

THE EXCHANGE OF ACTIN-BOUND ADP
IN MUSCULAR ACTIVITY

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ABSTRACT

Szent-Györgyi and Prior (1966) provided evidence for the exchange of actin-bound ADP during superprecipitation of actomyosin and myofibrils. Tentative experimental support was given by Cheesman and Whitehead (1968) to the suggestion that the exchange also occurred in vivo as a result of muscular contraction. Further experiments with improved techniques have confirmed these findings.

Injections of ^{14}C -glucose subcutaneously into frogs leads to a rapid incorporation of label into the total ATP by synthesis de novo. The total ATP therefore assumes a high specific activity, and, by an exchange of nucleotide, the bound ADP associated with actin also becomes labelled. In phasic muscles of active frogs the specific activity of the bound ADP soon exceeds that of the total ATP, which suggests that the myofibrils are in contact with a highly labelled nucleotide pool. The labelling of bound ADP in tonic muscles is much slower.

Curarisation, and therefore muscular paralysis, before the injection of ^{14}C -glucose produces an inhibition of uptake of radioactive isotope, which provides the first support for the view that the exchange of nucleotide is attendant upon contraction.

Stimulation, either by depolarisation with KCl or by direct electrical excitation, brings the bound nucleotide into exchange with the pool.

Since an exchange of nucleotide seems only to occur in vitro as a result of depolymerisation or a change in the conformation of actin, these results provide strong evidence for a similar change in the physical state of this protein during muscular activity

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ABBREVIATIONS

The abbreviations used in this account are those included in the instructions to authors provided by the Biochemical Journal, with the following exceptions:

CP-kinase	-	creatine kinase
PC	-	phosphoryl creatine
C	-	creatine

CHAPTER I, PART I.INTRODUCTION

There is now no doubt that muscular contraction occurs as a result of an interaction between ATP and the two major structural proteins of the myofibril, myosin and actin. The location of these proteins in the thick and thin filaments respectively has been established by extensive electron microscope studies. Light has also been thrown upon the nature of the cross-bridges which arise at intervals along the myosin filaments. These cross-bridges are formed from a part of the myosin molecule termed the head region or heavy meromyosin (HMM). They contain the actin-binding and ATPase sites of the whole protein and form the transitory connections with the thin filaments which result in the sliding of the interdigitating filaments relative to each other. This is the basis of the sliding filaments theory of Hanson & Huxley (1955), the essential features of which are now as follows: the formation of a link between the myosin cross-bridges and actin mediated by ATP, the hydrolysis of ATP by myosin ATPase and the breaking of the protein-protein link. This sequence of events results in the sliding of the actin filaments relative to the myosin filaments.

There are many detailed theories put forward to account for this sliding of the filaments. The most detailed of them by Davies (1963) involves the formation of a link between actin-bound ADP and ATP attached to the end of a polypeptide chain on the HMM, mediated by Ca^{++} ions. The neutralization of electrostatic charges causes the polypeptide chain to spring into an α -helix and drag the actin filament along. The link is broken by the ATPase activity of myosin. Another and more recent theory due to Huxley (1967) is based on the

positions of the cross-bridges in the different states of the muscle. It postulates the existence of a hinge region in the HMM which enables a change in the angle of attachment to exert a pull on the actin filaments. However, none of these theories involve the actin filament in any role other than being pulled along by the myosin.

One of the fundamental properties of actin is that it exists in two interconvertible forms, and several workers since the 1940's (Szent-Györgyi, 1943; Straub and Feuer, 1950; Mommaerts, 1950), have supposed that a $G \longleftrightarrow F$ transition of actin is not a wasted property but may be an essential feature of the contractile process. More recently, evidence has come to light in favour of a change in conformation of the actin during superprecipitation of actomyosin, measured by an exchange between bound and pool nucleotides which only accompanies such a change in conformation.

Discovery of actin.

Early in the 1940's Banga and Szent-Györgyi (1941) noticed that, depending on the extraction time of rabbit muscle mince, two different myosin solutions could be obtained, called myosin A and myosin B. Myosin B gave very viscous solutions and contracted when ATP was added, and myosin A, extracted over the shorter time, was less viscous and gave no mechanical response to added ATP. Straub (1942), studying the different properties of these solutions obtained from myosin B a highly viscous solution, which when added to myosin A converted it to B. This viscous protein he called actin. He subsequently discovered that actin could itself exist in two forms; a globular non-viscous form called inactive actin, (G-actin) and a fibrous viscous form called active

actin, (F-actin) and that the globular form could be converted to the fibrous form by the addition of neutral salts (Straub, 1943). Sodium chloride (NaCl) or potassium chloride (KCl) were most effective at 0.1 M concentrations, but magnesium (Mg^{++}) or calcium ions (Ca^{++}) would induce polymerisation at lower ionic strengths. In the simultaneous presence of monovalent ions Ca^{++} had an inhibitory effect on polymerisation, whereas Mg^{++} overcame the lag period characteristic of polymerisation with these ions. Actin preparations contained bound Mg^{++} and Ca^{++} ; if these were removed with ethylene diaminetetra-acetic acid (EDTA), polymerisation could be inhibited (Feuer et al., 1948).

Location and structure.

The existence of two kinds of filament making up the A and I bands of striated muscle was confirmed by the electron microscope studies of Huxley (1953), and the nature of the I substance in the thin filaments was initially identified by Hanson and Huxley (1957) as actin. Actin constitutes approx. 20% of the total dry weight of protein. The thin filaments, 55 Å in diameter, are constructed of two helically intertwined strands of actin monomers (Hanson & Lowy, 1963). X-ray diffraction studies of living frog sartorius muscles yield values of between 50.9 Å and 59.1 Å for the actin subunit repeat and of 2×370 Å for the pitch of the actin helix (Huxley and Brown, 1967). On this basis the helix contains between 13 and 14 monomers per turn. In the ionic conditions which exist in the muscle one would suppose that actin is present in the fibrous form. This is supported by X-ray diffraction data and electron microscopy (Hanson and Lowy, 1963), by the nature of the bound nucleotides (Perry, 1952), and by their resistance to enzyme action (Perry, 1954). The actin monomer is an ellipsoidal molecule composed of a single

covalently linked polypeptide chain (Rees and Young, 1967). It has a molecular weight of $47,000 \pm 4,700$, and has a 30% - 40% helix content (Asakura, 1961a).

Properties.

The two forms of actin differ considerably in a number of ways. G-actin contains ATP bound to it as prosthetic group, and F-actin contains bound ADP (Straub and Feuer, 1950; Laki and Clark, 1950; Szent-Györgyi, 1951; Perry, 1952; Mommaerts, 1952b). Indeed ADP is the only bound mononucleotide found in muscle to any significant extent and its concentration may be taken as a measure of actin content (Perry, 1952; Biro and Mühlrad, 1960). Estimation of bound nucleotides in the intact myofibril was originally undertaken by Perry (1952). The ATP bound to G-actin is loosely held and is free to exchange with ATP or ADP in the medium (Martonosi, Gouvea and Gergely, 1960a). It is available for attack by enzymes (myokinase, creatine kinase, heavy meromyosin and apyrase) (Straub and Feuer, 1950; Laki and Clark, 1950; Laki, Bowen and Clark, 1950; Strohman, 1961). Inactivation of G-actin by dialysis against bicarbonate ions can be prevented by the addition of reducing substances (Straub and Feuer, 1950), which suggests that some -SH groups are exposed. If the -SH groups of G-actin are fully reacted with arsenic compounds or mercurials, polymerisation is inhibited (Kuschinsky and Turba, 1951). Actin contains close to ~~seven~~ ^{five} -SH groups per mole and has ATP bound to it in a stoichiometric ratio (Rees and Young, 1967), (1 mole ATP per 47,000 g actin). The ADP associated with F-actin is very firmly bound and will not exchange with nucleotide in the medium (Martonosi, Gouvea and Gergely, 1960a). It is protected from enzymic attack (Perry, 1952; Perry, 1954;

Strohman, 1959). F-actin is far less susceptible than G-actin to -SH reagents, which suggests that it has some -SH groups protected (Mommaerts, 1951a; Barany, 1956; Katz and Mommaerts, 1962).

Polymerisation.

In ion-free solutions G-actin monomers carry electrostatic negative charges which keep the monomers apart by at least 250 Å (Mommaerts, 1952a), but on the addition of a salt or acid the negative charges are cancelled and the monomers polymerise. Accompanying this polymerisation is the non-enzymic splitting of bound ATP which is recovered as ADP and inorganic phosphate (Straub and Feuer, 1950; Laki and Clark, 1951). If bound ATP is removed, either by dialysis or by subjecting it to enzymic attack, actin permanently loses its ability to polymerise (Laki, Bowen and Clark, 1950; Straub and Feuer, 1950; Asakura, 1961a). The protective role of ATP is further demonstrated by the fact that G-actin, prepared by using a depolymerising agent like urea or 0.6 M-KCl, will not repolymerise under suitable ionic conditions unless ATP is present during the first depolymerisation. ATP added a few seconds after the depolymerising agent is ineffective (Szent-Györgyi, 1951). Clearly then it would seem that ATP bound in a specific way is essential for the polymerising property of actin. However, both G-actin-ADP (Hayashi and Rosenbluth, 1960; Grubhoffer and Weber, 1961) and nucleotide-free G-actin (Kasai et al., 1964) will polymerise under suitable ionic conditions. These forms of actin come out of solution differently (G-actin-AMP is amorphous), and it is possible that ATP may have an organising role.

In the presence of ATP, F-actin can be reversibly depolymerised by dialysis at high pH or by removal of ions. This reversal of polymerisation in the presence of ATP has formed the basis of the

purification procedure (Mommaerts, 1951b). We have seen that depolymerisation of actin in the absence of ATP leads to inactivation (Szent-Györgyi, 1951). In contrast to this, if F-actin is allowed to dialyse against 0.1 M-KCl for 20 days all the bound ADP can be recovered without the concomitant depolymerisation of F-actin (Mommaerts, 1952). Also sonication of F-actin (Asakura, 1961b), and elevated temperatures near the point of denaturation (55-60°C) (Asai and Tawada, 1966), lead to an exchange of bound nucleotide with ATP in the medium, and P_i liberation in excess of nucleotide incorporation, without the F-actin depolymerising. Bárány et al. (1966) have prepared nucleotide-free F-actin by replacing ADP with AMP and removing this with charcoal. This F-actin will reversibly polymerise in the presence of Mg^{++} or Ca^{++} , will interact with myosin and will dephosphorylate added ATP. These authors conclude that bound ADP is not absolutely essential for the physiological activity of F-actin (see also Bailin and Bárány, 1967).

