

SOME ASPECTS OF THE INTERACTION BETWEEN
THE PROTEINS OF THE MYOFIBRIL AND
THE ADENINE NUCLEOTIDES.

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SOME ASPECTS OF THE INTERACTION BETWEEN THE PROTEINS OF THE
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An investigation of the effect of 2:4-dinitrophenol (DNP) on the ATPase activity of myosin and actomyosin supported an earlier postulate that the protein was phosphorylated during interaction with adenosine triphosphate (ATP). The effect of DNP might be attributed to a breakdown of such an intermediate, or the inhibition of its formation, in a manner similar to that whereby DNP inhibits oxidative phosphorylation in mitochondria. Experiments were designed to detect any phosphate or adenine uptake during enzyme-substrate interaction in the bulk solution phase. No uptake was detected at 0°C, 19°C or 37°C, or when the reaction time was long (5 min.), short (15 sec.), or limited to 2 sec. followed by denaturation, in an attempt to "trap" any possible intermediate.

From the work of Hayashi with surface film techniques, it is known that actomyosin, when incompletely spread, retains its enzymic activity, at least partially, and therefore remains to some extent in the native state. A study of the physical changes of the film during enzyme-substrate interaction might be indicative of the chemical changes taking place at the interphase.

It has been shown by Cheesman and Sten-Knudsen that the mechanical rigidities of incompletely spread films of myosin

and actomyosin on M KCl are reduced when the substrate contains 0.0001 M ATP. This effect was confirmed, but found not to be given by ITP or ADP. It decreases as the ATPase activity of the protein is reduced by heating at 37°C. DNP inhibits the effect when present in the substrate. Since DNP promotes the ATPase activity, thus not inhibiting the formation of the enzyme-ATP complex, it seems probable that the ATP effect on films is due to the phosphorylation of myosin or actomyosin, unstable in the presence of DNP.

The antimonial drug fuadin has been found by Ulbrecht and Ulbrecht to accelerate the exchange of phosphate groups between ATP and ADP by actomyosin. Fuadin reinforces the effect on films of "natural" actomyosin but not on those of L-myosin. This is consistent with the findings of Ulbrecht and Ulbrecht who show that actin is essential to the exchange reaction.

The indirect evidence of these authors, together with the results of the present investigation, are strongly suggestive of the phosphorylation theory of muscular contraction, and also offer an explanation of the increased activity of ATPase in the presence of DNP.

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List of Abbreviations

The following abbreviations will be used throughout the text:

ATP	=	Adenosine-5'-triphosphate
ADP	=	Adenosine-5'-diphosphate
AMP	=	Adenosine-5'-monophosphate
ITP	=	Inosine-5'-triphosphate
DNP	=	2:4-Dinitrophenol
EDTA	=	Ethylenediaminetetraacetic acid
PCMB	=	p-Chloromercuribenzoic acid
Fuadin	=	Antimony bis-(catechol disulphonic acid), Na salt
Tris	=	Tris(hydroxymethyl)-aminomethane

Chapter I

INTRODUCTION

The basic phenomenon of all life is that function whereby the cells of organisms move from one state to another. Indeed Philosophers tell us, "Vita est in motu". Therefore, to know more of this function of motion would lead one to a greater understanding of that agent responsible for this fundamental process - the living cell, its structure, its properties, its mechanisms and changes in organization concurrent with the state of motion.

The cells associated with motion in the higher animals make up the muscular tissues. The striated muscle which is responsible for all voluntary motion consists of fibres, each composed of a column of fibrils 5 μ in diameter. These myofibrils, the structural units of striated muscle, are in turn composed of filaments 50 $m\mu$ in diameter. It is the proteins within these contractile elements of voluntary muscle which are the chief concern. In contraction, and its concomitant phenomenon relaxation, a series of chemical reactions ensue which last from the moment of excitation to the end of the recovery phase. Thus, a thorough knowledge of the interactions of those compounds which produce structural change in the native protein are essential for the full comprehension of the complete picture of the course of events during muscular activity.

HISTORY

Studies on the protein components of skeletal muscle began with the observations of Kühne in 1859, followed by those of von Fürth and Halliburton about 1890. These workers found that one half of the total protein content of minced muscle went into solution when extracted with water. This substance they called "myogen". The water insoluble material was called "myosinogen". This substance was soluble in neutral salt solutions and was coagulated upon raising the temperature above 45°C. The precipitated "myosinogen" they called "myosin". Little progress was made in connection with this protein until the work of Weber and Edsall in 1930, which showed that the water insoluble protein extracted with salt solutions was identical with von Fürth's myosinogen. The protein became known as myosin and became the object of Weber's, and his collaborators, very extensive investigations.

Soon after the discovery of creatine phosphate in muscle (Fiske & Subbarow, 1927) the importance of its role was demonstrated by Lundsgaard in 1930. He showed that, when a muscle was poisoned with iodoacetate, it continued to contract without the production of lactic acid, as it became not markedly acidic but slightly alkaline. In the course of the contraction, creatine phosphate was broken down, and this process continued until the supply was exhausted, at which point the muscle was said to be in a state of rigor. Lohmann in 1934 demonstrated

that the function of creatine phosphate was to act as a reservoir of phosphate groups for resynthesis of ATP from ADP. No creatine phosphate was split until ATP was split first. It soon became evident that the splitting of ATP might be the primary step in the development of muscular contraction.

Lohmann further observed that muscle brei shows high phosphatase activity with ATP as substrate, and that the two phosphate groups of ATP could be split off by the brei. He found that the water-insoluble fraction of muscle was capable of splitting off the first phosphate of the ATP, while the splitting of the second phosphate could be activated by Mg^{++} . This diphosphatase action could be eliminated by repeated reprecipitation, and it was concluded that the enzyme was water-soluble.

Thus the path was well paved when, in 1939, a major contribution to Biochemistry was made by Engelhardt and Ljubimova (1939) with their discovery that ATPase, the enzyme which liberates inorganic phosphate from ATP, could not be separated from myosin, and was in fact to be identified with it. Myosin was capable of breaking down the triphosphate to diphosphate, whereas the diphosphatase activity was associated with the water-soluble proteins. This finding was rapidly confirmed by Needham (Needham et al., 1941) and Bailey (Bailey, 1942), and although it has been questioned by many, no satisfactory evidence has been presented to demonstrate clearly a separation of the ATPase from myosin.

J. Needham et al. (1941) showed that when ATP was added to myosin solutions, the double refraction of flow of the solution was decreased to about two thirds its original value. The effect was not obtained with adenylate or pyrophosphate, and it was slowly reversible. The ATP effect was quite specific, for while it could be obtained with other substances, ITP and ADP, these were required in about ten times the molar concentration of ATP.

Carrying these observations still further, Schramm and Weber (1942) showed that myosin solutions are polydisperse, containing several rapidly sedimenting components with high DRF (S-myosin), and a slowly sedimenting component (L-myosin) with low DRF. The link necessary to connect these varied results was provided by Szent-Györgyi and Banga in 1942, who concluded that the so-called myosin was not a pure protein but a mixture of two proteins. They studied the duration of extraction and consequently discovered that after a short period extraction with a salt solution of KCl not exceeding a quarter of an hour, a myosin was extracted which showed low viscosity and was termed myosin A. Prolonged extraction, 3-12 hours, yielded a myosin with a high viscosity which was termed myosin B. The L-myosin of Schramm and Weber corresponded to the myosin A of Szent-Györgyi. ATP had no effect upon the double refraction of flow or the viscosity of myosin A, but only upon those of myosin B. Both the DRF and the viscosity of myosin B were reduced in the presence of ATP. The missing piece to the puzzle was found by

Straub (Straub, 1942) when he announced that myosin B was not merely a different type of myosin but a complex of two fibrous proteins, myosin and actin, now to be called actomyosin. The above effects with myosin B were due to the fact that ATP was able to dissociate the complex into its two components, actin and myosin. This discovery ended the purely analytical phase in the study of myosin, and the investigations were now designed to observe more closely the properties, structure and interactions of the proteins with ATP and other triphosphates, with and without the presence of various other biologically important substances.

THE PROTEINS OF THE MYOFIBRIL

Myosin

Our information regarding the properties of the myosin molecule come chiefly from the intensive work of Mommaerts (1950), A. Szent-Györgyi et al. (1941, 1942 and 1951), Weber & Portzehl (1951), Needham (1952), Bailey (1941 and 1954), A.G. Szent-Györgyi (1955) and Huxley (1956).

The myosin molecule may be characterized as one of the giant molecules belonging to the fibrous proteins having the alpha structure, its polypeptide chains being not fully extended. The best value for the molecular weight of myosin is given by Portzehl, Schramm and Weber (Portzehl et al., 1950) and reported to be approximately 850,000 by sedimentation and diffusion

methods. The molecule is rapidly destroyed by heat and extreme pH and is completely denatured by anhydrous solvents. When incubated at 37°C for 10 minutes, or stored for a long time at 0°C, its viscosity rises and its enzymic activity falls. Its isoelectric point is 5.2. It exhibits a splendid double refraction of flow in salt-free water, due to the association of particles. Pure myosin is soluble in KCl as low as 0.05 M. Evidence from H.E. Huxley's electron microscope investigations (Huxley, 1956) shows that the A band of muscle contains the myosin together with filaments of actin, while the I band possesses actin alone.

Myosin split by trypsin gives four units of L-meromyosin and two of H-meromyosin, the H units exhibiting the ATPase and ability to combine with actin, while the L units are the contractile elements (A.G. Szent-Györgyi, 1953). The L units have no contractile properties by themselves, and it is concluded that for contractile function the L and H units must be linked together, presumably by a peptide bond.

Actin

Since the discovery of this protein various methods for its purification have been presented. However, that of Szent-Györgyi as modified by Straub, to be described, seems to be the most accepted. The chief characteristics of actin are that it exists in two forms: G-actin, a typical globular protein, and F-actin,

the fibrous protein formed by polymerization. The addition of salt and some Mg^{**} causes the G-F transformation almost immediately. The suggestion by Feuer et al. (1948) that actin possess a prosthetic group led Pettko and Straub (1949) and others (Laki, Bowen and Clarke, 1949) to show that actin indeed contains ATP which is broken down during the polymerization. When ATP was removed from actin, by adding creatine containing creatine phosphokinase or hexokinase and glucose, the ability of actin to polymerize was lost and not restored upon subsequent addition of ATP. During the polymerization process 80% of the ATP disappears and there is an increase in ADP and inorganic phosphate (Straub and Feuer, 1950). However, it must be emphasized that actin is not acting as an ATPase since the reaction occurs only upon polymerization and the ADP formed remains attached to the actin polymer. As estimated by Feuer et al. (1948), the molecular weight of the actin monomer appears to be 70,000. Tsao (1953), using polarization-fluorescence techniques, obtained as a molecular weight of polymerized actin a value of 140,000 and therefore concluded that the polymer was, in fact, a dimer. Abolition of the SH groups in actin, as studied by Kuschinsky and Turba (1951), inhibits the polymerization. Removal of Mg^{**} with ethylenediamine-tetraacetic acid (EDTA) also prevents the polymerization process (Tsao, 1953). Actin represents 20-25% of the total protein of the myofibril.

The actin filaments are 4 μ in diameter as compared to the myosin filaments of 11 μ .

Actomyosin

Actomyosin may be prepared as "natural" actomyosin, extracted from minced muscle as such, or by mixing the purified proteins F-actin and L-myosin to give "artificial" actomyosin. Actomyosin is insoluble in media of ionic strength less than 0.3.

There is some doubt as to whether the actomyosin complex exists as such in muscle. From the electron microscope studies of Huxley (1956) we find that there are two filaments in the myofibril which are made up of the two structural proteins actin and myosin and which are clearly separated. The overlapping arrangement of these filaments would enable cross-linkages to be established between them (Huxley, 1956; Perry, 1956) by ATP. When ATP is added to an extracted solution of actomyosin, the actomyosin is dissociated into its components actin and myosin, with a consequent fall in viscosity. High ionic strength will produce the same effect. From the work of Mommaerts (1948) it is shown that the minimal amount of ATP required to give the maximum viscosity change at 0°C in actomyosin solutions is 1 mole ATP for 350,000 gms. actomyosin, and it is therefore concluded that there are 2-3 active myosin centres per molecular weight of 850,000. From the work of Szent-Györgyi (1953), who found that when myosin was digested with trypsin, two units of H-meromyosin were revealed, and assuming that each of these

units possesses one active centre, it follows that the intact myosin molecule must have at least two active centres. This is in good agreement with the work of Mommaerts (1945).

Bailey and Perry (1947) found that the sulphhydryl reagent PCMB, which inhibits the ATPase activity, also abolished any viscosity effects when adding actin to myosin. Since then the combination of actin and myosin depends on SH groups, it would seem that SH reagents could replace ATP in competing for these groups. This has been found to be true by Kuschinsky and Turba (1952) using salyrgan. Although actin also contains SH groups, all of which must be intact for polymerization, they are not all essential to actomyosin formation (Bailey & Perry, 1947). This suggests that the same active centres are involved in the enzymic and actomyosin-forming processes. The dissociation of the complex by ATP can be explained by displacement of actin by ATP which has a greater affinity for these centres on the myosin. ITP and UTP also dissociate the complex. The order of nucleotide activity with respect to dissociation is $ATP > ITP > UTP$.

Myokinase

Myokinase is closely associated with myosin in that it is always found in the myofibril and in myosin preparations and is not separated until the myosin is reprecipitated about 12 times. Its enzymic activity is confined to converting ADP to ATP and

adenylic acid. It is a very stable enzyme, highly resistant to heat or acid denaturation. Unlike myosin, it does not catalyse the splitting of other nucleotides. The enzyme is activated by Mg^{++} and is inhibited by any metal-binding compound such as EDTA.

5'-Adenylic Deaminase

This enzyme is also to be found within the myofibril and is specific for 5'-AMP which it deaminates and converts to inosinic acid. It is activated by citrate, chloride, acetate and lactate. Its activity is not increased by Mg^{++} or decreased by EDTA. Its pH optimum is 5.9, which is shifted to 6.5 when citrate is present.

THE ENZYMIC ACTIVITY OF MYOSIN AND ACTOMYOSIN

As has already been stated, myosin ATPase is extremely sensitive, is rapidly destroyed ~~when~~ by incubation at $37^{\circ}C$, by acid, storage or by SH reagents. Myosin exerts its maximal activity at pH 9, having another pH optimum in the region of the physiological pH (Bailey, 1942). The kinetic studies of Ouellet, Laidler and Morales (1952) reveal the Michaelis constant to be 1.4×10^{-5} at $24^{\circ}C$, pH 7, in 0.001 M- $CaCl_2$. The purified enzyme catalyzes the reaction $ATP + H_2O \longrightarrow ADP + H_3PO_4$. Other nucleoside triphosphates have been shown to be split by myosin - ITP (Kleinzeller, 1942) and UTP (Ranny, 1954; Kalckar,

1954; Bowen & Kerwin, 1954). Blum (1955) has determined the Michaelis constants for all the nucleoside triphosphates and found the reciprocals to be in the order of $ATP > UTP > ITP$, with Mg^{++} or Ca^{++} . Tripolyphosphate is also split (Dainty et al., 1944) but very much less rapidly than ATP (Weber, 1954).

The activity of myosin as an enzyme is markedly affected by the presence of ions, and again, the presence of actin influences or modifies these effects so that one must be very careful to distinguish between L-myosin and actomyosin. In the absence of salts, other than ATP, myosin has little enzymic activity. Ca^{++} greatly increase the activity of L-myosin and actomyosin. If Mg^{++} are also present there appears to be an antagonism set up and little ATPase activity is the result, the inhibition being 90% if the $Mg^{++}:Ca^{++}$ ratio is less than one (Mommaerts and Seraidarian, 1947). Cu^{++} produce 100% inhibition in concentrations as low as 10^{-5} M. Actomyosin is activated by Mg^{++} at low ionic strength (where actomyosin is not in solution $\mu < 0.1$), whereas myosin is inhibited (Banga & Szent-Györgyi, 1943). However, actomyosin is inhibited when in the sol form, for at this ionic strength, as explained by Szent-Györgyi (1945), it more closely resembles myosin - KCl exerting its dissociating effect. Perry (1956), working with myofibrillar and actomyosin systems, has performed extensive investigations on the $Mg^{++}-Ca^{++}$ antagonism and concluded that when the molar concentration of ATP exceeds that of Mg^{++} , inhibition begins to set in. This does

not occur with Ca^{++} -activated ATPase. If Ca^{++} and Mg^{++} are present together there is no inhibition when the ATP concentration exceeds that of Mg^{++} , here, the Ca^{++} preventing or relieving the normal inhibition. The marked ~~inhibition of~~ myosin ATPase produced when Mg^{++} and Ca^{++} are present in equal concentrations has led some workers (Mommaerts & Seraidarian, 1947; Barvermann & Morgulis, 1948) to question whether or not this ATPase is sufficient to account for the amount of inorganic phosphate produced during muscular activity. It is known that Mg^{++} and Ca^{++} concentrations within the cell are 0.012 and 0.006 M respectively (Perry, 1955), at which concentrations the myosin ATPase should be inhibited to an extent of 90%. However, the presence of actin alters the activity profoundly and must be taken into account before such questioning can be entirely justified. Weber and Portzehl (1954) suggest that in resting muscle, since the total concentration of Mg^{++} is greater than that of ATP (Dubuisson, 1950), Mg^{++} might be bound to the relaxing factor, that substance responsible for keeping the muscle in the resting state during which there is no enzymic activity (Marsh, 1952), and that the Mg^{++} is in some way released during activity, the Ca^{++} now becoming bound to the factor. At the end of activity the Mg^{++} once more becomes bound and inhibition of the ATPase sets in. However, inhibition will not take place as long as there are free Ca^{++} present.

