ATP Hydrolysis by Muscle and Related Topics

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ABSTRACT

Insect flight muscle is an advantageous preparation for energetic studies as it permits unambiguous measuring of the rate of ATP hydrolysis accompanying power output and tension production under different mechanical and chemical conditions.

The ATPase activity and mean tension of a single fibre incubated in activating solution were compared at different extensions under isometric and oscillatory conditions. Analysis of individual experiments showed that the relationship between ATPase activity and tension is linear and so the regression of ATPase activity upon tension gives a measure of the cost of tension production. This was found to be much higher when the fibres were oscillated at 2% amplitude than when they were stretched isometrically.

Power output at 2% amplitude was varied by changing extension and frequency of oscillation. ATPase activity rises approximately linearly with power output at moderate extensions and over a range of frequencies around that optimal for power production. The slope of the regression of ATPase activity upon power was taken as a measure of the efficiency of energy conversion by the muscle.

The monotonic relationship between energy usage and energy production breaks down when degree of extension or oscillatory frequency are too high. The fibres can be stretched beyond an optimum tension so that power output declines but ATPase activity and tension continue to rise. At frequencies too fast for work production, ATPase activity and tension may be high. Thus the rate of ATP hydrolysis is not inflexibly linked to the power output of the muscle and is more closely coupled with tension production.

From measurements of the mid-length tension difference over a wide range of frequencies the optimum frequency for work production at 2% and 0.2% amplitude was obtained.

The experimental conditions were varied to examine the effect of changes in temperature, pH, ionic strength and anion composition of incubating medium on five indices of the muscles' performance: tension cost under conditions of static stretch; tension cost under conditions of a 2% oscillatory length change; efficiency of power output; optimum frequency for work output at 0.2% amplitude, f_{max_1}; and at 2% amplitude, f_{max_2}.

The efficiency was found not to vary under a wide range of conditions and, despite a variation of one decade in the optimum frequency for work, a mean usage of 40% of the free energy available from ATP hydrolysis was observed with a range of ± 20%.

The cost of static and dynamic tension production varied proportionally as conditions changed. Static tension cost was half the dynamic tension cost.

Optimum frequency for work production at 2% and 0.2% also varied in proportion, the optimum frequency at 0.2% being 1.5 times that at 2%.

Tension cost and f_{max} under different conditions were plotted against each other and found to be proportional. On a model of muscular contraction based on a crossbridge cycle of attachment and detachment and involving hydrolysis of one ATP molecule per cycle, both tension cost and f_{max} depend on the duration of attachment. This is determined by the rate constant of the detachment step, and a proportionality between tension cost and optimum frequency would be predicted. As this was observed such a model can be accepted.

The change of tension cost when the muscle is oscillated will occur if the mean force of the bridge falls when interfilament movement occurs. The simplest model giving such a prediction is that of a stretched spring.

On this model, allowing a 10 nm movement of the crossbridge spring, the tension cost should double with 2% amplitude of oscillation, as is observed. The measurements of efficiency and tension cost at 0.2% amplitude are also as predicted by the model.

From the physical characteristics of a stretched spring, the force exerted by a crossbridge in isometric contraction is calculated as 12 pN. The rate constant, and hence time of attachment, varies with the external conditions; in phosphate buffered activating solution at 30°C, the conditions tested most resembling life, the rate constant was approximately 100 sec^{-1} and the duration of attachment 10 msec. The percentage of bridges attached during oscillatory work production was approximately 15%.

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ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
Cr, PCr	Creatine, Phosphocreatine
Arg, P Arg	Arginine, Phosphoarginine
P_i	Inorganic phosphate
PP	Inorganic pyrophosphate
DNFB	2,4-dinitro fluorobenzene
DNP	2,4-dinitrophenol
EGTA	Ethylene glycol bis-(β-aminoethyl ether)-N,N'-tetraacetic acid
PIPES	Piperazine-N,N'-bis(2-ethane sulphonic acid)
Tris	Trizma base
PEP	Phosphoenol pyruvate
LDH	Lactate dehydrogenase
PK	Pyruvate kinase
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
A	Actin
M	Myosin
AM	Actomyosin
HMM	Heavy meromyosin
LMM	Light meromyosin
SF1	Subfragment 1
SF2	Subfragment 2
OD	Optical density
I.S.	Ionic strength
g	Detachment probability
P	Crossbridge force/cycle
p	Crossbridge instantaneous force

t_{av}	Duration of attachment
f	Frequency
ΔG	Gibbs free energy
z	Extension

CONTENTS

CHAPTER 1

Introduction

Muscle is the contractile system used in the animal kingdom to bring about movement of parts of the body relative to one another or to the environment. In the form of individual cells, sheets or organs it is found in all types of animals from coelenterates to vertebrates. Partly because of its clearly identifiable nature and function, muscle has been a favourite subject for study for over a hundred years. Many different techniques: structural, mechanical, thermodynamic, biochemical and biophysical have all been applied to extend the field of knowledge. As a result of the duration and scope of study there is a vast literature on the subject, which cannot be reviewed in a brief survey. This thesis describes the results of mechanical and biochemical studies on muscle and the relevant work in those fields will be discussed.

Muscle tissue consists of specialised, highly contractile cells. Early work on muscle, particularly on its structure and mechanical behaviour, led to the division of anatomical muscles into classes with different appearances and behaviour. The work of the last twenty years, particularly in the biochemical field, has tended to emphasise the features which all types of muscle have in common. Indeed, recent work has shown that proteins similar or identical to the actin and myosin of muscle cells are found in most structures thought to be effectors of movement in animals including the microtubules and microfilaments associated with growth and shape change in individual cells (Nachmias et al., 1971; Pollard et al., 1971). Thus the usual approach is to look for a mechanism of contraction that is common to all muscles.

Figure 1a

Electron micrograph, L.S. through frog sartorius muscle.

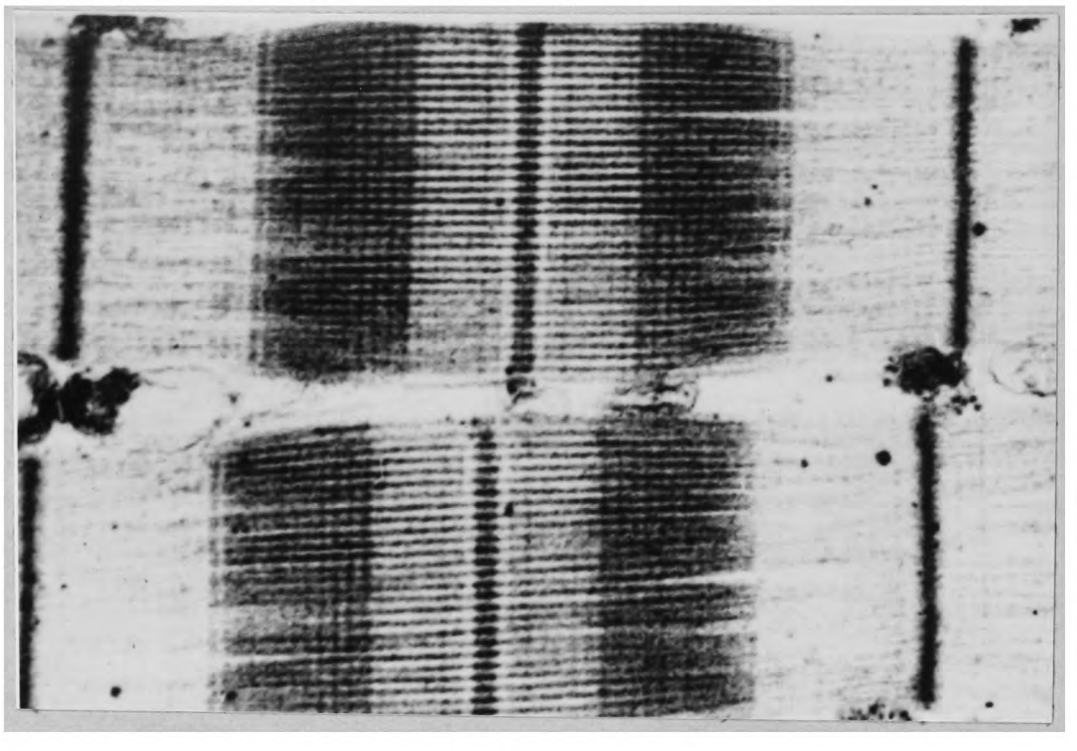
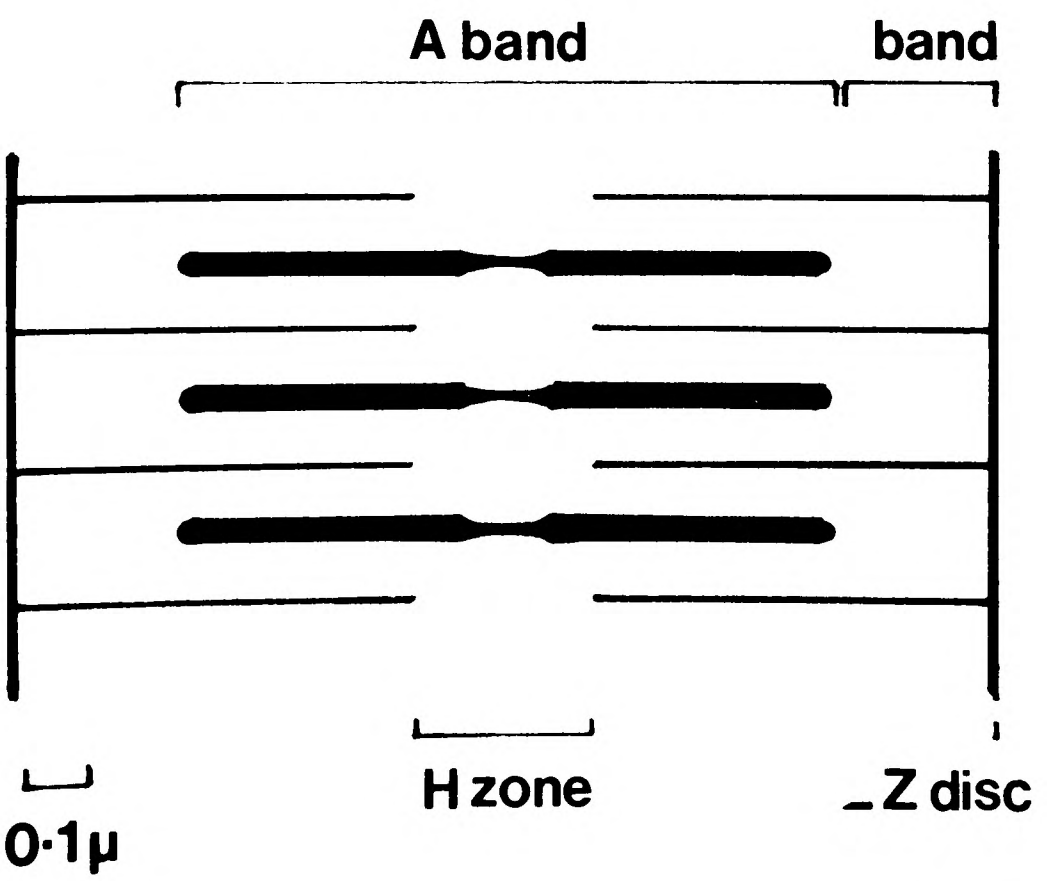


Figure 1b

Diagrammatic L.S. through sarcomere of frog sartorius muscle showing filament structure.



Muscles were originally typed according to differences in their appearance under the light microscope, a classification which was consistent with the division on the basis of their mechanical behaviour. Of the two main divisions, smooth and striated, it is the latter that, because of its structural and mechanical suitability for experiment, has been more fully investigated and most of the work reviewed here was performed using this tissue.

The typical appearance of striated muscle is shown in Fig. 1 by frog sartorius muscle. Like most striated muscles, it consists of long straight fibres running the length of the anatomical muscle and, in this case, about 100 μ in diameter. Each fibre is a syncytium of cells and is formed of hundreds of myofibrils (see Fig. 1a). The myofibrils contain all the elements necessary to muscle function: the ordered internal tubular arrangement of T-system and sarcoplasmic reticulum which is responsible for electrochemical coupling and thus control of contraction; large numbers of mitochondria which provide ATP, the fuel which is used to provide energy; and the filaments which transduce the chemical energy of ATP into mechanical energy and thus bring about contraction. The filaments are arranged in ordered units - sarcomeres - separated by dense Z discs. It is the repeated pattern of filaments and discs which gives rise to the striation visible in the light microscope.

All muscles contain the three elements which provide for powering, control and execution of contraction. Their arrangement and the degree to which they are present can be correlated with the performance and thus often with the function of the muscle. For example, muscles specialised for large and continuous power production are particularly rich in mitochondria. Instances include insect flight

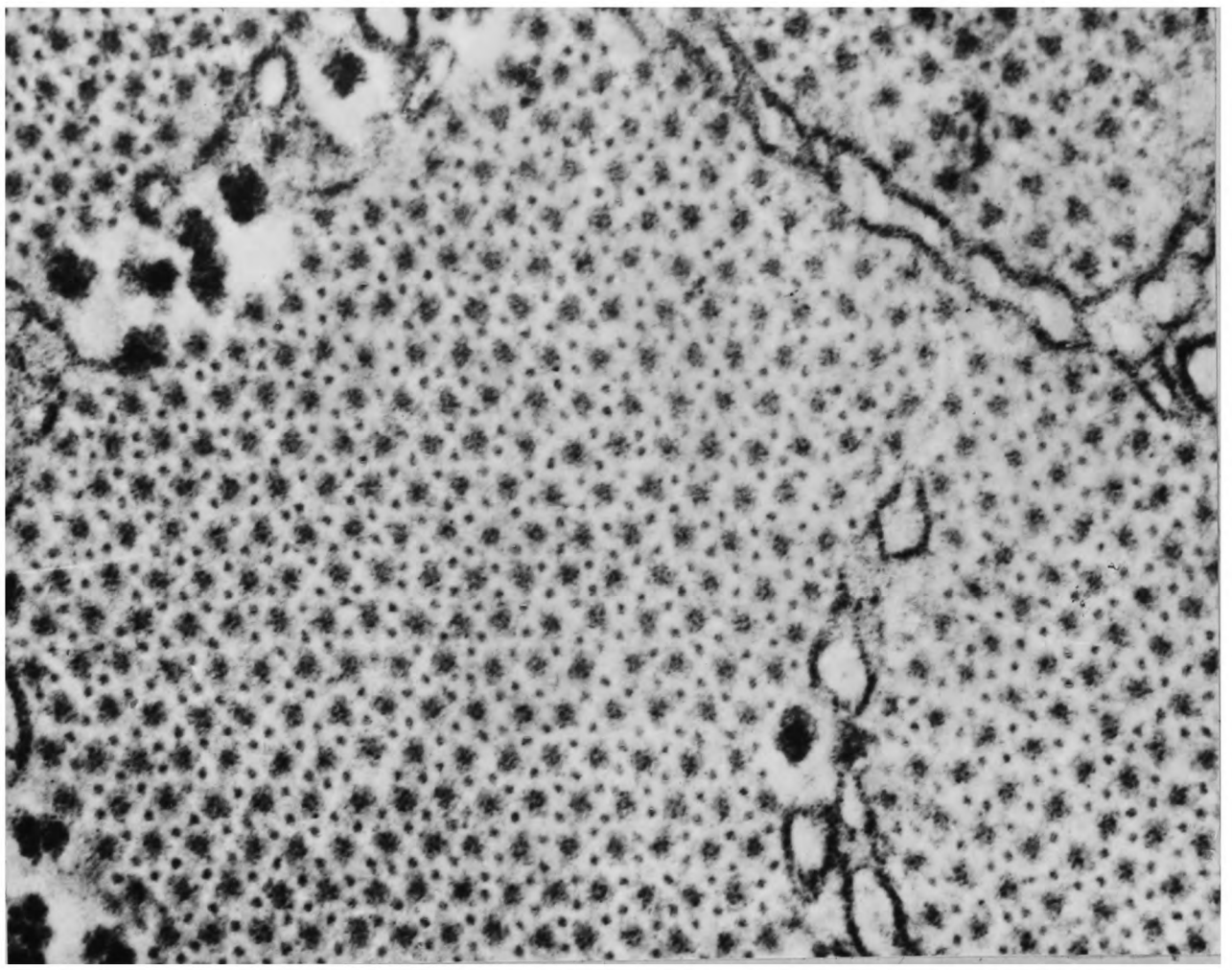
muscle (Neville, 1965), pigeon breast muscle (Ashhurst, 1969b) and squid mantle muscle (quoted by Hanson & Lowy, 1963a).

Investigation of the mechanism that controls muscle activity has shown that the contractile machinery is switched on by calcium. Ashley & Ridgeway (1970) used the luminescent protein aequorin to follow the course of calcium release from the sarcoplasmic reticulum, where it is stored (Ebashi & Endo, 1968), into the myofibrils. Calcium is released when depolarisation, either local or active, arising from stimulation of the motor end plate, spreads along the outer sarcolemma and down the associated transverse tubules to the 'triads' where the T system and sarcoplasmic reticulum are in intimate contact (Huxley & Taylor, 1958; Huxley, 1964). The degree to which this system is developed is correlated with the speed at which the muscle contracts - thus slowly shortening smooth muscle has few tubules and requires many nervous impulses before sufficient calcium is released to activate the contractile elements. Insect fibrillar flight muscle is an exception to the rule in that it has little sarcoplasmic reticulum (Smith, 1966), but can contract rapidly. However it has been shown that this muscle is peculiar in that it is activated by stretch rather than by calcium (Rüegg & Tregear, 1966).

The appearance of striated muscle in the light microscope is thus a consequence of its highly ordered arrangement at the filament and at the fibril level.

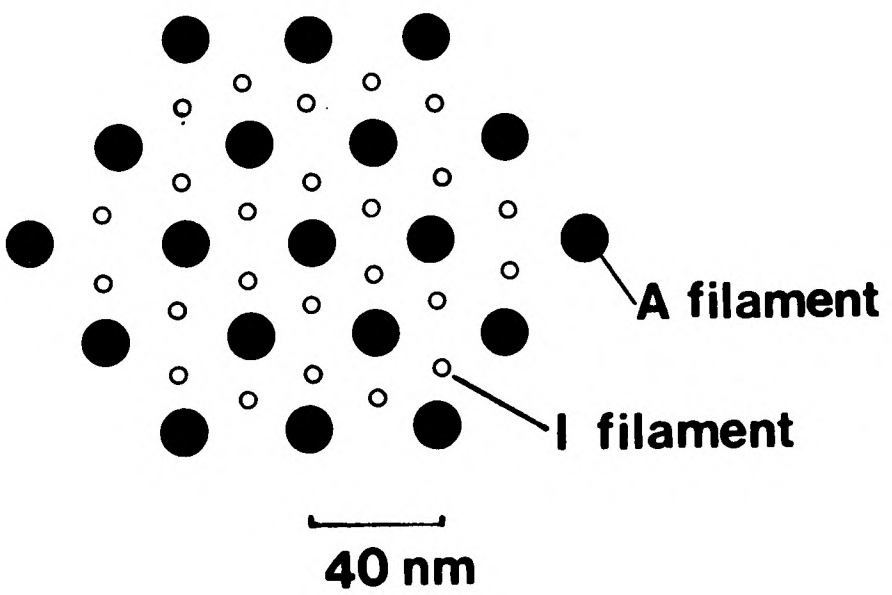
The original division of muscles into two distinct types, striated fibres and unstructured smooth cells has now been shown to be inappropriate when the ultrastructure is considered. Muscles originally classified as smooth can now be seen to have filaments of actin and myosin in more or less regularly ordered arrangements (Rice et al., 1971). The structure of the individual filaments may

Figure 2a Electron micrograph, T.S. through overlap region of A band of frog sartorius muscle



40 nm

Figure 2b Diagrammatic T.S. through overlap region of sarcomere showing filament array.



vary from type to type but it has been possible to derive all known thick filament structures from one arrangement of the constituent myosin molecules (Squire, 1971). The light-microscopic appearance of some muscles made their classification unclear as they lacked the characteristic banding pattern of striated muscle but nevertheless contained more order than true smooth types. Investigations on such muscles using the electron microscope have shown that overlapping sets of filaments are present, often with transverse order, but that the Z disc or equivalent is not always at right angles to the filament axis and the bundles of filaments themselves do not lie in the axis of the muscle, in contrast to striated muscle. Oyster adductor muscle (Hanson & Lowy, 1963a) and nematode muscle (Rosenbluth, 1965) both show such structure. The oblique arrangement of contractile units obscures the order when the tissue is studied at lower magnifications.

Thus the separation of muscles into different types on the grounds of light microscope appearance has much less rationale when the ultrastructure is considered. At this level, the divisions break down and it is seen that macroscopic structural differences reflect variations in the arrangment of a common basic pattern. It is likely that these differences reflect a gross but not molecular difference in action and that further investigation will reveal even greater similarities between muscles at the macromolecular level.

Their ordered structure and behaviour has made striated muscles the principal material for the study of the mechanism of contraction. Details of the arrangements of the bands creating the striated appearance of the fibres are shown in Figs. 1 and 2. Early theories, based on the recognition that muscles can pull but never push, attributed the contractile force to shortening of a gross stretched spring-like element between the Z discs. Early X-ray diffraction work

showed no spacing change compatible with an element shortening in such a way, and light and electron micrographs of muscle in various degrees of contraction showing changes in width of the various bands illustrated in Figs. 1 and 2, with associated X-ray data indicating that the filaments of the structure did not change length during shortening, led to the proposal of the sliding filament hypothesis (Hanson & Huxley, 1953; Huxley & Niedergerke, 1954). Improved microscopic resolution showed that the arrangement of the filaments in the sarcomere was as predicted by Hanson and Huxley and as shown in Figs. 1b and 2b, and that contraction was associated with increased interdigitation of the A filaments within the I filaments.

A number of criticisms of the theory have been made. The constancy of the length of the filaments during contraction was questioned by Carlsen et al. (1961). The electron microscope study of Page & Huxley (1963) of muscle under different conditions demonstrated that length changes observed were artefacts produced by preparative procedures and that filaments do not change length significantly during contraction. The X-ray diffraction work of Huxley & Brown (1967) confirmed this observation.

The theory predicted that overlap of the two types of filament, allowing interaction between them, should be proportional to the tension produced. The experiment of Gordon, Huxley & Julian (1966) in which tension and overlap of just a few sarcomeres of one fibre of frog muscle were compared and found to be directly related, showed this to be true. The exact sites of the interaction were thought to be projections that could be observed at regular intervals of 14.3 nm along the A filament (Huxley, 1957). It was suggested that these projections, called crossbridges, might by a cycle of attachment, pulling and detachment, bring about contraction. Evidence for this

widely accepted hypothesis has proved hard to come by although actomyosin attachment was seen by Huxley (1963). The work of Reedy, Holmes and Tregear (1965) showed that in a relaxed muscle, which has low tension, crossbridges stick out at 90° to the A filament and are unattached to the I filament, while in rigor muscle (the high tension state of a muscle deprived of ATP) the bridges are angled and apparently attached to the I filament as in actomyosin. Low angle X-ray diffraction patterns showed correlated changes, the spacing of the crossbridges along the filament modulating from one derived from the periodicity of the A filament to one related to the known distance of the I filament repeat (Miller & Tregear, 1972). Unfortunately on activation the intensities of diffraction spots attributable to the crossbridges are reduced, indicating disorder, but improved techniques have made possible the observation of changes in diffracted intensities signifying movement of crossbridges during active contraction and consistent with a crossbridge cycle of attachment, force generation and detachment (Tregear & Miller, 1969; Miller & Tregear, 1970).

Experiments on the mechanics of contraction have been performed with the aim of elucidating the behaviour of the crossbridge during the contractile events. Analysis of the rapid adjustments of length or tension of activated muscles subjected to quick changes in load or length shows that there is a brief period of non-linearity before the observed changes reach a steady-state condition of the type predicted by Hill (1938) force-velocity equation (Civan & Podolsky, 1966). Huxley and Simmons (1970, 1971a) have analysed tension transients consequent upon step length reductions during isometric tetanus. Initially there is an instantaneous reduction in tension followed by a partial recovery and then much slower full tension redevelopment. The first two stages are consistent with the existence of an undamped

elastic and a damped elastic element in series between the filaments and thus identifiable with the crossbridge. Recent work by Haselgrove (1970) gives support to the spring model of a crossbridge.

The length change necessary to reduce to zero the tension in the elastic element in Huxley & Simmons' experiments was 10 nm. This figure is a measure of the distance through which a crossbridge may move and is similar to estimates from other sources (Podolsky et al., 1969; White, 1970).

Thus structural and mechanical studies have provided evidence that muscle contraction involves the interdigitation of filaments brought about by repeated attachment and detachment of the filaments via the crossbridges. A repetitive cycling of the bridges must occur as they have an amplitude of movement much less than that of the overall structure. There is evidence that the crossbridges behave as elastic elements.

The likelihood that the site of production of mechanical energy would also be the site of generation of chemical energy led workers to try to identify the crossbridges with the site of ATP breakdown. Tice & Smith (1965) coupled the ATPase reaction to lead precipitation and produced micrographic evidence that ATPase activity was localised in the region of filament overlap. This work was repeated using rabbit psoas muscle by Gilles & Page (1967) who could not obtain a specific distribution of lead phosphate. Tice (1969), using indirect flight muscles of Phormia showed that non-enzymatically produced lead phosphate was randomly distributed whereas that by ATP hydrolysis was localised in the thick filaments. However it is really at this point that evidence obtained by biochemical techniques takes over from the more structural approach.

The characteristic proteins of muscle have been the subject of study for 30 years. Early workers were able to extract them from intact muscle using high ionic strength solution and by reprecipitating them in more dilute solution, reconstituted contractile threads consisting, at least in part, of filamentous protein with an alpha configuration. The extracted protein was found to be a mixture of two proteins: myosin and actin, which could combine to form a complex, actomyosin. The work of Hasselbach (1953) and Hanson & Huxley (1953), involving selective removal of the proteins from intact fibres showed that the A filaments were composed mainly or wholly of myosin and the I bands of actin.

Work on the myosin monomer has shown it to be a filamentous molecule of molecular weight 470,000 (Godfrey & Harrington, 1970). Physicochemical and X-ray diffraction studies reveal that it is composed of two strands but they are too intertwined to be separated. However the molecule can be split by tryptic digestion into two parts, light meromyosin (LMM), a supercoiled α-helix, and the much less helical globular heavy meromyosin, HMM (Lowey & Cohen, 1962). Further digestion, with trypsin or papain, splits HMM into two globular S-1 subfragments and a short α-helical stalk S-2. Electron microscopy of shadowed, negatively stained myosin molecules and fractions has revealed a long rod-like tail with two globular heads of a similar size to the S-1 fragments. The molecules appear to be flexible in the S-2 region (Slayter & Lowey, 1967; Mendelson et al., 1972). This area is particularly sensitive to proteases and it was postulated that it had an amorphous structure. However, Lowey et al., (1969) although finding it much more soluble than the rest of the rod, showed it to be equally helical in structure. The S-1 fragment has been identified as the site of actin binding (Mueller, 1965;

Moore et al., 1970). The active site of the myosin ATPase enzyme has also been located on the S-1 part of the molecule by the use of inhibitors of the enzyme activity (Schliselfeld & Bárány, 1968) and by employing ATP analogues. Murphy & Morales (1970) by this means were able to distinguish two equivalent, non-interacting binding sites for ATP on the HMM molecule. The separate identity of the sites for actin and ATP has been established by showing that agents which destroy the ability to bind one species can leave the other combining property unaffected (Perry & Cotterill, 1964; and others).

The heavy meromyosin portion of the myosin molecule has thus been shown to consist of two globular heads each with ability to combine with actin and with ATP, both joined to one end of a short helical region with flexibility possibly of an elastic nature. The LMM rod which, with this section, forms the myosin molecule tail, has the ability to combine with like molecules forming smooth filamentous structures (Huxley, 1963).

Aggregates of intact myosin molecules resemble natural A filaments, having a knobbly appearance except for a small region: the middle of the structure. Huxley (1963) showed that the monomers are arranged in each half of the filament in a regular parallel manner with the globular heads outside, the polarity reversing at the middle which, being composed only of the tail section of the molecules, appears smooth. Thus it is the HMM portion of the myosin monomer which forms the structures identified as crossbridges. Further evidence of their common identity was obtained by Huxley (1963) who showed that HMM molecules attached to actin filaments in rigor conditions adopt an arrowhead configuration similar to that which can be observed in intact muscle (Reedy et al., 1965).

Biochemical work on the proteins of muscle has thus led to the identification of a specific molecule with appropriate properties with the crossbridge implicated by the structural studies at the site of mechanochemical transduction. Parts of the molecule with the ability to aggregate to form polarised filaments, to split ATP, to combine with actin, and to form a flexible linkage have all been identified.

The work of Hanson & Lowy (1963b) showed that actin could exist either as a globular G-actin monomer, molecular weight 45,000, or at higher ionic strength as a fibrous F-actin polymer consisting of two strands of monomers helically twisted with 13 monomers per single strand turn of 380 Å and identical in structure to I filaments visualised in electron micrographs. X-ray diffraction has confirmed the existence of such a structure in living insect muscle (Miller & Tregear, 1972). High resolution electron microscopy and reconstruction techniques enabled Moore et al., (1970) to produce a detailed picture of the I filament with a reversal of polarity about the Z line reflected in the change of direction of the HMM or S-1 arrowheads. They showed that each actin monomer can combine with an S-1 unit so that the myosin fragment is angled axially and azimuthally to the I filament. Such angling of attached crossbridges is also deduced from X-ray diffraction results of Huxley & Brown (1967).

Later work has also revealed the existence of other proteins specific to muscle. Two supercoiled helical proteins have been identified; paramyosin, which may form a central core for some myosin filaments, e.g. smooth muscle (Szent-Györgyi et al., 1971) and arthropod striated muscle (Dewey et al., 1972), and tropomyosin, which combines with actin (Martonosi, 1962). Optical diffraction studies (O'Brien et al., 1971) and fluorescent antibody techniques

(Pepe, 1966) have been used to demonstrate the presence of tropomyosin in the grooves of the actin helix. It adopts a polar end-to-end arrangement with a 400 Å period (Caspar et al., 1969), which is observed by X-ray diffraction (Huxley & Brown, 1967).

The globular protein, troponin, which has been found at intervals of 400 Å along the tropomyosin, with which it combines (Ohtsuki et al., 1967; Hartshorne & Mueller, 1967), has been shown to consist of two compounds, A and B. Troponin B has the property of inhibiting actomyosin ATPase and troponin A, which binds calcium, of relieving the inhibition. In the presence of tropomyosin the inhibition is relieved only in the presence of calcium ions (Hartshorne, 1970). Thus the inhibition of actomyosin interaction requires the interaction of the three protein moeities. X-ray evidence shows that the response to calcium involves a slight movement of tropomyosin (Cohen et al., 1972; Parry & Squire, 1972).

The troponin-tropomyosin system has been shown to be responsible for calcium-reversed inhibition of actin-myosin interaction and ATPase activity by Ebashi & Endo (1968) in vertebrate systems and Maruyama et al., (1968) for arthropod muscle. Lehman & Szent-Györgyi (1972) and Kendrick-Jones et al., (1970) have demonstrated that thin filaments from a variety of vertebrate and arthropod species which contain actin, tropomyosin and other components show calcium dependent ATPase activity in the presence of myosin in contrast to molluscan filaments which lack the additional components and do not bind calcium. Meinrenken (1969) demonstrated, using intact glycerinated fibres, that removal of the troponin-tropomyosin system resulted in free-running ATPase activity and tension production in the absence of calcium. Combination of the extracted protein with actomyosin restored the calcium requirement for activation.

Thus structural and biochemical work has identified the myosin crossbridge as the site of transduction of energy from a chemical to a mechanical form. The experiments of Huxley & Simmons have given evidence concerning the possible nature of the mechanism of mechanical energy transfer from the crossbridge to the filament structure. The biochemical work concerned with the energetics of contraction will now be considered.

Early work on actomyosin gels showed that contraction required the presence of MgATP and that the rigor state which characterised the absence of ATP could only be relieved by adding more ATP (Szent-Györgyi, 1949). The structural analogue of this observation was demonstrated by Huxley (1963) who showed that the 'arrowhead' configuration of actin and HMM only appeared when ATP in the ambient solution was used up and that HMM was removed by addition of more ATP. However evidence of the necessity of ATP for contraction in intact muscle systems has proved more difficult to obtain. Carlson & Siger (1960) and others, were unable to demonstrate the net breakdown of any ATP in frog muscle contractions, although the splitting of phosphocreatine (PCr) proceeded to a degree comparable with the amount of work done in various contractions (Cain et al., 1962; Mommaerts et al., 1962). The use of 1-fluoro-2,4-dinitrobenzene (FDNB) for poisoning creatine kinase enabled Infante & Davies (1962) to demonstrate a net splitting of ATP during a contraction accompanied by no PCr breakdown. This having been shown, it was generally accepted that, as the experiments on actomyosin gels indicated, ATP was the primary source of energy for contraction, but that in vivo it was replaced as soon as it was hydrolysed by transphosphorylation of ADP from CrP, or arginine phosphate in insects, by the enzyme creatine kinase.

As this apparently accepted theory has been called into question (Banks, 1969) it is worth noting that the use of glycerinated fibrillar flight muscle in experiments such as those of Rüegg & Tregear (1966) and others, are a convincing demonstration that ATP is a necessary and sufficient source of energy for muscular contraction. Glycerinated fibrillar fibres, when supplied with sufficient calcium for activation and with magnesium ATP as the only possible energy source will, in suitable mechanical conditions, produce power for long periods with accompanying hydrolysis of ATP. In the absence of ATP fibres enter the rigor state and no work can be produced.

Estimates of the free energy available from one molecule of ATP for transduction into power are hard to obtain. It is generally assumed that the free energy, ΔG, is approximately equal to the enthalpy, ΔH, a quantity which, though easier to estimate, is nevertheless the subject of debate (Kushmerick & Davies, 1969; Woledge, 1971). Values calculated from *in vitro* experiments must take into account the many associated reactions that occur in the living cell; in consequence the estimates obtained vary widely from -4.7 kcal per mole (Podolsky & Morales, 1956) to -12 kcal per mole (Meyerhof & Lohmann, 1932). Experiments on muscle in which PCr or ATP usage and heat and work production were measured, giving, it was hoped, values of the total energy used and produced by the contractile elements, also gave dissimilar values of ΔH: 9.8 kcal/mole (Carlson *et al.*, 1963), 11.0 kcal/mole (Wilkie, 1968), 13.18 kcal/mole (Walsh & Woledge, 1970). The problem remains unresolved.

As all the energy produced from ATP should be observable and measurable, as either work or heat, it should be possible to chart the energy usage of muscle, although Woledge (1971) has pointed out that heat changes of entropic origin will not be accompanied by any

chemical activity. Changes should, however, be reversed in the course of a complete contraction-relaxation-recovery cycle and thus not affect overall heat measurements. Thermopiles and ergometers of various designs, and the development of rapid freezing techniques have made possible simultaneous measurements of heat and work output and chemical change in whole muscle preparations. PCr breakdown is often measured in preference to ATP splitting because the FDNB poisoned muscle is capable of far fewer contractions than the preparation used for PCr analysis as it does not have the benefit of the PCr energy store. The first notable step in muscle energetics can be attributed to Fenn (1923) who demonstrated a mobilisation of energy proportional to the work done by the muscle in the so-called 'Fenn effect'. He also noted a fairly constant term in the heat production which has been assumed to derive from energy used in activation. Later work has identified chemical breakdown apparently associated with both activation heat and work-related heat (Carlson et al., 1963; Cain et al., 1962). Analysis of heat production and chemical breakdown showed both to consist of a constant quantity attributable to the activation process and a quantity varying in proportion with the work done by the muscle. Estimates of the constant of proportionality between the total work plus heat produced and chemical energy split, which form the basis of the in vivo measurements of Δ H recorded above, vary considerably, as the quoted figures show. Neither do they tally with in vitro measurements (Woledge, 1971).

Estimates of the chemistry of activation also differ (Maréchal, 1964; Jöbsis & Duffield, 1967; Kushmerick et al., 1969). It is generally agreed that ATP or PCr splitting, where found to be positive, reflects the involvement of energy in the pumping back of calcium into the sarcoplasmic reticulum. Woledge (1971) suggests that the difficulty

of completely eliminating any actomyosin interactions may account for some of the discordant results.

No chemical equivalent has been found to the heat reported by Hill (1938, 1964) to be produced proportional to shortening in isotonic contractions (Mommaerts et al., 1962; Infante et al., 1965; and others). Carlson et al., (1963) were unable to find a net heat of shortening at the end of a complete cycle of contraction and relaxation, and on the basis of their chemical results Kushmerick & Davies (1969) attributed apparent production and resorption of shortening heat to the operation of an unknown, reversible reaction. Aubert & Lebacq (1971) find their heat results incompatible with both claims.

It is clear, therefore, that energy balance studies of such a type have revealed inconsistencies in the pattern of energy usage. If the in vivo enthalpy of ATP splitting is much less than 10 kcal/mole, most studies reveal heat unaccounted for by the known primary reactions of contraction. If only the contraction phase or, even more, just part of it, are considered, the discrepancies between ATP consumption and energy produced become increasingly marked (Carlson et al., 1967; Gilbert et al., 1971) as the initial excess of heat production over chemical change and the reverse situation after contraction, become manifest. It is known that the FDNB poisoned muscle is the site of a number of reactions apparently geared to the maintenance of a stable concentration of ATP (Kushmerick & Davies, 1969); one of these, hexose monophosphate formation, could be responsible for at least part of the inconsistency (Kushmerick & Davies, 1969). Hexose phosphate formation may occur in muscles in which phosphocreatine is measured (Mommaerts et al., 1962; Spronck, 1965). However, estimates of the degree to which these esterifications occur vary widely and it is improbable that the discrepancies are wholly accounted for by such reactions

(Woledge, 1971). At present it is not possible on this preparation to identify all the chemical reactions occurring or to account for the observed energy production by measurements of known chemical changes.

A completely different approach, derived from enzymology, has been to use muscle as an enzyme system and to seek to analyse its stoichiometry and kinetics, in particular looking for chemical steps which can be related to known events in the contraction cycle of the whole muscle. Recent work using actin, heavy meromyosin and labelled nucleotides in stopped flow apparatus and involving rapid separation of species on columns has shown convincingly that ATP is split by myosin while it is detached and that the separation of the product ADP from the enzyme constitutes a rate limiting step (Taylor et al., 1970).

Later work (Lymn & Taylor, 1971) showed that ATP induced extremely rapid dissociation of acto-heavy meromyosin and that actin combined with the intermediate myosin-product complex, M.ADP.P_i, at a site distinct from the nucleotide binding site, thus initiating the next step in the cycle. Whether the attachment of actin brings about displacement of the products of ATP hydrolysis is at present not clear. Eisenberg et al., (1972) conclude from measurements of ATP hydrolysis rates in solution that HMM exists in a refractory state, unable to combine with actin, for half the cycle time. The implication that the attachment step is rate-limiting is supported by evidence from X-ray diffraction studies showing a maximum of 20-30% of bridges attached at any one time (Miller & Tregear, 1970). Thus stages in a cycle of crossbridge activity deduced from structural work can be correlated with steps of an enzymatic cycle of actin-myosin interaction. A condition in muscle fibres which parallels the identification of M.ADP as a semistable species in solution has been established by

Marston & Tregear (1972) who observed the existence of a long lived M.ADP complex in relaxed fibrils. However a complete analogy between structural and biochemical cycles cannot be drawn as a number of discrepancies between the two still exist. Methods of observing configurational change in macromolecules, e.g. optical rotatory dispersion, have shown that substrate binding to myosin does not result in a large conformational change (Gratzer & Lowey, 1969). On structural grounds, considerable changes in myosin in the course of the cycle are predicted.

Biochemical studies have been linked with observations on intact muscle systems in another way. Bárány (1967), Buller et al., (1969) and Bárány & Close (1970) have measured the contraction speeds of a variety of anatomical muscles and found a positive correlation between these and the ATPase rates of myosin extracted from the muscles. Cross-innervation of fast and slow muscles resulted in appropriate change in contraction speeds of the muscles and associated rates of ATPase of their myosin. It has been postulated that light subunits, low molecular weight 15,000 - 25,000, fractions from heavy meromyosin which can be removed by treatment with dithio bis nitro-benzoic acid or alkali, are responsible for control of the ATPase activity of the myosin molecule. The number and composition, as judged by the thiol group sequences studied, are not species specific but related to the type of muscle of origin (Lowey & Risby, 1971; Sarkar et al., 1971). Weeds & Pope (1971) showed that ability to hydrolyse ATP could be reversibly removed from myosin by dissociation of the alkali-sensitive light chains.

To summarise, both the biochemical and the structural approach to the problem of muscular contraction have led to the concept of a transducing element going through a cycle of operations during activity.

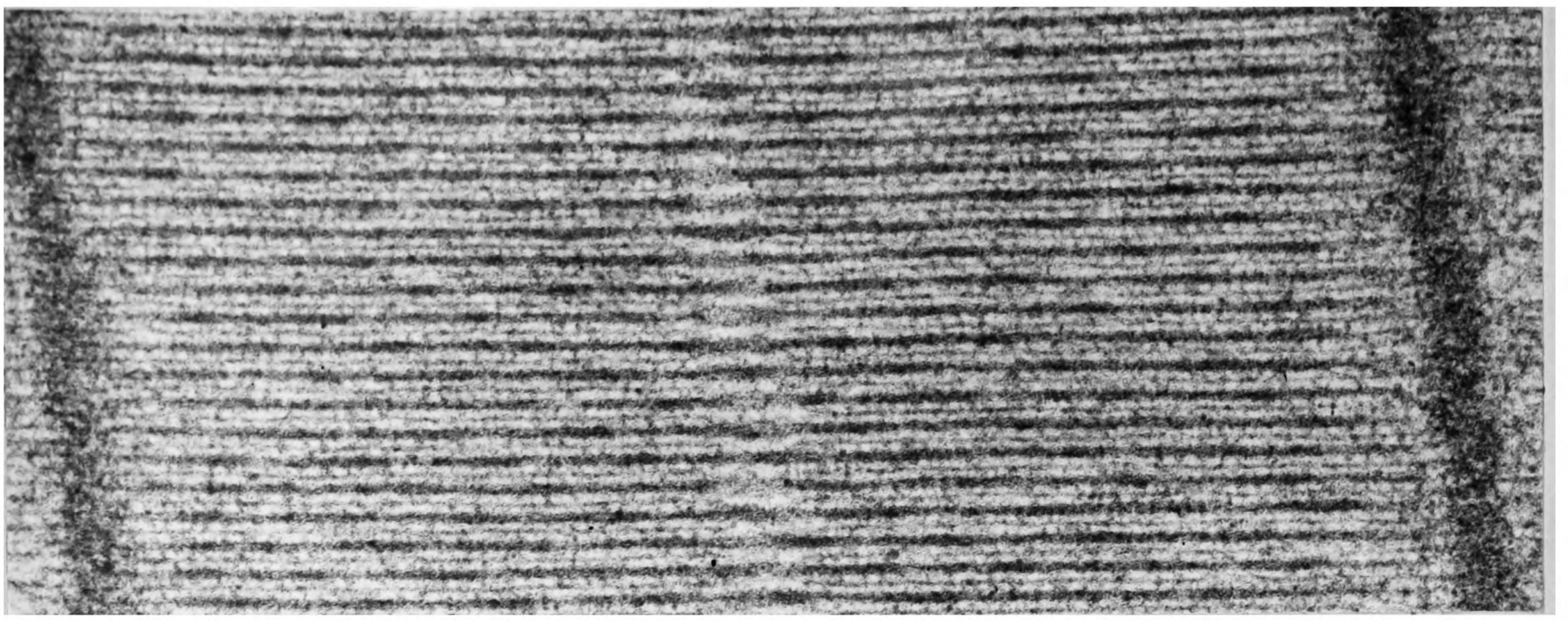
It has been possible to identify, structurally and biochemically, the transducing element as the myosin head crossbridge and work continues along both lines to elucidate the details of its operation.

That certain insect muscles, responsible for high frequency movement, were histologically distinct from the normal type of striated muscle was recognised by von Siebold in 1848. Insects in the orders Diptera, Coleoptera, Hymenoptera and Hemiptera may have flight, haltere, vibrating and tymbal muscles which are yellow coloured, only weakly striated, and very easy to separate into fibres (Pringle, 1957). Muscles of this type were found to have a common physiological peculiarity. Normal muscles have a one-to-one relationship between nervous stimuli and contractions but a fibrillar muscle is asynchronous: nervous impulses occur irregularly with respect to muscle contractions and it is length changes, after a delay, that instigate mechanical activity (Pringle, 1949). This property enables the muscle, when attached to a mechanically resonant load, in life the wings and thorax of an insect, to undergo oscillatory changes in length and tension at the resonant frequency of the system. By this means contraction frequency can far exceed that possible in normal muscles where the refractory period of the afferent nerve places an upper limit on the rate at which action potentials can stimulate (Pringle, 1957). Nervous input is necessary, but only to keep a sufficient concentration of calcium to maintain the muscle in an activated state.

Muscles of this type are termed 'asynchronous', 'fibrillar' or 'oscillatory' in view of their special electrophysiological, structural or mechanical characteristics (Cullen, 1971).

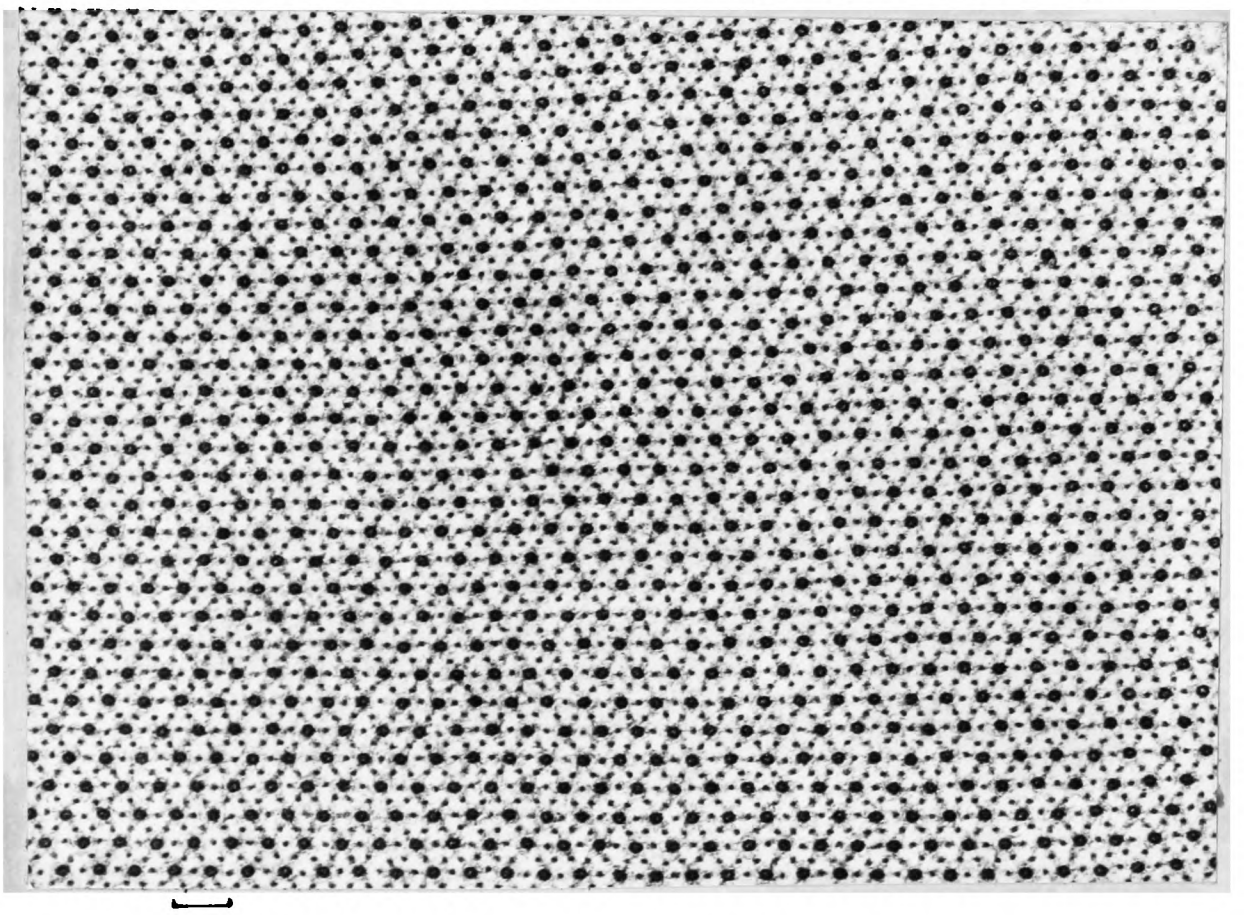
Morphologically, the peculiarities of fibrillar flight muscle are more quantitative than qualitative. The dorsal longitudinal muscle of Lethocerus cordofanus (see Fig. 3) is typical. The

Figure 3a Electron micrograph, L.S. through sarcomere of fibrillar flight



0·1 μ

Figure 3b Electron micrograph, T.S. through overlap region of sarcomere of fibrillar flight muscle, showing filament array.



0·1 μ

arrangements of actin filaments around, and crossbridges on, each myosin filament are different from those on vertebrate striated muscles (Ashhurst, 1967a; Reedy, 1968). The mitochondrial content is high, the tubular content low, as already noted. The A filaments are much longer than is commonly found, reaching nearly to the Z discs (Fig. 3a), an arrangement which is understandable as the muscle in vivo only undergoes small changes in length. (maximum Lethocerus dorsal longitudinal muscle about 10%, contrast frog sartorius more than 30%). Fibrillar flight muscle has a high resting stiffness (Machin & Pringle, 1960), the properties of which are consistent with the existence of a continuity between A filaments and Z line (White, 1967). Ashhurst (1967b) found no evidence for such connecting filaments in a study of flight muscles of Belostomatid water bugs, although they may exist in blowfly flight muscle (Auber & Couteaux, 1963).

Early work on fibrillar muscle was directed towards an understanding of its operation and significance in its anatomical situation (Pringle, 1957, Ch.3). Since 1960 its individual properties have recommended it as an advantageous preparation for the study of muscle mechanisms in general. Its ability to do work on a mechanically resonant system, a result of the delay between stimulus and tension changes, can be exploited. Sinusoidal stretching at an appropriate frequency results in a form of mechanical output suitable for analysis (Pringle, 1967). As activation is brought about by changes in length rather than changes in calcium ion concentration repeated contractions giving rise to measurable chemical breakdown can easily be obtained. As the control element is in the contractile elements rather than in the accessory structures, the latter (i.e. mitochondria and tubular systems) can be removed and the myofibrils alone, when provided with an energy source and appropriate ions (Mg^{2+}, Ca^{2+}) can perform oscillatory work (Jewell & Rüegg, 1966).

Glycerination provides a means of extracting the intracellular membranes from the contractile machinery. The process, described in Chapter 2, has been in use since 1949 when Szent-Györgyi discovered that extraction with glycerol did not remove the ability of muscle fibres to contract in the presence of ATP. Chemical and microscopical studies on glycerinated insect fibrillar muscle show that the membrane systems are not completely removed by glycerination (Abbott & Chaplain, 1966; Ashhurst, 1969b). However with due precaution all chemical activity but that of the myosin can be reduced to an insignificant level (vom Brocke & Rüegg, 1965). Thus the multiplicity of problems attendant upon the measuring and assignation of chemical changes in preparations such as frog muscle, where the membrane system must be retained to allow repetitive activation (Gilbert et al., 1971) do not arise.

The specialisations of insect fibrillar muscle thus make it particularly suitable material for mechanical and biochemical studies. The mechanical conditions and ambient medium of the muscle can be simply and accurately varied, and the resulting energy production and ATP usage unambiguously and easily analysed. But the general applicability of results from the preparation rests on evidence that its energy transduction mechanism is similar to that of ordinary striated muscle. Two lines of evidence suggest that this is so: firstly that fibrillar muscle shows many of the same properties as vertebrate examples, and secondly that an inherent but less developed ability of non-fibrillar muscles to show the same behaviour as the asynchronous types can be demonstrated.

Biochemical analyses (Maruyama, 1965), show that actomyosins from the two types of muscle have similar properties. The amino acid compositions and change of the proteins of rabbit and insect muscle

are very similar as are the properties of the proteins in solution. Actomyosins from the two sources show similar solubility, super-precipitation, birefringence and viscosity changes in the presence of calcium, magnesium and ITP. Insect muscle's greater turbidity is attributable to its larger particles. The ATPase activities show similar Q_{10}'s, although the heat lability and pH dependencies are not identical. Maruyama et al., (1968) examined the calcium sensitivity of fibrillar and non-fibrillar insect muscle and showed that the ATPase activity of myofibrils and actomyosin from fibrillar muscle is much less calcium sensitive than that of neurogenic types where a large degree of activation follows a small change in calcium concentration. Thus, except in its response to calcium which has already been discussed, fibrillar muscle has properties very similar to those of non-fibrillar muscle.

There is increasing evidence that, in the right conditions, many non-fibrillar muscles can be made to show delayed tension changes and oscillatory performance. Examples include frog semitendinosus (Armstrong et al., 1966), rabbit psoas (Goodall, 1956; Rüegg et al., 1970) and synchronous cicada tymbal muscles (Aidley & White, 1969). In all cases it was necessary to adjust the experimental conditions to obtain a high stiffness in the muscle before oscillatory behaviour could be observed. Finally, the taxonomic distribution of fibrillar muscle implies that it is not radically different from ordinary striated types. It has arisen in four insect classes not closely related and, in the Hemiptera it appears independently in several groups (Cullen, 1971). Aidley & White (1969) from their observations of the properties of fibrillar and non-fibrillar tymbal muscles of one family of cicadas, show that intermediate stages of evolution between the two could confer increasingly useful properties until the

full mechanism has developed. They suggest that fibrillar muscle may have arisen from an exploitation of the transient changes in force and velocity observed in vertebrate muscle before the normal steady-state relationship is obtained (Civan & Podolsky, 1966). Thus to a limited extent the peculiar properties of asynchronous muscle appear to be present in all muscles.

The two criteria cited for assuming the general validity of results from insect flight muscle are therefore answered: it is known to be very similar to non-fibrillar muscles and its specialisations can be shown to be inherent in all muscles tested. Consequently its particular experimental advantages can be exploited to obtain results applicable to all muscles. Firstly its regularity makes it particularly suitable for structural work where diffraction techniques are employed. Secondly its ability to produce consistent power outputs for long periods has made a good preparation for energetic studies of a mechanical nature. Thirdly, analysis of the chemical activity of muscle can be carried out much more easily on fibrillar muscle than other types as its ATP consumption can be simply measured.

Studies have been made of the energy relationships of muscle, employing the mechanical and chemical advantages of glycerinated fibrillar preparations.

Early work was concerned with the modes of activation of insect flight muscle and intact and in vitro experiments were performed which revealed the separate effects of calcium and stretch on ATPase activity (Rüegg & Tregear, 1966; Schädler, 1967; Maruyama et al., 1968). Parallel changes in length and tension in static muscle were reported by Rüegg & Stumpf (1969b). Work on oscilating muscle was hampered until an experimental technique was found which prevented development of the high tension state. In this condition the tension of the fibre increases

rapidly, work output falls off and the frequency response of the muscle is greatly reduced. The state can be reversed by lowering the power output of the muscle or reducing the calcium concentration of the incubating medium (Jewell & Rüegg, 1966). Evidence suggests that an inadequate concentration of ATP or a build-up of ADP in the contractile apparatus under conditions imposing a diffusional limitation on the fibres may be responsible for the high tension state (Abbott & Mannherz, 1970). It can be avoided by using single fibres rather than bundles in which ATP and ADP diffusion become rate-limiting, or by increasing the concentration of ATP to create a larger gradient and by addition of myokinase so that ADP formed is immediately reconverted to ATP (Rüegg & Stumpf, 1969a). Under these conditions it has been possible to obtain a performance from glycerinated fibres that resembles that found in life (Steiger & Rüegg, 1969).

The results of Rüegg and his co-workers demonstrate the activation of ATP splitting by oscillation at suitable amplitude and frequency. The parallel appearance of positive work and of extra ATPase activity is expressed as a biochemical Fenn effect - a manifestation of the recruitment of extra crossbridges in relation to the load presented to the muscle. Nevertheless a frequency dependence in the ATPase activity/power output relationship is recorded, showing a maximum efficiency at about 9Hz.

Later work has been concerned with the isometric oscillations in tension which can be observed when the fibres are subjected to a quick, high amplitude stretch (Schädler et al., 1969). Their frequency is observed to decrease in the presence of ADP but to increase when temperature or inorganic phosphate concentration are raised (Rüegg et al., 1971). The hypothesis that the oscillations result from the rapid and synchronous activation of a large percentage of available bridges and that their frequency reflects the natural lifetime of

attachment of myosin to actin has been investigated by Breull (1971). His evidence is consistent with the splitting of one ATP molecule by each active site (i.e. 2 ATP molecules/myosin crossbridge) during each cycle of isometric oscillation whether the muscle oscillates rapidly or more slowly. He finds also a correlation between speed of isometric and work-producing oscillation and the ATP hydrolysed per unit time per unit isometric tension. The relation between the ATPase activity and tension has not been extensively investigated although Schädler (1967) recorded, in insect flight, anterior byssus retractor and rabbit muscles, a correlation similar to that observed later by Breull.

Previous work on glycerinated insect flight muscle has thus emphasised the hypothesis that chemical and mechanical events are coupled in a rather inflexible way such as was first proposed by Fenn (1923). Power production and the hydrolysis of 'extra ATP' are shown to appear in parallel.

The work presented here is an extended analysis of the chemical and mechanical output of insect fibrillar muscle. The problems that underlie the study of the mechanism of muscle contraction are (i) how is the free energy of the ATP molecule transferred to the crossbridge? (ii) how does the bridge then pass that energy on to the filaments? The work in this thesis is an attempt to look at the second question. Modified experimental techniques have been used to give improved data. The analysis of results has been done in a different way. The relationships of the mean parameters of muscle activity have been compared in a number of different intrinsic and extrinsic conditions, and related to a structural-mechanical model.

CHAPTER 2

Materials and Methods

1. Muscle and its treatment

All the experiments were performed using fibres from the dorsal longitudinal muscles of the giant water bug Lethocerus cordofanus. The reasons for using fibrillar muscle, glycerinated, are presented in the previous chapter. L. cordofanus was used as a source of muscle as it is a large insect, having flight muscles about 1 cm long, and it can be kept in a laboratory tropical room without undergoing degeneration of the flight muscle (Cullen, 1971).

Live insects, either newly arrived from Uganda or from the tropical room tanks, were used. The dorsal flight muscles were removed intact with the notum and attached phragmata on which they insert. Both bug and instruments were dipped in 70% methanol prior to dissection to sterilise them. Remains of gut were removed carefully from the muscle and it was dropped into a pH-buffered antifreeze solution of glycerol. Dithiothreitol was added to the glycerol solution in later experiments to prevent oxidation of cysteine residues in the protein (Cleland, 1964).

The solution was usually used at pH 7.0. On one occasion solution at pH 6.7 was used. No significant difference was found. Jars of glycerol solution containing the muscles were removed to the cold room and placed in a desiccator attached to a rotary pump. The desiccator was evacuated until air bubbles ceased to emerge from the muscles and then air was readmitted. The process was repeated several times in the hope that the glycerol solution would better penetrate the structure of the muscles. The glycerol solution was changed at

intervals for 24 hours. Between changes the desiccator was evacuated and continuously shaken. After 24 hours the muscles were transferred to individual tubes of glycerol solution and stored at -18°C in the deep freeze.

Muscles were used for experiments after 1 week to 4 months of storage. A bundle of fibres was then removed from the muscle and placed on a microscope slide under a binocular microscope. Individual fibres, singly or in small bundles as required, were stripped from the bundle using mounted needles. Care was taken to select undamaged fibres and to touch only the ends with the instruments. They were then transferred to the apparatus.

All workers with glycerinated insect flight muscle have found it difficult to predict which muscles would work well and which would not. Generally between a quarter and a half of each batch of glycerinated muscles produce little or no work or tension. This is often because infection has led to decomposition. Examination of the myofibrils and ambient medium of useless muscles by phase contrast microscopy reveals the presence of large numbers of bacteria. The damage may show in the appearance of the muscle but this is not a sufficient condition for selection of material. Of the muscles which appear to be in good condition, having long, straight fibres of uniform colour and easily separable from each other using dissecting needles, some are unable to hold tension. The phase angle and the amount of work which is produced and sustained by muscles which can hold tension varies greatly. Thus it was found that only one criterion could be used in the selection of muscle fibres for experiment - ability to produce and maintain a good power output at 2% amplitude of oscillation for a length of time sufficient to do an experiment. Fibres which did not work, or could not sustain the performance, were rejected. It was

found that the fibres from the surface of one muscle generally gave similar performances. The muscle could be used for two to four weeks until the work output declined.

2. Solutions

The experimental media were based on those used by Jewell & Rüegg (1966). The exact composition of the media varied from experiment to experiment. The general features, showing which parameters were controlled are listed below. Both pH and pCa are buffered.

(i) ATP (disodium salt) 5 mM in all experiments.

(ii) $MgCl_2$ added in sufficient concentration to ensure that most of the ATP was present in the form of Mg^{2+}-ATP, generally 5 or 11 mM.

(iii) EGTA (Ethylenelgycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid) Ca^{2+} buffer, 4 or 5 mM.

(iv) $CaCl_2$ 0 mM Ca^{2+} ($< 1 \times 10^{-9}$ M Ca) = relaxing solution

2 mM Ca^{2+} ($> 1 \times 10^{-7}$ M Ca) = activating solution.

(v) KH_2PO_4)
) /histidine/PIPES (piperazine-N-N'-bis(2-ethane sulphonic acid)
K_2HPO_3)

One of these used as pH buffer.

(vi) KCl Added in varying quantities to adjust ionic strength.

(vii) NaN_3 added in approximately 10 mM concentrations to poison mitochondria (Sacktor, 1953; vom Brocke & Rüegg, 1965).

(viii) ADP/pyrophosphate/K_2SO_4 occasionally added.

The solutions were made up with distilled, deionised water. The chemicals used were obtained from the following sources:

ATP and other biochemicals and enzymes used in ADP analysis - Boehringer Corporation (London) Ltd.

EGTA, histidine and PIPES - Sigma London Chemical Co. Ltd.

Salts and other chemicals - BDH Chemicals Ltd. (Analar grade).

Figure 4 A Block diagram of the mechanical apparatus. Power supplies not shown.

