Purification and Properties of Frog Skeletal Muscle Phosphorylase*

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ABSTRACT: The purification of frog skeletal muscle phosphorylase is described. Frog muscle phosphorylase *b* can be converted into the phosphorylated form (*a*) by rabbit skeletal muscle phosphorylase *b* kinase, adenosine triphosphate (ATP), and Mg^{2+}. Both types (*b* and *a*) of frog muscle phosphorylase appear to be essentially homogenous by a variety of criteria. The molecular weight of frog muscle phosphorylase *b* was determined by sedimentation equilibrium and found to be 188,000 g. A redetermination of the molecular weight of rabbit muscle phosphorylase *b* by sedimentation equilibrium and by sedimentation velocity and diffusion gave a value of 185,000. There are 4 moles of pyridoxal 5′-phosphate and 4 moles of orthophosphate bound to each 370,000 g of frog skeletal muscle phosphorylase *a*. The amino acid compositions of frog, rabbit, and human skeletal muscle phosphorylases are quite similar, but frog and rabbit muscle enzymes differ immunologically. Several kinetic parameters of frog muscle phosphorylase *a* have been determined. As is the case with rabbit muscle phosphorylase *a* the dimer *a* is the active form of frog muscle phosphorylase *a* with glycogen as the substrate. The enzyme differs from mammalian muscle phosphorylase *a* in that the equilibrium between phosphorylase *a* dimer and phosphorylase *a* tetramer is shifted in the direction of the dimer. Furthermore the rate of dissociation at low temperatures is very much more rapid for the frog enzyme than for the rabbit enzyme. A possible correlation between these observations and the ability of frog muscle to carry out glycogenolysis at temperatures below 37° is discussed.

In a recent communication (Metzger *et al.*, 1967) we have presented data on the mechanism of activation of rabbit skeletal muscle phosphorylase *a* by various polysaccharide substrates, a phenomenon originally observed by Wang *et al.* (1965). On the basis of this evidence it was concluded that activation of phosphorylase *a* by glycogen results from the dissociation of the tetramer *a* to dimer *a* and that the dimer is the active form of the enzyme with glycogen as substrate. The greater affinity of polysaccharide substrates for the dimer *a* (as compared with the tetramer *a*) is responsible for this effect. In the case of rabbit skeletal muscle phosphorylase *a* the rate of activation by glycogen is very slow at low temperatures. At 15° at a concentration of enzyme of 3 mg/ml the half-time of activation is approximately 25 min. If activation of phosphorylase *a* by glycogen were to have physiological significance, one would expect that skeletal muscle phosphorylase *a* from a poikilothermic animal dissociates more rapidly at lower temperatures. Actually there is evidence suggesting that lobster phosphorylase, the only phosphorylase from a poikilothermic animal, which has previously been studied may exist mainly as a dimer (at least under the conditions studied) (Cowgill, 1959). Furthermore there exists a considerable body of evidence indicating that in frog muscle activation of phosphorylase is geared to muscle contraction (Danforth *et al.*, 1962; Helmreich and Cori, 1965). Since frogs are capable of sustained muscular activity at low temperatures, it seemed of interest to study the properties of frog muscle phosphorylase. For this purpose, skeletal muscle phosphorylase from *Rana pipiens* was purified to a state of apparent homogeneity. Some of the molecular and kinetic properties of this enzyme are described and compared with the corresponding parameters for the rabbit muscle phosphorylase. The effect of temperature on the activation of frog muscle phosphorylase *a* by glycogen was studied in some detail. The differences in the behavior of the frog *vs.* the rabbit muscle enzyme are discussed, and a hypothesis on the possible biological significance of the activation of phosphorylase by glycogen is presented.

Methods

Enzyme Assays. Routine assays of phosphorylase activity were carried out at 30° by measuring the release of inorganic phosphate (P_i) from α-D-glucose-1-P[1] in the presence of saturating concentrations of glycogen. The reaction mixtures contained 25 mM sodium glycerol-P, 1 mM EDTA, 1 mM 2-mercaptoethanol buffer (pH

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1446 (1966), are: glycerol-P, DL-β-glycerol phosphate; glucose-1-P, α-D-glucose 1-phosphate.

6.8), 25 mM α-D-glucose-1-P, 1% glycogen, 1.5 mM 5′-AMP, and 0.3 unit of enzyme/ml. One unit of enzyme is defined as the amount of enzyme that will cause release of 1 μmole of P_i/min under standard conditions. P_i was determined by the method of Fiske and Subbarow (1925). Protein was measured by the method of Lowry *et al.* (1951) or by absorbancy measurements at 280 mμ using an extinction coefficient of 12.8 (1% × cm^{-1}) for frog skeletal muscle phosphorylase. The evidence on which this value is based is presented in Results.

