C. W. Kellaway

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CHEMISTRY OF MUSCULAR CONTRACTION

BY

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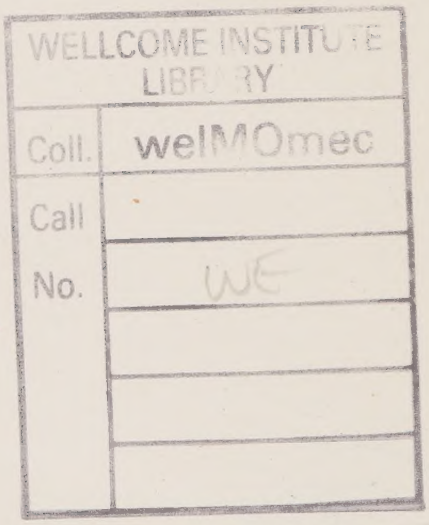

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FOREWORD

This little book contains my *Cameron-Prize* Lecture, held at the University of Edinburgh on July 23, 1946. Having been relieved of the danger of exhausting an audience rather than the subject, the text was completed.

I am conscious that the work to be reported here is very incomplete in many ways. This is because it is still in progress and means the opening up rather than the closing down of a field. The incompleteness of references may be excused by the completeness of my isolation for six years and the lack of a library at my present station.

The following abbreviations will be used:

ATP: Adenosine triphosphate, or adenyl diphosphate.
ADP: Adenosine diphosphate.
AMP: Adenosine monophosphate, or adenylic acid.
DR: Double refraction.
DRF: Double refraction of flow.
UW: Unit weight. For myosin and actin 17,600 g. will be taken arbitrarily as UW, whether such units actually exist or not.
MW: Molecular weight.
IP: Isoelectric point.

If not stated otherwise, my text relates to the cross-striated muscle of the rabbit.

I find it impossible to write on muscle without giving expression to my profound gratitude towards the *Josiah Macy, Jr. Foundation, New York*, whose help enabled me to start this work many years ago, and who came to my help again as soon as hostilities were over. It is equally impossible not to remember its late president, L. Kast, M.D., whose kind interest and encouragement doubled the value of the material help extended. My thanks are due to Professor J. W. McBain from Stanford University for giving me his fountain pen to write this book.

Arosa (Switzerland), August, 1946.

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Myosin, Actin, and Actomyosin

INTRODUCTION

Like most children, the biochemist, when he finds a toy, usually pulls it to pieces, and he can seldom keep his promise to put it together again. The loveliest toy ever provided by nature for the biochemist is the contractile muscle fibril. No wonder that biochemists of the fame of Danilewsky, Halliburton and v. Fürth tried to pull it to pieces as early as the eighties. They were fairly successful, showing that a considerable part of the fibril could be dissolved by strong salt solutions. A globulin-like protein was obtained which readily precipitated on dilution and was called "myosin" to distinguish it from the water-soluble "myogen."

Interest began to center around "myosin" again in the third decade of our century when H. H. Weber and, somewhat later, J. T. Edsall and A. v. Muralt took up its detailed study.

When my laboratory started work on muscle some seven years ago, our first step was to pull "myosin" to pieces. The earlier "myosin" turned out to be an undefined mixture of two proteins which united to form a compound. The properties of the pure components were quite different from those of "myosin" itself. For instance, they were quite soluble in water, whereas the most characteristic feature of "myosin" was its water-insolubility.

One of these new proteins will still be called "myosin." In retaining this name I wanted to pay tribute to the pioneers of this field. The other protein, discovered by F. B. Straub [16], will be called "actin."

Neither of these two proteins is, in itself, contractile. If put together in proper relations they unite to form the complex "actomyosin," which has the remarkable property of contractility. Contraction can be induced *in vitro* by ATP and ion constituents of the muscle fibre.

To see actomyosin contract was one of the greatest im-

pressions of my scientific career. Motion is one of the most basic biological phenomena, and has always been looked upon as the index of life. Now we could produce it *in vitro* with constituents of the cell.

Before embarking on the analysis of this phenomenon, I should like to make a general remark. Most of the properties and reactions of myosin, actin, and actomyosin seem unique, if not paradoxical. The reason is simple. As you know, all basic biological phenomena use up energy. The system performing these basic biological functions must have two states, rest and activity, each characterized by a different content of free energy. The proteins performing these reactions can also be expected to have two different states, and can be expected to be fibrous and to make part of the water-insoluble structure of the cell. These proteins generally refuse to be extracted without denaturation. Students of proteins thus limited their attention to the soluble, easily extractable globular proteins, which perform rather secondary functions around this basic, insoluble structure. What have been described as characteristics of "protein" are but qualities of the easily extractable globular fraction.

Owing to the specific mechanical function of muscle, its particles are fitted together in such a way that they can be taken apart without serious damage. Myosin can be obtained even in crystalline condition rather easily, so that most of the experiments referred to in this book were performed with recrystallized material. All the same, I hesitate to call myosin a true "substance." It is a whole world, a living little organism which releases and uses up energy, takes up and gives off water, leaning on a less reactive particle, actin, to translate these changes into motion.

A new fibrous protein called "tropomyosin" has been described recently by K. Bailey (1946). Its quantity is rather small as compared to that of actin or myosin, and since there is no evidence that it is involved in the contractile mechanism, its discussion will be omitted.

I. Myosin

General Properties

Myosin is a hydrophilic colloid: it dissolves in water giving a limpid solution. Its IP is pH 5.2. In the presence of small concentrations of KCl it readily crystallizes at pH 6.5–6.7 in the form of fine needles, forming small bundles (Figs. 1 and 2).

Fig. 1. Myosin crystals. Magn. 1:90.

Whether the crystals would satisfy the crystallographer, I do not know. I rather doubt it. Certainly they are beautiful enough to delight the heart of the biochemist. The first spinning experiments made with this material by Spellman and Erdös (personal communication) on the ultracentrifuge at Upsala show that it is perfectly homogeneous. The same is shown by the cataphoretic experiment of Erdös (personal communication).

On analysis, this recrystallized myosin showed the following composition:

C	50.04%	N	16.15%
H	7.70%	S	1.14%
	Ash	1.23%	

According to this analysis, myosin is a protein. It contains six S atoms for every UW, and no P. The myosin contained 3% lipoidic matter, partly insoluble in acetone. This was removed prior to combustion. Although myosin behaves like a globulin

Fig. 2. Myosin crystals. Magn. 1:300.

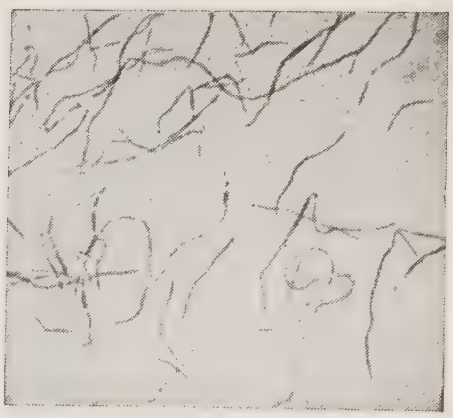

Fig. 3. Myosin with incipient acid denaturation. Magn. 1:90.

in that it precipitates with $(NH_4)_2SO_4$ as we pass half saturation, it cannot be classed with the globulins because it is soluble in water.

Myosin is very unstable, though not all of its properties are equally sensitive. It is completely denatured by acids or anhydrous solvents. Even at pH 7 its viscosity rises and becomes anomalous if it is incubated at 37° for ten minutes, or if it is stored for a long time at 0°. Salts and ATP, which increase its hydration, have a stabilizing influence. Slightly denatured myosin has a tendency to form threads (Fig. 3). Native, actin-free myosin readily spreads on water. On drying — even in the frozen state — myosin becomes insoluble.

If dissolved in salt-free water, myosin gives a limpid and rather viscous solution with a splendid DRF. Neither the high viscosity nor the DRF, however, are expressions of a fibrous

character, but are due to association, or swarm formation. If salt is added in higher concentration, or if the pH is raised, the viscosity drops and the DRF disappears. Myosin in many ways resembles soaps which, at certain pH's, also display a splendid DRF and high viscosity owing to association of the particles.

Myosin has a unique property: though hydrophilic, it is quantitatively precipitated from its watery solution by very small concentrations of neutral salts, like KCl. Such a small concentration as 0.001 M salt is sufficient to cause turbidity, and 0.025 M KCl causes complete precipitation. This reaction

TABLE I. *The Precipitating Action of Salts on Myosin*

	0.2	0.1	0.5	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: final molar concentration of the salt. 0 means no change, + means turbidity or precipitation.

is not specific for KCl and is shared by other neutral salts (Table I). If the salt concentration is increased the precipitate dissolves again.

For crystallization one generally starts with myosin dissolved in strong salt solution and precipitates the protein by dilution. A 5% myosin, dissolved in 0.5 M KCl, is somewhat opalescent, has a relatively low viscosity, and shows no DRF. If the salt is gradually diluted by the addition of water to about 0.3 M, the solution becomes more opalescent and begins to display DRF, and its viscosity rises as the result of gradual aggregation, but no particles can yet be seen under the microscope. On stirring, a silky sheen appears, indicating the forma-

tion of elongated particles and regular distribution. When the KCl concentration has been decreased to 0.05 M, visible particles appear in the form of long, fine, needle-shaped crystals. Crystallization occurs at the height of this gradual association of particles and the gradual decrease of intermicellar distances. These distances, however, must be rather large even in crystals because the crystalline mass, if separated on the centrifuge, always includes great quantities of water (95%) only a portion of which can be located between the crystals; hence a considerable part of the water must be located inside of the crystals. Crystallization is greatly promoted by stirring which provides the necessary coaxial orientation of particles. In many ways the properties of myosin also resemble those of vegetable viruses as revealed by the study of Bernal and Fankuchen (1941).

Naturally, association and consequently the DRF are functions of concentration. A certain concentration of myosin in salt solution will show DRF, whereas a more dilute solution may not.

The viscosity of myosin, dissolved in 0.5 M KCl, is low as compared to fibrous colloids but high as compared to globular proteins (Fig. 4). The viscosity of fresh myosin is normal even

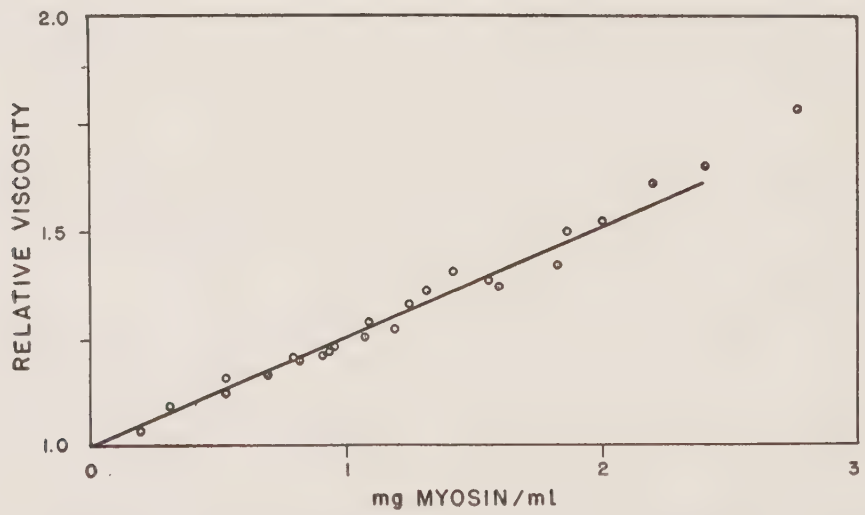

Fig. 4. Viscosity of recrystallized myosin in 0.6 M KCl at 0° (pH 7).

at pressures as low as 1 cm. water-pressure (Pittoni, unpublished). The viscosity seems to remain normal even at very high concentration, where the protein forms a soft, plastic gel.

All this indicates moderately elongated particles with a low degree of axial asymmetry.

According to the diffusion measurements of Weber and Stöver (1933) the MW of "myosin" is 10^6 g., the length of particles 500 Å, and their width 45 Å (maximum values, Weber, 1939). On treatment with strong urea the MW dropped to 10^5. As found by F. Guba (oral communication) the viscosity of such urea-treated myosin is as low as to indicate a globular shape, which suggests that the myosin micel is built of smaller globular units. Evidence will be given later, suggesting that units of MW of 17,000 and 150,000 are actually involved in the structure of the myosin micel. Unfortunately the measurements of Weber and Stöver, as well as later measurements of MW and molecular dimensions were made with impure myosin, more or less heavily contaminated with actin. More data are urgently needed.

Metal Myosinates

If a myosin solution is precipitated by KCl and the precipitate separated on the centrifuge, treated with alcohol, dried and analyzed, it will be found to contain no Cl but rather large quantities of K. The discharge and precipitation of myosin is thus due to the unequal adsorption of the anion and cation present. The absence of Cl shows that above the IP myosin has no affinity for anions, while the relatively great quantities of K show a strong affinity for this cation. The action of K is not specific: as shown by T. Erdös [47] the affinity of myosin is the same for K and Na. If preference is given to K throughout my discussion, it is because K is the main cation of muscle and is estimated with greater ease than is Na. The high adsorption power of "myosin" towards K was noted first by C. Montigel (1943) in Verzar's laboratory.

The adsorption of K to myosin has been studied more in detail by I. Banga [00]. The result of one of her typical experiments is reproduced in Fig. 5 (upper curve). This curve shows that myosin binds K strongly. The main feature of this curve is that it is composed of two parts of different gradient. Both parts are, on a logarithmic scale, linear, corresponding thus to an

exponential function, as adsorption processes usually do. The two parts meet at 0.025 M KCl, where the myosin has adsorbed five equivalents of K per UW, is isoelectric, and is maximally precipitated. We should thus distinguish between two processes of adsorption: (1) the adsorption of the five first equivalents of K^+ by the negative myosin, and (2) the further adsorption of K^+ by the electro-neutral K myosinate. Let us call the first flat right-hand side of the adsorption, taking place at the lower K ion concentration, primary adsorption, and call the second, steeper left-hand side, corresponding to the higher K ion concentration, secondary adsorption. The fifth valency, making the system isoelectric, lying at the breaking-point of the curve, may be counted with either zone of adsorption.

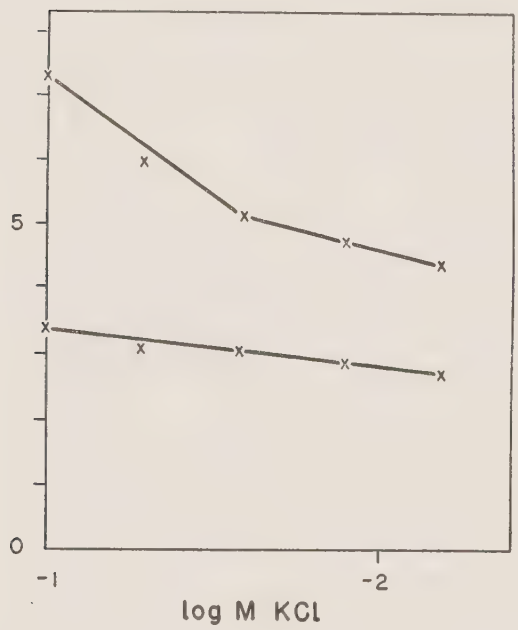

Fig. 5

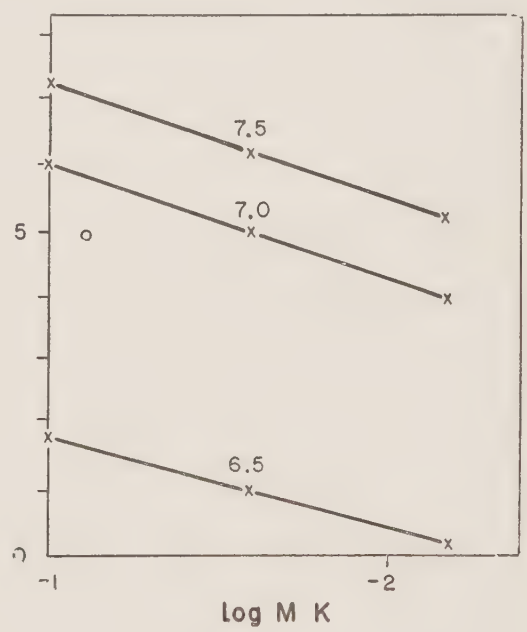

Fig. 6

Fig. 5. The adsorption of K on myosin in presence of KCl. Upper curve: fresh myosin 24 hours after the death of the animal. Lower curve: the same myosin 48 hours later. The ordinate shows the equivalents of K bound per UW of myosin.

Fig. 6. K-fixation of myosin at varied pH. Abscissa: log M KCl. Ordinate: equivalents of K bound per UW of myosin.

In the primary adsorption the basic negative charge of the protein prevails and the adsorption of K is supported by an electrostatic attraction which means that the K^+ is bound strongly, as expressed in the relative flatness of the curve; in

the secondary adsorption — as indicated by the steepness of the curve — the K is held loosely and K^+ is more readily taken up or given off on changes of K ion concentration in the surrounding fluid: a given change in the K ion concentration in the fluid will cause twice as much to be taken up or given off in this zone as in the primary one.

TABLE II. *Precipitation and Crystallization of Myosin*

$\frac{Na_2HPO_4}{KH_2PO_4}$	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 (Column 1). The final molar concentrations of PO_4 are given in the upper line: 0 = no change, + = precipitation. Asterisks mean crystallization.

K ion adsorption is a very labile property of the myosin. Even storage for a few hours makes an appreciable difference. The secondary adsorption suffers first and the curve flattens out. The curves in Fig. 6 are taken with such a myosin stored for 24 hours. On storage for 48 hours both processes suffer. The lower curve in Fig. 5 is taken with the same myosin as the top curve 48 hours later.

To come back for an instant to Fig. 6, it can be seen that myosin, in the presence of 0.025 M KCl (which precipitates it completely) is discharged and isoelectric, with one K^+ adsorbed at pH 6.5. Myosin, at this pH, seems to have one negative charge, compared to five negative charges at pH 7. Table II shows that crystallization of myosin is limited to a very narrow

range and has its maximum at pH 6.7, with probably two negative charges neutralized by two K^+. Table II and Fig. 6 also explain why different authors, working in the presence of salts (buffers), found the IP of myosin anywhere between 5.1–6.6. As pointed out by Hollwede and Weber, the IP of myosin is 5.1–5.3, whereas the IP's of alkali-myosinates are higher.

The real IP of myosin is thus at pH 5.2 but in the presence of 0.025 M KCl the myosin will be isoelectric up to pH 7. Thus KCl extends the IP of myosin. The extension of the IP of proteins by neutral salts is a well known phenomenon. In fact, my very first biochemical paper, written under the guidance of L. Michaelis (1920) twenty-six years ago, describes the shift of the IP of casein. The extension of the IP of casein under the influence of neutral salts, however, is exceedingly small, compared to that observed with myosin. While the behavior of myosin is thus not specific qualitatively and represents a basic reaction of proteins, it is quite specific quantitatively. This very high adsorption power towards K^+, and cations in general, seems to be intimately connected with the function of myosin. As shown, however, by A. Lajta (see page 86) the structural proteins of kidney and brain have a similar affinity to K. This specific adsorption power seems to be one of the basic properties of structural proteins, performing basic biological functions, and seems to be intimately connected with the very nature of life.

The curve of Fig. 5 could not be extended any further towards the larger or smaller K concentration for technical reasons. The K adsorption could be measured only if the myosinate was insoluble and could be separated on the centrifuge. Above 0.1 or below 0.006 M KCl, the myosin dissolves (pH 7). We can thus correlate the physical state of myosin with the number of K^+ bound: with three K^+ and two negative charges left, myosin is freely soluble; with four K^+ and one negative charge left, it is slightly soluble; when all negative charges are balanced by five K^+, myosin is insoluble; and it is soluble again when it has seven K^+ bound. Association ceases, DRF disappears, and viscosity reaches minimum with nine K^+, as extrapolated for 0.3 M KCl.

In Fig. 7 taken from Banga, the adsorption of Mg and Ca are compared. The curve shows that the adsorption of Ca and Mg is subjected to the same regularities as that of K and shows a rather pronounced break at the fifth equivalent adsorbed. Ca

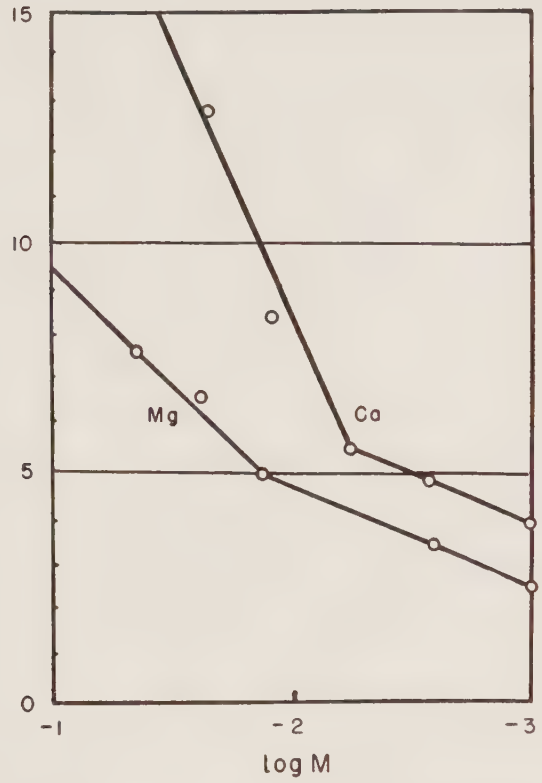

Fig. 7. Binding of Ca and Mg by myosin. Abscissa: log M concentration of the metal (as chloride). Ordinate: equivalents of metal bound per UW of myosin.

has the greatest affinity for myosin, which is demonstrated by the fact that the lowest concentration is needed from this ion to have five equivalents bound. Then Mg follows, and K is last. Therefore, if we have myosin in an isomolar solution of Ca and Mg, or Ca and K, the Ca will be adsorbed predominantly. Mg is preferentially adsorbed in an isomolar solution of Mg and K. (This was verified in the experiments of Banga.) The situation will be different if K is added in excess. If, for instance, myosin is brought into equilibrium with 0.12 M $MgCl_2$, from which five equivalents are bound, and KCl is added in 0.1 M concentration, two K^+ will be bound because 0.1 M KCl corresponds to the adsorption of seven equivalents, five of which are occupied by the Mg.

Myosin and Water

As has been shown in the previous chapters, the colloidal behavior of myosin is governed by its metal adsorption. Like other colloids, the myosin particle has at the alkaline side of its IP a negative charge due to the dissociation of its acidic groups. The hydrophilic nature of myosin is evidently due to this negative charge. As the outside action of this charge is balanced more and more by adsorbed K ions, intermicellar distances decrease and the quantity of intermicellar water becomes less and less. The minimum is reached when the number of positive charges equals that of the negative ones, at pH 7 in 0.025 M KCl with five K^+ adsorbed per UW. At this point myosin is quite insoluble. This water-insolubility shows that the myosin particle has no inherent "hydrophility" comparable to that of, say, glycerol, which is due to the electropolar nature of its OH groups. This is in agreement with results of Hill and Kupalow who showed that out of the 800 g. of water contained in 1000 g. of frog muscle, not more than 30 g., i.e., 4%, can be bound.

If the KCl concentration is increased above 0.025 M, more K is adsorbed, and intermicellar distances increase again. With two adsorbed K^+ in excess, myosin dissolves again but is still strongly associated, as shown by the high viscosity, strong DRF, and silky sheen. On further increase of charge, association gradually ceases, viscosity reaches a minimum, and DRF disappears. This shows that the colloidal behavior and "hydrophility" of myosin are governed mainly by electric forces, and the water bound by myosin is mainly intermicellar water filling the space between micels which mutually repel each other. The high water-content of myosin crystals or precipitates shows that in isoelectric myosin the electric forces are poorly balanced and prevent the very near approach of different particles or the approach of different parts of the same particle. The concentration of water around the charged structure may also play a role in these short-range relations.

ATP Myosinate

As mentioned before, myosin strongly binds metals but leaves (at neutral reaction) anions unbound. This property

holds even for trivalent anions, like ATP*, but occurs for metal-free ATP only. As K is adsorbed, myosin becomes more and more capable of binding ATP as well. As shown by Fig. 8, borrowed from W. Sz. Hermann [45], adsorption of K and adsorption of ATP are parallel. (The ATP-adsorption-curve in Fig. 8 could not be extended further to the left towards O K^+

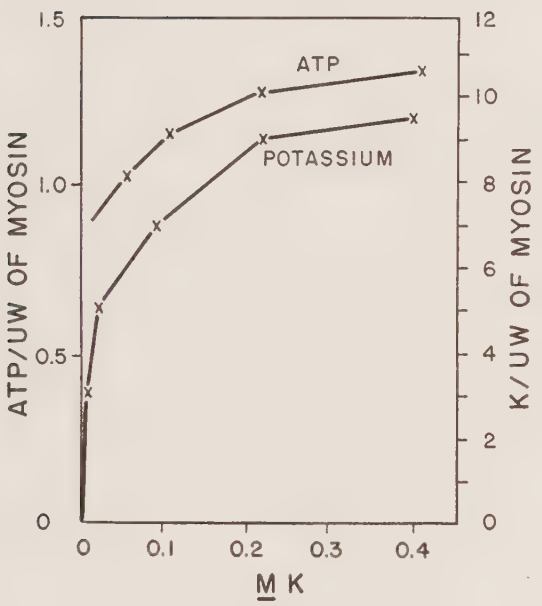

Fig. 8

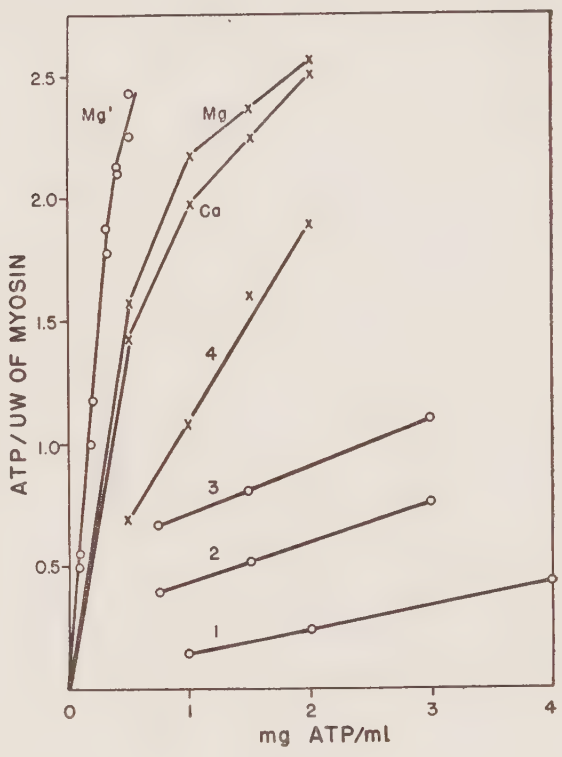

Fig. 9

Fig. 8. Adsorption of ATP and K by myosin. Left ordinate (relating to upper curve): molecules of ATP adsorbed per UW of myosin. Right ordinate: number of K adsorbed. Abscissa: [K] in the fluid. 0.17% ATP.

Fig. 9. Adsorption of ATP by myosin. Abscissa: ATP-concentration in the fluid. Ordinate: number of ATP-molecules adsorbed per UW of myosin.

because ATP, at neutral reaction, is a salt, containing in our experiments K as cation.)

The ATP adsorption is an exceedingly labile function of myosin and deteriorates quickly in storage. Curve 1 of Fig. 9 is an adsorption curve of ATP (Banga) taken at a time when no special care was exercised to use fresh preparations and no

*ATP is tetravalent but as shown by Banga the pK of the fourth acidic group is 8 and consequently, at neutral reaction, this group is not dissociated and ATP can be regarded as trivalent.

extra KCl was added. The curves 2 and 3 are corresponding curves of W. Sz. Hermann [45] using 24-hour-old myosin without and with, respectively, the addition of 0.2 M KCl. Curve 4 was taken by Hermann with myosin two hours after the death of the animal in the presence of 0.2 M KCl. This curve is much steeper than the previous ones, but all the same the adsorption at lower ATP concentrations is rather weak.

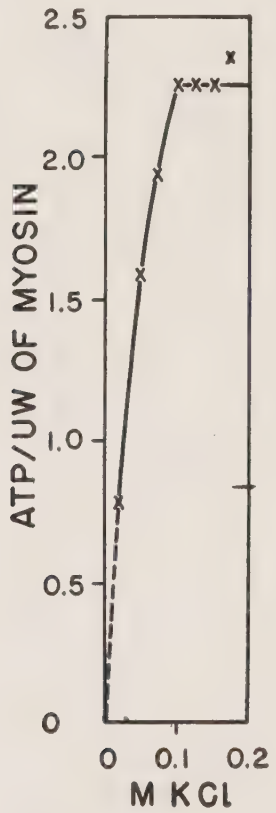

Fig. 10

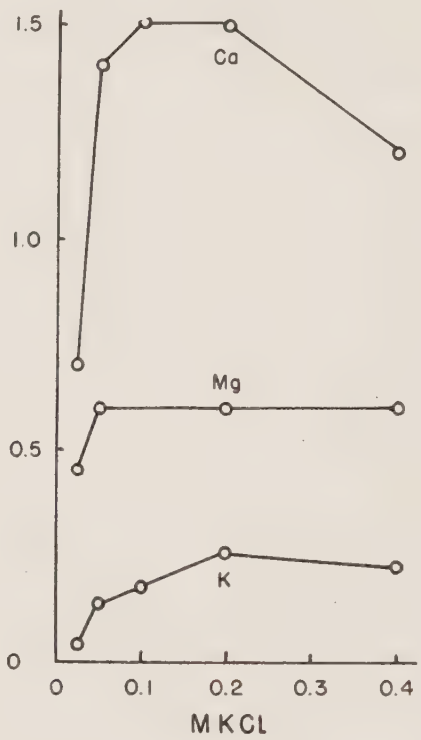

Fig. 11

Fig. 10. Adsorption of ATP in presence of 0.0005 M $CaCl_2$ and varied concentrations of KCl (abscissa). Ordinate: mols of ATP adsorbed by the UW of myosin in presence of 0.02% ATP. The broken line is extrapolated to O K.

Fig. 11. Adsorption of ATP at varied KCl concentrations in presence of 0.001 M $CaCl_2$, 0.001 M $MgCl_2$, and in the presence of K alone. Ordinate: molecules of ATP bound per UW of myosin. 0.2 mg ATP per ml.

Ca or Mg alone, in low concentration, does not cause ATP to be adsorbed. These ions cannot be tested in higher concentration because they precipitate ATP; in low concentrations (0.0005–0.001 M) they have no effect whatever on the ATP adsorption [Hermann, 45]. If added in the presence of KCl,

they very greatly increase the adsorption of the nucleotide. The Ca and Mg, adsorbed in the primary circle, change the general properties of the protein in such a way that it will intensely adsorb ATP if it has first adsorbed K ions, as shown by the curves Ca, Mg, and Mg of Fig. 9.

This rather striking effect of Ca and Mg is brought out more clearly if the KCl concentration is varied at constant Ca or Mg, and at a very low ATP concentration, such as 0.02%, as is the case in Fig. 10 [Hermann, 45]. The curve is exceedingly steep and reaches maximum critically at 0.05–0.1 M KCl. In spite of the low ATP concentration, much nucleotide is bound and the curve would have remained still straighter up to its maximum had not the ATP concentration in the fluid been reduced by the adsorption.

In these experiments performed by Hermann in the winter of 1945–46, Ca and Mg had the same effect. When these experiments were repeated in the spring of 1946, Ca showed the same effect as before but Mg was less active (Fig. 11). Whether the difference was due to seasonal variation or to methodical differences I am unable to state at present. It is not impossible either that in the earlier experiments a small quantity of Ca was retained by the myosin, careful purification being impossible in these very rapid preparations.

As shown before, myosin in 0.1 M KCl has two positive charges in excess. One would thus expect precipitation to occur on addition and adsorption of the negatively charged ATP. Instead, the opposite happens: ATP has a solvent action. If KCl is added to myosin in varied concentration with and without ATP, it will be observed that in the presence of ATP the precipitation is weaker, or no precipitate is formed at all, depending on the concentration of the ATP added. It can be deduced herefrom that the ATP adsorbed does not neutralize the positive charge of the adsorbed K ions.

