*J. Physiol.* (1984), **352**, *pp.* 575–599
*With 7 text-figures*
*Printed in Great Britain*



# THE KINETICS OF MAGNESIUM ADENOSINE TRIPHOSPHATE CLEAVAGE IN SKINNED MUSCLE FIBRES OF THE RABBIT

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(*Received 26 October 1983*)

SUMMARY

1. The time course of magnesium adenosine triphosphate (Mg ATP) cleavage in chemically skinned muscle fibres of the rabbit was measured by a method in which Mg ATP cleavage was initiated by photolytic release of ATP from $P^3$-1-(2-nitro)phenylethyladenosine 5′-triphosphate (caged ATP) and terminated by rapid freezing 50 ms to 8 s later. Up to 5 mM-ATP was released following a single 50 ns laser pulse at 347 nm. Mg ATP cleavage was measured at 19 °C in the presence and absence of calcium ions, for fibres near rest length and stretched beyond overlap of the myofilaments.

2. At full overlap and in the absence of calcium ($< 10^{-8}$ M) and nucleotide, the fibres developed rigor tension. Following the laser pulse the tension decreased to that of a relaxed fibre in two distinct phases. The first phase lasted about 40 ms and was followed by a second phase during which tension decreased to zero with an approximately exponential time course with a rate constant of 11 $s^{-1}$. In the presence of $2 \times 10^{-5}$ M-free calcium ions, the initial phase following the laser flash lasted approximately 13 ms, and was followed by an exponential rise of tension with a rate constant of 28 $s^{-1}$. The active tension reached by the muscle fibres was 54 $kN/m^2$. For fibres stretched beyond overlap, no change in tension was observed following the release of Mg ATP.

3. Under all conditions the time course of Mg ATP cleavage was biphasic, and consisted of a rapid initial burst of ADP formation, complete within 50 ms, followed by a slower steady-state rate of Mg ATP cleavage. The number of molecules of Mg ATP cleaved during the burst was approximately equal to the number of myosin subfragment 1 heads for fibres at full myofilament overlap, and equal to 0·7 molecules per myosin subfragment 1 head for fibres stretched beyond overlap. At full overlap in the presence of calcium ions, the steady-state rate equalled 1·8 mol Mg ATP cleaved per mole myosin subfragment 1 head per second. In all other cases the steady-state rate of Mg ATP cleavage was at least 10-fold less.

4. When fibres at full overlap were pre-incubated with 2 mM-ADP, the initial phase

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of the tension response was somewhat prolonged, but the burst of ADP formation was also complete within 50 ms. However, in the case of fibres stretched beyond overlap, the presence of ADP reduced the rate of the initial burst of ADP formation by at least a factor of 3.

5. A low steady-state rate of Mg ATP cleavage for fibres stretched beyond overlap in the presence of calcium ions showed that other ATPases such as the calcium ion-dependent ATPase of the sarcoplasmic reticulum did not cleave Mg ATP significantly. This conclusion was supported by control experiments in which fibres had been treated with Triton X-100.

6. The results show that during an isometric contraction most of the myosin heads are in the form of product intermediate states and that this steady state is reached rapidly ($< 50$ ms). These properties are similar to those of the Mg-dependent actomyosin subfragment 1 ATPase. The difference in the amount of rapidly formed ADP in fibres relaxed from rigor compared to when ATP was released in fibres stretched beyond overlap can be attributed to cycling of cross-bridges in the former case because of protein co-operativity in the myofilament.

## INTRODUCTION

The nature of the relationship between chemical change and the mechanical behaviour of muscle fibres is an important issue in the study of muscle contraction. The kinetic parameters of the reaction pathway for Mg ATP hydrolysis catalysed by myosin and actomyosin have been studied in several laboratories with rapid reaction techniques such as stopped flow and quenched flow (Taylor, 1979). These techniques are not applicable if the organized lattice of a muscle fibre is to be preserved or if the influence of mechanical constraints on the chemical steps is to be studied. Nor is it sufficient simply to add ATP to a skinned muscle fibre and to follow the time course of ATP hydrolysis and the mechanical response. Even when a fibre has its sarcolemma removed the rate of diffusion of Mg ATP from the bathing medium to the hydrolytic sites is slow compared to the rate of the elementary processes (Godt, 1974; Goldman, Hibberd & Trentham, 1984*a*). However, the synthesis of $P^3$-1-(2-nitro)phenylethyladenosine 5′-triphosphate (caged ATP) by Kaplan, Forbush & Hoffman (1978) and the use of laser flash photolysis to release ATP from caged ATP rapidly (McCray, Herbette, Kihara & Trentham, 1980) have made the study of the mechanical response as a function of ATP concentration in the organized muscle lattice possible (Goldman, Hibberd, McCray & Trentham, 1982*b*).

In the work described here, we measured the time course of Mg ATP cleavage and the accompanying tension changes occurring in chemically skinned fibres from rabbit psoas muscle by initiating the reaction with a laser pulse that caused tritiated ATP ([2-$^3$H]ATP) to be released from tritiated caged ATP ($P^3$-1-(2-nitro)phenylethyl-[2-$^3$H]adenosine 5′-triphosphate) termed [2-$^3$H]caged ATP. The reaction was stopped by freezing the fibres 50 ms to 8 s after the pulse of light, extracting the nucleotides from the frozen fibres and analysing the fibre extracts for tritiated AMP, ADP, ATP and caged ATP.

The kinetics of ATP cleavage and the accompanying tension response were analysed in fibres at full overlap of the thick and thin filaments in the presence and

absence of calcium ions. This provided a model for the study of the calcium-regulated actomyosin ATPase in living muscle. Also, fibres were stretched to long sarcomere lengths to prevent the interaction of myosin and actin. Under these conditions the preparation provided a means of studying the ATPase in the thick filament without interference from the thin filament. These experiments permitted us to relate the time course of ATP cleavage to the mechanical response of fibres, and to compare the kinetics of the ATPase in fibres with transient kinetic studies of the myosin and actomyosin ATPases in solution.

The approach described here depends particularly on the properties of caged ATP. In addition several other factors need to be carefully monitored or controlled, as follows.

(a) The rate of ATP liberation following photolysis of caged ATP is limited by a reaction which under our experimental conditions occurs with a rate of about 100 $s^{-1}$ (McCray *et al.* 1980; Goldman *et al.* 1982*b*). Kinetic processes occurring at rates comparable to, or faster than 100 $s^{-1}$ will be limited by the rate of appearance of ATP.

(b) Any binding of caged ATP to the myofibrils should not interfere with the analysis. In particular it is important to know whether caged ATP binds to the ATPase active site of myosin or actomyosin.

(c) The energy in the laser pulse must not be absorbed significantly by the fibre as this would result in damage to the muscle proteins or cause large temperature effects. Since laser pulse photolysis is carried out with the fibre suspended in air, the procedure has to be tested. At the same time enough ATP must be liberated by photolysis to provide excess ATP over the myosin ATPase sites and to approach the *in vivo* ATP concentration.

(d) The freeze-clamp method must rapidly halt Mg ATP cleavage to allow the study of the cleavage step, but must not destroy the physical integrity of the fibre so that the fibre can still be seen and handled.

(e) The extraction procedure has to extract the nucleotides and caged ATP completely from the fibre, but leave the protein *in situ*. The procedure must not cause breakdown of caged ATP or result in reactivation of the fibre ATPases.

(f) The contribution of other muscle ATPases has to be tested.

These points are considered below and several control experiments are described which address these issues.

Preliminary results of these studies have been presented to the Physiological Society (Ferenczi, Homsher & Trentham, 1983).

## METHODS

*Synthesis of [2-$^3$H]caged ATP.* 1 mCi [2-$^3$H]ADP (Amersham, code TRK 345) of specific activity 19 Ci $mmol^{-1}$ was transferred in water–ethanol to a pear-shaped flask and evaporated to dryness under vacuum. 2·5 $\mu$mol ADP, 20 $\mu$mol morpholine, 0·25 ml *t*-butanol and 0·1 ml water were added, followed by 50 mg dicyclohexylcarbodiimide dissolved in 0·5 ml *t*-butanol. The reaction mixture was kept under reflux for 7 h with two further additions of 50 mg dicyclohexylcarbodiimide in 0·5 ml *t*-butanol at 3 h and 6 h. The cooled solution was partitioned between ether and water. The aqueous phase was adjusted to pH 7·5 with HCl and diluted to 45 ml with water to reduce the ionic strength. [2-$^3$H]ADP morpholidate was isolated from the mixture in 65% yield by ion exchange chromatography with DEAE-cellulose (diethylaminoethyl-cellulose, column dimensions 28 cm × 1·5 cm diameter) and an 800 ml 10–410 mM gradient of triethylammonium bicarbonate

(TEAB) at pH 7·5. [2-$^3$H]ADP morpholidate, which was present in the 130–190 mM-TEAB fraction, was concentrated to dryness under vacuum. Methanol was added and evaporated under vacuum three times to remove TEAB.

The sodium salt of 1-(2-nitro)phenylethyl phosphate was synthesized from 1-(2-nitro)phenylethanol (prepared by sodium borohydride reduction of 2-nitroacetophenone) by the method used by Khwaja, Reese & Stewart (1970) to prepare 2-cyanoethyl phosphate by *o*-phenylene phosphorochloridate phosphorylation followed by bromine water treatment. 56 $\mu$mol 1-(2-nitro)phenylethyl phosphate (in $H^+$ form) and 56 $\mu$mol (13·4 $\mu$l) tri-*n*-butylamine were transferred in dry dimethylformamide to the [2-$^3$H]ADP morpholidate. The solvent was removed under vacuum and the condensation was carried out at 80 °C for 20 h in 0·3 ml dry dimethylsulphoxide under anhydrous conditions. This and all subsequent steps were performed in subdued daylight or in the dark.

[2-$^3$H]caged ATP was purified in three column steps. The cooled reaction mixture was taken up in 10 ml 10 mM-TEAB and loaded at pH 7·5 onto the same DEAE-cellulose column as used in the previous step. Elution was with a linear gradient of 800 ml 10–710 mM-TEAB. A broad radioactive band eluting at 185–425 mM-TEAB was concentrated to dryness and treated with methanol to remove TEAB. The sample was dissolved in water and adjusted to pH 6·5 with 1 N-HCl. The solution was concentrated to dryness, taken up in 1 ml water and loaded onto an analytical column (C-18 high performance liquid chromatography (h.p.l.c.), Waters Associates Inc. Milford, MA U.S.A). [2-$^3$H]caged ATP was eluted with a solvent of 10 mM-$KH_2PO_4$ at pH 5·5: methanol (85:15 v/v). Pure [2-$^3$H]caged ATP was obtained in fractions eluting 18–25 ml after the injection. The combined fractions were adjusted to pH 7·5 and [2-$^3$H]caged ATP was desalted on the same DEAE-cellulose column with the same eluting conditions as used in the first step of the [2-$^3$H]caged ATP purification. [2-$^3$H]caged ATP was present in the 240–330 mM-TEAB fraction. The dried triethylammonium salt of [2-$^3$H]caged ATP was dissolved to 1 mM in water and stored frozen at −18 °C. The over-all yield from [2-$^3$H]ADP was 11 %.

