THE ROLE OF SULPHYDRYL GROUPS IN THE INTERACTION OF MYOSIN AND ACTIN

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Much interest has recently been centred upon the work of the Szeged school under SZENT-GYÖRGYI concerning the interaction of myosin and a new protein, actin, first isolated by STRAUB[1]. Actin appears to be a fibrillar component which is extracted from minced muscle either in the form of an actin-myosin complex, or in its free form from muscle mince which, after appropriate treatment, is dried in acetone and then extracted with water. Of the nature and composition of actin, little is known. When first extracted, the aqueous solution is considered to contain corpuscular molecules which aggregate to a fibrous form on addition of salt (STRAUB[2]). This form of actin (F-actin) interacts with myosin in 0.5 *M*-KCl to give a system of greatly enhanced viscosity with all the properties of a weak gel. The addition of adenosinetriphosphate (ATP) ($\geqslant$ 0.00002 *M*) produces a sudden liquefaction and the viscosity falls to the additive effect of that of the two components. When, however, the actomyosin is precipitated as a filament in solutions of low ionic strength, the effect of ATP is to produce an *isodimensional* shrinkage or synaeresis. Although both effects will be discussed, only the interaction occurring at higher salt concentrations has been studied here. These phenomena are described in the detailed reports 'Studies from the Institute of Medical Chemistry University Szeged, S. KARGER: Basle and New York, 1941-2, 1942, 1943, Volumes 1, 2 and 3', and in several reviews (SZENT-GYÖRGYI[3, 4, 5]).

Electron microscope studies (PERRY and REED[6]; see also JAKUS and HALL[7]) have shown, as indeed could be predicted, that the actomyosin effect is one in which the discrete filaments of myosin anastomose freely with those of actin to give an interlocking network rather reminiscent of the fibrinogen-fibrin transformation (REED[8]). Why the two proteins interact in this way is of great interest, although the Hungarian workers have not essayed an explanation. In an attempt to solve the problem, we first explored the possibility that the interaction was of electrostatic type, and probably one between an acidic partner (myosin) and a more basic one (actin). On this view the role of ATP was to compete for the more basic protein. This line of attack was quite unsuccessful, as indeed the results of GUBA[9] had indicated they might be; he showed that the actin-myosin-ATP interrelations occurred at salt concentrations ($\mu = 2$) which might be expected to reduce very greatly the interaction of one protein with another. We were led therefore to the idea that the interaction was a specific one, rather like that between enzyme and substrate, and involving a special chemical grouping, although for this idea there was no precedent. It was fruitful, however, in the finding that the reactive SH groups of myosin are essential for the interaction: the same groups in fact which are

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also necessary for ATP-ase activity. This correlation of an enzyme and colloid reaction partially explains the action of ATP in preventing the formation of actomyosin. A preliminary account of some of the results has already appeared (BAILEY and PERRY[10]).

EXPERIMENTAL

Materials

Rabbit myosin (3 times precipitated) and ATP were prepared as described by BAILEY[11] and actin by the modified method of STRAUB[2]. We have further modified this latter method to reduce the amount of fatty material and to obtain less turbid solutions: after the muscle residue has been dried in acetone and in air, it is extracted twice with chloroform (4 vol. for 20 min) and quickly dried. The method then follows that of STRAUB.

o-Iodosobenzoic acid (used as the Na salt was synthesized by permanganate oxidation of *o*-iodobenzoic acid (ASKENASY and MEYER[12]) and recrystallised once from water; it was found to be 100% pure by iodometry. Iodoacetamide was prepared from chloroacetamide by the method of ANSON[13]; *p*-chloromercuribenzoate was donated by Prof. C. RIMINGTON.

Methods

Viscosity measurements. OSTWALD viscometers designed for a 5 ml vol. of liquid were used throughout (time of flow for 0.5 *M*-KCl, 60–90 sec at 0°). In the measurements with actomyosin, two difficulties arise: 1. actomyosin is thixotropic, and the flow-time of the first reading is somewhat greater than that of the second, and the latter slightly greater than that of the third. Since all determinations were comparative, the mean of readings 2,3 and 4 was taken. 2. The effect of ATP on the viscosity of actomyosin is complicated by uncertainties of ATP concentration due to enzymic breakdown. This difficulty is overcome by using an ATP concentration greatly in excess of the minimum amount necessary to dissociate actomyosin and by working at 0°. In this way, readings were quite constant over 5–10 min.

