The Exchange of the Actin-bound Nucleotide

A thesis presented by

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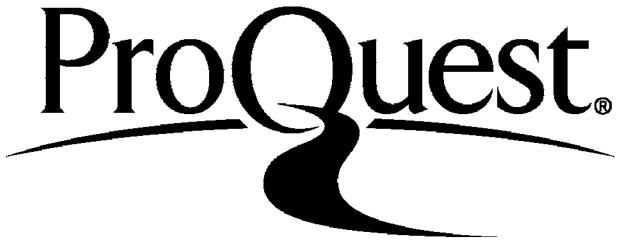


Abstract

Radioactive adenine is readily taken up by muscle and is incorporated into nucleotides as an intact unit. The rate of uptake is independant of the duration of incubation and increases with temperature.

About 1/6 of the actin-bound ADP is rapidly exchangeable with free nucleotide in resting muscle.

In isometric contraction there is a slight loss of tritium label from the 2 position of the actin-bound ADP.

In loaded contracture there is a slight exchange of the actin-bound nucleotide over and above the resting exchange. The extent of this additional exchange seems to parallel the peak power output of the muscle. For a muscle contracting at maximum power, the probability that a bound ADP will exchange during a single interaction with myosin is about 0.0005.

Experiments in vitro show that, at high temperatures, the F-actin-bound ADP is labile and that the ATPase of the actin is dependent on the preparation of the protein.

It is possible to calculate the length (along the thin filament) between the points at which any one cross bridge makes successive attachments. For the purposes of this calculation it is only necessary to know certain mechanical, energetic and geometric properties of muscle; no particular model of muscular contraction need be assumed. The length in question is about 38 nm, the length of the half turn of the thin filament.

Contents

INTRODUCTION

The structure of skeletal muscle

A muscle is composed of fibres; individual fibres are about 20-100 µm in diameter and their lengths vary from muscle to muscle. Each fibre is coated with connective tissue and the fibres are tied together in bundles of about 30 with further connective tissue. The bundles themselves are bound together to form the whole muscle and covered with more connective tissue.

Each fibre is bounded by the sarcolemma or cell membrane and has nuclei spaced at 5 µm intervals along its length. Each fibre also contains several thousand myofibrils. These are 1 to 2 µm in diameter and run the whole length of the fibre. Woven around the myofibrils is the sarcoplasmic reticulum, a structure which conducts the electrical impulse from the surface of the muscle to the myofibrils. Mitochondria are packed in rows between the myofibrils.

The structure of myofibrils

A myofibril contains arrays of thick and thin filaments. In most muscles these filaments are arranged in the hexagonal pattern shown in fig. 1.1. Longitudinally the filaments are organized into compartments, limited by the Z-discs, known as sarcomeres. A single thick filament is about 1.5 µm long and 12 nm in diameter and is an ordered aggregate of the protein myosin. Starr and Offer (1971) report that the thick filament also contains a second protein component - C-protein.

The proteins actin, troponin, tropomyosin and actinin make up the thin filament, the main component being actin. A thin filament is typically 2.2 μm long by 8 nm in diameter. Neighbouring thin filaments are attached to each other by the Z-discs.

The structure of the thick filament

The gross structure of a myosin molecule is shown in fig 1.2. The tail region is a rigid structure. The twin heads are the site of an ATPase and they show actin binding properties (Mueller and Perry, 1962). The myosin molecules are arranged so that the tails point toward the centre of the filament. The heads project from the surface of the filament and can form cross bridges to the thin filaments. These bridges are arranged in pairs[1], the members of each pair emerging on diametrically opposite sides of the filament. The spacing between pairs of bridges is 14.3 nm and each pair is rotated by 120° with respect to its neigbours. Each bridge in a resting muscle projects some 14 nm from the centre of the thick filament, i.e. just under half the distance to a neighbouring thin filament.

It is a consequence of the above structure that each thick filament has a region in its centre from which no bridges project.

The above picture of myofibrils and myosin has been built up by the electron microscopy, X-ray diffraction and chemical investigations of numerous authors including Hanson and Huxley (1957); Hanson and Lowy (1964); Huxley (1963 and 1963a); Huxley and Brown (1967); Ohtsuki et al.(1967); Page (1963); Pepe (1967); Perry (1967) and Morimoto and Harrington (1974).

1. See also discussion

The structure of the thin filament

The thin filament is composed of the proteins actin, tropomyosin and troponin. In addition α-actinin is present at or around the Z-disc (Masaki et al, 1967; Briskey and Fukazawa, 1970). β-actinin, another protein, seems to act as a terminator for the actin polymer (Maruyama, 1965 and 1971).

The actin monomers are ellipsoidal (5.5 x 5 x 3.5 nm) and form a double helix, in the grooves of which lies tropomyosin (Moore et al, 1970). Troponin (itself a three-component protein) has a specific binding site every 40 nm along the filament (Ohtsuki et al, 1967). Tropomyosin is a completely helical two stranded coiled coil molecule of length 40 nm (Woods, 1967 and 1969). α-actinin has a molecular weight of 180,000 (Robson et al, 1970), and has an amino acid composition distinct from that of actin, whereas β-actinin has a similar amino acid composition to that of actin and a molecular weight of 60,000 (Maruyama, 1971). Troponin is the protein which confers calcium sensitivity on the contractile apparatus and has a molecular weight of 80,000 (Ebashi and Endo, 1968). This protein consists of three components (Ebashi et al, 1971).

The double helix of the thin filament is right handed (Depue and Rice, 1965) and has a pitch of about 72 nm (Hanson, 1967; Elliott et al, 1967). There are therefore between 13 and 14 actin monomers per complete turn (in each chain).

A paper by Potter (1974) gives the molar ratios of the muscle proteins actin/myosin/tropomyosin/troponin as 7/1/1/1. This paper also states that the three components of troponin are present in equimolar proportions. The actin/troponin ratio

together with the known periodicity of troponin suggests that the latter molecules are attached (or co-polymerized) to the helix in pairs. The thin filament is shown in fig. 1.3.

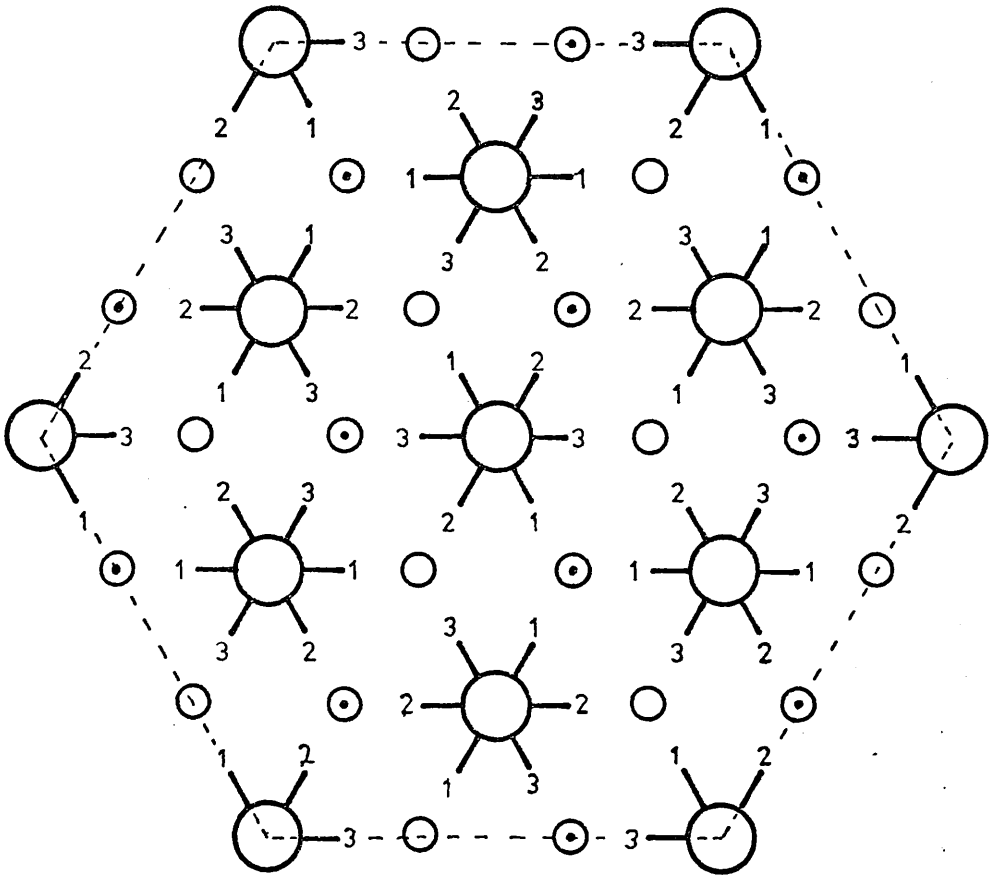
The sliding filament theory and the motion of the filaments during contraction

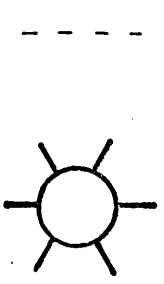
Hanson and Huxley (1955) first advanced the sliding filament model of muscular contraction, which has since become universally accepted. The main feature of the model is that in contraction the filaments slide past each other with no change in their individual lengths. X-ray diffraction studies on living muscle have shown that there is no gross change in the length or structure of either filament (Elliott et al, 1967; Huxley et al, 1965; Huxley and Brown, 1967).

Apart from the sliding of the filaments there is some information about the detailed molecular movements involved. As a muscle contracts its volume remains nearly constant. Rome (1967) has shown that changes in the length of a muscle are accompanied by lateral motion of the filaments, just sufficient to maintain constant volume. This fact has become the basis of a theory of contraction (Elliott et al, 1970) which does not demand direct contact between thick and thin filaments. Most other theories, e.g. those of Huxley (1966); Huxley and Simmons, (1971); Harrington (1971) and Loewy (1968) postulate direct contact between the cross bridges and the thin filaments. There is some evidence in vivo to support this postulate (Miller and Tregear, 1972; Huxley and Brown, 1967) and some indication that the cross bridges are, in vivo, the site of an ATPase

Fig. 1.1

Schematic cross-section of the muscle filaments.
The unit cell from Huxley and Brown (1967).
(For alternative possible arrangements of the bridges see discussion)





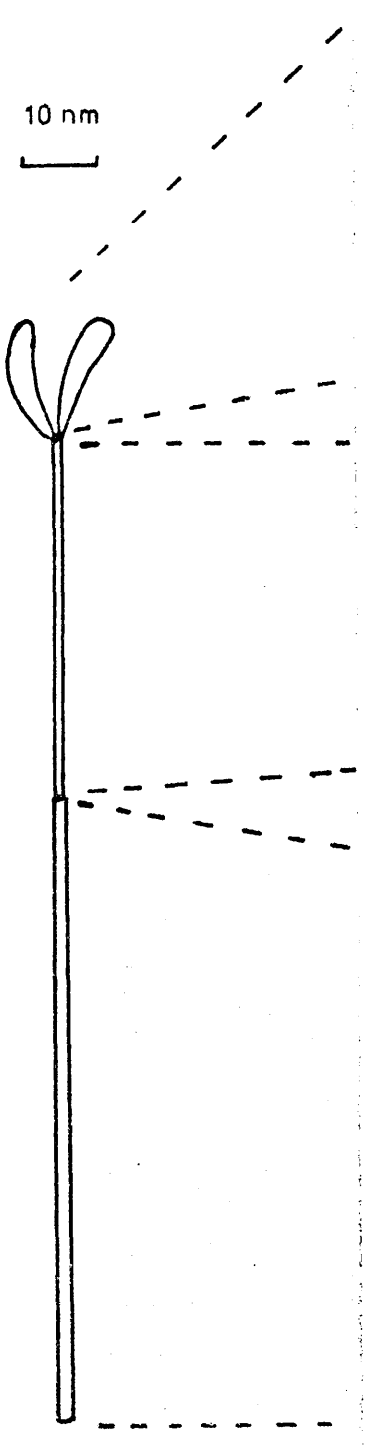
The boundary of the unit cell.

Myosin filament; the numbers refer to the level at which the bridges project. Those labeled '2' are 14.3 nm above those labeled '1', etc.

Actin filament; note those filaments shown with and without dots have different arrangements of bridges around them.

Fig. 1.2

The myosin molecule



Heavy meromyosin subfragment 1.
The head region of the molecule.
This is the site of the ATPase
and of the light polypeptide chains.
Molecular weight 120,000 (each head).
15 x 4.5 x 3 nm.

Flexible region.

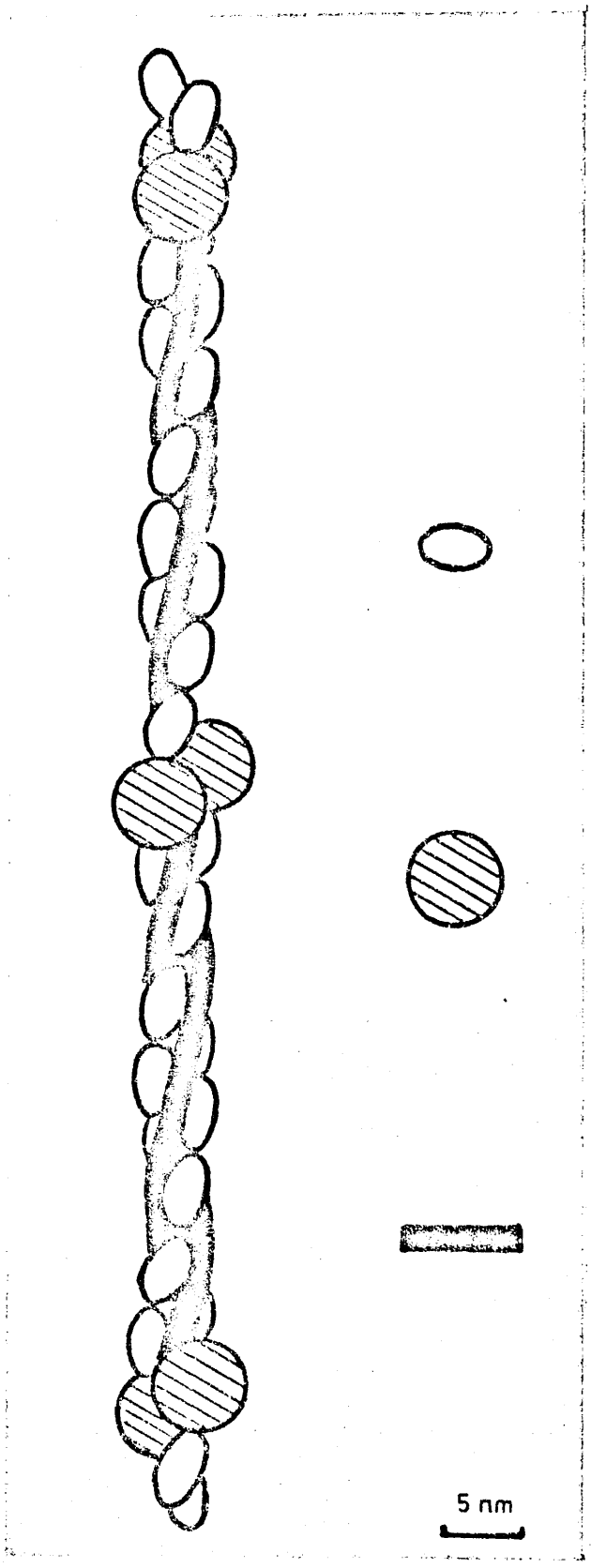
Heavy meromyosin subfragment 2.
Molecular weight 60,000.
47.5 x 1.8 (approx) nm.

Flexible 'hinge' region.

Light meromyosin.
The tail region.
This is the section of the
molecule which polymerizes to
form the backbone of the thick
filament.
Molecular weight 150,000.
84.5 x 2 nm.

Fig. 1.3

The thin filament



(Tice and Barrnett, 1962; Tice and Smith, 1965). There is overwhelming evidence *in vitro* for direct contact and for the ATPase (Moore *et al*, 1970; Engelhardt and Ljubimova, 1939).

On contraction the cross bridges move radially and azimuthally (with respect to the thick filament) and the motion is accompanied by a loss of regularity in their arrangement (Haselgrove and Huxley, 1973; Huxley and Brown, 1967). The latter authors did not exclude the possibility of a slight structural change in the thin filament on contraction. Such a change was reported by Vibert *et al*, (1972), who found that the change related to the stimulation of muscle rather than to its contraction.

G-actin

Under conditions of low ionic strength actin exists in the monomeric form and this is known as G-actin. It is a globular protein (single polypeptide chain) of molecular weight 43,000 (Akakibara and Yagi, 1970). The amino acid sequence of rabbit actin has been determined (Elzinger and Collins, 1972). Various actins vary little in their amino acid composition and physical properties (Carsten, 1965; Carsten and Katz, 1964) and contain the rare amino acid 3-methylhistidine (Johnson,1967). Myosin also contains this acid.

Each actin monomer binds one molecule of ATP and one divalent cation (Tsuboi, 1968). These are both freely exchangeable with the medium (Barany and Chrambach, 1962; Barany *et al*, 1962; Drabikowski and Strzelecka-Golaszewska, 1963). These authors found that the 'natural' bound cation was calcium, although Strzelecka-Golaszewska (1973) found that the affinity constant of actin for magnesium was much higher than previously reported.

The affinity constant for ATP has been measured by West (1971) as about 3×10^8 M^{-1}. This work also suggests that (although nucleotide and cation exchange separately) in the ternary complex there is interaction between the cation and the β- and γ-phosphate groups of ATP.

In addition to the tight cation-binding site of actin there are about seven weak binding sites (Martonosi et al, 1964).

The polymerization of actin

At moderate ionic strength (typically KCl 50 mM, $MgCl_2$ 2 mM) G-actin polymerizes to fibrous or F-actin. The polymerization proceeds with hydrolysis of the bound ATP, and the resulting ADP is the bound nucleotide of F-actin. The polymer does not have a well defined length (Arisaka et al, 1973), but in other respects it resembles the thin filament. If the ionic strength is lowered F-actin will depolymerize exchanging its bound ADP for ATP from the medium. If no ATP is available depolymerization still occurs and G-actin with bound ADP is formed (Gruhhoffer and Weber, 1961). However, the affinity of G-actin for ADP is only about 1.6×10^{-2} of that for ATP (Iyengar and Weber, 1964) and normally the nucleotide is lost and denaturation of the protein occurs. (Nucleotide-free G-actin can be prepared and is stable in 50 % sucrose (Kasai et al, 1965).)

Feuer et al. (1948) discovered that there were two distinct ionic effects on the polymerization of actin. Firstly, there was a non-specific effect due to monovalent cations; no differences were observed between the effects of Na^+ and K^+ when actin was polymerized in the presence of these ions. This non-specific effect is probably due to Debye-Hückel screening of

the electrostatic charge of the protein rather than any special property of monovalent ions. Secondly, the authors found that, in the presence of 100 mM KCl, the polymerization was accelerated by 10 mM $MgCl_2$ and retarded by 3 mM $CaCl_2$.

G- and F-actin can co-exist in a dynamic equilibrium (Kasai et al, 1962). Under any given conditions there is a critical concentration of G-actin. If the actual concentration of G-actin is below this critical value then there can be no F-actin. In the presence of F-actin, G-actin will exist at the critical concentration. The polymerization process is analagous to a phase change. The authors also found that the equilibrium could be shifted to favour the F- form by increasing the monovalent ion concentration, the magnesium concentration or the temperature or by decreasing the pH. The G- form could be favoured by increasing the calcium concentration (in the presence of monovalent ions). Neither divalent ion, however, had a large effect at high temperatures or at low pH.

The rate limiting step of the polymerization process is the formation of a nucleus of three or four monomers (Kasai et al, 1962). These authors also found that, for actin concentrations above the critical limit, the initial rate of polymerization $v = [k \text{ G-actin}]^n$. Where $3 < n < 4$ and the constant k was dependent on the ionic conditions:-

$k \propto (1 + 1.5 [Ca^{++}] / [Mg^{++}])^{-3.5}$.

However, Kasai (1969) found that neither the nature of the ions present, their concentration nor the pH affected the activation enthalpies of the nucleation or growth reactions of the polymer (both about 20 Kcal/mole).

Martonosi et al. (1964) found that the polymerization rate was half maximal when half the weak binding sites for divalent

cations were occupied.

The polymerization of actin is highly pH-dependent (Tsuboi et al, 1965). At pH 9 actin is virtually unpolymerizable, whereas at low pH it can be polymerized by the addition of acid alone. Interestingly, at pH 5.5 the bound nucleotide can be hydrolysed before polymerization occurs, but the resulting polymer has an abnormally high viscosity.

Most actins behave similarly in their response to their ionic environment, but an actin-like protein extracted from the slime mould plasmodium will polymerize to an F-actin-like structure in the presence of monovalent ions alone and to a globular structure if divalent ions are added (Hatano et al, 1967).

Although polymerization of actin is accompanied by hydrolysis of the bound ATP, this hydrolysis is not necessary for polymerization; G-actins prepared with other nucleotides, or even nucleotide-free actins, will polymerize (Kasai et al, 1965; Iyengar and Weber, 1964; Cooke and Murdoch, 1973). Nor is the nucleotide absolutely essential for the structure or stability of the polymer (Barany et al, 1966; Cooke and Murdoch, 1973).

The ADP and tightly bound cation of F-actin can only be removed with difficulty (Barany et al, 1966). In addition to the tight binding site for ADP, there seems to be a weak binding site for ATP (Suzuki et al, 1973).

Is the polymerization of actin linked to the contraction process ?

The hydrolysis of ATP is known to provide the immediate energy supply for muscular contraction. As polymerization and depolymerization of actin would result in the hydrolysis of ATP, it is tempting to speculate that this process (or a similar

one) might take place in contracting muscle. Straub and Feuer (1950) and Mommaerts (1951,1952) pointed out that if one ATP were hydrolysed for each actin in a muscle, then the energy released would be that of a single twitch. For convenience this calculation is repeated here.

Using the figures of Potter (1974) together with the dimensions of the filaments, the concentration of actin is 5.5×10^{-7} moles (monomer) / ml of myofibril. This figure is in agreement with the concentration of bound ADP in muscle. Taking the overall reaction to be phosphocreatine → creatine + phosphate and the free energy of this reaction (under physiological conditions) as 10 Kcal / mole, then the energy released would be 5.5×10^{-3} cal / ml of myofibril or about 4.5 mcal / g of muscle. Carlson et al (1963) give the energy output of frog sartorius muscle as 2.6 mcal / g (unloaded), 4.8 mcal / g (maximum) and 3.3 mcal / g (isometric) for a single twitch.

Oosawa et al.(1961) and Asakura et al.(1963) advanced a theory of muscular contraction based on the concept of an interrupted polymer of actin, which they called f-actin. This polymer was thought to be longer than F-actin (for the same number of monomers) and to be capable of binding ADP or ATP. The following was the proposed cycle of events:-

1. Myosin binds to an actin filament, causing the F- → f- transformation and leading to a localised stretch of the thin filament.
2. There is an exchange reaction between f-actin-ADP and free ATP. f-ADP + ATP → f-ATP + ADP
3. There is myosin binding to the other end of the extended section.
4. There is hydrolysis of the bound ATP and reconversion of

the thin filament to its normal form, with a return to its natural length. The first myosin link breaks at this time.

$$\text{f-ATP} \rightarrow \text{F-ADP} + P_i$$

Such a process would result in the relative movement of thin and thick filaments by the difference between the lengths of the extended and unextended thin filament.

A similar process had been amongst those postulated by Hanson and Huxley (1955). This theory, however, was not so detailed and involved neither the depolymerization of actin nor the change of polymer form.

More recently Laki (1971) has proposed another mechanism which depends on a length change in the thin filaments. In this model the thin filaments are likened to flagella and the z-disc to the basal body. The individual thin filaments are able to contract by up to a few percent, utilizing the hydrolysis of ATP and a linked conformational change in actin. Those thin filaments which are in a fovourable orientation to myosin are able to pull it toward the z-disc (but not push). It is also postulated that the conformational change of actin is co-operative, and capable of propagating down the filament after initiation in the z-disc.

If a polymerization-depolymerization cycle were to occur in muscle there would probably be an exchange of bound and free nucleotide on contraction; there is no evidence for the direct phosphorylation of ADP while it is bound to actin. There would also be exchange if the Oosawa model were correct and an exchange is likely under the Laki hypothesis with its conformational change.

The evidence for or against such an exchange reaction *in vivo* is sparse (see below), but under certain conditions

in vitro such a reaction has been observed.

F-actin as an ATPase

F-actin is not normally considered to be an ATPase, but under some conditions it will catalyse the hydrolysis of ATP.

At low concentrations of magnesium it is possible to achieve roughly equal concentrations of G- and F-actin in dynamic equilibrium. ATP is hydrolysed by a cyclic polymerization-depolymerization process (Asakura and Oosawa, 1960).

At a pH of 4.7 (the isoelectric point), actin will hydrolyse many nucleoside triphosphates, and this process is probably associated with the formation of actin paracrystals (Kuroda and Maruyama, 1972).

Asakura (1961) found that if F-actin was sonicated it became an ATPase and that the hydrolytic activity persisted for a short time after the removal of the sonic field. He attributed the phenomenon to the loosening of the rigid F-actin structure under sonication. The transient polymer he called f-actin (see above). However, Nakaoka and Kasai (1969) produced results implying that sonication caused a true depolymerization to G-actin, and they explained the ATPase as the result of a polymerization-depolymerization cycle.

At elevated temperature (50 - 60°C) F-actin is an ATPase (Asai and Tawada, 1966). Again a transient f-actin polymer was postulated. This result is especially interesting as an ATPase is observable at 45°C, a temperature approached by working mammalian muscle.

Ikkai et al.(1966) reported that the transformation of G-

to F-actin was accompanied by a volume increase of 391 ml / Mol (monomer). It would be expected therefore that hydrostatic pressure would favour depolymerization. This effect has been recorded (Ikkai and Ooi, 1966), but the authors also found other interesting behaviour. At pressures in the range 500 - 1500 Kg / cm^2 the bound calcium of F-actin became exchangeable, and the actin depolymerized at 1500 - 3500 Kg / cm^2 and denatured at pressures above 3500 Kg / cm^2. An ATPase activity was observed at pressures between 500 and 3000 Kg / cm^2. At the low end of this pressure range there was no evidence of depolmerization of the actin.

Nakaoka (1972) found that if salyrgan (mersalyl, neptal) were allowed to react with -SH groups in G-actin (in molar ratio of 2 or 3 : 1) the resulting protein would polymerize and the resulting polmer was an ATPase. The proceedure gave, however, a mixture of different polymeric forms of actin and the polymer responsible for the ATPase was not identified.

The F-actin used in all the above experimants had been purified by polymerization-depolymerization cycles. Laki (1971) is of the opinion that intact thin filaments might have a very much higher ATPase. He considers that the rate limiting step of the ATPase reaction is the release of phosphate from an active site and that one of the regulatory proteins could labilize the actin-phosphate bond.

Parrish and Mommaerts (1951) have provided some evidence that the rates of polymerization and depolymerization of actin are not always low. They freeze dried solutions of F-actin and dissolved the product in KCl-ATP solutions. The actin depolymerized instantaneously and immediately repolymerized.

Table 1.1

A comparison of the rates of enzymic and non-enzymic ATP splitting by actin.

1) Enzymic or continuous ATP splitting

Method	Turnover number min^{-1}	ATP mM	$MgCl_2$ mM	KCl mM	Temperature °C	pH
low Mg^{++}	0.001	0.5	0.5	0	20	9
low pH	0.01	1.0	0.0	100	45	4.7
sonication	0.22	1.0	0.0	100	20	8.3
high temperature	0.32	0.8	1.0	60	60	8.2
"	0.11	0.8	1.0	60	53	8.2
"	0.03	0.8	1.0	60	43	8.2
"	0.37	5.0	1 mM EDTA	60	53	8.2
salyrgan(3:1)	0.04	1.0	3.0	60	24	7.7
high pressure Kg / cm^2						
2400	0.1	0.5	0.0	60	25	8.1
1000	0.02	0.5	0.0	60	25	8.1

2) Non-enzymic or single-polymerization ATP splitting

Method	Reaction rate min^{-1}	ATP mM	$MgCl_2$ mM	KCl mM	Temperature °C	pH
normal	0.1	—	2.0	60	25	8.2

The references on which this table is based are to be found in the text.

The authors conclusion was that there existed a mechanism controlling the rate of the polymerization-depolymerization reaction and that this mechanism operated *in vivo*.

The rates of the various ATP splitting reactions are given in table 1.1. These rates should be contrasted with those from other systems. Ca^{++} activated myosin has a turnover number of (typically) 1000 min^{-1}; myofibril preparations:- 50 moles ATP / mole actin. min and intact muscle at 0°C:- 200 moles ATP / mole actin. min (calculated on the total energy production of a muscle).

Actin, as normally prepared, is not a good ATPase but it cannot be regarded as inert in this respect. The resting metabolic rate of frog sartorius at 20°C is about 2.5 mcal / g. min (aerobic); if this heat were produced solely by an actin ATPase it would correspond to a turnover number of about 0.3 min^{-1}.

Exchange of the F-actin nucleotide in vitro

Under the conditions in which F-actin is an ATPase there is exchange of the bound nucleotide with ATP of the medium. Martonosi *et al.*(1960) reported that the ADP of F-actin was inexchangable, but Moos *et al.* (1967) state that half the bound nucleotide is slowly exchangeable. Kitagawa *et al.* (1968) found that the nucleotide was exchanged and that the rate of nucleotide release from F-actin was greatly reduced by tropomyosin.

Szent-Györgyi and Prior (1966) found that half the bound ADP was exchangeable during superprecipitation of natural or

synthetic actomyosin gels, superprecipitation being necessary for the exchange. They also found a similar exchange using myofibrils. Moos and Eisenberg (1970) found that the rate of nucleotide exchange was increased by the binding of F-actin to myosin filaments; however, the effect seemed uncorrelated with either ATPase activity or superprecipitation. In addition they found that the bound nucleotide would exchange with free ADP as well as with ATP. Exchange in an actomyosin has also been reported by Moos et al. (1967).

Exchange of the F-actin bound nucleotide in vivo

Martonosi et al. (1960) treated rabbits, pigeons and rats with $^{32}P_i$ and found that the activity of the actin-bound nucleotide took 24 hours or longer to equilibrate with that of the pool. Exercise had no effect on the incorpoation of $^{32}P_i$ into bound ADP. The authors concluded that there was no (transient) G-actin in muscle. The 'test' animals were forcibly exercised, but muscular activity was not prevented in the control group. Moreover, inorganic phosphate enters muscle cells slowly and this may have contributed to the long apparent equilibration times.

Evidence for nucleotide exchange in vivo is provided by Cheesman et al. (1969); Cheesman and Priston (1970) and Priston (1970). In these experiments the free ATP of muscles was labeled by the injection of ^{14}C-glucose into live frogs, pre-curarized to prevent muscular activity. On electrical stimulation or potassium contracture there appeared to be some exchange of the bound nucleotide. However, the use of ^{14}C-

glucose in whole animals resulted in very low levels of label in the nucleotides and there was much scatter in the experimental results. There were also problems of interpretation arising from test-control asymmetry and from the unknown degree of labelling in the nucleotide precursors.

Purpose of the present study

The purpose of the present study was to investigate nucleotide exchange in resting and stimulated muscle in a system allowing improved accuracy and relatively simple interpretation.

MATERIALS AND METHODS

MATERIALS

Animals

The frogs used were of the species Rana temporaria and Rana pipiens. they were cold-acclimatized and unfed. No attempt was made to ensure a reproducible physiological condition.

Chemicals

Solvents and chemicals were of analytical grade where available. Other solvents were redistilled. (+)-tubocurarine was supplied by Wellcome Ltd. and insulin (80 I.U. / ml) was obtained from The Boots Co. Ltd., Nottingham.

Radioactive chemicals

The following carrier free substances were obtained from the Radiochemical Centre, Amersham:-

D-(U-14C) glucose

D-(1-14C) ribose

(8-14C) adenine

(2-3H) adenine

Enzymes

The following enzymes were obtained from Boehringer:-

1.1.1.27	lactate dehydrogenase (from pig heart)
1.1.1.49	glucose-6-phosphate dehydrogenase (from yeast)
2.7.1.1	hexokinase (from yeast)
2.7.1.40	pyruvate kinase (from rabbit muscle)
2.7.4.3	myokinase (from rabbit muscle)

Firefly lantern extract was supplied by Sigma Ltd..

Coenzymes and nucleotides

NADH and $NADP^+$ were supplied by Sigma Ltd.. British Drug Houses supplied ADP and AMP. The ATP was obtained from Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan. Phospho-enol-pyruvate was supplied by Sigma or Boehringer.

Ringer's solution

This had the following composition:-

NaCl	6.50 g / l	111 mM
KCl	0.14 g / l	1.88 mM
$CaCl_2$	0.15 g / l (anhydrous)	1.35 mM
$NaHCO_3$	0.20 g / l	2.4 mM
(+)-tubocurarine	40 mg / l	

The Ringer's solution was oxygenated with 95 % O_2 - 5 % CO_2

METHODS

Silylation

Glassware was silylated by filling with 5 - 10 % dimethyl-dichlorosilane (DMCS) in toluene. After 2 - 24 hours at room temperature, the DMCS solution was poured off and the tubes were rinsed quickly with toluene, filled with methanol and allowed to stand for at least ½ hour. The glassware was then thoroughly rinsed with distilled water.

