The Relationship of the Dissociation to the Catalytic Activity of Glycogen Phosphorylase a*

JERRY H. WANG AND DONALD J. GRAVES

From the Department of Biochemistry and Biophysics, Iowa State University, Ames

Received April 9, 1964; revised July 6, 1964

Dilution and rapid assay at 20° of phosphorylase *a* samples preincubated in 3.0 M NaCl yield high initial enzymic activity which rapidly decays to the activity of samples not incubated in NaCl. The more active species is preserved by dilution with glycogen. The decay at 6.5° follows second-order kinetics and the half-life for this conversion has been found to be dependent upon protein concentration. A dependence of specific activity upon protein concentration has been observed with phosphorylase *a* in contrast to phosphorylase *b*. Temperature, *p*H, and preincubation of enzyme with AMP have been found to alter the specific-activity profile. Activities at 15° are dependent upon preincubation temperature, and rapid cooling of enzyme from 34° to 9° results in a second-order decay of activity. A discontinuity in an Arrhenius plot has been observed for phosphorylase *a* which is altered by preincubation of enzyme with AMP. Ultracentrifugal patterns obtained in the presence of this nucleotide are symmetrical at 6°, skewed at 23°, and composed of two boundaries at 34° with $s_{20,w}$ of 8.3 and 12.7 S. In 3.0 M NaCl, phosphorylase *a* activity is dependent upon the presence of AMP and the preincubation time of enzyme in NaCl. These observations suggest that in solutions of high ionic strength or of low protein concentration the tetrameric form of phosphorylase *a* dissociates into a dimeric species of higher catalytic activity.

In solutions of high ionic strength, phosphorylase *a*, a tetramer with a molecular weight of 495,000 (Keller and Cori, 1953), was found to dissociate reversibly into a new molecular species with a molecular weight of 258,000 (Wang and Graves, 1963). In the present work study of the dimeric form of phosphorylase *a*[1] has been undertaken in order to (1) define the role of this species in the catalytic process; and (2) determine whether the appearance of enzyme activity that occurs in the conversion of phosphorylase *b*, a dimer with a molecular weight of 242,000 (Keller and Cori, 1953), to phosphorylase *a* is more directly related to the phosphorylation of the protein than to the doubling of molecular weight (Krebs *et al.*, 1958).

In order to fully understand the exact role of protein subunits in enzymic catalysis, it is essential to know what molecular form of enzyme is present under specific assay conditions. As activities are usually measured at low protein concentrations, the study of this relationship between molecular weight and activity is difficult. A new approach to the study of this relationship has been developed for glycogen phosphorylase *a* based on the measurement of its catalytic properties at different protein concentrations and temperatures.

Experimental Procedure

Materials.—The preparation of AMP-free phosphorylase *a* and the determination of protein concentration were as previously described (Wang and Graves, 1963). AMP and ATP were purchased from Pabst Laboratories, Milwaukee, Wisc. Cysteine-HCl, sodium glycerophosphate, potassium glucose-1-phosphate, and shellfish glycogen were obtained from Sigma Chemical Co., St. Louis, Mo. Glycogen was used without further purification unless otherwise described.[2]

Enzyme Assay.—Activities were measured according to the procedure of Illingworth and Cori (1953) unless otherwise stated. Specific activities were expressed as units/mg, where units were taken as the first-order rate constant multiplied by 1000 and by the appropriate dilution factor as described by Illingworth and Cori (1953). For the measurement of enzyme activities below 25° assays were conducted in a refrigerated water bath; in addition, the room temperature was controlled to within 1° of the assay temperature to minimize any change of temperature that might occur during the sampling of enzyme solutions. For enzyme assays at different protein concentrations, the assay time was varied from 30 seconds to 5 minutes as the protein concentration was varied approximately from 0.3 mg/ml to 0.03 mg/ml, respectively. As the release of inorganic phosphate from glucose-1-phosphate at *p*H 7.0 was found to follow first-order kinetics at 15° with a protein concentration of 0.12 mg/ml and the keq (glucose-1-phosphate)/(inorganic phosphate) = 0.37 was not significantly altered by decrease in temperature from 30 to 7°, enzyme activity was calculated at temperatures below 30° as described by Illingworth and Cori (1953).

Sedimentation Experiments.—Ultracentrifugation, measurement of sedimentation coefficients, and determination of percentage of different components were as previously described (Wang and Graves, 1963). For ultracentrifugation at 34°, the rotor was preheated for 1 hour in an oven to 36°. Temperature was controlled for these runs to within ±1°.

Results

Effect of Preincubation of Phosphorylase a in 3.0 M NaCl on Catalytic Activity.—In 3.0 M NaCl the tetra-

* Journal Paper J-48400 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa, Project No. 1510. This research was supported by a research grant (RG-9587) from the National Institutes of Health, U. S. Public Health Service. These results were presented at the 146th meeting of the American Chemical Society, Denver (Wang and Graves, 1964).

[1] As phosphorylase *a* has been found to be made up of four subunits (Madsen and Cori, 1956) this molecular species has been denoted as a tetramer. The dimer of phosphorylase *a* refers to a molecular species of one-half the molecular weight of the tetramer, and is distinguished from phosphorylase *b*, a dimer without phosphorylated seryl residues.

[2] Although commercial glycogen has been found to contain AMP (Fischer and Krebs, 1962), it was found that this contamination by AMP did not affect the dependence of specific activity upon protein concentration (Fig. 9) nor the kinetic analysis of the activity decay curve (Fig. 3).

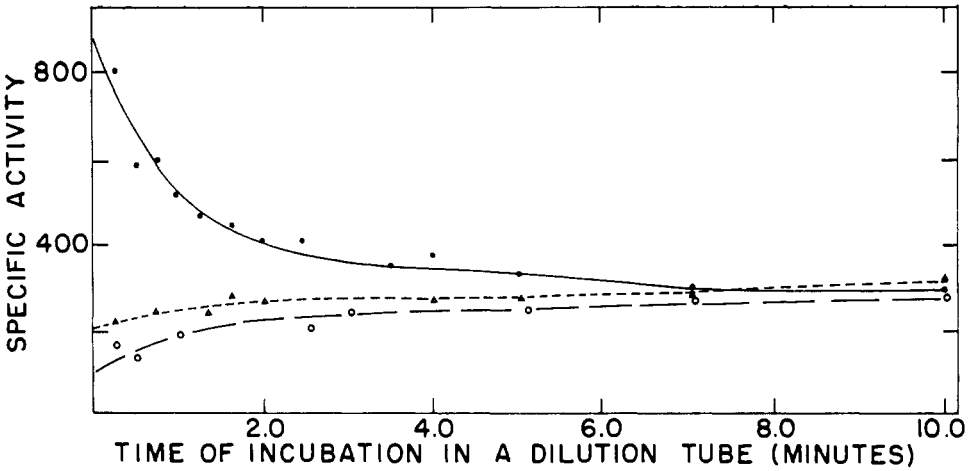


FIG. 1.—Decay of phosphorylase *a* activity. Solid circles, enzyme (7.5 mg/ml) was incubated for 1 hour at 20° in 0.04 M glycerophosphate–0.03 M cysteine, *p*H 7.0, with 3.03 M NaCl prior to a 30-fold dilution in 0.04 M glycerophosphate–0.0015 M EDTA, *p*H 7.0. Samples were withdrawn at various intervals after dilution and were assayed for 2 minutes at 20° with substrate without AMP, *p*H 7.0; open triangles, as solid circles except with the inclusion of 10^{-3} M AMP in the incubation solution. Open circles, as solid circles, with only 0.03 M NaCl in incubation buffer and 0.1 M NaCl in the dilution buffer.

