FRAGMENTATION OF THE MYOSIN MOLECULE

by

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Since the classical work of H. H. WEBER[1] on the chemistry and quantitative characterisation of myosin, a wide range of physical, physicochemical and biochemical methods have been applied to the study of the size, shape and enzymic properties of this globulin. The picture which emerges from the diverse investigations first on actomyosin, then on the actin-poor and so-called "crystalline" myosin and lately on the ultracentrifugally homogeneous preparation of PORTZEHL, SCHRAMM AND WEBER[2], is that of a highly elongated molecule. The size is of the order of 10^6 and the axial ratio about 100. Associated with the molecule are the activities of adenosine triphosphatase (ENGELHARDT AND LYUBIMOVA[3]) and to a greater or less degree adenylic acid deaminase (FERDMAN AND NICHIPORENKO[4]) and inosine triphosphatase (NEEDHAM, KLEINZELLER, MIALL, DAINTY, NEEDHAM AND LAWRENCE[5]).

It is difficult to imagine that a fibrous molecule of the dimensions and complexity of myosin could be synthesized fully formed from its template. The existence of subunits has been discussed from time to time and various values for the size of the subunits reported: 100,000 (H. H. WEBER AND STÖVER[6]; MOMMAERTS[7]) 1–300,000 (MOMMAERTS[8]), 17,000 and 150,000 (SZENT-GYÖRGYI[9]), 70,000 (SZENT-GYÖRGYI[10]—the "autones" of SZENT-GYÖRGYI[11]) and 40,000 and 80,000 (MIHALYI, see SZENT-GYÖRGYI[12]; A. G. SZENT-GYÖRGYI[13]). The direct estimate by WEBER AND STÖVER[6] using osmotic pressure technique was made on a myosin preparation which is now known to be impure, and that by MIHALYI[12] and by A. G. SZENT-GYÖRGYI[13] employing tryptic digestion, though extremely important in throwing new light on the location of the adenosine triphosphatase activity on the myosin molecule, is of unassessed value for subunit studies owing to the probable fission of peptide linkages. The other values were derived from indirect inferences or supported by little experimental evidence.

To conceive a molecular architecture for myosin and to correlate structure with function, more precise information on the submolecular level is obviously needed. The aims of the present investigation were to determine the quantitative trend of depolymerisation of a myosin preparation that is free from other protein contaminants, and to separate the subunits and characterise their individual dimensions by osmotic pressure and fluorescence-polarisation measurements. It was found that the myosin molecule is made up of two kinds of subunits: those with no identifiable terminal residue, possibly cyclic, of average particle weight 165,000, and open chain polypeptides of 16,000. There is no evidence as yet that tropomyosin forms part of the myosin molecule, nor has it been possible to separate the adenosine triphosphatase activity.

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MATERIAL AND METHODS

Preparation and purity of myosin. Myosin was prepared from rabbit skeletal muscle essentially by the method of SZENT-GYÖRGYI[9]. Before the second "crystallisation", the solution of myosin in 0.6 *M* KCl was filtered through paper pulp. To remove the small amount of actin present in the reprecipitated myosin, the ammonium sulphate fractionation procedure of DUBUISSON[14] as adopted by SZENT-GYÖRGYI[9] was employed. The fractionation was carried out at 0° C, and a saturated ammonium sulphate solution, the pH of which had been adjusted to 6.5 with ammonia, was added with stirring to the loosely packed precipitate of myosin. The initial addition brings the gel into solution, and salting-out begins at about 30% saturation when a slight turbidity first appears. The solution was brought to 40% saturation and centrifuged. The myosin in the supernatant was precipitated by further addition of ammonium sulphate to 50% saturation, separated on the centrifuge and dialyzed against rapid changes of distilled water in the ice-chest. The protein was brought into solution by dialyzing against several changes of the solvent to be used in the investigation.

Myosin prepared according to this procedure (henceforth denoted as myosin 40–50% for convenience) is electrophoretically homogeneous (Fig. 1). End-group assay (BAILEY[15]) by SANGER's technique[16] indicated that there is one mol of terminal residue for every 4–500,000 g of protein. The protein is readily soluble in 0.5 *M* KCl, giving a water-clear solution which shows neither fall nor rise in viscosity in the presence of adenosine triphosphate. In the fraction which came out at less than 40% saturation, on the other hand, a slight dependence of viscosity on shear rate and on the presence of ATP was evident; and such a fraction, after drying in ethanol-ether and extracting with *M* KCl at pH 7 or 13, showed a strong ultraviolet absorption at 260 mμ. All these may be regarded as indications of the presence of actin impurities in that fraction.

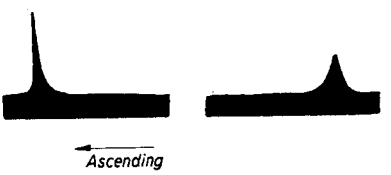

Fig. 1. Electrophoretic diagram of myosin 40–50%. In 0.1 *M* glycine, 0.2 *M* KCl, 0.08 *N* NaOH, pH 9.0; overall voltage 15 V, 5 mA, 30.5 hours.

It is perhaps worth mentioning in this connexion that when "crystalline" myosin was dehydrated and extracted in a similar way, it was possible to isolate about 0.9% of tropomyosin which was crystallised.

Myosin 40–50% was found to split off only one phosphate group from ATP and was therefore free from myokinase (see BAILEY[17]). The Qp value, however, was low compared with unfractionated myosin. Where a preparation of thrice precipitated and filtered myosin (also myokinase-free by the same criterion) showed a Qp 25° of 1,300, the value was reduced to 300 after fractionation. This is probably due to inactivation by traces of metallic contaminant in A.R. ammonium sulphate and through the long dialysis needed to remove it. Nevertheless, it has been thought worthwhile for the present investigation to sacrifice enzymic activity for electrophoretic homogeneity.

In the present procedure, any "denatured" (*i.e.* aggregated) myosin (H. H. WEBER[18]) which would have arisen from the two "crystallisation" steps, would most probably be salted out as a consequence of its greater mass, at 40% saturation, when some myosin too began to precipitate.

In concentrated urea solution (6.7 *M* urea, 0.075 *M* phosphate, pH 6.5) myosin 40–50% was found to give a water clear solution, while those for the once or twice "crystallised" myosin differed in degree of opalescence.

Reagents. The purification of urea and guanidine hydrochloride was carried out according to STEINHARDT[19] and GREENSTEIN (see NEURATH, COOPER AND ERICKSON[20]) respectively.

