Okubo, S., Strauss, B., and Stodolsky, M. (1964), *Virology 24*, 552.
Pène, J. J. (1967), *Biochem. Biophys. Res. Commun. 28*, 365.
Pène, J. J., and Marmur, J. (1964), *Federation Proc. 23*, 318.
Pène, J. J., and Marmur, J. (1967), *J. Virology 1*, 86.
Pizer, L. I., and Cohen, S. S. (1962), *J. Biol. Chem. 237*, 1251.
Reilly, B. E., and Spizizen, J. (1965), *J. Bacteriol. 89*, 782.
Romig, W. R. (1962), *Virology 16*, 452.
Roscoe, D. H., and Tucker, R. G. (1964), *Biochem. Biophys. Res. Commun. 16*, 106.
Roscoe, D. H., and Tucker, R. G. (1966), *Virology 29*, 157.
Spizizen, J., Reilly, B. E., and Evans, A. H. (1966), *Ann. Rev. Microbiol. 20*, 371.
Taylor, R. T., and Weissbach, H. (1965), *Anal. Biochem. 13*, 80.
Wilson, M. C., Farmer, J. L., and Rothman, F. (1966), *J. Bacteriol. 92*, 186.
Yeh, Y. C., and Greenberg, G. R. (1967), *J. Biol. Chem. 242*, 1307.
Young, F. E., and Spizizen, J. (1963), *J. Biol. Chem. 238*, 3126.
Young, F. E., Spizizen, J., and Crawford, I. P. (1963), *J. Biol. Chem. 238*, 3119.

# Subfragment 1 of Myosin: Adenosine Triphosphatase Activation by Actin*

Evan Eisenberg,† C. Richard Zobel, and Carl Moos

ABSTRACT: Adenosine triphosphatase measurements were used to study the interaction of actin with subfragment 1, a tryptic digestion product of heavy meromyosin. As is true for myosin, the adenosine triphosphatase activity of subfragment 1 is significantly activated by actin at low ionic strengths and this activation requires the presence of magnesium. Furthermore, as we previously found for heavy meromyosin, actin activation of the subfragment 1 adenosine triphosphatase increases markedly as the actin concentration is increased above the stoichiometric binding ratio of actin to subfragment 1. This shows that adenosine triphosphate causes marked dissociation of the acto-subfragment 1 complex even at low ionic strength; and if, as appears quite likely subfragment 1 has only a single binding site for nucleotide, then we can conclude that dissociation of the acto-subfragment 1 complex must be caused by the binding of adenosine triphosphate to the hydrolytic site of the subfragment 1 rather than to some separate dissociating site. This conclusion in turn leads to the possibility that motion is generated in muscle by repeated cycles consisting of formation of the rigor actin–myosin link in conjunction with adenosine triphosphate hydrolysis followed by dissociation of this actin–myosin link in conjunction with the rebinding of adenosine triphosphate at the hydrolytic site.

It has long been known that heavy meromyosin, a tryptic digestion product of myosin, retains both the ATPase activity and the actin-binding properties of myosin (Mihalyi and Szent-Györgyi, 1953). Recently a further tryptic digestion product of myosin, subfragment 1, was also found to retain the ATPase activity and actin-binding properties of myosin (Mueller and Perry, 1962); and since it has a molecular weight only one-third that of heavy meromyosin, several workers have suggested that a single molecule of heavy meromyosin might contain two or three identical subfragment 1 subunits (Mueller, 1965; Young *et al.*, 1965; Slayter and Lowey, 1967).

In relation to the role of myosin in muscle contraction, one of the most important questions concerning subfragment 1 is whether its ATPase activity, like that of myosin and heavy meromyosin, is activated by actin at low ionic strength in the presence of magnesium. There have been several reports that the subfragment 1 ATPase is activated very little, if at all, by actin and magnesium (Jones and Perry, 1966; Yagi and Yazawa, 1966); and the possibility therefore arises that the prop-

* From the Department of Biological Sciences, State University of New York, Stony Brook, New York, and the Department of Biophysics, State University of New York, Buffalo, New York. *Received April 29, 1968.* Supported by Grants AM-07635, GM-10249, and 5T1-GM-718 from the National Institutes of Health, and a grant from the Heart Association of Erie County, N. Y. Portions of this work have appeared in preliminary reports (Eisenberg *et al.*, 1966; Eisenberg and Moos, 1968a).

† Formerly a postdoctoral fellow of the National Heart Institute, and presently an advanced research fellow of the American Heart Association supported by the Heart Association of Suffolk County, N. Y.

† To whom to address requests for reprints. Present address: Department of Biological Sciences, State University of New York, Stony Brook, N. Y. 11790.

erty of actin activation might not reside in a single subfragment 1 molecule but rather might depend upon an interaction between the subfragment 1 subunits within each heavy meromyosin molecule (Hotta and Usami, 1967).

In the present study we have investigated the effect of actin on the ATPase activity of subfragment 1 and have found that, in fact, this ATPase activity is significantly activated by actin, and that, just as with myosin (Maruyama and Watanabe, 1962), this activation requires the presence of magnesium. Moreover, as we have found for acto-heavy meromyosin (Eisenberg and Moos, 1968b), the acto-subfragment 1 appears to be highly dissociated in the presence of magnesium and ATP even at low ionic strength so that the amount of ATPase activation increases markedly as the actin concentration is increased. This finding, in conjunction with the finding that subfragment 1 has only a single binding site for ADP (Young, 1967a) or pyrophosphate (Nauss and Gergely, 1967), suggests that the binding of ATP to the hydrolytic site itself causes dissociation of the acto-subfragment 1 complex. This conclusion in turn, together with the structural findings of Reedy *et al.* (1965), leads to a possible mechanism of muscle contraction where motion is generated by cyclic formation of the rigor actin–myosin link as recently proposed by Pringle (1967), but where dissociation of this actin–myosin link occurs in conjunction simply with the rebinding of ATP to the hydrolytic site of the myosin and does not involve any separate dissociating sites such as we and others have previously proposed (Weber and Portzehl, 1954; Eisenberg and Moos, 1965; Levy and Ryan, 1966, 1967).