Effect of 2:4-Dinitrophenol

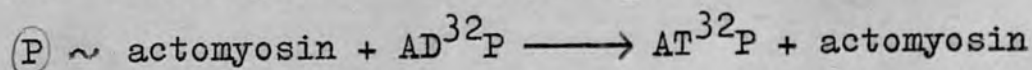
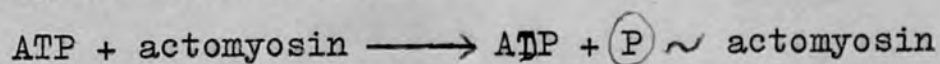
It has been reported that up to 150% stimulation of myosin ATPase could be obtained in the presence of DNP 10^{-3} M (Greville and Needham, 1955; Chappell and Perry, 1955; Perry, 1957). However, when actin was present, the degree of stimulation fell off progressively with increasing amounts of actin, and at low ionic strength the DNP became inhibitory. The stimulation effects disappeared at 0°C and could be obtained only by raising the ionic strength to 0.4 M KCl. Addition of Mg^{++} also decreases the degree of stimulation produced by DNP. There appears to be a great affinity of Mg^{++} for ATP and it is perhaps this Mg-ATP complex which prevents DNP from producing its stimulatory effect on myosin ATPase. With mitochondria, however, it is the Mg^{++} -activated ATPase which responds to the DNP stimulation. Green et al. (1949) found that cyclophorase preparations contained phosphate groups in highly labile combination with the enzyme complex, which they termed the "gel phosphate". Teplý (1949) noted that DNP was capable of promoting the dephosphorylation of this "gel phosphate".

The experiments of Teplý (1949) and Hunter (1951), endeavouring to elucidate the mechanism by which DNP induced this phosphatase-like process in mitochondria, led to the suggestion that DNP did not stimulate a previously existing phosphatase, but rather that it in some way cleft a trans-phosphorylase into two phosphatases, thus giving the apparent increase in ATPase activity. DNP is known to inhibit oxidative

phosphorylation in mitochondria. Thus it may well be that the tendency for DNP to further the breakdown of a phosphorylated compound is that factor responsible for the stimulatory effects on the ATPase, and this mechanism in the mitochondrial system may be similar to that of the myosin system.

The Possible Phosphorylation of Myosin

The ^{formation} ~~postulation~~ of a phosphorylated intermediate during the interaction between myosin and ATP has long been postulated (Needham, 1941; Kalckar, 1941). Buchthal et al. (1949) reported an appreciable uptake of phosphate and adenine by actomyosin in the presence of ATP. However, when these observations were extended (Buchthal et al., 1951), they observed that the uptake was entirely dependent on the concentration of iron in the system, no uptake being detectable in the absence of iron. Nevertheless, the recent experiments of Weber (1955), using Fuadin as an inhibitor to eliminate the release of inorganic phosphate from a possible phosphorylated myosin, indicates the presence of such a phosphorylated intermediate, since this worker was able to obtain a transfer of phosphate groups from ATP to $AD^{32}P$ catalysed by actomyosin.



This was confirmed by Ulbrecht and Ulbrecht (1957) who showed that actin, as present in "natural" actomyosin, was essential to the exchange reaction. Koshland et al. (1954) failed to observe

such a transfer without the use of this new inhibitor. The failure of Mommaerts (1950) to detect any such compound is also evidence for the transiency of such an intermediate.

Effect of Ethylenediaminetetraacetic Acid

EDTA has been found to inhibit myosin ATPase at low ionic strength, and activate to an extent of 400% at high ionic strength (Bowen & Kerwin, 1954). These effects were achieved with myosin A and myosin B, less KCl being necessary to obtain stimulation with myosin A. EDTA was found not to affect ITPase or UTPase (Bowen & Kerwin, 1954).

Contraction Theories

Contractile models are extremely useful in studying the mechanisms of contraction and relaxation and the chemical reactions in muscle responsible for structural change. In 1934 Weber introduced the actomyosin thread and studied its mechanical properties. This model aroused great interest among biochemists in 1942-43 when Szent-Györgyi (1942-43) showed that these threads were actually capable of contracting upon the addition of ATP. Threads made from artificial actomyosin develop considerable less tension than those made from "natural" actomyosin, which seems to suggest that in "natural" actomyosin preparations the actin and myosin have a better chance to become oriented with respect to one another, and so resemble, to a greater extent, the state in the myofibril. In artificial actomyosin the actin

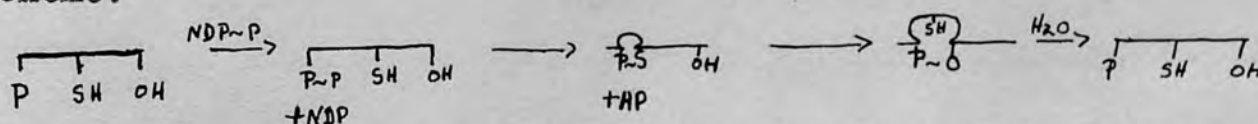
and myosin molecules are more randomly arranged and, as a consequence, less tension develops when ATP is applied during isometric contraction. In isotonic contraction both types of actomyosin contract to the same extent.

Weber et al. (1950) reported the use of glycerol-extracted muscle fibres, which had many advantages over the threads although the fundamental process of contraction in both systems appeared to be the same. These glycerated fibres contract upon the addition of ATP and remain so when ATP is washed out. Relaxation can be produced, when ATP is removed, by the addition of pyrophosphate. Inhibition of the ATPase prevents the contraction of the models, and it has been shown (Weber, 1952; Lundsgaard, 1950) that ATP is always broken down during contraction. The experiments of Buchthal (1947) showed that contraction could be produced in threads which were enzymically inactive. However, this contraction did not take place against a tension.

Even if experiments with models are a reflection of reactions taking place in contracting muscle, it remains to establish the sequence of events during the contraction, i.e. whether the contraction is the result of combination of protein with substrate, which in turn produces hydrolysis, or whether the contraction takes place simultaneously with the production of inorganic phosphate liberated from ATP, which process would supply the energy necessary for contraction. Morales et al.

(1955) support the former theory and postulate that the actomyosin possesses a net positive charge which keeps it in the relaxed or extended state. When ATP, which has a negative charge, is adsorbed, the net charge is reduced. The protein filament now shortens, hydrolysis of ATP takes place, thereby reducing the negative charge, and the filament begins to extend once more. The energy of binding of ADP is much less than that of ATP, thus permitting the desorption of ADP and the further adsorption of ATP, which continues the cycle. Here the contraction is independent of the splitting and merely due to the adsorption or binding of the ATP molecule.

The opposing theory, that contraction is produced simultaneously with the splitting of ATP, and therefore dependent upon it, is supported by Weber (1955). This author proposes a phosphorylation of the actomyosin molecule. Here the active sites of the enzyme can interact with the phosphate side chains of the nucleotide, this strong affinity producing the folding of the polypeptide chain and thus inducing contraction. Upon the liberation of inorganic phosphate, the protein is then free to unfold and relaxation ensues. Weber proposes the following scheme:



An earlier proposal by Riseman and Kirkwood (1948) suggests that the addition of ATP or the formation of phosphorylated myosin produces relaxation due to the repulsion of the charges,

assuming the myosin to be practically isoelectric. Upon the splitting of the phosphorylated intermediate, the myosin can contract, and thus the contraction is once again dependent on the splitting. However, it is difficult to reconcile this theory with the results of fibre model experiments of Weber (1952), which show that the models contract upon the addition of ATP.

The experiments of Perry (1955) provide certain evidence against the theory of Morales. This author, using well-washed myofibrils, studied their behaviour in the presence of a creatine phosphokinase system which **comprised** 10^{-5} - 10^{-6} M ATP or ADP and excess creatine phosphokinase and creatine phosphate. If the ATP were constantly being regenerated by this system, then the concentration of ATP was sufficient to cause contraction. However, in the absence of the creatine phosphokinase system no contraction occurred and it was necessary to increase the ATP concentration ten to one hundred times in order to obtain a contraction similar to that found with a lower nucleotide concentration maintained by the creatine phosphokinase system. That the contraction does not depend solely upon the concentration of ATP is a serious objection to the theory that binding of nucleotide alone is sufficient to cause contraction.

It is also known that increased ATP concentration inhibits shortening of fibre models (Weber, 1952). Lowering the

temperature to 0°C also inhibits shortening, while it should increase the possibility of binding. The presence of the relaxing factor also produces a similar inhibition. It is difficult to reconcile these facts with the theory that binding or adsorption of the nucleotide is the main cause of contraction. All of these inhibiting factors are likewise inhibitors of the ATPase.

Buchthal (1956) gives the following arguments against the adsorption theory: (1) Salyrgan inhibits ATPase and contraction but not relaxation; (2) ADP has an appreciable affinity for myosin and it is therefore difficult to assume a sufficient desorption rate for ADP; (3) a system so sensitive to changes in electrostatic charge over a wide range is unaffected by pH changes (Weber, 1955).

Nevertheless, there are also objections to the phosphorylation theory. Buchthal (1947) obtains contraction with threads possessing little ATPase activity and concludes that the contraction is independent of the enzymic activity. Bowen (1951, 1952) shows that under certain conditions there can be no direct connection between the rate of hydrolysis of ATP and that of shortening. Mg, he found, induces contraction yet inhibits ATPase. This effect has also been observed by Hasselbach (1952). However, Perry assesses these results (1956) and concludes that the low ionic strengths at which these

authors were working increase the degree of hydration which would necessarily alter the mechanical properties of the fibre. Owing to the method of preparation, Bowen's myofibrils used in enzymic experiments are considerably different from the glycerated fibres of contraction experiments and are therefore not strictly comparable.

The experiments of Fleckenstein, Janke, Davies and Krebs (1954), and Mommaerts (1955), showing that, in a single contraction of frog rectus abdominis, no significant change in ATP, ADP or CP takes place, do not rule out the possibility of ATP hydrolysis concurrent with contraction. In all experiments there is an increase in inorganic phosphate, the exact source of which is yet unidentified. It is suggested by Fleckenstein et al. that the ATP level is maintained by this unknown precursor of inorganic phosphate. These findings nevertheless appear to be consistent with the theory that the energy of contraction derives from the hydrolysis of an energy-rich phosphate compound which is not ATP but which may well be phosphorylated myosin or actomyosin.

Relaxation

It is apparent that when the ATPase is low, or when it is inhibited with Salyrgan (Weber, 1952), relaxation can be made to take place in fibre models in the presence of ATP. In this case the ATP does not exert its contraction effect but rather

acts as a "plasticizer", i.e. prevents the model from becoming rigid, enabling the molecules of the protein to move more freely with respect to one another. Although Salyrgan is an inhibitor of the ATPase, it does not inhibit the plasticizing action of ATP. This would imply that the relaxing action is due to the polyphosphate grouping of the ATP, since pyrophosphate produces the same effect and is not split by the ATPase (Weber, 1952).

Bailey and Marsh (1952) also observed that there was some factor present in the fibres which was responsible for relaxation, and which has subsequently become known as the "Marsh factor". Bendall (1954) succeeded in isolating the "Marsh factor" and reported it to be identical with myokinase.

Perry (1956) suggests that the contraction-relaxation cycle is very dependent on the ionic environment, particularly the Mg^{++} and Ca^{++} concentrations. When Mg^{++} is low in relation to that of ATP, inhibition of the ATPase appears to set in, and therefore relaxation takes place, the ATP now being available to exert its plasticizing action. The Mg^{++} concentration may be reduced by complexing with the relaxing factor. When the stimulus comes, the Mg is in all probability released, ATPase can take place at a high rate and contraction occurs. This contraction ceases when the Mg^{++} again becomes bound, thus increasing the relative amount of ATP, which is constantly maintained by the creatine phosphate system, and the ATP now exerts its plasticizing effect.

EDTA completely inhibits the contraction of actomyosin threads (Bozler, 1954; Watanabe, 1955). It is believed that EDTA, because of its strong affinity for Mg^{++} , reduces the Mg^{++} concentration, thereby increasing the relative ATP concentration and thus relaxation can take place.

In conclusion, shortening does not take place in the absence of ATPase, whereas the plasticizing action is independent of the enzymic activity and occurs even when sulphhydryl poisons are present (Weber, 1952). When the SH groups are so blocked, contraction cannot take place, and this may readily be explained by the theory that the contraction is directly dependent upon the ATPase activity of the enzyme.

THE PRESENT INVESTIGATION

The investigation was made in an attempt to obtain direct evidence for or against a phosphorylation of myosin and actomyosin during interaction of these proteins with adenosine triphosphate.

The thesis now submitted is that such a phosphorylation does, in fact, occur with both myosin and actomyosin. This process has not been detected by conventional chemical methods. Its occurrence has, however, been deduced from the properties of surface films of the myofibrillar proteins on substrates containing adenosine triphosphate.

Chapter II

EXPERIMENTALA. Reagents

Adenosine 5'-triphosphate (ATP)

Adenosine 5'-diphosphate (ADP) - Sigma Chemical Company, U.S.A., supplied as the disodium salts.

Inosine 5'-triphosphate (ITP) - synthesized according to the procedure of Kaplan (1955).

2:4-Dinitrophenol (DNP) - British Drug Houses, recrystallized once from distilled water.

Ethylenediaminetetraacetic acid (EDTA) - British Drug Houses.

Antimony bis-(catechol disulphonic acid) - (Fuadin), sodium salt, kindly provided by Bayer, Leverkusen, Germany.

p-Chloromercuribenzoic acid (PCMB) - Light & Co. Ltd.

Tris (hydroxymethyl)-aminomethane (Tris) - Light & Co. Ltd.

Sodium iodoacetate - La Roche & Cie.

Unless otherwise stated, all other reagents used in this investigation were of analytical grade.

B. Preparations

For all preparations of muscle proteins the distilled water employed was freed of heavy metal ions by treatment with ion exchange resins.

L-Myosin

L-Myosin was prepared according to the method of Mommaerts and Parrish (1951). A rabbit was injected with a series of injections of 20% $MgSO_4$ until the hind muscles were well paralysed, and death brought about by a blow on the head. The dorsal, hind and front leg muscles were quickly removed, dried and minced in a mincer. The mince was immediately extracted in 3 vols. of cold KCl - K phosphate buffer, 0.3 M KCl, 0.09 M KH_2PO_4 , 0.06 M Na_2HPO_4 . The extract was stirred for 15 minutes, then separated by straining through gauze and clarified by centrifugation. Successful precipitation in the first step requires a ten to twelve fold reduction in the ionic strength of the original extract. This was achieved by dialysis overnight without stirring in cellophane bags 1" in diameter. The bags were then emptied into a large beaker, and after centrifugation, cold deionized water was added slowly down the sides of the beaker with rapid constant stirring until precipitation occurred, which was indicated by strong opalescence of the solution. The solution was now centrifuged, the supernatant

being discarded. The precipitate of myosin, fat etc., was dissolved in KCl, centrifuged to remove the insoluble matter, and the supernatant was reprecipitated in like manner three times. The final precipitate of myosin was dissolved in 0.5 M KCl, a drop of toluene was added and the myosin was stored in the refrigerator at 4°C.

Actomyosin

Actomyosin was prepared according to the method of Weber (1944). A rabbit was killed by a blow on the head, and the dorsal, hind and front leg muscles were quickly excised and minced, and suspended in one litre of Weber solution (0.6 M KCl, 0.01 M Na₂CO₃, 0.04 M NaHCO₃) and allowed to stand at 4°C overnight. The extract was then strained, squeezed out through gauze and diluted to 3 times its volume with cold deionized water, thus precipitating the actomyosin. The solution was centrifuged and the precipitate was dissolved in 0.5 M KCl with some Weber solution added in order to maintain the pH. The actomyosin was reprecipitated in like manner three times, dissolved in 0.5 M KCl, treated with a drop of toluene and stored in the refrigerator at 4°C.

Actin

Actin was prepared according to the method of Szent-Györgyi as modified by Straub (1944). The residue from the L-myosin preparation was suspended in 0.4% NaHCO₃ solution, stirred for

thirty minutes, strained, pressed out and minced in a Waring Blendor. The residue was suspended in one volume of 0.05 M NaHCO_3 and 0.05 M Na_2CO_3 for ten minutes. It was then diluted with ten volumes of water, stirred for ten minutes and centrifuged. The sediment was mixed with equal volumes of acetone, and allowed to stand for ten minutes, strained and pressed out. The acetone treatment was repeated, and the final residue was spread out on filter paper and dried. The acetone powder was extracted with chloroform and dried in the incubator. It was then extracted with about ten volumes of cold, boiled, ~~out~~ water for twenty minutes, filtered on a Buchner funnel to give a solution of actin.

Myokinase

This enzyme was prepared according to the method of Colowick (1955). Rabbit muscle was ground in a mincer and extracted twice for 15 minute periods with one volume cold 0.03 M KOH - 0.002 M EDTA. The third extraction was performed with 0.5 vols. EDTA. The extracts were combined and acidified with 0.05 vols. 2N HCl and heated as rapidly as possible to 90°C. After three minutes at this temperature, the solution was cooled rapidly in ice water to room temperature and neutralized to pH 6.0 - 6.5 with 2N NaOH. The large white precipitate of denatured proteins was removed by filtration in the refrigerator and the filtrate of myokinase was stored at 4°C.

5'-Adenylic Acid deaminase

This enzyme was prepared according to Nikiforuk and Colowick (1955). Rabbit muscle was chilled and ground in a mincer. The mince was washed four times with 4 vols. cold 0.85% NaCl for twenty minutes, with occasional **stirring**, and squeezed out through cheesecloth. The deaminase was extracted from the colourless residue by stirring with 1 vol. cold 2% NaHCO₃ for one hour, and filtered through a folded Whatman No.12 filter paper. A solution of ammonium sulphate at 0°C, pH 7.6 and 0.01 vol. of 18% ammonia was added slowly to the filtrate to give 0.27 saturation. After ten minutes the precipitate was centrifuged at 2°C, 15000 r.p.m. The supernatant was brought to 0.45 saturation, and after centrifugation, the 0.27 to 0.45 fraction was dissolved in the least possible volume of 0.1 M Na₂HPO₄. The preparation was stored at 4°C in the refrigerator.