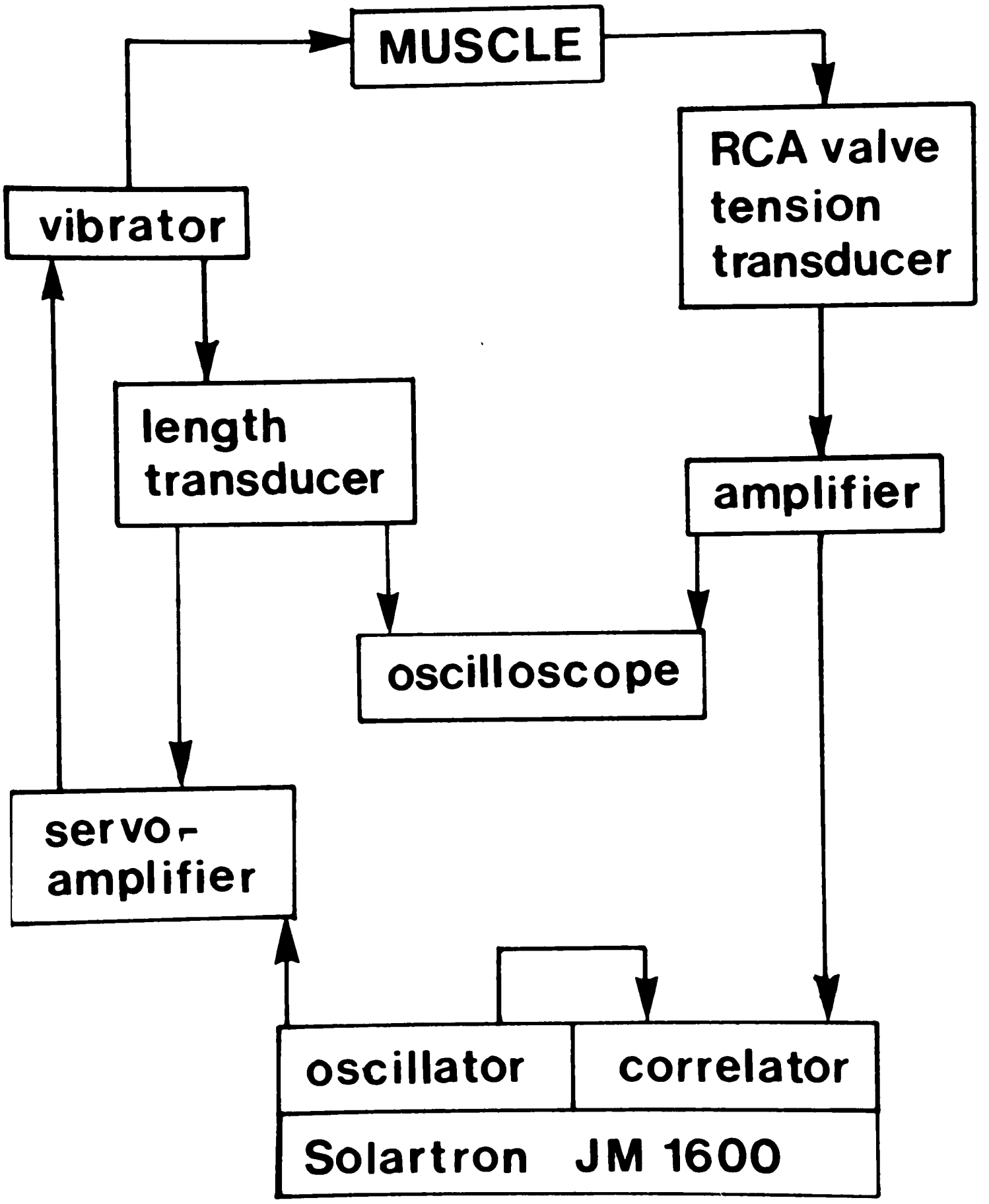


Figure 5 a Diagram giving general view of mechanical apparatus

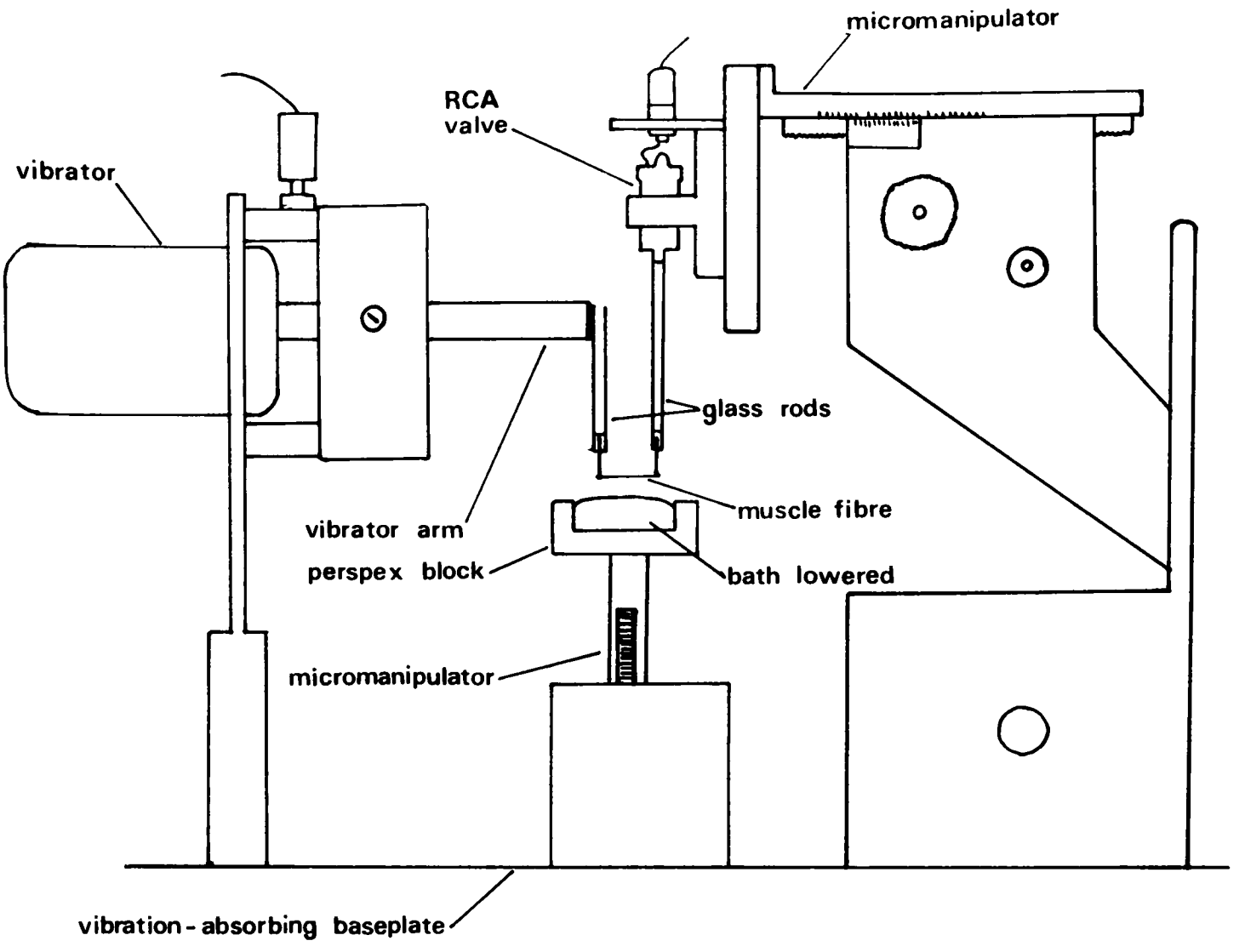
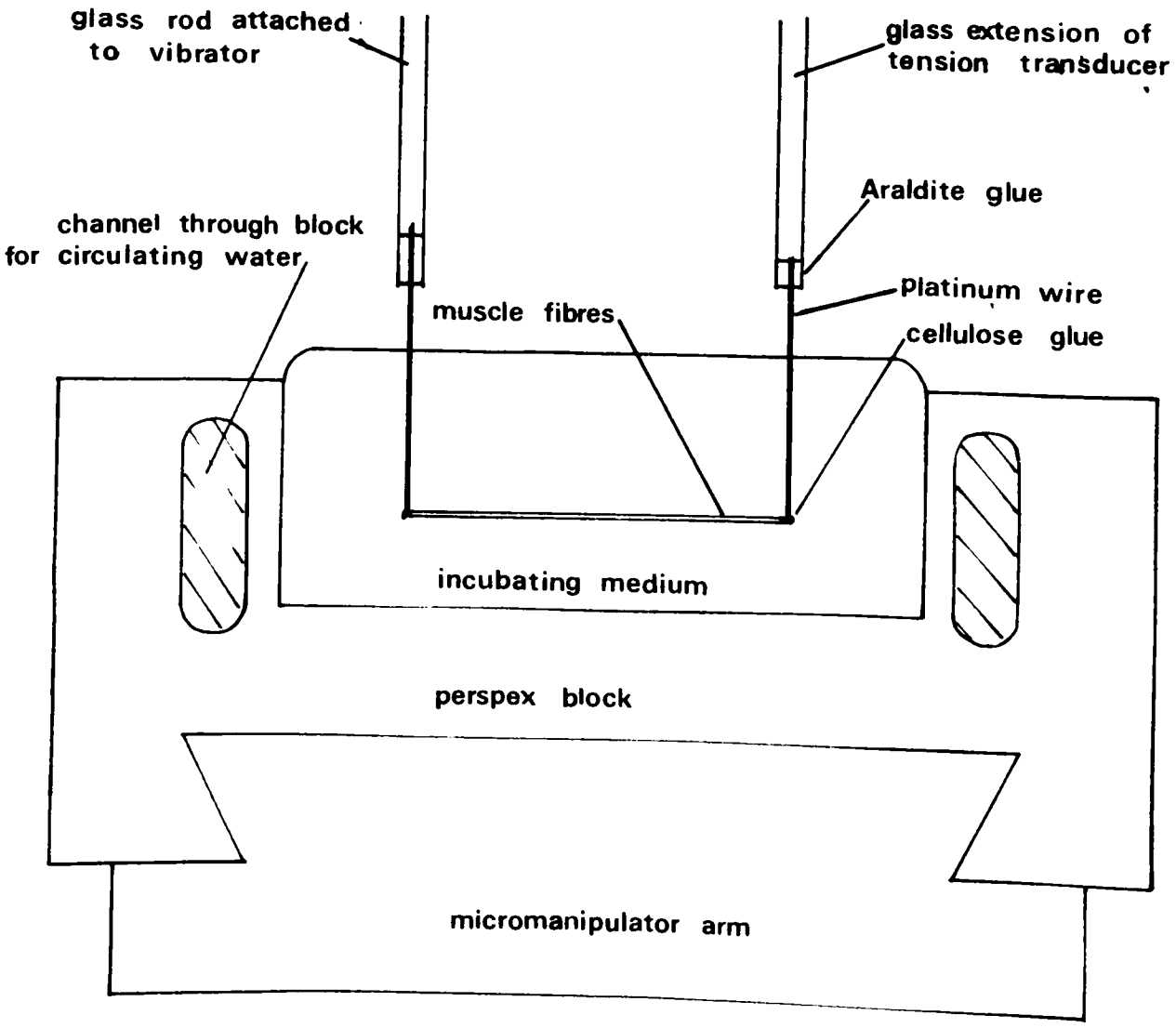


Figure 5 b Diagram showing relation of muscle fibre to attachments and to incubating bath.



Except where these parameters were specifically varied, the solutions were adjusted to pH 6.95 and the experiments performed at room temperature (20-21°C).

During the course of the work it became possible to monitor with accuracy the contents of the various solutions by using the computer programme of Perrin & Sayce (1967). The dissociation constants of species possibly present in the solutions were obtained from previous estimates of stability constants, e.g. Phillips et al., (1965). The programme calculates the actual concentrations of such species in the solutions given the pH and the concentrations of individual ligands and metal ions added. The exact composition of the solutions used varied, the details (computed by the above method unless otherwise stated) are given with the appropriate experiments.

3. Mechanical apparatus

The apparatus was similar to that described by Rüegg & Tregear (1966). A block diagram of the pieces of equipment involved is given in Fig. 4. Figure 5a shows the apparatus involved in changing and monitoring the length and tension of the fibres and its relationship to the fibres. Figure 5b shows the position of the fibres themselves. The fibre ends are wound round the wire pins and then stuck with glue made of cellulose nitrate dissolved in acetone. The tension transducer is an RCA 5734 triode transducer valve; the muscle is attached as shown to the extension of the plate shaft. The micromanipulator permits known length changes to be made. The Goodman's VP47 vibrator moves horizontally an arm to which the other ends of the fibres are attached as shown. A servo mechanism controls its position. The Solartron JM1600, as is shown in Fig. 4, both supplies a sinusoidal oscillation from a generator to the vibrator and, by receiving input from the

Figure 6 a

Calibration of RCA valve tension transducer

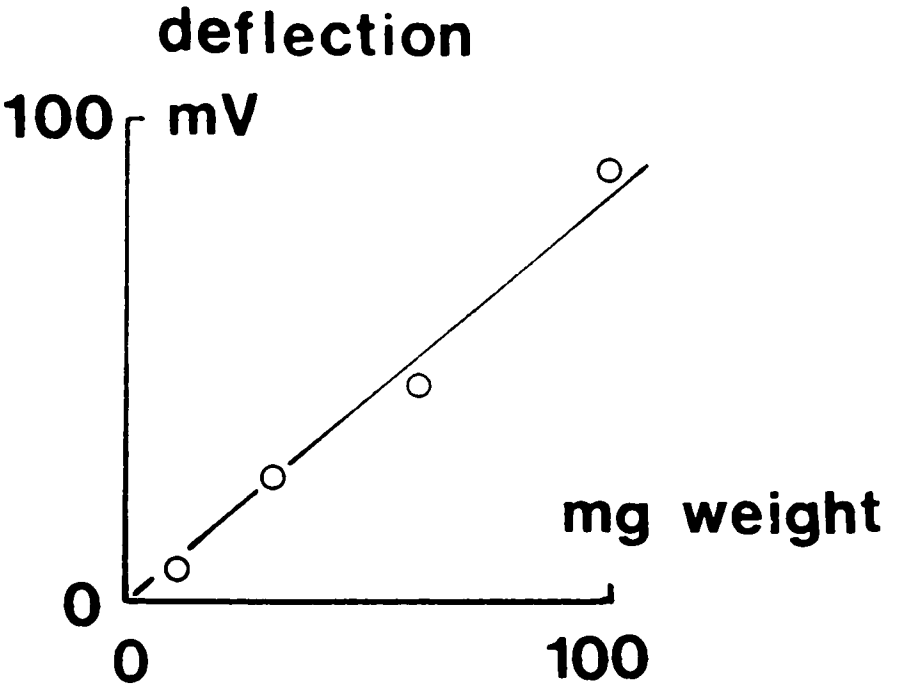


Figure 6 b

'Rustrack' record of transducer output showing shift.

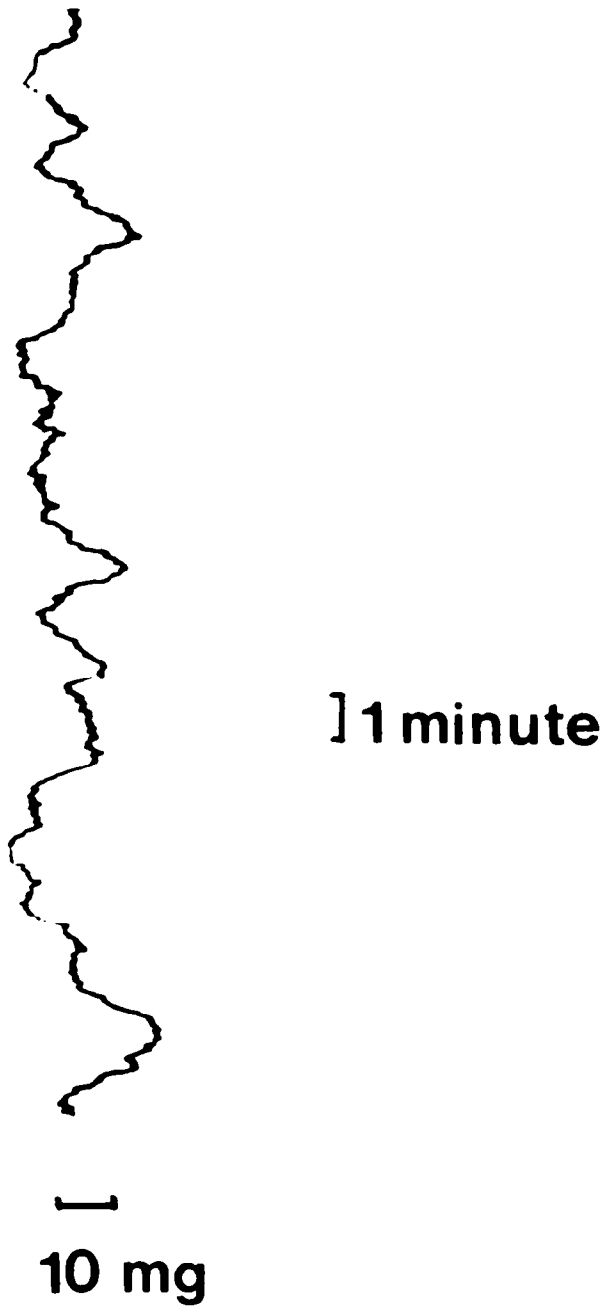
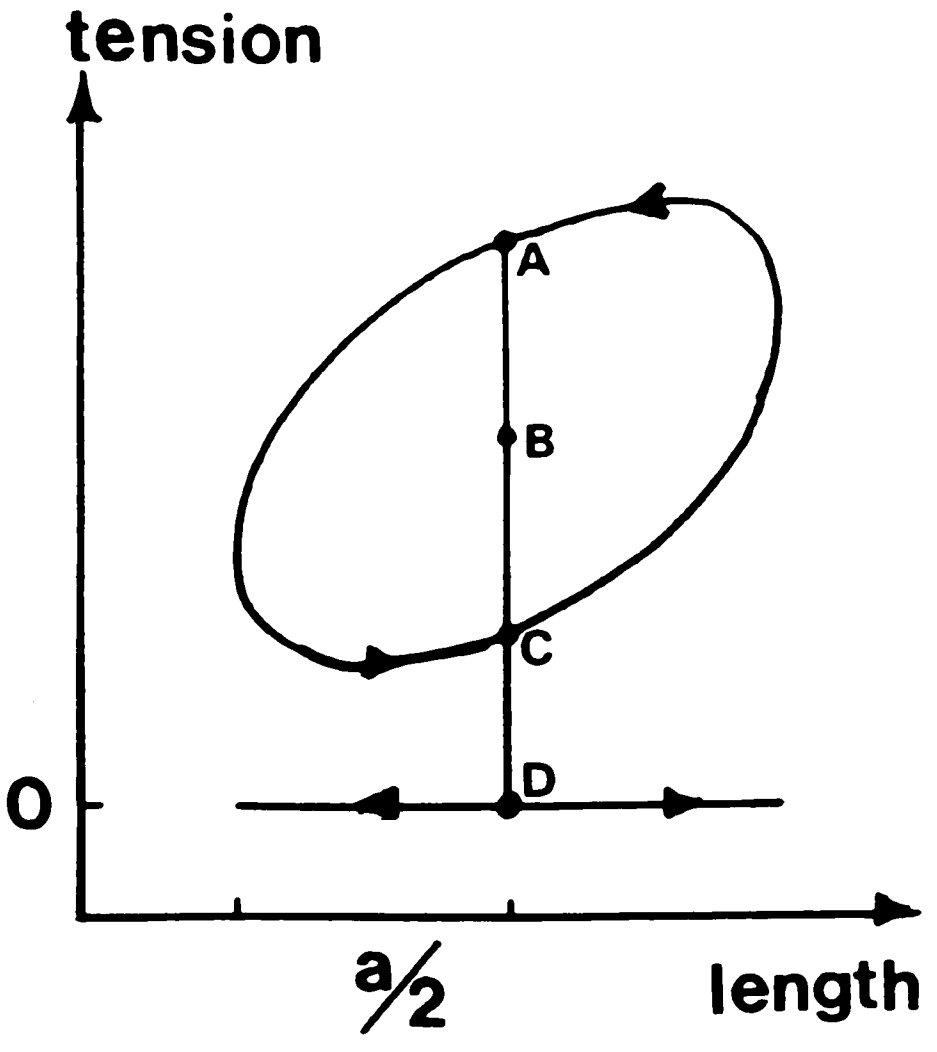


Figure 7



Length-tension loop of oscillated muscle.

Arrows indicate direction of spot around loop

a = amplitude of oscillation.

D indicates zero tension - fibre slack.

AC = mid-length tension difference.

r.m.s. AC = tension component in quadrature with length changes.

BD = mean tension of oscillating muscle.

generator and from the tension transducer, correlates the length and tension changes of the fibres. These are also displayed as an instantaneous X-Y plot on the screen of a Tektronix 502A dual beam oscilloscope.

4. Tension measurements

An RCA valve was used, as described, as a tension transducer. The valve has a number of advantages for the work - it has a good frequency response and high sensitivity (see Fig. 6a) - but it is subject to ineradicable drift. Figure 6b shows the output from a valve, not mounted with a muscle, recorded on a 'Rustrack' recorder over a period of 45 minutes. There are fluctuations every few seconds of ± 1 mg, and over the course of a few minutes superimposed fluctuations of ± 2-10 mg with a randomly drifting baseline. As a result the absolute tension in the muscle could not be obtained directly. Other transducers were tried, including a modified Endevco 'Pixie' strain gauge (Chapman, 1970), but the defects of the other systems made them less practical than the RCA valve. It was possible to establish a zero tension baseline by quickly reducing the length of the muscle until it was no longer producing tension. Mean tension of the muscle, in static and oscillatory experiments, was estimated this way at the end of each incubation. It was measured by observation of the oscilloscope spot in the case of static muscles, or from photographs in oscillatory experiments, where mean tension equals BD in Fig. 7. The tension at the beginning of each incubation was not used as an estimate of tension because in the static case, the tension on extension overshoots its final value considerably and only gradually - over a minute - assumes its final value (see Fig. 8a). In oscillatory muscle, it also takes several hundred cycles before the performance stabilises by which time the transducer drift has made accurate recording impossible.

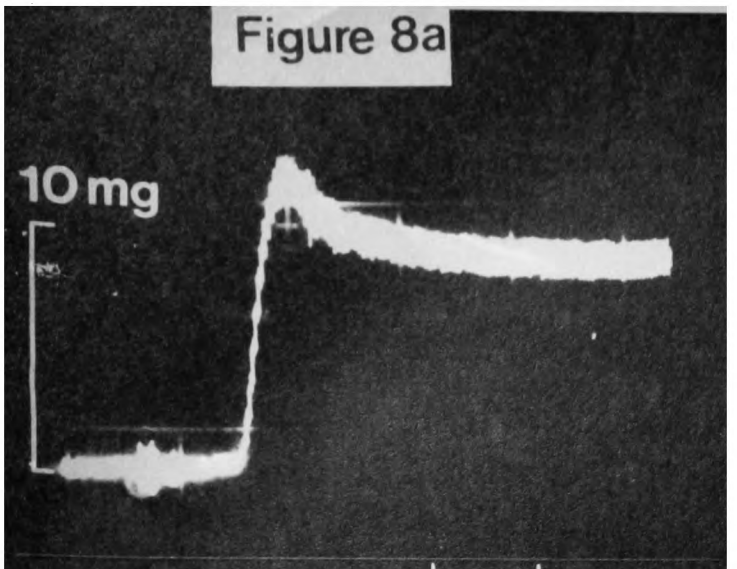
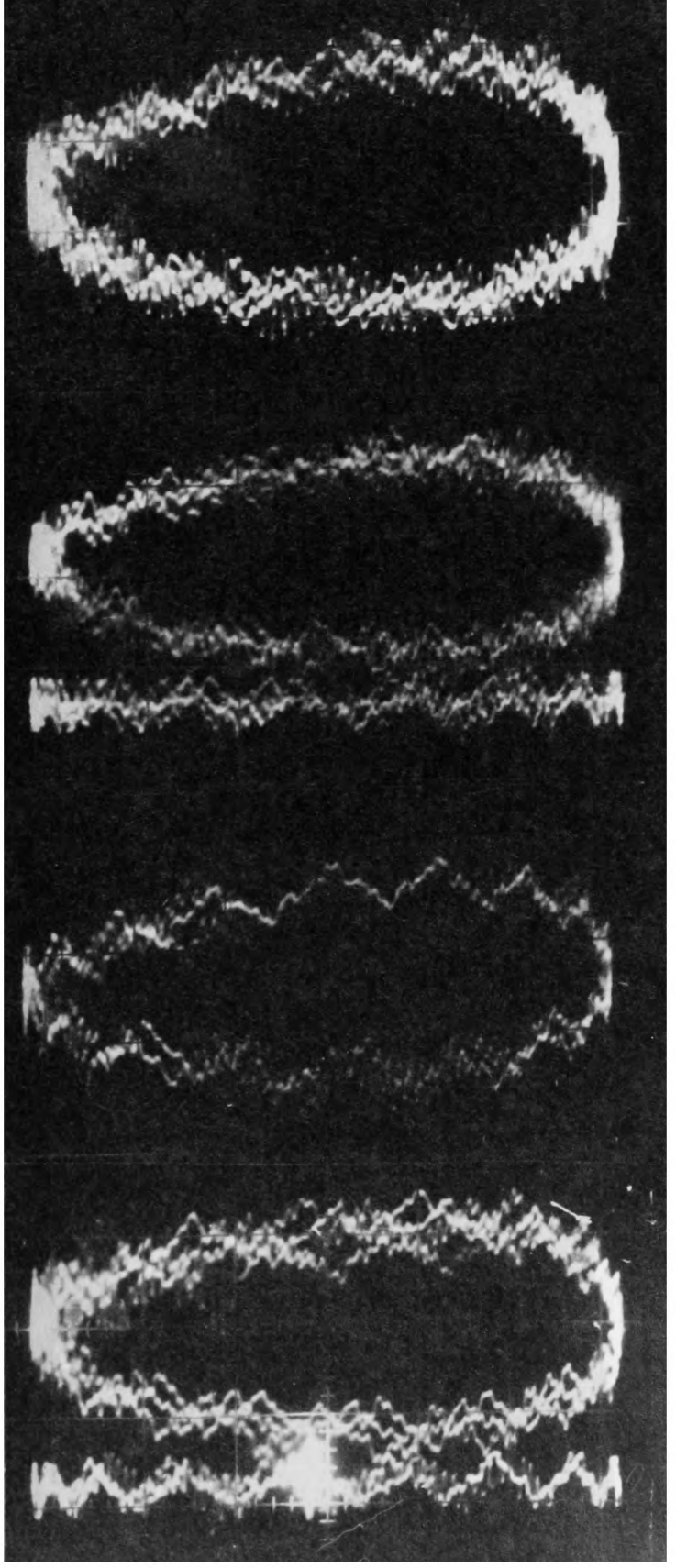


Figure 8a

Response of muscle fibre incubated in activating solution to step change in length, showing gradual decay of tension from initial high peak to steady level

Figure 8b



Effect of extension on relationship of power output to mean tension during the course of an experiment.

Histidine buffer 3 Hz
2.0% amplitude oscillation

(i) Time: 0 minutes

Power output: 10.8 nJoules/cm fibre/minute

Length: 5.1 mm

(ii) Time: 5 minutes

Power output: 8.6 nJ/cm/minute

Tension: 12.5 mg

Length: 5.1 mm

(iii) Time: 8 minutes

Power output: 6.4 nJ/cm/minute

Length: 5.1 mm

Fibre lengthened by 0.14 mm

(iv) Time: 23 minutes

Power output: 8.6 nJ/cm/minute

Length: 5.24 mm

Figure 8b shows length-tension loops from a typical experiment. The initial high mid-length tension difference is not maintained. The tension was measured by slackening the fibre to zero tension after 5 minutes. After 8 minutes the fibre was extended by a small amount to restore the power output to its previous value. When the tension was measured after 23 minutes it had not changed despite the extension. Thus, for a constant oscillatory power output the mean tension remains constant and so not affected by length changes. Consequently it is sufficient to measure tension only at the end of the incubation and not necessary to interrupt the experiment to slacken off the fibre in the middle.

In experiments on static muscle the tension of the fibres was recorded.

In the case of oscillating muscle a number of tension measurements were made. As described earlier, it is a feature of insect fibrillar muscle that tension changes lag behind length changes under appropriate conditions resulting in net positive power output. Thus when length is changed sinusoidally an anticlockwise moving hysteresis loop is seen on the oscilloscope screen. The tension changes around the loop can be analysed, by comparison with the length changes, into two components: tension changes in phase, and tension changes 90° out of phase ("in quadrature") with the length changes. The latter change represents the negative viscosity in the muscle system and is seen on the loop as the width across it at its mid-length (AC in Fig. 7). As the size of this tension is a measure of the work output of the muscle it was important to measure it accurately. Although it could have been estimated directly from the width of the loop it was quicker and more accurate to use the correlator of the JM1600 which, fed with length and tension change signals, performs the analysis of the tension

changes into in-phase and in-quadrature components by integration over a minimum of one cycle and displays their root mean square values and the phase angle between them.

5. Power measurements

As described earlier, the mid-length tension difference across the loop, AC, is a measure of the work output of the muscle and it can be read from the JM1600 correlator. Power can then be calculated thus:

Power = r.m.s. velocity x r.m.s. force

$= 2 \pi fa \times b$

where b = r.m.s. value of mid-length tension difference

f = frequency of oscillation

a = amplitude of oscillation, r.m.s. value

Alternatively the power output of the muscle can be calculated from the area of the length tension hysteresis loop. This was measured using a planimeter on enlarged photographs.

Power = k.A.f

where k = constant

A = area of loop

f = frequency of oscillation.

6. Pattern of experiments

0.2 ml of experimental medium was used per incubation. The solution was pipetted into small baths machined out of a perspex block which was mounted on a micromanipulator so that it could be moved vertically and horizontally to change the bath in which the muscle was immersed (see Fig. 5). The temperature could be controlled by pumping water at a thermostatically controlled temperature through the block (see Fig. 5). The muscle fibres were mounted as described between the two pins. The bath was then raised to immerse the fibres and, for an

experiment on isometric muscle, the fibres were stretched to give the desired tension. In the case of an oscillatory experiment the frequency was set and then the amplitude of oscillation was gradually increased from zero to the desired amplitude, 0.2% or 2% of rest length, and the fibre length adjusted to obtain the desired performance. Small adjustments were made as necessary to maintain constant performance throughout the course of the incubations which lasted 10-30 minutes. After the loop and zero tension had been photographed the bath was lowered from the fibres and they were reimmersed in another bath or removed from the pins using a paintbrush dipped in acetone to dissolve the glue, and replaced with fresh fibres. If significant evaporation from the bath appeared to have occurred the volume was made up to 0.2 ml with water using the appearance of the meniscus as a guide, and then 0.18 ml was removed into a small tube using Eppendorf automatic pipettes and the sample was kept for analysis. The volume of solution removed by this method is shown in Table 1.

TABLE 1

Weight of water delivered by pipette mg
0.165
0.176
0.173
0.169
0.174
0.172
0.168

The mean value and standard deviation is 0.171 ± 0.006 mg, the volume removed is thus consistently 5% lower than theoretical. Each fibre was used for 1 to 4 incubations, the number depending on how well the performance was maintained.

In previous work of this type on insect flight muscle (Rüegg & Tregear, 1966; Steiger & Rüegg, 1969) bundles of 5-10 fibres were used, as bundles suffer less from surface-tension pull when moved through the meniscus than single fibres and, for chemical investigations the larger titre for measurement is an advantage. However, bundles of fibres have two drawbacks - firstly there is evidence that there is a large diffusion gradient across a bundle of fibres resulting in ATP starvation at the centre of the bundle, manifesting itself as the appearance of the so-called 'high tension state' (Jewell & Rüegg, 1966; Steiger & Rüegg, 1969). The state is characterised by a rapid rise to two or three times the original tension in five to ten seconds accompanied by a reduced frequency response and loss of power production (Jewell & Rüegg, 1966). It can be temporarily reversed by switching off the oscillation briefly. It can be avoided by decreasing the fibre number, increasing the ATP concentration throughout or locally by stirring or lowering the activation of the muscle, i.e. being content with lower power outputs (Steiger & Rüegg, 1969). The last alternative was rejected.

Steiger & Rüegg (1969) found that using 15 mM ATP in their solutions prevented the muscle entering the high tension state. Such a high ATP concentration however has the disadvantage that the amount of phosphate and ADP already present in the solution becomes very high making accurate measurement of ATPase activity even more difficult so this course was not taken.

Stirring the incubating solution may aid diffusion of substrate and products into and out of the muscle. To test whether or not it had a significant effect on the ATPase activity of a single fibre, one fibre was incubated, statically and not under tension, conditions giving the most standard rate of chemical breakdown. In one of each pair of observations the bath was not shaken. In the other it was placed on the membrane of a loudspeaker which was coupled with a stiff vane on the core of a power oscillator so that the bath was subject to small rapid movements. The ATPase activity estimations are tabulated below.

TABLE 2

ATPase activity (pmoles/cm/min)	
shaken	still
87	89
147	115
150	140

The mean difference is +10 pmoles/cm/min, the standard deviation 17 pmoles/cm/min; thus the difference in ATPase activity produced by shaking is not significant.

The second disadvantage of fibre bundles is that it is not possible to ensure that the glue penetrates the bundle sufficiently to stick all the fibres to the pin and so centre fibres tend to slip giving tensions and power outputs that are not proportional to fibre number (see Table 3). The fibres were subjected to a 4 Hz oscillation at 2% amplitude.

TABLE 3

Fibre number	Width of loop (mV)	Work/cycle/fibre (mV)
6	20	3.3
5	25	5
4	34	8
2	18	9

In addition it is difficult to see if any fibres have broken.

Therefore, for the experiments described single fibres, or pairs of fibres, were used. Mechanical performance could be accurately measured, and at no time was there any evidence that the fibres were entering the 'high tension state'. The disadvantages of single fibres are that they are difficult to handle, that they tend to be stretched by being moved through the surface tension of the incubating solution, and that they give very low titres for analysis. The first objection was overcome with practice, the second was unavoidable; if the performance of the fibres was impaired they were replaced. It was found that by incubating for 15-20 minutes and estimating by the Adam method of ADP analysis the ATP breakdown could be satisfactorily measured.

7. Computer-controlled experiments

A full analysis of the mechanical performance of muscle requires a large number of measurements to be made on the performance under different conditions. The Solartron JM1600 makes possible the rapid measurement of the mid-length tension difference and hence work and power output, but manual manipulation of the apparatus and logging of results is a slow process and the operation should ideally be carried out fast to minimise variations in the muscle.

Abbott (1970) has developed a system for on-line control of the JM1600 by a PDP8-I computer so that frequency changes, measurements and logging can be performed rapidly. This system was employed for some experiments in which only the mechanical performance of fibres was studied.

The mechanical apparatus was similar to that described before except that the RCA valve tension transducer was replaced by a capacitance transducer of the type developed by Huxley & Simmons (1968).

The computer automatically controls the frequency and amplitude of the sinusoidal oscillations generated by the JM1600. The mean muscle length is controlled by a stepping motor which moves the micromanipulator on which the vibrator is mounted. The muscle fibres can be moved from bath to bath and thus solution to solution, by commands to the computer which then cause the baths to be moved vertically and horizontally by means of two motors.

Commands are issued to the computer via the teletype. The instantaneous length-tension plot is displayed on an oscilloscope, the DC tension on a digital voltmeter and the absolute length is recorded on paper by the teletype, as are the results of analyses of in-phase and quadrature tension which the computer can be commanded to make. It can make single measurements or a rapid series of measurements at a variety of frequencies and thus provide in 30 secs the data necessary for the construction of a graph showing the frequency response of the fibres. Depending on what information is required from the experiment the data may be displayed as a Bode plot, in which parameters are plotted against log frequency, or as a Nyquist plot, showing the in-phase and in-quadrature tension at various frequencies.

From a plot of the root mean square value of the mid-length tension difference, as logged by the computer, against the logarithm

of the frequency, estimates may be made of the optimum frequency for work and power production and the range of frequencies at which positive work is produced.

8. The estimation of ATPase activity

The hydrolysis of ATP results in the production of ADP, inorganic phosphate and hydrogen ions.

$$ATP \longrightarrow ADP + P_i + H^+$$

Theoretically, production of any of the three products can be estimated; in practice, the production of hydrogen ions is too small to be accurately measured. In most work, the production of phosphate is measured by one of a number of different methods. Recent workers have used the method of Marsh (1959) which avoids the inaccuracy found in other methods due to interference from ATP. The Marsh method involves the formation of molybdenum blue (P-molybdate complex) and its subsequent extraction into butanol. Excess molybdate is removed from the butanol layer as a citrate complex, thus minimising molybdate catalysed ATP breakdown. The density of the phosphomolybdate complex is determined spectrophotometrically at a wavelength of 310 nm. This method was used with the modifications described by Abbott & Mannherz (1970) to obtain an estimate to ATPase activity in early experiments.

Use of phosphate buffer in experimental solutions necessitated that ATP splitting be measured by a method not involving P_i investigation. The enzyme pull-off method developed by Adam (see Bergmeyer, 1965) was used. In this method, the ADP present is phosphorylated by phosphoenol-pyruvate, PEP, added in excess, via the enzyme pyruvate kinase, PK. The pyruvate thus formed in amounts equal to the quantity of ADP originally present is reduced to lactate by the lactate dehydrogenase

catalysed conversion of NADH to NAD^+. NADH absorbs strongly at 340 nm, NAD^+ does not, and the change in absorption at this wavelength, which is proportional to the ADP concentration of the test solution, can be measured spectrophotometrically.

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

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

In practice, triethanolamine buffer is added to the incubating solution plus excess NADH, PEP and lactate dehydrogenase, LDH. Thus any contaminant pyruvate in the PEP is reduced before ADP estimation begins. The sample is put in a glass cuvette in a Beckman spectrophotometer and the optical density recorded on a penrecorder. When a steady reading is obtained, excess PK is added, the cuvettes stirred and left until the output reaches a new steady level, the difference between the two levels representing the decrease in absorption due to oxidation of NADH to NAD^+. Blanks of unincubated solution are measured to estimate the quantity of ADP present as a contaminant in the activating medium before incubation with muscle fibres.

It was found that the enzyme pull off method of measuring ADP had a number of advantages over the Marsh method - samples do not have to be diluted on removal from the bath so that the danger of ATP hydrolysis in dilute solution is avoided. Once the Marsh estimation procedure has been started it must be continue without a break and the samples of phosphomolybdate in butanol must be read as quickly as possible after formation as decay becomes perceptible after 10 minutes. Therefore only a small number of samples can conveniently be analysed at one time. The enzyme method is quicker and more flexible in timing, samples can be kept before analysis and the OD measurements are much

steadier than those in the Marsh procedure. Use of the Marsh method requires extreme care about the cleanliness of glassware and other equipment used for handling samples as phosphate contamination can easily be introduced. The enzyme method is less sensitive to inaccuracies from this source.

A statistical analysis was performed to estimate the accuracy of the measuring methods. The variance of individual estimations, calculated from an analysis of the differences between the paired estimations of the blanks, was 0.63 nM ADP when the enzyme method was employed, or 1.48 nM P_i when phosphate production was measured using the Marsh method. More accurate determinations of ATPase activity are thus obtained by analysing ADP produced using the enzyme method.

Therefore, for a number of reasons the enzyme method of analysis was used. Analysis of ADP production is not possible in non-fibrillar muscle as under the conditions in which experiments must usually be performed, the preparation contains many enzymes handling ADP. Therefore the method has not previously been used. There are, however, several possible systematic errors.

(a) ADP production due to activity of other ATP hydrolysing enzymes, e.g. those concerned with providing energy for the active transport of calcium by the sarcoplasmic reticulum. The evidence of vom Brocke & Rüegg (1965) shows that most of non-fibrillar ATPase activity is removed from the fibres by glycerination. Certainly glycerination disrupts both the membrane systems and the mitochondria (Ashhurst, 1969b). vom Brocke & Rüegg showed that in the presence of the mitochondrial poison, sodium azide, the ATPase activity of non-fibrillar enzymes was reduced by 90%. 10 mM azide was therefore added to solutions to ensure that only actomyosin activity contributed to ADP produced.

(b) Adenylate deaminase activity

$$AMP \longrightarrow IMP + NH_3$$

Maruyama (1957) and Maruyama & Tonomura (1957) found that no AMP was deaminated to inosine monophosphate so interference from this source can be discounted.

(c) ADP removal due to the action of a phosphogen, arginine in insects.

$$ADP + P\ Arg \longrightarrow ATP + Arg$$

Arginine phosphate is known to be labile and thus unlikely to withstand the glycerination procedure. Arginine phosphokinase has a low activity in Lethocerus cordofanus (Leech, in preparation). The reaction is thus not likely to interfere greatly. The evidence presented below that ADP and phosphate estimations give the same results show that the reaction, if it occurs at all, proceeds at an imperceptible level.

(d) Adenylate kinase (myokinase) activity

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

This reaction is more likely to interfere. Myokinase is present in large quantity in muscle and is a very stable enzyme which survives the glycerination procedure (Abbott & Leech, in preparation).

The error in ADP measurements that the action of myokinase would produce under the conditions of the experiments can be calculated as follows.

The equilibrium equation for myokinase as computed by Krebs is

$$K_{eq} = \frac{ATP \quad AMP}{ADP^2} = 0.44$$

Therefore at equilibrium

$$AMP = \frac{0.44\ ADP^2}{ATP}$$

ATP is in excess at 5 mM concentration, so

$$AMP = \frac{0.44}{5}\ ADP^2$$

Assuming AMP is absent initially

$$2\ ADP \xrightarrow{\text{myokinase}} ATP + AMP$$

Thus every molecule of AMP formed requires the absorption of two ADP molecules so,

$$\text{actual ADP concentration} = \text{measured ADP} + 2\ AMP$$

$$= \text{measured ADP} + 2 \times \frac{0.44}{5}\ ADP^2$$

An average ADP titre is about 20 nM so,

$$\text{actual ADP concentration} = 20 \times 10^{-6} + \frac{2}{5} \times 0.44 \times (20 \times 10^{-6})^2\ \text{mM}$$

$$= 20 + \frac{2}{5} \times 0.44 \times 400 \times 10^{-6}\ \text{nM}$$

$$= 20 + 70.4 \times 10^{-6}\ \text{nM}$$

$$= 20.00007\ \text{nM}$$

The calculated error is thus extremely small: much less than the inherent experimental error.

Therefore on theoretical grounds no difference between estimations done by the two different methods would be expected. Experiments were performed to check this point. Fibres were incubated in histidine-buffered activating solution and subjected to a 4 Hz oscillation of 2% amplitude. At the end of the incubation the contents of the incubating

TABLE 4

Results of split estimation analysis

P_i produced nM	ADP produced nM	Difference (excess P_i positive)
18.25	11.2	7.05
11.2	6.55	4.65
3.5	6.55	-3.05
5.0	6.9	-1.9
4.0	10.52	-6.52
11.5	5.17	6.33
9.25	10.0	-0.75
5.5	3.28	2.22
13.25	4.66	8.69
4.25	9.85	-5.6
4.0	6.42	-2.42
9.5	7.9	1.6
2.5	1.92	0.58
9.5	11.55	-2.05
7.75	12.2	-4.55

bath were divided into two equal portions, one was used for phosphate analysis by the Marsh method, the other for analysis of ADP by the enzyme method. Table 4 shows the titres obtained using the two methods of analysis, blank values having been subtracted. It can be seen that the determinations in each pair differ widely from each other. The differences were analysed and a t-test for paired comparisons applied to discover whether the mean difference is signifcantly different from zero. The test showed that though the two methods of analysis gave different results, neither analysis gave consistently larger measurements.

In view of the wide differences between measurements, an analysis of variance was performed to determine whether the inequality could be attributed to variability in experimental measurement or whether there is evidence of another factor contributing to inaccuracy in such a way that neither method gives consistently too large or too small results. The variance of the differences between the observations is 22.3 nM. Each individual observation is subject to twice the variance of an individual point as a blank estimation has been subtracted. Thus, from the earlier estimations of variance of each method, a variance of the difference of 4.2 nM would be predicted. This is smaller than the observed variance. However analysis of the blank determinations from the split estimation experiments shows that the variance of the Marsh analyses in this series of experiments was much greater than when the method was being used regularly.

Five sets of three to five estimations of blank values of phosphate were analysed. They had a mean variance of 11.3 nM. Using this figure, the variance of the differences in Table 4 would be expected to be 2(11.3 + 0.6), i.e. 23.8 nM. The observed variance is 22.3 pmoles. Thus the dissimilarity between pairs of observations on identical samples can be attributed to errors inherent in the estimation methods.

Figure 9 a

Calibration graph, Marsh analysis of inorganic phosphate.

(Experimental titre 10 - 40 nm P_i)

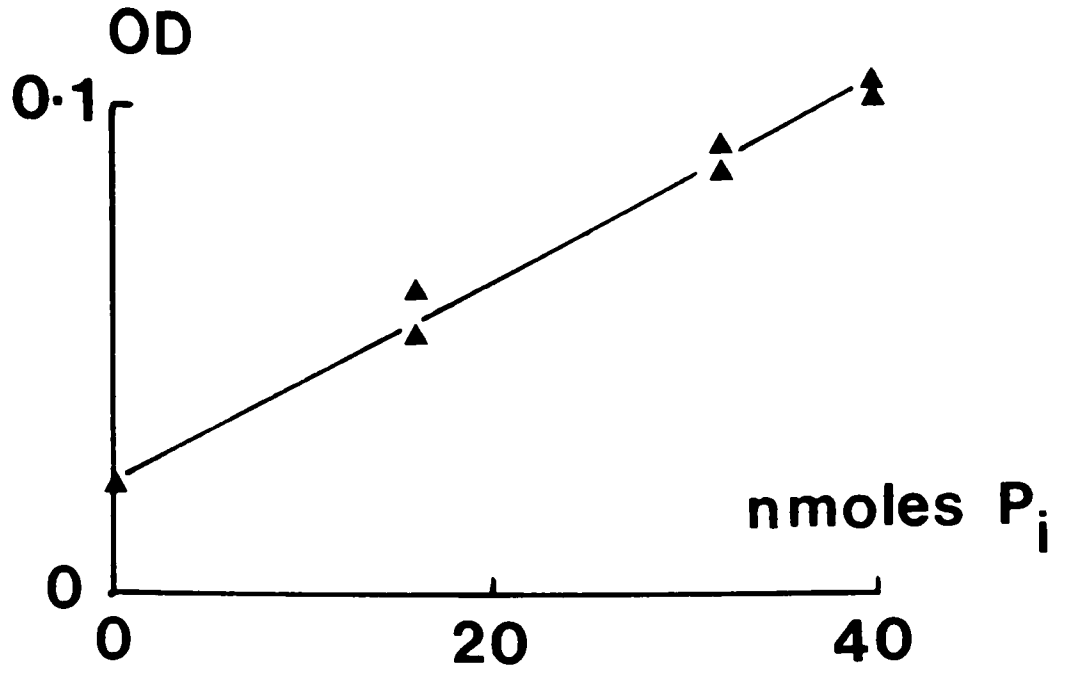


Figure 9 b

Calibration graph, enzymatic analysis of ADP.

(Experimental titre 10 - 40 nm ADP)

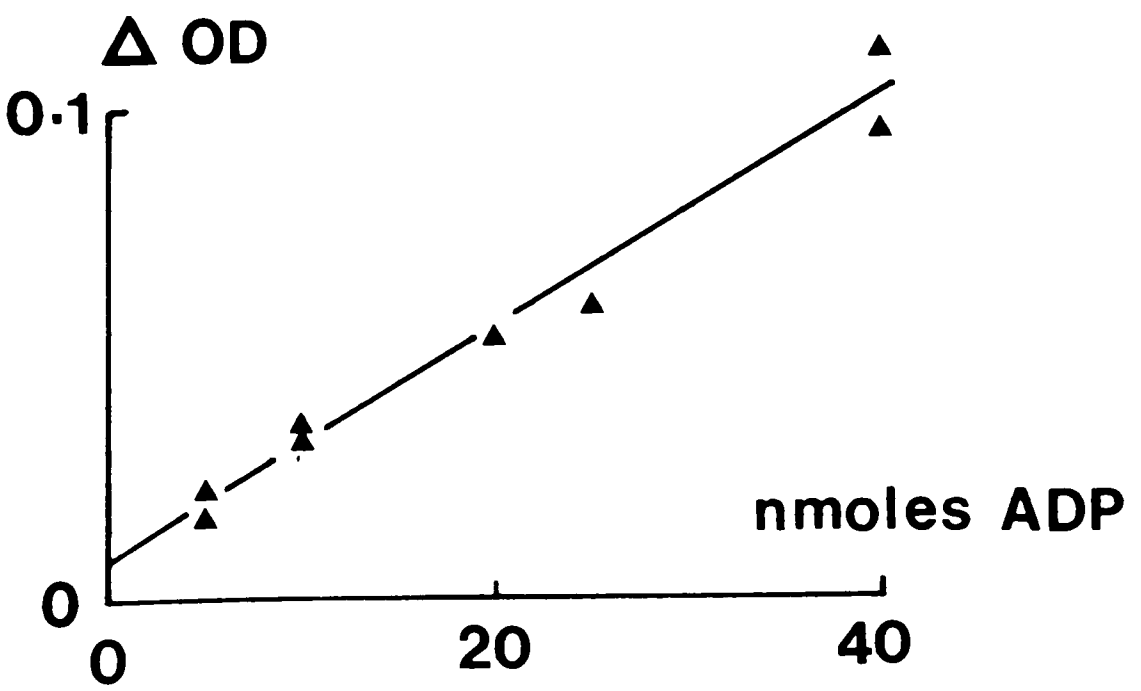


Figure10 a Relationship of P_i production by muscle fibre to duration of incubation

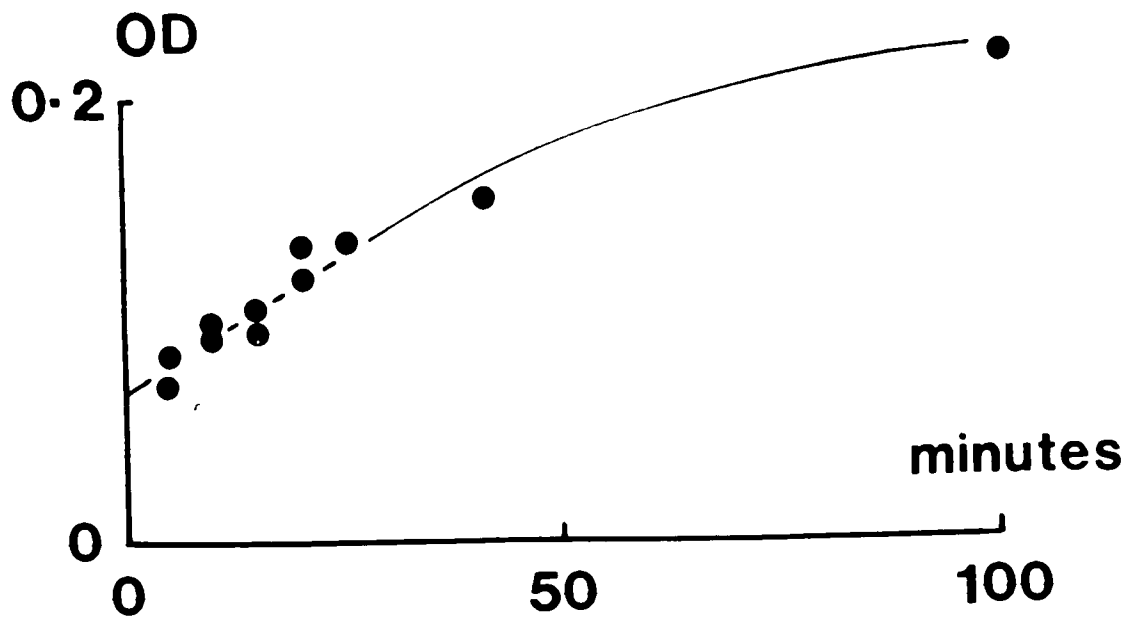
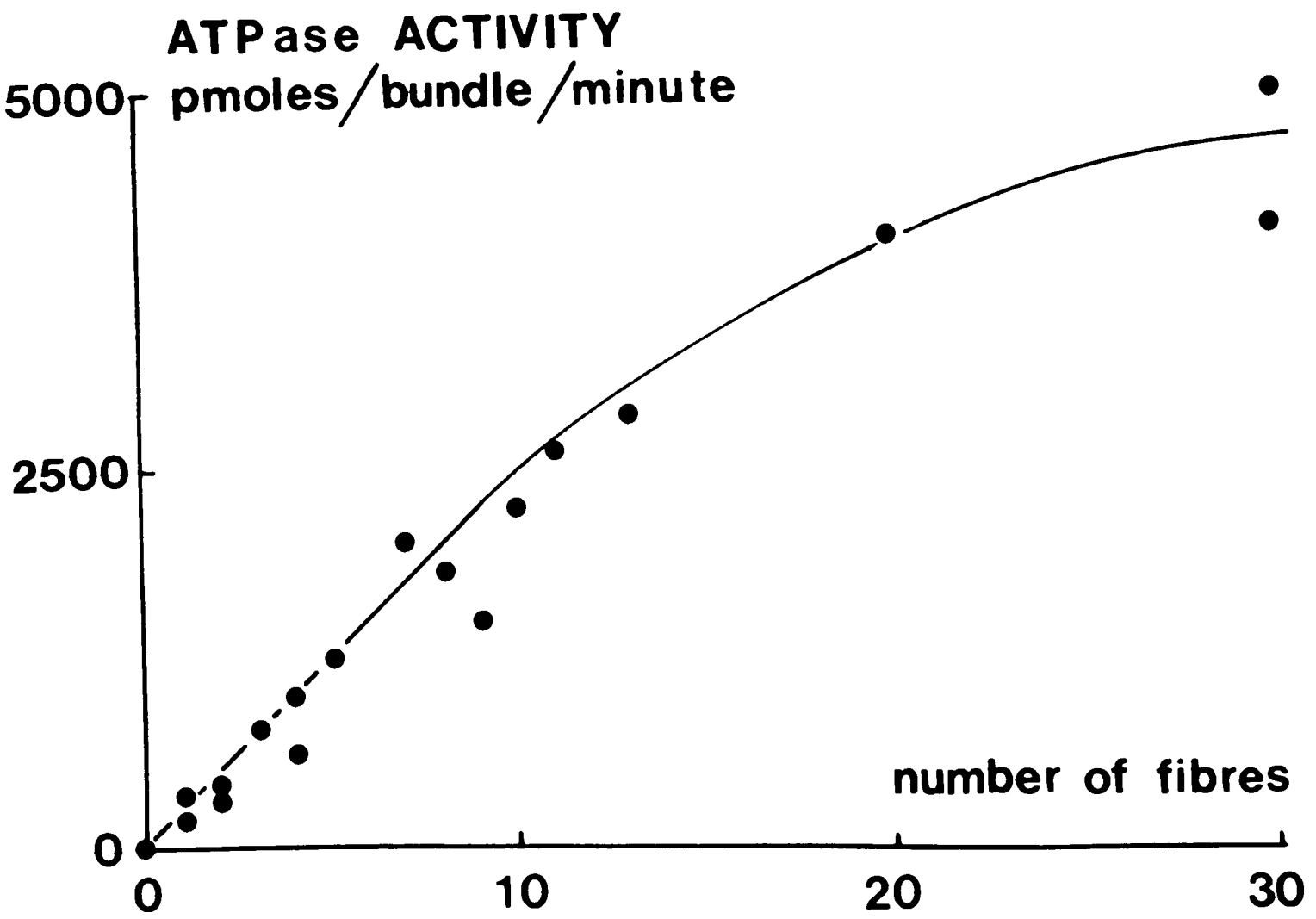


Figure 10 b Relationship of P_i production by muscle fibre to number of fibres in bundle incubated in solution.



The results of practical experiment therefore support the conclusion reached on theoretical grounds that analysis by the enzyme method of ADP produced by muscle gives the same results as analysis of phosphate by the Marsh method. As it is also more accurate and more convenient it is the preferred method in the absence of large concentrations of ADP.

Calibration graphs for the two methods are shown in Figure 9a & b. Estimations by both methods are linear over the range of experimental titres.

Control tests were performed to test the proportionality of ATPase activity to time of incubation and to fibre number. Figure 10a shows that phosphate production of six fibres stretched isometrically rises linearly with the duration of incubation up to 25 minutes. Most incubations used in experiments were of 15 to 20 minutes, some, where a low ATPase activity was expected, were of 20 to 30 minutes. Thus under the conditions of the experiments variations in incubation time will not affect the results. In Fig. 10b phosphate production is plotted against the number of fibres in the bundle. The muscle was incubated isometrically under no tension. ATPase activity is not affected at low fibre numbers. Under the conditions of the experiment, the rate of ATP hydrolysis is not high and thus even with large bundles, ATP starvation is not likely to occur in the middle fibres. When fibres are oscillated and the rate of ATP hydrolysis increases, this is likely to occur; it can be recognised as the muscle then enters the high tension state already discussed.

Thus control experiments show that estimations are reliable under the conditions of the experiments.

CHAPTER 3

The mechanical performance and ATPase activity of fibrillar muscle

1. Introduction

The aim of the work described in this thesis was to investigate the way in which different factors affect the ATPase activity of glycerinated muscle fibres. Before the effect of different conditions can be examined it is necessary to establish the relationship between the main parameters of muscle activity. In this chapter the effect of changes of extension, amplitude and frequency of oscillation on the mechanical and chemical output of the muscle are outlined.

2. Behaviour of the muscle in relaxing solution

It has been established (Rüegg & Tregear, 1966; Maruyama et al., 1968) that a threshold concentration of around 10^{-8} M calcium ions is necessary for activation of ATPase activity, expression of stretch activation and power production in insect flight muscle.

If fibres are placed in a solution containing Mg-ATP but no calcium - 'relaxing solution' - and subjected to oscillatory length changes of about 2% they show a characteristic non-linear rise and fall of tension with length, as shown in Fig. 11. No work is produced and typically there is a small absorption of work done by the apparatus.

The ATPase activity of relaxed fibres was measured to discover how much, if any, ATP is split in the absence of calcium activation. The fibres were immersed in phosphate buffered relaxing solution of the composition shown below.

Figure 11

Length-tension loop, calcium free relaxing solution. 6 Hz, 2% amplitude.

Arrowheads indicate direction of rotation of loop.

Figure 12

ATPase activity of fibres incubated in calcium-free relaxing solution.

Horizontal bars show duration of each incubation.

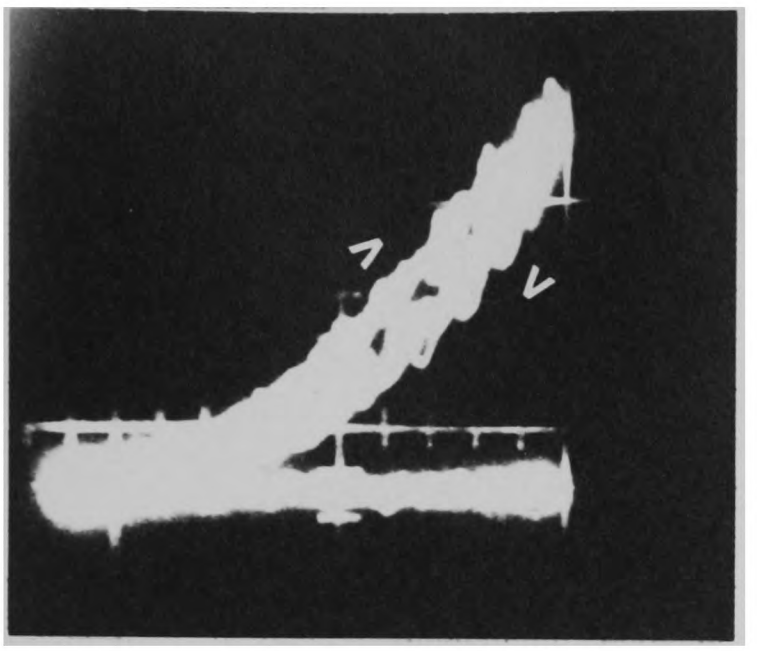
Waved bar ~~~~~ indicates that the fibres, when subjected to a brief test oscillatory length change, produced positive work.

Symbols represent individual experiments.

Each experiment was statistically analysed to find the regression coefficient of ATPase activity upon time.

Experiment	Temperature	Regression coefficient, slope	Standard error	Intercept
	°C	pmoles/minute	pmoles/minute	pmoles/cm/min
○	20	0.44	0.13	29.2
▽	20	0.62	0.19	22.3
□	20	0.26	0.07	45.9
●	30	1.93	0.42	15.0
▲	30	0.53	0.14	74.2

Figure 11



10 mg

Figure 12

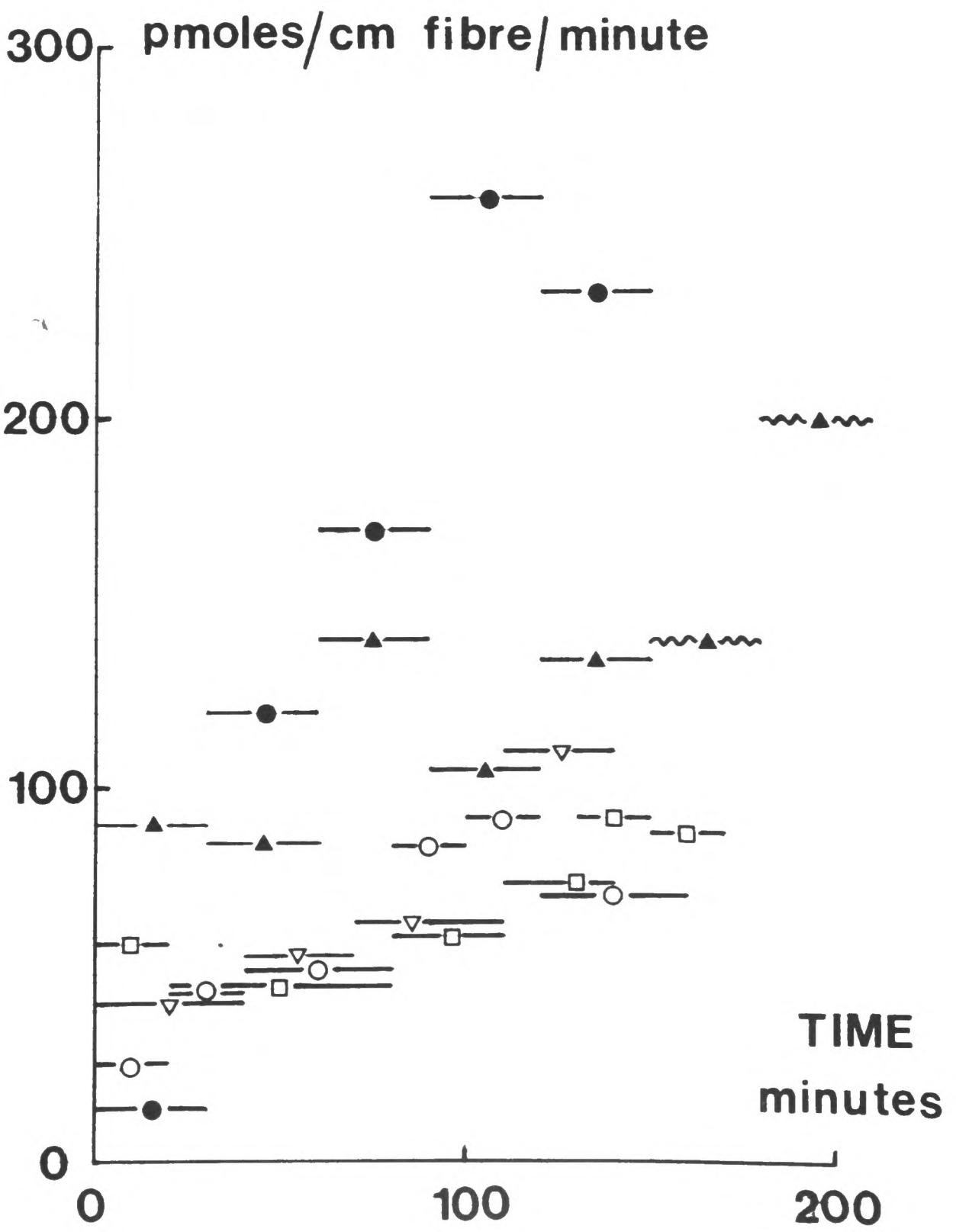


TABLE 5

Species	Concentration
Mg-ATP	4.6 mM
Ca^{2+}	$< 10^{-8}$ M
P_i buffer	10 mM
Ionic strength	0.09 M
pH	6.95
Temperature	20°C or 30°C

EGTA was added to the solution to remove any calcium ions that might be present as contaminants.

The fibres were incubated, unstretched, for consecutive periods of 20 to 30 minutes so that the ATP hydrolysis could be estimated. The graph in Fig. 12 shows the results of five individual experiments, each performed on a different fibre or pair of fibres. ATPase activity is plotted against immersion time and in all cases the rate at which ATP is hydrolysed increases with the length of time the fibre has been immersed. At 20°C the initial rate was on average 32.5 pmoles ATP hydrolysed/cm fibre/minute, rising after $2\frac{1}{2}$ hours to around 90 pmoles/cm/minute. The two experiments at 30°C differ but they show that ATP hydrolysis rate increases more steeply at 30°C than at 20°C. The lowest rates of ATP splitting in calcium containing solution are 131 ± 52 pmoles/cm/min (20°C) and 205 ± 70 pmoles/cm/min (30°C). Thus this rate was achieved within three hours' incubation at 30°C, but at 20°C the rate was not reached in that time.

