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REVIEW

The mechanochemistry of force production in muscle

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Introduction

During shortening of a striated muscle fibre, the thick myosin filaments slide past the thin actin filaments (Huxley & Hanson, 1954; Huxley & Niedergerke, 1954). The energy for this sliding filament movement is supplied when ATP is hydrolysed at the active centre of the myosin molecule; the force for filament movement is generated during the time that the heads of the myosin crossbridges are bound to the actin, whereas the hydrolysis step occurs when the myosin is detached from the actin. This article reviews experiments which made it possible to separate the step of force generation from the step of ATP hydrolysis and in which it was possible to study both the mechanical and biochemical events of the force generating step in the crossbridge cycle. The paper also provides a theoretical treatment of the relationship between mechanics and chemistry. The experiments were performed on skinned muscle fibres and made use of ATP analogues which are not cleaved by myosin but which bind to the active site on the myosin molecule, in particular β,γ-imido ATP (Yount *et al.*, 1971).

The mechanical crossbridge cycle

It is generally believed that the mechanical performance of a muscle can be accounted for by crossbridges acting between actin and myosin filaments and undergoing a mechanical cycle between an attached and a detached state (Huxley, 1957). This crossbridge concept was further supported by Huxley & Simmons (1971) who showed that in experiments where tension transients were induced by sudden length changes, the elastic tension responses of tetanized frog semitendinosus fibres

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vary in direct proportion to the degree of overlap between myosin and actin filaments, thus indicating that these transients are almost entirely properties of the crossbridges themselves. In particular, if a length change is applied sufficiently rapidly so that none of the biochemical reactions have time to take place, then the magnitude of the stiffness of the muscle measured with this high time resolution (called the *immediate stiffness* in this paper) will be a measure of the number of crossbridges attached to the actin at the instant the length change was applied (Huxley & Simmons, 1973; for fibrillar insect flight muscle, cf. Herzig, 1977; White *et al.*, 1977).

The nature of the initial relaxation of tension following a length step and the appearance of transiently produced heat (Hill, 1938; Woledge, 1971; cf. Huxley, 1974) led Huxley & Simmons (1971) to introduce a brilliantly simple model with several attached states rather than to extend Huxley's (1957) two state model [as did Podolsky *et al.* (1969)]. Fig. 1 shows a schematic representation of this model with two attached and one detached state: (a), (b) and (c) (cf. also Huxley, 1969). In state (a) crossbridges with unloaded elastic elements (springs) are attached to the actin in a perpendicular position, a conformation which Huxley (1969) postulated from X-ray diffraction studies. The bridges may then rotate to the acute-angled position (b) first described by Reedy *et al.* (1965) on the basis of electron micrograph and X-ray diffraction patterns. During the transition from state (a) to (b) the crossbridges remain attached to the actin filament. This rotation which may proceed in several steps stretches the elastic elements of the crossbridges thereby generating the elastic forces within them. Binding of ATP is involved in order to break the actin–myosin linkages of the acute-angled conformation (b); the detached crossbridges (c) are thereby produced which, after hydrolysis of ATP, reattach to the actin filament in the

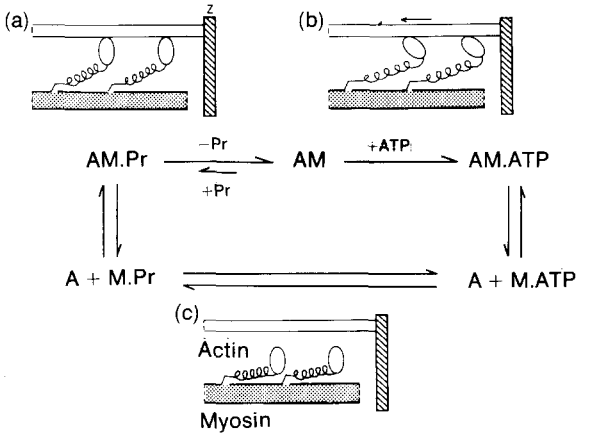

Fig. 1. Schematic representation of the crossbridge cycle in contracting muscle and the biochemical pathway of actin (A)-activated ATP splitting by the myosin heads (M, S-1 fraction). Sarcomere states a, b, c represent three crossbridge states of the Huxley & Simmons (1971) model.

perpendicular position (a). According to the proposal of Huxley & Simmons (1971), force generation in muscle takes place through a mere conformational change of crossbridges attached to actin; force generation and splitting of ATP constitute separate steps of the crossbridge cycle.

The biochemical cycle

The relationship between biochemical events involved in ATP-splitting and the mechanical events have been recently reviewed (White & Thorson, 1973; Eisenberg & Hill, 1978) and may be summarized as follows. ATP molecules combine with actomyosin (or acto S_1) giving rise to a transient ternary actomyosin–ATP complex (Fig. 1; Lymn & Taylor, 1971). This rapidly dissociates into actin and myosin–ATP (A + M–ATP), a process corresponding to detachment of crossbridges by ATP [(b) to (c), Fig. 1]. If actin concentration is not high (Stein *et al.*, 1979), it is M–ATP rather than AM–ATP which is reversibly hydrolysed to a myosin–product complex (product = $ADP.P_i$), that is, ATP is split when the crossbridges are detached from the actin (Bagshaw *et al.*, 1974) (Fig. 1c). This myosin–product complex has a long half-life in the absence of actin (Lymn & Taylor, 1970). There is evidence that this product complex can react rapidly and reversibly with actin (Lymn & Taylor, 1971). This process corresponds to attachment of crossbridges [(c) to (a), Fig. 1] in the mechanical cycle. The subsequent dissociation of the AM–Pr complex, which exhibits enhanced fluorescence by comparison with AM, presumably occurs in at least two stages, first, a change in conformation and, second, product release since both a conformational change and a product release are required to complete the cycle (White & Taylor, 1976; Johnson & Taylor, 1978). The latter change in conformation may be associated with the force generating step [(a) to (b), Fig. 1] in the Huxley & Simmons (1971) model (cf. also Lymn & Taylor, 1971). The ternary AM–ADP complex (Beinfeld & Martonosi, 1975; Schaub *et al.*, 1975) is presumably not equivalent to the ternary AM–Pr complex (White, 1977). (For further references on the biochemical cycle cf. Bagshaw & Trentham, 1973; Mannherz *et al.*, 1974; Wolcott & Boyer, 1974; Marston & Weber, 1975; Trentham *et al.*, 1976; Taylor, 1979.)

A model experiment

Force generation could be explored in isolation from other steps of the cycle if one could find a ligand which is not split by myosin and binding of which to the myosin heads induces an alteration of the state of attached crossbridges. ADP might constitute such a ligand. However, AM–ADP is not identical to the AM–Pr complex (White, 1977); furthermore, it might be a triphosphate nucleotide which changes the angular position of attached myosin heads. The ATP analogue AMPPNP (β, γ-imido ATP) is a substance which binds to the myosin heads (Yount *et al.*, 1971; Schaub *et al.*, 1975) but which is not cleaved by the myosin ATPase (Yount *et al.*, 1971; Bagshaw *et al.*, 1974). AMPPNP binds with a high affinity to the crossbridge heads in glycerinated muscle fibres (Marston *et al.*, 1976), when it causes muscle in rigor to

relax. However, this relaxation is a pseudorelaxation since it takes place without a concomitant change of immediate stiffness (Beinbrech *et al.*, 1976) or low frequency stiffness (Marston *et al.*, 1979). As revealed by X-ray diffraction and electron microscopic studies (Goody *et al.*, 1975; Beinbrech *et al.*, 1976; Marston *et al.*, 1976) binding of AMPPNP induces distinct structural alterations of the attached state of the crossbridges. These effects of AMPPNP and of other ATP analogues (cf. Barrington Leigh *et al.*, 1973) are described and discussed in the next section.

Isometric relaxation–contraction cycles in the absence of ATP hydrolysis

As mentioned in the Introduction the ATP analogue AMPPNP (β, γ-imido ATP) is suitable for experimentally testing whether force can be generated without concomitant ATP splitting. Since AMPPNP must be able to interact with the crossbridge heads within the fibre, the diffusion barrier of the sarcolemma must be removed so that the analogue can diffuse from the incubation bath to the fibre. This can be achieved by an osmotic shock technique in which the fibres are chemically skinned by glycerol extraction (Jewell & Rüegg, 1966). The muscle preparations used in the experiments discussed in this paper (for exceptions see below) were small bundles or single fibres from the dorsal longitudinal muscle of the giant water bug *Lethocerus maximus*. These muscles are particularly suitable for studying the mechanical (Jewell & Rüegg, 1966) and structural (Reedy *et al.*, 1965) aspects of muscular contraction (cf. Tregear, 1977; Pringle, 1978).

Isometric relaxation and contraction induced by addition and removal of AMPPNP

A state of rigor contraction is achieved when ATP is washed out from glycerol-extracted muscle fibres relaxed in an ATP saline with low Ca^{2+} concentration (White, 1970). Addition of AMPPNP to single fibres or small bundles of up to eight fibres from dorsal longitudinal muscle from *Lethocerus maximus* brings about a monotonic fall of isometric tension with increasing AMPPNP concentration (Barrington Leigh *et al.*, 1973; Kuhn, 1973). The immediate stiffness of the fibres measured by small length changes (up to $\pm 0.3\%$ L_i) completed within less than 1 ms remains nearly unaffected for AMPPNP concentrations up to 0.5 mM (Beinbrech *et al.*, 1976). These authors also showed that the slopes of the T_1 curves (cf. Huxley & Simmons, 1971) remain nearly unaffected by the size of the length step. The amount of the complex stiffness response to a 5 or 10 Hz sinusoidal modulation of length is also not altered by AMPPNP in the same concentration range (Marston *et al.*, 1976, 1979). However, there have been no systematic studies of the influence of AMPPNP on the elastic and viscous components of dynamic stiffness (cf. Fig. 5 in Marston *et al.*, 1979).

The first time that a fibre relaxed with AMPPNP is returned to a rigor solution there is a partial restoration of isometric tension (Fig. 2). Subsequent removal and readdition of AMPPNP leads to reversible lowering and raising of isometric tension,

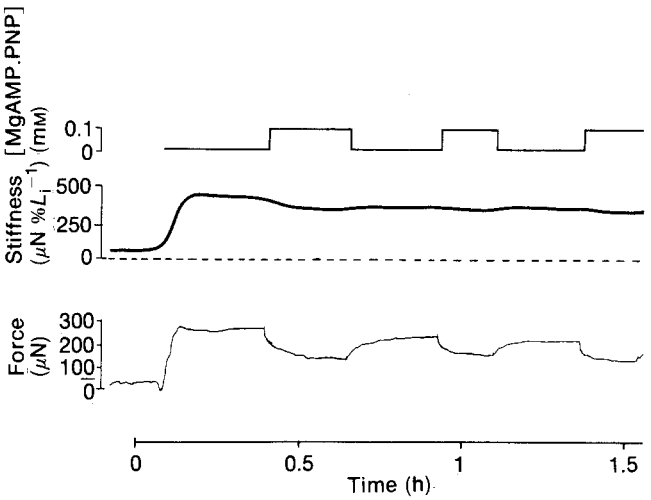

Fig. 2. Effect of 0.1 mM AMPPNP (top) on 1000 Hz stiffness (middle) and isometric force (below) of a single glycerinated fibre from the dorsal longitudinal muscle of the giant water bug *Lethocerus maximus*. The single fibre preparation was first relaxed by incubation in a saline containing 15 mM MgATP and 4 mM EGTA. Washing out the ATP from the fibre-induced rigor contraction, that is, a state of high isometric tension and high 1000 Hz stiffness. Addition and removal of AMPPNP induced isometric contraction–relaxation cycles which take place without appreciable alterations in 1000 Hz stiffness (pseudorelaxation). Conditions: pH 6.7; I = 0.06 M; 13° C.

but produces no appreciable changes in the values of immediate stiffness (dynamic 1000 Hz stiffness, Fig. 2). The effects of AMPPNP on glycerinated fibrillar fibres are thus mechanically reversible under isometric conditions (Kuhn, 1978b; Marston *et al.*, 1979). It follows that force in muscle may be maintained by an equilibrium between two distinct conformations of attached myosin heads and hence that force generation in muscle is *not a priori* correlated to ATP splitting.

