

STUDIES ON THE ANOMALOUS VISCOSITY AND FLOW-
BIREFRINGENCE OF PROTEIN SOLUTIONS

III. CHANGES IN THESE PROPERTIES OF MYOSIN SOLUTIONS IN RELATION TO
ADENOSINETRIPHOSPHATE AND MUSCULAR CONTRACTION

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INTRODUCTION

This paper contains the results of an intensive study of the particle shape of the globulin of muscle, myosin, by the methods of anomalous viscosity and flow-birefringence.¹ Our knowledge of the nature of muscular motion has hitherto been divided into two main fields of successful analysis. First, there was the discovery of the phosphorylation cycles whereby energy is transferred from carbohydrate breakdown to the muscle fibre; here the earliest landmarks were the classical investigations of Fletcher and Hopkins (1907) and of Harden and Young (1910)—the papers of Parnas (1937) and of D. M. Needham (1938) may be consulted for up-to-date reviews. Secondly, there was the discovery of the elongated or anisometric character of the particles of myosin, suggested by Brücke (1858) and demonstrated in the classical paper of von Muralt and Edsall (1930). This threw much light on the long known birefringence of intact muscle and its puzzling histology, and it was followed by the application of x-ray techniques to the problem, and the suggestion that muscular contractility is essentially a molecular contractility of protein chains (Astbury, 1933, 1939; Astbury and Bell, 1938; Astbury and Dickinson, 1940; Meyer and Picken, 1937) as Engelmann (1906) had surmised. The exact connection between these two great groups of observations, however, still remained obscure.

In order to bridge this gap, the most promising point of departure seemed to be the important finding of Engelhardt and Ljubimova (1939) that the enzyme adenosinetriphosphatase is either myosin itself or some protein very closely associated with it. This was confirmed by one of us in the following year (see

¹The present work was begun in 1940 by Joseph Needham, Shih-Chang Shen (Fellow of The Rockefeller Foundation), and Dorothy M. Needham, with A. S. C. Lawrence as rheological adviser. Arnöst Kleinzeller and Margaret Miall joined the group with Rockefeller Foundation grants in 1941, and Mary Dainty (Research Scholar of the Education Authority of the West Riding of Yorkshire) in 1942. Two preliminary reports have already appeared (Needham, Shen, Needham, and Lawrence, 1941; Needham, Kleinzeller, Miall, Dainty, Needham, and Lawrence, 1942).

D. M. Needham, 1942), and shortly afterwards by Szent-Györgyi and Banga, 1941; Edsall and Singher, 1941; Bailey, 1942). Its great importance lies in the following facts.

(1) Among the processes of intermediary metabolism in the muscle, that nearest in time to the contraction of the fibrils is the breakdown of adenosinetriphosphate² to adenosinediphosphate and inorganic phosphate.

(2) This is the only known reaction capable of supplying the bulk of the free energy for contraction. Although some ATP is broken down by transfer of phosphate to hexosemonophosphate (Neuberg ester) (by an enzyme not present in the myosin fraction of the proteins) this also probably liberating free energy, the quantitatively largest part of its breakdown occurs by splitting off of free phosphate under the influence of the enzyme adenosinetriphosphatase (D. M. Needham, 1942). It was considered of interest, therefore, to give careful study to the effect of ATP on the flow-birefringence and anomalous viscosity of myosin. This opinion was strengthened by the fact that after the work had begun, and as it proceeded, other investigators found it impossible, in spite of serious efforts, to separate the adenosinetriphosphatase activity from the protein myosin (Bailey, 1942, confirming the original work of Engelhardt and Ljubimova, 1939).

Since the data in the literature on the physicochemical properties of myosin, though numerous, are rather disjointed, we had to devote a good deal of time to the systematic examination of certain effects, as background for the interpretation of the action of ATP on myosin.

EXPERIMENTAL METHODS

Preparation of Myosin.—Myosin was prepared from rabbit muscle as follows (*cf.* Bailey, 1942). Fresh, ice-cooled muscle was finely minced and the brei extracted at 0° for 1 hour with 5 volumes 0.5 M LiCl containing 0.03 M NaHCO₃. Solid NaHCO₃ was added at intervals during the extraction in order to keep the pH alkaline to bromthymol blue and the mixture was mechanically stirred. After the extraction was finished the mixture was quickly centrifuged and the extractant cleared from suspended particles by filtration through filter paper pulp. The clear liquid was poured into 20 volumes ice cold distilled water; the myosin was precipitated by adjusting the pH of the whole to 6.8–7.0 (measured electrometrically), and leaving it to stand in the ice chest overnight. The precipitated myosin was then collected by decantation and centrifuging and dissolved either by addition of solid electrolyte to bring the final concentration to 0.5 M, or by adding an equal volume of 1 M solution of the electrolyte. Unless otherwise stated once precipitated myosin was used in the experiments. For some experiments the myosin was further purified, precipitating it twice by pouring the clear, filtered solution in 0.5 M NaCl into 20 volumes ice cold distilled water (the

² Hereinafter abbreviated as ATP; adenosinediphosphate as ADP; adenosinemonophosphate (adenylic acid) as AA.

pH of which was carefully adjusted to 6.8–7.0) and leaving the myosin standing for a further 24 hours in the ice chest to complete the precipitation. The protein content of the final solution of myosin in 0.5 M electrolyte was determined by estimating the N content (Kjeldahl) and multiplying the value obtained by 6.03 (Bailey, 1939). Myosin prepared in this way is translucent, with a honey-like appearance, readily splits off phosphate from added ATP, and is highly flow-birefringent. If precipitated at a lower pH (6.4–6.6), the myosin is whitish and pasty; at more alkaline pH (7.6 and higher) the myosin falls out in the form of a transparent gel, which in our experience was enzymically inactive although Bailey (private communication) has found such gels to be enzymically active over several weeks. It was found that the flow-birefringence of myosin prepared in the above way does not depend on the nutrition of the animal. Previous to the standardisation of the above method, myosin was prepared without the continuous checking of the pH; such preparations were occasionally non-birefringent (*cf.* Needham, Shen, Needham, and Lawrence, 1941) and the ratio Δ /protein³ varied widely, but with the method described we never failed to obtain flow-birefringence. Some of the experiments quoted below were carried out with myosin preparations which had been precipitated slightly on the acid side.

Preparation of Adenosinetriphosphate.—Adenosinetriphosphate was prepared from rabbit or horse muscle by a modification of the method of Lohmann (1931) as described by D. M. Needham (1942). In some cases the ATP was further purified as described by Kerr and Seraidarian (1941).

Preparation of Adenosinediphosphate.—ADP was prepared by the action of purified myosin on ATP as described by Ljubimova and Pevsner (1941) and Bailey (1942).

Preparation of Inosinetriphosphate.—Inosinetriphosphate was prepared from ATP by a modification of Lohmann's method (1932) as described by one of us (Kleinzeller, 1942).

Preparation of Inosinic Acid.—Inosinic acid was prepared from horse muscle as described by Ostern (1932).

Preparation of Sodium Triphosphate.— $\text{Na}_5\text{P}_3\text{O}_{10} \cdot 6\text{H}_2\text{O}$ was prepared as described by Huber (1937). Analysis: total P, found 19.95 per cent, calculated 19.55 per cent; 37.2 mg. titrated with 0.1 N HCl (methyl orange): found 1.557 ml., calculated 1.56 ml.; traces of inorganic P in 23.8 mg. Na triphosphate; no precipitate with 0.01 M CoCl_2 (see Neuberg and Fischer, 1937).

Estimation of P.—Inorganic P determinations were carried out according to Fiske and SubbaRow (1925).

FLOW-BIREFRINGENCE AND ANOMALOUS VISCOSITY

The apparatus used was exactly as described in a previous paper of this series (Lawrence, Needham, and Shen, 1944), consisting of a small annular cell with rotating external cylinder, mounted on the stage of a polarising microscope; and a coaxial viscosimeter with glass bottom permitting optical measurements of flow-birefringence. Four variables are observed: (a) the intensity

³ See p. 360.

of flow-birefringence itself; (*b*) the angle of isocline; (*c*) the extent of the anomalous viscosity, that is, the variation of apparent viscosity with rate of shear; (*d*) the relative viscosity, η/η_0 , where η is the lower limiting viscosity independent of rate of shear at higher rates of shear. The flow-birefringence and the anomalous viscosity of myosin solutions, are, of course, taken to indicate that the particles are of considerable asymmetry; *i.e.*, very much longer than they are broad.

The intensity of flow-birefringence in a protein sol subjected to shear stress depends upon the number of anisometric particles present (protein concentration), the degree of optical anisotropy of the particles themselves, the perfection of their orientation (a resultant of the opposing forces of shearing stress and thermal disorientation), the depth of solution through which the light beam passes, and the other conditions governing particle shape and size such as pH and salt concentration. We express it here in terms of Δ° ; the angle through which the analysing Nicol must be rotated to extinguish the plane-polarised light emerging from a quarter-wave plate. The double refraction itself can be obtained at once from this figure by means of the following relations: $\frac{\Delta^\circ}{180^\circ} = \Delta\rho$ the phase difference in wave-lengths between the two components of the elliptically polarised light; and $\frac{\Delta\rho\lambda}{S} = n_e - n_o$ the double refraction, where λ is the wave-length of the light source, and S the depth of solution under examination.

The angle of isocline ψ , is by definition the larger of the two angles which the cross of isocline makes with the crossed planes of polarisation of the polarising and analysing Nicols. It is here interpreted as a measure of the degree of perfection of the orientation of the anisometric particles in the stream lines under shear stress. It varies between 45° for nil orientation, and 90° for perfect orientation.

The relative viscosity η/η_0 is the ratio of the viscosity of the sol to that of distilled water or equivalent electrolyte solution, at the same temperature. Anomalous viscosity is the departure of viscosity from independence of the shear rate. Unfortunately no satisfactory means has yet been devised for its quantitative expression.

GENERAL INTERPRETATIONS

In order to avoid constant repetition in discussing the interpretation of the effects described in what follows, it is desirable to summarise the various logical possibilities at this point in the form of a table (Table I). The table assumes that flow birefringence, angle of isocline $> 45^\circ$, and anomalous viscosity, are present together, as they are in the case of myosin, and in most proteins which show (in bulk phase) any one of these properties. Anomalous viscosity has,

TABLE I
Possible Variations in Flow-Birefringence and Viscosity Properties of Protein Sols

Δ^0, ψ^0 , flow-birefringence; angle of isocline and anomalous viscosity	η/η_0 , relative viscosity	Interpretation
A Rise		Lengthening of axial ratio of rod-like or fibrillar particles by (1) linear aggregation of spherical particles or (2) sliding extension of parallel-aggregated micelles or (3) extension of aperiodic coil, such as denatured polypeptide chain or (4) true relaxation or extension of molecule or micelle analogous to β -, α -, and supercontracted keratin transformations
B Fall		(a) Shortening of axial ratio of rod-like or fibrillar particles, by reversal of any one of the processes (1), (2), (3), (4) above, or some other, or (b) Coalescence of rod-like or fibrillar particles into roughly symmetrical tangles of much larger size. This is probably not a reversible process
C	Rise	Either increase of intermicellar forces or, less probably, hydration, with increasing diameter of water shell
D) Rise	Fall	Converse of (C)
E) Rise	Rise	In practice, case (A) always takes this form, since relative viscosity is, in some way as yet not clear, proportional to axial ratio (e.g. ATP recovery; high temperatures)
F Fall	Fall	Converse of (E). Owing to intermicellar forces working in a contrary direction, however, rise or fall of relative viscosity may not be what would be expected from the flow-birefringence change which it accompanies (e.g. monovalent cations at low molarity; divalent cations at high molarity; PH < 6; ATP; urea at high molarity)
G) Rise	Fall	Not so far observed
H) Fall	Rise	Disaggregation of, or formation of tangles from, rod-like or fibrillar particles, accompanied by increase in intermicellar forces or hydration (e.g. monovalent cations at high molarity; divalent cations at low molarity; pH > 10)
I Constant	Rise or fall	Changes in intermicellar forces, unaccompanied by changes in the axial ratio of the particles; not so far observed
J) Rise	Constant	Not so far observed
K) Fall	Constant	Decrease of axial ratio of the particles, accompanied by compensatory increase in intermicellar forces. (pH 8.5-10; urea at low molarity)

however, been described without flow-birefringence, in the case of a liver nucleoprotein, by Greenstein and Jenrette (1940). Conversely, flow-birefrin-

gence has been described without anomalous viscosity in casein sols at alkaline pH (Nitschmann, 1938; Nitschmann and Guggisberg, 1941). These at present very exceptional cases have been discussed in the first paper of this series.

As will be seen below, most of the possible types of behaviour have been met with in the study of myosin sols. The importance of intermicellar forces is readily appreciated when one remembers the well known fact that during proteolysis, very minute changes in the chemical structure of a protein are accompanied by large changes in the solution's viscosity (Northrop, 1929; Cannan and Muntwyler, 1930). For example, in the peptic digestion of gelatin, the relative viscosity falls to half its original value in $1\frac{1}{2}$ hours but the number of amino groups set free per molecule does not attain its maximum till 356

TABLE II
Relation of Flow-Birefringence to Protein Concentration in Myosin Sols (Δ° /Protein Ratio)

Preparation No.	No. of precipitations	Protein content	Δ at 500 R.P.M.	Δ /protein ratio
		<i>per cent</i>		
1	1	3.02	81°	26.8
	3	2.29	63°	27.5
2	1	2.97	67°	22.6
	3	3.22	92°	28.6
3	1	1.90	49°	26.0
	3	2.02	54°	26.7
4	3	2.23	47°	21.1
	1	1.79	55°	30.7
5	3	0.81	27°	33.2
	1	1.54	31°	20.4
6	3	1.67	39°	23.4
	1	1.49	35°	23.5
7	1	1.49	35°	23.5
8	1	1.21	34-36°	29.0
9	1	2.44	70°	28.7
10	1	1.62	42°	26.0

hours and its half-value till about 40 hours. This subject has been interestingly discussed by Robertson (1928), Pauli and Valkó (1933), and recently by Mark (1941).

PHYSICOCHEMICAL PROPERTIES OF MYOSIN

The Relation of Flow-Birefringence to Protein Content

It was found that the Δ /protein ratio is of the same order in different preparations of rabbit myosin (see Table II) and does not change appreciably by several reprecipitations. The flow-birefringence was measured in the microscope cell at 500 R.P.M.