Dry Weight Determinations

The dry weights of L-myosin, actomyosin and actin were determined as follows: 5 ml. of stock solution was pipetted into a weighed centrifuge tube and 1 ml. 10% trichloroacetic acid was added. The precipitate was centrifuged down, ^{washed} twice with distilled water and once with absolute alcohol, and heated for two hours at 110°C. The tube was cooled in a desiccator and reweighed, the dry weight of the protein being found by difference.

C. The action of 2:4-Dinitrophenol upon the Enzymic Activities of Muscle Proteins

In view of the findings of Teplý (1949) and Hunter (1951) with reference to DNP, as discussed in the previous chapter, it may well be that if a phosphorylated myosin does exist, the action of DNP on the ATPase activity is analogous to that on mitochondrial systems, where the DNP inhibits the formation or accelerates the breakdown of phosphorylated compounds. If this were true, it would imply that there exists an important connection between the action of DNP and the phosphorylation process. Attempts were therefore made to reproduce the DNP-stimulatory effects on the ATPase activity of myosin and actomyosin observed by Greville and Needham (1955) and Chappell and Perry (1955), and to extend these observations in respect of these and other muscle enzymes.

Experiments were designed to show whether DNP affected the rate of splitting of ATP for all reaction times. ~~L-myosin was prepared according to the method of Mommaerts and Parrish (1951).~~ An assay of the enzymic activity was performed as follows: 0.25 mg. of actomyosin dissolved in 1 ml. 0.5 M KCl was incubated at 37°C with 1 ml. ATP (5×10^{-3} M), 1 ml. Tris buffer (0.05 M, pH 7.4), or 1 ml. DNP 10^{-3} M in Tris, and 0.05 ml. CaCl₂ (0.01 M). The total volume was 3.05 ml. The reaction was stopped by the rapid addition of 2.5 ml. of 8% trichloroacetic acid. The

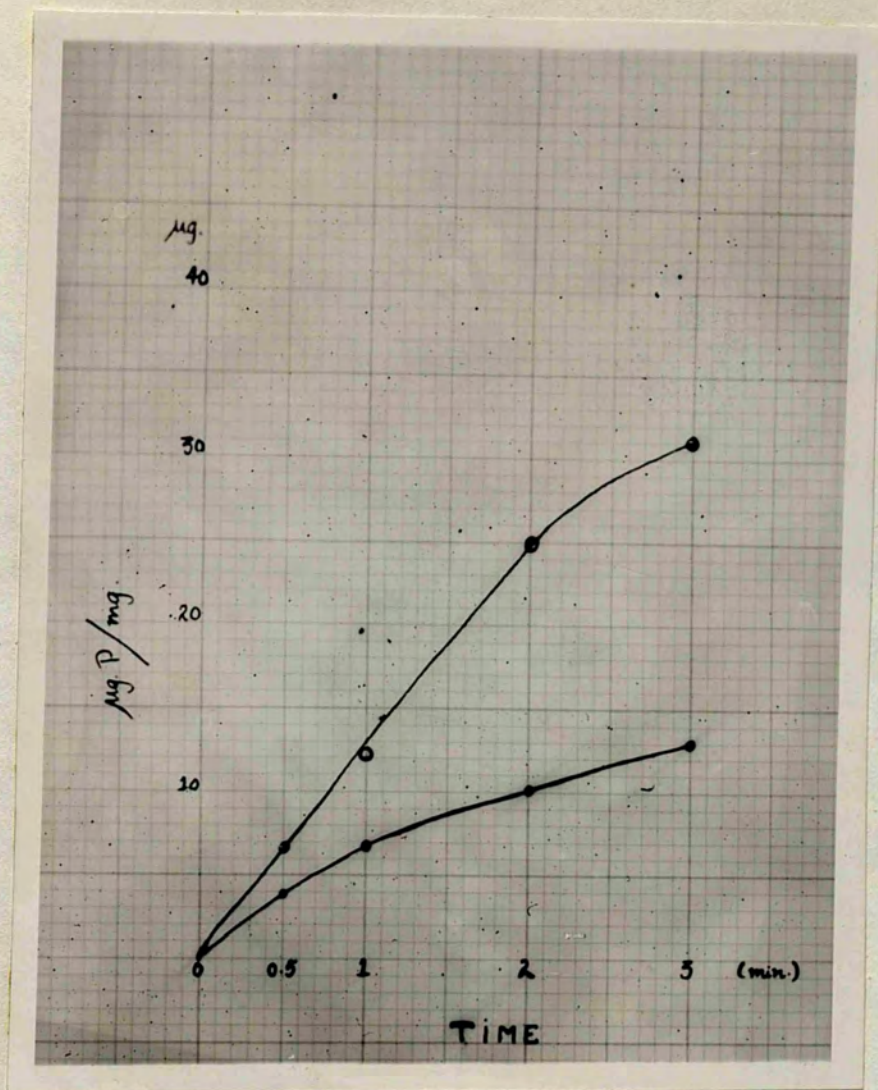


FIG. 1

The effect of 2,4 dinitrophenol on the ATPase activity of actomyosin

Incubations carried out in 0.05 M Tris buffer, pH 7.4, and 1×10^{-3} M ATP at 37°C.

· — · 1×10^{-3} M ATP

○ — ○ 1×10^{-3} M ATP and 1×10^{-3} M DNP

resulting precipitate was filtered through glass wool, washed once with 0.5 ml. 8% trichloroacetic acid and twice with water, and the filtrate was collected in 25 ml. graduated flasks. The amount of inorganic phosphate was estimated by the procedure of Fiske and Subbarow (1925) and measured in the Spekker absorptiometer. The reaction times were 30 sec., 1 min., 2 min., 3 min. The results of Fig.1 indicate that DNP, in exerting its stimulatory effect on the ATPase activity, increases the rate of splitting of ATP at all time periods of the reaction.

It was thought that the DNP effect on the ATPase activity might conceivably be due to a stimulation of the myokinase, which enzyme always contaminates myosin preparations unless special precautions are taken to eliminate it. The procedure for determining the DNP effect on the myokinase reaction is based on the rate of formation of 5'-AMP which could be detected by the deaminase reaction and was as follows: 0.05 ml. ADP (4×10^{-3} M) - Tris (pH 7.4, 0.05 M) mixture, or 0.05 ml. ADP and DNP (10^{-3} M) in Tris, was pipetted into a quartz cell of 1 cm. light path together with 0.02 ml. stock solution of myokinase. The reaction was allowed to proceed for 10 min., then stopped by the addition of 2.5 ml. citrate buffer (0.01 M, pH 6.4). The reading was immediately taken on the Unicam Spectrophotometer at 265 m μ and 0.03 ml. adenylic deaminase solution (prepared according to Nikiforuk and Colowick, 1955) was added. Readings were taken

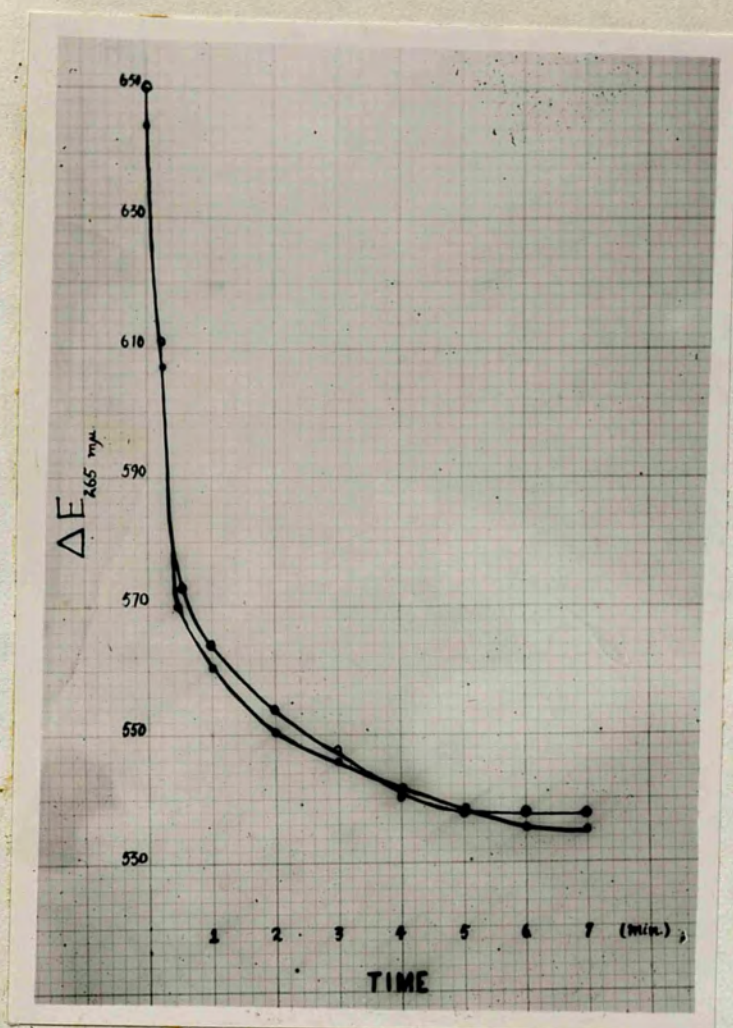


FIG. 2

The effect of 2:4-dinitrophenol on myokinase activity as determined by rate of deamination of adenylic acid

Incubations carried out in citrate buffer, 0.1 M, pH 6.4, and 4×10^{-3} ADP at 20°C.

• — • 4×10^{-3} M ADP

○ — ○ 4×10^{-3} M ADP and 1×10^{-3} M DNP

at exactly 15 sec. and 30 sec. after mixing, and at successive 1 min. intervals for 10 min. No significant differences between the DNP-treated enzyme and the control could be detected. It was concluded that DNP had no effect on the rate of formation of 5'-AMP, which reaction is catalysed by myokinase (Fig. 2). DNP also had no effect on the deaminase activity, thus confirming the results of Greville and Needham (1955).

D. Attempts to detect an uptake of phosphate groups by myosin in the presence of ATP

Although the existence of phosphorylated myosin has long been postulated, few attempts have been made to detect such a compound. The experiments of Buchthal et al. (1949-1951) were among the first to attempt a demonstration of myosin phosphorylation. Buchthal et al. showed that there was an uptake of phosphate and adenine by myosin and actomyosin when incubated with ATP, but that this uptake was proportional to the iron content of the system. It was evident that further experiment was needed before any satisfactory conclusions could be drawn about the possible phosphorylation of myosin. Buchthal's method was, in general, to allow actomyosin to react with ATP for a standard period of time. The reaction was then interrupted by dilution with water, the precipitate was centrifuged and washed at least 12 times before it was extracted with 10% perchloric acid. Phosphate and adenine estimations were then made on the

extract. Buchthal's method involved a rather drastic washing procedure and it was thought that a milder treatment, which would reduce the number of washings to a minimum, might be more effective in preserving any possible phosphorylated compound, which was, no doubt, of an extremely labile nature.

Preliminary experiments were designed, using essentially the procedure of Buchthal (1951), to re-examine the possibility of an uptake of phosphate groups by myosin and actomyosin. 10 mg. actomyosin, dissolved in ^{1.0 ml.} 0.5 M-KCl, was incubated at 37°C for 5 minutes with 2 ml. ATP (10^{-3} M) in Tris buffer (0.05 M, pH 7.4), and 0.05 ml. CaCl₂ (0.01 M). The protein was precipitated by the addition of 2 ml. deionized water. The precipitate was centrifuged down at 11,000 r.p.m. at 2°C, washed three times in cold water, and the supernatant decanted. The precipitate was suspended in 3 ml. water, and 0.5 ml. 60% perchloric acid was added to denature the protein. The extract was allowed to stand at 0°C for 30 minutes. The suspension was centrifuged and the supernatant decanted into a 10 ml. graduated test tube. The precipitate was washed twice with water, the washings being transferred to the graduated test tube.

Using the procedure of Fiske and Subbarow (1925), phosphate estimations differentiated with respect to total phosphate (P_T), determined after combustion with 0.5 ml. 60% perchloric acid, followed by hydrolysis at 100°C, "7 minute phosphate" (P_7), determined after 7 minutes hydrolysis in N hydrochloric acid at

100°C, and inorganic phosphate (P_0) were performed on 1 ml. portions of the extract. Table I shows no detectable phosphate uptake using this procedure.

TABLE I

$\mu\text{g. P/mg. actomyosin}$ using Buchthal's method (washings reduced to 3).

Additions	P_0	P_7	P_T
NONE	0.21	0.35	0.73
	0.24	0.35	0.54
ATP (10^{-3} M)	0.21	0.35	0.73
	0.28	0.36	0.55
ATP (10^{-3} M)	0.24	0.36	0.73
+ DNP (10^{-3} M)	0.21	0.37	0.74

Experiment carried out at pH 7.4 in Tris buffer (0.05 M) and 0.01 M CaCl_2 at 37°C.

P_0 = inorganic phosphorus.

P_7 = phosphorus liberated after hydrolysis at 100°C in N HCl

P_T = total extractable phosphorus. (for 7 minutes.)

The negative results indicated that no phosphate uptake was to be detected by this method.

1) Experiments to detect phosphate uptake by myosin in the native state.

An alternative procedure was adopted in which the washing of the protein was eliminated. After the precipitation and centrifugation, the supernatant was decanted off and the wet precipitate was weighed. Phosphate estimations were made on the supernatant and the precipitate, and corrections were made for the supernatant contaminating the latter.

Centrifuge tubes (15 ml.) were numbered and weighed. The incubation medium was as in the previous experiment, though the reaction time was reduced to 15 seconds. The reaction was allowed to proceed in an ice bath at 0°C , in order to reduce the activity and also to provide the best conditions for the stability of a phosphorylated compound, for a period of 15 seconds. The actomyosin was precipitated with 3.5 ml. of cold water and the suspension was mixed. After immediate centrifugation at 2°C (11,000 r.p.m.), phosphate estimations were made on 1 ml. samples of the supernatant thus obtained. The tubes were now drained as far as possible without disturbing the precipitate and these tubes containing the wet residue were then reweighed. The residue was suspended in 3 ml. of cold water, and 0.5 ml. of 60% perchloric acid was added to denature the protein. The perchloric acid extract was allowed to stand at 0°C for 30 minutes. The suspension was then centrifuged at 2°C , and 1 ml. samples were taken from the supernatant for phosphate

estimations. Table II clearly shows that no phosphate uptake could be detected by this procedure.

TABLE II

$\mu\text{g. P}$ present in supernatant

Additions	P_0	P_7	P_T
ATP (1×10^{-3} M)	59	188	253
+ DNP (1×10^{-3} M)	62	188	253
ATP (1×10^{-3} M)	45	188	253
	51	188	253
NONE	0.6	1.2	1.6
	0.5	1.1	1.6

$\mu\text{g. P}$ present in 1 mg. actomyosin precipitate

Additions	P_0	P_T
ATP (1×10^{-3} M)	0.7	1.5
+ DNP (1×10^{-3} M)	0.8	1.3
ATP (1×10^{-3} M)	0.8	1.4
	0.5	1.3
NONE	0.7	1.3
	0.6	1.2

Incubations carried out at pH 7.4 in Tris buffer (0.05 M) at 0°C for 15 sec.

P_0 = inorganic phosphorus

P_7 = phosphorus liberated after hydrolysis in N-HCl at 100°C for 7 min.

P_T = total extractable phosphorus

2) Denaturation Experiments.

All the foregoing negative results show that if phosphorylated myosin exists at all, its life is very transient and it is extremely unstable. A completely different experimental approach would therefore be necessary to render possible the detection of such a compound. It is known that monomolecular films of partially denatured myosin, when spread on surfaces containing ATP, exhibit a change in their elastic properties (Cheesman & Sten-Knudsen, quoted by Cheesman & Davies, 1954). This can only be explained by a change in the electrostatic charge or the structure of the protein, due to reaction with or adsorption of ATP or its degradation products. As proteins spread on surfaces are commonly regarded as denatured, these observations suggested the possibility that a phosphorylated myosin might display some stability in the denatured form.

The following experiments were designed in an attempt to find experimental support for this concept. 8 mg. of L-myosin (or 11 mg. of actomyosin) dissolved in 0.5 M KCl was incubated at 0°C in a 15 ml. centrifuge tube. 1 ml. ATP in Tris (0.05 M, pH 7.4) was rapidly blown through a 1 ml. pipette into the tube and was allowed to react for the short period of 2 seconds. The protein was then denatured by emulsification of the solution with 1 ml. chloroform, effected by rapid stirring with a mechanical stirrer for about 30 seconds. The system was diluted with 10 ml. cold water. The suspension was centrifuged, and the precipitate

was washed by mechanical stirring with small portions of cold water, diluted and centrifuged three times. 3 ml. water and 0.5 ml. of 60% perchloric acid were added to the precipitate, which was allowed to stand for 20 minutes. Phosphate estimations were made on 1 ml. portions of the extract. The results of Table III indicate that there was no significant difference in the phosphate content of the ATP treated tubes and the controls, and therefore no detectable phosphate uptake.

TABLE III

µg. P/mg. actomyosin after chloroform denaturation.

Expt. No.	Additions	P _T
I	NONE	1.5
		1.7
	ATP (10 ⁻³ M)	1.6
		1.2
II	NONE	2.0
		2.4
	ATP (10 ⁻³ M)	2.4
		2.1

Experiment carried out at pH 7.4 in Tris buffer (0.05 M) at 0°C.

P_T = total extractable phosphorus.

Urea, which was found to destroy ATPase irreversibly, was also employed as the denaturing agent. However, no phosphate uptake could be observed when myosin was incubated with urea either before the addition of ATP or after the initial enzyme-substrate interaction. The urea experiments are not strictly comparable with the chloroform experiments, owing to the difference in the denaturation process. As it became evident that no phosphate uptake was to be detected using this type of procedure, experiments designed to detect phosphate uptake by myosin using purely chemical methods were now abandoned.

