THE INCORPORATION OF INORGANIC PHOSPHATE

BY RABBIT SKELETAL MUSCLE

A thesis presented
by
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ABSTRACT

Cheesman & Hilton (1961, 1966) and Cheesman & Whitehead (1969) have demonstrated an uptake of $^{32}$P by frog muscle. A reversible 40% reduction in the specific activity of bound P was reported to occur on membrane depolarisation. A similar reduction was found by Whitehead (1970) when myofibrils prepared from $^{32}$P-labelled rabbit psoas were contracted. Mühlrad et al. (1963) have shown direct uptake of $^{32}$P by glycerol-extracted myofibrils and by myosin. The present report attempts to consolidate and expand the above observations.

A procedure allowing the resolution of myofibrils into P-containing fractions was developed; several modifications were made to existing methods for P determination. Methods for the direct investigation of myofibrillar ATPase, actomyosin ATPase, adenylate kinase and 5'-AMP deaminase activities in the pH range 7 - 8 and two methods for the estimation of IMP are presented. A circuit diagram for an integrator of pulses from a radioactivity counter is given. An appendix contains practical details of these methods.

The possibility was excluded that ATP synthesised from actin-bound ADP during water extraction of acetone-dried muscle derived its $\gamma$-phosphate group from a source other than the adenylate kinase reaction proposed by Tsuboi (1963). Changes in concentrations of ATP, ADP, AMP and IMP during the extraction were found to be consistent with adenylate kinase and 5'-AMP deaminase activities.

Contaminating bacteria were shown to be responsible for direct uptake of $^{32}$P by myofibrils and myosin.

The reduction, on contraction, in the specific activity of bound $^{32}$P in myofibrils prepared from labelled psoas was related to a decrease in extractability of protein from contracted myofibrils. Label was
distributed throughout the P-containing fractions although the nucleotides showed very low activity. Application of myofibrils to polyacrylamide gels yielded several labelled bands.

A new role for methylated amino acid residues in muscle is proposed. The roles of adenylate kinase and 5'-AMP deaminase and a contraction mechanism involving the reversible phosphorylation of actin-bound ADP are considered.

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A man once went looking for fire
With a lighted lantern.
Had he known what fire was
He could have cooked his rice much quicker.

A Zen Buddhist Teaching.
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<tr>
<td>A, Ac</td>
<td>actin</td>
</tr>
<tr>
<td>AK</td>
<td>adenylate kinase</td>
</tr>
<tr>
<td>ESA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaATP</td>
<td>ATP with bound calcium ion</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>c.p.m.</td>
<td>counts per min; kc.p.m. 1000 c.p.m.</td>
</tr>
<tr>
<td>c.p.s.</td>
<td>counts per s</td>
</tr>
<tr>
<td>DNP</td>
<td>2,4-dinitrophenol</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>eff.</td>
<td>efficiency</td>
</tr>
<tr>
<td>G, Glc</td>
<td>D-glucose</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>kBq</td>
<td>1000 c.p.s.</td>
</tr>
<tr>
<td>M, My</td>
<td>myosin</td>
</tr>
<tr>
<td>MgATP</td>
<td>ATP with bound magnesium ion</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MK</td>
<td>myokinase</td>
</tr>
<tr>
<td>NP</td>
<td>p-nitrophenol</td>
</tr>
<tr>
<td>NTP</td>
<td>p-nitrothiophenol</td>
</tr>
<tr>
<td>OAA</td>
<td>oxaloacetic acid</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethyleneimine</td>
</tr>
<tr>
<td>PYR</td>
<td>pyruvic acid</td>
</tr>
<tr>
<td>TM</td>
<td>tropomyosin</td>
</tr>
<tr>
<td>TN</td>
<td>troponin complex</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>$v_i$</td>
<td>initial velocity</td>
</tr>
<tr>
<td>$\Delta$</td>
<td>change</td>
</tr>
<tr>
<td>$c$</td>
<td>concentration</td>
</tr>
<tr>
<td>$\Delta P$</td>
<td>high-energy phosphate</td>
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<tr>
<td>%</td>
<td>implies % (w/v) for solid in liquid and % (v/v) for liquid in liquid</td>
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SECTION 1

SURVEY OF THE LITERATURE
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Tsuboi (1963), Tsuboi & Hayashi (1963), and Bencsáth & Biró (1963) have proposed that during the deionised water extraction of acetone-dried myosin-free rabbit muscle, the ATP found associated with the G-actin so formed originates through the dismutase action of endogenous adenylate kinase (E.C. 2.7.4.3) :

\[
2\text{ADP} \rightleftharpoons \text{ATP} + \text{AMP}
\]

The ADP was believed to originate from the prosthetic nucleotide of the polymerised actin in the dried muscle. Paper chromatography showed a spot identified as IMP in addition to spots for ATP, ADP, and AMP; IMP was said to be formed through the deamination of AMP by 5'-AMP deaminase (E.C. 3.5.4.6). ATP formation was shown to be accompanied by a nearly stoichiometric accumulation of AMP plus IMP. Changes in nucleotide concentration, in \( \mu \text{mol g}^{-1} \) dried muscle, were given by Tsuboi (1963) as : ADP, -1.24; ATP, +0.66; AMP, +0.03; IMP, +0.72.

Mühlrad et al. (1963) found that glycerol-extracted myofibrils would take up label when stored for up to 14 days in KCl-borate buffer at neutral pH (0°C) with \( ^{32} \text{P} \). Denaturation of the labelled myofibrils by treatment with cold dil. perchloric acid released about 20% of the label. Extraction with perchloric acid at 50°C for one hour appeared to hydrolyse about 50% of the label. The nature of the label not extractable with perchloric acid was undetermined. Extracted \( ^{32} \text{P} \) was in the form of \( \text{P}_1 \); no labelled nucleotides were isolated. Myosin was also found to take up \( ^{32} \text{P} \); myosin extracted from labelled myofibrils contained 80% of the total label. Heat-denatured myofibrils did not incorporate \( ^{32} \text{P} \). The authors concluded that although the \( ^{32} \text{P} \) may be
covalently bound to myosin it was not related to the supposed phosphorylated intermediate of ATP hydrolysis (1.5.4).

When frog muscle is suspended in aerated Ringer containing $^{32}\text{P}_i$ for 30 - 60 min, the bound P that cannot be removed by repeated washing of the minced muscle with water becomes labelled (Cheesman & Whitehead, 1969; Whitehead, 1970). The specific activity of the bound $^{32}\text{P}$ was reduced by 40% on membrane depolarisation, a result confirming the preliminary findings of Cheesman & Hilton (1961); the total bound P remained constant. The reduction in specific activity was observable after depolarisation whether or not the muscle contracted unloaded or produced tension isometrically or anisometrically. The effect was not temperature dependent nor caused by mechanical disruption of the tissue for analysis. The uptake of $^{32}\text{P}_i$ and the contraction effect were not attributed to calcium phosphate deposition. The specific activity could be restored to its resting value by relaxation under load whether or not the membrane was repolarised; repolarisation by itself had no effect. Whitehead (1970) obtained similar results with a membrane-free system of glycerol-extracted $^{32}\text{P}$-labelled rabbit psoas muscle. The muscle was labelled by incubation with $^{32}\text{P}_i$ in Ringer for 6 hrs, rinsed, and extracted with 50% aq. glycerol at $-15^\circ$C for several weeks. A suspension of myofibrils in buffered 0.1 M KCl was prepared by homogenisation. The psoas myofibrils were washed free of residual $^{32}\text{P}_i$ and metabolites. Actomyosin MgATPase (and hence contraction) reduced the specific activity of bound $^{32}\text{P}$ by 40%, but myosin CaATPase left it unchanged. The identity of the labelled fraction was unknown and no radioactive material other than $^{32}\text{P}_i$ could be isolated by acid extraction of minced and washed frog or rabbit muscle. These results suggested to Cheesman and Whitehead that the $^{32}\text{P}$ fraction they were dealing with could be the hypothetical phosphorylated myosin
intermediate of MgATP hydrolysis. Further, since the results of Cheesman et al. (1969), Priston (1970), and Cheesman & Priston (1972) indicated a turnover of bound ADP in frog muscle during contraction (confirmed by Shirley, 1978), it was proposed that actin-bound ADP acts as a short-lived phosphate acceptor, the donor being myosin.

This thesis is an attempt, with the aid of further experiments and theoretical considerations, to reconcile the above observations with respect to the existence of phosphorylated myosin. We have considered the possibility that the terminal phosphate of ATP found associated with G-actin in deionised water extracts of acetone-dried muscle might originate from the reducible $^{32}$P fraction. The feasibility of energy transfer from myosin to actin, possibly in the form of high-energy phosphate, is considered in relation to the chemical, quantum mechanical, and evolutionary aspects of molecules. A review of the relevant literature is presented.
1.2 CONCEPTS OF MOLECULES

1.2.1 CHEMICAL CONCEPT

The general principles of molecular structure and the nature of chemical bonding have originated from atomic theory, first formulated by Dalton in 1805. The notion of the interaction between atoms to form organised systems has followed from valence theory (Frankland, 1852; Kekulé, 1857; Kolbe, 1857; Couper, 1858; Butlerov, 1861) and more especially that of spatially orientated valence bonds from the work of van't Hoff (1874) and le Bel (1874). The modern concept of the chemical (Lewis, 1916) or covalent (Langmuir, 1919) bond is based on an electronic theory of valence (Lewis, 1916) wherein pairs of electrons are shared between 'bonded' atoms. The concept of the atom as a system of one nucleus and one or more electrons was developed through the studies of Planck (1901), Einstein (1907), and Bohr (1913). Spectral phenomena were shown to be related to the electronic aspects of atoms. Negatively charged electrons were considered to be present in concentric shells around the central nucleus composed of positive protons and uncharged neutrons; electron energy increased away from the centre. The viewpoint of Langmuir (1919) that known facts of chemistry could be substantiated by the application of the "new ideas", has been supported, since that time, by much accumulated evidence. However, the theory could not adequately explain or predict properties of aromatic, heterocyclic and conjugated compounds. Refinement of atomic theory has been largely due to the development of quantum mechanics.
1.2.2 QUANTUM MECHANICAL CONCEPT

Liebig (1842) suggested that it was the shape of the glucose molecule that gave it life-giving properties, i.e. it had the correct form to act as a vehicle for 'vital force'. The studied relationship between heat, vital force, and work culminated in the proposal of the Law of Conservation of Energy (Mayer, 1842), and the appreciation that "the ultimate cause of the heat produced (in muscle contraction) is therefore to be found in chemical changes" (Liebig, 1842). Heat changes, therefore, accompanied the making and breaking of bonds. Lewis's valence bond theory, however, left unanswered the fundamental questions as to the nature of the interactions involved and the source of the energy of chemical bonds. Development of covalent bond theory came through the quantum mechanical treatment of H₂ by Condon (1927) and by Heitler & London (1927).

The quantum mechanical concept of molecules is concerned with the energetics of selected electrons. Such studies give rise to a wave-mechanical description of the electron. The solution of the Schrödinger wave equation provides the values of permitted quantized energy levels and the spatial distribution of the electron. This concept has also provided the notion of electron spin. Each energy level represents a possible 'state' of the electron, the lowest value corresponding to the 'ground state' of maximum stability. All other levels are 'excited states' into which the electron can jump if sufficient energy is supplied. Quantum mechanics does not give the precise location of an electron but does give the probability of finding the electron with a particular energy at a chosen place. The boundary surface of the probability density provides a visual concept of atomic orbital (AO) shape; pairs
of electrons with antiparallel spins are fed into these AOs in order of stability.

The counterpart of the chemical single bond, created between two atoms by the sharing of two valency electrons, is the σ molecular orbital or bond. The σ orbital containing the two electrons is 'sausage-shaped' with its long axis lying along the line joining the two bonded atoms. This electron cloud surrounding the two atoms is densest in the region between them. The quantum mechanical structure of the double bond places two electrons in a σ orbital and the remaining pair in a π orbital. The π bond electron cloud lies above and below the plane of the σ bond. π bonding is weaker than σ. The triple bond of six electrons is created by one strong σ bond and two weak π bonds. The application of quantum mechanics to conjugated molecules of alternating single and double bonds has led to the concept of localised and delocalised electrons. Localised molecular orbitals are bicentric σ- and π-type bondings with bond properties essentially constant from one kind of molecule to another. The total bond energy of a localised system is the sum of the individual bond dissociation energies. Molecular orbital (MO) theory when applied to conjugated molecules views the π electrons involved to be merged into a single fluid distribution around the σ skeleton. Bond properties cannot be predicted with confidence from those observed in other types of molecule. Delocalisation of π electrons stabilises the molecule.

Many biochemicals are conjugated molecules and, therefore, quantum biochemistry attempts to provide information about the electronic distribution and energy levels in these molecules from which a more adequate explanation of molecular structure, behaviour, and function can be deduced (Pullman & Pullman, 1962, 1963; Kasha, 1962).
Application of quantum mechanical methods has provided some insights into the behaviour of macromolecules.

1.2.3 EVOLUTIONARY CONCEPT

Darwin (1849) has suggested that the patterns of life have evolved through a process of continual struggle and elimination whereby only the fittest survive. Under any given external condition and any internal organisation, a species can only survive if its progeny are adapted for adverse changes in the environment. This adaptation is through 'accidental' changes in the base sequence of nucleic acids. If mutations are the raw material of evolution then some of them must sometimes be, or become, advantageous; reproduction and mutation are the key processes of life (Kaplan, 1971). However, before the start of biological evolution decidedly simpler structures must have arisen by a circuitous long-drawn out yet selective replication mechanism (Gaffron, 1972). The thesis that molecules underwent selection processes prior to the first reproducing organisms has gained wide acceptance (Fox & Dose, 1972). A key question for the origin of cells, however, is that of the sequence of appearance of special properties in the system (Fox, 1973). For example, the properties of a given enzyme are phenotypic characteristics that have evolved on the basis of selective advantage (Atkinson, 1972).

Evolution requires the adaptation and improvement of bioenergetic processes. It is assumed that the properties of the biosphere changed to an important extent only as a consequence of the activities of expanding life itself. Spencer (1844) pointed out that "it was the
living organisms themselves that produced the atmospheric oxygen". Changes are unlikely to have involved large steps between intermediate stages, a state of affairs which Orgel (1968) associates with a Principle of Continuity. The capacity for any step can have established itself only when the external physico-chemical conditions for its operation were created. Every step in physiological and biochemical evolution must be (a) biologically useful, (b) thermodynamically possible, (c) mechanistically plausible (Orgel, 1968).

Living systems are far from equilibrium; the concentrations of most biochemicals even in so-called resting organisms are maintained dynamically, a mechanism that requires the constant input of energy (Schoenheimer, 1942; von Hevesy, 1948). Any non-equilibrium structure is a 'dissipative structure' that will transform structural energy into thermal motion (Prigogine & Babloyantz, 1971; Morowitz, 1971). Energy must be supplied in forms that will synthesise and maintain the integrity of the structure. In biological systems the mode of energy storage is in covalent bond energy, effectively the potential energy of electrons. Photosynthetic energy input serves to build up these high potential energy structures while catabolic processes act so as to degrade the energy into thermal motion. Delocalised electrons are readily implicated as the forms of molecular energy in biological systems. The flow of energy through the system leads to an ordering of the system (Morowitz, 1971). This flow of energy is mediated by the polyphosphates, predominantly ATP.
1.3 ENERGETICS OF PHOSPHATE COMPOUNDS

1.3.1 DISCOVERY OF P-CONTAINING METABOLITES

The observation by Harden & Young (1906) that phosphate esterification was coupled to fermentation is considered by Kalckar (1969) to represent the beginning of a new chemical development, the study of energetic coupling in enzyme systems. It had long been recognised through nutritional studies that restriction of P intake inhibited normal growth in both plants and animals (e.g.: Moleschott, 1850; Osbourne & Mendel, 1918; Sherman & Pappenheimer, 1921), and Hopkins (1910) had concluded that P was the only element required to be added to most common soils to ensure their fertility. It was also recognised that strenuous exercise led to increased excretion of P\textsubscript{i} in the urine (Engelmann, 1871; Embden & Grafe, 1921). The slow realisation in the 1930s that insertion of P into a molecule during the glycolytic transformation of glucose into lactic acid in muscle was associated with the energetic requirements of the cell, established the role of adenine nucleotides as energy carriers (Embden et al., 1933; Parnas et al., 1934; Lipmann, 1934; Lohmann & Meyerhof, 1934; Warburg & Christian, 1939). When the key compounds CP (Eggleton & Eggleton, 1926; Fiske & Subbarow, 1927) and ATP (Fiske & Subbarow, 1929; Lohmann, 1929) were discovered, their significance was not immediately apparent.

In 1907, Fletcher & Hopkins bridged the gap between the chemistry of dead or nonfunctional muscle and the modern biochemical approach to muscle function; they found that actively contracting muscle accumulated lactic acid. Hill (1912) related heat and lactate production. Meyerhof & Hill (1923) attempted to link data from chemical and biochemical
studies with those derived from thermal measurements and from the recording of mechanical work. Meyerhof (1925) viewed lactate production as mechanistically connected with mechanical response. During 1930 and 1931, Lundsgaard, using IAA-poisoned muscle, showed that lactate formation was not a necessary concomitant of contraction and that in unpoisoned muscle its production followed the cessation of tetanic contraction. In fact, Embden (1924) and Embden & Schwartz (1928) claimed to have shown that lactate formation was a relatively slow process which did not coincide with contraction. Lundsgaard (1933) began to argue that energy for contraction is supplied through CP breakdown, a mechanism more closely related to the contraction event than is lactate formation. The acidic hydrolysis of CP (Meyerhof & Suranyi, 1927; Meyerhof & Lohmann, 1928) and of ATP (Meyerhof & Lohmann, 1932) was found to be strongly exothermic. Lohmann (1934) confirmed his earlier (1929) notion of the reaction:

\[
CP + ADP \rightleftharpoons C + ATP
\]

Lehnartz (1933) had suggested that, at least theoretically, the energy from the fission of CP might be used for the synthesis of ATP. The significance of the high exothermicity of ATP and CP hydrolysis became readily accepted.

Lipmann, in his paper of 1941, formally recognised the utilisation of phosphate bonds as energy carriers and introduced the concept of energy-rich phosphate compounds (\(\gamma\)P). ATP, generated during the catabolism of phosphorylated glycolytic intermediates, was shown to stimulate muscle contraction (Engelhardt & Ljubimova, 1939; Needham et al., 1941; Szent-Györgyi et al., 1942).
1.3.2 ENERGY COUPLING AND THE UPTAKE OF $P_i$

In muscle, $P_i$ is incorporated into cellular components by two distinct methods:

1. by coupling to a metabolite, of which the known reactions are:

- **$\alpha$-glucan phosphorylase (E.C. 2.4.1.1)**
  
  \[ (\alpha-1,4\text{-glucosyl})_n + P_i \rightarrow (\alpha-1,4\text{-glucosyl})_{n-1} + G-1-P \]

- **glyceraldehyde phosphate dehydrogenase (E.C. 1.2.1.12)**
  
  \[ \text{glyceraldehyde} + P_i + \text{NAD}^+ \rightarrow 1,3\text{-diPglycerate} + \text{NADH} \]

Both enzymes are cytoplasmic. The bound P is eventually transferred, either directly or indirectly, to ADP.

2. by direct coupling to ADP in the mitochondria by either the reaction:

- **pyruvate carboxylase (E.C. 6.4.1.1)**

  \[
  \text{OAA} \quad \text{BIOTIN} \quad \text{AIP} + \text{CO}_2 \\
  \text{PYR} \quad \text{CARBOXY-BIOTIN} \quad \text{ADP} + P_i
  \]

  or through oxidative phosphorylation. Pyruvate carboxylase is of little importance in muscle since levels are low (Opie & Newsholme, 1967).

Oxidative phosphorylation (discussed below) accounts for most of the $P_i$ uptake in active muscle.
Mitochondrial energy transduction represents conversion of the energy linked with metabolite oxidation into phosphate bond energy (cf. Wilson et al., 1973). Oxidation is mediated by specific dehydrogenases that use either NAD\(^+\) or FAD\(^+\) as H acceptor. It is generally agreed that the total energy released through respiratory chain oxidation of NADH is divided into three parts, each providing energy for the synthesis of one ATP molecule; if FADH is the oxidised cofactor then only two ATPs are synthesised. The respiratory chain components and ATP synthase enzyme (or ATPase) are bound to the inner mitochondrial membrane.

At present there are two major hypotheses of the mechanism of mitochondrial phosphorylation:

1. **ELECTROCHEMICAL COUPLING THEORY**

   Mitchell (1966), in the revised version of his Chemiosmotic Hypothesis of 1961, proposed that the electrical potential across the mitochondrial membrane is generated by the electrogenic pumping of protons from the matrix by the respiratory chain. This transmembrane pH gradient is coupled to ATP synthesis. The distinctive feature of the 1966 hypothesis and of later supportive papers (Mitchell, 1976, 1979; Mitchell & Moyle, 1979) is the involvement of a phosphorylated intermediate in the membrane.

2. **CONFORMATIONAL ENERGY TRANSFER**

   Boyer et al. (1973) consider that the important function of respiratory electron energy is to promote ATP release from the active site of mitochondrial ATPase. In more recent papers (Boyer, 1975, 1977; Kayalar et al., 1976, 1977; Rosing et al., 1977) it is suggested, with supporting evidence, that there are two sites on the one ATPase which give rise to alternating
catalytic sites. There is an energy-linked conformational transition in which a tightly bound ATP at one catalytic site is released upon the binding of P\textsubscript{i} and ADP at the other and the influx of respiratory chain electron energy. Changes in protein conformation by proton gradients and an evaluation of Mitchell's interpretation are presented by Boyer (1975) and Boyer et al. (1977).

1.3.3 CONCEPT OF HIGH-ENERGY PHOSPHATE

The finding that the dephosphorylations of CP and ATP were highly exergonic presented a new development in the understanding of cellular energetics (1.3.1). Lipmann (1941) introduced the term 'high-energy phosphate', symbolically represented as ^P, and defined 'group potential' to emphasise the escaping tendency of a group due to the high bond energy of the linkage before cleavage. Klotz (1957) suggested the expression 'group-transfer potential' to stress the fundamental analogy with electrochemical potential and to demonstrate the interconvertibility of group and redox potential. Wilson et al. (1973) have related the apparent redox potentials of respiratory chain carriers with 'phosphorylation potential'.

The most strongly emphasised factor associated with the energetics of phosphate compounds is the standard free energy of hydrolysis of the O-P bond (\(\Delta G^0\)). Phosphates with high group-transfer potential are

\[\text{\dagger} \]

Since reactions are performed in solution the pressure-volume work done against the atmosphere is negligible. The difference between changes in Gibbs' and Helmholtz' free energy is disregarded. \(\Delta G\) is a change in Gibbs' free energy (cf. Wilkie, 1975).

characterised by $\Delta G^o < -30 \text{ kJ mol}^{-1}$, and those with low by $\Delta G = -12 \text{ kJ mol}^{-1}$ (cf. Jencks, 1968). Standard conditions are 1 M concentration at 25°C; $\Delta G^o$ is used to imply pH 7.0. As pointed out by Albery (1969, 1972), measurements of the thermodynamic quantities for:

$$\text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + \text{P}_i$$

have been made under a variety of conditions. He presents procedures for interconversion of data at a variety of temperatures, pH, and pMg. Values should also be corrected for actual concentrations of reactants and products under physiological conditions (Lipmann, 1960; Carpenter, 1960; Albery, 1972). Banks (1969) and Banks & Vernon (1970) consider that the preoccupation with free energy changes diverts attention away from the genuine problem of ATPase events. Pauling (1970), Huxley (1970), Wilkie (1970), and McClare (1972, 1975) have presented arguments in favour of ATP being a source of energy and have defended Lipmann's reference to a high-energy phosphate bond. Insight into the manner in which energy is stored by phosphate compounds and released for biochemical use as pyrophosphates has been gained by quantum mechanical methods.

In general, the common feature of energy-rich phosphates is that the phosphoryl group ($-\text{PO}_3^{2-}$) is attached either to another such group or to a resonating structure capable of conjugating with its mobile $\pi$ electrons. Those compounds of low phosphorylation potential have the phosphoryl group attached directly to a saturated group. The general structural arrangements of known biochemical phosphates are:

**LOW ENERGY FORM:**

$$\text{R} - \text{CH}_2 - \text{O} - \text{P} - \text{O}^-$$

$$\text{O}$$
HIGH ENERGY FORMS:

nucleoside polyphosphates:

\[
\text{purine or pyrimidine base - ribose - CH}_2 - O - \overset{\text{\(\text{P}\)}}{-\overset{\text{\(\text{P}\)}}{-\overset{\text{\(\text{P}\)}}{-\overset{\text{\(\text{O}\)}}{\text{O}}} -}}
\]

amidine phosphates:

\[
\text{NH}_2 \overset{\text{\(\text{P}\)}}{-\overset{\text{\(\text{O}\)}}{\text{O}}} - R_2N - C - N - \overset{\text{\(\text{P}\)}}{-\overset{\text{\(\text{O}\)}}{\text{O}}}
\]

carbonyl phosphates:

\[
\text{O} - \overset{\text{\(\text{P}\)}}{-\overset{\text{\(\text{O}\)}}{\text{O}}} - R - C - \overset{\text{\(\text{O}\)}}{-\overset{\text{\(\text{P}\)}}{-\overset{\text{\(\text{O}\)}}{\text{O}}}}
\]

phosphoenolpyruvate:

\[
\text{O} - \overset{\text{\(\text{CH}_2\)}}{-\overset{\text{\(\text{O}\)}}{\text{O}}} - \text{HO} - C - C - \overset{\text{\(\text{O}\)}}{-\overset{\text{\(\text{P}\)}}{-\overset{\text{\(\text{O}\)}}{\text{O}}}}
\]

The current understanding of the energy enrichment of the phosphates has been developed from the theory of 'opposing resonance' in carbonyl phosphates (Kalckar, 1941; Oespar, 1950). In this theory the high free energy of hydrolysis of the \(\text{O} \overset{\text{\(\text{N}\)}}{-\overset{\text{\(\text{P}\)}}{-\overset{\text{\(\text{O}\)}}{\text{O}}}}\) bond is considered to be due to competition between the component atoms of the phosphoryl and carbonyl groups for the lone pair of the central bridge \(\text{O}\). Cohn (1949) and Boyer et al. (1955) have shown that carbonyl phosphates can be split on either the C or the P side of the bridge \(\text{O}\). The moiety to which \(\text{O}\) belongs after cleavage is the low energy side:
Grabe (1958) has calculated the π electron distribution of the carbonyl phosphate group by considering a 6 π system composed of 2 electrons from C=O, 2 from the lone pair of -O-, and 2 from -PO₄³⁻. Comparison to the hydrolysis products -COO⁻ and PO₄³⁻ showed the C-O-P backbone of the molecule to be devoid of π electrons. The low π electron density of O₂⁻ shows a tendency for the electron cloud to be drawn towards Oₓ and Oᵧ; the cloud is evenly distributed between the oxygens in the products. Since the bridge O is strongly electronegative, it is probable that the σ electron distribution in the C=O and O-P bonds are disrupted:

\[ \text{O} \quad \text{C} \quad \text{O} \quad \text{O} \quad \text{P} \]

\[ \text{disrupted π electron system} \]

asymmetric σ bondings
Both C-O and O-P σ bonds are thereby weakened. The two forces representing the tendency for π electrons to move away from and σ to move towards O₂ will be balanced. There are probably, therefore, no activated groups in the free molecule, only a specific disturbance on one side rendering the required activation. This balance would be upset by the appropriate enzyme.

The above study provides the following general points:

(a) There exists a coupling between the delocalised π electrons and the σ skeleton, such that a decrease in energy of one part of the reacting molecule causes activation of the other.

(b) The possibility exists that either bridge O bond can be broken, even though internal disruptive forces are balanced.

A similar argument has been developed for polyphosphates (Pullman & Pullman, 1962; Alving & Laki, 1972). The utilisation of ATP requires a metal ion, usually Mg²⁺ (e.g. the complex MgATP⁺ is the substrate for actomyosin ATPase (Finlayson et al., 1969)). The Mg²⁺ ion electronically links the adenine ring delocalised system to the delocalised polyphosphate chain (Glassman et al., 1971). A more complete understanding of the electron distribution in MgATP must await more sophisticated quantum mechanical techniques.

The concept that opposing resonance is responsible for the high free energy of hydrolysis of some phosphates has been widely accepted (Boyd & Lipscomb, 1969). It in fact only accounts for 40 - 50% of the excess energies released from carbonyl phosphate and polyphosphates. Opposing resonances are calculated on the assumption that components fuse into
conjugated systems without the formation of any unique bonding. Energy changes resulting from new spatial arrangements of bonded atoms in the formation of these molecules contribute to their free energies of hydrolysis (Cruickshank, 1964). The tautomeric transformation of phosphoenolpyruvate into pyruvate on hydrolysis accounts for 80 – 90% of its free energy of hydrolysis. Electrostatic repulsion between negatively charged O atoms and their free energy of ionisation are additional factors that should be taken into account (Hill & Morales, 1951). The studies of Grabe (1958) and Pullman & Pullman (1962) have shown that in energy-rich phosphates there is always a chain of at least three adjacent atoms devoid of π electrons and that σ electrons are probably displaced towards bridge oxygens. This viewpoint is supported by the calculations of Alving & Laki (1972) on the combined σ and π distribution in polyphosphates. Their results show the backbone of this group to be one of alternating positive P and negative O charges. Alving & Laki (1972) consider the electrostatic attraction between the backbone O and P atoms and repulsion between the non-bridge O⁻ to be the major factors contributing to the high energy of ATP.

1.3.4 UTILISATION OF PHOSPHORYLATED METABOLITES

Under physiological conditions, phosphates clearly possess the particular combination of properties required for energy storage. Atkinson (1972) and Chapman & Fall (1972) have defined an energy charge:

\[
A_e = \frac{ATP + ADP/2}{ATP + ADP + AMP}
\]
such that the value of $A_e$ for cellular growth must lie between 0.75 and 0.95, but for cell maintenance without growth between 0.5 and 0.8. If $A_e$ falls below 0.5, death occurs. It can be readily calculated that if a 70 kg man requires 8 MJ per day to maintain body wt. then he must produce, metabolically, 40 nmol ATP per second per g body wt. The typical turnover time for ATP is 1 s (cf. Atkinson, 1972). In muscle, the ATP concentration is about 10 μmol g⁻¹, in other cells it is lower. Glycolysis and mitochondrial oxidative phosphorylation serve to build up and maintain the concentration of ATP in both resting and active muscle. Both these metabolic pathways are controlled by the relative amounts of ADP and ATP, and by many other factors. Hultman (1967) has shown that during exercise (man) the concentration of ATP remains constant until the person is on the point of exhaustion, whereupon it falls. Active muscle consumes ATP for contraction and the development of tension:

$$\text{MgATP} \rightarrow \text{MgADP} + \text{P}_1 \quad \text{(muscle ATPase)}$$

Since physiological response to the onset of contraction and to increasing levels of ADP may lag behind ATP demand, a temporary supply of high-energy phosphate is stored in the form of CP (about 20 μmol g⁻¹). This allows ADP to be rapidly rephosphorylated and muscle activity to continue:

$$\text{MgADP} + \text{CP} \rightarrow \text{MgATP} + \text{C} \quad \text{(creatine kinase)}$$

The CP level is restored to its resting value through the reverse reaction during the recovery period after cessation of muscle activity.

The accumulation of AMP in muscle is an indication that supplies of metabolic energy are depleted (Krebs, 1975; Wilkie, 1975). AMP is produced by the action of myokinase on ADP (Colowick & Kalckar, 1943):
\[
\text{MgADP} + \text{ADP} \rightleftharpoons \text{MgATP} + \text{AMP} \quad (\text{myokinase}^+) 
\]

AMP production may be induced artificially by poisoning the muscle with DNFB; this reagent is known to inhibit creatine kinase (Dydyńska & Wilkie, 1969; Curtin & Woledge, 1975). Lundsgaard (1930) had shown that IAA-poisoned muscle when stimulated to exhaustion produced ammonia; early theories of muscle contraction involving the degradation of protein were based on the observed increase in N excretion during prolonged exercise (see Needham, 1971, p. 36). Ammonia formation was shown by Schmidt (1928) to be due to the highly exergonic deamination of AMP to IMP by muscle 5'-AMP deaminase:

\[
\text{AMP} \rightarrow \text{IMP} + \text{NH}_3 \quad (\text{deaminase})
\]

The myokinase reaction is freely reversible (see p. 259) and can be driven into ATP production by the essentially irreversible deaminase reaction. These two reactions supply additional ATP at the expense of adenine when the concentration of ADP begins to build up due to inadequate resources of metabolites capable of producing high-energy phosphate. There is apparently little or no provision in muscle for the transformation of IMP into adenine nucleotide. Atkinson's energy charge does not take into account this loss of adenine, but as pointed out by Wilkie (1975) active muscle produces little AMP, and hence IMP, except just before the onset of rigor mortis.

^† Adenylate kinase found in liver mitochondria, for example, is a different enzyme to muscle cytosol myokinase although it catalyses the same reaction (cf. Klingenberg, 1975). Consequently the name myokinase will be used in this thesis when reference is made to the muscle cytosol enzyme.
ATP is made or used in every metabolic sequence. Equilibrium between $^{32}\text{P}_1$ and ATP occurs in about 30 min in rabbit muscle, both $\beta$ and $\gamma$ P atoms becoming labelled (Korzybski & Parnas, 1939). The utilisation of ATP, and other nucleoside triphosphates, may be divided into two classes:

(a) transfer to other forms of energy, i.e. mechanical work, protein translocation on the ribosome, ion and metabolite transport across membranes

(b) biosynthesis of macromolecules and phosphoryl transfer in metabolic regulation

Reactions involving phosphate compounds often require the participation of a metal ion.

The utilisation of nucleoside triphosphates for energy donation is not unique. A set of reactions in bacterial chromophores are those using inorganic pyrophosphate ($\text{PP}_i$) as an alternative energy donor to ATP (Baltscheffsky et al., 1971). A light-induced phosphorylation of $\text{P}_i$ to $\text{PP}_i$ was demonstrated with chromophores from *Rhodospirillum rubrum* in a reaction medium to which no ADP had been added (Baltscheffsky et al., 1966). Miller & Parris (1964) and Lipmann (1965) have suggested that $\text{PP}_i$ may have been the energy donor in early living cells. Higher polyphosphates have been shown to occur in a large number of microorganisms (reviewed by Harold, 1966) and in rat liver nuclei (Griffin et al., 1965). 2 – 3 $\mu$g polyphosphate of up to 5000 phosphate units have been isolated per g of various bovine tissues (Gabel & Thomas, 1971). The utilisation of higher phosphates as donors of activated P for the phosphorylation of glucose (as demonstrated in *E. coli*) may be
evolutionarily more ancient than that of ATP (Kulaev, 1971):

\[
\begin{align*}
&\text{glucose} \\
&\text{PolyP}_i \\ &\text{G-6-P} \\ &3-P\text{-glyceraldehyde} \\ &\text{1,3-diPGA} \\ &\text{3-P-GA} \\
&\text{P}_i \\
\end{align*}
\]

Kornberg et al. (1956) had shown that the following reaction apparently took place in *E. coli*:

\[
\text{ATP} + (\text{PO}_3^{2-})_n \rightarrow \text{ADP} + (\text{PO}_3^{2-})_{n+1}
\]

Griffin et al. (1965) have pointed out that such a reaction conserves \(\text{P}_i\) and stores energy without affecting the adenine balance.

Kulaev (1971) regards polyfunctionality to be one of the most important criteria for a compound to be selected in the course of evolution. He is of the opinion, therefore, that polyphosphate has additional roles as a localised pool of P, as a \(\text{P}_i\) scavenger, and as a cation-exchanger. Wald (1962) has considered the evolutionary advantages of P in the utilisation of energy in terms of the electronic exploitation of 3d orbitals, multiple bonding capacity, and atomic size.
1.4 PROTEINS AND THEIR INTERACTIONS

Knowledge of molecular conformation is central to the understanding of biochemical processes in living systems since molecule-molecule interactions depend on specific configurations (Zink et al., 1980). Proteins interact with small molecules and ions. The transient or stable complexes thus formed often have a biochemical function (Lipscomb, 1978). These functions include transport, protein stabilisation, conversion of the protein or binding molecule into an alternate conformation, chemical transformation of the bound species or of the active groups of the enzyme. In addition, proteins combine with other proteins and with nucleic acids, polysaccharides, and lipids. The interactions between macromolecules are basic to the organisation of structural entities as well as to the biological function of macromolecular aggregates (Frieden, 1971).

Further general discussion of the topic in this thesis will be restricted to protein-protein, protein-small molecule, and protein-solvent interactions.

Protein interactions, in their widest manifestations, provide the molecular mechanism for the operation and control of cellular reactions as well as the basis of many of the macroscopic functions of tissues and organs (Klotz et al., 1975). The interactions may be viewed as dynamic macromolecular rearrangements representing conformational changes within the tertiary structure, thereby altering properties and affecting the way in which the protein combines with other molecules. A protein may exist dynamically in more than one conformation, some of which may be short-lived. The binding of any molecule to the one state will disturb the equilibrium favouring a shift towards this state. The compact structures that result from the folding of polypeptide chains are
essentially free of solvent and are without large voids (cf. Richards & Richmond, 1978). Local thermal motion of amino acid side-chains may allow some covalent structural change to occur. Almost all protein chemical reactions are accompanied by considerable structural rearrangements of the whole protein molecule (Blumenfeld, 1978). Changes at the binding centre are characteristically fast, $10^{-12}$ to $10^{-13}$ s. The structure of the main bulk protein remains unchanged in this time and becomes conformationally out of equilibrium. The total duration of the rearrangement may be of the order of seconds. In some protein reactions studied (see Blumenfeld, 1978) two processes were observed to proceed, the fast chemical reaction and the slow conformational relaxation of the protein. Molecules of the same protein species at different stages of relaxation are in fact distinct molecules with dissimilar electronic and structural configurations and reactivity. Blumenfeld argues that the faster the initial reaction the more the protein molecules will be out of phase. This mechanism is in antithesis to the widely accepted schemes of Monod et al. (1965) and Koshland et al. (1966) which postulate that conformational changes occur rapidly and that intermediate stages are not manifested. Linderstrøm-Lang & Schellman (1959) had predicted that enzymes in solution would be found to have multiple interconverting conformations, a postulate borne out by recent experimental evidence (Vallee & Riorden, 1978).

That proteins in their native states might be built up of subunits seems to have been first recognised by T. Svedberg (cf. Klotz et al., 1975). The term 'quaternary structure', referring to these macromolecular systems composed of noncovalently linked subunits, was proposed by Eberl (1958). Such subunits that can be released without cleavage of covalent bonds are referred to as 'monomers'; the forces involved are perturbed
by urea, guanidine-HCl, and sodium dodecyl sulphate. The majority of proteins that possess quaternary structure are built up of 2 or 4 monomers; 15 - 20% of these proteins have non-identical subunits (cf. Klotz et al., 1975). One of the most striking features of the glycolytic and tricarboxylic pathways is the overwhelming presence of enzymes composed of subunits. Changes in enzyme activity are mediated through the binding of small molecules or ions ('effectors'). Quaternary aggregates, therefore, constitute regulatory units.

Known methods of regulation through modification of quaternary structure are as follows:

1. The binding of substrate to one subunit may cause the interaction of the protein with other members of the aggregate to alter.

2. The binding of effector to sites on a subunit may act positively or negatively.

3. Anions and cations may promote or inhibit association, or change molecular shape, or both.

4. The binding of cations that have functional roles are postulated to have the following roles (Vallee & Wacker, 1970):
   a. participation in substrate or cofactor binding
   b. activation of the enzyme-substrate complex
   c. formation of a coordination complex to hold the quaternary structure intact
   d. redox reactions
   e. change the protein conformation
   f. creation of an 'energetically-poised domain'.
(5) Solvent interactions often influence conformational stability and the activity of proteins. Three types of water are recognised (Franks, 1978):

(a) internal - structurally bound
(b) peripheral - bound to the surface as water of hydration
(c) free solvent

A protein communicates with its environment. The mechanism by which energy is transmitted through a macro-molecule and transferred to another is not immediately obvious. Szent-Györgyi (1941a) put forward the hypothesis that energy transfer in biological systems might take place by a mechanism analogous to semiconductivity in inorganic crystals. Proteins, when dry, show electrical conductivity (Eley et al., 1953; Eley & Spirey, 1960, 1962; Rosenberg, 1962) which is reduced on protein denaturation (Eley & Spirey, 1960). Ionic conductivity through adsorbed salt does not account for this phenomenon, although it has not been ruled out in some cases studied (cf. Kasha, 1962). Although the peptide bond is a delocalised system of $4\pi$ electrons, the polypeptide chain does not form one continuous system since the bonds are separated by saturated C atoms. It is improbable that electrons are transmitted along the chain. However, the peptide bonds are linked by H-bonds so that a degree of interaction does take place which might give rise to a general electronic delocalisation extending over large surfaces of the protein molecule (Szent-Györgyi, 1946; Evans & Gergely, 1949). The calculations by Evans & Gergely (1949) and by Suard et al. (1961), on the expected energy levels of peptide interaction through the H-bonds, predicted proteins to be good insulators. Nevertheless, Chandra et al. (1978) believe these organised arrays to be the primary mode of energy
transfer. Pullman & Pullman (1958) and Brillouin (1952) regard the amino acid side-chains of the protein as electron donors and acceptors which interact with the H-bond/peptide system. Kharkyanen et al. (1978), in their "donor-acceptor model of electron transfer through proteins", consider -COO" of glutamic and aspartic acid residues to be the major electron donors and to be acceptors in a -COO* form. The possibility of the formation of peptide free radicals (Gordy & Schields, 1960; cf. Pullman & Pullman, 1963) and the involvement of prosthetic groups of large polarizability, such as heme (Minton & Libby, 1968), have also been invoked. However, conventional semiconductivity theory does not account for the fact that electrical conductivity exceeds theoretically expected values by many orders of magnitude (cf. Kemeny & Goklany, 1974). Kemeny (1974) believes that the relevant property of biological systems is electron delocalisation. This is not sufficient, however, since the electron system occurs only in four types of amino acid residue and as an interrupted system in the polypeptide chain.

Blumenfeld & Chernavski (1973) view the prosthetic groups of chloroplast and mitochondrial transport-chain carriers as potential wells. Electron movement, from well to well, is accompanied by relaxation-type conformational changes in the protein. These authors believe that the structural rearrangement of the protein is linked to energy fixation. The concept of the 'conformon' has been introduced (1) to explain the dynamic relationship between electronic and conformational energies (Volkenstein, 1972), (2) to provide a new model of mitochondrial structure and function via the migration of packets of free energy (conformons) through a 'supermolecule' (Green & Ji, 1972), and (3) to explain protein conductivity (Kemeny & Goklany, 1973, 1974).
Propagation of the conformon through a macromolecule is related to the vibrational coupling of adjacent bonds. The energy released by the hydrolysis of ATP (say, 40 kJ mol$^{-1}$) is insufficient to excite the electronic states of molecules. Bond vibration energies, on the other hand, are of this order. Quantum mechanics views the crystal lattice as one large coupled system which is able to pulsate at various frequencies. The quanta of vibrational energy involve displacements of all the atoms rather than just one; a quantum of lattice vibrational energy is a 'phonon'. Changes in the energy of a macroatomic system alter the number of phonons. A slow, itinerant electron in an ionic crystal disturbs the charge balance and its movement is accompanied by a moving wave of lattice deformation. The electron becomes coupled to the phonons. The life-time of a vibration is usually small, $10^{-12}$ s; the propagation of such a vibration is called a dissipative wave. In organised systems, the duration of vibration is often longer and its propagation is as a non-dissipative wave or 'soliton'. Davydov (1977) believes that the collective excitation of a chain of successively arranged peptide groups constitutes a vibration soliton and that the formation of such stable excitation in the $\alpha$-helical portions of protein molecules is linked to high efficiency of energy transfer to proteins.
1.5 STRUCTURE AND FUNCTION OF MUSCLE

The structure of the different forms of muscle (i.e. cross-striated (skeletal), obliquely-striated, smooth, and heart) are closely related to their roles. This thesis is concerned with vertebrate cross-striated muscle and the word 'muscle' will, therefore, be used to imply that type of structure. One way of looking at the function of muscle is to regard it as a device analogous to a transformer that converts chemical forces, acting over very short distances \((10^{-10} \text{ m})\) into forces which act over a much greater range \((10^{-2} - 1 \text{ m})\); hypotheses of the contraction mechanism may be classed as ways either of subdividing movements or of amplifying the range of influence of short-range chemical events (Huxley, 1980, pp. 41 - 42). From the available evidence (phase-contrast light microscopy, electron microscopy, and X-ray diffraction studies on living muscle; cf. Huxley, 1972b, pp. 330 - 337), it is clear that changes in the length of a muscle are brought about by the relative sliding of two arrays of dissimilar interdigitating filaments. The lengths of both types of filament remain essentially constant.

It is believed that the chemical basis of contraction is the interaction of the two proteins, actin and myosin. Actin is present in one set of filaments and myosin in the other. This interaction is mediated by MgATP, the energy of its hydrolysis to MgADP and \(P_i\) being used to change the quality of the actin-myosin relationship. Force is generated at these sites of interaction through repeated cycles, 1 ATP molecule being hydrolysed per cycle per site. In relaxed muscle it is believed that MgATP is bound to the ATPase active site and that actin and myosin are dissociated. The interaction is prohibited through steric blockage by a protein complex called troponin-tropomyosin. Electrical stimulation
of the muscle releases small quantities of Ca\(^{2+}\) which bind to the complex. The resultant structural change in the protein causes the complex to move, allowing the actin-myosin interaction to take place repeatedly (with the concomitant hydrolysis of MgATP) until stimulation ceases and Ca\(^{2+}\) is removed. The state of rigor mortis occurs in the absence of ATP, the actin-myosin interaction taking place irreversibly regardless of the level of Ca\(^{2+}\).

1.5.1 STRIATED MUSCLE AND THE MOLECULAR BASIS OF THE CROSS-STRIATION IN SKELETAL MUSCLE

The structure of striated muscle and its subdivisions are shown in Fig. 1.1. The muscle is composed of fibres, 30 - 150 \(\mu\)m in diameter, arranged in bundles of 20 - 40; the length of a fibre is in the direction of contraction and runs from terminus to terminus of the muscle. Histologically, each fibre may be seen to be embedded in fine connective tissue (endomysium), each fibre bundle in a sheath of perimysium, and all the bundles invested by the muscle sheath, the épimysium (cf. Lockhart, 1972). These membranous connective tissues blend to form the tendon which is inserted into the structure to be moved or to which the muscle is to be anchored. The perimysium, as seen in cross-section by light microscopy, is a 40 - 70 nm thick translucent membrane which bridges the gap between sectioned muscle fibres. Bowman (1840) named this membrane the 'sarcolemma'. The sarcolemma in electron-micrographs is seen to be triple-layered and with an outer surface - the basement membrane - associated with a loose network of irregularly arranged collagen fibrils 25 - 35 nm in diameter (Robertson, 1956). The membrane component adjacent to the muscle fibre is the plasmalemma, 7 nm thick.
FIGURE 1.1

SUBDIVISIONS OF MUSCLE

- muscle
- tendon
- fibre bundles (< 0.7 mm dia.)
- fibre
- cross-striation

- myofibril (1 - 2 μm dia.)
- thick filament (1.6 μm)
- thin filament (1.0 μm)

- myosin head
- thick filament dia.: 15 - 20 nm
- thin filament dia.: 10 - 12 nm
- head projection: 10 - 40 nm
The plasmalemma is a permeability barrier and is separated from the basement membrane by a gap of 20 – 30 nm. The basement membrane is approx. 10 nm thick. The plasmalemma is decorated with numerous 'caveolae', 30 – 50 nm in diameter, which are invaginations of the membrane into the inter-membrane gap. The sarcolemma, taut in stretched fibres and forming loose folds in shortened ones, is responsible for some of the mechanical properties of muscle, notably for its parallel elastic component (cf. Franzini-Armstrong, 1973).

Muscle fibres are filled with columns of myofibrils, 1 - 2 μm in diameter, separated by a 0.1 - 0.2 μm layer of sarcoplasm. These myofibrils run the length of the fibre though some branching may occur (Goldspink, 1970). The most characteristic features of skeletal muscle in the light microscope are the cross-striations, a series of regular alternate light (I) and dark (A) transverse bands. Both bands are bisected by transverse lines, in the middle of the I-band is the Z-line and the A-band, the M-band. This cross-striation is a property of individual myofibrils and not of the fibre (Bowman, 1840). The myofibril bands, arising from a variation in submicroscopic density, are usually arranged in the fibre with the patterns in register. The elements of the myofibril are organised parallel to the fibre axis and to the direction of mechanical function (Brücke, 1858). The submicroscopic longitudinal elements were rendered visible by the electron microscope and were called 'myofilaments' (Hall et al., 1946). They were shown by X-ray diffraction studies (Huxley, 1952) to be arranged in a hexagonal array parallel to the fibre axis and 45 nm apart. Electron-micrographs of transverse and longitudinal sections of glycerinated rabbit psoas and frog sartorius muscles (cf. 1.5.2) clearly showed the presence of two kinds of filament, one thick and one thin (Huxley, 1953); the presence of thick and thin
filaments varied with the location of the transverse cut. Muscle contraction was thought to be elicited through the relative sliding of these interdigitating thick and thin filaments (Hanson & Huxley, 1955).

Pervading the sarcoplasm between myofibrils is a reticular membrane network, the sarcoplasmic reticulum (Porter & Palade, 1957). The interior of the sarcoplasmic reticulum (SR) is a compartment separate from the sarcoplasm; the lumen is continuous throughout the cell so that most of its components are probably equilibrated (Porter, 1961). The SR actively takes up Ca\(^{2+}\) from the sarcoplasm using ATP as an energy source (Nagai et al., 1960; Ebashi, 1961a) and serves as a storage area where Ca\(^{2+}\) is accumulated during rest (Hasselbach & Makinose, 1961; Pease et al., 1965; Page, 1969), a process linked to the control of contraction (see 1.5.5). The distribution of SR corresponds to a regular spacing in close relation to the cross-striation. At the level of the I-band, the SR forms dilated 'terminal sacs', but at the A-I junctions longitudinally oriented slender sacs run along the myofibril to form a palisade around the A-band. There is a 'fenestrated collar', containing pores, across the centre of the sarcomere, joining the longitudinal SR elements (Porter & Palade, 1957; Peachey, 1965). Autoradiography indicates that Ca\(^{2+}\) is taken up by the longitudinal elements and is subsequently accumulated in the terminal sacs (Winegard, 1970). The storage areas were also highlighted by calcium oxalate deposition (Costanin et al., 1965; Kornick, 1969). The SR may have some involvement in glycolytic activity (cf. Franzini-Armstrong, 1973).

The plasmalemma not only bounds the fibre longitudinally but also invaginates transversely as the 'T-tubule network' (Franzini-Armstrong & Porter, 1964 (fish); Walker & Schrodt, 1965 (rat)). The mouths of the
T-tubule network are open to the outside of the fibre; fibres show an uptake of ferritin (Huxley H.E., 1964) and fluorescent dye (Endo, 1964) through this system. The T-tubule network is extensive (Eisenberg & Eisenberg, 1968; Flitney, 1970). The T-tubules and SR interact at the terminal sacs of the SR; the two sacs and one T-tubule form the 'triad' visible in electron-micrographs (Porter & Palade, 1957). The relationship between T-system and SR varies from muscle to muscle and species to species (cf. Franzini-Armstrong, 1973).