Glycogen-Myosin Compounds

If a watery solution of myosin is mixed with a solution of glycogen, a precipitate is formed. If, for instance, equal parts of 0.2% myosin and 1% glycogen are mixed, the protein is

carried down quantitatively. The resulting glycogen-myosin complex is not soluble in dilute (0.1 M) KCl. If, however, a small amount (0.05%) of ATP is added, the compound dissociates, the myosin and glycogen dissolve, and the solution now behaves as a solution of free myosin. Soluble starch or dextrin gives no precipitate with myosin under similar conditions. The glycogen compound is thus specific to some extent and behaves, in relation to ATP, analogously to the actin compound (see p. 17). It is possible that the formation of this glycogen-myosin complex and its reaction to ATP are involved in the mechanism and regulation of glycogenolysis, fixation and fermentation of glycogen being promoted by decreasing ATP concentration.

Polysaccharide compounds of "myosin" were first observed by Przyleczky, Majmin, and Filipovics (1934, 1935).

II. Actin

The most interesting protein I have ever met is myosin; the second best is actin. The subtle reactivity of this latter and its interaction with ions are most fascinating objects of study. The actin particle is surrounded by a field of forces, governed by ionic balances, which forces determine the reactions of actin particles with one another and with myosin.

The most striking property of actin is its ability to exist in globular as well as in fibrous form. The properties of the two forms are widely different and so are the properties of the myosin compounds of both forms, the myosin compound of only the fibrous actin being contractile. The two forms can readily be transformed into each other reversibly, and this transformation from fibrous to globular and from globular back again to fibrous seems to occur in every contraction cycle.

The properties of actin are known — still rather incompletely — from the work of Straub and his collaborators, K. Balenović [17], G. Feuer and F. Molnár [62]. Actin is a protein and is readily denatured by heat. The preparation of actin, analyzed, showed the usual composition of proteins (51.3% C, 8.6% H, 15.1% N, 1.1% ash, and no P).

Unlike myosin, actin is not precipitated by alkali salts. It is less labile than myosin. Though it is readily denatured by salts below its IP, which is at pH 4.7, it can be precipitated at this pH, in the absence of salts, without considerable loss. It is readily destroyed by alkali or acid and is denatured by acetone but can be precipitated, under certain conditions, by strong alcohol or acetone in the native state. It is not precipitated by dilute (70%) alcohol.

Though present in resting muscle probably in the fibrous form, actin can be extracted from the tissue only in its globular form, and the method of extraction involves the change from the fibrous into the globular state.

Globular actin has all the earmarks of a typically globular

protein. Its viscosity is low and normal (Fig. 12); it has no DRF. On the subsequent pages it will be denoted by "G-actin," "G" standing for "globular."

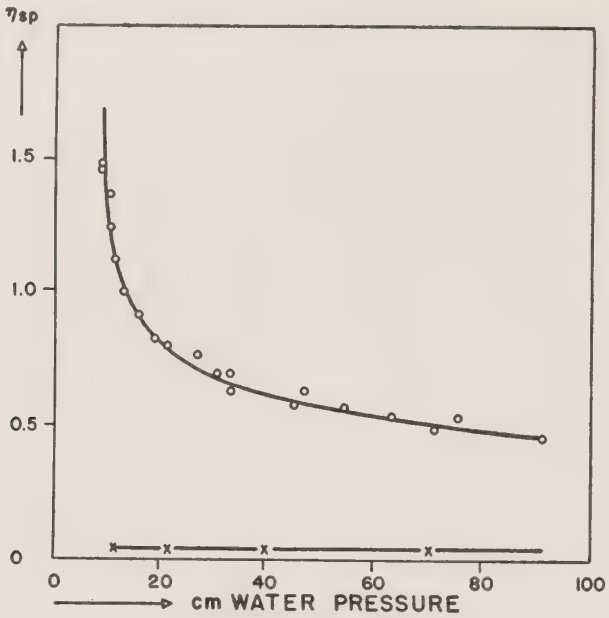

Fig. 12. Viscosity of globular and fibrous actin at various pressures. Crosses, 2 mg. globular actin per ml. in 0.2 M KCl. Circles, 3 mg. of fibrous actin per ml. in distilled water at 0° [Straub 16].

G-actin is freely soluble in water, giving a limpid solution. Its MW must be fairly low, 35,000–70,000 g. Its tryptophane content indicates that the MW is 68,000, but it passes readily through 10% collodion membranes which retain haemoglobin and let egg albumin through partially [62].

F-actin ("F" standing for "fibrous") has all the earmarks of a fibrous colloid. Its solution is opalescent and shows a strong Tyndall effect. This increase in light diffraction, as compared with that of G-actin, indicates that the formation of threads is not due to the unwinding of the globules of the G-actin but to their association in a row to something like a string of beads.

F-actin has a high and anomalous viscosity (Figs. 12 and 13). It shows a splendid DRF even on gentle shaking, which persists for some time after the fluid has come to rest (Fig. 14). It is fairly thixotropic, a property which contributes to the stabilization of the DRF. Stronger solutions set to a gel which liquefies on gentle shaking. Its DR is positive. It is completely retained by collodion filters.

Though thixotropy makes the exact measurement of viscosity impossible, it can be stated that the viscosity of F-actin is not too high; the range is the same as that of myosin; its axial asymmetry must therefore be in the same range too, and cannot be compared to the axial asymmetry of really fibrous, high-polymer colloids. The strong DRF and viscosity anomaly cannot be explained on the basis of long, thread-like form of particles, but must be due (as was the case with myosin) to association, or swarm formation, which is supported here by thixotropic forces. This conclusion is in agreement with the behavior of F-actin on drying. In the vacuum desiccator, actin dries down to a thin and hard sheet which can be pulverized and redissolved.

Metal Actinates

Actin is precipitated by very low concentrations of Ca, which reaction reveals a high affinity for this cation. It will be precipitated completely by 0.002 M $CaCl_2$. Straub's experiments (in which he estimated the decrease in concentration of

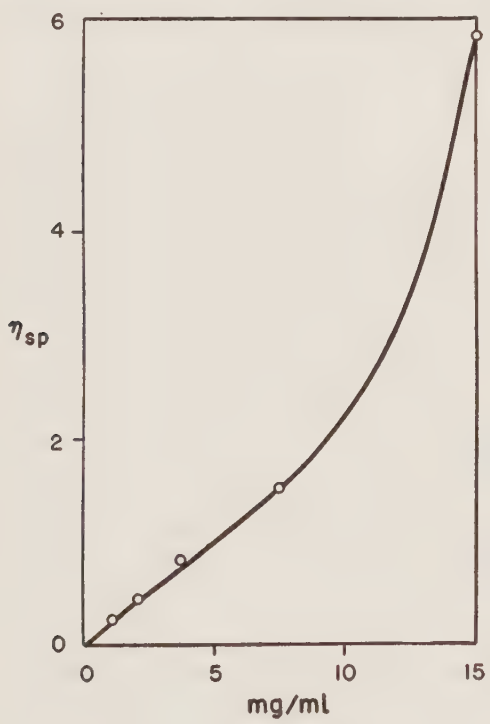

Fig. 13

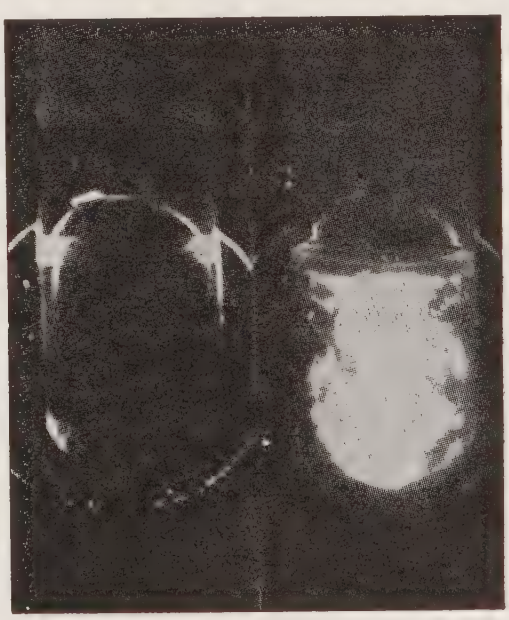

Fig. 14

Fig. 13. Specific viscosity of F actin as function of its concentration.
Fig. 14. Double refraction of actin solutions. Photographs taken through crossed nicols. Left: globular actin. Right: the same actin solution after polymerization to fibrous actin [Straub 16].

free Ca) show that the actin has bound six equivalents of the metal* per UW (pH 7). Erdos [57] studying the same phenomenon with Banga's somewhat modified method† (see "Metal Myosinates") found somewhat higher values (Fig. 15) and found no Cl in the precipitate. The curves obtained were

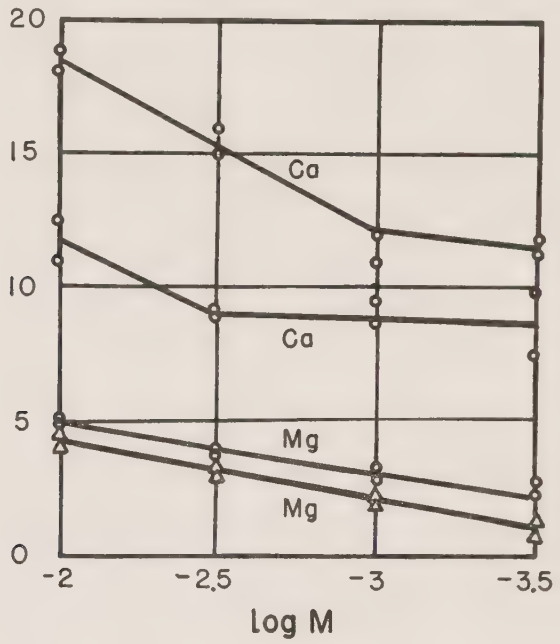

Fig. 15. Fixation of Ca and Mg by actin. Ordinate: equivalents of metal bound per UW of actin. Abscissa: log M Ca or Mg, present as chloride [Erdös 57].

analogous to the metal-binding curves of myosin in being composed of two straight parts and showing a break at the point where precipitation became maximal, indicating the complete "discharge" of particles. Accepting Straub's value, this happens with six equivalents of Ca per UW of actin as compared to the five equivalents found in myosin. The precipitation of actin by Ca salts is thus explained by the specific fixation of the metal and has the same mechanism as the precipitation of myosin. The precipitated Ca actinate can also be redissolved by an excess of the precipitating salt.

Actin is less readily precipitated by Mg which is explained by the lower affinity for this ion, borne out by the experiments of Erdös (Fig. 15). The affinity of actin for K and Na is very

*Ca fixation was found to have two optima, one at pH 7 and one at pH 9.

†Strong actin solutions were brought into equilibrium with different salt solutions through a cellophane membrane. After equilibrium was reached the actin was precipitated by acetone and the precipitate analyzed.

low and actin is not precipitated by these ions at all. The K bound in the experiments of Erdös was too low to be denoted in Fig. 15. In the presence of 0.1 M KCl, 2–2.5 K were bound, but no Cl.

There is a very fascinating antagonism between Ca and Na. If both ions are added to actin in the same relation as they are present in a physiologically "equilibrated" solution, as is Ringer's, no precipitate is formed at all, though Ca itself precipitates actin at these concentrations.

An explanation of this inhibitory effect is offered by Erdös [57] who found the fixation of Ca is inhibited by monovalent ions. The lower affinity of the latter may be compensated by their higher concentration. In the presence of 0.001 M $CaCl_2$ Erdös found ten equivalents of Ca bound in the absence of KCl, and three equivalents of Ca bound in the presence of 0.05 M KCl. This rather simple explanation probably does not reveal the whole situation, which will be shown later to be more complex.

The great affinity of actin for Ca is revealed also by the fact that actin preparations always contain one Ca per UW; so one Ca, at least, is bound very firmly. The relatively great affinity for Mg is revealed by the fact that actin preparations, obtained by Straub's original method, contain two Mg's per UW. Since both Ca and Mg are greatly diluted during the preparation, the conclusion seems to be warranted that these ions were not bound in the course of manipulation, but were linked to actin in the muscle, with their six equivalents, the probable number necessary for the complete neutralization of the outward charge of the particle. Since, in the course of preparation, the muscle is treated with strong KCl, the one Ca and two Mg's originally present seem not to be expelled by alkali-metals.

The G-F Transformation

The polymerization of G-actin into F-actin is connected with a rise in viscosity which makes it possible to follow this process with ease in the viscosimeter. Any salt that does not denature actin will cause its polymerization. The monovalent cations are active in the order $Rb < K < Na < Li$; the anions, $J < Br < Cl < F$. Bivalent ions are much more active than

monovalent ones. The latter cause strong polymerization in 0.1 M concentration, the former in 0.005 M. The most active cation is H^+ and polymerization at pH 6 is instantaneous.

Mg has a quite specific effect. In the absence of Mg there seems to be no polymerization at all. If the rate of polymerization is measured in the presence of 0.1 M NaCl (pH 7.4) and varied concentrations of Mg, and the rate is coördinated with the Mg concentration (counting also the quantity of bound Mg) the curve, extrapolated to zero Mg, shows that in the entire absence of Mg there would be no polymerization at all. If the actin is incubated for half an hour with 0.001 M Calgon (sodium hexa-metaphosphate) which strongly binds Mg, the actin will not polymerize at all.* This action of Mg is quite specific and is not duplicated by Ca, and polymerization is not inhibited by oxalate (oral communication of Straub). Very small concentrations of Mg, like 0.0005 M, which in themselves have no polymerizing action at all, suffice to secure rapid transformation in the presence of 0.1 M NaCl. Mg sensitizes G-actin, or, more exactly, Mg actinate only is capable of polymerization.

There is a most fascinating relation between the action of different ions which shows close analogies to physiological equilibration. First of all there is an antagonism between Ca and Na. Ca in 0.005 M or NaCl in 0.1 M concentration induces rapid polymerization. If given simultaneously there is no polymerization at all: they completely balance each other.†

KCl is less active than NaCl in inducing polymerization of actin, but when such small concentrations as 0.002 M KCl are added to actin in the presence of 0.1 M NaCl, the polymerization is enhanced. These relations correspond to the relation of Na and K in Ringer's solution. This reaction shows that polymerization and the development of the forces surrounding the actin particle are not merely questions of charge or mutual replacement, but that other, subtler properties are involved.

*If Mg is added now in excess, actin readily polymerizes, showing that Calgon, as such, has no inhibitory action.

†Observations made at 0°. At room temperature, results are less reproducible. The Ca inhibition, observed in earlier experiments, must have been such an inhibition of the action of Na or K, because Ca, in itself, does not inhibit but is active.

The polymerization of actin is autocatalytic and its curve has the typical S-shape; it shows a more or less long latent period, then starts up suddenly, and proceeds at a very high rate to completion. The length of the latent period depends on the quantity of polymerized actin present at the beginning. The latent period is needed for the accumulation of the first effective quantity of F-actin. If the actin is stored for twenty-four hours in the ice-box before the experiment and time is given for the polymerized actin present to disaggregate, the incubation period becomes rather long and constant, but once the reaction starts it proceeds at the same rate as in fresh preparations. Ca, in inhibiting the polymerization in the presence of Na, acts by lengthening the latent period, and once polymerization starts, it proceeds at the usual high rate in spite of the presence of Ca. These details may have physiological importance and suggest that ionic balances cannot be looked upon as static environmental factors permanently conditioning certain kinetics. Their slight changes might have trigger actions or might be the safety buttons which block the trigger.

The rate of the G-F transformation increases greatly with the actin concentration and is also greatly catalyzed by myosin. In muscle, where the concentration of actin is high and myosin is present, polymerization may be exceedingly fast. The catalytic action of myosin has some rather puzzling features: 0.1 M KCl promotes the polymerization of actin and 0.6 M KCl does so even more strongly. Myosin, in 0.1 M KCl, strongly catalyzes the reaction but completely inhibits it in 0.6 M KCl, so that G-actin, which readily polymerizes in 0.6 M KCl, is perfectly stable in this solution if myosin is present. In order to make G-actin polymerize under these conditions the G-actomyosin formed has to be brought to dissociation, whereupon the polymerization readily occurs.

F-actin depolymerizes to G-actin if the ions are eliminated by dialysis at slightly alkaline reaction.

KI has a remarkable action: in 0.1 M concentration it induces polymerization, similarly to other alkali-salts, while in 0.6 M concentration it irreversibly depolymerizes F-actin.

III. F-Actomyosin

If a dilute, watery solution of myosin and F-actin is mixed, the sudden rise of viscosity indicates the formation of a new substance. The viscosity is now not only high but also strongly anomalous (Fig. 16) and the solution displays a splendid DRF which is stronger than that of its components. If a more concentrated solution of actin and myosin is mixed, the liquid solidifies to an elastic gel. While myosin has an angle of isocline

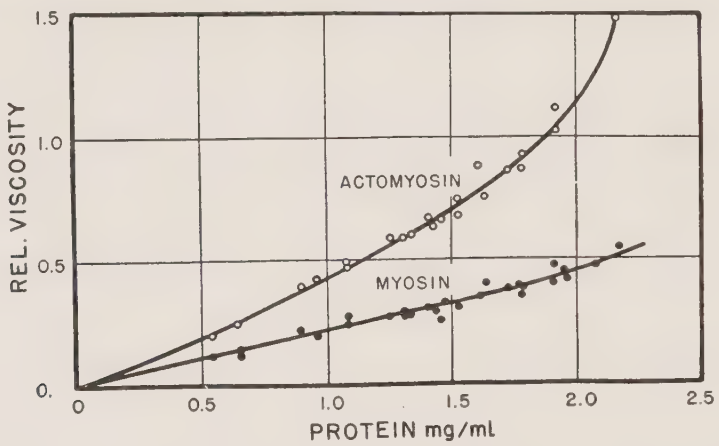

Fig. 16. Viscosity of actomyosin extracted from muscle. Upper curve: actomyosin. Lower curve: the same in presence of ATP. The latter curve is marked "myosin" since it is approximately identical with the curve of pure myosin. The actomyosin, in this case, was prepared from muscle as such and contained 1 part of actin to 5 parts of myosin.

approaching 78°, actomyosin, even at low velocities, readily orientates parallel to the wall (Mommaerts, 1945). All this indicates that out of two substances with relatively short particles a new substance has been formed, a typically *fibrous colloid with very long particles.* That this fibrous character is really due to the high axial asymmetry of the particles and not to some superficial association, is demonstrated by the fact that slight variation of pH or salt concentration does not affect these new properties. The substance, which is formed from actin and myosin, will be called "actomyosin."*

*Ardenne and Weber (1941) observed under the electron microscope very long threads in a "myosin" solution. These might have been actomyosin micels and were perhaps identical with the "heavy myosin" observed by Schramm and Weber (1942) in the ultracentrifuge. Similar threads were since observed by Hall, Jakus, and Schmitt (1946) in muscle extracts.

Actomyosin is not a stoichiometric compound; myosin and actin will unite to form actomyosin in all proportions, but the viscosity as well as other physical properties of these compounds will be different, depending on the relative concentration of the

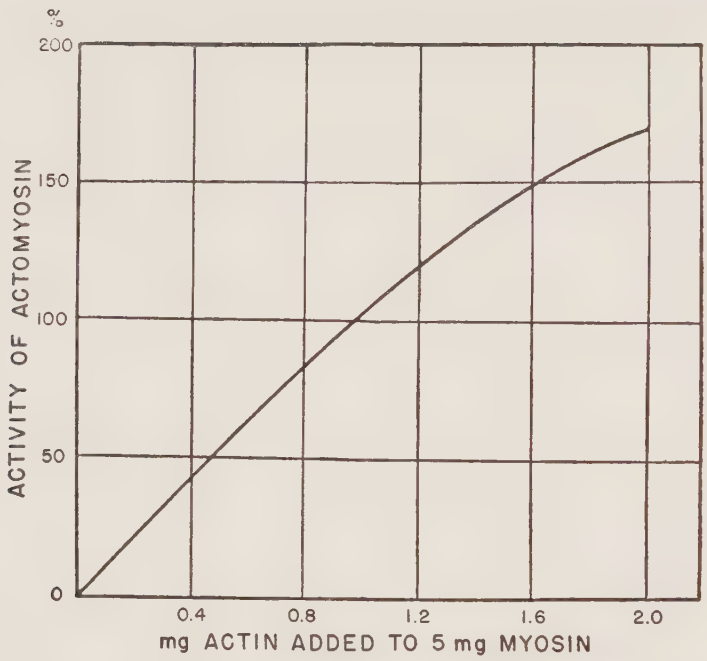

Fig. 17. Activity of F-actomyosin as function of the actin-myosin relation.

two proteins. The rise of viscosity, obtained on mixing actin and myosin, and the corresponding fall of viscosity obtained on dissociation of the compound have been termed "activity" in earlier papers and have been expressed numerically, 100% being

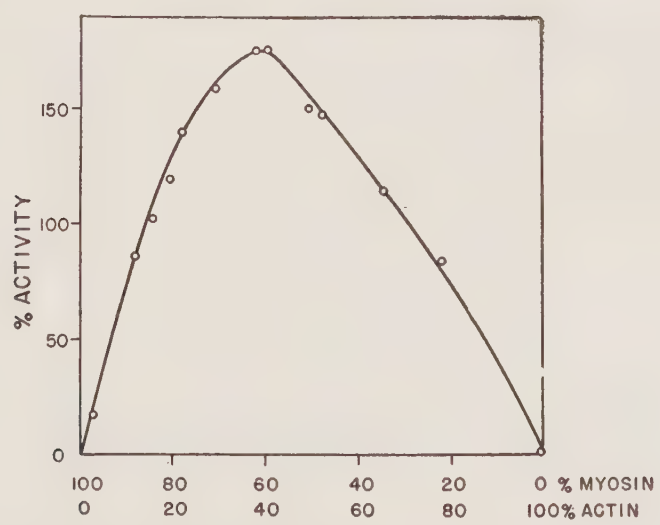

Fig. 18. Activity of F-actomyosin as function of the actin-myosin relation.

the viscosity of a certain standard preparation.* This expression of "activity" has since lost significance but was very useful at the beginning of this research as the first quantitative basis. Now it seems more adequate to express the composition of actomyosin with a ratio, giving the relative proportions of the two proteins.

Figs. 17 and 18, borrowed from Balenović and Straub [17], show the dependence of the "activity" on the actin: myosin ratio. The activity of the compound, at a given myosin concentration, increases at first proportionally to the concentration of actin, reaches a maximum, and then falls off again. The maximum is reached if the relative concentration of actin and myosin is 2:5. According to the measurements of Balenovic and Straub, muscle contains 2:5 or maximally "active" actomyosin. If not expressly stated otherwise, the term "actomyosin" will refer to such 2:5 actomyosin.

The colloidal properties of actomyosin are additive. Actomyosin is slightly thixotropic and exceedingly hydrophilic. Its solution is opalescent; the formation of actomyosin from its elements entails an increase of light diffraction.

Actin unites with myosin to form actomyosin only in the presence of small quantities of Mg (0.0005 M). In the entire absence of Mg, actomyosin is unstable and dissociates, but can be stabilized by the addition of this ion, which indicates that forces instrumental in linking actin to actin resemble those linking actin to myosin.

Actin, in many ways, modifies the enzymatic properties of myosin (see below). Under conditions in which Ca inhibits the polymerization of actin it neutralizes these influences.

Metal Actomyosinates

If neutral actin and myosin solutions are mixed in the presence of KCl and phenolphthalein, a red color appears

*100% was called the activity of "myosin B," the actomyosin obtained by extracting muscle at 0° with Weber's alkaline KCl for 24–48 hours. This actomyosin always had the same specific viscosity and contained one part of actin to five parts of myosin. Five parts of myosin are capable of bringing into solution and protecting from destruction one part of actin. Both 1:5 and the double of this, 2:5, seem to correspond to a special stoichiometric relation of actin to myosin.

showing that the reaction has become alkaline (Banga, oral communication). This alkaline reaction is evidently due to the release of bound K. Forces engaged in the adsorption of K seem to be used now for the adsorption of actin. Accordingly, the metal-binding capacity of actomyosin is somewhat smaller than would correspond to its myosin content. The difference is not considerable and the K-adsorption curve of myosin in free and bound condition shows no major difference. We can expect thus that the colloidal reactions of myosin, due to K fixation,

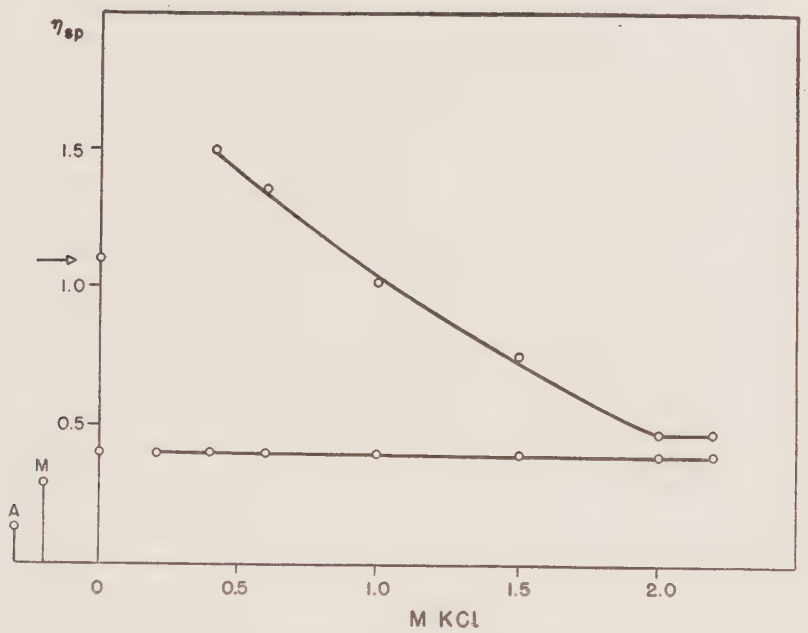

Fig. 19. Specific viscosity of actomyosin at varied KCl concentration in presence (lower curve) and absence of ATP (upper curve). 0.01 M veronal-acetate buffer of pH 7 in all samples, 0.015% ATP. The vertical lines on the left indicate the specific viscosity of the corresponding myosin and actin solutions. The viscosities of salt-free actomyosin with and without ATP (arrow) are marked on the ordinate [Guba, 58].

will be reflected in the reactions of actomyosin with the difference that a somewhat higher K ion concentration will be needed to produce maximal precipitation. This expectation is fully borne out by the experiment. The high hydrophility of metal-free myosin is reflected in the strong hydrophility of metal-free actomyosin, which causes extreme swelling of actomyosin gels in distilled water once their metal is completely washed out. This swelling can be prevented by 0.001 M KCl which also causes turbidity in free myosin. Increasing concentrations of KCl will cause more shrinking and turbidity

of actomyosin gels and increasing precipitation in actomyosin suspensions. Maximum of shrinking or precipitation will be reached (at pH 7) in the presence of 0.5 M KCl, as compared with the corresponding maximum of 0.025 M in free myosin. If the KCl concentration is further increased, shrinking or

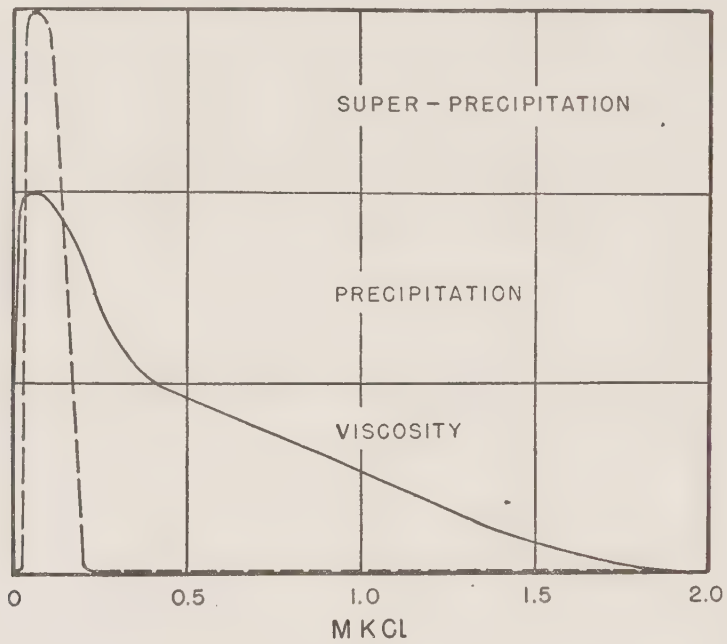

Fig. 20. Semiquantitative curve showing the behavior of actomyosin in presence (broken line) and absence (solid line) of ATP (0.1%) at varied KCl concentration.

precipitation becomes weaker again and 0.4 M KCl no longer precipitates; actomyosin remains dissolved and its viscosity can be measured. As the K ion concentration is further increased, the viscosity of the solution gradually drops, until at 2 M KCl it approaches asymptotically the additive value of actin and myosin, indicating the complete dissociation of the compound into the two free proteins. This is shown in the upper curve of Fig. 19. The solid-line curve, in Fig. 20, shows the whole course of events in a very rough, semi-quantitative way.

As can be expected from the properties of myosin, actomyosin, even at the maximum of its precipitation, is rather hydrophilic. Though it is precipitated completely, the precipitate includes rather great quantities of water which cannot all be located between the flocculi but must be enclosed within the particles. Accordingly, the shrinking of an actomyosin gel, caused by 0.05 M KCl, is but moderate. I have never succeeded

in preparing an actomyosin containing less than 95% water by precipitating the colloid with KCl and separating the precipitate on the centrifuge.

In a discussion about precipitating and redissolving actomyosin, one general remark cannot be suppressed. If actomyosin is precipitated (or its gel is brought to shrinking) cohesive links are soon formed between the particles, which makes redissolving increasingly difficult. The more the actomyosin is dehydrated, the sooner these links are formed and the stronger they are. In the case of maximum dehydration (obtained by ATP, as shown later) these cohesions develop within seconds.

Correlating the colloidal behavior with the curves of K fixation we may say, approximately, that actomyosin, with 3–9 K^+ bound by its myosin, is insoluble, remains in solution with more or less K^+ and dissociates completely with twelve K^+ bound (extrapolated). Dissociation of actomyosin thus depends on the charge of its myosin moiety; beyond a certain critical charge and hydration the myosin does not unite with actin. Actomyosin, on the whole, is more insoluble than free myosin and needs a higher charge to be dissolved. Like myosin, its colloidal behavior and hydrophilic character are governed by its charge.

Actin does not bind K; myosin does. So the K-fixation curve of actomyosin corresponds roughly to its myosin content. This does not hold for the case of Ca and Mg, these metals being bound by actin too. In this case the metal fixation is stronger than corresponds to the myosin content and the break in the curve is flattened out since the breaks of the myosin and actin curves do not overlap.

If actomyosin is dissolved in 0.5 M KCl and the salt is washed out by dialysis, the colloid solidifies to an elastic gel, provided its concentration is high enough (1–2%). This gelatinization of actomyosin solutions has been used by Weber (1933) in preparing his famous threads. He squirted his "myosin" solution in a thin jet into water. Since the salt was quickly washed out by the water, the actomyosin solidified in the form of a thin thread. Such threads have become classical objects of research. It is advisable to use a dilute (0.05 M) KCl

instead of water since, if the salt is washed out too thoroughly, the threads swell up enormously and become useless. The threads in 0.05 M KCl consist of actomyosin, maximally precipitated by KCl. They contain about the same quantity of water (about 98%) as the original solution, which shows their hydrophility. Actin-free myosin does not form such threads, so the "myosin" of Weber must have contained considerable quantities of actin. The threads, if kept for some time, become increasingly insoluble, even in M KCl.

One must be careful to use glass-distilled water, free of heavy metals. The heavy metals, usually present in common distilled water, are strongly bound by the threads which become denatured. Hereby they become elastic. While native actomyosin threads break when stretched by 10–15%, Cu-denatured threads may be stretched 300% which makes them suited for different physical studies but unsuited for the study of the subtler biological phenomena, like contraction.