In one of the experiments at 30°C the performance of the fibres was tested at intervals. Initially the performance was typical of

relaxed fibres, see Fig. 11, but after 150 minutes a few micrograms positive mid-length tension difference was obtained at low amplitude (0.2%) showing that the fibres had become capable of performing work. In the case of the other experiment at 30°C, the fibre was left in relaxing solution after the chemical analyses were completed and after a period of three hours work was obtained. The mechanical performance was not tested in the other experiments.

During prolonged incubation in relaxing solution, the ATPase activity of fibres increased and, in two cases tested, mechanical work was eventually produced. These results can be explained by postulating that the system which prevents crossbridge cycling in the absence of calcium is gradually being rendered inactive in some way. Meinrenken (1969) found that if the inhibition system, the troponin and tropomyosin of the I filament, is removed from glycerinated fibres in high ionic strength alkaline solution, there is an increase in the ATPase activity and ability of the fibres to produce tension. In these respects removal of the inhibiting system produced results similar to those following addition of calcium, although he was unable to obtain any work from his fibres. Nevertheless a more gradual inactivation or removal of the inhibitory proteins would result in both increased ATPase activity as more crossbridges become free to cycle, and also in ability to produce work as the whole molecular structure of the filaments was left intact. Abbott (1972, in preparation) has reported that freshly glycerinated fibres require much higher calcium concentration (10^{-8} M) for activation than do muscles that have been stored for three weeks or more, which require only 10^{-9} M calcium to reach the threshold of activation. This phenomenon also may represent the decay or removal or inactivation of the troponin-tropomyosin system, proceeding at a much slower rate at -18°C, the temperature at which glycerinated muscles are stored.

3. Behaviour of the muscle in phosphate-buffered activating solution

The details of the behaviour of muscle depend on the exact composition of the incubating medium. The results in this section were obtained using a solution of the composition shown below.

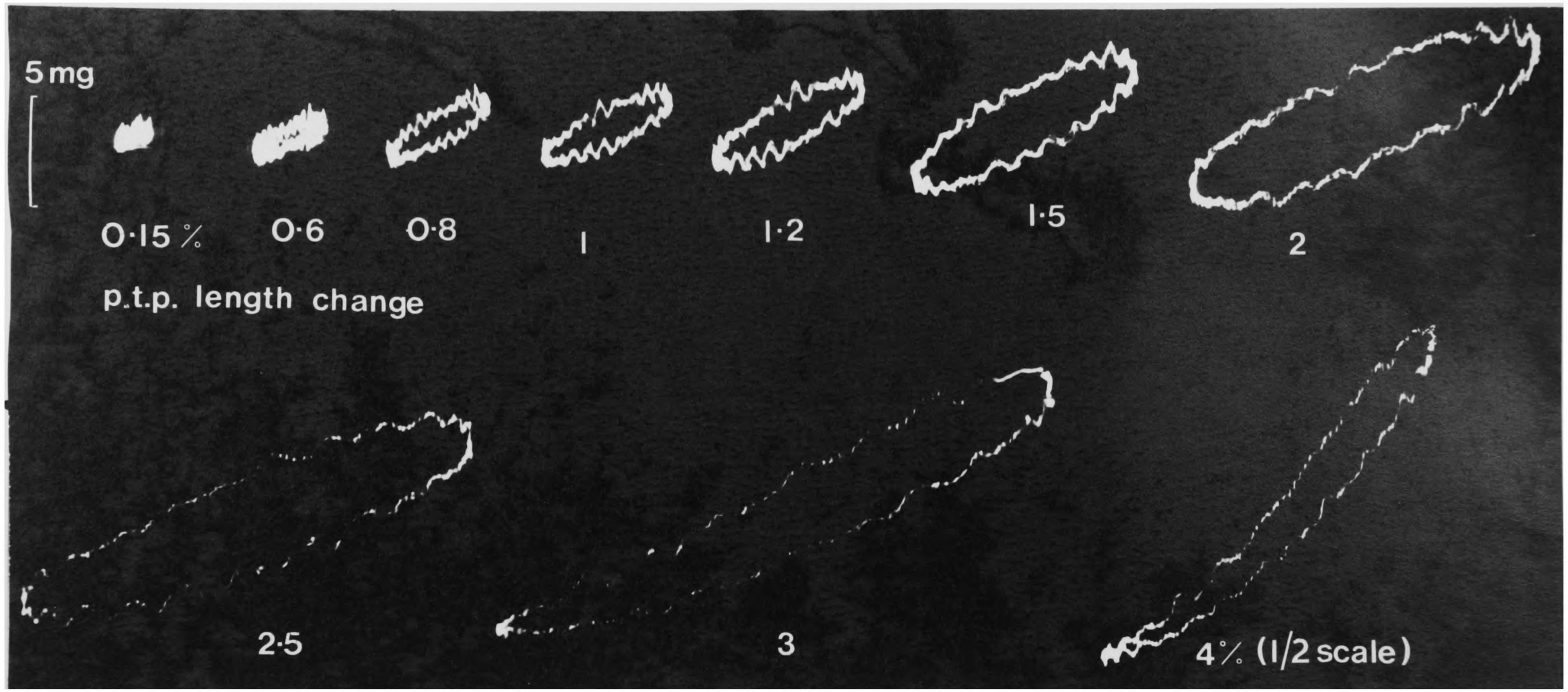
TABLE 6

Species	Concentration
Mg-ATP	4.7 mM
Mg^{2+}	3.5 mM
Ca^{2+}	0.0017 mM
P_i buffer	10 mM
Ionic strength	0.08 M
pH	6.95
Temperature	21°C

Many experiments were performed using solution of this composition so that a large amount of data was obtained. Therefore the details of the relationship of the parameters measured were deduced from the results of this work. As the conclusions form a framework of reference with which work described in subsequent chapters can be compared, the type of analysis and the relationships thus derived exist as a standard for this work. Thus the incubating medium can be regarded for this purpose as a standard solution.

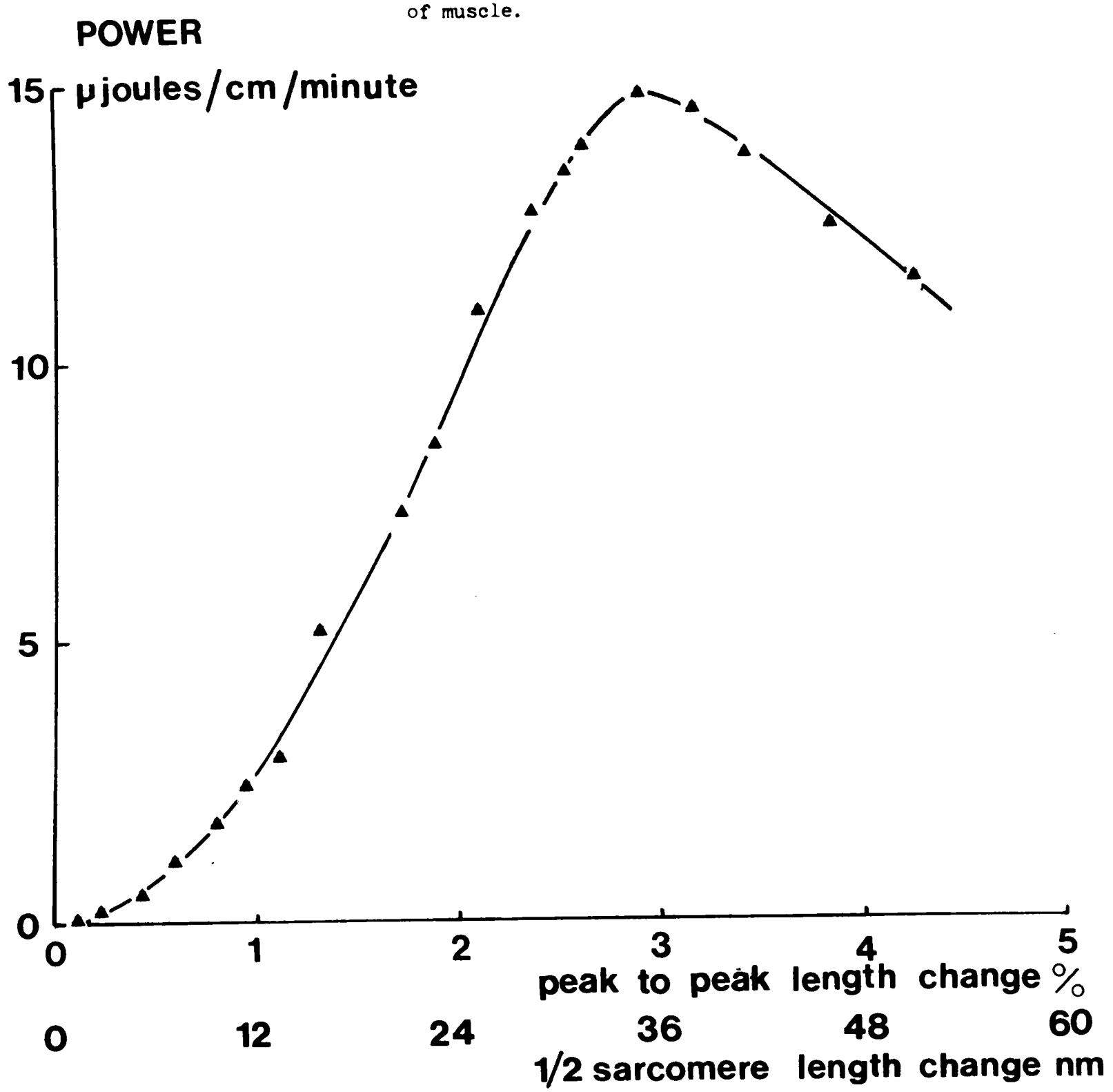
Single or paired fibres were mounted on the apparatus and immersed in activating solution. They sometimes showed evidence of contraction and it was necessary to shorten the distance between the pins to obtain zero tension. If the fibre is then elongated it shows

Figure 13 Effect of increasing amplitude of oscillation on performance of muscle.



6 Hz

Figure 14 Effect of increasing amplitude of oscillation on power output of muscle.



the characteristic delayed rise in tension which overshoots the final value and then gradually sinks back to a new steady level, see Fig. 8a.

The behaviour of the fibres described here is similar to that observed by other workers, e.g. Pringle & Tregear (1969). As already stated, the exact quantitative values differ from fibre to fibre and vary with age and details of chemical composition of incubating solutions and although the results quoted here do not match those of Pringle and Tregear exactly, the latter results are within the range of response observed during the performance of this work.

To obtain work from the fibre it is necessary to stretch it by approximately 1% of its resting length. Resting length is defined as the length of the fibre when it has been set so that just no tension is exerted. If an oscillatory length change of about 6 Hz is then imposed, gradual increase in amplitude will produce changes in the length tension diagram as shown in Fig. 13. The magnitude of both the mean tension and the mid-length tension difference increase as amplitude increases. Above an amplitude of about 0.5% the hysteresis loop becomes increasingly asymmetrical, sometimes becoming bottom pointed. Above about 3.5% work is lost and the loop may become top-pointed, see Fig. 14. At first the condition can be reversed by decreasing the amplitude, but if the amplitude is increased far beyond the point at which top-pointed loops appear, the fibre appears to sustain permanent damage and cannot work well even at lower amplitudes.

If an intermediate amplitude such as 2% is selected and the frequency of operation is varied, a pattern similar to that in Fig. 15a is found. The pattern of work per cycle and mean tension increasing, and then decreasing above 6-8 Hz is similar to that recorded by Pringle & Tregear (1969). The decrease in work per cycle at high frequency was characteristically manifested as a gradual symmetrical narrowing of

Figure 15a Effect of changes in frequency of oscillation on performance of muscle.

a. 2·0% amplitude

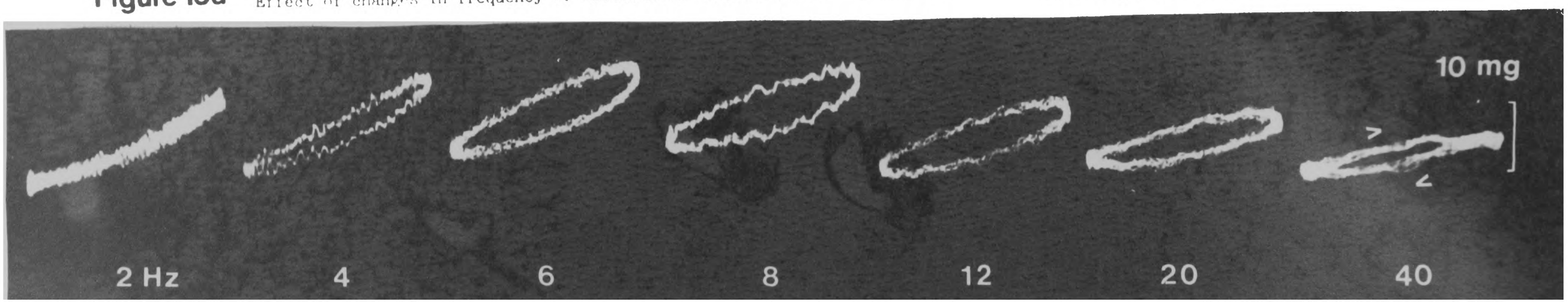
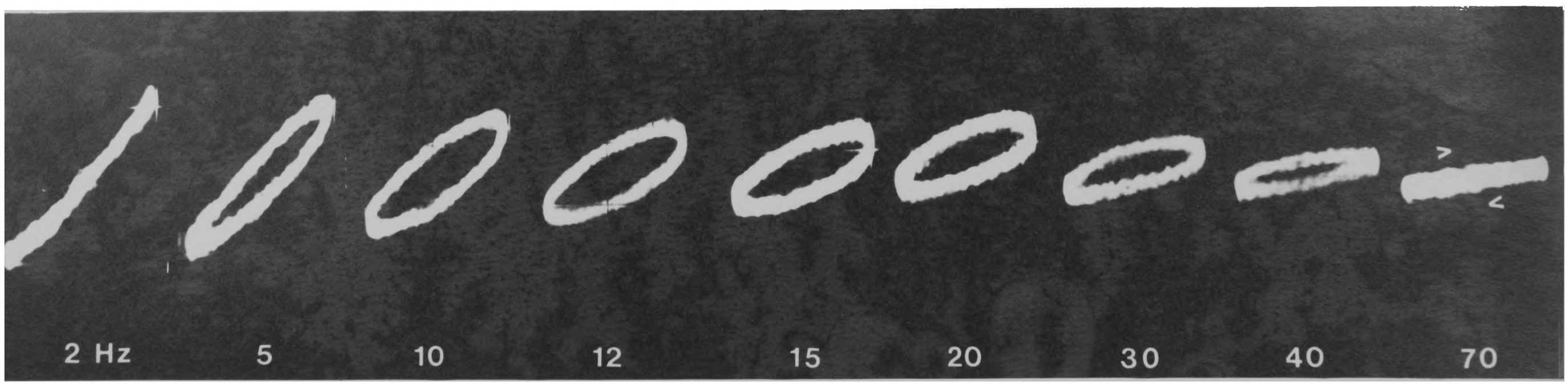


Figure 15b

b. 0·2% amplitude



Effect of frequency of oscillation on power production and work output.

Figure 16 a 2·0 % amplitude

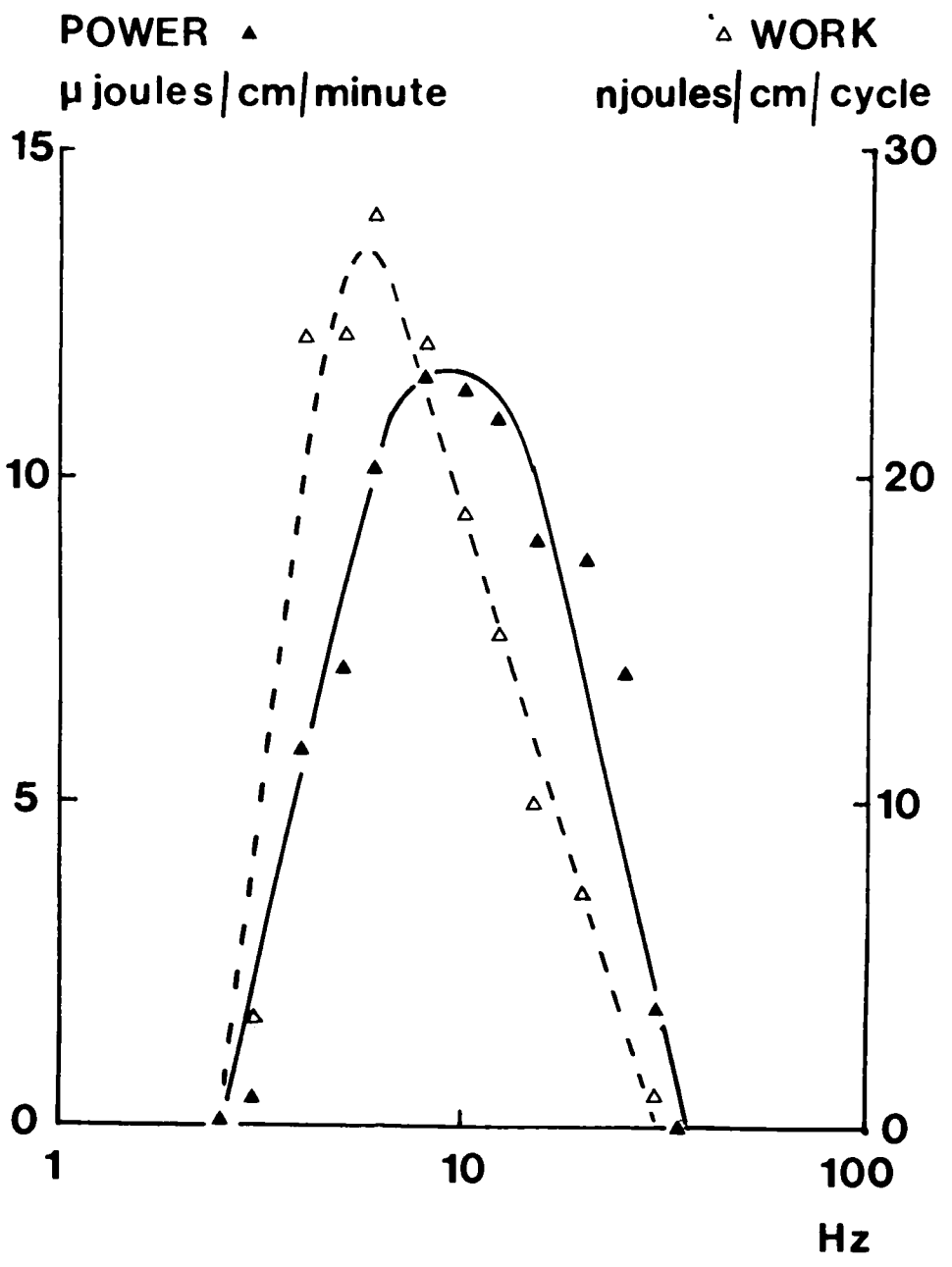


Figure 16b 0·2% amplitude

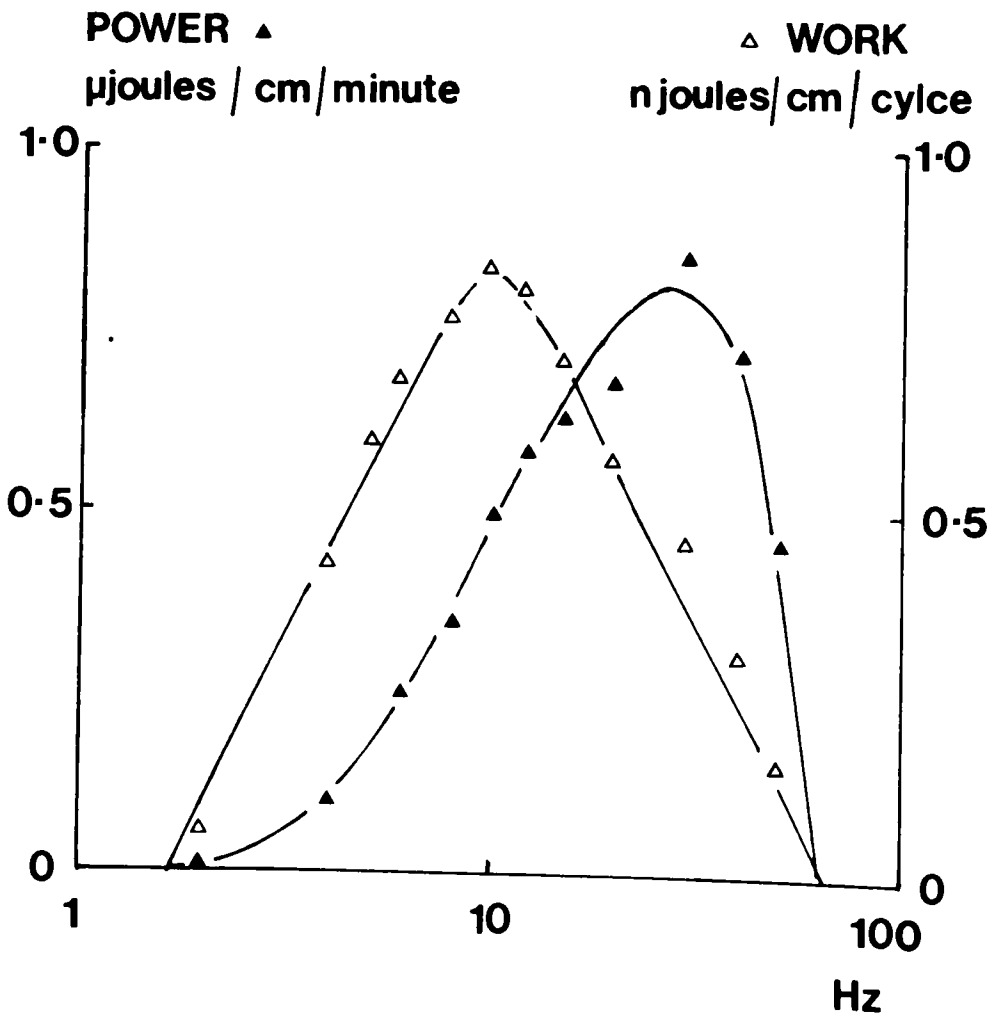
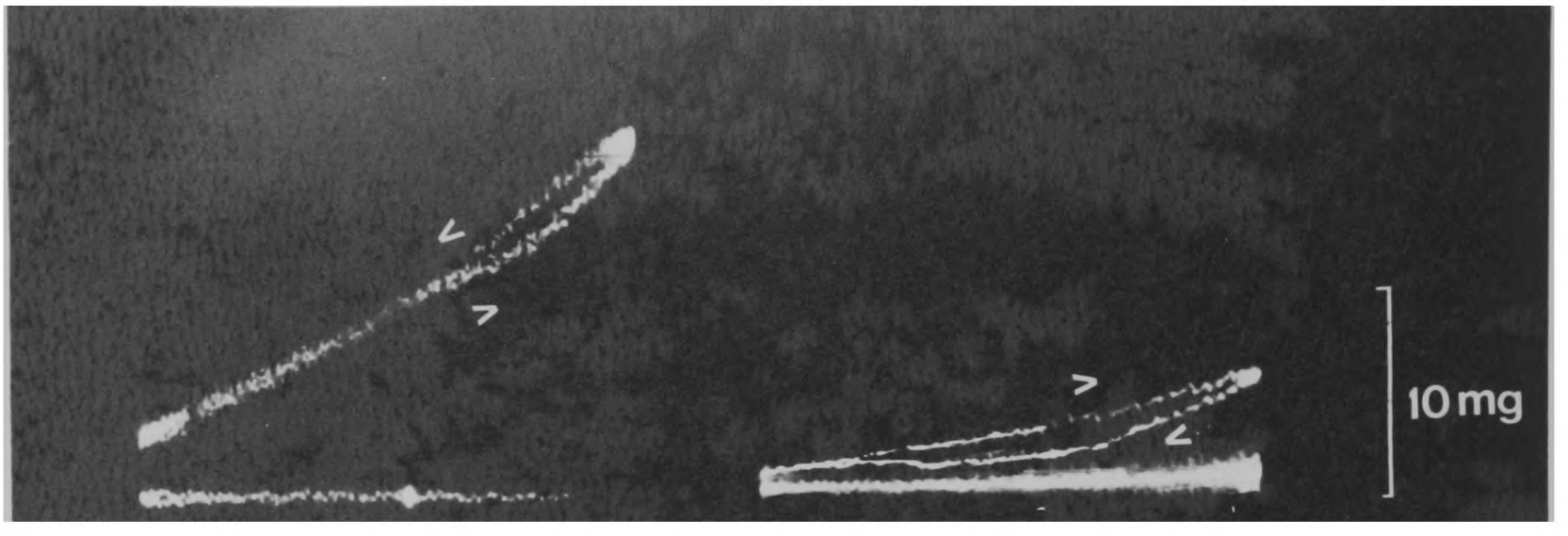


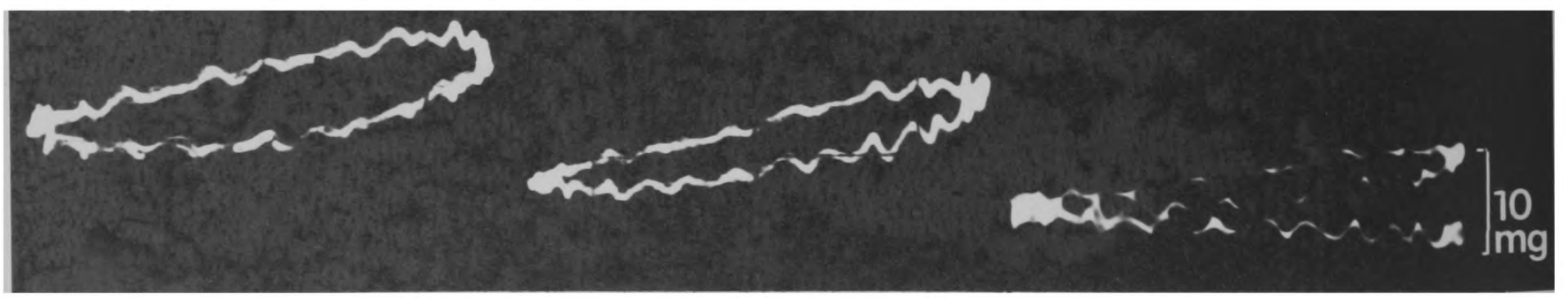
Figure 17 a Effect of prolonged oscillation at very low frequency.



0 minutes 10

2 Hz
2% amplitude

Figure 17 b Loss of power output of muscle fibre unable to maintain tension.



0 minutes	3	8
14 mg tension	10	4
4·2 μjoules power	2·3	1·3

6 Hz
2% amplitude
length constant

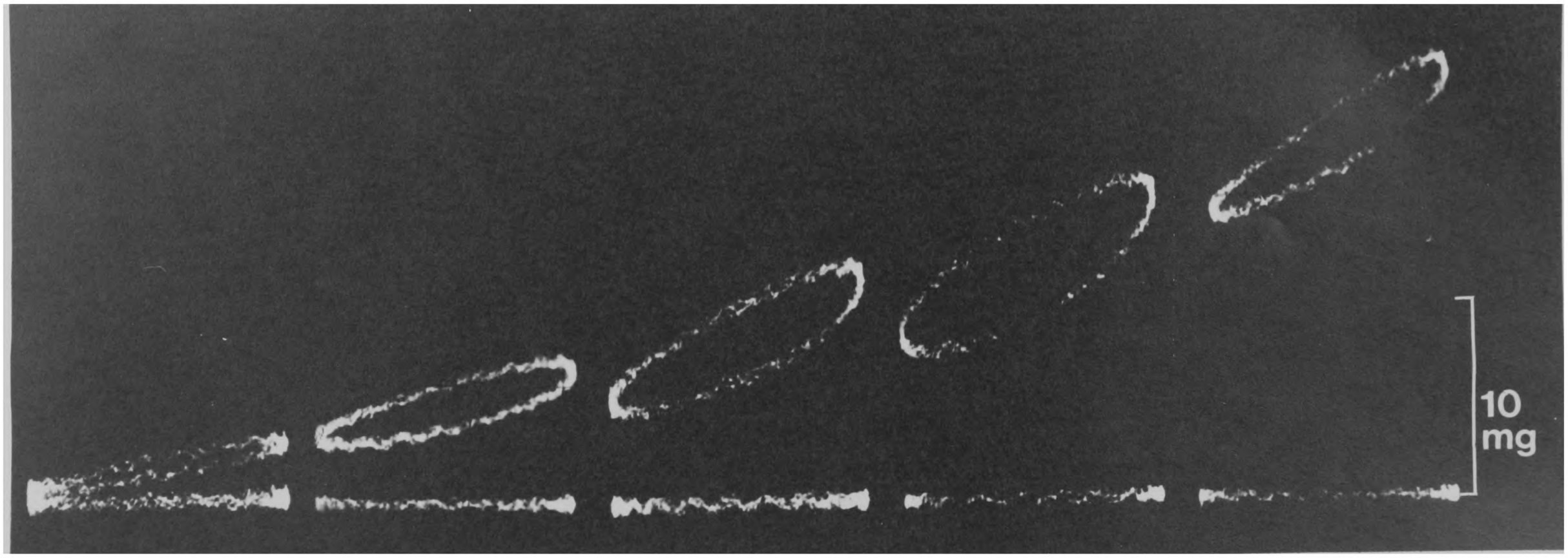
the loop but occasionally it was marked by the appearance of a figure-of-eight loop with a gradually expanding negative bottom portion. Prolonged oscillation at frequencies below 2 Hz damaged fibres; work, if initially obtained, would disappear after a few minutes, and usually the mean tension dropped to a very low value and the fibres ceased to work even at higher frequencies, see Fig. 17a. Very high frequency oscillation did not affect the muscle permanently.

The effect of variation in frequency at a low amplitude of oscillation such as 0.2% where the length-tension loop is symmetrical is shown in Fig. 15b by the hysteresis loops and in Fig. 16b by a graph of work and power output against frequency. Comparison with the data for 2% amplitude shows that at low amplitude work is produced up to much higher frequencies. The frequency at which most work is performed, and hence most power produced, is also higher at lower amplitude. Maximum work per cycle is greatly reduced at 0.2% and the fact that the fibres have their optimum performance at a much higher frequency does not compensate for the reduced mid-length tension difference, so that the maximum power output is also much lower. The mechanical characteristics estimated from the graphs are tabulated below.

TABLE 7

Amplitude	Optimum frequency for work	Maximum work/cycle	Optimum frequency for power	Maximum power	Frequency range
	Hz	nJoules/cm/cycle	Hz	μJoules/cm/minute	Hz
2.0%	5.6	27.0	9.2	11.7	2.4 - 34.0
0.2%	9.4	0.85	27.0	0.81	1.6 - 65.0

Figure 18 Effect of extension on performance of muscle.

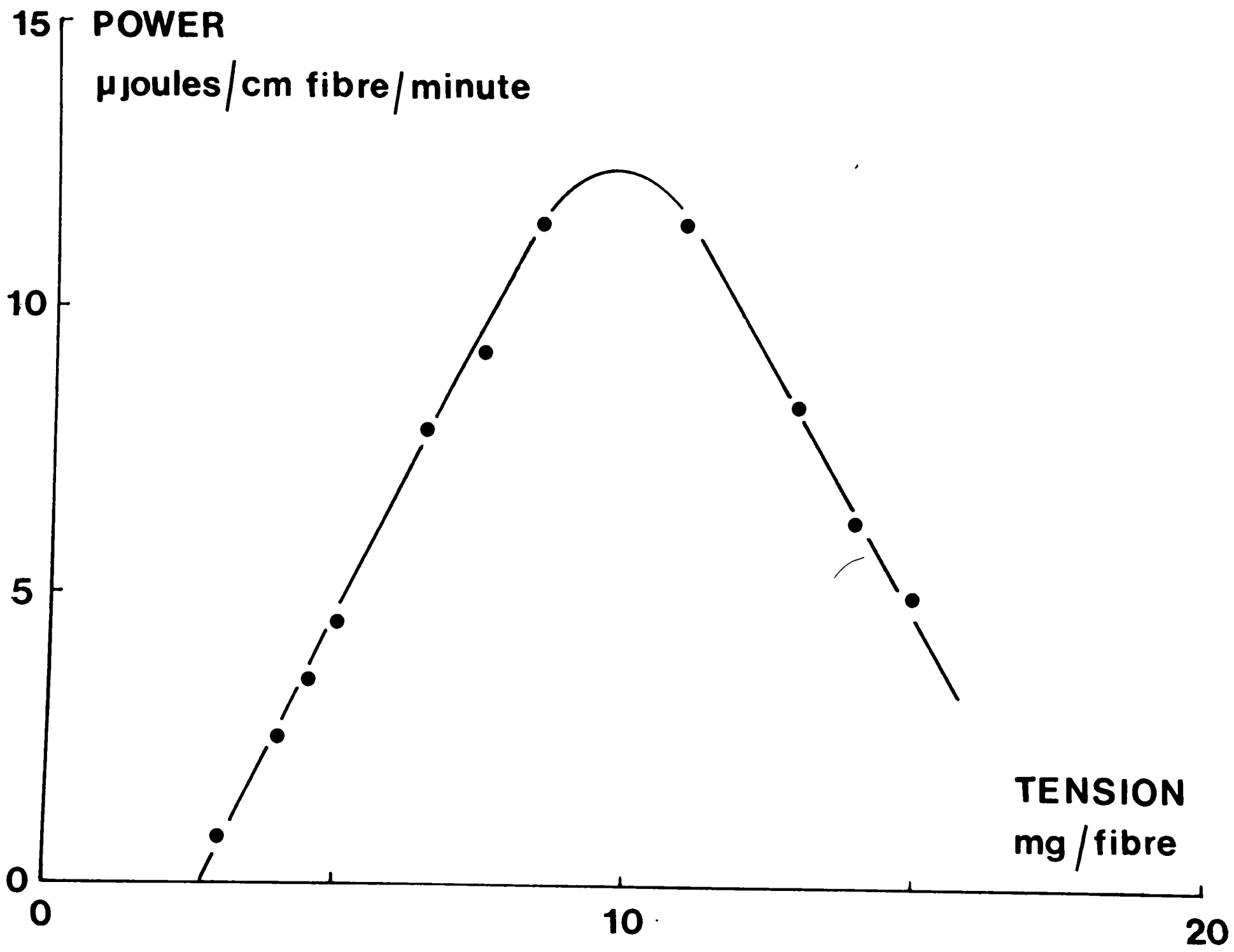


0·75 1 2 2·5 6% extension

6 Hz

2% amplitude

Figure 19 Effect of extension on power output of muscle.



The effect of extension of the fibre on the mechanical performance is recorded in Fig. 18. Extension results in an increase in the mean tension of the muscle. The mid-length tension difference, and thus the work, also increases up to a critical value, above which it falls. Such a position is generally not stable and, unless the fibre is continuously extended the tension will fall rapidly and the power output will rise, until a stable state is reached. The power output at this tension may be well below that which can be obtained, particularly at the start of oscillation when the first few dozen cycles may produce about twice as much work per cycle as is subsequently observed.

The stability of performance of fibres varies greatly. Some, if extended to any degree less than that for maximum power will maintain a steady performance for half an hour. Others, though capable of equally high work output, unless continuously extended, lose tension and power rapidly and stabilise at a very low performance (Fig. 17b). It was not possible to predict how constant a performance a particular fibre might maintain.

The effects of varying amplitude, frequency and extension on the performance of the muscle are shown graphically - Figs. 14, 16 and 19.

In selecting the mechanical state of the muscle for experiments in which the ATPase activity was measured certain factors had to be considered. Firstly, as chemical and mechanical activity were to be correlated it was necessary to have as stable a performance as possible for the duration of the incubations - 10 to 20 minutes, and preferably for several incubations. Secondly, a large power output was sought, as an indication that the fibres were working properly, and also as large titres of ADP were required for measurement and one was thought to accompany the other (Steiger & Rüegg, 1969). Thus as high an amplitude

as could regularly be obtained without producing top-pointed loops was used throughout the experiments, except where the effect of low amplitude was investigated. A frequency which gave a large power output was used, except where high frequency points were specifically required. The tension could then be adjusted by changing the length of the fibre to give the best performance. As this had to be maintained for 20 minutes, maximum power output was only rarely obtainable. The stability of the performance throughout the incubation was estimated by measuring the mid-length tension difference and the extension was adjusted to keep this as constant as possible, see Fig. 17b.

Tension, rather than extension, was chosen as a parameter for comparison because length did not prove a reliable measurement. Fibres were found to vary in their elasticity and so in the tension produced by a fixed extension. The fact that a stable tension could not always be maintained at a given length for the duration of an incubation and that constant performance can be maintained by extending the fibres during an experiment, also shows the unreliability of extension as a criterion of muscle performance, see Figs. 8b and 17b. Other workers have also found that, except in a limited experiment, tension is a more meaningful measurement to take than length (Pringle & Tregear, 1969).

ATPase activity was plotted against the mean tension of the muscle. In the case of an oscillatory experiment the mean tension is the tension at the centre of gravity of the loop (see Fig. 7). This is only one of a number of measurements of tension that could be made. It was chosen because it bears the closest relationship to the tension measured for isometric fibres as it represents the average force exerted by the muscle during its oscillatory cycle.

Figure 20 Effect of extension on ATPase activity of isometric muscle.

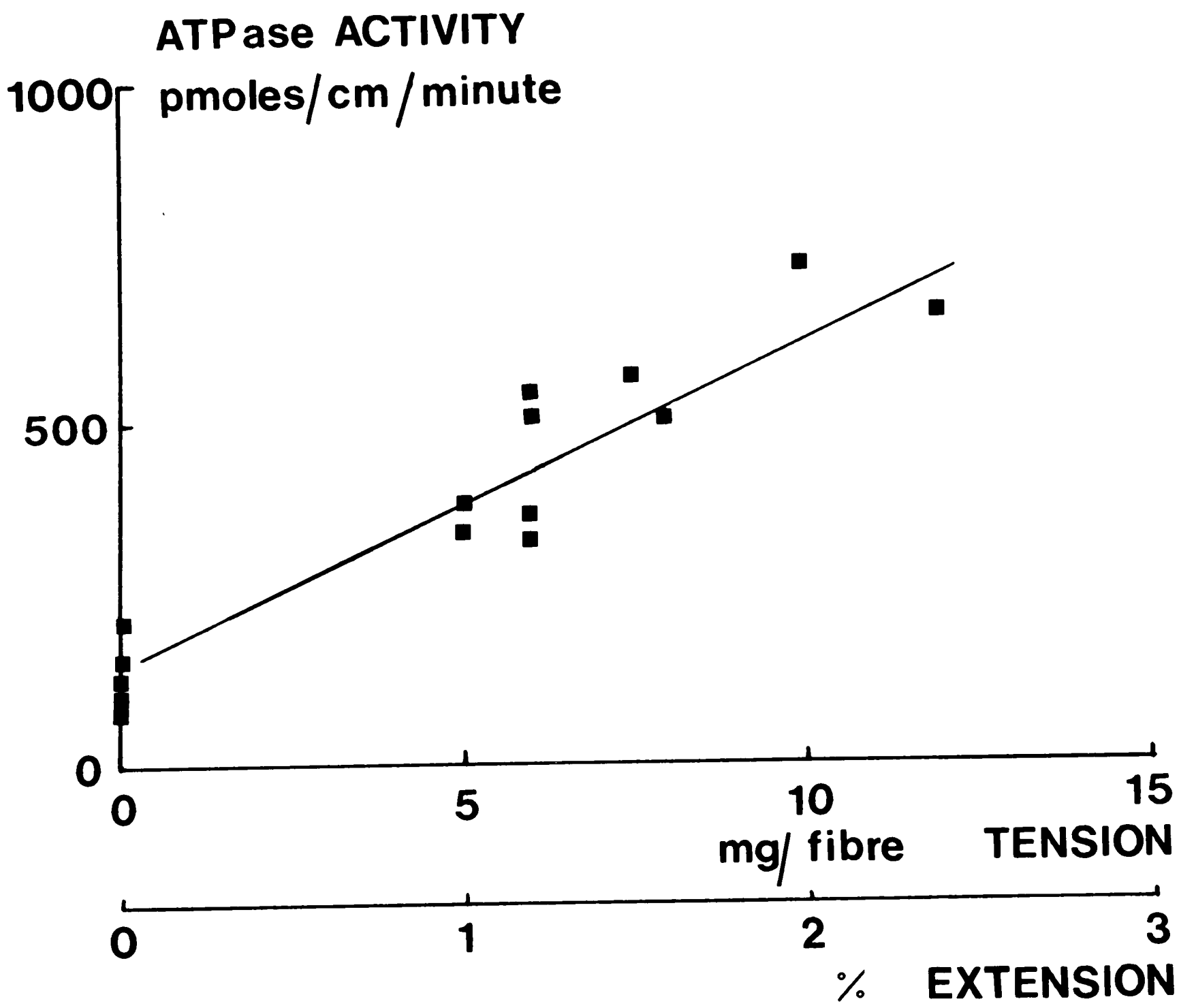


Figure 20 shows the effect of increased tension by extension on the ATPase activity of static muscle. Even when no tension at all is generated ATP is still split at the rate of 131 ± 52 pmoles/cm/minute. This is four to five times the rate of hydrolysis under similar conditions but in the absence of calcium, 32.5 ± 16.1 pmoles/cm/minute. When the muscle is stretched by a small amount tension is generated and the ATPase activity rises. An increase in ATPase activity with extension is not observed in relaxing solution (Rüegg & Tregear, 1966).

Figure 21 shows a similar plot for actively working fibres. The measurement of tension has already been described. The points on the Y axis, representing the production of no tension, are taken from the data for static fibres as a control experiment showed that the ATP hydrolysis rate when the fibre was unextended was the same whether or not an oscillation was imposed.

ATPase activity was also plotted against power output. Power was selected rather than work per cycle as power, representing the rate of doing work per minute, is comparable with the energy consumed by the fibre, expressed as picomoles of ATP split per minute. The same relationship could equally have been expressed in terms of rates of energy usage and production per cycle. The first alternative was selected as more convenient. The ratio between power output and rate of ATP hydrolysis gives a measure of the efficiency of the muscle, i.e. the quantity of mechanical energy it is extracting from a given amount of fuel.

The graph of ATPase activity against power (Fig. 22) shows that the rate of ATP hydrolysis rises as the amount of power produced increases. At very high frequencies, at which little or no power is produced, ATP hydrolysis still proceeds at a rate greater than that found when there is no power output at lower frequencies because there is insufficient tension. Table 8 gives experimental values for ATPase

Figure 21

Relationship of ATPase activity to tension in oscillating muscle.

Amplitude of oscillation 2%

- ● Oscillatory muscle
- O Points obtained from static muscle under no tension.

Line is best fit line calculated as described in Chapter.

Slope 95.6 pmoles/cm/min/mg tension

Error 14.5 pmoles/cm/min/mg tension

Intercept 101 pmoles/cm/minute

Figure 21

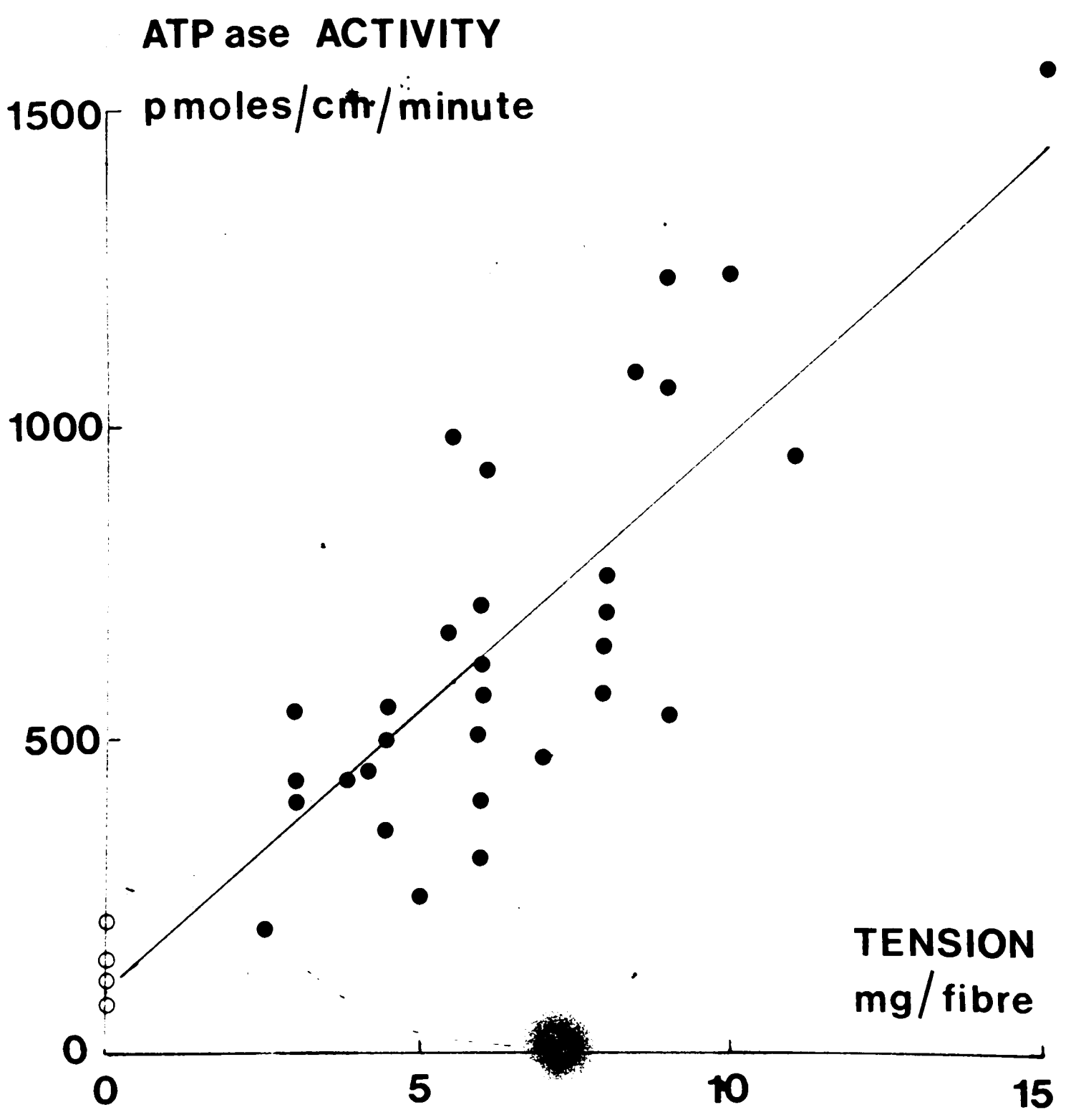

ATP ase ACTIVITY
p moles/cm/minute
1500
1000
500
0
0
5
10
15
TENSION
mg/fibre

Figure 22

Relationship of ATPase activity to power output

All points in which no power was produced excluded.

■ Points at which power and tension change in parallel.

□ Points at which extension and tension are greater than optimal and tension and power change in opposite directions.

Oscillation amplitude 2%

Frequency 3-20 Hz

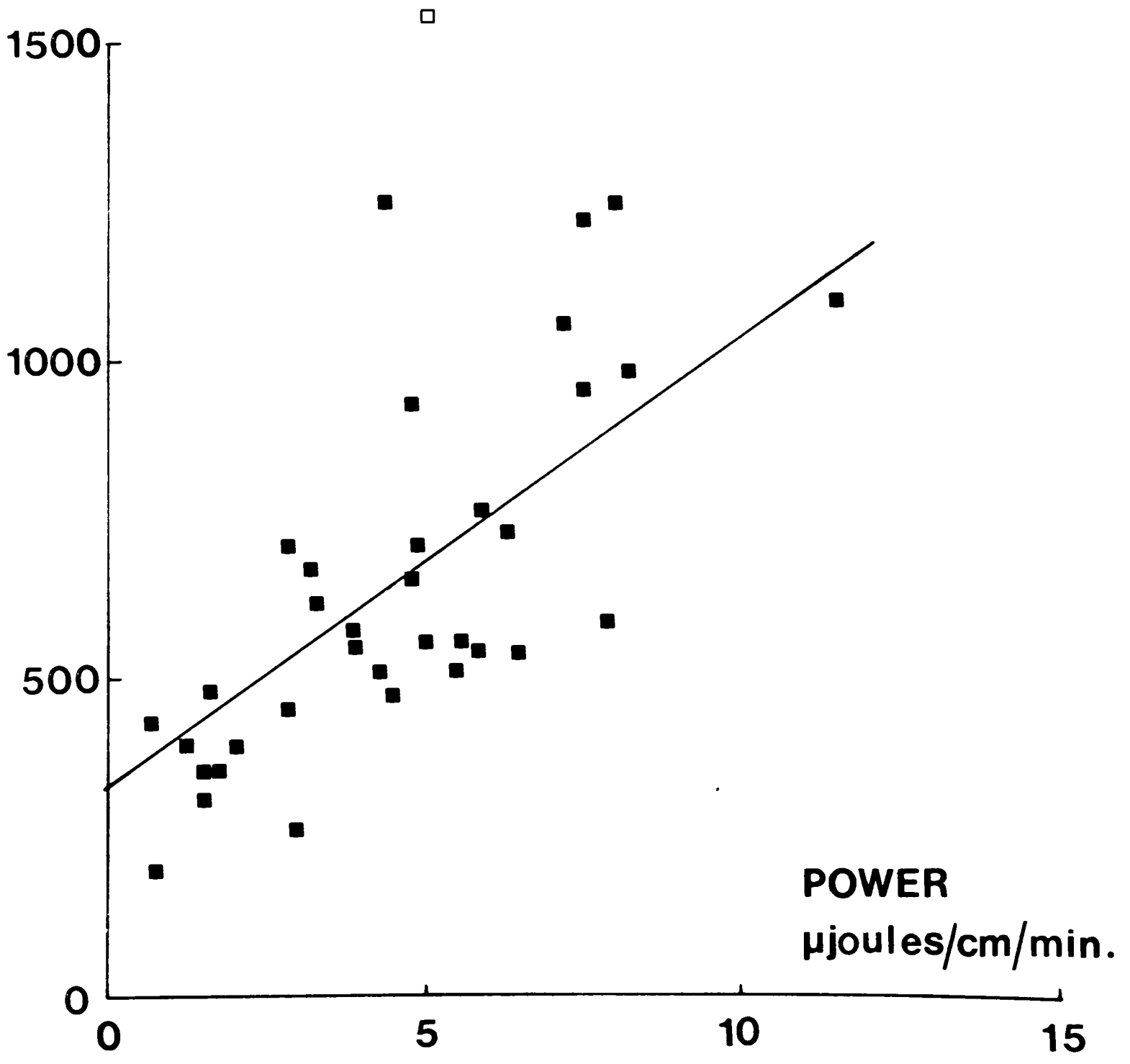
Line is best-fit line through ■ points calculated as described in Chapter 3.

Slope 72.0 pmoles/μJoule

Error 14.0 pmoles/μJoule

Intercept 327 pmoles/cm/minute

Figure 22


ATPase ACTIVITY
pmoles/cm/minute
1500
1000
500
0
0
5
10
15
POWER
µjoules/cm/min.

activity of a fibre exerting no tension and for fibres oscillated but not producing any power.

TABLE 8

ATPase activity of fibres producing no power

Slack fibres	Oscillated at high frequency
pmoles/cm/min	pmoles/cm/min
135	360
147	620
175	750
105	450
85	550
170	635
190	895
210	475
165	450
155	1200
	1250
	760

There is another condition which shows that power production and ATP hydrolysis are not always closely coupled. It has already been mentioned (see Fig. 18) that muscle fibres can be stretched to a point where tension increases but power decreases. Only occasionally can this state be maintained for long enough to allow the ATP hydrolysis to be measured. Figure 23 shows the results of two experiments in which this was possible. The length-tension loops of one experiment are shown. The rate of ATP hydrolysis rises linearly with tension, although power

Figure 23

ATPase activity, tension and power output in two experiments.

	Experiment A	Experiment B
Power output	●	■
ATPase activity	○	□

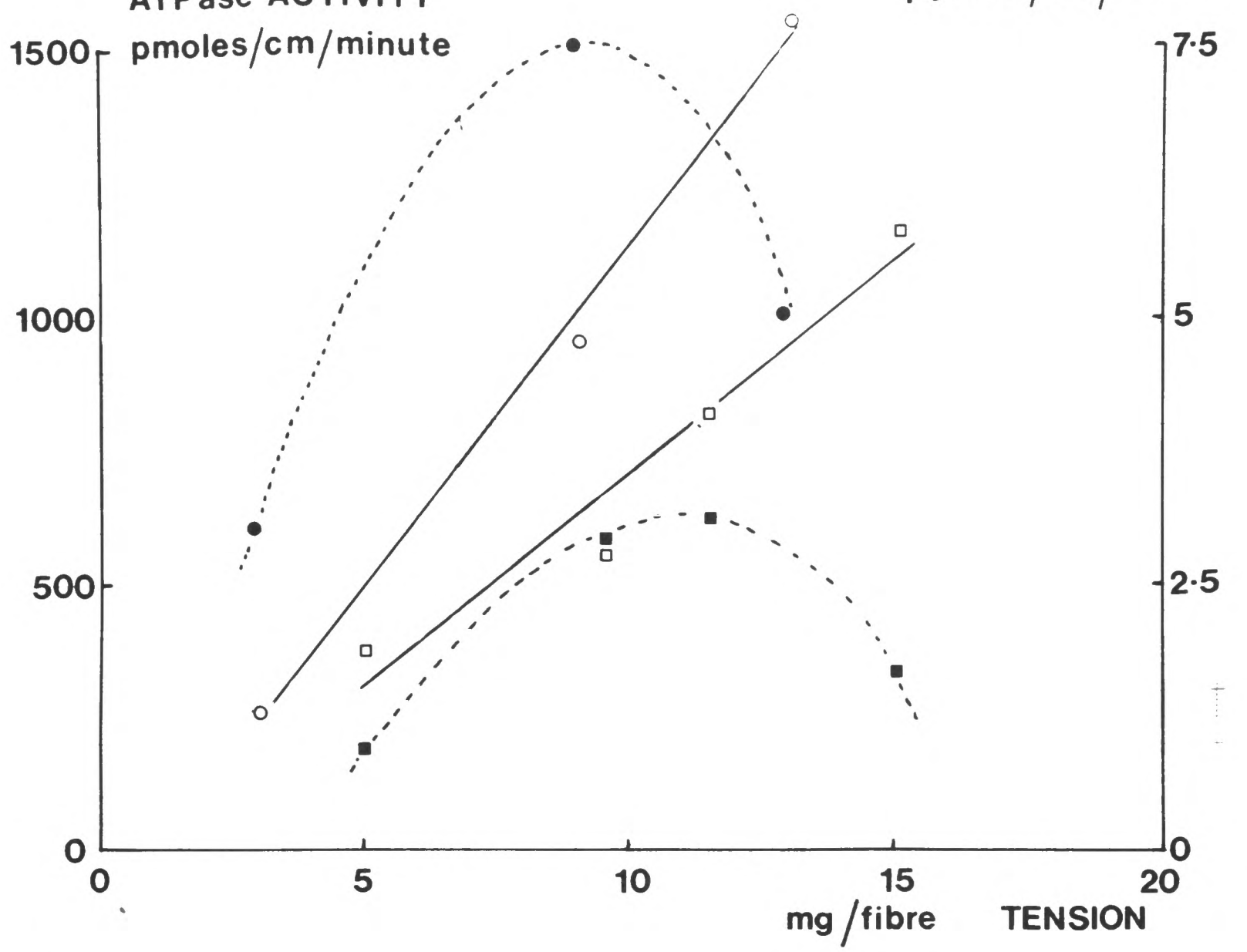
2% Amplitude oscillation both experiments

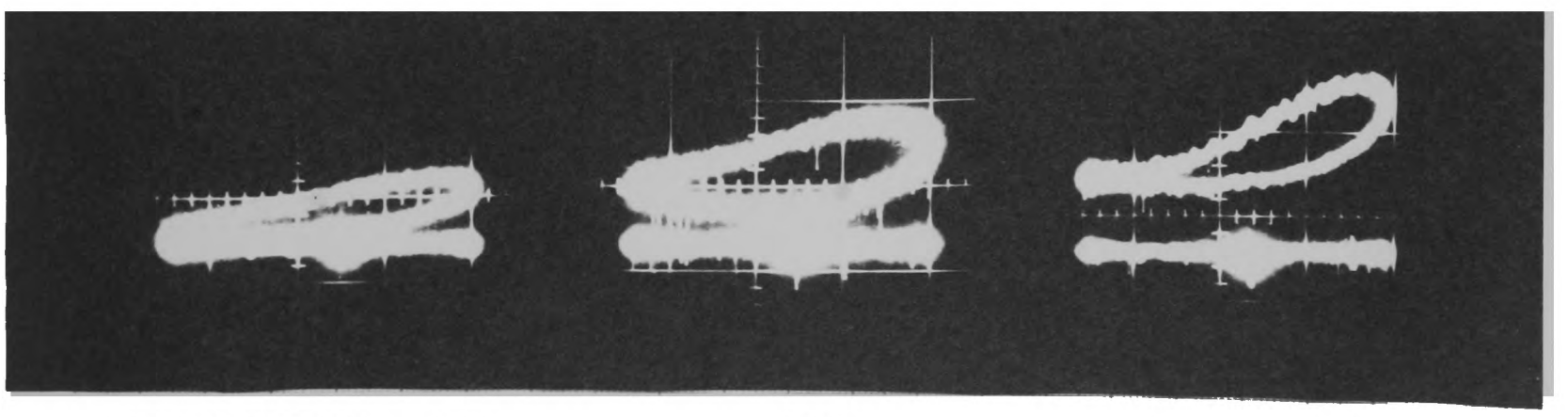
8 Hz experiment A

7 Hz experiment B

Length-tension loops and data from experiment A.

Figure 23





EXTENSION	1·5	2·5	5	%
TENSION	5	11	15	mg
POWER	3·0	7·5	5·0	μjoules
ATPase	260	955	1550	pmoles

Figure 24

Graph of ATPase activity against tension of fibres oscillated at high frequency and producing no positive work.

Oscillation amplitude 2%

Frequencies 10-25 Hz

Buffer: Histidine

Ca^{2+} concentration 0.0015 mM

MgATP concentration 4.6 mM

Line is best fit line through points, calculated by regression analysis.

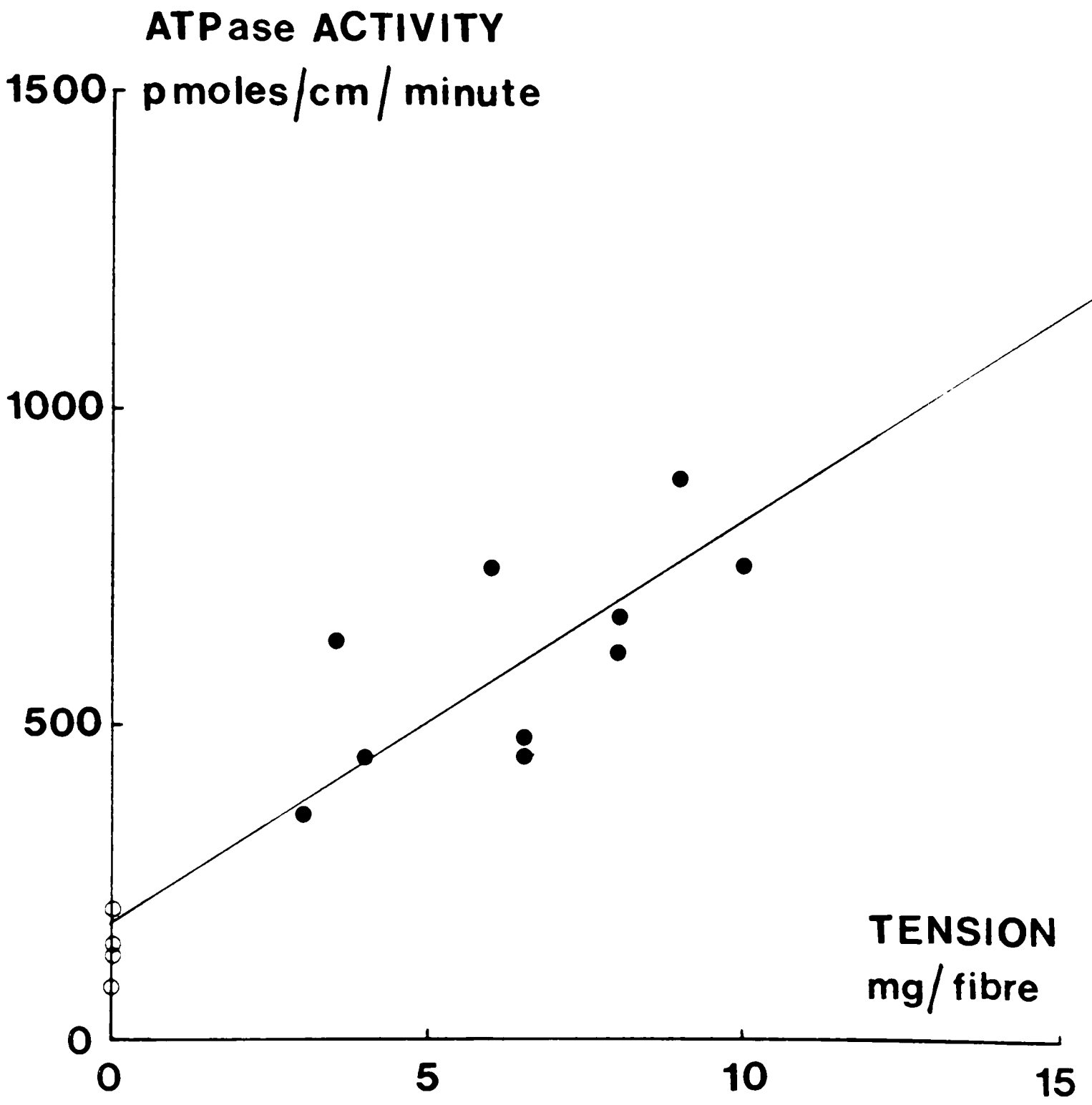
Regression coefficient 63.8 pmoles/mg

Standard error: 5.8

Intercept: 264 pmoles/cm/min.

Correlation coefficient 0.95

Figure 24


ATPase ACTIVITY
pmoles/cm/minute
1500
1000
500
0
0
5
10
15
TENSION
mg/fibre

reaches a maximum and then declines while tension continues to rise. The graph shows again that ATPase activity follows tension but not power production.

Thus in two mechanical conditions, high frequency non-power producing oscillations and very high tension, low power states, it has been demonstrated that ATPase activity and power production become disrelated. Under both conditions it has been shown that rate of ATP hydrolysis maintains its relationship to mean tension. This is demonstrated by Fig. 23 and also by Fig. 24, in which the values of ATPase activity in Table 8 are plotted against the force exerted by the fibres. The amount of ATP split rises with tension as was found earlier for fibres which were isometrically stretched or oscillated and work producing. (The slope of the relationship should not be compared with those of Figs. 20 and 21 as the data comes from experiments performed in histidine buffer as there was not sufficient data available for standard phosphate solution. The results described in Chapter 4 show that qualitatively relationships between parameters are similar in phosphate and histidine buffers.)

The results therefore show that the rate of ATP hydrolysis rises with power output as long as the power output of the muscle and the tension produced are positively correlated. When tension and power production do not change in the same direction, then the apparent relationship between energy usage and energy production ceases to be shown, as the ATPase activity continues to follow tension production.

It has been stated that the overextended state is not stable and that the tension of the fibres in most cases quickly dropped until a higher power output was restored. It is possible that at greater-than-optimal tensions an element in the muscle structure is overstretched and breaks, so lowering the tension in the fibre. It has been observed

(Tregear, personal communication) that in fibres subjected to high stretch a number of sarcomeres with a very wide I band can be seen, as if an element, such as the postulated C filaments, which normally maintains the length of the fibre, had broken, allowing the filament arrays to be pulled out from one another.

The aim of the work described in this chapter was to establish the nature of the relationships between various parameters of muscle activity. It has been shown that the rate of ATP hydrolysis rises with tension. ATPase activity has been shown to be related to tension under all conditions tested. The relationship to power output breaks down under conditions in which changes in tension and power production cease to be coupled.

