Studies on the Bound Nucleotide of Muscle Fibrils

A thesis presented by

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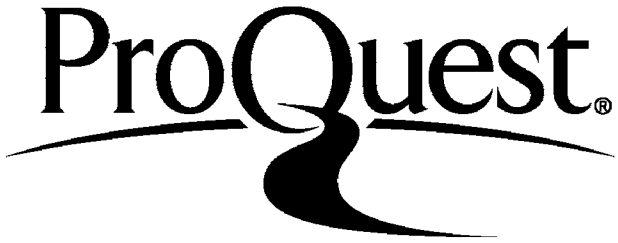


ABSTRACT

The possibility was investigated that phosphorus uptake from ATP or uptake of ATP molecules by the muscle proteins occurs during contraction and relaxation of rabbit myofibrils. It was found that the phosphorus content was the same in myofibrils contracted with ATP and the control myofibrils although phosphorylation of actin, if it occurred, may not have been detectable.

An examination by paper chromatography of the UV-absorbing substances in washed frog and rabbit myofibrils revealed the presence of the adenine nucleotides, IMP, hypoxanthine, and adenine. ADP gave the most intense UV-absorbing spot and was presumably derived from F-actin. An oligo - or polynucleotide, possibly derived from RNA, and a green pigment, were also found.

The adenine nucleotides in HCl and $HClO_4$ extracts of washed frog myofibrils were estimated. Approximately 2.90 - 3.10 μmoles/gm. dry weight of adenine nucleotides were present, most of which was ADP. Considerably more ATP was found in the HCl extracts than was present in the $HClO_4$ extracts, in which most of the nucleotide was ADP. The concentration of ATP in the HCl extracts was observed to alter after neutralization in association with a change in the ADP content. It is suggested that a phosphokinase is present in the HCl extracts, but not in the $HClO_4$ extracts, which becomes active after neutralization and synthesises the ATP from ADP and an unidentified substance XP.

A higher ATP/ADP ratio was found in the HCl extract from control myofibrils than in the extract from myofibrils contracted with ATP.

When a frog muscle homogenate was washed with successive small volumes of water, the myofibrils swelled and structural protein passed into solution, to a much greater extent when conditions favoured relaxation than contraction of the fibrils. The extracted protein was precipitated by Mgions and contained adenine nucleotide. It was concluded to be a form of actomyosin containing F-actin in a low state of polymerisation.

A theory of contraction is suggested which is consistent with these observations.

Contents

ABBREVIATIONS

ATP	adenosine triphosphate
ATPase	adenosine triphosphatase.
ADP	adenosine diphosphate
AMP	adenosine 5' phosphate
CP	creatine phosphate
CPkinase	creatine phosphokinase
CTP	cytidine triphosphate
DNP	2:4 dinitrophenol
DOC	deoxy-cholate
FDNB	1 fluoro 2: 4 dinibrobenzene
GTP	guanosine triphosphate
IAA	iodoacetic acid
IMP	inosinic acid
P_i	inorganic phosphate
RNA	ribonucleic acid
TCA	trichloracetic acid
UTP	uridine triphosphate

CHAPTER I

INTRODUCTION

(a) Early work on the chemistry of the contractile system.

The first indication of the importance of nucleotides in the process of muscular contraction came in 1931 when Lohmann isolated ATP from muscle and found that it contained two energy-rich phosphate bonds. Previously, in 1927, Eggleton and Eggleton found that CP present in muscle was decomposed during a long series of contractions. Meyerhof and Suranyi in the same year found that unexpectedly large amounts of heat were released during its enzymatic decomposition and it was concluded that the energy of hydrolysis of the phosphate bond must be used in some way by muscle during contraction. However, Lohmann (1934) discovered that CP hydrolysis in muscle apparently only occurred by way of an enzymatic reaction in which it was used as a source of P_i for ATP formation from ADP, the ATP being subsequently hydrolysed. He therefore suggested that ATP and not CP might be the source of energy most closely connected with muscular contraction.

Englehardt and Ljubimova (1939) identified the ATPase of muscle with one of the major muscle proteins, "myosin", which hydrolysed only the terminal high energy phosphate group of ATP to give ADP and P_i in the presence of Mg ions. This confirmed the view that ATP could be the primary source of energy which is replenished during contraction at the expense of CP.

The extensive work of Szent-Györgyi and Straub (1941-42) established techniques for the isolation and characterisation of the muscle protein

"myosin", and it was found to be a complex of two distinct proteins. Szent-Györgyi renamed the original "myosin" actomyosin, and its two components were named actin and myosin. It was found that threads of actomyosin contracted in the presence of ATP. Myosin itself was shown to be non-contractile although it catalysed the breakdown of ATP, but on addition of actin the contractile properties were restored. Szent-Györgyi suggested that the functional unit of muscle is the ATP complex of actomyosin. It was estimated that the adsorption of one ATP molecule to one molecular unit of myosin (100,000 g.) was possible from the calculated ATP and myosin contents of muscle.

A major discovery was the finding that actin could exist in two forms. Straub showed that actin extracted from an acetone powder of rabbit muscle as a clear aqueous solution (G-actin) became highly viscous and eventually formed a gel (F-actin) on the addition of Mg or Ca ions. Various physical measurements showed that a polymerisation of actin molecules had taken place, the molecules possibly being connected by metal atom bridges to form long rod-like fibres which would have sufficient strength to do mechanical work. Straub thought that the electric charge change during polymerisation might bring about contraction of a muscle.

(b) <u>The bound nucleotide of actin</u>.

Further work on actin by Straub and Feuer and also by Laki (1950) revealed that the polymerisation of isolated actin was accompanied by an apparently non-enzymic splitting of ATP which was bound to the actin

molecule. The first evidence that nucleotide was bound to muscle protein structure was obtained by Buchthal et al. (1947) who showed the presence of nucleotide in actomyosin threads which did not appear to be merely adsorbed. Two years later, Straub and Bettko found that ATP and ADP in actomyosin were inaccessible to enzymes which would attack free ATP and ADP. This work led to the discovery by Straub and Feuer (1950) that actin contains ATP in a bound form which disappears during polymerisation, P_i being released in a 1:1 ratio with the ATP utilised. Presumably ADP-actin was formed. Therefore G-actin is ATP-actin and F-actin is ADP-actin. The possibility that the protein was directly phosphorylated transiently during the polymerisation process was suggested. Depolymerisation was obtained by dialysis of F-actin against a dilute ATP solution.

The ATP content of the acetone powder used for extraction of the G-actin solution was estimated to be less than the ATP content of that solution and therefore ATP must have been formed during the extraction process. The conclusion was reached that in the acetone powder actin must be present in the polymerised form, F-ADP-actin. Since the acetone powder apparently contined no enzyme which would catalyse a reaction producing sufficient energy for the formation of ATP in G-actin, it was assumed that the energy for its formation was derived from the mechanical changes involved in depolymerisation.

Quantitative measurements by Mommaerts (1952) established that one molecule of ADP was bound to each molecular unit of F-actin having

a molecular weight of 57,000 gm. The initial heat of contraction of muscle had been estimated by Hill (1949) and it was calculated that if ATP hydrolysis does take place in a single twitch, 0.25 - 0.30 μM ATP/gm/twitch must be broken down to account for the heat production. Mommaerts calculated from this figure that if all the actin present in 1 gm. of muscle polymerised once, the amount of ATP dephosphorylated would be equivalent to the amount of dephosphorylation in the contractile phase of a single twitch. About 5×10^{-7} moles of ATP would be broken down. Hence there was evidence that actin polymerisation as well as myosin ATPase were, on energetic considerations, capable of providing the energy necessary for muscular contraction.

Estimation of the nucleotide bound in the intact myofibril was undertaken by Perry (1952). The spectra of TCA extracts of exhaustively washed rabbit myofibrils indicated that only adenine nucleotides were present and enzymic estimations of AMP, ADP and ATP yielded the following results:-

AMP μmoles/gm.	ADP μmoles/gm.	ATP μmoles/gm.
0.88	2.7	0.47

ADP therefore was the major adenine nucleotide present. Perry suggested that <u>in vivo</u> all bound nucleotide exists as ATP, 10% of the total cell ATP, since several powerful phosphorylating systems are present in an intact muscle, e.g. CP-CPkinase and oxidative phosphorylation. True binding of the nucleotide in the myofibrils was proved by the observation that ATP and ADP were stable in a system containing myokinase,

ATPase and adenylic deaminase, which would attack free nucleotides. Added ATP was not adsorbed but brought about contraction of the fibrils.

(c) Early attempts to detect ATP and CP breakdown during a single muscle twitch

Since both myosin ATPase activity and actin polymerisation would both presumably lead to an increase in ADP and P_i during contraction, several workers tried to show that this occurred, but the evidence obtained was not conclusive. Munch-Petersen (1953) found that dephosphorylation of ATP to ADP took place during the rising phase of a single twitch of electrically stimulated tortoise muscle. An increase in ADP of 6-7% was found after contraction, which would be sufficient to account for the heat production in a single twitch if this is dependent on dephosphorylation of ATP. However, Mommaerts (1955) could detect no such change, nor any change in the CP which, it was thought, might rephosphorylate ADP to ATP so quickly that one could not expect to detect any change in their concentrations. The work of Fleckenstein et al. (1954) provided evidence for the breakdown of CP after potassium contracture of frog rectus abdominis at 0° and 20°, but there was no change in the level of ATP or ADP. After electrical stimulation at 20° a similar result was obtained. However, in both experiments the amount of CP broken down would not have provided the amount of energy used by the muscle. After electrical stimulation at 0° the levels of ATP, ADP and CP remained constant, but an increase in P_i was obtained which was sufficient to account for the work done, assuming that it was derived from an energy-rich phosphate

compound. Fleckenstein concluded that there is a source of phosphate bond energy in muscle which is closer to the contraction process than either ATP or CP.

(d) The role of actin in the contractile process.

No further progress was made in the elucidation of the manner in which the adenine nucleotides might be used for energy during contraction until 1957. Attention was then again directed to the properties of the isolated proteins, particularly actin. Ulbrecht and Ulbrecht (1957) discovered that a transfer of phosphate took place between $AD^{32}P$ and ATP in isolated myofibrils and actomyosin. The rate of $AD^{32}P$ transfer was ten to twenty times the rate of ATP splitting and magnesium ions were required, Ca ions being inhibitory. The reaction could be explained to result from the phosphorylation of the contractile proteins by ATP in the following manner:-

$$ATP + \text{protein} \rightleftharpoons ADP + \text{protein} - P.$$

The finding that, in fibrils from which most of the myosin had been extracted, the exchange reaction still took place led to the suggestion that actin was phosphorylated by the ATP. Ulbrecht and Ulbrecht thought that in muscle, the energy from ATP might be transferred to the contractile mechanism in this way; after the release of P_i contraction would result from interaction between myosin and actin. The exchange reaction did not take place in actin from acetone powders, nor in reconstituted actomyosin, which indicated that structural organisation of the muscle proteins was essential for the transfer.

Changes in the striated structure of myofibrils after contraction induced by ATP, observed by Hanson and Huxley (1954), apparently occur as a result of changes in the relative positions of the actin and myosin filaments. Myosin and actin appear to be present as separate overlapping filaments in the myofibrils and contraction occurs by the sliding of the actin filaments across the myosin filaments so that shortening of the individual filaments does not take place. Hanson and H. E. Huxley (1955) suggested that cyclical changes in the actin filaments, perhaps occurring by successive depolymerisation and repolymerisation of actin molecules, could lead to small changes in their length and their successive attachment and detachment to the myosin filaments. This process would result in the movement of the actin filaments relative to the myosin filaments, but neither filament would be appreciably shortened.

A. F. Huxley and Niedergerke (1954) observed changes in the banded structure of a living fibre which could also be interpreted in this way. However, A. F. Huxley has suggested a mechanism to explain the sliding motion of the filaments which does not involve the polymerisation of actin. The existence of side pieces on the myosin filaments is postulated and between two such projections a group capable of combining with a group on the actin filaments oscillates back and forth between its equilibrium position as a result of thermal agitation. It is suggested that combination between the groups on actin and myosin takes place spontaneously when the oscillating groups on the myosin filaments are in a position where combination will result in a tension being exerted on the

actin filaments. The separation of the filaments is brought about when a high energy phosphate compound combines with sites on the actin filament close to the binding site with myosin. The compound is then dephosphorylated and its fragments detached from the filament, so that the initial conditions are restored and the combination of actin to points further along the myosin filament can be made.

The objection to the participation of actin polymerisation and depolymerisation in contraction and relaxation respectively until 1959 was that there was no evidence as to how a physiological depolymerisation could take place. Straub (1950) thought that it should occur by the phosphorylation of bound ADP but could find no evidence that ADP in actin was accessible to phosphorylating enzymes. In the depolymerisation of F-actin, which he obtained by dialysis against dilute ATP, ATP molecules replaced the ADP molecules of the F-actin. He thought that actin ATP might be resynthesised by mechanical forces involved in depolymerisation. This did not seem unlikely as actin polymerisation appeared to be a non-enzymic process also.

However, Strohman (1959) found that reversible depolymerisation could take place enzymically. When F-actin was dialysed against CP in the presence of CPkinase, the bound ADP was rephosphorylated at the expense of the CP and G-actin was thus formed. At no point was nucleotide released into the dialysis medium. The lowering of the ionic strength was necessary to obtain depolymerisation and in 0.1 M KCl, in which actin is maintained in the polymerised state, the ADP of F-actin was not phosphorylated by the CP-CPkinase system. In 1954, Perry had shown that in an intact fibril the

bound ADP was also unreactive in the presence of this system. However, he found that very low concentrations of added ADP brought about rapid contraction of the fibrils, presumably by means of the ATP synthesised in the presence of the CPkinase. It was remarkable therefore that a similar concentration of ATP added in the absence of the CP-CPkinase system did not induce contraction. Perry (1955) concluded that contraction depends on phosphate turnover rather thon on the presence of a given concentration of ATP; turnover could be maintined by the myofibrillar ATPase acting on catalytic amounts of ATP replenished from ADP by the CPkinase system.

If the reversible depolymerisation of actin demonstrated by Strohman (1959) took place <u>in vivo</u>, he suggested that it could explain why attempts to detect ATP breakdown in a single muscle twitch had been unsuccessful or had detected a smaller change in ATP and ADP concentrations than was expected. If in fact, ATP is split during contraction, it could take place through a dephosphorylation and rephosphorylation of only the adenine nucleotide bound in the myofibril to actin and no immediate change in sarcoplasmic ATP would be evident.

(e) <u>The nature of the nucleotide-actin bond</u>.

The results of Martonosi et al. (1960) have shown that when G-actin is incubated with ATP ($14C$), complete equilibration between bound and free nucleotide takes place in a very short time. Therefore the ATP of G-actin is not as tightly bound as Strohman originally thought. His conclusion was based on the finding that ATP in G-actin was non-dialysable,

but Martonosi et al. found evidence that this could be due to charge effects at the cellulose membrane, since ATP in G-actin is dialysable in the absence of salts through a collodion membrane. However, they agreed that ADP in F-actin is connected in a very strong bond with the protein, since no significant equilibration took place between ADP(^{14}C) and the ADP of F-actin. No evidence could be found that ADP is rephosphorylated from P_i during depolymerisation of actin or that transphosphorylation from added ATP to bound ADP took place.

Martonosi et al. suggested the following scheme to explain their results:-

$$G + ATP \rightleftharpoons G - ATP \qquad (1)$$

$$G - ATP \xrightarrow{\text{salt}} F - ADP + P_i \qquad (2)$$

$$F - ADP \xrightarrow{\text{salt removed}} G - ADP \qquad (3)$$

$$G - ADP + ATP(^{14}C) \rightleftharpoons G - ATP(^{14}C) + ADP \qquad (4)$$

The phosphorylation of ADP-actin during its dialysis against CP in the presence of CPkinase can be explained without postulating direct phosphorylation of the bound ADP. Instead, the CP-CPkinase system could phosphorylate the ADP formed in reaction (4) and the resulting ATP could combine with G-actin according to reaction (1).

However, experiments <u>in vivo</u> by the same workers (1960) failed to give support for the occurrence of the series of reactions given above or for the participation of polymerisation and depolymerisation of actin during contraction and relaxation respectively in the intact animal.

Injections of ^{32}P-orthophosphate were given to rabbits, rats or pigeons and the time course of radioactive incorporation into ADP bound to actomyosin was measured in periods ranging from one hour to 48 hours. According to the theory of Straub and Feuer (1950) relaxed muscle contains G-ATP-actin, and the *in vitro* results of Martonosi et al. indicate that its bound nucleotide should equilibrate with the nucleotide pool. However, when the specific activities of the α and β phosphates of the ADP isolated from actomyosin were compared with the specific activities of the α and β phosphates of ADP from TCA extracts of the whole muscle, it was obvious that equilibration had not occurred. Martonosi et al. therefore concluded that the presence of G-actin in relaxed muscle was unlikely.

However, experiments using injected radioactive isotopes may be criticised on the grounds of possible permeability barriers which could bring about a preferential use of the injected material by enzyme systems other than that under investigation.

