4. Reaction mechanisms for the second and third step can be formulated by analogy with homogeneous bifunctional catalysis.

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The Effects of Adenosine Triphosphate on the Mechanical Properties of Surface Films of L-Myosin and Actomyosin

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The earliest study of the effects of the adenine nucleotides on the properties of the myofibrillar proteins spread at the air-water interface was made by Munch-Petersen (1948), who found that when actomyosin was spread on 0.5 M-potassium chloride solution containing 0.1 mM-adenosine triphosphate, it occupied an area considerably greater, for a given surface pressure, than in the absence of adenosine triphosphate. The effect was later shown to be due, at least in part, to an increase by adenosine triphosphate in the rate of spreading of the protein (Lajtha & Rideal, 1952; Cheesman, 1952). Similar effects were found for actin-free myosin by Munch-Petersen, although Cheesman was unable to repeat this observation.

The acceleration by adenosine triphosphate of the spreading of actomyosin may be explained by the dissociation of the protein by this nucleotide into its constituent actin and myosin. A similar acceleration is produced by high salt concentrations, which also dissociate actomyosin in bulk

solution. For this reason, the results seemed to be of little biochemical interest, especially as no evidence of chemical alteration of the protein by adenosine triphosphate could be obtained from phase-boundary potential measurements (Cheesman, 1952).

Cheesman & Sten-Knudsen (1959) have developed a procedure for the study of the viscoelastic properties of protein films. It was found with this method that when actomyosin or L-myosin was spread on M-potassium chloride solution containing 0.1 mM-adenosine triphosphate, the mechanical rigidity of the resulting film was considerably lower than that of a similar film in the absence of adenosine triphosphate at the same surface pressure with the first protein, and at the same surface pressure and specific area with the second. Two explanations of these findings presented themselves. It was conceivable that a complex of adenosine triphosphate and denatured protein persisted in the spread film, with a consequent effect upon the mechanical properties of the latter. Alternatively, since the protein under the conditions of the experiments was not fully spread, the effect could have been due to an interaction between adenosine triphosphate and native

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protein forming an integral part of the film structure. The first explanation, if it proved to be correct, would be of some general interest. The second, on the other hand, would offer considerable possibilities in the study of the interaction of adenosine triphosphate and the muscle proteins, especially in relation to the much-disputed theory that the phosphorylation of actomyosin by adenosine triphosphate is an essential feature of the contraction cycle in living muscle (Weber, 1955; Morales, Botts, Blum & Hill, 1955). For this reason it was decided to undertake a more detailed study of the phenomenon, the results of which are now presented with earlier data which have not hitherto been published except in outline (Cheesman & Davies, 1954).

EXPERIMENTAL

Materials

Adenosine triphosphate (ATP). The material used in the earlier experiments was obtained from the Ba salt supplied by Boots Pure Drug Co. Ltd. (Nottingham) and purified by way of the Hg and Ba salts (LePage, 1949). All experiments were repeated, with similar outcome, with the crystalline disodium salt from the Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Adenosine diphosphate (ADP). This was the Na salt from the Sigma Chemical Co.

Inosine triphosphate (ITP). This was prepared from ATP by the method of Kaplan (1957).

2,4-Dinitrophenol (DNP). This was of analytical quality and was recrystallized from water and used in the form of the Na salt.

Fuadin (antimony bis-[catecholdisulphonic acid], Na salt). This was generously provided by Farbenfabriken Bayer, Leverkusen.

Ethylenediaminetetra-acetic acid (EDTA). This was obtained from British Drug Houses Ltd., and *p*-chloromercuribenzoic acid (PCMB) was obtained from L. Light and Co. Ltd. Solutions of both were brought to pH 7.4 with NaOH before use.

All inorganic salts and buffer substances were of analytical grade.

The water used in all experiments was from the laboratory distilled-water supply, further purified by passage over mixed anion- and cation-exchange resins in an Elgastat deionizer (Elga Products Ltd., London, S.W. 19).

