CROSS-BRIDGE CONFORMATION AS REVEALED BY X-RAY DIFFRACTION STUDIES OF INSECT FLIGHT MUSCLES WITH ATP ANALOGUES

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ABSTRACT The effects of three ATP analogues, α,β-methylene-ATP [ATP(α,β-CH_2)], adenosine 5'-0-(3-thiotriphosphate) [ATP(γ-S)], and β,γ-amino-ATP [ATP(β,γ-NH)] at various concentrations and temperatures on the X-ray fiber diagrams of glycerinated flight muscles from a water bug (*Lethocerus maximus*) have been investigated. It is shown that the "relaxed" state can be obtained with all three analogues at high concentrations, the result being particularly clear with ATP(γ-S). It is inferred that the binding of an ATP-like molecule suffices to produce the relaxed state. At low concentrations ATP(β,γ-NH) produces state intermediate between rigor and relaxed which is not simply a mixture of the two. The possible nature of the intermediate is discussed.

INTRODUCTION

The following facts about cross-striated muscle are well established: (*1*) That contraction comes about by the relative sliding of the thick and thin filaments (A. F. Huxley and Niedergerke, 1954; H. E. Huxley and Hanson, 1954). (*2*) That the thick filaments are composed mostly of the protein myosin (Hanson and H. E. Huxley, 1953) which consists of long rods each with two globular heads (Lowey et al., 1969). The myosin molecules assemble themselves into bipolar arrays (H. E. Huxley, 1963). (*3*) That the thin filaments are helical structures (Hanson and Lowey, 1963) the main component of which is the protein actin (Hanson and H. E. Huxley, 1953). The thin filaments connect to the *Z* line where the polarity of the filaments reverses (H. E. Huxley, 1963). (*4*) That the generation of force between the two kinds of filament is brought about by the "cross bridges," sets of protuberances originating on the myosin (thick) filaments which can attach to the actin (thin) filaments (H. E. Huxley, 1957). The generation of force is presumed to come about by the cross bridges altering their configuration while attached to the actin filaments (A. F. Huxley, 1957). Further, the cross bridges cyclically detach from the actin to allow sliding (H. E. Huxley, 1957; H. E. Huxley, 1969). The heads of the myosin molecule comprise the cross bridges although it is currently unclear whether one or two heads constitute one cross bridge. (*5*) During

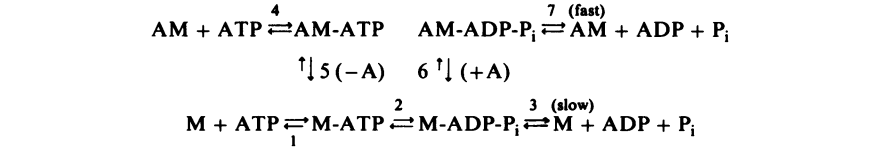
the cyclic activity of the cross bridges ATP is hydrolyzed (H. E. Huxley, 1969; Lynn and Taylor, 1971).

As an enzyme system the contractile apparatus of muscle consists of the two components actin and myosin. The active form of actin is polymerized actin (F-actin), the monomer being known as G-actin. The myosin filaments dissociate in high salt to give myosin monomers. Limited proteolysis of myosin yields the following fragments: LMM (light meromyosin) which is an entirely double α-helical (coiled-coil) rod, and HMM (heavy meromyosin) which contains a further portion of the rod and the two enzymatically active globular "heads" per molecule (Lowey et al., 1969). Limited digestion with papain yields single heads, HMM-subfragment 1 (S1), which show normal enzymatic properties. Preparations of S1 or HMM are freely soluble. In the presence of Mg^{++} they hydrolyze ATP slowly. In the absence of ATP, F-actin and myosin associate strongly and on addition of ATP this system (actomyosin) hydrolyzes ATP much faster than myosin, HMM, or S1 alone.

THE LYMN-TAYLOR KINETIC SCHEME

Insight into the complex process of ATP hydrolysis by actomyosin was provided by the studies of Lymn and Taylor (1970, 1971) and Taylor et al. (1970). It has long been recognized that the interaction of ATP with actomyosin has two main characteristics: (*a*) hydrolysis of ATP, and (*b*) the *dissociation* of F-actin and myosin.

Lymn and Taylor (1971) noted that the rate of *dissociation* of actomyosin by ATP at high ATP concentrations is at least a factor of 10 higher than the rate of cleavage of ATP. From this they concluded that the cleavage of ATP takes place *after* the dissociation of actomyosin. The much faster rate of turnover of ATP in the presence of actin is due to acceleration of the rate of release of products (ADP, phosphate) by the further interaction of actin with the myosin-product complex. In the absence of actin, the product release step is slow and rate controlling. The scheme advanced by Lymn and Taylor is shown in Fig. 1. In this scheme the cleavage of ATP by myosin alone is the important kinetic pathway and interest has centered on this aspect of the system (Fig. 1, step 2). Further work (Trentham et al., 1972); Bagshaw et al., 1974; Bagshaw and Trentham, 1974) has demonstrated a number of additional kinetically recog-



Forward Rate Constants				Rabbit Skeletal Muscle
k_1	$2 \times 10^6\ M^{-1} \cdot s^{-1}$	k_5	$>1{,}000\ s^{-1}$	A = F-actin
k_2	$100\text{–}150\ s^{-1}$	k_6	$3 \times 10^5\ M^{-1} \cdot s^{-1}$	M = HMM
k_3	$0.05\ s^{-1}$	k_7	$10\text{–}20\ s^{-1}$	
k_4	$1 \times 10^{-6}\ M^{-1} \cdot s^{-1}$			20°C, pH 8

FIGURE 1 The Lymn-Taylor kinetic scheme for the hydrolysis of ATP by actomyosin (see text).

TABLE I

THE HYDROLYSIS OF ATP BY RABBIT S1

$$M + ATP \underset{}{\overset{K_1}{\rightleftharpoons}} M\text{-}ATP \overset{K_2}{\rightleftharpoons} M^*\text{-}ATP \overset{K_3}{\rightleftharpoons} M^{**}\text{-}ADP\text{-}P_i \overset{K_4}{\rightleftharpoons} M^*\text{-}ADP\text{-}P_i \overset{K_5}{\rightleftharpoons}$$

$$M^*\text{-}ADP + P_i \overset{K_6}{\rightleftharpoons} M\text{-}ADP + P_i \overset{K_7}{\rightleftharpoons} M + ADP + P_i$$

Step	k_{+n}	k_{-n}	K_n
1	—	—	$4.5 \times 10^3\ M^{-1}$*
2	$400\ s^{-1}$*	$0.02\ s^{-1}$	10^8‖
3	$160\ s^{-1}$‡	18§	9§
4	$0.05\ s^{-1}$‡	$4.2 \times 10^{-3}\ s^{-1}$‖	16‖
5	—	—	$7 \times 10^{-3}\ M$‖
6	$1.4\ s^{-1}$¶	$400\ s^{-1}$*	3.5×10^{-3}*
7	—	—	$2.7 \times 10^{-4}\ M$*