Preparation of the chromatography papers

Whatman no. 1 papers were washed with 0.2 % EDTA (pH 8.5 with NaOH) and rinsed twice with distilled water and dried in

cold air before use.

PEI (polyethyleneimine) ion-exchange paper was produced by the method of Randerath (1963), modified to give a high-capacity paper.

Polymin P (a solution of polyethyleneimine, about 50 %) was treated with 3 volumes of water. Concentrated hydrochloric acid was added to give a pH of 1 - 2, and the solution made up to 6 volumes with water.

Half sheets of Whatman 3MM (with machine direction marked in pencil) were washed (as with Whatman no. 1 above) and dried. They were dipped into the PEI solution for 10 seconds, drained and dried in cold air. They were then washed twice in distilled water and dried in cold air.

On standing these papers turn brown (presumably by oxidation of PEI). The brown substance, which interferes with the running of the chromatogram, was removed immediately before use; two glass rods were used to clamp a leader strip of 3MM paper to the origin end of the PEI paper (fig. 2.1). The rods then served as support while running the paper as a descending chromatogram with water for 24 hours. The paper was finally dried in cold air. If a brown colouration remained at the edges of the paper it was cut off. During the coating process the weight of the paper increased from about 17.4 mg / cm^2 to 21.0 mg / cm^2, indicating that the concentration of the PEI monomer (exchange capacity) is about 50 µmole / cm^2.

Characteristics of PEI paper

There is some batch to batch variation in the PEI paper but the order of separation is:-

(fast) NAD^+

NADH, $NADP^+$, AMP

NADPH, ADP

(slow) ATP

Compounds separated by a comma run close together, but NADPH and ADP are well separated by Whatman no. 1.

Typical R_f values, obtained by the method on p. 28 , are:-

	1.4 M LiCl	1.9 M LiCl
AMP	0.9	1.0
ADP	0.4	0.7
ATP	0.1	0.3

These values apply to paper run in the open air. In a tank the concentrations of LiCl should be increased slightly to obtain the same separation. The use of a tank also reduces somewhat the streaking of the spots, but the resolution is sufficiently good that streaking is not a problem.

The nucleotides cannot be eluted from the paper with water. 2 M NaCl or 1 M $MgCl_2$ will remove the compounds. For elution in moderate ionic strength, the presence of a divalent cation and polyvalent anion are desirable, with as low a pH as possible. For this work 70 mM (Na^+) phosphate buffer (pH 7.4) containing 10 mM $MgCl_2$ was used, as this medium does not interfere with succeeding steps (occasionally there was precipitation in the scintillation fluid on long standing).

Incubation of muscles

Muscles were incubated in oxygenated Ringer's solution. Fig. 2.3 is a diagram of the bath. In practice twelve baths were used, with their water jackets connected in series.

Fig. 2.1

A leader strip attached to PEI paper for washing, without folding the PEI

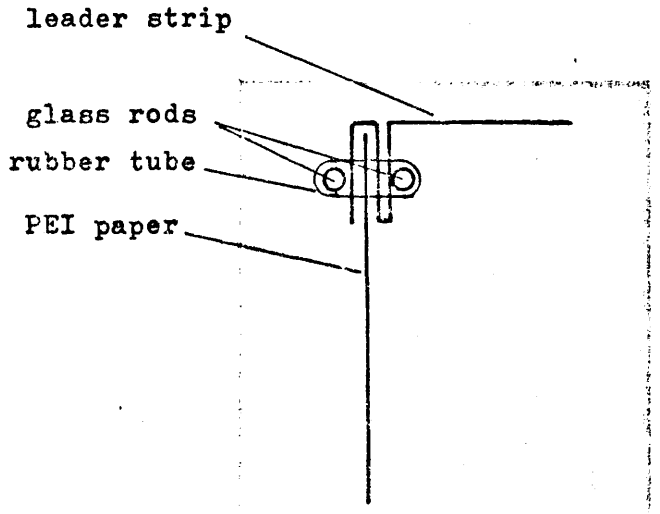
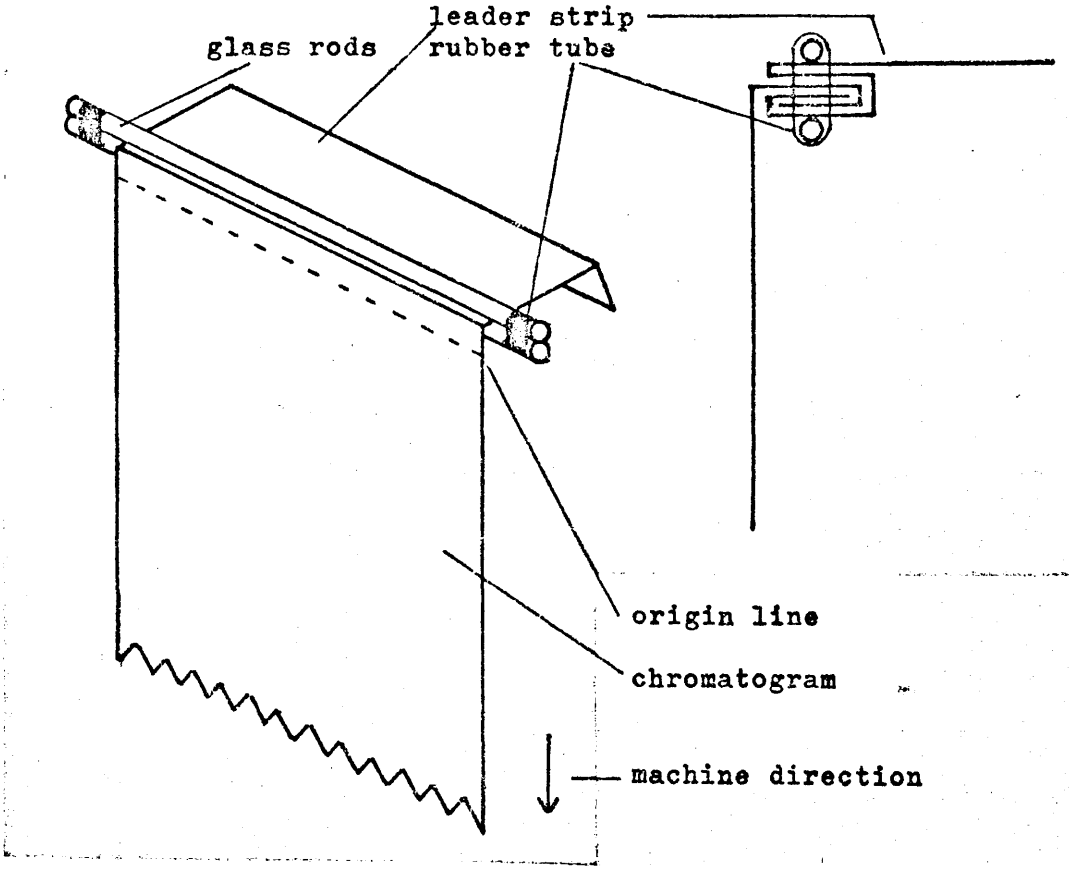


Fig 2.2

A leader strip attached to a chromatogram, allowing a straight solvent front



After equilibration the temperature difference between the first and last bath never exceeded 2°C.

The muscle support is shown in fig. 2.4, and its use during loaded contracture in fig. 2.5. In the experiments with the gastrocnemius, the muscle was removed with its connections to the bones intact. The points on the stainless steel wires were driven down the centres of the bones to support the muscle.

In isometric experiments the muscle was stimulated with the built-in electrodes. In experiments where the muscle shortened, a separate hand-held pair of electrodes was found to be more convenient. These electrodes were platinum wires held about 3 mm apart by a perspex block.

Preparation of nucleotide-containing extracts from muscle

Silylated glassware was used throughout the nucleotide isolation.

Muscles were moistened with cold Ringer's solution in a chilled beaker and finely minced with dissecting scissors. This process was completed within 30 seconds.

1) Total ATP fraction

About 1/8 of the mince was transferred to 1 ml of ice-cold 10 % (w/v) trichloroacetic acid and sonicated for 1 minute. The residue was centrifuged down and the supernatant shaken with about 5 ml of ice-cold ether. The washing was repeated with fresh ether, 10 times in all. The pH of the solution was adjusted to 8.5 - 10 (see Appendix 1) with 0.1 % NaOH and 0.3 ml of 10 % (w/v) barium acetate was added. After standing overnight in the cold, the precipitate was centrifuged down and dissolved in 20 µl of 2 N formic acid for chromatography.

Fig. 2.3

The muscle bath

Approx. 2/3 full size

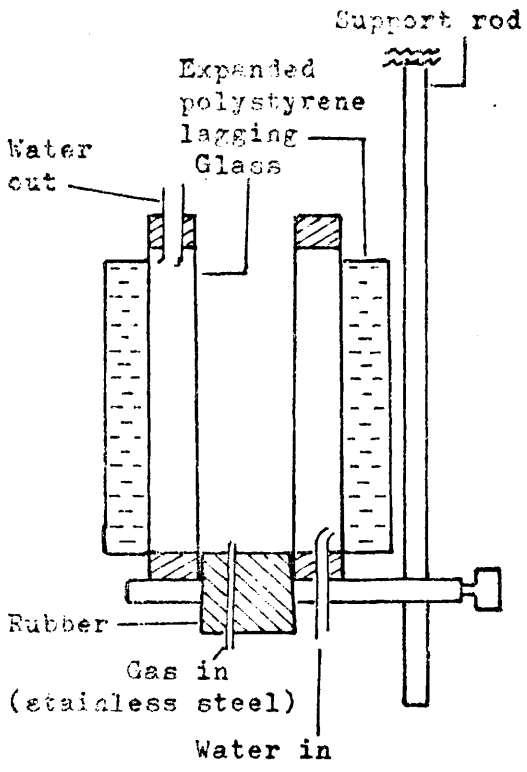
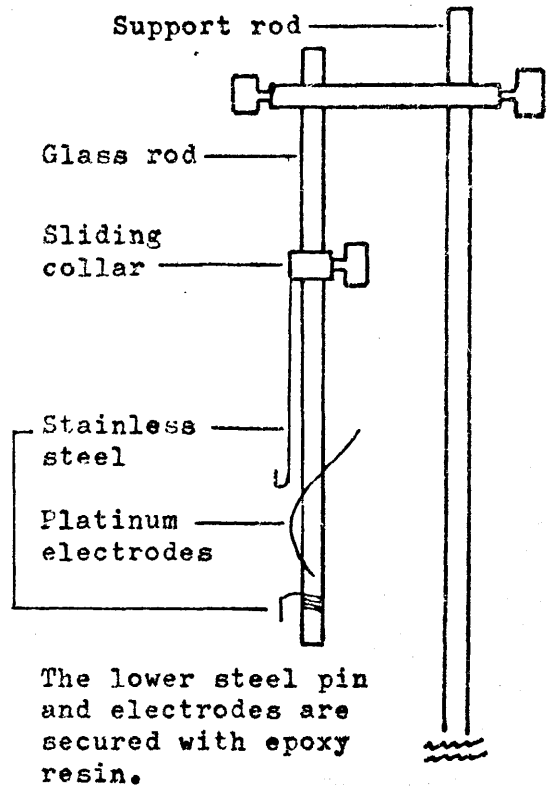


Fig. 2.4

The muscle support

Approx. ½ full size

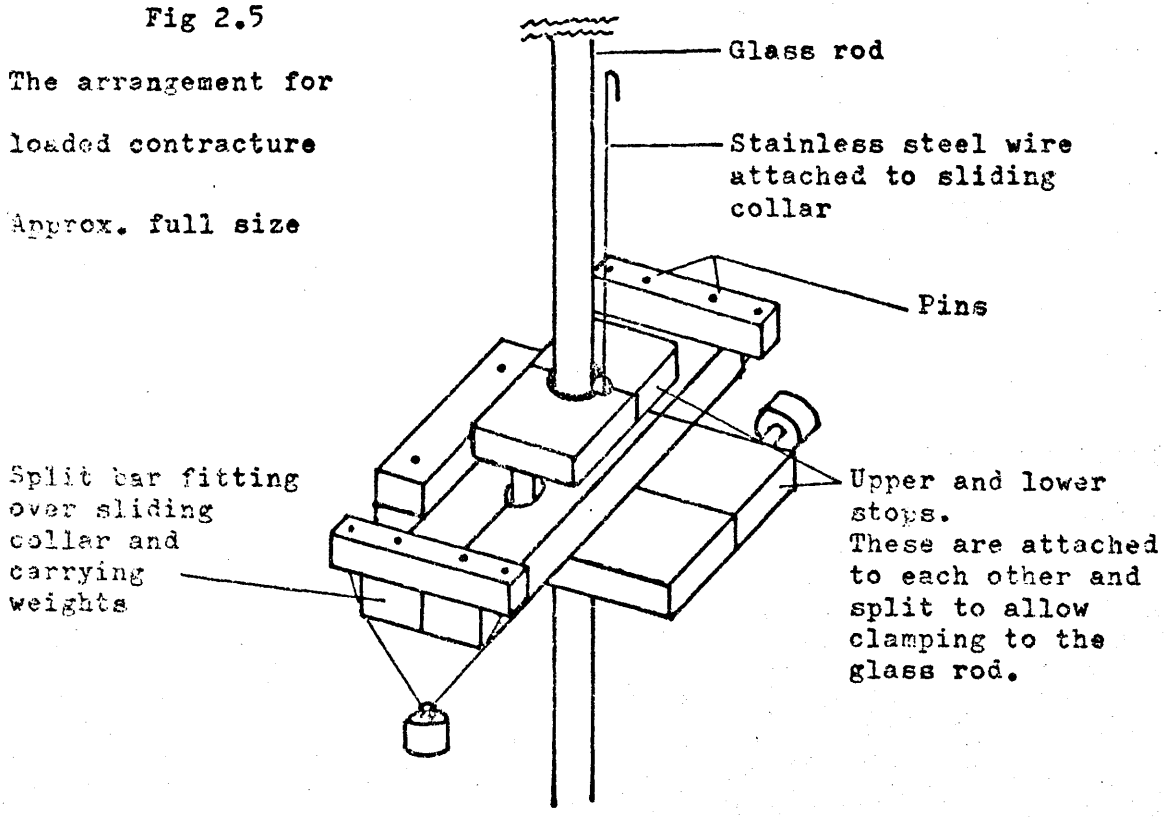


The lower steel pin and electrodes are secured with epoxy resin.

Fig 2.5

The arrangement for loaded contracture

Approx. full size



2) Bound ADP fraction

The rest of the mince was treated with 3 ml of cold 50 mM Na_2CO_3 / 50 mM $NaHCO_3$ and allowed to stand for 30 minutes. It was then washed 10 times with 3 ml portions of ice-cold water, over a period of between 2 and 4 hours. The mince was transferred to 3 ml of cold TCA and sonicated for 1 minute. The residue was centrifuged down and the supernatant washed 10 times with cold ether. The resulting solution was freeze-dried overnight. The dried material was dissolved in 20 µl of water for chromatography.

Chromatography of nucleotides

Nucleotides were initially isolated by the method of Krebs and Hems (1953). A sheet of Whatman no. 1 was folded in half, and an origin line drawn parallel to the fold and 4 cm from it. The nucleotide solutions were applied as spots, and the chromatogram developed (ascending) with isopropyl ether: formic acid (5: 3, v:v). Complete tank saturation is essential at this stage. The nucleotides remained at the origin while glycolytic intermediates migrated.

After 4 hours the paper was dried in cold air and cut about 4 cm above the origin line. The cut end and a fresh sheet of paper were folded together and clamped between two glass rods (fig. 2.2).

The nucleotides were then separated by an overnight descending run with isobutyric acid : 1 N NH_4OH : 0.1 M EDTA (100 : 60 : 1.6, v : v)

The paper was then dried at 60 - 80 °C and washed with ether to remove the last traces of butyric acid. The nucleotide spots were located under UV light, cut out and shaken with

1 ml of water for 1 hour.

The eluates were concentrated by freeze-drying. The residues were taken up in 20 µl of water and further purified by chromatography on PEI paper.

The PEI chromatograms were developed with 1.4 M LiCl for ADP, or with 1.9 M LiCl for ATP. The solvent run was 15 cm ascending in the open air. The papers were dried in warm air and washed with methanol to remove the LiCl. The spots were located under UV light, cut out and eluted with 1 ml of 10 mM $MgCl_2$ - 70 mM (Na^+) phosphate buffer, pH 7.4 (shaking for 1 hour).

Estimation of adenine nucleotides

The technique used for nucleotide estimation varied with the particular solution.

Solutions known to contain one nucleotide only and to be free from interfering substances were estimated photometrically using a molar extinction coefficient of 15.4×10^3 at 260 nm and neutral pH.

1) ATP

Solutions of ATP containing less than an equimolar amount of ADP were estimated by the method of Izzo (undated). This technique was used for all PEI eluates. 20 µl of solution (1 - 40 µM in ATP) was added to reconstituted firefly lantern extract in 20 mM $MgCl_2$ - 50 mM (Na^+) arsenate buffer pH 7.4. The mixture was stirred thoroughly and the light output followed with an Aminco 'Chem Glow' photometer and chart recorder. The emission at 30 seconds after mixing was taken as a measure of ATP concentration. Standards were run on every occasion. For concentrations of ATP below about 10 µM the standard line was

curved (concave upward), but reproducibly so. For ATP concentrations outside the range 1 - 40 µM, the amount of solution added was varied appropriately.

Solutions of ATP containing large amounts of ADP were estimated fluorimetrically, by the method of Estabrook *et al.* (1967). This method was also used exclusively in some of the earlier experiments. It utilises the following reactions:-

$$\text{glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP}$$

$$\text{glucose-6-phosphate} + \text{NADP}^+ \xrightarrow[\text{dehydrogenase}]{\text{glucose-6-phosphate}} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+$$

The fluorescence of the NADPH was followed with a Locarte fluorimeter Mk. 4 and a chart recorder (filters: primary 340 - 380 nm, secondary 440+ nm). The buffer was prepared fresh and prewarmed to the block temperature. It had the following composition :-

Triethanolamine / HCl	50 mM	pH 7.4
KCl	10 mM	
$MgCl_2$	10 mM	
EDTA	5 mM	
glucose	2 mM	
$NADP^+$	0.1 mM	

Dust was removed by millipore filtration.

A suitable amount of ATP solution was made up to 1.5 ml with buffer. 10 µl of glucose-6-phosphate dehydrogenase (0.2 mg / ml) was added to remove any endogenous glucose-6-phosphate. When a stable baseline had been achieved, 10 µl of hexokinase (0.4 mg / ml) was added. The increase in fluorescence

was mainly due to ATP. A second 10 µl of hexokinase was added to measure any fluorescence due to the enzyme. An ATP standard was then added.

This technique is limited by two factors. There is a slow side reaction between glucose and $NADP^+$ mediated by the dehydrogenase. The sensitivity falls off with increasing ATP concentration.

2) ADP

In the absence of ATP, ADP solutions were assayed by incubating 200 µl with 50 µl of myokinase (0.03 mg / ml). 100 µl samples of the mixture were then assayed by the firefly method. ADP standards were incubated concurrently.

In the presence of ATP, ADP was assayed by the method of Estabrook *et al.* (1967). Fluorescence changes were followed as above. The reactions were carried out in a buffer comprising :-

(K^+) phosphate	30 or 50 mM	pH 7.4
KCl	10 mM	
$MgCl_2$	5 mM	
NADH	10 µM	
phospho-enol-pyruvate (PEP)	0.8 mM	

The buffer was prepared fresh, freed from dust by millipore filtration and then warmed to the block temperature.

The reactions are :-

$$\text{ADP} + \text{PEP} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH}^+ \xrightarrow{\text{lactic dehydrogenase}} \text{lactate} + \text{NAD}^+$$

A suitable amount of solution was made up to 1.5 ml with prewarmed buffer and 10 µl of LDH (0.2 mg / ml) added, any fluorescence change being due to pyruvate (either endogenous or formed by decomposition of PEP). 10 µl of pyruvate kinase (2 mg / ml) was added; the decrease in fluorescence was due to ADP. A second 10 µl portion of pyruvate kinase was added to determine enzyme fluorescence. Successive 10 µl portions of ADP standards were then added (usually five in all). A least squares line was fitted to these internal standard readings. The slope of this line (change in fluorescence / unit ADP) was used to determine the unknown.

There seems to be a slow reaction between ADP and PEP in the absence of added pyruvate kinase (possibly due to an impurity in the lactate dehydrogenase). If this reaction is confused with a drifting baseline severe errors can result.

The readings of both fluorimetric techniques were corrected for the dilutions occuring as enzymes and standards were added.

Comparison of nucleotide measurements

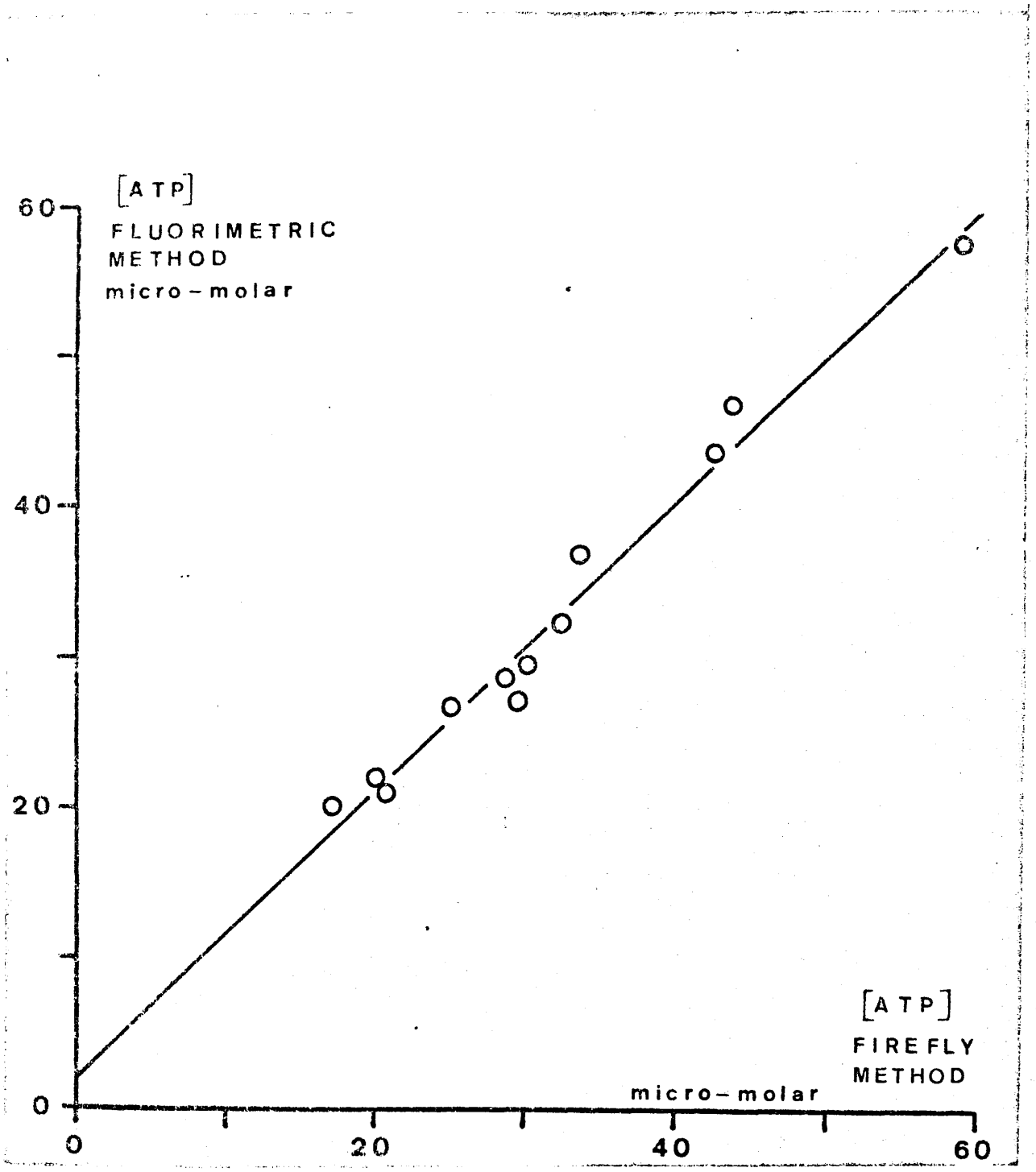
1) ATP

Fig. 2.6 shows the agreement between the firefly and fluorimetric methods of ATP determination. The points are for muscle extracts, not constructed standards.

Analysis by the firefly method was normally performed in duplicate. 138 pairs of readings were statistically analysed to determine the precision of the method. The standard error of the mean (of two duplicate readings) is directly proportional to the ATP concentration, and is 3.4 % of it.

Fig. 2.6

A comparison of the ATP concentrations of muscle extracts as measured by the firefly and fluorimetric techniques.



2) ADP

A solution of ADP will cause light emission from reconstituted firefly lantern extract. However, the time course of the output is different to that of ATP. In particular ADP gives an output which is increasing at 30 seconds after mixing, while that of ATP is falling. The deflection due to ADP at an added concentration of about 50 μM (a relatively high concentration) is only about 2 % of that of an equivalent amount of ATP.

It seemed probable that the ADP was converted to ATP by an impurity in the lantern extract. Fig. 2.7 shows that the deflection at 30 seconds is roughly proportional to the square of the ADP concentration. This indicates that a myokinase is the main impurity, but the curvature of the line suggests that there is also a pseudo-monomolecular phosphorylation observable at low ADP concentrations.

For measurement ADP was converted to ATP by incubation with myokinase at room temperature, for about one hour. The reaction is complete in a much shorter time.

Writing N for the total concentration of nucleotide, and K for the equilibrium constant of myokinase,

$$[ATP] \times [AMP] = K \times [ADP]^2$$

and $$N = [ATP] + [AMP] + [ADP]$$

but $$[ATP] = [AMP]$$

so $$N = 2 \times [ATP] + [ADP] \qquad \text{and} \quad [ATP] = K^{\frac{1}{2}} \times [ADP]$$

giving $$[ATP] = K^{\frac{1}{2}} / (1 + 2 \times K^{\frac{1}{2}}) \times N$$

for $K = 2.26$, $[ATP] = 0.38 \times N$

The ratio of the sensitivities of the technique to ADP and

ATP should therefore be 0.38.

In practice a ratio of 0.285 ± 0.016 was observed. This is probably due to a mass action effect in the luciferase reaction. Product inhibition would not lead to an almost straight standard curve. A typical standard curve is shown in fig. 2.8. It is very similar in shape to that obtained with ATP.

Radioactivity counting

Liquid scintillation counting was employed. The scintillant was that of Patterson and Greene (1965). The detergent Triton X 100 was purified by shaking with 1/10 volume of coarse silica gel for ½ hour and filtration through glass wool. One volume of detergent was added to two volumes of toluene containing 0.4 % (w/v) 2,5-diphenyloxazole (PPO) and 0.01 % 1,4-bis-2-(4-methyl-5-diphenyloxazolyl)-benzene (POPOP).

The scintillation counter used was a Packard 'Tri Carb'. This machine will count up to three radionuclides simultaneously and offers an automatic standardisation facility, which was used to correct for variable quenching.

As the particle energies of the external standard and the β-emitters used were different, the relationship between the standard count and sample count rate was determined experimentally.

Vials were set up containing identical amounts of scintillant and ^{3}H or ^{14}C or both. They also contained varying small amounts of nitromethane. The count rates on the ^{3}H or ^{14}C channels were plotted against the standardisation count (quench curve). Over the range of quenching found in practice, these lines were almost straight (though not through the origin).

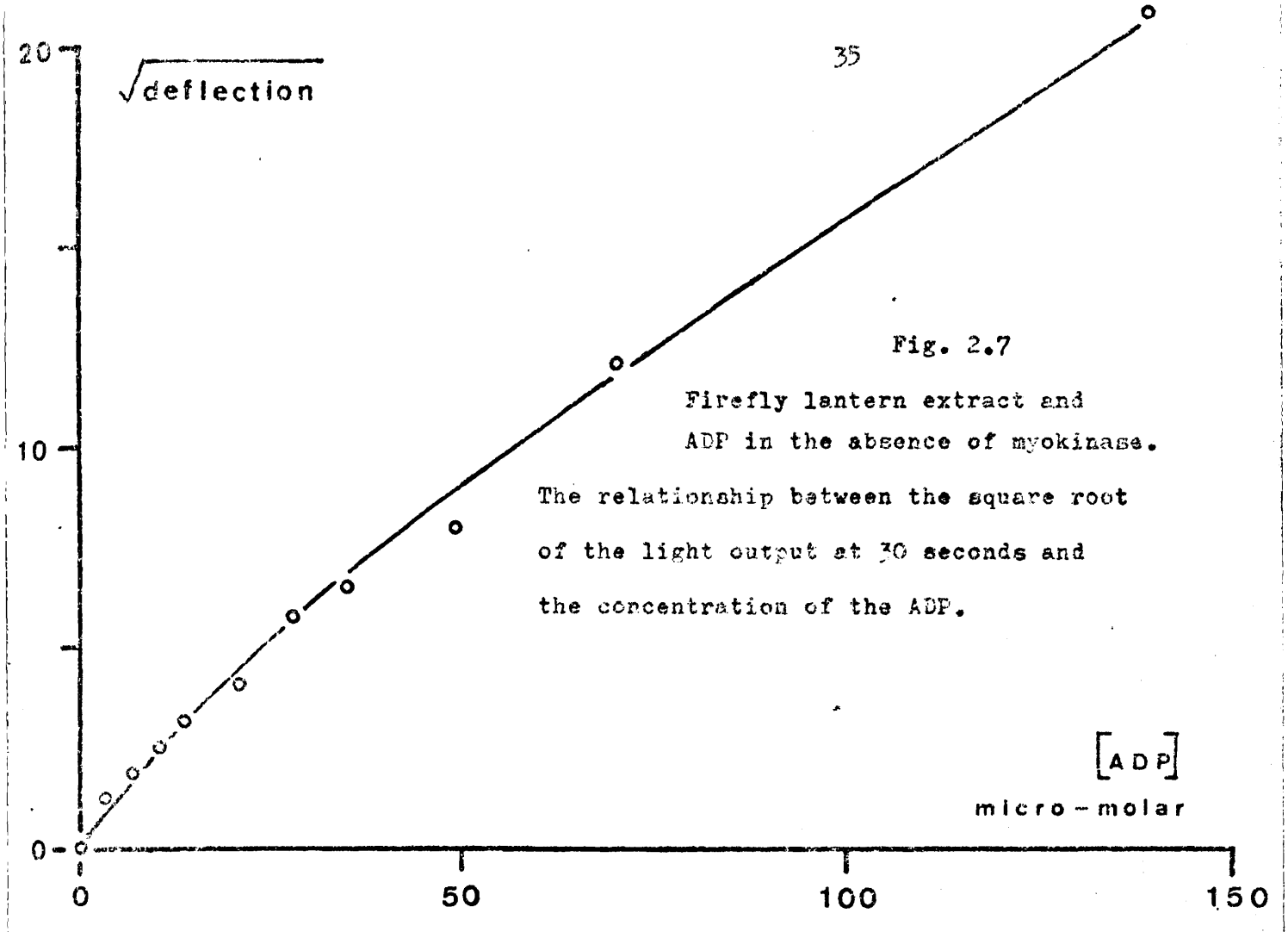


Fig. 2.7

Firefly lantern extract and ADP in the absence of myokinase.

The relationship between the square root of the light output at 30 seconds and the concentration of the ADP.

