A CONSIDERATION OF EXPERIMENTAL FACTS PERTAINING TO THE PRIMARY REACTION IN MUSCULAR ACTIVITY

by

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One of the most significant results of the investigations of MEYERHOF and his associates was the demonstration that, of all known metabolic processes the splitting of adenosine triphosphate (ATP) is most directly connected with the fundamental mechanical event in contracting muscle (LOHMANN[17]; MEYERHOF[20]; see[27], Chapter II). Notwithstanding its importance this result is subject to two limitations. For one thing, the nature of the breakdown of ATP is still not yet satisfactorily settled; the assumption now popular that it is due to a straightforward hydrolysis by the enzyme myosin-ATPase leads, at the present state of knowledge, to difficulties. On the other hand, the introductory statement as well as LOHMANN's original conclusion contained the restriction "of all *known* metabolic processes". It is possible that ATP, before becoming decomposed, engages in other more intimate reactions with the contractile structure, as will be emphasized in this paper. These restrictions do not diminish, they rather enhance the emphasis on ATP, and it is exactly here that the most direct link between the study of muscular metabolism and the modern analysis of its function exists.

An essential contribution to this latter category has been made by SZENT-GYÖRGYI[35] by his discovery of the contractility of actomyosin, his biochemical analysis of the components of this complex substance, and by the study of various aspects of its behaviour. This work has repeatedly been summarized in greater or lesser detail (*l.c.*[36, 37, 38; 27]). There are,however, a few points which may be discussed as a suitable introduction to the problem of this essay.

If ATP is indeed the ultimate action substance of muscle, as SZENT-GYÖRGYI in logical continuation of MEYERHOF's work assumes, it is to be expected that addition of this compound to a muscle will evoke contractions. This has been achieved. Contractions were obtained by BUCHTHAL *et al.*[2, 5, 6] by close arterial injection of ATP, and by its application to isolated muscle fibers. The latter effect was also studied in a quantitative manner by ROZSA[32], using a different method. Since BUCHTHAL finds the effect to persist after curarization, it may appear difficult to assume an indirect stimulation. Nevertheless, the possibility that ATP in such experiments activates the excitatory process of the muscle, rather than the contractile structure directly, has to be kept in mind. ROZSA's results indeed suggest this to be the case. Since the excitatory process in its turn activates or liberates the ATP present, this BUCHTHAL-ROZSA effect may play an essential rôle in the conduction of the contraction wave.

A simpler and more convincing system is what the writer proposes to call the fibril preparation, which has been introduced by SZENT-GYÖRGYI[35], I, page 24. Its great

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significance has been underlined by MEYERHOF[21]. If a muscle with parallel fibre arrangement is kept in distilled water for a prolonged time, and is frozen and thawed, one obtains a preparation which consists essentially of the original undisturbed fibrils, and from which the soluble constituents of the sarcoplasma, including all factors which have to do with irritability, have been removed. No stimulation will cause contraction of these fibrils. They shorten, however, promptly if ATP in a proper electrolytic medium is added. In this case there appears to be little doubt that ATP has directly acted upon the contractile structure itself.

The analysis has gone further. One can extract and fractionate the muscle, and obtain a crystalline protein, myosin (SZENT-GYÖRGYI, *l.c.*) and supposedly pure actin (STRAUB[33, 34]). Combined with each other, they form the complex actomyosin which can also be extracted directly (SZENT-GYÖRGYI, *l.c.*) and from which threads may be spun. These threads, suspended in the same solution of KCl and $MgCl_2$ as is used with the fibril preparation, will contract in response to the addition of ATP (SZENT-GYÖRGYI *l.c.*). It is true that these threads, unlike fibrils, become shorter and thinner instead of thicker. This is however merely a consequence of the fact that the actomyosin particles in such a thread are very imperfectly orientated. After initial difficulties (GERENDAS[13]), BUCHTHAL *et al.*[3] have succeeded in preparing well orientated threads, and these behave in accordance with the rule by becoming thicker during contraction. Two objections have been made. BUCHTHAL *et al.*, at the *International Congress of Physiology in Oxford* (1947)[3] (repeated by PERRY *et al.*[29]) raised the difficulty that such threads, when loaded, do not contract but become stretched upon addition of ATP. This may be due to the circumstance that in the formation of the threads very few and weak points of intermicellar attachment are formed, which are not able to carry any strain. Since the action of ATP upon actomyosin includes a disaggregative effect as well, the plasticity of the threads is actually increased by ATP. In the fibrils on the other hand, very strong intermicellar bonds exist in the densely packed system. The second objection, made by ASTBURY at the *Experimental Cytology Congress in Stockholm* (1947) (PERRY *et al.*[29]), was that upon electron-microscopical investigation actomyosin, after treatment with ATP at 0.05 M KCl, 0.005 M $MgCl_2$, showed a dispersion of the original aggregates, with no indication of a true contraction. Since however after the addition of ATP, during the drying of the preparation, the salt concentration had to increase and pass the limit above which the actomyosin dissolves and disaggregates, this experiment has no bearing upon the mechanism of contraction. Finally, the same authors[29], (p. 677) object that, even if the shortening of actomyosin threads may imitate the *contraction* of muscle, these threads show no relaxation. According to all we know about muscle, however, *relaxation* would seem to be the more complicated phenomenon. That this has not yet been reproduced *in vitro* is no objection against a contribution relevant to *contraction*. The objection is invalid the more so, since the contraction process in threads takes place to an extreme extent. Such extreme shortenings are irreversible even *in vivo* (RAMSEY's deltastate[31]). It seems thus that SZENT-GYÖRGYI's observations on the effect of ATP upon actomyosin are not subject to any serious inconsistency at this moment.