Role of actin.

So far we have discussed the behaviour of actin in solution and its location in the muscle cell. The role of actin in muscular contraction is a more obscure problem. Szent-Györgyi (1943) was the first to suggest that a change in the physical state of actin may be involved in contraction and there is now much evidence to support this view. It has been discussed by Straub and Feuer (1950) and by Mommaerts (1950; 1951a; 1951c) who pointed out that if all the actin in one gram of muscle polymerised, 5×10^{-7} moles ATP would be split. This is approximately equal to the amount of ATP thought to be split in the contractile phase of a single twitch. Owing to the ionic conditions which prevail in the muscle, myosin ATPase does

not function optimally, and the rate of P_i liberation in muscular activity ($200 \mu\text{g } P_i/\text{mg myosin}/\text{min}$) exceeds that produced by myosin ATPase under the same conditions in vitro ($3 \mu\text{g } P_i/\text{mg myosin}/\text{min}$) (Mommaerts and Seraidarian, 1947). Mommaerts suggests that a de- and repolymerisation of actin during muscular activity would circumvent these difficulties. Para-chloromercuribenzoate inhibits polymerisation of actin (Korey, 1950) and also inhibits the contraction of fibril preparations with ATP (Mommaerts, 1951a). Reversible depolymerisation is normally a slow process, although it can be speeded up under conditions more nearly representing those in living muscle. If a solution of F-actin, ATP and 0.1 M KCl is freeze-dried it will instantly depolymerise when dissolved in water and immediately repolymerises at a rate that depends on the pH. Mommaerts and Parrish (1951) therefore concluded that some mechanism exists which controls the rate of reaction, and that this presumably becomes operative in vivo. Further support for a change in the state of actin on contraction came from Laki and Clark (1951). When ATP is added to an already polymerised preparation of actomyosin, there is a fall in the viscosity below the levels of actin and myosin separately. This they attributed to dissociation of the actomyosin complex and the depolymerisation of actin.

Hanson and Huxley (1954), using rabbit psoas fibrils, and A.F. Huxley and Niedergengerke (1954), using frog fibres, presented evidence, based on observations with an interference microscope, to show that during passive stretching and contraction down to 65% of the resting length, almost the whole change is taken up by movement of the I band alone. Later, X-ray studies of muscle in rigor and contraction showed that the actin and myosin filaments remain unchanged, and all the change is located in the reflections which arise

from the cross-bridges (Huxley and Brown, 1967).

In order to check whether or not actin is undergoing a change in conformation during muscular contraction, several workers have used an indirect approach based on the properties of the protein, and using the protein both singly and in combination with myosin. The behaviour of the bound nucleotide of actin, the nature of which depends on the physical state of the protein, provides a convenient subject for study.

If such a change in conformation does occur, the bound nucleotide can be affected in one of two ways: either a direct phosphorylation of actin-bound ADP, or a release of bound nucleotide followed by an incorporation of new ATP. Using solutions of F-actin alone, evidence is conflicting. Strohman (1959) favours the former explanation. When F-actin, incubated with CP-kinase, was dialysed against a dilute solution of PC, Strohman obtained reversible depolymerisation accompanied by the direct phosphorylation of bound ADP by the CP-kinase system. No phosphorylation occurred when the actin was maintained in the polymerised state by the addition of 0.1 M-KCl to the dialysis fluid. Strohman found that the transphosphorylation occurred either during or after the depolymerisation process. Hayashi and Rosenbluth (1962) also provided evidence for the phosphorylation of actin-bound ADP. They observed that whereas solutions of G-actin-ADP at 0°C do not polymerise to any significant extent in the presence of KCl, polymerisation does occur when the same solution is incubated with the CP-kinase system. They attributed this phenomenon to the direct phosphorylation of G-actin-ADP. However, when West, Nagy and Gergely (1965) treated a G-actin-ADP solution with apyrase to remove free ADP and repeated the experiment with CP-kinase, they found no appreciable polymerisation.

The effect was reversed when ADP was added. They assumed that Hayashi and Rosenbluth's results were due to the phosphorylation of free and not bound ADP, and that polymerisation resulted from the rebinding of ATP. They found the free ADP in the actin solutions to be of the order of 7% of the total. Martonosi, Gouvea and Gergely (1960a) found no evidence for a transphosphorylation of bound ADP by ATP when F-actin was dialysed in the presence of ^{14}C -ATP. A considerable amount of ^{14}C -ADP was in fact formed in both the dialysed and the control samples, to which they attributed no significance. Perhaps a better interpretation would have been that the control was inadequate. They also ruled out the possibility of direct phosphorylation by P_i using ^{32}P in the dialysis fluid and measuring the incorporation of isotope into actin.

In experiments using ^{14}C -ADP-actin, Asakura, Taniguchi and Oosawa (1963) demonstrated an exchange between bound ADP and ATP in the medium when the F-actin was sonicated. This exchange occurred at the same rate as ATP-splitting, which suggested that the bound ADP was replaced directly with ATP which was then split, and that all the F-actin molecules participated equally. When F-actin was sonicated in the presence of CP-kinase, PC and low concentrations of ^{14}C -ATP, incorporation of labelled ADP into actin and PC-splitting occurred simultaneously, which adds further support to the suggestion that ADP is phosphorylated in the solvent and not when bound to the protein.

Lorand (1953, 1955) and Carlson and Siger (1960), studying actomyosin and iodoacetate-poisoned frog sartorius muscles respectively, both favour a transphosphorylation of bound ADP by the CP-kinase system. The latter observed, during a single isometric twitch, no net ATP-breakdown but a stoichiometry of one PC broken

down per G-actin per single twitch. They suggest that PC-breakdown is the net energy-yielding reaction, and that a $G \longleftrightarrow F$ transition is related to the contraction cycle. They believe that ADP and ATP are located in compartments as follows: ADP and ATP bound to actin, plus a PC-transphorylating enzyme, are located in the actin compartment and are not diffusible. Free ADP, ATP, P_i , C, PC and the enzyme are present in a sarcoplasmic compartment and are free to diffuse into the actin and mitochondrial compartments.

Perry (1955) found no evidence for conversion of bound ADP to ATP during incubation of myofibrils with the CP-kinase system, but he demonstrated that the addition of low concentrations of ADP caused rapid shortening of the fibrils (1954). When equally low concentrations of ATP were added to the system, without the CP-kinase system, he detected very little shortening, and therefore attributed the extent of contraction to phosphate turnover rather than to a given concentration of ATP, the ATP turnover in this case being maintained by the CP-kinase system.

By far the largest amount of evidence gathered is in favour of an exchange of nucleotide. Martonosi, Gouvea and Gergely (1960b) found little or no exchange between bound ADP and ATP in the medium in superprecipitated actomyosin, and concluded that reversible polymerisation and depolymerisation of actin was not related to the contraction cycle. However, more recent reports by two of these authors contradict this finding (Martonosi, Kitagawa and Gergely, 1965). Asakura et al. (1963b) believe that during muscular contraction a $G \longleftrightarrow F$ transition is not a likely mechanism. They find, in fact, that in a sonic field no change in the viscosity of the actin solution accompanies the exchange of nucleotide, even when the sonic vibrations are such as to induce the splitting of

ATP enzymically (Asakura, 1961b). Also, the ionic conditions which prevail in the muscle favour the formation of F-actin. Asakura et al. propose that an interruption occurs in the helix rather than a complete break, and a change takes place from the stable F-form where the nucleotide is protected, to the unstable f-form where the nucleotide is unmasked. The first real support for the suggestion that a conformational change in the actin was a part of the contractile process came from the experiments of Szent-Györgyi and Prior (1966). In a system where actomyosin contained ^3H -ADP bound to the F-actin, and the medium contained ^{14}C -ATP, they demonstrated an exchange of 50% between the bound and free nucleotides during superprecipitation. The incorporation of ^{14}C -ATP exactly equalled the loss of ^3H -ADP and occurred at the same time. Most important, superprecipitation, the in vitro analogy to muscular contraction, was an absolute requirement for this exchange. A rapid burst of the reaction led to 30% of the exchangeable nucleotide being released in the first 30 seconds. They showed that the same sites could undergo reactions more than once, and that the newly incorporated ATP was not available for enzymic attack; in other words the change which made the bound nucleotide available was not a permanent one. Szent-Györgyi also demonstrated the exchange in natural actomyosin, and, in contrast to previous workers (Biro and Mühlrad, 1960; Moos, 1964), he obtained a slow but extensive exchange in a glycerated myofibrillar preparation. Evidence against the formation of actin-bound ATP has been put forward (Szent-Györgyi, 1968) and like Asakura et al., Szent-Györgyi and Prior propose that the exchange occurs between F-actin ADP and ATP directly. They interpret their results in the following way: as a result of an interaction between myosin and actin at specific

sites, a change takes place in the actin which leads to the exposure of its bound nucleotide and a loosening of the bond between this and the protein. The change, which is of a cyclic nature, may be a depolymerisation of the actin filament or it may be a localised change at the binding site of the nucleotide.

These results give very strong support to, but do not provide direct evidence for, a similar cyclic event occurring as part of the normal contractile process.

CHAPTER I, PART IIPURPOSE AND PLAN OF THE INVESTIGATION

We have seen that there is now very strong evidence in favour of a change in the physical state of actin in superprecipitation of both natural and artificial actomyosin, and in contraction of myofibrils. In vitro, an exchange between bound and pool nucleotides is accompanied by some sort of change in actin, the precise nature of which is unknown. If we assume that the consistent behaviour of actin in vitro is indicative of its behaviour in vivo, then an exchange of actin-bound nucleotide detected as a result of muscular activity in vivo would provide valuable evidence for a change in actin during contraction.

The purpose of the work presented here was to provide evidence, more conclusive than that at present available, as to whether or not a change in the physical state of actin plays an important role in the process of muscular contraction.