## Methods

*Protein Preparations.* Actin, myosin, and heavy meromyosin were prepared as previously described (Eisenberg and Moos, 1967). Subfragment 1 was prepared by tryptic digestion of heavy meromyosin using a 10:1 weight ratio of trypsin (Worthington, twice crystallized) to heavy meromyosin and digesting for 20 min at 25° in 0.05 M KCl–0.1 M Tris buffer (pH 7.6). The digestion was stopped by adding soybean trypsin inhibitor (Worthington) at a 2:1 weight ratio of inhibitor to trypsin. The resulting digest was then purified either by the method of Mueller and Perry (1962) or that of Young *et al.* (1965). For the purification method of Mueller and Perry, the digest was cooled to 0° and then F-actin was added at a 1:3 weight ratio of F-actin to heavy meromyosin. The digest was then centrifuged at 30,000 rpm in a Spinco Model L ultracentrifuge, the resulting pellets were thoroughly homogenized in 0.15 M KCl, 10 mM sodium pyrophosphate, 5 mM $MgCl_2$, and 20 mM Tris buffer, and the pH was adjusted to 7.0. The suspension was then centrifuged again at 30,000 rpm for 4 hr and the resulting supernatant was dialyzed against 0.2 M KCl–10 mM imidazole buffer (pH 7). Finally the solution of subfragment 1 was concentrated using an Amicon ultrafiltration apparatus with a UM-1 membrane (Blatt *et al.*, 1965) and clarified by centrifugation at 30,000 rpm for 90 min.

For purification of subfragment 1 by the method of Young *et al.*, the tryptic digest of heavy meromyosin was cooled to 0° and concentrated using the Amicon ultrafiltration apparatus. The concentrated digest was then applied to a Sephadex G-200 column and eluted with 0.05 M KCl–0.1 M Tris (pH 7.6), and following elution the subfragment 1 fractions were combined, reconcentrated with the Amicon ultrafiltration apparatus, dialyzed against 0.2 M KCl–10 mM imidazole buffer (pH 7.0), and finally clarified by centrifugation for 90 min at 30,000 rpm. The subfragment 1 was always used within 3 days after preparation.

*Protein concentrations* were routinely determined by ultraviolet absorption at 280 mμ. The extinction coefficients used were 647 $cm^2/g$ for heavy meromyosin (Young *et al.*, 1964), 770 $cm^2/g$ for subfragment 1 (Young *et al.*, 1965), and 1149 $cm^2/g$ for F-actin. This extinction coefficient for actin was determined by J. E. Estes in this laboratory with the use of the micro-Kjeldahl technique and an assumed nitrogen content of 16%.

*ATPase activity* was measured at 25° using an automatic pH-Stat as previously described (Eisenberg and Moos, 1967). For studies of the effect of magnesium on the acto-subfragment 1 ATPase, the chelating agent DCTA[1] was added to reduce the free magnesium concentration. Since tetramethylammonium ion as opposed to potassium or sodium has essentially no effect on the myosin ATPase, the DCTA solution was brought to pH 7 with tetramethylammonium hydroxide in order to prevent variation in potassium or sodium concentration when the DCTA concentration was varied. For the same reason tetramethylammonium chloride was used to adjust the ionic strength in these experiments. In addition, EGTA was added in 1 mM concentration to avoid changes in the free calcium concentration when the DCTA concentration was varied.

*Sedimentation velocity studies* were carried out in a Spinco Model E analytical ultracentrifuge at 59,780 rpm with the rotor temperature maintained at about 4°. For correction of the sedimentation coefficient of subfragment 1 to standard conditions, a value for $\bar{v}_{20}$ of 0.7420 ml/g was used (Mueller, 1965).

*Viscosity measurements* on subfragment 1 were carried out at 20 ± 0.05° using a Cannon-Ubbelohde viscometer with an outflow time for water of 327 sec.

*Reagents.* All reagents were of analytical grade, and demineralized water was used for all solutions. The ATP was purchased from Sigma and used without further purification. The DCTA and EGTA were provided through the courtesy of Geigy Industrial Chemicals, Ardsley, N. Y.

## Results

*Effect of Actin on the Subfragment 1 ATPase.* Figure 1 shows the effect of actin on the ATPase rate of subfragment 1 preparations purified either by the method of

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: DCTA, 1,2-diaminocyclohexanetetraacetic acid; EGTA, ethyleneglycolbis(aminoethyl ether)tetraacetic acid.

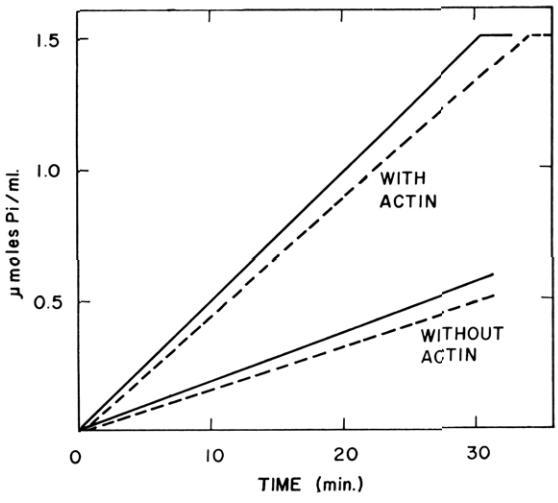



FIGURE 1: Actin activation of the subfragment 1 ATPase. Conditions: 1.5 mM ATP, 0.75 mM $MgCl_2$, 10 mM imidazole buffer (pH 7.0), 0.5 mg/ml actin, 0.5 mg/ml of subfragment 1, and 0.05 M KCl. (———) Subfragment 1 prepared by the method of Mueller and Perry using ultracentrifugation with actin; (– – – –) subfragment 1 prepared by the method of Young *et al.* using Sephadex chromatography.

Young *et al.* (1965) using Sephadex chromatography or that of Mueller and Perry (1962) using ultracentrifugation with actin. Clearly with both preparations of subfragment 1, actin increased the ATPase rate about threefold. To be certain that the actin was indeed affecting the ATPase rate of the subfragment 1 itself rather than that of contaminant heavy meromyosin which might have remained in the subfragment 1 preparation, we compared the actin activation of the subfragment 1 and heavy meromyosin ATPases under identical conditions.