The flow-birefringence of anisometric particles is a function of the particle length. It can therefore be concluded that in myosin solutions prepared in the

above way the particle length is of uniform order, and the Δ /protein ratio should be considered one of the physicochemical constants of the protein.

The flow-birefringence of myosin solutions increases in a linear manner with the protein concentration (see Fig. 1; Experiment app/347). This confirmed the results of von Muralt and Edsall (1930).

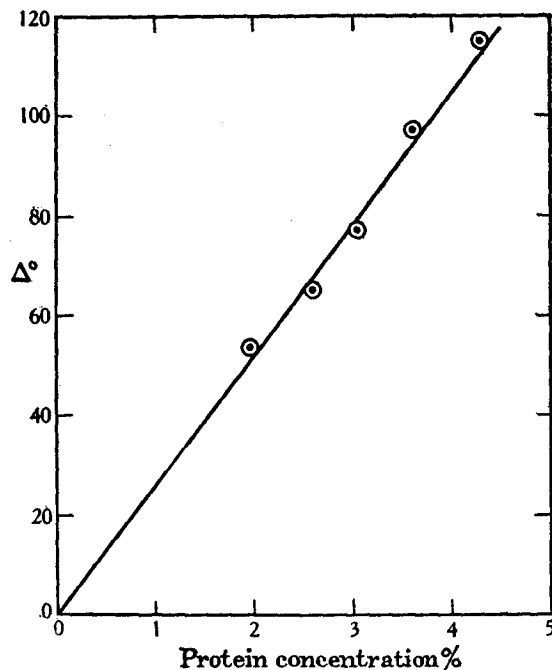


FIG. 1. Relation of flow-birefringence of myosin to protein concentration.

The Relation of Viscosity to Protein Content

As is known, myosin is a protein of extremely high viscosity. From many of our experiments, the relative viscosity can be plotted against the protein concentration (see Fig. 2); it can be seen that it is impracticable to work with myosin solutions of higher concentration than 1 per cent. The viscous anomaly also increases with rising protein concentration (Experiment II 127). Compared with the relative viscosities of other proteins, as in the inset plot in Fig. 2, (taken from the data in the literature, as summarised by Pauli and Valkó, 1933, p. 240), the order of decreasing viscosity is seen to be myosin > sodium caseinate > serum euglobulin > serum pseudoglobulin > serum albumin > ovalbumin; an order which roughly corresponds with the degree of

anisometry of their molecules. That the viscosity does correspond in this way we know from the work of Eirich, Margaretha, and Bunzl (1936) who obtained a graph similar to Fig. 2 using model particles of known lengths.

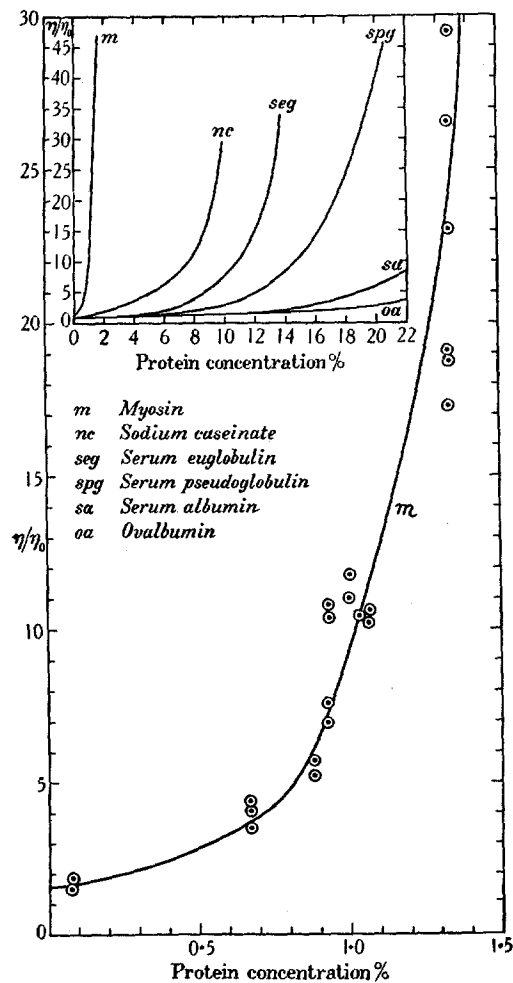


FIG. 2. Relation of relative viscosity of myosin and other proteins to protein concentration.

Effect of Electrolytes on the Flow-Birefringence and Viscosity of Myosin Solutions

The effect of electrolytes on the flow-birefringence of myosin was studied by Edsall and Mehl (1940). In our experiments a more systematic study of the effect of varying concentrations of different electrolytes on the (simultaneously measured) Δ and η/η_0 of myosin solutions was made. The effect of the electrolytes on the Δ was carried out either in the microscope cell, or, together with

measurements of η , in the Couette viscosimeter. The effect of electrolytes was studied either by comparing the Δ and η/η_0 in a number of myosin solutions containing different amounts of electrolyte, but the same protein content; or, where only small amounts of electrolytes were added, by addition of increasing amounts of the salts (in concentrated solutions) to the myosin solution in the viscosimeter, and correcting the values obtained for the dilution of the myosin solutions with the added electrolytes. It should be noted that some of the reported effects of the electrolytes may be due to changes of the pH in the little buffered protein solution.

Effect of Cations

Effect of 0.5 M Li⁺, Na⁺, K⁺, Rb⁺, Cs⁺, NH₄⁺.—The effect of Li⁺ and Na⁺ added to myosin solutions in 0.5 M KCl had been studied by Edsall and Mehl (1940), who found that both electrolytes had decreased the flow-birefringence of

TABLE III
Effect of Cations on the Flow-Birefringence and Relative Viscosity of Myosin (Experiment III 191)
Final concentration of myosin solution 1.86 per cent protein.

0.5 M electrolyte (final concentration)	Δ at 50 R.P.M.	η/η_0 at 2 R.P.M.
LiCl.....	54°	10.5
NaCl.....	53°	8.2
KCl.....	54°	10.5
RbCl.....	55°	10.5
CsCl.....	56°	12.4
NH ₄ Cl.....	42°	—

different myosin preparations. In our experiments the effect of 0.5 M LiCl, NaCl, KCl, RbCl, CsCl, and NH₄Cl on the Δ and η was examined on the same myosin solution. Equal amounts of once precipitated myosin were dissolved in equimolecular quantities of the above salts. The protein content of the solution was 1.86 per cent. The flow-birefringence was measured in the coaxial viscosimeter at 50 R.P.M.; the relative viscosity was calculated (from the values of viscosity obtained at different speeds) for 2 R.P.M. As shown in Table III, the 0.5 M electrolytes have no appreciable effect on the flow-birefringence, and the relative viscosity is affected to a slight degree only. The lowest values of η/η_0 were found for myosin dissolved in 0.5 M NaCl, the highest in 0.5 M CaCl.

Effect of Increasing Concentrations of Li⁺ and K⁺

The effects of increasing concentrations of LiCl and KCl on the Δ and η/η_0 of myosin solutions were studied. To equal volumes of stock myosin solution or myosin gel varying amounts of solid electrolyte were added and the volumes were adjusted by addition of distilled water.

Increasing concentrations of both LiCl and KCl decrease the flow-birefringence of myosin solutions. The highest values of Δ were always observed at about 0.5 M electrolyte; with increasing concentrations the Δ generally falls steadily and reaches 0° at approximately 3.5 to 4.0 M, when the myosin is nearly completely salted out. In a number of experiments a slight increase of flow-birefringence was observed, reaching a maximum somewhere between 0.5 to 1.5 M and then continuing its fall. No explanation can as yet be offered for this observation, which needs further confirmation on more purified myosin. The relative viscosity also decreases as the salt concentration rises, but only to 1.5 to 2.0 M; with higher concentrations a considerable increase of η/η_0 occurs. Here the viscous anomaly, however, is completely lost. The data

TABLE IV
Effect of Increasing Concentrations of LiCl on the Δ and η/η_0 of Myosin Solutions. (Experiment III 181).

Final Concentration of Myosin Solution 1.08 per cent Protein

LiCl concentration M	Δ at 50 R.P.M.	η/η_0 (2.5 R.P.M.)	Viscous anomaly
0.5	67°	9.0	+
0.75	66°	8.2	+
1.0	55°	7.9	+
1.25	20°	7.2	\pm
1.5	8°	6.8	-
2.0	10°	6.2	-
3.0	9°	8.9	-
4.0	0°	10.2	-

in Table IV represent a typical experiment where no transient increase of Δ between the concentrations 1.0 to 1.5 M was observed. Very similar pictures are obtained with potassium chloride. It is interesting that very concentrated salt solutions will decrease the birefringence of the intact muscle fibre also (Nasse, 1882; von Ebner, 1882).

The changes here described for myosin are not, it seems, paralleled very closely by TMD virus under similar conditions. According to Lauffer (1938 *b*) the higher the ionic strength of the electrolyte present, the lower falls the η/η_0 of the virus solution. There is no rise at the higher concentrations of salt, as here.

Effect of NH_4^+

It has long been known that in the presence of ammonia the birefringence of the intact muscle fibre decreases and disappears (Schipilov and Danilevsky, 1881; Biedermann, 1927; Liang, 1936). It has also been shown by Edsall and

Mehl (1940) that 1.4 M NH_4Cl or 1.6 M $(\text{NH}_4)_2\text{SO}_4$ rapidly decreases the flow-birefringence of myosin solutions. In our experiments the effect of increasing concentrations of NH_4Cl on the Δ of myosin solutions was studied in the microscope cell. As shown in Table V, 0.1 M NH_4Cl has no appreciable effect on Δ ; 0.5 M reduces it to 80 to 63 per cent of the control (myosin in 0.5 M LiCl), (see also Table III) whilst no birefringence was observed at 2.0 M NH_4Cl . The effect of NH_4^+ thus differs considerably from that of the other monovalent cations tested.

Effect of Mg^{++}

Mg^{++} is known to decrease the birefringence of the intact muscle fibre (von Ebner, 1882) and the flow-birefringence of myosin solutions at a concentration of 0.35 M (Edsall and Mehl, 1940). In our experiments carried out in the micro-

TABLE V
Effect of Increasing Concentrations of NH_4^+ on the Δ of Myosin Solutions

NH_4^+ concentration	Δ at 500 R.P.M.
0 (control in 0.5 M LiCl)	27°
0.1 M (+ 0.4 M LiCl)	27°
0.5 M	17°
1.0 M	4°
2.0 M	0°

scope cell 0.05 M MgCl_2 decreased the Δ to 57 per cent of the control (myosin in 0.5 M LiCl). In the coaxial viscosimeter the flow-birefringence decreased steadily with rising concentrations of Mg; the solution was nearly non-birefringent at a concentration of 0.225 M. The η/η_0 first increased with rising concentration of Mg^{++} and reached a maximum at 0.05 M; further additions of MgCl_2 decreased the relative viscosity (see Fig. 3). Anomalous flow was retained up to the highest of these concentrations. In another experiment (IV 195) the flow-birefringence reached zero value at 0.5 M, and the maximum relative viscosity was observed at 0.075 M.

Effect of Ca^{++}

The effect of Ca^{++} is somewhat similar to that of Mg^{++} . 0.05 M CaCl_2 reduces the Δ (measured in the microscope cell) to 65 per cent of the control, whilst 0.02 M had no effect. In the coaxial viscosimeter increasing concentrations of Ca^{++} (up to 0.4 M) produce a steady fall of Δ to zero, and a steady rise of relative viscosity (see Fig. 4).

In other experiments the same relationship was always found though the rate of rise of relative viscosity differed according to the myosin preparation used.

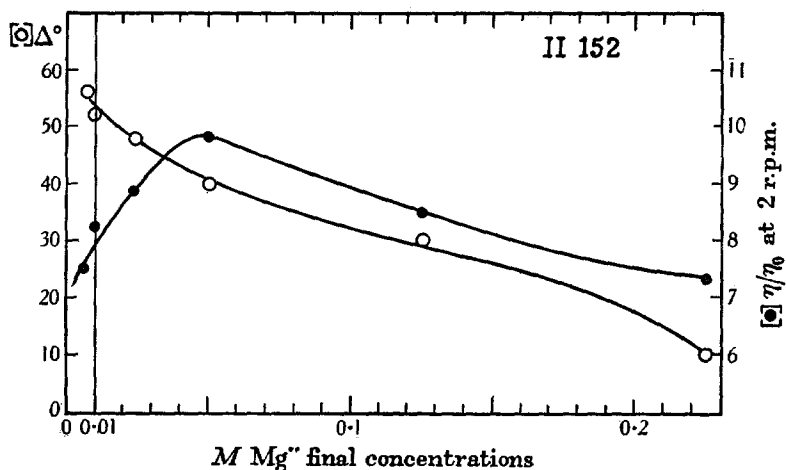


FIG. 3. Effect of Mg^{2+} on the flow-birefringence and relative viscosity of myosin. Final concentration of myosin 0.67 per cent (Experiment II 152).

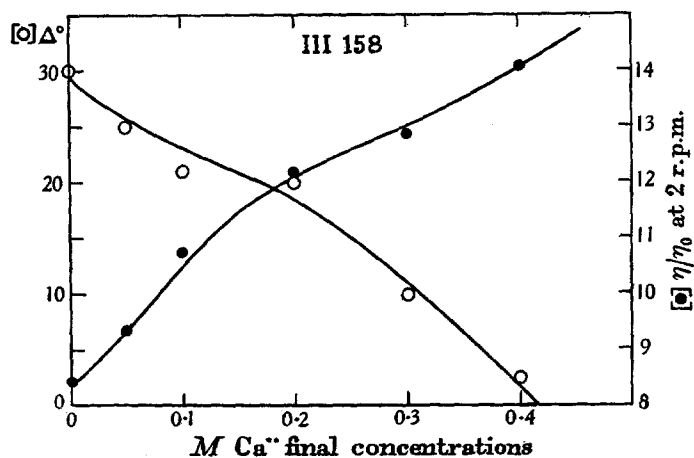


FIG. 4. Effect of Ca^{2+} on the flow-birefringence and relative viscosity of myosin. Final concentration of myosin 1.15 per cent (Experiment III 158).

