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THE NUCLEOTIDE COMPLEXES OF MYOSIN IN GLYCEROL-EXTRACTED MUSCLE FIBRES

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SUMMARY

Binding to single glycerol-extracted rabbit psoas muscle fibres at a low calcium concentration was measured in the presence of radioactive ATP and ADP.

In the presence of ATP, the fibres bound a maximum of 230 μM ADP and P_i and smaller amounts of ATP. The total amount of bound ADP and P_i, the rate of ADP and P_i efflux from the fibres and the K_m of bound ADP were consistent with ATP being cleaved by myosin in the fibres to form a myosin–ADP–P_i complex, whose decomposition was the rate limiting process of the myosin ATPase. The ATPase and the rate of bound ADP decomposition were activated in parallel by increasing concentrations of free calcium. The binding of ADP was associated with relaxation of the muscle fibres.

In the presence of ADP the fibres bound a maximum of 230 μM ADP, consistent with the ADP being bound to myosin. This complex differed from the ATP-generated complex since binding was not associated with relaxation of the fibres, and the rate of decomposition was faster than the ATPase.

INTRODUCTION

Much progress has been made in recent years in the investigation of the enzymic mechanism of myosin ATPase and the effects of actin upon it. Taylor and his associates[1,2] have shown that the bond between the terminal phosphate groups of ATP is rapidly cleaved by the globular head portion of myosin, heavy meromyosin, and the products held in an intermediate heavy meromyosin–ADP–P_i complex whose decomposition is slow and limits the overall ATP hydrolysis rate. Actin increases the rate of heavy meromyosin–ADP–P_i decomposition whilst not significantly affecting the rate of cleavage of the terminal phosphate bond of ATP[3]. Trentham *et al.*[4] have shown that although the decomposition of heavy meromyosin–ADP–P_i intermediate is rate limiting at 23 °C the dissociation of ADP and P_i bound directly to heavy meromyosin is fast. On the other hand, at low temperatures the dissociation of bound ADP from myosin may be rate limiting[30].

Abbreviations: PIPES, piperazine-*N*,*N*′-bis-(2-ethanesulphonic acid); EGTA, ethyleneglycol-bis-(β-aminoethyl ether) *N*,*N*′-tetraacetic acid.

The object of this study has been to relate the observations on purified proteins to the events which take place in the intact, nonactivated muscle. For these experiments single glycerol-extracted rabbit psoas muscle fibres have been used. These fibres closely resemble those of intact muscle[5]; they contain an intact myofibrillar matrix[6] which is sensitive to Ca^{2+}-activation and capable of exerting tension and doing work when incubated in the appropriate ATP and Ca^{2+} containing medium[7]. Single glycerol-extracted fibres are, in effect, miniature dialysis bags of actomyosin and as such permit rapid manipulation and easy measurement of the steady-state intermediates[8] and their rates of dissociation. In this work the binding, kinetic and mechanical properties of the two forms of nucleotide–myosin complexes formed in glycerol-extracted fibres in the presence of ATP and ADP respectively, have been investigated.

MATERIALS AND METHODS

Materials and solutions

Thin strips of psoas muscle were dissected out from a freshly killed rabbit and kept at −15 °C in glycerinating solution (50% glycerol, 100 mM KCl, 20 mM phosphate buffer, pH 7.0, 2.5 mM mercaptoethanol). Preparations were kept up to 4 months, and single fibres were dissected out as required.

Fibres were incubated in PIPES/EGTA buffer, unless otherwise stated (5 mM piperazine-*N*,*N*′-bis-(2-ethanesulphonic acid) (PIPES) buffer, pH 7.1, 5 mM ethyleneglycol-bis-(β-aminoethyl ether) *N*,*N*′-tetraacetic acid (EGTA), 5 mM $MgCl_2$, 50 mM KCl, 1.5 mM sodium azide, ionic strength 0.095). Free calcium levels in the buffer were adjusted by adding the appropriate amount of $CaCl_2$ (ref. 9). The concentration of the ionic species MgATP, MgADP and free Ca^{2+} were calculated using a modified version of the computer programme of Perrin and Sayce[10].

Radioactive nucleotides, [U-^{14}C]ATP, [γ-^{32}P]ATP, [U-^{14}C]ADP and [8-^{3}H]-IDP were obtained from the Radiochemical Centre Ltd, Amersham, Bucks. Specific activities were calculated from the manufacturer's data; these were checked by the absorbance at 260 nm and found to be correct.

When the [^{14}C]ATP-generated binding or dissociation rate was measured the [^{14}C]ADP formed due to hydrolysis by myosin was kept at a level $<0.5\ \mu M$ by addition of 2 mM creatine phosphate and 5 mg/ml creatine kinase (EC 2.7.3.2) as an ATP-regenerating system. When [γ-^{32}P]ATP-generated binding was measured, $^{32}P_i$ formed due to hydrolysis was removed by addition of 5 mM inosine and 2 mg/ml nucleoside phosphorylase (EC 2.4.2.1).

Binding measurements

The binding of nucleotides to glycerol-extracted fibres was measured by incubating the fibre at 1.5 °C in 30–50 μl of PIPES/EGTA buffer containing the appropriate radioactive nucleotide and [1-^{14}C]mannitol (1 mM) as a volume marker. The fibres were incubated for 1–2 min and then carefully withdrawn and rapidly transferred to 5 μl of 5% trichloroacetic acid to stop further reaction. The trichloroacetic acid was subsequently neutralised and the labelled compounds eluted from the fibres were separated and assayed.

In control experiments it was found that the fibre volume determined by the

amount of radioactive mannitol incorporated in the fibre, 25 nl/cm, corresponded closely to the physically measured volume, based on an observed mean diameter of 55 μm. The concentration of 'excess' ^{14}C-labelled nucleotide or $^{32}P_i$ was calculated from the cpm in the fibre per unit volume in excess of that in the incubating solution (see Table I).

Separation and assay of labelled compounds

The radioactive compounds in the neutralised trichloroacetic acid extracts of fibres and in aliquots of the incubating solution were separated by chromatography on polyethylimine impregnated cellulose thin layers. Chromatograms were run first in 50% methanol and then, after drying, in 0.25 M lithium chloride *plus* 2% formic acid[11]. The separated compounds were assayed by liquid scintillation counting (Table I).

