MUSCLE CONTRACTION

a Hungarian Perspective

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Miklós S.Z. Kellermayer
Editor

2018

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Electron micrograph of honeybee asynchronous flight muscle stretched in rigor to reveal connecting filaments. Courtesy of Károly Trombitás.

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Foreword

In 1996, when I was a postdoctoral research associate in the laboratory of Henk L. Granzier at Washington State University in Pullman, Sherwin S. Lehrer, professor at the famous Boston Biomedical Research Institute, visited our department to give a seminar. Following the seminar, as it was customary, Professor Lehrer met individually with the students and postdocs, including me, to discuss what we were researching on. As we stood in front of the optical tweezers apparatus which I built to manipulate titin, he asked me: „Did you build all of this?" „Yes", I replied. „Are you a physicist?", he went on. „No, I am a physician!", said I. Following a few perplexed moments, Professor Lehrer suddenly turned to me: „Aren't you Hungarian?!"

Never in my life, before and after this incident, have I been so deeply and affectionately touched through my nationality. Of course, Professor Lehrer didn't leave me in doubt regarding his question. He explained to me that for many years his boss, the director of the Boston Biomedical Research Institute, had been John Gergely, who was Hungarian. „John did not know the word *impossible*", explained to me Professor Lehrer, who happened to identify this characteristic with every Hungarian. This incident helped me understand and appreciate the anecdotal story that still lingered at the time. According to the anecdote, in the 1970s, during a Contractility Subgroup session of the Annual Meeting of the American Biophysical Society, someone stood up and exclaimed: „Gentlemen, shouldn't we continue our discussion in Hungarian?" Happened or not, a „Hungarian Muscle Maffia" existed or not, it is true that at the time scientists of Hungarian origin made dominating contributions to the field of muscle biochemistry and biophysics.

During and shortly after World War II, a wave of some of the most brilliant minds of the 20th century emigrated from

Hungary. Among them were the so-called *Martians* (Theodor von Kármán, John von Neumann, Leó Szilárd, Ede Teller, Eugene Wigner), who became the founders of the American aeronautical, nuclear and computational sciences. Also among them was the Nobel-laureate Albert Szent-Györgyi, who was in a league of his own, and who became an intellectual hub in Woods Hole, Massachusetts. It is less widely known, however, that an exodus of further outstanding scientists took place during the decade and half that followed, and these eminent dissidents contributed to the establishment of some of the most remarkable biochemistry and biophysics research schools and departments abroad. It is even less known that some of the talented scientists, many of them disciples of Szent-Györgyi, stayed behind, persisted and tried to excel through the intellectually deprivating years of the communist-era Hungary. This book intends to pay homage to this generation of scientists, most of whom, being intellectual descendants of the World-War-II era Szent-Györgyi group, were eminent muscle researchers. Their life is a testament to the notion that the pursuit of scientific truth is one of the noblest quests of humankind. Decades of intellectual exodus and deprivation notwithstanding, I would like to think that there is hope for a new generation of muscle research in Hungary. Hence, besides the biographical chapters, reviews on some of the most important current muscle topics, pursued in Hungarian research centers, are also included. Finally, the Appendix contains the facsimile version of the famous *Studies from the Institute of Medical Chemistry, University of Szeged*, the publication of which in 1942-1943 marks, in certain ways, the beginnings of modern muscle biochemistry and biophysics. Finally, my special thanks go to the authors of the chapters of this book for their outstanding work and the exceptional inspiration they radiated towards me!

Budapest, 5 August, 2018.

Miklós S.Z. Kellermayer
Editor

Ilona Banga — Research as Life Motif
(1906–1998)

Magdolna Hargittai
Budapest University of Technology and Economics

Ilona Banga
(all pictures courtesy of Mátyás Baló unless otherwise noted).

Ilona Banga was a strong and dedicated woman, who decided to pursue a scientific career at a time when this was not expected for a woman. During her career she managed to be highly successful in not only one but three different areas of research. At the same time, she was also lucky, right after graduating from the University of Szeged, to get, "under the wing"of such an exceptional scientist as the future Nobel laureate, Albert Szent-Györgyi (1893–1986).

Banga was born on February 3, 1906, in Hódmezővásárhely, a small town in Southeastern Hungary as the first child of Sámuel Banga, a high-school teacher and Mária Róza Banga (née Berényi). Ilona wanted to become a medical doctor but

her mother felt that it would not be a proper occupation for a girl so she decided to become a chemist. But first, she had to finish high-school, what she did as a private student because at that time girls were not allowed to attend high-school as regular pupils. In 1923, she enrolled at the Faculty of Science of the University of Szeged, majoring in chemistry. She spoke German beside Hungarian and after her first year in Szeged, she decided to continue her university studies at the University of Vienna for two years. She received her diploma in Budapest, in 1929, in chemistry. The topic of her diploma work was the chemistry of tropaic acid, a degradation product of the alkaloid atropine.

Banga graduated at the time of the Great Depression when it was difficult to find employment. She was lucky though, because Professor Frigyes Verzár, the head of the Department of Physiology at the University of Debrecen offered her an assistant's job. In another fortunate circumstance, in 1931, Albert Szent-Györgyi and his family moved to Szeged from Cambridge, England. Szent-Györgyi was offered an attractive position at the University. He started to build up his Medical Chemistry Institute and he needed co-workers. Verzár warmly recommended Ilona Banga to Szent-Györgyi, based on Banga's performance in Verzár's laboratory. For Banga, there was also geographical advantage as Szeged was much closer to Hódmezővásárhely, where Ilona's family lived, than Debrecen. Banga became Szent-Györgyi's first assistant in Szeged. Her appointment was for a 3-months trial period but Szent-Györgyi found her so good and reliable that after only 3 weeks she was appointed to a permanent position. She worked with Szent-Györgyi in Szeged until 1945. During this period of time she moved up in the academic ladder from assistant to the equivalent of associate professor (private docent) and then, to a close-to-professorial rank, which was of a lesser status than what Szent-Györgyi was in—the system at the time operated in a pyramidal hierarchy. During these years Szent-Györgyi and Banga managed to publish 25 scientific papers together, in international journals. Her work was instrumental in both research topics on which Szent-Györgyi and his team was working between 1931 and 1945 in Szeged. These two topics were, first, the Vitamin C/

fumaric acid research and, later, the muscle contraction research. In 1945, Szent-Györgyi moved from Szeged to Budapest, and Banga moved with him. When Szent-Györgyi left Hungary in 1947, she stayed and continued her career in Budapest in the medical school.

Szent-Györgyi had long been interested in the chemistry of cellular respiration and tried to determine the role of hexuronic acid for years during his stay in Cambridge. He called this molecule "godnose" – referring to the fact that for years nobody could determine the role of hexuronic acid in cell respiration. He was also interested in determining what Vitamin C was and how it was related to hexuronic acid. Thus, right after arriving in Szeged in early 1931, Banga started to work on these problems.

Albert Szent-Györgyi *and* **Ilona Banga** *during an experiment in the early 1930s in Szeged.*

Szent-Györgyi was full of ideas and he found a perfect experimenter in Banga. He called her Iluska, a tender nickname for Ilona. She was committed to and excited about her work; she was clever, meticulous, and ingenious about how to perform the experiments. She developed ways of running many parallel experiments as it was necessary for them to demonstrate the reproducibility of their findings. Her inventiveness was especially important because they had only the most basic equipment for their experiments. At first, they determined that the mysterious hexuronic acid was actually Vitamin C. There was still the problem that although the importance of Vitamin C as an anti-scorbutic material was well known by then, it was difficult to produce it, even in small amounts. Until then, they used to isolate it from adrenal glands, which was not readily available in large quantities. By sheer luck, they tested paprika—the green and red pepper for which Szeged was world-famous—and found that it was very rich in Vitamin C. There was plenty of paprika around Szeged – but this did not mean that the work was easy. For example, in order to prepare sufficient quantities of Vitamin C, Banga had to start from about a ton of paprika. As they worked out the necessary technology and made Vitamin C (i.e., hexuronic acid) available in large quantities, Szent-Györgyi continued his prior investigation of the catalytic properties of fumaric acid in cell respiration. In the Szeged laboratory, they determined that contrary to the general belief the dicarboxylic acids (such as fumaric acid) behaved as catalysts instead of being consumed in the process of muscle tissue respiration.

In 1937, Szent-Györgyi received the Nobel Prize in physiology or medicine, unshared, "for his discoveries in connection with the biological combustion processes, with special reference to vitamin C and the catalysis of fumaric acid." This Nobel Prize turned the attention of the scientific communities all over the world to the Szeged laboratory. Szent-Györgyi's associates, including Banga, received invitations from abroad. She spent some time in Liege, Belgium, and Oxford, England. She worked together with another, future, Nobel laureate, Severo Ochoa, in Oxford. They studied the biochemistry of Vitamin B1.[1]

The Nobel Prize greatly affected Szent-Györgyi's life. With his sudden fame, the world-wide attention, and the numerous invitations, for a while he appeared losing direction and determination and facing a dilemma of how to continue. At 44 years of age, he was too young to simply sit on his laurels. There was still much more to learn about the biological combustion processes he had addressed so brilliantly in his previous studies, but he was also looking for something different, something new. As his biographer, Ralph Moss writes, by the middle of 1938, Szent-Györgyi fell into depression in trying to figure out what to do next.[2] Then, in 1939, he noticed a paper in *Nature* by two Russian scientists Vladimir Engelhardt and Militza Lyubimova, and that article revitalized him.[3] In this paper, the Russian researchers showed that the material responsible for muscle contraction was myosin. It not only split adenosine-triphosphate (ATP) and hence released energy, but it was myosin itself that actually triggered this activity. This was an unexpected discovery, suggesting the possibility of further surprises in the field.

Both ATP and myosin had been discovered decades earlier but their roles were not known yet. Szent-Györgyi realized that if they understood the chemistry here, they might find the explanation of muscle contraction – and that would bring him closer to understanding life, the ultimate puzzle that kept intriguing him. He identified motion with life. He immediately decided to pursue this direction of research, and Banga was to do the experiments.

As it was their usual modus operandi, first they repeated the previous, classical experiments related to muscle from decades earlier. They also repeated the Engelhardt-Lyubimova experiment. They decided to use the internal loin muscle of the rabbit (psoas), which according to Moss was a clever choice since it "was a powerful engine in a small space."[4] In his book, *Chemistry of Muscular Contraction*,[5] Szent-Györgyi discusses in great detail why he chose the rabbit psoas. An attractive feature was, for example, that this rabbit has long fibers running parallel with a minimal amount of connective tissue. There were also more mundane reasons: "Since the rabbit is easily obtainable, does not bite or bark,

is neither too big nor too small, is rather cheap and very stupid, we will choose its *Musculus psoas* as the object of our study."[6] We should also mention that the equipment available in Szeged was rudimentary; they only had the simplest tools. Nonetheless, what was missing in the technology was compensated by Szent-Györgyi's ideas and the inventiveness and dedication of his associates. Quoting Moss again: "…it was with this 'fingertip science' that Szent-Györgyi proceeded to make what is probably the most fundamental breakthrough in twentieth century muscle physiology,"[7] – and Banga was an active participant in all this.

Banga finely minced the rabbit muscle and put it in a saline solution. After an hour or so, she extracted myosin from it. Once, late in the evening, she did not have time to do the extraction, so she just left the minced muscle in the saline solution overnight at room temperature. Next morning, she was surprised by what she saw in the bottle. Usually, the myosin was a thin liquid, while this time it looked like a thick viscous jelly-like substance. She understood immediately that they were into some-

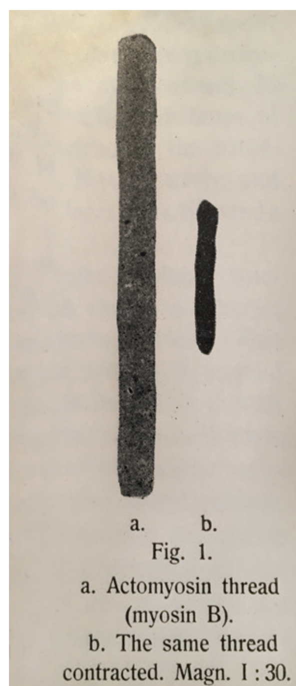
Laboratory scene in the Institute of Medical Chemistry in the early 1940s. **Szent-Györgyi** *and* **Banga** *are in the front,* **Bruno Straub** *is behind Banga and* **Koloman Laki** *is behind* **Szent-Györgyi.**

thing important. They analyzed this new substance, and found that it was also myosin, but different from the previously thin liquid. Eventually, they determined that if they added ATP to this "new" myosin, which they called myosin B (and the original one, myosin A), its viscosity decreased; it became the thin "old" myosin. At the same time, adding ATP to myosin A did not result in any viscosity change.

With further experiments, Banga and Szent-Györgyi determined that the appearance of the stickier, jelly-like myosin B was due to the presence of a new protein that they called "actin." Ilona used a very fine filter that let myosin A through but myosin B stayed on the filter. When she mixed myosin B with ATP, much of the mixed liquid went through the filter but part of it stayed. When she mixed this gel with myosin A, a thick jelly-like material formed. Later on, it was determined that this is an actomyosin-complex and this is what makes myosin B thick.[8] This last step of the experiment was carried out by Bruno Straub, a young research fellow, recently returning from Cambridge. Szent-Györgyi decided that he should join the team and he, rather than Banga, should continue the work on actin. Straub successfully isolated actin and determined its properties. Later in this paper, we will briefly return to this stage of the work on muscle contraction.

By this time they were confident that myosin was the contractile protein but it kept bothering Szent-Györgyi that *in vitro* they could not see it. So they made threads of actomyosin, the complex of myosin and actin, and put them under the microscope. Next, they added to it boiled muscle juice, as a source of ATP, and thus, finally, they could observe considerable shortening of the threads! In his autobiography, Szent-Györgyi remembered the moment: "To see them contract for the first time, and to have reproduced *in vitro* one of the oldest signs of life, motion, was perhaps the most thrilling moment of my life,"[9] (see below).

The significance of the results of the Szent-Györgyi group on muscle contraction is adequately described by Andrew Szent-Györgyi, Albert's cousin: "The demonstration that contraction can be reproduced *in vitro* by two proteins, actin and myosin,



Contraction of actomyosin from Ref. 28.

opened up the modern phase of muscle biochemistry. …. It simplified the study of contraction, allowed one to focus on the way the ATP energy is used and facilitated the beginning of the discussion that relates structural changes with biochemical events."[10] There were further discoveries regarding different aspects of muscle contraction by the Szeged group.

It is noteworthy that all this research was being carried out in Szeged, a relatively small town in southern Hungary, during World War II. Szent-Györgyi knew very well that even under war-time conditions it is essential that they publish their results. Although they kept receiving western scientific journals—thanks to the generosity of the Rockefeller Foundation—they could not publish their own manuscripts in them. Instead, Szent-Györgyi decided to summarize their results and publish them, in English, in the periodical of the University of Szeged, in three volumes (see also the Appendix of this book).[11, 12, 13]

Even with these precautions, Szent-Györgyi was afraid that their discoveries might not reach the scientific community. This was a realistic danger considering the times. Rall writes, "… the modern era of muscle biochemistry was established, but most of the rest of the world knew nothing about it."[14] Szent-Györgyi decided to send a copy of their summary to his friend Hugo Theorell, in Sweden, asking him to publish it in *Acta Physiologica Scandinavica*. There was one problem, though: according to the rules of the journal, only Scandinavian scientists could publish there. Theorell apparently understood Szent-Györgyi's frustration because he arranged for Swedish citizenship for him – and thus, the summary was published.[15] In the foreword of his book, *Chemistry of Muscular Contraction*, he thanks the Swedish legation for their help and also saving his life "without which he could hardly have lived to see his paper appear."[16]

There has been a lot of discussion about the history of the events leading to the understanding of muscle contraction. Why did Szent-Györgyi give the experiment that Banga had been carrying out so successfully suddenly to Straub? According to Banga's son, the renowned dermatologist Mátyás Baló, Straub had recently returned from Cambridge, where he learned new methods and Szent-Györgyi thought that this might be useful in determining what this new substance was. Szent-Györgyi used to emphasize Straub's role in these discoveries. He wrote, for example, "This study was undertaken in the author's laboratory by F. B. Straub (1942), who showed that the gradual increase in viscosity was due to the dissolution of another protein from the minced muscle, a protein distinct from myosin. This protein was termed "actin". Actin united with myosin to form the highly viscous "actomyosin."[17] Therefore, it has been generally accepted that Straub was the discoverer of actin.

Then, the story took an unexpected turn. In 1973, when the eighty-years old Szent-Györgyi visited Hungary for the first time after leaving in 1947, something strange happened. Suddenly, he claimed that it was not Straub, but Banga, who actually discovered actin and Straub started to work on it only afterwards. This caused quite a stir in Hungary among the scientists involved (and

even among those who were not). I may add that during the years since the discovery of actin, it had become a much more important substance than originally thought. Apparently, actin is responsible for movement not only in muscle but also in almost any cell.[18] It is hard to judge what happened. But many are of the opinion that coming home after a quarter of a century and seeing most of his students and assistants being professors, heads of departments, or occupying other high-level positions, it may have struck Szent-Györgyi that Banga was the only one whose career did not advance to the extent it should have. Moreover, Straub was by far the most successful career-wise among the lot; and Szent-Györgyi must have also remembered how good a scientist Banga was.

Straub and Banga had two rather different careers in post-war Hungary. Straub was elevated to high positions; he was elected to the Academy of Sciences and eventually served as its vice president. Toward the end of the communist regime, he was even charged to be the figure-head president of Hungary for a short while. Banga, on the other hand, was never elected member of the Hungarian Academy of Sciences, which she should have been. From various sources it appears that Straub did not use his positions to help his former colleagues in Szent-Györgyi's group. Mátyás Baló says that Szent-Györgyi probably acted the way he did because he saw that Banga did not receive the recognition that she deserved. Unfortunately, this strange encounter many years after the facts only opened up old wounds for Banga, which she had managed to overcome by now. In 1982, years after this problem resurfaced, Banga decided to write a letter to a well-known professor asking for advice, what to do?[19] She wrote that she had been receiving letters from Szent-Györgyi in which the aged scientist was urging her, to clear up the situation in which the discoverers had become entangled.

Banga tried to summarize the events. She and Szent-Györgyi were working on the isolation of the muscle myosin in the years 1940-1943, in Szeged. Since Banga was Szent-Györgyi's private assistant, they published the results of their experiments almost always together. They showed that the contractile ele-

ment of the muscle is not merely myosin as had been assumed before but what they called myosin B, later called actomyosin. Since during those war years it was impossible to publish their results in international periodicals, they communicated them in the Studies from the Institute of Medical Chemistry, University Szeged, Volumes I-III (see also the Appendix of this book).[12-14]

It was at that time that Szent-Györgyi was in house arrest and could not go to the University, so Banga alone continued the work, and each afternoon she communicated the results over the phone to him and they determined how to continue. During this work, she succeeded isolating another substance from myosin B, and they called it actin because it activated the reaction in which myosin A transformed into the contractile protein. This substance was isolated mostly by ultrafiltration and by several other techniques.

Banga describes in her letter that in the spring of 1942, Szent-Györgyi was again allowed to go to the laboratory. He told Banga that actomyosin was the real big discovery and they should give the less important actin to Straub, to make him interested in working with it. Banga had no say in this: "I agreed, what else I could have done if the professor wanted this. This is how the work "Actin" (Studies, II. pp. 3-15) that Szent-Györgyi and I had worked on together appeared under Straub's name alone." Banga notes that, by the way, Szent-Györgyi was mistaken about actomyosin being the "big thing" because actin, as a new protein, made the real splash in the literature.

Decades later, apparently, Szent-Györgyi changed his view on this matter. Again, quoting from Banga's 1982 letter: "Prof. Szent-Györgyi has been writing me letters since 1980 about how sorry he is that both he and I were left out of the recognition concerning actin. He suggested that I should turn to the [Hungarian] Academy of Sciences asking that they put together a committee for investigating what happened. He supports me all the way – and Straub has to confess that it was Szent-Györgyi and I who discovered actin. I wrote to Prof that I do not see any sense in doing all this after 40 years – on a problem that he created."

In contrast to Szent-Györgyi's late evaluation of what had happened, Straub narrated the actin discovery in the following way:[20] "Albert Szent-Györgyi suggested to me that I should leave my work on enzyme isolation and study the differences between myosin A and B. I never have regretted that I agreed – albeit with a bit of pang of heart – to join the team working on myosin. Albert had the idea that something must cause a colloidal change in myosin. My low opinion of colloid chemistry which I have already referred to, let me start on a different line. I thought that myosin B might contain another component which is not present in myosin A….. The protein present in this extract was given the name actin (Straub 1942b). Albert Szent-Györgyi acting as godfather and I as a happy parent."

Banga continued her letter: "Here is the trickery, because we with Szent-Györgyi already isolated actin before as this is clear from Studies I, "Discussion" by Szent-Györgyi on page 67. Not only was actin already mentioned here but, so was also the ATP-actomyosin complex." Banga stresses that Volume I. of the Studies went to press on July 6, 1942, whereas Straub's paper in Studies II went to press only on December 22, 1942.

However, reading Szent-Györgyi's "Discussion" in Vol. I, one gets somewhat confused: "Experiments of F. B. STRAUB, now in progress, definitely shows that myosin B is a stoichiometric compound of myosin A and another substance. We will call this other substance "actin" and the myosin-actin complex will be called "actomyosin".[21] This does not contradict directly what Banga stated in her 1982 letter, but does not rule out Straub's contribution to the discovery either.

There appears to be another important testimonial by another of Szent-Györgyi's associates, Koloman Laki (1909-1983). In his chapter entitled "Actin" in the book "Contractile Proteins and Muscle, he writes in 1971:[22] "Szent-Györgyi believed that during extraction another protein became extracted with myosin and that **extra protein** (here and further, emphasis by Banga) modified the properties of myosin. His belief received its first support when **Banga subjected myosin B** to Seitz-filtration in the presents of ATP and obtained a very viscous residue on the

filter which, when added to myosin A, changed it to myosin B. This was a good indication that myosin B was a complex of myosin A and another muscle protein. Szent-Györgyi named this other protein **actin**. At this stage, Straub, in Szent-Györgyi's laboratory, also began to study myosins A and B."

According to Moss,[23] though unbeknownst to Banga, Szent-Györgyi wrote also to Straub. He demanded that Straub acknowledge Banga's contribution to the actin saga. Szent-Györgyi was having increasingly strong feelings about the injustice to which he had also contributed in the past. He wrote a similar letter to the President of the Hungarian Academy of Sciences, the internationally renowned anatomist János Szentágothai. Neither Straub, nor Szentágothai responded. The contrast between Banga's and Straub's positions in the scientific hierarchy must have weighed heavily in this situation. Straub's attitude appears inelegant at best and an expression of misuse of authority at worst.

Banga was not only an outstanding scientist; she was also a brave woman and patriot. Toward the end of World War II, after Germany occupied Hungary, Szent-Györgyi was hiding from the GESTAPO that was out to arrest him for his anti-Nazi activities. Banga cleverly saved the equipment of the Institute for Medicinal Chemistry. She had notes posted on the door of the Institute in Hungarian, German, and Russian announcing that research on infectious materials was being conducted in the institute. To make the claim more credible, the notes indicated the receiving hours when such materials should be submitted. This kept away the departing German troops and the arriving Soviets, as well as Hungarian thieves.

In 1945, Ilona Banga married the internationally renowned pathology professor József Baló (1895−1979) in Szeged. Baló moved from Budapest to Szeged in 1928 to become the head of pathology at the University. When Szent-Györgyi and his group moved to Budapest in 1945, so did the Banga-Baló couple. Baló used a one-year Rockefeller fellowship to do research at the Johns Hopkins University in Baltimore and in the Wolbach Institute in Boston, in the United States. He had high university positions in Szeged. After the war he was professor of forensic medicine and

József Baló, Mátyás Baló, *and* **Ilona Banga**.

later of pathology in the medical school in Budapest. He was elected to the Hungarian Academy of Sciences before the war, but was demoted in 1949, only to be re-elected in 1956.

Banga was appointed head of the biochemistry laboratory of the pathology and cancer research institute of the medical school in Budapest. She collaborated with her husband in a research area that was new to her, the study of arteriosclerosis. They wanted to understand the causes of fiber degradation in vein-walls. They suspected that it was something that the organism itself produced. Following a great deal of experimental work, they determined that the culprit might be an enzyme that the pancreas produces, which they named *elastase*. The discovery was of major importance, but it sounded so new that many experts in the field doubted its validity. The doubts were dispelled when following another long stretch of experiments, Banga managed to crystallize elastase.[24] This was the third major research area

in which Banga produced exceptional results—this time it was a joint project with her husband.

In 1952, Ilona Banga received a telegram from the Parliament of Hungary that she was awarded the Kossuth Prize – the highest honor a scientist could receive in the country. However, when she learned that she was so honored alone, without her husband and partner in research, she declined it. The authorities warned her that her refusal would be considered a hostile act, inviting repercussions. At the time, the political system in Hungary was a Soviet-type dictatorship. Her response was that they worked together, and their achievements could not be separated. After Stalin's death in 1953, there was some relaxation in the political situation, and in 1955, Ilona Banga and József Baló shared the Kossuth Prize for their discovery of elastin and the enzyme elastase.

In her earlier career, Ilona Banga participated in two research areas with discoveries that could be considered Nobel Prize-worthy. One of the two areas was actually awarded the Prize—to Szent-Györgyi alone. Banga did most of the experiments and her name was also on the publications. The situation might, or might not, be different today, but assistants, let alone women rarely, if ever, shared Nobel Prizes at the time. My impression is that Szent-Györgyi himself found it quite natural that he alone was singled out for the honor of the Nobel Prize. In his Nobel lecture he spoke about his work conspicuously in first person, about "my work."[25]

The research of muscle contraction was at least as much Nobel Prize-worthy as the work on Vitamin C. It did not bring another Nobel Prize though, but it resulted in a Lasker Award for Szent-Györgyi in 1954. The Lasker Award by itself has great significance and it is often the precursor to an eventual Nobel Prize. It is a very rare occurrence that a Nobel laureate would get a Lasker Prize, which should be an indication of yet another major discovery independent of the previously earned Nobel Prize. Szent-Györgyi's Lasker was the expression of such an achievement. The controversy about the respective contributions by Banga and by Straub, did not help considerations for a shared

Lasker Award if such considerations were made at all in the first place. Of course, the three of them, that is, Szent-Györgyi, Banga, and Straub could have been considered jointly for the Lasker Award. In the early 1950s though it is doubtful whether Szent-Györgyi's former associates who stayed in Hungary might have been seriously considered by the Lasker judges for sharing the distinction. There are comments in the literature that a Nobel Prize could not be considered for the discoveries in the muscle research because the rules preclude a second Nobel in the same category. There is no such rule and there have been such Nobel Prizes, as were for John Bardeen in physics and Frederck Sanger in chemistry. The Nobel archives show that there was just one nomination for the muscle contraction topic, in 1951, for Szent-Györgyi. It was by an American professor, for "Muscular contraction and the role of myosin, actin, and adenosine-triphosphate". Neither Banga nor Straub received any nominations. In hindsight, it would have been justified for the three of them to share this highest recognition.

The description of the muscle contraction work carried out in Szeged in the 1940s on the website of the National Library of Medicine appears to be an impartial summary of what happened: "He [Szent-Györgyi] and his research team, notably Bruno Straub and Ilona Banga, went on to discover that muscle tissue contained a second protein, actin, which combined with myosin to form interlocking fibers; the higher the percentage of actin, the stronger the fiber's contraction response to ATP. By 1944, the team had elucidated the mechanism of muscle contraction and clarified the role of ATP in the process. They published a series of papers, *Studies on Muscle from the Institute of Medical Chemistry* which reported on their five years of research."[26]

Ilona Banga received recognition even if far less than her achievements would have warranted. In 1940, she became the first female "private docent" (comparable in rank to an associate professor) at the University in Szeged. In 1955, she acquired her higher doctorate, a prerequisite for professorial appointment, but she was never appointed professor. In 1962, she was elected member of the Deutsche Akademie der Naturforscher Leopoldina

(Halle, East Germany) and she was President of the Committee of Biochemistry of the Hungarian Academy of Sciences between 1968 and 1970. In 1986, she was the recipient of the first medal that the University of Szeged established in memory of Albert Szent-Györgyi. It is a sad oversight in Banga's recognition that she was never elected to be a member of the Hungarian Academy of Sciences, a membership she fully deserved. In contrast, Straub, who was elected member of the Leopoldina at the same time as Banga, had a brilliant career in academia. He was elected corresponding, then, full member of the Hungarian Academy of Sciences in 1946 and 1949, respectively and had a number of important positions in the Academy, including the vice presidency on two occasions for a total of 11 years, not to speak about his numerous other recognitions.

Ilona Banga, though, had a happy demeanor. From her early age, she engaged in what she loved best, working on challenging laboratory projects. She found ample rewards in these activities—significant discoveries. Few women could say in her time that they became successful in three unrelated research topics during their career. She used to say: "Research is my life motif, and it gives me fulfillment."[27]

Acknowledgement

I appreciate the invitation from Professor Miklós Kellermayer to prepare this contribution. I am grateful to Dr. Mátyás J. Baló for illuminating conversations about his mother and for providing me valuable documents. I thank Kornélia Papp of the Klebelsberg Library of the University of Szeged for her assistance in sending me copies of important papers.

Appendix

Books by ILona Banga

Structure and Function of Elastin and Collagen, Akadémiai Kiadó, Budapest, 1966.

Elasztin és elasztáz az atheroscloresis-kutatásban, Medicina, Budapest, 1978.

Selected publication list

Ilona Banga published well over 100 papers during her scientific career. According to her, the 10 most important papers she published are the following:

1 Banga, I., Szent-Györgyi , A., Über Atmungs-Coferment und Adenylpyrophosphorsare, *Biochem. Z.*, 247, 216, 1932.
2 Banga, I., Szent-Györgyi , A., The large scale preparation of ascorbic acid from Hungarian pepper. *Biochem. J.* 28, 1925, 1934.
3 Banga, I., Ochoa S., Peters, R.A., Pyruvate Oxydationssystem in Brain, *Nature*, 144, 74, 1939.
4 Banga, I., Szent-Györgyi , A., Structureproteine, *Science,* 92, 514, 1940.
5 Banga, I., Guba, F., Szent-Györgyi , A., Nature of myosin, *Nature*, 159, 194, 1947.
6 Baló, J., Banga, I. Elastase and elastase inhibitor, *Nature*, 164, 491, 1949
7 Banga, I., Baló, J. Elastin and elastase, *Nature*, 171, 44, 1953.
8 Banga, I., Baló, J. Szabó, D. Contraction and relaxation of collagen fibres. *Nature*, 174, 788, 1954.
9 Banga, I., Baló, J. Elastomucoproteinase and collagenmucoproteinase the mycolytic enzymes of the pancreas. *Nature*, 178, 310, 1956.
10 Banga, I., Ardelt, W., Studies on the elastolytic activity of the serum. *Biochim. Biophys. Acta*, 146, 284, 1967.

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1 Banga, I.; Ochoa, S.; Peters, R.A. *Biochem. J.* 1939, 33, 1109.

2 Moss, Ralph W. *Free Radical: Albert Szent-Györgyi and the Battle over Vitamin C*, Paragon House Publishers, New York, 1988, p. 117.

3 Engelhardt V. A., Ljubimova, M. N. Myosine and Adenosinetriphosphatase, *Nature*, 1939, 144, 668-669.

4 Moss, *Free Radical*, p. 120.

5 Szent-Györgyi , A., *Chemistry of Muscular Contraction*, 2nd revised ed., Academic Press, New York, 1951.

6 Ibid. p. 10.

7 Moss, *Free Radical*, p. 120.

8 See, e.g., Guba, F. Megkésett megemlékezés dr. Balóné, dr. Banga Ilonáról. (Belated reminiscences about Dr. Mrs. Baló Dr. Ilona Banga), *Orvosi Hetilap* (*Hungarian Medical Journal*, in Hungarian), 2000, Oct 1. Vol. 141. No. 40. *Vitalitas*, 2000, 141, http://www.vitalitas.hu/olvasosarok/online/oh/2000/40/51.htm

9 Szent-Györgyi, A. Lost in the Twentieth Century, *Annu. Rev. Biochem*. 1963, 32, 1-15, p. 9.

10 Szent-Györgyi, Andrew G., Milestones of Physiology: The Early History of the Biochemistry of Muscle Contraction, *J. Gen. Physiol.* 2004, 123, 631-641, p. 633.

11 Szent-Györgyi, A. *Studies from the Institute of Medical Chemistry University Szeged*, Vol. I (1941-42). Myosin and Muscular Contraction, by Banga, I. Erdős, T.; Gerendás, M; Mommaerts, W.F.H.M.; Straub, F.B.; Szent-Györgyi, A. Banga, I. Szent-Györgyi A (Sent to press on July the 6th, 1942.) 1. Banga, I.; Szent-Györgyi, A. Preparation and properties of myosin A and B. (5-15); 2. Szent-Györgyi, A. The contraction of myosin threads. (17-26); 3. Szent-Györgyi, A. Banga, I. The phosphatase activity of myosin, (27-36); 4. Mommaerts, W.F.H.M. Quantitative studies on some effects of adenyltriphosphate on myosin B, (37-42); 5. Straub, F.B. Reaction of myosin A with adenyotriphophate (43-46); 6. Gerendás, M. Technisches über Myosininfaden nebst einingen Beobachtungen über ihre Kontraktion (47-58); 7. Erdős, T.; The influence of K and Mg on the contraction of myosin (59-66); 8. Szent-Györgyi, A. Discussion. (67-71).

12 Straub, F.B. (1942) *Actin*. In: Studies from the Institute of Medical Chemistry, Vol. II. University of Szeged, Szeged, p. 3-15.

13 Straub, F.B. (1943) *Actin II*. In: Studies from the Institute of Medical Chemistry, Vol. III. University of Szeged, Szeged, p. 23-37.

14 Rall, Jack A. *Mechanism of Muscle Contraction*, In: Perspectives in Physiology, Springer, 2014. p 16.

15 Szent-Györgyi, A. Studies on Muscle. *Acta Physiol. Scand*. 1945, 9 (Suppl 25) 1-155.

16 Szent-Györgyi, *Chemistry of Muscular Contraction*, p. v.

17 Ibid. p. 32.

18 Moss, *Free Radical,* p. 228.

19 Letter of May 10, 1982, from Ilona Banga to Professor Dr. Tibor Trencsényi. Trencsényi (1907-1982) was the chief physician of the Hungarian Army at the time and the editor in chief of the Orvosi Hetilap (Medical Weekly).

20 Straub, F.B. *From Respiration of Muscle to Muscle Actin (1934-1943)*, In: Semenza, G.L. Ed. Of Oxygen Fuels and Living Matter (Evolving Life Science), John Wiley and Sons, 1981, 325-345, p. 335.
21 Szent-Györgyi, *Studies,* Vol. I, Discussion, p. 67.
22 Laki, K., *Actin*, In: Laki, K. Contractile Proteins and Muscle, M. Dekker, 1971, p. 98.
23 Moss*, Free radical*, pp. 226–227.
24 Banga, I., Baló, J., Elastin and Elastase, *Nature*, 1952, 171, 44.
25 Albert Szent-Gyorgyi – Nobel Lecture, Oxidation, Energy Transfer, and Vitamins, https://nobelprize.org/nobel-prizes/medicine/laureates/1937/szent-gyorgyi-lecture.html
26 „Profiles in Science: The Albert Szent-Györgyi papers," Szeged, 1931-1947: Vitamin C, Muscles, and WWII", National Library of Medicine, https://profiles.nlm.nih.gov/ps/retrieve/Narrative/WG/p-nid/149.
27 Guba, Megkésett megemlékezés (Belated reminiscences).
28 Szent-Györgyi, A. *Studies on Muscle*: From the Institute of Medical Chemistry, University of Szeged, 1944, p. 3.

Michael (Mihály) and Kate (Katalin) Bárány — A Scientist Couple

(Michael: 1921-2011; Kate: 1929-2011)

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Michael *and* **Kate Bárány** *working with the analytical ultracentrifuge at the Institute for Muscle Disease in New York, in the mid 1960s. (Photographs courtesy of George Barany).*

Michael and Kate Bárány chose "Strife and hope in the lives of a scientist couple" for the title of their 2000 scientific autobiography, a 77-page contribution to the *Selected Topics in the History of Biochemistry* series. Their lives were certainly marked by extremes of strife and hope, a phrase borrowed from the Lord's instructions to the biblical Adam in the closing line of Imre Madách's 1861 play *The Tragedy of Man*. In this shorter biogra-

phy, we emphasize the special significance of the term "scientist couple" in their title. Both words—*scientist* and *couple*—defined virtually every aspect of their lives, reinforcing each other in a remarkable partnership.

Their partnership began in postwar Budapest, where both Michael and Kate respectively worked to rebuild their lives through scientific education. Already Holocaust survivors, and later refugees, they found strength in each other through nearly 62 years together—51 of which in their adopted United States—and passed away a mere six weeks apart. Separately and as collaborators, the Báránys and their coworkers researched muscle biochemistry, biophysics, and physiology—making several fundamental contributions in these areas. In the lengthy latter parts of their careers, after moving from a series of research institutes to a university, they also became distinguished educators and were known around campus as "the professors who hold hands."

Michael (Mihály) was born in Budapest, Hungary on October 29, 1921, to Jewish farmers Angyalka (nee Schlichter) and Jozsef Fried. Despite graduating high school with highest honors (*summa cum laude*), Michael was prevented from advancing to the university due to restrictions on Jews ("numerus clausus"). Instead, he pursued a technical diploma, and then was drafted into a mechanical unit of the Hungarian army. Shortly before the German occupation of Hungary, Michael failed to convince his parents to go into hiding, and they perished in the death camps soon thereafter. Michael himself tried to hide but was exposed after a week, and subsequently deported to Buchenwald where he narrowly survived until the concentration camp was liberated in April 1945 by the American army. After a month recuperating from tuberculosis contracted in the camp, Michael returned to Hungary to study medicine at the University of Budapest, with financial support from the American Jewish Joint Distribution Committee.

Inspired at the start of his studies by the electric lectures of Albert Szent-Györgyi, Michael was thrilled later to be inducted into scientific research at Szent-Györgyi's Institute. He pursued two projects on blood coagulation by day, and by night read the

required texts for his medical degree. A recurrence of tuberculosis relegated him to a sanitorium, where he arranged to observe surgeries while keeping up his diet of reading. By the time Michael emerged from his treatment, Szent-Györgyi had decamped to the United States, so Michael continued his frenetic studies for a further year and then took his medical examinations with the aim of returning to research and pursuing a Ph.D.

Kate (Katalin) was born in Békéscsaba, Hungary on April 29, 1929, the younger child of Adél (nee Weisz) and Mihály Fóti (nee Fischmann), the latter a prominent Jewish physician. She excelled in her primary education despite the anti-Semitism of the time, and was an accomplished pianist who hoped for a concert career. At age 15, Kate was deported with her mother to the Auschwitz concentration camp, and at least twice found herself marked for the gas chambers before managing to elude these sentences. A third death-sentence was interrupted in January 1945 by Soviet liberators, who diverted Kate to a prisoner of war camp in Belorussia for another eight months. Returning to Hungary, Kate supported herself in Budapest by tutoring students in mathematics and by washing dishes, while completing her own high school education and then enrolling at the Eötvös University to study physics, physical chemistry, and mathematics.

The two had a chance encounter in June 1949, when Kate badly cut her finger attempting to slice a stale loaf of bread and Michael was called to administer first aid. They met again that year on August 1, at the start of a two-week student retreat sponsored by the American Jewish Joint Distribution Committee. By August 4, they decided that they would marry, and thereafter always celebrated August 1-4 as their anniversary. [The civil ceremony occurred that October.]

With the help of N.A. Biró, Michael returned to biochemical research at the former Szent-Györgyi Institute, now headed by F.B. Straub, discoverer of the contractile protein actin. Michael received his M.D. in 1951, and rapidly established himself as a researcher in Straub's orbit. His first scientific breakthrough soon followed: a precipitation method involving magnesium ions to isolate actin with unprecedented purity. This, in turn, was

Somewhere in Europe, in the mid 1950s.

the first evidence that actin itself, and not some external enzyme, catalyzed the ATP to ADP transformation during the protein's polymerization from G (globular) to F (fibrous). The result proved not just scientifically but politically important, securing the leadership of Straub's Western-oriented laboratory over the better-equipped one of Soviet-trained Communist biochemist Szörényi. Michael continued to pursue research related to his breakthrough, and obtained his Ph.D. in 1956.

During this same time period, Kate worked towards her M.Sc. in Physics (awarded in 1952) and simultaneously secured a position at the Electron Microscope Laboratory of the Hungarian Academy of Sciences, conveniently located in the basement of Straub's Institute. This allowed her to work in the same building as Michael, and furnished their first opportunities for scientific collaboration, eventually leading to their first co-authored work in *Nature* and the *Acta Physiologica* of the Hungarian Academy of Sciences. Within the Electron Microscope Laboratory, Kate and

her colleague László Hegedüs were given charge of the analytical ultracentrifuge, along with the diffusion apparatus—Western equipment that they had to set up and operate without being able to consult Western personnel. On her own, Kate devised a new cell to facilitate measurements of macromolecule diffusion.

Extracurricular collaboration with Michael, meanwhile, led to Kate's pregnancy in 1954—this despite admonitions from physicians that acute rheumatic fever contracted during the war would prevent her from ever having children. A paper resulting from her diffusion apparatus work was nearly ready for submission, with figures due to arrive the next day (February 19, 1955), when she went into labor. Kate managed to head to the laboratory on that Saturday morning, complete the manuscript, and take it to the postbox, less than two hours before delivering the couple's first son, George.

The Bárány couple was among the group of university students whose October 1956 march helped spark the Hungarian Revolution. Unable to teach under Communist rule because of his late father's prewar landholder status, Michael saw an opportunity to flee the country early in 1957 by walking 10 miles through the snow-covered border into Yugoslavia with then-pregnant Kate and their toddler son, who according to family lore remarked afterward "Jól sétáltunk [that was a nice walk]." After two weeks in a refugee camp, the family set off to Israel and moved in with relatives in Hayogev, an agricultural community near Haifa. Michael considered himself fortunate to find a position working with Ephraim Katchalski in the Biophysics Department of the Weizmann Institute, in Rehovoth (a half-day bus trip away). Kate remained at Hayogev, and son Francis (named after Straub) was born on April 4, 1957.

In Israel, Michael relished the opportunity to access the Weizmann Institute's library of Western journals and its state-of-the-art laboratory equipment, and to interact with numerous colleagues and visitors. These helped Michael lay the groundwork for further research on the structure and function of muscle contractile proteins, but both Michael and Kate came to find their situation in Israel limiting. Szent-Györgyi was unable to offer

them positions in the United States, but referred them to Hans Weber at the Max Planck Institute in Heidelberg, Germany.

The Báránys were anxious about moving to Germany after their wartime experience, but found Weber and the Institute there refreshingly supportive. Starting in Spring of 1958, Michael was able to work in the preeminent muscle laboratory in Europe, Kate was able to complete her Ph.D. in physical chemistry—awarded in 1959 by the Goethe University, Frankfurt am Main—and together they were able to renew their collaborative research started in Hungary.

In Germany, the Báránys discovered that urea reversibly inhibited live muscle contraction, and used this approach to elucidate the central role of the interaction of myosin, actin, and ATP in the contractile event both *in vitro* and *in vivo*. Importantly, they showed that the ATPase and the actin-binding sites of myosin were distinct. This work earned them international recognition and led to an invitation late in 1959 for Michael to head a department at the Institute for Muscle Disease, which had just been established in New York City by the Muscular Dystrophy Association of America, with Ade Milhorat as its founding Director. A position was created for Kate in a separate department headed by Hans Oppenheimer. In May 1960, the Bárány family immigrated to America, crossing the Atlantic Ocean on the S.S. Hanseatic.

While in New York, Michael made his most acclaimed discovery, finding a relationship between the speed of muscle contraction and the actin-activated ATPase activity of the myosin molecules in muscle. His 1967 *J. General Physiology* paper reporting this result has been cited over 1700 times and was chosen as a Citation Classic by the Institute for Scientific Information. This groundbreaking characterization of "fast-twitch" and "slow-twitch" muscles was followed by experiments in collaboration with Russell Close from Australia, showing that fast muscle could be changed into slow muscle and vice versa through cross-innervation, the first example of neural influence on gene expression in muscle.

Kate published a major finding of her own in 1967, in *Nature*, which became a pioneering result in the isolation and characterization of myosin light chains. She also took primary responsibility for raising the couple's sons, systematically incorporating academic and life lessons into their routines and cultivating interests in music and sports, as well as in science. Both joined their parents in the laboratory by their early teenage years and took on projects of their own. George entered the Ph.D. program at The Rockefeller University in New York at the age of 16, while Francis at age 17 was featured on the cover of *The New York Times* magazine as "Lord of the Venus' Flytraps." Both continued to distinguished scientific careers, respectively at the University of Minnesota, Twin Cities, and the Weill Cornell Medical College, New York City.

Working together, Michael and Kate published a series of important papers between 1969 and 1973 proposing that conformational changes in myosin are the driving force for muscle

Human Muscle Power Conference in 1984, Hamilton, Canada. **Kate** *and* **Michael** *are in the middle of the first row, staring, admiringly, at each other, rather than the camera.*

In their laboratory at the University of Illinois at Chicago, mid 1990s.

contraction. This work stopped with the sudden and unexpected closing in 1974 of the Institute for Muscle Disease. They were soon recruited to the University of Illinois at Chicago (UIC) College of Medicine as tenured faculty members—Michael in the Department of Biochemistry, Kate in the Department of Physiology and Biophysics.

At UIC, Michael quickly became a leader in the emerging field of biological nuclear magnetic resonance (NMR) spectroscopy and was the first researcher in the United States to conduct studies of live tissue with these methods. His co-authored 1977 *Science* review of biological ^{31}P NMR techniques was instrumental in sparking the development of non-invasive magnetic resonance diagnostic technologies, leading to the now-ubiquitous technology of medical Magnetic Resonance Imaging (MRI).

Through the end of the 1980s, Michael continued to study live muscle tissue using NMR, sorting out metabolites in healthy and diseased tissues, and contributing to the medical adaptation of the technology.

Together at UIC, Kate and Michael began a new collaborative research program on protein phosphorylation in healthy and diseased muscle in a range of physiological states. Among many discoveries, they established that myosin light chain phosphorylation was a prerequisite for smooth muscle contraction.

Having established their careers at research institutes, Kate and Michael responded to the challenges of university teaching (and of communicating with students unaccustomed to their Hungarian accents) by developing particularly helpful slides, handouts, and other supporting materials. Kate was a beloved educator of medical, dental, and graduate students at UIC, and received several prestigious teaching awards. Michael trained 9 Ph.D. students and 38 postdoctoral fellows, edited the 1996 textbook *Biochemistry of Smooth Muscle Contraction* which became a standard reference in the field, and learned to edit webpages in order to maintain a popular associated online resource. His service to the field was recognized by his 2006 election as a Fellow of the Biophysical Society (that Society's highest honor).

Both Báránys were also active in university service and faculty governance. Michael was elected the first president of UIC's Society of University Scholars, and Kate served for over two decades on the Chancellor's Committee on the Status of Women. Indeed, Kate became well known as a dedicated advocate for women in science and medicine, mentoring many young scientists, students, and colleagues while sharing her wisdom on balancing career, family, and health (she was an avid swimmer). She was instrumental in establishing the UIC Children's Center and fought for flexible tenure, career guidance for women in science, female-friendly facilities, and equal pensions for men and women. In recognition of her active efforts, as well as her history as a role model going back to a period when very few women could choose a scientific profession, Kate was named UIC's Woman of the Year in 1996.

George, *the 12-year-old* **Michael J.** *and* **Michael Bárány**
at the 16th American Peptide Symposium in Minneapolis, in 1999.

Michael and Kate supported awards at UIC, at the University of Minnesota, and with the Biophysical Society. The common thread to these awards was to recognize and honor students and scientists at early stages of their advanced education or professional careers. Throughout much of the 1990s, Kate was active in outreach activities that had the aim to inform American schoolchildren of the horrors of the Holocaust, while Michael was generally reserved on the subject of his Holocaust experiences. They each discussed their experiences in 1995 video interviews for the Shoah Foundation.

Michael's work continued, albeit at a slightly reduced pace, after he reached the UIC's mandatory retirement age in 1992 (two years before the rules were changed), and he continued to make discoveries and to author papers well into the start of the 21st century. His last paper was published in 2004, based on work that he carried out with his own hands, and funded out of his retirement savings. Kate was active in research, teaching, and service until impaired vision led her in 1998 to accept professor emerita status.

Kate *and* **Michael** *in their Chicago apartment in the early 2000s.*

As a scientist couple, the Báránys shared their passion for and commitment to science with their family, and created an extended family out of their many students and colleagues. For them, the highest standards of scholarship, integrity, and excellence were all core components of the effort to live well, and living well was a necessary precondition to doing good science. Science was logical and predictable in a world where they had experienced so much suffering based on the arbitrary prejudices and madness of human beings, and they strongly believed that the power of knowledge and understanding could improve the human condition. Strife and hardship taught them never to take science, family, or comfort for granted; it also instilled in them a deep sense of justice. Their lives exemplified resilience, hope, and the indomitable human spirit. They brought an inexhaustible love and devotion to what they considered truly important: science, family, and—most of all—each other.

Resources

All told, Michael Bárány published 164 full papers and 26 book chapters, while Kate Bárány published 82 full papers (all but 10 co-authored with Michael) and 12 book chapters (all co-authored with Michael). In 2006, they selected the following as their most significant papers (list modified slightly by the present authors):

1 Bárány, M. and Bárány, K. Studies on "Active Centers" of L-Myosin. *Biochim. Biophys. Acta* **35**, 293-309 (1959).

2 Bárány, K. and Oppenheimer, H. Succinylated Meromyosins. *Nature* **213**, 626-627 (1967).

3 Bárány, M. ATPase Activity of Myosin Correlated with Speed of Muscle Shortening. *J. Gen. Physiol.* **50**, 197-216 (1967).

4 Bárány, M. and Bárány, K. Change in the Reactivity of Myosin during Muscle Contraction. *J. Biol. Chem.* **245**, 2717-2721 (1970).

5 Bárány, M. and Close, R.I. The Transformation of Myosin in Cross-Innervated Rat Muscles. *J. Physiol.* **213**, 455-474 (1971).

6 Bárány, M. and Bárány, K. A Proposal for the Mechanism of Contraction in Intact Frog Muscle. *Cold Spring Harbor Symposium Quantitative Biology* **37**, 157-167 (1972).

7 Burt, C.T., Glonek, T., and Bárány, M. Analysis of Living Tissue by Phosphorus-31 Magnetic Resonance. *Science* **195**, 145-149 (1977).

8 Bárány, K. and Bárány, M. Phosphorylation of the 18,000-dalton Light Chain of Myosin during a Single Tetanus of Frog Muscle. *J. Biol. Chem.* **252**, 4752-4754 (1977).

9 Doyle, D.D., Chalovich, J.M., and Bárány, M. Natural Abundance ^{13}C NMR Spectra of Intact Muscle. *FEBS Lett.* **131**, 147-150 (1981).

10 Arus, C., Bárány, M., Westler, W.M., and Markley, J. L. ^{1}H NMR of Intact Tissues at 11.1 T. *J. Magnetic Resonance* **57**, 519-525 (1984).

11 Venkatasubramanian, P.N., Mafee, M.F., and Bárány, M. Quantitation of Phosphate Metabolites in Human Leg *in Vivo. Magn. Reson. Med.* **6**, 359-363 (1988).

12 Bárány, M., Langer, B.G., Glick, R.P., Venkatasubramanian, P.N., Wilbur, A.C., and Spigos, D.G. *In Vivo* H-1 Spectroscopy in Humans at 1.5 T. *Radiology* **167**, 839-844 (1988).

13 Bárány, M. and Bárány, K. Calponin Phosphorylation Does not Accompany Contraction of Various Smooth Muscles. *Biochim. Biophys. Acta* **1179**, 229-233 (1993).

14 Bárány, M. and Bárány, K. Strife and hope in the lives of a scientific couple. In: Semenza, G. and Jaenicke, R. (eds.) *Selected Topics in the History of Biochemistry. Personal Recollections VI, Comprehensive Biochemistry* **41**, 91-167 (2000).

15 Bárány, M. and de Tombe, P.P. Rapid exchange of actin-bound nucleotide in perfused rat heart. *Am. J. Physiol. Heart Circ. Physiol.* **286**, H1394-H1401 (2004).

After their deaths in 2011 (Kate: June 13; Michael: July 24), the family created separate websites for Kate at: http://www1.chem.umn.edu/groups/baranygp/katebarany/ and for Michael at: http://www1.chem.umn.edu/groups/baranygp/michaelbarany/ These pages, which were maintained for several years but as of this writing have a few no-longer-functioning links (fortunately, most still work), contain substantial biographical information, including each of their complete *curriculum vitae*, transcripts of interviews and talks about their Holocaust experiences and professional lives, numerous family photos, several endearing personal anecdotes, and a range of reminiscences from family and colleagues. The websites also encompass materials from their respective retirement events, as well as from various tributes and memorials.

Michael's first Ph.D. student, Joseph Chalovich, guest edited a special issue of *J. Muscle Research Cell Mobility* **33** (2012) in tribute to the life and science of Michael and Kate Bárány.

The most invaluable resource remains their own scientific autobiography, with many additional details, which was consulted extensively during the preparation of this chapter.

József Belágyi — Humble master of muscle EPR spectroscopy
(1932-2012)

Miklós S.Z. Kellermayer
Semmelweis University

József Belágyi *in 2004*
(Photo vitéz István Vadász).

In early September 1983, as a second-year medical student, I knocked on the front door of the Central Laboratory of the Medical University of Pécs to volunteer for part-time student research. I was taken to Professor József Belágyi, the chairman of the Central Laboratory[1]. Professor Belágyi was not unknown

1 The official name was Central *Theoretical* Laboratory in order to distinguish from the Central *Clinical* Laboratory.

to me: he had been giving us lectures on biometrics and biostatistics. In fact, he appeared to be one of the most knowledgeable professors who lectured to us, and this was the reason for which I naturally gravitated towards the research he may have had to offer. Yet, he cast the image of a very stern and strict man, therefore I approached his office with considerable anxiety and excitement. My anxiety instantly evaporated as we began to converse. He talked to me in the most frank, natural, straightforward and friendly manner, as if I had been his colleague. I could not have known at the time that later I was to indeed become his colleague, and that the next fifteen years of my life would be connected to the Central Laboratory. I was drawn naturally to the person of József Belágyi: his clear thinking and outstanding communication skills ever amazed me. Behind his humbleness and softly-spoken words stood a huge knowledge of muscle function, the applications of electron spin resonance spectroscopy (ESR or EPR) and a fundamental understanding of mathematics, physics and biophysics in general. Under his long leadership of the Central Laboratory (1970-1997) he managed to transform this service facility into a full-fledged university department with teaching duties and strong research activity. He was one of the last members of a classical generation of professors at the Medical University of Pécs, who were characterized not only by breadth of knowledge but also by charisma, affection, discipline, and a strong sense of responsibility.

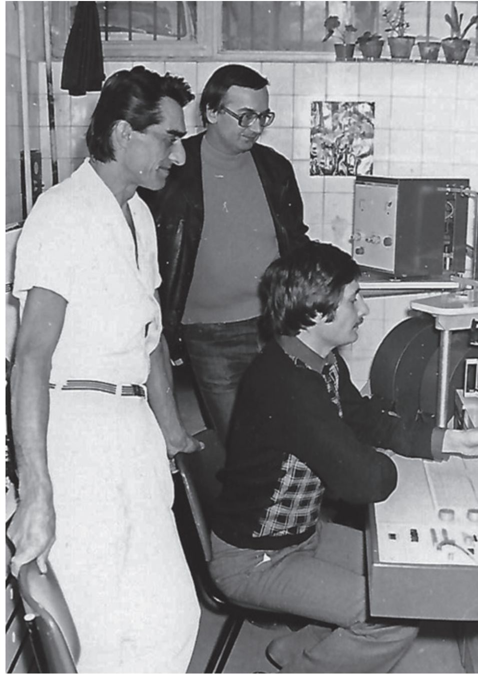
Biography

József Belágyi was born on December 12, 1932 in Kalocsa, a provincial agricultural town of ecclesiastical importance as the archbishop's center in southern Hungary near the Danube. His high-school years, spent at the outstanding Jesuit High School of Kalocsa, were influential in his life. The overtake of this excellent high school by the communist state in 1948 and the prosecution of the Jesuit priests and teachers in 1950 coincided with Belágyi's high-school years. These events have taken a toll on him, an

experience he spoke about rarely and only to his closest friends. He attended the University of Szeged where he obtained a mathematics and physics teacher degree in 1955. After graduation he accepted a physics teacher position at the Széchenyi High School in Pécs.

In 1959 Eugene Ernst, chairman of the Department of Biophysics at the Medical University of Pécs, invited him into his research group supported by the Hungarian Academy of Sciences. In his research Belágyi focused on the mechanics of striated muscle and worked in close collaboration with Miklós Garamvölgyi (see biographical chapter on Garamvögyi in this issue). Between 1963 and 1964 Belágyi, supported by a fellowship, was a scholar in Berlin (-Buch) to study the method of electron spin resonance spectroscopy (ESR, or electron paramagnetic spectroscopy, EPR). In 1968 he became adjunct faculty, and in 1969 he defended his CSc ("candidate of sciences") degree on the mechanics of muscle

The young **József Belágyi** *in 1955, in his final university year. (Photo courtesy of Tibor Belágyi).*



In the EPR Spectroscopy Laboratory in 1980, after installing the new EPR spectrometer from the German Democratic Republic. From left to right: **József Belágyi,** *installation engineer from the EPR company,* **Pál Gróf** *adjunct faculty. (Photo courtesy of Tibor Belágyi).*

contraction. Besides focusing on muscle mechanics, he devoted attention to the development of the subject of biomathematics and biometrics which was taught, initially as part of biophysics, to medical students. In 1970 the central theoretical building of the Medical University of Pécs was opened, and certain restructuring of the research facilities took place. The university established a facility called Central Laboratory with the intention that expensive, high-end research infrastructure could be centrally accessed and maintained. József Belágyi was appointed as director of Central Laboratory, a position he held until 1997. The concept of centralized or core facilities was quite novel and advanced at the time. The Central Laboratory of the Medical University of Pécs housed the following instrument facilities: electron

Award ceremony at the Medical University of Pécs, 1985. Left, the awardees: **Marietta Vértes,** *Department of Physiology,* **József Belágyi, Tibor Mérei,** *Department of Neurosurgery. Right, Belágyi receiving the Pro Universitate award from the rector* **Miklós Bauer.** *(Photo vitéz István Vadász).*

microscopy, EPR spectroscopy, NMR spectroscopy, gas chromatography, synthetic chemistry, computer center, photography and image processing laboratory. Each laboratory and instrument was maintained by a dedicated scientist, typically a prominent leader in the respective field. Besides directing the entire Central Laboratory, József Belágyi was the head of the EPR Spectroscopy Laboratory as well. In 1971 he became university docent.

In 1985 Belágyi defended his doctor of sciences (DSc) thesis which had the title "Molecular dynamics of contractile proteins". In his DSc thesis Belágyi summarized his results on the rotational motion of various muscle proteins (actin, myosin, actin-binding proteins) by using EPR spectroscopy. In 1987 he became full professor of the Medical University of Pécs. Between 1989 and 1990 he was a visiting professor in the Boston Biomedical Research Institute under the direction of John Gergely (see biographical chapter on John Gergely in this issue). In 1997 Belágyi resigned from the directorship of Central Laboratory but continued his research. In 2002 he became professor emeritus of the University of Pécs. In 2005 Belágyi departed the Central Laboratory and joined the Department of Biophysics. He was able to transfer his research group and instrumentation as well and carried on his research and teaching with rejuvenated effort. He

updated his textbook on Biomathematics which was published in three languages, Hungarian, English and German.

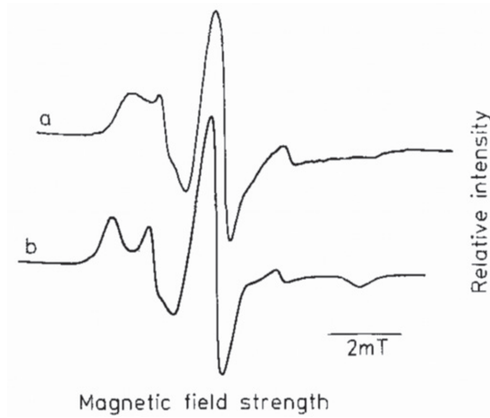
József Belágyi was a well-recognized scientist and teacher and hence the recipient of local and national prizes. In 1985 the Medical University of Pécs awarded him the Pro Universitate award. In 1998 he received the Knight of Cross from the Order of Merit of the Hungarian Republic for his lifetime achievement in science and higher education. In 2009 he received the Eugene Ernst Medal from the Hungarian Biophysical Society for his outstanding contributions to and lifetime achievements in Hungarian biophysics.

József Belágyi died on March 10, 2012 in Pécs. He was first buried in the cemetery of Győrújbarát, near the city of Győr in western Hungary. Later, as per his will, his ashes were transferred to the garden of his vacation house on Lake Orfű near Pécs. This garden was his favorite place for times of rest and seclusion.

Contributions to science and muscle research

József Belágyi was a devoted experimentalist and an excellent theoretician at the same time. Because he mastered mathematics, he always took care to set up physical and mathematical models based on his experimental results, which he tested by computer simulation. He was very critical not only with others but also with himself. His main interest was in the field of muscle contraction, and he is internationally recognized for his contributions towards the application of EPR spectroscopy for the understanding of the molecular dynamics of muscle's contractile proteins. He published more than 150 papers which received citations in excess of 2000.

As a member of Eugene Ernst's research group, József Belágyi initially worked on the biophysics of muscle contraction, and he explored the mechanical properties of striated muscle (Belágyi and Felker 1962; Belágyi and Biro 1962; Belágyi and Garamvölgyi 1968; Garamvölgyi and Belágyi 1968), although he he



EPR spectra of globular actin modified with maleimide spin label. a. Actin at low salt concentration. b. Actin depolymerized by the addition of 1 mM $MgCl_2$ and 15 mM ATP (Belágyi and Gróf 1983).

investigated the luminescence of biological tissue as well (Belágyi and Kutas 1972a; Kutas and Belágyi 1972b). His interest in the mechanics of contraction prevailed and later he studied the effect of velocity-dependent stretching on the kinetics of muscle force development (Belágyi et al. 1979). With the establishment of the EPR Spectroscopy Laboratory and an outstanding synthetic chemistry laboratory that produced various spin label compounds under the direction of Kálmán Hideg, Belágyi's attention turned towards the applications of EPR methods to understand the structural dynamics of contractile proteins. Initially he investigated the effect of ions and small molecules on globular and filamentous actin. Later he explored actin dynamics in the context of greater molecular complexity with actin-binding proteins. Particularly strong and fruitful collaborations were established with notable research groups at the Nencki Institute in Warsaw, Poland (Mossakowska, Belágyi, and Strzelecka-Gołaszewska 1988; Gałazkiewicz, Belágyi, and Dabrowska 1989a; Gałazkiewicz, Belágyi, and Dabrowska 1989b) and with György Hegyi in the Department of Biochemistry at Eötvös Loránd University in Budapest, Hungary (Hegyi, Szilágyi, and Belágyi 1988; Hegyi and Belágyi 2006).

Later Belágyi employed the spin labeling technique to investigate the rotational motion of myosin as well (Belágyi, Frey, and Pótó 1994; Lörinczy and Belágyi 1995; Belágyi and Lörinczy 1996). Although actin remained a favorite and important protein to investigate in subsequent years in ever increasing detail (Hild et al. 1996; Nyitrai et al. 1997; Hild et al. 1998; Nyitrai et al. 1999), Belágyi's attention was not focused strictly on muscle, but applied the EPR method to diverse biomolecular systems. For example, in my PhD diploma thesis and in a publication I investigated, with Belágyi, the rotational dynamics of tubulin (Kellermayer, Trombitás, and Belágyi 1989). Belágyi recognized that EPR could be well applied towards following redox processes in microorganisms. Hence he conducted numerous collaborative experiments to characterize the redox state in various fungi (Pesti, Gazdag, and Belágyi 2000; Pesti et al. 2002; Gazdag et al. 2003; Gazdag et al. 2011).

Belágyi managed his research group by both paying attention to instructing his students and carrying out experiments himself. He never stopped improving the instrumentation of his EPR spectrometer and he kept on writing software for driving the instrument and for data analysis. He was often the last to leave the laboratory in the evening. When in the midst of an experiment, his attention was so focused that he could not be bothered by phone calls, no matter whether it was from the dean or the rector.

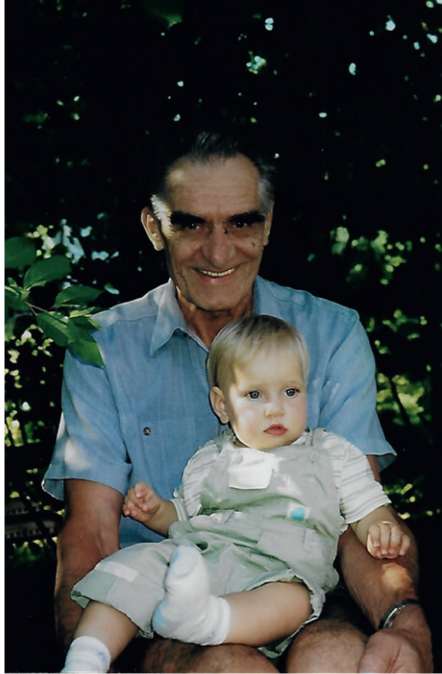
The person behind the man

József Belágyi was a man of intellectual integrity, honestly and faithfully serving the science of biophysics and the University of Pécs throughout his life. He was extremely polite and reserved. He was a very logical thinker and applied the clarity and logic of a physicist to every aspect of his life. He was an excellent lecturer and communicator, and his talks were often the highlight of the conference. Most of his active career, between 1959 and 1989,

fell under the socialist era of Hungarian history. This era left its deleterious effects on intellectual life, and the Medical University of Pécs was no exception. It was clear to all of us that Belágyi was often put under pressure by the communist party leaders, yet he never openly spoke about what emotional stress he had to withstand. His deeper thoughts and emotions were kept to himself. His private life was secluded. His everyday life was well structured and humble. After suffering a serious pancreatitis in 1986 he became even more ascetic and reserved. Yet, the few occasions I was able to converse with him about nature and life after some experimentation late into the evening were special, treasured moments of my life.

Belágyi's favorite pastime was attending his garden on Lake Orfű near Pécs, where he usually escaped when in need of silence and seclusion. He was happiest when among his immediate family. He is survived by his son, a surgeon, and four grandchildren.

József Belágyi *lecturing at the Eugene Ernst Memorial Symposium in 1986. (Photo courtesy of Tibor Belágyi).*



József Belágyi *with his third grandchild in 2003.*
(Photo courtesy of Tibor Belágyi).

Acknowledgements

I am indebted to László Kutas (associate professor at the University of Pécs), Pál Gróf (associate professor at Semmelweis University) and Tibor Belágyi (son of József Belágyi, Department of Surgery, Petz Aladár County Teaching Hospital, Győr) for information and photographs.

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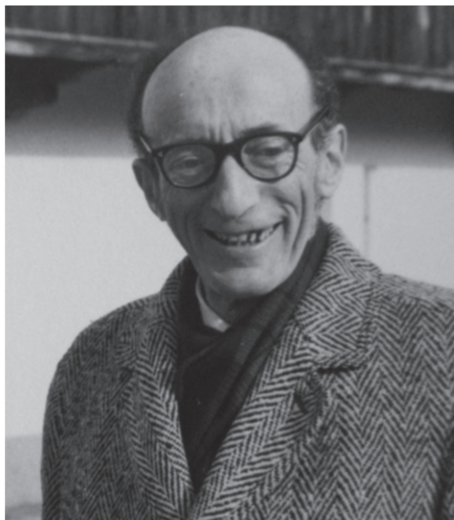
Endre Bíró — Following Albert Szent-Györgyi's footsteps in Budapest
(1919-1988)

Ágnes Jancsó[1] **and László Nyitray**
Eötvös Loránd University, Budapest



Endre Bíró
(Alpbach, 1974, courtesy of Marcus Schaub).

Endre Bíró and Albert Szent-Györgyi

Miklós Endre Bíró, Zebi for his family and fellow scientists, was born on April 22, 1919 in Budapest (His given names are translated to English as Nicholas Andrew, and that is why he is listed on some of his publications as N. A. Bíró.) His parents, Lipót Bíró and Emma Gráber were liberal-minded Hungarian

1 Current address, Madison, Wisconsin

Jews. Growing up in Hungary as a Jew in those years of the 20th century made even educational decisions difficult, since the numerus clausus law of 1920 limited the percentage of Jewish students at universities. Endre Bíró was accepted at Miklós Horthy University in Szeged. As explained in Ralph Moss's book about the life of Albert Szent-Györgyi [1]:

> *"Szeged presented its own peculiarities. Since the faculty at Budapest was notoriously pro-fascist, Jewish students in the thirties found it easier to get admitted to provincial colleges, working their way around the hated 'numerus clausus'. Thus, each September, Szeged University had more than the five percent quota of Jews. This, in turn, served as a good pretext for riots by right-wing students, mainly members of the Turul organization. Prof N. A. Bíró remembers those days well. Of Jewish origin and from a liberal family to boot, he was accepted into Szeged with the help of a Gentile family friend. In the fall of each year, rioting broke out in Szeged. Forewarned, Bíró and other Jewish students hid across the river in New Szeged until the fighting was over."*

Bíró majored in chemistry and physics, and took mathematics courses on the side. He graduated with a teacher's degree in Physics and Chemistry (equivalent of today's M.Sc. degree) in 1941. He certainly was talented, as indicated by an award from the university for his work titled "*Determination of Avogadro's Number Based on the Examination of Emulsions*", and by the solicitation by a medical student that he take the final chemistry exam for him. He never forgot the anxiety caused by his having taken that test… Endre Bíró earned a doctorate from the University in Szeged in organic chemistry and experimental physics in 1942.

As a fresh graduate he was immediately drafted into "labor service". (During World War II Jewish men were barred from serving in the regular Hungarian army; labor service units served as auxiliary troops.) In the summer of 1944 Bíró fled his labor service unit in Transylvania, and after a year in Bucharest he

finally returned to Hungary with the help of the American Jewish Joint Distribution Committee.

In the spring of 1945 he read in a newspaper that Albert Szent-Györgyi was planning to set up a new laboratory in Budapest, and wrote a motivation letter to him:[2]

"As indicated by the enclosed resume, even earlier, the reason for my going to university was my intention to devote myself to pure scientific research. I felt I had a scholarly aptitude. However, in the previous regime, due to circumstances beyond my control, I was not able to achieve much in scientific research. I am firmly committed to my decision of dealing with pure scientific research, although I am aware that being a scientist does not mean a high salary."

And so it was that he started a research career in Albert Szent-Györgyi's laboratory in the newly established Department of Biochemistry of the Medical Faculty of Pázmány Péter (today Eötvös Loránd) University. As told by Ralph Moss [1]:

"Most of Szent-Györgyi's students from Szeged had joined him in Budapest. (...) N.A. Bíró, who had survived a work team for Jews in Transylvania, was there as was Ilona Banga, who had saved most of the scientific instruments in Szeged from marauders."

Life was not easy in the lab, instruments were rudimentary, facilities were poor, and chemicals were scarce. And yet, two young associates of Albert Szent-Györgyi, Zebi and Csuli (Andrew Szent-Györgyi, see the chapter on his life) made an important discovery, showing that actin highly increases the Mg-ATP hydrolyzing activity of myosin [2]. This early work, published in 1949 in Hungarica Acta Physiologica, was among the first experi-

2 Biographical data are from Dávid Bíró (the son of Endre Bíró), a sociologist and teacher in Budapest. The letter dated April 9, 1945.

Endre „Zebi" Bíró *in 1946 (courtesy of Dávid Bíró).*

mental results leading to the concept that myosin ATPase activity is allosterically activated by actin.

The first international congress Endre Bíró attended together with Albert Szent-Györgyi was the 6th International Congress of Experimental Cytology in Stockholm. This is how Bíró reported on the reception of Albert Szent-Györgyi's talk at the meeting [3]:

> *"Professor Albert Szent-Györgyi's lecture reflected the triumph of comprehensive research concepts. Based on an extensive theory, it gave a clear review of all our knowledge on the functioning of the muscle. Used to doing research in small areas of detail only, researchers listened with enthusiasm to this comprehensive lecture. Although there were inexhaustible debates on specific issues, everybody agreed that Prof. Albert Szent-Györgyi's work is the first attempt at explaining the functioning of the muscle based on precise physical and chemical terms."*

Albert Szent-Györgyi did not return to Hungary after this meeting, he immigrated to the United States, and was followed by most of his associates. Among the few who decided to stay in Hungary was Endre Bíró, because at that time he was optimistic about the situation in the country [1]. In 1949 the new Institute of Medical Chemistry was formed under the leadership of Brunó F. Straub, who discovered actin in Szent-Györgyi's Szeged lab (see the chapter on his life). Endre Bíró, together with a few other members of the Department of Biochemistry, joined this institute.

In the meantime the political situation deteriorated – a communist regime got into power and hard times came for everyone in the country, even for those in a university laboratory. Dezső Prágay (1921-2011) was a colleague of Endre Bíró at the Institute of Medical Chemistry (he later became a clinical biochemist professor at SUNY Buffalo). This is how he remembered his encounter with Bíró [4]:

Endre Bíró *at a conference in Stockholm, July 1947 (next to him* **Tamás Erdős,** *opposite* **Albert Szent-Györgyi** *and* **W.F.H.M. Mommaerts;** *courtesy of Dávid Bíró).*

"I started my career as a fresh university graduate at the Medical Chemistry Institute in 1950. Endre Bíró (Zebi) was already on the faculty there. (…) We quickly got to know each other, and became good friends. (…)
His scientific approach could be characterized as kind of dignified, and extremely thorough. (...) He carefully thought through his research plan and methods, and only started to do experiments when – after some deliberation – he was convinced that the strategy was correct. Then he set out to prove his ideas with systematically executed experiments. It is safe to say that his results were rock solid. (…) He led an exemplary life. He respected science very much indeed, and he never treated it as a means for career advancement. (…)
In the Institute he did not spare time and effort when his younger colleagues needed something explained. A professor at the helm does not always have time for junior researchers. Zebi, however, was always available when needed. We had a maxim: «Don't worry, Zebi will explain it!». (…)
As far as I am concerned, it was primarily him who got me started to think like a scientist. (…) He created and incomparably warm, humane atmosphere, which, like a family hearth, radiated its warmth onto us."

The Bíró school at Eötvös Loránd University

In 1950 Pázmány Péter University adopted the name of the world-renowned physicist Loránd Eötvös, and in 1951 the Medical Faculty became a stand-alone medical school (today's Semmelweis University). In 1953, Endre Bíró was invited to organize the Animal Biochemistry Department at the Faculty of Natural Sciences of Eötvös Loránd University (ELTE). Shortly thereafter the authorities reversed this decision, and instead of an independent department, a small Biochemistry Research Unit was formed within the Department of Genetics, with only two members: Endre Bíró, and Béla Nagy (1927-2009). Nagy left

for the United States in 1956 (and later received a PhD from Brandeis University, worked at Boston Biomedical Research Institute, published many papers on muscle proteins, and retired as a professor from the University of Cincinnati). Nagy was replaced by András Mühlrad as faculty member. From 1962 on, the research unit was able to hire more scientists (Miklós Bálint, György Hegyi, among others).

> *"It was a unique phenomenon that all new faculty members were former Bíró disciples, and this circumstance made possible to maintain the Department's unity, its coherence. This may have been the most significant result of Professor Bíró's mentorship."[4]*

As Bíró himself noted [3]:

> *"I had enormous luck. I did not just receive new staff members, I selected and reared them."*

Back then the research group was poorly equipped, but this was largely offset by the ingenuity of Endre Bíró. Based on his group's patented invention (Patent number 145.827), the Hungarian Optical Works produced the first Hungarian photometer, called UVIFOT, which operated both at visible and ultraviolet wavelengths. Here is an anecdote about Zebi, the absent minded professor and the money he earned for the patent[3]: He has acquired a Citroen 2CV ("Deux Chevaux", or popularly The Ugly Duckling). On his first drive, being a heavy smoker, a cigarette from between his lips dropped to the floor. He leaned forward to pick it up, and crashed into a tree; that was the last time he drove.

Instrument building and rebuilding (later under the guidance of György Hegyi) did not end with the photometer – there was a shaker constructed from an old Warburg instrument, the

3 György Hegyi, personal communication

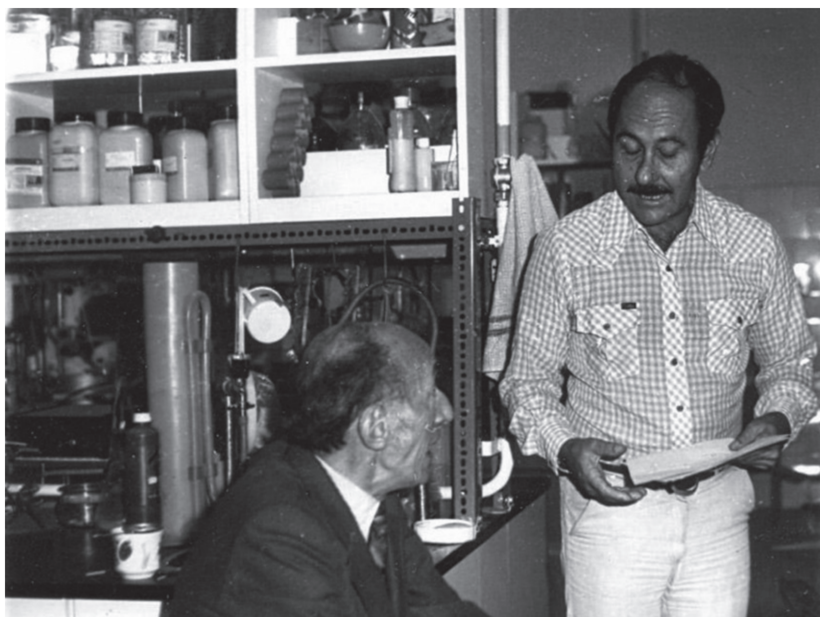
first UV lightbox in the 1980-s utilized the filter from an UVIFOT, and the Department's first spectrofluorometer started its life as a light scattering instrument. Finally, in 1968 the Biochemistry Research Unit became the Department of Biochemistry, independent of the Department of Genetics, and moved into spacious premises on the ground-floor and the basement of a university building at 3 Pushkin Street. The long-awaited event was enthusiastically celebrated by faculty, staff, and students. 2018 is the 50st anniversary that Endre Bíró became the head of the newly established Department of Biochemistry; he held that post until his retirement in 1986.

Endre Bíró's research interest remained constant during his whole career. He deliberately followed the footsteps of Albert Szent-Györgyi by focusing on the biochemistry of muscle proteins. He, together with all his colleagues at the Department, pursued structure-function studies of myosin and actin. Professor Bíró had a good judgement to choose the most dedicated students and postdocs to join his group and expand his experimental ideas. He gave great independence to his associates, and never added his name to the author list of publications where he was not directly involved in the project (same attitude as that of Albert Szent-Györgyi).

The scope of the present article does not allow to list the papers of all his colleagues, András Mühlrad, Miklós Bálint, György Hegyi, Gabriella Kelemen, László Szilágyi, Ferenc Fábián, Katalin Pintér, Ágnes Jancsó, Katalin Ajtai, Gábor Mócz; a few highlights of their results follow.

Experiments in the '60s mostly dealt with various aspects, e.g. metal ion dependence, of the ATPase of myofibrils, myosin and meromyosins (meromyosins were first described by Andrew Szent-Györgyi and John Gergely, see the chapters on their lives), as well as the role of the bound nucleotide of actin [5-7].

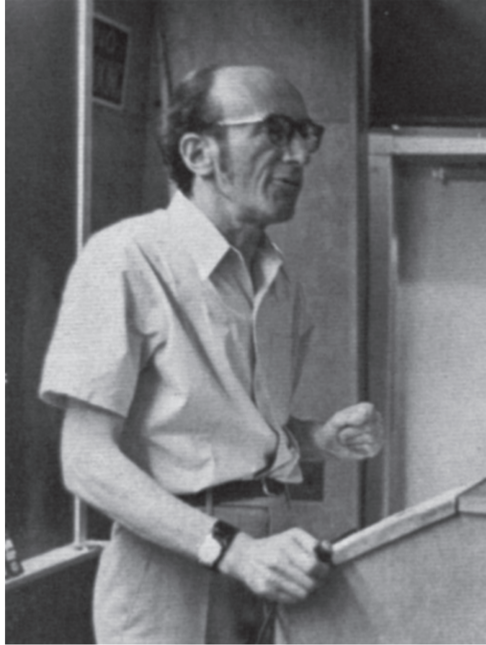
Of paramount importance were the systematic studies of the domain structure of myosin by limited proteolysis, initiated by Endre Bíró in 1964 [8, 9]. From the early 70s, a team led by Mikós Bálint published about 25 papers describing the „functional anatomy" of myosin: the relative position of the fragments and



Endre Bíró *and* **György Hegyi** *in the Pushkin Street laboratory (ca 1980).*

identification of their function [10]. László Szilágyi and György Hegyi used selective chemical modification to map the sites in actin that are involved in protein-protein interactions, and identified the nucleotide binding site by photolabeling [11]. All these achievements made the Bíró school highly recognized in the international muscle community. Even by today's strict standards, the output from Bíró's Department of Biochemistry contributed a great deal to the good reputation of Hungarian biochemists.

Certainly one of the high points in Bíró's scientific career was his attendance of the famous 1972 Cold Spring Harbor Symposium on Quantitative Biology: *The Mechanism of Muscle Contraction*, organized by James Watson. It was here that Endre Bíró met again Albert Szent-Györgyi, after 25 years of separation. At this meeting Bíró presented results showing for the first time that all helical tail fragments of myosin can be fully renatured and reassociated to two-stranded coiled coils [12, 13]. Excerpt from the publication (with co-authors László Szilágyi and Miklós Bálint): [12]



Endre Bíró *giving a talk at the Cold Spring Harbor Symposia in 1972 (courtesy of CSHL).*

> *"The functional role of the long, helical "tail" of the myosin molecule, however, can not be regarded as secondary. Aggregation of the myosin molecules to form the thick filaments secures the exact geometry of the heads relative to the thin filaments and thereby the transfer and "integration" of the mechanical force generated in the elementary cycles. This aggregation is based principally or perhaps uniquely on structural properties of the tail segment. Proteolytic fragmentation derives its interest from the fact that by this means one can make a sort of "anatomy" of this part of the molecule…"*

He also attended the first Alpbach Muscle Meeting in 1974, organized by Ken Holmes and Hugh Huxley, in the outstandingly beautiful Tirolean village. Holmes recalls the presence of Bíró at the conference:

"(The meeting) took place in an insalubrious cellar room that was used by the local brass band for rehearsals. Somewhat to our surprise everyone with a name in muscle research turned up. Fumio Oosawa and Setsuro Ebashi came from Japan. Endre (NA) Biro from Budapest chain-smoked at the back…"

Some of the scientists who once worked in Bíró's Department of Biochemistry have pursued muscle research till this day, and have gone on to distinguished careers in Hungary or abroad. András Mühlrád immigrated to Israel in the early 70s, worked there and at the University of California, and is currently professor emeritus at Hebrew University, with over 150 publications to his name. Over the years he collaborated extensively with former colleagues Katalin Ajtai and György Hegyi. Katalin Ajtai has been a researcher at the Mayo Clinic in Rochester, Minnesota since 1988. With Mühlrád they showed the role of reactive lysine residue, located at the interface of the catalytic and lever arm domains, in myosin activity [14].

"It is good to remember the days when as a beginner researcher I enthusiastically absorbed the knowledge that Professors Bíró and Mühlrád attempted to transfer from the school of Albert Szent-Györgyi to the new generation of muscle biochemists. I believe that this is how I got started on a lifelong career in research, and I managed to combine structural and functional approaches and apply them together when introducing new experimental methods."[4]

It is no exaggeration to say that the overall atmosphere and the working relationship between the people in Bíró's department were exceptional. His department felt like an intellectual haven in the 80's "socialist" Hungary. There has been a general consensus that this was the reflection of the personality of Endre Bíró. He was low-key, open-minded, honest, and wise. He never rode any

4 K. Ajtai, personal communication

political wave, and never offended anybody intentionally. („The problem with your Department is that moral standards there are too high"- said once a fellow scientist.) He led by example. He inspired respect for his knowledge of science well beyond his own field – the librarian could not hide her surprise when the professor asked to borrow a book on fractals.

He never raised his voice, but simply by his presence brought forth „the better angels of our nature". Here is Andras Mühlrad's recollection [4]:

> *"I have never known a researcher who was so much exempt from vanity or narrow-minded personal ambition. Bíró was always ready to help his students and colleagues with good advice. …His contact with people was characterized by the fact that he naively always assumed the best of all the people he came into contact with. Therefore, he hardly had any enemies. This is something rare in science where so much moral frailty can be observed even amongst the best researchers."*

Professor Bíró believed that spending a year in a Western laboratory („under civilized conditions", as he put it) was part of a scientist's proper training. Almost uniquely in those years (mid '70s and '80s) in Hungary the associates of "Bíró Prof" (as we colloquially called him), one after the other, went to the US. The generous host, who supported the visiting scholars at the Boston Biomedical Research Institute was John Gergely, another scientist from Albert Szent-Györgyi's group (see the chapter on his life). Interestingly, this was not a one way collaboration; a research scientist from BBRI, a Hungarian expatriate who left the country after 1956, Ferenc Sréter (1922-2012), spent a year at Bíró's Department in the '70s. The atmosphere at the Department of Biochemistry and the respect for Bíró had a major role in the fact that all of our colleagues who spent a year in the US between the mid-seventies and eighties returned to Hungary. Friendships were born that have lasted for many decades, often across borders.

It became a departmental tradition to have a New Year's Eve party on the last payday of the year, sometimes in the lab, later at somebody's house. It was on that occasion that Professor Bíró introduced his colleagues to the 12-year old kosher plum brandy, and eventually revealed the location of the store that carried it.

Besides research, teaching biochemistry to biology, and later also to chemistry and biophysics majors was a shared task in the Biochemistry Department. Bíró enjoyed working with smart students (he got along particularly well with the biophysicists), took lecturing very seriously, and occasionally listened to his associates' lectures and provided advice. We have to mention another "first in Hungary". He was the editor of the first really modern Biochemistry textbook for university students that was published in Hungarian [15].

Bíró received an Award from the Hungarian Academy of Science in 1977 and the State Order from the Presidential Council in 1988. He passed away in 1988 from complication of emphysema that was likely caused by his heavy smoking.

Endre Bíró *hands over the "baton" to* **László Gráf** *at a party on the occasion of his retirement in 1986.*

After his retirement László Gráf took over as Chairman of the Department of Biochemistry. Gráf used to work as a post-graduate student with Endre Bíró in the 60's studying the proteolytic fragments of the myosin rod. He returned to his alma mater after a successful career in the pharmaceutical industry and having spent years at UCSF in William Rutter's laboratory learning about protein engineering (he was also involved in the cloning of the insulin receptor there). After Gráf's term, László Nyitray followed as head of the Department. I also came from the school of Bíró, though indirectly; my thesis advisor was Miklós Bálint, who worked under Endre Bíró. Muscle protein studies remained at the forefront of research in the department, with newer and newer generations investigating the molecular basis of cell motility (our interest now goes beyond the main components of skeletal muscle, it includes non-muscle myosins and other motor proteins, like DNA helicases). Currently, László Nyitray, András Málnás-Csizmadia, and Mihály Kovács (former students of László Nyitray), György Hegyi, who is still doing active research, and many talented students successfully pursue research in this area, acknowledged by a recently received Academy Award from the

Student laboratory at Eötvös Loránd University named in honor of **Endre Bíró** *(inaugurated in 2008).*

Hungarian Academy of Sciences for their internationally recognized results in structure-function studies of motor proteins. We are proud to follow the footsteps of Endre Bíró, who followed the footsteps of Albert Szent-Györgyi in Budapest.

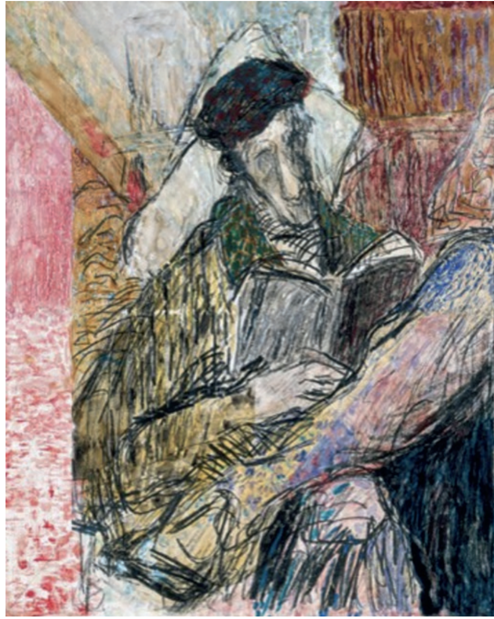
At the 40th anniversary of the foundation of Department of Biochemistry at Eötvös Loránd University, a student laboratory was named in honor of Endre Bíró.

Endre Bíró outside the laboratory

Endre Bíró met Ilka Gedő (1921-1985), a young painter and graphics artist, on New Year's Eve of 1945, and they got married next year. Mihály Bárány (see the chapter on his life) remembers the time he met Endre Bíró and her fiancée in the Szent-Györgyi lab [4]:

> *"Endre Bíró (Zebi) was Assistant Professor at that time and was a general favorite because he was friendly, unpretentious and enthusiastic. His painter fiancée visited him in the lab, while he was studying oxidative phosphorylation using kidney tissue slices. Ilka liked the way the Warburg flasks were swaying left and right, she found it amusing. I immediately recognized that if they get married they would remain poor. And indeed, when later I was invited to their apartment it was not easy to sit down since there were not enough chairs."*

(Many, many years later, their home was a great chaos of myriads of books on science and arts, all kinds of artifacts and artworks all around the rooms, but remained sparsely furnished.) Ilka Gedő was a highly talented artist and they lived a life together in harmony where the two cultures became one. They had two children, Daniel and David. "Ilka Gedő is one of the solitary masters of Hungarian art. She is bound to neither the avant-garde nor any traditional trends. Her matchless creative



Ilka Gedő: *The Reading Man (Portrait of E.B.), 1983*
(private collection, ©The Estate of Ilka Gedő).

method makes it impossible to compare her with other artists."[5] Today Ilka Gedő is an internationally highly acclaimed artist, with works in major museums throughout the world, but her recognition came only after her death in 1985. Endre Bíró, who himself was an expert in arts and art theory, kept fighting for her recognition in his modest way. He lived to see this happen: in 1987 he was present at the opening of Ilka Gedő's the first retrospective exhibition at Hungary's largest exhibition hall (Hall of Art, "Műcsarnok").

Endre Bíró's interests were not restricted to science and his wife's paintings. He was a member of a circle of artists, scientists and scholars that emerged around philosopher Lajos Szabó (1919-1988), who founded the Budapest School of the Philosophy of

5 In: István Hajdu - Dávid Bíró *The Art of Ilka Gedő (1921-1985) Oeuvre Catalogue and Documents*, Gondolat Kiadó, Budapest, 2003

Endre Bíró, *the founding Professor of the Department of Biochemistry at Eötvös Loránd University, Budapest (***Margit Gráber'***s*[6] *painting, which hangs in the library of the department.)*

Dialogue. This was a sort of open school of intellectuals with a multi-disciplinary approach (cf. the "open laboratory" concept of Albert Szent-Györgyi, and the openness of Bíró's department). Endre Bíró describes the circle around Lajos Szabó as a counter-cultural intellectuals:

6 Margit Gráber (1895-1993), an acclaimed Hungarian painter, the aunt of Endre Bíró

> *"This circle was a subculture, indeed I am often inclined to name it a sect. But maybe this is an exaggeration…Anyhow, the really talented intellectuals found the means for getting rid of the shackles of bourgeois morality"*[7]

Endre Bíró himself was a notable James Joyce scholar. He translated parts of the "non-translatable novel" Finnegans Wake. The translation with the accompanying scholarly essay first appeared in print in the former Yugoslavia, in the Hungarian literary monthly, Híd (Bridge) in 1964, then much later in Hungary, after the collapse of the old political system, in 1991. It was an impressive achievement to invent a Hungarian vocabulary for Joyce's idiosyncratic language (we do not dare to "re-translate" into English Bíró's masterpiece word inventions). According to literary experts, his translation had a significant impact on post-modern Hungarian prose.

Acknowledgements

We are thankful to Dávid Bíró for allowing us to use photos owned by the family and also for the written information on the life of Endre Bíró (*My Version of the Story (The Story of a Hungarian-Jewish Family)*. We are also indebted to him for reading the manuscript. Further thanks go to László Gráf, for writing an earlier biography on Endre Bíró in Hungarian.

7 Biographical interview with Endre Bíró, quoted in Dávid Bíró: Ilka Gedő – The Painter and Her Work (A Background Report), Budapest, 2014

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8 Endre Bíró's most important publications are highlighted in bold.

Eugene (Jenő) Ernst — The man behind „Little Heidelberg"
(1895-1981)

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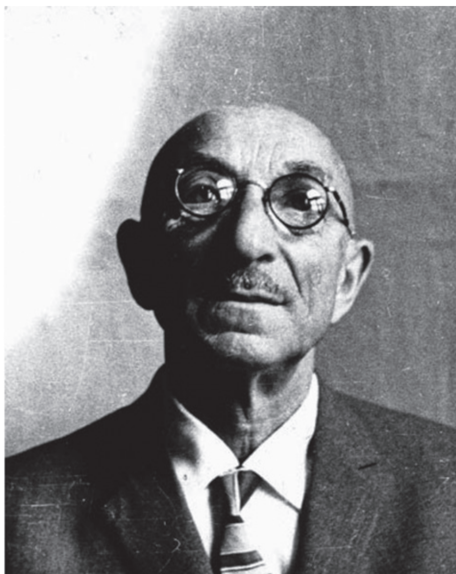


Eugene (Jenő) Ernst *in cca. 1970*
(photos courtesy of László Kutas and the University of Pécs photography collection).

I (MSZK) must have been about six years old when I first met him. It was a sunny summer Sunday morning as my father and I were waiting in the bus stop in front or our house in Pécs, Hungary. Suddenly, a funny-looking man approached us. He was short, bald, was wearing thick glasses and ridiculous Tyrolean shorts. His piercing eyes nearly frightened me. But it was the

utmost respect and honor with which my father, a pathologist and cell biologist himself, was speaking to him that raised my attention and curiosity. Who was this man? Only years later had I learned how powerful a man was Eugene (Jenő) Ernst, and it took a much longer time to understand and appreciate his true character, intellectual integrity and remarkable contributions.

Eugene Ernst was probably the most powerful person at the Medical University of Pécs and a decisive figure of Hungarian science in the post-war era (i.e., after World War II). His active years spanned six decades from the 1920s to the 1970s. While his main scientific interest was muscle biophysics, he was also interested in a variety of other topics such as water and potassium ions in the cell, and various aspects of radiation biophysics and cybernetics. He founded one of the first Biophysics Departments in the world, and played pivotal roles in the establishment of the Hungarian Biophysical Society and IUPAB (International Union of Pure and Applied Biophysics). His wise background orchestration and diplomacy led to an unparalleled intellectual development and the recruitment of a whole generation of internationally renowned scientists at the Medical University of Pécs, so that the golden years of the 1950s and 1960s of this university are often referred to as the “Little Heidelberg”. The heritage of Eugene Ernst prevails in the form of several prizes and fellowships named after him and maintained by the Hungarian Academy of Sciences and the Hungarian Biophysical Society.

Biography

Eugene Ernst was born on April 16, 1895 in the town of Baja in the county of Bács-Bodrog of greater Hungary as the eighth child of a poor merchant family. Although Jewish by birth, he attended the famous local Roman Catholic Cistercian secondary school from where he graduated with the highest marks in 1913. His exceptional talent towards mathematics and natural sciences was already noticed, as he won several awards

with his thesis works on various topics such as *"On integral calculus", "The Sun, the fountain of life", "Radium and the transformation of the elements"*, and *"Application of the laws of Archimedes"* (1). He entered the University of Budapest in 1913, but with the breakout of World War I he volunteered for service and fought on the eastern front. In 1915 he was captured by the Russian army and spent five years in Siberia as prisoner of war. It was only in 1920 when he could return to Hungary and continue his university studies by enrolling into Elisabeth University (named after "Sissi") which was relocated from Pozsony (Pressburg, today Bratislava) to Budapest following the war. In 1923 Elisabeth University was moved to the city of Pécs in southwest Hungary, and Eugene Ernst moved with it. He graduated here as an MD in 1924. Already in 1923 he started working as a student trainee in the Department of Pharmacology under the supervision of the famous Professor Géza Mansfeld, who was later nominated twice for the Nobel-prize in Physiology or Medicine, in 1928 and 1940. In 1924 Eugene Ernst joined the University of Pécs

Main building of the Elisabeth University (later University of Pécs) on a 1923 postcard. The laboratories and living quarters of Eugene Ernst were on the building's first floor, behind the third, arched window right of the corner facing us. Ernst essentially spent his life here until his death in 1981.

to stay at his *alma mater* for the next 57 years to come, until his death in 1981.

The nearly six decades that Eugene Ernst actively spent at the University of Pécs, the tumultous years of World War II notwithstanding, draw out a remarkable, productive scientific career that carved the course of Hungarian science for subsequent generations. Already in 1928 he habilitated to become a "private docent" by defending his thesis entitled *"Physical elements of organ function"*. Besides his private docent function he began working as an assistant professor in the Department of Medical Physics in 1932, and in the Department of Medical Chemistry in 1938. In 1935 he became extraordinary professor. In 1939, upon the announcement of the second *Numerus Clausus* that limited the participation of jews in economic and public life, Eugene Ernst was forced to give up his position at the University of Pécs. It was Albert Szent-Györgyi who offered him a position in these hard times and invited Ernst to work in his laboratory at the University of Szeged. This period lasted until 1943. In 1944 he was stranded in the ghetto of Pécs and was taken to work camp from where he escaped later that year. After the end of World War II, Eugene Ernst was appointed as lecturer at the University of Pécs, and in 1945 became full professor and chairman of the Department of Medical Physics. Even though in 1951 the Faculty of Medicine became an independent university under the name *"Medical University of Pécs"* (a status that lasted until 2000), and his own department was later changed to the Department of Biophysics. Ernst continued to direct the department for the following 26 years, until 1971.

Eugene Ernst was the recipient of a large number of different prizes and awards, and the number of prizes he declined was probably even greater. He was elected member of the Hungarian Academy of Sciences in 1946 and in 1959 the head of the Academy's Biology Section. He received the prestigious Kossuth-prize twice in 1948 and 1956, and was the recipient of the highest award of the Hungarian Academy of Sciences, the Academic Golden Medal in 1971. In 1980 he became Doctor Honoris

János Szentágothai *(left) and* **Eugene Ernst** *(right) during the Doctor Honoris Causa inauguration ceremony at the Medical University of Pécs, in 1980.*

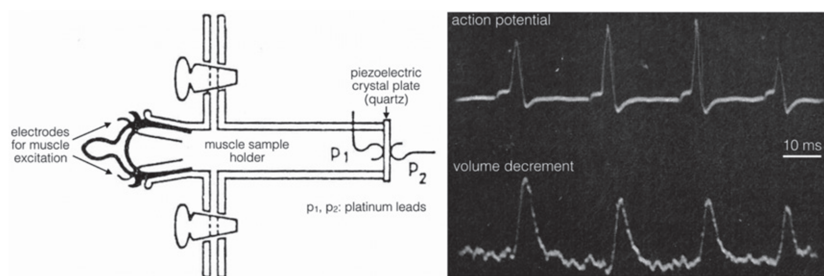
Causa of the Medical University of Pécs together with the neuroanatomist János Szentágothai.

Contributions to science, muscle research and Hungarian academic life

In the broad sense Eugene Ernst was interested in uncovering the physical principles behind biological and physiological processes. Accordingly, he investigated numerous phenomena including biological transport processes, diffusion, thermodiffusion, thermoosmosis, ion exchange, bound water and potassium, biological semiconductivity, cybernetics and radioactivity. But his favorite and most studied topic was by far the biophysics of muscle contraction. Of the nearly 160 original papers he published in his life more than 60 are on muscle from a variety of aspects. He explored the structure, mechanics, electrical and metabolic activity, and volume changes of striated muscle with the most modern methods of the time, and he even devised and constructed instrumentation himself to achieve measurements of the highest precision.

In the 1920s, the most accepted view on the mechanisms behind muscle contraction was the so-called *"lactic acid theory"* put forth by Otto Fritz Meyerhof (Nobel-prize in physiology or medicine, 1922). According to this theory, the mechanical response of muscle, seen as very small volume changes on the order of a few tens of nanoliters for the frog muscle specimens studied, is of metabolic origin related to the build-up of lactic acid. A fierce argument between Meyerhof and Ernst took place on the pages of the prestigious physiology journal *Pflügers Archiv (Pflügers Archiv für die gesamte Physiologie des Menschen und der Tiere)*, which went on for a number of years. Ernst took the effort to build ingenious instrumentation (2) to carry out precise time-correlated measurements of electrical excitation and volume changes and has shown that the volume decrement during contraction is the result of bioelectric processes (3). By the 1930s the case was settled and even Meyerhof acknowledged Ernst's convincing results and arguments (4). The muscle contraction-related volume decrement became known as the *"Ernst effect"*, which brought him international fame.

Eugene Ernst believed in the continuity of the filamentous elements of muscle, and in order to explore the fine structure of striated muscle he promoted the development of electron microscopy instrumentation and projects (see chapter on Miklós Garamvölgyi). Ernst was convinced that the molecular mechanisms behind muscle contraction are somehow related to the crystallization of the muscle proteins, much like how rubber-like



Muscle volume-measuring apparatus designed and constructed by Eugene Ernst (left) and the oscilloscope trace of the action potential and volume decrement measured (right).

polymer strands become structurally oriented when stretched. In retrospect, there is a great deal of ingenious thought behind this idea. In 1958 Eugene Ernst published an important, single-authored book (in German) entitled *"Die Muskeltätigkeit"* (second extended edition*:"Biophysics of the Striated Muscle"* in 1963), which was a comprehensive, state-of-the-art collection and discussion of experimental results on the structure and mechanics of muscle (5). In 1973 he wrote a survey of muscle biophysics as Congress report (6).

Eugene Ernst's efforts stretched much beyond the everyday laboratory experiments. He began an insightful and systematic development of his department, then his university, then the national and international biophysics community. In 1947 he established the first Biophysics Department of Hungary, which was the fourth such department globally. It was in this depart-

Eugene Ernst *with* **Arthur K. Solomon** *of Harvard Medical School, the first secretary general of the International Union for Pure and Applied Biophysics (IUPAB), in 1964 on the top-floor terrace of the then newly-built clinic of the Medical University of Pécs.*

ment where Hungary's first biological radioisotope laboratory was established. Ernst was instrumental in the establishment of the Hungarian Biophysical Society in 1961 of which he became the first president. The mission of the Society is the promotion of the research and higher education of biophysics nationally and internationally. Ernst reached out towards the international biophysics community as well and was instrumental in the establishment of IUPAB, the International Union of Pure and Applied Biophysics. Immediately following the II. IUPAB Congress (Vienna, 1966) Ernst organized a "*Symposium on Muscle*" in Budapest, which hasd drawn the prime muscle researchers of the time from around the world (7). József Tigyi, one of the most faithful students of Ernst and his successor as chairman of department, later became the secretary general of IUPAB (1984 -1993).

Probably one of the main, albeit informal, achievements of Eugene Ernst towards the improvement of Hungarian scientific life was the promotion of the utmost excellence at the Medical University of Pécs by means of recruiting faculty of the highest international renown. By using his network of connections that reached all the way to the top of the governmental and communist party establishment, he made every effort to reach out to, invite, and even protect those professors whom he thought of as being most talented and productive. Only intellectual quality mattered, and he was able to set aside the political priorities so common at the time. As a result, by the mid 1950s essentially all the departments, including the clinical ones, at the Medical University of Pécs were studded with the most remarkable professors who set the stage for medical training and research of the highest quality. Just to name a few: János Szentágothai (Anatomy), Kálmán Lissák (Physiology), György Romhányi (Pathology), Miklós Melczer (Dermatology), Gyula Méhes (Pharmacology), Ödön Kerpel-Fronius (Pediatrics), István Környey (Neurology), Tihamér Karlinger (Surgery), László Cholnoky (Medical Chemistry), Szilárd Donhoffer (Pathophysiology). The faculty of the Medical University of Pécs was looked up to and respected by the entire nation. These years are often remembered with sentiment as being the golden years, during which the Medical

Eugene Ernst *(middle) in the company of* **Albert Szent-Györgyi** *(front) and* **Zoltán Bay** *(back; he was the first to measure the earth-moon distance by using radio waves) at the press conference on the occasion of Szent-Györgyi's visit to Hungary in 1973. (photo: MTI).*

University of Pécs was called the "Little Heidelberg" of Hungary. Although noone spoke openly about it, everyone new that it was Eugene Ernst who was most influential in achieving this feat. Eugene Ernst stayed near the hub of Hungarian science even after retiring from the chairman position of the Department of Biophysics in 1971. In 1973, when Albert Szent-Györgyi returned from the United States after many years of self-imposed exile, Ernst was among the first to reach out towards him.

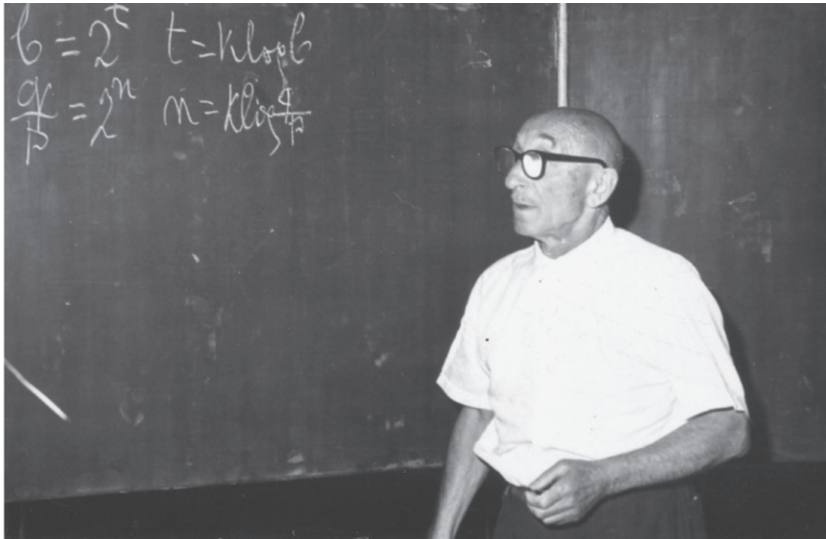
Most of the publications of Eugene Ernst were written in German and Hungarian, and only later in his life had he begun publishing in English. Apart from his more than 160 original papers he was the author and editor of several books. Generations of future biophysicists and physicians studied from his books *"In-*

troduction to Biophysics” and *“Biophysics”* (8). Together with Brunó Straub he was the chief editor of the periodical *“Acta Biochimica et Biophysica Hungarica”* (9).

The person beyond the scientist

Eugene Ernst was a devout believer of scientific truth and often declared: “I have facts, not fiction”. Although he lived through the most difficult times of the XX. century, his personal life was very reserved, puritanic, simple and strict. He lived almost like a monk in the room attached to his laboratory. Next to his plain bed the wall was covered with wrapping paper on which he wrote and drew his thoughts and ideas. He rose every day at 4:30 AM and spent the dawn hours reading and studying. He was punctually present in the lab by 8 AM, and allowed himself only a short noontime hour for relaxing. He walked a few miles every day and was in bed by 10 PM. The unimaginable hardships he must have witnessed in two world wars taught him to be humble and sparing. He knew hunger and poverty all too well. Thus, he knew very well that a student wandering in the hallway around noon is too poor for buying lunch. For them he kept an open petty cash account with his secretary. Since he was convinced that it was the communists who protected him from the Nazi horrors during World War II, he entertained communist ideals. However, unlike many in the 1950s Hungary, he never used his political connections for personal advantage. On the contrary, he often protected talented but politically troubled peers from being prosecuted.

Ernst entertained highly unusual approaches and ideas about many aspects of life. During exams he often tested the students’ associative and problem-solving abilities with simple, sometimes funny questions. An example goes like this: “A bear walks one mile south, then one mile west, then one mile north and finds itself at the starting point. What color is the bear?”



Eugene Ernst *lecturing. Medical University of Pécs, 1973.*

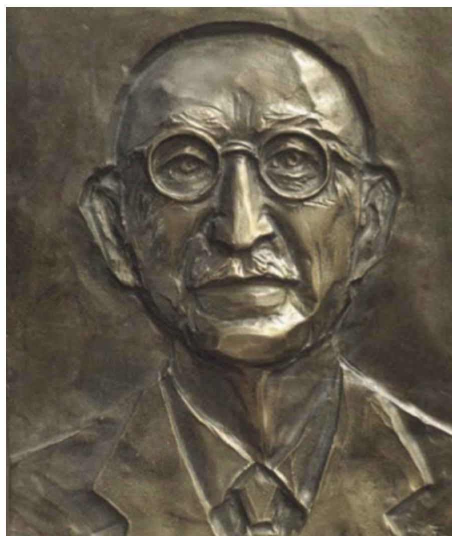
Although sparing with life's riches, one thing Ernst liked very much was Hungarian fruit brandy (so called *"pálinka"*). In fact, he himself distilled some of the strongest and finest plum brandy from the fruit harvested in his garden. No guest could leave Ernst's room without having tasted his plum brandy and having compared the different years' brands. Eugene Ernst never married and considered his students and colleagues his family. Certainly, everyone was always warmly welcome in his spartan room.

Heritage

Having lived a monk's life, Eugene Ernst hardly spent on himself. In his will he left all his savings on the Hungarian Academy of Sciences with the intention that the interests of the capital be spent on serving biophysics and helping young, talented scientists. In 1987 the Hungarian Academy of Sciences established three prizes from Ernst's inheritance: the *Ernst*

plaque to be awarded bi-annually to someone with outstanding career contributions to the research, development and education of biophysics; the *Ernst fellowship* to be awarded bi-annually to maximum six biophysicists under the age of 35 for their thesis works; the *Ernst-prize* to be awarded bi-annually to a maximum of two researchers who produced outstanding results in the field of biophysics. The awards are given out at the Congress of the Hungarian Biophysical Society, organized every two years. Professor Ernst was buried in the Central Cemetary of Pécs, and in 2005 his grave was declared a protected site by the National Committee on Memorial Sites. In the same year a bronze relief commemorating Eugene Ernst was installed and inaugurated within the Hall of Fame of Professors in the aula of the Medical Faculty at the University of Pécs.

1986 marked the fifth anniversary of Eugene Ernst's death. My father, who very much respected Eugene Ernst and in many ways considered himself Ernst's intellectual disciple, initiated the organization of a memorial symposium for Ernst. With the help of József Tigyi, professor of the Department of Biophysics at the



Bronze relief of **Eugene Ernst** *in the aula of the Medical Faculty at the University of Pécs. Sculptor: György Fábos, installed 2005.*

Eugene Ernst Memorial Symposium, July 3-5, 1986. First row: **József Tigyi** *(Department of Biophysics, Medical University of Pécs),* **Gilbert N. Ling** *(Cancer Research Foundation, FONAR Corporation),* **Mary Osborn** *(Max Planck Institute for Biophysical Chemistry, Göttingen Germany),* **Mrs. R. Lenk, Carlton F. Hazlewood** *(Department of Physiology, Baylor College of Medicine, USA),* **Ivan L. Cameron** *(Department of Cellular and Structural Biology, University of Texas, San Antonio, TX USA). Second row:* **Miklós Kellermayer Sr** *(Department of Clinical Chemistry, Medical University of Pécs),* **Ludwig Edelmann** *(Medizinische Biologie, Universität das Saarlandes, Homburg Germany),* **Zoltán Hummel** *(Department of Biohysics, Medical University of Pécs),* **Gerald H. Pollack** *(Center for Bioengineering, University of Washington, Seattle, WA USA),* **James S. Clegg** *(Bodega Marine Laboratory, Bodega Bay, CA USA),* **Rudolf Lenk** *(Laboratoire de Physiologie Végétale, Université Genéve, Geneve Switzerland),* **Árpád Mátrai** *(Department of Nuclear Medicine, Medical University of Pécs). Third row:* **Lajos Keszthelyi** *(Szeged Biological Center),* **Kázmér Jobst** *(Department of Clinical Chemistry, Medical University of Pécs),* **Béla Török** *(Central Laboratory, Medical University of Pécs),* **Gábor Bíró** *(Department of Biophysics, Medical University of Pécs),* **István Szalay** *(University of Pécs),* **Tamás Kőszegi** *(Department of Clinical Chemistry, Medical University of Pécs).*

University of Pécs and successor of Eugene Ernst as chairman, and Carlton F. Hazlewood from Baylor College of Medicine who was a follower of Ernst's ideas on bound water and potassium in the living cell, the Eugene Ernst Memorial Symposium took off on July 3, 1986.

As a third-year medical student, I naturally gravitated towards this fascinating symposium, and the buzzing intellectual activity profoundly affected me. Every talk of the symposium was recorded on tape, and I took on the task of transcribing the sound records onto paper (10). Thus, I digested every word spoken at the conference. It was then, when all of a sudden I found myself face-to-face with Eugene Ernst and his intellectual heritage. Albert Szent-Györgyi, long-time friend of Ernst was also invited to the symposium. Although he was unable to attend due to lingering health conditions (in fact he passed away later that year), he addressed the symposium with his ever unique words. Nothing can summarize better Eugene Ernst's character than Szent-Györgyi's words:

> *„Dr. Eugene Ernst was a very dear friend of mine and I am unable to accept in my mind really his death. His opinions and feelings were always so very vivid that it is difficult to believe that they will ever end. We often differed in our opinions which made the bond between us still stronger. The essential point of our opinions being always that science is a search for truth, and truth is the only thing that matters. I was fortunate enough that I was brought in near contact with him repeatedly and even had an opportunity to contribute to his safety during the disastrous Nazi period of Hungary under German occupation. He was a wonderful friend with a very deep understanding for everyone's opinion, though his own beliefs were often at variance with those of the majority. If science is a search for truth, then he was a real apostle of scientific knowledge."*

Some of Eugene Ernst's notable publications

Untersuchungen über Muskelkontraktion. 1–9. (Pflüglers Archiv, 1925–1929)
Osmosis in Physics and Biology. (Nature, 1938)
The State of Potassium in Muscle Investigated by High Frequency. (Acta Physiologica, 1962)
Biophysics of the Striated Muscle. (Bp., 1963)
Facts, Implications and Perspectives. (Bp., 1968)
Biology without Mysticism. A Biophysicist Reflections. (Bp., 1972)

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10. J. Tigyi – M. Kellermayer – C. F. Hazlewood: The Physical Aspect of the Living Cell. *Proceedings of the Eugene Ernst Memorial Symposium (1986. July 3-5. Pécs, Hungary)* Akadémiai Kiadó, Bp. (1991)

Miklós Garamvölgyi — Specialist of muscle electron microscopy
(1932-1980)

Miklós S.Z. Kellermayer
Semmelweis University

Miklós Garamvölgyi *in cca 1955*
(Source: Almanach of the University of Pécs).