AMPPNP-induced changes of crossbridge conformation

X-ray diffraction and electron microscopic investigations have shown distinct alterations of crossbridge states when a rigor muscle was irrigated with AMPPNP. The results of AMPPNP-induced changes in X-ray diffraction pattern both in fibrillar flight muscle of *Lethocerus* (Barrington Leigh *et al.*, 1973, 1977; Goody *et al.*, 1975; Holmes *et al.*, 1976; Marston *et al.*, 1976) and rabbit psoas muscle (Lymn & Huxley, 1973; Lymn, 1975) essentially show that the majority of AMPPNP-loaded bridges remain attached to the actin. This conclusion derives from the maintenance of a high intensity of the 38.8 nm reflections (actin periodicity intensified by attached crossbridges) in the presence and absence of AMPPNP. The main differences between rigor crossbridge states and AMPPNP states are that in the latter the 14.5 nm meridional reflection and the associated layer lines are strong, the (1.0) pattern on the 38.8 nm becomes much weaker and the rest of the actin-based

reflections become somewhat weaker (Barrington Leigh *et al.*, 1973; Lymn & Huxley, 1973; Goody *et al.*, 1975; Marston *et al.*, 1976). The equatorial intensity ratio (1.0)/(2.0) also alters in the direction of relaxed muscle (Lymn, 1975; Goody *et al.*, 1976; Marston *et al.*, 1979). Electron micrographs, the optical transforms of which exhibit the essential characteristics of the X-ray diffraction pattern, showed an increase in the number of crossbridges attached to the actin in a vertical position (cf. Huxley, 1969) when AMPPNP binds to the myosin heads (Beinbrech *et al.*, 1976; Marston *et al.*, 1976).

Interpretation of isometric relaxation–contraction cycles induced by AMPPNP in terms of the Huxley and Simmons model

The binding studies of Marston *et al.* (1976) have shown that in glycerinated fibrillar muscle preparations AMPPNP molecules bind to myosin heads in a 1 : 1 ratio. If it is assumed that the isometric relaxation effect of the myosin heads is proportional to the number of ternary AMPPNP-actomyosin complexes, the concentration dependence of isometric tension should follow a simple hyperbola.

$$\Delta f = f_R - f = \Delta f_0 c/(c + K_d) \quad (1)$$

Where f_R and f are the tensions observed in the rigor and AMPPNP states of the muscle, c is the AMPPNP concentration, the front factor Δf_0 is the amplitude of the

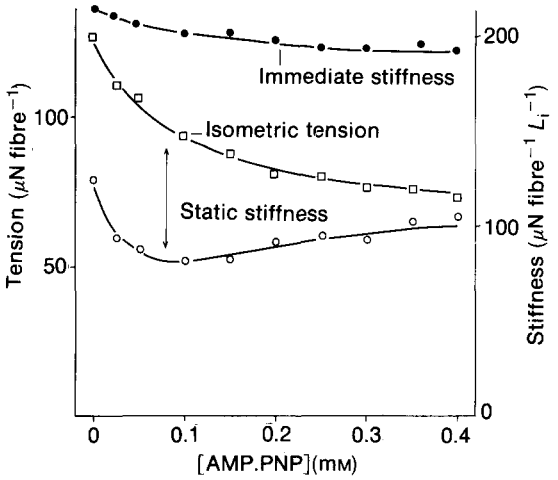

Fig. 3. Plots of isometric tension, immediate and static elastic modulus (mean values of 12 experiments) versus AMPPNP concentration in glycerinated bundles (5–6 fibres) from *Lethocerus maximus*. The decline of isometric tension with increasing AMPPNP concentration follows a hyperbola (solid line). Immediate elastic modulus is not altered significantly by AMPPNP whereas static elastic modulus values which exhibit a minimum near 0.1 mM AMPPNP are best fitted (solid line) by an increase in the affinity constant of AMPPNP binding to the myosin heads by a factor of 2 for a 0.5% L_i stretch of the fibre. Conditions: pH 6.7; I = 0.06 M; 18° C.

tension decreases observed between rigor conditions and saturating AMPPNP concentrations and K_d is the apparent dissociation constant of the ligand AMPPNP from the myosin heads. (A complete list of symbols and abbreviations is given on pp. 34–35.) The solid line through the isometric tension values of Fig. 3 is indeed a Gaussian fit with respect to a simple hyperbola (Equation 1). This fitting procedure gives a value for the dissociation constant $K_d = 85\ \mu M$ (Kuhn, 1978b; cf. also Marston *et al.*, 1979). Binding studies of AMPPNP to the active centre of myosin in glycerol-extracted dorsal longitudinal muscle of *Lethocerus cardofanus* gave a value of $K_d = 115\ \mu M$ (Marston *et al.*, 1976). From equatorial X-ray diffraction studies a value for K_d of $95\ \mu M$ was calculated (Goody *et al.*, 1976). All three methods of investigation give rise to approximately the same value for K_d (for a possible dependence of such K_d values on fibre tension, cf. section on stretch-dependence of binding). It may therefore be concluded that the isometric relaxation effect induced by AMPPNP is proportional to the number of myosin heads loaded with AMPPNP.

When AMPPNP is washed out from the fibres under isometric conditions tension increases but immediate stiffness does not change appreciably (Fig. 2). In accordance with the evidence given by Huxley & Simmons (1971), this indicates that the number of crossbridges attached to the actin is not altered appreciably when isometric force is generated by removal of AMPPNP from the fibres (Beinbrech *et al.*, 1976). This conclusion is confirmed by the maintenance of the intensities of the actin-based X-ray diffraction reflections (see above) as well as by the biochemical studies of Hoffman & Goody (1978) and Marston *et al.* (1979) who investigated in the presence and absence of AMPPNP the ternary association of actin, subfragment-1 (S-1) and nucleotide. From such experiments, Marston *et al.* (1979) concluded that little detachment should occur when AMPPNP binds to the attached myosin heads. *Force generation therefore seems to be correlated with a conformational change of attached bridges. Hence, generation of force by washing out AMPPNP from muscle is an experimental model for the force generating step of the 1971 contraction model of Huxley and Simmons* (Beinbrech *et al.*, 1976; Marston *et al.*, 1979). When AMPPNP is removed from the fibre that ligand dissociates from the myosin heads. Thereby the crossbridges rotate to an acute angled position (Beinbrech *et al.*, 1976) or they are bent by internal changes of the shape of the heads (Marston *et al.*, 1976) while they remain attached to the actin. Force is generated when such a conformational change within the crossbridges stretches their elastic elements and moves part of their mass from the actin filament towards the myosin filaments. However, in interpreting the results of their X-ray diffraction data Barrington Leigh *et al.* (1977) and Holmes (1977) concluded that this rotational model (Huxley, 1969) does not fit the whole information obtained from their structural results. Based on experiments in which the actin filament was decorated with molecules from the S-1 fraction of myosin Barrington Leigh *et al.* (1977) proposed a phenomenological model according to which crossbridges in muscle could be attached to the actin filament in at least two states which may be called r- and p-bridges (for similar notation, cf. Marston *et al.*,

1979; Tregear & Marston, 1979). These two states of crossbridge attachment would differ in their stereoaffinity to the actin. In rigor muscle the r-bridge would constitute the dominant species. These r-bridges are ordered in the 38.8 nm actin array which arises from an unequal distribution of attached crossbridges along the actin filament. Conversely, the p-bridges are distributed more equally along the actin filament, so that the 14.4 nm repeat arising from the myosin filament becomes the dominant array in p-bridge modulated muscle. In analysing their X-ray diffraction pattern by this model, Barrington Leigh *et al.* (1977) concluded that binding of the ligand AMPPNP to crossbridges in the r-state of rigor generates an at least partial redistribution of attached crossbridges on the actin filament, so that the bridges move to their p-state characteristics.

The effect of AMPPNP on other preparations

Other glycerol-extracted preparations from striated muscle (rabbit psoas muscle, tortoise ileofibularis muscle, alligator leg muscle) also monotonically relax without a concomitant change of immediate stiffness when the AMPPNP concentration is increased (to up to 1 mM) under isometric conditions. The tension in the leg muscle preparation from the alligator (but not the tension in the other preparations) was partially restored to the initial value in rigor when AMPPNP was removed from these fibres (Heinl & Kuhn, unpublished results).

When the Ca^{2+} concentration was varied over the range 0.01–10 μM by means of the Ca-buffer EGTA in the presence and absence of AMPPNP neither isometric tension nor immediate stiffness was affected. AMPPNP did not induce an isometric relaxation effect in the absence of Mg^{2+}, indicating that the Mg AMPPNP complex (Yount *et al.*, 1971) is responsible for pseudorelaxation of these fibre preparations. It is necessary that the ionic strength and pH of the rigor solutions and the solutions containing AMPPNP are the same since both in the absence and in the presence of AMPPNP isometric tension depends on ionic strength and pH (Kuhn, 1974; Kuhn *et al.*, 1973) presumably due to swelling of the filament lattice (Rome, 1972). As revealed by equatorial X-ray diffraction studies (Goody *et al.*, 1976) salines of different AMPPNP concentrations do not induce a swelling effect when adjusted to the same ionic strength.

Other nucleotides which induce isometric relaxation in glycerol-extracted fibres

Other ATP analogues [α, β-methylene-ATP (ADCPP, Miles Biochemicals) and 5′-*o*-3-thiotriphosphate (ATP-γ, S; Goody & Eckstein, 1971)] also exhibit an isometric relaxation effect (Barrington Leigh *et al.*, 1973). In contrast to AMPPNP, these two ATP analogues seem to dissociate crossbridges from the actin (Goody *et al.*, 1975). Neither estimates of immediate stiffness nor binding studies for these two analogues have been reported.

Use of ADP as a relaxant of isometric tension involves the problem of disproportionation into ATP and AMP by the adenylate kinase still present in a

glycerinated muscle preparation (Abbot & Leech, 1973). In the presence of a powerful myokinase inhibitor (diadenosine-pentaphosphate, Feldhaus *et al.*, 1975) and an ATP-exhausting enzymatic system (hexokinase and glucose) small but reversible isometric tension relaxations induced by 0.5 mM ADP at constant immediate stiffness could be observed (cf. also Marston *et al.*, 1979). But at higher ADP concentrations (for example, 1 mM) isometric tension was sensitive to the concentrations of myokinase inhibitor used. The results from mechanochemical and structural studies of ADP (cf. Rodger & Tregear, 1974) should therefore be interpreted with care: MgATP at low concentrations ($\sim 5\ \mu$M) induces contraction (White, 1970; Reuben *et al.*, 1971).

