V. 3. STUDIES ON THE FUNCTIONAL SULFHYDRYL GROUPS OF MYOSIN AND ACTIN

Michael Bárány

Department of Biophysics, The Weizmann Institute of Science, Rehovoth, Israel

I. Introduction

Since the pioneer studies of Mirsky, of Greenstein and Edsall, as well as of Todrick and Walker, who determined the number of sulfhydryl groups in native and denatured actomyosin (1–3), muscle biochemists have become interested in the —SH groups of the structural proteins. This interest was much increased when it was found later, that the characteristic reactions of myosin, actin, actomyosin, and fiber models can be abolished by different types of —SH reagents (4, 5).

It is generally assumed that contraction in muscle is essentially an interaction of myosin, actin, adenosine triphosphate (ATP), and ions. It is well known, on the other hand, that in many instances when the energy of ATP is transferred or used for biosynthesis protein —SH groups are necessary for the reactions. Based on this idea, Weber recently proposed a chemical theory of muscular contraction which is based on the participation of ATP splitting and reactions of the —SH groups of the contractile protein filaments (6). It seems that the elucidation of the role of the —SH

groups of myosin and actin should increase our understanding of the mechanism of muscle contraction. Such an approach will be presented in this paper.

II. The Functional Sulfhydryl Groups of Myosin

1. CURRENT THEORIES ON THE ROLE OF SULFHYDRYL GROUPS OF MYOSIN IN THE INTERACTION WITH ATP AND ACTIN.

Of the many publications dealing with the —SH groups of myosin (4, 5, 7) that of Bailey and Perry (8) is especially interesting. These authors have shown that a large number of —SH group reagents, which destroy the ATPase activity of myosin also destroy its affinity for actin. They found a close correlation between the loss of the ATPase activity of myosin and its ability to form actomyosin: "indicating that the same —SH groups are concerned in both the colloid and the enzyme reactions." This attractive hypothesis—emphasized repeatedly by Bailey (5)—has to be considered along with the knowledge that according to the revolutionary discovery of Huxley muscle fibrils are composed of two sets of filaments (9, 10), identified chemically as myosin filaments and actin filaments (11–14). In their new concept of contraction Hanson and Huxley propose a sliding of the actin filaments past the myosin filaments (15). This theory agrees with the old view of Szent-Györgyi that the resting muscle contains actin and myosin side by side, and that the contraction means actomyosin formation, while the ATP interacts with the myosin during the whole contraction cycle (see reference 16, p. 118). It is reasonable to assume that the chemical reactions of the myosin filaments with the actin filaments on the one hand, and with ATP on the other hand are carried out at the "active centers" of the myosin molecule, of which centers the —SH groups form presumably a part; hence any detailed theory of the molecular mechanism of muscle contraction must seriously consider the suggestion of Bailey and Perry.

Szent-Györgyi himself, however, discussing this theory some years ago objected to it. His objections were based on the following arguments: "If the theory were correct, then actomyosin should not be ATPase at all, its SH being blocked. The fact is that it is the most active one" (reference 16, p. 73). The discrepancy between the views of Bailey and Szent-Györgyi could be explained in two different ways. Bailey's idea agrees with the experiment, when actomyosin is dissociated by ATP at high ionic strength; it could be assumed that at low ionic strength actomyosin is similarly dissociated by ATP. If the view of Szent-Györgyi be accepted, it may be assumed that out of the many cysteine residues of the myosin molecule

certain —SH groups combine with actin, while others are responsible for ATPase activity.

2. THE EFFECT OF VARIOUS —SH GROUP REAGENTS ON CHARACTERISTIC PROPERTIES OF MYOSIN AND ACTOMYOSIN

a. Oxarsan

The concept that different—SH groups produce ATP splitting and actomyosin formation seems to be supported by some observations made by Turba and Kuschinsky in 1952 (17). These authors have found that treatment of myosin with oxarsan (m-amino-p-oxyphenyl-AsIII-oxide) inhibits subsequent actomyosin formation, however the reagent has little influence on the ATPase activity of actomyosin sols. Another statement of the authors that oxarsan inhibits the superprecipitation of actomyosin gel but does not affect its ATPase activity (17–20), was, however, recently rejected by Mugikura et al., who found a close correlation between the inhibition of superprecipitation and of ATP splitting (21). We have studied therefore the effect of oxarsan on characteristic properties of pure myosin; our results are shown in Fig. 1. The figure demonstrates that all effects of oxarsan depend in the same way on the oxarsan concentration. All the curves—representing Ca-activated myosin ATPase, Ca- and Mg-activated synthetic actomyosin ATPase (and observation of the superprecipitation), actomyosin formation, and the ATP-caused dissociation of the formed actomyosin—run fairly parallel; from which fact it can be concluded that oxarsan does not separate the ATPase activity from the actin combining properties of myosin.

A similar experiment performed with natural actomyosin instead of myosin is given in Fig. 2. The data, compared with those on myosin, show only one characteristic difference, i.e., actomyosin is not dissociated by oxarsan, a fact observed already by Turba and Kuschinsky (17). It should be stressed at the same time that there exists a close correlation between the ability of ATP to dissociate actomyosin and the ability of actomyosin to split ATP; in other words when the ATPase activity of actomyosin decreases, the dissociating effect of ATP on actomyosin also diminishes.

Explanation of the data of Figs. 1 and 2 can be made as follows: The sulfhydryl groups of myosin, which seem necessary for the interaction with both ATP and actin, are more sensitive to the attack of oxarsan when the myosin is separated, i.e., when it is not combined to actin. Thus all the characteristic properties of myosin have been lost simultaneously in the presence of oxarsan. Some of the —SH groups of myosin
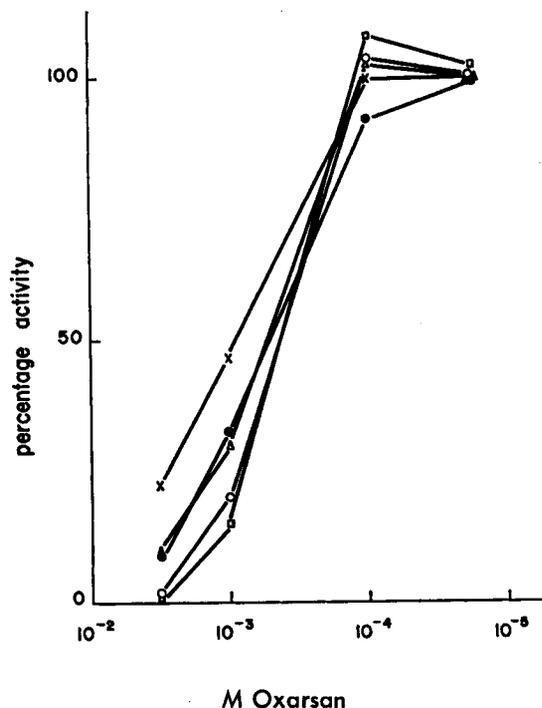
M Oxarsan

Fig. 1. The effect of oxarsan on myosin, inhibition of ATPase activity, of acto-myosin formation ability, and of the dissociating effect of ATP on the formed acto-myosin as a function of oxarsan concentration. The myosins are preincubated (at room temperature, for 10 minutes) with the oxarsan concentration indicated at the abscissa. This oxarsan concentration is kept constant when the myosins are diluted in course of experiments.

