THE RELATIONSHIP OF DISSOCIATION TO THE CATALYTIC ACTIVITY OF GLYCOGEN PHOSPHORYLASE

by

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Ames, Iowa

1965

TABLE OF CONTENTS

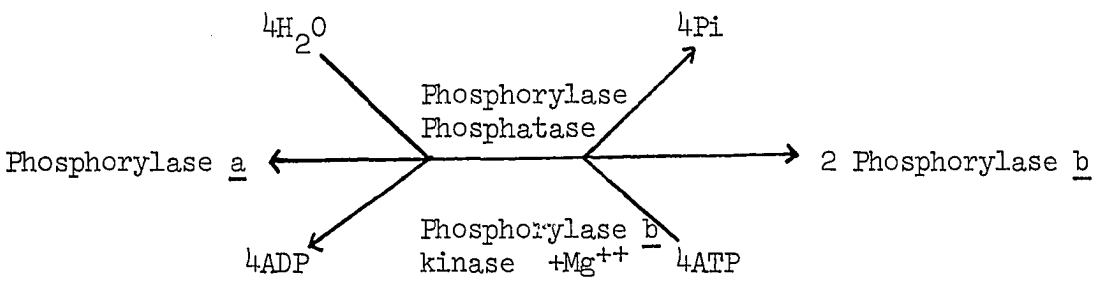
DEDICATION

To My Wife

INTRODUCTION

Glycogen phosphorylase has been isolated from rabbit muscle in two different molecular forms: phosphorylase a, molecular weight 495,000, is catalytically active (1, 2) and phosphorylase b, molecular weight 242,000, is active only in the presence of AMP[+] (2-4). The two forms are enzymically interconvertible as described in the following reaction scheme (5-7):

Scheme I



Although the alterations in chemical, physical and enzymic properties of the protein in the interconversion are well defined, their interrelationships, however, are far from clear. The possibility that phosphorylase a is formed through the bridging of two phosphorylase b molecules by phosphodiester bond appears remote since tryptic attack of phosphorylase a liberates all protein-bound phosphate as a monoester with serine (8). Furthermore, modification of cysteinyl residues of

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[+]Abbreviations used in this paper: SA, specific activity; AMP, adenosine-5'-monophosphate; ADP, adenosine-5'-diphosphate; ATP, adenosine-5'-triphosphate; IMP, inosine-5'-monophosphate; IDP, inosine-5'-diphosphate; ITP, inosine-5'-triphosphate; UMP, uridine-5'-monophosphate; PMB, paramercuribenzoate; Pi, inorganic phosphate; EDTA, Ethylenediaminetetra-acetate.

phosphorylase results in the cleavage of the molecule into subunits with molecular weight 125,000 (9).* If the phosphoryl groups are directly involved in the dimerization process, this also cannot be explained by chelation of four phosphoryl groups by a metal introduced in the phosphorylase b to a reaction (10). Trytic attack of phosphorylase a shows that the portion of the peptide chain at the site of phosphorylation is highly positively charged, and it has been suggested that dimerization follows phosphorylation by neutralization of the charge of this positive site, which would allow an interaction between interpeptide chains that were previously electrostatically repulsed (8).

As more and more proteins are subjected to detailed structural examination, it is becoming apparent that many of these proteins are composed of subunits. The wide occurrence of such structure suggests that there may be some biological significance in this phenomenon. Recent studies of cellular metabolic control mechanisms indicate the existence of regulatory enzymes as one of the most important devices in the regulation of metabolism (11, 12). The well studied regulatory enzymes have all been found to consist of subunits; furthermore, a model based on the interaction of subunits can account for all the abnormal kinetic properties of these enzymes (13). Since phosphorylase activity is known to be controlled by muscular activity (14, 15) and hormonal administration (16, 17), it is of interest to

*Assuming the subunit of phosphorylase with molecular weight 125,000 as fundamental unit of the enzyme, phosphorylase a can be considered as a tetramer; phosphorylase b, a dimer.

define the role of subunit interaction in the catalytic function of this enzyme.

Since the discovery of the difference in molecular weight of the two forms of phosphorylase, many attempts have been made to reveal the relationship of molecular weight to the catalytic activity of this enzyme. Although the observation that phosphorylase b might exist as a tetramer in the presence of AMP under certain conditions is suggestive of a correlation between enzymic activity and the association of the protein (18, 19), the question whether activation is directly related to the association, or the association is a result of the active configuration of the dimeric unit of the enzyme cannot be answered.

Many proteins have been shown to dissociate into their subunits with only minor alterations in the secondary and tertiary structure. It, thus, appears that phosphorylase a may also dissociate into smaller molecular weight units upon treatment with mild disruptive reagents or upon dilution. This thesis is concerned with work related to this theme with the following goals:

1. Characterization of catalytic properties of the subunit of phosphorylase a.
2. Delineation of forces involved in stabilization of the tetrameric form of phosphorylase a.

EXPERIMENTAL PROCEDURE

Materials

Rabbit Muscle Phosphorylase b was prepared according to the procedure of Fischer and Krebs (4) from commercial frozen rabbit muscle (Pel-freeze Biologicals, Inc., Rogers, Arkansas). Third or fourth crystals which were treated with Norit A to remove AMP were used for all experiments.

Rabbit Muscle Phosphorylase a was prepared with the use of partially purified phosphorylase b kinase (20) and crystalline phosphorylase b. Third or fourth crystals treated with Norit A were used throughout this work.

Potassium glucose-1-phosphate, sodium glycerophosphate, cysteine-HCl, dextrose, and shellfish glycogen were obtained from Sigma Chemical Co., St. Louis, Missouri.

AMP, ATP, IMP, IDP, and UMP were purchased from Pabst Laboratories, Milwaukee, Wisconsin.

Methods

Phosphorylase Concentration was determined spectrophotometrically with the use of an absorbancy index of 11.7 for a 1% solution of protein (21).

Enzyme Activities were measured according to the procedure of Illingworth and Cori (22) 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. 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 pH 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 (22).

Sedimentation Coefficients were determined with a Spinco model E analytical ultracentrifuge employing a 12-mm single sector cell at a rotor speed of 59,780 r.p.m. The temperature of the rotor during most runs was maintained at 20 ± 0.2°. Movement of boundaries was calculated from direct microcomparator measurements of the schlieren diagram. Corrections for viscosity and density of the various buffers were applied in calculation of sedimentation coefficients, although reported sedimentation coefficients were not extrapolated to zero protein concentration. For ultracentrifugation at 34°, the rotor was preheated for 1 hour in an oven to 36°. Temperature was controlled

for these runs to within $\pm 1^{\circ}$. The percentage of components with different sedimentation coefficients was determined by estimation of areas of empirically resolved components, as described by Ogston (23), from schlieren diagrams 45 to 50 minutes after centrifugation. Error in the estimation of areas of poorly resolved components was as high as 20%.

RESULTS

Effect of NaCl on the Structure and Catalytic Activity of Phosphorylase a

Dissociation of phosphorylase a in high concentrations of NaCl

Figure 1 shows that native phosphorylase a with an $s_{20,w}$ of 13.2 S is converted into a slower sedimenting component by solutions of increasing concentration of NaCl. At 2.8 M NaCl, only the slow moving component with an $s_{20,w}$ of 8.4 S could be detected in the ultracentrifuge. Increase in NaCl to 3.5 M did not further alter the sedimentation coefficient. At concentrations of NaCl at which both forms of the enzyme could be detected, phosphorylase a and the slow moving component were found to have average sedimentation coefficients of 12.4 S and 8.3 S, respectively.

The degree of transformation of phosphorylase a in salt solutions is not merely dependent upon the ionic strength. At pH 7.4, 2.0 M NaCl gave a 33% conversion, but 2.0 M KCl yielded only 8% conversion of phosphorylase a to a slow sedimenting component, and potassium phosphate at equal ionic strength was without effect.

Phosphorylase b, a dimer, in contrast to phosphorylase a is not transformed to a slower moving component in the ultracentrifuge by NaCl. The sedimentation coefficients of the enzyme in the absence and presence of 3.2 M NaCl were found to be 8.2 S and 8.4 S, respectively.

The molecular weight of the enzyme (8 mg per ml) in 3.0 M NaCl containing 0.02 M glycerophosphate-0.03 M cysteine, pH 7.4, was determined by the approach to equilibrium in the ultracentrifuge as described by Ehrenberg (24). The partial specific volume of phosphorylase a in 3.2 M NaCl was found to be 0.74, and the average molecular weight of the slow sedimenting component was calculated as 258,000 ± 7,000. Since phosphorylase a is a tetramer with subunits of a molecular weight of 125,000 (4), this new form of phosphorylase a may be considered to be a dimer.

Effect of AMP on the dissociation

AMP, a specific activator of muscle phosphorylase (2), has been found to prevent the dissociation of phosphorylase a in NaCl. Addition of AMP to a solution of phosphorylase a in 2.5 M NaCl (Figure 2A) yielded only one symmetrical ultracentrifugal component (Figure 2B) with an $s_{20,w}$ of 12.2 S. The protective effect afforded by AMP to dissociation of phosphorylase a was not dependent on the order of addition of NaCl and nucleotide. ATP, IDP, and UMP at 10^{-3} M all were without effect, whereas a 10^{-3} M solution of IMP provided a 5 to 10% protection to dissociation. A solution of 0.1 M potassium phosphate, a substrate, in 3.2 M NaCl with 0.02 M glycerophosphate-0.03 M cysteine, pH 7.4, did not prevent dissociation of the enzyme.

Enzymic activity of phosphorylase a in NaCl

Since phosphorylase a is converted in NaCl to a form with a molecular weight similar to phosphorylase b but with no loss of

covalently bound phosphate, it was of interest to study the kinetic characteristics of this new enzyme species. 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 (Figure 3). 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 reformation of the tetrameric species.

Reversal of the dissociation

The complete reversibility of transformation of phosphorylase a to a slow moving component could be demonstrated after removal of NaCl by dialysis against 0.03 M cysteine-0.02 M glycerophosphate, pH 7.4. Ultracentrifugal examination showed the presence of a symmetrical component with the sedimentation coefficient of native phosphorylase a. Although only little enzyme activity could be detected in 2.8 M NaCl in the absence of AMP, 90% of initial activity was regained after dialysis.

The reversal can also be achieved by dilution of 3 M NaCl

incubated phosphorylase a into 0.04 M glycerophosphate 0.0015 M EDTA buffer pH 7.0. At 30° full catalytic activity could be demonstrated following dilution. If dilution and activity tests were carried out at 20°, the appearance of an extremely active form of the enzyme could be demonstrated. Figure 4 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 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.

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 5A 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

(Figure 5B). With data obtained at 6.5° (Figure 6), 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 an $s_{20,w}$ 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 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

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

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.

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 (Figure 6). The activity-decay process, therefore, appears to conform to the kinetics of a second-order reaction. 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.

Subunit Structure of Phosphorylase *a* In Low Ionic Strength Solutions

Relationship between specific activity and protein concentration of phosphorylase *a*

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 7 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.

Effect of temperature

Figure 8 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 Figure 8 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 1). The effect of preincubation at 30° (column A) is not apparent if the preheated enzyme is first cooled before addition to a substrate solution (Table 1, column B). In order to examine the kinetics of the change of activity of phosphorylase a upon cooling, enzyme was first incubated at 34°

Table 1. Effect of incubation temperature on phosphorylase activity at 15°[a]

	Specific activities at 15°C			
	30°			
Enzyme	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, pH 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.

and then cooled rapidly at 9°. Analysis of this activity decay did not fit a first-order rate equation, but did comply with a second-order reaction (Figure 9, 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 AMP

Figure 10 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 (3). 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 demon-

strated by measurement of enzyme activities at different temperatures at one protein concentration. Without preincubation, as illustrated in Figure 11, 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 (25). As a working hypothesis to explain the results of Figure 11 and the lowering of the transition temperature of Figure 11, 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 12 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. 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%.

Activity of dimeric and tetrameric form of phosphorylase b

Although phosphorylase b exists ordinarily as a dimeric form, it can be converted to a tetrameric species in the presence of AMP and Mg^{++} at temperatures below 15° (18). Table 2 shows the effect of AMP and Mg^{++} on the catalytic activity of this enzyme at two different temperatures. At 26°, incubation of phosphorylase b with AMP, Mg^{++}, or Mg^{++} and AMP did not alter the specific activity of this enzyme; at 15°C incubation with AMP or Mg^{++} did not alter activity but incubation with both constituents lowered the catalytic activity of phosphorylase b significantly. These data suggest that the tetrameric form of phosphorylase b is less active than the dimeric species, in accord with results obtained with phosphorylase a. The insensitivity of phosphorylase b to either constituent alone, or to both at 26°C suggests that binding of these reagents alone cannot account for alteration of activity.

Table 2. Effect of AMP and Mg^{++} on catalytic activity of phosphorylase b

Additions in buffer*		None	10^{-2} M Mg^{++}	10^{-3} M AMP	10^{-2} M Mg^{++} and 10^{-3} M AMP
Specific	13°	397	409	381	179
Activity	26°	1166	1190	1159	1174

*Buffer contains 0.03 M cysteine and 0.04 M glycerophosphate at pH 6.8.