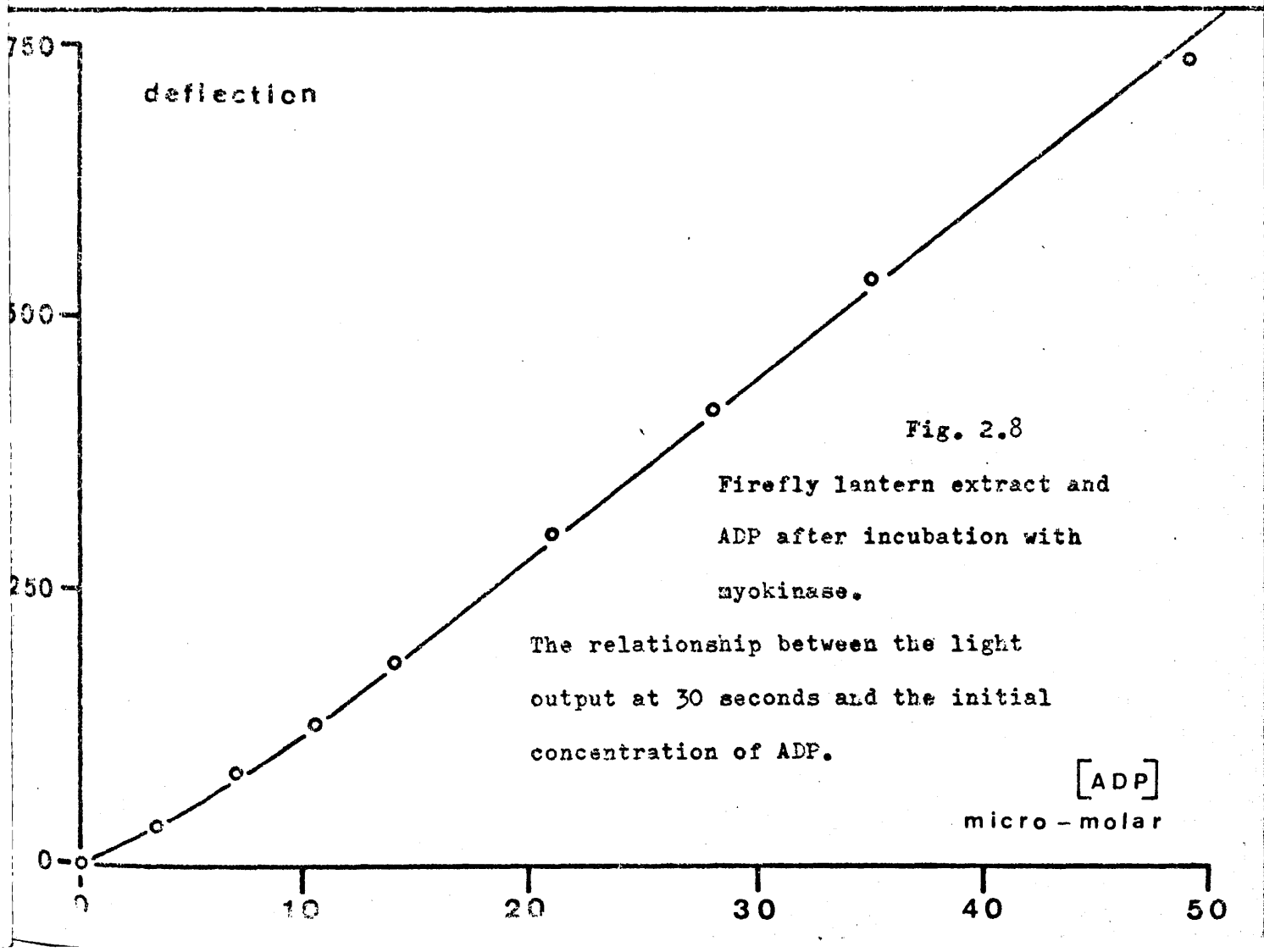


Fig. 2.8

Firefly lantern extract and ADP after incubation with myokinase.

The relationship between the light output at 30 seconds and the initial concentration of ADP.

The method of counting experimental samples was as follows. Vials were set up containing 10 ml of scintillant. These were standardised and counted for (at least) 50 minutes. Any showing a high background were rejected. $\frac{1}{2}$ ml of each radioactive solution was added to the corresponding vial; and to one vial (blank) was added $\frac{1}{2}$ ml of solvent. The vials were allowed to equilibrate at $4^{o}C$ in the dark for at least 4 hours and then restandardised and recounted.

A convenient value of standardisation count, in the middle of the observed range, was selected. All count rates were corrected to this value using the quench curve. The increase in count rate for the blank vial was then subtracted from the increase for each sample vial. This method of calculation was preferred for low count rates, as the increase in rate is found before the large (and possibly slightly inaccurate) correction to 100 % efficiency is made (to the separate readings).

Purification of nucleotides

Small quantities of ATP and ADP for use as standards were purified by PEI chromatography. Larger amounts of ATP were freed from ADP by precipitation from cold aqueous solution with cold ethanol.

Phosphate determination

Phosphate was estimated by the method of Ames (1966).

Protein estimation

A modified Folin-Ciocalteu procedure was used to determine protein (Hartree, 1972). It was standardised (on actin) by

micro-Kjeldahl nitrogen determination.

Titrations

Titrations were performed with a Radiometer 5BR 2C Titrigraph.

Preparation of actin

Acetone powders of muscle were prepared from freshly killed rabbit by the method of Szent-Györgyi (1944). The acetone powders were degreased with chloroform. Actin was prepared from these powders following the method of Spudich and Watt (1971). 0.2 mM ascorbate was used as the protective reducing agent.

RESULTS

PART 1. Experiments on isolated muscle

Preliminary experiments

1) The uptake of adenine

Prieton (1970) injected whole frogs with ^{14}C-glucose. The radioactive label was taken up by the muscle nucleotides, but low specific activities resulted (about 0.2 - 40 counts / nMol .min).

Count rates as low as 3 % above background were measured. It is very difficult to measure such low rates accurately, so a method was needed to introduce more label into the nucleotides.

A radioactive label, (especially in glucose) injected into a whole animal will tend to become distributed throughout the animal. However, if an isolated muscle is superfused with label no such dilution can occur (it is of course possible that the label will not be taken up by the muscle). Accordingly it was decided to search for a label which would enter an isolated muscle and be incorporated into nucleotides with high efficiency.

A label originating in glucose and entering an adenine nucleotide must follow a relatively long pathway. Also a label from glucose can potentially enter many other pathways. Thus radioactive glucose could give rise to relatively unspecific labelling, with adenine nucleotides no more highly labelled than many other compounds.

There is a biosynthetic reaction between adenine and 5-phospho-α-D-ribosylpyrophosphate to form AMP. If this reaction were to be found in frog muscle, it would provide a means of introducing a label into the adenine nucleotides with fewer side products, (assuming adenine will enter a muscle). Once AMP is labeled, the label should spread throughout the free nucleotides via the myokinase and phosphorylation reactions.

An experiment was designed to test adenine as a means of introducing a radioactive label.

The gastrocnemii, sartorii and rectus abdominis were dissected from a freshly killed frog. The rectus was split along the median line. Each of the six muscles was incubated in 5 ml of Ringer's solution containing 10 μCi of 2-^{3}H-adenine (carrier free).

After 1 hour at 4°C one muscle of each pair received an electrical stimulus: 0.5 ms single pulse at 15 V from platinum electrodes about 5 mm apart.

The nucleotides were isolated and their specific activities determined. The results are expressed in table 3.1.

In this experiment the nucleotides were purified by the method of Krebs and Hems (1953) only. The nucleotides were assayed by the fluorimetric method of Estabrook et al. (1967).

The following conclusions can be drawn:

a) The radioactive label is easily incorporated into ATP. A typical gastrocnemius of about 0.3 g might be expected to contain about 1 μMol ATP. If all the radioactive label were incorporated into this nucleotide, a specific activity of about 6000 counts / nMole.min would result (at 25 % counting efficiency). Observed values are about

TABLE 3.1

Specific activities and amounts of nucleotide recovered after incubation of muscles with 10 μCi of 2-^{3}H-adenine for 1 hour at 4°C. The test muscles also received a single electrical stimulus of 15 V for 0.5 ms.

Specific activities, counts / nMol..min

Muscle	Frog	total ATP		bound ADP	
		test	control	test	control
Gastrocnemius	1	570	600	28	28
"	2	740	780	130	60
Sartorius	1	660	680	49	46
"	2	1400	2400	57	52
Rectus	1	1400	2600	100	230
"	2	7100	5000	66	110

Nucleotide recovered, nMol

Muscle	Frog	total ATP		bound ADP	
		test	control	test	control
Gastrocnemius	1	1.5	1.6	4.0	7.1
"	2	12	27	1.4	4.1
Sartorius	1	0.45	0.32	0.85	1.2
"	2	0.64	0.40	2.1	1.2
Rectus	1	1.1	0.70	2.4	1.8
"	2	0.33	2.0	2.4	1.3

10 % of this.

b) The label is incorporated into ADP, this is in accord with the results of Priston (1970) and demonstrates that at least some of the ADP is not irreversibly bound to actin.

c) There is no obvious consistent change in the specific activities on stimulation.

d) Recoveries of ATP were poor and variable. 1/8 of a gastrocnemius should yield about 125 nMol of ATP. Actual recoveries were 1 - 20 % of this.

e) In no case did the specific activity of ADP approach that of ATP. This is in marked disagreement with the results of Priston (1970). A general feature of this present study was that the ADP activity never approached that of ATP. This, together with the radioactive contamination found on nucleotide chromatograms using the method of Krebs and Hems (1953), leads me to think that some of Priston's results may be artifacts. This is likely as she used glucose as a source of radioactive label.

f) Although the results derive from only two animals, it seems that asymmetry between test and control muscles is no worse for the gastrocnemius than for the other muscles. This again is in conflict with the results of Priston (1970). As the gastrocnemius can be dissected out with little chance of damage to its membrane it was used in subsequent experiments. (Damage to the membrane might be expected to affect the rate of uptake of label.) The rectus can also be dissected without damage, but must be split for incubation. The gastrocnemius also provides

more material.

The low recoveries of ATP were intolerable. By carrying pure ATP through the isolation process the losses were traced to the barium precipitation. The conditions of precipitation were changed to ensure good recoveries (see Appendix 1). All subsequent experiments included the improved precipitation step.

2) Electrical stimulation of frog muscle

Priston (1970) found that on stimulation the specific activity of the bound ADP changed to approach that of the ADP pool. Large changes in activity were found with the stimulus of a single twitch. To check this observation the following experiment was performed.

The gastrocnemii were dissected from a freshly killed frog and suspended in 10 ml of Ringer's solution at 4°C. After 10 minutes, 10 µCi 2-^{3}H-adenine were added and the incubation continued for a further hour. One muscle of each pair then received an electrical stimulus (isometric). The nucleotides were extracted and their specific activities determined. Table 3.2 shows the results and table 3.2a a statistical analysis thereof. There seemed to be no statistically significant changes brought about by stimulation.

If the specific activities of the test and control ATPs are plotted against each other (fig. 3.1), the results for the different stimuli fall on the same line. However, the line does not pass through the origin, nor does it have unit gradient. One possible explanation for this phenomenon is as

Table 3.2

The specific activities of the nucleotides of gastrocnemii incubated for one hour with 10 µCi of 2-^{3}H-adenine at 4°C and then electrically stimulated.

Specific activities, count / nMol . min

Stimulus	Frog	Total ATP		Bound ADP	
		test	control	test	control
single	1	554	464	4400	66
pulse	2	424	425	49	44
15 V	3(R.P.)	450	464	56	64
0.5 msec	4	885	526	75	90
"	5	456	327	77	53
"	6	333	357	74	62
"	7	327	387	59	55
"	8	178	212	37	77
"	9	104	194	63	108
"	10	138	165	200	26
pulse	11	320	259	162	131
as above	12	316	228	202	205
repeated	13	385	350	122	150
10 times	14	52	90	44	34
at 5 sec	15	61	126	25	33
intervals	16	112	124	102	102
5 sec	17	144	193	46	18
tetanus	18	111	122	25	66
15 V	19	121	156	28	20
0.5 msec	20	63	122	25	36
pulses	21	35	186	24	84
at 50 / sec	22	132	106	43	43

(R.P.) = Rana pipiens, the other animals were Rana temporaria

Table 3.2a

Analysis of the data given in table 3.2
The results of Student's t test on various functions of the data, ignoring the ADP figures on animals no. 1 and 10.

Function	Stimulus	t [1]	number of animals
$ADP_t - ADP_c$	st	-0.91	8
	tt	0.04	6
	tet	-0.95	6
	all	-1.20	20
$ATP_t - ATP_c$	st	0.78	10
	tt	0.47	6
	tet	-1.91	6
	all	0.25	22
$\frac{ADP_t - ADP_c}{ADP_c}$	st	-0.43	8
	tt	0.16	6
	tet	0.15	6
	all	-0.03	20
$\frac{ATP_t - ATP_c}{ATP_c}$	st	0.22	10
	tt	-0.35	6
	tet	-1.85	6
	all	-1.04	22
$\frac{ADP_t}{ATP_t} - \frac{ADP_c}{ATP_c}$	st	-0.06	8
	tt	0.09	6
	tet	0.09	6
	all	0.03	20

Stimuli:- st single twitch
tt ten twitches
tet tetanus
all all stimuli taken together

ADP_t = The specific activity of ADP in the test muscle, and similarly

Note, none of these functions are significantly different from zero

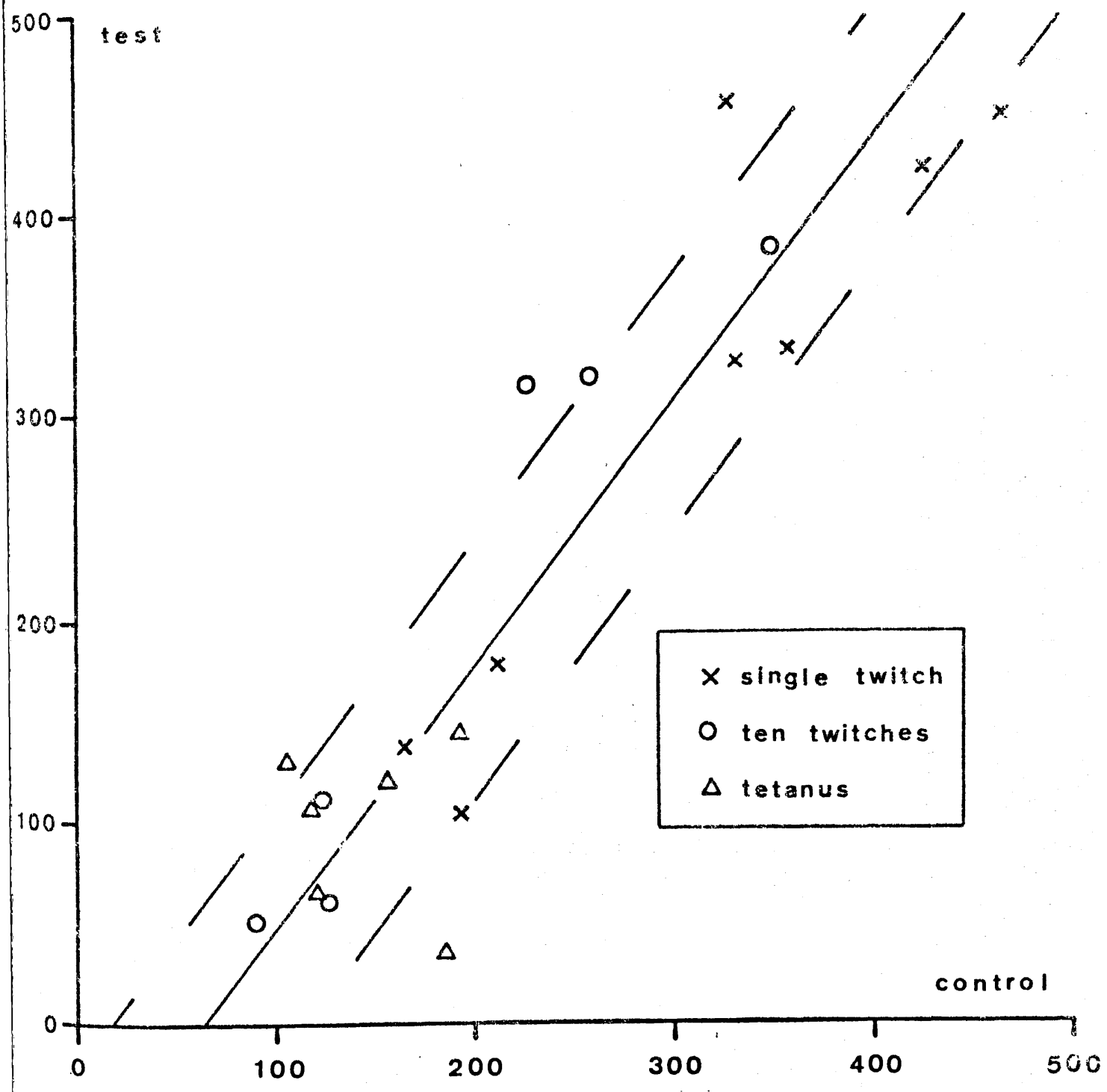
1 The t value has been given a sign to indicate the direction of change

Fig. 3.1

The change in specific activity of the free ATP of gastrocnemii on electrical stimulation. The radioactive label was introduced by incubation of the muscles with 10 μCi of 2-^{3}H-adenine at 4°C for 1 hour.

The units on both axies are counts / nMol . min

The broken lines indicate one standard deviation about the solid line.



follows.

a) Assume that in a muscle stimulated to contract, the metabolic pathways leading to adenine nucleotides are also stimulated.

b) The degree of labelling is dependent on the condition of the muscle. Specifically, a muscle from an animal captive for a long period is depleted of nucleotides. The specific activity in such a muscle will be higher than in that of a freshly caught animal. This effect was in fact observed (see p. 67)

c) Muscle depleted of nucleotides is also depleted of stores of energy.

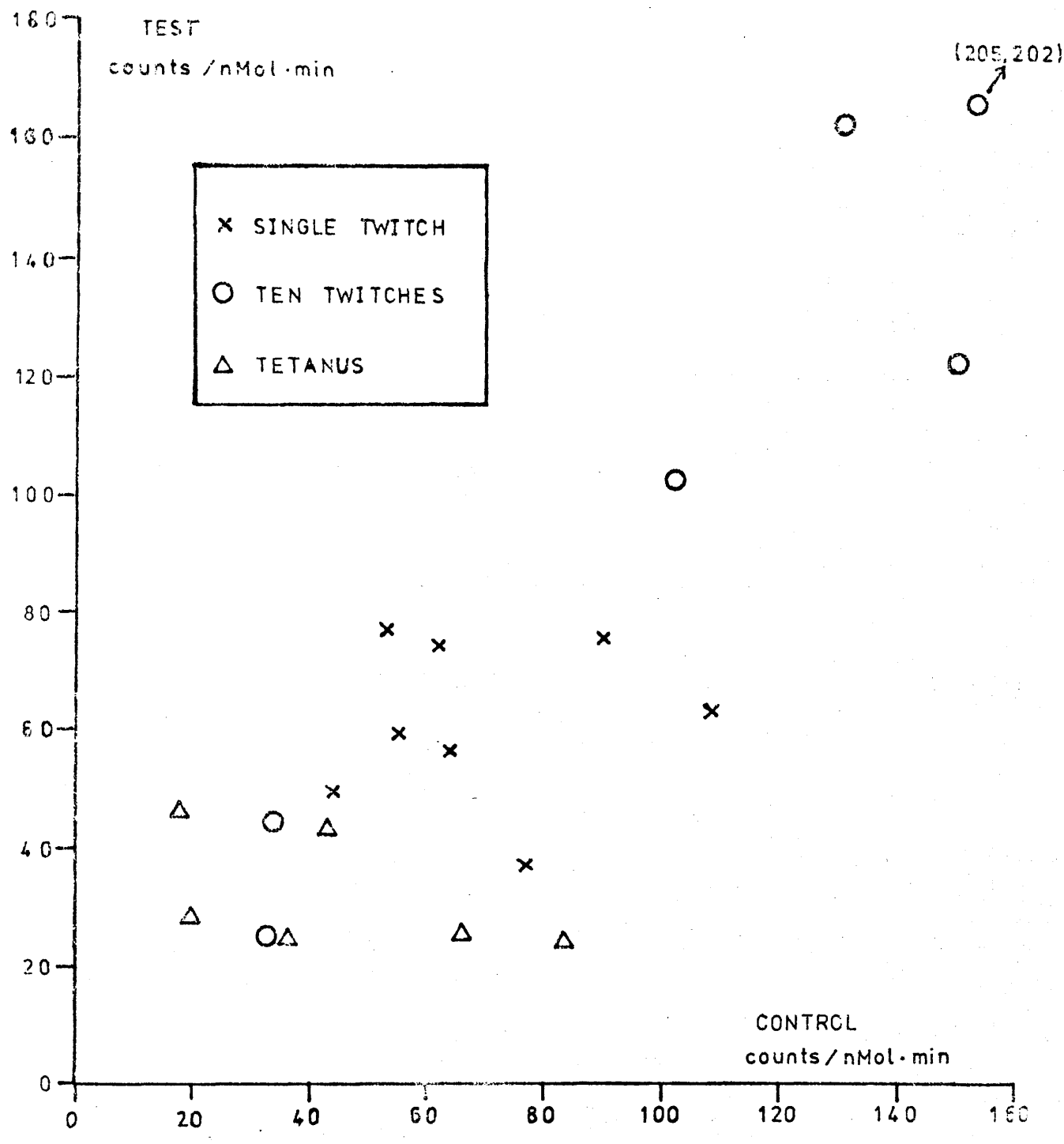
Then on stimulation a 'fresh' (low specific activity) muscle will generate ATP from the normal metabolic pathways (virtually unlabelled), while an 'old' (high specific activity) muscle will rely heavily on the scavenging (highly labelled) pathway.

On the above argument it might be expected that the more intense the stimulus the greater would be the change in labelling. This may well be what is happening (at high specific activities), but the differences are certainly not statistically significant.

A graph of the test specific activities vs. the control activities for ADP (fig. 3.2) shows no tendency to deviate from a unit gradient line through the origin. In a muscle there is roughly five times as much ATP as bound ADP. If a change in the ATP activity were brought about by interaction with ADP, there should be a larger change in ADP activity than in that of ATP. So the effect of stimulation on the specific activity of ATP is not mediated by the bound ADP.

Fig. 3.2

The specific activities of actin-bound ADP with and without electrical stimulation. The label was introduced by incubation of the gastrocnemii with 10 µCi of (2-^{3}H)-adenine at 4°C for one hour. The activities were determined after purification of the nucleotides by the method of Krebs and Hems (1953). Using this purification procedure, no change in specific activity can be observed.



The specific activities of ADP are poorly correlated (correlation coefficient = 0.32) with those of the corresponding ATP (fig. 3.3).

This, together with the presence of freak high readings (nos. 1 and 10) indicated that the ADP might be contaminated with radioactive material.

Refinement of techniques

1) Precipitation of ATP

As already mentioned (p. 42) losses of ATP were traced to the barium precipitation step in the isolation proceedure. The original barium precipitation had been based on the method of Cardini and Leloir (1957).

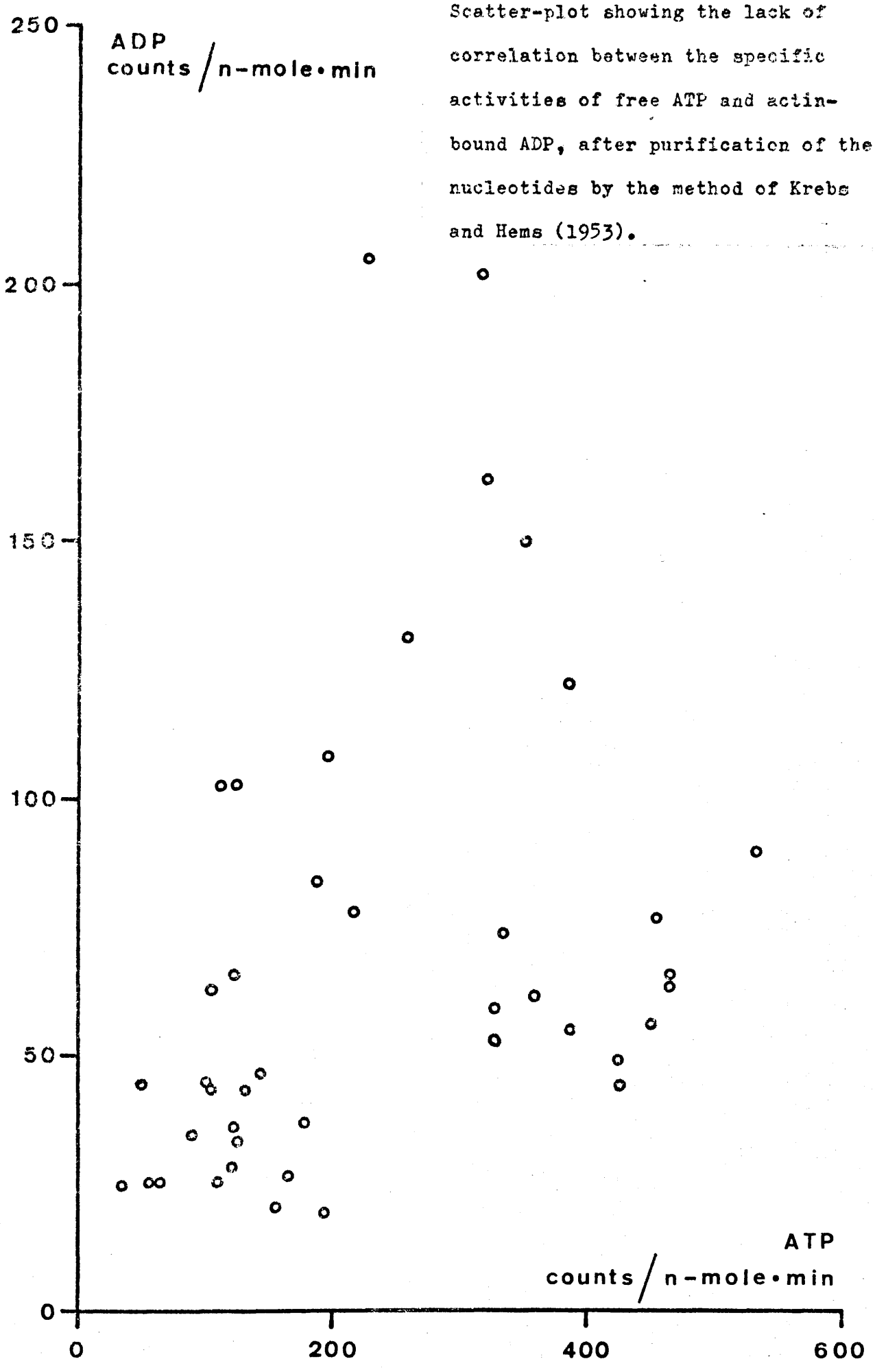
As a consequence of the results given in Appendix 1 this proceedure was modified to give better yields by a) minimizing the amount of divalent cation present (less Ringer's solution) and b) working at a higher pH.

2) Contamination of nucleotides with radioactive material

After descending chromatography by the method of Krebs and Hems (1953), the Whatman no. 1 papers were examined for radioactivity. Each chromatogram was cut into 1 cm^2 sections. Each section was eluted with 1 ml of water and the soluble radioactivity determined. The 3 x 1 cm section containing the visible spot (needed for specific activity determination) was eluted in one piece. From fig. 3.4 it can be seen that

Fig. 3.3

Scatter-plot showing the lack of correlation between the specific activities of free ATP and actin-bound ADP, after purification of the nucleotides by the method of Krebs and Hems (1953).



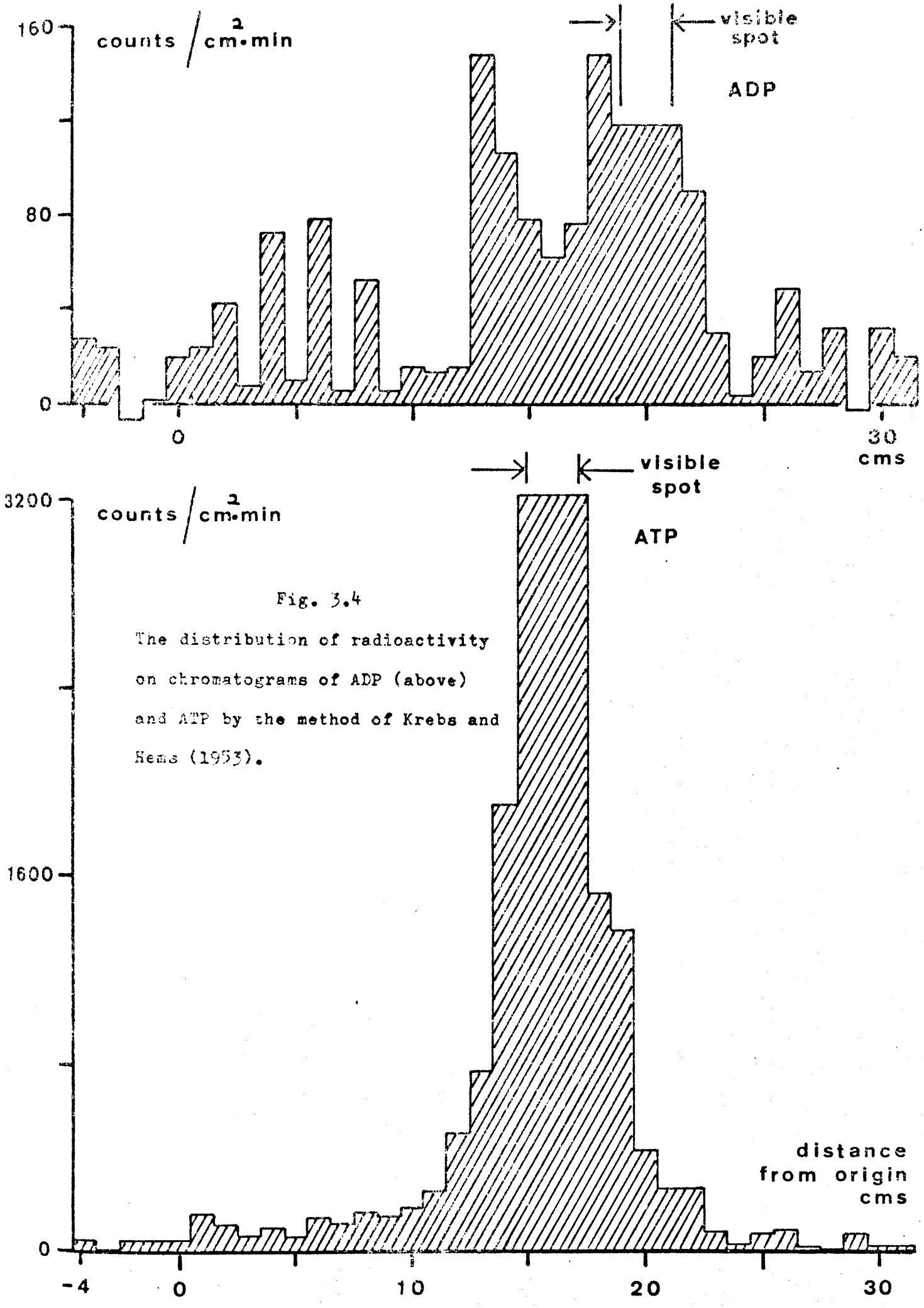


Fig. 3.4

The distribution of radioactivity on chromatograms of ADP (above) and ATP by the method of Krebs and Hems (1953).

there is considerable activity outside the visible nucleotide spot. Also, at least in the case of ADP, there seems to be a radioactive peak or peaks overlapping the visible spot.

The contaminants were thought likely to be adenine-derived nucleotides and dinucleotides. Accordingly a method was sought which gave good resolution of these compounds. Chromatography on PEI paper was the only suitable method of those tried.

Pairs of muscles were incubated in Ringer's solution containing (2-^{3}H-)adenine and (8-^{14}C-)adenine. The nucleotides were extracted and run on Whatman no. 1 paper. One visible spot from each pair was eluted with water, freeze-dried and re-run on PEI paper. Figs. 3.5a and 3.6a show the patterns of radioactivity found on the Whatman no. 1 chromatograms, while 3.5b and 3.6b show the pattern on PEI.

The tritiated ATP and ADP spots appear cleaner on PEI paper. The shapes of the ^{14}C-ATP and the ^{3}H-ATP peaks are similar, but even after PEI chromatography the ^{14}C background is relatively higher than, and may not be the same shape as, the ^{3}H background. The same is probably true of the ADP spots but the ^{14}C-ADP spot is lost in the background.

The ready appearance of the 2 and 8 positions of the adenine molecule in ATP suggest that adenine is incorporated as a whole, while the differences in shape and size of background suggest that adenine is degraded and resynthesised into the compounds which form the background. Carbon 8 is more widely distributed than hydrogen 2.