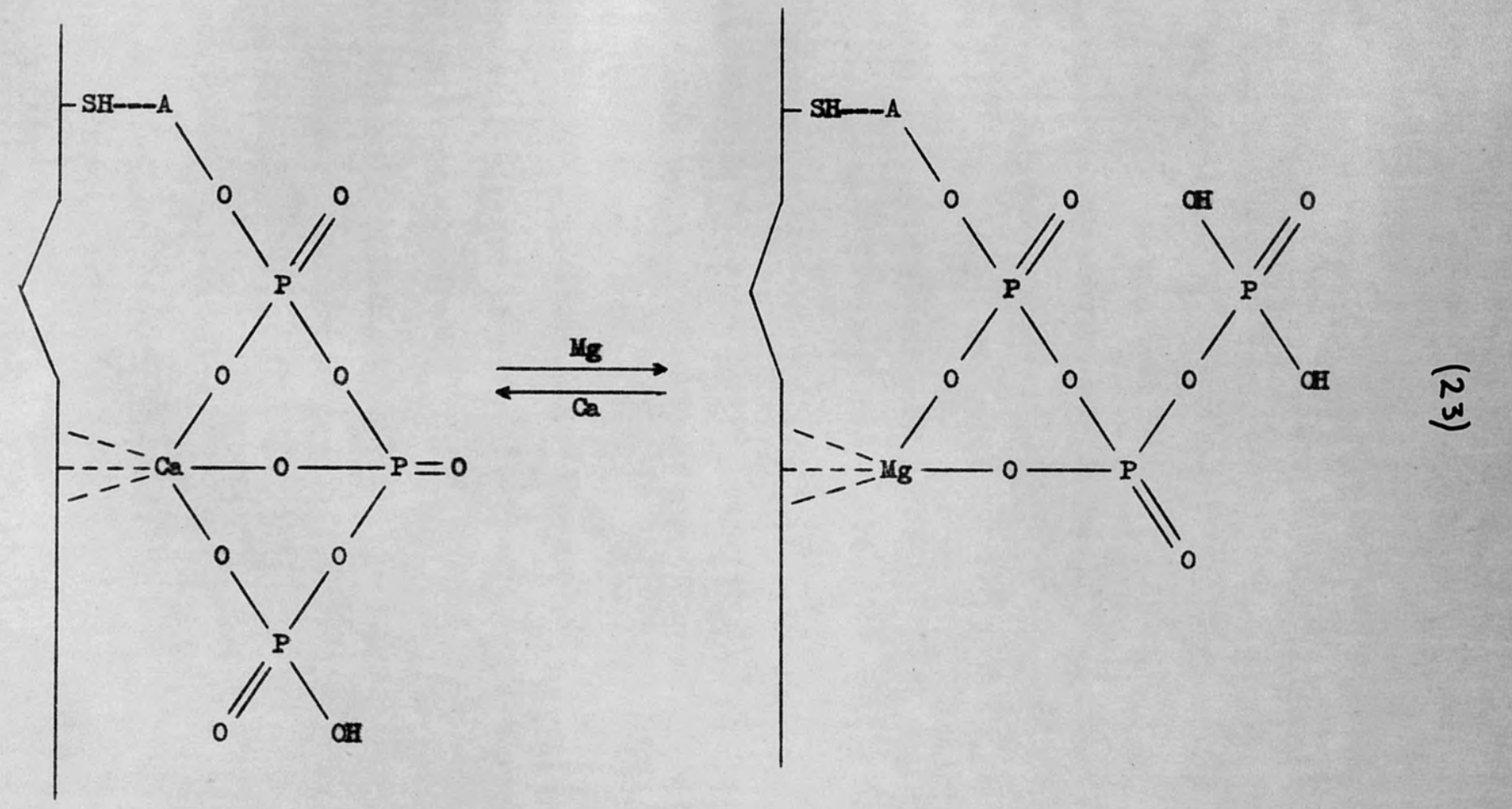
Strohman (1962) used the exchange reaction of Martonosi et al. to investigate the manner in which ATP is bound by G-actin. A study of the reaction:-

$$G - ATP(^{14}C) + XTP \rightleftharpoons G - XTP + ATP(^{14}C),$$

where XTP represents ATP, GTP, CTP, UTP or ITP, has shown that binding of ATP must occur through at least one point on the adenine ring since only ATP displaces all the bound $ATP(^{14}C)$. Very slight displacement occurred with the other nucleotides. Experiments with EDTA and

N-ethyl maleimide established a requirement for -SH groups and Mg or Ca ions in the binding of ATP to the protein. The rate of exchange of ATP was decreased by the presence of Ca ions but was only slightly affected by Mg ions, indicating that the Ca-ATP-actin complex was more stable than the Mg-ATP-actin complex. In 1959, Williams, from study of the entropy of hydration of Ca and Mg complexes of ATP, found that the Ca-ATP complex was more stable than the Mg-ATP complex, probably because the Ca ion was co-ordinated with more phosphoric acid groups than the Mg ion. This result therefore corresponded to Strohman's observations on the stability of the ion-ATP-actin complexes.

Strohman suggested the following schemes for the binding of the ATP-ion complexes to G-actin using the information given by Williams for the co-ordination of Ca or Mg to ATP alone.



(23)

(f) G-F actin equilibrium

The experiments of Asakura and Oosawa in 1960 have added a further complication to the interpretation of the manner in which actin polymerises. They found that at a very low Mg ion concentrations equilibrium states could exist between G and F-actin which would involve a continuous breakdown of the bound ATP in G-actin and combination with a new molecule in each polymerisation. During the process, P_i was slowly released and no enzymic impurity could be found in the actin which could be the cause of this release. Martonosi et al. in 1960 investigated the possibility of the reaction mechanism using ATP(^{14}C) and found that, although P_i was liberated as expected, radic-active incorporation was only 10-20% of the expected value, assuming Asakura's theory was correct. It is uncertain what the significance of this equilibrium could be *in vivo* but it does indicate that under certain conditions actin appears to act enzymically.

(g) Creatine-phosphate as a source of energy for contraction.

The work of Carlson and Siger (1960) has given more circumstantial evidence that the change in the bound nucleotide during polymerisation of actin may be physiologically significant. Their experiments were carried out on the isolated frog sartorius muscle in the presence of nitrogen and 0.5 mM IAA so that both glycolytic and oxidative systems were inhibited, but the CP-CPkinase system was still functional. The major result of their work was to show that there is a linear relationship between the

splitting of CP and the number of isometric twitches performed by a muscle. No net breakdown of ATP was found and it was concluded that CP hydrolysis is the net energy-yielding reaction during the performance of a series of muscle twitches. The link with actin polymerisation was suggested by the calculation that in one isometric twitch one CP molecule was dephosphorylated per actin molecule present in the muscle.

A scheme was suggested in which muscle ATP and ADP were located in separate compartments, an actin compartment, a sarcoplasmic compartment and a mitochondrial compartment. The actin compartment contained only bound nucleotide and the enzyme system CP-CPkinase which would depolymerise F-actin by rephosphorylation of its bound ADP. The CP and P_i in the actin compartment were visualised as being readily exchangeable with these substances in the sarcoplasm but ATP and ADP were not diffusible.

This idea finds support in the discovery by Strohman (1959) that F-actin can be depolymerised by dialysis against CP in the presence of CPkinase. Support for the location of the action of the CPkinase within an actin compartment is found in the work of Yagi and Noda (1960) They showed that in DOC-treated myofibrils or glycerinated myofibrils ADP was rephosphorylated at the expense of CP with the liberation of C, in the presence of CPkinase. Perry, as already mentioned (section d), was unable to show this in his experiements on untreated myofibrils.

Yagi and Noda attribute his result to failure of the enzyme to penetrate to the sites of bound ADP. In their experiments, the fibrils were presumably made more permeable by the DOC or glycerol treatment

but their structure was not drastically altered. Glycerinated fibrils retain the ability to contract and in these experiments were seen to shorten during the liberation of creatine.

However, the results of Yagi and Noda have recently been placed in doubt by Drabikowski and Gergely (1962) who think it is possible that ADP was released by the DOC treatment and hence made available to the CPkinase system. Another criticism was made by Moos at the Meeting of the American Society of Biochemists in 1962. He said that the phosphorylation of the bound ADP could be an artifact due to some kinase activity which persisted even after the addition of $HClO_4$ to stop the reaction.

In 1962, Mommaerts also detected CP breakdown into stoichimetric amounts of P_i and C occuring with no change in ADP or ATP concentrations, during a single muscle twitch of frog sartorius, in contrast to the experiments of Carlson and Siger (1960) where these substances were estimated after a series of twitches. However, this result was only obtained in a series of experiments on winter frogs. In summer frogs, only liberation of P_i was detected. In similar experiments with turtle sartorius muscle, Mommaerts obtained contractile activity without finding any change in ATP or CP following a single twitch. After a second period of contraction, an increase in creatine but not in P_i was detected. The rectus femoris muscle of the turtle gave similar results to the winter frog sartorius muscle. It is very difficult to interpret all these findings but they suggest that during the initial stages of contraction

the role of CP may be preceded by the activity of another factor, although after more prolonged activity this is reflected in a fall in the CP content of the muscle, as shown by Carlson and Siger (1960).

The liberation of P_i in the absence of C liberation in some of Mommaerts experiments, and also observed by Fleckenstein (1954), may eventually prove to be connected with the structurally bound phosphate found in frog skeletal muscle by Cheesman and Hilton (1960). After incubation of the frog rectus abdominis with ^{32}P - Ringer solution, this phosphate, which was extracted from the acetone powder of the muscle by TCA, contained all the activity, and if contracture were induced in the muscle, the specific activity of the P_i obtained from acetone powder was 50% lower than the specific activity of the bound phosphate of the unstimulated control. Chromatograms showed that the only other substance detectable in the TCA extract of the acetone powders was a trace of ADP which was not labelled. The conclusion reached was that the bound phosphate which was originally labelled had exchanged during contracture with unlabelled phosphate. It was suggested that the source of this phosphate may be CP since, in 1953 and 1957, Fleckenstein and Janke showed that in the rectus abdominis incubated with ^{32}P the CP had a lower degree of labelling than the ATP, while the CP and not the ATP was broken down during contracture. In contrast to the classical idea that CP participates in contraction only via the CP-CPkinase reaction for ATP resynthesis, CP would be acting directly as a source of phosphate for the phosphorylation of the contractile proteins.

(h) Phosphorylation of the myofibrillar proteins during contraction or relaxation

So far, no work has been done to investigate any possible relationship between the bound nucleotide of actin and the action of the relaxing factor shown to be present in muscle by Marsh (1952). The work of Weber (1952) on the glycerinated fibres of the rabbit psoas muscle showed that ATP and Mg ions could bring about relaxation as well as contraction, if the ATPase activity of the fibre were inhibited by an -SH reagent. He suggested that the contractile element must be phosphorylated by ATP before it can shorten. The phosphorylation is reversible and is followed by liberation of P_i from the protein with the simultaneous release of free energy for contraction. Energy from the dephosphorylation of ATP is not required in relaxation. This theory is compatible with the heat data of Hill (1949) which show that energy is required for the contraction of muscle only.

However, according to the theory of Riseman and Kirkwood (1948) the energy of hydrolysis of ATP is used to phosphorylate -OH groups on the myofibrillar protein during relaxation. The repulsion between the negatively charged phosphate groups would then maintain the fibrils in a relaxed state. The liberation of phosphate on stimulation of the muscle would result in an alteration in charge distribution leading to contraction.

The theory of Morales (1955) postulated that contraction takes place when ATP is bound to the contractile proteins and occurs as a result of the neutralisation of the positive charges which hold the

contractile element in an extended state. Energy for contraction is derived from the forces of thermal agitation when the charges are neutralised and not from the hydrolysis of ATP. When the ATP is dephosphorylated, the ADP is released and the contractile elements are free to extend.

However, the discovery of a physiological ATPase inhibitor in muscle by Marsh(1951), the relaxing factor, lends support to Weber's theory that relaxation occurs when the hydrolysis of ATP is inhibited. Bendall (1953) showed that the relaxing factor, like the -SH reagents used by Weber, prevents the hydrolysis of ATP by a contracted glycerinated fibre bundle and allows the ATP to exert a plasticising effect on the fibre which therefore relaxes.

Relaxing factor activity has been obtained in a number of protein fractions isolated from muscle but its exact nature is still unknown. Several suggestions have been made to account for its ability to inhibit the ATPase activity of muscle. Bendall (1953) thought that it could act by removing the Mg ions necessary for the ATPase activity of actomyosin. Hasselbach (1960) obtained indirect evidence that the relaxing factory accumulates Ca ions. Very small concentrations of Ca ions overcome the effect of the relaxing factor on an isolated fibre and it recontracts.

(i) Deamination in muscle.

Several observation have been made which suggest that deamination of nucleotide occurs during muscular contraction. The deamination of AMP to IMP in muscle by adenylic deaminase is well established, AMP

formation being possible through the activity of myokinase:-

$$2\ ADP \rightleftharpoons AMP + ATP$$

Myokinase activity can be removed from actomyosin preparations by extensive washing, but adenylic deaminase activity is extremely difficult to remove and may therefore be essential in muscular contraction. However, there is no real evidence for its participation. On the other hand, direct ADP deamination without the preliminary participation of myokinase to form AMP has been observed by Webster (1953). Chromatographic analysis and ionophoreses of extracts of well washed rabbit myofibrils after addition of ATP indicated that direct deamination of ADP had taken place. In 1954, Deutsch and Nilsson also found evidence for ADP deamination in actomyosin; the deaminase itself contained a nucleotide. U.V. light transmittance changes observed by Wajzer and Weber (1956) during the performance of mechanical work by the frog semi-tendinosus muscle were interpretated to result from a deamination of ATP to IMP. Deamination of Bound nucleotide has so far not been demonstrated.

(j) Conclusion

Deamination may eventually find a place in the explanation of the process of muscular contraction but, at the present time, the majority of experimental results suggest that the major processes, at least, are associated with changes in the CP and bound nucleotide of muscle, not involving deamination. The breakdown of CP after prolonged activity seems to be established and also its ability to rephosphorylate the

bound nucleotide of actin. However, the primary chemical events in contraction are not clear, nor is the manner in which such chemical events lead to the mechanical phenomenon of contraction. It seems likely that some essential factor has so far remained undetected and that the discovery of its role may help to reconcile many of the conflicting results which at the present time cannot all be incorporated into a single satisfactory theory.

CHAPTER II

OBJECTS OF THE INVESTIGATION

The tentative hypothesis which formed the basis for the experimental work described in this thesis was that the bound adenine nucleotides of actin are actively involved in processes occurring during contraction and relaxation of muscle.

The investigation of the chemical changes resulting from contraction or relaxation of whole muscle is complicated by the difficulties encountered in isolating the muscle in a definite physiological state. The influence of oxidative phosphorylation and glycolysis on the substances involved in the contractile process must be taken into account. To overcome these problems, our experiments were carried out on myofibrils or, in some cases, on actomyosin gels which can be conveniently examined in states of contraction or relaxation induced by ATP.

The changes in the banded structure of living muscle fibres during contraction observed by A.F.Huxley and Niedergerke (1954) by interference microscopy correspond closely to the changes observed by Hanson and H.E.Huxley (1954) in phase contrast microscope studies of glycerol-extracted fibrils contracted with ATP. Therefore, since the mechanical changes involved in the interaction of myosin and actin filaments are apparently the same in both the living fibres and the glycerol-extracted fibril, it seems reasonable to suppose that any chemical changes occurring will also be identical.

The experiments fell into three groups:-

(1) Since the participation of bound nucleotides in contraction and relaxation would most probably involve their phosphorylation and dephosphorylation, experiments were designed to detect changes in the overall phosphorus content of myofibrils or actomyosin gels during contraction or relaxation induced by ATP.

(2) Occasional reports that nucleotides other than ATP and ADP are bound to the myofibrillar proteins and are involved in contraction and relaxation have been made (see Chapter IV, Section 5). However, only the occurrence of the bound adenine nucleotides is well established. It was decided to make a qualitative study of the nucleotides associated with our preparations of myofibrils in view of these observations.

(3) An attempt was finally made to study possible quantitative changes in the bound adenine nucleotides of frog myofibrils during ATP-induced contraction and relaxation.

CHAPTER III

EXPERIMENTS TO DETECT CHANGES IN PHOSPHORUS CONTENT AFTER CONTRACTION AND RELAXATION OF RABBIT MYOFIBRILS, MYOFIBRILS DEPLETED OF MYOSIN AND ACTOMYOSIN

1. Preparation of materials and methods used

(a) Rabbit myofibrils

Rabbit myofibrils were prepared by the method of Ulbrecht and Ulbrecht (1957). The preparation involves homogenisation of skeletal muscle and subsequent extersive washing of the myofibrillar portion of the homogenate with 0.1 M-KCl at 0° C. Sarcoplasmic enzymes are removed from the myofibrils, yet the proteins actin and myosin are retained as in an intact muscle fibre. The myofibrils can be stored at -15° in a 50% glycerol/water mixture for several months and still retain the ability to contract when washed free of glycerol.

The phosphorus content of the myofibrils, estimated on an acetone powder prepared by extracting the fibrils three times with three volums of cold acetone, varies between 2.0 µg/mg and 1.4 µg/mg. The higher values are obtained on acetone powders of freshly prepared myofibrils; the lower values are found after the myofibrils have been kept for a long period in 50% glycerol. A large proportion of the phosphorus is associated with nucleotide (see Chapter IV).

(b) Rabbit myofibrils depleted of myosin

Myofibrils were prepared as described above. They were incubated with a solution containing 0·4 M-KCl, 0·1 M-pyrophosphate, 0·001 M-$MgCl_2$, pH 6·5 at 0° C., according to the method of Ulbrecht and Ulbrecht (1957).

The treatment removes 50% of the myosin in the fibrils; the extracted fibril has therefore a much higher proportion of actin. It was thought that if actin only were phosphorylated, it would be more easily detected under these conditions. The extracted fibril has only 10% of the ATPase activity of the original fibril so that more ATP would be available for phosphorylation if this process competed with the ATPase activity.

(c) Actomyosin

Actomyosin was prepared from rabbit muscle by the method of Szent-Györgyi (1942) in which the protein is extracted by a solution containing 0·6 M-KCl, 0·01 M-Na_2CO_3 and 0.04 M-$NaHCO_3$. It was dissolved and reprecipitated six times to obtain a pure preparation.