Cheesman and Whitehead (1968) had demonstrated, in this laboratory, in muscles of frogs labelled with ^{14}C -glucose, an apparent exchange of bound ADP during contraction, and these investigations were continued. The experiments fell into the following groups:

- i) control experiments were designed to check the agreement and reliability of the techniques used. Attempts were then made to demonstrate the exchange of nucleotide during muscular activity, induced by depolarisation with isotonic KCl and by electrical stimulation. The exchange during a 5-second tetanus and single twitch was measured.

- ii) the identity of the bound ADP with actin-bound ADP was confirmed.
- iii) experiments were made to follow the appearance of ^{14}C in the labelled nucleotide.
- iv) the observation had been made in this laboratory (unpublished) that contraction of glycerated rabbit psoas fibres, under load, could be reversibly inhibited by the presence of 2,4-dinitrophenol in the incubation medium. The exchange of bound ADP coupled to superprecipitation of myofibrils prepared from glycerated psoas muscles was demonstrated by Szent-Györgyi and Prior (1966). Experiments have therefore been designed to find the effect of 2,4-dinitrophenol on this exchange in myofibrillar preparations.

CHAPTER IIMATERIALS AND METHODS1. Materialsa) Animals

Cold-acclimatized frogs, Rana temporaria and Rana pipiens, were used throughout the experiments. The weights varied between 10 g and 45 g.

b) Chemicals

Enzymes for nucleotide assays were obtained from Boehringer, and NADH, NADP⁺, NAD⁺, ATP, ADP, AMP and IMP were supplied by Sigma. Chromatography solvents and all other reagents were of analytical grade where available. Whatman No.1 paper was used for chromatography, unwashed. A comparison was made of paper prepared by washing with 0.002 M-EDTA with unwashed paper, but the washed paper offered no advantages and was more difficult to handle.

Uniformly labelled ¹⁴C-glucose was obtained, freeze-dried, from the Radio-chemical Centre, Amersham. Solvents and materials for liquid scintillation counting were of scintillation grade except for the Triton-X 100 which was freed from phosphorescent contaminants by stirring with silica gel according to the method of Patterson and Greene.

2. Methodsa) Preparation of animals for experiments

The frogs were injected subcutaneously with 25 μ Ci of ¹⁴C-glucose and left at room temperature for the prescribed period. In the experiments where the animals were curarised (+)-tubocurarine chloride (approx. 20 μ g/g body weight) was injected

subcutaneously, either before or after the ^{14}C -glucose. After the prescribed period, the animals were pithed and the gastrocnemius, sartorius and rectus abdominis muscles were removed and placed immediately into cold Ringer's solution. One of the pair of muscles was brought either into a two-minute contracture by immersing it in cold isotonic (0.765% w/v) KCl and then returning it to Ringer, or into a 5-second tetanus or single twitch by electrical stimulation. The other muscle was treated as the control.

b) Preparation of nucleotide-containing extracts from whole muscle and washed muscle residues.

The individual muscles were cut with dissecting scissors very finely and rapidly in cold Ringer and each was divided into two portions. The larger portion was used for the preparation of actin-bound ADP as follows:

The finely cut muscle was allowed to stand for twenty minutes in 0.05 M- $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ at 0°C and was then washed ten times with ice-cold distilled water over a period of two hours. The residue was extracted for ten minutes with 3ml cold 10% (w/v) trichloroacetic acid, centrifuged and the trichloroacetic acid removed by shaking the extract ten times with cold ether. This solution was poured into siliconed tubes (prepared by coating the inside of 15ml conical centrifuge tubes twice with hot 1% dichlorosilane in benzene), frozen in a mixture of salt and ice, and dried in a desiccator over phosphorus pentoxide under reduced pressure for 16 hours. The small amount of dried material left was dissolved in 10 μl distilled H_2O and used for chromatography.

The smaller portion of muscle was extracted directly for total ATP content with 3 ml 10% (w/v) trichloroacetic acid for

ten minutes. It was centrifuged, and the supernatant shaken ten times with cold ether and brought to pH 8.2 with dilute NaOH. 0.3 ml 10% (w/v) barium acetate was added. After sixteen hours the precipitate, which included that of Ba-ATP, was centrifuged off and dissolved in 10 μ l 2 N-HCOOH. This solution was used for chromatography.

c) Isolation of the nucleotides

The nucleotides were isolated by two-directional chromatography according to the method of Krebs and Hems (1953). Separation of nucleotides from glycolytic intermediates with insoluble barium salts (i.e. fructose-1, 6-diphosphate, 3-phosphoglyceric acid, inorganic phosphate) was effected by a four-hour ascending run in isopropyl ether:formic acid (9:6, v/v). The nucleotides which remained at the origin were separated from each other by a subsequent 16-hour descending run in isobutyric acid, N-NH₄OH and 0.1 M-EDTA (100:60:1.6, v/v). The chromatograms were allowed to dry at room temperature, and the spots bearing the nucleotides were cut out. The nucleotides were eluted with 1 ml distilled H₂O and kept at 4°C.

ADP and ATP samples were run as standards with every chromatogram and the nucleotides were also run against NAD⁺, NADH and NADP⁺ as likely contaminants in the experiments. There was no migration of any of these substances in the ascending solvents. In a typical experiment, the distances from the origin in descending solvents were as follows:

AMP	—	23.5 cm
ADP	—	19 cm
ATP	—	16 cm
NAD ⁺	—	21 cm
NADH	—	16.5 and 23 cm
NADP ⁺	—	10.5 and 14 cm

The contaminating substances in the ascending run, move well away from the origin after four hours. In the descending run, the barium spot (which may be detected with sodium rhodizonate) is separated from the ATP which can be cut clear. IMP spots were always present in the test samples.

d) Estimation of the specific activity of the nucleotides

i) Counting techniques

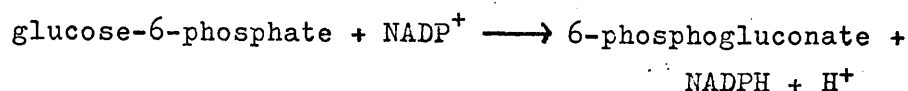
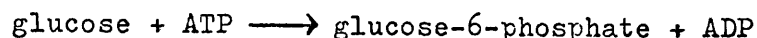
For low energy β -emitters, such as ^{14}C , liquid scintillation is the most accurate method of counting, and our method is based on the liquid emulsion counting technique of Patterson and Greene (1965). A Panax Dekatron scaler, type D657.C was used in conjunction with a Panax Scintillation counter, type SCA. The scaler was adjusted so as to give 85% efficiency, using a sealed ^{14}C source, and a linear relationship was established between counts and radio-activity using serially diluted ^{14}C -glucose solutions in the phosphor.

Aliquots (0.5 ml) of eluate were put into 10 ml phosphor (toluene:Triton-X 100, 2:1 v/v, containing 0.4% w/v 2,5-diphenyloxazole and 0.1% w/v 1, 4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene), at 4°C , shaken and allowed to equilibrate in the dark at 4°C for ten minutes. They were then counted for as long as practicable, but never less than 30 minutes. Blanks were prepared containing 0.5 ml H_2O in the same phosphor as above. Separate blanks were counted for each bottle on each day.

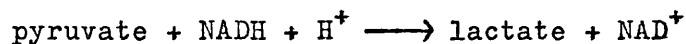
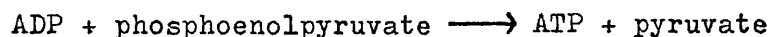
ii) Estimation of nucleotides

Further aliquots were taken for estimation of ATP and ADP by reactions coupled to NADP^+ reduction and NADH

oxidation respectively, according to the method of Estabrook et al. (1967). This is an indirect method of measuring nucleotides, based on the fact that NADPH and NADH are themselves fluorescent. In the presence of ATP and hexokinase, glucose is phosphorylated to glucose-6-phosphate. The formation of 6-phosphogluconic acid from this by glucose-6-phosphate dehydrogenase requires NADP⁺ as cofactor.



The estimation of ADP depends on the formation of ATP from ADP and phosphoenolpyruvate by pyruvate kinase, and the reduction of pyruvate at the expense of NADH.



The consequent increases and decreases in fluorescence were followed on a Locarte single sided fluorimeter, type Mk 4. In most cases 0.3 ml aliquots were used for both ADP and ATP estimations, in a total volume of 1.5 ml.

Where samples were very concentrated less eluate was used and the volume made constant with buffer.

Triethanolamine buffer, pH 7.4, was used for ATP determinations, and phosphate buffer, pH 7.0, for ADP determinations. In all other respects the original method was adhered to. Approximately 2 μ g of each enzyme was used. The reaction was carried out in 10 x 50 mm pyrex tubes, and using Locarte filters, LF/2 (340-380 m μ) as primary filter, and LF/5 (440 μ , photomultiplier cut off)

as secondary. The fluorimeter was first calibrated to obtain the optimum settings for our conditions. Standard curves were plotted for both ATP and ADP on new samples.

During the early stages of the investigations ATP and ADP were determined by the method of Kalckar (1947). This method is based on the stepwise conversion of ATP and ADP to AMP, by apyrase and myokinase, and the subsequent deamination of the AMP to IMP with adenylic deaminase. This reaction can be followed spectrophotometrically at 265 m μ .

e) Preparation of pure actin-bound ADP

The pooled leg muscles from eight frogs, previously injected with 25 μ C_i ¹⁴C-glucose, were used for the preparation of actin by the method originally described by Straub (1943). The acetone powder was extracted with a twenty-fold volume of water adjusted to pH 8.0 with NH₄OH, and the actin was purified according to the method of Mommaerts (1951b). The G \longrightarrow F transition was effected at pH 7.5-8.5 by the addition of sufficient KCl to produce a concentration of 0.1 M. The solution was centrifuged at room temperature for one hour at 140,000 x g and the pellet extracted directly with 10% w/v trichloroacetic acid for ten minutes. The bound ADP was isolated by the chromatographic method described.

f) Preparation and estimation of adenine from ¹⁴C-labelled ATP

The pooled leg muscles from ¹⁴C-labelled frogs were homogenised and used for the preparation of ATP as described previously. The ATP was eluted from the paper, after chromatography, in 2 ml distilled H₂O; 1 ml was used for ATP estimation and liquid scintillation counting, and the remaining 1 ml was made 0.1 N

with respect to HCl, and hydrolysed for one hour. The hydrolysate was freeze-dried and the resultant material dissolved in 10 μ l 2 N-HCOOH for chromatography. The chromatogram was allowed to run for four hours in the ascending solvents (isopropyl ether:formic acid), and for sixteen hours in the descending solvents (isobutyric acid:ammonia:EDTA). Care was taken that the solvent front did not run off the paper, as the adenine spot moves close to the solvent front. Extra long paper was used. The paper bearing the adenine spot was cut into small pieces and shaken at room temperature for two hours in 0.1 N-HCl to elute the adenine. The extract was centrifuged, and neutralised. Aliquots were taken for liquid scintillation counting and for enzymic estimation of adenine by the method of Klenow (1952). This involves the slow oxidation of adenine by xanthine oxidase to 2,8-dihydroxyadenine. The reaction was carried out at pH 7.0 and the increase in optical density at 305 m μ was followed. Aliquots of 0.5 ml were used in all cases, made up to a total volume of 3 ml with 0.2 M-phosphate buffer. 200 μ g xanthine oxidase was used and the reaction was allowed four hours to go to completion; the samples were read against a mixture of buffer and enzyme. Trial assays were initially carried out on a solution of adenine, 2 μ g/ml.

g) Preparation of myofibrils

Myofibrils were prepared from the back and leg muscles of a freshly killed rabbit, according to the method of Ulbrecht and Ulbrecht (1957).