*Bagshaw et al., 1974.
‡Lymn and Taylor, 1970.
§Bagshaw and Trentham, 1973.
‖Mannherz et al., 1974.
¶Bagshaw and Trentham, 1974.

nizable steps. In addition the back rate of the cleavage step (Bagshaw and Trentham, 1973), the rate-limiting step (Mannherz et al., 1974), and the dissociation constant for inorganic phosphate from the myosin-ADP complex (Mannherz et al., 1974) have been established. Since the equilibrium constant for ATP hydrolysis in aqueous solution under the appropriate conditions is known (Rosing and Slater, 1972) and the binding constant for ATP may be calculated, a full set of equilibrium constants is available for the hydrolysis of ATP by S1 from rabbit skeletal muscle (Table I). The starred states refer to physically or kinetically distinguishable complexes. The large value of $K_1 K_2$ demonstrates that the largest standard free energy change takes place on binding ATP. In comparison, the hydrolysis of ATP when bound (K_3) is accompanied only by a small standard free energy change.

STRUCTURAL STUDIES ON INSECT MUSCLE

In order to correlate structure and function it is necessary to relate the kinetically defined states of myosin to structural states. Two main methods of structure analysis may be used in muscle; electron microscopy and X-ray diffraction. Electron microscopy has the advantage of ease of interpretation and the disadvantage that samples must be fixed, embedded, stained, and sectioned, leading to distortions and artifacts. Low angle X-ray diffraction from muscle fibers is an ideal technique of observation in that measurements may be made on living muscle or on glycerinated muscle fibers bathed in solutions of chosen composition. The drawback is that the interpretation of diffraction patterns is not unequivocal and is often model dependent. The most favorable strategy is to combine both techniques.

Three different muscles have been studied extensively by electron microscopy and X-ray diffraction, namely frog (toad) sartorius, rabbit psoas, and the dorsal longitudinal flight muscles of the Belastomatid water bug *Lethocerus*. The frog sartorius

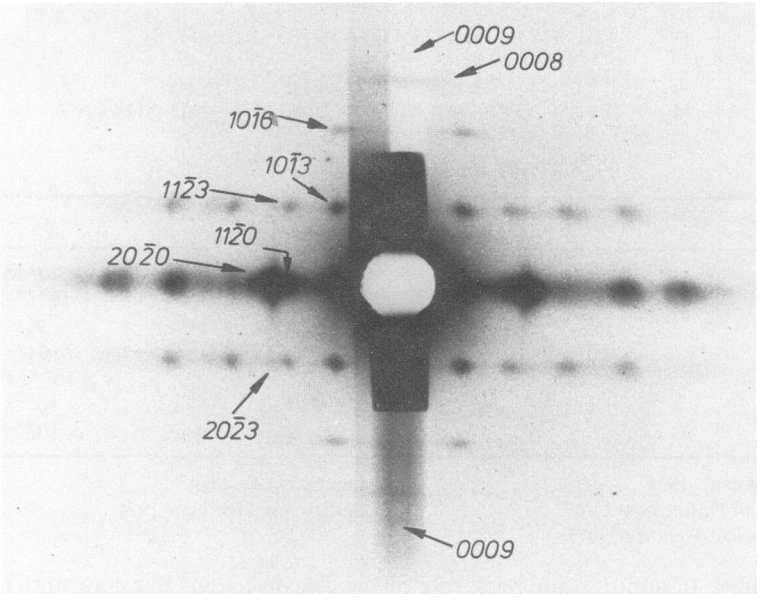


FIGURE 2 *a* The X-ray fiber diagram obtained from a bundle of ca. 100 glycerinated insect flight muscle fibers (*Lethocerus maximus*) irrigated in a flow cell with "rigor" solution (no ATP present) which additionally contains 20 mM $MgCl_2$, EGTA to complex traces of Ca^{++}, and buffer, pH 6.9. X-ray source: DESY electron synchrotron (Hamburg), 7.2 GeV, 9 mA. Focusing system: bent fused-silica mirror and bent quartz monochromator (10$\bar{1}$1 planes), λ = 1.5 Å; specimen-film distance 1.5 m, exposure time 1 h using Ilford Industrial G film. The hexagonal indexing is shown a = 520 Å, c = 1160 Å.

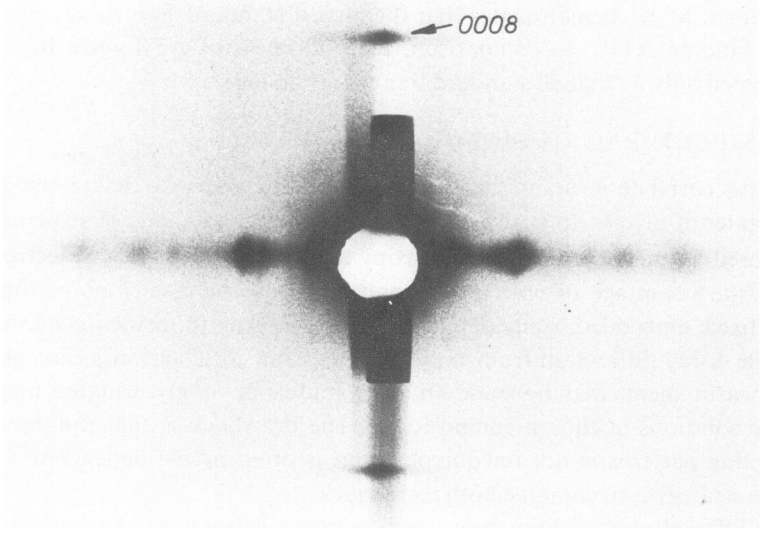


FIGURE 2 *b* As in Fig. 2 *a* but fibers are irrigated with a solution containing additionally 15 mM ATP. Exposure time 2 h.

is the muscle of choice for studies on living intact muscle by X-ray diffraction. This system has been investigated thoroughly by H. E. Huxley and his collaborators (e.g., H. E. Huxley and Brown, 1967; Haselgrove and H. E. Huxley, 1973). Glycerinated preparations of rabbit psoas muscle and insect flight muscle are usually made by soaking bundles of muscle fibers in 50% glycerol at −20°C for some days or weeks. This treatment destroys most of the membranes of the sarcoplasm thereby releasing soluble proteins without damaging the muscle filament arrays and without damaging the ability of these preparations to do work by hydrolysis of ATP under the appropriate conditions (Weber and Weber, 1950; Jewell et al., 1964).