Dissociation rate measurements

A single glycerol-extracted fibre was mounted in a frame between two needles 7 mm apart. The mounted fibre was incubated at 1.5 °C in a 10 μl drop of the appropriate radioactive solution (Fig. 1). After 1–2 min incubation the fibre was rapidly transferred (in about 0.5 s) to the first of a series of six 10 μl drops containing PIPES/EGTA buffer *plus* 2 mM non-radioactive ATP and ADP (ADP only for dissociation of ADP-generated complex). The fibre was incubated for 5 or 10 s in each drop and then rapidly transferred to the next one. The radioactive nucleotides and mannitol washed out into the drops in each 5 or 10 s interval were separated by polyethyleneimine impregnated cellulose chromatography and counted.

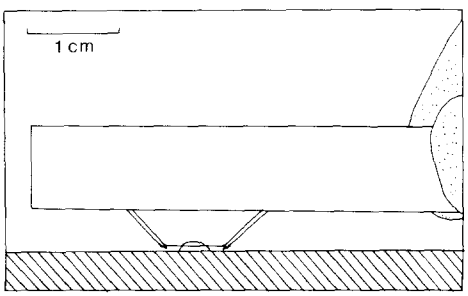

Fig. 1. Determination of the rate of release of radioactive materials from a single glycerol-extracted psoas fibre. The muscle fibre was fixed between the two needle points in the frame with a quick drying cellulose acetate glue. A 10-μl drop of the incubating solution and six 10-μl drops of washout solution were placed on a brass block coated with cellulose acetate which was maintained at 1.5 °C by contact with a 2 cm thick cooled aluminium plate containing a heating element regulated by a thermistor circuit. The fibre was incubated in the drop by holding the frame as shown, so that the points of the needles did not come into contact with the incubating solution

ATPase measurements

The maximum ATPase of a single fibre for a given concentration of free Ca^{2+} at 1.5 °C was determined by incubating fibres in 5 mM [^{14}C]ATP for 1–2 h. The [^{14}C]ADP released was separated by chromatography and counted. Fibre volume was determined by preincubation in [^{14}C]mannitol and hence ATPase per unit

volume was determined. The ATPase per ADP-binding site (turnover number) was calculated from the observed value of the concentration of binding sites within the fibres (see Table II).

Mechanical experiments

To measure fibre stiffness dependence on nucleotide concentration bundles of 3 or 4 muscle fibres were mounted with one end attached to an RCA.5734 mechano-electrical transducer and immersed in the appropriate solution. Tension changes were recorded by a Devices M2 pen recorder. The fibres were quickly stretched by 0.4–2% of their rest length, held for 700 ms and then returned to rest length. The tension changes when the fibres were released were plotted against the extension, Fig. 7a. The slope of the plot was estimated by a regression analysis of the data, from which the fibre stiffness was calculated.

RESULTS

Fibre binding in the presence of [^{14}C]*ATP*

When psoas fibres were incubated with [^{14}C]ATP and 10^{-9} M free Ca^{2+} at 1.5 °C, in the presence of an ATP regenerating system they were found to contain [^{14}C]ADP and smaller amounts of [^{14}C]ATP and [^{14}C]AMP in excess of that in the solution (Table I).

TABLE I

DETERMINATION OF EXCESS NUCLEOTIDE IN GLYCEROL-EXTRACTED RABBIT PSOAS FIBRES

The first column shows the cpm of radioactive mannitol, AMP, ADP and ATP separated from a 33-nl portion of incubating solution containing approximately 15 μM ATP. In the second column is shown the cpm of mannitol, ADP and ATP separated from an approx. 1.5 cm long rabbit psoas fibre; the calculated fibre volume is 33.5 nl. The number of moles of nucleotide is calculated from the cpm and specific activity, 810 cpm/pmole, and the concentration is determined from the volume and the number of moles. Columns 3–5 show the calculated concentrations of AMP, ADP and ATP in the incubating solution, in the fibre and the excess nucleotide in the fibre, respectively.

	Solution (cpm)	*Fibre (cpm)*	*Concn in solution (μM)*	*Concn in fibres (μM)*	*Excess concn in fibres (μM)*
Mannitol	989	1001			
AMP	4	76	0.2	3.8	3.6
ADP	3	3668	0.2	134	134
ATP	411	1275	15.3	46.8	31.5

Under the conditions of this experiment the dissociation rate experiments (described below) showed that the diffusion rate of nucleotides between fibres and solution was rapid compared with the ATPase, and hence the concentration gradients of ATP and ADP across the fibres were likely to be too small to account for the excess ADP (see discussion for numerical argument). The gradients would be further

reduced by the presence of the ATP regenerating enzyme, creatine kinase, inside the fibres. A control experiment showed that after incubation for 2 min at 1.5 °C with 5 mg/ml creatine kinase, the enzyme activity within the fibres was 10 μmoles/min per ml of fibres. This was approximately the same as the enzyme activity present in the incubating solution, which was 11 μmoles/min per ml, and thirty times the maximum ATPase activity of the fibres; thus the free ADP formed by myosin ATPase within the fibres would be effectively removed. For the purposes of this discussion all the excess ADP and ATP is assumed to be bound.

Bound nucleotide was measured at MgATP concentrations from 1 to 200 μM (Fig. 2). The 5% fiducial limits of bound nucleotide in a set of 4–10 fibres at a given ATP concentration were in the region of 10%. Bound ADP increased with concentration of MgATP in a roughly hyperbolic manner but the bound ATP did not fit a hyperbolic plot since it continued to increase almost linearly at high concentrations of MgATP. Bound AMP was low (2–5 μm) at ATP concentrations up to 200 μM. A similar result was obtained when nucleotide binding was measured in glycerol-extracted *Lethocerus* flight muscle fibres (Table II).

TABLE II

BINDING IN GLYCEROL-EXTRACTED MUSCLE FIBRES

Incubation conditions: pH 7.1, $I=0.095$, 5 mM Mg^{2+}, 10^{-9} M free Ca^{2+}, 1.5 °C except where otherwise stated. The mean and standard errors of the constants K_m and B_m were calculated for the statistical best fit of the assay results to the Michaelis–Menten equation.

Muscle	*Nucleotide in incubating medium*	*Species bound*	B_m (μM)	K_m (μM)	*No. of assays*
Rabbit psoas	[^{14}C]ATP	ADP	233 ± 6	15 ± 1	105
	[γ-^{32}P]ATP	P_I	226 ± 13	122 ± 26	42
	[^{14}C]ADP	ADP	231 ± 15	40 ± 6	98
	[^{14}C]ADP*	ADP	227 ± 15	32 ± 4	26
	[^{3}H]IDP	IDP	236 ± 32	280 ± 90	80
Lethocerus flight	[^{14}C]ATP	ADP	183 ± 14	12 ± 4	22
	[^{14}C]ADP	ADP	204 ± 8	11 ± 2	51

* 21 °C.