Kinetic experiments with glucose-1-P as substrate were carried out at 25° in 50 mM sodium glycerol-P buffer (pH 6.8) with the additions stated in the text. P_i was determined by the method of Lowry and Lopez (1946). The concentrations of substrates and 5′-AMP are given in Figure 8A–C. When phosphorolysis of glycogen was measured in kinetic experiments a coupled enzyme assay with phosphoglucomutase and glucose-6-P dehydrogenase as auxiliary enzymes was used. The assay was as described previously (Helmreich and Cori, 1964a).

Arsenolysis of glycogen in kinetic experiments was measured as follows. The reaction mixture used contained in a final volume of 1 ml, 25 μmoles of sodium glycerol-P, 1 μmole of EDTA, 0.5 μmole of 2-mercaptoethanol (pH 6.8), and enzyme, glycogen, and sodium arsenate (pH 6.8) at the concentrations indicated. The reaction was terminated by the addition of 0.14 ml of 4 N $HClO_4$. The samples were transferred to an ice bath and chilled for 15 min. The solution was then neutralized by addition of 0.14 ml of 4 N K_2CO_3. The precipitate which had formed at 45 min at close to 0° was removed by centrifugation. Glucose in the supernatant fluid formed from glucose 1-arsenate was determined with hexokinase and glucose-6-P dehydrogenase.

Activation of Enzyme by Glycogen. At high concentrations of enzyme (3 mg/ml), activation by glycogen was measured by using a modified arsenolysis procedure. The basal activity of the enzyme (*i.e.*, activity prior to preincubation with glycogen) was determined in the following way. To 0.6 ml of a solution containing enzyme (3 mg), 30 μmoles of sodium glycerol-P, 1 μmole of EDTA, 0.6 μmole of 2-mercaptoethanol (pH 6.8), 0.2 ml of H_2O were added. The reaction mixture was incubated for 30 min at the same temperature at which the actual activation experiment with glycogen was carried out (see below). The enzymatic reaction was started by the addition of 0.2 ml of a solution containing 200 μmoles of sodium arsenate (pH 6.8) and 20 mg of glycogen. The reaction was stopped after 30–60 sec by adding 0.4 ml of 0.14 N $HClO_4$. After neutralization of the reaction mixture with K_2CO_3, glucose was determined as described above. Activation of enzyme by glycogen was determined by measuring enzyme activity after preincubation with glycogen as follows. The enzyme was incubated in the absence of substrates for 30 min as described above for measurement of basal activity, then in place of H_2O 0.2 ml of a glycogen solution of the desired concentration was added. After preincubation of the enzyme with glycogen for the desired period of time the enzymatic reaction was started by addition of 0.2 ml of a solution containing 200 μmoles of sodium arsenate (pH 6.8) and if necessary enough glycogen to bring the final concentration to 1%. The reaction was stopped after 30–60 sec and the sample was analyzed for glucose as described above.

Light-Scattering Experiments. These experiments were carried out with a Brice-Phoenix photometer equipped with temperature control, as described by Frieden (1962). The light-scattering instrument was calibrated with crystalline rabbit skeletal muscle phosphorylase *b*, free of 5′-AMP. The experimental conditions for calibration were the same as for the actual experiments with frog skeletal muscle phosphorylase *a* (*i.e.*, the same concentration of protein, buffer system, and temperature was used; see above).

Ultracentrifugal Analysis. Sedimentation velocity and sedimentation equilibrium experiments were carried out in a Spinco Model E analytical ultracentrifuge. Sedimentation equilibrium measurements were performed using Yphantis cells (Yphantis, 1964) and either Rayleigh interference optics or an ultraviolet scanner (Schachman and Edelstein, 1966). The value used for the partial specific volume of phosphorylase was 0.75 (see Brown and Cori, 1961). The theory and the equations used for the calculation of the molecular weights by these techniques may be found in the literature cited. The experimental conditions are described in the legend to Figure 5. Only the data obtained with the ultraviolet scanner are shown.

Amino Acid Analysis. In preparation for amino acid analysis a solution of frog muscle phosphorylase *a* free of 5′-AMP was dialyzed repeatedly against a solution, 250 times its volume, of 5 mM sodium glycerol-P (pH 6.8). The solutions were prepared with deionized redistilled water. An aliquot of the dialyzed solution containing 1.25 mg of enzyme was evaporated to dryness *in vacuo*, 0.4 ml of 6 N HCl was then added, and the tube containing the sample was vacuum sealed and heated for 38 hr at 108°. The protein hydrolysate was evaporated to dryness and carefully dissolved in exactly 1.000 ml of the standard 0.2 N citrate buffer (pH 2.2), used for amino acid analyses. Aliquots (0.02 ml) were analyzed in a Spinco Automatic amino acid analyzer equipped with accelerated analysis accessories and expanded scale. Total nitrogen was determined by a micro-Kjeldhal method (*cf.* Kabat and Mayer, 1961). Cysteine was determined after performic acid oxidation (Moore, 1963). Tryptophan was determined spectrophotometrically (Goodwin and Morton, 1946).