X-ray diffraction and electron microscope studies of glycerinated insect flight muscle provided the first firm evidence that the myosin heads could exist in different conformations depending upon the presence or absence of the substrate ATP. Low angle diffraction pictures of glycerinated flight muscle from *Lethocerus maximus* bathed in a Ringer's solution and in a Ringer's solution with added ATP are shown in Fig. 2. These two rather different diffraction patterns were first described by Reedy et al. (1965), who were able to interpret the main features (Fig. 3) with the aid of electron

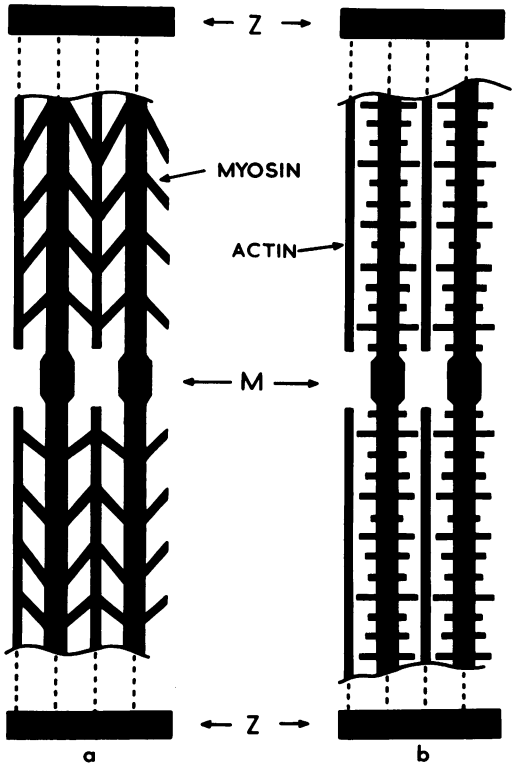


FIGURE 3 A summary of the characteristic differences between rigor and relaxed insect flight muscle. (Reedy et al., 1965). In rigor (*a*) the bridges bind tightly to the actin at an angle of 45° (only bridges in the plane of the diagram are shown). In the relaxed form most of the bridges are detached from the actin and stick out at right angles to the filament axis (the out-of-plane bridges are shown short in projection).

TABLE II

KINETIC CONSTANTS FOR INTERACTION OF ATP ANALOGUES WITH RABBIT S1

Analogue	$k_{ass} = K_1 k_{+2}$	$k_{+3} = k_{cat}$	$k_{diss} = k_{-2}$
	$M^{-1} \cdot s^{-1}$	s^{-1}	s^{-1}
ATP(γS)	10^6 (21°)*	0.24 (21°); 0.014 (4°)*§	1.4×10^{-3}§
ATP($\alpha,\beta CH_2$)	6.10^4 (21°)‡	0.1 (21°); 0.011 (4°)‡§	0.022 (4°)§
ATP(β,γ-NH)	6.10^4 (21°)*	Not cleaved	0.02 (21°)*

k_{ass}, association constant; k_{diss}, dissociation constant; k_{cat}, active center activity.

*Bagshaw et al., 1972.

‡Mannherz et al., 1973.

§Goody and Mannherz, unpublished results.

micrographs. In the rigor state (absence of ATP) the cross bridges make an angle of about 45° to the myosin rods and bind tightly to the thin filaments. In the relaxed state (ATP, no Ca^{++}—in intact fibers in the absence of Ca^{++} the pathway 6 [Fig. 1] is blocked by the troponin-tropomyosin system [see Weber and Murray, 1973]), the bridges are approximately at right angles to the fiber axis and are not attached to the actin filaments.

According to Lymn and Taylor's scheme (Fig. 1), the steady-state complex produced by adding ATP to myosin is myosin-ADP-P_i. This has been demonstrated by Marston and Tregear (1972) for both insect and rabbit muscle who showed directly that the dominant myosin-product complex present in *Lethocerus* flight muscle fibers bathed in ATP is myosin-ADP-P_i. Accordingly, we identify the configuration $3b$ with the myosin state M**-ADP-P_i (M** is defined in Table II).

THE USE OF ATP ANALOGUES

To extend this analysis we need to be able to define and examine other intermediate states. One approach to this problem is to examine actively contracting muscle fibers (Miller and Tregear, 1970). The success of such analysis, however, is dependent upon first identifying and characterizing different stationary states. Partly, therefore, as a prelude to such dynamic studies and also as a method in its own right we have been investigating the effects of three ATP analogues on the low angle X-ray fiber diagram of glycerinated insect flight muscle. Two of the analogues have rate constants for cleavage on the enzyme which are much lower than for ATP and the third analogue is not cleaved at all so that we expect the analogues to generate different steady-state complexes to that obtained with ATP. The three analogues we have used are:

(*i*) *α,β-methylene-ATP* [*ATP(α,β-CH_2); Miles Biochemical, Elkhart, Ind.*] It has been shown (Mannherz et al., 1973) that ATP(α,β-CH_2) is a substrate for rabbit myosin. It dissociates actomyosin as judged from ultracentrifuge studies. It was also shown that the rate of cleavage of ATP(α,β-CH_2) is ca. 1,000 times slower than that of ATP. The rate of release of products [ADP(α,β-CH_2) + PO_4] appears to be

fast compared with the rate of cleavage. The rate of turnover by myosin is higher than ATP at room temperature even though the cleavage is slow. In the presence of saturating concentrations of analogue the expected major species in the myosin steady-state complex should resemble M*-ATP rather than M**-ADP-P_i. If we assume that the kinetic constants for rabbit myosin and insect myosin are similar then in the X-ray experiments on insect flight muscle we expect to look at the state M*-ATP. The second-order rate constant for the association of ATP(α,β-CH_2) and S1 is considerably slower than that of ATP (see Table II).

(*ii*) *Adenosine 5'-0-(3-thiotriphosphate)* [*ATP*(γ-*S*)] (*Goody and Eckstein, 1971*). ATP(γ-S) has been shown to be a substrate for myosin with a cleavage rate considerably slower than that of ATP although the turnover is higher than that for the natural substrate (Bagshaw et al., 1974). It dissociates actomyosin in the ultracentrifuge: its binding parameters are similar to those of ATP. From the kinetic constants one expects the major component of the steady-state complex with myosin under saturating conditions to be like M*-ATP.

(*iii*) β,γ-*imino ATP* [*ATP*(β,γ-*NH*)] (*Boeringer Mannheim Corp., New York*). This analogue has been reported by Yount et al. (1971) to dissociate actomyosin. It is not cleaved by myosin. The rate of release of the uncleaved analogue is very temperature dependent (Goody and Mannherz, unpublished result). Under saturating conditions the steady-state complex is expected to be M*-ATP-like.

The kinetic constants for the three analogues on rabbit skeletal muscle so far as they are known are given in Table II.