The data for ADP binding as a function of MgATP concentration were analysed to give a statistical best fit to the Michaelis–Menten equation[12]. The Michaelis constant (K_m) of the bound ADP in psoas muscle was 15±1 μM, standard error and the maximum amount bound (B_m) was 233±6 μM. Thus, if ADP is bound in a simple hyperbolic manner, the determination of the constants for the curve is accurate. The possibility of heterogeneous binding or cooperativity was considered. At low substrate concentrations the Lineweaver–Burke plot (Fig. 2B) shows a deviation from the linear which might be due to positive cooperative binding of ADP; there was no evidence for heterogeneous binding. This observed deviation from linearity at ATP concentrations below 10 μM may, however, be an artefact due to an inadequate activity of ATP-regenerating enzyme resulting in the ATP concentration

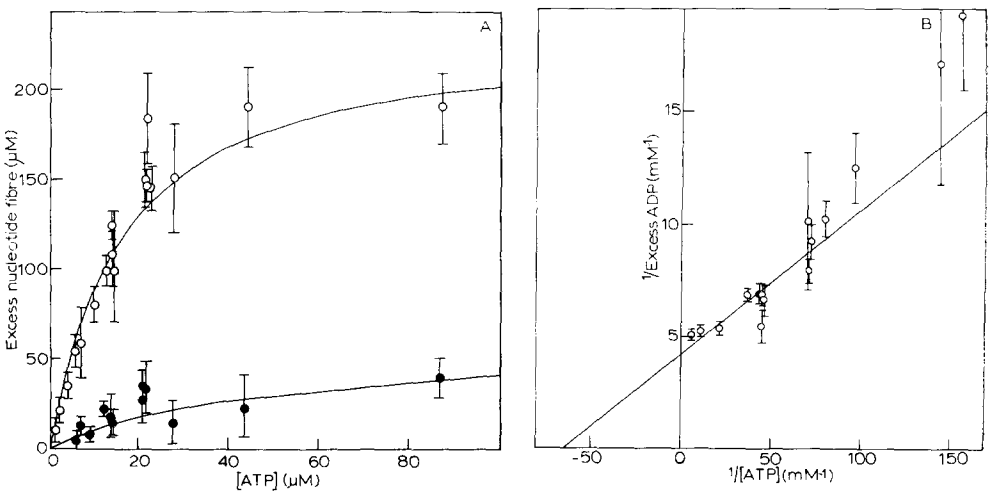

Fig. 2. Excess [^{14}C]ATP and [^{14}C]ADP in glycerol-extracted rabbit psoas fibres incubated with [^{14}C]ATP. (A) The dependance of excess nucleotide on MgATP concentration: The vertical bars are the 5% fiducial limits calculated by Student's t test. The line represents the best fit of the data to the Michaelis–Menten equation. (B) Lineweaver–Burke plot for ADP binding. The vertical bars are 5% fiducial limits. The line represents the statistical best fit to the Michaelis–Menten equation.

being lower in the fibres than in the incubating solution. In support of this it was found that when ADP binding was measured with myofibrils embedded in 1% agarose gels, where the concentration of ATPase sites was 1/10 that in fibres, no deviation from linearity occurred even at 0.5 μM ATP.

Binding in the presence of [γ-^{32}P]ATP

When fibres were incubated with [γ-^{32}P]ATP at 10^{-9} M Ca^{2+}, using nucleoside phosphorylase and inosine to remove any free P_i formed by ATP hydrolysis in the fibres, little or no excess [γ-^{32}P]ATP was found in the fibres, but there was a large excess of $^{32}P_i$ over that in the solution. Owing to the comparatively rapid rate of diffusion of small molecules from fibres, discussed above, only part of this excess could have been due to diffusional lag in the removal of phosphate from the fibres. With 2 mg/ml nucleoside phosphorylase the enzyme's activity within the fibre was at least 4 times the ATPase activity so it is likely that nearly all the excess P_i was bound. This is supported by the observation that the excess P_i is released from the fibres slowly (Fig. 3B) with no evident rapid release phase such as might be expected if unbound P_i were released. The graph of bound P_i in fibres against ATP concentration (Fig. 3A) is roughly hyperbolic and the calculated fit to the Michaelis–Menten equation gives reasonably precise values for K_m and B_m (Table II). B_m is about the same as B_m for ADP bound in the presence of ATP, but K_m is 8 times greater.

Binding in the presence of ADP

Glycerol-extracted fibres bound [^{14}C]ADP when incubated with [^{14}C]ADP (Fig. 4). Binding appeared to be simple, since no curvature of the Scatchard plot

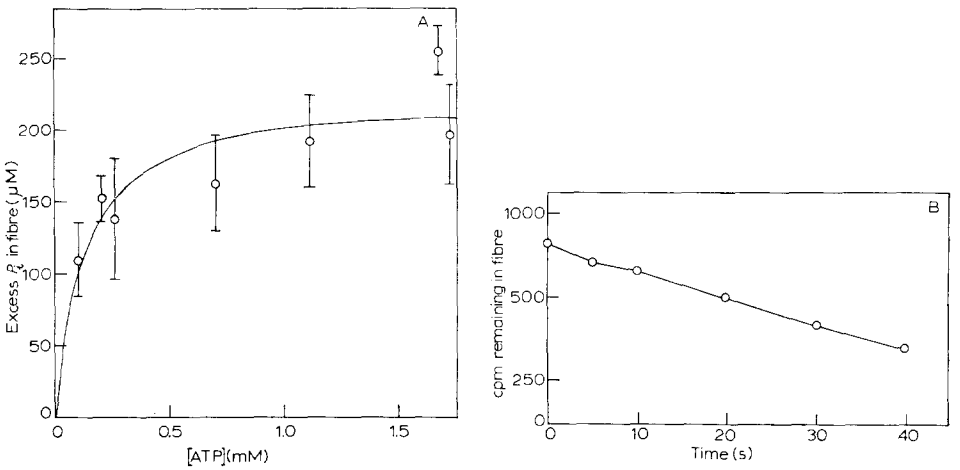

Fig. 3. Binding and release of $^{32}P_i$ by glycerol-extracted rabbit psoas fibres incubated with [γ-^{32}P]ATP. (A) The dependance of excess phosphate on MgATP concentration. The vertical bars are 5% fiducial limits. The line represents the best fit of the data to the Michaelis–Menten equation. (B) The release of $^{32}P_i$ from fibres pre-incubated in 1.1 mM[γ-^{32}P]ATP at 1.5 °C with 10^{-9} M free Ca^{2+}. The rate constant calculated from the slope of the semi-log plot is 0.020 s^{-1}, correlation coefficient as + 0.99.