Effect of Carbohydrates on the Structure and Activity of Phosphorylase a

Alteration in enzymic activity and sedimentation constant of phosphorylase a by glucose

The relationship between subunit structure and catalytic activity has been examined in the presence of glucose. Figure 13A shows that incubation of enzyme (1.2 mg/ml) with 0.05 M glucose at 20° results in approximately a threefold increase in enzymic activity. No increase in activity could be demonstrated with incubation in the absence of glucose. To relate alterations of enzymic activity to physical structure of glycogen phosphorylase, ultracentrifugation was carried out under the exact conditions of activation. Figure 13B, lower curve, shows that two diffuse boundaries with $s_{20,w}$ of 8.9 and 13.9 S appear during the course of centrifugation at 20°C with enzyme in 0.05 M glucose. In the absence of glucose only a component with an $s_{20,w}$ of 13.9 S is evident (Figure 13B, upper curve). Since

activation may be correlated with the formation of a component with an $s_{20,w}$ of 8.9 S, a sedimentation constant similar to that obtained for the dimeric form of phosphorylase a, these data suggest that activation is directly related to the conversion of the tetrameric form of phosphorylase a (2) to a dimeric species.

No activation of enzyme (0.03 mg/ml) could be demonstrated by preincubation with 0.05 M glucose at 30°. Since the preceding kinetic experiments suggested that phosphorylase a exists as a dimer at low protein concentrations at 30°, the present data further support the view that activation by glucose is directly related to enzyme dissociation.

In contrast to results obtained with phosphorylase a, incubation of phosphorylase b, at 1.0 mg/ml in 0.05 M glucose at 20° did not yield higher enzymic activity. No alteration of the ultracentrifugal characteristics of phosphorylase b (2.0 mg/ml) could be detected in 0.05 M glucose at 20°.

Reversal of action of glucose by AMP and glucose-1-phosphate

Since inhibition by glucose was found by Cori et al. (26) to be overcome by glucose-1-phosphate and AMP, the effect of these two compounds on the activation by glucose was tested. The results illustrated in Figure 14 show that addition of 0.032 M glucose-1-phosphate or 10^{-3} M AMP to enzyme which had been incubated for 30 minutes with glucose results in a slow decrease of catalytic activity and a decrease in amount of the component with an $s_{20,w}$ of 8.9 S.

It should be noted that if reversal of activation by glucose-1-phosphate were fast, no activation would have been detected since glucose-1-phosphate is a component of the assay system. Figure 14A, lower curve, shows that 10^{-3} M AMP had a greater effect on reversal of activation by glucose than 0.032 M glucose-1-phosphate and that the extent of reversal is paralleled by a further decrease in amount of the slow moving component (Figure 14B, lower curve). If glucose-1-phosphate were increased to 0.16 M, no activation or slow moving component could be detected in the ultracentrifuge. Inhibition kinetics reported by Cori et al. (26) and the present experiments with glucose-1-phosphate suggest that glucose and glucose-1-phosphate compete for a single binding site on the enzyme. The action of AMP on inhibition (26) and activation may be explained by binding of this nucleotide to a different site which results in a decreased affinity of enzyme for glucose and/or a decrease in Km for glucose-1-phosphate (27, 28). Binding of AMP at the substrate site for glucose-1-phosphate would not be expected since AMP potentiates rather than inhibits phosphorylase a activity.

Activation of phosphorylase a upon preincubation in glycogen

Since it was observed that glycogen effectively blocked the reassociation of a more active dimeric species of phosphorylase a into a less active tetrameric form, it was of interest to further characterize the action of this polysaccharide on the catalytic properties of phosphorylase a.

The effect of preincubation of phosphorylase a with three different glycogen concentrations at 20° is illustrated in Figure 15. A slow increase in enzymic activity is apparent with enzyme incubated in all glycogen concentrations, and it appears that the extent of activation by glycogen is a function of glycogen concentration. These data tend to exclude the view that activation by glycogen is catalytic.

As it has been known that the catalytic activity of glycogen phosphorylase depends strongly upon the structure of the priming polysaccharide, the possibility that activation results from formation of a better primer during the incubation was tested. Figure 16 shows that glycogen isolated from a phosphorylase a and glycogen incubation mixture had the same activation capacity as untreated glycogen. These data do not support the view that activation is due to the formation of a better primer.

Activation by glycogen and enzyme dissociation

Due to the high and heterogeneous molecular weight of glycogen the study of the relationship of glycogen activation and enzyme dissociation by analytical ultracentrifugation is untenable. An alternative approach is to study the response of the various species of phosphorylase to the incubation with glycogen. The effect of protein concentration on activation of phosphorylase a by glycogen was first studied. The results illustrated in Figure 17 show that specific activity of 20° is highly dependent upon protein concentration, but if enzyme is preincubated with glycogen no such dependence is apparent.

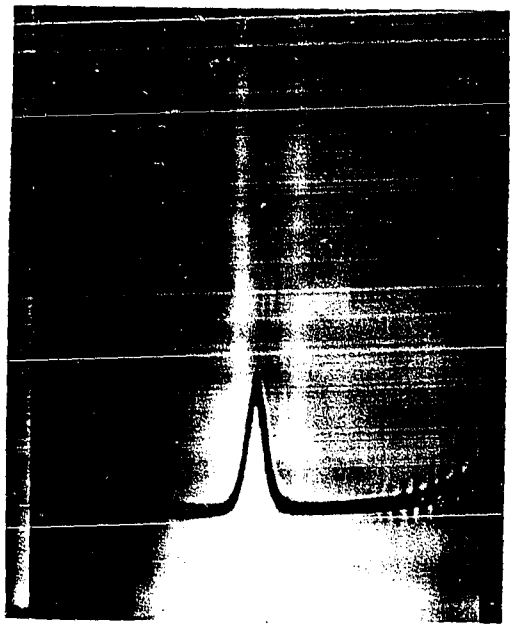
These data are in agreement with the postulate that glycogen induces the conversion of the less active tetramer to a more active dimer. With the hypothesis stated previously that increase of temperature favors enzyme dissociation, it might be expected that the extent of activation by glycogen would decrease with increase temperature of the incubation mixture. At a protein concentration of 0.03 mg/ml 46% activation was observed at 15°, 34% activation at 20° and only 2% activation at 30°.

In 3.0 M NaCl phosphorylase a exists as a dimer; in 3.0 M NaCl and 10^{-3} M AMP phosphorylase a exists as a tetramer. The action of glycogen on these two molecular forms is illustrated in Figure 18. The results show that the tetramer is markedly activated by glycogen, and the dimer is essentially insensitive to preincubation with glycogen. It is interesting to note that while the dimer is much more active than tetramer, the activity of the tetramer after incubation in glycogen appear to approach the activity of the dimer.

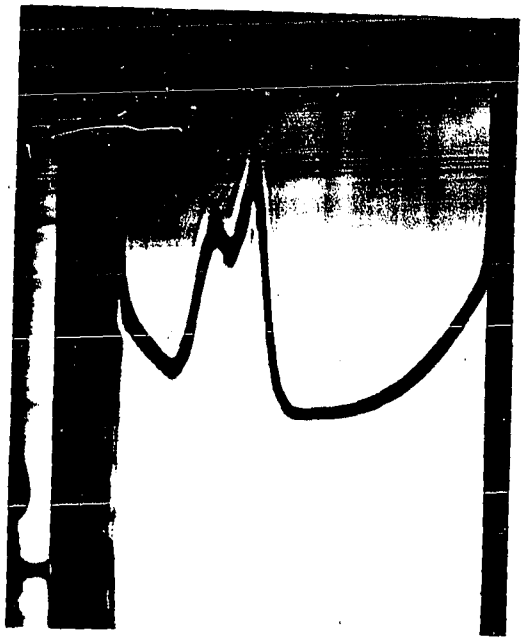
Effect of glycogen incubation on phosphorylase b activity

Since phosphorylase b can exist as a tetramer and a dimer and previous experiments indicated that in this case the dimer also possesses higher catalytic activity than tetramer, the effect of glycogen on these nonphosphorylated forms of the enzyme was studied. Figure 19 shows that only the tetrameric form of phosphorylase b can be activated by preincubation with glycogen and the catalytic activity rises to nearly the same level as the dimeric form.

Figure 1. Effect of NaCl on the sedimentation of phosphorylase a. Phosphorylase a in 0.02 M glycerophosphate-0.03 M cysteine pH 7.4 (A); with 2.0 M NaCl (B); with 2.8 M NaCl (C); and with 3.5 M NaCl (D). Pictures for (B), (C) and (D) were taken at 30 minutes after attainments of full speed at 59,780 and picture for (A) was taken 18 minutes after full speed.



A



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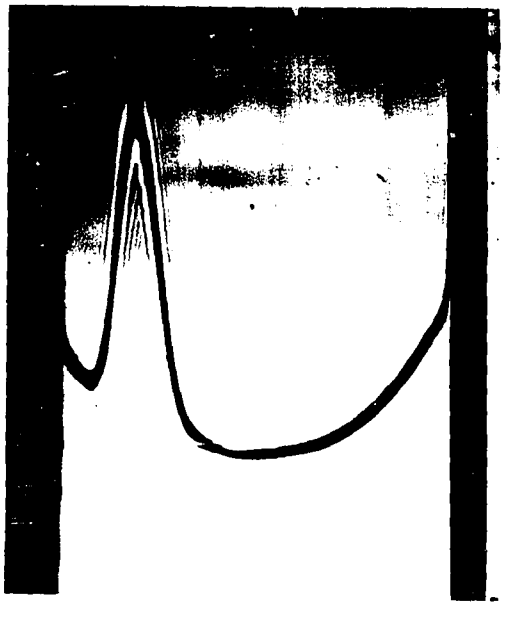

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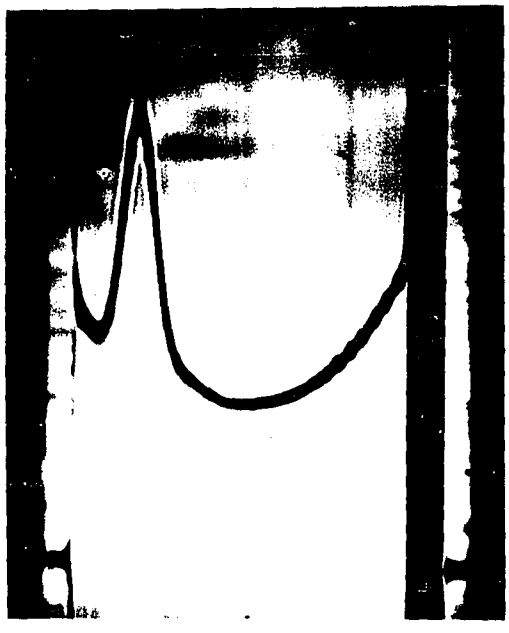
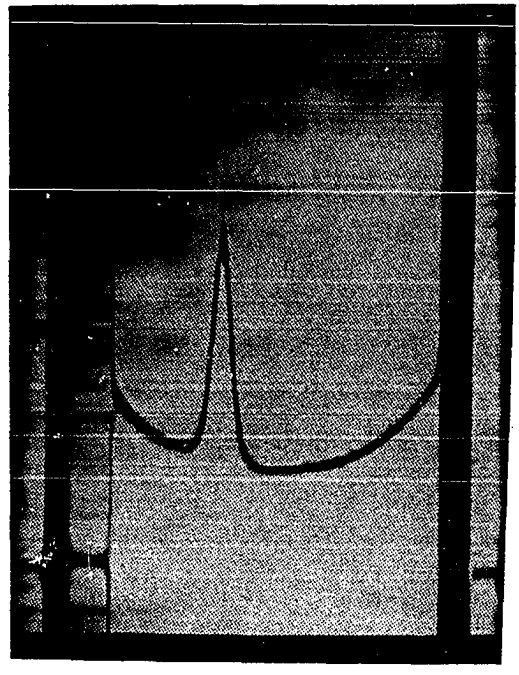


D

Figure 2. Effect of AMP on dissociation of phosphorylase a. A phosphorylase a (6 mg per ml) in 2.5 M NaCl, 44 minutes at 59,780 r.p.m., sedimentation coefficients from meniscus 8.3 S and 12.4 S, respectively; B, as in A with 10^{-3} M AMP, sedimentation coefficient 13.2 S. Other conditions were as described with Figure 1.

A



B

Figure 3. 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, pH 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.