L-Myosin. This was prepared from the leg and back muscles of rabbits by the method of Mommaerts & Parrish (1951) with five reprecipitations.

Actomyosin. This was made from similar material by extraction of the minced muscle for 16 hr. at 5° with Weber's solution (0.6M-KCl, 0.04M-NaHCO₃, 0.01M-Na₂CO₃; 300 ml./100 g. of muscle). The extract was strained through muslin and centrifuged at 1500 g for 20 min. Actomyosin was precipitated from the supernatant with 3 vol. of water at 5°; it was centrifuged and redissolved in 0.25 vol. of 2M-KCl. The protein was reprecipitated by dilution with 3 vol. of water. The reprecipitation was repeated four times with occasional small additions of Weber's solution to maintain a slightly alkaline pH.

Actin. This was obtained from the muscle residue in the L-myosin preparation by the method of Straub (1942) as modified by Bailey & Perry (1947).

L-Myosin and actomyosin were kept in solution in 0.5M-KCl at 5°. A few drops of toluene were added to delay bacterial attack. The preparations were used, unless otherwise stated, within 14 days.

Methods

Dry-weight determinations on protein solutions. These were made as follows: 5 ml. of solution was treated with 1 ml. of 10% (w/v) trichloroacetic acid in a weighed centrifuge tube. The precipitate was centrifuged, washed twice with water and once with ethanol on the centrifuge and dried to constant weight at 110°.

Determination of adenosine triphosphatase activity. The incubation mixture consisted of 1 ml. of 5 mM-ATP, 1 ml. of 0.05M-2-amino-2-hydroxymethylpropane-1:3-diol-HCl buffer, pH 7.4 (tris-HCl buffer: Gomori, 1946) in 0.5M-KCl and 0.05 ml. of 0.1M-CaCl₂ at 37°. At zero time about 0.25 mg. of L-myosin or actomyosin in 1 ml. of 0.5M-KCl at 37° was added. The reaction was stopped after 5 min. with 2.5 ml. of 8% (w/v) trichloroacetic acid. The precipitated protein was removed by filtration through cotton wool into a 25 ml. graduated flask. The cotton wool was washed once with 0.5 ml. of trichloroacetic acid and twice with 1 ml. of water. Inorganic phosphate in the filtrate was estimated by the method of Fiske & Subbarow (1925), the volume being made up to 25 ml. before the colorimetric reading in the Hilger Spekker absorptiometer. Reagent blanks and standards were run with all determinations.

Measurements of film plasticity. The modified Langmuir surface balance used for the study of the viscoelastic properties of the protein films was identical with that described by Cheesman & Sten-Knudsen (1959). The technique consisted in following the time course of the horizontal displacement in the film of a waxed mica float, of 6 mm. radius, bearing a saturated permanent magnet, during the application of an electric field from a solenoid. The solenoid was mounted near the trough with its axis along the surface. The force-current and force-distance relationships for solenoid and magnet were previously determined by direct measurement with the aid of a torsion balance. The experimental procedure was as follows, with occasional variations indicated where they are relevant.

Protein solution (40 μ l.) in 0.5M-KCl, containing about 0.04 mg. of actomyosin or L-myosin, was applied in small drops to the surface of the substrate in the trough. The available area was about 0.8 m.²/mg. of protein. During the spreading period the magnetic float was held in position in the mid-line of the trough at about 5 cm. from the floating mica boom of the balance by means of a small solenoid mounted above the surface. Five minutes after the application of the protein, the film was compressed to a surface pressure of 3 dynes/cm. as recorded on the torsion head. The current through the small solenoid was then switched off and the large displacing solenoid was adjusted to a standard distance (usually 18.5 cm.) from the centre of the magnetic float. The zero position of the float was recorded with a telescope bearing a micrometer eyepiece. The displacing current and a stop clock were turned on exactly 7 min. after the application of the protein. The positions of the float were read at intervals of 5 sec. The displacing current was interrupted at 30 sec. and the

readings were continued for a short time thereafter. During such experiments the surface pressure usually increased by about 0.5 dyne/cm.