The Design of the X-Ray Experiments

The present X-ray experiments have been performed with two new and much brighter X-ray sources:

(*1*) A much improved large rotating anode-mirror-quartz monochromator arrangement. The 18-in rotating anode was jointly developed by G. Rosenbaum (Heidelberg), Elliott Bros. (London), and H. E. Huxley (Cambridge). The camera consists of a very smooth 20 cm bent glass mirror (Franks and Breakwell, 1974), especially manufactured by the National Physical Laboratory (Teddington, London, England), and an asymmetrically cut quartz monochromator (Guinier, 1952) (Fa. Steeg und Reuter) arranged to give a 1,200 mm specimen-film distance. Exposure times with the apparatus are a factor of five better than are obtainable with a conventional fine focus rotating anode tube. The resolving power is much improved.

Details of the apparatus will be given elsewhere (Rosenbaum, 1975).

(*2*) A mirror-monochromator camera similar in principle to that described in (*1*) has been used together with the synchrotron radiation from an electron synchrotron (DESY, Hamburg) to provide exposures in about 1/20 of the time needed for the 18-in rotating anode X-ray tube described above. With a specimen-film distance of 2 m and using Ilford G film (Ilford Ltd., Ilford, Essex, England), a strong exposure with very

good angular resolution can be obtained in 1 h. Details will be published elsewhere (Barrington Leigh and Rosenbaum, 1975).

A further improvement we have made is to use much smaller fiber bundles (ca. 40 fibers) enclosed in a flow cell so that the bathing solution may be continually pumped past the fiber bundle. Typically a 5 mm length of the fiber bundle is illuminated with X-rays. In our previous work we were obliged, because of lack of intensity, to use large fiber bundles (1–1.5 mm diameter). This can result in the starvation of the inner fiber bundles (see below). The present flow cell is designed so as to bathe the fiber bundle and to prevent contact between the fiber bundle and the windows of the cell. The brighter sources make this design possible.

The Main Low Angle Features of the Rigor Structure

The distinctive features of the X-ray diffraction pattern from glycerinated fiber bundles of insect flight muscle in rigor have been analyzed by Reedy et al. (1965) and by Miller and Tregear (1972). The following summary is based upon their papers and upon the detailed electron microscope observations of Reedy (1968). The main low angle X-ray diffraction features (Fig. 2 *a*) are: (*1*) a very weak 129 Å meridional reflection (0009), (*2*) a very weak 145° meridional reflection (0008), (*3*) a weak reflection on the 194 Å layer line ($10\bar{1}6$), (*4*) a strong 388 Å layer line with a strong ($10\bar{1}3$), (*5*) a strong equatorial layer line with two very strong reflections ($10\bar{1}0$, $20\bar{2}0$), the $10\bar{1}0/20\bar{2}0$ ratio is about 0.8 (intensity). The *c*-axis repeat (1160 Å; Reedy, 1968) is the lowest common multiple between the actin helix pseudo-repeat and the myosin helix repeat.

The cross bridges are arranged along multi-start helices each of which repeats every 2,320 Å. Since the myosin helix has at least two-fold symmetry (and possible six-fold) parallel to the myosin filament axis the crystallographic repeat is 1,160 Å. The distance between cross bridges along the myosin filament is 145 Å.

The actin monomers are arranged on a helix which repeats every 776 Å but with a strong pseudo-repeat every 388 Å.

The characteristic low angle layer line structure ($\ell = 3, 6, 9$) in insect flight muscle in rigor (Fig. 2 *a*) apparently arises from the interaction of cross bridges with actin filaments. Under conditions where the myosin filaments have little affinity for actin (relaxed with ATP) these layer lines are very weak (Fig. 2 *b*). When the cross bridges attach to the actin filaments the layer lines are produced by a distortion of the helical symmetry of the attached cross bridges (e.g., see Squire, 1973).

The electron microscopic observations of Reedy (1968) may be interpreted to mean that the attached cross bridges form a P_{3_1} space group with a three-fold screw repeating every 388 Å. That class of the X-ray reflections which appear on going into rigor obey this symmetry. For example, the absence of meridional reflection on the third and sixth layer lines and the meridional 0009 are consequences of the P_{3_1} space group. The 0008 reflection is not explained by the P_{3_1} space group.

In rigor the 0009, 0008, and $10\bar{1}6$ reflections are very weak whereas the $10\bar{1}3$ reflection is strong. This can be explained if the rigor cross bridges are rod-like structures approximately at 45° to the filament axis (Reedy et al., 1965; Miller and Tregear, 1972).

This interpretation is also consistent with the intensity distribution in the wider angle part of the pattern (Miller and Tregear, 1972). On the equator the $10\bar{1}0/20\bar{2}0$ ratio is about 0.8 indicating that the main mass of the cross bridges is near to the actin filaments (c.f., Haselgrove and Huxley, 1973).

The Main Low Angle Features of the Relaxed Structure

The main features of the relaxed structure (Fig. 2 *b*) include: (*1*) a strong 145 Å meridional (0008); (*2*) a weak 388 Å layer line ($10\bar{1}3$); (*3*) two very strong equatorial reflections ($10\bar{1}0$, $20\bar{2}0$), the $10\bar{1}0/20\bar{2}0$ ratio is about 2.2 (intensity). These features can be explained on the basis of (*a*) very little cross-bridge-actin interaction, and (*b*) the myosin cross bridges being rods of about 120 Å length at right angles to the filament axis spaced vertically by 145 Å (Reedy et al., 1965; Miller and Tregear, 1972). Direct observations of the myosin filament by electron microscopy (Reedy, 1968) and arguments based on similarity of packing with other myosin filaments (Squire, 1973) suggest that the most probable myosin filament packing in rigor consists of six parallel helices each of which has 16 subunits spaced every 145 Å along one turn of the genetic helix of pitch 2,320 Å.

X-Ray Results with the Analogues

Details of solutions are given in Table III. pH 6.9 was chosen as the most alkaline value at which glycerinated insect fibers remain stable. Experiments were performed in the presence of an excess of $MgCl_2$ to ATP analogue.

ATP(α,β-CH_2). The fiber diagram obtained from muscle fiber irrigated with 30 mM ATP(α,β-CH_2) solution at 2°C is shown in Fig. 4 *a*.

The main diffraction features are identical to those of the relaxed pattern. The $10\bar{1}0/20\bar{2}0$ ratio is ca. 1.7; but this value is not accurately known and it could be as high

TABLE III

SOLUTIONS USED FOR LOW-ANGLE DIFFRACTION EXPERIMENTS

Nucleotide	Nucleotide concentration	Mg^{++}
	mM	*mM*
ATP	15	15
ATP(α,β-CH_2)	30	30
ATP(γ-S)	20	20
ATP(β,γ-NH)	1	10

All solutions contained additionally imidazol-HCl pH 6.9 (20 mM), EGTA (4 mM), and NaN_3 (10 mM) and were adjusted to pH 6.9 with 1 N KOH. The ATP(γ-S) solution also contained AP_5A (1 mM), DTT (4 mM), and acid phosphatase (0.1 mg/ml).