□—□—□, ATPase activity of myosin at pH 9.1, in presence of 5×10^{-3} M ATP and of 1×10^{-2} M Ca, temperature: 25°.

△—△—△, ATPase activity of synthetic actomyosin at pH 6.7 in presence of 1×10^{-3} M ATP and of 1×10^{-2} Ca, temperature 25°.

○—○—○, ATPase activity of synthetic actomyosin at pH 6.7 in presence of 1×10^{-3} M ATP and 1×10^{-3} M Mg, temperature: 25°.

Percentage activity defined as (v_i/v) v_i: velocity of ATP splitting with inhibitor; v: velocity of ATP splitting without inhibitor.

×—×—×, actomyosin formation ability of myosin in 0.5 M KCl at pH 7.0 in presence of 5×10^{-4} M Mg, temperature: 0°. Percentage activity defined as:

$$\left(\frac{\log \eta_{\text{rel}} i - \log \eta_{\text{rel}} \text{ATP}}{\log \eta_{\text{rel}} - \log \eta_{\text{rel}} \text{ATP}} \right) \times 100$$

$\log \eta_{\text{rel}}$ i: viscosity of actomyosin solution prepared from myosin treated with inhibitor; $\log \eta_{\text{rel}}$: viscosity of actomyosin solution prepared from myosin treated without inhibitor; $\log \eta_{\text{rel}}$ ATP: viscosity of actomyosin solution in presence of ATP prepared from myosin treated without inhibitor.

●—●—●, ATP sensitivity of the formed actomyosin in 0.5 M KCl, at pH 7.0, in

TABLE I

REVERSAL OF THE EFFECT OF OXARSAN ON MYOSIN AND NATURAL ACTOMYOSIN

| | M SH/10^5 gm. protein | Enzymatic activity μM P/mg. protein/minute/25° | | | Actomyosin formation log η_{rel} | | Superpre-cipitation |
| | | pH 9.1 | pH 6.7 | | | | |
		Ca	Ca	Mg	Before ATP	After ATP	
Myosin control	6.5	1.22	0.56[a]	0.18[a]	0.568	0.255	+
Myosin treated with oxarsan	6.3	1.12	0.50[a]	0.20[a]	0.579	0.271	+
Actomyosin control	6.3	0.64	0.42	0.30	0.456	0.235	+
Actomyosin treated with oxarsan	6.2	0.65	0.40	0.31	0.454	0.244	+

[a] Synthetic actomyosin.

NOTE: Myosin and actomyosin dissolved in 0.5 M KCl were incubated with 5 × 10^{-3} M oxarsan at room temperature for 10 minutes, they were precipitated by addition of 20 volumes of 0.02 M KCl solution in the cold; the precipitated proteins were brought into solution at 0.5 ionic strength and were reprecipitated twice as before by twentyfold dilution. Titration of protein —SH groups was performed according to Katchalski et al. (22); the average deviation for a given sample is not more than 5%.

seem, however, to be protected by actin in actomyosin from the dissociating effect of oxarsan (the affinity of these —SH groups for actin is much greater than for oxarsan); hence actomyosin remains unimpaired even at high oxarsan concentration. On the contrary the —SH groups of myosin responsible for the ATPase activity react in actomyosin with oxarsan just as in the pure myosin component, so that the splitting of ATPase decreases in a fashion strictly parallel to the increase in oxarsan concentration. These —SH groups are therefore not protected by actin against the influence of oxarsan but they are protected by ATP, as can

presence of 5 × 10^{-4} M Mg and of 5 × 10^{-4} M ATP, temperature: 0°. Percentage activity defined as:

$$\left(\frac{\text{ATP sensitivity of actomyosin solution with inhibitor}}{\text{ATP sensitivity of actomyosin solution without inhibitor}} \right) \times 100$$

ATP sensitivity calculated according to Weber and Portzehl (4) as:

$$\left(\frac{\log\eta_{rel} - \log\eta_{rel} \text{ ATP}}{\log\eta_{rel} \text{ ATP}} \right) \times 100$$

$\log\eta_{rel}$: viscosity of actomyosin solution before addition of ATP; $\log\eta_{rel}$ ATP: viscosity of actomyosin solution after addition of ATP.

be deduced from the experiments of Turba and Kuschinsky (*17*). It has been established that the extent to which ATP causes dissociation of actomyosin in solution depends on the ATPase activity of actomyosin sols or gels. This may be explained by the hypothesis that both reactions occur on the same part of the myosin molecule, containing —SH groups.
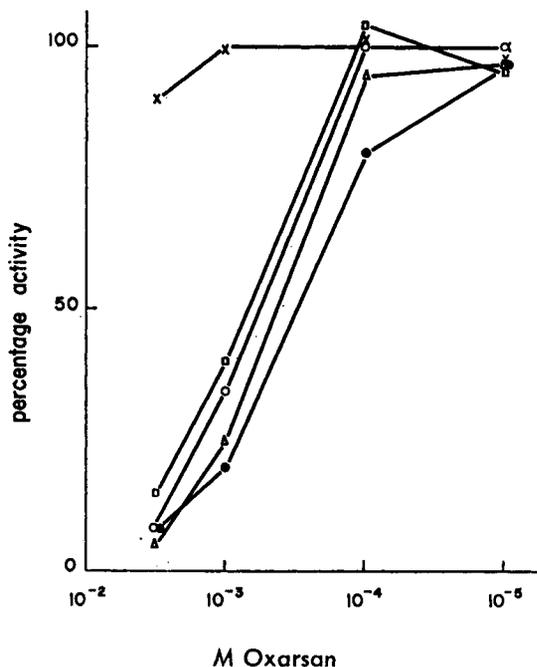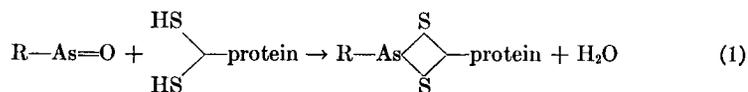

FIG. 2. The effect of oxarsan on natural actomyosin, inhibition of ATPase activity, of actomyosin formation, and of the dissociating effect of ATP on actomyosin as a function of oxarsan concentration. For reaction conditions, calculations, and symbols see Fig. 1.