In studies using pyrophosphate (PP_i), Weber (1951) and Portzehl (1952) showed that glycerinated fibres and reconstituted actomyosin fibrils in a state of isometric rigor tension relax when pyrophosphate is added (plasticiser effect, for review cf. Weber & Portzehl, 1954). White (1970) was able to show that PP_i induced relaxation is in fact a pseudorelaxation, that is, there is a reduction of isometric tension and static stiffness without reduction of 5 Hz dynamic stiffness. Kuhn *et al.* (1972) showed that washing out 0.5 mM PP_i from fibre bundles of fibrillar insect flight muscle caused a small contraction. Optical transforms of electron micrographs from PP_i-irrigated fibres showed similar patterns to those from fibre preparations fixed in the presence of AMPPNP (Beinbrech *et al.*, 1972; Beinbrech, 1977).

Teinochemistry of AMPPNP binding in glycerinated muscle

Teinochemical principles

A valuable concept in explaining the observed mechanochemical effect of AMPPNP in muscle is the teinochemical principle first formulated by Kuhn *et al.* (1960) (the Greek word τεινειν means to stretch or dilate). This principle can be summarized in the following two statements. (1) Contractile systems can fully and reversibly transform chemical energy into mechanical work provided that the system can be reversibly changed by the addition of chemical reagents and by length changes. (2) Such energy transformations are coupled with a teinochemical effect; reagents which induce relaxation under isometric conditions are absorbed by the contractile system when this is stretched at constant thermodynamic activity of that reagent.

In studies with ionized polyacrylic acid gels with a moderate degree of cross-linking, it was shown that these contractile systems exhibit thermodynamic equilibrium between osmotic forces and electrostatic repulsion (Kuhn, 1949; Katchalsky, 1949; Breitenbach & Karlinger, 1949). The gels shrunk by addition of H^+ ions (in the form of HCl) to the incubation bath which was originally held at neutral pH (Kuhn *et al.*, 1960). During this shrinkage the gel foils lifted a weight and performed mechanical work against gravitational forces (Fig. 4; Kuhn *et al.*, 1961). Removal of the H^+ ions by addition of NaOH induced the gels to extend; the original length was regained when the pH was adjusted to the original value. Pryor (1953)

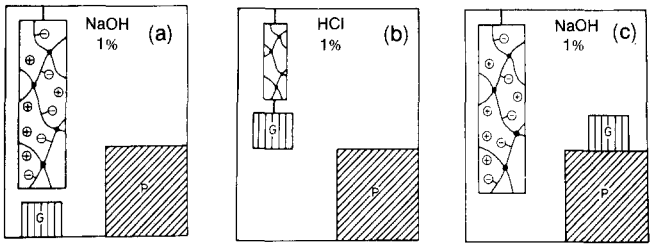

Fig. 4. Transformation of chemical energy into mechanical work with slightly cross-linked films of polyacrylic acid gels. (a) The film is immersed in a 1% sodium hydroxide solution whereupon it dilates. (b) If a weight (*G*) is attached to the film and the incubation solution replaced by 1% hydrochloric acid, the film shrinks lifting thereby the weight (*G*) to the height of the step (*P*). (c) By substitution of sodium hydroxide again the film regains its initial swollen state. The free energy supplied by the chemical reaction NaOH + HCl = NaCl + H_2O is turned during steps a–c into potential energy which is equal to the mechanical work performed when the weight (*G*) is lifted to the height of the step (*P*).

and Katchalsky (cf. Steinberg *et al.*, 1966) succeeded in constructing engines the power of which was supplied when LiBr dropped from a high to a low concentration; the transport of the LiBr occurred via a regenerated collagen fibre which contracted at high LiBr concentrations and relaxed at low LiBr concentrations. By means of such engines it was directly demonstrated that the free energy set free when a salt is diluted can be transformed into mechanical work. The H^+ ions thus acted as a teinochemical reagent inducing contraction. The polyelectrolyte gels are therefore a contractile system which fulfils the first condition of the teinochemical principle: they could be induced to perform reversible mechanical work. As pointed out by Kuhn *et al.* (1960) these gels should exhibit a teinochemical effect: the H^+ concentration in the incubation bath should increase when the gel is stretched. This prediction was verified experimentally when it was found that the pH of the incubation solution could be decreased in a reversible manner by stretching the gel foils.

Kuhn *et al.* (1960) and Katchalsky *et al.* (1960) quantified the teinochemical effect (cf. also Wiegand, 1925; Barkas, 1942; Warburton, 1946; Gee, 1946) on the basis of thermodynamic cross-relations presented by Gibbs (1961):

$$\Delta N_L = -(\Delta L/RT)[\partial f/\partial(\ln c)]_{T,L} \tag{2}$$

ΔN_L in Equation 2 is the number of moles of the teinochemical active substance (H^+ ions) which are absorbed by the contractile system (the gel) when it is stretched from initial length L_i to $L_i + \Delta L$. In the right hand expression of Equation 2, $(\partial f/\partial \ln c)_{T,L}$ is the increase of isometric force per unit of the change of ln*c* of the teinochemically active substance (for example, $c = H^+$ activity); RT is 2400 J mol^{-1} at 286° K.

The teinochemical relation (Equation 2) could be verified quantitatively for pH-sensitive polyacrylic acid gels (Kuhn *et al.*, 1961) and also for the same gels using

Ag^+ ions as the teinochemically active reagent (Pohl *et al.*, 1966). Teinochemical relations have also been studied for water absorption by hair (Treloar, 1952), for Ca^{2+}- and H^+-induced contraction of denatured actomyosin systems at pH 3 (Kuhn *et al.*, 1962) and for Ca^{2+}-induced contraction of the spasmoneme in vorticellids (Amos, 1971, 1975).

There is a direct link between the contractile structures of muscle and the polyelectrolyte gels (for reviews on polyelectrolytes see Katchalsky, 1954; Eisenberg, 1977). Both appear to be sensitive to variations of electrochemical parameters; ionic strength, pH changes or addition of divalent cations affect the interfilament spacing (Rome, 1967, 1968) and isometric rigor tension (Bozler, 1952; Kuhn *et al.*, 1973; Kuhn, 1974) in glycerinated muscle fibres as well as affecting the shape of ionized gels. An electric repulsion theory of muscle contraction which was partially based on such a Donnan osmotic effect was put forward by Elliott (1967, 1968; cf. also Matsubara & Elliott, 1972).

There is also a more indirect link between the findings of Kuhn *et al.* (1960) with polyelectrolyte gels and the results of studies on muscle contraction, in that the teinochemical principle experimentally verified in artificial contractile systems is derived from general thermodynamic laws without reference to the molecular mechanism involved in the contraction itself. This general aspect of the teinochemical principle will now be discussed.

Performance of mechanical work

The mechanical and structural studies reviewed above indicate that addition of AMPPNP relaxes the muscle preparations without concomitant loss in immediate stiffness (pseudorelaxation) and that removal of the analogue from the fibres generates tension. It was, therefore, appropriate to establish whether binding and dissociation of AMPPNP to and from the crossbridges is able to transform potential energy stored within the elastic elements of the crossbridges into free energy of AMPPNP binding and vice versa. Provided that the teinochemical principle can be applied to muscle preparations pseudorelaxed by AMPPNP, such a transformation of two kinds of free energies should be possible. In order to prove this proposition it will first be shown that AMPPNP induces reversible mechanical work cycles in a length–force diagram, and that performance of mechanical work is made possible by a stretch-dependent binding of AMPPNP to the myosin heads.

Fig. 5 shows, in a length–tension diagram, a work cycle obtained when a muscle preparation (a small bundle of glycerinated fibres from *Lethocerus maximus*) was released in rigor solution and restretched in 0.1 mM AMPPNP solution. The fibrillar muscle preparation in rigor was first released (Fig. 5) within 15 min by 0.5% L_i (A–B). At B, Mg.AMPPNP was added, inducing a partial relaxation (B–C). If the preparation is restretched within 15 min to L_i (C–D) in the presence of AMPPNP, there is a quasi-static increase in tension. If AMPPNP is now washed out, there occurs a recovery of isometric rigor tension to the original value (E); E almost

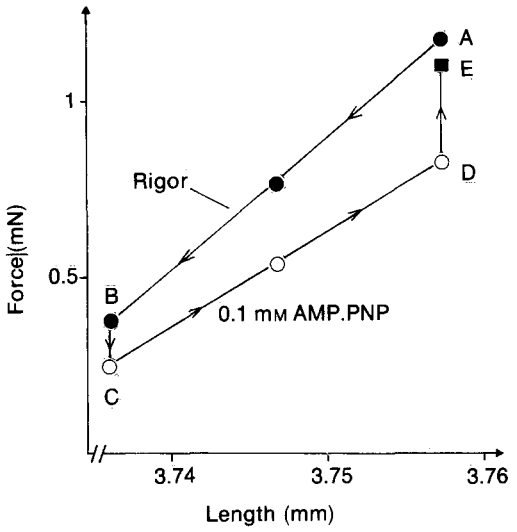

Fig. 5. Length–tension diagram obtained when a bundle (eight fibres) of glycerinated fibres from *Lethocerus maximus* was released by 0.5% L_i in rigor solution and restretched in the presence of 0.1 mM AMPPNP. Note that the isometric relaxation effect produced by AMPPNP is much smaller in the released state than at the initial length. Conditions: pH 6.7; I = 0.06 M; 10° C.

coincides with A. Note that the slope of the length–tension diagram is considerably higher in rigor than in the presence of AMPPNP. Consequently, the quasi-static stiffness of these fibres is lower in the presence of AMPPNP than in its absence, although AMPPNP does not alter immediate stiffness of the fibres (cf. Fig. 2).

During this sequence of events (A–B–C–D–E) the preparation does positive work corresponding to the area enclosed in the force–length diagram. The cycle could also be performed in the opposite direction (E–D–C–B–A); mechanical work was then performed on the fibre. At the end of the negative cycle the fibre bundle has returned to the same state as at the end of the positive cycle. The same mechanochemical state (same tension at the same length and at the same concentration of AMPPNP) is thus attained after positive and after negative work cycles.

This suggests that the chemical reaction between AMPPNP and the fibre bundle as well as the response to lengthening are reversible. It therefore fulfils the prerequisites of the teinochemical principle (Kuhn *et al.*, 1962), that is, a quantitative transformation of chemical free energy into mechanical work and vice versa. The theory of teinochemical systems predicts a teinochemical effect: that an additional amount of substance is bound to the fibre when it is stretched in a solution containing AMPPNP. Conversely, bound AMPPNP should be released into the solution when the fibre bundle is released.

Stretch dependent binding of AMPPNP

Direct AMPPNP binding studies in glycerol-extracted fibres (Marston *et al.*, 1976) have shown that this ligand binds to the active centre of the myosin heads. The apparent affinity constant of AMPPNP to the heads was estimated as $10^4\ M^{-1}$ and the concentration of sites accessible to AMPPNP binding was found to be about 200 μM, that is, all myosin heads seem to be accessible to AMPPNP in the concentration range below 1 mM AMPPNP (cf. also Marston & Tregear, 1972; Tregear & Squire, 1973).