(d) Relaxing factor

Relaxing factor was prepared from rabbit muscle by the method of Mueller (1960). It was extracted in a solution containing 0·05 M-KCl, 0.02 M-imidazole buffer and 0·005-M K-oxalate pH 7·2.

The preparation was shown to bring about the relaxation of an isolated glycerol-extracted fibre bundle under the conditions used by Bendall (1953), i.e. 6 mM-ATP, 4 mM-$MgCl_2$, 160 mM-KCl pH 6·9. Recontraction was obtained on the addition of 0·2 mM-$CaCl_2$.

(e) Creatine phosphokinase

The method of Stephen, Noda and Lardy (1954) was used for the preparation of creatine phosphokinase from rabbit muscle. The enzyme was extracted with 0·01 M-KCl and purified by ammonium sulphate and ethanol fractionation.

(f) Phosphorus estimations

Total phosphorus was estimated by the method of Allen (1940).

2. Data relevant to experiments on phosphorus uptake by muscle.

It was calculated that if ATP is bound by, or phosphorylates the myofibrillar proteins, the phosphorus uptake would amount to the following :-

(a) 0·1 µg/mg dry weight of protein if each actin molecule is phosphyorylated by the terminal phosphate group of one ATP molecule.

(b) 0·8 µg/mg dry weight of protein if one ATP molecule is also taken up by 100,000 g of myosin.

(c) 0.3 µg/mg dry weight of protein if myosin-bound ADP is displaced by ATP.

The calculations were based on the following data:-

i) Molecular weight of G-actin 70,000 g. (Tsao, 1953).

ii) Molecular weight of myosin 100,000 g. (Szent-Györgyi, 1947)

iii) Actin is 20% of the myofibrillar protein. (Perry, 1952).

iv) Actomyosin prepared by extracting muscle with alkaline KCl solution contains about 20% actin. (Szent-Gyorgyi, 1947).

Weights of myofibrils or actomyosin gel were used in experiments designed so that changes resulting from (b) and (c) should certainly be detectable. Changes caused by (a) alone would result in a change in the optical density of the phosphate solutions in the third figure only and therefore would probably not be detected.

3. Experimental Work

(a) Phosphorus estimations on acetone powders of ATP-treated myofibrils

Method. Samples of myofibrils with dry weights of 20-25 mg were incubated with 0·2 mM-ATP and 4 mM-Mg ions at pH 7·2 in a volume of 5 ml for 10 minutes at 37°. Contraction of the myofibrils occurred under these conditions. Samples were incubated in the absence of ATP to act as controls. The contracted fibrils were washed six times with 0·1 M-KCl to remove added ATP. The control fibres were similarly washed. Total phosphorus was then estimated on the acetone powders of the fibrils.

Result of six experiments.

Mean P content of ATP-treated fibrils 1·99 ± 0·9 µg/mg.

Mean P content of control fibrils 1·96 ± 0.15 µg/mg.

Conclusion. The differences in phosphorus content between contracted and uncontracted fibrils were not significant.

(b) Phosphorus estimations on acetone powders of ATP-treated myosin-depleted myofibrils

Method. Phosphorus estimations on the acetone powders of myosin-depleted myofibrils were made following the treatment of the fibrils described in (a).

Result of three experiments.

Mean P content of ATP-treated fibrils 1·64 ± 0·24 µg/mg

Mean P content of control fibrils 1·52 ± 0·33 µg/mg

Conclusion. The differences between the phosphorus content of the acetone powder of uncontracted and contracted myosin-depleted myofibrils were not significant.

(c) Estimation of the phosphorus content of wet myosin-depleted myofibrils treated with ATP.

Method. If myofibrillar protein were phosphorylated by ATP, it seemed likely that a very labile compound would be formed which might be broken down in the subsequent washing and acetone-drying of the myofibrils (later experiments, described in Chapter IV, showed that acetone does remove material which contains a polynucleotide and adenine).

To make allowance for this possibility, a series of experiments was carried out in which the phosphorus content of a measured part of the incubation medium containing 0·3 mM-ATP and 4 mM-Mg ions was estimated, and also the phosphorus content of the fibrils and the remaining ATP solution. Two controls were necessary, one containing the same concentration of ATP but no fibrils, the other containing fibrils and buffer instead of ATP. The fibrils were incubated for 10 minutes at 37° in a volume of 5 mls. Total phosphorus was estimated in 3 ml of the supernatant after centrifugation, and on the remaining 2 ml of solution containing the fibrils. Any decrease in phosphorus of the solution could be correlated with an increase in phosphorus in the fibrils.

The acetone dry weight of a known volume of myosin-depleted myofibrils was determined. The phosphorus content of the wet fibrils was then expressed in terms of this weight. Suspensions of myofibrils with dry weights of 30-35 mg were used.

Results of five experiments.

Mean P content of ATP-treated fibrils 2.01 ± 0.27 µg/mg

Mean P content of control fibrils 2.12 ± 0.30 µg/mg

Conclusion. A significant difference between the phosphorus content of contracted and uncontracted fibrils was not obtained.

(d) Estimation of the phosphorus content of myofibrils which had been actively relaxed of contracted with ATP

Introduction. According to Perry (1952) the position of the A and I bands in isolated myofibrils indicates that they are partially contracted. If this were so in our preparation, it seemed possible that a considerable amount of phosphate binding may have already occurred. It was hoped that, by causing the fibrils to relax fully and then recontracting them with ATP, a difference in the phosphorus content in the two states might be detected.

i) The effect of urea-induced relaxation of the phosphorus content of myofibrils

Barany (1960) found that the mechanical interaction of actin and myosin filaments was inhibited by 1 M-urea in the presence of ATP. The ATP-Mg-induced contraction of an isolated fibre was followed by relaxation on addition of 1 M-urea and the effect was reversed when the urea was washed out.

It was thought that urea would be a suitable non-physiological relaxing agent to use in experiments designed to detect uptake by myofibrils, since it presumably acts directly on the proteins to prevent the interaction of myosin and actin. Drastic denaturation does not take place however, since the proteins can recombine when the urea is washed out. It seemed likely that other artificial relaxing agents such as versene, fuadin and various polycations might combine with groups involved in phosphate or nucleotide binding if this occurs, or remove some ion essential for the process.

Method. Suspensions of fibrils with dry weights of 20-25 mg were incubated for 20 minutes at 37° in 3 ml of a medium containing 0·3 mM-ATP, 3×10^{-3} M-$MgSO_4$ and 5×10^{-2} M-KCL at pH 6·9, to induce contraction. To obtain relaxation, the fibrils were incubated in the same solution made 1 M with respect to urea.

Total phosphorus was estimated in 2 ml of the supernatant solution and the remaining 1 ml containing the fibrils after centrifugation at 0°.

Results of three experiments

P content of contracted fibrils. µg/mg	P content of relaxed fibrils µg/mg
1·60	1·60
1·10	1·37
1·73	1·60

Conclusion. The results were inconsistent and no definite conclusion could be reached.

ii) The phosphorus content of myofibrils relaxed or contracted by ATP in the presence of the relaxing factor.

Method. Suspensions of myofibrils with dry weights of 140-170 mg were incubated with 0·5 mM-ATP, 4 mM-$MgCl_2$ and relaxing factor pH 6.9 in a volume of 5 ml for 10 minutes at 20°. This treatment induced relaxation. Contracted fibrils were obtained by including 0.2 mM-$CaCl_2$ in the medium.

A large amount of fibrils was used in these experiments to increase the possibility of detecting phosphorus uptake. The

experiments were confined to detecting a possible change in the phosphorus content of the liquid phase. After centrifugation of the fibrils, the total phosphorus content of 3 ml of supernatant solution was estimated.

Result of nine experiments.

Mean ratio of optical densities in the colour reaction obtained with 3 ml of supernatant from relaxed and contracted myofibrils:-

$$\frac{\text{relaxed fibrils}}{\text{contracted fibrils}} = 0.98 \pm 0.02$$

Conclusion. A much higher ATP concentration is necessary to bring about the relaxation than the contraction of muscle. 6 mM-ATP is needed to relax an isolated fibre bundle but a lower concentration appears to relax isolated fibrils, or at least to prevent their full contraction in the presence of relaxing factor. Marsh (1952) found that maximum relaxation of fibrils was obtained with a concentration of 70 µg of phosphorus, from the terminal phosphate group of ATP, per gm of muscle, whereas 20 µg/gm would bring about maximum synaeresis. In our experiments the ATP concentration of 0·5 mM, which corresponded to 50 µg ATP-P/gm of muscle, was the highest that could be used in order to permit the detection of a difference in the final colour reaction corresponding to the phosphorylation of actin-bound ADP.

The ratios of the phosphate concentrations in the supernatants from the experiments with contracted and uncontracted fibres show no significant deviation from unity. Therefore, the phosphorus content of the fibrils in the relaxed and contracted states is apparently the same.

(e) The phosphorus content of actomyosin after contraction or relaxation induced by ATP in the presence of relaxing factor

i) Introduction. Buchthal (1949) carried out experiments in which he hoped to detect an uptake of phosphorus from ATP by actomyosin gels, resulting from phosphorylation of the protein or uptake of ATP by myosin. These two chemical changes in the protein had been suggested by Kalckar (1941) and Szent-Györgyi (1947) to account for the observed physical effect of ATP on actomyosin. Buchthal obtained an increase in the phosphorus content of actomyosin which was however due to the presence of iron in his ATP preparation, leading to the formation of ATP-iron-protein complexes. No uptake of phosphorus was found in the absence of iron. The experimental procedure involved washing the actomyosin 8-9 times after treatment with ATP before estimating the phosphorus content of the perchloric acid extract of the protein. It did not seem unlikely therefore that a labile phosphate compound, formed by the action of ATP on the actomyosin, may have been broken down during the washing procedure. We therefore planned our experiments so that this possibility was eliminated and also investigated a possible change in the phosphorus content of actomyosin relaxed, as well as contracted, by ATP.

The swelling of actomyosin gels by ATP and Mg ions in the presence of relaxing factor is thought to be analogous to relaxation of muscle fibrils. It was decided to repeat the experiments carried out on myofibrils with actomyosin, since the phosphate estimations can be made when the actomyosin is seen to be fully relaxed or contracted; the volume change between contracted and relaxed actomyosin is very marked.

ii) Method. Actomyosin gel having a dry weight of 45 mg. was incubated with 0·5 mM-ATP, 4 mM-$MgCl_2$ and relaxing factor pH 6·5 in a volume of 5 ml for 10 minutes at 20° C. Under these conditions, synaeresis of actomyosin was inhibited. Actomyosin incubated with the same solution but with 0.2 mM-$CaCl_2$ also, was completely contracted.

Total phosphorus was estimated in 3 ml of the supernatant solution after centrifugation of the protein.

Result of two experiments.

Ratios of phosphate concentrations in the supernatants from relaxed and contracted fibrils:

(i) 0·99; (ii) 1·01

Conclusion. No significant difference was found in the phosphorus content of actomyosin relaxed and contracted by ATP.

(f) The phosphorus content of actomyosin after treatment with ATP, relaxing factor, creatine and CP-kinase.

Introduction. It seems possible that the CP-CPkinase system is involved in the contraction-relaxation cycle of muscle. (Chapter I). Strohman (1959) found that the bound ADP of F-actin is phosphorylated by CP in the presence of CPkinase. Laki (1950) found CPkinase activity in his G-actin preparation and found that the addition of creatine inhibited polymerisation.

Experiments were designed to investigate whether, in the presence of creatine and CPkinase, phosphorus uptake could be detected by actomyosin gels after contraction or relaxation with ATP.

Method

i) Actomyosin gel having a dry weight of 40-45 mg was incubated for 10 minutes at 20° C. in a solution containing 0·5 mM-ATP, relaxing factor, 1 mM-creatine, CPkinase and 4 mM-$MgCl_2$ pH 6·9 in a volume of 5·5 ml, to obtain relaxation.

Contracted actomyosin was obtained by including 0.2 mM-$CaCl_2$ in the solution. Phosphorus estimations were made on 3 ml of the supernatant obtained after centrifugation of the protein.

ii) The experiment was repeated on dialysed actomyosin. Dialysis cuts down the ATPase activity without interfering with the mechanical properties of actomyosin; therefore, the use of ATP by a phosphorylation process rather than by ATPase might be favoured.

Result

i) Un-dialysed actomyosin

$$\frac{\text{Phosphate content of supernatant from relaxed actomyosin}}{\text{Phosphate content of supernatant from contracted actomyosin}}$$

Mean ratio from seven experiments = $0{\cdot}99 \pm 0{\cdot}02$

ii) Dialysed actomyosin

Ratio from two experiments:

(a) 0·99

(b) 1·00

Conclusion. The ratios of the phosphate concentrations in the supernatants from experiments with actomyosin contracted or relaxed by ATP in the presence of creatine and CPkinase showed no significant deviation from unity. The phosphorus content of the actomyosin gels

is apparently therefore the same in the contracted and relaxed states.

4. Conclusion. No significant difference was found in the phosphorus content of washed myofibrils, myosin-depleted myofibrils or actomyosin after contraction and relaxation induced by ATP.

It may therefore be concluded that if net phosphorus uptake occurs, it is only very small and certainly not greater than would correspond to the phosphorylation of actin-ADP

If bound phosphorus of myofibrils or actomyosin is involved at all in the physical changes brought about by ATP in the systems investigated, it is possible that it is redistributed rather than affected in quantity. However, it is also possible that the chemical composition of the contractile system is the same in the contracted and relaxed states and that only transient chemical modification of the protein occurs during the contraction process.

CHAPTER IV

PAPER CHROMATOGRAPHY OF UV-ABSORBING SUBSTANCES ASSOCIATED WITH MYOFIBRILS

1. MATERIALS AND METHODS

(a) Rabbit myofibrils.

Glycerinated rabbit myofibrils having dry weights of 30 - 40 mg were washed free of glycerol and the suspensions were extracted three times with three volumes of cold acetone.

A chromatographic examination of the UV-absorbing material in (i) the acetone extract and (ii) the acetone-dried myofibrils was made.

(i) The acetone extract of the myofibrils.

It was noticed that the total phosphorus content of myofibrils estimated on a wet suspension and expressed in terms of the acetone-dry weight was higher than the amount estimated on the acetone powder itself. A similar result was found with actomyosin, i.e.:-

	Fibrils	Actomyosin
Phosphorus content of wet suspension:	1.4 µg/mg.	0.95 µg/mg
Phosphorus content of acetone powder:	0.86 µg/mg.	0.59 µg/mg

Approximately 40% of the total phosphorus of the preparations was removed by the acetone/water mixture. The presence of UV-absorbing substances containing phosphorus in the acetone/water extract of myofibrils was investigated in the following manner:-

Method. The combined acetone extracts were concentrated under reduced pressure. After evaporation of the acetone, the aqueous residue which

remained contained denatured protein and lipid. The mixture was acidified with N-HCl and extracted with ether. The aqueous layer was freeze-dried and applied to a chromatogram in a very small volume of water.

(ii) The acetone powder of the myofibrils.

The following extracts were examined by paper chromatography for the presence of UV-absorbing substances:-

1. The extract obtained after treatment of the acetone powder for 15 minutes with cold water.

2. The material extracted by treating the acetone powder for 10 minutes in boiling water.

3. The cold N-HCl extract of the acetone powder treated with the acid for 16 hours at 4°C.

4. The cold N-HCl extract of the acetone-dried isoelectric precipitate of actin.

Actin prepared from rabbit muscle by the method of Tsao and Bailey (1953) was isoelectrically precipitated at pH 4.7 with M-acetic acid. According to Laki (1950), bound prosthetic ADP of actin is released into the supernatant together with small amounts of hypoxanthine nucleotide. The isoelectric precipitate was examined to see if any nucleotide remained bound to the protein.

The extracts in all cases were freeze-dried, taken up in a very small volume of water and dialysed. Dialysis was necessary to prevent

streaking on the chromatographs due to the presence of considerable amounts of protein in the extracts. The dialysate was freeze-dried, dissolved in the minimum amount of water and applied to chromatograms.

(b) Frog myofibrils.

(i) Frog skeletal muscle was homogenised for two minutes in five volumes of cold frog Ringer solution in a Waring Blender. It was then washed six times with ten volumes of cold water.

The myofibrils were extracted three times with three volumes of cold acetone; an examination of the acetone extracts and acetone powders was made, as with rabbit muscle, for the presence of UV-absorbing material.

(ii) Frog rectus abdominis muscle was incubated in ^{32}P-Ringer solution for 30 minutes. It was well washed, then cut into very small pieces and converted to an acetone powder. Chromatograms of the N-HCl extracts of the acetone powder were made. They were scanned to locate the distribution of ^{32}P between UV-absorbing substances, if any occurred.

(c) Solvents.

(i) Iso-butyric acid/N-NH_3/0.1 M-Versene.

The solvent used by Krebs and Hems (1953), which contains iso-butyric acid, N-ammonia and 0.1 M-versene in the ratio 100 : 60 : 1·6, was found to give satisfactory separation of adenine and its derivatives and of most other purine and pyrimidine bases, nucleosides and nucleotides.

They were separated by descending chromatography at room temperature, after 16 hours on Whatman No. 1 paper or after 14 hours on 3 mm paper. The thicker 3 mm paper was used to separate large amounts of UV-absorbing material; spots were then obtained which had sufficient UV-absorption to be identified, not only by their Rf, but by their UV-absorption spectrum when eluted from the paper.

(ii) Formic acid/diethyl ether.

In the isobutyric/ammonia solvent, P_i has an Rf very close to that of ATP. When the separation of P_i from UV-absorbing material was required, a solvent containing 50 parts of diethyl ether and 25 parts of 90% formic acid was used in an atmosphere saturated with ether. This solvent is an adaptation of that used by Krebs and Hems (1953). The separation of P_i was obtained by ascending chromatography on folded paper, using the arrangement of Fleckenstein and Janke (1953-54). The spots were applied at 2 inches from the central fold of the paper. P_i migrated about 4 inches in 3 hours in the formic acid/ether solvent; the UV-absorbing spots remained at the origin. The paper containing P_i was cut off and the purine and pyrimidine bases and their derivatives were then separated by paper chromatography in the iso-butyric/ammonia solvent.