The purity of [2-$^3$H]caged ATP was checked by h.p.l.c. on a C-18 column followed by scintillation counting of the eluted fractions and by an identical analysis of its photoproduct [2-$^3$H]ATP obtained by irradiation at 330 nm. [2-$^3$H]caged ATP was > 99 % pure and contained < 0·2 % ATP. The specific activity of [2-$^3$H]caged ATP was 250 mCi mmol$^{-1}$ and was diluted with non-radioactive caged ATP to 50 mCi mmol$^{-1}$ for the fibre experiments.

*Other reagents.* Unlabelled caged ATP was prepared as its triethylammonium salt on a 300 $\mu$mol scale by a modification of the above procedure and stored in aqueous solution at −18 °C. Its concentration was measured from $\epsilon_{260\,nm}$ = 19·6 mM$^{-1}$ cm$^{-1}$ at neutral pH.

ADP was purified by DEAE-cellulose chromatography with TEAB as eluting solvent to remove traces of ATP. The final product contained < 0·02 % ATP. ADP was stored as its triethylammonium salt in aqueous solution at −18 °C. The concentration of nucleotides other than caged ATP was determined from $\epsilon_{260\,nm}$ = 15·4 mM$^{-1}$ cm$^{-1}$ at neutral pH.

All other reagents were of analytical grade. Water was double-glass-distilled.

*ADP dissociation rate from subfragment 1.* The rate constant of ADP dissociation from subfragment 1 from rabbit skeletal muscle was measured in a stopped-flow spectrofluorimeter as described by Bagshaw, Eccleston, Trentham, Yates & Goody (1972) in a solution of 100 mM-*N*-Tris[hydroxymethyl]methyl-2-aminoethane sulphonic acid (TES) at 22 °C, pH 7·1 and other reagents as listed in Table 1 under Caged ATP ($2 \times 10^{-5}$ M-free $Ca^{2+}$). The observed rate constant for ADP dissociation from myosin subfragment 1 was 2·8 s$^{-1}$. Pre-incubation of subfragment 1 with 3 mM-caged ATP did not alter the rate of ADP dissociation. Similarly the rate of ADP dissociation was unaffected by the presence of 10 mM-glutathione.

*Caged ATP interaction with the ATPase active site of subfragment 1.* Caged ATP has already been shown not to bind to the ATPase active site of actomyosin (McCray *et al.* 1980). This was done by incubating actomyosin with caged ATP and looking for any effect of caged ATP on the kinetics of ATP binding. This method is sensitive to caged-ATP binding whether or not caged ATP dissociates slowly or rapidly from the protein (cf. a comparable study of $P_i$ and ADP binding to subfragment 1 (Bagshaw & Trentham, 1974)). However, since nucleotides bind less readily to myosin in the presence of actin, it was important to check the binding of caged ATP to subfragment 1 alone. A sensitive test of caged-ATP binding at the ATPase active site was to pre-incubate subfragment 1 with caged ATP and to study the binding kinetics of ATP and 6-mercapto-9-$\beta$-ribofuranosylpurine 5′-triphosphate (thioITP) with a stopped-flow spectrofluorimeter (by the approach of Bagshaw &

Trentham, 1974). (ThioITP quenches protein fluorescence on binding (Bagshaw *et al.* 1972).) There was no detectable binding (i.e. the dissociation constant was greater than 1 mM) of caged ATP at the ATPase active site. The solvent for the experiments was the rigor solution (Table 1).

*Rabbit muscle fibres.* Chemically skinned muscle fibre segments were obtained essentially by the method of Eastwood, Wood, Bock & Sorenson (1979). Strips of psoas muscle, 1–2 mm in diameter and 40–60 mm long were dissected from white male New Zealand rabbits weighing more than 5 kg. The muscle strips were tied at rest length to wood applicator sticks with surgical thread and chemically skinned by placing in a solution consisting of 170 mM-potassium propionate, 5 mM-EGTA, 2·5 mM-$Na_2ATP$, 2·5 mM-$MgCl_2$, 0·1 mM-phenylmethylsulphonyl fluoride, 5 mM-azide, and 10 mM-imidazole, pH 7·1 at 4 °C. The solution was gently and continuously stirred for 24 h with one exchange to fresh solution. The fibres were then transferred to a solution consisting of the above solution diluted with glycerol (50 % v/v) and stored at −18 °C for 2–6 weeks. Further dissection of the muscle strips was performed under silicone oil on a microscope stage cooled to 5 °C. Segments of single fibres or bundles of two to five fibres were isolated and cut to a length of 6–8 mm. The ends of the muscle fibres were crimped in aluminium T-clips (Goldman & Simmons, 1984) and transferred to the experimental cell on a small glass rod.

*The experimental apparatus.* The apparatus consisted of a ruby laser, two interchangeable cells in which the muscle fibres were immersed, and a freeze-clamping apparatus (Fig. 1). The ruby laser (694 nm emission, Holobeam Inc., FL, U.S.A.) was used in conjunction with a frequency-doubling crystal (temperature-phase heated rubidium dihydrogen arsenate crystal, Interactive Radiation, Inc., North Vale, NJ, U.S.A.) to provide 50 ns pulses of light at 347 nm. For the experiments on muscle fibres the energy contained in the pulse ranged from 30 to 50 mJ. The light beam was focused by a cylindrical quartz lens (L in Fig. 1) to a narrow band, 1–2 mm across and 8 mm long, in the plane of the muscle fibre. The laser pulse energy was limited to 50 mJ because higher pulse intensities resulted in tension artifacts, and in some cases the fibre was thrown off the hooks altogether. These effects, which were attributed to absorption of energy by the T-clips or the steel hooks and consequent movement of the hooks, were reduced by illuminating as little of the T-clips and transducer hooks as possible.

The fibres were attached by T-clips to two stainless-steel hooks (100 $\mu$m diameter) mounted on adjustable slides for adjustments of the sarcomere length. One hook, attached with epoxy resin to the semiconductor element of a strain gauge (model AE 801, AME, Horten, Norway; s.g. in Fig. 1*C*), was used to measure tension. The strain gauge was held in a housing designed by Dr Y. E. Goldman in which breakage of the strain gauge was prevented by limiting the excursion of the strain-sensitive element. The risk of breakage was further reduced by using a long hook (18 mm), so that clamping of the fibre by the copper blocks did not result in excessive strain, even when the blocks were not perfectly aligned. The hook and strain gauge assembly had a resonant frequency of 0·6 kHz and the sensitivity of the recording was 490 V/N. The fibre suspended between the two steel hooks was immersed in either one of two interchangeable cells ($c_1$ and $c_2$) with 0·5 mm wide slits at their ends to let the steel hooks through (Fig. 1). Cell $c_1$ had a volume of 12 $\mu$l ($8 \times 1{\cdot}5 \times 1$ mm$^3$); cell $c_2$ had a volume of 30 $\mu$l ($8 \times 1 \times 3{\cdot}75$ mm$^3$), a glass base to allow for illumination of the fibre and drain holes for rapid emptying by suction. The cells were mounted on a ball slide allowing them to slide in position. The ball slide itself was mounted on a stage which could be raised or lowered by means of a remote-control hydraulic piston as shown in Fig. 1 *A* and *B*. To change solutions, the stage was lowered, leaving the fibre suspended in air, and the new cell was then pushed into place or the solution in the cell was exchanged. The stage was then raised and the fibre immersed in the new solution. Although passage of the fibre through the air–water interface resulted in a tension transient, no damage to the fibre was observed.

The use of cold clamps to stop muscle metabolism rapidly was originally described by Wollenberger, Ristau & Schoffa (1960). The fibre clamp used in the present work is shown in Fig. 1. It consisted of two copper blocks, $5 \times 5 \times 6$ mm$^3$ each, which were attached by means of removable pins to two levers. Initially, the copper blocks were immersed in liquid nitrogen contained in polystyrene cups (Fig. 1*A*). At a pre-set time the activation of a solenoid caused a spring-loaded mechanism to lift the copper blocks out of the liquid nitrogen and to move the cups out of the path of the clamps. The lever arms, whose movements were coupled by gears, then fell, and the copper blocks squeezed a 5 mm length of muscle fibre m, and rapidly froze it. As the blocks came into contact, a knife-edge stainless-steel latch locked the lever arms in the clamped position. The time between the solenoid activation and contact between the copper blocks was 350 ms.