Physical measurements. Measurements of osmotic pressure, viscosity and protein concentration and calculation of particle weight and shape were carried out by methods described in an earlier paper (TSAO, BAILEY AND ADAIR[21]) and the same notation is employed here.

Polarisation measurements. The protein depolymerisation products were coupled with 1-dimethylaminonaphthalene-5-sulphonyl chloride at 0° and the polarisation of the fluorescent radiation of the complexes measured (G. WEBER[22,23,24]). The reciprocal of the polarisation $1/p$ was plotted against the temperature-viscosity ratio T/η, and the rotational relaxation times evaluated from the slope and intercept[22,23,24].

Electrophoresis. This was carried out in the Tiselius apparatus (Adam Hilger Ltd., London) at 4° C.

RESULTS

General considerations

It has been well known, since the pioneer work of H. H. WEBER AND STÖVER[6] and EDSALL, GREENSTEIN AND MEHL[25], that urea and a large variety of other substances cause depolymerisation of myosin. A possible, direct approach to the elucidation of the

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presence and the dimensions of subunits is to use such depolymerising media under conditions where no peptide linkage is broken and to study the resulting fragments. The pessimistic view (*cf.* SNELLMAN AND ERDÖS[26]) that, since depolymerised myosin is heterodisperse, methods such as osmotic pressure measurements give an average value which has no physico-chemical meaning is not altogether warranted. The polydispersity observed by SNELLMAN AND ERDÖS is at least in part due to the starting material, "crystalline myosin", which is impure. Besides some "denatured" myosin[18], it contains, as just shown, actin and tropomyosin. Although it is difficult to expect the degraded products of a pure myosin preparation to be homogeneous, physico-chemical measurements may nevertheless be of interest if the fragments happen to fall into distinct groups with regard to size, shape and chemical constitution. Furthermore, the observation of the course of depolymerisation as a function of time in one medium, and of pH or other variables in several media, may also yield valuable information concerning the structure of the macromolecule.

For convenience, the trend of fragmentation of myosin in a number of depolymerising media will first be described, followed by a study of the fractionation of the depolymerised units in concentrated urea solution. As depolymerisation progresses, it is possible in the first stages to effect the fractionation with ethanol and in the later with ammonium sulphate. The results obtained for the size and shape of the fragmented units from the two procedures are not identical but are complementary, and will be discussed in the above-mentioned order. Lastly, some preliminary results on attempts to degrade myosin to tropomyosin and to separate adenosine triphosphatase from myosin will be described.

Trend of depolymerisation of myosin

The following depolymerising media were used: concentrated guanidine solution (5.7 *M* guanidine HCl, 0.031 *M* Na_2HPO_4, 0.019 *M* KH_2PO_4, pH 5.4), dilute acid (0.1 *N* HCl, pH 1.25) and dilute alkali (0.104 *M* H_3BO_3, 0.1 *N* NaOH, pH 10.7; 0.2 *M* NaCl, 0.1 *N* NaOH, pH 13), as well as concentrated urea solution (6.7 *M* urea, 0.01 *M* Na_2HPO_4, 0.062 *M* KH_2PO_4, pH 6.5). On the acid side of the isoelectric point, the solubility of myosin is decreased in the presence of neutral salts, and opalescent or gel-like solutions were obtained. For the dilute acid medium, 0.1 *N* HCl alone was therefore chosen to yield a true solution. The solutions were kept at 0° and the average particle weights determined by osmotic pressure measurements. The electrophoretic behaviour of the products was examined after removing from the osmometers, and to ensure that no peptide bonds had been split during the period of the experiments, an assay of the terminal amino groups was also carried out (BAILEY[15]) both before and after the treatments.

The results of osmotic pressure measurements are given in Figs. 2, 3, 4 and 5 and the calculated particle weights summarised in Table I. Some viscosity data are also included. For comparison, some results obtained for that fraction of twice "crystallised" myosin precipitating between 30–50% saturation (*i.e.* rejecting the precipitate which first produces a turbidity) and for unfractionated myosin are also included.

For convenience, the significance of the values for the average particle size of depolymerised myosin in relation to subunit weight will be considered later. It may be of interest here to make the following observations on the qualitative trend of depolymerisation.

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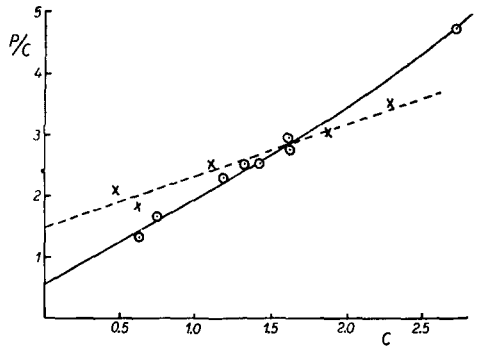

Fig. 2. Osmotic pressure of myosin in concentrated guanidine hydrochloride solution at 0° C. Solvent: 5.7 *M* guanidine HCl, 0.031 *M* Na_2HPO_4, 0.019 *M* KH_2PO_4, pH 5.4; —O— myosin 40–50%, – – × – – myosin 30–50%.

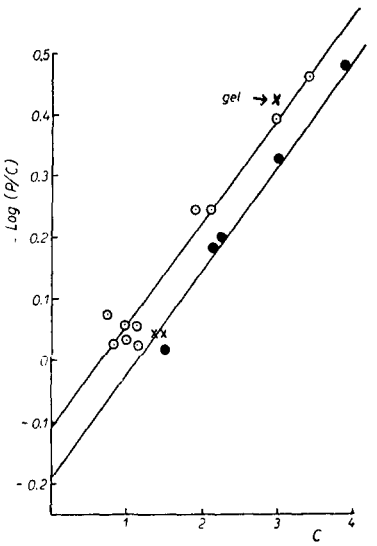

Fig. 3. Osmotic pressure of myosin in acid solution at 0° C. Solvent: 0.1 *N* HCl, pH 1.25; O myosin 40–50%, × myosin 30–50%, × myosin unfractionated.

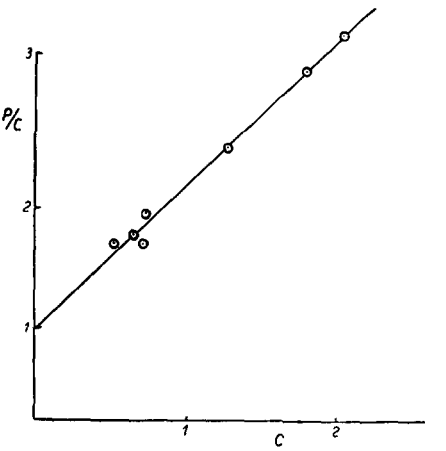

Fig. 4. Osmotic pressure of myosin in dilute alkali at 0° C. Solvent: 0.104 *M* boric acid, 0.1 *N* NaOH, pH 10.7.