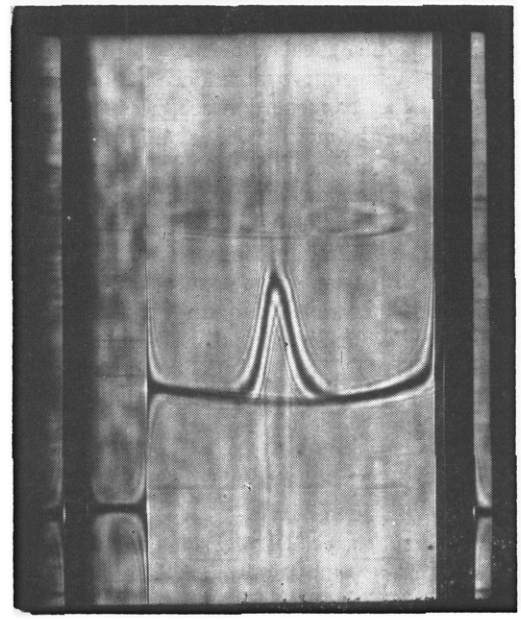


FIGURE 2: Ultracentrifugal schlieren pattern of subfragment 1 purified by the method of Mueller. Conditions: 6 mg/ml of subfragment 1, 0.2 M KCl–10 mM phosphate buffer (pH 6.8), temperature 3.5°, bar angle 55°, and rotor velocity 59,780 rpm. Picture was taken 100 min after reaching full speed.

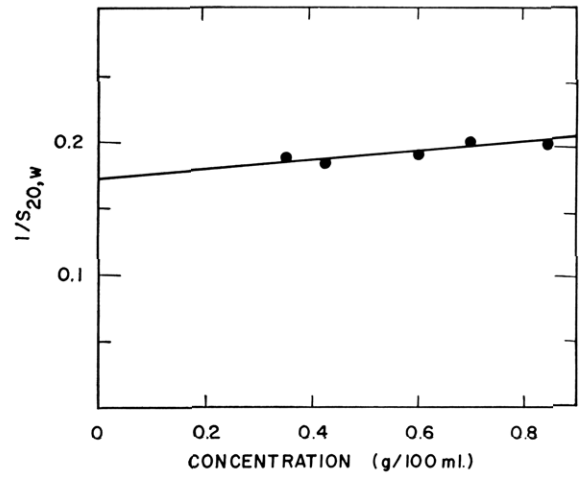



FIGURE 3: $1/s_{20,w}$ as a function of subfragment 1 concentration Conditions as given in Figure 2.

Table I shows that under conditions where the subfragment 1 is twofold activated by actin, the heavy meromyosin is fivefold activated. A simple calculation from these data shows that if all of the actin activation of the ATPase observed with our subfragment 1 preparation was due to contaminant heavy meromyosin, then about one-third of the subfragment 1 preparation would have to be heavy meromyosin. To rule out this possibility, several physicochemical assays of purity were performed. Figure 2 shows that our preparations of subfragment 1 gave a single peak in a sedimentation velocity run performed with a double-sector cell, and as shown in Figure 3, both the extrapolated value of $s_{20,w}$ (5.78 S) and its very low concentration dependence agree with previous reports for subfragment 1 (Mueller and Perry, 1962; Young *et al.*, 1965). We also carried out a viscosity study on subfragment 1 purified by the method of Mueller (Figure 4) and found it to have an intrinsic viscosity of 0.09 dl/g in agreement with other reported values for

TABLE I: Actin Activation of the Heavy Meromyosin and Subfragment 1 ATPases.[a]

| Enzyme | ATPase (μmoles/mg min) Without Actin | With Actin | Actin Activation |
|---|---|---|---|
| Subfragment 1 | 0.027 | 0.054 | Twofold |
| Heavy meromyosin | 0.020 | 0.10 | Fivefold |

[a] Conditions: 1.5 mM ATP, 0.75 mM $MgCl_2$, 10 mM imidazole buffer (pH 7.0), 0.1 M KCl, and 0.5 mg/ml of actin. Subfragment 1 concentration was 1 mg/ml in samples without actin and 0.5 mg/ml in samples with actin. Heavy meromyosin concentration was 1 mg/ml in samples without actin and 0.2 mg/ml in samples with actin.

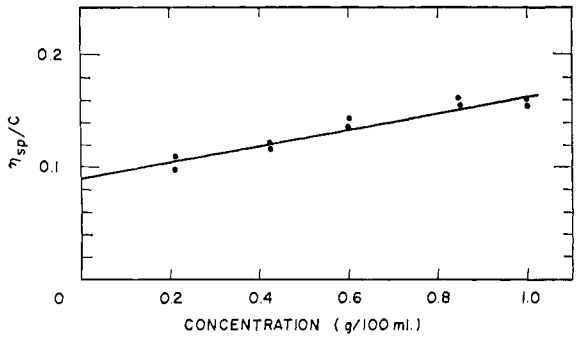



FIGURE 4: Reduced viscosity (deciliters per gram) as a function of subfragment 1 concentration. Conditions: 0.2 M KCl–10 mM phosphate buffer (pH 6.8), 20°.

subfragment 1 (Mueller, 1965; Young *et al.*, 1965). In contrast, heavy meromyosin has an intrinsic viscosity of about 0.5 dl/g (Lowey and Cohen, 1962; C. R. Zobel, unpublished data) and therefore if we assume that the subfragment 1 itself has an intrinsic viscosity of at least 0.06 dl/g then our subfragment 1 preparation could not be contaminated with more than 7% heavy meromyosin.

For the subfragment 1 isolated by the method of Young *et al.* (1965), purity was assayed by rechromatography on Sephadex G-200 (Figures 5 and 6). A small amount of material appeared near the void volume of the column even in the second chromatogram, but this

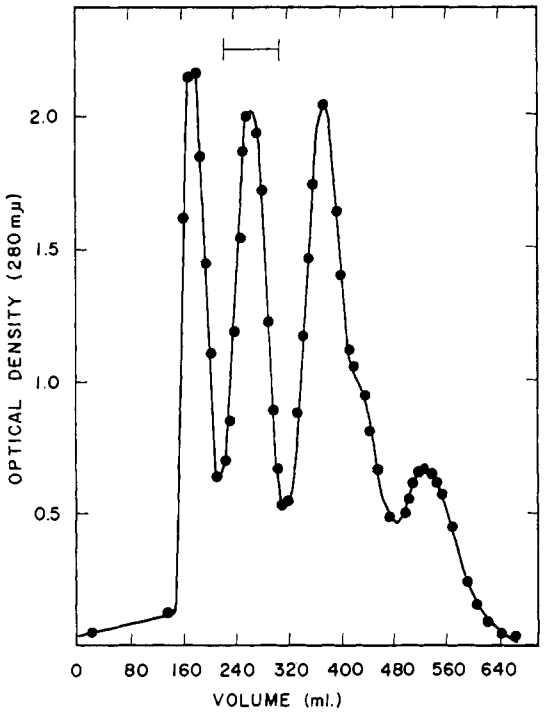



FIGURE 5: Fractionation of a tryptic digest of heavy meromyosin on Sephadex G-200. Heavy meromyosin was digested and concentrated as described in the Methods section. The concentrated digest (8 ml) was applied to a 4 × 50 cm Sephadex G-200 column which had previously been equilibrated with 0.05 M KCl–0.1 M Tris (pH 7.6). The column was developed at 3° with the same solvent at a flow rate of 15 ml/hr and 7-ml fractions were collected. The bar indicates the fractions combined for the rechromatography shown in Figure 6.