All solvents were redistilled before use.

(d) Identification of UV-absorbing spots.

(i) By Rf value and the presence or absence of phosphorus.

Spots were detected on the paper chromatograms by exposure to

UV-light. Markers of purine and pyrimidine bases, nucleosides and nucleotides were available for comparison of their Rf value with the Rf values of the substances in the muscle extracts.

Phosphorus-containing spots were located by the development of the chromatograms with the molybdate reagent of Hanes and Isherwood (1949).

(ii) By UV-absorption spectrum.

The UV-absorbing spots were eluted from the paper with water or 0.1 M-HCl. The spectra of the solutions were read in the Unicam SP 500 spectrophotometer. The spectra, after making the solutions alkaline were also read, since many purine and pyrimidine bases and their derivatives show characteristic changes in absorption spectrum as a result of changes in pH.

It was found by eluting nucleotide markers that the most complete elution was obtained by cutting out a spot, placing it in a Gooch funnel and allowing 3 ml of solvent to drip onto it slowly.

In order to obtain satisfactory spectra from eluted spots it was necessary to wash the paper twice on 0.1 M-HCl and ten times in water before use. Even after this treatment the paper still contained material having UV-absorption in the 230 - 240 $m\mu$ range. Since some purine and pyrimidine bases show minimum UV-absorption in this region, to obtain spectra showing characteristic minima, it was necessary to elute the paper adjacent to each UV-absorbing spot and use this solution as a blank.

2. RESULTS

The bases and nucleotides present in rabbit muscle were also found in the muscle of the frog.

Although water removed the same substances from the preparations as N-HCl, the removal was not complete and subsequent treatment with N-HCl released more of the same UV-absorbing substances. However, their presence in aqueous extracts indicated that they were not merely acid degradation products from a single nucleotide or polynucleotide.

(a) UV-absorbing substances in acetone extracts of washed frog and rabbit myofibrils.

(i) An oligo- or polynucleotide.

(ii) Adenine.

(b) UV-absorbing substances extracted by cold water from acetone powders of frog and rabbit myofibrils.

(i) Mainly ADP.

(ii) Traces of IMP, AMP and ATP.

(iii) A trace of adenine and a green pigment with a similar RF value.

(c) UV-absorbing substances in the isoelectric precipitate of actin.

(i) Mainly ADP.

(ii) Traces of AMP, ATP, adenine and an oligo- or polynucleotide.

(d) UV-absorbing materials in the hot water and HCl extracts of the acetone powders.

(i) Mainly ADP.

(ii) Small amounts of ATP and AMP.

(iii) An oligo- or polynucleotide.

(iv) P_i.

(v) Adenine and a green pigment with a similar Rf value.

(vi) Traces of IMP and hypoxanthine.

(c) Chromatograms of HCl extracts of the acetone powder of the frog rectus abdominis pre-incubated with ^{32}P Ringer solution.

The P_i spot was very strong; small amounts of the adenine nucleotides and adenine were present. It was found that virtually all the radioactivity was in the P_i spot, little or none was found in the nucleotide spots.

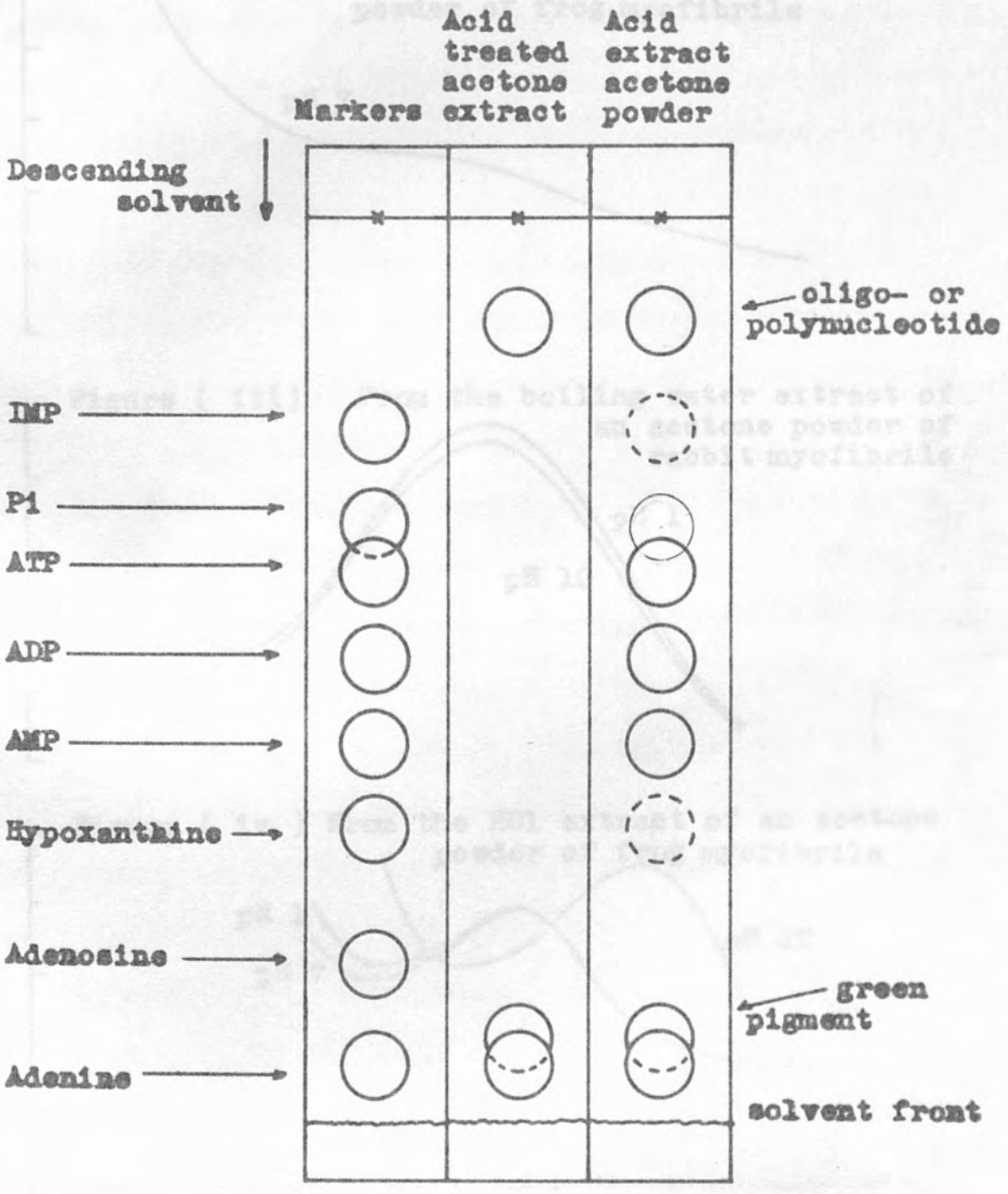
3. The oligo- or polynucleotide.

A UV- absorbing substance was found on our chromatograms in the position shown on figure (i). It was present in acetone extracts of frog and rabbit myofibrils and in water and acid extracts also. Development of the chromatograms with molybdate reagent showed that it contained phosphorus. Attempts to identify it by comparison with known purine or pyrimidine bases, nucleosides or nucleotides were unsuccessful. The spot was eluted on several occasions and seldom gave the same UV-absorption spectrum, although it always appeared in the same place on the chromatograms. The characteristics of the spot in three typical experiments are given here.

Figure (ii). A spectrum was most often obtained which showed no definite UV-absorption maximum but a slight inflection in the region λ 250 $m\mu$ to λ 260 $m\mu$. The form of the spectrum was not changed by altering the pH.

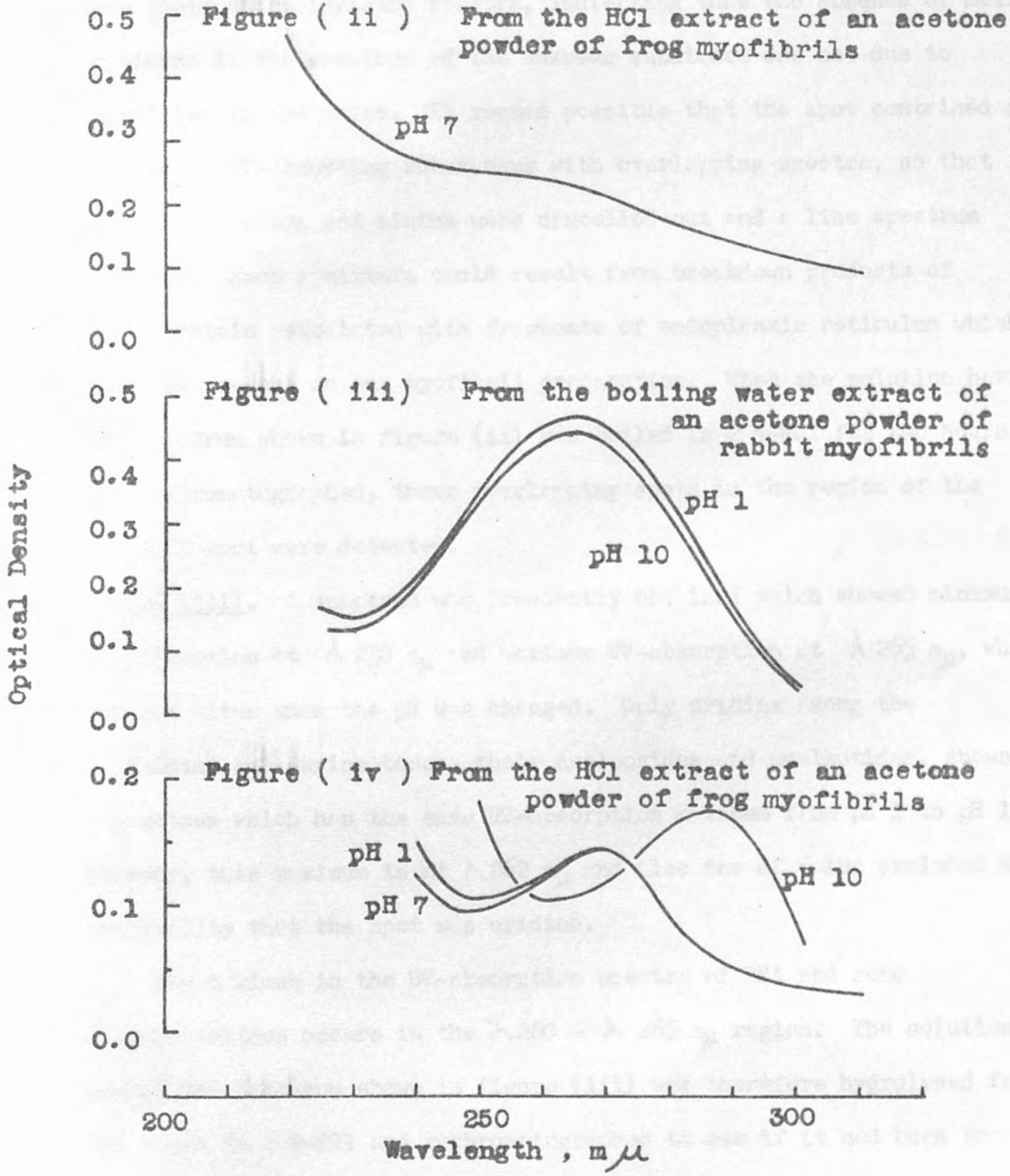
Figure (1)

Diagram of Chromatograms run in
isobutyric acid / ammonia



Figures (ii), (iii), (iv)

Spectra of the oligo- or polynucleotide



Nucleotide markers of similar intensity eluted from the same chromatograms gave their characteristic spectra, indicating that the absence of maxima or minima in the spectrum of the unknown substance was not due to impurities in the paper. It seemed possible that the spot contained a mixture of UV-absorbing substances with overlapping spectra, so that individual maxima and minima were cancelled out and a line spectrum obtained. Such a mixture could result from breakdown products of nucleoprotein associated with fragments of endoplasmic reticulum which might be present in our myofibril preparation. When the solution having the spectrum shown in figure (ii) was boiled in 2 N-HCl for two hours and rechromatographed, three overlapping spots in the region of the original spot were detected.

Figure (iii). A spectrum was frequently obtained which showed minimum UV-absorption at λ 230 m_{μ} and maximum UV-absorption at λ 265 m_{μ}, which did not alter when the pH was changed. Only uridine among the pyrimidine and purine bases, their nucleosides and nucleotides, shows a spectrum which has the same UV-absorption maximum from pH 1 to pH 14. However, this maximum is at λ 262 m_{μ} and also the Rf value excluded the possibility that the spot was uridine.

The maximum in the UV-absorption spectra of RNA and some polynucleotides occurs in the λ 260 - λ 265 m_{μ} region. The solution giving the spectrum shown in figure (iii) was therefore hydrolysed for two hours in 2 N-HCl and rechromatographed to see if it had been broken

down into smaller units. Five faint spots were obtained, presumably nucleotides or oligo-nucleotides derived from the original high molecular polynucleotide.

Figure (iv). A spot was sometimes obtained which showed minimum UV-absorption at λ 248 mμ and maximum UV-absorption at λ 270 mμ at neutral pH. These figures suggested that the substance might be a cytidine derivative; CTP was found to have an Rf very close to that of the unknown spot. The maximum and minimum were the same in acid solution, but in alkali minimum UV-absorption at λ 262 mμ and maximum UV-absorption at λ 285 mμ were obtained. No reference to a cytidine derivative showing these changes in spectra could be found.

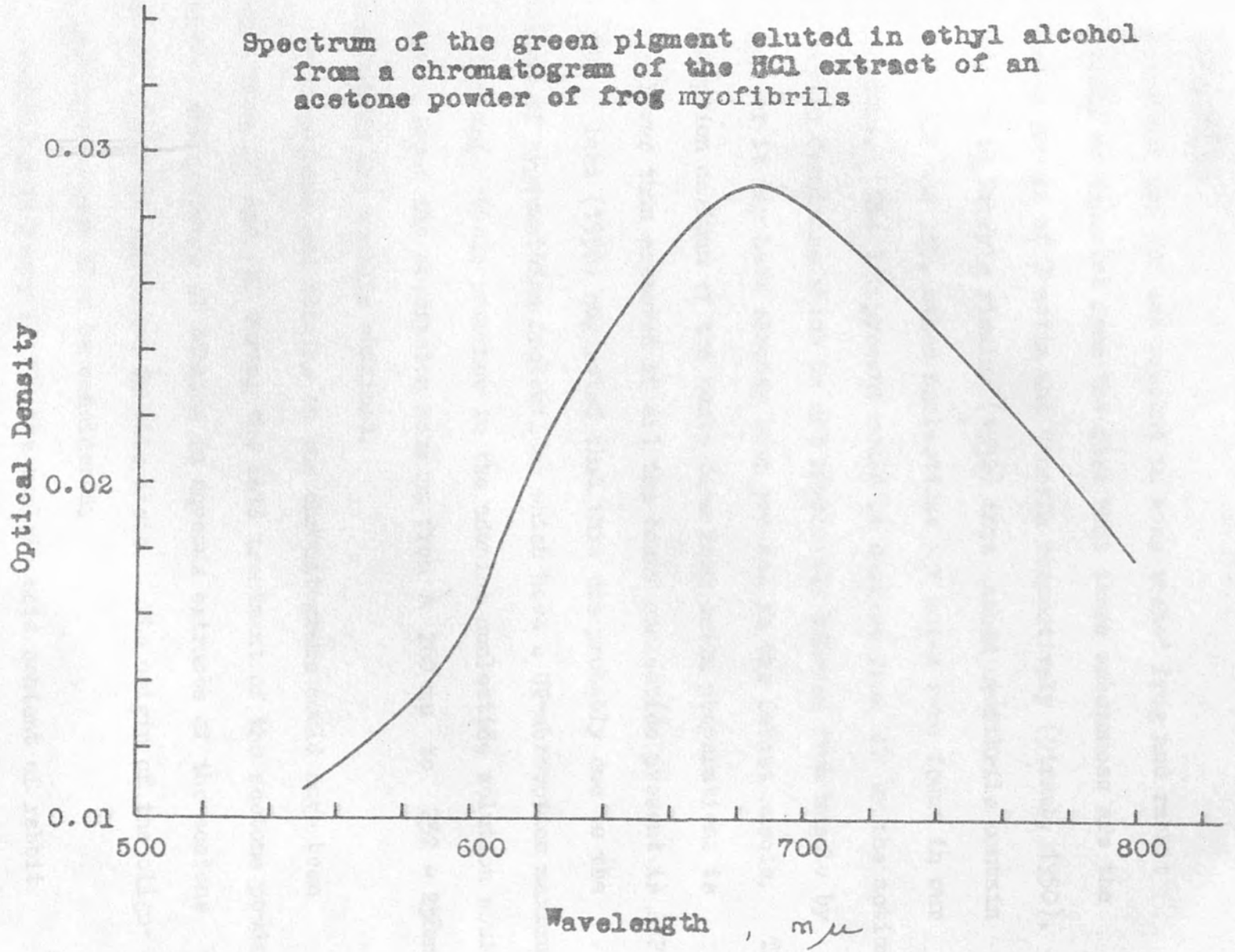
4. The green pigment - figure (v).

On several chromatograms of water or acid extracts of frog and rabbit myofibrils a green pigment having an Rf similar to that of adenine was found. It was extremely labile and the colour was destroyed when the spot was eluted with 0.1 M-HCl. However, a spectrum showing a minimum absorption at λ 560 mμ and maximum absorption at λ 683 mμ was obtained when the spot was eluted with ethyl alcohol. The green colour was very similar to that of biliverdin, which, according to Gray (1953), is characterised by the part of its spectrum showing minimum absorption at λ 500 mμ and maximum at λ 675 mμ.