Skeletal muscle fibres are electrically excitable. The electrical impulse causes membrane depolarisation which propagates along the whole fibre and causes contraction to occur. Physiologically the depolarisation results from an indirect activation of the muscle fibre through the motor nerve. A single motor nerve fibre sends a neurofilament to each of a group of 5 - 200 muscle fibres. Coers (1967) has described a variety of motor innervation patterns in various animals. These, and other patterns, have been reviewed by Couteaux (1973). In moderate action motor units work in relays, but as the demand for power increases, they work synchronously. The description of the motor unit and its relationship with the sarcolemma have been reviewed by Couteaux (1973), Franzini-Armstrong (1973), Nachmansohn (1973), and Thesleff (1976). Permeability changes along the sarcolemma, caused through events triggered by the nerve impulse reaching the motor unit, are propagated through the T-tubule network (cf. Huxley A.F., 1954) causing the release of stored Ca\(^{2+}\) from the SR. Released Ca\(^{2+}\) is picked up by specific protein molecules of the myofibril and directly initiate the onset of contraction; relaxation occurs with the return of Ca\(^{2+}\) to the SR terminal sacs on cessation of the stimulus.
FIGURE 1.2

SECTION THROUGH A MUSCLE FIBRE

Porter & Armstrong (1965)
1.5.2 PROTEINS OF THE MYOFIBRIL

The energy used by muscle in the performance of work and the maintenance of tension is ultimately derived from chemical reactions going on within it. The study of muscle contraction has been closely linked to that of the metabolism of phosphate compounds. The nature of the chemical reactions involved has been discussed in 1.2 & 1.3. Knowledge of the muscle proteins began with the extraction of "myosin" (Kühne, 1864), a protein capable of forming a gel that is soluble at high and precipitated at low ionic strengths. Methods for the extraction and purification of "myosin" and details of its physico-chemical properties were described by Edsall (1930) and von Muralt & Edsall (1930a, 1930b). Bate Smith (1934) investigated the dependence of "myosin" solubility on pH and ionic strength, finding that under physiological conditions it is in the gel form. "Myosin" when injected into water through a fine needle was shown to form threads that showed X-ray diffraction patterns characteristic of intact muscle (Boehm & Weber, 1932; Weber, 1933). "Myosin" could hydrolyse ATP in the presence of divalent ions (Engelhardt & Ljubimova, 1939). The tensile strength of its threads decreased in the presence of ATP (Engelhardt et al., 1941). Needham et al. (1941) and Dainty et al. 1944 showed that the viscosity and flow birefringence of "myosin" solutions dropped in the presence of ATP, only to increase slowly once the ATP was hydrolysed. During 1941 - 1943, A. Szent-Györgyi and his collaborators found that "myosin" was a complex of (at least) two proteins. The major component was called myosin and the other actin (Straub, 1942). Myosin showed the ATPase property and bound to actin,

"Myosin" is used to distinguish this crude extract from the purified protein.
the complex formed by actin and myosin was renamed actomyosin.

Szent-Györgyi et al. (1942) found that threads of actomyosin, in the presence of Mg$^{2+}$ and K$^+$, contracted rapidly upon the addition of ATP; partially dried and stretched threads would become shorter and wider (Buchthal et al., 1947). Mommaerts (1947) found that the viscosity drop in actomyosin solutions caused by the addition of ATP could occur without dephosphorylation. Straub (1943) and Mommaerts (1947) demonstrated that pyrophosphate, which is not hydrolysed (Bailey, 1942), and inorganic triphosphate, which is only slowly hydrolysed (Dainty et al., 1944), both cause an irreversible fall in actomyosin viscosity. Direct evidence that ATP would dissociate actin and myosin, thereby decreasing viscosity and flow birefringence, was not satisfactorily shown until the ultracentrifugation studies of Weber (1956) and the light-scattering observations of Gergely (1956).

The use of glycerol-extracted rabbit psoas muscle as a model system, and subsequently myofibril suspensions, was introduced by Szent-Györgyi in 1949. Glycerol solubilises the membranes thereby increasing the permeability of the muscle. Fibre bundle preparations were used by Weber & Portzehl (1952, 1954) to study the dual role of ATP in producing relaxation (actin-myosin dissociation) when hydrolysis was prevented and contraction (actin-myosin reassociation) when splitting took place. Buchthal & Kahlson (1944) claimed to have shown that ATP could elicit the contraction of intact muscle fibres, confirming the earlier notion of Lohmann (1934) that ATP was the immediate source of energy, but it was not until Cain et al. (1962) showed that DNFB-poisoned muscle (to inhibit creatine kinase) hydrolysed ATP during contraction, that this point of view was generally accepted.
### TABLE 1.1

**PROTEINS OF THE MYOFIBRIL**

<table>
<thead>
<tr>
<th>Protein</th>
<th>% Myofibril by wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>myosin</td>
<td>55 - 60</td>
</tr>
<tr>
<td>actin</td>
<td>20</td>
</tr>
<tr>
<td>tropomyosin</td>
<td>4.5</td>
</tr>
<tr>
<td>troponin</td>
<td>3 - 5</td>
</tr>
<tr>
<td>α-actinin</td>
<td>2</td>
</tr>
<tr>
<td>β-actinin</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>M-protein</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>C-protein</td>
<td>1 - 2</td>
</tr>
</tbody>
</table>

Myofibril proteins constitute 60% by wt. total muscle proteins.
1.5.2.1 MYOSIN

Myosin (mol. wt. 460,000) is composed of one pair of 'heavy' chains (mol. wt. 200,000) and two pairs of 'light' chains (mol. wt. 15,000 - 27,000) that vary in mass according to the muscle source (cf. Lowey, 1979). Treatment of myofibrils with high ionic strength solutions containing magnesium pyrophosphate produces 'ghosts', which when viewed with phase-contrast and with the electron microscope show the absence of A-bands (Hanson & Huxley, 1953; Hasselbach, 1953). Microscope studies combined with quantitative measurements show that myosin is confined to the A-band (Hanson & Huxley, 1957). Perry & Corsi (1958) found that prolonged extraction of myofibrils with low ionic strength solutions (e.g. 5 mM Tris-HCl pH 7.7) dissolved out the material of the Z-lines and I-bands but left the A-bands intact. The ATPase activity of the structure remained associated with the A-band. Direct evidence of the localisation of myosin in the thick filament came from fluorescein-antibody studies (Finck et al., 1956; Klatzó et al., 1958; Marshall et al., 1959) and through the use of heavy metal-labelled antibody (Pepe et al., 1961).

MYOSIN FILAMENTS

Myosin is soluble at an ionic strength of 0.6 M, but forms aggregates at 0.04 M (Portzehl et al., 1950). These aggregates are filamentous; Huxley (1963), in a detailed study of isolated thick filaments and synthetic myosin filaments, has shown that the structure of these aggregates is similar to that of thick filaments. Four distinct phases of myosin aggregation are recognised: (1) in the range of ionic strength 0.6 M - 0.3 M, myosin exists essentially as monomers but a small number of dimers exist in equilibrium with monomeric myosin (Godfrey & Harrington,
1970); (ii) between 0.3 M and 0.2 M, filament formation begins and is completed (Kaminer & Bell, 1966); (iii) between 0.2 M and 0.07 M, no further polymerisation takes place; (iv) <0.07 M, random aggregation of filaments occurs and sedimentation takes place. Myosin filaments have diameters between 10 and 20 nm and show many short projections on their surfaces; these projections can be seen in the A-bands of thin sections of myofibrils. The projections are absent from a 0.15 - 0.20 μm long 'bare zone' in the centre of the filament, again similar to the zone observed in the thick filament (see also 1.5.2.5). Synthetic filaments are formed by the antiparallel association of myosin molecules (Fig. 1.4) and are variable in length. Noda & Ebashi (1960) reported lengths of between 1 and 2 μm; Huxley (1972b, p. 349) reports 0.5 - 1.4 μm. The diameters of the filaments are in the range 14 - 18 nm, not including the cross-bridge projections. Miyahara & Noda (1977) suggest that length depends on speed of dilution of the myosin solution, the final ionic strength, pH, and protein concentration. The length of the thick filament is constant within any given muscle type and is 1.6 μm in vertebrate muscle (see 1.5.2.5).

**STRUCTURE OF MONOMERIC MYOSIN**

Myosin is an asymmetric molecule whose image in the electron microscope resembles a rod 134 nm in length and 2 nm in diameter to which are attached, at one end, two globular units each of diameter 9 nm (Slayter & Lowey, 1967; Lowey et al., 1969). The total length of the molecule appears to be between 140 and 155 nm (Cohen et al., 1969). The duplex nature of the myosin globular region was demonstrated by direct observation (Slayter & Lowey, 1967; Lowey et al., 1969). The heads are separated and each is attached to the rod by a long 'neck'. The details
of myosin structure have been elucidated by sectioning the molecule into discrete functional areas with the proteolytic enzymes trypsin (Mihályi & Szent-Györgyi, 1953; Szent-Györgyi, 1953; Mueller & Perry, 1961; Lowey et al., 1967), chymotryptic (Gergely, 1953; Jones & Perry, 1966; Hotta & Usami, 1967), papain (Kominz et al., 1965; Nihei & Kay, 1968; Lowey et al., 1969), and subtilisin (Middlebrook, 1958; Jones & Perry, 1966). These studies demonstrate the existence of three functional areas in the myosin molecule (Fig. 1.3).

The overall shape of the molecule is due to the two heavy chains. Each chain is continuous from one end of the molecule to the other. The fibrous LMM+ rod is composed of the two chains intertwined in a coiled-coil \( \alpha \)-helical configuration (Szent-Györgyi et al., 1960; Lowey & Cohen, 1962; Cohen & Holmes, 1963) and is the portion of the molecule responsible for the polymerisation properties of myosin; LMM is soluble at high and insoluble at low ionic strengths. In contrast, HMM, which is composed of the two functional areas S-1 and S-2 (Lowey et al., 1967, 1969), is water soluble. S-2 is nearly entirely a coiled-coil \( \alpha \)-helix (Cohen & Holmes, 1963) and is water-soluble above pH 5 (Lowey et al., 1967). S-1 is very water-soluble, has a low \( \alpha \)-helical content, and contains the ATPase and actin-binding properties of myosin; its dimensions are approximately \( 15 \times 4.5 \times 3 \) nm and it is in the shape of a 'bent finger' (Moore et al., 1970). The backbone of myosin filaments (and hence thick filaments) is believed to be made up of LMM portions of the molecules, with the HMMs projecting out sideways at regular intervals (Fig. 1.4).

† See Fig. 1.3 for abbreviations.
FIGURE 1.3

FUNCTIONAL AREAS OF MYOSIN

Offer & Elliott (1978)
Pepe (1971)

Lowey (1971)
Myosin contains the unusual amino acids 3-methylhistidine and N^6-methyllysine (cf. 1.5.2.2). The function of these residues is not known but Trayer et al. (1968) have found that slow muscle myosin does not contain 3-methylhistidine. This residue, at least, may be involved with the regulation of contraction.

MYOSIN AS AN ENZYME

Three forms of enzyme activity in myosin are recognised: (1) Mg(and Mn) ATPase which is linked to contractile responses and is of low activity; (2) Ca(and Sr) ATPase of high specific activity but does not induce contractile responses; (3) KATPase, markedly activated by magnesium-chelators such as EDTA (cf. Ebashi & Nonomura, 1973, pp. 292 - 295). Since Kielley & Bradley (1956) showed that the ATPase of myosin could be modified by blocking SH groups with PCMB, a number of reagents have been reported to alter the enzyme activity in various ways. The monitoring of the reactivity of SH groups in myosin has proved to be valuable for detecting conformational changes in myosin (and other proteins). Out of the 19 SH groups in each heavy chain, two (thiol-1 and thiol-2) are essential for enzyme activity (cf. Ebashi & Nonomura, 1973, pp. 296 - 301; Kunz et al., 1980). The chemical modification of these two SH groups shows that Mg, Ca, K, ATPases behave independently. Discussion of the kinetics of myosin ATPase is in 1.5.4.

LOW MOLECULAR WEIGHT SUBUNITS

Although the generally accepted view of myosin before 1970 was one in which a molecule of myosin was composed of two subunits (the heavy chains), it had been suggested as early as 1953 by Tsaö that a small amount of low molecular weight protein was present in myosin preparations.
Since myosin was known to be difficult to purify, it was generally assumed that minor components were contaminants. For example, Byrnes & Suelter (1965) comment that "no preparation of rabbit muscle myosin has been reported that is free of 5'-AMP deaminase". Even in more highly purified preparations (e.g. Harris & Suelter, 1967) low molecular weight components persisted. It was finally confirmed that 1 mole myosin contains 4 moles of 'light' chains (Weeds & Lowey, 1971). These light chains play regulatory roles. One pair is essential for ATPase activity, whilst the second pair regulates the actin-myosin interaction (Lowey, 1979). The former are often known as 'alkali light chains', since they are dissociated from myosin at pH > 10, and the latter as 'DTNB light chains', removable by treatment with DTNB. In rabbit muscle myosin, two types of alkali light chains are present, designated A-1 (mol. wt. 21,000 - 25,000) and A-2 (mol. wt. 16,000 - 17,000); DTNB has a mol. wt. in the range 18,000 - 19,000 (cf. Lowey, 1971).
The major component of the thin filament is actin (Hanson & Huxley, 1955, 1957) which can be removed from myofibrils by treatment with 0.6 M KI (Szent-Györgyi & Szentkirályi, 1960). Myosin-extracted myofibrils bind fluorescein-labelled HMM exclusively in the I-band (Aronson, 1965). Actin-antibody binds to the thin filaments (Marshall et al., 1959; Szent-Györgyi et al., 1964) as do the heavy metal-labelled antibody (Pepe et al., 1961). Actin is extractable from acetone-dried myosin-free muscle by treatment with water at pH 7 (Straub, 1943). Such actin is in a monomeric form (G-actin) that can be polymerised into a fibrous gel (F-actin) by the addition of salt. Direct evidence that F-actin consists of long fibrous chains was through the use of the electron microscope (Rózsa et al., 1949). G-actin loosely binds ATP which is split into ADP and P_i during polymerisation, actin molecules tightly binding ADP in the polymerised form (Straub & Feuer, 1950).

**STRUCTURE**

Monomeric actin is a water-soluble protein composed of a single polypeptide chain (mol. wt. 41,785) of 374 residues; the amino acid sequence of rabbit muscle actin (Elzinga & Collins, 1973, 1975) and actin from a variety of other skeletal, smooth, and heart muscles and non-muscle tissue have been determined (for reviews, see Vandekerckhove & Weber, 1978a, 1978b). Most of the sequence is highly conserved, but the N-terminal peptide of 17 - 18 residues carries a disproportionately high number of amino acid changes. Actin is not a spherical molecule; its dimensions are 4.5 nm tangential $\times$ 4.0 nm axial $\times$ 2.5 nm radial (Wakabayashi et al., 1975). Slightly larger figures are given by Moore et al. (1970), i.e. $5.5 \times 5.0 \times 3.5$ nm, values believed by Wakabayashi
et al. (1975) to be in error due to a contamination of purified actin with tropomyosin. There are 1 residue of 3-methylhistidine (3-MeHis$_{73}$), 5 SH groups, and 19 proline residues. Brandts et al. (1977) and Creighton (1978) suggest that large numbers of proline residues (i.e. about 20 per molecule) would have a special significance in the kinetic process of conformational change. Proline residues serve to break up the molecule into well-defined domains (Brandts et al., 1975). The occurrence of 3-MeHis in muscle proteins is restricted to myosin and actin (Perry et al., 1967). Laki (1969) has proposed that the function of 3-MeHis in actin is to establish the identity of the protein during cellular development thus permanently assigning the molecule to the building of thin filaments rather than to that of the mitotic spindle.

It is probably synthesised by enzymic methylation of the protein suggesting that this residue is on the surface and in contact with solvent (Asatoor & Armstrong, 1967; Hardy & Perry, 1969). There are no disulphide bridges in actin. Only 1 SH group is involved in polymerisation (Katz & Mommaerts, 1962; Martonosi, 1968). One residue (Cys$_{373}$, see Elzinga et al., 1973) is not available for reaction with iodoacetamide or iodoacetate in the native molecule (Bridgen, 1972). Cys$_{373}$, however, reacts quickly with NEM but has its reactivity reduced when actin interacts with myosin (Lusty & Fasold, 1969). Ishiwata (1976) has shown that Cys$_{373}$ becomes oxidised during the freezing of G-actin solutions and that the presence of dithiothreitol prevents the oxidation. This reagent would also reduce oxidised actin. This oxidation prevents polymerisation. Cohen et al. (1972) have commented that actin prepared from acetone-dried muscle becomes oxidised during its isolation more readily than actin obtained from frozen rabbit muscle.
BOUND NUCLEOTIDE

G-actin contains one exchangeable ATP per molecule (Straub & Feuer, 1950; Martonosi et al., 1960). Actin will bind other nucleotides in place of ATP, the individual binding constant depending on both base structure and phosphate content (Seidel et al., 1967). The binding constant for ATP is 30 - 100 times greater than that for ADP. G-actin will rapidly exchange bound ADP for free ATP, suggesting that G-(ATP)-actin is more stable than G-(ADP)-actin under the same environmental conditions (Strohman & Samarodin, 1962). The binding of any nucleotide causes major protein conformational changes (Standaert & Laki, 1962; Kominz, 1965).

BOUND DIVALENT ION

G-actin prepared from acetone-dried muscle by classical methods (e.g. Straub, 1943; Mommaerts, 1952; Spudich & Watt, 1971) contains 1 bound Ca\(^{2+}\) per protein molecule (Maruyama & Gergely, 1961; Bárány et al., 1962). G-actin will exchange bound Ca\(^{2+}\) for an alternative divalent ion in the solvent (Bárány et al., 1962; Kasai & Oosawa, 1968). The binding

\[+\] The following nomenclature for identifying actin species is used in this thesis:

- G-(ATP)-actin : monomeric actin with bound ATP
- G-( )-actin : nucleotide-free monomeric actin
- F-(ADP)-actin : polymeric actin with bound ADP
- F-( )-actin : nucleotide-free polymeric actin

Other types of bound nucleotide are shown in a similar way; bound metal ion is shown in parentheses after the nucleotide.
constant for Ca$^{2+}$ is 4 times greater than that for Mg$^{2+}$. Cation replacement does not alter the ability of actin to polymerise (Martonosi et al., 1964). The structure of actin depends, not only on the type of nucleotide, but also on the kind of bound divalent ion since differences in the denaturation rates of the various species of actin are observed (Strzelecka-Golaszewska et al., 1968). Both bound nucleotide and divalent ion are required for the retention of structural integrity in G-actin (Martonosi et al., 1960).

**POLYMERISATION AND DEPOLYMERISATION**

As reported above, addition of cations to G-actin results in polymerisation. Most cations that do not cause denaturation initiate the transformation of G to F-actin (Kasai & Oosawa, 1968; Strzelecka-Golaszewska & Drabikowski, 1968). Both bound nucleotide and divalent ion are retained in the polymer, 1 mole of each per mole actin; triphosphates are split during the transformation (Martonosi et al., 1960). Polymerisation follows immediately after cleavage of the phosphate-ADP bond (Tonomura, 1965; Stowring et al., 1966). ATPase activity is not a requirement for the polymerisation process. Thus, both G-(ADP)-actin (Hayashi & Rosenbluth, 1960; Grubhofer & Weber, 1961) and G-( )-actin (Némethy & Scheraga, 1963; Kasai et al., 1965) are capable of polymerising; G-( )-actin must be stabilised in 50% sucrose. The polymerisation rate of G-(ATP)-actin is much greater than that of other forms (Oosawa et al., 1965). Both nucleotide and divalent ion are firmly bound to F-actin (Martonosi et al., 1960; Bárány et al., 1962). If F-actin is dialysed against salt solution for 20 days then the polymer free of both nucleotide and divalent ion can be obtained (Mommaerts, 1952; Kasai & Oosawa, 1963). F-actin prepared from G-(ATP)(Mg)-actin is more stable
than the corresponding $\text{Ca}^{2+}$ type; actin in the thin filaments of muscle is possibly the $\text{Mg}^{2+}$ type (Weber, 1966; Kasai, 1969; cf. Ebashi & Nonomura, 1973, p. 322).

Depolymerisation of F-actin requires a decrease in ionic strength to less than 0.01 (Straub & Feuer, 1950; Mommaerts, 1951) or an increase above 0.5 (Guba, 1950; Szent-Györgyi & Szentkiralyi, 1960). Guba (1950) found that 0.5 M KI caused instantaneous depolymerisation, KCl and KBr producing a slower response. Cycles of depolymerisation and polymerisation are used in the purification of actin. Denaturation of G-actin occurs rapidly if ATP is not present in the medium. Hama et al. (1965, 1967) have prepared F-actin from myofibrils by direct isolation without the use of any depolymerisation process or acetone-drying of the muscle. Dialysis of this 'natural' F-actin against a low ionic strength medium containing ATP promoted depolymerisation but did not yield G-(ATP)-actin capable of repolymerisation. This G-(ATP)-actin was also unstable. 'Straub-type' G-actin (obtained by extraction of acetone-dried muscle by water or ATP solutions of low ionic strength; this type of procedure is the usual method, cf. 2.2.3) could be obtained by extraction of acetone-dried natural F-actin with a low ionic strength ATP solution, or by depolymerisation of natural F-actin with 0.6 M KI. Mechanical disruption of natural F-actin in a low ionic strength medium containing ATP also converts natural F-actin into Straub-type G-actin. Nearly all experimental work has been carried out on Straub-type actin. Natural F-actin filaments have been shown to be terminated by the protein actinin (see 1.5.2.5: Z-line).
CONFORMATIONAL CHANGES

Reversible structural deformations in F-actin have been proposed to account for the ability of F-actin to behave as an ATPase during ultrasonication (Asakura, 1961). These conformational changes may be due to a loosening or rupturing of portions of the polymer in such a manner that monomeric units created within the chain behave like G-actin molecules. The interaction between monomers appears to be essentially hydrophobic since increasing temperature favours depolymerisation (Asakura et al., 1960). Neither bound nucleotide nor divalent ion appear to be directly involved in holding the polymer together (cf. Mommaerts, 1952; Kasai & Oosawa, 1963). The addition of a small amount of F-actin to a solution of G-(ATP)-actin in the presence of cations was found to accelerate the polymerisation rate (Kasai et al., 1962). Much greater acceleration is caused by adding F-actin fragments obtained by ultrasonication (Asakura et al., 1963). Asakura et al. (1960) and Kasai et al. (1962) have demonstrated that at ionic strengths intermediate between salt-free and physiological, G-(ATP)-, G-(ADP)-, and F-(ADP)- actins are in dynamic equilibrium. It is believed that there is a critical concentration of monomer in equilibrium with polymerised actin. The critical concentration of monomers (0.95 μM) remains constant independent of the polymer concentration. When the total actin concentration is below 0.95 μM no polymers are formed (Oosawa & Kasai, 1971b cf. Loscalzo et al., 1975, 1977). Rich & Estes (1976) have proposed a scheme for polymerisation after experiments on the sensitivity of G-(ATP)-, G-(ADP)-, and F-(ADP)- actins to digestion by proteolytic enzymes. Their data support the existence of a species of actin called F-(ATP)-actin monomer. This species has the same physical properties of G-actin but F-actin-like low sensitivity to digestion; both G-(ATP)- and G-(ADP)-
actins have similar high rates of digestion. Rich & Estes conclude that the type of actin that must reach critical concentration in order to form polymers is not G-(ATP)- nor G-(ADP)- actin but F-(ATP)-actin monomer.

There is no appreciable difference in the u.v. absorption spectra of G-(ATP)- and G-(ADP)- actins (Higashi & Oosawa, 1965); CD and ORD measurements are similar (Nagy & Jencks, 1963). However, the existence of changes in structure during polymerisation is supported by the occurrence of differences in u.v. spectra of monomeric and polymeric actins. These differences apparently originate from changes in environment of tyrosine and tryptophan residues (Higashi & Oosawa, 1965; West, 1970). Further evidence for a change in conformation in the G-F transformation is provided by observed differences in CD spectra (Murphy, 1971) and in intrinsic fluorescence (Lehrer & Kerwar, 1972). Burley et al. (1972) have shown that the electron spin of Mn$^{2+}$ placed in the divalent site of actin interacts with the spin of a nitroxide label$^+$ attached to a fast reacting SH group. This SH group is Cys$^{373}$ (cf. Lusty & Fasold, 1969; Loscalzo et al., 1977). Propagated conformational changes in actin filaments are proposed by Loscalzo et al. (1975,1977) to account for changes in the e.s.r. signal of nitroxide-labelled F-(ADP)(Mn)-actin on the binding of S-1 or HMM, in the absence of tropomyosin and troponin. The changes do not occur in the presence of tropomyosin and troponin; the binding of Ca$^{2+}$ to the actin-tropomyosin-troponin complex causes a slight change in spin-spin interaction which is reversed by myosin binding.

$^+$ N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) maleimide
ACTIN FILAMENTS

The polymer formed in the G-F transformation described above, is a two-stranded helix with subunits repeated at approx. 5.5 nm intervals in the chain (Hanson & Huxley, 1963, 1964a). All the actin monomers are positioned in the filament with the same structural polarity; actin combined with HMM or S-1 is a composite helix which displays a characteristic 'arrowhead' appearance in the electron microscope since the myosin heads attach to the outside of the actin double helix in the same sense (Huxley, 1963). The sense of the helix is right-handed (Depue & Rice, 1965). Comparison of data from electron micrographs and X-ray diffraction studies show the monomers to be separated by 5.46 nm and the two strands to be staggered by 2.73 nm (cf. Huxley, 1972b; Wray et al., 1978). These values vary only slightly amongst different muscles, but the cross-over points (half-pitch) of the helix range from 35 - 41 nm (Huxley & Brown, 1967; Hanson & Lowy, 1964; Miller & Tregear, 1972). Such values set the number of actin monomers between cross-over points as 13, 14, or 15. Nonomura et al. (1975) have reported that filaments prepared from highly purified actin (Spudich & Watt, 1971) tended to be fragmented and disordered when polymerisation was achieved by 0.6 M KCl/2 mM MgCl2/10 mM Tris-HCl pH 7.6. However, well-formed filaments with a half-pitch of 36 nm were observed if 1 - 10 mM phosphate was present in the polymerisation medium. Ebashi & Endo (1968) had speculated that actin used by, for example, Hanson & Lowy (1963) would have been contaminated with tropomyosin and troponin (cf. 1.5.2.3 & 1.5.2.4) which would have probably regulated filament formation by binding to actin. Wray et al. (1978) have commented that "although the functions of the regulatory proteins are now becoming clear (1.5.2.3, 1.5.2.4, & 1.5.5.6), many questions still remain about their precise
structural relationships in the thin filament". Between 13 and 14 monomers per turn of 36 to 37 nm of the double helix are favoured (cf. Wray et al., 1978; Pepe, 1971; Huxley, 1972b).

The thin filament does not have a well-defined length in vitro, ranging from 0.1 - 20 μm with a distribution peak at about 1 - 2 μm (cf. Nonomura et al., 1975); filament length does not depend on the type of bound divalent ion (Arisaka et al., 1973). Natural actin filaments have lengths between 1 - 2 μm (Hama et al., 1965). In vertebrate muscles the length of the thin filament is approx. 1 μm (Page & Huxley, 1963). Further discussion of the lengths of thin filaments is in 1.4.2.5. Thin filaments are approx. 10 - 20 nm in diameter.

Evidence that bound adenine nucleotide was present in the thin filament was originally observed through the use of the quartz microscope. Muscle fibres irradiated with 260 nm light gave photomicrographs with absorption bands corresponding to I-bands (Caspersson & Thorell, 1942). This was later confirmed by Hill (1964) who obtained autoradiographs showing a concentration of radioactivity in the I-bands of toad muscle which had been incubated with tritiated adenine nucleotide. Pettko & Straub (1949) discovered that frog muscle contained approx. 3 μmol ADP per g dry muscle, in a form inaccessible to enzymes. Perry (1952) estimated that myofibrils contained 4.1 μmol adenine nucleotide per g, of which 22% was AMP, 67% ADP, and 12% ATP. Natural F-actin contains bound ADP (Hama et al., 1965, 1967). Nucleotide bound to actin in acetone-dried muscle is in the form of ADP (Tsuboi, 1963). It is generally believed, therefore, that the thin filament of muscle is composed of F-(ADP)-actin.
1.5.2.3 TROPONIN

The role of Ca$^{2+}$ as a mediator between nerve signal, surface membrane effects, and intracellular processes was recognised by Heilbrunn (1940). Bailey (1942), in pointing out the physiological importance of Ca$^{2+}$ as a stimulating agent of myosin ATPase, introduced the concept of specific regulators which promote relaxation and contraction. A crude muscle extract, the 'Marsh-Bendall factor' (Marsh, 1951, 1952; Bendall, 1953, 1954), was shown to convert glycerol-extracted muscle from a contracted to a relaxed state. Kumagai et al. (1955) suggested that the factor was identical to the Kielley-Meyerhof microsomal ATPase (Kielley & Meyerhof, 1948). The relaxing factor particles (or microsomes) were shown to accumulate Ca$^{2+}$ from the medium using ATP as a source of energy (Nagai et al., 1960; Ebashi, 1961a, 1962; Hasselbach & Makinose, 1961). The effective component of this factor is a suspension of vesicles created by the breakdown of the SR and T-tubules (Muscatello et al., 1961; Ebashi & Lipmann, 1962; cf. 1.5.1).

In the intact muscle, Ca$^{2+}$ is stored in the SR terminal sacs (1.5.1). Relaxation occurs when the calcium concentration is reduced below $10^{-7}$ M (Ebashi, 1962). A rise in free Ca$^{2+}$ to $10^{-5}$ M initiates contraction. Propagation of membrane depolarisation along the sarcolemma and T-system to the SR causes the terminal sacs to release Ca$^{2+}$. Pumping of Ca$^{2+}$ back into the SR occurs at the rate of 2 Ca$^{2+}$ per ATP hydrolysed (Weber et al., 1966). The unique effect of Ca$^{2+}$ in activating actomyosin MgATPase (Weber, 1959; Ebashi, 1961b), in initiating actomyosin superprecipitation (Ebashi, 1961b; Weber & Winicur, 1961), and in eliciting the MgATPase and contraction of myofilaments (Seidel & Gergely, 1963) and glycerinated fibre bundles (Ebashi, 1961a) has been clearly demonstrated.
However, the degree of calcium-sensitivity of actomyosin depended, for the most part, on actin purity; 'natural' actomyosin, prepared by high ionic strength extraction of minced muscle (cf. 1.5.2.1 & below), required Ca$^{2+}$ for activation (Maruyama et al., 1964; Kominz, 1966), whereas 'synthetic' actomyosin, obtained by mixing purified actin and myosin, did not (Schaub et al., 1967). Actomyosin, like myosin, is soluble at high ionic strength and is precipitated at low. Natural actomyosin may be desensitised by two to five cycles of dissolution and precipitation (Schaub et al., 1967). The Ca$^{2+}$-sensitising factor, protein in nature, was shown by Ebashi & Kodama (1965) to have two components, tropomyosin (cf. Bailey, 1946) and troponin. The Ca$^{2+}$-troponin-tropomyosin complex constitutes a regulatory system that activates or inhibits actomyosin MgATPase and muscle contraction. The role of tropomyosin (TM) is discussed in 1.5.2.4.

**SUBUNIT STRUCTURE OF TROPOVIN (TN)**

Purified TN (mol. wt. 80,000) is a complex of three subunits (Greaser & Gergely, 1971). These three components have unique structures and distinct functions and have been designated the abbreviations TN-T (tropomyosin binding, mol. wt. 31,000 - 36,000), TN-I (inhibitor of actomyosin ATPase, mol. wt. 21,000 - 24,000), and TN-C (calcium-binding, mol. wt. 17,850).

TN-T binds to TM and TN-C but only has a weak interaction with TN-I (Drabikowski et al., 1972; Greaser et al., 1972). It is a dumb-bell shaped molecule, approx. 10 nm in length and with a maximum width of 10 nm (Ohtsuki, 1975, 1979; Stewart, 1975). The sequence of rabbit muscle TN-T is known (Pearlstone et al., 1976). The TM interactive region of
TN-T is thought to be between residues 70 and 160 (Jackson et al., 1975) or 71 and 151 (Pearlstone & Smillie, 1977). Binding to TM is considered to be centred around Cys-190 of TM, with the main binding taking place on residues 197 to 217 of TM (McLachlan & Stewart, 1976b). Nagani et al. (1980) find that residues 90 to 148 of TN-T are in a triple-stranded coiled-coil with TM.

The mol. wt. of TN-I varies with muscle fibre type (Perry, 1979); the amino acid sequences of rabbit fast (Wilkinson & Grand, 1974) and slow (Grand & Wilkinson, 1977) muscle TN-I have been determined. The molecule is roughly spherical with a diameter of 1.0 - 1.5 nm (Dąbrowska et al., 1975).

The amino acid sequence of rabbit muscle TN-C has been determined (Collins et al., 1977). Like TN-I the general shape of the molecule is spherical (Dąbrowska et al., 1975). TN-C has 4 Ca\(^{2+}\) binding sites, two of which have binding constants \((K_{Ca})\) of \(3 \times 10^5\) M\(^{-1}\) (sites I and II) and the remaining pair (sites III and IV) with \(K_{Ca} = 2 \times 10^7\) M\(^{-1}\) (Potter & Gergely, 1975). Sites I and II are specific for Ca\(^{2+}\) but III and IV will bind Mg\(^{2+}\), \(K_{Mg} = 5 \times 10^3\) M\(^{-1}\). Under physiological conditions, these latter two sites will be occupied by Mg\(^{2+}\) and therefore sites I and II are the relevant Ca\(^{2+}\) binding sites. The metal ion binding constants for the TN-TM complex are considered to be 10-fold greater. The binding of divalent ion causes a major structural change to take place in TN-C with the formation of a more compact structure (Kretsinger & Barry, 1975). An increase in α-helical content occurs (Potter et al., 1976; Seamon et al., 1977). Kretsinger & Barry (1975) and Tsalkova & Privalov (1980) consider sites I and II to be a Ca\(^{2+}\)-specific co-operative block and III and IV a non-specific block. Tsalkova & Privalov (1980) have shown that
the specific block is very stable even in the absence of Ca but becomes more stable when the divalent ion(s) binds. The non-specific block, on the other hand, requires a divalent ion for stability.

TROPONIN COMPLEX

The TN complex is a combination of 1 molecule of each subunit; the various combinations of the three subunits with each other in vitro have been reviewed by Ebashi (1974, 1977). The inhibitory action of TN-I on actomyosin MgATPase and contraction of muscle is relieved by the binding of Ca^{2+} to the specific block of TN-C. The complex between TN-I and Ca^{2+}-TN-C is extremely stable (Head & Perry, 1974). Bárány & Bárány (1980) suggest that TN-I and TN-C may actually be complexed in both resting and contracting states and, therefore, it is the quality of interaction that alters on the binding of Ca^{2+}. TN-C has an affinity for TN-T; Ebashi (1977) assumes that the crucial process of TN function is the binding of TN-I to TM in the absence of Ca^{2+} and that the quality of the interaction between TN-I and TN-C is modified through the TN-T/TN-C complex.

1.5.2.4 TROPOMYOSIN

Tropomyosin (mol. wt. 66,000) is composed of two polypeptide chains, α and β, in a coiled-coil α-helical configuration (Cohen et al., 1972; Hodges et al., 1972). Preparations of rabbit skeletal muscle tropomyosin (TM) are heterogeneous mixtures of αα and αβ dimers (Lehrer, 1975). Cummins & Perry (1972) found that the ratio of α to β in these preparations was 4:1, this combination probably reflecting unequal distribution of dimers in different fibre types. The amino acid sequences of the α-chain (Stone & Smillie, 1978) and the β-chain (Mak et al., 1980) are known.
The dimer is 40 - 41 nm in length (Cohen & Longley, 1966; Stone et al., 1974; Longley, 1977) and 1.8 nm wide (Longley, 1977).

1.5.2.3 STRUCTURE OF THE SARCOMERE

The contractile unit of striated muscle, the sarcomere, is bounded longitudinally by the SR and transversely by the Z-line. The sarcomere is composed of the alternate, interdigitating, thick myosin-containing and thin actin filaments (Fig. 1.1). Thin filaments, found in each half-sarcomere, are attached to the Z-line boundaries; they are not continuous into the adjacent sarcomeres across this boundary. Thick filaments are located between opposing half-sarcomere thin filaments. In vertebrate skeletal muscle, the thick filaments are held in register by M-protein cross-links at the mid(M)-band region of these filaments (cf. Herasymowych et al., 1980). In three dimensions, the thick and thin filaments, M-band, and Z-line constitute a super-lattice.

Contraction and stretch occur through the relative sliding of the interdigitating thick and thin filaments (1.5.3). In fully contracted sarcomeres, the free ends of opposing thin filaments collide at the M-band; in fully stretched muscle, the thick and thin filaments no longer overlap. Resting and partially contracted muscle show intermediate degrees of overlap. Cross-bridges are evident between the two sets of filaments (Huxley, H.E., 1957); these projections from the myosin filament are the sites of energy transduction, i.e. ATPase activity. The bridges are not present along the entire length of the thick filament, there is a 'bare zone' in the middle (Huxley, 1963; see also 1.5.2.1). Skeletal muscle is a complex structure. This complexity is due to the
large numbers of different molecular interactions involved and, because the filaments are not very long with respect to molecular dimensions, these interactions are continually changing over much of the filament length (Craig, 1977). The elucidation of the molecular arrangements in the sarcomere has been the subject of intensive research.

**THICK AND THIN FILAMENTS**

The ratio of thick to thin filaments in vertebrate muscle is 2 : 1 and in insect flight muscle 3 : 1 (cf. Hoyle, 1969). In both types of muscle, the filaments are arranged in a highly ordered hexagonal lattice; in the overlap region, vertebrate thin filaments occur at the trigonal position between three thick filaments, but in insect flight they are positioned between only two. In Lepidoptera, however, there are between 7 and 10 thin filaments around each thick one (Reger & Cooper, 1967). Resting sarcomere lengths are inversely related to the maximum speed of contraction and relaxation; filament lengths vary accordingly. Two examples are given below:

- **Vertebrate fast**: sarcomere 2.3 - 2.8 μm; thick 1.6 μm; thin 1 μm.
- **Crab leg slow**: sarcomere 12 - 15 μm; thick 10 μm; thin 6 μm.

It is fundamental to the acceptance of sliding filaments that the lengths of the filaments remain constant between contracted and relaxed states (cf. Huxley, 1980). Carlson et al. (1961) and Page & Huxley (1963) observed small but significant changes in filament lengths. However, the latter authors found that if attempts were made to minimize distortions during the preparation of material suitable for observation, the changes were very much less. The most decisive evidence for constancy of filament length comes from X-ray diffraction observations on live muscle during
passive stretch and active contraction (see Huxley, 1972b, pp. 335 - 337).

The major protein of the thick filament (diameter 15 - 20 nm) is myosin (1.5-2.1). Other proteins are present in small amounts (Offer, 1972; Pepe & Drucker, 1975). One such protein, 'C-protein', has been isolated (Offer, 1972) and is known to bind to the surface of myosin filaments in vitro (Craig & Offer, 1976). C-protein also binds to LMM (Moos, 1972) and to S-2 (Starr & Offer, 1978). The X-ray diffraction studies of Elliott (1964) and Huxley & Brown (1967) and the electron microscope observations of Pepe (1967) and Moos et al. (1975) show a 14.3 nm and a 43 nm repeat along the myosin filaments; these are due to the emergence of myosin heads every 14.3 nm. These heads are visible as short projections of length 10 - 40 nm. The heads are absent from a central zone, 150 - 200 nm in length. Both the distribution of the myosin projections on the surface of the cylindrical myosin filament and the precise arrangement of the rod portions of the myosin molecules, constituting the backbone of the filament, are uncertain. Three models for the number of myosin (vertebrate) cross-bridges that emerge per 14.3 nm have been proposed (Fig. 1.4). The acceptance of any model depends on the quantity of myosin present in the thick filament. Estimates of the myosin content have been calculated on the basis of either the mean mass per unit length of a large number of filaments or the ratio of myosin to actin, where the latter is estimated from the ADP content of the sarcomere. Lamvik (1978) has introduced the use of the scanning transmission electron microscope for measuring the thick filament mass; the principle is that the number of electron-scattering events is proportional to the number of regularly arranged scattering units in the specimen. Lamvik believes his data to be consistent with 3 myosin molecules per 14.3 nm repeat. Craig (1977), in studying the structure of isolated A-segments from frog
and rabbit muscle, has observed the presence of 11 transverse stripes in each half-segment. The stripes are 43 nm apart. The distal 7 stripes in each half-segment are believed to be due to C-protein (cf. Craig & Offer, 1976). Craig (1977) argues that if C-protein and myosin molecules exhibit the same filament repeat (i.e. $3 \times 14.3 = 43$ nm), then the location of C-protein provides information on the packing of myosin.

Thin filaments (diameter 10 - 12 nm) are built up of actin monomers in a double-stranded helix of half-pitch 35 - 41 nm (1.5.2.2). TM dimers are associated head-to-tail to form filaments in both grooves between the actin chains (Haselgrove, 1972; Huxley, 1972a). Each dimer spans 7 actin monomers in each strand (Spudich et al., 1972; Weber & Murray, 1972). Thin filaments show an axial repeat of 38.5 nm (Huxley & Brown, 1967). Both TM and TN have been located in the thin filament by fluorescent antibody staining (Endo et al., 1966; Pepe, 1966; Ohtsuki et al., 1967); the location of TN coincides with the 38.5 nm repeat. The TN complexes are attached to TM filaments in diametrically opposed pairs. TN is bound to TM mainly through TN-T (cf. 1.5.2.3). Lehrer (1980) believes that a reversible S-S link forms between the two TM polypeptide chains at Cys-190 to alter the degree of folding and flexibility with TN-T. Wray et al. (1978), using X-ray diffraction patterns of fast lobster and crayfish muscle, calculate that the TN complex may be represented by 6 nm spheres centred at 8 nm from the thin filament axis. Wray et al. (1978) consider the 38.5 nm repeat sequence to be the functional unit of the regulatory system (Fig. 1.4).

M-BAND

The function of the M-band, observed in vertebrate muscle lying between
the centres of adjacent thick filaments, is believed to be to hold the
thick filaments in register (cf. Herasymowych et al., 1980). Some
invertebrate muscles have been shown, through X-ray diffraction studies,
to have random thick filament orientations, and correspondingly these
muscles have no M-band (Jahromi & Atwood, 1969). The M-band is built up
of a number of major and minor filaments; the major bridges cross-link
the thick filaments, the minor ones the M-bridges themselves. The
architecture of this band is debatable (Pepe, 1975; Luther & Squire,
1978).

M-protein is composed of two principal components (Trinick & Lowey, 1977;
Walliman et al., 1977a, 1977b). One protein, extractable from myofibrils
by repeated treatment with low ionic strength media, is a dimer of mol.
w t. 80,000 and has been identified as creatine kinase (Morimoto &
Harrington, 1972). This has been confirmed in situ by antibody staining
(Walliman et al., 1977a, 1977b). The second component (mol. wt. 165,000)
has been designated the name 'M-protein' (Masaki & Takaiti, 1974;
Trinick & Lowey, 1977). Herasymowych et al. (1980) have shown that
creatine kinase and purified M-protein will reconstitute the M-band of
M-protein depleted muscle.

Z-LINE AND ACTININS

A minor protein component of muscle was shown by Maruyama (1965) to
inhibit F-actin network formation. This protein, \( \beta \)-actinin, was found
in natural F-actin (1.5.2.2; Hama et al., 1965); natural F-actin will
not seed the polymerisation of G-actin (Kasai & Hama, 1969). The free
end of a thin filament is terminated with \( \beta \)-actinin. The thin filament
is attached to the Z-line through '\( \alpha \)-actinin' (Goll et al., 1969). Both
actinins have amino acid compositions similar to, but distinct from, that of actin (cf. Maruyama, 1971). Each type of actinin exists as a dimer of mol. wt. 180,000 (Suzuki et al., 1973). α-actinin has been located in the Z-line (Goll et al., 1967; Masaki et al., 1967). The Z-line is built up of other proteins in addition to α-actinin (Robson et al., 1970). The function of the Z-line is primarily to maintain the structural integrity of the sarcomere and thus the myofibril as a whole (Pepe, 1971).

Purified α-actinin has the ability to activate actomyosin MgATPase (Maruyama et al., 1966; Goll et al., 1969). It is difficult to reconcile the in vitro observations with the structural role of α-actinin (Suzuki et al., 1973).
FIGURE 1.4

CROSS-BRIDGE ARRANGEMENTS IN THE THICK FILAMENT
AND THE STRUCTURAL ARRANGEMENT OF THE THIN FILAMENT

cf. Lamvik (1978)

M

cf. Fig. 1.3

4.5 nm 2.5 nm actin monomer (Wakabayashi et al., 1975)

4.0 nm

troponin tropomyosin actin thin filament

38.5 nm
1.5.3 THEORIES OF CONTRACTION

There is now general agreement that the overall mechanism of muscle contraction is the relative sliding of the thick myosin filaments past the thin actin filaments, a process driven by the hydrolysis of MgATP. This sliding movement is caused by the cyclic interaction of cross-bridges, extending from the myosin filaments, with the actin filaments (Huxley, 1969). It is also commonly supposed that muscle generates force by a conformational change in the actin-myosin interaction on MgATP hydrolysis, akin to a rotation of the attached myosin head on the actin (Marston et al., 1976).

Before the concept of sliding filaments (Hanson & Huxley, 1953, 1955), it was generally assumed that contraction occurred through a 'folding filament' system (Hall et al., 1946). The important principle behind sliding filaments is that active or passive length changes in muscle occur through the relative sliding of the two sets of filaments and not by length changes within any of the filamentous structures, a requirement borne out by experimental observation (1.5.2.5). The concept of sliding filaments is not itself a theory of contraction (Huxley, 1975). The theories of contraction based on sliding filaments have been reviewed by Huxley (1975, 1980). Observations point towards one broad class of theories, those that postulate a series of cyclically-acting force-generators between adjacent thick and thin filaments within the overlap zone. MgATP is hydrolysed at these sites of interaction, the free energy of its hydrolysis to ADP and $P_i$ being used to generate the force.

Early evidence for the existence of force-generators (Huxley & Niedergerke, 1954) came from isometric length-tension measurements of frog muscle
(Ramsey & Street, 1940). The tension developed during tetanic stimulation is a maximum if the fibre is held at its 'resting length' (sarcomere length \( s = 2.1 \text{ pm} \)) and declines linearly as the muscle is stretched, reaching zero at \( s = 4.1 \text{ pm} \). This observation suggested to Huxley & Niedergerke (1954) that the total force on a filament is proportional to a number of sites equally spaced within the overlap zone, each site contributing to the tension, cyclically, through MgATP hydrolysis. Therefore, the total force was proportional to the length of the overlap zone. This theory was confirmed (Gordon et al., 1966).

Huxley A.F. (1957) suggested that the regularly spaced cross-bridges between myosin and actin were the sites of interaction; the relationship between actin, myosin, and cross-bridges has been discussed in 1.5.2.

The application of Ca\(^{2+}\) by micropipette to sarcomeres in order to initiate local MgATP hydrolysis and contraction (cf. 1.5.2 & 1.5.6) has shown that the force-generators are distributed along the overlap zone (Gillis, 1969). Gordon et al. (1966) found that the speed of unloaded contracture was constant when the sarcomere shortened from 3.0 \( \text{ pm} \) to 2.0 \( \text{ pm} \) and that the speed was the same whatever the initial degree of overlap. Thomas et al. (1978) have measured the force-velocity curve of skinned fibres in the presence of activating levels of Ca\(^{2+}\) sufficient to develop 20 - 80% maximum tension. Speed of shortening (at zero load) was found to be independent of the Ca\(^{2+}\) concentration (in the range used) and hence the degree of cross-bridge activation. These and other observations (cf. Huxley, 1975, 1980) suggest the existence of cyclically-acting force-generators.

The contraction of muscle is isovolumic (cf. Hill, 1948). When muscle is stretched, the spacings of equatorial X-ray reflections change indicating that the volume of the muscle filament lattice remains
constant; the centre-to-centre distance between thin and thick filaments depends on sarcomere length (Huxley, 1953b). Stretching muscle so that the sarcomere length increases from 2.1 μm to 3.6 μm decreases the thick-thin filament spacing by 8 nm (Elliott et al., 1967). The opposite effect is observed on contraction. Thus large sliding movements are linked to small radial shifts such that the sarcomere volume remains constant (cf. Fig.1.1). A 0.5 μm length change in a half-sarcomere entails a thick filament transverse shift of 10 nm (Huxley, 1980).

Several theories of contraction based on the isovolumic nature of muscle have been reported. Elliott et al. (1970) propose that the lateral expansion of muscle is caused by the electrostatic repulsion between fixed charges on the thin filaments. The charges become perturbed by cross-bridge movement. Morel et al. (1976) suggest that the cross-bridges in resting muscle resist the repulsion. Noble & Pollack (1977) believe that restraint is imposed by electrostatic attraction between cross-bridges and thin filaments. However, it has been found that a fibre skinned of its sarcolemma (Natori, 1954) does not behave in a constant volume manner but nevertheless contracts and generates a tension comparable to the intact fibre (Matsubara & Elliott, 1972; April & Wong, 1976).

Rome (1967) had observed that glycerol-extracted psoas fibres contracted isovolumically in 50% aq. glycerol but not in 0.1 M KCl. April & Wong (1976), therefore, propose that "the constant volume relationship in the intact fibre is a manifestation of a lattice which is subjected to a volume constraint imposed by the sarcolemma." "Skinned fibres do not shorten isovolumically." "Neither the isovolumic behaviour nor the sarcolemma is necessary for the generation of tension." The sarcolemma, however, does provide a series elastic component during stretch (1.5.1). Huxley (1980, p. 85) puts forward the view that "elongation of muscles is something that happens in very many of the movements that an animal
makes" and he imagines that "special features have been evolved which allow this elongation to take place without damaging the muscle."

1.5.4 MECHANICS AND KINETICS OF ACTIN-MYOSIN INTERACTION

There is little doubt that the interaction of actin with myosin cross-bridges is modified by MgATP. Kinetic and mechanical models for cross-bridge action assume the following (cf. Huxley, 1975, 1980):

(1) During a single contraction a cross-bridge performs repeated cycles of attachment to actin, pulling (force generation), and detachment. The total force on any one filament is made up of contributions from each cross-bridge that is within the overlap zone of thick and thin filaments.

(2) The cross-bridges act (more or less) independently of each other.

(3) The detachment step is a rapid consequence of interaction with MgATP (Lymn & Taylor, 1971). Bound MgATP is split to yield a myosin.MgADP.P complex.

(4) Each cross-bridge contains two elements in series, one of which behaves with a passive instantaneous elasticity while the other can do net mechanical work, maintaining tension as the filaments slide past each other for a distance of 10 - 15 nm (Civan & Podolsky, 1966; Huxley & Simmons, 1972; Ford et al., 1977).

(5) The angle made by a cross-bridge changes relative to the axis of the filaments (cf. Reedy, 1968).
KINETICS OF MYOSIN MgATPase

Although muscle and actomyosin readily hydrolyse MgATP, purified myosin is a poor MgATPase at physiological ionic strength (i.e. 0.15 M, pH 7.4) in marked contrast to its CaATPase activity (Szent-Györgyi, 1951; Mommaerts & Green, 1954). Seidel (1969) gives the ratio of Ca to Mg ATPase activity as 50 : 1. However, since the Ca\(^{2+}\) concentration is low under physiological conditions and Mg\(^{2+}\) is high, CaATPase has little intrinsic significance to muscle kinetics. The ATPase activity of myosin was first studied by Banga (1941). Since that time extensive research has been undertaken on the ATPase activities of actomyosin, myosin, HMM, and S-1 under many different conditions.