In the actual determinations of viscosity, myosin and actin stock solutions were diluted with 0.5 *M*-KCl containing veronal (final concentration 0.034 M, p_H 7.0) to contain 2 mg/ml and 4 mg/ml respectively. The myosin (5 ml) was mixed at 0° with 3 mg (0.7–1.0 ml) of actin and the relative viscosity (*a*) determined. The ATP solution (0.06 ml, 0.06–0.08 mg 7 min P) was then added via the wider tube of the viscometer and mixed well before the viscosity determination (*b*). Two parallel determinations were now made on actomyosin prepared from myosin treated with some chosen reagent, giving the relative viscosities (*c*) in absence and (*d*) in presence of ATP. Since the reagents employed had no effect on the relative viscosity of myosin itself, (*b*) and (*d*) do not differ significantly. The percentage inhibition of actomyosin formation, an arbitrary measure derived from these viscosity experiments to allow comparison with percentage inhibitions of ATP-ase activity, was defined as $\left(1-\frac{c-d}{a-b}\right)\cdot 100$.

Adenosinetriphosphatase activity. ATP-ase activities were always determined at 21°, a temperature as near as possible to that used for viscosity measurements but consistent with an adequate rate of enzyme action. To 1 ml of buffer were added 0.1 ml $CaCl_2$ (0.1 *M*), 0.1–0.5 ml (approx. 0.2–1.0 mg) of myosin, 0.5 *M*-KCl to a total vol. of 2 ml allowing for the final addition of 0.4 ml ATP solution (0.4 mg 7 min P). The buffer was 0.1 *M*-glycine p_H 8.9 except in the case of experiments with Hg-poisoned myosin when 0.1 M-veronal p_H 7.0 was used. Incubations (21°) were of 30–50 min duration and activities determined by the method of BAILEY[11]. Inhibitions were read from curves obtained by plotting PO_4 split from ATP against enzyme concentration (three points for each myosin sample).

Determination of SH groups. Since viscosity, enzyme activity and SH determinations had to be carried out almost simultaneously, a very rapid, though approximate, method for the determination of SH by simple iodine titration was developed. The reagent of HELLERMAN, CHINARD and RAMSDELL[14], *o*-iodosobenzoate , was adapted for this purpose. This substance (as Na salt) gives a stable solution which was found to liberate iodine quantitatively at p_H 6.3 in presence of KI; it may thus be used as a direct oxidant for SH groups (HELLERMAN *et al*[14]) or as a source of iodine at almost neutral or acid p_H.

As a selective oxidant for SH groups, iodine is by no means an ideal reagent. The detailed studies of ANSON (see ANSON[15]) show that the precise conditions of oxidation are important in defining the extent of the reaction between iodine and protein. Under our conditions, free histidine and tyrosine do not react, but tryptophan and methionine do. When present in proteins which do not contain cysteine and only very little cystine (caseinogen, tropomyosin) or no cysteine but much cystine (insulin), even these amino-acids are unaffected by iodine (Table I). Significantly therefore, the extra iodine uptake which cannot be accounted for in terms of cysteine occurs only in the case of SH-containing proteins and suggests an oxidation beyond the disulphide stage, but not necessarily via cystine. These considerations have some importance in the detailed interpretation of the iodine reaction and it is hoped to consider them in future work. In the case of myosin and actin, it is possible

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that the fatty material associated with the proteins in their native state may also consume iodine. This source of error has not generally been considered. It must be emphasized that the method is mainly of diagnostic value and the results interpreted as comparative rather absolute values; for this reason, they are recorded as 'apparent cysteine'.

TABLE I

APPARENT CYSTEINE CONTENT OF CERTAIN PROTEINS BY IODINE TITRATION

	Amino-acids present				Apparent cysteine (%)
	Tyrosine	Tryptophan	Methionine	Cysteine	
Caseinogen	+	+	+	nil	0.04
Tropomyosin . . .	+	nil	+	nil	0.2
Insulin (in urea) .	++	nil	nil	nil	nil
Ovalbumin	+	+	+	(1.2 %)	~ 1.6
Myosin (fresh) . .	+	+	+	(1.2 %)	~ 1.8

The advantages of the iodine method are its precision, rapidity, and suitability for the determination of SH groups before and after some chosen treatment; the values relevant to the present work are thus obtained by differential titration. The oxidants employed for the graded oxidation of SH groups in myosin were iodosobenzoate, H_2O_2 and iodine. At various levels of oxidation, excess reagent was removed, by cysteine in the case of iodosobenzoate, and catalase in the case of H_2O_2; with iodine, no excess was present. Protein samples treated with H_2O_2 or iodine were then subjected to differential titration, using untreated samples as controls. The SH groups remaining in iodosobenzoate-treated samples could not be estimated satisfactorily for two reasons: 1. the removal of excess reagent by cysteine and the necessity of subsequent dialysis before titration (during which cysteine is oxidized) gives rise to a complex oxido-reduction system in which more protein SH groups are finally oxidized than in experiments where excess iodosobenzoate is dialysed away without addition of cysteine; 2. dialysis of controls also effects a slight oxidation despite the use of oxygen-free diffusate. With this oxidant therefore, we have preferred to measure the inhibition effects as a function of iodosobenzoate concentration.

The action of alkylating reagents (iodoacetate, iodoacetamide, chloroacetophenone) can be studied if need be without removal of excess reagent. This is not the case with mercaptan-combining compounds (e.g., *p*-chloromercuribenzoate) which do not prevent the oxidation of SH groups by iodine; these reagents do nevertheless possess a high affinity for SH groups and are assumed to react stoichiometrically with them.