Actomyosin and ATP

Except for the formation of very long particles, actomyosin up to this point in the discussion has not revealed any reaction that could not have been predicted from the properties of its constituents. A highly specific and unexpected reaction occurs if ATP is added which, in the case of free myosin, had no other action than to increase solubility and hydration.

That ATP has a specific influence on the physical properties of "myosin" was demonstrated first by Engelhardt, Ljubimowa, and Meitina (1941) who showed that the extensibility of "myosin" threads is increased in a specific way by small concentrations of the nucleotide. J. Needham and his collaborators Shih-Chang-Shen, D. M. Needham, and Lawrence (1941) and later Kleinzeller, Miall, Dainty, D. M. Needham, and Lawrence (1941) found that DRF and viscosity of "myosin," dissolved in strong KCl, were decreased by small concentrations of ATP.

The action of ATP on actomyosin depends on the KCl concentration and is demonstrated, rather roughly, by the broken curve in Fig. 20. In this figure the abscissa corresponds to complete dissociation of the actomyosin into its

components. If we compare the two curves of actomyosin, obtained in the absence and the presence of ATP, we may say that ATP makes all reactions rather exaggerated and abrupt. Where KCl, in itself, caused maximal precipitation, ATP made this precipitation very much more intense. Outside this narrow range, ATP causes complete dissociation. What J. Needham and his collaborators have seen is evidently this dissociation.

Making the precipitation much more intense means that while KCl itself caused the actomyosin suspension to precipitate in the form of loose, voluminous flocculi, in the presence of ATP a granular precipitate is obtained which settles quickly to a small volume at the bottom of the test tube. If the ATP is added to the KCl precipitate, the loose flocculi change into a granular precipitate of small volume which indicates the extreme shrinking and dehydration of the particles. This precipitation, obtained in the presence of ATP, will be called "*super-precipitation*" to distinguish it from the simple salt precipitation obtained by KCl alone.

The visible effect of ATP depends on the physical state of the actomyosin. If this is present as an inhomogeneous suspension, super-precipitation will be observed; if it is present in the form of a continuous gel, the same action will express itself in an extreme degree of shrinking and dehydration. This shrinking is especially striking if the gel has the form of a thread (Fig. 21a). Owing to the relatively big surface, the ATP will diffuse into the gel quickly and so the shrinking will be very fast. For a few seconds nothing will happen, when the ATP pervades the gel; suddenly shrinking sets in, and within half a minute or so the thread shrinks to a small dense stick* (Fig. 21b). This shrinking is so fast that it gives the impression of active contraction and henceforth will be called "*contraction*" to distinguish it from the rather slow and moderate shrinking and dehydration caused by KCl alone.

*Sometimes the action is so violent that the outer layers contract before the interior can follow, with the consequence that the former break up, giving the appearance of a "crocodile skin" to our thread. This will more often be the case with actomyosin prepared from pure actin and myosin. The thread in Figure 21 was made from actomyosin, extracted as such from muscle.

Contraction of actomyosin can be observed even in solutions. If ATP is added to a 0.1% "solution" of actomyosin, containing 0.05 M KCl, the fluid becomes turbid and the substance contracts to a small plug (Fig. 22).

Figs. 21a, b

Fig. 22

Fig. 21. a. Actomyosin thread. b. The same contracted. Magn. 1:30.
Fig. 22. Contraction of an actomyosin solution.

All these reactions are perfectly reversible, as long as secondary cohesions do not disturb the picture. The contracted thread can be brought to relaxation by raising the KCl concentration to 0.25 M, especially in the presence of 0.001 $MgCl_2$ which makes both contraction and relaxation more intense and makes myosin less soluble.

Threads usually contain 98% water and 2% protein and consist, if suspended in 0.05 M KCl, of actomyosin, maximally precipitated by the salt present. On the addition of ATP they usually contract to about 66% of their former length. The thread now consists of 50% protein and 50% water. This is exceedingly close packing; closer packing can hardly be achieved. We may conclude herefrom that the thread consists now of anhydrous material with some entrapped water and that it contracted as far as was structurally possible.

As shown by Fig. 20, the transition from contraction to

dissociation, in the presence of ATP, is very abrupt. In the one tube the actomyosin may be found maximally super-precipitated, in the next completely dissolved and dissociated even if the KCl concentration differs in the two tubes by no more than 15%. The actomyosin is either contracted (super-precipitated) or dissociated, depending on the KCl concentration, with only a very narrow transition zone. Since dissociated actomyosin is not actomyosin at all, we may just as well say that actomyosin in the presence of ATP is stable only in a very narrow range of KCl concentrations and can exist here in the contracted state only. ATP has thus two effects: (1) in a wide range of KCl concentration it prevents the association of actin with myosin or makes actomyosin dissociate; (2) in the narrow range of KCl concentration, where actomyosin is most discharged and is stable, it causes the system to contract.*

The dissociating effect can easily be accounted for. As shown before, ATP has only a charging, hydrating effect on myosin. This charge adds on to the charge already present, whether the latter is due to the incomplete neutralization of the original negative charge of the protein by K^+, or whether it is due to the positive charge acquired by the adsorption of excess K^+. At a certain charge, the system will dissociate on addition of ATP when its hydration reaches the critical value. This happens, in the presence of 0.1% ATP, in KCl, more dilute than 0.02 M or stronger than 0.16 M, with less than 4 or more than 8 K^+ adsorbed. Between 0.02–0.16 M KCl the actomyosin is stable and contracted. It is easy to understand why increase of ATP concentration narrows down further this zone of stability, and why a decrease extends it.

In the experiment reproduced in Fig. 20, the zone of precipitation extended from 0.02 to 0.16 M KCl in the presence of 0.1% ATP. In the presence of 0.01% ATP the zone extended from 0.003–0.3 M KCl, while in the presence of 0.4% ATP it narrowed down to 0.03–0.1 M KCl. The same point is brought out nicely by the experiment reproduced in Table III. One can see the zone of precipitation widening as the ATP is gradually decom-

*Dissociation depends also on the relative concentration of actin and myosin. The more we deviate from the natural 1:2,5 relation, the more easily the complex dissociates.

posed. The border between contraction and dissociation is sharp and one K adsorbed or released brings the dissociated system to contraction and *vice versa.*

TABLE III. *Superprecipitation of Actomyosin at Varied KCl Concentrations*

	M KCl								
	0.100	0.106	0.112	0.118	0.124	0.130	0.136	0.142	0.148
1 Min.	X	–	–	–	–	–	–	–	–
2 Min.	XXX	X	–	–	–	–	–	–	–
3 Min.	XXX	XXX	XX	X	–	–	–	–	–
5 Min.	XXX	XXX	XXX	XXX	X	–	–	–	–
15 Min.	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	X

Parallel to the gradual decomposition of the ATP, the zone of superprecipitation widens. X = superprecipitation.

We thus have no difficulty in explaining the dissociating effect of ATP. The difficulty is with the contraction. Why should ATP which in the case of free myosin caused increase of hydration only, have the opposite effect and cause complete dehydration in the presence of actin? If hydration is a question of charge we must conclude that actomyosin, in this condition, is less charged. The experiments of Banga and Hermann have shown that the affinity of actomyosin for metals and ATP is not materially altered by contraction. Actomyosin while contracting does not thus release any of these substances, and the actual composition of the particles does not change during this process. If there is a decrease in charge, it can result only when the charges of the system balance each other. Contraction, in its essence, must be some sort of an intramolecular or intramicellar rearrangement within the system composed of myosin, actin, ATP, and adsorbed ions in which the charges become better balanced, losing their outward action. Such a rearrangement does not take place in the absence of actin or ATP. Above a certain charge and hydration, actomyosin dissociates, but if this critical charge is not reached and there is no dissociation, the spontaneous rearrangement into this better balanced and more stable structure occurs with the consequent agglutination of particles and dehydration. The observations indicate that the contracted-dehydrated and the dissociated-

hydrated condition are two distinct states, that the particles are either in the one or in the other, and there is no modified state between the two, a conclusion fully borne out by experiments to be discussed later.*

It follows from the reversible nature of the reactions concerned that it does not matter in which order we put the single constituents together. If we mix 0.05 M KCl, ATP, actin, and myosin, the result will be contracted actomyosin, regardless of the order in which we mixed them. This will be the case, at least, if the mixing is done at room temperature or above. If, however, we put these substances together at 0°, there will be no contraction. The high viscosity indicates that actin and myosin have united to form actomyosin, but in spite of the presence of ATP there is no contraction. As we allow the mixture to warm up, it contracts. At room temperature, contraction is complete. This shows that the formation of contracted actomyosin takes place in two steps: (1) the formation of uncontracted-hydrated actomyosin from its elements; and (2) the rearrangement of this system into the dehydrated-contracted structure. This latter is an equilibrium reaction depending on temperature.

Though unable to understand the nature and mechanism of this second reaction, we may study its relations: in the first place, its dependence on concentrations.

At the optimal 0.05 M KCl concentration the rate and intensity of contraction will depend on the ATP concentration. Contraction demands a fairly high ATP concentration. Below 0.01% ATP there is no contraction at all. Comparing this result with the ATP adsorption curves of Banga and Hermann the result suggests that there is no contraction if less ATP is adsorbed than one molecule per unit of 70,000–140,000 g. of myosin. Maximal effect is reached with 0.1% ATP which corresponds to one ATP adsorbed per UW. This is the quantity of ATP adsorbed to myosin *in vivo*. In 0.6 M KCl actomyo-

*The change cannot be explained either by a mutual discharge of actin and the myosin, for there is no stoichiometric relation between actin and myosin. It has been shown by Erdôs [33] that actomyosin threads may maximally contract with 7 or 30% actin. Moreover it is probable that actin, in muscle, is isoelectric.

sin is, in the absence of ATP, at the verge of dissociation. Mommaerts [11] has measured the quantity of ATP needed to produce complete dissociation of actomyosin at this KCl concentration and has found that dissociation is complete if there is one molecule of ATP for every 100,000 g. of actomyosin, which suggests that units of MW of 70,000 or 140,000 actually exist in the myosin micel.

Mg added in small concentration (0.001–0.0005 M) considerably narrows down the KCl zone of contraction, promoting dissociation. So, for instance, in the presence of 0.1% ATP, where the zone of contraction extends up to 0.16 M KCl, 0.001 M $MgCl_2$ will bring down the border of contraction and dissociation to 0.1 M KCl. $MgCl_2$ has the same action as increased ATP concentration. As will be remembered, Mg strongly enhances ATP adsorption, thus having the same effect as an increase of the ATP concentration. Mg also makes contraction much more intense and sensitizes actomyosin to the action of KCl. Contraction in 0.01 KCl is very weak and sluggish but if $MgCl_2$ is present in 0.0005–0.001 M concentration (which in itself does not induce contraction) contraction will be most intense. Possibly the increased ATP adsorption is involved here too, but it will be remembered that Mg is also instrumental in linking actin to actin and actin to myosin.

As found by Gerendás [13] Mn and Co have an action similar to that of Mg. Ca, on the other hand, has an opposite effect, raising somewhat the KCl limit of dissociation* [Erdös, 56]. If Mg and Ca are added simultaneously in equal concentration (0.001 M) Mg acts as if no Ca were present, lowering the limit of dissociation.

Ca, in higher concentration (0.01 M), reversibly inhibits the contraction in KCl. Mg may bring the thread into contraction in spite of the presence of the Ca.

Naturally, all these phenomena depend greatly on the freshness of the myosin preparation. Even a few hours' storage at 0° may make an appreciable difference. The reactions of older preparations are weak and sluggish, and maxima are hifted towards higher ionic concentrations.

*The antagonistic effect of Ca and Mg may be involved in Meltzer's narcosis.

IV. G-Actomyosin

Like F-actin, G-actin also unites with myosin to form actomyosin, but the union of the two substances is not accompanied by a rise of viscosity, the viscosity of G-actomyosin being equal to that of myosin. That a union has taken place at all is revealed by the fact that the subsequent addition of F-actin does not cause a rise of viscosity, the combining capacity of myosin being satisfied by the G-actin. That G-actin is formed is shown also by precipitating the protein with KCl: the precipitate is more opaque and granular than the precipitate of free myosin.

There is an important difference in the reaction of G- and F-actomyosin with ATP: while F-actomyosin is dissociated by ATP at only high or low salt concentration, G-actomyosin when ATP is added dissociates at any salt concentration. G-actin thus forms no compound with myosin-ATP, regardless of the K saturation. Consequently in the presence of ATP, myosin does not catalyze the G-F transformation of actin.

G-actomyosin forms no elastic gel and is not contractile. If F-actomyosin is brought to contraction by ATP and the contracted actomyosin is rapidly dissolved by adding salt in high concentration (0.6 M) the actin will be found to be present in the globular form, as indicated by the lack of DRF which appears only after time is allowed for the actin to polymerize again. F-actin is thus depolymerized during contraction of actomyosin.

Though G-actomyosin is not contractile, we may prepare contracted G-actomyosin by making F-actomyosin contract. This contracted G-actomyosin is dissociated by ATP, like F-actomyosin, only at high or low KCl concentrations.

V. A Theory of Contraction

"Super-precipitation" is but an extreme degree of precipitation, "contraction" an extreme degree of shrinking. Both phenomena are merely different expressions for one and the same reaction. Precipitation and shrinking are common occurrences in colloidal chemistry, and are usually due to loss of charge with the consequent agglutination of particles. The immediate cause of decrease in volume is due to the release and expulsion of water. As W. T. Astbury recently pointed out in his Croonian Lecture, the phenomena observed in actomyosin can be classed with colloidal synaeresis. But the rate and extent of these reactions as well as the colloidal and chemical inactivity of ATP are somewhat out of line. The fact that neither actin nor myosin shows similar reactions; that contraction and super-precipitation are specific reactions of the complex of the two materials, a complex built of two widely different and rather peculiar proteins, and representing a rather specific structure; these phenomena suggest that a special mechanism is involved, even though the forces at play may be identical with those generally responsible for analogous phenomena.

In an actomyosin thread the micels have a nearly random orientation and the contraction of the thread is almost isodiametric. The very slight DR indicates a very slight orientation parallel to the axis, and measurement shows that contraction is not perfectly isodiametric.

To quote a measurement of M. Gerendás, a thread which shortened by 62% became thinner by 58%. Gerendás tried to increase the orientation of micels within the thread by stretching. Stretching the thread by 10–15% increased the ratio to 62%:53%. Unfortunately, native actomyosin threads cannot be stretched any more because they break. Gerendás found that the threads can be stretched in glycerol as much as 200%. Threads hereby become doubly refracting and still show some contractility which is distinctly anisodiametric. To quote an example: stretching in 25% glycerol by 200% gave the thread a DR of $15{\cdot}6.10^{-4}$. Contraction became distinctly anisodiametric,

the thread shortening by 16% and becoming thicker by 20%.

Actomyosin threads, denatured by heavy metals, like Cu, are very elastic and can be stretched as much as 300%, but they do not contract any more under the influence of ATP. Gerendás found that lighter metals, like Zn, if applied in high dilution and for short periods, make threads stretchable without completely abolishing their contractility. For instance, a thread, treated with 0.001 M ZnSO4, could be extended by 200%. Its DR, after extension, was $11{\cdot}6.10^{-4}$. On extension the micels became thus oriented parallel to the axis; at the same time a considerable quantity of the intermicellar water was pressed out and appeared in the form of small droplets on the surface. The stretching is elastic but if the thread is kept in a stretched condition for some time it "sets" and does not contract any more if released. Suspended in 0.1 M KCl, the thread contracted on addition of ATP, becoming shorter by 30% and wider by 55%. We thus have a further insight into the mechanism of contraction. If, in the stretched thread, the actomyosin micels run parallel to the axis of the thread, then, evidently, the intermicellar splits run parallel too. But if the intermicellar splits, containing the intermicellar water, run parallel, then the loss of this water could not make the thread shorter, only thinner. Thus the shortening of the thread could not be due to the loss of water but must be due to the shortening of the particles themselves, and contraction of actomyosin cannot be dismissed as a simple case of shrinking due to loss of water. There must be a specific mechanism responsible for the shortening of the micels, a mechanism which explains why the actomyosin particle is built of two specific colloids, why contractility is a specific property of their complex and why these two colloids give off hydrate-water with different degrees of ease. The mechanism would also explain why one of the two colloids is capable of breaking up into globules and does so in every contraction, though a contractile complex is formed by its fibrous form only.

When trying to find a possible answer to these questions we may start from the most striking specific property of actomyosin: the strongly elongated shape of their particles. If we

picture the particle as a double rod, one half of which (actin) is composed of globules, we shall have something like the rod in Fig. 23. If in this system myosin (black) shrinks, or shrinks

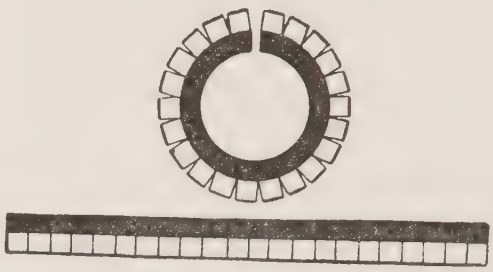

Fig. 23

Fig. 23. Model of relaxed and contracted actomyosin particle.

Fig. 24a

Fig. 24b

Fig. 24. Model of actomyosin micel. The myosin is represented by a rubber tubing which was gently stretched before its ends were fixed to the end of the wood. On release the system curls up. The model was kindly prepared in Prof. Kapitza's laboratory at Moscow.

more than its partner, the system must bend. Geometry demands that if such a system is, for instance, 100 units long and 1 wide, and if one of the partners shrinks by 3 units, then the system should curl up to a complete circle. Such a change would bring

the shrinking partner on the smaller inner circumference. But as the shrinking colloid becomes satisfied by this arrangement, then the other partner must tend to overcome being stretched by being distended on the longer, outer circumference. There will be no trouble, however, if this latter is capable of breaking up into globules, as shown in Fig. 23. The same is shown in Fig. 24. In this model the dehydrating myosin is represented by a rubber tubing which has been stretched gently before attaching it to the ends of the actin-rod. On release of the system, the rubber contracts, imitating the shrinking of myosin. By curling up, the system shortens its actual length by $\frac{2}{3}$. The mechanism is an amplifier in which slight changes of volume of two different substances are amplified, thus producing the very extensive motion of shortening.

If the atomic groups linking the actin globules are located on opposite poles of the particle, then bending of the system must disrupt these links even if there is no distention. This is schematically shown in Fig. 25 where the links are symbolized by strips.

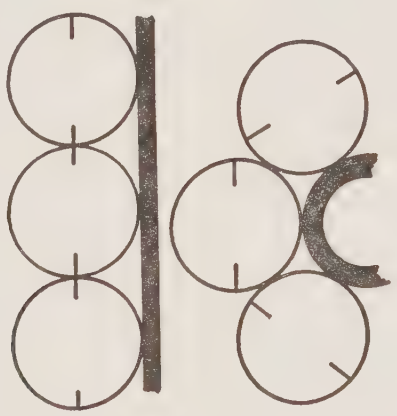

Fig. 25

These models or theories are to be taken with a grain of salt. They are designed to bring out the fact that an elongated system is prone to bend if composed of two parts which change their volume differently. This is nothing new. This simple principle is responsible for the bending of wooden boards or bending of "bimetals." Such a bending, or angular motion, would nicely explain the properties of the elongated actomyosin particle composed of two different colloids.

In Figs. 23 and 24, the actomyosin particle was pictured as a system composed of two partners, only one of which shrinks. The anhydrous nature of contracted actomyosin indicates that both proteins lose their hydrophility in the process of contraction. If the change in both is not equal, then, in addition to the general synaeresis, curving, or angular motion also occurs. How far angular motion and how far general synaeresis are responsible for contraction, remains to be shown. Angular motion may clarify considerably the dynamics of the system and explain the specific features of reactions and structure of actomyosin without introducing any new concepts, unknown in colloid chemistry or in everyday life. It also explains why our oriented thread becomes shorter and thicker on contraction and why it loses its DR, as has actually been found by experiment Gerendás 13].

The curling of unequally shrinking double rods can be nicely demonstrated in actomyosin threads, suspended in 0.1 M KCl. If ATP is added to the solution and the fluid is mixed, the ATP reaches the thread from all sides and the thread contracts. If, however, the ATP is allowed to reach the thread from only one side, then this side will contract first and the thread curls up. Ciliary motion possibly has such a mechanism, cilia being built of contractile matter in which waves of contraction pass along on one side, or alternately on the two opposite sides.

On the assumption that the actomyosin forms double rods the bending of which is involved in contraction, the question arises how these actin and myosin rods or threads are actually put together. One can try to approach this problem by constructing models. One will be led to the conclusion that there is only one arrangement which meets all demands: putting the two together in a spiral. Such a spiral may be obtained from the rod in Figs. 23 and 24 by twisting it. On bending, these twisted rods would no longer curl up into a circle but would form something similar to a corkscrew or spiral spring. Such a spiral is symbolized by the model in Fig. 26, in which a flat rod (supposed to consist of a flat actin and myosin half) is twisted. Figs. 27, 28, 29 show the same rod in increasingly bent condition. Fig. 30 shows the effect of different degrees of twist: a′, b′, c′ are the bent structures formed from a, b,

and c. It is evident that *c* will exert the greatest force on contraction, its angular motion being the greatest.

In an earlier paper [70] I tried to explain cross-striation in

Figs. 26-29

Fig. 30

Figs. 26–29. Model of an actin and myosin rod. Fig. 26 shows the twisted rod; Figs. 27–29 show the same rod in increasingly bent condition.

Fig. 30. Model of an actin and myosin rod, showing the effect of different degrees of twist. a′, b′, and c′ are the bent structures formed from a, b, and c.

muscle by this spiral twist, supposing that one turn corresponded to one period of cross-striation. Evidence obtained since that time on the electron microscope by Hall, Jakus, and Schmitt does not support this contention, and if the theory of the spiral and the angular motion is correct, the twist must be in molecular dimension so that the bent and shortened system, as shown in Fig. 30*c*, may still appear as a straight rod at the magnifications obtainable at present.

However nicely they explain many properties of muscle, spiral structure and angular motion are but theories and there are observations to suggest that the role of actin is not a geometrical one. MW measurements suggest that in muscle one actin particle unites with each sub-unit of the myosin micel. If this is the case in muscle then in "B-myosin" there is one actin particle for every two myosin sub-units. Erdös found that actomyosin shows maximum contractility only when its actin content is at least 8%. At this limit there would be one actin particle for four myosin sub-units (four being the greatest number of cubic particles which can be simultaneously in touch with one globular particle on the surface of their system). These relations are difficult to reconcile with a geometrical function of actin and rather suggest a direct chemical influence of actin on myosin.

VI. Energy Changes in Contraction

The observations discussed in the previous chapters suggested that the contracted and uncontracted conditions of actomyosin represent two distinct states of this matter, and that the transition from one to the other is an equilibrium reaction, dependent on temperature. The free energy change of an equilibrium reaction can be calculated from its equilibrium constant ($\Delta F = -RT \ln K$) while the heat of the reaction can be calculated from the dependence of the equilibrium constant on temperature by means of van't Hoff's equation:

$$\frac{dlnK}{dT} = \frac{W}{RT^2}$$

which, if integrated between the temperatures T_1 and T_2, gives

$$2.303(\log K_2 - \log K_1) = -\frac{W(T_2 - T_1)}{R\ T_2 T_1}$$

where W = heat of reaction, R = gas constant 1,986 cal., T = abs. temp. The problem is to find the equilibrium constant of the reaction (contraction) at different temperatures. If contraction and relaxation are two distinct states and the single micels are either fully relaxed or maximally contracted, we can draw conclusions about the equilibrium from the length of the thread. Maximal contraction in this case means that 100% of the micels are contracted, whereas no contraction means 0% of the micels are contracted. If the thread shortens maximally from 100 to 30, then this 70% contraction actually means that 100% of the micels contracted. Contraction half-way from 100 to 65 would mean that 50% of the micels contracted producing the equilibrium 1/1. Thus the length of the thread could give direct information about the equilibrium constant at any temperature; and in order to find this constant the length of the thread would simply have to be measured after the thread reached its equilibrium length, provided one had ideal threads of constant composition and orientation.

L. Varga considered the muscle fibre to be a similar perfect actomyosin thread and used as experimental material the psoas

of the rabbit, which is built of parallel-running fibres. He washed out the muscle *in situ* with ice water and cut it into slices on the freezing-microtome in such a way that each slice contained one sheet of parallel-running fibres. After the length was

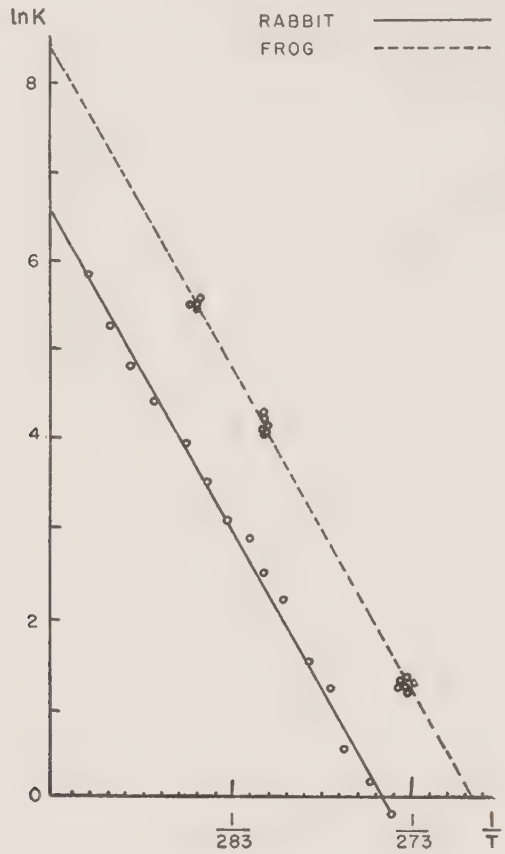

Fig. 31

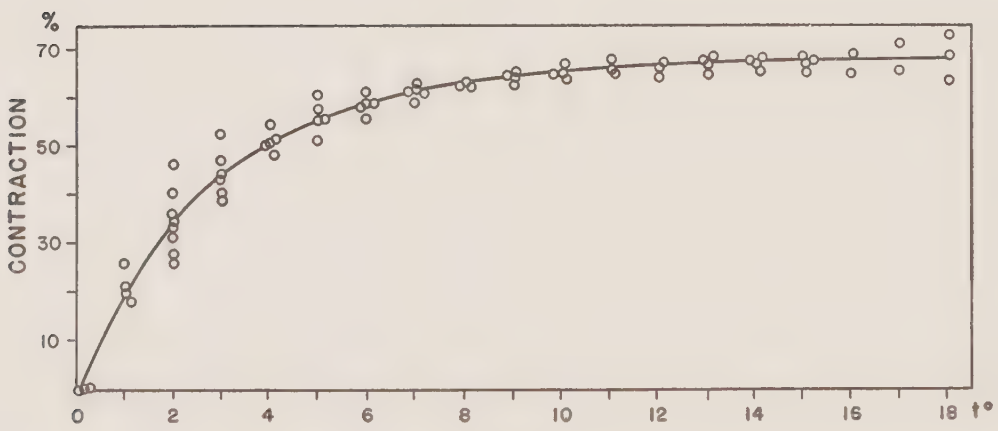

Fig. 32. Contraction of muscle slices at varied temperature in presence of KCl and ATP.

measured, the slice was transferred into a KCl-ATP solution of known temperature and its length measured again after maximal contraction was reached. The natural logarithm of

the equilibrium constants plotted against 1/T gave a straight line (Fig. 31) which proved both of Varga's suppositions to be correct: that muscle actually behaves as an ideal thread, that contraction and relaxation are distinct states; and that an actomyosin micel is either fully relaxed or fully contracted, there being no transitional states. If the muscle contracts only half-way it is not because the micels are half-contracted, but because only half of the micels are in the contracted state.

Figure 32 shows the result of the actual measurements. At 0° there is no contraction. With rising temperature the contraction becomes rapidly stronger to reach a maximum of

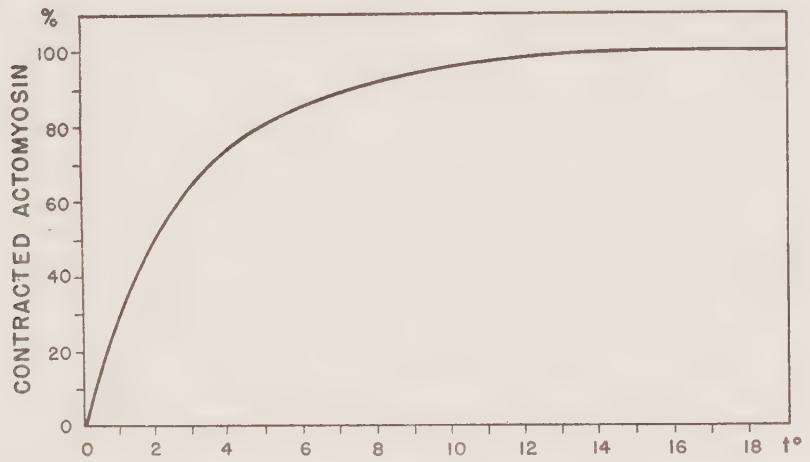

Fig. 33. Same curve as in Fig. 32, maximal (70%) contraction being marked as 100%.

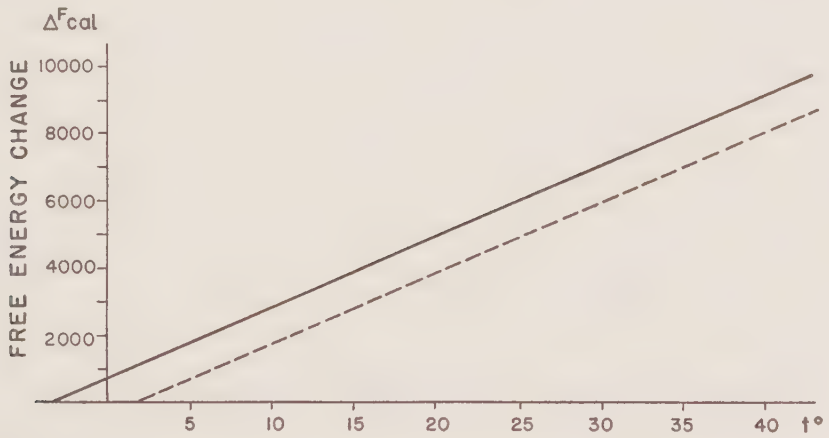

Fig. 34. Free energy change in muscle slices on contraction at varied temperatures. Upper curve: frog; lower (broken) curve: rabbit.

68–70% asymptotically at about 16°. Figure 33 is the same curve, the 70% maximal contraction being marked as 100% contraction. This 100% means that 100% of the micels are in

the contracted state: 50 on the ordinate means that the muscle is half-contracted and that 50% of the micels are in the contracted state. From this curve the equilibrium constants for different temperatures can be read. The equilibrium constants $K = \frac{\text{contracted actomyosin}}{\text{relaxed actomyosin}}$ are given in Table IV. The $\triangle F$ for different temperatures is given in the lower (broken) curve of Fig. 34. As can be seen, the $\triangle F$ decreases with decreasing temperature to cut the abscissa about 2°, which explains fully why muscle does not contract at 0°. The heat of the reaction,

TABLE IV

t°	T°	Contract. in %, max (68) = 100	K.	Log. K.	Ln. K.
1	274	30.66	0.43	0.634—1	—
2	275	51.00	1.04	0.017	0.039
3	276	64.00	1.69	0.230	0.53
4	277	75.00	3.00	0.477	1.09
5	278	82.00	4.55	0.658	1.52
6	279	86.10	6.19	0.792	1.82
7	280	88.99	8.07	0.907	2.09
8	281	92.00	11.05	1.060	2.45
9	282	93.72	14.79	1.170	2.69
10	283	95.73	22.40	1.351	3.12
11	284	96.50	30.55	1.485	3.42
12	285	97.44	38.11	1.581	3.65
13	286	98.19	54.33	1.735	4.00
14	287	98.63	72.11	1.858	4.28
15	288	98.99	98.86	1.995	4.60
16	289	99.40	165.00	2.218	5.05

calculated from these data, is −56,000 cal. Contraction is thus an endothermic process. At the same time the free energy drops at 37° by 7,000 cal. At 0° there is no drop of free energy and for this reason rabbit muscle does not contract at 0°.