Many reactions that utilise phosphate compounds involve an enzyme protein phosphorylation step (cf. 1.5.5). No phosphorylated myosin intermediate of ATP hydrolysis has been isolated, although its presence has often been inferred (cf. Needham, 1971, pp. 273 - 285). Weber & Hasselbach (1954) discovered that P\(_i\) liberation during the first 15 s of ATP hydrolysis was at least twice the rate of the steady state. It was suggested by Tonomura et al. (cf. Tonomura, 1972) that P\(_i\) appearing in the 'explosive' phase was actually derived from a phosphorylated intermediate of myosin, broken down on fixation with TCA. This early phase is commonly referred to as the 'initial burst'. The kinetic model for myosin MgATPase proposed by Lymn & Taylor (1970) and by Taylor et al. (1970) is based on the inferred phosphorylated myosin intermediate. In this model the rate limiting step is the slow liberation of ADP; the release of P\(_i\) precedes that of ADP. Although models for myosin ATPase have developed into more elaborate networks with the availability of more experimental information, the general outline of the Lymn &
Taylor (1970) and Taylor et al. (1970) model is still accepted. These models have been reviewed by Adelstein & Eisenberg (1980). The 'Modified Refractory State Model' of Stein et al. (1979) envelops a majority of previous concepts.

\[
\begin{align*}
M + ATP & \xrightarrow{k_1} M.ATP \xrightarrow{k_2} M \cdots \text{ADP} \xrightarrow{k_3} M + \text{ADP} + P_i \\
\end{align*}
\]

Lynn & Taylor (1970), Taylor et al. (1970)

\[
\begin{align*}
 k_1 &= 5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1} \\
 k_2 &= 50 - 75 \text{ s}^{-1} \\
 k_3 &= 0.02 \text{ s}^{-1} 
\end{align*}
\]

\[
\begin{align*}
 k_i &= 5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1} \\
 k_0 &= 50 - 75 \text{ s}^{-1} \\
 k_g &= 0.02 \text{ s}^{-1} 
\end{align*}
\]

Stein et al. (1979)

\[
\begin{align*}
 k_j &= \text{rate constant} \\
 K_j &= k_j / k_{-j} \quad (20^\circ \text{C}) \\
\end{align*}
\]

R - refractory state

N - non-refractory state

\[
\begin{align*}
 k_2 &= 20 - 200 \text{ s}^{-1} \\
 K_1 &= 10^{10} - 10^{11} \text{ M}^{-1} \\
 K_2 &= 1 - 10 \\
 K_3 &= ? + R \text{ state} \\
 K_4 &= 1 - 10 \text{ M}^{-1} \\
 K_5 &= 10^5 \text{ M}^{-1} 
\end{align*}
\]

M - myosin

ATP - MgATP

ADP - MgADP

P - phosphate
The concept of a conformational change from a 'refractory' (R) to a 'non-refractory' (N) state was introduced by Eisenberg & Kielley (1972). The equilibrium constant for this transition is not known but is believed to favour the R state. Bagshaw et al. (1974) have suggested that ATP binding is most probably at least a two-step process, i.e. formation of a collision complex followed by a protein structural change. The rate constant for protein relaxation is about $10^3$ s$^{-1}$ (cf. 1.4). The binding of ATP to myosin is irreversible, but measurable amounts of M. ADP.P and M. ATP form when high concentrations of $P_i$ are added to M. ADP (Cardon & Boyer, 1974; Mannherz et al., 1974; Wolcott & Boyer, 1974; Goody et al., 1977; Webb et al., 1978). The major decrease in free energy occurs during the ATP-induced conformational change. The release of $P_i$ is postulated to be a two-step process (Bagshaw et al., 1974; Webb et al., 1978); a conformational change associated with the release of $P_i$ is proposed as the rate-limiting step (Bagshaw & Trentham, 1974; cf. Lymn & Taylor, 1970). The release of ADP may also be a two-step process (Bagshaw et al., 1974) but Trybus & Taylor (1979) suggest three. The conformational change associated with ADP release is slow; below 5°C this step is rate-limiting (Webb et al., 1978).

**KINETICS OF ACTOMYOSIN MgATPase**

A mixture of purified myosin and actin forms an unphysiological state of aggregates which is called actomyosin; the combination of myosin and actin is visible in the electron microscope as a network of interconnected filaments (Huxley, 1963). Both HMM and S-1 also bind to actin (see 1.5.2.1 & 1.5.2.2).

At physiological ionic strength, MgATP both activates the interaction of
myosin and F-actin (Szent-Györgyi, 1951; Maruyama & Gergely, 1962; Eisenberg & Moos, 1967) and also depresses the interaction causing dissociation of the two proteins (Spicer, 1952; Maruyama & Gergely, 1962; Eisenberg & Moos, 1965). Maruyama & Gergely (1962) showed that the transition between associated and dissociated states occurs only over a narrow range of Mg$^{2+}$ and ATP concentrations. When the addition of MgATP causes dissociation, the myosin molecules aggregate into filaments; this state is believed to correspond to relaxation. Myosin splits MgATP at a slow rate but a critical concentration of MgATP is reached at which time F-actin filaments align parallel to and bind to myosin filaments; ATPase activity increases (Ikemoto et al., 1966). This state corresponds to the onset of contraction. As the concentration of ATP decreases, the alignment of thick and thin filaments becomes more extensive, and finally large aggregates are formed. The turbidity of the solution increases. The dissociated state is often called the 'cleared phase' and the formation of aggregates, 'superprecipitation' (Szent-Györgyi, 1951). The aggregated state can occur in one of two ways, as immediate precipitation or, if the original actomyosin was well dispersed, as a period of constant turbidity before precipitation (Ebashi, 1961b; Yasui & Watanabe, 1965a, 1965b). Under conditions when dissociation does take place, turbidity and ATPase activity are in unison (Watanabe, 1970).

Myosin has distinct binding sites for ATP and actin (Eisenberg & Moos, 1968). The binding of MgATP causes a conformational change to take place in myosin, reducing the binding to actin (cf. Kominz, 1970). Activation of MgATPase by actin is 100 - 200 times. The kinetic models for acto-S-1 MgATPase have been reviewed by Adelstein & Eisenberg (1980) and by Eisenberg & Greene (1980). The model of Stein et al. (1979) is
the counterpart of the Modified Refractory State Model for myosin MgATPase.

\[ \text{Lynn & Taylor (1971)} \]

\[ \text{Stein et al. (1979)} \]

K\(_1\) = \(10^7\) M\(^{-1}\)
K\(_2\) = \(10^4 - 10^5\) M\(^{-1}\)
K\(_5\) = \(10^4 - 10^5\) M\(^{-1}\)
K\(_6\) < K\(_4\)
K\(_7\) = \(10^4\) M\(^{-1}\)
K\(_8\) = ?
K\(_9\) = \(10^4 - 10^5\) M\(^{-1}\)
K\(_{10}\) = ?

K\(_3\) and K\(_4\) given on p. 89

A - actin

see p. 89 for other abbreviations
Stein et al. (1979) propose the existence of a \((A.M.ADP.P)_{R}\) state; step 8 in their model constitutes the hydrolysis of ATP by actomyosin without actin-myosin dissociation. Existence of such a step is supported by the data of Sleep & Hutton (1978) and Stein et al. (1979, 1980), but not by that of Taylor (1979). The rate of ADP release from actomyosin is fast. Lymn & Taylor (1970) postulate that \(P_i\) release from myosin is slow. Stein et al. (1979) believe that both ADP and \(P_i\) release must be faster than the corresponding steps for myosin alone, thus accounting for the actin-activation of ATPase.

In contrast to the situation in the absence of actin, where \(P_i\) binds to M.ADP to form M.ADP.P and M.ATP, there is no evidence that \(P_i\) can bind to A.M.ADP to form A.M.ADP.P and A.M.ATP. There is general agreement that a slow step occurs with S-1 attached to actin (Lymn & Taylor, 1971; Sleep & Taylor, 1976; Stein et al., 1979; Taylor, 1979). Stein et al. (1979) propose that this slow step is the conformational change, step 10, i.e. the transition from refractory to non-refractory state.

MODELS FOR CROSS-BRIDGE ACTION

The model of Eisenberg et al. (1968) for cross-bridge action was based on the observation that in relaxed insect flight muscle, the myosin heads were detached from actin and were at an angle of 90° to the filament axis, but that in the absence of ATP (rigor mortis) the bridges were bound at an angle of 45° towards the centre of the A-band (Reedy, 1968). In this model work was done, following ATP hydrolysis, when the angle of attachment changed from 90° to 45°. With the introduction of the concepts of actomyosin dissociation preceding ATP hydrolysis with the formation of a stable M.ADP.P complex, and of cross-bridge elasticity playing an
important role, Lymn & Taylor (1971) proposed the model shown below.

**MODEL FOR CROSS-BRIDGE ACTION**

Lymn & Taylor (1971)

see p. 92 for abbreviations
Modifications have been made to the model of Lymn & Taylor (1971) resulting in Refractory State (Eisenberg & Kielley, 1972) and Modified Refractory State (Eisenberg et al., 1980; Eisenberg & Greene, 1980) models. The models have been reviewed by Eisenberg & Greene (1980). The postulated transition from refractory to non-refractory state is believed to be a slow conformational change that occurs before S-1 reattaches to actin. After reattachment at an angle of 90°, the head rapidly rotates through 45°. Fast rates of transition between the attached states are postulated to explain rapidity of movement. Eisenberg & Greene (1980) make the assumption that ATP binding not only weakens the actin-myosin interaction, but also changes the 'preferred angle' from 45° back to 90°.

**MYOSIN HEAD-HEAD INTERACTION**

No model put forward gives any clearly defined role for the duplex nature of the myosin molecule. Evidence suggests that the two heads of the one myosin molecule interact with adjacent actin monomers (Margossian & Lowey, 1973; Greene & Eisenberg, 1980). Botts et al. (1972) have suggested that the binding of each head alternates and that the cross-bridge moves arm-over-arm along the thin filament. Offer & Elliott (1978) challenge these views by making a distinction between the in vitro studies on acto-HMM and the geometry of the highly ordered filament lattice of intact muscle; they conclude that the two heads of the one myosin molecule could bind to different actin filaments. The early work on the duplex nature of myosin centred on whether or not the two heads were functionally identical. Comparison of the ATPase activities of HMM and S-1 gave values of 1.7 to 2.4 (Jones & Perry, 1966; Lowey et al., 1969; Nauss et al., 1969), favouring the point of view that the two
heads are enzymically identical, in vitro. A later strategy has been to
determine whether or not the two heads act co-operatively or independently
of the other in cross-bridge action. Co-operativity implies an interaction between the two heads.

Each head interacts with actin and ATP at the same rate (Taylor & Weeds,
1977; Chock and Eisenberg, 1979). Cooke & Franks (1978) found that
single-headed myosin threads (obtained by papain digestion) produce half
the force of double-headed myosin, favouring independence of action.
The data presented by Shukla & Levy (1977)' and Eisenberg & Greene (1980)
show that HMM binds 600 times more strongly to actin than does S-1.
This effect was not observed by Highsmith (1978) nor by Margossian &
Lowey (1978). Reisler (1980) has put forward the suggestion that conflicting data probably reflect differences in preparation and heterogeneity
with respect to alkali light chains; this author's data support head independence. Inoue & Tonomura (1976a, 1976b) and Inoue et al. (1977a,
1977b) claim to have isolated two equimolar fractions of S-1 which show
different degrees of actin activation with respect to initial P_i burst
(see p. 88). They propose that one head, 'A', binds ATP without
immediate splitting, whereas head 'B' forms the M.ADP.P complex. Head A
is characterised by showing a low initial burst that is increased by
interaction with actin, and B by high burst kinetics unaffected by actin.
The differences were not attributed to light chain heterogeneity. The
MgATPase activities of heads A and B were similar. Kunz et al. (1980)
have used NEM as a reagent for detecting changes in the relative
reactivity of the SH groups in the two heads (1.5.2.1) with and without
PP_i, ADP, and ATP binding. They conclude that head co-operativity is
induced by nucleotide binding to the active site. The binding of
nucleotide to one head changes the reactivity of SH groups in the other
head. Any form of head-head interaction might imply an ATPase kinetic model along the lines proposed by Walz (1973). This mechanism requires that the binding of ATP to either site (i.e. head) prevents binding at the unoccupied site; the head-head interaction is assumed to be, therefore, negatively co-operative. The formation of products permits the binding of ATP at the alternate site.
1.5.5 MYOFIBRIL PHOSPHOPROTEINS

The regulation of enzyme activity through the phosphorylation and dephosphorylation of specific seryl residues (and to some extent threonyl, histidyl, and arginyl residues) in an enzyme is now recognised as an important control mechanism in metabolism (cf. Krebs & Stull, 1975). Reversible phosphorylation of an enzyme by substrate during phosphoryltransfer appears to be a necessary concomitant to the reaction. Before the phosphorylation of a given enzyme can be accepted as physiologically significant, several criteria must be met (Krebs, 1975):

(1) Does the phosphorylation of protein change a demonstratable function?

(2) Is the change reversed by dephosphorylation?

(3) Does phosphorylation occur in the intact cell?

In addition to enzymes, many other cellular proteins are phosphorylated or become so during metabolic processes. The enzymes that handle phosphoric monoesters fall into three categories:

(1) phosphatases, with water as the acceptor (ATPase is a special case where free energy of hydrolysis is coupled to metabolic function);

(2) kinases, with nucleoside triphosphate as donor and some molecule other than water as acceptor;

(3) mutases, for which the acceptor is another functional group on the donor molecule.

All three categories of reactions appear to involve a phosphorylated-
enzyme intermediate. The enzyme-catalysed phosphoryl-transfer reactions have been reviewed by Knowles (1980), protein phosphorylation and metabolic control by Rubin & Rosen (1975), and phosphorylation and de-phosphorylation of enzymes by Krebs & Beavo (1979). Bárany & Bárany (1980) have reviewed the phosphorylation of myofibrillar proteins and Adelstein & Eisenberg (1980), the regulation of contraction. The mechanism of phosphorylation has been discussed in 1.2.

Vertebrate skeletal myofibril proteins known to be phosphorylated are myosin DTNB-light chains, TN-I, TN-T, and TM. Commercially available \([\gamma^{32P}]ATP\) has been used for experiments in vitro; in vivo labelling has been carried out either by the injection of \(^{32}P\_1\) into the live animal or by the addition of \(^{32}P\_1\) to an incubation medium in which is suspended the intact muscle or fibre bundle. In live muscle, both \(\beta\) and \(\gamma\) phosphates of ATP become completely labelled in a few hours, and \(\alpha\) in about 24 hrs. Incorporation of \(^{32}P\) into proteins is catalysed by the appropriate protein kinase transfer of the \(\gamma\) phosphate of \([\gamma^{32P}]ATP\). The protein kinase may be specific. In vivo studies show that even after 2 - 3 days the muscle proteins are only partially labelled. Any changes in specific activity of a phosphoprotein caused by muscle stimulation must hence be related to the degree of protein phosphorylation rather than to turnover (Bárany & Bárany, 1980).

1.5.5.1 PHOSPHORYLATION OF MYOSIN LIGHT CHAINS

Phosphorylation in vitro of the myosin light chains in the red (Frearson & Perry, 1975) and white (Perrie et al., 1973) skeletal muscle of the rabbit have been demonstrated. Each DTNB-light chain is phosphorylated to the extent of 1 mole per mole; the nomenclature 'P-light chains' has been suggested by Frearson & Perry (1975). The amino acid sequence
around phosphorylated Ser-15 has been reported (Perrie et al., 1973). The general environment is cationic, a requirement in substrates of protein kinases (Williams, 1976). The P-light chains have been shown to become phosphorylated in vivo (Bárány & Bárány, 1977; Bárány et al., 1979).

Myosin light chain kinase is highly specific, has an absolute requirement for Ca$^{2+}$, and is independent of cAMP (cf. Rubin & Rosen, 1975; Krebs & Beavo, 1979). The active form of the enzyme has two subunits. One is a single polypeptide chain of mol. wt. 77,000 (Nairn & Perry, 1979) and is the subunit responsible for enzyme specificity (Hathaway & Adelstein, 1979). The second is a small Ca$^{2+}$-binding protein of mol. wt. 16,500 known as 'calmodulin' (Cheung, 1970), a protein common throughout the plant and animal kingdoms (for review, see Klee et al., 1980). The high mol. wt. subunit is inactive in the absence of the smaller Ca$^{2+}$-calmodulin complex (Yazawa & Yagi, 1978; Nairn & Perry, 1979).

Morgan et al. (1976) have isolated myosin light chain phosphatase (mol. wt. 70,000). The enzyme has high specificity and does not require a divalent ion; bound Mg$^{2+}$ has a protein stabilising effect. The phosphatase activity may be subject to some unknown form of regulation (Nimmo & Cohen, 1978).

1.5.5.2 PHOSPHORYLATION OF TN-I

Phosphorylation may be achieved in vitro by cAMP-dependent protein kinase (Bailey & Villar-Palasi, 1971) and by Ca$^{2+}$-dependent phosphorylase kinase (Stull et al., 1972). Thr-11 and Ser-117 are the sites of phosphorylation (Huang et al., 1974; Moir et al., 1974). Thr-11 is the main site of phosphorylase kinase attack, with Ser-117 as a minor. Ser-117 is the
specific site for cAMP-dependent protein kinase. Both sites are protected by TN-C binding (Cole & Perry, 1975). TN-I does not take up $^{32}\text{P}$ in vivo, suggesting the existence of a TN-I/TN-C complex (Ribolow et al., 1977).


1.5.5.3 PHOSPHORYLATION OF TN-T

A specific TN-T kinase has been isolated (Dobrovol'skii et al., 1976; Kumon & Villar-Palasi, 1978). There are three phosphorylation sites: Ser-1; Ser-149 (or -150); Ser-156 (or -157). The binding of TN-C to TN-T decreases the extent of phosphorylation (Moir et al., 1977). Ser-1 is phosphorylated in vivo (Bárány et al., 1979). No dephosphorylating enzyme has been found.

1.5.5.4 PHOSPHORYLATION OF TM

TM phosphorylation has not been demonstrated in vitro, phosphorylase kinase and cAMP-dependent protein kinase having no effect (Perry et al., 1975). However, incubation of frog muscle with $^{32}\text{P}_i$ yields phosphorylated Ser-283 α-TM (Ribolow & Bárány, 1977; Mak et al., 1978). β-TM is not phosphorylated.

1.5.5.5 EFFECT OF CONTRACTION ON $^{32}\text{P}$-LABELLED PHOSPHOPROTEINS

Of the three phosphorylated myofibril proteins found to be labelled in vivo (i.e. P-light chains, TN-T, α-TM) only the light chains exhibit a change in specific activity on tetanic stimulation (Bárány & Bárány,
1977; Bárány et al., 1979). The resting level was found to be 0.4 mol $^{32}$P per mol light chain; an additional 0.35 - 0.40 mol $^{32}$P mol$^{-1}$ is incorporated during a 20 - 30 s tetanus of frog muscle (25°C). Stull & High (1977) have also reported an uptake of 0.4 mol $^{32}$P per mol light chain in rabbit gracilis muscle after a 15 s tetanus; at the same time the phosphate content of the TN-TM fraction remained unaltered. Manning & Stull (1979), using fast twitch extensor digitorum longus muscle of rats, have followed the entire contraction-relaxation cycle, with respect to phosphorylated light chains, in detail. Resting values were 0.1 mol P per mol light chain. During 1 s tetanic stimulation the P content rose and continued to rise reaching 0.75 mol mol$^{-1}$ 7 s after cessation of the stimulus. The P content declined slowly to 0.1 mol mol$^{-1}$ after 300 s. Dephosphorylation did not correlate with removal of Ca$^{2+}$ and relaxation. The in vitro phosphorylation does not have an effect on actomyosin ATPase nor is any degree of phosphorylation required (Morgan et al., 1976; Perry, 1979; Stull et al., 1980). The phosphorylation of smooth muscle and non-muscle P-light chains, on the other hand, is essential for actomyosin ATPase (see Adelstein & Eisenberg, 1980). All the necessary elements of the myosin light chain phosphorylating system are present in skeletal, cardiac, and smooth muscle, but whereas phosphorylation is a major regulatory system in smooth muscle it may play only a modulatory and not obligatory role in skeletal and cardiac muscle (Perry, 1979; Stull et al., 1980).

The biological significance of TN and TM phosphorylations are unknown. Activation of cAMP-dependent protein kinase by the administration of 'isoproterenol' does not change the P contents (Stull & High, 1977). The extent of TN phosphorylation does not influence Ca$^{2+}$-binding properties (Perry, 1979; Stull et al., 1980). Cardiac muscle TN phosphorylation does appear to have a regulatory role (see Adelstein & Eisenberg, 1980).
1.5.6 REGULATION OF CONTRACTION AND RELAXATION

Regulation of the actin-myosin-MgATP interaction is either actin-based or myosin-based depending on which protein is the focus of the regulatory process. The regulatory process in vertebrate skeletal and cardiac muscle is actin-based; myosin-based systems are important in other types of muscle. Most invertebrates contain both systems. There are two known forms of myosin-based regulation. One is found in scallop muscle, wherein the binding of Ca$^{2+}$ to a specific myosin light chain relieves the inhibition of actin-myosin interaction. The second form found in smooth muscle and non-muscle myosin, requires phosphorylation of the P-light chains to activate actomyosin MgATPase. Ca$^{2+}$ regulates this latter system through activation of P-light chain kinase. Skeletal muscle actin-activated myosin MgATPase is repressed by the binding of TN-TM to actin and is derepressed by the binding of Ca$^{2+}$ to TN. Muscle stimulation releases Ca$^{2+}$ from the SR terminal sacs thereby changing the free pCa from $>7$ to $5$. Contraction is triggered by the binding of Ca$^{2+}$ to TN. Relaxation, after cessation of stimulation, is caused by removal of Ca$^{2+}$ by the ATP-driven SR Ca$^{2+}$ pump.

In the relaxed state, MgADP.P is believed to be bound to myosin (1.5.4). The muscle can be stretched because actin and myosin are prevented from interacting and generating force. Haselgrove & Huxley (1972) have proposed a 'steric-blocking model' to account for relaxation. The model suggests that TM physically blocks the myosin cross-bridge from interacting with actin; the TM position is thought to be controlled by the TN complex. In the thin filament, the binding of Ca$^{2+}$ to TN-C acts via TN-I and TN-T to permit a movement in TM (Haselgrove, 1972; Parry & Squire, 1973). Evidence indicates that each TM molecule spans 7 actin monomers (see p. 80); the movement in TM switches on the 7 actins
allowing interaction to take place between the thin filament, in this region, and a myosin head. There is evidence that TM has two modes of actin binding (cf. Haselgrove, 1972; Huxley, 1972a; Parry & Squire, 1973). McLachlan & Stewart (1976a) believe that each TM dimer has two sets of seven binding sites. Sets are switched by the rotation of the TM molecule through one quarter turn about its long axis. Binding of Ca$^{2+}$ to TN causes TM to rotate into the 'contraction position' allowing the actin-myosin interaction to take place repeatedly, with MgATP splitting and force generation, until Ca$^{2+}$ is removed. The formation of 'rigor bonds' occurs when myosin heads, free of MgADP-P or MgATP, bind to actin, pushing TM into the contraction position even when Ca$^{2+}$ is not bound to TN-C (Bremel et al., 1972).

An alternative model suggests that the cross-bridge remains loosely attached to actin in relaxed muscle and that the quality of the interaction between actin and myosin is affected by the TN-TM complex state; a conformational change is induced in actin by the binding of Ca$^{2+}$ to TN-TM and by the consequent movement of this complex (Adelstein & Eisenberg, 1980).
SECTION 2

MATERIALS AND METHODS
2.1 MATERIALS

acrylamide, specially prepared for electrophoresis (BDH)

ATP.2Na (Kyowa Hakko Kogyo, Japan)

casein, Hammersten (BDH)

chloramphenicol (Sigma London Chemical Co. Ltd.)

enzymes (Boehringer Corp. (London) Ltd.)

D-glucose (anhydrous), Micro-analytical Grade (BDH)

hypoxanthine (National Biochemicals Corp., Ohio, USA)

imidazole, Grade I (Sigma London Chemical Co. Ltd.)

IMP (Sigma London Chemical Co. Ltd.)

Neatan (Merck, West Germany)

p-nitrothiophenol (Koch-Light and Ralph N. Emmanuel Ltd.)

32P, in HCl (Radiochemical Centre Ltd., Amersham)

acetone and n-butanol, Reagent Grade (BDH), were redistilled.

All other reagents (Analytical Reagent Grade) were obtained from BDH Ltd. and Fisons Ltd.

Lactobacillus plantarum (pentosus; 124-2) was obtained from the National Collection of Dairy Organisms, Reading, Berks.

Rabbits, Dutch White (Female)

Yeast, DCL
Charcoal, sugar (a gift from Professor G. Pfleiderer), was purified by refluxing it with 2 M HCl (1 litre per 100 g) for 3 hrs and, after washing the charcoal free of acid, with ethanolic ammonia (0.1% ammonia in 50% aq. ethanol) for 1 hr. The purified charcoal was washed free of ethanolic ammonia with water and dried in air. 25 mg of charcoal was found to bind 1 μmol ATP. Treatment of purified charcoal with ethanolic ammonia was found to yield a small quantity of u.v. absorbing material. The extinction at 260 nm of the eluant was 0.002 per ml per 100 mg charcoal.
2.2 MUSCLE PROTEINS

2.2.1 MYOFIBRILS FROM MIXED RABBIT MUSCLE

2.2.1.1 PREPARATION AND STORAGE

A rabbit was killed by Nembutal injection, skinned, eviscerated and chilled on ice for 20 min. The back and leg muscles were removed and minced. The yield of mince from a typical adult rabbit was about 500g. 100 - 200 g portions of mince were homogenised for 2 min with about 1.5 l of 0.1 M KCl buffered with 50 mM Tris-HCl pH 7.5 (4°C) containing 15 mM 2-mercaptoethanol. The homogenates were combined and the volume adjusted to about 5 l with buffered KCl. The suspension was filtered through a single layer of surgical gauze to remove unhomogenised muscle and connective tissue. Myofibrils were collected by centrifugation at 30,000g using a continuous action rotor (MSE; 300 ml sediment container; flow rate of 60 - 70 ml per min). The sediment of myofibrils was homogenised for 1 min with buffered KCl and suspended in a final volume of 5 l KCl solution. The suspension was stirred for 1 - 2 hrs at 0 - 4°C and filtered through 2 - 4 layers of surgical gauze. The myofibrils were collected by centrifugation as before. The sequence of homogenisation, suspension in KCl and centrifugation was repeated 3 - 5 times. The final sediment (about 50 g wet packed wt. per 100 g of muscle mince) was homogenised into 50% (v/v) aq. glycerol at a concentration of 10 mg myofibrils ml⁻¹. The suspension was stirred for 12 - 16 hrs at 0 - 4°C and stored at -20°C for at least one week before being used.

Reference: Ulbrecht & Ulbrecht (1957)
2.2.1.2 SUSPENSION OF MYOFIBRILS

A suspension of myofibrils in 0.5% KCl buffered with 10 mM Tris-HCl pH 7.5 (4°C) was obtained by dilution of an aliquot of glycerol-extracted myofibrils with 10 volumes of buffered KCl. The suspension was stirred for 10 min and the myofibrils were collected by centrifugation at 1400g for 10 min. The myofibrils were washed 5 times by suspension in buffered KCl at 1 - 2 mg myofibrils ml⁻¹. The suspension was left at 0 - 4°C for 10 min each time and the myofibrils collected by centrifugation as before.

2.2.1.3 ACETONE-DRIED BUTANOL-EXTRACTED MYOFIBRILS

Myofibrils, prepared as described in 2.2.1.1 & 2.2.1.2, were collected by centrifugation at 1400g for 10 min. The sediment was suspended in 4 volumes of cold 0.4% NaHCO₃ and the suspension left for 20 min at 0 - 4°C, with occasional stirring. The myofibrils were centrifuged down at 1400g for 20 min, suspended in 10 - 15 volumes of water for 10 min and were collected by centrifugation at 1400g for 30 min. The sediment was homogenised for 1 min with 5 volumes of n-butanol (at -15°C) and left for 30 min with occasional stirring (cf. Tsao & Bailey, 1953). The butanol was filtered off through 4 layers of surgical gauze and the extracted myofibrils washed 3 times with ice-cold acetone over a period of 1 hr. Lipid material separated out as a white precipitate during the first acetone treatment {The effectiveness of n-butanol in the fission of lipoprotein complexes has been demonstrated by Morton (1950).}. The myofibrils were dried in air for 20 hrs and stored in a vacuum desiccator at -20°C.
2.2.2 ^32^P-LABELLED MYOFIBRILS FROM PSOAS MUSCLE

2.2.2.1 PREPARATION OF ^32^P-LABELLED PSOAS FIBRES

A rabbit was killed by Nembutal injection, skinned, eviscerated and chilled on ice for 20 min. The abdominal cavity was rinsed with aerated Locke's physiological saline at 4°C (0.9% NaCl, 0.042% KCl, 0.032% CaCl\(_2\).2H\(_2\)O, 0.021% MgCl\(_2\).6H\(_2\)O, 0.013% Na\(_2\)HPO\(_4\).12H\(_2\)O, 0.01% NaHCO\(_3\), and 0.1% glucose in sterilised water; the saline was aerated with 95% O\(_2\)/5% CO\(_2\) for 20 min before use). The psoas muscles were removed, washed free of blood with saline and finally immersed in saline. In all subsequent operations the muscles were kept wet by periodic applications of cold saline.

Each muscle was cut into two longitudinal sections and the fibres gently teased out into bundles of about 2 mm diameter. Each muscle section was suspended under light load in 200 ml of saline at 37°C; loading was necessary for keeping the muscle immersed. The muscle was tied to the support and weight with dye-free linen thread. The muscle-baths were maintained at 37°C and were continuously aerated with 95% O\(_2\)/5% CO\(_2\). After 10 min, 50 ml of saline containing 9250 kBq ^32^P\(_1\) was added to each bath (see 2.7). Incubation was continued for 4 hrs. The muscles were removed and rinsed 5 times with ice-cold saline to remove excess ^32^P\(_1\).

2.2.2.2 STORAGE OF LABELLED PSOAS

Fibre bundles of about 2 mm diameter were stretched around a glass frame, held in position with dye-free linen thread and the frame immersed in
ice-cold 50% (v/v) aq. glycerol. The frame was left in aq. glycerol for 16 - 20 hrs and then immersed in fresh aq. glycerol for storage at -20° C. The muscle was left for at least one week before being used.

2.2.2.3 **SUSPENSION OF PSOAS MYOFIBRILS FROM GLYCEROL-EXTRACTED FIBRE BUNDLES**

A length of fibres was removed from the frame, sectioned and immersed in 100 volumes ice-cold 0.5% KCl buffered with 10 mM Tris-HCl pH 7.5 (4° C). The muscle pieces were stirred for 30 min and were rinsed several times with buffered KCl. The muscle was homogenised into 100 ml buffered KCl. The suspension was left at 0 - 4° C for 10 min and the myofibrils collected by centrifugation at 1400g for 10 min. The homogenisation was repeated and the suspension filtered through 2 layers of surgical gauze. The myofibrils were collected by centrifugation and were washed 5 times by repeated suspension in buffered KCl. The final washed preparation was suspended in buffered KCl at a concentration of 10 mg myofibrils ml⁻¹.

2.2.3 **ACTIN**

Acetone-dried muscle was prepared from rabbit muscle mince by the method of Feuer et al. (1948). The dried powder was washed twice with 10 volumes of chloroform to remove lipid material. The organic solvent was removed from the acetone-dried muscle by evaporation. This preparation was ground in a coffee-mill, sieved and stored in a vacuum desiccator at -20° C. Purified actin was prepared from the acetone-dried muscle by the method of Spudich & Watt (1971).
2.2.4 MYOSIN

Myosin was prepared by the method of Harris & Seulter (1967). A solution of myosin in 0.5 M KCl buffered with 10 mM Tris-HCl pH 7.5 (4°C) was mixed with an equal volume of cold glycerol and stored at -20°C.

2.3 PREPARATION OF $\alpha_s$-CASEIN

1% aq. Hammersten casein at 0 - 4°C was adjusted to pH 7.0. 5 M CaCl$_2$ was added to a final concentration of 0.17 M. The suspension was left for 16 - 20 hrs at 0 - 4°C. The precipitate of Ca-casein was collected by centrifugation and the sediment dissolved in the original volume of 0.05 M citrate buffer pH 6.5 (4°C). 5 M CaCl$_2$ was added to a final concentration of 0.07 M. The precipitate was again collected and was dissolved in a minimum volume of citrate buffer. The solution was dialysed against cold distilled water and freeze-dried. The $\alpha_s$-casein was stored in a desiccator at least 24 hrs before use.

Reference: Thompson (1971)

2.4 PREPARATION OF SODIUM SILICOTUNGSTATE REAGENT

5.7 g sodium silicate.9H$_2$O and 79.4 g sodium tungstate.2H$_2$O were refluxed with 500 ml 3% H$_2$SO$_4$ for 5 hrs. The mixture was cooled, filtered and the volume adjusted to 1 l with water.

Reference: Martin & Doty (1949)
2.5 **SILANATION OF GLASSWARE**

Glassware for silanation was cleaned with chromic acid and washed with 1% phosphoric acid to remove chromate ions. The glassware was rinsed with 1% HCl and 3 times with distilled water. Dry glassware was immersed in benzene containing 1% (v/v) dimethyldichlorosilane and maintained at 60°C for 10 min. The silanated glassware was dried in an oven at 80°C, cooled and rinsed with methanol.

2.6 **ASSAY METHODS**

2.6.1 **NUCLEOTIDES**

2.6.1.1 **CONJOINT ASSAY OF ATP AND ADP**

**PRINCIPLE**

NADPH (and NADH) absorbs light in the near u.v. (λ_{max} = 339 nm) and re-emits the energy as a blue fluorescence (λ_{max} = 465 nm). Since NADP^+ (and NAD^+) does not absorb in this region, it is possible to relate the phenomenon to the rate of formation of the reduced compound (cf. Williamson & Corkey, 1969). By using the coupled enzyme reactions:

\[
\text{MgATP} + \text{Glc} \xrightarrow{\text{HK}} \text{MgADP} + \text{Glc-6-P} \\
\text{Glc-6-P} + \text{NADP}^+ \xrightarrow{\text{DH}} \text{6-Pgluconate} + \text{NADPH}
\]

HK : hexokinase (E.C. 2.7.1.1)

DH : Glc-6-P dehydrogenase (E.C. 1.1.1.49)
it is possible to relate the concentration of ATP to the increase in absorbance or fluorescence due to NADP⁺ reduction. The concentration of ADP may be determined by using the reaction:

\[ \text{Mg} + \text{ADP} \xrightarrow{\text{MK}} \text{MgATP} + \text{AMP} \]

MK : myokinase (E.C. 2.7.4.3)

in conjunction with the above reactions. If the MK reaction is carried out after HK and DH then the observed concentration of ADP will be greater than that initially present by an amount \( \equiv \) the initial quantity of ATP.

The increase in fluorescence (ΔF) was measured in a Locarte fluorimeter (Mk4; primary filter – Locarte stray light LF 2, secondary filter – Locarte 440 nm u.v. cut-off LF5).

**STANDARD LINE**

0 - 60 nmol ATP (ADP) in water pH 7.0 standardised by measurement of \( E_{260} \) (\( \varepsilon = 15.4 \text{ 1 mmol}^{-1} \text{ cm}^{-1} \))

**REAGENTS**

A reaction mixture containing:

- 50 mM glycylglycine-KOH buffer pH 7.5 (25°C)
- 1 mM MgCl₂
- 20 mM glucose (micro-analytical grade)
- 0.1 mM NADP⁺

The solution was filtered through a 3 µm filter (Millipore)
ENZYMES

DH 20 μg ml\(^{-1}\) buffer
HK 40 μg ml\(^{-1}\) buffer
MK 100 μg ml\(^{-1}\) buffer

PROCEDURE

To 0.3 ml of nucleotide-containing solution was added 1.2 ml reaction mixture. The solution was left at 25°C for 5 min. Standards and blanks (water) were run concurrently. 50 μl DH was added, the solution was thoroughly mixed and incubation at 25°C was continued for a further 5 min. The F reading was recorded and 50 μl HK added with thorough mixing. After 10 min the increase in F was recorded (\(\Delta F_{\text{ATP}}\)) and 50 μl MK added with thorough mixing. After 20 min the further increase in F (\(\Delta F_{\text{ADP}}\)) was recorded.

2.6.1.2 ESTIMATION OF AMP

PRINCIPLE

The deamination of AMP to IMP by 5'-AMP deaminase (E.C. 3.5.4.6):

\[
\text{AMP} \rightarrow \text{IMP}
\]

is accompanied by a u.v. spectral shift: \(\lambda_{\text{max}}\) AMP = 259 nm, \(\lambda_{\text{max}}\) IMP = 248 nm (pH 7.0). At 265 nm the molar extinction coefficient of IMP is 40% of that of AMP. Kalckar (1947) performed the reaction in 0.1 M succinate buffer pH 6.4; 0.1 M citrate pH 6.5 (Nikiforuk & Colowick, 1955), 0.1 M Tris-HCl pH 7 - 8 (Lee, 1957), and 0.1 M imidazole...
pH 6.4 containing 0.3 M KCl (Smiley et al., 1967) have also been used as buffers. The activity of the enzyme is dependent on pH and the buffer type. We found 50 mM glycylglycine-KOH pH 7.5 advantageous (see Appendix).

**STANDARD LINE**

0-70 nmol AMP in water pH 7.0 standardised by measurement of $E_{260}$ ($\varepsilon = 15.3 \text{ l mmol}^{-1} \text{ cm}^{-1}$)

**REAGENTS**

0.1 M glycylglycine-KOH pH 7.5 (30°C) (buffer)

**ENZYME**

200 μg deaminase ml$^{-1}$ buffer

**PROCEDURE**

To 2 ml AMP-containing solution was added 2 ml buffer. The mixture was incubated at 30°C for 5 min. Standards and blanks (water) were run concurrently. $E_{265}$ was recorded (vs. water) and 50 μl enzyme added with thorough mixing. After 20 min incubation at 30°C, the decrease in $E_{265}$ was recorded.

2.6.1.3 **ESTIMATION OF IMP**

2.6.1.3.1 **METHOD I (NUCLEOSIDASE)**

**PRINCIPLE**

The oxidation of hypoxanthine to uric acid by xanthine oxidase (E.C. 1.2.3.2) is accompanied by an increase in absorbance at
290 nm (cf. Gilbert & Bergel, 1964). IMP can be dephosphorylated to inosine by a 5'-nucleotidase (Hurst & Butler, 1951) and ribose removed by a nucleosidase to leave the base, hypoxanthine (Heppel & Hilmoe, 1952; Wang, 1955):

\[
\text{IMP} \xrightarrow{\text{NT}} \text{inosine} + P_i \\
\text{inosine} \xrightarrow{\text{NS}} \text{hypoxanthine} + \text{ribose}
\]

\[
\text{hypoxanthine} \xrightarrow{\text{XO}} \text{uric acid}
\]

\[\text{NT} : 5'\text{-nucleotidase} \]
\[\text{NS} : \text{nucleosidase} \]
\[\text{XO} : \text{xanthine oxidase} \]

Both NT and XO are reported, by the above respective authors, to be optimally active at pH 8.0 - 8.1; Wang (1955) carried out the NS reaction at pH 7.5. We found that a pH of 7.7 in our assay procedure to be satisfactory, although the activities of all three enzymes was reduced.

NT and NS will also degrade AMP to the free base, adenine. However, the oxidation of adenine to uric acid by XO is much slower than that of hypoxanthine. Consequently, we found that the presence of AMP did not contribute significantly to the value of \(\Delta E_{290}\).

**STANDARD LINE**

0 - 80 nmol IMP in water pH 7.0 standardised by measurement of \(E_{248}\) (\(\varepsilon = 12.3 \text{ l mmol}^{-1} \text{ cm}^{-1}\))
REAGENTS

0.1 M Tris-HCl pH 7.7 (37°C) (buffer)

ENZYMES

NT 1 mg Russell's Viper venom ml^-1 buffer
NS 0.1 ml of the preparation described on p. 303
XO 2 mg ml^-1 buffer

PROCEDURE

To 2 ml of IMP-containing solution was added 2 ml buffer, 0.1 ml NT and 0.1 ml NS. After thorough mixing, the reaction tubes were stoppered and incubated at 37°C for 20 hrs. Standards and blanks (water) were run concurrently. E_{290} (vs. water) was recorded. The solutions were aerated by vortex-mixing for 5 seconds and 10 µl XO added to each. After 20 min at 37°C the increase in E_{290} was recorded.

2.6.1.3.2 METHOD II (ACID HYDROLYSIS)

PRINCIPLE

All nucleotides are degraded to their component base, sugar, and phosphates by prolonged heating with aq. mineral acid. In the case of IMP, the hypoxanthine formed can be estimated using xanthine oxidase (cf. 2.6.1.3.1).

STANDARD LINE

0 - 25 nmol IMP in water pH 7.0 standardised by measurement of E_{248} (c = 12.3 l mmol^{-1} cm^{-1})
REAGENTS

5 M HCl
1.67 M KOH
0.1 M Tris-HCl pH 8.1 (30°C) (buffer)

ENZYME

2 mg XO ml⁻¹ buffer

PROCEDURE

To 1 ml of IMP-containing solution was added 0.25 ml HCl. Standards and blanks (water) were run concurrently. The reaction tubes were stoppered with dye-free cotton wool and incubated at 100°C for 1 hr, then cooled and neutralised with 0.75 ml KOH. 2 ml of buffer was added and the samples were incubated at 30°C for 5 min. $E_{290}$ was recorded (vs. water) and 10 μl enzyme added. After 20 min at 30°C, the increase in $E_{290}$ was recorded.

2.6.2 PHOSPHATE

2.6.2.1 INORGANIC PHOSPHATE ($P_i$)

PRINCIPLE

$P_i$ reacts with molybdate, in the presence of acid, to form a yellow phosphomolybdate complex. Reduction of the complex yields a blue one, the $E$ of which is proportional to the quantity of $P_i$ originally present. The procedure used in this thesis is a modification of the method of Martin & Doty (1949).

In those experiments that involve the measurement of $P$ liberated by hot conc. sulphuric acid digestion of muscle material (see 4.2), the
ammonium molybdate reagent is dissolved in water and not in acid. Under
these conditions, 3 ml of the acidified P-containing solution is mixed
with 1 ml water to obtain 4 ml for assay. This diluted solution contains
the requisite quantity of acid.

STANDARD LINE

0 - 1.5 μg P ml⁻¹ in water standardised as dry wt.

KH₂PO₄ (dried at 110°C for 30 min and stored in
a desiccator)

REAGENTS

1:1 (v/v) isobutanol/benzene
5% ammonium molybdate in 2 M H₂SO₄ or water
3% (v/v) conc. H₂SO₄ in absolute ethanol
0.2% acidified SnCl₂·2H₂O, prepared by 1:200 dilution
of 40% SnCl₂·2H₂O in conc. HCl with 0.5 M H₂SO₄.
The strong reagent is stored in the dark and the
cold.

PROCEDURE

To 4 ml of P-containing solution was added 5 ml isobutanol/benzene.
{To 3 ml of acidified P-containing solution were added 1 ml water and
5 ml isobutanol/benzene (cf. 4.2).} Standards and blanks were run
concurrently. 0.6 ml of the appropriate molybdate solution was added
and the solution mixed thoroughly by vortex-mixing. After leaving to
stand for 2 min, 2 ml of the organic (upper) layer was removed into a
tube containing 1 ml acidified ethanol. 0.3 ml diluted SnCl₂ was added
and the contents mixed thoroughly. E₆₅₀ was read after 15 min.
2.6.2.2 BOUND PHOSPHATE

The phosphate content of most materials can be determined by completely digesting the material with a hot oxidising acid and assaying the liberated $P_i$. The digestion mixture we used was based on that of Van Slyke et al. (1948), i.e. hot conc. sulphuric acid containing $HgSO_4$ as catalyst and $K_2SO_4$ to elevate the boiling point. These authors added the catalyst as a solution of $HgO$ in dil. sulphuric acid, but we found it convenient to add solid $HgSO_4$ to the digestion mixture. 100 mg (maximum) of acetone-dried myofibrils was digested in about 1½ hrs; the efficacy of $K_2SO_4$ as an aid to digestion is reflected in the time in its absence, 3 hrs. The disadvantage of using a $K$ salt is the contamination with radioactive $^{40}K$ (see 2.8.5); the Na salt cannot be used since it is insoluble in conc. sulphuric acid at the required concentration.

PROCEDURE

The material to be digested was stirred with water and immediately centrifuged (1400g; 20 min). The pellet was washed 3 times by repeated suspension in cold acetone (see 4.2.1.3), dried under reduced pressure (about 15 mm Hg), left over desiccant for 16 - 20 hrs and weighed into the digestion flask. 30 - 50 mg $HgSO_4$ and 1 ml conc. sulphuric acid containing 50% (w/v) $K_2SO_4$ were added. Digestion was carried out in the micro-Kjeldahl apparatus and continued for 30 min after decolourisation. The flasks were cooled and after the addition of 5 ml water, heated on a boiling water-bath for 10 min to hydrolyse pyrophosphate to orthophosphate. The contents were transferred to a 25 ml stoppered cylinder and the volume adjusted to 20 ml with water. $P_i$ in 3 ml of this acidified solution was estimated using aqueous molybdate (2.6.2.1).
2.6.3 **PROTEIN**

**PRINCIPLE**

The method is based on the Folin-Ciocalteu phenol reagent procedure as described by Lowry (1951), but modified to give a higher colour yield and direct proportionality between $E_{650}$ and up to 110 µg protein (Hartree, 1972). Protein precipitants (TCA, ammonium sulphate, sodium tungstate) at concentrations below 0.5% do not interfere with the assay; Cu$^{2+}$, detergents and sucrose do interfere.

**STANDARD LINE**

0 - 100 µg bovine serum albumin ml$^{-1}$ in water standardised by dry wt.

**REAGENTS**

Solution A:

- 2 g potassium sodium tartrate
- 100 g anhydrous sodium carbonate
- 20 g sodium hydroxide
- 1000 ml water

Solution B:

- 1 g potassium sodium tartrate
- 1 g copper sulphate, pentahydrate
- 0.4 g sodium hydroxide
- 100 ml water

Solution C:

- 1 vol. Folin-Ciocalteu phenol reagent (BDH)
- 15 vol. water

Solutions A and B were kept and used for up to 6 months after preparation; solution C was prepared fresh.
PROCEDURE

To 1 ml of protein-containing solution was added 0.9 ml A. The mixture was incubated at 50°C for 10 min. Standards and blanks were run concurrently. After allowing the solution to cool to room temp., 0.1 ml B was added and the mixture allowed to stand for 10 min. 3 ml C was rapidly forced into the solution. Incubation was carried out at 50°C for 10 min, after which time the $E_{550}$ (vs. water) was recorded.

2.6.4 PROTEIN NITROGEN

30 - 50 mg quantities of acetone-dried myofibrils (2.6.2.2) were digested with conc. sulphuric acid as described in 2.6.2.2. 1 ml of the solution was diluted to 250 ml with 0.4 M sodium citrate buffer pH 5.0. N was estimated in aliquots of the diluted solution by the method of Jacobs (1959).

2.6.5 2-D-DEOXYRIBOSE

1 g myofibrils was treated for 10 min twice with 160 ml cold 0.2 M perchloric acid. The denatured protein was incubated with 120 ml 0.3 M KOH for 1 hr at 37°C. After cooling on ice, 60 ml cold 1.2 M perchloric acid was added and the ppt. collected by centrifugation (1400g; 10 min). The pellet was washed with 100 ml cold 0.2 M perchloric acid and then was incubated with 100 ml 1 M perchloric acid at 70°C for 30 min. The ppt. was removed by centrifugation, the pellet washed once with 1 M acid and the supernatants pooled. The pellet was retained for
protein estimation. The pH of the supernatant was adjusted to 7 with KOH and 50 ml 1 M KOH was added. The suspension of KCIO₄ so formed was cooled on ice and centrifuged. The supernatant was concentrated to a few ml by rotary evaporation and made up to 10 ml with 1 M perchloric acid.

Deoxyribose was estimated by the diphenylamine-method of Giles & Myers (1965) was used; standards were 0 - 30 μg 2-D-deoxyribose per ml 1 M perchloric acid. The extracted protein pellet was dissolved in 100 ml 1 M KOH (100°C for 30 min). 80 ml water was added and, after cooling the suspension on ice, the ppt. of KCIO₄ was removed. The supernatant was adjusted to 1000 ml with water. The protein content in 1 ml of this solution was determined (2.6.3).

2.6.6 ENZYME ASSAYS

2.6.6.1 MYOSIN CaATPase

The Ca²⁺-dependent ATPase activity of a myosin solution was estimated by observing the rate of formation of Pᵢ. The reaction was carried out under the following conditions: 50 mM Tris-HCl pH 7.6 (25°C), 1 mM CaCl₂, 1 mM ATP, 0.1 M KCl. Incubation was carried out at 25°C. At time intervals, 2 ml of solution was removed and mixed with 0.6 ml sodium silicotungstate reagent to denature protein (2.4). After 10 min at room temp. and removal of the ppt. by centrifugation, the free phosphate was estimated as described in 2.6.2.1. Enzyme activity was
expressed as $\mu$mol ATP hydrolysed min$^{-1}$ mg$^{-1}$ protein. Protein was estimated as described in 2.6.3.

Reference: Harris & Seulter (1967)

2.6.6.2 MYOFIBRILLAR MgATPase

A suspension of myofibrils showed MgATPase activity under the following conditions: 50 mM Tris-HCl pH 7.5 (25°C), 1 mM MgCl$_2$, 1 mM ATP, 0.1 M KCl, 0.01 mM CaCl$_2$. Incubation was carried out at 25°C. The rate of formation of $P_i$ was used as a measure of enzyme activity (2.6.6.1).

2.6.6.3 MYOKINASE

The myokinase activity of a solution was estimated fluorimetrically as the rate of formation of NADPH (see 2.6.1.1).

REAGENTS

A reaction mixture containing:

- 50 mM glycylglycine-KOH pH 7.5 (25°C) (buffer)
- 1 mM MgCl$_2$
- 2 mM glucose (Micro-analytical Grade)
- 0.1 mM NADP$^+$
- 0.1 mM DTT

The solution was filtered through a 3 µm filter.

10 mM ADP in buffer
ENZYMES

DH† 100 µg ml⁻¹ buffer
HK 200 µg ml⁻¹ buffer

PROCEDURE

To 0.3 ml ADP solution was added 1.2 ml reaction mixture and 50 µl of DH and 50 µl HK. After 10 min incubation at 25°C, an aliquot of myokinase-containing solution was added and the rate of change of fluorescence followed on a chart recorder (Servoscribe IS). Activity was expressed as µmol ADP converted min⁻¹ mg⁻¹ protein. Protein was estimated as in 2.6.4.

2.6.6.4 5'-AMP DEAMINASE

The 5'-AMP deaminase activity of a solution was estimated spectrophotometrically as the rate of change of E₂₆₅ of an AMP solution (cf. 2.6.1.2). 50 mM glycylglycine-KOH pH 7.5 (30°C) was used as buffer (see Appendix). 2 ml AMP solution was incubated with 2 ml buffer for 5 min at 30°C. E₂₆₀ and E₂₆₅ were recorded. 50 µl enzyme was added and the decrease in E₂₆₅ was followed on a chart recorder (Servoscribe IS). Enzyme activity was expressed as µmol AMP deaminated min⁻¹ mg⁻¹ protein. Protein was estimated as described in 2.6.4.

†See 2.6.1.1 for abbreviations.
2.7 PREPARATION OF $^{32}\text{P}$ FOR USE

Causey & Harris (1951) found that polyphosphate compounds contaminated supplies of $^{32}\text{P}$-orthophosphate. The preparations we used were supplied in 1 M HCl and were stated to be free of these contaminants. As a precautionary measure, the acidified $^{32}\text{P}$-orthophosphate was heated in a boiling water-bath for 10 min to hydrolyse any polyphosphate compounds.

2.8 METHODS OF DETECTING $^{32}\text{P}$ DISINTEGRATIONS

2.8.1 FROM SOLID MATERIAL

Direct measurement of radioactivity of samples without destruction of the material was achieved using the Geiger-Müller tube (GEC, type GMH). Samples were contained in 25 mm aluminium planchets held in a Panax type LC/4 lead castle. The count rates of very active samples were reduced by interposing a metal filter of appropriate weight between sample and detector.