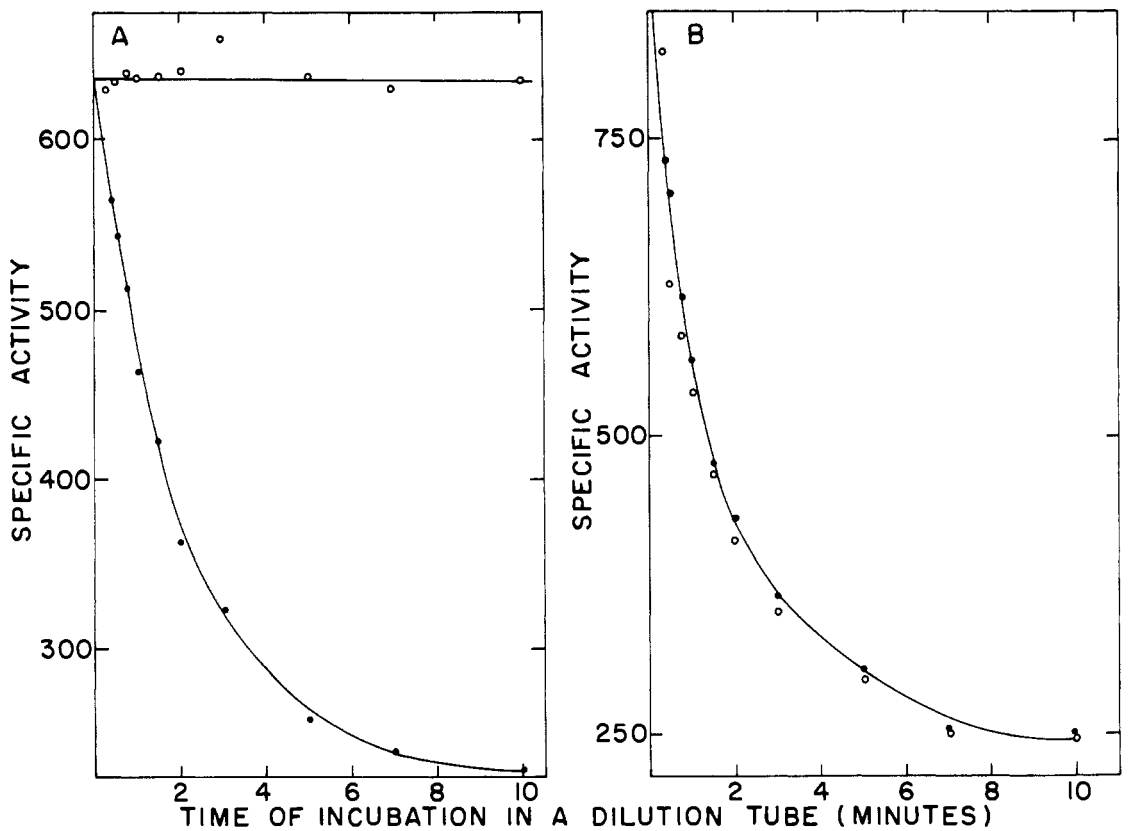


FIG. 2.—Effect of glycogen and glucose-1-phosphate on activity decay. Incubation as in solid circles of Fig. 1 with enzyme at 5 mg/ml. A, solid circles, dilution in 0.04 M glycerophosphate–0.0015 M EDTA, *p*H 7.0, at 20°; A, open circles, dilution with buffer containing 4% glycogen. A, open and solid circles, assay at 20° for 2 minutes with glycogen and glucose-1-phosphate at final concentrations of 2% and 0.016 M, respectively. B, solid circles, dilution as in A, open circles, and assay with glycogen and glucose-1-phosphate at final concentrations of 1% and 0.064 M, respectively; B, open circles, dilution as in A open circles with 0.128 M glucose-1-phosphate and assay as in B, solid circles.

meric form of phosphorylase *a* is converted to a dimeric species (Wang and Graves, 1963). Although only slight activity could be detected in 3.0 M NaCl, as measured in the absence of AMP, 90% of the original activity at 30° could be demonstrated following removal of NaCl by dialysis (Wang and Graves, 1963). At 30° full catalytic activity could be demonstrated following dilution and assay of phosphorylase *a* preincubated in 3.0 M NaCl. If dilution and activity tests were carried out at 20°, the appearance of an extremely active form of the enzyme could be demonstrated. Figure 1 illustrates the effect of time after dilution in the glycerophosphate-EDTA buffer on enzymic activity and shows that enzyme of high initial activity is rapidly transformed to the catalytic activity of control samples not exposed to high ionic strength. Preincubation of

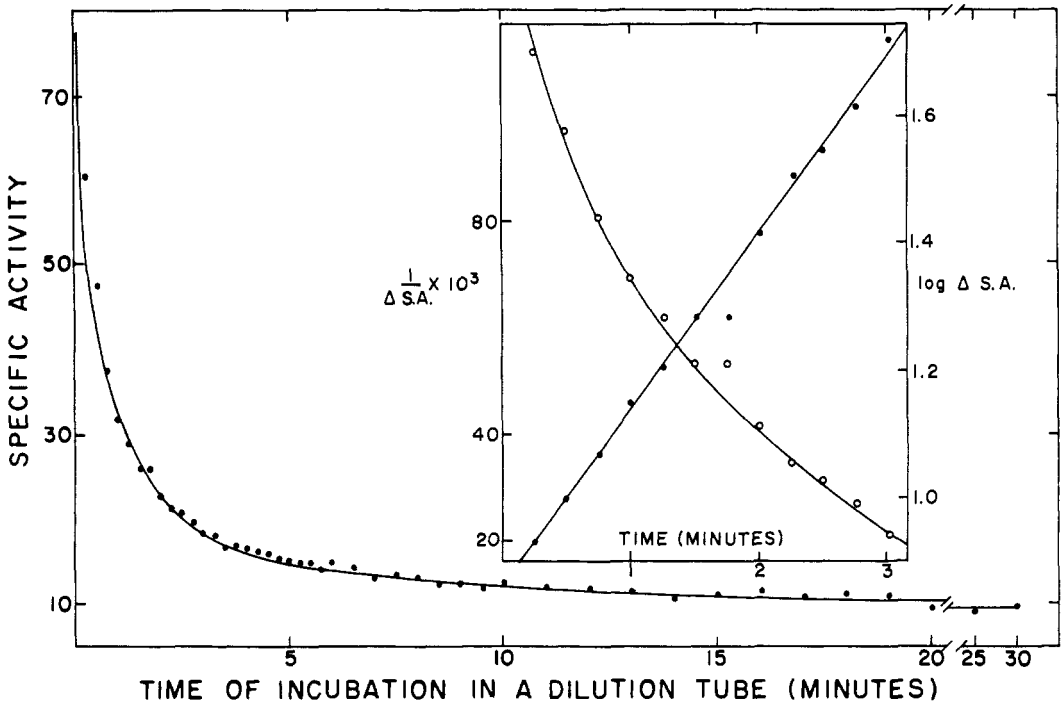


FIG. 3.—Kinetic order of activity decay. Incubation as in solid circles of Fig. 1 with enzyme at 10.3 mg/ml. After 1 hour a 15-fold dilution was made in 0.04 M glycerophosphate–0.001 M EDTA, *p*H 7.0, at 6.5°, and aliquots were then removed at various intervals for activity measurements at 6.5° for 5 minutes with glycogen and glucose-1-phosphate, at final concentrations of 2% and 0.016 M, respectively. Upper right rectangle: open circles, logarithm of ΔSA; and closed circles, reciprocal of ΔSA.