The pigment has not been further investigated; it may well be an artefact from a haem pigment of mitochondrial origin.

Figure (v)

Spectrum of the green pigment eluted in ethyl alcohol from a chromatogram of the HCl extract of an acetone powder of frog myofibrils



5. Conclusion.

Bound ADP and ATP are present in both washed frog and rabbit myofibrils, as expected from the fact that these substances are the prosthetic groups of F-actin and G-actin respectively (Straub, 1950). In contrast to Perry's finding (1952) that washed myofibrils contain only AMP, ADP and ATP, other nucleotides and bases were found in our preparations. The IMP present could be derived from AMP by the action of adenylic deaminase which is not completely removed from muscle by washing, or it may have already been present in the intact muscle. The UV-absorption maximum of the nucleotide from actin preparations is usually lower than expected if all the bound nucleotide present is ADP and ATP. Laki (1950) suggested that this was probably due to the presence of hypoxanthine nucleotides which have a UV-absorption maximum at λ 249 mμ; their presence in the adenine nucleotide solution could therefore lower the absorption maximum from λ 260 mμ to 252 - 258mμ values which are usually obtained.

Hypoxanthine and adenine on the chromatograms could have been formed from IMP and AMP during the acid treatment of the acetone powders. However, the presence of adenine in aqueous extracts of the acetone powders cannot be explained on this basis. The origin of the oligo- or polynucleotide must also be considered.

According to Perry (1955) the nucleic acid content of rabbit myofibrils is about 0.4% of the dry weight and is probably due to

microsomal contamination. The oligo- or polynucleotide in our preparations could therefore have been a random product of nucleic acid breakdown. Its presence in the acetone extracts suggested that it may have been attached to microsomal fragments from which the lipid in the extracts is derived. However, it was also present in the acetone powders of washed frog and rabbit myofibrils which indicated that it may also be firmly bound to protein. There was some evidence for believing that the adenine spot on the chromatograms was derived from the oligonucleotide since its intensity varied inversely with the intensity of the oligo- or polynucleotide spot. The origin of the green pigment with an Rf close to that of the adenine spot is uncertain.

Although it is possible that the oligo- or polynucleotide in our preparations was all derived from nucleic acid, the possibility that it may fulfil some purpose in the contractile process cannot be overlooked in view of the observations of other workers.

Fleckenstein and Janke (1954, 1955), using paper chromatography, found that two UV-absorbing phosphate esters were present in frog rectus abdominis muscle in amounts comparable to those of ATP and ADP. Although breakdown of ATP could not be detected during contraction, P_i was released from these two substances which have not yet been identified.

The existence of the oligo- or polynucleotide in several spectral forms suggested a resemblance to the substance found by Gelotte (1953) in actin preparations after removal of nucleic acid. It was extremely

labile and seemed to exist in several polymeric forms characterised by different spectra. However, the spectroscopic data given in the paper, i.e. maximum absorption at λ 210 mμ and λ 270 mμ in 0.01 M-HCl, and at λ 235 mμ and λ 287 mμ in 0.01 M-KOH, do not resemble figures obtained with our oligo- or polynucleotide.

The substance isolated from actin by Snellman and Gelotte (1951), apparently a dinucleotide, showed a maximum UV-absorption at λ 260 mμ in neutral and alkaline solution and at λ 263 mμ in acid, which shows some resemblance to one spectral form of our oligo- or polynucleotide (figure III). One component was identified as adenosine. The substance existed in two forms, one richer in phosphorus, the other poorer in phosphorus, and the suggestion was made that these forms were the prosthetic groups of G-actin and F-actin respectively, and not ATP and ADP which were absent in the preparation.

The P_i on our chromatograms of N-HCl extracts of frog and rabbit muscle may have been derived from nucleotide, or perhaps from phosphate bound in some other way to the contractile proteins. The total phosphorus in our preparation of rabbit myofibrils, 1.4-2.0 μg/mg dry weight, is five times the total phosphorus found by Perry (1952). He found 90-140 μg/gm dry weight of acid-labile phosphorus in his preparation which was apparently all derived from adenine nucleotide. It seems possible that the borate buffer used by Perry for the extensive washing of the muscle extracted phosphorus-containing

material not removed by the washing with 0.1 M-KCl in our preparation.

The fact that no radioactivity was found in any of the nucleotide spots, but only in the P_i spot, from extracts of washed frog myofibrils after short incubation of the whole muscle with ^{32}P-Ringer solution suggests that there is a source of phosphorus which is more readily exchangeable than the phosphorus of the bound adenine nucleotides.

Since the UV-absorbing substances identified on our chromatograms were present in the washed myofibrils of both the frog and the rabbit, it seems likely that they are present in the muscle of other species also. It would seem probable, therefore, that, like the proteins actin and myosin, they have a fundamental role in the contractile process.

CHAPTER V

ESTIMATION OF THE BOUND ADENINE NUCLEOTIDE IN WASHED FROG MYOFIBRILS AFTER TREATMENT WITH ATP

1. Preparation of materials and methods used

(a) The estimation of AMP, ADP and ATP.

The adaptation by Munch-Petersen (1953) of the spectrophotometric method of Kalckar (1947) was used for the estimation of the adenine nucleotides in acid extracts of the acetone powders of washed frog myofibrils. It is based on the fact that when AMP is deaminated to IMP by adenylic deaminase, there is a charge in the UV-absorption spectrum. At λ265 mμ, the extinction of IMP is only 40% of the extinction of AMP. The amount of AMP in a solution may be calculated from the estimation by Kalckar that 1γ/ml of AMP causes an average decrease in extinction at λ265mμ of 0.019 when converted to IMP. ADP and ATP are estimated in the presence of adenylic deaminase from the amounts of AMP derived from them by the successive use of myokinase and apyrase.

The following procedure was used:-

(i) Estimation of AMP

20μl of adenylic deaminase were added to 2.5 ml of the muscle extract containing 0.5M-succinate buffer pH6.5 in a quartz cell. Any AMP present would be converted to IMP in the following manner:-

$$AMP + H_2O \xrightarrow[\text{deaminase}]{\text{adenylic}} IMP + NH_3$$

The change in the optical density of λ265 mμ was read at intervals

in the Hilger "Uvispek" spectrophotometer. The value became constant after 5-10 minutes indicating that the reaction was complete.

(ii) Estimation of ADP

20 µl of myokinase were added to the extract after the deamination of AMP was complete. The ADP present was converted to ATP and AMP in the following reaction:-

$$2\ ADP \xrightleftharpoons[myokinase]{Mg^{++}} AMP + ATP$$

The AMP formed was deaminated to IMP by the adenylic deaminase, resulting in a reduction in optical density at λ265mµ equivalent to half the ADP present. The value became constant after 30-40 minutes.

(iii) Estimation of ATP

20 µl of apyrase were finally added to the extract to catalyse the following reaction:-

$$ATP \xrightarrow[apyrase]{Ca^{++}} AMP + PP$$

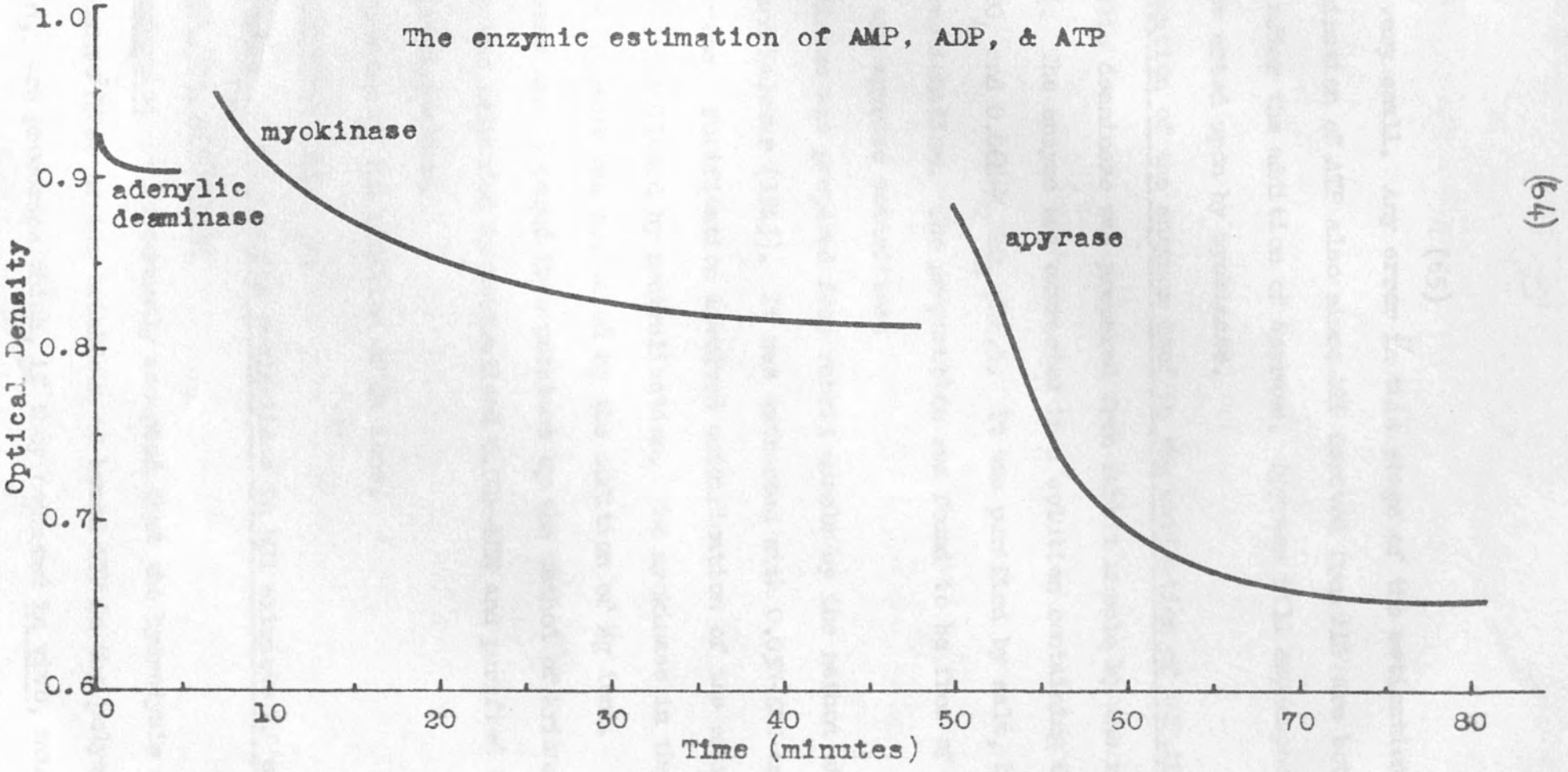
The reduction in the optical density at λ265mµ corresponded to all the ATP originally present in the extract and half the ADP which had been converted to ATP by the myokinase. The reaction was complete in 15-20 minutes.

The increases in the optical density due to each enzyme alone were measured in a buffer solution in order to obtain accurate values for the reductions in optical density in the muscle extracts. The time, course of a typical experiment is shown in figure (vi).

A disadvantage of the method is that the myokinase reaction is relatively slow, and the end-point indeterminate if the concentration

Figure (vi)

The enzymic estimation of AMP, ADP, & ATP



of ADP is very small. Any error in this stage of the estimation affects the determination of ATP also since ATP derived from ADP are both estimated after the addition of apyrase. Apyrase will dephosphorylate any ADP not acted upon by myokinase.

b. Preparation of the enzymes used in the estimation of AMP, ADP and ATP

(i) Adenylic deaminase was prepared from rabbit muscle by the method of Lee (1957). The enzyme was extracted in a solution containing 0.3M-KCl, 0.09M-KH_2PO_4 and 0.06M-K_2HPO_4 pH6.5. It was purified by salt, heat and ethanol fractionation. The preparation was found to be free of both myokinase and apyrase activities.

(ii) Myokinase was prepared from rabbit muscle by the method of Colowick and Kalckar (1943). It was extracted with 0.03N-KOH and 0.002M-versene. Purification involved acidification of the extract, heating to 90° followed by neutralisation. The myokinase in the neutralised extract was activated by the addition of Mg ions.

(iii) Apyrase was prepared from potatoes by the method of Krishnan (1949). The enzyme was extracted in neutralised 0.01M-KCN and purified by ammonium sulphate precipitation.

It was activated by the addition of Ca ions.

2. Experimental work

A Estimation of the adenine nucleotides in HCl extracts of water-washed frog myofibrils

(a) Introduction It is commonly accepted that the hydrolysis of ATP by actomyosin ATPase or the breakdown of bound ATP in the polymerisation of G-actin, are processes which, if they occurred in vivo, would be capable of providing the energy necessary for muscular contraction.

Several workers have looked for a reduction in the total ATP content of whole muscle after a single twitch (Chapter I) and conflicting results have been obtained. It seemed possible that if changes occurred only in the bound nucleotide of actin, the change in ATP concentration, being small compared with the total ATP content, might not have been detected. The bound nucleotide of rabbit myofibrils is calculated to be about 1/10th of the total adenine nucleotide (Perry 1952). In experiments on intact muscle, it is also possible that the rapid reconstitution of ATP by oxidative phosphorylation might mask any breakdown of ATP occurring during a single twitch. It was therefore decided to investigate the possibility that a reduction in the bound ATP of actin occurs during the contraction of the myofibrils, induced by ATP, in an homogenate of frog muscle.

(b) Estimation of the adenine nucleotides in the washed myofibrils of winter frogs

The leg muscles of a frog (Rana temporaria) which had been kept at 4^{o}C for several weeks were quickly removed and homogenised in 100ml. of ice-cold frog Ringer solution in a Waring Blendor for 2 minutes. The homogenate was divided into two 50ml portions. One half was made 10^{-4}M with respect to ATP pH 7.0 and 10^{-3}M with respect to $MgCl_2$ and $CaCl_2$ in a total volume of 60ml to bring about contraction of the fibrils. The other half, the control, was not treated with ATP and it was assumed that the muscle was in a relaxed or partially relaxed state. The homogenates were allowed to stand for 10 minutes at 4^{o}C and then each sample was washed 6 times with 500ml volumes of cold deionised water. The washed muscle was treated three times with 3 volumes of cold acetone and dried at room temperature for $\frac{1}{2}$ hour to 1 hour.

The acetone powders from the ATP-treated and untreated myofibrils were weighed and amounts from 90-150mg were extracted with 3-5ml. N-HCl at 4°C for 16 hours. A measured volume of the acid extract was neutralised with 2N-NaOH to approximately pH6.5. The nucleotides were immediately estimated using 2ml of the neutralised extract after the addition of 0.5ml of 0.5M-succinate buffer pH6.5

(c) Estimation of the adenine nucleotides in the washed myofibrils of summer frogs

The experiments described above were repeated using the muscle of large summer frogs which had not been kept at 4°C. The acetone powders were extracted for 1 hour with N-HCl instead of overnight.

(d) Results

(i) The adenine nucleotides in the myofibrils of winter frogs

Table I shows the results of 13 experiments on the nucleotide content of frog myofibrils washed after treatment with ATP to induce contraction, or left untreated in a state of partial relaxation. The values were obtained immediately after neutralisation of the acid extracts.

The mean values from these experiments are given here.

	Control	ATP treated
AMP µmoles/gm.	0.96	1.06
ADP µmoles/gm	1.14	1.70
ATP µmoles/gm	1.59	1.37
ATP + ADP µmoles/gm	2.73	3.07
ATP/ADP	1.40	0.80

The application of Student's t-test to the ATP and ADP levels and the ATP/ADP ratios to indicate the probability that the differences

between the controls and ATP-treated samples were due to chance, gave the following P values:-

ATP 0.05

ADP 0.01

$\frac{ATP}{ADP}$ 0.025

(ii) The adenine nucleotides in the myofibrils of summer frogs

The results of 10 experiments on the nucleotide content of the washed myofibrils of summer frogs are given on Table II. The mean values obtained are given here:-

	Control	ATP treated
AMP µmoles/gm.	0.23	0.25
ADP µmoles/gm	2.29	2.55
ATP µmoles/gm	0.78	0.63
ATP+ADP µmoles/gm	3.07	3.18
ATP/ADP	0.34	0.25

The following P values were estimated:-

ATP 0.40

ADP 0.05

$\frac{ATP}{ADP}$ 0.20

e. Conclusion A reduction in the ATP/ADP ratio after ATP treatment of the myofibrils indicated that contraction had led to a decrease in the bound ATP. The differences between the ATP/ADP ratios in the ATP treated and control myofibrils from summer frogs were not however, so significantly different as the results obtained from the myofibrils of winter frogs. The ATP content was less in both the control and ATP-treated myofibrils of the summer frogs than in the myofibrils of

TABLE I

Estimation of the adenine nucleotides in HCl extracts of washed myofibrils of winter frogs.