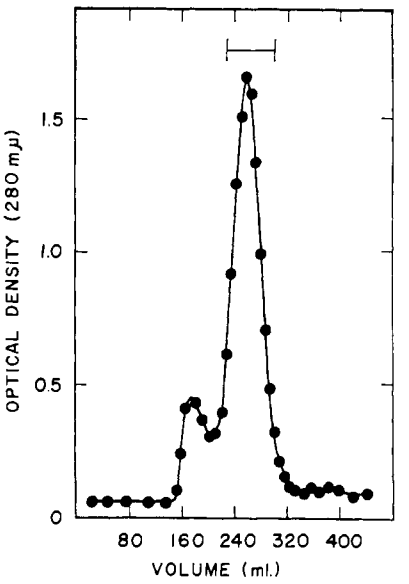



FIGURE 6: Rechromatography of subfragment 1 on Sephadex G-200. The fraction isolated in the chromatography shown in Figure 5 was concentrated and then 8 ml of this concentrated solution was reapplied to the column. Conditions: the same as given in Figure 5. The bar indicates the tubes which were combined, concentrated, and clarified for use in ATPase studies.

material may have been aggregated subfragment 1 rather than contaminant heavy meromyosin. At any rate, the subfragment 1 fraction isolated after the rechromatography was selected to avoid contamination with this material, but nevertheless, like the other subfragment 1 preparations, it too showed threefold activation of its ATPase under the conditions of Figure 1.

Even stronger evidence that activation of the subfragment 1 ATPase by actin was not due to contaminant heavy meromyosin is the demonstration of a difference in the effects of KCl concentration on the actin activation of the heavy meromyosin and subfragment 1 ATPases (Table II). As can be seen, a decrease in KCl concentration from 0.10 to 0.05 M caused almost a fivefold increase in the actin activation of the heavy meromyosin ATPase but only a twofold increase in the actin activation of the subfragment 1 ATPase. This difference in the effect of KCl could not have occurred if the actin activation of the subfragment 1 ATPase was in reality due to contaminant heavy meromyosin in the subfragment 1. In fact this difference must be due to some difference in the enzymatic properties of the heavy meromyosin and subfragment 1, and in this regard it is of interest that Dreizen *et al.* (1967) have also noted differences in the enzymatic activity of subfragment 1 and heavy meromyosin. Small differences in the properties of subfragment 1 and heavy meromyosin are certainly not unexpected considering the degradation to which the molecule has been subjected by trypsin. However, despite these small differences in behavior, our data clearly show that subfragment 1 still retains what is perhaps the principal feature of the myosin and heavy meromyosin ATPases, the ability to show actin activation.

TABLE II: Effect of KCl Concentration on the Acto-Heavy Meromyosin and Acto-Subfragment 1 ATPases.[a]

| Enzyme | KCl Concn (M) | ATPase (μmoles/mg min) Without Actin | ATPase (μmoles/mg min) With Actin | Actin Activation |
|---|---|---|---|---|
| Subfragment 1 | 0.10 | 0.02 | 0.06 | 3-fold |
| Subfragment 1 | 0.05 | 0.02 | 0.13 | 6-fold |
| Heavy meromyosin | 0.10 | 0.02 | 0.19 | 9-fold |
| Heavy meromyosin | 0.05 | 0.02 | 0.83 | 42-fold |

[a] Conditions: 2 mM ATP, 1 mM $MgCl_2$, 2.5 mM imidazole buffer (pH 7.0), and 1 mg/ml of actin. Subfragment 1 concentration, 3 mg/ml in samples without actin and 1 mg/ml in samples with actin. Heavy meromyosin concentration, 3 mg/ml in samples without actin and 0.2 mg/ml in samples with actin.

*Effect of Magnesium on the Acto-Subfragment 1 ATPase.* We next investigated whether the activation of the subfragment 1 ATPase by actin, like the actin activation of the myosin and heavy meromyosin ATPases, requires the presence of magnesium. Using DCTA as a magnesium buffer, we varied the free magnesium concentration, and as can be seen in Figure 7, at very low magnesium concentration there was almost no activation of the subfragment 1 ATPase, but as the magnesium concentration was increased there was a marked increase in the acto-subfragment 1 ATPase. To be certain that this increase in ATPase was indeed due to an increase in free magnesium concentration rather than to a direct effect of DCTA, we varied the DCTA concentration while holding the free magnesium concentration constant. As shown in Figure 8, almost no change in ATPase rate resulted from a change in free DCTA concentration much greater than the change from 5 to 3.7 mM which occurred in Figure 7 due to the complexing of free DCTA with added magnesium, confirming that the increase in the acto-subfragment 1 ATPase which was observed in Figure 7 was indeed due to the increase in free Mg concentration. We can therefore conclude that, as is true for myosin and heavy meromyosin, the actin activation of the subfragment 1 ATPase requires the presence of magnesium.