A length-dependent binding of AMPPNP to the crossbridge heads was observed in stretch-release experiments. The fibres were stretched in a compartment S and then released in a second compartment R (Kuhn, 1977). Both compartments initially contained the same AMPPNP concentration (100 μM). By repeating this release-stretch cycle a total of 150 times a concentration increase of AMPPNP in compartment R was observed (Fig. 6; Table 1, column 1). The stretch dependence of AMPPNP binding to the myosin heads was recently confirmed by Marston *et al.* (1979) using a different experimental approach. Fig. 6 also shows that a measurable amount of AMPPNP was transported against a concentration gradient when fibre was released after transferring to compartment R. In a control study, it could be demonstrated that no transfer of AMPPNP occurs if the bound AMPPNP is displaced by 2 mM pyrophosphate added to the incubation baths R and S.

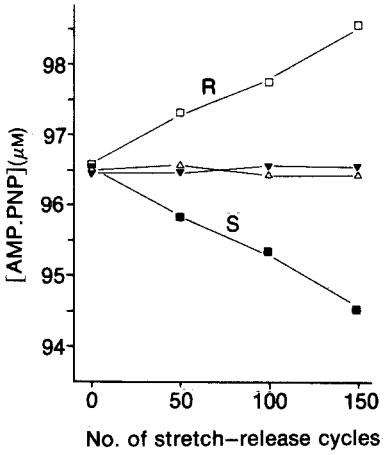

Fig. 6. Teinochemical effect of AMPPNP on release (open symbols) and restretch (full symbols) in absence (squares) and presence (triangles) of 2 mM pyrophosphate. The glycerinated fibre bundle (15 fibres) from *Lethocerus maximus* was first partially relaxed from the rigor state by 96.5 μM AMPPNP under isometric conditions before the bundle was released in compartment R, transferred to compartment S where it was restretched and then retransferred to R (duration of the cycle 3 min). Concentrations of AMPPNP determined from extinction measurements (at 260 nm) after correction for solvent evaporation. Amplitude of length change 60 μm, initial length 5 mm, volumes of compartments R and S 0.4 ml.

Table 1. Comparison of observation and theory in a teinochemical experiment.

	Δc (μM)	$\Delta N_{L,obs}$ (pmol fibre^{-1})	$-(\partial f/\partial c)_{T,L}$ (N M^{-1})	$\Delta N_{L,calc}$ (pmol fibre^{-1})
A	2.8 ± 0.4	0.42 ± 0.07	1.77 ± 0.15	0.43 ± 0.04
B	0.3 ± 0.5	0.05 ± 0.05	1.65 ± 0.1	0.00
C	0.1 ± 0.3	0.02 ± 0.07	0.03 ± 0.05	0.006 ± 0.06

Δc = Concentration difference between bath R where the fibre bundles were released by ΔL and bath S where the fibres were restretched (cf. Fig. 6). Concentrations were determined after 100 stretch–release cycles; $\Delta N_{L,obs}$ = molar amount of AMPPNP transferred per stretch of amplitude ΔL from the incubation bath to one fibre; $-(\partial f/\partial c)_{T,L}$ = slope of the AMPPNP concentration–isometric tension curve (cf. Fig. 3) at 100 μM AMPPNP. $\Delta N_{L,calc}$ = molar amount of AMPPNP transferred per stretch to one fibre as calculated from the teinochemical equation (Equation 2). In experiment B the amplitude of the length change ΔL was zero; in all other experiments it was 60 μm. In experiment C, 2 mM pyrophosphate was added to the solution. All figures are mean values ± S.D. (n = 5). Conditions: initial AMPPNP concentration 100 μM, initial volume 400 μl; pH 6.7; I = 60 mM; 14–20 fibres per bundle of length 5 mm; 13° C.

The molar amount of AMPPNP ($\Delta N_{L,obs}$, Table 1) transferred from the incubation bath to a single fibre by one stretch (amplitude 60 μm) was calculated from the observed concentration differences Δc of Table 1. The values in line A of Table 1 indicate that, at 100 μM AMPPNP, 0.42 pmol AMPPNP are bound to a fibre of length 0.5 cm when it is stretched by 1.2% L_i. Correspondingly, 0.7 pmol AMPPNP would be bound in excess to a fibre of 1 cm length by a 1% L_i stretch. When compared with the density of enzymatic sites (10 pmol cm^{-1}; Tregear & Squire, 1973) present in flight muscle of *Lethocerus*, this value reveals that about 7% of the sites are additionally loaded with AMPPNP by 1% L_i stretch. The amounts of AMPPNP transferred to the fibre during stretch ($\Delta N_{L,obs}$, Table 1) coincide fairly well with values derived using the independently determined expression $\Delta N_{L,calc} = -\Delta L/RT(\partial f/\partial \ln c)_{T,L}$ (Table 1). This provides full experimental proof (cf. Equation 2) that the teinochemical principle can be applied to muscle fibres which are still capable of active contraction (Kuhn, 1977; Marston *et al.*, 1979, obtained similar results by a different method).

AMPPNP is bound by the fibre during stretch (note the decrease of AMPPNP concentration in compartment S, Fig. 5) and AMPPNP is released again when the bundle shortens. This is taken to indicate that AMPPNP binding enables free energy to be stored during stretch (C–D, Fig. 5). This free energy of binding is converted into free energy of dilution during washing out the AMPPNP (D–A, Fig. 5).

Teinochemical aspects of static stiffness

Mechanical experiments should also demonstrate especially whether or not the binding of AMPPNP to the myosin heads is stretch dependent. The teinochemical

principle predicts that there will be a stretch-dependent increase in the amount of the teinochemically active substance, inducing isometric relaxation. This means that after a stretch has ceased there should be stress–strain relaxation due to the binding of the relaxant. In rubber strips swollen in benzene (which was in this case the teinochemically active 'reagent') this effect could be verified and related to the teinochemical relations. After a sudden stretch of that strip there was a gradual fall of the elastic modulus which reached the value of the static elastic modulus asymptotically. During this fall in stiffness the rubber strip absorbed additional benzene, that is, the substance which induced isometric relaxation was taken up into the contractile system following a stretch (Kuhn *et al.*, 1963).

In the case of AMPPNP, the experiments described above indicate that there is a stretch-dependent increase in the amount of AMPPNP bound to the myosin heads as predicted by the teinochemical principle. Further, the additional bound analogue brings about a rotation of attached bridges from an acute-angled to a perpendicular position. This rotation induces an enhanced stress–strain relaxation following a stretch. Conversely, when a fibre bundle is released, part of the AMPPNP is dissociated from the myosin heads. The discharged heads rotate to the acute-angled position and thereby increase the fibre tension. Tension recovery following a release of fibres incubated in AMPPNP solutions should consequently be enhanced relative to the recovery observed in rigor. The time dependence of this enhanced recovery of tension reflects the rates of AMPPNP dissociation from the myosin head and/or the rates of the conformational changes of the heads. If a length change is performed and one waits until stress–strain relaxation or recovery of tension has ceased, one obtains T_∞ curves. These correspond to teinochemical equilibrium reactions which are comparable to the T_2 curves of Huxley and Simmons. The slopes of these T_∞ curves (cf. also Fig. 5, A–B and C–D) are a measure of static stiffness. Static stiffness in rigor has only about 50% of the value of immediate stiffness in rigor. It falls further on adding AMPPNP up to 100 μM, but then rises again from 100 μM to 400 μM (see Fig. 3). Note however, that the immediate stiffness remains nearly unaffected by AMPPNP. Half of the decrease in isometric tension brought about by AMPPNP is reached at 100 μM, at which concentration the static stiffness is at a minimum.

Stretch-dependent AMPPNP binding to the myosin heads could be attributable either to a stretch-dependent recruitment of actomyosin sites accessible to AMPPNP binding and/or a stretch-dependent lowering of the dissociation constant. The curve of static stiffness with increasing AMPPNP concentration allows a discrimination between these alternatives. Following Kuhn (1978b) the difference of static stiffness (ΔS_{stat}) between the values of static stiffness in rigor (S_R) and static stiffness (S) at the AMPPNP concentration (c) used, can be calculated from the slope of the equilibrium force difference Δf (Equation 1) in a length–tension diagram, by assuming length-dependent values of the dissociation constant (K_d) and of the front factor (Δf_0). Since immediate stiffness is practically unaffected either by stretch (Beinbrech *et al.*, 1976) or by adding AMPPNP (Fig. 3), the difference (ΔS_{stat}) between static stiffness

in rigor and in the presence of AMPPNP becomes:

$$\Delta S_{stat} = S_R - S = \left(\frac{\partial \Delta f}{\partial \Delta L_r}\right)_{T,c} = \frac{c}{c + K_d}\left(\frac{\partial f_0}{\partial \Delta L_r} - \frac{f_0(\partial K_d/\partial \Delta L_r)}{c + K_d}\right) \quad (3)$$

The last function of Equation 3 is obtained by differentiation of Δf in Equation 1 at constant temperature and constant AMPPNP concentration with respect to the *relative* length change ($\Delta L_r = \Delta L/L$). Substituting Δf instead of the AMPPNP concentration (c) then gives:

$$\Delta S_{stat}/\Delta f = \partial(\ln \Delta f_0)\partial \Delta L_r - \{[1 - (\Delta f/\Delta f_0)][\partial(\ln K_d)/\partial \Delta L_r]\} \quad (4)$$

Fig. 7 demonstrates the validity of Equation 4. The values of the empirically determined ratio $\Delta S_{stat}/\Delta f$ versus $\Delta f/\Delta f_0$ are consistent with a linear regression line. The regression line intersects the abscissa at $\Delta f/\Delta f_0 \sim 1$. This indicates that $\partial \ln \Delta f_0/\partial \Delta L_r$ is nearly zero. $\partial \ln \Delta f_0/\partial \Delta L_r$ is the difference between static stiffness in rigor (that is, at zero AMPPNP concentration) and saturating AMPPNP concentration. As shown experimentally (cf. Fig. 3), the static stiffness difference between fibres at zero and 0.4 mM Mg.AMPPNP is indeed quite small. (Unfortunately static stiffness was not determined at AMPPNP concentrations >0.5 mM because of possible side effects of AMPPNP at high concentrations.) The slope of the regression line (Fig. 7) gives

$$[\partial(\ln K_d)/\partial \Delta L_r]_{T,c} = -140 \quad (5)$$

The fitting procedure (cf. Fig. 7) of the static stiffness–ligand concentration curve

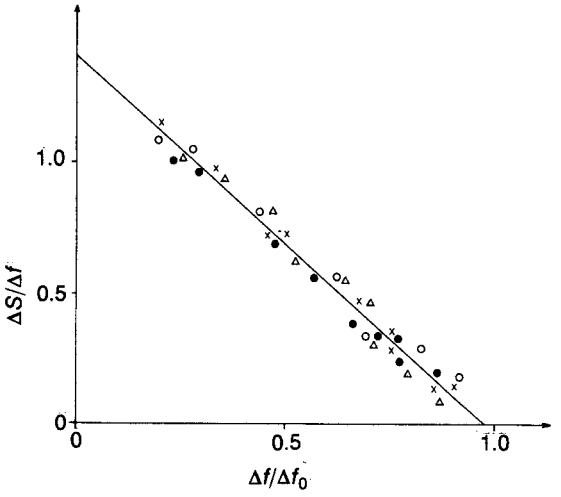

Fig. 7. Plot of the empirically determined ratio $\Delta S/\Delta f$ against $\Delta f/\Delta f_0$. In connection with Equation 4 in the text, the point of intersection of the linear regression line with the ordinate (1.4) is a measure of the dependence of dissociation constant of AMPPNP binding on sarcomere length. The symbols represent four different muscle preparations. The values of tension and static stiffness were obtained at concentrations from 0–0.4 mM AMPPNP. Conditions: bundles of 6–8 fibres, pH 6.7, I = 0.06, 18° C.