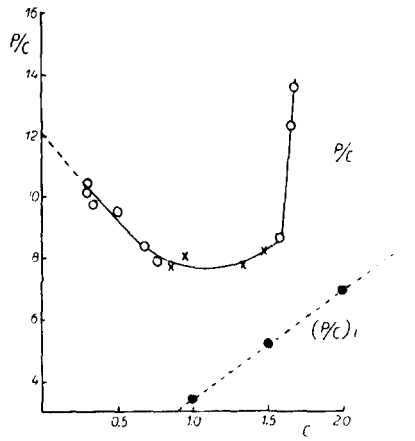

Fig. 5. Osmotic pressure of myosin in alkali at pH 13, 0° C. Solvent: 0.1 *N* NaOH, 0.2 *M* NaCl; O preparation (a), × preparation (b) –●– ion pressure difference across the semipermeable membrane.

TABLE I

AVERAGE PARTICLE WEIGHT OF DEPOLYMERISED MYOSIN BY OSMOTIC PRESSURE MEASUREMENTS

Myosin preparation	*Medium*	*pH*	*Duration of treatment in days*	$\left(\frac{\eta sp}{C}\right)_{c=o}$	$\left(\frac{P}{C}\right)_{c=o}$	*Particle weight*
Myosin 40–50%	acid	1.25	6	—	0.786	217,000 ± 16,000
	alkali	10.7	10	—	1.003	170,000 ± 5,000
	alkali	13	26–30	—	12.0	14,000
	guanidine	5.4	14	—	0.578	294,000 ± 10,000
	guanidine	5.4	750	0.81	—	—
	urea	6.5	60	1.12	—	—
	urea	6.5	240–485	0.61–0.70	—	—
Myosin 30–50%	acid	1.25	6	0.646		264,000
	guanidine	5.4	14	1.55		110,000

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1. Under identical conditions the osmotic behaviour of myosin precipitating between 40–50% saturation is different from that which includes the 30–40% fraction. In guanidine hydrochloride, the average particle weight and osmotic pressure-concentration dependence of the latter is smaller than that of the former; while in acid, the reverse is true. This may perhaps be attributed partly to the presence of actin impurity in the 30–40% fraction, and partly to some "denatured" or aggregated myosin which is dissociable by guanidine but not by acid. For unfractionated, twice "crystallised" myosin, the solution was slightly opalescent; the osmotic pressure-concentration dependence was even higher; and at 3% concentration, the solution set to a gel.

2. The depolymerisation of myosin 40–50% in concentrated urea solution seems to be a slow process. SNELLMAN AND ERDÖS[26] have shown that on treating "crystalline" myosin with concentrated urea solution for an hour, sedimentation-diffusion measurements indicated that the molecules possessed the same molecular weight but had become less symmetrical. After seven days' treatment, components with sedimentation constants less than 16 appeared as well.

As intrinsic viscosity or hydration are functions of the axial asymmetry of the particles, which may arise from either fragmentation or aggregation and from a change in configuration, it offers a qualitative measure as to the extent of changes in depolymerising media. It can be seen from Table I that changes continued after 60 days treatment at room temperature, and only appeared to reach a steady state after about 6 months. The solubility behaviour in urea-ethanol mixtures likewise showed the same trend. When myosin was freshly dispersed in the 6.7 *M* urea-phosphate buffer, the addition of 10–15 vol % of ethanol caused precipitation of the protein. This ethanol precipitation "threshold" was found to increase to about 30% after 1–2 months and to 65% after 6 months. Between 6 and 18 months there seemed to be no change. The lowest value of intrinsic viscosity reached was about 0.6–0.7, pertaining to particles of very high asymmetry, with axial ratios in the neighbourhood of 30–40.

3. Urea treated myosin showed a very strong tendency for gel formation. When the duration of urea treatment was lengthened, the strongly synaeresing gel, formed after dialysing away urea, gradually became limpid, and after six months, true solutions in water were obtained. These aqueous solutions of depolymerised myosin were viscous, showing no flow birefringence. On addition of neutral salts, the clear solutions became faintly turbid accompanied by a drop in viscosity. The magnitude of this drop, in contrast to that of tropomyosin denatured in urea, was rather small. For a 0.7% solution, the fall was from a relative viscosity of 3.49 to 1.37. It is therefore likely that this is due primarily to the reduction of electroviscous effects.

4. Under the conditions used in the present investigation, guanidine hydrochloride appeared to be less effective than urea as a depolymerising agent, although usually the reverse is true. After two years treatment in guanidine HCl at room temperature, the average intrinsic viscosity of myosin was higher than that reached in urea after comparatively much shorter treatments. After dialysing away the guanidine, the protein formed an opalescent gel.

5. Dilute alkali appeared to be a more effective depolymerising agent than either guanidine hydrochloride or dilute acid. At pH 10.7 end-group analysis revealed that there was no hydrolysis of peptide bonds.

The osmotic behaviour at pH 13 (Fig. 5) was rather peculiar. In the osmotic pressure determinations, the pressure showed a steady rise; the apparent equilibrium was not reached until after about four weeks at 0° C and peptide bonds were split; the N-terminal residues preferentially liberated were threonine, serine and the dicarboxylic acids (BAILEY[15]). The peculiar shape of the curve deserves special mention. The negative slope in the dilute region indicates dissociation by dilution. A positive slope may arise from particle asymmetry, solute-solute interaction and unequal distribution of ions across the semi-permeable membrane. U-shaped osmotic pressure curves have been obtained by Mr. ADAIR (personal communication) with human and horse serum albumin in acetate buffers of low ionic strength. They were also observed with actin in 0.6 *M* KI (TSAO[27]). The contribution of the charge effect can be estimated according to ADAIR AND ROBINSON[28] and is also plotted in Fig. 5. It is seen that this is far from being adequate in contributing to the extreme steepness of the right hand side of the curve. With these short fragments of average particle weight 14,000 the entropy effect and the solute-solute interactions would be expected to be rather low. The almost vertical trend of the curve therefore remains puzzling.