Total Phosphate and Pyridoxal-5-P Analyses. For analyses of pyridoxal-5-P, samples (3.5 mg) of frog skeletal muscle phosphorylase *b* were dialyzed against 10 mM sodium glycerol-P–1 mM EDTA buffer (pH 6.8). The pyridoxal-5-P analyses were carried out in triplicate after acid denaturation of the protein. A molar extinction coefficient of 6250 $mole^{-1}$ cm^{-1} at 295 mμ was used (Shaltiel *et al.*, 1966).

For analysis of total phosphate, samples of frog skeletal muscle phosphorylase *a* were exhaustively dialyzed for 48 hr against 20 mM Tris-Cl–1 mM 2-

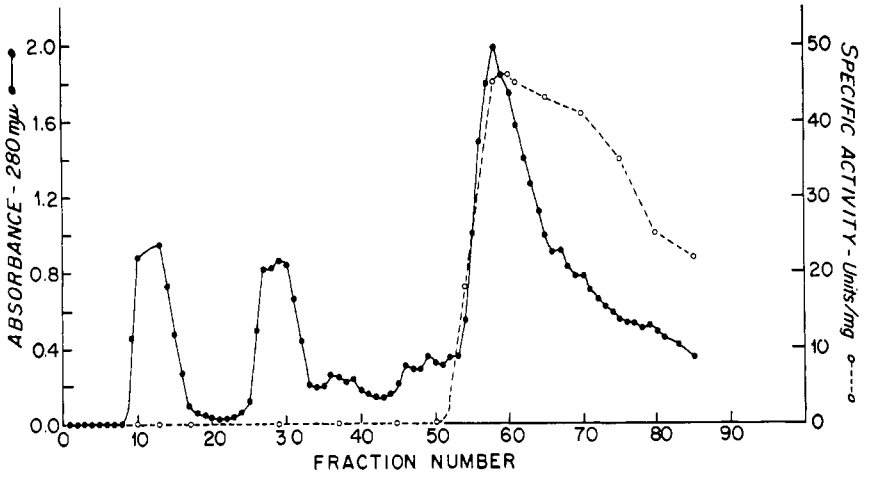

Figure 1: Chromatography of frog muscle phosphorylase *b* on DEAE-cellulose. For procedural details, see text. The enzyme preparation is the one described in Table I.

mercaptoethanol buffer (pH 7.3). Enzyme samples (1 mg) were ashed for the determination of total phosphate according to the procedure of Ames (1966).

Immunodiffusion Experiments. Double diffusion in agar according to the Ouchterlony technique was carried out as described by Crowle (1958). Oxoid Ionagar No. 2 was used. The plates were made with 8 ml of a solution containing 0.4% agar, 0.15 M NaCl, 0.5 M glycine, 1 mM 5′-AMP, and 2.0 mM dithiothreitol (2,3-dihydroxy-1,4-dithiolbutane). Antirabbit skeletal muscle phosphorylase *a* antisera from young Leghorn chickens were used (Michaelides *et al.*, 1964). The concentrations are given in the legend to Figure 4.

Materials. Frozen frog skeletal muscle from winter frogs (*R. pipiens*) was obtained from Schettle Frog Farms, Stillwater, Minn. Muscle can be stored for more than 1 year at −90° without loss of phosphorylase activity. The purification of frog muscle phosphorylase is described in Results.

Crystalline rabbit skeletal muscle phosphorylase *b* was prepared from frozen rabbit skeletal muscle by the method of Fischer and Krebs (1958). Phosphorylase *b* was converted to phosphorylase *a* with phosphorylase *b* kinase, Mg^{2+}, and ATP (Fischer and Krebs, 1962). Rabbit skeletal muscle phosphorylase *b* kinase was prepared according to a published procedure (Danforth and Helmreich, 1964). Preparations of frog and rabbit muscle phosphorylase were freed of 5′-AMP as described previously (Helmreich *et al.*, 1967). Hexokinase, glucose-6-P dehydrogenase, and phosphoglucomutase were purchased from Boehringer & Sons. These enzymes were dialyzed prior to use (*cf.* Helmreich and Cori, 1964a).

Adenine nucleotides, TPN, and sugar phosphates were products of Pabst Laboratories and the Sigma Chemical Co., respectively. Whatman microgranular DEAE-cellulose DE52 was purchased from Reeve Angel. Oxoid Ionagar No. 2 was a product of Oxo Ltd., London.

Rabbit liver glycogen (Mann Research Laboratories) was freed of nucleotide by dialysis for 24 hr against 20 volumes of a thin slurry of Dowex 1-X8 acetate (pH 4.0). Following dialysis the glycogen was precipitated with ethanol, washed with ethanol and ether, and dried. Phosphorylase limit dextrin was prepared by the method of Larner *et al.* (1952). All other reagents were analytical reagent grade and were used without further purification.

