Studies in the turnover of the bound phosphate of skeletal muscle during contraction and relaxation

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ABSTRACT

The possibility, already suggested by Cheesman and Hilton (J. Physiol. 155, 41 P 1961) that there is a turnover in active muscle of a phosphate fraction which remains attached to the muscle after extraction with aqueous media, was investigated further.

A reversible decrease in the specific activity was found when loaded or unloaded frog recti (briefly labelled with ^{32}P-orthophosphate) were contracted by treatment with KCl. Even in the absence of shortening (under isometric conditions or in the absence of calcium ions) a decrease in the specific activity occurred after depolarisation of the membranes.

A similar change in specific activity was found when ^{32}P-labelled glycerol-extracted psoas fibrils were treated with ATP or ITP in the presence of magnesium ions. No change occurred when the fibrils were treated with ATP and calcium ions. 2,4-Dinitrophenol did not inhibit the effect of ATP and magnesium ions on the bound phosphate of the myofibrils.

In the intact muscle the ATP-γP and the P of PC do not become equally labelled when incubated with ^{32}P-orthophosphate for 60 min. That material which is extracted as Pi by trichloracetic acid from acetone-dried washed muscle residues has a similar degree of labelling as the ATP-γP is isolated from whole muscles.

The only phosphorus-containing compounds isolated from trichloracetic acid-extracts of the washed ^{32}P-labelled muscle residues were ADP and Pi. Pi was labelled; ADP was unlabelled. An unidentified phosphorus-containing compound was detected on some chromatograms of neutralised HCl-extracts of acetone-dried muscle residues. This material was present in a reduced amount on chromatograms of contracted muscles.

It is suggested that the labelled phosphate fraction may derive from phosphorylated myosin, formed as an intermediate in the cycle of chemical events associated with muscular activity.

CONTENTS

ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
DNP	2,4-Dinitrophenol
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol tetraacetic acid
HMM	Heavy meromyosin
ITP	Inosine triphosphate
LMM	Light meromyosin
PC	Phosphorylcreatine
Pi	Inorganic phosphate
TCA	Trichloroacetic acid

CHAPTER 1

Introduction

The chemical investigation of muscular contraction began at the beginning of the present century. Fletcher and Hopkins (1907) discovered that lactic acid was produced in working muscles. They were able to show that the amount of lactic acid produced under anaerobic conditions was proportional to the work done by the muscle. Theories were developed for the conversion of chemical energy, e.g. the heat of neutralisation of lactic acid, to mechanical work. The "lactic acid theory" was finally discarded after the discovery by Lundsgaard (1930) that muscles poisoned with iodoacetate contracted normally for a considerable period without a concomitant lactic acid production, but with the splitting of PC.

In about 1910 A.V. Hill began his study of the heat changes which occur during the contraction cycle, which have dominated the field in the search for the initial energy source for muscular contraction. Between that year and 1926 Hill carried out experiments which together with those of Fenn led directly to the formulation of the requirements which must be satisfied by any theory of muscular contraction.

These requirements may be summarised:-

1. Any theory must take into account that no endothermic reaction is detectable at any stage in the process. If any do occur they must be balanced or overbalanced by exothermic reactions. (Hill 1926)

2. There is no heat production during relaxation if the muscle is not loaded. (Fenn 1924)

3. The heat activation is independent of all factors other than stimulation. The heat of activation starts at its maximum rate before any visible signs of contraction occur, declining to zero at about the time when the strength of contraction begins to fall off.

4. The maintenance heat in a prolonged contraction is the sum of the heats of activation following successive elements of the stimulus.

Eggleton and Eggleton (1927) published their work on the discovery of phosphorylcreatine and its breakdown to creatine and inorganic phosphate. Even in the absence of oxygen, they showed that PC was immediately resynthesised. Fiske and Subbarow (1929) published similar findings.

The following year (1930) Lundsgaard showed that the breakdown of PC was proportional to the work done by the muscle, when the muscle was poisoned with iodoacetate and later Lohmann (1934) while failing to show the breakdown of PC in aqueous muscle extracts did show that ATP was synthesised.

$$ADP + PC \rightleftharpoons ATP + C \qquad 1.$$

The fate of PC seemed to be the resynthesis of ATP and not the initial source of energy for contraction.

This discovery by Lohmann brought about a switch in the search for the energy source for muscular contraction from PC to an ATP-splitting system.

Engelhardt and Ljubimova (1939) discovered the ATPase activity of the muscle protein myosin and this led to unsuccessful attempts to isolate the enzyme from the structural protein. Szent-Györgyi and Straub (1941)

demonstrated that threads of myosin would contract in the presence of ATP. This was accompanied by the discovery that there is a drop in viscosity of myosin solutions in the presence of ATP.

Szent-Györgyi (1947) went on to show that myosin prepared by short term extraction of muscle showed a much smaller viscosity drop and a smaller contraction of its threads in the presence of ATP. The ATPase activity was high in both short and long term extracts of muscle.

Szent-Györgyi concluded that two proteins were present; he named the protein which conferred the mechanical properties to the complex, actin and the protein with the enzymic properties myosin. The original muscle protein was renamed actomyosin. Szent-Györgyi regarded the functional unit as the actomyosin-ATP complex.

Myosin has a molecular weight of about 500,000, artificial cleavage of the molecule with proteolytic enzymes breaks the molecules into two parts, the light (LMM) and heavy (HMM) meromyosins. LMM makes up the tail part of the myosin molecule, while HMM comes from the "neck" and fattened head regions and possesses all the enzymic properties of the parent molecule.

Actin can exist in two forms, referred to as F-actin and G-actin. This naming arises from the fact that in low salt concentrations, actin behaves as a globular protein with a molecular weight of about 70,000 or 140,000, whereas in the presence of higher salt concentrations it behaves like a fibrous protein. The polymerisation of the G-actin to the F-form may be brought about by the presence of divalent cations or more slowly by K^+ (Straub 1942). Petko and Straub (1949) discovered

that muscle contained about 3 μmoles ADP per g dry muscle, in a bound form which was inaccessible to enzymes which would split free nucleotides. Straub and Feuer (1950) showed that polymerisation of actin was accompanied by the dephosphorylation of the bound ATP to ADP.

Histologically skeletal muscle appears to have a banded structure. Under the light microscope the contractile unit. the myofibril consists of alternating light bands (I bands) and dark bands (A bands).

Huxley (1953) and Hanson and Huxley (1953) using the electron microscope, showed the myofibrils to be made up of two types of longitudinal filaments. The thick filaments (100 A^{o} in diameter) arranged hexagonally were confined to the I band but also projected into the A band. The thin filament also in hexagonal array, lay symmetrically between three thick filaments. A prominent feature of the A band is the system of cross bridges which project from the thick filament to the thin filaments, the bridges show a small variation in their spacing and in their angle of projection but are roughly at right angles to the filaments. No bridges appear on the thin filaments. In contraction the A bands were unaltered but the I bands disappeared. During contraction, the individual filaments remained unchanged in length; the shortening of the muscle was brought about by the interdigitation of the two types of filaments. The thick filaments appeared to be composed of myosin and the thin filaments of actin.

Attempts have been made by many workers to show the breakdown of ATP during contraction. Lundsgaard (1949) was able to show changes in the ATP content of muscles which had been brought into cold contracture

and fixed in liquid air at its boiling point. It seems, however, that in these experiments a layer of gas was formed about the muscle which prevented the rapid fixation of the muscle in a definite chemical state.

Munch-Petersen (1953) using paired tortoise muscles fixed in liquid propane cooled in liquid nitrogen showed a significant increase in ADP in the electrically stimulated muscle at half maximum contraction.

Lange (1955) was able to show both PC and ATP breakdown and an increase in ADP in frog muscles contracted at 0°C with KCl and acetycholine which had been fixed in cold alcohol (60%) at 0°C.

Fleckenstein et al. (1954 a and b) working with frog rectus abdominis which had been tetanised at 0°C were unable to show any changes in PC, ATP or ADP. Later experiments showed an increase in Pi in stimulated muscles. Fleckenstein and his collaborators (1957) in experiments with muscles labelled by incubation with ^{32}P-orthophosphate, showed that the phosphorus in the terminal phosphate of ATP did not show the same degree of labelling as that from PC. The latter had only 60% of the activity of the ATP-γP. This was somewhat unexpected in view of the high creatine kinase content of the muscle. They were unable to show any changes in the degrees of labelling after activity.

Mommaerts and Schilling (1955) carried out experiments which were similar in scope to those of Munch-Petersen (1953) on turtle sartorius and could show no changes in PC, ATP, ADP or phosphoenolpyruvate.

Fundamentally the problem is that the PC content of the muscle is high and the estimated changes to be found in a single twitch would be small. Based on the figures of A.V.Hill (1958) for the heat of contraction, about 1% of the total PC would be split during a single

twitch if the heat of hydrolysis of PC is about 10 Kcal. The ATP content of the muscle is much smaller, about 10% that of the PC, but it is possible that ATP would be immediately resynthesised from the PC pool.

It may be emphasised at this point that even when work is performed by muscle the breakdown of a phosphagen may not be detected. The experiments of Mommaerts have over a period of years given rise to a number of strange discrepancies. The first work on breakdown of ATP in turtle sartorius muscle (1955) gave no evidence of ATP splitting during contraction. In 1962 this work was repeated using turtle rectus femoris and sartorius. The rectus femoris on contraction gave results which indicated a breakdown of PC together with a corresponding increase in both Pi and creatine. The sartorius muscle gave different results. Contraction of the sartorius did not show PC breakdown even in a short tetanus, but after a recovery period followed by a second stimulation a measurable increase in creatine but no corresponding increase in Pi was shown. When these experiments were repeated using frog sartorius (1962), Mommaerts was able to show breakdown of PC during tetanus.

Mommaerts considered that a breakdown of ATP could be concealed by the transfer of phosphate from an unknown substance, possibly an unknown phosphagen. Perry (1952) pointed out that the myofibrils occupy two-thirds of the volume of the cell and the extramyofibrillar space occupied by the sarcoplasm contains both glycolytic and creatine kinase systems for the synthesis of ATP.

Carlson and Siger (1960) demonstrated with iodoacetate poisoned muscles, that there was a linear relationship between PC split and the number of isometric twitches. They could show no net ATP breakdown.

Davies (1963) using loaded frog muscles in which creatine kinase was inhibited by fluorodinitrobenzene was able to show breakdown of ATP during a short tetanus, which was proportional to the work done by the muscle. Davies' theory of muscular contraction proposes that the activation of the muscles releases Ca^{++} which diffuses and forms a linkage between the actin-bound ADP and ATP bound to the bridges of the myosin molecules. In the resting state ATP is bound to a polypeptide chain in the region of the active site of the myosin, this polypeptide in the absence of Ca^{++} is extended because the negative charge on the ATP induces repulsion between this negative charge and some other fixed negative charge in the region of the active site. When Ca^{++} enters the system, the formation of a linkage between actin-bound ADP and the ATP could neutralise the negative charge on the ATP, this might induce conformational changes in the polypeptide chain. The shortening of the polypeptide has the effect of pulling the actin, resulting in the contraction of the I band. The shortening of the polypeptide chain could also bring the bound ATP into contact with the ATPase site. Subsequent splitting of ATP relaxes the side chain and after rephosphorylation, a further linkage is formed between the bound nucleotide and the ATP binding site. This stepwise making and breaking of linkages between two types of filaments results in the interdigitation of the filaments and contraction of the muscle. However Davies (1967)

was unable to show changes in ATP either during activation or in unloaded shortening of muscles. Liberation of heat during activation must be accounted for in any theory of the energy sources for muscular contraction.

Some workers have investigated the possibility that the bound nucleotides are the immediate energy source for muscular contraction. Straub and Feuer (1950) showed that during the polymerisation of G-actin that Pi was released in a 1 : 1 ratio with the ATP split, ATP hence appeared to be the prosthetic group of G-actin and ADP that of F-actin. Straub suggested that the first stage in the contraction process was the phosphorylation of actin and that an unstable phosphoprotein was formed which yielded its phosphate during polymerisation. The polymerisation can be reversed by dialysis against dilute solutions of ATP.

F-actin-ADP + ATP $\rightleftharpoons$ G-actin-ATP + Pi 2.

Straub concluded that G-actin was the form characteristic of relaxed muscle. It would follow from equation (2) that the splitting of the bound ATP would accompany contraction. Martonosi, Gouvea and Gergely (1960) however maintain with some experimental support that F-actin is the form of actin <u>in vivo</u> and that the polymerisation is not a concomitant of the contraction process.

Other possible energy sources for muscular contraction have been postulated they include the possibility of a phosphorylated protein and the direct deamination of the nucleotides.

The existence of a phosphorylated protein as an intermediate of contraction could conceivably explain such anomalies as Mommaerts' failure to demonstrate breakdown of PC in turtle sartorius.

Levy and Ryan (1966) investigating the enzymic activity of myosin suggested that a phosphorylated myosin is formed as a stage in the hydrolysis on ATP. Tonomura and Kanazawa (1965) consider that the formation of a reactive myosin-phosphate complex was the key to muscular contraction. Tonomura proposed a mechanism of contraction based on the hydrolysis of ATP by actomyosin via the formation of a phosphorylated protein.

Burton and Lowenstein (1964) using HMM isolated an ATP-HMM complex on G 75 Sephadex. The binding was greatest in the presence of Mg^{++} and was reduced by Ca^{++} and K^{+}. This was the first real evidence of a phosphorylated myosin, which if it exists in vivo, might conceivably act as an energy source for muscular contraction.