The displacement of the float may be resolved into components representing viscous flow and elastic deformation in the film. The presence of the latter component is manifested in elastic recovery after removal of the stress. Since the results recorded here are meant to be only semi-quantitative, displacement curves are reproduced without mathematical treatment. In most instances only the displacements of the float at 30 and 40 sec. (10 sec. after interruption of the current) are given, the former quantity being taken as a rough measure of 'plasticity' (see below), the latter as an indication of the importance of the flow component. In the legends of Figs. 1 and 2, values are given for the elastic moduli of the films as calculated by the empirical procedure described by Cheesman & Sten-Knudsen (1959).

The term 'plasticity' is employed in this paper as an antonym to 'stiffness', which in turn is used to signify viscoelastic resistance to mechanical deformation. When therefore ATP is said to have a plasticizing (or stiffening) action on a film, it is implied that this substance has reduced (or increased) the sum of the viscous and elastic forces, determined by the film structure, which resist the movement of the float under a given stress.

Fig. 1. Plasticizing effect of ATP on an actomyosin film. This figure, with Figs. 2, 5 and 6, shows the time course of the displacement of the magnetic float under the stress imposed by the solenoid. This displacement may be resolved into a rapid initial movement against an elastic force and a much slower drift in a viscous medium. Elastic recovery occurs when the stress is removed, in this case at 400 sec. An empirical treatment of such curves permits the calculation of elastic moduli for the films (Cheesman & Sten-Knudsen, 1959). Substrate: m-KCl , 0.01M-veronal-HCl buffer, pH 7.0. Surface pressure: 10 dynes/cm. Specific areas: without ATP, 0.4 m^2/mg .; with ATP, 0.45 m^2/mg . Applied stress: 0.45 mg. weight, interrupted at 400 sec. ●, Without ATP (elastic modulus 5.6 dynes/cm.²); ○, with 0.1 mM-ATP (elastic modulus 3.0 dynes/cm.²).

RESULTS

Effects of adenosine triphosphate on films of L-myosin and actomyosin. The fundamental effect with which we are concerned is illustrated in Figs. 1 and 2, which represent measurements of the viscoelastic properties of films of L-myosin and actomyosin in the presence and absence of ATP. In both cases the plasticity of the film is increased by ATP.

It was observed in the early stages of the work that, after the proteins had been stored in the refrigerator for a few days, the effect of ATP upon the films decreased. Preparations of L-myosin more than 14 days old frequently showed no effect whatever. Films of aged actomyosin, on the other hand, were often stiffened by ATP. Since these aged-protein preparations had a greatly reduced adenosine triphosphatase (ATPase) activity, the matter appeared to merit further study. An experiment was made in which the enzymic activity of actomyosin was reduced progressively by incubation at 37°. Samples were withdrawn at intervals and used for film experiments which were made concurrently with estimations of ATPase activity. The results are shown in Fig. 3, where it will be seen that whereas the rigidity of the actomyosin film decreases with the reduction in the ATPase activity the presence of ATP reverses this effect, so that the plasticizing action of ATP is transformed

Fig. 2. Plasticizing effect of ATP on an L-myosin film (see legend to Fig. 1). Surface pressure: 15 dynes/cm. Specific area: 0.5 m^2/mg . Applied stress: 1.2 mg. weight, interrupted at 200 sec. Other conditions were as described for Fig. 1. ●, Without ATP (elastic modulus 10.6 dynes/cm.²); ○, with 0.1 mM-ATP (elastic modulus 5.1 dynes/cm.²).

into a stiffening action. The data in Table 1 show that the 'ageing' of the actomyosin corresponds to an increase in the area of the film at constant surface pressure in the absence of ATP, although this area remains unchanged in the presence of ATP.