A further simplification may be achieved by working not with carefully prepared actomyosin threads, but with a suspension of finely precipitated actomyosin flocks. Addition of ATP will cause their contraction as well. Since they are perfectly disoriented, their contraction will take place in all dimensions equally. It is manifested by an increased tendency of the flocks to settle (SZENT-GYÖRGYI's "superprecipitation"), and

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its extent can be quantitatively established by determining the volume of the gel pellet after centrifugation (MOMMAERTS[22]). One can thus study contraction at various levels of subcellular and supermolecular organization.

A still simpler system is a solution of actomyosin in 0.5 M KCl. As SZENT-GYÖRGYI has discribed[35, 37], the high viscosity of such a solution is greatly decreased by ATP. The analysis of this effect has shown that it is not due to a contraction of dissolved actomyosin micells[22, 23, 24, 25]. The true reason, as is well established now, is a disaggregation of the actomyosin into its components, myosin and actin. Although the immediate connection between this disaggregation and the contraction at lower ionic strengths is not clear, it may be presumed that the first effect of ATP is identical in both cases. One of the aspects of this first effect apparently is an elimination of certain intermolecular bonds. In the case of dissolved actomyosin, which is on the verge of disaggregation, the complex falls apart. At low salt concentration, where more or other bonds may exist, this dissociation cannot reveal itself, but the contraction can. It appears unlikely that in solutions of actomyosin contraction takes place side by side with the disaggregation. For it is an empirical fact (SZENT-GYÖRGYI, *l.c.*; ERDÖS[11]) that without actin myosin cannot contract; in 0.5 M KCl solution, ATP separates the actin and myosin so that no contractile complex then exists. Although the relation between the two effects is not understood, the study of the disaggregation in solution is highly useful, for it enables a great variety of experiments to be performed which would not be possible in strongly heterogeneous systems. As a result of the study of this viscosity effect, mainly three conclusions seem possible:

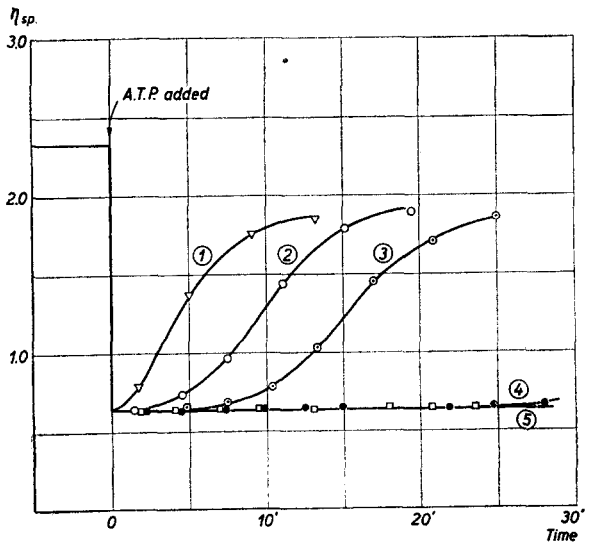

Fig. 1. Effect of ATP upon the viscosity of an actomyosin solution. At zero time, ATP is added. In all experiments, 2.5 mg actomyosin were present per ml, dissolved in 0.5 molar KCl at neutral reaction. Curve 1 (△) refers to an experiment in the presence of 0.001 molar $CaCl_2$, curve 5 (□) to an experiment with 0.001 molar $MgCl_2$. The amount of ATP added was $25 \cdot 10^{-8}$ moles in the experiments 1, 2 and 5; $50 \cdot 10^{-8}$ in 3; $200 \cdot 10^{-8}$ in 4 (see text).