Results

Preparation of Frog Muscle Phosphorylase b. Preparation of Extract. The purification described below is for 2000 g of muscle. For purifications on a smaller scale (from 500 to 2000 g), the quantities of reagents were reduced proportionally. Frozen muscle of *R. pipiens* was thawed in two times the volume (by weight) of distilled water. The beaker containing the frozen meat was placed in a water bath at 30° and the temperature of the water was allowed to rise to 8–10°. The muscle was thawed completely after about 1 hr. One-half the volume of water was decanted and saved. The muscle was ground at room temperature for 10–15 sec in a Waring Blendor to a coarse mince. The muscle mince was extracted at room temperature for 10 min with stirring by hand and the extract was separated from the pulp by filtering through cotton gauze. The muscle pulp was extracted once more for 10 min with the remaining half of the volume of water in which the muscle was thawed. Care was taken to recover as much extract as possible by squeezing the muscle pulp. From this step onward all operations were carried out in the cold room at 4°. The combined extracts were chilled to 4° and dialyzed for 3–4 hr against 15 times their volume of distilled water.

Acid Precipitation. After dialysis, the pH of the extract ranged from 6.1 to 6.4. The pH was lowered to 5.8 by addition of 5 N acetic acid and the acidified extract was allowed to stand for 30 min. A coarse precipitate which formed upon acidification was removed by centrifugation at 1600*g* for 15 min.

First $(NH_4)_2SO_4$ Fractionation. The clear supernatant was adjusted to pH 7.0 with solid $KHCO_3$ and neutral saturated $(NH_4)_2SO_4$ solution was added until 50%

TABLE I: Purification of Frog Skeletal Muscle Phosphorylase *b*.[a]

Purification Steps	Vol (ml)	Total Protein (mg)	Total Act. (units)	Sp Act. (units/mg)	Recov (%)
1. Crude extract	4100	52,800	50,800	0.96	100
2. Supernatant from acid precipitate	4100	50,800	50,800	1.00	100
3. First $(NH_4)_2SO_4$ precipitate	380	2,970	26,400	8.9	52
4. Heat treatment at pH 8.5	365	2,970	27,500	9.3	54
5. Third $(NH_4)_2SO_4$ precipitate	142	1,090	23,900	21.9	47
6. DEAE-cellulose column chromatography (peak fractions)[b]	40	280	12,600	45.0	25

[a] The data for purification of frog muscle phosphorylase starting from 2000 g of muscle are given (see text). Units of activity are expressed as micromoles of P_i per minute at 30° (see Methods) and specific activity is expressed as units per milligram of protein. [b] After concentration by $(NH_4)_2SO_4$ precipitation and dialysis.

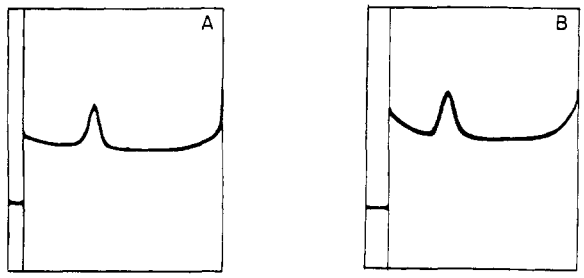

FIGURE 2: Sedimentation velocity measurements on frog muscle phosphorylase. (A) Frog muscle phosphorylase *a*, 3.0 mg/ml, in 50 mM sodium glycerol-P–2 mM EDTA–1 mM 2-mercaptoethanol buffer (pH 6.8). The run was carried out at 4° and at a rotor speed of 59,780 rpm. The picture was taken 38 min after the rotor had reached full speed. (B) Frog muscle phosphorylase *b*, 3.9 mg/ml, in 50 mM sodium glycerol-P–1 mM EDTA–1 mM 2-mercaptoethanol–5 mM magnesium acetate–0.5 mM 5′-AMP buffer (pH 6.8). The run was carried out at 8° and at a rotor speed of 59,780 rpm. The picture was taken 43 min after the rotor had reached full speed. Sedimentation in A and B was from left to right. The sedimentation velocity patterns were traced from the original photographs.

saturation was reached. The salt was added slowly so that the temperature did not rise above 10°. 2-Mercaptoethanol was added to a final concentration of 1 mM and the precipitate was allowed to form overnight. On the next day it was collected by centrifugation at 1600*g* for 15 min and the supernatant fluid was discarded. The precipitate was resuspended in 380 ml of 1 mM Tris-acetate–0.1 mM EDTA–1 mM 2-mercaptoethanol buffer (pH 7.4) and dialyzed for 38 hr against three changes of 2 l. each of the same buffer solution. Any precipitate formed during dialysis was removed by centrifugation at 20,000*g* for 15 min.[2]

Heat Precipitation. To the clear supernatant solution 5′-AMP and EDTA were added to a final concentration of 1 mM each. The pH was raised to 8.5 by addition of 2 M Tris base and the solution was quickly heated to 45°. It was kept at this temperature for 5 min and then rapidly cooled to 15° and readjusted to pH 7.0 with acetic acid.