phosphorylase *a* in high ionic strength in the presence of 10^{-3} M AMP does not yield enzyme of high initial activity. As AMP was previously shown to prevent formation of a dimeric species in 3.0 M NaCl, the data suggest that high initial enzyme activity is related to enzyme dissociation and is not due to mere exposure of enzyme to a solution of high ionic strength

Kinetic Analysis of the Activity-Decay Process.—Inclusion of glycogen, a substrate for this enzyme, in the glycerophosphate-EDTA dilution buffer was found to prevent the decay of activity of phosphorylase *a* samples preincubated in 3.0 M NaCl.

Figure 2A shows that the activity of enzyme diluted in the presence of 4% glycogen remains constant whereas enzyme diluted in glycerophosphate-EDTA buffer shows a marked dependence on time after dilution. With dilution in 2% glycogen approximately 90% of the more active species could be preserved. No protective effect could be afforded by dilution in the presence of glucose-1-phosphate, a second substrate for this enzyme even at 0.128 M, a concentration seven times greater than that used for ordinary activity measurements (Fig. 2B). With data obtained at 6.5° (Fig. 3), kinetic analyses of the decay of enzymic activity were made with the following assumptions: (1) The amount of the more active species does not change during the enzyme assay due to the presence of glycogen. (2) The activity of each enzyme species is not altered by the presence of other enzyme molecular forms.

The activity of enzyme after 30 minutes of incubation in the dilution buffer can be taken as the activity of the tetrameric form of phosphorylase *a*, since ultracentrifugation of enzyme diluted 15-fold from 3.0 M NaCl to 0.8 mg/ml at 7.0°, yielded only one component with a $s_{20,w}$ of 12.7 S. The specific activity of the more active species and the less active tetramer can be designated as A_x and A_y, respectively. The following equation results directly from assumption (2), where A is

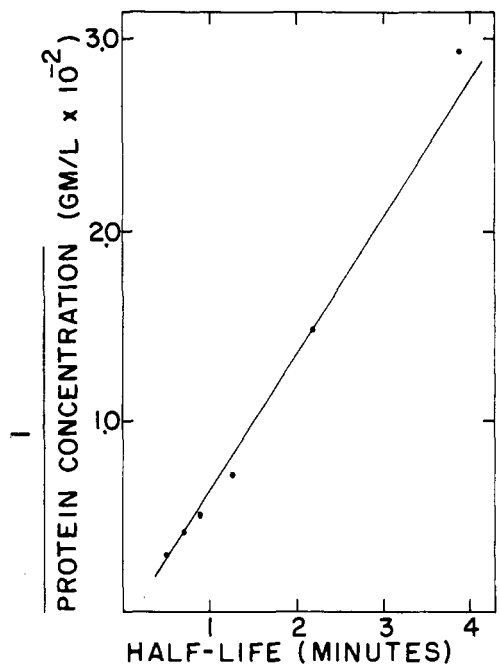


FIG. 4.—Effect of protein concentration on half-life. Enzyme preincubated at different protein concentrations in the incubation buffer of Fig. 1, solid circles, prior to a 30-fold dilution in 0.04 M glycerophosphate–0.001 M EDTA, *p*H 7.0, at 10°. Activities were measured at various intervals at 10° with substrate without AMP (see Methods). For the calculation of half-life, initial activity was determined by dilution in the given buffer containing 2% glycogen and assay at 10°.

the measured specific activity at time t with α and β as the fractions of the two forms, respectively.

$$A = \alpha A_x + \beta A_y \qquad (1)$$

with $\beta = 1 - \alpha$, equation (2) can be formulated

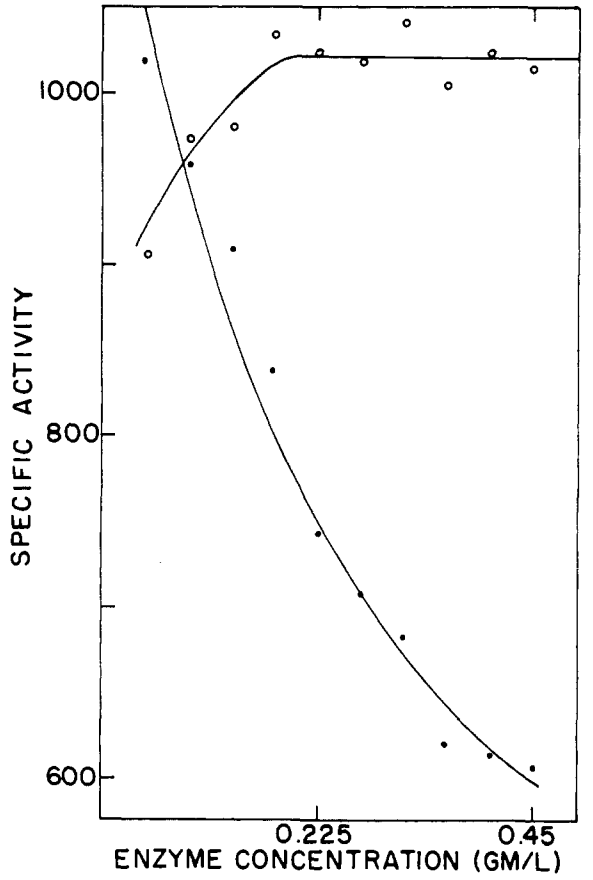


FIG. 5.—Dependence of specific activity of phosphorylase *a* and *b* upon protein concentration. Enzyme diluted to various concentrations in 0.03 M cysteine–0.04 M glycerophosphate, *p*H 7.0, and incubated for 10 minutes at 25° prior to assay with substrate containing 10^{-3} M AMP. Open circles, phosphorylase *b*; solid circles, phosphorylase *a*.

$$\alpha = \frac{A - A_y}{A_x - A_y} \qquad (2)$$

Since A_x and A_y are constants for a given experiment, the amount of the more active form is proportional to the difference of the observed enzyme activity and the activity of the native tetramer. The kinetic order of the transformation of the more active form to the tetramer, therefore, can be obtained following the rate of change of $A - A_y$, ΔSA.[3] A straight line is obtained when a reciprocal of ΔSA is plotted against the time of incubation in glycerophosphate-EDTA buffer prior to enzyme assay but not when the logarithm of ΔSA is used as the ordinate (Fig. 3). The activity-decay process, therefore, appears to conform to the kinetics of a second-order reaction. Figure 4 shows that the half-life for this conversion is dependent upon protein concentration also in accord with a second-order process. These studies suggest strongly that the more active species of phosphorylase *a* is a dimer. Decay of a more active tetrameric species with a different conformation from the native tetramer would have been expected to follow first-order kinetics.