It was rather shocking to find a frog swimming about in ice water after the conclusion of these experiments, and we had to decide whether the frog or Mr. Varga was wrong. The experiment was therefore repeated using a slightly modified technique with frog muscle. The heat of the reaction was found to be the same as in the rabbit experiment (53,500 cal.) but

the free-energy change was greater, 8,500 cal. as compared to the 7,000 cal. in the rabbit experiment (37°). In Fig. 34 the $\triangle F$ of the rabbit and frog are compared: the lines are parallel but in the frog there is still a decrease of the F at 0° which enables him to swim in ice water as he sometimes must do. Thus $-3°$, where his $\triangle F$ is 0, no longer interests him because at this temperature he is frozen anyway.

Varga's experiments allow different conclusions. Since the free energy decreases in contraction, we can postulate that this process, being a transition from an energy-rich relaxed state to a stabler state, poorer in free energy, occurs spontaneously. Relaxation however can occur only if the free energy of the system is increased by 7,000 cal. (if the process is carried out isothermally, as is true in the case of the rabbit). The splitting of ATP which, according to our present knowledge, is the source of muscular energy, is thus needed for relaxation. The free energy of ATP is unknown; the breaking of one phosphate link liberates 11,000 cal. Provided that this energy change is not very different from the $\triangle F$, then the breaking of one such link is enough to relax one actomyosin particle.

ATP thus has two functions. One is a static function, independent of splitting. ATP acts as a building stone of the contractile system. Without ATP, the actomyosin does not contract or relax: it is not reactive at all. The other function of ATP is to provide energy for relaxation.

VII. Enzymatic Functions of Myosin and Actomyosin

ATP-ase and ADP-ase Activity. Dephosphorylation and Deamination. Protein II.

The most important discovery in this field is that of Engelhardt and Ljubimowa who showed that it is myosin itself which splits off the first phosphate of ATP. This discovery was corroborated in many quarters (D. M. Needham, K. Bailey 1942) and was borne out by the very wide experience of my own laboratory with crystalline myosin. The enzymatic activity of myosin is not altered by repeated recrystallization.

According to Engelhardt and Ljubimova, only the first phosphate is attacked by myosin while the second phosphate is split off by a different, water-soluble enzyme. This latter view is undoubtedly erroneous and there can be little doubt that the second phosphate too is split off by the myosin itself, but this reaction is effected only in the presence of a water-soluble and acid-stable protein.

Kalckar was the first to describe an acid-stable protein involved in the splitting of the second phosphate. He called it "myokinase" and attributed to it the function of a dismutase which dismutates two ADP molecules into one molecule of AMP and one molecule of ATP. The ATP is dephosphorylated again to ADP and so the reaction goes on until all the ATP is dephosphorylated to AMP.

Independently of Kalckar's observation, Laki [26] also showed that an acid-stable, water-soluble protein is involved in the splitting of the second phosphate of ATP by the insoluble muscle-residue. The importance of this reaction was strongly enhanced by Laki's experiments which showed that the splitting of the first and the second phosphate had different pH optima, the former being at pH 7.4, the second at 8.6. This suggested that the splitting of the first and second phosphate were independent enzymatic functions of myosin and that there is no dismutation. If we assume dismutation occurs, then it is only ATP that is split.

Banga, taking up the work with crystalline myosin, found that this substance rapidly splits off one P from ATP but is capable of dephosphorylating the ADP formed only if acted on first by a soluble protein to which she attributed the function of an isomerase. Since no ATP or AMP was found, there could have been no dismutation.

In her later, extensive studies, pursued partly in collaboration with Josepovits, Banga was led to a different conception. She found that the water-soluble and acid-stable protein which she called "Protein II" did not act on ADP directly at all; it acted only in conjunction with myosin, completing and modifying the activity of this substance.

Whether Banga's protein II and Kalckar's dismutase were identical is difficult to say. It is possible that there is a whole group of water-soluble and acid-stable substances in muscle, acting with myosin.

These experiments of Banga furnished no conclusion about the chemical mechanism of the dephosphorylation of ADP. They simply showed that the second phosphate is split off only in the presence of myosin *and* protein II. At the same time they also showed that simultaneously with the second P, N is also detached and the ADP is deaminated. This reaction too takes place only in the presence of protein II *and* myosin. Efforts to fractionate protein II into two substances, one acting on dephosphorylation, the other on deamination, failed. The experiments thus indicate that ADP is deaminated under the combined action of myosin and protein II. Deamination occurs only after the first P is split off. Deamination and the splitting of the second P run so closely parallel that it is highly probable they do not represent different enzymatic functions, but are the result of one reaction. In this reaction, one of the phosphate groups in ADP is attached to the NH_2 group and is detached together with this NH_2 group. This ADP, with one phosphate attached to the NH_2 group, was called ADP_2. Its existence was supported by Banga's isolation of an ADP with its NH_2 group masked.

According to Banga, the ATP prepared from muscle and used in our experiments has the Lohmann formula, i.e., none of

its phosphates is attached to the amino group, which is free. The three phosphates are attached to the carbohydrate end of the molecule. If one phosphate is split off, an ADP results which has its two P's in its original position. This ADP is called by Banga "ADP_1." According to the observations of Banga, it is most probable that by the system myosin + protein II, one of the phosphates of ADP_1 is transferred to the amino group, whereby ADP_2 is formed. It is then this phosphorylated amino group which is split off by myosin + protein II. At the moment we must stress observations rather than comments. The observations could be equally well explained by supposing that protein II is an enzyme which transfers the phosphate in ADP to the amino group. We should have to suppose only that the equilibrium point of this reaction is rather on the side of ADP_1 and the concentration of ADP_2 is too low to be detected at present by direct chemical methods, but not too low to allow a rapid deamination of the phosphorylated amino group.

These results of Banga make it highly probable that deamination of ADP is a phospholytic reaction, that the NH_2 group is phosphorylated prior to its detachment. Such a transfer of phosphate from the carbohydrate to the NH_2 group seems to occur in ADP only, after the splitting off of the first P of ATP. These results settle the old problem of whether ADP is deaminated or dephosphorylated first: P and N go off together. Both the first and second phosphate of ATP are split off by myosin, but the second is attacked only after there has been a P transfer and the amino group has been phosphorylated. Whether the P transfer is an independent function of protein II and the P splitting an independent function of myosin, or whether myosin and protein II perform both functions together is not known.

If crystalline myosin acts on ATP in the absence of protein II the reaction stops after the first P has been split off. The products of this reaction have been analyzed by Banga and Josepovits [55]. Along with ADP_1 a dinucleotide was found, composed of one molecule of ATP and one molecule of ADP. It contained five P but had only one free NH_2 group. The simplest explanation is that there was some ADP_2 formed during the dephosphorylation which was dimerized with ATP,

or else that ADP_1 was formed and dimerized with ATP in such a way that the phosphate of the one molecule was linked to the NH_2 of the other. As shown before, if protein II is added to this mixture of ADP_1, dinucleotide, and myosin, the reaction proceeds and the second P is split off together with the N. One would be inclined thus to suppose that these substances, ADP_1 and dinucleotide, represent steps in the normal splitting of ATP. This, however, is definitely not the case, because the splitting of ADP_1 and dinucleotide is rather slow, and the splitting of dinucleotide even has a considerable latent period. But the action of crystalline myosin on ATP in the presence of protein II is rapid, and the phospholytic deamination follows the first dephosphorylation at a high speed without a latent period, ATP being split into inosinic acid, phosphate, and NH_4OH. Thus ADP_1 and dinucleotide are stabilization products and not normal intermediates. The normal primary product of the first dephosphorylation of ATP (which is formed if this dephosphorylation takes place in the presence of protein II) is a different substance which is attacked at once.

The presence of actin makes no difference if myosin and protein II are made to act on ATP. If, however, myosin and protein II are made to act on ADP_1 or dinucleotide, actin greatly speeds up the reaction and there is no latent period. In this case the system is thus fully active only if three proteins work together: myosin, protein (hence the name "protein II"), and actin. While myosin splits ADP_1 and dinucleotide slowly — the latter with a latent period — actomyosin, in the presence of protein II, attacks these substances readily.

Metals: Physical State and Enzymatic Activity

As Fig. 35 taken from the early work of Banga [10] shows, metals have a decisive influence on the enzymatic activity of actomyosin. All metals that do not denature protein have an activating effect. There is a distinct maximum of activity, both metal-free myosin and myosin with too much metal being inactive. The activation is of the same order with most metals, only Ca and Mg having a specially strong action.

Figure 36 shows the effect of varied KCl concentration on

the phosphatase activity of pure myosin and actomyosin. The very small enzymatic activity at the lowest salt concentration

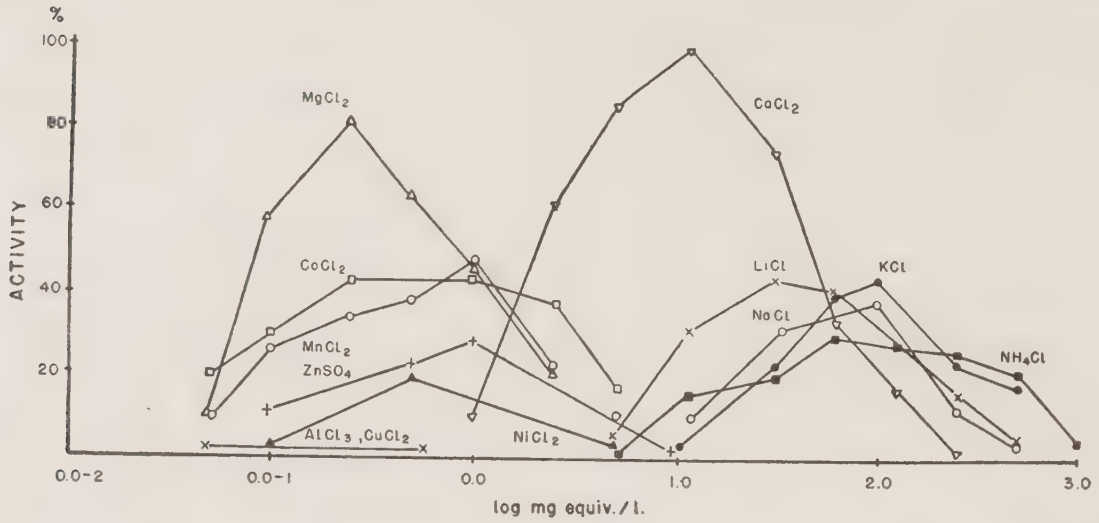

Fig. 35. Effect of various salts on the phosphatase activity of natural, impure actomyosin. 100% activity: maximal effect observed.

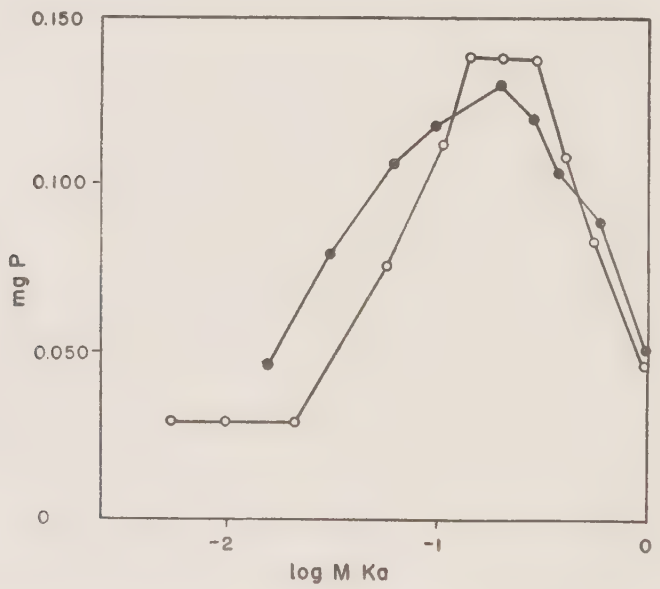

Fig. 36. Effect of KCl on the phosphatase activity of myosin and actomyosin. Ordinate: mg. P liberated. Incubation for 5 min. at 38°. Points: myosin. Circles: actomyosin [Banga and Szent-Györgyi, 36].

is probably due to the K introduced as cation of the ATP used as substrate. As can be seen, there is no real difference between myosin and actomyosin. Whether the two are really identical we do not know because in all these enzymatic experiments ATP has to be present as the substrate. In the presence of ATP, however, the actomyosin dissociates at the higher and lower

salt concentrations; hence at these concentrations both curves are but curves of myosin. The curves do not show any evident relation between the enzymatic activity of myosin and its physical state.

Ca very strongly activates both myosin and actomyosin. The highest enzymatic activities were observed in the presence of this metal. According to Banga's latest results Ca also strongly activates all ADP-ase activities in which protein II is involved.

A very unexpected effect is the activity of Mg. This metal (0.01–0.001 M) strongly enhances the ATP-ase action of actomyosin — being in this respect but little inferior to Ca — but very strongly inhibits the same activity of myosin: 0.001 M Mg is sufficient to cause almost complete inhibition. In the presence of Mg, actin thus has decisive influence on the activity of myosin, turning a strong inhibition into a strong activation.

This strong inhibitory action of Mg on myosin makes it possible to study the action of its varied concentrations on the enzymatic activity of actomyosin. In the case of other metals, for instance KCl, this was not possible because at higher or lower salt concentration the actomyosin dissociated, the free

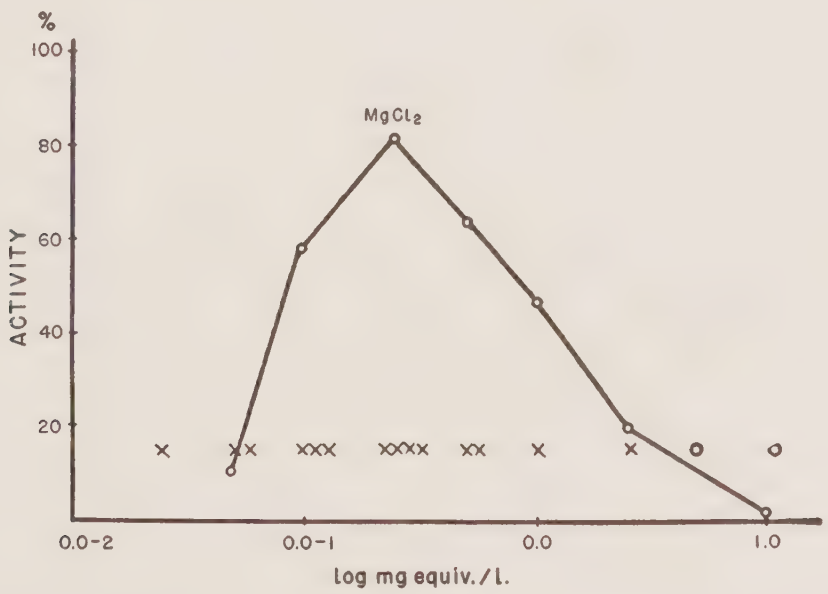

Fig. 37. Effect of varied concentrations of $MgCl_2$ on the phosphatase activity and physical state of actomyosin. Superprecipitation is noted with x 100% activity, maximum activity of the same preparation (obtained with Ca).

myosin took over the enzymatic activity, and it was impossible to say what was due to myosin and what to actomyosin. In the case of Mg the situation is different because the myosin formed on dissociation is inactive in the presence of Mg. So the ATP-ase action observed can only be action of the actomyosin.

Figure 37 shows the influence of varied concentration of Mg on the ATP-ase action of actomyosin from the early studies of Banga on impure, natural actomyosin. In a parallel experiment the physical state of the actomyosin was observed. Super-precipitation (contraction) was marked on the curve with crosses. As the curve shows, there is a complete parallel between super-precipitation and enzymatic activity. Where there is maximal contraction, there is maximal enzymatic activity; where there is partial or no contraction, there is partial or no enzymatic activity. But we know from the experiments of Varga that at the temperature employed (37°) the actomyosin particles could be either contracted or dissociated, and partial contraction when present does not result when the micels are partially contracted but when only part of the micels is contracted. The same must hold true for the enzymatic activity: partial enzymatic activity means that only part of the micels is active. Therefore we must conclude that only the contracted micels are enzymatically active. (This must necessarily be so since if they are not contracted they are dissociated and thus inactive.)

The myosin micel splits ATP thus in its contracted, dehydrated and energy-poor condition only, when it needs energy for its relaxation. As soon as it has liberated the energy necessary for its relaxation and has actually relaxed, it goes over into its enzymatically inactive, hydrated, relaxed, and dissociated state.

Since muscle contains Mg in rather high concentration, these observations seem to give the clue to one of the most basic biological problems: that of the regulation of energy liberation. In our system it is the need for energy which entails its liberation. The system is built in such a way that, in the presence of Mg, it is active only in its energy-poor state, when it needs energy in order to return to its energy-rich state, the state of rest. As soon as it has regained energy, splitting ceases, since the protein in this modification is enzymatically inactive.

It follows from the above data that KCl also must inhibit the ATP-ase action of actomyosin if added to the system in the presence of Mg, in a concentration sufficiently high to induce dissociation. While 0.001 M Mg and 0.2 M KCl both strongly promote enzymatic activity of actomyosin, together they will produce complete inhibition. The action, though striking, can be explained easily. This is not the case with the unexpected reaction obtained if Ca and Mg are added simultaneously to actomyosin. Both $CaCl_2$ and $MgCl_2$ strongly promote ATP-ase activity of actomyosin. One would expect a summation of this effect if both are added simultaneously. Instead, a very strong inhibition is observed which is almost complete if the two metals are added in 0.01 M concentrations, though when added one by one they produce maximal activation. It is difficult to explain this interaction. Possibly the Ca neutralizes the influence of actin on myosin and the myosin, released from the influence of actin, is now inhibited by the Mg. Whatever the explanation may be, it is possible that these subtle ionic synergisms and antagonisms may play an important role in the biological functions and regulations of the system. An ion-exchange between actin and myosin may completely change the reactions. The ATP-ase action and its relation to ions is a fascinating problem. Work here is facilitated by the great stability of phosphatase action of myosin which may be undiminished even after a fortnight's storage at 0°.

VIII. ADP Contraction, Protein I, and Fluochrome

If crystalline myosin is washed carefully and is allowed to act upon ATP, the ATP is dephosphorylated and the products of its splitting are stabilized in the form of ADP_1 and an ATP-ADP dinucleotide, substances which are no longer acted upon by myosin.

If pure myosin is mixed with actin to form actomyosin, this actomyosin will readily contract under the influence of ATP but not on addition of the mixture of ADP_1 and dinucleotide. Contraction occurs, as shown by Guba, if a small amount of a watery muscle extract is added. The substance, present in the aqueous extract and responsible for this action, is an acid-stable, thermolabile protein, which will be called here "protein I." In impure condition the substance can be boiled in 0.1 N HCl for 15 minutes without loss of activity. At neutral reaction it stands short boiling only. The purified substance at room temperature is not inactivated at pH 1 but is destroyed in ten minutes at neutral reaction at 55°. The substance can be precipitated by trichloracetic acid and redissolved by neutralization in active condition, though this treatment makes it rather labile. The IP of the protein is pH 4.6. It precipitates below pH 1.

The activity of the protein was expressed in units, one unit being that quantity which when added to 2 ml. fluid (containing 2 mg. actomyosin, 1.2 mg. ADP in 0.05 M KCl, and 0.001 M $MgCl_2$ at pH 7) caused contraction within 1–2 minutes at 22° C. The protein was purified in the following way: the meat of the freshly killed rabbit was minced and suspended in an equal weight of water and allowed to stand overnight at 0° C., then filtered through a cloth, pressed out, the juice centrifuged. The liquid was brought to pH 1 by means of concentrated HCl and neutralized with KOH. The heavy precipitate, formed on neutralization, was eliminated on the centrifuge, the fluid cooled to 0° and mixed with an equal volume of acetone at −15°. The mixture was centrifuged at once at 0°. The active substance was in the precipitate. The acetone was eliminated and the residue dissolved in water.

The insoluble fraction was separated on the centrifuge and rejected. The solution was then saturated to 0.5 with ammonium sulphate at 0°, the precipitate eliminated by centrifugation, the saturation increased (at 0°) to 0.7, and the precipitate eliminated again. Then the fluid was brought to pH

4.6 (at 0°) and the precipitate, which contained the active principle, was separated on the centrifuge.

In the course of this preparation half of the active substance was lost. While the primary muscle-extract contained 40 units and 30 mg. protein per ml., the end product contained one unit for 10–20 γ protein which corresponds to an average of 50 x purification.

If an active extract is added to the actomyosin-ADP mixture, there is no action for 10–120 seconds, and then contraction sets in. The length of time necessary to produce

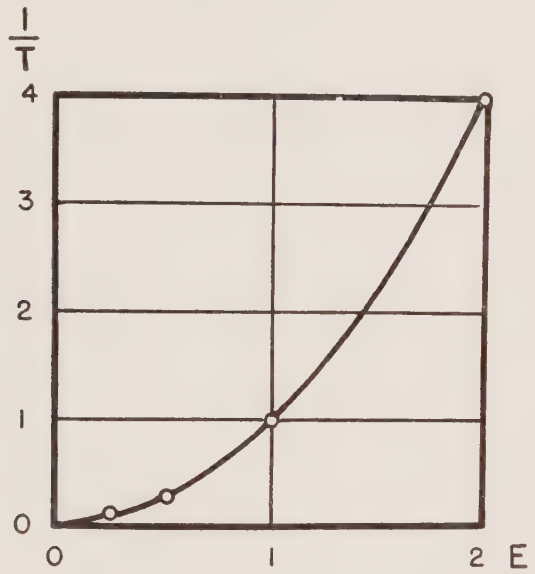

Fig. 38. Relation of number of units (E) of protein I to the reciprocal value of time necessary to produce contraction.

contraction depends on the activity of the extract (E). The relation between the two quantities is given in Fig. 38. The curve can be expressed by the equation $1/T = K.E^2$.

If the purified extract is brought to pH 9–10, within a few hours it turns yellow and loses its activity. If cysteine is added, the color disappears and the activity returns. Evidently the active substance is a chromoproteid with an auto-oxidizable prosthetic group which is active in its reduced state only. In the UV the substance shows a splendid fluorescence which disappears on reduction but not on acidification. This fluorescence is distinctly more bluish than that of the yellow enzyme or lactoflavin.

The chromatogram of the reversibly oxidized active substance, measured by means of the chromatograph of König-

Martens, is given in Fig. 30. As will be seen, the maximum, being at 460 mμ, is somewhat shifted towards the higher wavelength as compared to the chromatogram of lactoflavin, but the main difference is an absorption above 500 mμ not given by lactoflavin.

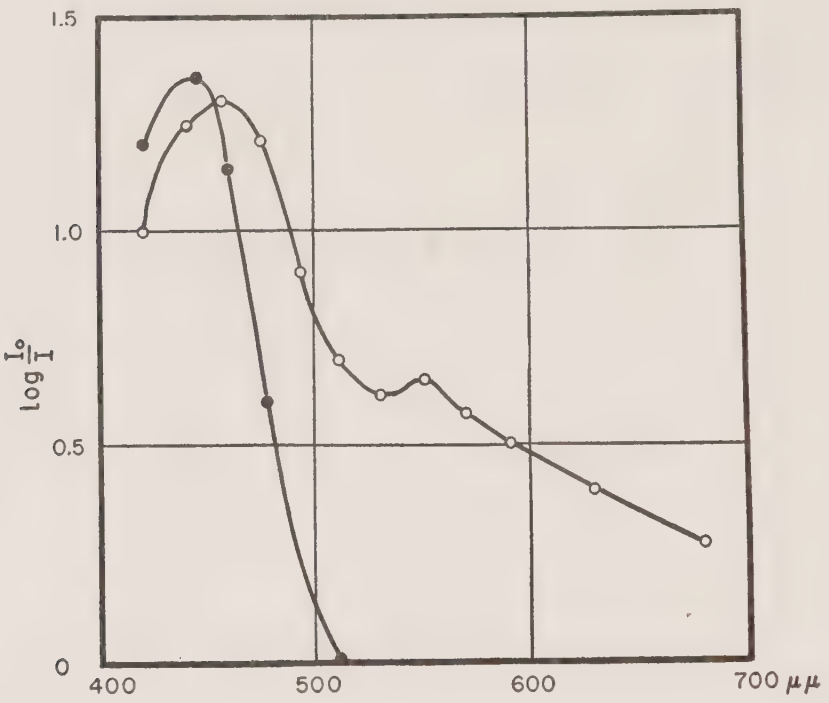

Fig. 39. Chromatogram of oxidized protein I (circles), as compared to the curve of lactoflavin (points).

The dye can be detached from the protein by dialysis at alkaline reaction or by treatment with 20–50% acetone. The detached dye is labile and, at alkaline reaction, readily goes over into a reddish-brown substance which no longer fluoresces. Possibly, the oxidation product adds to the extinction above 500. This irreversible oxidation occurs also in the chromoproteid on prolonged storage at alkaline reaction. The chemical behavior of the dye makes it probable that it is linked to a nucleotide.

The above data make it clear that the dye can be identical neither with lactoflavin nor with thiochrome. It seems likely that it has vitamin activity and is involved in muscular pathology.

If the ADP is incubated with protein I, the protein eliminated, and the ADP allowed to act on actomyosin, no contrac-

tion occurs, the situation being analogous to that with protein II of Banga. Though the reactions of both substances towards precipitating agents, as well as their lability, are very similar, they seem not to be identical, since Banga's protein II, if inactivated by alkali, cannot be reactivated by cysteine. Guba's purified extracts were always active in Banga's enzymatic test. Activity was lost in both tests by oxidation. If reduced again, activity returned in Guba's test but not in Banga's. Whether the protein I has any relation to Kalckar's myokinase, cannot be stated.

PART II

Conditions and Reactions in Muscle

I. Introduction

Up to this point we have discussed only the behavior of the contractile substance, extracted by different chemical manipulations. We came closest to the fibril by imitating its elongated shape and preparing threads from our actomyosin. In now discussing the fibril we must bear in mind that conditions in the muscle fibril differ widely from conditions in threads. Extracted actomyosin is no longer the same substance that existed in the muscle. Nor can the conditions of intermicellar spaces of muscle be imitated *in vitro*. The main difference is that this space, in muscle, is very limited and relatively small compared to the mass of actomyosin. So changes in adsorption of dissolved substances to actomyosin will change concentrations in the intermicellar space considerably. In the thread there is a little solid matter (2%) surrounded by a relatively large quantity of water (98%) so that the adsorption of substances to the solid will not alter appreciably the concentration of the solution.

Muscle contains around 10% actomyosin, located in the fibrils which occupy about half of the total volume.* The fibrils thus contain approximately 20% actomyosin. They probably contain 10% dissolved protein (myogen) which leaves 70% of their volume for intermicellar water. About half of the intermicellar water of the muscle fibre is located in the fibril.

Muscle contains 0.005 M myosin, calculated on the basis of the UW. It contains 0.005 M ATP bound to myosin. If there is no other protein to compete with myosin for the adsorption of ATP, then every UW of myosin holds one ATP molecule adsorbed. This is the quantity of ATP which, according to our previous experience, gives myosin optimal reactivity.

Muscle contains 0.002 M actin (calculated on the basis of the UW). This actin, if prepared according to Straub's method, contains one Ca and two Mg per UW. Since the method gives

* According to Dr. Buchthal's estimate, quoted by A. Krogh in his Croonian lecture, 60% of the volume of the muscle fiber is taken by fibrils. If the muscle contains 10% of blood vessels, fasciae, etc., then half of its total volume can be calculated as being occupied by the fibrils.

no opportunity for these ions to be bound in the course of preparations and since the actin, in this method, is treated with high concentrations of KCl, it is most probable that actin binds these metals in muscle too; that these cannot be expelled by alkali metals, and are bound in a specific way. It was indicated by Straub's experiments that six positive charges are needed to neutralize the basic charge of actin at pH 7. Actin is thus probably held in isoelectric condition *in vivo* by its bound bivalent ions.

According to Hill and Kupalow, frog muscle contains 0.007 M Ca and 0.014 M Mg. These values are somewhat higher than those given by M. Dubuisson (1942) for rabbits (0.005 M Ca and 0.01 M Mg). Taking Hill and Kupalow's values as a basis for calculation, the total concentration of Ca and Mg is 0.021 M of which 0.006 M is bound by actin, leaving 0.015 M free. If the 0.005 M myosin binds two M of this (four equivalents) per UW, 0.005 M thus becomes free, which will be largely Mg. Calculated for the 77% water contained in muscle, the concentration will be approximately 0.006 M. The curves of Banga show that myosin, with four equivalents of Mg or Ca bound, is actually in equilibrium with such a solution. The total concentration of free Ca^+ Mg cannot exceed 0.006 M, and in the muscle we can expect to find the basic negative charge of both actin and myosin balanced by the adsorbed bivalent ions, their primary adsorption being satisfied by Ca and Mg, the concentration and quantity of which is just sufficient to fulfill this role. On no account is there enough Ca or Mg to expel K from its secondary adsorption.

At the concentration of $K^+ + Na^+$ present in muscle, myosin can be expected to adsorb three alkali-metal ions in its secondary circle, which enables the myosin to adsorb ATP. I do not want to stress the actual numerical values which may be subject to modification. What is more important, at the moment, is the method of approach in calculating actual ionic concentrations which depend greatly on the adsorption by actin and myosin.

As mentioned before, there are considerable differences between the actomyosin found in threads and that found in

muscle. First, its distribution is different. The fibril is a highly organized structure with a high steric specificity. In the thread the myosin and actin particles are intermingled and distributed at random and, consequently, if the actomyosin dissociated, both actin and myosin dissolve. This behavior differs from that in muscle. If muscle is treated with strong salt-solution, the myosin dissolves and the actin remains. This suggests that in muscle, actin and myosin each forms a continuous system and it is not the myosin micels which unite with the actin micels to form actomyosin, but it is the myosin system which unites with the actin system. If the elementary filaments, which seem to build up the fibril (Hall, Jakus, and Schmitt) consist of actomyosin, they must be double threads, composed of a myosin and an actin thread.

As the experiments of Banga and Hermann have shown, the elementary processes, like adsorption by myosin, are not very different in extracted myosin from myosin in muscle. This, however, does not hold for more complex colloidal phenomena like dissociation. As shown, actomyosin — *in vitro* — dissociates in the presence of 0.1% ATP if the KCl concentration exceeds 0.16 M. Muscle fibres — washed or unwashed — contract up to 0.45 M KCl. They cease to contract and dissociate only if the KCl concentration reaches 0.5 M. A much higher charge and hydration is needed to separate actin from myosin in muscle, which shows that the forces holding these proteins together are considerably greater in muscle than in the extracted proteins and that these links, once disrupted, cannot be restored.

On extraction the myosin system disintegrates into small and uniform particles. This extracted myosin, in high concentrations, forms a soft, plastic mass which readily dissolves in water. This shows that *in vitro* the forces binding myosin to myosin are exceedingly low and do not explain the great resistance of muscle to tearing. It is highly probable that in muscle not only is actin linked to myosin by forces greater than those in the extracted condition, but also the myosin particles themselves form a continuous system, connected by links which once broken cannot be restored.

II. Rest

In discussing this basic condition of muscle, our first question may be whether actomyosin is dissociated or not: whether resting muscle contains actin and myosin side by side, or contains actomyosin. In my earlier papers I held the view that actomyosin must be dissociated. Resting muscle is soft, whereas a 20% actomyosin gel must be rigid. Moreover I was impressed by Hürthle's observation of a nematode moving with ease through the muscle fibre. No nematode could walk at ease through a 20% actomyosin gel. I did not then take into consideration two facts: one was the division of this actomyosin into very thin threads, which might make it easier for the nematode to get through; the other was the action of ATP. Resting muscle contains high concentrations of ATP, which must have a profound influence on the physical state of actomyosin turning the rigid gel into a soft, plastic mass. So both arguments in favor of dissociation have lost validity. The very strong resistance of muscle to tearing also indicates undissociated actomyosin.