Second and Third $(NH_4)_2SO_4$ Fractionations. The precipitate which formed upon heating was not routinely removed because the heat treatment was immediately followed by a second fractionation with $(NH_4)_2SO_4$ to give 25% saturation. After standing for 1 hr the precipitate was collected by centrifugation at 20,000*g* for 15 min and discarded. The supernatant solution was saturated to 50% with $(NH_4)_2SO_4$. A precipitate was allowed to form for 1–2 hr. It was collected by centrifugation at 20,000*g* for 15 min, taken up in 125 ml of 3 mM sodium glycerol-P–1 mM EDTA–1 mM 2-mercaptoethanol buffer (pH 7.2), and dialyzed for 18 hr against three changes of 1 l. each of the same buffer.

Chromatography on DEAE-cellulose. At this stage in the preparation, the enzyme was purified 25- to 30-fold over the extract. An additional 2- to 2.5-fold purification was achieved by chromatography on DEAE-cellulose. A column (30 × 3.0 cm) was equilibrated with 3 mM sodium glycerol-P–1 mM EDTA–1 mM 2-mercaptoethanol buffer (pH 7.2). The enzyme in the same buffer solution was added to the column and eluted with a linear ionic gradient. The reservoir flask contained 1000 ml of 200 mM sodium glycerol-P–0.1 mM EDTA–1 mM 2-mercaptoethanol buffer (pH 7.2), the mixing flask contained 1000 ml of 3 mM sodium glycerol-P–0.1 mM EDTA–1 mM 2-mercaptoethanol buffer (pH 7.2). The flow rate was adjusted to 75 ml/hr and the eluate was collected in 15-ml fractions. A typical elution diagram for phosphorylase *b* is shown in Figure 1. The phosphorylase activity was eluted between 60 and 80 mM sodium glycerol-P. The front of the activity peak is quite sharp although the enzyme activity trails over a rather broad range. The activity of the peak fractions measured under routine assay conditions was between 45 and 55 units/mg of protein. Frog muscle phosphorylase *b* eluted from the column was either concentrated by precipitation with $(NH_4)_2SO_4$ or converted into phosphorylase *a*.

[2] With some preparations centrifugation had to be carried out at 80,000*g* to obtain a clear supernatant fluid.

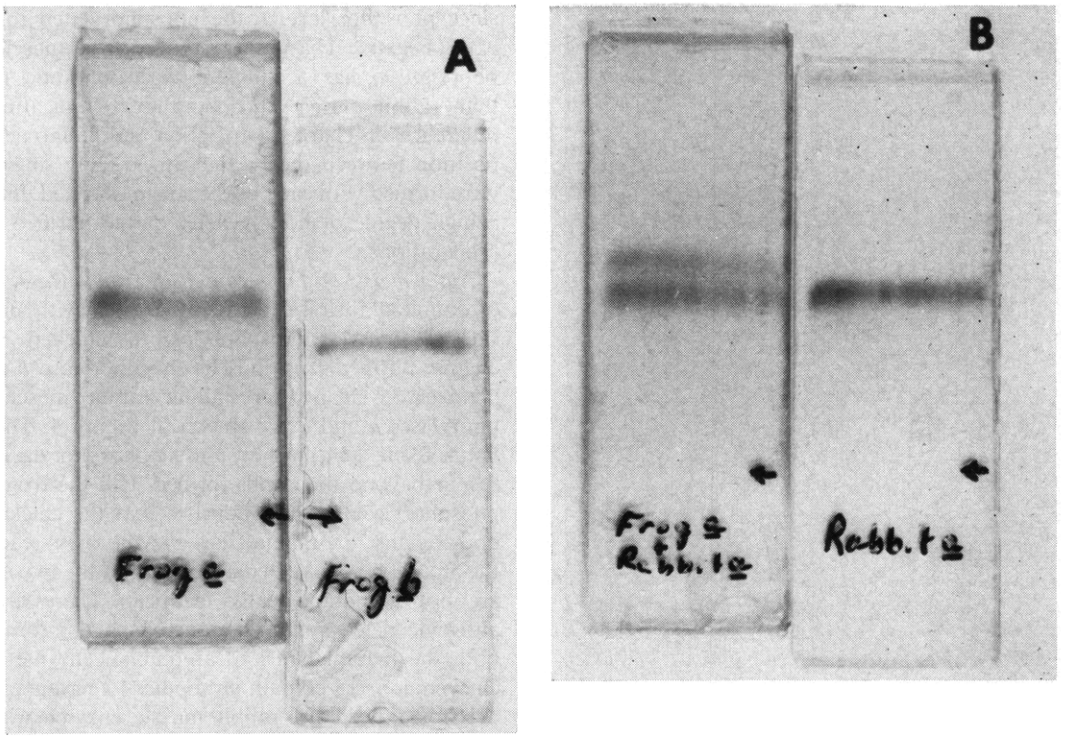

FIGURE 3: Electrophoresis of frog muscle phosphorylase on cellulose acetate. Aliquots (5 μl containing 5 μg) of the phosphorylase preparations indicated were subjected to electrophoresis on Sepharopore III (Gelman) equilibrated with 20 mM sodium glycerol-P (pH 6.4). The amount of protein which was applied was large enough in order to detect with certainty impurities in the range of 10% or less. Electrophoresis was carried out for 1 hr at 220 V. The strips were stained with ponceau-xylidine dye. (A) Frog muscle phosphorylases *a* and *b*. (B) Frog muscle phosphorylase *a* and rabbit muscle phosphorylase *a*.