CHAPTER IIIRESULTS1) Control experiments.

Before any exchange experiments could be carried out it was necessary to establish that paired muscles, given identical treatment, showed an agreement in their specific activities which was acceptable within the limits of the experimental procedures.

Mrs. A. Whitehead had earlier shown in this laboratory that this was the case, using different techniques applied to the rectus abdominis only. Control experiments were designed to check the agreement for all types of muscle used. The muscles were uncontracted, and bound and pool nucleotides were estimated and their radioactivity counted by the methods described in Chapter II. These results are given in Table 1. For all but one (Table 2, No.5) of the control experiments carried out on gastrocnemius muscles, the nucleotides were estimated by the method of Kalckar (1947).

It is important to mention that the term 'pool' nucleotide, as employed in this chapter, represents the total ATP of the tissue.

Table 1Control experiments on paired uncontracted muscles of Rana temporaria

No.	Type of muscle and period of labelling in hours	Specific activity (counts/ μ mole/min.)			
		Total ATP		Bound ADP	
		Left	Right	Left	Right
<u>Gastrocnemius</u>					
1	3	5520	5140	2450	3520
2	3	1540	1210	1180	1320
3	3	195	250	870	1340
4	3	4230	7000	1610	3520
5	3	11140	5160	11300	11900
<u>Sartorius</u>					
6	1	18100	19800	10050	7050
7	1.25	470	245	-	-
<u>Rectus</u>					
8	1	21900	19900	4125	3400
9	1.25	320	260	508(ATP)	570(ATP)

Of the results shown in Table 1, about half show reasonable agreement between the specific activities in the two muscles of the pool ATP and bound ADP. It was suspected that in some cases, handling of the muscles had given rise to mechanical stimulation. In order to reduce this source of error, an attempt was made to block the motor end plates by subcutaneous injection of (+)-tubocurarine (20 µg per g body weight), either before or after the administration of ^{14}C -glucose.

Table 2

Control experiments on paired uncontracted curarised muscles of
Rana temporaria

(20 µg (+)-tubocurarine per g body weight)

No.	Type of muscle and period of labelling in hours	Time of curarisation before or after ^{14}C -glucose		Specific activity (counts/µmole/min.)			
		Before	After	Total ATP		Bound ADP	
				Left	Right	Left	Right
<u>Gastrocnemius</u>							
1	3	-	1.5	3070	5230	7150	11100
2	1.5	1.5	-	30*	160	280	460
3	1.5	1.5	-	60*	170	90*	480
4	3	1.5	-	580	1100	1350	1510
5	3	1.5	-	735	730	3150	3780
<u>Sartorius</u>							
6	3	1.5	-	1000	1310	4760	5890
7	1	1.5	-	880	930	1550	1500
8	1	1.5	-	1340*	1650	2940	2760
<u>Rectus</u>							
9	1	1.5	-	1470	1400	640*	650
10	1	1.5	-	5000	5850	1760	1850

These experiments, the results of which are given in Table 2, also show differences, which are especially marked with the gastrocnemius muscles.

The results for sartorius and rectus were, in general, good, and

most of the later experiments were carried out with these. It is possible that the gastrocnemii show a greater tendency to asymmetry than the other muscles.

It will be seen (Table 2a) that the specific activities marked with an asterisk in Table 2 are subject to error because of a small amount of material estimated or a very low radioactive count.[†] In all future tables an asterisk will be used with similar significance.

Table 2a

Nucleotide assays and radioactive counts for some experiments in Table 2

No.	<u>Total ATP</u>				<u>Bound ADP</u>			
	<u>Left</u>		<u>Right</u>		<u>Left</u>		<u>Right</u>	
	<u>μmole/ml</u>	<u>cpm/ml</u>	<u>μmole/ml</u>	<u>cpm/ml</u>	<u>μmole/ml</u>	<u>cpm/ml</u>	<u>μmole/ml</u>	<u>cpm/ml</u>
6	0.0210	21.0	0.0165	21.6	0.0120	57.0	0.0090	53.0
7	0.0025	2.2	0.0028	2.6	0.0024	3.6	0.0036	5.6
8	0.0015	2.0	0.0034	5.6	0.0032	9.4	0.0065	18.0
9	0.0025	3.7	0.0030	4.2	0.0028	1.8	0.0040	2.6
10	0.0057	28.4	0.0066	38.6	0.0071	12.4	0.0092	17.0

Background count = 34.6 ± 0.4

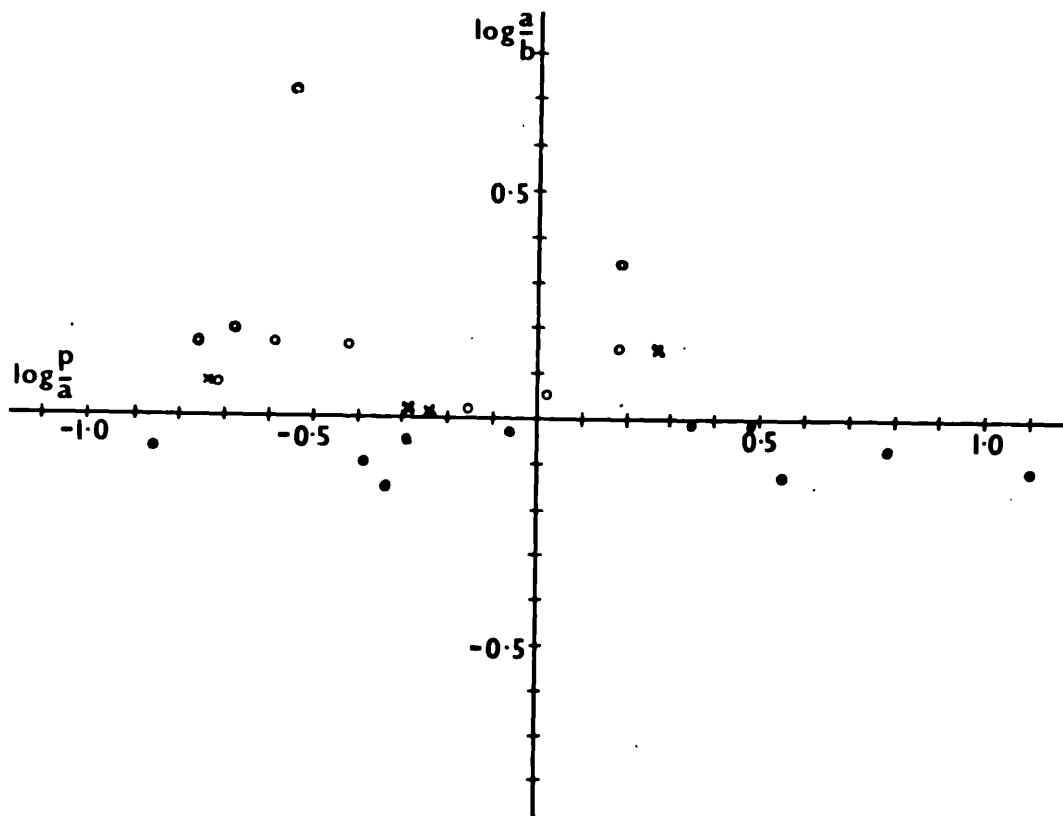
An impression of the magnitude of the error in these control determinations is given in fig.1.

Apart from establishing that there was an acceptable agreement between paired muscles, these control results showed another striking feature. In Table 1, with one exception, the specific activity of the bound ADP is of the same order of magnitude as that of the total ATP. This is in itself indicative of an exchange between bound and pool nucleotides. In Table 2, with gastrocnemius and sartorius muscles in all cases, the specific activity of the bound ADP exceeds that of the

[†] ≤ 0.0015 μmole ADP or ATP/ml (≤ 5 mm on fluorimeter) or ≤ 1 cpm.

fig. I.

Control experiments with paired uncontracted muscles of frogs
injected with ^{14}C -glucose.



Plot showing the results of the control experiments of Tables I & 2

Abscissae: $\log (p/a)$. Ordinates: $\log (a/b)$.

p = mean specific activities of total ATP in paired muscles.

a and b = specific activities of bound ADP in individual muscles.

o = gastrocnemius x = sartorius • = rectus.

Six results for the rectus were obtained by Mrs. A. Whitehead.

Error-free determinations on symmetrical paired muscles should give a line coincident with the abscissa axis.

The role of control muscle is so allocated that points for recti appear below those for other muscles above the abscissa axis.

pool. This observation provides very strong evidence for the existence of a pool of high turnover rate with which the myofibrils are in exchange

2) Experiments in potassium contracture.

In these experiments one of the pair of muscles was brought into an unloaded potassium contracture by immersing it in cold 0.765% KCl for two minutes. In all other respects the muscles received the same treatment, as previously described in Chapter II. Table 3 shows the results of six experiments on uncurarised gastrocnemius and sartorii.