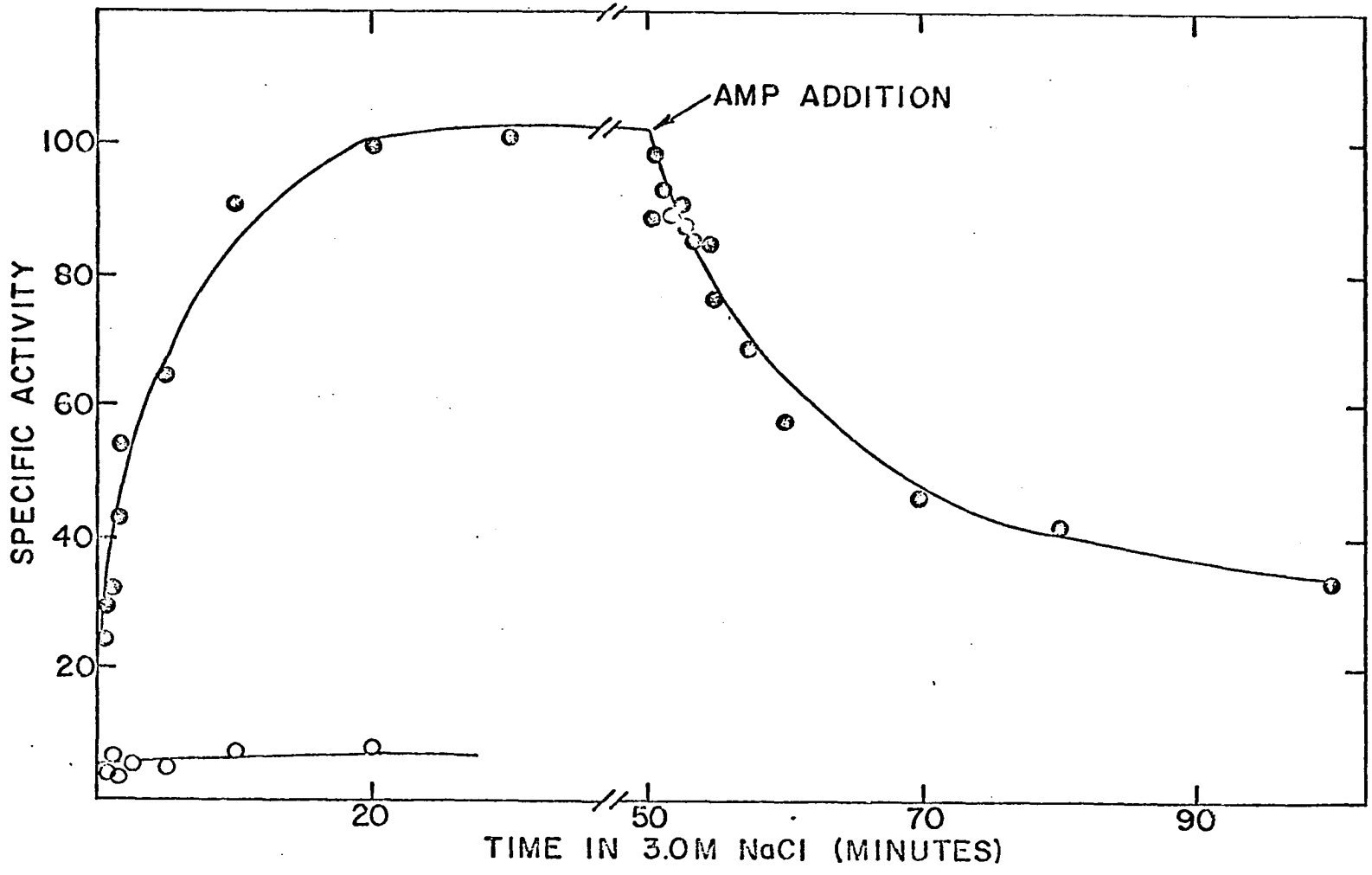

AMP ADDITION
SPECIFIC ACTIVITY
100
80
60
40
20
20
50
70
90
TIME IN 3.0M NaCl (MINUTES)

Figure 4. Decay of enzymic activity of NaCl incubated phosphorylase a following dilution. Solid circles, enzyme (7.5 mg/ml) was incubated for 1 hour at 20° in 0.04 M glycerophosphate-0.03 M cysteine, pH 7.0, with 3.03 M NaCl prior to a 30-fold dilution in 0.04 M glycerophosphate-0.0015 M EDTA, pH 7.0. Samples were withdrawn at various intervals after dilution and were assayed for 2 minutes at 20° with substrate without AMP, pH 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.

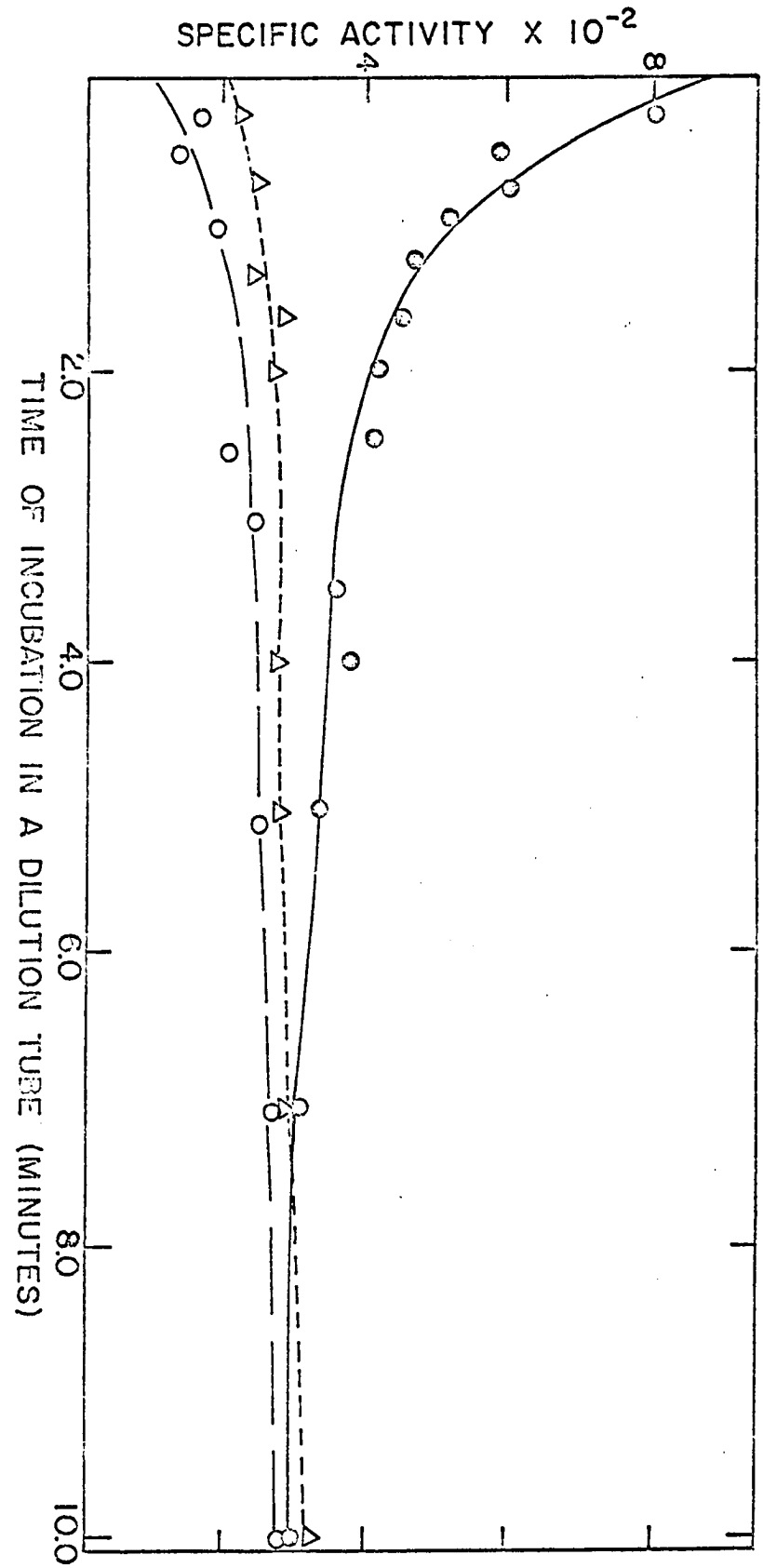

SPECIFIC ACTIVITY X 10^{-2}
4
8
2.0
4.0
6.0
8.0
10.0
TIME OF INCUBATION IN A DILUTION TUBE (MINUTES)

Figure 5. Effect of glycogen and glucose-1-phosphate on activity decay. Incubation as in solid circles of Figure 4 with enzyme at 5 mg/ml. A, solid circles, dilution in 0.04 M glycerophosphate-0.0015 M EDTA, pH 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.

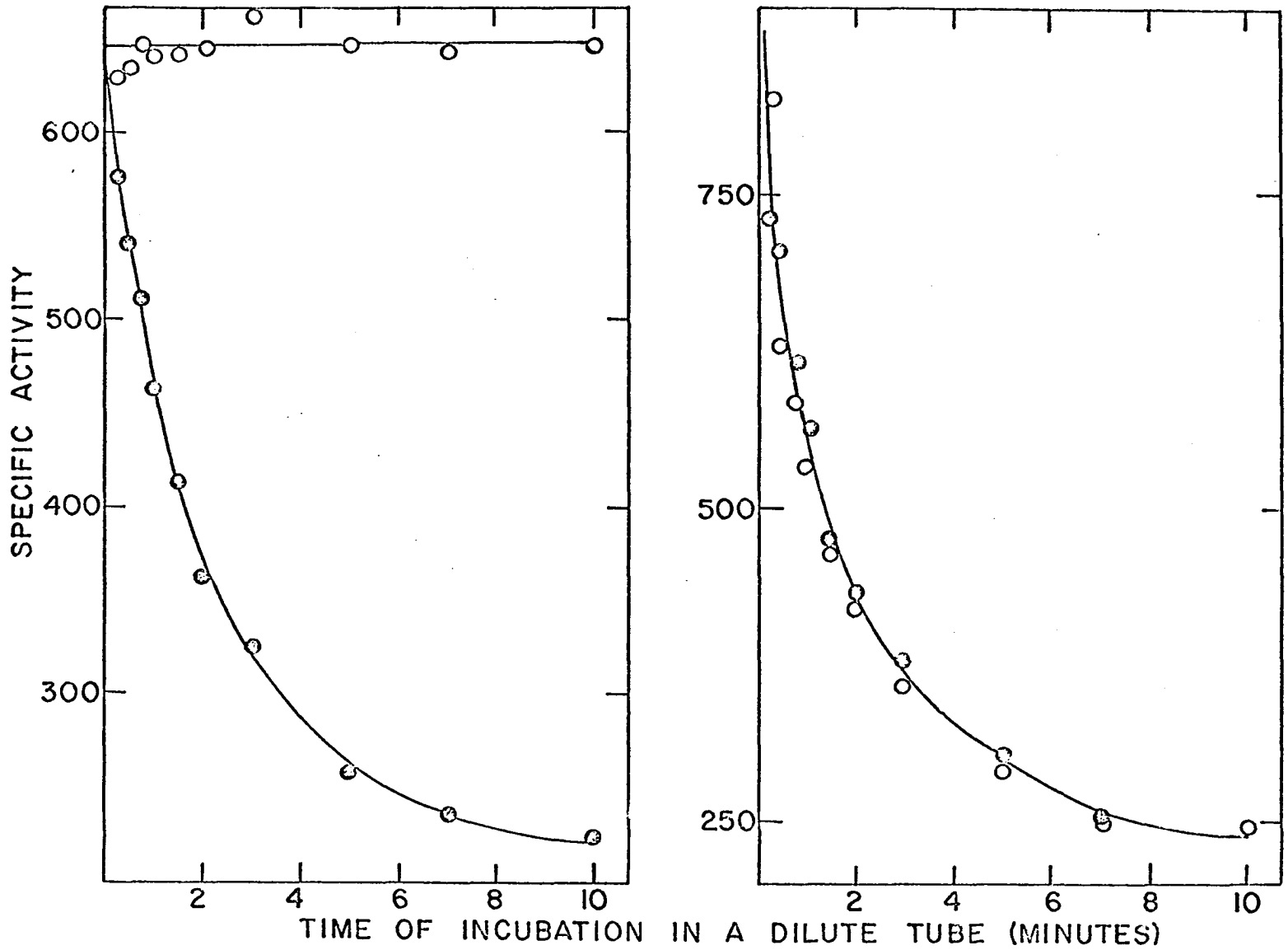

SPECIFIC ACTIVITY
600
500
400
300
750
500
250
2
4
6
8
10
TIME OF INCUBATION IN A DILUTE TUBE (MINUTES)

Figure 6. Kinetic order of activity decay. Incubation as in closed circles of Figure 4 with enzyme at 10.3 mg/ml. After one hour a fifteen fold dilution was made in 0.04 M glycerophosphate-0.001 M EDTA, pH 7.0 at 6.5°C, and aliquots were then removed at various intervals for activity measurements at 6.5°C 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 Δ S.A.; and closed circles, reciprocal of Δ S.A.

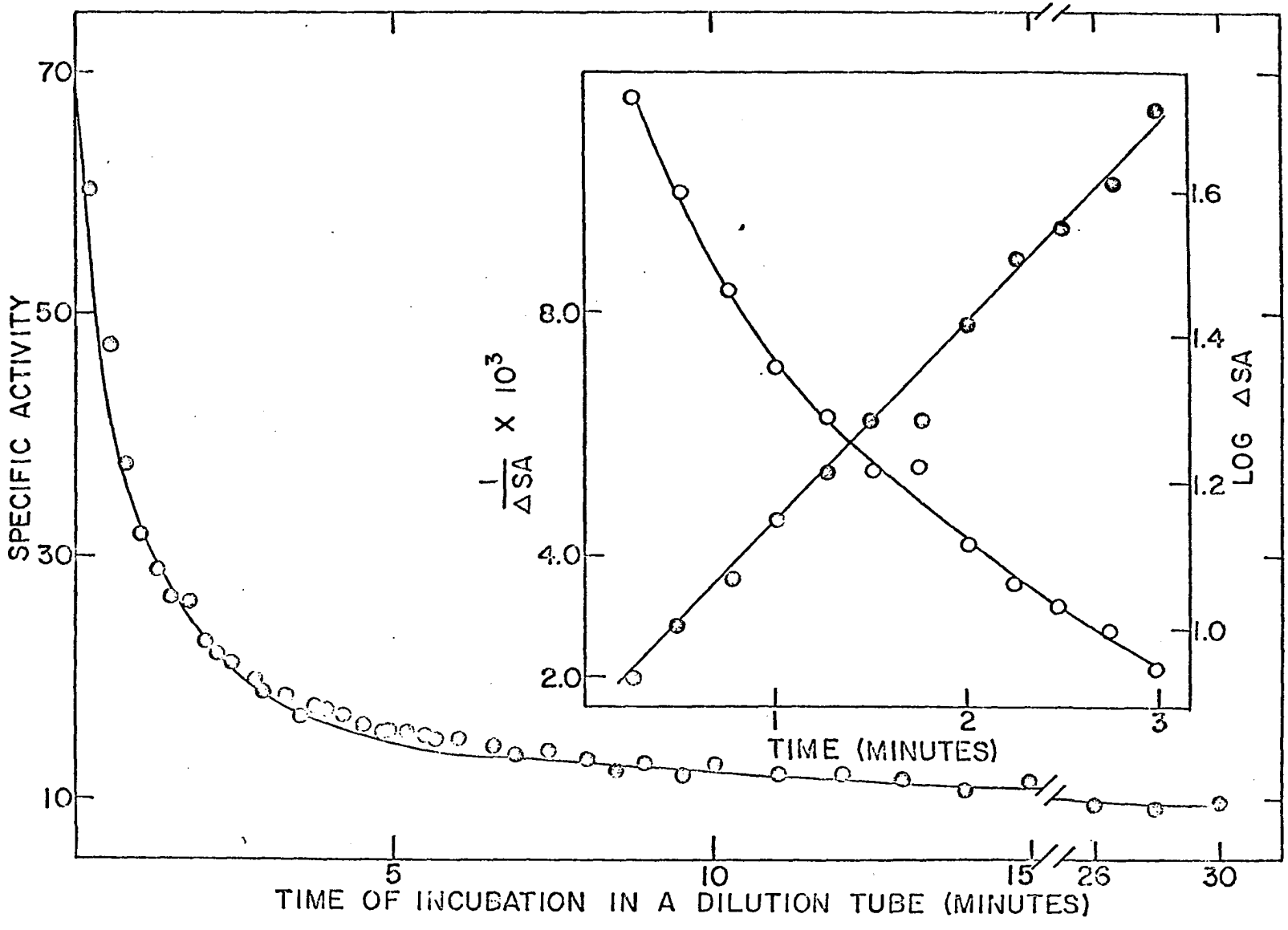

SPECIFIC ACTIVITY
70
50
30
10
5
10
15
26
30
TIME OF INCUBATION IN A DILUTION TUBE (MINUTES)
8.0
4.0
2.0
1/ΔSA x 10³
1.6
1.4
1.2
1.0
LOG ΔSA
1
2
3
TIME (MINUTES)