Table I summarizes the purification scheme of frog muscle phosphorylase *b*.

Purified frog muscle phosphorylase *b* was converted into phosphorylase *a* with rabbit muscle phosphorylase *b* kinase, ATP, and Mg^{2+} (Fischer and Krebs, 1962). Frog muscle phosphorylase *a* was isolated from the reaction mixture by rechromatography on DEAE-cellulose according to the procedure described above for phosphorylase *b*. A somewhat higher ionic strength (80–100 mM sodium glycerol-P) is required for the elution of phosphorylase *a*. Sixty per cent of the total phosphorylase *a* activity was recovered from the column. In some preparations the conversion reaction was carried out with phosphorylase *b* preparations obtained from the third $(NH_4)_2SO_4$ precipitate (step 5 in Table I).[3]

Frog muscle phosphorylase *a* eluted from the DEAE column and concentrated by ammonium sulfate precipitation had a specific activity of 50–58 units, *i.e.*, in the range of the activity of crystalline rabbit skeletal muscle phosphorylase. The specific activity of the enzyme in the absence of 5′-AMP was 80–85% of the activity in the presence of saturating concentrations of 5′-AMP. Repeated attempts to crystallize the enzyme were not successful, presumably because of the high solubility of frog muscle phosphorylase.

Frog muscle phosphorylase *b* prepared by this procedure frequently contained some glucosidase activity which caused release of glucose from glycogen or limit dextrin. The specificity of this enzyme was not determined. The glucosidase activity could be removed largely or completely after phosphorylase *b* was converted into *a* and the latter rechromatographed on DEAE-cellulose. Frog muscle phosphorylase *a* treated in this manner contained little or no glucosidase (less than 13 mμmoles of glucose was released from glycogen (1%)/hr per mg of phosphorylase *a*). Glucosidase activity was measured at 15° in 30 mM sodium glycerol-P buffer (pH 6.8). The glucose released from glycogen was determined with hexokinase and glucose-6-P dehydrogenase (see Methods).

Stability. The purified frog muscle phosphorylase preparations were rapidly inactivated if stored frozen. Frog muscle phosphorylase *a* was, however, quite stable when stored at 3° under toluene vapor at concentrations of 10–20 mg/ml in 50 mM sodium glycerol-P–2 mM EDTA–1 mM 2-mercaptoethanol buffer (pH 6.8). Under these conditions, the enzyme lost only 10–15% activity/month. Frog muscle phosphorylase *b* is quite unstable in the absence of 5′-AMP. Without 5′-AMP, irreversible losses of activity as high as 30% of the initial activity/week were encountered. The addition of 1 mM 5′-AMP stabilized phosphorylase *b* considerably. Purified frog muscle phosphorylase *b*,

[3] With some preparations at this stage of purification it has been found necessary to carry out the conversion of phosphorylase *b* into phosphorylase *a* in the presence of 0.2 M NaF in order to inhibit phosphorylase phosphatase activity.

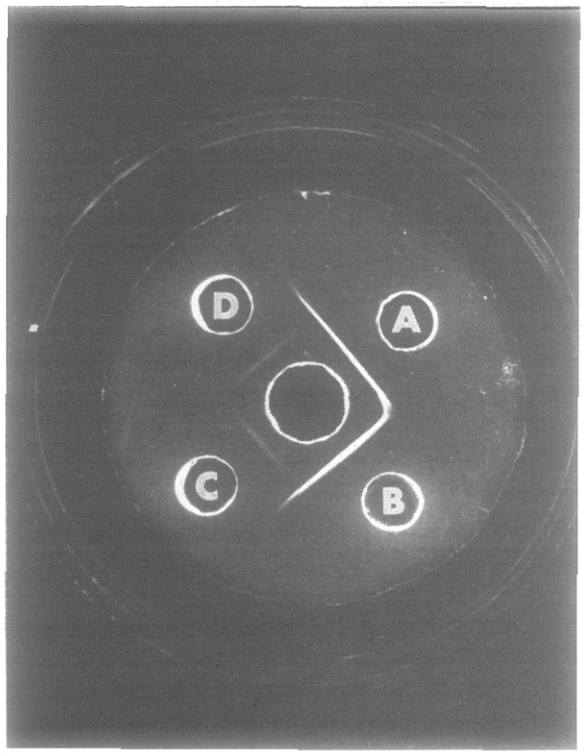

FIGURE 4: Comparison of antigenic determinants of frog and rabbit muscle phosphorylases *b* and *a*. Undiluted, decomplemented chicken antiserum (0.1 ml) against rabbit muscle phosphorylase *a* was added to the center well. Well A received 0.05 ml (112 μg) of crystalline rabbit muscle phosphorylase *a*. Well B received 0.02 ml (160 μg) of crystalline rabbit muscle phosphorylase *b*. C and D each received 0.04 ml (340 μg) of frog muscle phosphorylase *b*. The enzyme preparations were dissolved in 30 mM sodium glycerol-P–1.2 mM EDTA–0.6 mM 2-mercaptoethanol buffer (pH 6.8). The bands developed in 7 days at room temperature.