The Dependence of Phosphorylase a Activity upon Protein Concentration.—As it appears that both dimeric and tetrameric forms of phosphorylase *a* are catalytically active, the problem arises as to which of these enzyme species exists in dilute solutions normally used for activity measurements. By consideration of the principle of mass action, dilution would be expected to favor the dissociated species. If phosphorylase *a* tetramer dissociates upon dilution to a dimeric species, a higher specific activity might be expected with more dilute enzyme solutions. Figure 5 shows such a dependence of specific activity of phosphorylase *a* with concentration. Phosphorylase *b*, in contrast to phosphorylase *a*, did not show this increase in specific activity as measured over the same concentration range. Indeed, at very low concentrations of phosphorylase *b* a decrease rather than an increase in specific activity was observed.

[3] Abbreviation used in this work: SA, specific activity.

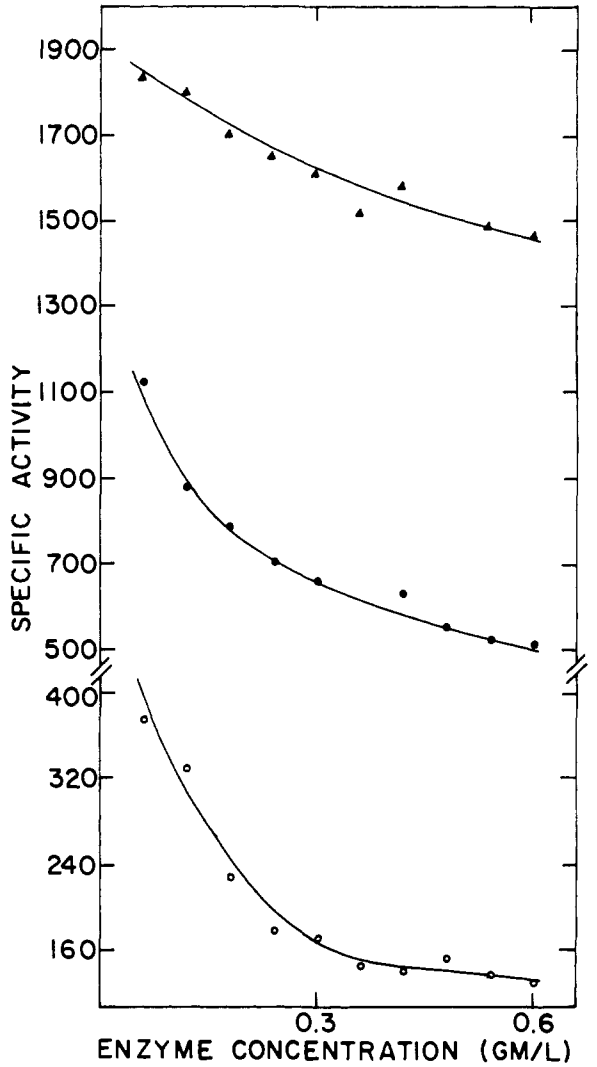


FIG. 6.—Effect of temperature on the specific activity of phosphorylase *a* at various protein concentrations. Enzyme diluted as in Fig. 5 and incubated at three different temperatures for 10 minutes prior to activity measurements at *p*H 7.0 with AMP substrate. Solid triangles, incubation and assay at 30°; solid circles, at 25°; open circles, at 18°.

Effect of Temperature.—Figure 6 shows the dependence of specific activity of phosphorylase *a* upon protein concentrations at three different temperatures. At 18° enzyme activity is markedly dependent upon protein concentration below 0.3 mg/ml, whereas at 25° a dependence of specific activity is observed at all protein concentrations. At 30°, the temperature normally used for activity measurements, enzymic activity is higher as expected but the variation of specific activity with protein concentration is less pronounced. If the variation of specific activity with protein concentration is interpreted on the basis of dissociation of a less active tetramer to a more active dimer, the effect of temperature on the shape of the curves illustrated in

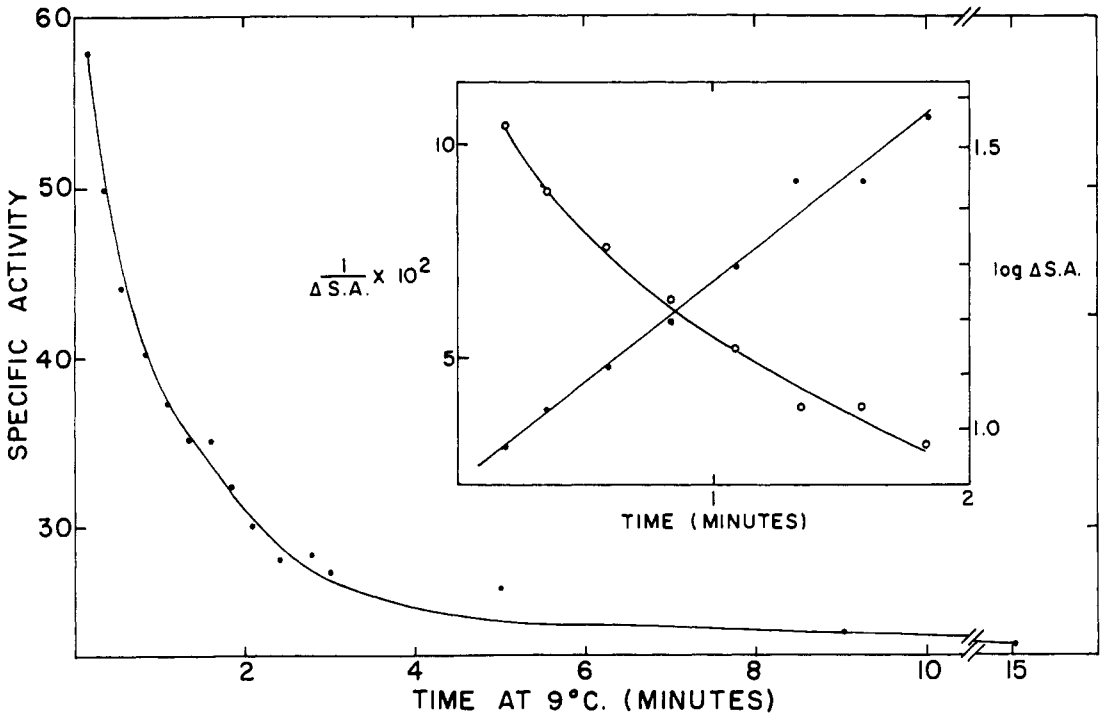


FIG. 7.—Kinetic analysis of decay of phosphorylase *a* activity upon rapid cooling. Phosphorylase *a* (0.4 mg/ml) was preincubated in 0.03 M cysteine–0.04 M glycerophosphate, *p*H 7.0, at 34° for 30 minutes prior to the rapid cooling to 9° in a dry ice–acetone bath (time for cooling, 12 seconds). After cooling, enzyme was transferred to a 9° water bath, incubated, and assayed for enzyme activity at 9° for 5 minutes with substrate without AMP. Inner rectangle: open circles, log ΔSA; solid circles, reciprocal of ΔSA.