Figure 7. 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, pH 7.0, and incubated for 10 minutes at 25°C prior to assay with substrate containing 10^{-3} M AMP; open circles, phosphorylase b; closed circles, phosphorylase a.

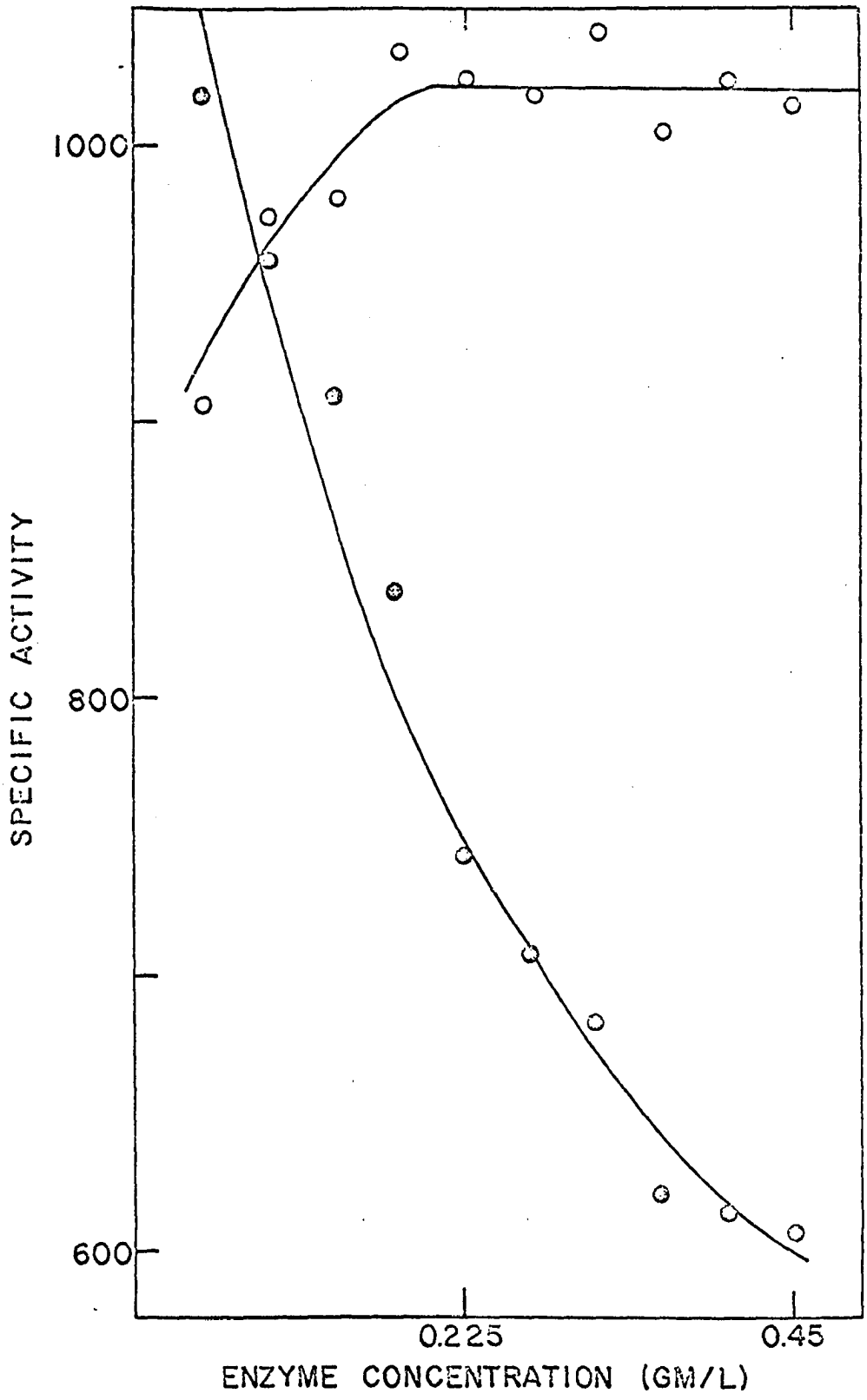

1000
800
600
SPECIFIC ACTIVITY
0.225
0.45
ENZYME CONCENTRATION (GM/L)

Figure 8. Effect of temperature on the specific activity of phosphorylase a at various protein concentrations. Enzyme diluted as in Figure 7 and incubated at three different temperatures for 10 minutes prior to activity measurements at pH 7.0 with AMP substrate; closed triangles, incubation and assay at 30°C; closed circles, at 25°C; open circles, at 18°C.

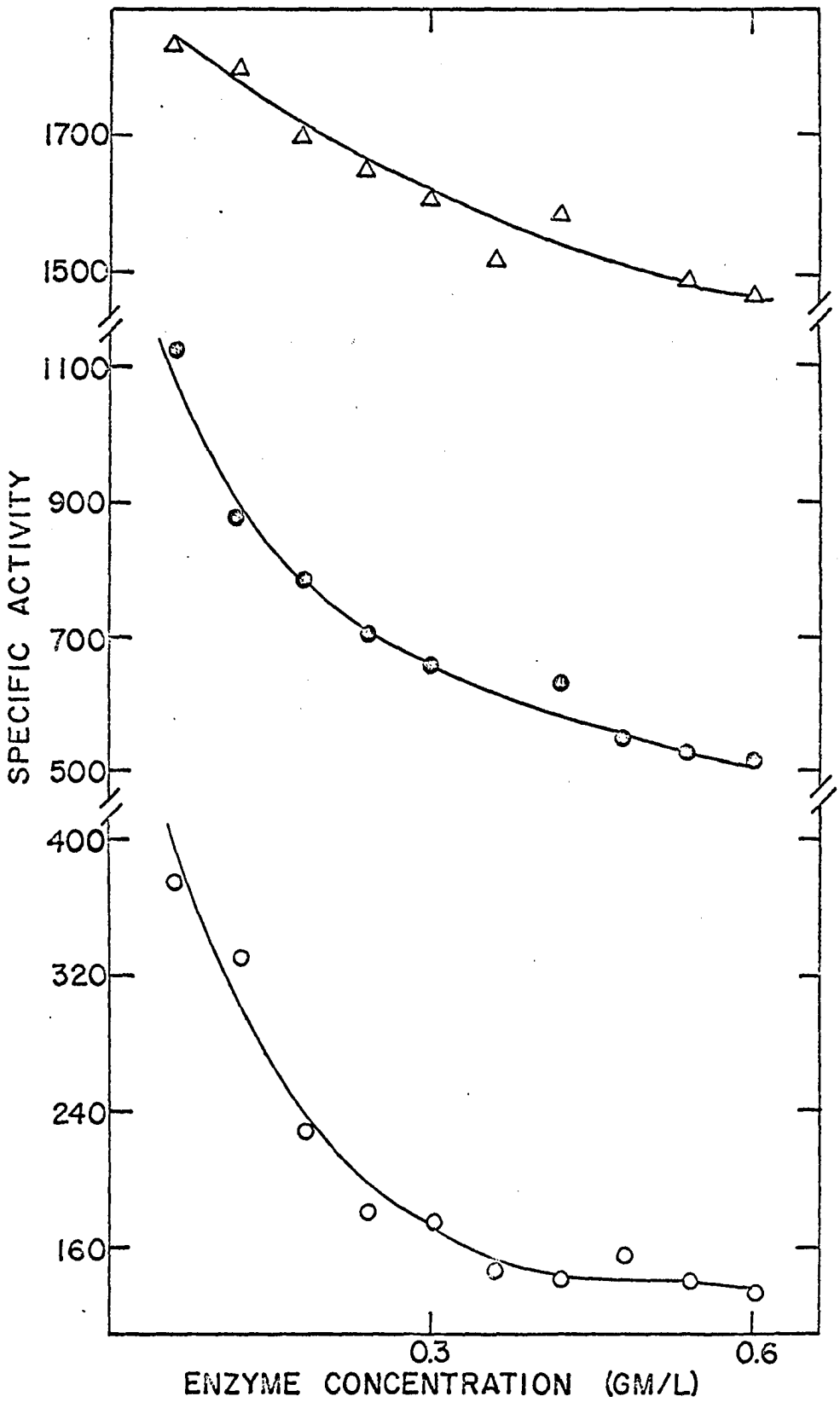

1700
1500
1100
900
700
500
400
320
240
160
SPECIFIC ACTIVITY
0.3
0.6
ENZYME CONCENTRATION (GM/L)

Figure 9. 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, pH 7.0 at 34°C for 30 minutes prior to the rapid cooling to 9°C in a dry ice acetone bath (time for cooling, 12 seconds). After cooling, enzyme was transferred to a 9°C water bath, incubated, and assayed for enzyme activity at 9°C for 5 minutes with substrate without AMP. Inner rectangle, open circles, log Δ S.A.; closed circles, reciprocal of Δ S.A.

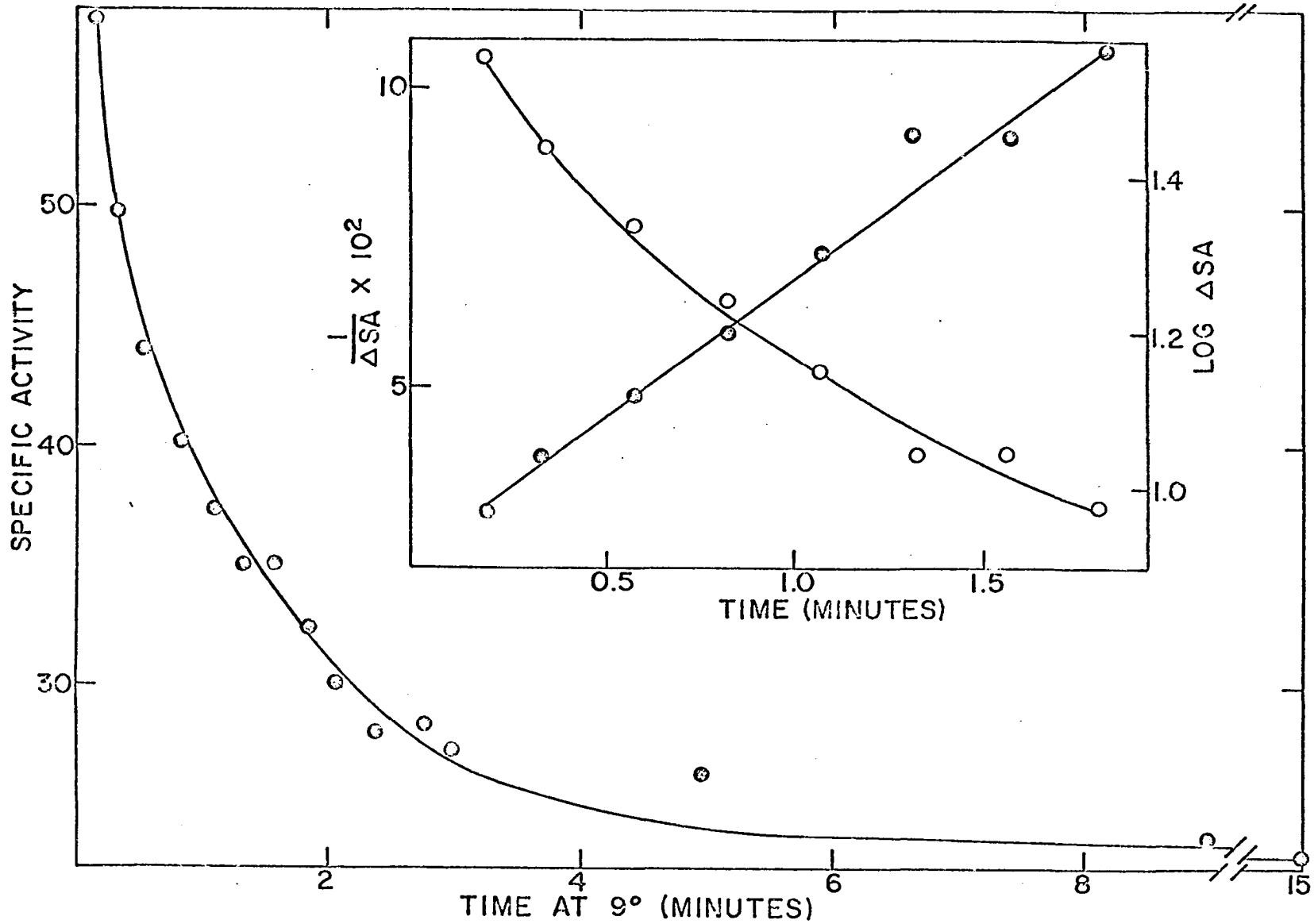

SPECIFIC ACTIVITY
50
40
30
2
4
6
8
15
TIME AT 9° (MINUTES)
10
5
$\frac{1}{\Delta SA} \times 10^2$
0.5
1.0
1.5
TIME (MINUTES)
1.4
1.2
1.0
LOG ΔSA

Figure 10. Effect of AMP on the specific activity profile. Open and closed circles, enzyme diluted in 0.03 M cysteine-0.04 M glycerophosphate, pH 7.0 and incubated for 10 minutes at 25°C. Open circles, assay with 10^{-3} M AMP at 25°C, closed circles, assay without AMP. Open and closed triangles, incubation as above with 10^{-3} M AMP. Open triangles assay with 1.5×10^{-3} M AMP; closed triangles, assay with 5×10^{-4} M AMP.