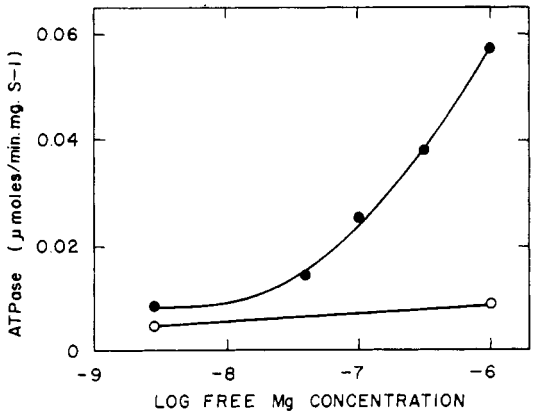



FIGURE 7: Effect of actin on the subfragment 1 ATPase at varying concentrations of magnesium ion. Conditions: 2 mM ATP, 0.05 M KCl, 5 mM DCTA, 1 mM EGTA, and 1 mg/ml of subfragment 1. (●) 0.5 mg/ml of actin added; (○) no actin added. The added magnesium concentration was varied and the free magnesium concentration was calculated by assuming that only ATP and DCTA bind magnesium, that the effective dissociation constants at pH 7 are $2.8 \times 10^{-6}$ M for MgDCTA (Maruyama and Watanabe, 1962) and $5 \times 10^{-5}$ M for MgATP (Nanninga, 1961), and that the total contaminant magnesium concentration is about 5 μM (Offer, 1964). Botts *et al.* (1967) recently reported data suggesting a stronger binding constant for MgDCTA at pH 7, but a change in this constant would not affect the shape of our curves.

*Dissociation of Acto-Subfragment 1 by ATP.* We have recently demonstrated that, at low ionic strength, in the presence of magnesium and ATP, acto-heavy meromyosin is largely dissociated and the observed ATPase is only a small fraction of the maximum attainable at high actin concentration (Eisenberg and Moos, 1968b). To test whether acto-subfragment 1 is similarly dissociated by ATP, we investigated the effect of increasing actin concentration on the subfragment 1 ATPase. As can be seen in Figure 9, rather than leveling off at the stoichiometric binding ratio of actin to subfragment 1, which is 1:2 (w/w) (Young, 1967b), the ATPase of the subfragment 1 steadily increases with increasing actin concen-

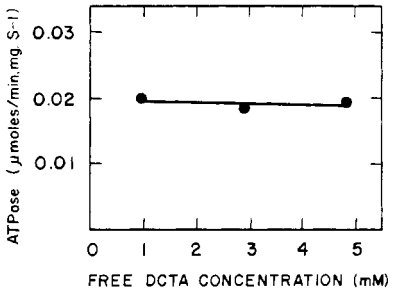



FIGURE 8: Effect of DCTA on the acto-subfragment 1 ATPase. Conditions: 2 mM ATP, 0.05 M KCl, 1 mM EGTA, 0.5 mg/ml of actin, and 1 mg/ml of subfragment 1. The added magnesium and DCTA concentrations were varied proportionately so that the free magnesium concentration calculated as described in Figure 7 remained constant at $1 \times 10^{-7}$ M. The DCTA used was neutralized with tetramethylammonium hydroxide and as the DCTA concentration was decreased, the ionic strength was held constant by adding tetramethylammonium chloride.

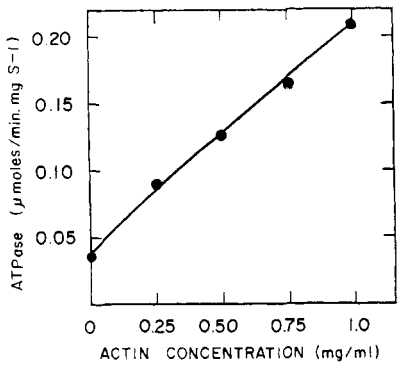



FIGURE 9: Dependence of the subfragment 1 ATPase upon actin concentration. Conditions: 2 mM ATP, 1 mM $MgCl_2$, 4 mM imidazole buffer (pH 7.0), 0.05 M KCl, and 1 mg/ml of subfragment 1.

tration up to at least a 1:1 weight ratio of actin to subfragment 1. This suggests that, like acto-heavy meromyosin, acto-subfragment 1 is also quite dissociated at low ionic strength in the presence of ATP and magnesium. Therefore it should be possible to apply to the actin–subfragment 1 interaction the same double-reciprocal plot analysis that we previously applied to the actin–heavy meromyosin interaction (Eisenberg and Moos, 1968b), that is to plot the reciprocal of the ATPase increment caused by addition of actin against the reciprocal of the added actin concentration and in this way to determine both the maximum ATPase of the acto-subfragment 1 and the apparent dissociation constant for the actin–subfragment 1 interaction in the presence of ATP. Figure 10 shows such plots for both heavy meromyosin and subfragment 1, and it is clear that a linear plot is obtained for subfragment 1 as well as for heavy meromyosin.

The intercept on the ordinate of Figure 10 gives a measure of the ATPase activity at infinite actin concentration, *i.e.*, that of the fully actin-bound subfragment 1, and although this appears to be only about one-half the maximum ATPase of the acto-heavy meromyosin, it represents about 100-fold activation of the subfragment 1 ATPase by actin. This result suggests that the low ATPase rates generally observed at low ratios of actin to subfragment 1 are due simply to the marked dissociation of the acto-subfragment 1 by magnesium and ATP at low ionic strength. In fact, the apparent dissociation constants for acto-subfragment 1 and acto-heavy meromyosin in the presence of magnesium and ATP can be determined from the intercepts on the abscissa of Figure 10. Assuming a binding ratio of $5 \times 10^4$ g of actin/mole of heavy meromyosin or subfragment 1 (Young, 1967b; Rizzino *et al.*, 1968), these dissociation constants are about $1 \times 10^{-4}$ M for the acto-heavy meromyosin and $3 \times 10^{-4}$ M for the acto-subfragment 1. This latter affinity represents about $^1/_{300}$ as strong a binding of actin to subfragment 1 as occurs in the absence of ATP (Young, 1967b), which shows that not only is the subfragment 1 ATPase activated by actin but in addition the binding of ATP to subfragment 1 causes marked dissociation of the acto-subfragment 1 complex at low ionic strength.