Figure 6 indicates that high temperature favors dissociation. The validity of this notion can be further tested by comparing the specific activities of phosphorylase *a* solutions incubated at different temperatures prior to the enzyme assay at 15°. The activity of phosphorylase *b*, in contrast to phosphorylase *a*, is not affected by preincubation at different temperatures prior to enzyme assay (Table I). The effect of preincubation at 30° (column A) is not apparent if the pre-

TABLE I

EFFECT OF INCUBATION TEMPERATURE ON PHOSPHORYLASE ACTIVITY AT 15°[a]

Enzyme	Specific Activities at 15°C			
	30°			
	A	B[b]	15°	0°
Phosphorylase *a*	234	95	95	56
Phosphorylase *b*	247		251	

[a] Enzyme (0.13 mg/ml) was incubated in 0.04 M glycerophosphate–0.03 M cysteine, *p*H 7.0, at 30, 15, and 0° for 10 minutes prior to addition to an equal volume of substrate at 0, 15, and 30°, respectively. The temperature after mixing under these conditions was found to be 15°.
[b] After a 30° incubation, enzyme was preincubated for an additional 10 minutes at 15° prior to assay.

heated enzyme is first cooled before addition to a substrate solution (Table I, column B). In order to examine the kinetics of the change of activity of phosphorylase *a* upon cooling, enzyme was first incubated at 34° and then cooled rapidly to 9°. Analysis of this activity decay did not fit a first-order rate equation, but did comply with a second-order reaction (Fig. 7, inner plot). The data suggest that phosphorylase *a* dissociates at 34° into two dimeric units which rapidly associate into a tetrameric species of lesser enzymic activity at 9°.

Effect of pH.—Figure 8 shows that at *p*H 7.0 and 6.0 there is a marked dependence of activity with concentration whereas at *p*H 8.0 the specific activity appears to be independent of protein concentration. In view of the effect of *p*H on the salt-dependent dissociation of phosphorylase *a* (Wang and Graves, 1963), it might be expected to find more tetramer at *p*H 8.0 than at *p*H 7.0. The lack of dependence of activity with protein concentration at *p*H 8.0 suggests that under these experimental conditions only the tetrameric form of the enzyme is present.

Effect of AMP.—Figure 9 shows that the inclusion of AMP in the substrate did not alter the shape of the activity curve significantly, but merely yielded higher enzyme activity as expected (Green and Cori, 1943). Preincubation of phosphorylase *a*, however, in 10^{-3} M AMP prior to the enzyme assay changed considerably the profile of the activity curve. The effect of preincubation of phosphorylase *a* with AMP can be further demonstrated by measurement of enzyme activities at different temperatures at one protein concentration. Without preincubation, as illustrated in Figure 10, there is a discontinuity in the Arrhenius plot with activation energies of 21,000 and 32,600 cal for the upper and lower limbs, respectively. With preincubation the transition temperature is lowered and the activation energies of 22,500 and 49,500 cal were calculated for the upper and lower limbs, respectively.

The discontinuity in the Arrhenius plot might be expected if the dimeric and tetrameric forms of phosphorylase *a* have different activation energies. As a working hypothesis to explain the results of Figure 9 and the lowering of the transition temperature of Figure 10, it has been assumed that preincubation with AMP enhances the dissociation of the tetrameric form of phosphorylase *a*. In order to test this hypothesis, ultracentrifugation of dilute phosphorylase *a* solution was carried out at different temperatures. Figure 11

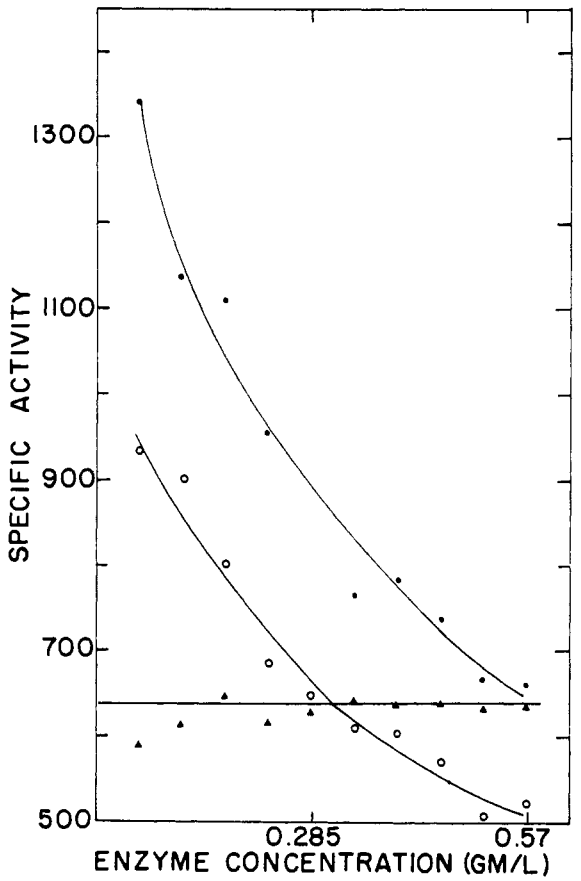


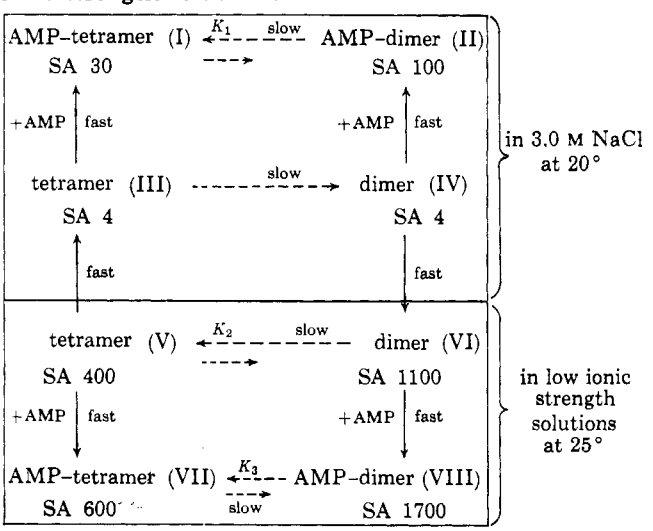
FIG. 8.—Effect of *p*H on the specific activity of phosphorylase *a* at various protein concentrations. Phosphorylase *a* was diluted and incubated at 25° at various protein concentrations in the following buffers for 10 minutes prior to assay at 25° with AMP substrate. Open circles, 0.03 M cysteine–0.04 M glycerophosphate, *p*H 6.0; solid circles, 0.03 M cysteine–0.04 M glycerophosphate, *p*H 7.0; open triangles, in 0.03 M cysteine–0.02 M Tris, *p*H 8.0.

shows schlieren patterns of six phosphorylase *a* samples. If ultracentrifugation of enzyme (1.2 mg/ml) is carried out at 6° without or with AMP (A) or at 23° without AMP (B), phosphorylase *a* appears to be monodisperse in contrast to the skewed schlieren pattern obtained in the presence of AMP at 23° (C). At 34° phosphorylase *a* does not appear to be monodisperse (D), and if 10^{-3} M AMP is included two diffuse boundaries develop during the course of the centrifugation with sedimentation constants of $s_{20,w}$ of 12.7 and 8.3 S (E), similar to those obtained for the dimeric and tetrameric form of enzyme in high ionic strength (Wang and Graves, 1963). The lack of resolution of these boundaries even after 42 minutes is characteristic of reaction between interacting components and not of a mixture of two noninterconvertible molecular forms with s_{20} values of 12.7 and 8.3 S. If these two boundaries are a result of an equilibrium between the tetrameric and the dimeric forms of phosphorylase *a*, the relative amounts of these two species should also depend upon protein concentration. Increasing the protein concentration from 2.1 mg/ml (E) to 12 mg/ml (F) decreases the slower-sedimenting component from 47 to 15%.