The third argument which made me believe that actomyosin in resting muscle is dissociated was Varga's observation which definitely showed that actomyosin at body temperature must be contracted if ATP is present. The fact that muscle contains ATP in rather high concentration seemed to exclude the presence of undissociated actomyosin.

All would have been well had not the muscle fibre, washed or unwashed, readily contracted under the influence of ATP added from without. If actomyosin, *in vitro*, contracts under the influence of ATP, it is natural that it should do the same in the muscle. But if the fibre contracts under the influence of ATP, then, evidently, it cannot be dissociated, or else we should have to suppose that the first action of ATP is to cause association, which is contrary to experience. Therefore in a later paper I held the view that actomyosin, in resting muscle, cannot be

dissociated. I will not take sides here but will simply describe what can be seen.

If ATP, in resting muscle, does not cause contraction of actomyosin, then evidently either the ATP or the actomyosin must be present in an inactive condition. It was natural that the first thought should have been that ATP is present in an inactive modification; its formula offers rich possibilities for such isomerism. Efforts, however, to isolate such an inactive ATP failed [Rózsa, 44].

Erdös [70] and Rózsa [44] found that the ATP, present in resting muscle, is not completely inactive, but that its activity depends on the salt concentration. In isotonic 0.16 M KCl, NaCl, or Ringer's solution, the ATP was inactive and did not cause contraction of freshly isolated muscle fibres, but did so if the fluid was slightly diluted. Dilution by no more than 10% sufficed to induce violent contraction. The behavior of the myosin, with its own ATP, was thus analogous to the behavior of actomyosin threads in ATP. This analogy is made clear by Fig. 40. The upper half of this figure is identical with Fig. 20, drawn on a logarithmic scale. In the lower half the behavior of freshly isolated muscle fibres is shown. The zone where all fibres contracted is black. This zone is bounded on the right by a very narrow zone, and on the left by a wider zone where some of the fibres contracted, evidently owing to imperfections of the technique and the unavoidable excitation: In the figure this contraction is marked with lines. Muscle is thus balanced between contraction and relaxation, just on the side of the latter, and the isotonic concentration is the critical concentration which just maintains relaxation.

Results with frog muscle were analogous to those obtained with rabbits, the difference being that here the critical salt concentration lay 30% lower.

If ATP is added to a fresh muscle fibre suspended in isotonic solution, contraction occurs, and as the curve in Fig. 40

With rabbits, advantage was taken of the fact that the muscle is unexcitable at 0°. Frog muscles are very excitable and readily contract at 0°, so the isolation of single fibres was omitted and the muscle (gastrocnemius) was perfused *in situ* from the artery. If the Ringer solution was diluted by 15%, single electric shocks provoked, instead of a twitch, a short contraction.

shows, contraction can be obtained up to 0.45 M KCl. This is not unexpected because washed muscle fibres also contract up to this limit. This limit is characteristic for actomyosin in muscle. The problem is rather why do the two ATP's, one present in muscle and one produced from without, behave differently, and why does the former cause contraction only below the isotonic concentration.

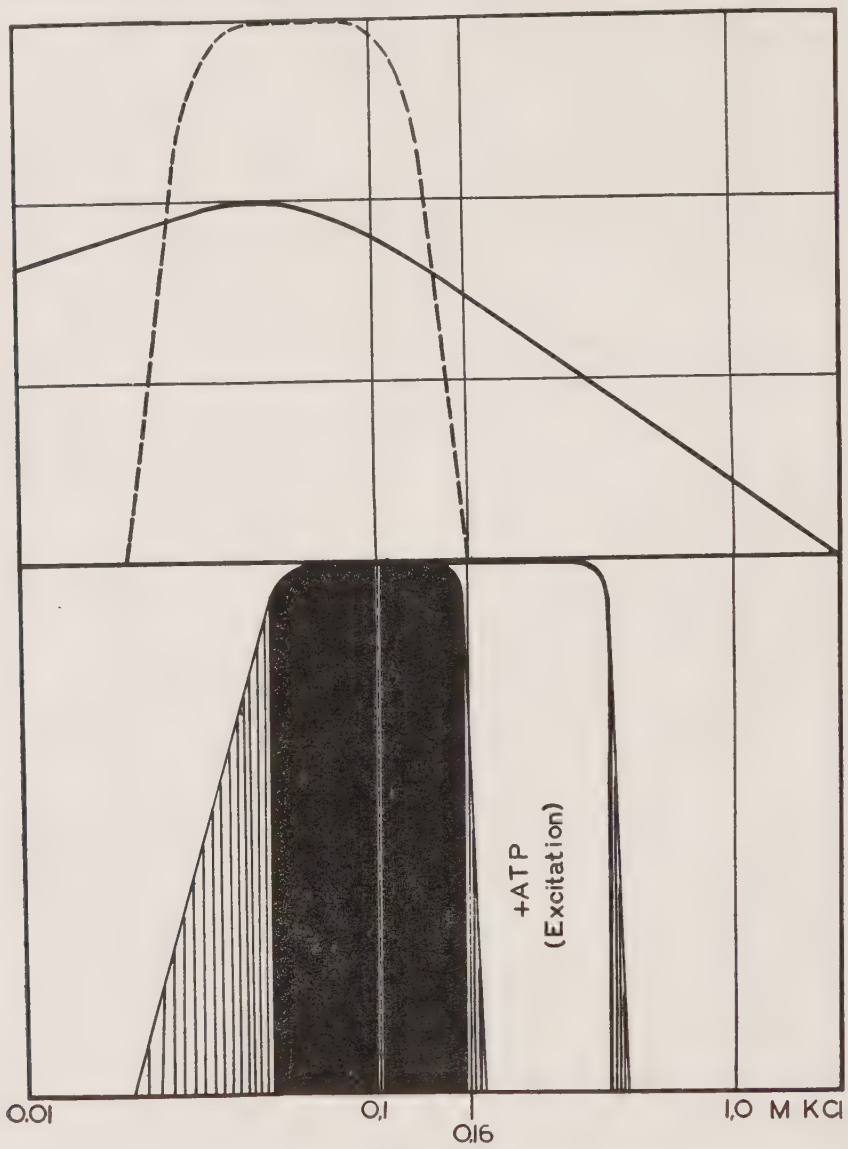

Fig. 40

If we suppose that ATP is responsible for the difference, then we must assume it to be in some inactive state in resting muscle. Knowing that the ATP present in muscle is adsorbed to myosin, we may formulate the situation by saying the adsorbed ATP causes contraction only below 0.16 M KCl, while free, added ATP does so up to 0.45 M. Hypotonicity activates the ATP by releasing it from its adsorption. It was shown

before that the adsorption of ATP is dependent on ionic concentration.

Why adsorbed ATP is unable to cause contraction is but a matter of speculation at this time.

The simplest explanation is to suppose that ATP is adsorbed in a specific steric orientation, in which its active group is kept out of touch with the protein, pointing away from it.

It is simple to convince oneself of the effect of steric orientation on biological activity, by sitting on drawingpins.* The effect will be different if the pins are oriented by means of their flat heads (adenyl group) or randomly distributed with some points (pyrophosphate group) towards our surface.

III. Excitation

Buchthal, Deutsch and Knappeis (1944) discovered that ATP, applied to the isolated muscle fibre or given intra-arterially, produces twitches or a tetanus-like contraction which, in many ways, resemble normal contraction. They arrived at the conclusion that ATP is involved in the process of excitation and that the ATP, present in resting muscle, is present in an inactive condition, conclusions supported by our observations. Buchthal and his collaborators applied the ATP from a micropipette to certain points of the muscle fibre; the quantity of ATP was small but its concentration high.

Unaware of these experiments, G. Rózsa started work on similar lines with a somewhat different technique. He suspended the isolated muscle fibre in Ringer's solution, or slightly hypertonic (0.2–0.25 M) KCl, and added ATP. So in these experiments ATP acted in a uniform concentration on the whole fibre and produced permanent shortening. Rózsa used freshly isolated muscle fibres and found that 0.5 γ of ATP per ml. sufficed to cause contraction. This result was very remarkable for several reasons. It showed that ATP, in resting muscle, must be adsorbed completely; the concentration of free ATP cannot exceed 0.5 γ per ml., or else it would cause contraction. But this result was in contradiction to our previous experience which showed that actomyosin needs a high ATP concentration.

*Thumb tacks.

for contraction; actomyosin will not contract if the ATP concentration sinks below 0.01% which is still more than one hundred times 0.5 γ per ml. This shows that these small concentrations of ATP added did not elicit contraction directly, but activated the relatively large quantity of ATP present in inactive condition, so that the ATP present in the fibre now behaved as free ATP, added from without, causing contraction up to 0.45 M KCl (Fig. 40).

The results were different if muscle fibres were used which have lost excitability. In these experiments the fibres were isolated after the muscle had lost electric excitability on storage. If such unexcitable fibres were used, ATP still produced contraction, but only in the high concentration usually needed to make actomyosin contract (0.01–0.1%). This suggests that the small amounts of ATP added to excitable muscle caused contraction by acting on the normal mechanism of excitation which activates the ATP present in resting muscle.

Whatever the mechanism may be, the observations presented make it sufficiently clear that the relaxed actomyosin micel, in muscle, has a very complex and labile structure, extremely delicately balanced. The stable, energy-poor condition is the contracted one. The muscle fibre contains 5,000 times more ATP than is necessary to elicit contraction. The adsorption (inactivation) of this ATP depends on K adsorption, which property is very labile. The effect of K adsorption, in its turn, depends on the previous adsorption of bivalent ions. Under certain conditions, the autocatalytic release of ATP may be started by the release of one single K ion and the loss of the bivalent ions may lead to the release of all the adsorbed ATP, K being inactive without bivalent ions. Sufficient evidence was given that these ions may influence each other's activities in a subtle way. An equally subtle property of actomyosin is its dissociation which depends both on ions and ATP. Moreover it has been shown that isotonicity means a critical ionic concentration and the whole equilibrium collapses on slight dilution. Thus, though we are unable to say what happens on addition of 0.5 γ ATP per ml., we know that resting muscle represents a most subtly balanced metastable condition and we cannot

be surprised if a small quantity of ATP is capable of upsetting it; or if the equilibrium is disturbed by an electric shock, which might tear off one K^+ or distort electric fields. Nor can we be surprised if the changes occurring in one micel upset conditions in its neighbor. Possibly the ATP released in one micel may also start the process in its neighbor, or it may be that the water released by one contracting micel causes dilution and herewith contraction of its neighbor.

There is one more point which should be mentioned. The observations suggest that the autocatalytic release of ATP plays a role in the normal process of excitation. It has been mentioned that a minute quantity of ATP, added from without, will cause contraction in the excitable muscle fibre even if 0.25 M KCl is used as suspension fluid. This is remarkable because muscle readily loses excitability in KCl. It may be deduced herefrom that KCl inactivates that part of the normal mechanism which liberates the first quantity of ATP but does not interfere with further action of this ATP. Possibly this KCl prevents the release of ATP by making its adsorption too strong.

Mention should be made, finally, of experiments of Rózsa, who has also studied the action of a series of poisons known to cause contraction in muscle. The freshly isolated muscle fibres were suspended in 0.25 M KCl and the poisons were added. Acetylcholine and nicotine caused no contraction. Eserine was active in $1:10^7$, veratrine in $1:10^6$, quinine and caffeine in $1:10^4$ dilution. What gave special interest to these experiments is the fact that these poisons were active only in excitable muscle. As soon as excitability was lost the poisons became inactive. These drugs seem thus to act by setting into action the normal mechanism of excitation, activating, in some way, the ATP present.

IV. Contraction

If the muscle fibre is subjected to a rather rude treatment, designed to destroy all finer structure, retaining only the rigid actomyosin system, and the fibre thus treated is suspended in dilute KCl and ATP is added, it contracts violently. So, for

instance, if the muscle is washed in distilled water for several days at 0°, then frozen on the freezing-microtome, cut into slices parallel to the fibres, thawed and suspended in KCl, it contracts violently if ATP is added, similarly to threads of actomyosin. This reaction is very specific and no other substance will elicit such contraction except ATP, the normal constituent muscle, or substances closely related to it. Evidence indicates that the two phenomena, the contraction of actomyosin threads and the contraction of muscle fibres in ATP, are related.

Contraction of actomyosin seems to be a simple colloid-chemical process, synaeresis, taking place in a specific structure, built of two specific colloids. In muscle the steric orientation, called "structure," may still add to the specificity of the reaction, but there can be little doubt that in essence both contraction of muscle and contraction of actomyosin are identical phenomena. By orienting the particles within threads the similarity of both processes can still be increased.

In the first part of this book a theory was evolved which attributes the special features of the contraction of actomyosin to the bending of particles, or angular motion. This theory, if applied to muscle, explains different features of muscular contraction in a rather satisfactory manner. It explains why muscle becomes shorter and thicker, and why it loses DR on contraction. It answers an old puzzle. In colloids, long-range forces are very weak and may lead to the accumulation of much intermicellar water. The loss of this water may give very extensive shrinking but is inadequate to do work. Short-range forces may be made to do work but the corresponding changes in volume are small. Muscle performs very extensive monodimensional shrinking but does considerable work throughout. This is nicely explained by our theory according to which this this motion is but the magnified action of the short-range forces.

Another puzzle is: why is the muscle strongest in its most stretched condition? If shortening is due to mutual attraction of atomic groups or particles, then why do these forces increase if we pull these groups or particles apart? It is in love only that attraction increases with the distance. But if muscular con-

traction is an angular motion, then, for a given shortening, the motion of any point of the rod (Figs. 23–24) will be most extensive and the product, force × distance, will be the greatest in the straight condition.

All this agreement is very gratifying but one would like to have more direct evidence about the basic correctness of the theory. Unfortunately the Röntgen method, in its present form, seems to be inadequate for the study of contraction. As far as I can judge, its results are in harmony with our theory but the evidence is negative. At low degrees of contraction, 10–20%, we can expect only part of the fibres to be contracted and the X-ray picture to be unchanged. At higher degrees of contraction we can expect the picture to become blurred. This is actually what has been found (Astbury, 1946). According to our theory, at the maximum of contraction, particles must be oriented at 90° to their original position at rest (Figs. 26 and 29). This degree of contraction throughout the muscle cannot be achieved by physiological stimulation. It may be achieved by heat, and Astbury actually found myosin in this position in heat-treated muscle. In histological slices of contracted muscle, at points of maximum contraction, DR can be found to have assumed an opposite sign.

It is difficult to arrive at final conclusions without being informed about the simplest basic questions, like the dimensions of the myosin and actin particle, as well as their orientations. Astbury finds the polypeptide chain parallel to the fibre axis, but Kratky, Secora, and Weber's* measurements suggest that the myosin particle is elongated and is formed by the collateral association of polypeptide chains, the long axis of the particle being perpendicular to these chains. If this would be so, then the myosin particle could stand radially, while the polypeptide chain stands coaxially. The angular theory of contraction makes no predictions on this line.

According to Varga's measurements, contraction is an endothermic process. This seems to be in contradiction to A. V. Hill, who has shown that contraction is exothermic. The contradiction is only apparent. What Varga measured was the

*Personal communication from Professor H. H. Weber.

heat change of the elementary primary process, the internal rearrangement of the particle. This is followed in a secondary way by dehydration, contraction, and also by the enzymatic splitting of ATP. What Hill measured is the heat change of the whole process in the whole muscle. If any prediction could be made from Varga's result, it would be that the function of the electric organ of fishes involves a cooling down, the electric organ being a sort of muscle without contraction.

V. Relaxation

As shown before, contraction and relaxation are two distinct states of actomyosin and there is no in-between state: it is either Jack or Jill. It has also been shown that the contracted state is the energy-poor state and at 37° the free energy of the particle decreases by 7,000 cal. on contraction. It follows that we have to impart 7,000 calories' worth of free energy to the system if we want it to relax. It would be simplest to picture relaxation as the reversal of contraction, starting as soon as the necessary quantity of energy has been invested. This picture, however, would be incorrect. It has been shown that only F-actomyosin is contractile and that the F-actin is broken up in contraction into globules. The properties of G-actin and G-actomyosin are very different from those of the F-form. We have to distinguish between two forms of G-actomyosin: the hydrated-relaxed, and the dehydrated-contracted form. (The latter can be obtained by making F-actomyosin contract.) The properties and reactions of these two forms are different. At a medium salt concentration the contracted-dehydrated, energy-poor, G-actomyosin is not dissociated by ATP. The relaxed-hydrated, energy-rich form is dissociated by ATP at any salt concentration. What we can expect to happen is the following: F-actomyosin is brought to contraction, going over into the energy-poor, dehydrated form. At the same time, the actin breaks up into globules. By going over into the contracted state the actomyosin becomes enzymatically active, splits ATP, going back into the energy-rich hydrated form. Under the action of ATP, this actomyosin dissociates into free G-actin

and free myosin. The G-actin then polymerizes into F-actin; we return to our starting point and the system is ready for a new contraction. This dissociation of the compound may greatly facilitate relaxation mechanically by decreasing the internal friction of the system which, in the contracted state, must be very high. But the dissociation may also facilitate return to rest chemically. It has been shown that free myosin has a greater adsorption power than has actomyosin. If the primary change in contraction is liberation of adsorbed ions or ATP, the readsorption and return to rest must be greatly facilitated by dissociation.

VI. Rigor and Contracture

In muscle every UW of myosin has one molecule of ATP adsorbed which contributes to the charge and hydration of the protein and herewith to the suppleness and plasticity of muscle. *Post mortem* this ATP is gradually decomposed and in the end we are left with a rather stiff, salt-precipitated actomyosin gel. Since dehydration means contraction, this relatively weak salt-precipitation will entail a weak contraction. This slightly contracted, rigid condition of actomyosin is *rigor mortis*. The actomyosin of such muscles is insoluble in 0.6 M KCl, no ATP being present; it can be rendered soluble again by the addition of ATP to the KCl solution [Erdös, 32].

Erdös has followed quantitatively the development of rigor and the disappearance of ATP. He has found that disappearance of ATP and stiffening of muscle set in immediately after death, progressing slowly until the complete exhaustion of ATP and the complete development of rigor. The two curves, that of the disappearance of ATP and that of the developing stiffness, were perfectly parallel. This shows that the whole physiological ATP saturation is needed to keep the muscle in a perfectly relaxed condition. The disappearance of the rigor, which occurs spontaneously later, was found to be due to the disintegration of the system, the actin becoming soluble. Exhaustion of ATP in an unexcitable muscle is rigor.

The picture will be different if ATP is exhausted *in vivo* in an excitable muscle. As shown before, less ATP is needed to produce contraction of actomyosin than is necessary to produce relaxation, and reduction of ATP concentration favors contraction at the expense of relaxation. If the concentration of ATP is decreased during excitation, the muscle will become more and more unable to relax and will stop at last in contraction. This condition also develops gradually. Erdös has compared the development of contraction and disappearance of ATP in different forms of contraction, like that developed under the action of monoiodo-acetate, chloroform vapor, caffeine, and prolonged labor. In all cases there was a parallel between contraction and ATP concentration. Though this parallel was less perfect than in the case of rigor, it was definite enough to show the close relation of the two processes. Exhaustion of ATP in excitable muscle is contraction.

The observations warranted the question whether certain pathological conditions, characterized by the failure of certain muscular elements to relax, are not due to the lack of ATP. Such conditions are, for instance, angina pectoris, certain vasospastic gangrenes, and dysmenorrheas. It was found at the medical clinic of Szeged that these conditions could be relieved by the administration of ATP. The experience showed that during treatment there were no attacks of angina; gangrenes which would have otherwise required amputation were healed; and dysmenorrheic pains were relieved. More extensive experience along these lines is desirable.

VII. Contraction, Fermentation, and Oxidation

The two sources of animal energy are fermentation and oxidation. The energy of fermentation is made available to the contracting mechanism in the form of the phosphate bonds of ATP. If the muscle needs energy it splits these bonds and the dephosphorylated ATP sets the mechanism of fermentation going by serving as phosphate-acceptor. Contraction sets off dephosphorylation and dephosphorylation starts fermentation.

The need for energy is the automatic starting mechanism for both processes.

In fermentation, the bound phosphate is not transported to myosin by the ATP. As shown, resting muscle can contain no free ATP: the ATP must be adsorbed. Most of it is linked to myosin; the rest, to the enzymes of fermentation. The ATP is released by myosin only after dephosphorylation and deamination to inosinic acid which has no more biological activity. The role of phosphate carrier is fulfilled by creatine. The phosphate is transferred from ATP to creatine and from creatine to ATP by a special enzyme, the function of which was described by Lohmann in the following equation:

$$\text{ATP} + 2 \text{ creatine} = \text{AMP} + 2 \text{ creatine-phosphate.}$$

It has been shown by Banga [34] that the Lohmann reaction is actually performed by two enzymes, one of which establishes equilibrium between ATP and creatine:

$$\text{ATP} + \text{creatine} \rightleftharpoons \text{ADP} + \text{creatine-phosphate}$$

and one which establishes equilibrium between ADP and creatine phosphate

$$\text{ADP} + \text{creatine} \rightleftharpoons \text{AMP} + \text{creatine-phosphate.}$$

She called the first ATP-creatine phosphopherase, the second ADP-creatine phosphopherase. The first was separated from the second and its equilibrium-constant established (Table V).

TABLE V. *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*

M creatine added	M ATP added	M P found	$K = \frac{\text{Cr.P.ADP}}{\text{ATP.Cr.}}$
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

Volume: 1.6 ml. Incubation at 38°, until state of equilibrium had been reached.

This constant varied with the pH and reached maximum at the pH-optimum (pH 9)of the reaction. These enzymes are very powerful and readily transfer the phosphate of ATP to creatine at the sites of its production (fermentation) and from creatine-phosphate to ADP at the sites of its utilization (contraction).

The relation of oxidation and contraction is less clearly understood. Oxidation could not be the sole source of the energy of contraction because the function of muscle is so abrupt that oxidation could not always keep up with it, and there has to be a readily accessible store of reserve energy, independent of 0_2 supply. This store is the bound phosphate of ATP and phosphagen (phosphocreatine). This, of course, is no reason for the muscle not to use oxidative energy too.

Other, more steadily working organs, like the kidney or brain, seem to depend entirely on oxidation for their energy. We can thus expect that muscle too will do the same and use phosphate if oxygen supply becomes inadequate. It becomes increasingly probable that muscle even uses its oxidative energy to synthesize bound phosphate, as seems to be the case in liver and kidney (Kalckar). It is even possible that there is a fluid equilibrium between phosphate splitting, phosphate synthesis, oxidation, and the energy state of actomyosin. If actomyosin is in its low-energy state of contraction, it splits phosphate; if it is in its high-energy state, it synthesizes it, taking the excess energy from oxidation. It may be brought from the low, contracted level to the higher, relaxed level either by phosphate splitting or oxidation.

These ideas are somewhat ahead of the experimental evidence, and coming back to earth, our first question may be: is there any close relation between the contractile system, its function, and oxidation?

The furnace in which the H of the foodstuff in muscle is burned is composed of the oxidases of succinic and citric acids. Both the succino- and citrico-dehydrogenase are linked to the water-insoluble structure and we have to suppose that either the oxidases are linked to the actin-myosin system, or else at the side of actomyosin there is another structural system to bear them.

Biró and A. E. Szent-Györgyi have undertaken to decide between these two possibilities. Knowing that the physical state of actomyosin is governed by ions and ATP in a rather specific way, they asked how far in washed muscle the oxidation of succinic acid depends on the presence of ions and ATP and whether there is any relation between oxidation and the physical state of actomyosin.

Actomyosin is precipitated (contracted, dehydrated) to some extent by KCl with a maximum of about 0.05 M. ATP very greatly increases this reaction up to 0.5 M KCl, where the system disintegrates. The results of Biró and Szent-Györgyi are summed up in Fig. 41. There is a complete parallelism between succinoxidation and the physical state of actomyosin. As the curve "K" shows, the oxidation of succinic acid depends on the K concentration and has a maximum about 0.05 M KCl where actomyosin is isoelectric and is maximally precipitated. The enzymatic activity is very greatly enhanced by ATP (curve ATP). Ca and Mg make the curve of enzymatic activity steeper, the zone narrower, as they do in the case of contraction. At the higher KCl concentrations, oxidation stops where the

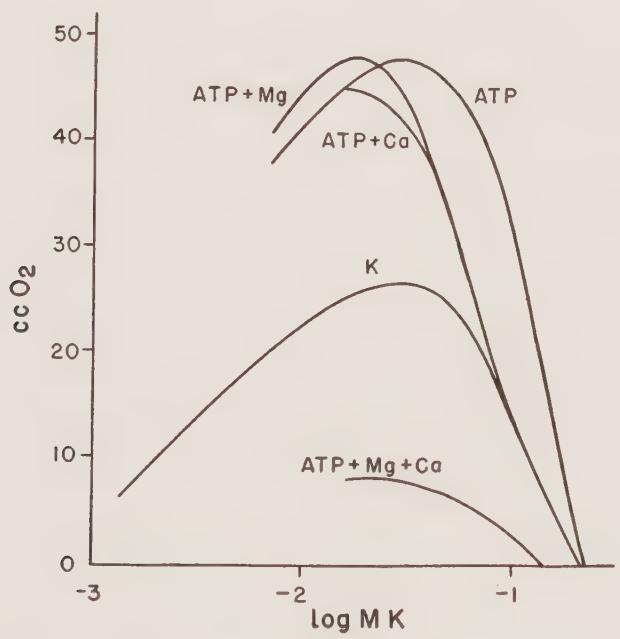

Fig. 41. Oxygen uptake of 300 mg. of washed muscle at varied K concentrations in absence and presence of 0.125% ATP, 0.005 M $MgCl_2$, 0.005 M $CaCl_2$, 1.2 mg. succinate. Abscissa: log M K present. Ordinate: cc. O_2 adsorbed.

actomyosin system disintegrates. The curve rapidly falls to the left of the maximum towards the smaller K concentration. (In the presence of ATP these curves could not be extended any further since K had to be introduced as cations of the ATP.)

The analogy of oxidation and the functional state of actomyosin is drawn still closer by the simultaneous action of Ca and Mg. Both ions have a strong promoting effect on the phosphatase activity of actomyosin, but have a very strong inhibitory effect if present simultaneously. This reaction is very specific and the curve Mg+Ca shows that oxidation of succinic acid is also very strongly inhibited by the combination of both ions.

As far as experience can take us, there is thus a complete analogy between the state of actomyosin and oxidase activity. Where there is partial contraction (KCl alone) there is partial activity; where there is maximal contraction, there is maximal activity; and where there is no contraction, there is no activity. In the presence of ATP there can be no partial contraction, only complete relaxation or complete contraction, as far as single micels are concerned; there can thus be no partial activity either, and only the contracted micels are oxidatively active.

These results show that actomyosin is involved in succinic oxidation. The oxidase and the contractile matter form one single functional system together. This must seem rather natural to the physiologist: what logic would there be in having the energy liberated by another system than that which uses it?

Our second conclusion, that the system is enzymatically active only in its contracted state, may seem still stranger to the enzymologist, but to the physiologist it will seem natural that the system should liberate energy only when needed, when in its lower energy-level. This is again an expression of the same fundamental principle we have met in phosphate hydrolysis: it is the need for energy which regulates its liberation. The liberation of energy starts automatically if energy is needed, and stops automatically as soon as energy becomes superfluous.

One wonders about the inner mechanism of this regulation. Biró and Szent-Györgyi have tried to approach this problem

by finding out which part of the succinoxidase system is responsible for it. The succinoxidase system is composed of three parts: at the one end there is the oxygen activation (*Atmungsferment*, cytochrome oxidase). At the other end there is the hydrogen activation (succino-dehydrogenase). In the middle there is the series of cytochromes which transmits the electrons, liberated by the dehydrogenase, to oxygen, activated by the cytochrome oxidase. The experiments showed that neither oxygen activation nor hydrogen activation are dependent on ATP or concentration of KCl. Naturally, this does not mean that there is no close relation between these enzymes and actomyosin; the experiment simply says that the enzymatic activity does not depend on the functional state of this protein.

Biró and Szent-Györgyi performed a great number of experiments to convince themselves either of the identity or the independence of the dehydrogenase and actomyosin. They followed two methods. They prepared "dehydrogenase" from heart by the standard method: the final preparation had all the properties of actomyosin. On the other hand, they tried to separate dehydrogenase activity from actomyosin and to prepare dehydrogenase-free myosin, or myosin-free dehydrogenase, but were unsuccessful. Their results, at this point, were rather complex and were largely in agreement with the assumption that dehydrogenase activity is linked to actomyosin which is the most active when present in its original structure, as it is in muscle. Once this structure is disrupted, myosin carries with it the activity, which becomes very labile. The activity cannot be increased by the addition of actin but is higher the more actin the myosin originally contains.

Having shown that it is neither the oxygen nor the hydrogen activation which depends on contraction, we are led to conclude that the electron-transport system is broken up in relaxation and restored in contraction. This means that there is always active oxygen and active hydrogen, but in resting muscle they do not react since the bridge between them is broken. As to the mechanism of the inactivation and activation of the electron transport, we can only guess. It may be that in the relaxed state the electronic structure of the system is simply such that it

will not work. But there is also a simpler and cruder mechanism possible, and we may think that by the stretching out and hydration of the myosin particle the cytochromes lose touch and become active again if the system goes back into its dehydrated-contracted state which brings the cytochromes into touch again.

Two objections may be raised: how is it possible that the action of metal ions has hitherto been overlooked? Succinoxidase is one of the most classical objects of research and many workers used distilled water as suspension fluid. The probable explanation is rather simple: succinate was added in these experiments as a K or Na salt and herewith the necessary metal was introduced, usually in optimal concentration.

The other objection may be this: the oxidative resynthesis of lactic acid takes place in the recovery period, when actomyosin is relaxed. It is possible that at the side of succino- and citrico-dehydrogenase there is another oxidative system (diaphorase?) involved in lactic oxidation which is independent of actomyosin or its functional state.

VIII. Lipins, Smooth Muscle, Heart Muscle, Kidney, and Brain

Myosin, recrystallized repeatedly, still contains a considerable quantity (3%) of lipid matter, partly soluble, partly insoluble in acetone. Lipids seem to play an important role in the structure of the contractile system, being closely associated with myosin. The acetone-insoluble fraction contains two main substances, one a cerebroside, which has been obtained in crystalline condition, and the other a substance which seems to be an alcohol-soluble cephalin. The latter, as shown by B. Jánszky (oral communication) is very active as thrombokinase while the former seems to be involved in the different functions of myosin. It is fairly difficult to liberate myosin from the cerebroside but, as shown by F. Guba, this can be achieved by washing the myosin with rather large volumes of fluid. Such a myosin will not contract with ADP but does so after the addition of the cerebroside, provided that protein II is present. A prelimi-

nary experiment of Biró and A. E. Szent-Györgyi indicated that its presence is necessary for dehydration of succinate also. All these are but suggestions, showing that the study of lipins is a rather large and fascinating field. Cortical hormones of the adrenal gland, which have such a profound influence on the development and function of muscle, will also have to be fitted into the picture.

Another most fascinating problem is to find out how far the relationships found in muscle represent general principles of living matter, or how far they are specific cases only. Some observations suggest that the different functions of different organs are closely related, with the same basic mechanism adapted to specific purposes. Caffeine, which produces contraction in muscle, produces increased nervous and renal activity. Veratrine,which provokes protracted contraction in muscle, produces prolonged flow of saliva. If the same key opens different slots, the mechanism of these slots cannot be any too different. The same holds true for the action of ions. It is the same ionic balance in different organs which conditions normal activity.

When trying to apply our experience gained on cross-striated muscle to other organs, the logical sequence is to start with the most closely related tissue, i.e., heart and smooth muscle. Rózsa [51] subjected these to careful study. He prepared actin and myosin from them, and studied the properties and reactions of the separate components as well as the actomyosin combination. He also tried to combine smooth-muscle myosin with cross-striated-muscle actin and *vice versa*. He studied the contractility and reactions with ions and ATP. His results can be summed up by saying that he found no real difference between the three sorts of muscle, cross-striated, heart, and smooth muscle. The difference in the function of these three different organs is thus not a difference of this basic contractile mechanism, but a difference in higher organization and regulation.