In order to get better information about the nature of these findings, we have tried to measure the number of those —SH groups that had not reacted in oxarsan-treated myosin and actomyosin. Unexpectedly, however, all the —SH groups were recovered when, after incubation with $5 \times 10^{-3}\ M$ oxarsan, the myosins were reprecipitated thrice. All the activities lost upon addition of oxarsan were regained at the same time (Table I).*
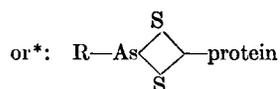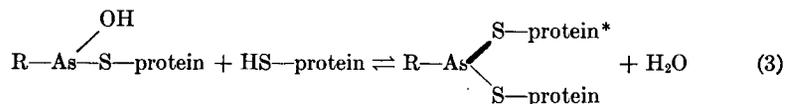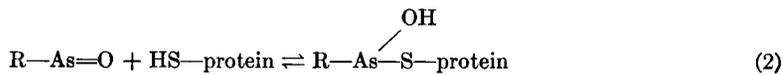
Turba and Kuschinsky gave the following reaction scheme to explain

* Since these results partly disagree with those of Staib and Turba (*20*), it should be noted that full reversibility of inactivation could be achieved by fiftyfold dilution of the oxarsan-containing reaction mixture as well.
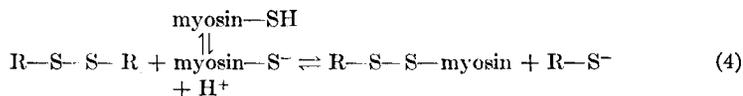
the reaction with oxarsan (17):

$$R—As{=}O + \overset{HS}{\underset{HS}{\diagup\diagdown}}\text{—protein} \rightarrow R—As\overset{S}{\underset{S}{\diamondsuit}}\text{—protein} + H_2O \qquad (1)$$

As it appears that the reaction is a reversible one, we suggest the following scheme instead:

$$R—As{=}O + HS—\text{protein} \rightleftharpoons R—As\overset{OH}{\diagup}—S—\text{protein} \qquad (2)$$

$$R—As\overset{OH}{\diagup}—S—\text{protein} + HS—\text{protein} \rightleftharpoons R—As\overset{S—\text{protein}^*}{\underset{S—\text{protein}}{\diagup}} + H_2O \qquad (3)$$

$$\text{or}^*:\ \ R—As\overset{S}{\underset{S}{\diamondsuit}}\text{—protein}$$

It is possible that the first reaction is sufficient to cause inactivation of myosins.

b. Disulfide Compounds

Cystine* was first applied for elimination of protein —SH groups by Mirsky and Anson (23). Some experiments were performed also with sodium dithioglycolate because of its better solubility than cystine. Moreover, since this reagent, unlike cystine, is soluble in the neutral pH range we could check its action in less alkaline conditions than those used with cystine. The reaction, however, which would have been the reverse of the reductive cleavage of disulfide bridges in proteins by thioglycolic acid (22, 24), did not proceed in the case of myosins below pH 9.0 at all. Thus the mechanism of the reaction is similar to the exchange reactions of cystine with glutathione (25), and the first step can be described by the following equation:

$$\underset{+\ H^+}{R—S—S—R + \overset{\text{myosin—SH}}{\underset{\Updownarrow}{\text{myosin—S}^-}} \rightleftharpoons R—S—S—\text{myosin} + R—S^-} \qquad (4)$$

whether in the course of the reaction any intra- or intermolecular bridges are also formed cannot be definitely answered.

The reactions between myosin and the R—S—S—R compounds were carried out in the cold, at pH 9.3–9.5, using concentrated protein solutions.

By varying the reagent concentration and the incubation time we were able to eliminate gradually the —SH groups of myosin, as shown in Fig. 3. As can be seen in the figure, a myosin sample possessing 2.7 M SH/10^5 gm. protein still retains all its characteristic properties, including its ability for superprecipitation. Below this SH level the loss of ATPase activity
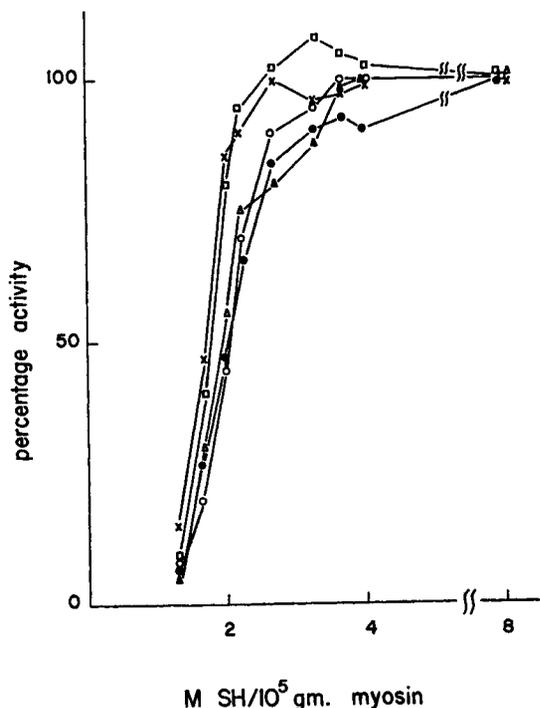

M SH/10^5 gm. myosin

FIG. 3. The effect of disulfide compounds on myosin, inhibition of ATPase activity, of actomyosin formation and of the dissociating effect of ATP on the formed actomyosin as a function of the remaining —SH groups of myosin. The myosin solutions were treated with graded amounts (20–100 equivalent concentration to —SH groups of myosin) of cystine resp. dithioglycolate for 1–15 hours at 0°, pH 9.3–9.5. Excess reagent was removed by threefold reprecipitation in the pH range 6.5 to 7.0. Activity determinations and symbols are given at Fig. 1.

and of the ability to form actomyosin can be observed, and this is complete at 1.3 M SH/10^5 gm. myosin. Addition of excess cysteine reverses these inhibitions.