Transience of the effect of adenosine triphosphate. At low surface pressures it is found that the plasticizing action of ATP upon films of L-myosin and actomyosin is of short duration. The data in Fig. 4, from an experiment with L-myosin, show an almost complete elimination of the effect after 15 min. The rise in surface pressure during this period suggests that increased surface denaturation is the chief contributory factor here. It is interesting to compare these data with Figs. 1 and 2,

Fig. 3. Influence of pre-heating of the protein on the effect of ATP. Fresh actomyosin was heated in 0.5M-KCl at 37°. Samples were taken at 3, 10, 20 and 30 min. for ATPase assays and for film experiments in the presence and absence of ATP. Substrate for films: 0.8M-KCl, 0.01M-tris-HCl buffer, pH 7.4. Spreading time: 5 min. Applied stress: 0.9 mg. weight. Displacement of float was recorded at 30 sec. ●, Without ATP; ○, with 0.1 mM-ATP.

Table 1. Changes in adenosine triphosphatase activity and rate of spreading of actomyosin incubated at 37°

See also Fig. 3.

Time of incubation (min.)	ATPase activity (µg. of P/mg./min.)	Area of film after 5 min. spreading and compression to 3 dynes/cm. (m. ² /mg. of protein)	
		With ATP	Without ATP
3	5.7	0.58	0.53
10	3.9	0.58	0.54
20	2.4	0.58	0.55
30	0.6	0.58	0.58

which show the persistence of the effect at higher surface pressures. These observations lend support to the view that the ATP effect is determined by the native component in the film.

Influence of p-chloromercuribenzoate on the effect of adenosine triphosphate. When the thiol reagent PCMB is present in the substrate, the effect of ATP on the plasticity of L-myosin and actomyosin films is entirely eliminated (Table 2). We have never found ATP to exert a stiffening effect in systems inhibited with PCMB, although the inhibitor itself gives such an effect in higher concentrations, presumably due to cross-linking over the carboxyl group by PCMB molecules which have combined with thiol groups in the protein.

Influence of 2,4-dinitrophenol on the effect of adenosine triphosphate. Greville & Needham (1955) and Chappell & Perry (1955) established that DNP increased the rate of liberation of inorganic phosphate from ATP by L-myosin (at all ionic strengths) and by actomyosin (at high ionic strengths). It was therefore of interest to study the effect of this substance in our systems. mM-DNP almost completely inhibits the plasticizing effect of ATP on films of L-myosin (Fig. 5). With actomyosin, the action of ATP is converted into a stiffening (Table 2).

Influence of ethylenediaminetetra-acetate on the effect of adenosine triphosphate. EDTA, which also activates the ATPase of L-myosin and actomyosin in a striking degree (Bowen & Kerwin, 1954), modifies the effect of ATP on films of the two proteins in the same sense as does DNP. With L-myosin this influence is not as marked as with DNP (Table 2).

Fig. 4. Decrease with time in the effect of ATP on films of L-myosin. Substrate was as for Fig. 3. Spreading time before compression: 5 min. Surface pressure increased from 3 to 5 dynes/cm. in 15 min. Applied stress: 0.9 mg. weight. Displacements were recorded at 20 sec. ●, Without ATP; ○, with 0.1 mM-ATP.

Table 2. Influence of various substances on the plasticizing action of adenosine triphosphate on films of L-myosin and actomyosin

Figures represent the means of at least two experimental values. Since several protein preparations were used, and these at different times after preparation, experiments are comparable only with those in the same group. The substrate in all cases was 0.8M-KCl buffered at pH 7.4 with 0.01 M-tris-HCl. ATP was added in a concentration of 0.1 mM; other substances, when not otherwise indicated, in concentrations of mM. Spreading time: 5 min. Surface pressure: 3 dynes/cm. Specific areas: actomyosin 0.45-0.55 m.²/mg. (increased by 10-18% with ATP); L-myosin 0.5-0.6 m.²/mg. (unaffected by ATP).