In the recent historical account about the discovery of titin filaments written by Cris dos Remedios and Darcy Gilmour (Remedios and Gilmour 2017), a reference is given to a 1964 paper by a certain Nikoli Garamvölgyi, who described the presence of slender filaments extending from the tip of the myosin thick filament to the Z disc in insect flight muscle (N. Garamvölgyi, Kerner, and Cser-Schultz 1964). The existence of these filaments, called C filaments, was a matter of controversy in the 1960s, but it later turned out that they are not an experimental artifact at all but are formed of giant elastic proteins related to titin. Who

was this Nikoli Garamvölgyi, who published the remarkable electron micrographs on C filaments? His real, Hungarian name was Miklós Garamvölgyi, and he was a talented muscle biophysist who happened to publish most of his papers under the name „N. Garamvölgyi". I first encountered his name in discussions with my late mentor József Belágyi (see biographical chapter on József Belágyi elsewhere in this volume), during general discussions on muscle and particularly on muscle ultrastructure. Belágyi considered Miklós Garamvölgyi one of the most outstanding muscle biophysicists and skilled electron microscopists. He was a most noted and respected member of a great generation of Hungarian muscle researchers. Although his short life was full of turmoil and, against his will and plan, he was unable to strengthen his own independent research group, his contributions to the understanding of the structure and function of muscle continue to prevail.

Biography

Miklós Garamvölgyi was born in Doboz, a small city in southeast Hungary near the Romanian border. He was educated in schools in Doboz, Novi Sad (Serbia) and Budapest. He graduated from the Eötvös Loránd University in Budapest in 1954 with a biology-chemistry teacher diploma. Garamvölgyi joined the research group of Eugene Ernst (see the biographical chapter on Eugene Ernst elsewhere in this volume) in the Department of Biophysics at the Medical University of Pécs in 1954. In the group of Eugene Ernst the main focus of Garamvölgyi's research was the structure, function and mechanics of striated muscle, a topic that he pursued with maximalism to the end of his life. In 1961 he defended his CSc ("candidate of sciences") degree at the Hungarian Academy of Sciences. The opponents (reviewers) of his CSc thesis were Ferenc Guba from the University of Szeged (see the biographical chapter on Ferenc Guba elsewhere in this volume) and György Romhányi, chairman of the Pathology Department at the Medical University of Pécs. With the support of

Some of the members of the muscle research group at the Department of Biophysics, Medical University of Pécs in cca 1958. Front row, assistants. Back row, from left to right: **Miklós Garamvölgyi, Antal Niedetzky, Ferenc Vető**. *(Photo courtesy of László Kutas)*

local fellowships, Garamvölgyi was able to take part in collaborative research abroad in Czechoslovakia (1964), France (1966) and Great Britain (1967). In 1966 he defended his doctor of sciences (DSc) degree, also at the Hungarian Academy of Sciences, under the title "Structure of striated muscle from a functional aspect". The opponents of his DSc thesis were János Szentágothai, the famous neuroanatomist, chairman of the Anatomy Department at Semmelweis University in Budapest, Ilona Banga the noted muscle biochemist (see biographical chapter on Ilona Banga elsewhere in this volume) and Ferenc Guba. In 1968, for disputed reasons, Garamvölgyi gave up his position at the Medical University of Pécs and transferred to Budapest, to the Department of Pathological Anatomy at the Postgraduate Medical School ("Orvostovábbképző Intézet"). He led the electron microscopy laboratory in this department. In 1969 he became associate professor. In 1973 Garamvölgyi changed appointment again and joined the Research Institute of the Hungarian College of Physi-

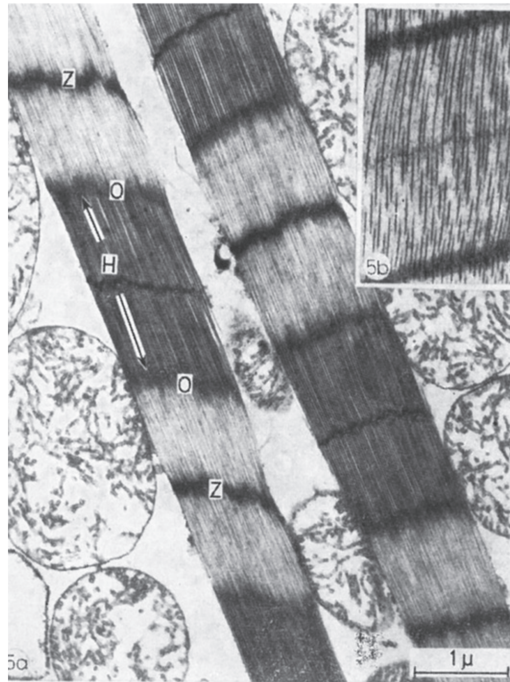
cal Education. Later he became full professor here, and this was his last appointment prior to his sudden death on April 15, 1980.

Miklós Garamvölgyi was a founding member of the Hungarian Biophysical Society. He was also the member of several thematic societies and committees at the Hungarian Academy of Sciences and the Scientific Council of Physical Education. Garamvölgyi was co-organizer (together with Endre Bíró) of the Third Conference of the European Muscle Society, held in Budapest in 1974.

Contributions to science and muscle research

Miklós Garamvölgyi was a remarkably talented experimentalist, and his papers reveal a pursuit of perfection. Besides mastering electron microscopy, a technique that became increasingly used to investigate biological problems in the late 1950s, he also skilfully applied light microscopic, micromanipulation and mechanical methods towards the understanding of the mechanisms of muscle contraction. He published about eighty papers, mostly under the name of N. Garamvölgyi. Some of his publications, particularly the ones that revealed, among the first, the presence of a mechanically continuous filament system in the sarcomere, are cited even today. He was an early proponent of the idea that the smallest unit of muscle contraction is the sarcomere. Even though he produced electron micrographs about the striated muscle of remarkable quality, he always stressed the relationship between structure and function. Thus, he did not investigate the fine structural detail of striated muscle *l'art pour l'art*, but he had a genuine interest in the fundamental understanding of the mechanisms behind its contraction.

In 1960, just six years after his arrival to the muscle research group of Eugene Ernst in the Department of Biophysics, Medical University of Pécs, Garamvölgyi wrote a short, concise review about the structure of striated muscle for the Biology Section of the Hungarian Academy of Sciences (M. Garamvölgyi

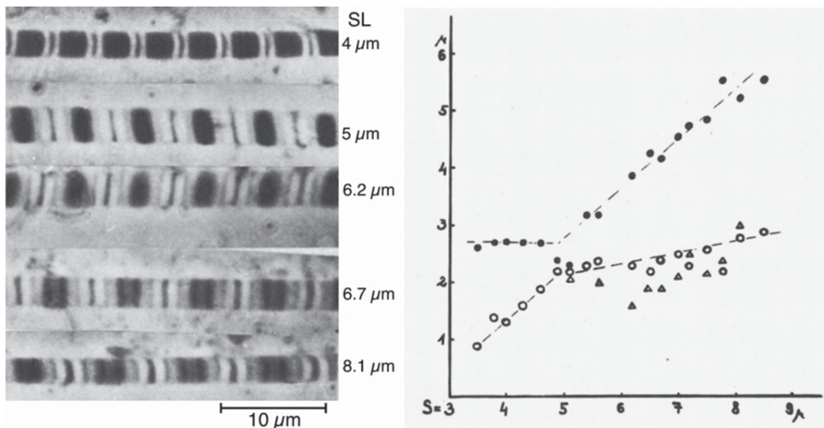


Example of a longitudinal electron microscopic section of honeybee flight muscle, prepared by **Miklós Garamvölgyi** *(N. Garamvölgyi 1972).*

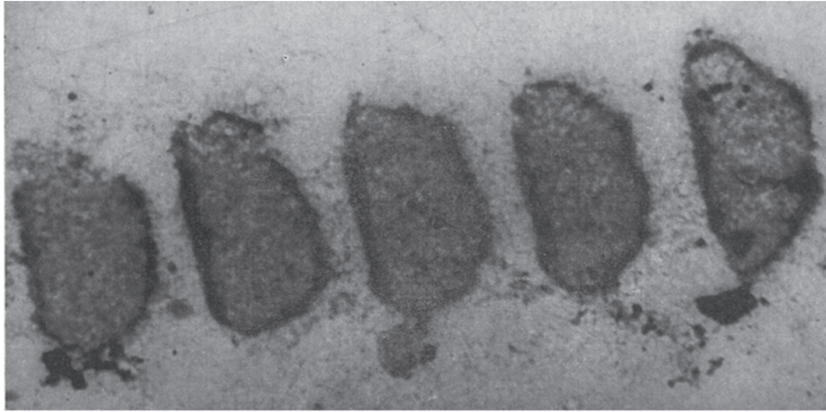
1960). This summary, which contained light (phase contrast) and electron microscopic data collected by Garamvölgyi himself, highlights his open-minded thinking about the mechanisms of contraction, and is a fresh reading even today. Garamvölgyi went on to manipulate single myofibrils from the honeybee flight muscle (N. Garamvölgyi 1959), and was able to record remarkable micrographs about highly stretched myofibrils, much beyond filament overlap (N. Garamvölgyi, Kerner, and Cser-Schultz 1964), coining the idea for the presence of a filamentous system that stretches along the sarcomere and maintains sarcomere integrity even beyond the point where actin and myosin filaments no longer interact.

Garamvölgyi was also interested in the structure and possible function of the Z- and M-lines. He made preparations in which everything except the Z-lines were removed (N. Garam-

Manipulation of a single myofibril from honeybee flight muscle (M. Garamvölgyi 1960).



Stretching honeybee flight muscle myofibrils beyond filament overlap. Left panel: phase contrast micrographs of myofibrils stretched to increasing sarcomere lengths (SL). Right panel: width of sarcomere bands as a function of sarcomere length. The „no overlap point" is at 5 μm sarcomere length. Filled circles refer to the sum of the A-band and the so called „E-band", a band of intermediate darkness at eigther edge of the A-band. Open circles refer to the I-band, and open triangles to the H-zone in the center of the A-band.



Z-disks of honeybee flight muscle prepared by treating the muscle with 0.1 % lactic acid (N. Garamvölgyi, Metzger-Török, and Tigyi-Sebes 1962).

Miklós Garamvölgyi *(front row) and* **József Belágyi** *(back) in the ring of assistants in the garden of the Medical University of Pécs in cca 1960. (Photo courtesy of László Kutas)*

völgyi, Metzger-Török, and Tigyi-Sebes 1962). Remarkably, the string of Z-disks displayed a continuous structure.

After moving to the Department of Pathological Anatomy at the Postgraduate Medical School in Budapest, Garamvölgyi collaborated with Elek S. Vizi and József Knoll in the Department of Pharmacology, Semmelweis University on the structure of smooth muscle. He was able to detect the presence of myosin thick filaments in guinea-pig vas deferens and ileal smooth mucle (Vizi, Garamvölgyi, and Knoll 1973; N. Garamvölgyi, Vizi, and Knoll 1973). After moving to the Research Institute of the Hungarian College of Physical Education in 1973, Garamvölgyi was involved in exploring the relationship between skeletal muscle structure, function and mechanics and their role in sports performance. Although he guided the thesis work of several students, his research and publishig activity declined.

The person behind the man

Miklós Garamvölgyi was a jovial, friendly and life-loving man. He was not only a determining figure of the scientific life of the Department of Biophysics at the Medical University of Pécs, but also a popular instructor of medical students. He was always ready for a joke. He married one of his assistants with whom they raised a daugther. In the early 1960s they purchased a house on the mountain slopes above the city of Pécs, as they became attached to this pleasant city. Apparently, Garamvölgyi was planning to build a career here. It is disputed what then made him decide to eventually transfer to Budapest. Some collegial jealousy may have arisen because of Garamvölgyi's scientific successes and rapid progression in the departmental hierarchy. Leadership issues of the electron microscope facility may have also played a role. In any case, in early 1968 Garamvölgyi was indicted to the local Communist Party committee. He was accused of a too relaxed treatment of his time sheet and tardiness at work. The political climate in these times was desperate, and every university in Hungary, among them the Medical University

Summer School in the Research Institute of the Hungarian College of Physical Education in the mid 1970s. From left to right: unidentified person, **Jenő Koltai,** *director of the Hungarian College of Physical Education,* **László Kutassi,** *director of the Research Institute, unidentified person,* **Miklós Garamvölgyi,** *and* **László Dobozy,** *research associate. (Photo from the archives of the Hungarian College of Physical Education)*

of Pécs particularly severely, was infiltrated with communist dogma, discipline and comrades. It is to the credit of Garamvölgyi that he was wise and courageous enough to boldly transfer to the Postgraduate Medical School in Budapest rather than argue and be humiliated in Pécs. Undoubtedly, however, the incident was detrimental for his subsequent life and career.

Miklós Garamvölgyi's favorite pastime was military history. He was not only knowledgeable about war history, but he collected military items, and he even painted militiary scenes. He had a vast collection of wartime artifacts and paintings. In the late 1970s he desperately fought alcohol problems, which eventually lead to his tragic, early death at the age of 48 years.

Acknowledgements

I am indebted to László Kutas (associate professor at the University of Pécs), József Tihanyi (professor emeritus of the Hungarian College of Physical Education) and László Molnár (director of the Archives of Semmelweis University) for information and photographs.

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John Gergely — Founder of the Boston school of muscle research
(1919-2013)

László Szilágyi, Ágnes Jancsó[1] and László Nyitray
Eötvös Loránd University, Budapest

John Gergely *(in Alpbach, ca. 1980, courtesy of Marcus Schaub).*

John (János) Gergely was born on May 15th, 1919 in Budapest and died in Danvers, Massachusetts on July 26th, 2013, at age 94.

Dr. Gergely received his medical degree in 1942 from the Medical School of Pázmány Péter University in Budapest. He started his scientific career in the Department of Pharmacology working with Béla Issekutz (1886-1979), a pioneer of modern

1 Currently in Madison, Wisconsin

experimental pharmacology in Hungary. He spent three years there until the fall of 1945 brought about important changes in his scientific outlook. He took a course at the Technical University given by Pál Gombás (1909-1971), an excellent theoretical physicist who was doing research on the application of quantum mechanics to complex systems. In a conversation with the professor, John mentioned his interest in the ideas outlined by Albert Szent-Györgyi in 1941 in *Science* [1]. Very briefly, in this paper Albert Szent-Györgyi suggested that full understanding of living systems requires not only molecular, but quantum mechanical studies as well. At that time Albert Szent-Györgyi, having moved from Szeged to Budapest, was the head of the Department of Biochemistry of Pázmány Péter University, and Professor Gombás introduced the talented young scientist to the Nobel laureate. John Gergely joined Szent-Györgyi's laboratory in January 1946. Shortly after this Albert Szent-Györgyi and Kolomon Laki arranged for John to visit Meredith G. Evans' laboratory at Leeds University in order to deepen his understanding of quantum chemistry. Evans used to work with Henry Eyring and Michael Polanyi, the founders of the transition state theory of chemical reactions. In Leeds, John Gergely registered as a graduate student, and received a PhD in Physical Chemistry by the end of 1948. The most important results of his studies were published in 1949, describing the first theoretical calculation for the energy bands in proteins [2].

In the late 1940 the political situation worsened in Hungary, there was a communist takeover, which caused Albert Szent-Györgyi to decide to leave the country. In 1947 he immigrated to the US. He settled in Woods Hole, and held a senior visiting fellowship at the NIH in Bethesda as well. In 1949, John Gergely together with his wife Nora Czoboly and, at that time, their two children moved to Bethesda. From there the family relocated to Wisconsin and John spent one year at the University of Wisconsin–Madison, in the Institute for Enzyme Research led by David Green, a biochemist who significantly contributed to the study of mitochondrial electron transport chain and oxidative phosphorylation. In the meantime, he received an American Heart

Association Established Investigatorship, which allowed him to take a research position in the Department of Neurology at Massachusetts General Hospital in 1950. He subsequently obtained a concurrent appointment as Associate Professor in the Department of Biological Chemistry and Molecular Pharmacology at Harvard Medical School. The Gergely family, which eventually included eight children, settled in Nahant, a small North Shore town, where John lived for the rest of his life. In 1961, an old friend and classmate from the Medical School in Budapest, Endre A Balazs (1920-2015) invited John Gergely to found the Department of Muscle Research in the Retina Foundation, established by the famous Belgian-American ophthalmologist Dr. Charles Schepens. In 1968, the Retina Foundation was divided into two separate institutions: the Eye Research Institute and the independent non-profit Boston Biomedical Research Institute. The

John Gergely *with* **Éva Szentkirályi** *(left;* **Andrew Szent-Györgyi***'s wife),* **Nora Gergely** *(John's wife) and* **Setsure Ebashi** *in Albert Szent-Györgyi's house in Woods Hole (ca. 1976, courtesy of NLM)*

Muscle Department became part of BBRI, and John Gergely was appointed its director. He held that position for 34 years, until he semi-retired in 2003. He remained active as member of the Board of Trusties till February 2013, when BBRI closed due to insufficient funding.

As we mentioned above, Albert Szent-Györgyi directed John towards quantum chemical studies, but during the time he spent at NIH he performed experiments, which drove him to the field of "wet" biochemistry. In a telephone interview by Adrian Kinnane, science historian, he remembers as follows [3]:

> *"I couldn't put my finger on direct developments that could be labeled as quantum-mechanical biochemistry . . . well spectroscopy, in a sense, yet, they're all quantum chemical concepts and techniques that are involved in fluorescent spectroscopy and other optical techniques. But when I came to Bethesda I was hoping that we could find something experimentally about these presumed molecular orbitals in proteins. Interestingly, you know how things happen serendipitously, the idea was to digest myosin. We did some spectrofluorescence measurements which, as I look back now were very primitive, I must say, hoping that this may reveal something, as a protein gets digested, that some of these phenomena which we hoped to attribute to these orbitals would change in a way that would show some connection. Well, interestingly, this experiment led me to a very important finding that you could digest the myosin and still have its ATPase activity intact, and this is an area where Andrew Szent-Györgyi then picked up and did some very important work."*

This pioneering work [4] opened the field on limited proteolysis of myosin. Although this was not a major direction in John's subsequent work, it became the basis of the collaboration between the Muscle Department and the Department of Biochemistry at Eötvös Loránd University (ELTE), headed by Professor N.A. Biró, also a former Szent-Györgyi disciple. John regularly visited his parents in Hungary. From the mid 1970, whenever he was in Budapest, he always found time to meet

John Gergely *and* **Andrew Szent-Györgyi** *at the XXXIV. European Muscle Conference at Hortobágy (on the Hungarian Puszta, near Debrecen) in 2005*

younger scientists in the Biochemistry Department. He was an important source of information and constant inspiration for us (including the authors of this article) in those years when personal contact with western scientists and availability of first-hand information was extremely limited. With time, the scope of the interaction between the Muscle Department of BBRI and the Biochemistry Department of ELTE expanded from Dr. Gergely's occasional visits to several researchers (Miklós Bálint, László Szilágyi, Katalin Pintér, Ágnes Jancsó, László Nyitray, István Boldogh) spending a year in John's department in Boston. This collaboration led to joint publications, e.g. describing the proteolytic substructure of heavy and light meromyosins and the localization of ATP binding site within subfragment-1 [5,6,7].

In the previously quoted interview, John summarizes the effect Albert Szent-Györgyi had on his scientific career [3]:

John Gergely *and* **Frank Sréter** *at the Eötvös Loránd University in Budapest, in 2008*

„Well, I certainly can say that he influenced both my science and my personal life in the end, you know, just getting me to this country and I've always thought very highly of him and that he was inspiring. As things developed, I thought that perhaps he was using too wide a paint brush, but then again in a way that's the attribute of a genius where many of us were more plodding and ended up working on the details."

John Gergely and his co-workers Jack Seidel and David Thomas, in the beginning of the seventies, were the first to apply saturation transfer electron paramagnetic resonance spectroscopy using spin-labels to study internal motions in F-actin, in the myosin head region of intact myosin molecules, heavy meromyosin, and subfragment-1 as well as in various actin-myosin complexes [8,9].

In the mid-sixties Prof. Ebashi's group in Japan isolated troponin, a new protein complex from skeletal muscle thought to play an important role in the Ca^{2+}-regulation of the contractile system. This work raised John's interest, and with Marion Greaser and Jim Potter they determined the subunit composition and metal binding properties of the troponin complex [10,11]. In the following years he and his colleagues devoted about 60 papers to the structural and functional aspects of Ca^{2+}-regulation in thin filaments, applying various spectroscopic, proteolytic and cross-linking techniques with meticulous precision [12-14]. John also coauthored papers with Noriaki Ikemoto on the sarcoplasmic reticulum [15], and with Frank Sreter[2], a fellow Hungarian, on myosin isozymes of various fiber types [16].

He was among the participants of the famous 1972 Cold Spring Harbor Symposium on Quantitative Biology titled "The Mechanism of Muscle Contraction" and was thanked in the Foreword of the published book by James Watson for helping him, together with Hugh and Andrew Huxley, Carolyn Cohen, and Andrew Szent-Györgyi, in choosing the best speakers [18].

Although John had no interest in commercializing his work, his group's research on troponin formed the basis of the high-sensitivity cardiac troponin assay, a gold standard in the diagnosis and prognosis of acute coronary syndrome, and of using calcium channel inhibitors to treat cardiac arrhythmia.

Altogether, John Gergely and his coworkers published about 180 scientific journal articles between 1941 and 2008, in addition, he wrote 63 book chapters and monographs. John was very active in the scientific community, participated in the editorial boards of more than ten scientific journals. He received multiple honors, among others an Established Investigatorship from the American Heart Association, and a Merit Award from the

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2 Ferenc Sréter (1922-2012) has graduated as a medical doctor at Pázmány Péter University in Budapest, and left Hungary during the 1956 Revolution. He was a faculty member of BBRI and also affiliated with the Department of Biological Chemistry, Harvard Medical School and the Department of Neurology, MGH.

John Gergely *in Cold Spring Habor in 1972 (courtesy of the CSHL Archives)*

National Heart, Lung, and Blood Institute. He became honorary Doctor of Medicine at the Semmelweis University Medical School, Budapest, and an elected Fellow of both the Biophysical Society "for fundamental contributions to the understanding of the mechanism of muscle contraction, in particular through pioneering studies of the structure and function of the troponin complex", and the American Association for the Advancement of Science. He was also elected an Honorary Member of the Hungarian Academy of Sciences in 2001.

John was a person of impressive intellect, and he loved discussing linguistics, politics, and religion, and solving math problems. The Friday "goodies" – an afternoon snack break – provided opportunity for these informal chats. The speed and acuity of his thinking was apparent at seminars – after seemingly having dozed off he asked questions which shed new light onto the topic, often surprising the speakers themselves with their originality. He had a great insight into the potential of new technologies. As Jim Potter reminisced [17]:

"John was at the forefront of computational capabilities at the time, utilizing a telephone connection to a remote computer at Harvard. We would enter the data via ticker tape (some taken directly from a scintillation counter) and this would be processed using programs John had developed. He had also developed programs to calculate free calcium concentrations, variants of which are still used today"

The establishment of the Analytical Ultracentrifugation Research Laboratory in BBRI with its instrumentation and world-class expertise is another testimony to John's technological savvy.

John's greatest contribution to the progress of muscle research was BBRI itself. For about five decades the Institute was a first-class workshop of excellent scientists. Due to John's

John Gergely *at a Friday goodies at BBRI in 1989*

open-minded approach to scientific co-operation, the Institute became an efficient training field for many visiting post-docs and collaborators from all over the world: Hungary, Poland, Czech Republic, England, Russia, Japan, Taiwan etc. He was a great mentor – he was supportive, held the researchers to very high standards, and did everything in his power to help their advancement. He believed that a true leader should be "the first among equals with his leadership". In the pre-PubMed era, in order to keep his colleagues up to date on the most recent developments in muscle research John compiled a weekly list of publications from Current Contents, and shared it with fellow scientists all over the world. A very significant aspect of his mentoring activity was helping younger scientists prepare manuscripts. He was relentless in suggesting revisions – objective, cautious, critical analysis of data were as important as the clarity of communication. It did not take long to figure out that when John told you that he did not understand something it was you who missed and important point, and not him.

Let us quote here personal memories from Sam Lehrer, one of his colleagues from BBRI about John's personality from an obituary article that appeared in *Journal of Muscle Research and Cell Motility,* titled "John Gergely: a pillar in the muscle protein field":

> *"John was a many-faceted person with multiple talents. He was first a mentor, particularly for those writing scientific grant proposals and journal articles. His post-docs and collaborators will remember long hours sitting at his desk discussing his red mark edits on their first drafts. Many versions got the same treatment which illustrated the proverb that "no piece of writing is ever finished". His English was the King's English that he learned in Hungary along with Latin before practicing it in England. Despite the apparent tediousness of the process, it was quite useful, particularly for the post-docs from abroad—they learned English writing in addition to clear scientific writing lessons. (…) His knowledge seemed to be endless. When apropos, he would quote Latin sayings and expect us to understand*

them. Or he would tell us of the interesting Hungarian word that was different from the Polish or the German that was being used"

John's caring, empathy, and concern for BBRI employees went way beyond the call of duty. In addition to creating a productive, collegial atmosphere he secured temporary funding sources and made sure that the Institute provided excellent benefits to faculty, post-docs and staff. While many of the researchers who were mentored by John moved on to other institutions and became leaders of the field, several researchers in the Muscle Department spent their entire career there. As Gale Strasburg put it [17]:

John Gergely *at the Alpbach Muscle Meeting in 2000*

BBRI retreat in Woods Hole in 1989 (John Gergely circled)

> *"John was largely responsible for fostering an environment where people wanted not only to join, but also to stay."*

Outside BBRI, in their family house in Nahant, "Nóra asszony" (Mrs. Gergely) took care of his life and the big family. When John passed away, they had been married for 68 years, had eight children, twelve grandchildren and seven great-grandchildren. John enjoyed having a big family (extended by his associates at BBRI) and imparted to his kids the pure joy of learning (as to his associates at BBRI). He was an accomplished runner, skier and sailor and he managed to pursue these activities well into his 80s. One author of this article (LNy) skied with him on the Alpbach slopes during the afternoons of the famous muscle meetings there; I vividly remember that the last time we did it was in 2007, and Nora chided him why such an old man should put on skis at all. But he was determined as all through his life.

For John, it was emotionally difficult time to witness the closing of BBRI. As described by Zenek Grabarek [17]:

He was very concerned about the future of the BBRI scientists and, despite his declining health; he wanted to know about every detail of our situation after the institute closed. John always had a positive outlook concerning people and events. He summed up the BBRI dissolution with his philosophical: "Tempora mutantur, nos et mutamur in illis"

John Gergely will always be remembered by the muscle community, as one of the Hungarian scientist coming from Albert Szent-Györgyi's school, as the father of BBRI and as an outstanding pillar of the muscle protein community.

Acknowledgements

I (LNy) am indebted for my co-authors for accepting my invitation to write this biography together. We are thankful for John Gergely and Endre Bíró (the late Head of Department of Biochemistry at Eötvös University) for allowing all of us to spend at least one year at BBRI. We also thank Phil Graceffa és Walter F Stafford III for helpful discussions.

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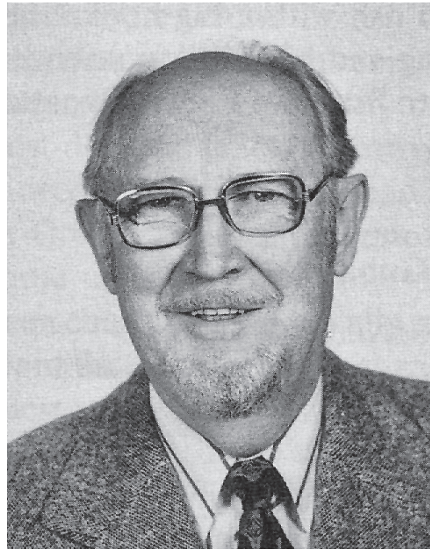
Ferenc Guba — A Fairy Tale in Muscle Research: the Way of the Son of a Steel-Worker to the Presidency of FEBS
(1919-2000)

László Dux
University of Szeged



Ferenc Guba, *1919-2000.*

Ferenc Guba was born as the son of a cannon factory worker in Győr, on March 20. 1919, just one day before the declaration of the so-called „Council-Republic" (Tanácsköztársaság), in fact a brutal communist terror period in Hungarian history. The chaos and poverty in the post World War I. country, together with the dismantling pressure toward the former Austro-Hungarian Monarchy left little space to resist this terror, and focus rather on educational and other social issues.

Right after his birth, the family of Ferenc Guba moved to the major city Arad in the southeast part of the country, which soon became separated from Hungary in the Trianon peace treaty, and part of the Kingdom of Romania. This was where he attended elementary and high schools. He was talented and diligent enough to get admitted to the Franz Joseph (later Miklós Horthy) University in Szeged, back in Hungary in the year 1938. That time an internationally recognized place to study, thanks to the unprecedented commitment and activity of Mr Kunó Klebelsberg, minister on education and religion between 1922-1931, in the post WWI country. Just one year after the Nobel-prize of Albert Szent-Györgyi, the school system was sound, elaborate and relatively well equipped in Hungary. Teacher's career was amongst the favorites for young intellectuals. Numerous subsequent Nobel laureates in physics and chemistry attended these schools that time. A sophisticated, superior level college system was established and operated (Eötvös Kollégium, dedicated to a former minister of education in the country during the XIXth century) mainly to collect, educate and socialize university students with lower social status but strong dedication in scientific and socio-economical issues. Ferenc Guba became a member of this college, making lifelong friendships and contacts with peers in different areas of research and education.

As part of the treaty in Vienna, the northern part of Transylvania, separated in 1920, returned to Hungary in 1940. This allowed the official return of the Franz Joseph University from Szeged to its original seat in Kolozsvár. Students and professors remaining in Szeged were incorporated into the newly founded Miklós Horthy University. The first rector of this old-new university become Albert Szent-Györgyi with enthusiastic support from the whole community. His commitment to motivate, educate and support young colleagues in science attracted a row of successful candidates amongst university students. Ferenc Guba as a left-wing student organization representative met Szent-Györgyi through his rectoral duties and developed mutual sympathy, particularly when he proved competence in playing volleyball as well.

Still as a chemistry student, he joined the Department of Medical Chemistry and mingled quickly into the everyday activities and social life there. The research interest already turned towards muscle contraction and the proteins behind it, after the successful completion of studies on biological oxidation, Vitamin C and fumarate catalysis, leading to the Nobel price in 1937. Thanks to the support of the Rockefeller Foundation and the former minister Mr Klebelsberg, the department was relatively well equipped with the necessary tools and chemicals for these studies. The approaching WWII created a dim atmosphere with the ever-increasing isolation of the scientific community, limiting access to communication both in person and in correspondence.

Ferenc Guba *commemorating at Albert Szent-Györgyi's memorial at his centennial anniversary in 1993.*

This increasing international segregation kept senior members of the Szent-Györgyi group together (like Banga, Straub, Laki etc) in Szeged, who otherwise would have been invited and appointed to leading positions in biochemistry worldwide. Under these conditions, the team entering into muscle research in Szeged was extremely strong and productive. The results were published in the series Studies form the Medical Chemistry Department University of Szeged, Vol I. (1941-42) Vol II. (1942), Vol III. (1943), and finally summarized by Szent-Györgyi before the end of the war in 1944, under the title Studies on Muscle from the Department of the Medical Chemistry University of Szeged in Acta Physiologica Scandinavica (see facsimile version in the Appendix of this book). These papers set the ground for the understanding how proteins actin and myosin, as well as ATP, can carry out the chemo-mechanical energy conversion in muscle. (The proper model, sliding-filament theory, was not within reach that time since the work of Huxley and the impact of muscle electron microscopy was to come a decade later.)

The chemistry student Ferenc Guba started to work on muscle biochemistry under the supervision of Bruno F. Straub, an assistant professor. His duty was to analyze the viscosity of different actin, myosin, actomyosin extracts under different conditions, i.e. pressure, pH, ionic composition and concentration. These works ended up in three papers in the Vol III. of the Studies. One, written together with Straub received particular attention and career later (Extraction of Myosin Vol III. 46-48.1943.). The description of the solution most appropriate to isolate myosin (0.15 M K-Phosphate buffer, pH 6.5 with 0.3 M KCl) was later called „Guba-Straub Solution". The label was present on bottles in the refrigerators of most muscle biochemistry laboratories still in the eighties all around the world, indicating a real impact on muscle science, more than just citation counts.

The turbulence in Hungary at the end of WWII starting from the spring-summer of 1944, enforced Szent-Györgyi into hiding, and the rest of the Department was not really in the position to carry out research afterward either. Ferenc Guba received his university diploma in chemistry in 1944. His only pa-

On the banquet at the Szent-Györgyi-centennial with **Ursula** *and* **Andrew Szent-Györgyi** *(right).*

per together with Albert Szent-Györgyi was published in Nature in 1946, on the fluorochromes in muscle.

Szent-Györgyi, becoming more and more involved in everyday political discussions in the post WWII Hungary moved to the capital, and became the head of the brand new Biochemistry Department at the Pázmány Péter University. Many of the young colleagues, including Ferenc Guba, followed him, moving most of the remaining equipment and chemicals with them, leaving an almost empty building for his successor Bruno F. Straub in Szeged. This way, Ferenc Guba became an assistant professor in the Biochemistry Department in Budapest by 1945. As Szent-Györgyi fled, form the more and more aggressive communist takeover, to the United States in 1947, Straub succeeded him in 1950 as the head of the Biochemistry Department in Budapest.

Ferenc Guba was protected from most of the communist atrocities, thanks to his previous left-wing connections, and became an associate professor by 1950. The Hungarian Academy of Sciences established the first central electron microscopic research facility in 1951. Guba was appointed as a senior associate and later the head of the laboratory until 1961. He got the opportu-

When „fibrillin met titin". First and only personal contact between **Ferenc Guba** *(right) and* **Kosack Maruyama** *(left) in Szeged, 1996 (in the middle:* **László Dux** *and* **Ernő Duda***).*

nity to work and study at the California Institute of Technology Molecular Biology Section in Pasadena during 1962-63.

In the next two decades, he was one of the founding fathers of electron microscopy, ultrastructure research in the country. His widespread profile of publications from this period indicated the eagerness of many scientific areas to exploit the new opportunities to see something from molecular structures that time.

His continuing interest in muscle research ended up in a significant observation regarding the ultrastructure of the muscle remnants after the extraction of all known contractile proteins. The fuzzy network of clearly visible proteins was called fibrillin. Almost ten years later it was identified and characterized in detail by professor Kosack Maruyama and his colleagues in Japan, and later named connectin/titin. Since then, titin is considered as an essential cytoskeletal protein controlling the length and the orientation as well as positioning of thin and thick filaments during contraction and relaxation. Unfortunately, the limited international communication during the sixties left the original

observation by Guba, Harsányi and Vajda and the name fibrillin mostly forgotten outside Hungary.

In 1968 the Medical University Szeged decided to establish a new, independent Biochemistry Department, separated from the previous Department of Medical Chemistry. This decision reflected the explosive developments in molecular life sciences by the sixties and put into the proper perspective the scientific and educational heritage of Albert-Szent Györgyi as well. The first professor and head of the Biochemistry Department became Ferenc Guba in 1968. Almost twenty years after the departure of the last muscle biochemists from Szeged in 1949, now a new era of muscle research was opened. As a remarkable support a brand new, top quality electron microscope (JEOL 100B) was installed in the Department. Unfortunately, the work on the muscle cytoskeletal network and fibrillin never continued in Szeged.

Senior colleagues Ödön Takács, Magda Mészáros, István Sohár, Györgyi Jakab, Attila Török, Zsuzsanna Kiss all joined the research projects. Molecular and ultrastructural studies were initiated on skeletal muscles in disuse and under other functional restraints. A close collaboration was developed with the Pathophysiology and Physiology Departments at the Medical University in Debrecen, with Professors Kesztyűs, Szilágyi, Szöőr and Rapcsák. During the seventies, and even more so in the eighties, all Eastern-bloc countries were recruited into the INTERKOZMOSZ programme organized and supervised by the Soviet Space Agency. Since muscle wasting was amongst the obvious, severe complications developing during extended weightlessness or micro-gravitation, Ferenc Guba and his colleagues became an essential part of these space-medicine research collaborations. Several animal muscle samples were collected and analyzed following space flights. Contractile protein, proteolytic enzyme, membrane composition, ultrastructure studies were carried out and published during these years. The involvement of the Research Institute of Aviation Medicine of the Hungarian Armed Forces in Kecskemét played a significant role in these studies as well, establishing perspectives for human studies in exercise and sports biomedicine in the future.

Joyful celebration of Christmas at the Biochemistry Department in 1997.

Another track for the survival of the muscle research heritage of Albert Szent-Györgyi's time remained alive in the Neurology Clinic in Szeged. Professor István Huszák, a former fellow in Szent-Györgyi's laboratory, kept research interest on muscle adaptation, plasticity and the normal and pathobiochemistry of muscle fiber types through his successors Professors Lajos Heiner and Jenő Domonkos. The wife of Professor Heiner, Dr Hortenzia Mazareán, a senior member of the Guba team proved to be really successful in collecting and motivating young medical students, recruiting them as early as possible into muscle research. Several professors of recent Hungarian medicine got the start form her laboratory in Ferenc Guba's institute (e.g., Professor István Édes, now cardiology professor in Debrecen, Professor Edit Buzás, professor of immunology in Budapest.)

The respect he earned through the Szent-Györgyi school, together with his empathic, flexible character and managerial skills put Ferenc Guba into the administration of scientific and university life in Hungary soon. He served as the dean of the Faculty of General Medicine (1971-1978), vice rector of scientific research (1978-1984). He was president, vice president, board member in several scientific societies i.e Hungarian Society of Biochemistry, Biophysics, Electron Microscopy, etc.

The last Christmas party with **Ferenc Guba** *in 1999.*

Most members of the Szent-Györgyi school left Hungary during the forties or after the 1956 revolution. Previous personal contacts remained somewhat active (it was called as the „Hungarian muscle maffia"), even during the years of the communist rule in Hungary. The somewhat eased oppression after the 1956 revolution allowed some forms of direct visits, communication in the late seventies-eighties already. Ferenc Guba was amongst the frontrunners to exploit these opportunities, not just for himself but for the interest of many young colleagues, and the muscle research in the country in general. Several major international muscle research conferences were organized in Szeged in 1977, 1980, 1983 and 1986, easing the isolation and limited access to new scientific information and expanding the horizons, the way of thinking tremendously. Many exchange visits, fellowships grew out of these conferences for the members of the Biochemistry Department. Ödön Takács spent some years in John Gergely's Muscle Research Institute in Boston, István Sohár at the Rutgers University in New Brunswick, Györgyi Jakab, István Édes and László Tálosi at Arnold Schwartz in Cincinnati, the author of this chapter and Elek Molnár in Syracuse in Anthony Martono-

si's laboratory, as well as in Dirk Pette's laboratory in Konstanz Germany.

The recognition of Ferenc Guba's professional career was assumed by becoming the president of the Federation of the European Biochemical Societies (FEBS) between 1974 and 1976. Upon his retirement in 1989 the Biochemistry Department of the Albert Szent-Györgyi Medical School, now called as the University of Szeged (with a short interruption) remained as a central place for muscle biochemistry and molecular biology research in Hungary and beyond.

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3 Guba F., F.B. Straub Note on the viscosity of myosin Studies from the Institute of Medical Chemistry University of Szeged 3. 49-50. 1943.

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Sándor Juhász-Nagy – Founder of the Cardiovascular Physiology School of Budapest

(1933–2007)

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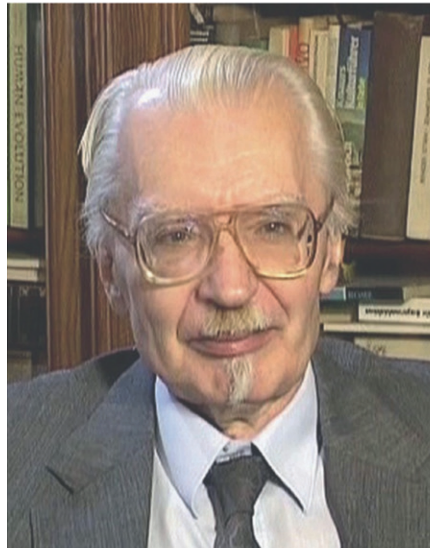

Sándor Juhász-Nagy.

Sándor Juhász-Nagy was an open-minded scientist with a broad spectrum of interest and special synthesis ability. His research work was essentially linked to the physiology and pathophysiology of cardiovascular function. He investigated the living organism in its inherent complexity; he was an excellent analyzer of individual facts and results, a great figure of systems physiology.

His interest ranged from questions of basic research to the scientific approach of the problems connected to practical med-

icine. His observations and results often served, in some cases even directly, medical therapy. (Nowadays this is called "translational" research.)

His students and colleagues remember him with high esteem and love as teacher, as paternal friend of the young, and the personal friend of the elder.

Juhász-Nagy was born on 1st of November, 1933 in Debrecen. His father was Sándor Juhász Nagy (1883-1946), a lawyer, a Presbyterian prelate, a university professor, and also a prominent civil democratic personality. (In 1944, he was elected for vice president of the new National Assembly of Hungary.) His mother, Klára Kótay (1899-1982) was a German-Hungarian teacher, and later the librarian of the University of Debrecen. His parents and all paternal and maternal predecessors were Calvinists and Hungarians, which was important for his intellectual development, and character formation. This intellectual inheritance encompassed the respect of true values, the love of truth and freedom, honesty and humanity.

Sándor and his brother Pál (Pál Juhász Nagy, 1935-1993, who became a famed theoretical botanist, and member of the Hungarian Academy of Sciences, HAS) were still children when their father died. Their mother brought them up alone.

Sándor Juhász-Nagy completed his secondary school at the Presbyterian College in Debrecen. After his graduation he learned medicine at the University of Medicine of Debrecen and received his diploma in 1952. It shows his excellence that he was the first to be awarded as Doctor of Medicine with the title *"Sub auspiciis Rei Publicae"* (Golden Ring of the President of Hungary). He already was involved in the work of the Institute of Physiology led by István Went, when he was a second-year student.

Went the Head of Department, as Sándor Juhász-Nagy later described (1), was a very good teacher: "he – being a very clear-minded scientist – knew, and, he could also teach 'where is God' in science... He always demanded unquestionable skilfulness in experimentation and did not tolerate obscure, confused thinking. At the same time, if he recognized talent what he considered a guarantee of the successful work, he respected the

Sándor Juhász-Nagy *(left) and* **Mátyás Szentiványi** *at the end of 1950s.*

autonomy of his students, what is more – encouraged them, indeed." Hence, Sándor Juhász-Nagy's carrier as a researcher started in the Institute of Physiology. Working with his colleague and good friend, Mátyás Szentiványi (who was also a brilliant, sparkling-minded young researcher), they had reached results already in the early years of their collaboration, that drew the attention of the scientific world to their work. They have shown for the first time that the coronary arteries have a functionally significant vasoconstrictor (sympathetic) innervation, which proved to be independent from the myocardial one.

This statement initiated a great reverberation, because it was in strong contradiction with the views denying the existence – or at least the significance – of direct sympathetic innervation of coronary vessels. It's worth to review briefly (2) how the image of neuronal regulation of coronary circulation had been formed and evolved over the years. The first coherent theory of the neural regulation of the heart and coronary function was published by Anrep and Segall in 1926, based on their results obtained on innervated heart-lung preparations. In this theory the sympathetic and parasympathetic innervation was imagined as equal and mirror-like reflections of each other (3). The model was log-

ical, but the influence of the myocardial O_2 consumption altered by sympathetic and vagal stimulation on the vascular responses could not be entirely excluded neither from these (nor from the later) results. Therefore, the next model, constructed by Gregg and Shipley in the middle forties, emphasized that the vegetative reactions of the heart function determine primarily the vascular response to autonomic neural activation (4). "Is that really so?" queried the two young researchers, Juhász-Nagy and Szentiványi.

Similarly to Gregg, they made their investigations on anesthetized animals, measuring all relevant hemodynamic variables (arterial pressure, coronary blood flow, myocardial oxygen consumption etc.) on *in situ* heart preparations. For sympathetic nerve stimulation, they stimulated the efferent fibres running from the *ganglion stellatum* to the heart by using fine differences in electrical threshold parameters to separate excitement of the neural branches reaching the heart muscle and the coronary arteries (5).

Figure 1. shows the model and their most important results presented on original tracings from an experiment. The old photograph shows that the coronary flow is greatly affected (reduced) by stimulation of one of the fibres with low voltage. The temporary decrease in blood pressure was maintained after administration of scopolamine (i.e. after *n. vagus* blockade), so it can be evaluated as a secondary consequence of coronary constriction. As the O_2 consumption (MVO_2) did not increase to sympathetic excitement, it meant that the stimulated fibres had a direct constrictor effect. At the same time, the arterio-venous O_2 difference (AVO_2) decreased, due to the compensatory O_2 extraction during the coronary constriction. These results have been published in leading international periodicals – mostly due to the fierce debate they have generated (6).

The idea on the role of the metabolic coronary relaxation connected to the increased myocardial metabolism under sympathetic heart stimulation, which was already considered in the Gregg's model, was developed by Robert Berne (in parallel with

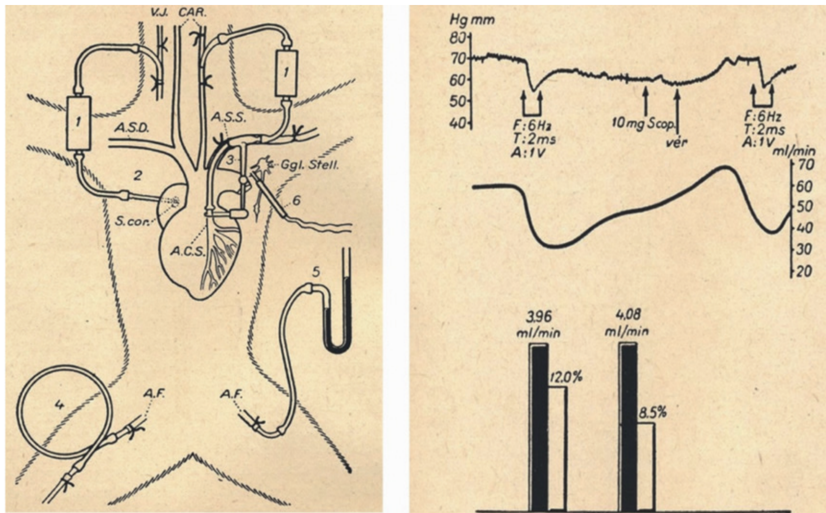

Figure 1. *The experimental model (left) and direct coronary constrictor effect of sympathetic nerve stimulation (right). Top curve: Blood pressure, bottom curve: coronary blood flow. Graphs:* MVO_2 *(black) and* AVO_2 *(white). For details see the text. (From Ref. 5.)*

LEFT panel: V.J. – vena jugularis, CAR. – arteria carotis communis, A.S.D. and A.S.S. – arteria subclavia dextra and sinistra, Ggl. Stell. – ganglion stellatum, S. cor. – sinus coronarius, A.C.S. – arteria coronaria sinistra, A.F. – arteria femoralis. 1 – rotameter, 2. – Morawitz cannula, 3. Eckstein cannula, 4. – rubber tube bridge to get arterial blood samples, 5. – mercury manometer, 6. nerve stimulator. RIGHT panel: Scop: scopolamine, "vér" means blood; F: 6Hz / T: 2 ms / A: 1 V parameters of stimulation.

Eckehard Gerlach) in the middle sixties with constructing the adenosine theory of coronary adaptation. Berne's theory shifted the centre of the sympathetic regulation to the direction of cardiac work-metabolism-coronary (indirect) dilatation, where the role of direct constrictor effect again was degraded (7.). The theoretical breakthrough in this field came only after the discovery of selective beta and alpha adrenergic receptor blocking agents, which were applied by Feigl, who – repeating the investigations of Juhász-Nagy and Szentiványi – came to the same conclusion; supporting without query the presence and physiological importance of sympathetic innervation in the coronary regulation (8.). The essence of different theories and changes in the regulatory

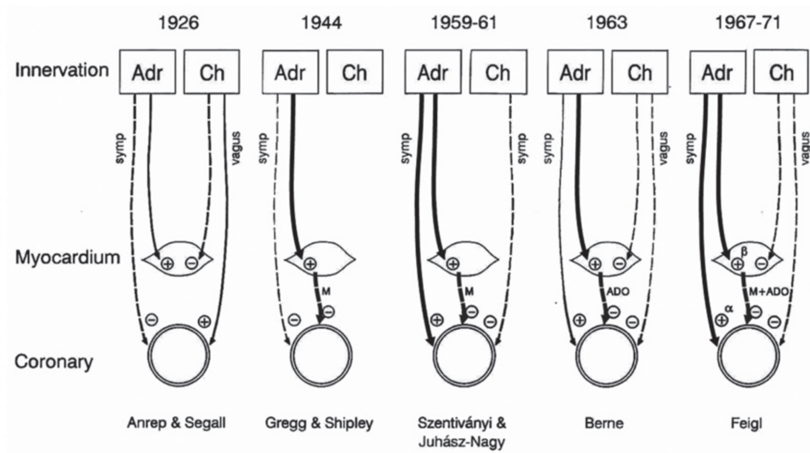

Figure 2. *Development of the models of the heart and coronary innervation, and their supposed significance in the cardiovascular regulation. Lines with plus sign (+): muscle-contracting effects (vasoconstrictor or positive inotropic action on the ventricles); dashed lines with minus sign (-): muscle relaxing effects (vasodilator or negative inotropic action on the ventricles). The thick lines indicate the effects considered to be significant, while the thin ones show the effects thought to be not. (From Ref. 2.)*
Adr – adrenerg, Ch – cholinerg, M and ADO – indirect vasorelaxing effects of different metabolites and adenosine released by the myocardium.

models is show on *Figure 2.* One can observe that the 4th scheme is basically a completed variant of the 2nd one, while the 5th model is the same as the 3rd from the point of view of the significance of the sympathetic innervation of coronary vessels. This was the way how the theory of cardiac and coronary sympathetic innervation was constructed – and it remained valid till this time.

The central nervous localization of the coronary constrictor system discovered by them (9.), and its activation in certain cardiovascular reflexes gave further proof of the importance of this regulatory function. Juhász-Nagy also demonstrated that the coronary constrictor function is more ancient in the phylogenesis of vertebrates (e.g., it is exclusive in the reptilian heart), but it is suppressed by the development of metabolic vasodilator mechanisms, which meets the blood supply to the higher cardiac load occurring in the more developed organisms (10, 11.). Moreover,

he described that injury of the sympathetic innervation leads to the loss of natural adaptability of the intact coronary vessels. The later examinations of Juhász-Nagy clarified that in the higher organisms the vasoconstrictor effects are in strong competition with the vasodilator mechanisms in the coronary bed, and among pathological conditions, such as myocardial ischemia, the constrictor effect may strengthen (12, 13). These investigations were already carried out in Budapest, at the Semmelweis University of Medicine.

Leaving the *alma mater* was motivated by the unexpected death of his tutor, professor Went (1963). The democratic atmosphere of the Institute of Physiology was changed significantly. The young researchers, who remained there were not helped in their work, and their unquestionable successes which made them known even beyond the borders of the country were not appreciated. Moreover, their international cooperation was almost fully blocked. Juhász-Nagy started to search a new workplace to continue his research.

The Director of the IVth Department of Surgery (present name: Heart and Vascular Center, Budapest), Professor József Kudász invited him to join the academic staff in 1966, and offered the possibility of organizing a research unit in his department. Juhász-Nagy accepted the invitation, and established there an independent research facility, which functioned as the only *in vivo* cardiovascular research place at the Semmelweis University for more than 40 years – and it still works there. Till the last year of his life, he studied the cardiac function and circulation in this scientific „citadel". Later he talked about this change in his carrier like this: "This was not a great position, but it fitted me like an old coat – first of all due to the absolute professional independence (which was not easily achieved). I could cooperate very well with professor Kudász, who wasn't an easy person but he was an original character and a genial surgeon; and I had also good connections with his successors based on mutual respect. The only condition from my side was that they should not mingle with my scientific activities." (14)

Juhász-Nagy looked to the problems of heart surgery from scientific perspectives: in his experimental investigations he clarified the pathological responses of the blood vessels of the ischemic heart; that is the disconnection between the myocardial O_2 demand and coronary blood supply. Based on these observations he created new types of angina models for the further research (15), and in cooperation with Kudász he demonstrated that during human heart operations such conditions could also appear (16).

From 1976 to 1977 he worked as a visiting researcher in the laboratory of Domingo Aviado at the Pennsylvania State University, where he participated in the studies investigating the effects of the endogenous agent inosine (an ATP breakdown product) on the heart and on the coronary vessels (17). He continued this work after returning to Budapest. He described for the first time the specific interactions of two nucleosides, inosine and adenosine, and recognized the role of inosine in the metabolic coronary adaptation. (I could join as a junior scientist and his PhD student to these studies. Note by V.K.)

On the basis of the interaction of adenosine and inosine in the coronary vasculature, the adenosine hypothesis of Berne could be converted to an adenine-nucleoside theory. This latter solved the fundamental contradiction of Berne's theory, namely the largely different sensitivity of the vasodilator effect of exogenous adenosine and endogenous adenosine-mediated vascular response (reactive hyperemia) to specific purinergic receptor blockade (18). In addition to that, through the accurate analysis of the nature of the inotropic and protective effect of inosine, the potential cardiologic and cardiosurgical importance of the agent was also clarified.

In the 1980s, to improve the intraoperative cardioprotection, he developed – with his outstanding disciple Lajos Papp, who was then a young heart surgeon – a new method to measure and to evaluate the blood supply and the metabolic status of the myocardial tissue. They demonstrated that, among certain environmental conditions, the infrared radiation of the heart (detected by an infrared camera) is not only proportional to myocardial blood supply, but it can be quantitatively evaluated. The recognition,

mathematics, and numerical display of this – seemingly simple – correlation opened a new field in the cardiosurgical research (19). The method was unique in that respect, that the blood supply of the heart could be estimated with large spatial and temporal resolution parallel. Its usefulness has been demonstrated in a high number of experimental and human heart studies, including the detection of normal or pathological inhomogeneity of myocardial blood supply, the estimation of the efficacy of application of cardioplegic solutions and also the assessment of beneficial or potentially harmful effects of different pharmacological agents. However, the most important result of the experimental research using this quantitative cardiothermographic method was the recognition that the use of stimulating catecholamines in certain cardiologic and cardiosurgical emergencies, such as heart operation and re-establishment of cardiac function, could lead to the complete energy depletion of the heart and ventricular asystolia. The minimalization of the perioperative administration of agents, which considerably increase the cardiac metabolism (especially Isuprel, widely used in those times) played a decisive role in that, that the efficacy of the surgical interventions improved significantly: the surgical lethality dropped to about a quarter (!) of the previous value in a year (20). Lajos Papp prepared his PhD thesis summarizing these results. Considering the significance of the results and their adaptability in human medication, the referees suggested that the PhD thesis should be accepted as an academic doctoral thesis (D.Sc.), which is a higher degree in the Hungarian scientific grading system. Their suggestion was accepted and – after defending his thesis – Lajos Papp received the degree of “Doctor of the Hungarian Academy of Sciences”. Later he had left the clinic and became head of department of cardiac surgery in three other institutions – two of the three was founded by him.

Besides the above works, Sándor Juhász-Nagy also got important findings on many other fields of cardiovascular research. He described, that the blood supply to the ischemic areas of the heart can be markedly improved by coronary sinus interventions (21). By the analysis of pericardial fluid he developed a method

to study the composition of the interstitial fluid of the myocardium. In the 90's with his student Miklós Tóth, who joined to his team as a freshly graduated postdoc, started to investigate the effects of different components (i.e. ANP, ET-1, ATII) of a new (peptiderg) type of myocardial regulation, and their potential role in the normal and pathological function of cardiovascular system (22). (Since that time he became full professor and head of different departments, Doctor of the Hungarian Academy of Sciences, and holders of several highly appreciated positions.) As a part of peptide research we (Professor Juhász-Nagy and me, as a young cardiologist and his PhD student – B.M.) recognized the very specific arrhythmogenic action of the endothelin-1 peptide, which was independent of its – otherwise strong – vasoconstrictor effect. Based on this, we constructed a new model of arrhythmogenesis for studying the mechanisms of life-threatening ventricular arrhythmias (23).

Another, perhaps not the most significant, but one of his most favorite field of research was the studying of the role of the Ca ion in vascular contraction/relaxation. It was known from the early times of vascular research – well before the discovery of any role of vascular endothelium in the mechanism of vasorelaxation – that the calcium ions may cause smooth muscle contraction as well as relaxation, depending on the circumstances. This relaxation was interpreted by David Bohr as a "membrane-stabilizing" effect of calcium (24).

Sándor Juhász-Nagy also presumed that Ca ions may have a dual role in the vascular function. His work on this subject was basically initiated by the contradiction of Berne's adenosine theory (see above). Seeking the common mechanism (and common inhibitors) of the adenosine-induced vasodilation and of the reactive hyperemic response, he found that – surprisingly – the blocking agents of the L-type Ca channels (called Ca-antagonists) equally and strongly inhibited both type of relaxations *(Figure 3.).* It was also confirmed that the diminution of the adenosine effect and of the reactive hyperemia was primarily not due to the moderation of the myocardial metabolism evoked by Ca-antagonist agents.

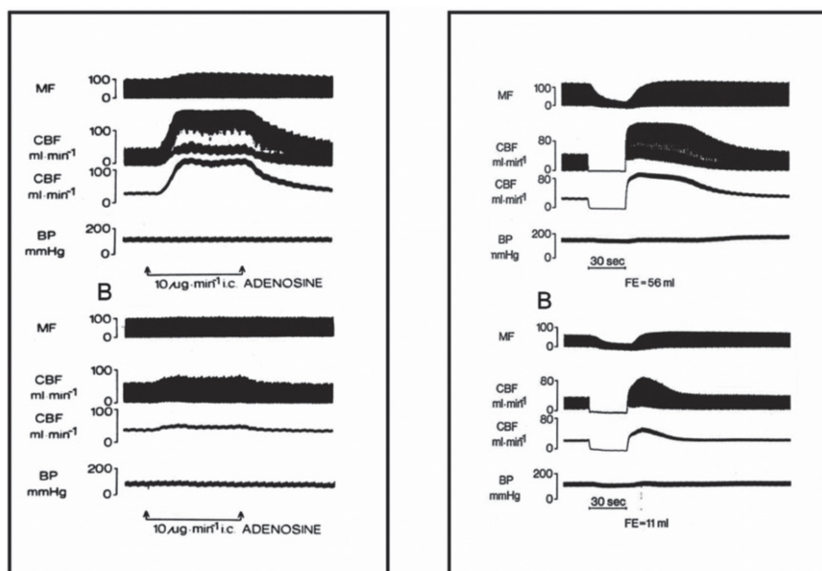

Figure 3. *Original tracings from the experiment of Juhász-Nagy. Vasodilatation to adenosine infused intracoronary (ic.) (left), and hyperemic response (right) after 30 sec occlusion of the left anterior descending coronary artery before (top) and after (bottom) verapamil in the in situ heart.*
MF: (regional) myocardial force, CBF: coronary blood flow, BP: (systemic) blood pressure. FE: hyperemic excess flow.

Parallel to this, he got another important result, namely that glibenclamide, an inhibitor of the ATP-dependent K channels (which are controlled metabolically by the decrease of intracellular ATP, and function as "O_2 sensors" of the cell) blocked the reactive hyperemic response effectively (25). What is more, the same blocking action of glibenclamide was proven in adenosine- and inosine-induced coronary relaxation as well. On the basis of the above results, he postulated that both the transmembrane Ca-uptake and K_{ATP} channel activation are involved in the metabolic coronary adaptation process. For the direct counteraction of these two mechanisms, the effect of L-type Ca channel inhibitors was investigated on the relaxation elicited by ATP-dependent K channel-opening agents. Following a simple logic, an enhancement of the coronary dilatation to the K_{ATP} channel opener pinacidil would be expected, when Ca-antagonist verapamil was

applied. However, the opposite happened: verapamil effectively inhibited the coronary effect of pinacidil. Thus, the concept of a bidirectional interaction between the opening of K_{ATP} channels and the activation of L-type Ca channels was born. The changes of coronary vasodilator responses to different inhibitors obtained from these studies and also from other investigations are summarized in the *Table.* It can be seen, that – beside the marked reduction of reactive hyperemia and dilatation induced by adenosine and pinacidil to the blockade of L-Ca and K_{ATP} channels – these effects were almost totally preserved during endothelial nitric oxide synthase (eNOS) inhibition (L-NAME administration), hence they proved to be largely eNOS-independent.

These results, and many other observation in this field were achieved during nearly two decades of his research period, and were published in a paper titled „Vasodilatatio paradoxica rediviva: modalities of the coronary adaptation" (26). The possible mechanism of interaction between the vascular membrane K_{ATP}

	Residual coronary effects after		
	L_{Ca}	K_{ATP}	eNOS
	blockade (% of control response)		
RH - FE	11[a]	40[b]	84[e]
Adenosine	19[a]	30[b]	80[d]
Pinacidil	40[b]	45[f]	81[f]
PTH	-	23[c]	85[g]

RH: reactive hyperemic excess flow, PTH: parathyroid hormone

a: Juhász-Nagy S, 1980; b: Juhász-Nagy S, 2004 c: Juhász-Nagy S, 2000; d: Buus NH, 2001; e: Smith TP jr, 1993; f: Deka DK, 1998; g: Schulze MR, 1993

Table. *Coronary dilatory effect of temporal occlusion, adenosine and pinacidil modified by the blockade of L_{Ca}, K_{ATP} channels and eNOS enzyme. The coronary effect of parathyroid hormone – a natural Ca-ionophore agent – was also studied. (From ref. 26.)*

channels and L-type Ca channels is shown on the schematic illustration of the **Figure 4.** The events of the outer circle are quite clear: the O_2 deficiency of the myocardium increases the ATP breakdown, and consequently, elevates the levels of the mediator metabolites (adenosine and inosine). The latter agents open the metabolically-regulated K_{ATP} channels, resulting in membrane hyperpolarization, and hence decrease the Ca-influx in one hand, and lead to smooth muscle relaxation by itself on the other. The vasorelaxation reduces O_2 deficiency, restores O_2 supply. At the same time, based on his results it seems that the activation of the L-type Ca channels may somehow open the K_{ATP} channels. This event may interpret the unexpected effect of Ca-antagonist agents on K_{ATP}-dependent vasodilatation. The mechanisms through which the change in the membrane Ca-fluxes induces K_{ATP} channel opening is not clear till now – and probably this remains so for a long time. It is assumed that either a small Ca-uptake (trigger Ca, $[Ca^{2+}]_o$) or local changes of the $[Ca^{2+}]_i$ (alterations of Ca compartmentalization in the submembrane space) may lead to the open-state stabilization of K_{ATP} channel, e.g. by

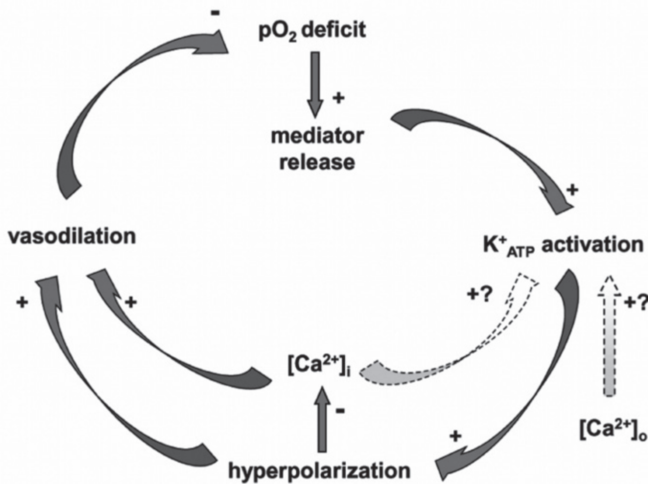

Figure 4. *The hypothesized mechanism of the interaction between membrane K_{ATP} channel activation and membrane Ca fluxes and/or intracellular Ca movements in the vascular relaxation. For the explanation see text.*

dissecting of the closing hydrogen bonds and supporting the electron transfer between the specific amino acids in the channel – as proposed by Lee et al. (27).

It is a great question whether the original ideas of Sándor Juhász-Nagy, for example this latter, may get *in vivo* evidences and, if the answer is yes, when. It is possible that much time will pass till then, since the focus of the research shifted from the smooth muscle to endothelial regulation, and – discovering the Ca-dependent eNOS activation and nitrogen monoxide release – the relaxation associated with Ca flux into the cells could be seen from another perspective.

Professor Juhász-Nagy was not only an originally thinking scientist but an excellent tutor as well. His laboratory was open for all, who were interested, and he tried to help all talented young people – not only his students – with his wise, benevolent, professional and personal advices. He raised many researchers and helped to start their career. His professional guidance and the transfer of knowledge was always aimed to press young people think originally. Just as Juhász-Nagy got from his master Went, he also offered the opportunity for his students to work and perform experiments independently. The corn of his activity was the curiosity and he tried to make them aware that without this there is no true research. This was connected to the respect for the facts, honesty and helpfulness. He gave a personal example of all of these.

The huge encyclopedic knowledge, freedom and self-reliance attracted many young people to his laboratory. Around fifty undergraduate students from the Students' Scientific Association studied under his personal guidance, who won many awards with their works at the University Conferences. In the postgraduate (PhD) program led by him 21 colleagues received their scientific degrees, young and elder ones as well. Moreover, the majority of the results of further 15 academic – among these 5 D.Sc. (Doctor of the Hungarian Academy of Sciences) – degrees has been worked out in this laboratory. He was asked several times to be the official referee of scientific theses written in other research institutions. His reviews were highly appreciated (several people

even collected them!) because they were so relevant, instructive, just and always benevolent.

As a reward of his work he received numerous awards, including the Jendrassik Award for Medical Research (1979), the Award for Excellence in Scientific Education (1991), the Albert Szent-Györgyi Award for Science (1994), the Széchenyi Award for Science (1999), the Semmelweis Award and Medal (2000), the Medal of the President of the Republic of Hungary (2003) and the title of "Laureatus Academiae" – Hungarian Academy of Sciences (2004).

Sándor Juhász-Nagy – although working primarily as a researcher – was also very active in the scientific organizing life. He worked for a long time as a member of different academic committees; he was a consulting member of the Section of Medical Sciences of the Hungarian Academy of Sciences (HAS), and for two cycles was deputy of the General Assembly of HAS. After the fall of the communist regime, throwing back his personal interests, he fought to renew the Hungarian research supporting system. He firmly committed himself to achieve the financial independence of scientific support, and that the scientific value should be a priority above anything else. As Director of Division of Life Sciences (Biology, Medicine, Agriculture) of National Science Research Foundation (OTKA) (1991-1996), he has made a continuous effort to keep the system based on objective decisions in the society, which was just changing. It is partially his merit that this organization in a milieu burdened with economic hardships could persist and became reliable. From 1997 he was elected President of the Board of the Széchenyi Professor Scholarship, and he remained in this function till 2001. Due to his broad scientific knowledge, he was also asked to accept the chairmanship of the Hungarian Biological Society. He performed all his duties with perception and without biases. According to him the original scientific result was the only important value. It was also undoubted that the stupidity and narrow-mindedness, especially when it came from „above", were not tolerated by him. By character he obviously was neither a politician, nor a diplomat; he never hid his opinion – which many did not like. The

Professor Sándor Juhász-Nagy *with his disciples.*

so called “politically correct” language was not his strength. This could be one, and maybe an important reason, why he did not receive the HAS membership, to which he was nominated three times – in spite of the fact that he was a much better scientist, and he had done much more to rise new generations of Hungarian researchers and to achieve a fair system to support the Hungarian scientists than several academicians.

The literacy of Sándor Juhász-Nagy was exceptional and very broad. He knew the literature so well, that he could easily give lectures on liberal arts at the university. Despite his scientific perspectives and great professional skills, he was basically a philosopher. This was reflected in his studies, notes and lectures, which were fascinating and enchanting. He could review excellently not only the works belonging to his scientific field: he had an instinctual sense to recognize real literary values and weaknesses. He considered taking care of the Hungarian professional language very important, especially in the education of new research generations. That is why he launched the series of

"Studia Physiologica" in 1995, in which outstanding Hungarian researchers could summarize their major results and oeuvre in Hungarian language. Thus, the younger generation could get acquainted with these and could realize that scientifically significant results could be produced not only abroad, but even in this country. Up to now, 22 volumes of the Studia were published, and the very existence of the series is the great merit of Sándor Juhász-Nagy. He chose a Latin proverb as motto for the series: Age quod agis! (If you do something, do it well!). "I would like, if this motto could be relevant to my personal activities" – was quoted in a book written on the basis of an interview made with him on his life not long before his death.

To the premier of this report book, which he already could not attend, he sent the following words: „Dear Participants! It is an old experience that the effect of indirectly transmitted truth is usually greater, than that of directly pronounced. This little book, which summarizes my career, has two main guiding motifs: love for scientific research and love for young scientists, disciples. I put the emphasis on love and not on proficiency. I would like to add to these the deep attachment to my family and the love for my friends and colleagues. My life, like most other people's, was rich in failures, but I had also some successes. It is not my duty to take the balance of these. However, I can confidently say that the sympathy of the benevolent people and the affection of my students, has been with me through my whole life."

Professor Sándor Juhász-Nagy died in a severe disease on January 8, 2007, at home, among his loved ones, quietly, peacefully, as he always wanted to.

Selected publications

Sándor Juhász-Nagy[1] published more than 250 original papers and close to 30 books and books chapters during his half-century lasting scientific work period. According to him, the most important papers he published are the following:

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1 In a number of the publications, his name appeared in English translation, as “Alexander” instead of “Sándor”

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Arisztid Kovách — Integrator of smooth- and skeletal-muscle physiology into clinical experimental research
(1920-1996)

Zoltán Benyó, Péter Sándor, Emil Monos
Semmelweis University, Institute of Clinical Experimental Research

Figure 1. Professor Arisztid Kovách *in his sixties.*

Dr. Arisztid György Balázs Kovách, M.D., physician, physiologist was born on 15 June 1920 in Budapest, Hungary. In the decades following World War II, he was an internationally renowned, prominent Hungarian representative of the physiological sciences. His activity was outstanding as a scientist, a public figure in the scientific community, a university professor and a mentor of future generations of scientists alike *(Figure 1)* – the authors of this chapter were also his students. At the beginning of his professional career he very soon achieved internationally recognized significant results in the experimental research of

traumatic circulatory shock and later the pathomechanism of haemorrhagic shock. His publication list for the years 1947–1982 contains the data of 361 works published in Hungarian and foreign languages; the number of his lectures given in the same period was 305. His first lecture was co-authored with Professor Aladár Beznák, later they published jointly several highly cited papers. Nearly a quarter of his publications describe his research on muscle, including both striated, cardiac and smooth muscles. Below is a list of some of the characteristic research topics expounded in his publications (the research was pursued in the Institute founded and headed by himself):

- Mechanism of the decrease in glucose uptake in skeletal muscle exposed to hemorrhagic shock (1952);
- Regeneration of biochemical, functional and histological muscle abnormalities manifesting in ischaemic shock (1956);
- The effect of dibenamine administered in various stages of ischaemic shock on the survival of dogs and on the oedema of the injured limb (1957);
- Changes taking place in denervated muscle (1959);
- Effects of gilutensins on isolated cardiac and smooth muscle preparations from animals exposed to experimental shock (1968);
- Kinetics of mitochondrial flavoprotein and pyridine nucleotide in perfused cardiac muscle (1972);
- Capillary liquid transfer in the musculature of the hind limb of cat in the course of haemorrhagic shock (1973);
- Kinetic localisation of activator calcium in gravid and post partum uterus of rabbits (1978);
- The effect of transitory ischaemia on the active and passive large deformation biomechanics of canine common carotid artery, post-ischaemic hypersensitivity (1978);
- Biomechanical properties of fibrosclerotic and normal human internal carotid artery (1979);
- Effect of smooth muscle activation on the biomechanical properties of porcine carotid arteries (1980);

- Effect of nickel ions on the ultrastructure of isolated perfused rat heart (1980);
- Protection of myocardial function in haemorrhagic shock (1981).

The Kovách family verified, in 1746, their nobility and the right to use the title "hodosi" (of Hódos) in front of their name as an indication thereof. His father, György Kovách (22 April 1894, Tiszafüred – 28 November 1955, Budapest), was Public Health Inspector of the town of Balassagyarmat. Later he worked as Head of the Department of Public Health at the Ministry of Welfare (1946–1949) and subsequently as member of the Institute of Sport's Medicine (1949–1955). His mother's maiden name was Márta Andrejovich. Arisztid Kovách was married in 1949 to Emma Morgen (Emília Morgen, "Milike"), who helped his career first as a laboratory technician and later as his secretary. His brother and sisters are: Dr. Egon Kovách, Mrs. Antalné Hudecz née Klarissza Kovách, Mrs. Dr. Istvánné Antalóczy née Georgina Kovách (M.D.) and Mrs. Románné Bogdán née Erzsébet Kovách. His sons are: György Kovách (1953–) and Balázs Kovách (1957–).

He completed his secondary education in Balassagyarmat, Kunszentmiklós and Budapest, and graduated from secondary school in the Madách Imre Gymnasium in Budapest (1938). He obtained his diploma as general physician at the Pázmány Péter University of Sciences in Budapest (1944). As a student, he received a scholarship to the University of Heidelberg, where he spent the years of 1942–1943. He had excellent manual dexterity: he was capable of preparing Starling heart lung preparations in anaesthetized dogs with the use of no more than one or two gauze sponges. This might have been the reason why he planned to become a brain surgeon at that time. It was in this period that his interest in irreversible haemorrhagic shock syndrome was first awakened. He received his general physician's diploma (MD) in the autumn of 1944, immediately before the siege of Budapest. He spent the siege in the cellars of the Institute of Physiology of the University at Puskin street, together with his Professor Aladár

Beznák, several colleagues and numerous interesting personages such as the writer László Passuth and the future Nobel Prize winner György Békésy (whom, to Beznák's request, he had earlier introduced to the anatomical structure of the inner ear). Here he participated in organizing an emergency aid station, rescuing persecuted people and treating the wounded. In the course of the siege the first group of Soviet soldiers to enter the building of the Institute was led by a young army doctor called Smirnov who, very fortunately, immediately realized that this was a medical research facility. It is especially interesting to note that several years later Smirnov, who was by then a professor and a member of the Soviet Academy of Sciences, repeatedly visited Arisztid Kovách (this time without a uniform) in Budapest, where Kovách was already the director of the Laboratory of Clinical Experimental Research. Moreover, one of the authors of this memoir returned Dr. Smirnov's visit and sought him out in Moscow.

After World War II Arisztid Kovách became Assistant Professor in the Institute of Physiology of the Pázmány Péter University of Sciences (1945–1946). He next moved to Tihany to follow his professor, Aladár Beznák, who was removed from Pázmány University to serve as Director of the Hungarian Biological Research Institute in Tihany (1). It was here that Kovách was appointed Adjunct Professor (1946–1948). After the emigration of Beznák, he was transferred from Tihany back to the Budapest Medical University (BOTE, from 1969: SOTE [Semmelweis Medical University]), to the Institute of Biochemistry (1948–1950), and from there to the Institute of Physiology (1950). He was appointed Senior Lecturer ("Docens") on 1 December 1951. Later, when he received his appointment as University Professor (1 July 1970), he was already Director of the Laboratory of Experimental Research ("KiKuLa", an institution that he founded himself) of the External Surgical Clinics of the university(1957–1990).

As a result of his scientific research activities, in 1952 he was awarded the scientific degree of "Candidate of Medical Sciences" by the Hungarian Academy of Sciences. In 1957 he was Visiting Professor at the University of Heidelberg and the University of Freiburg. In 1959 he acted as visiting lecturer in Cairo and Alex-

andria. In 1970–1971 he was visiting researcher at the University of Pennsylvania (Philadelphia, USA) and, later, appointed Professor at the Department of Neurology of the same University. In 1970–1991, for 20 years (renewed every 4 years) he continued to function at the University of Pennsylvania as co-Principal Investigator of a NIH Research Grant received together with the Cerebrovascular Research Center. Within the framework of this cooperation he was able to procure long-term research opportunities in Philadelphia for several young colleagues working in his Institute and other research institutes and clinics of our University.

Arisztid Kovách completed an exceptionally rich life-work as researcher, organizer and instructor in the field of science.

One of his most outstanding achievements was that in 1959 he founded, organized, directed and, up to 1990, developed the predecessors of the Institute of Clinical Experimental Research of the Semmelweis University *(Figure 2).* He established successful scientific cooperations with several dozen national and foreign research institutes and clinics; our Institute nearly continuously

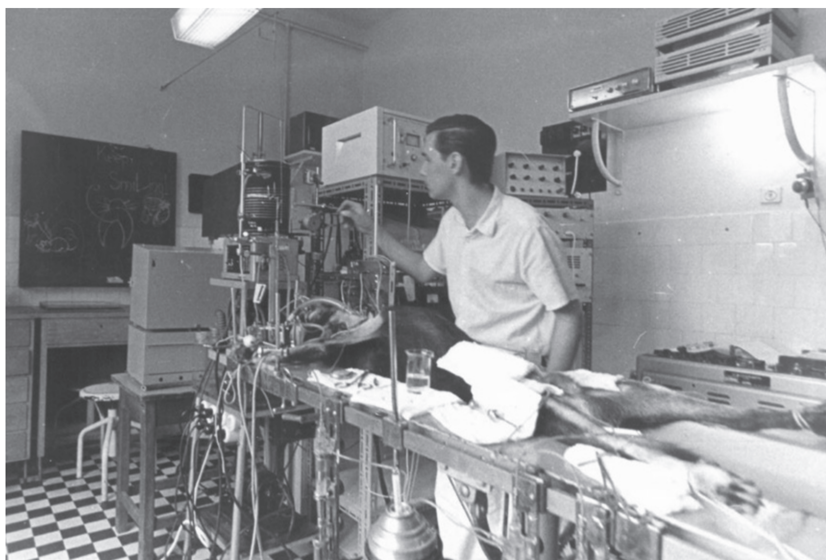
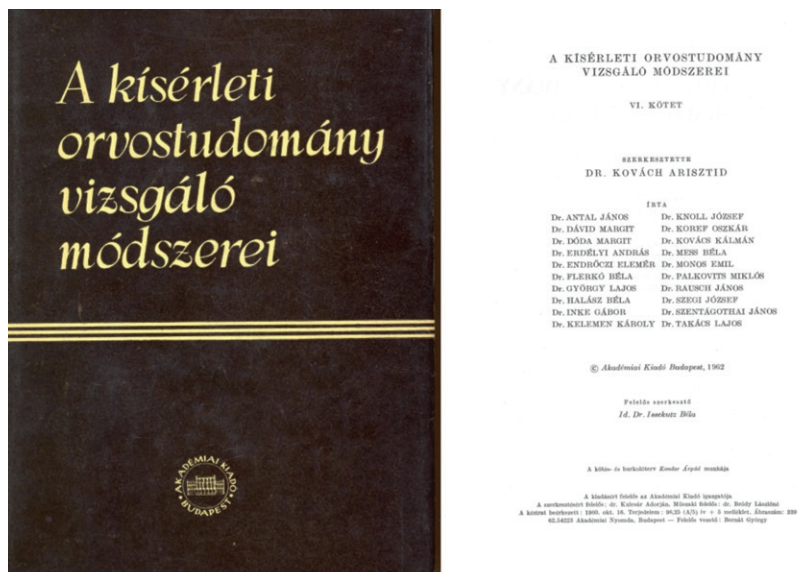


Figure 2. *Animal experiment in one of the haemodynamic laboratories of the Institute of Clinical Experimental Research in the nineteen-sixties.*

received visiting scientists from the research institutes, clinics and hospitals of Hungarian and foreign universities for extended work periods. He spared no effort to set up, in his Institute, laboratories equipped with the most up-to-date instruments of cardiovascular research, partly with help from his connections with foreign (Heidelberg, Leipzig, Stockholm, Philadelphia, Milwaukee) and Hungarian universities (e.g. Budapest University of Technology: Zoltán Benyó sr., Frigyes Csáki, Béla Szűcs) and partly by having an electric and precision mechanic workshop built within his Institute (manned by engineers Bocskai, Bölcshelyi, Szedlacsek and Németh, attended by a host of mechanicians).

Another monumental achievement was when he initiated, edited and, together with his coworkers and students, also partially authored the seven-volume methodical treatise "Methods in Experimental Medicine I-VII" (1954–63, Akadémiai Kiadó, Budapest) *(Figure 3).* This was an epoch-making contribution to the recovery of experimental medical research in Hungary, completed in an exceptionally difficult historic period of our country.



A KÍSÉRLETI ORVOSTUDOMÁNY
VIZSGÁLÓ MÓDSZEREI

VI. KÖTET

SZERKESZTETTE
DR. KOVÁCH ARISZTID

ÍRTA

Dr. ANTAL JÁNOS	Dr. KNOLL JÓZSEF
Dr. DÁVID MARGIT	Dr. KOREF OSZKÁR
Dr. DÓDA MARGIT	Dr. KOVÁCS KÁLMÁN
Dr. ERDÉLYI ANDRÁS	Dr. MESS BÉLA
Dr. ENDRŐCZI ELEMÉR	Dr. MONOS EMIL
Dr. FLERKÓ BÉLA	Dr. PALKOVITS MIKLÓS
Dr. GYÖRGY LAJOS	Dr. RAUSCH JÁNOS
Dr. HALÁSZ BÉLA	Dr. SZEGI JÓZSEF
Dr. INKE GÁBOR	Dr. SZENTÁGOTHAI JÁNOS
Dr. KELEMEN KÁROLY	Dr. TAKÁCS LAJOS

© *Akadémiai Kiadó Budapest*, 1962

Felelős szerkesztő
Id. Dr. Issekutz Béla

A kötés- és burkolóterv *Kondor Árpád* munkája

A kiadásért felelős az Akadémiai Kiadó igazgatója
A szerkesztésért felelős: dr. Kulcsár Adorján. Műszaki felelős: dr. Bródy Lászlóné
A kézirat beérkezett: 1960. okt. 16. Terjedelem: 98,25 (A/5) ív + 5 melléklet. Ábraszám: 339
62.54223 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

Figure 3. *Cover page and title page of Volume VI of the methodical book series edited by* **Arisztid Kovách** *(17×24 cm, 1099 pages), Akadémiai Kiadó, 1962, Budapest).*

It can be said of the work of Arisztid Kovách that he brought the standards of the experimental research of the physiology and pathophysiology of the cardiovascular system in Hungary to the level of international excellence. He obtained significant results in the research of the pathomechanisms of various types of circulatory shocks and in the elucidation of the neuro-humoral regulatory processes of the cardiovascular system (2, 3). He created a school of circulation research in the institute he founded, which helped launch the careers of more than a hundred young scientists, many of whom continued their work as university professors, department heads, institute directors and group leaders either in Hungary, or abroad. His energetic support for outstandingly talented young researchers sincerely devoted to research was widely known already in the nineteen-fifties. The performance of his carefully selected undergraduate students was always first class, and their lectures and publications ensured their position among the frontrunners of the conferences of the Students' Scientific Association both at the university and national levels. His determination as a leader and his remarkable qualities as a researcher and an instructor were acknowledged and respected by all his young colleagues (who were later associates also in founding own institute) working with him directly day by day: János Antal, János Menyhárt, András Erdélyi, Lajos Takács, Attila Fonyó, János Somogyi, Magdolna Irányi, Sándor Kiss, Edit Koltay, Pál Róheim, by a number of enthusiastic med students such as László Fedina, Attila Mitsányi, Margit Dóda and György Gosztonyi, as well as by his unforgettable chief technician and long-time friend, the ever helpful handyman Uncle Gyula Szilassy. Arisztid Kovách was an extremely severe instructor and highly demanding towards his fellow researchers; at the same time, however, he could create and maintain to the end an intimate, familial atmosphere in his Institute. The University had legends circulating about the enthusiasm and efficiency of Arisztid Kovách in motivating and mentoring talented young people committed to research, university students and young clinicians alike. He mentored the accomplishment and successful defence of several dozen candidate's and doctoral theses.

A great number of well-known and renowned Hungarian researchers were nurtured and started on their careers in the school founded and led by Arisztid Kovách, among them the future directors of our Institute: in chronological order, Emil Monos, Márk Kollai, Erzsébet Ligeti and Zoltán Benyó. Attila Fonyó later served as Director of the Institute of Physiology and Dean of the Faculty of Medicine, Semmelweis University.

Further eminent professors and instructors who started their careers in our Institute and later worked at Semmelweis and other universities and research institutions are: János Menyhárt, János Somogyi, Péter Sándor, László Tringer, Tivadar Tulassay, Viktor Bérczi, László Tretter, Ákos Koller, István Nyáry, János Hamar, Endre Cserháti, Gabriella Dörnyei, Ildikó Horváth, Mátyás Keltai, Erzsébet Makláry, György Nádasy, Rudolf Urbanics, Zsolt Lohinai, furthermore the late Eörs Dóra, Mária Kopp, László Ligeti, Lajos Matos and István Szlamka (in Hungary), and György Gosztonyi, Antal Hudetz, László Gyulai, Katalin Kauser, György Kunos, Gábor Rubányi, Csaba Szabó, Benedek Erdős and Katalin Komjáthy (abroad).

The political "iron curtain" (1948–1989) enormously hindered the activity of Hungarian scientists, who had great difficulties even in accessing the scientific results and publications of their "western" colleagues. Despite all these difficulties, within this period Arisztid Kovách succeeded in securing several dozen foreign fellowships, mostly in Western Europe and North America, for supporting the education of talented young Hungarian researchers. He was helped in this endeavour by his extremely wide circle of acquaintances he had established earlier: in the first half of the nineteen-forties he studied at the University of Heidelberg, and in 1970–71 he spent a long study visit at the University of Pennsylvania, Philadelphia. His professorship at the latter University aided his efforts to organize, a few years later, an exemplary official cooperation programme between the Semmelweis Medical University and the University of Pennsylvania. These opportunities for doing research abroad were of fundamental significance in the careers of all participating young researchers.

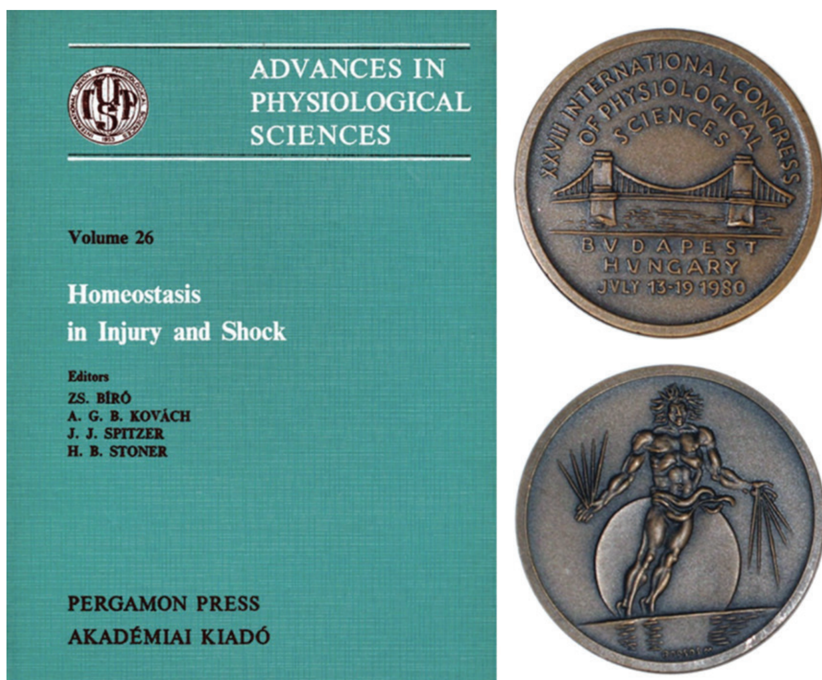


Figure 4. *The 26th volume of the "Proceedings" (1981) and the medallion (artwork by sculptor Miklós Borsos) of the IUPS Congress held in Budapest*

In 1972 Arisztid Kovách organized and initiated, in his Institute, the teaching of human physiology in Hungarian based on a modern system approach. He also played an essential role in the launch of the English teaching programme in 1990 in our University, including our Institute.

The international reputation of his work is confirmed by his numerous administrative positions held in the international scientific community. In 1967 he was invited to be editor of the journal "Newsletters" published by the International Union of Physiological Sciences (IUPS), and in 1974 he was elected Secretary-General of IUPS. As Secretary-General, he had a decisive role in granting the right to host the IUPS 28th World Congress of Physiology to the Hungarian Physiological Society *(Figure 4).* As Vice President of the Organizing Committee, he was one of the main leaders of the organization of the World Congress, which took place in 1980 in Budapest, with 5921 registered par-

ticipants. At this World Congress, Arisztid Kovách was elected 1st Vice President of IUPS.

He spared no effort to organize the publication of the entire material of this Congress in English in 36 volumes, in a joint project by Pergamon Press and Akadémiai Kiadó *(Figure 4).*

It is our firm conviction that Dr. Arisztid Kovách played a dominant role in laying the foundation of the modern integrative systems approach of physiology in Hungary. A good example is the comprehensive concept he used to direct the extremely diversified experimental research efforts for determining the pathomechanism of traumatic shock in the first decades of his career as a researcher. These research projects included the investigation of changes in metabolic processes, blood supply, transcapillary fluid transport and other processes in various muscle types (striated muscle of the extremities, cardiac muscle, uterine and vascular smooth muscles). A few representative publications published in English and German in the course of these studies are the following:

Kovách AGB, Takács L, Róheim P, Kiss S, Kovách E. The glucose uptake of muscle in shock. Acta Physiol Acad Sci Hung 3: 345-355, 1952

Kovách AGB, Takács L, Kiss S. Phosphorolytic and hydrolytic glycogen breakdown in the muscle of normal rats and those in shock. Acta Physiol Acad Sci Hung 10: 103-312, 1956.

Kovách AGB, Takács L, Szabó MT, Takács-Nagy L, Zachariev G, Hámori J. Regeneration in the biochemical, functional and histological changes found in the muscles of rats after ischaemic shock. Acta Physiol Acad Sci Hung 10: 313-325, 1956.

Sándor P, Kovách AGB. Untersuchungen über dieWirkung des Gilutensins an isolierten Herz- und Glattmuscelpreparaten in experimentallen Schock. In: Hypotonie. Wiener Symposion. Hrsg. Schneider KW, Stuttgart, New York, Schattauer Verlag. Pp. 27-38, 1968.

Chance B, Salkovitz IA, Kovách AGB. Kinetics of mitochondrial flavoprotein and pyridine nucleotide in perfused heart. Amer J Physiol 223: 207-218, 1972.
Rubányi G, Kovách AGB. Effect of ovarian steroid hormones on superficial activator calcium in the rabbit uterus. Acta Physiol Acad Sci Hung 53: 71-80, 1979.
Monos E, Kovách AGB. Effect of acute ischaemia on active and passive large deformation mechanics of canine carotid arteries. Acta Physiol Acad Sci Hung 54: 23-31, 1979.
Nádasy GL, Monos E, Mohácsi E, Csépli J, Kovách AGB. Effects of oxygen, hypoxia metabolic inhibitors, nitrite, indomethacin and adenosine on the spontaneous tone of a nerve-free vessel, the human umbilical artery. In: Adapability of Vascular Wall. Eds. Reinis Z, Pokorny J, Linhart J, Hild R, Schirger A. Avicenum and Springer-Verlag. Prague and Heidelberg, Pp. 159-160, 1980.
Hudetz AG, Márk G, Kovách AGB, Monos E. The effect of smooth muscle activation on the mechanical properties of pig carotid arteries. Acta Physiol Acad Sci Hung 56: 263-273, 1980.
Rubányi G, Balogh I, Somogyi E, Kovách AGB, Sótonyi P. Effect of nickel ions on ultrastructure of isolated perfused rat heart. J Molecular and Cellular Cardiol 12: 609-618, 1980.

Arisztid Kovách was an exemplary personality and an internationally prominent scientist. In all his life he was a devoted patriot, and actively pursued his Christian faith and humanism. He undertook a vast number of tasks and responsibilities, and completed and met them all, as indicated by the positions and honours he was awarded. The most important of these include, but are not limited to the following:

- Professor of the Department of Neurology, University of Pennsylvania at Philadelphia
- Member of the Executive Committee of the Hungarian Physiological Society

Figure 5. *On 12 September 2016, the 20th anniversary of the death of* **Arisztid Kovách** *a sculpted bronze plaque in memoriam of* **Arisztid Kovách** *was unveiled by* **Ágoston Szél,** *Rector of Semmelweis University and* **Zoltán Benyó,** *Director of the Institute of Clinical Experimental Research in the Basic Medical Science Centre of the University, the present location of the Institute founded by Professor Kovách. (photo Attila Kovács)*

- President of the Worldwide Hungarian Medical Academy
- First Secretary and later Vice President of the International Union of Physiological Sciences (IUPS)
- Honorary Member of the Royal Belgian Academy and the Polish Academy of Sciences
- Honorary Doctorate at Kuopio University (Finland)
- Honorary Member of the Czech Purkinje Society
- President of the International Society of Oxygen Transport to Tissues
- Member of the International Shock Society and numerous other prestigious national and foreign learned societies and scientific committees
- "Outstanding Tutor" of the Semmelweis Medical University
- Academy Award winner
- Grade I of the Semmelweis Award

- Award of the Swedish Medical Research Council
- Order of the Star of the Hungarian Republic with Laurel Wreath (1990)

Arisztid Kovách deceased on 6 February 1996 in Cologne, Germany. His passing closed a period of exceptional significance in the history of physiology in Hungary and in that of the research and educational institute he founded that has gained international renown in the course of its fifty years of operation (4). We as his students treasure, utilize and pass on, with solicitude, all the positive professional, spiritual and moral values represented by Arisztid Kovách to those who follow us.

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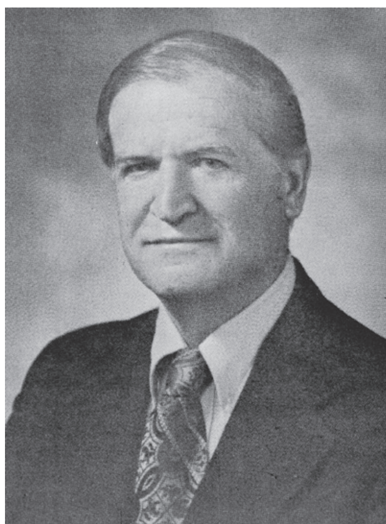
Kálmán Laki (Koloman Laki) — Discoverer of blood clotting factor XIII
(1909-1983)

László Muszbek
University of Debrecen, Department of Laboratory Medicine, Division of Clinical Laboratory Science



Kálmán Laki *in the mid 1970s.*

Biography

Kálmán Laki was born in Szolnok, but the family soon moved to a small village, Abádszalók. He lived most of his happy childhood and adolescence in this village close to the beautiful Hungarian river, Tisza. He considered this region, the Great Hungarian Plain, as his home of origin throughout his life. Once, already living in the US, he ordered from a well-known

Hungarian painter an oil canvas picturing the high, sandy riverbank of Tisza. Ever since I knew him, this picture had always been hanging on the wall of his study at home, looking over his desk. He completed high school studies in the small provincial town, Cegléd, then he was admitted to the Medical School at the University of Szeged in 1929. At the University, his ability was soon recognized by his chemistry professor Albert Szent-Györgyi, who was later, in 1937, awarded with the Nobel Prize in Physiology and Medicine "for his discoveries in connection with the biological combustion processes, with special reference to vitamin C and the catalysis of fumaric acid". For talented students with possible carrier in science, Szent-Györgyi invented a new program which after the first 4 years of medical studies included advanced courses in chemistry and physical chemistry at the Faculty of Science. Such combination provided Laki with expertise in the field of both medicine and chemistry. He graduated in chemistry in 1935 and one year later received PhD degree in biochemistry. He started doing research in the Department of Chemistry as a student and later as Assistant Professor. In 1938-39 receiving a Rockefeller fellowship he was working in the Department of Chemistry at the University of Manchester.

During the years of World War II, the dispersion of scientific achievements became hampered. As the group of Szent-Györgyi was unwilling to publish in German scientific journals, their results, particularly discoveries in muscle research including Laki's works, were published in pamphlets edited by their own institute (Studies from the Institute of Medical Chemistry of the University of Szeged). For these reason, the results failed to receive the recognition of the wider scientific community. Furthermore, Szent-Györgyi became engaged in unofficial anti-Hitler negotiations of the Hungarian government, and when it was discovered he had to go underground. In the absence of Szent-Györgyi, Laki had to replace him, but conditions in the last year of the war were highly unfavorable for experimental research. Instead, they were engaged in illegal activities, such as forging passports to help Jews escaping deportation. After the end of the war, Szent-Györgyi moved to Budapest and became the head of the

Kálmán Laki *(center) explaining to students, the 1953 finalists of the competition at the Society for Science and the Public.*

Department of Biochemistry at the Medical School of Pázmány University. Laki soon followed him and became appointed professor in Budapest. However, after the communist takeover the conditions for doing valuable research as well as the general political conditions in Hungary rapidly deteriorated, and Szent-Györgyi decided to emigrate to the United States. For a brief period of time, Laki became the head of the Department, but in 1948 he left the country for a guest professorship at the University of Leeds. One year later he moved to the US and was employed as a special fellow in the National Institutes of Health (NIH).

In 1954, he was appointed Chief of the Section of Physical Biochemistry at the National Institute of Arthritis and Metabolic Diseases, a position which he held until his death. During this period of time, for thirteen years, he was also the chief of the Laboratory of Biological Chemistry. In the last decade of his life, he was the Regional Director of the National Foundation for Cancer Research, the so-called Szent-Györgyi Foundation.

Kálmán Laki'*s last visit to Debrecen just a few weeks before his passing away in Bethesda. A portrait with his characteristic smile (left side). The last picture in his life, together with the author (right side).*

His relationship with Szent-Györgyi, which developed into a friendship, lasted throughout his life. He frequently visited Szent-Györgyi's laboratory and was a guest at his home in Woods Hole, MA. He was the guest professor at the Institute of Physical Chemistry of the University of Paris and the University of Erlangen, and had frequent contacts with scientists at the Weizmann Institute of Science, Rehovot, Israel.

He had a broad scientific interest, and linguistics particularly aroused his curiosity. In his middle age he completed a course in linguistics at the University of Washington, and then researched the origin of sexagesimal system and showed that it it was used in the proto-Finno-Ugric language (Laki K. The number system based on six in the proto-Finno-Ugric language. J Washington Acad Sci 1960;50:1. Laki K. On the origin of the sexagesimal system. J Washington Acad Sci 1969;59:24-9). He learned to read the Sumerian clay tablets and became a lecturer in sumerology.

He re-established connections with the Hungarian scientific community in 1970, when he visited the Department of Patho-

Kálmán Laki's meeting with scientists of the Department of Pathophysiology, University Medical School of Debrecen in 1970.
From left to right: **Prof. Tibor Szilágyi, Kálmán Laki, Hajnalka Szilágyi** *(secretary),* **Prof. Béla Csaba, Mária Kávai, László Muszbek, Juszupova Szaodat, Prof. Lóránd Kesztyűs** *(rector, chairman of the Department).*

physiology of the University Medical School in Debrecen. Following this occasion, he became a regular visitor at the Departments of Pathophysiology and, somewhat later, at the Department of Clinical Chemistry. His visits contributed to the increasingly important US connections at our University. He invited or helped in arranging the invitation of 25 young Hungarian scientists to the National Institute of Health and was instrumental in establishing a Regional Laboratory of the National Foundation of Cancer Research US in Debrecen.

His life was taken away by a serious heart attack following a minor surgery in 1973. He had a friendly warm personality and helped many people in their scientific carrier and in everyday life. He was best characterized by the following sentences from his mentor and friend, Albert Szent-Györgyi:

Kálmán Laki *(left) with* **Albert Szent-Győrgyi** *(middle) at Seven Winds, Woods Hole, Massachusetts (ca. 1976). (photo, The Albert Szent-Györgyi Papers, National Library of Medicine)*

> *"He had all the qualities of a genuine researcher and was driven in his very active research solely by the desire to understand nature. He was a faithful friend. He had strong imagination and an intensive knowledge connecting biology with theoretical physics and chemistry. Outside science, he had but one ambition – to help others."*

Laki edited three books and was the author on 159 scientific publications. In Hungary, he was elected to be a member of the Hungarian Academy of Sciences, the title he was deprived of after his emigration. His membership was re-established posthumously in 1989. He was awarded the Kossuth prize in 1948, just prior to his leaving the country. In 1973, he received the James F. Mitchell Foundation Award for the discovery of coagulation factor XIII together with László Lorand and Ariel G. Loewy. In

The plaquette established by the Hungarian Society of Thrombosis and Haemostasis to award scientists who contributed significantly to research on blood coagulation

The inauguration of Laki Kálmán's bronze statue on the main square of Abádszalók, where he spent most of his childhood

1976, the University Medical School of Debrecen conferred upon him honorary doctor's degree.

The memory of Kálmán Laki is still vividly living in the Hungarian scientific community. In 2006, the Hungarian Society on Thrombosis and Haemostasis established a Laki Kálmán award (see the photo) for those Hungarian scientists who contributed significantly to research on blood coagulation. In his favorite Hungarian village, Abádszalók, a street was given his name. Furthermore, an impressive statue, made by a leading Hungarian sculptor Sándor Győrfi, treasures the memory of Kálmán Laki on the main square of the village. Symbolically, the scultpure is made of 13 granite elements, referring to factor XIII discovered by Laki. At the University of Debrecen, a doctoral school was named after him. The council of the city of Szolnok (his birth place) established a Laki Kálmán scientific prize, and the recipients formed a table society to cherish his memory. His relief is in the Pantheon of Szolnok where a commemorative plaque was erected on the bank of the river Tisza. On the one hundredth anniversary of his birth, his mortal remains were brought to Hungary and found eternal rest in the cemetery of Debrecen; the exclusive burial place was donated by the city council.

Scientific achievements

Kálmán Laki excelled in two areas of biomedical science. Although the aim of this chapter is to recall his contribution to muscle science, one cannot resist to appreciate his highly valued contribution to our present understanding of blood coagulation. These contributions were summarized and emphasized by his collaborator Elemér Mihályi, another excellent NIH scientist in an obituary published in the journal Thrombosis Research (30:549-52). The followings are quotes from Mihályi's obituary:

> *"Laki discovered an accelerator of the prothrombin activation, which he called plasmakinin, much before Fantl and Nance, Seegers or Owren. His preparation was probably a mixture of*

factors V and VIII, but he thought it was the antihemophilic factor. Unfortunately, (during the 2nd World War) this was published in the Studies from the Institute of Medical Chemistry of the University of Szeged and remained unnoticed. Perhaps his most important contribution to the understanding of the fibrinogen-fibrin conversion was the demonstration of the two-step mechanism of this reaction. This clearly showed that thrombin has an enzymatic action on fibrinogen that modifies the molecule, but has no direct role in the polymerization itself. The latter occurs spontaneously with the fibrinogen molecules activated by thrombin if the conditions are favorable. Further work on these problems was performed in Bethesda with American collaborators. They demonstrated that thrombin is a proteolytic enzyme related to trypsin-chymotrypsin family of enzymes known today as serine proteases. The structure of fibrinopeptides also was determined in this period. In addition, important studies were made in Laki's section on the mechanism of fibrin gel formation and the cross-linking of these gels by what is now called activated factor XIII."

In fact this factor was discovered by Laki and his student, László Lóránd and was first published still from Budapest in a short article (Laki K, Lóránd L. On the solubility of fibrin clot. Science 1948;108:200). The factor was later acknowledged as the 13th coagulation factor by the International Committee for the Nomenclature of Blood Clotting Factors in 1963. Before it had been called Laki-Lorand factor. Laki edited the book Fibrinogen (Dekker, New York 1968) and the issue of the New York Academy of Sciences on "The biological role of clot-stabilizing enzymes: Transglutaminase and factor XIII" (1972).

The selected references demonstrate the highly valuable contribution of Laki to muscle research. It was very likely that Albert Szent-Györgyi and the atmosphere in his institute had inspired him to become interested in muscle proteins, although before his emigration to the US he had never worked or at least he had not published on this topic. His first and all subsequent publications on muscle proteins were submitted from NIH. The

first publication was a real breakthrough, demonstrating that G actin contains ATP, which is required for its polymerization, and de-phosphorylation of ATP affects actin so that it will not polymerize (1). This finding caused some tension between Laki and the scientists remaining in Hungary, led by Straub, who was also working on this topic and must have been close to such a discovery. Szent-Györgyi had to mediate between the two groups and as a result the following footnote was added to the publication:

> *"After the manuscript of this paper was submitted, Straub published an abstract for the First International Congress of Biochemistry in which he states that adenosine triphosphate must be regarded as the prosthetic group of actin, the removal of which renders actin non-polymerizable."*

I was able to detect a full-length follow-up article on this finding of Straub and Feuer only in Hungarian language. Investigating this phenomenon further Laki and Clark demonstrated that the mechanism of ATP disappearance during actin polymerization is due to its de-phosphorylation to ADP (5).

Laki and his coworkers were authors of several articles exploring the structure of actin and and its interactions with other muscle and non-muscle proteins (6,20,22,23,25,27,30-35). They reported the amino acid composition of actin myosin, tropomyosin and the meromyosins (7). They also estimated the molecular weight of actin with the use of carboxypeptidase A, although it was somewhat overestimated, still it was the best approximation available that time (20). Standaert and Laki demonstrated the presence of α-helices in G-actin and showed that the G- to F-actin transformation involves an internal structural change (22). The N-terminal amino acid sequence of actin was revealed by Alving and Laki (27). The investigations of Laki's group shed light on the interaction of actin with other contractile proteins, which was important for understanding the function of these proteins in the mechanism of muscle contraction. In a letter to

Nature, they provided evidence for a reversible equilibrium between actin, myosin and actomyosin (6). In an elegant paper they proved the stoichiometric combination of actin and tropomyosin and recommended a method for the purification of actin-free tropomyosin (23). The interaction of actin with fibrin was the first example for the interaction of actin with non-contractile proteins (33) and together with other related publications lead to the "sticky actin" concept introduced in an editorial of Nature (1975;256:616). A hypothesis on actin being an energy transducer was published in a theoretical journal (31).

Laki's group also devoted considerable attention to the muscle proteins tropomyosin and myosin. They prepared characterized and compared vertebrate and invertebrate tropomyosins and mammalian tropomyosins from different tissues ((11,12,15,18). They demonstrated that tropomyosin consists of two peptide chains, one of which has an N-terminal glutamic acid, while the N-terminus of the other one is N-acetylated alanine (26). By determining the amino acid composition of H-, and L-meromyosins and by analytical untracentrifugal analysis they established the approximate size of the myosin molecule (7,9). Their studies on meromyosins conrtributed to understanding the structure of myosin molecule (16,17,21). In another series of experiments they showed that KI solution induces contraction of glycerol-treated muscle fibers and compared such contraction with that induced by ATP (8,10,19,28). It should also be mentioned that in 1971 Laki edited the book Contractile Proteins and Muscle, which provided a state of the art summary of the field and introduced novel ideas fertilizing ongoing and future muscle research. The book was reviewed by Alfred Burger for the Journal of Medicinal Chemistry (1972;15:216). He closed his review by the following sentence:

> *"This book will do much to make available the literature and expert judgment of the background and status in this field".*

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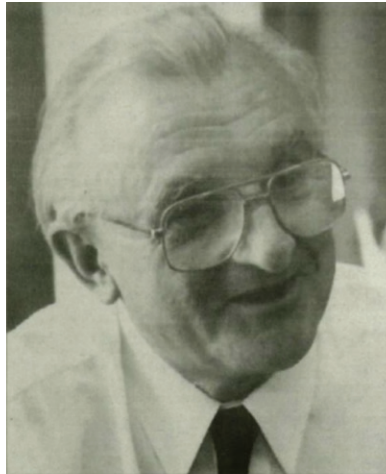
Anthony N. Martonosi — Lifelong Hard Work and Discipline in Muscle Research
(1928-2010)

László Dux
University of Szeged



Anthony Martonosi *(1928-2010).*

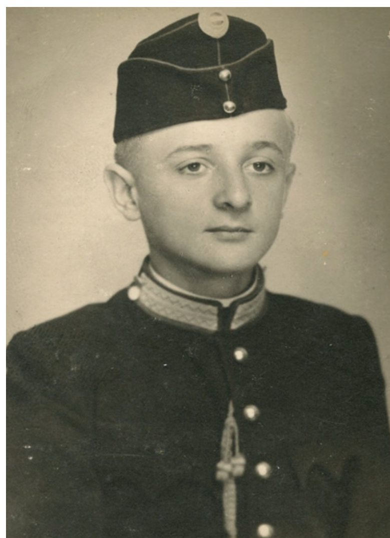
Anthony N. Martonosi's contribution to our understanding how muscle works was spread over decades and several areas. Although he was too young to meet Albert Szent-Györgyi in Szeged, and the years at the WWII were too chaotic for both of them, his life and career was definitely impacted by the muscle research school of Szeged, what many call the „Hungarian Muscle Maffia".

Anthony Martonosi was born into an almost typical middle-class family in 1928. Active in food-trading, his grandfather and the three brothers of his father owned and operated the largest central butchery shop in the center of Szeged right on the

Széchenyi square. His father Antal Martonosi (1901-1945) worked as an accountant at the City Savings Bank, but served the family business with book-keeping as well. His mother's side owned a small grocery store in Felsőváros (Upper Town) dominated then by people living on and from the river Tisza. The river played an important role in Anthony's life as well. Every time he came to Szeged even in the eighties-nineties, he regularly asked one of his nephews, István Kovács to take him on a boat trip there. The Zsótérs, the mother's family was present since the XIXth century life, involved in ship-trading and erecting some landmark buildings in the downtown area, one of which is still called as Zsótér-house.

The marriage of the parents Antal Martonosi and Anna Zsótér secured a safe middle-class life for the two children, Anthony, and his sister Éva, (born in 1934). In 1939, the parents decided to send their son Anthony in the famous military school in Kőszeg, a few miles from the Austrian border. The strict martial discipline and the company of other 5-600 schoolmates dressed in uniform made a long-lasting impact on everyone's life there. The school became world known and famous by the book of the writer Géza Ottlik „School at the Frontier". The title of the

The famous military school in Kőszeg, on a contemporary postcard.



Anthony Martonosi in 1940, as a student of the military school.

book in Hungarian „Iskola a határon" nicely exploits the double meaning of the word „határ", once the physical country-border with Austria, and at the same time the frontiers of strength, determination, patience, discipline, mental and physical capacity of the inmates there. Reading the book later, made for me easier to understand some reactions, gestures of Anthony when working together during the eighties and nineties.

1939 was the starting year of WWII. During the first three years, Hungary itself was relatively calm and safe. Although the disastrous defeat of the 2nd Hungarian Army at the Don River in January 1942 sent a chilling message to tens of thousands of families leaving sons and fathers dead or captured behind. After four years in Kőszeg in 1943, at the age of 14, Anthony was transferred to a military engineering school in Esztergom. The German and Soviet invasion of the country in 1944-45 completely demolished the tissue of the Hungarian society and the safe, prosperous environment developed over the previous decades. Anthony's father, as many innocent civilians, was captured by the Soviets and sent to a slave labor camp in Saratov (he died most probably in 1945).

The engineering school was evacuated to Western Hungary first, andlater to Dessau, Germany, as the soviet troops approached Budapest. Anthony, with a few of his classmates, decided to go rather to the capital and participate in the fighting there. As a fortunate event for later muscle research, they were identified soon by the Hungarian forces and sent after their emigrating school westward. Finally, they became prisoners of war of the American army near Passau, during the spring of 1945. The average age of the class was 15. In August 1945, the Americans handed them over to the Soviets. As an outcome, they were stripped of all belongings and started to march on foot to the east. At a point, Anthony and a few of his friends decided to jump into a swamp next to the road, and escape. They succeeded, and never met or heard from those comerades who stayed in the column. Most probably, none of them survived.

After weeks of an adventurous trip across postwar Europe, Anthony returned to Szeged, and enrolled to the almost 300-year-old catholic gymnasium of the Piarist order, to accomplish all the courses he missed during the military school years during the war. By 1947 he was admitted to the Medical School of the University of Szeged where he got his MD diploma in 1953. Anthony's university years were not that smooth either. Because of the „capitalist" roots of his family, and the military school education background, he was considered as an enemy of the „working class" and the more and more expansive communist regime in Hungary. Once refusing to march and celebrate on a Mayday parade with student fellows in 1948, he was arrested and interrogated for several days by the ÁVO (the Hungarian counterpart of KGB), being accused of organizing a plot to overthrow the regime. Not a bright perspective at the time. Fortunately, one of the guys who once worked in the Martonosi family's butchery shop, became a leading figure in the political police. Having good memories of his previous employers, he intervened, and Anthony was released and even allowed to continue his studies in medicine, although he remained under continuous surveillance.

After the completion of his medical studies, he joined the laboratory of the famous Professor Béla Issekutz at the Physiol-

Anthony Martonosi *graduating from the Piarist Gymnasium in 1947.*

ogy Department. He participated in research aimed at glucose uptake in frog muscles which resulted in some publications in local scientific journals. The revolution in 1956 electrified and mobilized the best of Hungarian youth inside and outside the universities. Anthony, together with some friend-colleagues, decided to organize an armored group and join fighting against the Soviet invasion. The disproportionate strength, and the relatively calm situation in Szeged convinced them to hide their weapons instead and give up the plan. (In 1983 when visiting Szeged Anthony showed me the place in the basement window recess of the 1st Clinic of Internal Medicine, where his Kalashnikov was buried under the autumn fallen leaves. It was not there anymore, of course.)

His former experience with ÁVO, and the pending harsh repercussions just started after the fall of the revolution, Anthony considered staying in Hungary unsafe. He managed to walk across the border to Yugoslavia, a few miles south of Szeged in January 1957. He spent several months in a refugee camp. When the authorities found out there are several well trained, talented

fellows among the refugees, they transferred them to Eszék for interview. The interviewer was the former senior associate of Albert Szent-Györgyi, John Gergely living in the US since 1948. He was delegated by the National Science Foundation to identify possible research personnel amongst the refugees, and transfer them to the US as quickly as possible. This way, Anthony was selected and arrived in New York. From several offers, he chose a position in his former interviewer, John Gergely's laboratory in Boston. First in the Massachusetts General Hospital, later the Retina Foundation, where he became the Assistant Director of the Muscle Research Department between 1962 and 1965, with a one-year sabbatical fellowship as visiting scientist in Birmingham England.

Settling in Boston meant a U-turn in Anthony's life. Upon John Gergely's suggestion, he changed his Hungarian surname Antal to Anthony. (As he complained later, many believed with the name Toni Martonosi to be an Italian). He met Mary Gouvea an assistant researcher in John Gergely's laboratory. She was born in a middle-class family with Portuguese origin living in the US since the early XXth century. She studied chemistry as major before. They married on May 2, 1959, and lived in a loving, warm family atmosphere with their four children, Mary Ann (1960), Anthony jr (1961), Margaret (1964) and Susan (1978), until his death in 2010.

The research activity Anthony opted for in Gergely's group was centered around muscle biochemistry. Starting back in the Szent-Györgyi laboratory in Szeged, the discovery of actin opened new avenues of questions for its composition, interactions and functional properties. They published a long series of papers as „Studies on Actin I.-VII". together with Mary Gouvea and in part with John Gergely between 1960-62, which meant a remarkable productivity even by American standards. (Note that these were the years when Anthony got, for the first time, real possibilities to carry out meaningful research in his life.)