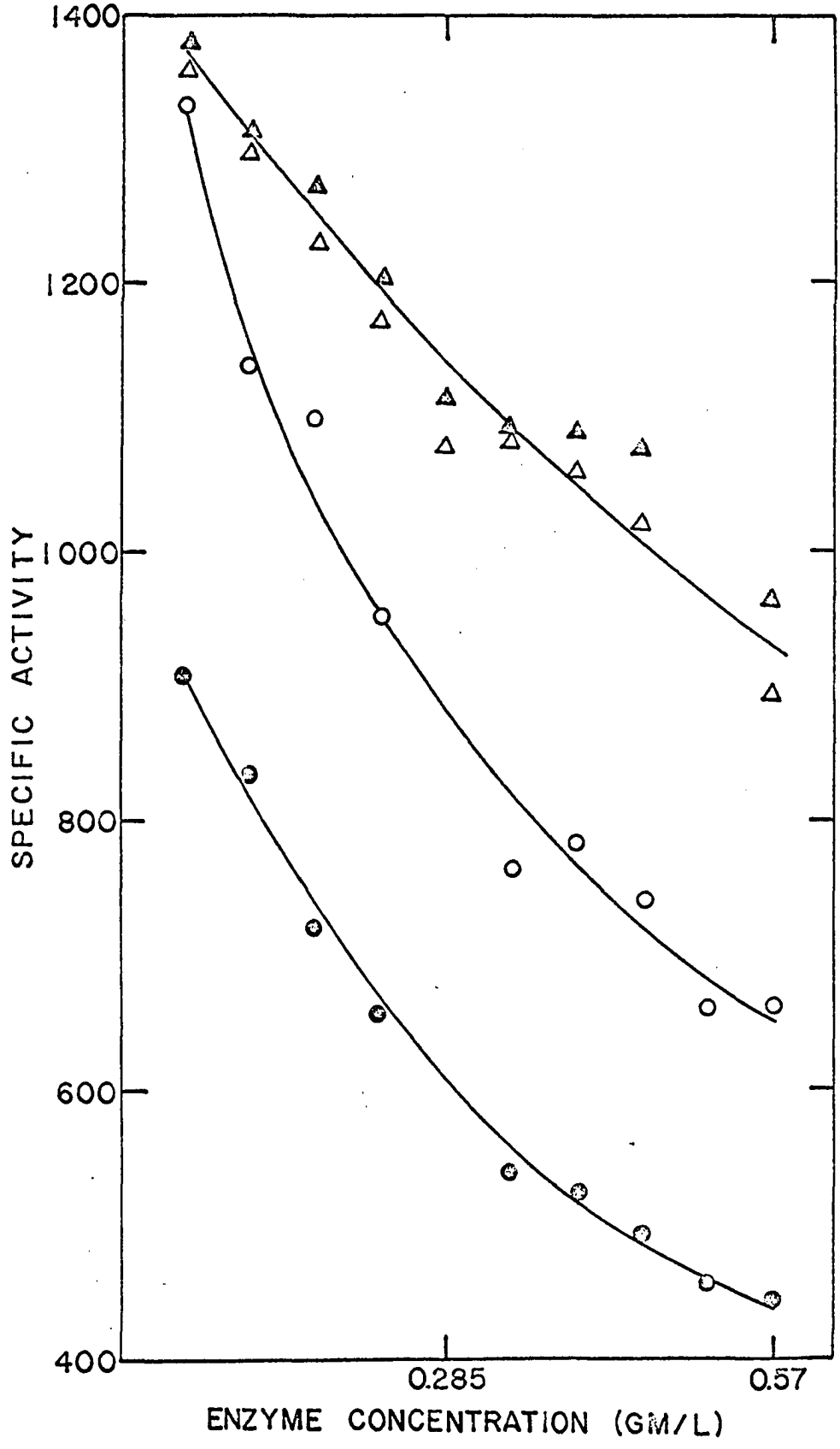

1400
1200
1000
800
600
400
SPECIFIC ACTIVITY
0.285
0.57
ENZYME CONCENTRATION (GM/L)

Figure 11. 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, pH 7.0 with 10^{-3} M AMP; closed circle, incubation without AMP.

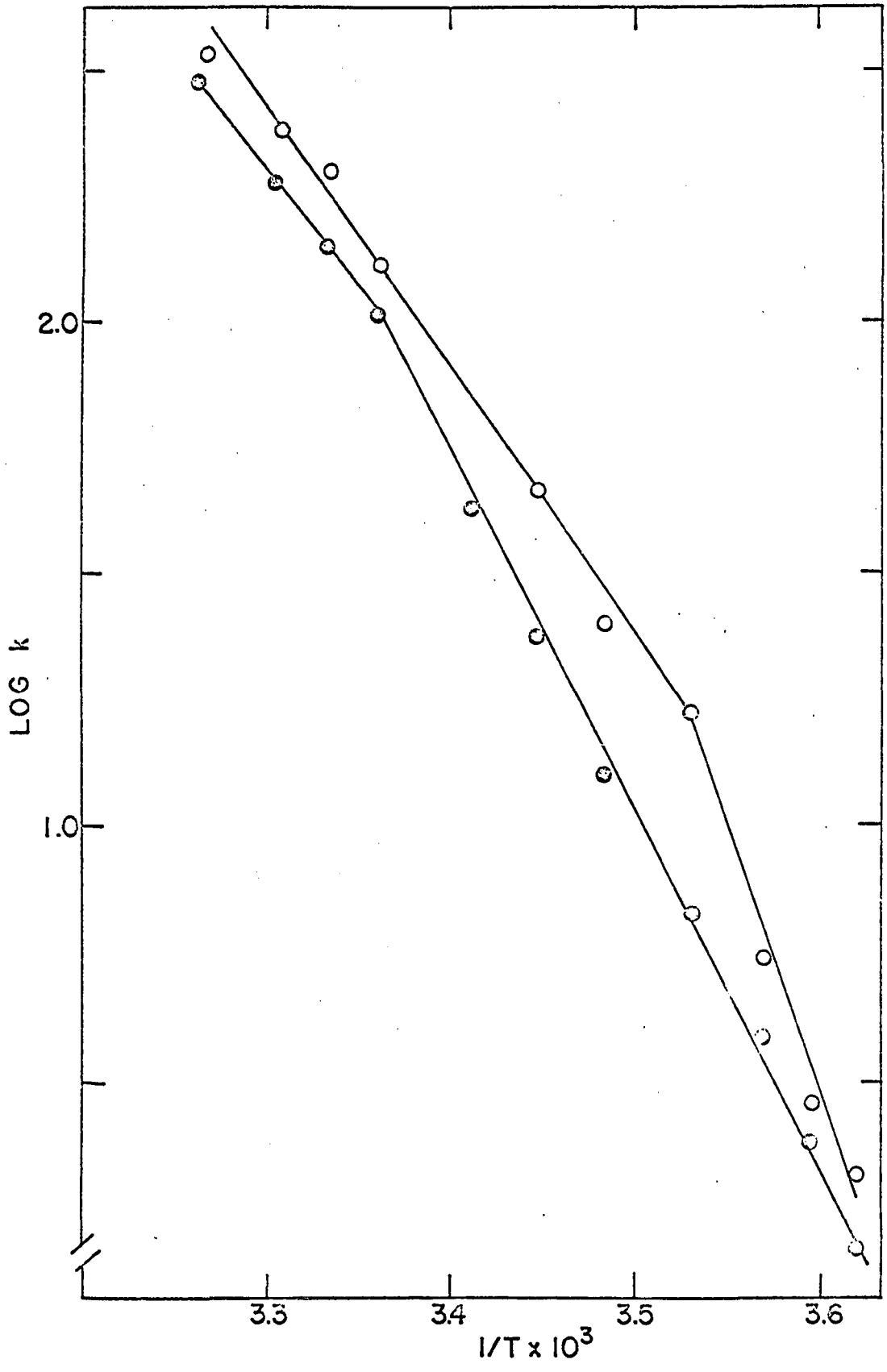

2.0
1.0
LOG k
3.3
3.4
3.5
3.6
1/T x 10³

Figure 12. Ultracentrifugal patterns of phosphorylase a at various temperatures. (A) Enzyme (1.2 mg/ml) in 0.04 M glycerophosphate - 0.03 M cysteine, pH 7.0, with 10^{-3} M AMP at 6°C. (B) Enzyme with buffer of A without AMP at 23°C. (C) As in B with 10^{-3} M AMP. (D) Enzyme (2.1 mg/ml) with buffer of A without AMP at 34°C. (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.

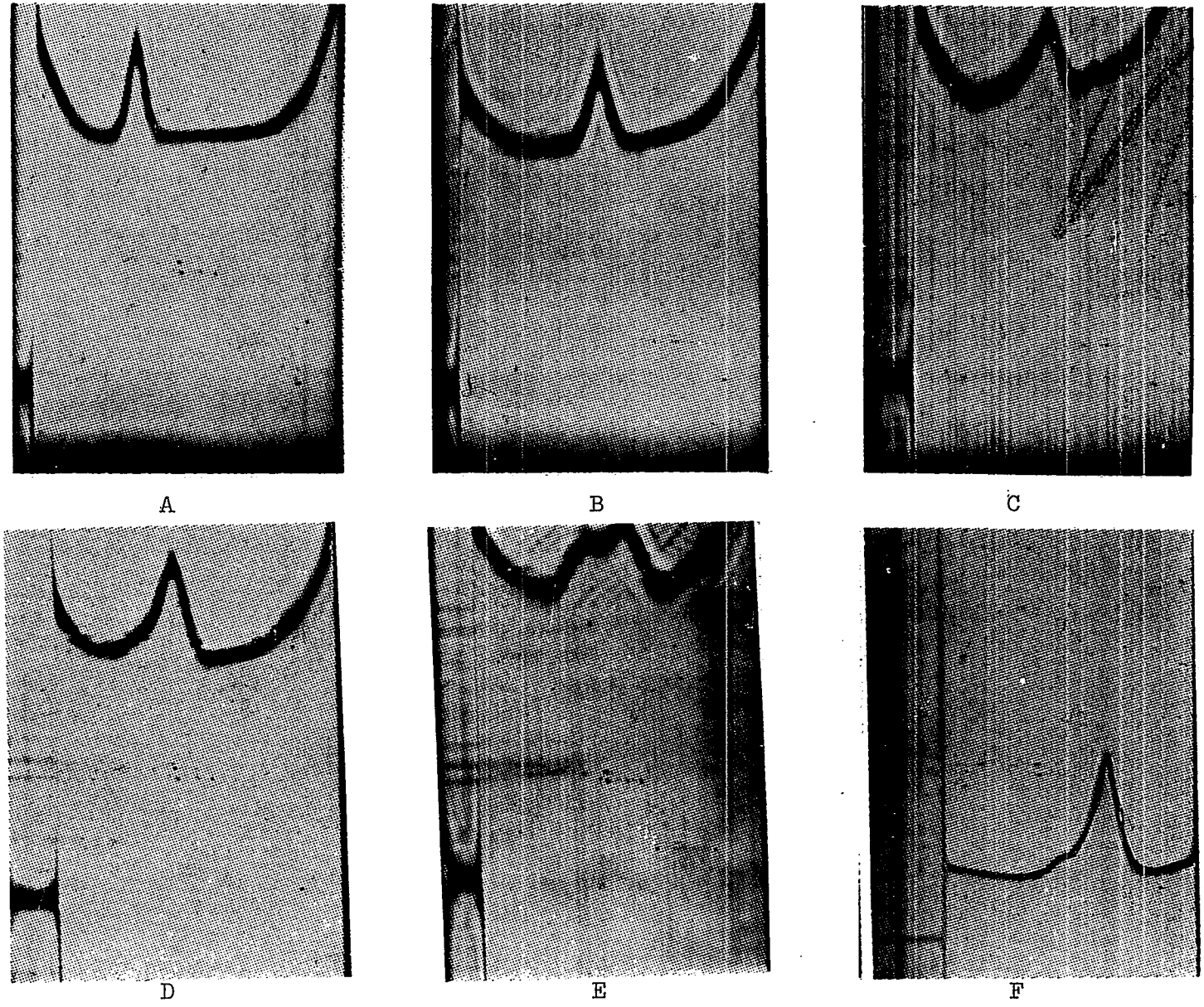
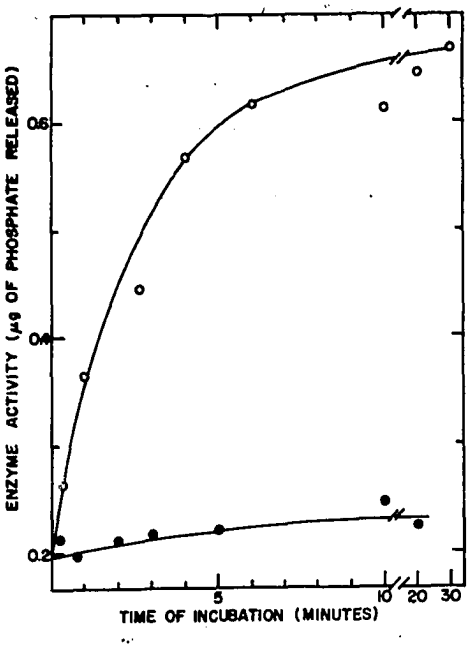
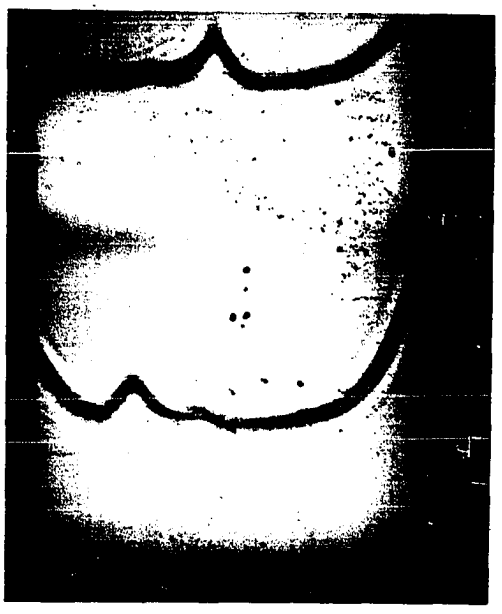