First, the effect is fast. It apparently takes a fraction of a second to reach completion. Methods for the exact study of its time course have not yet been available.

The second conclusion needs more elaborate explanation[25]. Fig. 1 shows a few examples of the viscosimetric measurement of the effect of ATP upon an actomyosin solution. It will be seen that, after the initial *viscosity response* a *recovery effect* sets in, which takes more time the more ATP had been added. It is inhibited by Mg^+ and activated by Ca^+-ions, and is to be identified with the removal of the ATP by the ATPase associated (POLIS AND MEYERHOF[30]) with the myosin. The viscosity response itself is not inhibited by Mg^+ and activated by Ca^+ (rather the opposite) and can also take place if no hydrolysis occurs. Hence the second conclusion: the effect of ATP upon the aggregation of actomyosin is not caused by any known breakdown

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of the ATP. In an attempt to specify the nature of this primary reaction between ATP and actomyosin, the quantitative relation between the amount of ATP added and the magnitude of the physical effect has been studied[22, 25]. Because of particular experimental difficulties, the results have not yet been satisfactory, but an example as that of Fig. 2 shows that one has to assume the formation of a sparingly dissociated compound between ATP and (acto)-myosin. Further quantitative researches are in progress. The relationships depend on whether Mg^+ or Ca^+ are present and the best result in the presence of the promoting Mg^+ showed that 1 mole of ATP causes the maximal change in as much as 300000 grams of myosin. The dotted line (Fig. 2) represents what would be expected if the ATP-actomyosin complex would be completely undissociated; the deviation of this from the actual curve is possibly still less than is indicated by the results, which are obtained by difficult measurements in a rapidly changing system. The third conclusion reads therefore: the effect of ATP upon a measured physical property of actomyosin is due to the formation of a sparingly dissociated ATP-(acto-) myosin complex. One is led to a similar conclusion by studies of the same combination in heterogeneous, contractile actomyosinsystems, but I had insufficient opportunity to study this in full detail. In solution, the measured effect was maximal when 1 mole of ATP was present for 300000 gram myosin. It is possible that upon addition of more ATP, more is bound and stronger physical changes are induced. This cannot be measured in solution, but may possibly be found in further studies with different methods. Not more can thus be stated than that 300000 gram myosin combine with at least 1 mole of ATP, or roughly that 100 mg myosin, present in one gram of muscle, combine with $3 \cdot 10^{-7}$ mole ATP or more.

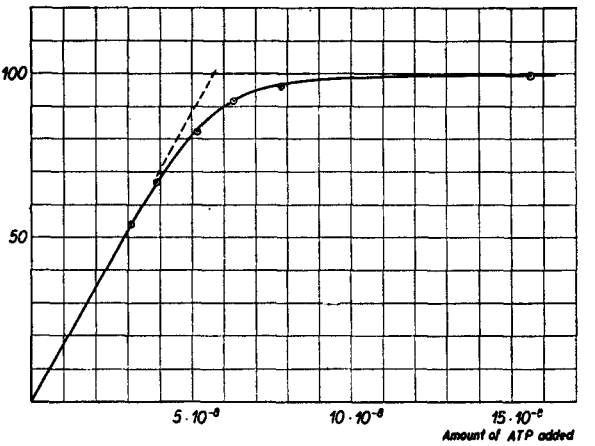

Fig. 2. Dissociation curve of the ATP-myosin complex. The effect of varying quantities of ATP upon the magnitude of the viscosity drop (at 0°; extrapolated to zero-time) of actomyosin was studied. System: 20 mg actomyosin in 10 ml 0.5 molar KCl, 0.02 molar $MgCl_2$. Abscissa: amounts of ATP added to this system. Ordinate: viscosity drop, expressed as percentage of the effect obtained with a large excess of ATP. The dotted line, tentatively drawn as representing complete absence of dissociation, indicates that the maximal effect is reached when $5.7 \cdot 10^{-8}$ moles ATP combine with 20 mg actomyosin, corresponding to 1 mol ATP per 300000 g myosin. The difference between the dotted and the experimental line indicates that at half-equilibrium the concentration of free ATP is much less than the total ATP concentration of $3 \cdot 10^{-6}$ moles per liter (see text)