(Fig. 2) indicates that the natural logarithm of the dissociation constant decreases by 1.4 when the fibre is stretched by 1% ΔL. The dissociation constant of Mg.AMPPNP binding thus increases by a factor of about two when the fibre is released by 0.5% ΔL (Kuhn, 1978b). Marston *et al.* (1979) obtained similar results; in addition these authors showed that the dissociation constant of H.AMPPNP or Mg.ADP binding are not affected by stretching the fibres.

Stretching of the fibre by 1% ΔL extends the half sarcomere (initial length 1.2 μm, Reedy, 1968) by ΔL_S = 12 nm. Thus the dependence of the natural logarithm of the dissociation constant can also be expressed by

$$[\partial(\ln K_d)/\partial \Delta L_S]_{T,c} = -120 \text{ half-sarcomere } \mu m^{-1} \quad (6)$$

The greater recovery of tension observed in AMPPNP loaded fibres than in rigor fibres is attributable to a transformation of binding energy of the ternary actomyosin–AMPPNP complex into mechanical potential energy. There are thus both a mechanical and a chemical energy store available to the fibre in the presence of the ligand AMPPNP. Teinochemical studies indicate that their energies are exchangeable reversibly. The store of binding energy is drawn on later in time than the store of potential energy which can only be converted into mechanical work (or heat) during the length change itself. A reversible length-dependent binding of a ligand to the myosin head thus enables a release of mechanical energy with a high degree of efficiency even after the length change is complete.

Molecular interpretation of the energetics of AMPPNP binding

It is the aim of this section to relate molecular parameters (that is, number of attached crossbridges, energy differences between different molecular states) to the empirically determined mechanical and chemical parameters of the fibre states achieved in the absence and in the presence of AMPPNP (that is, tension per fibre, stiffness per fibre and the length dependence of the affinity constant of AMPPNP binding).

A major difficulty arising here is that the number of crossbridges attached to the actin is not known. It must therefore be estimated by a complete statistical mechanical analysis of the phenomenological data. The discussion of the length–force diagrams observed in the presence and in the absence of AMPPNP will lead to estimates of the extension of the elastic elements within the crossbridges induced by dissociation of the AMPPNP from the heads under isometric conditions. The extension of the elastic elements and the stiffness of a single crossbridge determine the stretch-dependent binding equilibrium of AMPPNP to the heads. Consequently, analysis of these binding equilibria can result in an estimate of the stiffness of a single crossbridge and this is done in the third subsection. The number of crossbridges attached to the actin in a force maintaining conformation in the rigor state can then be estimated from isometric rigor tension, which is the sum of the

forces maintained by single crossbridges (equal to the stiffness of a single crossbridge times extension of the bridge).

Interpretations of the length–tension diagrams

The experiments described in the previous section demonstrated that the immediate stiffness of the muscle fibres is unchanged by the addition of AMPPNP to rigor muscle, and is independent of the size of the applied length step for smallish length changes. The simplest interpretation of this result is that the fraction of crossbridges attached to actin does not change when AMPPNP binds to them *and* that the stiffness of the crossbridges in the different attached conformations is the same (other possible interpretations require changes of stiffness to be exactly balanced by changes in the fraction of crossbridges attached).

When a fibre in rigor is stretched or released rapidly there is an elastic change of tension, giving rise to the immediate stiffness, and a subsequent stress-relaxation. The nature of this relaxation has been studied by Kuhn (1978a), who demonstrated that, for releases, the recovery during the relaxation was about 50% within 20 min and that the tension had then reached a new equilibrium that was stable for 24 h. A *static stiffness* can be defined to represent the stiffness measured at the end of the relaxation process. Fig. 3 includes the relationship between static stiffness and AMPPNP concentration. Note that the static stiffness measured in this way is different from the low-frequency (5 or 10 Hz) stiffness measured by Marston *et al.* (1976, 1979) which does not allow the full stress–relaxation to occur.

There is an abrupt change in the properties of the muscle fibres at the length at which the fibres developed rigor tension, as illustrated in Fig. 8. In this figure the fibres developed rigor from an ATP-relaxing solution to the point marked A. The static stiffness for releases is indicated by the length–tension relationship for small releases, following the curve A–B–A. When the fibres were stretched above A, then the stress relaxation was 100% and the static stiffness was therefore zero. A is known as the *yield point*. The rigor tension is maintained at a steady level over a 24 h period. Note that the yield point is very sharply defined. If after stretching the fibres are released (C–D–C in Fig. 8) then the static stiffness measured at the new length is identical to that measured from the initial length, and a new yield point (C) can be defined. Yielding of iodoacetic acid poisoned frog sartorius muscle in rigor was also reported by Maréchal (1960), Lowy & Mulvany (1973) and Mulvany (1975).

The value of tension at the yield point is lowered when AMPPNP binds to the crossbridges (Fig. 8); when the AMPPNP is removed the value of the yield point is raised towards its initial value, although the recovery is not complete.

The following conclusions can be made from these results. (1) Yield point and stress relaxation are properties of the crossbridges, rather than some other structure, since the yield point is changed by the binding of AMPPNP to the crossbridges. (2) Rigor muscle contains two classes of crossbridges: (a) maintained crossbridges and (b) slipping crossbridges. Maintained crossbridges are required to account for the

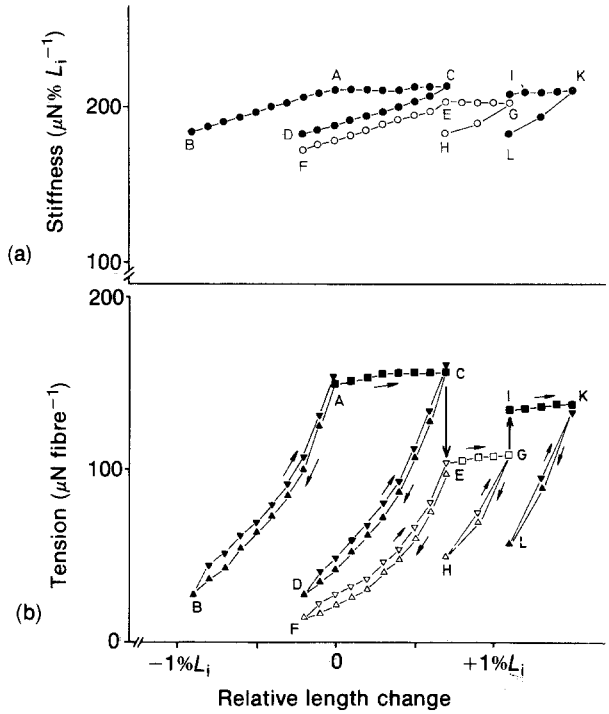

Fig. 8. Elastic and plastic phases of a glycerinated fibre bundle (six fibres) from a fibrillar insect flight muscle (*Lethocerus maximus*). (a) Plot of the immediate fibre stiffness versus relative length change. (b) The length–tension diagram shows reversible elastic tension responses to length changes only below the yield point E in the presence of 0.1 mM AMPPNP and the yield points A and I in its absence. Note the abrupt changes from elastic to plastic behaviour of the fibre to the applied length changes. Conditions: pH 6.7, I = 0.06 M, 12° C, initial fibre length 5 mm; immediate stiffness estimated from tension responses during quick releases; static tension values recorded 20 min after each quick length change.

long-term maintenance of tension of rigor muscle. The simplest interpretation of the stress relaxation is that this is caused by crossbridges which slip from one actin monomer to another. Since the immediate tension is unchanged the total fraction of bridges attached does not alter, and thus the relaxation cannot be accounted for by a net change in the attachment or detachment. (3) The yield point represents an abrupt change in the properties of the muscle. In statistical mechanical terms this is equivalent to a phase transition, and is described by cluster models or co-operative effects (cf. Becker, 1955; Mayer & Goeppert-Mayer, 1977). Accordingly the presence of the yield point is evidence that, in rigor, the crossbridges are arranged in clusters which allow co-operative interactions. The nature of the cluster is not apparent from the evidence but candidate explanations could be either in terms of short-range co-operative effects between crossbridges attached to adjacent actin monomers on the I-filament or in terms of all the attached crossbridges originating from a single A-filament, whose movements are linked by their common origin. (4) Above the yield point increases in length result in slippage of the force maintaining crossbridges

also, but since the tension equilibrates at its yield point value, and since releases from this point indicate that a new yield point has been established (Fig. 8), the distribution of bridges must contain the same proportion of force-maintaining and of slipping types as previously.

Because the properties of the muscle change at the yield point all the experiments described in the previous sections were performed below the yield point.

Estimates of crossbridge extension in rigor and in AMPPNP

A rapidly applied length change to rigor muscle below the yield point gives rise to an immediate tension change which then stress-relaxes, giving rise to the estimates of immediate stiffness and static stiffness discussed above. All crossbridges which are attached to the fibre at the moment of lengthening contribute to the tension change which is induced by straining the fibre (that is, all attached bridges contribute to the immediate stiffness). The slipping crossbridges then slip to actin monomers of which they exert zero force with rate constants determining the rate of stress-relaxation. Consequently only the non-slipping, force-maintaining crossbridges contribute to quasi-static stiffness (S_R) and to isometric tension (f_R). The elastic elements of the force-maintaining crossbridges underwent an extension (Z_R) when the fibre generated rigor tension from a relaxed state: $f_R = S_R Z_R$. The extension Z_R of the force-maintaining rigor bridges may then be calculated from the ratio of isometric tension (f_R = 125 μN fibre^{-1}) to static stiffness (S_R = 123 μN fibre^{-1} %L_i^{-1}, Fig. 3). As 1.02 L_i corresponds to a sarcomere length of 2.4 μm (Reedy, 1968)

$$Z_R = 12.2 \text{ nm half-sarcomere}^{-1} \quad (7)$$

This value of the zero tension extension indicates that the elastic elements of the force-maintaining rigor bridges were extended by 12 nm when the fibre underwent rigor contraction. The extension of the elastic elements could be ascribed to a rotation of myosin heads (length 15 nm, Elliott *et al.*, 1976) from 90° to 35°.

The T_2 curves reported by Huxley & Simmons (1971) and by Ford *et al.* (1977) for tetanized frog semitendinosus fibres and tibialis anterior fibres respectively extrapolate to zero tension at releases near 12 nm per half sarcomere. According to the Huxley & Simmons (1971) interpretation, the force generating crossbridges in these living preparations were extended by about 12 nm by isometric contraction, that is, the same value as found by mechanical studies of rigor bridges in glycerinated fibrillar fibres. Evidence for the slipping hypothesis of crossbridges in the rigor state of mechanically skinned tibialis anterior fibres of the frog was also provided by Yamamoto & Herzig (1978), who compared T_1 curves in contraction and in rigor (cf. also Goldman & Simmons, 1977).