4. Statistical treatment

(a) ATPase activity data

In this chapter the relationship of ATPase activity to mechanical output of muscle fibres was investigated and it was shown that the rate of ATP hydrolysis increases with increase in tension, in isometric and oscillatory conditions. ATPase activity rises with power output only as long as power output and tension changes are correlated.

The exact nature of the relationship between ATPase activity and tension will now be considered. The graphs in Figs. 20 and 21 show that there is considerable scatter in the data so that it is not justifiable simply by eye to draw a straight line or particular curve through the points. The estimates of both ATPase activity and tension are subject to random errors which will cause scatter of measured points about the true line. The magnitude of the error can be estimated.

The error in the tension measurements was estimated by repeatedly loading and unloading the transducer with a known mass and noting the

TABLE 9

Deflection produced by 50 mg weight mV
44
46
42
44
44
42
44
45
46

Mean and S.D. = 44.1 ± 1.5 mV
= 50.0 ± 1.7 mg

TABLE 10

ATPase activity isometric fibres. no tension pmoles/cm/min
210
80
150
125
150
165
130
95
120
90
130
185
140
90
150
85
190
110

Mean = 127.1 pmoles/cm/min
Variance = 2475.9 " " "
Standard deviation = 49.8 " " "

observed deflection on the oscilloscope screen. The results, presented in Table 9, were analysed and a standard deviation of 1.7 mg among them was calculated. Thus the tension measurements have an expected standard deviation of 1.7 mg/fibre. In experiments where two fibres were used the error will be half this value, i.e. 0.85 mg/fibre.

The error in the estimations of ATPase activity was calculated in Chapter 2. In the case of estimations by the enzyme method the standard deviation was 790 pmoles ADP measured. In order to see whether incubation with a fibre introduced an additional error, a number of determinations of ATPase activity of fibres under conditions of no tension, and therefore not subject to error from this cause, were analysed. The values are show in Table 10.

This deviation is equivalent to 1400 pmoles ADP measured, assuming a 20 minute incubation of two fibres 7 mm long, a typical situation. Thus incubation with a fibre introduces an additional source of error, and a standard deviation of 1400 pmoles measured, or 50 pmoles/cm fibre/minute represents the expected error in experimental situations.

The relative importance of the errors in the ATPase activity and tension measurements will depend on the slope of the line relating the two parameters. The smaller the change in ATPase activity with tension change, the more errors in estimations of ATP hydrolysis will affect the observed results. The steeper the relationship the more the effect of inaccuracy in tension measurements will dominate. When the slope of the relationship is about 50 pmoles ATP split/unit tension the two will have equal weight if two fibres are used in experiment.

The error in ATPase activity applies to measurements made on one fibre. When determinations using different fibres are made an additional source of error is introduced because, as already observed, all fibres do not behave identically. The performance of fibres from one muscle is similar in similar conditions over a short period of time, but comparison of results obtained using fibres from different bugs may

Figure 25

ATPase activity against tension of fibres

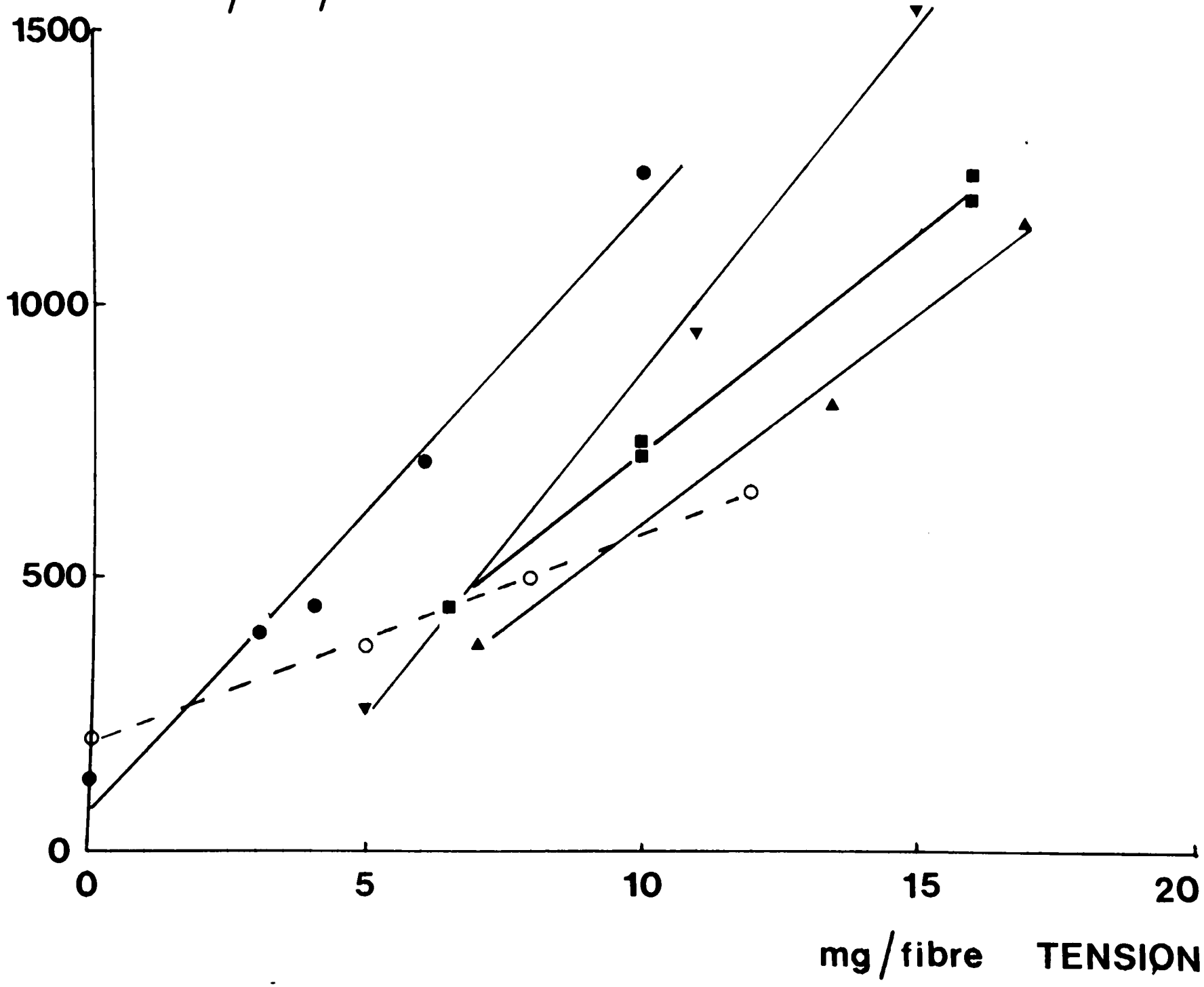
Each symbol represents an individual experiment, i.e. successive observations on one fibre.

Symbol	Amplitude of oscillation
○	isometric
▼	2%
■	2%
▲	2%
●	2%

Lines are best-fit lines through points, calculated by regression analysis.

Experiment	Regression coefficient pmoles/mg	Standard error	Correlation coefficient
○	38.4	0.8	1.00
▼	127.9	9.6	1.00
■	81.4	2.7	1.00
▲	75.8	6.8	1.00
●	113.1	8.7	0.99

Figure 25


ATPase ACTIVITY
p moles / cm / minute
1500
1000
500
0
0
5
10
15
20
mg / fibre TENSION

result in much larger error as completely different muscles vary greatly in their characteristics. Thus increasing error in results is introduced when more than one fibre is used, especially if the fibres come from different bugs.

A large scatter would therefore be expected under the conditions in which the points in Figs. 20 and 21 were obtained, as data from several days' experiments using more than one muscle were amalgamated. However it is possible to look at results from single experiments - several determinations on a single fibre. The error in the points will then be minimised and the exact nature of the relationship between the parameters will be better shown. There is only a small number of such experiments as single fibres cannot be used for more than about three incubations of which one was usually of an isometric fibre and two were under oscillatory conditions. Experiments of three or more points are shown in Fig. 25. In each case the points are closely fitted by a straight line, with correlation coefficients as shown.

Therefore under conditions in which the error is minimised the rise in ATPase activity with tension is linear. Consequently it is justifiable to take the data in Figs. 20 and 21 as lying on a set of straight lines as in Fig. 25.

Having established the linearity of the tension-ATPase activity relationship an estimate of the best line through the points can be made. There were insufficient experiments in which two or more points had been obtained for this to be done by averaging slopes and intercepts of individual experiments so all the points had to be taken together. A regression analysis was performed whereby the best fit line through the points was estimated by the method of least squares, giving a regression coefficient of ATPase activity upon tension and an intercept and a measure of the error associated with the estimate. Thus the best

fit line through the points was obtained. Coefficients from two independently obtained lines could then be compared using a t-test to see if they were significantly different.

The relationship between ATPase activity and power is more complex than that between ATPase activity and tension as it is not monotonic. Analysis of data from single experiments does not give evidence of linearity as it did in the case of the tension measurements. However an estimate of the best-fit line through the points was made, excluding points of high frequency oscillation in which no power was produced and those points where it was obvious that the fibre was at so high a tension that its power output was reduced below the maximum. Thus an estimate of the energetic cost of power production was obtained.

To illustrate the method the slope and intercepts of the best fit lines through the points in Figs. 20, 21 and 22 are shown in Table 11. The standard error of the slope and the intercepts of the limits are also shown.

TABLE 11

Conditions	Slope ± error	Intercept pmoles/cm/min
Isometric ATPase-tension	49.5 ± 4.0 pmoles/mg	140 ± 33
Oscillatory ATPase-tension	89.1 ± 9.7 pmoles/mg	101 ± 113
ATPase-power	72 ± 14.0 pmoles/μJoule	327 ± 125

(b) Mechanical data

In subsequent chapters the frequency response of the muscle will be considered in detail. Data concerning the ability of fibres to produce work at different frequencies were obtained in two ways.

(i) The PDP8-I computer was used to control the Solartron JM1600 oscillator generator so that rapid analyses of mid-length tension difference at many different frequencies could be made, as described in Chapter 2. The computer logs the jb value - the root mean square quadrature tension - from which the amount of work produced at each frequency can be calculated. Work was then plotted against the frequency on a logarithmic scale to give a Bode diagram such as that shown in Fig. 16. From this the frequency at which the fibres gave maximum work could be estimated, also the amount of work. The range of frequencies at which the fibres gave positive work could also be judged from the sign of the jb value, negative indicating a delay between length and tension changes, i.e. positive work, a positive jb value indicating work done by the system on the muscle.

(ii) The amount of work obtained at a particular frequency was calculated from the data obtained in the ATPase activity experiments. The different values relating to one frequency were then summed and averaged to give an estimate of work per cycle at each available frequency and the points thus produced plotted and the desired information obtained as described above.

It was desirable to have an estimate of the accuracy of the values obtained by these methods.

As already discussed, individual muscles may differ greatly in their mechanical output so this source of systematic variation is not considered. The experiments described were designed to evaluate the

random variation incurred in measurements on fibres from one muscle under conditions in which the actual performance was constant.

(i) Computer-controlled experiments. A bundle of four fibres was attached to the apparatus and subjected to oscillation at 2% and 0.2% amplitude. Over the space of 10-15 minutes three to five sets of measurements were made at each amplitude of oscillation. The experiment was repeated using many bundles of fibres from the same muscle. The data were then plotted. Each set of measurements yielded a value for the optimum frequency for work production, f_{max_1} at 0.2%, f_{max_2} at 2%. The estimates of f_{max} for each bundle were analysed statistically to evaluate the variation between individual measurements on one set of fibres. A residual variance was thus obtained. The mean values of f_{max} for each bundle were then analysed to produce an intertype variance. The variance ratio was then checked against F ratio tables for significance. The results obtained are tabulated below.

TABLE 12

Amplitude	Intertype variance	S.D. Hz	Residual variance	S.D. Hz	Variance ratio
2%	0.13	0.36	0.05	0.22	2.79
0.2%	9.56	3.09	0.88	0.94	10.84

It was found that there was a significant difference in the optimum frequency of different bundles of fibres oscillated at 0.2%. The difference was just not significant at 2%.

Values of f_{max} obtained from single observation on different fibres may thus differ significantly, a standard deviation of ± 3.1 Hz at 0.2% being expected, and ± 0.36 Hz at 2%. Individual estimations are subject to a large error; however the values of f_{max} which are

drawn from mechanical data obtained using the on-line system are not derived from single measurements. All involved a minimum of three estimates and in most cases several different fibre bundles were used, each bundle giving several measurements. Thus the errors in the quoted values of f_{max} are much less than those derived above.

(ii) Mechanical data obtained from ATPase activity experiments varied greatly in its completeness. In some cases more than twenty estimates of work were available at about four frequencies in the range at which the fibres worked well, plus smaller numbers of estimates at higher and lower frequencies. In other cases far fewer figures were available and at a limited number of frequencies. However the data were in most cases sufficiently complete in the region of f_{max} for evaluation of frequency optimum to be made. In all instances the data were derived from numerous fibre sets.

To conclude; as a result of statistical analysis, an estimate of the error inherent in the measurements made from the mechanical and chemical data has been obtained. By consideration of a number of individual cases, assumption of a linearity between tension and ATPase activity has been justified and hence the relationship between the two can be quantified in terms of pmoles ATP consumed/unit tension, i.e. measurement of the cost of tension production can be made. This measurement is inversely related to the holding economy parameter used by Schädler and others (1967). The energetic cost of power production can similarly be estimated and compared under different conditions.

CHAPTER 4

The effect of changes in the ambient medium

1. Introduction

In the previous chapter the mechanical performance of muscle fibres under different conditions of oscillation was examined and one amplitude was selected at which the muscle was able to produce continuous large power outputs. The ATPase activity of fibres at this amplitude of oscillation, and under isometric conditions, was studied, varying the frequency of oscillation and the tension in the fibres. The changes of ATPase activity with increases in tension and power output were examined and a statistical treatment employed to make a quantitative estimate of the relationship between the parameters. Mechanical experiments were also performed and from the results an index of mechanical response was measured.

The behaviour of a highly activated muscle producing large power outputs was therefore established. In the following chapter the conditions of incubation were modified in various ways and the effect of these changes on the indices of performance established in the previous chapter were analysed. The effects of temperature, pH, ionic strength and chemical constituents of the incubation medium were examined.

2. The effect of temperature

The effect of variation in temperature on the chemical and mechanical output of the muscle was studied. In these experiments the temperature of the incubating solution was regulated by circulating water. A small thermistor in the incubating medium showed that the temperature of the medium followed the temperature of the circulating water with

Figure 26 Bode plot showing effect of temperature on frequency response of muscle.

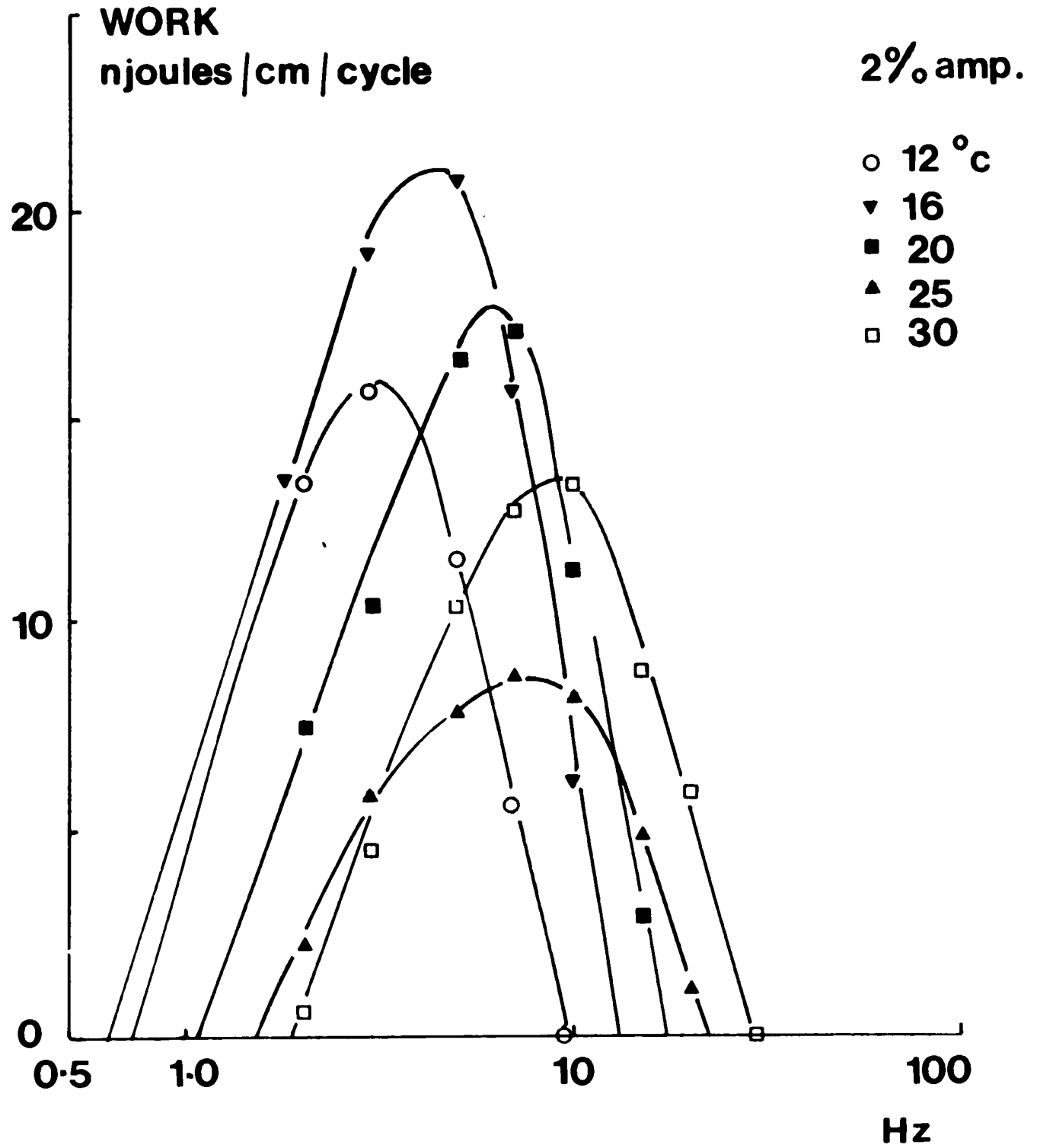


TABLE 13

Effect of temperature on the mechanical performance of muscle

Temperature	Oscillation amplitude	Optimum frequency	Maximum work/cycle	Frequency range of power production
°C	%	Hz	nJ/cm/cycle	Hz
12	2	3.1	15.6	0.6 - 9.0
16	2	4.6	21.0	0.8 - 14.0
20	2	6.3	17.2	1.0 - 18.5
25	2	8.0	18.0	1.5 - 22.5
30	2	9.5	13.5	1.8 - 30.0
12	0.2	6.5	0.75	1.0 - 35.0
16	0.2	7.6	0.86	1.0 - 35.0
20	0.2	8.7	0.80	1.0 - 40.0
25	0.2	10.5	0.81	1.0 - 45.0
30	0.2	16.8	1.00	1.0 - 72.0

negligible delay. Phosphate buffer was used for all experiments. It is known to be little affected by temperature changes over the range 10^{o}-30^{o}C. The effect of temperature on the complexes in solution was ignored; in the few instances where figures are available the effect was seen to be small (Grybowski, 1958).

The ATPase activity of muscle in different mechanical conditions over a range of temperatures was measured. Mechanical experiments using the PDP8-I computer on-line were also performed.

The mechanical data was plotted as work per cycle against log frequency as described in the previous chapter, and f_{max}, frequency range and maximum work estimated from the graphs.

The minimum frequency tested in the experiments was 2 Hz. This is above the minimum rate of oscillation at which positive work was obtained so that the value of this figure had to be judged from the graph. However as very low frequency oscillation impairs the performance of fibres it was not practical to use one set of fibres for repeated determinations in different conditions.

The results of the experiment are presented graphically in Fig. 26 and the values of the required parameters taken from the graph are shown in Table 13.

At 8^{o}C, which was not tested in these experiments, very little work was produced at any frequency or amplitude.

The table shows that, at both 2% and 0.2% amplitude, the optimum frequency and the frequency range increase with temperature. At 0.2%, as observed in the previous chapter, the span of working frequencies is much greater than at 2%, being extended at the top but not the bottom end of the range. The optimum frequency is also greater than that at 2%.

The size of the maximum work per cycle does not change regularly with temperature. Thus the maximum work obtainable is not affected by

Figure 27

Relation between mechanical performance and ATPase activity of muscle at 8°C.

a. Relationship of ATPase activity to tension.

O Isometric muscle

● Oscillatory muscle, 2% amplitude, work-producing frequencies.

- - - - Best-fit line through isometric points, calculated from regression coefficient.

———— Best-fit line through oscillatory points, calculated from regression coefficient.

b. Relationship of ATPase activity to power output.

———— Best-fit line through points.

High tension-low power points □

Not included in calculation of regression coefficient.

The above key and nomenclature apply to all similar graphs,

Figs. 27 - 32

34 - 43

46 - 52

Figure 27a 8°c

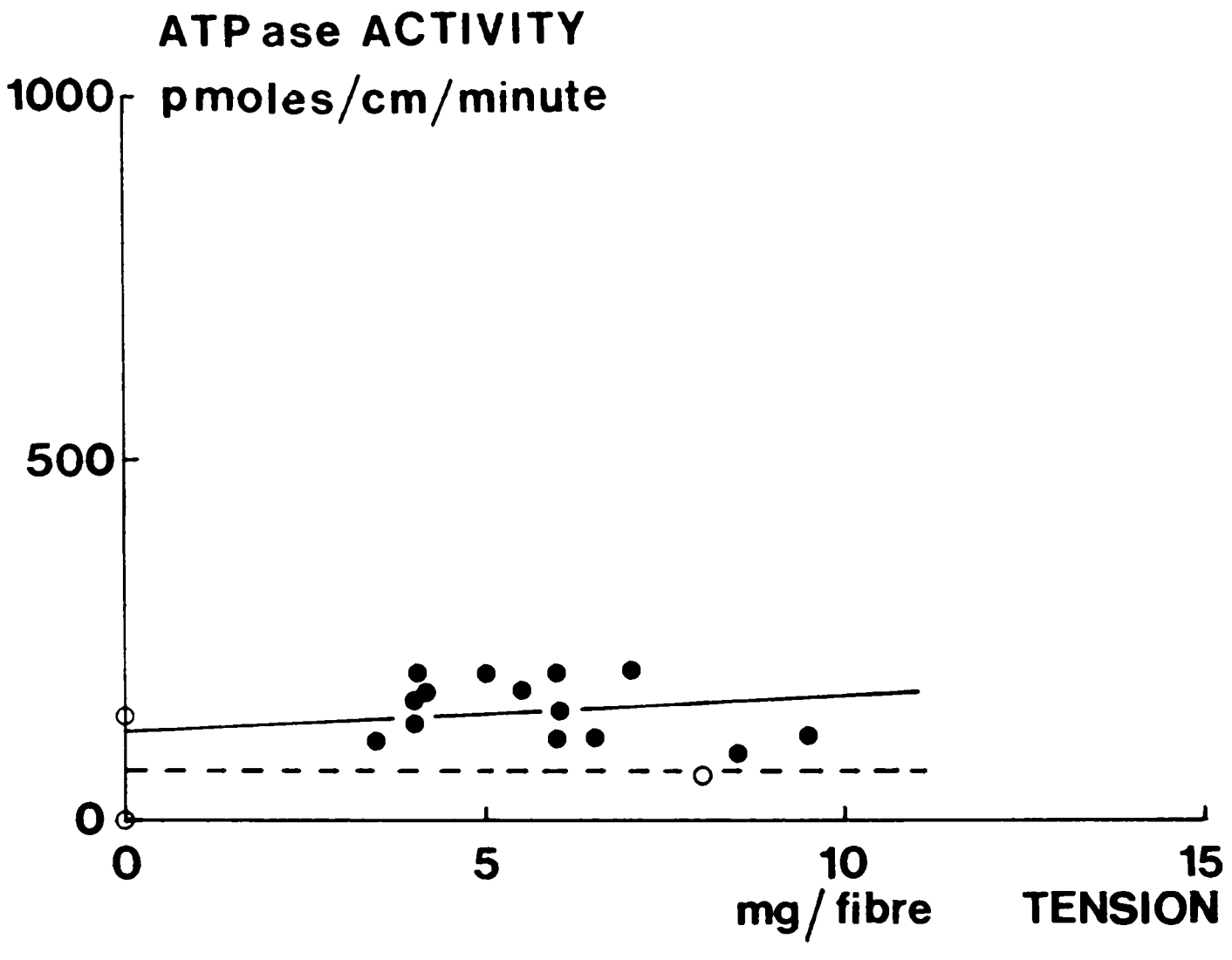


Figure 27b

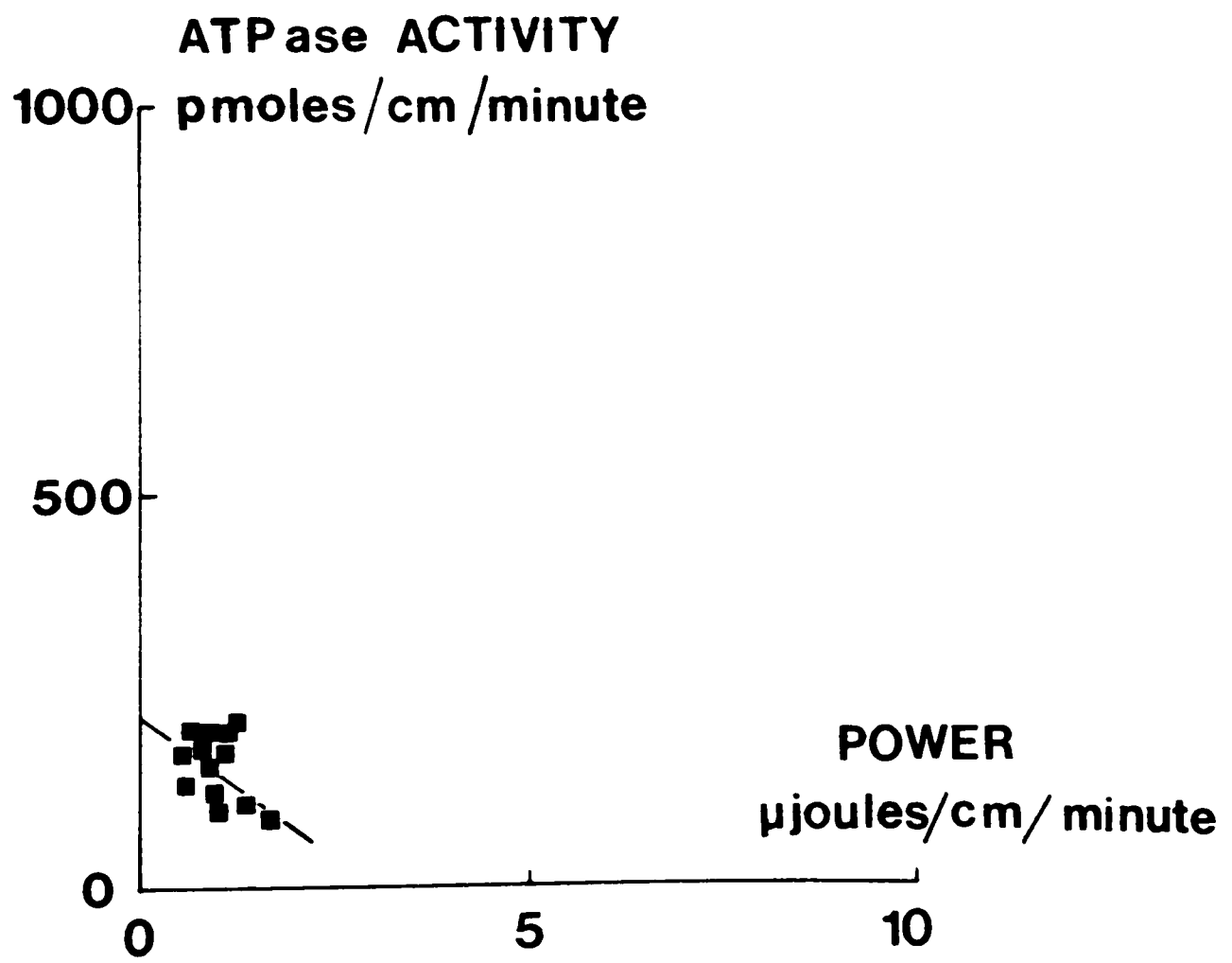


Figure 28a 12°c

Relation between mechanical performance and ATPase activity of muscle.

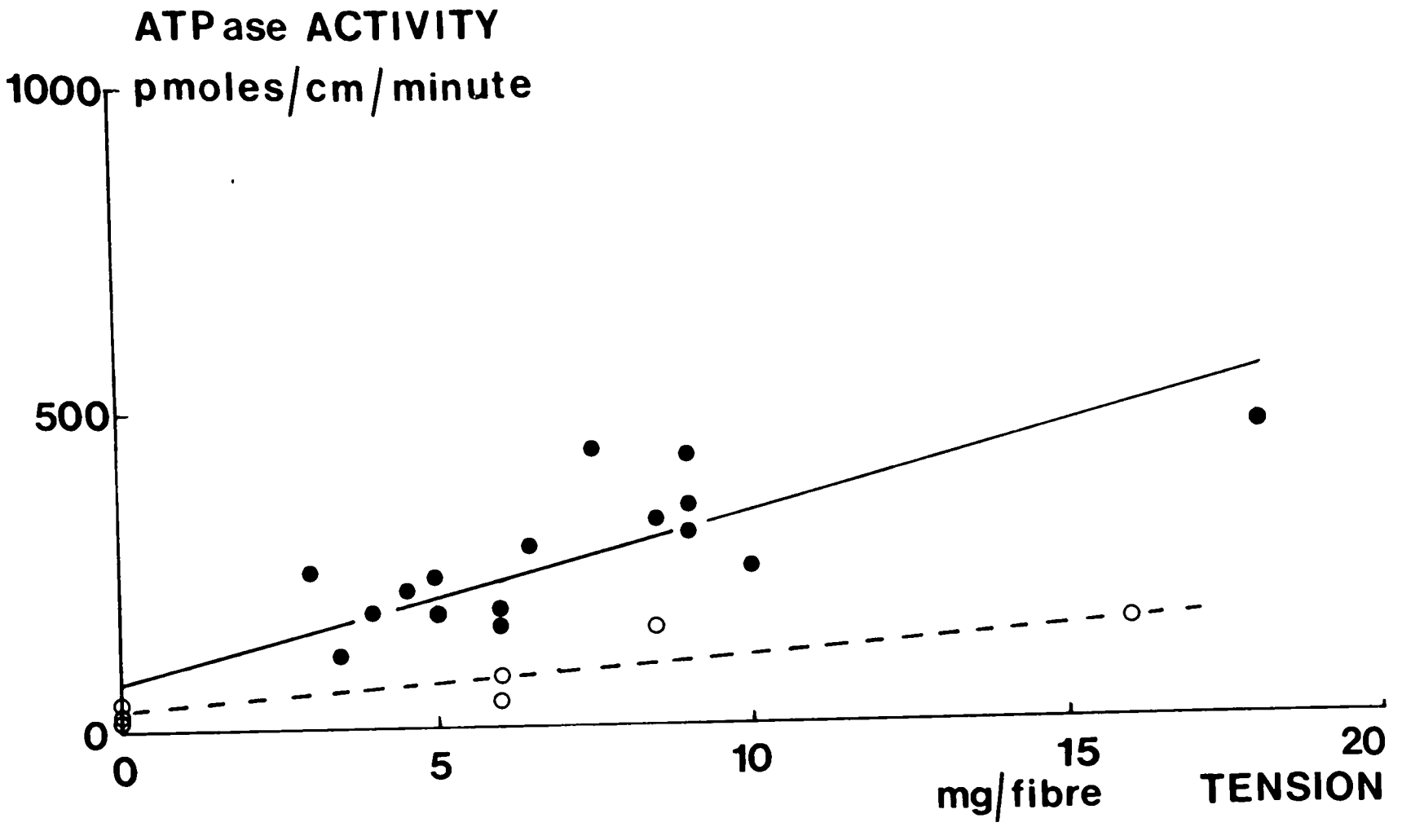


Figure 28b

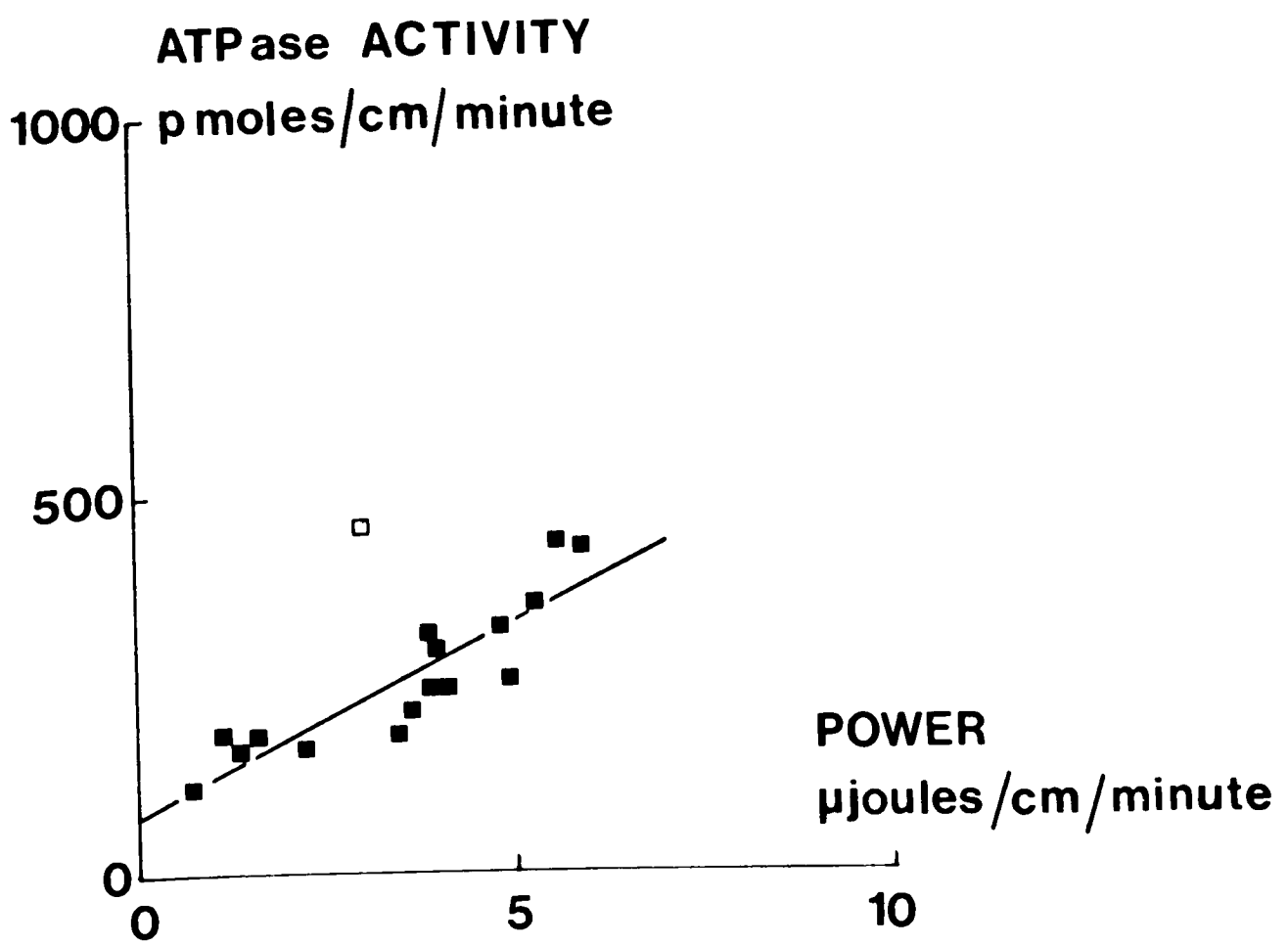


Figure 29a 16°c

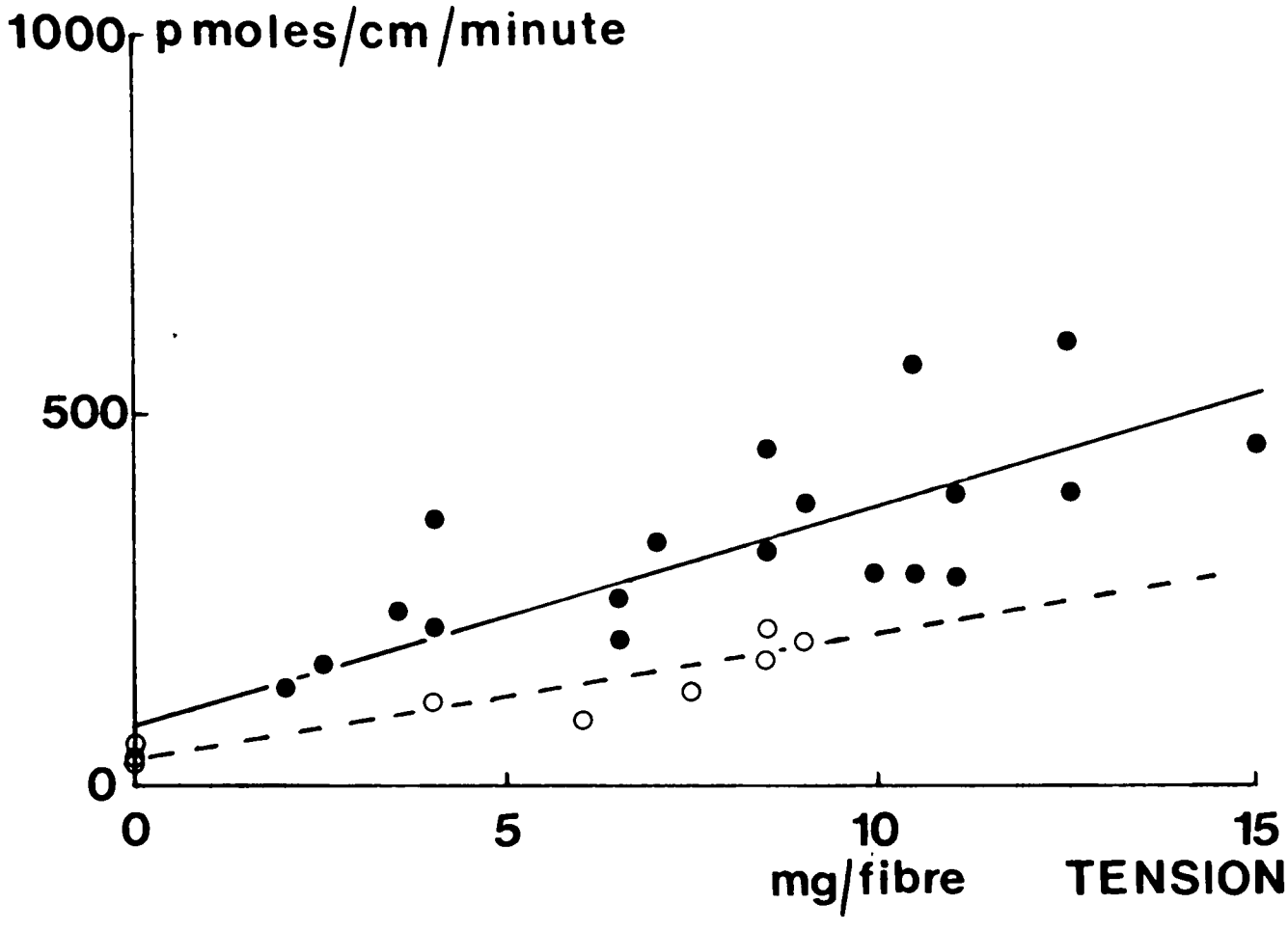


Figure 29b

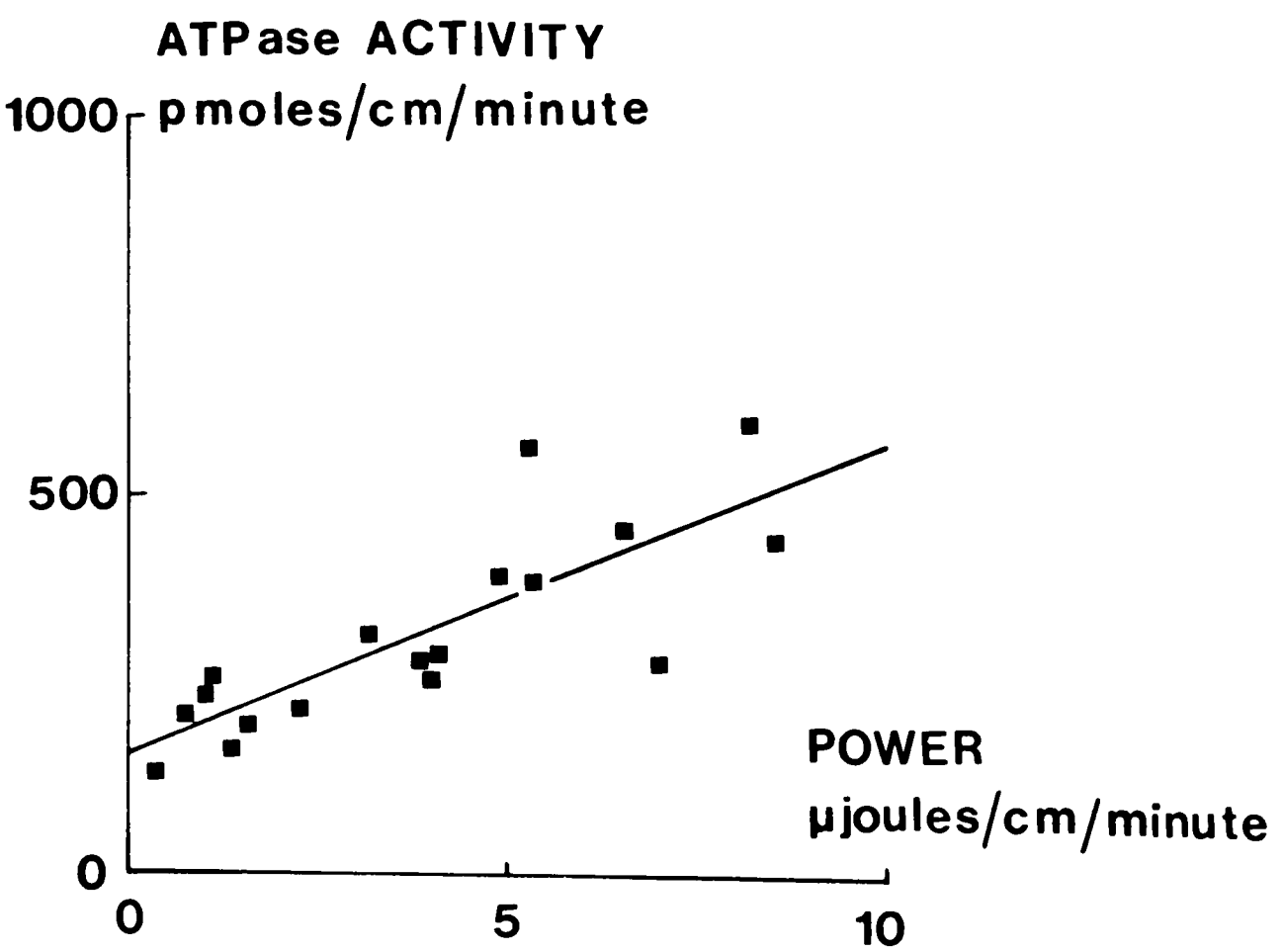


Figure 30 a 20° c

Relation between mechanical performance and ATPase activity of muscle.

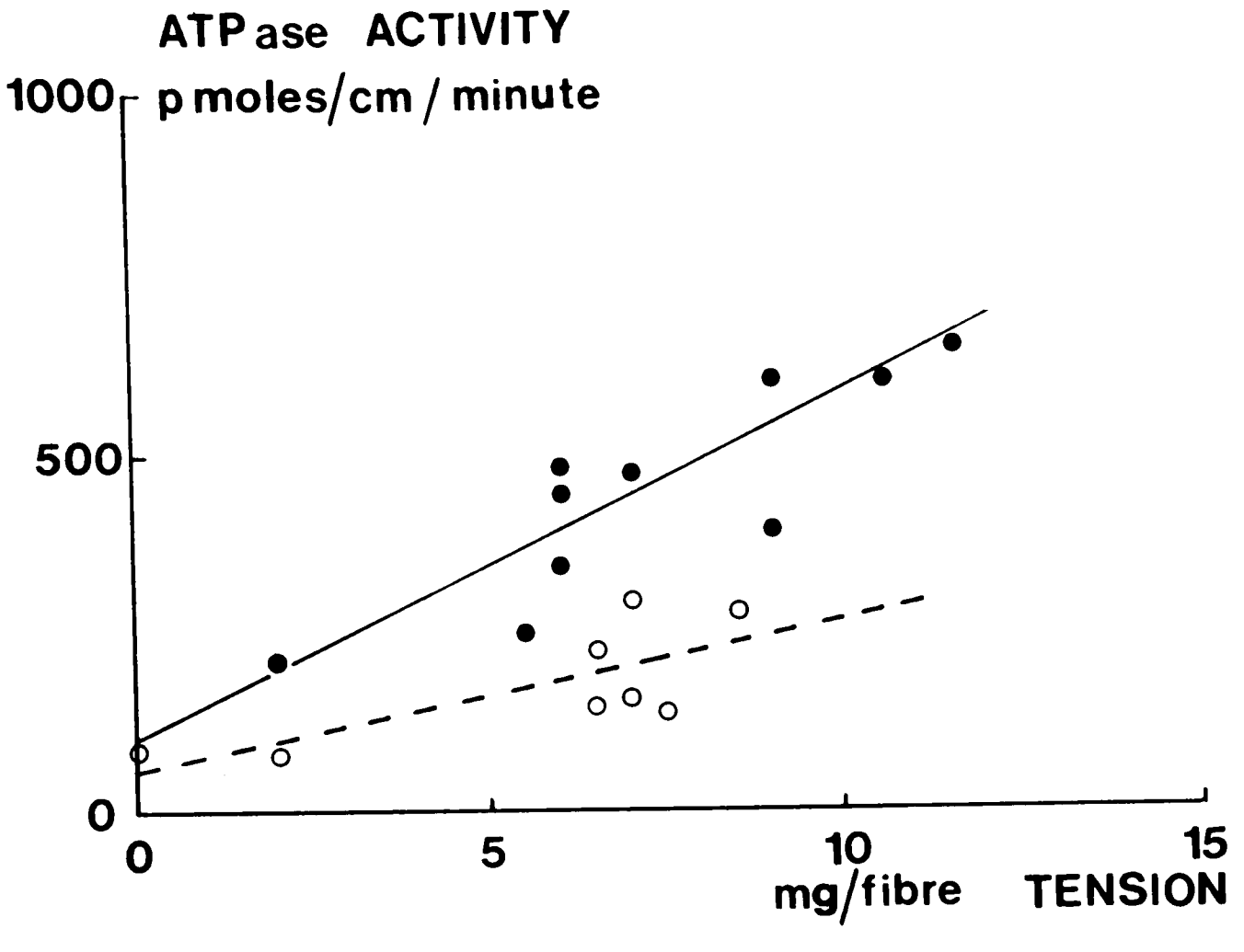


Figure 30 b

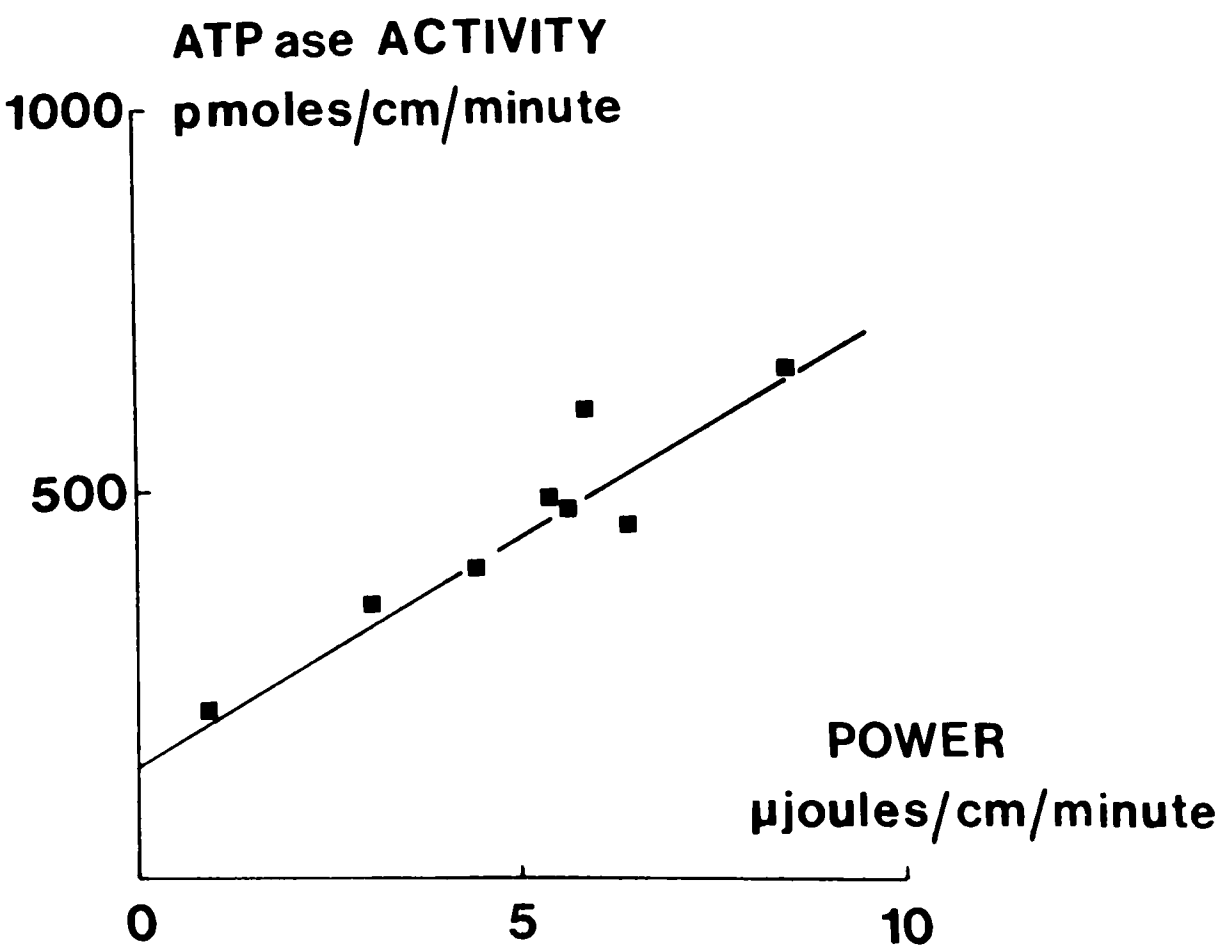


Figure 31 a $25^{\circ}c$ Relation between mechanical performance and ATPase activity

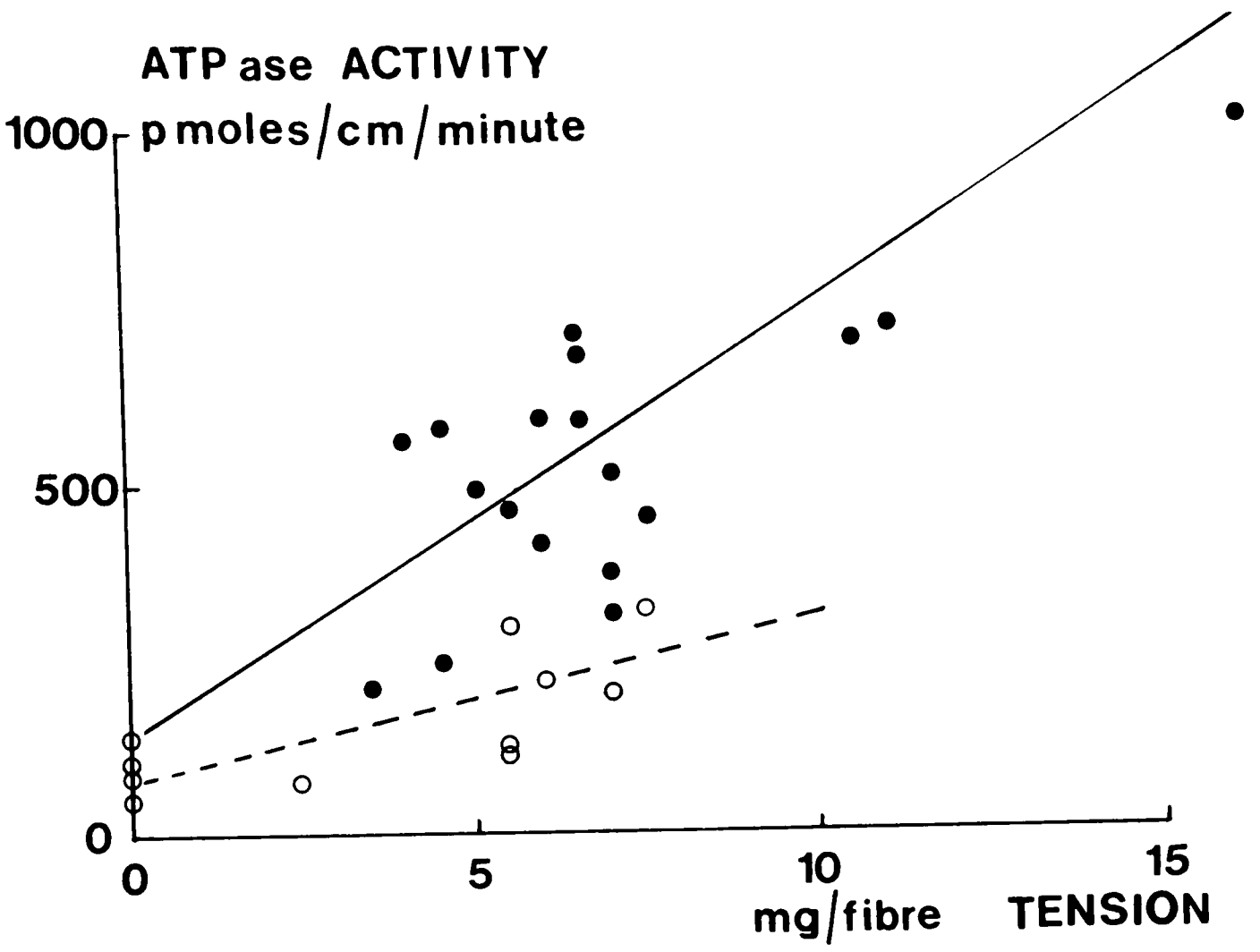


Figure 31b

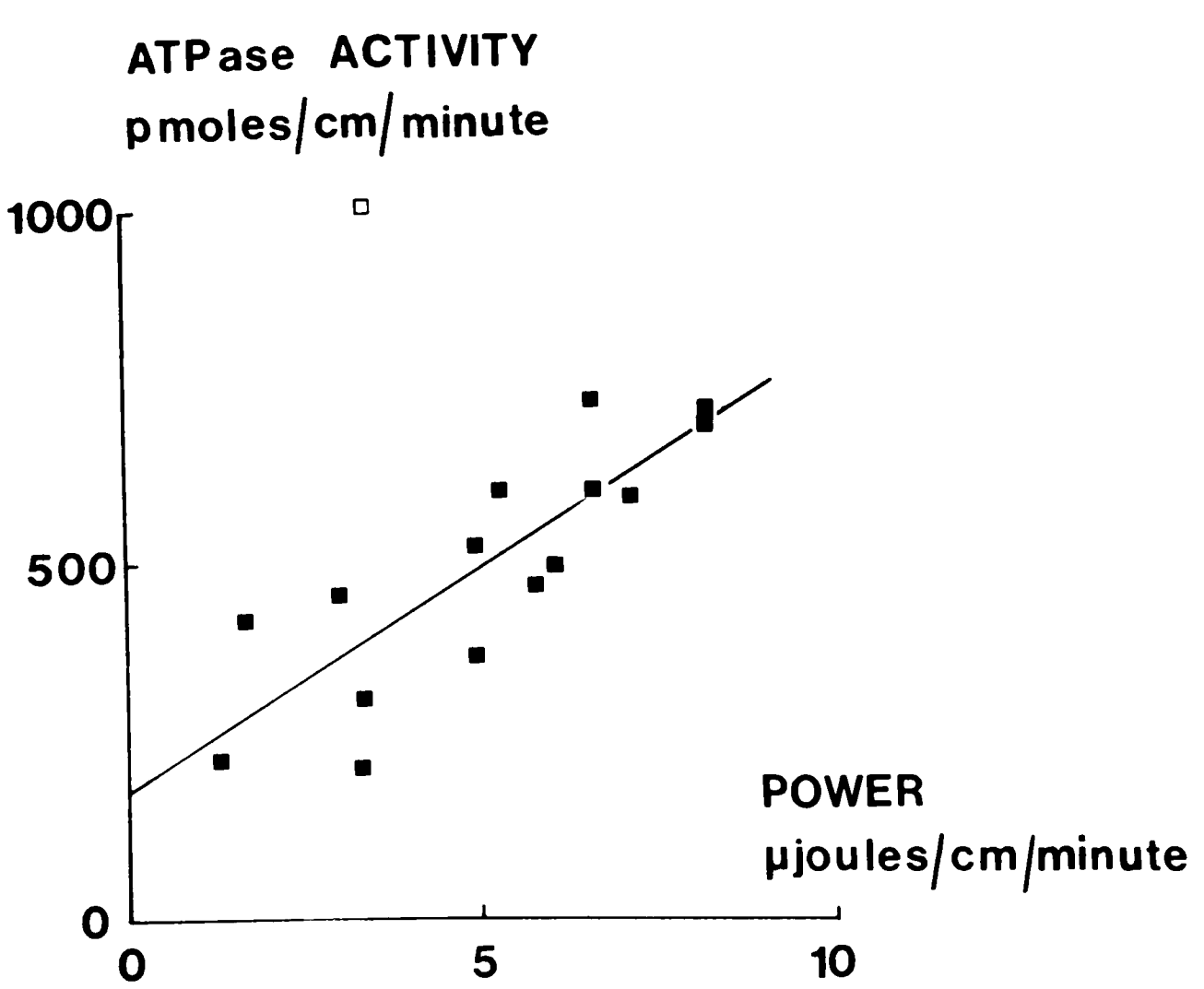


Figure32a 30°c

Relation between mechanical performance and ATPase activity of muscle.

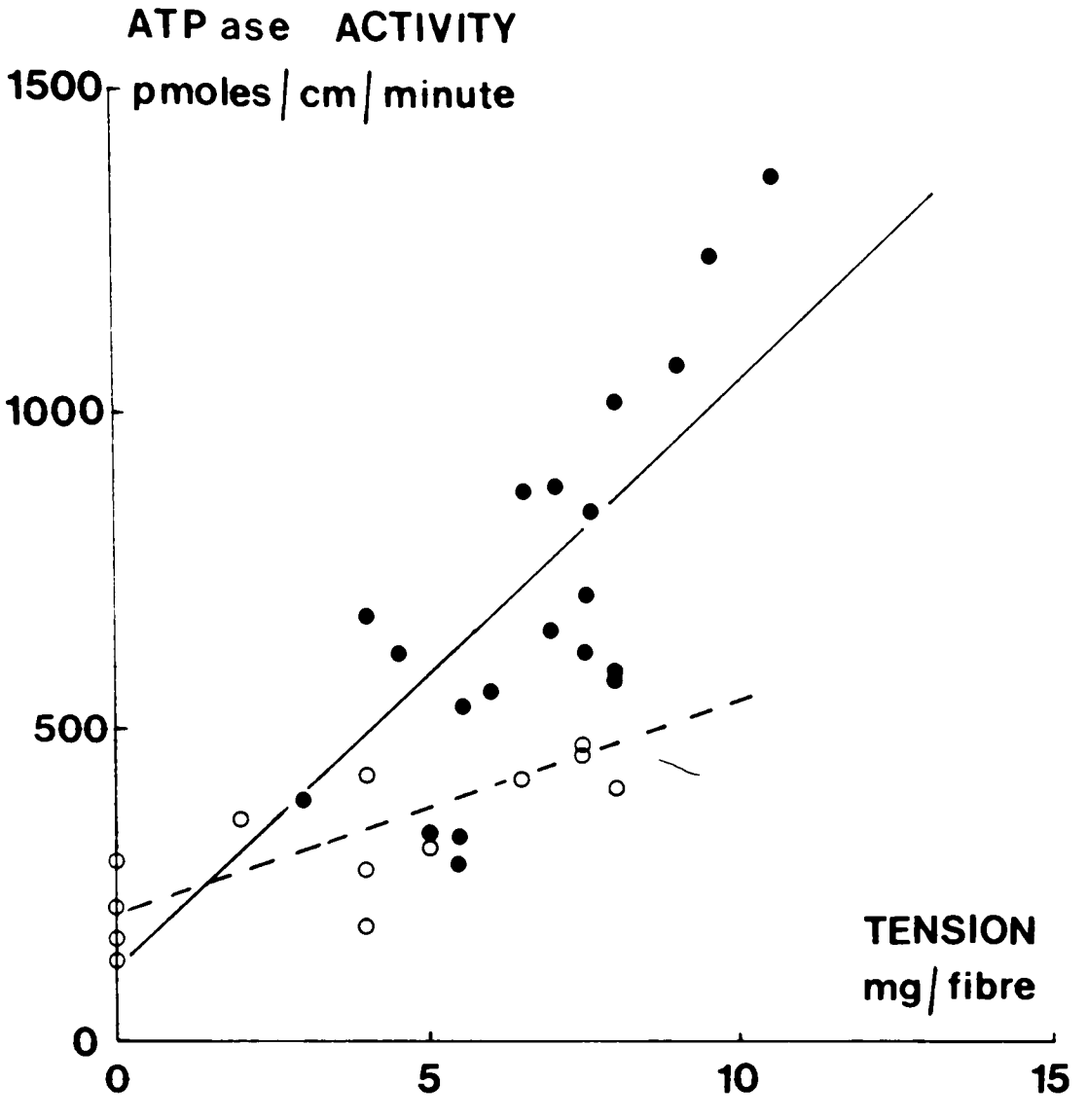


Figure 32b

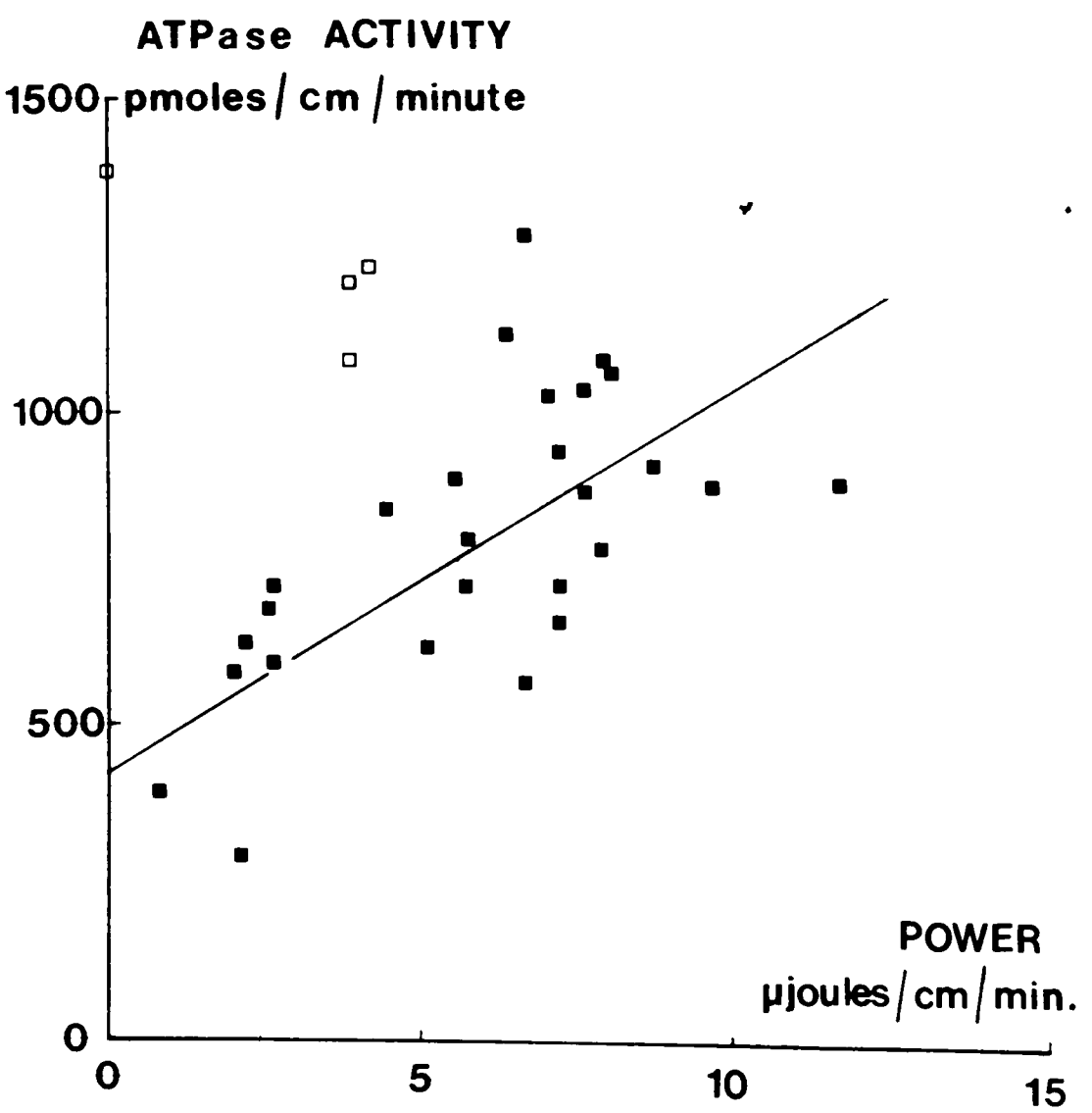


TABLE 14

Effect of temperature on ATPase activity of muscle

in relation to tension and power production

Regression coefficient of slopes

Temperature	Static ATPase/tension	Oscillatory ATPase/tension	ATPase/power
°C	pmoles/mg	pmoles/mg	pmoles/μJoule
8	-0.6 ± 15.0	3.3 ± 3.3	-57.0 ± 3.4
12	9.2 ± 2.2	26.4 ± 3.9	50.0 ± 7.0
16	15.2 ± 2.5	29.0 ± 4.4	41.0 ± 7.0
20	20.4 ± 7.9	47.8 ± 7.2	59.0 ± 14
25	22.3 ± 6.5	55.6 ± 7.5	63.0 ± 11
30	33.7 ± 8.3	93.0 ± 13.2	59.0 ± 12

TABLE 15

Effect of temperature on ATPase activity of muscle

in relation to tension and power production

Intercepts of best-fit lines and limits

Temperature	Static ATPase/tension	Oscillatory ATPase/tension	ATPase/power
°C	pmoles/cm/min	pmoles/cm/min	pmoles/cm/min
8	70 ± 85	126 ± 56	218 ± 66
12	28 ± 25	75 ± 50	78 ± 54
16	41 ± 26	80 ± 61	157 ± 57
20	60 ± 93	106 ± 99	146 ± 141
25	80 ± 49	147 ± 89	180 ± 121
30	207 ± 59	126 ± 158	462 ± 101

temperature although as the frequency at which greatest work appears rises with temperature, the maximum possible power output will be temperature-dependent. The finding that maximum work per cycle is unaffected by temperature is in contrast to the results reported by other workers. Aidley & White (1969) recorded an increase in maximum work/cycle with temperature. Steiger & Rüegg (1969) found a more complex behaviour: between 15°C and 20°C work per cycle doubled, but from 20°C to 35°C it gradually decreased.