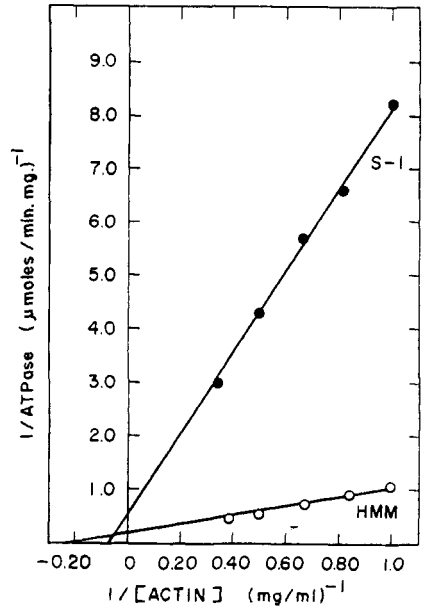



FIGURE 10: Reciprocal plots of acto-heavy meromyosin and acto-subfragment 1 ATPase *vs.* actin concentration. Conditions: 2 mM ATP, 1 mM $MgCl_2$, 5 mM imidazole buffer (pH 7.0), and 0.048 M KCl. (○) 0.08 mg/ml of heavy meromyosin; (●) 0.24 mg/ml of subfragment 1.

## Discussion

The results presented here demonstrate that, like the ATPase of myosin and heavy meromyosin, the ATPase activity of subfragment 1 is strongly activated by actin, and furthermore, that just as for myosin and heavy meromyosin, this actin activation requires the presence of magnesium. From these findings it appears that interaction between the individual subfragment 1 subunits in a myosin or heavy meromyosin molecule is not involved in the actin activation of the myosin or heavy meromyosin ATPase; rather the ability to bind actin and show an activated ATPase must reside within each single active subunit of the myosin molecule.

Our data also show that, as we found for acto-heavy meromyosin, acto-subfragment 1 is markedly dissociated by ATP and magnesium at low ionic strength. Of course, the dissociating effect of ATP on actomyosin, acto-heavy meromyosin, and acto-subfragment 1 at high ionic strength is well documented; however, the fact that ATP causes dissociation at low ionic strength as well leads to certain important consequences. First, it suggests that it may be a mistake to carry out ATPase studies at low ratios of actin to heavy meromyosin or subfragment 1 because at low actin concentrations very little of the heavy meromyosin or subfragment 1 is complexed with actin and to observe reasonable levels of ATPase activation, particularly with subfragment 1, much higher concentrations of actin must be employed. Second, it opens up the possibility that the dissociating effect of ATP may play a key role in the contractile mechanism since contraction itself occurs at a relatively low ionic strength. It therefore becomes of considerable importance to determine whether the dissociating effect of ATP is due to the binding of ATP at some separate

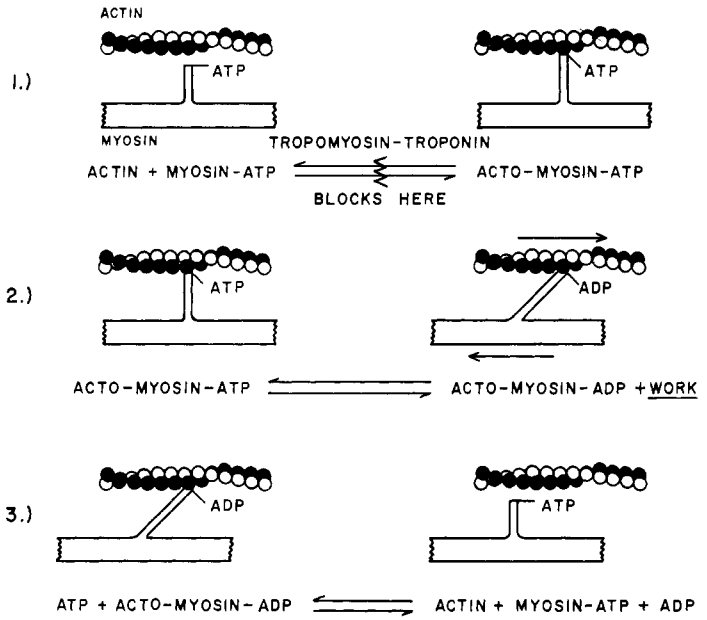
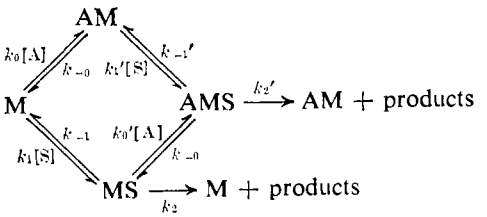



FIGURE 11: A possible scheme for muscle contraction.

dissociating site on the heavy meromyosin or subfragment 1 or whether it is due simply to the binding of ATP at the hydrolytic site.

In our previous study on the interaction of actin and heavy meromyosin (Eisenberg and Moos, 1968b) we presented a kinetic model based on the latter possibility, postulating that the binding of ATP at the hydrolytic site of the heavy meromyosin greatly weakens the binding of actin to its binding site on the heavy meromyosin. Direct evidence for this view is now provided by the data presented in this paper, in conjunction with the observations that subfragment 1 has only a single binding site for ADP (Young, 1967a) and pyrophosphate (Nauss and Gergely, 1967), assuming, of course, that we can extend these findings to ATP as well. It is clear from our data that at low ionic strength acto-subfragment 1 is capable of both hydrolyzing ATP and being dissociated by ATP. But if subfragment 1 has only a single binding site for ATP, then the same ATP must be involved in both of these processes, that is, the binding of ATP to the hydrolytic site must itself cause dissociation of the acto-subfragment 1 complex.

On this basis, the ATPase kinetics of the acto-subfragment 1 system can be most simply analyzed in terms of the general scheme for the interaction of enzyme, substrate, and modifier (Botts and Morales, 1953) which we have previously applied to the acto-heavy meromyosin system (Eisenberg and Moos, 1968b). Here M represents subfragment 1, A represents actin, and S represents ATP. As we described previously, at high ATP concentration the steady-state rate equation for this model reduces to a linear reciprocal form which is consistent with our finding for subfragment 1 that a plot of the reciprocal of the ATPase increment induced by actin *vs.* the reciprocal of the actin concentration is linear (Figure 10). Furthermore, as for acto-heavy meromyosin, the actin activation of the subfragment 1 ATPase is accounted for by assuming that $k_2' \gg k_2$; and the dissociating effect of ATP is readily explained by assuming that $k_0'/k_{-0}' \ll k_0/k_{-0}$, *i.e.*, that the binding affinity of actin to the subfragment 1–ATP complex is much less than the binding affinity of actin to subfragment 1 alone. Therefore this kinetic model explains the dissociating effect of ATP without postulating any separate dissociating site on the subfragment 1 distinct from the hydrolytic site for ATP; and as we noted above, such an explanation is certainly required if, as appears quite likely, subfragment 1 has only a single binding site for ATP.