	AMP µmoles/gm. dry weight		ADP µmoles/gm, dry weight		ATP µmoles/gm, dry weight		ATP/ADP		ATP+ADP µmoles/gm, dry weight	
	Control	ATP treated	Control	ATP treated	Control	ATP treated	Control	ATP treated	Control	ATP treated
1	1.84	3.05	0.94	0.77	1.69	1.77	1.79	2.29	2.63	2.54
2	1.00	1.00	1.29	1.78	1.50	1.12	1.17	0.63	2.79	2.90
3	0.84	0.98	0.66	1.97	2.21	1.28	3.34	0.65	2.87	3.25
4	0.89	0.92	1.45	1.64	1.66	1.52	1.14	0.92	3.11	3.16
5	1.23	1.23	0.40	2.20	1.38	1.04	3.45	0.47	1.78	3.24
6	0.86	0.86	1.05	2.04	1.93	1.28	1.83	0.63	2.98	3.32
7	1.41	1.15	1.29	1.69	0.81	0.93	0.62	0.55	2.10	2.62
8	0.86	0.92	1.10	1.71	1.60	1.22	1.45	0.71	2.70	2.93
9	1.54	1.80	1.10	2.16	0.91	0.93	0.83	0.43	2.01	3.09
10	0.28	0.27	0.55	0.59	2.42	2.34	4.40	4.00	2.97	2.93
11	0.15	0.33	1.92	2.92	1.20	0.93	0.62	0.32	3.12	3.85
12	0.18	0.20	0.75	0.96	2.62	2.49	3.50	2.60	3.37	3.45
13	1.40	1.15	1.29	1.68	0.71	0.93	0.55	0.55	2.00	2.61

TABLE II

Estimation of the adenine nucleotides in HCl extracts of washed myofibrils of summer frogs.

	AMP		ADP		ATP		ATP/ADP		ATP+ADP	
	µmoles/gm dry weight		µmoles/gm dry weight		µmoles/gm dry weight				µmoles/gm dry weight	
	Control	ATP treated	Control	ATP treated	Control	ATP treated	Control	ATP treated	Control	ATP treated
1	0.23	0.34	2.13	2.59	1.42	1.30	0.66	0.50	3.55	3.87
2	0.17	0.19	1.49	2.44	1.27	0.55	0.85	0.23	2.76	2.99
3	0.14	0.12	2.22	2.48	0.57	0.43	0.25	0.17	2.79	2.91
4	0.19	0.12	2.22	2.70	0.78	0.64	0.35	0.24	3.00	3.34
5	0.19	0.22	1.76	1.64	0.84	1.02	0.47	0.62	2.90	2.66
6	0.13	0.25	2.85	2.99	0.60	0.52	0.21	0.17	3.45	3.51
7	0.14	0.12	2.48	2.48	0.39	0.75	0.12	0.30	2.87	3.23
8	0.33	0.37	1.90	2.63	1.08	0.67	0.57	0.26	2.98	3.30
9	0.45	0.40	2.90	2.62	0.50	0.73	0.17	0.28	3.40	3.35
10	0.34	0.38	2.95	2.97	0.35	0.27	0.12	0.09	3.30	3.24

TABLE III

1st and 2nd estimations of the adenine nucleotides in the neutralised HCl extracts of washed myofibrils of winter frogs.

	AMP		ADP		ATP		ATP/ADP		ATP+ADP	
	µmoles/gm. dry weight		µmoles/gm. dry weight		µmoles/gm. dry weight				µmoles/gm. dry weight	
	Control	ATP treated	Control	ATP treated	Control	ATP treated	Control	ATP treated	Control	ATP treated
1 i	1.84	3.05	0.94	0.77	1.69	1.77	1.79	2.29	2.63	2.54
ii	0.89	2.56	0.75	1.26	1.93	1.75	2.57	1.38	2.68	3.01
2 i	1.00	1.00	1.29	1.78	1.50	1.12	1.17	0.63	2.79	2.90
ii	1.00	1.00	1.43	1.78	1.58	1.12	1.10	0.63	3.01	2.90
3 i	0.84	0.98	0.66	1.97	2.21	1.28	3.34	0.65	2.87	3.25
ii	0.89	1.12	1.15	2.78	1.58	0.49	1.37	0.17	2.73	3.27
4 i	0.89	0.92	1.45	1.64	1.66	1.52	1.14	0.92	3.11	3.16
ii	0.81	0.98	1.15	1.87	2.22	1.48	1.92	0.79	3.37	3.35
5 i	1.23	1.23	0.40	2.20	1.38	1.04	3.45	0.47	1.78	3.24
ii	1.23	1.27	0.68	1.64	1.14	1.34	1.67	0.82	1.82	2.98
6 i	0.86	0.86	1.05	2.04	1.93	1.28	1.83	0.63	2.98	3.32
ii	0.92	0.92	1.10	1.47	1.64	1.93	1.49	1.31	2.74	3.40
7 i	1.41	1.15	1.29	1.69	0.81	0.93	0.62	0.55	2.10	2.62
ii	0.46	0.52	0.96	1.19	1.12	1.38	1.16	1.16	2.08	2.57
8 i	0.86	0.92	1.10	1.71	1.60	1.22	1.45	0.71	2.70	2.93
ii	0.89	0.92	1.17	1.15	1.56	1.71	1.32	1.48	2.73	2.86

winter frogs, although the total ATP and ADP content of approximately 2.90 - 3.10μmoles/gm was the same.

It was thought that this difference could be due to the fact that some contraction was occurring in the untreated myofibrils. The muscles from the summer frogs were not wasted like those of the winter frogs which had been kept at 4°C for several weeks. It seemed likely, therefore, that the differences between the two series of results were due to the higher metabolic activity in the muscle homogenates from the summer frogs, which may well have given rise to ATP concentrations in the controls, not very much lower than in the experiments with added ATP. In all the experiments in fact the endogeonous ATP was probably sufficient to induce a certain degree of contraction in the presence of the added Mg and Ca ions.

The higher AMP content, 1.0μmoles/gm in the myofibrils of winter frogs compared with the AMP content of summer frogs, 0.24μmoles/gm, was probably due to breakdown of some ATP and ADP occurring during the longer acid extraction (16 hours) of the acetone powders of the winter-frogs. The acetone powders prepared from the myofibrils of summer frogs were extracted for 1 hour only.

f. Second estimations of the nucleotides in the neutralised HCl extracts of washed myofibrils from winter frogs

In the experiments on winter frogs, the adenine nucleotides were in some cases estimated a second time 2 hours after neutralisation of the HCl extracts in order to obtain confirmation of the first result. However, it was found that the ATP/ADP ratio had changed. The total adenine nucleotide estimated was the same within the limits of

experimental error. The results of the first and second estimations in 8 experiments are shown in Table III.

In such an experiment, an estimation of the adenine nucleotides after extraction of some of the acetone powder from the ATP-treated myofibrils not used in the original extraction, gave an ATP/ADP ratio immediately after neutralisation similar to that obtained in the original first estimation:-

	Estimation on original extract after neutralisation	2nd estimation of original extract	Estimation on other sample of same acetone powder after neutralisation
AMP µmoles/gm	0.86	0.92	0.75
ADP µmoles/gm	2.04	1.47	2.20
ATP µmoles/gm	1.28	1.93	1.26
ATP+ADP µmoles/gm	3.32	3.40	3.46
ATP/ADP	0.63	1.31	0.57

It seemed probable that the extracts contained an enzyme which had survived washing, acetone drying, and extraction with N-HCl and became active in the neutralised extracts.

In spite of the fact that the ATP/ADP ratio was altered by the activity of this enzyme, the results obtained after neutralisation were statistically significant. For this reason, it seemed worthwhile to continue the experiments analysing the data obtained from the first estimation in both the control and ATP-treated fibrols.

B. Comparison of the nucleotides in $HClO_4$ and HCl extracts of acetone powders of washed myofibrils

a) Introduction

It was decided to estimate the adenine nucleotides in $HClO_4$

extracts of the acetone powders of washed frog myofibrils, since it might be expected that the enzyme apparently present in the HCl extracts responsible for altering the ATP/ADP ratio after neutralisation would not be active. $HClO_4$ being an oxidising agent would probably complete inhibit any enzyme if oxidisable groups like the -SH group were required for its activity. A more exact estimation of the adenine nucleotides in the contracted and relaxed states of the myofibrils would therefore be obtained in $HClO_4$ extracts.

b) Comparison of the nucleotides in $HClO_4$ and HCl extracts of ATP-treated myofibrils

A sample of an acetone powder of frog myofibrils which had been treated with 10^{-3}M-ATP, $MgCl_2$ and $CaCl_2$ to induce contraction and washed 5 times with 500ml. of water, was extracted for 1 hour with cold N-HCl. Another sample of the same acetone powder was extracted for the same time with 0.5M-$HClO_4$. The adenine nucleotides in both acid extracts were estimated immediately after neutralisation and again 2 hours later.

Results

	$HClO_4$ extract		HCl extract	
	1st estimation	2nd estimation	1st estimation	2nd estimation
AMP µmoles/gm	0.09	0.07	0.25	0.21
ADP µmoles/gm	3.18	3.18	2.85	3.09
ATP µmoles/gm	none	none	0.34	0.03
ATP+ADP µmoles/gm	3.18	3.18	3.19	3.12
ATP/ADP	-	-	0.12	0.01

The absence of ATP in the $HClO_4$ extract was remarkable. The HCl extract contained a high proportion of ADP compared with ATP as

expected with an extract of ATP-treated myofibrils. The ATP/ADP ratio was altered in the second estimation.

c) The nucleotides in the HCl and $HClO_4$ extracts of untreated myofibrils

The adenine nucleotides in the HCl and $HClO_4$ extracts of an acetone powder of frog myofibrils which had not been treated with ATP but washed 5 times with 500 ml of water, were estimated.

Results

	$HClO_4$ extract		HCl extract	
	1st estimation	2nd estimation	1st estimation	2nd estimation
AMP µmoles/gm	0.30	0.28	0.46	0.54
ADP µmoles/gm	2.48	2.48	1.11	1.01
ATP µmoles/gm	0.07	trace	1.23	1.36
ATP+ADP µmoles/gm	2.55	2.48	2.34	2.37
ATP/ADP	0.03	-	1.11	1.34

ATP was present in trace amounts only, in the $HClO_4$ extract. A high ATP/ADP ratio was obtained in the HCl extract of the untreated myofibrils as expected from earlier results (A)

d) The nucleotides in the HCl and $HClO_4$ extracts of myofibrils homogenised in frog Ringer solution

Frog muscle was homogenised in 30ml of frog Ringer solution, washed 5 times with 500 ml volumes of water and converted to an acetone powder. The adenine nucleotide content of the acetone powder was estimated in HCl and $HClO_4$ extracts immediately after neutralisation and again 2 hours later.

Result

	$HClO_4$ extract 1st and 2nd estimations	HCl extract 1st and 2nd estimations
AMP µmoles/gm	0.16	0.26
ADP µmoles/gm	2.31	1.77
ATP µmoles/gm	none	0.32
ATP+ADP µmoles/gm	2.31	2.09
ATP/ADP	-	0.18

A low ATP/ADP ratio in the HCl extract indicated that the endogenous ATP may have caused some contraction of the myofibrils in the presence of the Ca ions in the Ringer solution. ATP was again absent in the $HClO_4$ extract.

e) Conclusion The total amount of adenine nucleotide in the HCl and $HClO_4$ extracts was comparable. However, the presence of only a trace of ATP, or its complete absence in the $HClO_4$ extracts of both ATP treated and untreated myofibrils was unexpected. It seemed probable that most of the ATP found in the HCl extracts was in fact synthesised after neutralisation by the enzyme which was responsible for the change in the ATP/ADP ratio in the second nucleotide estimation on a neutralised HCl extract observed in the first series of experiments. (A(f)).

It may therefore be concluded that in both the ATP-treated and control myofibrils, most of the bound adenine nucleotide is present as ADP. However, the fact that more ATP is synthesised in the extract of the control myofibrils may still be significant in the explanation of the changes occurring during the contractile process. A substance

capable of phosphorylating ADP must be present in the HCl extracts. It is probable that a kinase reaction of the type

$$XP + ADP \rightleftharpoons ATP + X$$

brings about the observed synthesis of ATP and the change in the ATP/ADP ratio. This possibility will be considered in the discussion.

It is noticeable that the AMP content is also less in the $HClO_4$ extracts than in the HCl extracts of the myofibrils, which indicates that myokinase may still be active in the HCl extract forming AMP and ATP thus...

$$2ADP \rightleftharpoons ATP + AMP$$

Myokinase is not easily removed by washing from myofibrils and is a very stable enzyme being able to withstand acid pHs and heating to 90°C. However, if myokinase activity were present in the HCl extracts it must be very weak since its presence would have been apparent during the enzymic estimation of the nucleotides; after the addition of adenylic deaminase to the extract, a reduction in optical density, representing ADP as well as AMP would have been obtained, the subsequent addition of myokinase would not have resulted in a further reduction in extinction. If the adenine nucleotide concentrations had been solely due to the activity of myokinase, equivalent amounts of AMP and ATP, would have been found in the extracts and this did not occur.

C. The extraction of water-soluble protein from washed frog myofibrils and the estimation of its nucleotide content.

a) Introduction.

It was planned to estimate the adenine nucleotide content of frog myofibrils relaxed as well as contracted with ATP. For this reason, the muscles from one hind leg of a frog were weighed and homogenised for 2 minutes in 30ml of ice-cold 0.7% NaCl solution. The homogenate was made 10^{-3}M with respect to ATP pH6.5, and $MgCl_2$ by the addition of 5 ml. of a solution containing these substances. It was assumed that in the small volume of homogenate, the relaxing factor would be sufficiently concentrated to maintain the fibrils in a relaxed state in the presence of Mg ions and the relatively high concentration of added ATP. No reduction in the volume of muscle did in fact occur. The homogenate of the muscle from the other leg of the frog was treated with 10^{-3}M-ATP and $MgCl_2$ and $CaCl_2$ to overcome the effect of the relaxing factor. A diminution in the volume of the muscle showed that contraction had occurred. After standing at 4°C for 10 minutes, the homogenates were centrifuged at 0° and then washed 6 times with water in 40ml centrifuge tubes.

It was during the washing procedure that a very unexpected observation was made. It was noticed that after the second water washing, swelling of the fibrils which had not been treated with Ca ions, took place and a jelly-like mass of protein which occupied half the total volume of the centrifuge tube, was obtained. On further washing the volume of the gel diminished and the supernatant became opalescent

indicating that the protein had gone into solution. The addition of Mg ions, to the aqueous extracts brought about the precipitation of the protein.

Swelling took place in the myofibrils treated with $CaCl_2$ as well as ATP and $MgCl_2$ but occurred after the fourth washing in water, and was not so marked as in the muscle treated with ATP and $MgCl_2$ only.

In the earlier experiments (A) in which the homogenates had been washed with large volumes of water (500ml.), this phenomenon was not observed. Experiments were therefore carried out to investigate the nature of the extracted protein, such as the presence of nucleotides and its extractability from myofibrils treated with Mg or Ca ions.

b) Estimation of the nucleotides in the protein extracted by water from Mg-ATP-treated myofibrils

A concentrated homogenate of frog muscle was treated with 10^{-3}M-ATP and $MgCl_2$ as described above. The myofibrils were washed with water in 40ml. centrifuge tubes and as soon as the fibrils began to swell, the washings were collected. Washing was continued until it seemed as though most of the protein had gone into solution. 3 volumes of cold acetone were added to the aqueous extract and the precipitated protein was treated twice with acetone and allowed to dry. The adenine nucleotides in the N-HCl extract were estimated.

Result.

AMP	0.63 μ moles/gm.
ADP	2.29 μ moles/gm.
ATP	1.04 μ moles/gm.

ATP+ADP	3.33 μ moles/gm.
ATP/ADP	0.45

The results showed that the extracted protein contained a considerable amount of adenine nucleotide. It was therefore decided to investigate whether any adenine nucleotide remained in the residue after extraction of the protein.

c) Estimation of the nucleotides in the extracted protein and residue from Mg-ATP-treated myofibrils

The experiment described above was repeated using weighed muscle (approximately 4.0gm wet weight from one frog), and the adenine nucleotides in the residue after extraction of the protein, as well as in the protein were estimated in HCl extracts of the acetone powders.

Result.

	Protein	Residue
% of wet weight	5.20	2.25
AMP μ moles/gm.	0.08	0.46
ADP μ moles/gm.	1.96	1.61
ATP μ moles/gm.	0.71	0.74
ATP+ADP μ moles/gm.	2.67	2.35
ATP/ADP	0.36	0.46

ATP and ADP were present in similar proportions in the residue and the extracted protein.

d) & e) Estimation of the nucleotides in the extracted protein and residues from Mg-ATP-treated and Mg-Ca-ATP-treated myofibrils.

The experiment described in (c) was repeated and estimations of the adenine nucleotides in the protein and residue from Mg-Ca-ATP-treated myofibrils were also made.

Result.

d)

	Mg treatment		Mg-Ca treatment	
	protein	residue	protein	residue
% of wet weight	9.20	2.60	2.45	5.90
AMP μ moles/gm.	trace	0.78	0.20	0.20
ADP μ moles/gm.	0.59	none	1.62	1.49
ATP μ moles/gm.	0.59	0.20	1.20	0.78
ATP+ADP μ moles/gm.	1.18	0.20	2.82	2.27
ATP/ADP	1.00	-	0.75	0.52

e)

	Mg treatment		Mg-Ca treatment	
	protein	residue	protein	residue
% of wet weight	5.80	1.50	1.16	8.70
AMP μ moles/gm.	0.14	0.11	0.07	0.15
ADP μ moles/gm.	0.78	1.29	1.15	1.46
ATP μ moles/gm.	2.40	0.69	2.18	0.46
ATP+ADP μ moles/gm.	3.18	1.98	3.33	2.92
ATP/ADP	3.10	0.53	1.91	0.31

In these two experiments washing was continued in the hope of extracting all the protein from the fibrils. In (d) after 15 washings, it seemed that all the protein from the Mg-treated myofibrils had gone into solution but was still being extracted from the Mg-Ca-treated myofibrils. The appearance of swelling was delayed in the Mg-Ca-treated myofibrils and the dry weight data show that after the same number of

washings, the amount of protein extracted from the Mg-treated myofibrils was greater than from the Mg-Ca-treated myofibrils. The low total nucleotide content of the extracted protein is probably due to the fact that the dry weight was high since the protein was difficult to dry. Most of the nucleotide content of the Mg-treated myofibrils was in fact present in the extracted protein, very little was present in the residue.