These successes earned him an Established Investigator Award support from the American Heart Association between 1961-66. This award supported a one year research stay at the

University of Birmingham in the Biochemistry Department headed by the recognized muscle expert S.V. (Vic) Perry. They spent the year 1963 in Europe. Besides deeper insights into research environment, cultural and university life in Europe's more fortunate half, they managed to see again Anthony's mother and sister Éva and her family the first time since right after the revolution. The time Anthony spent in Perry's laboratory was when the regulation of the actin-myosin interaction came more and more into the focus of research. Calcium ions as one of the key regulators were identified, while the „relaxing factor", first mentioned by Bendall, was identified by Wilfried Hasselbach in Germany and Setsuro Ebashi in Tokyo as the Calcium- transport ATPase enzyme in the muscle sarcoplasmic reticulum membrane. The discovery of the troponin complex added further momentum to the understanding, considered as a relevant and correct description of the process even in recent times.

These impressions turned Anthony's interest toward muscle calcium regulation and sarcoplasmic reticulum for the rest of his life. Soon after returning to the US from Birmingham, he accepted an associate professorship at the Doisy Department of Biochemistry at the St Louis University Medical School, in 1965. Since 1969 he continued to work as a full professor there.

With his talented students and postdoctoral fellows dominated the field of early sarcoplasmic reticulum research. With a large cohort of original research papers, he published a series called „Sarcoplasmic reticulum I-XVII". Amongst the students and postdoctoral fellows, he was particularly satisfied with and proud of the contributions and further career development of Jane Vanderkooi, later a professor of biophysics at the University of Pennsylvania. Almost everey aspect of the calcium transport, sarcoplasmic reticulum membrane structure and function was analyzed. The particular interest they placed on protein dynamics, protein-protein interactions, the role of membrane phospholipids, surface charge features and mobility in the regulation of muscle contraction and relaxation. A special field of interest was to understand the role of calcium in the regulation of muscle development, differentiation and protein synthesis. Not an easy

task in a time when everything was done at the protein level, and nucleic acid-based studies simply did not start at all yet.

After 14 years of calm and prosperous research in St Louis, Anthony was appointed as chairman and professor at the State University of New York Upstate Medical Center in Syracuse. His vibrant and maximalist attitude did not mix well with the state employee administrators at the SUNY. After a couple of years of tension and conflicts, he decided the concentrate rather on his own research.

This was the time when the author was given the opportunity to join Anthony's Laboratory in 1982. His former personal friendship to my previous supervisor as a medical student research fellow in the Biochemistry Department in Szeged, Associate Professor Hortenzia Mazareán, and her husband the neurology professor Lajos Heiner alleviated the difficult way of bureaucracy to get a permission to go and work in the US in 1982 Hungary. Scrutinizing such attempts worsened even just after the military regime suppressed Solidarity movement in Poland at the end of 1981. Coming from an „Eastern-bloc" country, the arrival to, and the initiation of professional and personal life in the US was a challenge. His wife Mary, the two younger daughters still at home and Anthony provided all the support and advice needed so much in such situations. For the first couple of weeks, I enjoyed living in their home in Manlius near Syracuse as well.

The life in the laboratory was just slowed down. Former colleagues left, a new postdoc couple was expected to arrive during the summer, to start or continue a project on calcium regulation of protein synthesis in muscle tissue cultures. To make something useful and interesting during the waiting time for the other postdocs, we decided to take a closer look at the protein-protein interactions of calcium ATPase molecules in the sarcoplasmic reticulum membrane. To promote the stability of the enzyme, we tried to use the known phosphate analogue vanadate, under different conditions. By increasing the vanadate concentration and making the medium free of calcium with EGTA, we managed to visualize the first two-dimensional arrays of SR calcium ATPase enzymes under the electron microscope. The same structures

were observed in artificial membrane vesicle reconstructed with the purified enzyme. These results initiated a complete turn in the research directions. The calcium regulation of muscle protein synthesis was forgotten, and all the activities were directed towards calcium ATPase molecular structure-function studies over the next two decades, resulting in over 70 publications.

During these years, the conformation-specific crystallization procedures were developed, other indicators like fluorescence signals, tryptic cleavage site availability etc, were tested. The molecular structure of different conformations of the enzyme was characterized, the effects of membrane potential, phospholipids, increased pressure were identified, SR Calcium ATPase, as well as sarcoplasmic reticulum membrane features, were characterized in different muscle types, in different species and under pathological conditions in muscle diseases as well.

A long list of excellent collaborators, research fellows were recruited into the project, which was considered by the National Science Foundation as the most prosperous international collaboration they supported in the eighties. Kenneth Taylor at the Duke University in Durham North Carolina contributed with the structure analysis and reconstruction. Even we got the opportunity to analyze our frozen hydrated protein crystals with cryo-electron microscopy there (in 1984 already). A cohort of young Hungarian researchers joined the team. It was the first opportunity for all of them to confront and meet the challenges in an internationally competitive research project. Their scientific career was definitely impacted and strengthened by the years they spent in Anthony's group. From Debrecen, István Jóna, now professor of Biophysics, Sándor Varga, Sándor Papp, Tamás Keresztes, from Budapest, Péter Csermely now professor of Biochemistry, member of the Hungarian Academy of Sciences, Miklós Végh, Nándor Müllner, from Szeged, the author of this chapter, the head of the Biochemistry Department (once led by Albert Szent-Györgyi) over the last 27 years, and Elek Molnár now professor of synaptic plasticity at the University of Bristol England, all got the jumpstart in Anthony's lab in Syracuse. Even the non-Hungarians, like Slawek Pikula, now a professor at the

Receiving his appointment as the first **Albert Szent-Györgyi** *visiting professor from the rector of the university in 1993.*

Anthony Martonosi *with* **John Gergely** *at the centennial celebration of Albert Szent-Györgyi in Szeged, 1993.*

Nencki Institute in Warsaw Poland, was taken as an „honorary Hungarian" into the team.

The concentrated efforts on the structural and functional characterization of a cation-transporting ATPase integral membrane protein resulted in the first three-dimensional crystals of the enzyme in 1986. (Note that it was in 1988 when Hartmuth Michel, Johann Deisenhofer and Robert Huber received the Nobel-prize in chemistry for the three-dimensional crystallization of the integral membrane proteins photosynthesis reaction center and bacteriorhodopsin). In the meantime in 1985 Christian Brandl and David MacLennan, the other giants in SR Calcium ATPase research, published the complete primary sequence of the enzyme. This and our structural data opened the way for generating folding models at better and better resolutions. A long series of site-directed mutagenesis experiments helped to identify the functionally important sites, domains in ATP dependent calcium transport These international joint efforts, partly carried out in Syracuse, were crowned after twenty years when Chikashi Toyoshima, at the Tokyo University in Japan published the first really high-resolution structure of the SR calcium ATPase enzyme in Nature. Additional studies since then made our understanding of the fine molecular model of the chemo-osmotic energy conversion nearly complete.

By the end of the nineties, Anthony's laboratory became more and more quiet. The Hungarians left, most of them got senior positions in the country's scientific and university system, just eliminating the remnants of 45 years of communist rule after 1990. Anthony followed and supported their steps even afterwards. He regularly participated and contributed to the traditional Membrane Transport Conferences in Sümeg and the annual conferences of the Hungarian Physiological Society. His main activity remained to summarize his lifelong scientific work in books. After editing several significant monographs on Membranes and Transport, Enzymes of Biological Membranes earlier, he started to write the main piece of his life's work (as we called the „Bible" of Sarcoplasmic Reticulum). The book was basically handwritten by Anthony and typed by the legendary secretary,

Lois Epstein (another „honorary Hungarian") afterwards, almost over 10 years. It was published in 2000, at Harwood Academic Publisher under the title „The Development of Sarcoplasmic Reticulum. Its 425 page text contains practically every information available about Sarcoplasmic Reticulum that time, with an additional 175 pages reference list, for those who want to take a deeper look. Those who are fortunate to have a copy of this book can get a real impression about Anthony's determination, perfectionism and unprecedented vigor and capacity to work hard. As another similar attempt he started to write the book „The Biochemistry of Sarcoplasmic Reticulum in Skeletal, Cardiac and Smooth Muscle, but it remained in preparation (according to my knowledge).

In 1993, at the centennial anniversary celebration of Albert-Szent-Györgyi's birth, the Medical University of Szeged established a guest professorship to commemorate the Nobel Laurate former professor. As the vice rector on education and the head of the Biochemistry Department then, I nominated Anthony to become the first appointed Szent-Györgyi guest pro-

Martonosi *lecturing at the first meeting of the American-Hungarian Medical Associaton in 1992 at Lake Balaton.*

Farewell party at the end of the Szent-Györgyi guest professorship semester in Szeged, 1994.

fessor in Szeged. As the university senate approved it, he received his appointment documents at the main centennial celebration event from the rector of the university in September 1993. His one-semester service was spent during the spring of 1994. Ha gave classes, seminars, consultations mainly on membrane and muscle biochemistry, but contributed toward the reorganization and strengthening of research activities after the collapse of the communist regime in his former Alma Mater institution. It was a tremendous help for me as well, having a busy time supervising the Biochemistry Department, the Central Laboratory of Clinical Chemistry and serving as vice rector of education at the same time. (One of the 2nd year medical students he tutored then, Attila Becskei become a professor of systems biology in Switzerland later.)

After his death, in January 2010 Anthony Martonosi was remembered as a loving husband, father and grandfather. His impact on world muscle research, with his189 publications, is indisputable. But in a book about Hungarians in Muscle Research, I must stress: despite his difficult, sometimes adventurous start,

Anthony *and* **Mary Martonosi** *with their family in Syracuse at Christmas, 1996.*

Anthony Martonosi had the longest lasting, broadest and strongest influence on the future generations in muscle biochemistry, biophysics research in Hungary.

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Andrew Paul Somlyo — A Life enriched by Science and The Arts
(1930-2004)

Avril V. Somlyo
University of Virginia

Andrew P. Somlyo *(1930–2004).*

Dr. Andrew Somlyo's life and career manifested his creativity, intellect, and powerful focus. His early life and experiences in the turbulent Hungary of 1930s and 40s shaped him profoundly. Born in Budapest, child of a physician, educated by the Jesuits, his life was profoundly disrupted by war and the subsequent political landscape. He escaped Hungary during the Soviet occupation in 1948, living in Rome for two years before immigrating to the United States, where he completed an undergraduate program and entered medical school. Dr. Somlyo received his Doctor of Medicine degree and a Master of Science in Pathology

from the University of Illinois, followed by internship and subsequently residency in Cardiology at Philadelphia General Hospital. He was Assistant Resident at Mount Sinai in New York City, Senior Resident in Medicine at Bellevue Hospital and served as Assistant Physician in Medicine at Columbia-Presbyterian Medical Center, New York City, where he held a fellowship in cardiology. While there, he was mentored by Dr. Dickinson W. Richards, who received the Nobel Prize for cardiac catheterization. At that time, Andrew became active in clinical research, writing a highly cited article on the toxicology of digitalis. Dr. Richards sent him to a conference on thrombolytic therapy at the Waldorf Astoria in New York, where we met, as I was attending as a graduate student studying blood coagulation at the University of Saskatchewan in Canada. His charming Hungarian ways and bright mind combined with great energy and enthusiasm won my heart and we were married shortly thereafter. At this time, publications applying engineering and mathematical approaches were appearing in the cardiovascular literature and a new NIH program was initiated in Philadelphia to bring physicians and engineers together. Highly technically inclined as many Hungarians of that generation were, Andrew joined this program and received a Master's degree in Biomedical Engineering from the Drexel Institute of Technology in Philadelphia and at the same time initiated research projects on the pulmonary vasculature as a Special NIH Research Fellow. This became the start of our own laboratory. Although he loved clinical medicine and kept up with advances in cardiology throughout his life, he received a NIH Research Career Development Award and research became his major focus. Andrew subsequently joined the faculty at the University of Pennsylvania, where he ascended the academic ladder to Professor of Physiology and Pathology. At the University of Pennsylvania, he founded the renowned Pennsylvania Muscle Institute, leading it as Director for nearly fifteen years. The institute grew out of the MyoBio Club, where faculty from universities in Philadelphia met monthly and consisted of a remarkable number of muscle oriented researchers, many internationally known.

Sessions were very lively, with only one rule for the speaker, "no questions until after the first slide". The environment in MyoBio set a very high bar and had a major impact on our research as young investigators. Advice and collaborations flowed- it was exciting and productive, continually funded by a large NIH Program Project grant that included fourteen Principle Investigators. Fifteen years later, in 1988, we were recruited to the University of Virginia, where Andrew served as Chairman of the Department Molecular Physiology and Biological Physics, until his sudden death in 2004. He built it into one of the preeminent physiology departments in the country, consistently ranking in the top five. He developed a structural biology group, internationally recognized for its contributions to the understanding of the structure and function of membrane and signaling proteins and DNA-binding molecules with special relevance to cardiovascular disease and cancer. There, he led a large program project that supported many of these research initiatives.

Andrew's research has won many accolades, including the CIBA Award for Hypertension Research from the American Heart Association, the Louis and Artur Lucian Award for Research in Circulatory Diseases, the Distinguished Scientist Award for Biological Sciences from the Microscopy Society of America, and the Presidential Award for Outstanding Scientific Contributions to the Theory and Practice of Microbeam Analysis. He was awarded an honorary doctorate from the Université Catholique de Louvain, Belgium, was a Fellow of the Biophysical Society and the American Association for the Advancement of Science. He was extremely pleased and honored to be named an Honorary Member of the Hungarian Physiological Society.

Through the course of his career, Dr. Andrew Somlyo trained over 58 postdoctoral fellows and students, who have gone on to careers in academia or industry around the world.

Contributions to the field of muscle

Spanning over more than five decades of research, Dr. Somlyo authored more than 300 articles in prestigious journals, as well as many reviews and book chapters. As we were very much a team with complementary skills, I was a co-author on the majority of these publications.

Our early research in the 1960s focused on vascular smooth muscle pharmacology and electrophysiology. Our electrophysiological studies showed that some vascular smooth muscles exhibited graded depolarization, contrary to the dogma at the time, claiming that contraction was triggered solely by action potentials. Because the field was fragmented into specialists in the various organs, we began with an exhaustive review of all the literature to date on smooth muscle, without the aid of today's searchable databases and bibliographic citation software. This re-

Andrew *and* **Avril Somlyo,** *in the early electrophysiology days.*

sulted in two extensive reviews in Pharmacological Reviews[1] that served as a source for many investigators in the field.

Moving forward, it was clear from the status of the smooth muscle field at that time that the organization of the contractile apparatus in smooth muscle was not known. Myosin filaments had not been visualized and the literature claimed that smooth muscle myosin was monomeric, dimeric, organized into large ribbons or assembled upon stimulation. Biochemical studies by Csapo, Laszt and Hamoir, Dorothy Needham, Kate Schoenberg and Caspar Ruegg had demonstrated an actomyosin-like complex regulated by calcium having higher solubility at low ionic strength than skeletal muscle, and the presence of myosin filaments. Our laboratory resolved this controversy, demonstrating a regular array of myosin filaments under relaxed and contracted conditions[2-4]. Andrew with Bob Rice showed that ribbons were formed from myosin filaments under hypertonic conditions and thus an artifact[5]. I believe our success in these electron microscopic studies stemmed from tissue preparation skills we developed for the viable muscles needed in our earlier electrophysiological and contractility studies. These results and the demonstration of crossbridges on myosin filaments[6] are now universally accepted and with mechanical studies from other laboratories provide a firm basis for the sliding filament mechanism of contraction in smooth muscle.

Research then progressed to a series of studies of the crossbridge mechanisms using flash photolysis of caged nucleotides with our collaborators Yale Goldman, David Trentham, Martin Webb, Mike Ferenczi, Taro Fujimori, Meredith Bond, S. Horiuti and Alexander Khromov[7-10]. The kinetics of crossbridge cycling and the use of a fluorescent phosphate binding protein for direct measurements of Pi release in smooth muscle fibers, as well as the application of fluorescent ATP to directly measure ADP dissociation from the cycling crossbridge were studied. Of the two classes of smooth muscle, the slow tonic type, unlike the faster phasic type, was found to have a higher affinity for MgADP and relatively low affinity for ATP, resulting in a prolongation of the strongly bound crossbridge state and slowing of muscle relaxa-

tion, even though myosin regulatory light chain phosphorylation had returned to resting levels. A significant finding, obtained by using a novel ADP analogue and monitoring fluorescent transients, was that regulatory light chain phosphorylation and strain on the crossbridge modulate ADP release in smooth muscle myosin[10]. Thus, strain was shown to alter chemomechanical coupling of these proteins. This slow ADP release prolongs the fraction of the duty cycle occupied by strongly bound actomyosin·ADP states and contributes to the well-known and characteristic high economy of force in smooth muscle and the catch/latch-like state.

Another major area of research focused on the source(s) of Ca^{2+} needed for contraction, whether from influx during depolarization or from internal stores. Smooth muscle, unlike the well-studied skeletal muscle, was thought not to have a sarcoplasmic reticulum (SR). We first demonstrated that there was a network of SR throughout smooth muscle cells and defined surface couplings where the SR was closely opposed to the plasma membrane much like the diads in cardiac muscle[11]. The volumes of SR varied from 1.5-7.5% of cell volume which correlated with their phasic or tonic behavior respectively. After defining the morphology, the question was whether this SR stored calcium and if so whether it was sufficient to lead to full activation of contraction. In order to directly measure elemental composition within organelles in cells we developed rapid freezing techniques and cryosectioning to preserve the elemental distribution and used electron probe x-ray microanalysis to detect and quantitate ions at high spatial resolution within these sections. The application of these techniques to biological specimens was in its infancy so we invested a huge effort into instrument development and quantitation of elements with Henry Shuman, Meredith Bond and others. By focusing the electron beam on elements of SR, mitochondria, cytoplasm, nuclei or extracellular space in muscle sections snap frozen in different states of contraction, we could follow the movement of ions over time. Outcomes showed that smooth muscle SR but not mitochondria stored Ca that was released upon stimulation[12].

X-ray microprobe and EElS analysis days.

Development of this technology allowed application to other tissues. We demonstrated that the endoplasmic reticulum in liver frozen in situ is a major intracellular store of Ca, while the concentration of Ca in mitochondria is low and compatible with the regulation of mitochondrial enzymes. Applications to frog skeletal muscle argued against a hypothesis held at the time, that EC-coupling depended on the existence of a large sustained trans-SR potential during tetanus. Following the Ca release from the terminal cisternae (TC) of the SR and counter ion uptake during a tetanus[(13)] approximately 60-70% of the total fiber calcium was localized in the TC in resting frog muscle. During a 1.2 s tetanus, 59% (69 mmol/kg dry TC) of the calcium content of the TC was released, enough to raise total cytoplasmic calcium concentration by approximately 1 mM. This is equivalent to the concentration of binding sites on the calcium-binding proteins (troponin and parvalbumin) in frog muscle. Importantly, there

was no significant change in the sodium or chlorine content of the TC during tetanus. The unchanged distribution of a permeant anion, chloride, argued against a hypothesis held at the time, that EC-coupling depended on the existence of a large sustained trans-SR potential during tetanus. With Hugo Gonzalez, Henry Shuman and George McClellan, we showed that at 400 ms following a 1.2-s tetanus, the force had relaxed to base-line, and 0.3 mmol of Ca^{2+}/liter of cytoplasmic H_2O had been pumped by the SR, This indicated that the in situ pumping of the SR Ca-ATPase is sufficiently high to account for the removal of Ca^{2+} from the Ca^{2+}-specific sites of troponin and for the rate of relaxation from a tetanus at room temperature. The half-time of the return of the total 1.0 mmol of Ca^{2+}/liter of cytoplasmic H_2O released during a tetanus was 1.1 s, comparable to the slow K_{off} rate of Ca^{2+} from (carp) parvalbumin (1.0 s-1) and consistent with the hypothesis that the return of this Ca^{2+} to the terminal cisternae is rate-limited by the Ca^{2+} off-rate from parvalbumin[14]. Andrew also worked with Bernd Walz on honey bee photoreceptors that contain large sacs of ER. The elemental composition of the ER was determined by electron probe x-ray microanalysis and was visualized in high-resolution x-ray maps. During a 3-sec nonsaturating light stimulus, approximately 50% of the Ca content was released from the ER accompanied by a highly significant increase in the Mg content of the ER; the ratio of Mg uptake to Ca released was approximately 0.7. These results show unambiguously that the ER is the source of Ca^{2+} release during cell stimulation and suggest that Mg^{2+} can nearly balance the charge movement of Ca^{2+} in this tissue.

A common thread throughout Andrew's research career was a fascination with excitation-contraction coupling (E-C coupling) and signal transduction. This began with the 1968 review[1] when we proposed that there were two mechanisms; electromechanical and pharmacomechanical coupling. Electromechanical coupling involving a change in membrane potential. Pharmacomechanical coupling, (a term originating during a conversation at a bar at a Gordon Conference with Alexander Sandow and Saul Winegrad) was not associated with any obligatory change in membrane po-

tential. We now know that multiple signal transduction pathways mediate pharmacomechanical coupling. Both mechanisms may occur simultaneously. Investigation of the underlying pathways have pervaded our research to the present day and progress in the field has been extensive, involving many laboratories and is relevant not only to smooth muscle signaling but also to signaling in non-muscle cells.

The development of a coupled skinned fiber[(15)], where receptors retained their in vivo coupling to downstream signaling cascades was critical to the discovery of different modes of signal transduction. In collaboration with Jeff Walker, Yale Goldman, and David Trentham, we demonstrated the temporal response to $InsP_3$ and also the concentration dependence[(16)] using flash photolysis of caged inositol 1,4,5-trisphosphate ($InsP_3$). Tellingly, the rate of tension rise was comparable to the in vitro rate of myosin light chain phosphorylation and thus an integral component of the in vivo activation mechanism. These findings strengthened a previous study with Toni Scarpa and Meredith Bond where we first had shown, using a Ca^{2+} electrode and permeabilized pulmonary artery, that $InsP_3$ could release Ca^{2+}[(17)]. Later studies with Graham Nixon localized $InsP_3$ receptors to smooth muscle surface couplings(18) and today $InsP_3$ is recognized as a second messenger in smooth muscle and is common textbook knowledge. Based on these studies of modes of pharmacomechanical coupling and the relationship between $[Ca^{2+}]_i$ and RLC_{20} phosphorylation, the concept of 'Ca^{2+} sensitization' and 'Ca^{2+} desensitization' emerged and efforts were directed towards uncovering the messengers, network pathways and molecular mechanisms, reviewed in 2003[(19)]. With Toshio Kitazawa, Ming Cui Gong and others, Andrew found that Ca^{2+} sensitization was linked through trimeric G proteins to the small GTPase, RhoA and Rho kinase leading to the inhibition of myosin light chain phosphatase(MLCP)[(20)]. Studies of the regulation of myosin phosphatase became a focus of several laboratories. A productive collaboration with Tim Haystead, Philippe Gailly and Phil Cohen demonstrated the necessity of the phosphatase regulatory subunit (MYPT1) for myosin light chain phosphatase (MLCP) activity in smooth mus-

cle. Cyclic AMP through phosphorylation of telokin was found to enhance MLCP activity resulting in Ca^{2+} desensitization and relaxation of force and reported in a series of publications with Joan Woo, Alexander Khromov, Laurie Walker and others. Thus, myosin phosphatase could be regulated independently of changes in Ca^{2+}. This regulation is relevant not just to smooth muscle contraction but to non-muscle cell migration such as occurs with metastasis. Collaborations with our structural biology colleagues, Zygmunt and Ursula Derewenda brought new insights into the structure and function of the RhoA-GDP(21) and the RhoA/RhoGDI complex(22). Efforts were directed towards uncovering the messengers, pathways and mechanisms of regulation of smooth muscle contraction and relaxation continue, driven by the rational that they will lead to the development of new targets for treatment of vascular disease, asthma and motility dysregulation of other hollow organs.

An active and engaged citizen in the scientific community, Andrew was well known for his insightful and often provocative discussions at scientific meetings. His training in debating by the Jesuits served him well, combined with a remarkable command of the literature. He loved discussing the pros and cons of new findings or theories. Striving for excellence in everything, he was as critical of his own work as he was of the work of others, and stimulated much collaboration, a valued mentor to fellows, students and colleagues. In a previous memorial, colleagues stated, "Andrew Somlyo will be remembered for his scientific rigor, intense work, pioneering spirit, infectious enthusiasm for research and great appreciation for the arts".

Family History

Andrew grew up in Budapest, raised as the studious only child of a physician until his life was disrupted by war, political upheaval, and personal loss. During the siege of Budapest, the family lived in a bomb shelter through the advance of the Soviet Army and into the occupation. The occupation was colored by

open violence and fear of the Soviet Army, which proved to be well justified for his family. The Minister of the Interior was in control of the police, aided by Russian Secret Service officers was headquartered on Andrassy Street, near their home. People opposing the Communist Party disappeared and were known to be sent to concentration camps. Andrew's father refused to join the Party and to accept the post as director of a nationalized group of hospitals. A new employee at the clinic denounced him as being against the People's Democracy, for which he was arrested and charged. Before he went to trial he died of a massive heart attack at the bedside of a patient, at the age of 48. During this time Andrew graduated from the Jesuit Szt. Istavan High School in Kalocsa, which was soon taken over by the state and the Jesuit priests, including the principal Fr. Alajos Tull, whom he much admired, were arrested or disappeared. Many of Andrew's anti-communist friends had been arrested, Andrew's mother was warned by police officers, who had been patients of her husband, that she was in danger and had to leave immediately, and Andrew escaped the country in 1948 crossing into Austria with the help of politically sympathetic people who knew the area well. Andrew's mother had a harrowing escape in a farmer's wagon under hay and sacks of potatoes. In Vienna, under false papers as Austrian citizens, they made their way to the American zone and then separately to French occupied Innsbruck. The goal was to make their way to Italy, where it was thought to be easier to obtain American visas. By hiring mountain climbers who knew the smuggling routes, but turned out to belong to Waffen-SS, they arrived at a small Italian village. Lacking visas, but having a letter of recommendation from a Vatican cardinal, they went to the Italian police and were granted political asylum. They lived in Rome, where Andrew was involved with the theater and studies. Life was pleasant until 1951 when they received visas to enter the United States and came by a cruise ship to New York and then settled in Chicago. Andrew then began his medical education. Andrew always claimed that experiencing these horrific events as a very young man made him fearless in confronting unjust authority in the academic and public sector. He was outraged

Andrew *and* **Andrew Jr.** *in the garden.*

by injustice and dishonesty, and fiercely stood for his friends and principles, knowing intimately the cost of silence.

Our son Andrew Paul Jr was born in Philadelphia in 1972. He grew up in the laboratory and traveled to meetings around the world with us. We thought he would be a writer of science fiction or a philosopher but to our surprise he suddenly announced he was applying to medical school! In later years this brought clinical medicine back into Andrew's life and he loved discussing cases with our son. Andrew Jr. works as a nephrologist in Seattle where he also coaches a variety of martial arts, including Historic European Martial Arts and an occasionally consults for a group of science-fiction and fantasy authors, confirming some of our early suspicions.

As a Student and Collector of the Arts

One of the great foundations of our relationship was Andrew and my shared love of the arts. Andrew and I were, and I continue to be, just as passionate about the arts as our science, which are profoundly complementary endeavors. They both require great passion, research into history, analysis of techniques, and much looking at data and objects. Discussions with others in the field hone one's analytical and observational skills to reach an in-depth understanding. Data from a well-executed experiment is as artistically satisfying as a fine piece of Kakiemon porcelain or Tibetan thangka. We dove into learning and collecting art together and our interest paralleled our scientific career as we explored several areas in depth. Japanese porcelain captivated us. Our research took us to numerous meetings in Japan where we visited many museums and met many knowledgeable art dealers, and we developed a network of friendships with collectors, art dealers, and scholars wherever we traveled. Setsuro Ebashi wrote in a memorial to Andrew, "Andrew was a lover of Oriental culture…. I sometimes felt that Andrew's sense was more Japanese than that of ordinary Japanese." Recently, an exhibition of our 17th C Japanese collection was shown in London in celebration of the four hundredth year since the first ships carrying Japanese porcelain for the stately homes and palaces arrived at Plymouth UK from Japan. We discovered of Himalyan art, paintings and 13th-17thC bronzes at Sotheby's in 1989 which opened up a new field of study and collecting for us. Many of these objects have been lent to museums over the years. Just as in science where scientific colleagues enrich one's life, so do the collectors, dealers and scholars in the art world. Somehow, the ability of humans to make beautiful meaningful art restores one's faith in mankind when the world news of wars and atrocities seem to dominate our lives.

Acknowledgements

I thank Dr. Miklos Kellermayer for inviting me to contribute to the volume Hungarians in Research and the opportunity to present my views on Andrew's life. In speaking for Andrew and myself, I would also like to express how grateful and fortunate we have been in having outstanding collaborators, fellows and students. None of this would have been possible without them. Unfortunately, I have not been able to cite all of their contributions and note that when I say "we" it includes all. Finally, I am very appreciative of the previous obituaries written by Drs. Marion Siegman, David Hartshorne, David Trentham and Richard Murphy, that I have expanded on for this article. I thank our son Andrew Paul Jr., M.D. for insights and editorial suggestions.

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Brunó F. Straub — Discoverer of actin
(1914-1996)

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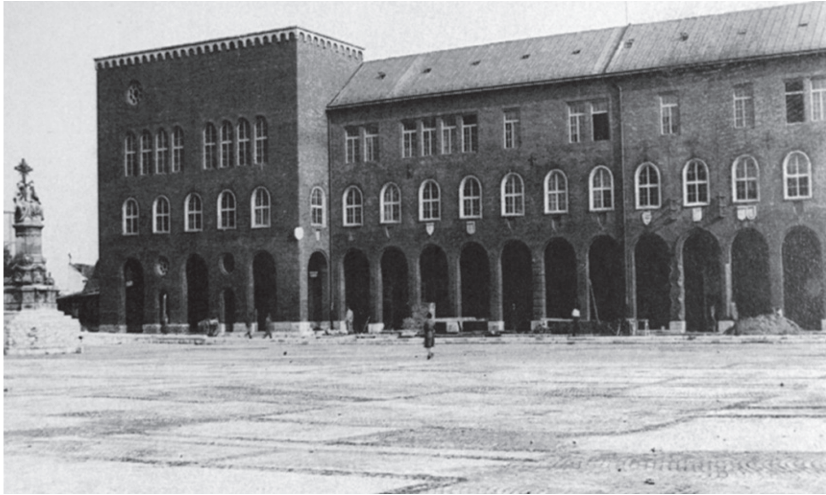
Brunó F. Straub *in 1988, as president of Hungary.*

Brunó F. Straub deserves a chapter in this book, and an eternal place in the history of muscle research, as the discoverer of actin, but he was much more than that. Undoubtedly, he was one of the most influential personalities of the scientific life of Hungary in the second half of the twentieth century. He belonged to that generation of great scientists, like Miklós Jancsó, György Ivánovics, Zoltán Bay, Győző Bruckner, János Szentágothai and others, whose career started before the second world war, who achieved international fame in their respective fields, who could maintain the high scientific standards of their research throughout the devastation of the war, the repression of the nazi and the communist terror systems, and who deserve

credit for the continuous presence of high-quality scientific life in Hungary.

Straub was born on 5th January 1914 in Nagyvárad, Bihar county (at present: Oradea, Romania); his father was a high-school teacher. Nagyvárad was one of the most cosmopolitan towns of pre-war Hungary, characterised by a high level of cultural, intellectual life. As a result of the Treaty of Trianon concluding World War I, Nagyvárad became part of Romania, and this resulted in a mass-exodus of Hungarian professionals and state-employees, among them the Straub family. They settled in Szeged, and in 1932 young Brunó enrolled in the University of Szeged as a student of medicine. There he became immediately fascinated by the brightest star of the faculty, the Professor of Biochemistry, Albert Szent-Györgyi, and in 1933 he joined his research-team. Szent-Györgyi was an unorthodox teacher, whose non-conformist personality differed strongly from the conservative, provincial atmosphere of the university, and he, recognizing the exceptional talent of his young student, suggested to him to leave medicine, and study chemistry instead. Straub followed the advice of the master, and obtained his degree in chemistry in 1936. All through his student years he was an active and productive member of Szent-Györgyi's research team involved in the study of the biochemistry of oxidation, which resulted in the awarding of the Nobel-Prize to Szent-Györgyi in 1937 (contrary to the popular belief in Hungary that he received the Nobel-Prize for the discovery of vitamin C, the prize was given "for his discoveries in connection with the biological combustion processes, with special reference to vitamin C and the catalysis of fumaric acid"). Straub's first scientific paper was published in 1935 on the role of fumaric acid in metabolism[1]. From 1937 he spent two years in Cambridge, in the laboratory of David Keilin, with the help of a Rockefeller fellowship. There he could finish several earlier experimental projects, and established his place among the pioneers of biological oxidation research. He purified and characterized two of the important enzymes of the oxidation cycle: lactic acid dehydrogenase[2] and malic dehydrogenase[3], and discovered an enzyme named after him: Straub's diaphorase[4].



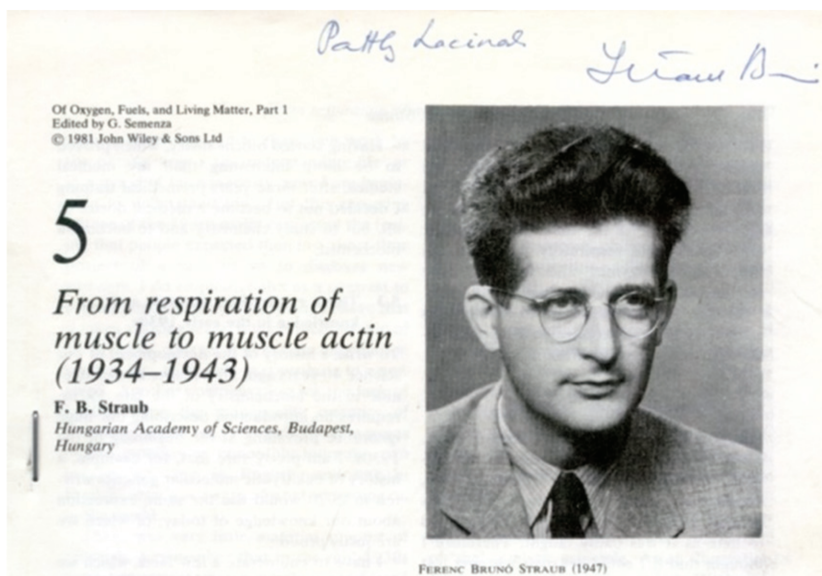
Szent-Györgyi's Institute in the University of Szeged.

After his return from Cambridge to Szeged, Straub, together with the entire team, followed Szent-Györgyi's turn of interest to muscle research. Despite the vicissitudes of doing research in a war-torn country, completely isolated from the scientists of the English-speaking world, the Szent-Györgyi laboratory became world-leader in this field during these years. As described in another chapter of this book (Magdolna Hargittai on Ilona Banga), Szent-Györgyi's interest in the problem of muscle contraction was aroused by the ground-breaking experiment of Engelhardt and Lyubimova showing the enzymatic activity of myosin, and this led eventually to the characterization of this substance as actomyosin, and then to the isolation of actin from this complex. The discovery of actin was published in papers authored by Straub and Szent-Györgyi[5,6,7], and every history of biochemistry ascribes the glory of this discovery to Straub. However, as discussed by Hargittai, a strange remark of the aged Szent-Györgyi cast doubt on this generally accepted view, by saying that the real discoverer was Ilona Banga. Hargittai's description of this controversy understandably reflects the point of view of Banga. I spent the first decade of my scientific career as a graduate student and close collaborator of Straub, and the next two decades under his

Szent-Györgyi's research team at the University of Szeged in cca 1933. First row: **Margaret Zétényi** *(secretary),* **Béla Gozsi** *(pharmacist),* **Albert Szent-Györgyi, Ernő Annau.** *Second row:* **Joseph Svirbely, Edit Joó** *(laboratory assistant),* **Nelly Szent-Györgyi** *(Szent-Györgyi's daughter),* **Ilona Banga**. *Third row:* **Brunó F. Straub** *(medical student),* **Kálmán Laki** *(medical student),* **Sándor Szalay** *(physicist).*

directorship, but as during these years I was not aware of this controversy, I never discussed it with him. However, after his death I put this question to Tamás Erdős, who was a member of the Szent-Györgyi team in this period, and his opinion was unambiguous and in complete agreement with Moss, the biographer of Szent-Györgyi: actin was discovered by Straub.

After the discovery of actin, the raging war determined the fate of the actors. Szent-Györgyi disappeared from public life as a participant of the resistance movement, and Straub was drafted into the army and was forced to withdraw with it to Germany. With the armistice, Szent-Györgyi was invited to Budapest University, and became a prominent personality in the public life of the new republic, while the 32-year-old Straub was appointed to his abandoned chair in Szeged as full professor. This marked the beginning of the second part of his career. Although he contin-



Of Oxygen, Fuels, and Living Matter, Part 1
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From respiration of muscle to muscle actin (1934–1943)

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FERENC BRUNÓ STRAUB (1947)

Portrait of the young professor in 1947.

ued to do experiments with his own hands in the field of muscle research, the centre of his activity became more and more that of the university professor, teacher and educator of a new generation of biochemists.

In 1947 Szent-Györgyi left Hungary (or more correctly: failed to return from a foreign trip, or with a word fashionable in a later period: defected from the communist-ruled Hungary), and in 1949 Straub was again appointed to replace him in Budapest. The Szent-Györgyi chair was divided into ”Biochemistry” and ”Medical Chemistry” and Straub became the head of the latter, a position he filled for more than two decades, until 1970. During his tenure there, he published several outstanding textbooks such as ”General, inorganic and analytical chemistry (1950)”, ”Organic chemistry (1951)”, ”General and inorganic chemistry (1958)” and ”Biochemistry (1958, 1965)” for generations of medical students. The three former books were concise introductory texts for undergraduates studying medicine, but Biochemistry was, in this period, the only comprehensive summary of this field of science available in Hungarian. Its second edition (1965), following the

development of the field, substantially differed from the first edition (1958) and was translated into several foreign languages. All these books bore the characteristic features of Straub's mindset: the ability to synthesize, the concentration on processes, not on data, the suggestive force of style, the clarity of expression. He did not like teaching, nevertheless he was an excellent lecturer, and generations of medical students remembered his lectures as the most exciting, impressive and also entertaining experiences of their student years. They were entertaining, because they were filled with fresh ideas, jokes, outlooks to other fields of the sciences and the arts, and radiated the authority of a great personality. In 1960 he became the director of the Institute of Biochemistry of the Hungarian Academy of Sciences, and in the following decade he kept both of these positions. In 1970 he left the University and took up the general directorship of the new Biological Research Centre of the Academy in Szeged, founded and organized by him. In 1978 he stepped down from the position of director-general, and kept only the directorship of his former institute, now the Institute of Enzymology, a fifth unit of the Centre. He retired from this position and from scientific activity in 1986.

In these post-war years his scientific productivity was much lower than in the previous decade. This was due partially to his other activities, partially to the extremely poor prevailing conditions for scientific research, and partially to the fact that, contrary to the then general practice of professors in Hungary, he never put his name on publications from his staff if he did not actually participate in the work. Here I mention only a few of these results. A new method of ATP assay (with M. Kramer)[8] is a good example of how creativity can overcome the severe difficulties of experimental work in a war-torn country. In an important paper (with G. Feuer)[9] they established the fact that ATP is the cofactor of actin. In a paper (with G. Gárdos)[10] that changed the direction of membrane research, they demonstrated the influence of ATP on the permeability of the red blood cell membrane. They discovered (with P. Venetianer)[11] the protein-disulphide-isomerase enzyme and characterized its mode of action. Perhaps his most

important (and unfortunately neglected) contribution in these late years was the formulation of the theory of „induced fit" of enzymes (with G. Szabolcsi, who was his second wife). This pioneering hypothesis was published only in Russian[12], and therefore ignored by the mainstream, only to be rediscovered later by several others who were then recognized as the original authors[13].

Straub's role as a master of generations of biochemists, as the "great old man of Hungarian biochemistry" was even more important than his own research. Today it is not easy to appreciate the importance of the fact that in 1949, one of the darkest periods of the cold war the editors of the "Annual Review of Biochemistry" asked a scientist from behind the Iron Curtain, from a country suffering under Stalinist terror, to write the chapter on muscle biochemistry[14]. After 1956, the Iron Curtain was partially lifted, and the first Western-European and American life scientists, who visited Hungary after years of nearly complete isolation, wanted to see Straub in the first place, and the young and aspiring biochemists from the neighbouring countries came to his laboratory to learn the tricks of the trade. In fact, several leading personalities in the scientific community of biochemists of Poland, Czechoslovakia and Bulgaria started their careers as graduate students, postdoctoral fellows, or visiting scientists in one of the two institutions led by Straub.

In an evaluation of the life and work of Straub, the most important aspect is probably his role in the science policy of his country. He was elected to the membership of the Hungarian Academy of Sciences as early as 1946 (he was then 32 years old), he was a member of its presidium between 1949 and 1958, and he was its vice-president for two periods (1967-73, 1985-88). His two most important and lasting achievements were the establishment of the biological section of the Academy (that is, the separation of the biological sciences from the section of medicine; he became the first secretary of this new section between 1962 and 1967), and the founding of the Biological Research Centre of the Academy in Szeged. This proved to be the largest scientific investment ever since in Hungary. An essential contribution to its success was a large grant from the United Nations

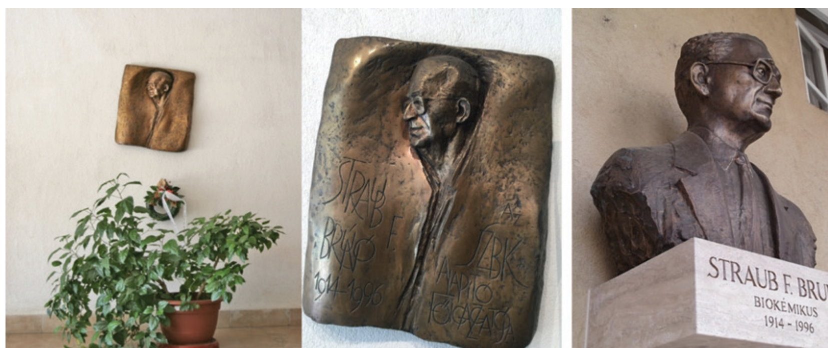
Development Program, obtained through the efforts of Straub. He became the first Director General of this new centre, and of course, his personality and his views on science were decisive factors in forming its direction and spirit. He was responsible for many characteristic features of the organization and working life of this institution, which were unique in this period behind the Iron Curtain. The intention to create the new centre was obviously to establish a home for research in molecular biology. This was unfortunately still a dirty word in the sixties, in Eastern Europe. Therefore, the four units of the new centre were called Institutes of Biochemistry, Genetics, Biophysics and Plant Physiology. The former three of these disciplines – in agreement with the opinion of most historians of the new branch of the tree of biology – represented the main sources of molecular biology. In contrast, plant molecular biology did not exist yet at that time. It was therefore a proof of Straub's foresight that he included this discipline when planning the structure of the centre. The next two decades completely supported the correctness of his vision, because most of the breakthrough results from the Szeged centre came from this field.

Among the other unique features, it is worth mentioning that, in contrast to the practice of that period, none of the newly appointed directors of the units were party members. The library had an open-shelf system (with The New York Times and Newsweek on the shelves, among others) and it was never closed. Young, inexperienced scientists could become principal investigators if they had an original idea. On Saint George's day, following an ancient Hungarian folk-tradition, any young researcher had the opportunity to opt for a change of boss, and join another group. These, and other factors contributed to the success of the new Centre, which was hailed in 1978 by reporters of *Nature* as the new "Warm Spring Harbor" of Europe[15].

Straub also played an important role in international science policy. He was the vice-president of the International Atomic Energy Agency (IAEA, 1967-68), a participant of the Pugwash Conferences in the sixties, and the president of the International Council of Scientific Unions (ICSU, 1976-78).

Straub *on the height of his career, in 1988.*

In the last active period of his life, after his resignation from the directorship of the Institute of Enzymology (in 1986), he also played a role in the public life of the country, not in direct contact with science. He was a Member of Parliament (1985-90), the President of the Council for Environmental Protection (1978-88) and finally the President of the Presidential Council (1988-89, in fact: the president of the country). His acceptance of this last position was disapproved by many of his friends and colleagues (including myself), because his predecessors in this position were insignificant puppets, faithful cadres of the communist system. Later, however, sensing the very favourable reaction to this appointment within the international scientific community (i.e. that after Israel's Ephraim Katzir, a well-known scientist became the president of a country again), I revised my opinion. After all, it was a welcome fact, and a harbinger of the coming political change that this position was filled not by a party-hack, but by an internationally respected, highly cultured European personality. As president, Straub represented Hungary at the burial of the emperor of Japan, he received the first visiting US president



The bas-relief in the hall of the Biological Research Centre (left two images). Straub's bust in the Szeged Pantheon (right).

in Hungary (Bush the elder) and he invited Pope John Paul II to Hungary.

Finally, just a few subjective words about his personality. When, after graduation, looking for a job, I first saw him, we did not talk more than five minutes, but I felt that for the first time in my life I met a really great man. It may sound preposterous, but I told myself what Napoleon was assumed to have said after meeting Goethe: ”Voilà un homme!”. Later I heard from several colleagues that they had similar feelings after the first encounter. As a person who wielded considerable power, he probably had enemies, some people obviously must have had various grudges against him, but everybody who worked with him in close contact in science or in various other organizations, admired and respected, and most of them also loved him. He radiated authority, nevertheless he not only tolerated, but positively appreciated contradiction, even impertinence. Remembering those 14 years, which I spent in his Institute of Medical Chemistry, I can characterize the atmosphere of that place with three features, all of which were determined by his personality. First: everybody was convinced that what we are doing is the most important thing in the world, in the progress of science (of course this was very far from the truth, the conditions were too bad for that). Second: the high culture was omnipresent, at the common lunch table every interesting new cultural event in Budapest was discussed,

and sometimes deep philosophical debates took place. Third: the presence of playfulness and humour (characteristic sentences and words from the books of Karinthy, Rejtő, Lewis Carroll, Milne and Wodehouse were permanent features of everyday communication).

Straub passed away on 15th February 1996. After his death a bas-relief of him was inaugurated in the hall of the Szeged Biological Research Centre. On this occasion, remembering him, I quoted Antoine de Saint-Exupéry:

> *"If you want to build a ship, don't drum up the men to gather wood, divide the work, and give orders. Instead, teach them to yearn for the vast and endless sea."*

Well, Straub was a person uniquely able to imprint two generations of scientists with this yearning for the vast and endless sea of scientific research. His bust was inaugurated in the Szeged Pantheon on the centennial of his birth in 2014.

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Albert Szent-Györgyi — In pursuit of understanding the living state
(1893-1986)

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To see what everyone has seen
and think what no one has thought.
(Albert Szent-Györgyi)

Albert Szent-Györgyi, *in the early 1930s in his laboratory at the University of Szeged. (photos from The Szent-Györgyi Papers, National Library of Medicine).*

Internationally, Albert Szent-Györgyi is one of the best-known Hungarians. His fame is mainly due to his winning the Nobel prize in 1937 "for his discoveries in connection with the biological combustion processes, with special references to vita-

min C and the catalysis of fumaric acid". This led to his becoming a living legend in Hungary.

He was one of the 14 Nobel laureates of Hungarian origin who was awarded the medal while still a Hungarian citizen [1]. In this way, one Nobel medal came to Hungary: the original gold medal (207 g) today remains in the Hungarian National Museum in Budapest. (Please, note that in 2002 Imre Kertész also received the Nobel prize, in literature, as a Hungarian citizen.)

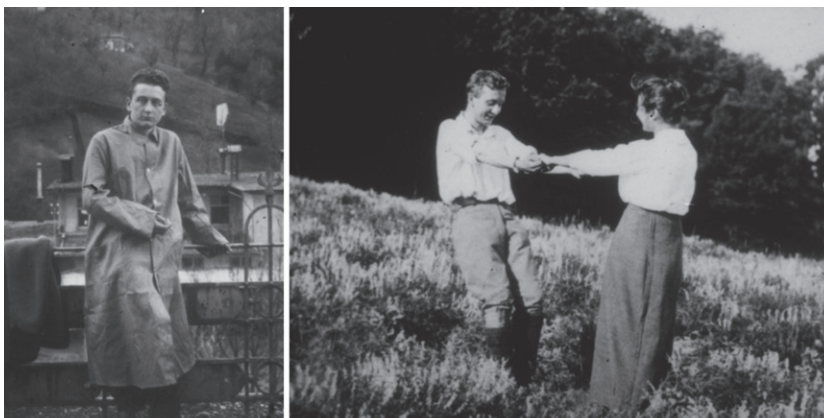
His scientific activities in Hungary and in Szeged, in particular are well known and well-documented [2], and an interview-based detailed biography entitled *Free Radical* was published in 1988 [3]. The title of this book refers to his independent personality and also to his increasingly acknowledged view of the decisive role of radicals in causing cancer. It has been translated into Hungarian 15 years later [4].

A concise biography of Albert Szent-Györgyi

Albert Szent-Györgyi was born Budapest in 1893 (see *Table 1* for important biographical facts). His father, Miklós Szent-Györgyi, was a landowner of noble descent, and his mother, Josefa Ludovika Lenhossék, was the descendant of the famous Lenhossék family member of anatomists. While his father had little infuence on Albert's life, his uncle, Mihály Lenhossék, was his role model. Szent-Györgyi had very weak relationship with his brothers, Pál and Imre, throughout his life.

Szent-Györgyi started his medical studies at Pázmány University (predecessor of Semmelweis University) in Budapest, 1911, and began experiments in his uncle's department. His studies were interrupted by World War I, during which he served in Ukraine. He was wounded (in fact, by his own gunshot through his arm), therefore invalidated and transferred back to Budapest. During his convalescence, he completed his medical studies, and was awarded his diploma in 1917. In the same year, he married Kornélia Demény, daughter of the Postmaster General. The end

Left: **Mihály Lenhossék,** *director of the Department of Anatomy at Pázmány University, Budapest. Right:* **Albert Szent-Györgyi** *(left) with his brothers* **Pál** *(middle) and* **Imre** *(right).*



Albert Szent-Györgyi *on the rooftop of a hospital in Udine, in 1917 (left).* **Albert** *with his wife* **Kornélia** *in Northern Italy, in 1917 (right).*

Albert Szent-Györgyi *and his daughter* **Cornelia** *(„Little Nelly") in Italy, in 1955.*

of the war saw him again at the front, this time in Italy. Their daughter (in fact, Szent-Györgyi's only direct descendant), Nelli was born in 1918.

The next few years were a hectic period for Hungary because of the war and the ensuing Trianon peace treaty. And it was just as hectic for Szent-Györgyi. He worked successively in Pozsony (today Bratislava, capital of Slovakia), Budapest, Prague and Berlin, just a few months in each. Then came a longer period in Hamburg. Later, he went to The Netherlands, where in Leiden and especially in Groningen he worked successfully on biological combustion. At that time, the main question in biochemistry was the subject of controversy between Heinrich Wieland and Otto Warburg and their followers. Heinrich Wieland held that the dehydrogenation of substrates was the basic process involved in biological oxidation, and that oxygen reacts directly with such activated hydrogen atoms. Otto Warburg suggested that activated

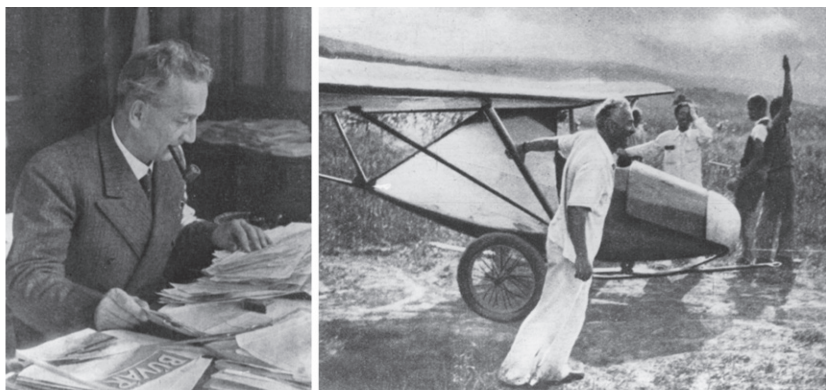
oxygen was essential for biological oxidation. Albert Szent-Györgyi recounted:

> *„I got interested in the controversy and found a very simple way to show that both were right, that active oxygen oxidized active hydrogen. I wrote a very nice paper about it" [3].*

This paper later became a landmark of 20th century biochemistry. Cambridge in England, his next workplace, became his scientific home, where he received his PhD in chemistry. In Groningen he had detected a strong reducing (anti-oxidant) substance in the adrenal gland. A similar material was found in lemon and orange juice and watery extract of cabbages. He extracted and purified it in Cambridge (about 1 g, good crystals), and it proved to be a carbohydrate, probably a sugar acid, with chemical formula $C_6H_8O_6$. At that time, 1 g was not enough for a detailed structural analysis.

His findings immediately aroused interest among biochemists worldwide, and he submitted a manuscript to the Biochemical Journal. In this paper he eventually had to decide on a name for what had simply been called „Szent-Györgyi's substance". He proposed calling it „Ignose", from the Latin „Ignosco" („I don't know"), while the ending „-ose" means a sugar compound. With this name, the manuscript was not accepted. Szent-Györgyi resubmitted it, renaming his compound „Godnose"(„God knows sugar"). Finally, the editor suggested the much more prosaic „hexuronic acid", and the paper was published with this nomenclature.

Szent-Györgyi received a professorship in Szeged, Hungary in September in 1928, but he got two sabbatical years to finish his work in Cambridge. In 1930 he moved to Szeged, where for the next fifteen years he was in the center of not only the scientific, but sports and intellectual life as well. ALreasy in 1931, it was proved that hexuronic acid and the vitamin C are one and the same, and in 1932 Szent-Györgyi found that paprika is „a regular mine of vitamin C". Since paprika is not a particularly sweet fruit, there was less difficulty in separating vitamin C from



Albert Szent-Györgyi *working at his desk in Szeged in 1937 (left). Preparing for takeoff with a glider (cca. 1937-1939) (right).*

other sugar compounds, than in the case of oranges, for example. 1.5 kg pure, crystalline vitamin C was produced from paprika within a week. This was ample for detailed chemical analysis. Some was sent to N. Haworth (Nobel prize in chemistry in 1937) in Birmingham, who quickly established its chemical nature.

The first thing to do was to give „hexuronic acid" a new name. Szent-Györgyi and Haworth decided to call it „a-scorbic acid", meaning that it prevented the scorbutic disease, scurvy.

Szent-Györgyi's idea was that the medically recommended daily adult intake of vitamin C, 60 mg, is sufficient to combat scurvy, but a higher amount will make the body more resistant to other diseases. The current megavitamin therapy or orthomolecular medicine, based on this concept, was developed by Linus Pauling [5] (1901-1994), two-time Nobel-Prize winner, in 1954 for chemistry and in 1962 for peace. These results and his role in resolving the Wieland-Warburg dispute over biological oxidation earned Szent-Györgyi the Nobel Prize in 1937.

Following an era of the most successful experimental work on biological oxidation, muscle contraction became the new research field of Szent-Györgyi's group during World War II and later in the US. The subject of the research work was the pigeon breast.

Contribution to muscle research

In 1938, one year after receiving the Nobel-prize, Szent-Györgyi set out to explore something more complex, muscle contraction. Magical years followed which led to profound discoveries and the establishment of modern muscle biochemistry and biophysics. Actin, which turned out later to be the most abundant protein in essentially every eukaryotic cell, was discovered by Brunó F. Straub (see chapter on Straub elsewhere in this volume) in Szent-Györgyi's laboratory. It was shown that the mixture of actin and myosin, upon the addition of ATP, goes through a certain contraction, which Szent-Györgyi called superprecipitation. Superprecipitation wsa the first model of biological motion reconstructed in a test-tube out of two proteins. No words can reflect the excitement of this time better than those of Szent-Györgyi himself [19]:

> *I felt I had now enough experience for attacking some more complex biological process, which could lead me closer to the understanding of life. I chose muscle contraction. With its violent physical, chemical, and dimensional changes, muscle is an ideal material to study. If one embarks on such a new field one usually does not know where to begin. There is one thing one can always do, and this I did: repeat the work of old masters. I repeated what W. Kuhne did a hundred years earlier. I extracted myosin with strong potassium chloride (KCl) and kept my eyes open. With my associate, I. Banga, we observed that if the extraction was prolonged, a more sticky extract was obtained without extracting much more protein. We soon found that this change was due to the appearance of a new protein "actin," isolated in a very elegant piece of work by my pupil, F. Straub, while I "crystallized" myosin. Myosin, evidently, was a contractile protein, but the trouble was that in vitro it would do nothing. A contractile protein should contract wherever it is. So we made threads of the highly viscous new complex of actin and myosin, "actomyosin," and added boiled muscle juice. The threads contracted. To see them contract for the first time,*

and to have reproduced in vitro one of the oldest signs of life, motion, was perhaps the most thrilling moment of my life. A little cookery soon showed that what made it contract was ATP and ions. My conclusion, that muscle contraction was essentially an interaction of actomyosin and ATP, was soon strongly attacked, so I developed (later at Woods Hole) the method of glycerination, and glycerinated (extracted with diluted glycerol at low temperature) the psoas muscle of the rabbit. This method is now widely used for conservation of biological material such as sperm. On addition of ATP, my glycerinated muscle contracted, developing the same tension as it developed maximally in vivo. This satisfied me and I was sure that in a few weeks' time the whole problem of muscle contraction would be cleared up, but ten years later I still did not understand muscle, which made me conclude that something had to be missing from our basic ideas, something that was essential for the understanding of energy transformation. So I left muscle to find what this something is. This took me, gradually, into my present field, that of electronic dimensions and mobility.

Albert Szent-Györgyi *and* **Ilona Banga** *working in the laboratory in Szeged in the 1930s. (photo University of Szeged, Klebelsberg Library).*

The enormous significance of Szent-Györgyi's experimental contributions and their widespread influence on muscle biochemistry and biophysics are reflected throughout the chapters of this book. The Appendix of this volume contains the facsimile edition of the Studies from the Institute of Medical Chemistry, University of Szeged, which is a collection of papers, mostly on muscle, which Szent-Györgyi was unable to publish in appropriate international journals because of the ongoing World War II.

Albert Szent-Györgyi in The New York Times

The author of this paper has collected all those articles published in one of the most respected American newspapers, The New York Times, which involve the personality, opinions or activities of Szent-Györgyi during his life in the US (and earlier). More than one hundred newspaper pieces written by or about him provide an excellent picture of his scientific activities and his contributions to the well-being of the wider community [6]. The journal was founded in 1851, and in the 20th century, especially after World War II, it became one of the best-known and most-respected American newspaper.

The first report in connection with Szent-Györgyi was published in 1933, in the „Week of science" section of the Sunday issue, entitled „The power of sound". „Astonishing things happened. Small animals and bacteria were killed by the vibrations. Liquids boiled and decomposed. Waves were set up in them of a strange order." ... „ ... physicists and chemists began to explore the new field. The latest of them is Professor A. Szent-Györgyi, known over the world for his work in trying to find out of what vitamins are composed. In fact, Szent-Györgyi is one of the leaders in organic chemistry" [7].

The second piece of news came just a week later from the Budapest correspondent of The New York Times, under the title „Vitamin C may be obtained from paprika, chemist finds".

„General scientific interest has been aroused by the claim of a prominent Hungarian chemist, Dr. Albert SzentGyoergyi, pro-

fessor at Szegedin University, to have discovered after ten years research a method of producing vitamin C artifically. Professor Szentgyoergyi says he has established that the vitamin is abundantly present in the Hungarian paprika, or sweet pepper, which he holds contains at least four times as much vitamin C as an orange or a lemon. In his experiments he has used 10 000 paprikas and claims that he has now extracted the vitamin, which can be administered in the form of powder or pills even to tiny babies. Professor Szentgyoergyi's research work has been financed by wealthy American friends. He has already been invited to lecture on his discoveries in Berlin, Stockholm and Copenhagen" [8].

The third article was the news of his winning the Nobel Prize from Stockholm in 1937: „NOBEL PRIZE GOES TO SZENT-GYORGYI; Hungarian wins the award in medicine for discoveries in biological combustion; He isolated vitamin C" [9].

In the following years The New York Times published a number of articles on his vitamin research, and from 1945 on new results concerning muscle contraction, his new research field. For example, at the end of 1947:

"At a protein symposium at the Polytechnic Institute of Brooklyn yesterday Prof. Albert Szent-Györgyi, Nobel Prize winner in medicine, presented a résumé of the important researches on the mechanism of muscle contraction which he carried on during the war at the University of Szeged, Hungary, and more recently at the Marine Biological Laboratory at Woods Hole, Mass. …The body muscle is built of thin fibers which are but bundles of still thinner fibres, fibrils, which, in turn, are built of still finer threads. The muscle contracts, because these smallest threads contract. They are built of two proteins, actin and myosin, which form a complex. In itself this conplex shows no sign of motion. It becomes contractile by absorbing ATP. …" [10].

In 1954, a further news item was published in connection with him, illustrated with his photo, indicating the importance of the news and the person: „The 1954 Albert Lasker Award of the American Heart Association will go to Dr. Albert Szent-Gyorgyi, it was announced yesterday. The 60-year-old biochemist was cited for his ‚distinguished research achievements in the field of

Marta *and* **Albert Szent-Györgyi,** *1955.*

cardiovascular diseases which have led to new understanding of the basic physiology of the heart'" [11].

A very important event in Szent-Györgyi's life, again making the pages of The New York Times in 1955, was when he received American citizenship after spending 8 years in the US. „The winner of the 1937 Nobel Prize in medicine became a United States citizen today. Dr. Albert Szent-Gyorgyi, a native of Hungary, and his wife, Marta, took the oath" [12].

After this, news about him and his work regularly appeared in The New York Times each year. *For example about his new idea, the quantum mechanics in Biology, with the title „New light is shed on muscle action" „Quantum mechanics, the mathematics of modern physics, appears now to have important new applications in understanding heart and cancer problems, a researcher said here yesterday. What is involved in muscle contractions not a chemical reaction, it is an electronic proces." Dr. Szent-Györgyi suggested [13].*

An another paper in the SCIENCE IN REVIEW of The New York Times, Sunday, October 5, 1958:

Muscle Mystery. Much is still unknown about vital tissue's function. A muscle is one of those everyday things which sem simple until looked at closely.The closer the look, according to an eminent scientist who has devoted two decades to its study, the more complex muscle and its functions become. There is no another tissue whose function involves such sweeping and intensive changes in chemistry, physical state, energy and dimensions, according to Dr. Albert Szent-Györgyi, director of the Institute for Muscle Research at the Marine Biological Laboratory in Woods Hole, Mass. In its contractions and relaxations, he said, muscle controls the very pulse of life for animals and man.
„The heart and uterus are both in a way, but bags of muscle" said Dr. Szent-Györgyi, „and our blood pressure is regulated by muscles that determine the lumen (interior open space) of our smaller blood vessels."….
Miscarriages in pregnancy can result from abnormalities in muscle function. Dr. Arpad Csapo of the Rockefeller Institute for Medical Research has shown these abnormalities to be linked in a complex but specific way to hormonal disturbances of function in the muscle cells of the uterus wall.
The structure of muscle fibers and some of the details of muscle chemistry in contraction were considered at a conference sponsored by the New York Academy of Sciences [14].

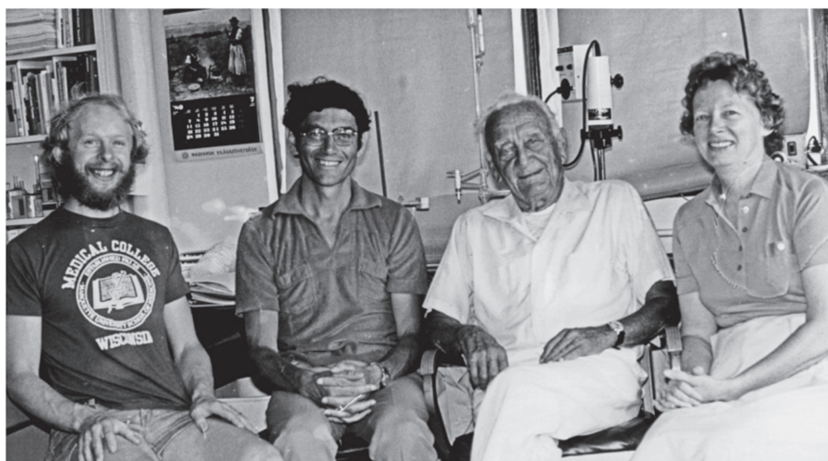
Arpad Csapo received his diploma at the University of Szeged in 1943. Albert Szent-Györgyi was his professor. Later they cooperated in the US, too. Arpad Csapo was professor of the Washington University in St. Louis from 1964. Marcia Houston was a young painter in St. Louis. The Csapo family introduced her to Szent-Györgyi, to prepare a portrait from a famous man. She became Szent-Györgyi's fourth wife.

As shown in *Table 2,* different items included scientific reports about his new research results (9 news from his cancer research), minor news announcements of different events, letters to the editor, philosophical articles of Szent-Györgyi about the future and interviews with him were published. Two maxima are revealed by the statistics. The first was in the early sixties, when,

Marcia *and* **Albert Szent-Györgyi,** *1976.*

besides the scientific reports, several „Letters to The Times" were published that warned against the danger of atomic war. The second maximum occurred in the early seventies, when Albert Szent-Györgyi was very active against the Vietnam War. For example, following the publication of his famous booklet „The Crazy Ape", he participated in a lengthy interview entitled „Biologist Doubts Man's Survival in a World Run by ‚Idiots' Too Old to Change" [15] and he wrote an article „15 Minutes to Zero" [16] at that time.

Later, Szent-Györgyi's activity decreased, and in 1986 The New York Times published his necrology [17]. He was 93 years old. It is symbolic that, one week after his death, the Patents section of the journal included a short news item: „Patent No. 4,620,014 was granted for compounds to help stimulate the body's immune system. The work was done by the late Albert Szent-Györgyi, a Nobel winner, and Gabor B. Fodor of the University of West Virginia" [18].



The last, faithful members of the research group: **Peter Gascoyne, Ronald Pethig, Albert Szent-Györgyi,** *and* **Jane McLaughlin, Woods Hole,** *1980.*

Albert Szent-Györgyi was one of the most remarkable thinkers of the twentieth century. Beyond his revolutionary discoveries that involved biological oxidation, muscle contraction, and cancer, in reality he was aways in pursuit of understanding the true nature of life and the human being. He left us with a wealth of inspiration and ideas, which extend beyond the mere laboratory wall and profoundly touch the intellect [19]:

> *"But is it true that science has no moral content? Is science not more than just a method of thinking, tools, or a collection of data and books? Is science not a living society? I think it is. To me, science, in the first place, is a society of men, which knows no limits in time and space. I am living in such a community, in which Lavoisier and Newton are my daily companions; an Indian or Chinese scientist is closer to me than my own milkman. The basic moral rule of this society is simple: mutual respect, intellectual honesty, and good will. So I think science does have its moral code which it offers as its third facet on which a new world order can be built. Science has raised man from stench and dirt, liberated him from the miasmas which decimated him in earlier times. It allows the bearing of children without fear. It has already shown the possibility of a*

dignified life, the expectation of which it has greatly extended. It is true, it has reduced man to a very modest place in Creation, but, then, why not try to lift ourselves, accepting the respo nsibility for our own fate? Why pull down one another, further poisoning our own atmosphere, showing how easily life can be wiped out? Science has opened endless possibilities for expansion if we work together instead of snatching small advantages from one another. Science has helped us to understand and master Nature. Maybe it will help us to understand and master ourselves, creating an elevated new form of human life, the wealth and beauty of which cannot be pictured today by the keenest imagination."

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[16] ibid, September 25, 1970,
[17] ibid, October 25, 1986,
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Table 1. Biographical details of Albert Szent-Györgyi

1893	born in Budapest
1911-	medical studies in Budapest
1914-	military service in World War I
1917	medical graduation, marriage
1918-1920	works in Pozsony, Budapest, Prague and Berlin
1921-1922	Hamburg
1922-1923	Leiden
1923-1926	Groningen, resolved dispute of Warburg and Wieland on biological combustion
1926-1930	Cambridge, PhD in chemistry hexuronic acid (later ascorbic acid, vitamin C)
1930	professor in Szeged
1932	vitamin C from paprika
1937	Nobel prize
1939-	muscle research
1942-	activity against Nazis
1944	avoided Gestapo, lived incognito in Budapest
1945	professor in Budapest
1946-1947	social activity
1948	move to USA, Director of Laboratory of Institute for Muscle Research, Woods Hole, Massachusetts
1955	American citizenship thymus gland research, cancer research
1970-	against the Vietnam War
1973	visit to Hungary after 26 years, opening ceremony of Biological Research Center in Szeged
1986	died in Woods Hole

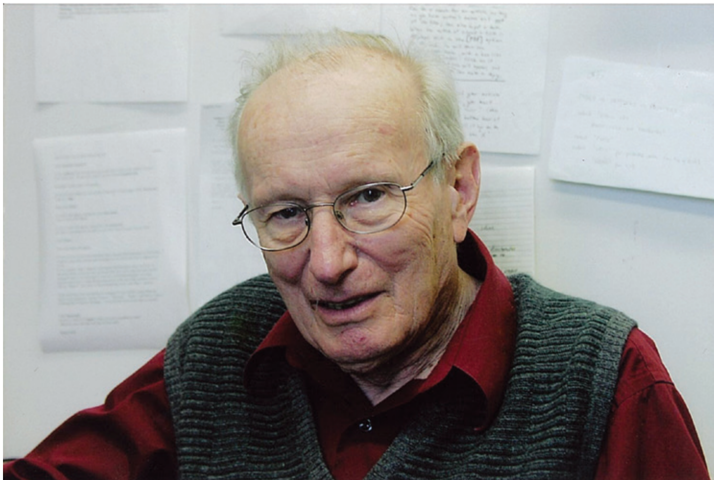
Table 2. Statistics of the articles on Szent-Györgyi which appeared, through the years, in The New York Times

year	number	year	number
1933	2	1961	7
1934	1	1962	10
1937	2	1963	11
1939	3	1964	2
1940	1	1965	5
1942	2	1966	8
1944	1	1967	5
1945	2	1968	1
1947	2	1969	3
1948	1	1970	8
1951	6	1971	7
1952	2	1972	6
1953	1	1973	3
1954	2	1974	2
1955	7	1975	3
1956	3	1976	2
1957	4	1978	1
1958	2	1981	1
1959	1	1986	2
			132

Andrew G. Szent-Györgyi — To be a Cousin of Albert and to Love Myosins
(1924-2015)

László Nyitray
Eötvös Loránd University, Budapest

„Ideas are cheap, but good data is hard to come by"
(Andrew Szent-Györgyi)



Andrew (Csuli) Szent-Györgyi.

Growing up in Budapest and joining Albert Szent-Györgyi's laboratory

Gábor András Szent-Györgyi, Csuli, as he was called by family members, friends and colleagues in science (a popular synonym for sparrow in Hungarian), was born in Budapest on May 16th, 1924. His father, a judge and excellent sportsman, was 56 years old when he was born, explaining why Albert Szent-Györgyi, his cousin, was so much his senior. That is why during his scientific career people often had mistaken him for Albert's son or nephew. Her mother, Lily Kronberger, "was a princess" (Andrew's words), a great figure skater, who won four successive ladies' world championships during the years 1907-1910. She was the first Hungarian woman to become world champion in any sport. Csuli's love of classical music, arts and sports was certainly greatly influenced by his parents (Zoltán Kodály was a family friend who later visited the Szent-Györgyis in the US).

After graduating from high school (Werbőczy Gimnázium), he became a medical student at Pázmány Péter University (where one of his classmates and lifelong friends was Georg Klein, the world-famous Hungarian-Swedish biologist and writer), the oldest continuously operating university in Hungary (founded in 1635, the medical faculty in 1769). He was contemplating to follow Albert Szent-Györgyi's footsteps. As he writes in his unpublished memoir [1][1]:

> *"I decided by the end of the first year in 1943 that I was more interested in doing research than practicing medicine. This was also greatly influenced by the book by Albert entitled: »Studies on Biological Oxidation and some of its Catalysts« published*

1 Quotes throughout the text are from the unpublished memoir of Andrew Szent-Györgyi with permisson from his daughter, Kathy Szent-Györgyi and his widow, Ursula Rowan.

in 1937, the year Albert received the Nobel Prize, which my mother used for teaching me English. It was certainly more interesting than any language book."

He started to work at the Department of Physiology. His first meeting with Professor Aladár Beznák was as follows:

"He asked me what I was interested in. I told him without knowing much: enzymes."

Later he was invited to Szeged by his cousin, but he first wanted to finish the third year of medical school. However, the course of history interrupted his plans:

"In retrospect the delay was an extremely lucky decision. The German army marched into the country in March of 1944. Albert was placed under house arrest and within a few months went into hiding from the Gestapo. (…) By that time Albert, together with his colleagues, had started to publish their fundamental studies on muscle biochemistry, which revolutionized the field, although because of the war this reached the western world with a five-year delay."

A detailed account on how Albert Szent-Györgyi survived the occupation of Hungary by Nazi Germany and how the results of the famous "Szeged Studies" were published was presented by Andrew at the European Muscle Conference in Hortobágy in September 2005 (it also appeared in print in [2]).

After surviving the hardships of war, Andrew finally joined Albert Szent-Györgyi, who became Professor of Biochemistry at the Medical Faculty of Pázmány University in Budapest. The laboratory became functional in September 1945. According to Andrew:

"Life in Albert's laboratory was an island of paradise. Discussions were free and interesting. Differing opinions were respected and, most importantly, there were no informers, which became more and more important as the Stalinist communist party grabbed more and more power over the country. The discussions at the table were wide ranging and free."

Szent-Györgyi's team continued to work on myosin. Andrew's first collaborator at the bench was a fresh university graduate and war survivor from Szeged, Endre Bíró (see the chapter about him in this book). Their most important finding, **the first major contribution** of Andrew Szent-Györgyi to muscle research, was that the steady-state ATPase activity was increased during the contraction of actomyosin or of minced and washed muscle [3]. This result is interpreted today that actin allosterically increases the Mg^{2+}-ATPase activity of myosin (see in this book the article by András Málnási-Csizmadia and Mihály Kovács).

Woods Hole years with Albert

After Albert Szent-Györgyi's departure from Hungary in 1947, Andrew managed to obtain his medical diploma and got married to the beautiful Éva Szentkirályi, his fellow medical student who was also interested in science.

Albert devised a plan on how his young investigators from Budapest could leave the country, where the political situation was getting worse, and the communist takeover was foreseeable. As Andrew writes:

"Albert wrote letters to his friends asking them to extend invitations for his young investigators to spend time in their laboratories, thereby facilitating their acquisition of passports to leave Hungary. Mihályi went to Stockholm to Theorell, Gergely to Evans in Manchester, Lajta to the Naples Biological Station, Lóránd to Astbury in Leeds, Laki to NIH in Washington.

Andrew Szent-Györgyi *working at MBL in Woods Hole, ca. 1954-56 (courtesy of MBL).*

I was invited by Fritz Buchthal in the Neurophysiological Institute of Copenhagen in Denmark."

After a short stay in Denmark the young couple arrived to the US in the summer of 1948, and went to Woods Hole, MA, where they stayed until 1962. Woods Hole was an extraordinary place to be and to work. The Institute for Muscle Research was established by Albert Szent-Györgyi at the Marine Biology Laboratory. MBL's strength was (and still is) the summer Physiology Course attracting the world's leading scientists and students. Nothing highlights this more than the fact that as many as 57 Nobel Prize laureates have significant affiliations with the MBL. As Andrew describes it:

> *"The high quality of science and the beauty of Woods Hole were stunning. The place was a hub of biology. (…) The 13 years that we stayed in Woods Hole were a particularly exciting time in biology. It encompassed the development of molecular biology. I remember walking back after the lectures following the extensive discussions of the step-by-step contributions that slowly clarified the understanding of a fundamental aspect of nature. The importance and relevance of the experiments and considerations of the proof required to make the story acceptable was a memorable experience for me."*

Although most members of the Budapest laboratory gathered there in the first year, they had all left in the following years to find permanent positions elsewhere in the US. Soon thereafter Albert Szent-Györgyi felt the need for new approaches to understand life, and switched to cancer research. According to Csuli:

> *"He switched from muscle in 1952 and I stayed in muscle, he didn't mind it. As a matter of fact, it was then that I found something important."*

It was **his second important contribution to muscle biochemistry**, characterizing the heavy and light meromyosins (terms coined by him) obtained by limited proteolysis of muscle myosin, together with Elemér Mihályi [4-6]:

> *"I stayed with research into muscle since I felt that its proper understanding would shed light on many different cellular functions. In 1952, with Mihályi, we made the important discovery showing that the proteolytic enzyme, trypsin, splits the myosin molecule into two well-defined fractions. I could show that these fragments had different functions. The heavy meromyosin was water soluble and interacted with actin, and was able to perform the reactions required for contraction in solution. The other fraction, light meromyosin, was responsible for forming the myosin filaments characteristic of myosin in muscle.*

The heavy meromyosin opened up new approaches in the study of contraction in solution and is extensively used even today."

I should mention here that the first experiments, which finally led to the discovery of meromyosins, were done by a fellow Hungarian, at that time in Bethesda at NIH, János Gergely [7] (see the chapter about his life). A few years later Andrew and Carolyn Cohen showed that LMM is fully alpha-helical [8].

Between 1953 and 1957 Andrew Szent-Györgyi was an instructor at the Physiology Course. Here he realized how important and exciting it was to teach eager and curious young students, often mesmerized by the secrets of life and life forms, especially those of marine creatures, the objects of their experiments.

"The student chose to work spending a week with three of the six instructors to learn the experimental methods and the thinking about the problems of a particular field on which the instructors worked. The course was very intensive, and the students spent long hours in the laboratory, in the informal discussions and at lectures. Following the formal six week-course the students could stay for the rest of the summer, working with the instructors or with scientists not directly involved in the course."

His fellow instructors were the leading biochemists and molecular biologist of those years: George Wald, Steve Kuffler[2], Herman Kalckar, Mat Meselson and Frank Stahl[3], and finally, Jim Watson. Besides doing long hours of teaching and research, they also enjoyed life. Here is Csuli's account of the so-called Gamow-Watson party:

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2 Born as Kuffler Vilmos, a pre-eminent Hungarian-American neurophysiologist, the „father of modern neourscience"

3 Their famous experiment to prove the semi-conservative replication mechanism was conceived at MBL.

"In 1954 Watson persuaded Francis Crick to spend a month in Woods Hole. Rosalind Franklin also visited for about two weeks or so. There was also George Gamow, a famous physicist who had escaped from Russia and was the first to propose the "big bang" theory to explain the birth of the universe. He became interested in how DNA can code amino acids that are the building blocks of proteins. (…) One late evening, Watson was in the Captain Kidd, the pub frequented by scientists, and he decided to play a joke on Gamow who was staying in Prof's cottage on Penzance Point. He wrote a letter, imitating Gamow's handwriting, inviting everyone to Muscle Beach adjoining the cottage: »To introduce Mr. Tomkins to a topsy-turvy RNA party on Muscle Beach (The cottage of Seven Winds)«. The party was »scheduled« for three weeks from the date on the letter, which was distributed to every mailbox. Gamow was somewhat shaken when within two days he received some 15-20 letters thanking him and accepting the invitation. However, within a day or two he recovered his equilibrium, ordered a couple of cases of whiskey, and, I guess, quite enjoyed the situation in which he was the center of attraction. Once people realized that all this was a joke, there was guessing as to who were the perpetrators. Eventually Wald guessed that it had to be Jim, although his helpers were not identified. Gamow built a collage out of Watson's dirty sneakers (stolen by a girl student), his awful hat, two ping-pong balls, representing eyes, all attached to the model of a DNA double helix. It looked rather awful; nevertheless it somehow reminded one of Jim. Gamow attached to the construct a note that »Instead of Mr. Tomkins I am introducing Dr. Watson«. At the time the housing at the MBL was rather crowded, tensions developed between families, and this gave them something else to talk about. All in all about two hundred people attended the party, and most of them brought drinks. It was one of the high points of the summer."

Andrew *gives a lecture at MBL, ca. 1966*
(courtesy of the National Library of Medicine).

Francis Crick adds to the story in his book, "What Mad Pursuit": *"The other hoaxer turned out to be Szent-Györgyi's nephew, Andrew Szent-Györgyi. I negotiated a treaty. Jim and Csuli, as he was known, would provide the beer, Joe (Gamow) would provide the whisky."* Jim Watson also features the young Csuli in his book, "Genes, Girls, and Gamow", as the joker of the company who was involved in all parties and events that helped to create a relaxing atmosphere. He writes: *"Andrew Szent-Györgyi and his super-attractive wife, Eve (…) let me know the local gossip and what to expect of dinner with the boring married couples. As a rule the most attractive girls took the invertebrate course"*.

Speaking of Eve (Éva), who, besides being the mother of their three children (Chris[4], Kathy, and David[5]), was fully committed to research. In the first summer of their stay in Woods Hole she helped the Prof (as Albert was called by almost everyone) in the experiments that led to the development of glycerol-extracted psoas muscle fiber preparation [9]. She considered those days as one of the high points of her scientific career, which is described by Csuli as follows:

> *"While she was an excellent collaborator who also produced important work based entirely on her own ideas and designs, her studies were in many ways part and parcel of my laboratory. She was much liked by the scientific community and friends, as shown by the large number of letters which I received from colleagues and friends after her death. She had publications of work of her own design and approach, in addition to a number of papers which represented our joint efforts. She was careful and a good critic and in every respect a great help."*

One additional biographical episode from this period as told by Andrew:

> *"In the summer of 1949, Marta's[6] children, Gábor and Orsi, received permits to leave Hungary and arrived in Woods Hole. Little did I know that I would marry Orsi nearly 40 years later after the death of our spouses in 1985 and in 1988."*

The last event I have to mention from the Woods Hole period is that Andrew and Éva bought a land near a cedar swamp

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4 Chris Szent-Györgyi is molecular biologist at Carnegie Mellon University, Pittsburgh.

5 David Szent-Györgyi is a computer scientist working for BioVision Technologies.

6 Márta Borbíró, Albert's devoted second wife who died of breast cancer in 1961. She was a great help to Albert in focusing on his science, and a great hostess for entertaining Albert's friends and guests in Woods Hole.

Csuli *with the Prof (***Éva Szentkirály** *in the background) in ca. 1976 (courtesy of NLM).*

and built a house on Wilson Road in 1957. Like the "Seven Winds", Albert Szent-Györgyi's house on Penzance Point, this house later became a summer escape for so many muscle people, all friends of Andrew (including the author of the present article, who feels privileged for having belonged to this circle). He and Éva were as great hosts and hostesses as Albert and Márta had been half a generation earlier.

Andrew's flagship research: scallop, as a model for myosin regulation

In 1962 Andrew accepted a position and became a Professor of Biophysics at Dartmouth Medical School in Hanover, NH. Four years later he joined Brandeis University in Waltham, MA, where he was a Full Professor of Biology for more than 30 years, and after officially retiring in 1997, Emeritus Professor. He kept visiting his lab until his death. From 1975 to 1979 he served as Chair of the Biology Department. While he was a faculty member at Brandeis, he lived in Wellesley with Eve until her premature death, then with his second wife, Ursula (Orsi) Rowan, who was not involved in science but was also a great hostess

Orsi (Ursula) *and* **Csuli** *in front of the Wilson Road house, 2003.*

and took fantastic care of Andrew and all the frequent visitors in their home.

During the summer months he moved down to Woods Hole and served as a faculty member of MBL from the early 1950s through the 1980s. He directed the Physiology Courses from 1967 through 1972, and, under his leadership, attracted a number of influential scientists to participate, including Sidney Brenner, Hugh Huxley, Julian Huxley, Alfred Sturtevant, Kenneth Van Holde, and Harvey Lodish. Practically all instructors were or became members of the National Academy of Sciences or the American Academy of Arts and Sciences. Some of the students were also extraordinary and later became leaders of their fields: Robert Waterstone, Harold Weintraub, Mary-Lou Pardue, Al Readfield and others. His interaction with the course continued as late as 2010.

Let me quote here a Physiology Course attendee from the 2000s:

> *"He attended every 9:00 am lecture in the Physiology Course and formed part of my morning ritual as I watched him glide into the bike lot in front of the Lillie Building and perform his mildly alarming, last-minute dismount. He often kibbitzed with students and instructors in the Physiology Course on the proper way to purify and assay myosin motors (…) And because of Albert, we still have Andrew tooling around Woods Hole on his bicycle, a palpable human connection to that work and to that time."* [7]

After setting up his lab at Brandeis he started to work with molluscan muscles, which turned out to be a lucky choice, but from a different point of view than they first thought [10, 11]:

7 From the blogspot of Dyche Mullins (now a Professor of Cellular and Molecular Pharmacology at USCF): http://kinetictrap.blogspot.hu/2012/07/dodging-nazis-and-discovering-actin.html?view=timeslide

Andrew *talking at the Physiology Course in Woods Hole in 2009 (courtesy of Dyche Mullins).*

> *"My collaboration with Carolyn intensified, working, of all things, on molluscan muscles such as scallops, clams, oysters and mussels. The reason for working on these shellfish was not gastronomical but rather their ability to maintain tension with little or no energy requirement. These muscles contain paramyosin, having a coiled-coil α-helical two-stranded structure, and we showed how they form filaments."*

Andrew was not quite accurate in saying that working with these sea creatures had no culinary aspects. When I worked in his lab, after isolating muscle proteins from shellfish, we had great seafood parties from the rest of the material; what a delicious clam chowder from quahogs (*Mercenaria mercenaria*)! Lobster from Maine was a different story in the Wilson Road house; it was not used as research material, but prepared personally by Csuli, and he was also a master of grilling.

His third major contribution to muscle research comes from 1969. He introduced scallop myosin as a model to study calcium regulation of myosins [12].

> *"In vertebrate skeletal striated and also heart muscles, contraction is inhibited by proteins bound on actin filaments in the absence, not in the presence, of calcium. The myosins of these muscles do not bind calcium, regulation is actin-linked. Surprisingly, in molluscan muscles regulation is myosin-linked due to the ability of myosin to bind calcium. We understand the structural and biochemical properties that account for these differences. The importance of these findings lies in the emerging observations of many cell functions where regulation depends on myosins that can directly bind calcium."*

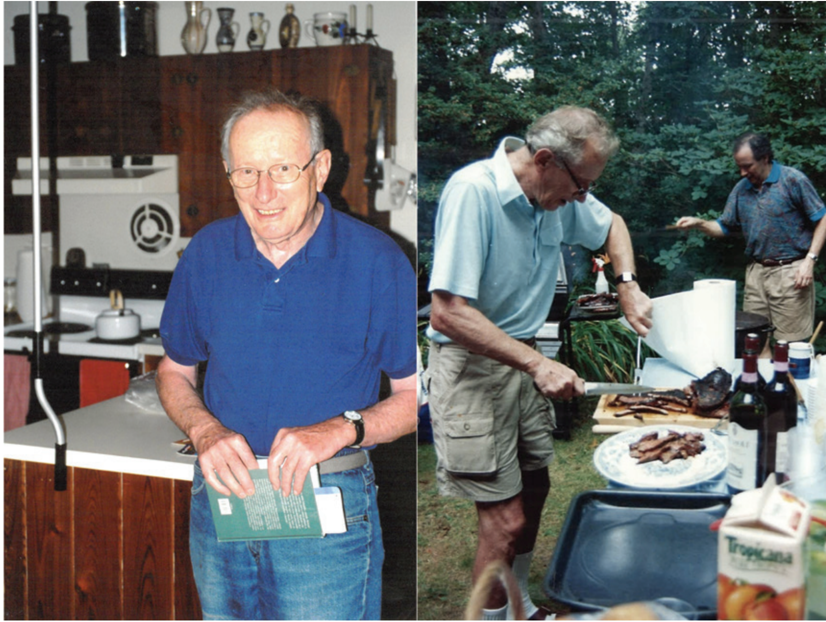
The original observation was followed by a series of seminal papers showing how calcium binding to the essential light chain of myosin works as an on-off switch to regulate scallop (and other molluscan) myosin activity [13-16]. Andrew Szent-Györgyi provided the muscle community with such an excellent model to study myosin regulation that it was sooner or later used by almost all important researchers in this field all around the world.

One of the ultimate goals of such structure-function studies is to see the structure at atomic resolution. This was a long (and still unfinished) tour de force with scallop myosin. In order to study the huge asymmetric myosin motor proteins by X-ray crystallography, it is a must to work with smaller domains. The first breakthrough towards structure determination was the isolation and characterization of the scallop myosin regulatory domain [17]. Afterwards came the first high-resolution structure of this key domain, followed by structures of the isolated head domain in several key enzymatic intermediate steps of the motor, as a result of collaboration with Carolyn Cohen and her nice and talented French postdoc, Anne Houdusse [18, 19]. These structures revealed part of the calcium regulatory mechanism, but also the mechanistic details of how a motor protein converts the chemical energy of ATP into mechanical work [20] . The hardest part

Brandeis alumni at a Gordon Conference in 1990 (first row from left: **Andrew Szent-Györgyi, Hugh Huxley, Carolyn Cohen***).*

of the myosin regulation story was the crystallization of heavy meromyosin from any molluscs (which is the minimal myosin fragment that is still regulated); no atomic resolution structure of this fragment has been reported yet. Andrew' last research paper is from 2013, together with Carolyn (who passed away last December) summarizes the current status of their decades of efforts [21].

The minor contribution of the author of this article to Andrew Szent-Györgyi's scallop years was the determination of the sequence of the scallop myosin heavy chain by cloning and characterizing its gene and transcript [22, 23]. My appearance as a postdoc in Csuli's lab initiated many years of fruitful collaboration with Hungarians, including my former students, András Málnási Csizmadia and Mihály Kovács, and also Miklós Nyitrai [24, 25]. Andrew frequently travelled to Hungary since the 80's, visiting family members, attending meetings and workshops, discussions with his young Hungarian collaborators, or events commemorating Albert Szent-Györgyi.



Csuli *smiling and grilling, 2003 and 1991.*

Csuli's personality

He followed his famous cousin's principles in many respects. "Scientific research is a passion" said Albert in 1943 and that was also Andrew's ars poetica in doing research. He really loved myosins, he loved to work in the lab himself and did experiments manually until his mid-eighties (when he developed trembling hands preventing manual work). He had a sense of humor with a little sarcasm, a great deal of wit, and highly democratic and liberal views of the world. He had a special gift for evoking an appreciation of science, echoing his own devotion and that of other scientists, first of all Albert Szent-Györgyi's. I have already mentioned the legendary hospitality of his two spouses. Their house both in Wellesley, and especially in Woods Hole, was a pilgrimage site for many of his scientist friends. I have pleasant memories being in both places, often together with "big names" of the muscle field.

Milestone in Physiology

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The Early History of the Biochemistry of Muscle Contraction

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Szent-Györgyi András Biokémia, 30: 2–6 (2006)

Az aktomiozin 64 éve

64 Years of actomyosin*

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Andrew Szent-Györgyi, *a historian of muscle research.*

Linus Pauling said about Albert Szent-Györgyi that he was "the most charming scientist in the world". Csuli was also liked by all his fellow scientists from all around the world. He knew almost everybody who counted in biochemistry and molecular biology in the past century. He was lucky to be in the right place at the right time, in the small fishing village at Cape Cod. As a matter of fact, he humbly attributed most of his scientific success to luck. As he notes:

> *"I happened to meet a large number of people who usually one wouldn't have occasion to meet. So there were many pluses in my connection with Albert."*

I have personally heard a great many insider stories and anecdotes about all the great names that I previously mentioned in the article. I should add a few more: Otto Warburg, Otto Meyerhof, Otto Loewi, Hans Krebs, Leonor Michaelis, Carl Neuburg, Fritz Lipmann, William Astbury, Andrew Huxley, Max Perutz and John Kendrew. Despite my continuous efforts I could not persuade Andrew to write these up for the next generations of young scientists.

One may ask whether Andrew was in the shadow of Albert. He answered such a question during an interview by the National Library of Medicine[8]:

“People asked me, »Is it hard to have a cousin« - they said »uncle« really - »who is so famous and such a personality and to work there? How did you feel?« I said, »Well, of course it was at first disturbing because I knew I would be on my own, but I had the feeling that I really hated all the old biochemists, because they already had discovered everything worthwhile to discover«.”

Although he is certainly not as famous as his elder cousin, he is respected scientist throughout the research community on his own right. He was certainly helped by Albert – in entering medical school, in leaving Hungary, in settling in Woods Hole, in getting introduced to numerous famous scientists. Andrew returned these favors after Albert’s death by being a historian of muscle research and telling the story of discovering actin and myosin in Szeged for the future generations, and as an ambassador to keep Albert’s legacy as a scientist and a humanist alive for the future [2, 26].

Andrew Szent-Györgyi was a great admirer of many other activities and lived a full life. He loved classical music. One of his best friends from high school throughout his life was Dénes Zsigmondy (1922-2014), a Hungarian violinist and music educator. He was a frequent visitor in Szent-Györgyi’s house, and I have fond memories of house concerts there together with the cream of Brandeis University scientists. One highlight was when he played Bartók’s sonata for solo violin, an extremely difficult piece.

Andrew had a keen interest in all kinds of arts and kept educating his students not only to love science but to appreciate

8 Available in Profiles of Science of NLM (https://profiles.nlm.nih.gov/ps/access/wgbblx.pdf)

Csuli *skiing in ca. 1960 and in Alpbach in 2004.*

high culture as well. He had a fantastic library of art albums, not to mention his great collection of classical music CDs. Just to name a few of his favorites: Schubert's piano sonatas played by András Schiff, as well as Wagner's and Mozart's operas.

He was also a good sportsman and a tough outdoors man. Tennis and skiing were his favorites, and he was also a good swimmer – many times together with Albert in the treacherous currents around Penzance Point in Woods Hole. Many from the muscle field could remember him from the ski slopes during Alpbach meetings or in Alta, Utah (where his family skied every winter with the Huxleys). We skied together the lift-free Tuckerman Ravine in the White Mountains. Living in New England, he climbed all the peaks above 3000 feet with his sons. Our best climb together was the picturesque Mount Lafayette in New Hampshire. Around 1990, I suggested the lab to go whitewater rafting. He accepted the idea immediately and we went to explore the rapids of the Penobscot and Kennebeck rivers in Maine.

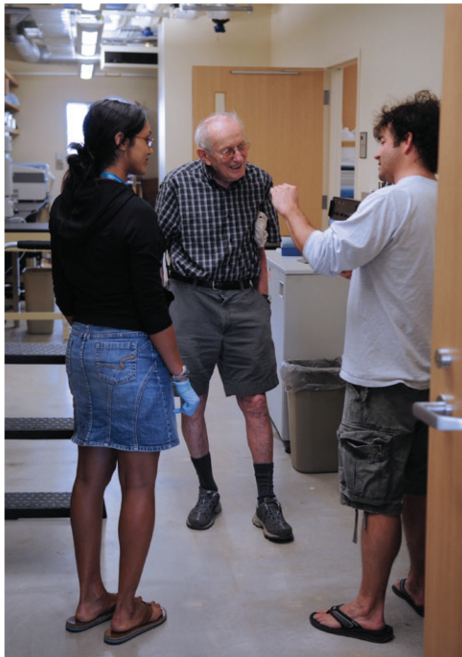
Finally, a short list of biographical data of his research career: Andrew Szent-Györgyi published more than 150 research articles and received numerous awards, including the Public Health Service Research Career Award (1962-1966), a Guggenheim Fellowship (1966), election to the American Academy of Arts and Sciences (1975), and a MERIT Award from the National Institutes of Health (1987-1997). He served as president of

the Society of General Physiologists (1970-197l), president of the Biophysical Society (1974-1975), and was an honorary member of the Hungarian Academy of Sciences from 1998.

At the age of 88, a symposium was organized in his honor at the MBL in Woods Hole. After his death, many of his close collaborators also attended the "Andrew Szent-Györgyi Memorial Symposium" organized by the Hungarian Academy of Sciences in Budapest in 2016.

As an epilogue, a couple of "Szentgyörgyisms", collected by Jim Sellers, Andrew's former PhD student at Brandeis:

- You're only as good as your proteins.
- The best work is that which you do with your own hands! A scientist works in the lab.
- Don't be afraid to be surprised.
- You must be willing to prove yourself wrong.
- A good biochemist should collaborate with a structural biologist.



Andrew *with students at MBL, ca. 2014 (courtesy of MBL).*

- Sometimes you have to be precise in your experiments and sometimes you don't. You can save a lot of time by knowing when!
- You can't control whether you're the smartest guy in the room, but you can control whether you're the hardest working guy in the room.
- If people think you're lucky, it's probably because you're working harder than they are.
- It is OK to have other passions in your life than science.
- The greatest service you can do to me is to prove me wrong.

To end with, here are two take home messages from Andrew Szent-Györgyi for all the readers:

- Recruit Hungarians to work in your lab!
- Not all Hungarians are geniuses!

Andrew Szent-Györgyi *at Penzance Point*
(Beach at the „Seven Winds"), 2012.

Acknowledgements

I am indebted to Miklos Kellermayer for inviting me to contribute to this book by writing a portrayal of Andrew Szent-Györgyi. I had the privilege to work with him as a postdoctoral fellow back in the '90s and later to collaborate with him for a decade. I always considered him as a mentor and role model. I am thankful to Ursula Rowan, Andrew's widow and Kathy Szent-Györgyi, for allowing me to use excerpts from Andrew's unpublished memoir. I am also thankful to Ágnes Jancsó (my former colleague both in Budapest and in Andrew's laboratory) for sharing with me some of her photos of Andrew, and to her, Erna Pap Nyitray, and Mihály Kovács for reading and correcting the text.

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Actin and actin-binding proteins

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Introduction

Muscle is evolved to adapt to physical laws to generate force that results in movement. Striated muscles are composed of myofibrils assembled from the repetitive arrangements of interconnected sarcomeres, their contractile units. At the heart of sarcomeric force production is the actomyosin machinery built from the actin-based thin and the myosin-based thick filaments. Albeit, actin and myosin on their own are sufficient to produce force – if ATP is present – inevitably, in the biological context of muscle, complex protein interactions are necessary to provide engineered architecture, as well as spatiotemporal functional control for efficient force generation and transmission.

Central to sarcomeric force generation is actin identified in Albert Szent-Györgyi's laboratory at the Institute of Medical Chemistry, University Szeged, Hungary at the beginning of 1940's. Since this seminal discovery, actin was established as an integral constituent of not only the muscle but the nonmuscle cytoskeleton of eukaryotic cells, where it functions in diverse cellular processes orchestrated by a large repertoire of actin binding proteins (ABPs) (reviewed in (Bugyi and Carlier 2010; Blanchoin, Boujemaa-Paterski et al. 2014; Pollard 2016; Carlier and Shekhar 2017)). During the years, actin's versatility is further extended by the realization that it is an essential component of the nucleus and linked to nucleoskeleton functioning, as well as that actin homologue proteins exist in prokaryotes (reviewed in (Cabeen and Jacobs-Wagner 2010; Jiang, Ghoshdastider et al. 2016; Kris-

to, Bajusz et al. 2016; Viita and Vartiainen 2017). Moreover, actin inspired engineers, mathematicians and computer scientists to use its intrinsic properties to assemble 3D electrical connections (Galland, Leduc et al. 2013) or use the actomyosin machinery for parallel computation in nanofabricated “biocomputers” (Nicolau, Lard et al. 2016).

Nevertheless, the early history of actin was paralleled with muscle research. The discovery of actin is credited to Szent-Györgyi’s laboratory in Szeged. Since the original documents published in the *“Studies from the Institute of Medical Chemistry University Szeged, Hungary”* between 1941-1943 are difficult to obtain from bibliographic databases[1], I make an attempt to recollect the findings of these studies performed by members of the laboratory leading to the discovery of actin, its ability to reversible polymerize and its ATPase activity, and how these findings started to portrayed our view of actin. Subsequent studies established our current mechanistic understanding of muscle contraction by formulating the classical theories of sliding filament and steric blocking mechanisms. The historical perspectives of how these theories were emerged, evolved and strengthened by experimental evidence provided by the continuously developing technical approaches was explored by excellent recent reviews (Lehman 2016; Trivedi, Adhikari et al. 2018). As a special emphasis of this chapter, the functioning of actin and ABPs in the adaptation and regulation of thin filaments for muscle contraction described by these classical, as well as recent models will be presented.

1 The original version of Studies was published between 1941-1943 and printed only in Hungary. Due to the 2nd World War, the seminal discoveries of the laboratory were not available to the international scientific community until their publication as “Studies on muscle” in Acta Physiologica Scandinavica 1945 (Szent-Györgyi, A. I. (1945). Studies on muscle. Stockholm.). The original papers with introductory notes provided by András Szent-Györgyi now can be found in the Archives of our department: http://actin.aok.pte.hu/archives/index.php.

Actin

Discovery of a *"compound"* that changes *"the qualities of myosin itself"* (Banga 1942)

After his pioneering "*discoveries in connection with the biological combustion processes, with special reference to vitamin C and the catalysis of fumaric acid*"[2] awarded by the Nobel Prize in 1937, Albert Szent-Györgyi decided to extend his research to the biology of muscle contraction, as he explains in his autobiography *"I felt I had now enough experience for attacking some more complex biological process, which could lead me closer to the understanding of life."* (Szent-Györgyi 1963; Szent-Györgyi 2004). Myosin was already established as an integral part of muscle and was also shown to possess ATPase activity (Kühne 1864; Engelhardt 1939). Szent-Györgyi set the course in the opening chapter entitled "*Preparation and properties of myosin A and B"* of the *"Studies from the Institute of Medical Chemistry University Szeged, Hungary"*: *"It is evident, then, that we need to understand myosin if we want to understand contraction"* (Banga 1942). To begin with Szent-Györgyi wanted to *"repeat the work of old masters"* (Szent-Györgyi 1963); with Ilona Banga they prepared myosin from rabbit skeletal muscle. Once, as Ilona Banga clarified the story in the Hungarian journal *"Orvosi Hetilap"* (Banga 1983), she finished with the usual 20 minute extraction late afternoon and since they needed to attend to a lecture, agreed by Szent-Györgyi, the myosin suspension was left overnight in the cold room. Then, their first remarkable observation was made; *"myosin can be obtained from muscle in two different forms"* depending on the exposure time to the Weber-Edsall solution (Banga 1942). The product of the 20 min extraction was called myosin A and the product of the 24 h extraction was called myosin B. Myosin A and B were characterized by different rheological properties; myosin B was more viscous and had higher turbidity as compared

2 Prize motivation cited from Nobelprize.org

to myosin A. A second important observation they made was that addition of ATP markedly decreased the viscosity of myosin B, while no such effects were detected for myosin A. They also noted that after some time, when ATP was depleted, myosin B *"gelatinises again"* (Banga 1942). These findings clearly indicated that the effect of ATP is specific for myosin B and reversible. The ATP effect was independently described by the Needham group in the same years (Needham 1942; Needham, Kleinzeller et al. 1942).

Szent-Györgyi defined *"activity"* as the measure of the ATP-induced decrease in the viscosity of myosin B (Banga 1942). The higher viscosity of myosin B corresponding to its higher activity was interpreted as the *"change in the qualities of myosin itself"* (Szent-Györgyi 1941; Banga 1942). The ongoing studies in Szent-Györgyi's laboratory performed by Brúnó F. Straub pointed towards that the quality changes can be explained as "*myosin B is a stoichiometric compound of myosin A and another substance"* (Szent-Györgyi 1941). Since this substance was an activator of myosin, they named this new compound **ACTIN** and introduced the following nomenclature *"we will call this other compound actin and the myosin-actin complex will be called acto-myosin*" (Szent-Györgyi 1941).

"To see them contract for the first time" **(Szent-Györgyi 1963)**

To understand the origin and the differences of the viscosity changes of the extracts and its relation to ATP, myosin threads – *"elongated pieces of myosin gels"* – were prepared from both myosin A and myosin B (Gerendás 1942; Szent-Györgyi 1942). Upon mixing the myosin threads with watery extract prepared from muscle Szent-Györgyi made a ground-breaking observation; he noted the shortening of longitudinal myosin B threads, as he described *"violent contraction"*, a similar behavior to intact muscle, while no such reaction could be detected for myosin A itself (Szent-Györgyi 1942). Szent-Györgyi remembers

to this discovery as *"To see them contract for the first time, and to have reproduced in vitro one of the oldest signs of life, motion, was perhaps the most thrilling moment of my life."* (Szent-Györgyi 1963). The observation made it clear that *"watery extract of the muscle contains thus something which causes violent contraction in myosin B threads"* (Szent-Györgyi 1942). The suspension left overnight failed to stimulate contraction, implying that the inactivity of the suspension is due to the depletion of ATP during the overnight storage. Indeed, when it was supplemented with fresh ATP the solution stimulated contraction again, proving that ATP is essential for contractile behavior *in vitro*. Based on these experiments Szent-Györgyi correlated the ATP induced reversible change in the viscosity of myosin B to the contractile behavior of the compound.

Straub's parallel work provided evidence that myosin B is *"a compound of myosin and actin"* in two ways. He succeeded to separately isolate actin from myosin B solutions (see below) (Straub 1942). Secondly, the viscous nature of myosin B was reconstituted by mixing actin with myosin A (Straub 1942). This showed that actin can transform the non-contractile form of myosin A to the contractile one of myosin B. As contractility, the biological functioning of muscle could be reconstituted from purified actin and myosin, whereby providing the first biomimetic system of the contractile machinery. In conclusion, these experiments led to the seminal discovery that isolated myosin is not sufficient for contraction, contractile behavior relies on the interaction of myosin and actin in the presence of ATP, setting actin as a central component of the contractile machinery.

Szent-Györgyi's proposal that *"muscle contraction was essentially an interaction of actomyosin and ATP"* was treated with criticisms (Szent-Györgyi 1963; Szent-Györgyi 2004). As he explained, loaded actomyosin threads contracted in the presence of ATP, but *"they will also slip and in spite of contraction…the system will lengthen"*, which led to the skepticism that *"phenomena in actomyosin threads are fundamentally different from those in muscle"* (Szent-Györgyi 1949). To stick up for himself, he started

to investigate rabbit psoas muscle fibers by using a seemingly straightforward strategy: *"In order to show whether an observed effect was actually due to an interaction of ATP and actomyosin, the latter had to be prepared free of ATP. The effects observed on addition of ATP could then safely be ascribed to an interaction of the protein and the nucleotide"* (Szent-Györgyi 1949). However, the method at that time to obtain contractile fibers from intact muscle was to subject them to freeze/thaw cycles to abolish the natural inhibition of contractility, however, this preparation contained physiological amount of ATP. Removal of ATP could be achieved by water extraction of muscle but this compromised contractility (Szent-Györgyi 1949). To overcome this technical barrier Szent-Györgyi used glycerol during muscle fiber extraction and preserved the fibers at -20°C. Using this strategy he succeeded to obtain ATP-free muscle fibers from rabbit psoas without compromised contractility (Szent-Györgyi 1949). When these glycerol-extracted fibers were combined with ATP they contracted rapidly. He was able to demonstrate that *"If connected to the isometric lever, on addition of ATP they develop tension comparable in intensity to that developed by intact muscle on maximal excitation. If loaded they will also lift weights isotonically, similarly to intact muscle fibre bundles of similar dimensions."* (Szent-Györgyi 1949), This work provided evidence for ATP-actomyosin to be the basic contractile machinery in muscle cells. The method developed by Szent-Györgyi, the preparation of glycerin-extracted muscle fibers provided an experimental model for studying muscle contraction that is still in use today.

Biochemical nature of actin: G-actin, F-actin, polymerization and ATPase activity

Identification of actin as an essential component of the contractile machinery of muscle urged subsequent studies to understand its properties. Straub was able to obtain isolated actin by extracting myosin B according to Weber's procedure followed by

the selective denaturation of myosin by acetone, which yielded acetone-dried muscle powder. Then, actin was extracted by salt-free solution (distilled water) (Straub 1942). The successful separation of actin and myosin was key to study *"the factors bringing about the transformation of myosin A into myosin B"*, moreover, it opened up the way to investigate the biochemical properties and activities of actin itself (Straub 1942).

Straub found that actin solution was highly viscous with large double refraction of flow even in the absence of myosin, suggesting that actin itself is some kind of *"aggregate"*, he assumed that "*actin has elongated, rod-shaped molecules of very great asymmetry*" (Straub 1942). As the second viscosity measurement systematically gave lower values, he proposed that mechanical effects can break the integrity of the actin gel, which was related to the thixotropic properties of actin. A modified purification of actin resulted in a protein that did not activate myosin; it did not change the viscosity of myosin when added to it, and ATP did not influence the properties of this actomyosin solution, either; it was called "*inactive actin*" (Straub 1943). The addition of ions to inactive actin before adding it to myosin resulted in increased viscosity of the actomyosin solution in an ATP sensitive manner, thus the salt-treated actin was called "*active actin*" (Straub 1943). The transformation between the two forms of actin was referred to first as *"actin activation"* that *"must be ascribed to ions"* (Straub 1943; Feuer, Molnar et al. 1948). Viscometry analysis suggested that the *"inactive actin is classed among the globular proteins"* (we call it G-actin), while *"active actin…has a very high viscosity which equals to the viscosity of polymer substances…it has therefore an extremely asymmetrical molecule of considerable length"* adopting a fibrous shape (we call it F-actin) (Straub 1943). Based on this result, Straub proposed that the G-F transition is manifested by polymerization. The effects of G-, and F-actin on myosin solutions in terms of viscosity, first led to the hypothesis that actin polymerization is important for muscle contraction, as relaxed muscle contains G-actin and contraction is induced by the formation of F-actin (Straub and Feuer 1950).

The kinetics of activation/polymerization is influenced by both the concentration of ions and actin, as well as the pH and the temperature, as revealed by viscometry measurements. It should be noted that the characteristic kinetic pattern of polymerization – composed of an initial, slow nucleation phase, a subsequent linear regime and ending in a steady-state plateau – that can be observed in pyrenyl-actin based bulk polymerization experiments, was already apparent in the kinetics of the salt-induced change in the viscosity of actin solutions (***Figure 2B***) (Feuer, Molnar et al. 1948; Laki, Bowen et al. 1950). They found evidence that the *"final product, of the polymerisation, polymerised actin does not catalyse the polymerisation"*, which led them to interpret the kinetic pattern as *"polymerization is a series of reactions, resulting in the overall picture of an autocatalytic reaction"* (Straub 1943; Feuer, Molnar et al. 1948). Indeed, while salt-induced, spontaneous actin polymerization is not autocatalytic, a remarkable cellular machinery the Actin-related protein (Arp) 2/3 complex catalyzes actin polymer formation in an autocatalytic manner, as it uses a pre-existing filament as a template to generate a new one (Pollard 2007; Bugyi and Carlier 2010).

At this time, Szent-Györgyi was a special fellow in National Institutes of Health, Bethesda. They developed an electron microscopy (EM) method where F-actin was polymerized from G-actin *in situ* to avoid the disturbance of F-actin threads after their formation by mechanical effects (e.g. pipetting) (Rózsa 1949). This methodology allowed to reveal finer structural details of actin filaments. Consistently with Straub's studies analysis of electron micrographs indicated that F-actin assembly is a multistep process that starts with the formation of ellipsoidal rodlets, called *"polymerization units"* followed by the *"lengthwise association of ellipsoidal rodlets"* (Rózsa 1949).

The ATP content of the actin solutions and that *"ATP is somehow involved in the polymerization of actin"* were realized

parallel by Straub's laboratory and Kálmán Laki[3], who worked as a special research fellow in the National Institutes of Health, Bethesda (Feuer, Molnar et al. 1948; Laki, Bowen et al. 1950; Straub and Feuer 1950). The discovery on one hand, originates from the observation that the maximum of the absorption spectrum of actin is shifted towards ~ 260 nm (Feuer, Molnar et al. 1948; Laki, Bowen et al. 1950). On the other hand, upon salt-induced polymerization of actin a large portion of its ATP content *"disappears"* that correlated with the increase in the amount of inorganic phosphate (Straub and Feuer 1950). These findings led to the conclusion that ATP is a functional group of actin and *"the polymerization of actin is connected with the simultaneous formation of ADP and inorganic phosphate from the ATP present in actin. In other words, globular actin is ATP-actin; ADP-actin, if formed, is in the fibrous form, i.e. polymerized"* (Straub and Feuer 1950). In a series of experiments based on dialysis and viscometry Straub and Feuer found that *"when dialysed against ATP…fibrous actin depolymerizes and acquires again bound ATP"* due to the removal of salts (Straub and Feuer 1950). This provided evidence that actin polymerization is a reversible process and *"it always coincidences with the change in the ATP content"* (Straub and Feuer 1950). Since the steps of the polymerization-depolymerization cycle could not be better resolved, they assumed a mechanism in which depolymerization results from the *"resynthesis of the protein-ATP complex"* that indicated that *"depolymerisation of actin should be the phosphorylation of actin-bound ATP"* (Straub and Feuer 1950). It was also noted that *"ADP is unable to replace ATP in the dialysis experiment"* reflecting the higher affinity of G-actin to ATP against ADP, as well as ATP-free solution resulted in

3 Kálmán Laki – or Koloman Laki as his name appears in publications – was a pupil of Albert Szent-Györgyi working with him between 1933-1941 at the Institute of Medical Chemistry, University Szeged, Hungary. He contributed to the *Studies*. As it is discussed later, when working at the National Institutes of Health, Bethesda, Laki also contributed to the realization that actin preparations are contaminated by tropomyosin, as well as to prove that tropomyosin is an actin filament binding protein.

the loss of the salt-induced polymerization ability of actin, which foresaw that ADP-actin is more prone to denaturation (Straub and Feuer 1950). The first indications that, besides ATP, actin binds a divalent cation came from the observation that actin preparations *"invariably contain a constant amount of calcium"*, which *"does not change even on prolonged dialysis"* (Straub and Feuer 1950; Barany, Biro et al. 1954). As they noted *"We believe that actin is a coordination complex in which Ca forms a bond both with the protein and with the ATP."* (Straub and Feuer 1950). Indeed, the atomic structure of actin revealed that the bound nucleotide (Ca^{2+}, Mg^{2+}), in part, is coordinated by the two terminal phosphoryl groups of ATP (Wang, Robinson et al. 2010).

Except dialysis, they were not able to identify other ways to revert polymerization, therefore, they noted intuitively *"One great problem of actin chemistry remains still unsolved, and that is the mode of physiological depolymerization."* (Straub and Feuer 1950). Now, it is well established that ATP hydrolysis and the dissociation of the γ-phosphate itself results in the destabilization of the contacts important for the integrity of actin filaments, leading to their disassembly. Importantly, the ATP-ADP conversion in the polymer forms a basis to regulate programmed and efficient actin network disassembly in the biological context. ADF/cofilin proteins are specifically targeted to the ADP-loaded portion of the polymer, moreover they can promote the ATP-ADP conversion within the polymer by increasing the rate of Pi dissociation [reviewed in (Blanchoin, Boujemaa-Paterski et al. 2014)].