Enzyme Activity in Solutions of High Ionic Strength.—Although only minor enzyme activity could be demonstrated in 3.0 M NaCl (approximately 1.0% of activity at low ionic strength), significant phosphorylase *a* activity could be demonstrated in 3.0 M NaCl with substrate solutions containing AMP (Fig. 12). The extent of enzyme activity with AMP substrate was found to be highly dependent upon preincubation time of enzyme in NaCl, in striking contrast to enzyme activities measured without AMP in the substrate solution. As the tetrameric form of phosphorylase *a* is converted to a dimeric species in 3.0 M NaCl, the data suggest that the slow rise in enzymic activity is related to dimer formation and the decrease in enzymic activity observed after addition of AMP to the incubation tube is related to re-formation of the tetrameric species.

DISCUSSION

Kinetic analyses of the alteration of the catalytic properties of phosphorylase *a* under different experimental conditions are consistent with the following reaction scheme for enzymic activity in low- and high-ionic-strength solutions:[4]



The kinetic and ultracentrifugal data for the conversion of dimer (IV) to tetramer (V) upon dilution of phosphorylase *a* preincubated in 3.0 M NaCl cannot be explained by rapid formation of an intermediate tetrameric species with a different conformation and higher enzymic activity than the tetramer (V). As the dilution of enzyme resulted in a second-order decay of enzymic activity, the present experiments support the concept that the transient form is dimer (VI) with high activity which is slowly transformed to a less active tetramer.

As these experiments suggest strongly that the dimeric form of phosphorylase *a*, mw 258,000, is catalytically active, the present work supports the notion that the appearance of enzymic activity that occurs in the conversion of phosphorylase *b*, mw 242,000, to phosphorylase *a* is more directly related to the phosphorylation of the protein than to molecular-weight alterations. Indeed, the kinetic experiments suggest that increase in molecular weight leads to a decrease rather than an increase in catalytic activity. The view that enzyme activity is primarily related to phosphorylation and that dimerization of the protein is only a secondary effect is consistent with the observation that the conversion of inactive liver phosphorylase

[4] The numerical values of activities of the different enzyme species in this scheme were not intended to be absolute, but only to serve the purpose of comparison. The activities for the enzyme species in low ionic strength were estimated from Figure 9 by extrapolation and for those in higher ionic strength were estimated from Figure 12. Since the temperature used in these two experiments was not the same, comparison of activities between enzymes in low-ionic-strength or higher-ionic-strength solutions may only be approximated.

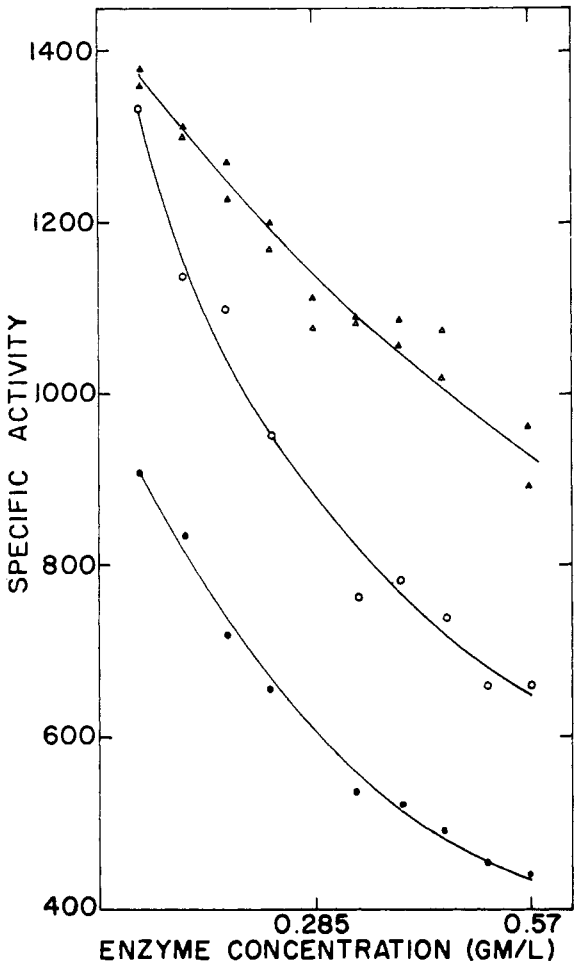


FIG. 9.—Effect of AMP on the specific activity profile Open and solid circles, enzyme diluted in 0.03 M cysteine–0.04 M glycerophosphate, *p*H 7.0, and incubated for 10 minutes at 25°. Open circles, assay with 10^{-3} M AMP at 25°; solid circles, assay without AMP. Open and solid triangles, incubation as above with 10^{-3} M AMP. Open triangles, assay with 1.5×10^{-3} M AMP; solid triangles, assay with 5×10^{-4} M AMP. Glycogen used in these experiments was purified according to Sutherland and Wosilait (1956).

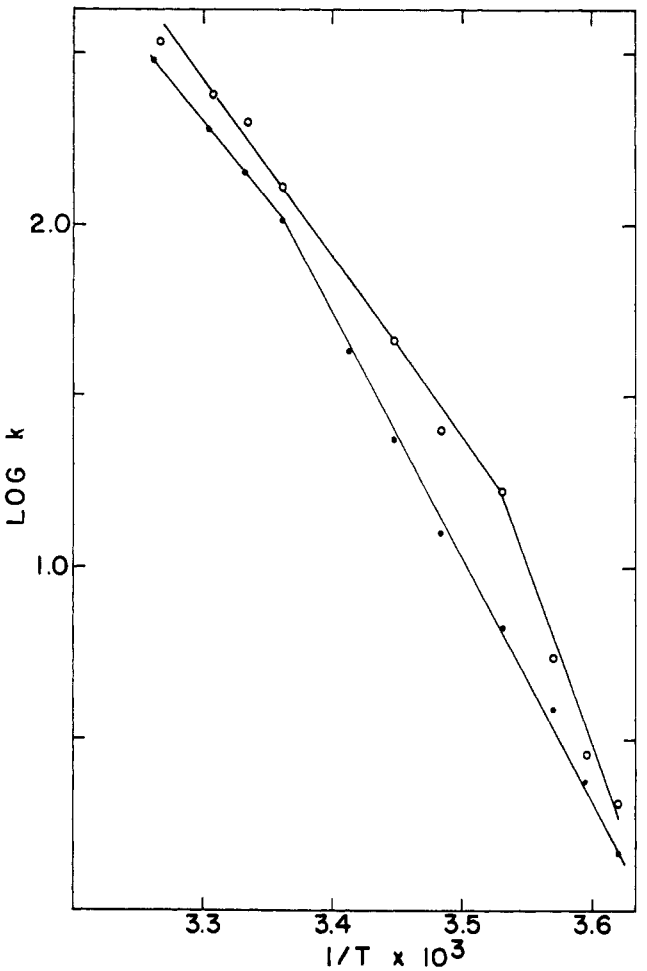


FIG. 10.—Effect of temperature on phosphorylase *a* activity. Phosphorylase *a* (0.43 mg/ml) was incubated at room temperature for 35 minutes and then incubated at the assay temperature for 5 more minutes prior to assay with AMP substrate. Open circles, incubation in 0.03 M cysteine–0.04 M glycerophosphate, *p*H 7.0, with 10^{-3} M AMP; solid circles, incubation without AMP.

to an active form involves phosphorylation but no molecular-weight alteration (Wosilait and Sutherland, 1956).