Method. The protein solution (10 ml; 20 mg myosin) is buffered with 0.5 ml *M*-phosphate pH 6.25, and 0.5 ml of 0.01 *N*-iodosobenzoate (Na salt, pH 8.5) added, followed by 0.5 ml of 10 % (w/v) KI. After mixing and standing for 15 sec, excess iodine is titrated with 0.01 *N*-thiosulphate using starch indicator. If the back-titre corresponds to < 0.1 or > 0.3 ml of 0.01 *N*-iodine, the titration should be repeated with an adjusted amount of iodosobenzoate to give a back titration of 0.2 ml. This procedure corrects for stoichiometrical deviation which within the limits of back-titration cited amounts to about 7 % error. It will be noted that the titration is carried out in homogeneous solution, and the addition of reagents in the order given avoids any local concentration of iodine which might otherwise cause precipitation of insoluble iodinated protein.

CHARACTERISTICS OF MYOSIN AND ACTIN WITH RESPECT TO IODINE TITRATION

Freshly prepared myosin after three precipitations reacts with iodine to an extent corresponding to 1.6–1.8 % apparent cysteine. On storage at 0° in presence of toluene, the titre is not affected over 1–2 weeks but thereafter decreases. Values of 0.8, 0.6 and 0.2 % have been found for aged preparations. The last figure supports the view that iodine reacts mainly with SH groups, since other possible reactants (apart from fatty material) are not likely to be autoxidizable. By porphyrindin titration, in presence and absence of guanidine HCl (GREENSTEIN and EDSALL[16]), the total cysteine of myosin is 1.2% and the reactive cysteine in the native protein which gives the nitroprusside test and is available to oxidants (SINGER and BARRON[17]) is 0.4 %. In diffarentiating the reactive and unreactive SH groups we have not attempted to correlate oxidation levels with the disappearance of the nitroprusside reaction which is difficult to assess, but have preferred to compare the extent of oxidation as determined by the iodine method with the concentration of oxidant (Figs. 1 and 2), when the two types of groups are readily distinguished.

Actin, like myosin, gives a strong nitroprusside test. The total apparent cysteine varies from 1.65–2.0 % in different samples. The SH character of actin requires further study, but is not of immediate interest since these groups are not concerned in actomyosin formation.

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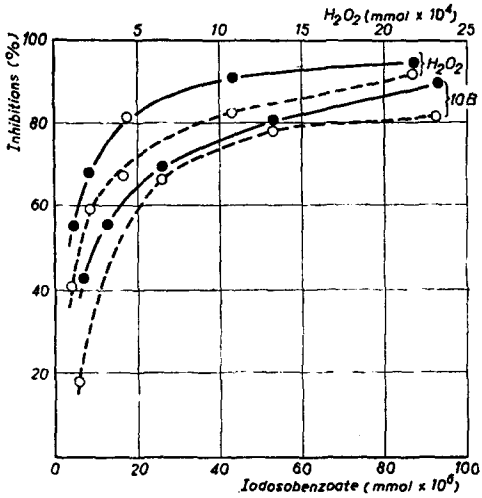

Fig. 1

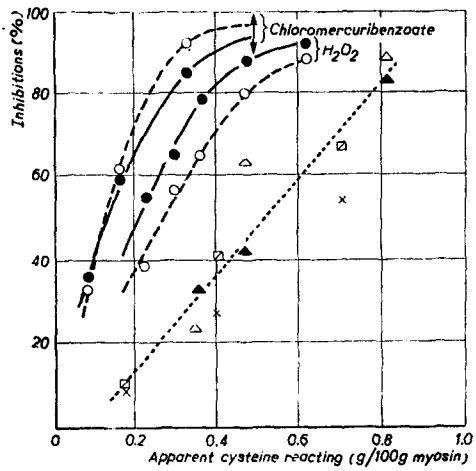

Fig. 2

Fig. 1. Inhibition of actomyosin formation and of ATP-ase activity as a function of oxidant concentration. —●—●—, actomyosin formation; –○–○– ATP-ase activity; IOB = iodosobenzoate

Fig. 2. Inhibition of actomyosin formation and of ATP-ase activity as a function of myosin SH groups oxidized or substituted. Curves for chloromercuribenzoate and H_2O_2: —●—●—, actomyosin formation; –○–○–, ATP-ase activity. Action of iodine: ▨ actomyosin formation, × ATP-ase activity. Action of iodoacetamide: ▲ actomyosin formation, △ ATP-ase activity.