In addition to ATP and a phosphorylated myosin, other possible sources of energy for muscular contraction have had their protagonists. Deamination of nucleotides has been regarded as a possible energy source by several workers. Deamination of AMP is well established as is its formation by myokinase. Webster (1954) found evidence for ADP deamination in actomyosin and a study by Wajzer et al.(1956) on the transmittance of UV light during mechanical work by the semitendinosus muscle of frog, led to the conclusion that deamination of ATP had occurred. Deamination of bound nucleotides has not so far been demonstrated.

In conclusion it is possible to summarise the evidence of possible energy sources as follows:-

The breakdown of PC during prolonged activity is established. The primary chemical event in contraction and its relationship to the

contraction process are still matters open to speculation.

It seems likely that some essential factor still remains undetected which could reconcile the apparent discrepancies and conflicting results hitherto published. The discovery of such a factor might lead to the incorporation of these divergent results in a single satisfactory theory of the energy sources and the nature of muscular contraction.

CHAPTER 2

Objectives of the present investigation

Cheesman and Hilton (1961) found that the specific activity of structurally bound phosphate of frog skeletal muscle preincubated with ^{32}P-orthophosphate was reduced by some 40% when the muscle was contracted with acetylcholine or isotonic potassium chloride. The specific activity was restored to the resting level by allowing the muscle to relax under load in normal Ringer's solution.

The objectives of the present investigation were to explore further the hypothesis proposed by Cheesman and Hilton, that some phosphorylated protein intermediate might be present in muscle and undergo hydrolysis during the first stages in the contraction cycle. Rephosphorylation of this unknown protein might occur by transfer of phosphate from ATP.

It is conceivable that a phosphorylated protein in resting muscle, might provide a source of energy adequate for a limited amount of activity and that, if its reconstitution from ATP were delayed, such activity could occur without a detectable reduction in high energy phosphate pool. Such a state of affairs would explain the anomalous findings of Mommaerts and Schilling (1955) and Mommaerts et al. (1962).

This investigation explored further the changes in specific activity of the bound phosphate associated with depolarisation, contraction and relaxation, with particular reference to the

conditions under which a reduction in specific activity might be achieved. The possibility of demonstrating a similar reduction in specific activity in a non-excitable system was also explored.

Attempts were made to discover the nature of the bound phosphate and to bring about an exchange between this substance and PC, ATP and ADP.

CHAPTER 3

Methods and Materials

Frog muscles

Slow and fast muscles from Rana temporaria were used. In 1953 Kuffler and Vaughan Williams published their findings on the differing physiological properties of the skeletal muscles of the amphibia. Certain muscles, particularly the rectus abdominis, iliofibularis and semitendinosus muscles show "tonic" properties. The "tonic" muscles respond to various stimuli e.g. mechanical and electrical stimulation and immersion in certain drugs such as acetylcholine with a maintained uncoordinated contraction called contracture. Depolarisation of the cell membranes with potassium chloride has the same effect. The contracture is maintained for a long period unless the loaded muscle is repolarised.

The use of the rectus abdominis for the present investigation was particularly useful since the muscle maintains its contraction during the subsequent treatment.

Fast muscles were used in those experiments where the investigation of relaxation in the absence of repolarisation was carried out. In an unloaded state of sartorius remains contracted after depolarisation. If a loaded sartorius is depolarised with KCl the muscle will contract but will rapidly relax without repolarisation of the membranes.

Rabbit psoas fibres

The psoas muscles were removed after cooling the skinned eviscerated animal on ice for 20 min. The muscles were washed in

Locke's physiological saline containing NaCl 0.9%, KCl 0.042%, $CaCl_2$ 0.024%, $MgCl_2$ 0.01%, Na_2HPO_4 0.005%, $NaHCO_3$ 0.01%, and glucose 0.1%. The muscles were teased out into fibre bundles approximately 0.2 mm diam. to facilitate the circulation of the medium. The fibres were subjected to a small load and suspended in Locke's solution containing 2 μCi/ml ^{32}P-orthophosphate for 6 hours. The material was rinsed with Locke's solution to remove excess radioactive phosphate, transferred to 50% (v/v) aqueous glycerol and stored overnight at 4°C. The fibre bundles were transferred to fresh 50% glycerol and stored under load at -15°C for a week before use.

Separation of adenine nucletides

The extracted nucleotides were separated by paper chromatography using the two-stage folded paper technique of Krebs and Hems (1953). Diethylether-formic acid (60:30 v/v) was used for the ascending stage and iso-butyric acid-ammonia-0.1M-EDTA-water(66:1:1.6:31.4) for the descending stage. The paper was washed before use with 2% EDTA then with water and dried before use.

Separation of adenine nucleotides from phosphorylcreatine and inorganic phosphate

The separation of PC and Pi and the adenine nucleotides was carried out using the method of Fleckenstein et al. (1960). Inorganic phosphate was removed with diethyl ether-formic acid. The adenine nucleotides and PC were separated with isopropanol-methanol-ammonia-water (40:30:10:20). The paper was washed with 2% formic acid followed by water and dried before use.

Location of phosphorus-containing compounds on chromatograms

Adenine nucleotides were located by means of a UV lamp with emmision at 247 mμ at which wavelength they quench the fluorescence of the chromatography paper. Suitable markers were used to identify the compounds. PC and Pi were located by the method of Hanes and Isherwood (1949): the paper was sprayed with acid molybdate and exposed to UV light.

Estimation of the specific activity of PC, Pi and ATP-γP

In those experiments where the specific activity of PC and ATP and Pi were compared, the compounds were located and cut out of the chromatograms as standard size discs to fit an 17 mm planchette. After counting the discs in a Panax castle with a solid counting tube, the paper was digested with H_2SO_4 and the phosphorus content was estimated by the method of Berenblum and Chain (1938) which is more sensitive than the Allen (1940) method.

The specific activity was expressed as counts/min./μg P for PC and Pi. For ATP; the specific activity was calculated in terms of ATP-γP.

Other materials used

Whatman No.1 paper was used for all chromatography. Supplies of ^{32}P-orthophosphate were brought from the Radio-chemical Centre, Amersham. The ^{32}P-orthophosphate was hydrolysed with 5 ml 2N-HCl before use to remove any polyphosphate compounds. Causey and Harris (1940) found that these compounds were present in early supplies of radioactive phosphate. Hydrolysis was discontinued when it was found to be unnecessary

Solvents were redistilled before use (ether, methanol, propanol, formic acid, acetone and TCA). All other chemicals were of analytical grades. Supplies of PC, ATP and ADP were bought from the Sigma Corporation.

Frogs were obtained from a number of different sources. The majority of animals were stored for several weeks before use at 4°C.

CHAPTER 4

The Contraction Effect

Experiments were carried out to study the effect of potassium contracture on the specific activity of the bound ^{32}P-labelled phosphate of frog skeletal muscle. Essential control experiments were carried out to establish that the two recti abdominis of frog have the same phosphorus content after exhaustive washing and exhibit a similar degree of labelling.

It had been suggested by Professor R.E. Davies in a personal communication to Professor Cheesman that the absence of Ca^{++} from the contraction medium might be responsible for the change in specific activity reported by Cheesman and Hilton (1966). Experiments were carried out to investigate the effect of Ca^{++} in the contraction medium.

SERIES A

The effect of contracture on the specific activity of the water-insoluble residues from ^{32}P-labelled recti

Method

The muscles were removed from the decapitated animals and were preincubated in Ringer's solution which contained: 0.65% NaCl, 0.02% KCl, 0.012% $CaCl_2$ and 0.1% $NaHCO_3$. The muscles were incubated in a 5 ml organ bath, attached to a small spring balance, the incubation medium being aerated at room temperature with a mixture of 95% O_2 : 5% CO_2. After 30 min. the muscles were labelled by incubation in Ringer's solution containing 0.02% μCi/ml ^{32}P-orthophosphate, with 2×10^{-5}M-KH_2PO_4 as carrier, for 30 to 60 min.

After labelling the muscles were washed 4 times with 5 ml saline solution to remove excess radioactive phosphate.

The control muscles remained in frog saline at room temperature. The test muscles were depolarised by immersion in isotonic (0.765%) KCl; contraction of the depolarised muscles was noted. Some muscles were depolarised while attached to the spring balance and some were depolarised in an unloaded state. The test and control muscles were cooled on ice, minced with fine scissors in their appropriate media (KCl for the tests and Ringer's solution for the controls). The minced tissue was extracted with 0.05 M $NaHCO_3$-Na_2CO_3 (pH 9.9) for 30 min. and washed 10-20 times over a period of 2 hours with ice-cold water. The residues were washed 4 times with 25 ml acetone and dried in air. The acetone powders were weighed and the phosphorus contents estimated by the method of Allen (1940). The radioactivity of the colorimetric solution was measured in a liquid counter of 12.5 ml capacity using a Panax scaler.

The specific activity was counts/min./µg phosphorus.

Results (Table 1.)

The effect of depolarisation of the rectus muscle with or without load is to reduce the radioactive count in the water-insoluble residue from ^{32}P-labelled recti by some 40%.

TABLE 1

Potassium contracture of ^{32}P-labelled recti

Effect on specific activity of bound, water-insoluble phosphate

Paired muscles incubated at room temperature for 30 min., Labelled with Ringer's solution containing ^{32}P-orthophosphate Unloaded or lightly loaded test muscles contracted in isotonic KCl. Test and control muscles minced and extracted by method described in text.

Control		Contracted		Ratio of specific activities
acetone powder		acetone powder		
µg P/mg	counts/min./µg P	µg P/mg	counts/min./µg P	Contracted/Control
2.25	79.0	2.49	64.0	0.81
2.44	113.0	2.57	74.0	0.66
2.16	103.0	2.07	82.0	0.80
1.55	31.0	1.95	15.0	0.52
1.32	74.0	1.51	53.0	0.71
1.91	50.0	1.81	21.5	0.43
2.51	17.2	2.14	12.6	0.73
2.14	110.5	2.48	44.0	0.40
2.71	40.0	2.32	22.0	0.55
2.08	79.3	2.51	61.5	0.78
1.85	91.5	1.88	68.0	0.74
1.78	38.0	1.76	29.5	0.78
1.81	50.0	1.48	16.1	0.32
1.86	155.0	1.88	130.0	0.84
1.46	122.0	1.85	65.0	0.53

Cont./over

1.82	39.4	1.87	24.5	0.62
1.61	19.9	1.68	12.3	0.62
1.49	7.2	1.68	4.5	0.63
2.30	89.0	2.12	44.0	0.50
1.84	137.0	1.62	56.0	0.41
1.31	10.5	1.69	6.0	0.57
1.45	23.0	1.52	13.0	0.56
1.59	34.5	1.79	22.0	0.64
Mean ± S.D.				
1.88 ± 0.38		1.94 ± 0.32		0.64 ± 0.14

23 experiments

SERIES B

The effect of alcohol fixation and extraction on the specific activity of the bound phosphate of ^{32}P-labelled frog rectus abdominis

Method

After labelling and contraction of the test muscles as in Series A, the test and control muscles were transferred to 96% ethanol at 0°C for 10 min. The fixed muscles were minced in ethanol and left in this medium for 5 min. The minces were extracted with alkali buffer, washed and dried following the procedure in series A and the specific activity of the bound phosphate estimated.

Results (Table 2.)

Alcohol fixation and extraction does not affect the ratio of the specific activities of control and contracted muscles. The phosphorus content of both test and control muscles was considerably reduced by alcohol fixation and extraction.

SERIES C

The effect of isometric contraction on the specific activity of the bound phosphate of ^{32}P-labelled frog rectus abdominis

Method

Isometric experiments could not be carried out without modification to the basic experimental procedure. Muscles were labelled following the method in series A. After rinsing with Ringer's solution they were measured, attached to an isometric lever and extended to about 130% of their resting length after extirpation.

TABLE 2

Potassium contracture of ^{32}P-labelled recti

Effect on specific activity of bound, water-insoluble phosphate

Experiments as in Table 1, but with fixation of muscle in alcohol before mincing and extraction of minced muscle with alcohol before extraction with aqueous solvents.

Total phosphorus content of acetone powder		
µg P/mg Control	µg P/mg Contracted	Ratio of specific activities Contracted/Control
0.87	1.06	0.56
1.02	1.25	0.61
0.97	1.17	0.70
1.04	1.09	0.87
1.17	1.15	0.60
0.97	1.00	0.48
1.08	1.04	0.64
0.77	0.68	0.49
Mean ± S.D.		
0.99 ± 0.12	1.03 ± 0.17	0.62 ± 0.11

8 experiments

The test muscles were depolarised with isotonic KCl; no shortening occurred. The control muscles were not contracted but were rinsed with Ringer's solution.

Before the muscles were detached from the lever they were fixed by immersion in cold ethanol for 10 min. If the depolarised rectus is detached from the lever before fixation, unloaded contracture will occur. The fixed muscles were minced with fine scissors in ethanol, drained well to remove the solvent and extracted with alkali buffer for 30 min. Subsequent treatment was as in series A.

Results (Table 3)

Since alcohol fixation does not affect the ratio of the specific activities of test and control muscles which have been contracted isotonically, it would appear that isometric contraction of frog rectus brings about reduction in the specific activity of the bound phosphate similar to that found with isotonic contraction.