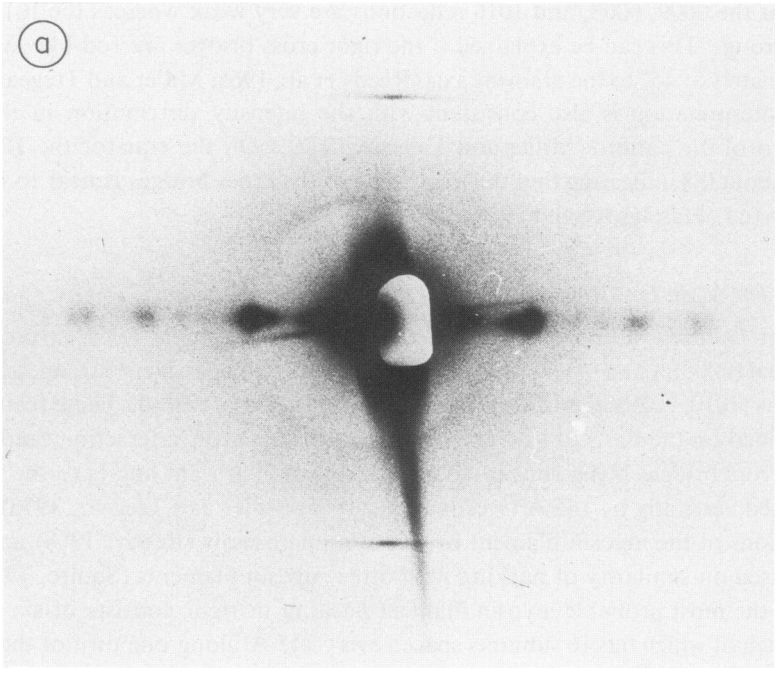


FIGURE 4 *a* X-ray fiber diagram from a small fiber bundle (ca. 40 fibers) in a flow cell irrigated with a solution containing 30 mM ATP(α,β-CH_2) at 2°C, pH 6.9. (18-in copper rotating anode X-ray generator, mirror-monochromator focusing system CuKα λ = 1.54 Å, specimen-film distance 1.2 m, exposure time [Ilford G film] 12 h.) This pattern is essentially identical to a relaxed diagram (Fig. 2 *b*).

as the relaxed value (2.2). At 21°C (Fig. 4 *b*) the $10\bar{1}6$ reflection and the 380 Å layer line become visible although the $10\bar{1}3$ remains very weak. The ratio $20\bar{2}3/11\bar{2}3$ is different from rigor. The 0009 becomes stronger. These changes are similar to those produced by low concentrations of ATP(β,γ-NH) (see Fig. 6) and their implications will be discussed below. The 145 Å meridional reflection (0008) is strong and the ratio of the equatorial intensities ($10\bar{1}0/20\bar{2}0$) is similar to that of ATP relaxed muscle. In an earlier publication (Mannherz et al., 1973) we reported that the (0008) reflexion was weak in the presence of ATP(α,β-CH_2). We now think this result to have been an artifact. On account of the relatively high turnover rate of ATP(α,β-CH_2) and the thickness of the fiber bundles previously used it is very likely that the inner parts of the thick fiber bundles were starved of analogue. Thus, the previous results probably reflected the coexistence of a mixture of states in the sample. The earlier conclusion that the joint absence of the ($10\bar{1}3$) and the (0008) 145 Å indicated a new state was premature. In our present series of X-ray experiments we have used thinner fiber bundles (ca. 300 μm diameter) and we have made parallel mechanical measurements on equivalent fiber bundles to check the relaxation of the fibers. At 30 mM ATP(α,β-CH_2) the fiber bundles are essentially as relaxed as can be achieved with ATP.

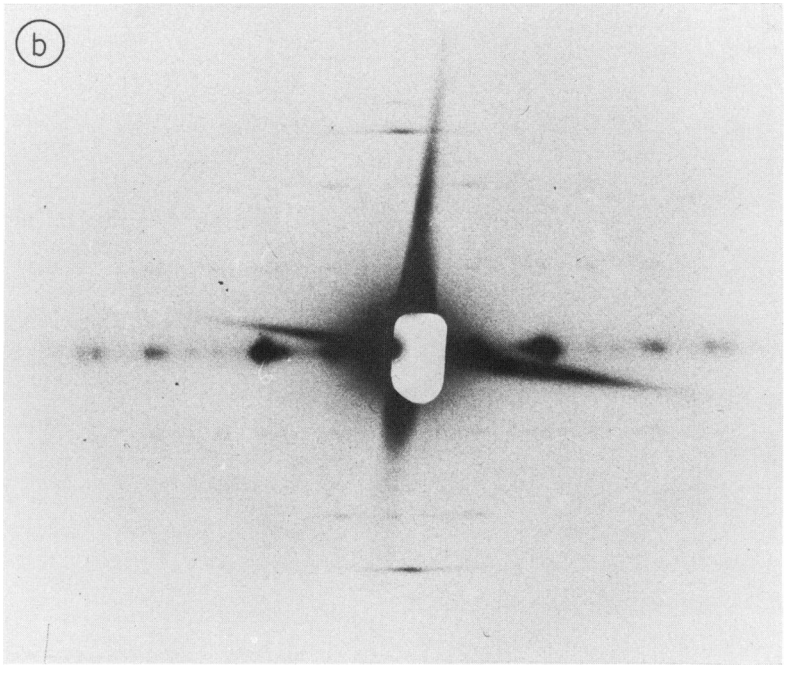


FIGURE 4 *b* Same as Fig. 4 *a* but 21°C. Note the presence of a weak $10\bar{1}6$ reflection. The 380 Å layer line is relatively stronger. This pattern bears some resemblance to the ATP(β, γ-NH) pattern (Fig. 6).

ATP(γ-S). As reported by Barrington Leigh et al. (1972) this analogue at 20 mM concentration gives a diffraction pattern (Fig. 5) very like that obtained from ATP (Fig. 2 *b*). Earlier we considered the possibility that this was due to regeneration of ATP by adenylate kinase from ADP formed by hydrolysis of ATP-γS. We have taken precautions to prevent this happening and still obtain an ATP-like X-ray diagram. We have employed: (*i*) A specific adenylate kinase inhibitor ($A\text{-}P_5\text{-}A$; Lienhard and Secemski, 1973). (*ii*) Acid phosphatase has been added to degrade the ATP produced. Experiments with ^{3}H-ATP(γ-S) under these conditions followed by analysis of the solution by thin layer chromatography on PEI-cellulose failed to show any ATP formation ($< 0.1\%$ of total nucleotide).

Mechanical experiments carried out on the fibers after the x-ray exposure showed them to be completely relaxed by the analogue solutions used. The diffraction pattern is essentially that of ATP relaxed muscle. In particular the measured $10\bar{1}0/20\bar{2}0$ ratio (2.25) is the same as for ATP.