The rate of reaction of natural actomyosin, in the same range of pH, is much slower. Half of the initial —SH groups could be eliminated without any loss of either activities.

c. Salyrgan

Salyrgan, an organic mercury compound [salicyl-(γ-hydroxymercuri-β-methoxypropyl)-amide-*O*-acetate] introduced by Kuschinsky and Turba (*26*), reacts readily with the —SH groups of myosin, as well as
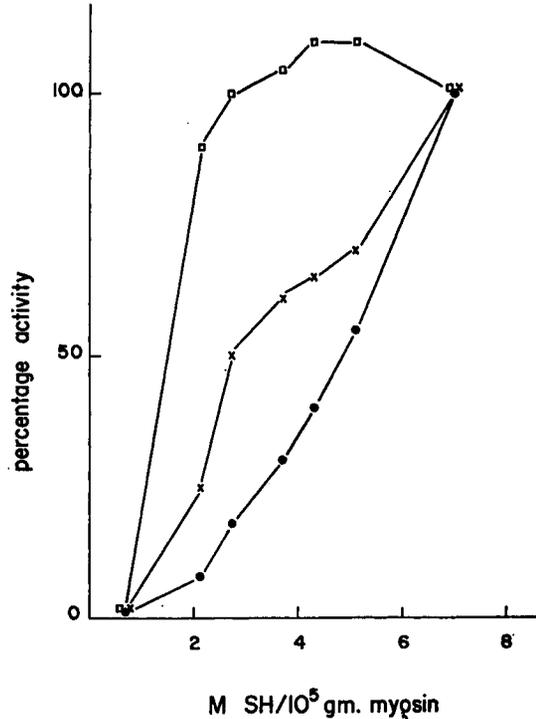

M SH/10⁵ gm. myosin

FIG. 4. The effect of salyrgan on myosin, inhibition of ATPase activity, of acto-myosin formation, and of the dissociating effect of ATP on the formed acto-myosin as a function of the remaining —SH groups of myosin. The myosin solutions were reacted with 0-8 *M* salyrgan/10⁵ gm. myosin at room temperature for 10 minutes, when samples were taken for determination of viscosity and enzymatic activity; the stock solutions were precipitated by addition of 20 volumes of glass-distilled water at the same time to remove the unreacted salyrgan, then centrifuged immediately in the cold; —SH groups were determined after dissolution of the precipitate. Symbols and calculations are shown in Fig. 1.

with those of actomyosin. The results are given in Figs. 4 and 5. Figure 4 shows that after removal of about two-thirds of the initial amount of the —SH groups of myosin, the full enzymatic activity is preserved, similar to the case of disulfide compounds. In contradistinction to these reagents,

however, salyrgan separates ATPase activity and actomyosin formation (e.g., at 2.2 M SH/10^5 gm. myosin the material possesses 90% of the former activity, but only 25% of the latter). Addition of excess cysteine reverses these inhibitions again.
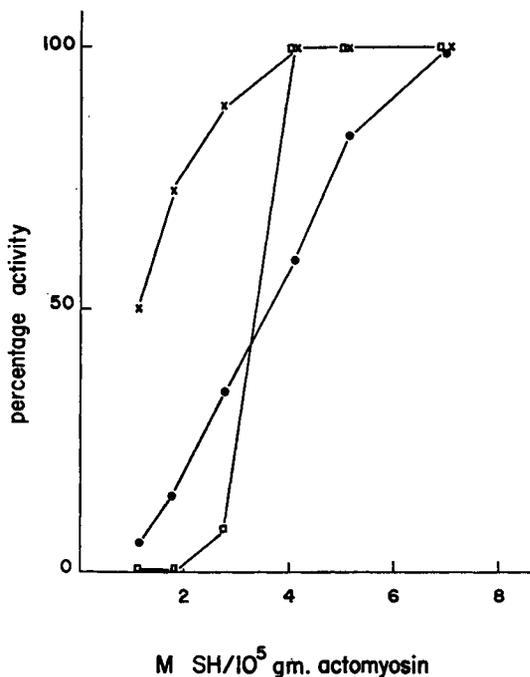

FIG. 5. The effect of salyrgan on natural actomyosin, inhibition of ATPase activity, of actomyosin formation, and of the dissociating effect of ATP on actomyosin as a function of the remaining —SH groups of actomyosin. Reaction conditions are the same as noted in Fig. 4.

Investigations on the reactions of salyrgan with natural actomyosin show a quite reversed feature (Fig. 5); first the ATPase activity is lost, while in the same range (1.8–2.7 M SH) actomyosin is only slightly dissociated by salyrgan. The effect is comparable to that of oxarsan, and it is reasonable to assume that the same mechanism takes place here also, i.e., actin protects those —SH groups of myosin which participate in the myosin-actin bonding. It can be hoped therefore, that isolation of the actin-free myosin component from an actomyosin, which has no ATPase activity but is not dissociated by salyrgan, will result in a myosin which would preserve its affinity for actin. All experiments—performed either with the KI method of A. G. Szent-Györgyi (27), or with the selective precipita-

tion of actin from natural actomyosin with $MgCl_2$ or $CaCl_2$ (*28*)—
pointed, however, to the contrary. It was assumed that the failure to ob-
tain this kind of myosin could be due to an interchange reaction between
the bound mercurial and the free thiol groups of actomyosin, considering
that the protein —S—Hg—R bonding is also slightly dissociable. In or-
der to avoid such dissociation we used iodoacetamide, known to react in
an irreversible way with the —SH groups of proteins, to yield the amide
of *S*-carboxymethylcysteine residue.

d. Iodoacetamide

We reacted iodoacetamide with samples of actomyosin in the cold, at
pH 8.0, at 0.1 *M* reagent concentration and succeeded in isolating mate-
rials bearing about 2.5 *M* SH/10^5 gm. actomyosin, without any ATPase
activity but completely undissociated. The myosin components isolated
from these materials formed a perfect actomyosin after addition of actin.
On the other hand they showed no ATPase activity whatsoever, either in
the absence or presence of actin.

3. Discussion on "Active Centers" of Myosin

The data presented above suggest the hypothesis that different sulfhy-
dryl groups of myosin are necessary for the interaction with ATP and
with actin.* This hypothesis is based on the fact that iodoacetamide-
treated actomyosin produces a myosin which reacts perfectly with actin,
but does not split ATP; salyrgan treated myosin, on the other hand, can
lose almost all its affinity for actin, while the ATPase activity is still un-
impaired (Fig. 4). Of the many —SH group reagents, only those contain-
ing heavy metals can dissociate preformed actomyosin (*17*), but all of
them can abolish the ATPase activity of actomyosin or myosin (Figs. 1–5,
and references *4*, *5*). Moreover, salyrgan affects the ATPase activity, and
only afterwards the dissociation of actomyosin (Fig. 5), suggesting that
first the "free" —SH groups of the myosin component are reacting and
only later on those which, protected by actin, take part in the actomyo-
sin formation.