SERIES D

The effect of depolarisation after preincubation and labelling in Ca^{++} -free Ringer's solution on the specific activity of the bound phosphate of ^{32}P-labelled frog rectus abdominis

Method

The loaded muscles were preincubated in Ca^{++} -free Ringer's solution for 30 min. Subsequent treatment followed the method in series A except that all solutions used were Ca^{++} -free, i.e. the medium in which the muscles were labelled and the Ringer's solution with which the muscles were rinsed to remove excess radioactivity. The test muscles were depolarised in a lightly loaded state.

TABLE 3

Isometric contracture of ^{32}P-labelled recti

Effect on specific activity of bound, water-insoluble phosphate

Fixation with alcohol after depolarisation.
Other conditions as in Table 1.

Total phosphorus content acetone powder		Ratio of specific activities
Control	Contracted	
µg/mg	µg/mg	Contracted/Control
1.51	1.45	0.57
1.22	1.57	0.60
1.30	1.37	0.86
1.84	1.74	0.48
1.63	1.73	0.40
1.41	1.61	0.68
1.30	1.30	0.32
1.80	2.00	0.81
1.97	1.93	0.62
1.89	1.55	0.38
Mean - S.D.		
1.59 ± 0.26	1.62 ± 0.21	0.57 ± 0.18

10 experiments

Results (Table 4)

Depolarisation of the cell membranes in the absence of Ca^{++} does not induce contraction; the specific activity of the bound phosphate is reduced.

SERIES E

The effect of contraction at 0°C on the specific activity of the bound phosphate in water-insoluble residues of ^{32}P-labelled recti

Method

The loaded muscles were preincubated and labelled at room temperature. After washing with Ringer's solution to remove excess radioactivity, the muscles were cooled by surrounding the organ bath with melting ice and by washing the muscles with ice-cold Ringer's solution. After 20 min. the test muscles were depolarised without load in ice-cold isotonic KCl. Subsequent treatment followed the procedure in series A.

Results (Table 5)

Test muscles contracted when depolarised with KCl at 0°C. The decrease in specific activity of the bound phosphate on contraction is similar to that found at room temperature.

SERIES F

The effect of contraction after brief labelling on the specific activity of the bound phosphate of water-insoluble residues of ^{32}P-labelled recti

Method

The experiments were carried out by the method described in series A except that labelling with ^{32}P-orthophosphate was reduced to 30 sec. The test muscles were depolarised without load.

TABLE 4

Potassium contracture of ^{32}P-labelled recti

Effect of depolarisation after incubation and labelling in Ca^{++}-free Ringer's solution.

Muscles preincubated and labelled in Ca^{++}-free media. Other conditions as in Table 1.

Total phosphorus content acetone powder		Ratio of specific activities
Control	Depolarised.	
µg P/mg	µg P/mg	Depolarised/Control
1.89	2.14	0.52
1.96	1.79	0.76
1.82	2.11	0.77
2.04	1.95	0.66
1.51	1.46	0.26
1.56	1.52	0.34
1.73	1.44	0.70
1.65	1.89	0.60
2.01	1.84	0.72
2.10	2.24	0.95
Mean ± S.D.		
1.83 ± 0.20	1.84 ± 0.27	0.62 ± 0.20

10 experiments

TABLE 5

Potassium contracture of ^{32}P-labelled recti

Effect of contracture at 0°C.
Other conditions as in Table 1.

Total phosphorus content acetone powder		
Control	Contracted	Ratio of specific activities
µg/mg	µg/mg	Contracted/Control
2.63	2.72	1.12
1.51	1.41	0.75
1.06	1.32	0.71
1.64	1.73	0.09
1.31	1.55	0.80
2.02	2.01	0.39
1.82	1.81	0.71
2.09	2.23	0.38
1.64	1.80	0.44
Mean ± S.D.		
1.73 ± 0.41	1.84 ± 0.41	0.69 ± 0.28

9 experiments

Results (Table 6)

The test muscles showed a decrease in specific activity similar to that shown by muscles labelled with ^{32}P-orthophosphate for 30 to 60 min.

SERIES G

The effect of a repeated contraction on the specific activity of the bound ^{32}P-labelled phosphate

Method

The test and control muscles were preincubated and depolarised as described in series A. Both muscles were repolarised by returning the muscles to Ringer's solution. The muscles were relaxed by stretching them between forcepses to restore the muscle to its original length. The test muscle was depolarised for a second time with isotonic KCl; the relaxed control muscle remained in Ringer's solution. Subsequent treatment was as described for series A.

Results (Table 7)

The control muscles passed through complete contraction - relaxation cycle, the test muscles depolarised for a second time appeared to contract normally. The results are similar to those obtained from a single depolarisation of the muscle.

SERIES H

Comparison of specific activities of the phosphate bound to the washed residues of paired recti abdominis

Method

An essential control experiment was to compare the specific activities of acetone powders of pairs of ^{32}P-labelled recti. The two

TABLE 6

Potassium contracture of ^{32}P-labelled recti

Brief labelling (30 sec.) with ^{32}P-orthophosphate.
Other conditions as in Table 1.

Total phosphorus content acetone powder		Ratio of specific activities
Control µg/mg	Contracted µg/mg	Contracted/Control
1.47	1.47	0.62
2.20	2.01	0.63
1.89	1.86	0.60
1.86	1.75	0.42
2.02	2.06	1.21
1.27	1.21	0.74
1.88	1.70	0.99
1.75	1.74	0.59
2.01	1.81	0.67
2.00	1.79	0.72
1.41	1.50	0.51
1.18	1.08	0.66
1.76	1.71	0.68
Mean ± S.D.		
1.75 ± 0.30	1.67 ± 0.28	0.70 ± 0.19

13 experiments

TABLE 7

Potassium contracture of ^{32}P-labelled recti

Effect of repeated depolarisation on a relaxed muscle.
Muscles (unloaded) contracted, relaxed and recontracted.
Other conditions as in Table 1.

Total phosphorus content acetone powder		Ratio of specific activities
Control µg/mg	Contracted µg/mg	Contracted/Control
1.98	1.80	0.41
2.08	1.81	0.62
2.06	1.72	0.83
1.72	1.93	0.54
2.57	2.29	0.42
1.42	1.67	0.99
1.60	1.44	0.83
1.81	1.74	0.24
1.22	1.06	0.43
1.32	1.38	0.76
Mean ± S.D.		
1.78 ± 0.39	1.68 ± 0.32	0.61 ± 0.22

10 experiments

muscles are anatomically distinct and are joined in the median line by the linea alba. The muscles were excised and after preincubation in Ringer's solution were labelled with ^{32}P-orthophosphate as described in series A. Both muscles were then minced with fine scissors and extracted with alkali buffer, subsequent treatment was as described for series A.

Results (Table 8)

These control experiments show good agreement between specific activities of the two muscles in four of the five experiments.

SERIES I

The effect of high concentrations of Ca^{++} in the contracture medium on the binding of ^{32}P-orthophosphate to frog skeletal muscle

Method

Paired recti were labelled with ^{32}P-orthophosphate as in series A. The test muscles were transferred to isotonic KCl containing 0.15% $CaCl_2$ for 15 min. Controls were depolarised in isotonic KCl. The subsequent treatment of the muscles followed the method described for series A.

Results (Table 9)

A white precipitate was formed in those tubes containing the high concentration of Ca^{++} on the addition of the alkali buffer. The tests showed a great increase in bound phosphate which was significantly greater than the established normal value for bound phosphate. Associated with this increase in phosphorus was an increase in count, which was greater than that which would have been found in a relaxed muscle, assuming that a ratio of specific activities of

TABLE 8

Control experiments to compare the specific activities of the bound phosphate of well washed pairs of frog recti

Both muscles were preincubated in Ringer's solution, labelled with ^{32}P-orthophosphate, minced with fine scissors, extracted with alkali buffer and exhaustively washed.

Total phosphorus content acetone powder		Specific activity	
µg P/mg	µg P/mg		
2.01	1.76	25.6	26.5
1.70	1.71	7.13	7.38
1.57	1.61	96.5	100.0
1.95	1.73	6.2	6.6
1.36	1.59	15.1	6.6

TABLE 9

The effect of high concentration of Ca^{++} in the contracture medium on the binding of ^{32}P-orthophosphate to frog skeletal muscle.

Paired muscles were labelled with ^{32}P-orthophosphate. Controls were depolarised in KCl. Tests were depolarised in KCl containing 0.15% $CaCl_2$. Tests and controls were minced with fine scissors, subsequent treatment as described in text.

Total phosphorus content acetone powder		Ratio of specific activities
Control	Test	Treated/Control
µg P/mg	µg P/mg	
1.81	2.58	2.09
1.73	2.86	3.36
2.19	4.78	2.15
2.03	2.88	2.15
2.08	3.35	2.03
1.98	2.96	2.10
Mean ± S.D.		
1.97 ± 0.15	3.23 ± 0.72	2.31 ± 0.47

6 experiments

contracted/control of 0.62.

SERIES J

The effect of normal tissue level of Ca^{++} in the contracture medium on the binding of ^{32}P-orthophosphate to frog skeletal muscle

Method

The method used was the same as that described in series A. except that the isotonic KCl contained 0.012% $CaCl_2$.

Results (Table 10)

There was precipitate on the addition of alkali buffer to the tests. The KCl effect is somewhat enhanced by the presence of Ca^{++} in the depolarisation medium. The ratio of the specific activities of tests and controls was 0.64 ± 0.14 (Table 1). The ratio from these experiments (6) was 0.48 ± 0.19.

SERIES K

The effect of a chelating agent on the bound ^{32}P-labelled phosphate of frog rectus abdominis

Method

Paired labelled muscles were minced and treated with alkali buffer as in series A, followed by 8 washed with cold water. Tests were washed 4 times over a period of 30 min. with neutralised cold 10^{-3}M-EGTA, controls were washed with water. Subsequent treatment as in series A.

Results (Table 11)

This was a small group of experiments which was not analysed. However all experiments show a reduction in phosphorus content and specific activity in the test muscles.

TABLE 10

The effect of the normal tissue level of Ca^{++} in the contracture medium on the binding of ^{32}P-labelled orthophosphate

Method as described in Table 1 except that the isotonic KCl used to depolarise the muscle contained 0.012% $CaCl_2$.

Total phosphorus content acetone powder		Ratio of specific activities
Control	Test	Contracted/Control
µg P/mg	µg P/mg	
1.54	1.54	0.23
1.81	1.87	0.76
1.56	1.59	0.47
1.42	1.57	0.28
1.74	1.74	0.68
1.54	1.50	0.45
Mean ± S.D.		
1.60 ± 0.13	1.63 ± 0.13	0.48 ± 0.19

6 experiments

TABLE 11

The effect of a chelating agent on the bound ^{32}P-labelled phosphate of frog rectus abdominis

Paired labelled muscles were minced and extracted with alkali buffer. Tests and controls were washed 8 times with cold water, Tests were washed 4 times with neutralised cold 10^{-3} M-EGTA. Subsequent treatment as in text.

Total phosphorus content acetone powder		Ratio of specific activities
Controls	Test	EGTA/Control
µg P/mg	µg P/mg	
1.68	1.38	0.39
1.87	1.44	0.74
1.58	1.34	0.40
1.67	1.42	0.51

4 experiments

SERIES L

Further study of the effect EGTA on the binding of ^{32}P-labelled phosphate

Method

As in series A, except that after 8 washes with water, tests and controls were washed 4 times with neutralised 10^{-3}M-EGTA.

Results (Table 12)

The total bound phosphate was lower in the EGTA-washed acetone powders than in normal powders.

Normal Tests 1.94 ± 0.38 Controls 1.88 ± 0.38 µg/mg

EGTA-washed Tests 1.75 ± 0.23 Controls 1.79 ± 0.27 µg/mg

The phosphate contents of the EGTA-washed muscle powders were not as low as had been anticipated from series K. The KCl effect appears to be slightly enhanced by the EGTA wash, but a comparison is difficult with such a small series of experiments.

	Ratio of specific activities Contracted/Control	
Normal (as in series A)	0.64 ± 0.14	23 experiments
EGTA wash (as in series L)	0.49 ± 0.06	6 experiments

SERIES M

The yield of acetone powder from wet muscles

Method

Test and control muscles were blotted to remove excess moisture and weighed. Test muscles were depolarised with isotonic KCl; controls remained in Ringer's solution. The muscles were minced with fine scissors and washed (series A). Care was taken to prevent any loss of material during washing. The residues were reduced to acetone

TABLE 12

The effect of washing with a chelating agent on the specific activity of the bound ^{32}P-labelled phosphate

Method as described in Table 1 except that tests and controls were washed 4 times with EGTA.

Total phosphorus content acetone powder		Ratio of specific activities
Control	Contracted	Contracted/Control
µg P/mg	µg P/mg	
2.12	1.98	0.40
1.74	1.67	0.48
1.90	1.82	0.51
1.79	1.77	0.53
1.23	1.30	0.59
1.97	1.98	0.44
Mean ± S.D.		
1.79 ± 0.27	1.75 ± 0.23	0.49 ± 0.06

6 experiments

powders and dried in air. The dry powders were further dried in a vacuum desiccator overnight, weighed, redesiccated and weighed again. Dry weights were expressed as a percentage of the wet weight of the muscle.

Results (Table 13)

There is no significant difference between the dry weights of acetone powders on contracted and relaxed muscles.