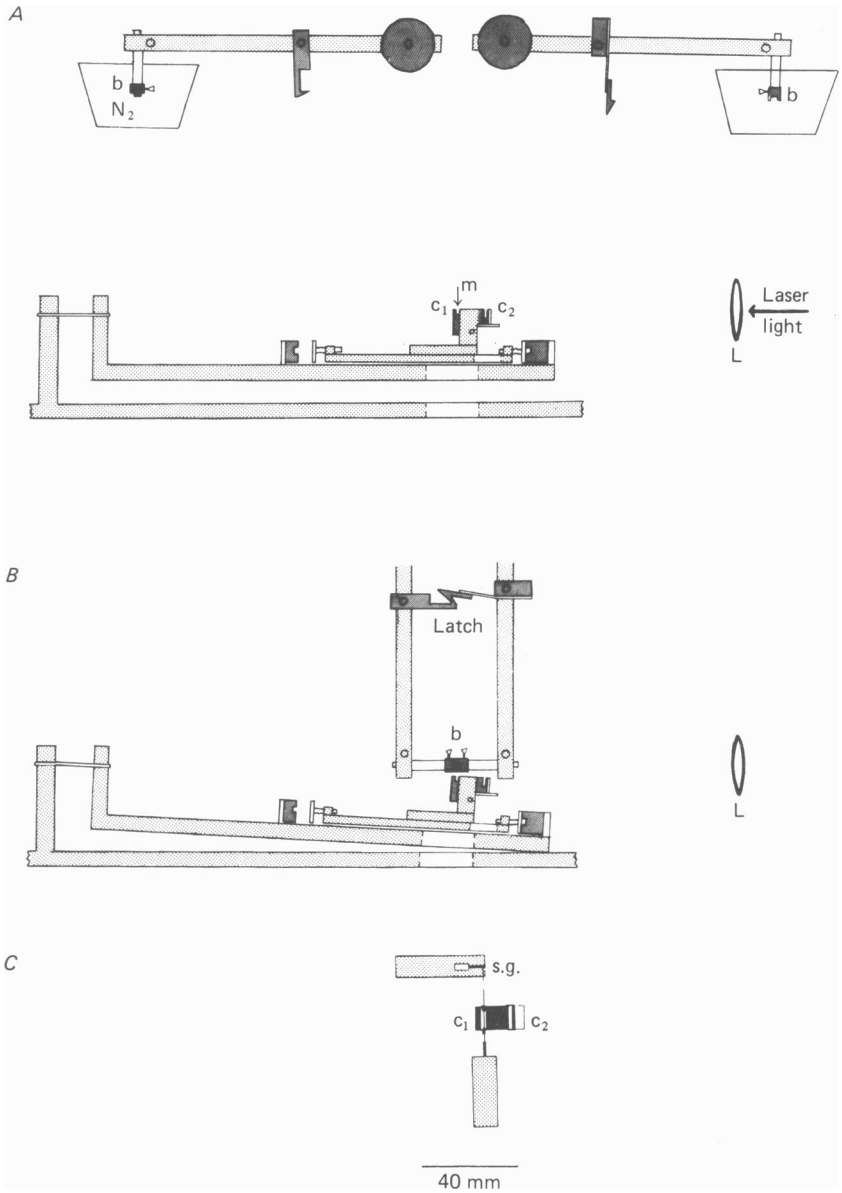


Fig. 1. Schematic representation of the apparatus. *A*, the cells ($c_1$ and $c_2$) are shown in their position prior to a pulse, with the muscle fibre (m) immersed in the cell containing [2-$^3$H]caged ATP. The copper blocks (b) are shown in the polystyrene cups containing liquid $N_2$. The cylindrical lens (L) which focuses the laser beam onto the fibre is also shown. *B*, the muscle apparatus is shown in its position at the time of the freeze-clamp. The movement from the position in *A* resulted in the muscle fibre being suspended in air until the freeze-clamp. *C*, cells $c_1$ and $c_2$ are shown from above. The strain gauge (s.g.) and the steel wires to which the T-clips and the fibres are attached are shown.

Monitoring the electrical contact between the two copper blocks provided a signal which indicated the time at which fibre freezing began and also provided a check of any bounce of the blocks. During the latter part of the lever swing, one of the copper blocks interrupted the path of the laser beam. This arrangement set a 35 ms limit as the shortest interval between the laser pulse and freezing of the fibre. Machining the copper blocks so as to leave a 100 $\mu$m gap between the clamping surfaces prevented the fibres from shattering during freezing. Fragmentation and grinding of the frozen fibres while removing the copper blocks was eliminated by minimizing the relative motion of the copper blocks. This was achieved by a small steel dowel in one of the blocks, which fitted into a hole drilled in the other block, and by locking the copper blocks together after the clamp by inserting a steel pin through a hole drilled in both blocks.

*Protocol*

The solutions used are as described in Table 1, except where noted in the text. They contained TES as a pH buffer and glutathione to protect sulphydryl groups on the fibre proteins from the photolysis biproduct (Kaplan *et al.* 1978; Goldman, Gutfreund, Hibberd, McCray & Trentham, 1982*a*).

TABLE 1. Composition of solutions (mM)

| Solution | Caged ATP | $Na_2ATP$ | $Na_2CP$ | $MgCl_2$ | CaEGTA | EGTA | HDTA | GSH |
|---|---|---|---|---|---|---|---|---|
| Relaxing | — | 6·0 | 16 | 7·3 | — | 25 | — | 10 |
| Rigor | — | — | — | 1·3 | 20 | 0·14 | 33 | 10 |
| Caged ATP ($< 10^{-8}$ M-free $Ca^{2+}$) | 1·7–3·0 | — | — | 3·2 | — | 13 | 30 | 30 |
| Caged ATP ($2 \times 10^{-5}$ M-free $Ca^{2+}$) | 1·7–3·0 | — | — | 12 | 20 | 0·21 | 24 | 30 |

In addition, each solution contained 100 mM-TES and was adjusted to pH 7·1 with KOH. Abbreviations: GSH, glutathione; $Na_2CP$, disodium creatine phosphate; HDTA, diaminohexanetetraacetate. Other abbreviations are given in the text. In some experiments the solvent conditions were altered slightly as indicated in the text.

*Tension development.* An example of the experimental protocol is shown in Fig. 3*A*. A fibre bundle was mounted on the apparatus with the fibre initially immersed in a solution containing 6 mM-Mg ATP and less than $10^{-8}$ M-free calcium ions. At time *a* (Fig. 3*A*) the fibre was transferred to a solution containing no Mg ATP (rigor solution). The process was repeated three times to remove all residual Mg ATP. A tension transient was seen each time the fibre passed through the air–water interface. After the third solution exchange, the tension rose as the fibre went into the rigor state. At *b* the fibre was relaxed by re-exposure to relaxing solution. This initial cycle in rigor solution provided a test of the ability of the fibres to sustain rigor tension. The fibres' sarcomere lengths were measured with a 40 × water-immersion objective (Zeiss) and a 25 × Periplan eyepiece (Leitz) while in relaxing solution and adjusted to the desired value. Measurements of the fibre width and thickness were used to calculate fibre cross-sectional area (Blinks, 1965). At *c* the process for removing Mg ATP was repeated and the fibre returned to the rigor state. At *d* the fibre was placed into a different cell ($c_1$) containing 2–3 mM-[2-$^3$H]caged ATP solution, but less than $10^{-8}$ M-calcium ions (Table 1). At *e* the pen recorder sensitivity was increased by a factor of 4 and at *f* the paper speed was increased by a factor of 60. At *g*, 2·6 min after immersion in caged ATP solution, the cell was dropped below the fibre, leaving the fibre suspended in air. 400 ms later at *h* the laser was triggered and the fibre illuminated. Released ATP caused the fibre to relax. At *i*, 1 s after the laser pulse, the copper blocks froze the fibre, ending meaningful tension recordings. The tension signal and electrical contact between the blocks were recorded on a faster time scale on storage oscilloscopes (Fig. 3*B* and *C*).

This general protocol was adhered to for each experiment. The temperature of the caged-ATP solution prior to lowering the bath and pulsing the laser was $19 \pm 1$ °C. Possible effect of the laser pulse on the fibre temperature is analysed below.

*Handling of the frozen fibre.* After freezing the fibre, a cold (near −195 °C) scalpel blade was used to cut the ends of the muscle fibre protruding from the copper blocks. The blocks were locked together, disengaged from the levers by removal of the locking pins and transferred to an extraction solution with an aluminium clamp cooled to near −195 °C. The extraction solution was pre-cooled to −50 °C by being placed in a glass vial in an aluminium block pre-cooled in liquid nitrogen. The solution comprised 1·5 ml methanol containing 0·02 % (v/v) concentrated phosphoric acid and 1 % (v/v) of an aqueous solution of 1 mM each of AMP, ADP, ATP and caged ATP, and had an apparent pH of 3·3 (measured at 20 °C with a glass electrode). The unlabelled nucleotides and caged ATP served as carriers to minimize extraneous surface adsorption of the radioactive compounds. The glass vial and its aluminium holder were placed on the stage of a dissection microscope and illuminated with yellow light ($\lambda > 400$ nm) to avoid photolysis of the caged ATP. The copper blocks were then separated and the muscle fibre, which had an opaque appearance, could be removed from the clamping surface of one of the blocks. At this stage, the fibres were usually in one to three fragments, but occasionally a large number of small pieces could be seen, indicating that some grinding of the frozen fibre had occurred. In these cases measurement of fibre protein was unreliable since not all of the fibre could be recovered, and these fibres could only be partially analysed as is described in the ADP determination section. Each copper block was rinsed with 0·2 ml extraction solution and removed from the glass vial. Finally, 0·1 ml 100 mM-EDTA was added to the vial to sequester divalent cations and to prevent enzyme-catalysed breakdown of nucleotides, particularly by residual adenylate kinase (Noda, 1973). The extraction solution was allowed to warm until the EDTA droplets thawed. The fibre and extraction solution were sucked into a plastic pipette tip and transferred to a conical plastic vial on ice. The glass vial was rinsed with an additional 0·1 ml extraction solution. Within 5 min of transfer the extraction solution and the fibre were stored at −18 °C and left for at least 16 h. A small volume (approximately 8 μl) 1 N-KOH was then added to bring the apparent pH of the solution to 6–8 at 0 °C and the solution was then sonicated at 0 °C for 10 min. Raising the pH to near neutrality was necessary for complete recovery of the nucleotides. Otherwise as much as 50 % of the radioactivity remained in the fibre. The fibre was then separated from the extraction solution by centrifugation in a bench-top microfuge and washed with a further 100 μl extraction solution at neutral pH, to which 5 mM-EDTA had been added. Sonication and centrifugation were repeated. After removing as much of the extraction solution from the extraction vial as possible under the dissecting microscope, the fibre was placed in 50 μl 50 % (v/v) methanol–water and stored at −18 °C for subsequent analysis. The extraction of radioactivity from the fibre was greater than 98 % (see below). All fibre transfers were carried out under the dissecting microscope (12 × magnification and under illumination with yellow light) to ensure recovery of all fibre segments.

The ability of the extraction solution to arrest hydrolysis of ATP by the fibre proteins was tested by treating fibres for 5 min in extraction solution before bathing in the caged ATP solution. After laser pulse photolysis and freeze clamp at 50 ms, the fibres were handled as above. The amount of ADP found in these fibres was equal to the background ADP contamination of the caged ATP solution.

*Analysis of fibre protein.* Protein was assayed by a micro-Lowry determination (Lowry, Rosebrough, Farr & Randall, 1951) with bovine serum albumin as standard. The following procedure was adopted to relate the colour yield of the fibre protein in the Lowry assay to that of the albumin standard. The colour yield of rabbit skeletal actin was measured and found to be 1·4-fold greater than that of albumin. The colour yield of rabbit myosin was equal to that of albumin and the colour yield of all other fibre proteins was assumed to be the same as that of albumin. Yates & Greaser (1983) reported that myofibrils from rabbit psoas contained 5·37 μmol actin/g protein. The molecular weight of G actin is 42000 (Elzinga, Collins, Keuhl & Adelstein, 1973). Therefore, based on these figures the colour yield from 1 g fibre protein will be 1·09-fold greater than that of 1 g bovine serum albumin.

For the protein assay, the fibre was treated with 50 μl 1 N-NaOH and dissolved by sonication of the vial containing the fibre for 1 h at 50 °C. After the micro-Lowry assay the contents of the optical cell were assayed for radioactivity to determine the efficiency of the nucleotide extraction. Less than 2 % radioactivity remained after the standard extraction procedure described above.