The catalytic activity of glycogen phosphorylase preincubated and assayed in 3.0 M NaCl is also consistent with the dimeric species' being more active than the tetrameric form. In this case, the data suggest that the tetramer (III) and the dimer (IV) are essentially inactive but in the presence of AMP may be rapidly converted to active species (I) and (II), respectively. As addition of AMP to enzyme preincubated in 3.0 M NaCl alters only slowly the activity as measured with substrate containing AMP, these data suggest that species (I) and (II) are only slowly interconverted.

In addition to a study of catalytic activity of the dimeric form of phosphorylase *a*, the present work was undertaken to define the molecular form of enzyme present under specific assay conditions. The observed dependence of phosphorylase *a* activity upon protein concentration suggest that phosphorylase *a* tetramer (V) dissociates into a smaller unit at low protein concentration and that this dissociation is favored by decreasing *p*H, increasing temperature, or preincubation of enzyme with AMP. As rapid cooling of a dilute phosphorylase *a* solution from 34 to 9° resulted in high initial activity at 9°, which rapidly decayed by a second-order reaction to the activity of enzyme not preincubated at 34°, these data suggest that at low protein concentration at 34° phosphorylase *a* exists as dimer (VI). The kinetic data do not exclude the possibility that the tetrameric form of phosphorylase *a* dissociates into an active monomeric species at low protein concentration, if it is assumed that upon cooling these units associate rapidly to dimer (VI) followed by slow formation of tetramer (V).

The protein concentration–dependence of specific activity and the kinetic data suggest that, although phosphorylase *a* is isolated from rabbit muscle as a tetrameric species, this molecular form is probably not important for *in vitro* measurements of enzyme activity above 30°, and causes strong doubt regarding the physiological significance of the isolated tetramer. As the dilution of phosphorylase *a* from 3.0 M NaCl at 30° did not yield enzyme of higher activity than control samples not exposed to NaCl, in contrast to results obtained at lower temperatures, the authors feel these results may also be best explained by assuming that at 30° only the dimeric form of phosphorylase *a* was present in control samples.

The effect of AMP on the structure and catalytic properties of the various molecular forms of glycogen phosphorylase is complex. Inclusion of AMP in the substrate solution activates phosphorylase *a* approxi-

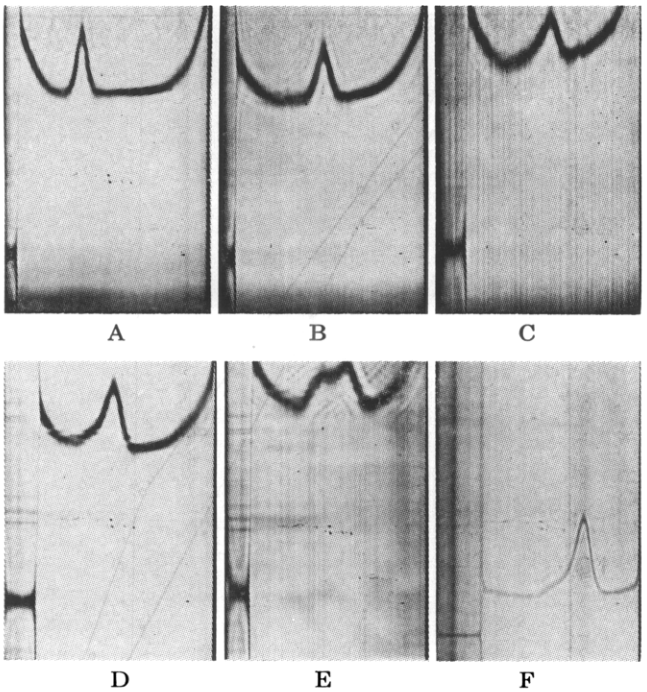


FIG. 11.—Ultracentrifugal patterns of phosphorylase *a* at various temperatures. (A) Enzyme (1.2 mg/ml) in 0.04 M glycerophosphate–0.03 M cysteine, *p*H 7.0, with 10^{-3} M AMP at 6°. (B) Enzyme with buffer of (A) without AMP at 23°. (C) As in (B) with 10^{-3} M AMP. (D) Enzyme (2.1 mg/ml) with buffer of (A) without M AMP at 34°. (E) As in (D) with 10^{-3} M AMP. (F) Enzyme (12 mg/ml); other conditions as in (E). All pictures were taken at 25–27 minutes after attainment of full speed at 59,780 rpm except picture (D), which was taken after 18 minutes.

mately 30% (Green and Cori, 1943). In the present work additional activation of this enzyme could be observed by preincubation of enzyme with this nucleotide in low-ionic-strength solutions. As the extent of this extra activation was found to be concentration and temperature dependent, the data can be explained by a dissociation hypothesis in which the dissociation constant for tetramer (V) to dimer (VI), K_2, is increased to K_3 for their respective nucleotide-enzyme complexes, (VII) and (VIII). The view that the dissociated species is a dimer is supported by ultracentrifugal analysis of phosphorylase *a* in the presence of AMP at 34°. Under these conditions two boundaries may be detected which correspond closely in $s_{20,w}$ to the dimeric and tetrameric forms of phosphorylase *a* in 3.0 M NaCl (Wang and Graves, 1963). With higher protein concentrations the relative amount of the slow-moving component decreases, consistent with a dissociation hypothesis. The question may be raised, "Is AMP activation of phosphorylase *a* in the assay also related to dissociation-association phenomena?" As activation of enzyme in the assay is rapid and independent of protein concentration, in contrast to the slow and protein-concentration-dependent activation that occurs during preincubation with AMP, these data suggest that the two effects of AMP may not be related. In contrast to the effect AMP has on phosphorylase *a* in low-ionic-strength solutions, addition of AMP to enzyme (IV) in high-ionic-strength solutions promotes formation of the tetrameric species (I). These two opposing effects may be reconciled since phosphorylase *a* exists as a tetramer in low-ionic-strength solutions and as a dimer in 3.0 M NaCl if it is assumed that the dissociation constants for $(\text{dimer})^2/(\text{tetramer})$ in the presence of AMP, K_1 and K_3, are similar.

The present work does not allow any conclusions in regard to the exact role of protein subunits in the catalysis by glycogen phosphorylase. Further experiments are in progress to relate differences in catalytic properties of the dimeric and tetrameric form of enzyme to differences in physical and chemical properties of these species.

ACKNOWLEDGMENT

The authors gratefully acknowledge the excellent technical assistance of Mr. Robert Sealock.