Not only is this kinetic model consistent with our data but in addition it can be used as a basis for a relatively simple scheme of muscle contraction which gives a role in the contractile mechanism to two of the major properties of the actin–myosin interaction *in vitro:* the strength of the actin–myosin bond in rigor, *i.e.*, in the absence of ATP, and the ability of ATP to cause dissociation of this bond. This scheme, which has certain features in common with the theory recently proposed by Pringle (1967) for insect flight muscle, is shown in Figure 11. The first reaction postulated to occur following activation of the muscle is the combination of myosin–ATP with actin to form a ternary actin–myosin–ATP complex. Although in our *in vitro* experiments relatively little actin binds to the heavy meromyosin–ATP or subfragment 1–ATP complex, considerable actin would probably bind to the myosin–ATP complex *in vivo* because the effective actin concentration is, in all likelihood, much higher *in vivo* than under our *in vitro* conditions.

In the second reaction shown in Figure 11, which is the motion-producing event, actin activates the splitting of the bound ATP and the ternary actin–myosin–ATP complex is transformed to the rigor actin–myosin–ADP complex. Reedy *et al.* (1965) have shown in insect muscle that during relaxation the cross bridges of the myosin extend perpendicular to the thick filament whereas during rigor the actin–myosin bridge forms an angle to the thick filament, and on this basis Reedy (1967) and Pringle (1967) have suggested that the cyclic transformation of a perpendicular actin–myosin bond to the angled rigor bond might push the actin filament relative to the myosin filament. We have applied this suggestion to our scheme by having the actin–myosin–ATP complex formed with the bridge in the perpendicular configuration so that motion will occur when it transforms to the rigor state. The driving force for this motion-producing reaction would come both from the conversion of the bound ATP into bound ADP and from the transformation of the relatively weak bond between actin and myosin–ATP to the much stronger rigor actin–myosin–ADP bond.

In the third reaction in our scheme, the dissociation of the rigor actin–myosin bond is coupled energetically with the replacement of ADP by ATP on the hydrolytic site of the myosin cross bridge. This reaction is postulated on the basis of the finding presented in this paper that apparently the binding of ATP at the hydrolytic site of subfragment 1 causes dissociation of actosubfragment 1 at low ionic strength. The dissociation of actomyosin by ATP at high ionic strength and the clearing response of actomyosin at low ionic strength (Maruyama and Gergely, 1962) would then be *in vitro* manifestations of this step in our scheme. Of course, in order for the cycle shown in Figure 11 to produce over-all relative motion of the thick and thin filaments, the cross bridge in the rigor actomyosin link must not return to its perpendicular configuration, *i.e.*, rebind ATP at the hydrolytic site, without first dissociating from the actin filament, and in terms of the kinetic model discussed earlier, this means that $k_1'$ must be very slow relative to the other rate constants in this model.

Finally, we can incorporate the relaxing effect of the tropomyosin–troponin complex (Ebashi and Kodama, 1966) into our scheme by postulating that, in the absence of calcium, it blocks formation of the ternary actin–myosin–ATP complex as is shown in the first reaction of Figure 11. This would explain the fact that magnesium and ATP are required for muscle relaxation (Watanabe and Sleator, 1957) because in their absence we would expect the system to revert to the rigor state even when the calcium concentration is very low.

We and several other workers have previously suggested that the ATP-induced dissociation of actomyosin is due to the binding of ATP to a dissociating site distinct from the hydrolytic site of the myosin (Weber and Protzehl, 1954; Eisenberg and Moos, 1965; Levy and Ryan, 1966, 1967). However all of the evidence for a separate dissociating site stems from experiments with actomyosin, and the interaction of actin and myosin might be complicated by the fact that at low ionic strength myosin occurs as filaments. In fact, we now have evidence that the ATPase of actomyosin is indeed affected by the way the myosin filaments are formed (E. Eisenberg and C. Moos, unpublished data). Therefore, we now believe that interaction of the individual myosin cross bridges with actin *in vivo* is probably better approximated *in vitro* by the interaction of actin with heavy meromyosin or subfragment 1 than by the interaction of actin with myosin itself.

In summary then, our data suggest that the binding of ATP to the hydrolytic site, rather than to some separate dissociating site on the myosin cross bridge, is involved in the dissociation of the rigor actin–myosin link; and on this basis we have suggested that motion might be generated in muscle by repeated cycles consisting of formation of the rigor actin–myosin link in conjunction with ATP hydrolysis followed by dissociation of this actin–myosin link in conjunction with the rebinding of ATP at the hydrolytic site.

Acknowledgments

We thank Mrs. Fay Ames, Miss Donaline Hayes, and Miss Deborah Schatz for their excellent technical assistance.

References

Blatt, W. F., Feinberg, M. P., Hopfenberg, H. B., and Saravis, C. A. (1965), *Science 150*, 224.

Botts, J., Chaskin, A., Schmidt, L., and Rasken, J. (1967), *Federation Proc. 26*, 818.

Botts, J., and Morales, M. (1953), *Trans. Faraday Soc. 49*, 696.

Dreizen, P., Gershman, L. C., Trotta, P. P., and Stracher, A. (1967), *J. Gen. Physiol. 50*, 85.

Ebashi, S., and Kodama, A. (1966), *J. Biochem. (Tokyo) 60*, 733.

Eisenberg, E., and Moos, C. (1965), *Arch. Biochem. Biophys. 110*, 568.

Eisenberg, E., and Moos, C. (1967), *J. Biol. Chem. 242*, 2945.

Eisenberg, E., and Moos, C. (1968a), *Biophys. J. 8*, A10.

Eisenberg, E., and Moos, C. (1968b), *Biochemistry 7*, 1486.

Eisenberg, E., Zobel, C. R., and Moos, C. (1966), *J. Cell Biol. 31*, 142A.

Hotta, K., and Usami, Y. (1967), *J. Biochem. (Tokyo) 61*, 415.

Jones, J. M., and Perry, S. V. (1966), *Biochem. J. 100*, 120.

Levy, H. M., and Ryan, E. M. (1966), *Biochem. Z. 345*, 132.

Levy, H. M., and Ryan, E. M. (1967), *J. Gen. Physiol. 50*, 2421.

Lowey, S., and Cohen, C. (1962), *J. Mol. Biol. 4*, 293.

Maruyama, K., and Gergely, J. (1962), *J. Biol. Chem. 237*, 1095.