Summary of experimental findings from study of the contraction effect

1. The effect of depolarisation of the rectus muscle with or without load is to reduce the radioactive count in the water-insoluble residue from ^{32}P-labelled recti. This was the effect demonstrated by Cheesman and Hilton (1961).
2. Alcohol fixation and extraction does not affect the ratio of the specific activities of control and contracted muscles.
3. When depolarisation is not accompanied by contraction i.e. without Ca^{++}, the specific activity of the labelled phosphate is still reduced.
4. The reduction in specific activity on contraction is not related to temperature. Fleckenstein and Janke (1954) showed that there is very little breakdown of PC at $0^{\circ}C$ so that the possibility of the reductions being caused by the breakdown of PC is lessened.
5. It may be concluded that the reduction in bound ^{32}P-labelled phosphate is associated neither with the shortening nor with the development of tension but rather with the activation process.

TABLE 13

Potassium contracture of frog rectus abdominis

Yield of acetone powder from wet muscle

Paired muscles blotted and weighed. Unloaded test muscles depolarised in isotonic KCl, controls remained in Ringer's solution. Tests and controls minced and extracted by method described in text.

Controls			Tests		
Wet weight mg	Weight of acetone powder mg	%	Wet weight mg	Weight of acetone powder mg	%
342	20.1	8.3	234	21.2	9.1
117	8.5	7.3	135	13.1	9.6
265	26.7	10.1	266	27.1	10.2
234	22.5	9.6	238	22.9	9.6
125	8.6	6.8	130	9.7	7.4
102	6.9	6.7	96	10.6	11.0
243	25.0	10.3	207	20.3	9.8
128	10.1	7.9	135	11.1	8.3
116	15.8	13.6	131	16.6	12.7
191	14.8	7.8	193	13.1	6.8
144	13.8	9.6	168	14.5	8.6
204	20.5	10.0	234	20.5	8.7

Mean ± S.D.

Tests 9.0 ± 1.5%

Controls 8.9 ± 1.9%

6. Occlusion of calcium phosphate in the control muscles is not responsible for the KCl effect.

7. There is no significant difference in the dry weights of acetone powder obtained from contracted and relaxed muscles.

CHAPTER 5

The Relaxation Effect

The effect of relaxation on the specific activity of the water-insoluble residues of ^{32}P-labelled recti was studied by Cheesman and Hilton (1965). Relaxation of the depolarised muscle restores the specific activity of the bound phosphate to its resting value.

In the present investigation it was planned to ascertain whether the restoration of specific activity was related to the relaxation process or simply to the repolarisation of the fibre membranes.

SERIES A

The effect of relaxation without repolarisation on the specific activity of the bound phosphate of ^{32}P-labelled frog sartorius

Method

Fast muscles relax rapidly without repolarisation when loaded. Tests and control muscles were labelled and depolarised by the procedure described in chapter 4, series A. The loaded muscles (attached to a spring balance) relaxed without repolarisation; the unloaded control muscles remained contracted in isotonic KCl. Test and control muscles were subsequently minced with fine scissors and reduced to acetone powders as described previously.

Results (Table 14)

Relaxation of the test muscles was noted in the absence of repolarisation. The ratio of specific activities of bound phosphate (contracted/relaxed) was 0.59 ± 0.14. This is similar to that found by Cheesman and Hilton (1965) for relaxation of recti with repolarisation.

Table 14

Relaxation of ^{32}P-labelled Sartorii

Effect of relaxation without repolarisation

Sartorii were contracted with isotonic KCl. Test muscles relaxed under load in KCl, controls remained in KCl unloaded. Test and control minced and extracted by the method described in the text.

Relaxed acetone powder µg/mg	Contracted µg/mg	Ratio of specific activities Contracted/control
1.64	1.65	0.64
1.67	1.66	0.71
2.36	2.50	0.65
1.52	1.41	0.72
1.24	1.34	0.72
2.37	2.26	0.67
1.84	2.07	0.26
Mean ± S.D. $\bar{1.94} \pm 0.41$	1.90 ± 0.39	0.59 ± 0.15

7 experiments

SERIES B

The effect of repolarisation without relaxation on the specific activity of the bound phosphate of ^{32}P-labelled frog recti

Method

After labelling, unloaded paired muscles were depolarised in isotonic KCl. Both muscles were repolarised in Ringer's solution. One of each pair was relaxed by stretching to its original length. Subsequent mincing and extraction followed the method in chapter 4, series A.

Results (Table 15)

Repolarisation of the muscle does not itself restore the specific activity of the bound phosphate to its resting value. It would appear that the mechanical effect of relaxation is related to the restoration of the resting value of the specific activity.

SERIES C

The effect of severe extension on the specific activity of bound phosphate in ^{32}P-labelled frog muscles

Method

Rectus and sartorius muscles were used for these experiments. The muscles were preincubated and labelled in ^{32}P-labelled Ringer's solution containing (+)-tubocurarine (1 in 10^5) following the procedure in chapter 4. The test muscles were severely extended with forcepses to about twice their original length. The control muscles were not stretched. The muscles were minced and extracted by the

TABLE 15

Repolarisation of ^{32}P-labelled recti

Effect of repolarisation without relaxation

Test and control muscles were depolarised in isotonic KCl. Tests and controls were repolarised by immersion in Ringer's solution, controls relaxed by stretching to original length, test muscles not relaxed.

Total phosphorus content acetone powder		Ratio of specific activities
Relaxed	Contracted	
µg/mg	µg/mg	Contracted/Relaxed
1.63	1.72	0.60
1.61	1.60	0.59
1.70	2.03	0.29
1.21	1.44	0.78
1.38	1.14	0.53
1.44	1.45	0.58
1.32	1.58	0.78
1.36	1.27	0.84
Mean ± S.D.		
1.46 ± 0.16	1.53 ± 0.26	0.62 ± 0.17

8 experiments

procedure in chapter 4.

Results (Table 16)

Curarisation was applied in an attempt to inhibit mechanical stimulation of the muscles. Severe extension of the muscles does not result in a significant alteration in the specific activity of the bound ^{32}P-labelled phosphate, although the scatter of the results is considerable.

Summary of experimental findings from the study of the relaxation effect

Cheesman and Hilton (1965) showed that relaxation of a contracted muscle restores the specific activity of the muscle to its original value. The total phosphorus content appeared to be unaltered within the limits of experimental error.

Control 1.78 ± 0.14 µg/P/mg acetone powder

Test 1.81 ± 0.22 µg/P/mg acetone powder

Further investigation shows that:-

1. Repolarisation of the muscle without relaxation does not restore the specific activity to its resting level.
2. Relaxation of a fast muscle without repolarisation restores the specific activity to its resting level.
3. Severe mechanical extension in itself has no effect on the specific activity of the bound phosphate. This tends to exclude the possibility that the effects are caused by mechanical means.

TABLE 16

Severe extension of ^{32}P-labelled muscles

Effect of stretch on curarised muscles.

Test and control recti and sartorii were incubated and labelled in Ringer's solution containing curare. Test muscle extended to about 2 times original length. Subsequent treatment as in Table 1.

	Total phosphorus content acetone powder		Ratio of specific activities
	Control µg/mg	Extended µg/mg	Extended/Control
Recti	1.92	1.98	1.02
	1.44	1.42	1.03
	1.96	1.76	0.88
	2.20	1.93	0.92
	1.78	1.76	2.27
	1.81	1.64	1.34
	1.42	1.54	0.49
	2.03	1.65	0.60
	1.37	1.49	0.86
	1.67	1.67	0.80
	1.60	1.73	0.55
	1.38	1.81	1.00

Cont./over

Sartorii

1.54	1.57	0.69
1.54	1.46	0.85
1.43	1.37	0.97
1.81	1.74	1.28
1.92	1.77	1.89

Mean ± S.D.

1.70 ± 0.25	1.66 ± 0.17	1.03 ± 0.36

17 experiments

12 recti 5 sartorii

CHAPTER 6

Investigation of Non-excitable Muscle Systems

A number of experiments were carried out on glycerol-extracted psoas fibres from rabbit, in an attempt to see whether the effect produced by isotonic KCl on rectus abdominis of frog could be produced by contraction of a non-excitable system with ATP and ITP. The myofibrils for these experiments were prepared from rabbit psoas muscles which had been previously labelled and glycerol-extracted (chapter 3). Myofibrils for the experiments with ITP were obtained from the muscles of frogs injected with ^{32}P-orthophosphate and left at room temperature overnight.

SERIES A

The effect of ATP in the presence of Mg^{++} on the specific activity of the bound ^{32}P-labelled phosphate of glycerol-extracted psoas fibres

Method

The labelled glycerol-extracted fibres bundles were rinsed with 0.5%-KCl to remove glycerol. Approximately 150 mg fibres were homogenised in 100 ml cold KCl in an M.S.E. homogeniser for 3 min. The homogenate was passed through a single layer of cheese-cloth and centrifuged at about 3,000 rpm for 10 min. The supernatent was discarded and the precipitate was resuspended in the same volume of KCl and rehomogenised. The fibrils were washed 6 times with KCl by resuspension and centrifugation, and twice with cold distilled water to remove KCl. They were then suspended in 20 ml 0.05 M-Tris-HCl buffer pH 7.0, containing 10^{-3}M $MgCl_2$ and divided into 2 parts of equal

volume. Tests were treated with an equal volume of Tris-HCl containing 10^{-3}M-$MgCl_2$ and 2 x 10^{-3}M-ATP. Controls were treated with an equal volume of Tris-$MgCl_2$. This gave a final concentration of 10^{-3}M-ATP in the tests and 10^{-3}M-$MgCl_2$ in both tests and controls. The material was kept at room temperature for 15 min. and spun gently for 3 min. at 1,000 rpm. The supernatant was discarded. Tests and controls were treated for 30 min. with 0.05M-alkali buffer (chapter 4, series A). The fibrils were spun gently as before and the supernatant discarded. The fibrils were washed 6 times with 0.5 M-KCl, 3 times with water and 4 times with 25 ml acetone. They were allowed to dry in air. The phosphorus contents and radioactive count were estimated on the weighed powder as described in chapter 4 series A.

Results (Table 17)

When ATP is added to the suspension of fibrils the well-known increase in the opacity of the fibrillar suspension, corresponding to contraction of the fibrils, is observed. The fibrils show a reduction in the specific activity of the bound phosphate identical, within the limits of experimental error, to that found when frog recti are contracted with KCl.

SERIES B

The effect of ATP in the presence of Ca^{++} on the specific activity of the bound phosphate of ^{32}P-labelled psoas myofibrils

Method

The method was identical to that used in series A above, except the $MgCl_2$ was replaced by $CaCl_2$.

TABLE 17

Effect of ATP in the presence of Mg^{++} on the specific activity of the bound ^{32}P-labelled phosphate of glycerol-extracted psoas fibrils

Extracted fibres homogenised in 0.5 MKCl washed 6 times with same medium and 3 times with water. Suspension divided into 2 parts, tests taken to 10^{-3}M with respect to Mg^{++} and ATP in Tris-HCl pH 7.0 (0.05M) and controls taken to 10^{-3}M with respect to Mg^{++} in Tris-HCl. Subsequent treatment as in text.

Total phosphorus content acetone powder		Ratio of specific activities
Control	ATP-treated	
µg P/mg	µg P/mg	ATP-treated/Control
0.97	1.01	0.59
1.07	1.02	0.81
0.94	0.98	0.52
0.89	0.85	0.64
0.80	0.92	0.61
Mean ± S.D.		
0.93 ± 0.09	0.96 ± 0.06	0.64 ± 0.09

5 experiments

Results (Table 18)

No reduction in the specific activity of the bound phosphate was detected when the fibrils were treated with ATP in the presence of Ca^{++}.

SERIES C

The effect of ITP on the presence of Mg^{++} on the specific activity of the bound ^{32}P-labelled myofibrils

Method

Frogs were injected with about 100 µCi ^{32}P-orthophosphate. The frogs remained at room temperature overnight and were killed by decapitation the following morning. The leg muscles were excised and homogenised in 0.5 M-KCl. The myofibrils were washed with KCl, rehomogenised and filtered through gauze to remove any unhomogenised muscle and connective material. Subsequent treatment was as in series A except that ATP was replaced by ITP.

Results (Table 19)

No change in the opacity of the fibrillar suspension was noted when ITP was added. Treatment with ITP gives a reduction in specific activity of the bound phosphate similar to that found when psoas fibrils are treated with ATP.

SERIES D

The effect of ATP in the presence of Mg^{++} on the specific activity of the bound phosphate of ^{32}P-labelled psoas myofibrils which have been treated with DNP

Method

A suspension of myofibrils was prepared as in series A and

TABLE 18

Effect of ATP in the presence of Ca^{++} on the specific activity of the bound ^{32}P-labelled phosphate of glycerol-extracted psoas fibrils

Extracted fibres treated as in Table 17 except that Mg^{++} was replaced by Ca^{++}.

Total phosphorus content acetone powder		Ratio of specific activities
Control	ATP-treated	
µg P/mg	µg P/mg	ATP-treated/Control
0.90	0.87	0.96
0.88	0.85	1.37
0.85	0.78	0.96
0.92	0.89	1.03
1.17	1.08	0.91
0.72	0.76	1.19
0.88	0.94	0.98
0.84	0.83	1.41
0.91	1.01	0.88
0.79	0.88	1.01
0.91	1.15	1.09
Mean ± S.D.		
0.99 ± 0.11	0.91 ± 0.12	1.08 ± 0.17

11 experiments

TABLE 19

Effect of ITP in the presence of Mg^{++} on the specific activity of the bound phosphate of myofibrils prepared from ^{32}P-labelled frogs.