2.8.2 SCINTILLATION COUNTING OF AQUEOUS SOLUTIONS

The scintillant used was that of Patterson & Greene (1965). 1 volume of Triton X100 (purified by stirring with 0.1 part coarse silica gel for 30 min) was mixed with 2 volumes of toluene containing 0.4% PPO and 0.01% POPOP. The scintillant was stored at 4 - 12°C in the dark for 24 hr before use. 1 ml aqueous, colourless, $^{32}\text{P}$-containing solution was mixed with 10 ml scintillant and, when cleared and dark-adapted for 30 min, counted in a Tri-Carb Model 3375 liquid scintillation spectrometer.
At least 2 counts for each sample were obtained; the % standard deviation of machine-accumulated counts was recorded for each count. At least 2 counts of background (± % standard deviation) for each scintillation vial, containing 10 ml scintillant only, were observed. The quenching of observed counts was by the external standard ratio method; counting efficiency is proportional to quench.

2.8.3 DIRECT COUNTING OF CERENKOV RADIATION EMISSION OF AQUEOUS SOLUTIONS

β-particles of energy greater than 0.26 MeV have a sufficiently high velocity to interact with the molecules of the medium through which they are travelling, resulting in the emission of light in the spectral region 300 - 700 nm. The particle energy for $^{32}$P is 1.71 MeV.

Cerenkov radiation emission could be conveniently detected by using the preset $^3$H energy window of the Tri-Carb Model 3375 liquid scintillation spectrometer (40% eff.). Up to 15 ml aqueous solution could be counted by this method; background counts for each vial were obtained with the appropriate volume of solvent (see 2.8.5). At least 2 counts for sample and background were obtained (± % standard deviation).

2.8.4 PAPER AND THIN-LAYER CHROMATOGRAMS AND POLYACRYLAMIDE GELS

Scanning of chromatograms and gel slices (see 2.12) for $^{32}$P distribution was carried out using a Panax RCMS2 chromatogram scanner in conjunction with a Panax PX Series Modular Control Unit. Thin layers were hardened by treatment with a 10% solution of Neatan in methanol.
The treated thin layer was dried for 16 - 20 hr, taken up on to Sellotape and attached to paper.

Radioactivity was detected through an anthracene crystal in intimate contact with the window of a photomultiplier tube (EMI type 6097F). Output pulses (counts) from the Panax equipment were integrated into a potential difference, measured on a chart recorder, by the apparatus described in the Appendix.

2.8.5 GENERAL CONSIDERATIONS

All sources of K\(^+\) ions contain the \(\beta\)-emitter \(^{40}\text{K}\) (1.35 MeV); its natural abundance is 0.0118\% and its half-life is \(1.28 \times 10^9\) years. \(^{40}\text{K}\) contamination corresponds to an absolute radioactive count rate of 50 c.p.m. mmol\(^{-1}\) K\(^+\); details of observed count rates under a variety of conditions are given in the Appendix.

The coating of glassware with dimethyldichlorsilane (2.5) is known to reduce the binding of \(^{32}\text{P}\) to the surface of the glass (cf. Petroff et al., 1964).

2.9 CHROMATOGRAPHY

Nucleotides were separated from P\(_1\) and other low molecular wt. cell components using the chromatographic procedures listed below. Nucleotides are known to bind to charcoal; P\(_1\) does not do so (Hurlbert, 1957). We have found that the treatment of a solution with charcoal to
separate nucleotides and $^{32}$P prior to chromatography to be an invaluable procedure. A small quantity of charcoal, suspended in 5 M HCl, was packed into a short glass column. The charcoal was washed with water until the pH of the effluent was about 3 and the sample applied. The charcoal was washed with water to remove unbound substances and the nucleotides eluted with ethanolic ammonia (0.1% ammonia in 50% aq. ethanol). Colloidal carbon was removed from the eluant by filtration (0.3 - 0.45 μm Millipore filter). The concentration of ethanol in the eluant was lowered by rotary-evaporation at reduced pressure and the solution freeze-dried.

**PAPER**

The method of Krebs & Hems (1953), as described by Priston (1970), was used.

**ION-EXCHANGE ON PAPER**

Paper impregnated with polyethyleneimine (PEI) was prepared and used as described by Shirley (1978). Aq. LiCl was used as solvent.

**ION-EXCHANGE ON THIN-LAYER**

Separation could be achieved on 0.25 - 0.50 mm layers of DEAE-cellulose (Whatman DE 41) using aq. HCl as solvent (e.g. 0.075 M).

**2.10 ULTRASONICATION**

Samples to be ultrasonicated were cooled to 0 - 4°C and subjected to
a frequency of 20 kHz (MSE Model 300W ultrasonicator, 9 mm titanium probe). The container was cooled in an alcohol-ice bath. Sonication was continued for 2 - 10 min.

2.11 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

REAGENTS

ELECTROPHORESIS BUFFER (Laemmli, 1970) :

0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3 (20°C)

GEL BUFFER (Laemmli, 1970) :

0.75 M Tris-HCl, 0.2% SDS, pH 8.8 (20°C)

SAMPLE INCUBATION BUFFER (Davis, 1964) :

0.125 M Tris-phosphate, 4% SDS, pH 6.5 (20°C)

ACRYLAMIDE :

30 g acrylamide, 0.8g N,N'-methylenbisacrylamide in 100 ml water

INITIATOR :

N,N,N',N'-tetramethylmethylenediamine (TEMED)

CATALYST :

15 mg ammonium persulphate ml\(^{-1}\) water

TRACKING DYE :

1% aq. bromophenol blue
FIXER-STAIN:
0.25% Coomassie Blue, 25% methanol, 4.6% acetic acid, 15% TCA

DESTAINER:
7.5% acetic acid, 5% methanol

STORAGE SOLUTION:
7% acetic acid

PREPARATION OF GELS

Glass gel tubes (11.5 x 0.7 cm and internal diameter 0.5 cm) were silanated (2.5). A 7.5% gel solution was prepared by mixing together 5 vol. acrylamide, 10 vol. gel buffer, 0.2 vol. TEMED, 4.48 vol. water. The solution was degassed under reduced pressure for 5 min, 0.04 vol. ammonium persulphate added and the solution transferred to the gel tubes to a depth of 10 cm. 2 - 3 drops of water were immediately layered onto the surface. Gelling time was about 20 min at room temperature.

PREPARATION OF SAMPLE

Protein samples were dialysed against 0.4% NaCl for 16 - 20 hrs to remove K⁺. K⁺ in the sample must be avoided, since potassium dodecyl sulphate is insoluble (below 30°C) and because of radioactive ⁴⁰K contamination (2.8.5 & 4.5). 1 ml dialysed protein sample, containing not more than 1 mg protein, was mixed with 0.4 ml sample incubation buffer, 0.2 ml 2-mercaptoethanol, 2.2 ml water. The mixture was heated at 50°C for 30 min and cooled. 0.2 ml tracking dye and 200 mg sucrose were added. This solution could be stored at -15°C; aliquots of stored samples were heated with 0.01 vol. 2-mercaptoethanol at 50°C for 2 min before being used. The solution was clarified at 25,000g.
ELECTROPHORESIS

The gel tubes were loaded into the electrophoresis apparatus (Shandon Southern Instruments Ltd.). Approx. 400 ml electrophoresis buffer was poured into both the upper and lower compartments of the apparatus. The tops of the gel tubes were flushed out with buffer and up to 100 µl sample (containing not more than 250 µg protein) was applied directly onto the surface of each gel. Current of 1.5 - 2.0 mA per gel was applied with the lower compartment as the positive pole (SDS-protein complexes are negatively charged under the conditions described). Electrophoresis was continued until the tracking dye was about 1 cm from the base of the gel tubes.

STAINING, DESTAINING AND STORAGE

The gels were immediately removed from their tubes, fixed and stained for 1 - 1.5 hrs, and rinsed free of excess stain with destainer. The stained gels were supported vertically in 12 cm perforated plastic tubes and immersed in 2 l destainer. Destaining was carried out for 24 hrs, under constant stirring, with one change of destainer. The destained gels were stored in 7% acetic acid.

SCANNING FOR PROTEIN BANDS

The gels were scanned by densitometry using a Vitatron densitometer (Vitatron Scientific Instruments, Holland) fitted with either a U5 filter (faint bands) or a U12 filter (dense bands).

DETECTION OF $^{32}p$

The detection of radioactivity in gels was achieved either by drying and
scanning (below) or by sectioning and counting in a scintillation spectrometer (4.5).

**DRYING OF GELS**

The gels were sliced in half, longitudinally, using the apparatus described by Lyons (1978). The slices were placed 'flat side up' on thin polythene sheet (wet) and packed side by side. Sections of blank gel were placed around the slices to prevent the outer ones from spreading during the drying process. A wet piece of Whatman No. 3 filter paper (15 cm) was place on the gels. The gels were dried on to the paper using the apparatus described by Lyons (1978).

**SCANNING OF DRIED GELS FOR **$^{32}$P

The apparatus described in 2.8.4 was used.

**2.12 AUTORADIOGRAPHY**

**2.12.1 MYOFIBRIL SMEARS**

A suspension of $^{32}$p-labelled myofibrils in 5 mM Tris-HCl pH 7.5 was smeared on the surface of a microscope slide and allowed to dry. The smear was fixed by mild heat treatment. The slide was coated with Ilford (Nuclear Research) K5 emulsion, stored and developed as described by Brown (1976). Slides of unlabelled myofibrils were run concurrently. The grain distribution was observed using a phase-contrast microscope (Wild Model M40, inverted).
2.12.2 GEL SLICES

Gels were sliced and dried on to Whatman No. 3 paper as described in 2.11. A sheet of Ilford Red Seal X-ray film was sandwiched between paired gel slices, enclosed in black plastic sheet and stored in the dark at room temperature for 28 days. The plate was developed with Ilford Phen-X. {A satisfactory autoradiograph can be obtained with $10^7 \beta$-particles cm$^{-2}$ (cf. Dawson, 1969).} The author is indebted to Mr. E. Hawkes (Department of Biochemistry, Bedford College, University of London) for technical assistance.
SECTION 3

STATISTICAL TREATMENT

OF RESULTS
3.1 TERMINOLOGY

Random errors in measurements are assumed to exhibit a normal distribution; systematic errors are believed to have been reduced to a low level. Greek letters are used to signify theoretical quantities; Latin letters are used to designate observations and observed sample statistics. The symbol 'Σ' implies summation from \( i = 1 \) to \( n \), where \( i \) is an incremental index denoting an observation and \( n \) is the number of observations in the sample. The 'number of degrees of freedom' is an integer related to \( n \). For a calculated number of degrees of freedom a quantity is 'significant' when the test statistic is accepted at a level of significance < 0.05.

<table>
<thead>
<tr>
<th>TABLE 3.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>STATISTICAL SYMBOLS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Theoretical</th>
<th>Observed</th>
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</thead>
<tbody>
<tr>
<td>mean</td>
<td>( \mu )</td>
<td>( \bar{x} )</td>
</tr>
<tr>
<td>variance</td>
<td>( \sigma^2 )</td>
<td>( s^2 )</td>
</tr>
<tr>
<td>standard deviation</td>
<td>( s )</td>
<td>( s )</td>
</tr>
<tr>
<td>standard error of mean</td>
<td>( s_{\bar{x}} )</td>
<td>( s_{\bar{x}} )</td>
</tr>
<tr>
<td>correlation coefficient</td>
<td>( \rho )</td>
<td>( r )</td>
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<tr>
<td>prediction from</td>
<td>( y )</td>
<td>( \hat{y} )</td>
</tr>
<tr>
<td>standard line</td>
<td>( x )</td>
<td>( \hat{x} )</td>
</tr>
<tr>
<td>level of significance</td>
<td>( \alpha )</td>
<td>( \alpha )</td>
</tr>
</tbody>
</table>
3.2 MEAN AND STANDARD ERROR OF MEAN

The most probable value of $x$ is the mean:

$$\bar{x} = \frac{\sum x_i / s_i^2}{\sum 1 / s_i^2}$$

The standard error of the mean is:

$$s_{\bar{x}} = \pm \sqrt{\frac{s^2}{\left(\sum 1 / s_i^2\right)}}$$

where $s^2$ is the variance of the sample:

$$s^2 = \frac{\sum 1 / s_i^2 \sum x_i^2 / s_i^4 - \left(\sum x_i / s_i^2\right)^2}{(n-1)\sum 1 / s_i^2}$$

$n$ is the number of observations. Each observation, $x_i$, is weighted in favour of the $x_i$ with the smallest standard error, i.e. by $1/s_i^2$.

3.3 TEST OF HYPOTHESIS

It is often necessary to decide whether or not a value '$x$' is significantly different from a chosen value '$x_0$'. We wish to test a 'null hypothesis', $H_0$, that $x = x_0$; we also define an 'alternative hypothesis', $H_1$, which we accept if we reject $H_0$. The decision to reject $H_0$ is based on there being only a small probability of rejection even if $H_0$ is true. The probability of rejection is $\alpha$, the 'level of significance'; the value '(1-$\alpha$)' is the 'power' of the test = the probability of accepting $H_1$ if $H_1$ is true. The selected level of significance is that value which makes the power of the test as large as possible; unless stated otherwise, $\alpha = 0.05$ to 0.01.
The hypothesis $H_0$ is tested by comparing a test statistic, $T$, calculated by:

$$T = \frac{|\bar{x} - x_0|}{s_x}$$

to a value $t$, from a theoretical probability distribution of $t$, for the calculated number of degrees of freedom and at the chosen level of significance.

### 3.4 COMPARISON OF MEANS

The experimental estimate of a mean, $\bar{x}$, is tested against a chosen value $x_0$ by:

$$H_0 : \mu = \mu_0 \quad H_1 : \mu \neq \mu_0$$

$$T = \frac{|\bar{x} - x_0|}{s_x}$$

$H_0$ is rejected if $T > \%$ for $(n-1)$ degrees of freedom.

The means of two sets of data, $\bar{x}_1$ and $\bar{x}_2$, are tested for equality by testing their difference against zero:

$$H_0 : \mu_1 - \mu_2 = 0 \quad H_1 : \mu_1 - \mu_2 \neq 0$$

$$T = \frac{|\bar{x}_1 - \bar{x}_2|}{\sqrt{(s^2_{\bar{x}_1} + s^2_{\bar{x}_2})}}$$

degrees of freedom = \frac{(s^2_{\bar{x}_1} + s^2_{\bar{x}_2})^2}{s^4_{\bar{x}_1} / (n_1-1) + s^4_{\bar{x}_2} / (n_2-1)}
3.5 LINEAR REGRESSION AND CORRELATION

\[ y = mx + c \] is the line of regression of \( y \) on \( x \), where, for \( n \) \((x, y)\) pairs of observations,

\[
m = \frac{\sum xy - \frac{\sum x \sum y}{n}}{\sum x^2 - \left(\frac{\sum x}{n}\right)^2} \quad c = \frac{\sum y - m \sum x}{n}
\]

Line statistics depend on the 'spread' of the \( x \) and \( y \) data. This spread is estimated by the respective variances:

\[
s^2_z = \frac{n \sum z^2 - (\sum z)^2}{n(n-1)} \quad z = x \text{ or } y
\]

The degree of correlation between the \( x \) and \( y \) data is given by the correlation coefficient:

\[
r = \frac{m}{s_x/s_y}
\]

where \( r = 1.0 \) indicates perfect correlation and \( r = 0.0 \), zero. A term called 'the sample variance from regression', \( s_{y|x} \), is defined:

\[
s^2_{y|x} = (n-1)s^2_y (1-r^2)/(n-2)
\]

If \( s_m \) and \( s_c \) are the standard errors of the slope (\( m \)) and \( y \) intercept (\( c \)), then:

\[
s_m = \pm \sqrt{s^2_{y|x} / (n-1)s^2_x} \quad s_c = \pm s_m \sqrt{\left(\sum x^2 / n\right)}
\]
Good correlation does not guarantee linearity; a test of linearity is given by $T = \frac{|m/s_m|}{s_m}$. Linearity is accepted if $T > t$ for $(n-2)$ degrees of freedom at a level of significance.

3.6 PREDICTION FROM STANDARD LINES

Creation of a standard line involves the use of known quantities of a controlled variable, $x$, and estimated values of $y$. Line statistics are calculated by the regression of $y$ on $x$. These statistics are usually used to predict $y$ for a known value of $x$; the standard line is used in reverse, i.e. prediction of $x$ for a measured value of $y$. If the line is linear, i.e. $m/s_m$ is large, then the standard error in the predicted value of $x$, $\hat{x}$, is $s_\hat{x}$ given by:

$$s_\hat{x} = s_y \sqrt{\left(1 + \frac{(\hat{x} - \bar{x})^2}{(n-1)s_x^2}\right)}$$

3.7 PROPAGATION OF ERRORS

The error in a quantity $Q$ is calculated from the errors in the values used to compute $Q$. If $Q = f(a,b,c,...)$, where $a$, $b$, $c$, ..... are observations with errors $s_a$, $s_b$, $s_c$, ..... then :

$$s_Q = \sqrt{\left(\frac{\partial Q}{\partial a}\right)^2 s_a^2 + \left(\frac{\partial Q}{\partial b}\right)^2 s_b^2 + \left(\frac{\partial Q}{\partial c}\right)^2 s_c^2 + \ldots}$$
SECTION 4

EXPERIMENTAL

AND

RESULTS
PRESENTATION OF DATA

1. Errors in prediction from a standard line are propagated through all calculations. Data in tables are shown with their individual propagated errors in parentheses.

2. Mean values are shown either in the form \( \bar{x} \pm s_x(n) \) or \( \bar{x}(s_x) \).

3. Estimates of dry wt. are assumed to be accurate. Material for weighing was washed several times with cold, redistilled acetone and dried under reduced pressure (see 2.6.2.2). The dry powder was stored in a desiccator for 16 - 20 hrs and weighed. Powder exposed to air steadily increased in wt. due to adsorption of atmospheric water.

4. Mean values of sets of data are shown in tables below their respective set.
We have considered the transformation of ADP → ATP, catalysed by acetone-dried muscle, in relation to the presence of adenylate kinase (E.C. 2.7.4.3) "fortuitously retained in muscle powder" (Tsuboi, 1963; cf. 1.1). Myokinase (E.C. 2.7.4.3) is located in the muscle cytosol (cf. Klingenberg, 1975) and its presence in most muscle preparations is generally regarded as a contamination. Adenylate kinase found in liver mitochondria, for example, is a different enzyme to muscle cytosol myokinase although it catalyses the same reaction (cf. Klingenberg, 1975):

\[ MgADP + ADP \rightleftharpoons MgATP + AMP \]

Consequently, the name 'myokinase' will be used in this thesis when reference is made to the muscle cytosol enzyme and 'adenylate kinase' when the enzyme is from some other source or its origin is unknown.

4.1.1 Deionised water extraction of acetone-dried muscle
   4.1.1.1 Minced muscle
   4.1.1.2 Myofibrils

4.1.2 Is adenylate kinase solublised during actin extraction or is it bound to the non-extractable muscle residue?

4.1.3 Isolation of a fraction from acetone-dried muscle showing adenylate kinase activity

4.1.4 Adenylate kinase activity of myofibrils
4.1.5 5'-AMP deaminase activity of myofibrils

4.1.6 General observations concerning myofibril preparations and the adenylate kinase activity of muscle fractions

4.1.7 Summary of experimental findings
4.1.1 DEIONISED WATER EXTRACTION OF ACETONE-DRIED MUSCLE

4.1.1.1 MINCED MUSCLE

Acetone-dried muscle was prepared by the method given in 2.2.3. 1 g dried muscle was suspended in 60 ml deionised water at 0 - 4°C. At time intervals, 5 ml of suspension was removed and added to 1 ml 18% TCA; an estimate of the concentration of nucleotide present in dried muscle was obtained by adding 6 ml 3% TCA to 80 mg dried muscle. The precipitated protein suspension was left on ice for 10 min and centrifuged at 1400g for 10 min. TCA was removed from the nucleotide-containing supernatant by 5 extractions with an equal volume of water-saturated diethyl ether. Residual ether was removed by aeration. The supernatants were maintained at 0 - 4°C throughout all operations. The sedimented, denatured residue was dried in an oven at 110°C for 2 hrs. The dried residue was stored in a desiccator for 16 - 20 hrs and weighed.

Nucleotides in the supernatant were estimated: ATP and ADP - method 2.6.1.1; AMP - method 2.6.1.2; IMP - method 2.6.1.3.1.

RESULTS

Table 4.1 gives the nucleotide content of a deionised water suspension of acetone-dried muscle (three preparations) at various times of extraction. The mean value of each nucleotide (nmol mg⁻¹ dried residue), at the extraction times, was calculated; these mean values are shown in Fig. 4.1. There is a correlative rise in the concentration of ATP and fall in that of ADP; the AMP level also rises but IMP falls. The low value for ADP at 'zero time' is probably due to ineffective penetration.
of dried muscle by aqueous TCA. The standard errors for ATP, AMP, and IMP are in the range ± 0.03 to 0.08 nmol mg\(^{-1}\) and for ADP, ± 0.13 to 0.36 nmol mg\(^{-1}\). The change in nucleotide concentrations (\(\Delta\) nmol mg\(^{-1}\)) over the period of extraction (5 to 180 min) are: \(\Delta\)ADP = -5.01 ± 0.38; \(\Delta\)ATP = +2.48 ± 0.08; \(\Delta\)AMP = +0.81 ± 0.06; \(\Delta\)IMP = -0.75 ± 0.08.

The sum of the concentrations of the four types of nucleotide extracted from the preparations at the times of extraction were calculated; the mean values are shown in Fig. 4.3. The fall in nucleotide level (5 to 180 min) suggests that further reactions were taking place during the extraction. Webster (1953) has reported that muscle can apparently convert ADP to IDP and Bárány & Bárány (1972) also report the presence of adenosine and inosine as well as ATP, ADP, AMP, and IMP in frog muscle.

4.1.1.2 MYOFIBRILS

Acetone-dried, butanol-extracted myofibrils were prepared as described in 2.2.1.3. 1 g dried myofibrils was suspended in 100 ml deionised water at 0 - 4°C. Subsequent procedures were performed as described in 4.1.1.1.

RESULTS

Table 4.2 gives the ATP and ADP content of a deionised water suspension of acetone-dried myofibrils (two preparations). The mean value of each nucleotide, at the extraction times, was calculated; these values are shown in Fig. 4.2. No AMP nor IMP could be detected under the conditions of this experiment. The u.v. absorption spectrum of the extract, at each time, showed a plateau between 250 nm and 260 nm, suggesting that inosine (phosphates) was present in the extract (\(\lambda_{\text{max}}\) adenosine = 259 -
260 nm and $\lambda_{\text{max}}$ inosine = 248 - 249 nm at pH 7). $\Delta$ADP (5 to 180 min) = -2.11 ± 0.41 nmol mg$^{-1}$ and $\Delta$ATP (5 to 180 min) = +1.25 ± 0.35 nmol mg$^{-1}$.

4.1.2 IS ADENYLAZE KINASE SOLUBILISED DURING ACTIN EXTRACTION OR IS IT BOUND TO THE NON-EXTRACTABLE MUSCLE RESIDUE?

We separated the muscle mass from the aqueous extract after a short period of time and monitored the nucleotide levels in the extract for 2 hrs. The muscle mass was re-introduced and observations were continued.

**PROCEDURE**

0.2 g acetone-dried, butanol-extracted myofibrils was suspended in 20 ml deionised water at 0 - 4°C. After 3 min, the aqueous extract was separated from the muscle mass by rapid filtration through scinered glass (Gooch No. 4). Both extract and muscle mass were kept at 0 - 4°C. The protein content of the extract was determined (2.6.4). At time intervals, 1 ml extract was removed, added to 0.02 ml 180% TCA and kept at 0 - 4°C for 10 min. Precipitated protein was removed by centrifugation (1400g; 10 min) and TCA removed from the supernatant as described in 4.1.1.1. ATP and ADP in the TCA-free supernatant were estimated (2.6.1.1). After 2 hrs, the muscle mass was resuspended in the extract. At time intervals, 1.5 ml of reformed suspension was filtered through scinered glass. 0.2 ml filtrate was used for protein estimation and 1 ml treated with TCA as above. ATP and ADP were estimated as before.

Concurrently with the above procedure, 0.5 g acetone-dried, butanol-extracted myofibrils was suspended in 50 ml cold deionised water and, at time intervals, 5 ml suspension was removed and filtered through scinered glass. 0.2 ml of this filtrate was used for protein estimation and 4 ml
treated with 0.08 ml 180% TCA. Subsequent operations were carried out as above and liberated nucleotides estimated.

RESULTS

Fig. 4.4 illustrates the changes in the concentrations of extracted ATP and ADP per mg extracted protein. The ADP $\rightarrow$ ATP transformation only takes place in the presence of the insoluble muscle mass. Fig. 4.5 shows the rate at which protein is solubilized; approx. 60% of the extractable protein is released from the muscle mass in the first 3 min. Electron microscope observations of material extracted in less than 3 min did not show F-actin chains.
TABLE 4.1

NUCLEOTIDE CONTENT OF A SUSPENSION OF ACETONE-DRIED MUSCLE IN DEIONISED WATER

For experimental details, see 4.1.1.

<table>
<thead>
<tr>
<th>TIME (min)</th>
<th>ATP  (nmol mg⁻¹)</th>
<th>ADP  (nmol mg⁻¹)</th>
<th>AMP  (nmol mg⁻¹)</th>
<th>IMP  (nmol mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.59(0.14)</td>
<td>3.55(0.30)</td>
<td>0.03(0.13)</td>
<td>0.01(0.16)</td>
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<td></td>
<td>0.71(0.15)</td>
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<td>0.03(0.11)</td>
<td>2.35(0.11)</td>
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<td>0.19(0.18)</td>
<td>4.84(0.50)</td>
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<td>5</td>
<td>0.56(0.10)</td>
<td>6.13(0.63)</td>
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<td>0.11(0.12)</td>
</tr>
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<td></td>
<td>0.52(0.10)</td>
<td>5.54(0.56)</td>
<td>0.83(0.06)</td>
<td>0.71(0.10)</td>
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<td></td>
<td>0.77(0.08)</td>
<td>5.59(0.59)</td>
<td>0.02(0.07)</td>
<td>2.37(0.08)</td>
</tr>
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<td>1.19(0.08)</td>
<td>0.14(0.11)</td>
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<td>4.45(0.51)</td>
<td>0.72(0.05)</td>
<td>3.02(0.10)</td>
</tr>
<tr>
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<td>1.39(0.08)</td>
<td>5.35(0.58)</td>
<td>1.03(0.06)</td>
<td>0.38(0.11)</td>
</tr>
<tr>
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<td>2.03(0.05)</td>
<td>3.20(0.39)</td>
<td>2.18(0.04)</td>
<td>0.05(0.10)</td>
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<td>2.75(0.05)</td>
<td>2.69(0.35)</td>
<td>2.24(0.04)</td>
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<td>2.86(0.37)</td>
<td>2.11(0.04)</td>
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</tr>
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<td>1.93(0.03)</td>
<td>2.01(0.07)</td>
</tr>
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<td>1.93(0.30)</td>
<td>3.94(0.09)</td>
<td>0.77(0.08)</td>
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<tr>
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<td>3.40(0.07)</td>
<td>1.89(0.30)</td>
<td>2.76(0.05)</td>
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<tr>
<td>40</td>
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<td>1.61(0.05)</td>
<td>0.06(0.09)</td>
</tr>
<tr>
<td></td>
<td>3.59(0.07)</td>
<td>1.69(0.28)</td>
<td>1.09(0.06)</td>
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<td>50</td>
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<tr>
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<td>0.57(0.09)</td>
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<td>1.41(0.05)</td>
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</tr>
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<td>2.09(0.04)</td>
<td>0.06(0.09)</td>
</tr>
<tr>
<td></td>
<td>3.35(0.07)</td>
<td>1.53(0.26)</td>
<td>1.80(0.04)</td>
<td>1.74(0.07)</td>
</tr>
<tr>
<td></td>
<td>3.56(0.07)</td>
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<td>2.43(0.08)</td>
<td>0.04(0.10)</td>
</tr>
<tr>
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</tr>
<tr>
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<td>3.06(0.06)</td>
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<td>0.05(0.10)</td>
</tr>
<tr>
<td>180</td>
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</tr>
<tr>
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<td>0.56(0.17)</td>
<td>1.40(0.04)</td>
<td>0.50(0.08)</td>
</tr>
</tbody>
</table>

The corresponding row at each time refers to data from the same preparation of acetone-dried muscle. Numbers in parentheses are the individual propagated standard errors from the respective nucleotide standard line. 'mg' is the wt. of acetone-dried muscle.
# TABLE 4.2

NUCLEOTIDE CONTENT OF A SUSPENSION OF ACETONE-DRIED BUTANOL-EXTRACTED MYOFIBRILS IN DEIONISED WATER

For experimental details, see 4.1.1.2.

<table>
<thead>
<tr>
<th>TIME (min)</th>
<th>ATP (nmol mg(^{-1}))</th>
<th>ADP (nmol mg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.18(0.27)</td>
<td>1.87(0.40)</td>
</tr>
<tr>
<td></td>
<td>0.47(0.34)</td>
<td>1.95(0.50)</td>
</tr>
<tr>
<td>5</td>
<td>0.55(0.35)</td>
<td>3.22(0.48)</td>
</tr>
<tr>
<td></td>
<td>0.90(0.47)</td>
<td>2.22(0.98)</td>
</tr>
<tr>
<td>10</td>
<td>0.63(0.25)</td>
<td>2.71(0.34)</td>
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<td></td>
<td>0.65(0.40)</td>
<td>2.15(0.83)</td>
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<tr>
<td>20</td>
<td>0.79(0.26)</td>
<td>2.61(0.36)</td>
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<tr>
<td></td>
<td>0.61(0.28)</td>
<td>2.35(0.57)</td>
</tr>
<tr>
<td>30</td>
<td>0.68(0.27)</td>
<td>2.84(0.37)</td>
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<tr>
<td></td>
<td>0.68(0.23)</td>
<td>2.21(0.54)</td>
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<tr>
<td>40</td>
<td>0.48(0.34)</td>
<td>2.48(0.32)</td>
</tr>
<tr>
<td></td>
<td>0.74(0.19)</td>
<td>2.22(0.36)</td>
</tr>
<tr>
<td>50</td>
<td>0.55(0.26)</td>
<td>2.44(0.37)</td>
</tr>
<tr>
<td></td>
<td>0.66(0.15)</td>
<td>1.94(0.32)</td>
</tr>
<tr>
<td>60</td>
<td>0.82(0.23)</td>
<td>2.21(0.33)</td>
</tr>
<tr>
<td></td>
<td>0.97(0.18)</td>
<td>1.93(0.38)</td>
</tr>
<tr>
<td>80</td>
<td>1.29(0.24)</td>
<td>2.38(0.36)</td>
</tr>
<tr>
<td></td>
<td>1.11(0.15)</td>
<td>1.91(0.32)</td>
</tr>
<tr>
<td>100</td>
<td>1.59(0.22)</td>
<td>2.03(0.35)</td>
</tr>
<tr>
<td></td>
<td>1.56(0.13)</td>
<td>1.71(0.32)</td>
</tr>
<tr>
<td>120</td>
<td>1.33(0.23)</td>
<td>1.36(0.18)</td>
</tr>
<tr>
<td></td>
<td>1.11(0.20)</td>
<td>1.55(0.25)</td>
</tr>
<tr>
<td>180</td>
<td>2.06(0.18)</td>
<td>0.83(0.37)</td>
</tr>
<tr>
<td></td>
<td>1.25(0.39)</td>
<td>1.01(0.30)</td>
</tr>
</tbody>
</table>

The corresponding row at each time refers to data from the same preparation of acetone-dried myofibrils; data at 120 min are mean values. Numbers in parentheses are the individual propagated standard errors of prediction from the respective nucleotide standard line. 'mg' is the wt. of acetone-dried myofibrils.
Acetone-dried muscle/myofibrils was suspended in deionised water at 0 - 4°C. At the times indicated, an aliquot of suspension was removed and reactions stopped by the addition of TCA. Precipitated material was removed by centrifugation and its dry wt. determined (mg). TCA was removed from the supernatant and nucleotides estimated. See 4.1.1.1 & 4.1.1.2 for experimental details. Values in the Figs. were calculated from the results in Tables 4.1 & 4.2.
Nucleotides in the deionised water suspension of acetone-dried muscle were estimated as described in 4.1.1.1. The mean sum of ATP, ADP, AMP, IMP was calculated for each time of extraction from the results given in Table 4.1; each error bar is the respective standard error of the mean.
Acetone-dried butanol-extracted myofibrils were suspended in deionised water at 0 - 4°C for 3 min. The muscle mass was collected by centrifugation and both sediment and supernatant were kept at 0 - 4°C for 2 hrs. The protein content of the supernatant was determined. The ATP and ADP content of the supernatant was monitored for the 2 hrs. The muscle mass was resuspended in the supernatant, indicated by arrows in the Fig. At time intervals, an aliquot of reformed suspension was removed, rapidly filtered, and protein, ATP, ADP in the filtrate determined. Over the initial 2 hr period, a whole suspension was kept at 0 - 4°C. At the time intervals indicated, an aliquot of suspension was filtered and protein (Fig. 4.5), ATP, ADP (Fig. 4.4) in the filtrate determined. See 4.1.2 for experimental details.

'mg' refers to wt. of extracted protein.
4.1.3 ISOLATION OF A FRACTION FROM MUSCLE THAT SHOWS
ADENYLATE KINASE ACTIVITY

Myokinase, in impure form, is known to be stable when heated to 90°C at
low pH but at neutral pH and high temp. it denatures (Colowick & Kalckar,
1943); the purified enzyme is unstable under any conditions (Bowen &
Kerwin, 1956). We treated acetone-dried myofibrils and myofibrils with
hot dilute mineral acid as described by Colowick (1955) for the isolation
of myokinase from rabbit muscle. We also extracted muscle mince with
0.15 M KCl and with water; these extracts were also treated with hot acid.

(a) ACETONE-DRIED MYOFIBRILS

A 1% suspension of acetone-dried myofibrils (2.2.1.3) in distilled water
was brought to pH 3.3 by the addition of approx. 0.05 vols. of 1 M HCl.
The suspension was heated at 90°C for 2 min. The suspension was cooled
rapidly to 20°C and the pH adjusted to 6.5 with KOH. After cooling the
suspension to 0 - 4°C, solid ammonium sulphate was added to 80% satur-
ation (0°C). The suspension was stirred for 10 min and the precipitate
collected by centrifugation (40,000g; 1 hr). The pellet was dissolved
in 2 ml cold 0.1 M glycylglycine-KOH buffer pH 7.5 (4°C) containing
0.1 mM DTT.

(b) MYOFIBRILS

Myofibrils, prepared as described in 2.2.1.2, were treated with hot HCl
etc. as in (a). Isolated protein was taken up in 5 ml buffer.

(c) MUSCLE MINCE

100 g (wet wt.) muscle mince was stirred with 400 ml cold 0.15 M KCl or
with cold water for 20 min. The extract was separated from the muscle residue by centrifugation (18,000g; 20 min), brought to pH 3.3 with 1 M HCl and heated, in small batches, at 90°C for 2 min. Subsequent operations were performed as in (a). Isolated protein was taken up in 5 ml buffer.

The adenylate kinase activity of each isolated protein fraction was determined (2.6.7.3); protein was estimated as described in 2.6.3.

RESULTS

| TABLE 4.3 |
| ADENYLATE KINASE ACTIVITY OF A PROTEIN FRACTION ISOLATED FROM MUSCLE MINCE, MYOFIBRILS AND ACETONE-DRIED MYOFIBRILS |

<table>
<thead>
<tr>
<th></th>
<th>muscle mince</th>
<th>acetone-dried</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KCl extract</td>
<td>H₂O extract</td>
</tr>
<tr>
<td>protein yield</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% dry wt.</td>
<td>0.25</td>
<td>0.85</td>
</tr>
<tr>
<td>endogenous rate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>μmol ADP converted</td>
<td>0.11</td>
<td>0.03</td>
</tr>
<tr>
<td>min⁻¹ mg⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>exogenous rate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>μmol ADP converted</td>
<td>11.72</td>
<td>4.59</td>
</tr>
<tr>
<td>min⁻¹ mg⁻¹</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Muscle mince was extracted with either 0.15 M KCl or water. The extracts, myofibrils and acetone-dried myofibrils were treated with hot mineral acid. The acid extract was fractionated with ammonium sulphate (80% sat.) and the adenylate kinase activity of the precipitated protein determined. Each rate shown above is the mean of two assays. % dry wt. refers to the dry wt. of source material. See 4.1.3 for experimental details.
4.1.4 ADENYLATE KINASE ACTIVITY OF MYOFIBRILS

MgADP is able to cause the contraction of glycerol-extracted psoas muscle (Bowen & Martin, 1963). This is believed to occur through the following sequence of reactions:

\[
\begin{align*}
\text{MgADP} + \text{ADP} & \rightleftharpoons \text{MgATP} + \text{AMP} \quad \text{(myokinase)} \\
\text{MgATP} & \rightarrow \text{MgADP} + P_i \quad \text{(actomyosin ATPase)}
\end{align*}
\]

We measured the rate of formation of \( P_i \) by myofibrils on the addition of MgADP.

PROCEDURE

A suspension of myofibrils (2 mg ml\(^{-1}\)) in 0.1 M KCl buffered with 50 mM glycylglycine-KOH pH 7.5 (25° C) was prepared (2.2.1.2). To an aliquot of suspension was added an equal volume of a solution containing 0.2 mM ADP, 0.1 mM MgCl\(_2\), 0.01 mM CaCl\(_2\), 0.1 mM DTT, in buffered KCl. At time intervals, an aliquot was removed and 0.2 ml sodium silicotungstate reagent (2.4) added per ml aliquot to stop the reaction and precipitate protein. After 10 min at room temp., the precipitate was removed by centrifugation (1400g; 10 min). \( P_i \), AMP, and IMP in the supernatant were estimated (2.6.2.1, 2.6.1.2, & 2.6.13.2 respectively).

RESULTS

Fig. 4.6 shows the rate of formation of \( P_i \) and AMP + IMP by myofibrils on addition of MgADP. The concentration of ADP at the start of the reaction was 107 nmol ml\(^{-1}\); we calculate that 1 mol ADP is 'hydrolysed' to 0.56 - 0.62 mol \( P_i \) in 30 min. This is not consistent with the stoichiometric equation ADP + AMP + \( P_i \).
MgADP was added to a suspension of myofibrils. At time intervals, the $P_i$, AMP, IMP content of the suspension was measured. See 4.1.4 for experimental details.

FIGURE 4.6

RATE OF FORMATION OF $P_i$, AMP and IMP FROM MgADP CATALYSED BY MYOFIBRILS
4.1.5 5′-AMP DEAMINASE ACTIVITY OF MYOFIBRILS

As reported in the Appendix, finely homogenised myofibrils will remain suspended in KCl solution for about 1 hr. We measured the 5′-AMP deaminase activity of myofibrils spectrophotometrically as the rate of decrease in $E_{265}$ on addition of AMP (cf. 2.6.1.2).

PROCEDURE

To 2.6 ml 0.1 M glycylglycine-EOH buffer pH 7.5 (20°C) was added 0.1 ml of a 1 mg ml$^{-1}$ suspension of myofibrils in buffer (2.2.1.2) and 0.4 ml water. The suspension was incubated at 30°C for 5 min. The reaction was initiated by the addition of 0.1 ml 1 mM AMP in buffer. The initial rate of decrease in $E_{265}$ was measured.

RESULTS

Under the above conditions, the 5′-AMP deaminase activity of myofibrils was found to be $0.19 \pm 0.05$ (2) μmol AMP deaminated min$^{-1}$ mg$^{-1}$.

4.1.6 GENERAL OBSERVATIONS CONCERNING MYOFIBRIL PREPARATIONS AND THE ADENYLATE KINASE ACTIVITY OF MUSCLE FRACTIONS

(1) Myofibrils, when viewed with phase-contrast, were observed to contract on the addition of a solution containing 1 mM ATP, 1 mM MgCl$_2$, 0.01 mM CaCl$_2$, 0.1 M KCl, 50 mM Tris-HCl pH 7.5 (20°C).

(2) Mitochondria were observed when a myofibril preparation was viewed with the fluorescence microscope. The author is indebted to Miss J. Vijayatunga and the Department of Zoology (Bedford
College, University of London) for technical assistance.

A suspension of myofibrils did not show any endogenous mitochondrial activity nor any \( O_2 \) uptake on the addition of \( \alpha \)-keto-glutarate (cf. Paul & Sperling, 1952). \( O_2 \) uptake was observed when NADH was added to a suspension of myofibrils.

(3) Both actin and myosin could be purified free of adenylate kinase activity (2.2.3 & 2.2.4). Natural actomyosin (NAM), prepared from myofibrils by the method of Schaub & Perry (1971), showed adenylate kinase (AK) activity. AK could be removed by repeated cycles of high ionic strength dissolution and low ionic strength precipitation of NAM (cf. 1.5.2.3); loss of AK activity paralleled removal of the relaxing protein system (cf. Bendall, 1954; Perry, 1956). We studied the superprecipitation of NAM and synthetic actomyosin (SAM), prepared by combining purified actin and myosin, under a variety of conditions. We found that the change in light-scattering of the suspension due to alteration in turbidity on the addition of ATP, to be a convenient indicator of the state of interaction between actin and myosin (cf. Yasui & Watanabe, 1965a, 1965b; Watanabe, 1970). Experimental conditions were adjusted so that [ATP] in the range 0.25 - 1.5 mM caused immediate superprecipitation of NAM and SAM (125 \( \mu \)g protein ml\(^{-1}\), 0.1 M KCl, 25 mM Tris-HCl pH 7.5, 2 mM MgCl\(_2\), 0.02 mM CaCl\(_2\), 0.1 mM DTT).

We observed the change in \( E_{350} \) of the actomyosin suspension on addition of ATP; some of our practical details are presented in the Appendix. We found that the initial speed of superprecipitation (\( v \)) of both NAM and SAM was linear and inversely proportional to the concentration of ATP: \( v = -1.5 \frac{[ATP]}{mM} \) for NAM. We subjected NAM to superprecipitation by 1.5 mM ATP (+ 2 mM MgCl\(_2\)) with the
simultaneous presence of AMP. We had thought that, if AK influenced superprecipitation by maintaining the active concentration of ATP through the forward reaction $2\text{ADP} \rightarrow \text{ATP} + \text{AMP}$, an effect might be induced by high concentrations of AMP. We found that at $30^\circ\text{C}$ AMP accelerated the superprecipitation by ATP ($6 \text{mM AMP}$: 60% increase; $3 \text{mM AMP}$: 16% increase) but at $10^\circ\text{C}$, AMP inhibited ($6 \text{mM AMP}$: 73% decrease; $3 \text{mM AMP}$: 41% decrease). AMP did not induce any changes in turbidity in the absence of ATP. AMP was also shown to inhibit NAM MgATPase (at both $10^\circ\text{C}$ and $30^\circ\text{C}$) but did not affect myosin CaATPase (2.6.6.1 & 2.6.6.2). AMP did not inhibit the Mg-ATPase of SAM but did inhibit the superprecipitation of this system at $10^\circ\text{C}$ and $30^\circ\text{C}$. De Weer & Lowe (1973) have reported that commercially available tetra-n-buty ammonium chloride (T3AC) contains an inhibitor of myokinase; the inhibitor is probably an oxidation product. We found that TBAC had no effect on the ATP-induced superprecipitation of NAM. TBAC (up to $10 \text{mM}$) did not inhibit actomyosin MgATPase. However, we found that the compound 'Fuadin' (sodium antimonyIII bis-pyrocatechol-2,4-disulphonate), which inhibited myokinase, did have an effect on the rate of superprecipitation of NAM; actomyosin MgATPase and myosin CaATPase were inhibited by Fuadin, but we were unable to demonstrate inhibition of myofibrillar-bound AK. $1.5 \text{mM ATP}$, when added to NAM in the presence of $1.7 \text{mM Fuadin}$, caused the dissociation of actin and myosin (decrease in turbidity). The turbidity of the NAM did not increase within 30 min after addition of ATP, i.e. superprecipitation did not occur. Lower concentrations of Fuadin (down to $0.3 \text{mM}$) delayed the onset of ATP-induced superprecipitation; initial dissociation of actin and myosin, nevertheless, occurred. $0.02 \text{mM Fuadin}$ was without effect. The addition of $1.7 \text{mM Fuadin}$
to superprecipitated NAM caused a gradual decrease in turbidity of the solution to a value approx. equal to that of the NAM before addition of ATP. This effect was also dependent on the concentration of Fuadin, i.e. the drop in turbidity depended on \([\text{Fuadin}]\); 0.02 mM Fuadin had only a slight effect. The 'Fuadin effect' was similar at \([\text{Mg}^{2+}]\) = 2 or 5 mM; Fuadin did not appear to act as a Mg-chelator.

In view of (1) the similarity in stability of myokinase (MK) and troponin-I (TN-I) to hot acid (cf. Colowick & Kalckar, 1943; Ebashi et al., 1971; Mani et al., 1973), (2) the susceptibility of TN-I to proteolytic digestion (Dąbrowska et al., 1973), (3) the similarity in amino acid composition of MK and TN-I (cf. Heil et al., 1974; Mani et al., 1973), (4) the correspondence between relaxing protein and contaminating AK activity (see above), and (5) the fact that no vertebrate skeletal muscle, or model system derived from such muscle, has been found to be capable of performing mechanical work in the proven absence of AK/MK, we have considered the possibility that AK found to contaminate muscle preparations, such as NAM and myofibrils, may be a modified version of one of the minor myofibrillar proteins (e.g. TN-I). We found that our troponin (TN) preparations (i.e. TN-I + TN-C + TN-T; cf. 1.5.2.3) always showed AK activity. The enzyme activity could not be increased by protein degradative treatments such as proteolytic digestion (acid or alkaline) or by heating with mineral acid. Neither phosphorylation nor dephosphorylation of TN components (1.5.5) altered the AK activity of a preparation.
4.1.7 SUMMARY OF EXPERIMENTAL FINDINGS

1. Both acetone-dried muscle and acetone-dried, butanol-extracted myofibrils were extracted with deionised water. The transformation of \( \text{ADP} + \text{ATP} + \text{AMP} + \text{IMP} \) was observed. The fall in ADP is not significantly different from twice the rise in ATP (muscle: \( t = 0.121, 2 \) degrees of freedom; myofibrils: \( t = 0.481, 1 \) degree of freedom) thus suggesting the transformation \( 2\text{ADP} + \text{ATP} \).

Values for AMP and IMP are not consistent with the reaction sequence \( 2\text{ADP} + \text{ATP} + \text{AMP}, \text{AMP} + \text{IMP} + \text{NH}_3 \); additional reactions involving nucleotides are suspected.

2. The 'contaminating' adenylate kinase, believed to be the enzyme responsible for catalysing the reaction \( 2\text{ADP} + \text{ATP} + \text{AMP} \), is firmly bound to the non-extractable muscle mass.

3. A fraction could be isolated from myofibrils and acetone-dried myofibrils that showed adenylate kinase activity.

4. Myofibrillar-bound adenylate kinase and \( 5'\)-AMP deaminase were shown to be active.

5. Adenylate kinase activity could be observed in many muscle protein preparations. Loss of adenylate kinase from the preparation paralleled removal of the relaxing protein system. Some reagents that inhibited purified myokinase also influenced the superprecipitation of natural actomyosin.

6. Intact mitochondria were observed in myofibril preparations.
SUB-SECTION 4.2

THE FRACTIONAL DISTRIBUTION OF PHOSPHATE IN MYOFIBRIL PREPARATIONS

The P-containing compounds in muscle can be resolved into a series of fractions. The procedure developed in this thesis, for the resolution of muscle tissue into P-containing fractions, was adapted from a method used for the determination of alkali-labile P (Weller, 1977, 1979).

- **cytoplasmic P**: extractable with aqueous solutions after cell disruption, e.g. metabolic intermediates, nucleotides, $P_i$.
- **cold acid-extractable P**: associated with cell components, e.g. actin-bound ADP, $P_i$.
- **chloroform/methanol-extractable P**: phospholipids, phosphoinositides.
- **hot acid-labile P**: DNA, RNA, histidyl phosphate, lysyl phosphate.
- **hot alkali-labile P**: RNA, seryl phosphate, threonyl phosphate, arginyl phosphate.

We measured the total bound P of the myofibril preparation. This P represents the sum of the last four fractions (above); myofibrils were washed free of cytoplasmic P components during preparation. Cold acid- and chloroform/methanol-extractable P and hot acid-labile P were not measured directly. We estimated the remaining P content of the protein residue ($\mu g$ P $mg^{-1}$ residue) after removal of a P fraction. Alkali-labile P was estimated as the P liberated by hot alkali after removal of all other types of (known) P.
4.2.1 Preliminary studies

4.2.1.1 Relationship between dry wt. of acetone-dried myofibrils and the protein content

4.2.1.2 Binding of TCA to denatured myofibrils

4.2.1.3 Treatment of myofibrils with reagents and 'control' experiments

4.2.2 Total bound phosphate

4.2.3 Cold acid-extracted residue phosphate

4.2.4 Chloroform/methanol-extracted residue phosphate

4.2.5 Hot acid-extracted residue phosphate

4.2.6 Alkali-labile phosphate

4.2.7 Summary of experimental findings
4.2.1 PRELIMINARY STUDIES

4.2.1.1 RELATIONSHIP BETWEEN DRY WT. OF ACETONE-DRIED MYOFIBRILS AND THE PROTEIN CONTENT

Myofibrils were dried with acetone as described in 2.6.2.2. 10 - 70 mg quantities of acetone-dried myofibrils were weighed and dissolved in 20 ml 1 M NaOH by heating at 100°C for 20 min. The alkaline solution of myofibrils was neutralised by the addition of 20 ml 1 M HCl. The volume was adjusted to 100 ml with water. The protein content of 0.1 ml solution was determined (2.6.3); volumes of 100 μg Bovine serum albumin (BSA) ml⁻¹ water were used as standards.

RESULTS

Fig. 4.7 shows the relationship between $E_{650}$, BSA protein, and myofibril protein and Table 4.4, the relationship between myofibril dry wt. and the protein content as predicted from the protein standard line.

TABLE 4.4

COMPARISON BETWEEN MYOFIBRIL DRY WT. AND MEASURED PROTEIN CONTENT

<table>
<thead>
<tr>
<th>myofibril dry wt. (mg)</th>
<th>protein content (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>63.5</td>
<td>62.2(0.4)</td>
</tr>
<tr>
<td>50.8</td>
<td>50.7(0.4)</td>
</tr>
<tr>
<td>38.1</td>
<td>41.1(0.4)</td>
</tr>
<tr>
<td>25.4</td>
<td>26.0(0.4)</td>
</tr>
<tr>
<td>12.7</td>
<td>12.6(0.5)</td>
</tr>
</tbody>
</table>
Numbers in parentheses (Table 4.4) represent the propagated standard errors of prediction from the protein standard line. The regression of protein content on myofibril dry wt. has a slope = 0.976 ± 0.044 (r = 0.997; t for linearity = 21.982). There is a 1:1 relationship between the dry wt. of acetone-dried myofibrils and the protein content as measured by the method of Hartree (1972).

4.2.1.2 THE BINDING OF TCA TO DENATURED MYOFIBRILS

During our preliminary studies into the extraction of myofibrils with cold TCA, for the determination of cold acid-extractable P, we found that TCA bound to the denatured protein. The dry wt. of untreated myofibrils was 43.48 ± 1.28 (4) mg and that of TCA-denatured myofibrils, 65.76 ± 2.07 (5) mg; the myofibrils were apportioned, initially, as equal volumes of suspension. Since we use dry wt. as a measure of protein content (cf. 4.2.1.1 & 2.6.2.2), the contamination of denatured myofibrils with bound TCA constitutes a systematic error. Weller (1977) suggested that TCA-denatured protein should be "washed once with 1:1 ethanol/diethyl ether to remove residual acid." We found this treatment to be ineffective for removing TCA from myofibrils. Schmidt & Thannhauser (1945) reported that they removed residual TCA from denatured protein with water; they used ethanol/diethyl ether to dry the material after this treatment.