The results presented above show that an increase in temperature allows the fibres to work faster.

ATPase activity estimations were performed at 8°, 12°, 16°, 20°, 25° and 30°C. The results are shown graphically in Figs. 27 to 32 and regression coefficients and associated statistical analysis presented in Table 14 and 15.

At 8°C the fibres produced very little work or tension and the results show that ATPase activity was not properly activated. The ATPase rate was uncorrelated with either tension or power production.

At 12°C the fibres were performing in a normal manner and the chemical activity, like the mechanical performance of the fibres, shows a gradual change from 12° to 30°C. The cost of tension production increases with temperature, more steeply in the case of oscillated muscle. The cost of power production, as measured by the slopes of the regression lines, does not vary significantly with temperature although as the intercept of the line on the Y-axis increases with temperature the absolute value of the quantity of ATP hydrolysed per unit power produced is higher at high temperature. At high temperatures, also, the fibres could be more easily stretched until the tension exceeded that sufficient for maximum power and so more high tension-low power output points, where power is inefficiently produced, were obtained. These points have not

TABLE 16

The effect of temperature on mechanical performance of muscle

Data obtained in conjunction with that on ATPase activity.

Temperature	f_{max}	Maximum work/cycle	Frequency range
°C	Hz	nJ/cm/cycle	Hz
8	2.5	6.0	1.2 - 12.5
12	3.4	20.5	1.5 - 17.0
16	4.8	20.5	2.0 - 23.0
20	6.5	26.8	1.5 - 25.0
25	7.6	17.0	1.5 - 29.0
30	10.5	24.5	4.0 - 32.0

been included in the data used to obtain an estimate of the efficiency of power production.

These experiments were performed on fibres from a different muscle from that used for the computer-controlled mechanical experiments. Some experiments were done in conjunction with the chemical experiments to examine the mechanical performance at different temperatures. Two percent amplitude only was studied. The data were plotted and the optimum frequency, range of working frequencies, and maximum work per cycle obtained from the graphs, see Table 16. The pattern observed is the same as that of the purely mechanical experiments: a rise in optimum frequency and frequency range with temperature, but, with the exception of the very low temperature, $8^{o}C$, no change in maximum work per cycle.

Thus the ability to work optimally at higher frequencies which increased temperature allows is accompanied by an increase in the cost of tension production, in both oscillatory and isometric contraction, although efficiency of power output is not affected.

In Fig. 33a the temperature-dependent parameters of f_{max} obtained at high and low amplitude and the static and dynamic tension costs, are plotted against the temperature, illustrating the rise of each with increasing temperature. Figure 33b is an Arrhenius plot. The data from all four parameters is fitted well by a straight line. The mean slope is $-2378^{o}K$. The slope of f_{max_1} is lower than that of the other three parameters which are very close, but the difference is not significant.

The fact that tension costs, f_{max_1} and f_{max_2} all have the same slope is evidence that all four are governed by the same rate process.

From the Arrhenius plot the activation energy of the rate-controlling reaction may be calculated.

Figure 33

a. Plot of static and dynamic tensions costs, f_{max_1} and f_{max_2}, against temperature showing rise with temperature of these parameters.

△ Static tension cost

▲ Oscillatory tension cost

■ f_{max_1} (0.2%)

□ f_{max_2} (2.0%)

b. Arrhenius plot to show effect of temperature. Ordinate: static and oscillatory tensions costs, f_{max_1}, f_{max_2}, plotted on a logarithmic scale.

Abscissa: 1/absolute temperature

Key as above

	Regression coefficient ^{o}K	Correlation coefficient
Tension cost	-2519 ± 316	0.9773
Oscillatory tension cost	-2726 ± 317	0.9804
f_{max_1}	-1897 ± 241	0.9767
f_{max_2}	-2346 ± 289	0.9780

Figure 33 a

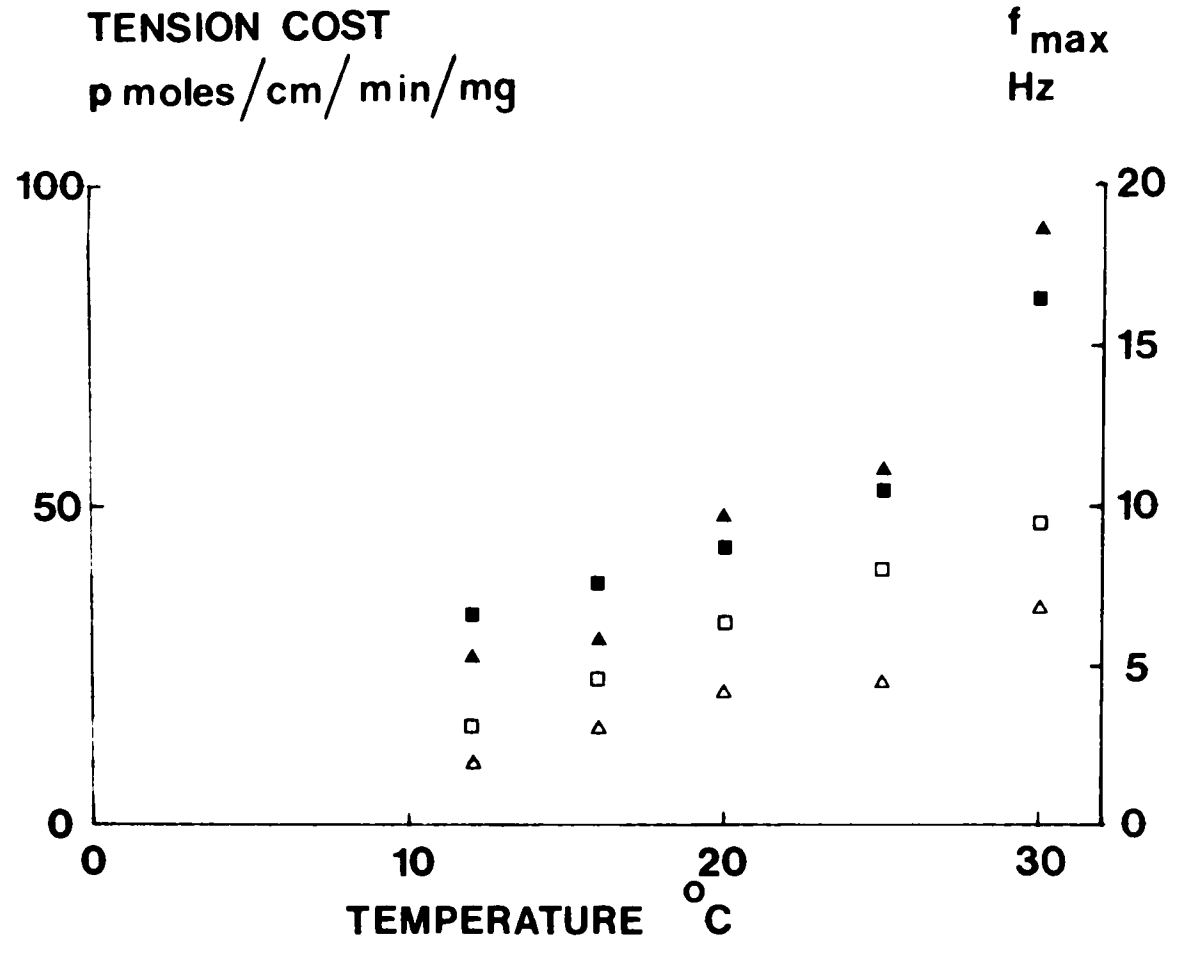
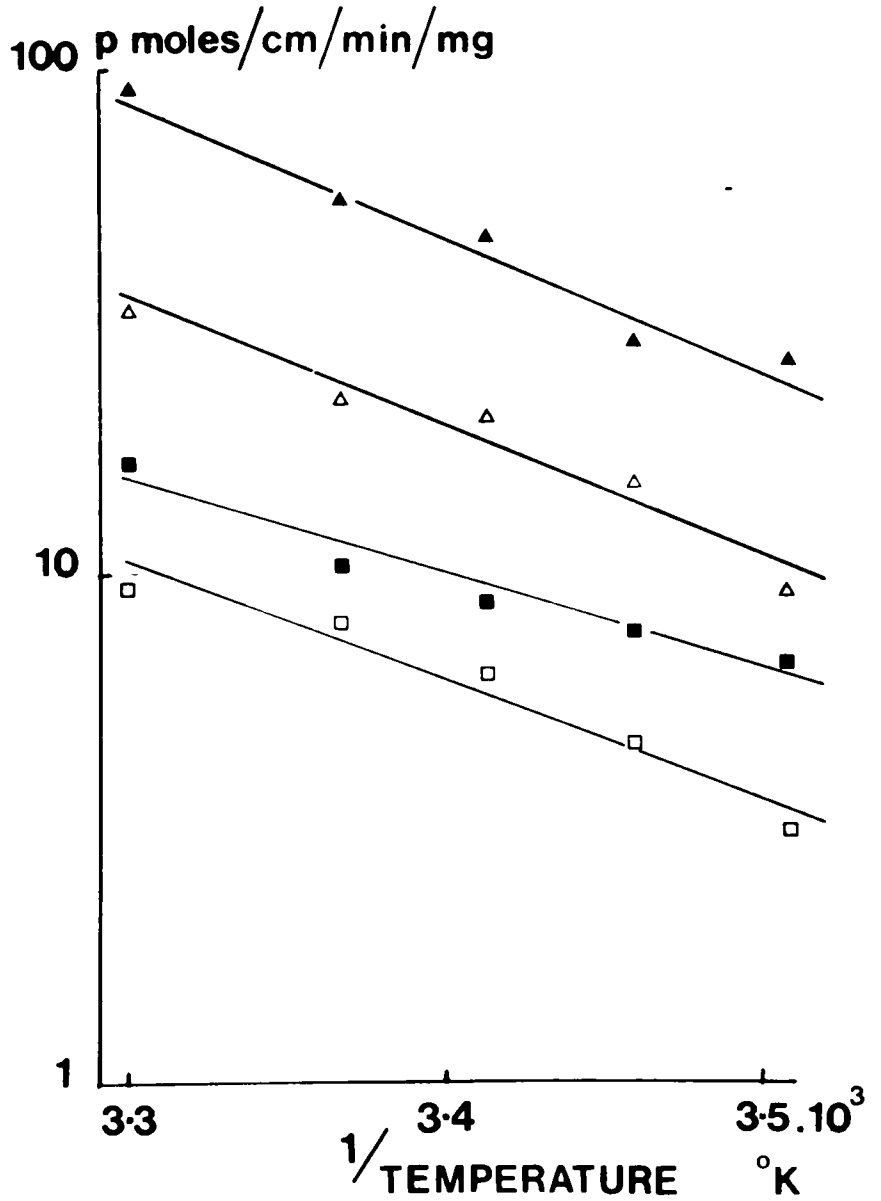


Figure 33b



$r = Ze^{-E/kT}$

r = rate

E = activation energy

k = Boltzmann constant

T = absolute temperature

Z = number of collisions

$\therefore \quad \log_e r = \log Z - \frac{E}{kT}$

Assuming E and log Z are constant over the range of temperatures studied,

$$\log r \propto -\frac{1}{T}$$

and slope $= \frac{-E}{2.3k}$ $\qquad k = 1.38 \times 10^{-23}$ J/°K/molecule

$\therefore \quad E = -$ slope $\times 2.3 \times 1.38 \times 10^{-23} \times 6 \times 10^{23} \times 10^{-3}$ kJ/mole

The mean slope is -2375°K

$\therefore \quad E = -(-2375) \times 2.3 \times 1.38 \times 6 \times 10^{-3}$ kJ/mole

$= 45.1$ kJ/mole

From the data an activation energy of 45 kJ/mole is obtained. This value is typical of activation energies of chemical reactions as opposed to physcial processes such as diffusion which have a much smaller value of E.

3. The effect of pH

In normal cells the proteins and other buffering systems and ion transport across the plasma membrane serve to keep the pH of the internal milieu constant at about 7.0 (Caldwell, 1958; Kushmerick & Davies, 1969, appendix), so that the changes in pH that accompany contraction (Distèche, 1960) are rapidly neutralised. Owing to the

regulatory effect of the cell membrane the effects of pH on the contractile elements cannot be reliably estimated unless it is removed, e.g. by glycerination.

The effect of pH variation on actomyosin systems has been studied by several workers. Typically the ATPase activity of myosin has a small maximum between pH 6.0 and 7.0, and a minimum around pH 7.5. Above pH 8.0 ATPase activity increases markedly, sometimes reaching a maximum around pH 9.0 (e.g. Mommaerts & Green, 1954), sometimes continuing to rise (Taylor et al., 1970). The difference was shown to be species specific (Bárány & Bárány, 1966). In all cases the activity of the myosin ATPase enzyme is relatively low at neutral pH, 0.2 μmoles/minute/μg enzyme.

Portzehl et al., (1969) found a pattern similar in extracted rabbit fibres when the calcium requirements for ATPase activity were investigated.

Glycerinated fibres are a suitable preparation for investigating the effect of pH on intact contractile apparatus. Different workers using Lethocerus flight muscle have used solutions of various pH's; Rüegg & Tregear (1965), 7.1; Pringle & Tregear (1969), 6.9; Steiger & Rüegg (1969), 6.5. High activation was achieved in each case. Schädler (1967) investigated the effect of pH upon the ATPase activity and tension of four different types of muscle and found that a decrease in pH from 7.0 to 6.0 decreased the calcium sensitivity, particularly in fibrillar flight muscle, but it did not affect the cost of tension production. pH did not change the frequency of the isometric oscillations observed after quick stretch (Schädler, et al., 1969).

A series of experiments was performed in which the pH of the incubating solution was varied between 6.5 and 7.3. The added calcium was adjusted so that at all pH's the free calcium ion concentration, as calculated using the Perrin programme, was constant. Purely mechanical

TABLE 17

Effect of pH on mechanical performance of muscle

pH	Oscillation amplitude	Optimum frequency	Maximum work/cycle	Frequency range,	Average Oscillatory mean tension
	%	Hz	nJ/cm/cycle	Hz	mg/fibre
6.5	2	4.6	0.14	2.8 - 7.5	4.25
6.7	2	5.6	1.1	1.8 - 13.0	6.10
6.95	2	6.6	4.6	1.5 - 20.0	6.30
7.3	2	6.0	42.0	1.4 - 25.0	9.45
6.5	0.2	7.8	0.04	0.8 - 26.0	
6.7	0.2	10.0	0.05	0.5 - 40.0	Not measured
6.95	0.2	8.5	0.13	1.0 - 44.0	
7.3	0.2	7.6	0.32	0.5 - 18.5	

experiments were performed using the PDP8-I computer on-line, and experiments involving the measurement of ATPase activity were also done.

It proved difficult to get consistent results in this series of experiments. The pH dependence of performance varied from muscle to muscle, some working well down to pH 6.5 and appearing to disintegrate at pH 7.3, others not producing any work at low pH but having their optimum output at pH 7.1 or even higher. All the experiments involving ATP estimations were done on muscle fibres from one bug; during the three week period for which it was used, its mechanical performance did not vary greatly and it was possible to do mechanical experiments and ATPase activity measurements on the same fibres.

The computer controlled experiments were similar to those described in the section on temperature. As before, the results were plotted as a Bode diagram and the optimum frequency, frequency range and maximum work per cycle taken from the graphs. These figures are presented in Table 17.

At 2% amplitude there is an increase in optimum frequency from 6.5 to 6.95. The frequency of maximum work at pH 7.3 is lower than that at 6.95. The range of working frequencies increases with pH over all the hydrogen ion concentrations tested. In contrast to the lack of effect of temperature on maximum work outputs, increased pH is accompanied by a marked increase in the work per cycle of the fibres, at both 2% and 0.2% amplitude. At low amplitude of oscillation the effect of pH on the optimum frequency is not clear-cut although there is, except at pH 7.3, an increase with pH in the range of frequencies at which work is obtainable. At pH 6.5 very little work is produced and the form of the Nyquist plot is typical of that observed when fibres are not properly activated.

Relation between mechanical performance and ATPase activity of muscle.

Figure 34 a pH 6·5

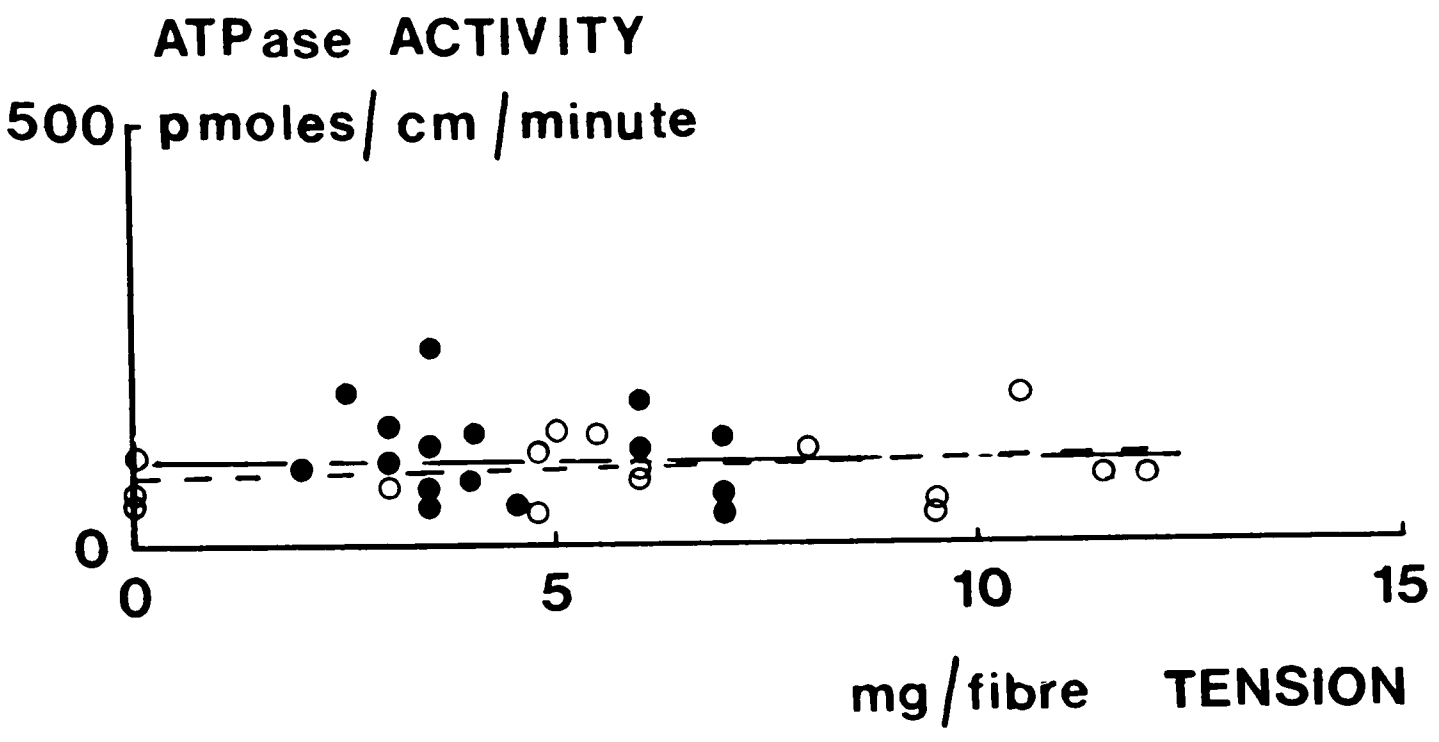
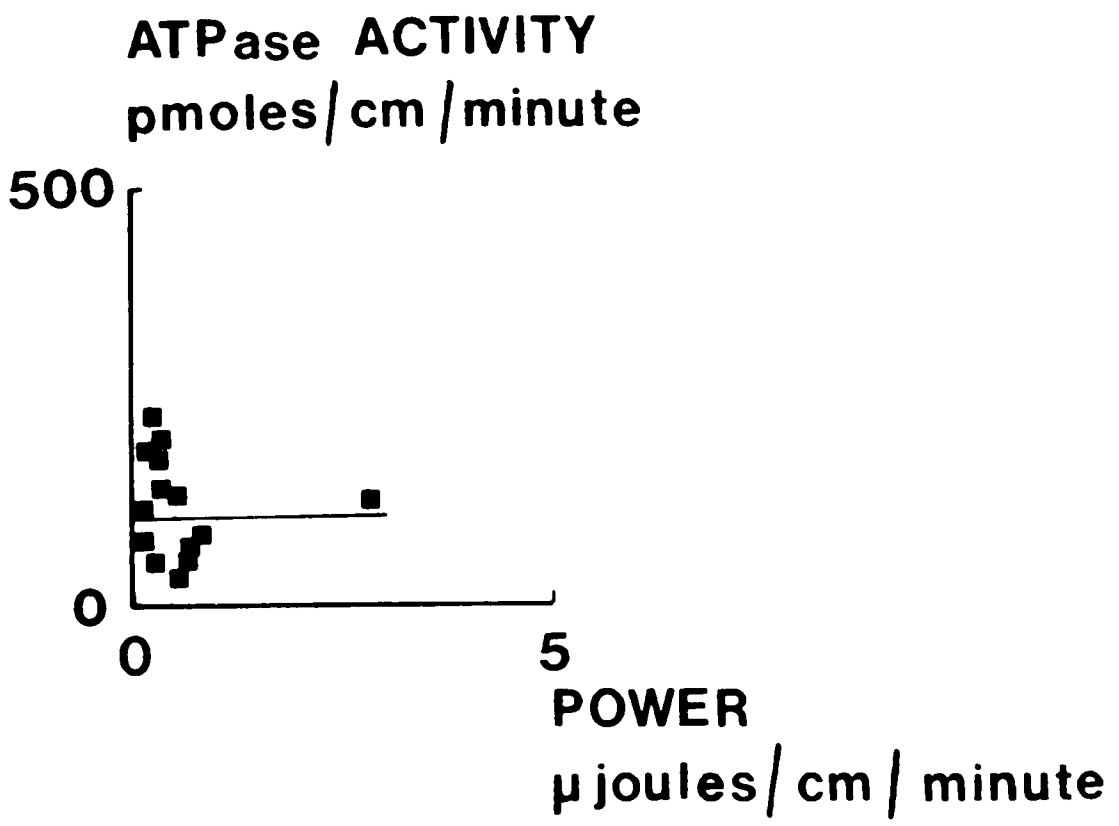


Figure 34b



Relation between mechanical performance and ATPase activity of muscle.

Figure35a pH 6·7

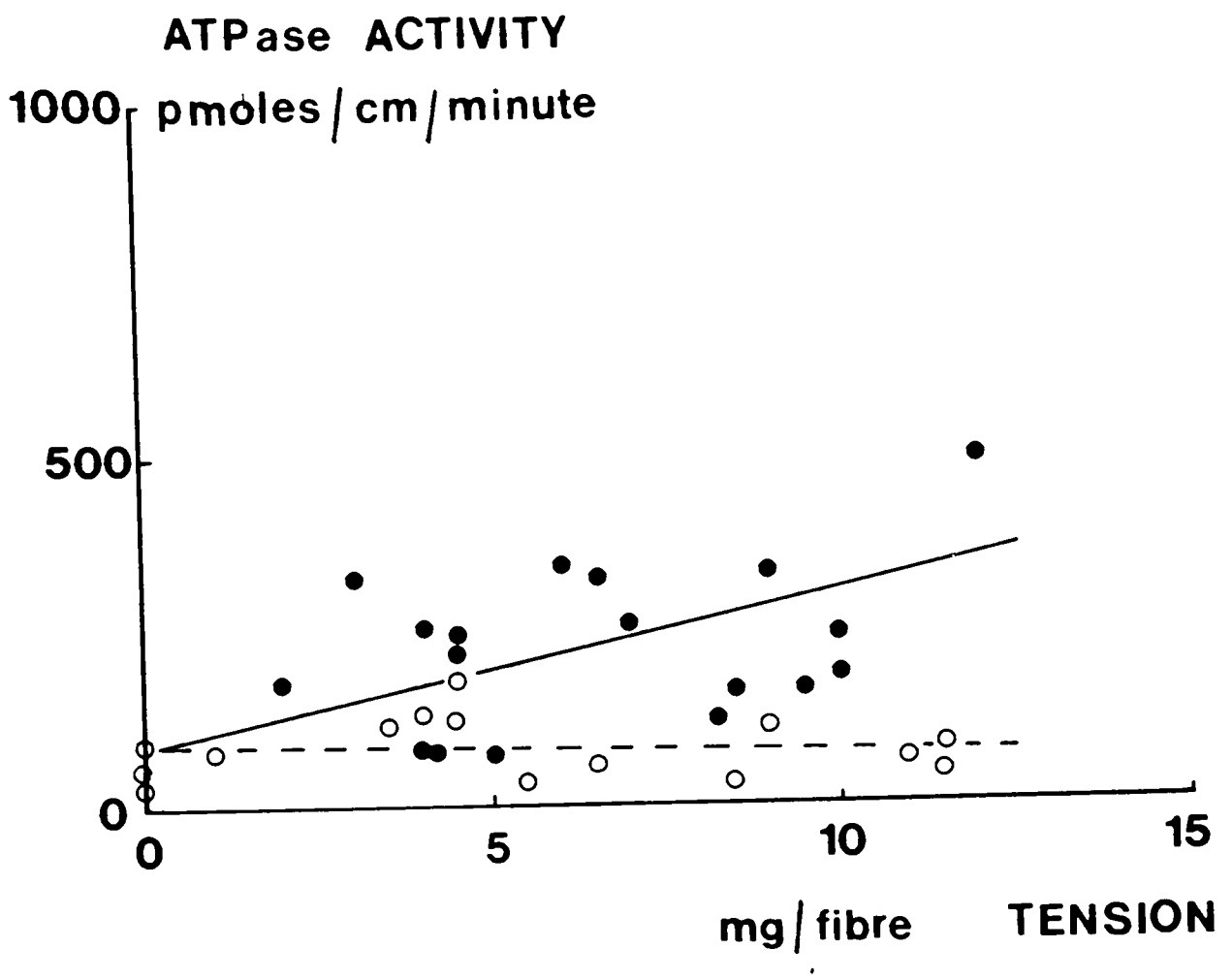


Figure 35b

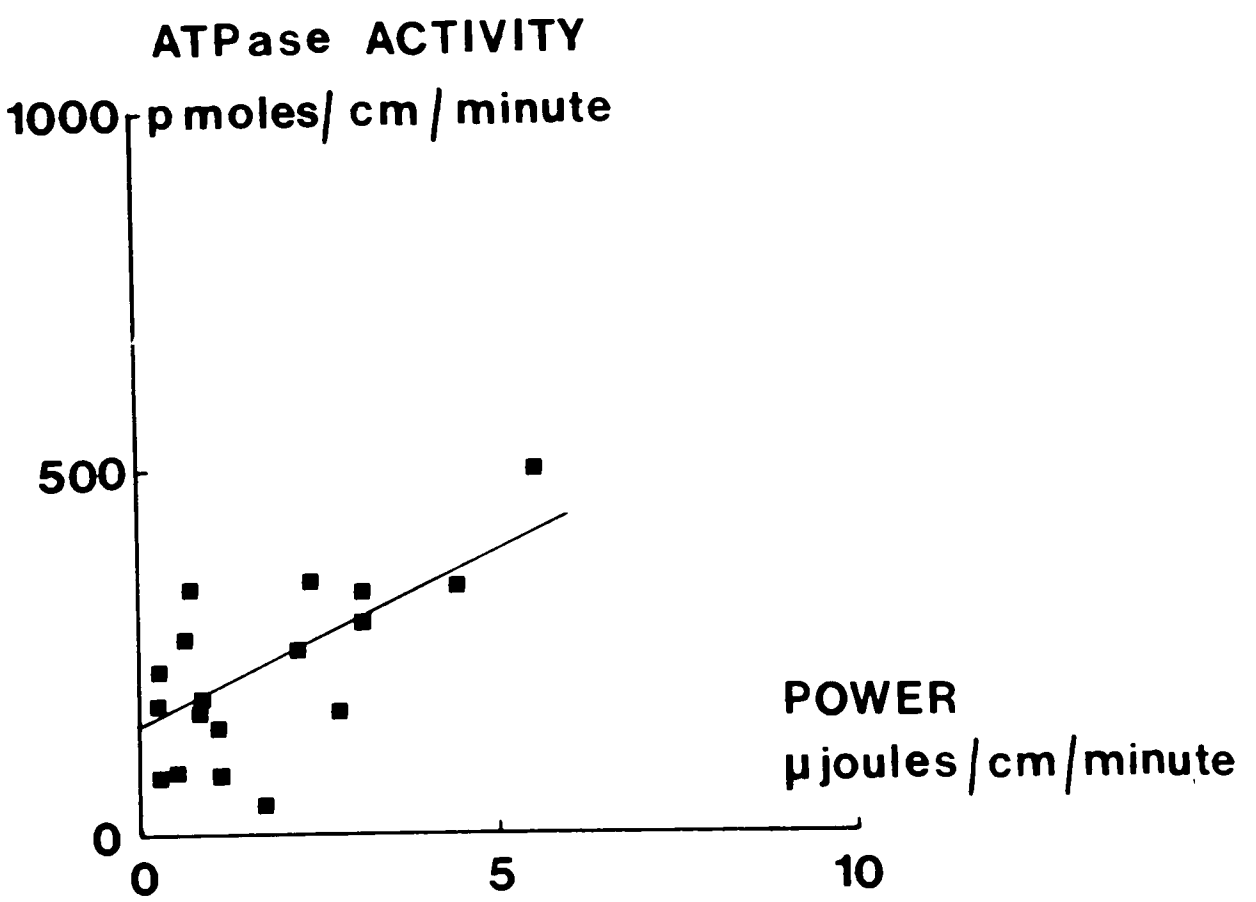


Figure 36a pH 6·95

Relation between mechanical performance and ATPase activity of muscle.

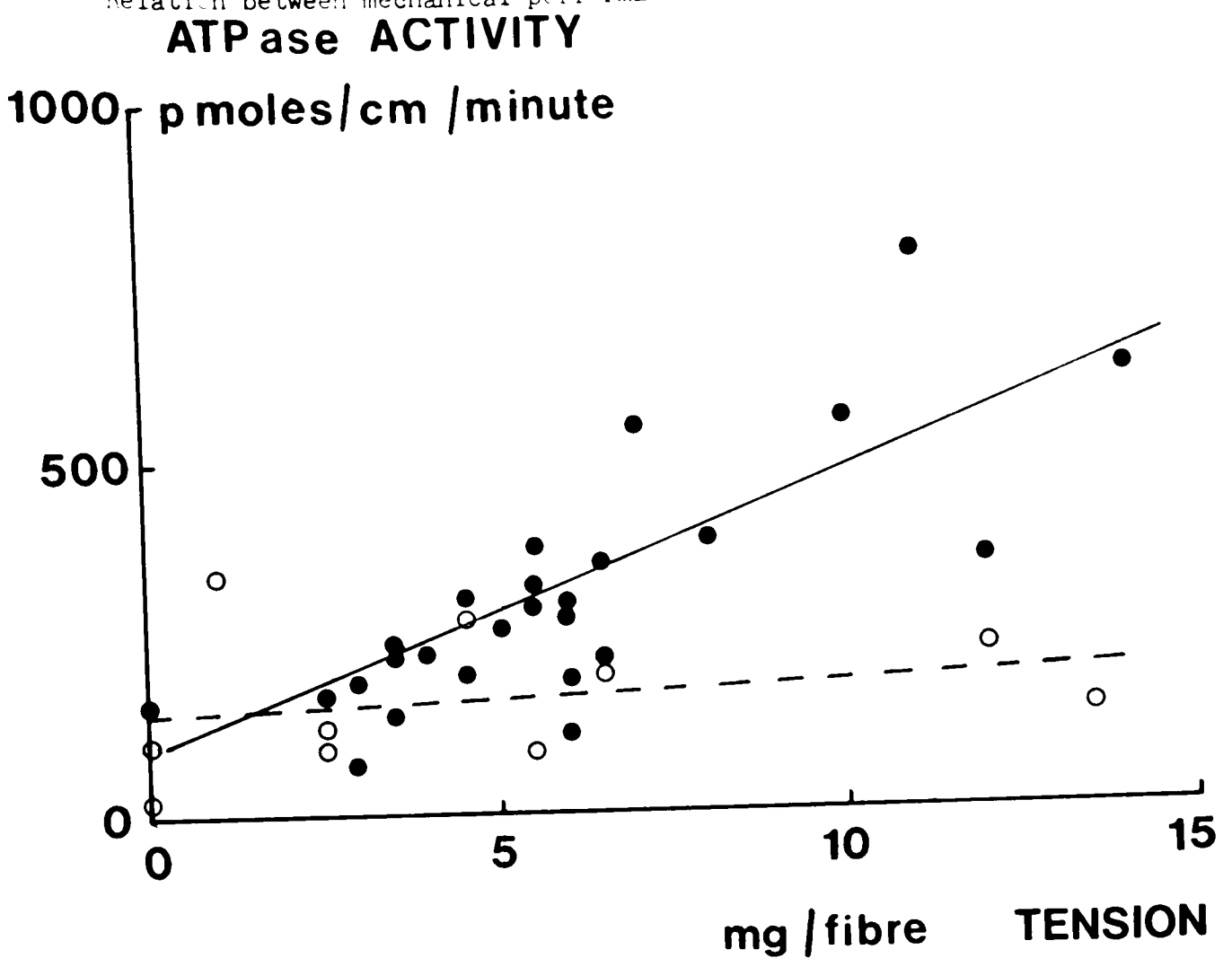


Figure 36b

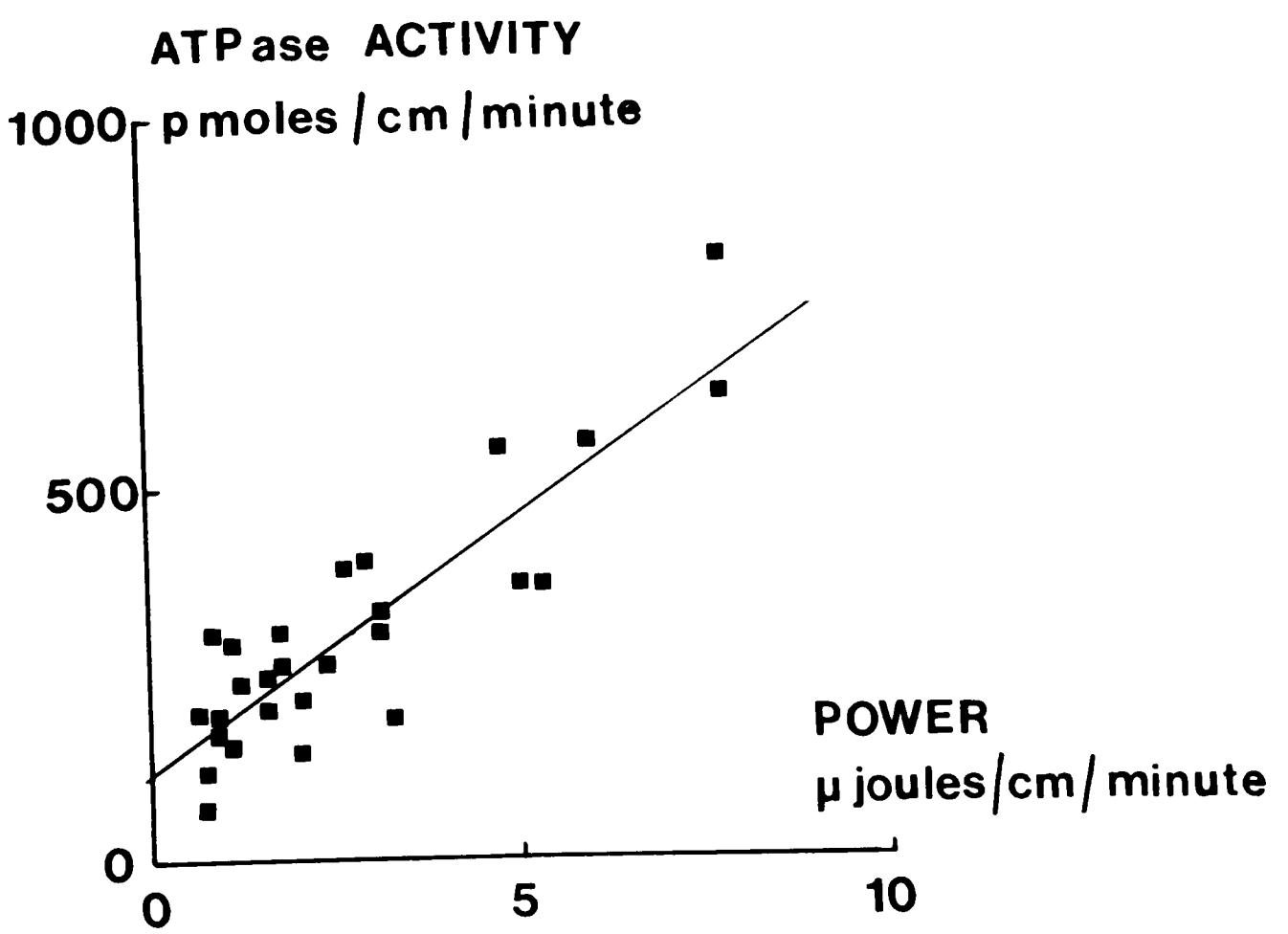


Figure37a pH 7·3

Relation between mechanical performance and ATPase activity of muscle.

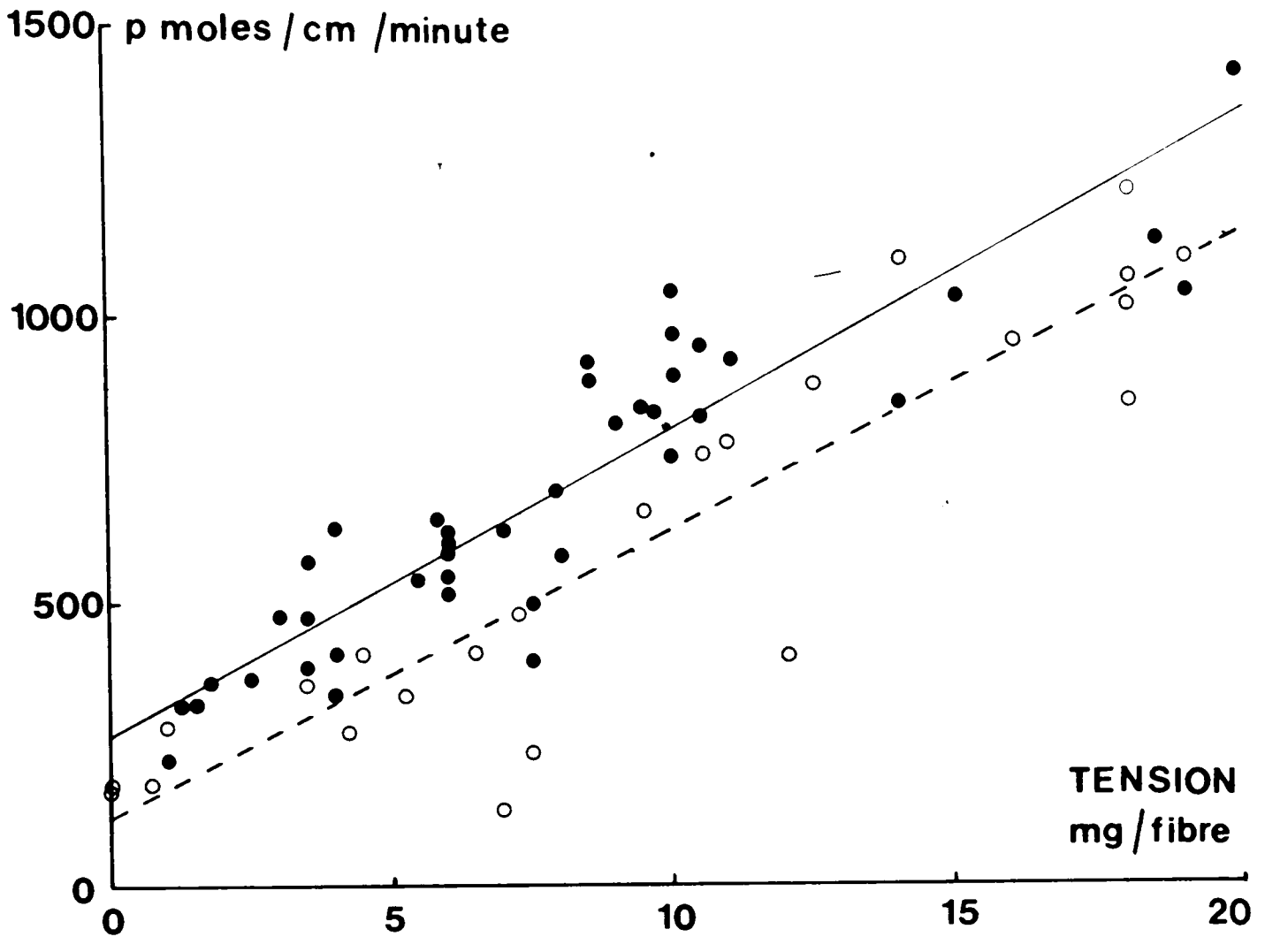


Figure 37b

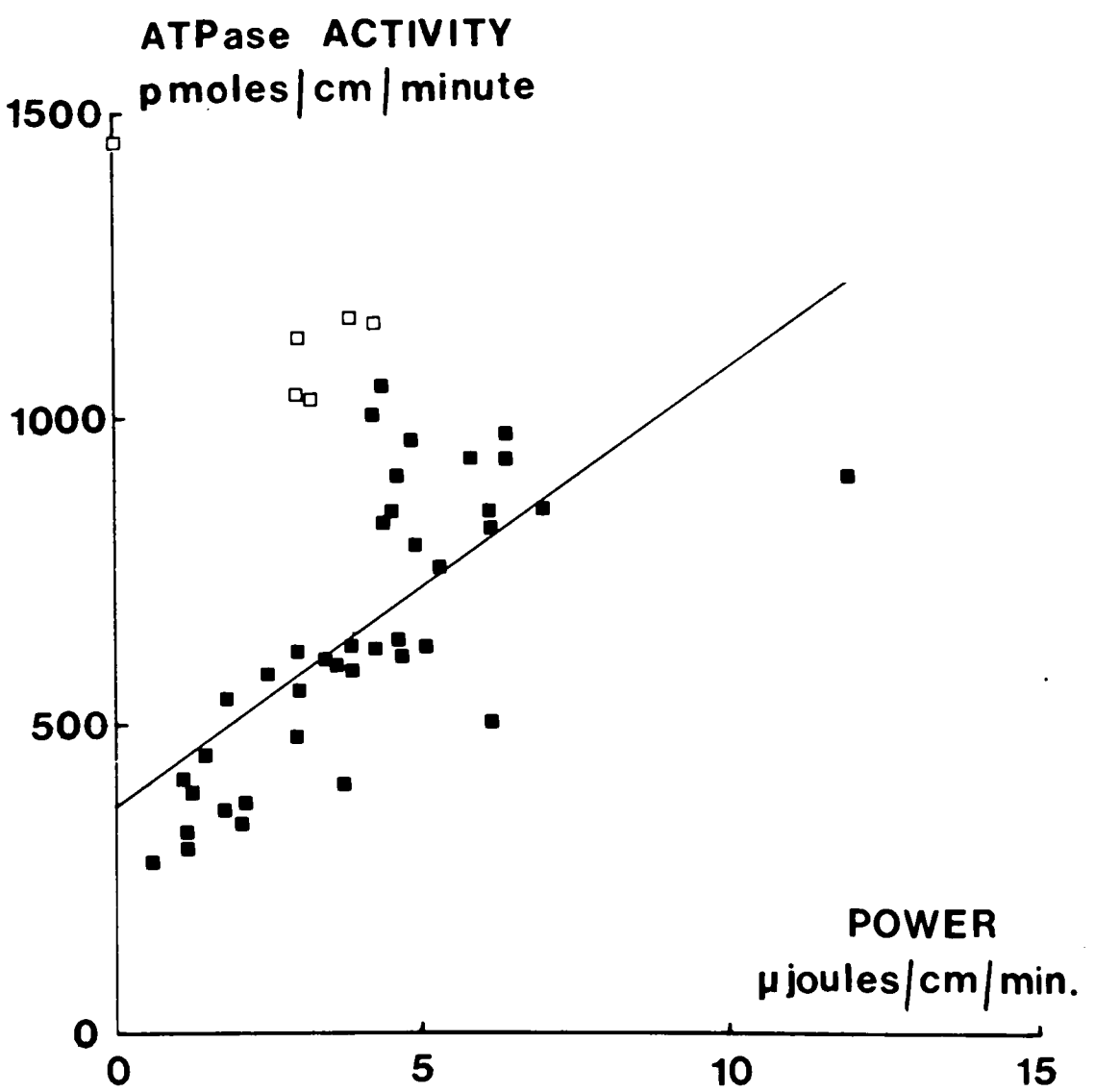


TABLE 18

Effect of pH on ATPase activity of muscles in relation to tension and power production

2% amplitude, frequencies 5-10 Hz

	pH	Isometric ATPase/tension	Oscillatory ATPase/tension	ATPase/power
		pmoles/mg	pmoles/mg	pmoles/μJoule
Regression coefficient	6.5	2.0 ± 2.8	-0.6 ± 5.0	3.0 ± 19.0
	6.7	-0.8 ± 2.8	22.5 ± 6.0	47.0 ± 14.0
	6.95	3.7 ± 7.2	38.5 ± 6.6	68.0 ± 7.0
	7.3	50.6 ± 5.2	53.6 ± 3.5	70.0 ± 11.0
		pmoles/cm/min	pmoles/cm/min	pmoles/cm/min
Intercepts	6.5	80 ± 35	101 ± 37	105 ± 20
	6.7	88 ± 30	86 ± 67	147 ± 47
	6.95	146 ± 73	95 ± 72	116 ± 40
	7.3	120 ± 103	265 ± 55	369 ± 97

The last column of Table 17 contains measurements of the maximum mean tension observed under oscillatory conditions at 2% at each pH. The figures were obtained from the photographed length-tension loops recording performance in the ATPase activity experiments. The mean tensions for all experiments performed at 5 to 10 Hz were averaged for each frequency to give a measure of the tension necessary to obtain as good an oscillatory power output as was possible. This tension increases with pH.

It was observed that at high pH, 7.3, the position at which tension is very high and power less than maximum could be obtained more easily by stretching the fibres than it can at pH 6.95. At pH 7.3 the position was often stable for at least twenty minutes whereas at pH 6.95 continuous extension of the fibres is necessary to maintain the high tension.

The graphs in Figs. 34-37 show the results of the chemical experiments and the details of regression coefficients and intercept are displayed in Table 18.

There is little or no stretch activation at pH 6.5, 6.7 and 6.95. Only at pH 7.3 is the slope of the regression of ATPase activity against tension significantly different from zero and at this pH ATPase activity increases steeply with tension at a rate similar to that observed in the oscillating muscle, in contrast to the situations in the previous chapters where cost of tension production in isometrically contracting fibres was much less than that in the power producing muscle. ATPase activity/unit oscillatory tension decreases with pH; at pH 6.5, where little power is produced and high tensions are not obtained, the slope is not significantly different from zero. Except at pH 6.5, the cost of power production does not vary significantly and is similar to that found in other conditions, i.e. about 16 kJoules power are

produced per mole of ATP consumed. The graph of ATPase activity against power at pH 7.3 contains many points derived from fibres stretched beyond the tension giving optimum power; power shows a maximum against ATPase activity. The regression coefficient of the points on the part of the graph where ATPase activity and power are positively correlated shows that in this range efficiency is not significantly different from that obtained under other conditions.

Thus, in the muscle used for study, decreasing hydrogen ion concentration was accompanied by increasing activation of the muscle, as observed by the increasing work per cycle and by the failure to show isometric stretch activation at low pH. The increase in oscillatory tension cost was not accompanied by a parallel increase in static tension cost or, except at low pH, by increase in f_{max}. In view of the known error in estimation of f_{max} it is not possible to identify any simple pattern in the values of f_{max} at different pH.

The inconstencies in results obtained when pH is raised are illustrated from other experiments. A series of measurements of mechanical performance at 0.2% amplitude was made at eight pH's from 6.5 to 7.3 on fibres from a different muscle from that used for the above work. f_{max} was constant over the whole range. This result is parallel to that of Schädler et al., (1969) demonstrating that pH did not affect the speed of isometric oscillations, if it is assumed that the optimum speed of isotonic oscillation and isometric oscillation are controlled by the same mechanism. In addition, no change in holding economy of statically stretched fibres was observed by Schädler (1967) as pH increased.

The mechanical behaviour of the fibres used for the ATPase rate measurements was therefore not typical of all fibres observed by me or by other workers.

4. The effect of ionic strength

A number of workers employing actomyosin systems or myofibrils to study the interaction of actin, myosin, ATP and activators have tested the effect of varying the ionic strength of their solutions on the measured parameters. Most results are consistent with those of Maruyama et al., (1968) who found that raising the ionic strength increased the calcium requirement of actomyosin and myofibrils of insect leg and fibrillar flight muscle. The effect was much more pronounced in actomyosin. A change of KCl concentration from 0.02 M to 0.06 M was accompanied by a 15% drop in ATPase activity at half-activation for myofibrils but a 70% change for actomyosin. Portzehl et al., (1969) observed no change in calcium requirement of rabbit fibrils in the range 0.1 M to 0.2 M ionic strength, but a reduced requirement at 0.05 M, and Weber & Herz (1963) reported a decrease in ATP hydrolysis of myofibrils of 20% as ionic strength was raised from 0.08 M to 0.16 M. A doubling of KCl concentration halved the ATPase activity of rabbit actomyosin in the experiments of Bárány & Bárány (1966). There is thus considerable evidence to show that increased ionic strength reduces the ATPase activity of actomyosin, and, to a smaller extent, myofibrils.

Work on the kinetics of actomyosin systems has demonstrated that raised ionic strength increases the dissociation of acto-heavy meromyosin, but does not affect the V_{max} or Michaelis constant (Eisenberg & Moos, 1970). The use of myofibrils for allied studies is hampered by the fact that at high ionic strength the proteins become soluble. Similarly in intact fibres the structural integrity of the filaments is damaged at high salt concentrations.

TABLE 19

Concentration in solution of ionic strength

	0.08 M	0.09	0.10	0.11	0.13	0.18
Species						
MgATP	4.7 mM	4.7	4.7	4.7	4.6	4.5
Ca^{2+}	0.0017 mM	0.0017	0.0017	0.0017	0.0017	0.0017
Mg^{2+}	3.5 mM	3.5	3.5	3.5	3.5	3.7
K^{+}	49 mM	59	69	79	99	148

It is therefore much more difficult to make measurements of the effect of ionic strength on muscle proteins which are associated out of solution than when they are dissolved, although the work quoted on myofibrils shows that the effects are much greater when actomyosin is in solution. In addition it is difficult to judge what ionic strengths are equivalent in dissolved protein, glycerinated fibre, and living muscle systems. The presence of a membrane around living fibres enables them to maintain their surroundings at a fairly constant ionic strength although whether that is as high (0.25 M) as the intracellular ionic composition would indicate is doubtful. Some of the ions may be bound, thus reducing ionic strength. The work of Hinke (1970) & Hinke et al, (1972) on barnacle muscle provides evidence that large fractions - in the case of Na^+ around half - of the monovalent ions are either compartmentalised or bound in intact fibres. Additionally only 68% of the water in the cells acts as a solvent for the ions. When the sarcolemma is destroyed by glycerination or other procedures the ions occupy the total water space. Living muscle behaves as a constant volume system, glycerinated muscle does so only at an ionic strength of 0.015 M, so this value may represent the ionic strength of the medium of living muscle (Rome, 1968). The intact membrane is therefore important in the control of ionic distribution and strength. It is not possible to make the ionic strength of the solution routinely used in this work as low as 0.015 M but experiments were performed in which the ionic strength of the phosphate-buffered activating solution was raised from 0.08 M to 0.18 M by altering the concentration of added KCl. The concentration of all other constituents was kept constant. The composition of the solutions was analysed using the Perrin programme and it was found that addition of KCl did not greatly affect the concentration of other species in the solution, see Table 19.

TABLE 20

Effect of ionic strength on mechanical performance of muscle fibres

Computer controlled experiments

Ionic strength	Oscillation amplitude	Optimum frequency	Maximum work/cycle	Working frequency range
M	%	Hz	nJoules/cm/cycle	Hz
0.08	2.0	6.6	23.2	0.3 - 29.0
0.09	2.0	6.8	23.4	0.3 - 34.0
0.10	2.0	6.2	16.8	0.25 - 33.0
0.11	2.0	5.8	17.9	0.3 - 32.0
0.13	2.0	6.8	19.2	0.2 - 32.0
0.18	2.0	6.4	2.8	0.2 - 12.0
0.08	0.2	9.7	0.87	1.0 - 44.0
0.09	0.2	10.5	0.86	0.5 - 38.0
0.10	0.2	9.6	0.87	1.6 - 42.0
0.11	0.2	9.9	0.91	1.6 - 42.0
0.13	0.2	10.0	0.65	1.5 - 58.0
0.18	0.2	-	-	-

Measurements of ATPase activity, tension and power output and frequency response were made at 2% amplitude using the conventional apparatus. In a later series of experiments the mechanical performance of fibres under conditions of varying ionic strength was examined at 2% and 0.2% amplitude using the computer controlled apparatus.

The data obtained from the computer-controlled experiments, the quadrature value at selected frequencies, was plotted as a Bode diagram and optimum frequency for work, range of positive work-producing frequencies and maximum work per cycle obtained from the graphs. The results are shown in Table 20.

At very high ionic strength, 0.18 M, very little work was observed at 2% amplitude and none at all at 0.2% and, if the fibres were left in the solution, they ceased to give any power on tension after several minutes, even when replaced in low ionic strength solution.

The mechanical performance at each amplitude did not change significantly in the range of ionic strength 0.08 M to 0.13 M, as the table shows. The characteristic increase in optimum frequency and range of working frequency with decreased amplitude is shown.

It was unfortunately necessary to use fibres from a number of bugs for the chemical experiments so the results are subject to error from this source. Particularly in the case of the 0.11 M investigation variability of performance and consequent large measured error in the results can be attributed to this cause.

The results of experiments in which ATPase was measured are shown in Figs. 38-43 and regression coefficients and intercepts calculated from the data are presented in Tables 21 and 22.

The results show that in the range of ionic strength 0.08 to 0.13 no significant change in chemical performance is found. The cost of tension and power production do not change significantly with ionic

Relation between mechanical performance and ATPase activity of muscle.

Figure 38 a Ionic strength 0·08 M

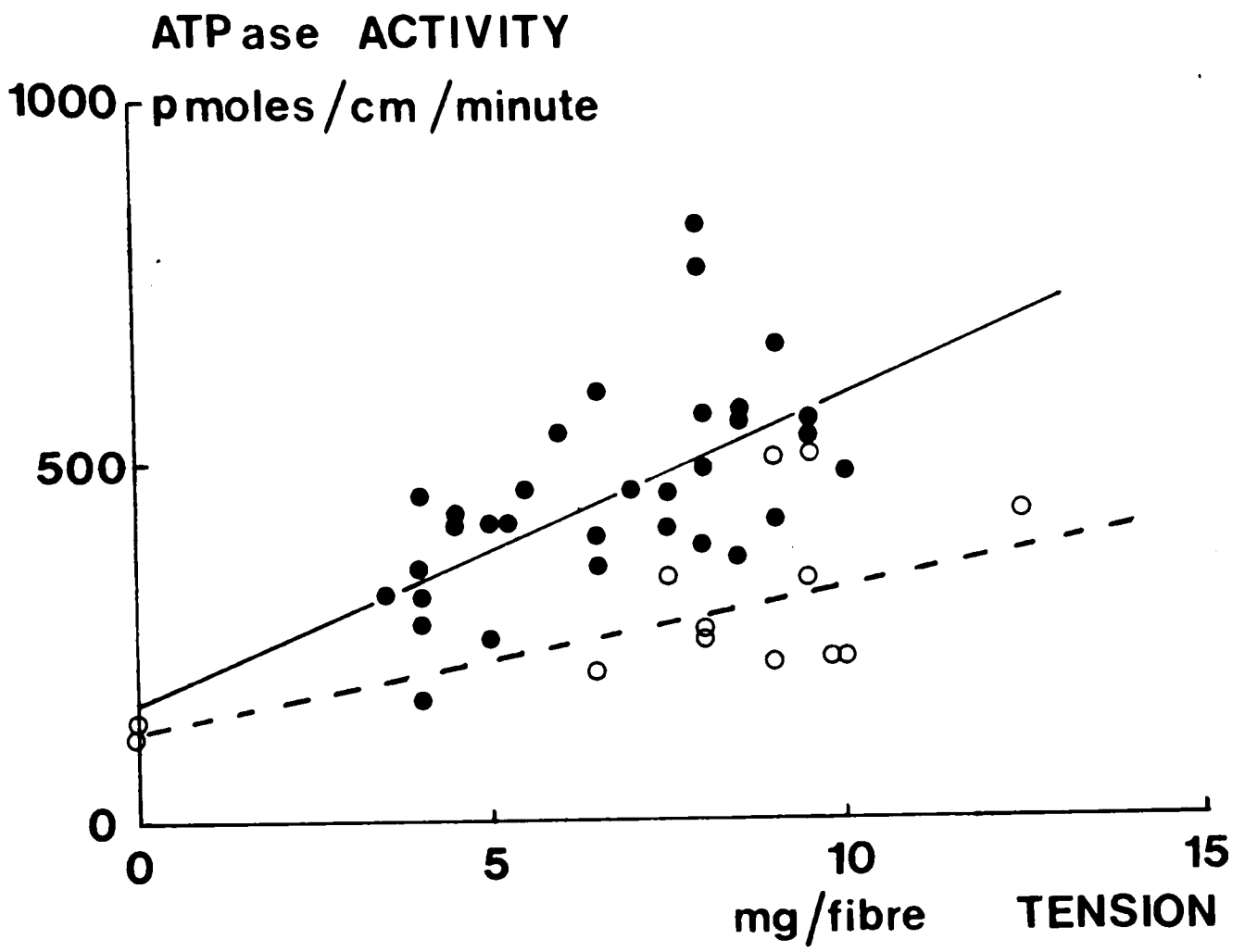


Figure 38b

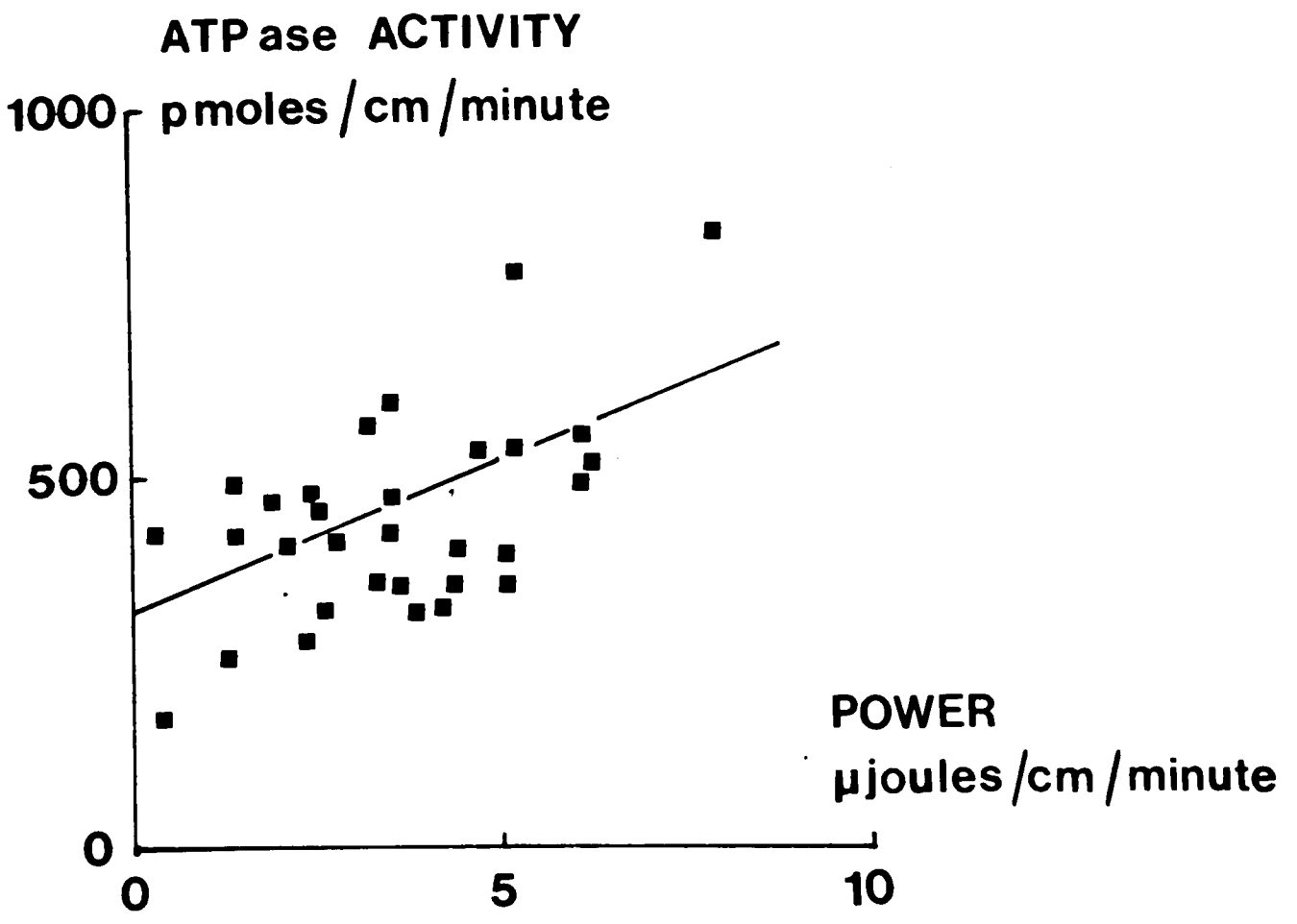


Figure 39a Ionic strength 0·09 M

Relation between mechanical performance and ATPase activity in muscle.