The binding of AMPPNP to the crossbridge heads induces a fall of isometric tension (f_P) to about 50% of its value in the rigor state at 400 μM AMPPNP where most of the bridges are loaded with AMPPNP (cf. Marston *et al.*, 1976). At this analogue concentration static stiffness (S_P) has nearly regained the value of rigor. Under the same assumptions as for the rigor linkages, the extension of the elastic

element in the AMPPNP loaded crossbridge conformation can be calculated from the ratio of isometric tension (f_P = 69 μN fibre^{-1}; Fig. 3) to static stiffness (S_P = 102 μN fibre^{-1} %L_i^{-1}) at 400 μM AMPPNP as

$$Z_P = 8.1 \text{ nm half-sarcomere}^{-1}. \quad (8)$$

At saturating AMPPNP concentrations the elastic elements of force-maintaining crossbridges are thus extended by 8.1 nm. This would correspond to an angle of 57° at which the myosin heads are attached to the actin when AMPPNP has bound to them. Thus addition of AMPPNP to a fibre in rigor may induce a rotation of crossbridges from 35° to 57°. Conversely, washing out the AMPPNP from a fibre induces a rotation of attached myosin heads to the rigor position (35°). Thereby an increase in tension is affected by an extension of the elastic elements within the force-maintaining crossbridges by (cf. Equations 7 and 8):

$$\Delta Z = Z_R - Z_P = 4.1 \text{ nm half-sarcomere}^{-1}. \quad (9)$$

Somewhat lower values of the extension of crossbridges induced by washing out the AMPPNP have been reported by Beinbrech *et al.* (1976; ΔZ = 3 nm per half-sarcomere) and by Marston *et al.* (1976, 1979; ΔZ = 2 nm per half-sarcomere). However, the values of these authors are based on the interpretation of parallel shifted T_1 curves (Beinbrech *et al.*, 1976; cf. also Marston *et al.*, 1976, 1979) or on the ratio of isometric tension to 5–10 Hz dynamic stiffness (Marston *et al.*, 1976, 1979). These interpretations neglect the recent demonstration of an appreciable recovery phase in rigor tension within 10–20 min following a release (Kuhn, 1978a).

Clearly, by means of mechanical experiments it is not possible to check the structural evidence in favour of the rotational hypothesis (Huxley, 1969) which is adopted in the Huxley & Simmons (1971) rotation model. Besides crossbridge rotation, another explanation for the AMPPNP-induced shortening of the elastic elements in crossbridges (cf. Equation 9) is given by the model proposed by Barrington Leigh *et al.* (1977, cf. section on AMPPNP-induced changes of crossbridge conformation). The slipping bridges in rigor muscle which readily detach and reattach to the actin filament could sweep from their r-state modulated crossbridge array in rigor into the p-state modulated array when AMPPNP binds to their heads. The changes in the stereo-affinity occurring during the transition from a p-state crossbridge array into a r-state array could then distort the force-maintaining crossbridges so that they shorten their elastic elements when the fibre is irrigated with AMPPNP under isometric conditions. It may be speculated that the first time a fibre is relaxed with AMPPNP some of the force-maintaining crossbridges could even be distorted by such an extent that they slip along the I-filament. Therefore the first restoration of tension following AMPPNP removal becomes only partial (Fig. 2).

An estimate of the stiffness of the crossbridge

The dependence of the AMPPNP dissociation constant upon length (cf. Equation 5) can be used to determine the stiffness of an individual crossbridge.

We have noted above that the magnitude of the immediate stiffness of the muscle in rigor and in AMPPNP-pseudorelaxed muscle are the same. If we denote the stiffness of an individual crossbridge by D, then the potential (free) energies of the two states will be parabolas with the formulae $D\lambda^2/2$ when the elastic elements of the bridges are extended by λ. Since the extensions of the elastic elements of the force-maintaining crossbridges in the two states differ by $\Delta Z = 4.1$ nm (cf. Equation 9) the extensions in the AMPPNP state and rigor state are $\lambda_P = \Delta L$ and $\lambda_R = \Delta L + \Delta Z$ respectively when the half-sarcomere is extended by ΔL. The chemical potentials μ_P and μ_R of the AMPPNP state and the rigor state are then (cf. Hill & Simmons, 1976a, b)

$$\mu_P = \mu_P^0 + [(D/2)\Delta L^2] + kT \ln p_P \tag{10}$$

$$\mu_R = \mu_R^0 + (D/2)[(\Delta L + \Delta Z)^2 - \Delta Z^2] + kT \ln p_R \tag{11}$$

Where μ_P^0 and μ_R^0 are the standard chemical potentials of the AMPPNP and rigor states in the unextended sarcomere ($\Delta L = 0$), and p_P and p_R are the molar fractions of these two attached states. At a specified concentration (c) of free AMPPNP molecules the chemical potential of this ligand becomes

$$\mu_L = \mu_L^0 + kT \ln c \tag{12}$$

The rigor states of the crossbridges can react with the ligand (L) to form the AMPPNP state: $P \rightleftharpoons R + L$. According to this reaction scheme the relation $\mu_P = \mu_R + \mu_L$, between the chemical potentials must hold when the reaction has attained equilibrium, and by means of Equations 10–12, it becomes possible to calculate the equilibrium ratio p_P/p_R of the AMPPNP and rigor states:

$$p_P/p_R = c \exp[(\mu_R^0 + \mu_L^0 - \mu_P^0)/kT] \exp(D\Delta Z \Delta L/kT) \tag{13}$$

Fig. 9 shows length–tension diagrams and potential energy diagrams of the two states. At zero length change ($\Delta L = 0$) the relative frequency of occurrence of the AMPPNP state and the rigor state is (cf. Equation 13) determined by the concentration (c) of the free AMPPNP molecules and by the difference $\mu_R^0 + \mu_L^0 - \mu_P^0$ of the standard chemical potentials at zero extension of the sarcomere. If the elastic elements of the crossbridges are extended by stretching the sarcomere then the elastic elements of the AMPPNP state store less potential energy than those of the rigor state (W, Fig. 9). Conversely, if the elastic elements of the crossbridges shorten by releasing the fibre ($\Delta L < 0$) then the elastic elements of the rigor state store less potential energy than those of the AMPPNP state. According to the Boltzmann principle the lower the potential energy of a state, the greater its frequency of occurrence. Consequently, the crossbridges to which the ligand AMPPNP has bound exhibit a greater probability to be formed in an extended sarcomere (W, Fig. 9) than in a shortened sarcomere (V, Fig. 9, cf. also Equation 13). When the AMPPNP

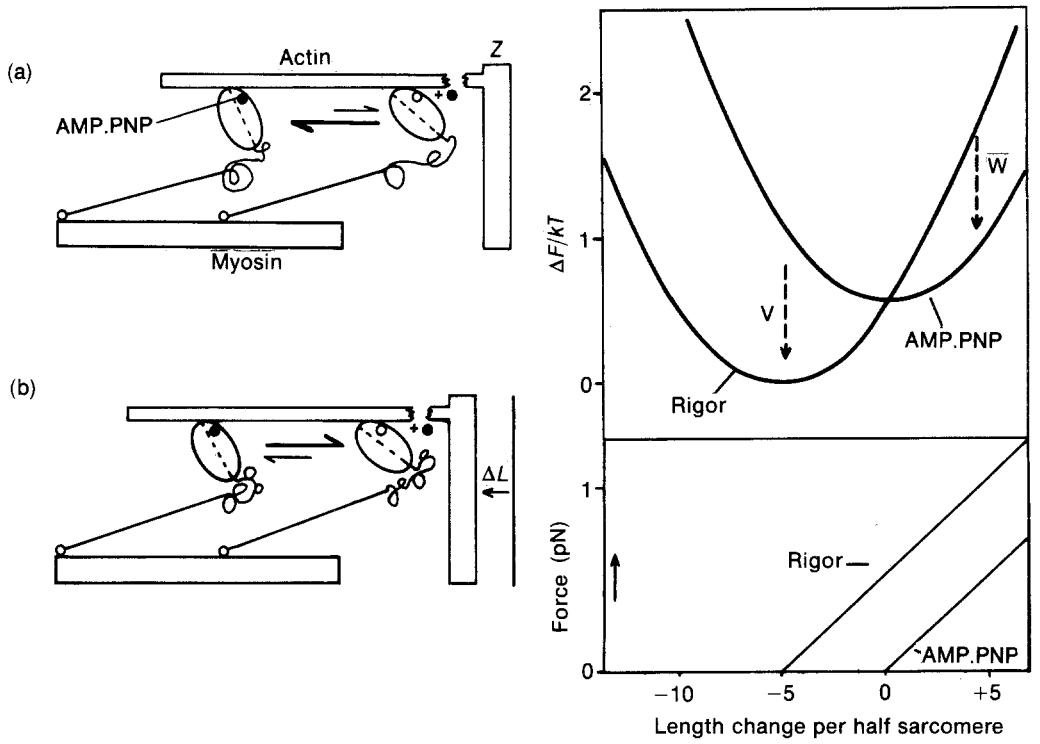

Fig. 9. A schematic representation of free energy (upper part) and force (lower part) plotted versus the length of the preparation for one crossbridge in rigor and in the presence of AMPPNP. The curve of force versus length is displaced in parallel to the right by a value of 5 nm in the presence of AMPPNP as compared to rigor. The stiffness per crossbridge is 1.6×10^{-4} Nm^{-1}. At $\Delta L = 5$ nm the free energy per crossbridge is lower in the presence of AMPPNP than in rigor (W). The equilibrium therefore favours the AMPPNP loaded crossbridges. When the fibre is released the energy situation is reversed and the direction of equilibrium is altered (V).

concentration $c = K_d$ then the two states are equally populated ($p_P/p_R = 1$), and the dissociation constant K_d can be expressed as

$$K_d(\Delta L) = \exp[(\mu_P^0 - \mu_R^0 - \mu_L^0)/kT]\exp(-D\Delta Z\Delta L/kT) \quad (14)$$

$$[\partial(\ln K_d)/\partial(\Delta L)]_{T,c} = -D\Delta Z/kT \quad (15)$$

The term on the left-hand side of Equation 15 was determined from the shape of the relationship between the static stiffness of the muscle fibre and the AMPPNP concentration (Fig. 3, cf. Equation 6). We know that $\Delta Z = 4.1$ nm (Equation 9) and $kT = 4.2 \times 10^{-21}$ J. giving

$$D = 1.2 \times 10^{-4}\ Nm^{-1}. \quad (16)$$

(A more rigorous derivation of this individual stiffness of a crossbridge is given in the appendix.)

The values of the zero tension length of the T_2 curves (12 nm) and the zero slopes of these T_2 curves at zero release amplitude reported by Huxley & Simmons (1971, 1973) and by Ford *et al.* (1977) suggest a value for the stiffness of a single crossbridge $D = 2.2 \times 10^{-4}$ Nm^{-1}).

These calculations are based on the assumption that the filaments are rigid and do not contribute to the fibre compliance. Oosawa *et al.* (1973) estimated the stiffness of a non-decorated F-actin molecule of length 1 μm to be 0.03 Nm^{-1}. In an insect flight muscle sarcomere the half length of the I-band is about 0.1 μm (cf. Reedy, 1968), so that the stiffness of the actin filament in the I-band zone could be about 0.3 Nm^{-1}. Since the stiffness of 500 crossbridges (6 per 14 nm) in the I-band (cf. Tregear & Squire, 1973) which could maximally act in series to the non-overlapping actin parts is about $500 \times 1.2 \times 10^{-4}$ Nm^{-1} = 0.06 Nm^{-1} (cf. Equation 16), the actin filament seems indeed to contribute less than 20% of total stiffness of fibrillar insect fibres.