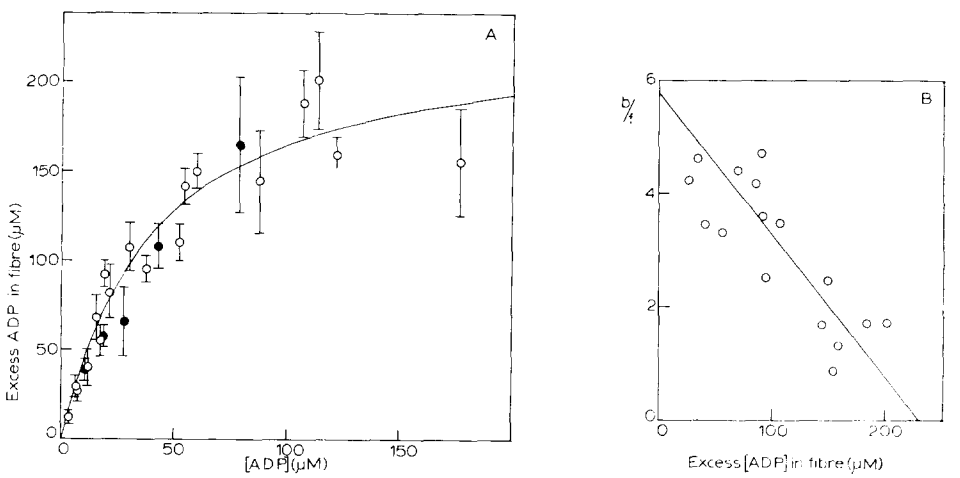

Fig. 4. Excess [^{14}C]ADP in glycerol-extracted rabbit psoas fibres incubated with [^{14}C]ADP. (A) The dependance of excess ADP on total ADP concentration. ○, in the presence of 5 mM Mg^{2+}; ●, no added Mg^{2+}. The vertical bars are the 5% fiducial limits. The line shows the best fit of the data to the Michaelis–Menten equation. (B) Scatchard plot of ADP binding with 5 mM Mg^{2+}. The line represents the best fit of the data to the Michaelis–Menten equation.

could be observed within the limits of the estimation (Fig. 4B). The dissociation constant calculated from the best fit to the Michaelis–Menten equation was $40 \pm 6\,\mu$M and the maximum amount bound was very similar to the maximum amount of bound ADP in the presence of ATP (Table II). Only 70% of the total free ADP

was complexed with magnesium in the PIPES/EGTA buffer used, which contained 5 mM $MgCl_2$, but complexing of Mg^{2+} with ADP seemed to be unnecessary for ADP binding, since the amount of ADP bound was not altered when Mg^{2+} was absent (see Fig. 4A). The dissociation constant of ADP-binding to fibres was almost independent of temperature over the range 1–21 °C (Table II).

The possibility that the bound ADP might be an artefact caused by conversion of ADP to ATP by endogenous myokinase instead of direct binding of ADP was investigated. The maximum initial myokinase activity of the fibres was 10 times the maximum activity of the ATPase. However since myokinase has a high K_m (ref. 13) the initial myokinase activity would have been about 1/40 to 1/4 of its maximum activity over the concentration range where ADP is bound (10 to 100 μM). The rate for formation of AMP from ADP in fibres at 20 μM ADP was measured and found to be less than one-tenth of the maximum ATPase rate; therefore the effect of myokinase was expected to be small. This was confirmed by the observations that the addition of 5 mM AMP, to 20 μM [^{14}C]ADP, which would have reduced ATP to less than 1 μM if myokinase had been at equilibrium, reduced the binding of ADP by only 20%, while the addition of 1 mg/ml hexokinase and 2 mM glucose to remove ATP, had no effect. The binding of ADP in the presence of ADP is, therefore, a direct binding and not due to a prior formation of ATP.

The binding of [^{3}H]inosine diphosphate was also measured. Its dissociation constant was much greater than for ADP, but the maximum amount bound was the same (Table II). IDP reacts with myokinase at least 100 times slower than ADP, therefore the binding of [^{3}H]IDP to fibres must also be direct.

Dissociation rates

The efflux of radioactive nucleotides and mannitol from glycerinated fibres approximated to a single exponential decay (Fig. 5). Results were analysed by least squares fit to the equation $\ln (C/C_0) = -rt$ where C is the cpm remaining inside the fibre at time t (s), C_0 is the initial cpm, and r the rate constant for release of the radioactive material from the fibres (s^{-1}). The correlation coefficients were usually about +0.99 for ATP and ADP but mannitol release appeared to be more erratic, the mean correlation coefficient being +0.94. The total amount of mannitol released from the fibres was that expected from the length of fibre used and the total amount of bound nucleotide relased (Figs 5A and 5C) was similar to the amount of nucleotide bound in the steady state experiments at the same substrate concentration (Figs 2A, 4A).

In fibres preincubated with 25 μM [^{14}C]ATP an ATP-regenerating system and 10^{-9} M free Ca^{2+} at 1.5 °C, the rate of release of ADP was slow: $r = 0.020\ s^{-1}$ (Fig. 5A). The P_i bound when fibres were preincubated in [γ-^{32}P]ATP with a P_i-removing system was released at the same rate as the bound ADP (Fig. 3B) (Table III). This rate agreed closely with the maximum ATP hydrolysis rate per ADP binding site (turnover number) $k_3 = 0.021\ s^{-1}$. The mannitol and ATP were released rapidly and with a common rate constant of $0.086\ s^{-1}$ (Table III). This rate of release was limited by the rate of diffusion from the fibres; a rate constant of $0.086\ s^{-1}$ for a 55 μm diameter fibre corresponded to a diffusion coefficient of $1.1 \cdot 10^{-7}\ cm^2/s$ (ref. 14), in accord with previous estimates[15].