Effect of Anions

It is known that anions have a considerable effect on the physicochemical properties of colloidal solutions (Hofmeister's series). In our experiments the effects of 0.5 M KCl, KBr, KI, KSCN, and K_2SO_4 on the Δ and η/η_0 were compared. Equal amounts of myosin were dissolved in equimolecular amounts

of the above salts and Δ and η/η_0 were measured in the coaxial viscosimeter. As shown in Table VI, Br⁻ and SO₄²⁻ have no appreciable effect on the Δ of myosin as compared with Cl⁻, whilst I⁻ and SCN⁻ abolish, or in some experiments, considerably reduce the Δ . The relative viscosity also is unaffected by Br⁻ and SO₄²⁻ as compared with Cl⁻. I⁻ was found to reduce the η/η_0 , but the values increase with time and within 30 minutes after the first measurements the values of η/η_0 are nearly as high as, or exceed those of the control. SCN⁻ generally increases the relative viscosity of myosin solutions. In one of our experiments (Experiment III 175) an increase of about 200 per cent was observed (η/η_0 in control 8.5, in KSCN 28.5). In the experiment quoted in Table VI, however, no appreciable effect of SCN⁻ on the viscosity of myosin was observed.

TABLE VI
Effect of 0.5 M Anions on the Flow-Birefringence and Viscosity of Myosin
Experiment III 189. Final concentration of myosin 1.86 per cent protein.

Electrolyte	Δ at 50 R.P.M.	η/η_0 at 2 R.P.M.
KCl.	70°	8.0
KBr.	65°	7.3
KI.	0° first reading	4.6
	after 15 min.	5.4
	" 30 "	5.8
KSCN.	0°	7.7
K ₂ SO ₄	70°	7.7

Effect of pH

Edsall and Mehl (1940) mentioned that variations of pH between 6–8 have no effect on the Δ of myosin, whilst they show that higher pH (9–11) irreversibly reduces the Δ . In our experiments the effect of pH on Δ and η/η_0 was examined on the same myosin solution in the coaxial viscosimeter. Equal amounts of myosin dissolved in 0.5 M KCl were mixed with phosphate buffer of varying pH. The final concentration was 0.1 M phosphate buffer. The pH of the myosin solutions was measured electrometrically.

As shown in Fig. 5 (result of four experiments) variations of pH between 6.0 and 8.5 have no appreciable effect on the Δ of myosin, thus confirming the results of Edsall and Mehl. If the pH is lowered below 6.0 a sudden increase of Δ is observed, simultaneous with an appearance of turbidity of the myosin solution. At pH 5.4–5.5 the myosin solution gels. It is therefore possible that the observed increase of Δ is due to a photoelastic effect of shear stress on myosin precipitating near the isoelectric point rather than to a genuine increase in flow-birefringence. At alkaline ranges Δ falls off steadily as the pH

increases from pH 8.5–11. This had early been noted (von Muralt and Edsall, 1930, p. 376; Edsall and Mehl, 1940), and it has, of course, long been known that the birefringence of the intact muscle fibre suffers a great, though reversible, reduction, in the presence of alkali (Nasse, 1882; von Ebner, 1882).

The relative viscosity is very high at pH lower than 6.0, and falls off parallel to Δ with increasing pH; it does not change between pH 6–8.5. But contrary

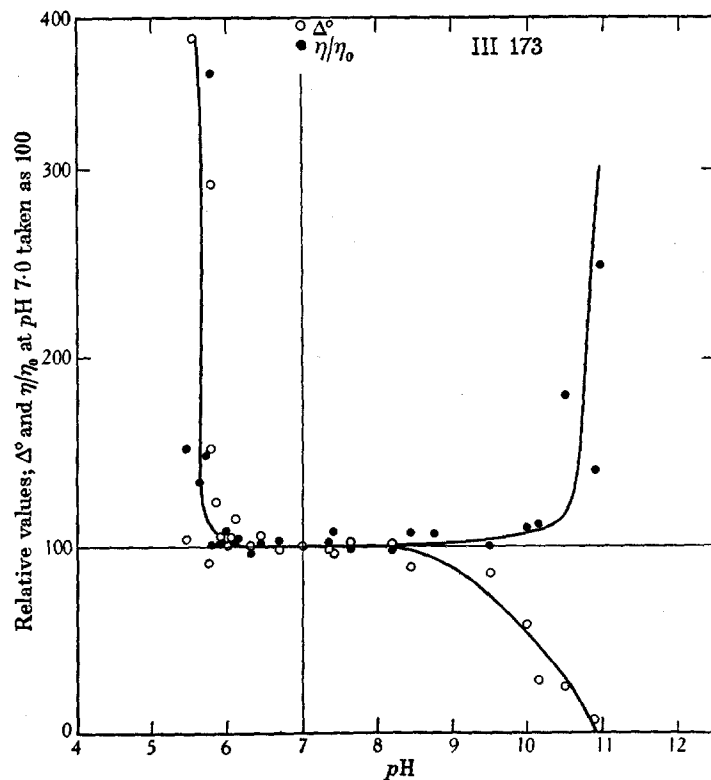


FIG. 5. Effect of pH on the flow-birefringence and relative viscosity of myosin (Experiments III 161–173).

to Δ the η/η_0 increases greatly with a further rise in pH. At the same time, above pH 10.5 the flow anomaly is lost.

It is interesting to contrast the behaviour of myosin at different pH with that of the other outstanding fibrillar corpuscular protein, TMD virus. According to Lauffer (1938 *b*) its Δ falls off with increasing pH and disappears between 9.5 and 10, but η/η_0 , instead of entering upon a marked rise, also falls off. Moreover, as the pH decreases there occurs about 5.5 a marked rise in η/η_0 as in the case of myosin, but this is not accompanied by a marked rise in Δ .

Effect of Urea

Urea is known to decrease the flow-birefringence of tobacco mosaic virus solutions (Bawden and Pirie, 1940) and of myosin (Edsall and Mehl, 1940). On the other hand, urea has been used as an extractant to confer flow-birefrin-

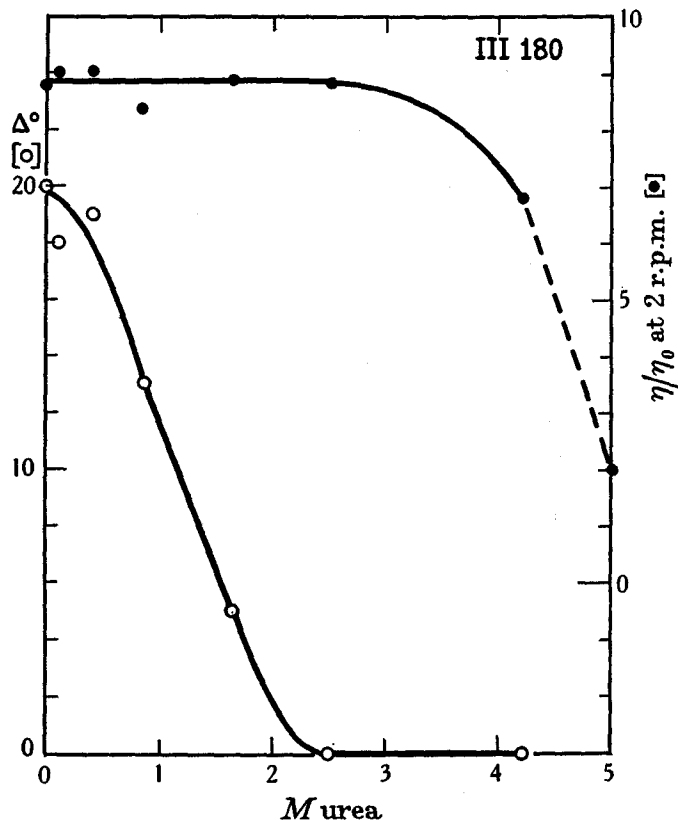


FIG. 6. Effect of increasing concentrations of urea on the flow-birefringence and relative viscosity of myosin. Final concentration of myosin 1.0 per cent (Experiment III 180).

gence on proteins from the liver and kidney (Banga and Szent-Györgyi, 1940; see Lawrence, Miall, Needham, and Shen, 1944). In our experiments the effect of increasing concentrations of urea on the Δ and η/η_0 of myosin solutions was studied. Varying amounts of urea were added to equal volumes of myosin dissolved in 0.5 M LiCl and Δ and η/η_0 were measured in the coaxial viscosimeter. As shown in Fig. 6, the flow-birefringence of myosin falls off when the concentration of urea is higher than 0.5 M, and reaches 0° at approximately 2.5 M.

The relative viscosity, however, is still unaffected at this concentration, and only higher concentrations (4.2 M) reduce it appreciably. The decrease of relative viscosity by high concentrations of urea had been already reported by Edsall and Mehl.

REVERSIBILITY OF THE REDUCTION OF FLOW-BIREFRINGENCE AND ANOMALOUS VISCOSITY

In view of the facts to be described below, the conception generally prevailing (*cf.* Edsall, 1942) that decreases in the flow-birefringence of myosin are irreversible and necessarily imply any far reaching denaturation, must now be abandoned. After the action of adenosinetriphosphate, as we shall see, Δ invariably returns to its initial value, and a variety of other effects which we encountered convinced us that this phenomenon does not stand alone. At the same time, we do not wish to suggest that the mechanism of the reversibility is in all cases the same.

Ageing and Temperature Effects.—If a flow-birefringent solution of myosin is allowed to stand at 0°C. for 10 days or more, under sterile conditions, it will often be found to have lost its flow-birefringence and its anomalous viscosity, while in all other characteristics it seems unaltered. We have found that if such a non-birefringent solution is placed for a short time at 37°C. it will rapidly become flow-birefringent again. If allowed to remain too long at 37°C. it will set to a thixotropic gel, as will any specimen of myosin if exposed to this temperature for more than about an hour. Fig. 7 (Experiment II 113) shows the restoration of flow-birefringence and anomalous viscosity to such an 8 day old solution in 0.5 M KCl; the flow-birefringence has risen after 2 hours at 37°C. from 0° to 55° Δ and bulk anomaly has returned. That this birefringence cannot be due to the photoelastic effect of a gel under strain is shown partly by the fact that the relative viscosity has risen very little, but more especially by the fact that under such conditions the angle of isocline is found to be unchanged. In another experiment of the same sort (II 118) the angle of isocline (ψ) was 52° before the heat treatment, and 54° after 2 hours at 37°C., indicating that the particles were still perfectly free to orient. At the same time Δ had risen from 20° to 60°. This evidence was all the more convincing because in this second experiment the relative viscosity rose rather more than in the first (η/η_0 before 2.02, after 3.15, at 7 R.P.M.).

Engelhardt and Ljubimova (1939), who studied the inactivation of adenosinetriphosphatase by heat, noticed that in the presence of its substrate, the enzyme was easily inactivated by temperatures such as 37°C. We accordingly made some observations on the effect of adenosinetriphosphate on the change in the physical properties of myosin at this temperature. From these figures several points emerge. In absence of ATP exposure to physiological temperature greatly increases the flow-birefringence, but without sensibly affecting the

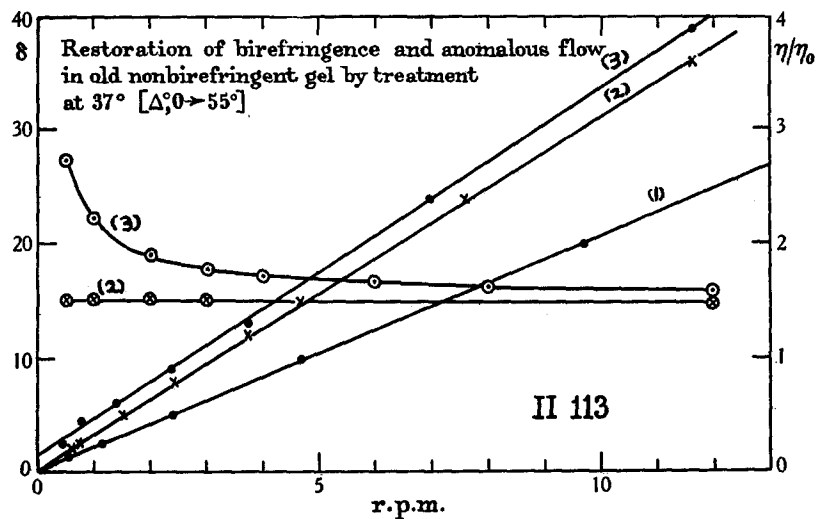


FIG. 7. Restoration of flow-birefringence and anomalous viscosity to an aged myosin sol by treatment at 37°C. (1) Curve for water; (2) curve for myosin before heating; (3) curve for myosin after heating 2 hours at 37°.

TABLE VII
Protective Effect of Adenosinetriphosphate on Myosin at 37°C.

		Δ	ψ	η/η_0 at 3 R.P.M.	Flow anomaly
Experiment II 120	Myosin in 0.5 M KCl before	40	45	3.8	Marked
	Myosin in 0.5 M KCl after 2½ hrs. at 37°C.	45	48	—	—
	Myosin in 0.5 M KCl after 5 hrs. at 37°C.	102	54	>4.6	Extremely marked; viscosity measure- ments difficult
	Myosin in 0.5 M KCl + 0.1 M ATP	40	42	3.8	Marked
	Myosin in 0.5 M KCl + 0.1 M ATP after 2½ hrs. at 37°C.	35	47	—	—
	Myosin in 0.5 M KCl + 0.1 M ATP after 5 hrs. at 37°C.	62	52	2.1	Marked
Experiment II	Myosin in 0.75 M KCl before	55	47	1.41	Marked
	Myosin in 0.75 M KCl after 1 hr. at 37°C.	80	50	1.64	Very marked
	Myosin in 0.75 M KCl + 0.1 M ATP	55	41	1.41	Marked
	Myosin in 0.75 M KCl + 0.1 M ATP after 1 hr. at 37°C.	50	44	1.45	Marked

angle of isocline; hence here again the phenomenon cannot be one of photo-elastic gel strain. At the same time, the relative viscosity rises. In the presence of the substrate, however, these effects are inhibited; the flow-birefringence and relative viscosity do not rise to the same extent and the intensification of the flow anomaly is not observed. In all these experiments the enzyme was actively splitting the substrate (in Experiment II 120 0.213 mg. inorganic P appeared after 2½ hours), and it is likely that when the substrate has been fully used up the usual physical changes will begin to take their course.