10–20 mg/ml, stored in 50 mM sodium-glycerol-P–1 mM 5′-AMP–2 mM EDTA–1 mM 2-mercaptoethanol buffer (pH 6.8) at 3° under toluene vapor was nearly as stable as frog muscle phosphorylase *a*.

Homogeneity. Sedimentation velocity measurements on frog muscle phosphorylase are shown in Figure 2. Frog muscle phosphorylase *b* travels as a single symmetrical peak with an s_{20} value of 8.13 S (Figure 2B). Figure 2A shows a sedimentation velocity picture of frog muscle phosphorylase *a* from which an s_{20} value of 11.2 S was estimated. This is lower than the corresponding value for rabbit muscle phosphorylase *a* (s_{20} = 13.2 S; *cf.* Brown and Cori, 1961). The lower value for frog muscle phosphorylase *a* probably represents the value for an equilibrating mixture of dimer *a* and tetramer *a* and thus reflects the ease with which frog muscle phosphorylase tetramer *a* dissociates to its dimeric form. This is discussed below.

The results of electrophoresis experiments on cellulose acetate with frog skeletal muscle phosphorylases *b* and *a* and for comparison with crystalline rabbit skeletal muscle phosphorylase *a* are shown in Figure 3. The electrophoretic mobility of frog skeletal muscle phosphorylases *b* and *a* is different as is to be expected since phosphorylase *a*, the phosphorylated form, has extra negative charges; moreover frog muscle phosphorylase *a* has a different electrophoretic mobility than rabbit muscle phosphorylase *a* indicating some differences in charge distribution and amino acid composition between the two enzymes. Only single bands were formed with each of the enzymes tested, indicating a high degree of homogeneity of the purified enzyme preparations.

Antigenicity of Frog Muscle Phosphorylase. Results of double-diffusion experiments in agar with antirabbit skeletal muscle phosphorylase *a* chicken antisera against purified frog muscle phosphorylase *b* and for comparison against crystalline rabbit muscle phosphorylases *a* and *b* are shown in Figure 4. The single bands formed with rabbit muscle phosphorylases *a* and *b* spur beyond the bands formed with the frog muscle phosphorylase *b*. This indicates that the chicken antibody against the mammalian enzyme cross-reacts with the amphibian muscle enzyme but that the two enzymes are not immunologically identical. There are some antigenic determinants present which are common to both the mammalian and amphibian enzyme but the chicken antisera contain antibodies to certain antigenic determinants in the rabbit muscle enzyme which the frog muscle enzyme lacks.

Molecular Weight. The molecular weight of purified frog muscle phosphorylase *b* was determined by sedimentation equilibrium measurements (Schachman and Edelstein, 1966). A set of typical data obtained by this method with frog muscle phosphorylase *b* is presented in Figure 5A. The linearity of the slope in Figure 5A again points to the homogeneity of the frog muscle enzyme. Based on these data the molecular weight of frog muscle phosphorylase *b* is 188,000. This is a much lower value than that for the molecular weight of rabbit skeletal muscle phosphorylase *b* reported by Keller and Cori (1953) which was based on sedimentation velocity and diffusion measurements. Because of these differences (188,000 *vs.* 242,000), we have redetermined the molecular weight of crystalline rabbit skeletal muscle phosphorylase *b* by sedimentation equilibrium. These data are given in part B of Figure 5.

The molecular weight of rabbit muscle phosphorylase *b* determined by this method is 185,000. A single redetermination of the diffusion constant by the method of Ehrenberg (1957), gave a value of 4.84 which when used in conjunction with an s_{20} value of 8.1 S would yield a molecular weight of 170,000. After this work was completed the results of extensive and careful redeterminations of the molecular weight of rabbit skeletal muscle phosphorylase were reported by Seery *et al.* (1967). These authors obtained essentially the same results as reported here and in recent publications by Buc and Buc, 1967) and by De Vincenzi and Hedrick (1967). There is therefore good agreement among the various groups who have recently redetermined the molecular weights of muscle phosphorylase. Furthermore it now appears that the molecular weight of highly purified phosphorylases from sources as phylogenetically different as mammalian heart (Davis *et al.*, 1967), rabbit and frog skeletal muscle, and even potato