*Myosin subfragment 1 head concentration in fibres.* To determine the myosin subfragment 1 head concentration in the fibres used in these experiments it was necessary to measure the mass of protein per unit fibre volume. Fibre volume was obtained by bathing the fibre in [2-$^3$H]caged ATP of known concentration and measuring the ratio of radioactivity in the fibre to the radioactivity in 1 μl

solution. The fibre volume of each fibre was plotted against the mass of protein in that fibre. A linear regression to the data gave a slope of $10{\cdot}7 \pm 1{\cdot}1$ nl/$\mu$g and an intercept of $7{\cdot}1 \pm 5{\cdot}6$ nl for sixty-nine fibres whose protein content ranged from 1·6 to 10·8 $\mu$g (average 5·1 $\mu$g). These data were obtained from the fibres of Fig. 6. The intercept of the regression line was not significantly different from zero (Student's $t$ test on the intercept, $P > 0{\cdot}2$). The slope corresponds to $93{\cdot}6 \pm 9{\cdot}2$ $\mu$g/$\mu$l of fibre. Yates & Greaser (1983) reported that myofibrils from rabbit psoas muscle contained 0·82 $\mu$mol myosin/g protein. Based on this value, the myosin concentration in our experiments was $76{\cdot}8 \pm 7{\cdot}8$ $\mu$M, and the myosin subfragment 1 head concentration was $154 \pm 16$ $\mu$M. Potential sources of error in the measurement of myosin subfragment 1 concentration are discussed below.

*Analysis of the nucleotide composition.* After removal of the fibre the extraction solution was placed in a 3·0 ml conical glass micro-reaction vessel fitted with a valve cap (Supelco, Inc., Bellefonte, PA, U.S.A.) and frozen in liquid nitrogen. The extraction solution was then freeze-dried with a liquid nitrogen-cooled trap between the vacuum chamber and pump. The residue was resuspended in a total of 450 $\mu$l water. A 10% aliquot was taken for scintillation counting to determine the total radioactivity in the fibre. The rest of the solution was analysed for nucleotide composition by ion-pair reverse phase h.p.l.c. (Darwish & Prichard, 1981). For this purpose an analytical C-18 h.p.l.c. column (5 $\mu$m spherical beads, 3·9 mm diameter × 15 cm) was equilibrated with a solution consisting of 92% (v/v) 65 mM-$KH_2PO_4$, pH 3·5, 0·9 mM-tetrabutylammonium dihydrogen phosphate (Eastman Kodak Co., Rochester, NY, U.S.A.) and 8% methanol. The elution was monitored at 254 nm. The fibre extract was loaded onto the column at a flow rate of 1·8 ml/min.

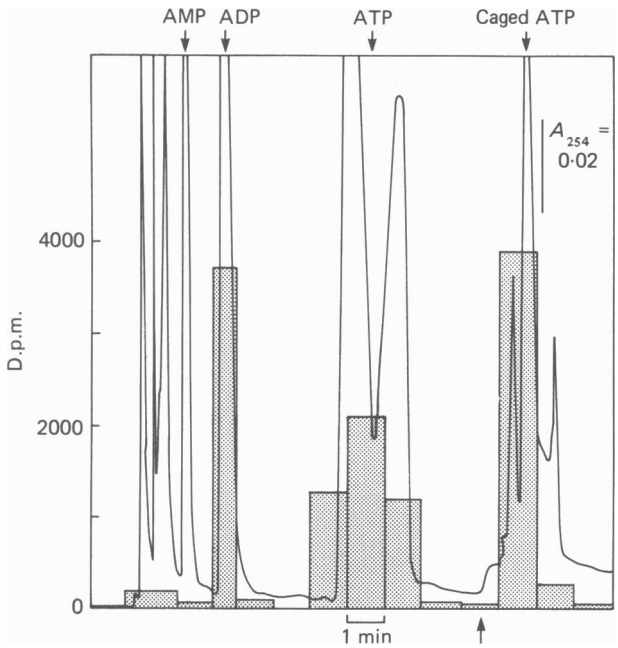


Fig. 2. H.p.l.c. elution profile of a fibre extract. The continuous line shows the absorption at 254 nm of the non-radioactive carrier AMP, ADP, ATP and caged ATP. The initial absorption peaks correspond to EDTA and other extraction constituents. ATP appeared as two peaks. This was due to an adsorption characteristic of the column which varied with the age of the column. The upward arrow indicates the point from which the eluate contained 50% methanol as indicated in the text. The bar graph indicates the radioactivity contained in the fractions. After correcting for radioactive impurities in the caged-ATP bathing solution, the radioactivity in the peaks was distributed as follows: ADP, 28·5%; ATP, 35·2%; caged ATP, 33·4%. A fibre bundle consisting of two fibres had been incubated in 2·0 mM-[2-$^3$H]caged ATP in the presence of $2 \times 10^{-5}$ M-calcium ions. The laser pulse released 1·29 mM-ATP. The fibre was frozen 1 s after the pulse. The tension signal obtained for this fibre bundle is shown in Fig. 7*A*.

The nucleotides eluted in well resolved peaks as shown in Fig. 2. EDTA was eluted close to the solvent front. Caged ATP was eluted by changing the eluant to a solution consisting of 50% (v/v) 65 mM-$KH_2PO_4$, pH 3·5, 0·9 mM-tetrabutylammonium dihydrogen phosphate and 50% methanol. Each fraction was then counted for 10 min in a scintillation counter (LS 7000, Beckman Instruments, Inc., Irvine, CA, U.S.A.) after addition of 10 ml ACS II scintillation fluid (Amersham). The radioactivity was corrected for quenching by the H-number method of quench monitoring (Beckman Instruments, Inc.). The total radioactivity incorporated in the fibre was determined from the aliquot of the extraction solution taken above and corresponded to 5000–50000 disintegrations per minute (d.p.m.). In control experiments to test whether the extraction procedure caused any breakdown of the caged ATP and to determine the background radioactivity in the nucleotide peaks, aliquots of the solution in which the fibres were bathed (as at *d* in Fig. 3) were treated in the same way as the fibres themselves. Radioactivity in the AMP, ADP, ATP and caged-ATP peaks did not differ from that observed in untreated samples of the [2-$^3$H]caged ATP solution. Values for background radioactivity were subtracted from the radioactivity in the nucleotide peaks derived from the fibres. Typically the radioactivity in the [2-$^3$H]ADP and [2-$^3$H]ATP peaks from fibre samples were 15-fold above background.

*ADP determination.* The amount of ADP formed in each experiment was calculated in two ways. First the concentration of ADP formed in a fibre was obtained directly from the h.p.l.c. analysis by multiplying the percentage of the radioactivity found in the ADP elution peak by the concentration of caged ATP in which the fibres were bathed (ordinate axis, Fig. 5). In the second method, the total amount of ADP formed was expressed as a fraction of the number of myosin subfragment 1 heads (ordinate axis, Fig. 6) present in the fibre. The latter figure was obtained from the micro-Lowry protein determination based on 1·65 $\mu$mol subfragment 1 heads/g fibre protein (Yates & Greaser, 1983) after correction for the high colour yield of actin (see above).

Each method had advantages and disadvantages. In the former case (data in Fig. 5) the concentration of ADP formed in each experiment was measured. There are potential errors in this measurement, as discussed in the next section, but this method had advantages in that it was less prone to error if the nucleotide extraction from the fibre was incomplete, and data from all experiments could be used. In the latter case (data in Fig. 6), the errors that could arise in the determination of ADP concentration were avoided. However, the data could only be used provided the amount of protein in each fibre could be measured. In some cases that was not possible due to fragmentation of the fibre in the freeze-clamp apparatus.

*Potential errors in the concentration measurements of subfragment 1 and ADP.* The first potential source of error which needs to be considered is the possibility that caged ATP binds to the myofibrillar protein. The evidence from stopped-flow experiments presented above and elsewhere (McCray *et al.* 1980) suggests that caged ATP does not bind at the ATPase active site of myosin. However, secondary binding sites on myosin have been detected or implicated for a wide range of nucleotides, aromatic molecules and polyanions (Nihei & Filipenko, 1975; Eccleston, 1980; Moss & Trentham, 1983; D. R. Trentham ($H^+$ dyes, unpublished results)). If it is assumed that each myosin molecule binds two caged-ATP molecules at secondary sites when the fibre is bathed in caged ATP, then the actual concentration of caged ATP in a fibre bathed in 2 mM-caged ATP would be 2·17 mM. Measurements of both subfragment 1 and ADP concentrations rely on the fact that the radioactive counts of caged ATP in the fibre can be correlated with the concentration of caged ATP in the fibre trough. So in the case cited the volume of the fibre would be over-estimated by a factor of 1·085 and hence the concentrations of both subfragment 1 and ADP would be too low. Fortunately the critical data derived from Fig. 5, namely the concentration of ADP formed in the burst relative to the subfragment 1 concentration and the steady-state ATPase rates, depend on the ratio of the ADP to subfragment 1 concentrations and secondary binding of caged ATP will not have introduced error into this ratio.

A second potential error is that introduced by a solvent film being left on the fibre when its solution trough is removed and the fibre is suspended in air. The estimate of ADP concentration is based on the fraction of radioactivity in the ADP peak to the total radioactivity in a fibre extract. The total radioactivity will be too high if a film of liquid is transferred and hence the concentration of ADP will be too low by a fraction equal to the ratio of the film volume to the volume of film plus fibre. The subfragment 1 concentration was determined from the slope of the regression line of a plot of fibre volume against fibre protein mass as described above. The estimate of subfragment

1 concentration would be too low if the slope was too high, as would be the case if a film was carried over with each fibre, and the volume of the film was proportional to the volume of the fibre. However, it is likely that thicker fibres would carry over a proportionately smaller fraction of liquid film. Consequently the error in the relative concentrations of ADP and subfragment 1 would not cancel out although the final error would probably be small.

The values obtained by Yates & Greaser (1983) for the myosin and actin contents of myofibrils were obtained after removal of the connective tissue and nuclei components from the myofibrils. Although we do not expect any connective tissue in our fibre preparations, the presence of nuclei will lead to a small error in the estimation of the myosin concentration.

Finally, any error in the estimate of the amount of myosin per gram protein will affect our estimate of the subfragment 1 concentration. The value of Yates & Greaser (1983) leads to the value of 2·5 myosin molecules per 143 Å repeat on the thick filament. On the other hand Tregear & Squire (1973) favour 3 rather than 2·5 on the grounds that there is an integral number of myosin molecules for each 143 Å repeat. This would suggest that the subfragment 1 concentration should be 185 $\mu$M (i.e. 20% higher) which is closer to other estimates of subfragment 1 concentration inferred from the concentration of protein-bound ADP in the myofibrils (Marston, 1973). Yates & Greaser (1983) argue that a value of 2·5 is possible if the number of myosin molecules per 143 Å repeat is not constant along the length of the thick filament.