Treatment of myosin with SH reagents

In all cases, reagents were added to a solution of myosin in 0.5 *M*-KCl, containing 0.034 *M*-veronal, pH 7.0.

o-*Iodosobenzoate.* Since HELLERMAN *et al.*[14] first recommended this reagent as an oxidant for SH groups, it has been successfully used for inhibition studies of urease (HELLERMAN, CHINARD and DEITZ[18]) and of myosin ATP-ase (SINGER and BARRON[17]). The myosin (2.5 mg/ml) was treated with graded amounts of 0.01 *N*-iodosobenzoate for 2 h at 21°. Excess reagent was reduced by 0.01 *M*-cysteine, added in equivalent concentration to the largest amount of iodosobenzoate used. (Under these conditions cysteine does not reverse the inhibitions produced by the oxidant). After 1 h at 21°, samples were taken for viscosity and enzyme experiments.

Hydrogen peroxide. Both ZIFF and MEHL[19] studied the inhibition of ATP-ase by H_2O_2, and observed a restoration of activity by addition of cysteine. No reversals have been attempted in our experiments. The myosin (1.85 mg/ml) was treated for 20 min at 0° with H_2O_2 in final concentration 0.0002–0.004 *M*. A trace of highly active catalase, supplied by Dr E. C. WEBB, was added, and the tubes left for 105 min at 0°, when samples were taken for determination of viscosity, enzyme experiments and for SH content. Under the experimental conditions, peroxide is entirely destroyed by catalase and does not therefore interfere in the iodine titration.

Iodine. To the myosin solution (1.84 mg/ml) was added with very rapid stirring a solution of iodine in 0.5 *M*-KCl (final concentration $1.0–3.5 \cdot 10^{-5}$ *M*). The reactants were added and maintained at 0°, and after 1 h, samples were taken for the determination of ATP-ase activity, actomyosin formation and SH titration.

p-*Chloromercuribenzoate.* This reagent was introduced by HELLERMAN *et al*[18] for the study of urease inhibitions and has since been employed by BARRON and SINGER[20] for the characterization of SH enzymes. The inhibitions are partly or wholly reversed by cysteine. The myosin (2.43 mg/ml) was treated for 1 h at 21° with chloromercuribenzoate in final concentrations of $1.6–19.2 \cdot 10^{-6}$ *M*. After cooling in ice, ATP-ase and actomyosin experiments were carried out, the former in 0.05 *M*-veronal buffer pH 7.0 instead of glycine in order to simulate the conditions of the parallel viscosity experiment. This deviation of procedure is necessary since we are here dealing with a dissociable system having a possible pH parameter; moreover, previous work had indicated (BAILEY[11]) that glycine buffer protects from heavy metal inhibition, particularly from Cu^{++}.

Reversal of inhibition was effected by addition of cysteine (final concentration 0.0005 *M*) and experiments carried out 10–60 min after the addition.

Iodoacetamide. The failure to obtain inhibition in presence of iodoacetate first led to the belief

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(D. M. NEEDHAM[21]) that SH groups played no part in ATP-ase activity. It has since become clear (SINGER and BARRON[17]) that myosin SH groups react readily with oxidants or mercurials but are resistant to alkylation. Even the powerful lachrymator chloroacetophenone has little effect upon the ATP-ase activity or SH titre of myosin (BAILEY, unpublished), whereas this and other similar reagents (bromobenzyl cyanide, ethyl iodoacetate) were inhibitors of certain enzymes studied by MACKWORTH[22] and of crystalline yeast hexokinase (BAILEY and WEBB[23]). There exist therefore not only differences in the reactivity of protein SH groups towards oxidants, to which most work has been directed, but also towards alkylating reagents. A study of SH enzymes from this point of view suggests the possibility of employing selective SH inhibitors for one or more components of a complex enzyme system.

The myosin (2.19 mg/ml) was treated for 15 h at 16° with iodoacetamide concentrations of 0.0017–0.042 *M* and then dialysed against the KCl-veronal solvent. Controls were subjected to the same treatment in absence of iodoacetamide and protein concentrations re-determined on all solutions. Samples were then taken for the usual experiments.

RESULTS

The myosin component

Action of oxidants. When inhibitions of ATP-ase activity and of actomyosin formation are plotted against oxidant concentration (Fig. 1) the lowest values of the latter produce marked inhibitions up to a level of 60–70 % and thereafter tail off over a very wide range of oxidant concentration. The meaning of this effect is clear by comparing with Fig. 2, where inhibitions (in the case of H_2O_2) are plotted against apparent cysteine oxidized; up to 0.4 % cysteine, inhibitions are linear with the number of groups oxidized, but between 0.4–0.6 % cysteine (the latter value giving almost 100 % inhibitions), the groups concerned are more difficult to oxidize. Previous workers have shown that the nitroprusside reaction is negative when about 0.4 % cysteine is oxidized by porphyrindin (GREENSTEIN and EDSALL[16]) or substituted by chloromercuribenzoate (SINGER and BARRON[17]). (The concurrence of oxidation levels marking the 'reactive' SH groups is not of great significance; our iodine method probably over-estimates, whilst the nitroprusside end-point methods in our experience are not very precise.) Taken together, these results indicate that much of the enzyme activity and actomyosin-forming ability is connected with the reactive, easily oxidized SH groups and a small residuum with the less reactive ones. The quantitative aspects of this conclusion are at variance with the work of SINGER and BARRON[17] on ATP-ase. They found that the disappearance of SH groups, giving the nitroprusside reaction, either by oxidation or substitution with mercurial, gave only 11–16 % inhibition, and concluded that the less reactive SH groups were mainly concerned with ATP-ase activity.