Table 3

Uncurarised muscles brought into unloaded potassium contracture

Type of muscle and period of labelling in hours	<u>Specific activity (counts/μmole/min.)</u>			
	Total ATP		Bound ADP	
	Control	Contracted	Control	Contracted
<u>Gastrocnemius</u>				
5.5	300	3130	2500	1000
<u>Sartorius</u>				
3	1600	2150	900	780
1.25	400*	2125	2430	430
1	365	620	1070	470
1.25	735	1200	1020	410
17.5	725	1300	1515	1100

With the exception of the frog labelled for three hours the activities of the bound ADP of the fast sartorii after short labelling times, and of the gastrocnemius after relatively long labelling times, exceed those of the pool. Contracture with KCl in all cases has reduced the activity of the bound ADP to a value nearer to the mean of the pool activities before and after contracture, and has increased the activity of the total ATP in all cases.

Experiments carried out on rectus abdominis after short labelling periods frequently showed a reduced activity of bound ADP, which increased on stimulation. Injection of (+)-tubocurarine prior to the ^{14}C -glucose further reduced the activity of the bound ADP (Table 4).

Table 4

Uncurarised and precurarised recti abdominis brought into unloaded potassium contracture (partly after Cheesman, Priston and Whitehead, (1969)).

Period of labelling in hours	Remarks	Specific activity (counts/ $\mu\text{mole}/\text{min.}$)			
		Total ATP		Bound ADP	
		Control	Contracted	Control	Contracted
3	uncurarised	1480	1850	720	1390
1.25		840	5800	1220	1840
1.25		2130	4800	70*	355
1		250	310*	60*	420
1	20 $\mu\text{g}/\text{g}$ body weight given one hour before ^{14}C -glucose	120*	330	-40*	300*
1		1140	1600	-10*	845
1		550	1550	0*	770
1		8550	16300	-470*	4350
1		1530	4650	85*	2690

Cheesman, Priston and Whitehead (1969), some of whose results are included in Table 5, used the figure 0 to denote specific activities which had negative values, but lay within the limits of error of the experimental procedures.

The rectus abdominis is a tonic muscle and contains a high percentage of slow fibres. It seems slow to become labelled. The sartorius on the other hand is a fast, phasic muscle. We have seen how rapidly the bound ADP becomes labelled in the sartorius muscle compared to the rectus; even after one hour the specific activity of the bound ADP is frequently higher than that of the pool ATP.

In order to achieve a higher count in the pool ATP than the bound ADP, the labelling time was reduced to ten minutes. Experiments were then carried out to see if precurarisation would inhibit the appearance of ^{14}C in the structural ADP of these fast sartorius muscles. The results are expressed in Table 5.

Table 5

Uncurarised and precurarised sartorii brought into unloaded potassium contracture after short labelling times.

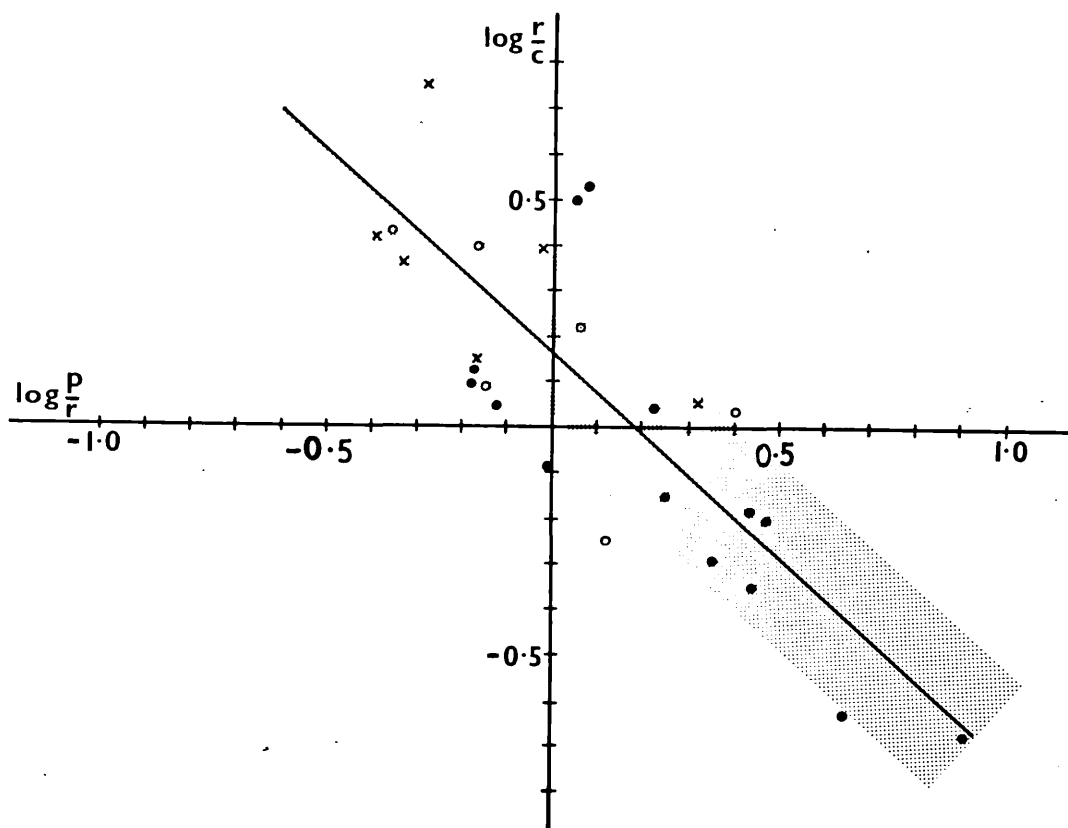
Period of labelling in minutes	Remarks	Specific activity (counts/ $\mu\text{mole}/\text{min.}$)			
		Total		Bound ADP	
		Control	Contracted	Control	Contracted
10	} uncurarised	5800	14170	3650	16100
10		17000	25770	13620	28780
10	} 20 $\mu\text{g}/\text{g}$ body wt given one hour before ^{14}C -glucose	44000*	10400	700	7630
10		35000	43000	280*	4540
60		110	210	410	160

Although a complete inhibition of label into the bound ADP was not obtained, it was considerably reduced by a combination of short labelling time and relatively long precurarisation. In a later series of experiments this fact is supported in every case - Tables 8 and 9. Contracture with KCl after 10 minutes labelling, in most cases causes an increase in the activities of both the bound and the pool nucleotides. The equilibration of the bound ADP with the pool during activity is illustrated in a graphical form in fig. II.

Subcutaneous injections of (+)-tubocurarine chloride given after the ^{14}C injection, and coupled with relatively long labelling periods lead to a situation in gastrocnemius and rectus where the activities

fig. II.

Effects of potassium contracture on the specific activity of actin-bound ADP in muscles of frogs injected with ^{14}C -glucose.



Abscissae: $\log(p/r)$. Ordinates: $\log(r/c)$.

p = mean of specific activities of total ATP in control and contracted muscles.

r = specific activity of bound ADP in control muscle.

c = specific activity of bound ADP in contracted muscle.

Six results are taken from Cheesman and Whitehead (1968).

The regression line is fitted by the method of least squares.

The shaded area represents the standard deviation about the line.

For $p = c$, the line should have a slope of -1 and pass through the origin. The concentration of points in the upper left-hand and lower right-hand sectors shows the tendency of the specific activity of the bound ADP to approach, in contracture, that of the total ATP.

of the bound ADP remain high as though they are arrested, while the pool values fall. These results are expressed in Table 6.

Table 6

Curarised muscles brought into unloaded potassium contracture after long labelling times.

(20 µg (+)-tubocurarine per g body weight injected after ^{14}C -glucose)

Type of muscle and period of labelling in hours	Time of curarisation after ^{14}C -glucose	<u>Specific activity (counts/µmole/min.)</u>			
		Total ATP		Bound ADP	
		Control	Contracted	Control	Contracted
<u>Gastrocnemius</u>					
5.5	3.5	350	275	7780	4250
5.5	1.5	640	1200	23800	64100
5.5	1.5	3240	2750	4300	3510
5.5	1.5	2030	750	3250	1200
<u>Rectus</u>					
3.5	2.5	5800	27000	25670	20160
20	3	850	2500	11300*	2750*

Depolarisation with KCl in most instances produces a decrease in the specific activities of bound ADP in both types of muscle, but the pool values on stimulation are varied.

The exchange of nucleotide clearly appears to be associated with stimulation of the muscle, but it is not clear whether this exchange is associated with activation or shortening. In Ca^{++} -free Ringer, the contracture of muscles by KCl can be inhibited (Niedergerke, 1956), although depolarisation of the membrane still occurs. The effect of potassium depolarisation on the exchange in the rectus abdominis after 30 minutes incubation in Ca^{++} -free Ringer was investigated. The frogs were given 20 µg (+)-tubocurarine per g body weight, subcutaneously, one hour before the injection of ^{14}C -glucose. The

labelling time was also one hour. The muscles were removed, divided and incubated in frequent changes of cold Ca^{++} -free Ringer in a shallow dish. The contracture was carried out by the usual method, and bound and pool nucleotides estimated as before. The results are shown in Table 7.

Table 7

The effect of potassium depolarisation on precurarised rectus abdominis after incubation in Ca^{++} -free Ringer

(20 μg (+)-tubocurarine per g body weight given one hour prior to ^{14}C -glucose)

Specific activity (counts/ $\mu\text{mole}/\text{min.}$)

<u>Total ATP</u>		<u>Bound ADP</u>	
<u>Control</u>	<u>Depolarised</u>	<u>Control</u>	<u>Depolarised</u>
740	2125	125*	2000
375*	1070	30*	700

An increase in the specific activities of both bound and pool nucleotides, occurred on stimulation. The very low activities for bound ADP before stimulation are consistent with our previous findings for this muscle when precurarised.

3) Experiments in electrical stimulation.

a) Short tetanus

Using the fast sartorius muscle from frogs precurarised and labelled for ten minutes, experiments were carried out to find the effect of a five-second isometric tetanus on the exchange of bound nucleotide. The muscle was stimulated electrically by application of platinum electrodes, approximately 0.5 cm apart, and carrying a potential of 1.5 V. The pulse rate was adjusted to 50 per second

and the pulse width to 0.5 msec. In some cases one end of the muscle was left in situ and the other end held to avoid damage to the muscle. In other cases the muscle was secured by fine pins to a cork board. The control muscle was given identical treatment, apart from the tetanus. For these experiments Rana pipiens were used when Rana temporaria were unavailable. The results of these experiments are shown in Table 8.