At the beginning of 1960's, János Gergely and Antal Martonosi at Harvard Medical School, Boston published series of reports entitled "Studies on actin" of reports in the Journal of Biological Chemistry. Using radiolabelled nucleotides, ultracentrifugation, equilibrium dialysis and light scattering approaches they confirmed the early findings of Szent-Györgyi's laboratory and reported on the interactions of actin with nucleotides and divalent cations, which refined the scheme of how the ATPase activity of actin is related to its polymerization/depolymerization cycle. They found evidence the recovery of the ATP-bound form of actin upon depolymerization is not the consequence of

rephosphorylation of the nucleotide as initially proposed (Straub and Feuer 1950), instead the bound ADP is exchanged to ATP in G-actin (Gergely, Gouvea et al. 1960; Martonosi, Gouvea et al. 1960; Martonosi, Gouvea et al. 1960; Martonosi and Gouvea 1961; Martonosi 1962; Martonosi 1962; Martonosii, Molino et al. 1964). Their *in vivo* analysis of P^{32}-injected rats suggested that actin is present in the form of filaments in the muscle and that the G-F transformation is not related to muscle contraction and relaxation (Martonosi, Gouvea et al. 1960).

The pioneering work by the Szent-Györgyi school laid the foundation of the modern era of both muscle and actin research. The significance of their work is evidenced by the test of time, as their initial findings on the properties and behavior of actin, myosin and actomyosin were corroborated by subsequent structural and functional investigations. These work started to sculpt the classical text book schemes and now contribute to our understanding of the biochemical and biological activities of actin. Of note, Szent-Györgyi received a second Nobel Prize nomination in 1951 motivated by the discoveries of his laboratory on *"Muscular contraction and the role of myosin, actin and adenosine-triphosphate"*[4]. Since the names of the nominees are kept in secret for 50 years, this was revealed at the beginning of the 2000's.

Towards the modern era of actin

The principles of Straub's actin purification protocol supplemented with a 0.6 M KCl wash to remove tropomyosin form the basis of the method to purify actin from muscle tissues today (Straub 1942; Spudich and Watt 1971). Despite the existence of the six different isoforms of actin encoded by separate genes ($\alpha_{skeletal}$, $\alpha_{cardiac}$, α_{smooth}, γ_{smooth} muscle and β, γ cytoplasmic isoforms) (Perrin and Ervasti 2010), it is widely accepted to use rabbit muscle actin in *in vitro* studies. This has mostly the following

4 Nomination cited from Nobelprize.org

reasons: the classic protocol provides a relatively simple mean to obtain actin in large quantities from muscle sources and there is a remarkably high amino acid conservation amongst actin isoforms (Perrin and Ervasti 2010). At the same time one has to consider the emerging evidence emphasizing that the functional diversity of actin networks may rely on isoform specific properties of actin, as well as the implications of actin mutations in both muscle and nonmuscle diseases (reviewed in (Ampe and Van Troys 2017)). These urge the development of isolation protocols to selectively obtain isoforms of actin or proteins carrying specific mutations. The isolation of β and γ cytoplasmic actins is difficult because they are expressed simultaneously in nonmuscle cells. Attempts based on recombinant strategies in bacterial systems proved to be mostly unsuccessful to obtain actin in its proper functional form. Alternatively, the baculovirus expression system offers isoform specific production of native or mutated actin isoforms, however, the yield falls off that of muscle sources (Ohki, Ohno et al. 2009; Bergeron, Zhu et al. 2010).

The first structural model of F-actin was proposed by Jane Hanson and Hugh Huxley described it as *"the filament consists of two helically-wound strands"* composed of *"single series of regularly spaced subunits which appear to be alike and approximately spherical"* that was attributed to actin monomers (Hanson and Lowy 1963; Huxley 1963). Importantly, it was also demonstrated that actin polymers assembled from isolated actin or isolated directly from muscle have identical structural features. The structural polarity of F-actin was evidenced by the EM visualization of the periodic binding of heavy-meromyosin (HMM) to F-actin in an obtrusive arrowhead configuration due to the regular orientation of myosin heads (Huxley 1963): the end in the direction of the arrowheads was named *"pointed end"*, while the opposite end was named *"barbed end"* (Woodrum, Rich et al. 1975). The first high resolution X-ray structure of actin in complex with DNaseI that prevents polymerization with resolution of 2.8 Å was published by the Holmes laboratory in 1990 (***Figure 1***) (PDB1ATN, (Kabsch, Mannherz et al. 1990)). Fitting this structure into X-ray fiber diagrams of oriented F-actin gels markedly improved the resolution

of the structural model of F-actin (Holmes, Popp et al. 1990). Notably, the X-ray structure of uncomplexed, isolated G-actin was reported only recently, which was achieved by the help of heat shock protein (***Figure 1***) [PDB3HBT, (Wang, Robinson et al. 2010)]. Due to the technical advances of X-ray fiber diffraction and cryo-EM techniques high resolution (3.7 Å) structures of F-actin and its complexes with muscle proteins are available, which opened up the investigations of molecular changes underlying the structural and functional regulation of force production (Oda, Iwasa et al. 2009; Fujii, Iwane et al. 2010; von der Ecken, Muller et al. 2015). Of note, EM analysis of F-actin:HMM complexes already suggested that actin polymerization is directional, monomer addition is preferred at barbed ends leading to the realization that, besides structural polarity, F-actin is endowed with kinetic polarity (***Figure 2A***) (Woodrum, Rich et al. 1975). Inspired by the pioneering work of Fumio Oosawa's laboratory proposing the nucleation-elongation mechanism of actin polymerization and purifying actin from nonmuscle sources (Oosawa and Asakura 1975), the understanding of the thermodynamic aspects of actin assembly and its regulation in the cellular context emerged the classical schools of actin biochemistry.

The actin molecule (G-, monomeric actin) adopts a flattened globule, doughnut-like shape that is divided into two main domains (inner and outer, based on their orientations as subunits in the polymer, respectively) which are subdivided into four subdomains (S1-4) (***Figure 1***). The S2-S4 subdomains enclose a cleft that binds a divalent cation (Ca^{2+} or Mg^{2+}) coordinated by the β-, and γ-phosphate of ATP. Actin monomers polymerize into filaments (F-actin) under physiological ionic conditions (called spontaneous polymerization) (***Figure 2***). The repetitive arrangement of the subunits building the polymer defines a single-stranded, left-handed helix – called genetic helix – with a rise of 27.6 Å/subunit and a twist of ~ -166°/subunit, as well as a long double-stranded, right-handed helix with a half-pitch of 370 Å. The ordered directionality of subunits within the filament give rise to its polarized structure (barbed and pointed ends). Kinetically, the *de novo* polymerization of actin filament starts with the association

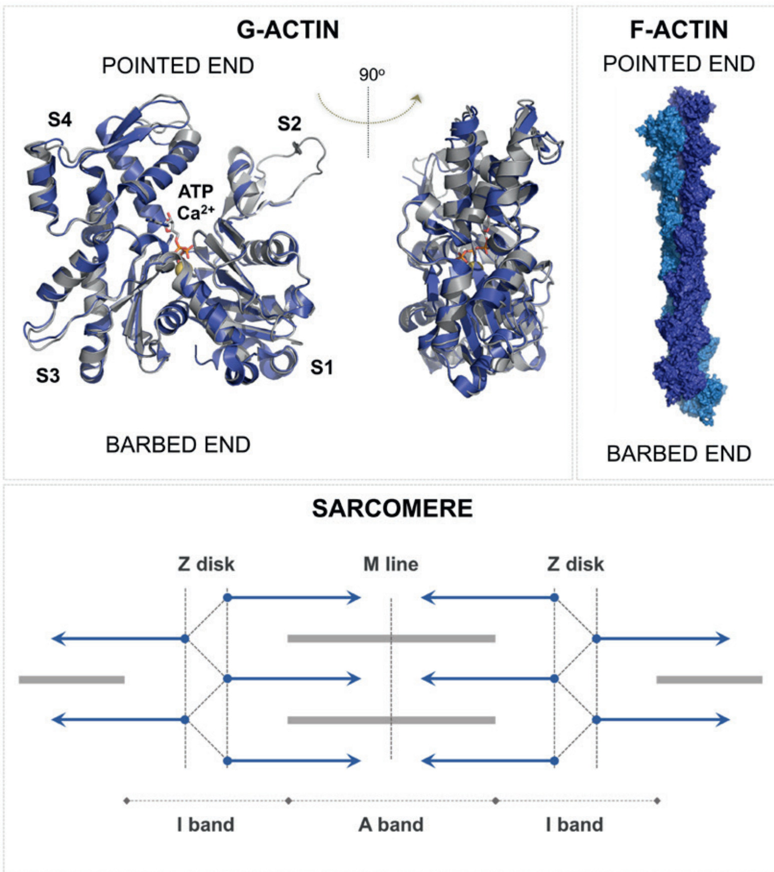

Figure 1. Structural organization of actin.
Ribbon representation of the X-ray structure of the actin molecule (G-actin) derived from its complex with DNAseI (gray) and its isolated state (blue) (PDB IDs: 1ATN, 3HBT) (Kabsch, Mannherz et al. 1990; Wang, Robinson et al. 2010). Structural model of the actin filament (F-actin) based on Oda et al. (Oda, Iwasa et al. 2009). The two strands building the double-stranded, right-handed helix are represented by subunits in light and dark blue, respectively. The single-stranded, left-handed helix assembled from alternating light and dark blue subunits. Schematic representation of the organization of thin (blue lines) and thick (gray lines) filaments in the sarcomere. The polarity of actin filaments is indicated by dots (barbed ends) and arrowheads (pointed ends).

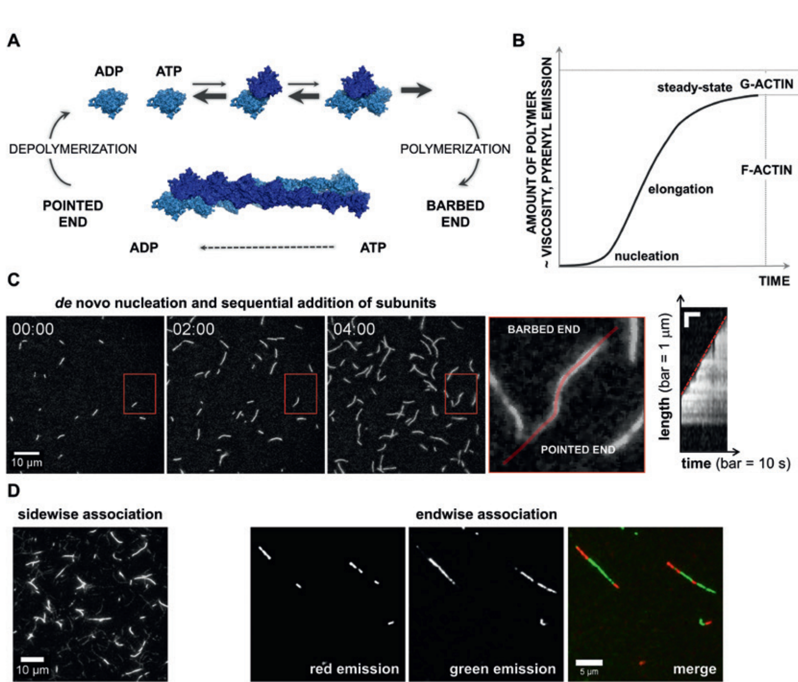

Figure 2. Spontaneous actin assembly dynamics.
(A) Schematics of spontaneous (salt-induced) actin dynamics. (B) Characteristic kinetic pattern of actin polymerization, as revealed by bulk pyrenyl fluorescence/viscometry measurements. (C-D) Visualization of actin assembly at the level of individual actin filaments by using total internal reflection fluorescence microscopy (TIRFM), as a result of de novo polymer formation (C), sidewise and endwise association of existing filaments (D). Of note to C, in agreement with the kinetic polarity of F-actin the preferred association of subunits at one end (barbed end) is readily revealed by kymograph analysis. Of note to D, the brighter-thicker and the dimmer-thinner segments corresponds to the sidewise association of filaments and individual filaments, respectively, the alternating green and red fluorescence emission along a filament reflects endwise association.

of two and three actin monomers called nucleation phase. These nucleation intermediates associate relatively fast (k_{ON} ~ 1 – 30 $\mu M^{-1}\times s^{-1}$), however, they are extremely prone to dissociate (k_{OFF} ~ 10^3 – 10^{10} s^{-1}), as predicted by Brownian dynamics simulations (Oosawa and Asakura 1975; Sept and McCammon 2001). Thus, nucleation by imposing a kinetic barrier is the rate-limiting step of polymer assembly, reflected by the initial lag phase in polymerization kinetics traces (***Figure 2B***). Nucleation is followed by the diffusion-limited sequential incorporation of subunits to filament ends, described by a reversible second-order reaction between the monomer and the filament end, which results in polymer lengthening (elongation phase). Due to the different kinetic constants, filament lengthening is dominated at barbed ends against pointed ends – alternatively named as plus and minus end – respectively, which confers kinetic polarity to the filament. At steady-state actin filaments coexist with free G-actin; defined as the critical concentration. It is different for the two ends, in a way that subunits assemble at barbed ends that is balanced by the subunit dissociation from the pointed ends in this regime, resulting treadmilling behaviour, that is observed in nonmuscle cell lamellipodia (Wegner 1976; Lai, Szczodrak et al. 2008). G-actin hydrolyses ATP very inefficiently (~ 10^{-6} s^{-1}), which is markedly increased upon its incorporation into the filament (~ 10^{-1} s^{-1}). The hydrolysis of the actin bound ATP is irreversible and hypothesized to occur stochastically within the filament. The subsequent dissociation of the γ-phosphate (k_{OFF} ~ 10^{-3} s^{-1}) results in ADP-subunits that dissociates faster from the polymer, as compared to ATP-subunit. (Carlier and Pantaloni 1986; Jégou, Niedermayer et al. 2011). The association and dissociation rate constants – characteristic to the spontaneous actin assembly – of actin in different nucleotide states (ATP, ADP-Pi, ADP) to and from barbed and pointed ends were derived from bulk polymerization experiments, as well as observing individual filaments by using total internal reflection fluorescence microscopy (TIRFM) assays. For the complete set of kinetic parameters I direct the readers to (Pollard 2007).

According to the classic scheme of the nucleation-elongation mechanism, actin filaments are formed by the association of in-

dividual subunits (*de novo* nucleation and sequential addition of subunits) (***Figure 2A-C***). It is however, noteworthy that alternative ways of building actin structures can occur spontaneously; by the lengthwise, end-to-end association of pre-existing actin filaments (annealing), which results in longitudinal growth of the filament, as well as by sideward assembly of pre-existing filaments (cross-linking/bundling), which results in lateral and transversal thickening of the actin network (***Figure 2D***).

ABPs in building a functional sarcomere

Sliding filament and steric blocking models

The seminal work done in Szent-Györgyi's laboratory revealed that the actomyosin is the basic contractile machinery in striated muscles, provided that ATP is present. As Szent-Györgyi remembers in his autobiography: *"This satisfied me and I was sure that in a few weeks' time the whole problem of muscle contraction would be cleared up, but ten years later I still did not understand muscle, which made me conclude that something had to be missing from our basic ideas, something that was essential for the understanding of energy transformation."* (Szent-Györgyi 1963). The relative movement of actin filaments and myosins due to the force generated by the isolated actomyosin machinery can be readily visualized nowadays by using fluorescence or TIRF microscopy in the classical *in vitro* motility assay developed in the 1980's (Sheetz and Spudich 1983; Spudich, Kron et al. 1985; Kron and Spudich 1986; Sheetz, Block et al. 1986). By inspecting the movement of actin filaments on myosin functionalized glass surface in such movies, the autonomous nature of isolated actomyosins is readily conspicuous. They generate force in an uncontrolled fashion both in space and time; their random orientation is against efficient force production at the level of sarcomeric actomyosin networks and they are continuously active provided that ATP is present. On the other hand, it was noted early in muscle research, that native muscles, even if ATP is present can

remain in their resting, not contracted state, which indicated that actomyosin is regulated in the physiological environment. To cite Szent-Györgyi's words on these: *"contraction should occur spontaneously wherever the ATP-actomyosin system is present in a suitable ionic milieu, and the system should persist in the low-energy stable contracted state"* and *"In the intact resting muscle, however, we find ATP in an active form, linked to actomyosin, but still the system does not contract-contraction being inhibited by some unknown mechanism. If we want the muscle to go over into the contracted state, we have to abolish this inhibition."* (Szent-Györgyi 1949). Altogether, this implied that both geometrical constrains and functional regulation must be considered to explain how the force generated at the microscopic scale by actomyosin causes the controlled shortening of sarcomeres, which results in muscle contraction at the macroscopic level. Compelling studies established the classical models of muscle contraction; the *sliding filament* and *steric blocking theories*. ABPs play important roles in these classical models extending to thin filament length regulations and anchoring, as well as to the control of actomyosin activity.

APBs and the classical models of sarcomeric contraction

Striated muscle myofibrils are assembled from the repeating units of sarcomeres, which appear as alternating regions of bright (I, isotropic) and dark (A, anisotropic) bands on longitudinal sections when observed by light and polarization microscopy (***Figure 1***) (Dobie 1849; Brücke 1858; Hanson and Huxley 1955). The A band is centered at the M line in the central H zone of the sarcomere, while the I band is equidistantly split by the Z disk that constitutes boundaries where neighboring sarcomeres adjoin. As our attention is attracted by recent papers, light microscopy studies from the 19th century already reported that upon contraction the length of the A band is unchanged, while the I band shortens (Krause 1869; Fredericq 1874; Huxley 1977; Huxley 1980; Rall 2014; Galler 2015). Structural and biochemical analysis of extracted components revealed that the I and A bands are formed

by actin and myosin, respectively; as they segregate into two distinct types of longitudinally oriented, parallel filaments with fixed lengths; the thin and thick filaments, respectively (Draper and Hodge 1949; Hanson and Huxley 1953; Huxley 1953; Hanson and Huxley 1955). These structural observations complemented with the knowledge on the molecular compositions of thin and thick filaments, as well as the contractile behavior of actomyosin led to formulation of the *sliding filament theory* (Huxley and Niedergerke 1954; Huxley and Hanson 1954). The realization of cross-bridges and the discovery of the ABP tropomyosin (Tpm) and the Tpm binding troponin (Tn) as novel constituents of the thin filaments established the *steric blocking model* (Huxley 1957; Huxley and Simmons 1971; Haselgrove and Huxley 1973; Parry and Squire 1973) evolving to the three-state model to explain the Ca^{2+}-responsive regulation of sarcomeric contraction (McKillop and Geeves 1993; Lehrer and Geeves 1998).

In sarcomeres, thin filaments are oriented in a way that the barbed ends of actin filaments are linked to the Z disk, while pointed ends point towards the thick filaments. Consequently, actin filaments are oriented in a strict polarized register accommodating to the orientation of myosin heads in the bipolar thick filament attached to the M line. This extremely highly-ordered, parallel organization of sarcomeric thin and thick filaments is critical for efficient force generation. According to the sliding filament theory the actin-based thin filaments are pulled by the myosin-based thick filaments towards the center of the sarcomere, thus the two intercalated myofilaments slide relative to each other, which results in sarcomere shortening. Cross-linking of actin filaments to Z disk is dominantly performed by the spectrin-family ABP α-actinin ((Sjoblom, Salmazo et al. 2008)). Evidently, the physical anchoring of the thin filaments to the Z disk (also the thick filaments to the M line) is essential for efficient force transmission to the sarcomere boundaries. The sarcomeric thin filaments has a well-defined constant length throughout the sarcomere supporting homogeneous force generation in transverse directions. The length of actin filaments is controlled on one hand by actin filament end binding ABPs. The barbed end of the

filament is capped by the heterodimeric capping protein (CapZ) composed of α and β subunits, which inhibits both association and dissociation of subunits (Narita, Takeda et al. 2006; Edwards, Zwolak et al. 2014). The pointed end dynamics is limited by the capping activity of tropomodulin (Tmod). Tmod also binds tropomyosin that strengthens its pointed end binding affinity (Fowler, Sussmann et al. 1993). Based on these activities, CapZ and Tmod by preventing their elongation, are expected to function to *maintain the length of thin filaments*. Nebulin, the giant ABP of ~800kDa composed of repeating modules that are considered to be actin binding regions, is also linked to thin filament length regulation. Nebulin spans the entire length of the thin filament, its C-terminus is an integral part of the Z disk and interacts with CapZ, while its N-terminal region is located near the pointed end where it interacts with Tmod (Witt, Burkart et al. 2006; Chu, Gregorio et al. 2016). Based on these unique properties nebulin was proposed to act as a sarcomeric *molecular ruler that defines thin filament length*. However, some new functions of nebulin emerge from recent studies (Chu, Gregorio et al. 2016). Nevertheless, in skeletal muscles of nebulin KO mice thin filaments was found to be shorter and less uniform accompanied by the compromised localization of CapZ and Tmod (Witt, Burkart et al. 2006). In contrast to skeletal muscle, in rat cardiomyocytes nebulin siRNA led to abnormal lengthening of thin filaments into the H zone (McElhinny, Schwach et al. 2005) suggesting different mechanisms of nebulin action in the two types of striated muscles. Nebulin was found to bind titin, which was proposed to have importance in the coordinated regulation of the length of thin and thick filaments by nebulin and titin, respectively (Witt, Burkart et al. 2006). Recombinant N-terminal fragments of nebulin were shown to interact directly with F-actin, as well as myosin with relatively high affinity ($K_d < 1\mu M$) and to inhibit actomyosin sliding velocity and ATPase activity *in vitro* motility assays (Root and Wang 1994). Indeed, muscle fibers obtained from *nebulin*$^{-/-}$ mice exhibited impaired mechanical performance, which was proposed to be the consequence of

the reduced number of actomyosin interaction and probability of cross-bridge formation (Bang, Caremani et al. 2009).

Skeletal muscle tropomyosin (Tpm), was identified in 1946 and named by considering to be a *"prototype of the much larger myosin"* molecule (Bailey 1946; Bailey 1948). Troponin was identified by Ebashi and colleagues as the Ca^{2+}-responsive element of the contractile system (Ebashi and Kodama 1965; Ebashi and Kodama 1966; Ebashi, Endo et al. 1969). Contributed by the work of Kálmán Laki it was realized that the Straub-type actin preparation contains a significant portion of tropomyosin and suggested that *"the presence of tropomyosin in actin preparations is not accidental, but, that there is an intimate interaction between actin and tropomyosin"* (Laki, Maruyama et al. 1962). Indeed, they provided evidence that Tpm binds actin, preferentially to its filamentous form (Laki, Maruyama et al. 1962). Extensive structural and functional investigations revealed that Tpm molecules composed of two α helical coiled-coils self-assemble to form a continuous chain stabilized by head-to-tail overlaps that wraps around both sides of the actin filament (Bailey 1948; Cohen 1955; Hitchcock-DeGregori and Singh 2010; von der Ecken, Muller et al. 2015). Troponin is composed of three subunits, the Ca^{2+} binding TnC, the Tpm binding TnT and the inhibitory TnI. The Tpm:Tn complex periodically decorates sarcomeric actin filaments ~ a few 10 nm from the edge of the Z disk to the pointed ends (Otsuki 1974; Trombitas, Frey et al. 1993) with a stoichiometry of the tripartite actin subunit:Tpm:Tn complex of 7:1:1 (Otsuki, Masaki et al. 1967; Spudich, Huxley et al. 1972; Otsuki 1974). In the absence of Ca^{2+} Tpm covers the myosin binding site on the actin filament thereby hindering myosin binding and cross-bridge formation (blocked (B) state). Ca^{2+} binding to TnC results in the movement of the Tpm polymer in a way that partially reveals myosin binding sites (closed (C) state, weak binding state). Subsequently, the competitive binding of myosin and Tpm stimulates further movement of Tpm to uncover the myosin binding sites (myosin (M) state, strong binding state).

ABPs in the dynamic regulation of thin filaments

As discussed above, actin filaments can spontaneously assemble by the sequential addition of subunits, as well as by the endwise or sidewise association of pre-existing filaments. Recent studies using temporally-controlled expression of GFP-G-actin in *Drosophila* and tracking its incorporation into thin filaments reveal that the formation and maintenance of thin filaments is achieved by a complex sequence of different assembly modes of actin filaments in different stages myofibrillogenesis (Shwartz, Dhanyasi et al. 2016). At the early stages of premyofibrils actin filaments are already present and homogeneously distributed in myofibrils, suggested to be generated by *de novo actin polymerization*. This indicates that the filament formation by nucleation occurs at very early stages of muscle development (Sanger, Wang et al. 2017). Subsequently, actin assembly is limited to the barbed (at Z disk) and pointed (towards M line) end regions of thin filaments by the 'patchy' incorporation of actin resulting in *endwise lengthening of thin filaments*. Later stages are characterized by a 'frame-like' assembly combining actin incorporation peripherally at both ends of the thin filaments and *sidewise addition of actin filaments to the thin filaments* that leads to circumferential sarcomere thickening. These observations suppose that the assembly and maintenance of thin filaments is dictated by a complex interplay amongst different ABPs.

Biochemically, *de novo* actin assembly is kinetically limited by the formation of nucleation intermediates, also it would lead to uncontrolled filament formation. On the other hand, striated muscle cells express actin monomer binding proteins including profilin and WH2/Tβ4, which are expected to suppress actin polymerization based on their *in vitro* activities. Profilin, in its complex with G-actin inhibits nucleation, but once polymers are formed the complex associates exclusively to barbed ends, albeit with somewhat lower efficiency as free G-actin. G-actin is sequestered in its complex with WH2/Tβ4 *in vitro*, which leads to the obligate inhibition of *de novo* polymerization. Altogether, these imply that the *de novo* initiation of actin filament assembly

from its subunits has to be promoted and spatiotemporally regulated during myofibrillogenesis to form functional sarcomeres. The molecular players and events responsible for the mechanism of *de novo* sarcomeric actin filament formation are still an unresolved issue in muscle research. Different groups of proteins were identified which, when purified, catalyze *de novo* filament formation, called assembly promoting factors (APFs) (reviewed in (Goode and Eck 2007; Pollard 2007; Renault, Bugyi et al. 2008; Bugyi and Carlier 2010; Fowler and Dominguez 2017)). The components of these machineries are expressed in striated muscles, which boosted muscle research to seek for the muscle actin nucleator. APFs include the Actin-related protein (Arp) 2/3 complex machinery, the members of the formin protein family and WH2 domain containing proteins. The catalytic units of the Arp2/3 complex, the Arp2 and Arp3 subunits by mimicking an actin dimer promote the *de novo* formation of an actin polymer that grows from the side of a pre-existing one. This machinery generates branched actin arrays with morphology characteristics to the nonmuscle cell lamellipodia (Vinzenz, Nemethova et al. 2012). Formins and the WH2 domain proteins Leiomodins (Lmod) can catalyze the formation of linear unbranched filaments, which are structurally more compatible to sarcomeric thin filaments, making them attractive candidates for being APFs in muscle. Formins exert multiple effects in actin dynamics *in vitro*, which rely on their actin binding domains comprising the conserved formin-homology domain 2 (FH2), as well as their more variable C-terminal (CT) regions. They catalyze nucleation by stabilizing nucleation intermediates, as well as influence polymer lengthening by processively associating to barbed ends. This provides the basis for their ability to antagonistically regulate barbed end dynamics with Capping Proteins (Bombardier, Eskin et al. 2015; Shekhar, Kerleau et al. 2015). These activities can be largely affected by the FH1 domain that binds profilin. Profilin suppresses the nucleation promoting ability of formins, but acts as a molecular switch that supports FH1-FH2-mediated barbed end elongation by increasing the association rate constants of formin-mediated profilin-actin assembly above the rate of form-

in-mediated free-G-actin assembly, which can even exceed the diffusion-limited rate (Kovar, Harris et al. 2006; Vig, Foldi et al. 2017). Fomins can bind to the side of actin filaments and promote their sidewise association (Esue, Harris et al. 2008). It has to be noted that activities related to monomer or filament binding differs both quantitatively and qualitatively amongst different formins. Notably, as recently reported, some formins possess actin isoform specific activities (Takeya, Taniguchi et al. 2008; Patel, Oztug Durer et al. 2018).

Many formin proteins are abundant in striated muscle cells including the FHOD, FMNL, Dia, INF and DAAM proteins. Genetic downregulation of the expression of formins in animal models reveals strong and complex morphological abnormalities; including disorganization of thin filaments and Z disk, as well as loss of the striated pattern, which are accompanied by functional defects both in embryos and adults. Since, thin filaments are not lost completely, the *in vivo* relevance of their nucleation ability was not demonstrated. In muscle cells formins exhibit a complex localization pattern depending on the formin type and the developmental stage. They localize to and near the Z disk, along thin filaments, as well as near pointed end regions, at the M line and along the sarcomere; the latter ones cannot be predicted from their *in vitro* actin interactions (Molnar, Migh et al. 2014; Sanger, Wang et al. 2017). Analysis of *Drosophila* Fhos (FHOD) knockdown myofibrils reveals that Fhos is involved in the patched actin incorporation at barbed ends and the sidewise assembly of filaments to the thin filaments (Shwartz, Dhanyasi et al. 2016). *Drosophila* DAAM was proposed to promote the formation of short actin filaments and their subsequent association to pointed ends in an antagonistic fashion with Tmod (Molnar, Migh et al. 2014).

The WH2 domain containing Leiomodins (Lmod) are vertebrate proteins, that consists of the cardiac and skeletal Lmod2 and Lmod3 and the smooth muscle Lmod1. Lmods can catalyze actin nucleation *in vitro* that relies on their actin binding sites (ABS1/2) and WH2 domains, which is enhanced by their binding to Tpm (Fowler and Dominguez 2017). Lmods can bind

to the side of actin filaments *in vitro* (Skwarek-Maruszewska, Boczkowska et al. 2010; Yuen, Sandaradura et al. 2014; Szatmari, Bugyi et al. 2017). In muscle sarcomeres, Lmods localize near the pointed ends, as well as are found diffusely along the thin filaments but excluded from the Z line. Genetic up-, and downregulation of Lmod2 in cardiac myocytes affected actin filament pointed end dynamics resulting in longer and shorter thin filaments, respectively (Pappas, Mayfield et al. 2015; Li, Mo et al. 2016). *Lmod2*$^{-/-}$ mice heart muscles develop shorter thin filaments and characterized by reduced performance, accompanied by the progressive development of dilated cardiomyopathy. Disruption of the expression of Lmod3 in mice led to sarcomeric disorganization and the development of nemaline myopathy (Cenik, Garg et al. 2015; Tian, Ding et al. 2015) Altogether, these observations indicate that Lmods are important for proper thin filament functioning by regulating assembly at pointed ends. A recent model proposes that Lmod nucleates actin filaments that assemble to the pointed ends thereby antagonizing the capping activity of Tmod and it contributes also to circumferential thin filament assembly (Fowler and Dominguez 2017). Recent *in vitro* data show that Lmod2 can decrease the actin activated Mg^{2+}-ATPase activity if skeletal muscle HMM implicating a role not only in thin filament length regulation but sarcomeric force generation, however, the biological importance of this observation awaits further investigations (Szatmari, Bugyi et al. 2017). Of note, the *Drosophila* Sarcomere length short (SALS) protein – not identified in vertebrates – was proposed to regulate sarcomeric thin filaments by a similar manner as Lmods (Bai, Hartwig et al. 2007; Shwartz, Dhanyasi et al. 2016). Biochemical analysis of the central part of the protein containing two actin binding WH2 domains reveals sequestration activity, implying that other regions and/or sarcomeric components can modulate the activities of SALS in the sarcomeric environment (Toth, Majoros et al. 2016)

In conclusions, albeit, we lack direct evidence for being nucleation factors in striated muscle, as both formins and Lmods are implicated in human diseases (DAAM1 in congenital heart

defects, FHOD3 in hyperthrophic cardiomyopathy, Lmod3 in nemaline myopathy, Lmod2 dilated cardiomyopathy) there is no cast on the importance of these proteins in striated muscle functioning (Bao, Zhang et al. 2012; Wooten, Hebl et al. 2013; Yuen, Sandaradura et al. 2014).

Concluding remarks

Actin and the large repertoire actin binding proteins both in muscle and nonmuscle cells inspired enormous and persistent efforts of almost 80 years, which led to the understanding of many of their versatile biochemical activities and biological functions (reviewed recently in (Blanchoin, Boujemaa-Paterski et al. 2014; Pollard 2016; Carlier and Shekhar 2017). Extensive studies of the muscle specific forms of APBs are continuously sculpting our view of sarcomeric thin filament assembly and force generation. Albeit, thin filaments show an overall static appearance, their components are dynamically assembled/disassembled and exchanged during development and throughout our entire life. ABPs play important roles in these processes, which is underlined by their implications in human muscle diseases. However, how the dynamic regulation of thin filaments is manifested without compromising the structural and mechanical integrity of the sarcomere essential for proper functioning awaits future investigations. On the other hand, similar set of ABPs exist in muscle and nonmuscle cells; what are their biochemical properties and activities in their interactions with actin and with other ABPs that specify their functioning in the unique structure and dynamics of the sarcomeric thin filament arrays have not been completely resolved. Many ABPs possess multifunctional nature in purified systems, which complicates to reconcile their biochemical activities and their biological functioning. As it is intuitively expected and supported by emerging evidence, in the complex biological environment, such as in sarcomeres the individual biochemical activities of ABPs is not only just added up, but are likely to influence each other through synergetic and antagonistic actions.

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Biophysical approaches that revealed the action of myosin as a molecular machine

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Abstract

Many of the concepts of biological structure-function relationships were pioneered in muscle research, resulting in mechanistic knowledge spanning from molecular actions to macroscopic phenomena. Due to its abundance and spatial organization, the actomyosin system powering muscle contraction could readily be investigated by a wide variety of biophysical methods, and also provided fertile ground for the development of these techniques. For decades, muscle actomyosin was the only known biological motor system. It was later discovered that muscle contraction represents a highly specialized form of actomyosin-based contractility. All eukaryotic cells express a variety of myosin isoforms, which drive cellular processes including cell division, differenti-

ation, movement, intracellular transport, exo- and endocytosis. In this chapter we discuss how various biophysical methods have been used to elucidate the structural and functional properties of the actomyosin system and the physiological processes driven by its motor activity. We provide an overview of techniques applied to study molecular and supramolecular features of diverse myosin motors including their structure, kinetics, conformational transitions, force generation, assembly, cooperation, regulation, and the linkage of these properties to cellular and physiological functions.

Seminal discoveries in muscle research contributed to general concepts of biology

All life forms exhibit some form of motility, the capability to perform active movement and associated work. Motility is driven by proteins called molecular motors. These enzymes can convert chemical energy, stored most commonly in the anhydride bonds of ATP or a proton concentration gradient, to mechanical energy. This direct conversion mechanism is fundamentally different from that of man-made combustion engines in which the free enthalpy liberated in a chemical reaction is first converted to heat, and heat is then used to produce work.

The most obvious macroscopic manifestation of motility is muscle action. It is driven principally by two kinds of protein, actin and myosin. For a long time, muscle was the only system for studying biological motility. Studies on muscle initiated the field of mechanobiochemistry, which paved the way for protein mechanical studies also in various non-muscle systems. With advances in cell biological and molecular genetic techniques, a wide spectrum of non-muscle myosins as well as tubulin- and nucleic acid-based molecular motors were discovered and mechanistically characterized.

Classical advantages of muscle research include (i) the abundance of proteins allowing large-scale homogeneous preparations for biochemical studies, (ii) the high level of spatial organization

allowing diffractional and microscopic investigations, and (iii) a macroscopic contractile phenomenon readily measurable by mechanical manipulation in physiological experiments (*1*).

Albert Szent-Györgyi introduced the idea of structure-function relationships in biological systems, and chose muscle as the most regularly organized specimen to study. His group was the first to purify actin and myosin in isolation (*2*). They made actomyosin threads from the isolated proteins, and investigated their contractile properties in correlation with the biochemical features of protein solutions and suspensions. In addition, they described the cyclic interaction between the two protein components and discovered that this interaction is coupled to the ATP hydrolytic cycle (*2*). Together with the subsequent discovery of the actin-induced activation of myosin's ATPase activity by Andrew G. Szent-Györgyi and Endre Bíró (*3*), these works lay the foundations for the concept of allosteric activation, which was later explored in detail in structural and kinetic studies (discussed in the next two sections). Decades later, the combination of structural and kinetic investigations with advanced force manipulation techniques revealed a special mode of large-scale allosteric communication in which the activities of individual catalytic sites are regulated and coordinated by external forces acting on proteins (*4-7*).

The principles of the allosteric regulation of specific events of the myosin ATPase cycle by myosin's interaction with actin proved to be generally applicable to a wide variety of motor-track and GTP-dependent signaling systems (*8-10*). In this framework, actin can be viewed as a nucleotide exchange factor for myosin, accelerating the release of hydrolysis products. Other biological phenomena discovered in muscle research include the principles governing supramolecular assembly leading to formation of actin and myosin filaments; and the discovery of Ca^{2+} as second messenger, initiated by Weber and colleagues (*1;11-13*).

Elucidation of molecular and supramolecular structure

H. E. Huxley and Hanson, in parallel with A. F. Huxley and Niedergerke, proposed the sliding filament model of muscle contraction based on their interference, phase contrast and electron microscopic (EM), and low-angle X-ray diffraction investigations (*14-16*). Among several competing theories, this model has emerged and remained the prevalent conceptual framework for muscle action. The model explained contraction as resulting from the sliding of intercalated thick (myosin) and thin (actin) filaments past each other. Sliding was proposed to be powered by the action of crossbridge structures emanating from thick filaments. Crossbridges were proposed to swing during the force-generating step (powerstroke), thus exerting a rowing-like action to perform mechanical work.

Thin and thick filaments are assemblies mainly consisting of actin and myosin molecules, respectively. Actin monomers (G-actin) have a molecular weight of 42 kDa and, at physiological conditions, polymerize into long, helically structured filaments (F-actin). The first X-ray structures of G-actin were of those crystallized in complex with DNase I and other actin binding proteins (*17-19*). These structures revealed that the actin monomer is organized into two domains with a bound nucleotide located between these two domains. The atomic structure of the actin filament was modeled by docking the G-actin structures into the cryo-EM envelope of F-actin (*20*). Recently, more refined structures were published, which revealed the fine details of conformational rearrangements associated with actin polymerization (*21;22*).

Myosin can be extracted from myofibrils at high ionic strength. The molecular weight of the myosin holoenzyme is around 500 kDa, as determined by analytical ultracentrifugation (*1*). Electrophoretic analysis performed under denaturing conditions showed that the holoenzyme consists of two heavy chain subunits with a molecular weight around 220 kDa, and two pairs of light chains

with molecular weights around 17-20 kDa. Historically, the light chains, which belong to the calmodulin family, have been termed as essential (ELC) and regulatory (RLC) light chains ***(Fig. 1A)***.

Electron micrographs of rotary shadowed myosin molecules showed that the molecule consists of two head-like structures (forming the crossbridges in muscle) and an elongated rod (*23*). Limited proteolytic experiments were fundamental in resolving the „gross anatomy" of the myosin holoenzyme. Myosin can be proteolytically digested to produce heavy and light meromyosin fragments (HMM and LMM, respectively) ***(Fig. 1A)*** (*1;24;25*). HMM contains the heads and the proximal part of the rod, whereas LMM forms the distal part of the rod. The actin binding property and ATPase activity reside in HMM, whereas LMM confers the capability of filament formation. Further proteolysis of HMM liberates myosin heads (subfragment 1, S1) from the proximal rod fragment (subfragment 2, S2) ***(Fig. 1A).*** S1 confers catalytic activity and can bind to actin, whereas the S2 portion holds the heads together and provides a spacer from rest of the tail, which forms the filament backbone (*26*). The flexibility of myosin at the S1-S2 and HMM-LMM junctions was detected by proteolysis, EM and time-resolved fluorescence anisotropy decay experiments (*23;27*).

X-ray diffraction, optical rotation, sequence analysis and structural modeling studies revealed that the myosin rod is an elongated coiled-coil made up of two long α-helical segments, mediating the dimerization of myosin heavy chains (*1*). The structure is held together by a medial hydrophobic stripe, strengthened by charged residues at its sides. The pattern of the latter elements defines a staggered arrangement of coiled-coil dimers in the bipolar myosin filament (Fig. 1B) (*28*). The double-headed arrangement and filament formation capability are shared by a large number of myosin isoforms classified as class 2 myosins, which are present in various forms of muscle and in the cytoplasm of non-muscle cells (*29*). The size and dynamics of myosin filaments varies considerably between sarcomeric (skeletal and cardiac muscle) and non-sarcomeric (smooth muscle and non-muscle) myosin 2 isoforms. Sarcomeric myosins form stable filaments comprising

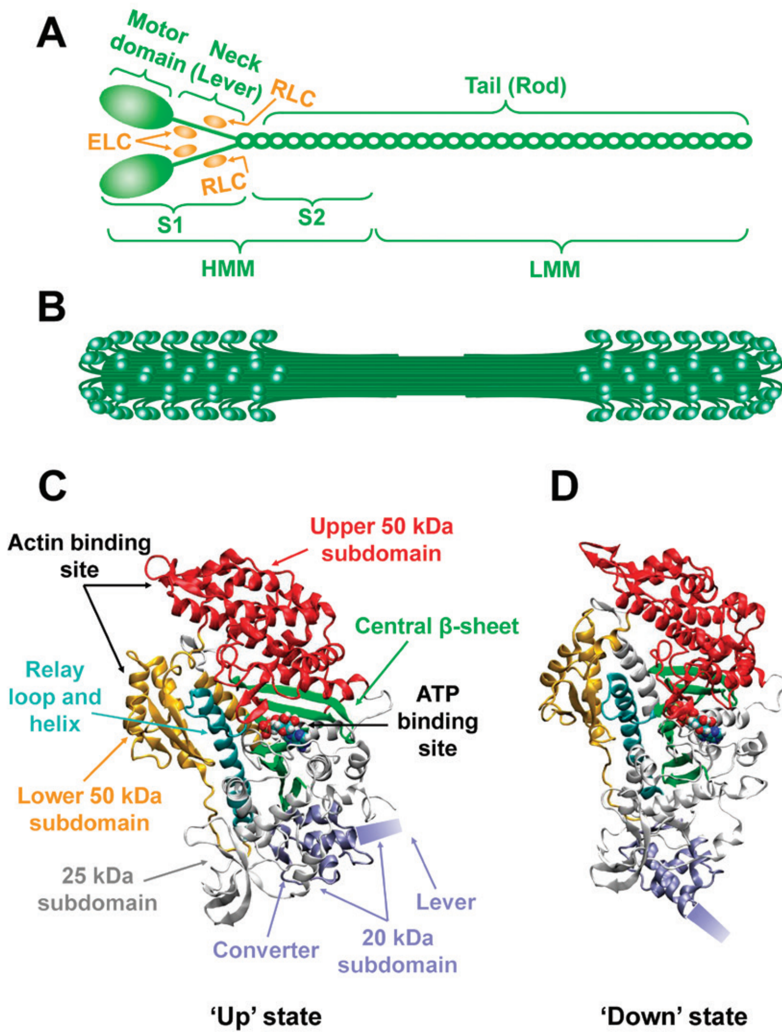

Figure 1: Overview of myosin structure

A, Structure of the myosin 2 holoenzyme. ***B,*** Schematic representation of a bipolar myosin 2 filament. ***C-D,*** Ribbon diagrams of the crystal structure of *Dictyostelium discoideum* myosin 2 motor domain in the up-lever (***C***) and down-lever (***D***) conformations (based on PDB structures 1VOM and 1MMD, respectively).

several hundred myosin holoenzymes, whereas non-sarcomeric ones are characterized by dynamic regulation of filament assembly and disassembly ***(Fig. 1B).*** Vertebrate non-muscle myosin 2 minifilaments consist of as few as 14 myosin molecules at each pole, as shown by EM both *in vitro* (*30*) and *in vivo* (*31*).

Unlike the myosin holoenzyme and the isolated myosin rod, the catalytic HMM and S1 fragments are soluble at physiological ionic strength, which has greatly facilitated their kinetic investigation. S1 was also further digested to produce three heavy chain fragments of 25, 50 and 20 kDa. These fragments were first considered as individual domains, but proved to be inactive (*32;33*). Two reactive sulfhydryl groups (SH1 and SH2) located in the 20 kDa segment proved to be useful in biophysical experiments as the attachment of spectroscopic probes to these groups enabled a wide range kinetic and structural investigations (*34*).

According to the swinging crossbridge theory, the bulk of the crossbridge would have to rotate during force generation. In early electron paramagnetic (EPR) spectroscopic studies on muscle fibers, however, probes attached to SH1 failed to show such a reorientation (*34*). This and several other unresolved questions regarding force generation were clarified upon the publication of the atomic structures of the myosin head. The first structure, solved by Rayment and coworkers, was that of chicken skeletal muscle S1 with methylated lysine side chains (*35*). The structure was tadpole-like in which a large globular motor domain (MD) was sequentially followed by a long α-helical segment of the heavy chain, to which the ELC and RLC were bound. The latter structural part (involving all three polypeptide chains) was termed as the neck, and its appearance immediately suggested a lever function. The previously identified proteolytic fragments turned out to be integral subdomains of S1, which were linked by flexible, protease-susceptible surface loops: loop 1 connecting the 25 and 50 kDa, and loop 2 connecting the 50 and 20 kDa fragments.

As depicted in the atomic structures of the MD shown in Fig. 1C-D, the ATP binding site is located at the interface of the 25 and 50 kDa subdomains. The 50 kDa subdomain is divided

by a large cleft; thus the "upper" (U50) and "lower" 50 kDa (L50) subdomains were defined. Parts of these subdomains were postulated to form the actin binding site. The 20 kDa fragment contains a region (termed the converter) connecting the nucleotide binding site with the lever. This fragment also contains the long α-helix of the heavy chain portion of the neck. SH1 turned out to be located at the pivot of the lever, which provided an explanation for the insensitivity of SH1-attached spectroscopic probes to lever orientation. Lever movement could be followed in later experiments using EPR and fluorescence spectroscopic probes attached to the light chains (*36-38*).

Subsequently, a series of crystal structures of a recombinantly expressed, catalytically active myosin 2 MD fragment from the amoeba *Dictyostelium discoideum*, and those of molluscan muscle S1 fragments were crystallized with a variety of nucleotide analogs mimicking different intermediates of the enzymatic cycle ***(Fig. 1C-D)*** (*39-44*). Some nucleotide analogs (including ADP, AMPPNP (adenylyl-imidodiphosphate) and ADP.BeF_x) induced a conformation that closely resembled the previously described chicken S1 structure (*40;41;43*). In contrast, other analogs (including ADP.AlF_4 and ADP.VO_4) induced a conformation in which the C-terminal part of the MD, which forms the base of the lever, pointed in a direction 70° different from that seen in the other structures (*40;42;43*). These findings were suggestive of the lever swing expected based on previous results. Some nucleotide analogs (chiefly ADP.BeF_x) were able to induce both states, which implied the reversibility of the lever swing in the absence of actin (see also in next section). However, the assignment of the detected structural states to the functional states of the mechanochemical cycle remained to be addressed by kinetic and spectroscopic studies.

Kinetic resolution of structural states

The model proposed by Lymn and Taylor in 1971 lay the groundwork for the coupling of the ATP hydrolytic and mechanical cycles of actomyosin and placed the major (then postulated) structural states of actomyosin in the first kinetic framework ***(Fig. 2)*** (*45*). The cycle was established by transient kinetic studies monitoring the interaction of F-actin, soluble myosin fragments (HMM and S1) and nucleotide. The cycle is usually described starting from the strongly actin-bound, nucleotide-free myosin (rigor) state. The rapid and high-affinity binding of ATP to the myosin head causes a drastic weakening of the acto-S1 interaction and the dissociation of the two proteins, as evidenced by a concomitant decrease in light scattering. The hydrolysis of ATP was found to occur mainly in the actin-detached state. A burst in phosphate (P_i) liberation from ATP, detected in quenched-flow transient kinetic experiments, indicated that ATP hydrolysis precedes the rate-limiting step of the chemical cycle. Although not directly evidenced by experiments at the time, ATP hydrolysis was proposed to be linked to the priming of the myosin head. The myosin.ADP.P_i products complex was shown to rebind to actin. The release of the hydrolysis products was thus proposed to be coupled to the strengthening of the actomyosin interaction and the force-generating powerstroke step, during which the crossbridge swings back to its rigor conformation.

A more detailed kinetic framework of the S1 ATPase cycle and the conformational changes occurring in the absence of actin was proposed some years later by Bagshaw and Trentham (*46;47*). In their detailed transient kinetic analysis they utilized the increase in the intrinsic tryptophan (Trp) fluorescence of rabbit skeletal muscle S1 occurring upon nucleotide binding and ATP hydrolysis. Their work revealed that the binding process occurs in two steps: a rapid formation of a weakly-bound myosin-nucleotide collision complex is followed by a conformational transition leading to the strengthening of the complex, associated with an increase in Trp fluorescence. ATP hydrolysis was associated with a further fluorescence enhancement, indicating the formation of

the primed (up-lever) crossbridge state. Quenched-flow experiments as well as isotope exchange studies (i.e. the incorporation of multiple ^{18}O atoms from water into the liberated P_i) revealed the reversibility of the hydrolysis step (*48*). The quasi-irreversible release of P_i was proposed to be the rate-limiting step in the absence of actin. ADP release was shown to be essentially the reversal of the two-step ATP binding process.

Almost three decades later, a series of kinetic and spectroscopic studies from various laboratories provided an extension of the Bagshaw-Trentham and Lymn-Taylor models by the determination of the precise correspondence between structural and kinetic states. Advances in molecular genetic techniques, recombinant protein expression and mutagenesis allowed the precise placement of spectroscopic probes into specific locations of the myosin head (*49-55*). The major source of Trp fluorescence changes upon nucleotide binding and ATP hydrolysis was assigned to conserved Trp residues located at the nucleotide binding site and the so-called relay loop, respectively. The relay loop is located at the interface of the L50 and converter subdomains ***(Fig. 1C-D)***. Trp fluorescence experiments showed that lever priming and ATP hydrolysis are distinct but coupled steps as hydrolysis can take place only in the primed (up-lever) myosin state (*54;56*). These findings were in line with structural results showing that the catalytic residues of the active site are in place for catalysis only in the up-lever conformation (*40;42*).

Trp signals originating from the relay loop allowed the experimental verification of the earlier concept that the up-to-down lever movement occurring after hydrolysis in the myosin.ADP.P_i complex is a distinct step preceding P_i release (*47;57*). In addition, it was shown that, in the absence of actin, the rate-limiting step of the enzymatic cycle is the up-to-down lever movement and not the actual release of P_i. The up-to-down lever movement is markedly accelerated by actin, which was pointed out as the key phenomenon to ensure that lever priming (down-to-up movement) and the force-generating powerstroke (up-to-down movement) occur in actin-detached and attached states, respectively. This feature leads to a kinetic pathway selection mecha-

nism enabling efficient force generation, and explains why the powerstroke can start from the myosin.ADP.P_i state, which has the lowest actin affinity among all intermediates of the enzymatic cycle ***(Fig. 2)*** (*58-60*).

Parallel advances in structural and kinetic-spectroscopic investigations also revealed many of the fine details of the allosteric linkage between the three most important functional parts of the myosin head: the actin and nucleotide binding sites and the lever. The ATPase active site contains three conserved loops termed the P-loop, switch-1 and switch-2. In the absence of actin, switch-2 and the lever show coupled movement. An open (non-catalytic) switch-2 conformation is associated with a down lever. Closure of switch-2, and thus the acquisition of catalytic competence, was found to coincide with lever priming (down-to-up movement). Kinetic studies revealed that lever priming is a rapid and reversible step, which is a prerequisite for the oncoming, relatively slower chemical step of ATP hydrolysis (*54;56*).

The concept of weak and strong actin-binding states of myosin emerged from kinetic and spectroscopic studies. Besides light scattering, the most useful signal used in these studies was site-specific labeling of actin by cysteine-reactive pyrene dyes at residue C^{374} close to the actin C-terminus (*61*). Quenching of pyrene fluorescence occurs upon the formation of the strongly-bound actomyosin complex (*62;63*).

The actin binding region of the myosin head involves several structural elements contributed from both sides of the large cleft separating the U50 and L50 subdomains (*64*). The incomplete fitting of the available atomic structures of the myosin head and actin into cryo-EM envelopes of the actomyosin rigor complex implied that the cleft must undergo closure in order to adopt a strongly actin-bound state (*65*). Structural studies also revealed that a class 5 myosin is able to adopt a closed-cleft structure even in the absence of actin, which can be well fitted into cryo-EM maps of the rigor complex (*66;67*). Correspondingly, it was found that this myosin isoform binds actin in a rapid, quasi-diffusion controlled manner (*68*). The kinetics of the pyrene fluorescence quench occurring upon actin binding by myosin 5 did not

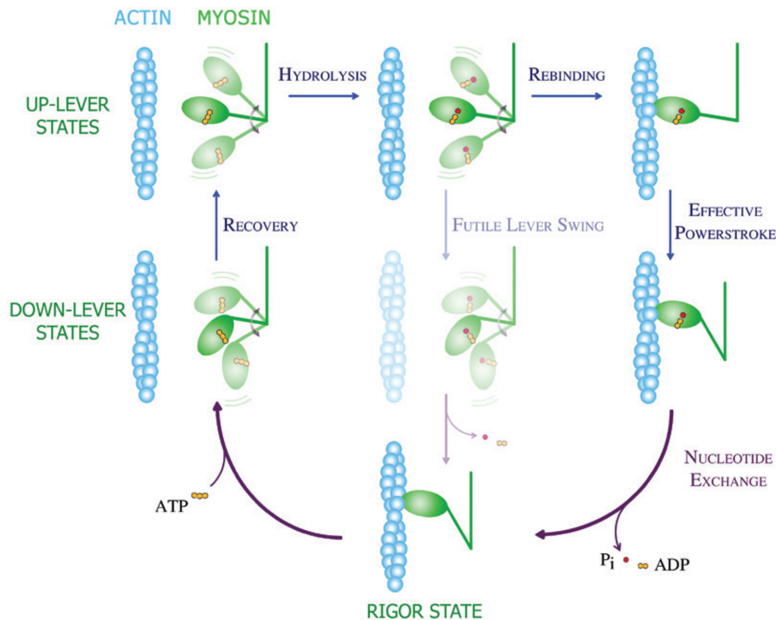

Figure 2: Mechanochemical cycle of the actomyosin motor

The mechanism shown incorporates the Lymn-Taylor model in conjunction with major conceptual advances gained from recent structural and kinetic investigations. Myosin and actin are shown in green and blue, respectively. During the working cycle, ATP binding to the myosin head dissociates the strongly bound actomyosin rigor complex (lowest panel). During the recovery step (left two panels), which occurs in ATP-bound myosin, the myosin lever moves from a 'down' to an 'up' (primed) position. Note the change in lever orientation relative to the motor domain. In the actin-detached states (upper left four panels), the myosin head (motor domain plus lever) is shown in several different orientations (connected by double-sided arrows) to indicate its free rotation about a flexible joint that connects it to the distal part of the molecule (and the thick filament in muscle). The hydrolysis of ATP to ADP and Pi (inorganic phosphate) occurs only in the up-lever state (upper left panel). The post-hydrolytic up-lever complex (upper middle panel) can continue the cycle in two pathways. If the lever swings back to a down position when the head is detached from actin (futile lever swing, middle panels), the ATP hydrolysis cycle is completed without work production. In order to undergo an effective powerstroke leading to force generation, the head must rebind to actin (upper right panel) before the lever swing (up-to-down movement, right two panels). Following the lever swing and the release of hydrolysis products (Pi and ADP), the myosin head binds a new ATP molecule. Note that this scheme does not indicate changes in actin affinity or motor domain structure, and thus it does not discriminate between alternative pathways of the powerstroke discussed in the text. Reproduced with permission from ref. (58).

show a saturating tendency with increasing actin concentration. Later it was found that muscle myosin 2 isoforms from various molluscan species are also able to adopt a rigor-like conformation in the absence of actin, and bind to actin rapidly in a manner similar to that observed in myosin 5 (*69*).

Coupling between the conformational changes in myosin's actin and nucleotide binding regions leads to an antagonistic relationship (negative thermodynamic coupling) between actin and nucleotide binding affinities. This coupling was characterized in detail and kinetically resolved by using a variety of intrinsic and extrinsic fluorescent and EPR probes (*51;70-72*). In addition, it was discovered that the conformation of the switch-1 loop of the active site is linked to changes in actin affinity, probably via cleft movement (*73;74*). An open switch-1 state is associated with low nucleotide affinity and a closed cleft conferring high actin affinity. Switch-1 closure upon nucleotide binding leads to cleft opening and weakening of myosin's actin affinity. The results also revealed the role of the γ-phosphate of ATP in switch closure, as the binding of ADP to actomyosin induces limited switch-1 closure.

As discussed above, the processes of nucleotide-induced actomyosin dissociation and the subsequent conformational changes of actin-detached myosin constitute fairly well-described segments of the mechanochemical cycle. However, the functionally most intriguing part of the mechanism, i.e. force generation via the powerstroke, has remained elusive, mainly due to the low abundance and kinetically inaccessible nature of its key intermediates. A comprehensive model describing myosin's structural transitions leading to the powerstroke was set forth by Geeves and Holmes (*64*). As a cardinal feature, the model proposes that the actin-attached powerstroke (up-to-down lever movement) occurs via a structural pathway that is markedly different from that of the well-characterized actin-detached lever priming process (down-to-up movement). In the actin-attached pathway, the lever swing starts from a closed-cleft, high actin-affinity "top-of-powerstroke" state that has a closed active site (switches 1 and 2) and the lever arm in the "up" orientation. The lever swing then occurs without

switch-2 opening (in contrast to the situation in the absence of actin), but switch-1 opens to release the hydrolysis products. This proposition challenges the long-standing "back door" hypothesis, which stated that, following ATP hydrolysis, P_i releases via a route different from that of ATP entry (*75*). The model of Geeves and Holmes also embraced the idea that the strengthening of the actomyosin interaction is associated with the movement of the P-loop and the twisting of the central β-sheet of the MD. This β-sheet, termed as the "transducer" together with associated elements, has been proposed to act as an energy-storing torsional spring within the MD ***(Fig. 1C-D)*** (*68;76*).

Force production is generally conceived to occur in strongly-bound actomyosin complexes. In line with this, the above model implies that the strengthening of the actomyosin interaction precedes the actual swing of the lever. However, the limited available evidence does not exclude that the force generation process could occur via parallel pathways – even in ones in which the lever swing occurs in weakly actin-attached myosin. The subsequent strengthening of the actomyosin interaction could thermodynamically stabilize the post-powerstroke state (*58*). The force dependence of the transition between weakly and strongly actin-bound myosin complexes can thus markedly influence the fluxes conveyed by the alternative pathways. This constitutes one of the key unresolved questions regarding force generation (see also in the next section).

The physical time scale of the kinetic transitions occurring during the working cycle (milliseconds to seconds) is several orders of magnitude slower than those accessible by computational simulation methods that could describe structural pathways, energy landscapes and transition states. However, significant advances in these approaches including conjugate peak refinement (CPR), normal mode analysis (NMA), umbrella methods and molecular dynamic simulations have enabled the description of structural trajectories of nucleotide-induced cleft opening, lever priming and ATP hydrolysis (*77-80*).

Force generation and motility

Mechanoenzymes confer the specific feature that the enzymatic reaction is linked to mechanical action, which can be followed and analyzed by force manipulation and particle tracking techniques. Muscle produces a macroscopic mechanical response, which was investigated for many decades on intact muscles and muscle fibers. These techniques generally measure the mechanical response of the specimen to the addition of chemical agents (by applying changes in solution conditions) or on mechanical stimulus (by applying rapid stretch or release). Demembranated (skinned) fibers with an exposed contractile apparatus, produced first by Albert Szent-Györgyi, allow the application of rapid changes in chemical conditions. Mechanical manipulation (length-jump) experiments have provided important insights into the kinetics and energetics of muscle contraction (*81*). A crucial aim of these studies is to establish the linkage between macroscopic parameters of force production and the underlying molecular mechanisms. For instance, the linkage of the force generating step to enzymatic product release steps has been a matter of great controversy. Based on the dependence of the mechanical response of muscle fibers on solute (mainly P_i) concentrations, the majority of groups argue that force generation occurs before P_i release (*82-85*). However, other workers propose that the force-generating step is a conformational transition between two ADP-bound states, and thus it occurs after P_i has released from the myosin head (*86;87*).

It was discovered as early as 1923 by Fenn that the heat output of muscle increases when it is allowed to shorten against a load, as compared to the situation when it only holds tension (isometric contraction) (*88*). Accordingly, force-velocity relationships of contracting muscles and fibers define an optimum for power output, generally around 1/3 of the maximal (unloaded) shortening velocity (*1*). Based on current knowledge of the molecular mechanisms, the Fenn effect is thought to arise from the load dependence of the kinetics of multiple key steps of the actomyosin ATPase cycle. As a result, lower loads will be associ-

ated with more rapid ATPase activity and shorter periods of actin attachment, whereas an increase in resistive load will prolong the actin attachment lifetime and slow down the enzymatic cycle of myosin heads (*5;7;89-91*). Fiber techniques have provided estimates of 1-10 pN for the unitary isometric force produced by a single myosin head and values around 7 nm for the unitary displacement occurring during a single powerstroke (*92;93*). These values were refined by single molecule techniques more directly measuring unitary displacement and force generation (see below).

The swinging lever hypothesis implies that force generation requires the movement of a lever whose length will directly determine the unitary displacement. *In vitro* motility assays were the first to allow quantitative measurement of motile properties of experimental systems assembled from isolated protein components (*94-97*). The most common form of these assays images and measures the velocity of the gliding of fluorescently-labeled actin filaments over a myosin-coated surface, monitored by fluorescence microscopy. *In vitro* motility assays also allowed the mechanical properties on non-muscle myosins to be studied, for which macroscopic forces would be difficult to assess *in vivo*.

The combination of *in vitro* motility assays with the genetic manipulation of lever length provided the first solid support for the lever arm hypothesis (*98;99*). Myosin constructs of varying lever length could be produced either by modification of the number of light chain binding regions in the neck region, or by appending artificial levers to the MD. The *in vitro* actin gliding velocity was found to increase in proportion with lever length. Further support for the lever theory came from experiments in which the orientation of the lever was redesigned by genetic manipulation. Myosin constructs with a reverse-oriented lever exhibited a reversal in the direction of the movement of actin filaments in the *in vitro* motility assay (*100*).

A further great technical breakthrough in the investigation of molecular motility was the application of the optical trap, which allowed direct measurement of forces produced by single molecules (*101-104*). In this technique, a focused laser beam is used to hold a micrometer-size bead in position, which allows the

measurement of molecular forces pulling the bead out of position. A widely used arrangement for the measurement of actin-myosin interactions is a three-bead dumbbell assay in which an actin filament is spanned between two beads, and a single myosin molecule is attached to a third bead serving as a pedestal. This arrangement also allows the application of rapid external force feedback to determine the effect of external load on the mechanical response of single myosin molecules. Besides the measurement of the main force-producing powerstroke step (*105*), these assays were also useful in determining the load dependent kinetics of ADP release of various myosin isoforms (*7;89-91;106*). This feature turned out to be an important mechanism regulating the actin attachment lifetime of myosin heads, which determines their sliding velocity and tension bearing properties.

Advances in the visualization of single molecules by fluorescence microscopic techniques have also been valuable in revealing motile mechanisms. To observe single molecules, one of the most widely applied techniques is total internal reflection fluorescence (TIRF) microscopy. This technique allows selective excitation of fluorophores in the vicinity of the microscope slide via an evanescent field, thereby eliminating background fluorescence originating from the bulk of the sample. Single enzymatic cycles were observed by following the interaction of fluorescently-labeled nucleotides with surface-attached single myosin molecules (*107;108*).

For the investigation of non-muscle myosin motility, the most widely applied TIRF arrangement involves the attachment of actin filaments to the slide surface via biotin-avidin bridges (*109*). This arrangement allows monitoring of the movement of fluorescently-labeled single myosin molecules on the actin tracks if they are processive, i.e. able to perform a series of steps along the actin filament before detachment. Movement observed in this assay is generally considered as direct evidence of the processive nature of a given type of motor protein.

A further sophistication of the TIRF assay is FIONA (fluorescence imaging with one nanometer accuracy), which allows precise determination of the position of a single fluorophore by applying two-dimensional Gaussian fits to the spatial distribution

of fluorescence emission intensity (*110*). Sizes and durations of individual steps during processive runs of various myosin isoforms have been resolved by FIONA (*111-114*).

Synthesis of the knowledge gained in biochemical and molecular mechanical investigations may provide clues regarding an important theoretical aspect of motor protein action, i.e. whether force generation is based on lever strain or a Brownian ratchet-like mechanism (*115*). In the lever strain model, one ATPase cycle leads to strictly one lever swing event and coupled translocation. In contrast, a Brownian ratchet-like motor can freely fluctuate between pre- and post-powerstroke states. This fluctuation is rectified by the nucleotide hydrolytic cycle, which imposes a bias as different lever conformations may be preferred for track binding and dissociation. Ratchet mechanisms can thus act at lower coupling between the chemical cycles and translocation. The current thinking on actomyosin action embraces both lever- and the ratchet-type concepts. This conceptual framework has been applied also to other systems including DNA-based motor proteins.

Correspondence between molecular properties and physiological functions

The first so-called unconventional myosin (myosin 1) was discovered in 1973 (*116*). This myosin does not contain sequences for dimerization and thus adopts a single-headed, non-filamentous structure. The large amount of sequence information gained in the subsequent decades revealed the existence of a multitude of myosin isoforms whose subunit composition and domain structure showed wide variations in line with their supramolecular assemblies, regulatory mechanisms and physiological functions (*29;117*). The activity of myosins is required for a variety of life processes including muscle contraction, cell migration, division, sensory functions, membrane trafficking, and formation of cellular protrusions such as brush borders and filopodia (*117*). The

myosin superfamily is currently classified into 35 classes (*118*). The MD has proved to be the most conserved part of myosin in terms of its sequence and structure, and therefore this domain was used as a basis for classification. Besides the MD, most myosins contain neck and tail domains. The neck is usually centered along an elongated α-helix of the heavy chain containing varying numbers of conserved sequence elements called IQ motifs, each of which can bind calmodulin or a calmodulin-family light chain. It was also proposed that myosin necks may be extended by stable charged single α-helices with no associated light chains (*119-121*). The tails of myosins from different classes contain various effector and partner binding domains and, in some classes, induce heavy chain dimerization.

The size and stability of filamentous assemblies varies widely between different myosin 2 isoforms (*1;29*). Sarcomeric (skeletal and cardiac) muscle myosin 2 isoenzymes form large and stable thick filaments. Regulation of the action of these myosins by Ca^{2+} is generally mediated by actin-associated protein complexes (mainly troponin and tropomyosin) by occluding or exposing the myosin binding sites on the actin filament (*13;122*). In smooth muscle and non-muscle myosin 2, filament assembly and myosin activity are dynamically regulated via myosin phosphorylation (*123*). In the off-state with unphosphorylated RLC, the two heads of these myosins adopt an asymmetrical arrangement in which the actin binding site of one head interacts with the nucleotide binding site of the other (*124*). Other myosin 2 isoforms, including those from molluscan muscle, are regulated by direct binding of Ca^{2+} ions to the ELC (*125*). Ca^{2+} binding also regulates the activity of many unconventional myosins (*117*).

Heavy chain dimerization is a prerequisite for the processive walking of cytoskeletal transporters acting as single holoenzymes. Dimerization of myosin 5 was obvious, but that of other transporters such as myosins 6 and 10 has been a contentious issue (*119;126;127*). In myosin 6, the importance of a cargo-induced dimerization mechanism has been pointed out (*126*). Myosin 6 displays other peculiar adaptations including reverse directionality (i.e. movement towards the minus end of

actin tracks) dictated by a class-specific insert at the base of its lever, which also has a reversibly extendable domain (*128;129*). Membrane attachment can also regulate the targeting and supramolecular organization of various unconventional myosins (*117*). Another intriguing adaptation is that other motor proteins or actin bundles can induce processive motility of some myosin isoforms that were previously shown to be non-processive on bare actin filaments (*130;131*).

Assemblies of multiple motor units into dimers or filaments raises the possibility of large-scale allosteric communication between motor units via exerting forces on each other. It has long been known from muscle physiological studies that rapid pulling or release of activated muscle fibers can produce partial synchronization of myosin heads, implying a load-dependent mechanism. Biochemical and single molecule experiments have revealed the importance of such mechanisms in most filamentous myosins (*117*). In the case of double-headed transporters, this form of communication may keep the mechanochemical cycles of the two heads out of phase. This mechanism enables efficient processive movement by preventing simultaneous actin detachment of both heads (*89*). In myosin 5 performing single-molecule motility, the length of the neck also determines the optimal arrangement of myosin heads to reach the next subunit on the actin track (*109*). Recent advances in time-resolved atomic force microscopy (AFM) have enabled the direct observation of the processive walking of myosin 5 molecules along actin filaments (*132*).

The general mechanokinetic framework set forth by Lymn and Taylor has proven to be applicable to most myosins, but there is a large variation between isoforms in the magnitude and ratio of the individual rate and equilibrium constants of transitions (*133*). Simplistically, the mechanochemical cycle time can be divided into the lifetimes of actin-attached (t_{on}) and actin-detached (t_{off}) states, governed by the steps of the ATPase cycle, which are linked to changes in actin interaction. The important concept of duty ratio (or duty cycle, r) can be defined by the fraction of cycle time spent in actin-bound states: r will thus equal $t_{on}/(t_{on}+t_{off})$. In the ergodic approximation, this will also equal the fraction of

motor units (heads) bound to actin in a population at a given time point during steady-state cycling.

It is characteristic of most myosins that ATP binding causes rapid dissociation from actin, and hydrolysis occurs in the actin-detached state. Following this, P_i release and ADP release are accelerated to various extents by actin. As the myosin.ADP.P_i and myosin.ADP states bind to actin weakly and strongly, respectively, the rate of P_i and ADP release will define various duty ratios ranging from 1-2 % in rapidly contracting fast skeletal muscle to more than 70 % in myosin 5. The physiological importance of the adaptable duty ratio is that (*i*) it must be sufficiently high to maintain continuous actin attachment of a supramolecular motor ensemble in order to produce prolonged translocation, (*ii*) it must be sufficiently low that the individual motor units do not pose a drag force opposing the contraction driven by other motors.

A key kinetic and thermodynamic determinant of the duty ratio is ADP-actin coupling, i.e. the allosteric effect of binding of actin and ADP to the same myosin head. If the coupling is highly negative, as in rapidly contracting muscle myosins, ADP release from (and subsequent ATP binding to) actin-bound myosin heads will occur rapidly and the heads will only spend a short time attached to actin. In contrast, in load-bearing myosins, the actin-ADP coupling is low because ADP release is not or only weakly accelerated by actin, which results in longer lifetimes of actin attachment. For instance, this occurs in the case of smooth muscle and non-muscle myosin 2 isoforms (*5;91;134*). The structural basis of load-dependent ADP release is that an additional lever swing occurs upon this step, first discovered by Milligan, Sweeney and colleagues in smooth muscle myosin (*135*). This structural feature varies greatly between isoforms, and is in correspondence with their mechanochemical properties (*136*).

In summary, the mechanokinetic properties of myosin motors are shaped by physiological demands. In general these features appear evolutionarily far less conserved than protein structure and sequence. These principles have proven generally applicable to various other motor-track systems and enzyme-nucleotide-effector ternary complexes (*8-10*).

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Unfolding the story of titin

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Abstract

The giant elastic muscle protein titin (also called connectin) was discovered forty years ago. Ideas about an elastic filamentous network, however, that somehow lends mechanical continuity to muscle, began sprouting much earlier. Ever since its discovery, titin has been the center of research and attention, and currently it is thought that its functions extend much beyond passive muscle mechanics. Here we review the significant events of titin research and discuss the properties and functions of this remarkable protein, which is associated with an even more remarkable molecular biology and biophysics.

Introduction

Self-propelled motion has always been considered to be one of the fundamental hallmarks of living systems. No wonder, therefore, that the exploration of the mechanisms of biological movement has always been in the center of attention of physiology, biology and biophysics. In multi-cellular organisms, muscle tissues are responsible for spatially directed motion. Among the different muscle tissues, striated muscle contains one of the most highly-ordered biomolecular systems, the myofibril and its structural-functional unit, the sarcomere. The main myofibril-

lar proteins myosin (Engelhardt and Ljubimova 1939) and actin (Straub 1943) were discovered and identified by the early 1940s, and, as muscle's main function is contraction, they were collectively named „contractile proteins". It has later been shown that the ATP-dependent interaction between actin and myosin, propelled by the enzymatic (ATPase) action within the myosin motor domain (Rayment et al. 1993), indeed leads to contraction (see superprecipitation, (Szent-Györgyi 1942)) and molecular motion (see *in vitro* motility, (Kron and Spudich 1986; Yanagida et al. 1984)). However, the mechanism behind this motion is the sliding of the actin filament (thin filament within sarcomeric context) past myosin molecules (thick filament within the sarcomeric context), rather than the contraction, *per se*, of either of the filaments (**Fig. 1**) (A. F. Huxley and Niedergerke 1954; H. Huxley and Hanson 1954). It has been very difficult to explain the remarkable structural order and integrity, and the passive, elastic mechanical properties of striated muscle based solely on two independent filament systems; models incorporating parallel elastic elements have been proposed, and experimental findings of filaments forming connections along the sarcomere have been noted. The related connectin (Maruyama 1976) and titin (Wang, McClure, and Tu 1979) proteins were discovered and first described in 1976 and 1979, respectively. Together with nebulin (Wang 1982), titin is now accepted to form a third filament system (besides actin and myosin) within the sarcomere (Wang 1996). In a way, the sarcomeric filaments constitute a highly specialized and distinctly ordered manifestation of the cytoskeletal system, in the center of which titin plays an important structural and functional role. Although the main function of titin is the determination and control of the fundamental elastic properties of striated muscle, it is thought to also play important roles in sensing the sarcomere's mechanical status, and in structurally integrating the different sarcomeric protein components by being the hub of an interaction network (Linke 2018). Titin was the first protein to be manipulated by single-molecule techniques, which led to the development of a whole new field of single-molecule force spectroscopy, an important tool in the exploration of

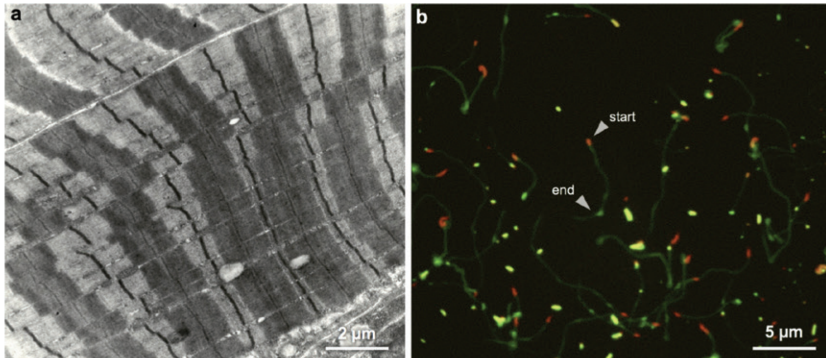

Figure 1. Mechanism of muscle contraction: sliding of actin past myosin. **a.** Electron micrograph of a fiber of *m. tibialis posterior* muscle, in which local contraction took place (Trombitás 2000). A sarcomere-length gradient emerges from sarcomeres with lengths beyond filament overlap (left upper corner) to lengths with full, or even double, overlap (lower right corner). The constancy of the A-band width and the variation of the H-zone and I-band in synchrony with the sarcomere length can be well visualized. **b.** The *in vitro* motility assay. Sliding of fluoreascent actin filaments on a lawn of heavy meromyosin molecules in the presence of 1 mM ATP. A summation image is shown here in which the trajectories, drawn between the start and end-points of the video recording, of the individual actin filaments are visualized.

protein folding and biomolecular interactions. The titin gene was first sequenced in 1995 (S. Labeit and Kolmerer 1995), and recent next-generation sequencing approaches began paving the way towards recognizing titin's so far hidden role in various muscle pathologies.

Early models of muscle – evolution of the three-filament concept

Prior to the emergence of high-resolution structural methods, it was generally thought that muscle contraction is propelled by some kind of a protein crystallization process (Ernst 1963). Upon the first applications of the electron microscope, it has been realized that the myofibrils, which are the histological

sub-cellular units of striated muscle, are composed of axially oriented filaments partitioned into the I- (isotropic) and A- (anisotropic) bands that make up the sarcomere, the structural unit along the myofibril (Draper and Hodge 1949; Rozsa, Szent-Györgyi, and Wyckoff 1950). Initially it has been though that these filaments shorten and lengthen in synchrony with the contraction and extension of muscle. In the early 1950s, based on elegant elctron microscopic and phase-contrast microscopic experiments, the sliding-filament theory of muscle contraction was developed (A. F. Huxley and Niedergerke 1954; H. Huxley and Hanson 1954). In this model, the discontinuous and inextensible thick and thin filaments slide past one another, hence overlap in the middle of the sarcomere. Sarcomere length then changes according to the varying degree of filament overlap (**Fig. 1.a**). The sliding of actin filaments past myosin could be reconstituted in the *in vitro* motility assay more than three decades later (**Fig. 1.b**) (Kron and Spudich 1986; Yanagida et al. 1984). In the two-filament sarcomere model of the sliding-filament theory (**Fig. 2.a**), there is no elastic component (except for the limited elasticity of the myosin cross-bridges). While the model was elegant, and it successfully explained numerous features of muscle contraction, it failed to explain the long-range elasticity of muscle. Furthermore, the model was unable to explain what kept the sarcomere together structurally, particularly when it was stretched beyond overlap. To explain these shortcomings, various three-filament models have been suggested (**Fig. 2**), in which an elastic filament was positioned in the sarcomere in addition to the thick and thin filaments (Trombitás 2000; Wang 1985). S-filaments (**Fig. 2.b**) were suggested to connect the tips of opposing thin filaments in the middle of the sarcomere (Hanson and Huxley 1956). Gap filaments, as proposed by Sjöstrand (**Fig. 2.c**), interconnect the tips of the thin and thick filaments (Sjöstrand 1962). C-filaments (**Fig. 2.d**) are structural connections between the ends of thick filaments and the Z-line in asynchronous insect flight muscle (Garamvölgyi 1965a; Garamvölgyi 1965b). T-filaments (**Fig. 2.f**) run in parallel with the thick and thin filaments and along the entire length of the sarcomere (McNeill and Hoyle 1967). The

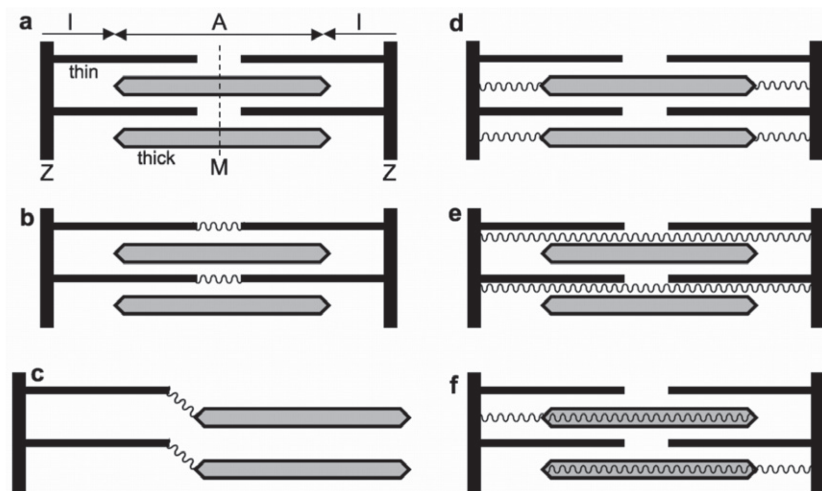

Figure 2. Early three-filament sarcomere models. The elastic third filament is indicated with a wavy line. **a.** The two-filament model on which the sliding-filament theory is founded (A. F. Huxley and Niedergerke 1954; H. Huxley and Hanson 1954). **b.** The S-filament, extending in between the tips of the thin (actin) filament (Hanson and Huxley 1956). **c.** The gap filament extending between the tips of the thin and thick filaments (Sjöstrand 1962). **d.** The C-filament model of Garamvölgyi (Garamvölgyi 1965a; Garamvölgyi 1965b). This model was a remarkable prediction of the current concept about the sarcomeric arrangement of titin. **e.** The T-filament stretching in between the Z-lines of the sarcomere (McNeill and Hoyle 1967). **f.** The gap filament of Locker and Leet (Locker and Leet 1975), which stretches along the core of the thick filament and connects it to one of the Z-lines.

G- or gap filaments of Locker and Leet form asymmetric connections between two thick filaments across the Z-line (Locker and Leet 1975).

Most of the above models were abandoned for lack of, or conflict with experimental evidence. The three-filament model proposed by Garamvölgyi, however, in which the elastic filaments were later named *connecting filaments* (Pringle 1967), quite closely described the structural features and the elastic behavior of the sarcomere. While the muscle field nearly abandoned the three-filament sarcomere model in the late 1960s and early 1970s, the existence of connecting filaments could clearly be demonstrated in insect flight muscle by experiments in which actin filaments

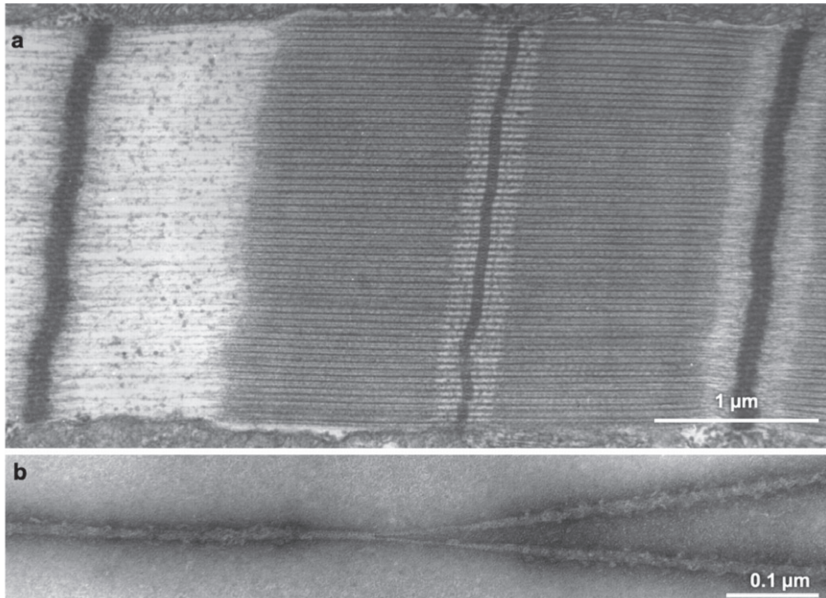

Figure 3. Connecting filaments in the honeybee flight muscle. **a.** Electron micrograph of a bee flight muscle sarcomere. The muscle was stretched in rigor, then fixed. In the left side of the sarcomere, the thin filaments were broken away from the Z-line, thereby allowing the visualization of the connecting filaments (titin-related projectin) (Trombitás and Tigyi-Sebes 1974). **b.** Direct demonstration of structural continuity between bee flight muscle thick-filaments. Electron micrograph of a negatively-stained sample. The tips of three thick filaments are linked by connecting filaments in such a way that two thick filaments from one sarcomere are connected with one thick filament from the adjacent sarcomer through the Z-line (Trombitás and Tigyi-Sebes 1986).

were eliminated from the sarcomere, or in which the muscle was stretched in the rigor state (**Fig. 3.a**) (Trombitás and Tigyi-Sebes 1974). Under such conditions, the thin filaments may break away from the Z-line, thereby exposing the thin, connecting filaments in the I-band. Furthermore, these connecting filaments can also bridge between thick filaments across the Z-line of the honeybee muscle sarcomere (**Fig. 3.b**) (Trombitás and Tigyi-Sebes 1986). The connecting filaments in insect flight muscle were later identified as projectin, a giant filamentous protein related to titin (Nave and Weber 1990; Saide 1981), which most likely has similar function in insects as titin in vertebrate species.

Titin/connectin as extra sarcomeric protein

By the mid 1970s, the protein composition of the striated-muscle sarcomere, which included myosin, actin, and their binding proteins, was thought to be well established (Squire 2012). When extracting these proteins from muscle with conventional methods, a certain stroma remained which was either discarded, or could not be analyzed with the usually available protein separation methods (Wang 1985). In 1964, Guba purified a proline-rich (5 %) protein, named fibrillin, from muscle residue (Guba and Harsanyi 1964). Although fibrillin was never further characterized in detail, it is suspected that it may have corresponded to or been part of titin. In 1976, Maruyama purified a protein from harshly extracted muscle stroma, which was named connectin (Maruyama 1976). It was hypothesized that connectin forms a filamentous network within and around the sarcomere (Ozaki and Maruyama 1980). In 1979, Wang discovered a set of very high-molecular weight proteins in muscle extracts analyzed on high-porosity SDS-polyacrylamide gels (SDS-PAGE). The two largest proteins were collectively named titin due to their enormous, titanic size that exceeded 1 MDa (Wang, McClure, and Tu 1979). It has been speculated that that the smaller of the two (T2) is a proteolytic fragment of the larger one (T1). Another protein, with a molecular weight of ~0.5 MDa, was also discovered and named nebulin after the nebulous N2 cross-striation in the sarcomere (Wang and Williamson 1980). Titin and nebulin are thought to collectively form the elastic third filament system of the sarcomere (Wang 1985). Connectin and titin are considered to be the same protein, but in recent years the name titin has become the sole term for it. The precise analysis of titin paved the way towards a more complete characterization of the protein, including the development of purification protocols (Soteriou, Gamage, and Trinick 1993; Trinick, Knight, and Whiting 1984; Wang 1982), the exploration of its ultrastructure, and the production of an array of different antibodies (Wang 1985). With the help of antibodies that bind at different places along the length of the molecule, titin's sarcomeric arrangement could

be established (Fürst et al. 1988). Thus, it has been shown that titin extends across the half sarcomere, from the Z-line all the way to the M-line. Furthermore, with the help of monoclonal anti-titin antibodies, it could be shown that the gap filaments that become visible in between the tips of thick and thin filaments in sarcomeres stretched beyond overlap, are in fact composed of titin (Trombitás et al. 1991).

Molecular biology of titin

In the early 1990s, Labeit and co-workers have reported the partial sequence of the complementary DNA of titin, which revealed the presence of repeating patterns of 100-amino-acid-long srtuctural motifs classified as immunoglobulin- and fibronectin-type domains (S. Labeit et al. 1992; S. Labeit et al. 1990). The finding is remarkable, considering that these domains are mostly found in extracellular proteins. In 1995, the complete cDNA sequence of titin was reported, which shed light on the existence of tissue-specific size isofoms and the possible mechanisms of obtaining these gene products (S. Labeit and Kolmerer 1995). Titin is encoded by a single gene (*TTN*) on chromosome 2 (locus 2q31) (Bang et al. 2001; Freiburg et al. 2000; S. Labeit and Kolmerer 1995). *TTN* encodes an enormous number of 364 exons and a maximal protein size reaching 4 MD. With this theoretical size, titin is the largest single polypeptide known to date in any biological system. The size of the titin gene is also enormous, and the processing of titin's huge transript appears to be a nearly unsurmountable task. *Via* alternative splicing, orchestrated by the splice factor RBM20 (Guo et al. 2012), different size isoforms may be expressed.

The human titin gene contains three mutually exclusive exons (novel exons – novex) called novex-1 (exon 45), novex-2 (exon 46) and novex-3 (exon 48) (D. Kellermayer, Smith, and Granzier 2017). The novex-3 transcript contains a stop codon that yields a small (~700 kDa) isoform called novex-3 titin. This unusually small titin isoform extends from the Z-disc and is

thought to be involved in stress-initiated sarcomeric restructuring processes.

Titin's I-band section is constructed of tandem-Ig-domain arrays interspersed with unique sequences such as the PEVK domain (so named for its abundance of proline, glutamic acid, valine and lysine) and the N2B unique sequence (Trombitás et al. 1999; Watanabe et al. 2002). In cardiac muscle, short isoforms (N2B and N2BA) are expressed by differential splicing (Freiburg et al. 2000). In skeletal muscles, N2A isoforms are expressed which lack the N2B element. An internal promoter, located at exon 241 that maps near the AI junction of the sarcomere, results in the expression of an N-terminally truncated titin named Cronos (J. Zou et al. 2015).

In the A-band, titin comprises super-repeats of Ig C2 and FN III domains (Bang et al. 2001; Freiburg et al. 2000; S. Labeit and Kolmerer 1995), and it is attached to myosin thick filaments *via* direct binding to myosin-II and indirect interaction *via* myosin-binding protein-C (MyBP-C) (Trinick 1996). Finally, near the M-line *TTN* codes for a kinase domain (S. Labeit and Kolmerer 1995), the sole enzymatic part of titin, which has been implicated in sensing sarcomeric stress. The different exons along *TTN* display different extent of usage (Linke 2018), within which the I-band region is the most variable, pointing at a transcript-level adaptation mechanism focused at the functionally elastic section of titin.

Mechanical properties of the titin molecule

By 1996, it could be suspected that at least three research groups were working towards the mechanical characterization of a single molecule of titin: the groups of John Trinick (University of Leeds, UK), Kuan Wang (University of Texas, Austin) and Henk L. Granzier (Washington State University, Pullman). Apart from the physiological significance of such an experiment, the stake was large from the biophysical aspects as well. By this time the nanomechanical behavior of DNA was characterized (S. B.

Smith, Cui, and Bustamante 1996), and titin, due to its sheer size, could have been the first protein to be extended mechanically, thereby obtaining an unprecedented insight into how a protein molecule responds to distorsions. Eventually, three papers were published in 1997, essentially on the same day (M. S. Kellermayer et al. 1997; Rief, Gautel, et al. 1997; Tskhovrebova et al. 1997). Two of the papers utilized optical tweezers (see **Fig 4.a**) (M. S. Kellermayer et al. 1997; Tskhovrebova et al. 1997), and the third used the atomic force microscope (AFM, see **Fig. 4.c**) (Rief, Gautel, et al. 1997). A common theme emerged from the experiments: titin behaves as a non-linear entropic spring in which domains unfold during stretch and refold during relaxation. The individual domain unfolding event is manifested in the appearance of a sawtooth in the force-extension function (see **Figs 4.b, d**), which is particularly well resolved in the AFM (Rief, Gautel, et al. 1997). The equilibrium (hence the rate) of domain folding and unfolding is set by the Boltzmann-distribution complemented with a mechanical-energy term determined by the external force and a geometrical (distance) parameter of the domain's energy landscape (Bell 1978; Evans 2001; Evans and Ritchie 1997; Evans and Calderwood 2007). The single-molecule experiments on titin were important not only in assigning molecular mechanisms to the explanation of the passive mechanical behavior of muscle, but they also set the stage for the development of a whole new biophysical method and field, single-molecule force spectroscopy (Rief, Oesterhelt, et al. 1997; Williams and Rouzina 2002). Furthermore, some domains in titin (I27, or I91 in the new nomenclature), by way of a poly-gene cloning sytem (Steward, Toca-Herrera, and Clarke 2002), have become popular models for exploring the mechanisms of protein folding (Best et al. 2003; Steward et al. 2011).

How the different domains contribute to overall elasticity and mechanical stability of titin has been an important question, which is still not completely resolved. Tandem globular-domain regions display the characteristic sawtooth-shape force spectrum (**Fig. 4.d**) (Grama et al. 2005; Rief, Gautel, et al. 1997). Unique sequences, such as the PEVK domain, with a putative unstruc-

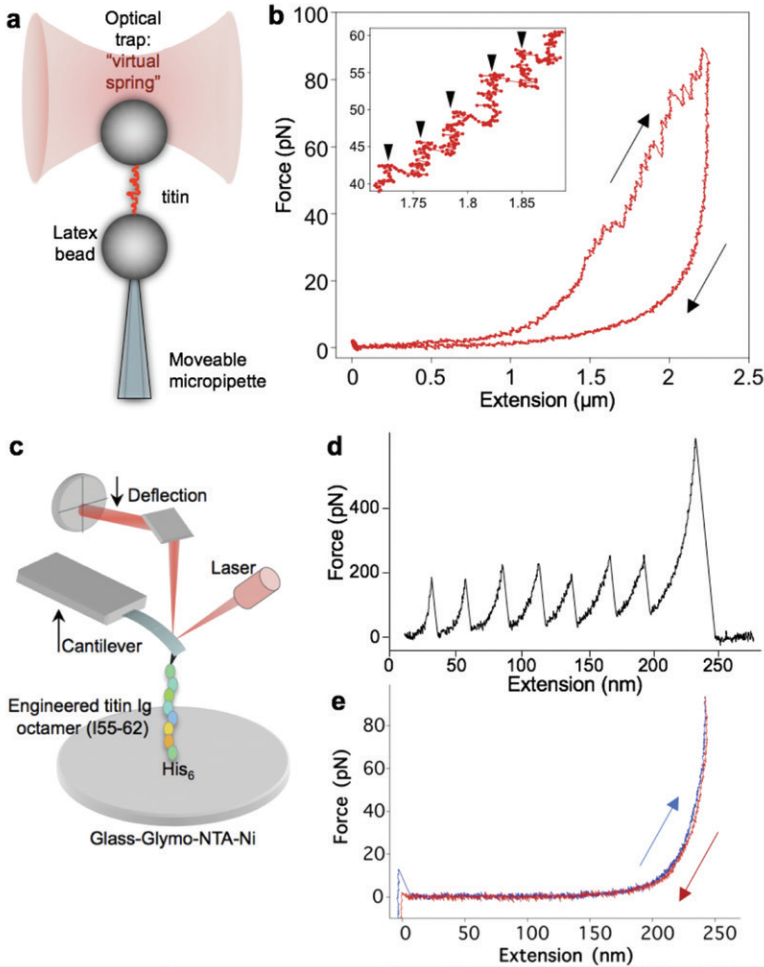

Figure 4. Single-molecule mechanics of titin. **a.** Schematics of titin manipulation with optical tweezers (M. S. Kellermayer et al. 1997). Titin is held in between two beads, one in the optical trap and the other captured with a moveable micropipette. Sequence-specific antibodies and photoreactive cross-linker on the respective beads aid the stable and specific handling of the molecule. Force is measured by using the optical trap as a virtual spring, by either stiffness-calibration or recording the changes in photonic momentum. **b.** Force *versus* extension curve of rabbit *m. longissuimus dorsi* titin measured with high-resolution optical tweezers. Arrows indicate the direction of data collection. Inset, magnified view of the stretch force-trace displaying stepwise (~28 nm step size) extensions that correspond to quantal domain unfolding events. **c.** Schematics of titin manipulation with the AFM. While full-length

tured conformation, display essentially featureless force curves which can be fitted with the non-linear, wormlike chan model of entropic elasticity (**Fig. 4.e**) (Li et al. 2001). Even though these force spectra point at the lack of stable three-dimensional protein structure, the variations in the local persistence length of the domain suggest that PEVK is likely to fluctuate between various conformational states (Huber et al. 2012; Li et al. 2001). Furthermore, the PEVK domain might be an element the mechanics of which could be modulated by calcium binding (D. Labeit et al. 2003). Given that we still know little about the mechanical features and the force-dependent dynamics of certain regions along titin, single-molecule mechanics continue to be an important experimental tool.

Mechanisms of *in situ* titin extensibility

Because titin has good antigenic properties, by the late 1980's numerous antibodies were developed against it (Maruyama 1994), which labeled specific epitopes along the molecule from its Z- to M-line ends (Fürst et al. 1988). Using these antibodies either in immunofluorescence (Linke et al. 1996) or immunoelectron microscopic (**Fig. 5**) experiments, the localization of titin's sequence elements, and hence the relative extensibility of regions demarkated by these boundaries, could be uncovered (Horowits

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titin molecules have been manipulated with AFM (M. S. Z. Kellermayer, Bustamante, and Granzier 2003), the method is more widely used for manipulating recombinant titin fragments such as the I55-62 octamer shown here (Grama et al. 2005). Vicinal cysteins and various chemical tags (e.g., His_6) engineered to the ends of the recombinant protein facilitate the specific capture of the molecule. Force is obtained from the bending of the stiffness-calibrated cantilever. **d.** Force spectrum of I55-62, an octameric fragment from the proximal tandemIg region of titin. Force sawteeth correspond to the unfolding of individual Ig domains. **e.** Force *versus* extension curve of a PEVK segment (N-terminal one third of the soleus PEVK) measured with AFM (Nagy et al. 2005). Arrows indicate the direction of data collection. Reversible, non-linear elastic curve is seen, which can be well fitted with the wormlike chain model of entropic elasticity.

1999; S. Labeit, Kolmerer, and Linke 1997; Maruyama 1997; Trinick 1996; Wang 1996). Furthermore, based on knowledge from single-titin-mechanics studies, from the relative extensibility of titin's distinct sections, local mechanical properties could be inferred. Accordingly, by following the extension of the tandem-Ig segments and the PEVK domain as a function of sarcomere length in human soleus muscle, their respective contribution to titin's elastic behavior was established (**Fig. 5.a**) (Trombitás et al. 1998). In short sarcomeres, both the tandem Ig and PEVK segments are contracted. Upon stretching sarcomeres from 2.0 µm to 2.7 µm, the intitially contracted tandem Ig segments extend to a maximum length calculated based on the number and structural dimensions of folded Ig domains. When sarcomeres are stretched further, the tandem Ig segments do not extend, suggesting that this region has reached its elastic limit, and the Ig domains remain in the folded state. Further titin exptensibility is made possible by the extension of the PEVK domain. This way, titin was thought to be a two-stage spring within the skeletal-muscle sarcomere (Linke, Ivemeyer, et al. 1998; Linke, Stockmeier, et al. 1998; Trombitás et al. 1998). Notably, with the help of a serendipitous intra-PEVK epitope, a hierarchical extensiblity within the PEVK domain could be uncovered (**Fig. 5.b**), suggesting that this domain extends in a telescopic manner (Nagy et al. 2005). In the heart muscle, by contrast, the PEVK domain is very short, but the N2B segment contains a large unique sequence. The short PEVK segment allows a rather limited extension, therefore additional elastic elements are likely to be invoked during sarcomere stretch. Indeed, the N2B segment was shown to be extensible. Thus, in the heart, titin functions as a serially linked three-spring system (Linke et al. 1999; Trombitás et al. 1999).

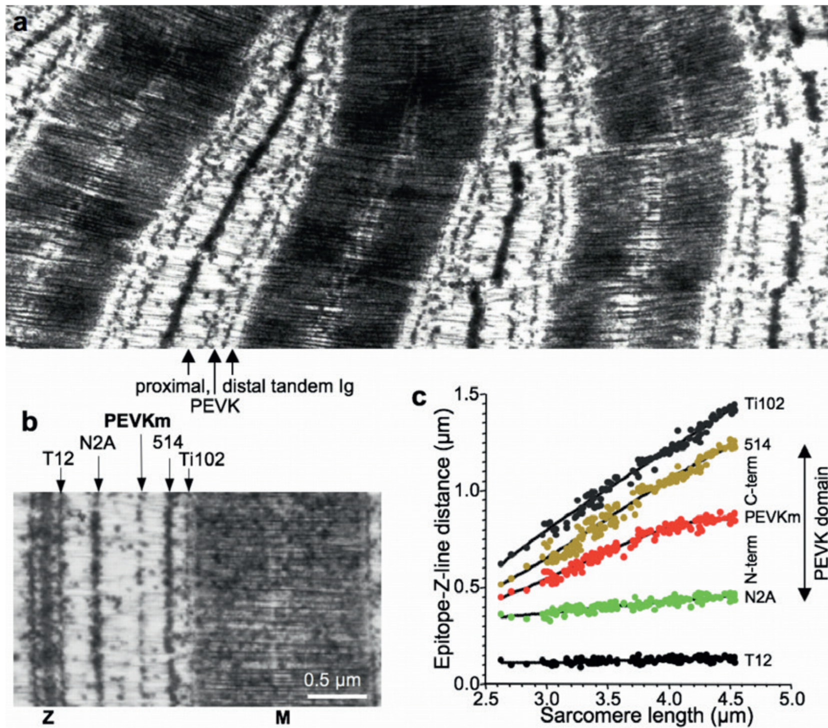

Figure 5. Exploration of titin extensibility by sequence-specific anti-titin antibody labeling. **a.** Electron micrograph of a human soleus muscle fiber labeled with two anti-titin antibodies (N2A, 514), which demarkate the ends of the PEVK domain. Because there is a gradient of sarcomere lengths due to a localized contraction in the fiber, the changes in the extension of the PEVK domain as a function of sarcomere length can be visualized. Upn increasing the sarcomere length, the tandem-Ig segments of titin become extended first, followed by the PEVK domain (Trombitás et al. 1998). **b.** Immunoelectron microscopic image of a soleus muscle sarcomere stretched to a sarcomere length of 3.8 μm. The fiber was labeled with a cocktail of T12, N2A, 514 and Ti102 antibodies (indicated above the figure). PEVKm indicates the extra epitope labeled with the 514 antibody. **c.** Z-line to epitope distance as a function of sarcomere length. The N-terminal region of the PEVK domain extends prior to the C-terminal region, pointing at a mechanical hierarchy within the domain (Nagy et al. 2005).

Multiplex functionality of titin: regulator of sarcomere mechanics, structural integrator, mechanosensor

Considering that titin spatially spans the half sarcomere from the Z- to the M-line, displays a highly repetitive domain structure (**Fig. 6.a**), and its contractile status closely matches that of the sarcomere (Granzier et al. 2005; Granzier and Labeit 2002; Roberts et al. 2015; Granzier and Labeit 2006; Granzier and Labeit 2004; Krüger and Linke 2011; Watkins 2015; Krüger and Linke 2009; LeWinter and Granzier 2013; LeWinter et al. 2007; Linke and Hamdani 2014; Linke and Krüger 2010; Tskhovrebova and Trinick 2010), it has been a nearly evident hypothesis that titin somehow senses and even regulates sarcomere mechanics (Krüger and Linke 2009; Mártonfalvi et al. 2014; van der Pijl et al. 2018) and provides a blueprint for sarcomere assembly (Trinick 1996). The sarcomeric arrangement of titin partitions the molecule into specific regions, the function and properties of which are briefly addressed below.

Z-line titin

Titin's N-terminal region, which contains nine globular domains (Z1-Z9) interspersed with unique sequences, is either integral part of Z-line or is tightly associated with it (Linke 2008). Z-line titin associates with a variety of ligands and forms a highly intricate, multi-level interaction complex which is part of what is now called the titin-interactome (Linke 2008). One of the most important interaction complexes is formed by titin's two N-terminal Ig-domains (Z1Z2) and telethonin (T-cap) (Gregorio et al. 1998; Valle et al. 1997). Telethonin anchors titin in the Z-line by forming a palindromic sandwich structure between the Z1Z2 domains of two anti-parallel titin molecules (P. Zou et al. 2006). MLP (muscle-LIM-protein), which can be translocated in the nucleus, may bind to the Z1Z2-telethonin complex, thereby

forming a mechanosensor (Hoshijima 2006). Studies in MLP-deficient mice forming dilated cardiomyopathy (DCM) provided evidence that MLP is part of the sarcomeric stress-sensing machinery (Knöll et al. 2002). Conceivably, mechanical forces induce structural changes in the Z1Z2-telethonin complex, thereby altering its association with MLP and consequently causing an imbalance between the Z-line-associated and nuclear MLP pools. Although dynamic force spectroscopic experiments have shown that the Z1Z2 domains alone are mechanically unstable (Kollár et al. 2010), molecular dynamics simulation suggests that the Z1Z2-telethonin complex may be able to withstand high mechanical forces, thereby providing mechanical strength to the Z-line meshwork (Lee et al. 2006).

I-band titin

The I-band section of titin is constructed of serially-linked Ig domains (proximal and distal tandem-Ig regions) interspersed with domains containing unique sequences. This section of titin is the most variable, and large elements may be spliced in or out depending on the tissue-specific isoform (Linke 2018). Molecular elements containing unique sequences are the N2B, N2A and PEVK, each of which has been implicated in mechanosensing by connecting to different metabolic, stress- or Ca^{2+}-dependent signaling pathways (Linke and Grützner 2008). The exact mechanisms, however, are largely unclear. As a result of extensive differential splicing (S. Labeit and Kolmerer 1995), different size-variants of the PEVK domain are expressed in different muscles. The local and global structure of PEVK is still largely enigmatic. The domain is thought to acquire random structure due to the preponderance of highly charged residues (S. Labeit and Kolmerer 1995). Immunoelectron microscopy has shown that PEVK indeed displays a quasi-unfolded chain behavior (Trombitás et al. 1998). Structural experiments have suggested that PEVK may contain left-handed poly-proline helices (Ma, Kan, and Wang 2001). A repetitive motif structure has been identified with sequence anal-

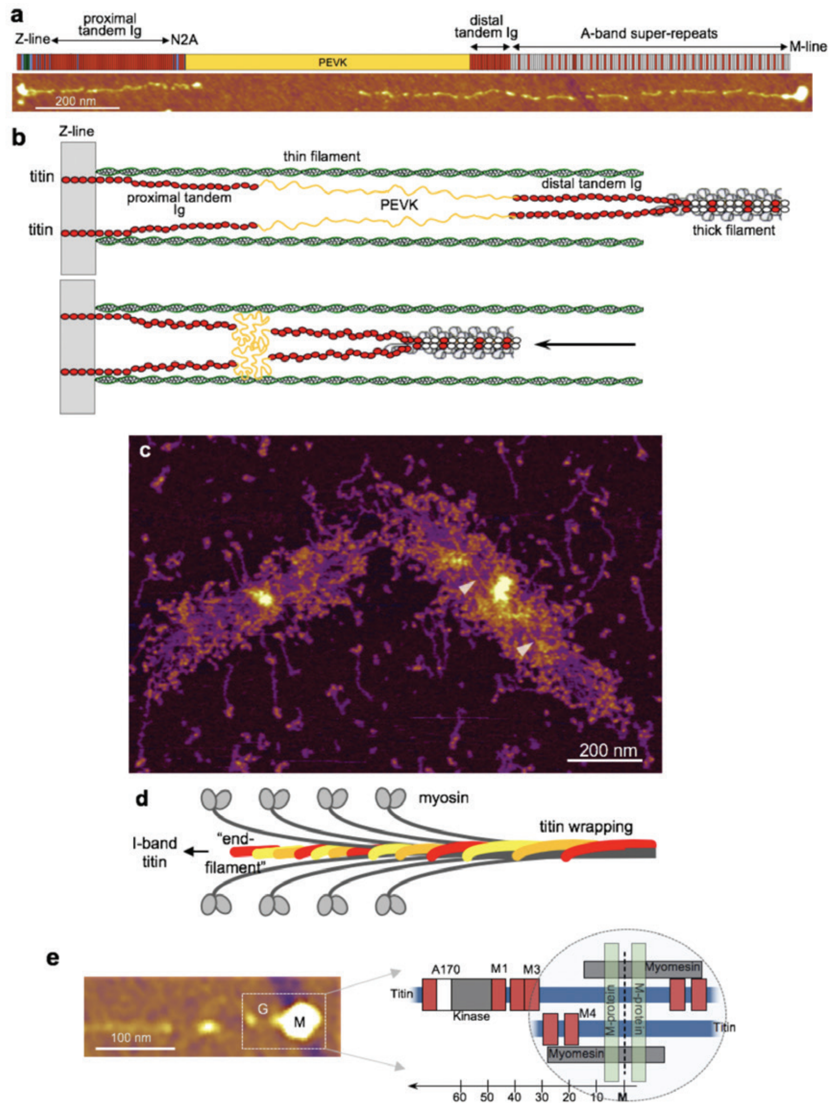

Figure 6. Anatomy and multiplex functionality of the giant protein titin. **a.** Domain layout and segmental structure of *m. soleus* titin (top) (Mártonfalvi et al. 2014). Ig and FN domains, the PEVK domain and unique sequences are indicated in red, white, yellow and blue, respectively. AFM image of a titin molecule stretched with receding meniscus (bottom) (Mártonfalvi and Kellermayer 2014). **b.** Model of the possible regulatory effect of PEVK-actin interaction on sarcomeric contraction (Nagy et al. 2004). **c.** AFM image of myosin synthetic filaments complexed with titin oligomers (M. Kellermayer et al. 2018). The titin oligomers bound to the surface of

ysis (Greaser 2001), which revealed two dominant motifs, PPAK and polyE. PPAK motifs are ~28-residue sequences which begin most often with amino acids PPAK. PolyE motifs contain a preponderance of glutamate. NMR and CD spectroscopy measurements indicate that PEVK might dynamically fluctuate between various conformational states: poly-proline helix, beta turn and unordered coil (Ma and Wang 2003). It was shown that parts of PEVK have Ca^{2+}-dependent elastic properties (D. Labeit et al. 2003). The large number of glutamates in polyE motifs indeed suggests Ca^{2+}-affinity. However, neither the stoichiometry, nor the pCa-dependence of Ca^{2+} binding are exactly known. Several different I-band elements of titin, including the PEVK segment (Kulke et al. 2001; Nagy et al. 2004; Yamasaki et al. 2001), have been demonstrated to possess actin-binding properties (Jin 1995; Trombitás and Granzier 1997). We have previously shown that titin, possibly via its I-band section, inhibits actin motility in a Ca^{2+}-dependent manner (M. S. Kellermayer and Granzier 1996). The implications of this finding are still unclear. A large proteolytic titin fragment, which includes the PEVK domain, binds thin filaments via direct tropomyosin interaction (Raynaud, Astier, and Benyamin 2004). The exact mechanisms of skeletal-muscle PEVK-actin binding and its physiological significance remain unclear. The PEVK domain might function as a non-canonical Ca^{2+}- and actin-binding element dedicated for sensing mechanical transients and forming a tight feedback between passive and active sarcomere mechanics (**Fig. 6.b**). Finally, correlation was

the thick filaments and stretched out along their axis (arrowheads). **d.** Schematics of the interaction between titin and the thick filament near the filament end (M. Kellermayer et al. 2018). The red, orange and yellow lines represent doublets of titin molecules spiraling along the thick-filament surface (AL-Khayat et al. 2013). Beyond the tip, titin molecules interact to form the end-filament (Houmeida et al. 2008; Trinick 1981), thereby spatially limiting to the thick-filament length. **e.** AFM image and schematics of titin's M-line region („M") (Mártonfalvi et al. 2014). The AFM image was collected on a titin molecule stretched with receding meniscus. „G" marks the topographical gap in titin which corresponds to the N-terminal ß-sheet of the titin kinase domain.

observed between passive tension and the stretch-induced shift in pCa_{50}, which marks the stretch sensitivity of active force, which suggests that titin might play a role in modulating contractile activity through somehow monitoring sarcomere length (Le Guennec et al. 2000).

A-band and M-line titin

In the A-band, titin is attached to the thick filament and is thought to function as a molecular scaffold (Trinick 1996). A-band titin comprises super-repeats of Ig and FN domains (S. Labeit and Kolmerer 1995). An FN domain has been shown to be mechanically less stable than an Ig (Rief et al. 1998), suggesting that external factors (e.g., anchorage to the thick filament) determine the overall high mechanical stability of A-band titin. Indeed, in purified titin, the progressively unfolded domains are distributed homogenously along the entire length of the molecule (Bianco et al. 2015). Because antibodies against the A-band section of titin do label sarcomeres, it has been thought that titin runs on the surface of the myosin thick filament. Single-particle analysis of negatively stained cardiac myosin filaments has indeed positioned titin on the surface of the thick filament as a gently winding pair of molecules (AL-Khayat et al. 2013). Recently, it was found that the deletion of two super-repeats from the C-zone of titin resulted in an exactly corresponding reduction of the A-band width (Tonino et al. 2017), causing a re-emergence of the titin-ruler hypothesis (Tskhovrebova and Trinick 2017). An AFM analysis of the structural arrangement in titin-thick filament co-assemblies, however, indicated that titin is unlikely to function as a simple geometric ruler that provides a template for the arrangement of myosin molecules. Rather, titin forms a wrap around the surface of the thick filament which is likely to control the structural dynamics at the end of the thick filament and place an upper limit on its length (**Figs. 6.c-d**) (M. Kellermayer et al. 2018). The mechanics of titin-myosin interaction in the A-band might be important, among others, in relaying forces

from the functionally elastic I-band titin segment towards the mechanosensor kinase domain near the M-line.

Towards titin's C-terminus, near the M-line, the molecule contains a ***kinase domain*** (titin kinase, "TK") (S. Labeit and Kolmerer 1995). It has been hypothesized that TK may play a role in intrasarcomeric signaling. For long, however, neither the activation mechanisms, nor the substrate of TK were known. A novel dual activation mechanism might take place, involving tyrosine phosphorylation and Ca^{2+}/calmodulin binding that induces the release of an autoinhibitory tail that blocks the ATP-binding site (Mayans et al. 1998). Surprisingly, it was demonstrated that TK phosphorylates telethonin which binds in the Z-line, over a micron away in the mature sarcomere (Gregorio et al. 1998). Therefore, TK might play an important role during myofibrillogenesis. Force-field molecular dynamics simulations have shown that the autoinhibitory tail of TK may be mechanically rearranged, thereby exposing the catalytic site (Gräter et al. 2005). The force-driven structural rearrangements are predicted to precede the complete unfolding of the enzyme. More recently it has been observed that a mechanically inducible conformation of TK interacts with Nbr1, a zinc-finger protein involved in muscle signaling cascades (Lange et al. 2005). The mechanoenzymatic properties of TK were characterized by single-molecule force spectroscopy, which indicated that the domain can indeed be activated by mechanical forces (Puchner et al. 2008). A topographical analysis, with AFM, allowed us to allocate the most distal unfolded titin region to the kinase domain, suggesting that the TK systematically unfolds when titin is exposed to overstretching forces (Mártonfalvi and Kellermayer 2014). The observations support the prediction that upon the action of stretching forces the N-terminal ß-sheet of the titin kinase unfolds, thus exposing the enzyme's ATP-binding site and hence contributing to the molecule's mechanosensory function.

Titin as an active contractile element

Whether globular domains of the titin I-band section dynamically unfold and refold under physiological conditions in the cardiac sarcomere has long been discussed and debated. Immunoelectron microscopic analyses using sequence-specific antibodies that demarcate the boundaries between canonical structured (e.g., tandem-Ig) and unstructured (e.g., PEVK) regions in titin (Trombitás et al. 1998) were unable to demonstrate domain unfolding, even under extensive stretch procedures. Recently, however, it was suggested that the folding of titin can produce mechanical work that assists active muscle contraction (Rivas-Pardo et al. 2016). In position-clamp optical tweezers experiments on the full-length titin molecule, we demonstrated that titin indeed can develop force *via* dynamic transitions between its unfolded and molten-globule structural states that proceed, overall, towards the native, folded conformation (**Fig. 7.a**) (Mártonfalvi et al. 2017). At low forces (in the range of 2-8 pN), where the mean free energy of the unfolded and molten-globule states are similar (**Fig. 7.b**), the dynamics of the molten-globule — unfolded transition control the overall length, hence extension of titin. Depending on the specific energy landscape of the domains in relation to the external force, some domains are selected, as if by a Maxwellian demon, for a transition from the unfolded to the molten-globule state, whereas others remain unfolded. This way titin shortens against force by gradually repopulating the domains from the longer unfolded state to the shorter molten-globule state. Thus, by controlling the microscopic state of the individual titin domains, the macroscopic mechanics of muscle may be regulated. While the number of domains connected in series along the axis of titin controls the overall length change, the number of titins connected in parallel in the cross-section of muscle sets the total force against which the legth change occurs. Altogether, the amount of work generated in the process can be finely regulated. Although work generation by a controlled repopulation of microscopic states appears to violate the second law of thermodynamics upon first glance, it must be taken into account that part of the

internal energy of the system comes from the work invested in the stretching (and unfolding) of titin in the preceding mechanical cycle, and that, within the context of the muscle sarcomere, the work of myosin molecules using chemical energy from ATP participate in controlling the force on titin. This ensemble molten-globule dynamics delivers significant added contractility that may assist sarcomere mechanics, and it may reduce the dissipative energy loss associated with titin unfolding/refolding during muscle contraction/relaxation cycles.