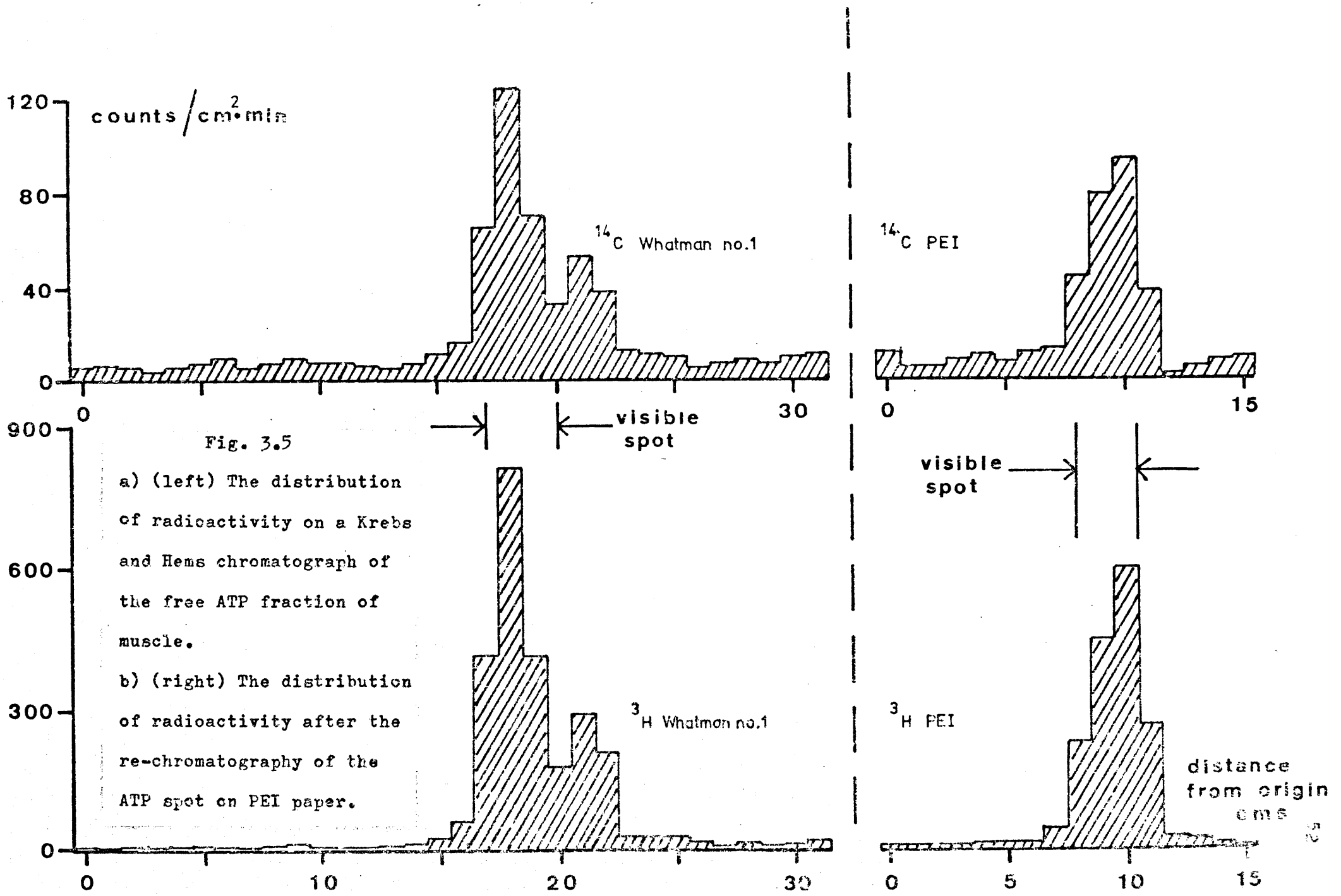


Fig. 3.5

a) (left) The distribution of radioactivity on a Krebs and Hems chromatograph of the free ATP fraction of muscle.

b) (right) The distribution of radioactivity after the re-chromatography of the ATP spot on PEI paper.

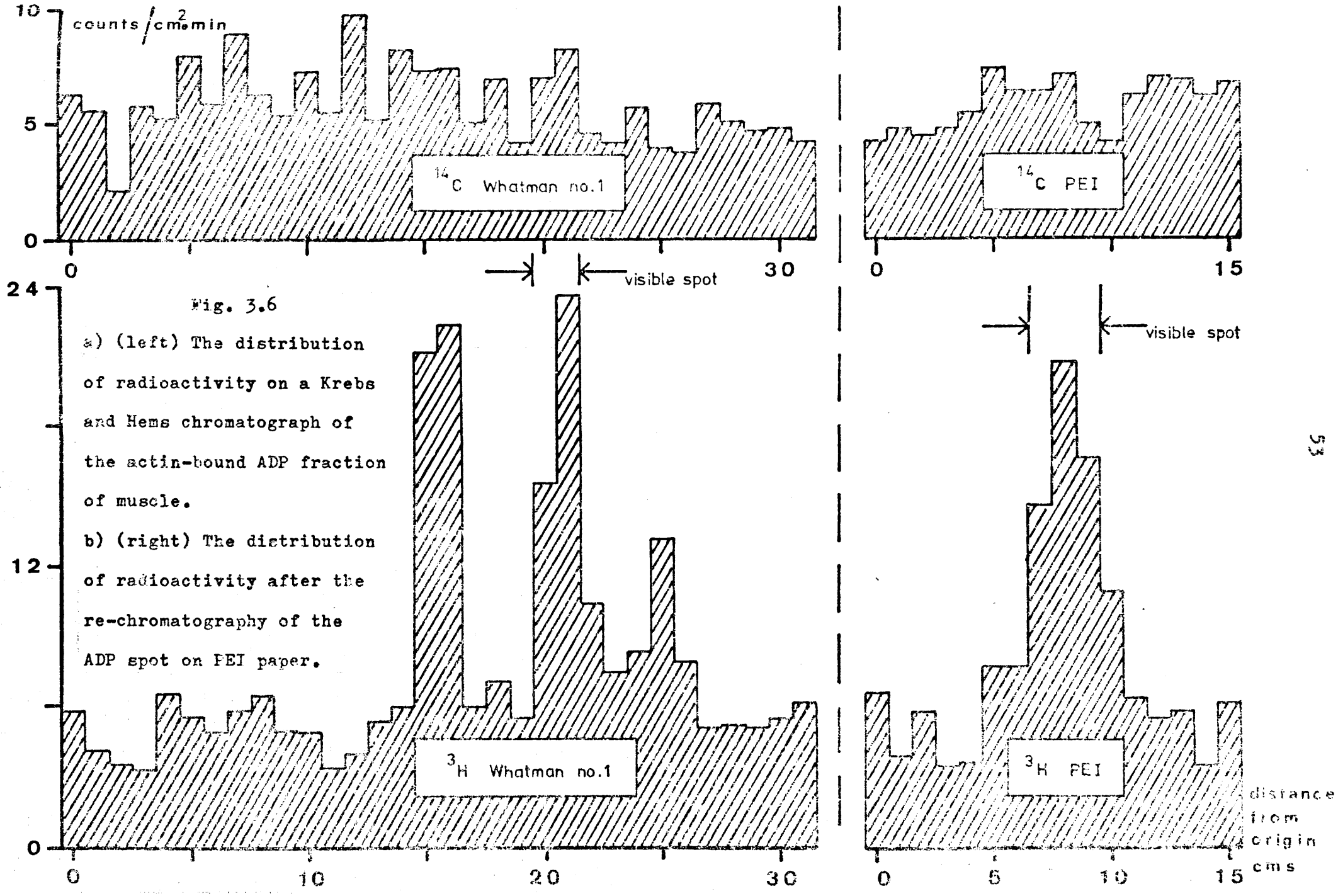


Fig. 3.6

a) (left) The distribution of radioactivity on a Krebs and Hems chromatograph of the actin-bound ADP fraction of muscle.

b) (right) The distribution of radioactivity after the re-chromatography of the ADP spot on PEI paper.

The details of label uptake

1) Introduction

The experimants described in the previous sections established that adenine was a suitable vehicle for introducing a label into the adenine nucleotides. They also tested the purification and assay techniques.

The experiments in this section were designed to investigate factors affecting the uptake of label by the gastrocnemius. A knowledge of these factors is necessary for the confident interpretation of later experiments involving stimulation of muscles.

The following experiments were all of the same general form. The gastrocnemii were dissected from a freshly killed frog and each was suspended in 7 ml of Ringer's solution. After 10 minutes a radioactive label was added and the incubation continued for a further period. The nucleotides were extracted, purified and their specific activities determined.

'Test' and 'control' muscles were always derived from the same animal. Frequently an experiment was designed to investigate two or more points simultaneously. The following results are grouped according to the points illustrated.

2) The uptake of adenine

So far it had been assumed that adenine was incorporated into nucleotides as an intact unit. However, other mechanisms were possible. The adenine molecule might be broken down

and resynthesised as nucleotide. Alternatively, tritium might enter the nucleotide by exchange.

In this experiment the radioactive label was a mixture of (2-^{3}H)-adenine and (8-^{14}C)-adenine (molar ratio 6 : 1). If adenine were incorporated as a unit, the labels should appear in the nucleotides in this ratio. In either alternative case it would be expected that the two labels would appear in the nucleotide at different rates. The rates would also vary from muscle to muscle. They would possibly vary with the duration and temperature of incubation, and perhaps be dependent on stimulation.

ATP The ratio of tritium to ^{14}C counts is in fact independent of time (see table 3.3), and stimulation (see table 3.4). From these tables it can also be seen that the muscle to muscle variation is no larger than the experimental error.

ADP In the case of ADP the results are less clear cut, since, even with fairly extravagant use of ^{14}C adenine, the bound nucleotide count was not much above background. However, some experiments showed a high incorporation of both labels into both nucleotides. Table 3.5 compares the average ^{3}H / ^{14}C ratio in ATP, ADP and the incubation medium in these high-yield experiments. Despite the low count there is fair agreement. All the ratios for ADP are low, but this could be due to a small systematic error rather than a selective mechanism.

The obvious conclusion is that the adenine molecule is incorporated into ~~nucleotide~~ ATP as a single unit. There is no sign of preference for hydrogen over tritium. It is possible that the 2-hydrogen in actin bound ADP may be very slightly exchangeable.

Table 3.3

The count rate ratio (^{3}H / ^{14}C) in ATP isolated from gastrocnemii after incubation with labelled adenine. The label was a mixture of (2-^{3}H)-adenine and (8-^{14}C)-adenine, both carrier free. The incubations were carried out at 2°C.

Duration of incubation minutes	Experiment number 1		2		3		4		5	
5	6.2	± 1.4	6.0	± 0.8						
10	10	7	7.6	0.6	15	± 5				
15	10	7	6.6	0.4	7	2				
25	7.6	1.2	6.3	0.4	8.9	1.0				
40	7.8	0.6	6.8	0.7	8.2	1.0				
60	6.5	0.6	6.5	0.4	6.4	0.8	6.8	± 0.8	6.7	± 0.4
"	6.9	0.5	5.8	0.2	7.7	0.6	6.2	0.3	6.8	0.4
"	6.9	0.4	7.0	0.4	6.9	0.6	6.6	0.5	5.9	0.5
"	7.6	0.5	6.6	0.3	7.8	0.5			7.0	0.5
"	6.9	0.3	7.4	0.4	7.1	0.6			6.5	0.4
"	6.5	0.6	6.6	0.4	7.7	0.8			6.3	0.4
90	6.5	0.3	6.9	0.5	7.3	0.6				
150							6.7	0.3	6.4	0.4
"							6.6	0.3	6.7	0.3
240							6.4	0.3	6.5	0.3
"									7.2	0.4
"									6.6	0.3
Mean	6.95		6.62		7.59		6.61		6.60	
Incubation mixture ratio	7.10		6.70		7.23		6.44		6.67	

The errors are calculated from the square roots of the total counts, and the mean ratios were calculated weighting each figure inversely with its error.

Table 3.4

The count rate ratio (^{3}H / ^{14}C) in ATP isolated from gastrocnemii after incubation with labelled adenine. The label was a mixture of (2-^{3}H)-adenine and (8-^{14}C)-adenine, both carrier free. The incubations were carried out at 2°C for one hour. The test muscle then received an electrical stimulus.

Stimulus	Count rate ratio (^{3}H / ^{14}C)		
	Test muscle	Control muscle	Incubation mixture
single twitch	6.6 ± 0.3	6.3 ± 0.3	6.7
5 sec tetanus	10.0 ± 0.3	10.7 ± 0.3	10.6
"	10.5 0.3	10.1 0.6	"
"	10.5 0.3	10.1 0.6	"
"	10.7 0.3	10.5 0.5	"
"	10.2 0.5	10.1 0.3	"
"	10.6 0.5	10.6 0.4	"
"	10.4 0.2	11.2 0.7	"

Table 3.5

The mean count rate ratio in nucleotides isolated from gastrocnemii after incubation with labelled adenine. The label was a mixture of (2-^{3}H)-adenine and (8-^{14}C)-adenine and the temperature of incubation 2°C.

Experiment number	Number of muscles	Count rate ratio (^{3}H / ^{14}C)		
		ATP	ADP	Incubation mixture
2	12	6.62	5.3	6.70
4	6	6.51	5.4	6.44
5	12	6.60	4.6	6.67
6	12	10.5	8.1	10.6

3.) The uptake of sugars

The label from adenine is incorporated into the base of the nucleotide. Attempts were made to introduce a label into the sugar moiety. The muscles were incubated with D-(U-^{14}C)-glucose or D-(1-^{14}C)-ribose, in the presence of tritiated adenine.

Table 3.6 shows that there was no incorporation of ^{14}C into nucleotide under the experimental conditions. But there was incorporation of tritium showing that the muscles were alive during the incubation.

In the case of ATP an incorporation rate of ^{14}C one-tenth that of ^{3}H would have been detectable.

This negative result can be explained in one of two ways. There may be a permeability barrier to the uptake of sugars, which is not overcome by the presence of insulin. Alternatively, and more probably, the glucose or ribose was taken up by the muscle, the label becoming distributed over a wide range of metabolic intermediates.

4) The time course of adenine uptake

Control muscles were incubated for 1 hour in the presence of 10 µCi of (2-^{3}H)-adenine. The test muscles were incubated for periods ranging from 5 minutes to 4 hours with the same label. The incubation temperature was 2°C.

The results of this experiment are given in Table 3.7. Fig. 3.7 shows the ratio of ATP specific activities in the test and control muscles. The incorporation rate of label into ATP

Table 3.6

The specific activities of ATP and ADP isolated from gastrocnemii after incubation (for one hour) with (2-^{3}H)-adenine and either D-(U-^{14}C)-glucose or D-(1-^{14}C)-ribose.

Incubation mixture					
^{3}H-adenine µCi	10	10	7.2	12.3	100
^{14}C-glucose	1	1	0	0	30
^{14}C-ribose	0	0	0.86	1.5	0
Conditions					
temperature ^{o}C	2	20	14	14	14
insulin IU / ml	0	0	0	0.4	0.4
Mean specific activities					
counts / nMol . min					
ADP ^{3}H	6.9	29	12	9.3	39
ADP ^{14}C	-0.12	-0.20	-0.29	-0.88	0.30
ATP ^{3}H	39	152	86	118	650
ATP ^{14}C	-0.02	0.03	0.11	0.05	0.14

Table 3.7

The time course of tritium incorporation into nucleotides. One gastrocnemius of a pair was incubated for 60 min at 2°C with (2-^{3}H)-adenine. The other member of the pair was incubated for a time t. The following ratios are tabulated :-

ATP_t / ATP_{60} - The specific activity of ATP after time t divided by the specific activity after 60 min.

ADP_t / ADP_{60} - Similarly defined.

ADP_t / ATP_t

Number of experiments	t min	ATP_t / ATP_{60}	ADP_t / ADP_{60}	ADP_t / ATP_t
5	5	0.18	0.35	0.21
6	10	0.23	0.34	0.14
5	15	0.23	0.28	0.18
5	25	0.39	0.53	0.15
5	40	0.63	0.97	0.18
37	60	-	-	0.12
3	90	1.49	1.55	0.12
4	150	1.71	1.37	0.10
4	240	5.32	1.61	0.08

Fig 3.7

The incorporation of (2-^{3}H)-adenine into ATP in the gastrocnemius at 2°C. The broken line is drawn through the origin and the control point (60,1). If the rate of incorporation were constant the points should lie on this line.

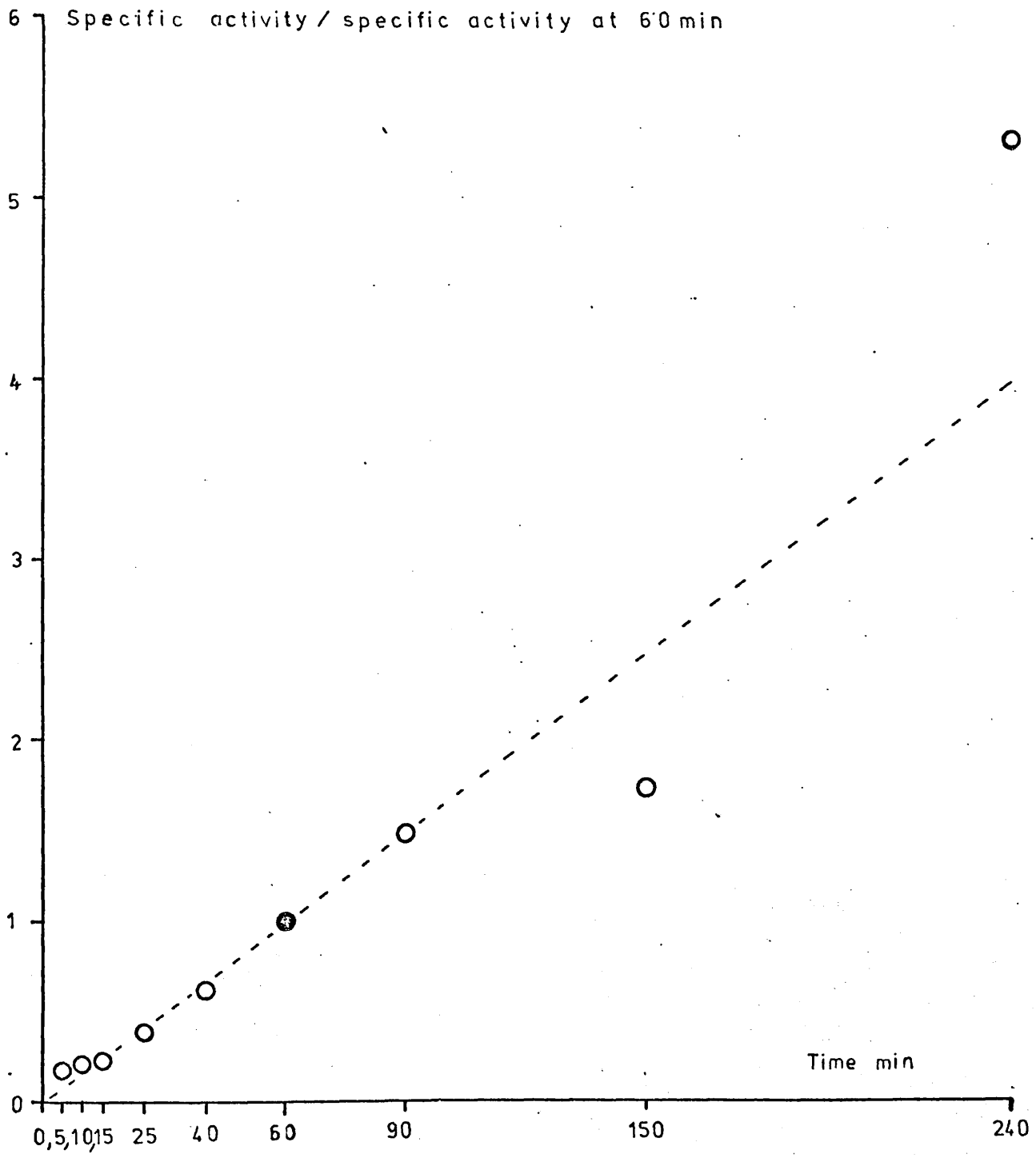


Fig 3.8

The incorporation of (2-^{3}H)-adenine into actin-bound ADP in the gastrocnemius at 2°C. The points for long incubations tend to lie below the line (coresponding to a constant incorporation rate) through the origin and the control point (60,1).

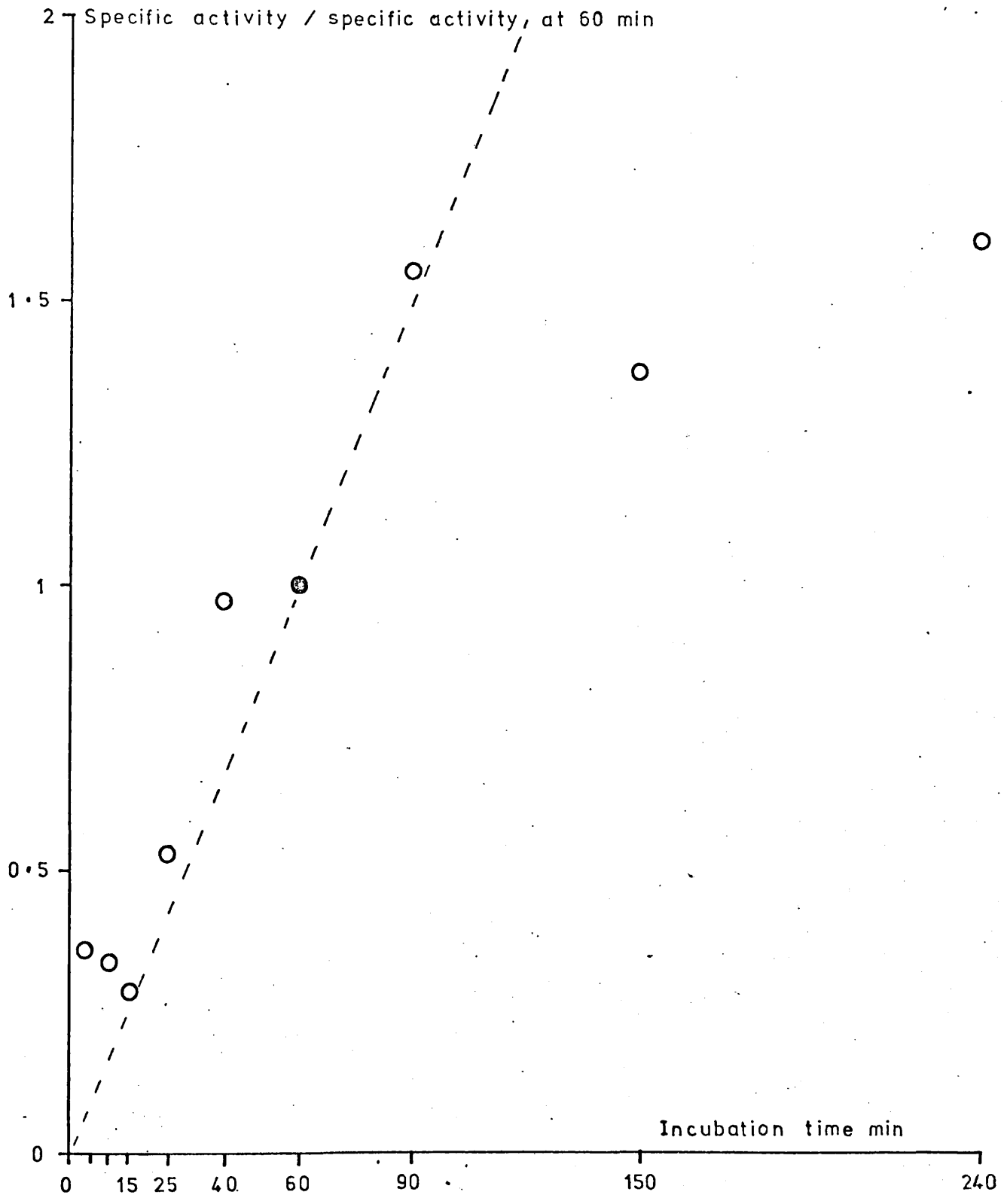
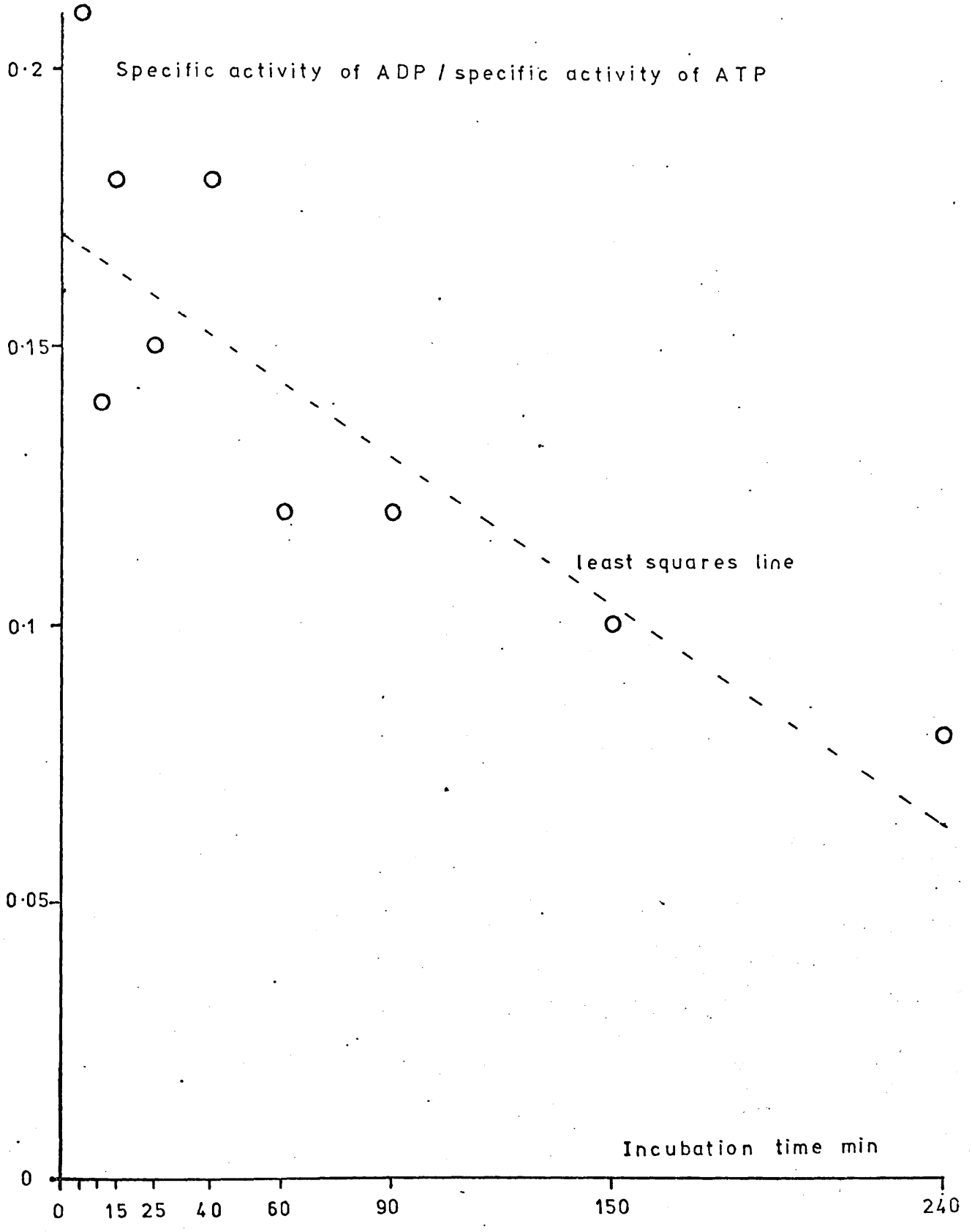


Fig 3.9

The ratio of the specific activities of actin-bound ADP and free ATP as a function of the incubation time. The gastrocnemii were incubated at 2°C. There is a tendency for the specific activity ratio to fall with increasing incubation time.



seems to be constant, giving a specific activity which increases linearly with the duration of incubation. At short incubation times, however, the specific activity seems a little higher than that predicted by this simple model. But, in a muscle as large as the gastrocnemius, the diffusion of adenine can hardly have reached a steady state within 10 minutes.

Fig. 3.8 is a similar graph for the actin-bound ADP. For short incubation times the relationship between specific activity and time is more or less linear. However, after long incubations the specific activity seems to be considerably less than that predicted by a linear model. This is shown more clearly by fig. 3.9 which is a plot of the ratio of the specific activities of ADP and ATP against time. This ratio falls with increasing incubation time (the correlation coefficient is -0.86).[1]

The main points to emerge from this experiment are that the label appears in the ATP pool very rapidly and in the bound ADP with no discernible lag.

Consider a simple model in which there is a fraction f of the bound ADP which is exchangeable with the free ATP pool. Let the specific activity of the labile ADP be a, and the size of the labile ADP pool be q. Let the specific activity of the free ATP be A.

Then the observed specific activity of the total bound ADP will be f x a.

If the exchange rate is n molecules / sec then

$$\frac{da}{dt} = \frac{n'}{q} x\ (A - a) \quad = \sigma\ x\ (A - a)$$

where 1 / σ can be regarded as the time taken to exchange all the labile ADP once.

1. Significant to the 0.5 % level.

If the specific activity of the ATP pool is increasing linearly with time, then

$$\frac{da}{dt} = \sigma \times (K \times t - a)$$

Substituting $Y = K \times t - a - K / \sigma$

$$\frac{dY}{dt} = K - \frac{da}{dt}$$

or $$\frac{dY}{dt} = K - \sigma \times (K \times t - a) = \sigma \times (K / \sigma - K \times t + a)$$

$$\frac{dY}{dt} = - \sigma \times Y$$

so $$Y = Y_o \times e^{-\sigma \times t}$$

or $$a = -(K / \sigma) + (K \times t) - Y_o \times e^{-\sigma \times t}$$

at $t = 0$, $a = 0$ so $Y_o = -K / \sigma$

so $$a = K \times t + (K / \sigma) \times (e^{-\sigma \times t} - 1)$$

$$a = A \times (1 + (e^{-\sigma \times t} - 1) / (\sigma \times t))$$

so for $t \gg 1 / \sigma$ the observed ratio of specific activities ($f \times a / A$) will asymptotically approach f.
Moreover the activity of the ADP will lag behind that of the ATP by a time = $1 / \sigma$

This is almost the exact opposite of what is actually observed, far from the ADP / ATP specific activity ratio rising to a limit it falls with increasing incubation time. Also the time $1 / \sigma$ is so short that it cannot be measured by this experiment.

As the initial specific activity of the ADP is 1/6 that of the ATP, the conclusion must be that 1/6 of the bound ADP is rapidly exchangeable with the nucleotide pool.($1 / \sigma < 5$ min).

Suppose that this exchange was solely with free ATP. Then the heat produced would be the product of the hydrolysis rate and the free energy of hydrolysis of ATP.

Taking the ADP concentration of bound ADP as 600 nMol / g and the free energy as 10 Kcal / Mol,

$$\text{heat rate} = \frac{600}{6} \times 10^{-9} \times \frac{1}{5} \times 10 \text{ Kcal / g.min}$$

(assuming $1 / \sigma = 5$ min)

heat rate = 0.2 mcal / g.min at $2^{\circ}C$

This figure should be regarded as a minimum, and compared to the metabolic rate of resting frog muscle:
2.5 mcal / g.min at $20^{\circ}C$ (in discussion after Weber (1964)).

Bearing in mind the difference in the temperatures, it can be seen that this exchange reaction would account for a large proportion of the resting metabolic rate of the muscle.

However, this heat would not be produced on either of the following assumptions.

a) The bound ADP exchanges with free ADP (free ADP should have the same specific activity as free ATP).

b) 1/6 of the actin bound nucleotide is ATP; and this is hydrolysed during TCA denaturation.

The fall in ADP / ATP specific activity ratio with increasing incubation time is probably best explained as follows; initially 1/6 of the actin-bound nucleotide is freely exchangeable, but, with prolonged incubation, the muscle deteriorates and the exchangeable fraction becomes smaller.

Compartmentalization of nucleotide within the cell is unlikely to explain the fall in ADP / ATP activity ratio. Consider, for instance, a cell containing a cold ATP pool and one whose activity increases with time (so the mean or measured activity increases linearly with time). Then to explain the progressive fall in ADP / ATP activity ratio it would be necessary to postulate that ADP initially exchangeable with the hot pool becomes exchangeable with the cold one as incubation proceeds.

5) The effect of temperature

Incubations were carried out at various temperatures, all for 1 hour. Although the data so gathered lack controls, the effect of temperature on the uptake of adenine is very marked. Fig. 3.10 shows the mean specific activities of nucleotides after incubation. The Q_{10} of the reaction is 1.9 for ATP, 2.2 for ADP.