*Rate of fibre cooling by the freeze-clamping apparatus.* The calculations and experiments of Heuser, Reese, Dennis, Jan, Jan & Evans (1979) indicate that a muscle will freeze to a depth of 50 $\mu$m in about 12 ms when placed in contact with a copper block cooled to liquid helium temperature. Although the copper blocks used here were only cooled to liquid nitrogen temperature, freezing will have occurred in less than 25 ms for a muscle fibre bundle 100 $\mu$m thick clamped between two cooled copper blocks. Most of the delay in cooling is associated with the latent heat of fusion of ice, and subsequent cooling to $-30°$ is fast. Travers & Hillaire (1979) have shown that at $-30$ °C the activity of the actomyosin ATPase is negligible.

*Changes of temperature in the fibre during an experiment.* Two mechanisms are likely to cause changes in the fibre temperature during an experiment: cooling of the fibre by evaporation as the fibre is lifted out of the solution and heating of the fibre by absorption of laser light.

Heating of the fibre by the laser light could not be measured directly with a thermocouple because the thermocouple in the beam absorbed considerable laser energy. However, it could be estimated by considering separately the absorption by caged ATP and that by the fibre itself. A 3 mM solution of caged ATP, 100 $\mu$m thick, will absorb 4·5% of the incident light ($\epsilon_{347\,nm} = 660\ M^{-1}\ cm^{-1}$). For a fibre with a diameter of 100 $\mu$m illuminated by a pulse of light 1 mm wide containing 50 mJ of energy, 10% of the light will fall on the fibre and 0·23 mJ will be absorbed. Thus the temperature of a fibre with a mass of 0·04 mg will rise by 1·4 °C (assuming that the fibre has the same specific heat as water).

For an estimate of heating by absorption of the fibre itself, Dr G. Dubyak observed that transmission loss through a single barnacle fibre at 400 nm corresponded to an optical density of 0·38 $mm^{-1}$. For a 100 $\mu$m thick fibre, this corresponds to an absorption of 8·4% of the light, which by the same calculations as above corresponds to heating of the fibre by 2·4 °C. If barnacle fibres absorb as much light as rabbit fibres and the absorption at 347 nm is identical to that at 400 nm, the total temperature change associated with absorption by the fibre is 4·1 °C. Since some of the transmission loss was due to light scattering, this value will be an over-estimate.

In addition, there is likely to be some effect due to laser light falling on the clips and hooks supporting the fibre. However, dissipation of this heat through the transducer is likely to be much greater than dissipation into the fibre. These possible causes for temperature increases are likely to be somewhat offset by cooling mechanisms.

Cooling of the fibre by evaporation depends on the fibre surface area, the air temperature, the degree of humidity, and on the air flow around the fibre during an experiment. The presence of filled liquid nitrogen containers situated above the apparatus caused about a 2 °C drop in the temperature of the air surrounding the fibre.

Over-all, we estimate that in the course of an experiment the fibre temperature changed less than 5 °C.

*Presentation of data.* The data are given as the mean plus or minus one standard error of the mean with the number of data points indicated as $n$. Student's $t$ tests for significance were applied where

required. The standard error for the slope and for the intercepts in Figs. 5 and 6, as well as Student's $t$ test for significance, were determined as described by Snedecor & Cochran (1967).

## RESULTS

*Amount of ATP released in muscle fibres.* In order to study the kinetics of the Mg ATP cleavage in muscle fibres, it was desirable to achieve pseudo-first-order conditions, namely a substrate concentration of about 5-fold excess over myosin subfragment 1 head concentration so that variation in the substrate concentration in the course of the experiment could be ignored. Since the enzyme concentration was 154 $\mu$M, a substrate concentration of about 1 mM was required. Such a substrate concentration was also desirable to achieve conditions resembling the *in vivo* situation where the concentration of Mg ATP is about 4 mM (frog muscle: Dawson, Gadian & Wilkie, 1977). It was found that by focusing the laser beam onto the fibre bundle, enough light was available to convert up to 77% of the [2-$^3$H]caged ATP to [2-$^3$H]ATP. For the experiments described in Figs. 5 and 6, an average of $57 \pm 2$% ($n = 78$) of the initial caged ATP was photolysed by a single pulse of light in incubation media that contained 1·7–3·0 mM-[2-$^3$H]caged ATP, so that [2-$^3$H]ATP was released in the millimolar range. In a few experiments where the incubation medium contained 10 mM-caged ATP, more than 5 mM-ATP was released.

When the fibre was replaced by a 200 $\mu$m diameter glass capillary tube containing 2 mM-[2-$^3$H]caged ATP, up to 64% of the caged ATP was photolysed to form ATP. Less than 1% ADP was formed, showing that ADP formed in the fibre experiments was due to ATP cleavage.

*Caged-ATP photolysis in the absence of calcium ions*

*Tension.* Fig. 3*A* shows the tension developed by a fibre bundle segment on a slow time scale, as described in the Methods section. The rigor tension developed by the fibres was $38{\cdot}1 \pm 2{\cdot}9$ kN/m$^2$ ($n = 64$). After incubating the fibre in the solution containing [2-$^3$H]caged ATP, the tension level slowly decreased to about half the rigor level. This drop in tension was always observed for fibres at full myofilament overlap, and was not affected by the presence of calcium ions. The presence of caged ATP caused tension to drop to $54 \pm 2$% ($n = 46$) of the rigor tension with a half-time of $17{\cdot}2 \pm 1{\cdot}4$ s ($n = 46$), and the rate of tension fall was not correlated with the fibre diameter.

Following the laser flash (*h* in Fig. 3*A–C*), 1·62 mM-ATP was released and the fibre relaxed. The relaxation is shown more clearly on the oscilloscope record of Fig. 3*B* and on a faster time scale in Fig. 3*C*. The burst of noise (*k*), which appears on the tension trace in Fig. 3*B* approximately 700 ms after the laser pulse (*h*), was caused by vibrations induced by triggering of the solenoid controlling the freeze-clamp. The top trace in Fig. 3*B* shows electrical contact between the copper blocks at the time of freezing (*i*). At that time the tension signal became meaningless. In Fig. 3*C*, the initial phase of relaxation can be seen in more detail. The laser pulse itself induced a resonance in the tension transducer which caused a large tension transient. Following an instantaneous drop in tension, the tension was maintained, or even rose a little during the first 40 ms following the pulse of light. Following this tension rise,

tension dropped with an approximately exponential time course until relaxation was complete.

The relaxation process was quantified in terms of two phases. The details of the initial phase were somewhat obscured by the noise in the tension signal so that only a duration was obtained for this phase. The second phase, which was roughly exponential as in Fig. 4 was quantified by a regression on the linearized data. In some

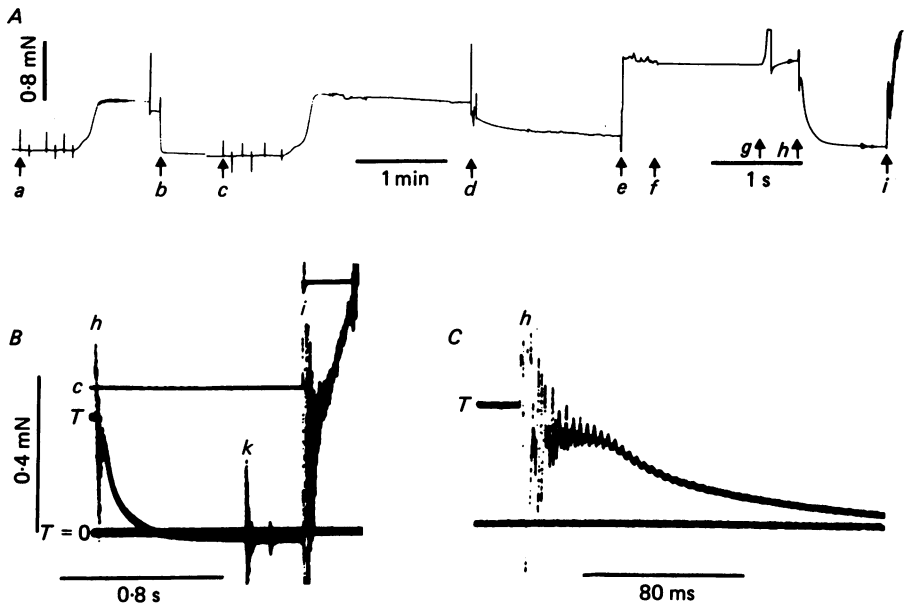


Fig. 3. Tension record during a laser-pulse photolysis–freeze-clamp experiment. In *A*, the letters indicate solution or recording changes as indicated in the text with *h* and *i* marking the time of the laser pulse and the time of freeze-clamp, respectively. *B* and *C*, time course of relaxation for the same fibre bundle at two different sweep speeds. The top sweep in *B* records the freeze-clamp through a 12 V signal caused by electrical contact of the copper blocks after 1 s (*c*). The middle trace records the tension of the fibre (*T*) and the bottom trace records tension when the fibre was fully relaxed, as at *c* in *A* ($T = 0$). The noise on the tension trace marked with *k* indicates the vibrations induced by the solenoid triggering the clamp. The fibre bundle consisted of three fibres with a total cross-sectional area (*A*) of $1{\cdot}3 \times 10^{-8}$ m$^2$ and a sarcomere length (*s.l.*) of 2·63 $\mu$m.

cases, as in Fig. 3*C*, the relaxation process could not be described by a single exponential phase. For simple exponential tension decays, the rate constant for the relaxation phase was $11 \pm 1$ s$^{-1}$ ($n = 11$). The duration of the initial phase was defined empirically as the time interval between the laser pulse and the time at which the exponential fit to the second phase had a value equal to the rigor tension just prior to the laser flash. The value obtained by this extrapolation for the duration of the initial phase was $39 \pm 7$ ms ($n = 11$). The initial phase of the relaxation process was quite variable: an initial hump in the tension trace was sometimes quite apparent, as in Fig. 3*C*. However, the prominence of such a hump did not depend on the

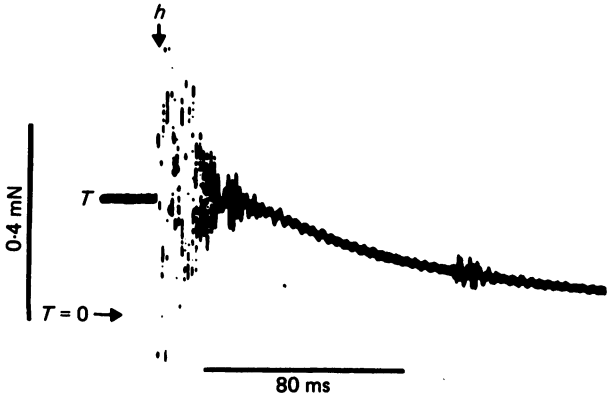


Fig. 4. Relaxation from rigor following photochemical release of ATP. The fibre bundle consisted of five fibres ($A = 3{\cdot}7 \times 10^{-8}$ m$^2$, $s.l.$ = 2·2 $\mu$m). The bundle was incubated in 3·0 mM-[2-$^3$H]caged ATP solution prior to the laser pulse and was freeze-clamped at 300 ms. The sweep is as described for Fig. 3*C* with *h* marking the time of the laser pulse. The rate constant for the exponential phase of relaxation was 8 s$^{-1}$ and the initial phase lasted 22 ms.

amount of Mg ATP liberated: 1·6 mM for the fibre of Fig. 3 compared to 1·5 mM for that of Fig. 4.