We compared the effectiveness of diethyl ether and dilute salt solution in removing TCA from denatured myofibrils (Fig. 4.8). We also denatured various quantities of myofibrils with TCA, washed the denatured protein 4 times with 0.5% KCl and dried the washed protein with acetone (cf. 2.6.2.2). The acetone-dried denatured protein was weighed and the protein
content determined (4.2.1.1). The regression of bound TCA (the difference between dry wt. and protein content of denatured myofibrils) on protein content is shown in Fig. 4.9; 1.1 ± 0.2 mg TCA is bound per mg denatured protein. We found that the most effective treatment for removing TCA was to stir TCA-denatured myofibrils with 0.5% KCl for 10 min; at least two further treatments were necessary. TCA contamination could be reduced to about 0.1 mg mg⁻¹ by this procedure.

4.2.1.3 TREATMENT OF MYOFIBRILS WITH REAGENTS AND 'CONTROL' EXPERIMENTS

Myofibrils were suspended with reagent at a concentration of 1 - 2 mg ml⁻¹; treatment with reagent was carried out at 0 - 4°C, unless stated otherwise. The term 'repeated suspension' implies that the following operations were carried out in sequence: (1) myofibrils were suspended and the suspension stirred frequently during the period indicated in the text; (2) the treated myofibrils were collected by centrifugation at 1400g for 10 min; (3) the suspension and centrifugation steps were performed the number of times indicated in the text.

'Control' experiments were carried out with myofibrils taken through the procedure without the addition of reagent.
FIGURE 4.7

PROTEIN STANDARD LINE (18 STANDARDS)

slope = 0.00546 ± 0.00007
y intercept = 0.070 ± 0.004
r = 0.999

Paired hyperbolic dotted lines are 95% confidence limits based on t = 2.120 (16 degrees of freedom). See 3.5 & 3.6 for terminology and details of regression.

See 4.2.1.1 for experimental details; protein was estimated by the method of Hartree (1972).
FIGURE 4.8

EFFECTIVENESS OF VARIOUS SOLVENTS IN REMOVING TCA BOUND TO DENATURED MYOFIBRILS

Myofibrils were denatured with 5% TCA. Aliquots of denatured myofibrils were washed several times with volumes of solvent. The treated myofibrils were dried with acetone each time (2.6.2.2), weighed and the protein content determined (4.2.1.1). The difference between the dry wt. and protein content is the quantity of bound TCA.
FIGURE 4.9

REGRESSION OF BOUND TCA ON PROTEIN (10 DATA PAIRS)

slope = 1.072 ± 0.163
y intercept = 3.96 ± 8.57

r = 0.919  t for linearity = 6.572

Paired hyperbolic dotted lines are 95% confidence limits based on t = 2.306 (8 degrees of freedom). See 3.5 & 3.6 for terminology and details of regression.

See 4.2.1.2 & Fig. 4.8 for experimental details.
4.2.2 TOTAL BOUND PHOSPHATE

Myofibrils were washed 5 times by repeated suspension in 0.5% KCl buffered with 10 mM Tris-HCl pH 7.5 (4°C). Aliquots of washed myofibrils were dried with acetone and the total bound P content determined (2.6.2.2).

RESULTS

Table 4.5.

4.2.3 COLD ACID-EXTRACTED RESIDUE PHOSPHATE

Myofibrils were washed 5 times with buffered 0.5% KCl (4.2.2) and suspended in 5% TCA. The suspension was ultrasonicated for 1 min (2.10) and left for 10 min. The TCA-denatured myofibrils were collected by centrifugation at 1400g for 10 min. The supernatant, containing 'cold acid-extractable P', was discarded and the denatured myofibrils ('cold acid-extracted residue') washed 3 times by repeated suspension in 0.5% KCl. Aliquots of the washed 'cold acid-extracted residue' were dried with acetone and the bound P content determined (2.6.2.2).

RESULTS

Table 4.6.
4.2.4 CHLOROFORM/METHANOL-EXTRACTED RESIDUE PHOSPHATE

Myofibrils were extracted with TCA to remove cold acid-extractable P (4.2.3). The TCA-extracted residue was dehydrated with methanol and extracted twice with 2:1:0.01 chloroform/methanol/conc. HCl for 10 min. The extracted residue was collected by filtration and washed once with methanol. Aliquots of the methanol-washed 'chloroform/methanol-extracted residue' were dried with acetone and the bound P content determined (2.6.2.2).

RESULTS

Table 4.7.

4.2.5 HOT ACID-EXTRACTED RESIDUE PHOSPHATE

Myofibrils were extracted with TCA (4.2.3) and chloroform/methanol (4.2.4). The extracted residue was washed with methanol and the residual solvent removed under reduced pressure (approx. 15 mm Hg). The protein was incubated with 1 M perchloric acid at 100°C for 15 min. The suspension was cooled to 0 - 4°C and the supernatant, containing 'hot acid-labile P', discarded. The protein residue was washed 3 times by repeated suspension in 0.5% KCl. Aliquots of the 'hot acid-extracted residue' were dried with acetone and the bound P content determined (2.6.2.2).

RESULTS

Table 4.8.
4.2.6 ALKALI-LABILE PHOSPHATE

Cold acid-extractable P, chloroform/methanol-extractable P, and hot acid-labile P were removed from myofibrils as described in 4.2.3, 4.2.4 & 4.2.5 respectively. The extracted residue was incubated with 2 ml 1 M NaOH at 100°C for 20 min. The solution was cooled to room temp., diluted to about 10 ml with water and 5 ml 0.2 M H₂SO₄ added. The volume was adjusted to 20 ml with water and 0.1 - 1.0 ml solution assayed for protein (2.6.3). To 5 ml solution was added 1.5 ml sodium silicotungstate reagent (2.4). The suspension was left at room temp. for 10 min and the ppt. removed by centrifugation (1400g; 10 min). 4 ml supernatant was used for P₁ estimation (2.6.2.1).

RESULTS

Table 4.9.

4.2.7 SUMMARY OF EXPERIMENTAL FINDINGS

See Table 4.10.
<table>
<thead>
<tr>
<th>μg P mg⁻¹</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.95 ± 0.02 (2)</td>
<td></td>
</tr>
<tr>
<td>0.91 ± 0.03 (6)</td>
<td></td>
</tr>
<tr>
<td>1.15 ± 0.04 (6)</td>
<td></td>
</tr>
<tr>
<td>1.14 ± 0.03 (4)</td>
<td></td>
</tr>
<tr>
<td>1.01 ± 0.01 (4)</td>
<td></td>
</tr>
<tr>
<td>1.01 ± 0.02 (4)</td>
<td></td>
</tr>
<tr>
<td>1.07 ± 0.01 (4)</td>
<td></td>
</tr>
<tr>
<td>1.00 ± 0.07 (4)</td>
<td></td>
</tr>
<tr>
<td>0.95 ± 0.05 (4)</td>
<td></td>
</tr>
<tr>
<td>1.10 ± 0.01 (4)</td>
<td></td>
</tr>
<tr>
<td>0.94 ± 0.02 (2)</td>
<td></td>
</tr>
<tr>
<td>1.01 ± 0.04 (1)</td>
<td></td>
</tr>
<tr>
<td>1.03 ± 0.01 (2)</td>
<td></td>
</tr>
</tbody>
</table>

See 4.2.2 for experimental details.
### TABLE 4.6

**P CONTENT OF PROTEIN RESIDUE AFTER COLD ACID EXTRACTION OF MYOFIBRILS**

For experimental details, see 4.2.3.

<table>
<thead>
<tr>
<th>Residue dry wt. (mg)</th>
<th>P content (µg P mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>81.00</td>
<td>0.79(0.03)</td>
</tr>
<tr>
<td>82.33</td>
<td>0.72(0.03)</td>
</tr>
<tr>
<td>86.66</td>
<td>0.74(0.03)</td>
</tr>
<tr>
<td>81.36</td>
<td>0.73(0.03)</td>
</tr>
<tr>
<td>72.25</td>
<td>0.78(0.03)</td>
</tr>
<tr>
<td>74.80</td>
<td>0.70(0.03)</td>
</tr>
<tr>
<td>79.72</td>
<td>0.72(0.03)</td>
</tr>
<tr>
<td>88.68</td>
<td>0.71(0.03)</td>
</tr>
<tr>
<td>66.64</td>
<td>0.72(0.03)</td>
</tr>
<tr>
<td>77.06</td>
<td>0.66(0.03)</td>
</tr>
<tr>
<td>69.75</td>
<td>0.77(0.03)</td>
</tr>
<tr>
<td>77.32</td>
<td>0.73(0.03)</td>
</tr>
<tr>
<td>76.94</td>
<td>0.81(0.03)</td>
</tr>
<tr>
<td>75.77</td>
<td>0.82(0.03)</td>
</tr>
<tr>
<td>75.09</td>
<td>0.78(0.03)</td>
</tr>
<tr>
<td>78.43</td>
<td>0.79(0.03)</td>
</tr>
<tr>
<td>55.16</td>
<td>0.72(0.03)</td>
</tr>
<tr>
<td>59.12</td>
<td>0.68(0.03)</td>
</tr>
<tr>
<td>59.84</td>
<td>0.71(0.03)</td>
</tr>
<tr>
<td>60.39</td>
<td>0.67(0.03)</td>
</tr>
<tr>
<td>59.72</td>
<td>0.65(0.02)</td>
</tr>
<tr>
<td>56.11</td>
<td>0.68(0.03)</td>
</tr>
<tr>
<td>55.17</td>
<td>0.68(0.03)</td>
</tr>
<tr>
<td>56.41</td>
<td>0.70(0.03)</td>
</tr>
</tbody>
</table>

The results are from three myofibril preparations. Numbers in parentheses are individual propagated standard errors of prediction from the P standard line.
TABLE 4.7

P CONTENT OF PROTEIN RESIDUE AFTER CHLOROFORM/METHANOL EXTRACTION OF COLD ACID-EXTRACTED RESIDUE

For experimental details, see 4.2.4

<table>
<thead>
<tr>
<th>residue dry wt. mg</th>
<th>P content µg P mg(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>70.94</td>
<td>0.54(0.03)</td>
</tr>
<tr>
<td>70.05</td>
<td>0.50(0.03)</td>
</tr>
<tr>
<td>71.83</td>
<td>0.50(0.03)</td>
</tr>
<tr>
<td>72.21</td>
<td>0.50(0.03)</td>
</tr>
<tr>
<td>69.32</td>
<td>0.56(0.03)</td>
</tr>
<tr>
<td>69.70</td>
<td>0.48(0.03)</td>
</tr>
<tr>
<td>67.58</td>
<td>0.49(0.03)</td>
</tr>
<tr>
<td>66.13</td>
<td>0.54(0.03)</td>
</tr>
<tr>
<td>39.67</td>
<td>0.50(0.03)</td>
</tr>
<tr>
<td>41.44</td>
<td>0.48(0.03)</td>
</tr>
<tr>
<td>57.27</td>
<td>0.54(0.03)</td>
</tr>
<tr>
<td>62.64</td>
<td>0.49(0.03)</td>
</tr>
<tr>
<td>51.59</td>
<td>0.48(0.03)</td>
</tr>
<tr>
<td>60.30</td>
<td>0.55(0.03)</td>
</tr>
<tr>
<td>61.11</td>
<td>0.54(0.03)</td>
</tr>
<tr>
<td>60.58</td>
<td>0.52(0.03)</td>
</tr>
</tbody>
</table>

The results are from three myofibril preparations. Numbers in parentheses are individual propagated standard errors of prediction from the P standard line.
### TABLE 4.8

**P CONTENT OF PROTEIN RESIDUE AFTER HOT ACID EXTRACTION OF CHLOROFORM/METHANOL-EXTRACTED RESIDUE**

For experimental details, see 4.2.5.

<table>
<thead>
<tr>
<th>residue dry wt. (mg)</th>
<th>P content (µg P mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24.89</td>
<td>0.071(0.008)</td>
</tr>
<tr>
<td>29.34</td>
<td>0.181(0.005)</td>
</tr>
<tr>
<td>37.89</td>
<td>0.112(0.004)</td>
</tr>
<tr>
<td>40.35</td>
<td>0.092(0.003)</td>
</tr>
<tr>
<td>42.07</td>
<td>0.129(0.003)</td>
</tr>
<tr>
<td>42.44</td>
<td>0.131(0.003)</td>
</tr>
<tr>
<td>43.47</td>
<td>0.119(0.003)</td>
</tr>
<tr>
<td>61.62</td>
<td>0.252(0.008)</td>
</tr>
<tr>
<td>62.57</td>
<td>0.185(0.005)</td>
</tr>
</tbody>
</table>

The results are from three myofibril preparations. Numbers in parentheses are individual propagated standard errors of prediction from the P standard line.
TABLE 4.9

ALKALI-LABILE P OF MYOFIBRILS

<table>
<thead>
<tr>
<th>protein mg</th>
<th>liberated P&lt;sub&gt;i&lt;/sub&gt; μg P mg&lt;sup&gt;-1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>33.46(0.30)</td>
<td>0.073(0.012)</td>
</tr>
<tr>
<td>29.80(0.26)</td>
<td>0.084(0.014)</td>
</tr>
<tr>
<td>30.06(0.27)</td>
<td>0.083(0.013)</td>
</tr>
<tr>
<td>32.95(0.30)</td>
<td>0.080(0.012)</td>
</tr>
<tr>
<td>26.87(0.23)</td>
<td>0.064(0.015)</td>
</tr>
<tr>
<td>33.06(0.30)</td>
<td>0.062(0.012)</td>
</tr>
<tr>
<td>27.97(0.24)</td>
<td>0.075(0.015)</td>
</tr>
</tbody>
</table>

Myofibrils were extracted with cold TCA, acidified chloroform/methanol and hot perchloric acid. The protein residue was dissolved in hot NaOH and the solution maintained at 100°C for 20 min. The protein and P<sub>i</sub> content of the solution was measured. See 4.2.6 for experimental details. The results are from three myofibril preparations. Numbers in parentheses are individual propagated standard errors of prediction from P and protein standard lines.
### TABLE 4.10

P-CONTAINING FRACTIONS OF MYOFIBRILS

<table>
<thead>
<tr>
<th>Category</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>total bound</td>
<td>1.03 ± 0.01 (48)</td>
</tr>
<tr>
<td>cold acid extracted residue</td>
<td>0.71 ± 0.01 (24)</td>
</tr>
<tr>
<td>chloroform/methanol extracted residue</td>
<td>0.52 ± 0.01 (16)</td>
</tr>
<tr>
<td>hot acid extracted residue</td>
<td>0.13 ± 0.01 (9)</td>
</tr>
<tr>
<td>cold acid-extractable</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>chloroform/methanol-extractable</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>hot acid-labile</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>alkali-labile</td>
<td>0.074 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>0.97 ± 0.02</td>
</tr>
</tbody>
</table>

See text of 4.2 for experimental details.
SUB-SECTION 4.3

THE DIRECT UPTAKE OF $^{32}\text{P}^{-}$
BY GLYCEROL-EXTRACTED MYOFIBRILS

Experiments reported in this sub-section are concerned with the causation of the uptake of $^{32}\text{P}$ by myofibrils as reported by Mühlrad et al. (1963) and discussed in 1.1.

4.3.1 General procedure for the labelling of glycerol-extracted myofibrils

4.3.2 Determination of P and $^{32}\text{P}$ content of labelled myofibrils

4.3.3 Some factors influencing $^{32}\text{P}$ uptake
   4.3.3.1 Concentration of $^{32}\text{P}$
   4.3.3.2 Time
   4.3.3.3 ATP and P$_i$
   4.3.3.4 Treatment of myofibrils with MgATP and CaATP prior to labelling
   4.3.3.5 Calcium ions
   4.3.3.6 p-nitrothiophenol, p-nitrophenol, 2,4-dinitrophenol
   4.3.3.7 Summary of experimental findings

4.3.4 Removal of bound $^{32}\text{P}$
   4.3.4.1 0.5% KCl treatment
   4.3.4.2 Cold TCA treatment
   4.3.4.3 Hot perchloric acid treatment
   4.3.4.4 MgATPase activity
   4.3.4.5 Effect of p-nitrothiophenol, p-nitrophenol, 2,4-dinitrophenol
   4.3.4.6 Summary of experimental findings

4.3.5 Fractionation of $^{32}\text{P}$-labelled myofibrils
4.3.6 Effect of the antibiotic 'chloramphenicol' on uptake of $^{32}\text{P}$

4.3.6.1 Extent of labelling of myofibrils in the presence of chloramphenicol

4.3.6.2 Effect of MgATPase activity on $^{32}\text{P}$ taken up by chloramphenicol-treated myofibrils

4.3.6.3 Summary of experimental findings

**DEFINITIONS**

$\text{mg} : \text{ dry wt. of myofibrils (p. 143)}$

$\mu\text{g P mg}^{-1} : \text{ bound P content of myofibrils (2.6.2.2)}$

$c.\text{p.m. mg}^{-1} : \text{ bound }^{32}\text{P content of myofibrils}$

$c.\text{p.m. }\mu\text{g}^{-1} \text{ P} : \text{ specific activity of }^{32}\text{P}$
4.3.1 **GENERAL PROCEDURE FOR THE LABELLING OF GLYCEROL-EXTRACTED MYOFIBRILS**

A 2 mg ml\(^{-1}\) suspension of myofibrils in 0.1 M KCl buffered with 50 mM Tris-HCl pH 7.5 (4°C) was prepared (2.2.1.2). To an aliquot of suspension was added an equal volume of buffered KCl containing 7.5 kBq \(^{32}\)P\(_1\) ml\(^{-1}\) (see 2.7). Storage in an air-tight container was carried out at 0 - 4°C for up to 48 hrs. The labelled myofibrils were collected by centrifugation (1400g; 10 min) and, except in experiments 4.3.4.1 & 4.3.4.2, were washed 5 times by repeated suspension (4.2.1.3) in 0.5% KCl buffered with 10 mM Tris-HCl pH 7.5 (4°C).

4.3.2 **DETERMINATION OF P AND \(^{32}\)P CONTENT OF LABELLED MYOFIBRILS**

Labelled myofibrils were washed free of P\(_1\) and reagents by 5 times repeated suspension in 0.5% KCl buffered with 10 mM Tris-HCl pH 7.5 (4°C). The KCl-washed, labelled myofibrils were dried with acetone, weighed, digested with conc. sulphuric acid and the P content determined as described in 2.6.2.2. Radioactivity was measured in 10 ml of digest (2.8.3).

4.3.3 **SOME FACTORS INFLUENCING \(^{32}\)P\(_1\) UPTAKE**

We have considered the possibility that some reagents and substrates known to influence phosphorylation reactions might also affect the uptake of \(^{32}\)P\(_1\) by myofibrils. For example: P\(_1\) as competitor; the state of the myofibril (relaxed or contracted, or undergoing ATPase in the presence of \(^{32}\)P\(_1\)); calcium ions (calcium phosphate deposition);
nitrophenols which uncouple oxidative phosphorylation (Hemker & Hülsmann, 1961); p-nitrothiophenol, which is believed to form p-nitrothiophenyl myosin by binding to a Glu residue (Kinoshita et al., 1969) or SH group (Wolcott & Boyer, 1973); SH group reducing agents. In the experiments which follow, myofibrils were either labelled in the presence of reagent or treated with and washed free of reagent before being labelled.

4.3.3.1 CONCENTRATION OF $^{32}$P

Myofibrils were stored for 48 hrs in the presence of different quantities of $^{32}$P (4.3.1). The bound $^{32}$P content was determined (4.3.2).

RESULTS

Fig. 4.10 shows the relationship between uptake of $^{32}$P and $[^{32}$P] initially present.

4.3.3.2 Time

Myofibrils were stored with $^{32}$P (4.3.1). The activity of bound $^{32}$P was determined at various time intervals; the $^{32}$P content was measured (2.8.1).

RESULTS

Fig. 4.11 shows the extent of $^{32}$P incorporation (two myofibril preparations).
4.3.3.3 ATP AND P\textsubscript{1}

Myofibrils were stored with \textsuperscript{32}P\textsubscript{1} for 48 hrs in the presence of either MgATP, ATP, or P\textsubscript{1}. The bound P and \textsuperscript{32}P content was determined. See Table 4.11 for experimental details and results.

4.3.3.4 TREATMENT OF MYOFIBRILS WITH MgATP AND CaATP PRIOR TO LABELLING

Myofibrils were incubated with MgATP or with CaATP at 25°C. The treated myofibrils were collected by centrifugation and washed free of reagent with 0.1 M KCl buffered with 50 mM Tris-HCl pH 7.5 (4°C). Labelling of the washed myofibrils was carried out for 48 hrs and the bound P and \textsuperscript{32}P content determined. See Table 4.12 for experimental details and results.

4.3.3.5 CALCIUM IONS

Myofibrils were stirred with either Ca\textsuperscript{2+} or EGTA; a portion of Ca\textsuperscript{2+}-treated myofibrils were washed free of reagent and stirred with EGTA. Myofibrils were washed free of reagent with 0.1 M KCl buffered with 50 mM Tris-HCl pH 7.5 (4°C) and the bound P and \textsuperscript{32}P content determined. See Table 4.13 for experimental details and results.

4.3.3.6 \textbf{p-NITROTHIOPHENOL, p-NITROPHENOL, 2,4-DINITROPHENOL, DITHIOTHREITOL}

Myofibrils were labelled for 48 hrs in the presence of either \textit{p}-nitrothiophenol (NTP), \textit{p}-nitrophenol (NP), 2,4-dinitrophenol (DNP). The bound P and \textsuperscript{32}P content was determined. Myofibrils were also stored with \textsuperscript{32}P and the above reagents in the presence of dithiothreitol (DTT). See Table 4.14 for experimental details and results.
FIGURE 4.10

RELATIONSHIP BETWEEN $^{32}\text{P}_i$ AND UPTAKE

FIGURE 4.11

TIME DEPENDENCE OF $^{32}\text{P}$ UPTAKE
INCUBATION TIME WITH $^{31}P_i$ (hr)

BOUND $^{31}P$ (ke$^{-1}$.P.m.-$^{-1}$.mg$^{-1}$)

log$_e$[bound $^{31}P$]

log$_e$[$^{31}P_i$]

[Graphs showing the relationship between log$_e$[bound $^{31}P$] and log$_e$[$^{31}P_i$] as well as the relationship between BOUND $^{31}P$ and INCUBATION TIME WITH $^{31}P_i$.]
Myofibrils, in 0.1 M KCl buffered with 50 mM Tris-HCl pH 7.5 (4°C), were incubated with \(^{32}\text{P}_\text{i}\) and one of the following solutions in buffered KCl:

1. **control**: 2 mM MgCl\(_2\), 0.02 mM CaCl\(_2\)
2. **ATP**: 2 mM ATP, 10 mM EDTA
3. **MgATP**: 2 mM ATP, 2 mM MgCl\(_2\), 0.02 mM CaCl\(_2\)
4. **P\(_i\)**: 2 mM \(\text{KH}_2\text{PO}_4\), 2 mM MgCl\(_2\), 0.02 mM CaCl\(_2\)

The myofibrils were stored for 48 hrs with \(^{32}\text{P}_\text{i}\) and reagent (4.3.1), washed free of residual label and the P and \(^{32}\text{P}\) content determined (4.3.2). See 4.3.3.3 for further information.
<table>
<thead>
<tr>
<th></th>
<th>myofibril dry wt.</th>
<th>$\mu g$ P mg(^{-1})</th>
<th>$^{32}$P content c.p.m. mg(^{-1})</th>
<th>specific activity c.p.m. $\mu g^{-1}$ P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>1.37(0.08)</td>
<td>5534(29)</td>
<td>4026(231)</td>
<td></td>
</tr>
<tr>
<td>11.8</td>
<td>1.08(0.05)</td>
<td>5623(20)</td>
<td>5206(312)</td>
<td></td>
</tr>
<tr>
<td>9.7(2.2)</td>
<td>1.23(0.15)</td>
<td>5594(42)</td>
<td>4444(564)</td>
<td></td>
</tr>
<tr>
<td><strong>ATP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.1</td>
<td>1.50(0.13)</td>
<td>879(8)</td>
<td>586(49)</td>
<td></td>
</tr>
<tr>
<td>9.3</td>
<td>1.06(0.06)</td>
<td>890(9)</td>
<td>843(52)</td>
<td></td>
</tr>
<tr>
<td>7.2(2.1)</td>
<td>1.14(0.17)</td>
<td>884(5)</td>
<td>707(128)</td>
<td></td>
</tr>
<tr>
<td><strong>MgATP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.2</td>
<td>1.19(0.07)</td>
<td>2245(39)</td>
<td>1881(120)</td>
<td></td>
</tr>
<tr>
<td>6.6</td>
<td>1.42(0.09)</td>
<td>1999(10)</td>
<td>1411(62)</td>
<td></td>
</tr>
<tr>
<td>7.4(0.8)</td>
<td>1.28(0.11)</td>
<td>2014(59)</td>
<td>1585(227)</td>
<td></td>
</tr>
<tr>
<td><strong>P(_{i})</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>1.26(0.07)</td>
<td>2361(22)</td>
<td>1827(111)</td>
<td></td>
</tr>
<tr>
<td>6.6</td>
<td>1.49(0.09)</td>
<td>2912(30)</td>
<td>1957(120)</td>
<td></td>
</tr>
<tr>
<td>7.3(0.7)</td>
<td>1.35(0.11)</td>
<td>2554(283)</td>
<td>1887(65)</td>
<td></td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.
Myofibrils, in 0.1 M KCl buffered with 50 mM Tris-HCl pH 7.5 (25°C), were incubated with an equal volume of one of the following solutions (in buffered KCl) at 25°C for 10 min:

1. control: 2 mM MgCl₂, 0.02 mM CaCl₂
2. Ca: 2 mM CaCl₂
3. MgATP: 2 mM ATP, 2 mM MgCl₂, 0.02 mM CaCl₂
4. CaATP: 2 mM ATP, 2 mM CaCl₂

The myofibrils were washed free of incubation medium and stored with ^32P⁻ (4.3.1). The bound P and ^32P content was determined (4.3.2). See 4.3.3.4 for further information.
TABLE 4.12

EFFECT OF THE MYOFIBRIL STATE ON UPTAKE OF $^{32}$P

<table>
<thead>
<tr>
<th></th>
<th>myofibril dry wt.</th>
<th>P content</th>
<th>$^{32}$P content</th>
<th>specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>µg P mg$^{-1}$</td>
<td>c.p.m. mg$^{-1}$</td>
<td>c.p.m. µg$^{-1}$ P</td>
</tr>
<tr>
<td>control</td>
<td>40.0</td>
<td>1.32(0.04)</td>
<td>58039(64)</td>
<td>43960(1415)</td>
</tr>
<tr>
<td></td>
<td>38.7</td>
<td>0.99(0.03)</td>
<td>43703(57)</td>
<td>44322(1239)</td>
</tr>
<tr>
<td></td>
<td>39.4(0.7)</td>
<td>1.11(0.08)</td>
<td>50044(7120)</td>
<td>44158(180)</td>
</tr>
<tr>
<td>Ca</td>
<td>41.6</td>
<td>0.83(0.02)</td>
<td>80874(75)</td>
<td>96871(2733)</td>
</tr>
<tr>
<td></td>
<td>38.7</td>
<td>1.01(0.03)</td>
<td>81830(78)</td>
<td>81035(2385)</td>
</tr>
<tr>
<td></td>
<td>40.2(1.5)</td>
<td>0.69(0.08)</td>
<td>81332(478)</td>
<td>87881(7645)</td>
</tr>
<tr>
<td>MgATP</td>
<td>35.0</td>
<td>1.15(0.03)</td>
<td>39942(57)</td>
<td>34758(1028)</td>
</tr>
<tr>
<td></td>
<td>35.6</td>
<td>0.96(0.03)</td>
<td>63400(71)</td>
<td>66305(1870)</td>
</tr>
<tr>
<td></td>
<td>35.3(0.3)</td>
<td>1.06(0.10)</td>
<td>49136(11452)</td>
<td>42079(13318)</td>
</tr>
<tr>
<td>CaATP</td>
<td>39.6</td>
<td>0.79(0.02)</td>
<td>46202(43)</td>
<td>58483(911)</td>
</tr>
<tr>
<td></td>
<td>36.0</td>
<td>0.84(0.02)</td>
<td>71135(75)</td>
<td>84937(2310)</td>
</tr>
<tr>
<td></td>
<td>37.8(1.8)</td>
<td>0.82(0.03)</td>
<td>52370(10758)</td>
<td>64043(9029)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.
Myofibrils, in 0.5% KCl buffered with 10 mM Tris-HCl pH 7.5 (4°C), were stirred with an equal volume of either 2 mM CaCl₂ in buffered KCl or 10 mM EGTA in buffered KCl for 30 min. Myofibrils were collected by centrifugation (1400g; 10 min) and a portion of calcium-treated stirred for 30 min with EGTA. The myofibrils were washed 5 times by repeated suspension in buffered KCl. Labelling was carried out for 48 hrs (4.3.1) and the bound P and ^32P content determined (4.3.2).

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>untreated myofibrils</td>
</tr>
<tr>
<td>Ca</td>
<td>myofibrils treated with CaCl₂</td>
</tr>
<tr>
<td>EGTA</td>
<td>myofibrils treated with EGTA</td>
</tr>
<tr>
<td>Ca/EGTA</td>
<td>calcium-treated myofibrils treated with EGTA</td>
</tr>
<tr>
<td>myofibril dry wt.</td>
<td>P content</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------</td>
</tr>
<tr>
<td></td>
<td>mg</td>
</tr>
<tr>
<td>control</td>
<td></td>
</tr>
<tr>
<td>50.7</td>
<td>0.67(0.02)</td>
</tr>
<tr>
<td>66.3</td>
<td>0.64(0.02)</td>
</tr>
<tr>
<td>49.9</td>
<td>0.66(0.02)</td>
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<td>62.3</td>
<td>0.67(0.02)</td>
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<tr>
<td>57.3(4.1)</td>
<td>0.66(0.01)</td>
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<td>Ca</td>
<td></td>
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<tr>
<td>56.4</td>
<td>0.67(0.02)</td>
</tr>
<tr>
<td>55.8</td>
<td>0.67(0.02)</td>
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<tr>
<td>57.7</td>
<td>0.69(0.02)</td>
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<tr>
<td>60.3</td>
<td>0.66(0.02)</td>
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<tr>
<td>57.6(1.0)</td>
<td>0.67(0.01)</td>
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<tr>
<td>EGTA</td>
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<tr>
<td>52.5</td>
<td>0.66(0.02)</td>
</tr>
<tr>
<td>59.0</td>
<td>0.67(0.02)</td>
</tr>
<tr>
<td>59.5</td>
<td>0.65(0.02)</td>
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<tr>
<td>57.7</td>
<td>0.64(0.02)</td>
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<tr>
<td>57.2(1.6)</td>
<td>0.66(0.01)</td>
</tr>
<tr>
<td>Ca/EGTA</td>
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<td>65.1</td>
<td>0.74(0.02)</td>
</tr>
<tr>
<td>55.6</td>
<td>0.67(0.02)</td>
</tr>
<tr>
<td>52.8</td>
<td>0.66(0.02)</td>
</tr>
<tr>
<td>57.5</td>
<td>0.68(0.02)</td>
</tr>
<tr>
<td>57.8(2.6)</td>
<td>0.69(0.01)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.
Myofibrils, in 0.1 M KCl buffered with 20 mM Tris-malate pH 6.5 (4°C), were incubated with $^{32}\text{P}_i$ and an equal volume of one of the following solutions:

1. control: buffered KCl
2. NTP: 0.05 mM p-nitrothiophenol in buffered KCl
3. NP: 0.05 mM p-nitrophenol in buffered KCl
4. DNP: 0.05 mM 2,4-dinitrophenol in buffered KCl

The myofibrils were stored with reagent and $^{32}\text{P}_i$ for 48 hrs in the presence or absence of dithiothreitol (DTT); see 4.3.1. Labelled myofibrils were washed free of reagent and residual label and the bound P and $^{32}\text{P}_i$ content determined (4.3.2).
**TABLE 4.14**

**EFFECT OF p-NITROTHIOPHENOL, p-NITROPHENOL, 2,4-DINITROPHENOL, DITHIOTHREITOL ON UPTAKE OF $^{32}$P**

<table>
<thead>
<tr>
<th>myofibril dry wt.</th>
<th>P content</th>
<th>$^{32}$P content</th>
<th>specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
<td>$\mu$g P $\text{mg}^{-1}$</td>
<td>c.p.m. $\text{mg}^{-1}$</td>
<td>c.p.m. $\mu$g$^{-1}$ P</td>
</tr>
<tr>
<td><strong>control</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24.40</td>
<td>0.92(0.03)</td>
<td>15651(16)</td>
<td>17018(584)</td>
</tr>
<tr>
<td>29.46</td>
<td>0.81(0.03)</td>
<td>15229(15)</td>
<td>18788(661)</td>
</tr>
<tr>
<td>28.30</td>
<td>0.82(0.03)</td>
<td>14962(15)</td>
<td>18330(635)</td>
</tr>
<tr>
<td><strong>NTP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25.99</td>
<td>1.02(0.04)</td>
<td>13790(14)</td>
<td>13525(485)</td>
</tr>
<tr>
<td>28.81</td>
<td>0.95(0.03)</td>
<td>13162(13)</td>
<td>13895(499)</td>
</tr>
<tr>
<td>28.62</td>
<td>0.84(0.03)</td>
<td>13328(13)</td>
<td>15795(556)</td>
</tr>
<tr>
<td><strong>NP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25.75</td>
<td>0.80(0.03)</td>
<td>10155(10)</td>
<td>12769(430)</td>
</tr>
<tr>
<td>28.19</td>
<td>0.88(0.03)</td>
<td>10088(10)</td>
<td>11407(403)</td>
</tr>
<tr>
<td>28.76</td>
<td>0.88(0.03)</td>
<td>10070(10)</td>
<td>11439(407)</td>
</tr>
<tr>
<td><strong>DNP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28.03</td>
<td>0.97(0.03)</td>
<td>11623(12)</td>
<td>11938(429)</td>
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<tr>
<td>28.86</td>
<td>0.87(0.03)</td>
<td>10849(11)</td>
<td>12430(439)</td>
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<tr>
<td>25.88</td>
<td>0.89(0.03)</td>
<td>11278(11)</td>
<td>12707(443)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>myofibril dry wt.</td>
<td>P content</td>
<td>$^{32}$P content</td>
</tr>
<tr>
<td>-------</td>
<td>------------------</td>
<td>-----------</td>
<td>------------------</td>
</tr>
<tr>
<td></td>
<td>mg</td>
<td>$\mu g$ P mg$^{-1}$</td>
<td>c.p.m. mg$^{-1}$</td>
</tr>
<tr>
<td>control</td>
<td>28.69</td>
<td>0.76(0.03)</td>
<td>12842(13)</td>
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<td>12346(12)</td>
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<td>25.66</td>
<td>0.97(0.03)</td>
<td>12579(13)</td>
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<td></td>
<td>27.30(0.92)</td>
<td>0.86(0.06)</td>
<td>12576(145)</td>
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<tr>
<td>+DTT</td>
<td>28.23</td>
<td>0.99(0.04)</td>
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<tr>
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<td></td>
<td>27.62(0.81)</td>
<td>0.85(0.05)</td>
<td>9631(104)</td>
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<tr>
<td>NTP</td>
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<tr>
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<td>8130(8)</td>
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<tr>
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<td>28.48</td>
<td>0.91(0.03)</td>
<td>8364(8)</td>
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<td></td>
<td>27.45(0.94)</td>
<td>0.90(0.04)</td>
<td>8386(172)</td>
</tr>
<tr>
<td>NP</td>
<td>25.96</td>
<td>0.75(0.03)</td>
<td>9101(9)</td>
</tr>
<tr>
<td>+DTT</td>
<td>25.49</td>
<td>0.99(0.04)</td>
<td>8942(9)</td>
</tr>
<tr>
<td></td>
<td>26.36</td>
<td>0.96(0.03)</td>
<td>8676(9)</td>
</tr>
<tr>
<td></td>
<td>25.94(0.25)</td>
<td>0.88(0.08)</td>
<td>8906(124)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.
4.3.3.7 SUMMARY OF EXPERIMENTAL FINDINGS

1. Incorporation of $^{32}$P is dependent on both $[^{32}\text{P}]_1$ and the time of incubation (Figs. 4.10 & 4.11). The extent of uptake was found to vary from preparation to preparation (Fig. 4.11 illustrates the time-dependent uptake by two different myofibril preparations) but was similar when a single preparation was labelled on different occasions. $^{32}$P contents of four different myofibril preparations were found to be 300, 5500, 25000, 50000 c.p.m. mg$^{-1}$; the standard deviation of an observed sample was between 17 and 22%.

2. The presence of either ATP (in the absence of divalent ion), MgATP,$^{32}$P$_1$, NTP, NP, DNP, or DTT during the labelling period reduces the quantity of $^{32}$P taken up by myofibrils (Tables 4.11 & 4.14).

3. The incorporation of $^{32}$P is not affected by myofibrillar ATPase activity or contraction prior to the addition of label (Table 4.12). However, the uptake is influenced by Ca$^{2+}$. The treatment of myofibrils with the Ca-chelating agent 'EGTA', prior to the addition of label, reduces the uptake (Table 4.13). The increase in $^{32}$P uptake due to Ca$^{2+}$ adsorbed on to the myofibrils is about 14% (Table 4.13); the presence of 1 mM Ca$^{2+}$ during labelling increases the uptake by 60% (Table 4.12).
4.3.4 REMOVAL OF BOUND $^{32}$P

4.3.4.1 0.5% KCl TREATMENT

Myofibrils were labelled for 24 hrs, collected by centrifugation but not washed with KCl solution (4.3.1). Labelled myofibrils were suspended in 30 ml 0.5% KCl buffered with 10 mM Tris-HCl pH 7.5 (4°C). After 10 min, the myofibrils were collected by centrifugation (1400g; 10 min) and the bound $^{32}$P content of an aliquot determined (4.3.2), without further KCl washing. The remaining myofibrils were resuspended in 0.5% KCl several times, the $^{32}$P content of an aliquot determined after each KCl treatment. After the final wash, the myofibrils were dialysed for 48 hrs vs. buffered 0.5% KCl and the bound $^{32}$P content determined.

RESULTS

Fig. 4.12 shows the effect of 0.5% KCl on the retention of $^{32}$P by myofibrils. The radioactive count could not be reduced below 10,000 c.p.m. mg$^{-1}$ by dialysis.

4.3.4.2 COLD TCA TREATMENT

Myofibrils were labelled for 24 hrs (4.3.1) and were extracted a number of times with 10 ml 5% TCA. The TCA-denatured myofibril suspension was left, each time, for 10 min and the bound $^{32}$P content determined (4.3.2; cf. 4.2.1.2). Protein was estimated as the N content of the acid digest.

RESULTS

Fig 4.13 shows the effectiveness of TCA in removing bound $^{32}$P.
FIGURE 4.12

EFFECT OF 0.5% KCl ON THE RETENTION OF $^{32}$P BY MYOFIBRILS

FIGURE 4.13

EFFECTIVENESS OF TCA IN REMOVING BOUND $^{32}$P
4.3.4.3 HOT PERCHLORIC ACID TREATMENT

Myofibrils were labelled for 24 hrs (4.3.1) and were extracted 5 times with 10 ml volumes of 1 M perchloric acid at 100°C for 15 min. The bound $^{32}$P content was determined (4.3.2).

RESULTS

Table 4.15.

4.3.4.4 MgATPase ACTIVITY

Myofibrils were labelled for 48 hrs (4.3.1) and were suspended in 0.1 M KCl buffered with 50 mM Tris-HCl pH 7.5 (25°C) containing 1 mM MgCl$_2$ and 0.01 mM CaCl$_2$. To one volume of myofibril suspension was added an equal volume of 2 mM ATP in buffered KCl/MgCl$_2$/CaCl$_2$. The suspension was incubated at 25°C for 10 min and the myofibrils collected by centrifugation (1400g; 10 min). The bound P and $^{32}$P content was determined (4.3.2).

RESULTS

Table 4.16.

4.3.4.5 EFFECT OF p-NITROTHIOPHENOL, p-NITROPHENOL, 2,4-DINITROPHENOL

Myofibrils were labelled for 48 hrs (4.3.1). Aliquots of labelled myofibrils were incubated at 4°C for 1 hr with a 0.025 mM concentration of either p-nitrothiophenol, p-nitrophenol, or 2,4-dinitrophenol in 20 mM Tris-malate pH 6.5 (4°C). The bound P and $^{32}$P content was
determined (4.3.2).

RESULTS

Table 4.17.

4.3.4.6 SUMMARY OF EXPERIMENTAL FINDINGS

1. $^{32}\text{P}$ is firmly bound. Washing of labelled myofibrils with saline removes the residual $^{32}\text{P}$ (Fig. 4.12). Treatment of KCl-washed, $^{32}\text{P}$-labelled myofibrils with cold 5% TCA reduces the bound count by about 60%. Hot perchloric acid removes about 90% of the label.

2. Neither NTP, NP, nor DNP removes any significant quantity of label.

3. The ratio of the specific activity of contracted $^{32}\text{P}$-labelled myofibrils to that of non-contracted is $0.874 \pm 0.046$ (Table 4.16). This apparent 13% reduction in specific activity of bound $^{32}\text{P}$ on contraction is believed, however, to be linked to the decreased aqueous extractability of contracted myofibrils; there is a 10% loss of protein in non-contracted myofibrils (see 4.4).
### TABLE 4.15

**HOT PERCHLORIC ACID EXTRACTION OF $^{32}$P-LABELLED MYOFIBRILS**

<table>
<thead>
<tr>
<th>myofibril dry wt.</th>
<th>P content</th>
<th>$^{32}$P content</th>
<th>specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
<td>$\mu$g P mg$^{-1}$</td>
<td>c.p.m. mg$^{-1}$</td>
<td>c.p.m. $\mu$g$^{-1}$ P</td>
</tr>
<tr>
<td>control</td>
<td>4.68</td>
<td>1.00(0.06)</td>
<td>2068(36)</td>
</tr>
<tr>
<td></td>
<td>3.76</td>
<td>0.98(0.06)</td>
<td>1493(23)</td>
</tr>
<tr>
<td></td>
<td>4.22(0.46)</td>
<td>0.99(0.01)</td>
<td>1660(261)</td>
</tr>
<tr>
<td>FCA</td>
<td>2.93</td>
<td>0.32(0.03)</td>
<td>140(5)</td>
</tr>
<tr>
<td></td>
<td>2.04</td>
<td>0.46(0.03)</td>
<td>191(1)</td>
</tr>
<tr>
<td></td>
<td>2.02</td>
<td>0.28(0.03)</td>
<td>191(7)</td>
</tr>
<tr>
<td></td>
<td>2.62</td>
<td>0.36(0.03)</td>
<td>143(6)</td>
</tr>
<tr>
<td></td>
<td>2.40(0.22)</td>
<td>0.36(0.04)</td>
<td>183(7)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.

Myofibrils were labelled and aliquots heated with perchloric acid at 100°C. See 4.3.4.3 for experimental details.
TABLE 4.16

EFFECT OF MgATP ON THE SPECIFIC ACTIVITY OF BOUND $^{32}$P

<table>
<thead>
<tr>
<th>myofibril dry wt.</th>
<th>P content</th>
<th>$^{32}$P content</th>
<th>specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>$\mu$g P mg$^{-1}$</td>
<td>c.p.m. mg$^{-1}$</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37.41</td>
<td>0.74(0.03)</td>
<td>3877(8)</td>
<td>5274(202)</td>
</tr>
<tr>
<td>34.13</td>
<td>0.53(0.02)</td>
<td>3989(8)</td>
<td>7459(246)</td>
</tr>
<tr>
<td>37.10</td>
<td>0.68(0.02)</td>
<td>4105(8)</td>
<td>6046(214)</td>
</tr>
<tr>
<td>38.39</td>
<td>0.64(0.02)</td>
<td>4099(8)</td>
<td>6447(228)</td>
</tr>
<tr>
<td>35.82</td>
<td>0.73(0.03)</td>
<td>3917(8)</td>
<td>5401(194)</td>
</tr>
<tr>
<td>35.14</td>
<td>0.68(0.02)</td>
<td>3918(8)</td>
<td>5766(203)</td>
</tr>
<tr>
<td>35.22</td>
<td>0.70(0.02)</td>
<td>4126(8)</td>
<td>5922(208)</td>
</tr>
<tr>
<td>36.20</td>
<td>0.85(0.03)</td>
<td>3961(8)</td>
<td>4673(172)</td>
</tr>
<tr>
<td>35.85</td>
<td>0.69(0.02)</td>
<td>4025(8)</td>
<td>5818(204)</td>
</tr>
<tr>
<td>35.97</td>
<td>0.82(0.03)</td>
<td>4067(8)</td>
<td>4979(182)</td>
</tr>
<tr>
<td>36.11(0.38)</td>
<td>0.68(0.03)</td>
<td>4008(28)</td>
<td>5641(237)</td>
</tr>
<tr>
<td>MgATP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40.20</td>
<td>0.72(0.03)</td>
<td>4156(9)</td>
<td>5790(211)</td>
</tr>
<tr>
<td>39.19</td>
<td>0.77(0.03)</td>
<td>4081(8)</td>
<td>5279(194)</td>
</tr>
<tr>
<td>40.07</td>
<td>0.79(0.03)</td>
<td>3896(8)</td>
<td>4959(183)</td>
</tr>
<tr>
<td>40.02</td>
<td>0.73(0.03)</td>
<td>3490(7)</td>
<td>4753(173)</td>
</tr>
<tr>
<td>40.09</td>
<td>0.61(0.03)</td>
<td>3906(8)</td>
<td>4835(179)</td>
</tr>
<tr>
<td>39.91</td>
<td>0.82(0.03)</td>
<td>4031(8)</td>
<td>4946(184)</td>
</tr>
<tr>
<td>40.50</td>
<td>0.70(0.03)</td>
<td>4197(8)</td>
<td>5970(216)</td>
</tr>
<tr>
<td>38.67</td>
<td>0.75(0.03)</td>
<td>3326(7)</td>
<td>4436(161)</td>
</tr>
<tr>
<td>39.74</td>
<td>0.85(0.03)</td>
<td>3921(8)</td>
<td>4623(173)</td>
</tr>
<tr>
<td>39.02</td>
<td>0.88(0.03)</td>
<td>3968(8)</td>
<td>4523(169)</td>
</tr>
<tr>
<td>39.74(0.19)</td>
<td>0.78(0.02)</td>
<td>3863(94)</td>
<td>4929(152)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.

Myofibrils were labelled and aliquots contracted with MgATP.
See 4.3.4.4 for experimental details.
### TABLE 4.17

**EFFECT OF p-NITROTHIOPHENOL, p-NITROPHENOL, 2,4-DINITROPHENOL ON THE SPECIFIC ACTIVITY OF BOUND $^{32}$P**

<table>
<thead>
<tr>
<th></th>
<th>myofibril dry wt. mg</th>
<th>P content $\mu$g P mg$^{-1}$</th>
<th>$^{32}$P content c.p.m. mg$^{-1}$</th>
<th>specific activity c.p.m. $\mu$g$^{-1}$ P</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>17.59</td>
<td>0.97(0.03)</td>
<td>406(2)</td>
<td>419(14)</td>
</tr>
<tr>
<td></td>
<td>14.70</td>
<td>0.98(0.03)</td>
<td>541(4)</td>
<td>549(17)</td>
</tr>
<tr>
<td></td>
<td>15.70</td>
<td>1.17(0.04)</td>
<td>396(3)</td>
<td>338(11)</td>
</tr>
<tr>
<td></td>
<td>17.36</td>
<td>1.19(0.04)</td>
<td>410(3)</td>
<td>346(12)</td>
</tr>
<tr>
<td></td>
<td>16.34(0.69)</td>
<td>1.05(0.06)</td>
<td>421(25)</td>
<td>388(42)</td>
</tr>
<tr>
<td>NTP</td>
<td>14.25</td>
<td>1.15(0.04)</td>
<td>392(3)</td>
<td>340(11)</td>
</tr>
<tr>
<td></td>
<td>12.62</td>
<td>1.12(0.03)</td>
<td>397(4)</td>
<td>353(11)</td>
</tr>
<tr>
<td></td>
<td>14.32</td>
<td>1.13(0.04)</td>
<td>419(3)</td>
<td>371(12)</td>
</tr>
<tr>
<td></td>
<td>13.94</td>
<td>1.18(0.04)</td>
<td>439(3)</td>
<td>373(12)</td>
</tr>
<tr>
<td></td>
<td>13.78(0.40)</td>
<td>1.14(0.01)</td>
<td>414(11)</td>
<td>358(8)</td>
</tr>
<tr>
<td>NP</td>
<td>16.46</td>
<td>1.10(0.03)</td>
<td>470(4)</td>
<td>426(14)</td>
</tr>
<tr>
<td></td>
<td>7.23</td>
<td>1.01(0.03)</td>
<td>399(4)</td>
<td>395(19)</td>
</tr>
<tr>
<td></td>
<td>11.40</td>
<td>1.17(0.04)</td>
<td>400(4)</td>
<td>344(12)</td>
</tr>
<tr>
<td></td>
<td>15.52</td>
<td>1.09(0.03)</td>
<td>465(5)</td>
<td>425(14)</td>
</tr>
<tr>
<td></td>
<td>12.65(2.12)</td>
<td>1.08(0.03)</td>
<td>430(20)</td>
<td>393(21)</td>
</tr>
<tr>
<td>DNP</td>
<td>14.91</td>
<td>1.05(0.03)</td>
<td>371(4)</td>
<td>356(12)</td>
</tr>
<tr>
<td></td>
<td>15.52</td>
<td>1.13(0.04)</td>
<td>480(5)</td>
<td>424(16)</td>
</tr>
<tr>
<td></td>
<td>14.12</td>
<td>1.07(0.03)</td>
<td>392(4)</td>
<td>367(11)</td>
</tr>
<tr>
<td></td>
<td>18.73</td>
<td>1.12(0.03)</td>
<td>387(3)</td>
<td>346(12)</td>
</tr>
<tr>
<td></td>
<td>15.82(1.01)</td>
<td>1.09(0.02)</td>
<td>398(20)</td>
<td>367(15)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.

Myofibrils were labelled and treated with reagent. See 4.3.4.5 for experimental details and Table 4.14 for abbreviations.
4.3.5 FRACTIONATION OF $^{32}$P-LABELLED MYOFIBRILS

Myofibrils were labelled for 24 hrs (4.3.1) and were resolved into P-containing fractions (4.2.2 - 4.2.6).