Fractionation of depolymerised myosin with urea-ethanol

Myosin was dispersed in concentrated urea solution (6.7 *M* urea, 0.062 *M* KH_2PO_4, 0.0127 *M* Na_2HPO_4, pH 6.5) and kept for over a month at room temperature. In a series of test tubes, ethanol and the protein solution were mixed with stirring in different ratios, keeping the final volume at 3 ml. After centrifuging, 2 ml of each supernatant were pipetted out and the protein remaining in solution precipitated with excess of

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ethanol. The precipitate was washed six times with 10 ml portions of ethanol which removed all the urea. It was transferred quantitatively to a micro-Kjeldahl flask and the total nitrogen determined. The precipitation follows a course as given in Fig. 6, where the fractional precipitation of protein by ethanol, $\frac{-\mathrm{d}N}{\mathrm{d}(\mathrm{Ethanol})}$ (N being mg nitrogen/2 ml supernatant; (Ethanol) in volume %), was plotted as a function of the volume per cent of ethanol added. It is seen that depolymerised myosin was resolved into two distinct fractions, *i.e.* I, that precipitated between 20–35% ethanol and II, that between 40–70%.

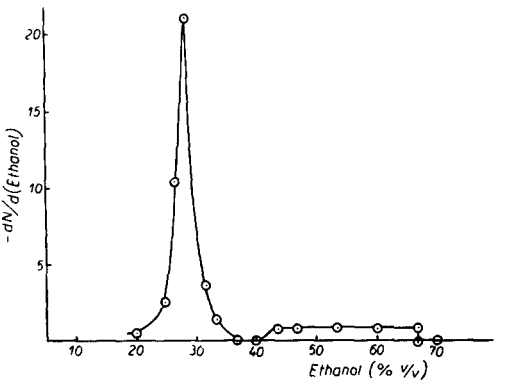

Fig. 6. Fractionation of urea-denatured myosin 40–50% with ethanol at 20° C.

For large scale fractionation, ethanol was added at 20° C with efficient stirring to a solution of myosin in concentrated urea until the ethanol concentration reached 40% by volume. The precipitate was separated on the centrifuge, and the supernatant brought to 70% with further addition of ethanol. Both ethanol and urea were removed by dialysis against water in the ice-chest.

Fraction I after dialysis was a strongly synaeresing gel, the aqueous phase of which gave a negative test for protein with trichloroacetic acid or by application of the biuret reaction. The 40–70% fraction was made up of some residual I and the aqueous phase contained a water-soluble protein (II). From the fractionation curve it can be estimated that the 40–70% fraction accounted for 18% of the total protein, and of this, roughly one quarter was in the aqueous phase. The water-soluble II therefore constituted 4–5% of the original myosin.

TABLE II

THE PHOSPHORUS AND NITROGEN CONTENT OF DEPOLYMERISED FRAGMENTS OF MYOSIN ON UREA-ETHANOL FRACTIONATION

	Myosin 40–50%	*I*	*II*
P (%)	0.05	0.032	0.023
N (%)	16.2	16.1	15.6

Some properties of the depolymerised fragments. The total N and P of the various fractions, freeze-dried and then dried at 120° C, were determined and given in Table II.

On dialysis against water I precipitated as large lumps of sponge-like material from which the water could be squeezed out to give a denatured coagulum. II presented some rather unusual properties. A neutral solution of II, either in water or 0.1 *M* KCl, was stable to heat. On the acid side of the isoelectric point, which was near 5, the presence of salt caused the protein to become permanently insoluble. However, in the absence of salt, the fragment was stable to acid. At room temperature, 10 vol of ethanol could be added to an aqueous solution of II without inducing precipitation. By ammonium sulphate fractionation at pH 7 the protein was precipitated at 48–61% saturation.

It is interesting to note that the solubility characteristics of II follow closely those

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of urea-denatured actin. But the quantity present in myosin (4–5%) seems too great to be considered in terms of an actin contaminating the parent myosin. To disprove this possibility, actin was treated in urea for over a month under the same conditions used for myosin, precipitated with ethanol and dialysed. Compared with II, two differences stood out: (1) Denatured actin, like untreated actin, showed a UV absorption maximum at 262 mμ. This is due to the presence of nucleotide prosthetic groups (STRAUB AND FEUER[29], LAKI, BOWEN AND CLARK[30]), which remained as an integral part of actin after urea denaturation. Of the eight preparations of II tested, only one gave the 262 mμ maximum, the rest a broad maximum near 275 mμ. It is likely that the one exception might arise from the presence of free actin or other nucleotide-containing protein impurities. (2) On ultrafiltration under pressure, the water soluble II passed freely through a collodion membrane which retained both native and denatured actin. Indeed osmotic pressure measurements to be described in this paper revealed that the size of II differs considerably from those of actin (TSAO[27]).

Structure of II. The electrophoretic diagram of II in phosphate buffer of pH 6.5 (0.02 *M* Na_2HPO_4, 0.04 *M* NaH_2PO_4, 0.2 *M* KCl) is shown in Fig. 7. There was some boundary spreading, suggesting a

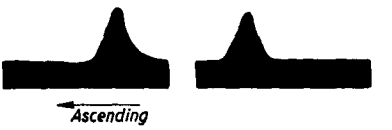

Fig. 7. Electrophoretic diagram of subunit II of myosin. In 0.02 *M* Na_2HPO_4, 0.04 *M* NaH_2PO_4, 0.2 *M* KCl, pH 6.5; overall voltage 35 V, 12 mA $4^3/_4$ hours.

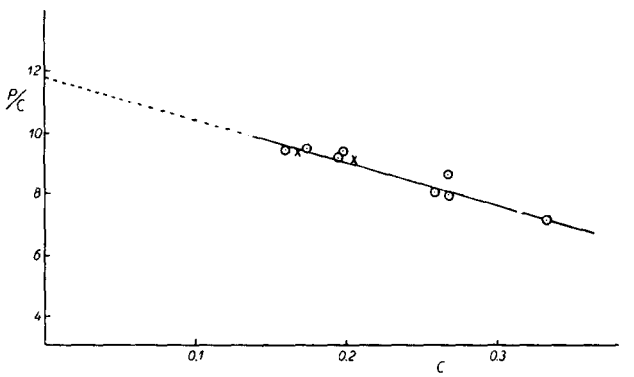

Fig. 8. Osmotic pressure of subunit II of myosin at 0° C. Solvent: as in Fig. 7; O× two different preparations.

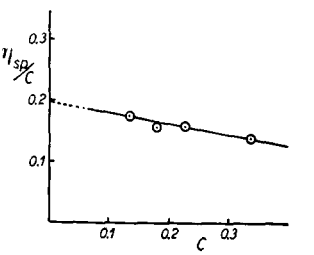

Fig. 9. Viscosity of subunit II of myosin at 20° C. Solvent as in Fig. 7.

polydisperse system, and the main peak constituted over 95% of the total area. The deduction of particle dimensions from osmotic pressure of such a system would nevertheless serve to give an indication of the order of magnitude of the particle sizes.