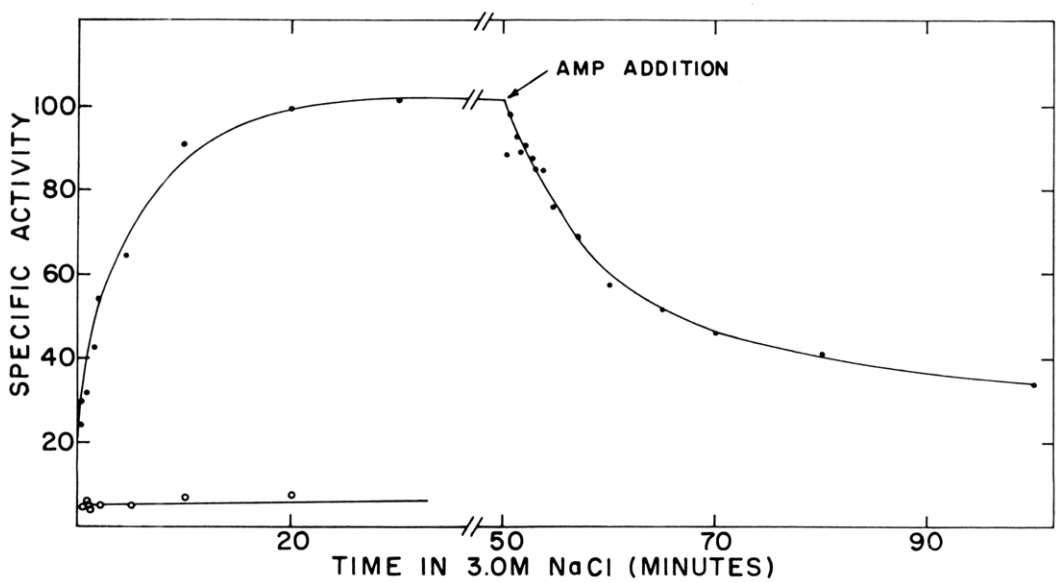


FIG. 12.—Effect of NaCl and AMP on the catalytic activity of phosphorylase *a*. Phosphorylase *a* (0.96 mg/ml) was incubated in 0.03 M cysteine–0.04 M glycerophosphate, *p*H 7.0, containing 3.0 M NaCl at 20°. Aliquots were withdrawn at various intervals for activity measurements in 3.0 M NaCl. The arrow indicates the time of addition of AMP to a final concentration of 10^{-3} M to the incubation tube. Open circles, assay without AMP; solid circles, assay with AMP.

References

Fischer, E. H., and Krebs, E. G. (1962), *Methods Enzymol. 5*, 369.

Green, A. A., and Cori, G. T. (1943), *J. Biol. Chem. 151*, 21.

Illingworth, B., and Cori, G. T. (1953), *Biochem. Prepn. 3*, 1.

Keller, P. J., and Cori, G. T. (1953), *Biochim. Biophys. Acta 12*, 235.

Krebs, E. G., Kent, A. B., and Fischer, E. H. (1958), *J. Biol. Chem. 231*, 73.

Madsen, N. B., and Cori, C. F. (1956), *J. Biol. Chem. 223*, 1055.

Sutherland, E. W., and Wosilait, W. D. (1956), *J. Biol. Chem. 218*, 459.

Wang, J. H., and Graves, D. J. (1963), *J. Biol. Chem. 238*, 2386.

Wosilait, W. D., and Sutherland, E. W. (1956), *J. Biol. Chem. 218*, 469.

Separation of Specific Glutamate- and Glutamine-activating Enzymes from *Escherichia coli*

Robert A. Lazzarini and Alan H. Mehler

From the U. S. Department of Health, Education and Welfare, U. S. Public Health Service, National Institutes of Health, National Institute of Dental Research, Bethesda, Md.

Received April 13, 1964; revised July 8, 1964

Two enzyme preparations have been obtained from *Escherichia coli* that exhibit specificity for esterifying glutamate and glutamine, respectively, to s-RNA. Each s-RNA species has been shown to be specific for the individual amino acid by determinations of capacity for acceptance of glutamate and glutamine alone and in combination, and by separation on columns of methylated albumin of the two species of s-RNA charged with the respective amino acids.

Recent investigations have given convincing evidence that the activation of amino acids as the enzyme-bound amino acid adenylate and the subsequent transfer to a soluble RNA molecule form a highly specific process in which the identity of the amino acid is conserved at each step. Thus, with few exceptions (Bergmann *et al.*, 1961; Sharon and Lipmann, 1957; Davie *et al.*, 1956), purified amino acid–activating enzymes catalyze a pyrophosphate-ATP exchange that is dependent upon a particular amino acid. Similarly, the transfer of the activated amino acid to soluble RNA is an equally specific process in which only certain s-RNA molecules can accept a particular amino acid. Chapeville *et al.* (1962) and Von Ehrenstein *et al.* (1963) have shown that the s-RNA moiety rather than the attached amino acid serves as the identifying character in the final step, the amino acid polymerization at the ribosomal sites.

Recently it has been proposed that glutamate, like hydroxyproline in collagen, is not directly incorporated into protein but arises there from the deamidation of glutamine (Zubay, 1962). This proposal was based on kinetic evidence that showed that glutamine was incorporated in the s-RNA fraction more rapidly than glutamate and the product of glutamate incorporation was glutaminyl-s-RNA. However, this proposal is not supported by the reports from the laboratories of Nirenberg (Matthaei *et al.*, 1962) and Ochoa (Wahba *et al.*, 1963), which show that the coding units for glutamine and glutamate are different. More direct evidence for the independence of glutamate and glutamine incorporation recently has been presented by Coles (Coles *et al.*, 1962; Coles and Meister, 1962). These data show that glutamate can be incorporated into yeast s-RNA without passing through a free glutamine intermediate, and that the product of glutamate incorporation is α-glutamyl-s-RNA.

In the course of our work on the activation and transfer of amino acids in protein synthesis we have separated and purified the activating enzymes for glutamate and glutamine from *Escherichia coli* and have obtained evidence for separate s-RNA's for each amino acid. The evidence presented in this communication strengthens and extends the argument (Coles and Meister, 1962) that glutamine and glutamate are incorporated into s-RNA independently.

Materials and Methods

Tritium and carbon-14-labeled glutamic acid were obtained from Nuclear Chicago Corp. and New England Nuclear Corp., respectively. Carbon-14-labeled glutamine was obtained from Schwartz Bioresearch, Inc. *E. coli* s-RNA was purchased from General Biochemicals. Protamine sulfate and ATP were obtained from Sigma Chemical Co. Diethylaminoethyl-cellulose was purchased from the Brown Co. Columns of hydroxylapatite and methylated albumin-kieselguhr were prepared as described by Levin (1962) and Mandell and Hershey (1960), respectively.

Estimation of Enzyme Activity.—Glutamyl- and glutaminyl-RNA synthetases were estimated by their ability to catalyze the incorporation of ^{14}C-labeled amino acid into a trichloroacetic acid–precipitable material. The assay mixture contained in 0.2 ml: 25 μmoles potassium cacodylate, *p*H 7.0; 0.2 μmole ATP; 1 μmole $MgCl_2$; 0.25 μmole mercaptoethanol; 0.5 mg s-RNA; 0.01 μmole ^{14}C-labeled amino acid (about 10 μc/μmole); and enzyme. The reactions were initiated by the addition of the enzyme and terminated after 10 minutes' incubation at 35° by the addition of 1 ml of 10% trichloroacetic acid. The resulting suspensions were chilled for 10 minutes in an ice bath, and filtered through a 25-mm Millipore filter. The precipitated material was washed with 50 ml of 5% trichloroacetic acid. The precipitates, together with the Millipore filters, were then neutralized with 1 ml 1.5 N NH_4OH, dissolved in 10 ml of the liquid scintillator described by Bray (1960), and assayed for radioactivity in a Packard Tricarb liquid scintillation spectrometer.

One unit of glutaminyl- or glutamyl-RNA synthetase is defined as the amount of enzyme that incorporates 1 mμmole of amino acid into RNA under the conditions of the standard assay.