RESULTS

Table 4.18 gives the results of labelling and fractionating three myofibril preparations. The TCA extract of $^{32}$P-labelled myofibrils contained no component, other than $P_i$, of significant radioactive count (2.9); $P_i$ represented 20% of the P in the extract. All three myofibril preparations show that the $^{32}$P-fraction extractable with hot perchloric acid is of high specific activity (Table 4.19). Two of the preparations also contained a bound $^{32}$P fraction that was in a form only susceptible to alkaline hydrolysis. If we assume that hot acid-labile P is removed from components such as nucleic acid and phosphorylated basic amino acids, and that alkali-labile P represents phosphorylated seryl and threonyl residues in proteins, then $^{32}$P present in these fractions is probably also in these metabolic forms. Myofibrils can apparently synthesise phosphorylated cell components. Synthesis and phosphorylation require the input of energy and the utilisation of nucleotides (1.3). We have observed the presence of mitochondria in our myofibril preparations and have shown that $O_2$ uptake occurs on the addition of NADH; $O_2$ uptake does not take place with the myofibril suspension alone. Unless there is an unsuspected store of oxidisable low mol. wt. substrates, it would seem unlikely that myofibrillar mitochondria can synthesise ATP, even though ADP (which might be released from actin, or even myosin) and $^{32}P_i$ are present. The system is also depleted of cytoplasmic enzymes and substrates so that $^{32}P_i$ is unlikely to be taken up via any anaerobic
TABLE 4.18

FRACTIONATION OF $^{32}$P-LABELLED MYOFIBRILS

<table>
<thead>
<tr>
<th>fraction</th>
<th>myofibril dry wt.</th>
<th>P content $\mu g$ P $mg^{-1}$</th>
<th>$^{32}$P content c.p.m. mg$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total bound</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19.82</td>
<td>0.86(0.02)</td>
<td>8158(8)</td>
<td></td>
</tr>
<tr>
<td>18.97</td>
<td>0.71(0.02)</td>
<td>7852(8)</td>
<td></td>
</tr>
<tr>
<td>42.31</td>
<td>0.88(0.02)</td>
<td>3800(4)</td>
<td></td>
</tr>
<tr>
<td>41.06</td>
<td>0.88(0.03)</td>
<td>4061(4)</td>
<td></td>
</tr>
<tr>
<td>104.33</td>
<td>0.93(0.04)</td>
<td>143.3(0.3)</td>
<td></td>
</tr>
<tr>
<td>103.90</td>
<td>0.97(0.04)</td>
<td>137.8(0.3)</td>
<td></td>
</tr>
<tr>
<td>cold acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.78</td>
<td>0.67(0.03)</td>
<td>7678(16)</td>
<td></td>
</tr>
<tr>
<td>19.71</td>
<td>0.60(0.02)</td>
<td>6995(7)</td>
<td></td>
</tr>
<tr>
<td>residue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46.22</td>
<td>0.52(0.02)</td>
<td>2758(3)</td>
<td></td>
</tr>
<tr>
<td>37.44</td>
<td>0.65(0.02)</td>
<td>2818(3)</td>
<td></td>
</tr>
<tr>
<td>100.96</td>
<td>0.77(0.03)</td>
<td>73.8(0.3)</td>
<td></td>
</tr>
<tr>
<td>130.01</td>
<td>0.68(0.03)</td>
<td>69.9(0.3)</td>
<td></td>
</tr>
<tr>
<td>chloroform/methanol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.35</td>
<td>0.61(0.04)</td>
<td>7921(16)</td>
<td></td>
</tr>
<tr>
<td>18.71</td>
<td>0.49(0.01)</td>
<td>6761(7)</td>
<td></td>
</tr>
<tr>
<td>residue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36.37</td>
<td>0.53(0.02)</td>
<td>2932(3)</td>
<td></td>
</tr>
<tr>
<td>32.40</td>
<td>0.46(0.01)</td>
<td>2837(3)</td>
<td></td>
</tr>
<tr>
<td>102.17</td>
<td>0.53(0.02)</td>
<td>63.5(0.3)</td>
<td></td>
</tr>
<tr>
<td>74.34</td>
<td>0.58(0.02)</td>
<td>68.7(0.3)</td>
<td></td>
</tr>
<tr>
<td>hot acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14.23</td>
<td>0.08(0.01)</td>
<td>263(2)</td>
<td></td>
</tr>
<tr>
<td>13.79</td>
<td>0.08(0.01)</td>
<td>263(2)</td>
<td></td>
</tr>
<tr>
<td>residue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27.17</td>
<td>0.13(0.01)</td>
<td>136(1)</td>
<td></td>
</tr>
<tr>
<td>24.65</td>
<td>0.08(0.01)</td>
<td>130(1)</td>
<td></td>
</tr>
<tr>
<td>68.46</td>
<td>0.29(0.01)</td>
<td>2.7(0.1)</td>
<td></td>
</tr>
<tr>
<td>67.27</td>
<td>0.17(0.01)</td>
<td>2.9(0.1)</td>
<td></td>
</tr>
<tr>
<td>alkali-labile</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.18(0.19)</td>
<td>0.11(0.05)</td>
<td>287(2)</td>
<td></td>
</tr>
<tr>
<td>8.77(0.18)</td>
<td>0.05(0.05)</td>
<td>230(2)</td>
<td></td>
</tr>
<tr>
<td>15.03(0.15)</td>
<td>0.05(0.03)</td>
<td>28(4)</td>
<td></td>
</tr>
<tr>
<td>16.57(0.15)</td>
<td>0.08(0.03)</td>
<td>141(1)</td>
<td></td>
</tr>
<tr>
<td>31.37(1.81)</td>
<td>0.08(0.01)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>31.35(1.44)</td>
<td>0.08(0.01)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
### Table 4.19

**P and \( ^{32}\text{P} \) Content of P-Containing Fractions of Labelled Myofibrils**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>( \mu g \text{ P mg}^{-1} )</th>
<th>( ^{32}\text{P} ) Content</th>
<th>Specific Activity ( \text{c.p.m. mg}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( ^{32}\text{P mg}^{-1} )</td>
<td></td>
<td>( ^{32}\text{P} \text{ c.p.m. mg}^{-1} )</td>
</tr>
<tr>
<td>Total bound P</td>
<td>0.79(0.08)</td>
<td>8005(153)</td>
<td>10151(797)</td>
</tr>
<tr>
<td></td>
<td>0.88(0.01)</td>
<td>3931(131)</td>
<td>4475(143)</td>
</tr>
<tr>
<td></td>
<td>0.95(0.02)</td>
<td>141(3)</td>
<td>147(5)</td>
</tr>
<tr>
<td>Cold acid</td>
<td>0.31(0.06)</td>
<td>1143(101)</td>
<td>3026(618)</td>
</tr>
<tr>
<td>Extractable P</td>
<td>0.14(0.04)</td>
<td>754(163)</td>
<td>2591(1320)</td>
</tr>
<tr>
<td></td>
<td>0.26(0.07)</td>
<td>69(1)</td>
<td>250(55)</td>
</tr>
<tr>
<td>Chloroform/methanol extractable P</td>
<td>0.13(0.09)</td>
<td>-97(78)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.10(0.02)</td>
<td>158(174)</td>
<td>2044(712)</td>
</tr>
<tr>
<td></td>
<td>0.17(0.07)</td>
<td>6(5)</td>
<td>25(15)</td>
</tr>
<tr>
<td>Hot acid</td>
<td>0.38(0.01)</td>
<td>2752(45)</td>
<td>7094(56)</td>
</tr>
<tr>
<td>Extractable P</td>
<td>0.43(0.05)</td>
<td>6680(428)</td>
<td>15373(655)</td>
</tr>
<tr>
<td></td>
<td>0.33(0.09)</td>
<td>63(3)</td>
<td>172(30)</td>
</tr>
<tr>
<td>Alkali-labile P</td>
<td>0.07(0.02)</td>
<td>118(45)</td>
<td>818(493)</td>
</tr>
<tr>
<td></td>
<td>0.08(0.03)</td>
<td>259(29)</td>
<td>2733(481)</td>
</tr>
<tr>
<td></td>
<td>0.08(0.01)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.

Data in the Table were calculated from the results given in Table 4.18. Corresponding rows in each fraction refer to the same myofibril preparation. See 4.3.5. for further information.
reaction (cf. 1.3.2). We suspected, therefore, the presence of an external agent in our myofibril preparations which contained the requisite enzyme systems, i.e. bacteria.

4.3.6 EFFECT OF THE ANTIBIOTIC 'CHLORAMPHENICOL' ON UPTAKE OF $^{32}P$

Chloramphenicol (D(-)-threo-2-dichloroacetamido-1-p-nitrophenyl-1,3-propandiol) inhibits bacterial growth by interfering with ribosomal function and thereby stopping protein synthesis (Rendi & Ochoa, 1962). The antibiotic does not affect processes of energy generation and transduction directly but inhibition of metabolism is a secondary event related to the inhibition of protein synthesis (cf. Hahn, 1967). We observed the effect of chloramphenicol on the uptake of $^{32}P$ by myofibrils.

4.3.6.1 EXTENT OF LABELLING OF MYOFIBRILS IN THE PRESENCE OF CHLORAMPHENICOL

A suspension of myofibrils (2 - 4 mg ml$^{-1}$) in 0.1 M KCl buffered with 50 mM Tris-HCl pH 7.5 ($4^\circ$C) was prepared (2.2.1.2) and stored in an air-tight container for 48 hrs with 0.1 mg chloramphenicol ml$^{-1}$.

A suspension of myofibrils was stored concurrently in the absence of antibiotic. The incubated myofibrils were labelled for 24 hrs in the continued presence of chloramphenicol (4.3.1). The bound P and $^{32}P$ content was determined (4.3.2). Aliquots of labelled myofibrils were extracted with 5% TCA (4.3.4.2) and the P and $^{32}P$ content determined.

RESULTS

Table 4.20.
### TABLE 4.20

THE UPTAKE OF $^{32}\text{P}$ BY CHLORAMPHENICOL-TREATED MYOFIBRILS

<table>
<thead>
<tr>
<th>myofibril dry wt.</th>
<th>P content</th>
<th>$^{32}\text{P}$ content</th>
<th>specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu g$ P $mg^{-1}$</td>
<td>c.p.m. $mg^{-1}$</td>
<td>c.p.m. $\mu g^{-1}$ P</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>49.96</td>
<td>0.69(0.03)</td>
<td>50845(14)</td>
<td>73374(2755)</td>
</tr>
<tr>
<td>50.51</td>
<td>0.75(0.03)</td>
<td>52967(10)</td>
<td>71097(2702)</td>
</tr>
<tr>
<td>49.87</td>
<td>0.93(0.04)</td>
<td>46633(24)</td>
<td>50120(1944)</td>
</tr>
<tr>
<td>51.62</td>
<td>0.99(0.04)</td>
<td>53172(81)</td>
<td>53967(2124)</td>
</tr>
<tr>
<td>45.17</td>
<td>0.77(0.03)</td>
<td>58311(17)</td>
<td>75795(2836)</td>
</tr>
<tr>
<td>control</td>
<td>49.43(1.11)</td>
<td>0.80(0.05)</td>
<td>52806(1528)</td>
</tr>
<tr>
<td>chloramphenicol</td>
<td>45.20</td>
<td>0.94(0.04)</td>
<td>925(1)</td>
</tr>
<tr>
<td>48.67</td>
<td>1.00(0.04)</td>
<td>815(1)</td>
<td>814(32)</td>
</tr>
<tr>
<td>50.10</td>
<td>0.89(0.03)</td>
<td>765(3)</td>
<td>862(34)</td>
</tr>
<tr>
<td>44.75</td>
<td>0.91(0.03)</td>
<td>849(1)</td>
<td>932(36)</td>
</tr>
<tr>
<td>47.94</td>
<td>0.89(0.03)</td>
<td>811(1)</td>
<td>915(35)</td>
</tr>
<tr>
<td>chloramphenicol</td>
<td>47.33(1.03)</td>
<td>0.92(0.02)</td>
<td>848(24)</td>
</tr>
<tr>
<td>TCA</td>
<td>55.83</td>
<td>0.65(0.03)</td>
<td>13133(8)</td>
</tr>
<tr>
<td>chloramphenicol</td>
<td>49.33</td>
<td>0.66(0.04)</td>
<td>619(2)</td>
</tr>
<tr>
<td>treated + TCA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.

Myofibrils were labelled either in the presence or absence of chloramphenicol. An aliquot of each type of labelled myofibrils was treated with TCA. See 4.3.6.1 for experimental details.
4.3.6.2 EFFECT OF MgATPase ACTIVITY ON $^{32}$P TAKEN UP BY CHLORAMPHENICOL-TREATED MYOFIBRILS

Myofibrils were labelled in the presence of antibiotic. Aliquots of labelled myofibrils were contracted with MgATP (4.3.4.5). The bound P and $^{32}$P content of contracted and non-contracted myofibrils was determined (4.3.2).

RESULTS

Table 4.21.

4.3.6.3 SUMMARY OF EXPERIMENTAL FINDINGS

1. The uptake of $^{32}$P by myofibrils is reduced by 98.4% when labelling is carried out in the presence of chloramphenicol (Table 4.20).

2. Contraction of chloramphenicol-treated myofibrils has no significant effect on the label.

3. TCA removes 27% of the $^{32}$P taken up by chloramphenicol-treated myofibrils.

4. Smears of myofibrils, on microscope slides, stained with Gram's Iodine, showed the presence of bacteria. Autoradiographs of smears (2.12.1) did not show any high local density of $^{32}$P; silver granule counts of randomly selected areas were not significantly different from background.
<table>
<thead>
<tr>
<th>myofibril dry wt.</th>
<th>P content</th>
<th>$^{32}$P content</th>
<th>specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>µg P mg$^{-1}$</td>
<td>c.p.m. mg$^{-1}$</td>
</tr>
<tr>
<td>control</td>
<td>37.40</td>
<td>0.65(0.02)</td>
<td>73701(10)</td>
</tr>
<tr>
<td>untreated</td>
<td>50.53</td>
<td>0.77(0.03)</td>
<td>56987(12)</td>
</tr>
<tr>
<td></td>
<td>50.69</td>
<td>0.74(0.03)</td>
<td>58426(45)</td>
</tr>
<tr>
<td></td>
<td>67.00</td>
<td>0.92(0.04)</td>
<td>49155(56)</td>
</tr>
<tr>
<td></td>
<td>63.75</td>
<td>0.65(0.02)</td>
<td>49694(28)</td>
</tr>
<tr>
<td></td>
<td>54.78</td>
<td>0.91(0.04)</td>
<td>52413(64)</td>
</tr>
<tr>
<td>MgATP untreated</td>
<td>43.23</td>
<td>0.89(0.03)</td>
<td>59777(15)</td>
</tr>
<tr>
<td></td>
<td>34.87</td>
<td>1.00(0.04)</td>
<td>70654(111)</td>
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<tr>
<td></td>
<td>37.51</td>
<td>0.96(0.04)</td>
<td>67815(33)</td>
</tr>
<tr>
<td></td>
<td>31.17</td>
<td>0.82(0.03)</td>
<td>79540(118)</td>
</tr>
<tr>
<td></td>
<td>33.44</td>
<td>0.95(0.03)</td>
<td>71754(73)</td>
</tr>
<tr>
<td></td>
<td>34.77</td>
<td>0.90(0.03)</td>
<td>69762(13)</td>
</tr>
<tr>
<td>chloramphenicol treated</td>
<td>35.83(1.70)</td>
<td>0.91(0.03)</td>
<td>65077(2246)</td>
</tr>
<tr>
<td>MgATP untreated</td>
<td>39.61</td>
<td>0.89(0.03)</td>
<td>807(1)</td>
</tr>
<tr>
<td>chloramphenicol treated</td>
<td>44.67</td>
<td>0.98(0.04)</td>
<td>747(1)</td>
</tr>
<tr>
<td></td>
<td>30.17</td>
<td>1.11(0.04)</td>
<td>973(1)</td>
</tr>
<tr>
<td></td>
<td>35.79</td>
<td>0.84(0.03)</td>
<td>845(2)</td>
</tr>
<tr>
<td></td>
<td>35.04</td>
<td>0.95(0.04)</td>
<td>863(1)</td>
</tr>
<tr>
<td></td>
<td>36.35</td>
<td>1.00(0.04)</td>
<td>942(5)</td>
</tr>
<tr>
<td>MgATP untreated</td>
<td>36.94(1.98)</td>
<td>0.94(0.04)</td>
<td>848(36)</td>
</tr>
</tbody>
</table>
A STUDY OF THE REDUCTION IN SPECIFIC ACTIVITY OF $^{32}\text{P}$ BOUND TO PSOAS MYOFIBRILS

As reported in 1.1, Whitehead (1970) has shown that the specific activity of $^{32}\text{P}$ bound to rabbit (and frog) muscle is reduced by about 40% on contraction ('the contraction effect'). This phenomenon, as evinced by glycerol-extracted myofibrils of labelled rabbit psoas muscle, appeared to be associated with actomyosin MgATPase, hence muscle contraction, and not with myosin CaATPase. Our preliminary investigations confirmed Whitehead's finding that the ratio of the specific activity of the $^{32}\text{P}$ bound to contracted and non-contracted myofibrils was about 0.6. However, in later studies no reduction in specific activity was observed to occur in any preparations. Our method of labelling psoas muscle was identical to that of Whitehead (cf. 2.2.2.1), as was the method of storage of glycerol-extracted psoas fibre bundles (cf. 2.2.2.2). We have looked for a reason for the disparity in results. Such an explanation may provide some insight into the contraction effect.

4.4.1 Determination of P and $^{32}\text{P}$ content of labelled psoas myofibrils

4.4.2 Effect of MgATPase and contraction on bound $^{32}\text{P}$

4.4.3 Water treatment of contracted and non-contracted $^{32}\text{P}$-labelled psoas myofibrils

4.4.4 Aqueous extraction of contracted and non-contracted psoas myofibrils
4.4.5 Fractionation of labelled psoas

4.4.6 Deionised water extraction of butanol-extracted acetone-dried psoas myofibrils

DEFINITIONS

mg : dry wt. of myofibrils (p. 143)

$\mu g \text{ P mg}^{-1}$ : bound P content of myofibrils (2.6.2.2)

$\text{c.p.m. mg}^{-1}$ : bound $^{32}\text{P}$ content of myofibrils

$\text{c.p.m. } \mu g^{-1} \text{ P}$ : specific activity of $^{32}\text{P}$
4.4.1 **DETERMINATION OF P AND \(^{32}P\) CONTENT OF LABELLED PSOAS MYOFIBRILS**

Labelled psoas myofibrils were washed free of P\(_{i}\) and reagents as described in the text. The washed myofibrils were acetone-dried, weighed, digested with conc. sulphuric acid and the P content determined as described in 2.6.2.2. Radioactivity was measured in 10 ml of digest (2.8.3).

4.4.2 **EFFECT OF MgATP\(_{ase}\) AND CONTRACTION ON BOUND \(^{32}P\)**

Labelled psoas myofibrils (2.2.2) were treated with MgATP as described in 4.3.4.4. Contracted and non-contracted myofibrils were washed 5 times by repeated suspension in 0.5% KCl buffered with 10 mM Tris-ECI pH 7.5 (4°C). The bound P and \(^{32}P\) content was determined (4.4.1).

**RESULTS**

Table 4.22 gives the bound P and \(^{32}P\) content of contracted and non-contracted myofibrils from three psoas preparations. The specific activities of contracted and non-contracted myofibrils are not significantly different (T for the preparations are 0.832, 0.966, 0.197, for 7, 1, and 1 degrees of freedom respectively). The ratios of the specific activity of contracted to that of non-contracted myofibrils are 1.08 ± 0.09, 1.27 ± 0.27, 1.04 ± 0.22.


<table>
<thead>
<tr>
<th></th>
<th>myofibril dry wt.</th>
<th>P content</th>
<th>$^{32}$P content</th>
<th>specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>$\mu$g P mg$^{-1}$</td>
<td>c.p.m. mg$^{-1}$</td>
<td>c.p.m. $\mu$g$^{-1}$ P</td>
</tr>
<tr>
<td><strong>control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>53.43</td>
<td>1.19(0.05)</td>
<td>222.6(0.8)</td>
<td>187.5(7.5)</td>
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<td>46.07</td>
<td>1.39(0.06)</td>
<td>231.5(0.7)</td>
<td>166.8(6.7)</td>
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<td>51.21</td>
<td>1.22(0.05)</td>
<td>224.5(0.6)</td>
<td>184.3(7.3)</td>
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<td>55.54</td>
<td>1.17(0.05)</td>
<td>214.2(0.1)</td>
<td>183.8(7.3)</td>
</tr>
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<td>46.34</td>
<td>1.27(0.05)</td>
<td>209.7(0.5)</td>
<td>164.8(6.5)</td>
</tr>
<tr>
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<td>1.11(0.04)</td>
<td>236.8(0.8)</td>
<td>214.2(8.4)</td>
</tr>
<tr>
<td></td>
<td>51.40(1.78)</td>
<td>1.21(0.04)</td>
<td>215.0(1.8)</td>
<td>181.0(6.9)</td>
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<tr>
<td><strong>MgATP</strong></td>
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<td></td>
</tr>
<tr>
<td>MgATP</td>
<td>54.90</td>
<td>1.41(0.06)</td>
<td>220.9(0.6)</td>
<td>156.5(6.3)</td>
</tr>
<tr>
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<td>57.32</td>
<td>1.21(0.05)</td>
<td>230.5(0.2)</td>
<td>190.0(7.5)</td>
</tr>
<tr>
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<td>48.62</td>
<td>1.15(0.05)</td>
<td>221.3(0.8)</td>
<td>193.1(7.6)</td>
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<tr>
<td></td>
<td>46.42</td>
<td>1.18(0.05)</td>
<td>234.2(1.1)</td>
<td>198.9(7.8)</td>
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<td>48.61</td>
<td>1.02(0.04)</td>
<td>261.4(0.2)</td>
<td>257.2(9.9)</td>
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<td>47.13</td>
<td>1.08(0.04)</td>
<td>245.2(0.2)</td>
<td>226.0(8.8)</td>
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<td>47.00(0.71)</td>
<td>1.14(0.05)</td>
<td>244.2(6.0)</td>
<td>195.1(13.4)</td>
</tr>
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<td><strong>control</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>59.70</td>
<td>1.18(0.05)</td>
<td>88.0(0.9)</td>
<td>74.8(3.1)</td>
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<td></td>
<td>58.25</td>
<td>1.22(0.05)</td>
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<td>73.5(3.0)</td>
</tr>
<tr>
<td></td>
<td>59.00(0.73)</td>
<td>1.20(0.02)</td>
<td>89.4(0.8)</td>
<td>74.1(0.6)</td>
</tr>
<tr>
<td><strong>MgATP</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgATP</td>
<td>56.02</td>
<td>0.72(0.03)</td>
<td>87.3(0.3)</td>
<td>121.2(4.6)</td>
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<td>59.12</td>
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<td>80.7(1.4)</td>
<td>79.1(3.4)</td>
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<td>57.57(1.55)</td>
<td>1.20(0.02)</td>
<td>89.4(0.8)</td>
<td>74.1(0.6)</td>
</tr>
<tr>
<td><strong>control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.50</td>
<td>1.05(0.03)</td>
<td>42.5(4.2)</td>
<td>40.4(4.2)</td>
</tr>
<tr>
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<td>10.03</td>
<td>1.26(0.04)</td>
<td>34.0(3.4)</td>
<td>27.0(3.3)</td>
</tr>
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<td>10.27(0.24)</td>
<td>1.13(0.10)</td>
<td>34.1(0.8)</td>
<td>32.1(6.5)</td>
</tr>
<tr>
<td><strong>MgATP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgATP</td>
<td>10.96</td>
<td>1.32(0.04)</td>
<td>40.8(1.2)</td>
<td>31.0(1.3)</td>
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<tr>
<td></td>
<td>10.67</td>
<td>1.18(0.03)</td>
<td>41.2(1.0)</td>
<td>34.9(1.0)</td>
</tr>
<tr>
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<td>10.82(0.15)</td>
<td>1.23(0.07)</td>
<td>41.2(0.1)</td>
<td>33.5(1.9)</td>
</tr>
</tbody>
</table>
4.4.3 WATER TREATMENT OF CONTRACTED AND NON-CONTRACTED
32P-LABELLED PSOAS MYOFIBRILS

The practice of removing unbound substances from myofibrils by washing the protein several times by repeated suspension in cold water, had been discontinued since myofibrils swelled considerably during such treatment. The swollen myofibrils were found to be difficult to sediment by centrifugation. The swelling became more pronounced as the number of water treatments was increased. This effect was greatly reduced when myofibrils were washed with dilute saline (e.g. 0.5% KCl) or buffer (e.g. 5 mM Tris-HCl pH 7). We treated KCl-washed contracted and non-contracted myofibrils with water.

PROCEDURE

Labelled psoas myofibrils were contracted and washed with buffered KCl as described in 4.4.2. Aliquots of KCl-washed contracted and non-contracted myofibrils were suspended in cold distilled water, at a concentration of 1 - 2 mg protein ml⁻¹ water, for 30 min and were collected by centrifugation at 1400g for 20 min. Similar quantities of KCl-washed contracted and non-contracted myofibrils were suspended in buffered KCl for 30 min and collected by centrifugation. The bound P and 32P content of the respectively-treated myofibrils was determined (4.4.1).

RESULTS

Table 4.23 gives the P and 32P content of water-treated contracted and non-contracted myofibrils. Comparative values of KCl-treated contracted and non-contracted myofibrils are also given. The specific activities
of KCl-washed contracted and non-contracted myofibrils are not significantly different \((T = 0.771, 9\) degrees of freedom\); the ratio is \(1.05 \pm 0.06\). However, the specific activities of water-washed contracted and non-contracted myofibrils are different \((T = 4.294, 10\) degrees of freedom\); the ratio of specific activities in this case is \(0.48 \pm 0.08\).

The specific activity of water-washed contracted myofibrils is not significantly different from that of KCl-washed contracted myofibrils \((T = 0.533, 8\) degrees of freedom\), whereas the specific activity of water-washed non-contracted myofibrils is significantly different from that of KCl-washed non-contracted \((T = 4.797, 6\) degrees of freedom\). The ratio of the specific activities of the two forms of non-contracted myofibrils is \(0.49 \pm 0.06\).

These observations show that a single water treatment has an effect on the specific activity of bound \(^{32}\text{P}\).

The data in Table 4.24 (mean values calculated from the results given in Table 4.23) show the following points.

1. The quantity of protein sedimented at 1400g in 20 min from suspensions of KCl-washed non-contracted and contracted myofibrils is similar. The quantities of protein-bound P and \(^{32}\text{P}\) are also comparable.

2. Water has extracted protein, P, and \(^{32}\text{P}\) from both non-contracted and contracted myofibrils; the quantities
<table>
<thead>
<tr>
<th></th>
<th>myofibril dry wt.</th>
<th>P content (μg P mg⁻¹)</th>
<th>³²P content (c.p.m. mg⁻¹)</th>
<th>specific activity (c.p.m. μg⁻¹ P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-contracted</td>
<td>2.36</td>
<td>1.02 (0.07)</td>
<td>107.7 (7.6)</td>
<td>105.5 (10.7)</td>
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<tr>
<td>water treated</td>
<td>2.52</td>
<td>1.68 (0.06)</td>
<td>93.5 (7.1)</td>
<td>55.6 (4.6)</td>
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<td>4.35</td>
<td>1.25 (0.03)</td>
<td>85.7 (4.1)</td>
<td>68.8 (3.7)</td>
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<tr>
<td></td>
<td>2.80</td>
<td>0.86 (0.06)</td>
<td>108.8 (5.7)</td>
<td>126.4 (10.6)</td>
</tr>
<tr>
<td></td>
<td>2.61</td>
<td>1.02 (0.07)</td>
<td>107.6 (4.7)</td>
<td>105.2 (8.1)</td>
</tr>
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<td></td>
<td>4.86</td>
<td>0.71 (0.03)</td>
<td>70.2 (2.4)</td>
<td>98.6 (5.4)</td>
</tr>
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<td>2.10</td>
<td>1.15 (0.08)</td>
<td>86.9 (3.7)</td>
<td>75.7 (6.2)</td>
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<td>3.09 (0.40)</td>
<td>1.04 (0.12)</td>
<td>84.8 (5.9)</td>
<td>78.0 (8.1)</td>
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<tr>
<td>contracted</td>
<td>9.55</td>
<td>1.25 (0.04)</td>
<td>33.8 (2.2)</td>
<td>27.0 (1.9)</td>
</tr>
<tr>
<td>water treated</td>
<td>9.31</td>
<td>1.37 (0.04)</td>
<td>29.6 (2.1)</td>
<td>21.6 (1.7)</td>
</tr>
<tr>
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<td>6.41</td>
<td>1.07 (0.02)</td>
<td>50.9 (3.6)</td>
<td>47.6 (3.6)</td>
</tr>
<tr>
<td></td>
<td>6.15</td>
<td>1.16 (0.03)</td>
<td>54.1 (3.3)</td>
<td>46.7 (3.0)</td>
</tr>
<tr>
<td></td>
<td>6.12</td>
<td>1.16 (0.03)</td>
<td>58.5 (5.6)</td>
<td>50.3 (4.9)</td>
</tr>
<tr>
<td></td>
<td>6.49</td>
<td>0.88 (0.02)</td>
<td>52.0 (2.5)</td>
<td>59.4 (3.2)</td>
</tr>
<tr>
<td></td>
<td>4.67</td>
<td>0.97 (0.03)</td>
<td>63.4 (3.5)</td>
<td>65.7 (3.7)</td>
</tr>
<tr>
<td></td>
<td>6.79</td>
<td>1.32 (0.03)</td>
<td>54.3 (1.5)</td>
<td>41.2 (1.5)</td>
</tr>
<tr>
<td></td>
<td>6.94 (0.59)</td>
<td>1.09 (0.06)</td>
<td>47.0 (4.2)</td>
<td>37.3 (5.0)</td>
</tr>
</tbody>
</table>

cont. next page.
### Table 4.23 (cont.)

COMPARISON OF THE EFFECTS OF 0.5% KCl AND WATER TREATMENTS ON THE $^{32}$P BOUND TO PSOAS MYOFIBRILS

<table>
<thead>
<tr>
<th></th>
<th>myofibril dry wt.</th>
<th>P content</th>
<th>$^{32}$P content</th>
<th>specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>$\mu$g P mg$^{-1}$</td>
<td>c.p.m. mg$^{-1}$</td>
<td>c.p.m. $\mu$g$^{-1}$ P</td>
</tr>
<tr>
<td>non-contracted</td>
<td>8.88</td>
<td>1.21(0.03)</td>
<td>40.4(2.3)</td>
<td>33.4(2.1)</td>
</tr>
<tr>
<td>KCl treated</td>
<td>8.86</td>
<td>1.09(0.04)</td>
<td>44.0(2.8)</td>
<td>40.4(3.0)</td>
</tr>
<tr>
<td></td>
<td>10.86</td>
<td>1.16(0.03)</td>
<td>41.0(2.2)</td>
<td>35.3(2.1)</td>
</tr>
<tr>
<td></td>
<td>8.42</td>
<td>1.01(0.03)</td>
<td>42.6(2.0)</td>
<td>42.2(2.3)</td>
</tr>
<tr>
<td></td>
<td>9.56</td>
<td>1.00(0.04)</td>
<td>41.2(2.4)</td>
<td>41.2(2.9)</td>
</tr>
<tr>
<td></td>
<td>9.18</td>
<td>1.02(0.03)</td>
<td>43.3(2.7)</td>
<td>42.5(2.9)</td>
</tr>
<tr>
<td></td>
<td>9.29(0.35)</td>
<td>1.09(0.04)</td>
<td>41.9(0.5)</td>
<td>38.3(1.7)</td>
</tr>
<tr>
<td>contracted</td>
<td>9.36</td>
<td>1.08(0.03)</td>
<td>45.2(2.6)</td>
<td>41.8(2.4)</td>
</tr>
<tr>
<td>KCl treated</td>
<td>10.72</td>
<td>0.93(0.04)</td>
<td>41.0(2.8)</td>
<td>44.1(2.6)</td>
</tr>
<tr>
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<td>8.63</td>
<td>1.21(0.03)</td>
<td>39.1(2.6)</td>
<td>32.1(2.8)</td>
</tr>
<tr>
<td></td>
<td>9.00</td>
<td>1.11(0.03)</td>
<td>43.3(2.3)</td>
<td>39.0(2.4)</td>
</tr>
<tr>
<td></td>
<td>8.96</td>
<td>1.08(0.03)</td>
<td>43.3(2.4)</td>
<td>40.1(2.4)</td>
</tr>
<tr>
<td></td>
<td>9.71</td>
<td>1.01(0.03)</td>
<td>42.8(2.6)</td>
<td>42.3(2.7)</td>
</tr>
<tr>
<td></td>
<td>9.40(0.30)</td>
<td>1.07(0.04)</td>
<td>42.5(0.8)</td>
<td>40.1(1.6)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.

Labelled psoas myofibrils were contracted with MgATP and washed free of P$_i$ and ADP by repeated suspension in 0.5% KCl buffered with 10 mM Tris HCl pH 7.5 (4°C). Aliquots of myofibrils were only washed with buffered KCl ('non-contracted'). Similar quantities of KCl-washed non-contracted and contracted myofibrils were either treated with water or with buffered KCl. See 4.4.3 for experimental details.
### TABLE 4.24

**P AND $^{32}$P BOUND TO NON-CONTRACTED AND CONTRACTED PSOAS MYOFIBRILS**

<table>
<thead>
<tr>
<th>treatment</th>
<th>myofibril state</th>
<th>dry wt. (mg)</th>
<th>bound P (μg)</th>
<th>bound $^{32}$P (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>non-contracted</td>
<td>9.29(0.35)</td>
<td>9.96(0.58)</td>
<td>385(13)</td>
</tr>
<tr>
<td></td>
<td>contracted</td>
<td>9.40(0.30)</td>
<td>10.01(0.12)</td>
<td>394(14)</td>
</tr>
<tr>
<td>water</td>
<td>non-contracted</td>
<td>3.09(0.40)</td>
<td>3.50(0.48)</td>
<td>257(28)</td>
</tr>
<tr>
<td></td>
<td>contracted</td>
<td>6.94(0.59)</td>
<td>6.70(0.69)</td>
<td>335(12)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.

Data calculated from the results given in Table 4.23. Myofibrils were originally apportioned as equal volumes of suspension. See 4.4.3 for experimental details.
sedimented are not comparable. 41 ± 9% more protein is extracted from non-contracted myofibrils. The bound P is evenly distributed since equivalent amounts are lost, with protein, from both myofibril states.

3. The difference in the amounts of protein-bound $^{32}\text{P}$ extracted from non-contracted and contracted myofibrils, 78 ± 30 c.p.m., is not as large as would be expected if $^{32}\text{P}$ was evenly distributed.

4. The 52 ± 7% reduction in specific activity of bound $^{32}\text{P}$ on contraction (Table 4.23) is linked to the extractability of protein.
4.4.4 AQUEOUS EXTRACTION OF CONTRACTED AND NON-CONTRACTED $^{32}$P-LABELLED PSOAS MYOFIBRILS

Labelled psoas myofibrils (2.2.2) were suspended in 0.1 M KCl buffered with 50 mM Tris-HCl pH 7.5 ($25^\circ C$). To an aliquot of myofibrils was added an equal volume of buffered KCl containing 2 mM ATP, 2 mM MgCl$_2$, and 0.02 mM CaCl$_2$. The suspension was incubated at $25^\circ C$ for 10 min and stirred occasionally. Non-contracted myofibril controls were incubated with buffered KCl/MgCl$_2$/CaCl$_2$ but without ATP. Myofibrils were collected by centrifugation (1400g; 10 min). The myofibril suspension was suspended in 0.5% KCl buffered with 10 mM Tris-HCl pH 7.5 ($4^\circ C$) and the myofibrils washed 5 times by repeated suspension in buffered KCl (4.2.1.3). The KCl-washed myofibrils were suspended, at a concentration of 1 - 2 mg ml$^{-1}$, in distilled water. The suspension was left at $0 - 4^\circ C$ for 10 min and stirred occasionally. The myofibrils were collected by centrifugation at 1400g for 20 min; both the sediment of myofibrils and the aqueous supernatant were retained ('first extraction'). The sedimented myofibrils were resuspended in water, as before, and both the sediment and supernatant were retained after centrifugation ('second extraction').

Subsequent treatments to the aqueous extracts and the second myofibril sediment ('residue') were as follows.

**RESIDUE**

The P and $^{32}$P content of the myofibril residue was determined without further treatment (4.4.1).
FIRST AND SECOND EXTRACTS

TOTAL EXTRACTED PROTEIN, P, AND $^{32}P$

The volume of extract was measured and the protein content determined (2.6.3). An aliquot was freeze-dried and the dried residue digested with hot conc. sulphuric acid; the $P$ and $^{32}P$ content of the digest was determined (cf. 2.6.2.2).

SOLUBLISED PROTEIN, P, AND $^{32}P$

An aliquot of extract was centrifuged at 40,000g for 1 hr. The protein content of the supernatant was measured. An aliquot of supernatant was freeze-dried and the dried residue digested with hot conc. sulphuric acid; the $P$ and $^{32}P$ content of the digest was determined.

$P_i$ IN THE SOLUBLISED PROTEIN FRACTION

An aliquot of the supernatant containing solublised protein (above) was deproteinated by the addition of 0.3 ml sodium silicotungstate reagent per ml (see 2.4). The suspension was left at room temp. for 10 min and precipitated material removed by centrifugation (1400g; 10 min). The $P_i$ content of the supernatant was determined (2.6.2.1). The $^{32}P$ activity of the supernatant was measured (2.8.3).

An aliquot of the deproteinated supernatant was freeze-dried and chromatographed on PEI paper (2.9)
SEDIMENTABLE PROTEIN, P, AND $^{32}$P

The protein sedimented at 40,000g in 1 hr was not measured directly but was calculated as the difference between total extracted and solubilised protein. The P and $^{32}$P sedimented with the protein were calculated in a similar way.

Aliquots of the first extract of non-contracted and contracted myofibrils were subjected to SDS-PAGE on 7.5% gels (see 2.11 for experimental details). The MgATPase activity of the first extract was investigated (cf. 2.6.7.2).

RESULTS

Table 4.25 gives the protein, P, and $^{32}$P contents of the various fractions of water-extracted non-contracted and contracted myofibrils. The Table designations are defined below.

\[
\text{supernatant} = \text{extract} - \text{P}_1 \\
\text{solubilised} = \text{supernatant} - \text{P}_1 \\
\text{sedimentable} = \text{extract} - \text{supernatant}
\]
**TABLE 4.25a**

PROTEIN FRACTIONS OF WATER-TREATED NON-CONTRACTED AND CONTRACTED PSOAS MYOFIBRILS

<table>
<thead>
<tr>
<th></th>
<th>residue dry wt.</th>
<th>extract solublised</th>
<th>sedimentable</th>
<th>total extract</th>
<th>s</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>mg</td>
<td>mg</td>
<td>mg</td>
<td></td>
</tr>
<tr>
<td>non-contracted</td>
<td>9.50</td>
<td>2.83(0.02)</td>
<td>2.47(0.14)</td>
<td>5.30(0.14)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>8.71</td>
<td>2.96(0.03)</td>
<td>2.66(0.13)</td>
<td>5.62(0.13)</td>
<td>2</td>
</tr>
<tr>
<td>first extract</td>
<td>10.15</td>
<td>2.81(0.02)</td>
<td>0.97(0.14)</td>
<td>3.78(0.14)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>10.23</td>
<td>2.84(0.02)</td>
<td>2.69(0.14)</td>
<td>5.53(0.14)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>9.62</td>
<td>2.66(0.02)</td>
<td>1.50(0.14)</td>
<td>4.16(0.14)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>9.56</td>
<td>2.68(0.02)</td>
<td>1.28(0.14)</td>
<td>3.96(0.14)</td>
<td>6</td>
</tr>
<tr>
<td>contracted</td>
<td>13.32</td>
<td>1.03(0.01)</td>
<td>0.26(0.21)</td>
<td>1.29(0.21)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>14.10</td>
<td>0.82(0.01)</td>
<td>0.25(0.21)</td>
<td>1.07(0.21)</td>
<td>2</td>
</tr>
<tr>
<td>first extract</td>
<td>15.35</td>
<td>1.01(0.01)</td>
<td>0.22(0.19)</td>
<td>1.23(0.19)</td>
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<tr>
<td></td>
<td>13.47</td>
<td>0.97(0.01)</td>
<td>0.27(0.20)</td>
<td>1.24(0.20)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>11.49</td>
<td>1.03(0.01)</td>
<td>0.31(0.20)</td>
<td>1.34(0.20)</td>
<td>5</td>
</tr>
<tr>
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<td>11.21</td>
<td>1.05(0.01)</td>
<td>0.14(0.21)</td>
<td>1.19(0.21)</td>
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<td>non-contracted</td>
<td>2.16(0.02)</td>
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<td>3.23(0.11)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>2.30(0.02)</td>
<td>0.66(0.12)</td>
<td>2.96(0.12)</td>
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<td>second extract</td>
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<td>3.70(0.11)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>2.18(0.02)</td>
<td>0.43(0.12)</td>
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<td>5</td>
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<td>2.66(0.02)</td>
<td>1.12(0.13)</td>
<td>3.78(0.13)</td>
<td>6</td>
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<tr>
<td>contracted</td>
<td>0.81(0.01)</td>
<td>1.47(0.18)</td>
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<td></td>
<td>1.17(0.01)</td>
<td>0.43(0.18)</td>
<td>1.60(0.18)</td>
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<td>second extract</td>
<td>1.19(0.01)</td>
<td>0.20(0.18)</td>
<td>1.39(0.18)</td>
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<tr>
<td></td>
<td>1.61(0.01)</td>
<td>0.53(0.18)</td>
<td>2.14(0.18)</td>
<td>4</td>
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<tr>
<td></td>
<td>1.36(0.01)</td>
<td>0.86(0.18)</td>
<td>2.22(0.18)</td>
<td>5</td>
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<td></td>
<td>1.15(0.01)</td>
<td>1.27(0.18)</td>
<td>2.42(0.18)</td>
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Numbers in parentheses are standard errors.

See 4.4.4 for experimental details and terminology.

s : sample number
TABLE 4.25b

P-CONTAINED INFRACTIONS OF WATER-TREATED NON-CONTRACTED AND CONTRACTED PSOAS MYOFIBRILS

<table>
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<th>residue</th>
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<th>extract-bound</th>
<th>total</th>
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<td></td>
<td></td>
<td>µg</td>
<td></td>
<td>µg</td>
<td>µg</td>
</tr>
<tr>
<td>non-c</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>10.66(0.29)</td>
<td>0.37(0.10)</td>
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<td>9.62(0.25)</td>
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<td>1.55(0.02)</td>
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</tr>
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<td>12.10(0.34)</td>
<td>0.70(0.08)</td>
<td>2.58(0.02)</td>
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<td>4.44(0.74)</td>
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<td>12.54(0.34)</td>
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<td>1.01(0.05)</td>
<td>3.28(0.75)</td>
<td>4.63(0.74)</td>
</tr>
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<td>0.70(0.08)</td>
<td>2.15(0.02)</td>
<td>1.78(0.74)</td>
<td>4.63(0.74)</td>
</tr>
<tr>
<td>6</td>
<td>11.58(0.32)</td>
<td>1.26(0.07)</td>
<td>2.35(0.02)</td>
<td>1.62(0.73)</td>
<td>5.23(0.73)</td>
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</tr>
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<td>0.55(0.07)</td>
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<td>1.51(0.80)</td>
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<td>0.06(1.02)</td>
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<td>0.29(0.98)</td>
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<td>1.06(0.06)</td>
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<td>2.65(0.79)</td>
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<td>0.30(0.13)</td>
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<td>0.71(0.11)</td>
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<td>3</td>
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<td>1.78(0.80)</td>
<td>3.52(0.79)</td>
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<tr>
<td>4</td>
<td>0.39(0.15)</td>
<td>0.82(0.03)</td>
<td>1.01(0.79)</td>
<td>2.22(0.78)</td>
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<tr>
<td>5</td>
<td>0.28(0.13)</td>
<td>1.13(0.05)</td>
<td>1.58(0.78)</td>
<td>2.99(0.77)</td>
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</tr>
<tr>
<td>c</td>
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<td>0.64(0.03)</td>
<td>0.73(0.87)</td>
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</tr>
<tr>
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<td>0.37(0.88)</td>
<td>1.44(0.85)</td>
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<tr>
<td>2</td>
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<td>0.96(0.02)</td>
<td>0.49(0.88)</td>
<td>1.06(0.85)</td>
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</tr>
<tr>
<td>3</td>
<td>0.39(0.15)</td>
<td>1.05(0.02)</td>
<td>1.02(0.87)</td>
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</tr>
<tr>
<td>4</td>
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<td>1.31(0.86)</td>
<td>2.46(0.85)</td>
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<tr>
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<td>0.03(0.25)</td>
<td>1.32(0.03)</td>
<td>0.85(0.86)</td>
<td>2.20(0.85)</td>
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</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.

See 4.4.4 for experimental details and terminology.

non-c : non-contracted

<p>| | | |</p>
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<th></th>
<th></th>
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</thead>
<tbody>
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<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>s</td>
<td></td>
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</tr>
</tbody>
</table>
|     |     |     | sample number
TABLE 4.25c

$^{32}$P-CONTAINED INFRATIONS OF WATER-TREATED NON-CONTRACTED AND CONTRACTED PSOAS MYOFIBRILS

<table>
<thead>
<tr>
<th>residue</th>
<th>$P_i$</th>
<th>extract-bound</th>
<th>total</th>
<th>s</th>
</tr>
</thead>
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<tr>
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<td>c.p.m.</td>
<td>c.p.m.</td>
<td>c.p.m.</td>
</tr>
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<tr>
<td>1</td>
<td>442.0(4.0)</td>
<td>596.5(35.7)</td>
<td>474.1(36.1)</td>
<td>219.3(82.1)</td>
</tr>
<tr>
<td></td>
<td>489.0(1.0)</td>
<td>701.6(47.4)</td>
<td>208.6(52.1)</td>
<td>142.2(49.2)</td>
</tr>
<tr>
<td></td>
<td>496.0(6.0)</td>
<td>556.0(55.0)</td>
<td>350.7(117.4)</td>
<td>-56.1(122.5)</td>
</tr>
<tr>
<td></td>
<td>421.0(9.0)</td>
<td>611.8(40.8)</td>
<td>448.7(48.1)</td>
<td>-61.2(71.1)</td>
</tr>
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<td>468.0(8.0)</td>
<td>530.3(30.6)</td>
<td>382.4(116.3)</td>
<td>-26.5(123.2)</td>
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<td>694.0(2.0)</td>
<td>545.6(20.4)</td>
<td>540.4(22.8)</td>
<td>-20.4(37.1)</td>
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<td>c</td>
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</tr>
<tr>
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<td>491.0(10.0)</td>
<td>738.9(36.2)</td>
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<td>87.5(75.5)</td>
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<td>576.0(14.0)</td>
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<td>234.5(52.7)</td>
<td>209.8(60.4)</td>
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<td>600.0(11.0)</td>
<td>801.5(11.4)</td>
<td>143.0(75.3)</td>
<td>326.3(97.5)</td>
</tr>
<tr>
<td></td>
<td>553.0(10.0)</td>
<td>527.4(29.9)</td>
<td>149.9(61.7)</td>
<td>443.4(80.6)</td>
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<td></td>
<td>623.0(13.0)</td>
<td>491.9(50.0)</td>
<td>371.1(83.7)</td>
<td>115.6(98.4)</td>
</tr>
<tr>
<td></td>
<td>466.0(16.0)</td>
<td>701.3(49.0)</td>
<td>119.0(66.6)</td>
<td>425.7(82.4)</td>
</tr>
<tr>
<td>non-c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>211.6(42.3)</td>
<td>34.6(43.0)</td>
<td>-103.9(31.7)</td>
<td>142.3(30.8)</td>
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<td></td>
<td>193.4(25.2)</td>
<td>-122.0(60.3)</td>
<td>122.0(64.3)</td>
<td>193.4(33.6)</td>
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<td></td>
<td>119.3(98.8)</td>
<td>214.0(104.1)</td>
<td>0.0(62.8)</td>
<td>333.3(53.5)</td>
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<td>19.7(70.8)</td>
<td>114.1(81.0)</td>
<td>169.3(54.6)</td>
<td>303.1(37.9)</td>
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<td>89.8(38.5)</td>
<td>185.3(54.4)</td>
<td>51.3(54.4)</td>
<td>384.8(38.5)</td>
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<tr>
<td></td>
<td>250.2(44.6)</td>
<td>-102.0(55.8)</td>
<td>143.7(78.6)</td>
<td>291.9(55.6)</td>
</tr>
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<td>c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10.7(53.7)</td>
<td>300.6(56.1)</td>
<td>-43.0(97.9)</td>
<td>268.3(96.6)</td>
</tr>
<tr>
<td></td>
<td>166.4(69.8)</td>
<td>26.8(119.2)</td>
<td>69.8(99.9)</td>
<td>263.0(25.4)</td>
</tr>
<tr>
<td></td>
<td>-1.7(79.1)</td>
<td>492.5(80.7)</td>
<td>-58.0(101.4)</td>
<td>432.8(100.2)</td>
</tr>
<tr>
<td></td>
<td>-4.4(37.6)</td>
<td>138.6(39.1)</td>
<td>177.1(28.9)</td>
<td>311.3(26.8)</td>
</tr>
<tr>
<td></td>
<td>93.4(50.2)</td>
<td>46.7(76.0)</td>
<td>332.0(92.4)</td>
<td>472.1(72.7)</td>
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<tr>
<td></td>
<td>221.9(77.6)</td>
<td>66.5(77.8)</td>
<td>-116.5(37.0)</td>
<td>171.9(36.6)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.

See 4.4.4 for experimental details and terminology.

non-c : non-contracted
c : contracted
1 : first extract
2 : second extract
s : sample number
TABLE 4.26a (part)

PROTEIN, P, AND $^{32}$P CONTENTS OF WATER EXTRACTS OF NON-CONTRACTED AND CONTRACTED PSOAS MYOFIBRILS

<table>
<thead>
<tr>
<th></th>
<th>non-contracted</th>
<th>contracted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total recovered (residue+extracts)</td>
<td></td>
</tr>
<tr>
<td>protein (mg)</td>
<td>17.72(0.42)</td>
<td>16.58(0.42)</td>
</tr>
<tr>
<td>$P$ (µg)</td>
<td>19.14(0.70)</td>
<td>18.57(0.81)</td>
</tr>
<tr>
<td>$^{32}$P (c.p.m.)</td>
<td>1809.2(87.4)</td>
<td>1953.1(82.7)</td>
</tr>
<tr>
<td>$P$ content (µg P mg$^{-1}$)</td>
<td>1.08(0.03)</td>
<td>1.12(0.04)</td>
</tr>
<tr>
<td>$^{32}$P content (c.p.m. mg$^{-1}$)</td>
<td>102.1(4.3)</td>
<td>117.8(4.0)</td>
</tr>
<tr>
<td>specific activity (c.p.m. µg$^{-1}$ P)</td>
<td>94.5(4.8)</td>
<td>105.2(5.2)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>protein (mg)</td>
<td>9.63(0.22)</td>
</tr>
<tr>
<td>$P$ (µg)</td>
<td>11.27(0.32)</td>
</tr>
<tr>
<td>$^{32}$P (c.p.m.)</td>
<td>561.4(33.4)</td>
</tr>
<tr>
<td>$P$ content (µg P mg$^{-1}$)</td>
<td>1.17(0.02)</td>
</tr>
<tr>
<td>$^{32}$P content (c.p.m. mg$^{-1}$)</td>
<td>58.3(3.2)</td>
</tr>
<tr>
<td>specific activity (c.p.m. µg$^{-1}$ P)</td>
<td>49.8(2.9)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors. See 4.4.4 for experimental details and terminology.
### TABLE 4.26a (part)

**PROTEIN, P, AND $^{32}$P CONTENTS OF WATER EXTRACTS OF NON-CONTRACTED AND CONTRACTED PSOAS MYOFIBRILS**

<table>
<thead>
<tr>
<th></th>
<th>non-contracted</th>
<th>contracted</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>first extract</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein (mg)</td>
<td>4.75(0.35)</td>
<td>1.23(0.04)</td>
</tr>
<tr>
<td>P (µg)</td>
<td>4.80(0.49)</td>
<td>1.33(0.19)</td>
</tr>
<tr>
<td>$^{32}$P (c.p.m.)</td>
<td>1005.6(100.5)</td>
<td>1067.8(78.5)</td>
</tr>
<tr>
<td>P content (µg P mg$^{-1}$)</td>
<td>1.01(0.07)</td>
<td>1.08(0.15)</td>
</tr>
<tr>
<td>$^{32}$P content (c.p.m. mg$^{-1}$)</td>
<td>211.7(14.3)</td>
<td>211.7(14.3)</td>
</tr>
<tr>
<td>specific activity (c.p.m. µg$^{-1}$ P)</td>
<td>209.6(20.3)</td>
<td>803.8(122.9)</td>
</tr>
<tr>
<td><strong>second extract</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein (mg)</td>
<td>3.36(0.20)</td>
<td>2.01(0.17)</td>
</tr>
<tr>
<td>P (µg)</td>
<td>2.99(0.22)</td>
<td>1.89(0.20)</td>
</tr>
<tr>
<td>$^{32}$P (c.p.m.)</td>
<td>252.3(44.9)</td>
<td>253.1(50.2)</td>
</tr>
<tr>
<td>P content (µg P mg$^{-1}$)</td>
<td>0.89(0.04)</td>
<td>0.94(0.06)</td>
</tr>
<tr>
<td>$^{32}$P content (c.p.m. mg$^{-1}$)</td>
<td>75.1(12.6)</td>
<td>125.9(22.6)</td>
</tr>
<tr>
<td>specific activity (c.p.m. µg$^{-1}$ P)</td>
<td>84.4(14.7)</td>
<td>133.9(25.5)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors. See 4.4.4 for experimental details and terminology.
Table 4.26 gives the mean values of various quantities calculated, as follows, from the data presented in Table 4.25.