It should be noted that the diffusion process from fibres also retards the rate

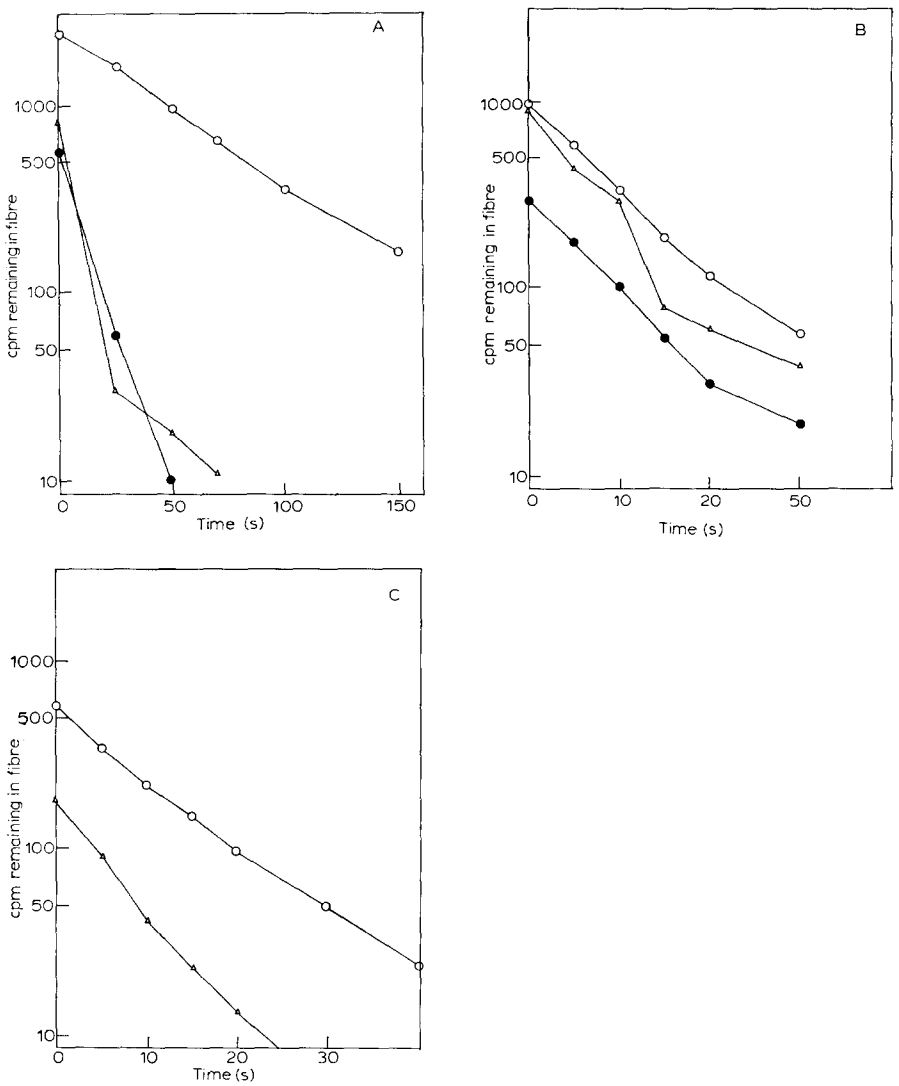

Fig. 5. Release of ADP, ATP and mannitol from rabbit psoas fibres at 1.5 °C. (A) Release of ADP (○), ATP (●) and mannitol (△) from a single muscle fibre preincubated in 26 μM [^{14}C]ATP with 10^{-9} M free Ca^{2+}. The rate constants were: ATP, 0.082 s^{-1}; ADP, 0.021 s^{-1}; mannitol 0.076 s^{-1}. The correlation coefficients were 0.99, 0.99 and 0.93, respectively. The fibre volume determined from the mannitol counts was 19.8 nl and the amount of ADP released corresponded to a concentration of 135 μM in the fibre. (B) Release of ADP, ATP and mannitol from a single muscle fibre preincubated in 26 μM [^{14}C]ATP with 10^{-6} M free Ca^{2+}. The rate constants were: ATP, 0.096 s^{-1}; ADP, 0.097 s^{-1}; mannitol, 0.112 s^{-1}. Correlation coefficients were 0.99, 0.99 and 0.96, respectively. The symbols are the same as in (A). (C) Release of ADP and mannitol from a single muscle fibre preincubated in 16 μM ADP with 10^{-9} M Ca^{2+}. Rate constants were: ADP, 0.077 s^{-1}; mannitol, 0.083 s^{-1} and the correlation coefficients were respectively 0.99 and 0.87. The fibre volume determined from the mannitol counts was 12.4 nl and the calculated total amount of ADP released was equivalent to 66 μM in the fibres (of which 16 μM, or 24% of the total ADP is free ADP). The symbols are the same as in (A).

TABLE III

RATE CONSTANTS FOR THE RELEASE OF LABELLED COMPOUNDS FROM GLYCEROL-EXTRACTED PSOAS FIBRES

Conditions: PIPES/EGTA buffer, pH 7.1, $I=0.095$, 1.5 °C. Rate constants shown are the mean and 5% fiducial limits of 8–12 determinations.

Preincubation medium	*Compound released*	*Rate constant of release, r, (s^{-1})*
26 μM [^{14}C]ATP, 10^{-9} M Ca^{2+}	[^{14}C]ATP	0.087 ± 0.013
	[^{14}C]ADP	0.020 ± 0.003
1.1 mM [γ-^{32}P]ATP, 10^{-9} M Ca^{2+}	$^{32}P_i$	0.020
26 μM [^{14}C]ATP, 10^{-6} M Ca^{2+}	[^{14}C]ATP	0.086 ± 0.016
	[^{14}C]ADP	0.088 ± 0.012
7 μM [^{14}C]ADP, 10^{-9} M Ca^{2+}	[^{14}C]ADP	0.084 ± 0.020
16 μM [^{14}C]ADP, 10^{-9} M Ca^{2+}	[^{14}C]ADP	0.080 ± 0.010
1 mM [^{14}C]ADP, 10^{-9} M Ca^{2+}	[^{14}C]ADP	0.089 ± 0.017
19 μM [^{3}H]IDP, 10^{-9} M Ca^{2+}	[^{3}H]IDP	0.083 ± 0.010
1 mM [^{14}C]mannitol	[^{14}C]mannitol	0.086 ± 0.015
0.8 mM [^{32}P]phosphate, 10^{-9} M Ca^{2+}	[^{32}P]phosphate	0.089 ± 0.012

of release of material from fibres even when that rate is less than the rate of diffusion. On the assumption that the decomposition of bound ADP and its diffusion out of the fibre are consecutive first-order processes the time course for the release of ADP into the solution was calculated[16]. With a diffusion rate of 0.087 s^{-1}, the model for ADP release gave a best fit to a single exponential decay with $r=0.020$ s^{-1} if the rate of decomposition of bound ADP was 0.022 s^{-1}. Therefore in the case of non-activated muscle the retarding effect of the diffusion on the rate of release of ADP from the fibres was probably small.