Protein	Additions to substrate	Stress (interrupted at 30 sec.) (mg.)	Displacement of float (mm.)	
			30 sec.	40 sec.
L-Myosin	None	0.4	0.57	0.43
	PCMB		0.37	0.28
	ATP		1.07	0.61
	ATP + PCMB		0.46	0.37
Actomyosin	None	1.2	1.34	0.75
	ATP		2.09	1.31
	ATP + PCMB (0.01 mM)		1.27	0.60
Actomyosin	None	0.9	1.16	0.62
	DNP		1.17	0.62
	ATP		1.42	0.73
	ATP + DNP		0.72	0.31
L-Myosin	None	0.9	1.34	0.76
	EDTA		1.37	0.78
	ATP		2.12	1.48
	ATP + EDTA		1.78	0.98
Actomyosin	None	0.4	0.69	0.42
	EDTA		0.69	0.42
	ATP		0.97	0.66
	ATP + EDTA		0.42	0.03
L-Myosin	None	0.4	0.57	0.43
	Fuadin		0.57	0.34
	ATP		1.07	0.52
	ATP + fuadin		0.64	0.47

Fig. 5. Inhibition by DNP of the effect of ATP on L-myosin films (see legend to Fig. 1). Substrate was as for Fig. 3. Spreading time: 5 min. Surface pressure: 3 dynes/cm. Applied stress: 0.9 mg. weight. Stress was removed at 30 sec. ●, Protein alone or with mM-DNP; +, with 0.1 mM-ATP; ○, with 0.1 mM-ATP and mM-DNP.

Fig. 6. Stimulation by fuadin of the effect of ATP on actomyosin films (see legend to Fig. 1). Conditions were as for Fig. 5. ●, Protein alone or with mM-fuadin; +, with 0.1 mM-ATP; ○, with 0.1 mM-ATP and mM-fuadin.

Influence of fuadin on the effect of adenosine triphosphate. Weber (1955) found that fuadin (antimony bis-[catecholdisulphonic acid], Na salt), a weak inhibitor of actomyosin ATPase, when added to a system containing adenosine [³²P]-diphosphate, ATP and actomyosin, made it possible to detect a transfer of phosphate groups between ATP and ADP.

This substance, when added to the substrate in our systems, had no influence on the elastic properties of L-myosin or actomyosin films. In the presence of ATP, the plasticizing action of the latter on L-myosin films was almost completely inhibited (Table 2). With actomyosin, on the other hand, the effect of ATP was greatly augmented by fuadin (Fig. 6).

Influence of calcium and magnesium ions on the effect of adenosine triphosphate. Calcium ions, added to the substrate as CaCl₂, have no significant effect

on the elastic properties of L-myosin or actomyosin films, in the presence or absence of ATP. Magnesium ions, on the other hand, although they are without influence upon the protein films themselves, modify the effect of ATP on films of both proteins. In concentrations of 0.01 mM, MgCl₂ converts the plasticizing action of 0.1 mM-ATP into a stiffening action (slight with L-myosin). When, however, the MgCl₂ concentration is increased to mM, the plasticizing action of ATP is restored (Table 3). It cannot at present be said how far these results are determined by the presence of Mg and Ca in the protein preparations.

Specificity of the effect of adenosine triphosphate. It has been established that neither ADP nor ITP has a significant effect upon the elasticity of films of L-myosin or actomyosin in a concentration of 0.1 mM (Table 4). The effect of ATP is detectable at μ M.

Table 3. *Influence of calcium and magnesium ions on the plasticizing action of adenosine triphosphate on films of L-myosin and actomyosin*

Conditions were as in Table 2.