Treatment at 37° will also restore flow-birefringence after its abolition by various ions. Typical figures are quoted in Table VIII (Experiment ST 314). From this it can be seen that the particles of myosin, having either been dis-aggregated into smaller more spherical fragments or tangled together into

TABLE VIII
Restoration of Flow-Birefringence of Myosin after its Abolition by Ions, at 37°C.

	At the beginning of experiment (before additions) (320 R.P.M.)		After 4 hrs. at room temperature (20°C.) (320 R.P.M.)		After 1 hr. at 37°C. (320 R.P.M.)		Increase Δ°
	Δ°	ψ°	Δ°	ψ°	Δ°	ψ°	
All at 1.1 M; 0.5 M KCl + 0.6 M other ion							
Control KCl	50	82	50	82	80	72	30
LiCl	42	72	0	—	65	62	65
NH ₄ Cl	50	80	0	—	50	58	50
MgCl ₂	50	80	16	—	55	62	39
CaCl ₂	50	80	15	—	62	73	47

larger more spherical clumps, are restored by exposure to 37° to something approaching their original condition. It is interesting that the angle of isocline tends to be less than it was originally while the birefringence tends to be higher. This suggests a small reduction in axial ratio, combined with more regular intramolecular packing and hence higher intrinsic birefringence.

Spontaneous Restoration.—Apart from the spontaneous restorations of flow-birefringence always found after the action of adenosinetriphosphate, as described below, we have sometimes observed a similar effect if myosin is treated with low concentrations of urea or certain ions. Fig. 8 illustrates two such experiments (ST 315). With the preparation of myosin here used 2 M urea was not sufficient to produce a permanent abolition of the flow-birefringence, but it reduced it to two-fifths of its original value, while the angle of isocline correspondingly fell; then in a very short time both began to return, and by half an hour had reached their initial positions. Further additions of urea were

required to abolish permanently the flow-birefringence. The reversibility in this experiment is not so surprising, for we know from numerous observations (e.g. those of Bawden and Pirie, 1940, on virus nucleoprotein, and many other workers on other proteins) that the initial stages of urea denaturation are generally reversible; what is remarkable is its spontaneity. Such effects may possibly be relevant to the reversible action of adenosinetriphosphate, for which they may perhaps be regarded as models. Fig. 8 also shows a similar curve for myosin in potassium chloride solution acted upon by lithium chloride.

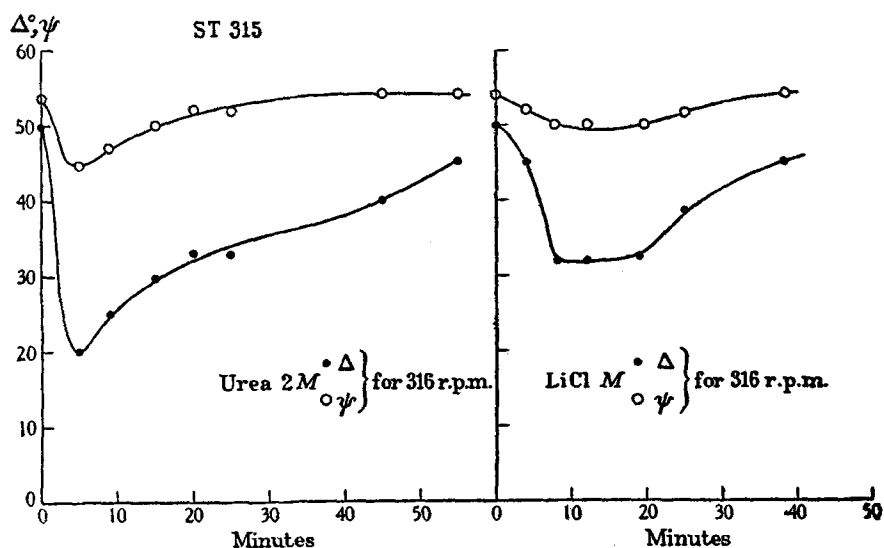


FIG. 8. Spontaneous restoration of flow-birefringence after treatment of a K^+ myosin sol with Li^+ and with urea.

These observations were extended by measurements in the coaxial viscosimeter (Experiment II 128). A myosin preparation showing $\Delta 65^\circ \psi 54$ (for 260 R.P.M.) was treated with 2 M urea; in 15 minutes its Δ had declined to 37° and ψ to 47° ; but by 80 minutes at $18^\circ C$. both values were back at their original positions. The myosin was then diluted four times with 2 M urea and examined in the coaxial viscosimeter. Its anomalous viscosity was considerably more pronounced than at the beginning of the experiment, and its relative viscosity had risen from 4.4 to 6.8 (at 3 R.P.M.). The preparation was then removed and boiled for 2 minutes, then re-examined in the coaxial viscosimeter. The treated protein, still fully in solution, now showed an anomalous viscosity and relative viscosity (4.4) exactly the same as that of the protein before urea had been added.

We do not wish at present to offer any explanation of the above observations, but it is sure that further work along these lines would be very profitable.

Reversibility by Removal of the Reduction Agent.—When the flow-birefringence of myosin solutions is reduced by addition of different electrolytes, a spontaneous return of Δ is not usually observed. If, however, the myosin solutions, the Δ of which has been reduced by addition of Mg^{++} or different concentrations of urea, are reprecipitated in water, the flow-birefringence returns. Experiments were set up as follows (Experiment app/349): to equal amounts of myosin dissolved in 0.5 M LiCl different salts and urea were added, and the Δ measured in the microscope cell. The myosin solutions were then precipitated by pouring them into ten volumes of ice cold distilled water; the precipitated myosin was collected by centrifuging, dissolved by addition of LiCl to the final concentration of 0.5 M, and the flow-birefringence measured again. As shown

TABLE IX
Restoration of Flow-Birefringence of Myosin by Removal of the Reduction Agents

Reduction agent	Δ in per cent of control	Δ in per cent of control after re-precipitation and re-solution
0.5 M LiCl (control).....	100	100
0.033 M adenosinetriphosphate.....	50	74
0.1 M $MgCl_2$	39	70
0.1 M urea.....	78	100
1.0 M urea.....	42	74
2.0 M urea.....	22	65

in Table IX, the flow-birefringence returned to a considerable extent, in some cases to the same extent as the controls. After 6.0 M urea, however, neither solubility nor flow-birefringence of the myosin were regained.

THE ADENOSINETRIPHOSPHATE EFFECT

If a myosin solution containing from 1 to 3 per cent of the protein is treated with adenosinetriphosphate at concentrations as low as 0.004 M, the flow-birefringence (Δ) is reduced at once by some 40 to 60 per cent (mean fall from Table X 48 per cent), the flow anomaly is unaffected, and the relative viscosity (η/η_0) is reduced by some 14 per cent. The subsequent return of both flow-birefringence and relative viscosity to their original values, which may take from 15 minutes to several hours, according to the conditions, is accompanied by the splitting off of inorganic phosphate from the ATP. The fall of flow-birefringence and relative viscosity is then exactly repeatable upon the same sample of myosin. The various features of this striking phenomenon will be taken up in turn in what follows.

TABLE X

Action of Adenosinetriphosphate and Inosinetriphosphate on the Flow-Birefringence of Myosin

Experiment No.	Conditions				Δ°_i	Δ°_{atp}	Fall of Δ°	Fall per cent of initial Δ	Time required for return of Δ
	Temperature	Reduction agent	Solvent	Method for determination of Δ°					
Once precipitated myosin									
	$^\circ\text{C.}$								<i>hrs. min.</i>
app/327	20	NaATP	KCl	mc350	43	15	28	65.1	19 30
app/328	20	NaATP	KCl	mc380	67	45	22	32.9	— —
app/329	20	KATP	KCl	mc380	68	42	26	38.4	— —
app/330	20	KATP	KCl	mc380	75	57	18	24.1	— —
app/332	37	NaATP	KCl	mc380	55	30	25	54.5	3 0
app/333a	37	KATP	KCl	mc380	48	27	21	43.8	3 10
app/333b	30	KATP	KCl	mc380	45	22	23	51.2	4 30
app/338	20	KATP	KCl	mc380	73	52	21	28.8	— —
app/339	37	KATP	KCl	mc380	37	23	14	38.0	2 0
app/341	20	KATP	KCl	mc380	28	15	13	46.5	— —
app/344 (dos)	20	KATP	KCl	mc560	75	20	55	73.5	>24 0
app/345 (P)	20	KATP	LiCl	mc570	50	25	25	50.0	20 0
app/350 (s 1)	20	NaATP	NaCl	mc514	65	25	40	61.5	— —
app/351 (s 2)	20	{ NaATP	NaCl	mc514	67	35	32	47.8	1 30
		{ NaITP	NaCl	mc514	67	44	23	34.3	0 15
app/352 (s 3)	20	NaATP	NaCl	mc514	64	37	27	42.3	— —
app/358	20	NaATP(KS)	NaCl	mc500	63	40	23	36.5	8 30
app/360	37	KATP	KCl	mc355	45	23	22	49.0	1 30
app/361	37	KATP	KCl	mc385	62	47	15	22.4	2 10
app/363	20	KATP	LiCl	mc204	75	40	35	46.8	— —
II 111	20	KATP	KCl	mc316	45	28	17	37.8	— —
II 141	20	KATP	LiCl	cv 50	62	22	40	64.5	0 45
II 143	20	KATP	LiCl	cv 50	37	7	30	81.1	3 15
II 148	20	KATP	LiCl	cv 50	52	24	18	53.9	2 30
III 176	20	KATP	LiCl	{ cv 50	48	17	31	64.8	1 55
				{ cv 50	48	25	23	48.0	1 0
				{ cv 50	48	22	26	54.1	1 0
Thrice precipitated myosin									
app/363(s 4)20		{ CaATP	NaCl	mc514	45	28	17	37.0	0 40
		{ NaITP	NaCl	mc514	45	25	20	44.5	0 40
		{ CaITP	NaCl	mc514	45	38	7	15.5	0 20
app/355(s 5)20		NaATP	NaCl	mc514	76	55	21	27.8	0 6
app/356(d 1)20		NaATP	NaCl	mc514	92	79	13	14.1	0 10

Δ° , flow-birefringence
mc, microscope cell

Δ°_i , initial
cv, viscosimeter

Δ°_{atp} after ATP

The immediate reduction of flow-birefringence when myosin and adenosinetriphosphate are brought together is illustrated in the curves of Fig. 9 (Experiment app/329)—a typical experiment, though the reduction is frequently greater. In order to avoid all ionic effects, K^+ or Na^+ was generally the only cation present, but as can be seen by inspection of Table X, various combinations of cations do not obscure the phenomenon. From the data already given in earlier sections of this paper, it is obvious that strict control of the salt con-

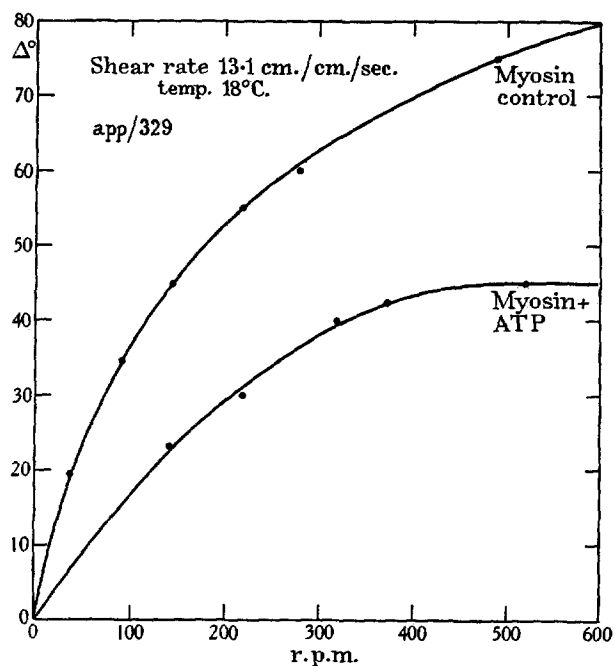


FIG. 9. Reduction of the flow-birefringence of myosin by addition of adenosinetriphosphate.

centration is essential, since the flow-birefringence of myosin varies with the molarity of the salt solution in which the protein is dissolved. Hence the ionic strength of the adenosinetriphosphate or other substances added was always compensated for in such a way as to bring all samples to a final concentration equivalent to a known ionic strength.

Close attention was paid to the stoichiometric aspect of the phenomenon. When the concentration of adenosinetriphosphate is varied at 20°C. and neutral pH, it is found that for a myosin solution of 0.86 per cent protein, approximately the full effect is obtained at 0.004 M, falling off very rapidly at lower concentrations, and being very little higher in amounts up to 0.2 M. These

relationships are illustrated in Fig. 10 (Experiment app/344). It should be emphasised that this concentration is much lower than that of any of the other classes of substances which affect myosin's flow-birefringence. To produce a

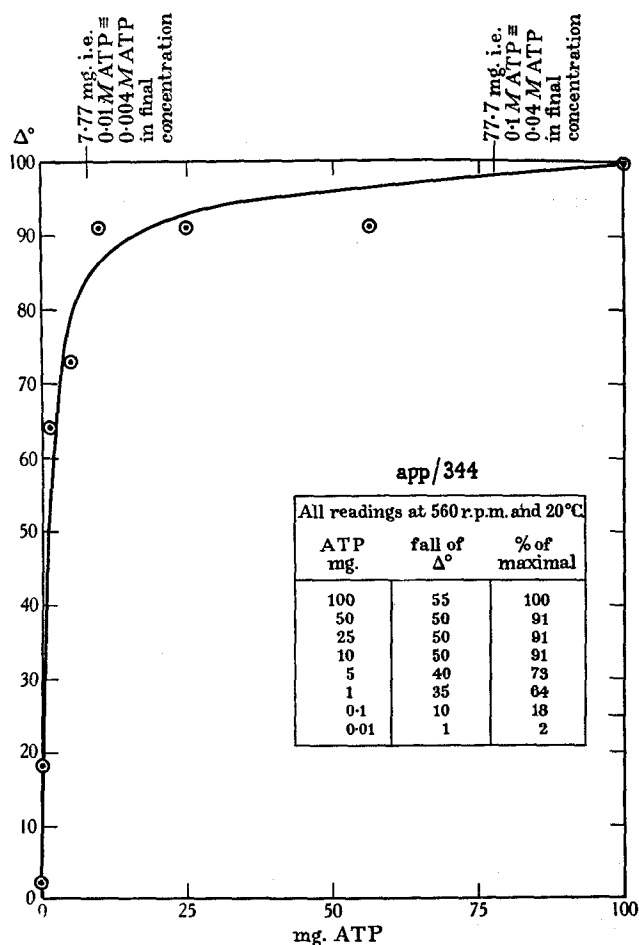


FIG. 10. Relation of myosin flow-birefringence reduction effect to concentration of adenosinetriphosphate.