Myofibrils washed with KCl and water, test suspension treated with ITP and Mg^{++} (10^{-3} M) in Tris-HCl pH 7.0 controls treated with 10^{-3} M Mg^{++} in Tris-HCl. Subsequent treatment as described in text.

Total phosphorus content acetone powder		Ratio of specific activities
Control	ITP-treated	
µg P/mg	µg P/mg	ITP-treated/Control
1.86	2.07	0.55
1.64	1.81	0.34
1.66	1.92	0.42
1.72	1.86	0.45
1.64	1.77	0.68
1.44	1.52	0.48
2.00	2.25	0.42
2.22	2.18	0.68
2.05	2.02	0.60
1.95	1.80	0.84
Mean ± S.D.		
1.82 ± 0.22	1.92 ± 0.21	0.55 ± 0.15

10 experiments

diluted with an equal of Tris-HCl buffer containing 3 x 10^{-3}M-$MgCl_2$ and 2.5 x 10^{-3}M-DNP. The fibrils were left to stand at room temperature for 30 min. The suspension of myofibrils was divided into 2 parts and the volumes were measured. The test suspension was brought to 10^{-3}M with respect to ATP by adding 0.5 vol. 3 x 10^{-3}M-ATP. The final concentrations in the test suspension were: 10^{-3}M-Mg^{++} and ATP and 8 x 10^{-4}M-DNP. The control suspension was diluted with 0.5 vol. water, this gave a final concentration to the control suspension: 10^{-3}M-Mg^{++} and 8 x 10^{-4}M-DNP. All subsequent treatment as described in series A.

Results (Table 20)

No change in the opacity of the myofibril suspension was detected when the DNP-treated myofibrils were treated with ATP. After the washing procedure, no DNP appeared to have remained attached to the myofibrils. The nyofibrils treated in this way gave a similar reduction in specific activity to that shown by untreated myofibrils.

Summary of experimental findings

1. Myofibrils contracted with ATP in the presence of Mg^{++} show a reduction in specific activity of the bound phosphate identical to that found when frog recti are contracted with KCl.
2. No reduction in the specific activity of the bound phosphate is found when myofibrils are treated with ATP in the presence of Ca^{++}.

TABLE 20

Effect of ATP in the presence of Mg^{++} and DNP on the specific activity of the bound ^{32}P-labelled phosphate of glycerol-extracted psoas fibrils

Homogenised and extracted fibres washed with 0.5M KCl and water, then treated with 0.05M Tris-HCl buffer pH 7.0 containing 3×10^{-3} M $MgCl_2$ and 2.5×10^{-3} M DNP. Suspension was divided into 2-3 parts, test suspension treated with 0.5 vol. 3×10^{-3} M-ATP in Tris-HCl and controls with 0.5 vol. Tris-HCl buffer. Subsequent treatment as in text.

Total phosphorus content acetone powder		Ratio of specific activities
Control	ATP-treated	
µg P/mg	µg P/mg	ATP-treated/Control
0.63	0.88	0.77
0.86	0.87	0.28
0.72	0.88	0.49
0.87	0.93	0.61
0.92	0.93	0.56
0.96	0.95	1.00
0.93	0.81	0.61
0.92	0.95	0.56
Mean ± S.D.		
0.86 ± 0.11	0.90 ± 0.04	0.61 ± 0.25

8 experiments

3. Treatment of myofibrils with ITP in the presence of Mg^{++} gives the same reduction in specific activity found with ATP.

4. Treatment with DNP does not prevent the reduction in specific activity when the myofibrils are treated with ATP.

CHAPTER 7

The nature of the phosphate bound to frog skeletal muscle after exhaustive washing

Cheesman and Hilton (1961) in their preliminary experiments showed that the ^{32}P-labelled phosphate that remained bound to the muscle after exhaustive washing at $0^{\circ}C$ was not exchanged with inorganic phosphate in the medium.

The effect of contraction on the specific activity of the bound phosphate has hitherto been expressed only in terms of the total bound phosphate. Experiments have been carried out to establish the distribution of activity among the fractions of the bound phosphate.

The yield of acetone powder was established (chapter 4 series M). The weight of acetone powder represents about 9% of the wet weight of the muscle. This was used as a basis for further study.

Cheesman (1964) had shown that the acetone drying of the exhaustively washed muscle-residue approximately 2.0 µg P/mg dry powder. The amount of count removed by acetone showed a considerable variation but was about 1/6 or 1/10 of the total count. There was no evidence that a higher proportion of the count had been removed from either resting or stimulated muscles.

SERIES A

The degree of labelling of the lipid phosphorus in frog rectus abdominis

Method

Weighed labelled acetone powders were refluxed with chloroform-

methanol (1 : 1 v/v) for 15 min. The solvent was decanted and the powder dried in the flask. The powder was removed from the flask, whereafter the solvent was returned and evaporated to dryness. The phosphorus content of the residue was estimated by the method of Allen (1940). The solvent was evaporated to dryness and the lipid phosphorus was dissolved by heating with $HClO_4$. The solution was transferred quantitatively to a Kjeldahl flask and the digestion with $HClO_4$ was completed. The lipid phosphorus was estimated by the Allen method. The colormetric solutions were counted for radioactivity in a Panax liquid counting tube and the specific activities of the extract and residue were estimated.

Results (Table 21)

About 0.5 µg P/mg acetone powder was extracted from the muscle by chloroform-methanol. This phosphorus was only lightly labelled. On further extraction with chloroform-methanol-HCl (60 : 30 : 1) only a very small amount of the count was removed and virtually no additional phosphorus.

SERIES B

The amount of material extracted with TCA as inorganic phosphates from washed residues of frog rectus abdominis

Method

Rectus muscles were excised and weighed, test muscles were depolarised with isotonic KCl and the controls were placed in Ringer's solution. After mincing in their respective media, tests and controls were exhaustively washed by the method described previously (chapter 4

TABLE 21

Extraction of lipid phosphorus from acetone-dried residues of washed frog recti

Labelled muscles were minced, extracted and acetone-dried as described in chapter 4 series A. Powders were refluxed for 15 min. with $CH_3OH:CHCl_3$ (1:1 v/v) Subsequent treatment as described in the text.

Residue		Extract	
µg P/mg acetone powder	counts/min./µg P	µg P/mg acetone powder	counts/min./µg P
1.17	104	0.49	9
1.45	99	0.50	9
1.10	105	0.56	13
1.37	13	0.52	< 1
1.47	10	0.49	< 1
1.71	6	0.43	< 1
1.29	8	0.50	< 1
1.62	6	0.53	< 1
1.37	164	0.54	14
Mean ± S.D.			
1.39 ± 0.18		0.51 ± 0.01	

9 experiments

series A).

The minces were homogenised in 4 ml 5% TCA and filtered through Whatman No.1 paper. The residues were washed twice with 0.5 ml TCA and the washing were combined with the extract.

Inorganic phosphorus was estimated by the method of Berenblum and Chain (1938). Results were calculated as μg Pi/mg acetone powder, the acetone powder being 9% of the wet weight of the muscle.

Results (Table 22)

Homogenisation of the muscle residues in TCA removes about 13% of the phosphorus in the form of inorganic phosphate. There is no significant difference in the amount of phosphorus extracted from contracted or relaxed muscle.

SERIES C

The amount of radioactivity extracted with TCA from the washed residues of frog rectus abdominis

Method

Rectus muscles were labelled with ^{32}P-orthophosphate; test muscles were depolarised with isotonic KCl. After mincing and washing exhaustively the residues were homogenised in 5 ml 5% TCA. The homogenates were filtered and the residues washed with 2 x 2 ml TCA; the washings were combined with the filtrate. The residues were digested in $HClO_4$ and were then diluted to 12.5 ml. The filtrate was diluted to the same volume and the solutions were counted in a Panax liquid counter of 12.5 ml capacity. The proportion of count in the extract and the residue was estimated.

TABLE 22

Inorganic phosphate extracted by TCA from washed residues in frog recti

Test muscles were depolarised with isotonic KCl. Subsequent treatment as described in the text. Results were calculated on the basis that 1g muscle yields 90 mg acetone powder.

Relaxed muscle	Depolarised muscle
µg P/mg acetone powder	µg P/mg acetone powder
0.23	0.21
0.22	0.37
0.25	(0.79)
0.30	0.23
0.25	0.25
0.22	(0.67)
0.27	0.27
0.32	0.37
0.26	0.39
0.36	0.41
0.12	0.12
0.16	0.10
0.11	0.23
0.19	0.17
0.25	0.22
0.17	0.19
0.33	0.27

Cont./over

0.34	0.26
0.11	0.13
0.18	0.43
0.22	0.29

Mean ± S.D.

0.23 ± 0.07	0.26 ± 0.09
Relaxed 21 experiments	Depolarised 19 experiments

Results (Table 23)

About 80% of the count is removed from the muscle residue of a relaxed muscle and about 72% from the contracted muscle. Contraction appears to reduce the quantity of material yielding ^{32}P-orthophosphate on extraction with TCA.

SERIES D

The relation between the extraction of ATPase activity and the radioactivity from washed residues of ^{32}P-labelled frog recti

Method

The washed labelled muscle residues were extracted with 0.5 ml NaCl at 0°C. Aliquots were removed at 10 min. intervals and incubated with a solution which contained 10^{-3}M-ATP, 10^{-3}M-$CaCl_2$ and 0.05M-glycine-NaOH pH 9.0 for 15 min. at 25°C. The incubation was stopped by the addition of 5% TCA. The inorganic phosphate in the supernatant was estimated by the method of Allen (1940). Radioactivity was counted on the colorimetric solutions in a Panax liquid counter.

Results (Figure 1)

Inorganic phosphate and myosin ATPase were extracted from muscle residues in approximately proportional amounts.

SERIES E

Attempted exchange of labelling between ^{32}P bound to washed frog recti and possible phosphate acceptors

Method

Labelled muscles were washed. Some preparations were dried

TABLE 23

The amount of radioactivity extracted with TCA from washed residues of frog recti

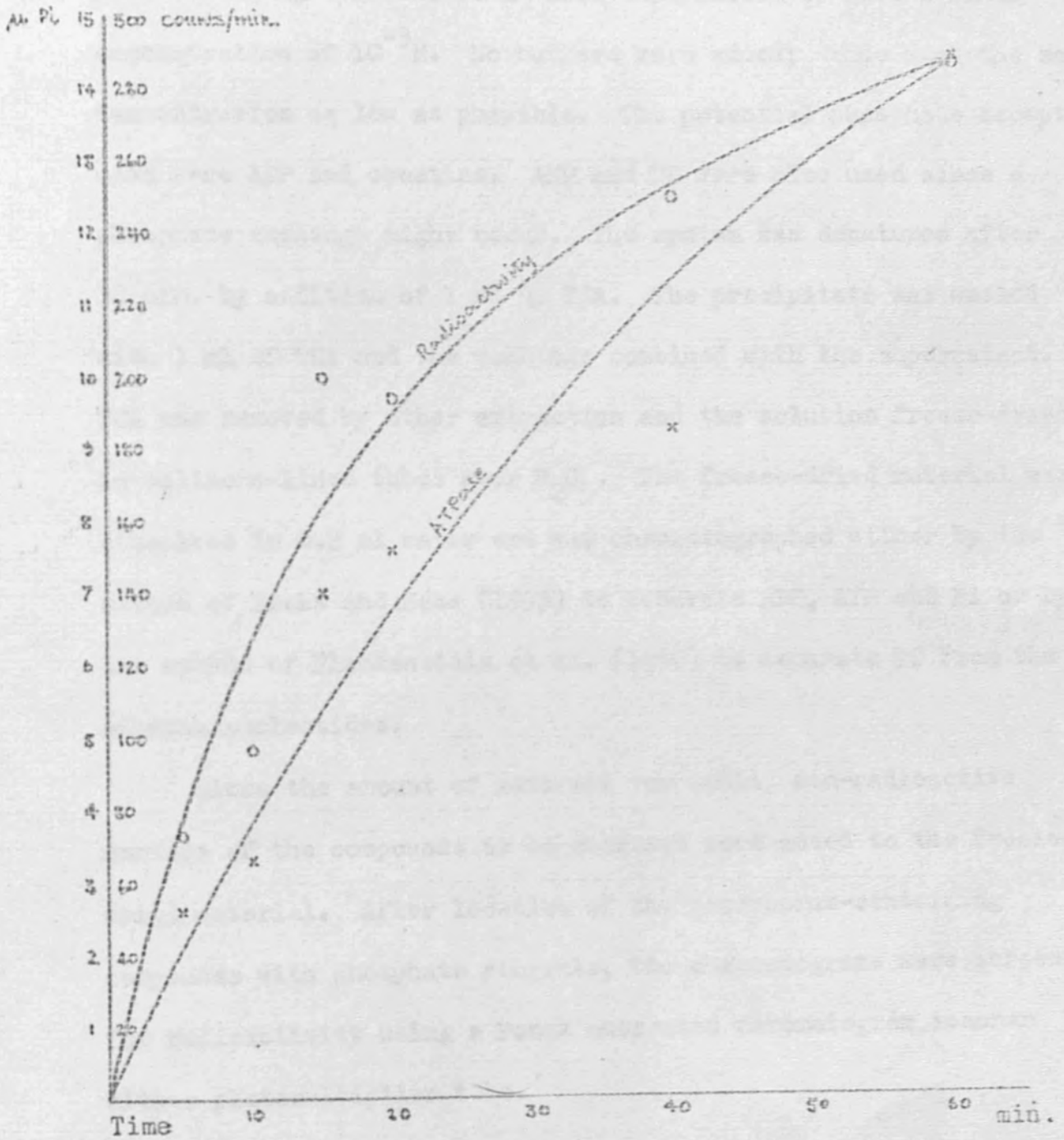
Rectus muscles were labelled with ^{32}P-orthophosphate; tests were depolarised with KCl. After washing residues were homogenised in TCA. Subsequent treatment as described in text.