Visking cellophan, found to be impermeable to the protein, was employed for osmotic pressure determinations. The results are given in Fig. 8 and the viscosity data are represented in Fig. 9. The extrapolated value of $(P/C)_{c=0}$, 11.8, indicates an average particle weight of 14,000; and the value of $(\eta sp/C)_{c=0}$, 0.20, gives, assuming a probable hydration of 30%, an approximate axial ratio of 12.

Structure of I. Osmotic pressure measurements were carried out in concentrated urea solution. ANSON[31] has shown that urea catalyses the oxidation of -SH groups, and it was thought that the addition of a reducing agent such as thioglycollate might prevent the linking up of particles through -S-S-linkages. The composition of the buffer was as follows: 6.7 *M* urea, 0.1 *M* Na_2HPO_4, 0.05 *M* thioglycollic acid, 0.1 *M* KCl,

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pH 7.0. The results are given in Fig. 10. The value $(P/C)_{c=0} = 1.39$ gives an average particle weight of 122,000 ± 6,000.

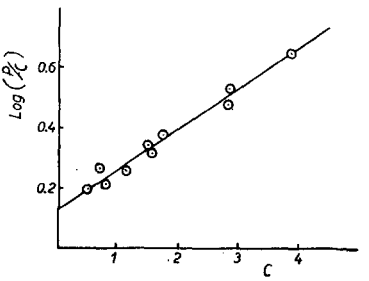

Fig. 10. Osmotic pressure of I (see text) in concentrated urea solution at 0° C. Solvent 6.7 *M* urea, 0.1 *M* Na_2HPO_4, 0.05 *M* thioglycollic acid, 0.1 *M* KCl, pH 7.0; straight line log $(P/C) = 0.1275C + 0.1444$.

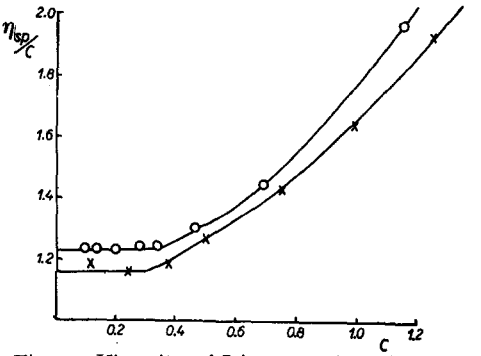

Fig. 11. Viscosity of I in concentrated urea at 20° C. Solvent: 6.7 *M* urea, 0.1 *M* Na_2HPO_4, 0.1 *M* KCl, pH 7.0. —O— with 0.05 *M* thioglycollic acid; —×— without thioglycollic acid.

Viscosity measurements are given in Fig. 11. The viscosity behaviour is the same either in the presence or in the absence of thioglycollate, therefore ruling out changes involving -SH groups. The respective values of $(\eta sp/C)_{c=0}$, 1.24 and 1.18, indicate, assuming 30% hydration, an approximate axial ratio 40.

No X-ray data are yet available on the intramolecular pattern of the fragments, nor was there other independent information about the shape factor.

Fractionation of depolymerised myosin with ammonium sulphate

In the foregoing urea-ethanol fractionation scheme, myosin had been left in concentrated urea solution for 1 month. When the urea treatment was extended beyond two months, the solubility behaviour of myosin underwent further changes, and a steady state was not reached until after six months. The electrophoretic pattern of such a system consists of two peaks: one large and sharp, the other small and diffused. At this stage all the protein components began to precipitate at 65% ethanol so that the ethanol urea fractionation procedure was no longer practical. It was found that, on dialysing away urea, fractionation could successfully be effected with ammonium sulphate. This was carried out by adding a saturated ammonium sulphate solution, the pH of which had previously been adjusted to 6.5, to the aqueous solution of depolymerised myosin under constant stirring. The salting out process appeared to be in two stages: 20–35% and 45–65%. The following fractions were therefore collected: A 0–40% and B 40–100%. The relative amounts of these fractions for two myosin preparations are given in Table III.

TABLE III

RELATIVE ABUNDANCE OF DEPOLYMERISED FRAGMENTS OF MYOSIN BY UREA TREATMENT AND AMMONIUM SULPHATE FRACTIONATION

Fraction	*Ammonium sulphate saturation*	*Relative amount (%)* *Experiment 1*	*Experiment 2*
A	0–40%	93	91
B	40–100%	7	9

Exp. 1: kept in urea for 12 months. Exp. 2: kept in urea for 16 months.

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Some properties of the depolymerised fragments. The aqueous solution of A is viscous. The properties of B are identical with those of II of the urea-ethanol procedure. When coupled with I: dimethylaminonaphthalene-5-sulphonyl chloride, A showed an orange-yellow fluorescence whereas B gave a greenish-yellow fluorescence.

Size and shape of A. Osmotic pressure measurements were carried out in a borate buffer of pH 10.7 (0.104 *M* boric acid, 0.1 *N* NaOH, 0.1 *M* KCl) and the results are given in Fig. 12. The value of $(P/C)_{c=0}$ = 1.03 gives an average particle weight of 165,000. The shape of the osmotic pressure-concentration curve is interesting. At $C = 2.1\%$ the solution became a gel. It is likely that partial association begins at a much lower concentration and would be reflected in a negative slope for the $P/C - C$ curve. At lower concentrations this effect may be exactly balanced by the contribution to a positive slope due to such factors as particle asymmetry and the unequal distribution of ions across the semi-permeable membrane; with the result that the concentration dependence becomes negligible.

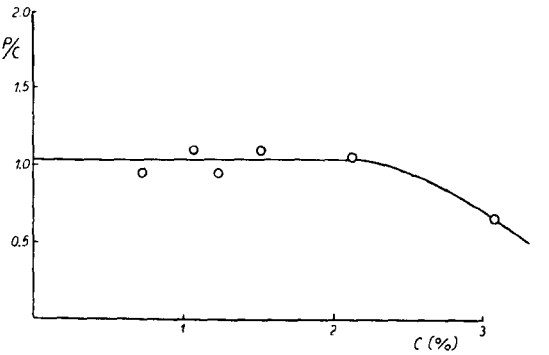

Fig. 12. Osmotic pressure of subunit A of myosin at 0° C. Solvent: 0.104 *M* boric acid, 0.1 *N* NaOH, 0.1 *M* KCl, pH 10.7.