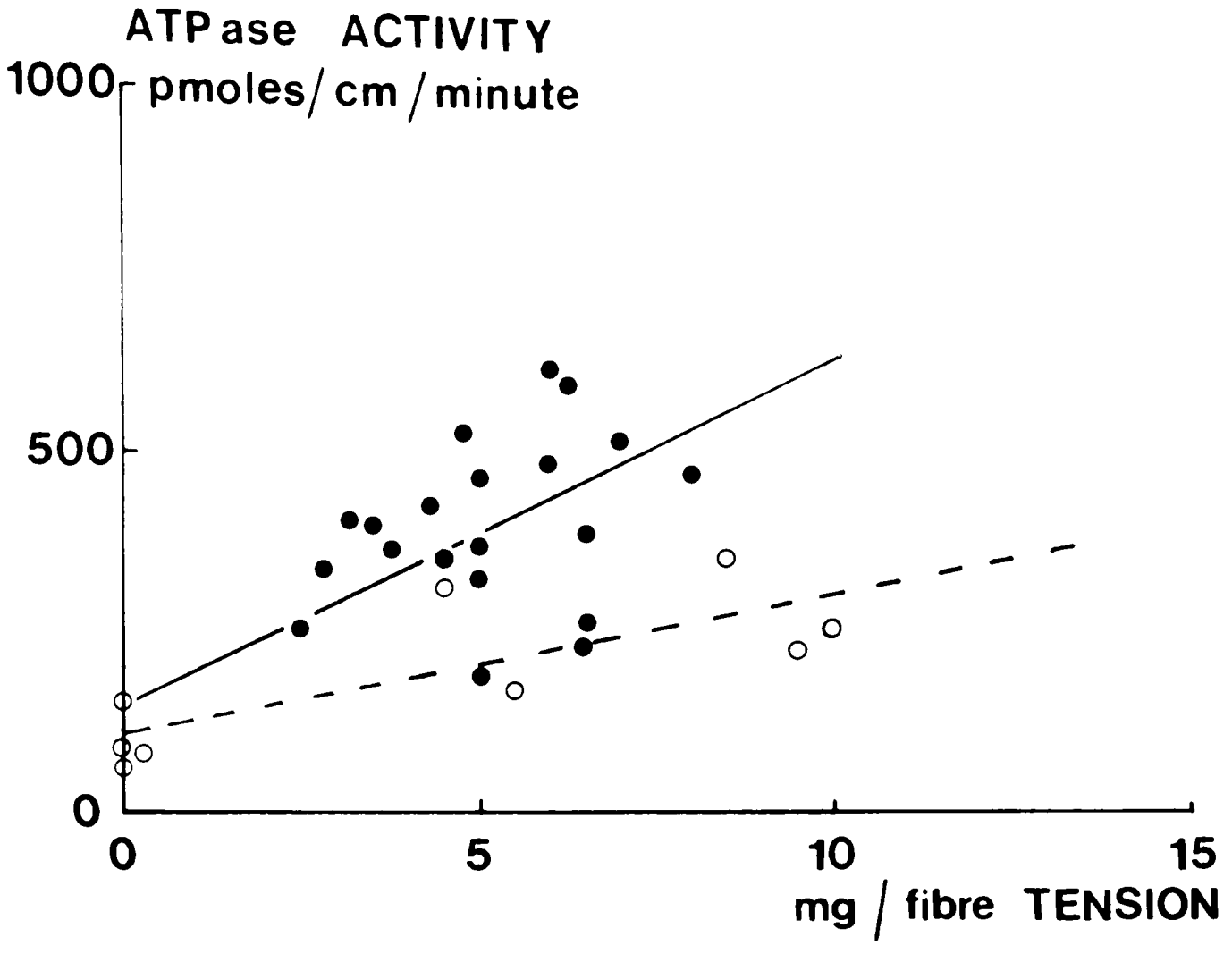


Figure 39b

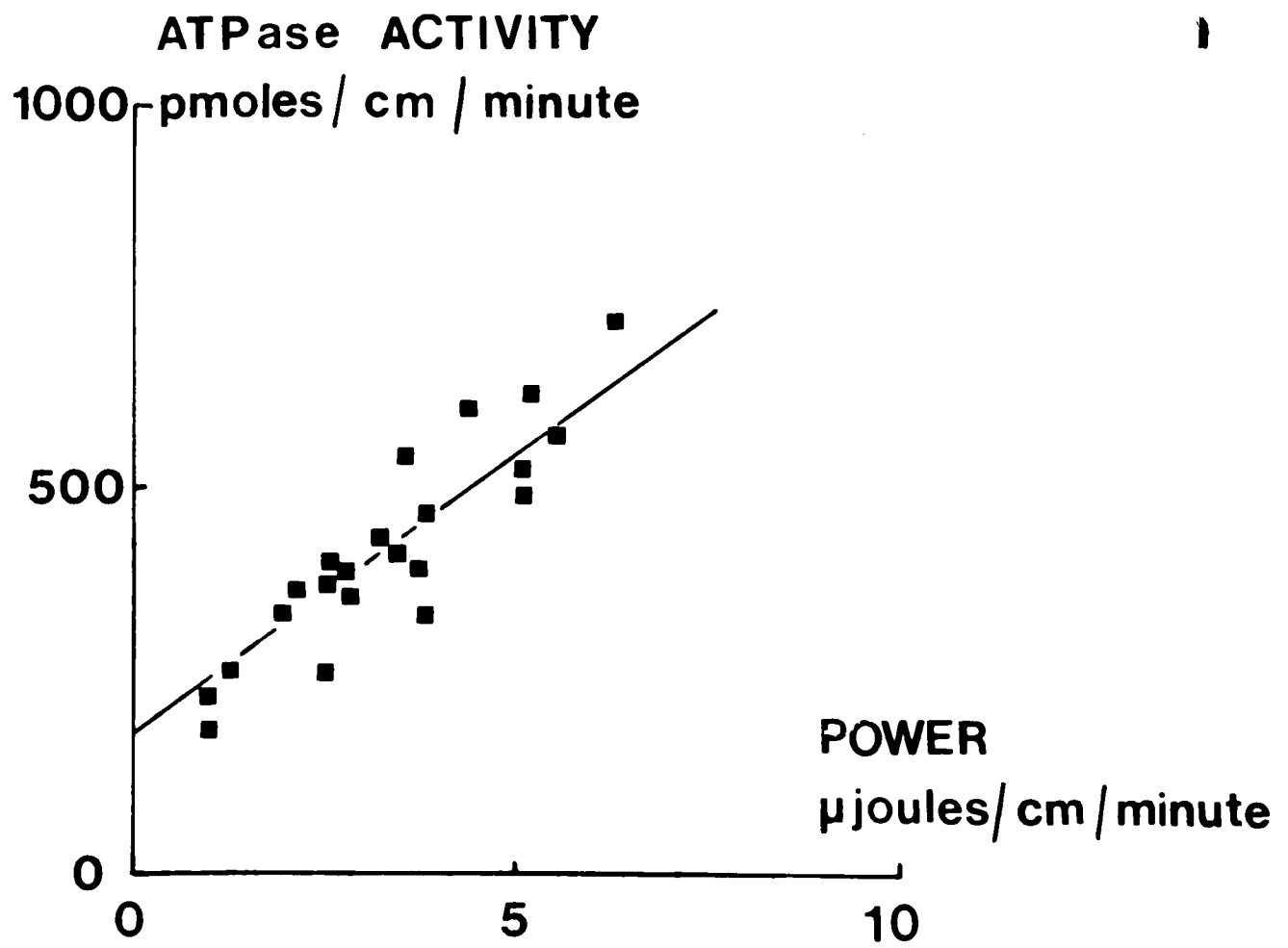


Figure 40a Ionic strength 0·10 M

Relation between mechanical performance and ATPase activity of muscle.

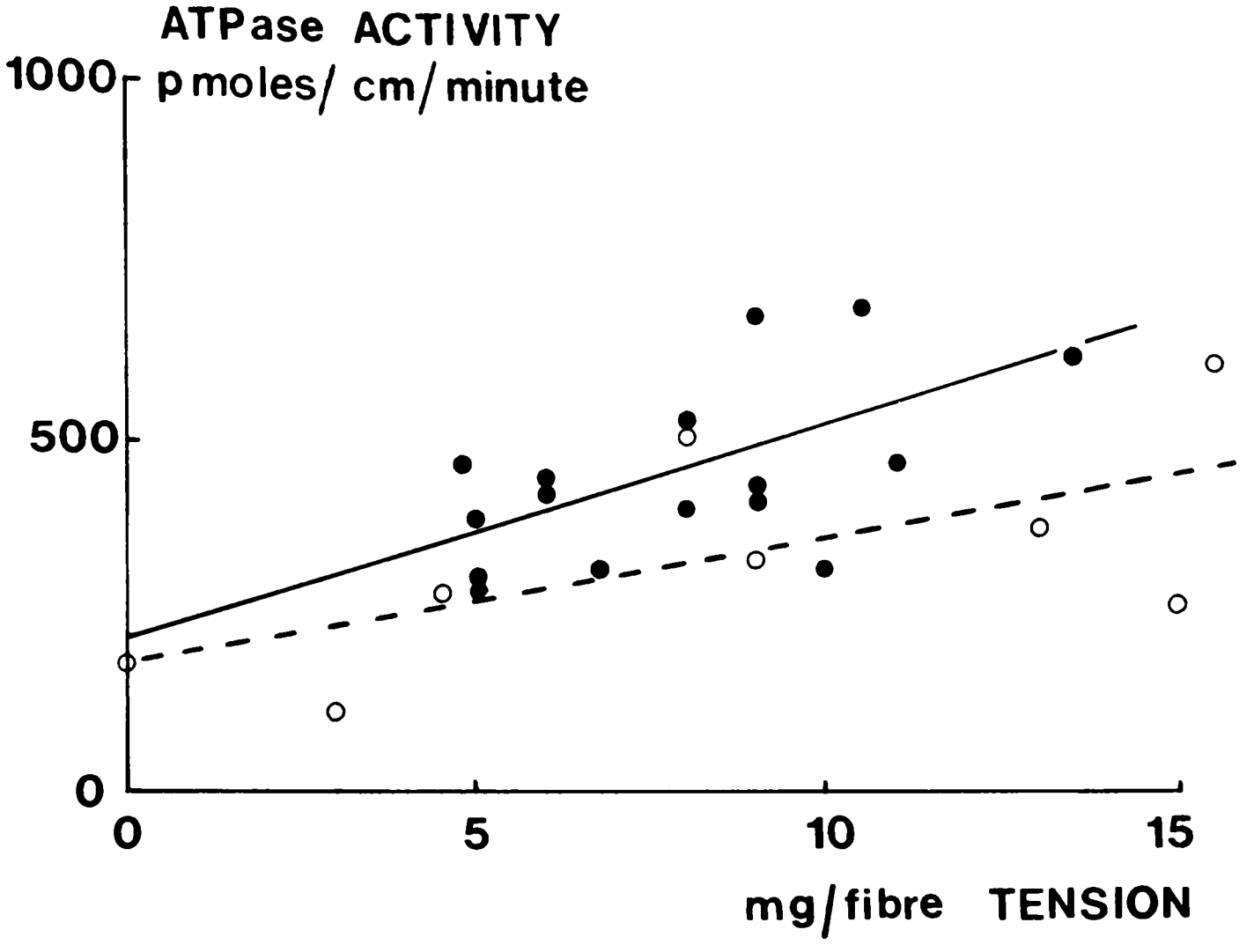


Figure 40b

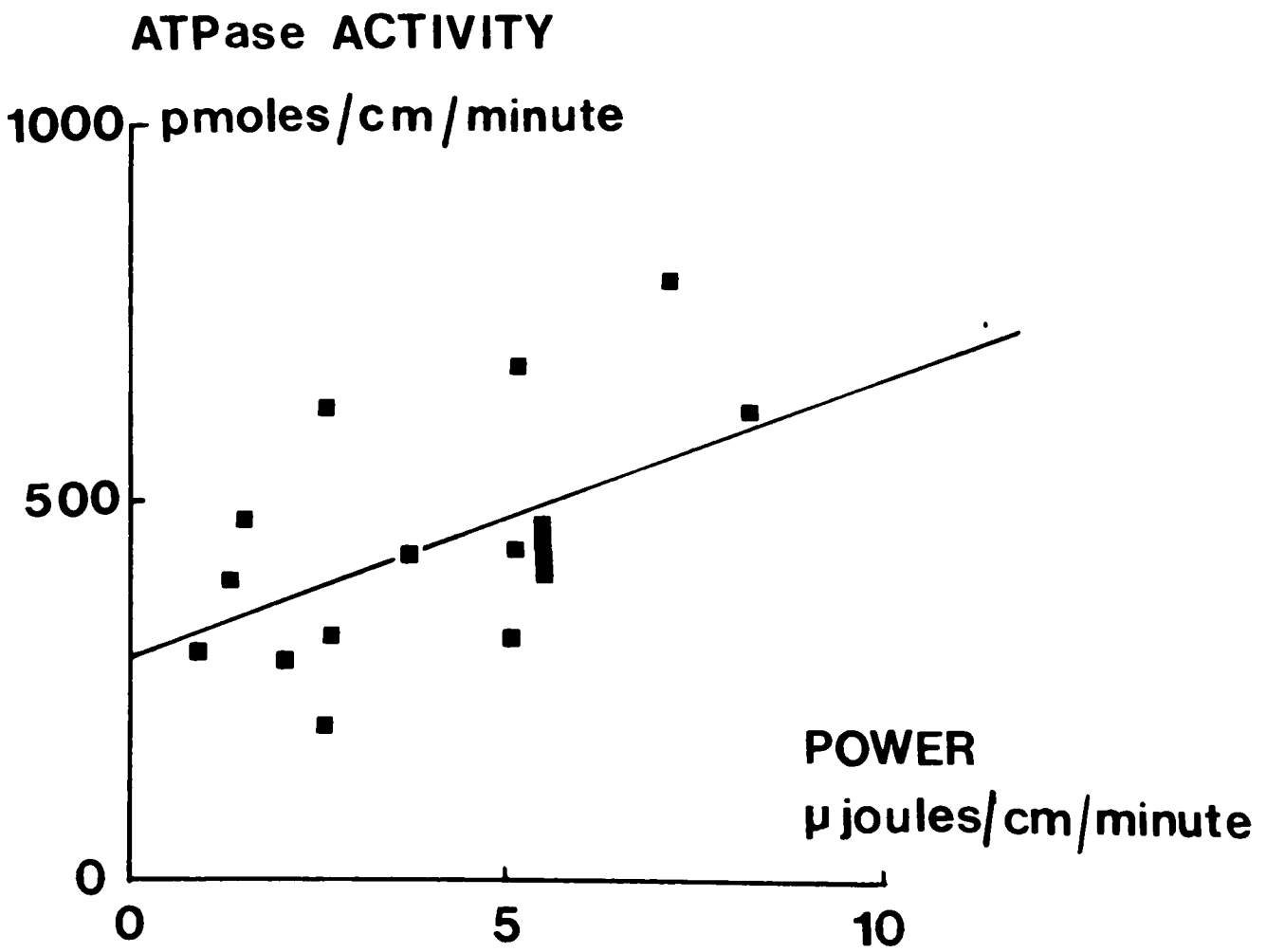


Figure 41a Relation between mechanical performance and ATPase activity in muscle.

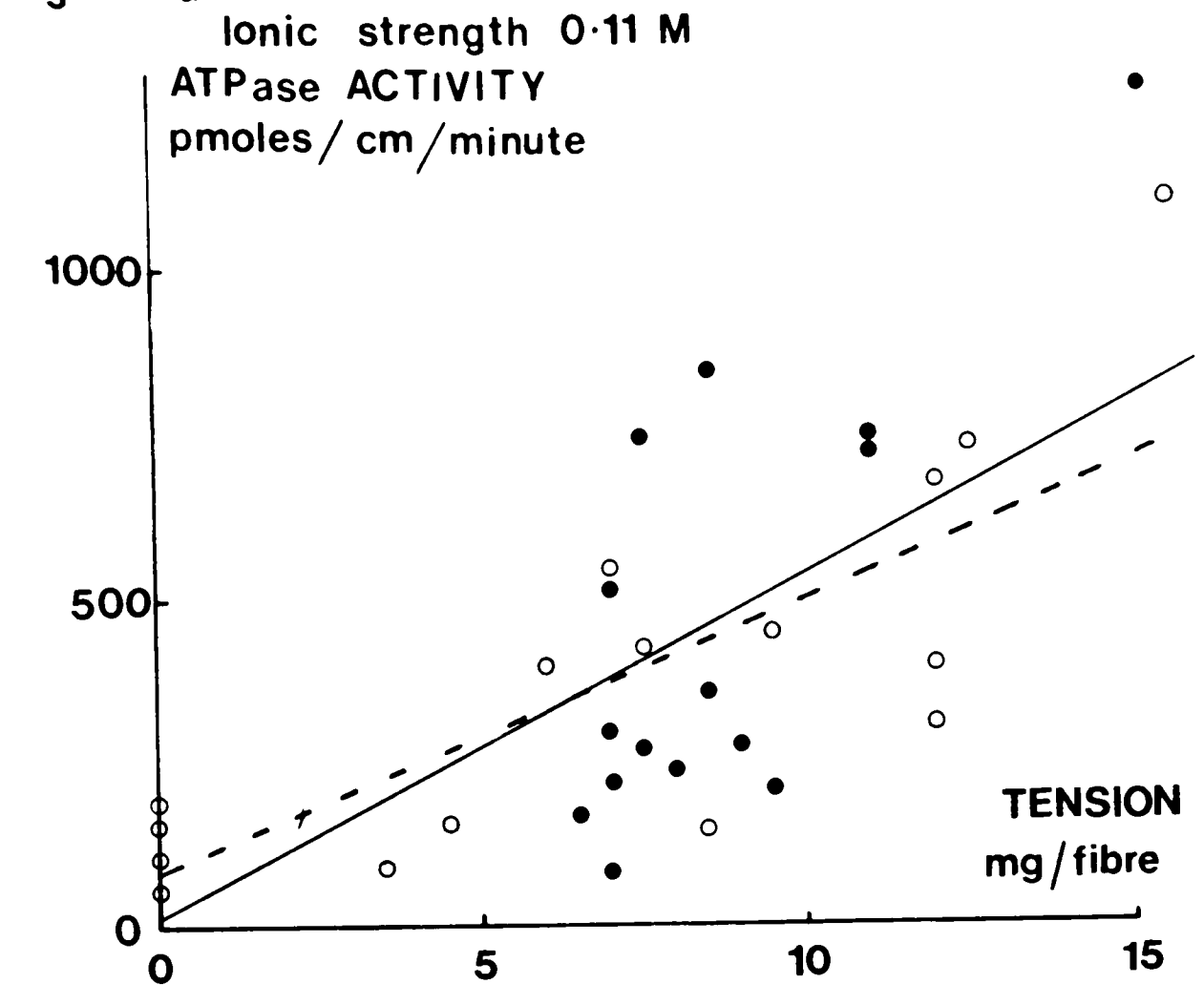


Figure 41b

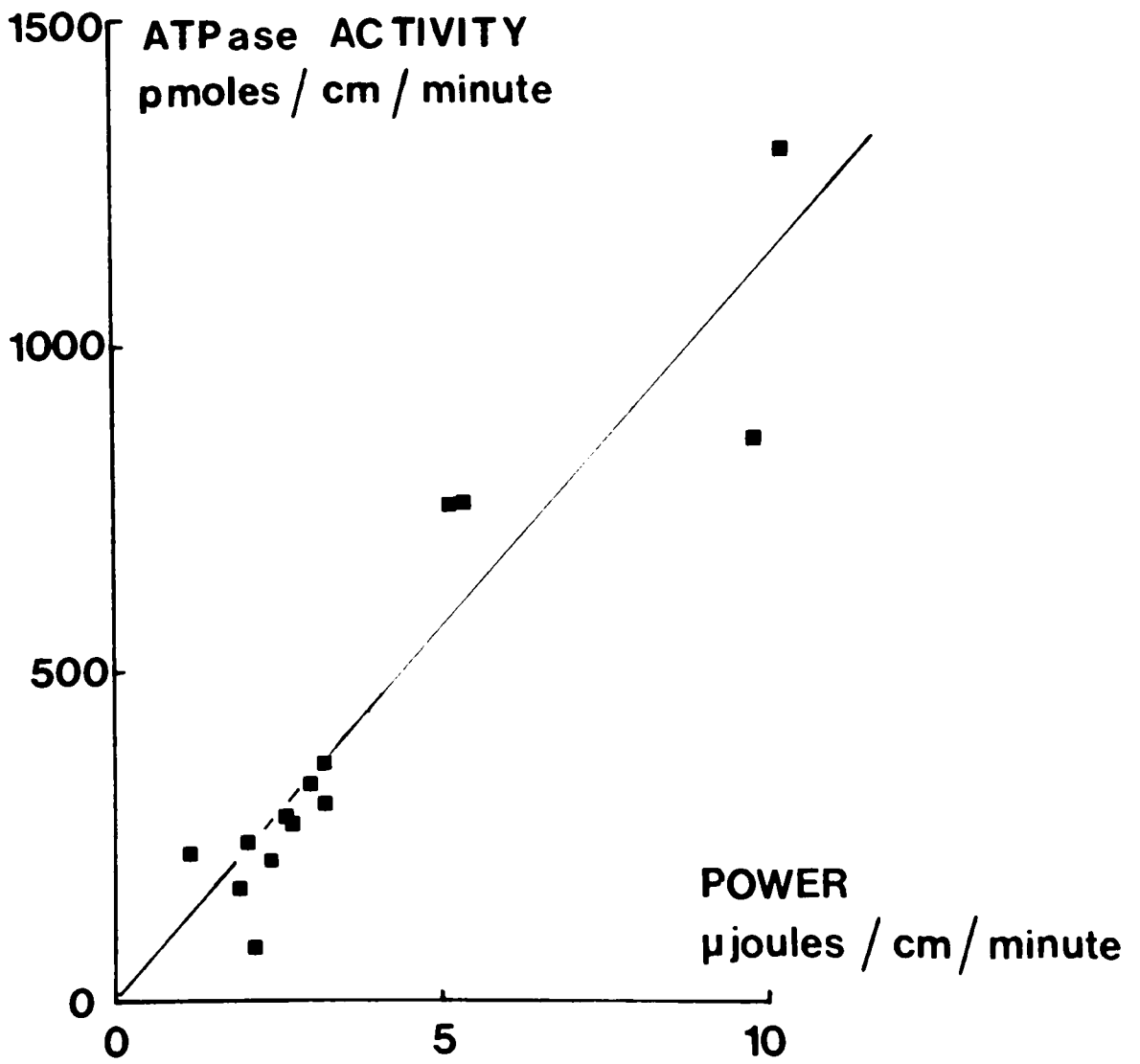


Figure 42a Ionic strength 0·13 M

Relation between mechanical performance and ATPase activity of muscle.

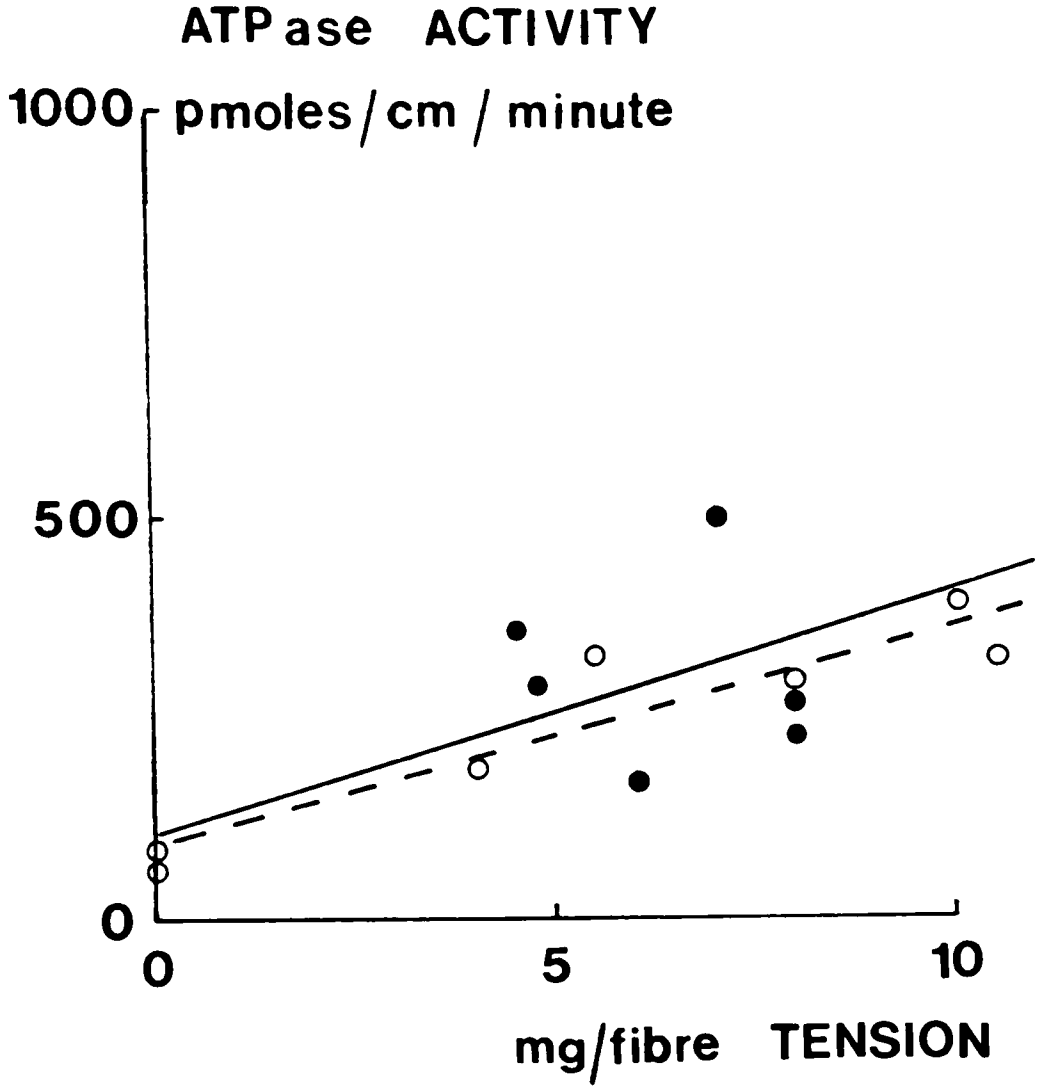


Figure 42b

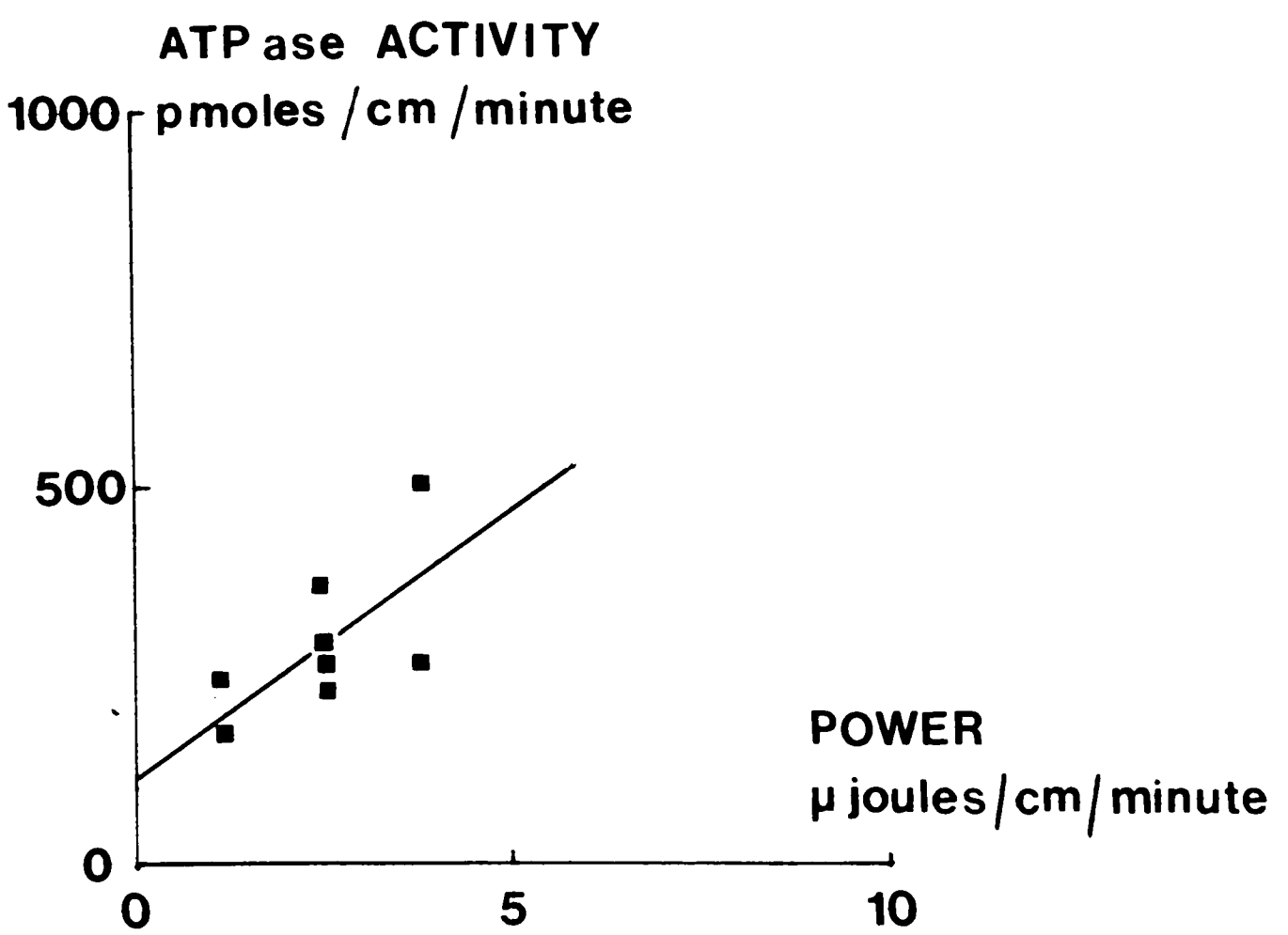


Figure 43 a Ionic strength 0·18 M

Relation between mechanical performance and ATPase activity of muscle.

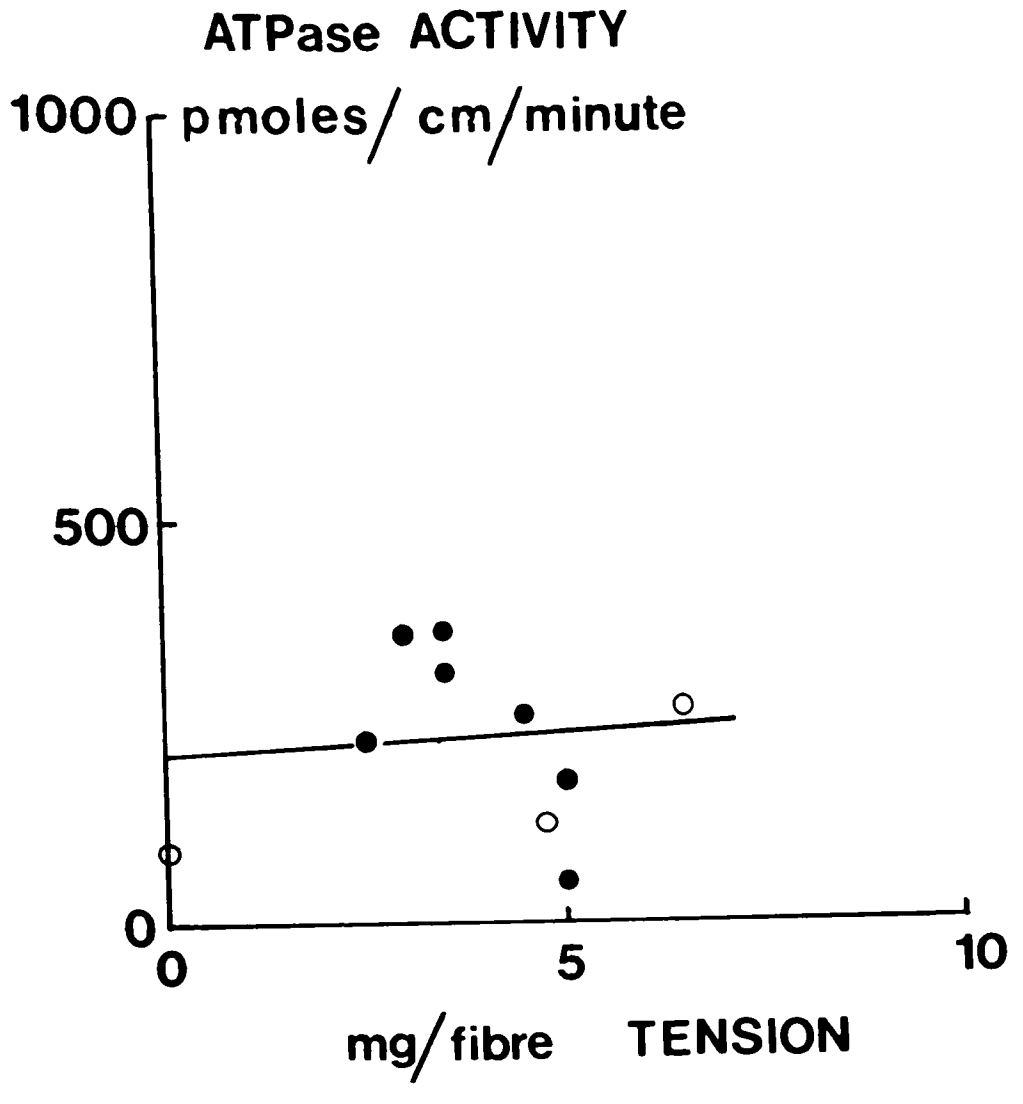


Figure 43 b

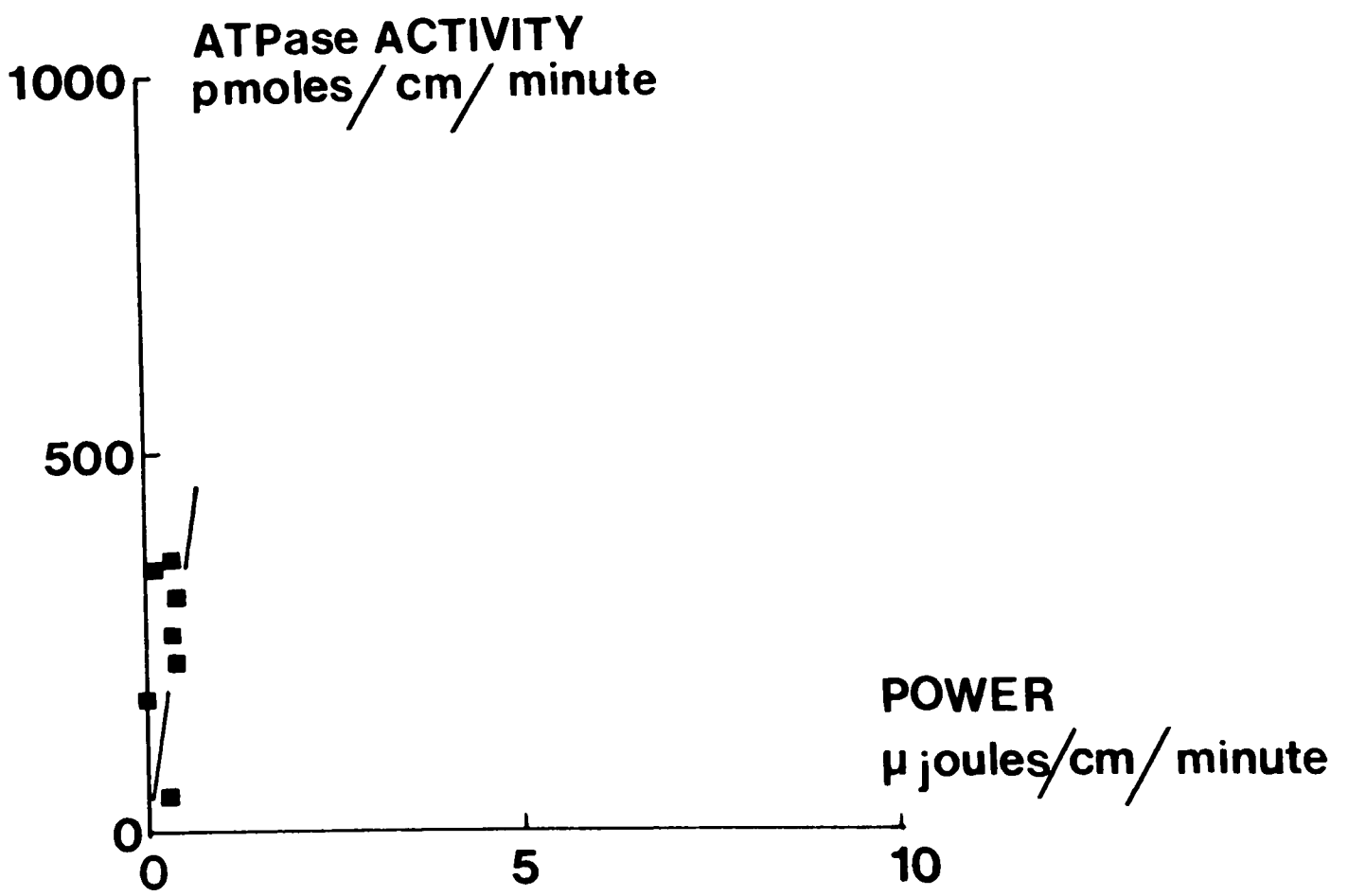


TABLE 21

Effect of ionic strength on ATPase activity of muscle

2% amplitude, 7 Hz

Regression coefficients and standard error

Ionic strength	Static ATPase/tension	Oscillatory ATPase/tension	ATPase/power
M	pmoles/mg	pmoles/mg	pmoles/μJoule
0.08	20.0 ± 7.1	44.2 ± 7.8	40 ± 11
0.09	19.1 ± 5.5	48.1 ± 9.8	70 ± 9
0.10	18.4 ± 8.8	30.4 ± 8.8	37 ± 14
0.11	44.8 ± 9.5	52.0 ± 12.5	113 ± 12
0.13	26.9 ± 4.7	29.5 ± 14.6	69 ± 32
0.18	No data	6.2 ± 29.3	97 ± 60

TABLE 22

Effect of ionic strength on ATPase activity of muscle

Intercepts

Ionic strength	Static ATPase/tension	Oscillatory ATPase/tension	ATPase/power
M	pmoles/cm/min	pmoles/cm/min	pmoles/cm/min
0.08	126 ± 111	164 ± 103	320 ± 84
0.09	110 ± 48	148 ± 88	186 ± 65
0.10	178 ± 156	215 ± 39	290 ± 121
0.11	67 ± 139	15 ± 193	-7 ± 97
0.13	94 ± 54	111 ± 145	114 ± 167
0.18	No data	211 ± 208	-99 ± 476

strength. The figures for ionic strength 0.11 lie out of the pattern but they are derived from a small amount of data from two experiments on fibres from different bugs and giving different results so they are subject to a large error. As the results obtained at 0.13 M ionic strength do not depart from the pattern at lower ionic strength it is unlikely that the results represent significant change in behaviour at 0.11 M. The only significant departure from the pattern is at very high ionic strength, 0.18 M. Very little work or tension was obtainable. As in the series of mechanical experiments, the damaging effect of incubation at ionic strength 0.18 M was observed. It was possible to make some observations on the ATPase activity of oscillating fibres but static muscles required incubations too long to be carried out.

The data on the mechanical performance of the fibres used for these chemical experiments was collected and analysed. It is tabulated below.

TABLE 23

Ionic strength	Optimum frequency	Maximum work/cycle	Frequency range
M	Hz	nJoules/cm/cycle	Hz
0.08	6.8	13.2	2.5 - 25.0
0.09	6.0	14.0	2.0 - 20.0
0.10	6.4	12.5	2.0 - 19.0
0.11	6.2	17.2	1.0 - 20.0
0.13	7.4	9.8	2.5 - 17.0
0.18	6.0	1.9	3.1 - 14.0

These results are derived from data less complete than that obtained using the computer on-line as far fewer frequencies are documented. However, from Table 23 it may be seen that the pattern is the same as that found in the mechanical experiments: except at 0.18 M

there is no change in frequency maximum, frequency range or maximum work per cycle with ionic strength.

On the evidence presented, therefore, except at very high salt concentrations variations in ionic strength do not affect the mechanical or chemical performance of muscle fibres.

5. The effect of various ions

(a) The omission of phosphate

In this section the results of experiments in which alterations or additions were made to the composition of the activating solution are presented.

All the results described in previous chapters were obtained using solutions buffered by phosphate. This buffer was not used by many workers as ATP hydrolysis rates could not be measured by analysis of phosphate produced in the presence of large excesses of buffering phosphate (e.g. Rüegg & Tregear, 1966). As a product of the hydrolysis of ATP, the effect of phosphate on muscle is of particular interest and more recently mechanical and chemical experiments have been performed to test this.

Rüegg et al., (1971) observed that 5 mm phosphate added to imidazole-buffered solutions reduced the isometric tension of fibres by about 50% without changing either the power output or the ATPase activity, so that the cost of isometric tension production was doubled in the presence of phosphate but the efficiency of power production was unchanged. The rise in tension of fibres following rapid step-changes in length was faster in the presence of phosphate and the speed of the isometric oscillations of tension approximately doubled. The optimum frequency of oscillation increased from 4 Hz to 6-7 Hz.

The conclusion that the presence of phosphate increases the speed of the contractile elements is supported by the work of White & Thorson (1972) who made many similar observations. They also studied qualitative differences in the response of fibres to step and oscillatory length changes in the presence and absence of phosphate and attributed the appearance of 'phosphate starvation tension transients' to the appearance of additional non-linearities of mechanical response in the absence of phosphate.

Thus there are many observations demonstrating that the performance of muscle differs in the presence and absence of phosphate.

For the experiments described below in which histidine buffer was used, the composition of the activating solution, computed using the Perrin programme, was as shown in Table 24.

TABLE 24

Species	Concentration
Mg-ATP	4.8 mM
Mg^{2+}	4.6 mM
Ca^{2+}	0.0015 mM
Histidine	20 mM
Ionic strength	0.081 M
pH	6.95
Temperature	$21^{o}C$

A large number of experiments were performed in which the ATPase activity of muscle was measured under isometric and oscillatory conditions and at 2% and 0.2% amplitude of oscillation. Many observations of mechanical performance were also made. Some involved use of the PDP8-I computer on-line.

Effect of frequency on power production and work output of fibres.

Histidine buffer.

Figure 44 a **2·0 % amplitude**

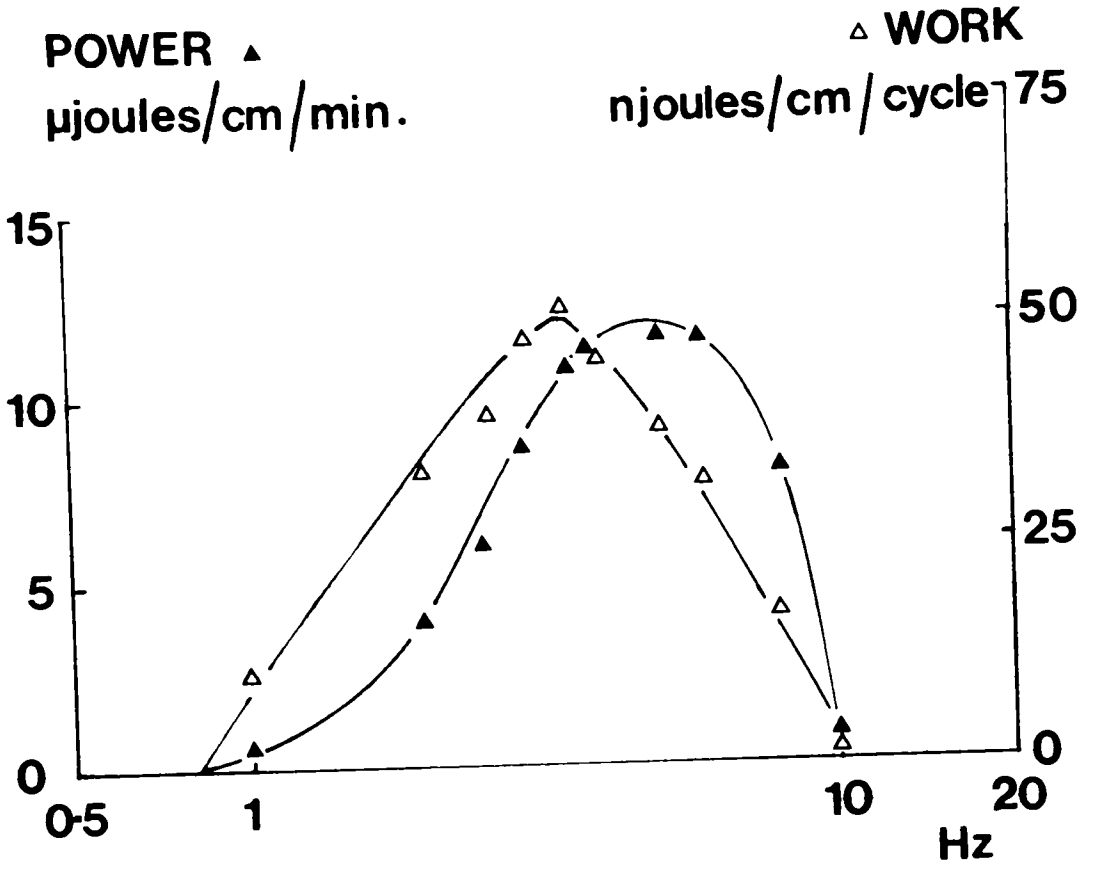


Figure 44 b **0·2 % amplitude**

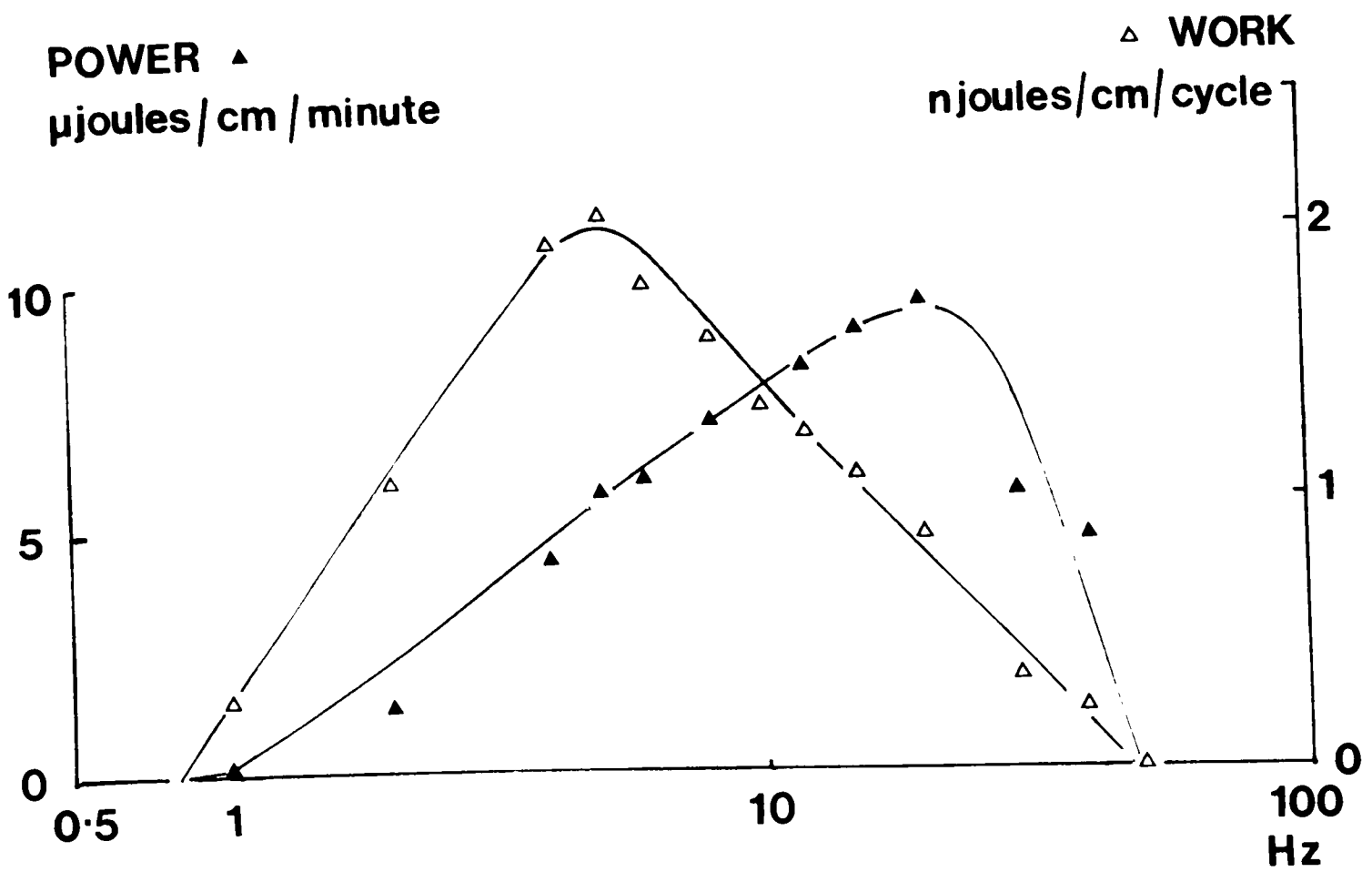
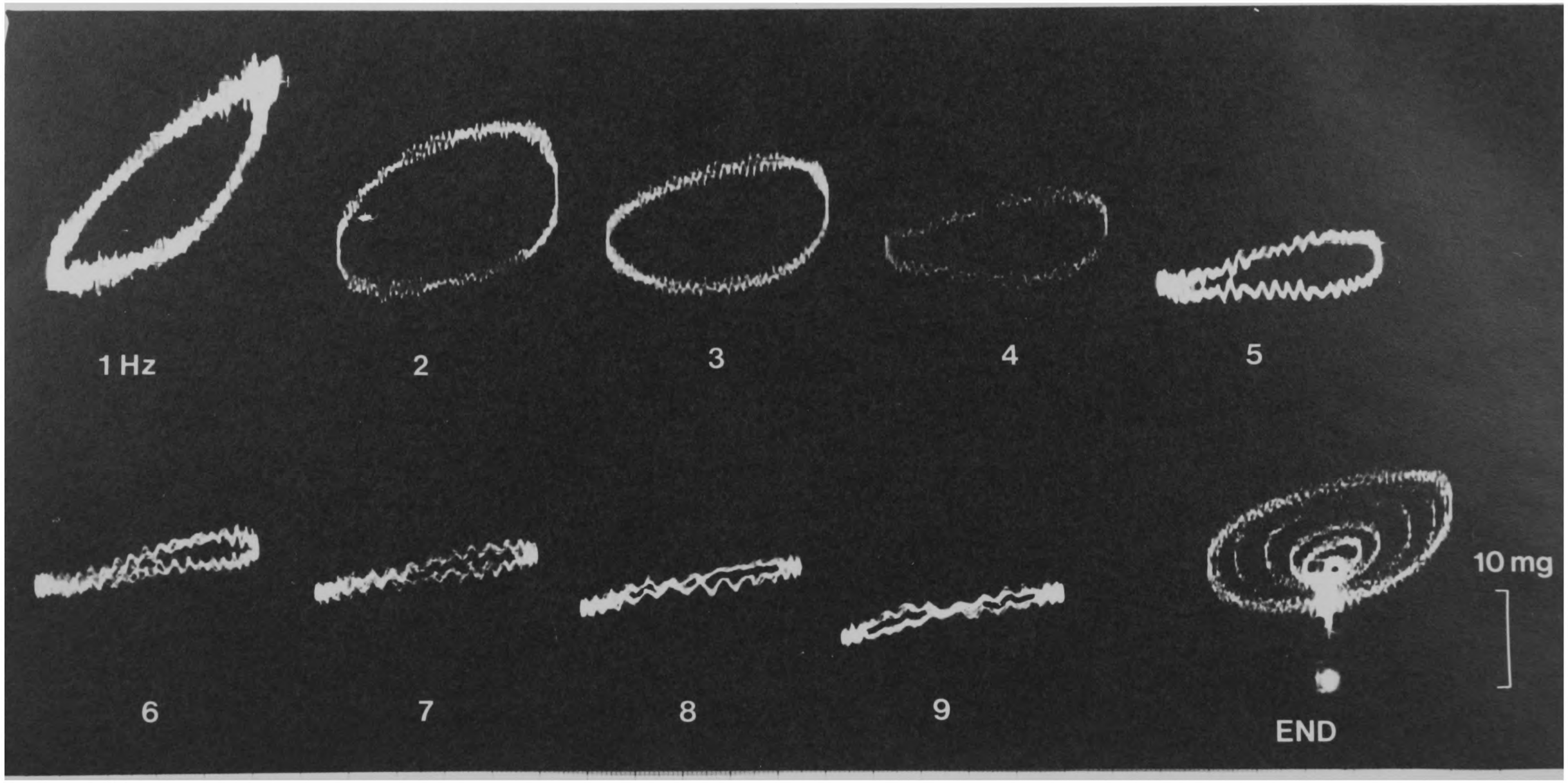


Figure 45

Effect of frequency on mechanical performance of muscle.

Histidine buffer.



2% amplitude

TABLE 25

Buffer	Oscillation amplitude	f_{max}	Maximum work/cycle	Working frequency range
	%	Hz	nJoules/cm/cycle	
Histidine	2	3.5	50.0	0.8 - 10.5
Phosphate	2	6.8	27.0	2.0 - 18.0
Histidine	0.2	7.5	2.0	1.0 - 55.0
Phosphate	0.2	10.0	0.8	1.6 - 64.0

The substitution of histidine buffer for phosphate had a marked and characteristic effect on the mechanical performance of muscle.

Figure 44 is a graph of the frequency dependence of power and work production of muscle fibres incubated in histidine and oscillated at 0.2% and 2.0% amplitude. By comparison of these results with those of similar experiments on fibres in phosphate, as in Fig. 16, it can be seen that optimum work per cycle, and thus maximum power output, occur at a lower frequency at each amplitude in histidine than in phosphate. The maximum work per cycle is much larger in histidine, compensating for the lower frequency at which it occurs, so that in both buffers the maximum power outputs observed are similar - around 10 μjoules/cm/min.

The mechanical performance in histidine is illustrated in Fig. 45 by a series of length-tension loops. As shown in Fig. 44 maximum work per cycle occurs at a much lower frequency of oscillation in histidine (2 Hz) than in phosphate (7 Hz). Power output begins to decline above 4 to 5 Hz and no power could be obtained above 8 to 11 Hz, at which frequency the loop routinely, as opposed to occasionally, has the form of a figure-of-eight. The mean tension of the muscle when producing oscillatory work is much higher than that in phosphate, and this tension is much further displaced (upward in the case of a work producing muscle, downward in the case of a work absorbing muscle) from the tension present before oscillation. These effects are the same as those reported by White & Thorson (1972). When the oscillation amplitude is quickly reduced to zero the tension changes are characteristic of the buffer in which the muscle is placed.

In Table 25, the results of an experiment are given in which one set of fibres was incubated in each buffer in turn. The effects described above are quantified.

Relation between mechanical performance and ATPase activity of muscle.

Figure 46 a Histidine buffer 2% amplitude

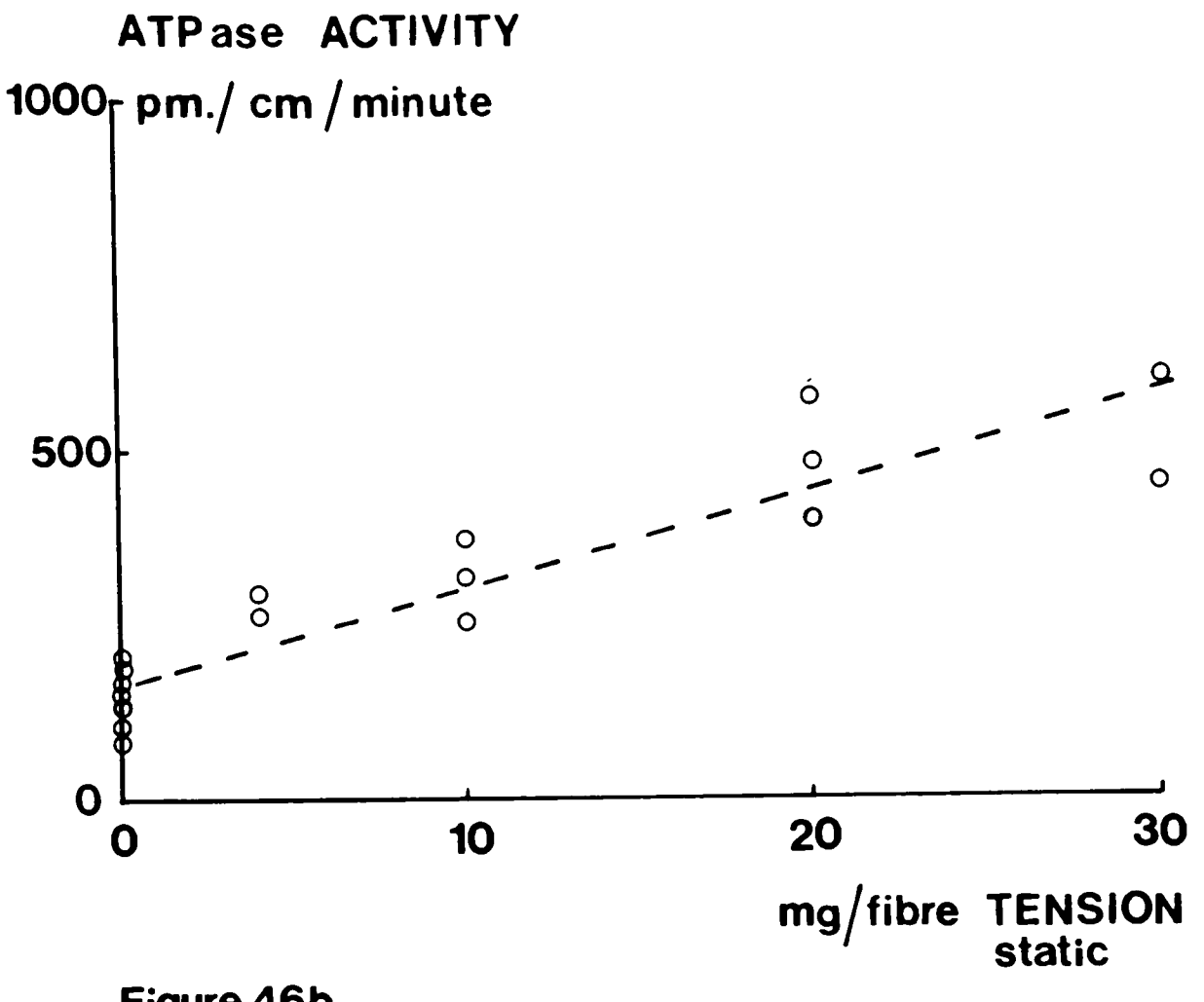


Figure 46b

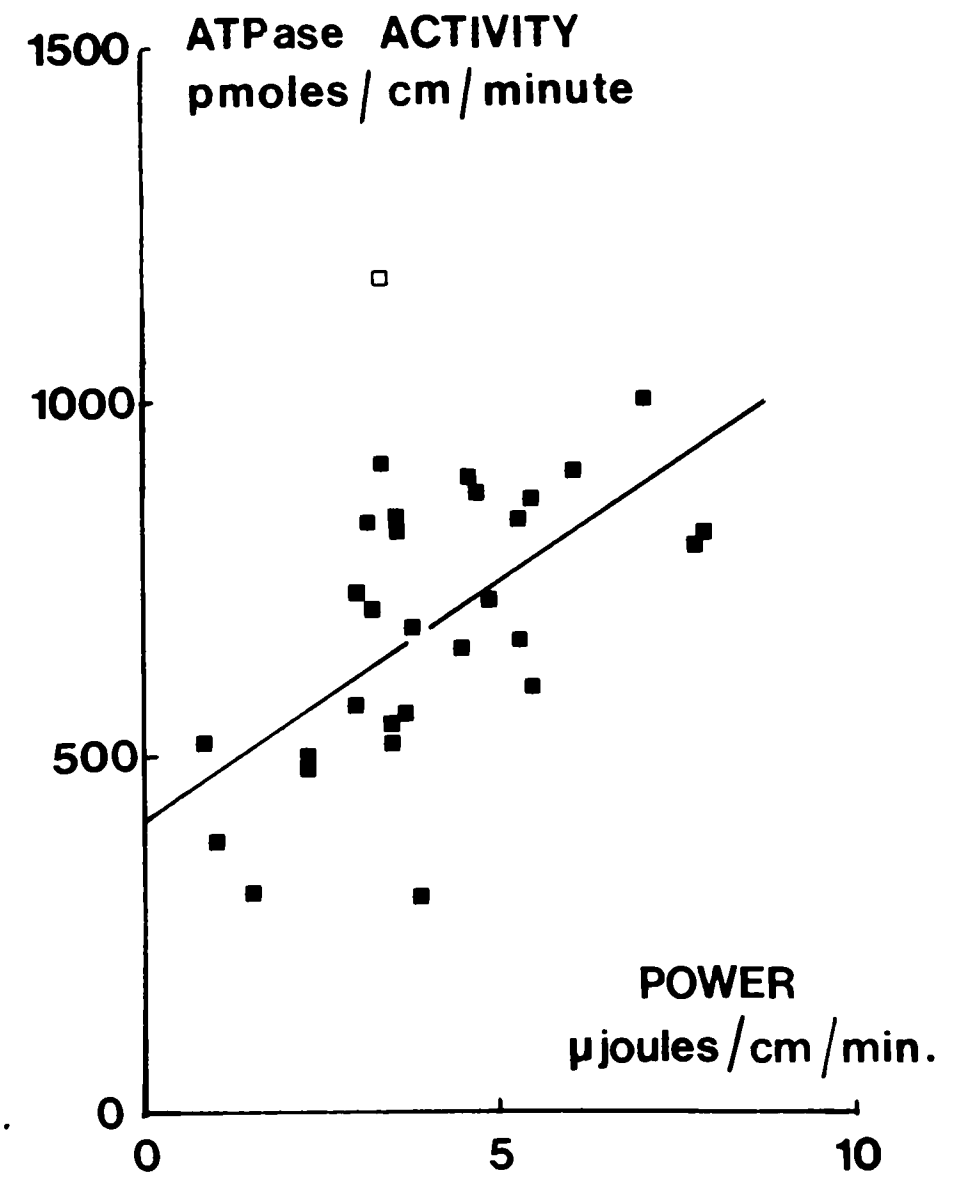
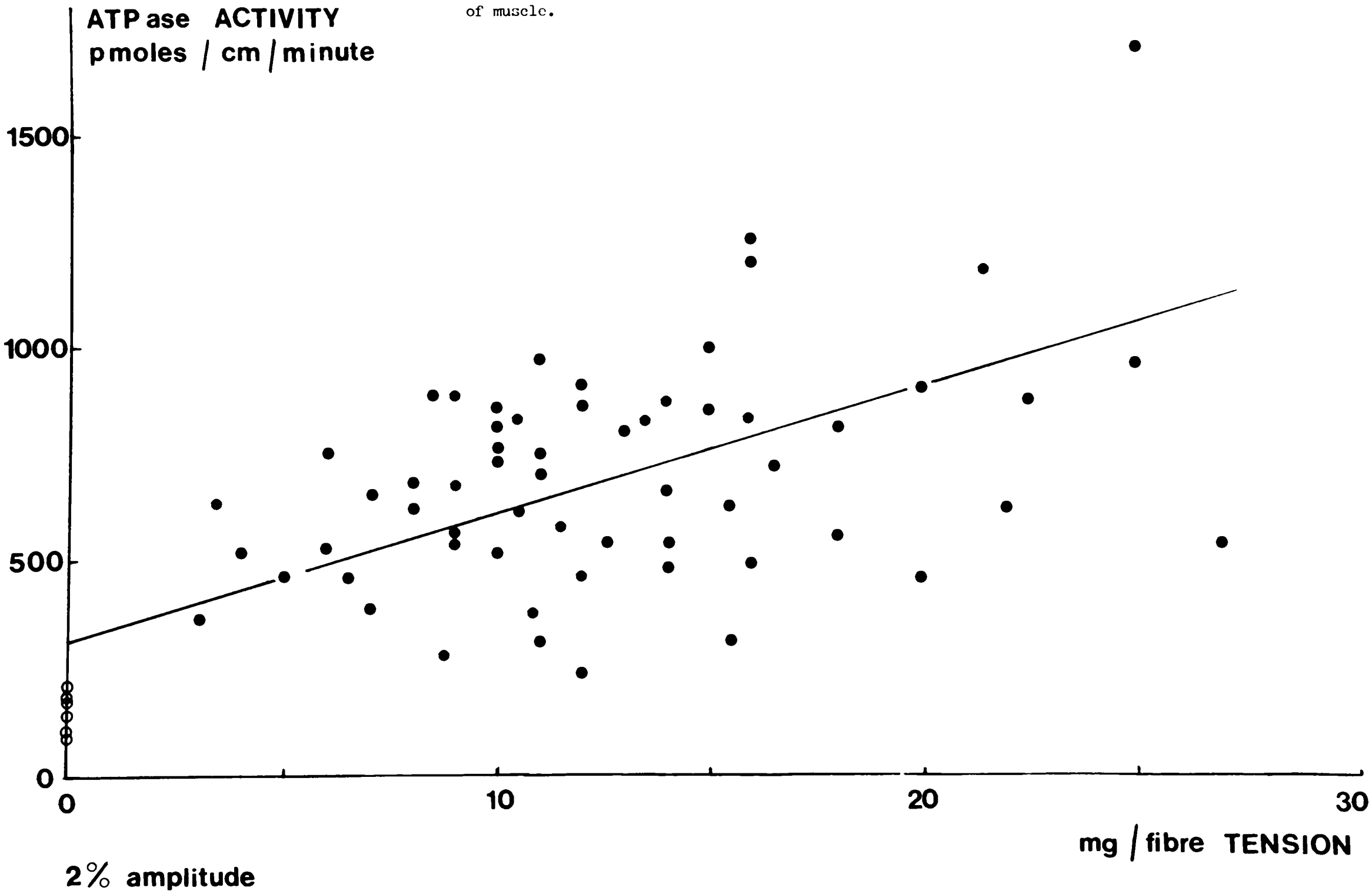


Figure 47 Histidine buffer Relationship between mechanical performance and ATPase activity of muscle.