These results as well as the results given below are summarized in Table 2.

TABLE 2. Summary of results

| | Tension | | | Mg ATP cleavage | | | |
|---|---|---|---|---|---|---|---|
| | | | | | Burst size | | |
| Conditions | Duration of initial phase (ms) | Rate of the tension fall (s$^{-1}$) | Rate of the tension rise (s$^{-1}$) | $k_{cat}$ (s$^{-1}$) | [ADP] ($\mu$m) | ADP/S-1 (mol/mol) | Rate of burst (s$^{-1}$) |
| Full overlap $< 10^{-8}$ M-Ca$^{2+}$ | 39 | 11 | — | < 0·1 | 152 | 1·16 | > 35 |
| Full overlap $2 \times 10^{-5}$ M-Ca$^{2+}$ | 13 | — | 28 | 1·9 | 164 | 1·31 | > 35 |
| No overlap $2 \times 10^{-5}$ M-Ca$^{2+}$ | — | — | — | < 0·2 | 122 | 0·71 | > 35 |
| Full overlap 2 mM-ADP $2 \times 10^{-5}$ M-Ca$^{2+}$ | 25 | — | 20 | — | — | — | > 35 |
| No overlap 2 mM-ADP $2 \times 10^{-5}$ M-Ca$^{2+}$ | — | — | — | — | — | — | 13 |

The laser pulse converted 2 mM-caged ATP into about 1 mM-ATP in the fibres. The subfragment 1 concentration was taken to be 154 $\mu$M (see text). Abbreviations: S-1: myosin subfragment 1 head. $k_{cat}$: steady-state ATPase rate in mol Mg ATP hydrolysed/mol S-1 per second.

*ATP cleavage.* The time course of ATP cleavage in the absence of calcium ions is shown in Figs. 5 and 6 by the filled triangles. In Fig. 5 the data are expressed as the concentration of ADP in the fibre at the time of freezing and in Fig. 6 as the ratio

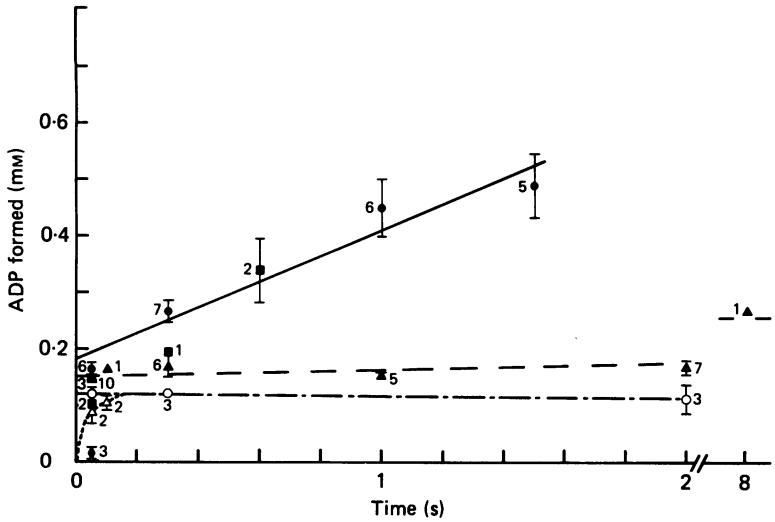


Fig. 5. Time courses of ADP formation following ATP release from caged ATP. Filled symbols indicate single fibres or bundles at full myofilament overlap and open symbols indicate fibres stretched to beyond overlap. Filled triangles, fibres were pre-incubated in the absence of calcium ions ($< 10^{-8}$ M). Circles, fibres were pre-incubated in $2 \times 10^{-5}$ M-calcium ions. Squares, fibres were incubated in solution containing calcium ions and with 2 mM-ADP added. Open triangles, fibres were incubated in the presence of calcium ions with 2 mM-ADP added and stretched beyond overlap. Diamonds, fibres were incubated in solution containing 10 mM-ATP ($< 10^{-8}$ M-calcium ions) before laser-pulse photolysis. Solvent conditions are listed in Table 1. The data points are the average of the values obtained for the number of experiments indicated. The error bars show ±one S.E. of mean.

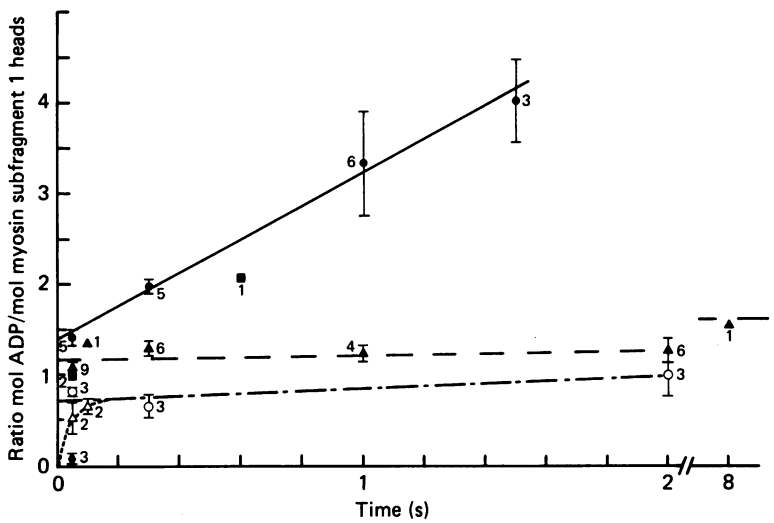


Fig. 6. Time courses of the ratio of mol ADP formed/mol myosin subfragment 1 heads following the laser pulse. Symbols have the same meaning as in Fig. 5.

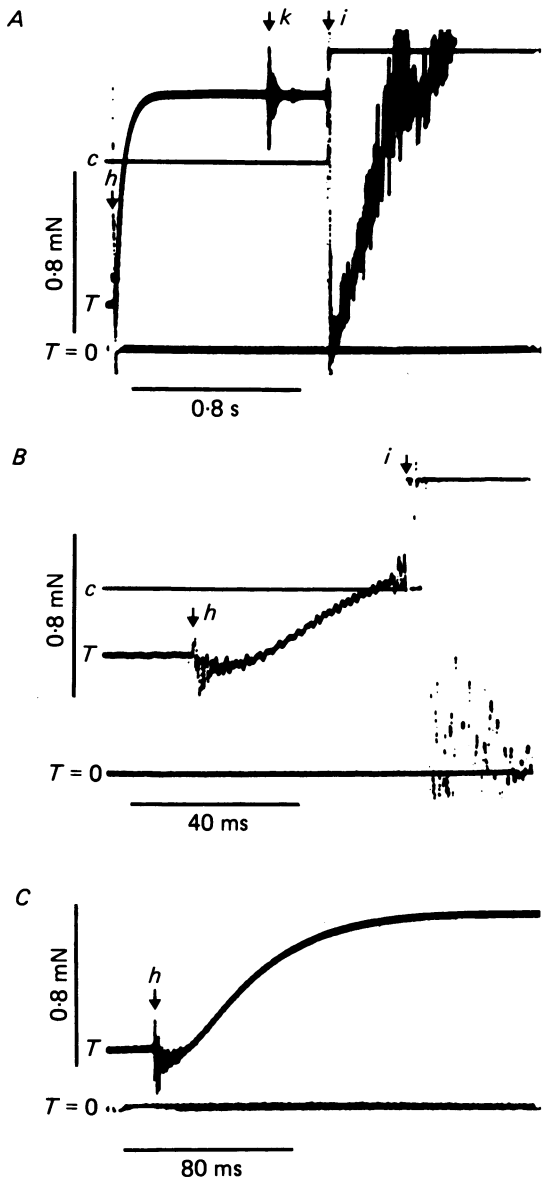


Fig. 7. Time course of tension development following photochemical release of ATP in fibres incubated in the presence of calcium ions. The bundles were in rigor prior to the laser pulse (*h*) and the freeze-clamp occurred at *i*. In *A* the top sweep (*c*) records the freeze-clamp through electrical contact of the copper blocks 1 s after the laser pulse. The middle trace records the tension of a fibre bundle (*T*) and the bottom trace the tension of the relaxed bundle ($T = 0$). *k* marks the time of triggering of the solenoid controlling the clamp. The fibre bundle consisted of two fibres (cross-sectional area ($A$) = $2{\cdot}4 \times 10^{-8}$ m$^2$, sarcomere length (*s.l.*) = 2·5 $\mu$m). From records at a faster sweep speed (not shown) measurements of the exponential rise of tension and of the initial phase were made. Values of 25 s$^{-1}$ and 6 ms were obtained, respectively. Analysis by h.p.l.c. gave the elution profile shown in Fig. 2. *B* shows a similar record for a fibre bundle that was freeze-clamped after 50 ms. The bundle consisted of two fibres ($A = 2{\cdot}0 \times 10^{-8}$ m$^2$,

of mol ADP formed/mol myosin subfragment 1 heads in the fibre. In both Figures the data points were fitted by regression lines which show positive intercepts. In Fig. 5 the intercept is $152 \pm 4$ $\mu$M which can be compared with the myosin subfragment 1 head concentration, $154 \pm 16$ $\mu$M, obtained in the Methods section. In Fig. 6 the intercept is $1{\cdot}16 \pm 0{\cdot}03$ mol ADP/mol myosin subfragment 1 heads. Both methods of analysis indicate that during the first 50 ms after the light pulse, cleavage of ATP has occurred, the magnitude of which corresponds to about 1 mol ADP/mol myosin subfragment 1 heads. It follows that, if the initial ATP cleavage can be described by an exponential process, then the rate constant for this process is greater than 35 $s^{-1}$.

The slope of the linear regression in Figs. 5 and 6 can be related to the steady-state rate of Mg ATP hydrolysis catalysed by myosin subfragment 1 ATPase in the absence of calcium ions. The slopes are not significantly different from zero, and upper limits for the steady-state rates are 0·10 and 0·09 mol Mg ATP hydrolysed/mol myosin subfragment 1 head per second for Figs. 5 and 6 respectively (95 % confidence limits).