6) Effect of the state of the animal

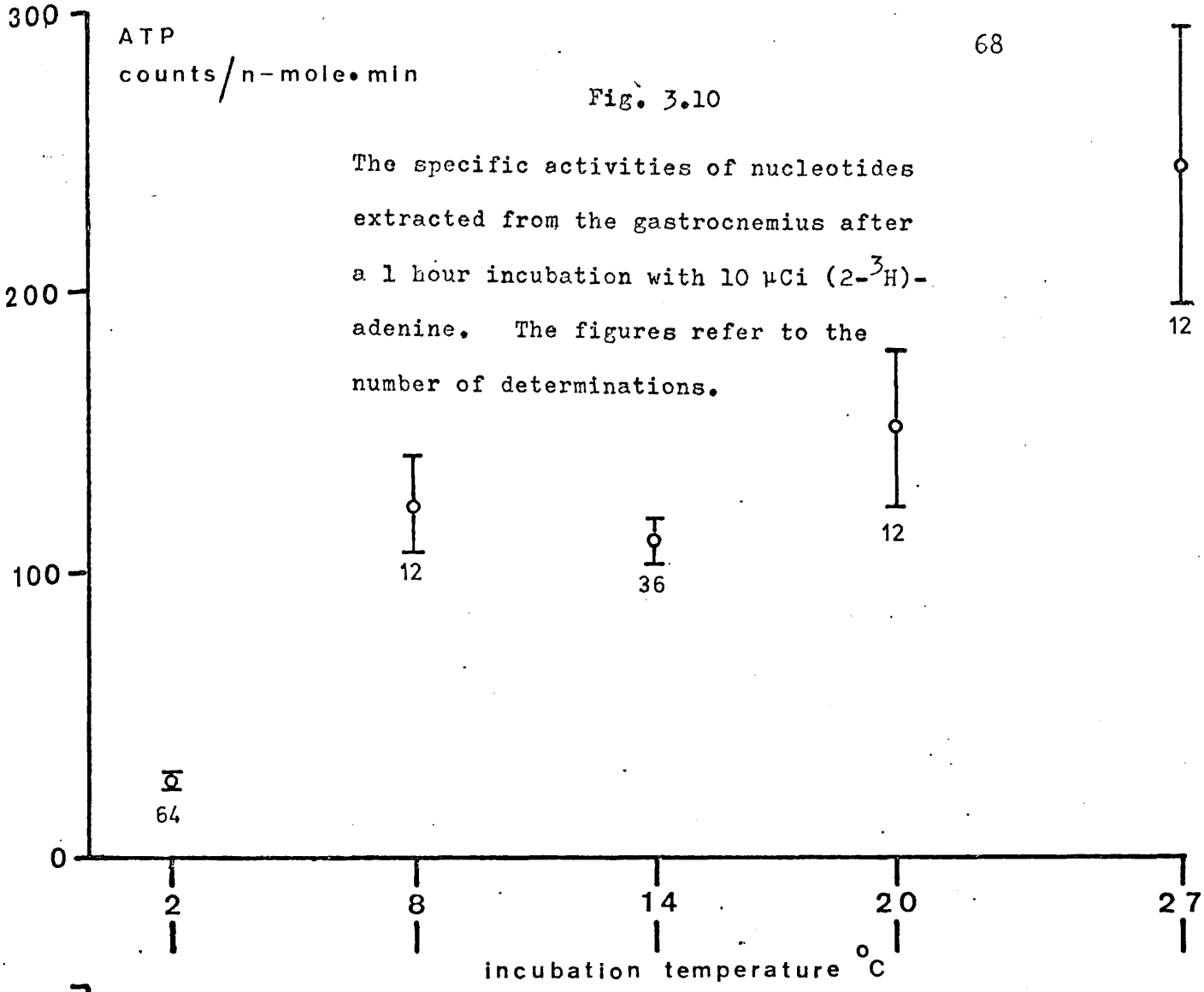
Frogs were delivered in batches, and kept in the cold without food. Fig. 3.11 shows the mean specific activity of ATP over a period of several months; also shown are the delivery dates of the frogs. Experiments performed on fresh frogs give lower values than those performed on animals captive for long periods.

The same effect is seen with the ADP activities.

A partial explanation is that the absolute amounts of nucleotides recovered tended to be less in the case of 'old' frogs, suggesting nucleotide depletion. Thus the same uptake of label would result in higher specific activities in 'old' animals.

Fig. 3.10

The specific activities of nucleotides extracted from the gastrocnemius after a 1 hour incubation with 10 μCi (2-^{3}H)-adenine. The figures refer to the number of determinations.



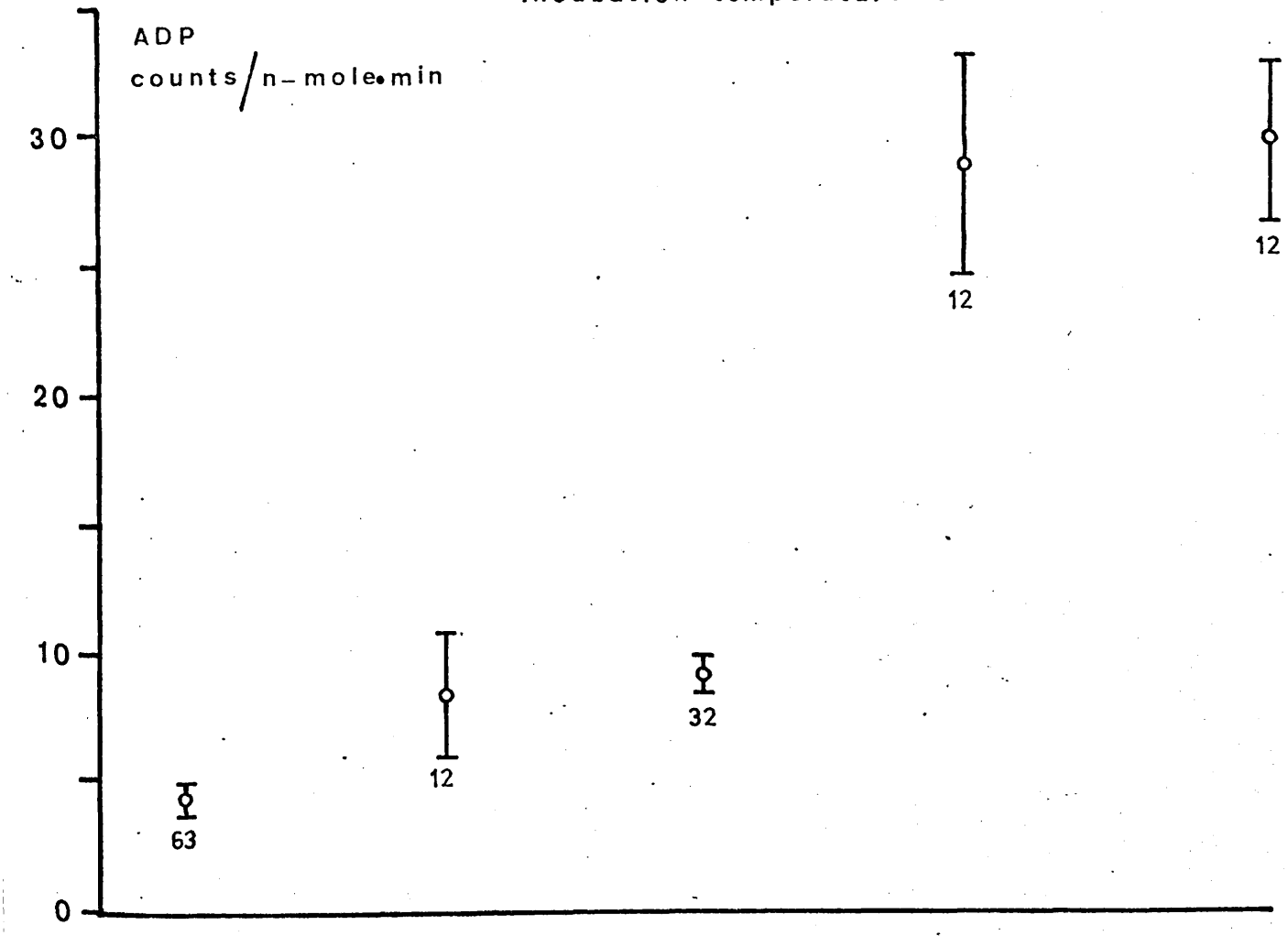
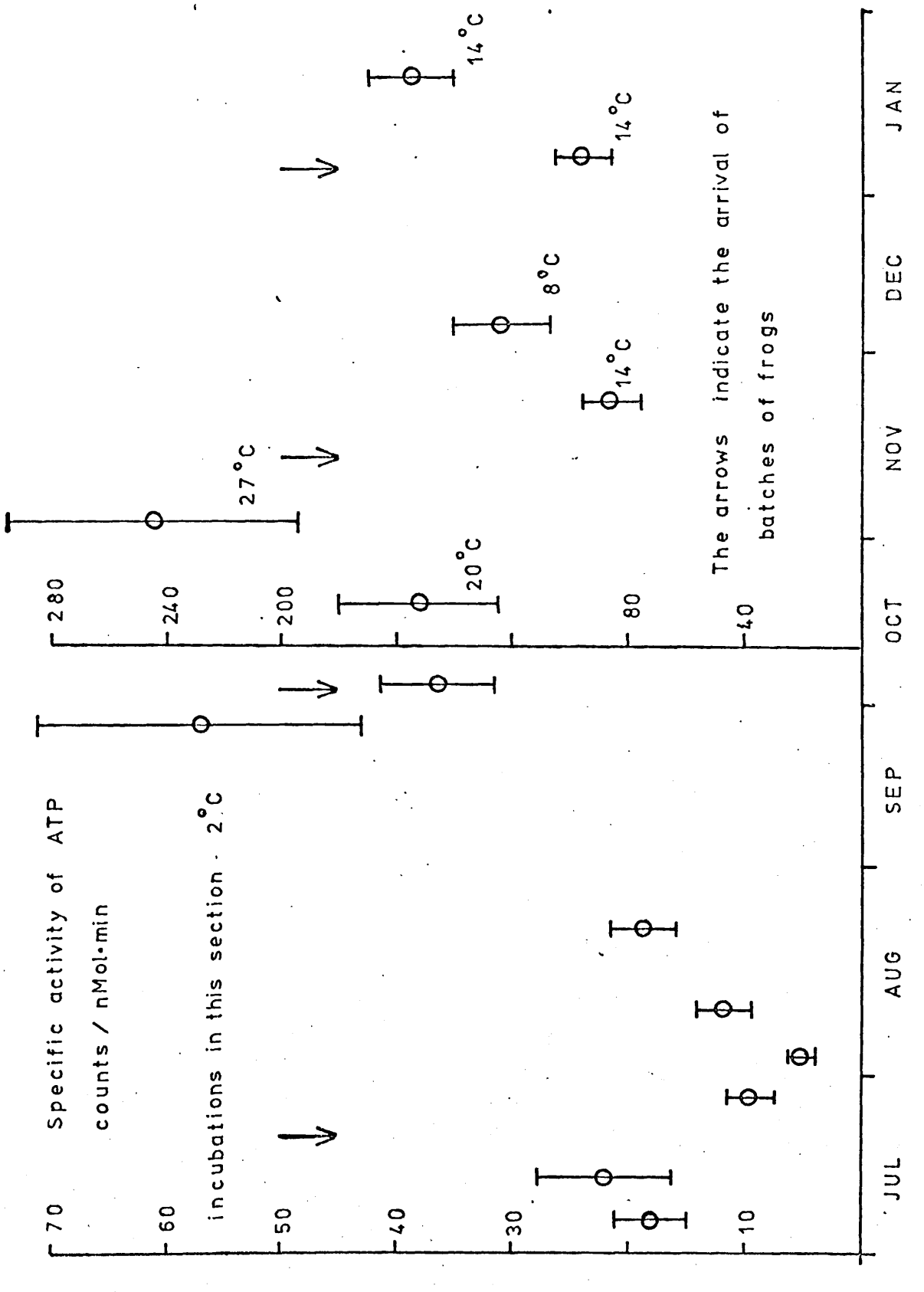


Fig. 3.11

The specific activity of ATP and its correlation with the length of time the animals spent in captivity. In each case the incubation was of one hour duration in the presence of 10 µCi of (2-^{3}H)-adenine. The temperatures of each incubation are as indicated on the diagram.



7.) The effect of muscle length

These experiments were, in fact, performed after those on electrical stimulation. They were originally designed to guard against the possibility that stimulated muscles might be slightly more stretched than the controls (to ensure good contact with the electrodes).

Muscles were incubated in a stretched or unstretched condition (test and control respectively). The incubations were for one hour at 14°C in the presence of 10 µCi of (2-^{3}H)-adenine. In some experiments the muscles were fixed in ice-cold alcohol before being released from their supports and minced.

The nucleotides were isolated, purified and their specific activities determined in the usual way.

The results are displayed in table 3.8. The ATP in those muscles which were stretched has a makedly lower specific activity than has that of the controls. The mean fall in specific activity is 33 % and the effect is significant to the 0.5 % level.

The specific activity of the actin-bound ADP in the test muscles is below that of the controls, but this change is not significant. Neither is the rise in ADP / ATP specific activity ratio significant.

At the extensions used, about 30 % of the rest length, there should still be overlap of the thick and thin filaments; about 15 - 25 % of the length of the thin filaments should be overlapped.

It is scarcely possible to explain the fall of ATP activity

Table 3.8

The labelling of nucleotides in stretched and unstretched gastrocnemii. The incubations were for 1 hour at 14°C. The initial label was, in each case, 10 μCi of (2-^{3}H)-adenine.

Alcohol fixation	Length of muscle mm in frog	test	control	Specific activities counts / nMol . min ADP test	ADP control	ATP test	ATP control
no	30	40	30	8.8	12.2	85	86
no	29	39	30	6.4	4.2	106	150
no	32	41	32	7.0	8.7	129	163
no	32	42	33	13	7.3	101	118
yes	29	36	27	15	9	120	230
yes	30	37	29	4.5	7.5	102	250
yes	24	33	25	12	10	104	180
yes	25	33	25	1.9	12	96	220
yes	28	40	28	4.5	7.4	120	130

Statistical tests on the above data.

Writing ATP_t for the specific activity of ATP in the test muscle etc.

	Mean value	Students t
$\frac{ATP_t - ATP_c}{ATP_t + ATP_c}$	-0.20	3.92
$\frac{ADP_t - ADP_c}{ADP_t + ADP_c}$	-0.07	0.68
$\frac{ADP_t/ATP_t - ADP_c/ATP_c}{ADP_t/ATP_t + ADP_c/ATP_c}$	0.09	0.89

by assuming that stretched muscle deteriorates faster than unstretched, as this would affect the activity of the ADP more than that of the ATP.

It is possible that the accumulation of adenine is an active process and stretching of the membrane inhibits it. Although the observed reduction in specific activity seems rather large to be caused by a rather mild extension.

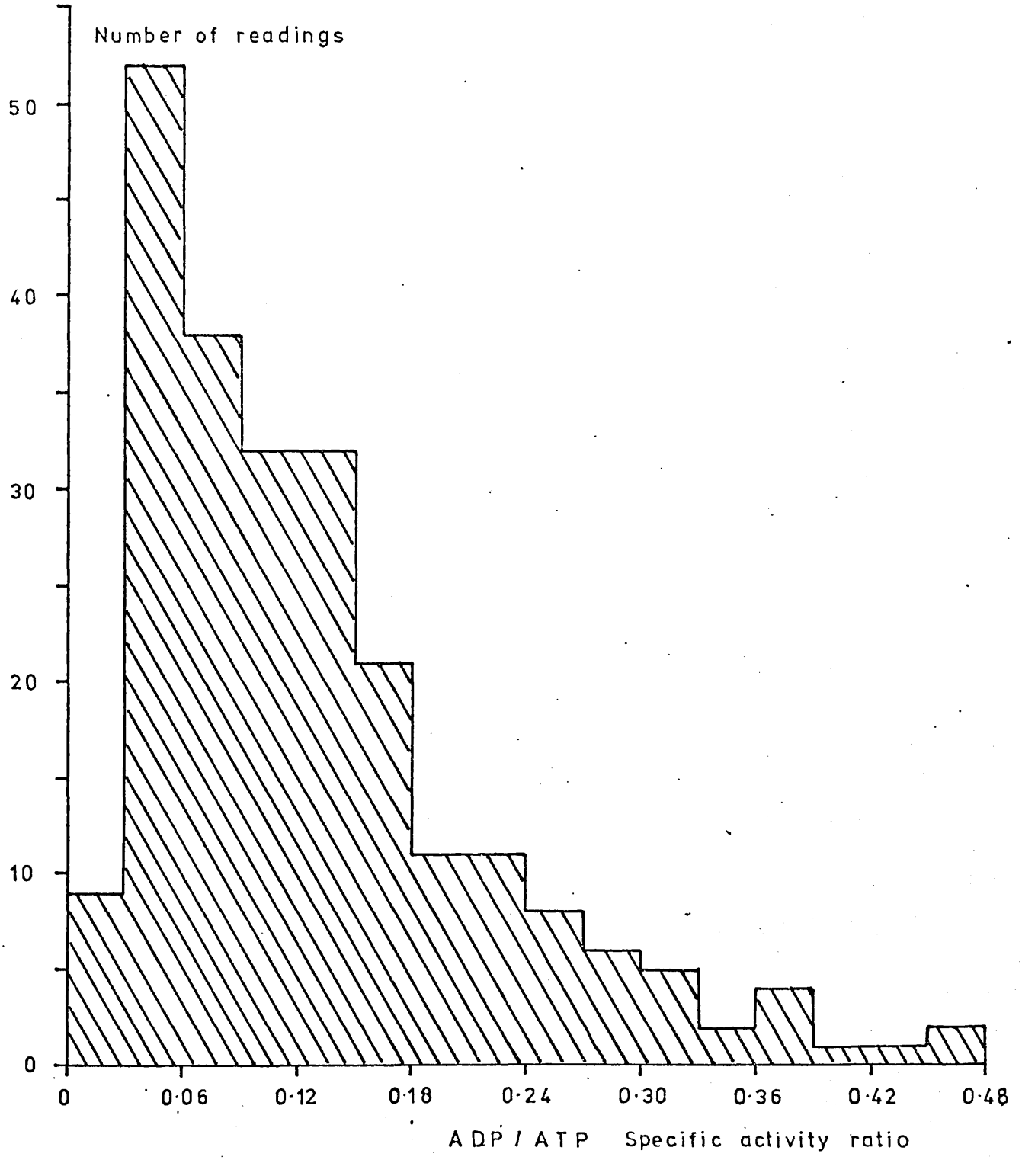
8) The consistancy of labelling

Fig. 3.12 is a histogram of the ratio between the specific activities of ADP and ATP. The histogram includes all the experimental results obtained with the improved purification techniques.

The time and temperature of the incubation affect this ratio, as do stretching and electrical stimulation. However, the ratio stays within fairly narrow limits.

Fig. 3.12

A histogram of the ratio of specific activities ADP / ATP. All determinations using improved purification techniques are included. The histogram includes measurements obtained after various conditions of incubation time, temperature and stimulation etc.



The effect of electrical stimulation

1) Under isometric conditions

The gastrocnemii were dissected from a freshly killed frog and suspended in 7 ml of Ringer's solution. After ten minutes, (2-^{3}H)-adenine was added and, after a further hour, one muscle of the pair received an electrical stimulus (15 V, 0.5 ms pulses at 50 / sec for 5 sec under isometric conditions). The nucleotides were extracted, purified and their specific activities determined. The experiment was repeated for various temperatures of the Ringer's solution.

The results are shown if table 3.9. Table 3.9a shows the outcome of t-tests on these data after the elimination of doubtful (low recovery) figures.

There is a fall in ADP specific activity due to stimulation of 22 $\pm$ 9 % (SEM) overall and the effect seems most pronounced at 8 - 14 ^{o}C. There is also an overall fall in the specific activity of the ATP, but the effect is less significant.

The specific activity of a nucleotide can only decrease in one of two ways:-

a) Selective loss of hot molecules, or of hot atoms from the nucleotide.

b) Addition to the pool of cold molecules.

Although the ratio of specific activities ADP / ATP does not fall significantly (to the 5 % level), it seems likely that such a fall does in fact take place. If this is so and the model so far advanced (1/6 of the ADP quickly exchangeable) is correct, then the fall cannot be explained by b) above.

Table 3.9

The specific activities of ATP and ADP isolated from stimulated and unstimulated gastrocnemii. The muscles were incubated for 1 hour at the temperatures given. The label was about 10 µCi of (2-^{3}H)-adenine, the exact quantity is given in the table. The stimulus was in each case 15 V 0.5 msec pulses at 50 /sec for 5 sec.

Conditions	Specific activities, counts / nMol.min			
	ADP		ATP	
	tetanus	control	tetanus	control
2 °C	2.1	1.6	16	15
11.6 µCi	3.1	3.1	13	15
	2.1	3.1	32	27
	2.9	2.5	11	23
	2.9	4.5	32	35
	3.9	4.3	25	16
2 °C	5.1	5.1	46	40
10 µCi	6.3	8.0	45	46
	12	14	52	31
	25	20	106	127
	8.2	7.1	32	40
	7.9	4.2	34	51
	3.4	9.3	26	43
	4.2	6.0	44	16
	12	5.4	34	34
	5.5	5.2	58	33
	7.6	13	50	42

(cont)

Table 3.9 (cont)

Conditions	Specific activities, counts / nMol.min			
	ADP		ATP	
	tetanus	control	tetanus	control
8 °C	4.6	18	82	107
10 µCi	2.7	6.5	90	150
	-	4.2	29	130
	1.6	20	150	160
	7	90*	110	270
14 °C	5.5	12	87	62
10.7 µCi	8.9	18	100	170
	9.2	10	103	57
	8.9	19	52	69
	11	6.3	87	97
	16	19*	76	70
20 °C	15	26	97	88
	27	44	88	210
	17	14	130	100
	33	33	85	120
	37	64	270	400
	24	14	130	120
27 °C	20	16	210	200
10 µCi	37	50	280	150
	20	34	190	690
	18	39	80	130
	31	31	460	250
	38	26	97	200

* high expected error

Table 3.9a

The results of Student's t test on the data of table 3.9.

$$Y = \frac{\text{Specific activity (test) - specific activity (control)}}{\text{Specific activity (test) + specific activity (control)}}$$

n = the number of determinations.

	Temperature	$\bar{Y}$	t	n	significance
ATP	2	0.02	0.44	17	no
"	8	-0.01	2.75	5	2.5 %
"	14	0.01	0.09	6	no
"	20	-0.09	1.12	6	no
"	27	-0.09	0.61	6	no
"	8 to 27 grouped together	-0.11	1.99	23	5 %
ADP	2	-0.03	0.56	17	no
"	8 and 14 grouped together	-0.34	2.84	8	2.5 %
"	20	-0.07	0.78	6	no
"	27	-0.08	0.90	6	no
"	8 to 27 grouped together	-0.18	2.77	20	1 %
"	all grouped together	-0.11	2.55	37	1 %
$\frac{ADP}{ATP}$	all grouped together	-0.08	1.53	37	no

In this model the only cold nucleotide is the unexchanged actin-bound ADP.

Loss of hot molecules seems unlikely, as there is nothing to take their place. One reasonable way of explaining the fall in ADP / ATP specific activity ratio is to assume that the label is lost from the ADP on stimulation; it has already been suggested (p. 55) that the 2-position hydrogen of adenine may be slightly exchangeable in F-actin-ADP. Stimulation might speed this exchange.

2) Under isotonic conditions

The gastrocnemii were dissected from a freshly killed frog and suspended in 7 ml of Ringer's solution. After 10 minutes (2-^{3}H)-adenine was added and after a further hour at 14^{o}C one muscle of each pair was stimulated. The stimulus was 15 V 0.5 msec pulses at 50 / sec for 5 sec. This caused the muscle to lift a weight vertically through 3 mm. The muscles were fixed in alcohol (ice-cold) before being removed from their supports and minced.

The results are given in table 3.10 and fig. 3.13. Unfortunately the masses of the muscles were not recorded, but it is clear that there is an increase in the specific activity of ADP especially at moderate loads. For the 21, 41 and 61 g loads taken together the average increase is 33 % $\pm$ 9.5 (SEM) and t = 3.44 on 14 degrees of freedom (this is significant to the 0.5 % level).

There seems to be no corresponding change in the ATP specific activity.

Table 3.10

The specific activities of ADP and ATP isolated from contracted and uncontracted gastrocnemii. The muscles had been incubated for 1 hour at 14 °C with (2-^{3}H)-adenine. The test muscles were stimulated to lift a load vertically through 3 mm. The stimulus was 15 V pulses 0.5 msec wide at 50 / sec for 5 sec.

Label	Load	Specific activities, counts / nMol.min			
µCi	g	ADP		ATP	
		contracted	control	contracted	control
40	3.5	16	21	410	400
"	"	37	24	830	880
"	"	53	46	610	1130
"	"	31	25	720	580
100	21	58	32	720	550
"	"	40	33	590	790
"	"	41	54	660	840
"	"	40	40	780	600
"	"	44	28	1090	890
"	"	37	29	810	530
100	41	310	190	2730	1860
"	"	90	75	1790	2140
"	"	100	65	1400	2560
"	"	42	30	890	770

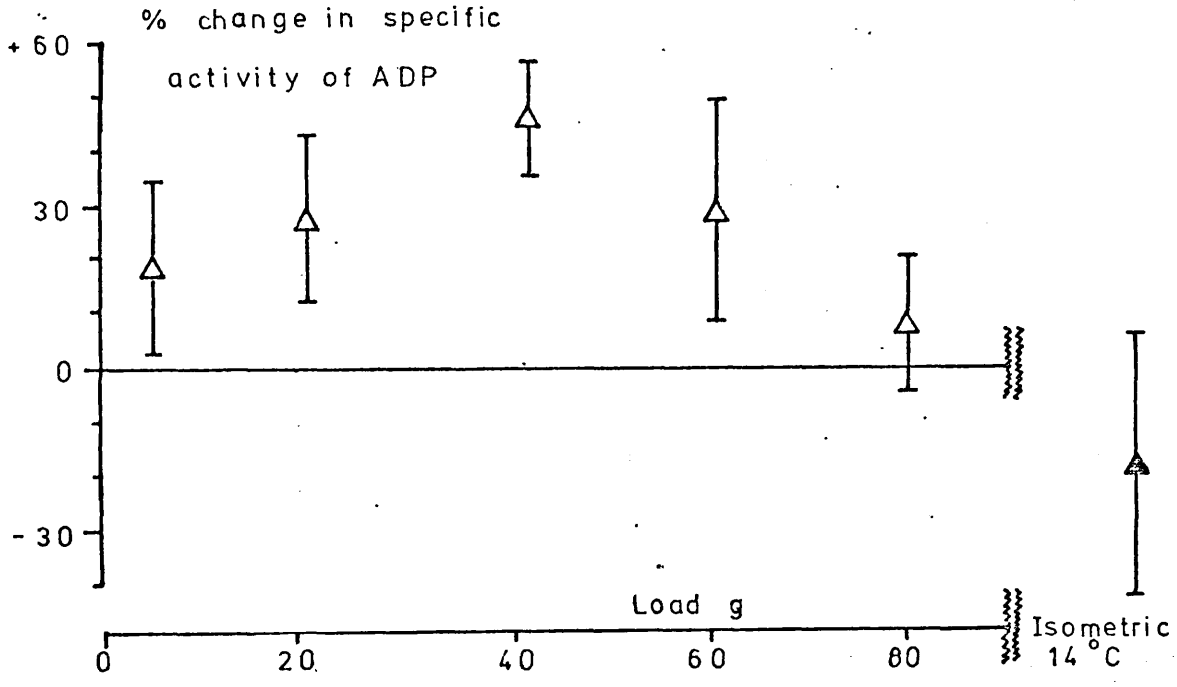
(cont)

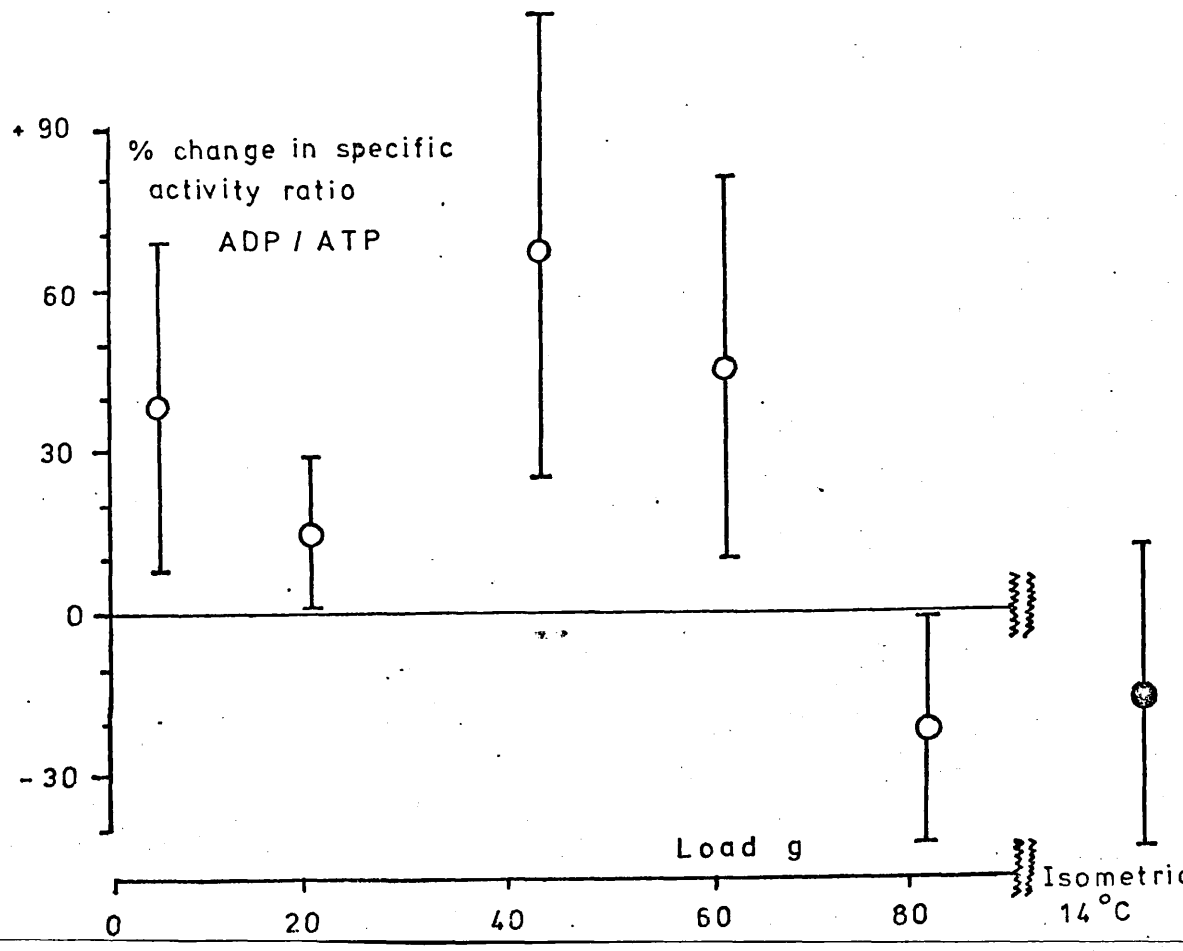
Table 3.10 (cont)

Label	Load	Specific activities, counts / nMol.min			
		ADP		ATP	
µCi	g	contracted	control	contracted	control
45	61	29	17	300	480
"	"	19	21	490	530
"	"	20	11	380	350
"	"	17	17	460	360
"	"	14	15	350	360
50	81	29	28	250	180
"	"	12	15	250	110
"	"	17	20	270	140
"	"	29	18	480	480
"	"	13	14	90	97
"	"	27	21	300	250

Fig. 3.13

The increase in specific activity of actin-bound ADP (top) and the ADP / ATP specific activity ratio (bottom) in loaded contracture. The label was introduced by incubation of the gastrocnemii with (2-^{3}H)-adenine at 14°C for 1 hour. In contraction the muscle lifted the load 3 mm vertically. The error bars indicate 1 standard error of the mean. For comparison the changes in isometric contraction are given.





As the muscle masses were not recorded, fig. 3.13 cannot be assumed to show the true relationship between changes in the activity of ADP and the load on the muscle, but the general form of the curve is probably correct i.e. it starts low, rises to a maximum and then falls with increasing load, eventually becoming negative.

Assuming that 1/6 of the bound ADP is freely exchangeable, then the only way in which the specific activity of the ADP can be increased is for the size of the exchangeable fraction to increase on stimulation. This increase in the size of the exchangeable fraction could only be transient and after contraction hot ADP molecules would be locked in inexchangeable positions.

The shape of the top curve in fig. 3.13 is reminiscent of the power vs. load curve of muscle. If this is so and the exchange of ADP is dependent on the mechanical power output of the muscle, the reaction must be coupled in some way to the fundamental energy transduction mechanism. But the extent of the reaction (the ADP / ATP specific activity ratio rarely exceeds 11 % after contraction) argues against there being an exchange for every ATP hydrolysis in working muscle.

Part 2. Experiments in vitro

1) The high temperature ATPase of actin

A solution of actin was prepared and it was polymerized by the addition of KCl to 60 mM. After standing at room temperature for 15 minutes, the solution was warmed to 53°C. At time zero the solution was mixed with prewarmed buffer. The buffer was such that the final concentrations were:-

Tris	10 mM
ATP	1 mM
(2-^{3}H)-ATP	1 µCi / ml
KCl	60 mM
EDTA	1 mM
Ascorbate	0.5 mM
pH	8.2 at 20°C

A sample of the mixture was used for viscosity measurements. Viscosity was measured at 53°C with an Oswald viscometer.

Further samples were were withdrawn into chilled tubes at times throughout the experiment. A small sub-sample of each was taken for ATP, ADP and protein determinations. The rest of each sample was filtered under pressure through a 0.22 µm millipore filter and washed through with 60 mM KCl. The filtrate was analysed for ATP, ADP, P_i and protein. The nucleotides were separated on PEI paper and their specific activities determined.

The filters were sonicated in ice-cold 10 % (w/v) TCA and centrifuged. The supernatent was analysed for ATP, ADP

and P_i. The TCA was removed by shaking six times with cold ether and the nucleotides were separated on PEI and their specific activities determined.

The "zero" time sample was formed by chilling the actin and buffer separately before mixing and the sample marked "b" in table 3.11 was formed by keeping seperate samples of actin and buffer at 53°C for the duration of the experiment, then chilling and mixing.

There was excellent agreement between the nucleotide contents of the solution before filtration and the filtrate. Only a trace of protein was found in the filtrate.