The low value of stiffness per crossbridge suggests that its elasticity can reasonably be compared with that of a randomly coiled protein. In his pioneering work, Wöhlisch (1926) introduced the concept of random coil elasticity to muscle mechanics. Recent theories of muscular contraction (Davies, 1963; Harrington, 1971; Holmes, 1977; Mason, 1978) make use of that concept in connection with helix–coil transitions within the crossbridges (cf. Flory, 1956; Doty *et al.*, 1957; Mandelkern *et al.*, 1958). Assuming that the number of amino acids within a random coil part of the crossbridge neck is constant, the stiffness of a single crossbridge could be discussed in terms of the dynamic theory of rubber elasticity (Kuhn, 1934; Guth & Mark, 1935). This theory gives a relation between stiffness (D) and mean coil radius (h) of the coil (cf. also Birshtein & Ptitsyn, 1966):

$$D = 3kT/h^2 \tag{17}$$

The empirically determined stiffness per crossbridge (Equation 16) could thus be described by a random coil of 8 nm radius ($kT = 4.2 \times 10^{-21}$ J). This speculation is taken to mean that the randomly coiled protein portion would be comparable in size to that of the myosin heads (length about 15 nm; cf. Elliott *et al.*, 1976). From studies of intrinsic viscosity and sedimentation of polypeptides in solution it is possible to estimate that a random coil of mean radius 8 nm comprises 60–80 amino acid residues (cf. Benoit, 1948). The coil could be stretched by about 25 nm without an appreciable increase of stiffness (cf. Kuhn & Grün, 1942; Treloar, 1956).

Tension of single crossbridges under isometric rigor conditions

Isometric rigor tension may represent the sum of tensions in N force-maintaining crossbridges per half sarcomere. If the elastic elements of each of these crossbridges are strained by isometric rigor contraction with displacement Z_R (cf. Equation 7) one force-maintaining crossbridge contributes the force

$$DZ_R = 1.3\,pN \tag{18}$$

to total rigor tension where D is the stiffness of a single crossbridge (cf. Equation 16).

From the isometric tension (125 μN fibre^{-1}) under rigor conditions it is thus possible to calculate the number of force-maintaining crossbridges per half-sarcomere:

$$M = f_R/DZ_R = 9.5 \times 10^7. \quad (19)$$

The density of force-maintaining crossbridges in a glycerinated insect flight muscle fibre is hence estimated as 1.3 pmol in 1 cm of a fibre. According to values stated by Tregear & Squire (1973) of 10 pmol cm^{-1} fibre this number amounts to about 13% of all the myosin heads present in these preparations. These results should be compared with the findings from electron microscope and X-ray diffraction studies. These showed that an appreciable portion of the myosin heads in rigor and in the presence of AMPPNP give rise to a continuous small angle X-ray diffraction pattern (Holmes *et al.*, 1976; Barrington Leigh *et al.*, 1977) which may reflect the unspecifically bound non-force-maintaining bridges observed in electron micrographs (Beinbrech *et al.*, 1976).

The mechanical work which a single crossbridge performs when it is displaced by zero-tension length Z_R from isometric tension to zero tension under rigor conditions is expressed by

$$W = DZ_R^2/2 = 7.3 \times 10^{-21} \text{ J}. \quad (20)$$

This amounts to only about 20% of the free energy set free by ATP cleavage in solution at 1 mM concentration of ATP, ADP and P_i (Benzinger *et al.*, 1959). Since the mechanical work specified by Equation 20 seems to be an upper limit for the external work done by the fibre per attached crossbridge, there arises a discrepancy between the high efficiency for the transformation of free energy released by ATP splitting and the mechanical work performed by muscular contraction (Wilkie, 1968; Kushmerick & Davies, 1969). A possible explanation which circumvents this discrepancy assumes that co-operativity between myosin heads could share the energy supplied per split ATP molecule (or parts of it) among two or more myosin heads. Another explanation would be to assume that crossbridge attachment is not a single-stage but at least a two-stage process: the crossbridges are easily detached and reattached without net ATP splitting until the last step has occurred (Huxley, 1974). Rapid attachment and detachment of myosin heads to the actin without concomitant ATP splitting [cf. the biochemical schemes of Lymn & Taylor (1971) and White & Taylor (1976)] were also discussed in a contraction theory of Abbott (1977) and are likely to occur when crossbridges slip along the actin filament in rigor (Maréchal, 1960; Lowy & Mulvany, 1973; Kuhn, 1978a) and in contraction (Flitney & Hirst, 1978; Güth & Kuhn, 1978; Güth *et al.*, 1979).

Physiological relevance of AMPPNP binding studies

The stretch-dependent increase of affinity in binding of AMPPNP to attached crossbridges suggests a possible role of the hydrolytic products of ATP splitting in

actively contracting muscle. If the AM.Pr complex (AM.Mg.ADP_i.P_i) behaves analogously to the Mg.AMPPNP actomyosin complex, a shortening of the elastic element would decrease the affinity of crossbridges for the products, whereas a stretch would cause it to increase. The analogy is suggested by an experiment (Marston *et al.*, 1979) showing that at 5 μM Mg.ATP the Mg.ATP binding (bound as the Mg.ADP_i.P_i complex; Marston, 1973) increases as the muscle is stretched.

The theory of irreversible thermodynamics (cf. Katchalsky & Curran, 1965) predicts that the changes in affinity following a release induce an enhanced rate of product dissociation from actomyosin linkages (the rate-limiting step of actomyosin ATPase): the decreased affinity for the products of the ternary actomyosin–product complex (AM.Pr; Fig. 1) will be followed by a decrease of its concentration, which in turn increases the concentration of the AM complex (Fig. 1). The following steps of the actomyosin ATPase (up to the AM–Pr complex, Fig. 1) would be enhanced in rate, since in each step the concentrations of the reactants become transiently increased relative to the concentrations of the products. The overall ATP-splitting rate would thus be increased when the elastic elements of the bridges are released and decreased when they are stretched (cf. Fig. 10). This would account for the extra release of energy observed in actively shortening muscle (Fenn effect; Fenn, 1924) and for the increase in the turnover number of ATP-splitting at high shortening

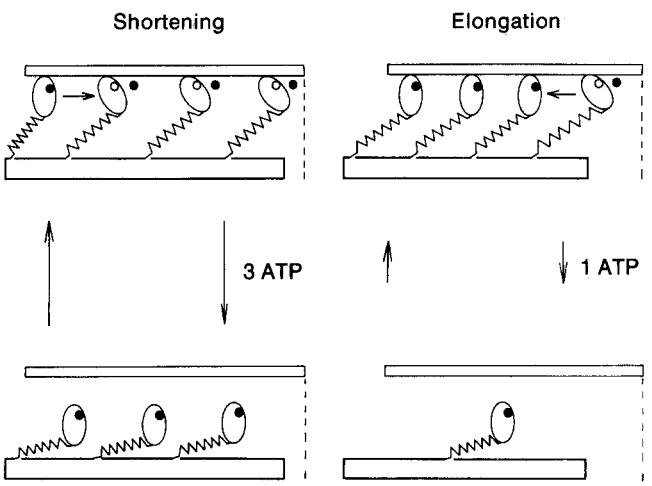

Fig. 10. Fenn effect by stretch-dependent product binding. Steady states of a schematic sarcomere during elongation and shortening are shown. During elongation the elastic elements of the attached crossbridges become stretched. These stretched bridges remain mainly attached in a perpendicular position in which the products (o) of ATP hydrolysis are bound to the active centre. In consequence, the number of crossbridges which are attached in an acute-angled position with low affinity to the products remains low, that is, in elongated fibres inhibition of product dissociation from the enzyme occurs. Conversely, in a steady state of a shortening fibre the crossbridge quickly rotates into the acute-angled position. This gives rise to an enhancement of product dissociation from the active centre.

velocities (Curtin *et al.*, 1974) as well as for the very low ATP-splitting rates found by Curtin & Davies (1975) in stretched muscle. [For the stretch activation of the ATPase in fibrillar insect flight muscle cf. Rüegg & Tregear (1966), Schädler (1967), Breull *et al.* (1973) and Pybus & Tregear (1975) as well as the theoretical aspects given by Thorson & White (1969).]

Appendix

THERMODYNAMICAL AND STATISTICAL MECHANICAL CONSIDERATIONS OF LIGAND BINDING TO ATTACHED CROSSBRIDGES

Thermodynamics of force generation by ligand binding

The quantitative aspects of the Huxley & Simmons (1971) model (cf. also Hill, 1974; Hill & Simmons, 1976a, b) are described in this section for four crossbridge conformations attached to the actin in perpendicular positions (Beinbrech *et al.*, 1976; Huxley, 1969) and in acute-angled positions (Reedy *et al.*, 1965) with ligand binding (AL, BL, Fig. 11) and without binding of a ligand (A, B, Fig. 11) in each case. It is assumed that detachment does not occur so that the number of attached crossbridges (M) is constant. Elastic elements of crossbridges in conformations (B, BL) are extended by ΔZ (Equation 9) relative to those in conformations (A, AL) the elastic elements of which are extended by Z_P (Equation 8) when the half-sarcomere as a whole is unstrained. The stiffness (D) of the elastic elements is the same in each conformation. The half sarcomere is strained when its length (λ) is increased.

This contractile system is in diffusional and thermal contact with the large incubation bath where ligand concentration is c. Its isothermal equilibrium is best described by the free energy function $F^{\S}$. This function of state is derived from ordinary free energy (Helmholtz energy) by the Legendre transformation $F^{\S} = F - N_L\mu_L$ (Katchalsky *et al.*, 1960; cf. also Callen, 1962), where N_L is the total molar amount of ligand in the contractile system and $\mu_L = \mu_L^0 + RT\ln c$ is the chemical potential of the ligand at free ligand concentration c. The energy potential $F^{\S}$ allows the equilibrium force (f) and molar amounts of bound ligand N_L to be calculated from

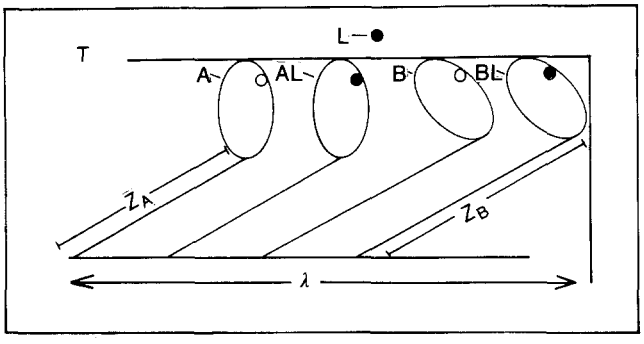

Fig. 11. Scheme of a half-sarcomere of length λ in contact with a ligand L reservoir at temperature T. Four conformations of crossbridges attached to the actin are shown: the perpendicular positions A, AL and acute-angled positions B, BL are in a ligand-binding equilibrium between the conformations A, B and AL, BL, respectively. The Hookean elastic elements (stiffness D) of the crossbridges are at any length (λ) of the half-sarcomere extended by $\lambda + Z_A$ and $\lambda + Z_B$ in the conformations A and B respectively.

derivatives of free energy $F^{\S}$ with respect to the natural independent variables length λ and concentration c of free ligand:

$$f = (\partial F^{\S}/\partial\lambda)_{T,c} \tag{21}$$

Equation 21 predicts that the force under equilibrium conditions should be a function of length and ligand concentration. This was verified when fibres incubated in various AMPPNP concentrations were made to perform mechanical work cycles in length–tension diagrams (Kuhn, 1973). Force in the fibre was indeed the same at the end of the cycle as at the beginning of the cycle, indicating that equilibrium force is a function of length and concentration.