*Caged-ATP photolysis in the presence of calcium ions*

*Tension.* When chemically skinned muscle fibres in the rigor state were placed in a trough containing caged ATP and $2 \times 10^{-5}$ M-free calcium ions, tension was observed to drop to about half the rigor level as described above (Fig. 3*A*). Following the laser pulse, tension rose rapidly to a plateau (Fig. 7*A*). Time courses of tension rise are shown in more detail in Fig. 7*B* and *C*. The freeze-clamps are recorded in Fig. 7*A* (1 s clamp) and in Fig. 7*B* (50 ms clamp). The laser pulse induced a resonance in the tension transducer that is less apparent than in Figs. 3 and 4, because of the lower recording sensitivity. The time course of tension development was essentially biphasic and consisted of an initial phase in which tension decreased slightly, and was followed by a phase in which tension rose with an exponential time course. For an average 1·4 mM-ATP released, the initial phase lasted $13 \pm 2$ ms ($n = 16$), and the exponential rise of tension occurred with a rate constant of $28 \pm 2$ $s^{-1}$ ($n = 16$). The active tension reached by the fibres was proportional to the fibre cross-sectional area and corresponded to $54 \pm 7$ kN/m$^2$ ($n = 21$), somewhat lower than the value of 80 kN/m$^2$ measured by Cooke & Bialek (1979) in 1 mM-Mg ATP and 10 °C. In the presence of calcium ions, the duration of the initial phase was one-third of that in the absence of calcium ions. The rate constant for tension rise was 2·5 times that for the relaxation of tension observed in the absence of calcium ions (Table 2).

*ATP cleavage.* The time course of ATP cleavage in the presence of $2 \times 10^{-5}$ M-calcium ions is shown by the filled circles in Figs. 5 and 6. The steady-state rates of the ATPase derived from Figs. 5 and 6 were $1{\cdot}5 \pm 0{\cdot}2$ and $1{\cdot}8 \pm 0{\cdot}4$ mol Mg ATP hydrolysed/mol

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*s.l.* = 2·48 $\mu$m). 1·31 mM-[2-$^3$H]ATP was released by the laser pulse. *C* shows the tension record of a fibre that was not freeze-clamped. The bundle consisted of two fibres ($A = 1{\cdot}8 \times 10^{-8}$ m$^2$, *s.l.* = 2·55 $\mu$m). The rise of tension had rate constant of 29 $s^{-1}$, an initial phase lasting 9 ms and the isometric tension reached was 56 kN/m$^2$. After the laser pulse, the fibre was returned to rigor solution and to caged-ATP solution. A second laser pulse was applied, following which the fibre was freeze-clamped at 300 ms. The second laser pulse caused a tension rise with a rate constant of 22 $s^{-1}$ and an initial phase lasting 15 ms. The second laser pulse released 1·66 mM-[2-$^3$H]ATP. The isometric tension reached after the second pulse was 50 kN/m$^2$.

myosin subfragment 1 heads per second. The ordinate intercepts show an initial burst of ADP formation of $182 \pm 22$ $\mu$M-ADP and $1{\cdot}36 \pm 0{\cdot}23$ mol ADP/mol myosin subfragment 1 heads for Figs. 5 and 6 respectively. The estimates of the initial burst of ADP formation are prone to greater error in the presence of calcium because of the steepness of the steady-state rate of ADP formation compared to the rate in the absence of calcium. The amplitude of the initial burst of ADP formation was not significantly different from that observed in the absence of calcium ions.

*Caged-ATP photolysis in fibres stretched beyond overlap.* With fibres stretched beyond overlap, it is possible to study the kinetics of the myosin ATPase without interference from actin. Furthermore, if present in skinned fibres, calcium-sensitive ATPases other than the actomyosin ATPase should be detectable. Fibres in relaxing solution were stretched to a sarcomere length greater than 3·9 $\mu$m. This was verified by the absence of tension development above resting tension when the fibres were placed in rigor solution. Furthermore no active tension developed when Mg ATP was liberated following a laser pulse in the presence of calcium.

The time course of ATP cleavage in the presence of $2 \times 10^{-5}$ M-free calcium in fibres stretched beyond overlap is shown by the open circles in Figs. 5 and 6. The ordinate intercepts of the regression lines show an initial burst of ADP formation of $122 \pm 8$ $\mu$M-ADP and $0{\cdot}71 \pm 0{\cdot}08$ mol ADP/mol myosin subfragment 1 head for Figs. 5 and 6 respectively. The ordinate intercepts are significantly different from those obtained from experiments done at full overlap in the presence and absence of calcium ions ($P < 0{\cdot}05$, except for the data with calcium ions in Fig. 5, for which a difference was observed but was not significant because of the size of the error).

The slopes of the regression lines in Figs. 5 and 6 are not significantly different from zero with upper limits on the ATPase steady-state rates of 0·04 and 0·24 mol Mg ATP hydrolysed/mol myosin subfragment 1 head per second. This suggests that at most 12 % of the ATPase activity in the presence of calcium could be attributed to ATPases other than actomyosin. The absence of other ATPases was confirmed by treating muscle fibres with Triton X-100, a non-ionic detergent which solubilizes both the calcium-dependent ATPase of sarcoplasmic reticulum and the mitochondrial ATPase (Solaro, Pang & Briggs, 1971). Fibres were bathed in relaxing solution containing 1 % Triton X-100 (v/v) for at least 30 min prior to transferring them to rigor and caged-ATP solutions. The amount of Mg ATP cleaved at 2 s in detergent-treated fibres in the presence of calcium ions and at full overlap equalled that in untreated fibres.

*Caged-ATP photolysis in the presence of ADP or ATP.* Experiments were carried out in the presence of 2 mM-ADP to see if it was possible to inhibit the rate of the burst of ADP formation and to measure the rate constant for this process. The first effect observed was that the slow tension drop which occurred when fibres were placed in the trough containing the caged-ATP solution (*d* in Fig. 3*A*) disappeared in the presence of 2 mM-ADP which did not itself induce any changes of tension of the fibres in rigor. This effect was seen in the presence and absence of calcium ions.

Following the laser pulse the time course of the tension rise in the presence of calcium ions was somewhat altered by the addition of ADP: the initial phase was prolonged from 13 to $25 \pm 10$ ms ($n = 3$) (Table 2). The exponential rise of tension which occurred with a rate constant of $20 \pm 5$ s$^{-1}$ ($n = 3$) was not significantly different from that in the absence of added ADP.

The time course of Mg ATP cleavage was not affected by the presence of ADP, both in the presence and absence of calcium ions, indicating that the initial rate of ADP formation was still at least 35 $s^{-1}$. However, when the fibres were stretched to beyond overlap, the time course of initial ADP formation was considerably slower. The data are shown by the open triangles in Figs. 5 and 6. The rapid phase of ADP formation can be fitted by an exponential with a rate constant of 13 $s^{-1}$. Thus the presence of 2 mM-Mg ATP slowed down the rate of the initial burst by at least a factor of 3.

As a control experiment [2-$^3$H]caged ATP was photolysed in relaxed fibres bathed in 10 mM-unlabelled ATP (Table 1; caged-ATP solution plus 10 mM-unlabelled ATP which gives approximately 3 mM-Mg ATP, $< 10^{-8}$ M-free calcium). Even though 1·25 mM-[2-$^3$H]ATP was released, no appreciable [2-$^3$H]ADP was detected at 50 ms (diamonds in Figs. 5 and 6). This is the expected result since the myosin sub-fragment 1 heads were saturated with non-radioactive nucleotide at the time of the laser pulse. It establishes that the rapid bursts of [2-$^3$H]ADP formation detected in the experiments described in Figs. 5 and 6 were due to an interaction between [2-$^3$H]ATP and myosin and were not an artifact of the experiments.

*Tension records and laser-induced damage to the fibres.* In their experiments Goldman *et al.* (1982*b*) exposed single fibres from rabbit psoas muscle to several laser pulses without deleterious effects. Since features such as suspending the fibre in air and greater focusing of the laser beam distinguish the experiments described here from those of Goldman *et al.* (1982*b*), it was important to determine whether laser-induced fibre damage gave rise to artifacts. This was done by comparing tension records between the two types of experiments and seeing whether a single fibre in air could withstand multiple laser pulses.

Relaxation records such as in Figs. 3*B* and 4 were similar to those obtained by Goldman *et al.* (1982*b*) (cf. figs. 2*a*, 3 and 5 of that paper). For most experiments the initial rigor tension per cross-sectional area of fibre was 15–30 kN/$m^2$ just prior to the laser pulse. At these low tension levels we did not observe the phase of rapid fall of tension which follows the release of 0·5 mM-ATP or more and that occurs with a half-time of about 5 ms when the initial tension is high (cf. figs. 1*b* and 3*a* of Goldman *et al.* 1982*b*). However, in some cases a more pronounced tension drop was observed. This phase was slower than would have been expected from the relaxation kinetics of the fibre in the trough. After an initial process masked by the transducer resonance, the half-time of the tension change associated with this phase was about 10 ms even when 1–2 mM-ATP was released. Probably the rate of fall of tension in this phase was not just limited by the detachment kinetics of the cross-bridges (Goldman *et al.* 1984*a*). A qualitative distinction (besides the resonance of the transducer) in the physiological response was that an instantaneous drop in tension sometimes occurred prior to the typical response. Small instantaneous tension drops are evident in some of the records of Goldman *et al.* (1982*b*). Activation records such as in Fig. 7 were similar to those of Goldman *et al.* (1982*b*) (see fig. 5 of that paper), although again instantaneous drops were sometimes observed.

Multiple cycles of tension development were obtained by laser-induced release of ATP in the presence of $2 \times 10^{-5}$ M-free calcium ions followed by wash-out of the fibre and restoration of the rigor state. Typically successive laser pulses caused up to a 25% increase in the half-time of tension development and a 15% drop in isometric tension (see the legend of Fig. 7*C*).

Since the fibres used in those experiments whose data are recorded in Figs. 5 and 6 were only exposed to a single laser pulse, it is unlikely that laser-induced damage would influence our experimental results significantly.

## DISCUSSION

*Release of ATP from caged ATP.* Conversion of up to 77 % caged ATP into ATP with a single 50 ns laser pulse is striking since the quantum yield for the photolysis of 1-(2-nitro)phenylethyl phosphate has been measured at 0·54 by Kaplan *et al.* (1978). It suggests either that the lifetime of the excited intermediate is less than 50 ns (so that a single laser pulse has the potential to excite a caged-ATP molecule more than once) or that the quantum yield for the photolysis of caged ATP is substantially larger than that of its parent phosphate ester.