Titin in cardiac-muscle pathology

Although titin has been implicated in numerous muscle pathologies (Linke 2018), in recent years it received special attention due to its association with dilated cardiomyopathy (DCM). Cardiomyopathy is defined by an abnormal myocardium (Jefferies and Towbin 2010; Akinrinade, Alastalo, and Koskenvuo 2016; Granzier et al. 2005; Hershberger, Hedges, and Morales 2013; Granzier and Labeit 2002; Begay et al. 2015; Granzier and Labeit 2006; Granzier and Labeit 2004; Gerull 2015; Krüger and Linke 2011; McNally, Golbus, and Puckelwartz 2013; Krüger and Linke 2009; Herman et al. 2012; LeWinter and Granzier 2013; LeWinter et al. 2007; Linke and Hamdani 2014; Linke and Krüger 2010; Tskhovrebova and Trinick 2010). There are four major types of cardiomyopathy: dilated (DCM), hypertrophic (HCM), restrictive (RCM) and arrythmogenic right ventricular (ARVC). DCM is the most common worldwide. The prevalence of DCM increases with age and previously has been underestimated. Its prevalence in the adult population is estimated as 1 in 500. Apart from ischemic DCM, which develops on the basis of myocardial infarct or ischemia, nearly half of the cases are familial and have a genetic origin. Approximately 30 genes have been associated with DCM (Jefferies and Towbin 2010; Haas et al. 2014; Hershberger, Hedges, and Morales 2013; McNally, Golbus, and Puckelwartz 2013; Hirtle-Lewis et al. 2013; Morales and Hershberger 2013; Punetha and Hoffman 2013).

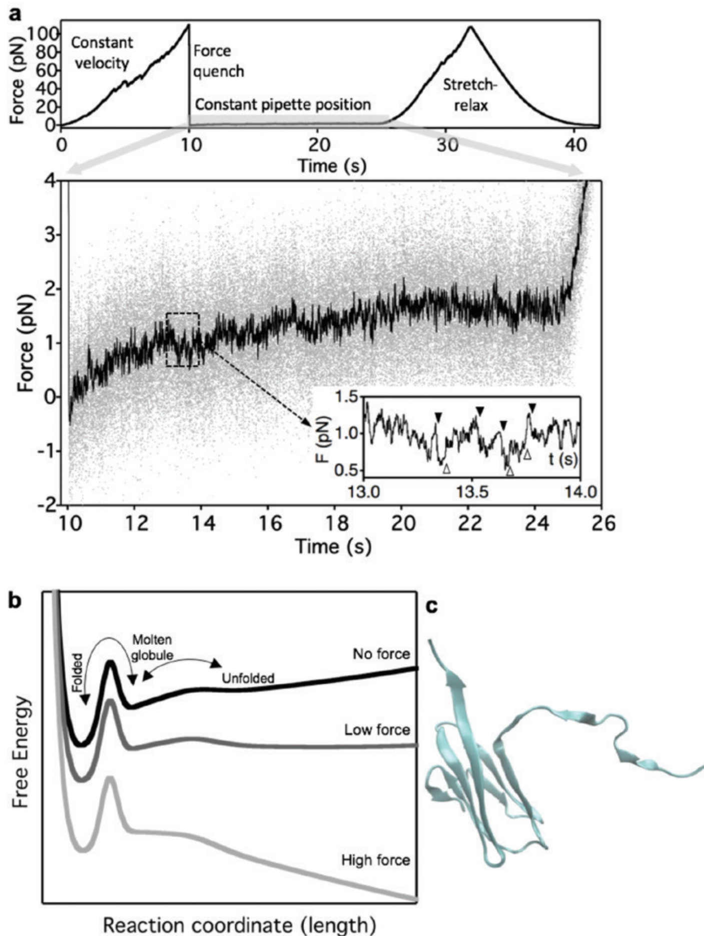

Figure 7. Force generation by titin folding. **a.** Force *versus* time plot (upper trace) displaying the position-clamp protocol. Following titin stretch with constant velocity, force is rapidly quenched, and the manipulated end of the molecule is held in a constant position while the force on the trapped bead is monitored. The protocol ends with a complete stretch-relaxation cycle to monitor refolding. Force *versus* time trace (lower trace) recorded during position clamp. Grey and black traces correspond to raw and 100-point median-filtered data, respectively. **Inset,** Enlarged view of the framed area showing force fluctuations consisting of contraction (filled arrowheads) and relaxation steps (empty arrowheads) with sub-pN amplitude. **b.** Schematics of the titin folding landscape based on a three-state model that contains the

The first observation that DCM might be linked to *TTN* mutation was reported in 1998 (Siu et al. 1999). Although several reports of *TTN* mutations associated with DCM appeared subsequently (Carmignac et al. 2007; Gerull et al. 2002; Gerull et al. 2006; Guo et al. 2012; Itoh-Satoh et al. 2002; Matsumoto et al. 2005; Xu et al. 2002), comprehensive analyses were greatly hampered by the enormous size of the gene. In 2012, a landmark analysis utilizing next-generation sequencing (NGS) methods demonstrated that 27% of familial DCM patients carry *TTN* mutations, most of which result in truncation and therefore an anticipated shortened isoform (Herman et al. 2012). As of early 2014, 127 disease-causing *TTN* mutations were known (Linke and Hamdani 2014). Thus, *TTN* has emerged as a major disease gene (Ceyhan-Birsoy et al. 2013; Chauveau, Rowell, and Ferreiro 2014; LeWinter and Granzier 2013; Linke and Hamdani 2014; Roberts et al. 2015). Most of the *TTN* mutations are linked to DCM, and a positional analysis indicates that most of them are located within the A-band section of titin (Herman et al. 2012; Linke and Hamdani 2014). It has been suggested, therefore, that small isoforms of titin with missing C-terminal ends might be present in the DCM sarcomere. It has been speculated that the consequently altered interaction of titin with the thick filament and the missing titin kinase domain might be behind DCM pathogenesis. However, the low penetrance of the mutations, the late onset of the disease and the difficulty of detecting short titin isoforms indicates that there are yet unknown rescuing mecha-

folded, unfolded, and molten-globule states of a titin domain. Arrows indicate reactions between the states. Depending on the magnitude of the force applied to the titin molecule, the landscape is tilted. Notably, at low forces the energies of the molten-globule and unfolded states are similar; therefore, a slight change in force may induce a large bias towards either of the ufolded or molten-globule states. **c.** Putative structure of the molten-globule state of the I27 (I91) titin domain. Steered molecular dynamics simulation of the domain exposed to a force field, with constant pulling velocity, to a maximum force value of 5 pN. The core of the domain, stabilized by a set of hydrogen bonds, remains relatively intact, whereas the C-terminal ß-strand was peeled off the core (Mártonfalvi et al. 2017).

nisms at work. Furthermore, a recent analysis indicated that the burden of *TTN* truncating variants on the general population is enormous, and every person is likely to carry at least one, albeit not disease-causing, *TTN* mutation (Roberts et al. 2015); by contrast, manifest DCM is less frequent. Thus, the molecular mechanisms of how *TTN* mutations lead to DCM symptomatology are still enigmatic.

Conclusions

Following the discovery of titin in the late 1970s, it took decades and a long line of physiological, biophysical and molecular biological experiments until this protein made its way into the textbook illustrations of the muscle sarcomere. By now, it is beyond doubt that titin plays a fundamental biophysical, biochemical, physiological and pathological role in muscle: through its three-dimensional structure and elasticity, titin determines the passive mechanical behavior of the muscle sarcomere, and, by transiently interacting with sarcomeric ligands (e.g., actin, myosin-binding protein C, calcium ions), it also contributes to the regulation of active muscle contraction; by providing a spatially coded array of ligand-binding sites, titin is thought to have a templating, hence structurally integrating function; and, titin is likely to sense the mechanical status of the sarcomere by multiple mechanisms which include its kinase domain. Although research on titin has accelerated in recent years and we learned much about the molecular genetics, structure and mechanics of this remarkable protein, many questions abound which await answering: is mechanical titin folding-unfolding part of the physiological muscle contractile cycle? How does force regulate the structure and dynamics of the titin interaction network? What are the mechanisms by which titin's gigantic gene transcript is spatially and temporally processed? How is titin turned over in the sarcomere? How are variations in the titin gene product manifested in disease? With these pressing questions at hand, an exciting future of titin research can be envisioned.

Acknowledgements

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Excitation-contraction coupling in skeletal muscle – a Hungarian perspective

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Introduction

The meaning of the term excitation-contraction (EC) coupling has changed considerably over time from when it was first realized that skeletal muscle fibres need electrical stimulation to initiate contraction. At that time EC-coupling referred to all events that take place between the electric stimulus, the action potential of the surface membrane as realized later, and the shortening of the muscle fibre. Recently – or not so recently as the transformation took place during the nineteen seventies and eighties –, as our understanding of the cellular and molecular events have grown, and the actual steps became more and more clear, the meaning of EC-coupling has distilled into describing the process how the change in membrane voltage initiates the release of calcium ions into the myoplasmic space. From here on this latter understanding of EC-coupling will be used. For those interested in the history of EC-coupling in more detail a recent review by Clara Franzini-Armstrong (2018) is a must.

During the past four decades a number of extensive and detailed reviews have been published on the topic of or related to EC-coupling (a simple search in PubMed with the keywords of "skeletal muscle", "EC-coupling", and "review" gave 45 hits, at the time when this text was prepared, with the earliest from

1984). This paper, therefore, is not attempting to give a complete description of how we reached the current understanding of the molecules involved in and their interactions needed for EC-coupling; rather, it tries to paint a picture how scientists from Hungary contributed to this process. Even with this restriction the task is enormous and the picture shall inevitably be faint with some details being left to the reader to complete, which is probably best illustrated by the fact that the aforementioned review from 1984 was written by Anthony N. Martonosi (and published in Physiological Reviews) a scientist who emigrated from Hungary to USA in 1957. And the list of Hungarian researchers working in the field of muscle contraction who have emigrated to the USA either following World War II, or the uprising in Hungary in 1956, or later could go on-and-on, so let me just mention here Michael and Kate Bárány, after whom (due to their endowment) the former Young Investigator Award of the Biophysical Society has been renamed in 1998.

The steps in EC-coupling

EC-coupling in vertebrate skeletal muscle takes place at the level of the triad where two terminal cisternae (TC) of the sarcoplasmic reticulum (SR) come in close apposition with a transverse (T-) tubule, the invagination of the surface membrane (as depicted in *Fig. 1;* for reviews consult e.g. Flucher, 1992; Franzini-Armstrong and Jorgensen, 1994). The depolarization of the muscle fibre results in the conformational change of the dihydropyridine receptors (DHPR-s) – L-type voltage-gated calcium channels (CaV1.1) – located in the T-tubular membrane serving as the voltage sensor of EC-coupling (e.g. Ríos and Pizarro, 1991). The change in membrane voltage is sensed by positively charged amino acids in the 4th transmembrane segment of the $\alpha 1$ subunit of DHPR. This conformational change will then initiate through a protein-protein interaction (see e.g., Ríos et al. 1992) the opening of the ryanodine receptor (RyR) calcium release channels (e.g., Franzini-Armstrong and Protasi, 1997) located in

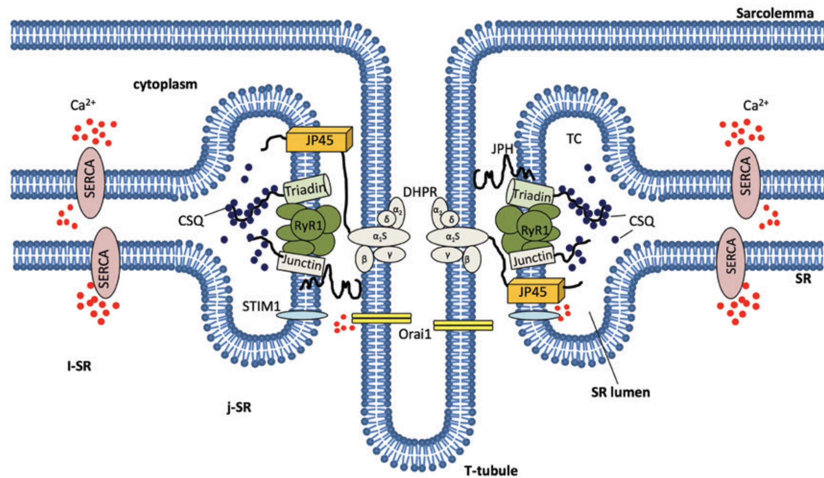

Fig. 1. Schematic representation of the EC-coupling machinery emphasizing the structural components at the level of the triad. Two terminal cisternae of the SR are in close apposition with the invagination of the surface membrane, the T-tubule. The calcium release channel, or RyR, is in the membrane of the former, while the voltage sensor of EC-coupling, the DHPR, is in the membrane of the latter. A number of accessory proteins – note that not all recognized proteins are shown –involved in the regulation of the coupling between DHPR and RyR and the release of calcium from the SR are also shown. Abbreviations: CSQ – calsequestrin, DHPR – dihydropyridine receptor, JPH – junctophilin, JP45 – 45 kDa junctional protein, j-SR – junctional SR, l-SR – longitudinal SR, RyR1 – ryanodine receptor type 1, SERCA – sarco(endo)-plasmic reticulum calcium ATP-ase, SR – sarcoplasmic reticulum, STIM1 – Stromal interaction molecule 1, TC – terminal cisternae, T-tubule – transverse-tubule.

the adjacent TC of the SR. The interaction between the DHPR and RyR likely involves the alteration of Mg^{2+} inhibition of the latter (Lamb, 2002). Once the calcium release channels are open Ca^{2+} will move into the myoplasm resulting in an increase in intracellular calcium concentration and, eventually contraction. As calcium within the SR is found mostly bound to the calcium buffer calsequestrin (CSQ), it must first dissociate from CSQ before it can be released. CSQ, however, is not just a simple calcium buffer it has important regulatory roles, too (see, e.g., Beard et al. 2004). Furthermore, both the interaction between DHPR and RyR and the opening of RyR are modulated by accessory proteins found in the membranes of the T-tubules and

TC including, among others, Orai1, STIM1, Junctate, JP45, Juntophilin, Triadin (for reviews see e.g. Treves et al. 2009; Barone et a. 2015; Marty, 2015).

The oncoming chapters will look at these events and highlight some advancement in the respective fields that were contributed by scientists from Hungary (or Hungarian by birth). I must acknowledge and, therefore, apologize in advance, that the selected references are thus, inevitably, biased; in certain cases I'm referring to papers that bear some Hungarian connection rather than citing those that were determinative in their time.

The introduction of the Vaseline-gap voltage clamp

First and foremost one should acknowledge that studies leading to the understanding of the steps in EC-coupling can thank their success to the rigorous mathematical and physical approach that has penetrated the field from its earliest. This could not have happened in any other way as these studies were a direct consequence and were following in the footsteps of the seminal work by Hodgkin and Huxley describing the ionic currents in the squid giant axon. Therefore, the earliest works focused on developing a proper method to record ionic currents in isolated skeletal muscle fibres.

There were, however, a number of obstacles to be overcome. First, skeletal muscle fibres are not as easy to dissociate from one another to obtain a single isolated cell as axons are. Second, the "classical" internal axial wire clamp inevitably damages the contractile apparatus and all internal membrane systems thus depriving the preparation from possibly important components. Thirdly, which directly follows from the previous, events that take place along the T-tubules are not well or not at all resolved. Finally, the large surface-to-volume ratio – due to the presence of the T-tubular network – requires the ability to pass large currents.

Although several other solutions, e.g. the “three microelectrode voltage-clamp” (Kao and Stanfield, 1968) or the double sucrose gap (Ildefonse and Rougier, 1972), were in use for some time, the Vaseline-gap voltage clamp clearly won the race. Its first reliable application in skeletal muscle research was presented in a series of papers that appeared in The Journal of General Physiology (the Reader is referred to the leading paper which describes the methods in detail; Hille and Campbell, 1976). Using the improved triple Vaseline-gap voltage clamp and the dissection and mounting of the isolated fibre into the chamber as described by Hille and Campbell albeit solved most of the problems mentioned above, the ability of the muscle fibre to contract once the normal resting membrane potential is restored remained and had to be addressed (as movement inevitably damages the Vaseline seal; note that the procedure used by Hille and Campbell resulted in mechanically inactive cells). Before turning to how this problem was addressed one should clarify that, at that time and for many years to come, the subject of study was the mechanically (i.e. under the microscope) dissected single fibre from the *m. semitendinosus* or *ileofibularis* of the frog. These fibres are large – usually around or more than 100 μm in diameter – and are relatively easy to dissociate due to the lack of intensive connective tissue. This is not the case for mammalian skeletal muscle as will be discussed later. In addition, one should be aware that using the Vaseline-gap voltage clamp requires cutting (or cutting a notch into) the fibre – hence the sometimes used “cut-fibre” expression for this technique – to access the intracellular space which will inevitably alter the intracellular environment.

When contraction is looked at from a theoretical point-of-view in case of the Vaseline-gap voltage clamp two possible alternatives can be considered. Either allow the fibre to contract and then utilize the additional information arising from force generation or prevent contraction completely. It was at this time – the middle of the 1970-s – when László Kovács (University of Debrecen, Hungary) spent his sabbatical at the University of Rochester (Rochester, NY, USA) working with Martin F. Schneider. They came up with a simple, yet effective solution for

the former case, the "single Vaseline-gap" (for the description of the system see e.g. Kovács and Schneider, 1978). In this arrangement the intact segment of the fibre is fixed through its tendon to a movable block allowing the setting of the sarcomere length and enabling the fibre to contract upon stimulation. The simple Vaseline-gap system was further improved/modified when László Kovács returned to Debrecen and the tendon was attached to a force transducer and the time course of the contraction was recorded in parallel with the other events of EC-coupling (Szentesi et al. 1997b; see later). Although allowing the fibre to contract can have its advantages – e.g., determination of the contractile threshold, working at physiological sarcomere lengths – there is an obvious disadvantage: optical signals, if measured, are distorted. As it will be discussed below (see Chapter "*Measuring changes in intracellular calcium concentration and calculating SR calcium release*") changes in intracellular calcium concentration ($[Ca^{2+}]_i$) are detected using calcium sensitive dyes, thus requiring an arrangement where the fibre cannot contract. This was achieved using the double Vaseline-gap voltage clamp (see e.g. Kovács et al. 1983), where the fibre can be stretched to sarcomere lengths beyond filament overlap (beyond 3.4-3.8 μm).

As mentioned above mammalian skeletal muscle fibres are not as easy to mechanically dissect as amphibian fibres mainly due to their smaller diameter (around 40 μm) and to the presence of a more extensive connective tissue. Only a handful of cases are documented where mechanical dissection of mammalian fibres was attempted and used in Vaseline-gap voltage clamp (e.g., Delbono and Stefani, 1993; Shirokova et al. 1996). After collagenase became commercially available and thus enzymatic dissociation of cells an easy task this approach seemed an ideal solution to obtain individual mammalian fibres for Vaseline-gap experiments. However, collagenase treated fibres did not seem to tolerate the contact with Vaseline. When Vincent Jacquemond (Claude Bernard University, Lyon, France) visited my lab in 1996 we developed a method to overcome this problem and have successfully implemented the use of enzymatically dissociated fibres in Vaseline-gap voltage clamp (Szentesi et al. 1997a).

Dr. Jacquemond went on to modify the technique which he coined as “Silicone-clamp” (see e.g. Pouvreau et al. 2007a).

Although these improvements in methodology might seem less important they paved the road to the understanding of the physiology of EC-coupling by enabling a precise measurement and rigorous description of the events that take place following the depolarization of the surface and T-tubular membranes.

Intramembrane charge movement and its components

After the work of Huxley and Taylor (1958) it became evident that the depolarization of the T-tubular network is needed to initiate contraction and this voltage change somehow (for an early review see Ebashi et al. 1969) plays an essential role in initiating the release of calcium from the SR which in turn is needed for contraction. This prompted Schneider and Chandler (1973) to speculate whether a charged entity in the membrane that moves (or re-orients) upon depolarization would be the voltage sensing step in EC-coupling. If this movement of the hypothesised charges in the membrane (thus the name intramembrane charge movement; ICM) indeed existed their displacement should be detected as part of the membrane current when the fibre is depolarized. Using the three microelectrode voltage clamp Chandler et al. (1976a) were able not only to demonstrate its existence but to also give a detailed description of its voltage dependence. With this seminal paper the “golden era” of ICM begun (for detailed reviews on ICM the Reader is referred to e.g. Huang, 1988; Ríos and Pizarro, 1991; Ríos et al. 1992).

Albeit the discovery of ICM was a clear advancement, a number of resolvable questions arose soon after its description. Some seemed merely technical, although not entirely negligible, while others more physiological. The former included the problems that the voltage dependence and pharmacology of ICM and the release of calcium from the SR seemed somewhat different

while the latter involved what the molecular identity of ICM was and the possible mode of action how it could control the release of calcium from the SR.

When Géza Szücs (from Debrecen, Hungary) visited the laboratory of Martin F. Schneider in the mid 1980-s they addressed, amongst others, the question of the voltage dependence. The original observation was that a considerable amount of intramembrane charge is moved at small depolarizations (to or below -50 mV) without any, or negligible, calcium release to accompany it. To address this apparent discrepancy they proposed that a portion of the charge movement is not associated directly with initiating calcium release (similarly as channel openings can involve transitions between closed states before the channel finally opens) and thus subtracted this "sub-threshold" charge from the total (Melzer et al. 1986). With this simple manoeuvre they could demonstrate a linear relationship between "suprathreshold charge" and peak rate of calcium release (for the definition and determination of the latter see Chapter "*Kinetics of SR calcium release*"). After returning to Debrecen Dr. Szücs and his co-workers went on to prove that in the presence of perchlorate – an anion that shifts the voltage dependence of both ICM and calcium release from the SR towards more negative membrane potentials – the linear relationship between "suprathreshold charge" and calcium release is maintained providing further evidence that a proportion of intramembrane charge is moved without initiating the release process (Csernoch et al. 1987).

In parallel with the studies on the voltage dependence of ICM, its pharmacology and kinetics were also investigated in detail. Two important findings should be mentioned here. Currents reflecting ICM measured close to the contraction threshold/intermediate voltages (around -50 to -30 mV) display a delayed, so called "hump" component (as presented in *Fig. 2A;* for early references see e.g., Adrian and Peres, 1979; Huang, 1982; Hui, 1983). The "hump" component (or Q_γ) gained a lot of attention as its pharmacology – e.g. its sensitivity to tetracaine – and its voltage dependence closely resembled that of SR calcium release. While its first reports (as referenced above) came from intact fi-

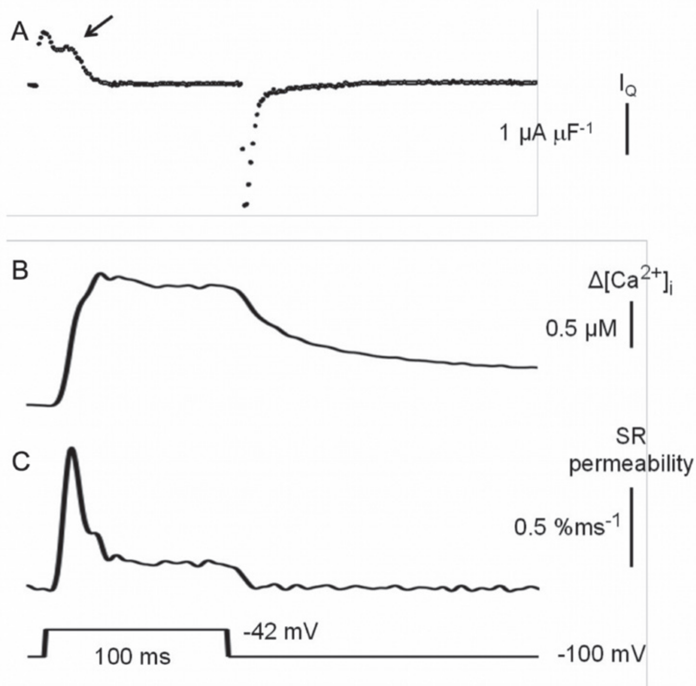

Fig. 2. Major steps in EC-coupling. (**A**) Non-linear capacitive current (I_Q) representing intramembrane charge movement measured in response to a voltage clamp depolarization to -42 mV from a holding potential of -100 mV for 100 ms as shown below the traces. The current was normalized to fibre capacitance. The arrow points to the delayed, so called "hump" component. (**B**) Change in intracellular calcium concentration ($\Delta[Ca^{2+}]_i$) calculated from the absorbance change of Antipyrylazo III accompanying the depolarization detailed above. (**C**) Time course of SR permeability change determined by fitting a removal model to the declining phase of the calcium transient (presented in panel B) after correcting for the depletion of calcium in the SR. The permeability was expressed as percent change in SR content in a millisecond. The measurement was carried out using a single Vaseline-gap voltage clamp.

bres using the three microelectrode voltage clamp, its description in the single Vaseline-gap voltage clamp was carried out by the group in Debrecen (stimulated by a visit by Christopher L-H Huang from the University of Cambridge, UK), with a number of papers also highlighting its pharmacology (Kovács and Szücs, 1983; Csernoch et al. 1988; Szücs et al. 1991).

Apart from the interesting voltage dependence and pharmacology mentioned above, the "hump" component of ICM also intrigued physiologists by its kinetics. Namely, a secondary rising phase on the current representing ICM can only be explained by non-linear dynamics (see e.g. Huang, 1983). This, and a series of additional experiments conducted in Eduardo Ríos's lab (Rush Medical University, Chicago, IL) during my visit in 1988 led us to suggest that the "hump" component was the consequence of calcium binding to the intracellular part of the molecule responsible for ICM following its release from the SR (Csernoch et al. 1991; Pizarro et al. 1991).

One must also acknowledge the role what studying ICM played in the identification of the molecular entity of the voltage sensor. Studying the pharmacology of ICM revealed its sensitivity to all classes of blockers of the voltage-gated L-type calcium channels, including the dihydropyridines (Ríos and Brum, 1987), phenylalkylamines (Feldmeyer et al. 1990), and benzothiazepins (Walsh et al. 1987). A series of papers from Kurt G Beam's laboratory (e.g. Tanabe et al. 1988; Adams et al. 1990) using myotubes from dysgenic mice and injected with plasmids encoding for the DHPR gave the final evidence that ICM originates from the DHPR and *vice versa* the DHPR is the voltage sensor of EC-coupling.

Measuring changes in intracellular calcium concentration and calculating SR calcium release

To detect changes in $[Ca^{2+}]_i$ (referred to as Ca^{2+} transients) optical methods have been introduced in parallel with the spreading of the different voltage clamp techniques. At first these included the use of either naturally occurring photoproteins having calcium-dependent luminescence or metallochromic indicator dyes as Arsenazo III or Antipyrylazo III (for review see e.g., Blinks et al. 1982). The application of the former was shortly

discontinued as their kinetics are slow and the relationship between calcium binding and light emission is complex rendering the calculation of $[Ca^{2+}]_i$ difficult if not impossible. While metallochromic dyes have fast reaction kinetics, can thus track the rapid changes in $[Ca^{2+}]_i$ associated with EC-coupling, and do not require complicated optics, the interpretation of the absorbance signals accompanying calcium binding is also not so straightforward. It was when László Kovács again visited Martin F. Schneider's lab that the detailed description how Antipyrylazo III absorbance signals reflecting the Ca^{2+} transients – measured using the double Vaseline-gap voltage clamp – should be interpreted was published (Kovács et al. 1983). This paper set the stage for a number of oncoming publications that used the appropriately determined time course and magnitude of depolarization-evoked changes in $[Ca^{2+}]_i$. An example of a depolarization-evoked Ca^{2+} transient recorded in a Vaseline-gap voltage clamp system using Antipyrylazo III is presented in *Fig. 2B.*

It should be noted here that detecting the changes in dye absorbance during a depolarizing pulse does not allow one to determine the resting $[Ca^{2+}]_i$. Thus in the early papers only changes in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) were presented (as in *Fig 2b*). In 1985 a new class of fluorescent calcium indicators from the laboratory of Roger Y. Tsien was introduced (Grynkiewicz et al. 1985). These fluorescent probes have high affinity to calcium and undergo a shift either in their emission (e.g. Indo-1) or excitation wavelengths (e.g. Fura-2) upon calcium binding. By measuring the fluorescence at two emission wavelengths (Indo-1) or with two excitation wavelengths (Fura-2) and calculating the fluorescence ratio allows one to determine the actual $[Ca^{2+}]_i$, if appropriate calibrations are performed, even in a resting muscle fibre. During the visit of Dr. Szücs in Martin F. Schneider's lab they have successfully used Antipyrylazo III and Fura-2 simultaneously to monitor myoplasmic $[Ca^{2+}]$ before, during, and after a depolarizing pulse (Klein et al. 1988). Utilizing this technique and the fact that fluorescence signals are considerably less sensitive to movement artefacts we were able to measure Ca^{2+} transients and force production simultaneously in a single Vaseline-gap voltage

clamp and use them to estimate the kinetics of calcium binding to the regulatory sites on troponinC and the time necessary for myofilament activation once troponinC has calcium bound (Szentesi et al. 1997b).

With the ability to detect changes in $[Ca^{2+}]_i$ during a depolarizing pulse came the desire to determine the underlying calcium flux through the SR membrane. This flux, termed rate of SR calcium release, could then be directly compared both in kinetics and in voltage dependence to ICM. The basic idea how to achieve this goal is to estimate the total amount of calcium outside the SR at any given point in time ($Ca_T[t]$) together with the amount transported back into the SR by the calcium pump since the beginning of the depolarization ($Ca_P[t]$). Naturally, the sum of $Ca_T[t]$ and $Ca_P[t]$ would give the total amount of calcium that was released from the SR until time t ($Ca_R[t]$). (Note that the above reasoning assumes that the calcium fluxes across the surface and T-tubular membranes are negligible as compared to that through the SR membrane.) Taking the first time derivative of $Ca_R[t]$ ($Rel = dCa_R[t]/dt$) will then give the desired flux. Essentially two independent approaches have been implemented to tackle the problem. Dr. Chandler's group used biochemical data available in the literature on the number of calcium binding sites together with their binding kinetics to estimate $Ca_T[t]$ and $Ca_P[t]$ (e.g., Baylor et al. 1983) while Dr. Schneider's group estimated these values by fitting the declining phase of the Ca^{2+} transients starting a few milliseconds after the membrane potential was returned to the resting value (e.g. Melzer et al. 1984). Both groups came to the unanticipated conclusion that SR calcium release flux (i.e. Rel) declines during a depolarizing pulse.

Kinetics of SR calcium release

By now it is firmly established that Rel displays an early peak and then declines first rapidly then slowly to a quasi-steady level during a depolarizing pulse of 100 ms in duration (see e.g., Baylor et al. 1983; Melzer et al. 1984; Ríos and Brum,

1987). Both the time-to-peak and the amplitude of the early peak displays a clear voltage dependence with the former getting smaller while the latter greater with increasing depolarizations. Although the quasi-steady level also increases with voltage the peak-to-steady ratio is not constant; rather, it has characteristic voltage dependence as will be discussed later. The decline of SR calcium release flux during a 100 ms long depolarizing pulse was unexpected mainly because the inactivation of ICM requires prolonged (several seconds long; see e.g., Chandler et al. 1976b) depolarization, i.e. the voltage sensor remains in its activating position throughout the entire pulse.

A simple reason for the decline in calcium release flux could be the depletion of calcium in the SR. Elegant experiments involving Géza Szücs and conducted in Dr. Schneider's lab have shown that depletion indeed occurs during calcium release and is responsible for the slow decline in Rel (Schneider et al. 1987). They also demonstrated that under partially depleted conditions Rel is identical in kinetics – only smaller in size – to that when SR is normally loaded with calcium. Taken this into account one can correct Rel for depletion and normalize it to SR content to obtain the time course of SR permeability (as shown in *Fig. 2c*).

As calcium release from SR vesicles was shown to be calcium dependent (Meissner, 1986), Schneider and Simon (1988) have proposed that the fast decline following the early peak in Rel is due to a calcium dependent inactivation of the channels responsible for the release process. This hypothesis has since been confirmed in a number of independent experiments using skinned fibres (i.e., sarcolemma removed) or intracellularly applied calcium buffers (e.g., Kwok and Best, 1991 and Hollingworth et al. 1992, respectively). Nevertheless, the details of inactivation of the calcium release channels has intrigued the scientific community ever since. One of, or likely the most striking behaviour of the inactivation process – the explanation of which is still elusive – is its deterministic nature (Pizarro et al, 1997). Namely, a depolarization to a given voltage seems to inactivate a given set of channels; in other words, repeating the same depolarization would not inactivate further channels even though $[Ca^{2+}]_i$ was again

elevated. This led to the suggestion that two groups of release channels exist, one that activates and then fully inactivates and another that doesn't inactivate at all. While in amphibians this could theoretically be linked to the two types of release channels present in their skeletal muscle, mammals expressing only one channel isoform (see Chapter "*The calcium release channel of the SR*") were not expected to show deterministic inactivation. We, on the other hand, have disproved this concept by demonstrating that mammals behave, in this respect, identically to frogs and suggested that the spatial arrangement of the voltage sensors and calcium release channels might be the underlying reason (Szentesi et al. 2000).

Even though there are significant differences (as will be discussed below) Rel in amphibians and mammals share a number of common features. These two classes of vertebrates are similar in, among others, that neither requires the entry of calcium from the extracellular space to initiate calcium release from the SR in their skeletal muscle. In this respect they are unlike as, e.g., crustaceans where calcium-induced calcium release (CICR) is the essential step in EC-coupling as demonstrated by Sándor Györke during his work at the University of Texas in Philip Palade's lab (Györke and Palade, 1992). Nevertheless, as RyR-s expressed in vertebrate skeletal muscle can also be activated by calcium (see Chapter "*The calcium release channel of the SR*") the question whether CICR – using the calcium released from the SR – would also operate in amphibians and mammals has surfaced repeatedly and a number of experiments were designed to address the question.

During the visit of Vincent Jacquemond and myself in Dr. Schneider's laboratory we injected large amount of strong calcium buffers, Fura-2 and BAPTA, into voltage clamped frog skeletal muscle fibres and found that the presence of the these buffers, together with preventing the rise in $[Ca^{2+}]_i$ preferentially suppressed the peak without affecting the steady component of Rel (Jacquemond et al. 1991; Csernoch et al. 1993). We went on to suggest that CICR should be responsible for the initiation of the peak while the interaction between DHPR and RyR for the

steady component of Rel. Looking at the peak-to-steady ratio of Rel in batrachian muscle Shirokova et al. (1996) showed that it had a voltage dependence incompatible with the simple calcium dependent inactivation model. Namely, it was the greatest at intermediate voltages (at around -30 mV) while the change in $[Ca^{2+}]_i$ increased monotonically with increasing depolarization (implying that the degree of inactivation and, consequently, the peak-to-steady ratio should be also increase monotonically). In contrast, the peak-to-steady ratio in mammalian skeletal muscle was found to be essentially constant or to increase slightly but monotonically over a wide voltage range. We in Debrecen have then confirmed that this monotonic relationship in mammalian muscle is maintained even in the presence of different potentiators – perchlorate and caffeine – of SR calcium release (Csernoch et al. 1999a). On the other hand, cardiac glycosides were able to further increase the peak-to-steady ratio in frog skeletal muscle (Sárközi et al. 1996). Recent experiments demonstrated that a sudden increase in $[Ca^{2+}]$ via flash photolysis of caged-Ca^{2+} can generate a propagating calcium wave in amphibian but not in mammalian skeletal muscle (Figueroa et al. 2012). All these results suggest that the two classes of vertebrates differ in their precise regulation of SR calcium release, namely, CICR is present in frogs but not in mammals. The most straightforward explanation for this lies in the different RyR isoforms expressed in these animals (see Ríos, 2018).

The calcium release channel of the SR

Our understanding of the SR calcium release channel was shaped by data obtained using very different techniques: purification and reconstruction of the channel into planar lipid bilayers, molecular cloning, and studying its location and morphology using electron microscopy. Its identification was aided by the observation that ryanodine (a plant alkaloid from *Ryania speciosa* Vahl; note that ryanodine was known for its action on calcium homeostasis as early as the 1960-s, see e.g., Hajdu and Leon-

ard, 1961; Jenden and Fairhurst, 1969) specifically influences SR calcium release in skeletal muscle (Sutko et al. 1985; for early and recent reviews see e.g. McPherson and Campbell, 1993 and Meissner, 2017). Based on the above the presence of a cationic channel with large single channel conductance was demonstrated when heavy SR vesicles or purified [^{3}H]ryanodine-binding protein complexes were fused with lipid bilayers (Smith et al. 1985; Imagawa et al. 1987, Lai et al. 1988). This was then followed by the cloning and sequencing of the channel from a number of mammalian tissues revealing three closely related isoforms, namely, RyR1 (from skeletal muscle, Takeshima et al. 1989; Zorzato et al. 1990), RyR2 (from cardiac muscle, Nakai et al. 1990; Otsu et al. 1990), and RyR3 (from brain, Hakamata et al. 1992). RyRs are homotetrameric channels where each subunit is comprised of ~5,000 amino acids. Their tissue distribution has since been described in more detail and, while RyR1 is the major isoform in mammalian skeletal muscle, RyR3 has been shown to be expressed in developing and, albeit in small quantities (the diaphragm and the extraocular muscles expressing the largest amounts), in adult skeletal muscle, too (e.g. Conti et al. 1996; Sekulic-Jablanovic et al. 2015). In amphibian skeletal muscle, on the other hand, two isoforms (termed RyRα and RyRβ) of the calcium release channel are expressed in approximately equal amounts (Olivares et al. 1991), where the former is homologous to RyR1 while the latter to RyR3.

In the meantime electron microscopy studies established the presence of electron dense structures, termed "feet" in the junctional space between the membranes of the T-tubules and of the TC of the SR (Franzini-Armstrong, 1970). These feet were later identified as RyR embedded in the SR membrane and shown to be organized in a double row, displaying fourfold symmetry (Inui et al. 1987; Block et al. 1988; for review see e.g., Franzini-Armstrong and Jorgensen, 1994). In addition, assemblies of tetrads – later recognized as four DHPR – were visualized in the T-tubules of fish and amphibian muscle (Franzini-Armstrong and Nunzi, 1993; Franzini-Armstrong, 1984) and their relevance to EC-coupling becoming obvious when their spatial organization

in respect to the feet was established (Block et al. 1988). Namely, every other RyR in the TC faces a T-tubule tetrad. This arrangement gave the structural foundation for all models to come which described the steps of EC-coupling. The observation that while RyR1 is localized to junctional, RyR3 is present in parajunctional membranes of SR (Felder and Franzini-Armstrong, 2002) suggested a different regulation for these two isoforms.

Since their first description a number of studies have addressed the pharmacology as well as the role of disease related mutations in the functioning of the calcium release channel. Experiments on single channels incorporated into planar lipid bilayers have established, among others, the calcium, magnesium, adenine nucleotide as well as redox sensitivity of RyR (for an early and a recent review see e.g. Meissner, 1994; 2017). While intracellular ATP promotes, Mg^{2+} inhibits channel opening. Calcium ions, on the other hand, have a complex regulatory role with activating and inhibiting binding sites (the latter shared with Mg^{2+}) on the cytosolic – with the activating site being within the channel vestibule (see e.g., Sárközi et al. 2017) –, and an activating site on the luminal surface of RyR. In many cases the parallel sensitivity of Rel and RyR to pharmacological agents, including those used in therapy, have been confirmed in a number of laboratories, including that in Hungary (e.g. Csernoch et al. 1999b; Szentesi et al. 2001; Szentesi et al. 2004). Several small molecular weight peptides isolated from the venom of different scorpions – the so called calcins: e.g. imperacalcin (*Pandanus imperatus*) and maurocalcin (*Scorpio maurus palmatus*) – display a high affinity for RyR (Valdivia et al. 1992; Fajloun et al. 2000; Xiao et al. 2016). They share a common action by locking the channel in a sub-conducting state (e.g., Tripathy et al. 1998; Lukács et al. 2008).

Although not directly connected to EC-coupling, the presence and role of inositol 1,4,5-trisphosphate receptors (IP_3R-s; the other class of intracellular calcium release channels) in mammalian skeletal muscle should also be acknowledged. The seminal work of the group led by Dr. Jaimovich has established the role of IP_3R-mediated calcium signalling in skeletal muscle (Jaimov-

ich and Rojas, 1994; for review see Carrasco et al. 2004). IP_3R-s are found in the longitudinal SR as well as around the nuclei of the motor end plate (Salanova et al. 2002; Powell et al. 2003) and play a fundamental role in muscle development, regeneration, and gene transcription.

Elementary, or not so elementary events of calcium release

If the spatial resolution is good enough one expects that not only the changes in global $[Ca^{2+}]_i$ can be detected, but localised increases in calcium concentration due to the opening of – in theory – a single RyR should be resolved. Shortly after the introduction of confocal laser scanning microscopy and of novel fluorescence indicators (e.g., Fluo-3) that could be excited by appropriate laser lines, localized – both in space (full width at half maximum being 1.5-2 μm) and time (with rise times around 10 ms) – calcium signals were demonstrated in cardiac cells (Cheng et al. 1993) and then in frog skeletal muscle (Tsugorka et al. 1995; Klein et al. 1996). These events were termed calcium sparks and believed to be the elementary building blocks of SR calcium release (see *Fig. 3*). Indeed, in frog skeletal muscle they were shown to be stimulated by membrane voltage and, furthermore, as depolarization increased the frequency of their occurrence increased in parallel (Klein et al. 1996). These events thus became the focus when studying RyR function *in situ*.

RyR agonist and antagonists were shown to alter the frequency and spatio-temporal characteristics of calcium sparks (e.g. González et al. 2000a; Szentesi et al. 2004; Szappanos et al. 2005; Zhang et al. 2005; Hollingworth et al. 2006) in frog skeletal muscle fibres as expected substantiating further that RyR functions as the calcium release channel of the SR and shedding light on its regulation. On the other hand, the description of smaller amplitude events (termed embers – long lasting events with small, but constant amplitude –; González et al. 2000b) as

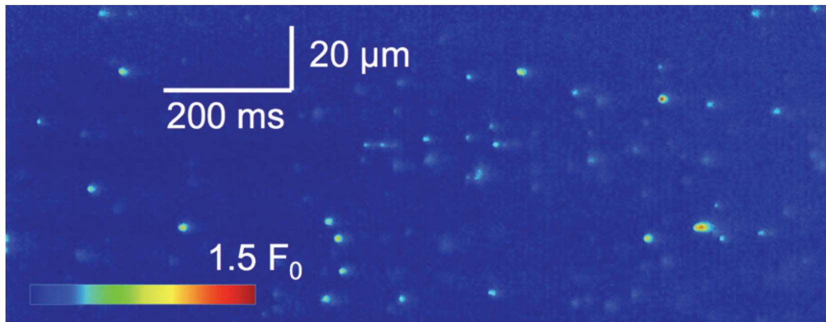

Fig. 3. Spontaneous calcium release events (calcium sparks) recorded in an intact isolated frog skeletal muscle fibre using a laser scanning confocal microscope operating in the line-scan mode. The changes in fluo-4 fluorescence (F) were normalized to background fluorescence (F_0) and the image was than artificially coloured as indicated.

well as the calculation of the calcium flux underlying an individual spark (González et al. 2000a) has led to the conclusion that not the opening of a single rather the concerted activation of a group of RyR-s is responsible for the spark.

As Rel was found to be qualitatively different in amphibian and mammalian skeletal muscle with different RyR isoforms expressed (see, respectively, Chapters "*Kinetics of SR calcium release*" and "*The calcium release channel of the SR*" above) the question arose how the elementary events in mammalian skeletal muscle would look. When the question was carefully investigated striking differences were found. While in frogs depolarizing the T-tubular membrane elicited calcium sparks, a seemingly eventless increase in $[Ca^{2+}]_i$ accompanied the change in membrane potential in rats (Shirokova et al. 1998). Interestingly though, on skinned – either mechanically or chemically with Saponin – mammalian fibres Kirsch and Fink (2001) were able to show calcium release events resembling the sparks and the embers of batrachian muscle, with the latter being relatively more numerous. Since their first description, a number of experiments, including some from Hungary, have proven the existence of these events on skinned (see e.g. Zhou et al. 2003; Szappanos et al. 2005; Von Wegner et al. 2007) or osmotically stressed (Wang et al. 2005) mammalian fibres.

As these (i.e. skinned fibres) preparations were considered as non-physiological the question whether sparks or embers would be the "elementary" events in mammals remained, for some time, unanswered. During my visit to Dr. Ríos's lab we were able to record calcium release events in enzymatically isolated rat fibres under voltage clamp conditions using a modified double Vaseline-gap set-up (Csernoch et al. 2004). The events resembled the embers of frog muscle fibres having small, but constant amplitudes. The presence of these events was confirmed in mice using the Silicone-clamp technique when I worked in Lyon in Dr. Jacquemond's lab (Csernoch et al. 2008). It is important, however, to stress that even though events were identified in both cases, they were scarce and the response to a depolarising pulse was in most of the cases a homogenous, eventless increase in fluorescence confirming the original observation of Shirokova and colleagues (1998).

In the meanwhile spontaneous calcium release events remained elusive in intact adult mammalian skeletal muscle fibres (with an exception, e.g., from Conklin et al. 1999a), while readily observed in embryonic cells or cultured myotubes (e.g. Conklin et al. 1999b; Shirokova et al. 1999; Chun et al. 2003; Fodor et al. 2008). The work of Shirokova et al. (1999) demonstrating that in myotubes regions with developed T-tubules do not, while regions that lack the T-tubular network display calcium sparks led to the suggestion that the interaction with the DHPR prevents RyR1 from giving rise to spontaneous calcium release events (Zhou et al. 2006). Further experiments pointed towards the role of RYR3 in the generation of calcium sparks in mammalian muscle (e.g. Ward et al. 2001; Pouvreau et al. 2007b; Weisleder et al. 2007; Legrand et al. 2008). Recently we have been able to demonstrate that spontaneous calcium release events occur on intact adult mammalian skeletal muscle fibres that solely express the embryonic isoform (CaV1.1Δ29) of the α1 subunit of DHPR also suggesting that the direct interaction between the voltage sensor and the SR calcium release channel plays an inhibitory role in spark generation (Sultana et al. 2016). All these observations raised the concern whether these calcium release events

could be considered as the "elementary" steps of EC-coupling in mammalian muscle, or not.

Accessory proteins of the SR terminal cisternae

Together with the demonstration of RyR in junctional SR preparations it became clear that other molecules are also present in the triadic space (for an early review see e.g. Caswell and Brandt, 1989). Since then the number of identified proteins – often referred to as accessory proteins – has increased considerably and their interaction with RyR or DHPR and role in EC-coupling have been studied in detail. It is far beyond the scope of this review to discuss or even enlist all members of this illustrious group. They include, among others (please note that the following list is far from being complete and the reader is advised to consult recent reviews, e.g. Treves et al. 2009; Barone et al. 2015; Dulhunty et al. 2017; Meissner, 2017), channels in the T-tubular and SR membranes – as Orai1 and TRIC (trimeric intracellular cationic), respectively –, calcium buffers in the TC – calsequestrin (CASQ) –, transmembrane proteins of the SR – as, e.g., triadin, junctin, JP45, STIM1 –, structural proteins that seem to link the membranes of the T-tubule and the TC or play an important role in the organization of the triad – as junctophilins and small ankyrins (sAnk), respectively –, and certain kinases and phosphatases. Some members – as FK506-binding protein (FKBP12) – are tightly bound to RyR and can thus co-purify with the channel and be present in single channel measurements unless special care is taken to dissociate them.

The 95 kDa isoform of triadin (Trisk95) was one of the first of accessory proteins to be recognized and originally believed to be responsible for EC-coupling (Caswell et al. 1991). Trisk95 has since been defined as the largest member of the triadin family (or reviews see Marty et al. 2009; Marty 2015) having multiple roles in skeletal and cardiac muscle. It seems to regulate SR calcium release as its overexpression suppressed the depolarization-evoked calcium transients in myotubes (Rezgui et al. 2005). We have,

in addition, shown that Trisk95 overexpression not only reduces the global calcium transients but also suppresses the frequency of spontaneous calcium release events, while shRNA-mediated downregulation of the protein results in augmented calcium release and increased event frequency in cultured myotubes (Fodor et al. 2008). Furthermore, a triadin knock-out mouse displayed reduced force production and altered triad and SR morphology (Oddoux et al. 2009).

FKBP12 is a 12 kDa molecular weight protein bound to RyR – each subunit of RyR has one FKBP bound – that was identified in skeletal muscle by its sensitivity to FK506 or rapamycin (Jayaraman et al 1992). In the presence of these immunosuppressants FKBP dissociates from RyR which in turn alters the interaction between the individual subunits resulting in increased open probability of the channel (e.g. Mayrleitner et al. 1994) and the appearance of characteristic subconductance states (e.g. Marks, 1996; Ahern et al. 1997) indicating that FKBP12 stabilizes the closed conformation of RyR. Single channel measurements conducted in Debrecen have also revealed that the interaction between the intracellular loop of DHPR (using synthetic peptides corresponding to the amino acid sequence of $DHPR^{671-690}$) and RyR are also modulated by FKBP12; namely, the activating effect of this peptide is absent if FKBP12 is dissociated from the release channel (O'Reilly et al. 2002).

Although IP_3 doesn't seem to play a direct role in EC-coupling, this is not true for phospholipids. Evidence connecting X-linked myotubular myopathy (characterized by very severe muscle weakness often with fatal outcome) to the phosphatidylinositol phosphate phosphatase myotubularin (encoded by the *MTM1* gene and present in the junctional space) deficiency (Laporte et al. 1996; Biancalana et al. 2003) has brought the role of phospholipids in EC-coupling into the forefront of studies (Al-Qusairi et al. 2009; discussed in our recent review Csernoch and Jacquemond, 2015). It has since been demonstrated that phosphoinositide substrates of myotubularin suppress voltage-gated SR calcium release and depress the frequency of calcium release events in permeabilized muscle fibres (Rodríguez et

al. 2014) while the inhibition of phosphatydilinositol 3-kinase restores these defects (Kutchukian et al. 2016). The crucial role of phospholipids in EC-coupling was further strengthened when we have shown impaired calcium release and spontaneous calcium release events in intact fibres in a mouse model of autosomal centronuclear myopathy (Kutchukian et al. 2017).

The sarcoplasmic reticulum calcium ATP-ase (SERCA)

Although the function of the SR calcium pump is not directly part of EC-coupling, the calculation of Rel would have been less accurate if our knowledge on the transport process had not been as precise. As the group led by Anthony N Martonosi and the number of scientist from Hungary who worked in his lab contributed significantly to our understanding I felt obliged to acknowledge some of their input. During the visit of László Dux in Dr. Martonosi's lab at the State University of New York (Syracuse, NY) they succeeded in obtaining large, two-dimensional crystals of the SR calcium pump (see e.g. Dux and Martonosi, 1983) which later enabled the determination of its three-dimensional structure (see e.g. Toyoshima et al. 2000). From those members of the University of Debrecen working in the Martonosi lab, István Jóna specifically contributed to the description of the now widely used SERCA inhibitor cyclopiazonic acid (Buchet et al. 1992) and to the identification of its interacting partner sarcolipin (e.g. Wawrzynow et al. 1992). The expression and tissue distribution in a number of species of sarcolipin was further characterized in Frank Wuytack's lab (Catholic University Leuven, Belgium) by, among others, Ernő Zádor (e.g. Vangheluwe et al. 2005). In addition to the work mentioned above, Vaseline-gap experiments were also carried out in Dr. Schneider's lab involving László Kovács to specifically address the transport properties of SERCA (Klein et al. 1991). Their data were consistent with the model in which individual pump molecules have two calcium binding sites and form functional dimers in the SR membrane.

Conclusion

The past fifty years have seen a tremendous increase in human knowledge and the field of EC-coupling is not an exception. From the earliest observations where externally placed glass microelectrodes were used to stimulate the muscle fibre we arrived to routinely using super-resolution imaging and genetic modification, always working with a clear objective in our minds: to help those in need and to advance the understanding of how the human body, in this special case its skeletal muscles work. Together with colleagues from all over the World scientists from Hungary have contributed to this goal. I hope that this short review, more a historical perspective, has highlighted some of this contribution and acknowledged the remarkable effort.

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Appendix

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STUDIES

FROM THE INSTITUTE OF MEDICAL CHEMISTRY UNIVERSITY SZEGED.

VOL I..

(1941—42)

MYOSIN AND MUSCULAR CONTRACTION

BY

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To the memory of

L. KAST,

president of the *Josiah Macy Jr.*

Foundation, *New York.*

Preparation and properties of myosin A and B.

by

I. Banga and A. Szent-Györgyi.

The contractile element of muscle is the muscle fibril and the chief building stone of the muscle fibril is myosin. It is evident, then, that we have to understand myosin if we want to understand contraction.

Myosin is defined, according to J. T. EDSALL (1) as the protein fraction of muscle which is extracted by a pH 8,5—9 mol 0,6 KCl solution and precipitates at pH 7 when the solution is diluted to 0,1 mol KCl.

It will be shown in this paper that myosin can be obtained from muscle in two different forms which we will call myosin A and B. The myosin obtained by EDSALL's method, and generally used by investigators is a solution of myosin A with a small admixture of B.

Material.

We used rabbit's muscle as material and EDSALL's salt solution for the extraction, which we will briefly call „salt solution." This contains 0,6 mol KCl, 0,01 mol Na_2CO_3 and 0,04 mol $NaHCO_3$. Only reagents of high purity were used.

As solvent and for other purposes (dilution, precipitation, etc.) we used distilled water exclusively which was redistilled twice from glass vessels. The importance of this will be evident later from the paper of M. GERENDÁS.

The adenyltriphosphate („ATP") used in this work was prepared in our laboratory as the Ca salt and was of 100% purity. Only in spcial cases, where absolute purity was immaterial, did we use preparations less pure (60%). The ATP was liberated from its Ca salt with HCl, then treated with the calculated amount of K-oxalate. The Ca-oxalate was separated on the centrifuge and the solution neutralised with

KOH. The solution obtained contained the K salt of ATP and about 0,3% KCl.[1]

The extraction of myosin A and B.

The animals head was cut off, its body rapidly skinned, then eviscerated and dipped, for a minute or so, in ice-water. Then the muscles of the hind legs, the belly and the back were quickly cut out, cooled for a short time by packing in ice, and minced in a cooled meat mincer with holes of 2 mm diameter.[2] 40 g of the mince were suspended in 120 ml salt solution of 0°C and stirred energetically for 20 minutes. The suspension was then quickly centrifuged in the cold room (0°C) and the supernatant thin fluid poured off. This contained about 20 mg of myosin per ml. It was stored over night at 0° during which time the adenyltriphosphate (ATP) present was split and the fluid became slightly more viscous. We will briefly call this fluid „the 20 min. extract".

If the suspension, after having been stirred for 20 min., is stored over night at 0° or for 6 hours at room temperature, without having been centrifuged, it turns into a semi-solid gel from which the muscle particles cannot be separated on the centrifuge any more.[3] If, however, a small quantity (0,014%) of ATP is added, the solution liquifies, assuming the appearance of a 20 min. extract and the muscle particles can be separated on the centrifuge. After a few minutes the ATP is split and the fluid gelatinises again. Thus, when the

[1] A preparation of equal properties is brought on the market by „Magyar Gyógyszer RT." Budapest.

[2] Mincing on a finer mincer like LATAPIE's did not improve the extraction.

[3] During the winter 1941—42 this transformation of myosin within the given time took place with absolute regularity. Simultaneously with the beginning of the hot season results began to be less regular and it was found repeatedly that the muscle suspension needed more, sometimes twice as much time for this transformation. The suspensions prepared from the muscle of animals that had been kept for 48 hours in the cold room, showed a tendency to imitate our winter results but the experience was not quite regular and could not always be reproduced.

Different species of animals may behave differently. For example the transformation of myosin in the pike is very rapid, taking place within the 20 min. of stirring.

extract stands in contact with muscle particles, a change takes place characterised by a greatly increased viscosity which can be lowered reversibly by the addition of small amounts of ATP.

This change in the qualities of the extract is not due to an increased myosin concentration since this latter rises only by 5—10% during storage. It is not due to the presence of some highly viscous new substance either but to a change of the qualities of the myosin itself, as can be shown by isolation of the myosin. If the myosin is precipitated by dilution and neutralisation, washed and redissolved in salt solution, it has the same qualities as the extract from which it was prepared.

We will call this highly viscous extract obtained by 24 hours storage of the stirred but uncentrifuged muscle suspension „the 24 h. extract".

Viscosity.

To compare the viscosity of a 20 min. and a 24 h. extract the latter must also be freed from the suspended muscle particles. This can be done on the centrifuge after dilution with salt solution. 80 vol. salt solution to 100 vol. of the suspension mostly suffices to allow centrifugation.

The extracts can be compared by plotting their viscosity against their myosin concentration.

The myosin concentration was determined as follows: 2 ml. of the extract was placed in a weighed centrifuge tube, 10 ml. of water added and the solution neutralized with 5% acetic acid. The myosin precipitate was then separated on the centrifuge, washed twice with 10 ml. of water, the tube dried at 120° C and weighed.

The viscosity was determined in OSTWALD's viscosimeter at 0°C. The relative viscosity is defined as t/t_0, t being the time required for the outflow of the extract, t_0 the time required for the outflow of a pure salt solution. Naturally such a crude salt extract of muscle contains other proteins besides myosin, like myoalbumin and globulin X. These proteins, being globular, have relatively little influence on the viscosity which is governed by the viscosity of the fibrous myosin. That other proteins present do not materially affect viscosity can be demonstrated by purifying the myosin. If this is precipitated, washed and redissolved in salt solution, it still has the same viscosity and gives the same reaction with ATP as the extract.

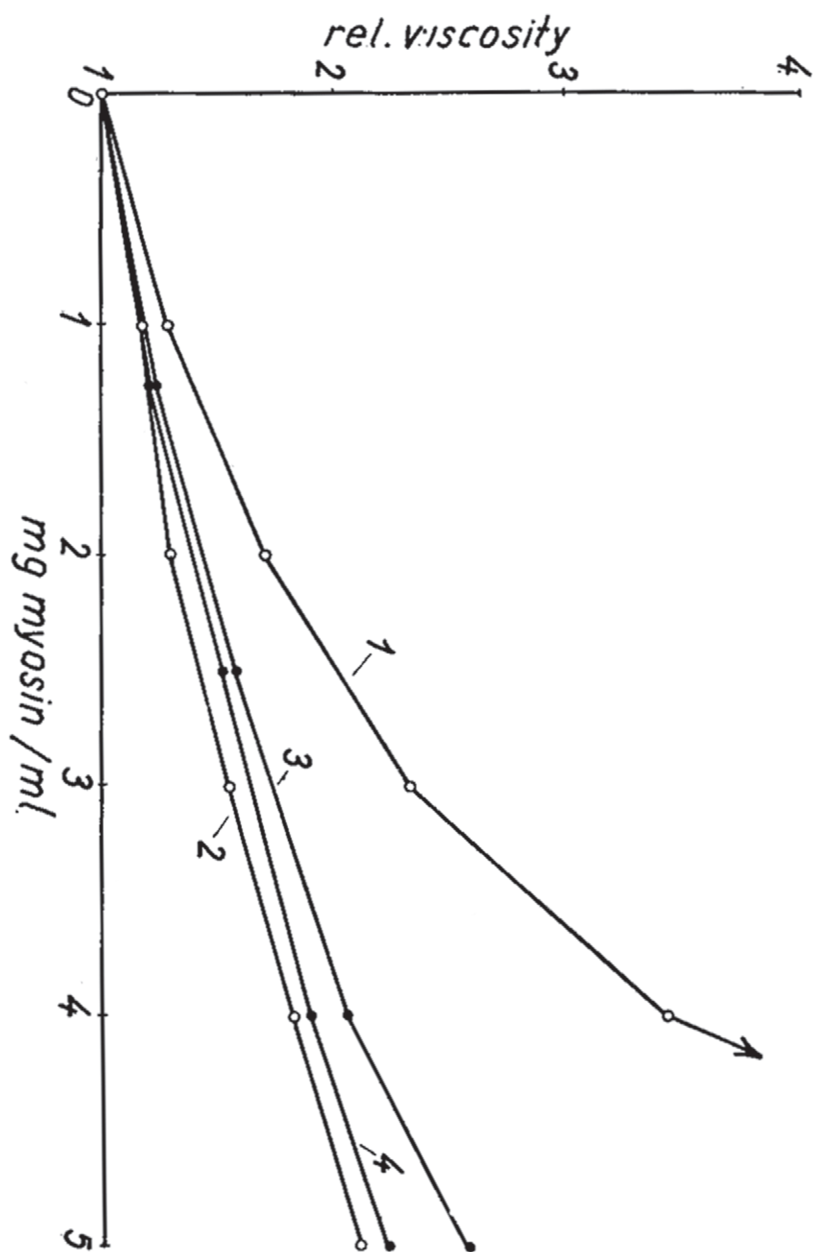

Fig. 1.

In Fig. 1 the relative viscosities of a 20 min. and a 24 h. extract are plotted against the myosin concentration, with and without the additon of ATP. Curve 1 gives the viscosity of a 24 h. extract; curve 2 the same after the addition of 0,014% ATP.; curve 3 gives the viscosity of a 20 min. extract; curve 4 the same after the addition of ATP. It will be seen that the viscosity of the 24 h. extract is much higher than that of the 20 min. extract and is anomalous even at low concentrations. The viscosity of a 20 min. extract is much lower and becomes anomalous only above 0,4%. The viscosity of both extracts falls on addition of ATP to about the same value, the viscosity of the 24 h. extract being somewhat lower. Both ATP curves are up to 0,5% roughly linear. By means of such a curve conclusions can be drawn on the myosin concentration of an extract.

By the „activity" of the myosin we will mean the fall of viscosity on addition of ATP.[4]

The myosin prevailing in the 20 min. extract and characterised by its low viscosity, will be called „myosin A"; the predominant myosin of the 24 h. extract, characterised by its relatively high viscosity and great „activity" will be called „myosin B."

It seemed likely that the low activity of the 20 min. extract is not due to myosin A but to the admixture of smaller quantities of myosin B. This supposition was verified by experiment. It was found that the myosin of the 24 h. extract is retained by the SEITZ K filter up to 90% while the 20 min. extract can be filtered without apreciable loss. Myosin B is thus retained by the filter to a much higher degree than myosin A. If the 20 min. extract is filtered repeatedly its „activity" disappears completely while its viscosity comes down to the level of curve 4. in fig. 1.

We cannot exclude the possibility that just as the 20 min. extract contains some myosin B so the 24 h. extract may contain some A. But in spite of this uncertainty there is no doubt that there is a distinct difference between myosin

[4] The relative viscosity itself also gives some indication of the „activity" but is not a reliable guide for it has been found that on incubation at 37° C the myosin solution becomes more viscous while the fall obtained on addition of ATP remains unchanged.

A and B. The latter is characterised by its higher viscosity. According to physical chemistry increased viscosity must be explained by an aggregation, and thus the lowering effect of ATP, by a disaggregation.. This is supported also by the lesser filterability of myosin B. On addition of ATP the latter also becomes more filterable, about 40% passing the filter.

The higher aggregation of myosin B must be due to increased cohesive forces and we can thus expect myosin B to be less soluble, and in this gel-form more solid than. A. This lesser solubility of B can be noticed at its preparation. While the myosin of the 24 h. extract precipitates already on dilution, the myosin of the 20 min. extract will come down only after the diluted extract has been neutralised.

It is most convenient to study the solubility of myosin on myosin gels, that is,' on myosin threads which contain the myosin in the gel-form. Experiment shows that threads prepared from the 20 min. extract are very loose and dissolve even in a neutral 0,6 mol KCl solution. In fact it is quite difficult to obtain well formed threads from this extract. The 24 h. extract gives relatively strong, solid threads which do not dissolve even in mol. KCl or EDSALL's fluid, especially after the threads have been standing for some time and new links have developed between micells.

Turbidity.

It seems desirable to characterise the difference of A and B myosin with as many physical factors as possible. One of the physical factors easily measured, though less easily interpreted, is turbidity. If a 20 min. and a 24 h. extract of equal myosin concentration are compared, the latter will be found to be much more turbid. A small fraction of this turbidity is due to suspended particles but the major part is due to the turbidity of the myosin itself.

The turbidity was measured in the Stupho-nephelometer and the values obtained plotted against the myosin concentration. The 24 h. myosin was found to be 4 times more turbid than the 20 min. myosin.

A similar difference can be observed in a precipitated and redissolved myosin preparation. 0,1—0,5 mg. (per ml)

myosin of the 20 min. extract gives an almost limpid solution while the equally concentrated solution of 24 h. myosin is turbid. Almost limpid 1% solutions of myosin can be obtained if the 20 min. extract is passed through a Seitz K filter, precipitated and redissolved.

Addition of ATP greatly dicreases the turbidity of myosin B though it does not bring it down to the values of myosin A. (see MOMMAERST).

Double refraction of flow (DRF).

As known from EDSALL and v. MURALT's (2) publication, myosin shows a strong double refraction of flow. We have compared the DRF of a 20 min. and 24 h. extract at different dilutions, *i. e.* at different myosin and KCl concentrations. The results are given in table I. The intensity of the DRF is marked with crosses. We used the chamber of GERENDÁS. (3)

Myosin content	Mol. KCl	Double refraction of flow without ATP	with 0.5 mg ATP/ml.
	20 min. extract		
8 mg/ccm	0.24	++++	++++
10 mg/ccm	0.30	++++	++++
4 mg/ccm	0.12	++++	++++
2 mg/ccm	0,05	++	+++
1 mg/ccm	0.02	+	++
	24 h. extract.		
4 mg/ccm	0.24	++++	++++
5 mg/ccm	0.30	+	++++
2 mg/ccm	0.12	0	+
0.8 mg/ccm	0.06	0	0
0.4 mg/ccm	0.03	0	0

The table shows that both extracts give a strong DRF but on dilution the DRF of the 24 h. extract is more readily lost, probably owing to the lesser solubility and the precipitation of the myosin. At 0,24 mol KCl the ATP brings the disappearing DRF back. This effect is reversible: if the extract is kept at room temperature the ATP is split in 20—30 minutes and the DRF disappears but can be brought back again by the addition of ATP. Precipitated and redissolved myosin behaves, on the whole, in a similar way.

The results given above are not quite constant. In some

experiments we observed a decreased DRF on addition of ATP in a 0,6 mol KCl solution (20 min. extract.) We also observed a decrease or disappearance of the DRF on addition of ATP at a lower KCl concentration (0,12—0,03 mol. KCl.) in 24 h. myosin solutions.

Miscellaneous observations.

ATP and Extraction. The muscle was minced and suspended in 0,1 mol KCl (2 ml per g. of muscle). After 0,20,40,80 and 160 minutes samples were taken. To these samples a salt solution was added (1 ml per g. of muscle) which contained the salts in a three times higher concentration than the EDSALL solution. The final salt concentration of our samples corresponded thus to Edsall's solution. After 20 min. of stirring at 0°C the samples were centrifuged and the ATP and myosin in the fluid estimated. The sample taken at 0 min. contained 5,4 mg. ATP per g of muscle and 19 mg myosin per ml. That taken at 20 min. contained 3 mg. ATP and 21 mg. myosin. In the 40 min. sample we found only traces of ATP which could not be estimated with accuracy any more and the myosin concentration dropped to 4 mg. per ml. In the 80 min. sample the myosin equalled 2 mg. per ml. and in the 160 min. we found no myosin at all. After 160 min. the original ATP concentration was restored by the addion of this substanc. The extract now contained 10 mg. myosin per ml.

This experiment shows that the ATP present in fresh muscle has a deciding influence on the solubility of myosin and that the dissolution of the myosin of fresh muscle in EDSALL's solution is due to a combined effect of the salts and the ATP present.

The A—B transformation and ATP. Several experiments were performed to learn at what rate the A—B transformation of myosin takes place in muscle suspension and how this rate depends on the concentration of the ATP present. In these experiments the muscle was suspended in 0° salt solution and stirred for twenty minutes. Then a sample was taken, quickly centrifuged at 0° and the liquid poured off. The ATP and myosin were immediately estimated. The

liquid was then stored over night at 0°C and after the ATP had disappeared its „activity" was determined. The muscle suspension itself was kept at different temperatures and samples were taken and treated in the same way at different intervals.

The result of such an experiment is illustrated by fig 2. The muscle suspension was kept at room temperature and samples were taken at 0, 30, 60, 90, 120, 180, 240, 360, 480 min... The abscissa gives the time in hours. The ordinate

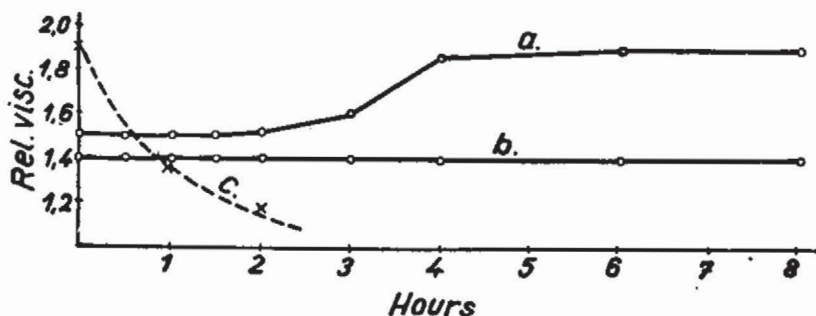

Fig. 2.

gives the relative viscosity. Curve a gives the viscosity without and curvee b the viscosity with 14 mg % ATP. Curve c gives the ATP concentration on a voluntary scale. The highest point of this curve corresponds to 1 mg ATP per ml of the extract. (3.5 mg ATP per g of muscle) For technical reasons the estimation of the last, smallest quantities of ATP is rather doubtful.

In agreement with other experiments this fig. shows that while there is ATP present in appreciable concentration there is no increase in „activity" at all. The activity begins to rise and becomes maximal within a fairly short time after the ATP has sunk to low values or has disappeared altogether.

At 0°C ATP needs about 8 hours to disappear during which time there is no activation. Then only the activation begins.

P h o s p h o r y l a t i o n o f m y o s i n. It has been shown that, in the presence of 0,6 mol KCl, ATP disaggregates myosin B. Later MOMMAERTS will show that 1 molecule of ATP for every molecule of myosin (MW 100.000) suffices

for this action. It could bee thought that ATP acts either as a whole or by transferring its phosphate on to the myosin. Our experiments permit to exclude this latter possibility and there is no transference of one molecule of phosphoric acid per 100,0C0 g of myosin. We cannot exclude the possibility that one phosphate is transferred to some higher unit.

Such experiments were made possible by the circumstance that the reaction between myosin and ATP is instantaneous and ATP, as shown by MOMMAERTS, need not be present in excess to cause maximal effect.

We added 1,5 mg. ATP to 300 mg. 24 h. myosin dissolved in salt solution *i. e.* one mol. ATP for every 100,000 g myosin. The fluid was thoroughly mixed. 15 seconds after the addition of the ATP the myosin was inactivated by the addition of acetic acid which brought the pH to 4—4,5. At this pH the myosin coagulated and could be separated on a cloth by filtration. Only about 20% of the myosin remained in solution and was precipitated by the addition of trichloroacetic acid. In the control experiment we added the acetic acid first, the ATP afterwards.

We estimated the free phosphate and the ATP in the filtrates (+ free phosphate after 7 min. hydrolysis at 100° in N HCl.)

Out of the four experiments performed we found the ATP quantitatively in the fluid of two while in the two others we found the ATP with the loss of 0,11 and 0,25 mg.

Adenylic acid. If ATP looses its pyrophosphate, adenylic acid is formed and it is of considerable interest to know how this adenylic acid behaves with regard to myosin. Adenylic acid was found to be completely inactive and had no influence on the viscosity of myosin.

Summary.

It has been shown that myosin can be obtained from muscle in two different forms which were called myosin A and B.

Myosin A has, dissolved in Edsall's fluid, a relatively low viscosity which is not influenced by ATP (adenyltriphosphate).

Myosin B has, under the same conditions, a relatively high viscosity which is brought down to the level of the viscosity of myosin A by the addition of small amounts of ATP.

Myosin B is less soluble and gives more solid gels than myosin A and also has a greater turbidity which is descreased by ATP.

The behaviour of myosin under different conditions of extraction has been discussed.

No phosphorylation of the myosin could be detected.

Adenylic acid was found to be inactive.

Literature.

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2. *J. T. Edsall* and *A. v. Muralt.* Jl. biol. Chem. *89*, 315, 1930.
3. *M. Gerendás.* Enzymologia 9, 123, 1940.

The contraction of myosin threads.

by

A. Szent-Györgyi.

It has been shown be H. H. WEBER, (1) that a myosin solution, if squirted in a thin jet into water, solidifies in the form of a thread. In this way the myosin can be brought into a form which resembles the muscle fibril in some respects. The myosin thread is an elongated piece of myosin gel.

It has been shown in the preceding paper by BANGA and myself that myosin can be obtained from muscle in two different forms which were called myosin A and B. Threads can be prepared from both. For the sake of convenience I will call the threads prepared from the 20 min. extract (see BANGA and Sz.) „myosin A threads“ while the threads prepared from the 24 h. extract will be called „myosin B threads“.

The threads used by previous investigators correspond, in all probability, to our myosin A threads.

The technique of the preparation and observation of threads will be described by M. GERENDÁS.

A watery extract of muscle was made in the following way) the rabbits muscle was cut out and minced (as described in the previous paper), suspended in water (1 ml per g of muscle), stirred for 5 minutes at 0°C and squeezed through a cloth. The fluid was then filtered through paper at 0°C.

If a myosin B thread is suspended in this extract and observed under the microscope, a violent contraction will be seen. The thread contracts within 30 seconds to less than half of its length and within 2—3 minutes it reaches a maximum contraction of 66%, $^{1}/_{3}$ of its original length. (Fig. 1.) At the same time the thread becomes proportionately thinner, and is seen to become quite dark.[1] Watched in lateral illumina-

[1] Frequently the contraction is so violent that the interior of the thread cannot keep pace with the contraction of the outer sheets, so that these latter break up, like a crocodile's skin. Fig. 2. For the same reason

tion the transparent thread is seen to turn white. opaque. The rate of contraction depends on the diameter of the thread. Fig. 3. curve a gives the time-curve of the contraction of a relatively thick thread of 0,3 mm diameter. Thinner threads, like those of 0,1 diameter, contract faster but their rate of contraction cannot be measured by the same method because it is too fast. Furthermore thin threads mostly curl up and stretch out again only after they have reached maximum contraction.

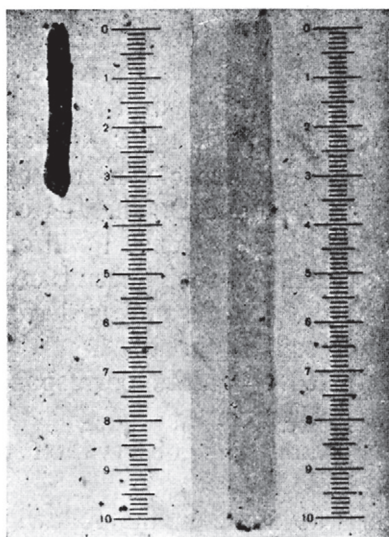

Fig. 1.

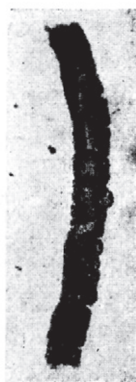

Fig. 2.

If a myosin A thread is suspended in the same fluid no striking change will be observed. (Fig. 4.) Measurement by the ocular micrometer will reveal a weak and slow contraction. (Fig. 3 curve b). If the myosin solution is filtered twice through a Seitz K filter before the thread has beene pulled, the contraction becomes still weaker (Fig. 3 curve c). As shown in the previous paper, the Seitz filter retains the myosin B present in our myosin A preparations as an impurity.

The frest, watery extract of thee muscle contains thus

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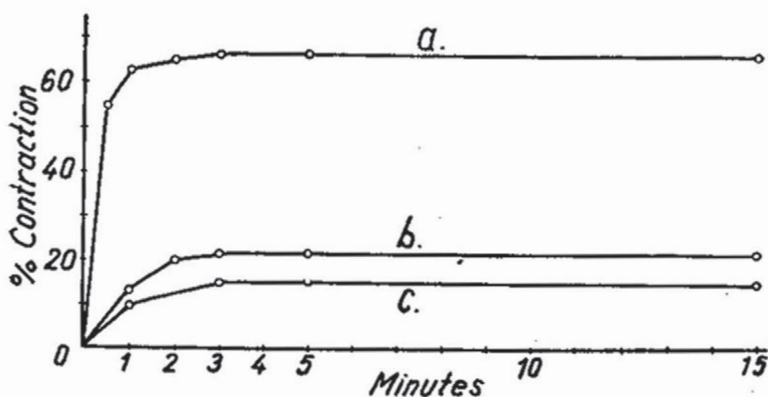
the whole thread sometimes breaks up into dark, solid lumps of myosin instead of giving a contraction.

something which causes a violent contraction in myosin B threads but has little influence on myosin A. The active agent seems to be present in excess for the extract can be diluted to 1:4 with water and will still give the same contraction with thinner threads.

If the muscle suspension is stored over night at 0°C and filtered only the next day, the extract obtained will found to be entirely inactive. The myosin B thread, suspended in this extract, will show no change at all.

Rabbits muscle contains on the average 3,5 mg adenyl-



Fig, 3.

triphosphate („ATP") per g., thus the fresh extract contains ATP in about $^1/_2$ of this concentration. This ATP is split during storage by the phosphatase present. If the original ATP concentration is restored to the inactivated extract, again the same violent contraction will be obtained as in the fresh extract. Even half of this ATP concentration (0,9 mg per ml) is sufficient to give a maximum effect. This shows that ATP is involved in the observed contraction.

If the same quantity of ATP (0,09%) is dissolved in water and the myosin B thread suspended herein, no contraction will occur and the thread remains entirely unchanged. If we dissolve our ATP in the boiled extract instead of water we obtain a violent contraction again. Even incinerated juice will produce contraction with ATP. This makes it evident that, apart from ATP, inorganic constituents of the extract are also involved in the reaction.

If we use a 0,1 mol KCl solution as solvent for our ATP instead of water the contraction will be much slower. (see Fig. 5 curve b.)

Muscle contains 0,01 mol Mg. If we add 0,01—0,001 mol $MgCl_2$ to our 0,1 mol KCl and dissolve the ATP in this, the myosin thread suspended in this fluid will give the same violent contraction as in the fresh, watery extract (Fig. 5 curve a). It is evident thus that three factors were involved

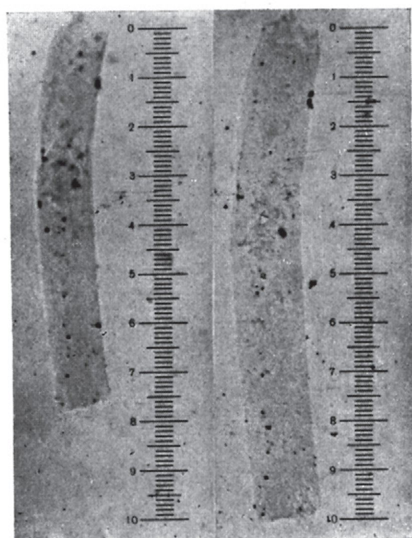


Fig. 4.

in the production of the contraction of our myosin B thread: ATP, K and Mg.

The myosin A thread will give the same weak and sluggish contraction (Fig. 5 curve c) with ATP in pure KCl or KCl plus $MgCl_2$. If the myosin solution is filtered twice through a Seitz K filter, the thread prepared from this solution will give a somewhat weaker contraction in KCl (Fig. 5. curve d). If, in addition to the 0,1 mol. KCl, 0,001 Mg is also present there will be practically no contraction at all (Fig. curve e). The contraction of myosin A is not only not enhanced but is almost completely inhibited by $MgCl_2$. If the thread, prepared from unfiltered myosin, gave the same contraction in KCl and $MgCl_2$ (curve c) this was due to the myosin B present as

an impurity: the contraction of this myosin B was enhanced and thus compensated the inhibition caused by Mg in the contraction of myosin A.

Myosin B gives thus a strong contraction with 0,1 mol. KCl and ATP and the contraction is greathy enhanced by Mg. Myosin A gives a weak and sluggish contraction with KCl and ATP and the contraction is suppressed by Mg.

If the concentration of KCl is increased, at 0,2 mol. the same reactions are still obtained. But if the concentration is raised to 0,04 mol., the thread, instead of giving a contraction,

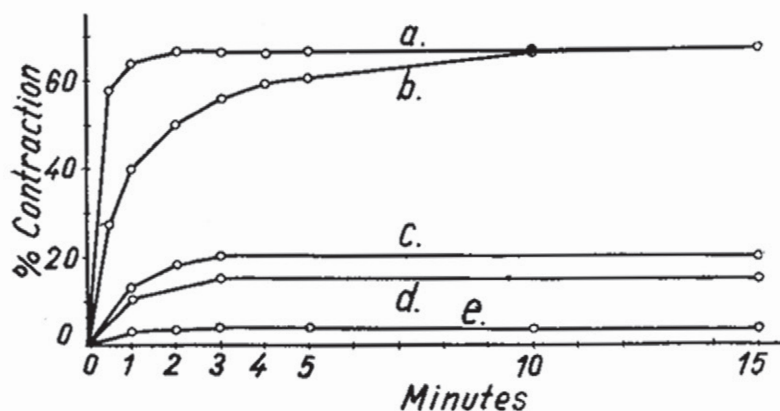


Fig. 5.

will dissolve. The action of our ATP—KCl—$MgCl_2$ mixture will depend on the concentration of the KCl present and at higher concentrations the action will be reverted; instead of contraction we will obtain dissolution and instead of aggregation, disaggregation. KCl without ATP will not dissolve the myosin B thread not even in a molar concentration.

Threads prepared from the precipitated, washed and redissolved myosin of these extracts gave identical results.

A d e n y l i c a c i d, if employed instead of its pyrophosphate ester, ATP, was found to be entirely inactive. It does not give contraction or dissolution.

Neither the effect of KCl, nor that of $MgCl_2$ is specific and can be reproduced by other ions.

In fig. 6 the effect of KCl is compared with the effect of other halogen salts of K. The abscissa gives the log of the

molar concentration of the salt, the ordinate the % of shortening. By bringing the curve under the abscissa I wanted to express dissolution. The broken line means that the dissolution already takes place wihout the addition of ATP. The threads were prepared from precipitated and washed myosin B and were placed for 5 min. into the salt solution before the addition of ATP (0,018%). No value should be attached to the relative hight of the curves, which, in this respect, are not strictly comparable because the results were obtained with different myosin preparations. Readings ware made 5 min. after the addition of ATP.

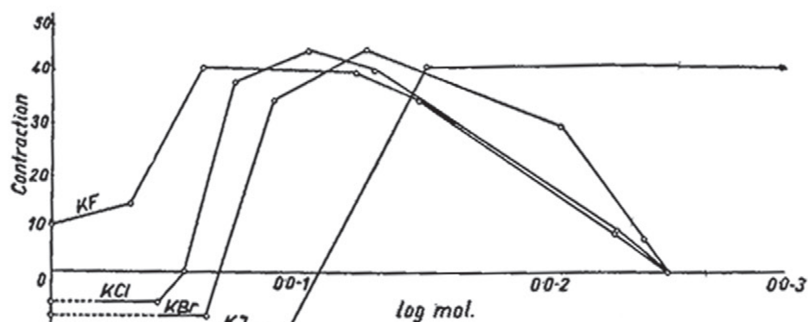


Fig. 6.

It will be seen that the effect of KCl is not specific and that the effect of a salt is not dependent on the kation only but depends on the anion too. With the increasing weight of the anion the curves are shifted more and more to the right. KJ has a very strong effect at relatively high dilutions.

In fig. 7. the anion (Cl) is kept constant and the kation varied. The differences are not very marked but there is a tendency to the opposite effect, a shift to the right with the decreasing weight of the kation. With Li the effect is distinct. In this curve NH_4Cl (neutralised with NH_4OH) and potassium phosphate (pH 7) are also given. This latter is effective at very high dilutions.

The enhancing effect of Mg can be reproduced by Co and Mn as will be shown by M. GERENDÁS. A detailed study on the effect of varied concentrations and pH will be given by T. ERDŐS.

Reversibility. The question presents itself whether

the contraction observed is reversible or whether it is connected with an irrevesible change of the myosin.

If the contracted myosin B thread is transferred into pure water it will remeain contracted. This naturally does not mean that the change is irreversible, for the contracted muscle has no reason to relax. Conditions in the muscle, where every myosin micell is fixed within a certain pattern, are different.

If the contracted thread is transferred into Edsall's salt solution, it swells up again. When the thread has reached its

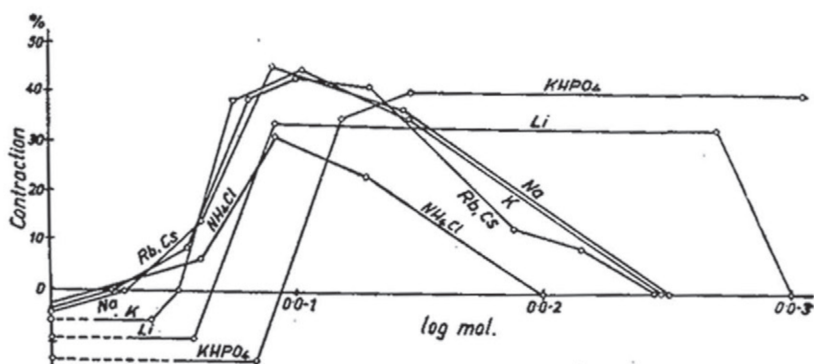


Fig. 7.

original dimensions the swelling can be stopped by transferring it into 0,1 mol. KCl. Such a thread has a perfectly normal appearance and does not contract spontaneously. If ATP is added it contracts again in the same way, as it did the first time. This shows that the contraction is reversible.

Experiments with myosin *in stitu.* We may ask whether the contraction obtained with myosin threads can have any bearing on muscular contraction at all and whether in muscle myosin may behave also as myosin B. It has been shown that myosin gives a contraction even after it has been dissolved and precipitated, once the necessary ions and ATP are present. The contractility of muscle is a very sensitive process and seems to be thus the expression of a subtle organisation. Naturally it is just as possible that a higher or-organisation is not needed for the contraction itself, but for those changes that elicit this contraction or bring the muscle back to rest again.

To obtain some information on this question, I tried to destroy the finer structure of the muscle as far as possible without destroying the myosin. It is known that the excitability of the muscle is lost in distilled water and also by feezing. Neither of these destroy myosin.

The broad neck muscle of the rabbit was cut into 2 mm. wide strips parallel to the muscle fibres. The strips were placed into distiled water. After one to several hours the strips, if contracted, were stretched to their original length, frozen in solid CO_2 and cut into slices on the freezing microtome. The slices were made parallel to the muscle fibres and were about one fibre thick. Thus they contained one sheet of muscle fibres running through the whole length of the preparation. The slices were put into distilled water and transferred after one to several hours into 0,1 mol. KCl, then placed on a slide under the microscope. After their length had been measured a drop of 0,14% ATP vas dropped on them. Immediately a strong contraction began, which reached a maximum within 15—120 seconds and shortened the fibres by 50—60%. The myosin behaved thus as myosin B.

Experiments with myosin suspensions. The last question I want to touch in this paper, is, whether myosin suspensions give changes which are analogous to the contraction of threads.

The muscle extract containing myosin was neutralised and diluted till the KCl concentration went down to 0,1 mol. The myosin precipitate was centrifuged, washed and reddissolved in Edsall's fluid, precipitated and washed thoroughly again.

The myosin obtained in this way is a fairly stable suspension which settles slowly. Salts is smaller concentration cause precipitation and the suspension will settle somewhat faster. Salt in higher contcentration will tend to dissolve the mysin. There is great difference in the behaviour of myosin A and B. The former is much less turbid and has a greater tendency for dissolution.

If, in addition to 0,1 KCl, a small quantity, say 14 mg % of ATP is also added to the myosin B suspension, the precipitation, will be greatly intensified. The precipitate immediately becomes roughly granular and settles quickly leaving a clear

fluid behind. The effect is very striking. We may call it a „superprecipitation", contrary to the precipitation caused by KCl alone. Mg still enhances the reaction.

4 mol. KCl has no appreciable dissolving action on the myosin B suspension. If ATP is added in addition to this KCl., the myosin dissolves. We can thus say that ATP greatly enhances the effect of salts, bringing about dissolution at concentrations at wich the salt by itself is inactive and it also greatly intensifies precipitation.

The phenomena seen in the myosin B suspension are analogous to the phenomena observed on myosin B threads. ATP and higher KCl concentrations dissolve both. ATP and lower salt concentrations, which produce a contraction in the thread, cause a superprecipitation in the suspension.

The analogy is lacking in one point. While smaller salt-concentrations cause by themselves a precipitation in the suspension, salts without ATP never give a contraction in threads. There seems to be a qualitative difference between the precipitating action of salts alone and the precipitation observed in the presence of ATP. Salts alone seem only to cause an aggregation of the myosin micells, while in the presence of ATP they seem to cause some deeper change within the single units, which change expresses itself in the superprecipitation of suspensions and the contraction of threads. GERENDÁS has found that while salts by themselves have no influence on the double refraction of oriented threads, salts + ATP cause, besides contraction, a complete disappearance of double refraction. Double refraction disappears in musculur contraction also.

Myosin A suspensions behave in an analogous way to myosin A threads. They dissolve without ATP at lower salt concentrations (0,4 mol. KCl) and ATP has only a very slight precipitating action which is not enhanced by Mg.

Summary.

It is shown that a myosin B thread, if suspended in a fresh, watery extract of muscle, gives a violent contraction. Myosin A is relatively inactive.

It is shown that three factors are involved in the contraction of myosin: ATP, K and Mg.

At higher salt concentrations, in the presence of ATP, dissolution is obtained.

The action of ions is not specific.

Under the same conditions which cause the myosin thread to contract, the myosin suspensions give a precipitate.

Literature.

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The phosphatase activity of myosin.

by

I. Banga.

ENGELHARDT and LJUBIMOVA (1,2) found close correspodence between phosphatase activity and the myosin content of muscle extracts. They concluded that myosin itself is the phosphatase which splits, according to K. LOHMANS (3) equation, adenyltriphosphate (ATP) into adenylic acid and two molecules of o phosphate. Two years ago I undertook to control this statement and could fully corroborate it. My myosin preparation showed different enzymic activities like that of a succinodehydrase, cytochromoxydase, desamidase, lactic acid formation etc., but in agreement with the russian authors I also found that on fractionation only the phosphatase activity went parallel to the myosin content and the myosin could be separated eventually from other enzymic activity.

By repeated extraction with EDSALL's salt solution the myosin can be extracted exhaustively. On the first extraction one obtains 2—2,2% myosin. The second extraction gives a solution containing about 0,7%, the third extraction about 0,3% myosin. Further extractions are practically free of myosin and the residue contains only traces of it. The phosphatase activity of these fractions was found to be proportional to the myosin content.

The desamidase can be separated from myosin by precipitation with half saturated ammonium suphate which leaves the desamidase in solution. Desamidase can be extracted from muscle, contrary to myosin, by isotonic NaCl or KCl.

The enzymes belonging to lactic acid fermentation can be separated from myosin by repeated precipitation with water at ph 7.

The succinodehydrogenase and cytochromoxydase are dissolved by the salt solution only to a small extent. The first extraction will bring out about 1/6 of these enzymes, further extracts will have no activity at all. It is doubtful whether this small quantity of the enzymes is really dissolved or only suspended. Both enzymes seem to be bound to the less soluble fraction of the muscle.

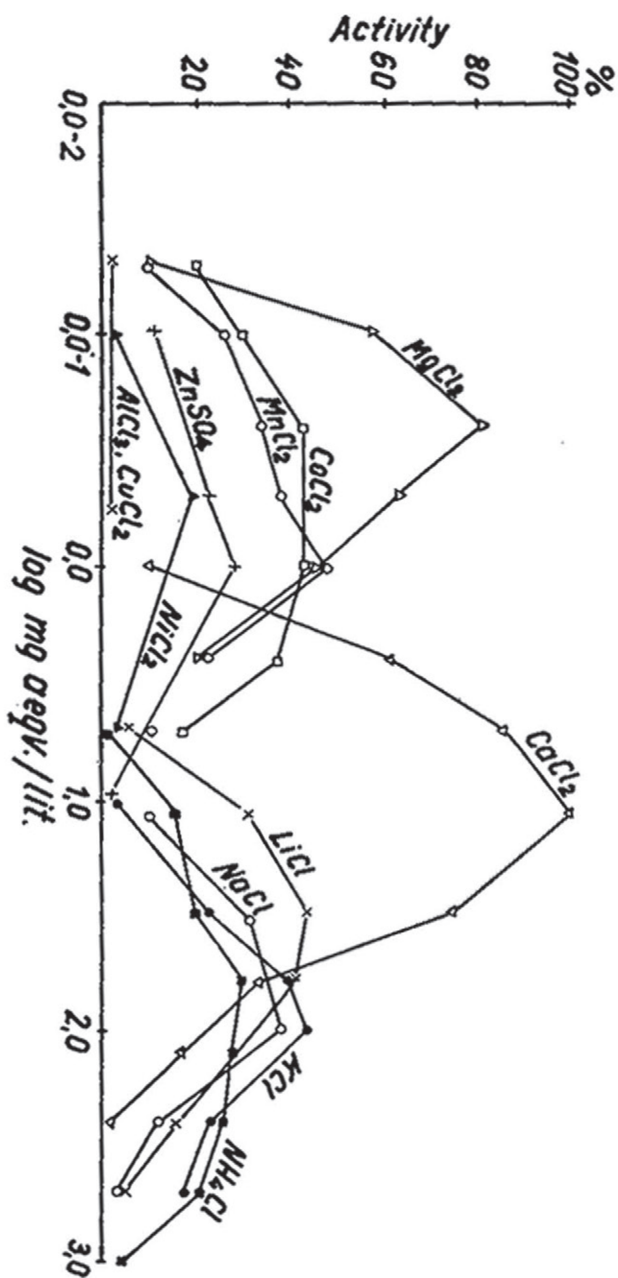

Fig. 1.

In these experiments I found that by repeated precipitation and washing with water, or by protracted dialysis, the phosphatase activity of myosin was reduced to about 10% of its original value. This activity could be restored by muscle extract. Boiling or incineration did not annihilate this reaction and it was evident that by the purification I had removed some inorganic constituent which served as an activator to the myosin.

Further experiments showed that the action of salts is not specific and that a great number of different ions are capable of this reactivation.[1]

Our earlier experiments were made with rabbits muscle minced on LATAPIES mincer and extracted for 30 min. according to EDSALL. Later we used 24 and 20 min. extracts as described in the preceding paper of BANGA and SZENT-GYÖRGYI. The extracts were precipitated by dilution and neutralisation, washed with water, redissolved and precipitated and washed again. Then the myosin content of the suspension was determined. My original experiments gave the same results as the later ones on the whole and so some of these original observations will be quoted too at the end of this paper.

The reaction mixture had a final volume of 3 ml and contained 1 mg of mysoin, 4.6 of ATP and the salt in question. The mixture was placed into small flasks of 50 ml and incubated in the waterbath under constant shaking at 38° for 5 min. Then 1 ml. of 20% trichloroacetic acid was added and the inorganic phosphate estimated after FISKE and SUBBAROW. We used no buffer, the ATP itself buffers to some extent. Salts, which dissociate hydrolitically, were neutralised.

My results obtained with 24 h. myosin are summed up in Fig. 1. As will be seen, analogous to contraction, the phosphatase activity of myosin is also activated by a great variety of ions: most salts studied had a definite activity with distinct optima. White the salt is not active below a certain concentration, its excess inhibits the reaction. Activation was

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[1] A short note about these results was sent to SCIENCE but I am unaware whether our letter reached the editor. Definite publication was delayed by external circumstances and retained later because of complications which culminated in the discovery of the two different myosins.

In the mean time R. CLOETENS found that the alcaline phosphatase of kidney was activated by different metals, like Ca, Mg, Mn, Co, Ni, Zn, Hg. (Enzymologia *7*, 157, 1939. Naturwiss. *28*, 252, 1940. Biochem. Z. *307*, 352. 1941. Ibid. *308*, 37, and *310*, 400. 1941.)

hardly detectable with $AlCl_3$ and $CuCl_2$ which salts, as well as Ni, inactivate myosin irreversibly.

The great variety of ions which were found to be active allow to axclude the possibility that the metal acts as a prosthetic group of the enzyme.

It should be noted that I always found some free phosphate without the addition of salts, which is subtracted in Fig. 1. It is, to a small extent, due to the free phosphate present at the beginning of the experiment but mainly to enzymic activity. It seemed likely that this activity was due to the activating effect of the K ions introduced with the ATP partly as its cation partly as its KCl impurity. To decide this question the phosphatase activity was measured in the presence of varied ATP concentrations. Results of such an experiment are given in Tab. I. The experiment was made with 24 h. myosin precipitated once and washed twice. The numbers give the P split off from ATP in mg.

Tab. I.

Added mg ATP	no salt added	0.1 mol. KCl	0.001 mol. $MgCl_2$	0.01 mol. $CaCl_2$	0.01 mol. KCl. 0.001 mol. $MgCl_2$
1.4	0.003	0.037	0.074	0.055	0.074
2.8	0.006	0.078	0.104	0.100	0.120
4.2	0.019	0.104	0.162	0.140	0.134
5.6	0.038	0.134	0.168	0.162	0.180

As will be seen from col. II. the quantity of P rapidly falls with the decreasing quantity of ATP added in the absence of added salt. With 2.8 mg of ATP the activity is very small but the next column shows that this is not due to the lack of ATP but to the lack of ions, since in the presence of KCl the activity is still considerable. Thus the higher activity in presence of 5.6 mg of ATP must have been conditioned by the K ions introduced with the ATP. In this experiment the O value i. e. the value obtained without addition of salt, was fairly low. In other experiments the O value was found to be as high as 0.050 and it seems likely that here also other ions absorbed on the myosin play some role.

Tab. II gives the results of the experiment performed with the myosin of the 20 min. extract of the same muscle which was used in Tab. I. The columns and numbers have the same meaning as before.

Tab. II.

Added mg ATP	no salt added	0.1 mol. KCl	0.001 mol. $MgCl_2$	0.01 mol. $CaCl_2$	0.01 mol. KCl 0.001 mol. $MgCl_2$
1.4	0.002	0.023	0.004	0.064	0.028
2.8	0.004	0.044	0.008	0.109	—0.037
4.2	0.022	0.066	0.026	0.165	0.044

As can be seen the differences between A and B myosin (See BANGA and SZENT-GYÖRGYI) are the following: the O value of myosin A is somewhat lower than that of myosin B. Myosin A is activated by KCl to a lesser extent than myosin B. $MgCl_2$ which has a maximum effect on myosin B, has no activating affect on myosin A at all. Both A and B myosins are equally activated by $CaCl_2$. The KCl-activation is enhanced by Mg in myosin B and is inhibited in myosin A.[2]

This experiment shows the difference in the reaction of myosin A and B found in the majority of cases. There is, however, a certain variation in the results obtained with different preparations. Sometimes the K activation is equally strong in the 20 min. and in the 24 h. myosin. Sometimes the K activation of myosin A is depressed by Mg to a greater, sometimes to a lesser extent than was the case in the quoted experiment. In none of the experiments, however, had Mg any activiting effect on the 20 min. myosin while it always strongly activated the 24 h. preparation.

On the whole the results obtained with the phosphatase activity show a close analogy to the results obtained in the contraction of myosin threads. The analogy between phosphatase activity and contraction breaks down in the case of $CaCl_2$. Ca has no activating effect on contraction but activates phosphorilysis maximally in both A and B myosin. Ca even inhibits the contraction caused by ATP and other ions (see ERDŐS).

[2] The inhibition of KCl activation by $MgCl_2$ in myosin A becomes stronger if the myosin is sent twice through a SEITZ K filter. As has been shown 20 min. myosin always contains some myosin B, the activity of which is enhanced by Mg, while the activity of myosin A is inhibited. These two effects may compensate each others. (The Seitz filter retains myosin B.)

Repeated filtration through the SEITZ K filter reduced the KCl activation of the myosin of the 20 min. extract to one third of its original value as it also reduced the contractility and „activity" of this preparation (for definition of „activity" see BANGA and SZENT-GYÖRGYI.)

Apparently Ca induces some change in the myosin which renders its contraction impossible. This Ca activation of phosphorilysis is important because it proves that phosphatase activity is inherent to that part of the myosin which is the basic constituent of both A and B forms.

Apart from the case of Ca there are many and close analogies between the phosphatase activity and the contraction of myosin. My next question was thus, how far this analogy can

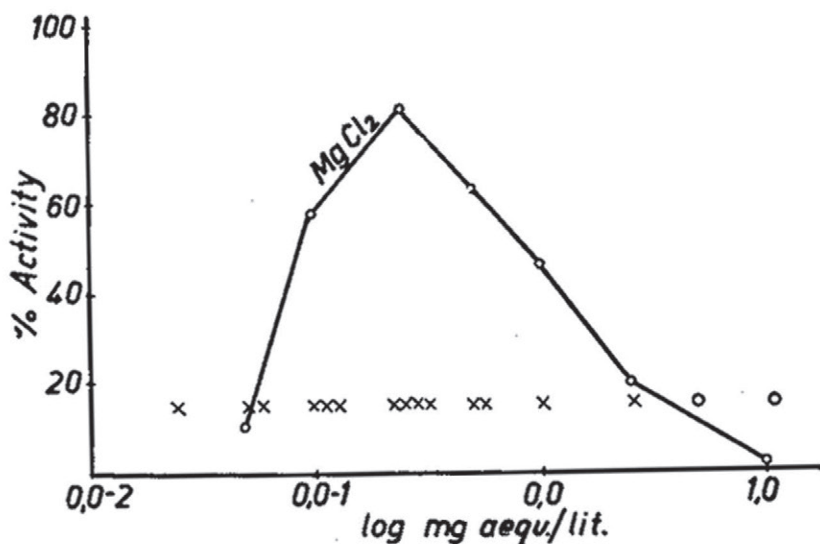

Fig. 2.

be drawn and whether there is not an even more intimate relation between both phenomena. As has been shown (SZENT-GYÖRGYI) contraction of threads and precipitation of myosin suspensions are but different expressions of the same process. For technical reasons it seemed more convenient to compare phosphorilysis with precipitation than with contraction. Maxima can be judged more easily in precipitation since contraction is very fast, its extent depends on time, and diffusion complicates the situation.

I therefore compared precipitation and phosphatase action. The results of one experiment with $MgCl_2$ (fig. 2.) and KCl (fig. 3.) is summed up in the curves which are analogous to fig. 1. Two identical sets of dilutions were prepared with both salts. One of the sets was prepared in flasks and served to

measure the phosphorilysis, while the other set was prepared in reagent tubes and served to observe the precipitation. Maximum precipitation is denoted with four crosses, 1—3 crosses mean weaker precipitation while O means the dissolution of the myosin. (24 h. extract).

As the figures show there is close connection between the physical state of the myosin and phosphorilysis: while the maximum of precipitation coincides with maximal

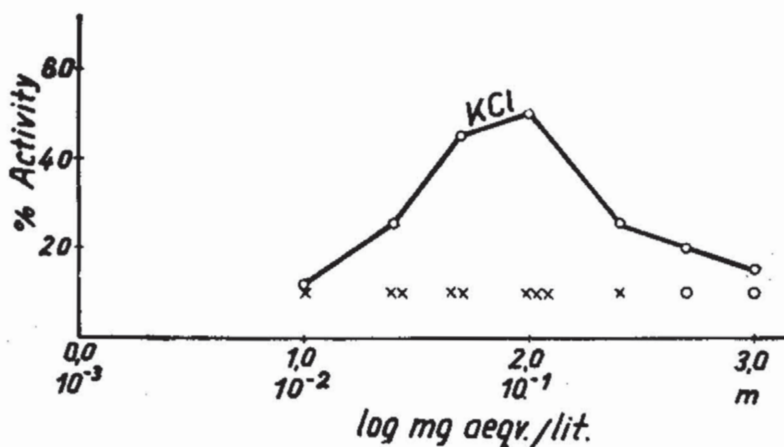

Fig. 3.

enzymic activity, dissolved myosin is inactive. Myosin is thus fully active in its most contracted state only.

The dependence on pH of the phosphatase activity of myosin B (24 h.) is shown in fig. 4. K and Mg were used in optimal concentration as activator. There is a sharp optimum at pH 6, the isoelectric point of myosin, which is additional evidence for the identity of myosin and phosphatase. Unfortunately the curve is not free of criticism. I did not dispose of a suitable buffer between pH 6 and 7, as the use of phosphate was excluded, since it interfered with the P estimations. Furthermore buffers are salts themselves and have their own activating effect. Up to pH 7 I used an acetate buffer, between Ph 7.60—10 a $NaHCO_3$—Na_2CO_3 buffer, both in 0.05 mol concentration. The buffers were controlled electrometrically. The ATP solution was adjusted colorimetrically, and did not materially influence the pH of the buffer.

I want to close this chapter with a few observations taken from my unpublished paper of two years ago.

Phosphatase activity at varied temperatures. Results are given in fig. 5. The abscissa gives the time, the ordinate the log. of the ATP still present. As will be seen

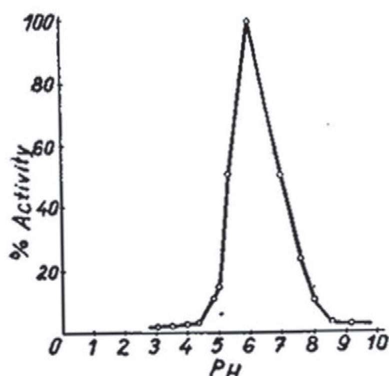

Fig. 4.

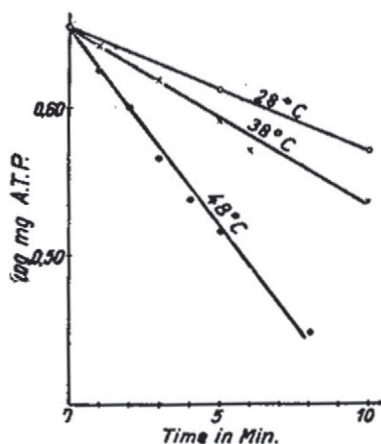

Fig. 5.

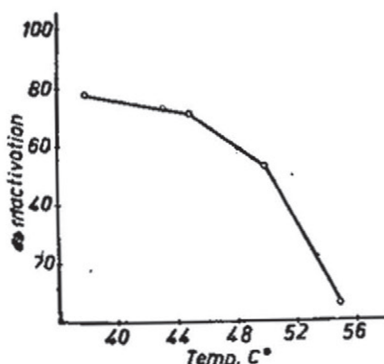

Fig. 6.

the curves are straight which is in agreement with a monomolecular type of reaction.

Inactivation of myosin at elevated temperatures. The results are given in fig 6. Incubation for 10 minutes. pH 7. The activity measured at 38°C.

Reversibility. If our myosin contains no other en-

zyme which acts on ATP or its splitting-product we san study the kinetics of its phosphatase activity. According to Lohmann[5], ATP is split by muscle into adenylic acid and two molecules of o-phosphate. If this reaction would be reversible we would always have to arrive at the same equilibrium mixture at varied ATP concentrations. On the other side addition of adenylic acid or phosphate should inhibit the reaction and ATP should be formed from the mixture of both, provided the equilibrium is not too far on the side of the splitting products. I found that the % of the ATP split varies at varied ATP concentrations (the myosin concentration remaining constant). The more ATP I added the higher was the % of it that was split, as shown in Tab. III.

Tab. III.

mg. ATP added	mg. ATP split	% AT Psplit
1.15	0.36	31.2
2.30	0.91	39.2
3.45	1.34	39.0
4.60	1.95	42.0
5.75	2.60	45.0
6.90	3.20	46.2

On the other hand addition of adenylic acid or phosphate had no influence on the splitting of ATP and no ATP could be detected in the mixture of the two substances. (Tab. IV.)

Tab. IV.

Added mg. ATP	Adddd mg Aden. acid	Added mg. P (as PO_4)	mg. ATP split	mg ATP formed
4.6	—	—	2.65	—
4.6	—	0.148	2.73	—
4.6	4.6	—	2.65	—
—	4.0	0.104	—	0.0

A few experiments were made on the accelerating or inhibiting effect of pharmacologically active substances (in the presence of 0.1 mol. KCl or 0.01 mol. $CaCl_2$). Aconitin, veratrin, acetylcholin (in presence of phisostigmin), adrenalin, coffein, chloroform, urethan, guanidin-sulfate were found to be inactive at a dilution of 1:1000 and inhibited at higher concentrations. Quinine's inhibition was 50% at 1:10,000 dilu-

tion. Higher concentrations could not be studied since the alcaloid interfered with the P estimation. Nicotine inhibits in 1:1000 completely, in 10^{-4} concentration to 25%. NaF does not inhibit, not even at a mol/30 concentration, in which concenration it inhibitis glycolysis completely. Mol/500 Na-oxalate inhibitis 100%. Monojodacetate and maleinic acid were inactive.

I also tried to find some connection between the functional states of myosin and its activity. I hoped to be able to increase the enzymic activity by a tetanisation of the muscle before the extraction. Frog-muscle was subjected to a short, in other experiments to an exhaustive tetanization (indirect electrical stimulation), then frozen in CO_2, minced in the frozen condition and extracted. The best results i. e. the highest phosphatase activity, could be obtained with resting muscle cooled to 0°C, minced and treated carefully at a low temperature.

Summary.

ENGELHARDT and LJUBIMOWA are corroborated.

It is shown that the phosphatase activity of myosin depends on the presence of ions. Differences between the activation of myosin A and B are described.

Myosin activated by K and Mg is most active at the maximum of its precipitation or contraction.

Dependence of the phosphatase activity of myosin on pH and temperature are described and the reversibility of phosphatase action is discussed.

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2. *M. N. Ljubimowa* and *U. A. Engelhardt:* Biokhimija *4,* 716, 1939.
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Quantitative Studies on some Effects of Adenyltriphosphate on Myosin B.

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The question discussed in this paper is the following: which is the minimal quality of adenyltriposphate („ATP") which still gives rise to a maximal effect on myosin?

The effects of ATP on viscosity, on light scattering and on precipitation have been studied.

1. The viscosity-lowering effect. The relative viscosity of a solution of myosin B is high; addition of ATP causes a marked fall. It is, however, not possible to determine the quantitative relations between ATP and myosin simply from this, because the viscosity-lowering effect of very small quantities of ATP decreases with time, apparently due to the splitting of ATP by myosin. The influence of time has therefore to be taken into account.

0,2—0,3% solution of myosin in Edsall's fluid were used and measurements taken with a viscosimeter of the Ostwald type, which was immersed in ice-water.

Results are given as specific viscosity; the differences in density of the solution and the solvent have not been taken into account in the calculations, this correction being of no importance, as all determinations of one series of experiments were made with solutions of the same concentration.

First the viscosity of the myosin solution was determined. The fluid was then poured out of the viscosimeter into a tube, likewise suspended in ice-water and the viscosimeter immediately put back in its place. After a short time dissolved ATP was added in an amount which did not cause dilution of the myosin solution by more than 0.1%. The time of complete mixing was noted. The solution was then put back

into the viscosimeter, with a pipette surrounded by a mantle of ice-water. If care is taken to avoid warming of the solution or instruments, the first reading can be made immediately, without error due to a rise in temperature. Measurements were repeated every 3 or 4 minutes, usually for 20 to 40 minutes.

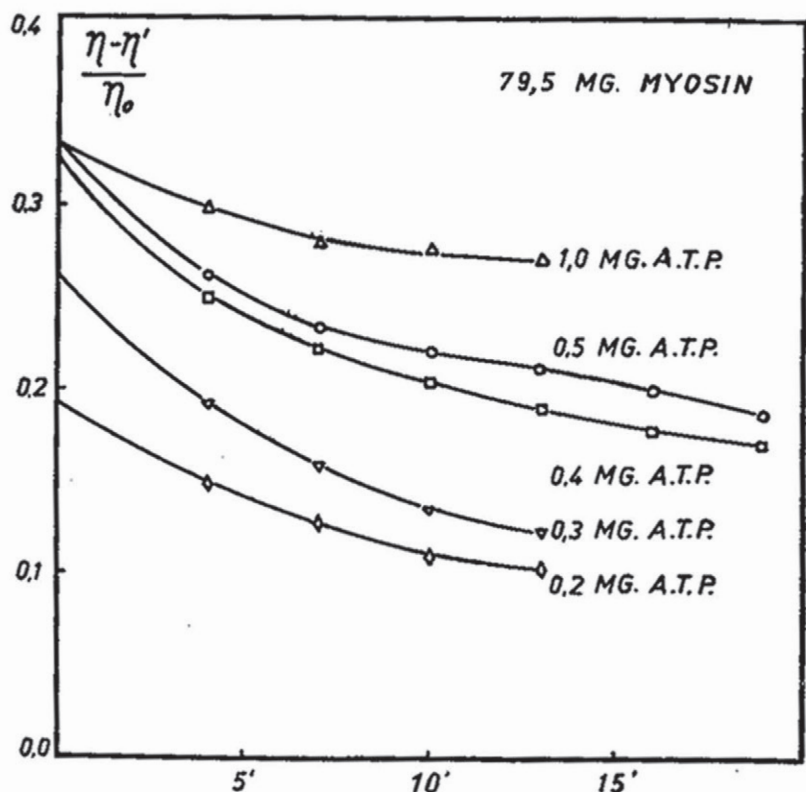

Fig. 1.

The graphs show the viscosity-lowering effect as the difference between the specific or relative viscosities of the solution with and without ATP at different moments after mixing.

An example of a series of experiments is shown in fig. I. As will be seen, the viscosity-lowering effect decreases regulary with time, an end-value usually being reached within 15 to 45 minutes. Extrapolation of the curves towards

zero time gives the magnitude of the initial effects. The figures in this example show that 1,0 and 0,5 mg ATP had the same effect; 0,4 mg acts slightly less and still smaller doses of ATP are clearly submaximal.

In Fig. 2 the results of a number of series of experiments have been plotted in the following way: the ordinate shows the effect of each ATP quantity as the percentage of the maximal effect in the same series; the abscissa gives the ATP quantity present per 100 mg of myosin.

In the range of lower concentrations the spreading of the

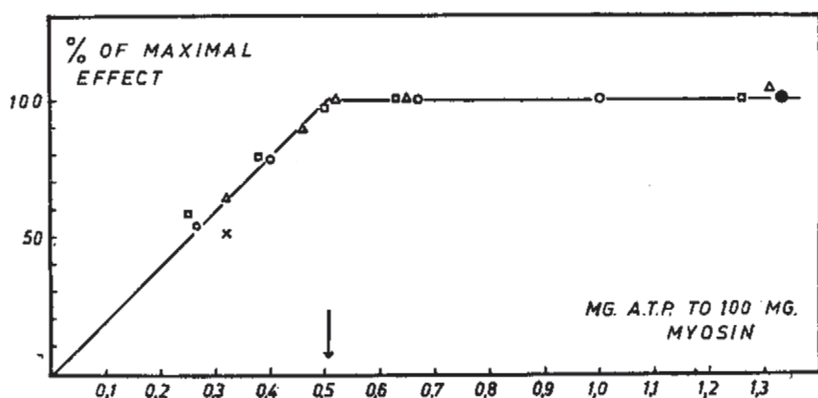

Fig. 2.

points is considerable owing to the fact that in the case of actions far below the maximum the extrapolation of the time-curves is difficult. In the neighbourhood of the maximal effect it can be made with sufficient accuracy.