Protein	Additions to substrate	Stress (interrupted at 30 sec.) (mg.)	Displacement of float (mm.)	
			30 sec.	40 sec.
L-Myosin	None	1.4	1.60	—
	CaCl ₂ (mM)		1.71	—
	ATP		2.77	—
	ATP + CaCl ₂		2.71	—
L-Myosin	None	1.4	1.75	0.85
	MgCl ₂ (0.01 mM)		1.75	0.85
	MgCl ₂ (mM)		1.75	0.85
	ATP		2.90	1.92
	ATP + MgCl ₂ (0.01 mM)		1.48	0.75
	ATP + MgCl ₂ (mM)		2.28	1.20
Actomyosin	None	1.4	1.34	—
	CaCl ₂ (mM)		1.31	—
	ATP		2.77	—
	ATP + CaCl ₂ (mM)		2.71	—
	ATP + CaCl ₂ (0.01 mM)		2.68	—
Actomyosin	None	1.4	1.09	0.60
	ATP		1.35	0.69
	ATP + MgCl ₂ (0.01 mM)		0.71	0.23
	ATP + MgCl ₂ (mM)		1.23	0.67

Table 4. *Comparison of the effects of different nucleotides*

Conditions were as in Table 2. Nucleotides were added in concentrations of 0.1 mM.

Protein	Additions to substrate	Stress (interrupted at 30 sec.) (mg.)	Displacement of float (mm.)	
			30 sec.	40 sec.
L-Myosin	None	0.4	0.87	0.36
	ADP		0.71	0.40
	ITP		0.71	0.43
	ATP		1.27	0.72
Actomyosin	None	0.4	0.69	0.37
	ITP		0.64	0.30
	ATP		1.03	0.50

Actin films. G-Actin spreads readily at water surfaces (Lajtha & Rideal, 1952). Although we have not yet studied the properties of its films in any detail, preliminary experiments have shown that their elastic properties are unaffected by ATP in the presence or absence of magnesium.

DISCUSSION

Structure of the protein films

Actomyosin and L-myosin spread on the surfaces of salt solutions at a low rate. The experiments described in this paper were made under conditions where the area was of the order of $0.5 \text{ m.}^2/\text{mg.}$ at a surface pressure of 1 dyne/cm. , or little more than half that for a protein film in which the unfolding of the polypeptide structure in the surface has proceeded to completion (cf. Bull, 1947). In protein films where the molecules are not fully unfolded, a proportion of these retain their native properties, as has been demonstrated for actomyosin by Hayashi (1952). Since native molecules of corpuscular proteins are likely to show only small surface activity, it appears to be a reasonable assumption that an incompletely spread film consists of an upper layer of unfolded molecules with an adsorbed layer of native molecules beneath it. Evidence in support of this view is presented by Cheesman & Schuller (1954). It may be conceived that the adsorbed, native component of the film, through the polar groups on the surface of its molecules, will establish cross-linkages between the unfolded polypeptide chains of the upper, denatured layer and thus contribute to the mechanical rigidity of the film as a whole (Cheesman & Sten-Knudsen, 1959). Conditions for this effect would be especially favourable with molecules possessing high axial ratios and high contents of ionizable side chains. L-Myosin and actomyosin have these characteristics in a marked degree (Bailey, 1954). The linkages between the actin and myosin components of actomyosin in the native layer might themselves contribute to the mechanical strength of the film.

Effects of adenosine triphosphate

It seems probable, in view of the above considerations, that the plasticizing action of ATP on films of actomyosin is due in part to the acceleration of the spreading of this protein by ATP. Thus under the conditions of our experiments, the area of actomyosin films was nearly 20% greater at 3 dynes/cm. surface pressure in the presence than in the absence of ATP (in a typical case, 0.55 as against $0.47 \text{ m.}^2/\text{mg.}$), with a correspondingly lower surface concentration. Although the importance of this factor is difficult to investigate directly, it may be pointed out that substances

such as DNP which, in small concentrations, greatly modify the plasticizing action of ATP on actomyosin films, are without significant effect on the rate of spreading of this protein in the presence or absence of ATP. We have not found the rate of spreading of L-myosin to be measurably altered by ATP under the conditions of our experiments.

Since the effect of ATP on the mechanical rigidity of the films is transient at low surface pressures, there can be little doubt that it reflects an interaction between the nucleotide and native-protein molecules forming part of the film structure. This effect might be due either to the presence of a protein-nucleotide complex or to that of an altered protein resulting from the decomposition of such a complex. In either event ATP, or groups introduced by ATP, could modify the viscoelastic properties of the film by competition for polar groups in the protein and alteration of the charge distribution.