In experiment (e) after 13 washings of the myofibrils, it seemed that a steady state was reached when no more protein dissolved from the swollen fibrils. The dry weights and nucleotides were estimated at this point. It was again evident that the protein was not so easily extracted from the Mg-Ca-treated fibrils as from the fibrils treated with Mg ions only.

f) Estimation of the nucleotides in the $HClO_4$ extract of Mg-treated-extracted protein and untreated-extracted protein

Although the extracted protein apparently had a high ATP content, it seemed possible in view of the earlier results that some, if not all of the ATP found in both the protein and residues was synthesised after neutralisation of the HCl extracts. It was therefore decided to estimate the nucleotide content of the $HClO_4$ extracts of the water-soluble protein in the following manner :-

The aqueous extracts of Mg-ATP-treated myofibrils were collected and a small amount of $MgCl_2$ was added to half the volume. Precipitation of protein occurred. Both halves were treated with acetone, and the nucleotides in the acetone powder of both the Mg-treated and

untreated water-soluble protein were estimated in $HClO_4$ extracts.

Result.

	Untreated protein	Mg-treated protein
AMP μ moles/gm.	0.09	0.06
ADP μ moles/gm.	0.89	1.05
ATP μ moles/gm.	0.19	none
ATP+ADP μ moles/gm.	1.08	1.05

The presence of some ATP, about 1/5th of the ADP, in the $HClO_4$ extract of the protein indicated that ATP was in fact, present in the extracted protein. The precipitation of the protein by Mg ions did however, result in the disappearance of the ATP corresponding to an increase in the ADP content.

g) Conclusion. The absence of ATP in the $HClO_4$ extract of the Mg-treated protein indicated that the ATP present in the $HClO_4$ extract of the untreated protein was derived from G-action which was polymerised by the Mg ions forming ADP-actin.

$$\text{ATP-actin} \xrightarrow{Mg^{++}} \text{ADP-actin} + P_i$$

However, the appearance of the protein indicated that it did not consist entirely of actin. A solution of G-actin is clear, and has a low viscosity and on the addition of Mg or Ca ions forms a thixotropic gel. The extracted protein gave a viscous opalescent solution from which the protein was precipitated by the addition of small concentrations of electrolytes ($MgCl_2$, KCl). The presence of actin was indicated not only by the considerable adenine nucleotide

content of the protein, but also by the fact that the latter after precipitation with $MgCl_2$, super-precipitated on addition of ATP in the manner typical of actomyosin. The high viscosity of the protein solution suggested the presence of a soluble form of actomyosin before the treatment with $MgCl_2$. This would differ from the form of a actomyosin usually encountered in the relatively low degree of polymerisation of the actin.

D. Estimation of the adenine nucleotides in acid extracts of myofibrils relaxed or contracted with ATP and washed with 0.5% KCl solution.

Since washing with small volumes of water was found to extract protein from the myofibrils, it was decided to estimate the adenine nucleotides in myofibrils washed with 0.5% KCl to maintain their structure. Homogenates of frog muscle were treated with ATP and $MgCl_2$ or with ATP, $MgCl_2$ and $CaCl_2$ to induce relaxation or contraction respectively, as described in C(a). The myofibrils were then washed 6 times in 40ml. centrifuge tubes with 0.5% KCl and once with water to remove the salt. Acetone powders were made and the adenine nucleotide content was estimated in HCl extracts. A single estimation in an $HClO_4$ extract of contracted and relaxed myofibrils was made.

Result.

The results of 5 experiments on HCl extracts are shown on Table III. Two out of the five experiments showed a lower ATP/ADP ratio after contraction of the myofibrils with ATP, $MgCl_2$ and $CaCl_2$, as expected from the earlier results (A). In two experiments, the ratios were approximately the same in the contracted and relaxed myofibrils. In a single experiment a higher ATP/ADP ratio was obtained after contraction.

The mean results are given here :-

	Relaxed	Contracted
% dry weight of wet weight	11	11
AMP μ moles/gm.	0.14	0.14
ADP μ moles/gm.	2.12	2.29
ATP μ moles/gm.	0.69	0.72
ATP+ADP μ moles/gm.	2.81	3.01
ATP/ADP	0.32	0.31

The estimation on the $HClO_4$ extract gave the following result :-

	Relaxed	Contracted
% dry weight of wet weight	10.5	10.5
AMP μ moles/gm.	0.11	0.06
ADP μ moles/gm.	3.32	2.78
ATP μ moles/gm.	0.11	0.07
ATP+ADP μ moles/gm.	3.43	2.85
ATP/ADP	0.33	0.25

The ATP concentrations in the $HClO_4$ extracts from contracted and relaxed myofibrils were both very low compared with the results on the HCl extracts. The presence of an enzyme in the HCl extracts capable of synthesising ATP was again indicated.

The variability in the differences between the ATP/ADP ratios in the HCl extracts from contracted and relaxed myofibrils will be considered in the discussion. (Chapter VI(c))

TABLE IV

Estimation of the adenine nucleotides in HCl extracts of frog myofibrils washed with 0.5% KCl

	AMP µmoles/gm. dry weight		ADP µmoles/gm. dry weight		ATP µmoles/gm. dry weight		ATP/ADP		ATP+ADP µmoles/gm. dry weight	
	Mg	MgCa	Mg	MgCa	Mg	MgCa	Mg	MgCa	Mg	MgCa
1	0.14	0.16	1.60	2.19	1.14	0.94	0.71	0.43	2.74	3.13
2	0.14	0.17	1.74	2.38	0.64	0.34	0.34	0.14	2.38	2.72
3	0.12	0.10	2.59	2.58	0.58	0.57	0.22	0.22	3.17	3.15
4	0.17	0.14	2.06	2.18	0.48	0.56	0.23	0.26	2.54	2.74
5	0.13	0.14	2.62	1.85	0.66	1.18	0.25	0.64	3.28	3.03

CHAPTER VI

DISCUSSION

(a) Summary of Experimental Findings.

The major findings from the experimental work described in this thesis are the following:-

(i) No evidence was found that phosphorylation of myosin or actomyosin, or that ATP binding by the muscle proteins takes place during contractile activity. The possibility that actin is phosphorylated was not eliminated.

(ii) Most of the adenine nuoleotide in washed frog and rabbit myofibrils is ADP, smaller amounts of ATP and AMP being present. The total amount of adenine nucleotide bound in frog myobrils is approximately 2.90 - 3.10 μmoles/gm. dry weight, and most of this is maintained as ADP in the contracted and relaxed states.

(iii) An enzyme which is presumably bound in the frog myofibrils is capable of synthesising ATP from ADP in neutralised HCl extracts of the myofibrils. Although there is some indication that myokinase may also be present which could synthesise ATP from ADP, the results cannot be fully explained as being due to myokinase activity only. The formation of more ATP in the acid extracts of the control myofibrils than in the extracts of ATP-treated myofibrils, is detectable immediately after neutralisation.

(iv) A substance readily yielding inorganic phosphate is present

in washed frog and rabbit myofibrils. The use of ^{32}P showed that this phosphate fraction in frog muscle is more readily exchangeable than the phosphate groups of the adenine nucleotides.

(v) A form of actomyosin containing G-actin which is not present in the form of actomyosin usually extracted from muscle by salt solutions, was extracted from the ATP-treated myofibrils when they were washed with small volumes of water.

A description of the possible changes taking place during contraction and relaxation which could explain the observations described in this thesis will be discussed. The results of other workers which have any bearing on the suggested mechanism will be considered.

(b) A theory of Contraction and Relaxation.

The results have shown that the bound adenine nucleotide of actin in both relaxed and contracted myofibrils is mainly ADP but that an enzyme, which is active in neutralised HCl extracts of the acetone powders of washed myofibrils is capable of synthesising ATP from ADP released from the acetone powders by the acid treatment. Since the formation of ATP by myokinase activity does not occur to any great extent, a substance capable of phosphorylating ADP must also be present in the extracts. It seems possible that in the intact muscle, the reaction which maintains actin in the polymerised form is a reversible reaction, catalysed by a phosphokinase, which takes place between the bound ADP of actin and a bound phosphate containing substance XP.

XP would be capable of phosphorylating actin-ADP in the following manner:-

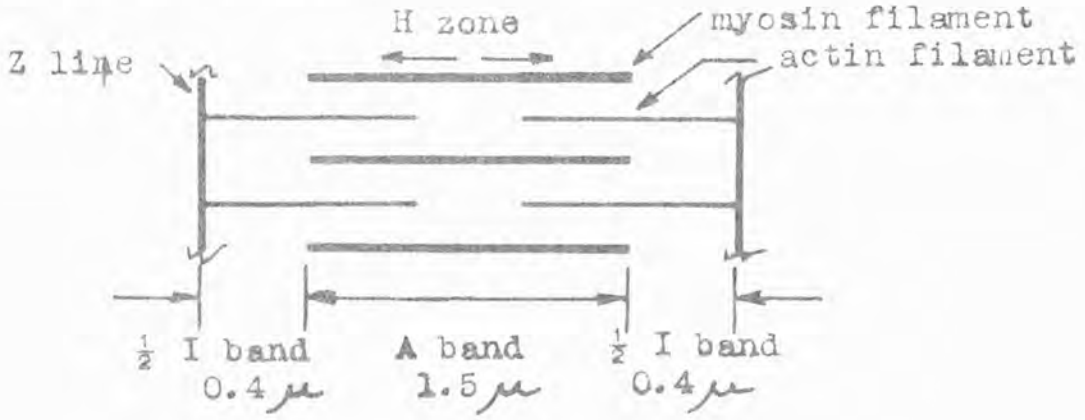
$$\text{ADP-actin} + \text{XP} \xleftrightarrow{\text{phosphokinase}} \text{ATP-actin} + \text{X}$$

The ionic conditions *in vivo* would probably favour the existence of actin in the polymerised form (Perry 1952) so that the amount of ATP-actin present at any given moment would be very small compared with the amount of ADP-actin present, assuming that the above reaction takes place.

It is generally accepted that contraction occurs as a result of the relative movement of the actin and myosin filaments in the myofibrils. It is postulated that in relaxed muscle, the actin filaments would contain mainly bound ADP and XP. Stimulation of the muscle would result in a change in ionic concentrations favouring the phosphorylation of actin-ADP by XP and the combination of the ATP formed with an active site on the myosin filaments, in the regions of the myofibrils where the actin and myosin filaments overlap. In the combination of ATP with its substrate myosin, it has been calculated from the K_m, that more energy would be available for the contraction of muscle than is made available in the hydrolysis of ATP by myosin ATPase. However, the hydrolysis of ATP must take place in order that the filaments may separate. ATP formed between the filaments would therefore have only a momentary existence. When hydrolysis had occurred, because ATP would also be formed in the regions of the actin filaments not adjacent to myosin filaments, there would be a tendency for movement to occur in order that this ATP may also combine with its substrate.

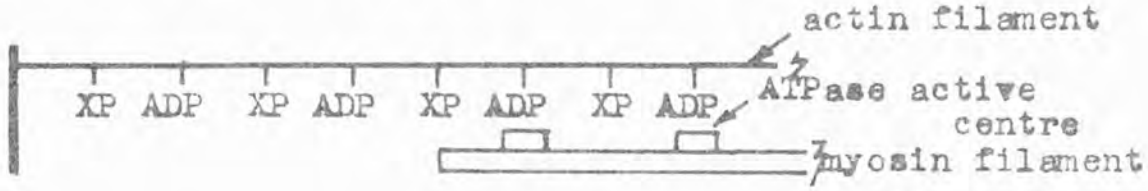
Figure (vii)

(a) Arrangement of filaments in one sarcomere of a myofibril at rest-length



(b) Relationship between actin and myosin filaments in resting myofibril.

(ATP-actin formed in the reversible reaction between ADP-actin and XP not shown, being very small compared with ADP-actin.)



(c) Changes resulting from stimulation.

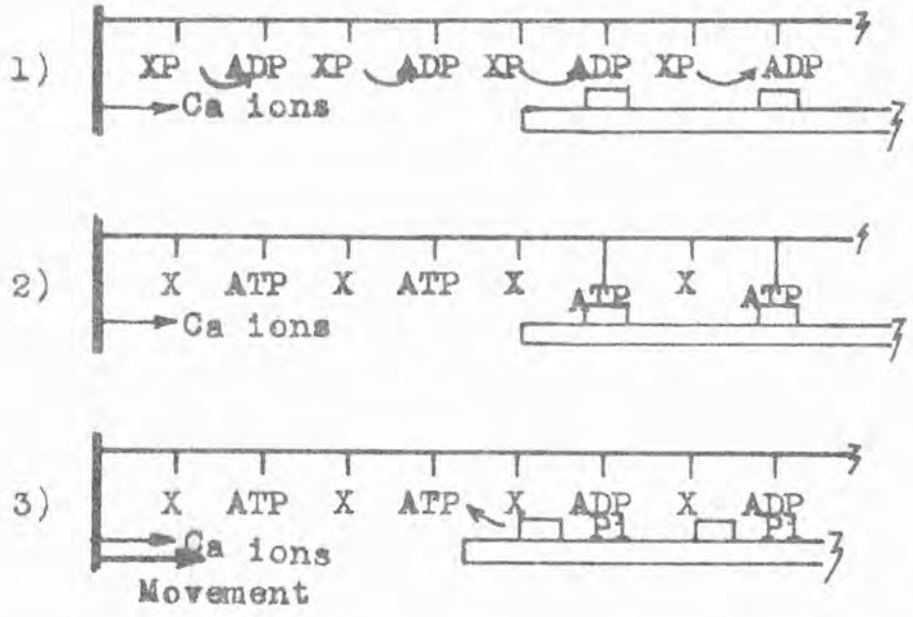


Figure (vii) represents the reactions which may take place between the actin and myosin filaments during contractile activity.

In the contracted state as in the resting state most of the nucleotide of actin would be maintained as ADP by means of the phosphokinase reaction. However, the phosphate group of XP would be utilised during contraction and in order that relaxation may occur, the rephosphorylation of X must take place. This could be brought about by sarcoplasmic ATP thus:-

$$ATP + X \rightleftharpoons XP + ADP$$

The reaction could presumably also be catalysed by the phosphokinase. When XP was again formed, the filaments could slide back into their original resting positions. Relaxation would therefore be a passive process as indicated by the heat data of Hill (1949 Chapter I (d)).

(c) Control of the Contractile Processes.

It is suggested in the present theory that a change in the ionic concentrations in the myofibrils could lead to their contraction. Contraction of an isolated glycerinated fibre only occurs when it is treated with ATP and Mg ions and is accompanied by the hydrolysis of the ATP. However, ten times more ATP is hydrolysed than would be required to supply the energy needed for the work done by the fibre in contracting. (Bowen and Martin 1962). It therefore seems as though, in an isolated system, some factor which prevents this inefficient process in vivo is absent. According to the present theory, the ATP added to a glycerinated fibre would bring about the phosphorylation of X, thus:-

$$ATP + X \rightleftharpoons ADP + XP$$

In the presence of suitable ionic concentrations the formation of ATP-actin from XP and the combination of the ATP with myosin would be favoured, leading to contraction. It seems possible that the phosphokinase reaction, which it has been suggested maintains actin in the polymerised form *in vivo* may not be the only reaction leading to the formation of ADP-actin in isolated contractile systems. It is well established that actin solutions polymerise on the addition of Mg ions in the following manner:-

$$ATP\ actin \xrightarrow{Mg++} ADP\text{-}actin + P_i$$

It is not unlikely that this reaction, as well as the phosphokinase reaction takes place in isolated glycerinated fibres. Some ATP-actin formed in the phosphokinase reaction may therefore be polymerised in the above reaction and not utilised in the contractile process. More ATP would therefore be broken down than would be necessary to supply the energy for contraction.

ATP and Mg ions in the presence of relaxing factor bring about the relaxation and not the contraction of actomyosin, glycerinated fibres and fibrils. Ca ions in very low concentrations will, however, bring about contraction in these systems. Since the behaviour of contractile systems in the presence of relaxing factor is presumably a closer approximation of their behaviour *in vivo* it seems probable that an increase in the Ca ion concentration in the myofibrils may bring about the contraction of living muscle. Ca ions may in fact activate the combination of the ATP of actin formed in the

phosphokinase reaction, with its substrate myosin.

It has been suggested by A.F. Huxley (1957) that the change in membrane potential which leads to contraction of a muscle fibre, is propagated through the whole structure by means of the Z-line. The Z-line is apparently a continuous membrane which runs through the whole muscle uniting individual fibrils to each other and to the sarcolemma. It seems possible that the impulse is propagated to the contractile proteins by means of the release of Ca ions from the Z-lines. Relaxing factor activity is associated with the microsomal particles of the endoplasmic and reticulum in muscle. As the endoplasmic reticulum may be present as a network running between the actin and myosin filaments, it is possible that the relaxing factor provides a mechanism for pumping Ca ions back to the Z-lines so that relaxation can take place. According to Hasselbach (1960) the relaxing factor has the ability to accumulate Ca ions so that this function does not seem impossible.

(d) The ATP/ADP Ratios in HCl Extracts of Myofibrils contracted with ATP.

Although the ATP content of the HCl extracts of washed myofibrils is apparently an artefact due to the presence of phosphokinase activity, it was found that the ATP/ADP ratio in the extracts from myofibrils treated with ATP to induce contraction was lower immediately after neutralisation than in the extract from the control myofibrils. If it has not been found that the ATP was an artefact, this would have suggested that the energy for contraction had been supplied by the breakdown of

of bound ATP.