Relaxed muscle			Contracted muscle		
counts per min. Extract	counts per min. Residue	% count extracted	counts per min. Extract	counts per min. Residue	% count extracted
2679	860	75.7	1855	605	75.4
2856	809	78.0	2004	700	74.1
2025	500	80.2	1888	497	79.1
800	121	86.9	564	248	69.4
1094	141	88.5	750	98	88.3
788	318	71.2	511	345	59.6
862	206	80.7	1064	400	72.7
1094	258	81.0	1581	643	68.0
1086	380	74.0	1205	345	77.3
Mean ± S.D.					
79.5 ± 5.2			72.5 ± 7.6		

9 experiments

Figure 1

The relationship between the extraction of ATPase activity and radioactivity from washed residues of ^{32}P-labelled recti



with acetone; others were used wet. The preparations (approximately 50-100 mg dry weight) were incubated with 200 µg phosphate acceptor in a total incubation volume of 2 ml. The pH was adjusted to neutral and Mg^{++} was added in some experiments to give a final concentration of 10^{-3}M. No buffers were added; this kept the salt concentration as low as possible. The potential phosphate acceptors used were ADP and creatine. ATP and PC were also used since a phosphate exchange might occur. The system was denatured after 30 min. by addition of 1 ml 5% TCA. The precipitate was washed with 1 ml of TCA and the washings combined with the supernatent. TCA was removed by ether extraction and the solution freeze-dried in silicone-lined tubes over P_2O_5. The freeze-dried material was dissolved in 0.2 ml water and was chromatographed either by the method of Krebs and Hems (1953) to separate ADP, ATP and Pi or by the method of Fleckenstein et al. (1960) to separate PC from the adenine nucleotides.

Since the amount of material was small, non-radioactive markers of the compounds to be detected were added to the freeze-dried material. After location of the phosphorus-containing compounds with phosphate reagents, the chromatograms were screened for radioactivity using a Panax automatic chromatogram scanner with a photomultiplier tube.

Results

No transfer of phosphate was detected in any experiments. The only radioactive material located on chromatograms was inorganic

phosphate. In some of the early experiments it was thought that some labelling of ATP had occurred but since later experiments failed to confirm this it seems probable that trailing of the inorganic phosphate had contaminated the ATP.

Summary of experimental findings

1. No significant part of the count is removed from the muscle residues by lipid solvents although some 30% of the total phosphorus is extracted with chloroform-methanol.
2. The material which is extracted by TCA as inorganic phosphate represents about 13% of the total bound phosphate.
3. About 80% of the count is removed from the washed residues of relaxed muscle and about 72% from contracted muscle.
   Hence it appears that contraction may reduce the quantity of material yielding ^{32}P-orthophosphate on extraction with TCA.
4. There was complete failure to extract the variable phosphate in a form other than inorganic phosphate.
5. Under the conditions employed in these experiments, no transfer of labelled phosphate to a number of possible acceptors was achieved.

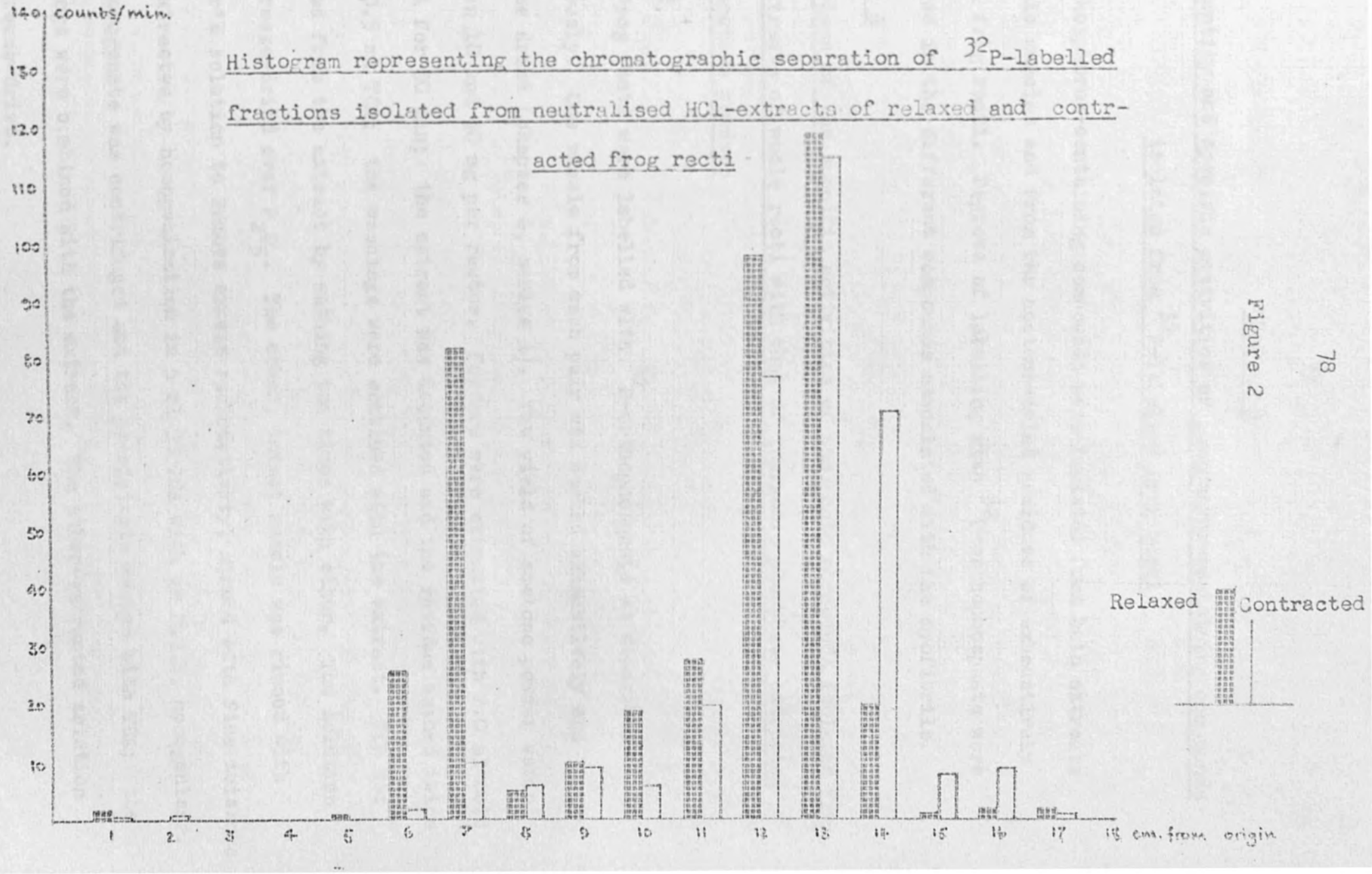


Figure 2

Histogram representing the chromatographic separation of ^{32}P-labelled fractions isolated from neutralised HCl-extracts of relaxed and contracted frog recti

CHAPTER 8

The identity and specific activities of phosphorus-containing compounds isolated from ^{32}P-labelled frog recti

Phosphorus-containing compounds were isolated from both extracts of whole muscles and from the acetone-dried residues of exhaustively washed frog recti. Degrees of labelling with ^{32}P-orthophosphate were compared in the different compounds associated with the myofibrils.

SERIES A

Comparison of the specific activities of phosphate compounds isolated from TCA extracts of whole recti with that of inorganic phosphate extracted from acetone powders

Method

Frog recti were labelled with ^{32}P-orthophosphate as described previously. One muscle from each pair was washed exhaustively and acetone dried (chapter 4, series A). The yield of acetone powder varied between 10 and 30 mg per rectus. Powders were extracted with 2.0 ml cold 5% TCA for 30 min; the extract was decanted and the residue washed twice with 0.5 ml TCA; the washings were combined with the extract. TCA was removed from the extract by washing ten times with ether. The solution was freeze-dried over P_2O_5. The other, intact muscle was rinsed with Ringer's solution to remove excess radioactivity, minced with fine scissors and extracted by homogenisation in 5 ml 5% TCA with an M.S.E. homogeniser. The homogenate was centrifuged and the precipitate washed with TCA; the washings were combined with the extract. The ether-extracted solution was freeze-dried.

The phosphate compounds were separated on Whatman No.1 paper using the method of Fleckenstein et al. (1960) for PC, ADP and ATP and the method of Krebs and Hems (1953) to isolate the inorganic phosphate.

After detection of the compounds (chapter 3) the spots were cut from the chromatograms as standard size discs to fit 17 mm planchettes. The radioactivity was counted in a Panax castle with an end window tube. After counting the samples were digested with H_2SO_4 and the phosphorus content estimated by the method of Berenblum and Chain (1938). Phosphorus content was converted to µg ATP or PC and the specific activity of the total inorganic phosphate, PC and ATP-γP and of the bound inorganic phosphate were calculated.

Results (Table 24)

Some labelled material from the whole muscle extract remained at the origin, migrating in neither solvent system. No attempt was made to identify this material since it represented only a small fraction of the count. No count was extracted by the ether.

The estimations of specific activity show a very considerable variation. The results are expressed in terms of the ratios of specific actviities between ATP-γP (100) and the other phosphate compounds. The results calculated on the assumption that 85% count in ATP is associated with the γP (cf Fleckenstein and Janke 1957). In all cases the labelling of the PC is lower than that of the ATP-γP (mean value 62.5 ± 0.16 for ATP-γP = 100). This agrees with the findings of Fleckenstein and Janke (1957), who with a much larger group of experiments

TABLE 24a

The relative specific activities of PC, ATP-γP and Inorganic phosphate isolated from TCA extracts of whole ^{32}P-labelled frog recti

Muscles were labelled with ^{32}P-orthophosphate for 60 min. Muscles were homogenised immediately in TCA. The subsequent treatment as described in the text.

Whole muscles

ATP-γP	PC	Inorganic phosphate
counts/min./ µg P	counts/min./ µg P	counts/min./ µg P
100	86.4	140
100	61.8	40
100	49.4	85
100	54.8	1000
100	51.0	1100
100	75.5	4600
100	54.4	3145
100	88.5	39000
100	94.0	760
100	67.4	1910

Mean ± S.D. for PC 0.65 ± 0.16

TABLE 24b

The relative specific activities of ATP-γP isolated from whole TCA extracts of ^{32}P-labelled frog recti and inorganic phosphate from acetone powders of washed residues of ^{32}P-labelled recti

Muscles were labelled with ^{32}P-orthophosphate for 60 min. One muscle was homogenised immediately in TCA; the other was washed as described previously, converted to an acetone powder and extracted with TCA. Subsequent treatment as described in the text.

Whole muscle	Acetone powder
ATP-γP	Pi
counts/min./ μg P	counts/min./ μg P
100	135
100	401
100	452
100	154
100	153

Ratio of specific activities of ATP-γP and Pi $\pm$ S.D. 2.5 ± 1.4

found a ratio of 60 : 100.

The labelling of inorganic phosphate from whole muscles show wide discrepancies, probably caused by variations in washing with Ringer's solution before homogenising the muscle.

Only a few values were obtained for the labelling of P released as inorganic phosphate from the washed muscle residue and again these vary widely. The degree of labelling in this fraction is in general higher, but of the same order as that found in ATP-γP.

SERIES B

Isolation and identification of phosphate compounds extracted by aqueous solutions from acetone powders

Method

Acetone powders of contracted and relaxed labelled frog recti prepared as described previously. The powders (10-30 mg) were extr with 2.0 ml ice-cold solutions for 30 mins. The solutions used wer

1. 5% TCA
2. 6% $HClO_4$
3. deionised water
4. 0.2 N-HCl

The extracts were decanted and the residues were washed twice a further 0.5 ml solution. The washings were combined with the ext

Solutions containing $HClO_4$ were neutralised with ice-cold KO care was taken to prevent heating. The neutralised solution was l at 0°C for 30 min. to give maximum precipitation of $KClO_4$. The so was centrifuged and the supernatant decanted. The precipitate was with 1 ml water and the washings were combined with the extract.

The water extracts were treated in three ways. Group 1 was freeze-dried without further treatment. Group 2 was treated with 5% TCA to remove the protein and Group 3 was taken to 10^{-3}M with respect to $MgCl_2$. The Mg^{++} treated solution was allowed to stand at room temperature (22°C). for 15 min. to polymerise G-actin in solution. The extract was then deproteinised with TCA which also extracts the bound nucleotide. TCA was removed from all solutions by ether extraction.

HCl extracts were neutralised with ice-cold LiOH and the LiCl removed by ether extraction repeated 15 times. The extracted passed through a short column containing Zeocarb.

All solutions were freeze-dried in silicone-treated tubes over P_2O_5. The freeze-dried material was dissolved in the minimum volume of water (0.2 ml) and chromatographed on Whatman No.1 paper using the two-stage separation of inorganic phosphate and adenine nucleotides of Eggleston and Hems (1952). The chromatograms were screened for radio-activity using a Panax automatic chromatogram scanner.