There are differences in the extractibility of myosin and actin in different varieties of muscles but, as shown by Guba [70] there are considerable differences in this respect between the cross-striated muscles of different animals or even between the different body muscles of the same animal.

Lajta [59] undertook to study a quite different organ, the kidney. On first approach there is a great similarity between the behavior of kidney and muscle. If the freshly minced muscle is suspended in a strong salt solution (0.6 M KCl) a viscous extract is obtained (containing myosin and actomyosin). The dissolution of the viscous structural protein is due not merely to an action of the salt but also to the action of the bound phosphate of the ATP present. If the minced muscle is stored, the ATP decomposes and the protein becomes insoluble. Instead of a viscous extract, a thin liquid of low viscosity is obtained. Parallel to this change, the bound phosphate disappears and is found now in free condition.

If freshly minced kidney is suspended in 0.6 M KCl, a very viscous and thixotropic extract is obtained. If the minced kidney is stored before extraction for an hour at 37° or for twelve hours at 0°, the extract will not show great viscosity. At the same time the free phosphate increases at the expense of bound phosphate to about the same extent as in muscle. The difference between fresh and stored kidney is still more marked if, instead of 0.6 M KCl, a 2 M KCl or alkaline KCl containing 30% urea is employed.

Rabbit muscle contains, on the average, 2.5 mg. ATP per g. with about 0.25 mg. labile phosphate, which is liberated on storage. In kidney, during storage for one hour at 37°, an average of 0.3 mg. P was liberated per g. of tissue. This phosphate, however, is not derived from ATP (there can be but very little ATP in kidney!) but from the protein and is linked in the fresh tissue to the structure itself, being found in the trichloracetic-acid-insoluble fraction.

These results indicate that, as in muscle, the physical state of the structural protein of kidney is decided by the bound phosphate, but contrary to muscle this bound phosphate is not linked to a small molecule adsorbed to protein, but to the protein itself.

The K ion plays a basic role in muscle, and actomyosin has a very high adsorption power for this metal. Is high K-adsorption power connected with the specific function of actomyosin or is it a general property of structural proteins, connected

with the nature of life itself rather than with a specific function only? In order to approach this question, Lajta measured the K-binding capacity of kidney and brain. He washed the freshly minced organs with 0° water, suspended the mince

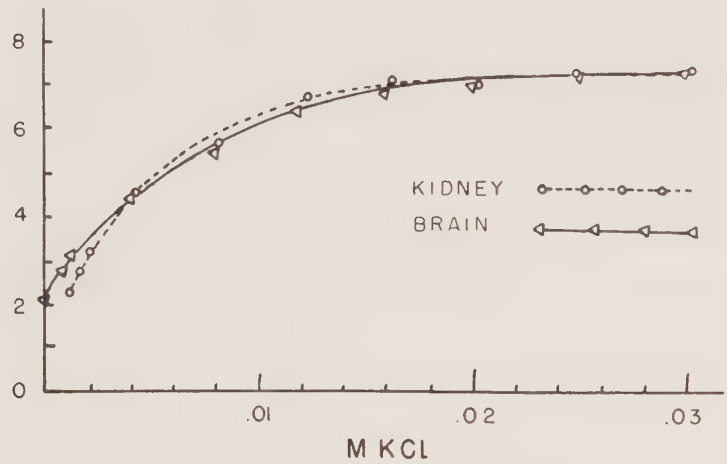

Fig. 42. K adsorption curve of washed kidney and brain. Ordinate: equivalents of K bound per UW of water-insoluble structural protein.

in KCl solution of different concentrations, and precipitated the suspension with alcohol, as done by Banga and Hermann in estimating the K fixation by myosin or muscle. Above 0.3 M the precipitate contained Cl, so the K fixation was followed only up to this concentration. His results are summed up in Figures 42 and 43. The curve, like that of myosin, corresponds

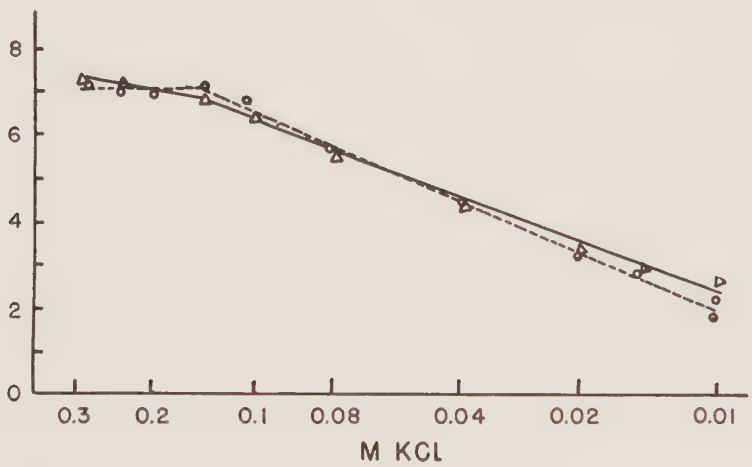

Fig. 43. Same as Fig. 42 on logarithmic scale.

to an exponential equation, being straight on logarithmic scale. It shows a break at the isotonic concentration. Here too the break probably occurs at the IP, but unlike myosin there is no more adsorption above this point. The break is

reached with seven equivalents of metal adsorbed. If fresh kidney or brain is precipitated with alcohol, three K ions in kidney and four in brain are carried down with the protein. If the K adsorption, in washed tissue, is effected in the presence of 0.001 M Mg, one K less will be bound.

The results show that similarly to muscle, the structural proteins of kidney and brain also have a great affinity for K and are capable of binding this metal, and that here too the isotonic, ionic concentration marks a turning point, though the rules governing this adsorption are somewhat different than in muscle, according to the different special functions of these organs.

IX. On the Nature of Cross-Striation

There are two trends at present in explaining the cross-striation of muscle. The morphologist's view is that muscle is what it looks like: a structure built of segments, comparable to a roll of different coins put together in regular sequence. If this is correct, cross-striated muscle represents a very highly differentiated structure. It is difficult to see how such a highly differentiated structure could appear so suddenly, without any transitory forms, in the phylogenetic scale and how and why heart muscle fibres, in tissue cultures, should first begin contracting and later develop cross-striation. The anatomist's view has found the support of the biochemists who showed that the different segments have different chemical composition. These differences, however, were detected by means of optical methods — microscope, spectroscope, or polarimeter — and might have thus been subjected to the same error as direct visual observation. They cannot be adduced as independent arguments.

The other view is that cross-striation is but an optical phenomenon due to some sort of periodically changing property of a continuous structure. Bernal explained cross-striation tentatively, ascribing to fibrils the structure of reversed spirals. I myself have tried to explain cross-striation by a spiral

structure of elementary fibrils [70]. Neither of these theories can be maintained in the light of recent observations with the electron microscope (Hall, Jakus, and Schmitt) and there is no use in speculating about the nature of cross-striation until the possibilities offered by this splendid, new method are exhausted, and we are better informed about the dimensions of the actin and myosin particle.

I myself tried to decide between the spiral and segmental theory by rotating the insect muscle-fibril under the microscope: if the cross-striation is due to segmental structure, rotation should make no difference; if it is due to the spiral structure, striation should move on rotation along the axis. I have seen it moving, and moving just the distance demanded by the theory (two segments on a full rotation). These experiments, however, were done under very bad conditions which did not allow repetition or spotless objective registration; and knowing the difficulties of the experiment I must say myself that the result cannot be accepted as final until corroborated. I published the results, not expecting to live to repeat them [70]. I found the striation in half of the fibres moving from left to right, in the other half from right to left, which indicates partly left, partly right spirals.

At the moment the whole problem is in flux. The electron microscope pictures of Hall, Jakus, and Schmitt indicate that the fibril is built of a great number of still smaller filaments of 50–250 Å diameter, similar to the threads, observed by Ardenne and Weber as well as by the American workers on "myosin." The pictures suggest that cross-striation is rather a property of the substance located between filaments.

I was often amazed to find under the microscope cross-striation and fibrillary structure still present, apparently unaltered, in muscle fibres from which the bulk of myosin has been extracted.

It is possible that cross-striation is the result rather than the cause of motion, like the cross-striation in van Iterson's streaming colloids and Bernal and Fankuchen's vibrating virus solutions. That something is wrong with the old morphologist's view is clearly borne out by the simple observation

that I-bands do not contract, but rather expand in contraction. Since they make half of the length, muscle could never contract by more than 50% if the I-bands were anatomical structures. Muscle may contract to one-fifth.

This much may suffice to bring out the interest of the prob-

TABLE VI

Name of Insect	Musculus adductor mandibulae	Musculus extensor tibiae	Musculus dorso-ventralis	Remarks
1. *Decticus verrucivorus* ♂	5.4	2.7	2.0–2.7	wings unfit for flying; jumper legs
2. *Locusta viridissima* ♀	5.4	2.7	2.7	larva, unable to fly, jumper legs
3. *Stenobothrus sp.*	—	1.4–2.7	—	larva, jumper legs
4. *Liogryllus campestris*	5.4	4.0–5.4	2.7	jumper legs
5. *Carabus Scheidleri jucundus*	2.7–4.0	5.4	5.4	no wings
Carabus Scheidleri jucundus	5.4–8.1	5.4	4.0	no wings
6. *Cybister laterimarginalis*	5.4–6.7	2.7–4.0	2.0–2.7	swimmer legs
Cybister laterimarginalis	4.0–6.7	2.7–4.0	2.7	swimmer legs
Cybister laterimarginalis	5.4–8.1	4.0	2.0–2.7	swimmer legs
7. *Hydrous piceus*	8.1–10.8	5.4	4.0	
Hydrous piceus	5.4–8.1	5.4–6.7	1.3–2.7	
8. *Gnaptor spinimanus*	5.4	5.4	4.0–5.4	no wings
9. *Anoxia orientalis*	8.1	3.5–5.4	—	
10. *Lucanus cervus* ♂	6.8–8.1	4.0–5.4	1.7	big
Lucanus cervus ♂	8.1	5.4	2.0–2.7	medium size
Lucanus cervus ♂	8.1	5.4	1.3–1.7	small (ab. capreolus)
Lucanus cervus ♀	8.1–10.8	5.4	1.0–1.5	
11. *Cerambyx cerdo* ♂	5.4	4.0	1.3–2.7	
Cerambyx cerdo ♂	5.4	5.4	1.3	
12. *Sphinx convolvuli*	/5.4/*	2.7–4.5	1.3–1.7	*Musculus dilatator pharyngis
13. *Apis mellifica*	—	2.7–4.0	2.0–2.7	
Apis mellifica	—	2.7–4.0	2.7–4.0	
14. *Polistes gallica*	5.4–6.7	2.7–4.0	2.7–4.0	
15. *Bombus lapidarius*	—	4.0–4.5	2.0	
16. *Aedes vexans*	—	1.7	1.7	
Aedes vexans	—	1.5–2.0	1.7	

1–4 Orthoptera, 5–11 Coleoptera, 12 Lepidoptera, 13–15 Hymenoptera, 16 Diptera. Numbers indicate the diameter of one Q and I band in 0.001 mm.

lem as well as our ignorance. More definite data are urgently needed.

I will close this part of my book with the very fascinating data of W. Szekessy [52] who asked himself whether there is any relation between width of cross-striation and the rate of motion. There is a very wide range of variation in the rate of motion of different insect muscles. The mandibular muscles, moving the jaw, move exceedingly slowly. The legs are much faster, and the wings are the fastest. Székessy's measurements, summed up in Table VI, show that the widest striae are found in mandibular muscles, where the diameter of one period may exceed 10 μ; the average diameter was found to be 6 μ. The diameter in leg muscle averages 4 μ and that of thoracic muscles, moving the wings, is 2 μ, on the average. Thoracic muscles in animals which do not fly (*Carabus*, *Gnaptor*) have considerably wider striation.

PART III

The Continuum Theory

I. Introduction

The observations presented can be explained in a more or less satisfactory manner in terms of colloidal chemistry, as long as we are dealing with the particles as a whole. But all the reactions described are but outward manifestations, consequences of changes inside the particles. In explaining changes inside molecules we have to be led by chemical concepts, but classical chemical concepts fail to give us any lead.

This failure is not limited to muscular contraction. Discounting a few cases of electron- or H-transfer we are unable to explain any biological reaction. The biochemist, by means of his active substances, like hormones, vitamins, or physiological or pharmacological agents, is capable of setting off the most astounding biological reactions, but if asked about the molecular mechanism of these reactions he will have to confess his complete ignorance. Not only are we unable to explain these reactions by means of our chemical concepts, but we can state that the properties of living matter, to which these amazing biological reactions are due, are not expressed in our chemical symbols and are thus not taken account of in our theory of matter. A phenanthrene, for instance, acting as sex hormone, is, according to its chemical formula, a perfectly stable compound and so is the polypeptide chain of the protein, which the hormone probably acts upon. Judged by their chemical formula, both require a rather drastic chemical treatment in order to be made to react. In the body they react spontaneously at low temperature. The same holds true for other biological agents, most of which are, chemically, perfectly stable. This failure to explain biological reactions suggests that our basic theory of the structure of living matter is inadequate. This theory finds expression in our structural formulae in which each atom is denoted by a special symbol. The theory could be summed up by saying that living matter, like other substances, is built of molecules, the molecules of discrete atoms, each atom consisting of a nucleus and the surrounding electrons. Each nucleus has its electrons

and each electron belongs to a nucleus, only valency electrons being shared by neighboring atoms. (In exceptional cases, as that of conjugated double bonds, single electrons are shared by a greater number of atoms.)

The study of electric conductivity of metals has led to a new idea of the structure of matter which explains electric conductivity by the pooling of valency electrons. In this theory the energy-terms of the single valency electrons fuse to common, quasi-continuous bands. Thus the single electron ceases to be, so to say, the private property of an atom but belongs to the whole system in which it is capable of moving more or less freely. This concept has since been extended to dielectrics, which might have such a continuous band-spectrum without being conductors.* Whether a substance is a conductor or not does not depend solely on whether it has a continuous band-spectrum but also depends on the number of electrons within this band. Any band contains N terms if N is the number of unit-cells taking part in the formation of that band. The Pauli exclusion principle does not allow more than two electrons (of opposite spin) to occupy the same level; so if the number of valency electrons in the single atoms is odd, then only half the terms will be occupied in the highest band and half will be free; if the number is even, then all terms will be occupied. In the first case the system will be a conductor, in the last, an insulator, because for every electron moving in any direction there will be another moving in the opposite direction.

The single bands or "zones" are separated by forbidden zones. If the forbidden zones are narrow, heat agitation may suffice to raise an electron from the highest filled band into the next higher, empty zone, making the substance a "semi-conductor."

In order to be able to develop band spectra, the atoms or other units taking part in the building of that system must comply with certain demands. First, there must be a certain, rather large number of them. Second, their energy-terms must not be too different. Third, they must not be too far apart

*An admirably clear summary is given by Seitz and Johnson in the *J. Appl. Physics* (1937). *8*, 84, 156, 260.

in space and must be arranged according to a pattern with very high regularity, a condition fulfilled in crystals.

It occurred to me several years ago that if this theory of matter could be applied to living structures, we might be able to explain biological reactions. For instance, if we could look upon a protein particle as upon a formation in which electrons are shared by the whole system, then we should also have to suppose that the common electrons within this system have a well-defined statistical distribution. In order to disturb this it would suffice to approach this system by any molecule, the specific steric configuration of which allows it to come close enough. The basic problem was whether the protein molecule has all the qualities required for the formation of continuous energy bands, whether there is a sufficient number of atoms, or atomic groups, whether the energy-terms of these are sufficiently similar, whether they are situated sufficiently close, and, last but not least, whether their arrangement has the required high degree of regularity.

In the course of my career as a biochemist I was more and more depressed by the feeling of complete failure, not being able to explain to myself a single biological reaction. Much relieved by this new theory of matter, I gave vent to my joy in an article in *Science* entitled "Towards a New Biochemistry?" By this rather ambitious title I wanted to emphasize that the application of this new theory to living systems might mean a new period in biochemistry. The reaction to this article was rather amazing: there was none at all. The reason is evident: as long as no evidence of such band spectra in protein or living systems is produced, all this is but speculation, although P. Jordan has pointed out that certain observations of Kubovitz on enzyme inactivation were in agreement with the existence of energy bands in proteins, and Warburg and Negelein's research, together with that of Gaffron and Wohl, also showed that in chloroplasts, energy may be transmitted over a large distance. Timoféef-Ressowsky's experiments on x-ray mutation also pointed in the same direction.

My first efforts to produce fresh evidence bore no fruits and soon work had to be discontinued altogether. After the war was

over, I found myself in close proximity to P. Gombás, professor of physics, one of the pioneers of the new theory of matter, who became my faithful guide in this field. I also had Dr. K. Laki within my reach, who was partly responsible for the development of these ideas. New experiments were begun which, I think, have led to the demonstration of continuous energy bands in proteins.

II. Inner Photoelectric Effect in Protein

M. Gerendás was the first in my laboratory to try to approach the problem experimentally. He measured the dependence of electric conductivity in oriented actomyosin threads on temperature, expecting to find a Hall effect. His results were inconclusive.

J. Boros and D. König based their experiments on a research of Gudden and Völkl who, using K. W. Wagner's theory, measured the electric conductivity of dispersed semi-conductors, embedded in a dielectric. From the dielectric angular loss, the conductivity of the semi-conductor could be calculated. Boros and König tried to measure in this way the conductivity of proteins suspended in water, but the complications, introduced by the solvent, were too great. Powdered casein was embedded in paraffin, the conductivity measured, and was found to parallel the water content of the protein. On complete drying, the conductivity became too low to make the method applicable.

Having failed on this line Boros [48] was led to study the properties of gelatin phosphors.* It has been known since the end of the last century that gelatin, if dried with certain dyes and illuminated, emitted light after illumination was discontinued. The history of these gelatin phosphors is described in the paper of Frölich and Mischung. To Professor Frölich I am indebted for most of the colored gelatin films used in these experiments. The problem of these gelatin phosphors is closely related to the problem of phosphorescence in crystals. As is

*For the general theory of phosphorescence see "Luminescence, a general discussion" held by the Faraday Soc. 1938. Published by Guerney and Jackson, London (reprinted from Transactions of the Faraday Soc.)

generally known, many metal sulfides, carbides, and oxides show phosphorescence, the classical example being ZnS. On illumination, electrons are raised to a higher level, causing light absorption. When the electron falls back to the lower level, it may emit light, causing phosphorescence. There is a certain time-interval between light absorption and light emission. It was recognized that metallic impurities play a decisive role in these phenomena. (The metal may be derived from the crystal itself.) The forbidden zone between filled energy band and the next empty level is too great to allow electrons to be lifted across. The terms of the metallic impurity bridge the gap, since they lie close to the filled band. If an electron is raised by illumination to a higher level it will leave an empty place, a "hole," and the excited electron and the "hole" may travel independently, and at different speeds,* both contributing to conductivity. Eventually the "hole" and the raised electron will have to find one another, the excess energy causing light emission. This theory of phosphorescence involves energy bands. The presence of such a structure is greatly supported by the concomitant electric phenomena, phosphorescence being accompanied by an increased conductivity, the photoelectric current having the same spectral distribution as the light adsorption. This photoelectric current can hardly be explained differently except by supposing the existence of quasi-continuous energy bands.

In gelatin phosphors, the place of the metallic impurity is taken by the dyestuff. Phosphorescence is neither the property of gelatin nor that of the dyes but a property of the system formed by both. The concentration of the dye being rather low, the continuity of the system is evidently due to the gelatin. The mere fact of phosphorescence makes the presence of band-spectra highly probable but cannot in itself be taken as final evidence of such a structure. Phosphorescence can be taken as evidence of a continuous band structure if during illumination the conductivity is raised, i.e., if the electrons in a higher level can move more or less freely.

This has been demonstrated to be the case by Boros in

*Holes may move very slowly.

gelatin phosphors. He dried his colored gelatin films carefully above P_2O_5 *in vacuo*. The films were clamped between the electrodes in a small chamber, provided with a glass window, the air within the chamber being kept dry by P_2O_5. The film was illuminated with an electric arc. The current was measured with a single-threaded electrometer, by the charging method

TABLE VII. *Conductivity of Different Gelatin Phosphors in Dark and Illumination*

Dye	*Dark*	*Illuminated*
Rodulin orange	1.24	3.85
	1.37	2.75
Rhodamin B	6.65	32.2
	6.65	12.8
	13.8	56.5
Methylviolet 5B	12.7	20.7
	10.9	35.3
	10.9	16.0
	14.9	35.3
Methyleosin	10.9	45.5
	6.3	29.7
	9.2	32.6
Rhodamin 3B	8.5	51.7
	8.5	24.4
Eosin W	12.1	33.7
	12.1	17.9

with a voltage of 10–200. The whole instrument was placed in a metallic cage. Cooling of the light, by passing it through water, did not affect the results, which are summed up in Table VII. The single numbers give the average of five readings. The numbers give relative intensity of current in dark and during illumination.

The table shows that there was a considerable increase of conductivity during illumination in all experiments, ranging from 50–500%.

This internal photoelectric effect of the protein shows that the system has a band-spectrum and this structure is actually involved in its phosphorescence. This latter conclusion allows the extension of the study to watery solutions where conductivity of the solvent makes the measurement of the very weak photoelectric current impossible.

III. Phosphorescence of Chromoproteids

J. Gergely [49] undertook, as first orientation, to make observations of phosphorescence of a number of different substances. He dried their solution down with a small quantity of rodulin orange, other dyes like fuchsine, orange, and methylene blue being less satisfactory. As a beginning, the following low-molecular weight substances were tested:

*Phosphorescent**	*Non-phosphorescent*
Carbamide	Urethane
Guanidine sulphate	l-aspartic acid
Orcine	l-asparagine
Pyrocatechol	Hesperidine (from alkaline watery solution, noncrystalline)
Various sugars	Quinone (from water or alcohol)
Glycocoll	Cholesterol (from ether or chloroform)
Glutamic acid HCl	Stearic acid (from chloroform)
Hydroquinone	Resorcinol (from alkaline water, not crystalline)
Sodium acetate	α-and β-naphthols (from alkaline water, not crystalline).
Resorcinol (from alcohol, crystalline)	
α-and β-naphthols (from alcohol, crystalline)	

In solution none of these substances showed a phosphorescence, which is natural if we assume that phosphorescence is an expression of band-spectra and a rather great number of particles is needed for their formation. The table shows the well-known fact that the crystalline condition, in itself, is not sufficient for the establishment of this structure, but is still essential for its development. The intensity of phosphorescence depended on the well-formed nature of the crystals. The more perfect the crystals were, the more intense was the phosphorescence. If the dried mass was formed from a rather concentrated solution by rapid evaporation, crystals were imperfect and phosphorescence was weak. The extreme of this difference is represented in the two last members of the two columns. The same substances, resorcinol and α and β-naphthols were dried down in crystalline and amorphous conditions. In the first case they showed a strong phosphorescence, in the latter they were non-phospho-

*Schmitt (*Wied. Ann.* 58, 102, 1896) found hippuric acid, sulpho-carbamid, anilottic acid, quinine-bisulphate and sugars to be phosphorescent.

rescent. These observations are expressions of the well-known fact that a very regular arrangement in space, such as a crystal lattice, is necessary for the formation of continuous energy levels.

As the next step, a series of proteins was dried down in like manner. They were: gelatin, casein, serum-proteins, egg-white, actin, myosin, actomyosin, fibrinogen, and fibrin. Myosin and fibrin were found previously to be fluorescent by Boros, who prepared films of them.*

All of these proteins showed an intense phosphorescence although none of them was crystalline. The protein molecule has thus in its own structure that high degree of regularity which is necessary for the development of continuous energy bands. It was this difference between proteins and other substances, giving phosphorescence only in crystalline condition, which caused physicists to call such dye-protein systems collectively "gelatin-phosphors" to distinguish them from the "crystal-phosphors."

If the dried gelatin-phosphor is moistened, it loses its phosphorescence, showing that in water either the close association of the gelatin particles or the association of the gelatin particles with the dyestuff is disturbed. This absence of phosphorescence in a watery medium is, however, not a general property of proteins. Actomyosin, for instance, in a 2% solution shows (with rodulin orange) a fairly strong fluorescence if observed in a sufficiently deep (4 mm.) layer. With 0.25 mg. dye per ml. the phosphorescence was weak, reddish-brown. On diluting the dye, the phosphorescence became stronger and was yellowish-green. It reached a maximum with 0.01–0.06 mg. dye per ml. and ceased with 0.003–0.004 mg. In observing phosphorescence, the optimal dye concentration was used and the latent period of phosphorescence was taken into account, the result being declared negative only if no light emission appeared after five minutes of illumination.

*Films were prepared by pouring the watery protein-solution on Hg in the desiccator. Fibrin films were prepared in a like manner, the thrombin being added to the fibrin solution immediately before this was poured on the Hg.

The phosphorescence in a watery solution is important because it shows that even in water, small molecules, like dyes, can enter with the protein in sufficiently intimate relation to allow the development of a common electronic structure.

The results of the first observations on different proteins were the following;

Myosin: to the crystalline mass of myosin, containing 20–40 mg. protein per ml., KCl was added to make the final concentration 0.1–0.5 M for this salt. Phosphorescence was intense, as was the DRF. Then the myosin was diluted with 0.5 M KCl. The period of latency gradually increased from 30 sec. to 4–5 min. When the myosin concentration decreased to 1%, phosphorescence and DRF disappeared. If one part of actin was now added to every five parts of myosin, the fluorescence and DRF reappeared and remained observable down to a dilution of 0.3–0.6% actomyosin. The reappearance of phosphorescence on the addition of actin is important because it shows that the lack of phosphorescence in a 1% myosin solution was not due to the low protein content but to the lack of association of these particles. (In the low concentration used, the actin itself has no phosphorescence.)

Actin showed phosphorescence and DRF in 1–2% solution.

Egg white was concentrated to half its volume. 20% protein. No DRF, phosphorescence.

Serum: 7% protein. No DRF, no phosphorescence.

Casein: below 10%. No DRF, no phosphorescence. Above 10%, both positive.

Casein is globular, myosin is slightly elongated. The axial asymmetry of neither is big enough to orient the particle in streaming fluids at moderate gradients. Their DRF is not due to the orientation of the single particles but to their loose association. This is reached with the slightly elongated myosin above 1%, and demonstrates itself in the DRF. The simultaneous appearance or disappearance of phosphorescence and DRF brings out two rather important points: (1) the single protein particle, myosin or casein, gives no phosphorescence—evidently it is not big enough to contain a sufficient number of atoms or atomic groups to make common bands; (2) even a

superficial association of particles is sufficient for the establishment of a common electronic band-structure stretching over the whole system.

If the superficial association is intimate enough to provide fusion of energy-terms then we can expect the same at a still higher degree from fibrous colloids forming the insoluble basic cellular structure. So the whole elementary fibril can be expected to form a single unit with common energy bands, whatever its length. The same can be expected to be true for structural proteins of other cells, the cell membrane denoting the probable border of the single energy-units. This may perhaps give an adequate explanation of the basic biological fact of the division of higher organisms into such small units, cells.

P. Gombás and Hofmann have calculated the stability and thereby the upper limit of the number of atoms forming such energy-units in proteins. Their results agreed closely with the number of atoms in the structural proteins of one single, average animal cell. The calculation, involving one rather fortuitous assumption, will not be reproduced here, but it is not impossible that calculations can be produced in time without any fortuitous elements, and quantum-mechanical reasons can be given for the existence and dimensions of a cell. It is remarkable that most animal cells have approximately the same dimensions. The dimensions of plant cells, protozoa, or egg cells may be very different, but it would be interesting to know whether the quantity of structural proteins in these cells is not similar to the quantity of these elements in the animal cell.

A few observations of G. Rózsa may be quoted about phosphoresence in animal organs. Their incompleteness is due to the fact that this whole line of research has been taken up very recently and there was no possibility yet for the development of more quantitative methods or the study of more extensive material. Rózsa's experiments show that animal tissues, e. g., dried muscle lamellae of rabbit's diaphragm of the abdominal wall, show an intense fluorescence in the Becquerel phosphoroscope if dyed with rodulin orange, lactoflavine, or haematoporphyrine. The most intense was the effect with rodulin orange. Hydroquinone reduced, quinone extinguished phosphorescence.

Muscles were cut on the freezing-microtome into slices and the slices dyed and dried on non-phosphorescent celluloid plates. The best results were obtained with slices 0.02–0.05 mm.

thick and dyed with a solution of rodulin orange containing 0.03–0.05 mg. of dye per ml. Slices of heart muscle, liver, kidney, brain showed phosphorescence. The wet, fresh, dyed slices showed a distinct though weaker phosphorescence.

Extraction with distilled water or treatment with different drugs, like chloroform water, digitoxin, or thyroxine seemed not to influence phosphorescence which could be observed even in organs affected by parenchymatous degeneration (heart, liver), though differences may be revealed later by more quantitative methods. Incipient *post mortem* decomposition did not seem to affect phosphorescence.

Finally, different organs were studied without any dyeing or drying. A distinct, though rather weak, yellowish phosphorescence was observed.

IV. Structural and Time Relations

It is evident that if there is a continuous band structure of energy-terms in proteins, its backbone is the polypeptide chain with its high regularity of pattern. Side chains tend to decrease the regularity of the structure. Their position is regular but their nature is different, though the atoms taking part in their construction are the same as those building the polypeptide chain.

The terms of C, N, O, and H are given in the first columns of Fig. 44, the highest filled levels being marked with a solid line, empty levels with a broken line. The filled levels of H, N, and O are rather similar and can be expected to fuse into a common band, the width and position of which are tentatively given in the fifth column. The term of C is different and could be expected to fuse rather with the first empty levels of H, N, and O into a common band, the position of which is given tentatively in the fifth column. Naturally, these are but guesses, and it might be that not the single atoms but atomic groups like CO, NH, and CH, act as unit cells, in which case the term structure will be quite different. Nor do we know to what extent the side chains take part in the common structure.

There is no reason to suppose that the short side chains, bearing the COOH groups, should remain excluded.

There is no reason to suppose, a priori, that the peptide link is the only carrier of structural continuity. The significance of H bonds has been recognized more and more in the last

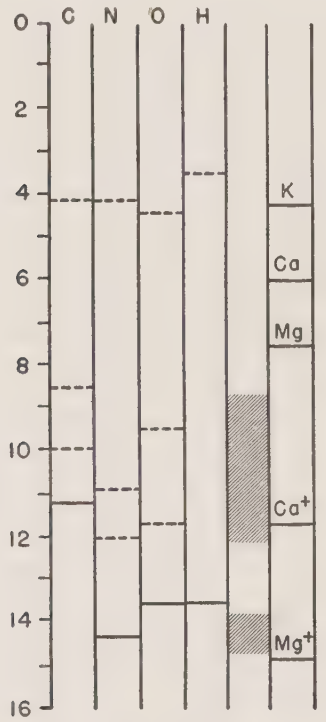

Fig. 44

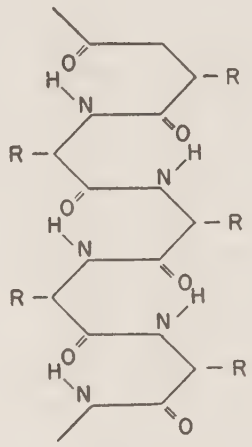

Fig. 45

decade. The supposition of H bonds between CO and NH groups of the polypeptide chain offers many new possibilities. If, for instance, we write the peptide chain in the form shown in Fig. 45, the establishment of such H bonds could transform the whole chain into a cyclic structure.* The energy of the bands is not inconsiderable, and may be in the range of 10,000–50,000 cal., which energy may contribute to the stability of the whole structure and also to the stability of the H bonds along which it extends. On the other hand the H bond, preventing rotation around C-C and C-N axis, may contribute to the spatial regularity of the structure and thereby to the establishment of common energy levels.

The extent of the continua need not be a fixed magnitude, and may depend on the state of the structure and may increase

*The H, not being directed in space, need not be counted with the members of the ring, which thus make six in number.

in the dehydrated (precipitated, contracted) state when different molecules or different parts of the same molecule come into closer contact, as has been postulated in the case of the succinoxidase.