E. Experiments with Surface Films

The denaturation procedure was not wholly abandoned but carried over to surface film experiments where monomolecular films of partially denatured myosin could be studied with relative ease. The experiments of Hayashi (1952), showing that films of actomyosin, which were subsequently found to be incompletely spread, retain much of their enzymic activity, are good evidence for the possible study of enzymic behaviour using surface film techniques. Since the conditions offered by the surface would allow enzyme-substrate interaction at the moment of application of the enzyme to the surface, so that a possible phosphorylated myosin might have a transient existence in the resulting film, it was thought that studies on surface films of myosin and actomyosin might be helpful in elucidating the interaction between ATP and these proteins.

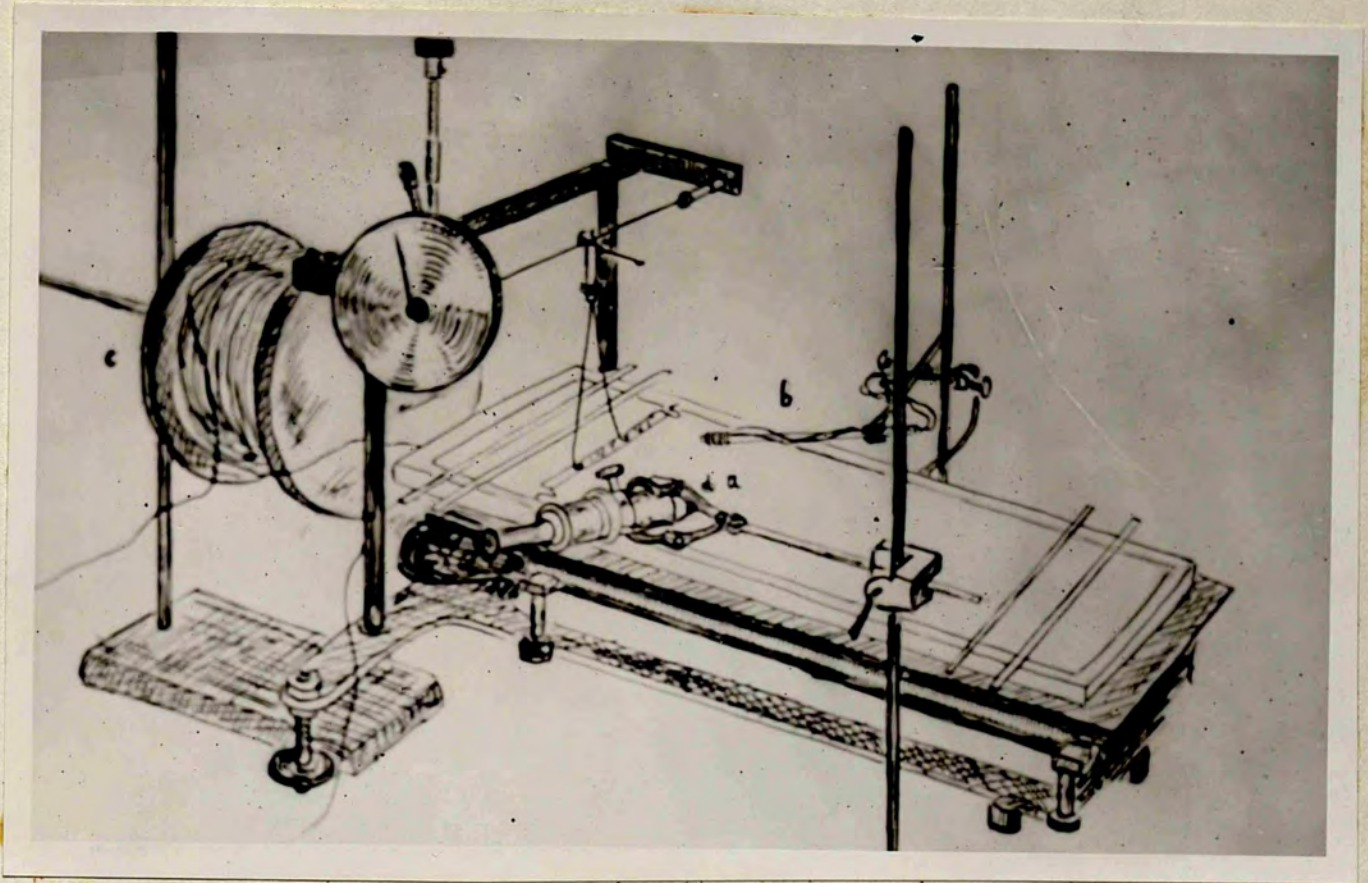


FIG. 3

Apparatus: Langmuir trough as assembled for elasticity measurements on surface films.

a = mica disc bearing magnet and fine glass fibre

b = positioning solenoid

c = large solenoid

A trough of standard Langmuir pattern (35 x 15 x 1.7 cm. and 250 ml. capacity) was assembled as in Fig. 3 (with the exception of the solenoids and microscope). The torsion wire was calibrated and each scale division on the torsion head was found to correspond to 0.52 dynes per cm. surface pressure. The glass barriers and the mica barrier were waxed with paraffin before each experiment. Silk threads attached with paraffin to the mica barrier enabled that barrier to move freely and enclosed the area in which the enzyme was to be spread. The threads were treated with paraffin wax, dissolved in petroleum ether, as also were the sides of the trough. During all measurements it was of extreme importance to have the surface entirely free of all contaminating surface active material. Before each run the surface was therefore "swept" several times with the waxed glass barriers. As a final check against dirt or dust the glass barrier was moved up to the mica barrier; the surface was taken as clean if only a slight pressure developed at very small areas due to the head of water against the mica barrier. Addition of talc (free from all surface active material) to the film, followed by compression, made possible the detection of leaks around the threads attached to the mica barrier.

1) Effect of ATP on the area of L-myosin and actomyosin films.

Preliminary experiments were carried out to note the effect of ATP upon the spreading area of L-myosin and actomyosin films.

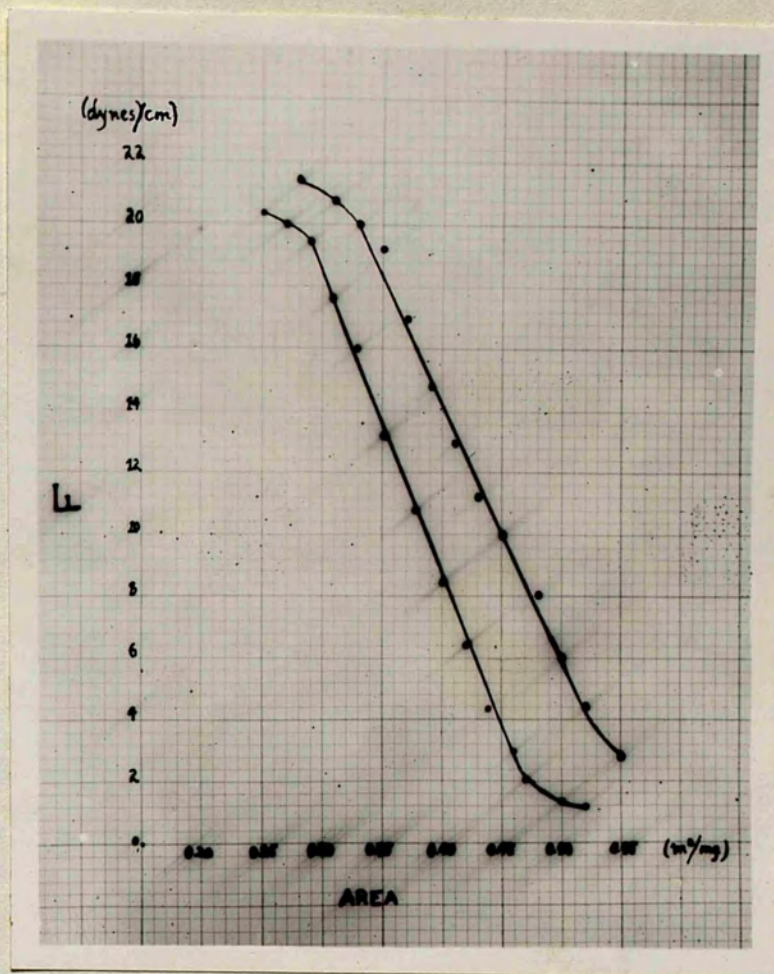


FIG. 4

The effect of ATP on the rate of spreading of actomyosin films.

0.07 mg. actomyosin spread on substrate containing 0.8 M KCl buffered to pH 7.4 with 0.01 M Tris buffer. Measurements were taken after 6 minutes spreading time.

● — ● actomyosin film.

(upper curve) ○ — ○ actomyosin film with 0.0001 M ATP.

With the aid of an Agla micrometer syringe, 40 μ l. of protein, 0.036 mg. L-myosin or 0.07 mg. actomyosin, was spread on a surface containing 0.8 M KCl buffered to pH 7.4 with 0.01 M Tris buffer. The film was allowed to spread for 5 minutes. Film pressure readings were taken after every 0.5 cm. displacement of the barrier and such force-area curves as in Fig. 4 were obtained. In this experiment the limiting area of an actomyosin film, found by extrapolating the straight portion of the curve to zero pressure, was about 0.47 m^2/mg . and was increased to 0.57 m^2/mg . in the presence of 0.0001 M ATP. Similar effects were first noted by Munch-Petersen (1948) and were later confirmed by Cheesman (1952) for actomyosin but not for L-myosin. No such ATP effects upon the area of L-myosin films were noted in the present investigation. The effect of ATP upon the area of actomyosin films seemed to be specific for that nucleotide, for it was not given by ITP (0.0001 M) or ADP (0.0001 M).

Addition of 0.001 M MgCaCl_2 or CaCl_2 did not alter the rate of spreading of actomyosin films with or without ATP. Neither did the antimonial compound fuadin or 2:4-DNP affect the degree of spreading with or without the presence of ATP.

EDTA, however, greatly accelerated the spreading of both myosin and actomyosin films, but did not exceed the ATP effect on actomyosin films. EDTA did not modify the normal ATP effect on actomyosin films. Upon the addition of Mg^{++} (0.001 M) to the EDTA-ATP system, marked inhibition of the spreading resulted.

Cu^{++} (0.0001 M) completely inhibited the spreading of both L-myosin and actomyosin films with or without ATP present in the substrate. Urea (0.1%) also greatly increased the rate of spreading of actomyosin films and did not modify the normal ATP effect.

The effect of ATP on the area of actomyosin films has been explained (Cheesman & Davies, 1954) by some interaction taking place between enzyme and substrate which greatly accelerates the degree of unfolding within the myosin molecule.

2) Effect of ATP on the elasticity of L-myosin and actomyosin films.

It is known that at sufficiently high pressures a protein film becomes rigid and forms a two-dimensional gel which exhibits very strong elastic properties. When foreign substances are added to the substrate, the elastic properties of the film may, or may not, be altered. It has been shown (Cheesman & Sten-Knudsen, in preparation) that the mechanical rigidities of L-myosin and actomyosin films are greatly reduced by 0.0001 M ATP present in the substrate. At this stage of the work it was therefore thought that an investigation of the elastic properties of myosin films might be of value in detecting some compound or complex formed when the protein was spread on substrate containing ATP. In the ATP-myosin interaction, whether this involves phosphorylation of the protein or merely the formation of an enzyme-substrate complex, there is presumably some change

in charge distribution which may be expected to have some effect on the elastic properties of the film. The following experiments were therefore designed to study the effect of ATP on the elasticities of L-myosin and actomyosin films.

A small saturated permanent magnet (6 x 1 x 1 mm.) was mounted in wax on a waxed mica disc of 1.2 cm. diameter. It was placed in the trough and held in position by a small solenoid operating just overhead. Another large solenoid, composed of 7 lbs. of 22 gauge copper wire, was placed at the end of the trough with its axis along the surface of the trough as in Fig. 3. Both solenoids were operated from a single two-way switch. When the larger solenoid was turned on, the time course of the movement of the disc was determined by a microscope with micrometer eyepiece, which viewed a fine glass fibre mounted in wax perpendicular to the plane of the disc.

(i) The ATP effect on the elasticity of myosin films.

With the disc in a fixed position, 40 μ l. ^(0.01 mg) of L-myosin or actomyosin was spread from an Agla syringe on the standard substrate (0.8 M KCl in 0.01 M Tris buffer, pH 7.4) and on this substrate containing 0.0001 M ATP, and allowed to remain for 7 minutes. After 5 minutes, the current leading to the positioning solenoid was switched off, the float now being held in position by the surrounding film. A reading on the microscope was taken a few seconds before the 7 minutes had elapsed, and at exactly 7 minutes time the current leading to the larger solenoid was

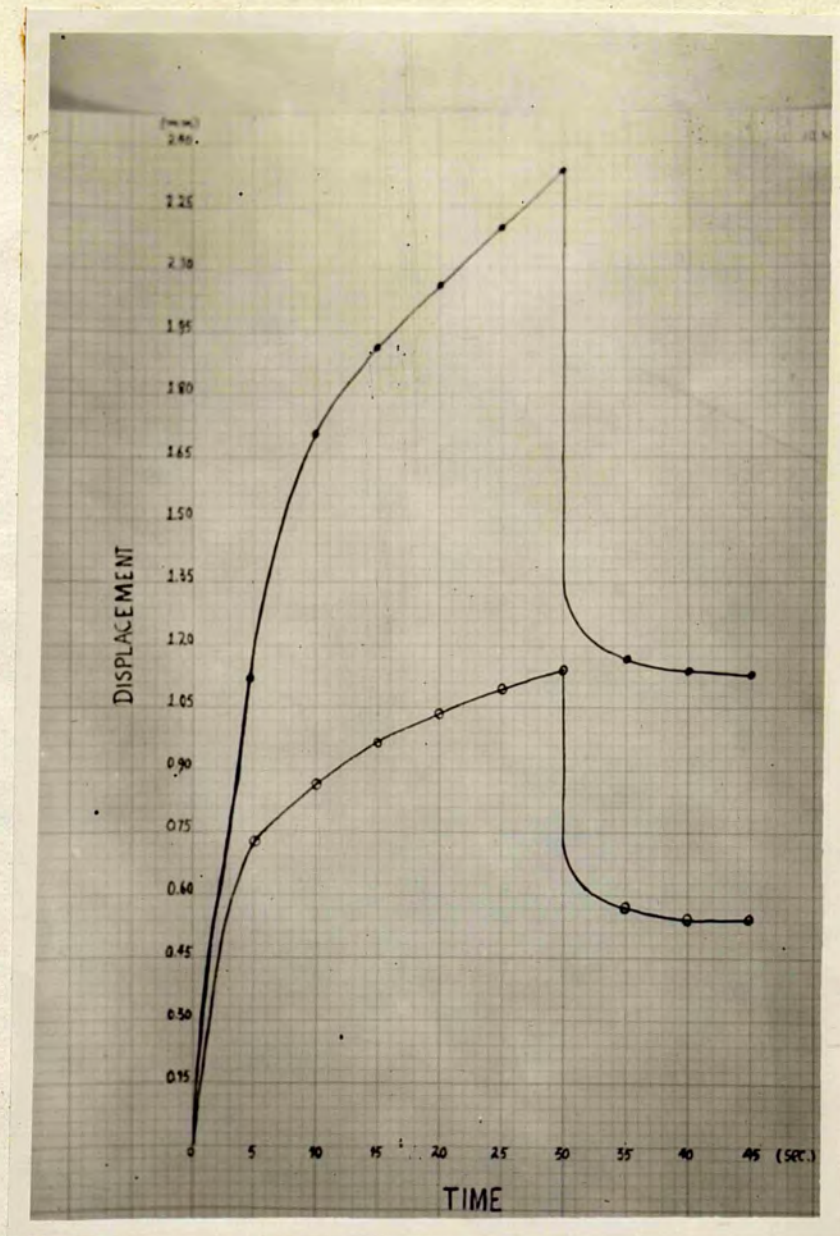


FIG. 5

The effect of ATP on plasticity of surface films of L-myosin.

(Substrate as in Fig. 4)

*Current through solenoid	260 mA.
Applied force	0.9 mg. weight.
Film pressure	3.0 dynes per cm.
ATP concentration	0.0001 M.

○ — ○ actomyosin alone.
 ● — ● actomyosin with ATP.

The qualitative significance of these and subsequent transient curves is immediately evident. An empirical treatment which permits a resolution of the curves into viscous and elastic components, and hence makes it possible to derive values for the elastic moduli of the films, is given in the Appendix.

* The current was turned off at 30 seconds.

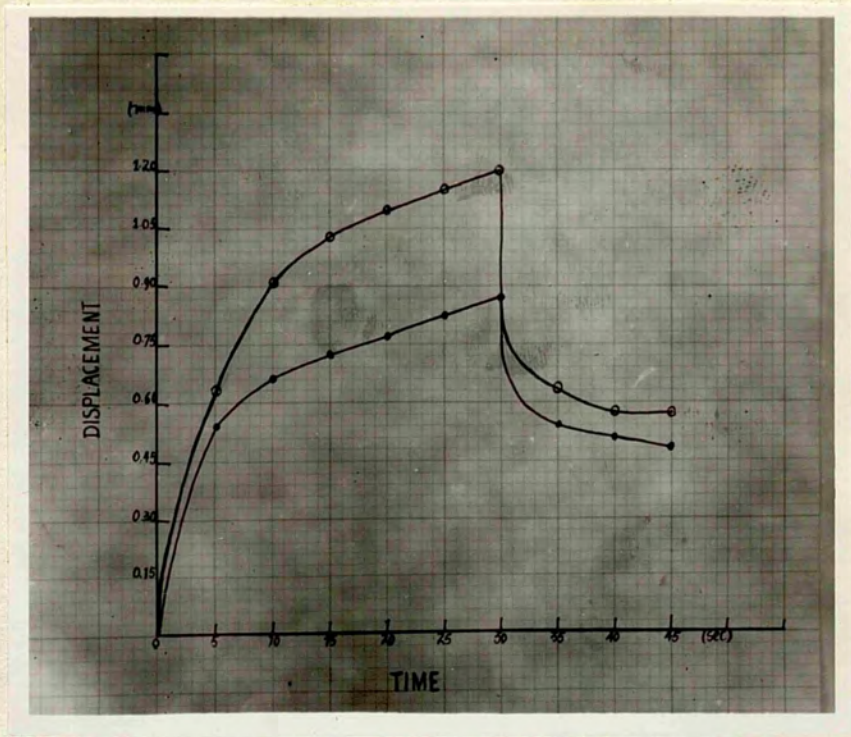


FIG. 6

The effect of ATP on plasticity of surface films of actomyosin.