Phosphorus-containing compounds were located by means of a Panax automatic scanner, conversion to molybdenum blue or in the case of nucleotides, by means of a UV lamp with emission at 247 mu.

Results

In all experiments, except some where HCl was used, only two phosphorus-containing compounds were detected, inorganic phosphate and ADP. Only inorganic phosphate was significantly labelled.

When acetone powders were extracted with HCl a small amount of acid-soluble protein was extracted, this was subsequently precipitated

by neutralisation and ether extraction. No count was retained by the Zeocarb.

The chromatograms of HCl-extracts showed a single unlabelled nucleotide spot indentified as ADP. In addition in some experiments where control and contracted muscles were compared, a second radioactive spot was detected which was not visible in UV light. This spot was separated from inorganic phosphate in the ether-formic acid system (figure 2 on page 78). In this system the inorganic phosphate travels close to the solvent front. The second compound, which was rarely observed with the contracted muscles, had an R_f value approximately two-thirds that of inorganic phosphate.

The amount of count in the second peak in these experiments is sufficient to account for the difference in count between acetone powders of relaxed and contracted muscles.

This unidentified compound could not be distinguished as a separate spot by staining with phosphate reagents but was detected by scanning the chromatogram for radioactivity.

Summary of Experimental Findings

1. Comparison of the specific activities of P in PC and ATP-γP isolated from TCA extracts of ^{32}P-labelled whole muscles show a ratio of 0.65 ± 0.16.
2. The specific activity of the P isolated from acid extracts of acetone powders of washed muscle residues was of the same order as that found in ATP-γP.

3. In all experiments, except some where HCl was used, only two phosphorus-containing compounds were isolated from acetone powders of washed muscle residues, inorganic phosphate and ADP.

4. The ADP was not labelled with ^{32}P.

CHAPTER 9

Discussion of Results

At an early stage in their investigations Cheesman and Hilton (1961) showed that the bound ^{32}P-labelled phosphate of the washed muscle residue was not exchangeable with inorganic phosphate. This suggests that the binding of the ^{32}P-orthophosphate is in the form of a stable (though not necessarily covalent) linkage and not merely absorbed by the myofibrils. The failure to remove ^{32}P-labelled phosphate quantitatively with EGTA tends to support this view, particularly since washing with EGTA slightly enhances the KCl effect (Table 12). This enhancing of the effect could be brought about by the removal of a small amount of accluded $Ca_3(PO_4)_2$ which raised the specific activity of the total bound phosphate in both tests and controls. The amount of material involved must be small since the EGTA-wash does not appreciably alter the total bound phosphate content of the acetone powder (Table 12: 1.79 ± 0.27 ug P/mg as compared with 1.84 ± 0.32 ug P/mg acetone powder). If the calcium phosphate were highly labelled, its removal would enhance the KCl effect.

Dr. R.E.Davies suggested (1967) that the KCl effect was caused by the adsorption of calcium phosphate. He pointed out that no Ca^{++} was included in the isotonic KCl and that he was able to bring about a reversal of the KCl effect by adding Ca^{++} to the depolarised medium. It is clear from those experiments in which Dr. Davies'

method was held that a concentration of Ca^{++} much greater than that found in Ringer's solution will increase the specific activity of the bound phosphate (Table 9). However, this treatment also causes the precipitate of calcium phosphate in the muscle residue and the phosphorus contents of muscle powders treated in this way are greatly increased (3.23 ± 0.72 µg P/mg as against 1.97 ± 0.15 µg P/mg for normal controls). When the KCl contained the amount of Ca^{++} that is found in Ringer's solution, no phosphate binding occurred but the KCl effect in a short series of experiments appeared to be enhanced (Table 10). The ratio of the specific activities of contracted and control muscle was decreased to 0.48 ± 0.19 as against 0.64 ± 0.14. The phosphorus contents of the acetone powders were normal (1.75 ± 0.23 and 1.79 ± 0.27 µg P/mg respectively).

Experiments were, in fact, carried out on frog sartorius in which test and control muscles remained in identical media throughout the experimental procedure. These experiments showed the increase in specific activity after relaxation as was found when frog recti were depolarised in KCl and relaxed in Ringer's solution. The ratios of the specific activities of contracted and relaxed muscles were (0.60 ± 0.22 for rectus abdominis and 0.59 ± 0.14 for sartorius Table 1 and Table 14).

The reduction in specific activity of the bound phosphate is found in both loaded and unloaded contractures (Table 1). The change in specific activity occurs even in the absence of Ca^{++} where

depolarisation of the membranes takes place but is not followed by contraction (Niedergerke 1956). The experiments on KCl-induced isometric contractures gave similar results to the isotonic experiments (Table 3). These findings taken in conjunction, lend support to the view that the change in specific activity is not related to the shortening of, or to development of tension in, the muscle.

Very brief (30 sec.) labelling of the muscle before contraction gave a similar change in the ratio of specific activities in contracted and control muscles, although the absolute activities were lower than in experiments with longer labelling times (Table 6). This suggests that the contracture effect is not due to changes in a phosphate fraction readily accessible to the medium.

At all times the muscles were labelled when lightly loaded, except in some experiments with myofibrils from frog muscle which was labelled when unloaded. These experiments did not give anomalous results.

Fleckenstein et al. (1954) have shown that PC breakdown in frog muscles is much reduced at 0°C. In our experiments, contraction of frog recti at 0°C gave a similar decrease in specific activity to that found at 20°C. At an early stage in the present investigation it was thought possible that the reduction on specific activity was caused by an exchange of bound phosphate with a pool of low specific activity during contraction. Fleckenstein and his coworkers (1957)

have shown that ATP-^{32}P and the P of PC are not equally labelled in whole muscle extracts but the failure to detect PC breakdown at low temperatures does tend to discount a theory of renewal of the bound phosphate fraction from PC during contraction.

Contraction followed by repolarisation, relaxation and a second contraction gives the same change in the specific activity of the ^{32}P-labelled bound phosphate that is found with a single contraction. Relaxation may hence cause a rephosphorylation of some compound from the same highly labelled pool from which the initial labelling occurred. If rephosphorylation took place from a less highly labelled pool, the KCl effect would be enhanced by a second contracture

The relaxation effect was established by Cheesman and Hilton (1966). Although no experiments have been carried out to compare a control muscle with a contracted and relaxed muscle, it is clear that relaxation of a repolarised muscle restores the specific activity of the bound phosphate to its resting level. Further experiments have established that repolarisation without relaxation does not restore the specific activity to its original level but relaxation without repolarisation (experiments on frog sartorius relaxed under load, Table 14) does bring about an increase in specific activity. These results suggest that depolarisation initiates contraction and the decrease in the specific activity but that repolarisation in itself does not cause the restoration in count. The contraction effect is not related to the muscle

shortening (experiments with Ca^{++} free media, Table 4) but the increase in specific activity is clearly concomitant with the lengthening, i.e. related to the mechanical extension of the muscle, so that it is only when the muscle relaxes that a "rephosphorylation" occurs. It is significant that severe mechanical extension of resting muscle does not bring about an increased binding of ^{32}P (Table 16) which suggests that such uptake only occurs during relaxation of contracted muscles.

The contraction of rabbit psoas fibrils with ATP in the presence of Mg^{++} reduces the specific activity of the bound phosphate by an amount which is very similar to that found after potassium contracture of frog recti (Table 17). When Mg^{++} is replaced by Ca^{++} in similar experiments, no reduction in count occurs.(Table 18). Mg^{++} is required for the performance of work by the myofibrils in the presence of ATP (Weber and Portzehl 1952), even though Ca^{++} activates the actomyosin-ATPase

The difference in the effect of ATP in the presence of Mg^{++} and Ca^{++} tends to discount the possibility that the decrease in specific activity is caused by a mass effect independent of the cations presents.

Experiments with DNP-treated myofibrils (Table 20) give different results from those obtained by Cheesman and Hilton (1966) for frog recti. These muscles, when treated with 5×10^{-4}M-DNP and subsequently contracted with KCl, showed an increase in the

specific activity of the bound phosphate. DNP-treated myofibrils contracted with ATP and Mg^{++} show the same decrease in specific activity as the untreated fibrils. The two experiments are of quite different types. The treatment of the intact muscle with DNP will result in the uncoupling of oxidating phosphorylation, (cf Lardy and Elvehjem 1955), this may well cause a change in the relative labelling of the various phosphate pools in the muscle. Fleckenstein and Janke (1957) have shown that these pools are not in equilibrium. The increased labelling of the pool with which the bound phosphate exchanges during contraction would result in a raised specific activity of the total bound phosphate. Since DNP presumably increases the rate of turnover of a reduced high-energy phosphate pool, it is understandable that anomalous results were obtained by the experiments of Cheesman and Hilton, since the reproducibility of the contracture effect in the experiments with unpoisoned muscles may well be due to the relative constancy in the labelling of the high-energy phosphate pool during contracture.

The myofibrillar preparation contracted with ATP is not strictly analogous to a relaxed muscle contracted with KCl since in the absence of ATP the fibrils will be rigor. However, the introduction of ATP to the system may result in a state of the fibrils corresponding to that in relaxed muscle. The subsequent contraction could, under these circumstances, have an effect on the bound phosphate quite analogous to that found in the intact tissue.

The ITP used for these experiments was prepared by Mrs.A.Priston by the method of Kaplan (1953). When experiments were carried out on loaded glycerated psoas fibre bundles by Mrs. Priston no contraction of the fibres was brought about by this preparation, or by a commercial preparation by Sigma, in the presence of Mg^{++}.

The repeated failure in this laboratory to reproduce the contraction of glycerated-fibre bundles and myofibrils with ITP and Mg^{++} as observed by other workers (Hasselbach 1956) is difficult to explain.

A nucleotide diphosphokinase catalyses the conversion of ADP to ATP:

$$ADP + ITP \rightleftharpoons ATP + IDP$$

The synthesis of ATP may be responsible for the contraction found by other workers. It is possible that some synthesis of ATP may occur by the reaction between the bound nucleotide of the glycerated-myofibrils and ITP, catalysed by an enzyme not inactivated by prolonged treatment with glycerol.

It seems reasonable in general to relate the decrease in specific activity of myofibrils treated with ATP to the KCl effect in frog recti. and both phenomena to possible events in living muscle.

There has been failure to isolate a significant part of the ^{32}P-labelled phosphate from muscle residues in a form other than inorganic phosphate. The only exception to this was the erratic appearance of an unidentified phosphorus-containing material on

chromatograms of neutralised HCl-extracts of acetone powders (chapter 8, series B). This substance was probably an artefact but since it appeared principally on chromatograms of relaxed muscle and only in reduced amounts on those of contracted muscle, it may indicate a potential for phosphate transfer on the part of some fraction of the bound phosphate. Rodnight (1966) found that under certain conditions a similar compound present in brain tissue its phosphate group to methanol. It is possible that some impurity present in the acetone used to prepare the muscle powders might be capable of phosphate acceptance. The ^{32}P-orthophosphate used in these experiments was hydrolysed before use, this removed any of the polyphosphate compounds which Causey and Harris (1951) have reported as being present in preparations of ^{32}P and which might appear on chromatograms.

Attempts were made to isolate and identify the second phosphate fraction found in the HCl-extracts of acetone powders. Although much time was devoted to this, the compound eluded isolation. Column chromatography of the extract on Dowex-1 chloride following the method of Cohn and Carter (1951) and on Norite-K charcoal was carried out but no labelled phosphate fraction other than inorganic phosphate was obtained. A very small amount of labelled material (about 1% of the total labelling) was adsorbed by charcoal, subsequent experiments using ^{14}C-ADP suggested that his might have been bound labelled ADP. Although the unknown compound was not identified it was possible to exclude its being PC, phosphoarginine,

inorganic pyrophosphate or metaphosphate by its chromatographic behaviour.

The compound could be analogous to the one reported by Rodnight (1966) in brain tissues. Since the compound was rarely found in contracted muscle, and then always in a much reduced amount, it would be tempting to suppose that it, or its precursor, was related to the process of contracture.

Extraction of muscle powders with boiling chloroform-methanol (1 : 1 v/v) removes about 30% of the bound phosphate which is only slightly labelled (Table 21). Further extraction with chloroform-methanol-HCl (66 : 33 : 1 v/v) failed to remove a substantial part of the count.

There is no significant difference between the total phosphorus contents of the washed residues of resting and contracted muscles (Table 1: 1.94 ± 0.32 and 1.84 ± 0.37 µg P/mg acetone powder).

The dry weights of the acetone-dried residues of exhaustively washed resting and contracted muscles are similar (Table 13: 8.9 ± 1.9 and 9.0 ± 1.5% of the wet weights respectively). When the washed residues were extracted with 5% TCA, about 13% of the phosphorus was extracted as inorganic phosphate (Table 22: 0.24 ± 0.08 and 0.24 ± 0.09 µg P/mg acetone powder for resting and contracted muscles). Some 80% of the ^{32}P is removed by this treatment from resting muscles and about 72% from contracted muscles (Table 23). Contraction hence appears to reduce the quantity of material yielding ^{32}P-orthophosphate on extraction with TCA.

From experimental findings it is not immediately evident whether the contraction of the muscle brings about an exchange of phosphate with a pool of low specific activity or the breakdown and elimination of a phosphorus-containing substance. However the evidence tends to support the latter alternative since:

1. The effect is reproducible. If contraction involved an exchange with a lowly labelled pool, the ratio of the labelling of the different pools must be constant in different experiments. This seems unlikely since the animals used were highly differing metabolic states; some were freshly caught, while others had been stored at $4^{o}C$ for several weeks.