Table 8

Electrical stimulation; five-second tetanus on precurarised sartorii

(20 µg (+)-tubocurarine per g body weight given one hour before ¹⁴C-glucose)

Species and period of labelling in minutes	<u>Specific activity (counts/µmole/min.)</u>			
	Total ATP		Bound ADP	
	Control	Contracted	Control	Contracted
<u>Rana pipiens</u>				
10	1330*	8330*	-600*	1540
10	19400*	5460	110*	770
10	35670*	14000*	800	1830
<u>Rana temporaria</u>				
10	1830	3700	-350*	4400
10	1160	8670	-250*	1400
10	11600*	23200	1440	1500
10	12400*	5470	1550	3550
10	960	3070	370	1820
10	1460	5930	700	3380
10	860	2640	590*	1160

In all cases the specific activity of the bound ADP is increased on stimulation, but the pool results vary.

Unexpected results were obtained when the frogs contained eggs. The neuromuscular block produced by tubocurarine seemed incomplete

in these frogs even to the extent that extremely high doses of tubocurarine (up to 40 μg per g body weight) failed to paralyse the muscles completely. In most instances the animals were discarded but in those where experiments were completed the results were those shown in Table 9.

Table 9

Five-second tetanus on precurarised frogs containing eggs

(20 μg (+)-tubocurarine per g body weight given one hour before ^{14}C -glucose)

Species and period of labelling in minutes	<u>Specific activity (counts/$\mu\text{mole}/\text{min.}$)</u>			
	<u>Total ATP</u>		<u>Bound ADP</u>	
	Control	Contracted	Control	Contracted
<u>Rana pipiens</u>				
10	7000*	3000*	3000	0*
<u>Rana temporaria</u>				
10	1180	150*	1770	1300
10	7120	2600	1440	150*
10	6030	1620	2050	990

Some fibrillation occurred with most of these frogs and in all cases the specific activities of both the bound and pool nucleotides fell on stimulation.

b) Single twitch

Rectus abdominis muscles were brought into an isometric twitch by application of a single pulse. The frogs were precurarised for one hour and labelled for one hour. The muscles were secured by fine pins through the xiphisternum and the base of the muscle. Rana pipiens and Rana temporaria were used for these experiments, the results of which are given in Table 10.

Table 10

Precurarised rectus abdominis brought into a single twitch(20 µg (+)-tubocurarine per g body weight given one hour before ¹⁴C-glucose)Specific activity (counts/µmole/min.)

Species	Total ATP		Bound ADP	
	Control	Contracted	Control	Contracted
<u>Rana pipiens</u>				
	29150	46800*	1890	6150*
	2930*	4450	1240	7620
	2220	5180	380	1950
	3700	7660	-360*	1250
<u>Rana temporaria</u>				
	1000	1630	110*	430
	3600*	4900	290	535
	530	1270	200*	250
	280	385	110*	460
	2600	4800	295*	1395
	2000	3600	150	1640
	2470	4870	260	1650
	1960	4490	390	1130

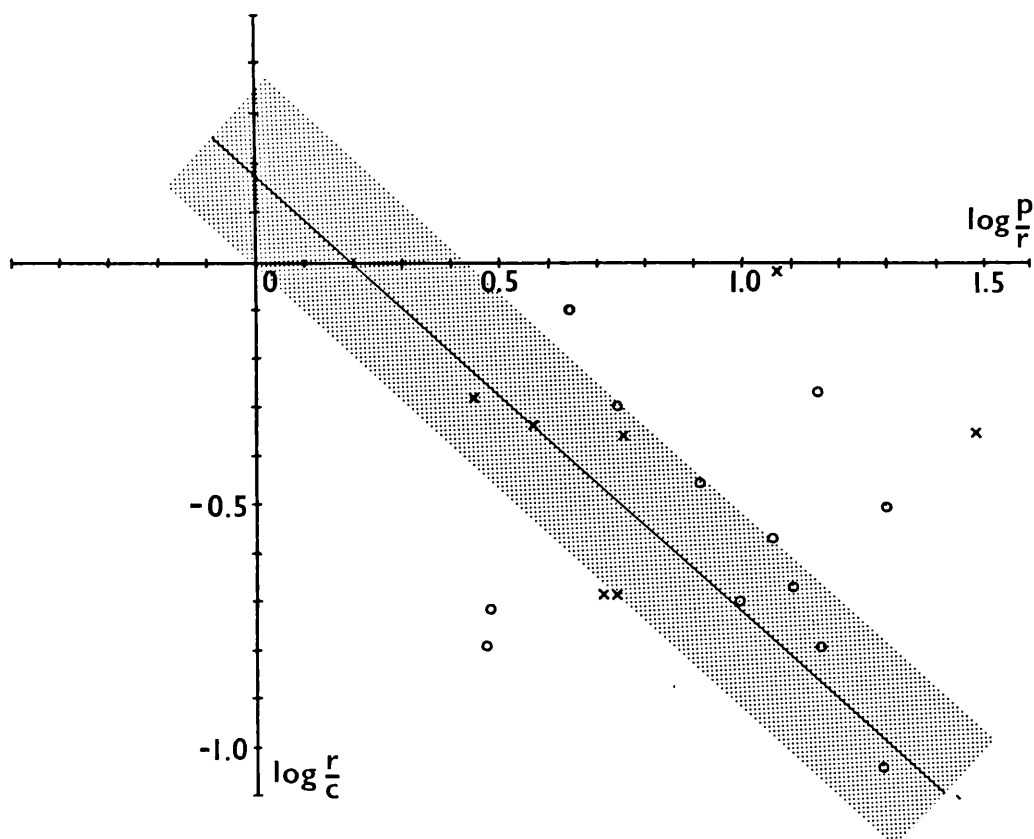
Stimulation of these muscles in every instance brought about an increase in the activities of the pool and the bound nucleotides, although there was great variety in the size of the increase. The specific activity of the bound ADP becomes closer to the mean of the pool activities before and after stimulation. Electrical experiments are expressed in a graphical form in fig. III. The regression line and standard deviation are those from fig. II superimposed.

4) The identity of bound ADP with actin-ADP.

The combined muscles from eight frogs, labelled with 25 µCi ¹⁴C-glucose for three hours were homogenised twice and divided into two portions. The smaller portion was used for the preparation of bound

fig. III

Effects of electrical stimulation on the specific activity of actin-bound ADP in muscle of frogs injected with ^{14}C -glucose.



Plot analogous to that in fig. II of the results shown in Tables 8 and 10.

o = rectus abdominis, single twitch (1.5 V, 0.5 msec.)

x = sartorius, 5-second tetanus (1.5 V, 0.5 msec., 50 pulse/sec.)

The regression line and standard deviation are taken from fig. II.

ADP, and the remainder was used for the preparation of actin as described in Chapter II. Bound ADP was extracted from this. The specific activity from each of these was estimated. The experiment was repeated on another eight frogs.

The results are as follows:

Experiment 1	Bound ADP	-	2500 counts/ μ mole/min.
	Actin-ADP	-	2260 counts/ μ mole/min.
Experiment 2	Bound ADP	-	1870 counts/ μ mole/min.
	Actin-ADP	-	2220 counts/ μ mole/min.

These results were accepted as indicating the identity with the prosthetic group of actin of the bound ADP associated with the washed muscle residue in our experiments.

5) Contamination with fluorescent material.

In all the extracts of washed muscle residue, and in the nucleotide preparations from pure actin, a contaminating material was always present on the chromatogram. This was not found in the chromatograms of pool ATP. The fluorescence of this material at the origin was counteracted by the adenine-containing materials. In the ascending solvent the material migrates slowly and after a four-hour run can be cut away. If allowed to run in the descending solvent, the material migrates at the same speed as ADP and interferes with ADP estimation by fluorimetry. The material has a light blue fluorescence and is water-soluble. When eluted after the ascending run and scanned, it shows three absorption peaks at 198, 248 and 270 m μ . The material appears to be most abundant in the rectus abdominis and least abundant in the sartorius. The concentration in fact seems to vary with the slow fibre content, but this may be fortuitous.

6) The site of labelling of the nucleotide.

Experiments were carried out to find the initial site of labelling in the nucleotide molecule. Frogs were injected with 25 μCi ^{14}C -glucose; some were left for 1.5 hours and some for 17 hours. The leg muscles were pooled. ATP was isolated and, in part, used for the preparation of adenine. The specific activities of adenine and ATP were determined by the methods described in Chapter II.

The results were as follows:

1.5 hour labelling

1. Specific activity of ATP - 540 counts/ $\mu\text{mole}/\text{min}$.
Specific activity of adenine - 26 counts/ $\mu\text{mole}/\text{min}$.
2. Specific activity of ATP - 1120 counts/ $\mu\text{mole}/\text{min}$.
Specific activity of adenine - 66 counts/ $\mu\text{mole}/\text{min}$.

17 hours labelling

1. Specific activity of ATP - 700 counts/ $\mu\text{mole}/\text{min}$.
Specific activity of adenine - 126 counts/ $\mu\text{mole}/\text{min}$.
2. Specific activity of ATP - 870 counts/ $\mu\text{mole}/\text{min}$.
Specific activity of adenine - 157 counts/ $\mu\text{mole}/\text{min}$.

After 1.5 hours the adenine contains 6% of the total labelling, and after overnight labelling it contains 17.5%. The rest of the radioactivity must therefore be in the sugar moiety. If the labelling only occurs in carbon-6 (derived from CO_2), the specific activity of the adenine would be 10% of the total. Our results after

one hour seem to be consistent, within the limits of error, with this one carbon being labelled. After 17 hours incubation, the purine moiety contains 17.5% of the activity. This represents two labelled carbon atoms, the theoretical activity for which is 20%. Labelling in positions other than carbon-6 must depend on the turnover of the labelled precursor pools. Of the other four carbons two are derived from formate and two from glycine, and the labelling of each will depend on the turnover of tetrahydrofolic acid. The distribution of labelling in the ATP provides evidence for synthesis de novo of ATP from ribose-5-phosphate.