Relationship between mechanical performance and ATPase activity of muscle.

Figure 48 a

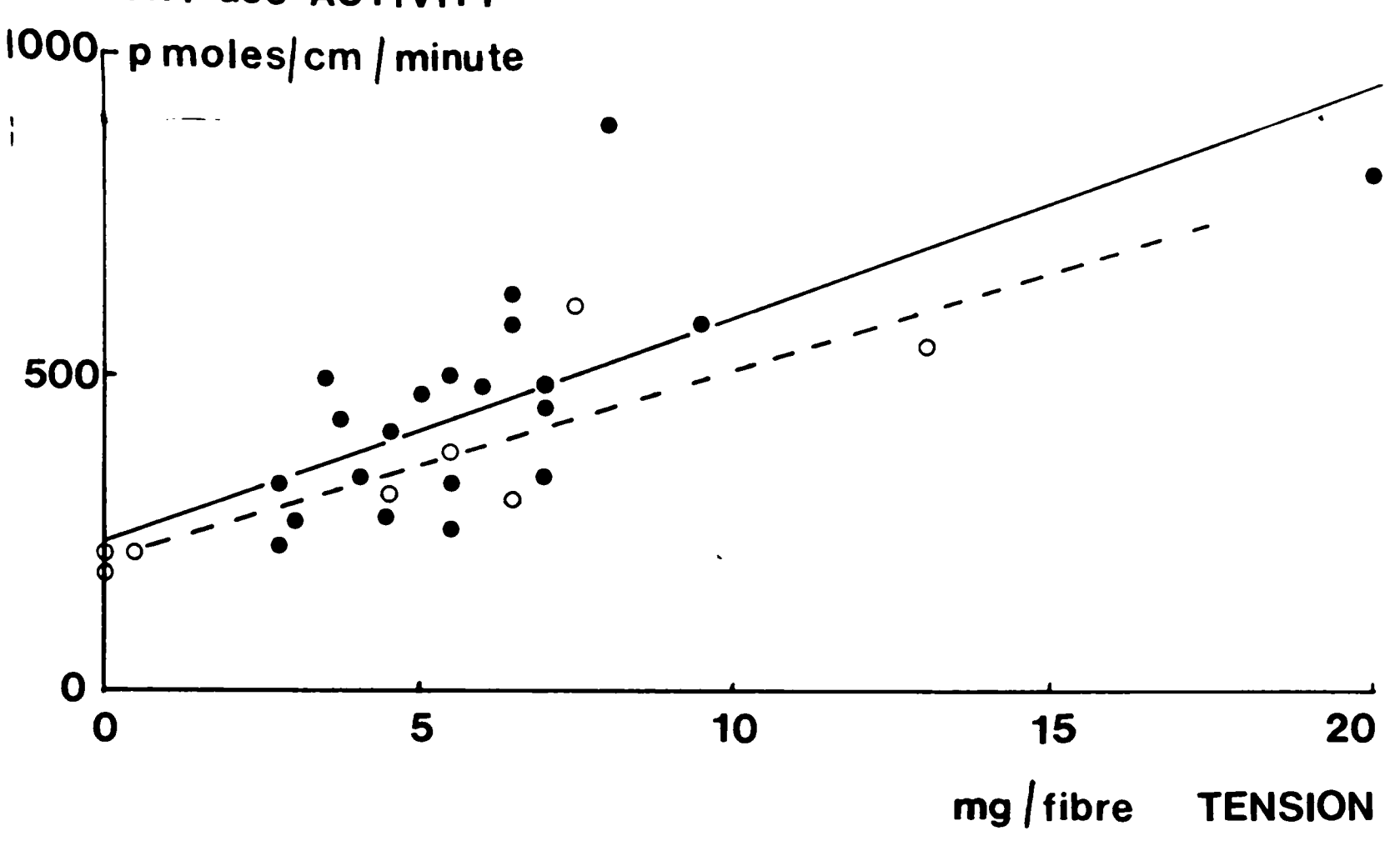


Figure 48b

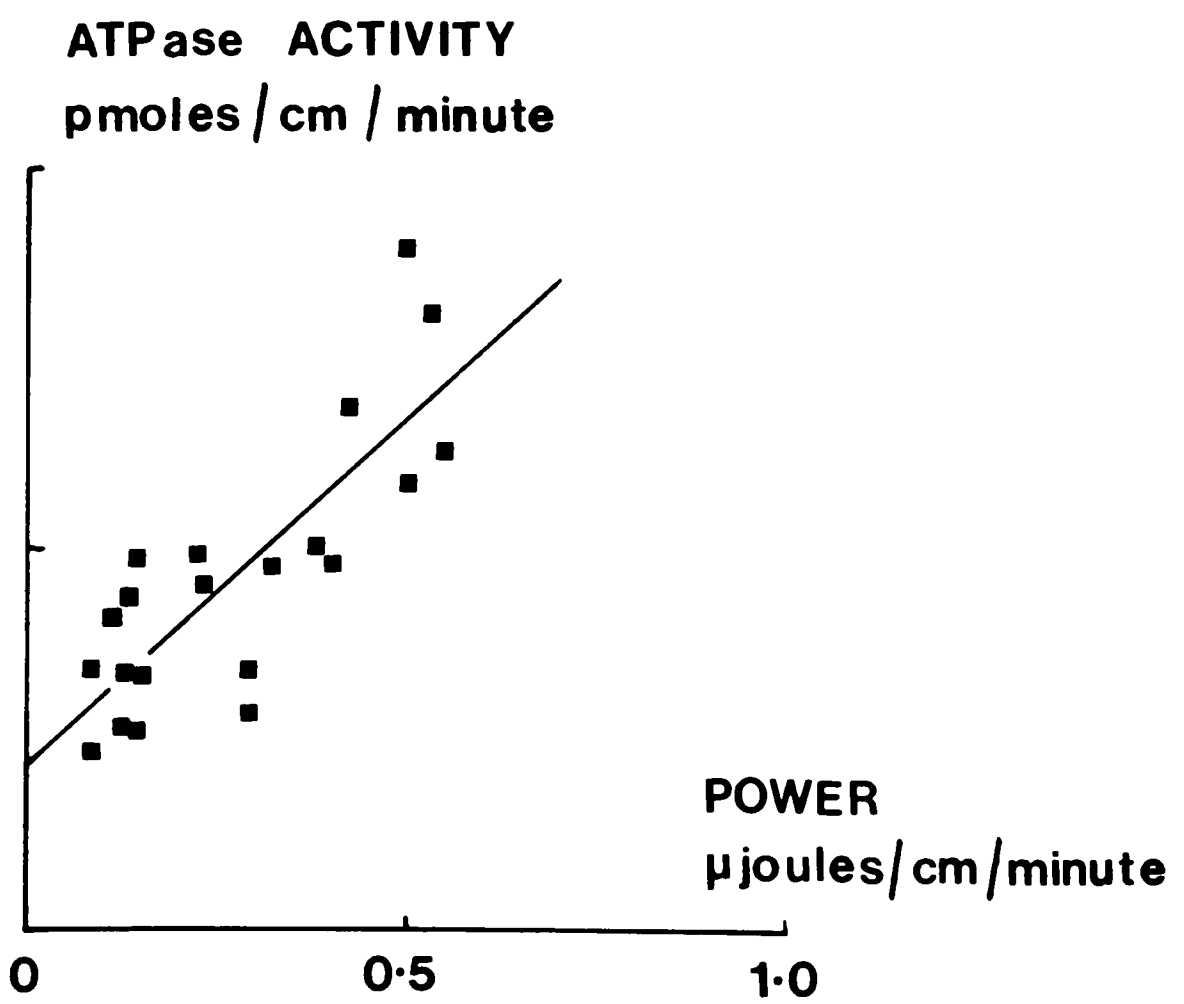


TABLE 26

Effect of the presence and absence of phosphate on

ATPase activity. Regression coefficient and standard error

Buffer	Oscillation amplitude	Static ATPase/ tension	Oscillatory ATPase/ tension	ATPase/ power	Optimum frequency of muscle	
	%	pm/mg	pm/mg	pm/μJoule	2%	0.2%
Histidine	0.2	29.5 ± 7.4	35.0 ± 6.8	89.2 ± 149	4.0	6.5
Histidine	2.0	13.7 ± 1.6	29.4 ± 4.8	66.0 ± 16.0	2.0	?
Phosphate	2.0	49.4 ± 4.0	89.1 ± 9.7	72.0 ± 14.0	7.6	?

TABLE 27

Effect of presence and absence of phosphate

Intercepts of regression lines

Buffer	Amplitude	Static ATPase/tension	Oscillatory ATPse/tension	ATPase/power
	%	pm/cm/min	pm/cm/min	pm/cm/min
Histidine	0.2	211 ± 73	236 ± 79	221 ± 85
Histidine	2.0	170 ± 31	314 ± 109	411 ± 135
Phosphate	2.0	140 ± 34	101 ± 113	327 ± 126

ATPase activity was measured for fibres incubated statically and oscillating at 2% and 0.2% amplitude. The results of the experiments are shown in Figs. 46-48 and in Tables 26 and 27. The results of the experiments on phosphate buffer described in Chapter 3 are included for comparison.

The experiments at 2% amplitude were performed using, in part, muscles which were also used for the ATPase activity investigations on phosphate-buffered fibres described in Chapter 3. These results are therefore directly comparable with each other. The experiments at low-amplitude were done separately. The mechanical data included in the table shows that the muscle used for the low amplitude experiments had a higher optimum frequency at 2% than those used in the high amplitude work. Therefore the results should not be compared directly.

At 2% amplitude, but not at 0.2%, the cost of tension production rises when the fibres are subjected to oscillatory length changes. Such a difference in tension cost betwen static and oscillating muscle has been demonstrated in the previous chapters to occur at 2% amplitude in almost all cases examined.

Comparison of the regression coefficients obtained in these experiments with those from experiments performed in phosphate buffer shows that at 2% amplitude the use of histidine buffer reduces the cost of tension production in both oscillating and isometric muscles, but the efficiency of power production is not significantly changed. At 0.2% the increased frequency response does not wholly compensate for the reduction in work per cycle at low amplitude so that power outputs are approximately one-tenth those at 2.0%. The mean tension required to optimise power output at low amplitude is similar to that of muscles at 2%, so the efficiency of power output is very low compared with the efficiency observed under a wide range of conditions at high amplitude.

Substitution of histidine for phosphate therefore has a variety of effects on the activity of muscle fibres. There are characteristic qualitative changes in the mechanical performance and also quantitative changes. The reduction in frequency response which is shown at both 2% and 0.2% is accompanied by a reduction in the cost of tension production, isometric and oscillatory, at 2%. Efficiency of power production is not affected.

The results obtained here are in accord with those observed by other workers.

Evidence shows that the changes arise as a consequence of the absence of phosphate from the medium rather than because histidine is present. White & Thorson (1972) found that mechanical responses typical of phosphate were found when the incubating medium contained both buffers in concentrations above 5 mM.

When the concentration of phosphate was reduced from 5 mM to zero in the presence of 10 mM histidine the non-linearities characteristic of histidine gradually appeared.

The concentration of phosphate buffer used in the experiments described in Chapter 3 was 10 mM, so on the evidence above the full effect of the presence of phosphate would be seen.

The contrast in performance is observed when phosphate is exchanged for buffers other than histidine. Rüegg et al., (1971) used imidazole buffer and White (personal communication) has reported that in Tris buffer the performance is similar to that in histidine and not phosphate.

Some mechanical experiments were performed in which piperazine-N,N'-bis-2-ethane-sulphonic acid buffer (PIPES) was used in place of either phosphate or histidine. The overall performance resembled that of fibres in histidine rather than in phosphate. The frequency response was low and at the highest frequencies at which work was obtained

figure-of-eight loops regularly occurred. Rapid reduction in amplitude of oscillation from 2% to zero resulted in the spiralling reduction of tension characteristic of histidine buffer.

TABLE 28

Buffer	Amplitude	f_{max}	Maximum work/cycle	Frequency range
		Hz	nJoules/cm/cycle	Hz
Histidine	2%	2.5	20.2	0.5 - 6.8
PIPES	2%	2.8	1.2	1 - 12.0
Phosphate	2%	6.8	11.0	0.5 - 20

This evidence and that of White & Thorson (1972) demonstrates that the difference in mechanical performance in histidine and phosphate is a consequence of the absence of phosphate.

Analysis of fibrillar flight muscle of Phormia has shown that it contains 8 mM inorganic phosphate(Sacktor & Hurlbut, 1966); estimates for rabbit muscle are similar (Potop et al., 1966). What percentage of this phosphate is free and how much bound is not known, but it is clear that living muscle contains millimolar quantities of phosphate whereas the concentration of free histidine, for instance, is very small. So it is probable that the phosphate buffer is more physiological than histidine or other solutions, and therefore the properties of the muscle in phosphate are more likely to resemble those observed in vivo.

The discrepancy that has been observed between the mechanical response of fresh flight muscle and its glycerinated model must be reviewed in the light of this observation. It has long been recorded that the optimum frequency of power output of glycerinated fibres is much lower than the natural wing beat frequency of L. cordofanus which is about 30 Hz (Jewell & Rüegg, 1966). The discrepancy is reduced if high ATP concentrations (Steiger & Rüegg, 1969) and a temperature

similar to that of the thoracic cavity during flight - 40°C - (Pringle & Tregear, 1969) are employed. Another factor which would bring the response of the glycerinated preparation into line with that of live muscle would be the substitution of phosphate buffer for histidine or Tris which was used by the authors quoted above. Clearly the more physiological the conditions in which glycerinated fibres are tested, the more their performance resembles that of living muscle in vivo.

(b) The effect of sulphate

Sulphate has been suggested as a possible analogue to phosphate in biological systems. To see whether sulphate ions produced any of the known effects of phosphate ions on muscle, 10 mM potassium sulphate was dissolved in normal histidine activating solution and, as a control, also in phosphate solution. The composition of the solutions is tabulated below.

TABLE 29

Species	Concentration	
	Phosphate	Histidine
Mg-ATP	4.5 mM	4.7 mM
Ca^{2+}	0.0016 mM	0.0015 mM
KSO_4^-	3.5 mM	1.8 mM
$NaSO_4^-$	0.23 mM	0.26 mM
$MgSo_4$	1.76 mM	2.76 mM
Ionic strength	0.11 M	0.094 M
pH	6.95	6.95
Temperature	21°C	21°C

Mechanical and chemical experiments were performed to elucidate the effect of sulphate.

The computer was used on-line to control mechanical experiments. The results of experiments performed concurrently on a phosphate-buffered

solution containing no sulphate and of ionic strength similar to that of the phosphate-sulphate solution are included for comparison. The indices of mechanical performance are shown in Table 30.

TABLE 30

Solution	Oscillation amplitude	Optimum frequency	Maximum work/cycle	Working frequency range
	%	Hz	nJ/cm/cycle	Hz
P + SO_4	2.0	7.8	22.0	0.7 - 33.0
Hist. + SO_4	2.0	1.1	10.0	0.25 - 13.0
Phosphate	2.0	6.8	23.4	0.3 - 34.0
P + SO_4	0.2	10.8	0.72	1.2 - 38.0
Hist. + SO_4	0.2	2.7	0.38	0.3 - 16.0
Phosphate	0.2	10.5	0.86	0.5 - 38.0

From the tabulated results and observation of length-tension loops, there is no significant change in mechanical performance when sulphate is added to phosphate buffer.

The addition of sulphate to histidine buffer, however, has a profound and reversible effect on performance. The fibres continue to behave in a manner typical of the absence of phosphate, i.e. figure-of-eight loops at the frequencies at the top of the working range and the characteristic spiral reduction in tension when amplitude is rapidly reduced to zero. The frequency response of the fibres is affected by sulphate as the figures in the table show. The range of frequencies and the optimum frequency for work production are both reduced.

The results of measurements of ATPase activity in the presence of 10 mM sulphate are shown graphically in Figures 49 and 50. The regression coefficients and associated statistics derived from the data are given in Tables 31 and 32.

Figure 49a P_i + sulphate

Relation between mechanical performance and ATPase activity of muscle.

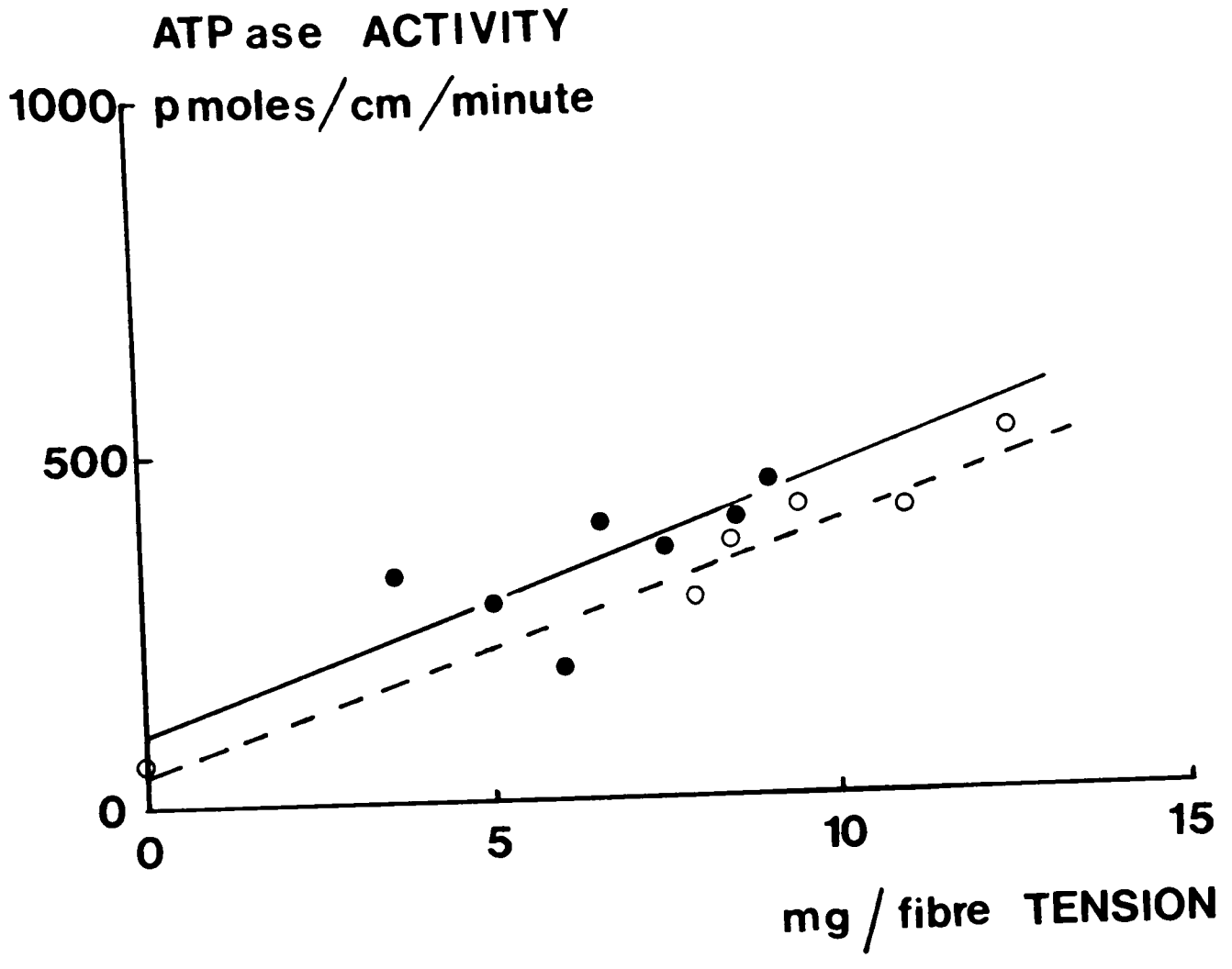


Figure 49b

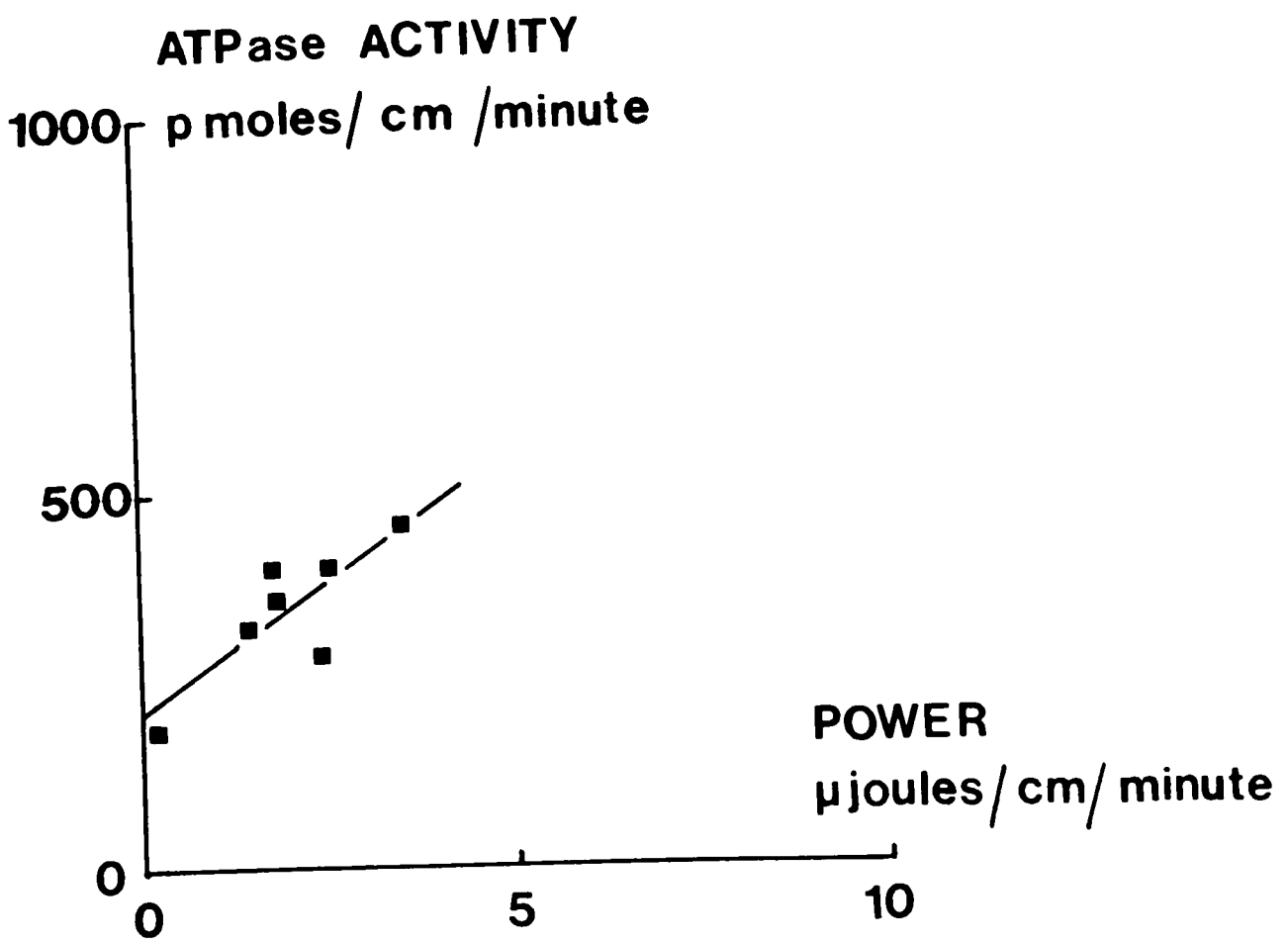


Figure 50a Histidine + sulphate

Relation between mechanical performance and ATPase activity of muscle.

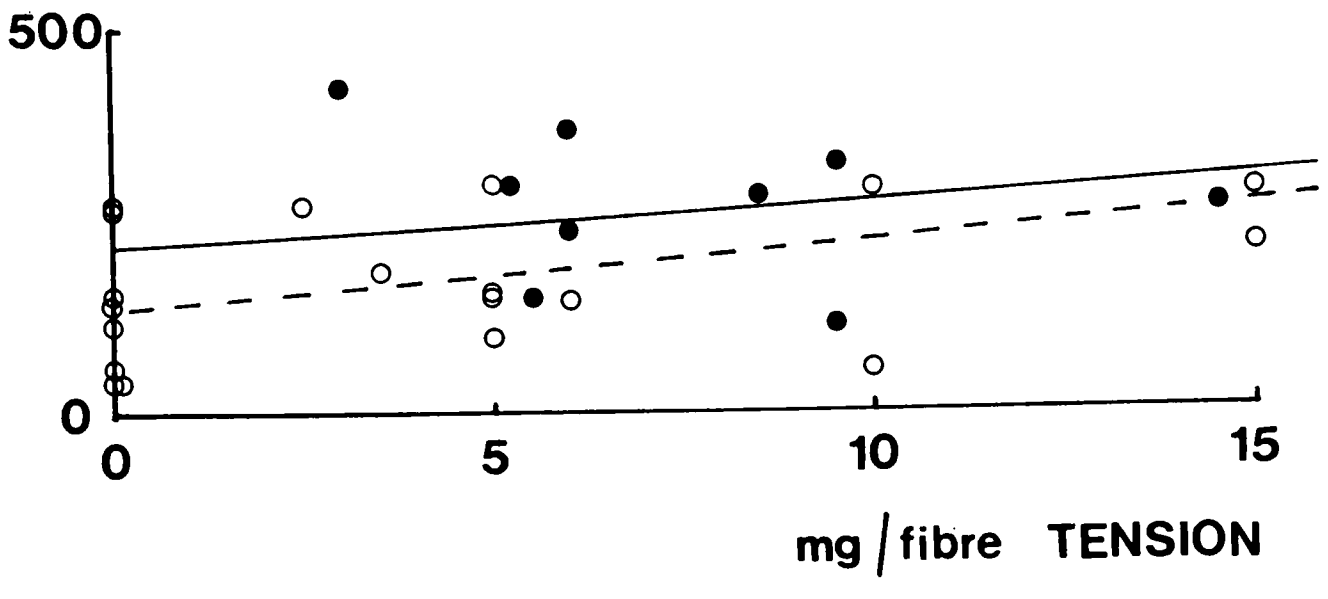


Figure 50b

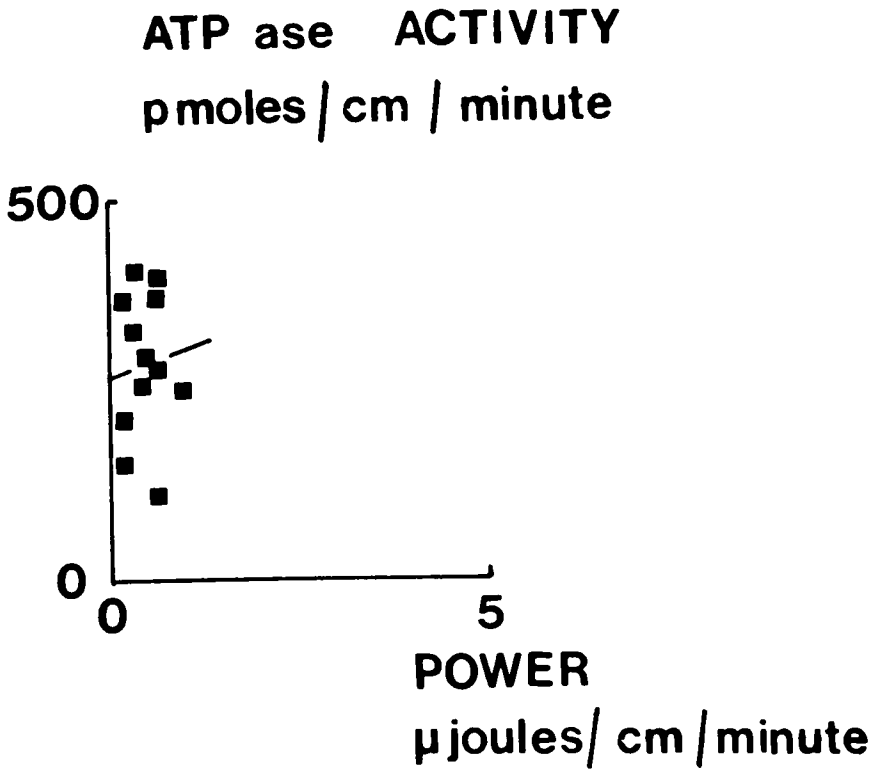


TABLE 31

Regression coefficients and errors,

tension cost and power cost, Figs. 49 & 50

Solution	Static tension cost	Oscillatory tension cost	Power cost
	pmoles/cm/min/mg	pmoles/cm/min/mg	pmoles/cm/min/μJoule
P + SO_4	35.2 ± 3.5	36.2 ± 10.4	69.0 ± 21.0
H + SO_4	5.7 ± 3.8	4.6 ± 10.3	59.0 ± 87.0
Phosphate	19.1 ± 5.5	49.1 ± 9.8	70.0 ± 9.0

TABLE 32

Intercepts of regression lines

Solution	Static tension cost	Oscillatory tension cost	Power cost
	pmoles/cm/min	pmoles/cm/min	pmoles/cm/min
P + SO_4	47 ± 33	106 ± 126	210 ± 86
H + SO_4	147 ± 31	222 ± 146	267 + 67
Phosphate	110 ± 48	148 ± 88	186 ± 65

From the results it can be seen that, as in the mechanical case, sulphate in the presence of phosphate has no observable effect on muscle. Cost of tension and power production are not significantly changed.

When sulphate is added to histidine buffer, there is a reduction in frequency response compared with that observed in sulphate-free histidine. There is also a change in the chemical performance of the muscle. From the figures in Table 31 a very low cost of tension production is found in both isometric and oscillatory conditions. In the latter case the slope of the regression line is not significantly different from zero.

Power production was greatly reduced so that the points are not sufficiently spread for a meaningful line to be drawn through them. Nevertheless the graph in Fig. 50b shows that the power, though very low, was not more inefficiently produced than in the absence of sulphate.

The experiments on the ATPase activity of fibres in the presence of sulphate were done on fibres different from those used for the mechanical study. However the performance of the fibres used for chemical experiments was examined and in both cases the effects of sulphate were similar, i.e. no effect on phosphate and a reduction of the frequency response in histidine. The figures are tabulated below.

TABLE 33

Buffer	Optimum frequency 2%	Maximum work/cycle	Frequency range
	Hz	nJ/cm/cycle	Hz
P + SO_4	6.2	10.5	1.1 - 29.0
H + SO_4	0.9	8.4	0.1 - 7.0

The addition of sulphate to phosphate buffered activating solution has no observable effect on the fibres. When it is added to histidine there is a significant effect but it in no way resembles the effect produced by addition of phosphate. F_{max} and cost of tension production are both reduced instead of being increased.

From the evidence presented it is clear that sulphate does not imitate the action of phosphate as it does not increase the speed of contraction, but reduces it.

(c) The effect of pyrophosphate

Pyrophosphate is one of the many phosphate compounds which have been used to mimic the action of ATP. It has long been known as a plasticiser of muscle. In muscle fibres and actomyosin systems it can bring about breakage of the actomyosin link, apparently by attaching at the hydrolytic site on the myosin bridge in place of ATP (Nauss et al., 1969; White, 1970). However the actions of ATP and pyrophosphate are not completely analogous. When bound to myosin they produce different changes in the secondary and tertiary structure of the protein as revealed by optical rotatory dispersion studies (Tonomura et al., 1963). Pyrophosphate is not split subsequent to attachment as is ATP (Lymn & Taylor, 1971) and although it relieves rigor the high frequency dynamic stiffness of glycerinated fibrillar muscle is not greatly reduced by pyrophosphate relaxation as it is by ATP (White, 1970).

Recent analysis of the optical transforms of E.M.s of pyrophosphate-relaxed muscles (Beinbrech et al., in preparation) supports the hypothesis that pyrophosphate, while able to induce detachment, cannot, because it is not hydrolysed to provide energy, return the crossbridge to its commonly observed ATP relaxed position, 90° to the filament. Consequently the bridges remain angled, giving rise to a diffraction picture with

characteristics of both the relaxed and rigor patterns. Bridges so positioned could cause increased viscosity, leading to a large high frequency dynamic stiffness but not changing the low frequency or static stiffness, i.e. isometric tension, from the normal relaxed value. Polarisation studies give similar results (dos Remedios et al., 1972). The action of pyrophosphate is therefore of considerable interest and has been little studied for the actively contracting muscle.

Experiments were performed to test the effect of pyrophosphate on activated muscle.

It was found that the calcium in activating solution made inclusion of large concentrations of pyrophosphate impossible as calcium pyrophosphate is extremely insoluble. Solutions could be made not more than 1.5 mM with respect to pyrophosphate. The exact composition of solutions is given in Table 34.

TABLE 34

Species	Concentration	
	P_i buffer	Histidine buffer
Mg-ATP	4.6 mM	4.8 mM
Ca^{2+}	0.0014 mM	0.0015 mM
PP^{4-}	0.115 mM	0.075 mM
$Mg\text{-}PP^{2-}$	0.852 mM	0.921 mM
$MgH\ PP^{-}$	0.364 mM	0.393 mM
Ionic strength	0.105 M	0.081 M
pH	6.95	6.95
Temperature	21°C	21°C

Experiments in which power, tension and ATPase activity were measured were performed. The mechanical performance was also examined.

Relation between mechanical performance and ATPase activity of muscle.

Figure 51 a

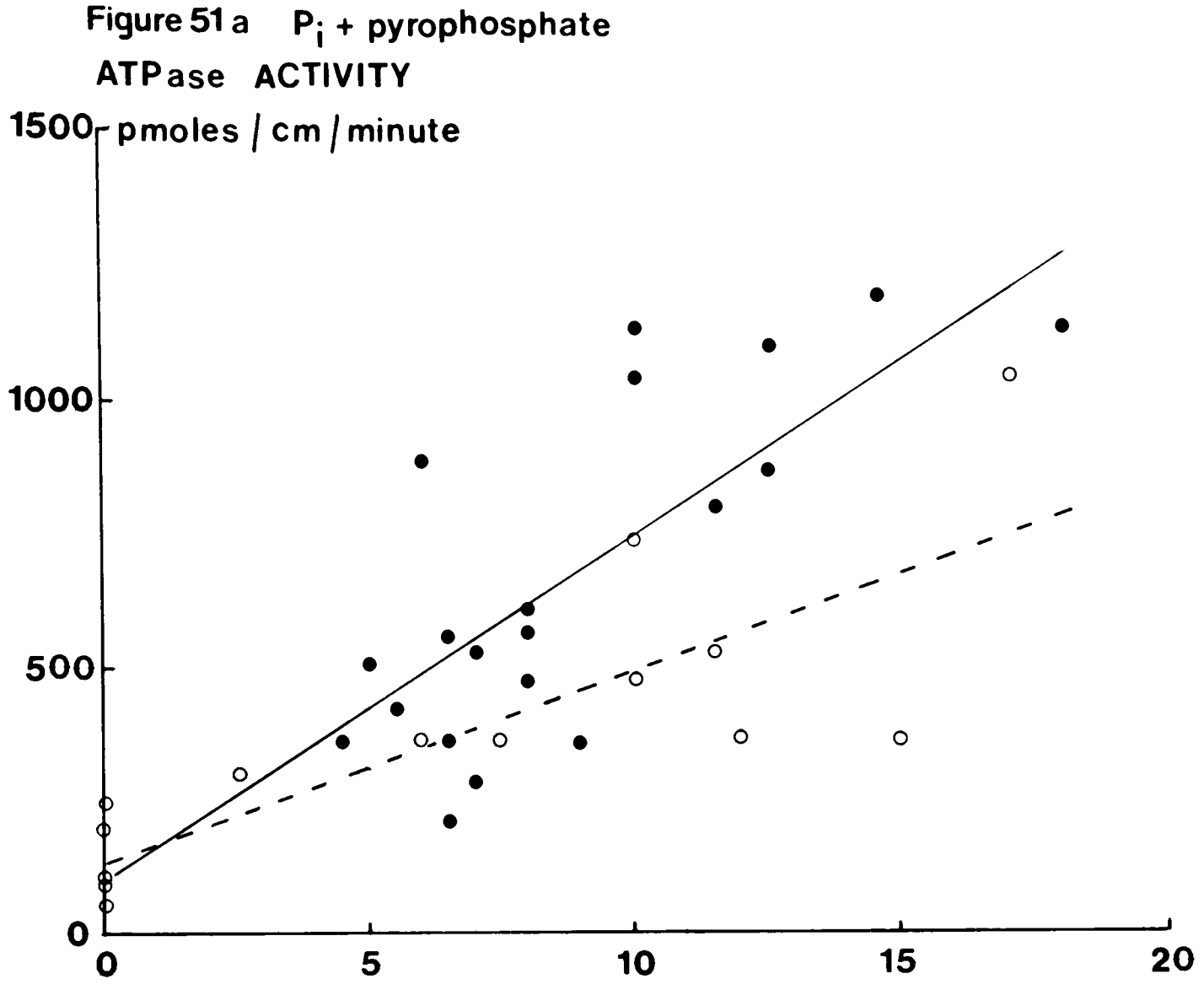


Figure 51b

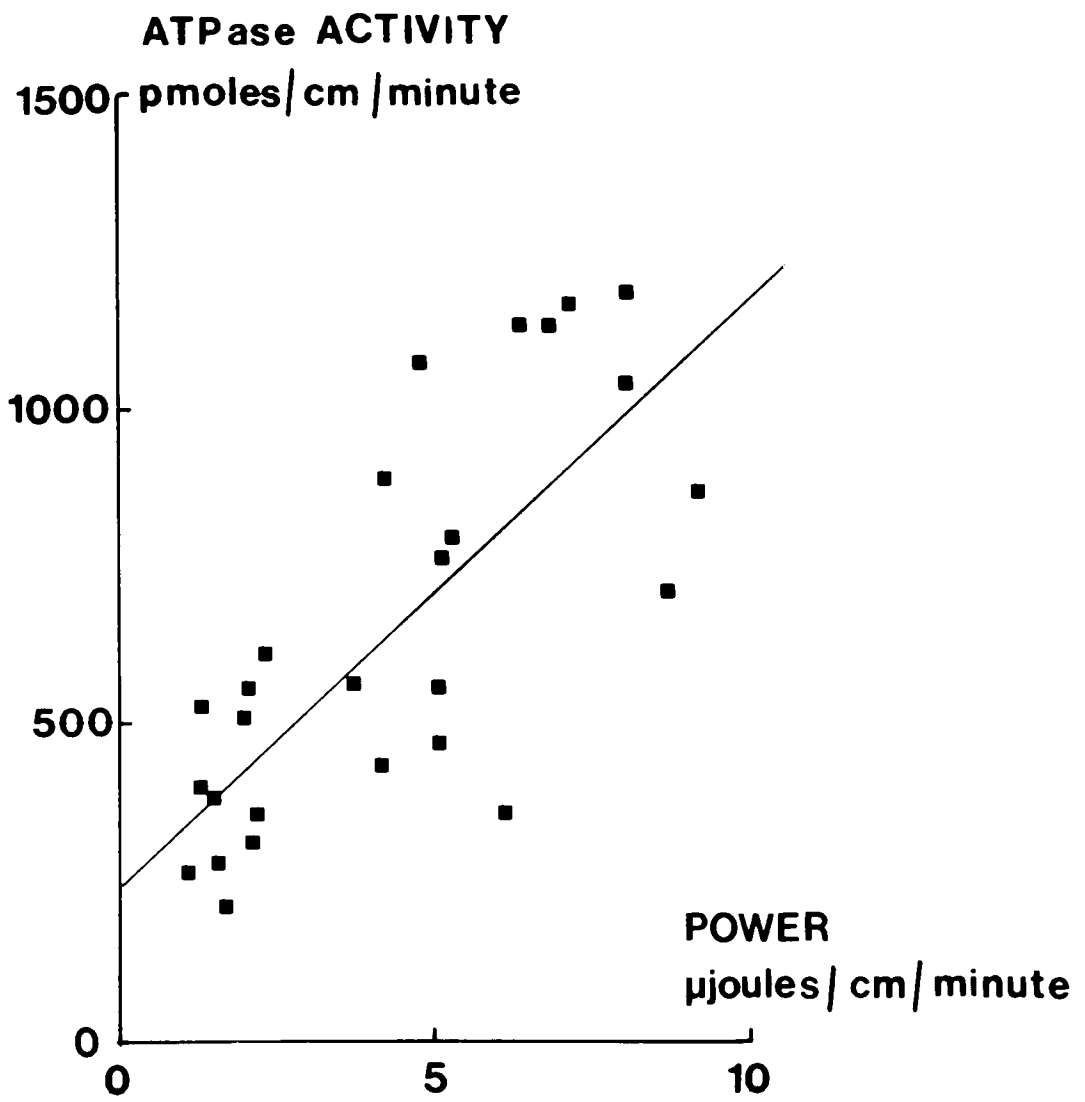


Figure 52 a Histidine + pyrophosphate

Relation between mechanical performance and ATPase activity of muscle.

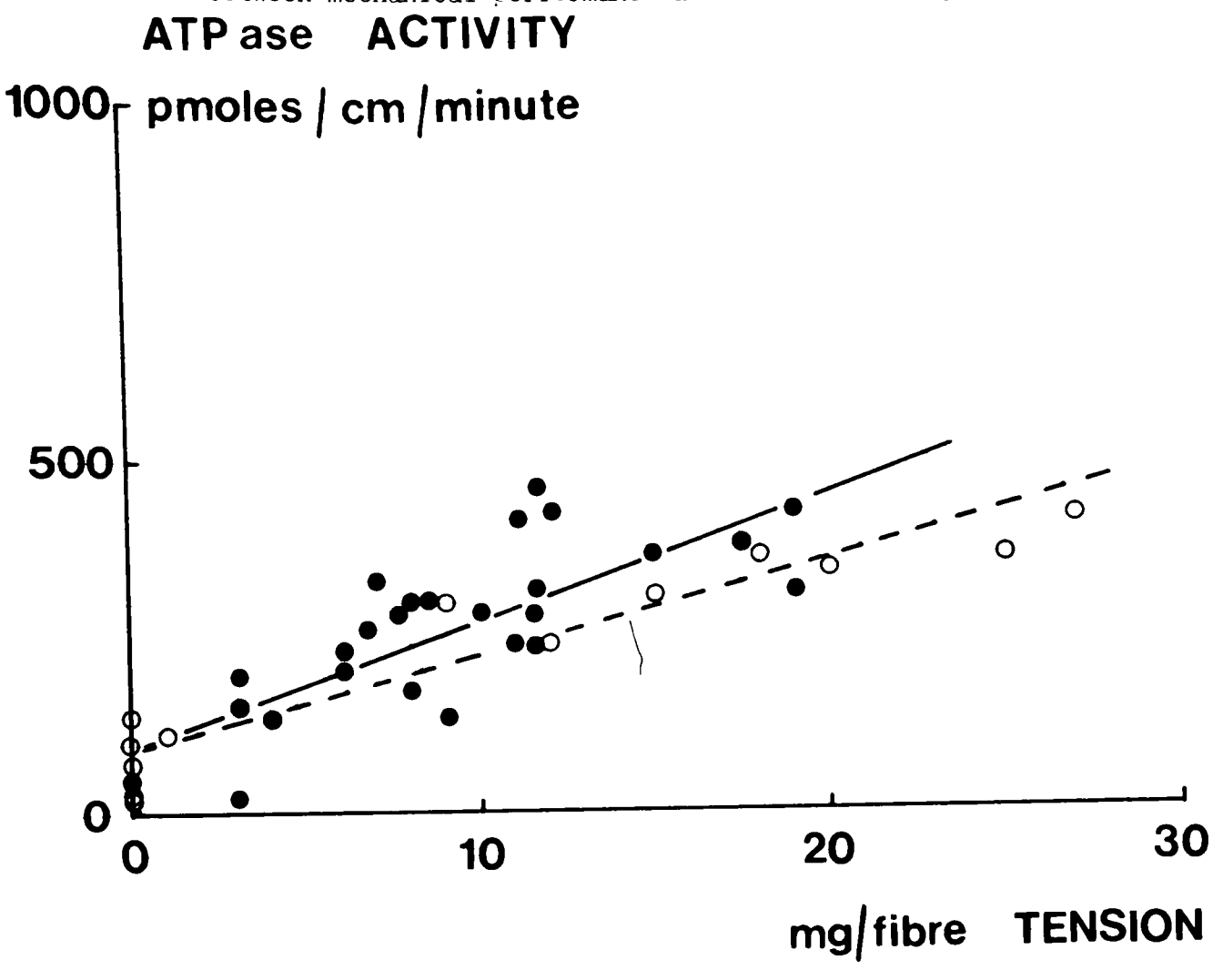


Figure 52b

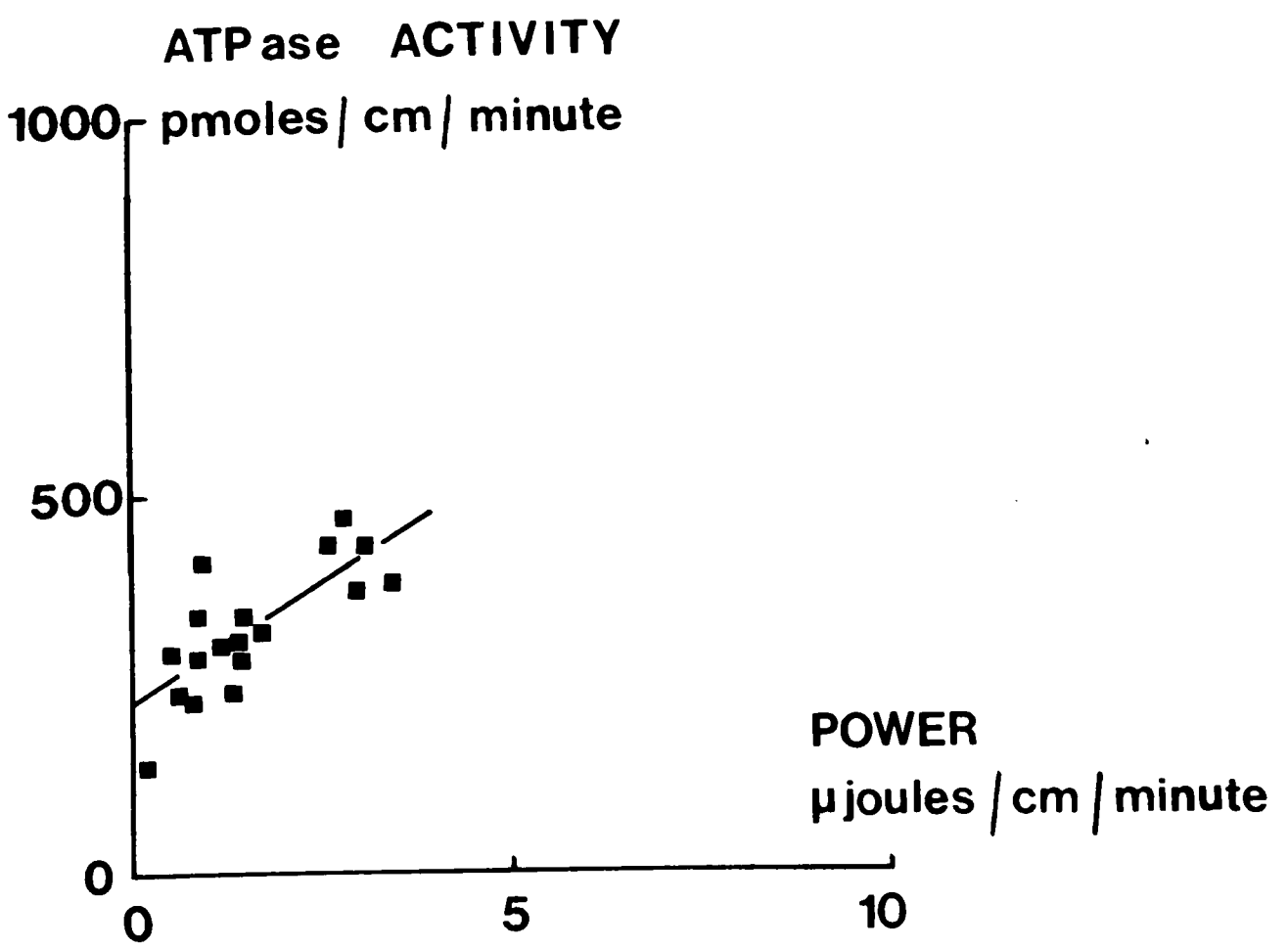


TABLE 35

The effect of pyrophosphate

Regression coefficients and standard errors

Solution	Static tension cost	Oscillatory tension cost	Power cost	f_{max}	Range
	pmoles/cm/min/mg	pmoles/cm/min/mg	pmoles/cm/min/μJ	Hz	Hz
P + PP	34.6 ± 7.6	64.8 ± 8.3	53 ± 13	7.6	2 - 21
H + PP	13.4 ± 1.7	18.9 ± 2.4	62 ± 13	1.4	0.2 - 9.0

TABLE 36

The effect of pyrophosphate

Intercepts of regression lines

Solution	Static tension cost	Oscillatory tension cost	Power cost
	pmoles/cm/min	pmoles/cm/min	pmoles/cm/min
P + PP	144 ± 104	96 ± 122	254 ± 175
H + PP	91 ± 30	89 ± 40	228 ± 40

The graphs in Figs. 51 & 52 show the results of experiments in which ATPase activity was measured. The regression coefficients and other statistical data are tabulated. The details of the mechanical performance of the fibres used for the chemical experiments is also included in Tables 35 and 36.

The addition of pyrophosphate to phosphate buffer does not change the cost of tension or power production. The mechanical performance is also unchanged from that typical of phosphate.

In the case of histidine buffer the cost of tension production is reduced by the addition of pyrophosphate although as in previous cases in which tension cost was found to change, the efficiency of power output is not affected. The mechanical data shows that the reduction in the tension cost is accompanied by a reduction in the f_{max} and frequency range below those for pyrophosphate-free histidine solution.

(Note: As was the case with previous experiments, the mechanical performance was more fully analysed at a later date using the computer facilities. The performance in phosphate was not affected by the presence of pyrophosphate, as observed above. However, the effect of a reduction of frequency response in histidine could not be reproduced. In all later experiments fibres in histidine/pyrophosphate solution showed a raised frequency optimum for work production around 7 Hz, and a narrow frequency range - 4 to 12 Hz at 2%. However the data from the original experiment has been used in the later analysis where the functional relationship between f_{max} and tension cost is considered and not the instigator of changes in any one case.)

From the results obtained, therefore, pyrophosphate, when it is added to activating solution, only affects muscle in the absence of phosphate.

TABLE 37

(1) Conditions	(2) Cost of power production	(3) Static tension cost	(4) Dynamic tension cost	(5) f_{max_2}	(6) f_{max_1}
	µmoles/Joule	pmoles/mg	pmoles/mg	Hz	Hz
P_i bfr 12°C	50.0	9.2	26.4	3.1	6.6
16°C	41.0	15.2	29.0	4.6	7.6
20°C	59.0	20.4	47.8	6.3	8.7
25°C	63.0	22.3	55.6	8.0	10.5
30°C	59.0	33.7	93.0	9.5	16.8
I.S. 0.08 M	40.0	20.0	44.2	6.8	9.7
0.09 M	70.0	19.1	48.1	6.0	10.5
0.10 M	37.0	18.4	30.4	6.4	9.6
0.11 M	113.0	44.8	52.0	6.2	9.9
0.13 M	69.0	26.9	29.5	7.4	10.0
P_i + SO_4	69.0	35.2	36.2	6.2	-
Hist. + SO_4	59.0	5.7	4.6	0.9	-
P_i + PP	90.0	34.6	64.8	7.6	-
Hist + PP	62.0	13.4	18.9	1.4	-
P_i	72.0	49.5	89.1	7.6	20.0
Histidine	59.0	13.7	26.9	2.0	5.5

Conditions unless otherwise stated: Phosphate buffer
pH 6.95
I.S. 0.8 - 0.11
Temperature 20°C
2% Amplitude

Columns (5) and (6) results obtained from fibres used for ATPase activity measurements where sufficient data available to give reliable estimate and where full mechanical analysis on different muscle gave discrepant results.

(6) Summary

In this chapter the effect of a number of agents on the mechanical and chemical behaviour of glycerinated fibres has been considered. The results have shown that at 2% amplitude of oscillation, the efficiency of power production as measured by the slope of the regression of ATPase activity upon power is fairly constant through a wide range of conditions. The cost of tension production and the frequency response of the fibres, however, are much more sensitive and were affected by changes in temperature, pH and ionic composition of the incubating medium. In general, the faster the shortening velocity at which the muscle was able to produce positive work, the greater the cost of tension production. The changes in speed of the operation of the muscle brought about by alterations in the ambient medium, therefore, appear to be linked with changes in the cost of tension production but they do not affect the cost of producing power.

In subsequent chapters these empirical observations will be analysed and examined with reference to possible theories of the underlying mechanism. In Table 37 the results which will be considered are displayed.

CHAPTER 5

The Efficiency of Muscle

The rate of ATP hydrolysis was related to power production in most of the experiments performed and the resulting measurements of efficiency will now be discussed in detail.

The importance of a high efficiency of mechanical work production to an animal, particularly a flying one, is obvious. For this reason insect flight muscle is a particularly good preparation to use for efficiency measurements.

The efficiency of glycerinated flight muscle oscillated at an amplitude of 2.0% was measured under a variety of conditions as described in the previous chapter. A typical plot is that obtained for phosphate buffer at 20°C, Figure 22. As has been pointed out, power output increases with extension up to a maximum and above an optimum value decreases while the ATPase activity, which is correlated with tension rather than power, continues to rise. Thus the Fenn effect, the mobilisation of chemical energy in response to the work required of the muscle, is only strictly observed when power is produced at moderate extension.

Regression analysis of the points obtained when tension was not greater than the optimum gives a value for the slope of the regression of ATPase activity upon power output, and an intercept. In all cases the intercept is positive. The slope of the regression line is a measurement of the rate of ATP splitting required per unit power produced. Column 2 in Table 37 shows that over the wide range of conditions studied this figure is remarkably constant, having a mean value of 60 μmoles/Joule with a range of approximately ± 20

μmoles/Joule. However the slope of the regression line only serves as a measurement of efficiency if the ATPase activity observed when no power is produced, i.e. the intercept, is subtracted from the hydrolysis rates accompanying power production. The validity of such a subtraction is a moot point.

If it could be shown that the basic ATPase activity arose from elements not responsible for the production of power so that the slope of the regression line were measuring the efficiency of all the transducing elements, subtraction would clearly be fully justified. However there is no evidence that this is so. It is known that a tension in the fibre of several milligrams is necessary for power production and it is likely that the basic level of ATP hydrolysis represents the activity of crossbridges exerting this tension. When conditions are suitable for power production such bridges would not remain in parallel with work-producing elements but all bridges would form a uniform population whose exact mechanical output is determined by the imposed conditions.

Thus the validity of subtracting the basic ATPase activity cannot be assumed although the uniformity of the slopes of ATPase activity upon power implies that the intercept ATPase rate, which is much less constant in different conditions, should not be included. If it is, there is then under any one set of external conditions no single measurement of efficiency as it will increase as the power output increases and the relative size of the basic ATPase activity is reduced. At a power output of 10 μJoules/cm fibre/minute, about the maximum observed in ATPase activity estimations, inclusion of the basic ATP hydrolysis rate reduces the efficiency from 24.5 kJ/mole to 17.5 kJ/mole at 16°C. Thus in practice the effect on efficiency of

the subtraction is not large at high power outputs. If the muscle were working as *in vivo* the effect would be very small. The maximum power output obtained here is much less than that recorded by other workers using glycerinated fibres. Pringle & Tregear (1969) observed a maximum power output of 18 μJoules/cm/minute from freshly glycerinated fibres at high amplitude. Steiger & Rüegg (1969), at 2% amplitude using high ATP concentration and temperature, obtained power outputs of 10^5 erg/g muscle/sec. This value is comparable with the power production of living basalar fibrillar flight muscle of the beetle *Oryctes rhinoceros* from which 3×10^5 erg/g/sec were obtained (Machin & Pringle, 1959). No measurements on live muscle are available for *Lethocerus*. In the same units, a power output of 10 μJoules/cm/min, the maximum involved in the efficiency measurements, is 0.4×10^5 erg/g/sec, only 13% of the *in vivo* figure. At *in vivo* rates of working, the efficiency would be very nearly equal to the slope of the regression line, assuming that ATPase activity and power continue to rise linearly.

Consequently, whether or not the basic ATPase activity may validly be subtracted the slope of the regression line may be taken as a measure of the efficiency. The results show that the amplitude of oscillation greatly affects the cost of power production: the slope at 0.2% is 890 μmoles/Joule, that at 2% 66 μmoles/Joule. The frequency clearly is not critical. The frequency optimum at 2% varied from 0.9 to 7.8 Hz when temperature and ionic environment were changed, a factor of 8.5, but the efficiency did not alter. So, at one amplitude, the power producing mechanism of the muscle is such that it may work over a wide range of contraction velocities without change in the efficiency.

So far, the efficiency has been measured as the amount of power produced per unit ATP hydrolysed, an optimum figure of 24.5 KJoules/mole

being obtained at 16°C. To obtain a measure of the efficiency of energy conversion it is necessary to know how much free energy is released when a molecule of ATP is hydrolysed under the conditions of the muscle's operation.

As has been discussed in the introduction, it has proved very hard to obtain reliable measurements of the <u>in vivo</u> free energy of hydrolysis of ATP, ΔG. George <u>et al.</u>, (1963) obtained a figure of -44.2 kJoules/mole for $\Delta G'_o$ at pH 10.0, 25°C and zero ionic strength. The estimate of Benzinger <u>et al.</u>, (1959) of -29.4 kJoule/mole at pH 7.0, 37°C and 0.2 M ionic strength is not significantly different when ΔG under one set of conditions is considered.

<u>In vivo</u> ΔG is calculated from the standard figure using the following equation (Kushmerick & Davies, 1969).

$$\Delta G_{available} = \Delta G'_o - 2.3\ RT \left\{ pH_{cell} - pH_{\substack{\text{appropriate to} \\ \text{thermodynamic} \\ \text{data}}} - \log\left(\frac{[ADP][P_i]}{[ATP]} \right) \right\}$$

The free energy available to the muscle is greater than G'_o at a similar pH as the concentrations of reactants and products are millimolar, not molar.

In the experiments described here, the ATP for hydrolysis came from an external source. ADP was produced by the muscle and also was introduced as a contaminant in the ATP. The expected free energy of ATP hydrolysis under these conditions can be calculated.

ambient pH = 6.95

$[ATP]$ = 5 mM added, but assuming a concentration gradient through each fibre of 1 mM (Rüegg & Tregear, 1966) the actual concentration at the active site will be 4 mM.

$[ADP]$ = 1 mM (Rüegg & Tregear, 1966)

$[P_i]$ = 10 mM added.

using the data of Benzinger et al., (1959)

$$\Delta G_{available} = -29.4 - 5.25 \left\{ 6.95 - 7.0 - \log \left(\frac{[1 \times 10^{-3}][10 \times 10^{-3}]}{[4 \times 10^{-3}]} \right) \right\}$$

$$= -42.7 \text{ kJoules/mole.}$$

Using this value for ΔG, the mean efficiency is found to be 39%. In two cases, 16°C and ionic strength 0.08 M, the rate of power production was 40 kJ/mole, and the efficiency 58%. So at least half the free energy available to the muscle from ATP hydrolysis can be converted to useful work.

These figures should be compared with estimates by other workers. Taking first work on the same preparation, Steiger & Rüegg (1969) obtained 12.6 to 21.0 kJ/mole, efficiencies which are similar to those recorded above. In their calculations they employ the concept of an 'oscillation-induced extra ATPase' and subtract from the total ATP split at one power output the ATPase activity of a muscle stretched isometrically to the extension imposed on the experimental fibre before oscillation, 101% to 102% rest length. As there is always a rise in mean tension when oscillation is imposed, the subtraction is not equivalent to subtraction of the chemical activity of an isometric fibre at similar mean tension. It was postulated that the intercept ATPase activity, observed when the data were handled as described here, is a consequence of production by the crossbridges of the low tension necessary in the fibre for oscillation to produce positive work. The subtraction employed by Steiger & Rüegg to obtain 'extra ATPase activity' and that of the intercept ATPase rate are therefore approximately equivalent.