The myofibrillar ATPase was activated as the free calcium concentration increased. The maximum turnover number which was obtained at calcium concentrations above $5\cdot10^{-7}$ M, was 0.14 s^{-1} (Fig. 6). The rate of release of bound ADP from fibres likewise increased as the free calcium concentration was raised (Fig. 5B) and over the range 10^{-9}–10^{-7} M free Ca^{2+} the rate constant is similar to that for ATP hydrolysis (Fig. 6). Unfortunately, the rate of ADP release is limited by the rate of diffusion in activated fibres and no increase in the rate constant can be observed at free calcium concentrations above $2\cdot10^{-7}$ M.

In contrast, the rate of release of ADP from fibres pre-incubated in [^{14}C]ADP was fast at 10^{-9} M Ca^{2+} (Fig. 5C). At 7, 16 and 1000 μM ADP where bound ADP was 80, 70 and 20% of the total ADP, respectively, the rate constants for ADP release were the same (Table III); hence the directly-bound ADP was released at the same rate as free ADP, both being limited by the rate of diffusion. The release of bound [^{3}H]IDP was also rapid at low calcium concentrations (Table III).

The effect of ATP and ADP on fibre stiffness

When bundles of 3 or 4 glycerol-extracted psoas muscle fibres were incubated at 2 °C in PIPES/EGTA buffer with 10^{-9} M Ca^{2+} and stretched by 0.4–2% of their

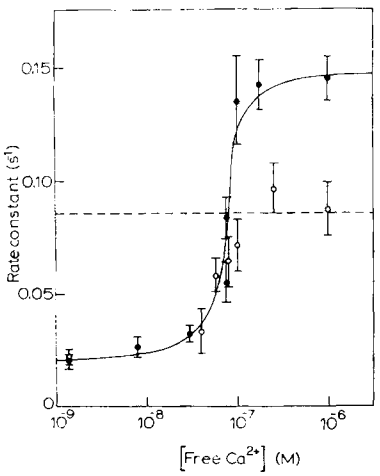

Fig. 6. Dependance of the rate constant, r, for bound ADP release from fibres (○) and the ATPase turnover number, k_3, (●) on free calcium concentration. The bars are 5% fiducial limits. The dotted line is the diffusion-limited rate of efflux of materials from fibres; 0.087 s^{-1}.

length, the tension increased approximately linearly with their extension (Fig. 7A); thus for short extensions the fibres behaved elastically. The elastic modulus (stiffness) was calculated as about 5.4 N/mm^2 (Fig. 7B).

When ADP at concentrations up to 2 mM was added, no change in stiffness occurred (Fig. 7A). However when low concentrations of ATP with an ATP-regenerating system were added the stiffness decreased (Fig. 7B). Stiffness dropped to about half its initial value at 10–20 μM ATP (Fig. 7B), which is similar to the ATP concentration where ADP is 50% bound (Fig. 2). Indeed the fall in stiffness closely mirrored the binding of ADP. At high ATP concentrations the residual stiffness was very low (about 0.7 N/mm^2).

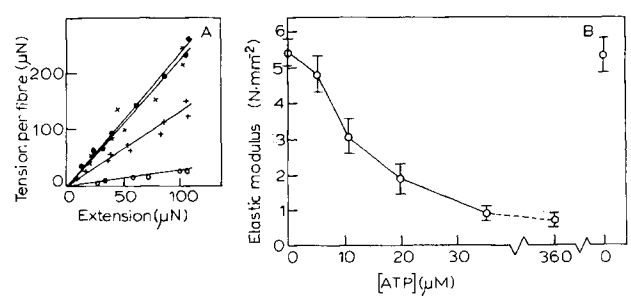

Fig. 7. Dependance of fibre stiffness on ATP and ADP. (A) Change of fibre tension with extension in rapidly stretched 6 mm long bundles of glycerol-extracted psoas muscle fibres at 2 °C. Incubation media: ●, PIPES/EGTA buffer, 10^{-9} M Ca^{2+}; ×, 2 mM ADP, PIPES/EGTA buffer, 10^{-9} M Ca^{2+}; +, 10 μM ATP, PIPES/EGTA buffer, 10^{-9} M Ca^{2+}, 5 mg/ml creatine kinase, 1 mM creatine phosphate; ○, 360 μM ATP, PIPES/EGTA buffer, 10^{-9} M Ca^{2+}, 5 mg/ml creatine kinase, 1 mM creatine phosphate. The lines are least squares fits obtained by a regression analysis of the experimental points. (B) Dependance of elastic modulus (stiffness) of glycerol-extracted psoas muscle fibres on ATP concentration at 2 °C with 10^{-9} M Ca^{2+}. Elastic modulus, defined as the ratio of stress (tension/unit area of fibre) to strain (length change per unit length), was calculated from the slopes of the length–tension plots. The vertical bars are 5% fiducial limits.

The stiffness changes with ATP concentration were fully reversible and the elastic modulus of a fibre bundle returned to its initial value when incubated in the absence of ATP.

DISCUSSION

Characteristics of the myosin–ADP–P_i intermediate complex

The maximum amount of excess ADP in fibres incubated in ATP was 233 μM. Diffusion calculations predict that, at a maximum, less than one-third of this could be free ADP. The release of free nucleotide and mannitol from fibres approximated to a single exponential decay with a rate constant of 0.087 s^{-1} (Table III) and if one assumes the fibre to behave as if it were an infinitely long cylinder of diameter 55 μm the diffusion coefficient may be calculated from the equation $r = 5.8\ D/a^2$ where r is the rate constant for diffusion and a is the radius of the cylinder[14]. The value obtained is $1.1 \cdot 10^{-7}$ cm^2/s which is similar to previous estimates[15]. The concentration gradient between the solution and the centre of the fibre, ΔC, can now be determined from the equation $\Delta C = m \cdot a^2/4D$, where m is the rate of the ATPase within the fibres, a is the fibre radius, and D is the diffusion coefficient[17]. It was calculated that at the maximum ATPase rate (4.3 $nmole \cdot ml^{-1} \cdot s^{-1}$) the ADP or ATP concentration gradient would be 70 μM. This was a maximum value; it would be reduced at ATPase rates less than the maximum and assumed that no ATP regeneration could occur within the fibres.

Since recent reports indicate that large molecules and proteins can be diffused into the interior of glycerinated fibres[18], it might be expected that creatine kinase can diffuse into the fibres and remove any free ADP present, and indeed it was found that the creatine kinase activity within glycerinated fibres approached the activity in the incubating medium after 2 min incubation at 1.5 °C. The absence of significant free ADP in the fibres was confirmed by the rate experiments (Fig. 5A); unbound ATP and mannitol were released from the fibres rapidly ($r = 0.087\ s^{-1}$) and the semi-log plot showed that all the excess ADP in the fibres was released slowly; it follows that all the ADP in the fibres was bound, since if some of it were free the ADP release plot would have an additional fast phase. This argument is also valid for the binding of the γ-phosphate moiety of ATP (Fig. 3B). Since the free ADP concentration was too low for direct binding of ADP, the bound ADP must have originated from the ATP in the solution.