The results are shown in table 3.11 and in fig. 3.14. Several points emerge from this experiment:

a) The rate of ATP hydrolysis is in accord with that reported by other workers, but

b) The viscosity decreased by a factor of ten over 90 minutes; although the rate of hydrolysis fell, it did not do so by this amount.

c) Immediately on mixing there was a decrease in P_i and an increase in ATP, but there was no corresponding decrease in ADP.

d) The bound ADP seems to correspond to protein in the molar ratio 1 : 2. ATP also appears to be bound in this ratio. There is also bound P_i.

e) The radioactive label appeared immediately in the free ADP. It is possible that the tritiated ATP contained ADP as an impurity, but the blank "b" shows the lowest free ADP specific activity.

f) The radioactive label appeared very rapidly in the bound

Table 3.11

The viscosity and ATPase activity of a solution of F-actin at 53°C. The reaction was carried out in 10 mM tris-HCl buffer at pH 8.2 and 60 mM KCl, 1 mM EDTA and 0.5 mM ascorbate. All concentrations are given as μM in the reaction mixture, actin being assumed to have a molecular weight of 43,000.

Time	Specific viscosity	phosphate free	phosphate bound	ATP free	ATP bound	ADP free	ADP bound
min	cSk.ml/mg	μM	μM	μM	μM	μM	μM
0		433	53	1228	6	43	9
2		225	20	1264	6	45	11
6½		195	15	1280	14	68	9
10		205	15	1256	18	91	11
12	15.7						
17	12.9						
24	10.0						
25		280	13	1176	8	182	7
40		325	28	1124	17	255	11
41	6.4						
51	4.9						
59	2.9						
60		410	20	1072	7	315	8
69	1.7						
81	0.9						
89	1.3						
90		443	15	1024	11	382	7
"b"		180	123	1240	11	50	12

(cont)

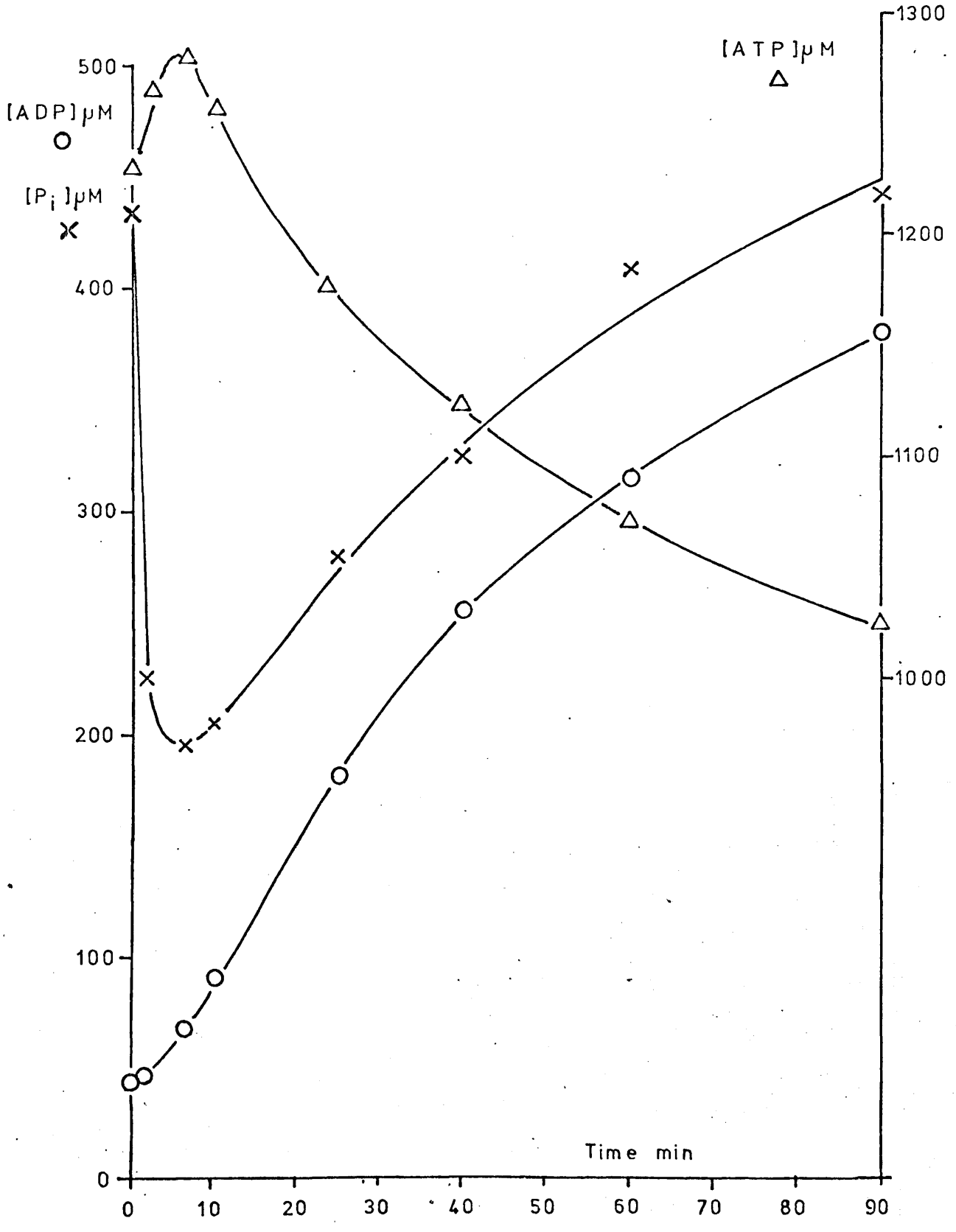
Table 3.11 (cont)

Protein, total adenine and total phosphate concentrations and the specific activities of nucleotides in a solution of F-actin at 53°C. Total adenine is the sum of free and bound ATP and ADP. Total phosphate is the sum of free and bound phosphate, 2 x ADP and 3 x ATP.

Time	Protein	Total adenine	Total phosphate	Specific activities			
				counts / nMol.min			
				ATP		ADP	
min	µM	µM	µM	free	bound	free	bound
0	21.8	1286	4292	193	206	206	129
2	21.2	1326	4167	166	226	176	128
6½	21.4	1371	4246	169	208	221	156
10	20.7	1376	4246	167	225	199	229
25	21.2	1373	4223	163	218	219	236
40	20.9	1407	4318	154	185	205	221
60	20.7	1402	4313	166	179	207	252
90	20.4	1424	4341	147	201	210	243
"b"	21.8	1313	4180	185	258	148	187

Fig. 3.14

The hydrolysis of ATP by actin at 53°C. The experimental conditions were:- tris-HCl 10 mM pH 8.2, 60 mM KCl, 1 mM EDTA 0.5 mM ascorbate.



ADP.

g) The specific activity of the free ATP fell during the experiment.

The loss of label from the free ATP probably reflects a catalysed exchange of the 2-hydrogen of adenine (with water). This reaction would also explain the low specific activity of the free ATP; 1µCi / ml in 1.3 mM ATP corresponds to a specific activity of about 430 counts / nMol.min (at 25 % counting efficiency).

The rapid spread of label throughout all the nucleotides means that actin in vitro shows different behaviour from that in isolated muscle. In vitro the specific activity of the bound ADP is roughly equal to that of the free ATP, whereas, in the muscle, the bound ADP activity is very much less.

Immediately after mixing there seems to be a synthesis of ATP as the change in ATP and P_i is more than stoichiometric with actin. If there is a synthesis the only possible source of energy is the ascorbate.

2) Effect of the purity of actin

The results of the previous section have far-reaching implications. Attempts were made to repeat them. A new batch of actin was prepared from the same acetone powder. This actin was shown to be very pure by polyacrylamide gel electrophoresis. It polymerized rapidly in the presence of KCl. However, it produced no hydrolysis of ATP under the following conditions (all experiments were made in 60 mM KCl, 10 mM

tris-HCl pH 8.2, with actin at 1 mg / ml)

a) At 45°C with 0.3 mM ATP, in the presence of 1 mM $CaCl_2$ or 1 mM EDTA or neither, both before and after the addition of 0.5 mM ascorbate. The polymerization took place at room temperature.

b) At 53°C, 0.4 mM ATP, 1 mM EDTA in the presence or absence of 0.5 mM ascorbate. The polymerization took place at 53 °C.

c) At 53°C, 0.4 mM ATP, 1 mM EDTA. The actin was initially oxidized with 0.5 mM H_2O_2 and polymerized at room temperature. There was no hydrolysis at 53°C before or after the addition of ascorbate to 0.5 mM.

The sensitivity and reproducibility of the phosphate assay was such that a hydrolysis of 10 µM / hr, about 1 / 20 of that seen in the first experiment, would have been observable.

One possible conclusion is that the high temperature ATPase of actin is critically dependent on the presence of an impurity absent from the second batch of actin. To test this idea myokinase (a muscle enzyme that is notoriously impure) was added to the actin preparation.

Actin was polymerized in a buffer consisting of:-

Tris	10 mM pH 8.2 at 20°C
EDTA	1 mM
ATP	0.4 mM
ADP	0.2 mM
AMP	0.2 mM
KCl	60 mM

(The nucleotides are approximately an equilibrium mixture with respect to myokinase, which should be inactive in the

presrnce of EDTA.)

The mixture was warmed to 53°C. A solution of myokinase was also incubated. Samples were taken from both solutions, de-proteinised with cold 10 % (w/v) TCA and assayed for phosphate. The protein solutions were then mixed and further samples taken. The final protein concentrations were:- actin 1 mg / ml, myokinase 0.2 mg / ml.

On mixing there was a slow hydrolysis (about 9 µM / hr, compared with about 180 µM / hr in the first experiment).

This was the only hydrolysis seen with this batch of actin. The fact that the actin was, in this case, contaminated gives support to the notion that the actin ATPase is dependent on an impurity. The active substance is probably not the myokinase itself but an impurity therein.

Discussion

The incorporation of adenine into ATP

The ready and simultaneous appearance of labels from the 2- and 8- positions of adenine in ATP in the isolated muscle is very strong evidence that the adenine is incorporated intact into ATP via one of the salvage mechanisms. The reason for the inhibition of the incorporation when the muscle is stretched is not clear. It is hardly possible that a 30 % extension of a muscle would cause it to leak PRPP or ribose-1-phosphate. Neither does there seem to be any reason for there to be a control mechanism of the salvage pathway triggered by muscle length. If the salvage enzymes were located (or active only) in the region of overlapped filaments there would be a reduction of incorporation in the stretched muscle. The fall in incorporation rate is 33 % when the length of the overlapped region falls from about 700 nm to 150- 250 nm, a percentage decrease of 65-80. The decrease in incorporation rate and the decrease in overlapped length are not so disparate that the latter could not explain the former.

Another possible explanation of the phenomenon is that mechanical stress on the muscle membranes inhibits either the accumulation of adenine (supposing an active uptake) or the salvage enzymes (assuming them to be membrane bound).

Nucleotide exchange in resting muscle

The experiments described in this thesis leave little

doubt that some of the actin-bound nucleotide is exchangeable. Priston (1970) showed that the ADP associated with well washed muscle residues and the actin-bound nucleotide were identical. Also, if the observed specific activities were due to contamination, there would have to be a pool of ADP of about 100 nMol / g which was in equilibrium with the free ATP yet firmly enough bound to resist washing; this pool could hardly have escaped detection. Alternatively a very small, very highly labelled pool of bound ADP could be imagined. However, contamination from such a source is hardly likely to produce readings as consistent as those actually obtained.

As already pointed out (pp 64-66) a fraction of the bound ADP is rapidly exchangeable. This fraction is initially about 1/6 of the total. This fraction falls with increasing incubation time as the muscle deteriorates. The fraction also falls as the temperature of incubation is raised. The comparison of data for 1 hour incubation times at $2^{o}C$ and $14^{o}C$ yields mean ADP / ATP specific activity ratios of 0.155 at $2^{o}C$ and 0.093 at $14^{o}C$ with $t = 3.61$ on 99 degrees of freedom; this is significant to better than the 0.1 % level. This is exactly what would be expected if high temperatures accelerated the deterioration of muscle.

The constancy of the ADP / ATP specific activity ratio (under a given set of incubation conditions) strongly suggests that this ratio reflects some underlying property of the actin. As the ratio is 1 : 6 at short incubation times at $2^{o}C$ (when the muscle is in the best condition) and the ratio of troponin to actin is 1 : 7, it seems likely that it is one actin monomer in a particular geometric relationship with

troponin which carries the exchangeable ADP. If troponin is co-polymerized with actin in the thin filament then there are three types of bond parallel to the axis of the filament:- actin-actin, actin-troponin and troponin-actin. Troponin is not known to carry a bound nucleotide, so either the actin-troponin or troponin-actin bond cannot be associated with nucleotide. The other bond might well be associated with exchangeable nucleotide.

Nucleotide exchange in stimulated muscle

On stimulation under isometric conditions, the ADP / ATP specific activity ratio may fall. One explanation for this has already been advanced, i.e. the 2-hydrogen of actin-ADP becomes slightly exchangeable on stimulation. An alternative hypothesis is that, on stimulation, the (exchangeable) ADP becomes totally free; and on removal of the stimulus the ADP is rebound. If the time taken for the rebinding were of the same order as the time taken to mince the muscle (30 sec) then a slight reduction in ADP / ATP specific activity ratio would be observed.

If 1/6 of the actin-bound ADP is fully labelled then the increase in ADP / ATP specific activity ratio in loaded contracture can only be explained by labelling of some of the remaining bound ADP. Now, the concentration of actin monomers in muscle is about 5.5×10^{-7} mole / ml and the energy rate during a tetanus is about 30 mcal / g.sec. if the free energy of hydrolysis of ATP is taken as 10 Kcal / Mol then the mean number of hydrolyses is $\frac{30 \times 10^{-3} \times 10^{-4}}{5.5 \times 10^{-7}}$ per monomer.sec

so in a 5 sec tetanus there are about 30 ATP hydrolyses for each actin monomer. Therefore if every hydrolysis were accompanied by nucleotide exchange, the ADP would become fully labelled. However, the mean increase in ADP / ATP ratio (21, 41 and 61 g loads) is only 0.013. This means that only one hydrolysis in 2300 is accompanied by exchange. Alternatavely it is possible that the exchange takes place only at the 1 site in 7 where the nucleotide is loosely bound, the increase in labelling being explained by a reversal of the deterioration by the motion of the filaments. It seems, therefore, that the nucleotide exchange, although it is related to the mechanical power output of the muscle, is only a side reaction and not an essential feature of contraction.

Moos et al (1967) put forward the idea that the actin-bound nucleotide served a repair function. This hardly seems likely as no additional labelling was found in isometric tetanus, but it was found in loaded contracture. The isometric tetanus would be expected to inflict at least as much damage on the contractile machinery as would contraction.

It is most probable that motion is accompanied by a distortion of the thin filament and that this distortion renders the actin-bound nucleotide very slightly exchangeable.

Doubts on the identity of the actin-bound nucleotide

As pointed out on p. 66, if the nucleotide exchange in resting muscle were accompanied by hydrolysis of ATP, the

reaction would account for a very large proportion of the cells' metabolic rate. It is possible that the bound nucleotide exchanges with free ADP. But an alternative explanation is that the exchangeable nucleotide is ATP. There is no reason to suppose that an actin adjacent to troponin has ADP as its bound nucleotide. As such an actin does not participate in the normal actin-actin bond, it is possible that the bound nucleotide is ATP. Radioactivity is found in ADP after acid denaturation of the protein, but it is possible that hydrolysis of the nucleotide accompanies such denaturation. Yagi and Noda (1960) found that 15-20 % of myofibril-bound nucleotide was ATP and that this could be labelled by creatine-^{32}P. It is possible that this ATP could be the exchangeable nucleotide found in the present experiments.

The apparent nucleotide binding of actin found <u>in vitro</u>, a ratio of 2 : 1 : 1, actin : ATP : ADP can be explained in one of several ways:-

a) There was a "fault" in the preparation which gave a polymer that did bind nucleotide in this ratio.

b) Under the conditions of the experiment, actin existed as a mixture of the F- and G- forms. However, the nucleotide binding ratio did not change systematically with the viscosity of the solution.

c) About half the actin denatured during filtration, losing its ADP. The ATP that was found would be contamination from the solution.

On the evidence presented here it is not possible to distinguish between these possibiliies. But it must be remembered that the experiment was performed at 53°C and the nucleotide

content of actin has not been investigated at this temperature, so a) is not impossible.

The re-attachment length

1) Definitions

The re-attachment length is the length (measured along the thin filament) between points of successive attachment of the same cross bridge. This length can be calculated from the dimensions and energetics of a muscle, with few assumptions.

In the calculation which follows it is calculated for a muscle working at maximum power.

Let

n be the mean number of bridges attached to $\frac{1}{2}$ thin filament simultaneously. ($\frac{1}{2}$ thin filament is from z-disc to tip)

N the number of bridges / sec attaching to $\frac{1}{2}$ thin filament

F the force (parallel to the thin filament) generated by each bridge.

x the displacement occuring while a single bridge is attached.

E the chemical energy released during each attachment.

M the mechanical energy produced during each attachment.

β the fundamental efficiency = M / E

v the relative velocity of the thick and thin filaments.

t the length of time for which a bridge is attached on any one occasion.

z the total force sustained by a thin filament.

σ the time between successive attachments of any one bridge.

l the re-attachment length.

α the number of cross bridges dedicated to any $\frac{1}{2}$ thin filament.

P the mechanical power generated during one attachment.

also let

V be the velocity of shortening of a muscle.

Z the force / unit area of a muscle.

Q the power (mechanical) output of a muscle.

2) The basic relationships

The following relationships are obvious:-

$x = t.v$ (1) $l = \sigma.v$ (1a)

$n.F = z$ (2)

N is the rate of attachments to $\frac{1}{2}$ thin filament in the steady state and n bridges are attached simultaneously, each for time t. Therefore during time t, n new bridges attach.

$N = n / t$ (3)

Each bridge attaches every σ seconds, therefore the total rate of attachments is

$N = \alpha / \sigma$ (4)

$l = v.\alpha / N$ (5) from (1a)

also

$P = M / N = E.\beta / N$ (6)

From (5) the re-attachment length can be calculated from the velocity, the number of potential bridges, and the hydrolysis rate.

3) The number of thin filaments in unit area and volume

A hexagon of side 28 nm surrounding a thick filament contains 6/3 thin filaments. The area of this hexagon is $6 \times \frac{1}{2} \times 28^2 \times \cos 30^\circ$ nm^2. There fore the area surrounding each thin filament is 1018 nm^2 or 1×10^{-11} cm^2.

If a sarcomere is 2.5 µm in length then the volume surrounding $\frac{1}{2}$ thin filament is 1.25×10^{-15} cm^2.

If the free ATP is 3 µMol / g, then this volume contains some 2300 ATP molecules.

4) The mechanical parameters

Using the Hill equation and the constants given by Hill (1938), for a muscle contracting at maximum power:-

$(Z + a).V = b.(Z_o - Z)$

where $a = 357$ g wt / cm^2

$a / Z_o = 0.22$

$b = 0.27$ muscle lengths / second

$Q = Z.V = V.(b.Z_o - a.V) / (V + b)$

$$\frac{dQ}{dV} = \frac{(V + b).(b.Z_o - 2.a.V) - (b.V.Z_o - a.V^2)}{(V + b)^2}$$

at maximum power

$V.b.Z_o - 2.a.V^2 + b^2.Z_o - 2.a.b.V - b.V.Z_o + a.V^2 = 0$

or $V^2.a + 2.a.b.V - b^2.Z_o = 0$

$$V = \frac{-2.a.b \pm \sqrt{4.a^2.b^2 + 4.a.b^2.Z_o}}{2.a}$$

$$= -b \pm b.\sqrt{1 + Z_o / a}$$

$$= 0.27\ (\sqrt{1 + 1 / 0.22} - 1)$$

$= 0.366$ muscle lengths / second

$$\text{so } Z = \frac{a.(b.Z_o/a - V)}{V + b}$$

$$= \frac{357\ (0.27/0.22 - 0.366)}{0.366 + 0.27}$$

$$= 483 \text{ g wt / cm}^2$$

This gives $v = 4.6 \cdot 10^{-7}$ m/s (assuming a half sarcomere length of 1.25 μm) and $z = 5.7\ 10^{-11}$ Newtons / thin filament (assuming 80 % of the cross section of the muscle consists of contractile apparatus). The mechanical power output $N.P = 2.6\ 10^{-17}$ Watts / $\frac{1}{2}$ thin filament.

5) The cross bridge arrangement

The exact number and arrangement of cross bridges in a thick filament is still a matter of contention. According to the model of Huxley and Brown (1967), double stranded six bridge / turn helix, there would be some 196 bridges / thick filament, or 48 or 49 bridges / $\frac{1}{2}$ thin filament.

More recently the model of Squire (1973) has gained some acceptance. This model is a triple stranded nine bridge / turn helix and predicts 264 bridges / thick filament, or 66 bridges / thin filament.

If the molar ratio of myosin : troponin is 1 : 1 as stated by Potter (1974), then the number of bridges / thin filament is the same as the number of troponins / thin filament. The length of $\frac{1}{2}$ thin filament is between 1 and 1.1 μm and there are 2 troponins every 40 nm. This gives between 50 and 55 troponins / thin filament. This is in closer agreement with the Huxley and Brown model.

In the remainder of this calculation numerical values

assuming 48 bridges / thin filament will be given, with the corresponding values for 66 bridges in parentheses, the true values must lie somewhere between these two.

6) Energetics

The overall efficiency of a muscle (mechanical work / heat + work) can be expressed as the product of three terms:-

a) The chemical efficiency (the chemical energy stored in form of ATP / the free energy of combustion of glycogen). This is about 0.6 (aerobic)

b) The transduction efficiency (mechanical energy output of a single event / free energy of hydrolysis of ATP). This is β as defined above.

c) The mechanical efficiency (the mechanical output of a whole muscle / the sum of the mechanical outputs of the fundamental events). Let this be μ.

The efficiency of frog muscle (at maximum power) is 0.22. For frog muscle, therefore, the minimum value of β is given by;-

$$0.22 = 0.6 \times \beta \times \mu$$

(at maximum power the muscle is utilizing aerobic and anaerobic mechanisms and 0.6 is an overestimate of the chemical efficiency)

Taking $\mu = 0.9$, $\quad 0.41 < \beta < 1$

A more precise estimate of β can be obtained from tortoise muscle which can be considered to be aerobic and sufficiently slow for μ to be close to unity. This assumes that muscles from the two species share the same fundamental event. The overall efficiency of tortoise muscle is one of the highest known and is 0.37.

$$0.37 = 0.6 \times \beta \times 1$$

which gives $\beta = 0.62$.

Taking the free energy of hydrolysis of ATP to be 10 Kcal / Mol, the mechanical energy produced at each event is

$0.62 \times 10^{4} \times 4.18 / 6 \times 10^{23}$ Joules $= 4.3 \times 10^{-20}$ Joules.

At maximum power 2.6×10^{-17} Watts / $\frac{1}{2}$ thin filament are produced (see above), so the number of events / $\frac{1}{2}$ filament.sec is $N = 2.6\ 10^{-17} / 4.3\ 10^{-20} = 605$

In view of the assumptions in the above argument it is probably reasonable to estimate the value of N as between 500 and 700 per $\frac{1}{2}$ filament.sec.

7) The values of the fundamental parameters.

From the equations given in 2)

l (the reattachment length) = $v.\alpha / N$

= 32 to 44 nm (43 to 61 nm for the 66 bridge model)

σ (the cycle time of a single bridge) = α / N

= 69 to 96 msec (94 to 130 msec)

These cycle times are compatible with the results of Ritchie (1954) which indicate that the active state of frog muscle (single twitch at $0^{\circ}C$) lasts some 400 msec. This would mean that there were three to six events at each bridge in a single twitch. Ritchie (1954) estimates the period of maximum activity to be 25 msec in frog sartorius at $0^{\circ}C$.

On the Huxley model, the reattachment length lies between 32 and 44 nm. It may be reasonable to assume therefore, that this length is 37 nm, the distance between crossover points of the actin helix. If this is so $\beta = 0.63$ (0.46)

Using these values of β, it is possible to construct a

table giving the force, duration and displacement of each event for various values of n, the number of simultaneous attachments: table 4.1.

Table 4.1

n is the number of bridges simultaneously attached to $\frac{1}{2}$ thin filament.

F is the force generated by each fundamental event.

t is the length of time for which each bridge is attached.

x is the displacement occurring during each attachment.

n	F	t		x	
	Newtons	msec		nm	
1	$5.7\ 10^{-11}$	1.7	(1.2)	0.77	(0.56)
2	$2.9\ 10^{-11}$	3.4	(2.5)	1.5	(1.1)
5	$1.1\ 10^{-11}$	8.4	(6.1)	3.9	(2.9)
10	$5.7\ 10^{-12}$	17	(12)	7.7	(5.6)
20	$2.9\ 10^{-12}$	34	(25)	15	(11)
50	$1.1\ 10^{-12}$	-	(61)	-	(29)

Doubts on the structure of the thin filament

1) Symmetry

Naively, it would be expected that the thin filament would show three-fold screw symmetry, so that it could interact with any of the surrounding thick filaments.

2) Geometry and efficiency

If a particular bridge can attach to a thin filament

every 37 nm or so down the whole length of a thin filament, then either

a) there is one actin monomer every 37 nm in exactly the same favourable orientation to the thick filament,

or b) attachment can occur to a monomer which is nearly in the optimum orientation, such monomers occurring at average intervals of 37 nm.

On the basis of the accepted picture of the thin filament, with its non-integral number of monomers per turn, b) must be true.

However, if bridges can attach to inexactly aligned actin monomers, then each bridge must have one more degree of freedom than it would need if the monomers were aligned precisely. The more degrees of freedom a bridge has, the more it will distort under stress and the less efficient it will be.

Moreover, if we take one particular actin monomer as being in an optimum angular orientation, then, at a distance of about 37 nm, the actin nearest to the optimum is 7° away from it. At one full turn of the helix from the given actin, the monomers closest to the optimum are $\pm 13^{\circ}$ from it. This is as far from the optimum as it is possible for them to be, as the angular displacement between neighbouring monomers is 27° (calculated on $13\frac{1}{2}$ monomers per full turn in a single chain).

3) Troponin

Troponin occurs in the thin filament with a periodicity of 40 nm. If actin and troponin are in molar ratio of 7 : 1,

then either the troponin is attached to the outside of the filament or it is co-polymerized, occupying some 1.5 nm of the length of the chain (40 - 7 x 5.5 nm). In either case the troponin would project considerably beyond the actin of the thin filament and might hinder bridge attachment. In fact, as the troponin - troponin spacing and the half pitch of the thin filament are believed to be different, such a projection almost certainly would cause steric hinderance at some point along the filament.

4) A model of the active state of the thin filament

On activation, the thin filament is known to become more ordered (Huxley and Brown, 1967). Vibert et al (1972) found that the ordering and a possible slight increase in the pitch of the helix were linked to stimulation of the muscle rather than to contraction. If the increase in pitch were rather larger than usually believed, then it would be possible that the thin filament, in its active state, possessed an integral number of monomers per turn and three-fold screw symmetry.

If the pitch increased to 41.3 nm, keeping its basic structure of two chains staggered by $\frac{1}{2}$ monomer, then there would be 15 monomers in each chain in a full turn. Three-fold screw symmetry would arise naturally out of such a structure; and a single bridge could re-attach at distances of 41.3 nm to perfectly aligned actin monomers.

Replacing one of the actin monomers with two molecules of troponin would still result in a bulky projection, but it

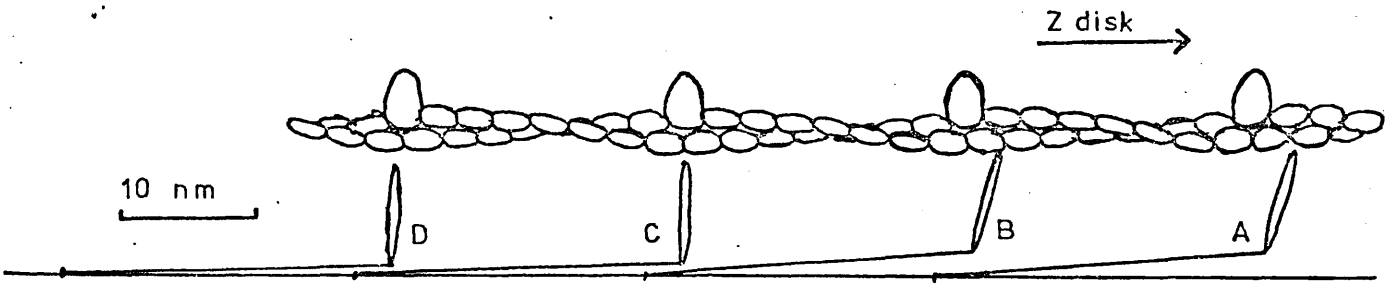
give the correct 7 : 1 molar ratio of actin to troponin. As the troponin projections would occur exactly once per half turn of the helix, they could be positioned so as not to interfere with bridge formation.

The actin-actin spacing in such a structure would be 5.47 nm and there would be X-ray diffraction lines at 5.13 and 5.85 nm.

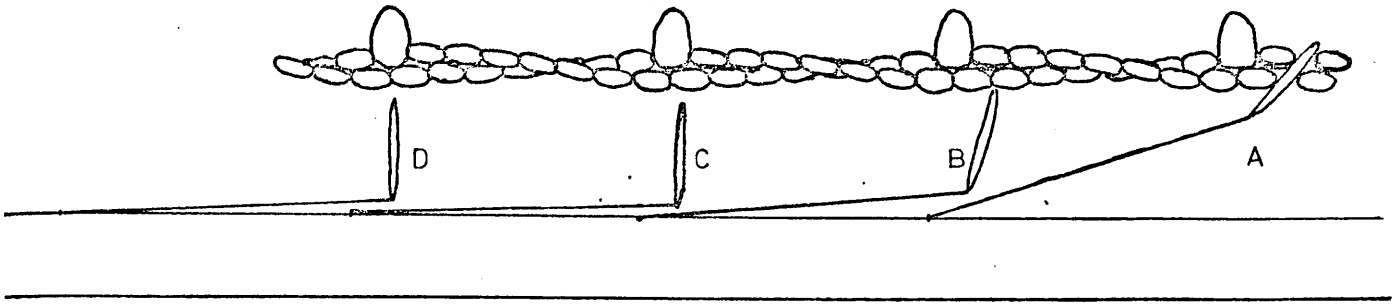
A theory of muscular contraction

This theory is described using the structure of the thin filament postulated above, but it can fairly readily be adapted to other structures.

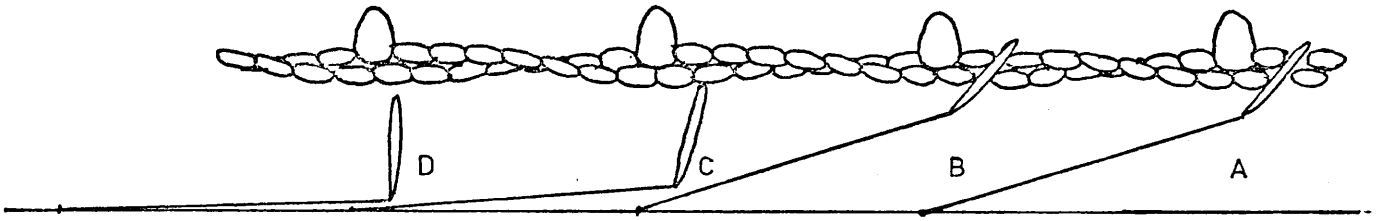
1) On stimulation the thin filament assumes its active configuration. One myosin head (A) is aligned directly opposite to its binding site. At this stage all the myosin heads carry ADP and inorganic (energy rich) phosphate.



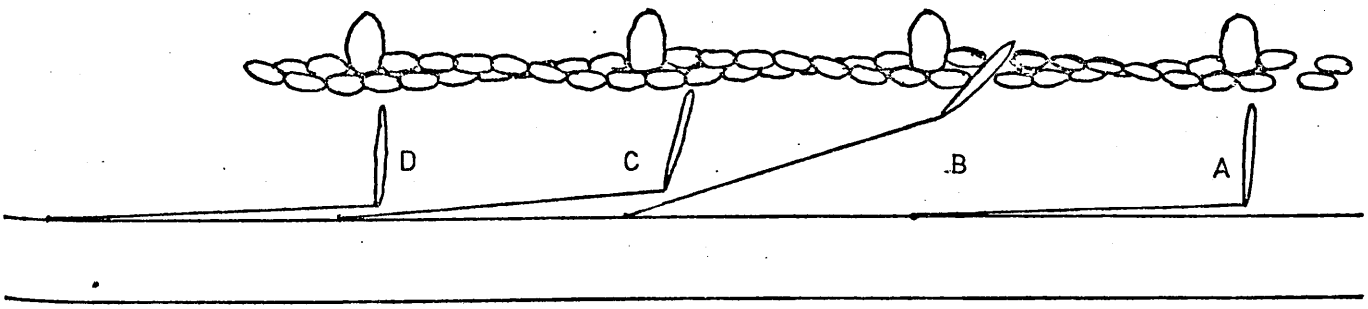
2) The myosin co-polymerizes into the actin chain. Simultaneously the phosphate is released, the energy being stored in the distortion of the tropomyosin (not shown for reasons of clarity). This binding brings head (B) into alignment with its binding site.