7) The effect of 2,4-dinitrophenol on the exchange of bound ADP in contracting myofibrils.

The exchange of bound ADP in myofibrils was shown by Szent-Györgyi and Prior (1966) to be associated with contraction in the presence of ATP and Mg^{++} . The inhibition of superprecipitation of actomyosin by 2,4-dinitrophenol was demonstrated by Levy and Ryan (1966), and was shown to be of a competitive nature. We have demonstrated in this laboratory in experiments as yet unpublished, that contraction of loaded glycerated psoas fibres can be reversibly inhibited by DNP, and therefore it seemed of interest to study the effect of DNP on the exchange in contracting myofibrils.

Experiments were carried out in the presence of both Ca^{++} and Mg^{++} as follows:

tubes containing 20 mM-Tris-HCl (pH 7.0); 50 mM-KCl;
3 mM- $MgCl_2$ or $CaCl_2$; 1 mM-DNP or 5 mM-DNP;
0.037 mM-ATP containing ^{14}C -ATP and 1.7% myofibrils
(calculated as protein, determined by the Folin-Lowry method), were incubated at 25°C.

The reaction was carried out for four hours.

The results are given in Table 11.

Table 11

The effect of 2,4-dinitrophenol on exchange of bound ADP
in contracting myofibrils.

Activating ion	Remarks	Specific activity (counts/ μ mole/min.)	
		Control	DNP
Mg ⁺⁺	DNP = 1 mM ¹⁴ C-ATP = 0.5 μ Ci per tube	29300	48100
		-	58400
Ca ⁺⁺		46100	57600
		37000	37000
Mg ⁺⁺	DNP = 5 mM ¹⁴ C-ATP = 0.3 μ Ci per tube. Myofibrils pre- incubated with DNP for 30 min.	20865	23235
		15230	19480
Ca ⁺⁺		29070	22070
		21000	22460

From these few experiments, it appears that the exchange is not inhibited, but rather somewhat increased, under conditions where contraction is greatly inhibited. Levy and Ryan (1966) record the relevant dissociation constant for the actomyosin-DNP complex as 1.9×10^{-3} M. Szent-Györgyi and Prior (1966) claim that superprecipitation is an absolute requirement for exchange, although in their modified system, when myosin is incubated with p-nitrothiophenol prior to combination with actin, superprecipitation does not accompany ADP exchange.

CHAPTER IVDISCUSSION

Incorporation of ^{14}C into the pool nucleotide occurs extremely rapidly after the injection of ^{14}C -glucose, even in cold-acclimatized frogs whose metabolic activity is much reduced. The actin-bound ADP also becomes rapidly labelled and is clearly in exchange with a highly labelled nucleotide pool. Injection of labelled glucose ten minutes before the removal of the muscles leads to comparatively high levels of radioactivity in the bound ADP of the fast sartorius muscles. In the majority of experiments when the muscles were activated either by electrical stimulation or by potassium chloride, the specific activities of the total ATP increased. This implies an increase in the metabolic rate of muscle after stimulation, which seems, from our experiments in the distribution of label to involve a de novo synthesis of nucleotide.

The time curve for the incorporation of isotope into the nucleotide varies from frog to frog, and from muscle to muscle. In experiments where the bound specific activities were high and the pool low, whether in curarised or uncurarised frogs, a potassium contracture nearly always resulted in an approach to equilibration between the pool activities and those of the bound, so that the bound specific activities fell and the pool activities increased. This occurred after one hour in the fast muscles and after long labelling times in the other muscles. The bound activity after stimulation approached a value near to the mean of the pool activities before and after stimulation, which meant a reduction in the bound activity when it exceeded the pool. In the experiments where the pool activity exceeded the bound, stimulation led to an increase in the bound activity and also an increase in the

pool activity owing to synthesis of ATP. The bound activity after stimulation still approached a value closer to the mean of the pool activities.

In experiments using the rectus abdominis muscle where postural activity is inhibited by an injection of (+)-tubocurarine chloride administered before the isotope, the incorporation of ^{14}C into the bound ADP can be inhibited. This inhibition can be relieved by bringing the muscle into a contracture or single twitch, when the bound ADP becomes labelled. This experiment gives direct evidence for the exchange of bound nucleotide during muscular activity. The values of the absolute activity show the usual biological variation for frogs given identical treatment, and we have therefore confined our attention to comparison of results from paired muscles, contracted and uncontracted, within the same frog rather than to absolute values between individuals. Nevertheless, when the rectus abdominis was brought into a single twitch, in most experiments, a very great increase in the bound and pool activities did occur, often as great as after a five-second tetanus. This implies that even a single contractile event brings about a large increase in ATP synthesis and therefore in metabolic activity. This can be interpreted to mean that during repeated contractile cycles the same sites are undergoing an exchange more than once with a pool of relatively constant specific activity. Szent-Györgyi and Prior (1966) did indeed show this to be the case in their experiments in vitro.

Tubocurarine causes muscular paralysis by competing for the acetylcholine binding sites at the neuromuscular junction. In the muscles containing a high proportion of slow fibres, injection of (+)-tubocurarine chloride produced the most effective neuromuscular block, if it be assumed that exchange of bound nucleotide in resting

muscle is determined by postural activity. In the mixed and fast muscles it was impossible to obtain such consistent and effective blocking as measured by nucleotide exchange, even though the animal's movement appeared to have ceased completely. These effects may be due to the higher amount of acetylcholine released in fast muscles (Hník et al 1967), thereby more effectively overcoming the competition by curare (although the doses administered in these experiments were in excess), or to physiological differences between phasic and tonic muscles, either in their structure and properties (Page and Slater, 1965; Sréter and Gergely, 1964), or in their excitation coupling (Bianchi, 1969). Differences have also been reported in the contractile proteins of slow and fast muscles (Bárány et al. 1965). For the relative insensitivity to curare of frogs with egg masses I can offer no explanation. The possibility of the egg mass absorbing most of the curare was considered but was discounted after massive doses and long incubation times proved to be ineffective. As to the fall in specific activities on stimulation it is possible that these animals are in a very high metabolic state and the incorporation of isotope into the nucleotide is on the decline even after so brief a labelling period.

From our few experiments in potassium contracture using Ca^{++} -free Ringer, we conclude that the change in actin during muscular activity accompanies activation rather than shortening.

It is now known that muscular contraction occurs as a result of an interaction of actin and myosin with ATP, although neither of these proteins is contractile when treated by itself with ATP. The localisation of the muscle proteins in the different filaments and the presence of cross-bridges provide evidence for the sliding filament theory of muscular contraction. For contraction to occur,

specific sites on both proteins must react in the presence of ATP, and the fact that the proteins are arranged in their filaments as they do so imposes the stress on the system necessary for development of tension. X-ray diffraction studies and electronmicroscope studies have clearly shown the different positions of the cross-bridges during active contraction, relaxation and rigor (Huxley and Brown, 1967; Reedy et al. 1965; Huxley, 1967). During a single twitch the changes in sarcomere length are far greater than can be accounted for by a single movement of the cross-bridges, and it would be foolish to try and relate changes in the conformation of the proteins to the different muscle lengths. The length of the sarcomere during contraction depends on the speed with which the cross-bridges make and break their connections. X-ray diffraction studies have shown that the cross-bridges are in contact with actin for a very short period of time (Huxley, 1967), and that only a few of them are in contact at any one moment. Therefore they must react many times during a single contractile event, and their reaction must be of a cyclic nature. What is more, the time for the cycle must be very short indeed, probably no longer than a millisecond. During this reaction sequence, some structural change must occur which enables a pull to be exerted on the actin filaments, and relatively little is known about the conformational change which takes place in the proteins as a result of their interaction with ATP. It is certain, however, that the altered conformation can only last for a brief part of the lifetime of the cycle. This is supported by an experiment by Szent-Györgyi (1968). If myosin is treated with unpurified p-nitrothiophenol, it will combine with actin, but the actomyosin formed loses the power of superprecipitation. It shows no difference from untreated actomyosin with respect to the exchange between bound ADP and medium ATP.

Since this exchange probably occurs normally as a result of a change in conformation, it seems reasonable to assume that the p-nitrothiophenol takes effect in such a way that the brief conformational change is not followed by the events which result in superprecipitation. A similar conclusion can be drawn from our experiments in which myofibrils were incubated with 2,4-dinitrophenol. An exchange was detected even though superprecipitation was inhibited.

Most of the hypotheses put forward as explanations of muscular contraction in the early years assumed that the protein chains shortened either by coiling or folding, and hence brought about a shortening of the muscle as a whole. However, when Hanson and Huxley (1955) showed that the filaments do not change their length during activity, new hypotheses had to be devised and the folding filaments of a single actomyosin complex were replaced by a system of interdigitating filaments of actin and myosin. Hanson and Huxley entertained the possibility that one of the filaments underwent a small cyclic change in length at several points of attachment, which resulted in an overall change in length of the filament. They went further to suggest that the cyclic changes might be a successive depolymerisation and repolymerisation of actin. Following this, A.F. Huxley (1957) put forward a theory which involved oscillating side pieces on the myosin molecule. These formed spontaneous temporary connections with adjacent actin filaments and exerted a pull on the actin as they slid backwards and forwards along the backbone of the myosin filaments, the extent of the movement being limited by elastic connections. The connections would be broken by the combination of a high-energy phosphate compound with actin near to the site of reaction with myosin. This phosphate compound would provide the energy for the breaking of the actin-myosin link, would be dephosphorylated, and detach from the protein, restoring the

initial conditions. More recently, as we have mentioned, a detailed theory was put forward by Davies (1963), involving contractile side chains.