(Conditions as in Fig. 5)

- — • actomyosin alone.
- — ◦ actomyosin with ATP.

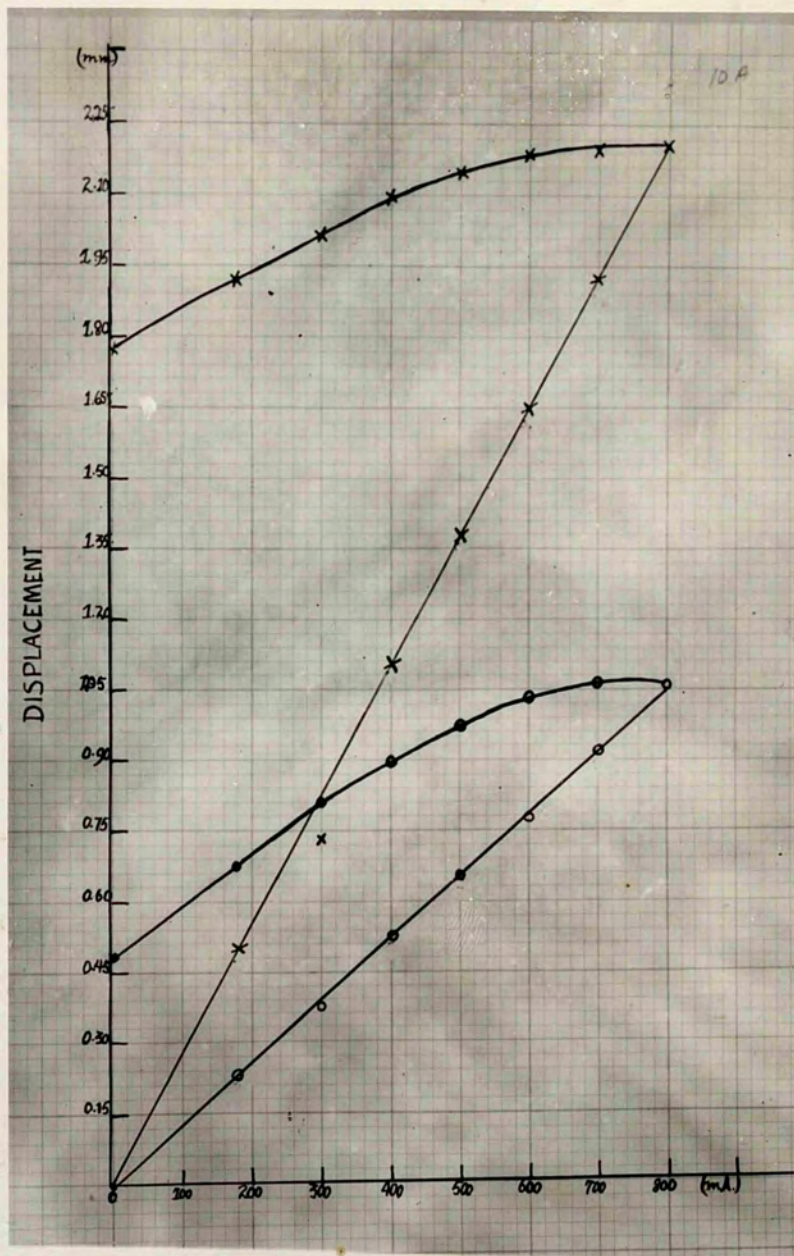


FIG. 7

Alternative procedure for the effect of ATP on surface films of L-myosin.

Measurements recorded 5 sec. after increase & decrease of current.

Applied force : 0.85 mg. weight per amp.

Film pressure : 3 dynes per cm.

ATP concentration: 0.0001 M.

o — o L-myosin alone.

x — x L-myosin with ATP.

switched on, thus causing a displacement of the disc in a forward direction towards the larger solenoid. Readings were taken of these distances every 5 seconds over a period of 30 seconds. At the end of this time, the current was switched off and readings were taken for another 30 seconds at 5 second intervals, during which the disc receded owing to the attracting force being withdrawn. Transient curves, as in Figs. 5 & 6, were obtained for L-myosin and actomyosin films. ATP is shown to have a marked effect in reducing the elasticity of L-myosin films and, to a lesser extent, of actomyosin films. No such effect was observed using films made from "artificial" actomyosin.

An alternative method consisted of allowing the current to increase by 100 mA every 5 seconds for 30 seconds, then decrease by the same amount to zero mA. An example of such stress-strain curves given by this method is shown in Fig. 7, where myosin was spread on the usual substrate with and without ATP and allowed to remain for 7 minutes before any measurements were recorded. Because of the relative ease of the first method, where the current is kept constant throughout the experiment, it was finally adopted as the standard procedure.

(ii) The ATP effect on the elasticity as a function of time.

When several readings were made on the same film, it was soon noted that films became stiffer with each reading. These time effects were investigated more closely, the initial readings being taken at various time intervals from the moment of

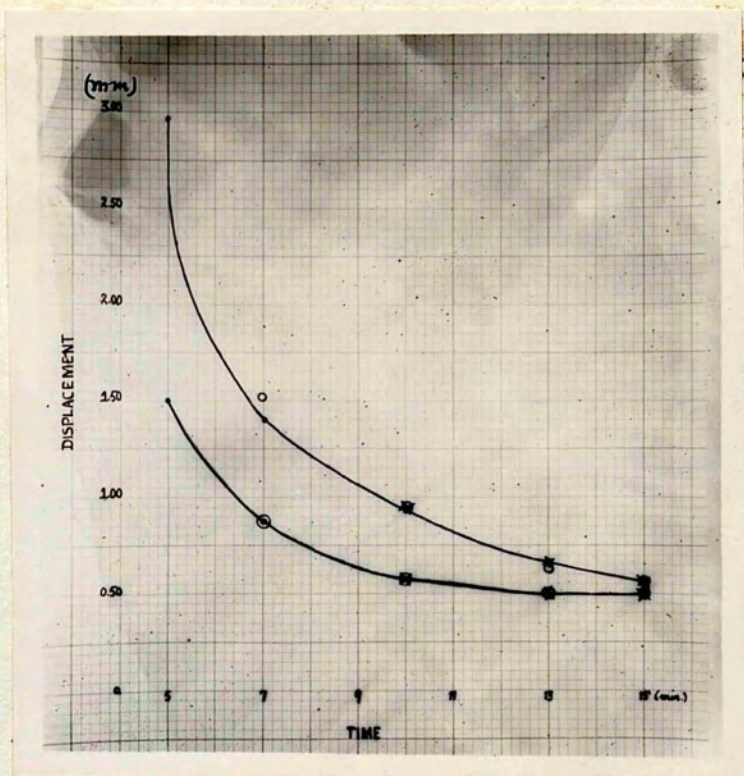


FIG. 8

The time course of the ATP effect on surface films of L-myosin.

Displacements recorded after 20 seconds.

Applied force : 0.42 mg. weight

Film pressure : 5 min. = 3, 7 min. = 3.5,
10 min. = 4, 13 min. = 4.4,
15 min. = 5 dynes per cm.

ATP concentration: 0.0001 M

Lower curve : L-myosin alone

Upper curve : L-myosin with ATP

• — • readings begun after 5 min. spreading time
 ○ — ○ " " " 7 " " "
 x — x " " " 10 " " "

application, i.e. elasticity measurements would be taken on one film after 7, 10, 13 and 15 minutes spreading time. On another freshly spread film measurements were started at 7 minutes and continued at the standard intervals as indicated above. The next series of measurements would be started after 10 minutes, and the next after 13 minutes. As illustrated in Fig. 8, reproducible values were obtained at standard times for L-myosin films spread on the usual substrate with and without ATP. This eliminated the possibility of the ATP effects being due to the initial disturbance of the film during the first reading, in which case the successive readings might have settled to the readings of the control at the corresponding time intervals. This experiment also showed that the ATP effect on L-myosin and actomyosin films was short-lived and disappeared at about the fourth or fifth determination.

(iii) Specificity of ATP plasticizing effect.

The effect of ATP in reducing the elastic modulus of L-myosin and actomyosin films was specific for adenosine triphosphate. No such effects were observed when L-myosin or actomyosin films were spread on 0.0001 M ITP or 0.0001 M ADP.

(iv) Specificity of the ATP effect for myosin and actomyosin.

To eliminate the possibility of a general ATP effect upon all protein films, bovine haemoglobin, kindly provided by Miss D.A. Norden, was spread on a substrate containing 0.4 M KCl, 0.01 M Tris (pH 7.4), in the presence and the absence of 0.0001 M

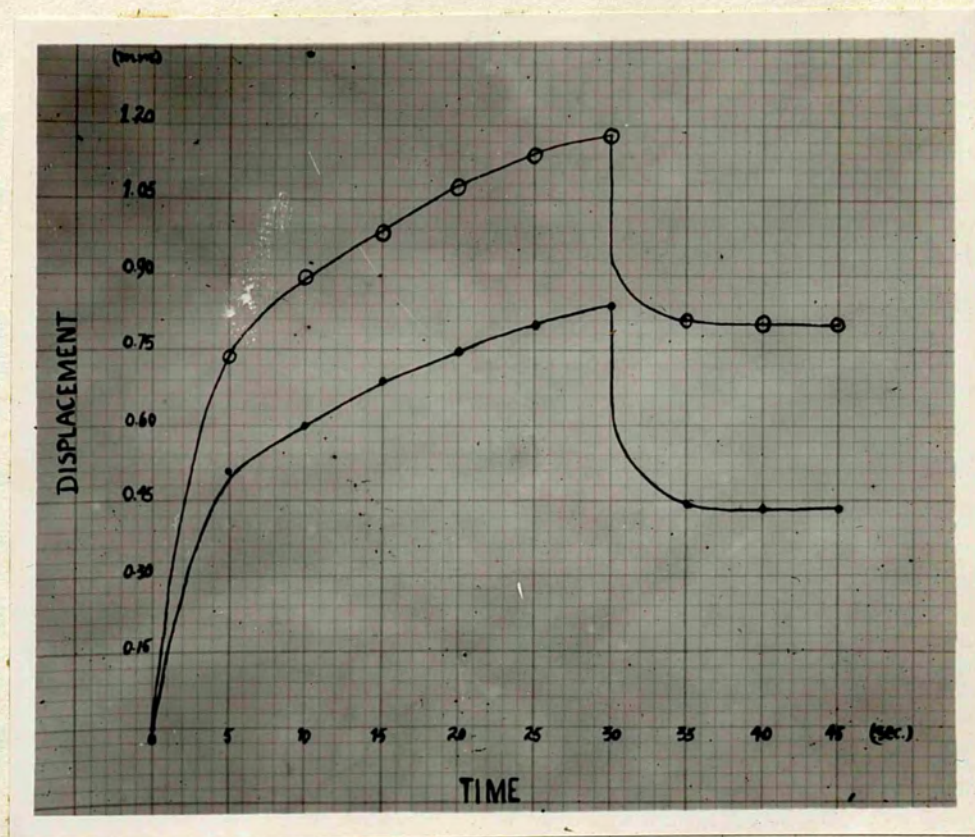


FIG. 9

The effect of ATP on films of aged actomyosin.

Current through solenoid: 260 mA.
 Applied force : 0.9 mg. weight
 Film pressure : 3.0 dynes per cm.
 ATP concentration : 0.0001 M

○ — ○ aged actomyosin alone
 ● — ● aged actomyosin with ATP

ATP. No effects of ATP either on the rate of spreading or on the elasticity of haemoglobin films could be detected. It was concluded that the ATP effect on L-myosin and actomyosin films might well be taken as a reflection of enzyme-substrate interaction.

(v) Correlation between ATPase, elasticity and area.

During the course of the present investigation it was noted that the ATP effect on the elasticity of L-myosin and actomyosin films was in some way related to the enzymic activity. With old preparations (10-14 days) the effect began to fall off until it was no longer detectable, and in fact with actomyosin films ATP produced a "stiffening" effect where the elastic modulus was actually increased. ^(Fig. 9) It was also noted that with aged preparations of actomyosin, no increase in the rate of spreading could be detected in the presence of 0.0001 M ATP. It was thought that there might be a correlation between the ATPase activity and the elasticity effects, and also between the ATPase activity and the area effects. A correlation between the ATPase activity and the degree of spreading is suggested by the findings of Hayashi (1952), working with actomyosin films, and also by Cheesman & Schuller (1953), experimenting with pepsin films. Both investigations show that films of these proteins do retain partial enzymic activity providing they are incompletely spread on a surface. The following experiments were designed to illustrate such a postulated correlation which might

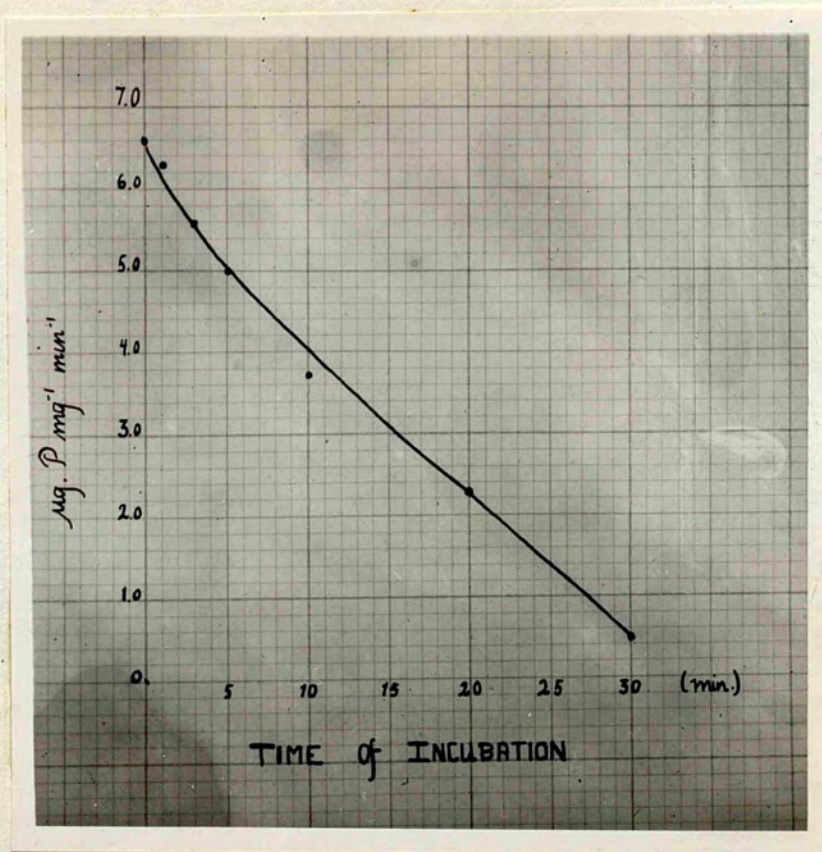


FIG. 10

The effect of incubation at 37°C on
ATP activity of actomyosin.

10 mg. actomyosin dissolved in 0.5 M KCl (1 mg/ml.)
 incubated at 37°C for various time intervals before determination
 of ATPase activity.

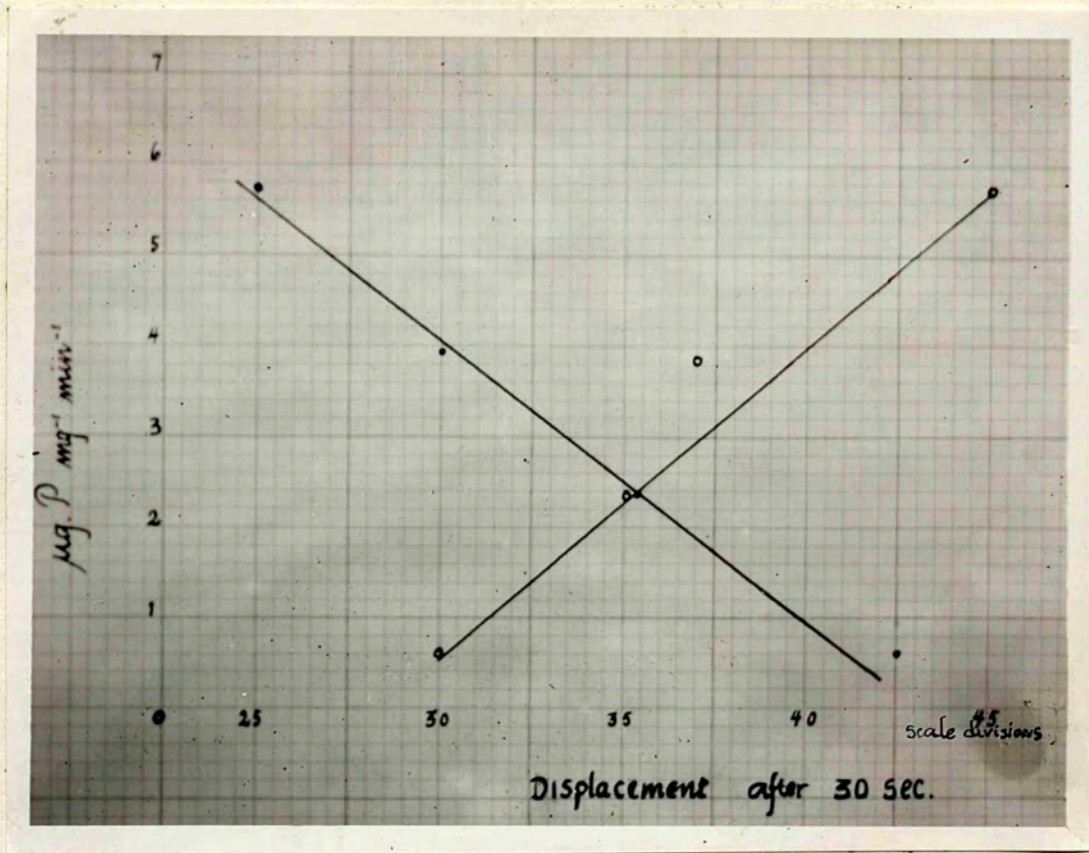


FIG. 11

Relation between ATPase activity and film plasticity of actomyosin.