Using frog rectus abdominis preparations and comparing phosphocreatine levels in a contacting muscle and an unstimulated pair Cain et al., (1962) obtained an efficiency of 11 kJ/mole. In these

experiments 2,4-dinitrophenol (DNP) was used to reduce the initial PCr levels so that the difference between experiment and control was measurable. When this was not done, and changes in P_i and creatine were measured, the efficiency was greater: 13.5 KJ/mole. No work-independent 'maintenance metabolism' was detectable.

Maréchal (1964), employing iodoacetate-poisoned frog sartorii, obtained a maximum efficiency of 100%. However in these experiments the excess creatine production of a shortening muscle over an activated pair at the same mean isometric tension was used as the measure of the ATPase activity. Such a mode of measurement is qualitatively different from the subtraction employed by either Steiger & Rüegg or myself and involves taking away a large proportion of the total chemical breakdown. The creatine produced in the isometric contractions was 83% of that of the work producing case. The phosphocreatine level in muscles after a contraction producing 126.8 mJoule/g work was 9.24 μM/g; stable metabolism was evaluated as -0.5 μM/g and average resting PCr level at 18.24 μM/g. Therefore 9.0 μM/g PCr were consumed during the contraction, of which 0.5 μM/g can be attributed to the calcium pump ATPase activity and other ancillary reactions. So 128.6 mJoule work derives from 8.5 μM PCr hydrolysis, giving an efficiency of 15 kJoules/mole, a figure comparable with the mean obtained from fibrillar muscles. The results from experiments in which the control was unstimulated are slightly lower - 11.5 kJoule/mole.

Carlson et al., (1963) performed experiments of a similar type and obtained a slope of work against Δ PCr of 25 KJ/mole. However, the metabolism of equivalent-tension isometric twitches was such that, for an isotonic contraction producing about the highest work output observed, total chemical breakdown was equivalent to 12.6 KJ/mole. Allowing for

TABLE 38

Author	Preparation	Details	Efficiency kJoules/mole	Efficiency %
Self	Insect flight muscle	Mean	16.5	39
		Best	24.5	58
Steiger & Rüegg (1969)	Insect flight muscle	Mean	16.8	33
		Best	21.0	42
Cain et al., (1962)	Frog rectus abdominus		13.5	27
Carlson et al., (1963)	Frog sartorius, iodoacetate	Activation metab. not subtracted	12.6 - 25.0	25 - 50
Maréchal (1964)	Frog sartorius, iodoacetate	Recalc. from author's data	15.0	30
Kushmerick & Davies (1969)	Frog sartorius, DNFB poisoned	Plateau	28.0	67
		Best (K + D)	42.0	100
		Best (recalc.)	32.0	76

consumption of some energy in producing and maintaining the activated state, the true efficiency will lie between 12.5 and 25 kJ/mole. If, as Woledge (1971) suggests, most calculated estimates of non-contractile ATP consumptions are overestimates as actomyosin interaction has not been completely eliminated, the efficiency is likely to be nearer the lower figure than the higher.

Kushmerick & Davies (1969) measured ATP hydrolysis of DNFB-poisoned frog sartorii over a range of velocities of contraction, using an unstimulated control for comparison. They subtracted from the total chemical breakdown a figure of 0.25 μmol ATP/g/sec which they calculated from their experiments (Kushmerick et al., 1969) to be attributable to the operation of the calcium pump and other ancillary processes. The work rate was then found by them to be around 25 kJ/mole at velocities of 0.5 to 3 cm/sec, with a peak at 2 cm/sec of 42 kJ/mole. However, recalculation from the experimental data presented in the paper, while reconfirming the first figure, gives a lower value of 32 kJ/mole for the optimum velocity.

The efficiencies obtained by different authors are listed in Table 38. Most calculations are based on a free energy of ATP hydrolysis of -50 kJoules/mole, calculated by Kushmerick (Kushmerick & Davies, 1969) for aerobic muscles. The exceptions are those in which the muscle was poisoned and anaerobic, or in which ATP was added from an external source. Kushmerick & Davies estimate that the value of ΔG falls to -42 kJoules/mole in a muscle poisoned with DNFB as the concentration of ADP is abnormally high - 36% of the ATP concentration, as opposed to 0.9% in untreated muscles.

The value of ΔG under the conditions of the present experiments has already been calculated as -42.7 kJoules/mole.

Substituting the appropriate figures from their experimental data into the equation for ΔG, a value for the free energy of ATP hydrolysis of -50 kJ/mole is obtained for the conditions employed by Steiger & Rüegg. Their low pH, high ATP and low ADP due to the use of myokinase, are responsible for the high value.

The lowered efficiency observed by Cain et al., (1962) of muscles treated with DNP to reduce the PCr level may be attributable to a lowering of the free energy of PCr available in abnormally low concentration.

Both vertebrate and insect muscle under a range of experimental conditions are at least 30% efficient and 50% usage of available free energy has been obtained in advantageous conditions. The 76% efficiency recorded by Kushmerick & Davies is the best experimentally recorded without subtracting large proportions of observed chemical breakdown. It is now known that the heat evolution during contractions under the conditions used is much greater than can be accounted for by the known chemical change, indicating the existence of previously undetected reactions (Gilbert et al., 1971; Woledge, 1971). Consequently these results must be regarded as unreliable.

The efficiency measurements in this work are therefore consistent with those made on the same and other muscles in showing a minimum conversion by the crossbridge of 50% of the free energy of ATP into work.

CHAPTER 6

The Mechanism of Contraction

1. Basic assumptions

In this chapter the significance of the measurements of tension cost will be considered in detail, and the results will be interpreted in terms of a particular model of muscular contraction.

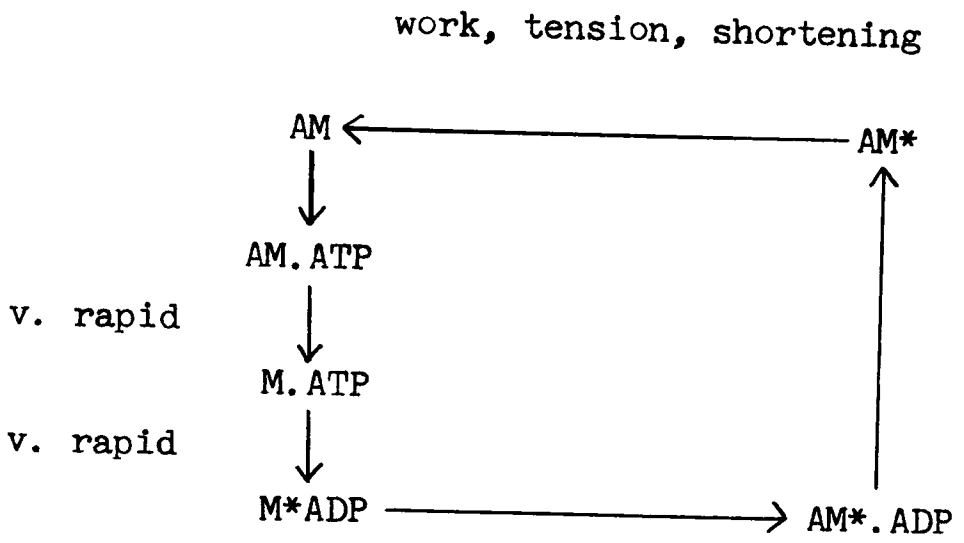
The interpretation is based on a number of assumptions which will be outlined. Firstly, it is assumed that the active contraction of muscle involves attachment to actin of the myosin heads and their subsequent detachment. It has been established that in the presence of MgATP and the absence of calcium there are few or no connections between the filaments and that the muscle is therefore in a low-tension, relaxed, state. In the absence of ATP, the artificial state of rigor is induced and electron microscopy and X-ray diffraction provide evidence in the form of a changed ordering of the myosin heads that crossbridges are attached to the actin filaments (Reedy et al., 1965).

The attachment of crossbridges during active contraction has not been conclusively established but has been inferred from work on the association of actin and myosin in solution (Szent -Györgyi, 1949) and from diffraction studies, the results of which are consistent with attachment of 10-30% of myosin heads during normal operation (Miller & Tregear, 1970; Parry & Squire, 1972).

To this first assumption of a crossbridge cycle of attachment and detachment is added a second: that one molecule of ATP is hydrolysed per cycle. This is based on the work of Lymn & Taylor (1971).

Binding of one ATP molecule at an active site on the myosin head induces rapid detachment followed by hydrolysis of the ATP, leaving a

semistable M.ADP complex which has been identified in myofibrils (Marston & Tregear, 1972). Attachment of actin is followed at some time by dissociation of the product, freeing the site for another ATP. Therefore the two assumptions can be represented as shown below:



On such a scheme, hydrolysis of one molecule of ATP accompanies each attachment. Each bridge, except under ideally isotonic conditions, generates tension during attachment. The ATPase activity, which is a measure of the number of crossbridge cycles occurring, can therefore be related to tension in the fibre to give an estimate of the amount of force exerted per cycle. This is limited by the duration of the attachment phase, which, in turn, depends on the probability of detachment, g. So it follows from the assumptions made that the rate of ATP hydrolysis per unit fibre tension, i.e. tension cost, is a measure of the attachment time, and therefore of g.

2. Proportionality between tension cost and optimum frequency for work, and its significance

Measurements of the cost of tension production by muscles statically stretched or subjected to a 2% oscillatory length change gave, under a wide range of conditions, a pattern of results of which Figs. 20 and 21 are typical.

ATPase activity rises linearly with tension, there being a higher cost of tension production when the fibres are oscillated. The justification for assuming a linear relationship has been given in Chapter 3. In all instances the regression line of ATPase activity upon tension has a positive intercept on the ATPase activity axis. As in the case of the measurements of efficiency, the question arises whether this basic ATPase activity may be justifiably subtracted from values when tension is produced, leaving the slope as the measure of tension cost. In contrast to the basic ATPase activity measured on power graphs, no observable mechanical output accompanies this ATP hydrolysis; the intercept on the tension graphs is in almost all cases lower than the equivalent power intercept. However, there is evidence that the basic ATPase activity arises from the crossbridges. If a fibre is set at such a length that tension is just not produced, after about twenty minutes a few milligrams of tension are frequently observed, implying that the activity of a few crossbridges has caused the slack in the fibre to be taken up by contraction until a small isometric tension is registered. However, whatever the origin of the basic ATPase activity, the argument that was used in the case of the power cost applies here: the effect of including the basic ATPase activity in the measurements of tension cost will be small at high tensions and thus at high power outputs. For this reason, the cost of tension production was measured by the slope of the regression line.

Tension cost measurements, available from the experimental data as outlined above, give one measurement of g. A second measure of this quantity can be obtained from the mechanical experiments. Thorson & White (1969) showed that the effect of stretch is to induce an increase in the probability of attachment in fibrillar muscle; the rate at which the tension reaches a new equilibrium is then dependent upon g

Figure 53

Graph of f_{max_1} against f_{max_2}; independent variable: temperature, ionic environment.

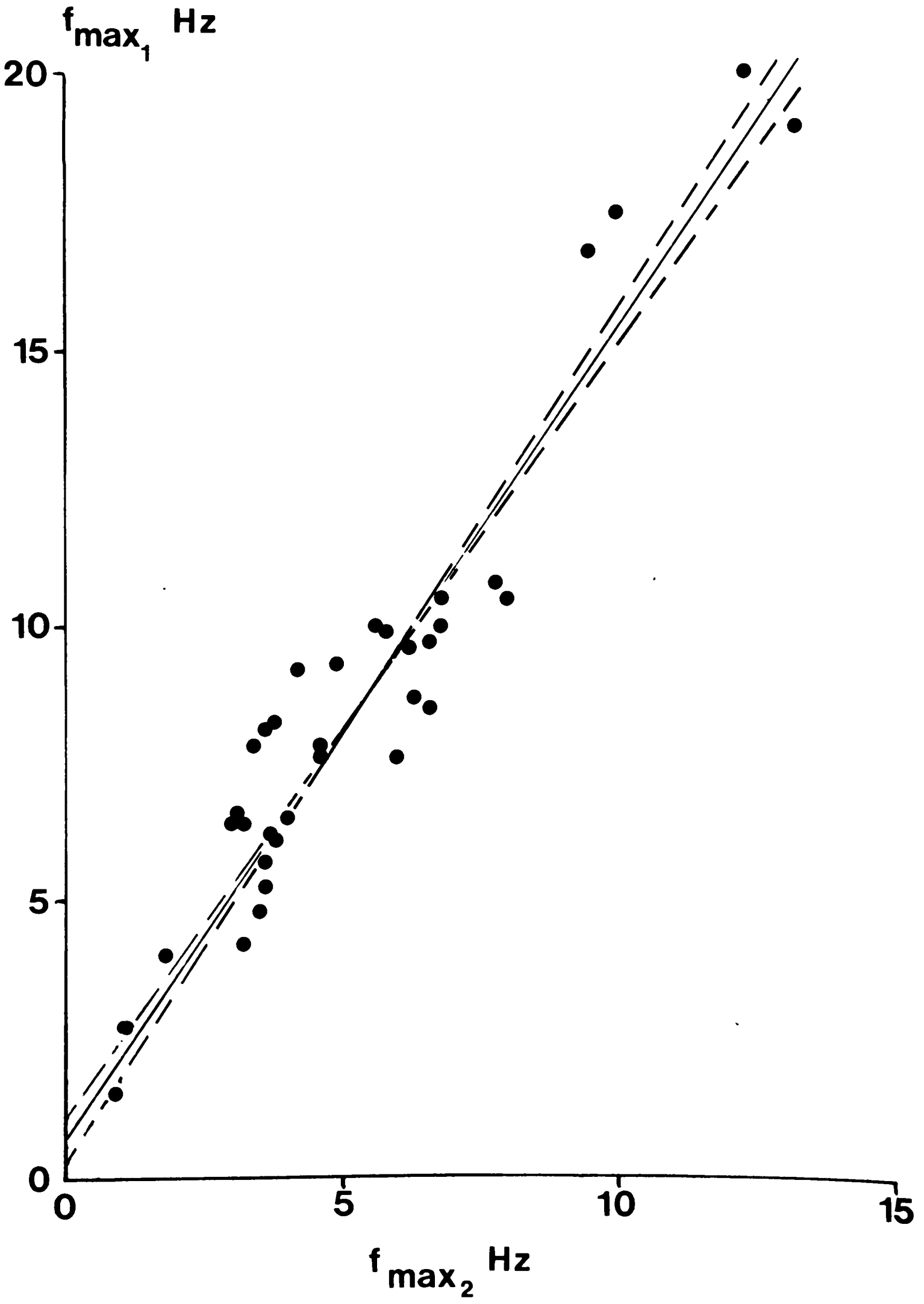
- - - Regression lines of (a) f_{max_1} upon f_{max_2} and (b) f_{max_2} upon f_{max_1}

——— Best fit line calculated by averaging two regression coefficients.

Regression	Regression coefficient	Standard error	Intercept on y axis Hz
f_{max_1} on f_{max_2}	1.4	0.1	1.1
f_{max_2} on f_{max_1}	1.6	0.1	0.3
Mean	1.5	0.1	0.7

Correlation coefficient 0.95

Figure 53



in the small signal case where the response is symmetrical. As work production requires that the rate of the delayed tension change be commensurate with the imposed frequency of oscillatory length change (Tregear, 1967), the frequency of maximum work will depend directly on g. At low amplitude of oscillation, e.g. 0.2%, the rate constant of detachment equals $2\pi f_{max_1}$, i.e. the optimum angular frequency (Thorson & White, 1969). So f_{max_1} is a direct measure of g. As the system becomes non-linear and mathematically complex at 2%, f_{max_2} cannot be directly interpreted. However, a plot of f_{max_1} against f_{max_2} of fibres under a variety of conditions of temperature and ionic environment is closely fitted by a straight line, Fig. 53. The regressions of x upon y and y upon x were calculated and the best fit line was taken from the average of the two coefficients, as the error in the two measurements was similar. The line has an intercept insignificantly different from zero so the two are proportional with a coefficient of 1.5. Thus g can be calculated from f_{max_2}.

The experiments therefore yield two separate indices of muscle performance, tension cost and f_{max}, both of which, on the crossbridge cycle theory, are limited by, and thus measure, the same thing, namely the duration of attachment, which in turn is directly determined by g. Consequently a change in one should be accompanied by a change in the other. In the previous chapter experiments were described in which the conditions of activity were varied in a number of ways, including ambient temperature, which will change the rate of any chemical reaction. It was found that the ionic environment, and, predictably, temperature affected the tension cost and f_{max} of fibres, see Table 37. The isometric tension cost and the tension cost at 2% amplitude of oscillation were plotted against the optimum frequency for work production at 2%. Figure 55 shows that there is quite a lot of scatter around the best-fit

Figure 55

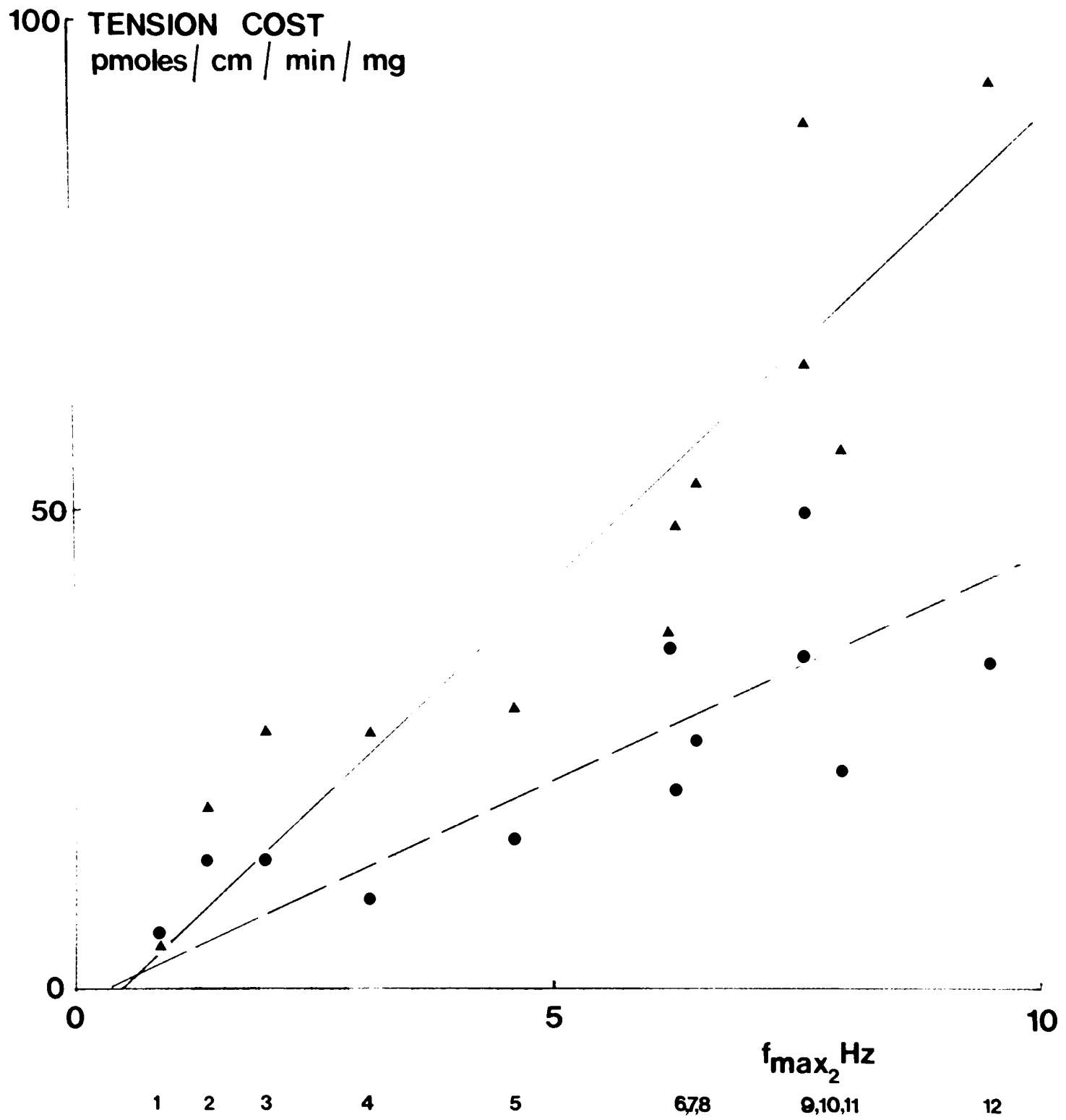

100
TENSION COST
pmoles / cm / min / mg
50
0
0
5
10
f_{max_2} Hz
1
2
3
4
5
6,7,8
9,10,11
12

Figure 55

Graph of dynamic tension cost , ▲ , and static tension cost, ● ,

against f_{max_2}

Data from Table 37.

Best-fit line of dynamic tension cost against f_{max_2} ————

" " " " static " " " " — — —

Regression of tension cost on f_{max_2} and f_{max_2} on tension cost calculated

and averaged to obtain best-fit line.

Regression	Regression coefficient	Standard error	Intercept on y axis pmoles/mg	Correlation coefficient
Dynamic				
Tension cost on f_{max}	8.6	1.3	-0.4	0.90
f_{max} on tension cost	10.5	1.3	-10.5	0.90
Mean	9.6	1.3	-4.0	0.90
Static				
Tension cost on f_{max}	3.6	0.9	4.1	0.80
f_{max} on tension cost	5.7	0.9	-7.1	0.80
Mean	4.7	0.9	-1.5	0.80

Key to points

1. Histidine buffer, + sulphate
2. Histidine buffer, + PP
3. Histidine buffer
4. Phosphate buffer, 12°C
5. " " 16°C
6. " " + sulphate
7. Phosphate buffer, 20°C
8. " " all ionic strengths
9. " " + PP
10. " "
11. " " 25°C
12. " " 30°C

Figure 54

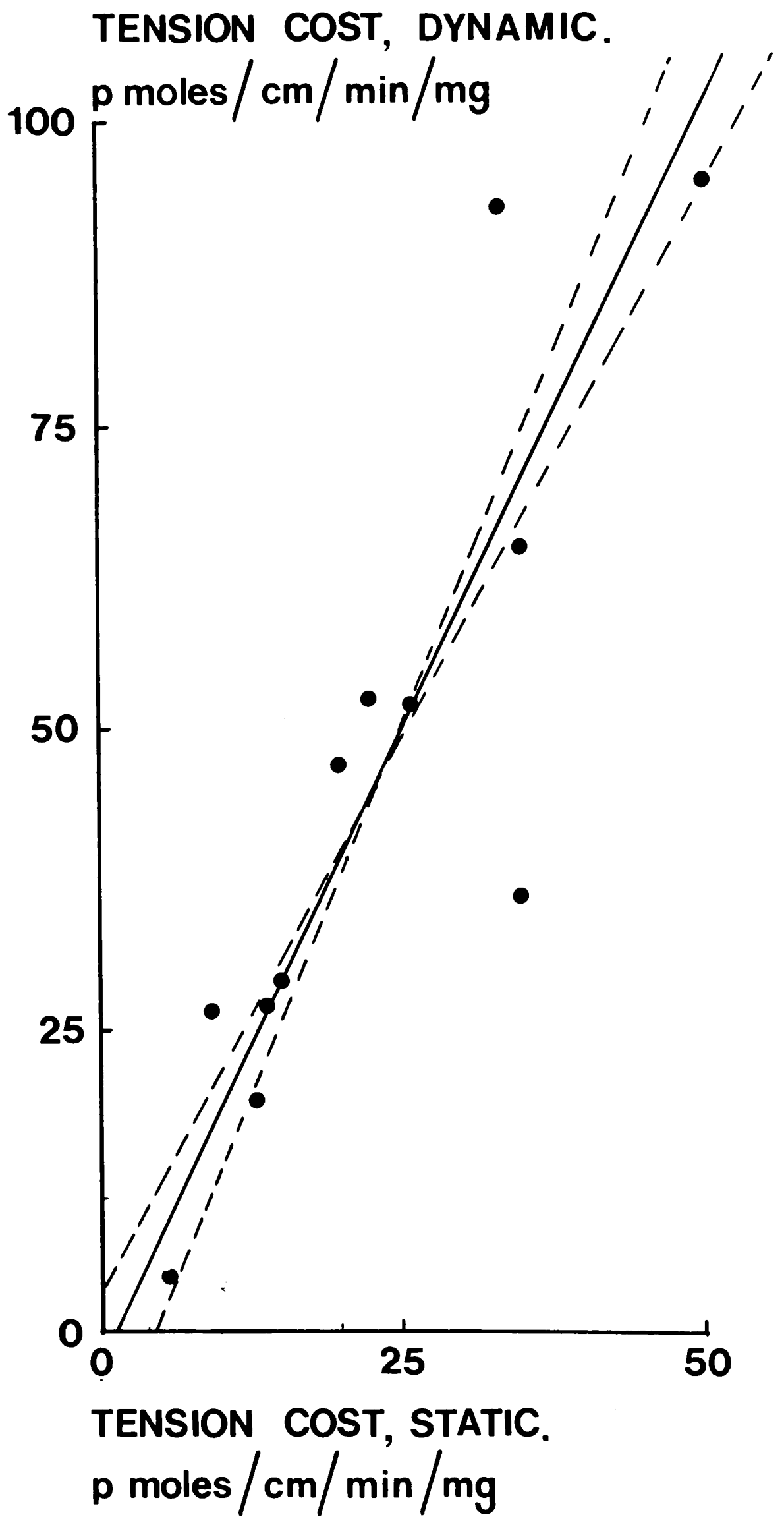

TENSION COST, DYNAMIC.
p moles / cm / min / mg
100
75
50
25
0
0
25
50
TENSION COST, STATIC.
p moles / cm / min / mg

Figure 54

Graph of tension cost for static muscle against tension cost for muscle oscillated at 2% under identical conditions.

Independent variable: temperature, ionic environment

Data taken from Table 37.

- - - Regression lines of static cost upon dynamic cost and vice versa.

——— Best-fit line calculated by averaging two regression coefficients.

Regression	Regression coefficient	Standard error	Intercept on y axis pmoles/min/cm/mg
Dynamic cost upon static cost	1.8	0.03	4.0
Static cost upon dynamic cost	2.4	0.03	-12.0
Average	2.1	0.03	-3.0

Correlation coefficient 0.85

lines. In each case the line is derived from the average of the two regression coefficients as both tension cost parameters and f_{max} are subject to large standard errors. The regression lines both have intercepts on the f_{max} axis which are very small and the plots are consistent with a proportionality between f_{max} and tension cost, but the error is large, ± 20% for the tension cost, ± 25% for mechanical measurements.

In view of the evidence in favour of proportionality and thus of the postulate that tension cost and f_{max} are both governed by the same event, the data was reorganised in an attempt to reduce the scatter observed in Fig. 55. Two measurements of each parameter are available: static and dynamic tension cost and f_{max} at two amplitudes. From Fig. 53 it is clear that f_{max_1} and f_{max_2} are linearly related by a factor of 1.5. Therefore f_{max_2} was multiplied by 1.5 to attain its equivalent f_{max_1} value, and this and the experimentally obtained f_{max_1} were added and averaged. From Fig. 54 static and dynamic tension cost are by the same arguments proportional with a coefficient of 2.1. This was applied to obtain a single measurement of static tension cost. In view of the fact that at all values of ionic strength between 0.08 and 0.13 M, no net change in tension cost or frequency optimum could be observed, the data for all ionic strengths were combined to give a single point.

The point for 8°C was excluded as the fibres were not activated. The data obtained from pH variation were also omitted. They did not fit in the pattern of the other experiments as, although oscillatory tension cost rose with pH, the frequency response either did not change or decreased. Only at pH 7.3 were the fibres working well, and only at this pH was any static stretch activation shown. In view of the unsatisfactory nature of the muscles' performance the results were excluded from later analysis.

Figure 56

Plot of static tension cost against f_{max_1}

Ordinate in tension cost obtained thus:

$$x = \frac{1}{2} \text{ (tension cost, static) + (tension cost, dynamic } \times 1/2.1)$$

Abscissa: f_{max_1} obtained thus:

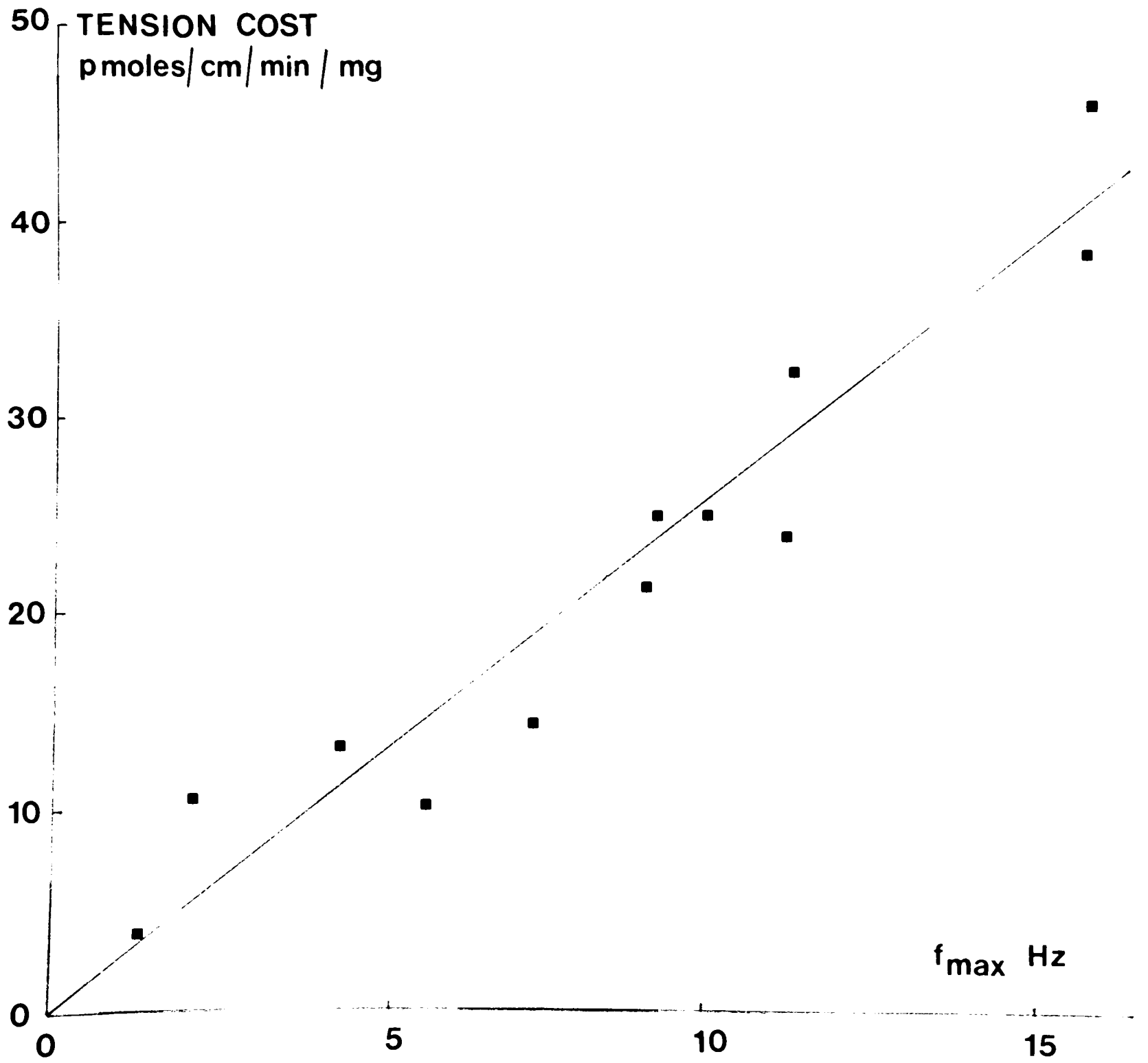
$$y = \frac{1}{2} (f_{max_1}) + (f_{max_2} \times 1.5)$$

Independent variable: temperature, ionic environment

Regression	Regression coefficient	Standard error	Intercept on y axis
Tension cost on f_{max}	3.8	0.4	0.65
f_{max} on tension cost	4.1	0.4	-1.37
Mean	3.9	0.4	0.0

Correlation coefficient 0.96

Figure 56



The data obtained by varying temperature from 12° to 30° by changing the anions present, and the single point from the ionic strength experiments were then plotted as in Fig. 56. The points show far less scatter than was shown by the uncombined data in Fig. 55, a fact which statistical analysis illustrates. The regression line has a correlation coefficient of 0.96 and an intercept insignificantly different from zero. The points are thus very well fitted by a straight line. (A concave curve with a positive y intercept would also fit the points, a convex curve would not.)

The justification for the amalgamation of data in this fashion lies in two facts. Firstly, there is clear evidence that the two measurements of each type are proportional to one another, Figs. 53 and 54. As the manipulations have been applied uniformly to all points they cannot have introduced a bias, and therefore the apparent linearity cannot be a product of the handling of the data. Secondly the linearity only becomes visible when the data are put together because of the aforementioned scatter arising from error in individual measurements.

The observed proportionality between f_{max} and tension cost is very good evidence that, as proposed, both parameters are controlled by the same process. On the theory outlined, both depend on g as a consequence of the interaction of actin and myosin during a cycle of crossbridge activity. Therefore the coupling of tension cost and optimum frequency of work is support for such a mechanism of contraction, rather than one in which interaction does not occur, such as has been proposed by Elliott et al., (1970).

3. The increase with oscillation in tension cost

This having been established, the relationships between the principal parameters of muscular activity, ATPase activity, tension and

power can be further analysed so that the theoretical framework can be extended.

The linear relation between tension and ATPase activity has been established and it has been found that the cost of tension production in an oscillating muscle is 2.1 times that of a static muscle (Fig. 54). In other words, the number of cycles needed to maintain a given tension is approximately doubled when the muscle is allowed to oscillate. That the change in tension cost is a consequence of shortening rather than of oscillation which involves shortening and extension phases is shown by several pieces of evidence. Firstly the majority of the actomyosin interactions occur during shortening, as is demonstrated by two facts. The efficiency of over 50% is proof that few crossbridges are attached during extension: if they were, the stretching of the links would absorb much of the work imparted during shortening. In addition, it may be seen from the length tension loops, e.g. Figs. 15 and 45, that there is little increase in tension during extension; at work producing frequencies the increase occurs overwhelmingly around the peak length. The second piece of evidence that it is shortening which is the cause of increased tension cost and not oscillation is that the same phenomenon is observed in vertebrate muscles which contract isotonically but do not oscillate. Carlson et al., (1963) and Maréchal (1964) performed experiments in which the chemical activity of isometric frog sartorii was compared with that of isotonically contracting, work producing muscles at the same mean tension. The muscles allowed to shorten had a higher PCr breakdown per contraction so that in vertebrate as in fibrillar muscles tension cost is increased when shortening is permitted.

The total force, P, produced by a crossbridge in one cycle, equals the product of the average instantaneous tension exerted during attachment, P_{av}, and the average time during which it is exerted, t:

$$P = \int pdt = p_{av}\, t_{av}$$

The results show that P in a static fibre is twice P for a dynamic contraction in which half as much tension is produced per unit ATP split. From the equation, the reduction in P could be brought about by a change in p_{av} or t_{av}, or both.

Considering first the hypothesis of a constant force in the crossbridge throughout its attachment phase, the time of attachment will vary if g changes when the filaments move. The oscillatory force per crossbridge cycle would be less than the static force if, at the peak of the oscillatory length change, detachment were much more probable than at minimum extension, so that the overall probability were greater than that at the mean length. Such a hypothesis requires a non-linear increase in g with stretch. The experiments of Abbott (1969), measuring the rate constant of tension change following quick stretches, show that over a range of amplitude changes from 10 nm to 100 μm, no change in g is observed. Therefore g does not vary with position. It might, alternatively, be velocity dependent, in which case the optimum frequency of work should increase with amplitude. That it does not is shown by Pringle & Tregear (1969), and, for two amplitudes, in Fig. 16. However, as is clear from Fig. 13, increasing amplitude of oscillation is accompanied by the appearance of non-linearities in the muscle response. These may be the consequence of the advent of another limiting step. But it can be seen from Fig. 53 that at 0.2% amplitude, where the response is linear, and at 2%, where it is not, the optimum frequencies for work are proportional. For this to arise despite different rate limiting steps at the two amplitudes requires complex postulates. Thus, while the point cannot be proved, it is unlikely that g is velocity dependent.

Therefore it can be assumed that the movement of the filaments does not involve an increase in g so that the decrease in P with isotonic contraction is not a consequence of increased detachment probability. Consequently, the change in tension cost must be due to a change in p with movement.

The simplest model of a crossbridge which predicts a fall in force during shortening is that of a stretched spring (Huxley & Simmons, 1971a). Assuming that the energy of ATP hydrolysis is used to extend the spring on attachment so that it exerts its maximum force,

$p_{max} = kz$ $\quad$ k = spring constant

z = extension.

During isometric contraction the spring will be unable to shorten and its instantaneous force will be unchanged so that

$$P = p_{max}\, t_{av}$$

If the filaments can move relative to one another the crossbridge movement will be brought about by shortening of the spring and as the force in a spring is proportional to its extension, the tension in the bridge will fall.

If the spring is able to transfer all its energy by shortening until it exerts no tension, the average force in it will be $p_{max}/2$, as long as it spends an equal time in all positions.

Therefore, if it can be shown that under conditions of a 2% oscillatory length change crossbridges with a spring structure would undergo full shortening, then such a model would account for the observed changes in tension cost, assuming, as has been shown to be justified, that few bridges are attached during extension.

As has been outlined, the work production of oscillatory muscle depends on g because it is necessary that the attachment of bridges be at least partially synchronised with the length changes. Assuming the ideal case in which attachment occurs only at the peak length, the the crossbridge may detach, (a) while it is still under tension, (b) at the point at which it has released all its energy, or (c) after this point. In the first case its mean force will be greater than $p_{max}/2$; in the second, $p_{max}/2$. Its mean tension in (c) will depend on whether it can exert a pushing force when pulled backwards. Assuming that the bridge goes slack when it has transferred its energy, and that the amplitude is such that it is pulled backwards before extension begins, the average force, $\int p\ dt$, must be less than $p_{max}/2$ but can never be negative. Consequently the mean tension in the population of attached bridges will be approximately $p_{max}/2$ and the tension cost in the oscillatory, work producing muscle will be twice that in the static muscle. At very low frequencies of oscillation, as Fig. 17a shows, attachment becomes much more distributed with respect to length. As the bridges will then stand an equal probability of being stretched as of contracting, so the average force will tend to p_{max}. When the frequency is too high for work production, as a consequence of the fast cycling time, many bridges will remain attached during extension and again be subjected to stretch. Therefore a decreased in tension cost may be expected.

The assumption has been made that the bridges do not exert a resistive force. If they did, unless the 2% amplitude used in the experiments were exactly that at which the point of no tension of a bridge attaching at peak length coincided with the reversal of direction of length change, the effect of pushing bridges would be to create negative tensions and to increase the cost of tension production.

Efficiency of power production will also be greatly reduced as power is absorbed by resisting bridges.

The efficiency of power production at 2.0% was optimally nearly 60%. Negative tensions were not encountered and the cost of tension production in the dynamic muscle was only twice that of the static muscle. Unless the optimal frequency was by chance selected, it is improbable that the bridges were exerting negative tension.

Measurements of the degree of extension of the crossbridge should show if the length change at 2% oscillation amplitude, equivalent to 24 nm/half sarcomere, is (a) sufficient to allow the bridge to shorten to zero tension, and (b) if it is such that bridges, if capable of pushing, would have an opportunity to do so.

In the quick-release experiments of Huxley & Simmons (1971a) an 8-12 nm step change was required to reduce the tension attributable to the elasticity in the crossbridge to zero. Step changes in load led Podolsky et al., (1969) to a similar estimate. These estimates were performed on vertebrate muscle but White (1970) found a movement of 8-16 nm was required to reduce the rigor tension of Lethocerus flight muscle fibres to zero. These measurements are estimates of the crossbridge extension as long as crossbridges are not pushing. In the experiments of Huxley & Simmons the quick-release was superimposed on the rising phase of isometric tension development so that the bridges will be biased towards the extended state and they will have measured the true extension of the bridge. White, releasing the length of a rigor muscle, would, if bridges can push, be measuring the distance necessary to reach equilibrium between bridges pulling in and out. As his estimate is not smaller than that of Huxley & Simmons and Podolsky et al., this is unlikely to be the case.

A degree of extension of approximately 10 nm is obtained from these experiments. Therefore at 2% bridges would be able to shorten completely and would have the opportunity to push. The observed efficiency and tension cost measurements make it unlikely that they do, so that a crossbridge with an elasticity stretched to 10 nm but not capable of being forced backwards would give half as much tension under oscillatory as under static conditions.

At an amplitude of oscillation of much less than 10 nm/half sarcomere the bridge should not be able to shorten sufficiently to release all its energy, so a low tension cost and efficiency would be predicted. In the experiments at 0.2% amplitude, equivalent to a movement of 2.4 nm/half sarcomere, the cost of dynamic tension production was not significantly different from static and the efficiency was very low, 7% of the mean value at 2%.

An instantaneous elasticity in the crossbridge extending over 10 nm would therefore give rise to the observed changes in tension cost.

4. Other evidence for the spring hypothesis

Huxley & Simmons (1970, 1971a,b) performed quick-length change experiments on frog sartorius muscle and with high time resolution were able to distinguish a tension change simultaneous with the length change, followed by a quick partial recovery of variable time course and degree. Full tension recovery takes place slowly. Measurement of the size of the instantaneous tension change and partial recovery step in response to different length changes gave evidence for elastic elements between the filaments giving rise to the instantaneous tension changes and visco-elastic elements responsible for the partial recovery. The experiments were performed at two different overlaps; the results were consistent with an alteration in the number of elements in parallel

Figure 57

Diagram of the crossbridge cycle to illustrate the operation of a spring-like energy transducing element.

State 1 Relaxed, no calcium.

M.ADP.

State 2 Attachment: presence of Ca^{2+}. Rapidly converts to

State 3 The attachment to actin permits stored energy of ATP hydrolysis to induce conformational change in myosin SF1 relative to SF2 and I filament, thus stretching spring.

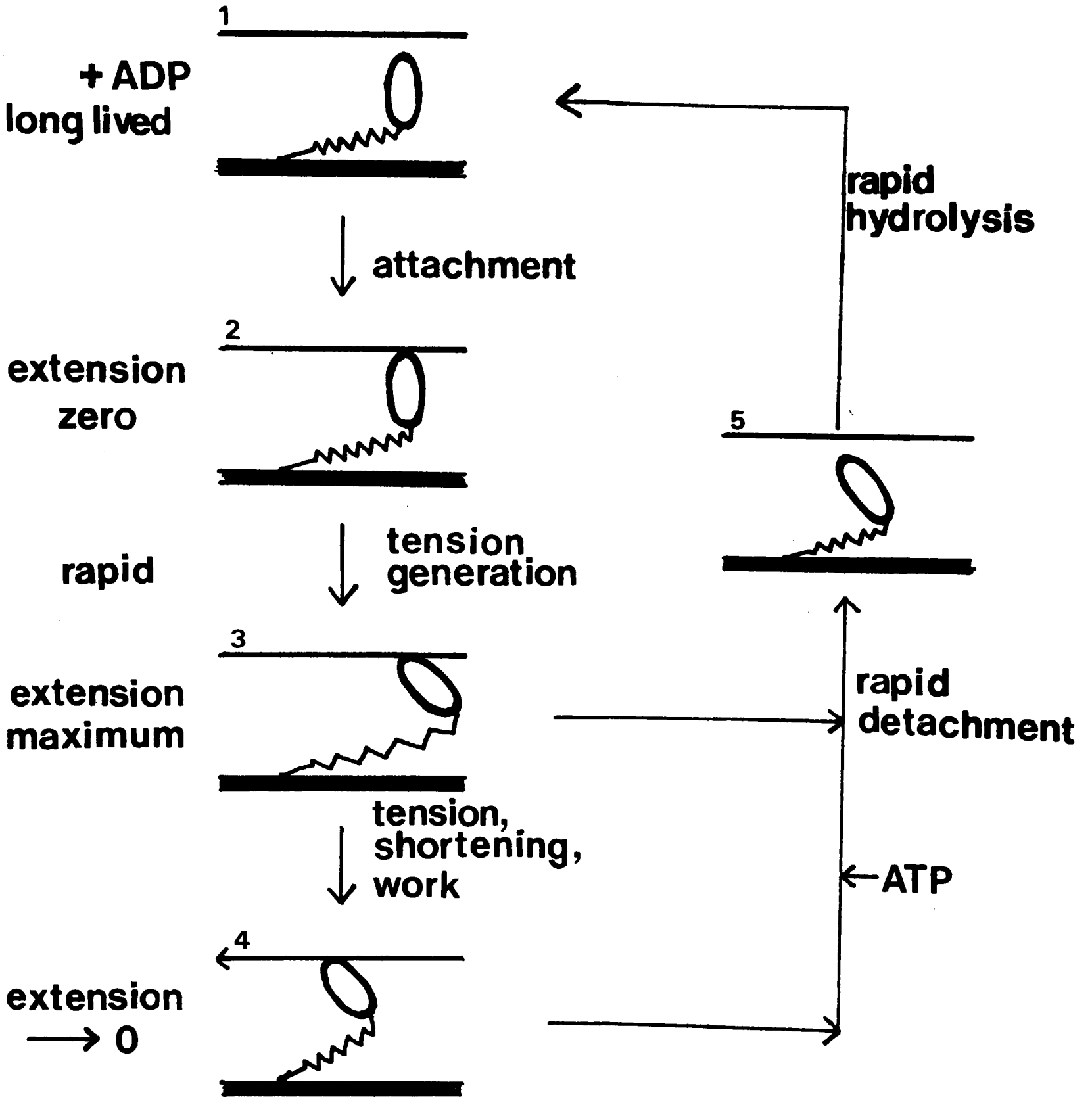
Tension proportional to extension of spring is exerted. If mechanical conditions are suitable, force brings about sliding of filaments and spring shortens until

State 4 No tension is exerted. During course of attachment, ADP has dissociated.

Attachment of ATP to active site rapidly induces detachment, with loss of energy if spring is still stretched.

State 5 ATP is very rapidly hydrolysed, returning myosin head to resting orientation.

Figure 57


1
+ ADP
long lived
attachment
2
extension
zero
tension
generation
rapid
3
extension
maximum
tension,
shortening,
work
4
extension
→ 0
rapid
hydrolysis
5
rapid
detachment
ATP

and thus with the hypothesis that the elastic elements reside in the crossbridges.

This model of muscular contraction is consistent with the structural picture derived from X-ray diffraction studies of H.E. Huxley and his collaborators (Huxley & Brown, 1967; Huxley, 1968; Moore et al., 1970). The changes in the meridional reflections from the A filament show that the dense, and therefore heavily diffracting, globular myosin heads maintain most of their axial arrangement on activation, so that their movement as they attach is radial and circumferential with respect to their parent filament. Similar results have been obtained using insect flight muscle (Tregear & Miller, 1969; Miller & Tregear, 1970). Study of equatorial reflections has shown that the mass moved from A to I filament does not form a continuum but has the form of a dense globule, oval rather than round, attached to the A filament via a thin connector (Miller & Tregear, 1972). The X-ray data, and the evidence of a flexible non-combining SF2 portion in the myosin molecule (Lowey et al., 1969), led to a crossbridge model in which the head end maintains its axial spacing because it is joined to the myosin rod by a 400 Å arm with flexible joints at either end, allowing radial and azimuthal angling (Huxley, 1969). The exact site of, or nature of, a spring-like element in the molecule is not immediately apparent from the known structure of myosin but its mode of operation can be visualised as depicted in Fig. 57. A similar scheme is envisaged by Huxley & Simmons (1971b). As the diagram shows, the attachment of the myosin head to actin is followed by tilting so that the spring element is stretched. The energy of ATP hydrolysis is in this way, by the tilting of the head, converted to a mechanical form. Contraction involves the shortening of the spring against the rigid angled myosin head and hence relative filament movement. The direction of angling of the myosin head necessitated by

the model is that observed by Reedy et al., (1965); other mechanical models have implied a reversed tilt.

The spring model of the crossbridge is therefore consistent with the picture of the crossbridge in relaxation, activity and rigor obtained from structural studies. On the molecular level the physical identity of the spring is not obvious, but as its manifestation necessitates provision of two rigid attachment points, it may not be observable by present means in the absence of actin.

5. Calculations from the theory, using experimental data

The experimental observations having been explained on the basis of a spring model of a crossbridge, estimates can be made of the value of different parameters of the model's operation. A crossbridge is here assumed to be one myosin molecule. The elastic element is assumed to originate from the SF2 portion in such a way that it is fully stretched when one SF1 hydrolyses ATP and attaches.

The tension exerted by one crossbridge can be calculated. The relations between energy, force and extension of a stretched spring are well known.

$$E = \frac{1}{2}kz^2$$

$$p = kz$$

$$\therefore p = \frac{2E}{z}$$

E = potential energy

k = spring constant

z = extension

p = force

10 nm is the average of a number of measurements of extension z.

From the data in this thesis, the minimum value of E is 25 kJoules/mole, as this amount of mechanical energy can be produced by the muscles. 42 kJoules/mole, the free energy of ATP hydrolysis, is the maximum value. On the spring theory, detachment is a first order rate process and so some bridges will detach before they have

fully shortened, so that some of their energy will be wasted. In view of this, and allowing for frictional inefficiencies in transduction of power, it is probable that the energy actually transferred to the bridge in the form of spring extension is greater than that which is obtained as useful work. Consequently E is almost certainly nearer the upper limit , 42 kJoules/mole, than the lower, 25 kJoules/mole.

$$E = 42 \text{ kJ/mole}$$

$$= \frac{42 \times 10^3}{6 \times 10^{23}} \text{ Joules/molecule}$$

$$\therefore p = 2 \times \frac{42 \times 10^3}{6 \times 10^{23}} \times \frac{10^{12}}{10 \times 10^{-9}} \text{ pN/molecule}$$

$$= 14 \text{ pN/molecule (maximum)}$$

or 8 pN/molecule (minimum)

Therefore a figure for crossbridge tension at maximum extension of around 12 pN is obtained.

Another estimate of p, made by Huxley & Simmons (1971b) gave a figure of 2 pN. This is much too small to give the efficiency obtained in practice, as the authors point out.

With an estimate of p_{max}, the rate constant of detachment which is given by the measurements of tension cost can be calculated and then compared with the estimate from mechanical measurements.

From Fig. 56, the cost of static tension production of a muscle with a frequency optimum of 15 Hz, at 0.2% amplitude is

39 pmoles/cm/min/mg

$$= 39 \times 10^{-12} \times 6 \times 10^{23} \times \frac{1}{60} \times 10^{-4} \times 1.2 \times 10^{-12} \times 10^5$$

molecules/half sarcomere/second/pN

$$= 4.7 \text{ molecules/half sarcomere/sec/pN}$$

(taking sarcomere length as 2.4 μ)

i.e. assuming one ATP hydrolysis per cycle

4.7 cycles per second yield 1 pN tension

or 56 "" " " " 12 pN "

each bridge yields 12 pN tension during attachment, therefore duration of attachment is 1/56 sec, or 18 msec, and the rate constant is thus 56 sec^{-1}.

The rate constant can also be deduced from the mechanical response of the fibre.

A muscle with a low amplitude frequency optimum of 15 Hz has

a rate constant = 2π 15 sec^{-1}

= 94 sec^{-1}

The duration of attachment is 10.5 msec.

The two estimates of the rate constant of the controlling process, identified as detachment, are 56 and 94 sec^{-1}. The discrepancy between the two values, which differ by a factor of two, may be attributed to the high degree of error associated with the data on which the calculations are based. Small changes in the values assumed for z or E make a large difference to the estimates derived from them. The two methods give attachment times of 18 or 10.5 msec under these conditions, so that the attachment times vary, taking the estimate from the mechanical study from 10 msec at high f_{max} to 120 msec when the frequency response is very low.

The percentage of crossbridges attached during oscillation can be calculated.

There are 6 crossbridges every 14.5 nm along the filaments, from the estimates of myosin content of Chaplain & Tregear (1966) and the geometrical analysis of Squire (1971).

The length of half an A filament is 1200 nm, of which 90% bears bridges.

The unit cell is 53 nm square (Miller & Tregear, 1970).

The fibre diameter is 70 μ (Jewell & Rüegg, 1966).

40% of its cross sectional area is myofibrillar (White, 1967).

$\therefore$ Number of myosin molecules/half sarcomere/fibre

$$= 6 \times \frac{1200}{14.4} \times \frac{90}{100} \times \frac{\pi\, 35^2 \times 10^6}{0.85 \quad 53^2} \times \frac{40}{100}$$

$$= 30 \times 10^7 \text{ molecules/half sarcomere/fibre}$$

p_{max} has been calculated as 12 pN/bridge; if the muscle is oscillating the mean tension exerted will be $p_{max}/2$, i.e. 6 pN

$\therefore$ total force of all bridges attached $= 30 \times 6 \times 10^7$

$$= 180 \times 10^7 \text{ pN/fibre}$$

Actual maximum force observed is approximately 30 mg/fibre, i.e. 30×10^7 pN/fibre

$$\therefore \text{ \% bridges attached} = \frac{30 \times 10^7 \times 100}{180 \times 10^7}$$

$$= 17\%$$

The calculation is based on the assumption that only one myosin head can operate at a time. If they are capable of attaching and pulling independently the estimated is halved, i.e. 8.5% of bridges are attached.

From measurements of equatorial reflections interpreted as the transfer of matter from the myosin to the actin lattice points, Miller & Tregear (1970) estimated a 10-20% attachment during oscillatory contraction so the calculation based on this work gives a similar figure.

6. Analogy with other work

Other work in similar fields must be reviewed. The concept of tension cost, or its inverse function, holding economy, is not new. Schädler (1967) measured the ATPase activity of a variety of muscles at different tensions, altering calcium concentration rather than stretch. He observed that the holding economy was much higher, i.e. the tension cost was lower by a factor of ten, in striated muscle than in tonic smooth muscle. Rüegg & Stümpf (1969b) observed a proportional increase in ATPase activity when the isometric fibre tension was increased by stretching or by addition of calcium. The tension cost observed is similar to that obtained here. Breull (1971) measured the ATPase activity accompanying various degrees of quick stretch, and so various tensions, of fibres of glycerinated L. annulipes and L. maximus. He correlated the higher tension cost in L. annulipes with the faster natural wing beat speed, rate of rise of delayed tension and frequency of isometric oscillation. In each case the figure for L. annulipes was about 1.7 times that for L. maximus. So in this work, as in that of Schädler (1967), the tension cost has been found to be greater in muscle with a faster contraction velocity. In Breull's work the differences in *in vivo* speed of operation are reflected in the experimental indices of mechanical characteristics.

Goldspink, Larson & Davies (1970a,b) measured the holding economy of a variety of vertebrate muscles from chick and hamster and found that the cost of tension maintenance was correlated with the measured speeds of contraction.

Bárány (1967) measured the speed of contraction of vertebrate muscles and then extracted the myosin and estimated the activity of the calcium-activated actomyosin ATPase. Bárány & Close (1971) performed similar experiments on rat fast and slow muscles, self- and

cross-innervated. Both the mechanical and biochemical characteristics of the cross-innervated muscles were found to change to suit the innervating nerve. The speed of contraction was altered, and similarly the pH profile curve of the extracted myosin and its AM-ATPase activity.

It was assumed that as the myosin was fully saturated with actin, the myosin ATPase activity was a measure of detachment, rather than attachment probability. Rate constants can be deduced from (i) the mechanical and, (ii) the chemical data obtained by Bárány & Close.
(i) A maximum speed of sarcomere shortening of 35 μm/second is equivalent, from the force-velocity curve, to a shortening speed of approximately 11 μm/second, i.e. 5 nm/msec.half sarcomere, at maximum power production. A 10 nm movement takes 2 msec so the rate constant is 500 sec^{-1}.
(ii) This speed of shortening is equated with a myosin ATPase activity of 1.0 μmole P_i/min.mg myosin, i.e. 8 moles/mole.sec. Thus the rate constant derived from the myosin ATPase activity measurements, 8 sec^{-1}, is very much lower than that from the mechanical data. One possible explanation is that the myosin was not fully activated. The work of Eisenberg & Moos (1968) shows that the maximum rate of ATPase activity of actomyosin is one-tenth that of acto-heavy meromyosin, so that the rate-constant measured may after all be that of attachment rather than detachment.

Nevertheless there is evidence, as has been shown, in a variety of preparations, that optimum velocity of contraction can be related to the cost of tension production. The work of Carlson et al., (1963) and of Maréchal (1964) which demonstrates that increase in tension cost with shortening occurs in vertebrate preparations has already been quoted. The general applicability of the results described in this thesis is shown by the identification of similar phenomena in a variety of other muscles.

7. Summary of conclusions

(a) On a model of contraction involving a crossbridge cycle of detachment and attachment, the duration of the attachment step, and hence the tension produced, are determined by g, the probability of detachment. The velocity of optimum work is also governed by g. Assuming one ATP hydrolysis per cycle, the tension cost is a measure of the tension produced per cycle. f_{max} is a measure of the optimum velocity for work in oscillatory muscle. Therefore these two should be proportional. Analysis of the measurements of tension cost under a variety of conditions which produced change in f_{max} from 2 Hz to 10 Hz shows that f_{max} and tension cost are indeed proportional.

Therefore the theory of a crossbridge cycle involving actomyosin interaction stands.

(b) Tension cost in a muscle allowed to shorten is double that in a static muscle. As $P = \int_0^t p\,dt$ this may be brought about because either p_{av} or t_{av} changes when shortening is permitted. Evidence shows that g, and therefore t_{av}, does not change with movement.

(i) A change in g with position is discounted from results showing that g is constant over a wide range of stretch amplitudes.

(ii) A change in g with velocity should lead to an increase in f_{max} with amplitude of oscillation. This is not observed.

Therefore p_{av} must fall when movement is allowed.

On the hypothesis of a springlike element in the crossbridge with a 10 nm movement a doubling of tension cost in a 2% oscillation is predicted if the crossbridges do not push. Such a doubling of the tension cost is observed.

In view of this, the absence of negative tensions and the high efficiency of over 50% it is assumed that the bridges do not push.

The predicted low tension cost and low efficiency for a 0.2% oscillation are observed in practice.

Therefore the observations confirm the existence of a spring-like element in the crossbridge responsible for the transduction of energy.

(c) From the measurements of ΔG and of extension a force of 12 pN per crossbridge when the spring is maximally extended as in static muscle is calculated.

From this, and from the tension cost, an estimate of the rate constant of detachment is obtained, and from the measurements of f_{max}, a second estimate is obtained.

The duration of attachment is the reciprocal of the attachment rate constant. For a muscle incubated in the presence of P_i at 30°C it is approximately 10 msec.

From the force per individual crossbridge, the percentage of attached bridges is estimated at approximately 15%.

8. Further work

There are a number of ways in which the work in this thesis could be extended. They fall into three categories:

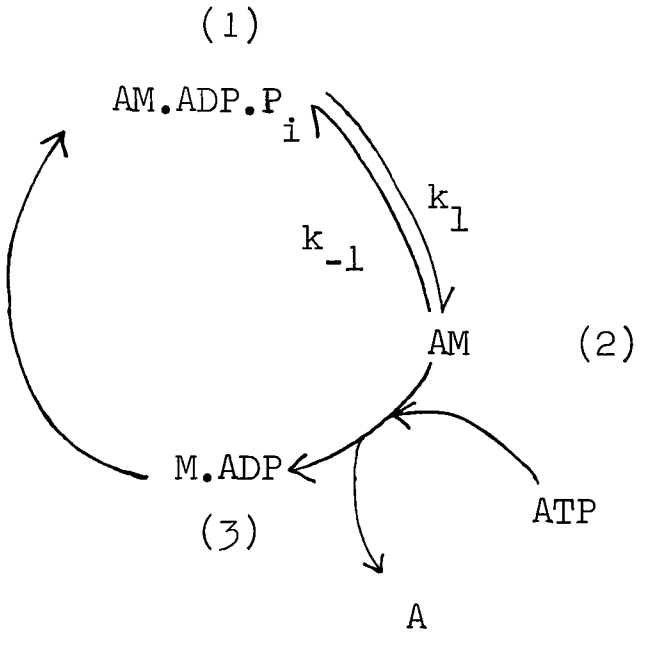
(i) Examination of the explanation for the effect of the different ions on the detachment rate constant.

(ii) Parallel experiments to those performed to test the effect of varying other parameters and thus to add more data to that already obtained.

(iii) Experiments to seek an independent confirmation of the hypothesis and to look at other consequences of it.

These points will be considered in turn.

(i) As inorganic phosphate is one of the products of ATP hydrolysis, it is possible that it has its effect on the probability of detachment by influencing one of the steps in the crossbridge cycle. White, in unpublished results, has shown that the delayed tension changes following changes in length can be modelled by a three-state system:



State 1 is the tension generating step. In the absence of P_i k_{-1} is low and consequently non-linearities arise. It is necessary that P_i should increase not only the rate of the basic reaction, k_{-1}, which would be expected from simple equilibrium kinetics, but also that it should increase k_1, a less straightforward effect, for the increase in detachment rate constant to arise. It can be postulated that there is a second attachment site for phosphate in addition to the active site and that k_1 and g are greatly reduced in the absence of P_i. The action of pyrophosphate and sulphate, which lower the rate constant in the absence of phosphate, can be interpreted on such a postulate. If binding of P_i at an allosteric site is necessary for detachment, then pyrophosphate and sulphate, both of which are possible phosphate mimics, may, if the concentration of P_i is sufficiently low, temporarily block the binding site and therefore delay detachment.

There is no evidence for a phosphate binding site on myosin distinct from the active site. Similarly pyrophosphate binding sites have all been identified as the site where ATP normally binds (e.g. Nauss & Gergely, 1967). It is not necessary for operation of the postulated scheme that the phosphate allosteric site be on the myosin; it could affect the reaction step equally well from a site on the actin subunit.

There is therefore room for investigation into the binding properties of phosphate on both actin and myosin with a particular view to discovering how it exerts its effect on the rate constant of detachment.

(ii) There are a number of ways in which the type of experiments described here could be continued. It is clear that the effect of pH needs further investigation and other parameters can be varied. The effect of calcium is a particularly important factor to examine as it has been shown that it increases the rate constant of detachment (Abbott, 1972). ATP analogues which are split by myosin and permit oscillatory work output might prove a fruitful field of investigation.

On the spring theory outlined the amplitude of oscillation is critical. Only two amplitudes were investigated in this work, 0.2% and 2%. The examination of tension cost and efficiency at 1% would be particularly valuable as at this amplitude the peak to peak movement of length, 12 nm, is similar to the estimated distance over which the crossbridge has to move to deliver all its potential energy. Experiments at 3% amplitude would present technical problems but the tension cost at such high amplitude should provide definite results to show whether or not bridges are capable of pushing as the total length change at 3% is much larger than the work producing crossbridge movement.

(iii) There are numerous ways in which other types of experiment could be employed specifically to investigate the particular hypothesis represented here.

Possible independent checks on the tenet that it is the rate constant of detachment that is changed under the different conditions might be carried out using biochemical kinetic analyses. Using intact muscle preparations and proportional counting techniques X-ray diffraction could be employed. If detachment occurs earlier in conditions producing a high f_{max}, then this should be detectable from analysis of the timing of the shift in density from I filament lattice to A filament lattice during the cycle of oscillation.

On the spring model, the presence of actin is necessary for the manifestation of the transducing mechanism, so it is not surprising if the investigation of the molecular structure of isolated myosin, though it has shown the molecule to be flexible, has not permitted the identification of an elastic component. Therefore allied studies should be continued but looking at myosin in situ. X-ray diffraction is a possible technique. A number of very high angle reflections have been identified which are characteristic of coiled α-helices. Miller & Tregear (1972) also recognise interference maxima on reflections either side of the equator as deriving from the regular arrangement of light meromyosin chains. These and other features of the very high angle pattern of muscle, relating to the internal structure of the constituent molecules, present a new and promising field of investigation.

To conclude, there are a number of obvious experiments in the series with which this thesis is concerned which remain to be done, in particular the effect of calcium and of other oscillation amplitudes. If the results are consistent with the hypothesis of muscular contraction which has been

described and which the results already obtained are evidence for, then further steps in the investigation may most profitably take other lines of approach.

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