Naturally, the mere demonstration that ATP, when interacting with actomyosin, actually combines with it (most probably with the myosin component only), is yet no explanation of the mechanism of its action. In this connection, the question arises whether ATP is only bound to myosin, or whether any further reaction takes place between them. More precisely the question may be asked whether myosin is phosphorylated by ATP. The author spent a summer trying to demonstrate such a phosphorylation. Actomyosin and ATP were allowed to react in a proper medium, and were

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then separated by centrifugation. Considerable quantities of P were found in the precipitate. Eventually it was found out, however, that the apparent phosphorylation was proportional to the amount of calcium in the system, and what appeared to be a phosphorylation turned out to be nothing else than a coprecipitation of actomyosin, Ca^+ and inorganic phosphate, the latter being formed by enzymatic splitting of the ATP*. It is true that without Ca^+ very small amounts of P were found in the sediment, but those were neglected at that time.

Meanwhile, however, BUCHTHAL, DEUTSCH *et al.*[4] conducted their study of just this small effect. They find amounts of about or above 15 μ gram P per 100 mg myosin (Professor BUCHTHAL kindly provided me with additional data not given in the preliminary paper), which would correspond to $5 \cdot 10^{-7}$ mole or more of P transferred to 100 mg myosin (1 gram muscle). This is the same order of magnitude as that of the combination between ATP and myosin. Indeed, BUCHTHAL, DEUTSCH *et al.* also measured an uptake of nucleotide. It thus seems likely that the primary reaction between ATP and myosin does not remain restricted to a mere combination, but is followed by more intricate interactions as well.

In spite of the insufficient information available, some further quantitative aspects of the (acto-)myosin-ATP dissociation curve just referred to may be discussed. We indicate the molar concentrations of the myosin (taking the relative weight of the unit combining with one ATP), the complex, and the ATP with c_M, c_{MA} and c_A. From viscosity measurements, as described above, it would be possible to determine the value of K, most easily by measuring the c_A at which half the maximal viscosity response is obtained (for $c_{MA} = c_M$, $K = c_A^{-1}$). This problem is now under investigation, but previously no values for c_A have been obtained due to experimental difficulties. Naturally, only the concentration of *free* ATP is relevant here; ENGELHARDT[10] (page 189), who attempted to calculate an equilibrium constant from my earlier measurements[22] erroneously took the total ATP amount present in the system. If the total ATP concentration is below 10^{-5}, (see Fig. 2) c_A is very much smaller, perhaps around 10^{-8}. Thus, K will be of the order of 10^7 or more, and the value of RT ln K will be in the range of 10000 calories, a very considerable free energy effect.

There is an independent way of estimating the quantitative relationships between ATP and myosin in a single elementary contractile event. As is well known (comp. LUNDSGAARD, *l.c.*), in iodoacetate poisoning, where the muscle uses up its stores of $\sim$P, some seventy contractions are possible. Such a muscle, before beginning its activity, contains some $2.5 \cdot 10^{-5}$ moles of $\sim$ P per gram, counting only the terminal P of the ATP. One can look upon every twitch as one elementary event involving a fraction of this $\sim$ P in the form of ATP, which first combines with myosin, and is thereupon decomposed. For simplicity of argument, it will be assumed that the poisoned muscle performs some 50 full, rather than 70 decreasing twitches. Since $2.5 \cdot 10^{-5}$ moles $\sim$ P enable to 50 full twitches, one elementary event involves the reaction of $5 \cdot 10^{-7}$ moles of $\sim$ P with the contractile structure, followed by direct or indirect degradation into inorganic phosphate. Since this same amount of muscle contains nearly 100 mg myosin, it is found that in every complete elementary process 1 mole of $\sim$ P reacts with 200000 gram myosin. This value is so close to the proportion of 1 ATP to 1–3 hundred thousands myosin which I regularly found *in vitro* that it would be hard to consider it as a mere coincidence.

* The critical attitude of DR. GERHARD SCHMIDT is gratefully acknowledged.

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It is still difficult to judge the exact physiological meaning of the described reaction, but it is of obvious interest to see whether a theory ascribing to it the significance of the primary event in muscular activity would meet the standards set by HILL's thermal measurements. As is well known, a single anaerobic twitch, in which the primary event would take place only once, is accompanied by an appearance of about $3 \cdot 10^{-3}$ calories per gram muscle[14]. In the given picture, this primary event would involve the combination of $3 \cdot 10^{-7}$ or more moles of ATP with the structure protein. Thus the heat effect of this combination per mole ATP would have to be 10000 calories or less. This has not yet been measured, but the requirement seems to be quite in line with what could be expected. It seems a permissible hypothesis therefore to identify the primary event of contraction with a combination and further reaction between ATP and (acto-) myosin.