The most striking difference between actomyosin and L-myosin films is that, whereas both are plasticized by ATP, this effect on actomyosin is converted into a stiffening by a variety of agents such as DNP, EDTA and preliminary ageing of the protein; the same agents tend merely to inhibit the effect of ATP on L-myosin films. Another agent, fuadin, enhances the ATP effect on actomyosin and eliminates that on L-myosin. The simplest explanation of these phenomena is that actomyosin interacts with ATP in two manners which are antagonistic in their effects on the viscoelastic properties of the film, one of which is inhibited by DNP, the other by fuadin. With L-myosin, on the other hand, there is no evidence of such antagonism: it suffices to assume a single mode of interaction with the nucleotide, the effect of which is reduced by all the agents enumerated above.

It is known from the work of Weber (1955) and Ulbrecht & Ulbrecht (1957) that actomyosin, glycerol-extracted myofibrils and such myofibrils from which most of the L-myosin has been removed catalyse an exchange of phosphate groups between ATP and adenosine [^{32}P]diphosphate activated by magnesium but not by calcium. This reaction was first detected in a medium containing fuadin, a weak inhibitor of the ATPase. It probably involves an intermediate phosphorylation of the protein which, if it occurred in actomyosin films, might well exert a plasticizing action. This would be expected to be unimpaired by fuadin and, indeed, to be enhanced if the latter substance inhibited an interaction between protein and ATP which tended to stiffen the films. Although the effect of DNP on the phosphate-exchange reaction catalysed by actomyosin has apparently not been studied, an

analogous reaction in mitochondria is inhibited by this compound (Cooper & Lehninger, 1957). It is hence conceivable that DNP inhibits the exchange reaction in actomyosin films, so that ATP has a resultant stiffening effect.

The stiffening action of ATP might be due to a charge effect or to a cross-linking by the nucleotide between components of the actomyosin complex. It becomes evident when the ATPase activity of the protein is decreased by heating. If the mechanism of the exchange reaction were simultaneously inactivated, this would in itself explain the progressive decrease in film plasticity. The outcome of the heating experiments would suggest a binding of ATP at sites not associated with enzymic activity, although it cannot be excluded that active centres will continue to bind nucleotide after their catalytic function has been lost, with a consequent increase in the stability of the protein-ATP complex. The high ionic strength used in our experiments presumably brought about a considerable dissociation of actomyosin; the effects of ATP, DNP and EDTA are hence probably due in part to the mechanisms suggested below for films of L-myosin.

The phosphate-exchange reaction is not catalysed by L-myosin and hence cannot be responsible for the plasticizing effect of ATP on this protein. An irreversible phosphorylation of the protein by ATP, such as was suggested in a preliminary communication (Cheesman & Keeler, 1958), might nevertheless be imagined. Brahms & Rzyzsko (1958) claim to have detected by chemical means a phosphorylation of H-meromyosin by ATP. A more probable explanation of our results, suggested to one of us by Dr J. Gergely, is that the increase in film plasticity is due to the presence of the enzyme-substrate complex and that the effects of DNP and EDTA consist of a reduction in the steady-state concentration of that complex. Fuadin, as an ATPase inhibitor, might be expected to have the same effect as PCMB in blocking the formation of the complex, thus inhibiting the ATP effect. This is indeed observed.