Phosphorescence in "gelatin-phosphors" has a latent induction period which may last for several minutes. Corresponding to the latent period there is an after-effect, observed and studied by Frölich and Gyulay, which may last for hours and is demonstrated in the reduction of the latent period in repeated illumination. It looks as if we have to fill up certain energy levels with excited electrons until phosphorescence occurs, or fill up a certain level to a certain degree. As shown by the duration of the after-effect, these non-phosphorescent, excited electrons seem to be quite stable and persist in the excited condition for hours. This may have the greatest importance for biology, at the same time opening new technical possibilities. Boros had the impression that the latent period is reduced by preceding illumination not only in the illuminated spot but also in its vicinity. If this is borne out by quantitative measurement, it means that this "basic excitation" spreads and that the excited electrons move away by measurable distances. This may open the way to the study of the spontaneous motion of electrons along different structures, such as the muscle or nerve fibrils, and their arrest by formations like cell membranes, etc.

V. Biological Aspects

It may be objected that there is no point in discussing biological implications until the energy levels of the protein are known. Certainly the knowledge of the term structure is of basic importance, and this only can bring biological problems into the realm of quantum mechanics. Meanwhile, the general consequences of a band structure can be considered and may even lead to new experiments. As will be found by those who try to apply this theory of continuous band spectra to biological problems, the first result of this application is a freedom of thought which allows an approach to problems that seemed

beyond reach before. With the continuum theory in the back of one's mind, most of the biological problems appear in a new light. One might be in danger of being carried away by fantasy but there is no reason, even at the price of this danger, not to review biological problems in the light of this theory. I shall try to limit myself on this subject to a few problems which have occupied my mind in the past a great deal and with little result.

The problem of the oxidation of succinate has already been discussed. The electrons, released from the H of succinate by the succinodehydrogenase, must pass the cytochromes of Keilin before reaching oxygen. By passing from one cytochrome to the other, the electrons gradually lose their energy, as indicated by the decreasing redox potential of the cytochrome series. The redox potentials of the single members of this system lie about 200 mv. apart, dividing the drop of energy into four more or less equal parts. The single cytochromes are fixed in space, being linked to the insoluble structure. My difficulty with these cytochromes always was that I could never understand how they could react at all. According to classical chemical concepts their Fe atoms would have to collide in order to react. It is almost impossible to place three such big atomic structures in space in such a way that their Fe atoms should touch. They would have to touch not only each other, but also the metal of the cytochrome oxidase and the active group of the succinodehydrogenase as well. But if we managed to arrange these substances in this required position then the whole system would make no sense, for the shooting of the electron through this series would represent a chain of spontaneous reactions the energy of which would be liberated as heat and lost as a source of work. The continuum theory offers a simple explanation. In this theory the Fe atoms of the single cytochromes play the same role as of the "metallic impurity" in phosphorescence, which allows excited electrons of higher energy levels to drop to lower levels. One would have to picture each Fe atom as being coördinated to a certain energy level. The electron of the H of succinate is placed by the succinodehydrogenase on the highest of these levels which electron

would have to move there until it finds a cytochrome-Fe codiörnated to this level, which allows it to drop to the next lower level. Here it would have to find its next cytochrome-Fe and so forth. It is easy to believe too that these single cytochromes represent mechanisms which allow the energy, given up by the dropping electron, to be employed usefully. The question whether these supposed energy levels are empty levels of the protein, or are levels of the cytochrome system (formed in analogy to the energy continuum found in chlorophyl) must be left open. The latter possibility seems more probable; if the energy levels of protein would not lie further apart than by 200 mv. (as is the case with cytochromes) then thermal agitation would suffice to raise electrons from lower to higher levels and proteins would have to be colored, as are the cytochromes, if they have their extra electron.

In this picture the real sense of oxidation is to place excited, energy-rich electrons on the "structure" which performs the basic biological functions. The real food of this system is the excited electron. Since the whole living unit, the cell, bacterial or virus body, forms one energy unit, it is immaterial where we feed in the H or electron, at the head or at the tail end. The highest degree of symbiosis would be that where one organism is devoid of the whole metabolic apparatus and feeds simply by touching upon another living structure, taking its excited electrons. Possibly this highest degree of parasitism is found in viruses.*

There is one sort of cell in the animal body which has no access to blood or lymph: the outer cells of multilayered epithelium. These cells could thus be expected to feed on excited electrons of deeper cells. Accordingly, this is the only animal tissue (to my knowledge) in which the cell membranes are broken through by bridges.

Naturally, one could explain this by supposing that the connections serve as channels for the cell fluid. The former assumption, however, is supported by the rather mysterious behavior of hairs (a form of multilayered epithelium) the condition of which depends on the animal's health, though they have no circulation, and cell fluids cannot be expected to diffuse at such

*In many ways certain viruses, like tobacco mosaic virus, resemble the fibrous structural proteins. If the myosin particle were stripped of its whole metabolic apparatus and if the remaining frame liberated itself from the rules of its community, retaining its reproducibility, it would behave as a virus.

distances in an anhydrous system. The appearance of the hair changes in a short time if the animal is taken ill, its shininess disappearing; this is a truer indicator of the animal's health than the thermometer. According to the experience of hairdressers, permanent waves do not hold if made during the menstrual period. The change sets in one day before bleeding starts. L. Varga has tried to measure these differences between menstrual and intermenstrual hair by physical methods measuring extensibility, strength, and torsion modulus. Hitherto these methods have been inadequate. One would be inclined to connect these observations with the latent induction period of phosphorescence which suggests that there may be in protein stable electrons in a lower degree of excitation, and one may even start speculation about the relation of health to the degree of saturation of these energy levels.

The common electron bands of the protein particle may make the peptide links inaccessible to enzymes, which might explain the resistance of native globular proteins to trypsin. The connection of the band structure with the native state may explain the all-or-none nature of denaturation. Once a band breaks down and rearrangement takes place, this denaturation must be complete, exposing at the same time the peptide link to the attack of enzymes. The higher the energy level of the bands, and the more electrons these bands contain, the more complete may be their protecting action against trypsin or bacterial enzymes.

The continuum theory allows us to consider ideas as abstruse as those of health and disease, but it may take us even one step further into mysterious domains like that of the origin of life. It is not impossible that the gelatin phosphor, a chromoproteid, represents the simplest form of life in which energy of radiation is made accessible to the protein by a dyestuff. Flavins and probably fluochromes and cytochromes are capable of acting this way. The present-day participation of these dyes in metabolic processes may be the continuation of this ancient function, with the difference that it is not radiation but metabolic energy which they transfer onto the protein.

PART IV

Recent Advances

1946–47

The foregoing pages were completed during the summer of 1946. The galleys arrived as progress continued into 1947; and while at first the author considered incorporating into the original text the advances achieved, it seemed preferable for technical and other reasons simply to add a supplement. A section on methods has also been added.

I. Molecular Weight

O. Snellman and T. Erdös are engaged in measuring the MW of crystalline myosin in the ultracentrifuge. The first results suggest that crystalline myosin is homogeneous, and its MW is of the order of 10^6, in agreement with earlier results of Weber and Stöver.

II. Actin Content and Activity of Myosin

Actin and myosin, if brought together, unite to form the highly viscous actomyosin, which if dissolved in 0.6 M KCl at pH 7, dissociates again on the addition of small amounts of ATP. This dissociation is accompanied by a corresponding drop of viscosity which, at earlier stages of our work, was termed "activity." The drop observed in an actomyosin containing one part of actin to five parts of myosin was called 100% activity. If the myosin showed no drop of viscosity on addition of ATP (activity $=0$), it was assumed that the myosin contained no actin.

B. Horváth, repeating experiments of M. Dubuisson,* fractionated myosin with ammonium sulphate. Actin and actomyosin are precipitated at a lower ammonium sulphate saturation than myosin. He thus obtained myosin which on addition of ATP showed an increase in viscosity of 5–20% instead of a drop. If actin-free myosin shows a rise in viscosity on addition of ATP, evidently the "inactive" myosin, obtained previously, might have contained actin, the drop of viscosity on addition of ATP being compensated. In this case our earlier "0 activity" could have been in fact a 20% activity which might have corresponded to an actin content of 3%. If the MW of myosin is 10_g^6 and that of actin 35,000, then 3% actin would mean one actin for every myosin particle, which might have a profound influence on certain reactions of the latter.

* *Experientia*, 2, 413, 1946.

Since these data are rather recent, no final statement can be made. The question must be reinvestigated and earlier results carefully revised.

III. The Fixation of Ions by Myosin

Salt-free myosin is anodic. If KCl is added in increasing concentration, K ions are adsorbed and compensate the negative charge. In the presence of 0.025 M KCl, the myosin is discharged and precipitates. If the KCl concentration is further increased, more K ions are adsorbed and the myosin dissolved again. It was logical to think that the myosin dissolves with a positive charge. Unfortunately the author's laboratory did not have the apparatus necessary for measurement of cataphoretic motion at high salt concentrations.

M. Dubuisson* found myosin dissolved in 0.5 M KCl to be anodic. T. Erdös took measurements of this material in Svedberg's laboratory at Upsala and found crystalline myosin dissolved in 0.5 M KCl also to be anodic with a very weak negative charge;† this necessitated a reinvestigation of the problem of ion fixation, giving special attention to the adsorption of anions.

The work was taken up by W. Sz. Hermann. The main results of one of her experiments are reproduced in Figure 46. (Ordinates are equivalents of K adsorbed by the UW of myosin.) In corroboration of Banga, the figure shows that K is adsorbed according to a curve which, on logarithmic scale, is composed of two straight parts of different gradients. The break, separating "primary" and "secondary" adsorption, corresponds to the maximum of precipitation at 0.025 M KCl. Up to the breaking point, no Cl was adsorbed. At this point, at pH 7.4, three equivalents of K^+ were found to be adsorbed

*Dubuisson, *Experientia* **2**, 258, 1946.

†The first experiments suggest three elementary charges per particle having a MW of the order of 10^6. Possibly this negative charge is of different origin than the original negative charge of the protein, due mainly to its dissociation. Myosin, dissolved in an excess of $CaCl_2$ has a positive charge at neutral reaction. This charge, acquired in strong salt solution, may be an expression of difference of the electro-negativity of the anion and the cation, as suggested by Laki [21] in the case of casein.

per UW as compared with the six K found by Banga at pH 7.5. If the KCl concentration was increased, K was bound still more intensely, as expressed by the steeper gradient. At the same time an equivalent quantity of Cl was bound. The

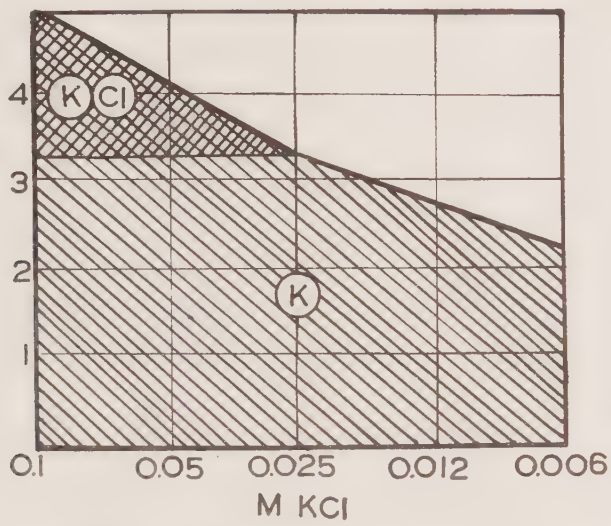

Fig. 46. Fixation of K and Cl ions by myosin in presence of varied concentrations of KCl at pH 7.4. Ordinate: equivalents of K or Cl bound by the UW (17,600 g.) of myosin. In the singly hatched zone only K is bound. In the doubly hatched zone K and Cl are bound in equivalent quantities.

single-hatched area in Figure 46 corresponds to the binding of K^+, the double-hatched area to the binding of equivalent quantities of K^+ and Cl^-.

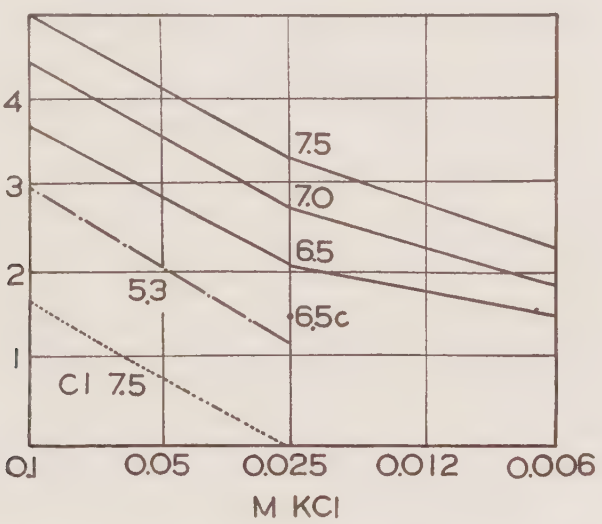

Fig. 47. Fixation of ions by myosin at varied pH. Ordinate: equivalents of ions bound by the UW of myosin. Full lines: fixation of K. Broken line: fixation of K and fixation of Cl. Curve "Cl 7.5" shows fixation of Cl at pH 7.5. The point 6.5 c indicates the quantity of K bound in 0.025 M KCl at pH 6.5 after subtraction of the quantity equivalent to the Cl bound simultaneously.

We may summarize by saying that the negatively charged myosin adsorbs only K ions from KCl. The adsorption of K ions continues until the myosin becomes roughly isoelectric. If the KCl concentration is further increased, the isoelectric myosin binds K^+ and Cl^- ions in equal numbers.

This result is in agreement with the experiment reproduced in Figure 47. The top curve here shows K fixation at pH 7.5. No Cl was found up to the breaking point. From here on, the quantity of K and Cl adsorbed was equivalent (Cl curve, see lower left corner).

The curve 5.3 shows the K fixation of myosin at its IP (pH 5.3). The quantity of adsorbed Cl was equivalent to the adsorbed K throughout, also at 0.025 M KCl.

The curves for pH 7.5 and 5.3 are parallel. The identical gradients indicate that the adsorption of ions is equally intense at either pH. The curve of pH 7.5 is higher, showing that the original negative charge of the protein not only prevents the adsorption of Cl ions, but also promotes that of K ions.

At pH 7 the situation was similar to that in 7.5. At pH 6.5 in the vicinity of the IP, a small quantity of adsorbed Cl appeared at the breaking point, its quantity corresponding to the distance between 6.5 and 6.5c.

The quantity of K^+ adsorbed at the breaking point at different pH's in excess of the adsorbed Cl, corresponds roughly to the number of dissociated COOH groups present, as calculated from the dissociation curve of myosin of Dubuisson* and Dubuisson and Hamoir.†

We may deduce herefrom that myosin is equally capable of binding positive and negative ions and that the fixation of these ions is not due to the dissociation of COOH or NH_2 groups. The dissociation of the COOH group plays a role only as far as the resulting negative charge of the protein prevents the adsorption of anions and promotes that of cations. "Primary adsorption" is the selective adsorption of the cation by the negatively charged protein. "Secondary adsorption" is the adsorption of anions and cations in equivalent quantities by

*M. Dubuisson, *Arch. Internat. Physiol.* **51**, 38, 1943.

†G. Hamoir, *ibid.* **53**, 38, 1943.

the myosin, rendered isoelectric by the adsorbed K^+ or the decrease of pH. The nature of the forces responsible for the binding of ions is one of the most exciting and basic problems of biology.

As has been emphasized, there are reasons to believe that the adsorbed ions retain their charge and the K ions neutralize only the outward charge of myosin. The K and Cl ions, adsorbed in the secondary zone, increase the number of charged points on the protein and promote herewith its hydration and dissolution.

These relations are brought out more clearly by the adsorption of Ca and Cl in the presence of $CaCl_2$ as shown in Figure 48 of W. Sz. Hermann. Up to 0.003 N Ca, no Cl was bound.

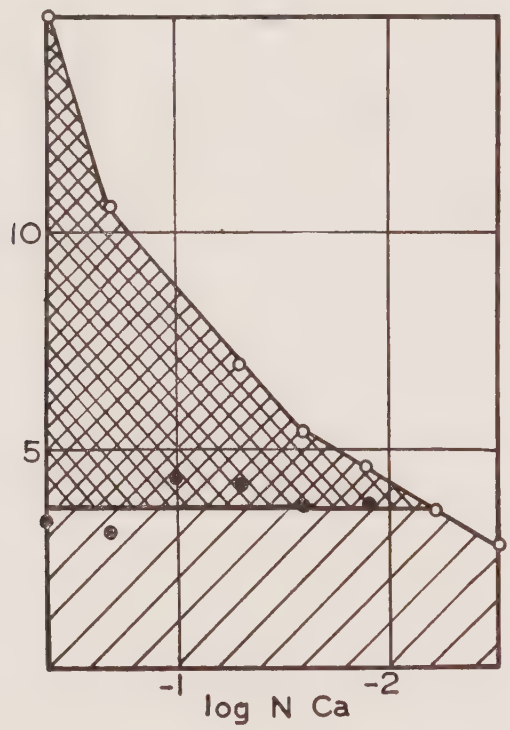

Fig. 48. Fixation of Ca and Cl by myosin in presence of varied concentrations of $CaCl_2$. Ordinate: equivalents of Ca and Cl bound by the UW of myosin. Circles: Ca adsorbed. Points: same after subtraction of a quantity equivalent to the adsorbed Cl. Singly hatched area: Ca adsorption. Doubly hatched area: adsorption of Ca and of the equivalent Cl.

At this $CaCl_2$ concentration the protein has bound four equivalents of metal, thus being approximately isoelectric. From here on, Ca and Cl were bound in equivalent quantities. The upper curve (circles) shows the fixation of Ca, the lower curve (points) shows the same curve after the subtraction of the

quantity equivalent to the adsorbed Cl. The double-hatched area corresponds thus to the adsorption of $CaCl_2$, while the single-hatched area corresponds to that of Ca^{++} without Cl^-.

Figure 49 shows the result of an experiment of Hermann in which KCl was added to myosin in increasing concentration in the presence of 0.001 M $CaCl_2$. The adsorbed Ca, K, and Cl were measured. In the presence of only 0.001 M $CaCl_2$, the myosin was made isoelectric through the selective adsorption of Ca, and was found to bind no Cl. Up to 0.025 M KCl, no K or Cl was bound. At a higher KCl concentration, however, K and Cl were adsorbed by the isoelectric protein in equivalent and increasing quantities. Above 0.1 M KCl, where the K^+ concentration became more than one hundred times greater

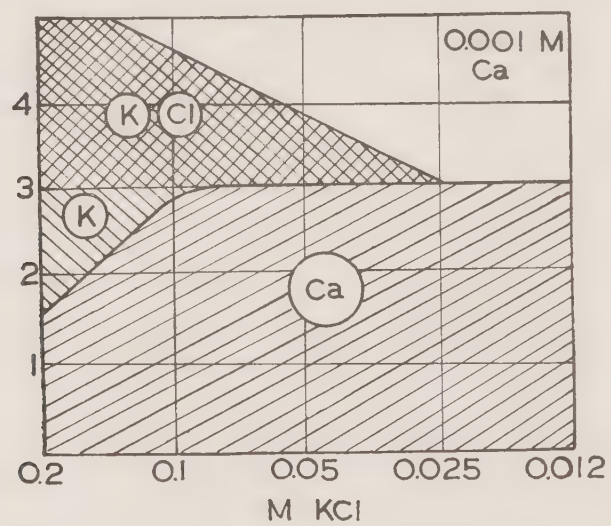

Fig. 49. Adsorption of ions by myosin in presence of 0.001 M $CaCl_2$ and varied concentrations of KCl. Ordinate: equivalents of ions bound by the UW of myosin. "Ca" area: adsorption of Ca. "K" area: adsorption of K. Doubly hatched area: adsorption of equivalent quantities of K and Cl.*

than the Ca^{++} concentration, the K^+ began to expel the Ca from its adsorption.

The question may be raised whether in all these reactions the ions act merely as electrically charged balls, distinguished only by the number of charges and ionic radius, or does their further individuality, their finer structure also come into play. Although physicists may vigorously object to this latter

*In a number of later experiments the adsorption of Ca began to decrease in the presence of 0.025 M KCl to become asymptotic with one Ca adsorbed.

possibility, the observations support it, the ions having a specific influence on the protein which can hardly be explained either by the ionic radius or the number of charges.

Mg, for instance, greatly enhances contraction of actomyosin in the presence of KCl and ATP. G. Rózsa compared the effect of Mg with that of Be, Ca, Sr, and Ba, and found it rather specific. Cu completely changes the properties of myosin: it makes the protein very elastic and unable to contract. If the Cu is washed out with cyanide, original physical properties return [Dubuisson] and the protein becomes contractile again.* Ca myosinate is not contractile, in contrast with Mg myosinate. If part of the Ca is replaced by Mg, the myosin regains contractility. Ca myosinate is very active as ATP-ase; Mg myosinate is completely inactive. In the presence of actin, both are equally active.

Actin offers still more striking examples of the specific activity of ions (e.g., K^+ and Na^+).

IV. Enzymatic Activity and Contractility of Myosin. Protins

The main achievement reported in the first part of this book was that old "myosin" could be pulled to pieces, into actin and myosin proper. Lately we succeeded in pulling myosin to pieces, in spite of the fact that it can readily be crystallized and behaves on recrystallization as a homogeneous substance. Since the foregoing parts of this book were written, my laboratory was led to regard myosin as a rather involved system in which a number of protein-like substances are attached to a skeletal substance. The skeleton as well as the adsorbed proteins are, in themselves, inactive, their system being capable only of enzymatic reactions or contraction. Even lipids seem to be an integral part of this system.

The adsorbed proteins all seem to belong to the same remarkable group of substances, characterized in impure condition by their stability in 0.1 N HCl at 100° (15 minutes). They can be treated without loss with N HCl or precipitated

*G. Rózsa, unpublished.

with trichloracetic acid. These substances will be called "protins" (prosthetic-proteins) to distinguish them from other proteins. H. M. Kalckar was the first to describe in muscle an acid- and heat-stable catalytic protein, his "myokinase."

This problem was pursued along two different lines. F. Guba studied contractility of actomyosin. He prepared protin-free actomyosin. Such actomyosin does not contract with ATP and ADP but can be reactivated with watery muscle extract. I. Banga studied the enzymatic activity of myosin, namely dephosphorylation and deamination of ATP, and dephosphorylation and deamination of ADP. Both workers agree that the protins, involved in the reaction with ADP ("ADP-protins") are much more easily removed from myosin than the protins involved in reactions with ATP ("ATP-protins"). Accordingly, they succeeded much sooner in detaching the ADP-protins from the skeletal substance and reactivating this latter by watery extracts of the muscle: protein II of Banga and the chromoproteid of Guba, described in the first part of the book, are what we now call ADP-protins.

The protins involved in the reaction with ATP are detached from the myosin skeleton, but under specific conditions. Actin, which renders removal of ADP-protins difficult, makes the removal of ATP-protins impossible; the myosin must thus be entirely free of it. The dissociation of myosin into skeletal substance and protins is promoted by acid reaction. The skeletal substance, however, is very labile and denaturates readily under the influence of H ions. Thus one is rather limited in the pH range, the more so because the dissociation of the complex is a rather slow reaction and demands protracted treatment. There is but a narrow margin between the range of denaturation and dissociation. Protins, adsorbed to the skeletal substance, greatly increase the solubility of the latter. Myosin, rich in protins, is clearly soluble in 0.5 M KCl, becoming more insoluble and cloudy as the protins are removed. The solubility of the skeletal substance can be increased again by the re-adsorption of protins, but if the protins are completely removed, changes become irreversible. So Banga was able to reactivate the ATP-ase activity of her myosin only if this was not reduced by more than 85%.

The results of the contraction test and the phosphatase test are not yet sufficiently co-ordinated. In one case the myosin of Guba, which gave no more contraction, still showed 50% ATP-ase activity. It seems probable that both protins, the one involved in contraction ("ATP-c-protin") and the one involved in dephosphorylation are identical, but contractility disappears if the protin concentration drops below a critical value, which corresponds to 50% inactivation of the phosphatase activity.

These results are very recent and do not allow more quantitative statements at this time. This is true especially for the protin involved in deamination of ATP ("ATP-N-protin"). This reaction has been discovered lately by Banga, whose results allow only the statement that this protin is different from that involved in dephosphorylation of ATP or in deamination of ADP.

Singher and Meister* reported the preparation of myosin, inactive as phosphatase. It is difficult to say whether the inactivity was due to the removal of protins since myosin can be inactivated in many ways. Their method being similar to ours, it seems likely that they removed protins, provided their myosin was free of actin.

Myosin can also be inactivated for the ADP contraction test by the removal of a lipid, which has been obtained in crystals and seems to be a cerebroside. Its addition to the myosin reactivates the latter. Myosin can also be inactivated by storage at alkaline reaction in contact with air [Guba]. Such a myosin contracts no more but can be reactivated by cysteine. Inactivation, in this case, is due probably to the oxidation of SH groups. Bailey and Perry† found SH groups essential for the actin-myosin link, while Singer and Barron‡ found SH groups involved in phosphatase activity. Naturally, it is important to distinguish between autoxidative inactivation of protins and of the skeletal substance.

The quantities of actin, which may make ATP-protin

J. Biol. Chem. **159**, 491, 1945.

†K. Bailey and S. V. Perry, *Proc. Biochem. Soc.*, 24th meeting, 1946.

† *Proc. Soc. Exp. Biol. Med.* **56**, 120, 1944, quoted after Bailey and Perry.

inseparable from the myosin skeleton, are too small (3%) to explain this effect in a rough physical way by supposing that the protin is entrapped between the myosin and actin particles. We must suppose that the actin increases the affinity of myosin to protin.*

V. ATP-ase Activity of Muscle at 0°C

A. Biro and A. E. Szent-Györgyi, measuring the ATP-ase activity of muscle and myosin at varied temperature, have found an exceedingly low temperature quotient. The increase in rate pro 10° was but 30% instead of the 250–300% usually observed in other reactions. This suggests that the enzymatic activity has a rather involved mechanism and is limited by a reaction with a low quotient. The relatively low activity of myosin as an enzyme is also suggestive of such an involved mechanism. Other systems in the realm of physiology make such a mechanism rather probable. The function of classical enzymes like trypsin or pepsin is to decompose their substrate as rapidly as possible. The decomposition of ATP would have no sense if it would not fit into a more complex pattern of reactions which together form the contraction cycle.

But whatever the reason for such a low temperature quotient may be, it opens valuable experimental possibilities for studying the ATP-ase action of muscle at 0° where there is no contraction. So the enzymatic activity can be studied without being complicated by such gross physical changes as contraction. Once we have determined what uncontracted muscle does, it will be easier to find out what is due to contraction itself. If both the physical state and the rate of the enzymatic reaction would change with temperature, the two variables would make interpretation of results rather difficult.

Biro and Szent-Györgyi studied first the ATP-ase activity of freshly minced muscle, no ATP being added. Such a muscle

*This effect may have its physiological bearing. It is not unlikely that the influence is mutual and that changes in the protin (e.g., its phosphorification) after the affinity of myosin to actin, inducing association or dissociation. The latest results of Guba suggest that protins are involved in actomyosin formation.

contains ATP in high concentration. They found that at a lower KCl concentration the muscle splits its own ATP rapidly; at a higher concentration it does not. The borderline lies somewhat below 0.16 M. The transition is quite critical: at 0.15 M, splitting is maximal; at 0.16 M KCl, there is almost none. The same was found to be true for the two instances when ATP was added from without to the suspended and washed muscle.

A solution of 0.16 M KCl is roughly isotonic with Ringer, serum, or resting muscle. Just below isotony there lies thus a critical ionic concentration at which muscle begins to split its own ATP, or ATP added from without. Resting muscle is just above this limit.

It will be remembered that the same ionic concentration, between 0.15 and 0.16 M KCl, was found to be critical also for contraction, as shown by the lower half of Figure 40. Below this concentration the ATP, present in resting muscle, induces contraction; above this concentration it does not. The ionic balance of resting muscle is such that the muscle does not contract and does not split its ATP, being just beyond the critical level below which it splits and contracts.

If ATP is added to washed or unwashed muscle at 37°, the muscle contracts up to 0.45 M KCl. The first experiments of Biro and Szent-Györgyi show that it also splits ATP. If any conclusion may be drawn from these first results, one would have to conclude that it is contraction which enables the muscle to split ATP above the critical level.

VI. Energetics

In the first part of this book, experiments of L. Varga were described which permitted calculations of the heat- and free-energy change in muscular contraction. The numeric values obtained related to the molar quantity of the contractile matter, but we did not know what this molar quantity was. The molar quantity of extracted myosin is in the range of 10^6 g. It has been shown, however, that the myosin particles, in muscle, are associated with higher structures. Moreover the

contractile substance is not myosin but actomyosin, containing in muscle 1 g. of actin to 2.5 g. of myosin. If the relation of the MW of actin and myosin is 70,000:1,000,000, this means that about six actin particles unite with one myosin particle. The situation is complicated further by the association of the actin particles; thus we are at a loss as to what to call the molar quantity.

To approach this problem, Varga repeated his muscle experiments on actomyosin threads. The curves obtained were identical with those obtained on muscle, which shows that the association of myosin particles in muscle to higher structures does not change the molar quantity, which is the same in muscle and in extracted myosin, where the myosin system is disintegrated to particles of MW of the order of 10^6.

The next question to be answered was, to what extent did actin comprise the molar quantity of contractile matter. For this reason actomyosins of different relative actin and myosin content were prepared. As shown previously by Erdös [33] an actomyosin containing 8% of actin is as contractile as an actomyosin containing 30%. The experiment showed that the variation of the actin content did not appreciably alter the curves obtained. The energy change of the system is governed thus by changes within the myosin particle, the molar quantity of which is in the range of 10^6 g.

It is generally accepted that the energy of the acid anhydride link of the pyrophosphate group of ATP is used in muscular contraction. The free-energy change of the splitting of this link is unknown; its total energy change is 11,000 cal. If the free-energy change and the change of total energy are not very different, then the splitting of one link suffices to supply the energy for the relaxation of one myosin particle which, as shown by Varga, is 7,000 cal. (37°).

Professor Lundsgaard was good enough to inform me that a muscle is capable of seventy contractions in iodoacetic acid poisoning when the regeneration of the ATP is inhibited. If the MW of myosin is 10^6, there are about seventy molecules of ATP in resting muscle for every molecule of myosin. If muscle contains 10% myosin,* then 10,000,000 g. of muscle contains a

*Banga has recently found 10% myosin in muscle on exhaustive extraction.

molar quantity of it. If the free-energy change of contraction is 7,000 cal., then in a single twitch 1 g. of muscle should be able to lift about 30 g. a distance of 1 cm., which is in fair agreement with physiological experience.

VII. The Continuum Theory

J. Boros obtained the photoelectric effect in gelatin phosphors by spectrally decomposed light. The spectral distribution of this effect has not been measured yet quantitatively, but the first observations suggest that its maximum coincides with the maximum of the light adsorption of the dye. There has been found a linear relationship between the logarithm of the concentration of the dye in the gelatin phosphor and the photoelectric effect. These results do not constitute any progress in the theory, but both results are rather reassuring that the photoelectric effect, observed on illumination, was not due to thermal changes.

J. Gergely made a few observations on the phosphorescence of myosin solutions in the presence of rhodulin-orange. Myosin was employed in a 4-mm.-thick layer. The KCl concentration was varied between 0 and 2 M and the pH from 7 to 9. At the concentration used (1–2%) all solutions showed DRF and phosphorescence. On dilution, phosphorescence always disappeared before the DRF.

If the myosin dissolved in 0.5 M KCl was diluted until it gave no more phosphorescence (0.5–1%) and then an actin solution was added which in itself showed no phosphorescence (one part of actin for five parts of myosin) the DRF and the phosphorescence appeared again. If the actomyosin was brought to dissociation by the addition of ATP or by the raising of the salt concentration above 1 M, the phosphorescence disappeared. This shows that actin and myosin, when uniting to form actomyosin, pool their valency electrons into one common system. Actomyosin is thus not simply actin plus myosin; it is a new substance.