That up to 5 mM-ATP can be released in a single laser pulse has implications for the use of laser-pulse photolysis in the study of muscle and other biological systems. For example, reactions in skinned muscle fibres can be initiated with ATP (and/or ADP and/or $P_i$) concentrations as close to those *in vivo* as the experimentalist chooses. Since relatively few metabolites exist *in vivo* at above 5 mM concentration, it should be possible to initiate photochemically a wide range of reactions in biological systems when one of the reactants is the photoproduct of a biologically inert *o*-nitrobenzyl derivative.

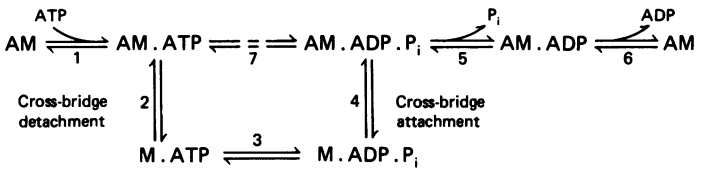
*Effect of caged ATP on fibre tension.* The drop in tension of muscle in rigor on addition of caged ATP suggests there is an interaction between caged ATP and the muscle fibre. The most probable explanation is that caged ATP binds weakly at a site other than the ATPase active site. This possibility has already been referred to in the Methods section. The presence of ADP overcomes the caged-ATP effect which could be a result of ADP binding at the ATPase active site or at a hypothetical secondary binding site. The drop in tension was not observed for fibres prepared from the flight muscles of the water bug *Lethocerus Americanus* (M. A. Ferenczi & D. C. S. White, unpublished observations).

3 mM-caged ATP does not modify the kinetics of ADP dissociation from myosin subfragment 1 (see Methods section) or the ATP-induced actomyosin dissociation (McCray *et al.* 1980), suggesting that it will not influence the kinetics of ATP cleavage. It is unlikely that the drop in tension of a fibre in rigor is due to ATP contamination of caged ATP since this would be much more likely to result in a small rise of tension (Goldman *et al.* 1982*b*).

The possibility that binding of caged ATP to other sites on myosin or on other myofibrillar proteins modifies the cross-bridge cycle cannot however be eliminated. Similarly, we have not eliminated the possibility that some degree of evaporation occurred while the fibres were suspended in air which would have resulted in hypertonic fibres.

*Implications for the mechanism of muscle contraction.* Lymn & Taylor (1971) proposed a scheme relating the biochemistry of the actomyosin ATPase to muscle contraction. Goldman *et al.* (1982*b*) used this scheme together with some recently characterized steps to interpret the tension responses they obtained when ATP was released by laser-pulse photolysis of caged ATP in skinned muscle fibres. That scheme

is reproduced as a basis for discussing whether the results obtained here can be incorporated into it. Each step in the scheme is characterized by a number as shown. The symbol AM with or without nucleotide denotes a cross-bridge state of actomyosin; M.ATP and M.ADP.$P_i$ denote detached states.




The pathway of a cross-bridge cycle envisaged by Lymn & Taylor (1971) was via steps 1, 2, 3, 4, 5 and 6. Data from Kassab's and Eisenberg's laboratories (Mornet, Bertrand, Pantel, Audemard & Kassab, 1981; Stein, Chock & Eisenberg, 1981) indicate that an alternative direct hydrolysis pathway is possible via steps 1, 7, 5 and 6. Cross-bridge detachment could occur in this pathway via rapid reversal of steps 2 and 4 (Sleep & Hutton, 1978). Such a rapid reversal may be able to relieve strain or stress in a cross-bridge, even if ATP were being hydrolysed via the direct pathway.

When ATP is released in a fibre in rigor in the absence of calcium ions, the resulting cross-bridge detachment can be described by a process that is first-order in ATP concentration with a second-order rate constant of at least $1 \times 10^6$ M$^{-1}$ s$^{-1}$ (Goldman *et al.* 1984*a*). This means that in the corresponding experiment (Figs. 5 and 6, ▲) ATP, which was typically 1 mM, would have bound to and caused an initial detachment within 50 ms, even when the relatively slow rate of release of ATP on photolysis is taken into account. Thus, when at 50 ms the first measurement of ATP cleavage was made, most cross-bridges (or myosin subfragment 1 molecules) were expected to have bound one ATP molecule. The fact that about one molecule of ADP is formed at a rate greater than 35 s$^{-1}$ per myosin subfragment 1 head is consistent with this. It is also consistent with the transient kinetics of muscle proteins in solution. Lymn & Taylor (1971) showed that the rate of ATP cleavage in the first ATPase cycle of acto-heavy meromyosin was 50 s$^{-1}$.

Goldman *et al.* (1982*b*) showed that when ATP was released in a fibre in rigor in the absence of calcium ions, there was a tension change which extended in time well beyond the initial detachment process, so that complete relaxation took about 200 ms. They interpreted this as being caused by a certain fraction of cross-bridges cycling because of protein co-operativity in the myofilament. Accordingly, besides the ADP formed within the first 50 ms, there could be additional ADP formation in a process with a half-time of about 50 ms (see Figs. 3*C* and 4) before the system is fully relaxed, at which time ADP will be formed at the steady-state ATPase rate of a relaxed fibre. It is not possible to calculate, without several assumptions, how much ADP might be expected in this second phase. However, the fraction of cross-bridges which are involved in recycling is likely to be relatively small since the stiffness in the second phase of relaxation is low compared to the stiffness of muscle in rigor (Goldman *et al.* 1982*b*; Hibberd, Goldman & Trentham, 1983), and it is not clear that all cycling is accompanied by ATP hydrolysis.

Cross-bridge recycling would not occur in fibres stretched beyond overlap. Therefore

the recycling cross-bridge hypothesis predicts that the regression lines obtained beyond overlap should have ordinate intercepts below those obtained from relaxed fibres. The observed differences are 30 $\mu$M and 0·45 mol ADP formed/mol subfragment 1 for Figs. 5 and 6 respectively so that as many as half the cross-bridges might be recycling with accompanying Mg ATP hydrolysis.

*The equilibrium constant of Mg ATP cleavage in fibres stretched beyond overlap.* The most likely explanation for formation of less than one mol of ADP/mol subfragment 1 being formed in the burst phase is that this reflects the ready reversibility of the ATP cleavage step (Bagshaw & Trentham, 1973). With subfragment 1 in solution the equilibrium constant for this step varies between 1 and 10 depending on the reaction conditions (Taylor, 1977).

*Effect of calcium ions.* When ATP is released in a fibre in rigor in the presence of calcium ions, the kinetics of initial cross-bridge detachment as detected from tension changes are similar to those in the absence of calcium (Goldman, Hibberd & Trentham, 1984*b*). In the experiments described here, initial detachment was followed by tension development to the level of an isometric contraction that was achieved in 150 ms (Fig. 7*C*). In terms of scheme 1 and of the transient kinetic results of Lymn & Taylor (1971), cleavage of up to 1 mol ADP/mol myosin subfragment 1 heads is predicted within 50 ms, as is observed (Figs. 5 and 6).

It would not be surprising if more than one cross-bridge cycle was necessary during the period of development of isometric tension and for the ATPase activity to vary with tension. So there may be a further burst of ADP formation during the first 150 ms characterized by a rate constant of about 25 $s^{-1}$ (Fig. 7*C*). Our data are insufficient to test for this possibility. The ordinate intercepts of the regression lines in Figs. 5 and 6 (open and filled circles) are consistent with the hypothesis if no more than about one mol of ADP/mol myosin heads was formed in this way, but a detailed time course of ADP formation over the first 150 ms is needed to provide a critical test.

The steady-state rate of the ATPase in the presence of calcium ions in fibres developing isometric tension is at least 10-fold less than the ATPase activity of actosubfragment 1 at low ionic strength (Weeds & Taylor, 1975) or cross-linked actosubfragment 1 at more physiological ionic strength (Mornet *et al.* 1981). However, the steady-state rate observed here is comparable to that observed in skinned rabbit psoas fibres by Takashi & Putnam (1979).

We believe the key feature is that, since a burst of ADP formation of about 1 mol per myosin head has been observed, almost all the myosin heads in the isometric state must be in the form of product complexes and correspondingly be in that part of the scheme around steps 5 and 6. This conclusion is consistent with evidence derived from a number of experimental approaches (Marston & Tregear, 1974; Goldman *et al.* 1982*b* and references therein).

*ADP dissociation in fibres.* That ADP bound to cross-bridges in rigor did not have a measurable effect on the amount of ATP cleaved within 50 ms of ATP release is expected in view of the known stimulation by actin of the dissociation rate of ADP from heavy meromyosin (White, 1977).

In the case of fibres stretched beyond overlap no such actin stimulation was possible and the data (open triangles in Figs. 5 and 6) suggest an ADP dissociation rate constant for myosin of about 13 $s^{-1}$. In fact the rate constant may be somewhat

larger than this as the 2 mM-ADP in the medium will compete with the ATP for the ATPase site, thus slowing net ADP dissociation. The value of 13 $s^{-1}$ compares with an ADP dissociation rate constant of 2·8 $s^{-1}$ for subfragment 1 measured under identical solvent conditions. The rate constant of ADP dissociation from subfragment 1 is temperature sensitive, increasing by a factor of 2·8 for a 5 °C temperature rise (Bagshaw & Trentham, 1974). If the laser pulse had increased the temperature by as much as 7·5 °C immediately following the flash, the disparity between the two ADP-dissociation rate constants could be explained though, as noted in the Methods section, such a large temperature rise is unlikely. However, it is possible that the lattice of the myofilament modifies the ADP dissociation kinetics, but further experiments are required to establish this point.

*Conclusion.* The results obtained here show that it is possible to obtain kinetic data about Mg ATP cleavage catalysed by calcium-regulated actomyosin in chemically skinned muscle fibres. On addition of ATP to a fibre in the presence or absence of calcium, a steady state is reached rapidly in which the dominant intermediates are protein–product complexes. These observations closely parallel those for the isolated muscle protein actin and myosin in solution. The tension changes observed in these experiments are complex and it will be interesting to find out if they are accompanied by biochemical changes on similar time scales.

Support for this project was provided by NIH Grants AM 23030, AM 30988, HL 15835 to the Pennsylvania Muscle Institute and C79112 from the Muscular Dystrophy Association of America. For part of this work M.A.F. was an MDA Post-doctoral Fellow. We thank Mrs F. Itshak for technical assistance, Mr N. V. Ricchiuti for assistance in building the apparatus, Drs Y. E. Goldman and J. A. McCray for advice on many aspects of working with laser-pulse photolysis of caged ATP in muscle fibres, and Dr G. Dubyak for measuring fibre absorption.

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