It seems to be established that all the —SH group reagents when block-
ing the ATPase activity of actomyosin or myosin diminish also the disso-
ciating effect of ATP on actomyosin (Figs. 1–5); this suggests that the
"ATPase centers" and the "dissociation centers" of actomyosin are identi-
cal. Since the dissociation of actomyosin brought about by ATP in the
presence of some —SH group reagents takes an appreciable time (*17*), it

* There are also some indications in Bailey and Perry's paper that iodoacetamide
inhibits first the ATPase activity and only afterwards the actomyosin-formation
ability of pure myosin (*8*).

can be assumed that the binding of ATP to the myosin component is incomplete in such cases [it may be that the —SH groups of myosin, responsible for the ATPase activity, are binding the ATP at the adenine moiety as suggested already in the case of actin (29, 30), or in the case of yeast alcohol dehydrogenase with respect to the binding of DPN (31)].

We could show, however, that in the presence of some —SH group reagents this delay of dissociation of actomyosin does not occur if sodium pyrophosphate is added as the dissociating agent instead of ATP. It seems, therefore, that ATP dissociates actomyosin normally with its pyrophosphate moiety and that the "pyrophosphate-binding part" of myosin shares in the interaction with ATP and with actin. As to the nature of these groups of the myosin molecule, which should be identical in the "ATPase centers" and "Actin-binding centers," the following facts should be mentioned: strong salt solutions favor the dissociation of actomyosin (32, 33) and also inhibit its ATPase activity (34, 35). It seems possible, thus, that these groups are ionized. Neutralization of the dissociated carboxylic groups of myosin by excess polylysine does not result in any loss of ATPase activity (36).

Binding the amino groups of actomyosin or myosin with benzaldehyde (37), with formaldehyde, or by acetylation (36), however, stops the enzymatic properties at once. Since in the latter case the net anionic charge of the myosin molecule increases enormously, the specificity of these reactions must be doubted. In order to overcome this difficulty in the interpretation of the results we have prepared a N-carboxy-L-cysteine anhydride (38) modified derivative of actomyosin; the free amino groups of the protein serve as initiators of a polymerization that proceeds according to the scheme shown (5).

$$
AM\left[\begin{array}{l} -NH_2 \\ \\ -NH_2 \end{array}\right. + n \begin{array}{l} NH-CO \\ | \quad\quad \diagdown O \\ CH-CO \diagup \\ | \\ CH_2SH \end{array} \rightarrow AM\left[\begin{array}{l} -NH-(CO-CH-NH)_qH \\ \quad\quad\quad\quad\quad | \\ \quad\quad\quad\quad\quad CH_2 \\ \quad\quad\quad\quad\quad SH \\ \\ -NH-(CO-CH-NH)_pH \\ \quad\quad\quad\quad\quad | \\ \quad\quad\quad\quad\quad CH_2 \\ \quad\quad\quad\quad\quad SH \end{array}\right. + n\ CO_2 \quad (5)
$$
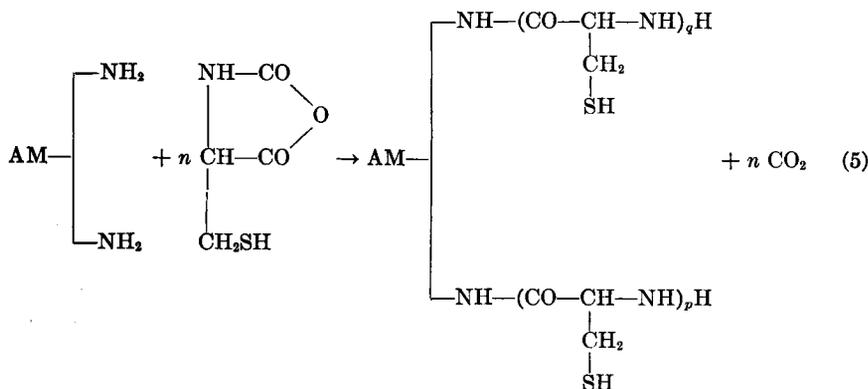
Hence each amino group that has reacted is replaced in the end product by a new one formed in the reaction. Again no ATPase activity could be

found in the modified actomyosin (compared with the 15% aqueous di-
oxane-treated control), despite the preservation of —SH and —NH₂
groups in the altered myosin molecule.

III. The Functional Sulfhydryl Groups of Actin

1. THE ROLE OF SULFHYDRYL GROUPS IN THE POLYMERIZATION OF ACTIN

Straub *et al.* were first to observe that certain oxidizing agents pre-
vented the polymerization of globular actin (*39*). Consequently the dialy-
sis or the precipitation of actin is usually carried out in the presence of
ascorbic acid (*40–43*). Kuschinsky and Turba reported that the conversion
of globular (G-) actin into fibrous (F-) actin is inhibited by salyrgan and
is reactivated by cysteine (*26*), whereas Tsao and Bailey pointed out that
N-ethylmaleimide has little effect on this process (*41*). It was shown by
our group in Hungary that of the four —SH groups of G-actin there are
only two which are necessary for the transformation of G-actin to F-actin.
These two functional —SH groups are presumably taking part in the
actin-actin bonding, as they do not react with excess salyrgan in F-actin
at room temperature (*29*, *44*).

2. A PROBABILITY FOR THE GLOBULAR → FIBROUS TRANSFORMATION OF ACTIN IN MUSCLE CONTRACTION

This difference between the reactivity of G-actin and F-actin with
salyrgan encouraged us to study the form of actin (i.e., globular or fibrous)
in muscle. Taking advantage of our method of preparing actin without
previous extraction of myosin from muscle (*45*), we treated fresh muscle
mince with 10 volumes of a solution containing $1 \times 10^{-3} M$ salyrgan and
0.4% $NaHCO_3$, at room temperature for 15 minutes, and after washing out
the excess of salyrgan we isolated an actin that contained only half of the
—SH groups of the control actin, prepared from the untreated muscle.
Furthermore, we obtained the same diminution of —SH groups of actin
when natural actomyosin was treated with salyrgan. Since natural acto-
myosin is known to contain F-actin, this led us to the conclusion that in
the freshly minced muscle the actin also exists in the fibrous form (*46*).

This experiment does not refer, however, to a well defined functional
state of muscle (i.e., relaxed or contracted). Even a freshly minced muscle
can contract by tenfold dilution and washing at room temperature, de-
pending on the activity of the relaxing factor, discovered by Marsh (*47*).
It was soon realized, furthermore, that a salyrgan concentration of $1 \times
10^{-3} M$ may be insufficient for preventing G → F transformation of actin
in muscle; although this salyrgan concentration inhibits completely poly-
merization of 0.3–0.5% actin solutions *in vitro*, this process may be fun-

damentally different *in vivo* at 5% actin content in the myofibrils themselves. Moreover, since our salyrgan solution also contained $NaHCO_3$ at the same time, the diffusion of salts could have preceded that of salyrgan to the actin in muscle. Thus polymerization of actin could have already taken place before the salyrgan had diffused sufficiently into the fibers.