ATP(β, γ-NH). We have examined the diffraction pattern of fiber bundles in the presence of a number of concentrations of ATP(β, γ-NH). In the presence of 1 mM ATP(β, γ-NH) at 4°C (Fig. 6 *a*) the main features of the X-ray diagram are: (*1*) a medium 129 Å meridional reflection (0009), (*2*) a strong 145 Å meridional reflection (0008), (*3*) a strong reflection on the 194 Å layer line ($10\bar{1}6$), (*4*) a strong

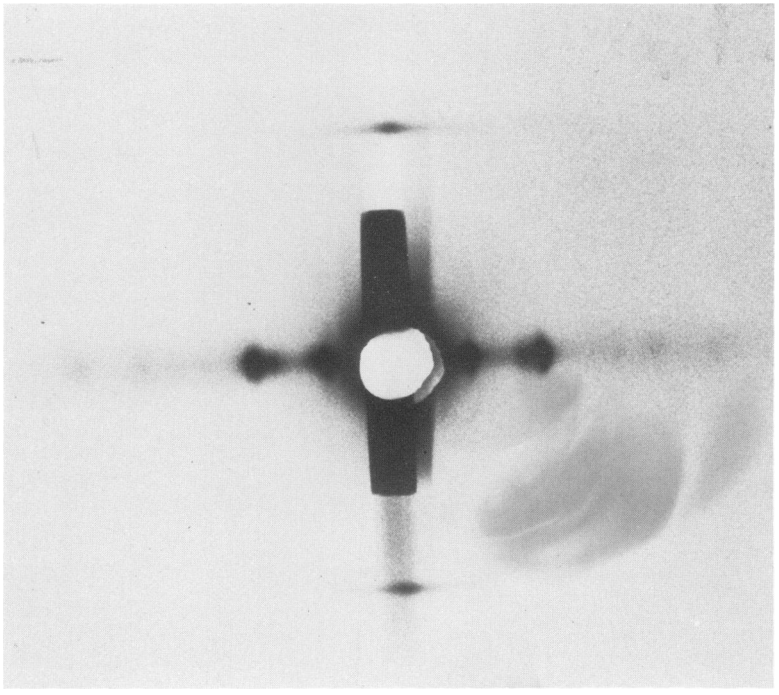


FIGURE 5 X-ray fiber diagram (details as in Fig. 2) from glycerinated muscle fibers irrigated with 20 mM ATP(γ-S) 4°C. Possible regeneration of ATP by adenylate kinase was avoided by adding AP_5A and acid phosphatase. Note the similarity of this diagram to Fig. 2 *b*.

388 Å layer line but with a weak ($10\bar{1}3$), (*5*) a strong $20\bar{2}3$ and a weak $11\bar{2}3$ (c.f. rigor where the $11\bar{2}3$ is strong and the $20\bar{2}3$ is weak), (*6*) strong equatorial reflections ($10\bar{1}0$, $20\bar{2}0$, etc.). The ratio $10\bar{1}0/20\bar{2}0$ is ca. 1.6.

On warming up the muscle to 23°C the diffraction patterns assume a more rigor-like appearance (Fig. 6 *b*, 6 *c*); on cooling to 4° it reverts to the diagram shown in Fig. 6 *a*.

In the presence of 1 mM ATP (β, γ-NH), pH 6.9, 4°C, we see that the 388 Å layer line system is well developed (similar to rigor). As discussed above, the strong 388, 194, and 127 Å layer lines apparently arise from attached cross bridges. Therefore, many of the cross bridges which are bound in rigor may still be attached to the actin filaments in the presence of 1 mM (β, γ-NH). Compared with rigor, however, the intensity distribution is different. The 0009 and 0008 are strong (although the 0008 is not as strong as in relaxed muscle). The $10\bar{1}6$ is strong and the $10\bar{1}3$ is weak. The $10\bar{1}0/20\bar{2}0$ ratio is 1.62. In rigor muscle the $11\bar{2}3$ is strong and $20\bar{2}3$ weak whereas in ATP(β, γ-NH) the $11\bar{2}3$ is very weak and the $20\bar{2}3$ is strong. This indicates that we are dealing with a state which is not simply a *mixture* of relaxed and rigor since such a distribution of intensities cannot be obtained by mixing.

We can go further and try to estimate the structural characteristics of the new state from the diffraction pattern. The $10\bar{1}3$ reflection arises from Bragg planes running

at about 45° to the fiber axis. In rigor the $10\bar{1}3$ is strong and is characteristic of the 45° angled cross bridges (Miller and Tregear, 1970). Since this reflection is weak in the new state one expects the bridges no longer to be strongly angled. Furthermore the $10\bar{1}6$ and 0009 reflections arise, respectively, from Bragg planes making angles of 70° and 90° to the fiber axis. Their weakness in the rigor is naturally explained by the simple model of rod-like cross bridges angled at 45°. They would be strengthened as found for ATP(β, γ-NH) if the bridges were more nearly at right angles to the fiber axis because more electron scattering mass would then lie in these planes. The strength of the 0008 (145 Å meridional) indicates that more of the cross-bridge mass has taken up the regular myosin filament period than in rigor muscle. This could be explained by supposing that a proportion of the bridges were now free or from the fact that a greater proportion of the mass of the bridge now lies near the myosin filament. The ratio of the strong equatorial reflections (1.6) is nearer the relaxed value than the rigor value. This may indicate that a considerable transfer of mass in the direction of the thick filament can take place without the cross bridges letting go of the actin.

In similar experiments on glycerinated rabbit psoas muscle Lymn and Huxley (1972, 1975) found a marked alteration from the rigor intensity distribution to be brought about by ATP(β, γ-NH). They found the effects to be markedly temperature sensitive. The diffraction pattern at pH 8.0 and 2°C shows characteristics of both rigor and

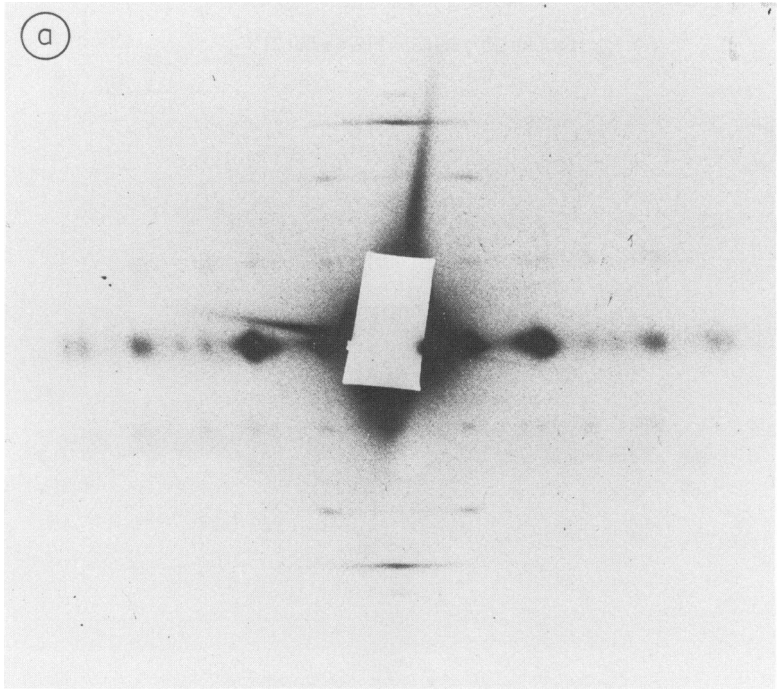


FIGURE 6 *a* Conditions as in Fig. 4: muscle irrigated with 1 mM ATP(β, γ-NH), 4°C. Exposure time 10 h.