There are in muscle a large number of proteins to which the ADP might be bound. Actin is an unlikely candidate because the ADP exchange of F-actin takes several hours[19]. Mitochondria are also unlikely because *Lethocerus* flight muscle, which contains a large mass of mitochondrial protein, bound no more ADP than rabbit psoas muscle, which contains little. No other proteins have been excluded, but the maximum amount of bound ADP is much greater than the other myofibrillar proteins[20], except for myosin.

It is believed that the ADP and P_i bound in the presence of ATP are bound to the active site of myosin since they exhibit the characteristics expected of the myosin–ADP–P_i intermediate complex first postulated by Taylor and co-workers[1,2].

$$\text{Myosin} + \text{ATP} \overset{K_m}{\rightleftharpoons} \text{myosin–ADP–P}_i \overset{k_3}{\rightarrow} \text{myosin} + \text{ADP} + \text{P}_i$$

If ADP and P_i are bound in an myosin–ADP–P_i complex then the maximum amount bound, B_m, should equal the concentration of myosin active sites in the fibres, the K_m values for binding should be the same as the K_m of the myofibrillar ATPase and the ATPase turnover number, k_3, should be the same as the rate constant for decomposition of bound ADP and P_i, r.

According to recent estimates[33] the concentration of myosin molecules is 120 μM in rabbit psoas fibres and 100 μM in *Lethocerus* flight muscle fibres. Thus the B_m of ADP and P_i in rabbit psoas muscle (Table II) corresponds to two molecules bound per myosin molecule, or one per myosin active site. There is also approximately one ADP bound per active site in *Lethocerus* flight muscle (Table II).

The Michaelis constant for ADP binding, 15 μM, was larger than the K_m of the myofibrillar ATPase which was about 6 μM with 10^{-9} M free Ca^{2+} and excess Mg^{2+} (refs 8, 21). Theoretically the two values should be the same, and the higher K_m for ADP binding may be due to the ATP-regenerating enzyme being unable to maintain the ATP concentrations in the fibres when the concentration of ATP in the incubating solution was low.

The apparent K_m for the binding of the γ-phosphate, 120 μM, was much greater than that for ADP binding. However, the binding of the γ-phosphate of ATP had to be measured without an ATP regenerating system and consequently the concentration of ATP within the fibres would have been less than that in solution, due to a diffusional lag in ATP entry[15]. Thus at an ATP concentration in solution of 120 μM (where bound P_i is 110 μM (Fig. 3)) there is an excess P_i concentration of 210 μM within the fibre if the nucleoside phosphorylase is not present. Hence the diffusional lag in free P_i release causes a concentration difference of 100 μM between the fibre and the solution. If one may assume that the concentration gradient of ATP between the solution and fibre is equal and opposite to the P_i concentration gradient (this is a reasonable assumption, since small molecules diffuse from fibres at the same rate (Table III)), then the concentration of ATP inside the fibre is about 20 μM. The K_m observed was therefore probably not the true K_m, which must be in the region of 20 μM.

The rate constant for release of bound ADP and P_i, r, was the same as the ATPase turnover number, k_3, at 10^{-9} M Ca^{2+} (Table III) and both bound ADP release and the ATPase were activated by free calcium over the same range of concentrations (Fig. 6).

From these experiments it is concluded that in relaxed glycerinated fibres, as in isolated myosin [1,2], the terminal phosphate bond of ATP is rapidly cleaved by myosin and the ADP and γ-phosphate moieties are retained as a myosin–ADP–P_i intermediate complex. The decomposition of this complex is slow compared with the other steps in the reaction, and limits the overall ATP hydrolysis rate.

Similar results have been obtained by Maruyama and Weber in myofibrils[22]. They found that activated or non-activated myofibrils incubated in radioactive ATP bound 2 moles of isotope per myosin molecule, though they did not distinguish between bound ATP and ADP. The major part of the isotope was bound with a K_m similar to the K_m of the ATPase, but a fraction binding at higher affinity was also observed. This was not found for ADP bound to glycerol extracted fibres, but its occurrence cannot be ruled out since such heterogeneous binding might have been masked if the ATP regenerating system had not been sufficiently active; on the

other hand, the difference may be due to the different preparations investigated.

In the absence of ATP, glycerol extracted fibres are in rigor and have a high elastic modulus (Fig. 7). It is believed that in rigor nearly all the myosin molecules are attached to actin in the thin filaments[6,23,24], whereas in relaxed fibres, when the stiffness of the fibres is very low, most of the myosin molecules are dissociated from actin. It is therefore reasonable to assume that the fibre stiffness gives a measure of the proportion of myosin molecules attached to actin.

The mechanical experiments (Fig. 7B) showed that at 10^{-9} M Ca^{2+} stiffness fell over the same ATP concentration range as that in which ADP was bound. Thus, under these conditions, the binding of actin and ATP (with formation of myosin–ADP–P_i) to myosin appears to be mutually exclusive and relaxation takes place as a consequence of ATP reacting with the myosin ATPase active site, thereby displacing the actin.

In addition to ADP, ATP was also bound in fibres in the presence of ATP (Fig. 2A). It is not known where this is bound, but some of it may be bound to myosin, since a small amount of myosin–ATP in addition to myosin–ADP–P_i has been observed in heavy meromyosin in the presence of ATP[34].

Evidence for an actomyosin–ADP complex

The maximum excess [^{14}C]ADP found in fibres incubated with [^{14}C]ATP was 231 μM (Fig. 4; Table II). ADP is not metabolised by the myosin ATPase and it has been shown (see results section) that there was no significant reaction of ADP with myokinase in the glycerol extracted fibres. As a result there should be no concentration gradients of ADP between the fibre and the incubating solution due to diffusional lag, so any excess ADP found within the fibres must be bound.