The phosphorescence of muscle (dyed with rhodulin-orange) was not altered by contraction or heat denaturation.

The experience collected with actin, myosin, and protins cannot be taken as direct evidence for the correctness of the continuum theory. Without this theory, however, it would be difficult to explain how these proteins, when uniting to a complex, develop entirely new qualities, as enzymatic activity or contractility, and how a small actin particle, attached to a relatively huge myosin particle, should be able to change the affinity of this latter toward a protin.

VIII. Conditions in Muscle

Since it has been shown that myosin adsorbs in its secondary zone cations and anions in equivalent amounts, the question may be raised which is the anion in muscle to balance the K^+. There can be little doubt that the primary zone is occupied by bivalent ions, chiefly if not wholly by Mg. According to the curves of Hermann, at the K- and Na- ion concentration of muscle we can expect to find three alkali metal ions bound per UW in the secondary zone.

In the presence of KCl, myosin adsorbs K and Cl in its secondary zone. Muscle, however, contains but very little Cl; most of the anions are organic, chiefly PO_4 bound to organic radicles.

Muscle, under normal conditions, contains about 0.005 M ATP, the greatest part of which is bound to myosin. This is equal to one ATP bound per UW of myosin, which would be just sufficient to balance the three K ions adsorbed, ATP having three negative charges at neutral reaction. It is true that Banga and Hermann have found evidence indicating that the negative charge of the adsorbed ATP is balanced by K ions of the surrounding solution, but I am inclined to believe that these results do not reflect conditions *in vivo* and are due to specific conditions prevailing during precipitation.

Assuming thus that the anion adsorbed by myosin in the secondary zone is actually ATP, a number of observations become intelligible.

There are three critical concentrations of KCl in regard to

ATP adsorption: 0, 0.1, and 0.16 M. At concentration 0, there is practically no ATP adsorbed to myosin, even if the primary zone is occupied and the negative charge of myosin compensated by bivalent ions. If there is no free positive charge on myosin, it is unable to bind any ATP. At 0.1 M KCl, in most experiments, the adsorbed quantity of ATP reaches maximum. At this KCl concentration there are two K ions bound in the secondary zone. This suggests that myosin is capable of binding the maximum amount of ATP when it has at least two alkali metal ions adsorbed to balance two out of three negative charges of ATP (at neutral reaction ATP has three negative charges). At the third critical concentration, lying somewhat below 0.16 M KCl, the adsorbed ATP anions seem to be bound most strongly. At this concentration three K ions are adsorbed to balance completely the three negative charges of ATP. Now the ATP seems to be bound very strongly and to be unable to react with the ATP-protins adsorbed. If the KCl concentration falls below this critical level, the number of K ions adsorbed falls below three and the ATP is no more fully balanced, its adsorptive power is decreased; it interacts with the protin, is split, and induces those changes which eventually lead to contraction.

This may also explain the difference in the action of ATP present in muscle and the ATP added from without. It has been shown in the second part of this book that above 0.16 M KCl the relatively great quantities of ATP present in muscle are unable to induce contraction while minimal amounts of ATP, added from without, do so, though there is no reason to suppose that there is any difference in chemical structure. According to the above experiences, the adsorbed ATP may be unable to interact with the protin, while the free ATP is free to do so.

At last the question may be raised concerning the nature of the interaction of protin and ATP. The simplest and most probable assumption is that there is a transphosphorylation, the "myosin" being phosphorylated. This phosphorification of "myosin" could induce the rearrangement of charges which leads eventually to contraction, the whole system losing 7,000

cal. of free energy. If the contracted system would now detach its acquired phosphate, it could return to its original state, taking the 7,000 cal. necessary from the splitting of the pyrophosphate link. If the free energy of this latter is 11,000 cal., relaxation also would go hand in hand with a fall of free energy by 4,000 cal. Thus both contraction and relaxation would be spontaneous processes accompanied by a loss in free energy, and the splitting of ATP would seem logical. It is not even necessary to suppose a transphosphorification to be able to accept this picture. An adsorption of the ATP to the protin may do the same, having been shown that substances, adsorbed to proteins, may take part with their electrons in the structure of the whole; thus it is possible that a mere adsorption of the ATP to the protin may induce the rearrangement of electronic distribution and allow the energy of the pyrophosphate link to be communicated to the whole system.

Naturally, until we know more about conditions in resting muscle we must not adhere too strongly to the details of any such picture. It is still possible that actomyosin, in resting muscle, is in the dissociated condition and that phosphorification of the protin (or the adsorption of the ATP to protin) only increases the affinity of myosin to actin, causing association to actomyosin which, in its turn, might entail the intramolecular rearrangement of charges leading eventually to contraction. If this electric disturbance (or the consecutive release of water) disturbs the K adsorption in the neighboring micels, thereby causing release of ATP, the change becomes self-propagating.

If muscle is stored for a few hours at room temperature, it loses its excitability. G. Rozsa found that excitability is lost when the ATP concentration of muscle falls below ½ of its original level. It is possible that in this case the predominating K^+ adsorption prevents the release of the adsorbed ATP.

IX. The Nature of Cross-Striation

M. Gerendás and G. Matoltsy have tried to repeat the author's experiment in which insect muscle fibers were rotated

under the microscope. Under these conditions, the author has observed a shift of the cross-striation and concluded therefrom that cross-striation must be an optical phenomenon and cannot be due to a segmental differentiation of the fiber.

Gerendás and Matoltsy, working under better conditions, were able to register the results photographically. They showed that under the conditions of the author's experiments a continuous shift of cross-striation can actually be observed on rotation but that this shift is an optical phenomenon only and there is no real shift of striations. The explanation is this: since the fibers never lie perfectly axially, rotation shifts them out of focus. To facilitate observation during rotation, the author did not focus his microscope on the equator of the fiber but as far from the equator as visibility permitted, say above the equator. The fiber was rotated and moved upwards through the focus as far as possible. Gerandás and Matoltsy showed that if we focus the microscope in this manner above the equator and then move it below the equator, there is always a shift of the cross-striation by half a period. If the above manipulation is repeated, one gets the impression of a continuous motion. This, however, is but an optical phenomenon and there is no real shift of cross-striation, which can be shown if we use objective methods of registration, focus consistently in the same plane, and use as fixed point some point in our preparation outside the fiber. The complete series of pictures obtained on a full rotation, if put together, reveals that no shift has occurred.

To obtain further information about the nature of cross-striation, Gerendás and Matoltsy undertook the microscopic examination of mammalian cross-striated muscle, the myosin of which was removed by thorough extraction with Weber's salt solution containing ATP. In plain light, cross-striation was found unchanged, but the DR had greatly diminished or had disappeared completely.

They now treated the extracted muscle with strong (0.6 M) KI, which depolymerizes and dissolves the actin. The result was unexpected: the cross-striation was still present but the positive DR of the muscle turned into a negative one and the

former I-band became (negatively) double-refracting, while the former Q-bands became isotropic. The negative double refraction of the I-bands was equal (4.10^{-4}) to the positive DR of the Q-bands of untreated fibers.

These observations admit a very simple explanation: the I-bands contain a negative double-breaking substance, which compensates the positive DR of the continuous, positively double-breaking contractile actomyosin filaments. This substance cannot be identical with Bailey's tropomyosin, which has a positive DR. The negative double-breaking substance can be dissolved with urea salt solution and displays, after alkalization with NaOH, a negative DRF.

This protein has been described previously by I. Banga and myself [2]. We showed that the structure of all animal tissues examined is built of fibrous proteins which can be dissolved partly in urea salt solution and partly in alkaline urea salt solution.* It was also shown that muscle contains such a protein which can be detected after the extraction of myosin. Heart muscle was found to be especially rich in this protein.

It is hoped that these findings will open the way to the understanding of the function of the muscle fiber as a whole. At the same time they open the question again, whether cross-striation corresponds to a segmental differentiation, or whether it is the result of the periodically changing optical properties of a continuous structure. Possibly the negative double-breaking substance of the I-bands fills the space between the actomyosin filaments continuously, but its particles have a periodically changing orientation, as the particles of the fibrous colloid in van Itterson's capillaries.

*The urea salt solution had the following composition: 0.6 M KCl, 0.01 M Na_2CO_3, and 0.04 M $NaHCO_3$ and 30% urea; thus it corresponded to Weber fluid + urea. The alkaline urea salt solution had the same composition but contained 2% NaOH in addition.

PART V

Methods

I. The Preparation of ATP*

Research on muscular contraction is greatly dependent on a generous supply of ATP. The methods hitherto employed are rather expensive and their yield is low. In these methods the tissue is usually extracted with trichloracetic acid which precipitates the proteins and extracts the ATP which is then isolated as the Hg, Ca, or Ba salt.

The method to be described, worked out by J. Banga and myself and somewhat modified later by W. Sz. Hermann, is based on the observation that ATP, in muscle, is adsorbed to the structure and is precipitated with it if anhydrous solvents (alcohol) are used. The denatured protein releases the ATP on extraction with water. In this way the ATP is obtained in relatively high concentration and purity, the major part of soluble substances having been removed by the alcohol. This method allowed us to prepare about 1 kg. of ATP, used in the research described in this book. The method is cheap, the alcohol being recovered with little loss.

The procedure is the following:

The animal (rabbit or horse) is killed. Its muscles are rapidly minced and dropped from the mincer into 96% alcohol, 4–5 liters of 96% ethyl alcohol being used for every kg. of meat. After one or two hours of occasional stirring, the muscle residue is separated from the alcohol either on a press or by centrifugation. The muscle residue is suspended again in alcohol, one liter being used for every kg. of muscle. The suspension is brought to boiling, allowed to cool, and the alcohol again separated. This second alcohol extracts some more soluble matter and makes the protein quite insoluble. The muscle residue is dried in air and reduced to a powder in a mill. The dry muscle can be stored in a dry place indefinitely.

The powdered muscle is extracted with water. At the beginning, we extracted 1 kg. of powder with five liters of water

*The industrial use of the method is covered by patents both in Europe and the Americas.

for ten minutes, pressed out the water, suspended the muscle in three liters of water, pressed again after ten minutes, repeating this last procedure a second time. In this way about ten liters of extract was obtained.

Later it was found more convenient to separate the muscle on a centrifuge, a type being used which is found in laundries. By this method the volume of the extract could be reduced to six liters, three liters being used for the first and one to two liters for the second and third extractions.

To the six liters of fluid obtained on extracting one kg. of muscle-powder, four ml. of glacial acetic acid was added per liter. The precipitate formed carried down the glycogen present and was removed. Then 200–250 ml. of 20% mercuric acetate was added (dissolved in 2% acetic acid) depending on the quantity of ATP present. After a short sedimentation th , precipitate was separated on the centrifuge, the inorganic phosphates being left in solution. The loss in this precipitation amounted to 10%.

The precipitate was suspended in 250 ml. 0° 0.5 M HCl and decomposed with H_2S at 0°. It was separated on the centrifuge in the cold, washed with 50 ml. of 0.5 N cold HCl, and centrifuged again. The extracts were combined, treated once more with H_2S, and centrifuged. The H_2S was eliminated by aeration. Then 100 ml. of 10% $CaCl_2$ was added (prepared by dissolving 10% $CaCl_2$ in 96% alcohol). Four vols. of alcohol were added and the fluid kept overnight in the icebox. The precipitate was separated, washed with alcohol until free of Cl, and dried; it contained the ATP in the form of its acid Ca-salt, with one Ca per mol ATP.

About half of the ATP present in muscle (2.5 mg. per g. muscle) was obtained this way in high (about 90%) purity. Further purification could be effected by standard methods.

The ATP was liberated for experimental use in the following way: 1 g. Ca salt was dissolved in 30 ml. water, then 10% K oxalate was added until the fluid contained neither Ca nor excess oxalate. Then the fluid was neutralized with KOH, made up to 60 ml., and centrifuged. One ml. contained about 12 mg. ATP.

II. The Preparation of Myosin*

General Remarks

Myosin is very sensitive to the action of heavy metals: it strongly binds and accumulates them, undergoing denaturation. Working with myosin, metal-free water must be used, distilled from glass vessels through glass coolers. The salts used for the extraction of myosin in high concentration must be of high purity. KCl has been preferred in this work as the neutral salt, but its action is not specific.

Some properties of myosin are very labile, thus its extraction and preparation must be done at a low temperature (0°). Unfortunately, it is not possible to work throughout at this temperature because actomyosin is not precipitated by ATP at 0°. ATP has a stabilizing action, as do salts, in the absence of which myosin is more unstable.

Extraction

As described before, there are reasons to believe that in muscle both actin and myosin form a continuous system, and it is the actin system which unites with the myosin system to form actomyosin. To extract myosin, it must be separated from actin and the links connecting the myosin micels to a continuous system must be disrupted. To do this the myosin particle must be charged and hydrated. To reach the necessary degree of charge and hydration, the simultaneous action of ATP and of a high concentration of salts is needed. ATP is present in fresh muscle in a sufficiently high concentration, but if the muscle is stored the ATP is decomposed and the myosin becomes insoluble. If myosin is kept in the absence of ATP at a lower salt concentration, cohesive links develop which make dissolution increasingly difficult.

To bring myosin of muscle into solution, KCl must be present in at least 0.5 M concentration. Once the myosin was dissolved and the myosin system broken up into smaller units, it is soluble even in 0.1 M KCl.

*Crystalline myosin was prepared routinely by this method in the author's laboratory for a long period, with very few failures.

To precipitate myosin from its solution, the salt must be diluted strongly; thus any excess of salt during extraction involves additional dilution and therewith loss of material. The solvatizing action of ATP is greatly enhanced by the Mg and the protins adsorbed to myosin. The separation of dissolved actin from the dissolved myosin involves a rather heavy loss of myosin. Hence, if crystalline, actin-free myosin is needed, it should, if possible, be free of actin in the first extract.

Increased pH favors the dissolution of actin. The solubility of actin varies also in different animal species. The rabbit, as shown by Guba (70, page 56) is an especially favorable material for the preparation of pure myosin. The experiments reported in this book relate to this material.*

The animals were killed by decapitation, rapidly skinned, eviscerated, and dipped into ice water for 2–3 minutes. Then the muscles were excised and covered with minced ice. The muscles were treated 2–3 minutes later in a mincer with a sieve plate having holes of 2 mm. diameter. The mince was suspended at once in the ice-cold salt solution.

The dissolutions of myosin and actin follow different time curves. Myosin dissolves rather rapidly, while actin dissolves slowly; therefore, the extraction should be of short duration.

The alkaline KCl of Weber,† used by most previous research workers, yields a myosin rather heavily contaminated with actin. KCl alone (0.6 M) extracts myosin with varying actin content.

The purest myosin of fairly reproducible qualities could be obtained with the acid KCl-phosphate solution of Guba and Straub. This fluid contains 0.3 M KCl and 0.15 M K-phosphate of pH 6.5. Every 100 g. of the minced muscle were suspended in 300 ml. of this fluid of 0°. The mince was extracted for ten minutes under constant, gentle stirring. After this time the suspension was diluted with water of room temperature, four volumes being added for every volume of salt solution used. The suspension was then rapidly strained

*Since glycogen also precipitates myosin, it is advisable to use rabbits which have fasted for 1–2 days.

†0.6 M KCl, 0.04 M $NaHCO_3$ and 0.01 M $NaHCO_3$.

through a cloth and pressed out. The resulting turbid liquid was warmed to 20° and stirred very gently. The fluid now contained myosin, some actin, and ATP. At this ionic concentration, the ATP present keeps the actomyosin in dissociated condition. The ATP is gradually split by the myosin, and its concentration decreases within 1–2 hours to such an extent that it no longer dissociates the actomyosin but causes it to undergo a floccular precipitation. The precipitate consists mostly of actomyosin containing 1.5 parts of actin to 98.5 parts of myosin. The excess of myosin is left in solution. If the muscle extract contains more actin than 1.5 parts to 98.5 parts of myosin, no actin free myosin can be obtained by this method, the whole myosin being precipitated as actomyosin.*

During the formation of the precipitate, the fluid must be stirred gently. If the stirring is omitted, the actomyosin forms a very fine colloidal precipitate which cannot be separated on centrifugation. The result is the same if the stirring is too violent.

Crystallization

The precipitated actomyosin is separated on the centrifuge at once and rejected. The fluid is rapidly cooled to 0°. The myosin is now, in the absence of ATP, rather labile. The fluid is strongly stirred and diluted with 1.5 volumes of ice-cold distilled water. The water is run in slowly, its addition being effected in about 10 minutes. The myosin precipitates in the form of fine needles. The silky sheen of the stirred fluid indicates crystallization. The strong stirring provides coaxial orientation of particles and favors crystallization.

The crystalline suspension is allowed to stand for one to two hours at 0°, decantated, and the crystals separated by centrifugation at 0°. In spite of its crystalline nature, the precipitate is very voluminous and usually contains no more than 5% of solid matter.

Muscle contains 8–10% myosin. About ⅓ of this quantity

*The relative proportion of actin : myosin in the precipitate depends on the salt concentration. The smaller the ionic concentration, the lower the ratio in the precipitate.

is extracted by the phosphate; 50% is lost on crystallization, being left behind in the mother liquor.

Further Purification

The crystalline precipitate may be purified in various ways. It may be suspended in various volumes of 0.025 M KCl (the author usually used one liter if the crystals were obtained from 10 liters of fluid.)

The myosin may be recrystallized by dissolving it in 0.6 M KCl, powdered salt or 2 M KCl solution being added to the precipitate. The solution should contain no more than 3% myosin and should not be very viscous. The solution is diluted very gradually under strong stirring at 0° until the KCl concentration is decreased to 0.04–0.025 M.

The myosin at this stage still contains a small quantity of actin from which it cannot be liberated by recrystallization. Myosin may crystallize with as much as 3% actin, a quantity which may considerably influence the enzymatic reactions or physical properties of myosin.

To separate myosin from actin, it was dissolved by the gradual addition of 0.02 M K_2CO_3, containing 0.01% phenolphthalein. Carbonate was added until the solution retained a faint rose color (pH 8.3). Then, for every g. of myosin present, 4 ml. of 2 M KCl were added and the fluid homogenized by stirring. Then, for every ml. of KCl used, 50 ml. of water at room temperature (20°) were added under strong stirring. This water contained 0.001% phenolphthalein and a sufficient quantity of K_2CO_3 to give it a faint rose color. If the rose color disappeared, it was re-established by the addition of carbonate-phenolphthalein mixture. The voluminous precipitate contained the actomyosin present and was separated on the centrifuge at room temperature. The faint rose, opalescent fluid was poured off and cooled. The precipitate still included considerable quantities of myosin; again 2 M KCl was added, this time half as much as before, followed by the addition of the corresponding quantity of water, and centrifugation. The united fluids were cooled to 0°, then 1% acetic acid was added very gradually with strong stirring until the pH fell to 7. The

myosin separated in the form of somewhat irregular needles, which showed no "activity" (no drop of viscosity on addition of ATP to the 0.5 M KCl solution).

The myosin obtained on the first crystallization, followed by washing with 0.025 M KCl, contracts energetically if converted into actomyosin by the addition of actin. It deaminates ATP and ADP and splits off both phosphates from ATP. The same holds for recrystallized myosin and most preparations of the actin-free myosin purified by the carbonate method.

Ammonium Sulphate Method

The carbonate method previously described for liberating myosin from actin is rather unsatisfactory, however valuable its services were. The manipulations are too complicated, involve a large loss of material, and expose myosin to denaturation. The development of another method was shown by M. Dubuisson* who fractionated "myosin" by ammonium sulphate into two different fractions which he called α- and β-myosin. There were reasons to believe that Dubuisson's α-myosin was actomyosin, his β-myosin a rather actin-free myosin, actin or actomyosin being precipitated at a lower degree of $(NH_4)_2SO_4$ saturation than myosin.† B. Horvath undertook the study of α- and β-myosin and found that α-myosin had all the properties of actomyosin while β-myosin was a rather actin-free myosin. The precipitability of actomyosin depends on its actin content. The higher the actin content, the lower the saturation at which the actomyosin precipitated.

Our experience with this method is not extensive enough to allow very accurate directions. The method found most convenient was to subject ½% myosin solution to the $(NH_4)_2SO_4$ fractionation. The actomyosin being precipitated according to its actin content at 27–40% saturation, the solution is brought to 40% saturation at 0° (pH 6–6.5), the precipitate separated on the centrifuge, the fluid brought to 45–47% saturation, the myosin centrifuged out, dialyzed overnight at 0°, and precip-

*M. Dubuisson. *Experientia*, **2**, 413, 1946.

†Pure actin is precipitated at pH 5.5 at 20%, at pH 6–8 at 25–35% ammonium sulphate saturation.

itated by dilution. The myosin thus obtained is clearly soluble in 0.5 M KCl, is enzymatically very active, and contracts (with actin and ATP) most energetically. It seems to be very rich in protins.

Though our experience with this method is rather limited, certain of its advantages may be indicated. As mentioned before, there are reasons to believe that the myosin thus prepared is more actin-free than the myosin obtained with earlier methods. High salt concentrations dissociate actomyosin, and ammonium sulphate denatures actin,* which might also contribute to rendering the myosin free of actin. Another evident advantage of the $(NH_4)_2SO_4$ method is that it permits preparing actin-free myosin from material contaminated with actin rather heavily, which was not the case with our earlier method.

III. The Separation of Protins and Myosin

The Contraction Test

The myosin is dissolved in 0.5 M KCl. A fibrous and protin-free actin solution is prepared. Myosin, KCl, and $MgCl_2$ solution and water are pipetted into a small test tube in such a way that the 1.8 ml. fluid contains 2 mg. myosin, 0.05 M KCl, 0.001 M $MgCl_2$, and 2 mg. ATP or ADP. The test tube is shaken thoroughly and placed into a water bath at room temperature. Then 0.2 ml. 0.2% fibrous actin is added and the tube shaken thoroughly once. If a sufficient quantity of protin is present, contraction ensues within ½–3 minutes. Contraction can best be observed in a water bath with flat glass walls, illuminated from behind through an opaque screen. The neutrality of the myosin, actin, and ATP or ADP solution provides the neutral reaction. Reactivation of inactive myosin preparations is effected by adding watery muscle extract to the myosin solution.

Protin-free Myosin

To prepare protin-free myosin for his ADP contraction test, Guba recrystallized his myosin prepared by the acid

*Straub, unpublished.

phosphate method. The crystalline myosin precipitate formed on dilution was not separated on the centrifuge at once but was allowed to stand in both crystallizations at 0° with gentle stirring for six hours, time being given to the protin to become dissociated. The actomyosin prepared from this myosin did not contract on addition of ADP.

In preparing myosin for the ATP-contraction test, the "activity" (drop of viscosity on addition of ATP in 0.5 M KCl) was tested after the first crystallization. If the myosin showed any "activity," indicating the presence of actin, it was put through the carbonate purification (see preparation of myosin, page 138). After the second crystallization, the myosin was suspended in a large volume of 0.025 M KCl brought to pH 6 by 0.01 M acetate buffer. For every g. of myosin about 1 l. of fluid was taken. The suspension was stored at 0° with gentle stirring for 5–36 hours and tested repeatedly for contraction. The rate at which it became unable to contract depended on the quantity of actin present. The best results were obtained with myosin prepared by the ammonium sulphate method. In the case of myosin, showing a negative "activity" (rise of viscosity with ATP in 0.5 M KCl), and being thus probably completely free of actin, the protein could be deprived of its contractility within five hours.

Banga, to prepare myosin unable to dephosphorylate ADP, suspended recrystallized myosin for a few hours in a larger volume of 0.025 M KCl, then subjected it to the carbonate treatment. The myosin thus obtained dephosphorylated and deaminated ADP no more but did so after the addition of watery muscle extract provided that actin was present. In absence of actin, ADP was dephosphorylated only after a long incubation period. Possibly, in the absence of actin, myosin is unable to bind the protin.

To prepare myosin for the ATP-ase test, Banga dissolved the recrystallized myosin, which had been put through the carbonate treatment, in 0.5-1.0 M KCl, containing 0.1 M acetate buffer of pH 6, and allowed the solution to stand for 1–2 days at room temperature. Her solutions contained 1 mg. of myosin per ml. The myosin was separated by precipitating it through

dilution with eight volumes of water. Banga has also recently used myosin prepared by the ammonium sulphate method and found this myosin distinctly superior to that prepared by the earlier procedure.

The myosin thus treated showed strongly reduced phospholytic activity with ATP. To judge the result of reactivation, the phospholytic activity must be studied in detail. Not only the quantity of free phosphate must be measured but also the initial rate of dephosphorylation. If only the quantity is measured, it is easy to mistake the action of the ADP-P-protin for that of the ATP-protin.

Whereas in ADP, the P and the N are detached together, they are split off independently from ATP. Testing for dephosphorylation, a relatively large quantity of myosin and little protin (watery extract) must be taken. To test for deamination, relatively little myosin and much protin must be used.

Cerebroside

To test for the action of the cerebroside, the myosin must contain all protins but must be free of this lipid. To obtain such a preparation, F. Guba extracted muscle with 0.6 M KCl, instead of with acid phosphate. Similarly to the standard method, he diluted with four volumes of water, allowed the actomyosin to precipitate, and then diluted the liquid with equal volumes of water at 0°. The precipitated myosin was kept in suspension by gentle stirring overnight. The myosin thus obtained did not contract on addition of ADP but did so if the very finely suspended cerebroside of the muscle was added.

IV. The Preparation of Actin

Actin present in minced muscle in its fibrous form is linked to myosin. To extract it, the myosin must be eliminated, its unextracted residue denaturated, and the actin depolymerized. The myosin is extracted by strong salt solutions, its residue denaturated by acetone which, at the same time, may split H bonds, linking actin to the structure. The fibrous actin is depolymerized by OH ions in the absence of salts.

Two methods of preparation have hitherto been used in the study of actin: the original borate method of F. B. Straub, and the same method as modified by F. Guba, remodified by Straub. The two methods do not yield the same actin. Both methods yield an actin containing one Ca atom per UW of actin, but the actin prepared by Straub's original method contains two Mg atoms in addition, while the actin prepared by the modified method contains but fractions of one Mg per UW.

The modified method permits using the same muscle for actin preparation which has been used for the preparation of myosin. This method is the only one now used in the author's and Professor Straub's laboratory; hence, it will be the only one described here.

The muscle is minced and extracted with the acid K phosphate, the suspension diluted with water, filtered, and pressed out through a cloth as described in the preparation of myosin. The filtrate is used for the preparation of myosin, the residue for the preparation of actin. The residue is weighed and suspended in four volumes of 0.4% $NaHCO_3$, stirred for 20 minutes, then filtered and pressed out again. The residue is minced once more on a mincer with small holes (1–2mm.).*

Every 100 g. of the mince is suspended in 100 ml. of a solution containing 0.05 M $NaHCO_3$ and 0.05 M Na_2CO_3. The suspension is stored for ten minutes and then diluted with ten volumes of water containing 0.0005 M $CaCl_2$, stirred for ten minutes and then centrifuged.†

The muscle is suspended in three volumes of acetone, strained, and pressed out. The residue is suspended in its equal weight of acetone, allowed to stand for 20 minutes, strained, and pressed out. Now the residue is suspended once more in its equal weight of acetone containing 5 ml. 0.1 M Na_2CO_3 per liter, strained, and pressed out, spread on filter paper, and dried in air for 10–15 hours. The dried muscle may be stored for weeks unchanged in the desiccator. If the muscle contained much fat, the extracted actin may be turbid, owing

*If the myosin was extracted with KCl or the alkaline KCl of Weber then it may be washed with water instead of $NaHCO_3$.

†The $CaCl_2$ considerably increases the later yield of actin (Straub).

to the presence of lipoids. This turbidity may be prevented by extracting the muscle with Na_2CO_3-acetone a fourth time.

In the author's laboratory, this method is used in a slightly modified form (Guba). The muscle, extracted with $NaHCO_3$ and minced a second time, is not suspended in Na_2CO_3-. $NaHCO_3$ but placed in the refrigerator at −12°C overnight. (The muscle may be kept here for any length of time.) When thawed, it is treated with acetone as described above.

The dried muscle is ground in a coffee mill and is then extracted with CO_2-free water, 20 ml. being used for every g. of muscle. The powder is mixed with the water and allowed to stand for 10–15 minutes,* poured on a Buchner funnel, and the solution sucked off. The extract is limpid and contains the actin in globular form in high purity, usually 10 mg. per ml.†

Further Purification

Nine volumes of acetone, added to the globular actin solution, does not precipitate the protein. On addition of 0.0025 ml. of M pH 4.6 acetate-buffer per ml, actin precipitates in globular form (Straub). The actin may be precipitated from its watery solution at 0° at its IP by acetate. A very dilute buffer must be used, since actin at its IP is very sensitive to ions (Straub). The precipitate is formed instantaneously if the actin solution was not too dilute and is separated rapidly with the centrifuge at 0° and redissolved at once by addition of $NaHCO_3$. It contains the actin partly polymerized. Actin may also be precipitated by small concentrations of $CaCl_2$ and redissolved in oxalate.

The primary solution of actin usually contains traces of protins. Protin-free actin can be obtained if the muscle, before acetone treatment, is repeatedly washed with stirring, using great volumes of water.

*Mixing increases the yield but may make the solution cloudy and prone to spontaneous polymerization.

†The extract should not color phenolphthalein. If it does, mincing was not sufficiently fine and the removal of alkali was incomplete. Alkali destroys actin in solution.

V. Actomyosin Threads

H. H. Weber has discovered that "myosin," dissolved in strong salt solution and squirted in a thin jet into water, forms threads. Myosin forms no threads, and what Weber actually used was actomyosin, or myosin denaturated by copper.

If one simply wants to observe the contractility of actomyosin, freshly minced muscle is extracted with the alkaline KCl of Weber (0.6 M KCl, 0.04 M $NaHCO_3$, and 0.01 M Na_2CO_3) for 24 hours at 0°, three volumes of fluid being used for every g. of muscle. The actomyosin thus extracted is a highly viscous fluid. Usually, one must dilute it with 0.6 M KCl to permit separation of the undissolved muscle particles on the centrifuge. After centrifugation, the sticky fluid is sucked into a glass tube, one end of which is mounted with a rubber tube, while the other end is drawn out to a gradually narrowed capillary. By breaking off more and more of the point, one finds the proper diameter. A basin is filled with 0.05 M KCl and the capillary end of the tube is dipped into the fluid. The tube is moved right and left as we blow into the rubber tube. With a little practice, fairly uniform threads can be pulled. Threads of about 0.2 mm. diameter are the most convenient. Thinner ones easily curl up, thicker ones are rather sluggish. For the demonstration of contraction, small pieces of 2–3 mm. length are cut out and observed on a hollow ground microscopic slide under the microscope mounted with a low-power lens and an ocular micrometer scale.

A small home-made celluloid spatula of 2–3 mm. diameter can be used with advantage. If this is put under the thread and lifted to some extent, a 2–3 mm. wide piece of the thread may be cut out by striking with a sharp instrument over the edge of the spatula. The thread does not stick to celluloid and, lying upon it, may be transferred into the fluid in which the contraction is to be observed. Contraction is very active in a 0.5–0.1 M KCl, containing 0.0001 M $MgCl_2$. Contraction occurs on addition of 0.1% of ATP. The ATP should reach the thread simultaneously from all sides; otherwise the thread curls up. The author used to squirt the ATP into the suspension fluid under strong pressure from a small pipette with

a capillary end. Blowing on the surface of the fluid from this pipette, after the ATP has been squirted in, might serve for mixing. Solutions of 2% actomyosin are the most suited for preparing threads. Threads prepared from thinner solution may be too fragile, those prepared from more concentrated solution too sluggish.*

For quantitative studies, more uniform threads should be used, prepared mechanically. Apparatus for mechanical preparation of threads has been described by M. Gerendás [13] and M. and A. Dubuisson.†

Arch. Internat. Physiol.* **53, 29, 1943.

†Difficulty may be experienced with very dilute actomyosin solutions or actomyosin of very low actin content. Neutralization of the alkaline solution or the use of 25% glycerol instead of water may facilitate the preparation of threads.

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