60 per cent fall of flow-birefringence, 1.5 M urea is required, 1.0 M monovalent cation, and 0.2 M divalent cation; or a pH of 10. Moreover, concentrations of adenosinetriphosphate as low as 5.10^{-5} M will bring about a fall of flow-birefringence of some 15 per cent of the maximum fall.

The temperature coefficient of the fall of flow-birefringence of myosin caused by adenosinetriphosphate is a high one. In the experiment just quoted, at

the highest ATP concentration, the flow-birefringence fell from Δ 75° to Δ 25° within 5 minutes from the moment of mixing, and in other experiments we always found that the fall goes to completion in under 5 minutes. Such are the figures for room temperature (16–20°C.), but if the experiment is carried out at 0°C. then the fall is greatly prolonged. Fig. 11 (Experiment app/335) illustrates such an experiment. Since the temperature coefficient is thus appropriate for a chemical reaction, we have adopted the working hypothesis that the birefringence reduction signifies the union of adenosinetriphosphate with its substrate.

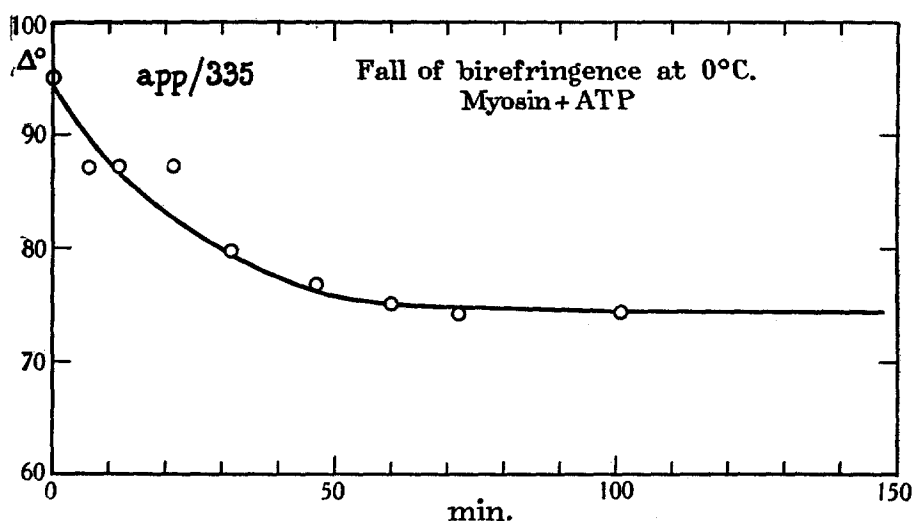


FIG. 11. Fall of flow-birefringence at 0°C.

The time required for the complete spontaneous return of the flow-birefringence of myosin to its original value before the addition of adenosinetriphosphate varied considerably in different experiments (see Table X). It was much shortened by allowing the phosphorolysis of ATP to proceed at 37°C. instead of 20°C. Especially notable is the fact that with thrice precipitated myosin the return time of Δ is extremely short, *i.e.* less than three-quarters of an hour as compared with the 1 to 3 hours taken by once precipitated myosin at 37° and the 20 hours or so at 20°C. This behaviour is readily explained by the fact that myosin, unless purified by repeated precipitations, contains the myokinase of Kalckar (1942), which converts two molecules of ADP to one of ATP and one of adenylic acid. In presence of this agent, then, there will be a continuous, if slow, formation of adenosinetriphosphate, which will provide further quotas of substrate for the myosin as the breakdown of the ATP

originally added goes on. So rapid, indeed, is the return in the absence of myokinase that it is difficult to obtain the lowest point reached by Δ immediately after mixing, and the per cent falls appear therefore to be less for thrice precipitated myosin samples than for the rest.

At the same time as the fall in flow-birefringence, there occurs a marked change in the viscosity properties of the myosin sol. While the anomaly of

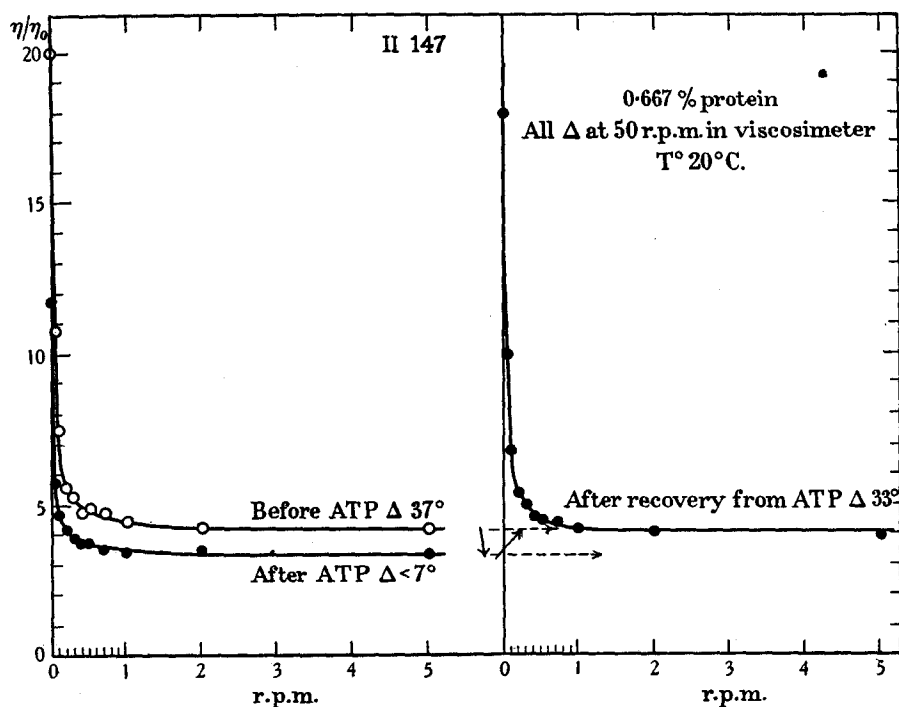


FIG. 12. Retention of flow anomaly and reversible reduction of the relative viscosity of myosin by addition of adenosinetriphosphate.

flow is not appreciably changed, the relative viscosity declines some 14 per cent—*cf.* Fig. 12 (Experiment II 147) in which η/η_0 falls 20 per cent, from 4.4 to 3.5 at 1 R.P.M. upon the addition of adenosinetriphosphate, rising again spontaneously to 4.3 when recovery to the initial flow-birefringence has almost been completed, and no further ATP remains to be split. This is a typical experiment out of many in which flow-birefringence and viscosity properties were measured upon the same sample of myosin in the coaxial viscosimeter, though necessarily at different rates of shear.

Among the most demonstrative of such experiments, we may give one in

which three successive falls and returns of flow-birefringence were observed in the same sample of myosin upon three successive additions of adenosinetri-

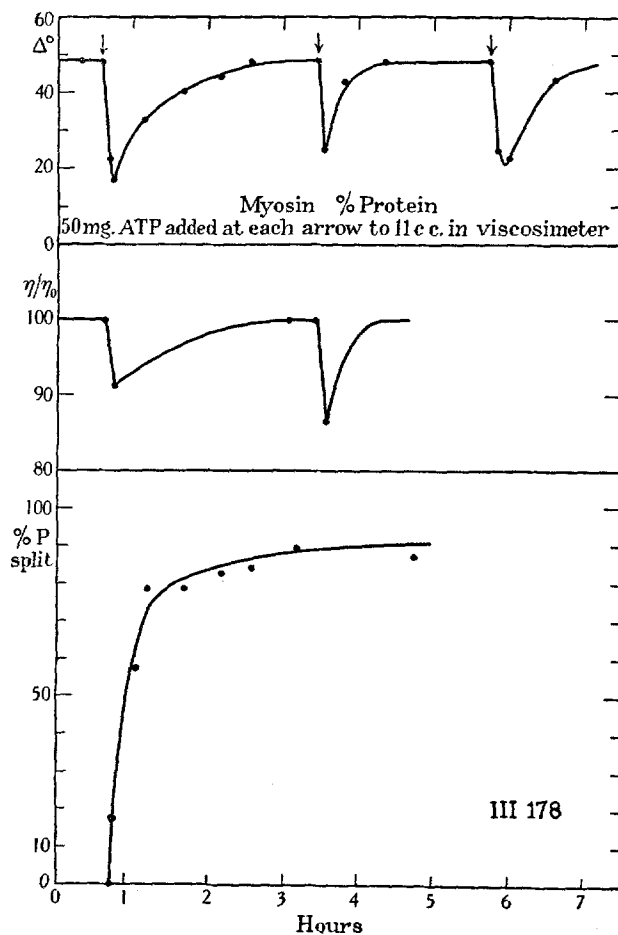


FIG. 13. Three successive falls and recoveries of flow-birefringence of a myosin sol treated three times with adenosinetriphosphate, with two successive falls and recoveries of relative viscosity (expressed in percentage of initial value) and estimations of inorganic phosphate liberated by adenosinetriphosphatase activity of the myosin during the first cycle.

phosphate, with two successive falls and returns of relative viscosity, while over the first period the splitting-off of inorganic phosphate was measured. As in many other experiments, the phosphorolysis proceeded normally in the viscosimeter while readings of the protein's physical properties were being taken. From Fig. 13 (Experiment III 178) and Table XI it may be seen that

the first fall of Δ was 64 per cent, the second 48 per cent, while the third was 54 per cent. The first viscosity fall was just under 10 per cent and the second just under 15 per cent. It was noticeable here—as also in several other experiments of the same kind—that the curve of the time relationship of the splitting of ATP does not agree over its whole course with the curves of re-

TABLE XI
Action of Adenosinetriphosphate upon the Relative Viscosity and Flow-Birefringence of Myosin. Temperature 20°C.

Experiment No.	Conditions		Protein concentrations	η/η_0 at 1 R.P.M.			Ratio η_i/η_{atp}	f		R.P.M. used for Δ	Δ°		$(n_e - n_o) \times 10^6$		Ratio Δ_i/Δ_{atp}
	Solvent	Reduction agent		Initial	atp.	Recovery		Initial	atp.		Initial	atp.	Initial	atp.	
II 111	KCl	KATP	0.078	1.79	1.67	Not measured	1.07	62.0	57.5	316	45	28	148	87	1.70
II 147	LiCl	KATP	0.667	4.4	3.5	4.3	1.25	45.0	39.0	50	37	7	121	24	5.05
II 149	LiCl	KATP	0.932	10.4	7.6	10.8	1.37	63.5	53.0	50	52	24	170	78	2.19
II 141	LiCl	KATP	1.01	11.8	11.0	Not measured	1.07	66.0	63.0	50	62	22	204	72	2.82
III 178a	LiCl	KATP	1.33	18.8	17.4	19.0	1.08	73.0	70.0	50	48	17	158	56	2.82
III 178b	LiCl	KATP	1.33	26.6	23.0	29.5	1.16	87.7	81.0	50	48	25	158	83	1.90

f , length/ratio of particles
 η/η_0 , relative viscosity
 η_i , relative viscosity, initial
 η_{atp} , relative viscosity, after ATP
 Δ° , observed extinction angle
 Δ_i , observed extinction angle, initial
 Δ_{atp} , observed extinction angle after ATP
 $n_e - n_o$, birefringence

turn of flow-birefringence and relative viscosity. Interpretation of this fact must await further investigation; more than enough ATP may have been present at the beginning than was required to saturate the enzyme.

When our relevant data for relative viscosity are summarised and compared with the simultaneously measured flow-birefringence—as in Table XI—a further perspective is gained. The experiments are arranged in order of increasing protein concentration over a nearly twentyfold range, but it is seen that the ratios of falls of relative viscosity and flow-birefringence are more or less independent of conditions such as protein concentration and ions present.

The axial ratio of the particles, f , has been calculated according to the simplified formula of Robinson (1939):

$$\eta/\eta_0 = 1 + \frac{Vf^2}{4}$$

where V is the volume fraction in gm. (cc.)/cc., and f is the length/radius of the particle (see Lawrence, Needham, and Shen, 1944, p. 208). The birefringence has also been calculated from the observed extinction angles (Δ°).

The interesting point which the table presents is that the decrease in axial ratio of the particles caused by ATP, is not, as judged from viscosimetry alone, very large. The η_i/η_{atp} ratio is of the order of 1.1. But the corresponding measurements of flow-birefringence show a much larger fall, the $\Delta_i/\Delta_{\text{atp}}$ ratio being of the order of 2.7.

At least two possible explanations are available for this. It must be remembered that the birefringence measurements are all taken perforce at considerably higher shear rates than the viscosimetric measurements. On the one hand, then, we may accept the hypothesis of sliding parallel extension and retraction in the micelle, as given by Lawrence, Needham, and Shen (1944, p. 228), and assume that no marked changes take place in intermicellar forces. In this case, the main contractive effect of ATP would be on the parallel sliding motion (whether or not exerted in conjunction with a keratin-like configurational folding process) and this would obviously manifest itself much more markedly on the high shear rate birefringence properties than upon the low shear rate viscosity properties. The pulled-out anisometric micelles would contract, and hence the flow-birefringence would drop, but they would remain anisometric micelles, and hence the relative viscosity would remain fairly high. Thus about three-quarters of the ATP effect would be on the parallel sliding system, and one-quarter of it would be on the closed micelle itself. The former might be called an "anti-plasticiser" effect, and the latter might be the configurational effect.⁴

An alternative interpretation would assume that some factor, probably connected with changes in intermicellar forces, is masking the fall in relative viscosity which the solution should, according to its flow-birefringence, show. This factor also might not be without physiological significance.