A
B
C
D
E
F

Figure 13. Effect of glucose on the activity and sedimentation of phosphorylase a. (A) Phosphorylase a (12 mg/ml) was diluted 10 fold at 20° in 0.03 M cysteine - 0.04 M glycerophosphate, pH 6.8, solid circles; in buffer containing 0.056 M glucose, open circles. Aliquots were removed at various intervals for measurement of enzymic activity* (30 second assay) at 20°. The assay contained 0.6 mg/ml of enzyme, 0.016 M glucose-1-phosphate, 1% glycogen, and 0.025 M glucose. (B) Ultracentrifugation at 20° of enzyme (1.2 mg/ml) in glycerophosphate-cysteine, upper curve; in buffer with glucose, lower curve. Picture was taken at 18 minutes after attainment of 59,780 rpm. Sedimentation is from left to right.

*Enzymic activity in this case is expressed as μ moles or phosphate released after 30 seconds of reaction.

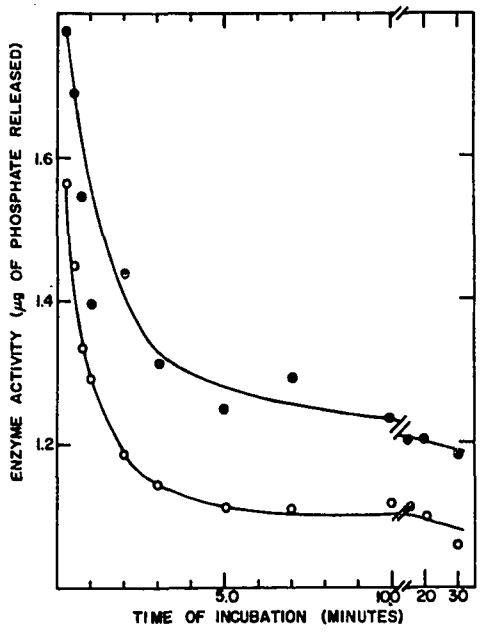

ENZYME ACTIVITY (µg OF PHOSPHATE RELEASED)
0.6
0.4
0.2
5
10
20
30
TIME OF INCUBATION (MINUTES)

A

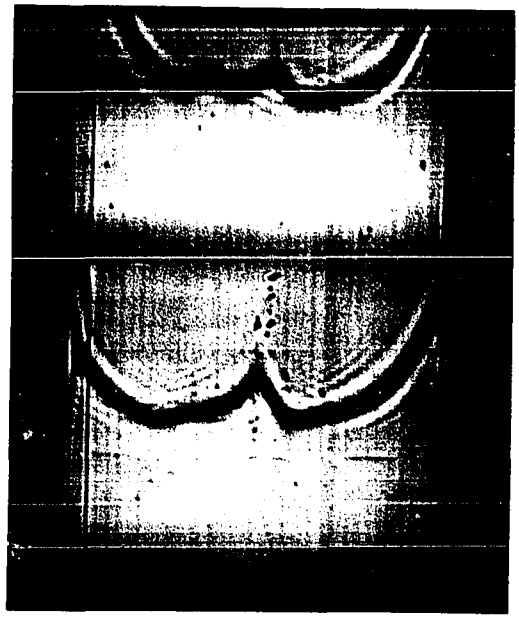


B

Figure 14. Effect of glucose-1-phosphate and AMP on activation and dissociation of phosphorylase a by glucose. (A) Phosphorylase a (1.33 mg/ml) was preincubated at 20° for 30 minutes in 0.03 M cysteine - 0.04 M glycerophosphate, pH 6.8, containing 0.056 M glucose before addition of glucose-1-phosphate (solid circles) or AMP (open circles) to a final concentration of 0.032 M and 10^{-3} M, respectively. Aliquots were withdrawn at various intervals and assayed for activity as in Figure 13 with 10^{-3} M AMP. (B) Ultracentrifugation at 20° of enzyme (1.2 mg/ml) in 0.03 M cysteine - 0.04 M glycerophosphate, pH 6.8, containing 0.05 M glucose. Upper curve, with 0.032 M glucose-1-phosphate. Lower curve, with 10^{-3} M AMP. Picture was taken as in Figure 13B.


ENZYME ACTIVITY (μg OF PHOSPHATE RELEASED)
1.6
1.4
1.2
5.0
100
20
30
TIME OF INCUBATION (MINUTES)

A



B

Figure 15. Activation of phosphorylase a upon incubation in glycogen. Phosphorlase a (0.67 mg/ml) was incubated in 0.03 M cysteine - 0.04 M glycerophosphate, pH 6.8 at 20°, solid circles; open triangles, with 0.1% glycogen; open circles with 0.5% glycogen and solid triangles with 1% glycogen. At various intervals aliquots were removed for enzymic activity for 30 seconds.

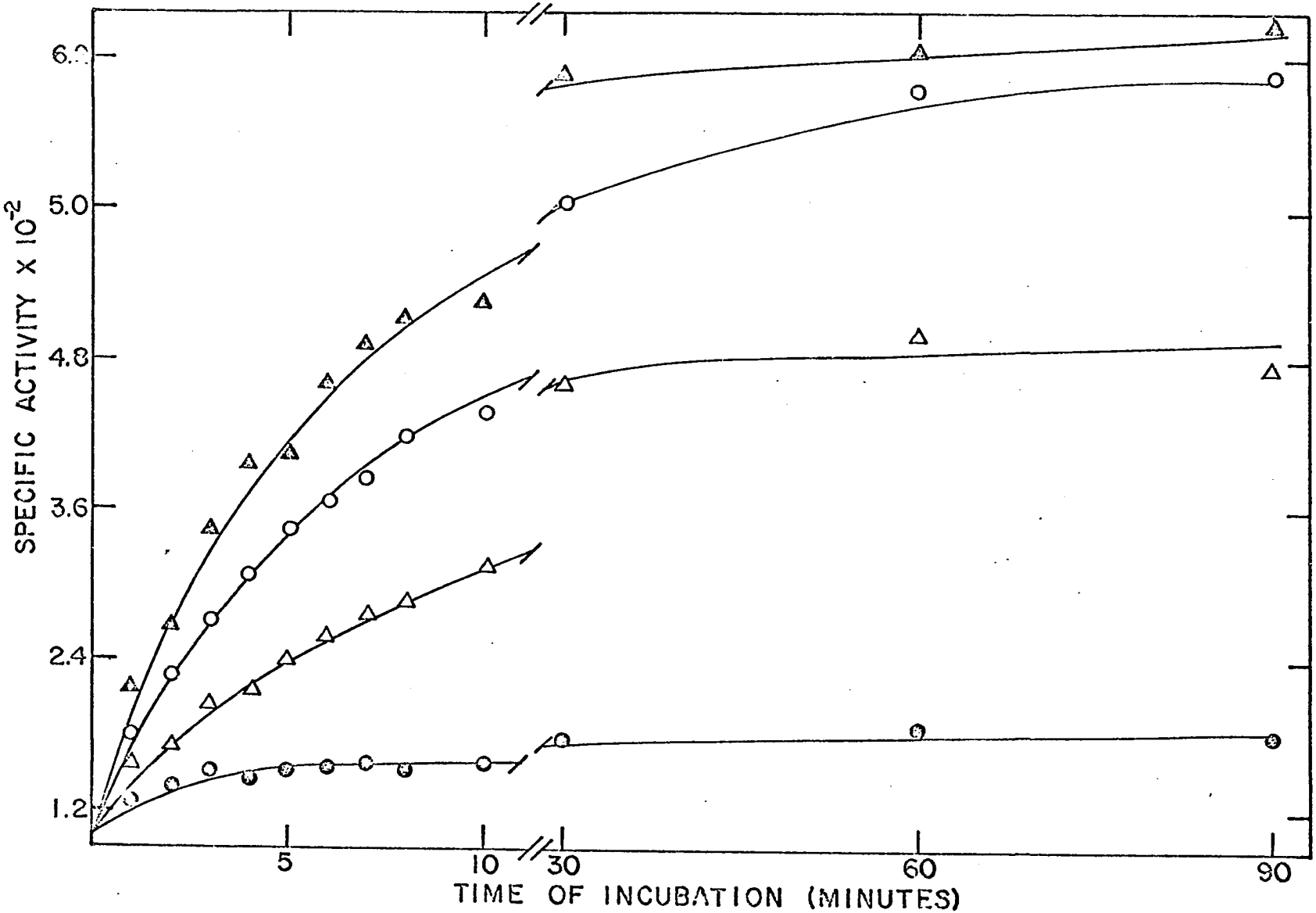

SPECIFIC ACTIVITY X 10^{-2}
6.0
5.0
4.8
3.6
2.4
1.2
5
10
30
60
90
TIME OF INCUBATION (MINUTES)

Figure 16. Effect of preincubation with phosphorylase a upon activation capacity of glycogen. Phosphorylase a (0.3 mg/ml) was incubated in 0.03 M cysteine - 0.04 M glycerophosphate, pH 6.8, at 15°, lower line; upper line, with 2% glycogen. At various intervals, aliquots were removed and tested for enzymic activity for 2 minutes. Upper line, open circles, glycogen used for this experiment was preincubated for 50 minutes with phosphorylase a. At this time the reaction mixture was placed in a boiling water bath for 15 minutes to inactivate enzyme. Fresh enzyme was added at t_0 of this figure; upper line, open squares, as in open circles without preincubation with phosphorylase a.

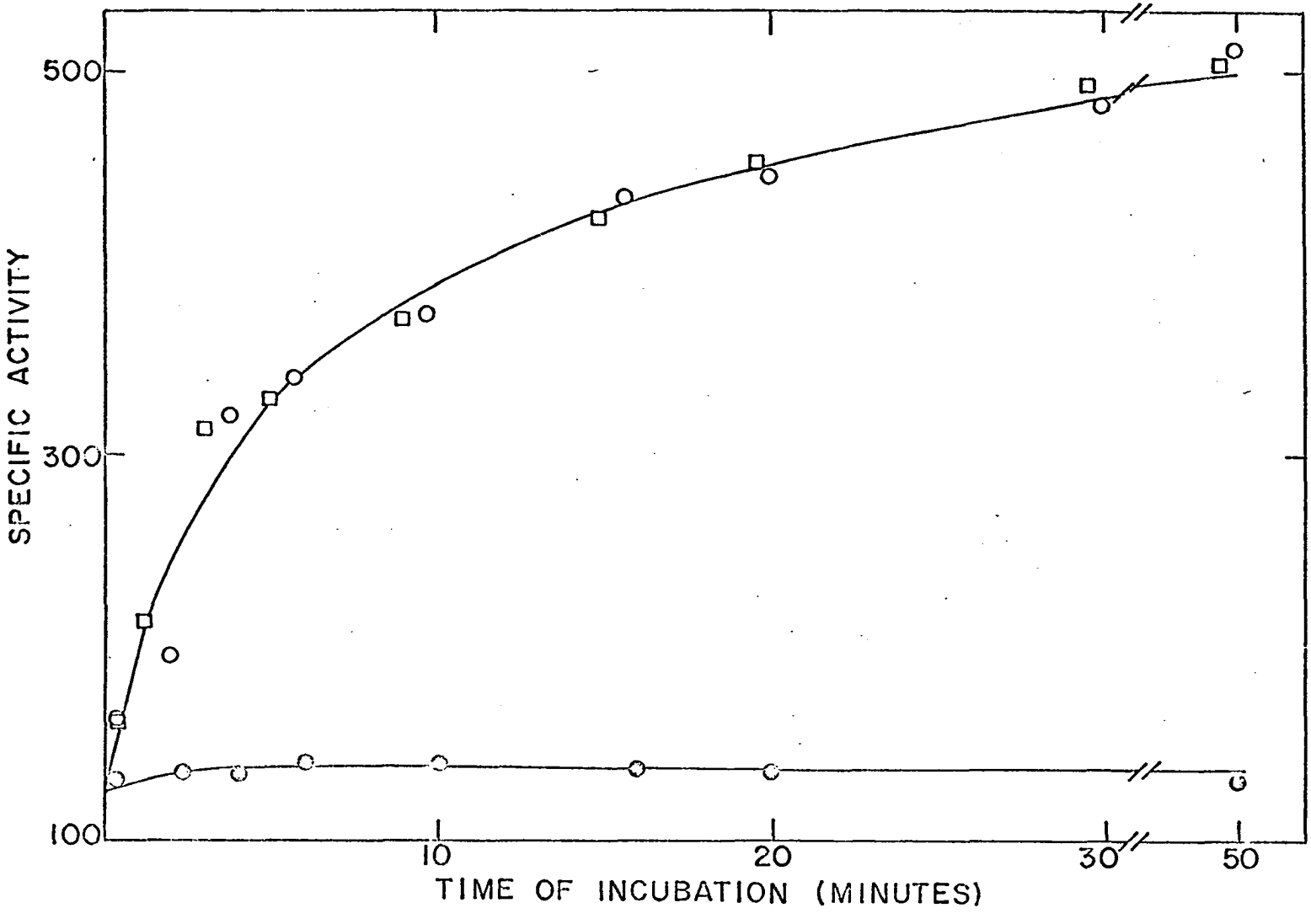

SPECIFIC ACTIVITY
500
300
100
10
20
30
50
TIME OF INCUBATION (MINUTES)