Based on these considerations new experiments were carried out accordingly: Freshly minced muscle was divided into three parts; the first was treated immediately in the cold in the Waring blendor with 4 volumes of a $1-2 \times 10^{-2} M$ salyrgan solution (and in some cases with solutions containing $1-2 \times 10^{-2} M$ salyrgan in 20% aqueous acetone) for one minute and stirred mechanically for an additional 30 minutes; the two other parts were suspended in 2 volumes of $0.05 M$ KCl and left to stand at room temperature for one hour; after cooling, one of them was mixed thoroughly with 3 mg. ATP/gm. muscle, and both were treated at once with a final concentration of $1-2 \times 10^{-2} M$ salyrgan, as in the case of the freshly minced muscle sample mentioned above. The muscle residues were washed 3–4 times with 10 volumes of distilled water (in one case with $0.05 M$ KCl) in order to remove the excess of salyrgan and after drying with acetone the actins were extracted with 10–12 volumes of a solution containing $0.3 M$ KI and $5 \times 10^{-4} M$ ATP, pH 7.0.

TABLE II

REMAINING —SH GROUPS IN ACTINS ISOLATED FROM DIFFERENTLY TREATED
MUSCLE MINCES AFTER REACTING WITH SALYRGAN[a,b]

	Treatment of the freshly minced muscle before addition of salyrgan		
		Suspended in 0.05 M KCl	
Experiment	No treatment	Without ATP	3 mg. ATP/gm. muscle
1[c]	3.0	3.4	3.0
2[c,d]	2.7	3.0	2.3
3	2.8	3.8	2.9
4[d]	2.2	3.4	2.5
5[d,e]	2.1	2.4	1.6
control (without salyrgan)	6.6	6.5	6.5

[a] The numbers represent M SH/10^5 gm. actin.

[b] SH groups were titrated according to Katchalski *et al.* (*22*); the average deviation for a given sample is not more than 5%.

[c] Actomyosin formation measurements (*42*) were performed: actins prepared from the muscle minces suspended in $0.05 M$ KCl showed a 10–15% increase in actomyosin formation compared with the actins of the two other kinds of muscle preparations.

[d] Salyrgan and simultaneous acetone treatment.

[e] Washing the muscle residues with $0.05 M$ KCl.

The number of —SH groups of the different actin preparations are given in Table II.

As can be seen in the table the number of —SH groups in the three different actin preparations do not change if salyrgan has not been added to the muscle mince; it can be seen, furthermore, that this number decreases to about half of the initial —SH groups in the salyrgan-treated samples, suggesting that most of the actin in the muscle minces is in the fibrous form, since excess salyrgan reacts with about half of the total —SH groups, a reaction characteristic of the fibrous form of actin (*29, 44*). An additional decrease in the number of —SH groups of actin is found, however, between the middle column and the two other ones; that is, in actin isolated from muscles containing a high level of ATP more —SH groups are accessible for the reaction with salyrgan than in the muscle minces suspended in 0.05 *M* KCl. This difference in some of the experiments is very significant, while it is less pronounced in others; but even in these cases the difference exceeds the average deviation of the —SH group titrations and can be judged, on the other hand, by actomyosin formation measurements as well. Thus, remembering that salyrgan prevents $G \to F$ transformation and that there is a characteristic difference between the reactivity of G-actin and F-actin with salyrgan, our data permit the conclusion that the increased reaction of salyrgan with the —SH groups of actin in muscles containing ATP is due to the fact that a part of the actin occurs in the globular form in these muscle preparations.

We observed that the volume of the muscle mince during centrifugation showed an increase at a high level of ATP compared with that at a low level of ATP; thus our experiment imitates a whole contraction cycle, as was shown first by Bailey and Marsh (*5, 48*). Microscope examinations, on the other hand, revealed that fibers of fresh and of ATP-treated muscles were also partly in the contracted state, thus a part of the actin in these minces cannot be regarded as that which might occur in the relaxed state. Our data therefore favor the view of Straub that G-actin occurs in the contraction cycle (*40*). They do not give a definite answer, whether all or only a part of actin can exist in the globular form in muscle; this G-actin, however, would correspond either to the relaxed state or to the early phase of contraction.

Two data of the literature support the findings of Table II: Perry (*49*) and recently Hasselbach (*50*) have found that the bound nucleotide of muscle, discovered by Pettko and Straub (*51*), consists of adenosine diphosphate (ADP) and, to a lesser extent, ATP. Whether actin in muscle exists in globular or fibrous form can be judged from the nature of the bound nucleotide as well, since, as was shown by Straub and Feuer (*40*), G-actin contains bound ATP (i.e., G-actin = ATP-actin), and, as shown

by Mommaerts (*52*), F-actin contains bound ADP (i.e., F-actin = ADP-actin). Perry and Hasselbach have in fact demonstrated that the ADP content in the washed fibrils is just as large as the ADP content of isolated F-actin. Hence their findings about the bound ATP of myofibrils are convincing for some G-actin in the fibrils at the same time. Since washed myofibrils represent a contracted or partly contracted state of muscle, as was pointed out by Perry (*9*), these data are in good accordance with ours (in Table II) and if we consider all these data together, a probability is given for the existence of G-actin in muscle.

It is important to keep in mind the principal discovery of Straub and Feuer, that during polymerization of G-actin its bound ATP is transformed to ADP and inorganic phosphate (*40*), confirmed by many other authors (*52–56*). This change is not catalyzed by any enzyme which would transfer or split phosphate from ATP or ADP (*40, 42*).

3. A POSSIBLE CONNECTION BETWEEN THE ATPASE ACTIVITY OF MYOSIN AND THE G → F TRANSFORMATION OF ACTIN IN MUSCLE CONTRACTION

Since the brilliant discovery of Engelhardt and Ljubimova that the main structural muscle protein, the myosin, splits the ATP (*57, 58*), it is usually accepted that transformation of chemical energy into mechanical work in muscular contraction is performed by the ATPase activity of myosin, which would act in the form of actomyosin. The nature of the energetic coupling to the contractile proteins is, however, still a mystery. No explanation is yet available as to the possible meaning of the bound nucleotide of actin in muscle, the importance of which was recently reemphasized by Biró (*59*). In the course of our discussions with Dr. A. N. Biró in Hungary some time ago, the question arose whether there is any connection between the ATPase activity of myosin and the bound nucleotide of actin (*60, 61*).