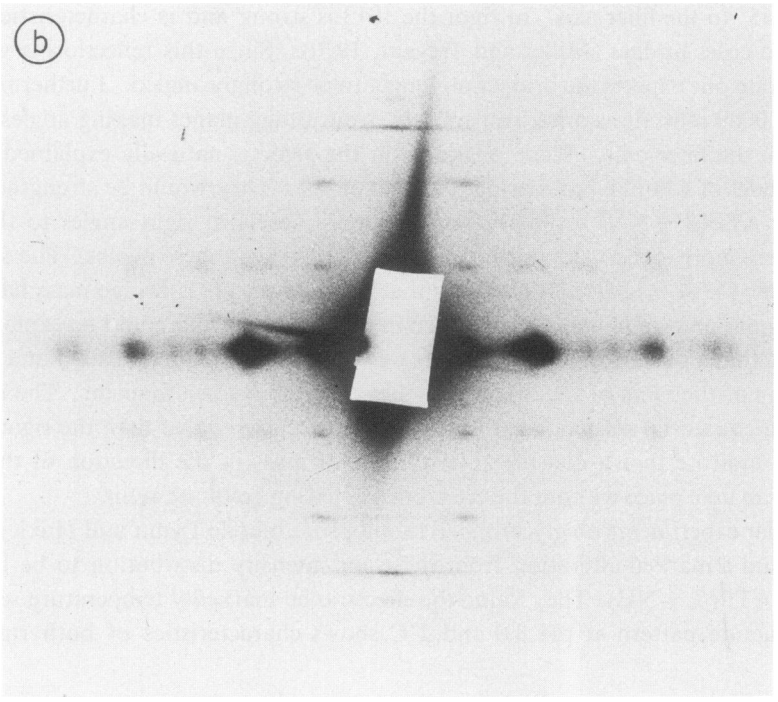


FIGURE 6 *b* Same as Fig. 6 *a* but 21°C.

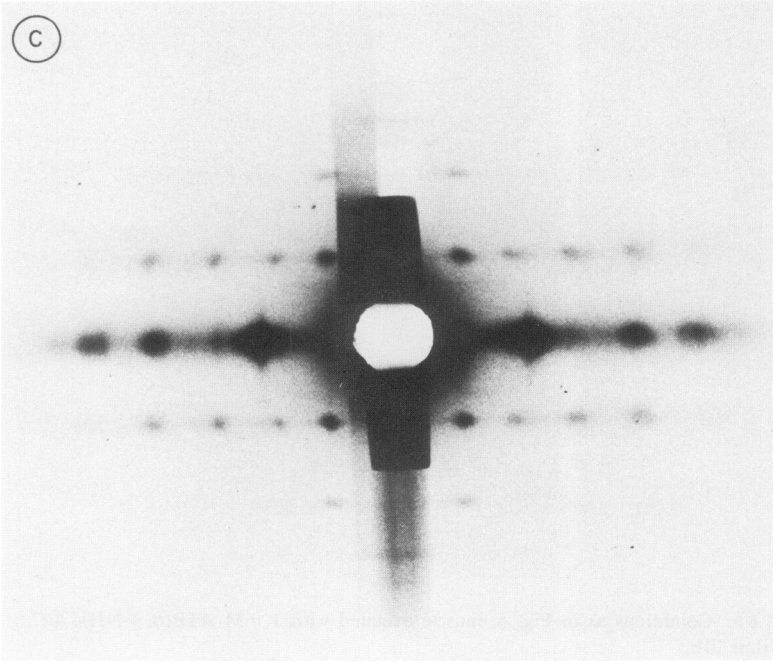


FIGURE 6 *c* A similar preparation to 6 *b* but photographed with the DESY synchrotron (conditions as in Fig. 2). Exposure time 1 h.

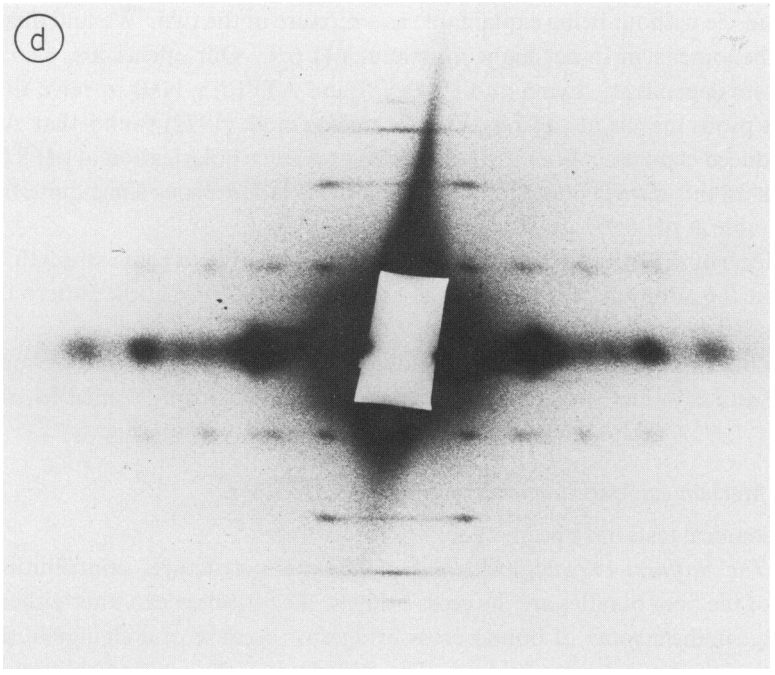


FIGURE 6 *d* A repeat of experiment 6 *c* with the addition of 0.1 M KCl.