P content (μg mg⁻¹) and ³²P content (c.p.m. mg⁻¹) were derived for each sample and the means calculated from these individual values.

Protein (mg) is the mean value of the individual protein wts.

P (μg) is the mean P content (μg mg⁻¹) x mean protein (mg).

³²P (c.p.m.) is the mean ³²P content (c.p.m. mg⁻¹) x mean protein (mg).

Specific activity (c.p.m. μg⁻¹ P) is the mean ³²P content (c.p.m. mg⁻¹) / mean P content (μg mg⁻¹).

The total protein, P, and ³²P recovered from contracted myofibrils (i.e. the combined values of residue and the two extracts) are not significantly different from the equivalent quantities measured for non-contracted myofibrils. However, contraction leads to a highly significant increase in the amount of non-extractable residue (T = 5.216, 6 degrees of freedom) and a consequent decrease in the quantity of extractable protein. The specific activities of the ³²P bound to the non-contracted and contracted myofibril residues are significantly different (T = 3.874, 9 degrees of freedom); the ratio of contracted to non-contracted is 0.72 ± 0.06. The quantity of ³²P bound to the two types of residue are not significantly different (T = 0.404, 9 degrees of freedom). However, the ratio of the wts. of non-contracted and contracted residue is 0.73 ± 0.04, suggesting that it is this ratio which contributes to the difference in specific activity of bound ³²P. There is a highly significant increase in the specific activity of the first extract due to contraction (T = 4.770, 5 degrees of freedom).
**TABLE 4.26b (part)**

**DISTRIBUTION OF PROTEIN, P, AND $^{32}$P IN THE FRACTIONS OF WATER EXTRACTS OF NON-CONTRACTED AND CONTRACTED PSOAS MYOFIBRILS**

<table>
<thead>
<tr>
<th></th>
<th>non-contracted</th>
<th>contracted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>first extract</td>
<td></td>
</tr>
<tr>
<td>$P$ (µg)</td>
<td>0.76(0.14)</td>
<td>0.37(0.08)</td>
</tr>
<tr>
<td>$^{32}$P (c.p.m.)</td>
<td>582.4(16.8)</td>
<td>758.3(69.7)</td>
</tr>
<tr>
<td>Specific activity (c.p.m. µg$^{-1}$ P)</td>
<td>770.4(143.7)</td>
<td>2025.3(413.7)</td>
</tr>
</tbody>
</table>

|                  | solubilised   |            |
| protein (mg)     | 2.80(0.05)    | 1.04(0.07) |
| $P$ (µg)         | 1.99(0.25)    | 0.87(0.13) |
| $^{32}$P (c.p.m.)| 477.1(52.8)   | 208.4(43.6)|
| $P$ content (µg P mg$^{-1}$) | 0.71(0.09) | 0.84(0.11) |
| $^{32}$P content (c.p.m. mg$^{-1}$) | 170.4(18.6) | 200.4(39.7) |
| Specific activity (c.p.m. µg$^{-1}$ P) | 240.0(40.1) | 238.6(56.7) |

|                  | sedimentable |            |
| protein (mg)     | 1.95(0.31)   | 0.27(0.14) |
| $P$ (µg)         | 2.40(0.38)   | 0.18(0.10) |
| $^{32}$P (c.p.m.)| 51.7(37.9)   | 137.8(84.2)|
| $P$ content (µg P mg$^{-1}$) | 1.23(0.02) | 0.68(0.16) |
| $^{32}$P content (c.p.m. mg$^{-1}$) | 26.5(19.0) | 510.2(165.3)|
| Specific activity (c.p.m. µg$^{-1}$ P) | 21.5(15.5) | 750.3(300.4) |

Numbers in parentheses are standard errors. See 4.4.4 for experimental details and terminology.
### TABLE 4.26b (part)

**DISTRIBUTION OF PROTEIN, P, AND 32P IN THE FRACTIONS OF WATER EXTRACTS OF NON-CONTRACTED AND CONTRACTED PSOAS MYOFIBRILS**

<table>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>second extract</strong></td>
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<td></td>
</tr>
<tr>
<td>P (µg)</td>
<td>0.41(0.08)</td>
<td>0.31(0.07)</td>
</tr>
<tr>
<td>32P (c.p.m.)</td>
<td>180.8(28.7)</td>
<td>45.3(32.8)</td>
</tr>
<tr>
<td>Specific activity (c.p.m. µg⁻¹ P)</td>
<td>441.2(102.4)</td>
<td>148.4(110.8)</td>
</tr>
<tr>
<td><strong>solubilised</strong></td>
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</tr>
<tr>
<td>Protein (mg)</td>
<td>2.41(0.12)</td>
<td>1.22(0.11)</td>
</tr>
<tr>
<td>P (µg)</td>
<td>1.13(0.13)</td>
<td>0.89(0.12)</td>
</tr>
<tr>
<td>32P (c.p.m.)</td>
<td>28.0(55.2)</td>
<td>150.1(65.3)</td>
</tr>
<tr>
<td>P content (µg P mg⁻¹)</td>
<td>0.47(0.05)</td>
<td>0.73(0.07)</td>
</tr>
<tr>
<td>32P content (c.p.m. mg⁻¹)</td>
<td>11.6(22.9)</td>
<td>723.0(52.4)</td>
</tr>
<tr>
<td>Specific activity (c.p.m. µg⁻¹ P)</td>
<td>24.7(48.8)</td>
<td>168.5(73.6)</td>
</tr>
<tr>
<td><strong>sedimentable</strong></td>
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<tr>
<td>Protein (mg)</td>
<td>0.97(0.15)</td>
<td>0.79(0.20)</td>
</tr>
<tr>
<td>P (µg)</td>
<td>1.40(0.24)</td>
<td>0.62(0.22)</td>
</tr>
<tr>
<td>32P (c.p.m.)</td>
<td>16.1(45.8)</td>
<td>-54.7(34.1)</td>
</tr>
<tr>
<td>P content (µg P mg⁻¹)</td>
<td>1.44(0.11)</td>
<td>0.79(0.20)</td>
</tr>
<tr>
<td>32P content (c.p.m. mg⁻¹)</td>
<td>16.6(47.1)</td>
<td>-69.2(39.4)</td>
</tr>
<tr>
<td>Specific activity (c.p.m. µg⁻¹ P)</td>
<td>11.5(32.7)</td>
<td>-87.6(54.6)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors. See 4.4.4 for experimental details and terminology.
Analysis of the distribution of protein, P, and $^{32}$P between solubilised and sedimentable material in the first extract (Table 4.26b) shows that:

1. the quantity of $^{32}$P extracted from contracted myofibrils is greater than from non-contracted ($T = 2.453$, 5 degrees of freedom); the ratio of contracted to non-contracted is $1.30 \pm 0.13$;

2. the specific activity of the $P_i$ fraction released from solubilised protein in the extract by deproteination has increased significantly ($T = 2.865$, 6 degrees of freedom) on contraction;

3. the specific activity of the P bound to extracted sedimentable protein has increased significantly on contraction ($T = 2.423$, 5 degrees of freedom).

No labelled small molecules other than $^{32}P_i$ could be detected in the sodium silicotungstate extract of solubilised material. The $^{32}$P measured in the $P_i$ fraction is assumed to be, therefore, only in the form of $^{32}P_i$.

Fig. 4.14 (over page) shows the results of SDS-PAGE of whole myofibrils and the aqueous extracts of contracted and non-contracted myofibrils.
The extract of non-contracted myofibrils contains a representative proportion of the myofibril proteins. The extract of contracted myofibrils, however, contains a relatively higher proportion of actin, myosin and tropomyosin. Both the extract of non-contracted and contracted myofibrils (i.e. solubilised plus sedimentable protein) showed MgATPase activity and superprecipitated. The clear extract became cloudy on the addition of ions (ionic strength > 0.1 M).
4.4.5 FRACTIONATION OF LABELLED PSOAS

Labelled psoas myofibrils (2.2.2) were resolved into P-containing fractions (4.2.2 - 4.2.6). The P and \(^{32}P\) content of each fraction was determined (4.3.2).

Portions of labelled psoas myofibrils were extracted with 5% TCA and the nucleotides, released by protein denaturation, estimated (see 4.1.1.1 for experimental details). The \(P_i\) content of the extract was measured (2.6.2.1). Nucleotides and \(P_i\) in the extract were separated by chromatography (2.9) and the chromatogram scanned for radioactivity (2.8.4).

RESULTS

Table 4.27 gives the results of fractionating one \(^{32}P\)-labelled psoas preparation. The mean values of the P and \(^{32}P\) content, and the specific activity of each fraction were calculated (Table 4.28).

The nucleotide and \(P_i\) contents of the psoas myofibril were found to be:

- \(0.23 \pm 0.02\) (3) nmol ATP mg\(^{-1}\);
- \(5.1 \pm 0.2\) (3) nmol ADP mg\(^{-1}\);
- \(0.11 \pm 0.01\) (3) nmol AMP mg\(^{-1}\);
- \(0.17 \pm 0.09\) (3) nmol IMP mg\(^{-1}\);
- \(1.65 \pm 0.39\) (3) nmol \(P_i\) mg\(^{-1}\). These molecules contribute \(0.35 \pm 0.02\) \(\mu g\) P mg\(^{-1}\) to the myofibril. The P content of the myofibril was found to be \(1.15 \pm 0.03\) (3) \(\mu g\) P mg\(^{-1}\) and that of the residue after TCA extraction, \(0.81 \pm 0.03\) (3) \(\mu g\) P mg\(^{-1}\). Only \(P_i\) was significantly labelled.
TABLE 4.27

FRACTIONATION OF $^{32}$P-LABELLED PSOAS MYOFIBRILS

<table>
<thead>
<tr>
<th>fraction</th>
<th>myofibril-dry wt.</th>
<th>P content $\mu g$ P $mg^{-1}$</th>
<th>$^{32}$P content c.p.m. $mg^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total bound</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>46.74</td>
<td>1.14(0.05)</td>
<td>261.2(4.0)</td>
</tr>
<tr>
<td></td>
<td>50.06</td>
<td>1.20(0.05)</td>
<td>241.0(3.7)</td>
</tr>
<tr>
<td></td>
<td>50.35</td>
<td>1.15(0.05)</td>
<td>230.1(3.5)</td>
</tr>
<tr>
<td></td>
<td>54.05</td>
<td>1.30(0.05)</td>
<td>245.5(1.8)</td>
</tr>
<tr>
<td></td>
<td>54.89</td>
<td>1.19(0.05)</td>
<td>247.7(1.0)</td>
</tr>
<tr>
<td></td>
<td>63.64</td>
<td>1.14(0.05)</td>
<td>254.2(0.6)</td>
</tr>
<tr>
<td>cold acid</td>
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</tr>
<tr>
<td>extracted residue</td>
<td>56.01</td>
<td>0.81(0.04)</td>
<td>37.8(1.1)</td>
</tr>
<tr>
<td></td>
<td>43.96</td>
<td>0.86(0.04)</td>
<td>32.1(1.0)</td>
</tr>
<tr>
<td></td>
<td>50.09</td>
<td>0.87(0.04)</td>
<td>32.1(1.0)</td>
</tr>
<tr>
<td>chloroform/methanol extracted residue</td>
<td>54.14</td>
<td>0.59(0.02)</td>
<td>59.9(1.0)</td>
</tr>
<tr>
<td></td>
<td>49.39</td>
<td>0.52(0.02)</td>
<td>28.4(0.9)</td>
</tr>
<tr>
<td></td>
<td>57.87</td>
<td>0.58(0.02)</td>
<td>28.7(0.6)</td>
</tr>
<tr>
<td></td>
<td>52.49</td>
<td>0.58(0.02)</td>
<td>29.4(0.9)</td>
</tr>
<tr>
<td>hot acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>extracted residue</td>
<td>36.27</td>
<td>0.17(0.004)</td>
<td>11.3(0.5)</td>
</tr>
<tr>
<td></td>
<td>43.33</td>
<td>0.15(0.003)</td>
<td>8.3(0.5)</td>
</tr>
<tr>
<td></td>
<td>43.02</td>
<td>0.07(0.003)</td>
<td>10.1(0.5)</td>
</tr>
<tr>
<td></td>
<td>34.36</td>
<td>0.08(0.005)</td>
<td>10.8(0.6)</td>
</tr>
<tr>
<td></td>
<td>43.20</td>
<td>0.14(0.003)</td>
<td>9.5(0.5)</td>
</tr>
<tr>
<td></td>
<td>40.10</td>
<td>0.09(0.004)</td>
<td>11.1(0.6)</td>
</tr>
<tr>
<td>alkali-labile</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>41.79(0.38)</td>
<td>0.08(0.01)</td>
<td>0.9(0.1)</td>
</tr>
<tr>
<td></td>
<td>46.28(0.38)</td>
<td>0.07(0.01)</td>
<td>1.0(0.2)</td>
</tr>
<tr>
<td></td>
<td>46.19(0.38)</td>
<td>0.07(0.01)</td>
<td>2.9(0.1)</td>
</tr>
<tr>
<td></td>
<td>48.66(0.38)</td>
<td>0.05(0.01)</td>
<td>0.5(0.1)</td>
</tr>
<tr>
<td></td>
<td>49.48(0.39)</td>
<td>0.06(0.01)</td>
<td>1.5(0.1)</td>
</tr>
<tr>
<td></td>
<td>41.24(0.38)</td>
<td>0.10(0.01)</td>
<td>0.1(0.1)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.

See 4.4.5 & 4.2 for experimental details and the description of the P-containing fractions.
Table 4.28

$^{32}$P content of P-containing fractions of labelled psoas myofibrils

<table>
<thead>
<tr>
<th>Fraction</th>
<th>P content $\mu g$ P mg$^{-1}$</th>
<th>$^{32}$P content c.p.m. mg$^{-1}$</th>
<th>Specific activity c.p.m. $\mu g^{-1}$ P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bound P</td>
<td>1.19 (0.02)</td>
<td>251.5 (2.1)</td>
<td>207.3 (2.4)</td>
</tr>
<tr>
<td>Cold acid extractable P</td>
<td>0.34 (0.03)</td>
<td>217.7 (2.8)</td>
<td>640.3 (57.1)</td>
</tr>
<tr>
<td>Chloroform/methanol extractable P</td>
<td>0.28 (0.03)</td>
<td>0.0 (6.8)</td>
<td>0</td>
</tr>
<tr>
<td>Hot acid extractable P</td>
<td>0.45 (0.03)</td>
<td>22.8 (6.6)</td>
<td>50.7 (15.1)</td>
</tr>
<tr>
<td>Alkali-labile P</td>
<td>0.07 (0.01)</td>
<td>1.2 (0.4)</td>
<td>17.1 (6.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.14 (0.05)</td>
<td>241.7 (0.06)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.

Labelled psoas myofibrils were fractionated into P-containing components (4.2). The P and $^{32}$P content of each fraction was determined. Values (above) were calculated from the results given in Table 4.27. See 4.4.5 for further information.
4.4.6 DEIONISED WATER EXTRACTION OF BUTANOL-EXTRACTED ACETONE-DRIED PSOAS MYOFIBRILS

$^{32}$P-labelled psoas myofibrils were extracted with butanol and dried with acetone (cf. 2.2.1.3). The acetone-dried myofibrils were extracted with deionised water (4.1.1). Liberated nucleotides were separated by chromatography (2.9) and the chromatogram scanned for radioactivity (2.8.4).

RESULTS

No labelled nucleotides were isolated.
Sub-Section 4.5

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of $^{32}$P-Labelled Myofibrils

Protein samples, in 0.4% NaCl buffered with 10 mM Tris-HCl pH 7.5 (4°C), were dialysed for 16 - 20 hrs vs. buffered NaCl. Samples were incubated with SDS and 2-mercaptoethanol in Tris-phosphate buffer pH 6.5 as described in 2.11: 'Preparation of sample'. Potassium salts were not used in any of the media in order to eliminate the precipitation of potassium dodecyl sulphate and to reduce radioactive $^{40}$K contamination (see 2.8.5). Prepared samples were mixed with tracking dye (bromophenol blue) and electrophoresed at 1.5 - 2.0 mA per gel using the discontinuous pH 8.3 / pH 8.8 SDS-Tris-glycine buffer system of Laemmli (1970). Gels were stained for protein with Coomassie Blue. Stained gels were stored under 7% acetic acid. Experimental details of gel preparation, electrophoresis and staining are given in 2.11.

Stained gels were either sliced longitudinally and dried on to filter paper (2.11: 'Drying of gels') or were sectioned transversely and transferred to silicone-coated glass scintillation vials (cf. 2.5). Transverse sections were selected to represent mol. wt. ranges containing known protein components. Dried gel slices were scanned for radioactivity using the apparatus described in 2.8.4 and were autoradiographed using X-ray film (2.12.2). Gel sections were immersed in 10 to 15 ml scintillant and counted in a scintillation spectrometer (2.8.2). Alternatively, gel sections were disintegrated by oxidation (1 - 2 ml 30% aq. hydrogen peroxide at 90°C for 2 - 3 hrs) and the resulting solution counted as the rate of emission of Cherenkov radiation (2.8.3).
Scintillation counting (2.8.2) of oxidised gel sections proved to be unsatisfactory because of the high quenching caused by dissolved $O_2$. Polyacrylamide gels need not be disintegrated to obtain a high counting efficiency of $^{32}P \beta$-particles (cf. Gordon, 1969).

**FIGURE 4.15**

**SDS-PAGE SECTIONS**
4.5.1 \(^{32}\text{P}\)-LABELLED GLYCEROL-EXTRACTED MYOFIBRILS (cf. 4.3)

Myofibrils were prepared from the back and leg muscles of a rabbit and were extracted with 50% aq. glycerol (2.2.1.1). The glycerol-extracted myofibrils were incubated with \(^{32}\text{P}\) for 24 hrs (4.3.1). Aliquots of the labelled myofibrils were dialysed against buffered 0.4% NaCl and electrophoresed. Gels were sectioned transversely (Fig. 4.15) and oxidised with hydrogen peroxide. Radioactivity in the solution was measured by method 2.8.3 (40% counting eff.). Quantities of labelled myofibrils were dried with acetone and the bound P and \(^{32}\text{P}\) content determined (4.3.2).

RESULTS

See Tables 4.29 & 4.30.

4.5.2 MYOFIBRILS FROM \(^{32}\text{P}\)-LABELLED PSOAS MUSCLE (cf. 4.4)

Psoas muscle was labelled with \(^{32}\text{P}\) (2.2.2.1). Labelled muscle was extracted with 50% aq. glycerol (2.2.2.2) and a suspension of myofibrils in buffered 0.4% NaCl prepared (2.2.2.3). Aliquots of myofibrils were dialysed against NaCl and were electrophoresed. Gels were sectioned transversely (Fig. 4.15) and suspended in 10 - 15 ml scintillant. Radioactivity was measured by method 2.8.2 (90 - 95% counting eff.). Quantities of myofibrils were dried with acetone and the bound P and \(^{32}\text{P}\) content determined (4.4.1).

RESULTS

See Table 4.31.
4.5.3 UNLABELLED PSOAS MYOFIBRILS

A suspension of psoas myofibrils in buffered 0.4% NaCl was prepared from glycerol-extracted muscle (2.2.2.2 & 2.2.2.3). Aliquots of myofibrils were dialysed against NaCl and electrophoresed. Gels were sectioned transversely (Fig. 4.15) and suspended in 10 - 15 ml scintillant. Radioactivity was measured by method 2.8.2 (90 - 95% counting eff.).

100 µg aliquots of dialysed unlabelled myofibrils were mixed with $^{32}$P$_i$ (25,000 c.p.m.) and electrophoresed. Gels were sectioned and radioactivity in the sections measured as above.

RESULTS

The count rate in each gel section was not above background. Gel electrophoresis of myofibrils mixed with $^{32}$P$_i$ gave one labelled section, the front of the gel (zone 10). The activity was $16.2 \pm 1.0$ (4) c.p.m.
TABLE 4.29

SDS-PAGE OF $^{32}$P-LABELLED GLYCEROL-EXTRACTED MYOFIBRILS
ON 7.5% GELS

<table>
<thead>
<tr>
<th>gel zone</th>
<th>loaded protein per gel:</th>
<th>75 µg</th>
<th>94 µg</th>
<th>125 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>bound $^{32}$P (c.p.m.)</td>
<td>1000</td>
<td>1253</td>
<td>1500</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c.p.m.</td>
<td>c.p.m.</td>
<td>c.p.m.</td>
</tr>
<tr>
<td>top</td>
<td>1</td>
<td>76.1(1.1)</td>
<td>109.3(1.2)</td>
<td>137.4(1.4)</td>
</tr>
<tr>
<td>My+M</td>
<td>2</td>
<td>9.6(0.9)</td>
<td>9.9(1.1)</td>
<td>15.4(1.0)</td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>2.0(1.0)</td>
<td>-0.3(1.0)</td>
<td>9.8(1.1)</td>
</tr>
<tr>
<td>α-Ac</td>
<td>4</td>
<td>1.7(0.9)</td>
<td>5.1(1.1)</td>
<td>3.2(0.7)</td>
</tr>
<tr>
<td>gap</td>
<td>5</td>
<td>6.5(1.1)</td>
<td>19.4(1.1)</td>
<td>18.0(0.9)</td>
</tr>
<tr>
<td>Ac</td>
<td>6</td>
<td>-1.1(1.0)</td>
<td>-1.0(1.0)</td>
<td>-2.1(1.2)</td>
</tr>
<tr>
<td>TN-T+TM</td>
<td>7</td>
<td>5.3(1.0)</td>
<td>6.3(1.2)</td>
<td>3.0(1.1)</td>
</tr>
<tr>
<td>TN-I+TN-C+light</td>
<td>8</td>
<td>35.5(0.9)</td>
<td>28.0(0.9)</td>
<td>16.1(1.1)</td>
</tr>
<tr>
<td>front</td>
<td>9</td>
<td>96.9(1.2)</td>
<td>88.9(1.2)</td>
<td>104.1(1.3)</td>
</tr>
<tr>
<td>observed gel c.p.m.</td>
<td></td>
<td>232.5(3.0)</td>
<td>265.6(3.3)</td>
<td>304.9(0.2)</td>
</tr>
<tr>
<td>as % load c.p.m.</td>
<td></td>
<td>23.3(0.3)</td>
<td>21.2(0.3)</td>
<td>20.3(0.2)</td>
</tr>
</tbody>
</table>

Radioactivity counts were corrected for background; numbers in parentheses are the combined standard errors of the means of 10 sample and 10 background counts. Sample counts were corrected for decay during the counting period. See Fig. 4.15 for definitions of gel zones.
TABLE 4.30 (part)

SDS-PAGE OF \(^{32}\)P-LABELLED GLYCEROL-EXTRACTED MYOFIBRILS ON 7.5% GELS

<table>
<thead>
<tr>
<th>Loaded protein per gel</th>
<th>75 μg</th>
<th>75 μg</th>
<th>125 μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>bound (^{32})P (c.p.m.)</td>
<td>5250</td>
<td>5250</td>
<td>8750</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gel zone</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top</td>
<td>19.4(0.8)</td>
<td>15.8(0.7)</td>
<td>34.5(1.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>My+M</td>
<td>3.0(0.9)</td>
<td>9.2(0.9)</td>
<td>7.6(1.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>5.1(1.2)</td>
<td>2.1(0.9)</td>
<td>17.0(0.8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Ac</td>
<td>3.9(0.9)</td>
<td>5.2(1.1)</td>
<td>9.8(1.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gap</td>
<td>19.5(0.7)</td>
<td>13.5(0.8)</td>
<td>34.9(1.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ac</td>
<td>0.1(1.1)</td>
<td>1.2(0.9)</td>
<td>2.0(0.9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TN-T+TN</td>
<td>5.3(0.9)</td>
<td>0.8(0.9)</td>
<td>2.0(0.9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TN-I+TN-C+light</td>
<td>6.2(0.9)</td>
<td>7.2(0.9)</td>
<td>15.0(0.8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Front</td>
<td>18.1(0.9)</td>
<td>17.8(0.9)</td>
<td>40.2(1.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observed gel c.p.m.</td>
<td>80.6(2.8)</td>
<td>72.8(2.7)</td>
<td>163.0(3.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>As % load c.p.m.</td>
<td>1.54(0.05)</td>
<td>1.39(0.05)</td>
<td>1.86(0.03)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Radioactivity counts were corrected for background; numbers in parentheses are the combined standard errors of the means of 10 sample and 10 background counts. Sample counts were corrected for decay during the counting period. See Fig. 4.15 for definitions of gel zones.
<table>
<thead>
<tr>
<th>gel zone</th>
<th>250 µg</th>
<th>250 µg</th>
<th>250 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>top</td>
<td>64.5(1.2)</td>
<td>53.5(1.0)</td>
<td>67.2(1.1)</td>
</tr>
<tr>
<td>My+M</td>
<td>19.1(0.8)</td>
<td>19.4(0.8)</td>
<td>24.8(0.8)</td>
</tr>
<tr>
<td>C</td>
<td>39.3(1.1)</td>
<td>25.7(0.9)</td>
<td>33.8(0.9)</td>
</tr>
<tr>
<td>α-Ac</td>
<td>11.6(0.7)</td>
<td>8.4(1.0)</td>
<td>15.3(0.8)</td>
</tr>
<tr>
<td>gap</td>
<td>67.7(1.3)</td>
<td>53.7(1.1)</td>
<td>71.0(1.2)</td>
</tr>
<tr>
<td>Ac</td>
<td>8.2(0.9)</td>
<td>2.9(0.9)</td>
<td>15.0(0.8)</td>
</tr>
<tr>
<td>TN-T+TM</td>
<td>12.4(0.7)</td>
<td>8.3(1.1)</td>
<td>18.9(0.6)</td>
</tr>
<tr>
<td>TN-I+TN-C+light</td>
<td>44.2(1.1)</td>
<td>39.2(1.1)</td>
<td>47.0(1.2)</td>
</tr>
<tr>
<td>front</td>
<td>76.3(1.1)</td>
<td>53.3(1.0)</td>
<td>85.7(1.1)</td>
</tr>
<tr>
<td>observed gel c.p.m.</td>
<td>343.3(3.0)</td>
<td>264.4(3.0)</td>
<td>378.7(2.9)</td>
</tr>
<tr>
<td>as % load c.p.m.</td>
<td>1.96(0.02)</td>
<td>1.51(0.02)</td>
<td>2.16(0.02)</td>
</tr>
</tbody>
</table>

Radioactivity counts were corrected for background; numbers in parentheses are the combined standard errors of the means of 10 sample and 10 background counts. Sample counts were corrected for decay during the counting period. See Fig. 4.15 for definitions of gel zones.
## TABLE 4.31

SDS-PAGE OF $^{32}$P-LABELLED PSOAS MYOFIBRILS ON 7.5% GELS

<table>
<thead>
<tr>
<th>loaded protein per gel:</th>
<th>200 µg</th>
<th>200 µg</th>
<th>500 µg</th>
<th>500 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of gels</td>
<td>18</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>total bound c.p.m.</td>
<td>560</td>
<td>400</td>
<td>1240</td>
<td>1240</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>gel zone</th>
<th>c.p.m.</th>
<th>c.p.m.</th>
<th>c.p.m.</th>
<th>c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>top</td>
<td>7.9(0.8)</td>
<td>4.9(0.8)</td>
<td>39.0(1.5)</td>
<td>43.8(1.4)</td>
</tr>
<tr>
<td>My</td>
<td>6.1(0.8)</td>
<td>3.5(0.7)</td>
<td>13.4(1.9)</td>
<td>12.0(1.4)</td>
</tr>
<tr>
<td>M</td>
<td>0.8(0.4)</td>
<td>3.3(0.8)</td>
<td>2.5(0.5)</td>
<td>1.9(0.9)</td>
</tr>
<tr>
<td>C</td>
<td>-0.5(0.6)</td>
<td>0.4(0.9)</td>
<td>2.5(1.0)</td>
<td>1.8(0.5)</td>
</tr>
<tr>
<td>α-Ac</td>
<td>-0.6(0.6)</td>
<td>0.6(1.0)</td>
<td>1.6(1.6)</td>
<td>-1.8(0.9)</td>
</tr>
<tr>
<td>gap</td>
<td>1.0(0.7)</td>
<td>0.6(0.4)</td>
<td>1.5(1.7)</td>
<td>-3.9(0.9)</td>
</tr>
<tr>
<td>Ac</td>
<td>1.5(0.5)</td>
<td>3.4(0.9)</td>
<td>-2.6(1.2)</td>
<td>3.9(0.9)</td>
</tr>
<tr>
<td>TN-T+T</td>
<td>3.7(0.4)</td>
<td>2.8(0.7)</td>
<td>2.9(1.2)</td>
<td>-0.1(1.0)</td>
</tr>
<tr>
<td>TN-I+light</td>
<td>-0.5(0.8)</td>
<td>3.4(0.7)</td>
<td>-0.5(0.8)</td>
<td>-1.5(1.1)</td>
</tr>
<tr>
<td>front</td>
<td>16.2(1.0)</td>
<td>22.0(0.9)</td>
<td>12.6(3.1)</td>
<td>18.8(1.6)</td>
</tr>
</tbody>
</table>

| observed gel c.p.m. | 35.6(2.4) | 44.9(2.5) | 72.9(5.0) | 75.1(3.5) |
| as % load c.p.m.     | 6.4(0.4)  | 11.2(0.6) | 5.9(0.4)  | 6.1(0.3)  |

Radioactivity counts were corrected for background; numbers in parentheses are the combined standard errors of the means of 10 sample and 10 background counts. Sample counts were corrected for decay during the counting period. See Fig. 4.15 for definitions of gel zones.
SECTION 5

DISCUSSION
5.1 ADP → ATP TRANSFORMATION IN A DEIONISED WATER SUSPENSION OF ACETONE-DRIED MUSCLE

ADENYLATE KINASE IN ACETONE-DRIED MUSCLE PREPARATIONS

The thin filament of muscle is believed to be composed of F-actin, one molecule of ADP being bound to each molecule of protein (1.5.2.2 & 1.5.2.5). Monomeric G-actin is released from the thin filament of acetone-dried muscle on the addition of a low ionic strength medium, e.g. deionised water. The extraction of protein is accompanied by the transformation of ADP → ATP. It has been suggested that ADP is converted to ATP by the action of adenylate kinase (Tsuboi, 1963; Tsuboi & Hayashi, 1963; Bencsáth & Biró, 1963):

\[
\text{2ADP} \rightarrow \text{ATP} + \text{AMP}
\]

Tsuboi (1963) has also proposed that AMP, formed in the above reaction, is converted to IMP by:

\[
\text{AMP} \rightarrow \text{IMP} + \text{NH}_3 \quad (5'\text{-AMP deaminase})
\]

The deamination reaction is highly exergonic and hence would drive the reversible adenylate kinase (AK) reaction in the forward direction (1.3.4). Tsuboi measured the quantity of each nucleotide present in the dried muscle and the amounts found in the aqueous suspension of dried muscle after 30 min. Changes in nucleotide, mmol mg\(^{-1}\) dried muscle, were found to be: ADP, -1.24; ATP, +0.66; AMP, +0.03; IMP, +0.72. The values are consistent with the AK and deaminase reactions. Our experimental evidence, reported in 4.1 (cf. 4.1.7), suggests the presence of enzymically active AK not only in acetone-dried muscle but also in acetone-dried, butanol-extracted myofibrils. AK is bound to the myofibril
structure or to some component in the myofibril preparation (Fig. 4.4); Bowen & Martin (1963) have observed AK activity in homogenates of psoas muscle. We also demonstrated 5'-AMP deaminase activity in myofibrils (4.1.5); this enzyme is firmly bound to myosin (cf. Byrnes & Suelter, 1965). However, Tsuboi & Hayashi (1963) found that the addition of ADP or ATP, together with AK, did not influence the ADP + ATP transformation nor the yield of actin. If endogenous ADP is converted to ATP via the AK reaction then the yield of native G-(ATP)-actin cannot exceed 50% since 1 mole of ADP is converted to AMP. The monophosphate is not capable of binding to G-actin and maintaining the integrity of the protein structure.

We followed the changes in ATP, ADP, AMP, and IMP concentrations during 3 hrs of deionised water extraction of acetone-dried muscle and myofibrils (4.1.1). The results show that additional reactions, involving the nucleotides, occur during the extraction period (Fig. 4.3). We also believe that TCA, used to denature protein and facilitate isolation of nucleotides for estimation, is not completely effective in removing all the bound nucleotide from dry muscle powder (Figs. 4.1 & 4.3 at 0 min); we calculate that about 30% of the bound nucleotide may not be extracted by TCA. The poor extraction is more pronounced when acetone-dried myofibrils are TCA-extracted (Fig. 4.2). The change in ATP and ADP concentrations (nmol mg⁻¹ myofibrils) after 3 hrs extraction (muscle: ADP = -5.01 ± 0.39; ATP = +2.48 ± 0.08; myofibrils: ADP = -2.11 ± 0.41; ATP = +1.25 ± 0.35) suggests the transformation 2ADP → ATP.

We have, however, considered the possibility that the γ-phosphate of ATP might be derived from an immediate source other than ADP.
The equation:

\[ 2\text{ADP} \rightarrow \text{ATP} + \text{AMP} \quad (1) \]

suggests that \( -\Delta[\text{ADP}] = +2\Delta[\text{ATP}] \)

whereas:

\[ \text{ADP} + R-O-\text{PO}_3 \rightarrow \text{ATP} + R-O-H \quad (2) \]

(\( R \) is any group other than AMP)

requires that \( -\Delta[\text{ADP}] = +\Delta[\text{ATP}] \).

Thus, the regression of \([\text{ATP}]\) on \([\text{ADP}]\) at corresponding times of extraction (Table 4.1) should be linear with a slope \( (m_0) = -0.5 \) in the case of equation 1 and \(-1.0\) if ATP formation is best represented by equation 2. The slope of the regression line was found to be \(-0.579 \pm 0.053\) (Fig. 5.1).

The following pairs of hypotheses were tested:

\begin{align*}
H_0 : m_0 &= -0.50 & H_0 : m_0 &= -1.00 \\
H_0 : m_0 &< -0.50 & H_0 : m_0 &> -1.00
\end{align*}

Using the methods given in Section 3, we can show (for 23 degrees of freedom) that:

1. the line is linear
2. for \( m_0 = -0.50 \), \( T = 1.491 \);
   for \( m_0 = -1.00 \), \( T = 7.943 \).

We reject \( m_0 = -1.00 \) and accept \( m_0 = -0.50 \) at the 0.0005 level of significance.
FIGURE 5.1

DEIONISED WATER EXTRACTION OF ACETONE-DRIED MUSCLE;
REGRESSION OF ATP ON ADP (28 DATA PAIRS)

\[
slope = -0.579 \pm 0.053
\]
\[
y \text{ intercept} = 4.026 \pm 0.175
\]
\[
r = -0.905 \quad t \text{ for linearity} = 10.925
\]

Paired hyperbolic dotted lines are 95% confidence limits based on \( t = 2.056 \) (26 degrees of freedom). See 3.5 & 3.6 for terminology and details of regression.

See 4.1.1.1 for experimental details; values taken from Table 4.1. See 5.1 for discussion.
FIGURE 5.2

DEIONISED WATER EXTRACTION OF ACETONE-DRIED MYOFIBRILS;
REGRESSION OF ATP ON ADP (29 DATA PAIRS)

slope = $-0.481 \pm 0.110$

$y$ intercept = $1.917 \pm 0.225$

$r = -0.645 \quad t$ for linearity = 4.373

Paired hyperbolic dotted lines are 95% confidence limits based on $t = 2.052$ (27 degrees of freedom). See 3.5 & 3.6 for terminology and details of regression.

See 4.1.1.2 for experimental details; values taken from Table 4.2. See 5.1 for discussion.
A similar regression analysis has been carried out on the [ATP] and [ADP] isolated during water extraction of acetone-dried myofibrils (Table 4.2) and is shown in Fig. 5.2. The slope of the regression line was found to be \(-0.481 \pm 0.110\). For 27 degrees of freedom:

1. the line is linear
2. for \(m_0 = -0.50\), \(T = 0.173\);
   for \(m_0 = -1.00\), \(T = 4.718\).

We reject \(m_0 = -1.00\) and accept \(m_0 = -0.50\) at the 0.0005 level of significance. On the basis of our measurements of changes in ATP and ADP concentrations, we can be, therefore, 99.95% confident that both acetone-dried muscle and acetone-dried butanol-extracted myofibrils convert ADP into ATP via a reaction consistent with 2ADP + ATP.

If we now consider the pair of equations:

\[
\begin{align*}
2\text{ADP} & \rightarrow \text{ATP} + \text{AMP} \\
\text{AMP} & \rightarrow \text{IMP} + \text{NH}_3
\end{align*}
\]

we would expect that the regression of [ATP+AMP+IMP] on [ADP] to be linear with a slope \((m_0) = -1.0\) (Table 4.1). If the conversion of ADP to ATP is independent of AMP and IMP, then \(m_0 = -0.50\). The slope of the regression line was found to be \(-0.841 \pm 0.159\) (Fig. 5.3).

\[
\begin{align*}
H_0 : m_0 &= -1.00 & H_0 : m_0 &= -0.50 \\
H_0 : m_0 &> -1.00 & H_0 : m_0 &< -0.50
\end{align*}
\]

For 26 degrees of freedom:

1. the line is linear \((T = 5.289)\);
2. for \(m_0 = -1.00\), \(T = 1.000\);
   for \(m_0 = -0.50\), \(T = 2.145\).
FIGURE 5.3

DEIONISED WATER EXTRACTION OF ACETONE-DRIED MUSCLE;
REGRESSION OF ATP+AMP+IMP ON ADP (24 DATA PAIRS)

slope = -0.983 ± 0.178
y intercept = 7.737 ± 0.628

r = -0.762  t for linearity = 5.522

Paired hyperbolic dotted lines are 95% confidence limits based on t = 2.074 (22 degrees of freedom). See 3.5 & 3.6 for terminology and details of regression.

See 4.1.1.1 for experimental details; values taken from Table 4.1. Points marked '◇' are not included in the analysis. See 5.1 for discussion.
We, therefore, reject $m_0 = -0.50$ and accept $m_0 = -1.00$ at the 0.01 level of significance. As the results in Fig. 4.3 show, during protracted periods of water extraction the sum of the four measured nucleotides falls. As reported in 4.1.1.1, we believe that this drop is caused by additional reactions utilizing some or all of the four types of nucleotide. The sum at 120 min is 83% and at 180 min 75% of the mean sum of the nucleotides isolated between 5 and 60 min; the points marked '○' in Fig. 5.3 are the values for 120 and 180 min. The regression analysis carried out on the 24 points marked 'Δ', representing data obtained during the first 60 min of extraction, gives a slope = $-0.983 \pm 0.178$. For 22 degrees of freedom (Fig 5.3):

1. the line is linear
2. for $m_0 = -1.00$, $T = 0.096$;
   for $m_0 = -0.50$, $T = 2.713$.

We reject $m_0 = -0.50$ and accept $m_0 = -1.00$ at the 0.005 level of significance. On the basis of our measurements of changes in ATP, ADP, AMP, and IMP concentrations, we can be 99.5% confident that the AK reaction is taking place during the deionised water extraction of acetone-dried muscle, accounting for the conversion of ADP into ATP.

Our analysis of the deionised water extracts of acetone-dried, butanol-extracted myofibrils did not provide any information on the concentrations of AMP and IMP (4.1.1.2). We did suspect the presence of inosine (nucleotide) since the u.v. absorption spectrum of the extract had shown a plateau between 250 and 260 nm ($\lambda_{\text{max}}$ adenosine = 259 - 260 nm and $\lambda_{\text{max}}$ inosine = 248 - 249 nm, at pH 7). Webster (1953) has reported that muscle can apparently convert ADP to IDP and Bárány & Bárány (1972)
also report the presence of adenosine and inosine, as well as ATP, ADP, AMP, and IMP, in frog muscle. We have provided evidence that our myofibril preparations do contain a bound AK fraction (4.1.3 & 4.1.4) and show 5'-AMP deaminase activity (4.1.5). It is highly probable that the conversion of AMP into IMP is complete at all stages of water extraction and that the latter nucleotide is transformed into other inosine compounds by unknown myofibrillar-bound enzymes. We are of the opinion, therefore, that the proven presence of AK and deaminase in myofibril preparations provides supportive evidence for the involvement of these enzymes in the ADP→ATP transformation.

The conditions in the deionised water suspension of acetone-dried muscle and myofibrils are not optimal for AK activity. The presence of divalent ions is an absolute requirement for the activity of muscle cytosol AK, i.e. myokinase. The optimal rate is observed when the ratio of ADP to Mg$^{2+}$ is 2 (Noda, 1958). The apparent equilibrium constant, $K_{eq} = \frac{[ADP]}{[ATP] \cdot [AMP]}$, is dependent on $[\text{Mg}^{2+}]$, being near unity in vivo (Eggleston & Hems, 1962). ADP formation is favoured at low concentrations of divalent ion, $K_{eq}$ having a value of about 2 at $p\text{Mg}$ between 5 and 7 (DeWeer & Lowe, 1973). It is generally believed that the reaction catalysed by the enzyme is:

$$\text{MgADP} + \text{ADP} \rightleftharpoons \text{MgATP} + \text{AMP}$$

Ca$^{2+}$ can substitute for Mg$^{2+}$; at the optimal ADP/divalent ion ratio, the enzyme activity with Ca$^{2+}$ is 10% of the rate in the presence of Mg$^{2+}$. Actin in the thin filament not only has bound ADP but also has a bound divalent ion; the type of divalent ion (Ca$^{2+}$ or Mg$^{2+}$) bound to actin in vivo is unclear (see 1.5.2.2). Placing actin in a low ionic
strength medium not only has the effect of releasing the bound nucleotide but also of liberating the divalent ion. We may assume, therefore, that actin is not just the source of the nucleotide observed in deionised water extracts of acetone-dried muscle but also of the divalent ion required for the activation of AK. The availability of divalent ions may be the limiting factor in the ADP $\rightarrow$ ATP transformation, thereby accounting for the fact that Tsuboi & Hayashi (1963) were unable to show any AK activation. $p$(divalent ion) is about 5.

**DIRECT TCA EXTRACTION OF ACETONE-DRIED MUSCLE**

Figs. 5.1, 5.2 & 5.3 show that the points representing nucleotide concentrations at 'zero time' (cf. Tables 4.1 & 4.2), i.e. nucleotides obtained by direct extraction of acetone-dried muscle by TCA, lie well outside the 95% confidence bands. Experiments performed in the investigation of the P distribution in myofibrils (4.2) have shown that the effectiveness of TCA, as a phosphate compound extractor, is enhanced by disrupting the cell with ultrasonication. (The study of the TCA-extraction of myofibrils has shown that TCA remains bound to denatured myofibrils (4.2.1.2). As a result, the value of the wt. of acetone-dried denatured myofibrils, as an estimate of the protein content (cf. 4.2.1.1), was found to be in error. We found that it was necessary to wash the denatured myofibrils several times with saline in order to remove bound TCA. The TCA contamination could be reduced to 0.1 mg mg$^{-1}$ protein. In our analysis of the nucleotides present in a deionised water suspension of acetone-dried muscle, we denatured the protein with TCA and used the wt. of oven-dried TCA-denatured protein in calculations. However, the residual protein-bound TCA evaporates at 110°C. Our wts. of oven-dried
denatured myofibrils are considered to be accurate.) There is a 10% increase in the yield of ADP from acetone-dried muscle when the TCA-denatured suspension is briefly ultrasonicated.

AN ESTIMATE OF THE ACTIN CONTENT OF ACETONE-DRIED MUSCLE

The regression of $\text{[ATP+AMP+IMP]}$ on $\text{[ADP]}$ (Fig. 5.3) predicts $\frac{\text{ADP}}{} = 8.46 \pm 0.97 \ (28) \text{ nmol mg}^{-1}$ (x axis intercept) as an estimate of the bound ADP content of acetone-dried muscle. If we assume that the ADP originates from actin (1 mole per mole) then we predict 8.46 nmol actin per mg acetone-dried (myosin-free) muscle. Using mol wt. actin = 42,000, we calculate that there is 35.5 ± 4.1% actin by wt. in acetone-dried (myosin-free) muscle.

The predicted ADP content of acetone-dried, butanol-extracted myofibrils is 3.99 ± 0.48 (29) nmol mg$^{-1}$ (Fig. 5.2), equivalent to 16.8 ± 2.0% actin by wt. in the myofibril. We did not remove myosin from myofibrils since acetone butanol treatment denatures myosin and its continued presence in that state no longer interferes with actin extraction (2.2.1.3). Our estimate is consistent with the reported value of 20% (Table 1.1).

The release of soluble protein from the insoluble muscle mass, during water extraction of acetone-dried butanol-extracted myofibrils, is 80 - 90% complete within 10 min (Fig. 4.5); 0.097 ± 0.006 (8) mg protein is released per mg myofibrils. The aq. extraction was carried out at 0 - 4°C, hence the contamination of liberated actin by tropomyosin and troponin was probably minimised since these myofibril components are not extracted in any great quantity at low temperature (cf. Drabikowski & Gergely, 1962; Ebashi & Ebashi, 1964). If released protein is almost entirely actin,
then 57.7 ± 7.7% of the myofibril actin has been extracted by deionised water. The ADP → ATP transformation continues after protein is extracted (Fig. 4.4); ADP is released from the myofibril rapidly. If the ADP content of acetone-dried myofibrils is, as discussed above, 3.99 ± 0.48 nmol mg⁻¹ and if this quantity of nucleotide enters solution, then the deionised water extract would contain 41.1 ± 5.5 nmol ADP per mg released protein. We calculate, from this data, that 1.73 ± 0.23 mole ADP should be released per mole of actin extracted and hence that there should be

$$0.87 ± 0.12 \text{ mole ATP per mole of actin after the ADP → ATP transformation is complete.}$$

From the results shown in Fig. 4.4, we calculate that 30.85 ± 1.23 (9) nmol ADP mg⁻¹ extracted protein was released from the myofibril. (The value of $2 \left[\frac{\text{ATP}}{\text{ADP}}\right]$, at equivalent times, is constant.) This value is equivalent to 1.3 ± 0.1 mole ADP released per mole of extracted actin, and hence 0.65 ± 0.05 mole ATP per mole of actin.

These results suggest that 1 in every 2 actin-ADP molecules is released from the myofibril; the actin which remains in the myofibril retains its bound ADP. We did not observe, in the electron microscope, any F-actin chains in extracts of acetone-dried myofibrils which had been treated with deionised water for 3 min (4.1.2). Actin-ADP is probably released from the myofibril as monomers. The acetone-drying process, a necessary requirement for the release of G-actin from the thin filament in a stable form (see p. 68), influences only half the monomers in the thin filament. We cannot exclude the possibility that butanol-treatment also has an effect on the extractability of actin in this context. The release of only half of the actin monomers probably reflects a natural sequence within the filament. Since the thin filament is composed of two actin chains, it is conceivable that acetone ruptures the bonds
between the monomers in the two chains in such a way that half the filament remains intact and the other half disintegrates. This kind of process may reflect dissimilarity in the states of actin monomers in the intact native thin filament. Three possible models for thin filament structure based on the extractability of half the monomers are given in Fig. 5.4.

5.2 UPTAKE OF $^{32}$P BY GLYCEROL-EXTRACTED MYOFIBRILS AND BACTERIAL CONTAMINATION OF MYOFIBRIL PREPARATIONS

P CONTENT OF THE MYOFIBRIL

Table 5.1 shows the distribution of bound P in the myofibril as % total bound P. Data was calculated from the results presented in 4.2. The observed total bound P is significantly different from the sum of P in the various fractions ($T = 2.683$, 77 degrees of freedom; see Table 4.10). The difference, $0.06 \pm 0.02 \mu g \ P \ mg^{-1}$, is the phosphate which remains bound to the denatured myofibril residue after removal of the known P fractions.

EXPERIMENTAL AND THEORETICAL ESTIMATES OF KNOWN P COMPONENTS OF THE MYOFIBRIL

The TCA extract of acetone-dried, butanol-extracted myofibrils contains $0.29 \pm 0.14$ (2) nmol ATP and $1.90 \pm 0.04$ (2) nmol ADP per mg of myofibrils (Table 4.2, 0 min). As discussed in 5.1, these two values are inaccurate since TCA does not remove all the nucleotide from a dry powder of myofibrils. The predicted ADP content of myofibrils is $3.99 \pm 0.48$ nmol mg$^{-1}$ (p. 261), hence $52.4 \pm 13.6\%$ of the myofibrillar-bound ADP,
FIGURE 5.4
MODELS FOR THIN FILAMENT STRUCTURE BASED ON THE EXTRACTABILITY OF HALF THE ACTIN MONOMERS

I

II

III

• actin monomer

× 2 actin + 2 troponin

not extractable ——— acetone-resistant state

extractable ———— acetone-sensitive state
and presumably ATP, is not extracted under the experimental conditions described in 4.1.1.1. The value for ATP, corrected for loss, is 0.55 ± 0.30 nmol mg⁻¹. The TCA extraction of wet myofibrils yields 0.90 ± 0.05 (2) nmol ATP, 3.42 ± 0.30 (2) nmol ADP, and 1.95 ± 0.35 (2) nmol Pᵢ per mg of myofibrils (cf. 4.4.5). The most probable values for ATP and ADP are 0.89 ± 0.06 nmol mg⁻¹ and 3.58 ± 0.26 nmol mg⁻¹ respectively. The ratio of ATP to ADP in the myofibril is calculated to be 0.25 ± 0.02. Perry (1952) has reported that the myofibril contains 0.47 nmol ATP, 2.7 nmol ADP, and 0.88 nmol AMP per mg; the ratio of ATP to ADP is 0.17. Nucleotides and Pᵢ are removed from the myofibril with TCA and, therefore, contribute to the cold acid-extractable P.

We found the 2-D-deoxyribose content of myofibrils to be 0.418 ± 0.017 (4) μg mg⁻¹ (see 2.6.6). The source of deoxyribose is DNA and, therefore, is an estimate of the DNA content of the myofibril. DNA contributes to the hot acid-labile P fraction.

Myofibril proteins known to be phosphorylated are the DTNB-light chains of myosin (1.5.2.1 & 1.5.5.1), TN-I (1.5.2.3 & 1.5.5.2), TN-T (1.5.2.3 & 1.5.5.3), α-TM (1.5.2.4 & 1.5.5.4). The proteins are phosphorylated on specific Ser and Thr residues.

<table>
<thead>
<tr>
<th></th>
<th>nmol mg⁻¹ myofibrils</th>
<th>mol P mol⁻¹ protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTNB-light chains</td>
<td>2.39</td>
<td>1</td>
</tr>
<tr>
<td>TN-I</td>
<td>0.63</td>
<td>2 or none</td>
</tr>
<tr>
<td>TN-T</td>
<td>0.63</td>
<td>3 or 1</td>
</tr>
<tr>
<td>α-TM</td>
<td>0.85</td>
<td>1</td>
</tr>
</tbody>
</table>
The concentration of each protein was calculated from the % myosin, troponin and tropomyosin present in the myofibril (Table 1.1). Myosin contains 2 mol DTNB-light chains mol$^{-1}$, troponin 1 mol each TN-I and TN-T mol$^{-1}$. Not all the phosphorylation sites of TN-I and TN-T may be phosphorylated in the myofibril. As reported in 1.5.5, the TN-I sites are protected from phosphorylation in vitro by the binding of TN-C (Cole & Perry, 1975). Ribolow et al. (1977) report that TN-I does not take up $^{32}$P in vivo, thereby suggesting the existence of a TN-I/TN-C complex. Only 1 TN-T site has been shown to become phosphorylated in vivo (Bárány et al., 1979). TM is present in muscle as either the $\alpha\alpha$ or $\alpha\beta$ dimer; in a mixture of muscle fibre types, the ratio of $\alpha$- to $\beta$-TM is 4:1 (cf. 1.5.2.4). $\beta$-TM is not phosphorylated. The phosphorylated muscle proteins contribute to the alkali-labile P of the myofibril.