Molar amounts of ligand bound to the contractile system

$$N_L = -1/RT\{\partial F^{\S}/[\partial(\ln c)]_{T,\lambda}\} \tag{22}$$

should also be a function of λ and c. This was verified by studies of AMPPNP binding to glycerinated insect flight muscle fibres (Marston *et al.*, 1976) and by studies of the stretch dependence of AMPPNP binding to the same muscle preparations (Kuhn, 1977; Marston *et al.*, 1979).

Provided that the free energy $F^{\S}$ is a function of state L and c the second derivatives $\partial^2F^{\S}/\partial\lambda\partial(\ln c)$ and $\partial^2F^{\S}/\partial(\ln c)\partial\lambda$ should be equal. This gives the relation (cf. also the teinochemical relation (Equation 2).

$$\{\partial f/[\partial(\ln c)]\}_{T,\lambda} = -RT(\partial NL/\partial\lambda)_{T,c} \tag{23}$$

which must be demonstrated experimentally in order to test the existence of the potential $F^{\S}$. Table 1 shows that glycerinated insect flight muscle fibres to which AMPPNP binds do indeed obey Equation 23. This gives experimental proof that this system can be described by a free energy function $F^{\S}$.

Statistical mechanics of stretch-dependent ligand binding

In statistical mechanics the isothermal equilibrium between the four conformations (Fig. 11) and the free ligand is best described by means of the grand canonical ensemble introduced by Gibbs (cf. Denbigh, 1955). Knowledge of the partition function Q for the distinct states A, AL, B, BL in this statistical ensemble allows the free energy function $F^{\S}$ to be predicted (M = number of attached heads):

$$F^{\S} = -kTM \ln Q \tag{24}$$

In the absence of co-operativity between the four conformations (Fig. 11) the partition function (Q) is equal to the sum of the partition function of the single components multiplied by the concentration (molar fraction) of free ligand for those components which bind the ligand. The partition function of a single component entails the whole isothermal thermodynamic information for its quantal states. In the unstrained state of their elastic elements these partition functions will be denoted by Q_A, Q_{AL}, Q_B, Q_{BL}, for the conformations A to BL. Straining of the elastic elements within the conformations decreases their partition functions. When an elastic element of a conformation is strained by displacement λ_i its potential energy becomes $D\lambda_i^2/2$. Provided that the energy of each quantal state of a conformation is increased on stretching by this potential energy, the partition function factorizes into that of the unstrained state times that due to the potential energy $D\lambda_i^2/2$.

The latter factor gives the Boltzmann factor $q_i = \exp(-D\lambda_i^2/2kT)$.

The elastic elements of crossbridge conformations A, AL are stretched by $\lambda_A = \lambda + Z_A$ whereas those of conformations B, BL are stretched by $\lambda_B = \lambda + Z_B = \lambda + Z_A + \Delta Z$. The Boltzmann

factors then become:

$$q_A = q_{AL} = \exp[-D(\lambda + Z_A)^2/2kT] \tag{25}$$

$$q_B = q_{BL} = \exp[-D(\lambda + Z_A + \Delta Z)^2/2kT] \tag{26}$$

The partition function of an equilibrium mixture of the four crossbridge conformation of Fig. 11 becomes:

$$Q = q_A(Q_A + cQ_{AL}) + q_B(Q_B + cQ_{BL}) \tag{27}$$

or with slight changes in the notation of the partition function

$$Q_B = \exp[(Z_A^2 - Z_B^2)/2kT]Q'_B$$

$$Q_{BL} = \exp[(Z_A^2 - Z_B^2)/2kT]Q'_{BL}$$

and with

$$q(\lambda) = \exp(-D\lambda\Delta Z/kT) \tag{28}$$

$$Q = q_A(\lambda)[Q'_A + cQ'_{AL} + q(\lambda)(Q'_B + cQ'_{BL})] \tag{29}$$

The molecular amount of ligand bound to crossbridges is now easily calculated from equations 22, 24, 26:

$$ML = M\frac{c[Q'_{AL} + q(\lambda)Q'_{BL}]}{Q'_A + q(\lambda)Q'_B + c[Q'_{AL} + q(\lambda)Q'_{BL}]} \tag{30}$$

$$ML = M[c/(c + K_d)] \tag{31}$$

Comparisons of Equations 30 and 31 yields the apparent dissociation constant of the ligand from the crossbridge conformations AL and BL:

$$K_d = \frac{Q'_A + q(\lambda)Q'_B}{Q'_{AL} + q(\lambda)Q'_{BL}} \tag{32}$$

The dependence of the apparent dissociation constant on the length is expressed by the Boltzmann factor $q(\lambda)$ (Equation 28). It was shown experimentally that ligand dissociation decreases when the fibre is stretched ($\partial K_d/\partial\lambda < 0$). This implies the following inequality for the partition function:

$$Q'_B Q'_{AL} > Q'_{BL} Q'_A \tag{33}$$

Hence the frequency of occurrence of a logical AND pair of crossbridges (B, AL) which is expressed by the product $Q'_B Q'_{AL}$ must be greater than the frequency of occurrence of the logical AND pair (BL, A) if binding of ligand is to be enhanced by stretching the fibre.

The partition function Q (Equation 29) also allows the equilibrium force to be calculated (cf. Equation 21, 24):

$$f = MD\left[\lambda + Z_A + \Delta Z\left(\frac{Q'_B + cQ'_{BL}}{Q'_A + cQ'_{AL} + q(Q'_B + cQ'_{BL})}\right)\right] \tag{34}$$

Equation 34 can be simplified by introducing the dissociation constant K_d (Equation 32) and by discussing the difference in equilibrium force (Δf) between force in rigor ($c = 0$) and the force f established at ligand concentration c.

$$\Delta f = MD\Delta Z\left(\frac{Q'_B Q'_{AL} - Q'_{BL} Q'_A}{(Q'_B + Q'_A/q)(Q'_{AL} + Q'_{BL} q)}\right)\left(\frac{c}{c + K_d}\right) \tag{35}$$

Equation 35 shows that isometric force falls on increasing the ligand concentration, provided that condition 33 for the frequencies of the pairs (B, AL) and (BL, A) is fulfilled. This demonstrates that isometric relaxation of force by addition of a ligand and an increase of the affinity for ligand binding by stretch are closely related. Equation 35 shows further that isometric force should follow a hyperbola when plotted in a ligand concentration–force diagram. This is consistent with the experimental findings (cf. Fig. 3).

However, Equation 35 and the Boltzmann factor further predicts that at high ligand concentration ($c \gg K_d$) the force should depend via the Boltzmann factor $q(\lambda)$ on the length of the fibre provided that neither Q'_A nor Q_{BL} are zero. The absence of an appreciable change of static stiffness (that is, $\partial\Delta f/\partial L$) between high (0.4 mM AMPPNP) ligand concentrations and static stiffness under rigor conditions (cf. Fig. 3) implies that both Q'_A and Q_{BL} are small. The frequency of occurrence of the perpendicular rigor conformation (Q_A) as well as the frequency of occurrence of an acute-angled ligand charged conformation (Q'_{BL}) must be small compared to the two other conformations in order to fit the static stiffness–AMPPNP concentration curve of Fig. 3. Under these conditions ($Q'_A \ll qQ'_B$; $qQ'_{BL} \ll Q'_{AL}$) Δf and K_d become:

$$\Delta f = MD\Delta Z[c/(c + K_d)] \tag{36}$$

$$K_d = Q'_B/Q'_{AL} \exp(-D\lambda\Delta Z/kT) \tag{37}$$

Static stiffness (ΔS_{stat}) is then straightforwardly calculated (cf. also Equation 3)

$$\Delta S_{stat} = (\partial\Delta f/\partial\lambda)_{T,c} = [-MDZc(\partial K_d/\partial\lambda)_{T,c}]/(c + K_d)^2 \tag{38}$$

Static stiffness values in fibres loaded with AMPPNP at different concentrations (Fig. 3) provided a reasonable fit by assuming a constant value for

$$(\partial \ln K_d/\partial\lambda)_{T,c} = -D\Delta Z/kT = -120\ \mu m^{-1} \tag{39}$$

Since the difference of zero tension length $\Delta Z = 4.1$ nm could be estimated from interpreting length-force diagrams (Equation 3) and since the value of $K_d = 85\ \mu M$ was estimated by fitting the isometric force–AMPPNP concentration curve, the stiffness of a single crossbridge could be calculated from Equation 39 as $D = 1.2 \times 10^{-4}$ N m^{-1} ($kT = 4.2 \times 10^{-21}$ J at 286 K). In turn the number of attached force maintaining crossbridges (M) per half-sarcomere was estimated from the empirically determined values of isometric rigor tension as 9.5×10^7.

LIST OF SYMBOLS AND ABBREVIATIONS

Chemical

A – Actin
ADCPP – α, β-Methylene ATP
ADP – Adenosine diphosphate
AM – Actomyosin
AMPPNP – β, γ-Imido ATP
ATP – Adenosine triphosphate
ATP-γ, S – 5′-o-3-Thiotriphosphate
M – Myosin
Pr – $ADP_i.P_i$

P_i – Inorganic phosphate
PP_i – Pyrophosphate

Indices
A – Perpendicular unloaded conformation of crossbridges (cf. Fig. 7)
AL – Perpendicular conformation loaded with ligand
B – Acute-angled unloaded crossbridge conformation
BL – Acute-angled crossbridge conformation loaded with ligand
L – Ligand
P – Conditions of saturating ligand concentration
R – Rigor conditions (zero ligand concentration)

Expressions used in formulae
c – Bath concentration of ligand
D – Stiffness of a single crossbridge
F – Free energy
$F^{\S}$ – Legendre transformation of free energy
f – Force of the fibre or force per fibre in a fibre bundle
$\Delta f = f_R - f$ – Difference between force of fibre in rigor and force of fibre at actual ligand concentration
$\Delta f_0 = f_R - f_P$ – Difference between force of fibre in rigor and force of fibre at saturating ligand concentration
h – Mean length of statistical coil (cf. 17)
K_d – Dissociation constant of ligand from myosin heads
k – Boltzmann constant
L – Fibre length
ΔL – Length change of fibre
λ – Difference between actual sarcomere length and sarcomere length under isometric conditions
M – Number of myosin heads
ML – Number of ligand molecules bound to the myosin heads
μ – Chemical potential
N_L – Molar amount of ligand bound to the myosin heads
p_P, p_R – Molar fraction of myosin heads bound to the actin ($p_P + p_R = 1$)
Q, Q' – Partition function of a grand canonical ensemble
q – Boltzmann factors (cf. Equation 29)
R – Gas constant
S – Static stiffness
$\Delta S_{stat} = S_R - S$ – Difference between static stiffness in rigor and static stiffness at actual ligand concentration
W – Mechanical work
Z – Extension of the elastic element of crossbridges under isometric conditions (cf. 'rest length' in Marston *et al.*, 1976, 1979)

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