The important feature of the curves is that the inhibition both of ATP-ase and of actomyosin formation increase at the same rate, indicating that the same SH groups are responsible for, or intimately connected with, both types of activity. The absolute value of each type of inhibition for any given level of oxidation is not identical, and this is true also for Hg-poisoned myosin; the differences are probably methodological. It is indeed surprising that the viscosity experiments can be so precisely interpreted into inhibition measurements; it indicates that the viscosity decrease of actomyosin is related to SH groups and to no other factor.

In Fig. 2, the use of iodine as oxidant leads to a different result in that inhibitions are by no means complete when 0.7 % of apparent cysteine is oxidized. This anomaly is discussed below in relation to the action of iodoacetamide.

Action of chloromercuribenzoate. The two types of inhibition (Fig. 2) again follow

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a similar course and are complete when 0.5 % cysteine is substituted. This figure is derived by assuming a stoichiometric relation between reagent added and SH reacting; the great affinity of thiol groups for heavy metals justifies this procedure. In comparing the effect of oxidant with that of mercurial, the inhibitions by the latter for any one level of cysteine groups undergoing reaction are greater. The difference is not to be regarded as absolute since we have already indicated that the iodine titre may give results which are rather high. The results indicate that the SH groups available for oxidation are precisely those which are first substituted by mercury, though one might suppose that the latter could react indiscriminately with all SH groups rather than those which respond to oxidation by virtue of the proximity of two cysteine residues. In the case of urease also, however, HELLERMAN *et al.*[18] found that mercury first substituted the oxidizable 'a' groups and then the less easily oxidizable '*b*' groups.

On addition of cysteine to mercury-poisoned myosin, the inhibitions are reversed by 90–100 % in the ability to form actomyosin and by about 80 % with respect to ATP-ase.

Action of alkylating reagents. The reaction with iodoacetamide is so slow that a long reaction time and relatively high concentration of reagent are necessary (see experimental section). The inhibition curves (recorded as points in Fig. 2) do not fall closely together as in previous examples, due partly to the more difficult technique of these experiments and have not attained 100 % inhibition even when 0.8 % of apparent cysteine groups are substituted. They have the same characteristics of the curves for iodine (Fig. 2), and in our view, may have the same explanation: It is probable that the SH groups of myosin are uniformly resistant to alkylation, and in this respect, iodoacetamide does not distinguish between the easily-oxidized groups and the more resistant ones. For the complete substitution of these former, which are mainly connected with enzyme and actomyosin-forming properties, a more extensive substitution of all SH groups is necessary. With respect to iodine, there is evidence that its reaction with protein SH like iodoacetamide, involves an initial reaction between one molecule of reagent and one SH group (ANSON[24]). It is not proven, however, that the end product of oxidation is entirely cystine, as ANSON[15] supposes; the ability of iodine to react with all SH groups of proteins may reflect its ability to oxidize cysteine to a cysteine acid without requiring that two SH groups shall be adjacent before oxidation can proceed.

TABLE II

EFFECT OF STORAGE OF MYOSIN ON ACTOMYOSIN FORMATION, ATP-ASE ACTIVITY AND APPARENT CYSTEINE OXIDIZED

Myosin (prep. no.)	Age in days	Relative viscosity of actomyosin		Actomyosin-forming ability	ATP-ase activity	Approx. apparent cysteine oxidized (% of myosin wt)
		No ATP	+ ATP			
21	22	3.40	1.86	(100)	(100)	(0)
20	49	2.74	2.08	43	44	0.3–0.4
19	77	2.13	2.08	3*	1*	0.9
16	122	1.91	1.88	nil	nil	0.7

* Not significant

Effect of storage. The decrease in ATP-ase activity of myosin after storage under aseptic conditions at 0° was noted by BAILEY[11]. Extending this study to include SH

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titre and the ability to form actomyosin, it is found that all three are decreased on storage. Table II shows the results for different myosin preparations using the same actin and compared at the same time with the most active myosin then available. In practice, the rate of ageing is not uniform from one preparation to another, the autoxidation depending perhaps on trace impurities of heavy metals.