(Conditions as in Fig. 5)

The ATPase activity was reduced by incubation at 37°C (see Fig. 10).

- — • actomyosin alone
- — ○ actomyosin with 0.0001 M ATP

ATPase activity µg.P/mg/min	Area of actomyosin film at pressure of 3 dynes/cm.	
	without ATP	with ATP
	m ² /mg.	m ² /mg.
5.7	0.530	0.577
3.9	0.537	0.577
2.4	0.545	0.577
0.6	0.577	0.577

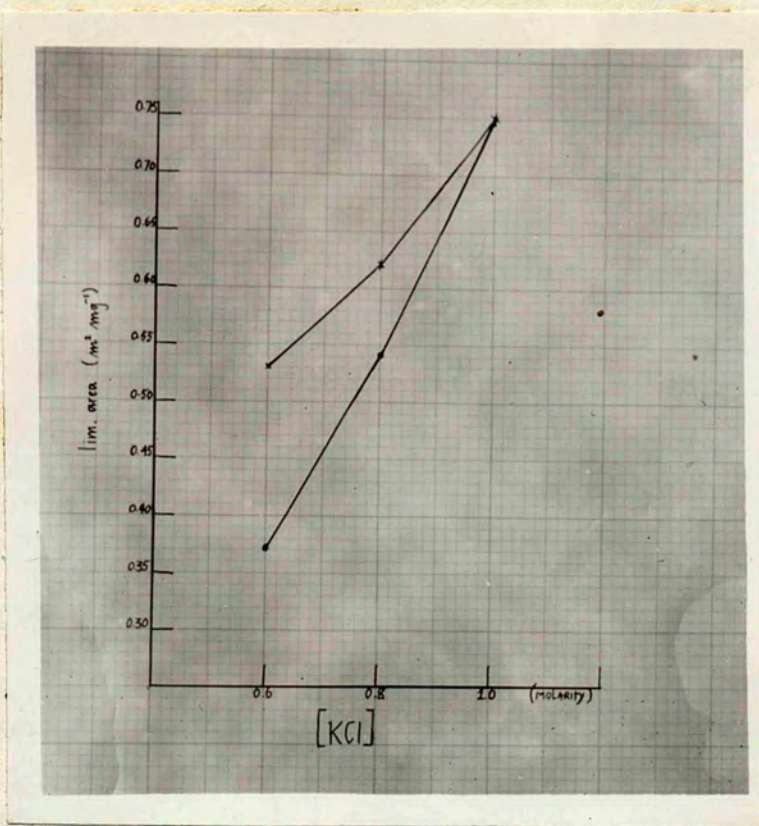


FIG. 12

The effect of KCl concentration on the rate of spreading of actomyosin films.

Films spread on substrate of 0.6, 0.8 or 1.0 M. KCl buffered to pH 7.4 with 0.01 M Tris buffer.

Measurements recorded after 7 minutes spreading time.

- — • actomyosin alone
- x — x actomyosin with 0.0001 M ATP

be of value in justifying the use of surface film techniques as a means of detecting chemical changes occurring at an interphase.

Samples of actomyosin (0.01% solution in 0.5 M KCl) were incubated at 37°C for 10, 20 and 30 minute periods (Fig. 10). Portions of each, 40 μ l (0.040 mg.), were then spread from an Agla syringe on the usual substrate (0.8 M KCl, Tris, 0.01 M, pH 7.4) and on this substrate containing 0.0001 M ATP. Force-area curves and elasticity measurements were taken, and the ATPase activity in bulk solution was determined for each set. The results, given in Fig. 11, show clearly that the higher the ATPase activity, the less fully spread is the protein and the greater is the plasticizing action of ATP. In this experiment an ATPase was obtained where ATP exerts no plasticizing action, and still a lower ATPase where ATP actually stiffens the actomyosin film.

From this experiment it is also shown that as the enzyme is aged at 37°C, thus decreasing the ATPase activity, there is a corresponding increase in the area of spreading (Fig. 12). Increasing KCl concentrations have also been long known to accelerate the spreading and so increase the spreading area. It was thought that a greater ATP effect on the elasticity of myosin films might be given if the protein were spread on a substrate of lower KCl concentration, where the enzyme would not be as fully spread. A KCl concentration curve was therefore determined for the spreading and elasticity effect

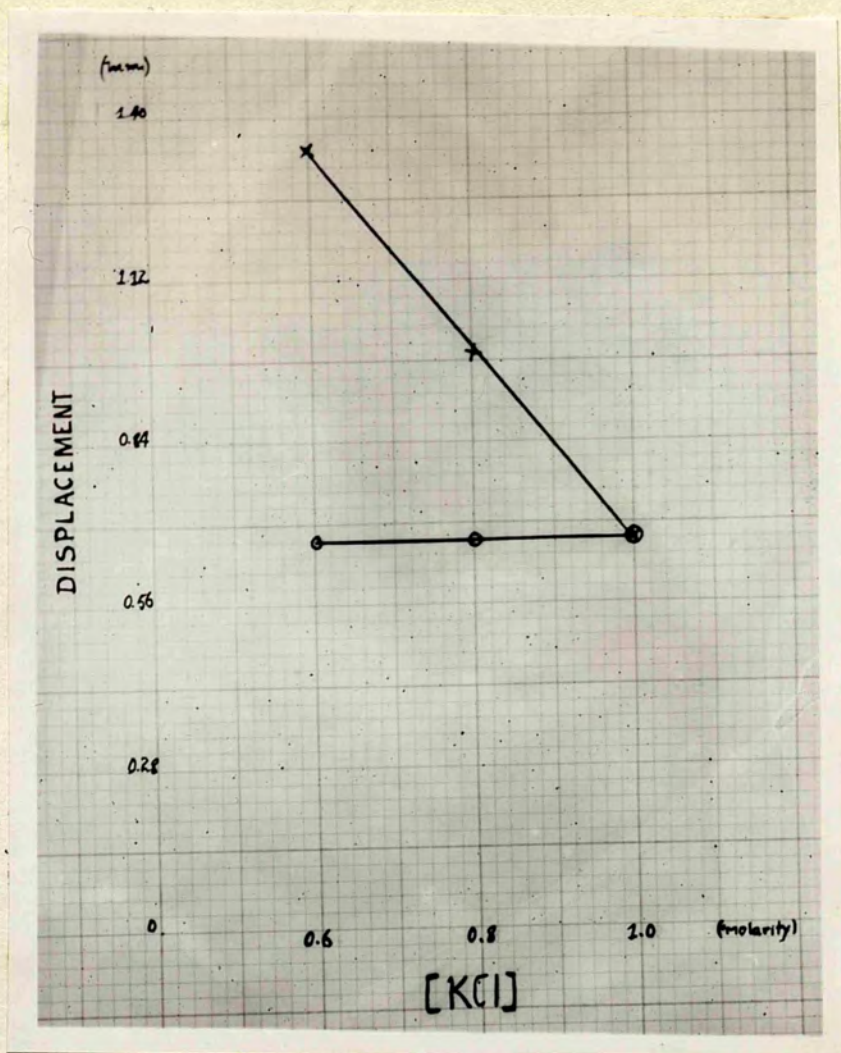


FIG. 13

The influence of KCl concentration on the ATP effect.

Films spread on KCl buffered to pH 7.4 with 0.01 M Tris buffer. Measurements for total displacements recorded after 7 minutes spreading time.

- o — o actomyosin alone
 x — x actomyosin with 0.0001 M ATP

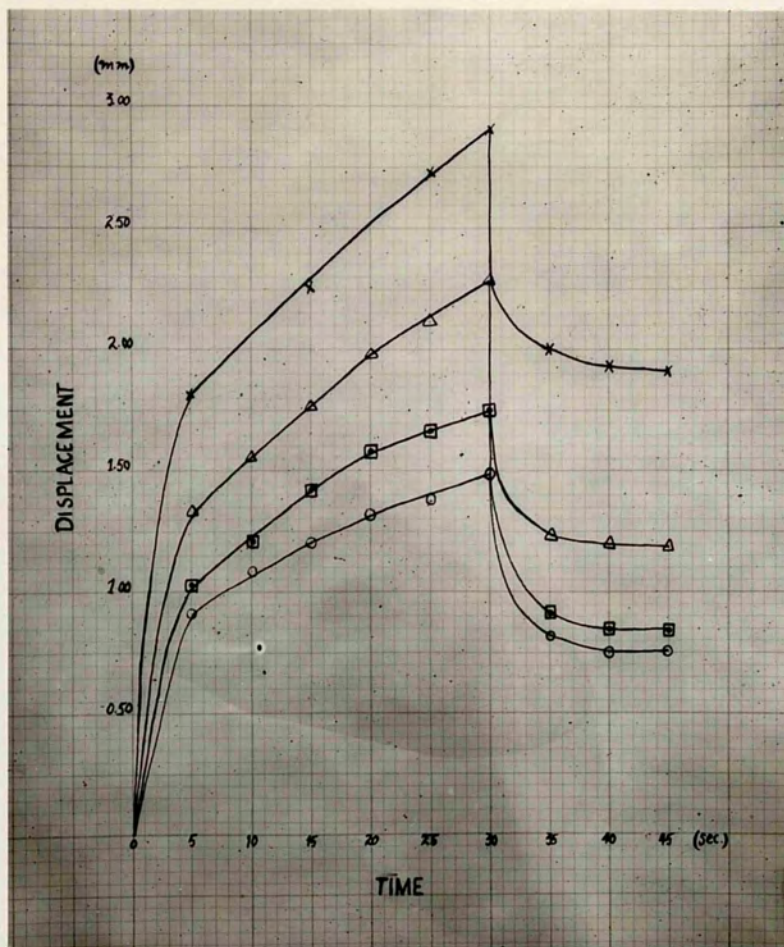


FIG. 14

The effect of magnesium ions on the ATP effect.

Current through solenoid: 400 mA.
 Applied force : 1.4 mg. weight
 Film pressure : 3 dynes per cm.
 ATP concentration : 10^{-4} M.

• — • L-myosin alone
 □ — □ L-myosin with Mg²⁺ (10^{-3} M or 10^{-5} M) } coincident
 x — x L-myosin with ATP
 Δ — Δ L-myosin with ATP and 10^{-3} M Mg²⁺
 o — o L-myosin with ATP and 10^{-5} M Mg²⁺

on actomyosin. 40 μ l. of actomyosin (0.001 mg.) was spread on the usual substrate containing 0.6, 0.8 or 1.0 M KCl with and without 0.0001 M ATP. As indicated in Fig. 13, the enzyme spreads more fully with increasing KCl concentration, but the ATP effect on the elasticity is not as great at higher KCl concentrations as at lower ones. The effect is eliminated completely with M KCl. It was concluded that the effect of ATP in reducing the modulus of elasticity of myosin films was dependent upon incompleteness of spreading, with partial retention of the protein in its native state, so that it continues to show a specific affinity for ATP.

(vi) The effect of Mg^{++} on the plasticizing action of ATP.

The plasticizing action of ATP on films of L-myosin and actomyosin was curiously affected by the addition of Mg^{++} . When the Mg^{++} concentration was less than that of ATP, a marked stiffening of the film resulted. When the Mg^{++} concentration was raised above that of ATP, the plasticizing effect of ATP was restored (Fig. 14). This observation served to support previous results where the elasticity effects were shown to be dependent upon the ATPase activity of the enzyme: when the Mg^{++} concentration is lower than ATP, inhibition of the ATPase sets in (Perry, 1956), when the opposite is the case the ATPase is active. Thus it would seem that these results are a reflection of ATPase inhibition and provide further evidence to show that the ATP effect on the elasticity of L-myosin and actomyosin films is largely dependent upon the ATPase of the enzyme.

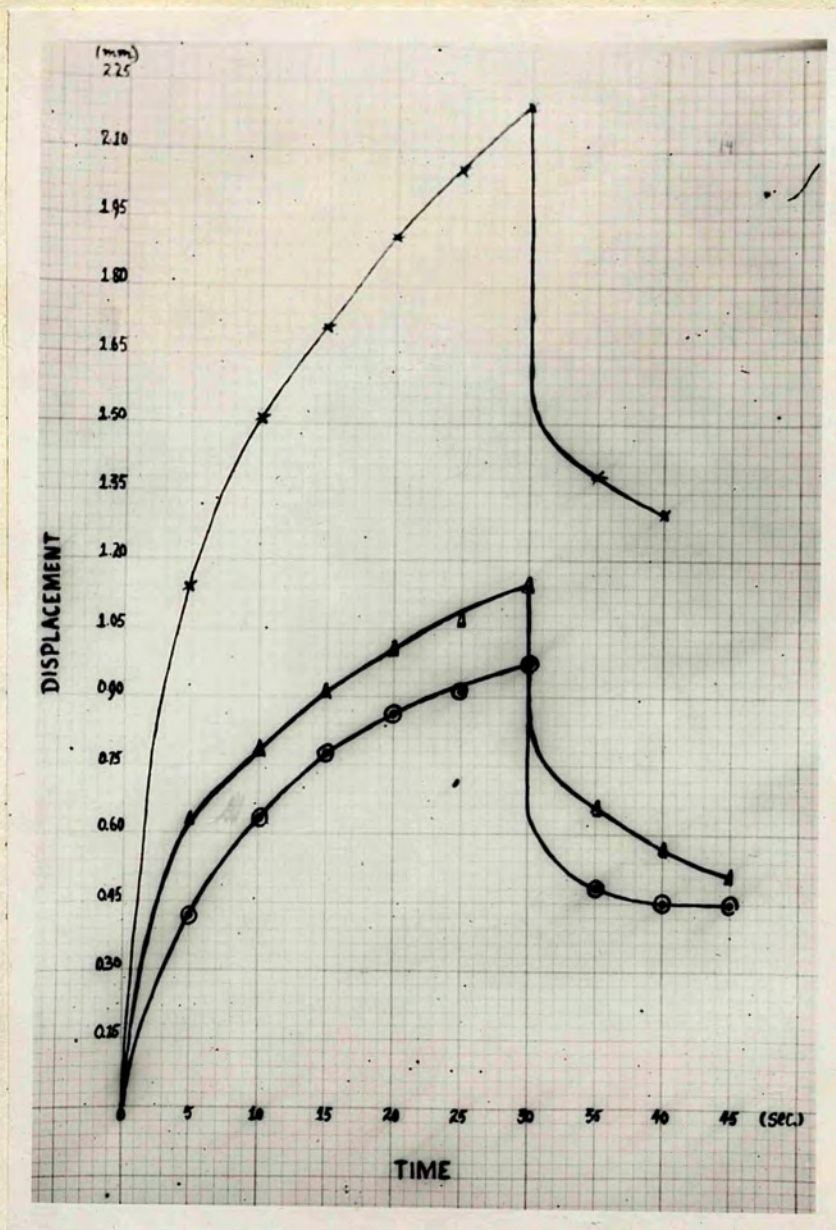


FIG. 15

Effect of ATP and ATP + DNP on films of L-myosin.

Current through solenoid: 260 mA.
 Applied force : 0.9 mg. weight
 Film pressure : 3.0 dynes per cm.
 ATP concentration : 0.0001 M.
 DNP concentration : 0.0009 M.

Calculated elastic moduli for films: (see Appendix)

• — •	L-myosin alone	:	3.3	dynes per cm ² .	} coincident
o — o	L-myosin and DNP	:	3.3	" " "	
x — x	L-myosin with ATP	:	1.5	" " "	
Δ — Δ	L-myosin with ATP + DNP	:	2.8	" " "	

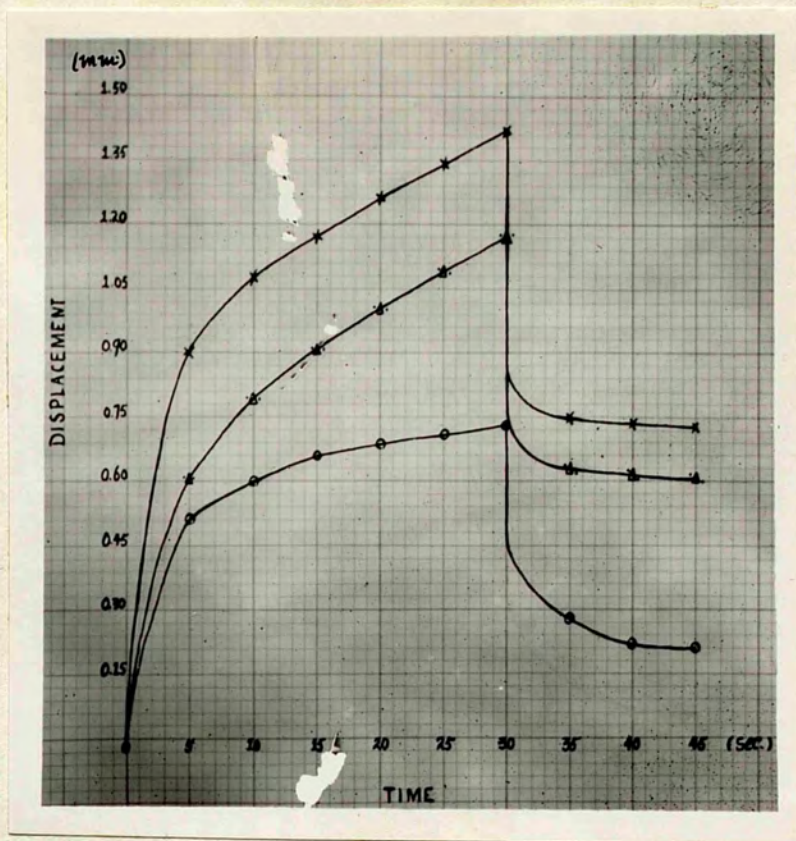


FIG. 16

Effect of ATP and ATP + DNP on films of actomyosin.