The great advances made during the last few years in research on enzyme action have shown, as M. Cohn writes: "Many hydrolytic enzymes, including the phosphatases, catalyze group-transfer reactions so that hydrolysis may be considered a particular case of the general reaction when water is the acceptor" (*62*). Concerning this hypothesis it is very interesting to recall the observations of Koshland *et al.* that, when washed muscle or purified actomyosin split ATP, in the presence of H_2O^{18}, the cleavage of the bond occurs between O and the terminal P (*63, 64*), which could be anticipated from the fact that they are phosphoryl-transferring enzymes. In other words the mechanism of hydrolysis of ATP-catalyzed by myosin is just the same as in the case of creatinephosphokinase, hexo-
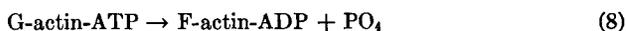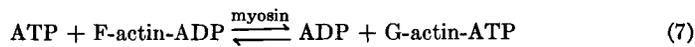
kinase, phosphoglycerokinase, or enolpyruvatephosphokinase (*65*), i.e., of those enzymes which are classified as phosphoryl-transferring enzymes. According to the recent work of Ulbrecht and Ulbrecht, however, ATP may be considered as a donor of phosphoryl groups in the reactions between actomyosin and ATP as well (*66*), since these authors have shown that preparations of highly purified natural actomyosin or myofibrils transfer phosphate between ADP^{32} and ATP, represented in the following equation:

$$ATP + protein \rightleftharpoons protein—P + ADP \tag{6}$$
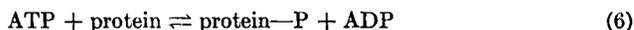
and it was concluded by them that the phosphate transfer occurs by means of the actin component of actomyosin.

Because of the great interest in the role of ATP in muscular contraction, several workers have looked for changes of ATP level in pairs of muscles, one of which has been stimulated (*67–73*). The latest results favor the idea that ATP need not be broken down during the contraction phase of muscle activity (*70–73*), although Fleckenstein *et al.* have found inorganic phosphate liberation during contraction (*70*).

The connection between these varied results could be easily explained now in terms of the data of the present paper summarized in the two following reactions:

$$ATP + F\text{-actin-}ADP \underset{}{\overset{myosin}{\rightleftharpoons}} ADP + G\text{-actin-}ATP \tag{7}$$

$$G\text{-actin-}ATP \rightarrow F\text{-actin-}ADP + PO_4 \tag{8}$$

Thus it is assumed that the ATPase activity of isolated myosin is an artifact; under physiological conditions myosin transfers phosphate from ATP to the bound ADP of F-actin. This process, which can be considered as the connecting link between contraction and the "enzyme system" furnishing the energy for it, would result through the phosphorylation of the bound ADP in depolymerization of F-actin. The formed G-actin would immediately polymerize to F-actin with simultaneous splitting of the bound ATP to bound ADP and liberating inorganic phosphate; the breakdown of ATP would be suitable for the energy requirement of a single muscle twitch, as calculated by Mommaerts (*74*). Rephosphorylation of the F-actin-ADP would be carried out again by the myosin.

REFERENCES

1. A. E. Mirsky, *J. Gen. Physiol.* **19**, 559 (1936).
2. J. P. Greenstein and J. T. Edsall, *J. Biol. Chem.* **133**, 397 (1940).
3. A. Todrick and E. Walker, *Biochem. J.* **31**, 292 (1937).
4. H. H. Weber and H. Portzehl, *Advances in Protein Chem.* **7**, 161 (1952).
5. K. Bailey, in "The Proteins" (H. Neurath and K. Bailey, eds.), Vol. 2, Part B, p. 951. Academic Press, New York, 1954.
6. H. H. Weber, "The Motility of Muscle and Cells," Chapter 2. Harvard Univ. Press, Cambridge, Massachusetts, 1958.
7. H. H. Weber, *Ann. Rev. Biochem.* **26**, 667 (1957).
8. K. Bailey and S. V. Perry, *Biochim. et Biophys. Acta* **1**, 506 (1947).
9. H. E. Huxley, *Biochim. et Biophys. Acta* **12**, 387 (1953).
10. H. E. Huxley, *Proc. Roy. Soc.* **B141**, 59 (1953).
11. W. Hasselbach, *Z. Naturforsch.* **8b**, 449 (1953).
12. J. Hanson and H. E. Huxley, *Nature* **172**, 530 (1953).
13. F. Guba, Ph.D. Dissertation, Budapest, Hungary, 1956.
14. A. Corsi and S. V. Perry, *Biochem. J.* **68**, 12 (1958).
15. J. Hanson and H. E. Huxley, *Symposia Soc. Exptl. Biol.* **9**, 228 (1955).
16. A. Szent-Györgyi, "Chemistry of Muscular Contraction," 2nd ed. Academic Press, New York, 1951.
17. F. Turba and G. Kuschinsky, *Biochim. et Biophys. Acta* **8**, 76 (1952).
18. G. Kuschinsky, F. Turba, and I. Kohler, *Experientia* **8**, 23 (1952).
19. G. Kuschinsky, G. Lange, and F. Turba, *Biochem. Z.* **325**, 321 (1954).
20. W. Staib and F. Turba, *Biochem. Z.* **327**, 473 (1956).
21. H. Mugikura, E. Miyazaki, and T. Nagai, *Enzymologia* **17**, 321 (1956).
22. E. Katchalski, G. S. Benjamin, and V. Gross, *J. Am. Chem. Soc.* **79**, 4096 (1957).
23. A. E. Mirsky and M. L. Anson, *J. Gen. Physiol.* **18**, 307 (1935); **19**, 439 (1936).
24. M. Sela, F. H. White, Jr., and C. B. Anfinsen, *Science* **125**, 691 (1957).
25. I .M. Kolthoff, W. Stricks, and R. C. Kapoor, *J. Am. Chem. Soc.* **77**, 4333 (1955).
26. G. Kuschinsky and F. Turba, *Biochim. et Biophys. Acta* **6**, 426 (1951).
27. A. G. Szent-Györgyi, *J. Biol. Chem.* **192**, 361 (1951).
28. M. Bárány, N. A. Bíró and J. Molnár, *Acta Physiol. Acad. Sci. Hung.* **5**, 63 (1954).
29. M. Bárány, *Biochim. et Biophys. Acta* **19**, 560 (1956).
30. M. Bárány, J. Spiró, G. Köteles, and E. Nagy, *Acta Physiol. Acad. Sci. Hung.* **10**, 159 (1956).
31. J. van Eys and N. O. Kaplan, *Biochim. et Biophys. Acta* **23**, 574 (1957).
32. F. Guba, *Studies Inst. Med. Chem. Univ. Szeged* **3**, 40 (1943).
33. H. Portzehl, G. Schramm, and H. H. Weber, *Z. Naturforsch.* **5b**, 61 (1950).
34. J. W. Mehl and E. Sexton, *Proc. Soc. Exptl. Biol. Med.* **52**, 38 (1943).
35. H. O. Singer and A. Meister, *J. Biol. Chem.* **159**, 491 (1945).
36. M. Bárány, unpublished data, 1957.
37. G. Kuschinsky and F. Turba, *Experientia* **6**, 103 (1950).
38. A. Berger, J. Kurtz, T. Sadeh, A. Yaron, R. Arnon, and Y. Lapidoth, *Bull. Israel Research Council* **7A**, 98 (1958).
39. G. Feuer, F. Molnár, E. Pettkó, and F. B. Straub, *Hung. Acta Physiol.* **1**, 150 (1948).
40. F. B. Straub and G. Feuer, *Biochim. et Biophys. Acta* **4**, 455 (1950).
41. T. C. Tsao and K. Bailey, *Biochim. et Biophys. Acta* **11**, 102 (1953).