The relation between the ATP quantity and the magnitude of the effect in the submaximal range seems to be a linear one: the transition towards the horizontal part of the curve is very sharp; the transition-point, which indicates the minimal fully effective ATP quantity, seems to lie between 0,50 and 0,51 mg ATP per 100 g myosin. From this the weight of the reacting myosin-unit may be calculated to be about 100,000.

2. The effect on light-scattering. As has been decribed in another paper of this series (BANGA and SZ.) the turbidity of myosin B solutions is higher than the turbidity of solutions of myosin A. Addition of ATP in sufficient

quantity to the former causes a decrease of turbidity by 20—30%.

Measurements were made with the Zeiss Stupho-nephelometer using the S. 53 greenfilter. The lightscattering of different samples of myosin solution was measured, before and after the addition of a known amount of ATP. Like in the case of viscosity, the ATP-effect depends on time and the real value can be found by the extrapolation of the time-curve towards zero; however, the mean value of 5 readings made

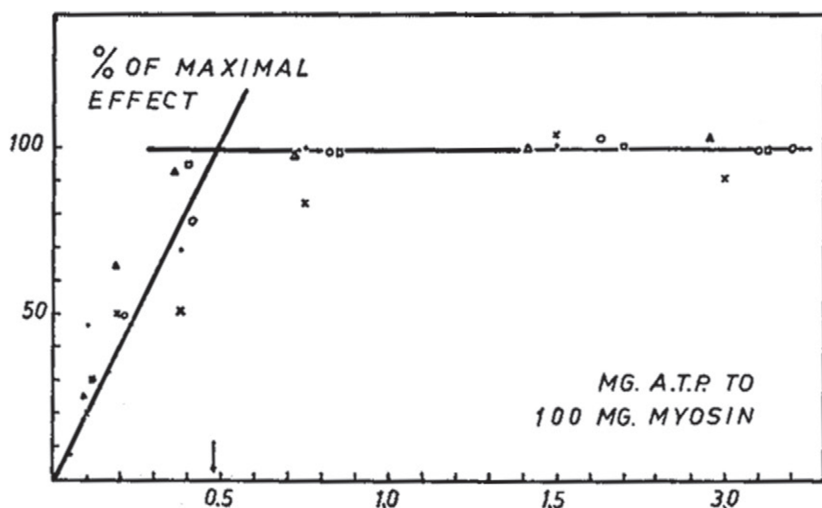

Fig. 3.

within 1,5 minutes after the addition of ATP, can be used as a base of comparison just as well as the extrapolated value. All measurements were made at room temperature (30°C.)

The results of a number of series of experiments, made with different preparations containing 0,4—2,0 mg myosin per ml are given in fig. 3. The spreading of points is considerable owing to the smallness of the effects; it is even difficult to decide about the exact form and situation of the curve. The line which has been chosen as the most probable one points to 0,47 mg ATP per 100 mg of myosin as the minimal quantity giving full effect; this value is almost the same as that got with the viscosimentric method (0,51).

It may, therefore, be concluded that the study of the

effect of ATP on light-scattering in myosin solutions is not in disagreement with the results of the viscosity measurements.

3. The effect on gel-volume. If a myosin preparation, dissolved in Edsall's fluid, is diluted with a five-fold volume of distilled water and neutralised with acetic acid, a precipitate is formed. The precipitation becomes more intense on addition of ATP and correspondingly the volume of the precipitate becomes smaller.

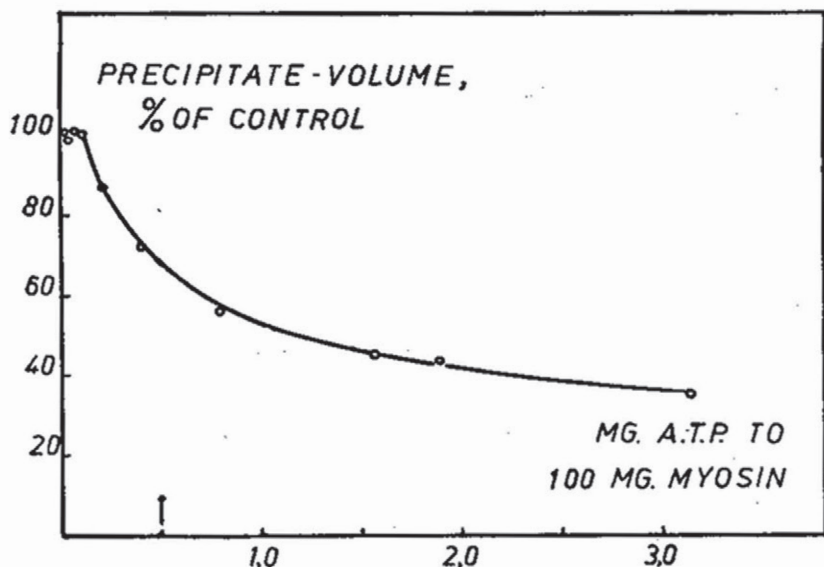

Fig. 4.

For the determination of the volume of the precipitate, 1 ml of myosin solution (in most cases containing 12 mg per ml) was mixed with 5 ml of distilled water and different amounts of ATP in a weighed cetrifuge tube of 10 ml. The volume of the precipitate was determined by weighing it in the wet state, the tube having been dried carefully with filter paper. Neutralisation was found not to be essential for precipitation, although the gel settled more easily and with a smaller volume after neutralisation; this is in agreement with the results of Edsall (1). Common distilled water had to be used because no well-formed gel could be obtained in glass-distilled water. The most regular results were obtained by precipitating the myosin first and then adding the ATP; similar results, only

with somewhat scattered data, were obtained if the myosin solution was diluted with water already containing ATP. If first the ATP was added to the myosin and dilution carried out after the elapse of a few seconds or minutes, higher ATP concentrations were needed in order to get the same effect, which was doubtless due to the splitting of the ATP in the concentrated myosin solution. This method seemed, therefore, to be less suited for the present purposes.

Fig. 4 shows the mean values of the results of six series of experiments. As will be seen, a clear maximal effect was not reached, not even in the case of the highest ATP doses. For the explanation of this fact it should be mentioned that between the addition of ATP and the determination of the gel-volume a time of 20 to 25 minutes elapses. Therefore the ATP added will in greater part be split during this time, thus making addition of a larger amount necessary and smoothening the sharp curve. It will be seen, however that at 0,5 mg ATP per 100 mg of myosin the effect is considerable and differs from that of the higher doses by an amount which can be expected to be caused by the splitting of ATP during the experiment. It can be concluded therefore, that the results of these experiments agree in order of magnitude with the results obtained by other methods (The arrow marks the value corresponding to 1 mol. ATP per 100,000 g myosin).

4. Conclusion. From the quantitative study of the effects of ATP on the physical properties of myosin one can conclude that myosin reacts in units of 100.000, a number which seems to be related to the molecular weight in urea solution as found by WEBER and STÖVER (2). Every single unit reacts with one molecule of ATP. This result is based on three entirely different methods of investigation in which myosin was studied at 0°C and at room temperature, in solution and as a precipitate, in the streaming as well as the resting condition. From the sharpness of the transition point of curve 2 it must be concluded that the myosin-ATP-compound is, at least at 0°C, not dissociated to a marked degree.

Literature.

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Reaction of Adenyltriphosphate with Myosin A.

by

F. B. Straub.

It has been described by BANGA and SZENT-GYÖRGYI that the viscosity[1] of a 24 h. extract of muscle, containing myosin chiefly in the B form, decreases very strongly on addition of adenyltriphosphate (ATP). Under similar conditions the viscosity of a 20 min. extract, containing mainly the A form, decreases but slightly, the cange being only about 10% of the change observed in the 24 h. extract. It has also been shown that this slight decrease of viscosity is not due to myosin A, but to the small quantitities of myosin B present as an impurity.

These experiments have been done with extracts of muscle. Edsall's fluid was used as solvent which is an alkaline 0,6 mol. KCl solution. If the 24 h. extract, which I will call for the sake of convenience „myosin B", is tested in a solution of lower pH and KCl concentration, the viscosity will show an even more pronounced decrease (40% more) on addition of ATP.

Myosin A behaves in an interesting way if the pH and salt concentration are varied. If e. g. myosin A is dissolved in a veronal-acetate buffer of pH 5,3 and the potassium ion concentration is varied by adding different amounts of KCl, ATP will cause a great decrease of viscosity, the magnitude of this effect varying with the salt concentration. Such results are shown in Fig. 1. The maximal value of this ATP effect is quantitatively the same as that which would be obtained with a myosin B solution having the same viscosity. The same

[1] The term viscosity is used in this paper knowing that, in the type of viscometer used in these experiments, deviations from POISEUILLE's law are perceptible. The effects described here, however, are not materially influenced by possible corrections in the viscosity values.

type of curve is obtained if the potassium ion concentration is kept constant and the pH varied.

While myosin A gives a clear solution in EDSALL's alkaline KCl, in solvents in which the ATP effect in' increased,

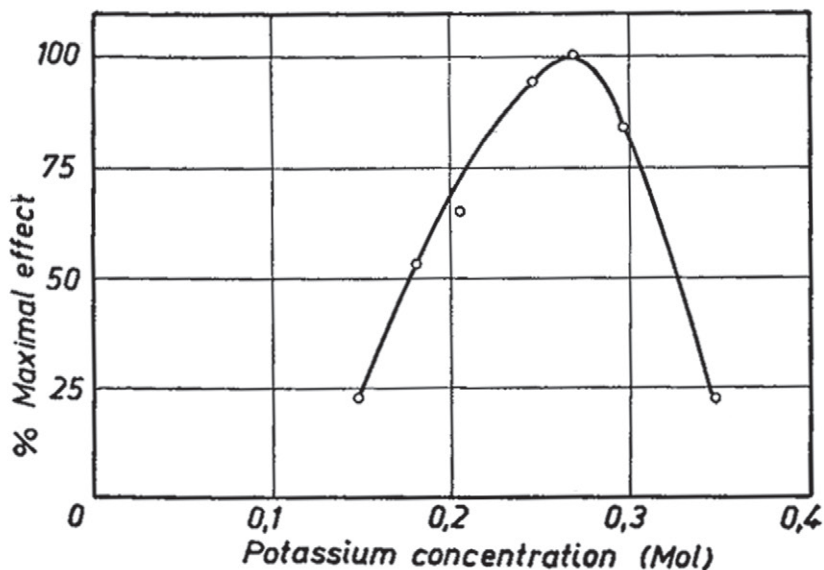

Fig. 1.

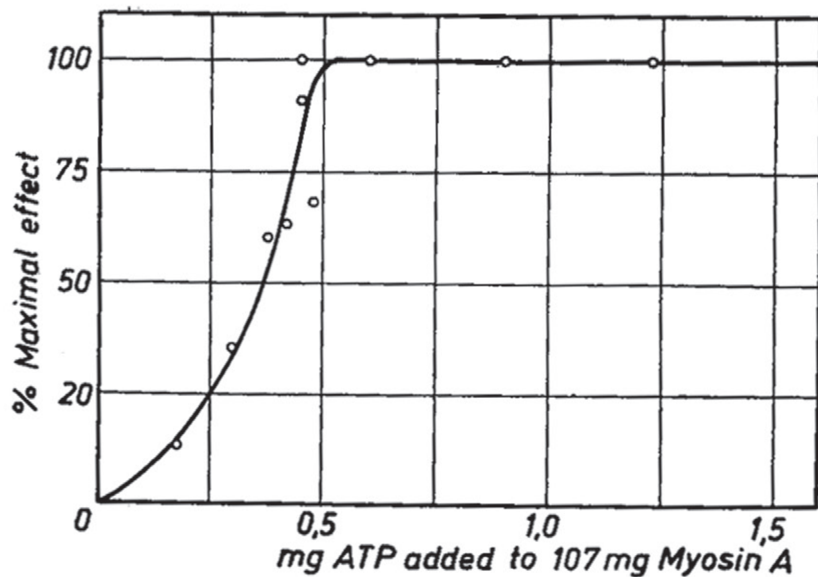

Fig. 2.

the opalescence increases roughly in proportion. Finally, decreasing the potassium ion concentration below its optimum (which gave maximal ATP effect) will result in inreasing flockulation. Considring the combined action of pH and salt concentration, one is led to suppose. that the ATP effect is the function of a particular colloidal state and not of the difference between the two myosin.

It was interesting to see what concentration of ATP is required to give a maximal effect (maximal decrease of viscosity) in these experiments with myosin A. It will be seen from Fig. 2. that 0,5 mg ATP in 30 ml are needed to give a maximal decrease of viscosity with 107 mg myosin A. The combination between ATP and myosin A is therefore just as strong as the combination between ATP and B. Moreover it should be pointed out that in spite of a difference in solubility and viscosity, suggesting a difference in size of the myosin A and B particles, the equivalent reacting weight of myosin with ATP is the same in both cases.

Experiments.

In the experiments recorded in Fig. 1., 5 ml of myosin A solution (100 mg myosin in 0,6 mol. KCl) were added to 25 ml of a buffer solution and the viscosity determined in a capillary vicometer at 0°C. The buffer solution consisted of 2,5 ml of 0,28 mol. veronal-K, 2,5 ml of potassium acetate of the same concentration, 8,0 ml n/10 HCl, varying amounts of KCl and distilled water to make up 25 ml. If no KCl was added, the potassium concentration in the final volume of 30 ml was 0,147 mol, resulting from the potassium of the buffer and myosin solutions.

A similar mixture was used in the experiment from which Fig. 2. was constructed. The 30 ml of mixture, analysed in the viscometer, contained the same buffer and 5 ml of a myosin A solution (107 mg myosin), together with so much KCl, as to bring the concentration of potassium ions to 0,25 mol. When the amount of ATP was too small, successive readings in the viscometer did not agree but showed an ever decreasing ATP effect. This is due to the splitting of ATP by myosin. In these cases the viscosity at zero time (at the moment

of adding the ATP) was evaluated by linear extrapolation from the values of 3—4 successive determinations. The errors inherent in this extrapolation may account for the fact that the rising part of the curve is not linear, as could be expected.

Summary.

It is shown that, at a lower pH and a lower salt concentration, myosin A also has a high viscosity which is decreased by ATP. ATP reacts with myosin A in the same proportion as with myosin B (one g. mol. ATP per 100,000 g of myosin).

Technisches über Myosinfäden nebst einigen Beobachtungen über ihre Kontraktion.

von

M. Gerendás.

H. H. WEBER[1] zeigte, dass wenn eine genügend konzentrierte Myosinlösung durch eine Kapillare in Wasser eingelassen wird, das ausströmende Myosin in Form eines fadenförmigen Gels erstarrt. Nach WEBER sind diese Fäden elastisch und 100—300% dehnbar.

Ich habe mich bereits früher 2.) 3.) mit den Eigenschaften solcher Fäden beschäftigt und konnte WEBER's Angaben vollauf bestätigen. Bei der Wiederaufnahme des Problemes hatten wir Fäden nőtig die ATP. spalteten. Obwohl die Phosphatase-Aktivität des Myosins äusseren Eingriffen gegenüber nicht empfindlich ist und Myosin bei 0° ohne Verlust der Fermentaktivität wiederholt pezipitiert werden kann, fand ich unsere Fäden fermentativ unwirksam. Die Analyse zeigte, dass unser Myosin durch die, im destillierten Wasser anwesenden Metalle, in erster Linie durch das Kupfer inaktiviert wurde. Cu wird durch das Myosin gebunden und angereichert. Aus diesem Grunde wurden die Versuche derart wiederholt, dass für alle Zwecke nur destilliertes Wasser zur Verwendung kam, das aus Glasgefässen zweimal umdestilliert wurde. Diese Fäden, auf die sich alle Angaben dieses Bandes beziehen, sind fermentativ aktiv, kontrahieren mit ATP in Gegenwart von Salzen, sind aber locker, brüchig, unelastisch, können nicht mehr als 10—15% gestreckt werden ohne zu zerreissen.

Wird dem Wasser, in dem man die Fäden zieht, 0,001 mol $CuCl_2$ zugefügt, so erhält man wieder Fäden, die elastisch, 100—300% streckbar, aber fermentativ unwirksam sind.

Methodisches über die Herstellung von Myosinfäden.

Myosinfäden können am einfachsten derart hergesstellt werden, dass man die Myosinlősung in ein Glassrohr aufsaugt dessen eines Ende zur Kapillare ausgezogen ist. Man lässt nun das Myosin in einer flachen Schale in Wasser oder in verdünnte Salzlősung einlaufen und bewegt das Glasrohr hin her. Bei visköseren Lősungen muss noch ein Druck aufgesetzt werden: am einfachsten verbindet man das dicke Ende des Rohres mit einem Gummischlauch, in den man einbläst. Mit einiger Übung

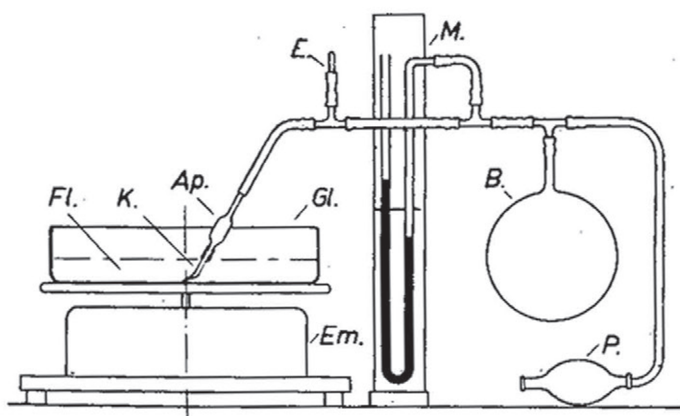

Fig. 1.

kann man in dieser Weise ziemlich gerade und gleichmässige Fäden herstellen. Die Dicke der Fäden hängt vom Diameter der Kapillare, der Viscosität der Lősung, der Auslaufsgeschwindigkeit und von der Geschwindigkeit ab, mit der man das Rohr bewegt. Am bessten eigneten sich für unsere Zwecke Fäden von etwa 0,2—0,3 mm. Diameter.

Für viele Fragen sind aber die, in dieser Weise hergestellten Fäden nicht geichmässig genug. In solchen Fällen müssen die Fäden mechanisch gezogen werden. Die von uns gebrauchte Einrichtung ist in beistehender Figur 1 abgebildet. Diese besteht aus einem drehbaren Tisch der durch einen Motor (Em) langsam ca 3 Rev. per Min. gedreht wird. Die Geschwindigkeit ist regulierebar. Auf diesen Tisch wird, wohl zentriert, das Wasser oder die vedünnte Salzlősung in einer weiten, flachen Schale (Ge) gelegt. Die Auslaufskapillare (K) wird mit

einem kleinen Reservoir verbunden (Ap), das mit der Myosinlösung gefüllt wird. Der Druck über der Myosinlösung wird mit einer kleinen Gummipumpe (P) hergestellt, am eingeschalteten Manometer (M) kontrolliert und mit Hilfe eines eingeschalteten Gummiballes (B, innere eines Fussballen) stabilisiert. Der Druck konnte durch öffnen bei E aufgehoben werden. Die Kapillare und das Reservoir sind verschiebbar auf einen Querbalken montiert so, dass sie nach einen jeden Umlauf des Tisches verschoben werden können. Der Druck wird erst aufgesetzt und das Fadenziehen begonnen nachdem die Flüssigkeit im Gefäss die Bewegung des Tisches aufgenommen hat. Die Kapillare ist schräge gestellt, es ist aber auch vorteilhaft ihr Ende etwas umzubiegen, um dieses noch mehr in die Auslaufsrichtung zu bringen. Das Ende der Kapillare ist etwa 1—2 cm. vom Boden der Glasschale entfernt. Der Faden setzt sich in Form einer Spirale auf den Boden des Gefässes und kann von diesem, nachdem er genügend erstarrt is, leicht abgetrennt werden.

Je nach Natur des Myosins können Fäden aus 0,5—5% Myosin gezogen werden. Zur Untersuchung der Kontraktion eignen sich Fäden vom 1—2% am bessten. Die aus konzentrierteren Myosinlösungen hergestellten Fäden ziehen sich minder gut zusammen, verdünntere Lösungen geben zu lockere Fäden. Myosin B Lösungen über 5% sind zu viskös um überhaupt verarbeitet zu werden.

Material.

Fäden können unmittelbar aus dem 24 stündigen Extract (s. BANGA and SZENT-GYÖRGYI) gezogen werden, wenn der Extrakt mit Salzlösung im Verhältniss von 100:80 werdünnt und zentrifugiert wurde. Eine konzentrierte Myosinlösung lässt sich erhalten, wenn man den Extrakt mit einer geringen Menge von ATP (0,014%) verflüssigt, rasch zentrifugiert, die Flüssigkeit abgiesst und wartet bis diese wieder gelatinisiert d. h. das zugesetzte ATP gespallten ist.

Der 20 Min. Extrakt, wenn frisch zubereitet, gibt überhaupt keine Fäden obwohl es etwa 2% Myosin enthält. Das Myosin fällt flockig aus, oder zerfliesst am Boden des Gefässes. Der Grund hierfür liegt in der Anwesenheit von ATP das in Ge-

genwart der hohen Salzkonzentration veflüssigend wirkt. Um Fäden aus dem 20 Min Extrakt zu erhalten muss man diesen erst über Nacht im Eisschrank stehen lassen, in welcher Zeit das ATP gespalten wird. Aber selbst dann geben die Auszüge meistens keine Fäden, da das Myosin A, aus dem das Myosin dieser Auszüge vorwiegend besteht, viel schwächere Kohäsionskräfte hat, wie das Myosin B des 24 Stündigen Extraktes. Um aus dem 20 Min. Extrakt Fäden zu erhalten muss der Extrakt neutralisiert werden. Ich gebrauchte zur Neutralisierung 5% Essigsäure. Die berechnete Menge der Säure wurde zugesetzt und die Lösung sehr energisch umgerührt. Die Neutralisierung hat keinen Einfluss auf die Kontraktionsfähigkeit der Fäden. Ohne Neutralisierung lassen sich nur sehr dünne Fäden u. zw. bei sehr kleiner Auslaufsgeschwindigkeit herstellen.

Die Myosinextrakte sowie die Fäden können bei 0° mehrere Tage bis über eine Woche ohne Verlust an Aktivität bewahrt werden.

Sollten Fäden aus gereinigtem Myosin hergestellt werden, so wurden die Extrakte anlehnend an J. T. EDSALL, mit 5-fachem Vol. Wasser verdünnt und neutralisiert, das Prezipitat abzentrifugiert, mit Wasser gewaschen, dann wieder in Salzlösung gelöst. Das Salz wurde in Form eines feinen Pulvers oder in Form einer konzertrierten Lösung zugesetzt. Zur Homogenisierung liess ich die Lösungen vor Gebrauch einige Stunden stehen und entfernte die Luftbläschen durch Zentrifugieren. Alle Manipulationen wurden bei 0° ausgeführt. Das Myosin kann unter diesen Umständen wiederholt umgefällt werden, ohne seine Aktivität zu verlieren.

Das an der Zentrifuge abgeschiedene Myosin ist zu locker um eine genügend konzentrierte (1—2%) Myosinlösung zu geben. Um eine solche zu erhalten wurde das Prezipitat bei -15° befroren. Nach auftauen lässt sich das Myosin leicht auf ein geringes Volum bringen. Oft genügt es schon das aufgetaute Prezipitat mit einem Glasstabe zu zerschlagen, wonach man das Wasser einfach ablaufen lassen kann. Das Myosin soll aber nicht zulange in gefrorenem Zustande gehalten werden da es sonst körnig wird.

Die Fäden, die aus derart gereinigtem Myosin hergestellt wurden, kontrahieren ebenso als di aus dem primären Extrakt hergestellten. Werden aber die Fäden aus derart gereinigtem

Myosin in destilliertem Wasser hergestellt, so schwellen diese nach einiger Zeit enorm an. Dieses Schwellen kann durch geringe Mengen von Neutralsalzen hintangehalten werden. 0,002 mol KCl oder 0,0001 mol CsCl ist deutlich aktiv. Aus diesen Gründen haben wir Fäden aus prezipitiertem Myosin stäts in 0,1 mol KCl und nicht in Wasser hergestellt. Wird die Myosinlősung vor dem Fadenziehen neutralisiert, so wird kein Schwellen beobachtet und der Faden kann besser in Wasser gezogen werden.

Untersuchung der Fäden.

Zur Beobachtung und Messung der Kontraktionerscheinungen bringt man am bessten kurze, etwa 3 mm lange Stückchen des Fadens in der betreffenden Lősungen auf ein ausgehöhltes Objektglas. Der Faden kann dann unter dem Mikroskop beobachtet und mit dem Okularmikrometer gemessen werden. Wir gebrauchten besonders dicke, tief ausgehölte Objektgläser.

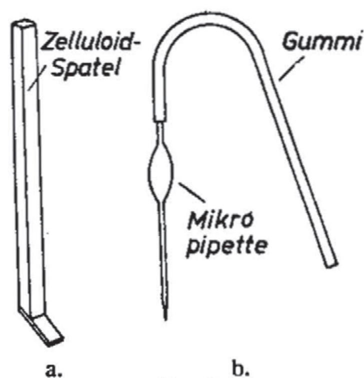

Fig. 2.

Soll die Länge des Stückes gemessen werden, so ist es wichtig dass die Schnittfläche an den Enden scharf sei. Zur Herstellung und Übertragung derartiger Faderstücke eignet sich ein aus Celluloid hergestellter Spatel besonders gut. (Fig. 2. a.) Das Myosin klebt zum Celuloid nicht. Der etwa 3 mm breite Spatel wird unter den Faden gelegt, etwas aufgehoben, dann streift man mit einem gebogenen, dünnen Galsstäbchen über die Ränder, wobei man den Faden abschneidet. Das abgeschnittene kleine Stückchen liegt am Spatel und kann mit diesem leicht aus der Flüssigkeit gehoben und auf das Objektglas übertagen werden.

Sollte die Kontraktion auf Zugabe von ATP in verschiedenen Salzen verfolg werden, so wurde die abgemessene Menge der Salzlősung (0,35 ml.) auf das Objektglas gebracht und das ATP (0,05 ml) aus einer kleinen, zur Kapillare ausgezogenen Pipette zugesetzt, (Fig. 2 b.) u. zw. derart, dass die Lősung

in einem dünnen Strahl energisch eingeblasen wurde. Wird dann noch Luft auf die Oberfläche der Flüssigkeit geblasen so wird die Lösung hierdurch energisch durchgerührt, was sehr wichtig ist. Es ist auch wichtig darauf zu achten, dass der Faden sicht nicht an das Glas anhafte und nicht am Rande, sondern frei in der Flüssigkeit liege, also von allen Seiten der Diffusion frei zugänglich sei.

In den Arbeiten vorliegenden Bandes wurde die Kontraktion stäts mit % der Verkürzung ausgedrückt. Diese Ausdruckweise ist bequem aber irreführend. Die richtige, aber mehr komplizierte Weise wäre die Kontraktion durch Messen des Volumes des Fadens auszudrücken.

Dies sei mit folgendem Beispiel erleuchtet: nehmen wir an dass wir die Kontraktion zwier Fäden vergleichen. Der eine Faden verkürzt sich (isodiametrisch) um 25, der ander um 66%. Man wäre also geneigt zu denken dass letzterer sich bloss 2,5-mal mehr kontrahierte als ersterer. Wird aber das Volum berechnet, so findet man, dass während der erste Faden sich nur um etwa 40% zusammenzog, letzterer eine Kontraktion von 97% zeigte. Handelte es sich um Fäden, die ursprünglich 1,5% Myosin enthielten so verlor der erste Faden 40% letzterer 98,5% seines Wassers. Während der Myosingehalt im ersteren Faden von 1,5 auf 2,5 stieg, stieg der Myosingehalt im letzteren auf 50%. Dieser letztere Faden besteht nun also praktisch genommen aus festem, benetztem Myosin.

Diese Berechnung erklärt auch warum in dem Vessuchen über die in diesem Bande berichtet wird, die maximale Kontraktion nie 66—70% überschritt. 66% Kontraktion wird sehr oft gefunden, aber kaum überschritten. Zugleich verstehen wir warum der kontrachierte Faden undurchsichtig wird.

Doppelbrechung und Kontraktion.

Die Myosinfäden, die in der beschriebenen Weise hergestellt wurden ziehen sich in der entsprechenden Salzlösung auf Zugabe von ATP in allen Richtungen gleichmässig zusammen, d. h. die Kontraktion ist isodiametrisch. Fig. 3. gibt die % Kontraktion eines Myosinfadens in 0,1 M KCl. Wie ersichtlich bleibt Kontraktion in der Querrichtung kaum hinter der Kontraktion der Längsrichtung zurück. (Abszisse: Zeit in Min.)

Dieses von der Muskelfaser abweichende Verhalen der Myosinfäden ist dem Umstande zuzuschreiben, dass die Myosinmicellen im Faden, im Gegensatzt zum Muskel, nicht, oder nur ganz schwach geordnet sind. Selbst wenn sich die einzelnen Micellen anisodiametrisch zusammenziehen muss die Kontraktion des Fadens isodiametrisch werden wenn die Micellen nicht geordnet sind. Meistens hat aber der Faden eine sehr geringe Doppelbrechung, also eine ganz schwache Orientierung der Micellen und darum bleibt auch die Kontraction im Querdurchmesser hinter der Verkürzung meistens etwas zurück (Fig 3).

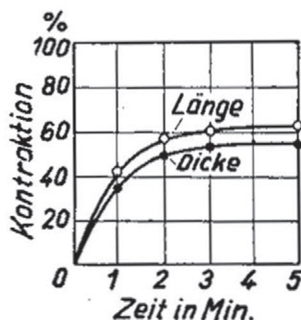

Fig. 3.

Um die Micellen im Faden zu orientieren und somit die Verhältnisse des Muskels, so gut wie möglich, nachzuahmen, muss man den Faden spannen. Dann ordnen sich die Micellen koaxial mit dem Faden. Diese Orientierung kann durch Beobachtung der Doppelbrechung messend verfolgt werden.

Leider aber können unsere, in Wasser gezogenen Fäden nicht oder nur sehr unwesentlich (10—15%) gesteckt werden, da sie zerreissen. Die in gewöhnlichem destillierten Wasser oder $CuCl_2$ hergestellten Fäden sind dehnbar sind aber inaktiv und geben keine Kontraktion mit ATP (s. Tab. 1.). Bis zu einem gewissen Grade können die in KCl gezogenen Myosin Fäden derart orientirt werden, dass man sie auf ein Objektglas bringt, mit einem Deckglas bedeckt und durch Bewegen des letzteren die Fäden hin und herrollt. Der Faden zerreist hierbei, aber die entstandenen fadenförmigen Fragmente zeigen eine ausgesprochene Doppelbrechung. Auf Einwirkung von ATP werden diese Fadenstücke kürzer und gleichzeitig um etwa 15—20% dicker. (S. Tab. I.)

Ich bestrebte mich Verhältnisse zu finden unter denen dehnbare und doch aktive Fäden erhalten werden können. Ich versuchte Fäden an Stelle von Wasser, in verschiedenen Salzlösungen oder organischen Lösungsmitteln herzustellen. Nachdem die frisch gezogenen Fäden 5 Min. lang in der betreffenden Lösung verweilten untersuchte ich ihre Dehnbarkeit, dann über-

Tabelle I.

Lösung	Dehnung	Doppelbr. vor der Dehnung	Doppelbr. nach der Dehnung	ATP — Wirkung	
				Länge %	Dicke %
0,1 KCl	10—15%	—	In Spuren	— 62	— 53
0,001 $CuCl_2$	200%	0,33.10^{-4}	18,5.10^{-4}	—	—
0,1 KCl gewalzt	—	—	7,5.10^{-4}	— 7	+ 15
25% glycerin	200%	—	15,6.10^{-4}	— 16	+ 20
0,001 $ZnSO_4$	200%	In Spuren	11,6.10^{-4}	— 30	+ 55

trug ich sie in 0,1 mol KCl und untersuchte nach weiteren fünf Minuten unter ATP Zugabe (0,18%) ihre Kontraktonsfähigkeit. Zur Untersuchung der Dehnbarkeit gebrauchte ich den in Fig.

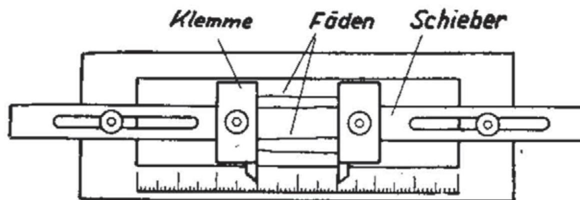

Fig. 4.

4 abgebildeten Apparat. Die Fäden wurden an beiden Ende zwischen zwei Plättchen eingeklemmt. Dann wurden die Schieber auseinander gezogen. An der Skala konnte die Dehnung abgelesen werden, bei der die Fäden rissen.

In Glycerin gezogen, werden die Fäden dehnbar ohne ihre Kontraktilität zu verlieren. Am bessten eignet sich 25% Glycerin, die Kontraktilität geht aber auch in reinem Glycerin nicht verloren. In reinem Glycerin steigt aber die Dobbelbrechung auf Dehnen nicht.

Die untersuchten Katione können in drei Gruppen geteilt werden (s. Tab. II.):

1. Die die Aktivität nicht vermindern aber auch die Dehnbarkeit nicht steigern (Li, Na, K, Mg, Mn.)

2. Die die Dehnbarkeit steigern aber die Aktivität vernichten, also das Myosin denaturieren (Cu.)

3. Die die Dehnbarkeit bei gewissen Konzertrationen stei-

Tabelle II.

Lösung	Norm. Konz.	Dehnbarkeit	ATP — Kontraktion % (Länge)
LiCl	0,1 0,01 0,001	20 20 30	52 60 58
NaCl	0,1 0,01 0,001	— — 20	55 51 58
KCl	0,1 0,01 0,001	10 10 10	62 62 62
$CaCl_2$	0,1 0,01 0,001	10 20 20	59 55 55
$MgCl_2$	0,1 0,01 0,001	Zerfliesst 15 20	— 58 55
$MnSO_4$	0,1 0,01 0,001	— — —	43 50 00
$CuCl_2$	0,1 0,01 0,001	203 230 80	— — 6
$ZnSO_4$	0,1 0,01 0,001	130 220 75	10 38 40
$Al_2(SO_4)_3$	0,1 0,01 0,001	100 100 10	14 21 47
$FeCl_3$	0,1 0,01 0,001	40 95 30	17 23 40
$Co(NO_3)_2$	0,1 0,01 0,001	30 30 20	44 54 50
$NiSO_4$	0,1 0,01 0,001	70 60 50	42 44 40

gern aber die Aktivität nur in geringerem Masse vermindern (Zn, Al, Fe, Co, Ni). Natürlich ist der Unterschied kein absoluter und bei höherer Konzentration wirken auch diese Katione denaturierend.

Es ist scheinbar unmöglich Ione zu finden, die den Faden dehnbar machen und die Kontraktionsfähigkeit ganz unbeeinflusst lassen. Die Dehnbarkeit scheint bereits ein Ausdruck der Denaturierung zu sein.

Am günstigen fand ich die Verhältnisse bei 0,001 N $ZnSO_4$,

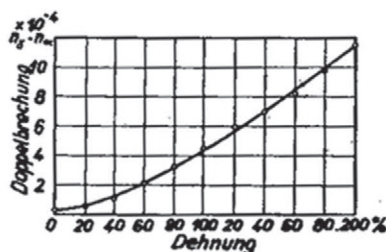

Fig. 5.

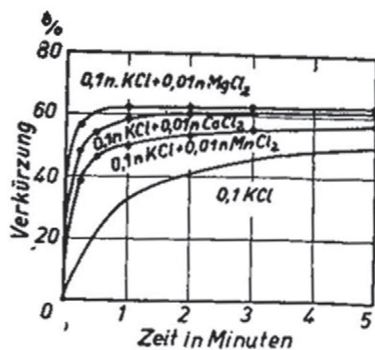

Fig. 6.

In der Fig. 5. wird der Zusammenhang zwischen Dehnung und Doppelbrechung gegeben.

Werden die gestreckten Fäden losgelassen, so ziehen sie sich wieder zusammen, die Dehnung ist also elastisch. Werden aber die Fäden 10 minuten lang in gedehntem Zustande gehalten, dann losgelassen, so behalten sie ihre länge. Offenbar entwickeln sich zwischen den benachbarten Micellen neue Verbände. Werden nun die also „gesetzen" Fäden in 0.1 KCl übertragen, hier 5 Min. lang belassen, dann ATP zugesetzt, so verkürzen sie sich, so wie der Muskel, unter gleichzeitiger Zuhnahme ihres Querdurchmessers (s. Tab. 1.) Gleichzeitig verschwindet ihre Doppelbrechung.

Anlässlich dieser Versuche untersuchte ich ob die Wirkung von Mg auf die Kontraktion spezifisch sie. Wie durch Szent-Györgyi beschrieben, kann die aktivierende Wirkung von KCl durch $MgCl_2$ befördert werden. Ich fand, dass Mg in diesen Versuchen durch Mn and Co ersetzt werden kann. Das Ergebniss meiner Versuche ist in beistehender Fig. 6 zusammenge-

fasst. Ohne KCl geben $CoCl_2$ and $MnCl_2$, so wie das $MgCl_2$, mit ATP nur eine schwache und langsame kontraction.

Zusammenfassung.

Technik der Herstellung und Untersuchung von Myosinfäden wird beschrieben.

Es wird gezeigt, dass Myosinfäden, in üblicher Weise hergestellt, sich isodiametral zusammenziehen. Gesteckte Fäden, in denen die Myosinmicellen koaxial zur Fadenaxe orientiert sind, ziehen sich anisodiamatral zusammen: werden gleichzeitig kürzer und dicker.

Ähnlich wie im Muskel verschwindet die Doppelbrechung während der Kontraktion.

Die Wirkung verschiedener Ione auf Dehnbarkeit und Kontraktilität wird untersucht.

Es wird gezeigt dass Mn und Co bei der Kontraktion das Mg ersätzen können.

Litteratur.

1. *H. H. Weber:* Arch. ges. Physiol. 235, 205, 1934.
2. *M. Gerendás* and *A. Szent-Györgyi:* Enzymologia, 9, 117, 1940.
3. *M. Gerendás:* Ibid. p. 123.

The influence of K and Mg on the contraction of myosin.

by

T. Erdős

(Chinoin research student).

It has been shown by SZENT-GYÖRGYI that myosin B threads are capable of giving a contraction in the presence of adenyltriphosphate („ATP") and KCl. The rate of contraction was greatly increased by $MgCl_2$. At higher salt concentrations and in the presence of ATP, dissolution was obtained instead of contraction. Myosin A was found to be relatively inactive.

The object of this paper is to obtain more detailed information about the influence of salt concentration. For the sake of convenience I also will call the threads prepared from the 20 min. extract (see BANGA and SZ.) myosin A threads and those prepared from the 24 h. extract, myosin B threads, although the 20 min. extract always contains small quantities of myosin B and the 24 h. extract may contain some myosin A.

For the preparation and measurement of threads I used the technique of M. GERENDÁS.

Effect of varied KCl concentrations.

Table 1 gives the result of an experiment with varied KCl concentrations. The experiment was made on two different threads which I will call *a* and *b*. *a* was prepared from muscle extract which had been stored for two days, *b* from an extract which had been stored for ten days at 0°C. It will be seen that storage makes no difference.

The freshly prepared threads were allowed to stand for 30 min. at room temperature and were then transceferred into the corresponding salt solution and measured. After 5 min. ATP (0,09%) was added and readings made 5 min. after that.

Table I.

Mol KCl	Myosin B I. a	Myosin B I. b	Myosin B II. + 0,001 mol $MgCl_2$ a	Myosin B II. + 0,001 mol $MgCl_2$ b	Myosin A III. a	Myosin A III. b	Myosin A IV. ATP + KCl simult a	Myosin A IV. ATP + KCl simult b
0.10	66	66	66	66	23	20	14	23
0.17	70	58	50	66	19	9	17	16
0.18	59	62	64*	56*	16	14	13	15
0.19	69	66	59*	66*	—	—	—	—
0.20	60	59	?	?	—	—	—	—
0.21	45	66	—	—	—	—	—	—
0.22	66	60	—	—	—	—	—	—
0.23	58	62	—	—	—	—	—	—
0.24	67	62	x	—	—	—	—	—
0.25	66	66	x	x	—	—	—	—
0.26	61	63	x	x	—	—	—	—
0.27	69	57	x	x	—	—	—	—
0.28	58	61	x	x	—	—	—	—
0.29	66*	70*	x	x	—	—	—	—
0.30	52*	59*	x	x	—	—	—	—
0.31	30*	30*	x	x	—	—	—	—
0.32	?	?	x	x	x !	x !	—	—
0.33	?	x	x	x	x !	x !	—	—
0.34	x	x	x	x	x !	x !	—	—
0.40	x	x	x	x	x !	x !	—	—
0.41	x	x	x	x	x !	x !	x !	x !

The whole experiment was repeated also in the following way: the thread was put directly into the salt-ATP mixture instead of putting it into the salt solution first and adding ATP afterwards. This made no appreciable difference with myosin B but changed the behaviour of the myosin A threads considerably. For this reason I am giving the effect of ATP and varied KCl concentrations on myosin A with the simultaneous addition of both substances.

The result of this experiment agrees with the result of other similar experiments except for a small variation of the KCl concentration at which the changes from inactivity to contraction or to dissolution occure. The experiment was also repeated with purified myosin which gave the same results except for the slighlty greater solubility of myosin A which dissolved in KCl at 0,28 mol. instead of 0,32. The experiment was also repeated with threads which had been strored for 12 hours at room temperature (25° C). This made no difference to myosin B. Myosin A threads lost their contractility.

Columm I and II give the data for myosin B, col. I. with pure KCl, col. II in the presence of 0,001 mol. $MgCl_2$. Col. III and IV relate to myosin A in pure KCl. In col. III the thread was put into KCl and ATP added afterwards. In col. IV KCl was added simultaneously with ATP.

The numbers give the % of shortening. The asterisks mean that the threads were very fragile and prone to fall into pieces if agitated. *x* means dissolution. *x!* means that the thread dissolved in the salt solution before the addition of ATP. ? means a doubtful result: partial contraction and partial dissolution. — means inactivity, i. e. that there was no contraction or dissolution..

If we go over col. I we will notice a variation in the numbers, in spite of the fact that the contraction is evidently equally strong from 0,1 to 0,29 mol. KCl. This variation is due partly to errors of measurement and to a greater extent, to the different reaction of different threads; also to local conditions surrounding the thread which inhibited diffusion (too close proximity of the glass). So no great value should be attached to single measurements, only the general trend is of importance.

Now if we go over col. I we will find equally strong contractions from 0,1 mol. KCl up to 0,30. At 0,31 mol. the contraction is weaker and at 0,32 part of the thread dissolves part of it contracts. At 0,34 mol. KCl there is dissolution only. (Whithout ATP the thread is not dissolved, not even by mol. KCl).

It will be seen from this that the change from maximal contraction to complete dissolution is not any too sharp and takes place within a range of 0,04 mol. (0,3—0,34). The KCl concentration, at which the change takes place, is fairly high.

Now if we compare this with col. II it will be seen that the 0,001 mol $MgCl_2$ present decreased the KCl concentration. necessary for the dissolution of the thread, greatly. An entirely new phenomenon appears also: between contraction and dissolution there is a zone in which the thread neither contracts, nor dissolves, but is just inactive. The myosin has thus three different states: the contracted, the inactive and the dissolved state. The transition from one state into the

other is sharp and takes place within a concentration difference of 0,01—0,02 mol. The experiment gave the same result in the presence of 0,01 mol. $MgCl_2$ as with 0,001.

Col. III shows that myosin A behaves quite differently. With smaller KCl concentrations it gives the usual weak contraction. Then follows a wide zone of inactivity even without $MgCl_2$. At 0,32 mol KCl we find dissolution without the addition of ATP. From 0,19 mol KCl upwards ATP has thus no effect at all. The KCl concentration at which, in the presence of Mg, myosin B still gives a maximum contraction (0,19 mol.) myosin A is entirely inactive. Addition of 0,001 Mg made no difference.

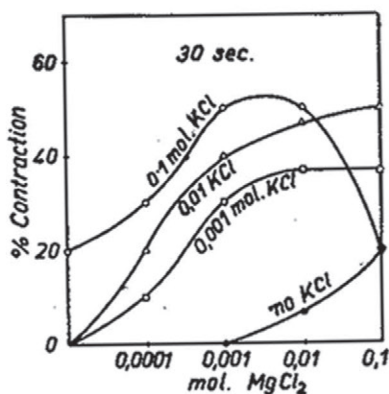

Fig. 1.

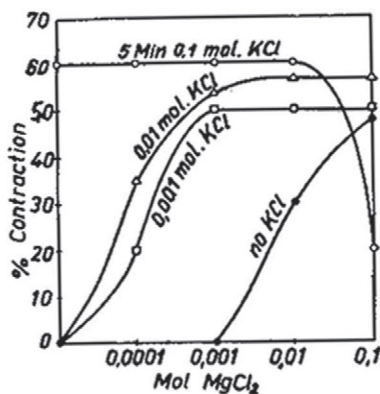

Fig. 2.

Col. IV shows that if ATP is added simultaneously with KCl, the zone of inactivity is very much extended and much higher KCl concentrations are necessary for the dissolution of the thread.

If the ATP is added first, and allowed to act for 5 minutes and the KCl then added, the effect of sequence is stronger still and the thread dissolves only in 0,47 mol. KCl while it is still inactive in 0,46.

Varied KCl and $MgCl_2$ concentrations.

In fig. 1. and 2. I am giving the effect of varied KCl and $MgCl_2$ concentrations on a myosin B thread. The thread was allowed to stand for five minutes in the salt solution and the ATP then added (0,09%). Readings were made 30 sec. (fig. 1) and 5 min. (fig. 2) later.

It will be seen that KCl, without $MgCl_2$ is inactive in a 0,01 or a 0,001 mol. concentration. 0,1 mol. KCl gives a relatively slow but strong contraction. On the other hand 0,001 mol. $MgCl_2$, without KCl is inactive, while 0,01 mol. $MgCl_2$ shows a slow, weak action and 0,1 gives a slow, but fairly strong contraction.

In the presence of 0,001 mol. $MgCl_2$ even 0,001 mol. KCl becomes strongly active while in the presence of 0,01 mol KCl even 0,0001 mol. $MgCl_2$ gives a strong contraction.

Effect of varied ATP concentrations.

I am giving the effect of varied KCl and ATP concentrations on a myosin B thread prepared from precipitated myosin in Tab. II. Owing to the higher myosin content the thread was somewhat less contractile than the thread of Tab. I.

Table II.

% ATP	0,4 KCl	0,3 KCl	0,2 KCl	0,1 KCl	0
0,7	x	x	x	48	50
0,35	x	x	41	47	35
0,18	x	x	46	47	8
0,09	x	x	42	44	0
0,045	x	x	32	21	0
0,022	x	x	22	15	0
0,011	x	2	12	6	0
0,006	4	0	8	0	0
0,003	0	1			

The thread was allowed to stand for 5 minutes in the salt solution, then the ATP was added and readings made 5 min. later. It will be seen that at a certain KCl concentration we obtain the same effect at all ATP concentrations, *i. e.*, contraction or dissolution. (The dissolution in 0,7% ATP at 0,2 mol. KCl, ist due to the additional KCl introduced with the ATP.) The nature of the action is thus independent ot the ATP concentration. Either we obtain contraction or dissolution or no effect at all.

In the absence of KCl, ATP is inactive in concentrations in which it still gives a maximal effect in the presence of KCl. This justifies the conclusion that the contraction observed in

the highest concentrations of ATP is due to the K present partly as the kation of the ATP and partly as a KCl impurity and that ATP in itself, without K, is inactive.

Varied concentrations of the muscle extract.

The fresh, minced muscle was suspended in water, 1 ml. of water being taken per g of muscle. The suspension was stored over night at 0° and filtered next day, first through a cloth and then through paper. The neutral juice was diluted with water; the dilution is given on the abscissa of Fig. 3. The myosin B threads were placed into the solution for 5 minutes and then 0,09% of ATP was added. Readings were made after 30 seconds. As will be seen the juice gives, even in $^1/_4$ dilution, a maximal contraction. With further dilution the effect becomes wearker and eventually disappears. It may be concluded thus that the juice obtained in the described way contains the ions necessary for maximal contraction in excess.

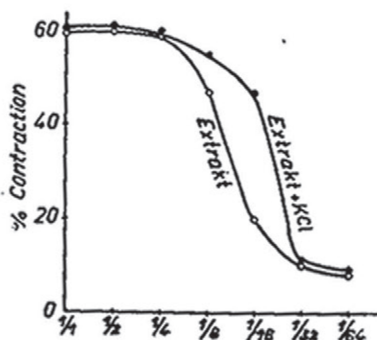

Fig. 3.

In a second experiment 0,1 mol KCl was added to the same juice. It will be seen (Fig. 3) that even at a dilution of $^1/_4$ the effect of the extract is not increased by this addition of KCl thus there was K present in sufficient concentration to give maximal contraction. At a higher dilution of the extract the contraction is increased showing that there is still a sufficient concentration of Mg to enhance the action of KCl. At a dilution of $^1/_{32}$ the Mg becomes insufficient too.

Variation of pH.

Some time ago I studied the action of salts on the isoelectric point of casein. The isoelectric point of casein is shifted by neutral salts and a precipitation or a dissolution can be obtained by their addition, at certain pH-s. It seemed

possible that the precipitating and dissolving action of salts and ATP in the case of myosin could be explained in a similar way. For this reason I studied the influence of pH on the physical state of myosin in the presence of different concentrations of KCl, with and without ATP, Mg and muscle extract. Contrary to my expectation I found that KCl did not shift the range of insolubility at lower concentrations to either side of the pH scale, nor did the ATP or Mg or muscle extract. The contraction, precipitation and dissolution of myosin can thus not be explained by a shift of the insolubility range on the pH scale.

I want to give the chief result of this very extensive work in a few words only. Threads prepared from precipitated, washed and redissolved myosin A and B were placed into the corresponding solutions, the pH of which was adjusted by the addition of HCl or KOH and controlled colorimetrically. Solutions of pH 2-pH 11 were prepared. After 5 min. the effect was observed.

Myosin B threads begin to dissolve above pH 8,5 and below pH 4. At pH 9,5 and 2,5 dissolution is complete. The range of insolubility lies thus between pH 4 and 8,5. This range is not shifted to either side by KCl not even by KCl in a 0,2 mol. concentration. At 0,4 mol. there is a slight shift towards the smaller pH: the thread begins to dissolve above pH 8 and is insoluble even at pH 2. ATP alone or in the presence of 0,25 mol KCl makes the thread insoluble on the acid side but has no effect on the alkaline side. At higher KCl concentrations ATP makes the thread somewhat more insoluble on the alkaline side too. The thread dissolves only at a somewhat (0,5—1) higher pH than without ATP. At 0,4 mol KCl the ATP dissolves the thread at all pH-s except on the extreme acid side, pH 2—3. Mg or muscle extracts increase the effect of ATP without shifting the insolubility range to either side of the pH scale.

Myosin A is insoluble between pH 4—7.5 in the absence of KCl and ATP; its insolubility range is somewhat narrower than that of myosin B. Up to 0,3 KCl the range remains unchanged. At 0,4 mol KCl the thread is insoluble between pH 2—5,5 and soluble at higher pH's. Here too the ATP makes the thread insoluble on the acid side but has otherwise

little effect. Mg or extract is inactive, they only broaden the insolubility-range to some extent.

Miscellaneous observations.

A number of experiments were made with $CaCl_2$. In 0,01 mol concentration this salt inhibits the contraction given by ATP in the presence of KCl or KCl + Mg. This inhibition may be complete but could not be reproduced regularly. Several of our myosin pereparations were not inhibited or inhibited only if precipitated myosin was used for the preparation of the myosin threads.

The Ca-inhibition is reversible. If the Ca is washed out again the thread behaves normally.

The action of 0,1% nicotine, quinine and K-oxalate were also studied. None of these reagents had any inhibitory action on contraction. This is important because BANGA found that the splitting of ATP by myosin is completely inhibited by these reagents. Myosin can thus contract without splitting ATP.

Summary.

The contraction of myosin A and B threads is studied at varied KCl, $MgCl_2$, ATP and H^+ concentrations. Differences in the behaviour of A and B myosin are pointed out. It is shown that in the presence of ATP and Mg very slight changes in KCl concentration are sufficient to change the physical state of the myosin B thread and make the inactive thread contract or the contracted thread become inactive or dissolve.

Contraction is not inhibited by the complete inhibition of the phosphatase activity of myosin.

Discussion.

by

A. Szent-Györgyi.

It has been shown in the previous papers that myosin can be extracted from muscle in two forms: as the relatively inactive myosin A and the very reactive myosin B. The myosin of previous investigators, prepared by EDSALL's method, corresponds to our myosin A with a small admixture of myosin B as an impurity. We have shown that myosin A is transformed into B if it stands in prolonged contact with muscle particles.

If we want to correlate our data with muscle physiology, our first question must be: what is the relation of the two substances that we called myosin A and B and what is the nature of the A→B transformation.

Experiments of F. B. STRAUB, now in progress, difinitely show that myosin B is a stoichiometric compound of myosin A and another substance. We will call this other substance „actin" and the myosin-actin complex will be called „acto-myosin". Actin, in itself, is insoluble in salt solution. It is, together with cytochromoxidase and succinodehydrogenase. part of the insoluble muscle residue.

There in thus, according to these results of F. B. STRAUB which will be presented later, but one myosin, and the substance that we called myosin B is acto-myosin.

As has been shown by MOMMAERTS and STRAUB myosin B, just as myosin A, forms a well defined complex with ATP also. Myosin can therefore exist in four different forms: 1. as free myosin (myosin A), 2. as ATP myosin, 3. as acto-myosin (myosin B), 4. as ATP-acto-myosin. If we want to discuss the influence of salts on myosin we do better if we talk about the influence of salts on these four different forms.

In table I. I am giving the table of T. ERDŐS somewhat

Table I.

Mol. KCl + 0,001 mol. $MgCl_2$	Myosin	ATP-Myosin	Acto-Myosin	ATP-Acto-Myosin
0	—	—	—	—
0,01	—	+	—	+++
0,1	—	+	—	+++
0,17	—	+	—	+++
0,18	—	+	—	+++
0,19	—	+	—	+++
0,20	—	—	—	?
0,21	—	—	—	—
0,22	—	—	—	—
0,23	—	—	—	—
0,24	—	—	—	x
0,25	—	—	—	x
0,30	—	—	—	x
0,31	—	—	—	x
0,32	x	—	—	x
0,33	x	—	—	x
0,45	x	—	—	x
0,46	x	—	—	x
0,47	x	x	—	x
0,48	x	x	—	x
0,60	x	x	—	x

completed and translated into these new terms. The signs mean the same as in ERDŐS's paper: — means inactivity *i. e.* no contraction and no dissolution, × means dissolution; ? means partial contraction, partial dissolution. The % of contraction has been marked with crosses, +++ meaning maximal, + weak contraction. There was 0,001 mol $MgCl_2$ present everywhere.

It will be seen that free myosin is fairly soluble in KCl and gives no contraction. ATP-myosin is less soluble in salt and gives a weak contraction at a low KCl concentration. Acto-myosin is insoluble in salt and gives no contraction. By forming the ATP compound this complex becomes most sensitive to salts: according to the concentration of the salt present the ATP-acto-myosin may be inactive, maximally contracted, inactive again or dissolved, and all this at a KCl concentration below 0,25 mol.

If we try to apply this experience to muscle, the first question is whether in muscle the myosin is in sufficiently intimate contact with the actin to form a complex. It has been shown by BANGA and myself that in the absence of ATP the myosin of the muscle cannot be extracted with EDSALL's fluid, which means that it is insoluble in salt solutions and behaves thus like acto-myosin. I have shown that the myosin in the frozen and extracted muscle reacts as myosin B. It can therefore be stated that myosin is in sufficiently intimate touch with the actin in muscle to form acto-myosin.

In living muscle, under normal conditions, there is always a fairly high concentration of ATP which is kept constant throughout life. The living as well as the freshly minced muscle contains thus the highly sensitive ATP-acto-myosin.

Our viscosity measurements indicate that, at the high salt concentration of EDSALL's salt solution (0,6 mol KCl), the complex dissociates into actin and ATP-myosin. This helps us to understand what happens in the 24 h. extraction. If we suspend the muscle in EDSALL's fluid, the ATP-acto-myosin dissociates into actin and ATP-myosin. The former is insoluble in salt solution or is linked to the insoluble residue and will therefore remain undissolved while the ATP-myosin goes into solution. For this reason we always obtain myosin A from fresh muscle containing ATP whether the myosin, present in muscle, was bound to the actin and was thus present as myosin B or not. On storage the ATP is split and the dissolved ATP-myosin goes over into free myosin which forms acto-myosin with actin. This complex being stable even in the presence of 0,6 mol. KCl, the myosin which is already dissolved will, by the formation of this complex, bring the actin into solution. (Possibly the connections of the actin with the residue are loosened up meanwhile by the alkaline reaction). If we start with muscle free from ATP, the myosin will be present in the form of the stable and insoluble acto-myosin which is insensitive to salts, and so will not be extracted by the salt solution.

Evidence indicates thus that myosin is present in muscle as ATP-acto-myosin. As shown by the last column of the table, this complex can exist in different states which depend on the KCl concentration: if there is no salt present the

complex will neither contract, nor dissolve, i. e. it will be inactive; on addition of very small amounts of KCl (0,01 mol) we will get (in the presence of Mg) maximal contraction. The change from 0 to 0,01 mol. concentration will be sufficient to cause the inactive myosin to contract maximally. A similar jump in the opposite direction will be observed between 0,18 and 0,20 mol, the change of 0,02 mol in the KCl concentration being sufficient to cause a maximal effect. Any of these two changes might be analogous to what happens in muscle when the wave of excitation arrives and the muscle goes over from relaxation to contraction. It is not impossible either that relaxed muscle corresponds to our dissolved myosin which is formed at 0,24 mol. KCl.

Naturally I do not mean to say that there is KCl in muscle and that the change of the KCl concentration is the cause of contraction. This would be in contradiction with primitive facts. What I mean to say is that our experiments show that slight changes in ionic concentration are capable of inducing changes in the structure of the protein and that analogous changes in the protein might occur in muscular contraction.

As contraction is brought about in our threads by a disturbance of the ionic equilibrium, so, in its turn, contraction might act on the ionic balance. It does not seem impossible that in muscle the contraction of one ATP-acto-myosin unit might cause the disturbance responsible for the contraction of the next unit and so forth. In this case the sharp distinction between the wave of excitation and contraction would be unjustified.

No attempt has been made in this series of papers to explain the inner molecular mechanism of the contraction of myosin. In our experiments the changes in the physical state of the ATP-acto-myosin-complex have been brought about by salts and are thus, to some extent, analogous to other colloidal reactions. As has been shown by BANGA and ERDŐS the splitting of ATP is not involved in the developement of the contraction. The myosin becomes fermentatively active only when contracted. Translated into terms of muscle physiology this would mean that the energy of the splitting of ATP is used for relaxation; relaxed muscle again is inactive. This causes

adequate quantities of ATP to be split and only adequate quantities of energy to be liberated, since it is the function itself (contraction) which liberates energy. Naturally I do not mean to say that contraction, as such, activates phosphorilysis. The contraction as well as the enzymic activity are consequences and expressions of the same change in the finer structure of the molecule.

I want to close with a rather subjective remark. I was always led in research by my conviction that the primitive, basic functions of living matter are brought about by ions, ions being the only powerful tools which life found in the sea water where it originated. Contraction is one of the basic primitive functions and the results reported in this volume corroborate me in my conviction.

PS. After this volume was sent to press reprints have been received from *U. D.'Ancona* (Protoplasma *17*,388,1932) who emphasises the dehydration of the muscle fibril during contraction. This is in agreement with our observations on myosin.

A reprint has also been received from *Fr. Verzár* (Schweiz. Med. Wochenschr. *72*, 661,1942). He stresses the importance of K in contraction and brings the known facts about K, carbohydrates and adrenals into one ingenious theory. He also quotes *J. Needham* and collaborators (Nature 147,766,1941) who found that the double refraction of flow of myosin disappears in presence of small concentrations of KCl and ATP. Very unfortunately this paper is not accessible here at present.

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STUDIES

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Actin.

by

F. B. Straub.

It has been shown by *Banga* and *Szent-Györgyi*[1] that myosin can be extracted from rabbit's muscle in two different forms.

Myosin A is obtained by extracting the muscle tissue with three volumes of *Weber*'s solution* for 20 minutes in the cold and centrifuged immediately thereafter. The solution of myosin A obtained in this way is viscous and threads may be prepared from it. Neither the viscosity of the solution, nor the threads prepared from it, show any significant change on adding adenyltriphosphate (ATP).

If the muscle is extracted in a similar way for 20 minutes with the same solution, but left to stand for 24 hours in the cold and centrifuged only thereafter, a turbid and very viscous solution is obtained. On addition of ATP the viscosity of such a myosin B solution is decreased to a great extent. Threads prepared from myosin B show a vigorous contraction on addition of ATP and in presence of definite amounts of salts such as KCl and $MgCl_2$.

There was another difference between the two myosin modifications. Whereas both of them would split adenyltriphosphate with the appearence of free posphate, this enzyme action was increased by Mg ions in the case of myosin B, and not increased but rather inhibited in the case of myosin A.

The problem of understanding the difference between these two modifications of myosin was taken up by studying

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* This solution contains 0,6 M KCl, 0,04 M $NaHCO_3$ and 0,01 M Na_2CO_3. In previous communications from this Institute (Studies from the Institute of Medical Chemistry, Szeged, vol *1)* this solution was referred to as „*Edsall*'s solution". Since then it was brought to our attention that this solution was first used by *Weber* and *Meyer*[2].

the factors bringing about the transformation of myosin A into myosin B. These investigations led to the discovery of a new protein present in the muscle stroma. The name *actin* was given to this protein. In combination with myosin it gives the contractile protein of the muscle. As shown later in this paper, myosin B is formed if a certain amount of myosin A and actin are mixed. It follows that myosin A is what was termed by earlier investigators as myosin, myosin B on the other hand is a mixture of a definite amount of actin and myosin.

The ability of a myosin preparation to react with a decrease of viscosity on addition of ATP, was termed by *Banga* and *Szent-Györgyi* the „activity" of the respective myosin. It will be shown in this paper that apart from myosin B other mixtures of myosin and actin can be prepared, which show varying degrees of activity. Myosin B is only one of the possible combinations, its significance being only that if the muscle is extracted in the way described above, myosin B vill be invariably extracted. But muscle does not contain myosin B, instead it contains an actin-myosin complex with a higher activity than myosin B. We therefore think it advisable to modify the nomenclature put forward by *Szent-Györgyi*[3] in such a way that *actomyosin* is generally a mixture or compound of actin and myosin, there being many possible actomyosins. One of them is myosin B.

Methods.

The viscosimeters used in this work had the following measurements: capillary diameter 0,060 cm, length of capillary 210 mm, diameter of the cylindrical reservoir tube 1,65 cm, amount of outflowing fluid 1,2—1,7 ml. The viscosimeters have been placed in an icebath, which was vigorously stirred. The time of outflow of the solution was referred to the time of outflow of the solvent. No correction was taken for the change of specific weight by the presence of the proteins, as the protein content was maximally 3 mg/ml. All measurements have been performed using a buffered KCl solution of pH 7 as solvent. (Its composition see in the following paper of *Balenovič* and *Straub.)* 4 ml of myosin solution were placed in the viscosimeter. ATP was added in the form of its K salt, 0,1 ml of a 1,4% solution were added to 4 ml solution.

Determination of actin in solution.

Any specific property of actin which is in any way proportional to its quantity, may be utilized for its quantitative determination. The combination of actin with myosin to form an actomyosin is such a specific property. The activity of the resulting actomyosin is the higher, the more actin is added to the myosin. It remains to define the measure of the activity of actomyosin. This is complicated by the fact that the decrease of viscosity on addition of ATP depends not only on the actin content but also on the viscosity of the actomyosin. This is cleary brought out in Fig. 1. It shows the decrease of the specific viscosity ($\Delta\eta_{sp}$) on addition of ATP as the function of the specific viscosity in presence of ATP (η_{ATP}). The curve is valid for myosin B and it was constructed from the data of *Balenovič* and *Straub* (Fig. 1. of the following paper).

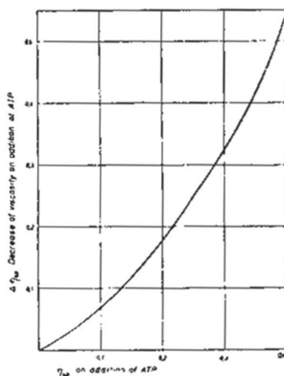

Fig. 1.

The activity of an unknown myosin solution is defined as the relation of its $\Delta\eta_{sp}$ to the $\Delta\eta_{sp}$ of a myosin B solution having the same η_{ATP} value.

As example let us take a myosin solution, which has the specific viscosity $(\eta) = 0{,}625$, in presence of ATP $(\eta_{ATP}) = 0{,}35$. Fig. 1. shows that in case of myosin B to this value of η_{ATP} corresponds a $\Delta\eta_{sp}$ of 0,41. After the above definition the activity is

$$\frac{0{,}625 - 0{,}35}{0{,}41} \cdot 100 = 67\,\%$$

To a myosin A solution,* which contains 6 mg of myosin, and has no activity (no decrease of viscosity on addition of ATP) different amounts of an actin solution are added, the solution is made up to 6 ml so as to have 0,6 M KCl concentration and pH 7, and the activity of the resulting mixture is

* All experiments described in this paper have been performed using crude myosin A solution, obtained by extracting the muscle tissue with the *Weber* solution.

determined in every case. The results are plotted against the amount of actin as in Fig. 2. From the curve it is easy to obtain the amount of actin which is necessary to transform the 6 mg of myosin present in the experiment into myosin B (100% activity). From the purest actin so far obtained 1 mg protein is needed to transform 6 mg of myosin into a 100% active myosin. The actin content of an unknown actin solution can be therefore evaluated if we determine the amount of the solution necessary to activate 6 mg myosin from 0 to 100%. In this way the element of arbitrariness introduced by the arbitrary measure of activity, is again eliminated.

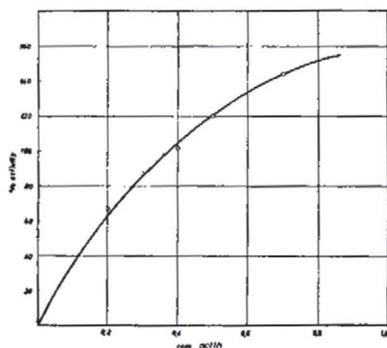

Fig. 2.

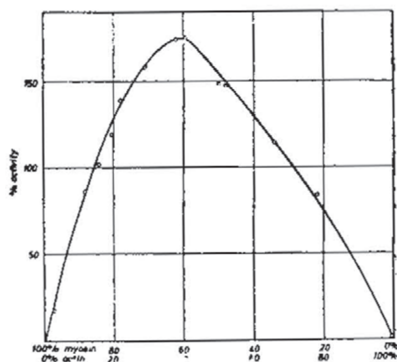

Fig. 3.

There are good reasons to believe that the actin preparations, of which 1 mg activates 6 mg myosin to a 100% active actomyosin, represent the pure actin. This means that myosin B is a compound of 6 mg myosin and 1 mg actin.

That myosin B is a compound of myosin and actin, is supported by the following experiment, in which actin was prepared from myosin B. In the usual way a myosin B was obtained, dissolved in *Weber*'s solution. It was diluted with 5 volumes of distilled water and centrifuged. The precipitate was then treated first with 4 and then with one volume of acetone and left to dry at room temperature. By extracting the dried myosin with distilled water a viscous solution of actin is obtained, which does not contain any myosin. $^1/_4$-$^1/_3$ of the estimated actin contents of myosin B can be extracted in this way.

It is seen from Fig. 2. that on addition of more actin than necessary to bring about 100% activation, actomyosins may be

obtained with more than 100% activity. Fig. 3. shows the extension of these studies. It is seen that addition of more and more actin, i. e. increasing the ratio actin: myosin, results first in an increase of activity and later in a decrease of it. Finally, actin alone like myosin alone does not show any change on addition of ATP.

The existence of actomyosins more active than myosin B is rather important. It will be described in the following paper that muscle contains an actomyosin with 170% activity. It is seen from Fig. 3. that this is the maximal activity to be achieved at pH 7.

Preparation of actin.

Rabbit's muscle from the legs and of the back are minced in a cooled Latapie mincer. To every 100 g are added 300 ml of *Weber*'s solution and stirred mechanically for 20 minutes at 0°. The mixture is centrifuged and the supernatant solution discarded. The residue is left to stand in the cold room for one or two days. It is then stirred up with 5 times its volume of distilled water and centrifuged again. The solution is again discarded and the residue mixed with 4 volumes of acetone. After 10 minutes the acetone is sucked off and replaced by a fresh lot of 1 volume of acetone. After 10 minutes this is sucked off again and the residue is spread over a filter paper and left to dry at room temperature over night.

The acetone dried muscle is extracted with 20 volumes of distilled water. The extraction can be carried out in either of the two following ways:

a) The dried muscle is mixed with the distilled water and then left to stand in itself without stirring for 4 hours at room temperature.

b) The dried muscle is ground with the distilled water in a mechanical mortar. In about $^{1}/_{2}$—1 hour, depending on the amount to be extracted, the mixture will become a rather rigid mass of foam. Further grinding is then inadvisable as the actin is denatured when present in the foam. During the subsequent centrifugation the foam is reduced to a viscous liquid.

After the extraction the undissolved muscle particles are centrifuged off; the resulting solution contains the actin. There

is no difference in the purity of the actin obtained by either of these procedures. But whereas by the thorough grinding nearly all of the actin is extracted, by procedure a) only about $^1/_6$-$^1/_4$ of the estimated actin of the muscle can be obtained in solution. On the other hand, actin obtained by a) is a clear solution, that obtained by b) is a stable milky suspension. Usually these solutions contain 3—4 mg protein per ml and the purity is such that 1,5—2 mg activate 6 mg myosin to 100%. From these solutions of the actin further purification can be achieved by precipitating it isoelectrically or by Ca ions.

The actin solution, obtained by procedure a) is diluted 8 times by distilled water and then an acetate buffer of pH 4,8 is added. The buffer concentration will be 0,01 M. The precipitate is centrifuged off at room temperature and dissolved by the addition of bicarbonate to neutral reaction.

Actin can be precipitated by adding $CaCl_2$ solution to the actin solution. The amount of Ca necessary to precipitate the actin however depends largely on the concentration of univalent ions already present. Thus about 0,02 M $CaCl_2$ is needed to precipitate the actin of the first extract (which still contains some of the KCl of the *Weber*'s solution), but 0,002 M or less is sufficient to give complete precipitation from isoelectrically purified actin solutions, which are poor in salt.

The Ca-precipitate of actin is inactive. Its activity will however return if KCl is added to the solution. At the same time it is observed that the Ca precipitate will form a stable suspension if KCl is added to it. To ensure this dissolution and reactivation, 40—50 times as much K^+ ion should be added as there was Ca^{++} ion present.

Obviously, the facts recorded here, can be described as a Ca : K antagonism. As the action of Ca is very pronounced and is observed with small, physiological concentrations, moreover the ratio Ca : K, where the effect of Ca is abolished is close to the physiological values, further, considering that actin is a part of the contractile element of the muscle, we may suppose that these observations are related to the Ca : K antagonism in muscle physiology. Further work is planned along this line.

It should be mentioned here that not only Ca^{++}, but also Mg^{++}, Mn^{++} or Sr^{++} ions can precipitate the actin in the

same way. On the other hand the effect of Ca^{++} can be overcome by Na^{+} ions just as well as by K^{+}.

It appears that the colloidal state of actin does not materially influence its activity. It was mentioned before that a clear solution obtained by extraction a) activates myosin to the same extent as the suspension obtained by extraction b), if both have an equal protein content. For this reason we prefer to prepare actin by the first method and such preparations have been used throughout this study. If the clear actin solution is treated with acid to precipitate it at pH 5—6, when dissolved at ph 7 the actin does not form a clear solution any more, but is more or less cloudy. When precipitated with Ca and redissolved with KCl, it is always a suspension.

The isoelectric precipitation results in removing 10—15% of the protein and the activity of the preparation is raised sometimes by more than 50%. Whether this is due to the removal of inhibitors or some other factor could not be decided. An actin, purified through the Ca-salt, has the maximal activity and all of its protein is precipitated at pH 6 or by new addition of Ca.

As other purification procedures, like precipitation with alcohol or salting out with KCl did not lead to further purification and because all of the protein was precipitated by minimal Ca concentrations, we think that such preparations are homogeneous and do not contain any significant amount of impurities.

Actin is easily destroyed by heat over 50°. It is stable in a narrow pH zone between pH 7—7,5 but is rapidly destroyed even at 0° by more acid and alkaline reactions. It is precipitated from the solution at pH 6. Dialysis at pH 7 does not diminish its activity. From the solution it can be precipitated by cold alcohol but it is completely destroyed if treated with cold acetone. (This is the more interesting as it is very resistent to acetone when still in the muscle tissue.) If precipitated without loss of activity, the precipitate is voluminous. After centrifuging for 20 minutes at 3000 r. p. m. it still contains more than 99,5% of water.

Thixotropy. The most remarkable property of actin solutions is their very strong thixotropy. A dilute solution of neutral actin containing as little as 3—5 mg protein per ml sets

to a gel if left alone at 0° for a few hours. A slight shaking however breaks up the gel immediately. As common with thixotropic gels, the viscosity of actin solutions cannot be determined with accuracy. So much can be said only that it is roughly the same as that of myosin. The time of outflow is determined largely by the shearing forces present in the capillary viscosimeter. For this reason successive determinations of the time of outflow show strongly decreasing values. If the solution is left to stand for a while before the next determination, again a higher value of viscosity will be found. A quantitative study of these phenomena is planned in a system, in which the pressure can be taken into consideration.

Strictly speaking it would be better to use the term resistance (Strömungswiederstand) instead of viscosity. The resistance of a solution results from the true viscosity, which can be measured at high shearing forces and from the elasticity of the solution. That indeed elasticity plays an important part is shown by the fact that the viscosity increases if the time of outflow is longer, i. e. the shearing forces are smaller. The resistance of actin solutions decreases assymptotically with higher shearing forces. It is interesting to point out two parallelisms between actin and thixotropic inorganic sols. One is the permanent double refraction to be dealt with later. The other is the great influence, which inorganic salts exercise on the resistance (apparent viscosity) of actin solutions. It is known from the experiment of ***Freundlich***[4] and the theoretical treatment by *Szegvári*[5] that the elasticity but not the viscosity is influenced by the salt concentration.

Not only the viscosity of actin, but also the viscosity of actomyosin is influenced by the strong thixotropy of actin. Whereas myosin A solutions show no variation in viscosity during successive determinations, myosin B shows already such an effect, a second run in the viscosimeter gives always lower value than the first one. This results in some ambiguity in the determination of viscosities. The more active an actomyosin is, the less reliable is the determination of viscosity. Therefore we have accepted a certain routine, which would give comparable results. The actin solution was kept at 0° for at least 1 hour prior to use, then it was gently shaken up and the desired amount mixed with the myosin, the mixture imme-

diately put into the icebath and its viscosity determined 5 minutes later. Immediately thereafter two more readings were taken. After the addition of the ATP, two more determinations were made. For the calculation of the activity the first readings were always used. The others served only to see the extent of the thixotropic effect. The solutions are to some extent thixotropic even after the addition of ATP.

Whereas the difficulties caused by the thixotropic effect make the viscosity values uncertain, the determination of activity by this routine procedure can be carried out with satisfying reproducibility, except for actomyosins containing more actin than myosin.

A thixotropic effect with salt free myosin has been observed already by *Muralt* and *Edsall*.[6] It is clear that these authors dealt with a myosin A preparation, that is an actomyosin of a few per cent activity. This is shown by the very small dependence of the viscosity of their solutions on the shear rate. *Freundlich*[7] has suggested that the interior of the muscle cell is a thixotropic gel, as evidenced by the observation of *Kühne*.[8] Knowing that the myosin in the muscle fibril is not the myosin studied by *Muralt* and *Edsall*, but an actomyosin of about 170% activity, which is a rather strongly thixotropic substance, this assumption is now experimentally supported.

Thixotropic behaviour is an expression of certain properties of actomyosin, which might be of considerable significance in understanding the architecture and the mechanical properties of muscle.

If we assume that the most active actin, described in this paper, is the pure actin, it was calculated (see *Balenovič* and *Straub*) that 1 gramm of muscle contains 25—30 mg actin. It is impossible to make such a concentrated solution of actin, which requires a very close packing. Actin reveals by its thixotropy and permanent double refraction (see later) very strong intermolecular forces. At the close packing present in muscle, these intermolecular forces must be very strong and must play an important rôle in determining the mechanical properties of muscle.

High viscosity and thixotropy are two phenomena each pointing to the fact that actin has elongated, rod-shaped molecules of very great assymetry. Double refraction of flow is

another typical characteristic of actin solutions, bearing evidence to the same point. The sign of double refraction is identical with that of myosin. Myosin already has a strong double refraction of flow, which appears at very low shearing forces. But its double refraction is far weaker than that of actin solutions. A slight movement of the actin solution gives rise to the appearence of doubly refracting patches, which persist long after the fluid had ceased to move. If there is so much actin in solution that it turns into a thixotropic gel, the double refraction is permanent. It is interesting to remember that according to *Freundlich* and *Schalek,*[4] thixotropy is observed among those inorganic sols (like aged V_2O_5 sols) which show a permanent double refraction.

Of the physicochemical properties touched upon in this paper, viscosity and double refraction of flow show a parallelism with the actin content of the solution.

The process of activation.

If actin is mixed with myosin in solution, an actomyosin will be formed instantaneously. When in contact with muscle, myosin will be activated at 0^0 only in the course of many hours. It has been pointed out by *Banga* and *Szent-Györgyi*[1] that there is no activation at all, until the ATP of the muscle is split. That the disappearence of ATP is not the only factor involved in determining the rate of activation, is demonstrated by the following experiment. Rabbit's muscle is extracted at 0^0 several times in succession with *Weber*'s salt solution. By 3—4 extractions, practically all of the myosin is extracted. On adding an ATP-free myosin solution to the residue, which is by now likewise free of ATP, activation of myosin takes again nearly 24 hours at 0^0. The rate of activation can be, however, strongly increased by grinding the muscle residue with sand, prior to the incubation with myosin. In this case activation will be complete within several minutes. From these experiments it follows that the rate determining process of the activation in muscle is the extraction of the actin from the muscle residue.

In absence of myosin, the *Weber* solution will extract no actin from the muscle residue. The presence of myosin is

therefore essential for the extraction of actin. In absence of ATP, as shown by *Banga* and *Szent-Györgyi*,[1] neither myosin, nor actin are extracted.

To account for these phenomena, we must assume that the actomyosin present in the muscle, dissociates into its components in presence of ATP and the salts of the *Weber* solution. The myosin is dissolved but the actin is retained by the strong intermolecular forces. After the ATP has disappeared, the myosin already in solution forms a compound again with the actin. By forming this compound, the forces binding actin in its place are overcome and the dissolved myosin dissolves the actin.

The only other way, by which I succeeded to liberate actin is the acetone treatment described above. But even acetone is capable of breaking up the structure only if the latter has been loosened up by a prolonged treatment with the alkaline salt solution.

Actin is not extracted by the usual salt solutions which have been used to study the distribution of muscle proteins. Therefore it is clear that only a fraction of it goes into solution (this gives the few % activity of myosin A), whereas the greater bulk of it remains in the so called muscle stroma. This insoluble protein fraction has been estimated as 15—20% of the muscle proteins. We find (see *Balenovič* and *Straub*) that actin represents about 12—15% of the muscle protein. Making an allowance for some connective tissue and the nucleoproteids of the nuclei there is not much protein left unaccounted. We therefore find that there is no other protein left in the muscle stroma, to which the rôle of a structure-protein could be assigned. The possibility that actin is the main structure-protein, receives strong support by its properties, discussed above.

The formation of the myosin-ATP complex.

It has been shown by *Mommaerts*[9] that about one gramm molecule of ATP are needed to give a maximal effect with 100,000 gramm of myosin. From the experiments of the author[10] on myosin A at acid reaction, it became obvious that ATP is bound to the myosin part of the actomyosin complex. This

is supported also by the fact that myosin is the carrier of the adenyltriphosphatase activity.

On a 100% active actomyosin, prepared from myosin A and actin, I have reinvestigated this problem. As *Mommaerts'* experiments were performed at the alkaline reaction of the *Weber* solution, it was important to know the conditions at physiological pH. The viscosities have been determined at pH 7,0 in a solution containing 0,6 M K ions and veronal acetate buffer. The decrease of viscosity on addition of ATP was

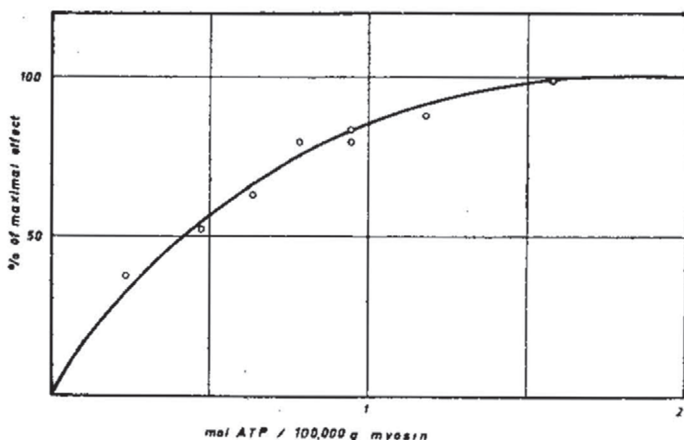

Fig. 4.

measured and the results are expressed as % maximal effect and plotted against the amount of ATP in Fig. 4. 0,1 ml ATP solution was added in each case to the 6 mg of actomyosin present in 4 ml salt solution. From four successive determinations after the addition of ATP, the viscosity of the solution at the moment of adding the ATP was determined by linear extrapolation. It is evident, from the nature of the curve that the bond between myosin and ATP is very strong, less than 2 g molecules of ATP being needed for 100,000 g of myosin B to give a maximal effect. There is some dissociation of the myosin-ATP complex, but the results are essentially in agreement with the assumption that 1 g molecule of ATP reacts with 100,000 weight of myosin.

It is quite convenient to work with 6 mg of myosin in the 4 ml of the solution placed in the viscosimeter. In this case about 0,015 mg ATP would give a 50% effect. In our experi-

ments such an amount of ATP caused the viscosity of the myosin to decrease from 1,83 to 1,61. Such a change in viscosity can be determined with satisfactory precision. Considering the absolute specificity of ATP in decreasing the viscosity of myosin, these facts may be utilized for determining very small amounts of ATP.

Summary.

A method for extracting a new protein, actin, from muscle tissue is described. Actin forms, together with myosin, actomyosin, the contractile protein of muscle. The most conspicuous properties of actin are its high viscosity, thixotropy and strong double refraction, all proving a great molecular assymetry.

The antagonistic effect of Ca and K ions on actin is described.

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Über das Aktomyosin des Kaninchenmuskels.

von

K. Balenovic und F. B. Straub.

Aktomyosin ist ein Komplex von Myosin und Aktin. BANGA und SZENT-GYÖRGYI[1] haben einen solchen Komplex aus Kaninchenmuskulatur erhalten, den sie Myosin B benannten. Es wurde von STRAUB[2] bewiesen, dass dieser Komplex aus Myosin und Aktin besteht. Nach den Angaben der vorangehenden Arbeit können durch Mischen von Myosin mit verschiedenen Mengen von Aktin Aktomyosine erhalten werden, die durch verschieden grosse Viskositätsänderungen auf Zusatz von Adenyltriphosphat (ATP) reagieren. Die Grösse dieser Viskositätserniedrigung wurde als Mass der „Aktivität" eines Aktomyosins definiert. Die Aktivität von Myosin B, die bei den verschiedenen Präparaten konstant ist, wurde willkürlich 100% Aktivität genannt. STRAUB beobachtete, dass auch Aktomyosine von mehr als 100% Aktivität entstehen können, wenn man mehr Aktin dem Myosin zusetzt.

Wir haben gefunden, dass in einigen Fällen bei der Darstellung des Aktins aus dem Muskel soviel Aktin ausgelöst werden kann, dass dieses das im selben Muskel vorhandenen Myosin mehr als 100% zu aktivieren vermag. Dadurch müssten wir die Möglichkeit in Betracht ziehen, dass das im Muskel vorhandene Aktomyosin, also das eigentliche Kontraktionsprotein, aktiver ist als Myosin B. In dieser Arbeit wird gezeigt, dass das Aktomyosin des Kaninchenmuskels wahrscheinlich 170% aktiv ist und enthält 1 mg Aktin pro 3 mg Myosin. Ist dem aber so, dann müsste eine weitere Frage beantwortet werden: warum erhält man bei der Extraktion des Muskels mit der WEBER-Lösung ein Aktomyosin (Myosin B) das im Muskel nicht vorkommt?

Da Aktin in saurer oder alkalischer Lösung rasch zer-

stört wird, schien es zweckmässig die Viskositätsmessungen nicht in WEBER-Lösung, sondern bei pH 7 auszuführen. Deshalb haben wir die Viskositätsbestimmungen von BANGA und SZENT-GYÖRGYI,[1] die in WEBER-Lösung ausgeführt wurden, wiederholt. Dieselben Kapillarviscosimeter, die in der vorangehenden Arbeit beschrieben wurden, dienten zu diesen Messungen. Myosin wurde in einer 0,6 m KCl Lösung gelöst, die aber durch Veronal-Azetat auf pH 7,0 gepuffert war. Diese Lösung wurde folgenderweise bereitet: zu 200 ccm der Veronal-Azetat Mischung nach MICHAELIS (die aber Kalium an Stelle von Natrium enthält) wurden 24 ccm n-HCl zugesetzt, dann 271 ccm einer 2 m KCl Lösung und auf 1 Liter aufgefüllt. Die K Ionenkonzentration war dann 0,6 m.

Myosin A und B wurden folgenderweise hergestellt: Kaninchenmuskulatur wurde durch einer eisgekühlten Latapie-Mühle gemahlen und bei 0° mit 3 Volumen WEBER-Lösung unter mechanischen Rühren 20 Minuten lang extrahiert. Zentrifugiert man die Mischung nach dieser Zeit, so erhält man eine Myosin A Lösung. Wird die Mischung nach dem Extrahieren 24 Stunden lang bei 0° stehen gelassen, mit dem gleichen Volumen WEBER-Lösung verdünnt und dann zentrifugiert, so bekommt man eine Lösung von Myosin B. Solche Lösungen wurden durch vorsichtige Zugabe von einer 2 m Azetatpuffer-Lösung auf pH 7 neutralisiert und mit der oben beschriebenen gepufferten KCl Lösung von pH 7 entsprechend verdünnt.

Der Myosingehalt der Myosin B Lösungen wurde in der von BANGA und SZENT-GYÖRGYI angegebenen Weise bestimmt. Bei der Bestimmung des Myosingehaltes von Myosin A Lösungen haben wir aber beobachtet, dass die Trockengewichtsbestimmung in der von BANGA und SZENT-GYÖRGYI angegebenen Weise schwankende Ergebnisse gibt. Bei der Neutralisierung der verdünnten Myosinlösung kann nämlich das pH schwer auf pH 7 eingestellt werden. Wir haben versucht, diese Trockengewichtsbestimmung zu standardisieren. Gibt man zu je 1 ccm einer Myosin A Lösung (in der WEBER-Lösung gelöst) 5 ccm 0,1 m Azetatpufferlösungen von verschiedenen pH, so erhält man Niederschläge von Myosin, deren Menge mit dem pH variiert und einen maximalen Wert bei Zugabe einer pH 4,8 Azetatpufferlösung erreicht. Das pH der Lösung ist

wegen der Alkalinität der Myosinlösung etwas höher. 5,2. Tabelle 1 zeigt die Ergebnisse dieser Versuche. Myosin I ist ein gereinigtes Myosin, Myosin II ein ungereinigtes Myosin, beide in WEBER-Lösung gelöst.

Tabelle I.

pH der zugegebenen Puffers	Trockengewicht mg/ccm Myosin I.	Trockengewicht mg/ccm Myosin II.
3,6	0,7	1.3
4,0	1,1	3.7
4,4	8,0	21,9
4,8	9,1	22,5
5,2	8,4	19,0
5,6	8,6	17,4

Wir haben deshalb die Myosin A Trockengewichte derart bestimmt, dass wir immer 1 ccm des sich in WEBER-Lösung befindenden Myosins mit 0,5 ccm 0,1 m Azetatpuffer von pH 4,8 versetzten. Der Niederschlag wurde auf der Zentrifuge zweimal mit Wasser gewaschen, bei 105° getrocknet und gewogen.

Fig. 1. zeigt die Ergebnisse unserer Viskositätsmessungen. Im Wesentlichen erhält man dasselbe Bild, wie in der Arbeit von BANGA und SZENT-GYÖRGYI. Es gibt nur den Unterschied, dass wir die Viskosität von Myosin A und B in Gegenwart von ATP gleich gefunden haben.

Versuchsvolum in dem Viskosimeter war 4,0 ccm, dazu wurden 0,1 ccm einer 1,4%-ger ATP-Lösung gegeben. Temperatur: 0°.

In die Figur wurde die Viskosität von Myosin A ohne Zugabe von ATP nicht eingezeichnet. Dieser Wert variiert. Wir hatten Präparate die keine Aktivität besassen (keine Viskositätsänderung auf Zugabe von ATP) und andere die 20% aktiv waren. Meistens findet man aber eine Aktivität von 15% bei Myosin A. Aus verschiedenen Gründen nehmen wir an, dass diese Aktivität durch ein wenig Aktin hervorgerufen wurde, das als Verunreinigung in Myosin A Präparaten vorkommt. Reines Myosin hat demgemäss keine Aktivität.

Den Zusammenhang zwischen Viskosität von Myosin B in Gegenwart von ATP und die durch ATP hervorgerufene Viskositätserniedrigung stellt Fig. 1. der voranstehenden Ar-

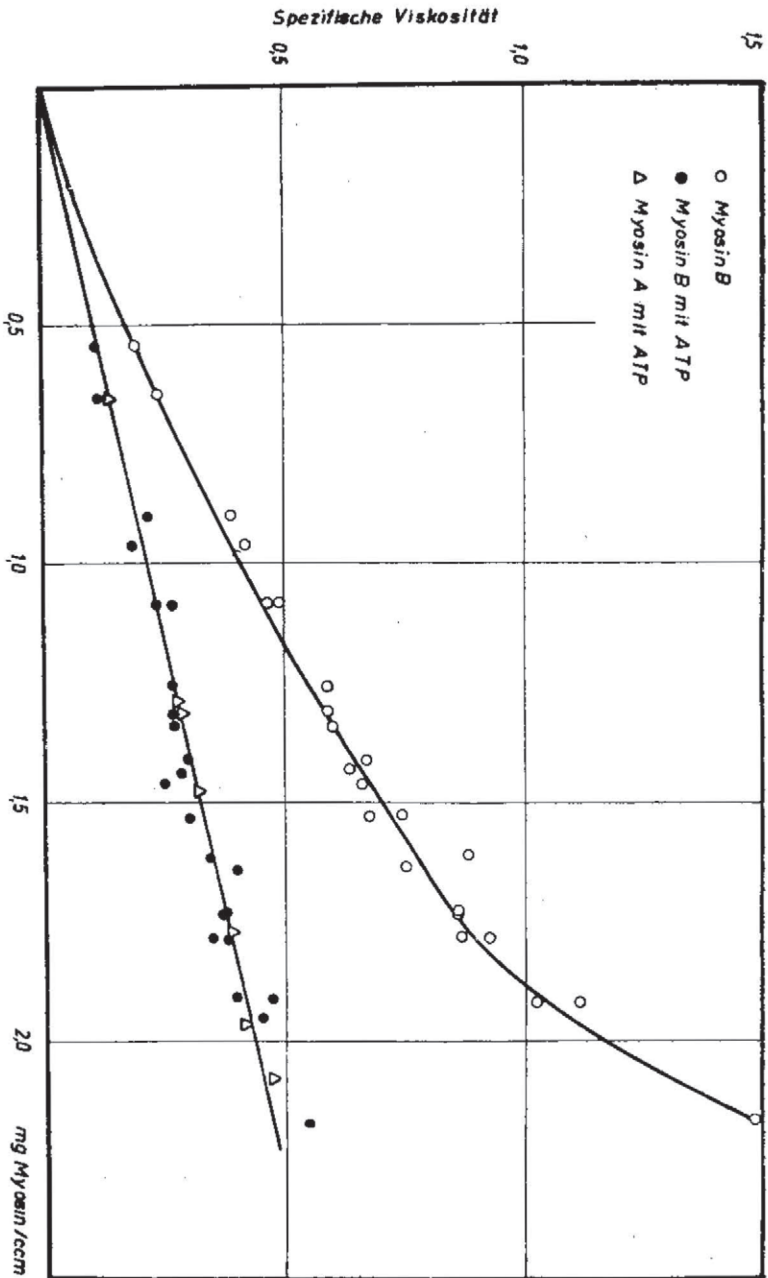

Fig. 1.

beit dar. Auf diesem Grunde wurde die Methode der Auswertung der Aktivität eines unbekannten Aktomyosins ausgearbeitet. (Näheres s. bei STRAUB[2].)

Bestimmt man die Aktivität eines Myosin B Präparates, das nach der oben beschriebenen Methode dargestellt wurde, so findet man innerhalb der Fehlengrenzen der Methode immer eine 100% Aktivität. Wurde das Myosin mit dem Muskel nicht 24 sondern 48 Stunden lang stehen gelassen, dann verdünnt und zentrifugiert, so findet man meistens eine 100%, manchmal aber eine kleinere Aktivität. Das Myosin, das nach drei Tagen vom Muskel abgetrennt wird, ist schon immer weniger als 100% aktiv, z. B. in einem Falle nur 60%. Abgesehen von dieser Aenderung der Aktivität nach längerem Stehen, ist es klar, dass in erster Linie nicht eine Zeitfaktor in der Entstehung von Myosin B eine Rolle spielt. Myosin B, wenigstens nach der beschriebenen Methode dargestellt, ist ein wohl definiertes Produkt und reagiert bei Zugabe von ATP mit einer konstanten Viskositätserniedrigung.

B e s t i m m u n g d e s A k t i n s i m K a n i n c h e n m u s k e l. Bei der Darstellung des Aktins wird die Muskelsubstanz zuerst bei alkalischer Reaktion gehalten und dann mit Azeton behandelt. Beide Operationen können zu unkontrollierbaren Verlusten führen. Deshalb kann man aus Ausbeute bei der Isolierung auf die Menge des Aktins keinen Rückschluss ziehen. Mit der Methode von STRAUB wird durchschnittlich soviel Aktin aus dem Muskel isoliert, wie zur 100% Aktivierung von 30—60% des im Muskel vorhandenen Myosins nötig wäre.

Folgendes Verfahren ist aber für die Bestimmung des im Muskel gebundenen Aktins geeignet: man bestimmt die Menge an Myosin, die durch 1 g Muskel 100% aktiviert werden kann.

Kaninchenmuskel wurde wie üblich mit 3 Volumen WEBER-Lösung 20 Minuten lang extrahiert. Die Mischung wurde zentrifugiert und das Myosin A vom Muskelrückstand abgetrennt. Aus 150 g Muskel (Frischgewicht) erhielten wir somit 340 ccm von Myosin A Lösung und 240 g Rückstand. Die Lösung enthielt 22,5 mg/ccm Myosin.

Nun wurde die Hälfte des Rückstandes (der das Aktin enthält) mit der gesamten Menge Myosin zusammengebracht,

24 Stunden lang bei 0° stehen gelassen, dann mit WEBER Lösung verdünnt und zentrifugiert. Die Aktivität der überstehenden Myosinlösung wurde aus dem ATP Effekt viskosimetrisch bestimmt. Solche Versuche wurden auch derart eingestellt, dass 20, 25, 30, 33, 40, 100 und 200% des Rückstandes mit der gesamten Myosinlösung gemischt wurden. In jedem Falle wurde die Aktivität nach 24 Stunden bestimmt. Die Ergebnisse solcher Experimenten mit verschiedenen Muskelpräparaten sind in der Fig. 2. zusammengestellt. Man sieht, dass ungefähr $^1/_3$ des Muskelrückstandes genügt, um die ganze Myosinlösung 100% zu aktivieren. Ist mehr Rückstand, also mehr Aktin vorhan-

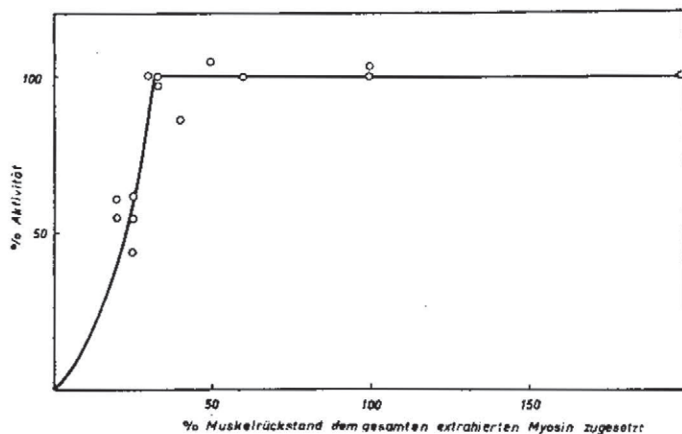

Fig. 2.

den, so tritt keine weitere Aktivierung ein. Dies ist im Gegensatz zur Aktivierung bei pH 7 mit gelöstem Aktin wo die Aktivierung bei weiterem Zusatz von Aktin bis 170% weitergeht.

Bei der zahlenmässigen Auswertung dieser Ergebnisse darf man nicht ausser Achtung lassen, dass ein Teil des Myosins durch die einfache Extraktion mit 3 Volumen WEBER-Lösung im Rückstand zurückbleibt. Die Schwankungen in der Menge des extrahierten Myosins sind für die Streuung der Punkte in Fig. 2. verantwortlich. Ist M die Menge des Myosins pro g Muskel und werden davon M' pro g extrahiert, so bleibt im Rückstand $M—M'$ pro g. Bekommt man mit 33% des Rückstandes noch eben eine 100% aktive Myosinlösung, so bedeutet das, dass das Aktin von 0,33 g Muskel 0,33 (M—M') + M' Myosin 100% aktivierte. In einem unserer Versuche aktivier-

ten 33% des Rückstandes noch eben zu 100%. *M'* war 51 mg/g, *M* ist bekanntlich 80 mg/g. Das Aktin des 0,33 g Muskels aktivierte also

$$0{,}33\ (80-51) + 51 = 60 \text{ mg Myosin},$$

1 g Muskel dreimal soviel, also 180 mg. Nach den Angaben von STRAUB braucht man 1 mg Aktin um 6 mg Myosin 100% zu aktiveren. Daraus folgt, dass im Muskel 180/6 also 30 mg Aktin pro g (Frischgewicht) gibt. In ähnlichen Versuchen fanden wir den Wert 26—30 mg/g Muskel. Zu einer 100% Aktivierung, also zur Entstehung von Myosin B wäre die Hälfte dieser Menge nötig. Nach den Angaben von STRAUB[2] bekommt man ein 170% aktives Myosin, wenn man Myosin und Aktin in der Proportion, wie sie im Kaninchenmuskel vorkommen, bei pH 7 zusammensetzt. Das ist eben der maximale Wert der Aktivität, der bei pH 7 erreicht werden kann. Demgemäss können wir sagen, dass sich im Muskel nicht ein 100% aktives Aktomyosin (Myosin B) sondern ein maximal aktives Aktomyosin mit 170% Aktivität befindet.*

Es frägt sich nun, warum man aus dem Muskel durch Extraktion mit WEBER-Lösung das 100% aktive Myosin B an Stelle des natürlichen 170% aktiven Aktomyosins erhält. Aus den in dieser Arbeit mitgeteilten Versuchen geht hervor, dass wenn auch das in WEBER-Lösung gelöste Myosin mehrere Tage lang mit dem Muskel in Berührung steht, keine Aktivierung über 100% stattfindet. Folgender Versuch zeigt, dass dabei freies Aktin im Muskel zurückgeblieben ist. Es wurde wie üblich Myosin B bereitet und vom Muskelrückstand abgetrennt. Der so erhaltene Muskelrückstand wurde nun mit soviel Myosin A versetzt, wie ursprünglich im Muskel vorhanden war. In 24 Stunden wurde auch dieses Myosin zu 100% aktiviert.

Myosin B bekommt man demgemäss darum, weil das Myosin unter den Bedingungen der WEBER'schen Lösung nur soviel Aktin aus dem Muskel extrahieren kann, das eben zur 100% Aktivierung nötig ist. Wie in der vorangehenden Arbeit erwähnt, ist Aktin im Muskel festgebunden, seine Auslösung geschieht in der Weise, dass es zu dem in Lösung vorhande-

* Fäden, die aus Myosin B und aus künstlich hergestelltem 160% aktivem Myosin ausgezogen wurden, kontrahierten in Gegenwart von K, Mg und ATP in annähernd gleicher Weise.

nen Myosin fester gebunden wird, als zum Muskelrückstand. Es ist leicht möglich, dass die Bindung zwischen Myosin und Aktin nur bis zu 100% Aktivität fest genug ist, um das Aktin aus seinem Verbande im Muskel loszulösen. Gibt man noch *gelöstes* Aktin zu einem 100% aktiven Aktomyosin, so entsteht ein aktiveres Aktomyosin.

Zusammenfassung.

1. Kaninchenmuskel enthält ungefähr 25—30 mg Aktin pro Gramm Frischgewicht. 12—15% des Gesamtproteins ist also Aktin. Diese Menge des Aktins vermag das anwesende Myosin maximal zu aktivieren (170% Aktivität).

2. Durch die alkalische Salzlösung von WEBER wird ein minder aktives (100%) Aktomyosin extrahiert.

Litteratur.

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The reversibility of the contraction of myosin threads.

by

A. Szent-Györgyi.

It has been shown in a previous paper (1) that threads of myosin B show a violent contraction if suspended in a solution containing KCl (0,05 M), $MgCl_2$ (0,001 M) and ATP (Adenyltriphosphate 0,17%). If such a contracted thread is washed out with water and supended in a solution containing 0,25 M KCl and 0,001 M $MgCl_2$ no appreciable change is observed. If ATP is added now to the solution the thread swells up within a few minutes to its original size; it becomes transparent and similar to the original uncontracted thread in all respects. If the liquid is replaced by the salt solution in which the contraction was obtained, the thread contracts again. The contraction is thus reversible and ATP is essential not only for the contraction but also for the relaxation. The thread can be brought to contraction and relaxation by the variation of the KCl concentration

Mg is essential for the contraction as well as for the relaxation. In absence of Mg the contraction is sluggish and there is no relaxation at all. Only a very slight swelling is obtained as revealed by the somewhat increased transparency. At higher KCl concentrations (in presence of ATP) the thread desintegrates without much swelling.

The Mg can be replaced by a dialysed extract of the muscle. Whether this action is due to the traces of Mg, possibly bound by the protein, or by some other substance, cannot be stated at present.

In presence of 0,1% quinine the same contraction and relaxation is obtained as in absence of this substance. As shown by I. BANGA this alcaloid greatly inhibits the phosphatase action of myosin. Both the contraction and relaxation can thus take place without the splitting of the ATP.

The same experiment were also repeated with threads prepared from 160% active myosin (see Straub's paper) prepared from myosin A and Actin. The results were similar to those obtained with myosin B with the one difference that in presence of ATP these threads dissolve at a lower KCl concentration. While a myosin B thread contracts still in a 0,15 M KCl solution (in presence of ATP and 0,001 M $MgCl_2$), is inactive in 0,2 M KCl and dissolves in 0,25 M KCl, the thread prepared from the 160% active myosin contracts in 0,05 M KCl, is inactive in 0,1 M KCl and dissolves in 0,15 KCl, is thus more sensitive to the action of this salt. As shown by STRAUB and BALENOVIČ, the myosin of the muscle is about 170% active.

The effect of ATP depends thus on the concentration of the KCl present. But ATP added in form of its K salt may increase the salt concentration sufficiently to convert its own action from a contracting to a dissolving one. This may be illustrated by the following experment: Threads, prepared from 160% myosin were suspended in 0,35 ml. of a 0,05 M KCl solution containing 0,001 M $MgCl_2$. Then 0,05 ml. of an ATP solution were added, containing 1,4% ATP, 0,05 KCl and 0,001 $MgCl_2$. A slow contraction is obtained. In a second experiment the threads were suspended in 0,3 ml of the KCl $MgCl_2$ solution and 0,1 ml of ATP was added. The threads dissolved. The concentration of the ATP thus determines its own effect, whether it will cause contraction or relaxation.

The reversibility of the contraction of myosin threads was also demonstrated by M. GERENDÁS and D. VARSÁNYI in a very neat way. (Oral communication). T. ERDÖS has shown that ATP, in presence of lower concentrations of KCl (+ Mg) causes a contraction of the threads while in presence of higher concentration it causes a dissolution. Between the two ranges of concentration there is a range in which the thread neither contracts, - nor dissolves: is inactive. GERENDÁS and VARSÁNYI have shown that such an inactive thread can be brought to a rapid contraction by the simple dilution of the solvent or by the addition of Ca-permutit which binds the K.

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Über die Fibrinogen-Fibrinumwandlung.

von

K. Laki.

(Arbeit aus den Mitteln des fürstl. Esterházyschen Stipendiums.)

Vorliegende Arbeit bezweckt das Studium des zweiten Abschnittes der Blutgerinnung, der Umwandlung des Fibrinogens in Fibrin. Vor allem erwies es sich als erforderlich das Fibrinogen in möglichts reiner Form, frei von sonstigen Proteinen darzustellen und zu untersuchen welche Veränderungen durch die Einwirkung von Thrombin zustande kommen.

Isolierung des Fibrinogens.

Für die Reindarstellung des Fibrinogens verwandte ich das Oxalatplasma von Schweineblut.

Oxalatplasma. Das Gemisch von 200 ml 0,7%iger Kochsalz- und 100 ml 2%iger Na-Oxalatlösung wurde auf dem Schlachthofe unter mässigem Rühren mit Schweineblut auf 1 l aufgefüllt. Im Laboratorium wurde das Blut im Kühlraum (0° C) abzentrifugiert. Ich erhielt 500 ml Plasma.

Fällung durch Ammoniumsulphat. Dem kalten Plasma wurde 125 ml gesättigter Ammoniumsulphatlösung in kleinen Portionen zugefügt (0,20 Sättigung). Der Niederschlag wurde in der Kälte abzentrifugiert, sodann in dem Gemische von 90 ml 0,7 %iger NaCl- und 10 ml 2%iger Na-Oxalatlösung aufgelöst. Der kuchenartige Niederschlag löst sich langsam bei andauerndem Digerieren. Die erhaltene trübe Lösung wird durch Zentrifugieren geklärt.

Tricalciumphosphatadsorption. 100 ml 10 %iger Trinatriumphosphatlösung wurden 100 ml 10%iger $CaCl_2$-Lösung zugefügt. Der enstandene Niederschlag wurde abzentrifugiert, sodann in der Zentrifuge mit dest. Wasser

zweimal ausgewaschen, schliesslich in 0,7%iger NaCl-Lösung so suspendiert, dass das Gesamtvolumen 150 ml betrug. Das Fibrinogen wurde aus seiner Lösung in NaCl an 25 ml frisch bereitetem Tricalciumphosphatgel adsorbiert. Nach dem Hinzufügen des Phosphatgels zentrifugiert man die Lösung. Das das Fibrinogen mit sich führende Phosphatgel wurde mit 50 ml 0,7%iger NaCl-Lösung in der Zentrifuge gewaschen, das Fibrinogen mittels 80 ml eines Gemisches von gleichen Teilen M/5 primären und sekundärem Phosphats eluiert.

Zweite Fällung durch Ammoniumsulphat. Zwecks weiterer Reinigung wurde das Fibrinogen aus dieser Phosphatlösung mit einer 1/4 Vol. gesättigter Ammoniumsulphatlösung (0,20 Sättigung) aufs Neue gefällt, sodann wurde der Niederschlag nach Abzentrifugieren in 30 ml des oben erwänten Phosphatpuffers unter andauerndem mässigem Digerieren aufgelöst. In der Regel erhielt ich eine wasserklare Lösung.

Die quantitativen Daten der Isolierung werden durch folgende Tabelle veranschaulicht.

	Fibrinogen mg in 1 ml	Fibrinogen %	Ausbeute an Fibrinogen
Oxalatplasma	3,6	10	1,800 g
Nach der ersten Ammoniumsulphatfällung	12,5	50	1,250 g
Nach der Ca-Phosphatadsorption	8,2	88,6	0,660 g
Nach der zweiten Ammoniumsulphatfällung	12,6	96,5	0,380 g

Bemerkt sei, dass von den Angaben dieser Tabelle oft auch grössere Abweichungen möglich sind, indem bereits die erste NaCl-Lösung nicht nur 50, sondern auch 65% Fibrinogen enthalten kann. Durch die Adsorption und Eluation vermag die Reinheit in der Regel 88—90% zu erreichen, wogegen dieselbe im letzten Schritte im allgemeinen 96—98% beträgt. Erwähnenswert ist, dass das Tricalciumphosphatgel unter den besprochenen Umständen aus der NaCl-Lösung 80% des Fibrinogens adsorbiert, wobei der Reinheitsgrad auf der Oberfläche des Adsorbens 80% ist.

Kristallisierung. Giesst man 10 ml der im letzten Schritte gewonnenen Lösung in die 10fache Menge dest. Wassers von 38—40° C, dann scheidet sich das Fibrinogen während des Abkühlens im Eisschranke in der Form eines schneeweissen flockigen Niederschlags ab, der unter dem Mikroskope aus

nadelförmigen Kristallen besteht.[1] Nach dem Abzentrifugieren löst sich der Niederschlag bei gelindem Erwärmen (38° C) in 8 ml Phosphatpuffer obiger Zusammensetzung langsam auf. Beim Impfen mit Thrombin gerinnt die Lösung. 1 ml dieser Lösung enthält 7,4 mg Fibrinogen und 7,4 mg Gesamteiweiss, d. h. das in der Lösung befindliche Fibrinogen hat sich vollständig in Fibrin umgewandelt. Diese Lösung ist auf die vorhergehende Weise aufs Neue kristallisierbar, die neuen Kristalle sind indessen schwerer löslich, was darauf hinweist, dass das Fibrinogen sich einigermassen verändert hat.

Fibrinogenbestimmung.

Wir bestimmten das Fibrinogen durch Wägen des auf Einwirkung von Thrombin entstandenen Fibrins. Zu diesem Zwecke wird die zu untersuchende Lösung in ein abgewogenes Zentrifugenröhrchen (65 × 11 mm) gefüllt und durch Hinzufügen von Phosphatpuffer und Wasser auf das entsprechende pH und die nötige Salzkonzentration gebracht. Dann wird dem Gemisch Thrombinlösung zusetzt (Lösung A). Binnen einigen Minuten vollzieht sich die Umwandlung des Fibrinogens. Das nach weiteren 10—15 Minuten entstandene Fibrin ist mittels Spatel von der Rörchenwand leicht ablösbar und zu einer flachen Masse knetbar. Nach mehrmaligem gründlichen Waschen mit NaCl-Lösung und danach mit Wasser trocknet man das Fibrin in den Röhrchen und wägt. Bei der Bestimmung des Fibrinogengehalts des Oxalatplasmas wird zwecks Erreichung des entsprechenden pH 1 ml Plasma 1 ml M/5 primäres K-Phosphat und 1 ml Wasser zugesetzt. Im Falle der Fibrinogenlösung in NaCl fügt man 1 ml Lösung 1 ml M/5 Phosphatpuffer (primär : sekundär = 3 : 1) und Wasser zu. Den Lösungen, in denen das Fibrinogen sich in einem Phosphatpuffer befindet, wird den zu bestimmenden 1 ml Fibrinogenlösung primäres Phosphat und 1 ml Wasser zugegeben. Bei solchen Zusammenstellungen löst sich das Fibrin von der Gefässchenwand leicht ab und kann mit einer Spatel zu einer flachen Masse gedrückt werden. Die Daten der Tabelle zeigen die auf diese Weise entstandenen und gewogenen Fibrinmengen. Das in den Lösungen befindliche Gesamteiweiss wird mittels warmer Trichloressigsäure ausgefällt und sein Gewicht

nach gründlichem Waschen des Niederschlags mit Wasser und darauffolgendem Trocknen in den tarierten Rörchen bestimmt.

Thrombinherstellung.

Das für die Umwandlung von Fibrinogen in Fibrin verwandte Thrombin wurde analog der oben besprochenen Schweineplasmaherstellung aus Rinderoxalatplasma gewonnen. Bei der Thrombinherstellung verfuhr ich im wesentlichen nach der Methode von Astrup.[2] Ich goss 1 l Plasma in die 10fache Menge angesäuerten dest. Wassers; der Niederschlag setzte sich bei 0° C über Nacht ab. Nach dem Zentrifugieren löste ich diesen in calciumfreier Ringerscher Lösung (300 ml) auf und liess ihn nach Hinzufügung von $CaCl_2$ und Hirnextrakt (s. Astrup) gerinnen. Nach Auspressen und Entfernung des entstandenen Fibrins diente die auf diese Weise gewonnene Lösung bei einem grossen Teil der Versuche als Thrombinlösung (Lösung A). Wird in einer solchen Thrombinlösung mittels 2 Vol. Aceton bei Zimmertemperatur ein Niederschlag erzeugt und dieser abzentrifugiert, sodann aus diesem mit 80 ml einer 0,7%iger NaCl-Lösung das Thrombin herausgelöst, so kann eine etwa 10fache Reinigung erzielt werden. Dialysiert man die auf solche Art gewonnene gereinigte Lösung gegen dest. Wasser, dann entsteht ein Niederschlag, der sich in einigen ml Ringerscher Lösung gut löst und starke Thrombinaktivität aufweist (Lösung B), Fibrinogen jedoch nicht allein zum Gerinnen bringt, vielmehr solches auch auflöst.

Kinetik der Thrombinwirkung.