(Conditions as in Fig. 15)

- Δ — Δ actomyosin alone.
- Δ — Δ actomyosin with DNP. } coincident
- x — x actomyosin with ATP.
- o — o actomyosin with ATP + DNP.

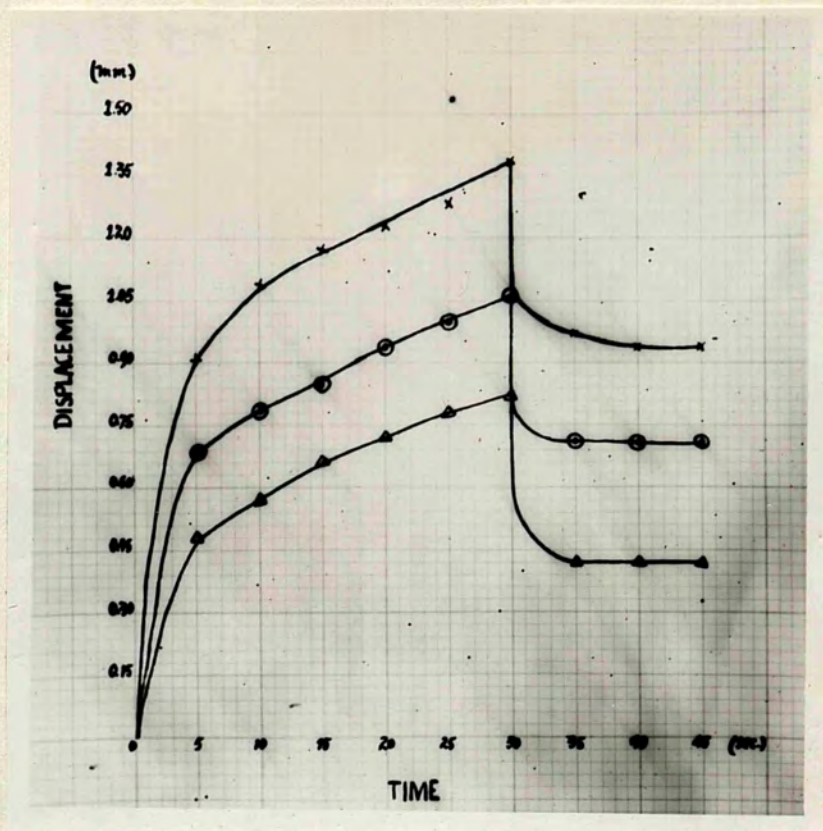


FIG. 17

Effect of ATP and ATP + DNP on films of aged actomyosin.

Current through solenoid: 260 mA.

Applied force : 0.9 mg. weight

Film pressure : 3.0 dynes per cm.

ATP concentration : 0.0001 M

DNP concentration : 0.0009 M

•—•	A—A	actomyosin alone
o—o	Δ—Δ	actomyosin with DNP
Δ—Δ	X—X	actomyosin with ATP
x—x	⊙—⊙	actomyosin with ATP and DNP

3) The influence of 2:4-Dinitrophenol on the ATP effect.

The results thus far obtained would indicate that either there is involved an actual phosphorylation of the myosin or the formation of an enzyme-substrate complex, which, in either case, would tend to reduce the elastic modulus of the film by a change in charge distribution. In view of the effect of DNP in **uncoupling** phosphorylation in mitochondrial systems, it seemed advisable to investigate the effect of this compound on the elasticity effects of L-myosin and actomyosin films. DNP (9×10^{-4} M) was therefore introduced into the substrate and the effects determined in the usual way. The effect of ATP on the elasticity was greatly reduced in the case of L-myosin (Fig. 15), and completely eliminated in the case of actomyosin, and produced a "stiffening" effect (Fig. 16). With aged preparations of actomyosin, where ATP showed a stiffening action, the DNP relieved this effect and permitted the ATP to exert its plasticizing action (Fig. 17). Mg^{++} ^(10^{-3} or 10^{-5} M) had no effect on DNP systems. These results can scarcely be taken to imply that a myosin-ATP complex is rendered unstable by DNP, since it is well known that DNP does, in fact, activate the ATPase activity of myosin (Greville & Needham, 1955; Chappell & Perry, 1955; Perry, 1957), which is inconsistent with a labilization of the enzyme-substrate complex. It seems far more likely that the myosin is phosphorylated by ATP, and that the phosphate groups are easily removed, or perhaps never introduced, in the presence of DNP.

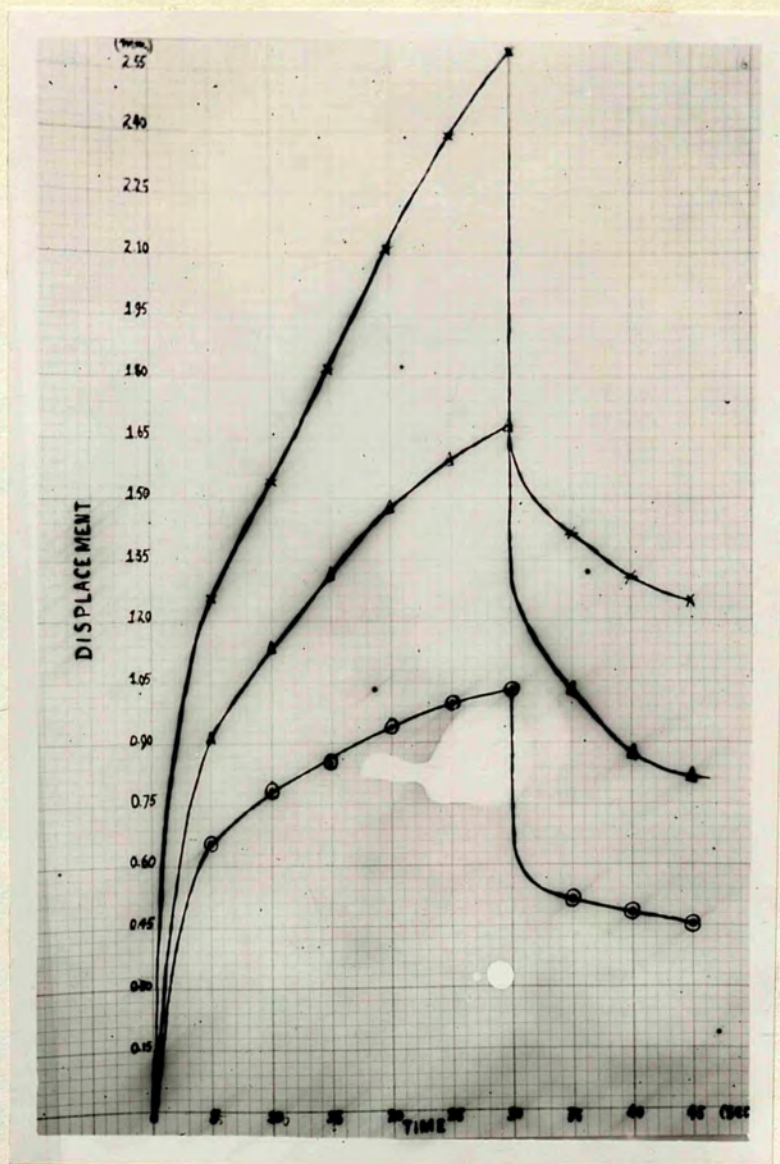


FIG. 18

Effect of ATP and ATP + fuadin on films of actomyosin.

Current through solenoid: 260 mA.
 Applied force : 0.9 mg. weight
 Film pressure : 3.0 dynes per cm.
 ATP concentration : 0.0001 M
 Fuadin concentration : 0.001 M

Calculated elastic moduli: (see Appendix)

•—• actomyosin alone	:	4.0	dynes	per	cm ²	}*
o—o actomyosin with fuadin	:	4.0	"	"	"	
Δ—Δ actomyosin with ATP	:	2.2	"	"	"	
x—x actomyosin with ATP and fuadin:	:	1.7	"	"	"	

* coincident

4) The effect of fuadin on the ATP effect.

The experiments of Weber (1955) show that a transfer of phosphate groups between ATP and $AD^{32}P$ by actomyosin becomes detectable in the presence of fuadin (antimony-bis [catechol disulphonic acid], Na salt). Weber suggests that an intermediate phosphorylated myosin is stabilized by fuadin. If this hypothesis were, in fact, correct, and if the effect of ATP on films of actomyosin and myosin were due to phosphorylation of the protein, then one might anticipate an augmentation of this effect in the presence of fuadin, always provided that this substance did not in itself affect the mechanical properties of the protein film.

Fuadin (10^{-3} M) was therefore introduced into the substrate and readings were taken on films of L-myosin and actomyosin with and without ATP (0.0001 M) in the usual way. As shown in Fig. 18, fuadin greatly increased the ATP effect on films of actomyosin. The effect, however, was completely eliminated on L-myosin films. It is interesting to compare this finding with the results of Ulbrecht and Ulbrecht (1957) who showed that actin was essential to the exchange reaction. Fuadin was found not to affect the rate of spreading or the elasticity of L-myosin or actomyosin films.

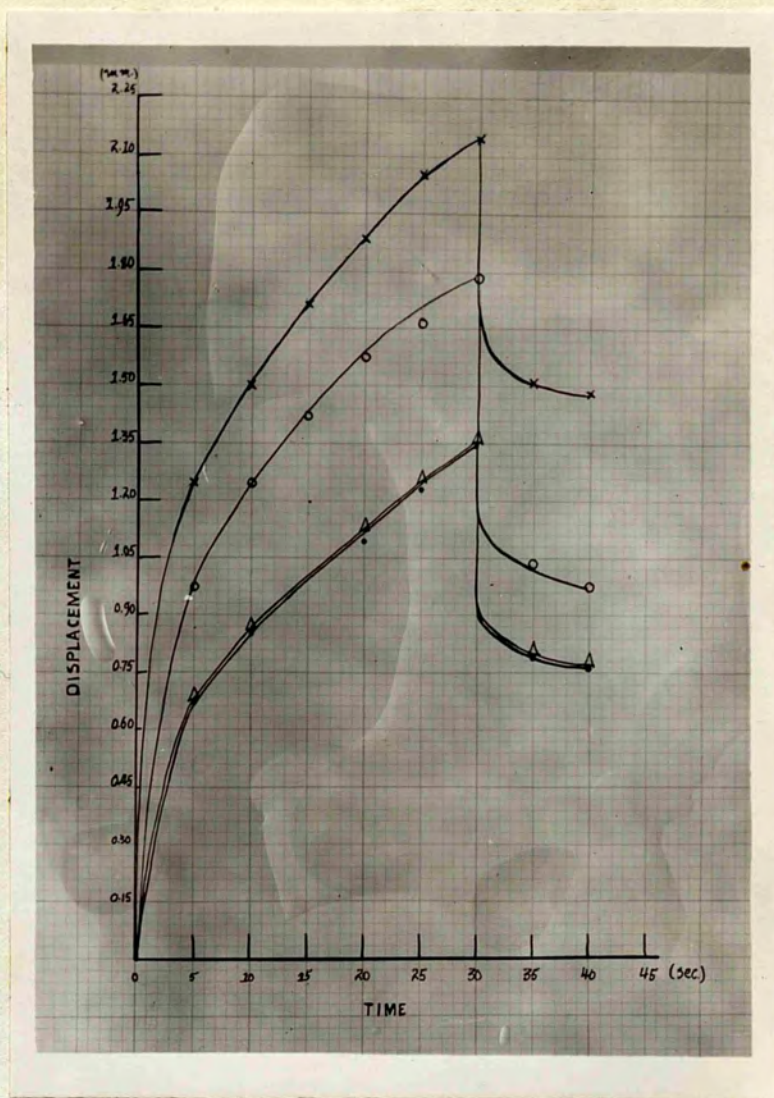


FIG. 19

Effect of ATP and ATP + EDTA on films of L-myosin.

Current through solenoid: 260 mA.
 Applied force : 0.9 mg. weight
 Film pressure : 3.0 dynes per cm.
 ATP concentration : 0.0001 M
 EDTA : 0.001 M

• — • L-myosin alone
 Δ — Δ L-myosin with EDTA
 x — x L-myosin with ATP
 o — o L-myosin with ATP and EDTA

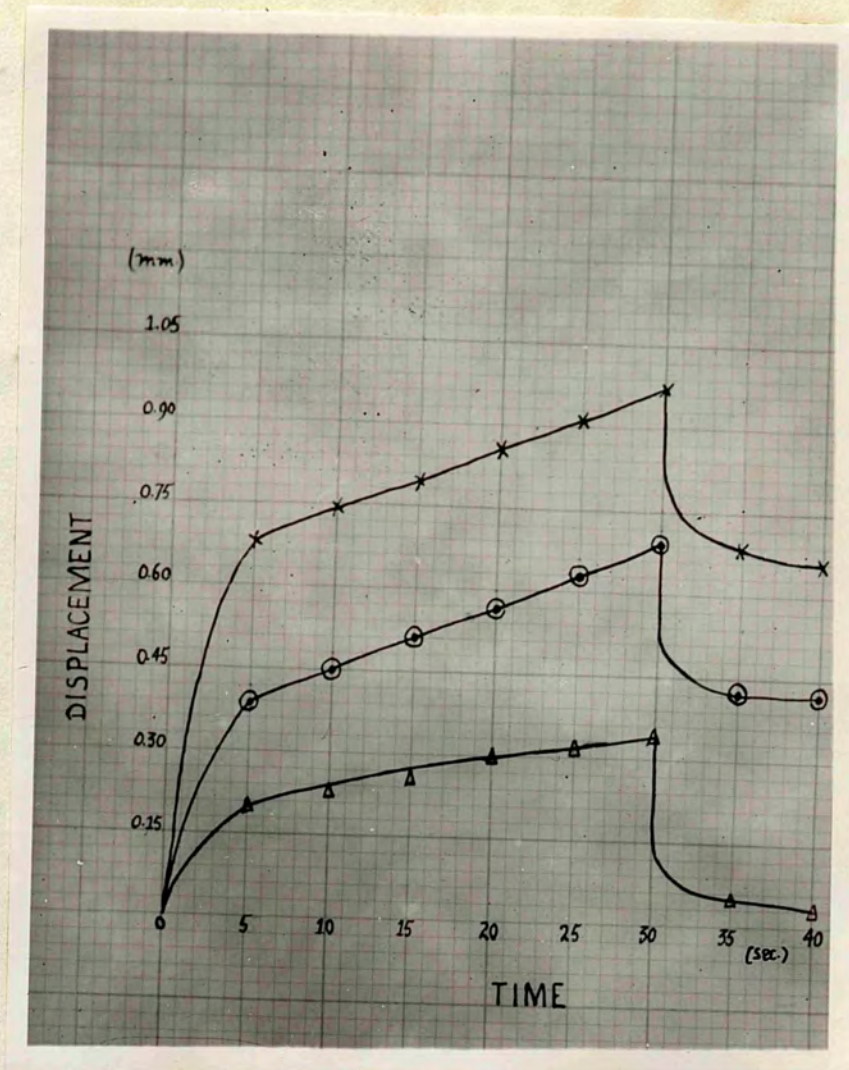


FIG. 20

Effect of ATP and ATP + EDTA on films of actomyosin.

Current through solenoid: 110 mA.
 Applied force : 0.4 mg. weight
 Film pressure : 3 dynes per cm.
 ATP concentration : 0.0001 M
 EDTA concentration : 0.001 M

• — • actomyosin alone
 o — o actomyosin with EDTA } coincident
 x — x actomyosin with ATP
 Δ — Δ actomyosin with ATP and EDTA

5) The effect of EDTA and PCMB on the ATP effect.

EDTA (10^{-3} M), which is known greatly to enhance the ATPase activity of L-myosin and actomyosin, also reduced the plasticizing effect of ATP on spread films of these proteins (Figs. 19 & 20). When Mg^{++} was added to the EDTA-ATP system so that the Mg^{++} concentration exceeded that of ATP, a marked stiffening of the film and great inhibition of the spreading resulted.

PCMB, an inhibitor of the ATPase at concentrations above 10^{-5} M, was found at 10^{-3} M concentration to eliminate the effect of ATP on the elasticity.

Chapter III

DISCUSSION OF RESULTS

It is known that myosin contains 0.04 - 0.01% phosphate (Bailey, 1942; Lajtha, 1948) which is not removed during the purification procedure. Buchthal et al. (1949) have shown that 30% of the total phosphate is present as orthophosphate, 30% as readily hydrolysable phosphate and 40% as difficultly hydrolysable phosphate. The possibility of any alteration by ATP or DNP in the phosphate content of myosin has been examined more closely with the earlier experiments of this investigation. The results are in agreement with those of Mommaerts (1950) and Buchthal et al. (1949), who show that although there is some phosphate present in myosin, this content remains unchanged when myosin is brought in contact with ATP. In Buchthal's earlier experiments (1949) he found that there was an uptake of phosphate and adenine by myosin and actomyosin; however, he later showed (1951) that this uptake was entirely due and dependent on the presence of iron, which he found contaminating his ATP and protein preparations. In the absence of iron no phosphate or adenine uptake was detectable. Thus it appears that if a phosphorylated myosin exists at all, its life is very transient and it is extremely difficult to isolate or detect. This can readily be seen from the results of Tables II and III, where measures were adopted to provide the most favourable conditions

for the stability of this labile complex, and yet no uptake was to be observed.