The actin component

Accepting that the actin-myosin interaction involves a special SH grouping in myosin, the possibility of some reciprocal group in the actin partner is suggested. We have considered seven possibilities: 1. a pyrophosphate group which attaches to myosin in the same way as ATP; 2. a carbonyl group, capable of forming the dissociable $>C<^{OH}_{S\text{–}\underline{R}}$ (c.f. cysteine-pyruvic acid compound (SCHUBERT[25]) with SH groups; 3. SH groups; 4. SS groups, which might give rise to an oxido-reduction system between myosin SH and actin SS; 5. a divalent heavy metal component of the actin which might link to myosin by mercaptide formation; 6. a dicarboxylic acid grouping of the type inhibitory to the succinic dehydrogenase system; 7. some type of interaction between myosin and the fatty material which accompanies actin. None of these has been examined in such detail as the contribution of myosin SH to the interaction, but the results thus far have not given positive evidence of their participation. The pertinent experiments are outlined below.

Pyrophosphate. The total P of aqueous actin preparations varies from 0.31–0.35 % of the weight as deduced from N content (total N 16.2 %). This P value decreases on dialysis (against 0.5 *M*-KCl) to approx. 0.15 % without affecting the myosin-combining property; subsequent extraction of dialysed actin with ethanol (to remove lipin) further reduces the value to 0.09 %. This figure represents the maximum amount of pyro P which could be present, and is rather higher than that obtained by direct determination (0.06 %) following the method of LAWACZEK[26]. In terms of ATP and in relation to test conditions, such an amount corresponds to about 1/6 of the minimal ATP concentration (0.00003 *M*) required to dissociate actomyosin, and cannot therefore be responsible for the effect.

Carbonyl group. A precedent for such a group is to be found in the enzyme disulphurase (FROMAGEOT, WOOKEY and CHAIX[27]), shown to be inhibited by reagents such as phenylhydrazine, semicarbazide and hydroxylamine (LAWRENCE and SMYTHE[28]). After treatment with hydroxylamine in 0.01 *M* concentration, actin was unaffected in its actomyosin-forming capacity. Moreover, pyruvate (0.017 *M*), used in the sense of a competitive inhibitor, was without effect on the interaction.

SH groups. On addition of aqueous iodine at p_H 7, the nitroprusside reaction disappears when 0.8–0.9 % apparent cysteine has been oxidized. At this point, actomyosin formation is inhibited by only 5 %; when 1.3 % is oxidized, the inhibition is still only 33 %. A concentration of H_2O_2 which, acting on myosin gave 75 % inhibition of actomyosin formation, gave < 10 % when actin was so treated. Likewise, chloromercuribenzoate equivalent to 0.3 % cysteine may be added before inhibition is apparent, and the equivalent of 1.2 % gives an inhibition of 60 %, but this latter figure most probably results from transfer of reagent to the myosin component. These results do not suggest that actin SH groups are important in the interaction.

SS groups. The possible role of SS groups has not been fully explored. Since actomyosin formation proceeds, and is somewhat enhanced in presence of KCN (0.009–0.017 *M*), a reagent which causes dismutation of the SS bond, it seems improbable that these groups have any importance.

Heavy metal. Since the interaction is unimpaired in presence of cysteine, which must certainly complete for heavy metals such as Cu^{++}, it is unlikely that metals play any role.

A dicarboxylic acid grouping. Succinic dehydrogenase contains an active enzymic centre consisting of one SH group and of two other groups with an affinity for certain dicarboxylic acids. Malonate is thus a competitive inhibitor in the dehydrogenation of succinate (QUASTEL and WOOLDRIDGE[29]; see also POTTER and DUBOIS[30]). A similar grouping in myosin might interact with two closely-spaced COOH groups arising from the free acid groups of the dicarboxylic amino-acids. This hypothesis is particularly attractive, since the action of ATP (and of pyrophosphate (STRAUB[31]) on actomyosin might be explained as a competition of two adjacent acidic (phosphoryl) groups for the active centre of the myosin; indeed, LELOIR and DIXON[32] found that pyrophosphate inhibited succinic dehydro-

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genase in the same way as malonate, and explained its action in the same terms. Both malonate and succinate might thus be expected to produce with actomyosin the ATP effect, but in practice they have no action.

Lipin/lipid material. Actin often contains about 5 % of fatty material which imparts a turbidity to its solutions. If, before extraction with water, the muscle residue is twice extracted with chloroform, much clearer solutions are obtained, and the actin prepared from them is equally active. On freeze-drying, STRAUB's preparations lose about 50 % of their activity, and this is only slightly reduced after chloroform extraction. The formation of actomyosin does not therefore appear to require the mediation of fatty substance, and the chloroform extraction of the muscle residue is recommended to produce a purer actin.