Table 5.1 shows that:

1. we can account for the cold acid-extractable P on the basis of the nucleotide and P$_4$ content of myofibrils,
2. the origin of only 31% of the hot acid-labile P is known,
3. since we found only 0.07 $\mu$g alkali-labile P per mg myofibrils, the lower value of 0.12 $\mu$g P mg$^{-1}$ contributed by phosphorylated muscle proteins may be more representative.

The origin of 0.06 $\mu$g bound P mg$^{-1}$ is unknown (Table 5.1). 0.0123 $\mu$g P mg$^{-1}$ remains bound to the myofibril residue after cold acid, chloroform/methanol and hot acid extractions (Table 4.10). Treatment of the protein residue with hot alkali liberates 0.07 $\mu$g P mg$^{-1}$. The unknown fraction might represent protein-bound P not hydrolysed by alkali.
### TABLE 5.1

**COMPARISON OF P CONTENT OF MYOFIBRIL FRACTIONS WITH KNOWN PHOSPHORYLATED MYOFIBRIL COMPONENTS**

<table>
<thead>
<tr>
<th>phosphorylated component</th>
<th>P contribution (theoretical)</th>
<th>extraction</th>
<th>P fraction (observed)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg P mg⁻¹</td>
<td></td>
<td>µg P mg⁻¹</td>
</tr>
<tr>
<td>ATP</td>
<td>0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pi</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.36 cold acid</td>
<td></td>
<td>0.32(0.01)</td>
</tr>
<tr>
<td>not measured</td>
<td>chloroform/methanol</td>
<td></td>
<td>0.19(0.01)</td>
</tr>
<tr>
<td>DNA</td>
<td>0.12 hot acid</td>
<td></td>
<td>0.39(0.01)</td>
</tr>
<tr>
<td>light chains</td>
<td>0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TN-I</td>
<td>0.04 or none</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TN-T</td>
<td>0.06 or 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-TM</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.20 or 0.12 alkali</td>
<td></td>
<td>0.074(0.003)</td>
</tr>
<tr>
<td>sum of P fractions</td>
<td></td>
<td></td>
<td>0.97(0.02)</td>
</tr>
<tr>
<td>total measured P</td>
<td></td>
<td></td>
<td>1.03(0.01)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.

Data for P fractions are taken from Table 4.10. Values for theoretical P components are calculated through considerations proposed in the text (5.2).
(under the experimental conditions given in 4.2.6), suggesting, with some justification, that a better estimate of alkali-labile P is 0.13 μg mg⁻¹. If this is the case, then the two TN-I and two of the three TN-T sites might not be phosphorylated in the myofibril. Ribolow et al. (1977), Moir et al. (1977) and Bárány et al. (1979) have shown that these sites do not take up label in vivo.

**BACTERIAL CONTAMINATION**

The experiments reported in 4.3 show: (1) the incorporation of ³²P into glycerol-extracted myofibrils is time dependent; (2) the extent of phosphorylation varies from preparation to preparation, but is similar when a preparation is labelled on different occasions; (3) the presence of various reagents during the labelling period reduces the uptake; (4) ³²P is firmly bound. See 4.3.3.7 & 4.3.4.6 for summaries of experimental findings. The uptake of ³²P by myofibrils was found to be reduced by 98% when myofibrils were treated with the antibiotic 'chloramphenicol' (4.3.6). This antibiotic inhibits the growth of bacteria by stopping protein synthesis, but does not affect the processes of energy generation and transduction (cf. Hahn, 1967). The staining of myofibril smears with Gram's Iodine has provided a visual proof of bacterial contamination (4.3.6.3).

TCA extracts of labelled myofibrils contained no radioactive component of significant count other than P⁺; P⁺ represents 20% of the P in the TCA extract (Table 5.1). The distribution of ³²P in the myofibril, labelled in the absence of chloramphenicol (4.3.5), shows that 46 ± 4% of the ³²P uptake can be isolated from the myofibril in the form of ³²P⁺ by TCA extraction (Table 5.2 & Table 4.19). 60 ± 7% is found in
### TABLE 5.2

**P AND $^{32}$P CONTENT OF P-CONTAINING FRACTIONS OF LABELLED GLYCEROL-EXTRACTED MYOFIBRILS**

<table>
<thead>
<tr>
<th>fraction</th>
<th>P content</th>
<th>$^{32}$P content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% total</td>
<td>% total</td>
</tr>
<tr>
<td>cold acid extractable</td>
<td>23.4(3.9)</td>
<td>46.4(3.8)</td>
</tr>
<tr>
<td>chloroform/methanol extractable</td>
<td>14.3(0.6)</td>
<td>0.9(1.2)</td>
</tr>
<tr>
<td>hot acid labile</td>
<td>44.0(3.7)</td>
<td>60.1(7.2)</td>
</tr>
<tr>
<td>alkali labile</td>
<td>8.1(0.1)</td>
<td>2.9(0.3)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.

Values are calculated from the results given in Table 4.19.
a form susceptible to hydrolysis by hot acid; this fraction is of high specific activity. Part (or all) of this fraction is nucleic acid. Since label is taken up into this fraction, we assume the incorporation to be an indicator of bacterial proliferation. A significant quantity of $^{32}$P can only be isolated by hot alkaline dissolution of the myofibrils ($T = 9.667, 7$ degrees of freedom); this uptake represents protein phosphorylation. Of the small quantity of $^{32}$P taken up by chloramphenicol-treated myofibrils, $27\%$ of the label is extractable as $P_i$ (4.3.6.3).

The quantity of $^{32}$P that could be removed from labelled myofibrils appeared to be preparation dependent (cf. Tables 4.18 & 4.20). Labelling myofibrils in the presence of $1$ mM Ca$^{2+}$ increases the uptake by $60\%$ (Table 4.12). Washing myofibrils with calcium chloride and then several times with $0.5\%$ KCl, prior to the addition of $^{32}$P, increases the uptake by $14\%$ (Table 4.13); the treatment of myofibrils with the Ca-chelating agent 'EGTA' reduces the uptake by $30\%$. It is probable that the varying quantities of TCA-extractable $^{32}$P reflect the amount of myofibrillar-bound Ca$^{2+}$ (or other divalent ions) in accessible sites.

The application of labelled myofibrils to polyacrylamide gels demonstrates the existence of protein-bound $^{32}$P (4.5.1). Tables 4.29 & 4.30 give the observed $^{32}$P distribution along the gels (two different myofibril preparations); the gel sections and the major proteins in each mol. wt. zone are illustrated in Fig. 4.15. The total $^{32}$P bound to the myofibril components in the gel as a percentage of the $^{32}$P content of the labelled myofibrils (c.p.m. mg$^{-1}$) is $21.2 \pm 0.9$ (3)$\%$ for the first preparation (Table 4.29) and $1.8 \pm 0.1$ (6)$\%$ for the second (Table 4.30). The components, bound to the gel, of mol. wt. > 200,000 are nucleic acids and those < 10,000 are low mol. wt. phospholipids (cf. Bárány & Bárány,
$^{32}$P travels at the anion front during electrophoresis and would be leached out of the gel during protein staining and destaining. Since the results in Table 5.2 show that phospholipids do not carry a high proportion of label, the high count at the dye front cannot be accounted for; some RNA is known to travel as a single zone at the front (cf. Smith, 1962). If unlabelled myofibrils are electrophoresed concurrently with $^{32}$P (100 μg protein with 25,000 c.p.m.), a small proportion of count is present in the gel at the dye front after staining of the gel (16.2 ± 1.0 c.p.m.). The remainder of the gel gives a count rate indistinguishable from background.

If the observed count at the top of the gel and at the dye front (zones 1 and 9) are subtracted from the count in the gel as a whole, then this value represents the protein-bound count. Thus, the protein-bound $^{32}$P for the two preparations, as % of the respective $^{32}$P content of the myofibril, are 5.02 ± 0.51 (3)% and 1.08 ± 0.08 (6)%. The mean of these two values is 1.18 ± 0.21 (9)%. The estimate of the alkali-labile $^{32}$P in labelled myofibrils, representing phosphoproteins, is 2.9 ± 0.3 (8)% of the total count taken up by myofibrils (Table 5.2). The value for the protein-bound $^{32}$P in the gel and the alkali-labile $^{32}$P fraction in the myofibril are significantly different at the 0.1 level ($T = 1.443$, 14 degrees of freedom); the difference is 0.71 ± 0.49%. High mol. wt. $^{32}$P compounds may have been bound to material that was not solubilised during gel sample preparation and was subsequently removed by centrifugation (2.11).

Although the amount of bacterial protein present in the myofibril preparations must be low, the labelled protein may be of high specific
activity. Protein-bound $^{32}$P may reflect the presence of these proteins in mol. wt. ranges corresponding to known myofibril phosphoproteins. The conclusion that phosphorylation of myofibril proteins has taken place may be erroneous. Alternatively, it could be argued that, since the bacteria are active and that there is a supply of nucleotide, phosphate, and oxidisable material, $[\gamma-{^32}P]$ ATP could have been synthesised. Assuming the presence of the appropriate protein kinases, phosphorylation of some of the myofibril proteins might have taken place. We cannot, at this stage, distinguish between the effects of substrates (ATP, $P_i$, MgATP, CaATP) and of reagents (NTP, NP, DNP, DTT) on myofibril proteins and bacterial proteins. It may be necessary to reinvestigate some of the properties of myofibril preparations in the presence of an antibiotic.

We have stored various muscle protein preparations with $^{32}$P for several days, dried aliquots of protein and looked for $^{32}$P uptake. Both myosin preparations and actomyosin showed high uptake but actin showed little. Chloramphenicol reduced the uptake in all cases.

5.3 THE REDUCTION IN THE SPECIFIC ACTIVITY OF $^{32}$P BOUND TO PSOAS MYOFIBRILS

The experiments reported in 4.3, and discussed in 5.2, demonstrate that the uptake of $^{32}$P by glycerol-extracted myofibrils is almost entirely due to bacterial contamination. Quantities taken up vary from $10^2$ to $10^4$ c.p.m. $mg^{-1}$ myofibrils (4.3.3.7). Up to $10^3$ c.p.m. $mg^{-1}$ is in the form of TCA-extractable $P_i$. The degree of labelling, probably, reflects the level of bacterial contamination.
However, the labelling of psoas myofibrils was performed on the intact muscle in an effectively sterile state and under conditions in which aerobic respiration was encouraged (i.e. oxygenated-physiological saline containing glucose). The muscle was suspended in oxygenated-physiological saline containing $^{32}\text{P}$ at 37°C. After incubation, the psoas muscle was washed free of $^{32}\text{P}$ with physiological saline at 0 - 4°C and stored in 50% aq. glycerol at -20°C for 1 week (see 2.2.2 for experimental details). Sections of glycerol-extracted muscle were removed and homogenised in cold buffered 0.5% KCl. The myofibrils were washed several times by repeated suspension in buffered 0.5% KCl. Experiments were carried out on this labelled psoas myofibril preparation. The degree of labelling of four preparations was found to be about 30, 80, 200, 250 c.p.m. mg$^{-1}$. Each value is the $^{32}\text{P}$ content of the myofibril two weeks after labelling of the muscle.

**DISTRIBUTION OF $^{32}\text{P}$ IN THE PSOAS MYOFIBRIL**

Fractionation of labelled psoas muscle (4.4.5) revealed that $^{32}\text{P}$ was distributed throughout the P-containing components; Table 5.3 gives the P and $^{32}\text{P}$ distribution in psoas myofibrils as % total bound P and $^{32}\text{P}$ contents. Separation of psoas myofibril components on polyacrylamide gels shows the presence of labelled material (Table 4.31). The c.p.m. found in the gels was compared to the quantity in the loaded protein. 6.7 ± 0.9 (4)% load protein-bound $^{32}\text{P}$ was found in the gel. As discussed:

† In this discussion, 'load' or '% load' refers to the protein, $^{32}\text{P}$, etc. applied to the gel and 'gel' or '% gel' to the quantity of material found in the gel after electrophoresis etc.
In 5.2, the top band of the gel is mainly nucleic acid and the front composed of phospholipids and some RNA. The mean sum of the counts in the protein bands (zones 2 to 9, Fig. 5.6) of the 4 sets of gels was calculated as % load $^{32}\text{P}$ and % gel $^{32}\text{P}$. $1.5 \pm 0.5$ (4)% load c.p.m. was found in the gels. $0.5 \pm 0.2$ (6)% of the total $^{32}\text{P}$ content of a psoas myofibril preparation was released by hot alkali, after removal of other P fractions (Table 5.3); this fraction represents labelled phosphoproteins (Table 5.1). The % gel protein-bound $^{32}\text{P}$ and the % label in the alkali-labile fraction are significantly different at the 0.1 level ($T = 1.857$, 3 degrees of freedom). For reasons given in 5.2, the estimate for alkali-labile P may be 54% too low because of losses that occur during isolation of the fraction. If the loss is taken into account, then the alkali-labile $^{32}\text{P}$ is $0.9 \pm 0.4\%$, a value which is not significantly different from the % gel protein-bound $^{32}\text{P}$ ($T = 0.937$, 6 degrees of freedom).

Fig. 5.5 shows the distribution of $^{32}\text{P}$ in the gel (mean values of 4 experiments, Table 4.31) as % gel c.p.m. Four protein zones (2,3,4,8) were found to be significantly labelled. The major proteins in these zones are myosin, M-protein, C-protein, TN-T, TM; other protein bands are discernible within each zone. The presence of a particular protein in a region of the gel does not guarantee the possibility that a distinct protein of similar mol. wt. moves with or in close proximity to the protein. As recently shown by Pepe & Drucker (1979), a protein band is located on each side of the actin band in gels and are so close, in fact, that it is almost impossible to distinguish them. The fact that we found it necessary to load each gel with very large quantities of protein, in order to obtain measurable radioactivity, accentuates the enigma.
TABLE 5.3

P AND $^{32}$P CONTENT OF P-CONTAINING FRACTIONS
OF LABELLED PSOAS MYOFIBRILS

<table>
<thead>
<tr>
<th>fraction</th>
<th>P content</th>
<th>$^{32}$P content</th>
</tr>
</thead>
<tbody>
<tr>
<td>cold acid extractable</td>
<td>28.6(2.6)</td>
<td>86.6(1.3)</td>
</tr>
<tr>
<td>chloroform/methanol extractable</td>
<td>23.5(2.6)</td>
<td>0.0(2.7)</td>
</tr>
<tr>
<td>hot acid labile</td>
<td>37.8(2.6)</td>
<td>9.1(2.6)</td>
</tr>
<tr>
<td>alkali labile</td>
<td>5.9(0.8)</td>
<td>0.5(0.2)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.

Values are calculated from the results given in Table 4.28.
See Fig. 4.15 for abbreviations.

s = significant count
The most active of our psoas myofibril preparations was 250 - 300 c.p.m. mg\(^{-1}\). The protein load per gel was 200 - 500 µg per gel. If the c.p.m. bound to the protein bands in the gel is 2% of the load \(^{32}\text{P}\), then there are about 2 c.p.m. per gel. It was necessary, therefore, to measure the radioactivity in pooled, equivalent sections from 16 or more gels. Zone 8 contains two known phosphoproteins, TN-T and TM, both of which are known to be phosphorylated in vivo; intact frog muscle incorporates \(^{32}\text{P}\) into TM (1.5.5.3 & 1.5.5.4). Phospholipids are not labelled (Table 5.3); \(^{32}\text{P}\) at the front of the gel is probably high mobility RNA. Nucleic acid (zone 1) also shows a relatively high proportion of the gel count. The sum of the % load c.p.m. in zones 1 and 10 is 4.9 ± 0.5%; the % \(^{32}\text{P}\) content of the hot acid-labile fraction is 9.1 ± 2.6% (Table 5.3).

THE CONTRACTION EFFECT

Contraction of psoas myofibrils (1 mM MgATP in 0.1 M KCl buffered with 50 mM Tris-HCl pH 7.5 containing 0.01 mM CaCl\(_2\)) reduces the specific activity of bound \(^{32}\text{P}\) by 52.2 ± 8.1% (Table 4.23); Whitehead (1970) has reported 36 ± 9%. This phenomenon (the 'contraction effect') appears, from the experiments reported in 4.3.2 & 4.3.3, to be linked to the solublisation of myofibrillar proteins. The contraction effect was not observed when contracted and control myofibrils were washed with 0.5% KCl unless at least one treatment with water was carried out (Tables 4.22 & 4.23). Control myofibrils ('non-contracted') were not treated with ATP but were incubated with buffered KCl containing Mg\(^{2+}\) and Ca\(^{2+}\). Subsequent treatments of non-contracted and contracted myofibrils were identical (i.e. KCl and water treatments, acetone-drying, P and \(^{32}\text{P}\) estimation). The dry wts. of non-contracted and contracted myofibrils
collected after water treatment (Table 4.24) were significantly different 
\((T = 5.401, 11 \text{ degrees of freedom})\) as were the quantities of bound \(P\) 
\((T = 4.523, 12 \text{ degrees of freedom})\) and \(^{32}\text{P} (T = 2.560, 8 \text{ degrees of} \ \text{freedom}).\) A summary of some of the conclusions that have been drawn is 
given on p. 224. Bound \(P\) is evenly distributed throughout the myofibril; 
the ratio of \(P\) to dry wt. of protein is the same in both non-contracted 
and contracted myofibrils even though protein and \(P\) are extracted 
together by water from the two myofibril states to different extents. 
But the difference in the quantity of \(^{32}\text{P}\) extracted from the two states 
is less than would be expected if the isotope was evenly distributed 
(Table 5.4).

### Table 5.4

<table>
<thead>
<tr>
<th></th>
<th>Protein</th>
<th>(P)</th>
<th>(^{32}\text{P})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-contracted</td>
<td>66.6(4.5)</td>
<td>64.9(5.2)</td>
<td>13.0(4.3)</td>
</tr>
<tr>
<td>Contracted</td>
<td>26.2(6.7)</td>
<td>33.7(6.9)</td>
<td>15.0(4.3)</td>
</tr>
</tbody>
</table>

The decrease in extractability of protein-bound \(P\) on contraction is 
the major factor contributing towards the contraction effect.
DISTRIBUTION OF $^{32}$P IN THE AQUEOUS EXTRACT

Approx. 90% of the $^{32}$P bound to the psoas myofibril is in a form that is extractable with cold acid (Table 5.3). We analysed a TCA extract for $P_i$ and nucleotides. ATP, ADP, AMP, IMP and $P_i$ were found in the extract (4.4.5). The $P$ contributed to the myofibril $P$ content by these five types of molecule accounts for all the TCA-extractable $P$, 0.34 µg P mg$^{-1}$ (cf. 4.4.5 & Table 5.3). 15% of the extracted $P$ is $P_i$; only $P_i$ is significantly labelled and, therefore, TCA-extractable $^{32}$P ($86.6 \pm 1.3\%$) is in this form. We analysed the aq. extracts of non-contracted and contracted myofibrils for $^{32}$P distribution (4.4.4).

Changes in the distribution of $^{32}$P were observed to occur on contraction (see pp. 233, 236 & Table 4.26b for summaries of experimental findings).

The various fractions of the extract have already been defined (pp. 225 - 227); a résumé is given below.

<table>
<thead>
<tr>
<th>RESIDUE</th>
<th>acetone-dried myofibril protein residue after two consecutive water extractions.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOLUBLISED</td>
<td>the protein which remains in solution after centrifugation of the extract at 40,000g for 1 hr.</td>
</tr>
<tr>
<td>SEDIMENTABLE</td>
<td>the protein sedimented from the extract at 40,000g in 1 hr.</td>
</tr>
<tr>
<td>ACID-EXTRACTABLE $P_i$</td>
<td>the $P_i$ found in solution after deproteination of the solublised fraction.</td>
</tr>
<tr>
<td>SOLUBLISED BOUND P</td>
<td>the bound $P$ of solublised protein not including acid-extractable $P_i$.</td>
</tr>
<tr>
<td>SEDIMENTABLE BOUND P</td>
<td>the $P$ bound to sedimentable protein including acid-extractable $P_i$.</td>
</tr>
</tbody>
</table>
We also define SEDIMENTABLE $P_i$ and RESIDUE $P_i$ as the acid-extractable $P_i$ sedimented with protein and the $P_i$ present in the residue respectively. These two fractions were not measured but may be deduced from the total $P_i$ content of the myofibril, $0.051 \pm 0.012$ (3) $\mu g P mg^{-1}$, and the $P_i$ released from solubilised protein in both the first and second extracts (Table 4.26). Sedimentable $P_i$ and residue $P_i$ are negligible. Water apparently extracts all the $P_i$ bound to the myofibril.

The protein (mg), $P$ ($\mu g$), and $^{32}P$ (c.p.m.) present in the various fractions of the extract were calculated as percentages of the respective total myofibril and of the respective extract protein, $P$, and $^{32}P$ contents (Table 4.25); see Table 5.5. In the discussion that follows, the term 'extract' refers exclusively to the first of the two consecutive aq. extractions of myofibrils.

The non-contracted state represents the original protein, $P$, and $^{32}P$ distribution in the myofibril. There are significant changes in all the measured quantities after contraction except in the amounts of $^{32}P$ bound to the extracted residue and acid-extractable $^{32}P_i$. On contraction, there are significant increases in the specific activities of acid-extractable $P_i$ and $P$ bound to sedimentable protein. The specific activity of $P$ bound to solubilised protein remains constant, as does the $P$ and $^{32}P$ content per unit wt. of myofibrils. Data for the $P$ content of solubilised protein represents nucleotides, phospholipids, nucleic acids and phosphoproteins. No detectable turnover of these $P$-containing molecules has been shown to occur in the water-soluble fraction. Turnover of acid-extractable $P_i$ in this fraction does occur during contraction. The sedimentable $P_i$ in both myofibril states is
<table>
<thead>
<tr>
<th></th>
<th>non-contracted</th>
<th>contracted</th>
</tr>
</thead>
<tbody>
<tr>
<td>protein residue</td>
<td>54.0(1.2)</td>
<td>79.4(2.2)</td>
</tr>
<tr>
<td>residue-bound P</td>
<td>58.9(2.7)</td>
<td>81.5(6.0)</td>
</tr>
<tr>
<td>residue-bound $^{32}$P</td>
<td>31.0(2.4)</td>
<td>27.7(2.3)</td>
</tr>
</tbody>
</table>

**first extract**

<table>
<thead>
<tr>
<th></th>
<th>non-contracted</th>
<th>contracted</th>
</tr>
</thead>
<tbody>
<tr>
<td>solublised protein</td>
<td>15.4(0.5)</td>
<td>6.0(0.3)</td>
</tr>
<tr>
<td>solublised-bound P</td>
<td>10.4(1.4)</td>
<td>4.7(0.7)</td>
</tr>
<tr>
<td>solublised-bound $^{32}$P</td>
<td>26.4(3.2)</td>
<td>10.7(2.3)</td>
</tr>
<tr>
<td>sedimentable protein</td>
<td>11.0(1.7)</td>
<td>1.5(0.2)</td>
</tr>
<tr>
<td>sedimentable-bound P</td>
<td>12.5(2.0)</td>
<td>1.0(0.5)</td>
</tr>
<tr>
<td>sedimentable-bound $^{32}$P</td>
<td>2.9(2.1)</td>
<td>7.1(4.3)</td>
</tr>
<tr>
<td>acid-extractable $P_1$</td>
<td>4.0(0.8)</td>
<td>2.0(0.4)</td>
</tr>
<tr>
<td>acid-extractable $^{32}P_1$</td>
<td>32.2(1.1)</td>
<td>38.8(3.9)</td>
</tr>
</tbody>
</table>

**second extract**

<table>
<thead>
<tr>
<th></th>
<th>non-contracted</th>
<th>contracted</th>
</tr>
</thead>
<tbody>
<tr>
<td>solublised protein</td>
<td>12.9(0.8)</td>
<td>6.7(0.6)</td>
</tr>
<tr>
<td>solublised-bound P</td>
<td>5.9(0.7)</td>
<td>4.8(0.7)</td>
</tr>
<tr>
<td>solublised-bound $^{32}$P</td>
<td>1.5(3.1)</td>
<td>7.7(3.4)</td>
</tr>
<tr>
<td>sedimentable protein</td>
<td>5.4(0.8)</td>
<td>4.7(1.3)</td>
</tr>
<tr>
<td>sedimentable-bound P</td>
<td>7.3(1.3)</td>
<td>3.3(1.2)</td>
</tr>
<tr>
<td>sedimentable-bound $^{32}$P</td>
<td>0.8(2.3)</td>
<td>-2.8(1.7)</td>
</tr>
<tr>
<td>acid-extractable $P_1$</td>
<td>2.1(0.4)</td>
<td>1.7(0.4)</td>
</tr>
<tr>
<td>acid-extractable $^{32}P_1$</td>
<td>10.0(1.1)</td>
<td>2.3(1.7)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are standard errors.

Values are calculated from the results given in Table 4.25.
**TABLE 5.5b**

**PROTEIN, P, AND $^{32}$P DISTRIBUTION IN THE AQUEOUS EXTRACTS**

**OF NON-CONTRACTED AND CONTRACTED PSOAS MYOFIBRILS**

**(% EXTRACT)**

<table>
<thead>
<tr>
<th></th>
<th>non-contracted</th>
<th>contracted</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>first extract</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>solublised protein</td>
<td>56.1(3.1)</td>
<td>80.2(1.6)</td>
</tr>
<tr>
<td>solublised-bound P</td>
<td>30.7(4.3)</td>
<td>56.9(4.7)</td>
</tr>
<tr>
<td>solublised-bound $^{32}$P</td>
<td>41.7(4.7)</td>
<td>16.7(3.3)</td>
</tr>
<tr>
<td>sedimentable protein</td>
<td>41.8(3.6)</td>
<td>19.7(1.7)</td>
</tr>
<tr>
<td>sedimentable-bound P</td>
<td>46.6(7.3)</td>
<td>12.9(1.9)</td>
</tr>
<tr>
<td>sedimentable-bound $^{32}$P</td>
<td>3.8(3.9)</td>
<td>24.4(4.7)</td>
</tr>
<tr>
<td>acid-extractable $P_i$</td>
<td>12.3(2.3)</td>
<td>22.0(0.5)</td>
</tr>
<tr>
<td>acid-extractable $^{32}P_i$</td>
<td>54.5(3.0)</td>
<td>57.2(3.6)</td>
</tr>
</tbody>
</table>

|                          |                |            |
| **second extract**       |                |            |
| solublised protein       | 69.3(3.2)      | 47.5(3.6)  |
| solublised-bound P        | 36.1(3.1)      | 40.0(5.4)  |
| solublised-bound $^{32}$P | 18.7(18.7)     | 40.2(14.0) |
| sedimentable protein     | 29.2(3.2)      | 52.5(8.4)  |
| sedimentable-bound P     | 47.3(4.2)      | 42.8(3.6)  |
| sedimentable-bound $^{32}$P | 20.9(18.0)     | 34.1(19.1) |
| acid-extractable $P_i$   | 12.4(1.7)      | 13.8(3.2)  |
| acid-extractable $^{32}P_i$ | 43.1(16.3)     | 13.2(9.9)  |

Numbers in parentheses are standard errors

Values are calculated from the results given in Table 4.25.
insignificant, hence bound P and $^{32}$P is present in other forms. The P content of this fraction in the non-contracted state is approx. equal to that of the protein residue; sedimentable-bound $^{32}$P is insignificant. Contraction decreases the extractability of both soluble and sedimentable protein (Table 5.5a) but decreases that of sedimentable to a much greater extent (Table 5.5b).

The extracts of both myofibril states contain representative proportions of the myofibril proteins (Fig. 4.14). Since both extracts show MgATPase activity and also superprecipitate, we conclude that water extracts actomyosin from myofibrils. SDS-PAGE was not carried out on the solubilised protein, only the whole extract, therefore we are unsure as to the types of protein present in this fraction. Prolonged treatment of myofibrils with 5 mM Tris-HCl pH 7 removes material from the I-band and Z-line but not from the A-band (Perry & Corsi, 1958). Actin is solubilised at low ionic strength only from the non-overlap region of the sarcomere (cf. pp. 67 - 69). The 'rigor' bonding between actin and myosin in the region of overlap of the two filaments (pp. 93, 103), the inherent insolubility of myosin at low ionic strength, in vitro (pp. 58 - 59) and the cross-linking of thick filaments by M-protein (p. 81) must maintain the integrity of the A-band. Since our aq. extracts contained both thick and thin filament material, we conclude that a more complete dissolution of the sarcomere occurs when myofibrils are treated with water. The two protein fractions that compose the extract, solubilised and sedimentable, might correspond to I + Z and A material respectively. We make the assumptions, therefore, that the soluble protein fraction of the aq. extract contains actin, TN, TN, Z-protein and that the sedimentable protein is composed of actomyosin,
myosin, TM, TN; additional thick and thin filament proteins will also be present in the fractions (1.5.2.5). We cannot exclude the possibility that some myosin might be present in the soluble fraction although, in view of its aggregation properties at low ionic strength (pp. 58 - 59), it is unlikely. Contraction increases the degree of overlap of thin and thick filaments. About 20% less protein is extracted from contracted myofibrils. As discussed above, contraction has the effect of decreasing the extractability of sedimentable protein (thick + thin filament) to a greater extent than it does soluble (thin filament); a higher proportion of soluble protein is in the extract of contracted myofibrils. The bound P and 32P content per unit wt. of the soluble thin filament fraction remains constant on contraction (Table 4.26b). Since ADP and phospho-lipids are not labelled, 32P in this fraction is probably bound to TN and TM components and nucleic acid (if extracted). The increase in sedimentable bound 32P on contraction must be due to the increase in the proportion of 32P-labelled thin filament material.

The labelled psoas preparation that was used to determine the distribution of bound 32P had a 32P1 content of 87% (Table 5.3). Water extracts 42% of the myofibril label as 32P1, i.e. 50% of the 32P1 content of the myofibril. The ratio of the specific activity of 32P1 in the contracted state to that in the non-contracted myofibrils is 2.63 ± 0.73. The hot acid- and alkali-labile 32P accounts for only 10 - 13% of the bound label. This suggests that the 26% 32P found bound to soluble protein has a 32P1 component. Protein was precipitated from the soluble fraction by the addition of acidified sodium silicotungstate (p. 226) and the 32P1 in the supernatant was estimated. The combination of the strong acid (5% TCA) and ultrasonication procedure used in the determination of cold acid-extractable P releases more 32P1. It is possible that the 32P1
apparently bound to the soluble protein is sufficiently bonded to the thin filament and/or Z-line components to resist solublisation on protein precipitation but not so tightly bound that it cannot be removed by harsher treatment. One possibility is that there is a weak complex between $P_1$ and actin-ADP. Sedimentable protein contains no $^{32}P$ in the non-contracted state; water-extractable $^{32}P_1$ might have originated from this protein fraction. Contraction, and the consequent increased thick-thin filament overlap, has the effects of increasing the quantity of water-extractable $^{32}P_1$ and depositing bound $^{32}P$ in sedimentable protein. It is likely that the change in thick and thin filament overlap due to contraction alters the degree of $P_1$ bonding.

The second extract contains only one $^{32}P$ fraction, i.e. water-extractable $^{32}P_1$ from non-contracted myofibrils. No $^{32}P$ was found in any nucleotides isolated during deionised water extraction of acetone-dried, butanol-extracted psoas myofibrils. The $^{32}P_1$ fraction is not transferable to ADP under these conditions. The contraction effect was originally observed with frog muscle. Whitehead (1970) has reported that the dry wts. of contracted and control muscles, as % wet wt., were similar. In other words, no protein was extracted differentially from the two muscle states. $^{32}P_1$ was removed. The intact sarcolemma of this system prevents the extraction of protein but not small molecules.

The contraction effect appears to be linked to the interaction of actin and myosin and the increased extractability of a bound $^{32}P_1$ fraction due to contraction (Fig. 5.6).
FIGURE 5.6

THE PROTEIN FRACTIONS OF AN AQUEOUS EXTRACT OF MYOFIBRILS

initial state

Z + I

A-band

I + Z

solubilised

sedimentable

solubilised

contracted

state
5.4 AN EXPLANATION OF THE BASIS OF MOLECULAR INTERACTIONS

The structures of protein molecules are constantly changing due to interaction with their surroundings (1.4). Conformational changes have biochemical functions and are often caused by the binding of small molecules or ions, not necessarily through a high-energy phosphorylation of the protein (cf. 1.3.3). Proteins also combine with other proteins with the concomitant change in conformation of either or both molecules. Muscle is a highly complex protein system in which many different interactions take place within short molecular distances (pp. 77 - 78).

The interactions that are directly concerned with the transduction of energy from the electronic to the physical level (relative movement of the thick and thin filaments) are (1) the binding and hydrolysis of MgATP at the myosin active site, and (2) the binding of the myosin-nucleotide complex to actin (1.5.3 & 1.5.4). It is "commonly supposed that muscle generates force by a conformational change in the actin-myosin interaction on MgATP hydrolysis, akin to a rotation of the attached myosin head on the actin" (p. 84). The question that we have formulated is not how muscle works but why?

Biological systems require the constant input of energy to maintain the system; energy flows through the system (p. 27). Structures that are not in equilibrium with their surroundings transform structural energy into thermal motion (Prigogine & Babloyanz, 1971; Morowitz, 1971). Stored molecular energy must not be allowed to decay into heat if something useful is to be done with it (McClure, 1975). In other words, proteins are 'dissipative structures' that require the input of energy in order to maintain structural integrity. The energy
required to keep the structure intact is less than 4 kJ mol\(^{-1}\) (cf. Franks, 1978, pp. 1 -18). This energy could easily be acquired through the binding of a small molecule (cf. Lipscomb, 1978). During an enzyme reaction it is highly probable that energy is transmitted from the substrate to the enzyme either through binding or transformation to product and that this energy is used to maintain the protein structure. Conformational changes in the protein are known to take place on the binding of substrate (p. 43). It is conceivable that an enzyme and substrate have evolved and exist in a 'symbiotic' relationship with respect to structural energy. The transformation of substrate to product provides the energy, at some stage, that is required to maintain protein structure. In the absence of substrate the protein denatures; structural energy is dissipated and not replaced. We conclude, therefore, that the primary role of the myosin-actin interaction is to maintain the structure of actin through the transfer of energy from myosin to actin. The energy of maintenance of myosin and actin is provided through the transformation of MgATP. The energy transferred to actin is dissipated as heat at some later stage thereby creating an energy flow through the system.

\[
\text{MgADP} + P_1 \xrightarrow{\text{electron energy}} \text{MgATP} \xrightarrow{\text{M}^*} \text{A} \xrightarrow{\text{heat}} \text{M} \xrightarrow{\text{A}^*}
\]

The interactions between MgATP, myosin and actin are of crucial importance for the structural integrity of the proteins. The protein structures deteriorate in the absence of the energy supply.
5.5 POSSIBLE MECHANISMS FOR THE TRANSFER OF ENERGY FROM MYOSIN TO ACTIN DURING MUSCLE CONTRACTION

There is increasing evidence that propagated conformational changes take place in the thin filament through interaction with myosin (pp. 69 - 70; Fujima & Ishiwata, 1975; Loscalzo et al., 1975, 1977; Ando & Asai, 1979; Yanagida & Oosawa, 1980). The role of the bound ADP of F-actin in vivo remains elusive (cf. 1.5.2.2). It has been proposed that the binding of ATP and not the splitting provides the energy for protein interactions, the free energy of hydrolysis being used to remove the products (cf. Boyer, 1977). Cooke (1975) has calculated that the binding of nucleotide to G-actin provides 3 kJ mol⁻¹.

Since the available free energy of hydrolysis of ATP to ADP and Pᵢ is at least 10 times greater than this, Cooke concludes that dephosphorylation plays little or no role in the energetics of polymerisation. ATP is required, however, for the stability of G-actin. F-actin, on the other hand, is stable without bound nucleotide. Kasai & Oosawa (1963) have suggested that ADP becomes 'trapped' during polymerisation. There is evidence that the bound nucleotide of F-actin exchanges with free nucleotide during the superprecipitation of actomyosin (Szent-Györgyi & Prior, 1966; Kitagawa et al., 1968; Szent-Györgyi, 1968). Moos & Eisenberg (1970) have also shown that the bound nucleotide exchanges on the addition of ADP to actomyosin, a phenomenon found not to be linked to a myokinase-catalysed transformation of ADP to ATP.

Inhibition of actomyosin ATPase does not diminish the exchange (Szent-Györgyi, 1968) but is prevented by the presence of TN-TM in the absence of Ca²⁺. It is thought that the exchange is not directly related to ATPase activity but is caused by actin-myosin binding. Martonosi et al. (1960) could not demonstrate the exchange in vivo. However, Cheesman
et al. (1969), Priston (1970), and Cheesman & Priston (1972) believe that the increased turnover of actin-bound adenine nucleotide which they have observed in contracted frog muscle is proof of the exchange. The increased turnover has been confirmed by Shirley (1978).

It has been proposed that actin-ADP, in the thin filament, acts as a short-lived phosphate acceptor, the phosphate being transferred from myosin after MgATPase activity and during the actin-myosin interaction (cf. Cheesman et al., 1969; Whitehead, 1970). The phosphorylated bound ADP (i.e., ATP) becomes exchangeable with free ATP. Cooke (1975) has speculated that "the bound nucleotide plays an as yet unrecognised role in the interaction of actin with myosin or with the relaxing proteins". The role of the bound divalent ion similarly is unclear; Kasai & Oosawa (1969) have shown that the divalent ion exchanges during actomyosin superprecipitation. The present author inclines towards the viewpoint that "evolution does not favour purposeless function" (Frey, 1980) and that the bound nucleotide and divalent ion play important, and not minor, roles in the actin-myosin interaction.

If, as proposed in 5.4, energy must be transferred from myosin to actin it might occur through the phosphorylation of actin-bound ADP or through the energy associated with a conformational change analogous to the passage of a 'conformon' through a macromolecule (see pp. 46 - 47). Evidence suggests that when MgATP binds to myosin (M), the nucleotide is split immediately yielding the M.ADP.P complex (1.5.4). Phosphorylation of the prosthetic group of actin (A) could occur in one of two ways. Either myosin directly phosphorylates ADP or a dismutase reaction between myosin-bound MgADP and actin-bound ADP occurs, both mechanisms resulting in the
formation of A.ATP. Structural changes would accompany the phosphorylation and subsequent dephosphorylation of actin-bound nucleotide. The dismutase reaction should result in the formation not only of ATP but also AMP.

**SCHEME 1**

\[
M + MgATP \rightarrow M\cdot MgADP\cdot P \\
M\cdot MgADP\cdot P + A\cdot ADP \rightarrow M\cdot (MgADP) + A\cdot ATP \\
A\cdot ATP \rightarrow A\cdot ADP + P_i \\
MgATP \rightarrow MgADP + P_i
\]

**SCHEME 2**

\[
M + MgATP \rightarrow M\cdot MgADP\cdot P \\
M\cdot MgADP\cdot P + A\cdot ADP \rightarrow M\cdot (AMP\cdot P) + A\cdot ATP(Mg) \\
A\cdot ATP(Mg) \rightarrow A\cdot ADP + P_i + Mg \\
MgATP \rightarrow AMP + 2P_i + Mg
\]

The Mg\(^{2+}\) carried with ADP, necessary for a dismutase reaction (cf. the myokinase reaction, p. 258), might exchange for actin-bound Ca\(^{2+}\).

Actomyosin MgATPase has not been shown to liberate AMP in stoichiometric quantities, as would be required by Scheme 2. Any AMP formation that has been observed has been attributed to myokinase contamination. However, no vertebrate skeletal muscle, or model system derived from such muscle, has been found to be capable of performing mechanical work in the proven absence of the enzyme (cf. pp. 164 - 165). It is conceivable that actomyosin prepared with 'Straub-type' actin isolated
from acetone-dried muscle does not manifest AMP formation because of the conformational state of the actin (Fig. 5.1); the transient step M.AMP...P...ADP.A might not go to completion. In the intact muscle, with native actin, this scheme could very well operate. It has been demonstrated that two different proteins are capable of catalysing the Mg$^{2+}$-dependent reaction 2ADP $\rightarrow$ ATP + AMP (cf. Klingenberg, 1975).

Lagunas & Sols (1970) have isolated two species of adenylate kinase from skeletal muscle. One is soluble, myokinase (135 units per g), and the other is bound to 'particulate structures' (37 units per g). We have also shown that myofibrillar-bound adenylate kinase is responsible for the ADP $\rightarrow$ ATP transformation during deionised water extraction of acetone-dried muscle and myofibrils. We have also demonstrated, to a limited extent, that some reagents which inhibit purified myokinase also influence the actin-myosin interaction (4.1.6). If, as proposed above, the actin-myosin interaction involves the dismutation of 2ADP molecules then it is possible that the actomyosin complex is the origin of the adenylate kinase reaction in acetone-dried preparations and in the particulate structures. It is of interest that Drabikowski & Gergely (1962) found that the yield of actin from acetone-dried (myosin-free) muscle was only 1 - 2 g per 100g, constituting 4½% of the available actin in the muscle, whereas we obtained a yield from acetone-dried (butanol-extracted) myofibrils that was 50 - 60% of the actin content of myofibrils (see 5.1). The fact that we did not remove myosin from the myofibrils may be indicative of the participation of this protein in the ADP $\rightarrow$ ATP transformation. Spudich & Watt (1971), for example, treat acetone-dried muscle with a low ionic strength ATP solution to isolate actin. The yield is still about 4½%.
The mechanism for myosin-ATP-actin interaction in muscle, based on a
dismutase reaction, would be (Mg\textsuperscript{2+} not shown) :

\[
\begin{align*}
M + ATP & \rightarrow M.ADP.P \\
M.ADP.P + A ADP & \rightarrow M + P_1 AMP + A.ATP \\
A.ATP & \rightarrow A.ADP + P_1 \\
\text{muscle ATPase} \\
\text{ATP} & \rightarrow \text{AMP} + 2P_1 + \text{work} \\
\text{cytosol myokinase} \\
\text{AMP} + \text{ATP} & \rightleftharpoons 2 \text{ADP} \\
\text{glycolysis, mitochondria} \\
2 \text{ATP} & \rightarrow 2 \text{ADP} + 2P_1 .
\end{align*}
\]

It is a fact that little AMP is found in active muscle except just
before the onset of rigor mortis and that the accumulation of AMP is an
indicator that supplies of metabolic energy are depleted (pp. 38 - 39).
The last step in the above sequence is blocked by lack of metabolic
(electron) energy. Myosin-bound 5'-AMP deaminase removes AMP generated
by actomyosin ATPase to prevent the accumulation of ADP and to drive the
myokinase reaction towards ATP synthesis. The activity of deaminase may
be sensitive to pH changes; accumulation of lactic acid might trigger
the enzyme.

It is also proposed that one of the key amino acid residues in actin
involved with energy transfer is MeHis-73 (see pp. 64 - 65). In
solution, the pK of His is 6.04 and that of MeHis, 6.48. At the
physiological pH of 7.4, the ratio of His to His\textsuperscript{+} is 23 and that of
MeHis to MeHis\textsuperscript{+} is 8. It is possible that the local environment of
MeHis-73 is such that the charged form is favoured for a particular protein conformation. The change from one form to the other might be involved with the phosphorylation of ADP, or exchangeability of nucleotide or divalent ion. MeHis-73 is found on the surface of the protein (p. 65). We propose that the methyl group is present to provide steric protection of a fundamentally important positively charged ionic species from the environment, especially P$_i$.

The results discussed in 5.1 & 5.3 suggest that:

1. the thin filament is a sequence of alternating actin conformations;
2. there is a labile P$_i$ fraction associated with the myosin-actin interaction.

The interaction between myosin and actin and the flow of energy into the thin filament probably alters the conformational state of actin. Wray et al. (1978) consider the regulatory unit of muscle to be composed of 7 actin monomers, 1 TN complex, 1 TM dimer. The interaction between one myosin molecule (one or both heads, pp. 95 - 97) and a regulatory unit may cause a concerted change in all 7 actin monomers even though only 1 monomer interacts directly (the two heads of the one myosin might be attached to adjacent actin monomers). Approx. 50% of the actin-bound nucleotide exchanges instantaneously on the addition of ATP to the F-actin-myosin complex (Szent-Györgyi, 1966; Kitagawa et al., 1968). This effect is thought to be due to a transient loosening of the bound nucleotide. If phosphorylation of actin-bound ADP does occur then it would be this event which triggers the concerted conformational change in 6 or 7 actin monomers.
Molecules are electronic in nature and not mechanical. Quantum mechanics views a delocalised π electron system to be a single fluid distribution and the underlying skeleton of nuclei, non-bonding and σ electrons as pulling on the cloud thereby creating differential π electron densities (pp. 24–27, 35–36). Interaction with such a system distorts the π electron distribution creating ruptures in the σ framework, i.e. bond-breaking. The interaction of myosin and actin could conceivably cause a mixing of mobile electrons between the two types of macromolecule. The passage of an energetic 'superparticle', generated by the myosin–actin–nucleotide interaction, along a short stretch of thin filament, for example between TN complexes, might carry with it the electronically-interlocked myosin head.

\[ \text{Z-line} \quad \text{actin} \quad \text{myosin} \quad \text{MgATP} \quad \text{energy transfer} \quad \text{energy flow} \quad \text{TN} \quad \text{energy dissipation} \]
APPENDIX
FIGURE A.1

CIRCUIT DIAGRAM OF PULSE INTEGRATOR

Pulses arriving at capacitor C create a voltage difference across it and the input terminals of the chart recorder. The voltage depends on C, R, and pulse rate. The higher the pulse rate the greater the voltage, up to the capacity of C. Resistance R acts as a voltage regulator. Selection of R and C provides a means of controlling the response of the chart recorder (Fig. A.2, \( \tau = RC \)). With \( \tau = 50 \) sec, the background voltage swing is \( \pm 20 \) mV. SC is provided to reset the voltage to 0 V. IC 1 amplifies the input signal; IC 2 is an output stage to offset the recorder impedance.
Pulses from a Model SD9 Stimulator (Grass Instruments, Quincy, Mass., USA).

- pulse height: 12 V
- pulse duration: 20 μsec
- C: 50 μF
- R: 1 M and 100 kΩ
To 10 ml 250 μM IMP (in water) or 250 μM hypoxanthine (in water) was added 2.5 ml 5 M HCl. The solution was heated at 100°C. 1 ml aliquots were removed at time intervals and assayed for hypoxanthine by the xanthine oxidase-catalysed oxidation to uric acid (2.6.1.3.1).

\[
\text{IMP} \rightarrow \text{hypoxanthine + ribose + phosphate}
\]
\[
\text{hypoxanthine} \rightarrow \text{uric acid}
\]

Hypoxanthine adsorbs maximally at 248 nm, uric acid at 290 nm.
The principle of the hydrolysis of IMP to hypoxanthine, ribose and phosphate is discussed in 2.6.1.3. The procedures for both methods of IMP estimation are given in 2.6.1.3. Nucleosidase used to hydrolyse inosine to hypoxanthine and ribose was prepared either from a lactobacillus or from yeast (p. 303).
Acid hydrolysis

Enzymic hydrolysis
PREPARATION OF NUCLEOSIDASE FROM *Lactobacillus plantarum*

The commercial source of the lactobacillus is given in 2.1. Cells were grown and harvested by the method of Wang (1955). Cell-free extracts were prepared by ultrasonic disintegration (2.10). The cell residue was removed by centrifugation at 40,000g for 20 min. The extract was stored at -20°C.

PREPARATION OF NUCLEOSIDASE FROM YEAST

40 g of dried yeast (DCL) was incubated at 37°C in 140 ml 0.2 M acetate buffer pH 5.1 for 20 hrs. The cell debris was removed by centrifugation. The supernatant was brought to 35% sat. with respect to ammonium sulphate and the pH adjusted to 4.6 with 2 M acetic acid. The ammonium sulphate saturation was brought to 45% and, after 15 min, the ppt. was removed. Saturation was brought to 70% and the ppt. dissolved in 0.1 M acetate buffer pH 6.0. The suspension was dialysed against 0.01 M acetate buffer pH 6.0. The solution of nucleosidase was stored at -20°C. (Reference: Heppel & Hilmoe, 1952).

Both preparations of enzyme were unstable.
FIGURE A.5

EFFECT OF SOME BUFFERS ON 5'-AMP DEAMINASE ACTIVITY

To 2.6 ml buffer was added 0.5 ml AMP (200 μM AMP in appropriate buffer plus water). The solution was incubated at 30°C for 5 min. $E_{260}$ was measured (against buffer) to obtain the concentration of AMP. The wavelength was adjusted to 265 nm and 0.1 ml of buffer containing 5 μg 5'-AMP deaminase (Sigma London Chemical Co. Ltd.) added. The initial rate of decrease in $E_{265}$ was measured. See 2.6.1.2 for further information.
Ebashi (1961), Yasui & Watanabe (1965a, 1965b), and Watanabe (1970) have studied the superprecipitation of actomyosin by observing the increase in turbidity spectrophotometrically. Some of our experimental findings using natural and synthetic actomyosins are reported in 4.1.6. We have found that a finely homogenised suspension of myofibrils, prepared by 5 min maceration of myofibrils (2.2.1.1) in a Silverson Mixer-Emulsifier (Silverson Machines Ltd., Chesham, Bucks.), would remain suspended in solution for about 1 hr if left undisturbed. If the homogenate is placed in the light-path of a spectrophotometer ($\lambda = 350$ nm, for example), then $E$ remains constant. The addition of $\text{MgATP}$ causes $E$ to increase. The $E$ remains constant at the new level for about 15 min (Fig. A.5). The speed at which the new value of $E$ was reached was proportional to the concentration of ATP (cf. 4.6.1).

By a suitable arrangement of experimental conditions, it is possible to measure the adenylate kinase and 5'-AMP deaminase activities of a myofibril suspension by observing $E_{265}$. The addition of $\text{MgADP}$, however, causes a sudden decrease in $E$ (not due to dilution) which gradually increase; this phenomenon was not investigated further. AMP formation by myofibrils due to adenylate kinase activity was studied by observing the 5'-AMP deaminase activity of myofibrils as follows. To 2.6 ml buffer (see Fig. A.4) was added 0.1 mg of myofibrils (prepared as described above) in 0.1 ml buffer and 0.4 ml water. Spectrophotometric adjustment was made for the opacity of the suspension at 265 nm. The suspension was incubated at $30^\circ C$ for 5 min.
FIGURE A.6

EFFECT OF MgATP ON THE OPACITY OF A MYOFIBRIL SUSPENSION
UNDER CONDITIONS WHEN CONTRACTION OCCURS

3.0 ml 0.1 M glycylglycine-KOH buffer pH 7.5 (25°C)
0.1 ml 10 mM MgCl$_2$
0.1 ml 0.3 mM CaCl$_2$
0.1 ml 1 mg ml$^{-1}$ myofibril suspension in buffer

The above mixture was placed in a quartz cuvette (1 cm light-path) and incubated at 25°C for 5 min. Contraction was initiated by the addition of 0.1 ml 10 mM ATP in buffer.
Reaction was initiated by the addition of 0.1 ml 1 mM AMP in buffer. The initial rate of decrease in $E_{265}$ was recorded.

<table>
<thead>
<tr>
<th>buffer</th>
<th>pH</th>
<th>$\mu$mol AMP deaminated $\text{min}^{-1} \text{mg}^{-1}$</th>
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<tr>
<td>imidazole-HCl</td>
<td>6.5</td>
<td>0.14 ± 0.05 (3)</td>
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<tr>
<td>glycylglycine-KOH</td>
<td>7.5</td>
<td>0.19 ± 0.05 (3)</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>7.5</td>
<td>0.10 ± 0.03 (3)</td>
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<tr>
<td>glycylglycine-KOH</td>
<td>8.0</td>
<td>0.08 ± 0.04 (3)</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>8.0</td>
<td>0.05 ± 0.03 (3)</td>
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### BIBLIOGRAPHY

**Non-Standard Abbreviations**

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<td>ABB</td>
<td>Archives of Biochemistry and Biophysics</td>
</tr>
<tr>
<td>BBA</td>
<td>Biochimica et Biophysica Acta</td>
</tr>
<tr>
<td>BBRC</td>
<td>Biochemical and Biophysical Research Communications</td>
</tr>
<tr>
<td>BJ</td>
<td>Biochemical Journal</td>
</tr>
<tr>
<td>BZ</td>
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<tr>
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<td>Journal of the American Chemical Society</td>
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