Lastly, it will be noticed from Table XI that the axial ratio of the myosin particles before the addition of ATP is in rough agreement with values in the literature now widely accepted. Our average figure makes them 31.7 times as long as they are broad; this may be compared with a figure from x-ray analysis

⁴In practice, the ATP was always added when the sol was at rest. Hence the 14 per cent effect on viscosity would appear to be a true contraction while the 48 per cent effect on birefringence would appear to be an inhibition of the pulling-out effect otherwise seen at the higher shear rates.

of 27.3 (2050 Å long, 75 Å broad) given by Worschitz (1935) although in the present state of our knowledge such an agreement may be but fortuitous. Compare also the axial ratio estimates for TMD virus of 36.8 (Frampton and Neurath, 1938), 35 (Lauffer, 1938*a*), and 55 (Neurath and Saum, 1938).

From the observations just summarised, the question immediately arises whether we have to deal here with a true contractility of the myosin molecules in the sol, analogous to the reversible configuration changes in keratin and myosin which have been revealed by the x-ray techniques. Data such as those of Fig. 12 do, after all, recall the classical twitch diagrams of intact muscles. Since the flow anomaly is retained, the particles must clearly continue to be rod-like or fibrillar, but since the flow-birefringence is reduced, their axial ratio must be less than before. This view is supported by the fact that the angle of isocline also decreases slightly, as shown in a few typical figures collected in Table XII, and returns again to its original value when the

TABLE XII
Action of Adenosinetriphosphate upon the Angle of Isocline of Myosin

Experiment No.	ψ_i	ψ_{atp}
app/329	62	52
app/330	58	52
app/341	66	64
app/363	76	67

flow-birefringence likewise returns. Von Muralt and Edsall's figure (1930) for ψ on normal myosin preparations was around 70°. The decrease in relative viscosity would then be expected, since we know from work on polymers (Kraemer and van Natta, 1932; Powell and Eyring, 1942; Mark and Simha, 1940) that for highly asymmetric molecules, the relative viscosity is proportional to the polymer length or axial ratio at equal molar concentrations.

At the same time, in the absence of some reliable estimate of the number of particles present before and after the treatment with adenosinetriphosphate, changes of aggregation and even of hydration are also conceivable. Rod-like particles might split transversely so that their axial ratio would greatly decrease. The perfection of the spontaneous return of the physical properties after the accomplishment of the enzyme action, however, seems to argue against disaggregation of the myosin micelles or molecules. But even if the phenomenon were wholly one of reversible disaggregation, rather than of true contraction and extension, the physiological significance of the effect might probably be none the less.

There is, moreover, the third possibility, namely intramicellar configurational

changes. In the first paper of this series (Lawrence, Needham, and Shen, 1944), it was pointed out that myosin molecules in a myosin micelle may be free to move one upon another like the opening and closing of slide-rules. Adenosinetriphosphate might then have an inhibitory effect on the opening.

From the stoichiometric data given above, a tentative calculation may be attempted, adopting the figures of Astbury and Bell (1941), including an equivalent weight of 67,000 for myosin, that 30 molecules of adenosinetriphosphate are required for every molecule of myosin, or one for every 19 amino acids in the myosin chain. Whether the fact that arginine occurs 32 times in the repeat unit, or once for 18 amino acids, may have any significance in this connection, remains for further consideration. But the possibility now arises that adenosinetriphosphate, the actual energy-providing substrate of the contractile protein itself, may in fact be the agent of contraction, which it would bring about by its initial combination with the enzyme.

Following this train of thought, it would be desirable to have experiments designed to show that if the enzyme-substrate combination is inhibited no change in flow-birefringence occurs; and if the subsequent enzymic action is inhibited, no restoration of the original flow-birefringence occurs. As will be seen below, we have obtained much evidence that the fall of flow-birefringence and the enzyme action may be inhibited together by substances which would be expected to be competitive inhibitors for the enzyme's normal substrate. We also carried out a few experiments, however, in which attempts were made to inactivate the enzyme by exposure to 37°C. before the substrate was added. These were not very clear cut because it is difficult to inactivate the enzyme-protein completely without bringing it to the thixotropic gel stage; however, in one case (Experiment app/337) after myosin had been held at 37°C. for 20 minutes before the addition of ATP, the fall of Δ was reduced from 50 per cent to 26.5 per cent, the return to the original value was completed in 1 hour, and the phosphorolysis inhibited 62 per cent. Since we know (D. M. Needham, 1942; Bailey, 1942) that Ca^{++} greatly activates adenosinetriphosphatase, a better way of inactivating the enzyme would be to free it entirely from this ion, perhaps by dissolving it as a sol in oxalate and vigorously centrifuging, but we have not so far succeeded in experiments along this line.⁵ It will, however, be seen from Table X that the return time with CaITP is shorter than with NaITP .

A control of some importance which may find mention at this point is that of the refractive index of the medium. Flow-birefringence may be caused either by orientation in stream lines of rods or plates themselves intrinsically bire-

⁵ According to a private communication from Dr. Bailey oxalate does not prevent splitting of ATP, when added to Ca myosin. Probably calcium oxalate is fairly soluble in presence of protein.

fringent, or by such orientation of rods or plates themselves isotropic if they are suspended in a medium of different refractive index to themselves. It was therefore necessary to check the refractive indices of solutions of ATP and myosin at different salt concentrations. As will be seen from the accompanying figures, the differences are very small and cannot account for the 50 per cent fall in Δ which myosin suffers when in the presence of adenosinetriphosphate.

	Refractive index at 17.2°C.
Distilled water	1.3335
0.5 M KCl	1.33855
1.0 M KCl	1.3435
KATP 50 mg./cc. molarity equivalent 0.5 M KCl	1.34015
KATP 100 mg./cc. molarity equivalent 0.625 M KCl	1.3437
Myosin 1 per cent in 0.5 M KCl	1.3420
Myosin 1 per cent in 0.95 M KCl	1.3460
Myosin 1 per cent in 1.43 M KCl	1.3545

In Stübel's experiment (1923) a range of refractive index of from 1.3 to 1.7 was shown to be necessary to bring about marked changes in the birefringence of the intact muscle fibre.

PROTEIN SPECIFICITY OF ADENOSINETRIPHOSPHATE

The question may be asked whether adenosinetriphosphate might possibly affect other fibrous corpuscular proteins in the same way as myosin. The only other member of this group with which we have so far worked is the virus nucleoprotein of tobacco mosaic disease, and we therefore tested the action of adenosinetriphosphate on its flow-birefringence. No fall of flow-birefringence was observed.

SUBSTRATE SPECIFICITY OF MYOSIN

On approaching this subject we should like to emphasise that the viscosity data themselves indicated a certain specificity in the action of adenosinetriphosphate on myosin. With cations and at alkaline pH ranges Δ falls while η/η_0 rises; with urea Δ falls while η/η_0 remains constant; only with adenosinetriphosphate do Δ and η/η_0 fall together to a limited and well defined extent, which larger doses will not increase, and which is spontaneously reversed during the enzyme action. It therefore seemed important to explore more thoroughly the action of other substances which might have the same effect as adenosinetriphosphate.

A number of intermediate compounds in carbohydrate breakdown and substances related to them were examined. The investigations were carried out

in the microscope cell by comparing the effect of 0.03 M solutions of the substances (final concentration) on myosin solutions in 0.5 M NaCl or LiCl with that of an equimolecular amount of ATP and with a control without any additions. The following substances had no effect on the Δ of once precipitated myosin solutions: lactate, glucose, glucose-*l*-phosphate (Cori ester), hexose-6-phosphate (Embden ester), hexosediphosphate (Harden-Young ester), glycerophosphate, phosphoglycerate, creatinephosphate, adenylic acid, inosinic acid, aneurin, aneurin-pyrophosphate (cocarboxylase), diphenylpyrophosphate (Neuberg and Wagner, 1926), inorganic pyrophosphate, inorganic triphosphate, metaphosphate, hexametaphosphate.⁶

TABLE XIII

Action of Adenosinediphosphate and Inosinetriphosphate on the Flow-Birefringence of Myosin

Experiment No.	No. of myosin precipitations	Final concentration of protein	Substrate 0.03 M	Δ at 514 R.P.M.
app/351	1	3.58 <i>per cent</i>	Control	67°
			Adenosinetriphosphate	35°
			Adenylic acid	62°
			Adenosinediphosphate	46°
			Inosinic acid	65°
			Inosinetriphosphate	44°
app/353	3	2.29	Control	45°
			Adenosinetriphosphate	27°
			Adenosinediphosphate	46°
			Inosinetriphosphate	25°

The only substances which we have so far found to affect the Δ of once precipitated myosin solutions to an extent comparable with ATP are adenosinediphosphate and inosinetriphosphate (see Table XIII). The former compound, however, has no effect on the Δ of thrice precipitated myosin. It may therefore be concluded that the effect of ADP is due to the formation of ATP from the diphosphate by myokinase (Kalckar, 1942).

Myosin and Sodium Triphosphate.—Although the sodium triphosphate was without effect on the Δ of myosin solutions, the observations of Neuberg and Fischer (1937) on the formation of orthophosphate from inorganic triphosphate by muscle and kidney extracts invited a further investigation of the effect of myosin on this salt.

Experiments in which myosin and $\text{Na}_5\text{P}_3\text{O}_{10} \cdot 6 \text{H}_2\text{O}$ were incubated at 37° and

⁶ In view of the fact that certain drugs are believed to have a direct effect on the muscle cell, we also examined the action of varying concentrations of acetylcholine, adrenalin, veratrine, nicotine, and iodoacetate on the flow-birefringence of myosin. No effects on Δ were observed, (*cf.* the work of Mirsky, 1938).

20° show that the inorganic triphosphate is enzymatically split by myosin although at a slower rate than ATP (see Tables XIV and XVII).⁷

The extent to which PO₄³⁻ is formed from Na₅P₃O₁₀ in the presence of myosin was examined in the following way: thrice precipitated myosin was incubated at 37°C. with Na triphosphate and CaCl₂ (which is known to activate the splitting of ATP by myosin; Szent-Györgyi and Banga, 1941; D. M. Needham, 1942; Bailey, 1942); the whole volume being adjusted to 0.5 M NaCl. Samples were withdrawn at intervals, the myosin precipitated by addition of 4 per cent trichloroacetic acid, and inorganic P was estimated in an aliquot of the filtrate. A control in which the myosin was replaced by distilled water was made in each case. It was shown in one experiment that a mixture containing Ca⁺⁺ but otherwise identical with a control showed twice the amount of inorganic P split from added Na₅P₃O₁₀ in the same time (see Table XV,

TABLE XIV

Enzymatic Splitting of Sodium Triphosphate by Myosin

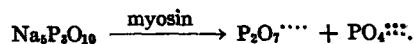
Final concentration of myosin 3.58 per cent protein; volume in tube adjusted to 3.0 ml-0.5 M NaCl; incubated 1 hour.

Additions (final concentration)	Temperature	Inorganic P found	Inorganic P formed
	°C.	mg.	mg.
0.025 M sodium triphosphate.....	20	0.298	
0.025 M sodium triphosphate + myosin.....	20	0.396	0.098
0.025 M sodium triphosphate.....	37	0.363	
0.025 M sodium triphosphate + myosin.....	37	1.134	0.771

Experiment 1). As shown in Table XIV (Experiment 2), about 28.4 per cent of the total P in Na₅P₃O₁₀ is split off as PO₄³⁻. No inorganic P was found in controls where inorganic triphosphate was incubated without myosin.

It should be noted that acids split sodium triphosphate to pyrophosphate and metaphosphate; the latter is then slowly converted at room temperature into orthophosphate. All the inorganic P estimations were therefore carried out at the end of the experiment as rapidly as possible, the deproteinised samples being kept in ice.

Since it was shown in a number of experiments that 22 to 29 per cent of the total P of added Na₅P₃O₁₀ appears as orthophosphate when incubated to completion of the reaction with myosin, the reaction would appear to be:



⁷ Dr. Bailey (private communication) found no splitting of Na triphosphate by very low concentrations of myosin, although these readily split ATP. Our observation may therefore be due to another enzyme.

Attempts were made to separate sodium pyrophosphate crystals from the incubation mixture, since the properties of the inorganic phosphates are so similar that a chemical separation of pyrophosphate appeared to be cumbersome. Although experiments using as much as 0.5 gm. of the sodium triphosphate were made, the results were inconclusive, chiefly due to the large amount of NaCl which it is necessary to have present and which also crystallises out.

Competitive Inhibition of the Effect of ATP on Myosin Solutions.—The investigation of the effect of ATP on the physical properties of myosin solutions suggests that it may be due to enzyme-substrate combination (*cf.* p. 384).

TABLE XV

Effect of Ca⁺⁺ on the Enzymatic Splitting of Sodium Triphosphate by Myosin

Final concentration: myosin 1.85 per cent protein; 0.01 M Na₃P₃O₁₀; 0.5 M NaCl. Incubated at 37°.

Experiment No.	Time	Addition	Inorganic P found
	<i>min.</i>		<i>mg.</i>
1	0	0	Trace
	30	0	0.315
	60	0	0.562
	0	0.0045 M CaCl ₂	0.270
	30	0.0045 M CaCl ₂	0.720
	60	0.0045 M CaCl ₂	1.282
2	0	0.0045 M CaCl ₂	0.278
	10	0.0045 M CaCl ₂	1.110
	20	0.0045 M CaCl ₂	2.10
	30	0.0045 M CaCl ₂	2.18
	60	0.0045 M CaCl ₂	2.64

If this were so, any related substances which were competitive inhibitors for the enzyme action should also block the fall of Δ produced by ATP.

Experiments set up to investigate this problem were carried out as follows: To a set of tubes containing myosin solution in 0.5 M NaCl, different substances (0.15 M final concentration) were added, and their effect on Δ was compared in the microscope cell with controls with and without 0.03 M ATP. After the examination 2 ml. of the various solutions were pipetted into 0.25 ml. ATP solution (0.03 M final concentration) and the Δ was measured again immediately after mixing. Controls were set up to measure the fall of Δ due to dilution. As shown in Table XVI inorganic orthophosphate, pyrophosphate, metaphosphate, hexametaphosphate, diphenylpyrophosphate, adenylic acid, and inosinic acid, have no appreciable effect on the fall of flow-birefringence brought about by ATP. Inorganic triphosphate and ADP however do show an inhibition of the ATP fall of Δ in thrice precipitated myosin.