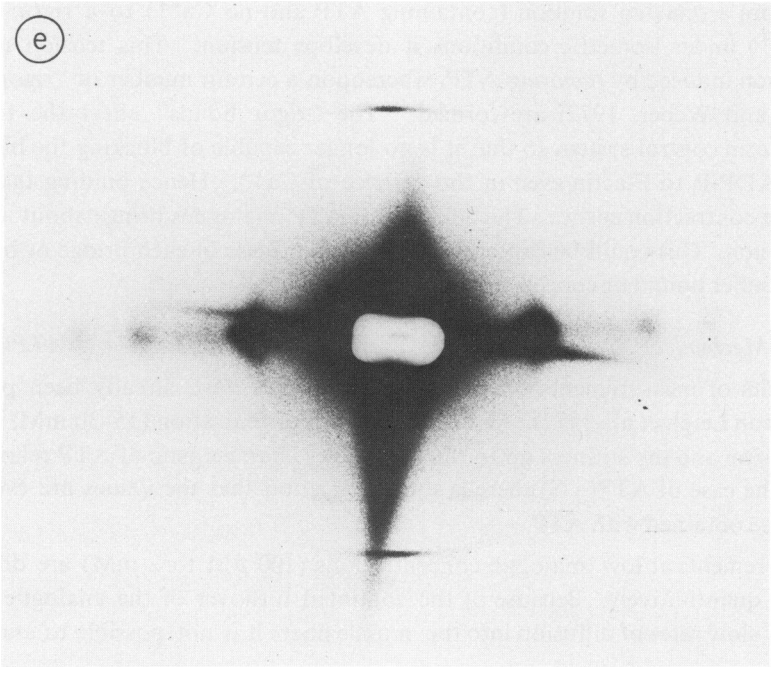


FIGURE 6 *e* Conditions as in Fig. 4: muscle irrigated with 20 mM ATP(β, γ-NH), 4°C. Exposure time 12 h.

relaxed muscle without being explainable as a mixture of the two. We find qualitatively similar phenomena in insect flight muscle at pH 6.9. Our effects are also strongly temperature dependent. Lymn and Huxley found ATP(β, γ-NH) to have little effect on rabbit psoas muscle at pH 7.0. Dos Remedios et al. (1972) found that ATP(β, γ-NH) produced changes in fiber stiffness and fluorescence polarization at pH 8.0 but not at pH 7.0. In this respect insect muscle differs from rabbit psoas since quite big effects are detectable at pH 6.9.

The effects of ATP(β, γ-NH) do not seem to be sensitive to ionic strength. Fig. 6 *d* shows that the addition of 0.1 M KCl does not alter the diffraction pattern (c.f., Fig. 6 *d*, 6 *b*, and 6 *c*).

On adding 20 mM ATP(β, γ-NH) the diagram (Fig. 6 *e*) begins to look much more like ATP and is in fact rather like the diagram obtained from 30 mM (α, β-CH_2) at 21°C.

Mechanical Measurements on Glycerinated Fibers

Two mechanical tests have been made.

The Stiffness (Young's Modulus). The main structures contributing to the stiffness of the fiber bundles are the cross bridges. The stiffness can alter either because of a change in the *number* of bound cross bridges or because of a change in the *elastic properties* of the bound cross bridges. The stiffness (i.e. the mechanical impedance) is in general a function of frequency. Present measurements were made at ca. 1 Hz and low amplitude (< 1%).

Development and Relaxation of Rigor Tension. When a fiber bundle is transferred from a relaxing solution (containing ATP and no Ca^{++}) to a rigor solution (no Ca^{++}) under isometric conditions it develops tension. This tension reflects a contraction induced by *removing* ATP, whereupon a certain number of "rigor bonds" (Bremel and Weber, 1972) are formed. The "rigor bonds" effect the troponin-tropomyosin control system so that it is no longer capable of blocking the binding of myosin-ADP-P_i to F-actin even in the absence of Ca^{++}. Hence binding takes place and some contraction ensues. The addition of ATP analogues brings about a drop of rigor tension. This could be caused by a drop of stiffness of each bridge or by a drop in the number bound or conceivably by an induced length change.

Mechanical Measurements in the Presence of ATP(α, β-CH_2) + ATP(γ, S)

The results of measurements on these two analogues have already been published (Barrington Leigh et al., 1972). At high analogue concentration (15–30 mM) both the rigor tension and the stiffness go to the low values characteristic of ATP relaxed muscle. In the case of ATP(γ-S) there is some indication that the values are even lower than those obtained with ATP.

Measurements at low analogue concentrations (100 μM to 2 mM) are difficult to interpret quantitatively. Because of the continual turnover of the analogue and the relatively slow rates of diffusion into the muscle fibers it is not possible to atain a well

defined concentration within the fibers. However, qualitatively, the drop of rigor tension seems to saturate at 5–10 mM analogue concentrations.

The drop of stiffness seems to be complete (i.e. ATP-like) at analogue concentrations between 20 and 30 mM.

Mechanical Measurements in the Presence of ATP(β,γ-NH)

A drop of stiffness and rigor tension is induced by adding ATP(β,γ-NH) to muscle fiber bundles in the presence of an excess (20 mM) of $MgCl_2$ (pH 6.9, 22°C). From stiffness titration curves the binding constant is estimated to be 200 μM. The stiffness typically drops to between 50 and 70% of the rigor value and then reaches a plateau. It only decreases very gradually on adding further analogue.

DISCUSSION

Two conclusions may be drawn from these studies:

(*1*) The results obtained with ATP(α,β-CH_2) and ATP(γ-S) indicate that the structure of the cross bridges in insect flight muscle does not differ greatly, at the presently obtainable resolution of the low-angle X-ray diffraction methods, in the presence of ATP or ATP-analogues which are cleaved by myosin but which give the steady-state complex M*-ATP. This indicates that the change of cross-bridge angle from 45° to 90° occurs before ATP cleavage occurs, presumably associated with a step in the binding process. These results must be interpreted with some reservation, since it is not certain that the kinetics of hydrolysis of these analogues by insect myosin have the same characteristics as with rabbit muscle myosin.

(*2*) Low concentration of ATP(β,γ-NH) [and ATP(α,β-CH_2) under some conditions] produces an intermediate state which is not a simple mixture of rigor and relaxed. Two classes of explanation may be advanced to account for the X-ray results reported on ATP(β,γ-NH), namely:

(*a*) The intermediate state consists of a mixture of detached cross bridges in the "90°" position *but restrained* by their actin-bound neighbours *from assuming the relaxed helical configuration* and some fully rigor-like attached bridges. A similar model has been put forward by Lymn and Huxley (1975) as a possible explanation of their results on rabbit psoas muscle.

(*b*) The intermediate state arises through the cross bridges undergoing a conformational change like that involved in the rigor-relaxation transition (i.e. towards the "90°" position) *without letting go of the actin filament.* It is not clear that the new position is identical to the relaxed configuration but it is similar.

On balance our present evidence does not allow one to reject one or the other of these classes of hypothesis. One expects a clear distinction will be possible when our data is supplemented by electron microscopic investigations and by binding studies.

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