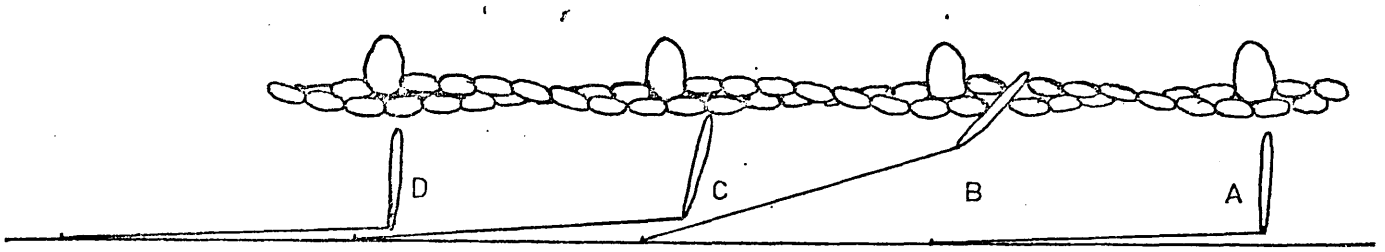
3) The head (B) co-polymerizes in a similar fashion, releasing phosphate and bringing (C) into the correct orientation.



4) Head (A) binds ATP, releasing ADP and freeing itself from the chain. Normally there would be some interaction between (A) and (B) so that (A) could not release until (B) bound.



5) . With the strain imposed by the head (A) released, the actin helix reforms, moving the thick filament to the right. At about this time the ATP carried by (A) is hydrolysed, the products remaining bound.



6) The cycle now repeats from stage 2) with heads (B) and (C) active. The wave of activity ripples down the thin filament away from the z-disc.

Since there is a displacement of 1.6 nm while each bridge is attached there must be an average of just under 2 bridges attached to the thin filament. (The table on page 102 gives values based on a re-attachment length of 37 nm; in this case the re-attachment length is 41.3 nm and the displacement corresponding to two bridges is therefore 1.7 nm.) This suggests that only one thick filament interacts with a given thin filament at any given time, on this model.

In the above scheme rigor is easily explicable as, with no ATP, bridges cannot release and the binding of one bridge aligns the next one. Potentially all the bridges could become bound simultaneously, as the actin and myosin periodicities are automatically made equal.

Although exchange of the actin-bound ADP is not an

essential part of the above scheme, the distortions involved and the break in the helix might well render this nucleotide more exchangeable. However, as each bridge is attached for only about 4 msec and the break in the helix is exposed for a much shorter time than this, the experimentally determined value of 1 nucleotide in 2000 exchangeing in a 5 sec tetanus is not unreasonable.

Summary

1) The structure of muscle and the literature leading to its elucidation are discussed. Particular regard is paid to the protein actin, its ATPase activity and its possible active role in muscular contraction.

2) The methods and materials utilized in the experiments are described.

3) Experiments are described which throw light on the uptake of adenine into muscle cells and its incorporation into nucleotides. The following are the main conclusions which can be drawn from these experiments :-

a) Adenine is readily taken up by resting muscle and it is incorporated into ATP as an intact unit.

b) The rate of adenine uptake is independent of the period of incubation; it increases with temperature, having a Q_{10} of about 2.

c) About 1/6 of the actin bound ADP in a resting muscle is rapidly exchangeable with the free nucleotide of the cell.

d) The fraction of the bound ADP which is exchangeable falls with increasing time of incubation and with increasing temperature. This suggests that some deterioration of the muscle is observable within a few tens of minutes of its removal from the animal.

e) The rate of adenine uptake is very much higher in muscles from animals captive for long periods than it is in freshly caught animals.

4) Experiments on stimulated muscle showed that :-

a) In isometric contraction, the main change in the

actin-bound nucleotide is a slight loss of tritium label from the 2 position of the molecule.

b) Loaded contracture brings about a slight exchange of the previously unlabelled 5/6 ADP with free nucleotide. The extent of this exchange seems to parallel the mechanical power output of the muscle.

c) Only about one bound ADP molecule in 2000 exchanges, at each event, in a muscle contracting at maxiumum power (over and above the resting exchange).

5) Experiments in vitro show that, at high temperatures, the actin-bound nucleotide is labile and the ATPase activity of actin depends on the preparation of the protein.

6) The results and their relation to other published work are discussed. The following are the main conclusions :-

a) Each troponin molecule modifies the properties of one of its neighbouring actins to such an extent that the actin-bound nucleotide becomes exchangeable in resting muscle.

b) There appears to be a very slight exchange of the 2-hydrogen of the actin-bound ADP in resting muscle; this exchange is increased by stimulation of the muscle.

c) In loaded contracture the (normally inexchangeable) actin-bound ADP becomes slightly exchangeable, the extent of the exchange depends on the number of fundamental events and hence on the power output of the muscle.

7) From the mechanical properties and energetics of muscle, it is possible to calculate the length (along the thin filament) between the points at which any one cross bridge makes successive attachments. This length is close to one

half turn of the actin helix.

8) A theory of contraction is proposed which is based upon the co-polymerization of actin with the myosin head.

Appendix 1

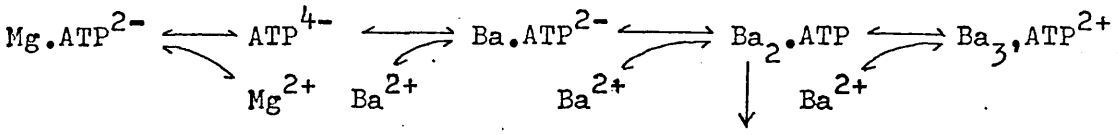
The solubility of ATP in the presence of barium

1) Experimental

Tubes were set up containing 100 mM tris - HCl buffer, pH 8.0 and approximately 2 mM ATP (disodium salt). The tubes also contained a range of concentrations of barium acetate and magnesium chloride. After standing overnight in the cold, the solutions were brought to 0°C for 1 hour. The precipitates were centrifuged down an the supernatants diluted with tris - HCl buffer. The ATP concentrations were then determined photometrically.

2) Results

The ATP solubilities are shown in fig. X.1. From the general shape of the curve, it might be thought that the reaction was simply :-



with $\left[Ba.ATP^{2-}\right] = k_1 / Ba^{2+}$ and $\left[Ba_3.ATP^{2+}\right] = k_2 \times Ba^{2+}$

or

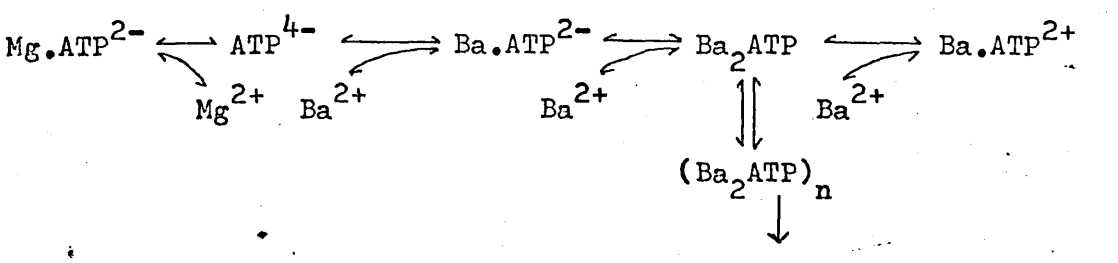
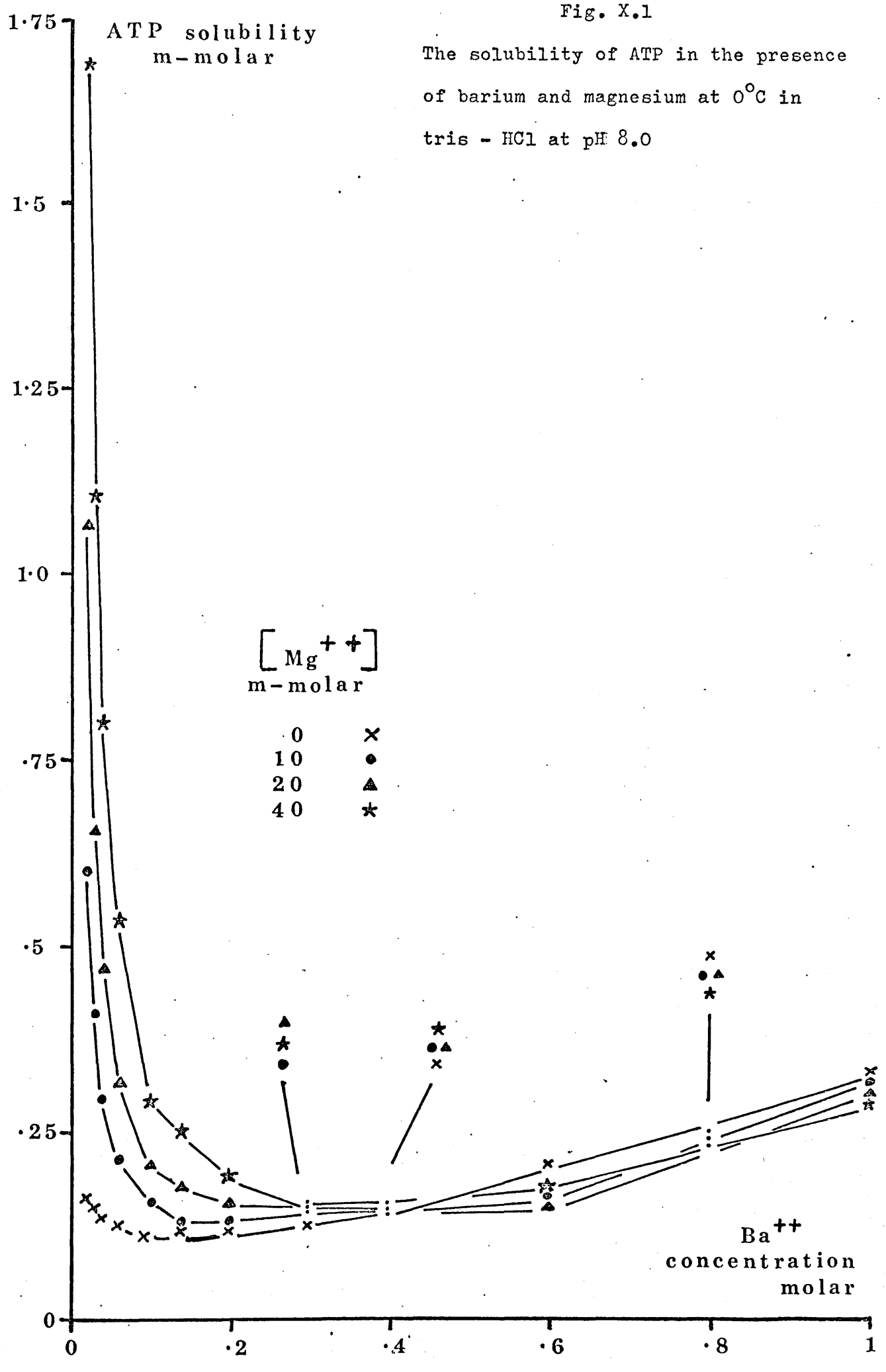


Fig. X.1

The solubility of ATP in the presence of barium and magnesium at 0°C in tris - HCl at pH 8.0



In either case, $$\frac{[Mg.ATP^{2-}]}{[Ba.ATP^{2-}]} = K \times \frac{[Mg^{2+}]}{[Ba^{2+}]} \qquad (1)$$

Both schemes predict the solubility of ATP to be of the form

$$[ATP] = A \times \frac{[Mg^{2+}]}{[Ba^{2+}]^2} + \frac{B}{[Ba^{2+}]} + C + D \times [Ba^{2+}] \qquad (2)$$

where A, B, C, and D are constants. C is zero in the case where $Ba_2.ATP$ is the insoluble species.

The coefficients were determined by a least squares method; the resulting curve is shown in fig. X.2, for zero concentration of magnesium. It can be seen that the fit is not good, especially at low $[Ba^{2+}]$.

3) Curve fitting

a) At low $[Ba^{2+}]$, the main soluble ATP complexes are probably $Ba.ATP^{2-}$ and $Mg.ATP^{2-}$. The concentrations of these species will be related by eqn. 1.

Writing $[ATP]$ to mean the total concentration of all the soluble ATP complexes :-

$$[ATP] \simeq [Mg.ATP^{2-}] + [Ba.ATP^{2-}] \quad \text{at low } [Ba^{2+}]$$

or $$[ATP] = [Ba.ATP^{2-}] \times (1 + K \times [Mg^{2+}]/[Ba^{2+}]) \qquad (3)$$

Writing $[ATP]_{Mg}$ to denote the solubility of ATP in the presence of magnesium :-

$$[ATP]_{Mg} / [ATP]_0 = 1 + K \times [Mg^{2+}] / [Ba^{2+}]$$

or $$\log([ATP]_{Mg} / ([ATP]_0 - 1)) = \log K + \log([Mg^{2+}]/[Ba^{2+}]) \qquad (4)$$

Fig. X.2

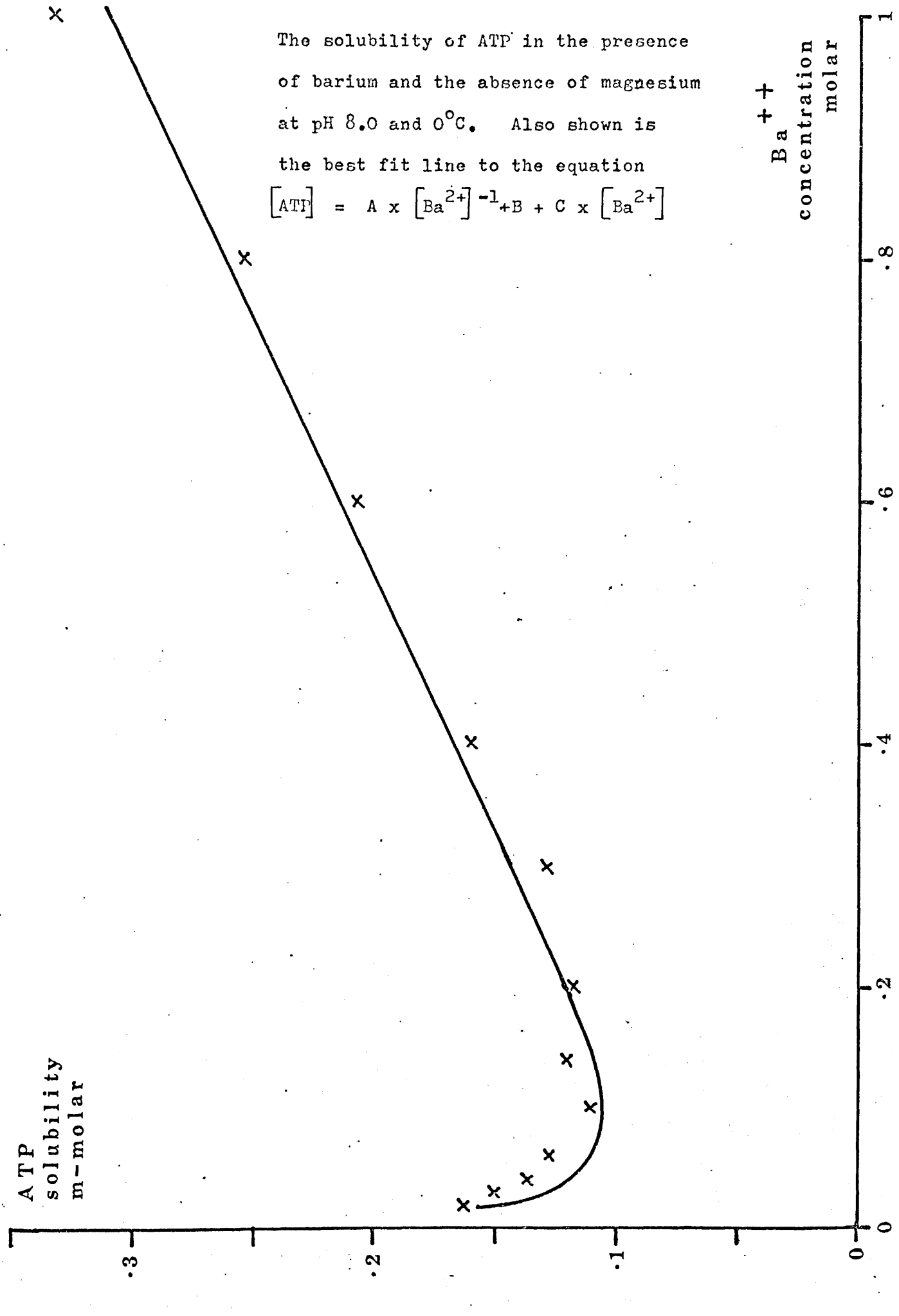

The solubility of ATP in the presence of barium and the absence of magnesium at pH 8.0 and 0°C. Also shown is the best fit line to the equation
$[ATP] = A \times [Ba^{2+}]^{-1} + B + C \times [Ba^{2+}]$
ATP solubility m-molar
Ba^{++} concentration molar
0
.1
.2
.3
.2
.4
.6
.8
1

Fig. X.3 is a plot of this function for low values of $[Ba^{2+}]$. It has the expected gradient of +1 and an intercept of 0.66. This intercept is the logarithm of the ratio of the affinity constants of ATP for magnesium and barium.

b) At any given concentration of barium, $[ATP]$ seems to be a linear function of $[Mg^{2+}]$.

From eqn. (3) :-

$$\left.\frac{\partial [ATP]}{\partial [Mg^{2+}]}\right|_{[Ba^{2+}]} = K \times \frac{[Ba.ATP^{2-}]}{[Ba^{2+}]}$$

now if $[Ba.ATP^{2-}] = k \times [Ba^{2+}]^{-1/n}$

where k and n are constants,

then $\log [ATP]_0 = \log k - (\log [Ba^{2+}]) / n$

Also $\log \left.\frac{\partial [ATP]}{\partial [Mg^{2+}]}\right|_{[Ba^{2+}]} = \log k + \log K - (1 + 1/n) \times \log [Ba^{2+}]$

So the graphs of $\log \left.\frac{\partial [ATP]}{\partial [Mg^{2+}]}\right|_{[Ba^{2+}]}$ and $\log [ATP]_0$ against $\log [Ba^{2+}]$ should have a difference in slope of 1 and a difference in intercept of log K. These graphs are shown in fig. X.4. The difference in the intercepts is 0.7. The gradients are -1.26 and -0.23 respectively, which gives a value for n of 4.

This corresponds to the reaction :-

$$4(Ba.ATP^{2-}) + Ba^{2+} \rightleftharpoons (Ba_4(Ba.ATP)_4)^{6-} \downarrow$$

The precipitate must, therefore, contain some positively charged species as well as barium. The only other ions present in any quantity are $tris^+$, Na^+ (ATP was added as the

Fig. X.3

The relationship between log $\frac{[ATP]_{Mg}}{[ATP]_0} - 1$ and

log $[Mg^{2+}] / [Ba^{2+}]$, where $[ATP]_{Mg}$ is the solubility of ATP in the presence of magnesium and barium and $[ATP]_0$ is the solubility of ATP in the presence of barium alone. The solubilities were measured at 0°C and at pH 8.0. This graph should have unit gradient and an intercept on the "y" axis of log K, where K is the ratio of the affinity constants of ATP for magnesium and barium.

The gradient is, in fact, 1.03 and the intercept is 0.66.

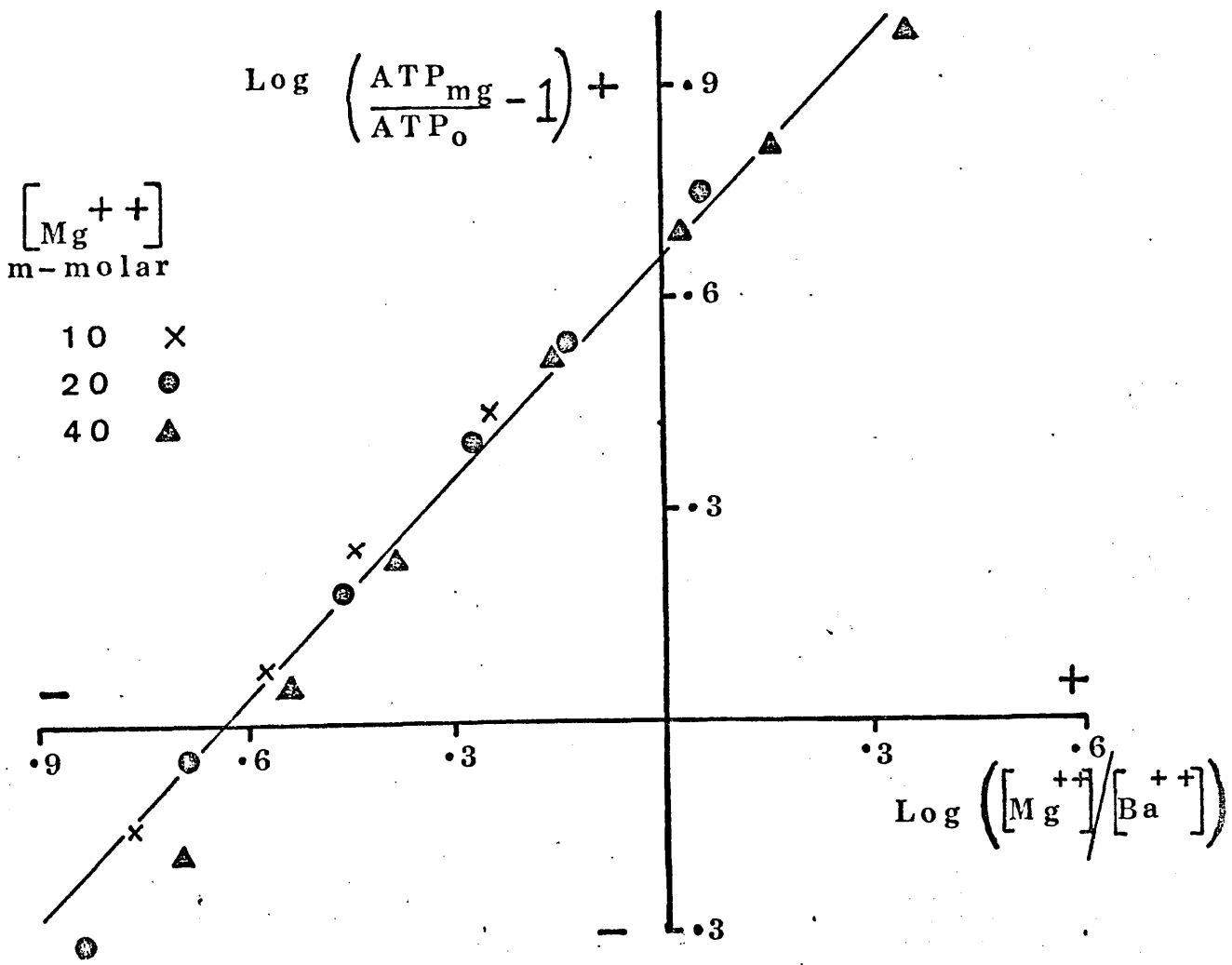


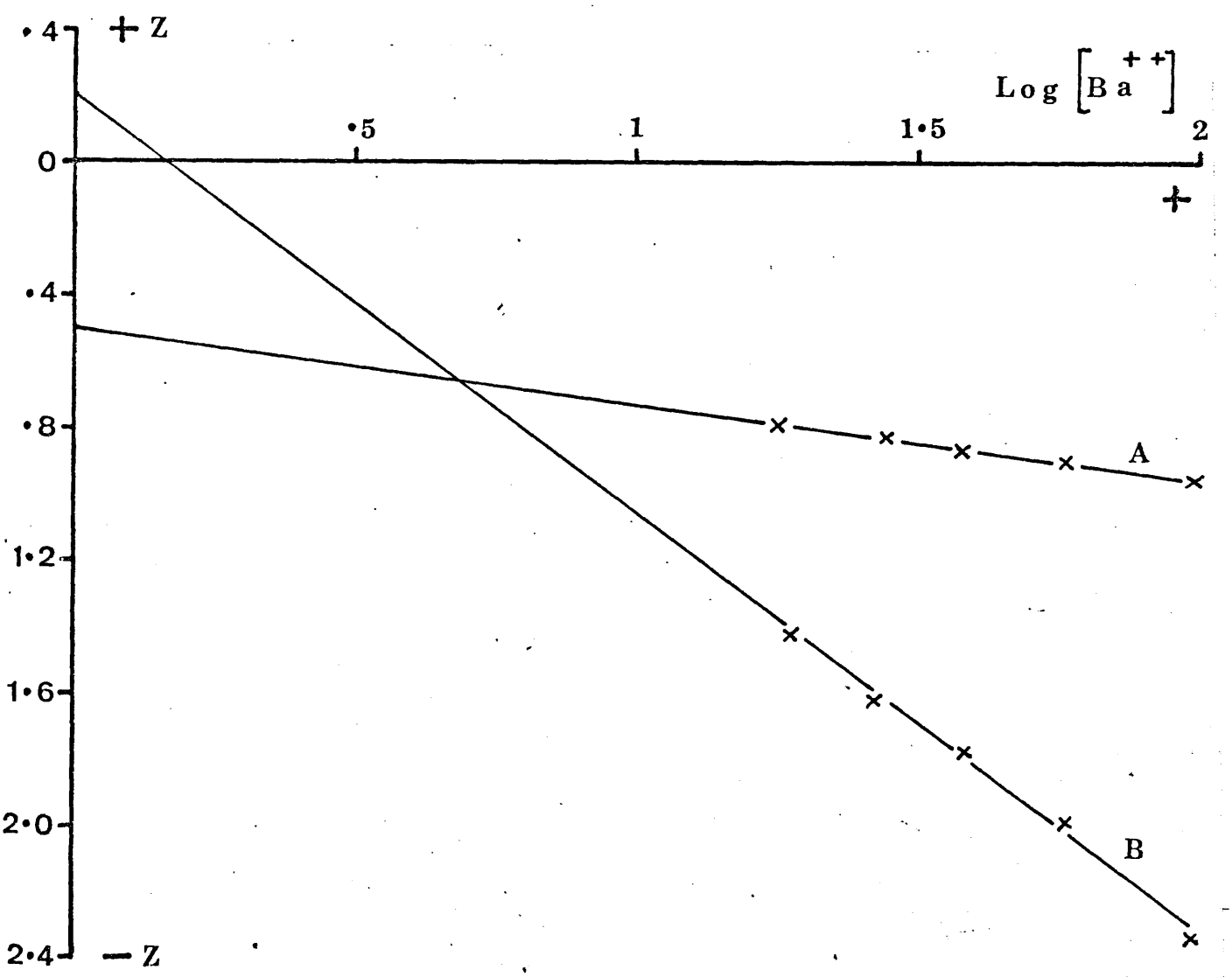
Fig. X.4

The relationships between log Ba^{2+} and the functions :-

A, $Z = \log [ATP]_0$ and B, $Z = \log \left.\frac{\partial [ATP]}{\partial [Mg^{2+}]}\right|_{[Ba^{2+}]}$

$[ATP]_0$ and $\left.\frac{\partial [ATP]}{\partial [Mg^{2+}]}\right|_{[Ba^{2+}]}$ were determined by fitting best straight lines to data for ATP solubility at varying magnesium concentrations for each of five barium concentrations. The solubilities were measured at pH 8.0 and 0°C.

The gradients of the lines (-0.23 and -1.26) should be -1/n and -(1 + 1/n) respectively; where n is the ratio of $Ba.ATP^{2-}$ to Ba^{2+} in the initial barium-ATP precipitate.



disodium salt) and sometimes magnesium.

c) The precipitate starts to redissolve at high concentrations of barium. The precipitate must be converted to the dibarium salt at a critical concentration of barium. Above this concentration the solubility should follow eqn. (2). Fig. X.5 shows the best fit curve of the form of eqn. (2) for the higher values of Ba^{2+} .

At high concentrations of barium, ATP seems slightly less soluble if magnesium is present. It is possible that there is some hydrolysis of the tribarium-ATP complex or the dibarium salt; and that the rate of hydrolysis is reduced by the presence of magnesium. But even if the whole of the rising tail of the solubility curve were due to ADP produced by hydrolysis it would not affect the general conclusion of section b). If a straight line is drawn through the origin of fig. X.1 tangent to the tail of the curve and its value subtracted from the experimental points, then the analysis of section b) yields a value of n between 3 and 4.

4) Summary of the effect of barium concentration on the solubility of ATP

As the concentration of Ba^{2+} is increased, the first complex to be formed is $Ba.ATP^{2-}$. The ATP is then precipitated as $Ba_5.ATP_4^{6-}$. At a critical concentration of barium (about 150 to 200 mM) the precipitate is converted to the dibarium salt of ATP. At higher concentrations of barium the precipitate starts to redissolve, probably as $Ba_3.ATP^{2+}$. Fogt and Rechnitz (1974) report the existence of positively charged metal-nucleotide

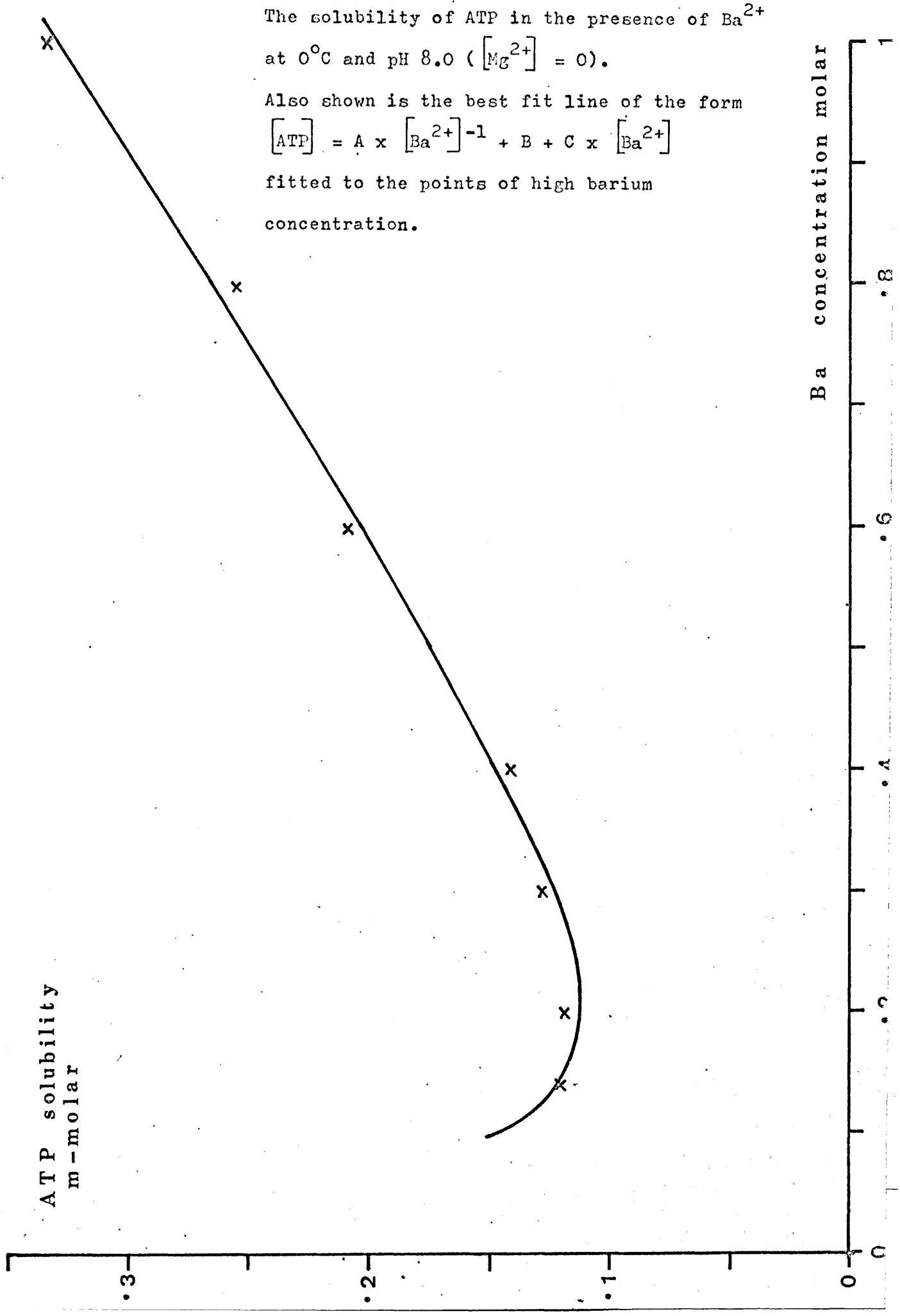
Fig. X.5

The solubility of ATP in the presence of Ba^{2+} at 0°C and pH 8.0 ($[Mg^{2+}] = 0$).