No full hypothesis has so far been suggested that involves a brief and reversible conformational change of actin, in spite of the gathering evidence in vitro that this may be the case (Szent-Györgyi and Prior, 1966). In their experiments with actomyosin these authors obtained an almost immediate exchange of nucleotides with the onset of superprecipitation. This is consistent with our findings in vivo. However, Martonosi et al. (1960b) reported that equilibration between the bound and pool nucleotides in vivo takes nearly 24 hours, and that exercise and electrical stimulation have no effect on the exchange. They also reported that repeated superprecipitation of labelled actomyosin did not induce an exchange. This is in direct conflict with the results obtained by Szent-Györgyi and Prior. However, in their comparisons of ^{14}C incorporation into actomyosin in vitro, and ^{32}P incorporation into actin-bound ADP in vivo, it may be significant to point out that they were comparing different isotopes. For the experiments in vivo the isotope used was ^{32}P in the form of the difficulty permeating orthophosphate. For their experiments in vitro they were using G-actin labelled with ^{14}C , and obtained nearly 100% equilibration within 5 minutes (1960a). The results for superprecipitation, however, cannot be explained on this basis, since both sets of workers were using ^{14}C . Incorporation of bound ADP from medium ATP by actomyosin and myofibrils is reported to be very slow. Moos (1964) observed an amount of ADP incorporated of up to 0.2 $\mu\text{moles/g}$ with actomyosin and far less with myofibrils. Szent-Györgyi and Prior (1966) report that Noda and Bono found a variable amount of incorporation, usually less than 10%, while they themselves obtained only 30% exchange after four hours.

Szent-Györgyi attributes the limitation to the availability of the sites. An attempt to explain the lack of exchange during repeated superprecipitation (Martonosi et al., 1960b) has been made by Martonosi, Kitagawa and Gergely (1965). They found a decrease in the ^{14}C -ADP released from actomyosin as the protein concentration increased from 0.3 mg/ml to 0.5 mg/ml. The ^{14}C -ADP release fell from 65% to 22%. They suggest that this may explain the differences between Martonosi's previous results and those of Szent-Györgyi and Prior. However, although the former do not indicate the protein concentrations used, the latter used concentrations far exceeding 0.5%. Martonosi et al. did in fact obtain a 15% exchange, but the difference between this and Szent-Györgyi's 50% may be due either to the method of preparation or to storage.

The experiments of Szent-Györgyi and Prior do not provide evidence for a conformational change in vivo, nor do they suggest the nature of the change in vitro. In view of the results of Asakura (1961a) where no change in the viscosity of a sonicated solution accompanied the exchange of bound nucleotide, a distortion of the helix rather than a break in the polymer structure seems more likely and indeed preferable. In their model to account for this, Asakura, Taniguchi and Oosawa (1963b) suggest that an $F \longrightarrow f$ transition spreads throughout the actin units owing to the stretching of one unit by the transition and reformation of another. In the presence of ATP this cycling produces a smooth shortening force along the filament.

Using X-ray diffraction techniques, Huxley and Brown (1967) studied the patterns of muscles in rigor, rest and relaxation. During rigor the actin reflections undergo some small changes in spacing and intensity although at wide angles the rigor pattern is

unchanged. The subunit spacing of the actin monomers is unchanged, showing that no appreciable distortion of the actin structure in a longitudinal direction results on combination with myosin. A slight change in the pitch of the actin helix from 370 Å to 380 Å when the muscle goes into rigor cannot be ruled out. No changes were detected in contracting muscles except that the clarity of the actin reflections improved as though the actin filaments were becoming more ordered. Patterns were the same for both isotonic and isometric contractions and the measurements were accurate to within 1 part in 500. If the changes in the conformation were of the F \longrightarrow f type as proposed by Asakura et al., (1963b), and if only a few of the actin units were involved at any given moment, it is possible that pronounced changes in reflections would not occur.

There now seems no doubt that some change in conformation leading to the exchange of actin-bound nucleotide does take place. There are two ways in which this exchange could be brought about; either by a transfer of phosphate to the ADP while it is still bound to form ATP which is then free to exchange, or by direct exchange between bound ADP and free ATP as a result of a loosening of the actin structure. The arguments concerning this mechanism have been discussed. Szent-Györgyi and Prior (1966), in their scheme for the cycle, favour a direct exchange between bound ADP and free ATP, followed by an immediate dephosphorylation of the bound ATP. Szent-Györgyi (1968) finds no accumulation of actin-bound ATP during the exchange in actomyosin systems. Using unlabelled ATP incubated with (^3H -ADP)-actomyosin, he puts the limits of the formation of bound ATP by transphosphorylation at 1%. However, even these experiments are carried out on artificial systems and therefore do not necessarily represent the behaviour in vivo. Actin exists in vivo in conjunction

with tropomyosin, troponin and α -actinin (Ebashi and Kodama, 1966) all of which exert an influence on the F-actin. It is therefore extremely likely that anomolous results in isolated preparations are due to the lack of the effects from other proteins. A transphosphorylation from, say myosin, must not be ruled out as a result of these experiments in vitro.

In short, then, the general pattern of labelling, which seems to be consistent in all types of muscle studied, seems to be as follows: ^{14}C -glucose injected into frogs subcutaneously is rapidly absorbed and incorporated into the newly synthesised ATP, largely in the sugar moiety. The pool ATP therefore assumes a high specific activity, and by exchange of nucleotide with the pool, the bound nucleotide also becomes labelled. In active frogs, the bound activity eventually exceeds the activity of the total ATP of the tissue. This occurs very rapidly in phasic muscles and more slowly in tonic muscles. The exchange between the bound and pool nucleotides can be inhibited by inducing muscular paralysis with (+)-tubocurarine chloride either before the isotope is administered, in which case the bound ADP remains virtually unlabelled, or after the administration of the isotope. In this case the bound activity remains high, while the activity of the total ATP falls as a consequence of metabolism. The pattern of labelling resulting from stimulation of the muscle depends on the state of the nucleotide pool, which in turn depends on the time factor and the metabolic state of the animal. When the bound activity exceeds that of the pool, stimulation causes a reduction in the activity of the bound to a level between the stimulated and unstimulated pool values. ATP synthesis occurs as a result of muscular activity but its specific activity will only increase if the precursor pools are still labelled. In most experiments this is the case.

When the pool activity exceeds the bound, stimulation produces an increase in the specific activity of the bound ADP.

The term 'pool' is applied in this account to the total ATP of the muscle tissue. In many instances the bound ADP has assumed a specific activity which exceeds that of the total ATP, and this provides evidence for the existence of a highly labelled pool, undergoing rapid turnover, in contact with the contractile elements of the myofibril. We have here excellent direct evidence for compartmentalisation of nucleotide within the cell.

Exchange of actin-bound ADP in vitro occurs only when F-actin depolymerises. The entire nucleotide then exchanges with free ATP and G-actin is formed. On repolymerisation of the protein, ATP is split and F-actin-ADP is reformed. We have clearly demonstrated that this exchange occurs in the living animal as a direct result of muscular activity, and have thus provided evidence for a change in the actin molecule during contraction. To decide whether the change is a complete break in the actin filament or a localised distortion in the region of the binding site is beyond the scope of this work.

SUMMARY

1. The literature describing the discovery and properties of actin and its role in living muscle is discussed. Previous theories attributing to actin an active part in muscular contraction are reviewed.
2. The purpose of the investigation is described.
3. An account is given of the materials used and the methods employed in carrying out the experiments.
4. Experiments are described which led to the following observations:
 - a) After subcutaneous injections into frogs of uniformly labelled ^{14}C -glucose, the isotope can be detected in the total ATP of the cell and in the actin-bound ADP.
 - b) An acceptable agreement was obtained between the specific activities of the adenine nucleotides in paired muscles given identical treatment.
 - c) In gastrocnemius and sartorius muscles, after relatively long periods (60 min.) of labelling, the specific activities of bound ADP were found to be higher than those of the pool (total) ATP. Even ten minutes after injection of ^{14}C -glucose, the bound ADP of the fast sartorii becomes highly labelled, although its activity does not exceed that of the pool.
 - d) In the slow rectus abdominis muscle the bound ADP usually acquires only slight activity after one hour.
 - e) Potassium chloride contracture causes an increase in the specific activity of the bound ADP in the rectus muscles and

a decrease in that of the gastrocnemius and sartorius muscles. Contracture almost invariably results in the activity of the bound ADP becoming closer to the mean of the activities of the pool ATP before and after contracture. The specific activity of the pool ATP is greatly increased in contracture.

f) Subcutaneous injections of (+)-tubocurarine chloride given one hour before the isotope inhibits, often completely, the appearance of labelling in the bound ADP of the slow rectus abdominis, and considerably reduces the bound activity in the fast sartorius. Depolarisation with KCl brings about an increase in the activities of the bound and pool nucleotides in every instance with the rectus abdominis, and in most instances with the sartorius.

g) Injection of (+)-tubocurarine chloride given after the isotope leads, after long labelling times, to a situation in gastrocnemius and rectus muscles where the activities of the bound ADP remain high when those of the pool have fallen off.

h) Electrical stimulation (5-second tetanus) of briefly labelled, precurarised sartorii showed a marked increase in the specific activities of the bound ADP and, in most cases, of the pool ATP. Incomplete curarisation occurred in all the female frogs containing eggs, and in every case where the experiment was not discarded, the activities of both bound and pool nucleotides fell on stimulation.

i) Precurarised rectus muscles, brought by electrical stimulation into a single twitch, showed a large increase in the activities of the bound nucleotide and of the pool nucleotide. The bound activity after stimulation became

closer to that of the pool.

j) The specific activity of the bound nucleotide prepared directly from washed muscle residues was shown to be, within the limits of experimental error, identical with that of the ADP from actin isolated from the same muscles.

k) Experiments designed to find the distribution of labelling in the nucleotide molecule showed that the isotope is initially found in the highest concentration in the ribose moiety, being incorporated much more slowly in the purine ring.

l) Experiments on recti preincubated in Ca^{++} -free Ringer showed an exchange between bound and pool nucleotide on depolarisation with KCl.

m) Exchange of bound nucleotide during contraction of washed rabbit myofibrils by ATP and Mg^{++} was not inhibited by 2,4-dinitrophenol although the contraction was partially inhibited by this substance.

5. These results are discussed in relation to other published work and the following conclusions are drawn:

(i) Stimulation of the skeletal muscles of the frog, either by depolarisation with KCl or by electrical excitation, results in an exchange of actin-bound ADP, probably associated with a change in the configuration of the actin.

(ii) The labelling of actin-bound nucleotide in the muscles of inactive frogs is probably due to postural activity or to fibrillation.

(iii) Limited activity of the muscles, even that producing a

single twitch, results in a marked increase in the turnover of ATP, which involves synthesis de novo.

(iv) The myofibrils, during activity, are in exchange with a nucleotide pool with a turnover rate higher than that of the total ATP of the tissue.

(v) The change in the actin during muscular activity is associated with activation rather than with shortening.

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