According to the present theory, during contraction the phosphorylation of actin-ADP by XP takes place by means of the phosphokinase reaction, and X is rephosphorylated in order that relaxation may occur. Therefore, in the contracted myofibrils, less XP may be present at any given moment than is present in the relaxed myofibrils. XP as well as ADP may presumably be released from the acetone powder treatment and participate in the reaction

$$XP + ADP \rightleftharpoons ATP + X$$

assuming that the phosphokinase is also released. The higher concentration of XP present in the extracts from the control myofibrils will favour the synthesis of more ATP from ADP in the phosphokinase reaction immediately after neutralisation than may occur in the extract from the ATP-treated myofibrils. This will account for the higher ATP/ADP ratio in the extracts from the control myofibrils than in the extracts from the ATP treated myofibrils immediately after neutralisation, and for the change in the ratio in the second estimation on the neutralised extract.

However, this explanation does not completely explain the results which were obtained. It was also noticed that although the ATP/ADP ratio was lower in the ATP-treated myofibrils the ATP plus ADP was slightly higher than in the controls, e.g. Mean of 13 experiments on myofibrils from winter frogs:-

	Control	ATP treated
ATP + ADP	2.73 μmoles/gm	3.07 μmoles/gm.

In the experiments on fibrils treated with ATP, $MgCl_2$ and $CaCl_2$ to induce contraction, or ATP and $MgCl_2$ only to induce relaxation (Chapter V.D.), it was noticeable that the single experiment which gave a much higher ATP/ADP ratio in the fibrils treated to bring about contraction - the opposite of the expected result, a lower ATP plus ADP was found than in the fibrils treated to induce relaxation (Table IV.5). In the experiments giving the expected lower ATP/ADP ratio after treatment of the myofibrils to induce contraction, the ATP plus ADP was higher in agreement with the earlier results. Further investigation is needed to explain these observations.

(e) The Water Soluble Protein.

It was found that when frog myofibrils were washed with small volumes of water, protein was extracted in such quantities that even in the absence of other evidence it must be concluded that it was derived from the structural porteins. The precipitation of the protein by Mg ions and the super-precipitation of the gel on the addition of ATP, suggested the presence of actomyosin. However, actomyosin containing only F-actin is extracted from muscle by 0.5 M KCl and is not generally considered to be water-soluble. The estimation of the nucleotide content in the $HClO_4$ extract of the protein showed that it contained only ADP when the protein was precipitated with Mg ions. The ATP was probably derived from G-actin formed in the phosphokinase reaction.

$$\text{ADP actin} + \text{XP} \rightleftharpoons \text{ATP-actin} + \text{X}$$

It seems possible that in the lowering of the ionic strength by the washing procedure, the equilibrium in the above reaction which *in vivo* favours the formation if ADP-actin was displaced so that at any given moment, more ATP-actin was formed. The disappearance of ATP on the addition of Mg ions probably resulted from the polymerisation of the ATP-actin catalysed by Mg ions, which occurs *in vitro*.

$$\text{ATP-actin} \xrightarrow{Mg + +} \text{ADP-actin} + P_i$$

It is not unlikely that the sites on the actin filaments where ATP is a prosthetic group at a given moment, may be points of instability so that the disturbance in the structure of the myofibrils resulting from the washing procedure may have led to the release of actin fragments at points where ATP is the prosthetic group. Chains of F-actin molecules with terminal ATP-actin groups would therefore be extracted, at the same time releasing the myosin molecules which would no longer be held in a fixed position by the actin filaments. It is questionable whether the extracted protein was dissociated G and F-actin and myosin, or actomyosin containing actin in a low state of polymerisation. The latter seems more likely in view of the super-precipitation of the Mg-precipitated gel on the addition of ATP. According to Szent-Györgyi (1947) myosin is soluble in distilled water and F-actomyosin is dissociated in the absence of very low concentrations of Mg ions. The myofibrillar proteins themselves contain bound Ca and Mg ions, and it seems probable that if, in fact, the washing procedure led to the complete elimination of free ions, the bound Mg of the proteins could possibly bring about the association of actin and myosin.

It was found that in the myofibrils treated with ATP, $MgCl_2$ and $CaCl_2$ to induce contraction, the extraction of the protein was delayed, and did not occur to the same extent as in the myofibrils treated with ATP and $MgCl_2$ to induce relaxation. However, the nucleotide composition and therefore probably the actin/myosin ratio, was similar in the contracted and relaxed myofibrils. It is conceivable that the concentration of XP which will be different in the contracted and relaxed states, determines the extractability of the protein. In the earlier experiments where the same amount of muscle was washed with 500 mls of water, the swelling of the fibrils was not observed and could not have passed un-noticed. It seems that there may be some factor in the relaxing factor system which facilitates the extraction of the protein when not excessively diluted.

(f) AMP and Myokinase Activity in the Myofibrils.

A comparison of the adenine nucleotides in the $HClO_4$ extract of washed frog myofibrils with the adenine nucleotides in TCA extracts of washed rabbit myofibrils estimated by Perry (1952) shows that the amounts are of the same order of magnitude in both species:-

Units:- μmoles/gm.

	AMP	ADP	ATP	Total Nucleotide
Rabbit	0.88	2.70	0.47	4.05
Frog	0.30	2.48	0.07	2.85 (Chapter V.B(c))

ADP is present in the greatest amount in both washed frog and rabbit myofibrils; the proportions of AMP and ATP are greater in rabbit myofibrils. However, a strict comparison cannot be made as the fibrils

were prepared under different conditions, those of the rabbit being prepared by extensive washing of an homogenate of rabbit muscle in 0.1-M borate buffer pH7. Breakdown of some ATP and ADP to AMP may have taken place as apparently occurred in frog myofibrils when they were extracted with N-HCl for 16 hours. After this time, the AMP content was found to be 1.0 μmoles/gm compared with a value of 0.24 μmoles/gm when the fibrils were extracted for 1 hour only.

AMP isformed in muscle from ADP in the myokinase reaction.

$$2ADP \rightleftharpoons ATP + AMP$$

Myokinase is a very stable enzyme which survives low pHs, and a temperature of 90°C. It is also not easily extracted by washing from rabbit myofibrils. Myokinase activity in frog muscle has apparently not been investigated. The possibility that survival of myokinase in the HCl extracts of frog myofibrils could lead to the synthesis of ATP and the change in ATP/ADP ratio has been considered (Chapter V.B(e)). However, if the enzyme had been significantly active in the neutralised HCl extracts, its presence would have been obvious during the enzymic assay of the nucleotides.

The possible significance of bound AMP in muscle, if, in fact, it is present <u>in vivo</u> is not clear. It is probably derived from ADP by myokinase activity, its subsequent deamination by adenylic deaminase providing a pathway for the elimination of nucleotides from the body. Recently Cain, Infante and Davies (1962) obtained evidence which led them to suggest that synthesis of ATP from ADP by myokinase activity, may occur in extreme conditions to provide the energy necessary for

muscular contraction. They found that CP breakdown could be detected during a single twitch of frog abdominis when the CP content had first been reduced by treatment of the muscle with DNP. When the CPkinase reaction was inhibited by FDNB, ATP was split during a single contraction, but the net amount of ADP produced was less than expected from the ATPase reaction. However, the discrepancy could be explained if myokinase were active in reconstituting ATP from the ADP produced. The presence of DNP and FDNB would alter the normal metabolic activity of the muscle drastically; it seems possible that the formation of ATP from ADP by myokinase may not be quantitatively important for contractile activity under normal conditions. The nucleotides were estimated in whole muscle so that it is not known whether the bound ADP of actin or only the sarcoplasmic ADP, or both, were converted to AMP and ATP under these conditions.

(g) Evidence for the Role of Actin in Contractile Activity.

Straub (1950, Chapter I(b)) found in preparing actin from acetone powders of muscle that although the acetone powder contained only ADP, the actin solution obtained from it by distilled water was ATP-actin which polymerises on the addition of Mg ions. Since at the time, there was no evidence that an enzyme was present which could synthesise ATP, he concluded that it must be formed in the depolymerisation which accompanied the extraction of the protein.

It seems possible that, in fact, ATP was synthesised from XP by the phosphokinase which we have suggested is present in the acetone powders of washed frog myofibrils. Straub suggested that in relaxed muscle actin is present as ATP-actin which plymerises on contraction

giving ADP-actin. Mommaerts, (1952, Chapter I(b)) calculated that this would provide sufficient energy for the contractile process. The finding by Strohmann (1959, Chapter I(d)) that ADP-actin can be phosphorylated by CP in the presence of CPkinase, thus suggesting a mechanism for physiological depolymerisation, gave support to Straub's theory. However, Martonosi (1960, Chapter I(e)) after examining the nucleotides in the actomyosin from animals injected with ^{32}P, could find no evidence that actin polymerisation and depolymerisation takes place in vivo. He concluded that F-actin is present in both relaxed and contracted muscle as is indicated by the experiments described in this thesis.

According to the present theory, the phosphorylation of actin would take place during contraction, a reaction which would probably not have been detectable in the experiments on phosphorus uptake by myofibrils, (Chapter III). However, the proposal is supported by the work of Ulbrecht and Ulbrecht (1957, Chapter I(d)). It was found that a transfer of phosphate between AD^{32}P and ATP took place in myofibrils from which myosin had been extracted as well as in intact myofibrils, which could have resulted from a phosphorylation of the actin.

In the present experimental work (Chapter IV, 2(e)) it was shown, after pre-incubation of the frog rectus abdominis with ^{32}P-Ringer solution, that a substance yielding P_i on treatment of the acetone powder of the washed myofibrils with acid was present, and that its phosphate group was more readily exchangeable than the phosphate of the bound adenine nucleotide. It seems possible that in fact this substance

was XP.

Martonosi (1960, Chapter I(f)) made an investigation of the steady-state reaction occurring between G and F-actin at low Mg ion concentrations. P_i was released during this process at a much greater rate than seemed possible if the mechanism involved a continuous breakdown of ATP and the combination of a new molecule of ATP with actin in each polymerisation step. The suggestion was made that a dephosphorylating enzyme might be present, dependent on the Mg ion concentration in the same way as the equilibrium between G and F-actin. It is possible that this observation may have some bearing on the proposed presence of XP bound to actin in the myofibrils.

(h) The Sources of Energy for Muscular Contraction.

The breakdown of CP in muscle after prolonged contractile activity observed by Eggleton and Eggleton (1927) is well established. It has been suggested by several workers that CP is used to reconstitute ATP by means of the CPkinase reaction, ATP being presumably a more immediate source of energy than CP.

$$ADP + CP \overset{\text{CPkinase}}{\rightleftharpoons} ATP + C$$

According to the present theory, CP would replenish sarcoplasmic ATP used in the rephosphorylation of X bound to actin. A decrease in ATP and increase in ADP in extracts of whole muscle would not therefore be detected after a few contractions unless the CPkinase reaction were inhibited. The results of attempts to detect changes in ATP, ADP and CP concentrations during a single twitch have been discussed (Chapter I(c) and (g)).

In the recent experiments of Crain, Infante and Davies (1962) mentioned in this Chapter (section (e)), after the inhibition of CPkinase by FDNB, the breakdown of ATP was in fact found to occur during a single twitch. Under normal conditions, it was concluded that the ATP would be replenished by the action of myokinase and CPkinase. However, Mommaerts (1962, Chapter I(g)) could find no change in the ATP or ADP concentrations in whole muscle after a single twitch. In one series of experiments, an increase in P_i and C after contraction was obtained. In another series of experiments, a liberation of P_i only occurred, and in a third series, C and not P_i was liberated after contraction. Fleckenstein (1954, Chapter I(c)) also found that contraction could occur in the absence of detectable breakdown of CP or ATP. However, P_i was liberated in such an amount that it could account for the work done, assuming it was derived from an energy-rich source. Two unidentified UV-absorbing phosphate-containing substances were found in the muscle in amounts comparable to ATP and ADP which could be sources of this phosphate bond energy.

There is therefore, a considerable amount of evidence that a source of energy may be present in muscle apart from that which may be derived from ATP and CP in known enzymic reactions. The participation of a substance such as XP in the contractile process does not therefore seem unlikely. Although its energy would only be used indirectly, since its utilisation would involve the transient formation and breakdown of ATP, only the liberation of P_i and not a reduction in ATP concentration would be detectable after contraction. It would be interesting to investigate the possible presence of bound CP in the washed myofibrils.

The reaction proposed to occur between ADP-actin and bound XP is very similar to that occurring between CP and ADP-actin catalysed by CPkinase in vivo.

$$CP + \text{ADP-actin} \rightleftharpoons C + \text{ATP-actin}.$$

Carlson and Siger (1960, Chapter I(g)) estimated that in a single isometric twitch, one CP molecule was dephosphorylated per actin molecule present in muscle, and suggested that the above reaction may take place in vivo between ADP-actin and CP which diffused into the "actin department". It does not seem impossible that XP may in fact be bound CP.

However, the substances yielding phosphate bond energy in Fleckenstein's experiments (1960, Chapter I(c)) showed strong UV-absorption. It also seems possible therefore that the oligo- or polynucleotide found in the extracts of washed frog and rabbit myofibrils (Chapter IV.3) may not be merely derived from contaminating microsomal RNA, but, in fact, be derived from XP, which may be a phosphorylated oligo-nucleotide.

SUMMARY

Chapter I

The literature is reviewed concerning the bound nucleotide of actin and its possible significance in muscular contraction.

Chapter II

The objects of the investigation are described.

Chapter III

1. Attempts are described to detect the expected increase in the phosphorus content of washed myofibrils, myosin-depleted myofibrils and actomyosin after treatment with ATP, if ATP is bound by or phosphorylates the myofibrillar proteins during contractile activity.

2. It is concluded that if net phosphorus uptake occurs it is only very small and not greater than would correspond to the phosphorylation of actin-ADP.

Chapter IV

1. The examination by paper chromatograph of the UV-absorbing substances in the acetone extracts, and water and HCl extracts of the acetone powders of washed frog and rabbit myofibrils is described.

2. It is concluded that mostly ADP and smaller amounts of ATP and AMP are present in both frog and rabbit myofibrils and are presumably derived from the prosthetic group of actin.

3. The possible derivation from the adenine nucleotides of IMP, hypoxanthine and adenine which are also present is discussed.

4. It is concluded that an oligo- or polynucleotride present in both frog and rabbit myofibrils, may be derived from microsomal RNA or may in fact have some importance in muscular activity.

5. The suggestion is made that a green pigment in extracts of both frog and rabbit myofibrils may be an artefact from a haem pigment of nitochondrial origin.

6. It is found that a phosphate fraction is present in washed frog and rabbit myofibrils which is not associated with any UV-absorbing substance. Chromatograms of the HCl extract from the washed myofibrils of the frog rectus abdominis pre-incubated with ^{32}P-Ringer solution reveals that this phosphate fraction is more readily exchangeable than the phosphate of the adenine nucleotides.

Chapter V

1. The enzymic estimation of AMP, ADP and ATP in neutralised acid extracts of acetone powders of washed frog myofibrils is described.

2. It is found that the ATP/ADP ratio in the HCl extracts of myofibrils which have been treated with ATP to induce contraction is greater immediately after neutralisation than in the extract of the control myofibrils. The ratio is altered in both extracts when the nucleotides are estimated again 2 hours later.

3. Estimations of the nucleotides in $HClO_4$ extracts of the myofibrils reveals that in both ATP treated and control myofibrils nearly all the adenine nucleotide is ADP. It is concluded that the ATP in the HCl extracts must be synthesised from ADP by an enzyme which is also responsible for the change in ATP/ADP ratio after neutralisation, and which is inactivated by $HClO_4$.

Since the ATP is not apparently synthesised from ADP by myokinase, it is suggested that it must be formed in a phosphokinase reaction between ADP and an unknown phosphate - containing substance, XP, thus:-

$$ADP + XP \rightleftharpoons X + ATP$$

4. Although the lower ATP/ADP ratio is the HCl extract from ATP-treated myofibrils than in the extract from the control myofibrils does not show that bound ATP is broken down in contraction, it is suggested that the difference between the amounts of ATP synthesised from ADP may be significant in the explanation of the changes occurring in the myofibrils during contractile activity.

5. The extraction of protein from frog myofibrils treated with ATP and $MgCl_2$ to induce relaxation, by washing with small volumes of water is desribed. It is found that the protein is more readily extractable from myofibrils treated under conditions favouring relaxation. The protein contains ADP and some ATP which appears as ADP when the protein is precipitated with $MgCl_2$. The suggestion is made that it may be a form of actomyosin differing from the actomyosin extracted from muscle by salt solutions in that the actin is not completely polymerised.

6. The nucleotides are estimated in extracts of Mg-ATP-treated myofibrils washed with 0·5% KCl to preserve their structure. The results again indicate that the ATP in the HCl extracts is synthesised enzymically from ADP.

Chapter VI

1. The main findings from the experimental work are summarised and a theory of contraction and relaxation is proposed which could explain the results. The theory involves the presence of an unknown phosphate-containing substance XP bound to actin in the myofibrils which is able to phosphorylate the bound ADP of actin thus :-

$$XP + \text{ADP-actin} \rightleftharpoons X + \text{ATP-actin}$$

It is postulated that since the ionic conditions *in vivo* probably favour the existence of actin in the polymerised form, the amount of ATP-actin present at any given moment would be minute compared with the amount of ADP-actin present. A series of events is described which could lead to contraction even though the bound nucleotide of actin is maintained as ADP in both contracted and relaxed myofibrils. However, more XP would be present in relaxed myofibrils at a given moment than would be present in contracted myofibrils.

2. It is concluded that the ATP in the neutralised HCl extracts is synthesised from ADP and XP released from the acetone powders of the myofibrils, in a reaction catalysed by the phosphokinase which survives the acid treatment.

$$ADP + XP \rightleftharpoons ATP + X$$

It is suggested that the ATP/ADP ratio is higher in the HCl extract from control myofibrils than in the extract from ATP-treated myofibrils, because more XP is present in the control myofibrils.

3. The nature of the water-soluble protein and the possible changes leading to its extraction from the myofibrils are considered.

4. The observations of other workers concerning the state of actin in living muscle and which support the presence of a substance such as XP, are discussed.

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