Maruyama, K., and Watanabe, S. (1962), *J. Biol. Chem. 237*, 3437.

Mihalyi, E., and Szent-Györgyi, A. G. (1953), *J. Biol. Chem. 201*, 211.

Mueller, H. (1965), *J. Biol. Chem. 240*, 3816.

Mueller, H., and Perry, S. V. (1962), *Biochem. J. 85*, 431.

Nanninga, L. (1961), *Biochim. Biophys. Acta 54*, 330.

Nauss, K. M., and Gergely, J. (1967), *Federation Proc. 26*, 727.

Offer, G. (1964), *Biochim. Biophys. Acta 89*, 566.

Pringle, J. W. S. (1967), *Progr. Biophys. 17*, 3.

Reedy, M. K. (1967), *Am. Zoologist 7*, 465.

Reedy, M. K., Holmes, K. C., and Tregear, R. T. (1965), *Nature 207*, 1276.

Rizzino, A. A., Eisenberg, E., Wong, K. K., and Moos, C. (1968), *Federation Proc. 27*, 519.

Slayter, H. S. and Lowey, S. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1611.

Watanabe, S. and Sleator, W., Jr. (1957), *Arch. Biochem. Biophys. 68*, 81.

Weber, H. H., and Portzehl, H. (1954), *Progr. Biophys. 4*, 60.

Yagi, K., and Yazawa, Y. (1966), *J. Biochem. (Tokyo) 60*, 450.

Young, M. (1967a), *J. Biol. Chem. 242*, 2790.

Young, M. (1967b), *Proc. Natl. Acad. Sci. U. S. 58*, 2393.

Young, M., Himmelfarb, S., and Harrington, W. F. (1964), *J. Biol. Chem. 239*, 2822.

Young, M., Himmelfarb, S., and Harrington, W. F. (1965), *J. Biol. Chem. 240*, 2428.

# On the Kinetics of Hydrogen Exchange in Deoxyribonucleic Acid. pH and Salt Effects*

Morton P. Printz and Peter H. von Hippel

ABSTRACT: The hydrogen-exchange kinetics of native calf thymus deoxyribonucleic acid have been studied as a function of pH and salt concentration using a tritium–Sephadex method. Previous work had demonstrated that only hydrogens involved in interchain hydrogen bonds in deoxyribonucleic acid exchange sufficiently slowly ($t_{1/2}$ = >50 sec) to be visualized by such gel filtration techniques. It is shown in this study that deoxyribonucleic acid hydrogen exchange proceeds at a minimum rate at 0° and pH values close to neutrality ($pH_{min}$); increasing or decreasing the pH from $pH_{min}$ increases the rate of exchange. Furthermore, the rate of exchange at any given pH, as well as the value of $pH_{min}$, depends upon the salt concentration. At pH values greater than $pH_{min}$, the rate of exchange increases directly with log [$Na^+$]; at pH values below $pH_{min}$ the rate decreases with log [$Na^+$]. $pH_{min}$ also shifts to lower pH values as the salt concentration is increased. These findings correlate directly with salt-induced changes in the pH–titration curve of DNA. It is shown that $pH_{min}$ for hydrogen exchange corresponds closely at a given salt concentration to the midpoint of the plateau of the pH–titration curve, and the rate of exchange at a pH removed from $pH_{min}$ is inversely related to the absolute value of the difference between the experimental pH and the $pK_{app}$ of the nearer limb of the titration curve. The results of this study are discussed in terms of a simple two-step exchange model (see eq 4 of text) involving a structure-controlled opening–closing reaction characterized by forward and reverse rate constants, $k_1$ and $k_2$, and an over-all rate constant for the chemical exchange process, $k_3$. Attempts are made to separate pH- and salt-induced changes in exchange rate into structural ($k_1$ and $k_2$) and chemical ($k_3$) effects.

Hydrogen exchange as a potential method of macromolecular conformation analysis was introduced and first developed by Linderstrom-Lang and his colleagues (*e.g.*, see Linderstrom-Lang, 1955). In the intervening years, conceptual and methodological refinements have improved this approach to the point that several variants of it are now being extensively used in protein structure studies (for reviews see Hvidt and Nielsen (1966), Harrington *et al.* (1966), and Englander (1967)).

More recently, this approach has also been extended to an analysis of DNA structure (Printz and von Hippel, 1965; von Hippel and Printz, 1965), using the tritium–Sephadex method introduced by Englander (1963). It has been shown that the potentially exchangeable hydrogens of DNA (hydrogens attached to nitrogen and oxygen; carbon-bound hydrogens do not exchange with solvent tritium under the conditions used) can be fully labeled by brief incubation in tritiated water, and that after passage of the sample through a Sephadex column to remove most of the free tritium, the exchange-out of the hydrogens involved in interchain hydrogen bonding can be followed (after another gel filtration step) on a time scale calibrated in seconds. Similar findings have been obtained by Englander and Englander (1965) with tRNA.

In DNA, exchangeable hydrogens are generally calculated as hydrogens/nucleotide pair (H/np) and the data are presented as plots of log $(H/np)_t$ *vs.* $t$ (exchange-out time). Under conditions where exchange is moderately slow, such graphs can be extrapolated to zero time of exchange to determine the total measurable hydrogens per nucleotide pair, $(H/np)_0$, originally present in the structure. It has been shown (at pH 7–8 in 0.1 M NaCl and at temperatures below 4°), that exchange-out data obtained with calf thymus and bacteriophage T4 DNA (Printz and von Hippel, 1965) and various bacterial DNAs ranging in base composition from 30 to

* From the Rockefeller University, New York, New York 10021, and the Department of Chemistry and Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403. *Received June 24, 1968.* Most of the experimental work reported here was performed while the authors were members of the staff of the Department of Biochemistry, Dartmouth Medical School, Hanover, N. H. 03755. Presented in part at the 49th Annual Meeting of the Federation of American Societies for Experimental Biology, April 1965, Atlantic City, N. J. This research was supported by U. S. Public Health Service Research Grants AM-03412, AM-12215, and AM-02493 from the National Institute of Arthritis and Metabolic Diseases, and by Postdoctoral Fellowship 5-F2-GM-13,156 (M. P. P.), Research Career Program Award 2-GM-K3-5479 (P. H. von H.), and Research Grant GM-15792 from the National Institute of General Medical Sciences.