42. M. Bárány, N. A. Biró, J. Molnár, and F. B. Straub, *Acta Physiol. Acad. Sci. Hung.* **5,** 369 (1954).

43. M. Bárány, G. Köteles, E. Nagy, and J. Spiró, *Kisérletes Orvostudomány* **8,** 491 (1956).

44. M. Bárány, J. Spiró, G. Köteles, and E. Nagy, *Acta Physiol. Acad. Sci. Hung.* **10,** 145 (1956).

45. M. Bárány, K. Bárány, and F. Guba, *Nature* **179,** 818 (1957).

46. M. Bárány, K. Bárány, F. Guba, G. Köteles, and E. Nagy, *Acta Physiol. Acad. Sci. Hung.* **11,** 145 (1957).

47. B. B. Marsh, *Biochim. et Biophys. Acta* **9,** 247 (1952).

48. K. Bailey and B. B. Marsh, *Biochim. et Biophys. Acta* **9,** 133 (1952).

49. S. V. Perry, *Biochem. J.* **51,** 495 (1952).

50. W. Hasselbach, *Biochim. et Biophys. Acta* **25,** 562 (1957).

51. E. Pettko and F. B. Straub, *Hung. Acta Physiol.* **2,** 114 (1949).

52. W. F. H. M. Mommaerts, *J. Biol. Chem.* **198,** 469 (1952).

53. K. Laki, W. J. Bowen, and A. M. Clark, *J. Gen. Physiol.* **33,** 437 (1950).

54. K. Laki and A. M. Clark, *J. Biol. Chem.* **191,** 599 (1951).

55. A. G. Szent-Györgyi, *Arch. Biochem. Biophys.* **31,** 97 (1951).

56. G. Feuer and M. Wolleman, *Acta Physiol. Acad. Sci. Hung.* **3,** 277 (1952).

57. W. A. Engelhardt and M. N. Ljubimova, *Nature* **144,** 669 (1939).

58. M. N. Ljubimova and W. A. Engelhardt, *Biokhimiya* **4,** 716 (1939).

59. N. A. Biró and B. Nagy, *Acta Physiol. Acad. Sci. Hung.* **8,** 313 (1955).

60. N. A. Biró, Ph.D. Dissertation, Budapest, Hungary, 1954.

61. M. Bárány, Ph.D. Dissertation, Budapest, Hungary, 1956.

62. M. Cohn and G. A. Meek, *Biochem. J.* **66,** 128 (1957).

63. E. Clarke and D. E. Koshland, Jr., *Nature* **171,** 1223 (1953).

64. D. E. Koshland, Jr., Z. Budenstein, and A. Kovalsky, *J. Biol. Chem.* **211,** 279 (1954).

65. B. Axelrod, *Advances in Enzymol.* **17,** 159 (1956).

66. G. Ulbrecht and M. Ulbrecht, *Biochim. et Biophys. Acta* **25,** 100 (1957).

67. E. Lundsgaard, *Proc. Roy. Soc.* **B137,** 74 (1950).

68. W. F. H. M. Mommaerts and J. C. Rupp, *Nature* **168,** 957 (1951).

69. A. Munch-Petersen, *Acta Physiol. Scand.* **29,** 202 (1953).

70. A. Fleckenstein, J. Janke, R. E. Davies, and H. A. Krebs, *Nature* **174,** 1081 (1954).

71. W. F. H. M. Mommaerts, *Nature* **174,** 1083 (1954).

72. W. F. H. M. Mommaerts, *Am. J. Physiol.* **182,** 585 (1955).

73. B. Chance and C. M. Connelly, *Nature* **179,** 1235 (1957).

74. W. F. H. M. Mommaerts, *in* "Phosphorus Metabolism" (W. McElroy and B. Glass, eds.), Vol. I, p. 551. Johns Hopkins Press, Baltimore, Maryland, 1951.

Discussion

MORALES: I have a couple of questions. In that particular material in what kind of linkage would you say an SH is involved? What sort of linkage are you imagining?

BÁRÁNY: I have no evidence for the linkage. As I understood here at the conference there was a discussion whether the SH groups are directly involved in the "active centers" or not. I cannot say they are. I can say that it is possible to isolate a myosin which contains SH groups and which combines with actin, but it does not combine with ATP.

MORALES: I understand that. What kind of linkage? If you had some idea . . .

BÁRÁNY: It is a question whether these SH groups are directly involved in the binding of actin or whether they are indirectly involved in the maintenance of the structure of the "active centers."

MORALES: The other thing is that I think that Koshland's observation is certainly a very interesting one, but it is not obvious to me that this fits the scheme you put there. In particular, people have tried this ADP-P^{32} exchange into ATP, and I gather from Dr. Gergely this is not a clear-cut situation either. Since you mentioned the Koshland effect, how do you account for the Koshland results? You seem to think that it fits with your scheme. We would be very grateful to know why you think this because a number of us have been trying to understand the Koshland report.

BARANY: I think this question goes beyond the scope of this conference since it is a question of the mechanism of muscle contraction. I would finally mention again that I have put forth a working hypothesis which is not based directly on Koshland's experiments. I have tried to find a connection between the ATPase activity of myosin and the bound nucleotide of actin. This connection is supported by the experiments of Koshland, by the experiments of Ulbrecht, and by the experiments of Fleckenstein. But the "main support" for it is that both the structural proteins which are responsible for contraction are somehow connected with ATP, one of them splits ATP, the other contains bound ATP which is converted to ADP during the polymerization. The question is what the connection is.