Using surface film techniques in an endeavour to study the interaction between enzyme and substrate at an interface, it was observed that ATP had a marked effect upon the rate of spreading of actomyosin films. Now it is known from the work of Hayashi (1952) that films of actomyosin do retain, at least partially, their enzymic activity when they are incompletely spread. Cheesman and Schuller (1953) have confirmed this observation for films of pepsin. This would imply that although denatured protein contributes to the physical properties of the film, some protein has retained its native configuration and contributes to the mechanical properties of the film. When actomyosin films are spread on substances containing ATP, the ATP may have little affinity for the denatured portions of the enzyme, but will have great affinity for those molecules which are still in their native state. This interaction produces an acceleration of the spreading. That this acceleration of spreading by ATP is not observed on films of L-myosin would imply that the complex actomyosin is split into its two components, actin and myosin, at the moment of application. As Cheesman & Davies suggest (1954), the actin will now have a chance to spread at a faster rate, although the myosin spreading may be somewhat retarded owing to its interaction with ATP, which now becomes bound, at least transiently, to some sites on

the enzyme molecule, thus tending to stabilize the enzyme to some extent against denaturation.

The acceleration in the spreading of actomyosin films produced by EDTA might well be due to a similar affinity of EDTA for some sites on the enzyme surface, the increase in the rate of spreading resulting from polar effects. Urea could be expected to exert its accelerating effect by the fission of intramolecular hydrogen bonds.

The effects on the elastic properties of films are also a reflection of enzyme-substrate interaction. As has already been suggested, a decrease in the modulus of elasticity of L-myosin and actomyosin films, brought about by ATP, may be explained by a change in electrostatic charge or structural change due to interaction with ATP. The effect is greater on L-myosin films than on actomyosin films and is not given at all with films made from "artificial" actomyosin. This suggests the disorganization of the actin and myosin components in relation to each other. If the effect is due to the contribution of ionized groups, either phosphorylation of the myosin molecule or adsorption of the ATP molecule on the protein surface would provide a possible explanation.

The fact that DNP eliminates the ATP effect on films of L-myosin and actomyosin strongly suggests that the intermediate compound presumably responsible for the plasticizing effect is either not formed or broken down. The evidence suggests that

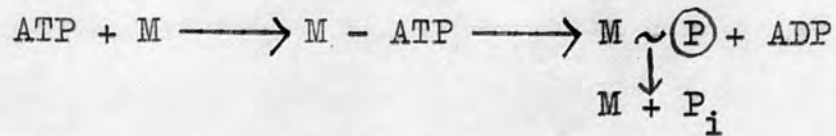
the effect of ATP on films of myosin and actomyosin in reducing the elastic modulus is due to the formation of a phosphorylated myosin, unstable in the presence of DNP.

If the mechanism of the DNP stimulation of the ATPase activity involves a breakdown of phosphorylated myosin, then an analogy may be drawn between this mechanism and that in which DNP inhibits oxidative phosphorylation in mitochondrial systems. As the result of his work with cyclophorase preparations, Teply (1949) concludes that DNP increases the dephosphorylation of a primary ester, and suggests that a phosphatase activity has been induced in such systems by DNP. Hunter (1951) proposes that DNP does not stimulate a previously existing phosphatase, but that a transphosphorylase acting between the primary ester and ATP is cleaved into two phosphatases in the presence of DNP.

Working with digitonin extracts of mitochondria, Cooper and Lehninger (1957) have shown that DNP inhibits the ATP- $^{32}\text{P}_i$ exchange reaction and oxidative phosphorylation, and at the same time stimulates the ATPase. They suggest that DNP may have a general effect on phosphatases and on phosphate-transferring enzymes.

With the L-myosin system it would seem that phosphate groups are transferred from ATP to the enzyme, thus producing a phosphorylated myosin. When DNP is present, a labilization of these phosphate groups takes place, which results in an

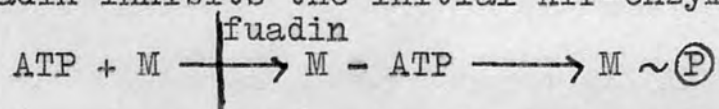
acceleration of the rate of the ATPase reaction:



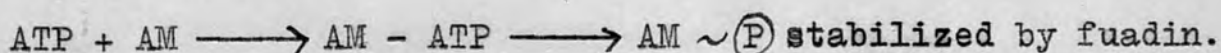
With the actomyosin system, however, it is conceivable that the ATP effect is, in fact, a combination of two mechanisms: one by which phosphate groups are transferred from ATP to the myosin, and the other by which actin acts as a receptor for these phosphate groups. It has recently been suggested by Weber (1957) that F-actin plays a major role as a receptor of phosphate groups after the interaction of myosin and ATP. In his modified phosphorylation theory, Weber proposes that actin accepts the phosphate groups resulting from hydrolysis, which are in turn transferred to functional groups on the L-myosin filament. This is in agreement with the experiments of Ulbrecht and Ulbrecht (1957) who show that actin is essential to the exchange reaction, i.e. an exchange of phosphate groups catalyzed by actomyosin which these authors have shown to occur between AD^{32}P and ATP in the presence of fuadin, and to a lesser degree in the absence of this inhibitor. If in this alternative mechanism of phosphorylated myosin actin is essential to obtain a transfer of phosphate groups from ATP to myosin, the effect of DNP in eliminating the ATP effect on actomyosin films may be explained in view of the above mentioned findings of Teply and Hunter, i.e. DNP converts the transferring enzyme responsible for the exchange reaction into a phosphatase and thereby increases the ATPase activity of actomyosin.

The possible function of actin as an acceptor of phosphate groups might also explain the finding that the elasticities of films of "artificial" actomyosin are not affected by ATP; it might be suggested that in this case the actin is unable to play its part in accepting phosphate groups because of the alteration in the spatial arrangement of the two proteins. Ulbrecht and Ulbrecht (1957) have found that the exchange reaction does not occur with "artificial" actomyosin preparations.

Fuadin was also found to augment the ATP effect on actomyosin films, but to eliminate the effect on L-myosin films. This is in keeping with the above mentioned experiments of Ulbrecht and Ulbrecht (1957). It would seem that with L-myosin films, fuadin inhibits the initial ATP-enzyme reaction



However, the presence of actin in "natural" actomyosin in some way prevents the fuadin from exerting its inhibitory effect. The fuadin is capable now only of preventing the release of phosphate from phosphorylated actomyosin, and so the ATP effect is greatly reinforced



Because of the effects of DNP eliminating the effects of ATP on myosin and actomyosin films, it would seem that the fuadin effects were due to a phosphorylated actomyosin and not merely to an adsorption of the ATP molecule.

The elasticity effects on films of L-myosin and actomyosin are dependent on the ATPase activity and on the degree of spreading (Figs. 11 & 12). Aged films of actomyosin when spread on substrates containing ATP become more rigid. It is possible that certain specific groups are still capable of binding ATP, even though the enzymic function has been eliminated. Under these circumstances it seems reasonable to suppose that ATP can act as a cross-linking agent. When samples of actomyosin were incubated at 37°C, in order to decrease the ATPase activity, a consequent change in the elasticity of the films could be correlated with the disorganization of the protein as reflected in an increase in the rate of spreading and a diminution in the ATPase activity.

The effect of DNP in relieving the stiffening effect of ATP on films of aged actomyosin may suggest that when the ATPase is very low, or even absent, the ATP-actomyosin complex may be labilized by DNP. Thus any residual ATP effect observed may be due to a residual ATPase.

The magnesium ion effects on the elasticities of films of myosin and actomyosin might be explained in the light of the proposal of Perry (1956) that when the Mg^{++} concentration exceeds that of ATP, ATPase activity can be exhibited. When the ATP concentration exceeds that of Mg^{++} , inhibition of the ATPase results. At low Mg^{++} concentration (ATP exceeds Mg^{++}), an elimination of the ATP effect and indeed a stiffening of films

of L-myosin and actomyosin is brought about. This might be taken as a reflection of inhibition of the ATPase activity with consequent establishment of crosslinkages by the ATP. When the Mg^{++} concentration is raised above that of ATP, ATPase activity can be displayed and ATP can again exert its plasticizing action.

The effect of EDTA in reducing the ATP effect on films of L-myosin and actomyosin might also be explained by a postulation that EDTA causes the breakdown of a phosphorylated myosin intermediate, which mechanism would also explain the increased ATPase activity in the presence of EDTA.

PCMB, an inhibitor of myosin ATPase at concentrations above 10^{-5} M, in decreasing the ATP effect on the elasticity of L-myosin and actomyosin films, probably inhibits the formation of the initial ATP-enzyme complex and thus would tend to reduce the ATP effect.

Although direct chemical evidence is still lacking, the behaviour of surface films of myosin and actomyosin lends considerable support to the view that these proteins undergo phosphorylation by ATP. The conditions of the experiments were such as to permit the detection of altered protein molecules of extremely high lability, since ATP was necessarily always present in great excess for their reconstitution. If further work should

indicate that phosphorylated actomyosin is so transient as to be detectable only by indirect techniques, this would be no argument against its participation in muscular contraction. It must, however, be emphasized that no immediate relevance to the physiological problem is claimed for the results of this investigation, which are, at the most, only suggestive.

Chapter IV

SUMMARY

The purpose of this investigation has been to provide evidence for or against the phosphorylation of myosin and actomyosin during interaction of these proteins with adenosine triphosphate.

1. In Chapter I the historical aspects of the proteins of the myofibril are summarised. The enzymic properties of these proteins are discussed. The theories relevant to muscular contraction and relaxation are outlined. The possibility of the phosphorylation of myosin, together with experiments designed to detect such phosphorylation, are likewise examined. The effect of 2:4-dinitrophenol on mitochondrial ATPase is considered and a possible correlation between the mechanisms by which DNP stimulates the ATPases of mitochondria and myosin is suggested. The effects of EDTA and of Mg^{++} on myosin ATPase are also discussed.

2. The experimental results are presented in Chapter II.

(1) No phosphate uptake by L-myosin or actomyosin in the presence of ATP could be detected using purely chemical methods.

(2) The properties of surface films of L-myosin and actomyosin have been studied, and the rate of spreading of actomyosin has been found, in agreement with earlier observations, to be greatly increased in the presence of ATP. ITP and ADP have no

effect on actomyosin films. DNP, fuadin, Mg^{++} and Ca^{++} likewise have no effect on the rate of spreading of L-myosin or actomyosin films with and without the presence of ATP. EDTA, however, greatly accelerates the rate of spreading of actomyosin films.

(3) The moduli of elasticity for films of L-myosin and actomyosin are greatly reduced in the presence of 0.0001 M ATP. This effect is not given with films of artificial actomyosin.

(4) This effect appears only when the protein is incompletely spread and when it shows, in bulk solution, a considerable ATPase activity.

(5) The effect is specific for ATP, and not given by ITP or ADP. ATP does not give this effect on films of haemoglobin.

(6) The effect is short-lived and transient, disappearing after about 13 minutes spreading time.

(7) DNP greatly reduces the effect with films of L-myosin and completely eliminates it with films of actomyosin.

(8) Fuadin eliminates the effect with L-myosin films and reinforces it with films of actomyosin.

(9) The effect is reduced by EDTA and completely inhibited by PCMB.

3. The results are discussed in Chapter III. From the above mentioned observations it would seem that phosphorylated myosin has been detected indirectly from the properties of surface films of L-myosin and actomyosin in the presence of ATP. The

fact that phosphorylated myosin and actomyosin are unstable in the presence of DNP would suggest that DNP produces a labilization of phosphate groups on the protein in a manner analogous to that in which it inhibits phosphorylation in mitochondrial systems. The effect of fuadin in reinforcing the ATP effect on films of actomyosin suggests that the protein is phosphorylated, as this compound also promotes the exchange of phosphate groups by actomyosin between ATP and $AD^{32}P$. That fuadin eliminates the ATP effect on films of L-myosin suggests the inhibition of the formation of the enzyme-substrate complex in the absence of actin.

It is suggested that EDTA removes the ATP effect in a manner similar to that of DNP, by labilizing the phosphate groups of the phosphorylated protein. The action of PCMB in completely eliminating the ATP effect on films of both L-myosin and actomyosin no doubt reflects the inhibition of ATPase in films of L-myosin and actomyosin.

APPENDIX

The Elastic Moduli of Protein Monolayers.

A. An empirical treatment of the transient curves.

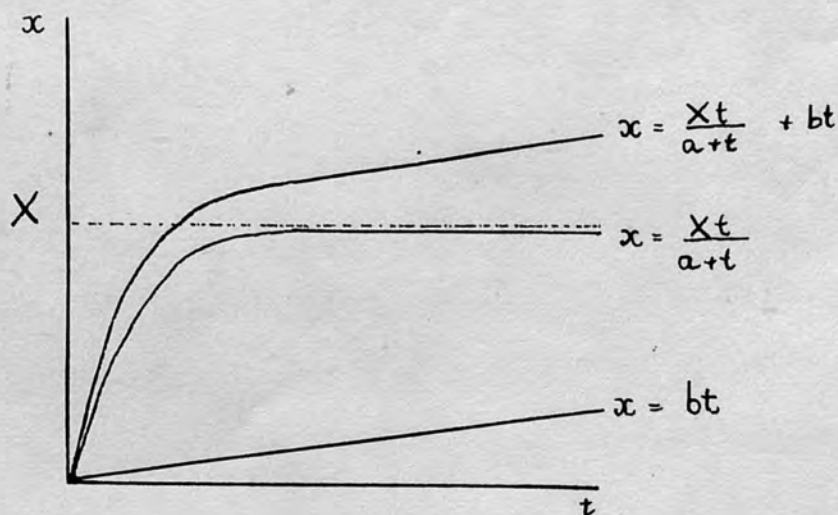
I am indebted to Dr. D.F. Cheesman for the following considerations.

Since protein monolayers display mixed viscoelastic properties, it is reasonable to suppose that the transient curves may be resolved into two components, representing the time courses of movement against an elastic and a viscous resistance respectively. Such a resolution, quite empirical in nature, has been found possible.

It is found that, with a suitable choice of constants, the experimental curves can be fitted to an expression of the type:

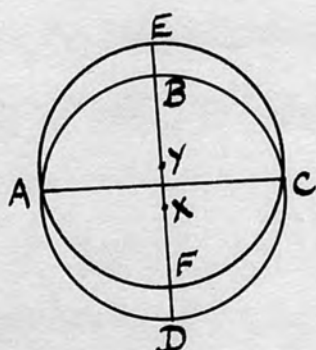
$$x = \frac{Xt}{a+t} + bt$$

where x represents the displacement and t the time of transit, while a , b and X are constants.



A reasonable interpretation of this relationship appears to be that the term $\frac{Xt}{a+bt}$ represents the movement against an elasticity, with limiting displacement X , whereas bt gives a movement of constant velocity against a viscosity which is an inverse function of b .

B. Calculation of elastic moduli.



Let the disc of radius r move from a position ABCD to a position AECF, the centre being displaced from X to Y .

$$\text{Let } XY = 2x$$

The crescent AECB represents the strain on the film in the direction of compression, the

crescent ADCF that in the direction of extension.

$$\text{Crescent AECB} = \text{segment AEC} - \text{segment ABC}$$

$$\begin{aligned} &= \frac{\pi r^2}{2} + \left[x\sqrt{r^2-x^2} + r^2 \sin^{-1}\left(\frac{x}{r}\right) \right] \\ &\quad - \frac{\pi r^2}{2} + \left[x\sqrt{r^2-x^2} + r^2 \sin^{-1}\left(\frac{x}{r}\right) \right] \\ &= 2x\sqrt{r^2-x^2} + 2r^2 \sin^{-1}\left(\frac{x}{r}\right) \end{aligned} \quad (1)$$

When x is small with relation to r this becomes

$$\text{Crescent AECB} = 4xr \quad (2)$$

$$\text{Total strain (compression and extension)} = 8xr.$$

Let $r = 6$ mm. and $x = 0.5$ mm.

$$\text{According to (1) Strain} = 2 \left(\sqrt{35.75} + 72 \sin^{-1} \frac{1}{12} \right) = 23.90$$

$$\text{According to (2) Strain} = 24 \text{ mm}^2.$$

The error involved in the approximation is hence negligible for the displacements in the present investigation.

Let the force applied to the float be mg dynes, and the radius of the float r cm.; then, if the displacement $2x$ and the limiting displacement "X" be measured in cm., an elastic modulus E of the film is given by

$$E = \frac{\text{stress}}{\text{strain}} = \frac{mg}{8xr} \text{ dynes per cm}^2.$$

The elastic modulus thus derived is evidently of a composite nature. It nevertheless offers a provisional basis for the comparison of film elasticities.

C. Determination of the experimental stress.

The force exerted by the field of the solenoid on the float was found by direct measurement with a torsion balance sensitive to 0.2 mg. For this purpose the float was attached by a silk thread to the arm of the torsion balance. The solenoid was placed with its axis vertical, directly beneath the suspended float. Calibration curves were made for force against distance at constant current, and force against current at constant distance. An inverse square law applied in the former case and a direct linear relationship in the latter.

D. The experimental curves.

In the text of Figs. 15 & 18, values are given for the elastic moduli of the corresponding films. In these cases the constants in the empirical equation were determined by trial, since the experiments had not been continued to times sufficiently long to permit the use of an extrapolation procedure.

ACKNOWLEDGEMENTS

I wish to acknowledge my great indebtedness to Dr. D.F. Cheesman for his unceasing interest and devoted guidance during the course of research which has led to the writing of this thesis. His constant advice and numerous helpful suggestions have been a great source of encouragement to me throughout its entire progress.

I should also like to thank Professor M.M. Murray of the Physiology Department for having so generously allowed me the use of the laboratories in her department.

Thanks are also due to Mr. J. Gibbons who has helped with the photographic reproductions, and to Mrs. J. Dunn for the typing of the manuscript.

To my superiors, I am deeply grateful for the time and the opportunity they have given me to perform this research.

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