DISCUSSION

Quite apart from its significance in muscle biochemistry, the myosin-actin interaction is unusual in that it is not of a non-specific electrostatic type, but necessitates the presence of a special chemical grouping in the myosin partner. For lack of precise interpretation, it may be thought of as a specific interaction, rather like that between enzyme and substrate. Although the specificity of the grouping depends upon the presence of SH groups, its affinity for actin or for ATP may necessitate the participation of other unidentified groups. The SH groups concerned are mainly those available to oxidants in the native protein and giving the nitroprusside test; however, the less easily oxidized groups are responsible for a small residuum of activity. The inhibition of actomyosin formation as derived from viscosity measurements increases at the same rate as the inhibition of ATP-ase activity, indicating that the same SH groups are concerned in both the colloid and the enzyme reactions. These facts argue strongly for the identity of myosin and ATP-ase: if, for example, the latter were an adsorbed impurity, it would be most unlikely that the two types of inhibition should be stoichiometrically related to SH groups which indubitably belong to myosin itself and not to some small fraction, By our method of estimation, the SH groups concerned represent as cysteine about 0.6 % of the protein weight, though the true value is probably somewhat lower. These groups are readily accessible to heavy metal poisons, but resist alkylation. In this latter respect, myosin differs from some of the accredited SH enzymes.

The participation of ATP in preventing the formation of actomyosin and as substrate in the enzyme reaction gives additional support for the view that the same special grouping is involved in both colloid and enzyme reaction, and clarifies the mechanism of the action of ATP on actomyosin. The enzyme centres combine either with actin or with ATP, but the affinity of the latter is so great that actomyosin cannot exist in its presence, nor indeed can any diminution of enzyme activity be observed when actin is present. The latter does not therefore behave as a competitive inhibitor, though we may interpret its combination with myosin as something of the nature of an enzyme-substrate compound. Whether a comparable specific grouping is involved on the actin side of the partnership is not known, but neither pyrophosphate nor SH groups play any part.

The interpretation of the action of ATP on actomyosin envisages the possibility that enzymes using ATP as substrate are always of SH character. Examples are to found in yeast hexokinase (BAILEY and WEBB[23]), myokinase (COLOWICK and KALCKAR[33]); the properties of creatine phosphokinase (LEHMANN and POLLAK[34] NEEDHAM[35]), of phosphopyruvate phosphokinase (NEEDHAM[35]) and of the choline acetylase system (NACHMANSOHN and JOHN[36]) are also suggestive in this respect.

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Thus far, the actomyosin interaction has been discussed in the light of experiments in which all components are maintained in solution by a high (0.5 M) concentration of salt. The Szeged workers, however, have been concerned with the manifestation of the reaction which simulates more closely the ionic environment of muscle itself. When an actomyosin gel in 0.5 *M*-KCl is squirted into solutions of lower ionic strength (0.05), a 'fibre' is produced which on transfer to other solutions will shrink or expand isodimensionally according to the concentration and nature of the ions present. The addition ot ATP causes an enhanced shrinkage ('contraction') which is the basis of the various hypotheses advanced by SZENT-GYÖRGYI for physiological contraction (SZENT-GYÖRGYI[3, 5]) According to electron microscope studies, actomyosin consists of an anastomosis of giant fibres enclosing large amounts of intermicellar liquid, and the action of ATP is to break down this network into its component molecules by the mechanism we have discussed. In the stronger salt solutions, all components are maintained in solution, and the system becomes less viscous; at the lower ionic strengths where myosin is no longer soluble, it would follow that the actin is released into solution (or interacts normally with myosin) and the myosin is precipitated. After precipitation in water, myosin gels usually contain, even after hard centrifuging, about 2 % of protein, whereas actomyosin fibres after the action of ATP contain about 50 %. It is clear therefore that this enhanced shrinkage cannot be explained in terms of the ordinary properties of myosin, nor wholly in the light of our present results*.

SZENT-GYÖRGYI[5] has interpreted the effect as due either to intramolecular or intramicellar re-arrangement. For the first view, there is no evidence, and the second has no precise meaning. Many types of denaturation lead to the formation of dense, cohesive precipitates in which loss of solubility is partly due to a strengthening of the backbone linkage and partly due to the aggregation of polar side-chains (ASTBURY and LOMAX[37]). The synaeresis of a fibrin clot is an excellent example of this process which is known to occur without intramolecular rearrangement of the reacting molecules (BAILEY, ASTBURY and RUDALL[38]). By analogy therefore, the dense actomyosin precipitates must arise by a somewhat extensive interaction of mutually attracting groups. Since both proteins remain in the native condition, the occurrence of the interaction suggests that such groups are favourably disposed configurationally, particularly when the myosin component is in combination with ATP. Where the configurational factor is lacking, aggregation is best accomplished by thermal agitation as in heat denaturation, and indeed it should be noted that the synaeresis of actomyosin is also increased by a rise in temperature. This explanation is complementary to the mechanism we have proposed for the interaction of actin and myosin at higher salt concentrations. In the latter case, only the very special interaction involving SH centres can occur, since the presence of salt diminishes the normal interaction of the two proteins, and the addition of ATP thus resolves the system into its freely-moving components. At low salt concentrations both interactions are possible, but here the addition of ATP, whilst preventing the SH interaction, intensifies the other.