We shall now turn to a discussion of the chemical basis of relaxation, and will have to correlate this event, by exclusion, with the enzymatic breakdown of ATP or its myosincomplex. In connection with the close association between ATPase and myosin, the current assumption is that it is the myosin-ATPase itself which hydrolyses the ATP, and thus makes the energy of this reaction available to the contractile structure. After an extensive study of the activity of myosin-ATPase it has been estimated[26] that in muscle the overall speed of hydrolysis by this enzyme can amount to only about $3 \cdot 10^{-3}$ mg P per mg myosin per minute. The actual speed of ATP breakdown in active mammalian muscle is much higher. From LUNDSGAARD's[18] results with frog muscles the writer estimated the speed of this process to be around $2 \cdot 10^{-1}$ mg P per mg myosin per minute, and a reinvestigation of all relevant data ([27]Chapter III) gave rise to the same or even higher values. Likewise, BRAVERMAN AND MORGULIS[1] essentially confirmed these results and reported the same disproportion. To reformulate the difficulty: the actual speed of breakdown of ATP in active muscle proceeds a hundred times faster than the myosin-ATPase under the given circumstances can account for. Several explanations of this discrepancy seem possible. Either, intact muscle contains unknown potent activators of the myosin-ATPase. Or, the true reaction is not at all a hydrolysis of ATP, but a phosphorus transfer to some acceptor; in fact there are indications (LUNDSGAARD[18]; CORI AND CORI[7]) that a P-transfer of ATP to fructose-6-phosphate under formation of hexose-diphosphate is a significant reaction. Further, it is not yet possible to judge which rôle the new ATPase described by KIELLEY AND MEYERHOF[16] has. Several possibilities for a solution of the dilemma thus seem to exist, and the identification of the exact course of ATP breakdown may throw a significant light upon the question of relaxation. At this moment however, no suggestions seem to be indicated.

The above considerations have been developed on the basis of *in vitro* experiments only, and the task remains of identifying the sequence of events in the contraction cycle of a living muscle. In this field, we owe most direct and illuminating experiments to DUBUISSON[8, 9], who studied the rapid p_H changes which accompany a contraction. It was found that first an acidification occurs which in favourable specimens was preceded by a small reaction change in the opposite direction. Then there is an alkalinization, followed in turn again by an acidification. The last two changes could be identified convincingly: they are due to the dephosphorylation of phosphocreatine, and to the formation of lactic acid. The latter process takes place only after the mechanical events, the former is coincident with the relaxation. The initial acidification is correlated with the initiation of the contraction process, and is therefore of great interest. DUBUISSON assumes it to be due to hydrolysis of ATP, but this conclusion is tentative; acidification

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might likewise be caused by the binding of ATP by myosin followed by phosphorylation of the latter. On this point therefore, no decision seems possible as yet.

With respect to the moment at which the energy of metabolism is made available to the contractile apparatus, it is now customary (see[27]) to distinguish two possible mechanisms. In the first of these, chemical energy may be transferred at the very moment of contraction, when it is necessary. The alternative possibility is that the primary event merely releases, by a trigger action, a spontaneous contractile process (often paralleled with the shortening of stretched rubber), and that it is the event of relaxation which is linked with exergonic metabolic reactions in order to restore the active state. The latter category, the so called postenergization mechanisms, seems difficult to reconcile with the results of FENN AND HILL[12, 15] indicating rather the existence of contraction-coupling. Nevertheless, postenergization hypotheses are rather in demand at present, and the opinion seems to prevail that SZENT-GYÖRGYI's work may lead to this type of inter probation, a viewpoint taken, *e.g.*, in the speculations of MORALES[28]. As the present communication shows, the analysis of the effects discovered by SZENT-GYÖRGYI gives, on the contrary, rise to a preenergizationtheory.

It has been the purpose of this discussion to show where the actual experimental analysis of the contractile event, in terms of ATP-actomyosin interaction, at present stands. No detailed theory seems warranted, or, as MEYERHOF said in 1930[19] (p. 280): "Es soll daher hier weniger eine bestimmte Theorie ausgearbeitet werden als die festgestellten Tatsachen und die sich daraus ergebenden mehr oder weniger wahrscheinlichen Folgerungen zusammengefasst sowie Missverständnisse gegenüber der Auslegung dieses Tatbestandes beseitigt werden". But neither should the impression prevail that ". . . (man) auch heute eigentlich noch gar nichts weiss".

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