The ADP is apparently bound to a single class of sites (Fig. 3B) with an affinity which is almost as great as the affinity of the myosin–ADP–P_i complex. However, the accuracy of the assays does not permit the complete exclusion of heterogeneous binding or cooperative effects. The estimated maximum amount of ADP (or IDP) bound (Table II) was the same as the maximum amount of the ADP and P_i bound in the presence of ATP, which as has been already discussed approximates to the number of myosin active sites present in the fibre; consequently it is proposed that the ADP-generated bound ADP is bound to myosin

ADP has been reported to be a competitive inhibitor of actomyosin ATPase in both rabbit psoas and insect flight muscle[25,26] which suggests that the bound ADP may be closely associated with the active site of myosin ATPase. It was not possible to measure the effects of ADP on ATP-generated binding since myosin–ADP–P_i could only be measured in the absence of free ADP. However, it was found that adenylyl imidodiphosphate, a competitive inhibitor of myosin ATPase[31], inhibited both ATP- and ADP-generated binding, K_i being about 1 mM in both cases. The simplest explanation for these observations is that ADP binds to the active site of myosin ATPase in fibres; it is possible that the ADP could bind to a separate site, but if this were so the interaction between the ATPase site and the ADP-binding site must be very strong[27].

The stiffness measurements (Fig. 7) showed that there was no relaxation of the glycerol extracted fibres, even in the presence of 2 mM ADP. Similarly it has been observed that ADP does not dissociate actomyosin except at very high concen-

trations[28]. It must be concluded that ADP can bind to glycerol-extracted fibres without dissociating the actin–myosin links.

In summary, it is proposed that, in the presence of ADP, actomyosin is in rapid equilibrium with a complex in which ADP is bound to myosin, whilst the actin–myosin links remain intact.

$$\text{Actomyosin} + \text{ADP} \rightleftharpoons \text{actomyosin–ADP}$$

It is uncertain whether or not the actomyosin–ADP complex is an intermediate in the decomposition of myosin–ADP–P_i in the myosin ATPase cycle in non-activated fibres. Since pure myosin and its subfragments hydrolyse ATP, apparently in the same way as myosin in fibres[1,2,4], actin-containing complexes are not necessarily included in the cycle. The dissociation of actomyosin–ADP is fast (Table III), therefore if actomyosin–ADP is part of the ATPase cycle in non-activated fibres, its decomposition is not the rate-limiting step.

The properties of the actomyosin–ADP complex differ substantially from those of ADP bound to myosin alone (myosin–ADP). The dissociation constant of actomyosin–ADP is considerably greater than that of myosin–ADP which is 0.7 μM at low ionic strength and 2.5–7 μM at high ionic strength with high Mg^{2+} concentrations[29,32]. The high K_d is probably due to the presence of actin in the actomyosin–ADP complex, since actin is known to inhibit ADP binding to myosin[29]. Unlike myosin–ADP whose dissociation constant increases 6-fold over temperature range 6–25 °C[30], the dissociation constant of actomyosin–ADP is not greatly affected by temperature (see Table II).

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REFERENCES

1 Lymn, R. W. and Taylor, E. W. (1970) *Biochemistry* 9, 2975–2983
2 Taylor, E. W., Lymn, R. W. and Moll, G. (1970) *Biochemistry* 9, 2984–2991
3 Lymn, R. W. and Taylor, E. W. (1971) *Biochemistry* 10, 4617–4624
4 Trentham, D. R., Bardsley, R. G., Eccleston, J. F. and Weeds, A. G. (1972) *Biochem. J.* 126, 635–644
5 Szent-Gyorgyi, A. (1949) *Biol. Bull.* 96, 140–161
6 Rome, E. (1972) *J. Mol. Biol.* 65, 331–345
7 Bendall, J. R. (1969) *Muscles, Molecules and Movement*, pp. 58–73, Heineman Educational Books, London
8 Marston, S. B. and Tregear, R. T. (1972) *Nat. New Biol.* 235, 23–24
9 Portzehl, H., Caldwell, P. C. and Ruegg, J. C. (1964) *Biochim. Biophys. Acta* 79, 581–591
10 Perrin, D. D. and Sayce, I. G. (1967) *Talanta* 14, 833–842
11 Randerath, K. and Randerath, E. (1967) in *Methods in Enzymology* (Grossman, L. and Moldave, S., eds), Vol. XII, pp. 323–347, Academic Press, New York
12 Wilkinson, G. N. (1961) *Biochem. J.* 80, 324–332
13 Noda, L. (1958) *J. Biol. Chem.* 232, 237–250
14 Carslaw, H. S. and Jaegar, J. C. (1959) *Conduction of Heat in Solids*, pp. 199–202, Oxford University Press, Oxford
15 Mannherz, H. G. (1968) *Pfluegers Arch.* 303, 230–248
16 Moore, W. J. (1963) *Physical Chemistry*, 4th edn, pp. 266–267, Longmans Green, London

17 Meyerhof, O. and Schulz, W. (1927) *Pfluegers Arch.* 217, 547–573
18 Rome, E. (1973) in *Cold Spring Harbor symp. Quant. Biol.* 37, 331–340
19 Szent-Gyorgyi, A. G. and Prior, G. (1966) *J. Mol. Biol.* 15, 515–538
20 Ebashi, S. and Endo, M. (1968) *Prog. Biophys.* 18, 123–183
21 Kominz, D. R. (1970) *Biochemistry* 9, 1792–1800
22 Maruyama, K. and Weber, A. (1972) *Biochemistry* 11, 2990–2998
23 Huxley, H. E. (1968) *J. Mol. Biol.* 37, 507–520
24 Reedy, M. K., Holmes, K. C. and Tregear, R. T. (1965) *Nature* 207, 1276–1280
25 Green, I. and Mommaerts, W. F. H. M. (1954) *J. Biol. Chem.* 210, 695–702
26 Maruyama, K. and Pringle, J. W. S. (1967) *Arch. Biochem. Biophys.* 120, 225–226
27 Monod, J., Changeux, J-P., and Jacob, F. (1963) *J. Mol. Biol.* 6, 306–329
28 Gergely, J., Martonosi, A. and Gouvea, M. A. (1959) in *Symposium on Sulfur in Proteins* (Benesch, R., Benesch, R. E., Boyer, P. D., Klotz, I. M., Middlebrook, W. R., Szent-Gyorgyi A. G. and Schwarz, D. R., eds), pp. 297–313, Academic Press, New York
29 Kiely, B. and Martinosi, A. (1968) *Biochim. Biophys. Acta* 172, 158–170
30 Malik, M. N. and Martinosi, A. (1972) *Arch. Biochem. Biophys.* 152, 243–257
31 Yount, R. G., Ojala, D. and Babcock, D. (1971) *Biochemistry* 10, 2490–2495
32 Lowey, S. and Luck, S. M. (1969) *Biochemistry* 8, 3195–3199
33 Tregear, R. T. and Squire, J. M. (1973) *J. Mol. Biol.*, in the press
34 Bagshaw, C. R. and Trentham, D. R. (1973) *Biochem. J.*, in the press