Figure 17. Enzyme concentration and glycogen activation. Phosphorylase a was diluted to various concentrations in 0.03 M cysteine - 0.04 M glycerophosphate, pH 6.8, and incubated at 20°, solid circles; with 2% glycogen, open circles. After 30 minutes, activity was measured.

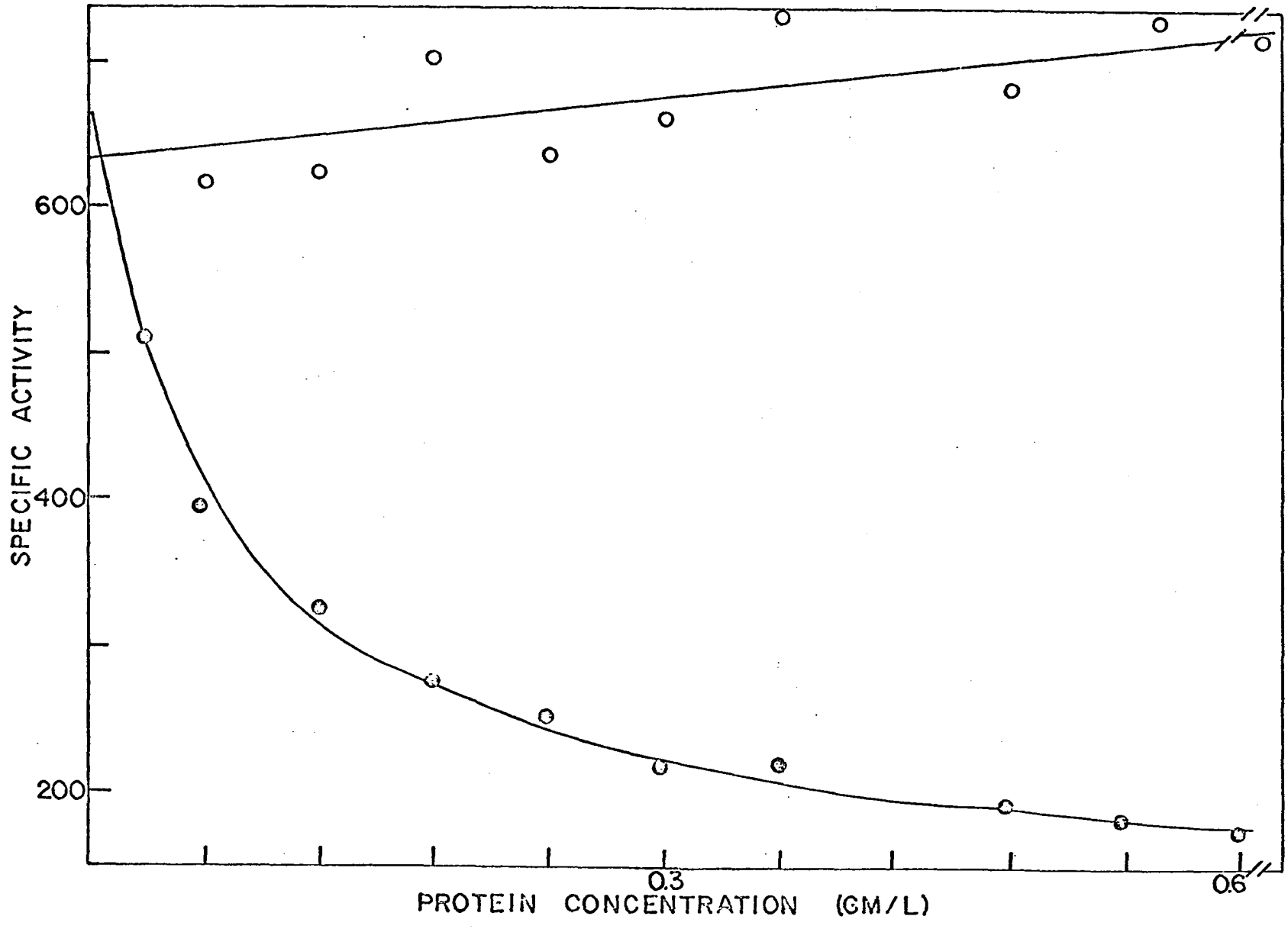

SPECIFIC ACTIVITY
600
400
200
0.3
0.6
PROTEIN CONCENTRATION (GM/L)

Figure 18. Effect of NaCl on glycogen activation. Phosphorylase a (6.0 mg/ml) was preincubated at 20° in (A) 0.03 M cysteine - 0.04 M glycerophosphate, pH 6.8, containing 3.0 M NaCl, (B) with 10^{-3} M AMP. After 50 minutes (t_o of this figure), enzymes were diluted 10 fold at 20°. Open circles, enzyme (A) diluted in buffer with NaCl; solid circles, enzyme (A) in buffer, NaCl, and 2% glycogen; open triangles, enzyme (B) in buffer, NaCl, and AMP; solid triangles, enzyme (B) in buffer, NaCl, AMP and 2% glycogen. At various intervals aliquots were removed and tested for enzymic activity at 20° (5 minutes) with substrate containing AMP at a final concentration of 10^{-3} M.

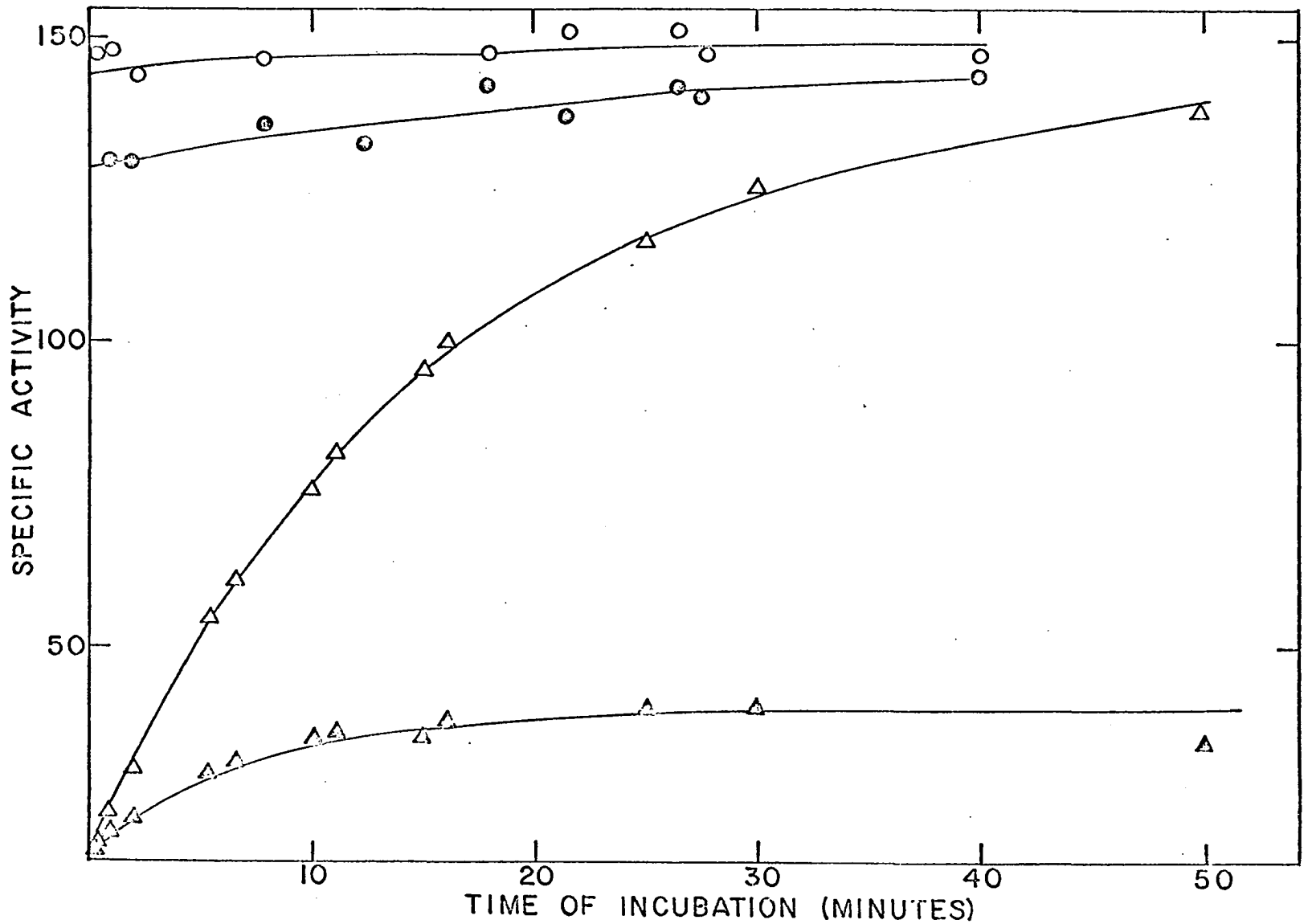
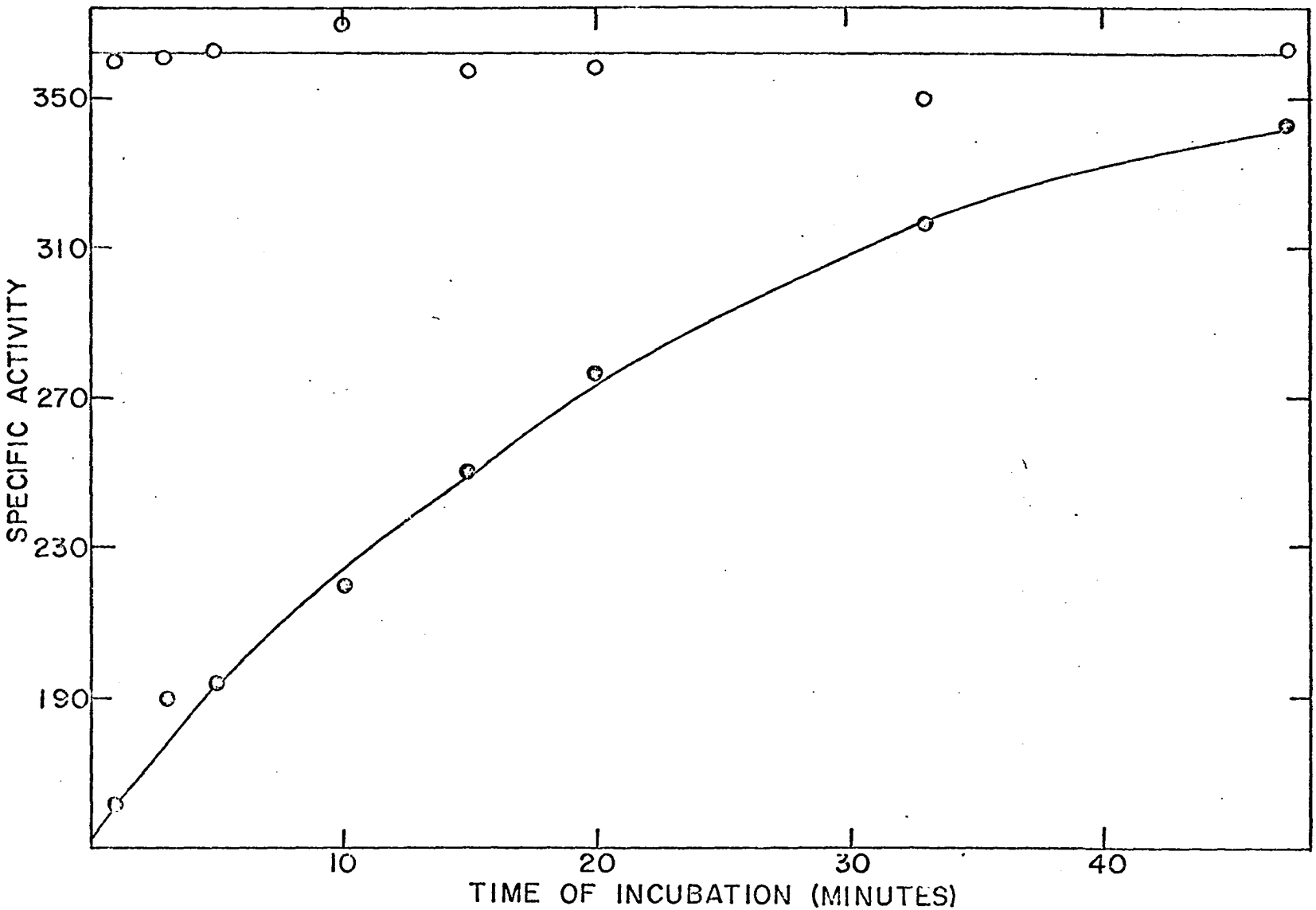

150
100
50
SPECIFIC ACTIVITY
10
20
30
40
50
TIME OF INCUBATION (MINUTES)

Figure 19. Effect of glycogen on the activity of phosphorylase b. Phosphorylase b (5 mg/ml) was preincubated at 20° in 0.03 M cysteine - 0.04 M glycerophosphate, pH 6.8, open circles; in buffer containing 10^{-2} M Mg^{++} and 10^{-3} M AMP, solid circles. After 50 minutes enzyme samples were diluted 10 fold in their respective buffers containing 2% glycogen (t_o of this figure). Aliquots were removed at various intervals for measurements of enzymic activity (1 minute) at 12°. All activities were tested with substrate containing 10^{-3} M AMP and 10^{-2} M Mg^{++}.