Table XVI requires a few words of explanation. The values in the first column will be seen to be all a little lower than that of the control without any addition—this is almost certainly due to a salt-like effect of the high concentration of the substances added. We have already seen that at similar molarity to the ATP they have no effect, but in this experiment it was necessary

TABLE XVI
Competitive Inhibition of the Flow-Birefringence Reduction by Sodium Triphosphate and Adenosinediphosphate

Thrice precipitated myosin; ATP at 0.03 M; all other substances at 0.15 M.

Experiment No.	Protein concentration	Substances	Δ° at 514 R.P.M.			Per cent of best possible fall
			Δ_i	Δ_{atp}	Fall of Δ	
app/352	3.58	Control; no addition	64	58	27	100
		Control; ATP	37	31		
		Orthophosphate	53	33	25	93
		Pyrophosphate	52	30	28	104
		Metaphosphate	64	33	25	93
		Hexametaphosphate	65	30	28	104
		Triphosphate	50	37	21	77
		Phenylpyrophosphate	48	28	30	111
		Adenylic acid	58	30	28	104
		Inosinic acid	58	30	28	104
app/355		Control; no addition	76	67	20	100
		Control; ATP	55	47		
		Adenosinediphosphate	80	57	10	50
		Triphosphate	78	61	6	30
		Orthophosphate	66	46	21	103
		Pyrophosphate	72	45	22	107
		Phenylpyrophosphate	71	46	21	103

to raise their concentration in order to demonstrate any inhibition. It will also be noticed that the values for the controls in the second column are less than those in the first; this is because of the extra dilution involved in adding ATP or its dilution control.

Since triphosphate is able to block the fall in the flow-birefringence of myosin brought about by ATP, it was of interest to ascertain whether it would also inhibit the phosphorolysis of ATP. The formation of inorganic P from ATP does in fact appear to be inhibited by the presence of triphosphate, which is

also split itself (see Table XVII). In each case the values obtained from samples taken from the tube containing both sodium triphosphate and ATP were significantly lower than the sum of the controls (*e. g.* 0.956 instead of 1.58 mg. P).

All these results seem to be further evidence in favour of the view that the observable changes of the optical properties of myosin solutions in the presence of ATP are connected with the enzymatic properties of myosin as adenosinetriphosphatase. Myosin splits off inorganic P from adenosinetriphosphate and inosinetriphosphate (Kleinzeller, 1942), acting on the triphosphate grouping:

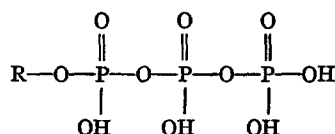


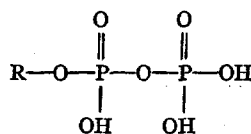
TABLE XVII

Competitive Inhibition of Adenosinetriphosphatase by Sodium Triphosphate

Final concentrations: myosin 1.48 per cent protein, 0.5 M NaCl; 0.009 CaCl₂. Final volume 2.5 ml. Incubated at 37°.

Time	Inorganic phosphorus				
	Substrate added				
	0.01 M ATP	0.015 M Na ₂ P ₂ O ₁₀	0.01 M ATP + 0.015 M Na ₂ P ₂ O ₁₀	0.01 M ATP + 0.03 M Na ₂ P ₂ O ₁₀	0.01 M ATP + 0.06 M Na ₂ P ₂ O ₁₀
min.	mg.	mg.	mg.	mg.	mg.
2	0.630	Trace	0.53	0.315	0.338
15	0.755	0.506	0.855	0.775	0.653
30	0.843	0.742	1.057	0.956	0.935

(where R can be adenosine or inosine); but does not attack the pyrophosphate grouping:



Since inorganic triphosphate has no effect on the flow-birefringence, it is concluded that both the triphosphate group and the adenosine or inosine nucleoside group are necessary to bring about the optically observable changes in myosin solutions.

DISCUSSION

About ten years ago there was much debate on what were called the "physical" and the "chemical" theories of muscular contraction (*cf.* Ritchie, 1932).

In discussions at that time, it was felt by many that "topochemical" would have been a better word than "physical." The point was very clearly put by Bernal (1938). "Do the chemical reactions (in muscle)" he said, "have to do directly or indirectly with the actual contraction process? If the first is true, they are probably of a topochemical nature, that is, reactions in which one of the reagents is not a free molecule but a radical bound in a fixed position on a chain molecule. On the other hand . . . the chemical reactions may bring about contraction indirectly by building up a potential field, probably of an electrical nature, in which the chemically inert myosin molecule contracts reversibly." The new facts seem to be showing that the first alternative is the right one. And it should perhaps be emphasised that this has further implications than the very obvious phenomenon of muscle contraction itself. It is not wide of the mark to say that all morphogenesis is a problem of protein chemistry, since we can credit only the proteins with the burden of change and maintenance of cell architecture. Hence the relation between the smaller energy-rich molecules in true solution or dispersion and the enormous protein molecules which are the bricks of cell structure is one of vital importance for the embryologist no less than the physiologist.

From the data reported in the present paper, it seems fair to say that the interaction between adenosinetriphosphatase and its natural substrate brings about deep seated and optically observable effects upon the enzyme's physical state. Whether these changes are micellar in character, or whether they are analogous to the configurational changes established for the keratins, remains as yet undetermined. Could adenosinetriphosphate itself (or, in view of the results of Kleinzeller (1942), inosinetriphosphate) then be normally *in vivo* the agent of contraction? Were this so, the reception of the nervous or other stimulus would essentially allow the enzyme and substrate to come in contact. The exact location of inorganic ions at this stage may also be important, *e.g.* Ca⁺⁺ (Bailey, 1942), K⁺ (Verzar, 1942). In this connection it may be significant that Caspersson and Theorell (1941, 1942) have been able by ultraviolet microspectroscopy to localise the adenosinetriphosphate in the non-birefringent bands of the *resting* muscle fibre. By this enzyme-substrate combination, then, the configuration of the contractile enzyme would itself immediately be changed (as pictured by Astbury and Dickinson, 1940; Astbury, 1942). Further experiments upon the quantitative relationship between the ATP present and the change produced in double refraction and upon the time relationship of the optical changes and the enzymic breakdown are still needed to decide between the two possibilities: (a) That the combination between myosin and ATP so alters the myosin molecule, that reaction between some groups along the length of the chain becomes possible and provides energy. The subsequent splitting off of phosphate from the substrate would then supply energy needed for relaxation and recharging of the myosin fibril. (b) That

the splitting off of phosphate and setting free of energy from the ATP accompanies the contraction. In any case, it must be remembered that, *in vitro*, the contraction of the myosin particles does no work, and the conditions are therefore very different from those *in vivo*. This objection might be met if it should prove possible to do enzyme experiments with myosin fibrils strong enough to withstand a measurable tension without breaking. (See the experiments of Engelhardt, Ljubimova, and Meitina (1941) referred to later.)

From the facts already known arises another alternative suggestion (entertained for some time past by one of us and now put forward in a rather different form by Kalckar, 1941), that myosin itself might be phosphorylated and dephosphorylated, occupying thus the last link in the chain of simultaneous transfers of phosphate ions and energy. As is well known, adenosinediphosphate can be phosphorylated according to circumstances by creatinephosphate, phosphopyruvic acid, or diphosphoglyceric acid. One may suggest that while adenosinediphosphate acts as a phosphate acceptor towards these substances, adenosinetriphosphate acts as a phosphate donator towards the contractile protein itself. Contracted myosin would thus have adenosinetriphosphatase activity, and phosphate would be transferred from the adenosinetriphosphate to some part of the protein molecule, which would simultaneously extend. Extended myosin would then be in phosphorylated form and charged with energy. When the physical changes touched off by the nerve stimulus occur, inorganic phosphate would be set free, and the energy available from the dephosphorylation used in contraction. Experiments on the possible phosphorylation of myosin are in progress by one of us.

Very occasionally, in two myosin preparations in our experience, adenosinetriphosphate was found to exert no effect on the flow-birefringence of the protein. We can offer nothing to account for this observation, but it may be remarked that in one of these cases the myosin had been prepared from a rabbit which had been subjected to convulsions before being killed.

In considering the effect of ATP on myosin it must always be remembered that the enzyme action and the physical changes are, under certain conditions, separable. In early preparations which were not flow-birefringent, the enzyme was active. Conversely, after treatment at 37°C. the flow-birefringence is retained or enhanced but the enzyme action is largely destroyed. The first case may have several explanations (*a*) the protein may be already "set" in the fully contracted state; (*b*) its particles may have too small an axial ratio to allow of the normally occurring changes being seen; (*c*) the ATP may only link on at the phosphate end and be split like inorganic triphosphate. In the second case, the protein micelles may be "set" in the fully extended state. The possibility, therefore, must still be borne in mind that the enzymic activity (adenosinetriphosphatase) may involve some grouping separable from the contractile protein itself. In this case the optically observable effects of ATP on myosin would be the result of changes conveyed through this grouping.

If it should prove that the changes are molecular rather than micellar, then one might picture the molecular length of adenosinetriphosphate as being shorter than the length between the groups on the myosin chain to which it becomes attached. Contraction would then necessarily accompany combination. This would be in line with the general two-affinity theory of enzyme action, only in globular corpuscular proteins configurational changes in the enzyme protein would not be so readily observable as in linear corpuscular (soluble fibrous) proteins.

The evidence for the identity of myosin and adenosinetriphosphatase, resting upon the failure of all attempts to separate them, must remain of a negative character. But the fact that far reaching optically observable changes in the myosin accompany the enzyme-substrate combination and the subsequent enzyme action, gives positive support to the view that the enzyme is myosin itself. It may therefore now be allowable to speak for the first time of a "contractile enzyme." And the present work provides suggestive evidence that the enzyme-protein contracts "in solution."

One cannot, of course, compare too closely the behaviour of myosin in the muscle cell and myosin isolated from it in the form of a sol. In the muscle the contracting particles do work against resistance; in the sol this does not happen. In the muscle, only a very small proportion of the myosin can be in true sol form, such as we see it *in vitro* (Bate-Smith, 1934). It is interesting, however, to compare the birefringence effects seen on isolated purified myosin *in vitro* with those which may be observed in the intact muscle fibre. It is known from older work, put on a firm basis by von Muralt (1932) that during a single isometric twitch the birefringence of the intact muscle fibre decreases by some 35 per cent, returning to its original value by the end of relaxation. It has also been found by Buchtal and Knappeis (1938) that in the absence of contraction, changes in pH and salt concentration in the medium surrounding a contractile isolated muscle fibre, bring about slower reversible changes in its birefringence. We have seen, too, in the first part of this paper, that many chemical agents exert effects on the flow-birefringence of myosin similar to those which they exert on the birefringence of the intact muscle fibre.

From the important work of Stübel (1923) we know that this birefringence of the intact muscle is due to three components (*a*) a positive rod form-birefringence, (*b*) a positive intrinsic birefringence, and (*c*) a negative intrinsic birefringence. The first two are certainly due to the protein, the third to associated lipins. Already in 1923 Stübel could confidently write "In den Muskelfaser finden sich gleichsinnig orientierte, stäbchenförmige, kristallinische, doppelbrechende, Eiweissteilchen." Later, Stübel and Liang (1928) showed that the birefringence of intact muscle decreases and is lost in rigor or contractures, whether isotonic or isometric.

The "viscosity" or viscous elasticity of the intact muscle has also been the subject of important investigations (Hill, 1922; Gasser and Hill, 1924; Levin and Wyman, 1927; Stevens and Metcalf, 1934; Fenn and Marsh, 1935). They seem to show that the viscosity of a muscle on tetanus or even during a single twitch is at least ten times that of the same muscle at rest. But it is impossible to compare these data on the

highly packed myosin gel within the cell membrane with ours on the freely orientable particles of the myosin sol. If indeed the intracellular pH shifts slightly towards the acid side, the *in vivo* phenomenon might be connected with the high viscosity seen on the left-hand side of Fig. 5 in the present paper.

We doubt if there is anything to be gained by referring to the myosin sol after its flow-birefringence has fallen and it has begun to dephosphorylate adenosinetriphosphate as "denatured." Such a "denaturation" (which involves neither changes of solubility, nor as has been shown by Greenstein and Edsall (1940) in the case of the salt effects, changes in titratable —SH linkages) has not gone beyond an extremely early reversible stage at which it is compatible with active enzymic breakdown of the adenosinetriphosphate. As we have noted in Paper II of this series (1944) enzymic activity may continue actively even after an enzyme-protein has undergone denaturation by uncoiling in the surface film at an air-water interface. But the evidence we bring forward here shows that the relative viscosity of myosin falls under the action of ATP, whereas in denaturation it should rise; and the flow anomaly is retained, whereas it should be lost.

A case which is perhaps relevant to the present one, though not closely analogous to it, is the fall in flow-birefringence and anomalous viscosity of polymerised sodium thymonucleate brought about by proteins and the "depolymerase" (Greenstein and Jenrette, 1941, 1942; Greenstein, 1942) and by ultraviolet irradiation (Hollaender, Greenstein, and Jenrette, 1941). These effects, however, are not reversible. It does not seem as yet clear that they are due to disaggregation (depolymerisation) as against deformation or tangling of the micelles, though admittedly we know of other, purely chemical, evidence for "ribonucleo-depolymerase" (Schmidt and Levene, 1938).

If, as we assume, the change in flow-birefringence (and hence in particle shape) of myosin in presence of adenosinetriphosphate is a manifestation of the enzyme-substrate combination, it would take its place among the few other such directly observable processes, such as the spectral shifts of catalase (Keilin and Hartree, 1937) and peroxidase (Keilin and Mann, 1937) and the visible colour changes of certain flavoproteins (Haas, 1937). It would be, however, the first example of such an effect observable by polarised light. Little else is known about the combination of myosin and adenosinetriphosphate, save that no change in the ultraviolet absorption spectrum of the protein is to be seen; the curves of the two substances together are summative (Ljubimova and Shipalov, 1940). A non-summative change, however, would not necessarily be expected, since ultraviolet absorption primarily indicates ring structures, and no changes in these are likely to happen when myosin and adenosinetriphosphate combine.

From the data reported above, the conclusion is probably justified that myosin is a specific triphosphatase. Since inorganic triphosphate combines with

the protein and is split without causing any fall of Δ , we may say with some plausibility that the purine-ribose end of the nucleoside-triphosphate molecule is also of much importance in changing the shape of the protein molecule or micelle.