Also shown is the best fit line of the form

$$[ATP] = A \times [Ba^{2+}]^{-1} + B + C \times [Ba^{2+}]$$

fitted to the points of high barium concentration.



complexes.

At low concentrations of barium, magnesium increases the solubility of ATP, but at high barium concentrations magnesium reduces the solubility slightly.

At low concentrations of barium, the logarithm of the ratio of the affinity constants of ATP for magnesium and barium is 0.66.

The effect of pH

The solubility of ATP in 200 mM tris-HCl buffer and 100 mM barium acetate was measured over the pH range 6 to 9.5. The solubilities were measured by the same technique as above.

The results are shown in fig. X.6. If the curve can be interpreted as the ionization of a single group, then the pK is about 8.4 which corresponds neither to tris nor to ATP.

The value obtained for the solubility at pH 8.0 is rather higher than that found in the previous section (100 mM buffer).

These two facts point to the formation of a complex between ATP and tris buffer.

Titration of tris-ATP

In order to test for the formation of a tris-ATP complex, solutions of tris and ATP were titrated with sodium hydroxide. A mixture of the two was also titrated.

Fig. X.7 shows the region in which there was most difference. There was also a difference in the shape of the ATP titration curve at pH 5 to 5.5 in the presence of tris. From fig. X.7

Fig. X.6

The solubility of ATP in 200 mM tris-HCl and 100 mM barium acetate over the pH range 6 to 9.5. The solubilities were measured at 0°C.

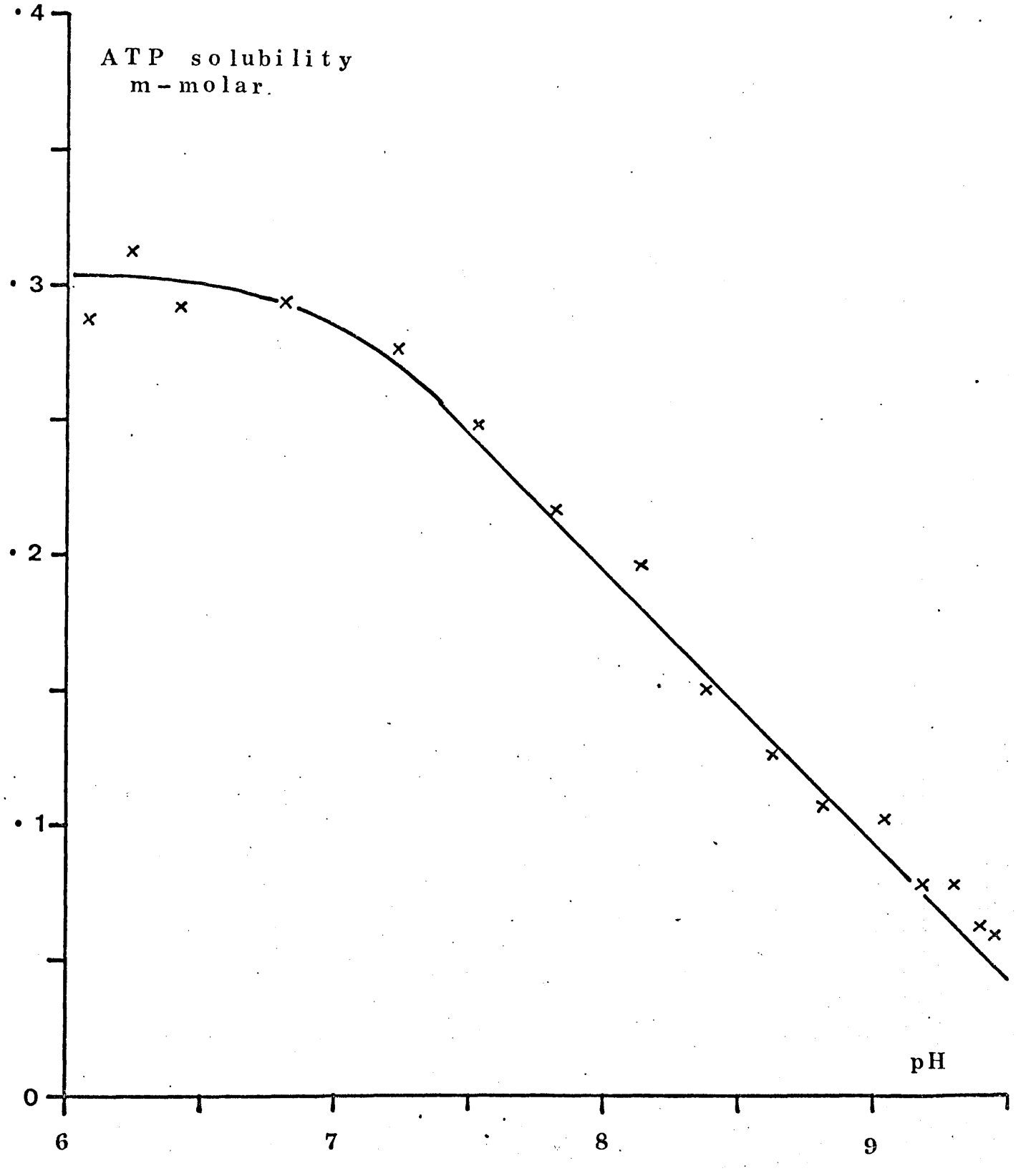
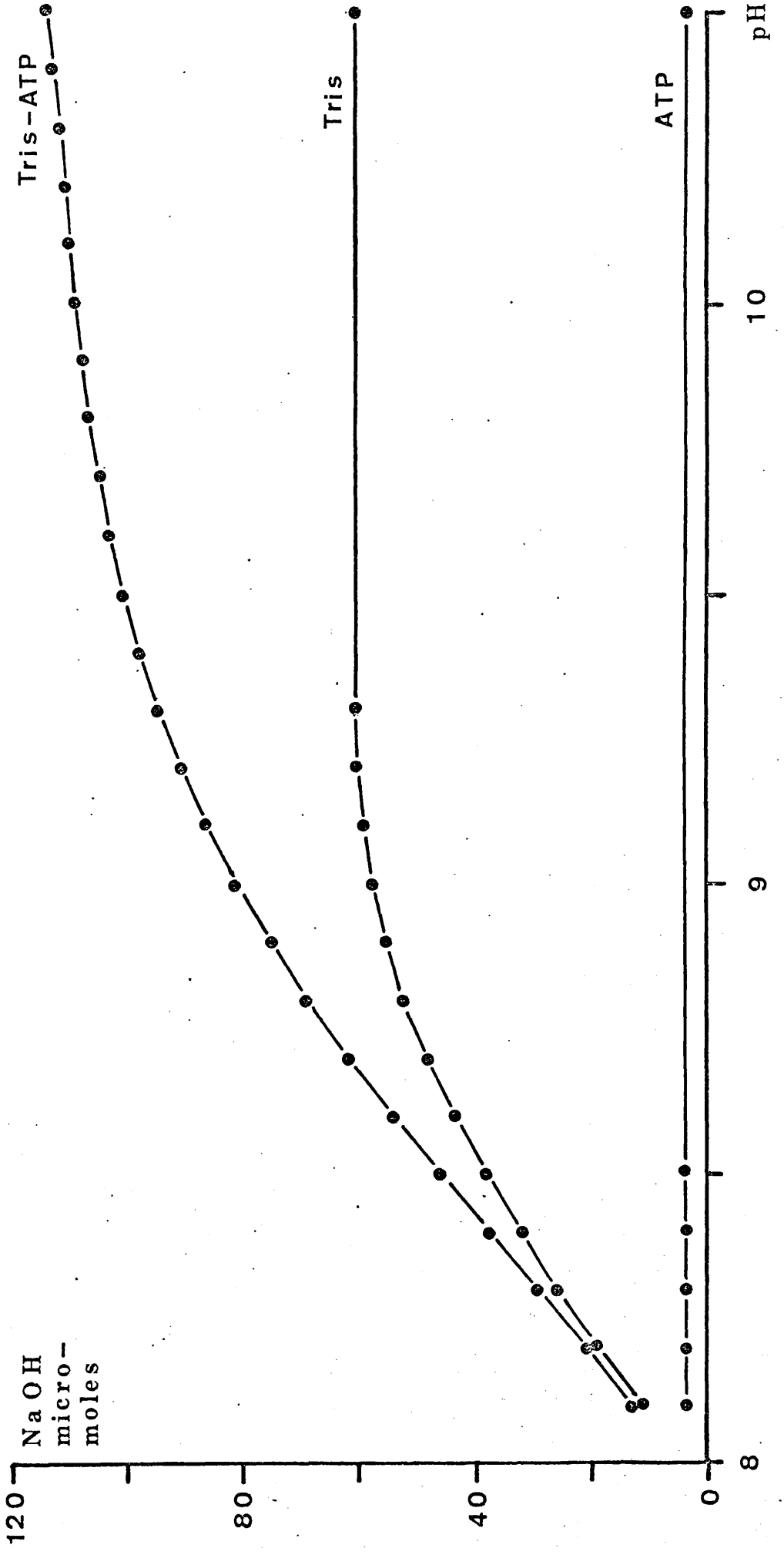


Fig. X.7

Titration curves of tris, ATP and tris-ATP.



it seems that the pK of tris has shifted alkaline (as would be expected if a complex were formed with a negative ion, such as ATP).

The shape of the tris-ATP titration curve over the pH range 8 to 10 may not be the simple superposition of a curve for tris and one for the complex. However, it would take a more detailed experiment and analysis to decide this.

Titration of tris-ATP with metal ions

If tris can form a complex with ATP (of pK 8.4) and if tris can be displaced from the complex, then the change in pK of the buffer should give rise to acidification of the solution.

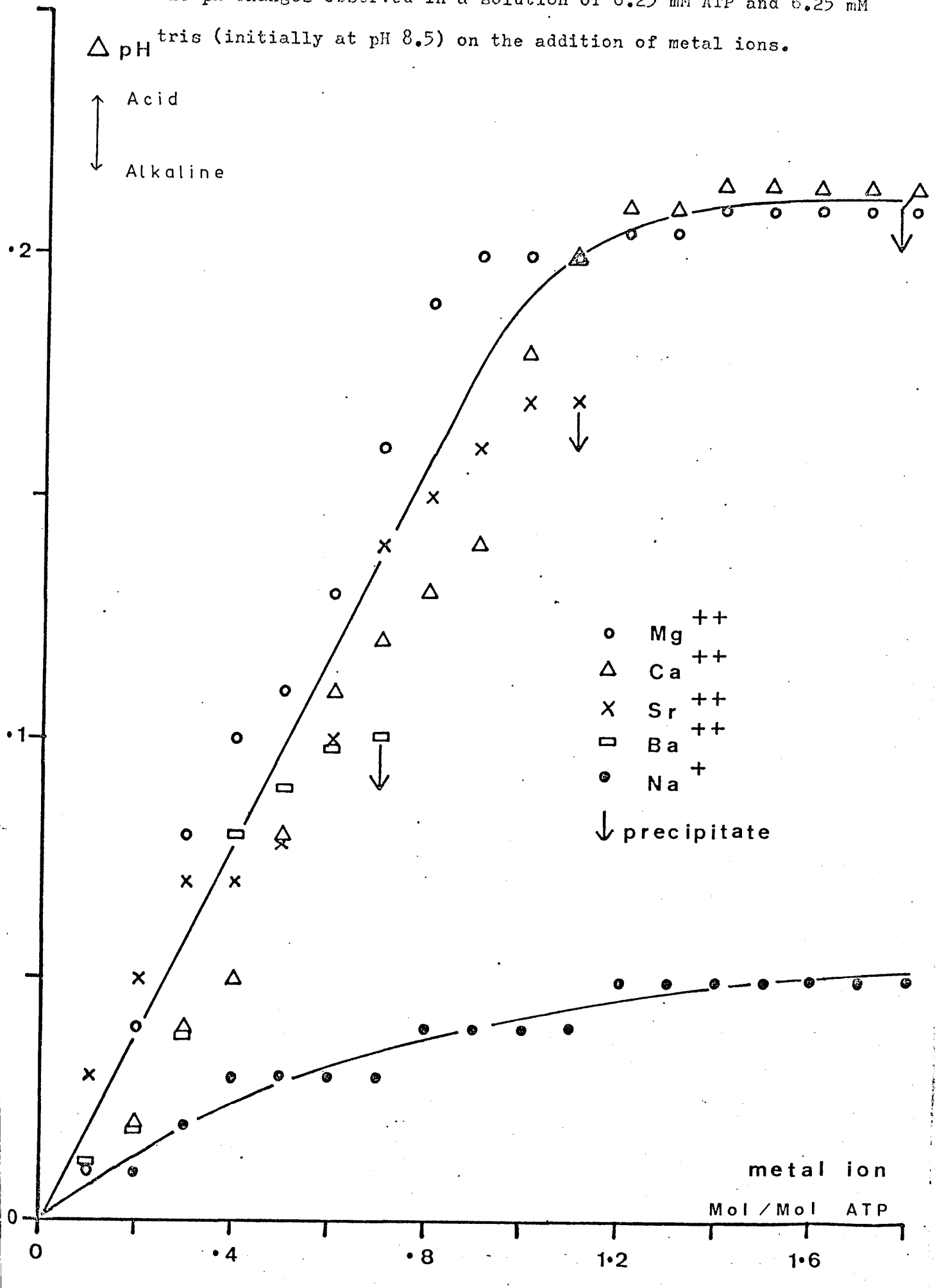
Fig. X.8 shows the observed pH shift on the addition of sodium and various alkaline earth ions. The pH shift is virtually complete after the addition of one Mol of divalent ions / Mol ATP. So the stability constant of tris-ATP must be at least an order of magnitude less than that of the metal-ATP complexes. With sodium, the pH shift is much smaller and it is not complete after one Mol of ions / Mol ATP has been added. This indicates that the tris-ATP stability constant is of the same order as, and higher than that of sodium-ATP.

The barium precipitate appeared after the addition of only 0.7 Mol of ions / Mol ATP. This is in line with the results of the solubility experiment; i.e. the initial precipitate is not the dibarium salt of ATP, but a complex poorer in barium. The strontium precipitate appeared after the addition of only 1.1 Mol of ions / Mol ATP.

In order to remove any ambiguity arising from the use

Fig X.8

The pH changes observed in a solution of 6.25 mM ATP and 6.25 mM tris (initially at pH 8.5) on the addition of metal ions.



of the disodium salt of ATP, the tris salt of ATP was prepared on an ion exchange column.

During the addition of the first Mol of ions / Mol ATP, there was acidification; see fig. X.9. But during the addition of the next one or two Mol / Mol, the solution became more alkaline. Barium gave a curve with a second pH minimum at $2\frac{1}{2}$ Mol of Ba^{++} / Mol ATP, but at this time a precipitate was present.

This secondary alkaline shift was most unexpected, as the addition of a positive ion to a complex is likely to encourage the release of protons rather than their uptake.

Further confirmation of the tris-ATP complex

As the pK of tris-ATP (8.4) is higher than that of tris and much higher than that of ATP, the mixing of the two solutions at pH 8 to 8.5 should give a product more alkaline than either component. Fig. X.10 shows the pH as tris (pH 8.2) is added to neutralised ATP. The pH rises to a maximum after the addition of 1.4 Mol of tris / Mol ATP and then falls (rather slowly) as the added tris swamps the pH change induced by complex formation.

Effect of sodium chloride

The solubility of ATP at various sodium chloride concentrations in 100 mM barium acetate, 200 mM tris-HCl at pH 8.0 was measured. Fig. X.11 shows that the solubility increases with sodium chloride concentration in a non-linear fashion.

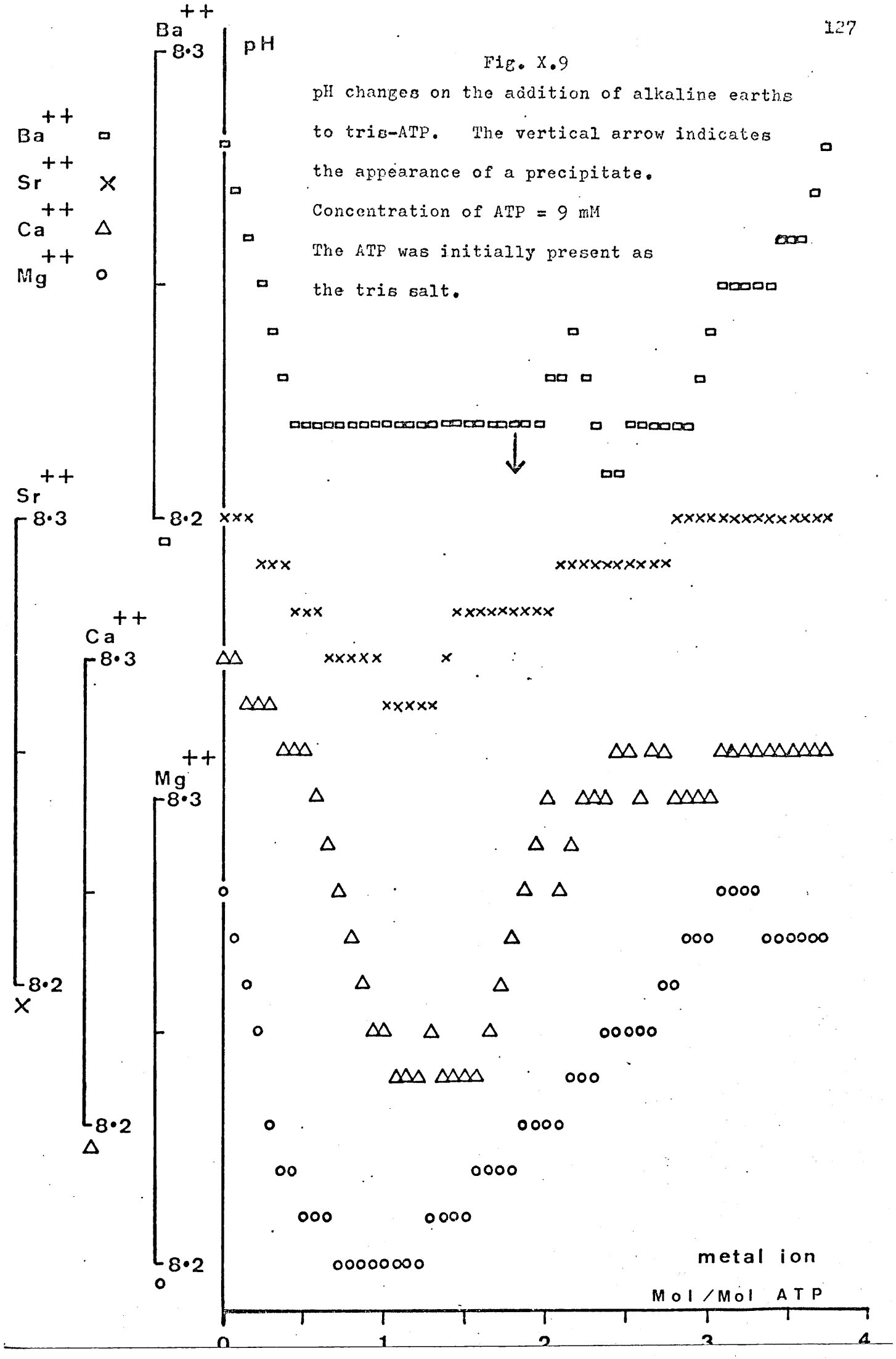


Fig. X.9

pH changes on the addition of alkaline earths to tris-ATP. The vertical arrow indicates the appearance of a precipitate.

Concentration of ATP = 9 mM

The ATP was initially present as the tris salt.

Fig. X.10

The change of pH as tris (pH 8.2) is added to ATP (initially at pH 8.13.

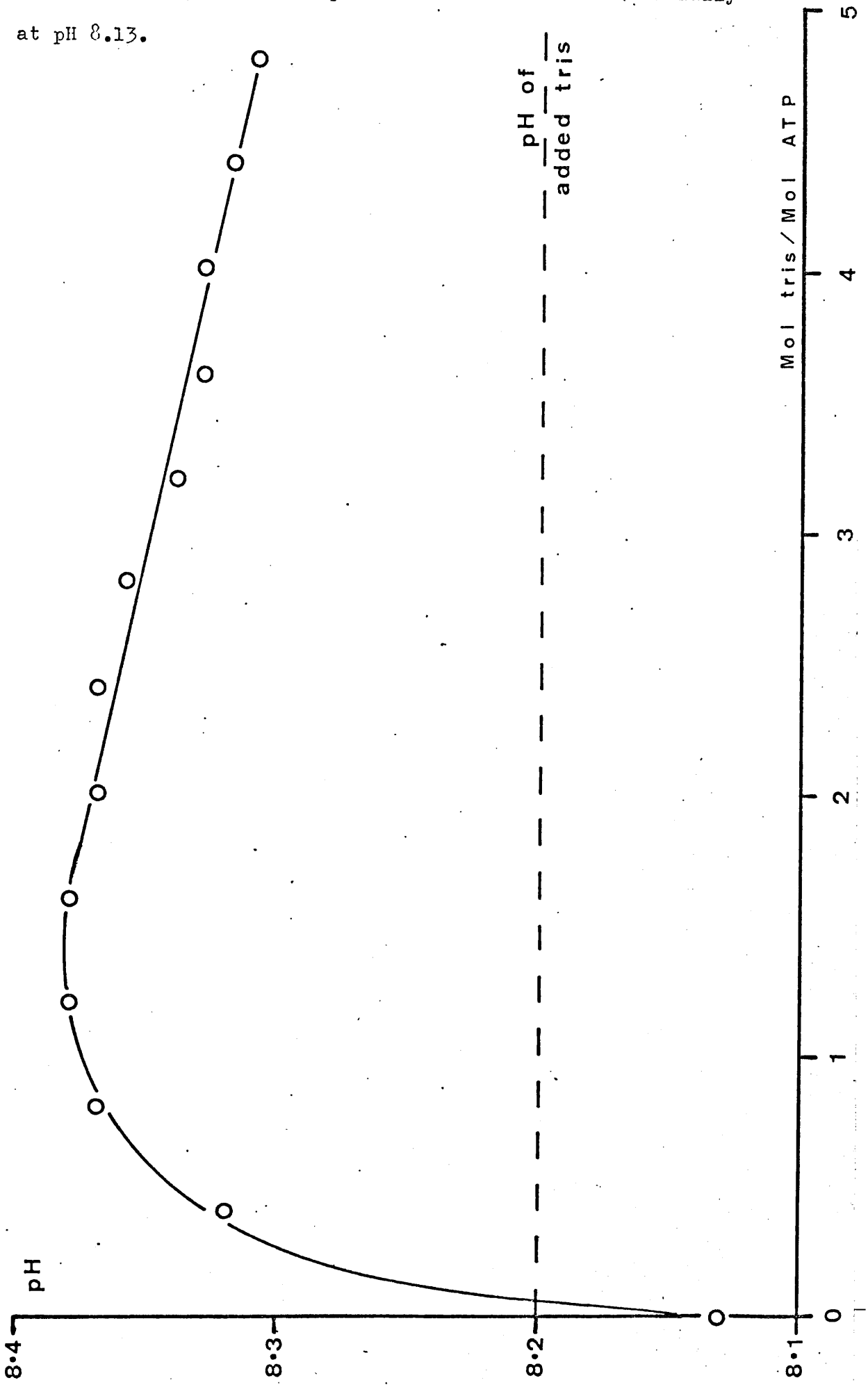
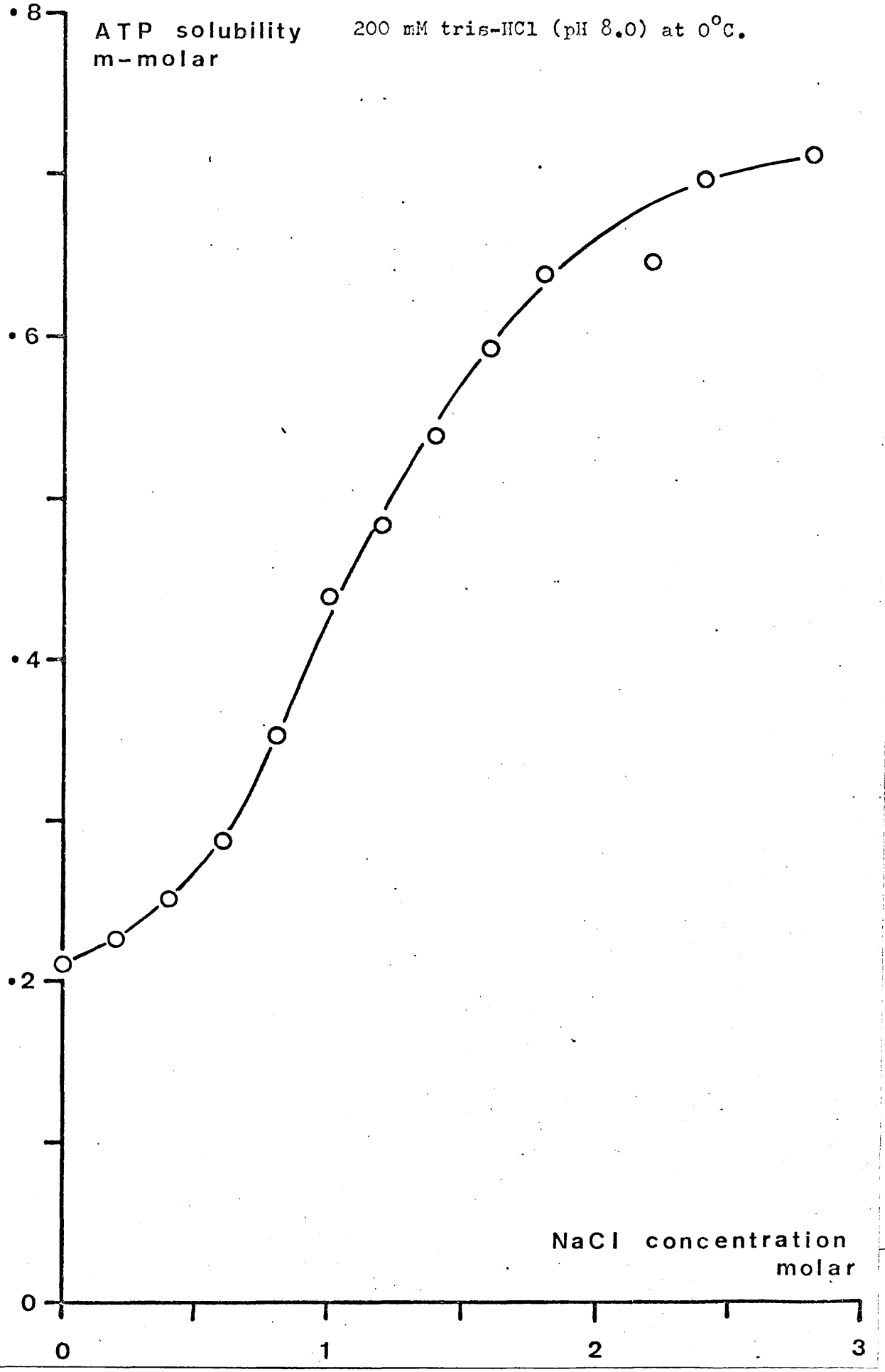


Fig. X.11

The effect of NaCl on the solubility of ATP in 100 mM barium acetate-200 mM tris-HCl (pH 8.0) at 0°C.



Triethanolamine

Since ATP forms a complex with tris, its solubility was measured in another nitrogenous buffer: triethanolamine. The solubilities were determined in 100 mM triethanolamine-HCl pH 8.0, 78 mM barium acetate and various concentrations of magnesium chloride. Fig. X.12 shows that the solubility is not quite a linear function of magnesium concentration. The solubility is rather higher that in the corresponding mixture containing tris buffer. This suggests that there is a soluble triethanolamine-ATP complex with an affinity constant higher than that of tris-ATP.

Conclusions

1) At low concentrations of barium the ATP precipitate is poorer in barium than the dibarium salt. It is probably of the form $Ba.(Ba.ATP)_4^{6-}$ with complexed cations.

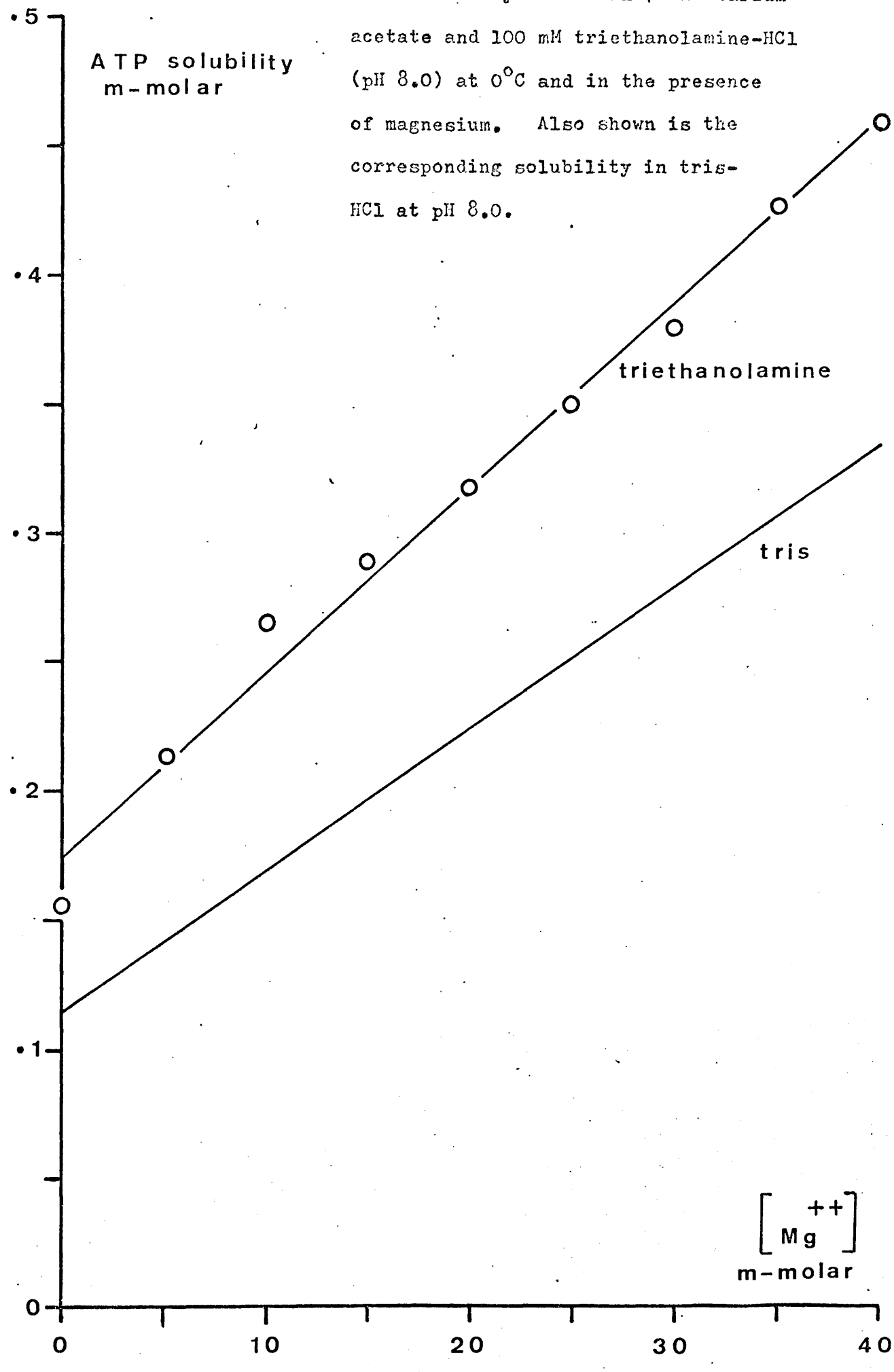
2) At high concentrations of barium the ATP precipitate redissolves slightly. The new soluble species is probably $Ba_3.ATP^{2+}$.

3) ATP forms a complex with tris buffer (and with triethanolamine). The stability constant of tris-ATP is between that of Na-ATP (log K = 1.1) and that of Sr-ATP (log K = 3.0); and is probably nearer the former. The pK of the tris-ATP complex is about 8.4.

4) In the presence of barium, the solubility of ATP is increased by the presence of any of the cations investigated.

Fig. X.12

The solubility of ATP in 78 mM barium acetate and 100 mM triethanolamine-HCl (pH 8.0) at 0°C and in the presence of magnesium. Also shown is the corresponding solubility in tris-HCl at pH 8.0.



Application to barium precipitation in the preparation of ATP

Extraction of a muscle mince results in a solution of ATP of about 0.1 to 0.2 mM in TCA. This is of the same order as the minimum solubilities found on fig. X.1.

In order to precipitate ATP with barium and obtain a good recovery, it is necessary :-

1) To use the minimum possible volume of TCA, ensuring as concentrated solution as possible.
2) To avoid the introduction of any extra cations (especially divalent cations). The muscle should only be moistened with Ringer's solution.
3) To use the optimum concentration of barium (100 to 200 mM)
4) To work at as high a pH as possible (in practice pH 9.5 to 10, there is excessive hydrolysis of ATP at higher values of pH).

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