SPECIFIC ACTIVITY
350
310
270
230
190
10
20
30
40
TIME OF INCUBATION (MINUTES)

DISCUSSION

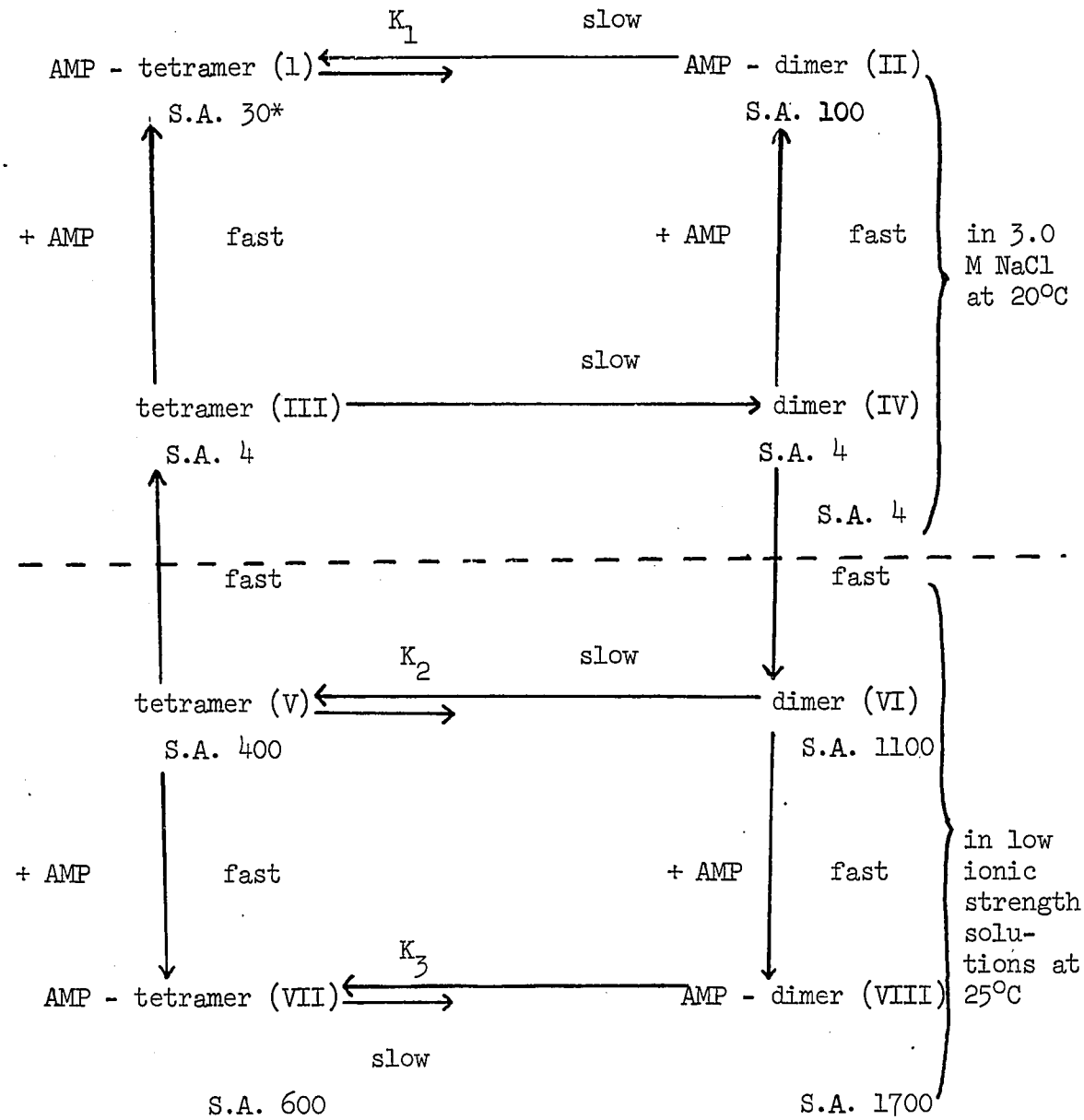
In order to correlate the activity of an enzyme to its subunit structure, it is necessary to carry out the activity and the molecular weight measurements under the same conditions. As the enzyme activity is ordinarily measured at very low protein concentration, the determination of the exact molecular species present in the usual assay procedure is difficult.

A new approach is developed in this work for the study of the relationship of dissociation to the catalytic activity. This method circumvents the determination of the molecular weight of the enzyme at its activity assay concentration. The method is based on the measurement of enzyme activity under various conditions and the kinetic analysis of change of enzyme activity upon sudden alteration in environment.

Kinetic analyses of the alteration of the catalytic properties of phosphorylase a under different experimental conditions are consistent with reaction scheme II.

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)

Scheme II



*The numerical values of activities of the different enzyme species in Scheme II 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 10 by extrapolation, and for those in high ionic strength were estimated from Figure 3. Since the temperature used in these two experiments are not the same, comparison of activities between enzymes in low-ionic-strength and in high-ionic-strength solutions may only be approximated.

with high activity which is slowly transformed to a less active tetramer.

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 suggests that phosphorylase a tetramer (V) dissociates into a smaller unit at low protein concentration and that this dissociation is favored by 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 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 approximately 30% (2). 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 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 $\frac{(\text{dimer})^2}{(\text{tetramer})}$ in the presence of AMP, K_1 and K_3, are similar.

Although it appears from the preceding analysis that Scheme II can be established purely by catalytic activity measurements under various conditions, its validity, however, has to be tested by other means. The hypothesis that the dimer of phosphorylase a is more active than tetramer was supported by the correlation of the modification of ultracentrifugal patterns and activity determinations of phosphorylase a incubated in glucose. Furthermore, the comparison of activities of tetramer and dimer of phosphorylase b is also in agreement with this view.

As these experiments suggest strongly that the dimeric form of phosphorylase a, M.W., 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 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 (29) or lobster (30) phosphorylase to an active form involves phosphorylation but no molecular weight alteration

Experimental evidences which support the view that the main

molecular form phosphorylase a under the ordinary assay conditions is a dimer are (1) incubation of enzyme with glycogen or glucose was shown to activate the tetrameric species, but not the dimeric forms, (2) little effect of preincubation with glycogen on phosphorylase a activity was observed at protein concentrations and temperature used for usual activity measurements, and (3) ultracentrifugal analysis of phosphorylase a at 34° showed a nonsymmetrical schilieren pattern suggestive of enzyme dissociation. Although the dissociation can result in the monomeric unit as indicated in the preceding analysis, the fact that only the dimeric form of phosphorylase a and b are detected in glucose, AMP or 3 M NaCl solutions suggests that this is not the case. Furthermore, kinetic study of the inhibition of phosphorylase by glucose NH_4SO_4 (26, 31) and ATP (32) also favors the hypothesis that the dimer is the smallest unit in the ordinary assay. Confirmation of this may perhaps be obtained by light scattering measurement at low protein concentrations.

The correlation of ultracentrifugal patterns and activity of phosphorylase a in glucose leaves little doubt to the relation of enzyme dissociation to activation by glucose. The same scheme for activation by glycogen is not firmly established. However, among the various phosphorylase species tested with glycogen only the tetramers could be activated by preincubation with glycogen and the maximum activities of these tetramers after glycogen incubation were found to be equal to those of their corresponding dimeric forms. These observations indicate that the activation of phosphorylase by

glycogen is due to dissociation of the enzyme into dimeric units. The results, however, agree equally well with the interpretation that the tetrameric form complexes with glycogen to form an activated tetramer with specific activity similar to that of the dimer. Detailed kinetic study of the activation process may distinguish these two possibilities.

To determine what major forces are responsible for stabilizing the tetrameric form of phosphorylase a is no easy task. In a recent article on protein subunit structure, Tanford (33) pointed out that since protein structure is stabilized cooperatively by various types of interactions (34, 35), it is more meaningful to ask about the nature of newly exposed groups upon dissociation of the protein than about the most important force in association of subunits. From solubilities of various amino acids and peptide in 3.0 M NaCl, Tanford concluded that the dissociation can be favored in high ionic strength solutions only when the newly exposed groups are mainly charged (33). The results presented here, thus, suggest that dissociation of the tetrameric form of phosphorylase a into a dimeric form in high concentrations of NaCl results in the exposure of more charged groups of the protein to the solvent. The data alone, however, do not prove that the combination of dimeric form of phosphorylase a into tetramer is through these charged groups. It is possible that free dimer of phosphorylase a and the dimeric unit in the tetramer of the enzyme assume different conformations. The alteration in catalytic properties of phosphorylase a in 3.0 M NaCl suggests that dissociation of

the enzyme under these conditions may involve the conformational rearrangement of the dimeric unit. The requirement for AMP for this form of phosphorylase a supports this view.

Although the incorporation of phosphate groups into the protein during conversion of phosphorylase b to phosphorylase a results in the neutralization of a highly positive site of the molecule (8), the direct participation of this portion of the molecule in the association of the dimeric unit of phosphorylase a is not clear. The high accessibility of this portion of the enzyme to proteolytic action suggests that this phosphorylated site is exposed to the solvent (8, 36) and, therefore, is not likely to be involved directly in the association. Since it has been shown that the tetramer of phosphorylase a may dissociate into a dimeric species under a wide variety of conditions, it appears that this conclusion may not be justified. Tryptic action can be explained equally well with the phosphorylated site involved in the association if it is assumed that the tetramer dissociates into a dimer with exposure of this site under the conditions of the tryptic attack. Kinetic studies on the proteolysis of various species of phosphorylase a may facilitate further understanding of subunit structure of phosphorylase a.

The fact that no monomeric form of phosphorylase a was detected in solution of high concentrations of NaCl or in glucose solution suggests that the types of interaction between the individual monomeric units of the enzyme are not identical. Similar observation was obtained with hemoglobin (37, 38) where the interactions between monomeric units are known to differ.

SUMMARY

Phosphorylase a, a tetramer, has been found to dissociate in 3.0 M NaCl into a dimeric species. No dissociation was observed with inclusion of 10^{-3} M AMP in the solution. Significant activity of phosphorylase a could be demonstrated in 3.0 M NaCl only when AMP is present in the assay mixture; the activity has been found to increase gradually with time of incubation of the enzyme in NaCl. Addition of AMP to the incubation solution results in a slow decrease in enzymic activity. These results suggest that dimeric form of phosphorylase a is more active than the tetrameric form of the enzyme in high concentrations of NaCl.

The dissociation and alteration of phosphorylase a activity in 3.0 M NaCl can be reversed by dialysis or by dilution of NaCl-incubated enzyme in low ionic strength solutions. Dilution and rapid assay at 20° of phosphorylase a preincubated in 3.0 M NaCl yields high initial enzymic activity which rapidly decays to the activity of sample not incubated in NaCl. The decay of the activity follows second order kinetics, suggesting that the enzyme form with high initial enzymic activity which rapidly decays to the activity of sample not incubated in NaCl. The decay of the activity follows second order kinetics, suggesting that the enzyme form with high catalytic activity is a dimer.

Specific activity of phosphorylase a has been found to increase with decreasing protein concentration with a profile resembling a

dissociation reaction. Increase of temperature or addition of AMP was found to alter the specific activity profile. Rapid cooling of phosphorylase a from 34° to 9° results in a second order decay of activity. The dimeric form of phosphorylase b shows a higher activity than the tetramer of the enzyme. These data suggest that phosphorylase a in low ionic strength can also dissociate into smaller subunits with higher specific activity, and that in the ordinary assay the enzyme exists predominantly as the dissociated form.

Incubation of phosphorylase a with glucose or glycogen results in an increase in catalytic activity. Ultracentrifugal analysis with phosphorylase a in glucose shows the appearance of a slow moving component with a sedimentation constant of 8.95 similar to that of the dimeric form of this enzyme. Among various phosphorylase species tested by incubation with glycogen, only the tetrameric species can be activated. At protein concentrations and temperature used for ordinary assay of phosphorylase a no significant activation can be observed with preincubation in glucose or glycogen. These observations further support the view that phosphorylase a in the ordinary assay conditions exists as a dimeric unit.

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