Further evidence for this view may be found in the failure of ITP to plasticize films of L-myosin in a concentration of 0.1 mM. It is difficult to suggest a plausible mechanism whereby the splitting of ATP by L-myosin, but not that of ITP, would result in phosphorylation of the protein. Kielley, Kalckar & Bradley (1956) have shown, however, that the Michaelis-Menten constant (K_m) for the ATPase reaction of calcium-activated L-myosin is much lower than that for the inosine triphosphatase (ITPase) reaction ($< 3 \times 10^{-5}$ as against 1.9×10^{-4}) whereas the ITPase activity is some 4.5 times greater in the presence of mM-substrate. Furthermore, the ATPase reaction for L-myosin activated

with mM-EDTA and 0.4 M-KCl gave K_m 2.9×10^{-4} and an activity about three times that for the calcium-activated enzyme. The steady-state concentration of enzyme-substrate complex in our experiments would hence be expected on the basis of simple Michaelis-Menten theory to be very much lower for ITP than for ATP at nucleotide concentrations of 0.1 mM. Similarly, EDTA might reasonably be supposed to reduce the concentration in the films of the L-myosin-ATP complex.

A puzzling feature of our results is the failure of magnesium and calcium ions in mM-concentration to affect significantly the plasticizing action of ATP, whereas the former have an inhibitory action at 0.01 mM. If the phosphate-exchange reaction contributes to the effect of ATP on actomyosin, it might be expected that calcium would remove this contribution. The behaviour of actomyosin and L-myosin towards bivalent cations in a heterogeneous system at high ionic strength is, however, unknown and cannot readily be predicted.

SUMMARY

1. Films of L-myosin and actomyosin at the air-water interface, when incompletely spread, incorporate native-protein molecules which probably contribute to their mechanical properties.

2. When such films are spread on potassium chloride solutions containing adenosine triphosphate (0.1 mM), the latter causes a marked decrease in their mechanical strength. This effect is not given by adenosine diphosphate or inosine triphosphate in the same concentration and is inhibited by *p*-chloromercuribenzoate.

3. With L-myosin, the effect is reduced or eliminated by 2:4-dinitrophenol, ethylenediamine-tetra-acetate and fuadin.

4. With actomyosin the effect is enhanced by fuadin, and it is converted into a stiffening action by 2:4-dinitrophenol and ethylenediaminetetra-acetate.

5. Calcium ions do not modify the effect of adenosine triphosphate on either protein. Magnesium ions (0.01 mM) produce stiffening of both types of films in the presence of adenosine triphosphate, but the plasticizing effect reappears with higher concentrations of these ions (mM).

6. Aged or heated actomyosin solutions yield films which are stiffened by adenosine triphosphate.

7. It is concluded that adenosine triphosphate interacts with actomyosin films in at least two manners, one of which may involve phosphorylation of the protein. The effect of adenosine triphosphate on L-myosin films may be explained by the formation of an enzyme-substrate complex.

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The Copper Content and Cytochrome Oxidase Activity of Tissues from Normal and Swayback Lambs

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Swayback is a non-febrile, ataxic disorder of newborn and young lambs. The pathological changes may include symmetrical cerebral demyelination, degeneration of tracts in the spinal cord and chromatolytic changes in the neurones of the brain-stem nuclei and ventral horn (Innes & Shearer, 1940). Both swayback and the related condition enzootic ataxia, which occurs in Australian lambs, are associated with a deficiency of copper. In Australia this deficiency is due to the low level of copper in the pasture (Bennetts & Beck, 1942). The 'swayback' pastures of this country contain adequate levels of copper (Allcroft, 1952), and it is thought that swayback is caused by a failure in the intermediary metabolism of copper in the ewe, or by the copper in the pasture being in an unavailable form (Hunter, Eden & Green, 1945).

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Recently Gallagher, Judah & Rees (1956*a, b*) have examined the effects of copper deficiency in young rats and hens. It was found that although the copper content of liver, kidney and brain was reduced no demyelination was produced. The two major metabolic disturbances which resulted from this deficiency were loss of cytochrome oxidase activity and the suppression of phospholipid synthesis. This suggested that cytochrome oxidase activity may be reduced in swayback. The present investigation was undertaken to determine the copper content and cytochrome oxidase activity of tissues from normal and swayback animals with particular reference to the brain.

MATERIALS AND METHODS

Tissues were obtained as soon as possible after the animal had been killed and they were sent to us immediately by rail, either in a fresh state or packed in ice. Material was