The results of viscosity measurements are given in Fig. 13. The extrapolated value of $\eta sp/C_{c=0} = 0.7$ gives, assuming a probable hydration of 30%, an approximate axial ratio 30.

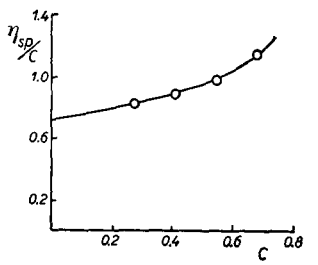

Fig. 13. Viscosity of subunit A of myosin at 20° C. Solvent as in Fig. 12.

The results of fluorescence-polarisation measurements in water and in the borate buffer at pH 10.7 are given in Fig. 14 and the evaluation of the harmonic mean of the principle relaxation times of rotation ϱ_h, the molecular volume P and the particle size *M* from the slope *S* and intercept $1/p_0$ is summarised in Table IV. The correction factor ϱ_0/ϱ_h, where ϱ_0 is the relaxation time of a sphere of volume equal to that of the protein fragments, are taken from G. WEBER[22,24]. With axial ratios near 30 the factor is practically the same as the limiting value of 3/8 for an ellipsoid of infinite elongation.

In water, A appeared to be of such a large size that the evaluation by means of fluorescence-polarisation technique proved to be unprofitable. In the borate buffer of pH 10.7, there seemed to be some dissociation of the protein-fluorescent dye complex at higher temperatures, giving rise to the

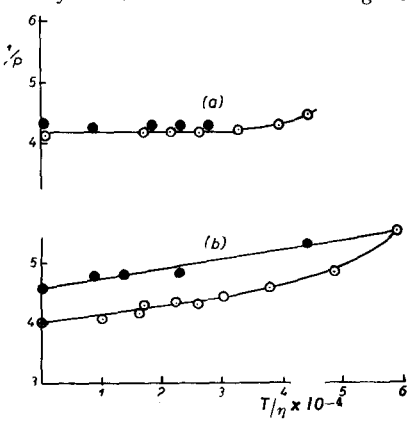

Fig. 14. Fluorescence-polarisation of subunit A of myosin labelled with 1-dimethylaminonaphthalene-5-sulphonyl chloride (a) in water at pH 7; (b) in 0.104 *M* boric acid, 0.1 *N* NaOH, 0.1 *M* KCl, pH 10.7.

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TABLE IV

AVERAGE PARTICLE WEIGHT OF DEPOLYMERISED FRAGMENTS OF MYOSIN BY FLUORESCENCE-POLARISATION MEASUREMENTS

Fraction	*Condition*	*Slope* $S \cdot 10^4$	$1/p_0$	$\varrho_h \cdot 10^8$*	a/b**	ϱ_0/ϱ_h	$\varrho_0 \cdot 10^8$	$V \cdot 10^5$	d***	M
A	Water, pH 7	0	4.21	—	30	0.375	—	very large		very large
A	Borate, pH 10.7, cooling	0.166	4.62	37.9	30	0.375	14.2	1.31	1.35 1.25	177,000 164,000
A	Borate, pH 10.7, heating	0.140	4.02	39.4	30	0.375	14.8	1.36	1.35 1.25	184,000 170,000
B	Water, pH 7, (initial slope)	2.09	5.32	3.43	12	0.410	1.4	0.129	1.35 1.25	17,400 16,000

* calculated for 25° C.
** from viscosity measurements.
*** assuming density of protein $d = 1.35$ (partial specific volume 0.74). Upper value calculated for anhydrous particles, lower value for particles with 30% hydration.

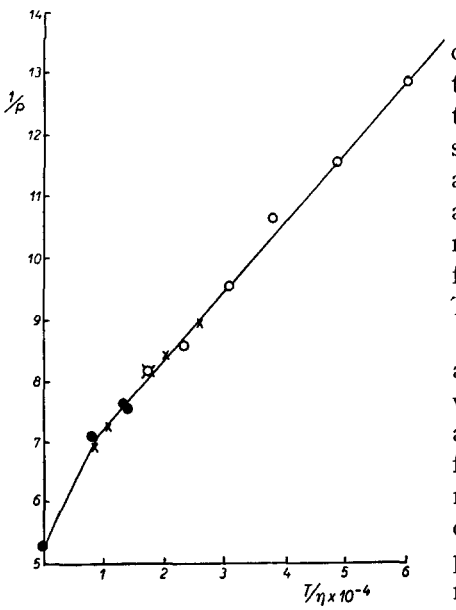

Fig. 15. Fluorescence-polarisation of labelled subunit B of myosin in water.
○ polarisation on heating
● polarisation on cooling
× polarisation in 20% sucrose.

curvature of the heating-up curve. The values for the particle weight, deduced from the slope and the intercept of the cooling curve and the initial slope and intercept of the heating curve, are 164,000 and 170,000 respectively. As these are number averages derived from the harmonic mean ϱ_h, the magnitude is directly comparable to that obtained from osmotic pressure measurements, *i.e.*, 165,000. The agreement seems to be satisfactory.

Size of B. The solubility, viscosity, stability and ultrafiltration behaviours of B are identical with those of II of the urea-ethanol procedure, and there were no osmotic pressure data on this fraction. The fluorescence-polarisation data are represented in Fig. 15. The curve shows a definite curvature towards the T/η axis, an indication of polydispersity. The initial slope gives the harmonic mean for the relaxation times while the limiting slope at higher values of T/η the arithmetic mean (WEBER[22]). The average particle weight deduced from the harmonic mean for the relaxation times is 16,000.

Subunits of myosin

The foregoing qualitative and quantitative results on the depolymerisation of myosin, obtained under a wide variety of conditions, are of interest in the elucidation of the structure of the subunits of myosin. The seeming confusion can readily be disentangled if it is accepted that the action of urea is to split off the fragments of small

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particle weight from the myosin molecule, leaving the skeleton intact in size, though more hydrophilic and less asymmetric; while the action of dilute alkali is to depolymerise the skeleton into units on which the small fragments are still firmly attached. The first reaction is very slow, whilst the latter appears to be almost instantaneous. The reason for this very marked difference is not yet clear. Since both processes involve no cleavage of peptide bonds and since the value for the particle sizes obtained are minimal and reproducible, it is highly probable that the 15,000 and the 165,000 fragments are in fact the subunits of myosin.

The foregoing results, together with some subsidiary experimental evidence, can now be examined in this light.