At this stage it seems untimely to interpret the actomyosin interaction as the basis of physiological contraction. SZENT-GYÖRGYI's theory and modifications of it are variants

* Since the completion of this manuscript, Prof. F. BUCHTHAL (personal communication) has drawn our attention to a recent paper (F. BUCHTHAL, A. DEUTSCH, C. G. KNAPPEIS and A. PETERSEN, *Acta physiol. Scand.*, 13 (1947) 158) which reports that SH reagents prevent the shrinkage of actomyosin fibres in the presence of ATP. They find, however, that the inactivation of ATP-ase by acid does not diminish this property.

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of BERNAL's original speculation on muscle (BERNAL[39]). Both invoke a spiral arrangement of contractile elements, and for the mechanism of contraction, a dehydration or synaeresis. Against this type of theory, structural evidence, as noted by ASTBURY[40] weighs heavily. At the same time it must be recognized that such an unusual type of interaction, linked as it is with the enzyme character of myosin, must find an important place in the ultimate explanation of muscle contraction.

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SUMMARY

1. The interaction of actin and myosin in 0.5 *M*-KCl is not of normal electrostatic type but depends upon the presence of SH groups in the myosin partner.

2. The SH groups concerned are also connected with the adenosinetriphosphatase activity of myosin. Their oxidation by reagents such as iodosobenzoate, hydrogen peroxide, iodine, substitution by chloromercuribenzoate, or alkylation by iodoacetamide lead to a diminution of enzyme activity and of actomyosin-forming ability, the two properties declining at the same rate.

3. The groups concerned are mainly, but not entirely, those which give the nitroprusside test and are accessible to oxidants.

4. Whether a specific chemical grouping is necessary in the actin partner is not known, though several possibilities have been explored.

5. The findings elucidate why adenosinetriphosphate, which is the substrate for the enzyme reaction, so profoundly modifies the colloid reaction. It is suggested that enzymes utilizing adenosinetriphosphate are always of SH character.

6. The results are discussed in relation to the earlier work of SZENT-GYÖRGYI and the Szeged school.

RÉSUMÉ

1. L'interaction de l'actine et de la myosine dans une solution de KCl 0.5 M ne s'explique pas par les forces électrostatiques ordinaires, mais exige la présence de groupes SH dans la myosine.

2. De ces même groupes SH, dépend également l'activité enzymatique (adénosinetriphosphatase) de la myosine. L'oxydation des groupes SH par l'iodosobenzoate, l'eau oxygénée ou l'iode; la substitution par action du chloromercuribenzoate, ou l'alkylation par l'iodoacétamide, ont pour effet de diminuer simultanément l'activité enzymatique et le pouvoir de former l'actomyosine.

3. Les groupes SH impliqués sont pour la plupart, mais pas tous, ceux qui réagissent au nitroprussiate et qui sont sensibles aux oxydants.

4. Rien ne montre la nécessité d'un groupe spécifique dans l'actine pour que celle-ci réagisse avec la myosine. Les auteurs discutent plusieurs possibilités permettant d'expliquer cette interaction.

5. Les résultats expérimentaux permettent d'expliquer pourquoi l'ATP, qui est le substratum de la réaction enzymatique, exerce une influence si profonde sur la réaction colloïdale. Il est probable que les enzymes qui utilisent l'ATP doivent toujours posséder des groupes SH.

6. La signification de ces résultats est discutée par comparaison à ceux obtenus par SZENT-GYÖRGYI et le laboratoire de Szeged.

ZUSAMMENFASSUNG

1. Die Wechselwirkung zwischen Actin und Myosin unterliegt nicht den Gesetzen des normalen elektrostatischen Reaktionstypes, sondern hängt von der Anwesenheit von SH-Gruppen im Myosinpartner ab.

2. Die betreffenden SH-Gruppen sind auch mit der Adenosintriphosphataseaktivität des Myosins verbunden. Ihre Oxydation durch Reagentien, wie Jodosobenzoat, Wasserstoffsuperoxyd und Jod, ihre Substitution durch Chlormercuribenzoat, oder ihre Alkylierung mit Jodazetamid führen zu einer Verminderung der Enzymaktivität und der Fähigkeit, Actomyosin zu bilden. Dabei nehmen beide Eigenschaften in gleichem Masse ab.

3. Die betreffenden Gruppen sind vor allem diejenigen, welche die Nitroprussidreaktion geben und für die Oxydation zugänglich sind.

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4. Ob eine spezifische chemische Gruppierung im Aktinpartner nötig ist, ist nicht bekannt, obgleich verschiedene Möglichkeiten zur Erklärung dieser Zwischenreaktion untersucht wurden.

5. Eie Ergebnisse zeigen, warum Adenosintriphosphat, welches das Substrat für die Enzymreaktion ist, die Kolloidreaktion so stark verändert. Es wird angenommen, dass Enzyme, die Adenosintriphosphat gebrauchen, immer durch SH-Gruppen charakterisiert sind.

6. Die Resultate werden mit denjenigen verglichen, welche aus den früheren Arbeiten von SZENT-GYÖRGYI und der Szegediner Schule hervorgegangen sind.

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