Zeitlicher Verlauf der Reaktion. Mittels Zugabe von Kaliumpermanganat kann die Fibrinogenumwandlung zu jedem Zeitpunkte zum Stillstand gebracht werden. Das bis dahin enstandene Fibrin kann auf die oben beschriebene Weise von dem unveränderten Fibrinogen getrennt und getrocknet abgewogen werden. An Hand dieses einfachen Verfahrens kann auch der zeitliche Verlauf der Reaktion verfolgt werden. Nachstehender Versuch bezweckt eine solche Untersuchung: In abgewogene Zentrifugenrörchen füllte ich 2 ml Fibrinogenlösung (enthaltend 9,2 mg Fibrinogen) und 1 ml dest. Wasser. Nach

dem Hinzufügen und Vermischen mit 0,3 ml 3fach verdünnter Thrombinlösung (Lösung A) stellte ich mittels 0,5 ml N/10 Kaliumpermanganatlösung die Reaktion zu verschiedenen Zeitpunkten ab. Nach dem Zusetzen des Permanganats drückte ich das entstandene Fibrin zusammen, währenddessen sich das Permanganat mit der Flüssigkeit vermischt, und die Reaktion zum Stillstand kam. Die Flüssigkeit wurde vom Fibrin abpipettiert, das Fibrin auf der bereits erwähnte Weise gewaschen, getrocknet und gewogen. Das Abstellen der Reaktion ist

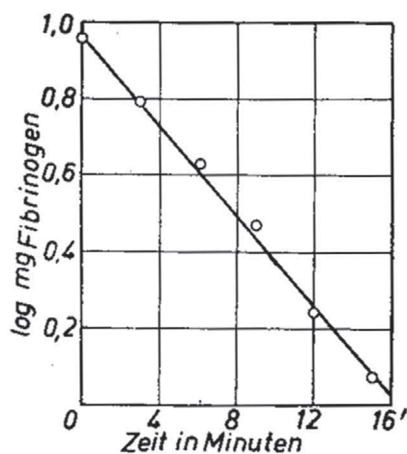


Abb. 1.

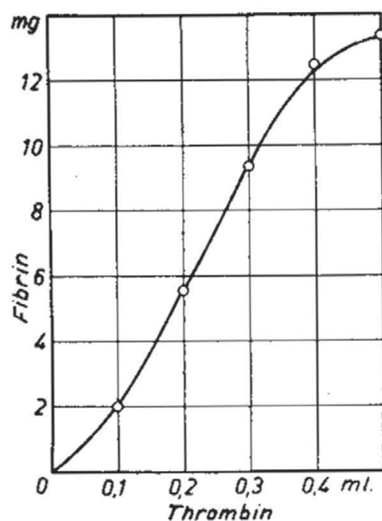


Abb. 2.

auf diese Weise binnen 15—20 Sekunden durchführbar. Abb. 1 zeigt das Versuchsergebnis: auf die Ordinate sind die Logarithmen der nicht umgewandelten Fibrinogenmengen in mg aufgetragen, während die Abszisse die bis zum Abstellen mittels Permanganat verstrichene Reaktionszeit angibt. Wie auf der Abbildung ersichtlich, scheint die Fbrinogenumwandlung, oder zumindest die Fibrinausscheidung — wie die Eiweissdenaturierungsreaktionen im allgemeinen — den monomolekularen Reaktionsstypus zu befolgen.

E i n f l u s s d e r T h r o m b i n m e n g e a u f d i e R e a k t i o n. Abb. 2 zeigt, wieviel Fibrin von verschiedenen Thrombinmengen aus der Fibrinogenlösung in gleichen Zeiten hergestellt werden. Der Versuch war in folgender Weise zusammengestellt. Ich beschickte auf die vorhergehende Weise kleine Zent-

rifugierröhrchen mit 1 ml Fibrinogenlösung (enthaltend 16,6 mg Fibrinogen), 1 ml M/5 primärem Phosphat und 1,5 ml dest. Wasser und mit wechselnden Mengen Thrombin; in das eine Röhrchen kam 0,5 ml Thrombinlösung (Lösung A), in das andre 0,4 ml Thrombinlösung + 0,1 ml Ringersche Lösung usw. Nach dem Hinzufügen der Thrombinlösurg stellte ich die Reaktion nach 13 minuten mittels 1 ml der beschriebenen Permanganatlösung ab. Auf der Abbildung stellt die Ordinate das entstandene Fibrin in mg, die Abszisse die Thrombinmengen dar. Die ergebnisse zeigen, dass die entstandene Fibrin-

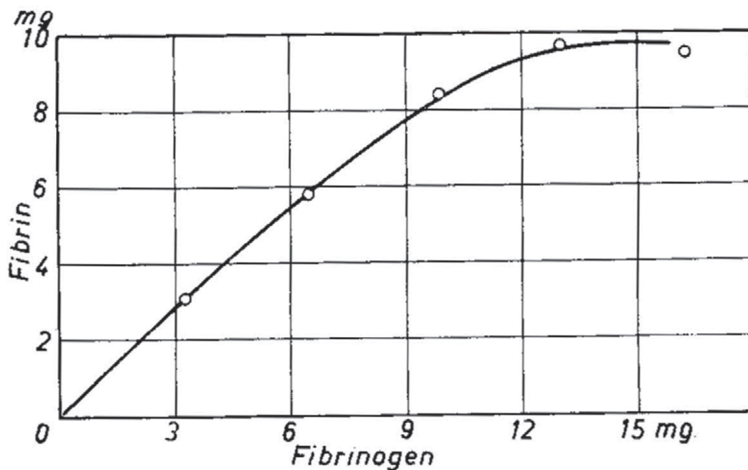


Abb. 3.

menge bis zu einer gewissen Grenze in geradem Verhältnis zu der zugefügten Thrombinmenge steht.

Einfluss der Fibrinogenmenge auf die Reaktion. Ähnlich dem vorhergehenden Versuche zeigt Abb. 3 wieviel Fibrin aus verschiedenen Fibrinogenmengen auf die Wirkung gleicher Thrombinmengen hin entsteht. Ich pipettierte in kleine Zentrifugierröhrchen 1 ml (enthaltend 16,3 mg Fibrinogen) und 0,8, 0,6, 0,4 ml Fibrinogenlösung und füllte diese in allen Gefässen mit Phosphatpufferlösung auf 1 ml auf. Nach Hinzufügen von 1 ml M/5 primären Phosphat und 1 ml dest. Wasser wurde dem Gemisch 0,2 ml Thrombinlösung (Lösung A) zugesetzt. Jedes Röhrchen wurde 5 Minuten nach dem Beimischen von Thrombinlösung mittels Permanganat versetzt.

Auf der Abbildung zeigt die Ordinate das entstandene Fibrin in mg, die Abszisse die Fibrinogenausgangsmengen in mg.

Alle diese Versusche weisen auf das enzymartige Verhalten des Thrombins hin, allein die endgültige Entscheidung der Frage ist ohne Isolierung des Thrombins nicht möglich.

Fibrinolyse. Die fibrinlösende Wirkung von Thrombinpräparaten wird von nachstehendem Versuche veranschaulicht. Ein kleines Zentrifugierröhrchen wird mit 0,3 ml Fibrinogenlösung (4 mg Fibrinogen enthaltend), 0,3 ml M/5 primären Phosphat, 0,6 ml dest. Wasser und 0,5 ml gereinigter und konzentrierter Thrombinlösung (Lösung B) beschickt. Das Fibrinogen gerinnt binnen einigen Sekunden. Setzt man nunmehr dieses, das geronnene Fibrinogen enthaltende Röhrchen in ein Wasserbad von 45° C, dann löst sich das Fibrin binnen 1—1½ Stunden vollständig auf. Das gelöste Fibrin koaguliert nach dem Zusetzen weitern Thrombins nicht aufs Neue. Scheinbar ist diese lösende Wirkung des Thrombins ganz spezifisch für Fibrin, denn bringt man die Fibrinogenlösung obiger Zusammensetzung durch Wärme zur Gerinnung, dann löst sich das gefällte Fibrinogen durch Thrombin nicht auf. In gleicher Weise bleibt auch das durch Trichloressigsäure gefällte und gewaschene Fibrinogen unlöslich.

Die Absorptionsspektren von Fibrinogen und Fibrin.

Die Fibrinogenlösung gerinnt bei pH 6,2 auf die Wirkung von Thrombin hin zu einer undurchsichtigen weissen Masse. Bei pH 7,2 bleibt Fibrin in M/5 Phosphatpufferlösung vollkommen durchscheinend, nur eine geringe Zunahme der Trübung ist zu beobachten. Unter solchen Umständen ist auch das Ultraviolettabsorptionsspektrum des Fibrins messbar. Das Absorptionsspektrum des Fibrinogens und des Fibrins wurde von P *Csokán* aufgenommen.[3] Die Versuche erwiesen, dass das Fibrinogenspektrum im allgemeinen die Eigenschaften der Eiweisspektra aufweist: einen Absorptionsstreifen bei 279$\mu\mu$ und eine Beugung bei 255$\mu\mu$. Das Fibrinspektrum zeigt nebst den in Fibrinogen erscheinenden Streifen auch das Auftreten eines neuern Streifens: einen niedrigen solchen um 400$\mu\mu$ herum. Aus dem Auftreten dieses neuen Maximums kann man bezüglich der

Struktur des Fibrins einen interessanten Schluss ableiten. Nach KISS u. MITARB.[4] tritt bei den Schiffschen Basen vom Typus *a* unserer Abb. 4 in Fällen, in denen der Stickstoff der Azomethingruppe (— H C = N —) durch Vermittlung einer Wasserstoffbindung an eine OH-Gruppe gebunden ist, neben den Streifen der Azomethingruppe ein neuerer Streifen auf; als Reaktion der durch die Wasserstoffbindung gestörten Azomethingruppe erscheint dieser Streifen genau auf derselben Stelle wie der oben erwähnte neue Streifen des Fibrins.

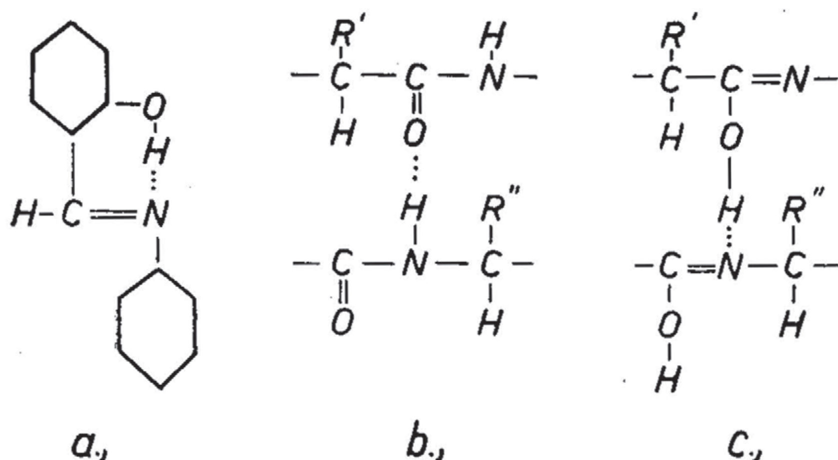
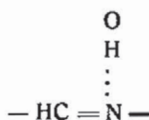


Abb. 4.

Auf der Abbildung 4. zeigt die Formel *b* zwei Polypeptidkettenstücke, wie sie sich im Eiweissmolekül mittels Wasserstoffbindungen verbinden. Nach den Infrarotabsorptionsuntersuchungen von RODEBUSH u. MITARB.[5] kommt diese Wasserstoffbindung zwischen der NH-Gruppe und dem Carbonyl zustande.

Ordnet man die Doppelbindungen nach Formel *c* um, dann erhält man in der Polypeptidkette genau die gleiche Struktur bzw. Umlagerung, die nach BERGMANN[6] auf der Oberfläche der peptidspaltenden Enzyme zustande kommt. Die dergestalt umgebildete Formel enthält zugleich genau dieselbe Atomgruppierung, die bei den vorhin erwähnten Schiffschen Basen den auf das Auftreten der Wasserstoffbindung hinweisenden neuen Streifen hervorbringt.



Die Analogie würde, soweit sie zutreffend ist, zeigen, dass beim Unwandeln des Fibrinogens in Fibrin diese Umlagerung der Doppelbindungen stattfindet. Als Folge der Umlagerung würde mit den in die Polypeptidkette geratenen Doppelbindungen einigermassen eine Versteifung sowie eine Verkürzung der Kette einhergehen. Nach den Röntgenuntersuchungen von KATZ u. ROOY[7] ist die Identitätsperiode im orientierten Fibrinfaden entlang dem Faden 6,7 Å anstatt 7,0 Å.

Zusammenfassung.

In gereinigten Fibrinogenlösungen bewirkt Thrombin eine vollständige Umwandlung des Fibrinogens in Fibrin. Die Umwandlung scheint eine monomolekulare Reaktionstypus zu befolgen. „Fibrinolysin“ macht einen scharfen Unterschied zwischen Fibrin und wärmedenaturiertem Fibrinogen. Das Ultraviolettabsorptionsspektrum des Fibrins zeigt neben den Absorptionsstreifen des Fibrinogens auch einen neuern Streifen, der auf das Auftreten von Wasserstoffbindungen von gewissem Typus hinweist.

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The effect of salts on the isoelectric point of casein.

by

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(Chinoin research fellow).

It has been described by MICHAELIS and SZENT-GYÖRGYI[1] that small concentrations of neutral salts shift the pH range, in which casein is insoluble, either to the acid or to the alkaline side. I have attempted a more detailed study of this phenomenon.

Hammarsten's casein (Merck) was used in the experiments. A stock solution was prepared by dissolving 0.1 g casein in 100 ml of a 0,004 M Na-acetate solution. The buffer solutions used were acetic acid - Na-acetate mixtures, covering the pH range of 3,0 — 6,0 the difference between each two members of the series being 0,05 pH units.

The experiments were made in the following way: 1 ml of the 0,1% casein solution was given to 8 ml of buffer and made up to 10 ml with distilled water. The contents of the tubes were mixed thoroughly, left to stand at room temperature (21—23°) for 24 hours, and the results noted. If sedimentation or visible flocculation was observed, the result was marked „precipitated", if there was only opalescence but no flocculation, it was marked „not precipitated". After reading the results, the pH of the solutions was controlled electrometrically.

It is seen from Table I that an increase in the concentration of the buffer will shift the precipitation zone to the acid side. There is no difference between the effect of 0,02 and 0,01 M buffers. In these buffer solutions casein is precipitated between pH 4,32—5,00. The middle of this range, pH 4,66, agrees well with the known values of the isoelectric point of casein:

Table I.

M concentration of the buffer	Range of precipitation
1.00	3.70 — 4.70
0.50	3.92 — 4.78
0.20	4.15 — 4.85
0.10	4.20 — 4.90
0.04	4.27 — 4.96
0.02	4.32 — 5.00
0.01	4.32 — 5.00

pH 4,7 according to RONA and MICHAELIS,[2] 4,6 according to MICHAELIS and PECHSTEIN.[3]

In the following experiments I have used a 0,02 M buffer to adjust the pH of the casein solutions. In certain cases, where a higher concentration of the buffer was needed, I have made allowance for the modifying action of the buffer concentration.

1 ml of a 1,0 M salt solution or the corresponding amount of salt in substance was added to 8 ml acetate buffer and 1 ml 0,1% casein. The volume was made up to 10 ml with dist. water. After standing at room temperature for 24 hours the precipitation was noted. In Fig. 1. the results of these experiments are summarized. The horizontal lines indicate the pH-range in which casein was precipitated. The salt added to the buffered casein is indicated by its formula over the precipitation zone. The final concentration of these added salts was 0,1 M except in the case of CH_3COOAg, whose final concentration was 0,05 M and $AgNO_3$ which was studied in both of these concentrations. Whereas in most cases the concentration of the buffer was 0,02 M, in some cases the concentration was 0,1 M and in others the buffer was a 0,5 M acetate buffer. In these cases allowance was made for the shift in the precipitation zone due to the buffer and this being substracted the net effect of the salt is shown in Fig. 1. In the case of Na_2HPO_4 there is no acetate buffer, but phosphoric acid was added to give the desired pH. The horizontal line marked „0“ shows the precipitation pH-range of casein in 0,02 M acetate buffer.

Special attention was paid to the effect of the alkali haloids.

It is seen at first sight from Fig. 1. that most of the salts shift the precipitation zone towards the acid side. This shift is obviously due to the anion, its extent being the function of the atomic weight. Cations have a pronounced secondary

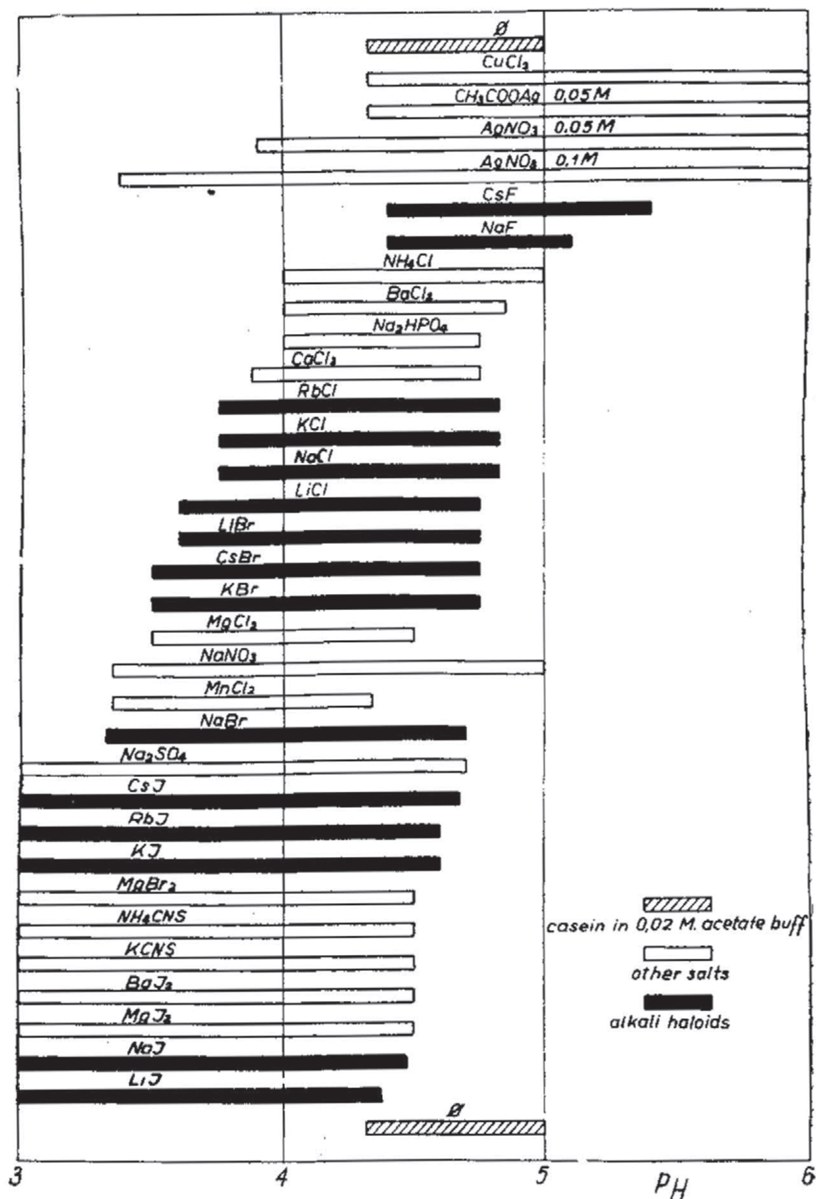

Fig. 1.

influence, shifting the precipitation zone towards the alkaline side. In this case too, the effect is proportional to the atomic weight. In two cases (the two fluorides) the precipitation zone is shifted altogether towards the alkaline side, showing that in these salts the cations take the upper hand. The iodides have a remarkable precipitating action, they would precipitate casein even at pH 1 (not shown in the figure). Similar results have been obtained with the Mg, Ca and Ba haloids. LiBr had exceptional influence, the effect of this salt would not fit into the system.

We may draw the conclusion that the effect of a salt results from the effect of its anion and cation. If the effect of its anion is stronger, the precipitation zone will be shifted to the acid side. If the effect of the cation is stronger the reverse will happen. On the whole the results confirm the data of MICHAELIS and SZENT-GYÖRGYI.

The precipitations obtained at these different pH values in presence of salts are all reversible and do not change the properties of casein. This is demonstrated by the following experiment. The precipitate obtained at pH 3,0 in presence of NaJ was centrifuged off and the small volume of precipitate dissolved in a 0,02 M acetate buffer: its precipitation zone was now identical with the original precipitation zone of casein. i. e. 4,32—5,00.

Next I have studied the influence of the salts on the acid-base binding capacity of casein. Two salts, NaCl and NaJ have been chosen for these experiments, the former giving a small effect, the latter a strong effect in the precipitation experiments. It was found that both salts have identical effects, both of them increase the acid-base binding capacity to the same extent and neither of them changes the isoionic point of casein.

First I made an electrometric titration curve by adding 0,1 N HCl to a 0,01 N NaOH solution and a similar curve by adding 0,1 N NaOH to a 0,01 N HCl solution. 1% casein solutions were made in 0,01 N NaOH and 0,01 N HCl, and again the electrometric titration curve was made by adding different quantities of NaOH or HCl. The titration curve was naturally different of the titration curve of the pure acid or base owing to the acid-base binding capacity of the casein. The difference of the two curves gives the acid-base binding capacity, the

minimum of which (i. e. the isoionic point) was in my experiments at pH 4,6 which agrees well with the accepted value in the literature. Then the experiments have been repeated in the presence of 0,1 and 1,0 M NaCl and NaJ respectively. The acid-base binding capacity was increased by the presence of either salt the more, the higher the concentration of the salt was in the solution. There was however no difference between the

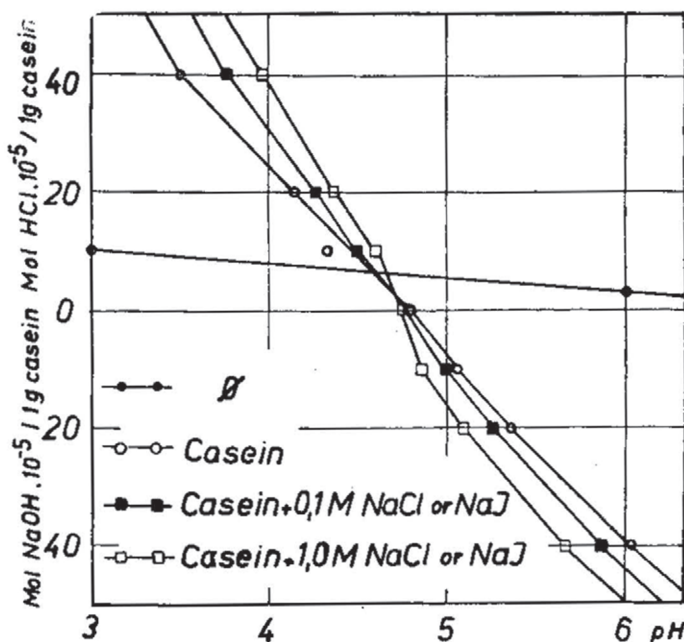

Fig. 2.

effect of NaCl and NaJ. The isoionic point was not changed by the presence of the salts. (Fig. 2.)

We may conclude therefore that the change in the isoelectric pH range of casein due to the presence of different salts in the solution cannot be explained by assuming an effect of the salts on the acid-base binding capacity of casein.

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On the electronegativity of atoms and their influence on the isoelectric point of casein.

by

K. Laki.

The electric charge of a protein particle in absence ot salts is determined by the pH of the solution. At pH below the isoelectric point the protein particle takes up hydrogen ions in excess and aquires hereby a positive charge. At the opposite side of the isoelectric point the protein particle aquires a negative charge the hydroxyl ions being in excess. The quantity of the charge taken up by the protein particle increases as we depart from the isoelectric point. The charge has its maximum at a pH where the protein particle reaches the maximum of its acid or base combining capacity.

Curve A in figure 1. shows schematically how the acid combining capacity of casein (Hammarsten) changes with the pH, the shape of the curve being determined from the data of titrations combined with pH measurements. The curve reaches the abscissa at pH 4,8 (the isoelectric point of the casein) and becomes assymptotic at pH 2 showing that the protein reached here the maximum of its acid combining capacity and also the maximum of the positive charge that can be obtained.

If, at a pH where the casein particle has a positive charge, we add a salt which neutralises this charge,[1] then the casein particle will have no charge in excess and will have its isoelectric point at this pH. If this neutralisation happened at a pH at which the charge of the casein is not yet maximal, on further acidification the protein particle becomes positively charged again. Curve B in figure 1. gives an example of this case. The charge of the casein is neutralised at pH 4 by the addition of a salt. On further acidification the casein particle takes up a positive charge again. It is clear that if we neutra-

lisę the charge of the casein at a pH where the charge maximum has already been reached (pH 2), on further acidification it aquires no charge again. It follows that in presence of salt the isoelectric point depends on the extent to which this salt can neutralise the charge of the casein particle. On the pH scale the isoelectric point becomes thus displaced from its original position. This displacement is a measure of the electric charge by which the salt neutralises the casein particle.

At the isoelectric point the casein precipitates from the

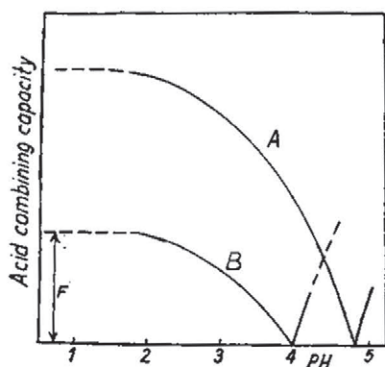

Fig. 1.

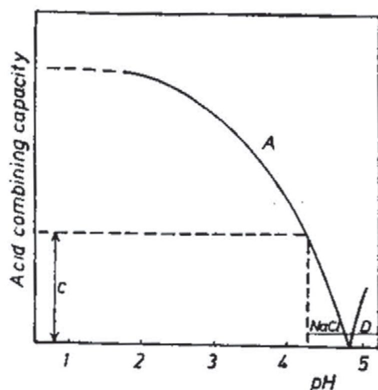

Fig. 2.

solution, and also precipitates in the neighbourhood of it indicating that the casein particle needs a certain quantity of charge to remain in solution. Correspondingly the casein has, instead of a point, a zone of precipitation on the pH scale.

Some precipitation zones, obtained in the presence of halogen salts of alkali metals, can be seen on fig. 3 taken from the preceding paper (Erdős). Strict comparison of the single zones is impossible because the left end of some of these zones is not defined, and so the exact position of the isoelectric point can not be given (calling the middle of the zones the isoelectric point).

The fact that in case of certain salts only the right end of these zones is defined can be explained in the following way. In figure 2 the line D represents the precipitation zone of the casein in the presence of NaCl. It can be seen from the figure that the casein remaines precipitated in a fairly large

pH zone until its charge exceeds a certain quantity (C) which is necessery to bring the casein into solution. Now let us suppose that a certain salt displaces the zone and the isoelectric point. In this case curve B in fig. 1. shows how the casein can be charged again by altering the pH. The comparison of the charge quantities (C and F in fig. 2. and 1.) shows that the amount of charge in this case is insufficient even at pH 2 to charge the casein properly and to bring it into solution.

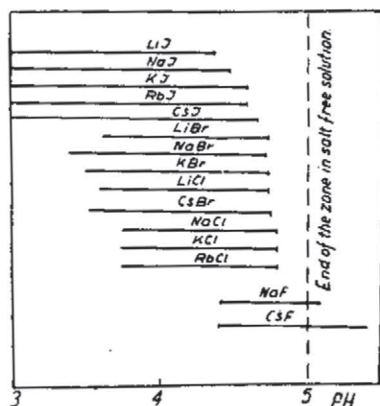

Fig. 3.

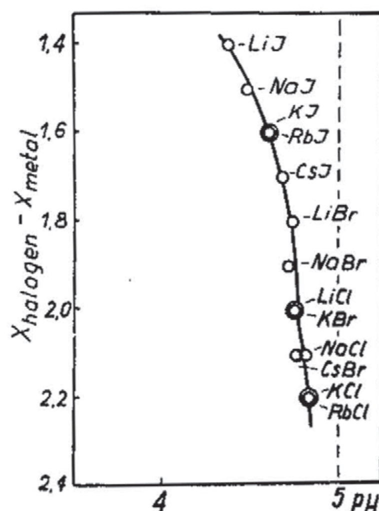

Fig. 4.

The casein thus would remain undissolved throughout the whole acid pH range. It follows from these that in cases where the precipitation zones are displaced to some extent, the zones will extend to cover the whole acide pH range having no defined end to the left. This is the case e. g. with NaJ and LiJ in fig. 3.

Since the left end of the zones in many cases is not defined the middle of the zones can not be ascertained and given as the isoelectric point. We are thus confined to the defined right end of the zones if we want to use them as a basis for comparison.

In figure 4. the circles represent the right ends of the precipitation zones of fig. 3. The abscissa gives the pH scale, the ordinata the values of electronegativity differences of the atoms composing the salt. It can be seen that the displacement

of the precipitation zones is correlated to the values of electronegativity differences.

The electronegativity represents the power of an atom in a molecule to attract electrons,[2] and MULLICAN[3] pointed out that the average of the first ionisation energy and the electronaffinity of an atom can be the measure of its electronegativity.

This correlation of electronegativity values with the power of salts to displace the isoelectric point suggests that the ionisation energy and electronaffinity of atoms play a role in the chargeing process in which the charge of the casein particle is neutralised.

In the following discussion an attempt will be made to explain — at least qualitatively — how the electronegativity differences can be brought into connection with the charge given to the protein particle.

In the past few years a new general theory of the solid matter has been developing.[4] According to this theory the solid matter can be regarded as a large molecule containing a great number of atoms which are arranged in a fashion which gives a regular lattice system. Because of this regular arrangement the valence electrons of these atoms belong to the whole system of atoms having common energy bands. This theory has been successful in interpreting many of the various properties of solids.

There is an increasing number of observations about the behaviour of the protein molecules which can be explained by picturing the protein praticle as a piece of solid matter[5] built up by C, N, O, H atoms. These atoms or a part of them are arranged in such a manner as to give a regular lattice at least in one dimension. The H atom in the hydrogen bond plays an important part in building up the lattice.

In the following treatment this new theory of solid matter will be accepted for the casein particle and discussed what sort of changes occur when a new atom is brought to the surface of the particle.

Suppose, that an atom such as Na having a low ionisation energy is brought on the surface of the casein particle the lattice atoms of which have a higher ionisation energy. Then it might happen that the electron of the Na atom leaves its core and enters into the continuum built up by the atoms

of the casein particle. The result of this is that the Na will be ionised and positively charged and the lattice atom nearest to the surface negatively charged.

In a case where the difference between the ionisation energies is small, the chargeing of the casein also may occur if the lattice atom has a greater electron affinity compared with that of the adsorbed atom. Then the lattice atom attracts an electron from the adsorbed atom, thus the surface atom of the casein will have a negative charge, the adsorbed atom a positive charge.

It follows therefore that two factors determine the magnitude of the charge given to the surface of the casein particle by the adsorption of a foreign atom: 1) the difference between the ionisation energies and 2) the difference between the electron affinities of the adsorbed atom and the lattice atoms.

These two factors can be symbolised in the following way: 1.) $J_l - J_a$ symbolises the magnitude of the charge caused by the difference in the ionisation energies of the adsorbed atom (J_a) and the lattice atom (J_l). 2.) $E_l - E_a$ represents the magnitude of the charge caused by the difference of the electron affinities of the lattice (E_l) and adsorbed (E_a) atom.

Adding up these two symbols we get the following expression:

$$J_l - J_a + E_l - E_a = J_l + E_l - (J_a + E_a)$$

$J_l + E_l - (J_a + E_a)$ represents the total charge given to the casein molecule.

The sum of $J + E$ (ionisation enregy and electron affinity) devided by 130 is identical with the electronegativity values (X) of the atoms.[2] Taking this into consideration the charge given to the casein particle can be represented by the differences of the electronegativities of the adsorbed (X_a) and the lattice composing (X_l) atoms: $X_l - X_a$.

Now if a Cl atom is brought to the surface of the casein particle and this atom behaves in the opposite way as the Na atom (it has a higher ionisation energy and greater electron-affinity than the lattice atom,) the magnitude of the charge given to the casein particle can be represented by the following symbole: $X_b - X_l$. Where X_l means the electronegativity of the lattice atom and X_b represents the electronegativity of the adsorbed Cl atom.

If both Na and Cl atoms are simultaneously on the surface, the resulting charge can be given by adding up these two expressions:

$$X_l - X_a + X_b - X_l = X_b - X_a$$

The symboles representing the electronegativities of the lattice atoms fall out and there remains the difference of the electronegativities of the Cl and the Na atoms: $X_b - X_a$.

The Na and Cl atoms according to this picture are ionised on the surface as Na and Cl ions.

The symbole $X_b - X_a$ says that the magnitude of the charge given to the casein particle is related to the values of the electronegativity differences of the salt composing atoms, and so gives the same result which is born out by the experiment.

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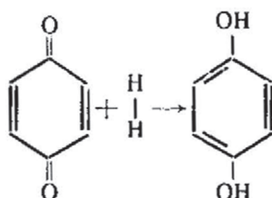
Redoxpotential and resonance energy of certain quinones.

by

K. Laki.

The reaction which takes place when a quinone is hydrated to the corresponding hydroquinone is symbolised by the following equation:



The energy change accompanying this reaction can be calculated from bond energy data. According to the above equation one H—H, two C=O, two C=C and four C—C bonds must be broken and two O—H, two C—O, three C=C and three C—C bonds must be rebuilt. Since in all reactions in which a paraquinone is hydrated to hydroquinone the same structural change occurs, the energy change calculated from bond energies will have the same value. Correspondingly the redoxpotential calculated from this value would have the same value in all cases. (The entropy in these reactions has a standard value[1].) Various quinone-hydroquinone systems however have different redoxpontential values. The resonance energies are responsible for this fact. These energy values must be included into calculations useing bond energy data.

In the following table some data are given to show how the resonance energy values are related to the redoxpotential values of some quinone-hydroquinone redox-systems. In col. 1. the redoxpotential values[2], in col. 2. the resonance energy

values of quinones and hydroquinones, and in col. 3 the difference of these resonance energy values are given. On comparing col. 1. and 3 it can be seen that the redoxpotential becomes smaller and smaller as the difference of the resonance energies between quinones and hydroquinones diminishes. Thus the redoxpotential seems to be a function of the resonance energy.

Table.

	Redoxpot. in Volts	Resonance energy in kcal./mole	Difference of resonance energies.
Quinone Hydro „	0,681	— 3.3 47,7	51,0
Toluquinone Hydro „	0,623	5,4 51,3	45,0
Thymoquinone Hydro „	0,579	6,0 49,0	43,0
Naphtoquinone Hydro „	0,492	47,5 89,0	41,5
Antraquinone Hydro „	0,155	99,0 122,0	23,0

The values of resonance energies given in col. 2. were calculated from thermochemical data comparing the values of heat of formation with the values of the sum of bond energies. Most of the bond energies were taken from the book of L. PAULING.[3] For the energy of the C—O bond a 20% higher value[4] was used than that given by PAULING. The C = O bond energy value used in these calculations was 16 kcal higher than that given for ketons. This seemed justified since the distance of the C = O bond in quinones[5] and the CO_2 molecule[6] is nearly the same.

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Das Oxydationsferment des Kartoffelgewebes.

von

W. F. H. M. Mommaerts.*

Die Oxydationssysteme der höheren Pflanzen sind nur noch sehr unvollkommen bekannt. Es gibt Systeme die durch Vorherrschen von Peroxydase, Askorbinsäureoxydase, oder Phenoloxydase gekennzeichnet sind (1), während neuere Arbeiten auf die Bedeutung der Cytochrome und der Cytochromoxydase hinweisen (2, 3; vergl. auch schon Keilin 4). Keines dieser Systeme ist eingehend erforscht. Es wurde daher das Studium der Kartoffelatmung, als Beispiel einer Pflanzenatmung, vorgenommen

Kartoffeln enthalten bekanntlich eine hochaktive Phenoloxydase, die von Kubowitz (5) als ein Kupferproteid erkannt wurde. Die Deutung der physiologischen Rolle des Ferments ist schwer: im zerkleinerten Gewebe findet die Sauerstoffaufnahme 15 bis 20 mal schneller statt wie in Gewebeschnitten (6); (in diesen Versuchen mit Gewebebrei wurde das bei der Oxydation des Katechinderivats gebildete Chinon mittels Askorbinsäure reduziert.) Dieser Befund zeigt dasz die Oxydase, dem reduzierenden System gegenüber, in Überschusz anwesend ist; man würde also erwarten dasz in der Zelle das gesamte Katechinderivat als Chinon vorliegen würde; dies trifft aber nicht zu, weil dann das Gewebe sich braunfärben würde, und die Oxydase gehemmt sein sollte. (Vergl. 7, 8) Zur Erklärung dieser Diskrepanz hat man angenommen dasz das Ferment in der Zelle in inaktiver Form vorliege, oder dasz in vivo Ferment und Substrat getrennt seien.

Um zwischen diese beide Möglichkeiten eine Entscheidung zu treffen habe ich zuerst versucht das Ferment in in-

* Stipendiat des „De Groot — Fonds", den Haag.

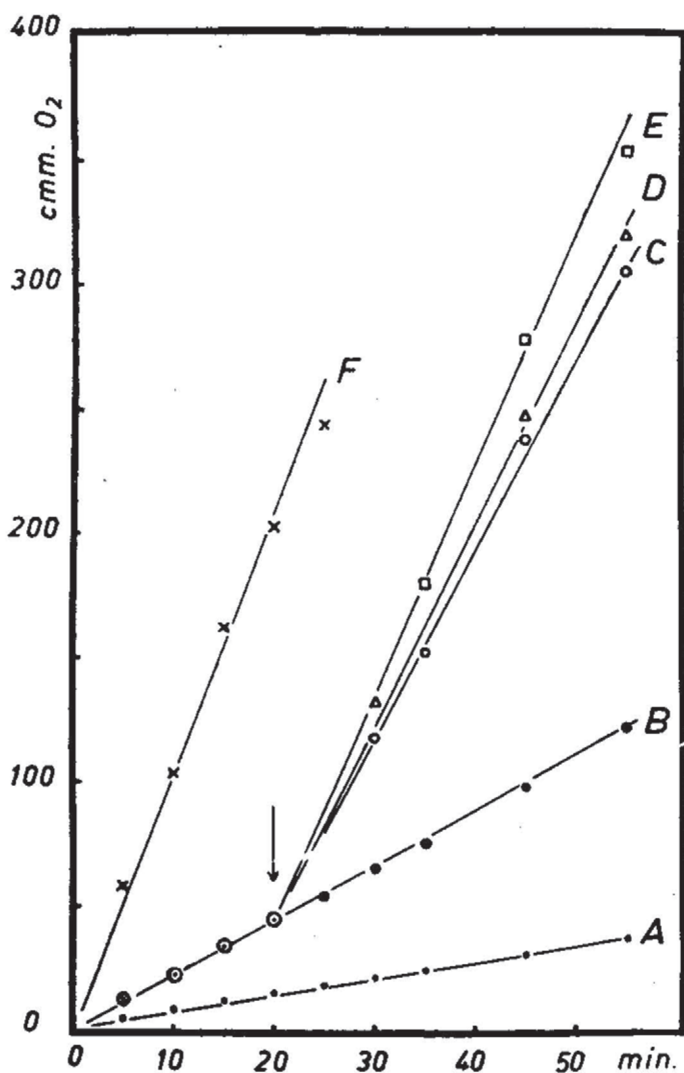

Fig. 1.

A. bis E.: Atmung von 400 mgr. Kartoffelgewebeschnitte in 2.5 cm.³ $\frac{m}{15}$ Phosphatpuffer p_H 6.4 bei 26°.

A: ohne Zusatz; B bis E mit Askorbinsäure in Überschusz; in C, D. und E nach 20 Min. Zugabe von resp. 4.10^{-7}, $2. 10^{-6}$ und 10^{-5} gr. mol. Brenzkatechin.

F.: Atmung von 400 mgr. Kartoffelbrei in Phosphatpuffer mit Askorbinsäure in Überschusz.

aktiver Form zu extrahieren; dies blieb aber ohne Erfolg. Hingegen sprechen folgende Befunde zugunsten der zweiten Deutung:

Askorbinsäure steigert die Sauerstoffaufnahme von Kartoffelschnitten um mehrere hundert Prozente; wird jetzt überdies noch Brenzkatechin zugegeben, so wird der Sauerstoffverbrauch aufs neue erheblich gesteigert, und zwar bis zu einem Betrag der dem der Gewebebreiatmung (mit Askorbinsäure!) ungefähr gleich ist (Fig. 1.)

Dies spricht für die Annahme dasz in der Zelle das natürliche Brenzkatechinderivat, wenigstens zum grószten Teil, vom Ferment getrennt, und für die Atmung ohne Bedeutung ist. Damit wird natürlich auch die Bedeutung der Phenoloxydase für die Atmung zweifelhaft. Um zu entscheiden ob sich die Phenoloxydase überhaupt an der Atmung beteiligt, habe ich untersucht ob die Atmung und die Phenoloxydase sich in Hemmungsversuchen übereinstimmend verhalten. Um die Aktivität der Phenoloxydase in der Zelle zu messen, kann man nicht einfach die Oxydationsgeschwindigkeit zugegebenen Brenzkatechins bestimmen; das Ferment wird dann nl. schnell inaktiviert, und bei Anwesendheit von Giften werden die Verhältnisse recht undurchsichtig; es wurde daher den Sauerstoffverbrauch nach Zusatz von p-Phenylendiamin mit wenig Brenzkatechin gemessen; das Diamin reduziert das gebildete o-Dichinon, wird aber ohne Katechinzusatz vom Ferment nicht angegriffen.

Es wurde gefunden dasz die Atmung, wie die Phenoloxydase, von Schwermetallantikatalysatoren wie Kohlenmonoxyd, Cyanid, Sulfid, Hydroxylamin, Fluorid, und Azid kräftig bis fast vollständig gehemmt wird. Als Beispiel sei ein Hemmungsversuch mit Hydroxylamin angeführt (Fig. 2.) Hier besteht eine vollständige Übereinstimmung zwischen Atmungshemmung und Hemmung der Phenoloxydase. Nicht immer ist die Übereinstimmung so gut; Abweichungen können von verschiedenen Faktoren verursacht werden n. l. *1.* Meistens ist das Atmungsferment ungesättigt, weil ein anderes Glied der Fermentkette die Atmungsintensität beschränkt. 2. Es kann zwischen Katechin und Hemmungssubstanz Konkurrenz um das Fermentkupfer auftreten 3. Besonders bei höheren Konzentrationen von z. B. Cyanid, Azid und Fluorid kön-

nen auch andere Glieder der Katalysatorkette gehemmt werden.

Die Blausäurehemmung wird durch Kupferionen aufgehoben; Fe, Mn, Zn, Co, und Mg reaktivieren nicht;[1] dies steht in Übereinstimmung mit den Befunden von Kubowitz über die Phenoloxydase. Die Kohlenmonoxydhemmung der Atmung ist

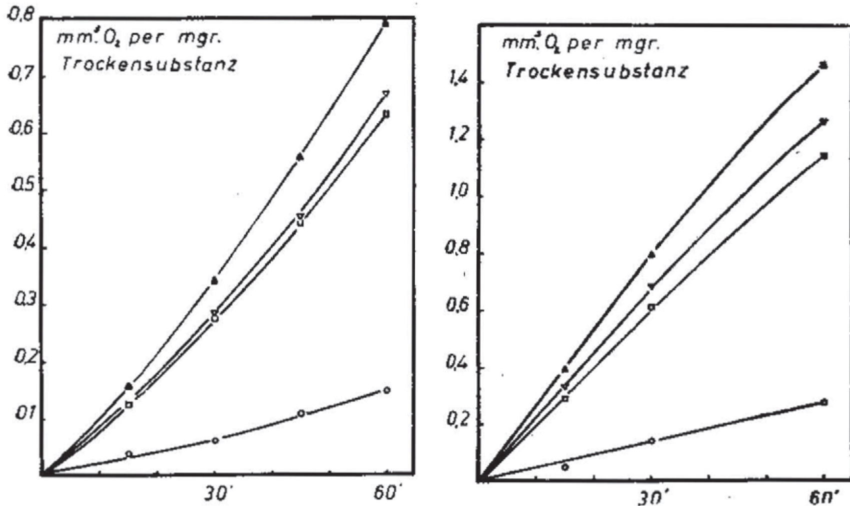

Fig. 2a. Atmung, und Fig. 2b. Phenoloxydaseaktivität unter Einflusz von Hydroxylamin.

▲ Kontrolversuch.
△ Hydroxylamin, 4×10^{-7} gr. mol. per gram Gewebe. (mol/100.)
□ „ 4×10^{-6} „ (mol/1000.)
○ „ 4×10^{-5} „ (mol/10000.)

nicht lichtreversibel, was nach den bisherigen Kenntnissen ebenfalls für Kupferproteide bezeichnend ist.

Starke Atmungshemmungen wurden mit Salicylaldoxim, α-Benzoinoxim, und Natriumdiaethyldithiocarbaminat erhalten. Diese Substanzen reagieren mit Kupfer, z. T. spezifisch, unter Komplexbildung.

[1] Auch Ni erhöht die Cyanidgehemmte Atmung, aber nach einem anderen Mechanismus. Während der Kupfereffekt, als eine echte Reaktivierung, bei höheren Konzentrationen des Cyanids und des Metalls immer schwächer ist, nimmt der Nickeleffekt bei höheren Dosierungen zu; offenbar wird durch den Nickel-Cyanidkomplex irgend eine Autoxydation katalysiert.

Die Versuche zeigen also dasz ein Kupferproteid, allem Anschein nach mit der Phenoloxydase identisch, als Atmungsferment des Kartoffelgewebes dient; die Frage welches Substrat dabei primär oxydiert wird, und aus welchen Komponenten das desmolytische System noch besteht, ist der Gegenstand weiterer Arbeit.

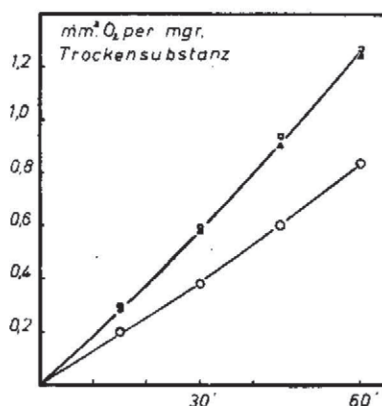

Fig. 3. Hemmung der Atmung durch Cyanid, und Reaktivierung durch Kupfer.

▲ Kontrolversuch.

□ Kaliumcyanid 8×10^{-7} gr. mol. per Gram Gewebe (mol/5000) und $CuCl_2$ 8×10^{-7} gr. mol. per Gram Gewebe.

○ Kaliumcyanid 8×10^{-7} gr. mol. per Gram Gewebe, mit $FeCl_3$, $MnSO_4$, $ZnSO_4$, $Co(NO_3)_2$ und ohne Metallzusatz.

M e t h o d i s c h e s. Die Versuche wurden in Warburg-Manometer bei 28,5° C ausgeführt. Es wurde die Atmung von etwa 500 mgr. Kartoffelschnitte (Dicke ungefähr 0,4 mm.) in mol/15 Phosphatpuffer pH 6,4 gemessen. Die Schnitte wurden aus gekühlten Kartoffeln hergestellt und vor dem Versuch 6 Stunden in stehenden Leitungswasser belassen; derartig vorbehandelte Schnitte geben, wie in einer ausführlichen Mitteilung gezeigt werden wird, ein zuverlässiges Bild der Atmung des intakten Gewebes.

Der Hauptraum der Gefässe enthielt die Schnitte in 2 cm.³ Pufferlösung mit etwaige Zusätze; der Einsatz enthielt 0,1 cm.³ KOH 20% (bei Cyanidversuche 2%), die Birne 0,2 cm³ mol/25 p-Phenylendiamin, mol/250 Brenzkatechin, das nach 60 minuten zugegeben wurde.

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Aminopherase.

by

P. Lénárd and F. B. Straub.

I.

D. M. NEEDHAM[1] was the first to observe that muscle tissue can decompose l (+) glutamic acid in such a way that the amino group remained in the amino-N fraction. BANGA and SZENT-GYÖRGYI[2] have discovered that glutamic acid reacts with oxaloacetic acid in the presence of muscle tissue. This reaction is so fast and complete that it could be used to trap oxaloacetic acid. The nature of the reaction was not investigated in either of these earlier investigations. It was BRAUNSTEIN and his associates'[3] merit to elucidate the mechanism of the very intensive intermediate metabolism of some natural aminoacids in the muscle. They have shown that a number of aminoacids are able to react with some ketoacids with the exchange of the amino and keto groups. According to these studies such reactions take place in many different organs, the most active among them being skeletal and heart muscle. The significance of these reactions is not yet clear.

No detailed information is available about these reactions from the point of enzyme chemistry. Neither the purification procedures elaborated by BRAUNSTEIN and KRITZMANN,[3] nor that described by COHEN,[4] could bring the question closer to its solution. Studies made on such preparations, assuming that they are purified, proved to be misleading.

We have undertaken the purification of such an enzyme in an attempt to study the mechanism of its action, whether it needs a coenzyme as suggested by BRAUNSTEIN. Moreover, the kinetics of such a reversible reaction between four substrates are of considerable interest from the point of enzyme-

substrate complex formation. Unfortunately, owing to external conditions, this work had to be abandoned at a rather early stage and therefore we decided to describe the purification of the enzyme along with some kinetic measurements.

II.

It is known from the work of BRAUNSTEIN that the following reactions occur most actively in the presence of muscle tissue:

(1) oxaloacetic acid + l(+) glutamic acid $\rightleftharpoons$ l(—) aspartic acid + α-ketoglutaric acid.
(2) pyruvic acid + l (+) glutamic acid $\rightleftharpoons$ l (+) alanine + α-ketoglutaric acid.

There are considerable difficulties connected with the measurement of reaction rates in reaction (1), owing to the relative instability of oxaloacetic acid in muscle tissue. Its reduction to malic acid, decarboxylation to pyruvic acid and condensation to citric acid lead to unreliable results unless the complete balance sheet is drawn up for all of the substances concerned. There are no such difficulties in determining the reaction rate in reaction (2). We have chosen this reaction as the subject of our studies.

In principle we accept the nomenclature advanced by BRAUNSTEIN and call the enzyme aminopherase. As the aminopherase in question catalyzes only reaction (2), we think it advisable to call this enzyme glutamino-pyruvic aminopherase.

With the method, described below, we were able to purify glutamino-pyruvic aminopherase 550 *times as compared with the original activity of the heart muscle tissue. The most* active preparations are colorless. Different preparations obtained by somewhat altered procedures yielded the same maximal activity. Therefore, as attempts to purify the enzyme any farther have all failed, we believe that such preparations are practically pure.

We had no indication that the enzyme would require a coenzyme. The slight yellow colour invariably present in our preparations, shows no specific absorption bands and we do

not think its having anything to do with the enzymic activity.

Glutamino-pyruvic aminopherase brings about an equilibrium in reaction (2), there is less pyruvic acid than alanine in equilibrium.

We have determined two of the four enzyme-substrate dissociation constants, which are necessary to describe the reaction. The enzyme-alanine dissociation constant was found to be $1{,}5 \cdot 10^{-2}$ the enzyme-α-ketoglutaric acid dissociation constant: $1{,}7 \cdot 10^{-3}$. In spite of great deal of experimental work, we could not determine the similar values of the enzyme-glutamic acid and enzyme-pyruvic acid dissociation constants. This was mainly due to the lack of reliable and exact methods for the determination of other substrates than pyruvic acid.

III.

Methods.

Throughout this work the enzyme activity was determined by the amount of pyruvic acid formed or disappeared. STRAUB's salicilic aldehyde method[5] was used and we found it very satisfactory for the present purpose. None of the other three components gave any reaction with salicylic aldehyde, thus ensuring complete specificity. When muscle tissue, or a still impure enzyme was used, the protein was removed with the tungstate-sulfuric acid mixture (see STRAUB[5]). But at a rather low degree of purity, this deproteinization became superfluous as the amount of protein present in the test was negligible. In these cases 1 ml of the reaction mixture was pipetted into 1 ml of the concentrated KOH solution and 0,5 ml of the salicylic aldehyde reagent added. The protein originally present, was dissolved in the strong potash solution, without giving any opalescence. Nor did it give any colour with salicylic aldehyde.

We found that the method would give reliable results within 3% of error, if the following procedure was rigorously adhered to: The pyruvic acid solution (1 ml) was pipetted into a test tube, containing already 1 ml of the potash solution. 0,5 ml salicylic aldehyde solution was immediately added and the mixture thoroughly shaken up. Within a minute the test

tube was placed into a water bath of 38°, kept there exactly for 10 min., placed in an icebath for 10 minutes and the result read after this time. Determinations on pure pyruvic acid solutions have shown that in this case the extinction values (log I_0—log I) are directly proportional to the pyruvic acid concentration. The linear relationship will however break down if more than 0,3 mg pyruvic acid is taken for the determination. Should there be more pyruvic acid in solution, it should be correspondingly diluted. 0,1—0,3 mg pyruvic acid per ml is the ideal concentration to be taken for a determination. The readings were made with a Pulfrich photometer, using a cuvette of 1 mm thickness and the blue filter S47.

The following preparations were used as substrates: 1. l(+) glutamic acid. Its optical rotation was controlled in the presence of ten mols of HCl and was found to agree with the described value of $\alpha^{20}_{D} = +31°$. 2. Pyruvic acid was redistilled from the commercial product and a solution made up by acidimetric titration. 3. Alanine was used in the d, l form. As the d (—) form does not react at all, the concentration of l (+) alanine was taken only into consideration. 4. α-ketoglutaric acid was prepared synthetically from oxalosuccinic ester by the method of GABRIEL[6]. Its melting point was close to the theoretical value (111° instead of 112—113°). These preparations contained some impurity, which gave a colour with the salicylic aldehyde reagent. In the preparation mostly used in these studies, 1 mol/liter ketoglutaric acid gave a colour corresponding to 0,014 mol/liter pyruvic acid. The impurity, however, was not pyruvic acid, and corrections were taken for this value.

IV.

Test for the enzyme activity.

We have defined, as the glutamino-pyruvic aminopherase u n i t, the amount of enzyme, which formed 1 mg of pyruvic acid in 4 ml of the test solution within 15 minutes at pH 7,3 and 38°, when the initial concentration of l-alanine and α-ketoglutaric acid was 0,017 mol/liter each, the initial concentration of pyruvic acid and glutamic acid nil.

From 3—4 determinations with different quantities of an

unknown enzyme solution, this value is obtained by graphic interpolation. Fig. 1. shows such a determination. It is seen that the curve obtained is far from being linear. This is due to the fact that equilibrium is reached under these conditions if 2 mg pyruvic acid are formed.

The test solution was made up in the following way: Phosphate buffer of pH 7,3 to give a final concentration of 0,1 M, the enzyme solution and 0,5 ml of a 0,137 M l-alanine solution were placed in a test tube and filled up to 3.5 ml with dis-

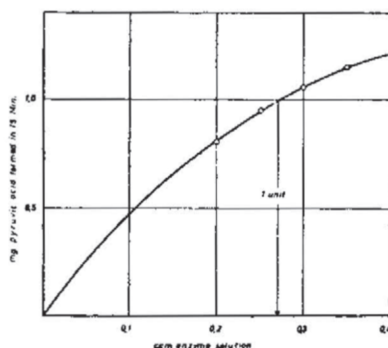

Fig. 1.

tilled water. The tube was then placed in a waterbath of 38°. The α-ketoglutaric acid solution (neutralized with NaOH) was placed in a test tube in the same waterbath and after 5 minutes, 0,5 ml of it was given to the test, mixed thoroughly and then left to stand in the waterbath for 15 minutes. It was found unnecessary to employ any shaking during the incubation. After 15 minutes the solution was either deproteinized by letting the required amount of sulfuric acid run into the tube, while still in the waterbath, or 1 ml of it were taken out and given immediately into 1 ml of potash solution. If there was too much pyruvic acid present, 1 ml of the test solution was taken out, mixed with 1 ml potash solution as before and then diluted with a twice diluted potash solution to reach a convenient pyruvic acid concentration. From this dilution 2 ml were pipetted into a test tube and mixed with 0,5 ml salicylic aldehyde for the determination.

V.

Purification of the enzyme.

Pig's hearts were minced with an ordinary meat mincer. 800 g of the mince were mixed with 800 ml of a 0,1 M acetate buffer of pH 3,8 and left to stand at room temperature for 24 hours. The mixture was then warmed up to 60° and kept at this temperature for 15 minutes. Then the fluid was squeezed through a thin cloth. If neutralized to pH 7 by the addition of a 10% NaOH solution, a precipitate was formed. This is centrifuged off, leaving a clear, pink solution (A).

To 1080 ml of solution (A) thus obtained, 242 g ammonium sulfate were added and the precipitate, which contained the enzyme, was filtered off through fluted filters. (Ammonium sulfate: 0,375 saturation.) The filter paper, together with the precipitate was suspended in 80 ml of distilled water. The enzyme solution was sucked off from the filter paper pulp and the residue was washed with 20 ml of distilled water. The solution and washing were combined and dialyzed against distilled water at 0° for 24 hours. After dialysis we obtained 149 ml of a clear brown solution (B).

130 ml of solution B were acidified by the addition of 6,5 ml of a 2 M acetate buffer of pH 4,6 and then mixed with 12 ml of an alumina Cγ suspension (280 mg Al_2O_3). The enzyme was adsorbed on the alumina gel and separated from the solution in the centrifuge. The precipitate was stirred up with 20 ml of a 0,1 M phosphate buffer of pH 7,3 and the solution was adjusted to pH 6,8 by the addition of a few drops of a dilute NaOH solution. The precipitate was eluted for a second time by adding 20 ml of a 0,1 M phosphate buffer of pH 6,8 to the gel. After thorough mixing it was centrifuged off again and this elution was combined with the first one. We obtained in this step 39 ml of enzyme solution (C). It is advisable to make a preliminary test in order to find the optimal amount of alumina gel, i. e. the amount of alumina necessary to bring down about 60—70% of the enzyme.

33 ml of the solution (C) obtained in the previous step, were refractionated with ammonium sulfate between 0,30 and 0,375 saturation values. Both precipitates settled well in the centrifuge. The fraction, which precipitated between 0,3 and

0,375 saturation, was dissolved in distilled water, giving 6 ml of a pale yellow solution. It contained the enzyme with maximal activity. (D).

The purified enzyme keeps well in solution, especially well in an ammonium sulfate solution. In some cases we found no loss of activity during a period of two months, if the enzyme was kept in a 0.15 saturated ammonium sulfate solution in the icechest.

In Table I. the numerical details of the purification procedure are summarized:

Table I.

Step	Enzyme units/ml	Total units	Enzyme unit/mg
A	4,1	4450	1,03
B	16,5	2150	4,17
C	28,4	937	12,6
D	103	618	28,6

It is seen that from the first solution 14% of the activity are retained in the last fraction and the activity per dry weight is 28 times higher. Actually the most successful step of the purification is the first one, in which at the very acid pH and high temperature, most of the muscle proteins are denatured and removed. We find that 1 g of fresh muscle tissue contains 10 units of glutamino-pyruvic aminopherase, i. e. about 0,05 units per mg protein (dry weight). Therefore in the present case the first step means a 20-fold increase in activity and the whole procedure a 570-fold purification.

In different preparations, the final value of the purified enzyme lay always between 28—29 units/mg.

P r o p e r t i e s o f t h e e n z y m e. Glutamino-pyruvic aminopherase shows a remarkable stability towards heat and a considerable resistance towards acid pH. The first point was already observed by BRAUNSTEIN. If kept at room temperature for half an hour, the enzyme is inactivated by pH 3 and pH 11. It is stable between these pH values. Alcohol, but not acetone destroys the enzyme even at 0°. Dialysis against distilled water does not diminish the activity of the enzyme. We have tested at different stages of the purification the effect of boiled muscle juice and of boiled enzyme solutions on the activity of the enzyme. We could never find any rise in the activity.

It is consequently plain that the enzyme does not have a dissociable prosthetic group.

Part of the enzyme can be removed by extracting the muscle with distilled water or salt solution, part of it will, however, remain in the residue even after prolonged washing. It is the heat treatment with — or without — the acid ph which brings the enzyme into solution.

The specificity of the enzyme was not studied extensively. We found that if alanine is replaced by aspartic acid in the

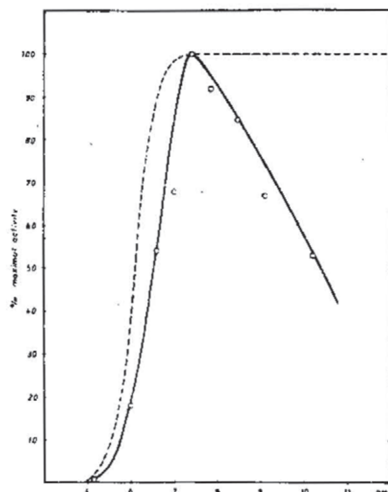

Fig. 2.

test, or aspartic acid and pyruvic acid are added to the enzyme, only traces of oxaloacetic acid are formed and no pyruvic acid disappears in the second case.

Fig. 2. shows the pH activity curve of the enzyme, when the reaction starts from alanine and ketoglutaric acid. The assimetry of the curve is an interesting feature. It is probable that on the acid side the activity is connected with the charge of the substrate, as shown by NORTHROP[7] for he pH-activity curves of pepsin and trypsin. The dotted line in Fig. 2. shows the percentage of negatively charged alanine ions as the function of pH. The enzyme seems to be able to react only with the negatively charged alanine ion and not with the positive ion or the zwitterion. It should be remembered, that the enzyme is not destroyed by even much lower pH values, than those at

which there is already no reaction. Preliminary experiments on the pH activity-curve of the enzyme for the reverse reaction show that this is shifted towards the alkaline side as compared with the curve of Fig. 2.

The experiments to determine the pH activity curve have been performed so as to get initial velocities. Therefore 2 units of enzyme were taken for the usual test solution of 4 ml. The reaction was started 5 minutes after the other ingredients were placed in the waterbath, by the addition of the ketoglutaric acid solution. It was stopped after 4 minutes by pipetting 1 ml of the solution into 1 ml of concentrated KOH solution. The buffers used were: glycine-HCl in the acid region, citrate-phosphate mixtures between pH 4,6—7,8 and glycine-NaOH on the alkaline side. All of them were present in 0,1 M final concentration. The pH values were controlled colorimetrically.

VI.

Kinetics of the reaction.

1. Equilibrium. The position of the equilibrium in the reversible reaction (2) was studied for the case of equal concentration of the two reacting substrates, under varyng enzyme concentration and varying initial concentration of the substrates. Table II. contains some of these results. It is seen that the equilibrium position does not depend on the concentration of the enzyme, nor on that of the substrate. From the mean value of 45,5% pyruvic acid in equilibrium, the equilibrium costant can be calculated:

$$K = \frac{C_{alanine} \cdot C_{ketoglutaric\ acid}}{C_{glutamic\ acid} \cdot C_{pyruvic\ acid}} = \frac{54,5^2}{45,5^2} = 1,43$$

2. Reaction velocity. The aim of kinetic investigations is the analysis of the mechanism of enzyme reactions. If the reactions, which take place during the enzyme action are set up correctly, we should be able to obtain an equation, which tells us the composition of the system at any given time. provided the initial concentrations of substrates and enzyme are given at a fixed pH and temperature.

There is no enzyme reaction for which such an equation

Table II.

Enzyme units/ml	Initial concentration mol/lit				pyruvic acid mol/lit found in equilibrium	% pyruvic acid in equilibrium
	l(+) glutamic acid	pruvic acid	l(+) alanine	α-ketoglutaric acid		
1,0	0,100	0,100	0	0	0,044	44,0
1,0	0,070	0,070	0	0	0,0309	44,1
1,0	0,040	0,040	0	0	0,0167	41,8
1,0	0,0200	0,0200	0	0	0,0094	47,0
1,0	0,0090	0,0090	0	0	0,00398	44,3
1,0	0,0050	0,0050	0	0	0,00241	48,2
1,0	0	0	0,0050	0,0050	0,00230	46,0
1,0	0,0025	0,0025	0	0	0,00122	48,8
1,0	0,0010	0,0010	0	0	0,00043	43
2,5	0,0060	0,0060	0	0	0,00278	46,4
0,82	0,0060	0,0060	0	0	0,00281	46,8
0,25	0,0060	0,0060	0	0	0,00276	46,0
					mean value:	45,5

is elaborated, owing no doubt to the complexity of the question. Several attempts were made earlier on kinetic studies of enzyme reactions, but most of them were connected with proving or disproving empirical laws. It was first realized by MICHAELIS and MENTEN[8] and later by WARBURG and his associates[9] that the mathematical solution of this question can be achieved by assuming that the enzyme reaction rate depends on the concentration of the enzyme-substrate complex. By determining the enzyme substrate dissociation constants they were able to obtain time-laws for some enzyme actions. But even from these studies no generalized time law could be constructed, owing no doubt to the aproximative nature of the calculations.

The necessity of work on these lines is emphasized by the fact that one often encounters kinetic studies in which the simple non catalytical time laws of monomolecular or bimolecular reactions are shown to hold for an enzyme reaction. COHEN for instance in his paper on transamination states that the transamination can be described by a bimolecular reaction constant. This can only be due to experimental error. Apart

from the fact that not even the reversibility of the system is taken into account, such an attempt is meaningless. This is illustrated by Fig. 3. in which the experimental time curve for the alanine-ketoglutaric acid reaction is indicated by the amount of pyruvic acid formed. Substrate concentration: 0,00685 mol/liter for each substrate, enzyme concentration 0,56 units/ml glutamino-pyruvic aminopherase. PH 7,3, temperature 38°. The time curves of a simple bimolecular reaction and that

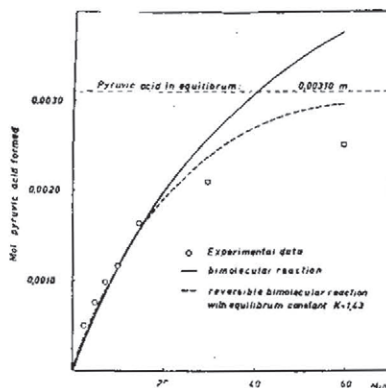

Fig. 3.

of a reversible bimolecular reaction (with equilibrium constant $K = 1,43$) are drawn into the same figure so as to meet the facts at one point. (10 min.). It is seen that they do not meet the facts at any other point.

If we assume that the substrates form dissociating complexes with the enzyme, it is possible to obtain an integral equation as the time law of this reversible bimolecular enzyme reaction. This is rather a complicated equation even for the case of equal initial substrate concentration, but it contains only the following quantities: initial concentration of substrates, enzyme concentration, the four enzyme-substrate dissociation constants, the equilibrium constant and the substrate concentration at time *t*. Owing to reasons described above, two of the enzyme-substrate dissociation constants are still missing and we were not able to prove our equation, thus not described here. But it is believed that only on these lines can the kinetic laws of catalyzed reactions be developed.

Determination of the enzyme-substrate

dissociation constants. We suppose that alanine and ketoglutaric acid, which react with one another are bound to different points of the enzyme. In this case the dissociation

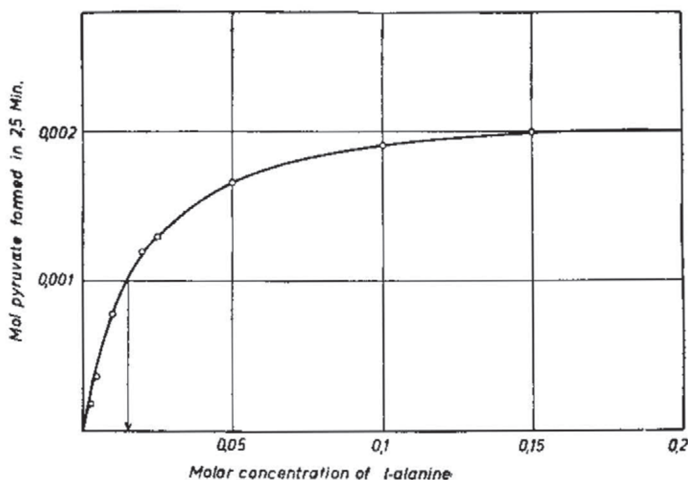

Fig. 4.

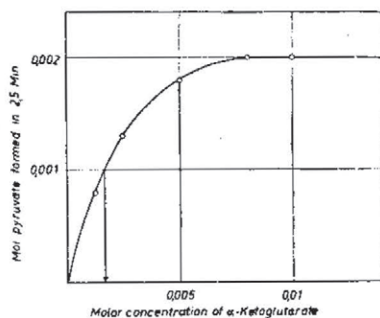

Fig. 5.

of the enzyme-alanine complex is independent of the concentration of the ketoglutaric acid and vice versa. If therefore the concentration of ketoglutaric acid is kept constant and so high that it saturates the enzyme (0,05 M), then the initial velocity is proportional to the concentration of the enzyme-alanine complex. If the initial velocities are now determined for varied alanine concentrations, the curve of Fig. 4. is obtained. The alanine concentration, at which half of the maximum velocity

is observed, is equal to the enzyme-alanine dissociation constant. In a similar way, the enzyme-ketoglutaric acid dissociation constant can be evaluated from Fig. 5. Here the concentration of alanine was kept constant and high (0,1 *M*) and the concentration of α-ketoglutaric acid varied.

We are indebted to Prof. V. BRUCKNER and Mr. C. AUTHERIED for a generous supply of synthetic α-ketoglutaric acid.

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STUDIES

FROM THE INSTITUTE OF MEDICAL CHEMISTRY UNIVERSITY SZEGED

VOL III.

(1943)

MUSCULAR CONTRACTION, BLOOD COAGULATION.

EDITED BY

A SZENT-GYÖRGYI

(Sent to press on Dec. the 13th 1943)

S. KARGER

BASEL, NEW YORK.

SZEGED VÁROSI NYOMDA ÉS KÖNYVKIADÓ RT
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Abbreviations used in this volume.

ATP = Adenylpyrophosphate or adenosinetriphosphate.

ADP = Adenylphosphate or adenosinediphosphate.

DRF = Double refraction of flow.

In this volume two expressions are used for the characterisation of actomyosin:

1. *% Actomyosin.* By % actomyosin is meant the quantity of actin in 100 parts of actomyosin. So for instance, 16,7% actomyosin contains 16,7 g actin in every 100 g of actomyosin.

2. *% Activity.* By % activity of actomyosin is meant the fall of viscosity of actomyosin, (dissolved in neutral 0,6 M KCl) on addition of ATP — relative to the fall of viscosity of a 16,7% actomyosin. If we say, for instance, that an actomyosin is 100% active, this means, it shows the same fall of viscosity as a 16,7% actomyosin. (16,7% is the activity of our myosin B, see vol. I. of these studies.)

The actin content of an actomyosin can be calculated from its % activity by means of the curves given by F. B. STRAUB in this volume.

The autocatalytic formation of thrombin and the clotting defect of hemophilic blood.*

by

K. LAKI

The coagulation of blood is the conversion of a soluble protein, the fibrinogen, into the insoluble fibrin. This conversion of fibrinogen is brought about by the action of thrombin, which is formed from prothrombin during the process of blood clotting. The formatin of thrombin increases during the process of clotting and follows the type of an autocatalytic reaction.

This autocatalytic type of the formation of thrombin has aroused considerable interest among students of blood coagulation and many theories have been put forward to

* This work was aided by a grant from the Duke of ESTERHÁZY.

explain it. As knowledge about blood coagulation has increased and more and more components taking part in it have been isolated and investigated, it has become evident that the classical theory of blood coagulation is inadequate as an explanation of this autocatalytic type of the formation of thrombin. The processes known at present to be taking part in coagulation are not autocatalytic. Purified thrombin is not able to catalyse the thrombin formation from purified prothrombin. The conversion of fibrinogen into fibrin[1] is not autocatalytic nor is the formation of thrombin from prothrombin by the action of tissue kinase.[2]

The possibility that some hitherto unknown substance or mechanism is responsible for the autocatalytic formation of thrombin was indicated by the experiments of FISCHER[3] and especially of ASTRUP.[2] ASTRUP demonstrated that in the so called Mellanby-fibrinogen solutions the autocatalytic formation of thrombin was broken down and that even a slight acidification of the normal plasma was enough to destroy the autocatalytic formation of thrombin.

In a series of investigations performed in recent years on the hemophilic patients of the Clinic of Internal Medicin of Szeged, in collaboration with L. SZÉCSÉNYI NAGY, we compared the blood coagulation of normal persons with that of hemophilic patients.* Following quantitatively the formation of thrombin during the whole process of coagulation we came to the conclusion that hemophilic blood lacked the autocatalytic formation of thrombin and that the solution of the problem involved the necessity of elucidating the mechanism responsible for the autocatalytic formation of thrombin.

According to ASTRUP's and our findings Mellanby-fibrinogen and hemophilic blood resemble each other, both lacking the autocatalytic formation of thrombin.

It was found in the course of the investigation on the clotting of hemophilic blood that small amounts of fibrinogen prepared according to the method of the author in vitro restored not only the time of coagulation to normal but also

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* A brief account of this work was read before the meeting of the Hungarian Physiological Society in 1942, and the detailed account will appear elsewhere.

the formation of thrombin. 0,01 cc. of fibrinogen solution containing 17 γ of protein was enough to restore the clotting of 1 cc. of hemophilic blood to normal.

Experiments soon revealed that some contaminating substance present in fibrinogen solutions is responsible for the effect. I call this substance *„plasmakinin."*

The resemblance between hemophilic blood and Mellanby-fibrinogen became closer when it was found that the same plasmakinin solution which restored the clotting of hemophilic blood to normal also accelerated the clotting of Mellanby-fibrinogen solution. The Mellanby-fibrinogen thus seemed to be a suitable material for the investigation of the mode of action of plasmakinin.

In the present communication the preparation of a crude solution containing plasmakinin will be described together with experiments showing the action of plasmakinin on Mellanby-fibrinogen. It will be seen that the addition of plasmakinin to Mellanby-fibrinogen leads to the formation of kinase, and that this formation of kinase increases in time. With the increase of kinase also increases the quantity of thrombin. Thus in view of these findings plasmakinin shows itself as an important factor in the formation of thrombin and the lack of it explains the hemophilic clotting defect.

Experimental.

Preparation of plasmakinin.

Oxalated cattle blood (containing 0,2% Na-oxalate) collected in the slaughter house was centrifuged and the clear plasma was sucked off. To the clear plasma saturated ammoniumsulfate was added in small portions until 0,25 saturation was reached. The precipitate which consisted mainly of fibrinogen was centrifuged. After discarding the supernatant plasma, the precipitate was brought into a solution of 0,7% NaCl (containing 0,2% Na-oxalate). Starting with 300 cc. of plasma, the precipitate was brought into 80 cc. of the NaCl solution. The precipitate dissolved slowly and gave a turbid solution. To remove the ammoniumsulfate this solution was dialysed in a cellophane tube against ten volumes of NaCl

solution for about 6 hours in the cold. The solution was then thoroughly centrifuged and used as PK_1 (first plasmakinin solution) in the experiments. 0,01 cc. of this solution given to 1 cc. of hemophilic blood is usually enough to restore the clotting time to normal.

Further purification was achieved by coagulating the fibrinogen. For this purpose 4 cc. of purified thrombin* solution were added to 80 cc. of PK_1 solution. Within an hour a firm clot was formed. The fibrin clot, after standing for a few hours in the ice chest was broken into pieces by means of a spatel then put on a cloth and the liquid, enclosed in the clot, pressed out. The fibrin-free liquid was left to stand in the ice chest; some further formation of a small amount of fibrin occured. Removing the clot by centrifugation a clear solution was obtained, which was almost free of fibrinogen and contained practically no thrombin. (The thrombin was probably adsorbed by the fibrin and was removed with it.) This solution (PK_2) often has the same activity as PK_1 though usually the activity is somewhat less. The preliminary experiments about the nature of this substance show that boiling to 100° destroys the activity of plasmakinin solutions. Warming to 50° for 5 minutes in neutral or slightly alkaline solution does not influence the activity but warming to this temperature in acid solution (N/40 acetic acid) or precipitation with alcohol or with acetone completely destroys the activity.

Preparation of Mellanby-fibrinogen (MF).

Cattle oxalate plasma obtained as above was gradually acidified by adding *N* acetic acid. During the course of acidification the pH was controlled colorimetrically. In order to test the pH 1 cc. of plasma was pipetted into test tube and 1 drop of bromocresolpurple** was added to it. Acetic acid was added till the colour of the dye became greenish-yellow. The acidified plasma was then poured into ten volumes of distilled water; a voluminous precipitate appeared which was allowed to settle

* 0,1 cc. of the thrombin solution gave a clotting time of 1' 30" when added to 1 cc. of oxalated plasma.

** 0,1 g. of bromocresolpurple dissolved in 18,5 cc. of *N*/100 NaOH was diluted with water to 250 cc.

overnight in the cold. The fluid was then sucked off and 15 cc. of Ca-free Ringer* solution were added to the precipitate for every 100 cc. of plasma which was used for the preparation. This turbid solution, (— made up to 40 cc. with water —) was allowed to stand at room temperature for about 12 hours and then centrifuged. The precipitate was discarded and the solution placed into the ice chest overnight during which time some formation of fibrin clot occured. This clot can easily be removed and after centrifugation a clear slightly yellow solution is obtained. This solution contains 3—6 mg. of fibrinogen and yields, on the addition of tissue kinase and calcium, as much thrombin per cc. as 1 cc. of the original plasma. 0,03 cc. of this solution added to 1 cc. of hemophilic blood reduces the clotting time only slightly, which shows that the active substance responsible for the normal clotting is greatly reduced in MF solutions.

The Mellanby-fibrinogen can be kept for weeks in frozen state and gives a clear solution when thawed. Storing in an ice chest is not very convenient owing to the formation of fibrin clot which must be removed before using the solution. In order to serve as a test solution for the action of kinase or plasmakinin the MF was diluted five times with *M*/20 borate buffer. This clear solution clots only after the addition of Ca and it takes usually 25 minutes before the first fibrin clot is detectable. If the clotting time is much shorter, then the original undiluted MF solution should be left overnight in an ice chest. After removing the fibrin clot which has been formed the solution can be treated as above and a suitable test solution will be obtained.

Kinase and plasmakinin have a similar effect on MF: both reduce the clotting time.

The action of tissue kinase on MF.

The clotting time of MF depends on the quantity of kinase used as demonstrated by fig. 1. The curve was obtained by plotting the clotting time against the quantities of kinase. The experiment was performed in the following way: Into small test tubes different quantities of very diluted brain

* The Ringer solution contained 0,23% $NaHCO_3$.

kinase* were pipetted. Then 1 cc. of recalcified MF solution was added after being diluted with borate buffer of pH 7,8. (The concentration of $CaCl_2$ was 10^{-} *M*.) One tube without kinase served as control. By shaking the tubes gently from time to time the appearance of the first fibrin clot can be observed sharply and the time of its formation measured. It is necessery to use test tubes made of the same glass because the glass wall

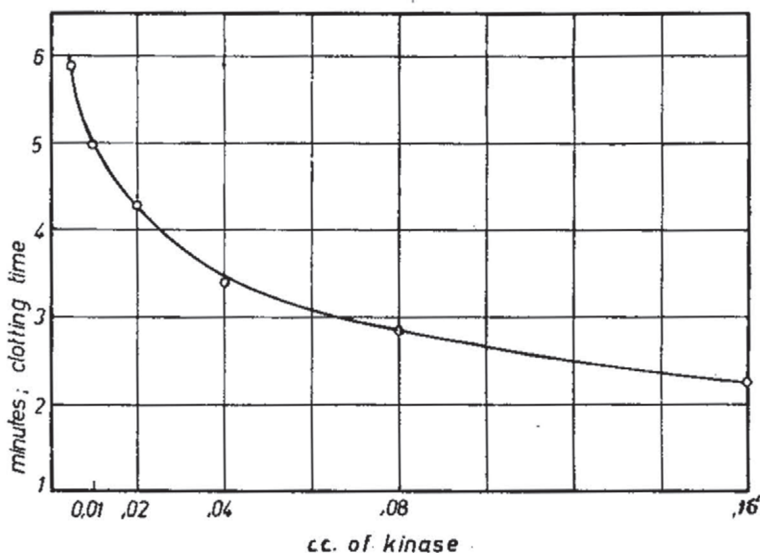

Fig. 1.

somewhat influences the clotting time of MF solutions. In all the experiments presented in this paper glass of Jena was used.

The concentration of Ca influences the clotting time to a great extent, the optimum is at 2×10^{-3} *M* $CaCl_2$. Fig. 2. gives the data of an experiment in which the concentration of Ca was varied and the kinase quantity kept constant. The experimental technic was the same as described above. The $CaCl_2$ solution and the kinase was pipetted into the tubes first, then 1 cc. of MF solution, which was diluted with borate buffer of pH 8,0 was added and the clotting time observed. In fig. 2. the clotting time is plotted against the $CaCl_2$ concentrations.

---

* Rabbit's brain was extracted with an equal volume of 0,9% NaCl solution.

The action of plasmakinin on MF.

Like kinase, plasmakinin also catalyses the clotting of MF. Data of a similar experiment which was performed as above, using various quantities of PK_1 and PK_2 instead of kinase, are presented in table 1. It can be seen how the clotting time depends on the quantity of plasmakinin solutions. In the first column the dilution of plasmakinin is given and in the second the clotting time. PK_2 has somewhat longer clotting times indicating some loss in activity.

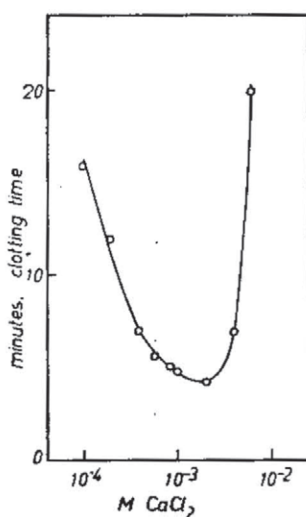

Fig. 2.

Table 1.

The clotting time of Mellanby-fibrinogen on the addition of various quantities of PK_1 and PK_2.

Quantity of PK_1 or PK_2 added to 1 cc. of MF.	Clotting time in the presence of PK_1	or PK_2
0,05 cc. of . . . . PK_1 or PK_2	10 min.	10 min.
„ 5 times diluted . . . . „	15 „	16 „
„ 25 „ . . . . . „	25 „	29 „

Although the simplest explanation of the action of plasmakinin is that it acts as a kinase, it may be of interest to discuss briefly the possibility that other factor present in plasmakinin solutions might be responsible for the observed effect. The possibility that thrombin is responsible can easily be ruled out because PK_1 does not contain thrombin at all. The accelerating effect can hardly be attributed to prothrombin. MF contains much more prothrombin and the slight increase in prothrombin

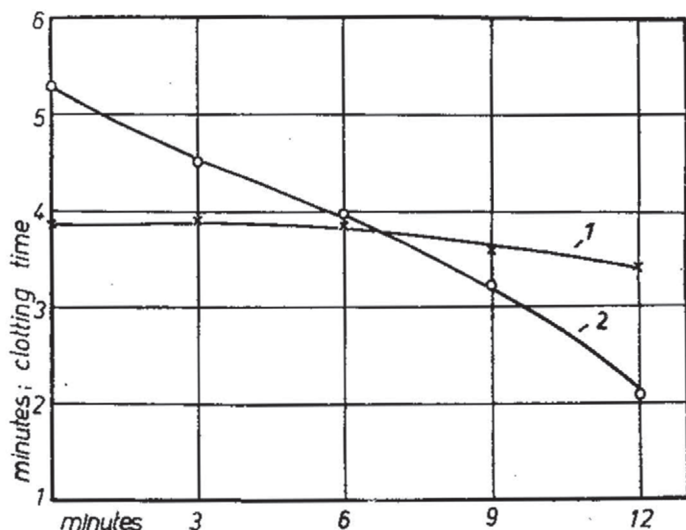

Fig. 3.

due to the addition of plasmakinin cannot be responsible for the effect. There was found no correlation between the fibrinogen quantities of plasmakinin solutions and their action on MF. The most plausible explanation remains that plasmakinin acts as kinase.

There is now increasing evidence that blood plasma contains some kind of soluble kinase. HOWELL,[4] using hemophilic plasma as a test, isolated a substance which, according to him, represents a kinase very similar in its action to the kinase in platelets or in tissues. According to FEISSLY[5] the kinase activity is connected with a viscous protein fraction of the serum. LENGGENHAGER[6] studying the clotting defect in hemophilic blood, postulated the existence of an inactive kinase present in normal plasma but lacking in hemophilic plasma. APITZ[7]

calls this plasma substance which the hemophilic plasma lacks, X-factor. WIDENBAUER and REICHEL[8] also came to the conclusion that blood plasma contained a kinaselike substance in an inactive prestadium, and whinh becomes active only when the clotting of blood starts.

It was found in our recent experiments that cattle plasma does not contain kinase whereas after clotting the serum does contain. Further experiments revealed that the kinase present in serum appears when the clotting starts and that this formation of kinase depends on the presence of Ca ions.

In view of these findings it seemed surprising that plasmakinin obtained by a simple ammoniumsulfate fractionation of plasma should act as kinase when tested with MF. The experiment in which the action of kinase and plasmakinin was compared showed that plasmakinin is not an active kinase but becomes kinase after it has been added to MF.

Comparison of the action of kinase with plasmakinin.

In the following experiment recalcified MF was allowed to stand at room temperature and at various times samples were taken out to test the activity of tissue kinase and of plasmakinin. The experimental procedure was the following: At various times samples of 1 cc. were taken out from the recalcinated MF and brought into small test tubes, one part of which contained kinase while the otthers plasmakinin solution. There were thus two series of test tubes. The tubes in one series contained 0,04 cc. of suitably diluted brain kinase and the tubes in the other 0,04 cc. of PK_1. Into the first tube of each series the MF solution was pipetted in the moment of recalcination. After 3, 6, 9, 12 minutes had passed MF was pipetted into the 2nd, 3rd, 4th and 5th tubes of each series. Thus there were tubes in which MF was added after it had stood for 0, 3, 6, 9, 12 minutes at room temperature. The appearance of the first fibrin clot in the tubes was observed and recorded as clotting time. The clotting time of MF without PK_1 or kinase was 25 minutes. The clotting times in the presence of PK_1 and kinase were much shorter as can be seen on fig. 3 where the clotting time is plotted against the time which elapsed after recalcination. Curve 1 represents the results obtained with kinase and curve 2 the results with PK_1.

In can be seen that it is almost immaterial for the action of tissue kinase how old the MF was. The clotting time in the first tube is nearly the same as in the last where MF stood for 12 minutes after recalcination. There was no substance formed to activate tissue kinase. In the case of PK_1 however the age of MF had marked effect. The older the MF solution is the more pronounced the effect. The first clotting time is much longer than the last. This shortening of the clotting time shows that in MF a substance was formed which acted on PK_1 in such

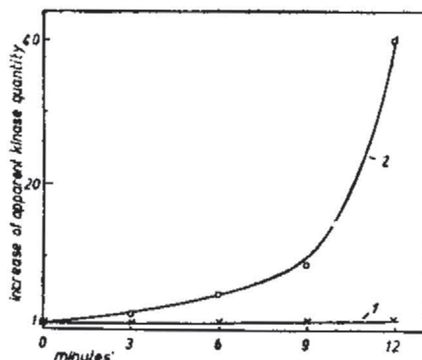

Fig. 4.

a way as if its kinase content had been increased. This apparent increase of kinase quantity is more pronounced when instead of the clotting time the apparent kinase quantity is represented. If the action of PK_1 on MF is equivalent to a certain quantity of kinase then every clotting time obtained with PK_1 corresponds to a certain quantity of kinase. This quantity of kinase can be read from the curve of fig. 1. Taking the kinase quantity corresponding to the first clotting time as a unit it can readily be calculated how many times the apparent kinase quintity has increased. If the increase of kinase quantity, calculated this way, is plotted against the time which passed after recalcination curve 2 of fig. 4 results. The data for curve 1 were calculated in a similar way: the first clotting time obtained with tissue kinase being taken as a unit, the increase in kinase quantity was calculated. As fig. 4 shows a certain quantity of kinase always represents the same quantity, independent of how old the recalcinated MF is. But the apparent kinase quantity of plasmakinin increases

according to the time which has passed after recalcination. In the light of this experiment plasmakinin seems to be an inactive substance which only gives rise to the formation of kinase upon the action of an other factor. This factor formed in MF is probably the thrombin itself, if not, it must be an as yet unknown substance, the formation of which strictly follows the formation of thrombin.

Summing up the results of the above experiment two facts emerge: a) During coagulation kinase is formed. b) For this formation of kinase the plasmakinin is responsible plus an other substance which is the thrombin or an unknown substance.

Discussion.

In view of the findings described in this paper the clotting of blood can be pictured in the following way: The reaction starts with the conversion of a small amount of prothrombin into thrombin by the action of platelets or tissue kinase. The thrombin converts fibrinogen into fibrin, parallel with this process kinase is formed from plasmakinin. This new kinase converts more prothrombin into thrombin, which is followed again by the formation of an other amount of kinase. Thus during the clotting process there is an increased formation of kinase and consequently an increased formation of thrombin too. This simultaneous formation of thrombin and kinase explains the autocatalytic type of the formation of thrombin.

The clotting system in hemophilic blood lacks this mechanism due to the lack of the plasmakinin. The addition of this substance restores the autocatalytic mechanism and with it the normal clotting.

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Adenosinetriphosphatase of muscle.

by

K. LAKI.

JACOBSEN,[1] BARRENSCHEN and LÁNG[2] demonstrated the existence of a phosphate splitting enzyme in animal tissues which exhibited great substrate specificity towards ATP. This enzyme, called ATP-ase liberates two inorganic phosphates from the ATP thus forming adenylic acid. Later T. SATOH[3] held the view that the dephosphorylation of ATP is accomplished by two enzymes, one is a pyrophosphatase producing ADP, the other is a phosphomonoesterase. One of them needs an activator while the other working at pH 9,0 needs Mg ions. LOHMANN[4] found that in crab's muscle only one phosphate is split off from the ATP. He found also that even in muscle tissues in which the ATP is broken down to adenylic acid, the washing of the muscle renders it incapable of splitting more than one phosphate group, and that the addition of Mg restores the full activity.

The ATP-ase attracted much attention when ENGELHARDT and LJUBIMOWA[5] concluded from their experiments, that myosin, the contractile element of the muscle, might be the ATP-ase itself. According to these authors the myosin splits one phosphate group from the ATP and the splitting of the second phosphate is brought about by a water soluble enzyme.

The experiments presented in this paper show that the enzymatic hydrolysis of two inorganic phosphate groups of the ATP molecule in the muscle tissue is not due to a single enzyme, but is a joint action of various factors present in the muscle. A muscle juice obtained by extracting the minced muscle with water is very active in splitting off two labile phosphate groups

from the ATP molecule. This turbid muscle juice can be separated into two parts by centrifugation. One of them is the precipitate, the other is the supernatant fluid. The phosphatase activity resides in the precipitate, the supernatant clear fluid. containing the water soluble part of the muscle, is inactive. It was found that the precipitate, when washed four or five times with water did not only lose some of its original activity but became altered in such a manner that it was able to hydrolyse only one phosphate group from the ATP molecule. It was also found that the full activity (the splitting of two phosphate groups) could be restored by bringing the precipitate and the inactive water soluble part of the muscle together.

It is clear that the insoluble muscle particles which consist mainly of actomyosin contain the enzyme responsible for the splitting of one phosphate from the ATP. The splitting of the second phosphate group is brought about by the joint action of the insoluble muscle particles and the water soluble part of the muscle. (Fig. 1.) This water soluble part of the muscle which I call aqueous extract exhibits phosphatase activity neither on ATP nor on ADP.

It can be seen from fig. 2 that the splitting of the first phosphate group has a pH-optimum at 7,4 (curve 1) and the splitting of the second phosphate at pH 8,6 (curve 3).

The analysis of the action of the aqueous extract showed that two factors were responsible for the reactivating effect of the extract: one of them is a protein like substance, the other is the Mg ion, which can be substituted by Co^{++} and Mn^{++}

Pyrophosphate inhibits the splitting of the second phosphate group.

Experimental.

Preparation of the washed muscle suspension which splits one phosphate group from the ATP.: 50 gr. of pigeon breast muscle were minced in the Latapie mincer and mixed with 150 cc. of dist. water. The mixture was allowed to stand in the cold for two hours, then it was squeezed through a cloth. The turbid fluid obtained was centrifuged and the precipitate washed in the centrifuge four or five times, each time with about 150 cc. of dist. water. The pecipitate was finally suspended in 40 cc. of 0,6 *M* KCl solution.

It often happened that even after the fifth washing the muscle suspension retained some activity in regard to the splitting of the second phosphate group. In such cases it was enough to allow the muscle suspension to stand in the ice chest in order to obtain a suspension which splits only one phosphate group.

Preparation of the aqueous extract which activates the splitting of the second phosphate group: The muscle juice, from which the insoluble particles were separated by centrifuga-

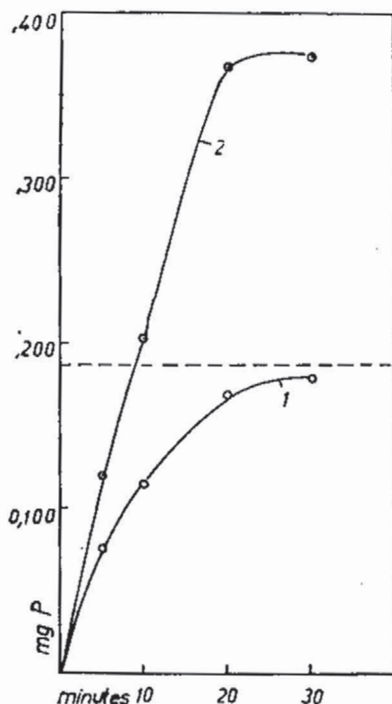

Fig. 1. Liberation of phosphate from ATP by the washed muscle suspension (curve 1), and washed muscle suspension + aqueous extrct (curve 2). The dotted line represents the quantity of P corresponding to the splitting of one phosphate group from ATP.

tion, was dialysed overnight in the cold against dist. water. After centrifugation a clear fluid was obtained which showed no phosphatase activity but restored the full activity of the above muscle suspension. So this aqueous extract contains a factor which is necessary to activate the splitting of the second phosphate group.

Fig. 1. shows the result of a typical experiment demonstrating the role of the two factors. The quantities of the inorganic phosphate marked on the ordinate are plotted against the time on the abscissa. Curve 1 shows that the muscle suspension

splits one phosphate group. From curve 2 it can be seen that in the presence of the aqueous extract two phosphate groups are split off.

The experiment presented on fig. 1 was performed in the following way: The reaction mixture was pipetted into two Erlenmeyer flasks placed in the water bath (36⁰). The composition of the reaction mixture in the flasks was the folloving: into the first flask 5 cc. of M/20 borate buffer pH 8,5, 4 cc. of water, 2 cc. of muscle suspencion and 1.5 cc. of ATP (15 mg.) solution were pipetted. In the second flask in addition to the above components 1 cc. of aqueous extract was added replacing 1 cc. of water. The flasks were gently shaken during the experiment. The reaction started when the ATP solution was added to the reaction mixture. The reaction was followed by the estimation of the inorganic phosphate liberated. For this purpose from time to time 2,5 cc. were pipetted out of the reaction mixture into test tubes containing 1 cc. of 20% trichloroacetic acid. Then the solution in the test tubes was filtered and the inorganic phosphate was estimaed in the filtrate.

In all the experiments presented in this paper the inorganic phosphate was determined by the FISKE and SUBBAROW method with the modification of LOHMANN and JENDRASSIK.

Inhibition of the splitting of the second phosphate group. It was found that pyrophosphate had a strong inhibiting effect on the dephosphorylation of the ATP. Pyrophosphate in *M*/100 concentration prevented the liberation of the second phosphate group, but it had no effect on the splitting of the first phosphate group. The inhibiting effect of pyrophosphate can be paralysed by the addition of Mg.

Effect of pH on the dephosphorylation of the ATP. It can be seen from fig. 2 that the splitting of the first phosphate group accomplished by the muscle suspension has a pH optimum at 7,4 (curve 1). When two phosphate groups are hydrolysed the pH optimum is at pH 8,6 (curve 2). Subtracting curve 1 from curve 2, curve 3 results. This curve represents the activity —pH curve of the action of the aqueous extract.

The experiments which gave the results shown in curve 1 and 2 were performed in test tubes. Two sets of test tubes were used. The first set conisted of 8 tubes. Into each tube the felowing solutions were pipetted: 1 cc. of veronalacetate buffer with different pH-s, 0,3 cc. of the muscle suspension, 0,5 cc. of *M*/20 Na-pyrophosphate solution adjusted prior to the pH of the buffer solutions, and 0,4 cc. of water. The rôle of pyrophosphate in these experiments was to assure that no second phosphate splitting occured. In the second set of test tubes the reaction mixture was similar to that of

the first except that pyrophosphate was not added, but there was added 0,3 cc. of aqueous extract instead to achieve the splitting of two phosphate groups. The total volume of the solutions was brought up to 2,2 cc. by the addition of the necessary volume of water. The tubes were placed into the water bath (36°). After temperature equilibrium was reached to each tube 0,3 cc. of ATP (3,8 mg.) solution was pipetted. The reaction time was 16 minutes. Then the reaction was stopped by the addition of 1 cc. of

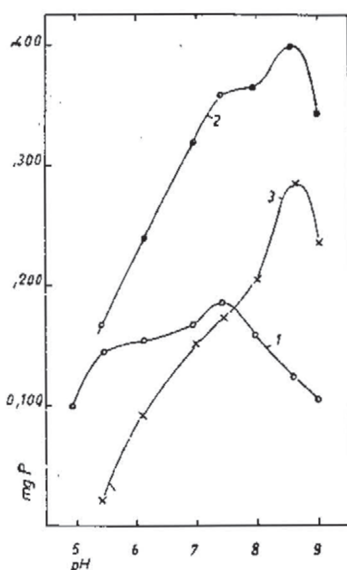

Fig. 2. The action of pH on the liberation of phosphate when only one phosphate group (curve 1), and when two phosphate groups are split off from ATP (curve 2). Curve 3 results by subtracting curve 1 from curve 2 and thus reprecents the activity pH curve of the oqueous extract.

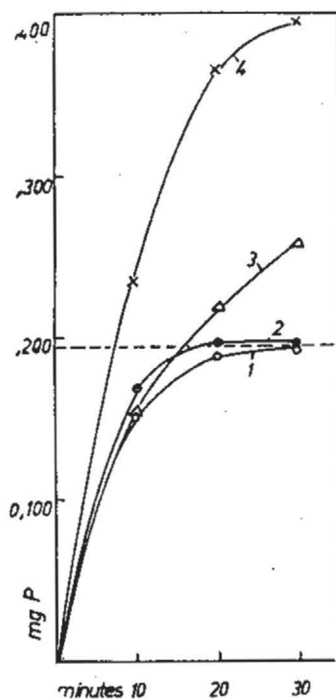

Fig. 3. The rate of liberation of phosphate from ATP by the muscle suspension (curve 1). Curve 2 represents the same in the presence of Mg, curve 3 in the presence of the aqueous extract and curve 4 in the presence of Mg and the aqueous extract together. The dotted line represents the quantity of P corresponding to the splitting of one phosphate group from ATP.

20% trichloroacetic acid. After filtration the inorganic phosphate was determined. In fig. 2 the curves are drawn by plotting the phosphate quantities against the pH-s at which the reaction proceeded.

Purification of the aqueous extract. 220 gr. of pigeon

breast muscle were minced in the Latapie mincer, then 440 cc. of dist. water was added and the mixture was allowed to stand in the cold. After four hours it was squeezed through a cloth. The 410 cc. of turbid fluid were centrifuged. To the supernatant fluid (400 cc.) 40 cc. of *N* acetic acid were added and the solution placed in a water bath (36°) for 15 minutes. 37,5 cc. of *N* KOH were then added and the great quantity of precipitate formed was discarded. To the clear reddish fluid ammoniumsulfat was added to reach a 0,6 saturation. The solution was filtered through fluted filters and the precipitate discarded. By the addition of more ammoniumsulfate to the solution the saturation was brought to 1,0. The precipitate, which contains the active substance, was collected on the filter paper and dissolved in 30 cc. of dist. water. The reddish solution was dialysed overnight against dist. water in the cold and then used for the experiments.

The activating effect of magnesium ions. The experiment, presented in fig. 3 shwos that the aqueous extract has almost lost its activity by this purification process (curve 3) and that it activates the muscle suspension only in the presence of Mg ions (curve 4), thus demonstrating that the activating effect of the original aqueous extract is due to two factors: one of them being a protein, the other the Mg ion. Curve 2 shows that Mg has no effect on the splitting of the first phosphate group. (The slight activation is very likely due to incomplete removal of the protein component of the extract). The Mg must be bound to the protein because it cannot be removed even by prolonged dialysis and the purification procedure was needed to remove it from the protein. Co, Mn ions can substitute the Mg ions in the same concentration, but Fe, Ni, Cu, Cn cannot: they even inhibit the reaction.

The experiment presented in fig. 3 was performed in Erlenmeyer flasks. Four flasks were used each containing the following solution: 5 cc. of borate buffer pH 8,5, 2 cc. of muscle suspension, 1,5 cc. of ATP (15,2 mg) solution which was added to the reaction mixture only when temperature equilibrium was reached. The addition of ATP marked the starting of the reaction. In addition to the above solutions 1,2 cc. of *M*/100 $MgCl_2$ solution were added to the second and fourth flask, 1,5 cc. of ten times diluted purified aqueous extract to the third and the fourth flask. With dist. water the total volume in each flask was adjusted to 12,5 cc.

At the 0, 10, 20 and 30th minute 2,5 cc. samples were removed from the flasks and the phosphate determined as above.

Summary.

The insoluble muscle particle in itself is only capable of splitting one phosphate group from the ATP molecule. The splitting of a second phosphate group is due to the joint action of the insoluble muscle particle, a soluble protein and Mg ion.

The splitting of the second phosphate group can be inhibited by the addition of pyrophosphate.

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Actin. II.*

by

F. B. STRAUB.

I. Introduction.

An improved method for the preparation of actin from rabbit's muscle is described in the present paper. During the course of these investigations it has been found that actin may exist in two different forms. In the absence of salts it is present in solution as *inactive actin,* in the presence of salts as *active actin.*

Both forms of actin would combine with myosin, but the properties of the resulting actomyosins are different. Active actin, when added to myosin, forms the actomyosin, the characteristics of which have already been described in earlier communications.[1] This is the actomyosin, which has a high viscosity and which reacts, on adding ATP, with a decrease of viscosity, the decrease being proportional to its actin contents. For the sake of clarity we shall now term this compound, *active actomyosin,* in contrast to the complex of *inactive actomyosin,* which is formed when inactive actin and myosin are brought together in salt solution. This latter complex has a viscosity practically equal to that of the myosin which is present in it. The viscosity of an inactive actomyosin is not influenced by the addition of ATP.

Inactive actin is easily transformed into active actin by the addition of small amounts of any salt which does not destroy the protein. Active actin partly reverts into inactive

* This work was aided by a grant from the Duke ESTERHÁZY.

actin on dialysis under certain conditions. Owing to the destruction of actin during dialysis, this tansformation has not yet been realized in a quantitative manner. But there is no doubt that the reaction: inactive actin $\rightleftarrows$ active actin is a reversible one.

Profound changes are observed in viscosity and in double refraction of flow during the transformation of inactive actin into active actin.

On addition of salt, the viscosity of an inactive actin solution rises rapidly and the readily flowing thin solution of inactive actin sets to a gel of active actin.

The viscosity of an inactive actin solution is very low compared with that of active actin. We found in one case the specific viscosity to be 0,032 for a 4 mg/ml solution of inactive actin, in an other case 0,03 for 3 mg/ml. In many other cases somewhat higher values were obtained. The variations must be ascribed to the presence of small amounts of active actin.

As the determination of active actin in small amounts can be made only with considerable error, we cannot state definitely what the viscosity of a completely inactive actin may be. It is either identical or lower than the values given above.

The viscosity of hemoglobin is somewhat lower, that of serumalbumin somewhat higher than the viscosity of inactive actin. Thus, by its viscosity, inactive actin is classed among the globular proteins.

Active actin, on the other hand, has a very high viscosity which equals the viscosity of polymer substances, such as nitrocellulose and rubber. It has therefore an extremely assymetrical molecule of considerable length.

The change of shape of the molecule during the activation of actin is quite unique. A trace of salt changes a globular protein into a highly assymetrical, fibrous one.

The great rise in viscosity of actin during its activation is partly due to the elastic anomaly of the viscosity of active actin. The relation between the viscosity and the pressure in the viscosimeter has been studied in the capillary viscosimeter previously specified.[1] A 12 l flask of air served to maintain uniform pressure during the measurement; the pressure was read on a water manometer. The results are shown in Fig. 1.

Even in dilute solution and at rather high pressures the orientation of the molecules is not yet complete. Contrary to earlier statements,[1] the results presented in Fig. 1. show that actin is not thixotropic; readings at different pressures were taken at random and yet no effect of previous treatment could be found.

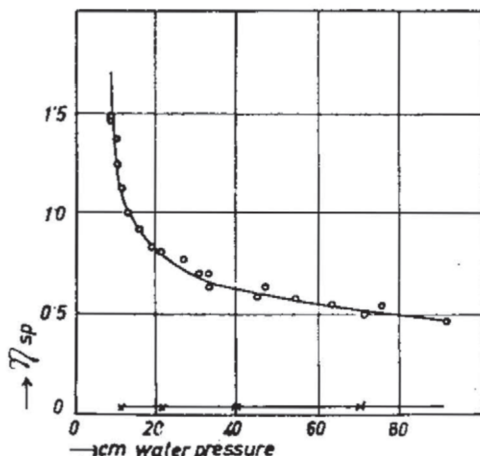

Fig. 1. Viscosity of inactive and active actin at different pressures. o——o 2 mg/ml active actin in 0,2 M KCl. ×——× 3 mg/ml inactive actin in dist. water at 0°.

That inactive actin is a globular protein whereas active actin consists of rod-shaped particles is born out by the study of the double refraction of flow of actin solutions.

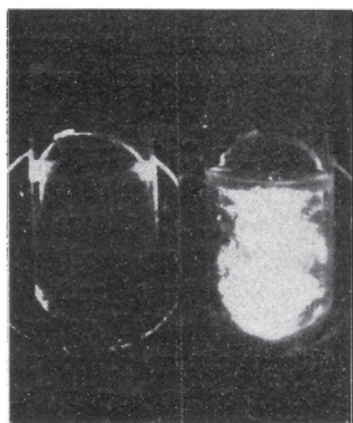

Fig. 2. Double refraction of actin solutions. Photographs taken through crossed nicols. Left: inactive actin. Right: the same solution after activation.

If a solution of inactive actin is sucked through a capillary and the rapidly moving fluid is observed between crossed nicols.

there is no double refraction of flow to be observed. Active actin has, on the other hand, a very strong double refraction of flow. Furthermore, a solution containing 3—5 mg active actin per ml remains doubly refracting more than half an hour after it has been gently stirred. (Fig. 2.)

It is obvious from these observations that the actin preparations described in the earlier communication[1] were solutions of active actin with occasional admixture of some inactive actin.

The transformation: inactive actin ——→ active actin, which we will call the ***activation of actin,*** may be brought about by any salt which does not destroy the protein. Non-ionizable substances have no effect on inactive actin. With the method to be described later in this paper, we have studied the velocity and the extent of the activation under different conditions. It was found:

1. that the activation proceeds to the same end point regardless of the nature of the salt concerned and of the temperature and pH, or — beyond a certain limiting concentration — of the concentration of the salt;

2. that the velocity of the transformation depends a/ on the purity of the actin, b/ on the concentration of the actin, c/ on the concentration of the salt, d/ on the temperature, e/ on the pH and f/ on the nature of salt used.

The conclusions which may be drawn from these results are the following. As non-dissociating substances have no effect on actin, the activation must be ascribed to ***ions.*** The concentration of salt needed for the same activation is inversely proportional to the ***charge*** of the positive ion.

About 10 times more KCl is needed than $CaCl_2$ to produce the same effect. Of the alkali chlorides the velocity of activation increases in the order: $Rb < K < Na < Li$, if all are used in the same concentration. Of the haloids, if all are used in the same concentration as Na salts, the velocity of activation increases in the order: $J < Br < Cl < F$. Thus NaF activates very fast, KJ very slowly. The effect of the charge of the anion is opposite to the effect of the charge of the cation, e. g. sulfates activate much slower than chlorides. Hydrogen ion is a more potent activator than any other ion, there is instantaneous activation when the pH is lower than pH 6.

II. Method of preparation.

The main features of the method previously described[1] have been retained. Slight alterations, however, were introduced so as to obtain the actin in the inactive form.

Rabbit's muscles from the back and from the legs are rapidly excised and cooled immediately by packing them into ice. They are minced first through a cold meat chopper (diameter of holes 2 mm) and then through a cold Latapie mincer. 300 ml of an alkaline KCl solution* are added to every 100g of the mince and stirred mechanically together at 0° for 20 minutes. The mixture is then centrifuged and the supernatant fluid, which contains the greater part of the myosin and some actin, is discarded. The residue is left to stand at 0° for 24 hours. At the end of this period it is weighed and mixed with 5 volumes of its weight of distilled water at room temperature. After standing for 1 hour at room temperature, the mixture is centrifuged. The washing of the residue with distilled water at room temperature is repeated, with the same amount of water as before, again standing for 1 hour. After this second washing the muscle residue is treated with 4 volumes of acetone at room temperature. After 20 minutes' standing at room temperature, the acetone is removed by pressing it out through a filter cloth. The residue is now mixed with a fresh lot of acetone (1/4 of the former volume) and left to stand again at room temperature for 20 minutes. The acetone is again pressed out and the residue is spread over filter paper and left to dry.

The acetone dried muscle powder (after 10—15 hours of drying) is extracted with 20 volumes of CO_2-free distilled water at room temperature. Neither grinding of the muscle powder before the extraction, nor grinding nor stirring during the extraction is advantageous, as in either case the solution will become opalescent and, moreover, the actin will be partly or mostly activated. Therefore the muscle powder is mixed with the extracting water and then left to stand alone at room temperature for 10—15 minutes. At the end of this

* This is prepared by mixing 800 ml 0,1 *M* potassium borate with 200 ml 2 *M* KCl. — The potassium borate solution is made by dissolving 12,4 g boric acid in 100 ml *N* KOH, then making the solution up to 1 l with dist. water.

period the resulting pulp is poured in to a Buchner funnel and the solution is sucked off. It contains the actin in its inactive form. The protein content varies between 3—6 mg/ml. The purity of the actin in such a solution is mostly maximal (1,0)* but rarely below 0,7. If the purity of the preparation is not maximal, it cannot be purified any further. It has been found that the purification procedures described in the previous paper[1] do not lead to purification but to the activation of the actin. which was in an inactive form.

The yield in this new procedure is not very high. It might be increased by a more thorough extraction of the dry muscle. Yet in such cases the resulting solution will be opalescent, and the actin in it will become activated. Therefore higher yields have been sacrificed for the advantage of obtaining inactive actin in clear solution.

Solutions of the actin show varying degrees of stability. Inactive actin is less stable than active actin. The former would lose on the average 10—20 % of its potential activity in 24 hours at 0°.

Actin of the maximal purity has been analysed by DR M. KOVÁCS-OSKOLÁS. She found:

51,3 % C, 8,6 % H 15,09 % N

The ash content was 1,1 %. After wet ashing the P content was determined colorimetrically. Less than 0,07 % were found. These results indicate the actin to be a protein. The orcin test for pentoses is negative, the Millon and Pauly tests are positive.

III. Determination of active and inactive actin.

In the previous communication a method for the determination of actin has been described.[1] The method was based on the determination of the activity of the actomyosin, which results when the actin solution in question was mixed with a certain amount of myosin. The definition of the activity of actomyosin and its determination have been described in the same paper. The technique used there was followed in the experiments described in the present paper. Viscosity was

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* The purity of actin is maximal (1.0) if 1 mg of it gives with 5 mg pure myosin a 100% active actomyosin.

always determined in a 0,6 *M* KCl solution which contains a veronal-acetate buffer of pH 7. Temperature: 0°.

When using a solution of crystallised myosin,[2] the formation of actomyosin was found to be the same as with the impure myosin solutions, used in earlier investigations. It was found, however, that of the purest actin preparations 1 mg is needed to activate 5 mg of myosin to 100 % activity. (With impure myosin we found a ratio of 1:6. The discrepancy is due to the fact that the myosin contents of impure myosin solutions were estimated wrongly.)

The method of determination of actin in solution, referred to above, is a determination of the active actin content only. The total actin content (active + inactive) can be determined only if the actin solution is first activated by salt solution. This is shown by the following observations.

If a solution of inactive actin is mixed with myosin, there is no rise in the viscosity of myosin (the viscosity of the added inactive actin being negligible compared with that of the myosin). If ATP is added, there is no decrease of viscosity. If, however, the inactive actin solution is first kept at room temperature (22°) in presence of at least 0,1 *M* KCl for 15 minutes and after that added to the myosin, the viscosity will be found to be high and ATP causes a drop of viscosity almost to the level of the viscosity of the myosin present in the solution. This is illustrated by the following experiment (Table I.):

Table I.

	Relative viscosity	
	alone	with ATP
6 mg myosin + 0,3 ml inactive actin, made up to 6 ml with 0,6 *M* KCl	1,30	1,30
6 mg myosin + 0,3 ml of the same actin after it has been standing at 22° in 0,1 *M* KCl for 5 minutes, made up to 6 ml with 0,6 *M* KCl	1,89	1,39

The result is somewhat surprising in view of the fact that during the determination of the viscosity there is 0,6 *M* KCl in

both solutions, which could at least partially activate the actin at 0°. It follows therefore that a combination must have taken place between inactive actin and myosin and that this combination prevents the inactive actin from becoming activated.

As an explanation of the effect of ATP on the viscosity of actomyosin it has been suggested that ATP splits the complex into its components: myosin and actin. For the case of the inactive actomyosin this is supported by the following observation.

18 mg myosin were mixed with a solution of actin, which contained the actin mostly in its inactive form. KCl was added to bring the KCl concentration to 0,6 *M*. The volume was then 4,5 ml. This mixture was divided into three parts. One part of it was immediately diluted with 0,6 *M* KCl to 6 ml and the activity of the actomyosin was determined. The other two samples were left to stand at room temperature for 1 hour, one of them with the addition of 0,05 mg ATP. During this time the small amount of ATP added was obviously completely split by the myosin, as addition of fresh ATP caused a marked decrease in viscosity. The determination of the viscosity was made in all cases at 0°.

Table II.

	Relative viscosity		% activity of actomyosin
	alone	with ATP	
6 mg myosin + 2 mg inactive actin determined immediately	1,325	1,25	30
Same after 1 hour at 22°	1,415	1,325	25
Same + 0,05 mg ATP after 1 hour at 22°	1,755	1,303	138

The activation of the inactive actin in the experiment, which contained ATP, is no doubt due to the fact that ATP has split the complex of inactive actomyosin, setting free the actin thus making it accessible to the activating effect of the salt. This effect is very likely instantaneous but it cannot be measured experimentally as one has to wait until the ATP is completely split, before one can measure the viscosity and the activity of the actomyosin.

As, according to the former experiment, there is no

activation of the inactive actin under the experimental conditions prevailing during the determination of actin, it follows that only the active actin content is determined by the method.* If we want to know the sum of active + inactive actin, we have first to activate all the actin. This was usually achieved by adding KCl in 0,1 *M* concentration to the actin solution and keeping it at 22° for 10—15 minutes. A determination of the actin after this treatment will give the total actin contents. An example of such a determination is shown in Table III.

Table III.