The smaller subunits of myosin. It has not been possible to obtain the small units in any other depolymerising media besides urea (and guanidine). After treatment in urea for one or two months, *i.e.* at the stage where the ethanol procedure is applicable, there are some 4% of the small fragments liberated. This amount is increased to 8% on further prolonged treatment, when the properties of the remaining framework have been so altered that the ammonium sulphate procedure has to be adopted to effect fractionation.

The value of 16,000 for the size of B derived from fluorescence-polarisation measurements agrees well with that deduced from osmotic pressure determinations, 14,000. Viewed in conjunction with the results of end-group assay for II[32] which revealed approximately one terminal amino residue for 16,000 g of protein, it is most probable that the small fragments depleted from myosin upon urea treatment consist of particles of average weight 16,000. The heterogeneity as revealed by electrophoresis and fluorescence-polarisation is further demonstrated[32] by the presence of several kinds of terminal residues. Although the physico-chemical interpretation of the results can in no sense be exact, nevertheless, from the molecular point of view, it is significant that the particle size lies within a rather narrow range around a value markedly different from that of the original molecule or of the other depolymerised fragments. The component particles are, in addition, characterised by common properties such as the relative stability to heat.

The larger subunits of myosin. Fraction A obtained by ammonium sulphate fractionation of urea-treated myosin consists of elongated particles of average weight 165,000. It is interesting to note that this value is identical with that for myosin 40–50%, also in borate buffer at pH 10.7. In this latter case there was no evidence that any small molecular weight fragments, like II, were split off from the macromolecule. As B constitutes about 8% of the total weight of protein, it can be calculated that for every 165,000 fragment, there is one of 16,000 which would not sensibly alter the magnitude of the size of the former. The agreement between the two series of experiments is therefore not fortuitous. Depleted of the small fragments, the skeleton showed 1 terminal residue per 900,000 g of protein and it is therefore possible that the 165,000 units are cyclic (BAILEY[15]).

Other values for the size of the depolymerised units. The higher values for the average particle weight obtained for depolymerised myosin 40–50% in acid, 217,000, and in guanidine, 294,000, probably indicate incomplete depolymerisation, and the lower value for I in urea, 122,000, (*i.e.* myosin 40–50% in which 4% of the small fragments have been removed) can be understood partly in terms of the 165,000 units accompanying some of the remaining 4% of 16,000 units which become dissociated in the course of osmotic pressure measurements.

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Tropomyosin as a subunit of myosin. The striking similarity between myosin and tropomyosin in amino acid composition and physico-chemical characteristics, as well as its mode of occurrence at the same histological site and its ability to aggregate into fibres have led to the suggestion (BAILEY[33, 34]) that tropomyosin may be one of the ultimate subunits of which myosin is composed and elaborated. This postulate implies two aspects which are amenable to experimental approach, *i.e.*, either tropomyosin may be a "building stone" in readiness to form the myosin complex, or it may be a breakdown product from such a complex. If the former view is correct, it might be expected that in embryonic muscle, where the elaboration of the myofibril and the component protein molecules is at an initial stage, tropomyosin would be in relative abundance. Current work of ROBINSON[35] on chick embryo muscle has shown, however, that there is no detectable difference of the relative ratio of myosin and tropomyosin in embryonic and adult muscles. With regard to the breakdown concept, BAILEY[34] has shown, on the basis of the quantitative aspect of the extraction of tropomyosin from muscle, that the possible effect of salt solutions in the production of tropomyosin is unlikely.

The following considerations may be of interest in this connection: (1) From the foregoing results, it appears that none of the depolymerised units fall near 53,000, the size of tropomyosin. Unlike tropomyosin, whether native or denatured in urea, the fragments do not exhibit any tendency to associate in aqueous media.

(2) When ethanol-ether dried myosin, prepared according to BAILEY[36], was treated with the procedure for tropomyosin extraction, a small amount of material was obtained, which, if tentatively considered to be tropomyosin, amounted to 0.1% of the myosin taken (BAILEY[34]). It was mentioned in a previous section that once "crystallised" myosin contained about 0.9% tropomyosin. In twice "crystallised" myosin, this was found to be absent. When twice "crystallised" myosin, ethanol-ether or butanol-acetone dried, was extracted with *M* KCl, *M* KI, and *M* LiCl at pH 7 or 13, some 2–17% of protein went up into solution. There was no evidence that tropomyosin was present.

(3) It has been possible to isolate and crystallise nucleotropomyosins (TSAO[37]), first demonstrated to be co-existent with tropomyosin in fish muscle by HAMOIR[38], from skeletal as well as smooth and cardiac muscles. The complex isolated from rabbit skeletal muscle was found to be stable to the action of strong salt solutions, dilute acid and alkali, and organic solvents such as ethanol, ether and acetone. Quantitative extraction suggests that all the tropomyosin exists in muscle as the nucleo-complex. It may be that there is a dynamic aspect of the biological role of tropomyosin quite apart from the suggested possibility as the structural "monomer" of the macromolecular myosin.

Attempts to separate adenosine triphosphatase from myosin. The attempt to separate ATPase from myosin has so far been unsuccessful (see reviews by ENGELHARDT[39], and by NEEDHAM[40]). MORTON[41] claimed an apparent separation of the Ca-activated enzyme from myosin into a salt free solution using butanol treatment. His experiments have been repeated and greatly extended (MORTON AND TSAO[42], TSAO[43]). The results indicated that the native properties of myosin, such as the solubility in 0.5 *M* KCl, and ATPase activity always run parallel. In the presence of butanol, inactivation and denaturation occur unless protected with ATP or actin. The residual activity observed in a salt free medium after butanol treatment can be adequately interpreted as arising from a trace of the undenatured myosin which remains in solution. There is no positive indication therefore that a small molecule of ATPase has been detached from myosin.

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DISCUSSION

The present investigation on the depolymerisation of a purified preparation of myosin under a wide range of conditions, coupled with the end group assay applied by Dr. K. BAILEY provides unambiguous evidence for the existence of subunits in myosin. By applying ethanol and ammonium sulphate fractionation, it has been possible to isolate and characterise two types of subunits: 92% of possibly cyclic fragments of average particle weight 165,000 and axial ratio $\sim$ 30 and 8% open chain peptide of average particle weight 16,000 and axial ratio $\sim$ 12, containing an average of 1 terminal amino group per chain. These two types of fragments were liberated under different conditions, an indication that perhaps more than one kind of secondary bond is involved in cementing the subunits together. Accepting the best value, obtained by PORTZEHL, SCHRAMM AND WEBER[2] for myosin, 840,000, it appears that there are 4–5 each of the subunits per molecule.