An interesting analogous case to this has recently been published by Buchman, Heegaard, and Bonner (1940). Thiazol-diphosphate (thiazol-pyrophosphate), which has a similar constitution to aneurin (thiamin) diphosphate, save that it lacks the methyl-amino-pyrimidine ring, inhibits the activity of carboxylase, competing with thiamin-diphosphate itself, if the latter is present. Here a coenzyme, cocarboxylase, therefore, which is thought normally to become attached to the enzyme-protein by two bonds (Stern and Melnick, 1939), is kept away from the protein, and hence the activity of the whole system destroyed, by a closely related substance which becomes attached by only one bond and has no cozymic activity. In the case of myosin, of course, the smaller molecule is substrate, not coenzyme; but there is an analogy in the blockage of optical effect and phosphorolysis by inorganic triphosphate.

The only other attempt which has been made to elucidate the relationships of adenosinetriphosphate and myosin, which today seem crucial for any understanding of protein contractility, is that of Engelhardt, Ljubimova, and Meitina (1941). Partially dried fibres of myosin, prepared by injection from extruders into distilled water, and still retaining some adenosinetriphosphatase activity, were treated with a variety of substances, of which alone adenosinetriphosphate showed any effect—an increase of extensibility. The concentration of adenosinetriphosphate was similar to that found by us to be minimal for complete effectivity, that is, 0.0025 M. It is, of course, impossible to compare readily the results of two techniques so different, especially as the full details of the Russian work are not yet available. Much may be expected from the x-ray analysis of such fibres parallel with determinations of enzyme activity, if this should be technically possible. The effects of electrolytes on partially dried myosin fibres have recently been examined by Gerendas and Szent-Györgyi (1941), but the paper has so far been available to us only in the form of an abstract.

The observations and experiments discussed in this paper may at first sight seem to have little bearing on the problems of interest to the embryologist with which the first and second papers of the series partly dealt. But the significance of protein contractility is far wider than the muscle problem alone. The contractility of intra- and intercellular fibrils in various embryonic processes has often been pointed out (*cf.* J. Needham, 1942). The problem of energy transfer and change in configuration of protein fibrils is not without relevance to inductor-reactor systems in embryos undergoing morphogenesis. The possibility should now be borne in mind that an inductor substance might be, not a hormone, nor a coenzyme nor an autocatalytically active protein,

but a substrate, and that the changes in protein configuration which will lead to a specific path of histogenesis and all that that implies, might be the result of the unavoidable action of an enzyme-protein in the competent cell upon the inductor itself.

SUMMARY

1. An investigation of the physicochemical properties of myosin has been carried out. Prepared under standard conditions, the ratio of flow-birefringence to protein concentration is uniform. The effect of electrolytes, pH, and urea on the flow-birefringence and viscosity (relative and anomalous) of myosin has been examined.

2. Decrease or abolition of flow-birefringence does not necessarily imply far reaching denaturation, since such effects can be reversed by a variety of means.

3. When a myosin solution is treated with adenosinetriphosphate, its flow-birefringence is decreased (average 48 per cent), its anomalous viscosity is retained, and its relative viscosity is decreased (average 14 per cent). The full effect of adenosinetriphosphate is obtained at 0.004 M; a molarity very much less than that of other substances which decrease the flow-birefringence of myosin.

4. The changes in the physicochemical properties of myosin brought about by adenosinetriphosphate are spontaneously reversible, and are connected with the enzymatic action of the protein as adenosinetriphosphatase.

5. Effects similar to those of adenosinetriphosphate on the physicochemical properties of purified myosin have been obtained so far only with inosinetriphosphate.

6. Inorganic phosphate is split off by myosin from inosinetriphosphate as well as from adenosinetriphosphate. Inorganic triphosphate is split by 1 to 2 per cent solution of three times precipitated myosin.

7. Adenosinediphosphate and inorganic triphosphate act as competitive inhibitors with adenosinetriphosphate, blocking the fall of flow-birefringence.

8. The implications of the results, and the conception of active enzymic groups attached to proteins participating in cell structure, whether contractile or non-contractile, are discussed in relation to present views on muscle physiology and other biological problems.

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REFERENCES

- Ambronn, H., 1914, cited in Ambronn, H., and Frey, A., 1926, *Das Polarisationsmikroskop. Seine Anwendung in der Kolloidforschung und in der Färberei*, Leipzig, Akademische Verlagsgesellschaft, M.B.H., 169.
- Astbury, W. T., 1933, *Fundamentals of fibre structure*, Oxford University Press.
- Astbury, W. T., 1939, *Proc. Roy. Soc. London, Series B*, **127**, 30; in Annual review of biochemistry, (J. M. Luck, and J. H. C. Smith, editors), Stanford University, Annual Reviews, Inc., 1939, **8**, 113.
- Astbury, W. T., 1942, *J. Chem. Soc.*, 337.
- Astbury, W. T., and Bell, F. O., 1938, Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, **6**, 109.
- Astbury, W. T., and Bell, F. O., 1941, *Nature*, **147**, 696.
- Astbury, W. T., and Dickinson, S., 1940, *Proc. Roy. Soc. London, Series B*, **129**, 307.
- Bailey, K., 1939, *Biochem. J.*, **33**, 255.
- Bailey, K., 1942, *Biochem. J.*, **36**, 121.
- Banga, I., and Szent-Györgyi, A., 1940, *Science*, **92**, 514.
- Bate-Smith, E. C., 1934, *Proc. Roy. Soc. London, Series B*, **114**, 494.
- Bawden, F. C., and Pirie, N. W., 1940, *Biochem. J.*, **34**, 1258.
- Bernal, J. D., 1938, *Perspective in biochemistry*, Hopkins Presentation Volume, Cambridge, University Press, p. 50.
- Biedermann, W., 1927, *Ergebn. Physiol.*, **2**, 416.
- Brücke, E., 1858, *Sitzungsber. k. Akad. Wissensch., Math.-naturwissensch. Cl., Wien*, **15**, 69.
- Buchman, E. R., Heegaard, E., and Bonner, J., 1940, *Proc. Nat. Acad. Sc.*, **26**, 561.
- Buchtal, F., and Knappeis, G. G., 1938, *Skand. Arch. Physiol.*, **78**, 97.
- Buck, J. B., 1942, *J. Hered.*, **33**, 3.
- Cannan, R. K., and Muntwyler, E., 1930, *Biochem. J.*, **24**, 1013.
- Caspersson, T., and Theorell, B., 1941, *Naturwissenschaften*, **29**, 364.
- Caspersson, T., and Theorell, B., 1942, *Acta physiol. Skand.*, **4**, 97.
- von Ebner, V., 1882, *Untersuchungen über die Ursachen der Anisotropie organisirter Substanzen*, Leipzig, W. Engelmann.
- Edsall, J. T., 1942, *Advances Coll. Sc.*, **1**, 269.
- Edsall, J. T., and Mehl, J. W., 1940, *J. Biol. Chem.*, **133**, 409.
- Edsall, J. T., and Singher, H. O., 1941, private communication to the authors.
- Eirich, F., Margaretha, H., and Bunzl, M., 1936, *Kolloid-Z.*, **74**, 276; **75**, 20.
- Engelhardt, W. A., and Ljubimova, M. N., 1939, *Nature*, **144**, 668; *Biochemia*, **4**, 716.
- Engelhardt, W. A., Ljubimova, M. N., and Meitina, R. A., 1941, *Compt. rend. Acad. sc., U. R. S. S.*, **30**, 644.

- Engelmann, T. W., 1906, *Sitzungsber. k. Akad. Wissensch. Berlin*, 694.
- Fenn, W. O., and Marsh, B. S., 1935, *J. Physiol.*, **85**, 277.
- Fiske, C. E., and SubbaRow, Y., 1925, *J. Biol. Chem.*, **66**, 375.
- Fletcher, W. M., and Hopkins, F. G., 1907, *J. Physiol.*, **35**, 247.
- Frampton, V. L., and Neurath, H., 1938, *Science*, **87**, 468.
- Gasser, H. S., and Hill, A. V., 1924, *Proc. Roy. Soc. London, Series B*, **96**, 398.
- Gerendas, M., and Szent-Györgyi, A., 1941, *Enzymologia*, **9**, 117; *Chem. Abstr.*, **36**, 788.
- Greenstein, J. P., 1942, *J. Nat. Cancer Inst.*, **2**, 357.
- Greenstein, J. P., and Edsall, J. T., 1940, *J. Biol. Chem.*, **133**, 397.
- Greenstein, J. P., and Jenrette, W. V., 1940, *J. Nat. Cancer Inst.*, **1**, 91.
- Greenstein, J. P., and Jenrette, W. V., 1941, *J. Nat. Cancer Inst.*, **1**, 845.
- Greenstein, J. P., and Jenrette, W. V., 1942, *J. Nat. Cancer Inst.*, **2**, 301.
- Haas, E., 1937, *Biochem. Z.*, **290**, 291.
- Harden, A., and Young, W. J., 1910, *Proc. Roy. Soc. London, Series B*, **82**, 321.
- Hill, A. V., 1922, *J. Physiol.*, **56**, 19.
- Huber, H., 1937, *Z. angew. Chem.*, **50**, 323.
- Kalckar, H., 1941, *Chem. Rev.*, **28**, 71; *Biol. Rev.*, **17**, 28.
- Kalckar, H., 1942, *J. Biol. Chem.*, **143**, 299.
- Keilin, D., and Hartree, E. F., 1937, *Proc. Roy. Soc. London, Series B*, **124**, 397.
- Keilin, D., and Mann, T., 1937, *Proc. Roy. Soc. London, Series B*, **122**, 119.
- Kerr, S. E., and Seraidarian, K., 1941, *J. Biol. Chem.*, **139**, 121.
- Kleinzeller, A., 1942, *Biochem. J.*, **36**, 729.
- Kraemer, E. O., and van Natta, F. J., 1932, Colloid Symposium Monograph, **10**, 327.
- Lauffer, M. A., 1938a, *Science*, **87**, 469.
- Lauffer, M. A., 1938b, *J. Biol. Chem.*, **126**, 443.
- Lawrence, A. S. C., Miall, M., Needham, J., and Shen, S. C., 1944, *J. Gen. Physiol.*, **27**, 233.
- Lawrence, A. S. C., Needham, J., and Shen, S.-C., 1944, *J. Gen. Physiol.*, **27**, 201.
- Levin, A., and Wyman, J., 1927, *Proc. Roy. Soc. London, Series B*, **101**, 218.
- Liang, T.-Y., 1936, *Chinese J. Physiol.*, **10**, 327.
- Ljubimova, M. N., and Pevsner, D., 1941, *Biochemia*, **6**, 178.
- Ljubimova, M. N., and Shipalov, M. S., 1940, *Biochemia*, **5**, 144.
- Lohmann, K., 1931, *Biochem. Z.*, **233**, 460.
- Lohmann, K., 1932, *Biochem. Z.*, **254**, 381.
- Mark, H., 1941, Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, **9**, 204.
- Mark, H., and Simha, R., 1940, *Nature*, **145**, 571.
- Meyer, K. H., and Picken, L. E. R., 1937, *Proc. Roy. Soc. London, Series B*, **124**, 29.
- Mirsky, A. E., 1938, Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, **6**, 150.
- von Muralt, A., 1932, *Arch. ges. Physiol.*, **230**, 299.
- von Muralt, A., and Edsall, J. T., 1930, *J. Biol. Chem.*, **89**, 315, 351.
- Nasse, O., 1882, *Zur Anatomie und Physiologie der quergestreiften Muskelsubstanz*, Leipzig.
- Needham, D. M., 1938, *Enzymologia*, **5**, 158.

- Needham, D. M., 1942, *Biochem. J.*, **36**, 113.
- Needham, J., 1936, *Order and life*, New Haven, Yale University Press.
- Needham, J., Kleinzeller, A., Miall, M., Dainty, M., Needham, D. M., and Lawrence, A. S. C., 1942, *Nature*, **150**, 46.
- Needham, J., Shen, S.-C., Needham, D. M., and Lawrence, A. S. C., 1941, *Nature*, **147**, 766.
- Neuberg, C., and Fischer, H. A., 1937, *Enzymologia*, **2**, 191, 241, 360.
- Neuberg, C., and Wagner, J., 1926, *Biochem. Z.*, **171**, 485.
- Neurath, H., and Saum, A. M., 1938, *J. Biol. Chem.*, **126**, 435.
- Nitschmann, H., 1938, *Helv. chim. acta*, **21**, 315.
- Nitschmann, H., and Guggisberg, H., 1941, *Helv. chim. acta*, **24**, 434, 574.
- Northrop, J. H., 1929, *J. Gen. Physiol.*, **12**, 529.
- Ostern, P., 1932, *Biochem. Z.*, **254**, 65.
- Parnas, J. K., 1937, *Ergebn. Enzymforsch.*, **6**, 57.
- Pauli, W., and Valkó, E., 1933, *Kolloidchemie der Eiweisskörper*, 2nd edition, Dresden and Leipzig, Theodor Steinkopff.
- Powell, R. E., and Eyring, H., 1942, *Advances Coll. Sc.*, **1**, 183.
- Ritchie, A. D., 1932, *Nature*, **129**, 165.
- Robertson, T. B., 1928, *Colloid Chem.*, **2**, 255.
- Robinson, J. R., 1939, *Proc. Roy. Soc. London, Series A*, **170**, 519.
- Schipilov, C., and Danilevsky, A., 1881, *Z. physiol. Chem.*, **5**, 349.
- Schmidt, G., and Levene, P. A., 1938, *J. Biol. Chem.*, **126**, 423.
- Stern, K. G., and Melnick, J. L., 1939, *J. Biol. Chem.*, **131**, 610.
- Stevens, H. C., and Metcalf, R. P., 1934, *Am. J. Physiol.*, **107**, 568.
- Stübel, H., 1923, *Arch. ges. Physiol.*, **201**, 629.
- Stübel, H., and Liang, T.-Y., 1928, *Chinese J. Physiol.*, **2**, 139.
- Szent-Györgyi, A., and Banga, I., 1941, *Science*, **93**, 158.
- Verzar, F., 1942, *Schweiz. Med. Woch.*, **72**, 661.
- Worschitz, F., 1935, *Fortschr. Geb. Röntgenstrahlen*, **51**, 81.