	Relative viscosity		% activity of actomyosin
	alone	with ATP	
5 mg myosin + 0,3 ml actin "258"	1,545	1,31	69
5 " " + 0,3 " activated actin "258"	1,87	1,313	164
5 " " + 0,15 ml " " "258"	1,464	1,225	114

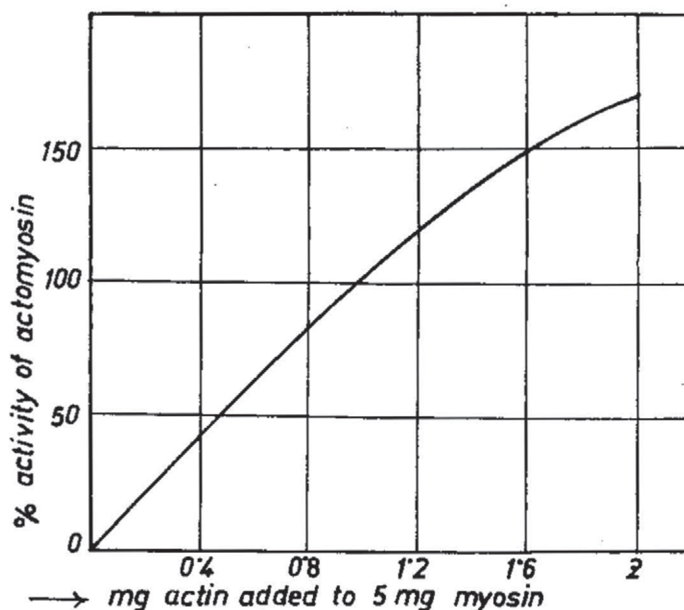

Fig. 3.

* Naturally, if an excess of inactive actin is added to the myosin, e. g. more than 3 mg to 5 mg myosin, it will become partly activated as the myosin will not be able to bind it completely.

To evaluate these data, we have to refer to the curve of Fig. 3. which shows the activity of the actomyosin as the function of the amount of active actin, added to 5 mg myosin. The curve was determined by using crystallised myosin and actin of the highest purity. It is seen from this curve that 0,67 mg actin are needed to produce a 69 % active actomyosin, and 1,15 mg to produce an 114 % active actomyosin from 5 mg myosin. It follows that the actin preparation „258" contains $\frac{0,67}{0,30} = 2,23$ mg/ml active actin and the total actin content is $\frac{1,15}{0,15} = 7,70$ mg/ml The active actin content of the preparation is therefore $\frac{2,23}{7,70} \times 100 = 29$ %, whereas the remaining 71 % is inactive actin. We say that in this preparation there is 29 % active actin.

It might be mentioned here that a given actin solution, which is partially active, gives varying activity according to the temperature at which it was kept before the determination. An example of this is found in Table IV.

Table IV.

	% active actin found
Actin "275" kept 1 hour at 23°	33
Same „ „ „ „ 35,5°	17

This is due to the fact that there is an equilibrium between the salts present and the fraction of salt bound to the actin. This equilibrum changes with the temperature and thus the active actin content changes also. If the actin solution is brought to another temperature, the new equilibrium will be reached only after considerable time. For this reason we made the activity determinations on partially active actin only after at least 2 hours standing ot 0°.

There is an even more pronounced change in the activity of a partially active actin solution after it has been frozen. Even a nearly completely inactive solution will appear to be completely active after it has been frozen and then left to thaw. If the solution is then kept at 0° this apparent activity

will be lost within one or two hours. The phenomenon may be repeatedly observed, that after freezing and thawing there is always a high activity to be found. During freezing there is a local increase in the concentrations of both actin and salts, which leads to activation. When the ice has melted, the thermal equilibrium is reached only after a considerable time.

IV. The factors influencing the activation of actin.

The endpoint of activation. That an actin preparation is activated to the same endpoint regardless of the nature of the salt used, is shown in the following table. A „100 % active actin“ means the active actin content which was found when activated with KCl. Rather high concentrations were used to obtain, as far as possible, real endpoints of activation.

Table V.

2 mg/ml actin during activation, pH 7,0 Temperature: 22°			
Salt	final concentration of salt, Mol/liter	incubation time, min.	% active actin found
KCl	0.2	15	(100)
„	0,2	120	100
LiCl	0,4	30	102
NaBr	0,4	30	100
NaJ	0.1	60	95
„	0,5	60	0
$CaCl_2$	0,02	15	92
Na_2SO_4	0,2	15	48
„	0,2	120	92
NaF	0,2	30	98

$CaCl_2$ gives a somewhat lower value in spite of fast activation, this is due to a visible denaturation. The reason why 0,5 *M* NaJ fails to activate at all, will be discussed later.

Time curve of activation. The velocity of activation depends upon the concentration of the salt. If a low concentration of salt is used, the time curve of activation can be studied. It is found that after a rapid rise the activation is considerably slowed down and the end point is reached very slowly. This fact points to the multimolecular nature of the reaction. (Fig. 4.)

Temperature effect. Table VI. shows the rapid rise in activation velocity by increasing temperature. 2 mg/ml actin in 0,05 *M* KCl were incubated at the temperature indicated and after 5 minutes 0,6 ml of this solution were added to 5 mg myosin and rapidly cooled to 0° at which temperature the determination of activity was done.

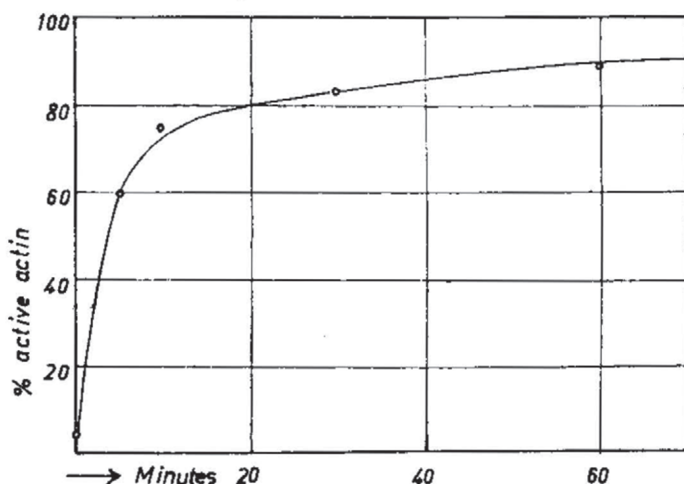

Fig. 4. Activation time curve in 0,02 *M* NaF at pH 6,5 and 35,5°.

Table VI.

Temperature	% active actin found
0	4
12	70
20	82
30	100
35,5	100

Effect of salt concentration. It is almost impossible to get a true picture of the effect of salt concentration because the endpoint and the velocity of activation both vary with the salt-concentration. It has already been shown that the endpoint is reached very slowly with smaller concentrations of salt. Therefore we believe that the curves presented in Fig. 5. do not express the true activation endpoints at smaller salt concentrations, the points indicated by the experiment being probably too low.

Activation by H ions. To 5 ml of an inactive actin solution various amounts of an 0,01 *N* HCl solution were added. The pH was determined colorimetrically and the % active actin content was determined with the usual procedure after the mixture had stood 15 minutes at room temperature. As the

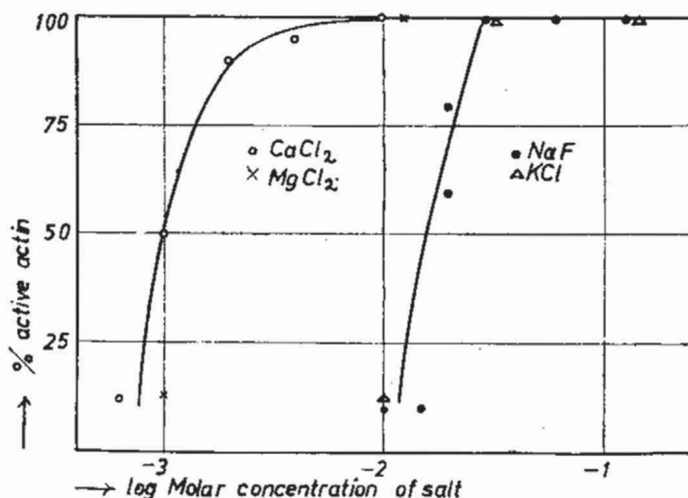

Fig. 5. Effect of concentration of salt on the activation of actin. 2,5 mg/ml actin incubated at 35.5° with salt of the concentration indicated on the abscissa. pH 7,4. The % active actin content was determined after 60 minutes incubation.

myosin solution used for the determination was buffered, the acidified actin solution was not neutralised before adding it to the myosin. During the determination of the activity of the resulting actomyosin the pH was 7.

Table VII.

Actin incubated for 15 min. at 22° at pH	% active actin found
7,0	6
6,0	20
5,7	75
5,3	100

The reversibility of the activation. If addition of salt causes activation, it could be expected that removal of the salt would effect inactivation of active actin. In spite of this, if

an actin solution, which contains just enough salt to keep its actin content in the activated form, is subjected to dialysis at 0°, no inactive actin will be formed. There will be a loss in the total actin content, but what remains is active actin. Inactive actin is however formed during dialysis if the actin solution is first made alkaline. An actin solution which contained 70 % of the actin in the active form, was mixed with $^{1}/_{10}$ of its volume of 0,1 *M* borate buffer of pH 10 and dialysed against CO_2 free distilled water for 24 hours. The pH of the dialysed solution was adjusted to pH 7. It contained only 2 % of the actin in its active form, the rest being inactive actin, which could be activated by the addition of salt. The total actin content decreased by 30 % during dialysis.

V. Secondary salt effects.

Any salt, if given in great excess over the concentration which brings about the activation of actin, will precipitate the protein from its solution. KCl will precipitate over 2 *M*, $CaCl_2$ and $MgCl_2$ already precipitate at 0,002 *M*. The precipitation usually does not set in immediately and it is always connected with partial denaturation.

If we add a KCl solution to a precipitate of actin, caused by the addition of small amounts of Ca ions, the precipitate may be redissolved. If the operations are not carried out rapidly enough, this redissolution will be incomplete and there will be a loss of activity.

Actin is precipitated from its solution at pH 5. This precipitate is redissolved by neutralizing the precipitate to pH 7. Minute amounts of salts, even KCl, if present during this precipitation, will cause denaturation of the actin.

Addition of dilute acid to the actin will first bring about its activation, the solution will become highly viscous, but transparent. Addition of more acid will cause precipitation, and of still more, dissolution of the precipitate. It is interesting to note that if the precipitate is only just dissolved by acid, it will not be viscous, neither does it show any double refraction of flow, comparable to that of the solutions which are obtained if the precipitate is dissolved by adding alkali. Actin dissolved

in acid is very sensitive towards anions: chlorides cause immediate denaturation, whereas phosphates do not precipitate.

At neutral pH the iodides like NaJ show an exceptional behaviour. At concentrations up to 0,1 *M* NaJ activates like any other salt. At 0,5 *M* it does not activate at all.(See Table V.) If an actin solution is activated by the addition of 0,1 *M* KCl and then NaJ is added to the solution to give a 0,5 *M* solution of NaJ, within half an hour, at room temperature, all activity of the actin is abolished. There is, however, no precipitation of protein. The viscosity and the double refraction of flow of the active actin disappear together with its ability to form active actomyosin. If an actin in 0,5 *M* NaJ was diluted 5 times or if the excess of salt was removed by dialysis, the inactivation was not reversed or if there was some reversal, this was only to a very small extent.

My thanks are due to Mr K. BALENOVIĆ for his helpful cooperation during part of this work.

References.

1. *F. B. Straub,* These studies **2, 3** (1942).
2. *A. Szent-Györgyi,* These studies **3** (1943).

On the specificity of the ATP-effect.*

by

F. B. STRAUB.

Actomyosin, in 0,6 *M* KCl, is split into actin and myosin by the addition of small amounts of ATP. This is evidenced by the fall of the viscosity, the original high viscosity returning if the ATP is removed. We find that the same effect is also produced by inorganic pyrophosphate. This is shown in Table I. Adenylic acid, ortophosphate and metaphosphate were found to be without any effect. ADP did not show any effect if pure myosin and actin were used for the preparation of actomyosin, it was however active, if impure myosin and actin were used. This is most likely due to contamination of myosin in the latter case by the enzyme system, which brings about the transformation of ADP into some other compound with a pyrophosphate group.

Table I.

Added substance	Relative viscosity of actomyosin in 0,6 *M* KCl at 0°
None	1,70
0,0007 Mol/lit ATP	1,28
0,00002 „ „	1,28
0,0006 „ Na-pyrophosphate	1,28
0,00006 „ „	1,29
0,00001 „ „	1,375
0,000003 „ „	1,425

There is some difference between the effect of ATP and inorganic pyrophosphate. Whereas the effect of ATP is almost

* This work was aided by a grant from the Duke ESTERHÁZY.

instantaneous, the effect of pyrophosphate takes some time to develop, especially so at smaller pyrophosphate concentrations. As pyrophosphate is not split by the myosin, its effect is permanent, in contrast to the effect of ATP, which is soon abolished, due to the splitting of ATP by the myosin. If, however, the actomyosin, to which pyrophosphate was added, is precipitated and washed with dilute saline solution, pyrophosphate can be removed and the actomyosin shows again a high viscosity. Thus the effect of inorganic pyrophosphate is reversible.

The action of pyrophosphate depends on the temperature. At 0° and at 6,5° it acts like ATP in very dilute solutions. At 23° however, inorganic pyrophosphate has no effect at all, even in 3.10^{-3} *M* concentration. ATP is fully active not only at this temperature, but at 37,5° also.

If 6.10^{-4} *M* inorganic pyrophosphate is added to a solution of actomyosin at room temperature (22°), there will be no change in its viscosity. If this solution is cooled to 0° its viscosity will become low, just as if ATP would be present. Bringing the solution back to 22°, its viscosity will rise again and can be lowered by the addition of ATP.

From these data it appears that it is the pyrophosphate group of the ATP which is responsible for the viscosity effect. It might therefore be concluded that adenosinediphosphate, prepared through dephosphorylation of ATP by myosin, does not contain a pyrophosphate residue.

It may also be concluded that the splitting of ATP is not involved in its viscosity decreasing effect, since pyrophosphate has the same effect and is not split by myosin.

DR T. ERDŐS has found in this laboratory (unpublished) that on addition of inorganic pyrophosphate actomyosin threads do not contract at 1,3°, whereas addition of ATP brings about their contraction.

Observations on myosin and actomyosin.

by

F. GUBA.

1. *Comparative Study.*

I compared the behaviour of the striated muscle of different animal species during extraction. I used the muscle of the rabbit, pigeon (breast muscle), frog (Rana esc.), fish (amiurus nebulosus), cattle, hen (leg and breast) and the heart of the rabbit. The muscles of the freshly killed animal were quickly cooled, minced on a cooled Latapie mincer, suspended in 0,6 *M* KCl, 3 ml being taken per g of muscle. The suspension was stirred at 0° C and then centrifuged. My problem was to see how much myosin is extracted and how active it is when the time of extraction is varied. The results are summed up in the two curves.

Fig. 1a. and 1b. show the relation between activity and the time of extraction. As can be seen the extract of rabbit muscle shows little activity during the first 15 min. and begins to rise after this time. This rise is continuous and after 3 hours the rabbit (as well as the pigeon) muscle has given a 120% active myosin. The activity of frog muscle extract rises steep in the beginning to remain unchanged afterwards. Very remarkable is the behaviour of the fish which gives in 20 min. a 120% active myosin. Other fishes (esox mucius) behave in like manner. The actin thus releases the stucture very easily. This might explain the easy digestibility of fish meat.

Fig. 2a and b. shows the relation between the time of extraction and myosin content of the extract. These fig. show

that the rabbit is an especially favorable object. The values obtained in 20 min. in the other animals seem to lie close to

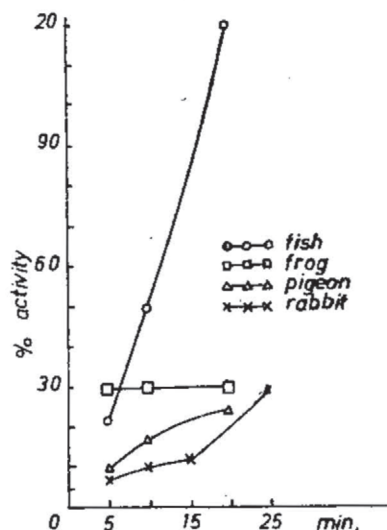

Fig. 1. a.

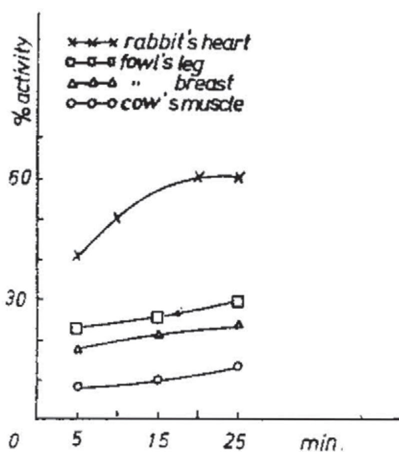

Fig. 1. b.

the limit, for, in the pigeon even after 3 hours, no more than 9 mg per ml was extracted.

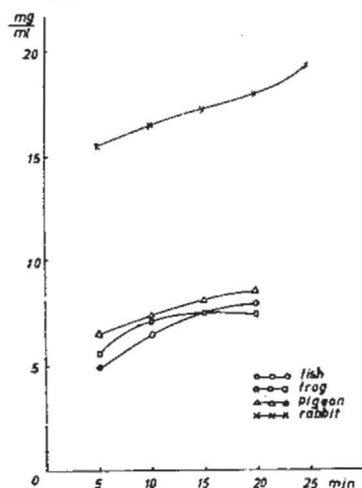

Fig. 2. a.

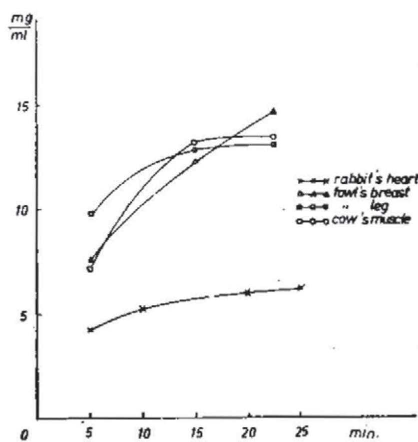

Fig. 2. b.

2. KCl curve and activity.

In these experiments I compared the viscosity of myosins of different activity — thus different actin content — at varied

KCl concentration with and without the addition of ATP. The 10% and 90% active myosin were prepared directly from muscle. The 50 and 150 % myosin were prepared by mixing actin and myosin. My solutions contained 1,6—1,8 mg myosin per ml. The pH was stabilised with veronal acetate buffer of pH 7 (see BALENOVIĆ and STRAUB[1]). The solution contained 0,001 M $MgCl_2$. To 4 ml of fluid 0,05 ml of a 1,2 % ATP solution

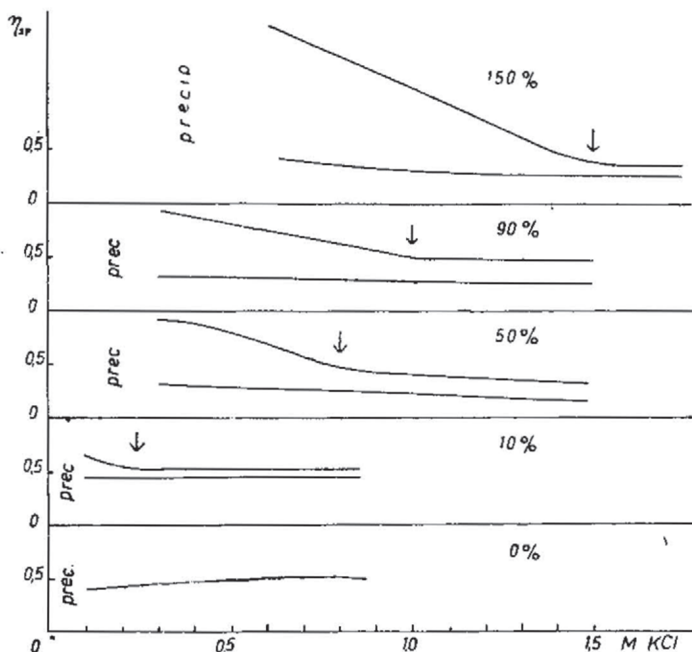

Fig. 3.

was added. Foaming was prevented by the addition of one drop of caprylic alcohol. The viscosity was determined in the capillary viscosimeter. The time of outflow of the salt solution varied between 70 and 120 sec. First the viscosity was determined without and then with ATP. Temp. 0°.

My results are summed up in Fig. 3. The blank zone on the left means precipitation. In every case the lower line is the ATP curve.

In the case of 0% active myosin ATP had no effect and the two curves were identical. F. B. STRAUB[2] found that ATP at a lower pH greatly affects the viscosity of myosin A. At that time, when those experiments were made, however, we

were not yet able to prepare entirely inactive myosin. Myosin A was not quite inactive; it had only a low actin content. Now, repeating these experiments with completely inactive myosin, ATP is found to have no effect at all.

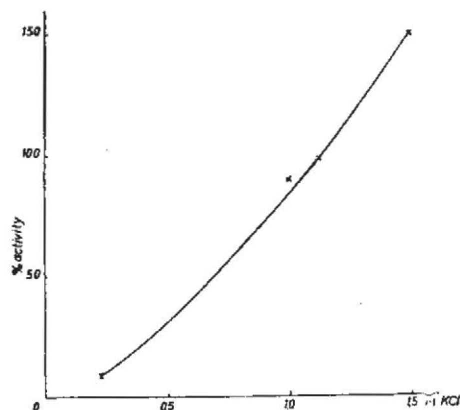

Fig. 4.

The other curves show that at a high KCl concentration the two curves lie close to each other and are parallel. A high

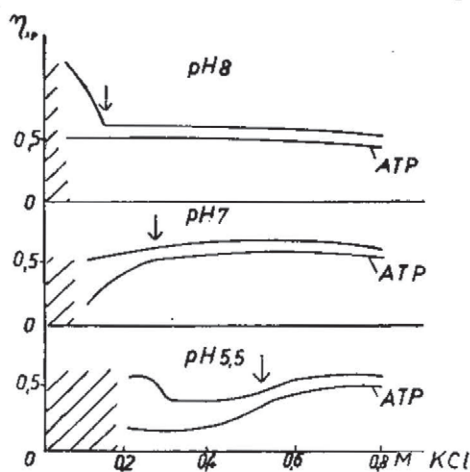

Fig. 5.

salt concentration has thus the same effect as ATP, i. e. it is capable of dissociating the actomyosin.

The curves show that according to the activity (actin content) of the actomyosin, the parting of the two curves lies at different KCl concentrations i. e. actomyosins of different

activity are dissociated by the salt alonce at different concentrations. This means that actomyosins of different % composition behave as individuals. The less actin they contain the less KCl is needed for their dissociation.

If the points, at which the two curves part, are plotted against the activity, Fig. 4. is obtained which is, in fact, the curve of complete hydration and dissociation of actomyosins of different actin content.

In the case of 10% active actomyosin I also studied the effect of pH (see curve Fig. 5.) I found that higher pH shifts the point of parting to the left, lower pH to the right. At pH 8 this point lies at 0,15 *M* KCl, at pH 5,5 at 0,6 *M* KCl. In both cases the pH was stabilised by a veronal acetate buffer.

As a practical consequence we may deduce that the measurement of activity of actomyosins of low actin content can be made with advantage at low KCl concentration and at lower pH, which, so to say, magnifies the effect of ATP on viscosity.

The theoretical consequence of these curves is still more interesting. We have seen that the 10% active myosin behaves, in absence of ATP, as an individual and not as myosin plus actomyosin. This 10% active actomyosin contains only 1,6% actin to 98.4% myosin. It can be concluded herefrom that actin is capable of coating itself with several layers of myosin. Myosin in itself has a tendency towards coaxial association and the actin seems to act as the nucleus of these associated particles. The force, by which actin holds this great mass of myosin can be but small because 0,25 *M* KCl suffices to cause dissociation. As has been shown elsewhere (F. B. STRAUB[3]) maximum activity is 170%. Also fermentatively this complex is the most active (BANGA[4]). It corresponds to the natural actomyosin of muscle and contains actin and myosin in the proportion of 1:3. Very probably this is the actomyosin in which the actin micels are coated with one layer of myosin. This one layer is held very firmly and a very high KCl concentration is needed for dissociation.

The smaller the % activity, the thicker this layer of myosin held by the actin, but the smaller the force by which myosin is held and, correspondingly, the smaller the KCl concentration which is capable of effecting dissociation.

If the salt concentration of the actomyosin solutions of different activity is gradually reduced at a certain point the solution precipitates. The more active the actomyosin, the more salt is needed to keep it in solution. This precipitation is reversible. If the salt concentration is increased again the precipitate dissolves. One very interesting fact, which emerges from these experiments of Fig. 3, is that if the precipitate is dissolved by increasing the salt concentration in presence of ATP the actomyosin not only dissolves but also dissociates at the same time into actin and myosin. Actomyosin, dissolved thus in presence of salt and ATP means dissociated actin and myosin.

If the KCl concentrations, at which the actomyosin dissociates without ATP, are plotted against the activity of the actomyosin, a straight line is obtained (Fig. 5).

References.

1. *K. Balenović* and *F. B. Straub.* These studies **1**, 43, 1941—42.
2. *F. B. Straub.* Ibid **2**, 6, 1942.
3. *F. B. Straub.* Ibid **1**, 43, 1941 42.
4. *I. Banga.* Oral comm.

Extraction of myosin.

by

F. GUBA AND F. B. STRAUB.

If rabbit's muscle is extracted with salt solution an actomyosin of varying activity is obtained. In order to prepare pure myosin it is essential to start from a possibly inactive material.

We have studied the amount and the activity of the extracted myosin under varying conditions. The mode of mincing, the pH, the salt concentration and the time of extraction were varied. Activity was determined by the method of STRAUB[1], myosin contents were calculated from the viscosity in presence of ATP.

We find that the extraction of coarsely minced rabbit's muscle for 10 minutes at 0° with 3 volumes of a phosphate-KCl mixture of pH 6,5 (which contains 0,15 *M* K-phosphate buffer of pH 6,5 and 0,3 *M* KCl) will give an actomyosin of low (3 %) activity.

1. The effect of mincing and time of extraction. In the first series the muscle tissue was minced through an ordinary meat chopper only* (= coarse mincing), in the second it was minced first through the meat chopper and then minced again through a Latapie mincer (= fine mincing). In both cases the mince was extracted at 0° with 3 volumes of a 0,6 *M* KCl solution under constant stirring for different lengths of time, as indicated in Table I.

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* Diameter of holes: 2 mm.

Table I.

Time of extraction minutes	Coarse mincing		Fine mincing	
	% activity	mg myosin/ml	% activity	mg myosin/ml
5	4,5	12	7	15,6
10	4,8	3,8	9	16,5
15	5,0	15,5	13	17,2
20	6,6	17,7	20	18
25	9,0	20,2	22	19
60	—	—	40	—
120	—	—	80	—

Table I. shows that there is a rise in the activity of the extracted myosin on prolonged extraction. We therefore extract the muscle tissue only for 10 minutes. Fine mincing results in somewhat more myosin being extracted, but the activity of the extracted myosin is in this case more than twice of that obtained from coarsely minced muscle. It is further seen from the table that even from the coarsely minced muscle KCl extracts a fairly active myosin. This extracting agent is not satisfactory also because the results are not strictly reproducible and show considerable variations.

2. *The effect of salt concentration.* The muscle was minced through a meat chopper and extracted for 10 minutes at 0° with 3 volumes of the KCl concentration indicated in Table II.

Table II.

Salt concentration of extracting fluid, *M*	% activity	mg myosin/ml
0,2	—	0,8
0,3	17	3,5
0,4	10	8,7
0,5	11	10
0,6	12	11,8

Table II. shows that a minimal KCl concentration of 0,4 *M* is needed to extract myosin. It is to be seen moreover that the activity of the extracted myosin has a minimum value at 0,4 *M*.

The effect of pH. The muscle tissue was extracted at 0° for 10 minutes with 3 volumes of a phosphate-KCl mixture. This contained 0,15 *M* phosphate (KH_2PO_4 and K_2HPO_4) of varying pH and 0,3 *M* KCl.

Table III.

pH	% activity	mg myosin/ml
5,9	12	4,3
6,3	7	10,5
6,5	3,5	12
7,0	4,5	10,7
7,5	5	10,3

The activity was found to be 15—20 % when the muscle tissue was extracted with WEBER's solution* under similar conditions and 1% activity if it was extracted similarly with a 0,6 *M* KCl buffered to pH 10 with borate buffer.

Phosphate ions seem to have a depressing effect on the activity of the extracted myosin as extracts made without phosphate at the same pH and ionic strength are always more active than those prepared with phosphate. That the low activity is due to the lower actin content and not to the phosphate present is shown by the fact that the myosin of the phosphate extract, if precipitated and redissolved in a phosphate free KCl solution, will show the same low activity.

4. Salt concentration of phosphate-KCl mixtures. The extraction was made at 0° for 10 minutes with 3 volumes of phosphate-KCl mixture. The latter always contained 0,15 *M* phosphate of pH 6,5 and varying concentrations of KCl.

Table IV.

M KCl	% activity	mg myosin/ml
0,1	19	2,8
0,2	3	7,1
0,3	3	11,9
0,4	4	12,8

The table shows that apart from the phosphate 0,3 *M* KCl is needed to give optimal results. In a great number of experiments identical results were obtained.

References.

1. *F. B. Straub,* These studies **2**, 3, (1942).

* This solution contains 0,6 *M* KCl, 0,01 *M* Na_2CO_3 and 0,04 *M* $NaHCO_3$.

Note on the viscosity of myosin.

by

F. GUBA AND F. B. STRAUB.

Several workers have, in the past, studied the viscosity of myosin and found it to be anomalous. As it is now clear that in all these cases impure myosin was used, these data must be revised. A small amount of actin influences the viscosity of myosin very strongly.

We have determined the viscosity of twice recrystallised

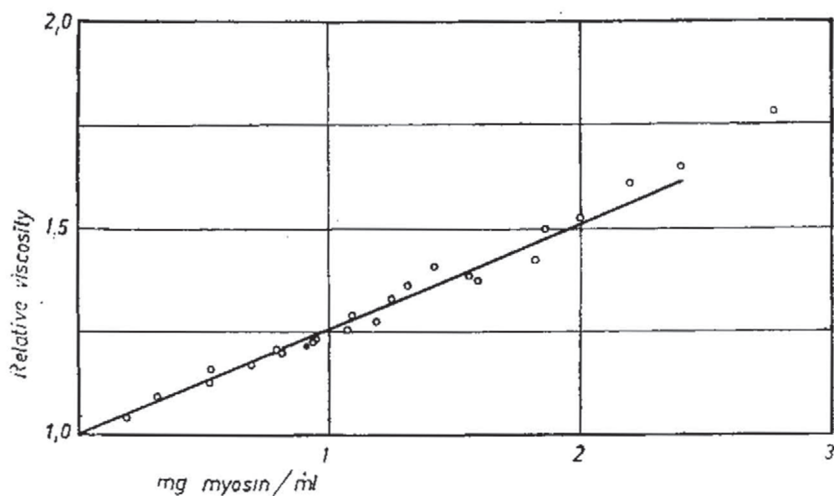

Fig. 1. Viscosity of recrystallised myosin in 0.6 M KCl at 0°.

myosin at 0° in 0,6 M KCl solution buffered with veronal acetate to pH 7. (For the composition of this solution see BALENOVIĆ and STRAUB[1].) The myosin content was determined in the following way: 1 ml of the myosin solution was diluted so as

to contain less than 0,2 *M* salt and then an equal volume of alcohol was added and the mixture was heated to 70° for 10 minutes. The precipitate was centrifuged off and washed with a 50 % alcohol, centrifuged and dried at 105°.

The viscosity of myosin solutions in relation to the concentration of myosin is shown in Fig. 1. No correction was taken for the change of specific weight on addition of myosin to the salt solution. The points were obtained with four different myosin preparations.

The dependence of the viscosity on the pressure was studied with the method described in this volume.[2] It was found that, between 5—100 cm water pressure, the viscosity is independent of the pressure within the limits of experimental errors. Thus it appears that the viscosity of myosin in 0,6 *M* KCl is not anomalous. The same conclusion can be drawn from Fig. 1. where it is to be seen that the viscosity of dilute solutions is proportional to the myosin content.

References.

1. *K. Balenović* and *F. B. Straub,* These studies **2,** 17 **(**1942**).**
2. *F. B. Straub,* ibid., **3,** (1943).

Rigor, contracture and ATP.

by

T. ERDŐS.

It has been shown in previous papers of this laboratory that the contractile substance of the muscle fibril is actomyosin, a complex composed of two proteins, actin and myosin. Actin is bound to the solid structure or rather forms the solid structure of the fibril itself. Myosin is attached to the actin in a loose, dissociable form. It has also been shown that ATP has a decisive influence on the physical state of actomyosin which, according to conditions, is contracted, relaxed or dissolved by ATP.

The object of this work was to see, whether in muscle ATP has any influence on the physical state of the actomyosin, especially, whether the relaxed state of the muscle is dependent on its presence.

Muscle contains the rather high concentration of 300—350 mg % of ATP. CASPERRSSON and THORRELL[1] have shown that a considerable part of the ATP is bound in the *I* band. One can thus expect no close parallelism between ATP concentration and the state of relaxation because the ATP is not evenly distributed. A really close parallel can be expected in rigor mortis only, where there is a post mortal disorganisation and sufficient time for the ATP to be distributed evenly within the muscle fiber. In other cases, the more the muscle is damaged and the more time given for even distribution of ATP the closer we may expect the parallelism to be. Apart from rigor mortis I also studied other forms of contracture, such as monoiodoacetic acid, caffeine, chloroform-contracture and the contracture of electrically stimulated muscle.

It has been found by several investigators (2, 3, 4) that the solubility of muscle proteins decreases during activity and in different forms of contracture. In the case of monoiodoacetic

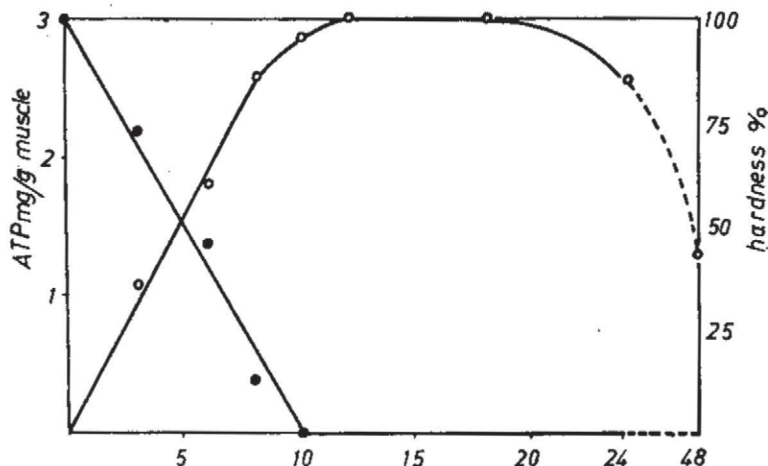

Fig. 1. a. ATP content during development of rigor mortis, in rabbit muscle.
● Hardness %/0%: fresh muscle. 100%: rigor mortis.
○ ATP mg/muscle

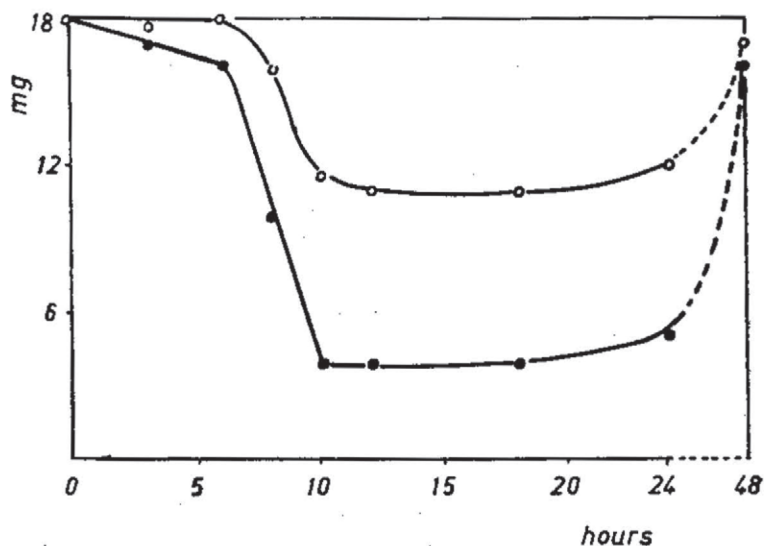

Fig. 1. b. Solubility of myosin during the development of rigor mortis. (Same experiment as in Fig. 1. a.)
● Myosin mg/ml, extracted with 3 vols. of. 0.6 *M* KCl.
○ Myosin mg/ml, extracted with 3 vols. of 0.6 *M* KCl, in presence of 100 mg% ATP.

acid, MIRSKY[5] found that the solubility of myosin is decreased. For this reason I also undertook to measure, parallel with the rigor and the ATP concentration, the solubility of myosin. My results were the following:

Rigor mortis: There is a close parallelism between hardness of the muscle and the ATP concentration. The rigor develops at the same rate as the ATP disappears. At the maximum of rigor there is no ATP at all. (Fig. 1.)

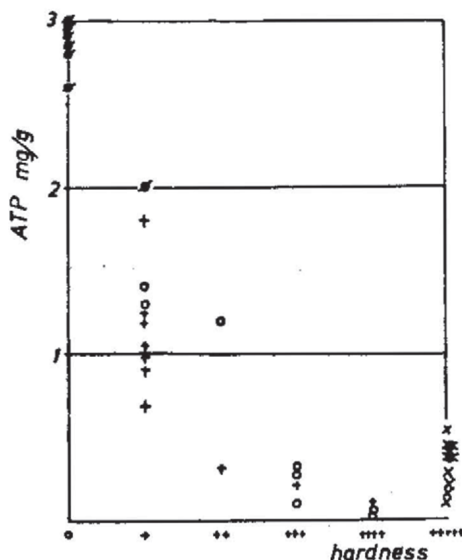

Fig. 2. ATP content and hardness of frog muscle, in different forms of contractures
0 is the hardness of fresh muscle.
+++++ is the hardness of muscle in maximal rigor.
∅ Fresh muscle.
O Muscle 24—48 hours after decapitation. (At 18—20°)
+ Electrically stimulated muscle.
× Isolated gastrocnemius exposed to chloroform vapours for 12 mins.
● Left leg
● Right leg, stimulated of frog poisoned with iodoacetate.
The hardness was measured *in situ.* The same muscles were used for ATP determination.

Parallel with the development of the rigor the myosin becomes more and more insoluble showing that both phenomena, rigor and insolubility are closely connected. By addition of ATP the solubility of myosin could be restored. This supports the conclusion that both the rigor and the decreased

solubility were, in fact, due to the reduction of the ATP concentration. In absence of ATP, myosin is practically insoluble.

Parallel to the relaxation of the rigor the myosin becomes soluble again and goes into solution even without addition of ATP. In this case, however, it is not myosin that goes into solution but actomyosin. It can be concluded that the relaxation of rigor mortis is thus due to desorganisation, i. e. to the release of actin from the structure.

In the other forms of rigor there is less close parallelism between contracture and ATP concentration than in the case of rigor mortis. (Fig. 2.)

Experimental part.

The hardness of the muscle was measured by MANGOLD'S* method. I worked throughout with 10 g weight.

Myosin was extracted with 3 ml 0,6 *M* KCl pro g muscle. The muscle was minced in the Latapie mincer, suspended in the KCl solution, stirred for ten minutes and centrifuged. In order to estimate the myosin content of this extract 1 ml. 0,5 *M*, pH 5,2 acetate buffer and 8 ml water were added to 1 ml of it. The precipitate was centrifuged off, washed twice, dried and weighed.

The ATP was estimated in extracts of muscle according to SZENT-GYÖRGYI (see page 93.).

Rigor mortis: The hardness was measured on one and the same place of the thigh of the decapitated and skinned rabbit. From the other thigh I excised, after certain periods, 10—10 g samples. 3 g were used to estimate the solubility of myosin without addition, 3 g with addition of ATP. (3 mg ATP was added pro g. muscle). In 3 g of the muscle the ATP was estimated. The results are summed up in Table 1. The data of the first series of experiments are shown also in Fig. 1, where the correlation between the different factors can be seen.

I was unable to pull threads from the myosin extracted from the muscle in rigor, which shows that there was very little actin present. The myosin extracted after relaxation can readily be pulled to threads. It was a 9% actomyosin and contracted on addition of K, Mg and ATP: 66%.

In the case of monoiodoacetic acid, and caffeine I injected the solutions into the lymphsac of the frog. (0.4 mg iodoacetate

Table I.

Hours	Hardness %	Soluble myosin mg/ml	Soluble myosin mg/ml in presence of 100 mg % ATP	ATP mg/g muscle
0	0	18	18	3
3	36	17	17.5	2.2
6	60	16	18	1.4
8	86	10	16	0.4
10	96	4	11.5	0
12	100	4	11	
18	100	4	11	
24	86	5	12	
48	43	16	17	
0	0.	14	15	2.8
5	50	15	15	1.4
6	70	10	15	0.7
9	100	4	13	0
0	0	13	15	3
6	50	13	16	1.1
24	100	3	16	0
0	0	18	18	2.9
6	100	4	17	0
0	0	16	17	
10 days At 0°	40	14	14	
0	0	15	16	
8 days At 0°	45	14	15	

The hardness of fresh muscle: 0%.
Rigor mortis: 100%.

per g, 0,15 mg caffeine per g). After the rigor had developed I estimated the solubility of myosin with and without the addition of ATP. The results are summed up in Tab. 2. In one experiment the lower half of the spinal cord, innervating the lower limb, was destroyed. When rigor began to develop in the fore limb I stimulated one hind limb electrically for a minute and then used both limbs for estimation of rigor and ATP. The result of this experiment is summed up with other experiments in Fig. 2.

Table II.

Soluble myosin mg/ml extracted with:					
3 ml 0.6 *M* KCl/g muscle			3 ml 0.6 *M* KCl/g muscle in presence of 100 mg % ATP		
Fresh resting muscle	Muscle in iodoacetate rigor	Muscle in caffeine rigor	Fresh resting muscle	Muscle in iodoacetate rigor	Muscle in caffeine rigor
7	1.1	5	8	8.5	11
11	2.5	5.5	12	11	12
11.1	4		11	11	
9.5	3.6		10	9.2	
10	5		11	22	

The ATP content of muscles in rigor was 0.10—0.20 mg/g.

I am indebted to the CHINOIN Foundation for a grant.

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On the relation of the activity and contraction of actomyosin threads.

by

T. ERDŐS.

The object of my experiments was to determine the relation of the % activity of the actomyosin and the contraction of the threads. In order to obtain comparable results threads of equal diameter have to be used and the thread should have the same concentration of protein. It is not possible to use in the whole series of experiments threads of the same protein content. Threads of low protein concentration cannot be pulled from an actomyosin of low activity. Threads of highly active actomyosin contract rapidly only at a low protein concentration and become sluggish if the protein concentration is high. For this reason under 50 % activity I used threads of 1,6 %, above 50 % threads of 0,9 % protein content. The diameter was throughout 0.25 mm. The actomyosin was prepared from recrystallised myosin and actin. The actomyosin contained 0,5 *M* KCl and was squirted through a capillary into 0,05 *M* KCl containing 0,001 *M* $MgCl_2$. The contraction was measured by following the length of the thread in a 0,02 *M* pH 6,7 potassium phosphate solution containing 0,001 *M* $MgCl_2$ to which 0,16 mg ATP was added after the thread was soaked in the salt solution.

Two series of experiments were performed. In the first I compared threads made from actomyosin of 10, 20, 30, 40 and 50 % activity. In the second I compared threads made from actomyosin of 50, 100, 170 % activity.

The results were the following: up to 40 % activity the contractibility increased with the activity (see fig. 1.). Threads prepared from 40 and 50% active myosin give the same contraction. Threads of 50, 100 and 170 % active myosin, also give identical results.

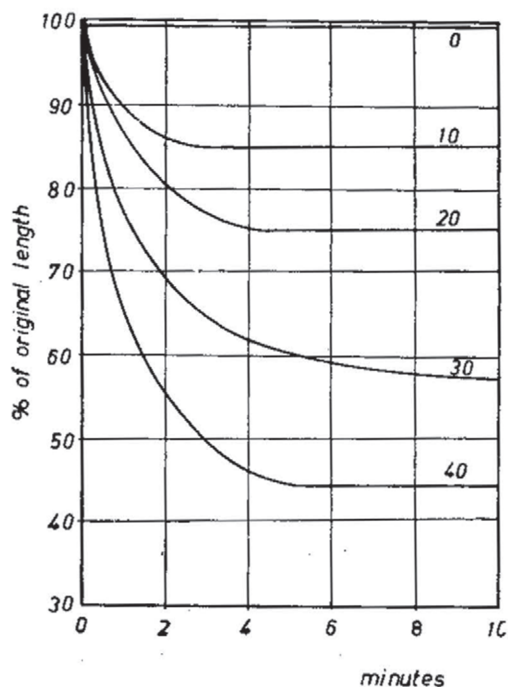


Fig. 1. Contraction of actomyosin threads of actomyosin of different activity. The numbers on the curves indicate the % activity of the actomyosin.

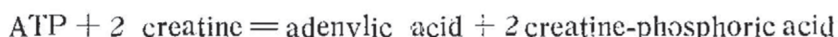
Summing up, thus, it can be said that the contractibility of actomyosin increases with increasing activity up to 40 % activity; 40 % actve myosin containing 6,5 parts of actin to 93,5 parts of myosin gives maximal contraction.

The ATP-creatine phosphopherase.

by

I. BANGA.

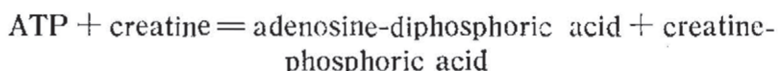
It has been shown by K. LOHMANN[1] that in muscle the easily hydrolysable phosphate of ATP is taken over by creatine. In dialysed muscle extract therefore the reaction taking place can be expressed by the equation:



ATP + 2 creatine = adenylic acid + 2 creatine-phosphoric acid

This reaction was investigated by H. LEHMANN[2] who demonstrated its reversibility, but the equilibrium constant = K calculated for a trimolecular reaction was not constant.

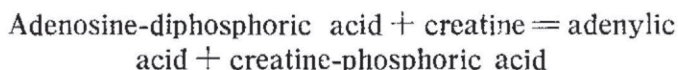
My investigations have proved that the above reaction takes place in two distinct steps and that the presence of two distinct proteins is necessary for this catalysis. The first reaction is:



ATP + creatine = adenosine-diphosphoric acid + creatine-phosphoric acid

The enzyme catalysing the reaction was called the ATP-creatine phosphopherase and was purified to a degree at which no secondary reactions occured. If the kinetics of this reaction were examined at varied concentrations of the reactants, the equilibrium constant calculated for a bimolecular reaction was found to be constant.

The second step of the reaction



Adenosine-diphosphoric acid + creatine = adenylic acid + creatine-phosphoric acid

is catalysed by another protein, which I called ADP-creatine phosphopherase, the isolation of which is in progress.

Experimental:

Test: Unit of the ATP-creatine phosphopherase is the quantity of enzyme which is capable of transferring 0,00213 moles P from ATP to creatine in a period of five minutes in the presence of 0.00475 moles of ATP and 0.0215 moles of creatine in a pH 8.55 veronal-acetate buffer. Half that quantity of enzyme transmits 0.00106 moles and a quarter of it 0.00053 moles of P during the same interval. The resulting creatine-phosphate was determined after FISKE and SUBBAROW.* In this method creatine-phosphate hydrolyses during the five minutes of incubation and appears as inorganic phosphate. There was no preformed inorganic phosphate in my experiments.

The activity of the enzyme is defined as: enzyme units divided by the quantity of the protein dry material, $a = \frac{\text{units}}{\text{mg protein}}$ The protein dry material was determined by placing a given quantity of enzyme in a weighed centrifuge tube and precipitating it with 5% of trichloro-acetic acid. The precipitate was washed twice in a tenfold volume of water, dried and weighed.

The process of isolation: The muscle of a freshly killed rabbit was minced on a Latapie mincer, suspended in 1.5 ml 0.1 *M* KCl per g and was then extracted by stirring it at 0° for 10 min. The muscle was then strained through a cloth and once more extracted in ⅓ of the former volume of 0.1 *M* KCl for a period of ten min. The extracts were united, left alone for 1—2 hours and then centrifuged. The ATP-creatine phosphopherase activity of this extract could not be determined with accuracy because it also contained ADP-creatine phosphopherase. On the whole it was found that 1 g of fresh muscle of the rabbit contained 100—150, that of the pigeon 200—250 units of ATP-creatine phosphopherase.

Preparation of ATP-creatine phosphopherase:**

First step: To every liter of the above fluid were added 80 ml of 0.5 *M* $NaHCO_3$. Then the fluid was half saturated with ammoniumsulfate and centrifuged once more. The precipitate was discarded. Activity of the fluid = 6.

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* as modified by LOHMANN and JENDRASSIK.

** All operations were carried out at 0°.

Second step: For every liter 30 ml of 0.5 *M* $NaHCO_3$ were added and as much ammoniumsulfate as was needed to bring it up to 0.7 saturation. The precipitate was filtered and dissolved in 250 ml buffer solution of 0.025 *M* borate of pH 8.55. Dry weight 30mg/ml. a=7. No loss of activity.

Third step: To 1 liter of enzyme solution 2.5 litres of alcohol of —20° were added. The precipitate was centrifuged off at 0° and dissolved in 250 ml of 0.02 *M* borate buffer of pH 8.55. The insoluble material was filtered off and the solution

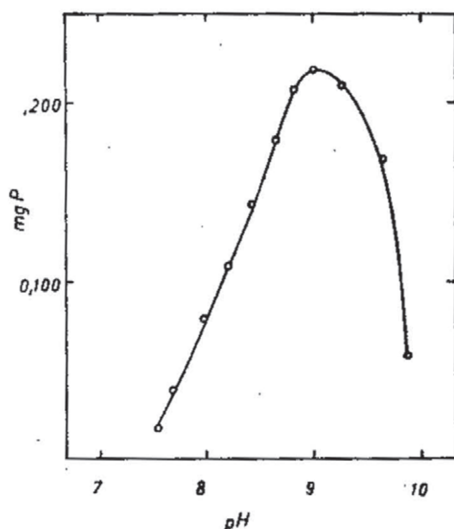

Fig. 1.

was dialysed for a period of five hours against thirty volumes of water. Dry material 7—10 mg/ml; a = 30. Loss 30 %.

Fourth step: Adsorption to γ $Al(OH)_3$. To every liter of dialysed fluid 300 ml of $Al(OH)_3$ suspension was added. This contained 20 mg of $Al(OH)_3$ per ml. After shaking for five minutes the solution was centrifuged. Elution with 250 ml of pH 8.55 borate buffer containing *M*/10 borate and 20 % ammoniumsulfate. Dry material 3—4 mg/ml; a = 95. Loss 45 %.

Fifth step: *M*/50 NH_3 and ammoniumsulfate was added till 0.5 saturation. The resulting precipitate was filtered and dissolved in *M*/10 borate buffer. a=100. Loss 10%.

1 γ of this purest enzyme preparation (a=100) transferred at pH 8.55 in one minute 2 γ P from ATP onto creatine. If the

molecular weight of the enzyme is taken to be 70,000, this would mean, that one molecule of enzyme did react upon 4.400 molecules of the substrate per min.

In the course of this isolation the ADP-creatine phosphopherase disappeared at step 3. together with ADP-isomerase (see page 66.).

With ATP-creatine phosphopherase, thus isolated. I determined how far the activity was dependent on the pH. As can be seen from Fig. 1., in presence of borate buffer the pH optimum was 9.05.

Determining the equilibrium constant at different pH, I found that the value of K depended to a great extent on the pH and reached its maximum at the pH optimum of the reaction. Table I. shows the equilibrium constant $= K$ at varied pH and with constant creatine and ATP concentrations. These investigations were complicated by the fact that the ATP itself acted as a buffer, and thus changed the pH of the original buffer solution. For this reason the pH of the ATP and creatine solutions were colorimetrically brought to the necessary pH and the buffers were used in high concentrations.

Table I.

Experimental technique: 1 ml of borate buffer solution of varying pH, 0.0195 *M* creatine, 0.00890 *M* ATP, 0.1 ml $= 60\ \gamma$ of ATP-phosphopherase. Volume 1.6 ml. Incubation at 38° until the state of equilibrium had been reached.

pH	Measured *M* creatine P	$K = \frac{Cr.P.ADP}{ATP.Cr.}$
7.50	0.00059	0.0023
7.60	0.00085	0.0049
7.80	0.00172	0.0239
8.20	0.00289	0.0925
8.50	0.00383	0.1910
9.05	0.00459	0.3651
9.50	0.00344	0.1382
9.70	0.00094	0.0061

In establishing the pH curve the following experimental technique was employed: 2 ml of buffer solution were mixed with 1 ml (0.01915 *M*) of creatine + 0.5 ml (0.00890 *M*) of ATP + 0.5 ml H_2O. Of this mixture 0.5 ml were removed for the pH determination. 1.5 ml were used to determine the rate of the reaction. 0.05 ml enzyme (30 γ) was added and the mixture was incubated for 5 min. at 38°. Another 1.5 ml in the presence

of enzyme were incubated for 30 min. at 38° in order to determine the equilibrium constant.

Table 2 shows the values of the equilibrium constant (K) at identical pH (veronal acetate buffer of pH 8.55) and with varied ATP and creatine concentrations. As can be seen, the equilibrium constant has the same value throughout. The value is lower in veronal acetate buffer than in borate buffer. This might be attributed to the complex formation of borate and ATP which complex might react faster with creatine than free ATP. To avoid such complications I employed for the estimation of the equilibrium constant a buffer solution of veronal acetate.

Table II.

Experimental technique: 1 ml of veronal-acetate buffer solution of pH 8.55 + 0.1 ml = 60 γ ATP-phosphopherase + varying quantities of ATP and of creatine. Volume: 1.6 ml. Incubation at 38°, until state of equilibrum had been reached.

M creatine added	*M* ATP added	*M* P found	$K = \frac{Cr.P.ADP}{ATP.Cr.}$
0.02390	0.00222	0.00104	0.040
0.02390	0.00444	0.00158	0.042
0.02390	0.00890	0.00232	0.038
0.02390	0.01331	0.00292	0.039
0.01030	0.00236	0.00078	0.040
0.01531	0.00236	0.00090	0.038
0.02040	0.00236	0.00104	0.040
0.02542	0.00236	0.00108	0.038

Further experiments with ATP-creatine phosphopherase showed that the pH stability curve did not coincide with the pH activity curve. After a storage for a longer period, the enzyme had changed its pH optimum.

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Enzyme Studies.

by

I. BANGA.

I. Enzymic activity of crystallised myosin.

SZENT-GYÖRGYI[1] described a method for the preparation of myosin free of actin. This myosin could be crystallised and purified by repeated recrystallisations. I have found that, in presence of KCl, this myosin, recrystallised twice, has the same enzymic activity towards ATP as earlier impure preparations. This can be seen from Fig. 1.

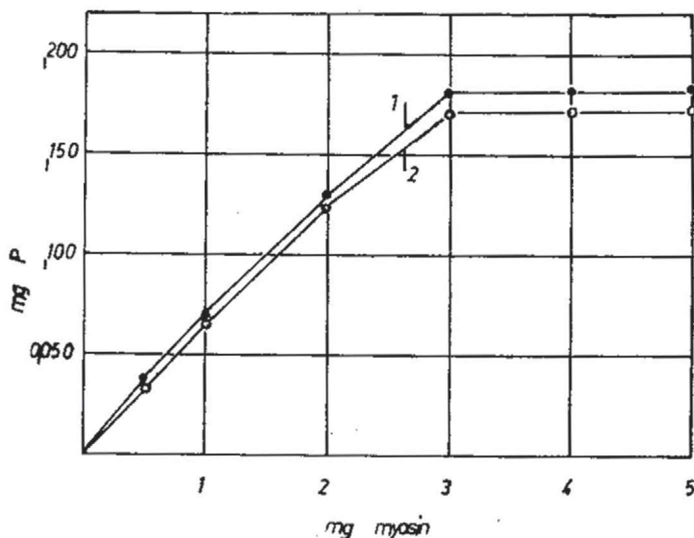

Fig. 1. The splitting of ATP by impure and recrystalliseed myosin. mg P split off at 38° in 5 min. from a mixture of 1 ml of 0.1 *M* veronal acetate buffer of pH 7.0. 0.05 *M* KCl. 3.6 mg ATP as neutral K salt and varying amounts of myosin. Total volum 3 ml.
Curve 1: purified myosin before crystallisation.
Curve 2: the same myosin recrystallised twice.

The reaction mixture was always deproteinised by $^1/_9$ volume of 20% trichloroacetic acid and P was determined in the filtrate according to the method of FISKE and SUBBAROW, modified by JENDRASSIK and LOHMANN.

ATP has two phosphate groups which are readily hydrolysed in 15 minutes at 100° by *N* HCl. Only one of these is split off by crystallised myosin. This process of splitting off of phosphate is activated by KCl (see page 69.) and is inhibited by Mg. The Mg inhibition is shown in Fig. 2. The resulting ADP

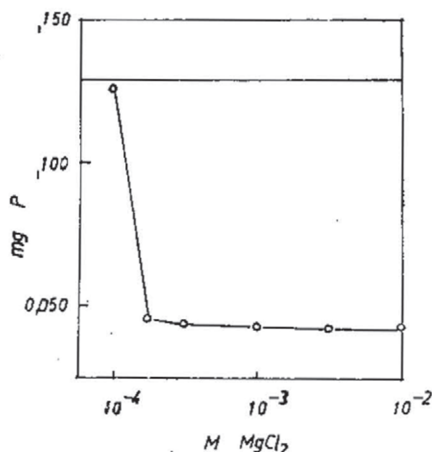

Fig. 2. Effect of $MgCl_2$ on splitting of ATP by crystallised myosin. mg P split off at 38° in 5 min. from a mixture of 1 mg myosin, 0.1 *M* KCl, 3.6 mg ATP and varying concentrations of $MgCl_2$. Total volume 3 ml.

was isolated from the reaction mixture. The Ba salt contained $^3/_2$ Ba atom per molecule of ADP. From the two phosphate groups only one was readily hydrolysable.

The ADP was isolated by precipitating the inorganic phosphate by ammoniacal $MgCl_2$ and precipitating the ADP by $BaCl_2$. I proceeded as follows: 100 mg crystallised myosin and 1400 mg of ATP, in the form of neutral K salt, were dissolved in 300 ml 0.1*M* KCl. Incubation at 38° until samples taken showed that one P had already been split off. The protein was then removed by adding 30 ml of 20% trichloroacetic acid. Thereafter I added 3 g $MgCl_2$ and NH_4OH until the solution began to turn red in presence of phenolphtalein. The resulting mixture was stored overnight at 0° during which period the inorganic P had separated quantitatively in the form of $Mg(NH_4)PO_4$. In order to precipitate the neutral Ba salt of ADP. 3 g of $BaCl_2$ and $^1/_5$ volume of alcohol were added to the liquid. The precipitate was washed first in 50% alcohol, then in

absolute alcohol and dried. The yield was 65 % of the theoretical value.

In order to obtain the ADP free of Mg, the Ba salt was dissolved in 5 % acetic acid and precipitated by Hg acetate. The salt was dissolved in 0.5 N HCl and the Hg removed as HgS. H_2S was removed by ventillation. By adding $BaCl_2$ and alcohol to the resulting liquid at neutral reaction the ADP was precipitated as a Ba salt.

Analysis of P: 100 mg Ba salt of ADP were dissolved in presence of HCl in 8 ml of H_2O and K_2SO_4 was added (one molecule of K_2SO_4 to every molecule of Ba salt). Thereafter I neutralised with KOH and brought the volume up to 10 ml with water and centrifuged. 0.1 ml of this solution contained 0.002 mg of inorganic P, 0,046 mg readily hydrolysable P and a total P of 0.093 mg. The proportion of the readily hydrolysable P to the stable one, was found to be 1 : 1.

II. ADP-isomerase.

When adenosine triphosphate was added to impure actomyosin (myosin B)[2] then both of the readily hydrolysable phosphate groups were split off. When all the soluble protein had been removed from actomyosin by four consecutive precipitations, it was found that only one of the two readily hydrolysable phosphates was split off. The other phosphate would split off upon addition of a muscle extract from which all substances, insoluble in water, had previously been removed. K. LAKI[3] showed that in addition to an enzyme system, insoluble in water and liberating only one phosphate group from ATP, a soluble protein and Mg were necessary to split off a second phosphate. My investigations confirmed LAKI's findings. The soluble factor must have been a protein since it was thermolabile and could be precipitated by trichloroacetic acid. In presence of this protein actomyosin readily attacked ADP. This protein was named ADP-isomerase for reasons to be shown hereafter.

By the following method the ADP-isomerase can be obtained 50 times purer than the original muscle juice. Freshly minced muscle of rabbit was suspended in 0.1 M KCl, 1.5 ml being taken per g of the muscle. Two hours later the insoluble

parts were removed by centrifugation. Ammonium sulfate was added to 0.5 saturation and the precipitate discarded. The liquid was neutralised and ammonium sulfate added to 0.7 saturation. The resulting precipitate contained the ADP-isomerase. It was dissolved in 0.1 *M* borate buffer of pH 8.5, dialysed for 24 hours and the insoluble parts removed by centrifugation. The isomerase was adsorbed to $\gamma Al(OH)_3$. From the latter it was eluted with 0.1 M borate buffer of pH 8.5. From this solution the isomerase was purified by precipitating it between 0.5—0.6 saturation of ammoniumsulfate. 50 γ of this protein in 3 ml. activated the splitting of ADP by actomyosin.

The question might be raised whether this new protein is not identical with the myokinase described by KALCKAR,[*] which dismutates ADP into adenosinetriphosphate and adenylic acid. My experiments showed that this is not the case since the new protein when incubated with ADP, did neither produce ATP nor adenylic acid. The product of incubation was not split by crystallised myosin, thus it was not ATP, nor was it reacted upon by deaminase, therefore the product could not have been adenylic acid either. When ADP had been incubated with isomerase, the product was split by actomyosin which suggested that isomerase had changed the molecular structure of ADP. It was for this reason that the name isomerase was given to this new protein since its effect consisted not in dismutation but in isomerisation. There was no appreciable change observed in the relation between the labile and the stable phosphate of ADP if incubated with isomerase.

Actomyosin is able to split off phosphate from the compound formed by the action of isomerase on ADP, whereas it is unable to attack ADP itself. If therefore ADP is incubated first with isomerase and the reaction product is added afterwards to actomyosin, inorganic phosphate will be formed to the extent to which the isomerisation reaction has proceeded. By this method I was able to study the isomerisation reaction itself.

Isomerisation of ADP: 3 mg K-ADP were incubated at 38° for 10 minutes at pH 8.5 in the presence of 50 γ isomerase in 1 ml. I deproteinised with 2 ml of 20% trichloroacetic acid, centrifuged and neutralised it by addition of *M* KOH.

Splitting of the isomerised product: The reaction mixture contained 2 mg of 25% actomyosin produced from cryst. myosin and actin, 1 ml 0.1 *M* veronal acetate buffer. To this mixture I added the substances noted in Col. I. Total volume 3 ml. Incubation for 10 min. at 38°. The results are shown in Table I.

Table I.

Splitting of the isomerised product of ADP by actomyosin

	mg P split off	Percentage ADP converted
Actomyosin + 3 mg ADP	0.00	
" + 3 mg ADP + 50 γ isomerase	0.104	(100)
" + 3 mg ADP previously incubated with isomerase	0.022	20
" + 3 mg ADP previously incubated with isomerase + Mg	0.064	60
" + 50 γ isomerase + 3 mg ADP previously incubated with isomerase + Mg	0.107	

Upon addition of Mg, the effect of the isomerase could be increased by 200 per cent. Thus while in a certain interval isomerase alone turned only 20% of ADP into a product which was split directly by actomyosin, 60 % were transformed in the presence of 0.001 *M* $MgCl_2$.

As to the question what molecular changes ADP had undergone upon the effect of isomerase, we must remember on one hand that isomerisation did not affect the relation of the labile and stable P of ADP. It is seen on the other hand from Table II. that only one half of the readily hydrolysable phosphate of ADP is split off by actomyosin + isomerase, i. e. only one quarter of the total P.

Table II.

10 mg of 25% actomyosin, 0.5 mg isomerase, 10 ml 0.1 M veronal-acetate buffer of pH 8.5, 30 mg ADP containing 2.25 mg readily hydrolysable P and 4.50 mg total P. The mixture was made up with water to 30 ml. Incubated at 38°. At definite time intervals samples of 3 ml were taken out and deproteinised with 1 ml of trichloroacetic acid. The experiment was finished when no more P was liberated. This usually was reached at about the end of 20 min. No more P was liberated even if the experiment was protracted to an hour.

	Inorg. P	Readily hydr. P	Total P
At the outset of experiment in 3 ml	0.00	0.225	0.450
At the end of incubation in 3 ml	0.115	0.110	0.450

III. Adenosinediphosphatase.

When ADP was added to crystallised myosin no reaction whatever took place. ADP was not split by crystallised myosin, not even when isomerase had been added. But if a third protein, — extracted in a soluble form from a washed, acetone

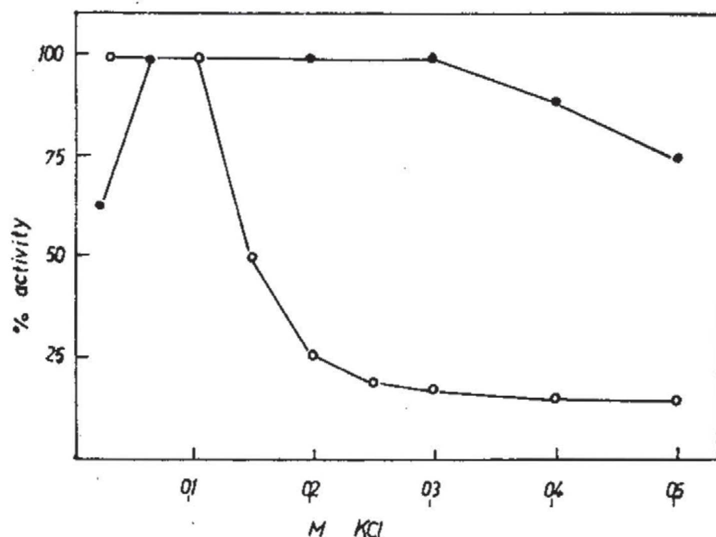

Fig. 3. Effect of KCl concentration on splitting of ATP and ADP. 100% is the maximal amount of P split off under optimal conditions.
o — o — P liberated from ADP
• — • — P liberated from ATP
2 mg of myosin, 3 mg of K ATP or ADP, KCl of varying concentrations. Total volume 3 ml. Incubated for 5 min. at 38°. In the case of ADP, isomerase and third protein were added, too.

dried muscle — was added to a mixture of crystallised myosin + isomerase, then one half of the readily hydrolysable phosphate of ADP was liberated. The optimum pH of the ADP splitting was at 8.5. The reaction was very sensitive to the KCl concentration. While the ATP-phosphatase activity of crystallised myosin was constant along a wide range of KCl concentration, the ADP splitting of the above three-enzyme system was optimal only within a narrow range of KCl concentration. (Fig. 3, curves 1, 2.)

The third protein could be replaced by actin prepared according to F. B. STRAUB's[5] method, therefore it seemed likely that the two proteins were identical. The experiments, numbered 1—6 below, seem to indicate, that the protein which splits

ADP is actomyosin itself. This, however, could not be proved conclusively.

1. No solution of the third protein could be extracted from a fresh muscle in water or in weak salt solution, since it was always the insoluble part of the fresh muscle which contained the third protein. But, similarly to actin, this protein became soluble in water when the muscle had previously been washed, treated with acetone and dried. When this washed and acetone dried muscle had been extracted in a tenfold volume of water for about 10—20 minutes, a 0.1% solution of protein was obtained of which 0.1 mg manifested the third-protein-activity. In case of small amounts the activation was

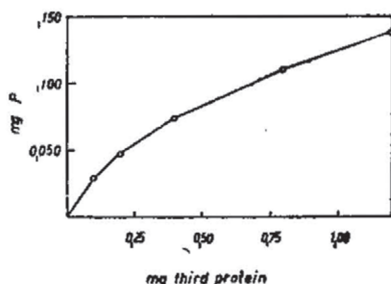

Fig. 4. Effect of varying amounts of the third protein on the splitting of ADP by myosin in presence of isomerase. 1 mg of crystallised myosin, 50 γ isomerase, 1 ml veronal acetate buffer of pH 8.5, 3 mg ADP as K salt and varying amounts of third protein. Total volume 3 ml. Incubated for 5 min. at 38°.

proportional to the quantity of the protein, whereas with greater amounts the curve for the activity flattened as shown by Fig. 4.

Actin prepared by the method of F. B. STRAUB shows a third-protein-activity proportional to its actin content.

2. If 0.01 *M* $CaCl_2$ had been added to a solution of the third protein, the Ca precipitated the third protein quantitatively just as it precipitated actin.

3. When I added the third protein to crystallised myosin in a 0.5 *M* KCl, actomyosin was formed which could be precipitated upon a 5—10 fold dilution. This actomyosin could again be dissolved in a 0.5 *M* of KCl and once more precipitated by dilution. Actomyosin, thus produced, splits ADP in the presence of isomerase and shows the same activity as myosin + actin, or myosin + third protein. Since the third protein as well as the myosin were soluble in water, it was evident that a combination had taken place between myosin and the third protein, making the former insoluble in water.

It is clear from all this that the third protein produced the same effect as actin.

4. Similarly to actin the third protein could be precipitated from its solution by addition of weak acids (pH of about 4.8).

5. The KCl sensitivity (Fig. 3) of the ADP splitting, also corroborated the surmise that it was the actomyosin, which played a part in the reaction since actomyosin, in a 0.2 *M* KCl solution, dissociates into myosin and actin, as proved by the experiments of F. GUBA.[6]

6. The various impure myosin preparations split ADP in the presence of isomerase proportional to their actin content.

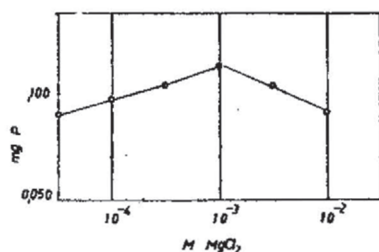

Fig. 5. Effect of varying amounts of $MgCl_2$ on the splitting of ADP by the three-enzyme-system. 1 mg of crystallised myosin, 50 γ isomerase, 0.5 mg third protein 1 ml 0.1 *M* veronal acetate buffer of pH 8.5 and varying concentrations of Mg Cl_2. Total volume 3 ml. Incubated for 5 min. at 38.°

The ADP splitting of the three-enzyme system was activated by $MgCl_2$ to about 30% (see Fig. 5) whereas isomerisation itself was activated to a considerable greater degree (200 %) upon addition of Mg. The reason of this is probably that the whole reaction is limited by the relatively slow splitting off of phosphate, which is not activated by Mg.

The ADP splitting in presence of actomyosin and isomerase was inhibited by 0.01 *M* pyrophosphate to an extent of 50—75%, depending on the length of incubation with the inhibitor. Initial velocities were inhibited by 75%, whereas in case of longer incubations the inhibition fell to 50%.

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1. *A. Szent-Györgyi,* This Vol. page 76.
2. *I. Banga* and *A. Szent-Györgyi,* These studies **1**, 5, (1942).
3. *K. Laki,* This Vol. page 16.
4. *H. M. Kalckar,* J. Biol. Chem. **143**, 299, (1942).
5. *F. B. Straub,* These studies **2**, 3. (1942).
6. *F. Guba,* This Vol. page 40.

The influence of salts on the phosphatase action of myosin.

by

I. BANGA and A. SZENT-GYÖRGYI

One of us has studied before the influence of salts on the phosphatase activity of myosin.[1] It seemed desirable to repeat part of this work with crystallised myosin.

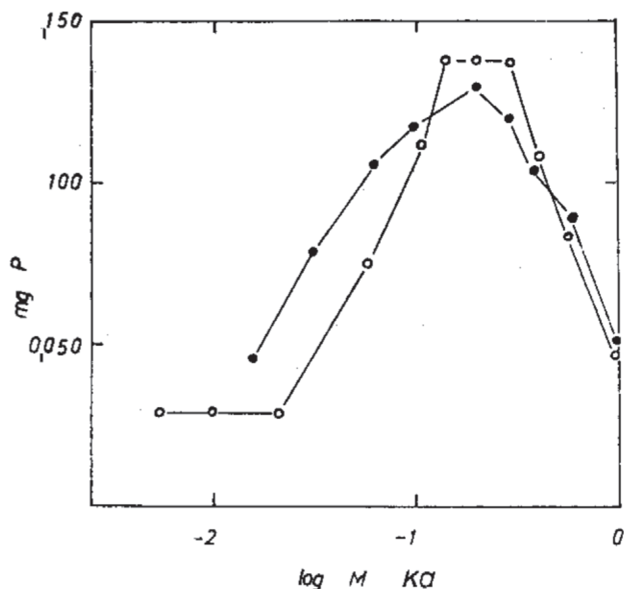

Fig. 1. Effect of KCl on the phosphatase activity of myosin and actomyosin. Formation of inorganic P determined by the method of *Fiske* and *Subbarow*. 1 mg myosin or 1 mg myosin plus 0.3 mg actin in 3 ml of water, 3,6 mg ATP as neutral K salt. Incubation for 5 min. at 38°.
Points = myosin, circles = actomyosin.

The influence of the KCl concentration on the phosphatase activity of myosin and actomyosin is given in Fig. 1.

It can be seen that there is no great difference between the KCl curve of myosin and acto-myosin. Both have a distinct KCl optimum at 0,2 *M* KCl.

The influence of $MgCl_2$ concentration at two different KCl concentrations is given in Fig. 2. (0,01 *M* KCl) and Fig. 3 (0.1 *M* KCl).

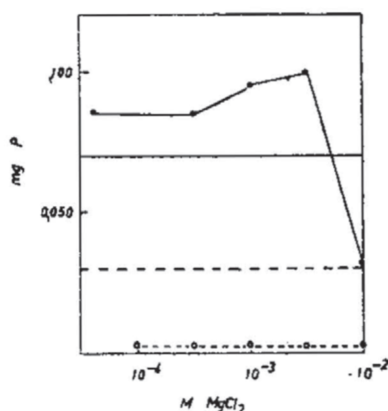

Fig. 2. Effect of $MgCl_2$ on the phosphatase activity of myosin in the presence of 0.01 *M* KCl. Broken line = myosin, full line = actomyosin. The straight lines indicate the phosphatase activity without $MgCl_2$.

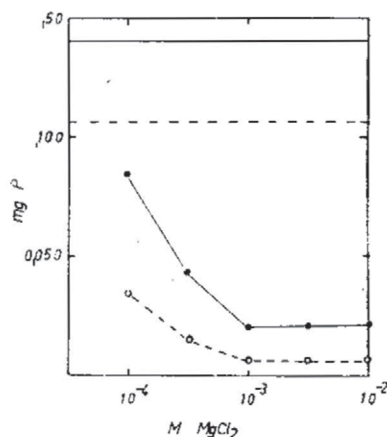

Fig. 3. Effect of $MgCl_2$ on the phosphatase activity of myosin in the presence of 0.1 *M* KCl. Broken line = myosin, full line = actomyosin. The straight lines indicate the phosphatase activity without $MgCl_2$.

It can be seen that the phosphatase activity of myosin is completely inhibited even by the smallest $MgCl_2$ concentrations regardless of the KCl concentration. The influence of $MgCl_2$ on the activity of actomyosin depends on the KCl concentration. In the presence of very small KCl concentrations $MgCl_2$ enhances the phosphatase activity with a maximum between 10^{-3} and 5.10^{-3} *M* $MgCl_2$. Higher concentrations inhibit. In presence of 0,1 *M* KCl the activity of actomyosin is inhibited by all Mg concentrations, similar to the action of $MgCl_2$ on myosin. (The intermediary KCl concentrations of 0,025 and 0,045 had an intermediary effect: Up to 5.10^{-3} *M* Mg had no effect at all, higher concentrations inhibited.)

The question arises how this action of KCl should be

explained? The results of this laboratory obtained by viscosimetric methods[2] allow of a simple explanation. It has been shown that in the presence of KCl and ATP the actomyosin dissociates into actin and myosin. This dissociation depends on both the KCl and ATP concentrations. In our experiment this dissociation took place at 0,1 *M* KCl, hence Mg had at this KCl concentration on actomyosin the same inhibiting effect as on myosin.

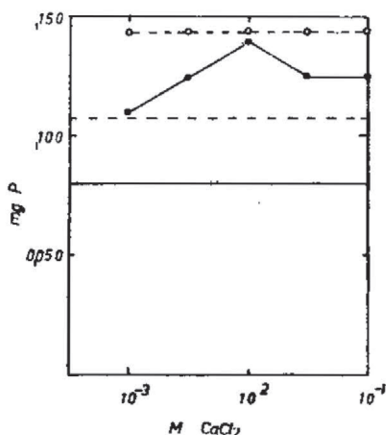

Fig. 4. Effect of $CaCl_2$ on the phosphatase activity of myosin in the presence of 0.01 *M* KCl. Broken line = myosin, full line = actomyosin. The straight lines indicate the phosphatase activity without $CaCl_2$

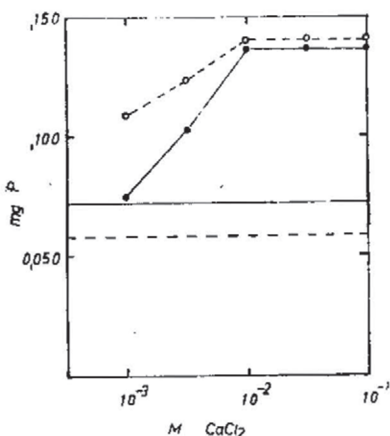

Fig. 5. Effect of $CaCl_2$ on the phosphatase activity of myosin in the presence of 0.1 *M* KCl. Broken line = myosin, full line = actomyosin. The straight lines indicate the phosphatase activity without $CaCl_2$.

Fig. 4 and 5 show the action of varied $CaCl_2$ concentrations in presence of 0,01 and 0,1 *M* KCl respectively. The experimental conditions are the same as in the experiments of Fig. 2. It will be seen from this curve that $CaCl_2$ has a strong enhancing influence at all concentrations between 10^- and 10^{-1} *M*. The effect is identical at both KCl concentrations. Since myosin and actomyosin are equally activated by $CaCl_2$ dissociation will have no effect and identical curves will be obtained with myosin and actomyosin at both KCl concentrations.

In Fig. 6 is given the effect of varying concentrations of $MgCl_2$ in presence of 0,01 *M* $CaCl_2$ and 0,01 *M* KCl. It will be seen that the phosphatase activity which has been increased

by $CaCl_2$ is depressed by all concentrations of $MgCl_2$. The experiment gave in presence of 0,1 *M* KCl the same results. Mg and Ca, though in themselves both capable of increasing the phosphatase activity of actomyosin are, if given together, not synergetic but antagonistic.

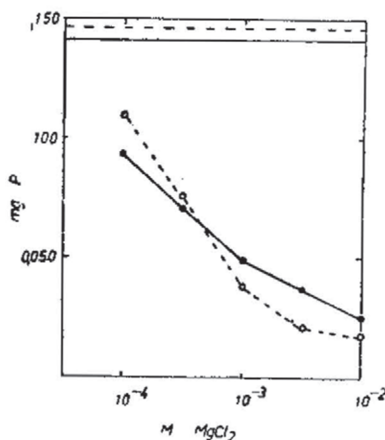

Fig. 6. Effect of $MgCl_2$ on the phosphatase activity of myosin and actomyosin in presence of 0.01 *M* $CaCl_2$ and 0.01 *M* KCl. Broken line = myosin, full line = actomyosin. The lines on the top give the phosphatase activity in absence of $MgCl_2$.

We want to use this occasion to correct an earlier statement. One of us (B.) has found before[1] that quinine and nicotine strongly inhibited the phosphatase action of myosin. This was a rather important statement because these poisons do not inhibit the contraction of actomyosin. It was found since that quinine and nicotine do not inhibit the enzymic action of myosin. They only inhibit the detection of the phosphate hydrolysed. We could not corroborate either the earlier finding that oxalate inhibits the enzymic action of myosin. The question, thus, whether splitting of ATP is necessary for contraction, is still open.

1. *I. Banga.* These studies 1, 27, 1941 – 42.
2. *F. Guba.* This vol. p. 40.

The crystallisation of myosin and some of its properties and reactions.

by

A. SZENT-GYÖRGYI.

It has been shown in the previous papers of this laboratory (1. 2.) that the contractile substance of striated muscle is „actomyosin", a compound of two proteins: actin and myosin.

The methods, hitherto employed by different authors for the preparation of „myosin" always yield a myosin more or less heavily (1—3%) contaminated with actin. All the data of literature relate to such undefined mixtures. Since even small traces of actin greatly modify the properties and reactions of myosin it seemed desirable to obtain myosin free of actin.

In this paper I will describe the preparation of actin-free myosin, its crystallisation, will describe some of its properties and reactions.

The preparation of myosin.

In all our experiments only chemicals of high purity were employed. The distilled water was redistilled from glass vessels. As material the striated muscle of the rabbit was used.

The animal was killed by decapitation, quickly skinned, eviscerated and dipped into ice-water. After a few minutes the body-muscles were cut out and packed between ice. Then they were minced on a cooled meat mincer with a sieve plate of holes of 2 mm diameter.

Principle. The separation of myosin from actin is based

on the observation that ATP will precipitate, in presence of different salt concentrations, actomyosins of different composition. The higher the salt concentration the less myosin is taken down by a given amount of actin but at the same time the less complete the precipitation. We have to work at the salt concentration at which the precipitation is fairly complete and not too much myosin is taken down by the actin. So for instance, in presence of 0,05 *M* KCl and at pH 6,5 a 0,5 % actomyosin will precipitate as such, that will say that one part of actin will take down with it 200 parts of myosin. In presence of 0,1 *M* KCl a 1,5 % actomyosin will be precipitated from the same solution and one part of actin will take down only 66 parts of myosin. Above 0,2 *M* KCl the precipitation becomes incomplete. The separation of actin involves always the loss of a relatively great quantity of myosin. It is thus essential to start with myosin of possibly little activity.

This precipitation with ATP will not yield a completely actin-free preparation (0.1% actomyosin). The muscle extract seems to contain also some inactive actomyosin which is not precipitated by ATP. This actomyosin can be precipitated by diluting the KCl to 0.04 *M* at a slightly alkaline reaction. Myosin is soluble, actomyosin insoluble in this solvent. The relative loss in myosin in still bigger than in the case of the ATP precipitation and still more myosin is taken down by the same amount of actin. This method can thus be used only with extracts which have been rendered very poor in actin by the preceding ATP-precipitation.

Myosin cannot be freed from actin by crystallisation since actomyosin behaves as an individual substance. As shown by F. GUBA (oral comm.) even a 2,5% actomyosin can be brought to crystallisation as such.

First step. The muscle is suspended in the ice-cold KCl-Phosphate mixture of GUBA and STRAUB which contains 0,3 *M* KCl and 0,15 *M* K-phosphate of pH 6,5. The muscle is extracted for ten minutes under constant stirring and then centrifuged at 0°. The precipitate is discarded, the fluid diluted with four volumes of water of room temperature (22°) and stirred gently. After one or two hours suddenly a flocculent precipitate is formed. What happened was that the ATP present was used up to such an extent that it caused no

more a dissolution but a precipitation of the actomyosin. (If this precipitation takes place without stirring a very fine colloidal precipitate is formed which cannot be separated on the centrifuge.)

Myosin is rather sensitive to heat and even short (10 min.) incubation at 37° causes a rise of its viscosity and partial loss of its enzymic activity. Therefore it is important to work at low temperature. Unfortunately this is not possible throughout, for in two of the steps the precipitaion would be incomplete at 0°. Fortunately myosin is stabilized by ATP, as observed by ENGELHARDT and LJUBIMOWA (3) and corroborated by myself. In the first steps of our preparation the myosin is protected by the ATP present. The fluid contains even at the moment of the precipitation some ATP.

The precipitate thus formed is separated by rapid centrifugation at room temperature and the opalescent fluid is diluted with 1,5 vols. of ice-cold water. This water is run in slowly, in about 10 minutes under constant energetic stirring. The myosin separates in the form of fine, needle-shaped crystals. If the water is added suddenly and without stirring an amorphous precipitate is obtained.

The fluid is allowed to stand for an hour or two at 0°, decanted and the myosin separated on the centrifuge.

If necessary this crystalline precipitate can be washed by suspending it in 0,04 *M* KCl and separating the myosin again on the centrifuge.

Example: 357 g minced muscle. Extract 750 ml. Contains 7 g 0,35% actomyosin. The first precipitate contains 2 g 1,5% actomyosin. The final myosin precipitate contains 3,4 g myosin. Thus only about $^1/_2$ of the myosin present in the extract is isolated. The other half is lost as actomyosin or is left behind in the fluid.

Second step. The crystalline myosin precipitate of step 1 is dissolved in a 0,02 *M* K_2CO_3 containing 10 ml of 1% alcoholic phenolphthalein in every liter. We add the carbonate solution in small quantities and homogenise carefully with strong stirring. Carbonate is added till the fluid retains a faint rose colour (pH 8,3) and add for every g of myosin present 4 ml of 2 *M* KCl. Then we dilute with water adding 50 ml for every ml of KCl solution. This water is of room tempera-

ture (22°) and contains 0,001% phenolphtalein and sufficient K_2CO_3 to give it a faint rose colour. The water is added under strong stirring. A voluminous, loose precipitate is formed which is separated on the centrifuge. The faint rose coloured opalescent fluid is poured off and cooled. The precipitate is treated once more in the above way, *i. e.* if its colour has faded out we restore it by adding K_2CO_3, then we add KCl and finally water and centrifuge, the only difference being that this second time we add only half as much KCl and water as the first time. The precipitate is discarded and the fluids united. From this point on the preparation is continued at 0°.

The fluid is stirred energetically and 1% acetic acid is run in very slowly till the fluid is neutralised. The myosin separates in the form of somewhat irregular needles and is centrifuged.

Recrystallisation.

The precipitate is dissolved by adding 2 *M* KCl in small kuantitites. The fluid is carefully homogenised after each addition. KCl is added till the concentration of the KCl reaches 0.6 *M*. Then we dilute further with 0,6 *M* KCl till the fluid loses its very high viscosity and contains about 3 % myosin.

The myosin solution is stirred very energetically and water is run in very slowly till the KCl concentration drops to 0,04 *M*. The addition of this amount of water should take about one hour. The myosin separates in the form of beautiful needleshaped crystals. About half of the myosin isolated in the first step will be obtained in this form. This myosin contains no actin or only negligible traces of it (0—0,02 %).

Some properties and reactions of myosin.

Myosin crystallises in the form of very fine needles (Fig. 1.) with a strong tendency of lateral association. Needles, observed with high power, will often be found to be a bundle of finer needles. Sometimes the needles associate into fine, long threads, fibrils (Fig. 2.).

Analysis. Recrystallised myosin was precipitated with alcohol and extracted with boiling abs. alcohol for two hours.

The alcohol was evaporated, the residue extracted with chloroform. The chloroform was evaporated and the residue

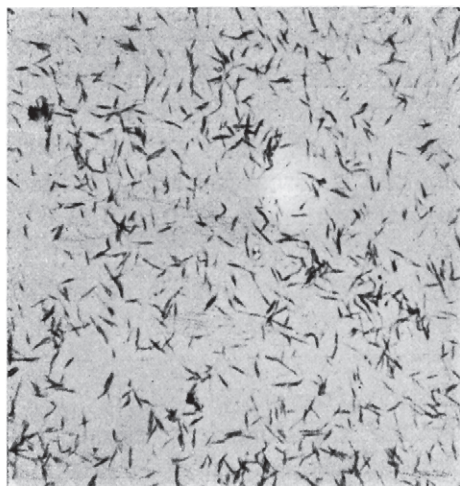

Fig. 1. Myosin crystals. Magn. 1:90.

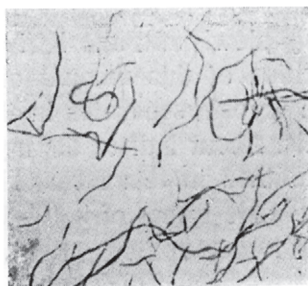

Fig. 2. Myosin threads. Magn. 1:40.

extracted with ether. After evaporation of the ether the residue was weighed. The lipoid matter was partly insoluble in acetone and constituted 3% of the dry weight of myosin.

The lipoid-free myosin was dried *in vacuo* at 120° C. The elementary analysis was kindly made by DR. M. KOVÁTS OSKOLÁS. It showed the following result:

C	50,04%
H	7,70%
N	16.15%
S	1.14%
Ash	1.23%

According to this analysis myosin is a protein. It contains 6 atoms of S for every 17000 g.

Stability. Myosin, if dissolved in 0,5 *M* KCl and neutralised, can be kept at 0° C without loss of enzymic activity for a fortnight. Finer colloidal properties, however, seem to be impaired after a few days storage, as indicated by the sluggishness of the fall of viscosity of an actomyosin, prepared from it, on addition of ATP (oral comm. of F. B. STRAUB).

Solubility. If the crystalline myosin is suspended in water and is rendered salt-free by dialysis it swells up to a glassy mass which shows only a slight opalescence. On dilution it dissolves in water giving a clear and very viscous solution from which no myosin can be separated by centrifugation. According to the water-solubility in entire absence of salts myosin is not a globulin. On the other hand, on adding ammoniumsulfate, the myosin precipitates as we pass half saturation. In this respect myosin conforms with globulins.

Myosin is precipitated by small concentrations of neutral salts and dissolves in presence of higher salt concentrations. If dissolved in water or in 0,1--0,2 *M* KCl it shows a very strong DRF. This DRF, however, is not due to the single myosin particles but to their coaxial association, for the DRF becomes weaker at 0.3 *M* KCl and disappears entirely at 0.4 M KCl. It also disappears if the solution is rendered alkaline. Evidently the higher salt concentration or pH prevents association. At the low velocity gradients of our experiments myosin shows, in 0,6 *M* KCl, no DRF.

Hand in hand with the association the limpid myosin solution becomes more and more opalescent.

If a sufficiently strong 0.5 *M* KCl-solution of myosin is diluted with water and is stirred, the turbidity assumes a silky appearance as the concentration of the KCl falls below 0,4 *M*. Between crossed Nicolls the fluid shows a strong double refraction. This indicates that the myosin particles aggregate

coaxilly to anisodimensional particles. As we dilute the solution more and more the aggregation becomes stronger and as we reach the limit of the solubility of myosin about 0,05 *M* KCl the particles become visible in the form of crystals. Strong mixing is essential if we want to obtain well formed crystals, for this mixing provides the coaxial orientation of particles which is necessary for crystallisation.

Reaction with salts. The most striking property of myosin is that it is readily precipitated by small concentrations of alkali salts. It is almost quantitatively precipitated by 0,05—0,01 *M* KCl; even 0,0015 *M* KCl causes precipitation.

In table 1. the precipitating action of different salts is compared.

Table I.

	0,2	0,1	0,05	0,025	0,0125	0,006	0,003	0,0015	0,0008	0,0004
KCl	0	0	+	++	++	++	+	+	0	0
KF	+	+	+	++	++	++	++	++	+	0
KJ	0	0	+	++	++	+	0	0	0	0
LiCl	0	0	+	+	++	++	++	+	+	0
NaCl	0	+	+	++	++	++	+	+	0	0
$MgCl_2$	0	++	++	++	++	++	++	++	++	++
$CaCl_2$	0	++	++	++	++	++	++	++	++	++

0,5 ml. of the salt solution was added to 2 ml of a 0,1% salt free myosin solution. Upper line: the final molar concentration of the salt. O means no change, + means turbidity or precipitation.

The table shows that the action of the different alkali halogens is rather similar. Bivalent cations give a rather voluminous precipitate through the whole range without a well defined maximum. This makes it evident that the precipitation is rather the action of the cation than that of the anion. The valency of the anion has less influence and even the trivalent phosphate as K salt has about the same action as KCl.

It was interesting to know whether the precipitation of myosin by salts is connected with a loss of charge. On my request K. LAKI has kindly undertaken to investigate this question. His report reads as follows:

„The migration of charcoal particles coated with myosin was followed in the microscopic cataphoretic apparatus described by J. H. NORTHROP und M. KUNITZ (J. gen. Physiol. 7. 729, 1925). It was found that the myosin, dissolved in dist. water, is negatively charged and readily migrates under the influence of an electric field. In KCl solution of 0,015 *M* the migration is slowed down. At 0,025 *M* the migration is still detectable but at 0,05 *M* the particles ceased to move. Tests were made also at higher KCl concentrations up to 0,2 *M* but no migration was detectable.

From these preliminary experiments the conclusion can be drawn that the charge of the myosin particle is diminished or lost at KCl concentrations where the myosin precipitates. The results obtained are presented in the following table:

M KCl	Conc. of the myosin	
0,00	6,0 mg/cc	Moves to the pos. pole.
0,012	0,6 „	Moves slowly „ „
0,025	3,0 „	Moves slowly „ „
0,05	0,6 „	No migration
0,05	0,3 „	„
0,08	2,0 „	„
0,10	6,0 „	„
0,10	3,0 „	„
0,20	2,0 „	„

Casein, treated in the same way, migrates in 0,2 *M* KCl to the positive pole".

The effect of pH on precipitation is shown in Tab. II. The buffer acted as salt.

The table shows that at a higher pH the precipitation is limited to a smaller range and is weaker. If we raise the pH further there will be no precipitation at all. At low pH (the three lowest lines), where we approach the isoelectric point, the myosin separates in form of a gelatinous mass instead of giving a precipitate. The maximum precipitation is observed at pH 6,4 and 6,7.

The asterisks mean that the precipitate, on mincing, has a silky appearance and shows a strong DRF, is thus crystalline.

Table II.

$\frac{Na_2HPO_4}{KH_2PO_4}$	0,1	0,05	0,025	0,0125	0,006	0,003	0,0015	0,0008	pH
1/0	0	0	0	0	+	+	+	0	
16/1	0	0	0	++	++	+	+	0	8
8/1	0	0	0	++	++	+	+	0	7.7
4/1	0	0	+	++	++	+	+	0	7.3
2/1	0	0	+*	++	++	+	+	0	7
1/1	0	0	++**	+++	+++	+	+	0	6.7
1/2	0	+**	++**	++*	++	+	0	0	6.4
1/4	+	+	++	++	++	+	0	0	6.1
1/8	+	+	+	+	+	+	0	0	5.8
1/16	+	+	+	+	+	+	+	0	5.5
0/1	+	+	+	+	+	+	+	0	

Isomolar Na_2HPO_4 and KH_2PO_4 were mixed in different proportions (Col 1). The final molar concentration of PO_4 is given in the upper line.

This shows that crystallisation is limited to a narrow pH range and has its maximum about pH 6,5.

This pH of 6,5, at which the myosin precipitates and crystallises most readily, at which the most inactive myosin can be extracted (see GUBA and STRAUB), seems to correspond to the pH of thoroughly washed myosin, as determined by SLATER (5), the flocculation maximum as found by COLLIP (6) and the minimum of acid-base binding capacity as given by EDSALL (4).

It seemed to be interesting to know whether myosin binds any K at those KCl concentrations at which it is precipitated. This question was answered in the following way: the crystalline myosin, after its recrystallisation, was centrifuged, K was estimated in the supernatant fluid. The crystalline precipitate was weighed, dried, weighed again, combusted and its K estimated. I am indebted to Prof. E. ERNST for the K estimations.

The precipitate contained 4,5—7% crystalline myosin and a KCl solution the concentration of which was identical with that of the supernatant fluid and varied between 0,3—0,1 *M*. Knowing the quantity of the water in the precipitate and the

KCl concentration in the supernatant fluid, it can be calculated how much of the total K of the precipitate falls to the water and how much to the myosin. If the K of the water is subtracted from the total K the difference is the amount held by the myosin These data show that one g atom of K is held in the different experiments by the following quantities of myosin: 10000, 12000, 12000, 12000, 14000. On average 12000 g myosin bind one atom of K. This K can be removed by dialysis.

Reaction with ATP. If ATP is added to the watery solution of myosin the viscosity will decrease and the DRF will become somewhat weaker. This indicates that ATP disaggregates to some extent the associated myosin.

ATP will show a solvatising action also in presence of salts. If ATP is added along with the salts the precipitate formation will be weaker, or no precipitate will be formed at all. So, for instance, 0,05 mg ATP per ml will be sufficient to prevent any precipitate-formation by neutral potassium-phosphate.

If the salt precipitates the myosin in amorphous form the DRF disappears and can be brought back by the addition of ATP which dissolves the precipitate.

These experiments indicate that the myosin particles are present in their solution in a straight, distended form. They do not change their shape not even if discharged or precipitated or if ATP is added. This conclusion is also supported by our earlier observation that myosin threads do not contract.

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Observations on Actomyosin.

by

A. SZENT-GYÖRGYI.

If a 0,5 *M* KCl-solution of myosin and a solution of actin are mixed actomyosin is formed. If the solution is sufficiently strong it can be pulled into threads.* If it is diluted with water it precipitates and can be washed salt-free giving a fine and fairly stable suspension.

Actomyosin is much less soluble than myosin or actin is. While myosin dissolves in water actomyosin only swells up. While 0,2 *M* KCl dissolves myosin 0,6 *M* KCl will be needed to dissolve actomyosin. But on the whole the collodial reactions of actomyosin are a reflection of the reactions of myosin.

Free actin is not precipitated by alkali salts, myosin is. Actomyosin will be precipitated similarly to myosin. In Tab. I. the precipitation of myosin and actomyosin by KCl are compared and it will be seen that the precipitation of both, at small salt concentrations, go parallel.

ATP has a solvatising action on myosin and will, in absence of salts, dissolve actomyosin also.

While myosin and actomyosin react thus to salts and ATP in a similar way; they will respond in a different way to the joint action of salt and ATP. While in the case of myosin the sole effect of ATP was to weaken the precipitating action of salts, in the case of actomyosin small amounts of ATP will greatly intensify the action of the salt. If the salt, in itself, had a precipitating action, ATP will make this precipitation much

* Technic of threads see vol. 1 of these studies.

Table I.

M . KCl	0,2	0,1	0,05	0,025	0,0125	0,006	0,003	0,0015	0,0008	0
Acto-myosin	++ 15	++ 14	+++ 11	+++ 12	++ 16	++ 20	+ 28	+ 44	0 65	0 65
Myosin	0	0	+	++	++	++	+	+	0	0

Upper line: molar concentration of KC. Middle line: volume of the actomyosin precipitate in an arbitrary unit. Crosses mean precipitation. 0,1% solution of myosin and 0,1% suspension of a 25% actomyosin.

Table II.

The action of varied KCl and ATP concentration on an actomyosin suspension

M KCl	mg ATP per ml						
	0,4	0,2	0,1	0,05	0,025	0,0125	0
0,4	!!!	!!!	!!!	!!!	!!!	!!!	!!
0,3	!!!	!!!	!!	!!	!	0	!
0,2	!!	!	xx	xx	xx	x	++
0,05	xxx	xxx	xxx	xxx	xx	xx	+++
0,0125	!	!	0	0	x	x	+
0,003	!!	!!	!	!	!	x	+
0,0008	!!!	!!!	!!!	!!	!	0	0

0, 1 % suspension of 25% actomyosin.

+ = precipitation, x = superprecipitation, ! means clearing up of the suspension. !!! means complete dissolution. O means no change. In this experiment first the KCl was added to the actomyosin suspension, the resul noted and then the ATP introduced.

stronger. If the salt (owing to its higher concentration) solvatised actomyosin in, ATP will make the dissolution complete. (Tab. II.).

Not only will the precipitation, caused by KCl in presence of ATP, be much intenser. The whole character of the precipitate will be changed: it will become granular and settle quickly to a small volume. This change is due not only to the intenser precipitation but at the same time to the strong shrinking of the particles. I will call this effect „super-precipitation" to distinguish it from the simple precipitation given by salts alone.

The shrinking of actomyosin can better be observed on actomyosin threads which give under the same conditions a violent contraction which is, in fact, but an extreme degree of shrinking. Superprecipitation and contraction are identical phenomena caused by the shortening of the actomyosin micels, as proved by the anisodimensional contraction of oriented myosin threads (M. GERENDÁS[1]). We can thus sum up our results by saying that if salts act on an actomyosin particle, they will simply discharge and precipitate it. If they act on the ATP complex of actomyosin they will cause not only discharging and precipitation of the particles but also their shortening. This is what has been called superprecipitation and what is observed in threads as contraction.

We must distinguish between the action of small and high concentrations of ATP. Small concentrations will have the effect described. Big doses will have a solvatising action only. Comparing the solvatising action of this excess of ATP at different salt concentrations (Tab. II.) we will find that the stronger the KCl precipitation and thus the stronger the KCl—ATP superprecipitation, the more ATP will be needed to cause dissolution.

Similarly to precipitation also superprecipitation is reversible. Any agent that brings about a dissolution and dissociation of actomyosin reverts it to its uncontracted form. It has been shown earlier (3) that contracted actomyosin threads can be brought to relaxation by alkaline salt solutions or by the combined action of salts and ATP (4). These relaxed threads are capable of contracting again.

Superprecipitated or contracted myosin can be dissolved by 0,05 % ATP in absence of salts. It is also dissolved by

higher salt concentrations or salt plus ATP. Substances that will dissolve it will also restore to it its DRF, restore thus also the original shape and dimensions of its particles.

ADP.* Threads prepared form synthetic actomyosin contract energetically in presence of salts and ATP. They are somewhat more labile than threads prepared from the impure natural actomyosin (our earlier myosin B). It was repeatedly observed that a freshly pulled thread gave only a sluggish contraction and became very active spontaneously in a few hours. The initial sluggish contraction could be speeded up by soaking the thread in an aqueous muscle extract.

There is one very sharp difference between the reaction of threads prepared from pure actomyosin and from myosin B. If the myosin B thread is suspended in a 0,05 *M* KCl containing 0,001 *M* $MgCl_2$, and 0,1% ADP is added, the thread will contract in the same way as if ATP had been added. If the pure actomyosin thread is treated likewise no contraction occures. If, however, in addition to ADP also $^1/_{10}$ parts of an aqueous muscle extract** are added, the thread will contract.

The aqueous extract can be inactivated by 20 minutes boiling. The contraction of the thread will be faster if the extract is mixed with the ADP solution before this latter is added to the thread. If the extract is allowed to stand with the ADP for a few minutes and the mixture then boiled for twenty minutes, it will cause rapid contraction if added to the thread. This makes it evident that the aqueous muscle extract contains a thermolabile substance which alters the ADP in such a way that it is capable of causing contraction. Evidently this catalyst is identical with K. LAKI's watersoluble factor and I. BANGA's isomerase. In all probability it restores to the nucleotide the active pyrophosphate configuration.

* The ADP was prepared by I. BANGA from ATP by having one phosphate split off by myosin.

** The aqueous extract was prepared in the following way: The muscle was minced, suspended in water, 3 ml being taken for every g of muscle. The suspension was stored over night at 0° during which time the ATP present was split. The clear filtrate contains no ATP and added to threads, gives, in itself, no contraction.

This experiment was repeated with a purified isomerase solution of I. BANGA. It was found that the protein solution reactivated the ADP only in presence of Mg (0,001 *M*). If isomerase and ADP were allowed to stand in absence of Mg, boiled and then added to the muscle, no contraction ensued.

We can thus say that the difference between myosin B and pure actomyosin is due the presence of isomerase in myosin B As shown by I. BANGA, myosin B can be freed from isomerase by repeated washing. Threads, prepared from such myosin B. behave in respect to ADP as the synthetic actomyosin. As shown by I. BANGA, such an isomerase-free actomyosin does not split ADP either. There is thus, at this point, a close analogy between splitting and contraction.

Salts. It has been shown before that there is a close analogy between the precipitation of actomyosin by KCl and the superprecipitation elicited by KCl and ATP. This analogy between salt-precipitation and superprecipitation breaks down in the case of Ca and Mg. Both ions have an equally strong precipitating action on actomyosin and could both be expected to act as K and give contraction in presence of ATP and enhance the K-ATP contraction. But contrary to this expectation Ca will give with ATP no contraction at all and Mg will do so only in presence of higher ATP concentrations which makes it probable that the contraction, in this case, is also due to the joint action of the Mg and K, the latter being present as cation of the ATP. Ca inhibits the K-Mg-ATP contraction also. There is thus an antagonism between K and Ca on the one side and Ca and Mg on the other side which makes it probable that Ca and Mg exert their influence by some other mechanism than K does. The antagonism of K and Ca in living muscle has been known for a long time. The predominance of K causes tetany, Ca suppresses it. Also the antagonism of Ca and Mg has been known from pharmacology (*Melzer* narcosis).

Example: actomyosin thread prepared from 12,5% actomyosin. The thread was soaked for a few minutes in the salt solution and then 0,125 % ATP was added in the form of its neutral K salt. The numbers give the % linear contraction reached in one minute, as measured by the length of the thread.

KCl 0,05*M*	38
KCl 0,05*M* and $MgCl_2$ 0,001 *M*.	52

KCl 0,05M and $CaCl_2$ 0,01 M	2
KCl 0,05M, $MgCl_2$ 0,001 M and $CaCl_2$ 0,01 M	18

The bending of actomyosin threads as a model of contraction. It has been shown that, under action of salts and ATP, actomyosin threads shrink rapidly i.e. become shorter and thinner: they contract. If, however, there is an assymmetry in the system which makes that the two long sides of the thread do not contract equally, then the thread, before

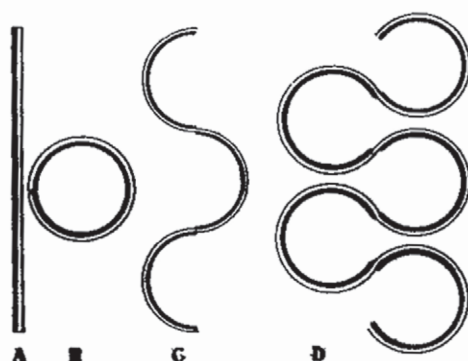

Fig. 1.

contracting, will bend or curl up. This is shown in fig. 1A and B where the more reactive side is marked with black. Such an assymmetry in the reaction can be produced by introducing the ATP on the one side of the thread. An assymmetry may also be found in the substance of the thread. Threads, if not pulled under special precautions, have some such assymmetry in their composition and tend to bend before they contract. One very often observes the curling up as shown in fig. 1B. It is immaterial what the source of the assymmetry is, the result will be the same: the bending of the thread. This is not a new phenomenon, it is an undesirable experience of every-day life: if two boards are stuck together and shrink or swell unequally then humidity will bend them.

Such an assymmetrically built myosin thread may serve as a model of the actomyosin micel. If a myosin and actin micel stick together side by side an assymmetrical structure will be obtained, as symbolised in fig. 1A. But myosin and actin

are both hydrophil colloids and rather similar in their constitution and react'ons. A high degree of assymmetry will be introduced by ATP which reacts only with the myosin moiety of actomyosin forming with it an ATP-myosin* complex which is most sensitive to ions. The one sided shrinking of the actomyosin micel will have to cause its bending. This bending will make the micel effectively shorter and so the whole mass of the actomyosin gel will shrink and the thread, made herefrom, shorten. The maximum contraction will be reached when the micels have curled up as in fig 1B. In this case the thread has to contract to $^1/_\pi$ of its original length (more exactly $l = \frac{l_0}{\pi} + d$ *d* being the diameter of the thread). The maximum contraction of actomyosin threads was found to be 66%.

It is also evident that in threads in which the actomyosin micels have been arranged coaxially to the thread the curling of the micels must give anisodimensional contraction: the thread must become shorter and thicker and at the same time its double refraction must be lost.

Figure 1B makes it evident that, if a contracted actomyosin is brought to dissociation, we obtain the original uncontracted actin and myosin which, if put together, will give uncontracted actomyosin, as is actually the case in the experiment.

The geometrical limitations given above do not hold if the actin forms a continuous structure, as is the case in muscle. Combination with myosin and ATP will give shortening also here but in this case the limit will not be $1/\pi$. The contraction will theoretically have no limits. In fig. 1C is pictured a shortening of $^1/_3$, in fig. 1D a shortening of $^3/_4$.

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* Should it be found that myosin is phosphorylated then the only change will be in regard of this model that we will have to write P-myosin in stead of ATP-myosin.

Observations on muscle extracts.

by

A. SZENT-GYÖRGYI.

If the minced muscle is extracted for ten minutes with 0,6 *M* KCl and centrifuged a fluid is obtained which contains about 1—1,2% of actomyosin with a 0,5—1% actin content. If the extract is stored over night at 0° the ATP is split. If the extract is diluted now with 4 vol. of water containing 0,001 *M* $MgCl_2$, a slightly turbid fluid is obtained which contains about 0,1 *M* KCl and the actomyosin in the form of a stable suspension.* If ATP is added now, according to its concentration, a turbidity, or a clearing up will be seen. It seems to be logical to observe this before a black back-ground with side illumination. This mode of observation, however, gives in this case erroneous result for flocculation often goes hand in hand with a decrease of luminosity.

Good results are obtained if the flocculation is observed before a black background with light falling in from behind the test tube at a very small angle. To make this into a simple method the test tubes were immersed into a waterbath with glass walls. At the back wall a paper-screen with black lines was fixed (Fig. 1). The source of light was placed behind this screen. Now the light falls in from between the black lines at a very small angle and the black lines form the black background. Precipitation will cause the black strips to appear hazy and grey. Clearing up will have the opposite effect.

The ATP was always introduced with a small spoon

* The water should be added suddenly. If it is run in slowly the actomyosin flocculates.

(Fig. 1) in a volume of 0,1 ml. If the spoon is pulled once or twice through the fluid within the fractions of a second complete mixing can be obtained. Stirring was continued throughout the observation.

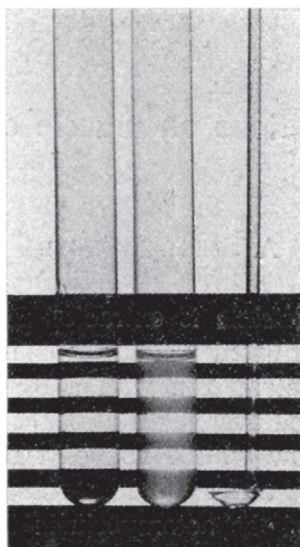

Fig. 1.

What will happen on addition of ATP depends on the first place on the quantity of the ATP added. If a very small quantity is added a precipitate will be formed at once. If more ATP is added the fluid will clear up. After the excess of ATP has been split the fluid will suddenly become turbid and precipitate will be formed.

If we plot the quantity of ATP against the time required for the formation of the precipitate we obtain the time curve of the splitting of ATP. Such an experiment is reproduced in the fig. 2. (0,1 *M* KCl). The splitting proceeds at a low ATP concentration at a fairly high rate to slow down suddenly at a somewhat higher ATP concentration. At a high ATP concentration the splitting becomes faster again. An increase of the KCl concentration from 0,1 to 0,15 and 0,2 *M* caused a flattening of the curve while the decrease of the KCl concentration from 0,1 *M* to 0,075 *M* made the curve much steeper. At 0,05 *M* KCl

the curve is still steeper. All the curves were S shaped. (On the curves of 0,15 and 0,2 *M* KCl the right half of the S falls outside the figure.)

The explanation of this change of slope is given by the experiments of I. BANGA who is showing in her paper that Mg very greatly inhibits the splitting of ATP by myosin and enhances the splitting of ATP by actomyosin. The increasing KCl concentration, as shown by F. GUBA makes the actomyosin dissociate into actin and myosin; the phosphatase action of the

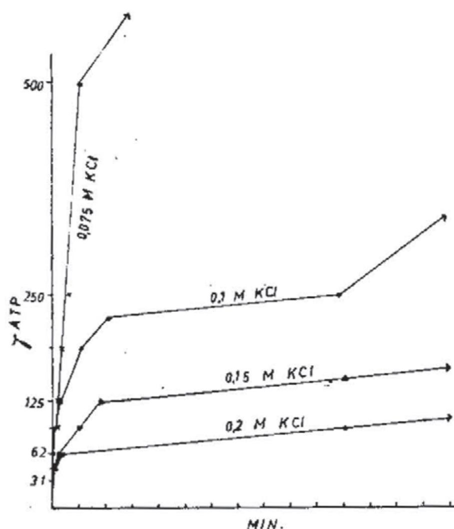

Fig. 2.

latter is then inhibited by Mg. The flattening of the curve indicates thus the dissociation of actomyosin.

This explains also the paradoxical flattening of the curve caused by the increase of ATP concentration. Evidently the excess of ATP also promotes dissociation.

A number of minor problems can be approached by the method described.

1. *ATP estimation.* If an unknown ATP solution is added to the 0,1 *M* KCl extract and its effect is compared with the effect of a known ATP solution, conclusion can be drawn on the ATP content of the unknown solution, provided it did not materially change the salt concentration.

2. *Maximum rate of splitting of ATP by myosin.* If we

measure phosphatase action by estimating the P liberated we have to take greater amounts of ATP and longer periods. Higher concentrations of ATP, as shown, inhibit the enzymic action. The described experiment allows to work with small ATP concentrations. In our experiment, working with small ATP concentrations at 0,05 *M* KCl, 100,000 g of myosin split 10 g molecules of ATP per second.

3. *Reversibility.* A small quantity of ATP causes precipitation, a bigger dissolution (0,1 *M* KCl). If we add a small quantity of ATP and, as soon as the precipitate is formed, we add a bigger dose, the precipitate will dissolve. If, however, we wait for a minute or so before adding the second ATP there will be no dissolution or the dissolution will be incomplete. This shows that very quickly unspecific cohesive links are formed in an actomyosin precipitate.

Note on plasmakinin.

by

K. LAKI.

Since the preceding papers have been closed down the experiments on plasmakinin showed that all the different globuline fractions of oxalated plasma obtained by ammoniumsulfate fractionation were active. If, however, the tests were made quantitatively it was found that the bulk of the active substance was brought down at 0,25—0,30 ammoniumsulfate saturation at which the fibrinogen precipitates. At the other saturations only 1 or 2 percent of this quantity came down.

If the fibrinogen, precipitated by the ammoniumsulfate is redissolved and then treated with alcohol or acetone, the precipitate is found to be inactive even if the precipitation was performed at low temperature where the proteins were not denatured.

Further experiments showed that an alcohol-soluble lipoid could be obtained from plasmakinin which showed almost the original activity when tested on Mellanby-fibrinogen.

The lipoid can be obtained in alcoholic solution in the following way: Fibrinogen is precipitated from the oxalated cattle plasma by 0,25 ammoniumsulfate saturation. The precipitate is centrifuged down and thoroughly washed with acetone and extracted with alcohol at room temperature. The alcohol is evaporated in a vacuum and the residue emulsified with water and tested on Mellanby-fibrinogen.

The active substance can also be extracted from the acetone-treated powder with petroleum ether. If the petroleum ether solution is concentrated in a vacuum, the addition of absolute alcohol produces a small quantity of an inactive precipitate, while the active substance remains in the alcoholic solution. This lipoid thus behaves similarly to the analogous alcohol-soluble lipoid fraction of brain. When this lipoid is exposed to air it looses the activity, probably owing to oxidation.

Note on actomyosin.

by

A. SZENT-GYÖRGYI.

Since the preceding papers have been closed down a new method has been worked out for the study of contraction. This method shows that actomyosin contracts also under the sole influence of alkali salts. This contraction is much weaker than the contraction caused by the joint action of salt and ATP. Its maximum lies at the same salt concentration as the maximum of the contraction caused by salt plus ATP.

It has also been found that, during contraction, active actomyosin goes over into inactive actomyosin. The change: inactive actin $\rightleftarrows$ active actin seems thus to be involved in contraction. The breaking up of the fibrous active actin micels into the globular inactive actin can be explained by the model described in my second paper. If the actin forms the outer circle it will be stretched. If it could not break up it would resist bending.