It is generally believed that urea or guanidine disrupt secondary linkages such as the H-bonds between the side chains by competing with the amidinium ions or between or along the backbones by competing with the imino groups. If this is so, it is difficult to imagine why the depolymerisation of myosin in these reagents is so slow. STANLEY AND LAUFFER[44] also reported a large difference in the average particle weight of tobacco mosaic virus depolymerised in concentrated urea solution for 5 days (100,000) as compared with that for 4 weeks (40,000). It is not certain whether the slowness of depolymerisation is restricted to the more complicated macro-molecules such as myosin and tobacco mosaic virus; but it seems that in the earlier studies of the subunits or protein by fragmentation (see review by NEURATH, GREENSTEIN, PUTNAM AND ERICKSON[45]), the importance of the time factor appears not to have been sufficiently stressed.

The attempt to separate ATPase from myosin has not been successful. It remains for future work to demonstrate whether it is possible to obtain the 16,000 units in "native" form in order to characterise unambiguously their chemical and enzymic (if any) nature. The possibility also exists that these may be primitive chains in the biological elaboration of the macromolecular myosin.

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SUMMARY

1. Evidence is given for the existence of actin and tropomyosin impurities in "crystalline" myosin.

2. The existence of subunits in myosin has been demonstrated by studying the depolymerisation of purified myosin in concentrated urea and guanidine hydrochloride solutions, dilute acid and alkali. Collateral data from end-group assay (BAILEY[15]) show that in all cases no peptide bonds are split except in alkali at pH 13.

3. The fractionation of subunits of myosin has been effected in a medium of urea and ethanol or with ammonium sulphate after complete depolymerisation in urea; and the size and shape of the fragments studied with osmotic pressure, viscosity and fluorescence-polarisation techniques.

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4. Purified myosin appears to contain two types of subunits: about 92% of highly asymmetric units of average particle weight 165,000 which may be cyclic, and about 8% of units of average weight 16,000 and axial ratio ~ 12.

5. The small fragments could only be detached from the myosin macromolecule by treatment with urea, but not by alkali or acid, while maximum depolymerisation of the myosin "skeleton" seems to occur at pH 10.7.

6. The depolymerisation of myosin with 6.7 *M* urea or 5.7 *M* guanidine hydrochloride is a very slow process. At 20° C, pH 6.5, apparent equilibrium was not reached until after several months treatment in urea. Guanidine hydrochloride appears to be less effective.

7. There is no evidence as yet that tropomyosin forms a part of the myosin molecule, nor has it been possible to separate the adenosine triphosphatase activity.

RÉSUMÉ

1. La myosine "cristallisée" renferme de l'actine et de la tropomyosine à l'état d'impuretés.

2. La dépolymérisation de la myosine purifiée, dans des solutions concentrées d'urée et de chlorhydrate de guanidine, ou dans les acides ou les alcalis dilués, montre qu'il existe des subunités dans la myosine. Des déterminations de bouts de chaînes, effectuées parallèlement à ce travail (BAILEY[15]) indiquent que dans aucun cas il n'y a de rupture de liaisons peptides, sauf dans les alcalis à pH 13.

3. Après dépolymérisation complète dans l'urée, les subunités de myosine sont fractionnées en présence d'urée et d'éthanol ou de sulfate d'ammonium. La taille et la forme des fragments ont été étudiées par pression osmotique, viscosité et fluorescence-polarisation.

4. La myosine purifiée contient deux types de subunités: 92% des particules sont fortement asymétriques, de poids moyen 165,000 et peut-être cycliques; 8% ont un poids moyen de 16,000 et un rapport axial ~ 12.

5. Les fragments de petite taille sont détachés de la macromolécule de myosine, uniquement par traitement à l'urée, et non par les alcalis et les acides, alors que la dépolymérisation maximum du "squelette" de la myosine se produit à pH 13.

6. La dépolymérisation de la myosine par l'urée 6.7 *M* ou le chlorhydrate de guanidine 5.7 *M* est très lente. A 20° C, et pH 6.5, l'équilibre apparent n'est pas atteint après plusieurs mois de traitement par l'urée. Le chlorhydrate de guanidine semble moins efficace.

7. Il n'est pas encore prouvé que la tropomyosine fasse partie de la molécule de myosine. Il n'a pas été possible non plus de séparer l'activité adénosine-triphosphatasique.

ZUSAMMENFASSUNG

1. Die Existenz von Aktin und Tropomyosinverunreinigungen in "kristallisiertem" Myosin wurde augenscheinlich gemacht.

2. Die Existenz von Untereinheiten in Myosin wurde durch die Untersuchung der Depolymerisierung von gereinigtem Myosin in konzentrierten Harnstoff- und Guanidin-hydrochloridlösungen, verdünnter Säure und Alkali gezeigt. Gleichlaufende Daten der Endgruppen-bestimmung (BAILEY[15]) zeigen, dass in allen Fällen, ausser in Alkali bei pH 13, keine Peptidbindungen gesprengt werden.

3. Die Fraktionierung der Untereinheiten des Myosins wurde in Harnstoff und Äthanolmedium oder mit Ammoniumsulfat nach der vollständigen Depolymerisation in Harnstoff bewirkt und die Grösse und Form der Bruchstücke durch osmotische Druck-, Viskositäts- und Fluoreszenzpolarisationsmessungen untersucht.

4. Gereinigtes Myosin scheint zwei Typen von Untereinheiten zu enthalten; ungefähr 92% von höchst asymmetrischen Einheiten mit einem durchschnittlichen Teilchengewicht von 165,000, die cyclisch sein können, und ungefähr 8% von Einheiten mit einem Durchschnittsgewicht von 16,000 und einem Achsenverhältnis von ~ 12.

5. Die kleinen Bruchstücke konnten nur durch Behandlung mit Harnstoff vom Myosinmakromolekül losgelöst werden, aber nicht mit Alkali oder Säure, während die maximale Depolymerisation des Myosin-"Skeletts" scheinbar bei pH 10.7 stattfindet.

6. Die Depolymerisation des Myosins mit 6.7 *M* Harnstoff oder 5.7 *M* Guanidin-hydrochlorid ist ein sehr langsam verlaufender Prozess. Das augenscheinliche Gleichgewicht wurde erst nach einer mehrmonatigen Behandlung mit Harnstoff bei 20° C und pH 6.5 erreicht. Guanidin-hydrochlorid scheint weniger wirksam zu sein.

7. Bis jetzt liegt noch kein Beweis vor, dass Tropomyosin einen Teil des Myosinmoleküls bildet, noch war es möglich gewesen, die Adenosintriphosphataseaktivität abzutrennen.

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