2. The ratio of labelling for untreated rabbit psoas myofibrils and those contracted with ATP was similar to that for frog muscles in contracture experiments. This also, suggests, in view of the absence of labelled pools for exchange in the former case, that the change is caused by breakdown rather than exchange.

3. In experiments where a resting muscle was compared with a muscle that had been contracted, relaxed and recontracted, the ratio remained constant after a second contraction (Table 7), a finding difficult to reconcile with exchange unless the pools are large.

The bound phosphate does not transfer groups to a number of possible phosphate acceptors under the experimental conditions employed (chapter 8). The only exception to this was the appearance of a labelled phosphate-containing substance on chromatograms of

neutralised HCl-extracts of acetone powders from resting muscles, which has been discussed previously.

When the specific activities of the material extracted as inorganic phosphate by the method of Fleckenstein et al. (1960) was compared with the ATP-γP in whole muscle, the specific activity of the Pi was greater by a factor of 2.5 $\pm$ 1.4 (Table 24) in 5 experiments. This was calculated on the assumption that 85% of the labelling of the ATP was in the ATP-γP (Fleckenstein et al. 1957). If contraction involves a decrease in bound phosphate, smaller than the standard error of the phosphate determinations, i.e. a decrease of less than 0.2 ug P with respect to the total phosphorus, the decrease of 40% in the specific activity must represent the breakdown of a phosphate fraction with a specific activity at least 5 times greater than that of the total ATP-γP (Cheesman et al. 1969).

Extraction of wet muscle residue with 0.5 M-NaCl releases ^{32}P (as orthophosphate) and actomyosin-ATPase in approximately proportional amounts (chapter 7, series D, figure 1). Treatment with NaCl to extract the actomyosin may simply release occluded inorganic phosphate which has become more accessible after extraction of the protein. Alternatively the active phosphate may be associated with one of the structural proteins, in such a way that treatment with TCA brings about liberation of ^{32}P as inorganic phosphate.

The material extracted as inorganic phosphate, determined by the method of Berenblum and Chain (1938) showed considerable variation (Table 22). The values, expressed in terms of dry weight of muscle (1g muscle gives 90 ± 15 mg acetone powder: Table 13) were 0.24 ± 0.08 and 0.24 ± 0.09 µg P per mg for resting and contracted muscle. The variation in these figures is still sufficiently high to hide a breakdown of some phosphate compound.

Investigation by Tonomura and his colleagues (1965) of a phosphorylated myosin as an intermediate to myosin-ATPase activity and the earlier work of Levy and Koshland (1958) and Levy, Ryan, Springhorn and Koshland (1963) makes it tempting to identify a phosphorylated myosin with that material which breaks down to form inorganic phosphate in aqueous media.

The attempts to isolate a phosphorylated myosin have not been completely successful, although Burton and Lowenstein (1964) claim to have isolated ATP-HMM on Sephadex. These workers were able to show that this complex was labile in vitro. Such a sompound might be stabilized in vivo and be normal intermediate in the contraction process.

If a phosphorylated myosin existed as the normal intermediate in contraction, it seems possible that, in the relaxed state, phosphorylation of the actin-binding sites could prevent actin-myosin interactions. Bailey and Perry (1947) and Poglazov, Bilushi and Baev (1958) produced evidence that the same -SH groups of myosin

participate in the ATPase activity and in the reaction of myosin with actin. Levy and Ryan (1966) have shown that the ATP-binding sites on myosin are distinct from hydrolytic and actin-binding sites. Perry and Cotterill (1964) using phenyl - mercuric acetate have selectively inhibited the ATPase activity of HMM; the actin-binding sites remain active. Similar findings by Barany and Barany (1959), Barany et al. (1963) and Kaldor et al. (1964) tend to confirm that the ATPase and actin-binding sites are distinct in myosin. However, it seems probable that the steric effects of ATP-binding or phosphorylation could prevent interactions between the two proteins.

Excitation of the muscle and associated changes in ionic conditions could bring about dephosphorylation of the myosin, so initiating the formation of cross linkages between thin and thick filaments which is thought to constitute the actual contraction process (Huxley 1960). This might occur without apparent breakdown of ATP, which does not seem to have been detected in a single twitch. Infante and Davies (1962) have shown ATP breakdown in a "single contraction" of frog sartorius, which was, in fact, a short tetanus.

The decrease in specific activity of the bound phosphate on contraction is about 40% with respect to the resting control. If, however, the decrease is related to the readily extracted activity, i.e. that activity which is extracted with TCA (Table 23), the decrease would represent a 50% change. This could mean that muscles contracted have about 50% of their active sites on the myosin bridges engaged in

linkages with actin.

The breakage of the cross linkages formed on contraction is not caused by repolarisation but by the extension of the muscle. Buchthal and Kaiser (1951) have shown that in frog semitendinosus muscle, removal of excitation does not initiate the breaking of crosslinkages. Failure to increase the specific activity of the bound phosphate by severe mechanical extension of resting muscles supports the view that no actin-myosin interactions occur in the resting state. In experiments on frog sartorius (Table 14) no repolarisation occurs, but the specific activity returns to its resting level. In those experiments where repolarisation was not accompanied by mechanical extension of the muscle (Table 15) no restoration of the count was observed. These two series of experiments tend to strengthen the view that rephosphorylation only occurs during the relaxation process.

It has been shown by A.V.Hill (1958) that the activation heat for single twitches of frog skeletal muscle is about 1 mcal per gram of muscle. R.E.Davies (1967) although demonstrating ATP breakdown in a short tetanus, has not been able to show any chemical change coincident with the activation heat.

The turnover of bound phosphate has not been studied during a single twitch, but only after KCl-induced contracture which seems to be analogous to a tetanus. Experiments where contracture, but not depolarisation, was inhibited by the use of Ca^{++}-free Ringer's solution have shown that the decrease in the specific activity of the bound phosphate is associated with the activation process rather than the shortening.

It has been calculated by Cheesman et al. (1969) that, if the change in enthalpy accompanying the hydrolysis of a phosphorylated myosin is similar to that for ATP (say 10 Cal./mole) the dephosphorylation of one site for each myosin molecule would give a heat output of 1 mcal/g, corresponding to A.V. Hill's (1958) activation heat for single twitches of frog skeletal muscle. (Muscle is here assumed to contain 5% myosin of mol.wt. 500,000).

The requirements which must be satisfied by a viable theory of muscular contraction were formulated by A.V.Hill in 1950. These were based on the observations of Hill on the liberation of heat from muscle during the various phases of the contraction and relaxation cycle.

Heat production during contraction may be divided into 3 distinct parts: activation heat, which begins before the onset of the tension in the muscle, shortening heat, which is proportional to the amount of shortening, and the work heat, which only appears if work is done by the muscle. No heat is liberated during relaxation, except that equivalent to work done on the muscle during this process.

If a phosphorylated myosin is a normal "high energy" intermediate in muscular contraction it could satisfy all the requirements as an immediate energy source and fulfill the conditions laid down by Hill for such a compound (1950).

Niedergerke (1956) demonstrated that, in the absence of Ca^{++} depolarisation of the membranes occurs but is not accompanied by contraction. From the present investigation it has been indicated that some phosphate compound is broken down without actual contraction of the muscle (Table 4). The experiments on frog muscle in Ca^{++}-free

Ringer's solution show the same decrease in specific activity of the bound phosphate on depolarisation that occurs when the muscles contract (cf Table 1 and Table 4). These results tend to indicate that the changes in specific activity are associated with the activation process rather than with the shortening. The breakdown of some phosphate-containing compound during the activation of the muscle could contribute to the activation heat recorded by A.V.Hill (1958).

Fenn (1924) was the first to show that there was no heat production during the relaxation of an unloaded muscle. Under conditions of constant entropy, the rephosphorylation of the protein during relaxation would only bring about liberation of heat if the enthalpy changes associated with hydrolysis of a phosphorylated protein was lower than that of ATP.

The final requirements for any theory of muscular contraction is that there can be no endothermic processes, unless they are balanced or overbalanced by exothermic reactions. The formation of a phosphorylated myosin as a normal intermediate in contraction need not involve any endothermic reactions.

If a phosphorylated myosin exists in resting muscle an an energy source for a limited amount of contractile activity, it is possible to explain the failure of many workers to detect the breakdown of ATP during a single twitch.

Hydrolysis of a phosphorylated protein as the primary event in contraction could reconcile some of the apparent discrepancies and conflicting results hitherto published. Fleckenstein and his coworkers

(1954) were able to show an increase in Pi in stimulated muscles but no corresponding decrease in ATP or PC. Mommaerts (1955) has shown increases in Pi in leg muscles of tortoise without decrease in ATP or PC. Later experiments by Mommaerts showed in a short tetanus PC breakdown in frog sartorius (1962a) and in turtle rectus femoris but not in turtle sartorius (1962b).

Failure to isolate bound ^{32}P-labelled nucleotides indicate the permeability barrier that exists for ^{32}P-orthophosphate in muscle. Later experiments by Cheesman and Whitehead (1968) and Cheesman, Priston and Whitehead (1969a) using frogs injected with ^{14}C-glucose have shown that the actin-bound ADP rapidly becomes labelled and that in fact muscle (sartorius and gastrocnemius) the ADP soon has a specific activity higher than that of the total ATP pool. In the rectus abdominis, labelling of the bound nucleotide occurs less rapidly than in fast muscles. When the muscles are brought into isotonic contracture with KCl the increased activity induced by contraction gives an increased labelling of the ATP. The specific activity of the bound nucleotide is altered on contraction. With the sartorius the change tends to be a decrease but with rectus abdominis an increase.

It has been shown by Martonosi et al. (1960) that when labelled ATP is added to a solution of G-actin exchange occurs between the ^{14}C-ATP and the bound nucleotide. However the bound ADP of F-actin is inexchangeable.

Szent-Györgyi and Prior (1966) showed that bound ADP of myofibrils and actomyosin become exchangeable during contraction and super-precipitation respectively in presence of ATP and Mg^{++}.

From the work of Straub and Feuer (1950) it is known that G-actin has ATP as a prosthetic group and that F-actin has ADP. It is therefore possible that contraction involves repetitive change in the physical state of actin, involving phosphorylation and dephosphorylation of the actin-bound nucleotide. Phosphorylation might occur by transfer from a high-energy intermediate in the breakdown of ATP by myosin.

SUMMARY

Chapter 1

A review is made of the literature concerning the immediate source of chemical energy for muscular contraction.

Chapter 2

The objectives of the investigation are described.

Chapter 3

The methods and materials used in the experiments are described.

Chapter 4

When the rectus abdominis of the frog, briefly labelled (up to 1 hour) with ^{32}P-orthophosphate, is subjected to loaded or unloaded contracture with isotonic KCl, the radioactive count of the water-insoluble muscle residue is reduced by some 40%.

Fixation and extraction with alcohol does not affect the ratio of the specific activities of control and contracted muscles.

Isometric contraction and depolarisation without contraction (Ca^{++}-free media) results in a similar decrease in specific activity.

The reduction in specific activity on contraction is not related to the temperature.

Occlusion of Ca_3PO_4 in the control muscles is not responsible for the effect.

There is no significant difference in the dry weights of acetone powders obtained from contracted and relaxed muscles.

Chapter 5

Repolarisation of the muscle without relaxation does not restore the specific activity to its resting level.

Relaxation of fast muscle (sartorius) without repolarisation restores the specific activity to its resting level.

Severe mechanical extension in itself has no affect on the specific activity of the bound phosphate.

Chapter 6

Myofibrils contracted with ATP in the presence of Mg^{++} show a reduction in specific activity of the bound phosphate similar to that found when frog recti are contracted with KCl.

No reduction in specific activity of the bound phosphate is found when myofibrils are treated with ATP in the presence of Ca^{++}.

Treatment of myofibrils with ITP in the presence of Mg^{++} gives the same reduction in specific activity as that found with ATP.

Treatment with DNP does not prevent the reduction in specific activity when myofibrils are treated with ATP and Mg^{++}.

Chapter 7

No significant part of the count is removed from the muscle residues by lipid solvents, although some 30% of the total phosphorus is extracted with chloroform-methanol.

The material which is extracted as inorganic phosphate by treatment with TCA represents about 1% of the total bound phosphate.

About 80% of the count is removed by TCA from the washed residues of relaxed muscle and about 72% from those of contracted muscle.

Chapter 8

Comparison of the specific activities of the P in PC and ATP-γP isolated from TCA extracts of ^{32}P-labelled whole muscles show a ratio of 0.65 $\pm$ 0.16.

The specific activity of P isolated as inorganic phosphate from acid extracts of acetone powders of washed muscle residues was of the same order as that found in the ATP-γP.

In all experiments, except some where HCl was used, only two phosphorus-containing compounds, inorganic phosphate and ADP, were found in acid extracts of the acetone powders from washed muscle residues.

The ADP was not labelled with ^{32}P.

Chapter 9

The significance of the turnover of bound phosphate during contraction and relaxation is considered.

The possible existence of a phosphorylated myosin as an intermediate in the contraction process and as a primary source for muscular activity is discussed.

In conclusion it is suggested that the labelled phosphate fraction may derive from a phosphorylated myosin, formed as an intermediate in the cycle of chemical events associated with muscular activity.

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