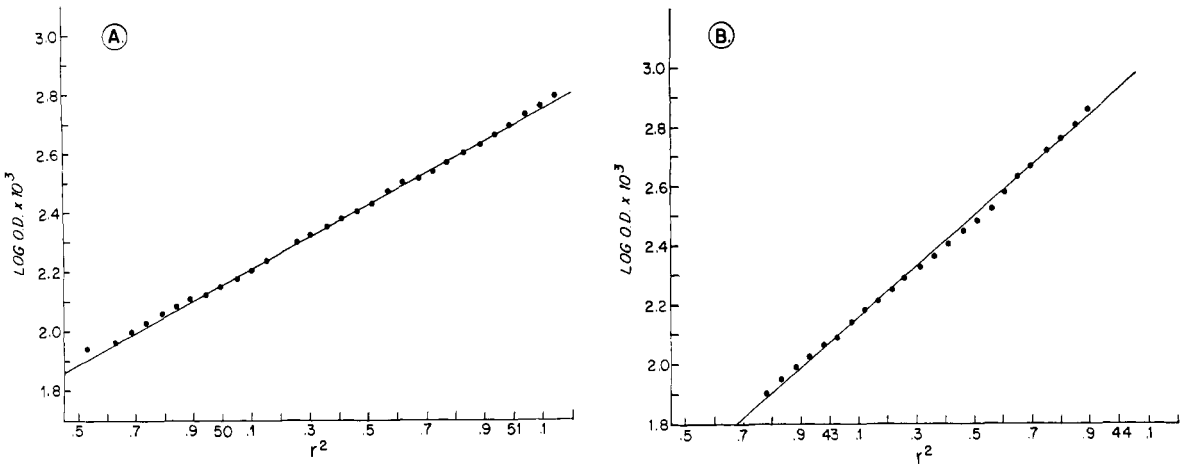

FIGURE 5: Sedimentation equilibrium measurements on frog muscle phosphorylase *b* and rabbit muscle phosphorylase *b*. (A) Sedimentation equilibrium measurement with 0.298 mg/ml of frog muscle phosphorylase *b* in 20 mM sodium glycerol-P–2 mM EDTA–1 mM 2-mercaptoethanol–100 mM NaCl buffer (pH 6.8). (B) Measurements with 0.211 mg/ml of rabbit muscle phosphorylase *b* in 17.9 mM sodium glycerol-P–1.7 mM EDTA–0.9 mM 2-mercaptoethanol–87 mM NaCl buffer (pH 6.8). The speed of centrifugation was 10,589 rpm for 20 hr. The measurements were carried out according to the procedure of Schachman and Edelstein (1966). The data are plotted as log optical density as a measure of concentration against the square of the distance from the axis of rotation (r^2).

TABLE II: Amino Acid Composition of Rabbit, Human, and Frog Muscle Phosphorylases.[g]

Amino Acid	Residues/Molecule (monomer)[a]		
	Rabbit[b]	Human[b]	Frog[c]
Lysine	46.8 (53.6)[d]	45.7 (50.0)[d]	39.2 (46.4)[d]
Histidine	21.6 (24.7)	22.7 (24.8)	18.1 (21.4)
Ammonia	(69) (79.0)	(66.5) (72.8)	(60.9) (72.0)
Arginine	61 (69.8)	61.7 (67.5)	48.4 (57.2)
Aspartic acid	87.4 (100)	91.4 (100)	84.6 (100)
Threonine	30.5 (34.9)	30.9 (33.8)	31.4 (37.1)
Serine	22.7 (26.0)	23.8 (26.0)	31.2 (36.9)
Glutamic acid	86.2 (98.6)	85.5 (93.6)	77.7 (91.8)
Proline	32.3 (37.0)	32.0 (35.0)	39.8 (47.0)
Glycine	43.1 (49.5)	44.6 (48.8)	43.2 (51.1)
Alanine	56.9 (65.1)	59.9 (65.5)	50.7 (60.0)
Half-cystine[e]	8.2 (9.4)	7.8 (8.5)	9.8 (11.6)
Valine	55.4 (63.4)	54.7 (59.8)	53.2 (62.9)
Methionine	19.3 (22.1)	20.8 (22.8)	18.3 (21.6)
Isoleucine	44.6 (51.0)	45.0 (49.3)	39.5 (46.7)
Leucine	72.9 (83.4)	72.9 (79.8)	64.2 (75.9)
Tyrosine	32.7 (37.4)	31.6 (34.6)	31.6 (37.7)
Phenylalanine	35.7 (40.8)	38.3 (41.9)	32.4 (38.3)
Tryptophan[f]	11.2 (12.8)	10.8 (11.8)	11.4 (13.5)

[a] Calculated on the basis of a molecular weight of 90,000 g for each monomeric subunit (see text). [b] From data of Appleman *et al.* (1963). [c] The values for the frog enzyme are calculated on the basis of *A* 12.8 (see text), while the data of Appleman *et al.* (1963) were calculated on the basis of *A* 11.9. Using instead a value for *A* 13.2 (see text) would minimize the differences between the frog and the human and rabbit muscle phosphorylases. [d] The values in parentheses give the amino acid compositions expressed in percentiles relative to the values for aspartic acid which were set arbitrarily as 100. Therefore the differences in amino acid compositions of the frog, rabbit, and human muscle phosphorylases which are found by this method of calculation are not due to differences in molecular weights and/or in absorbancy indices. [e] Determined as cysteic acid (see Methods). [f] From spectrophotometric measurements (see Methods). [g] For procedural details see Methods.

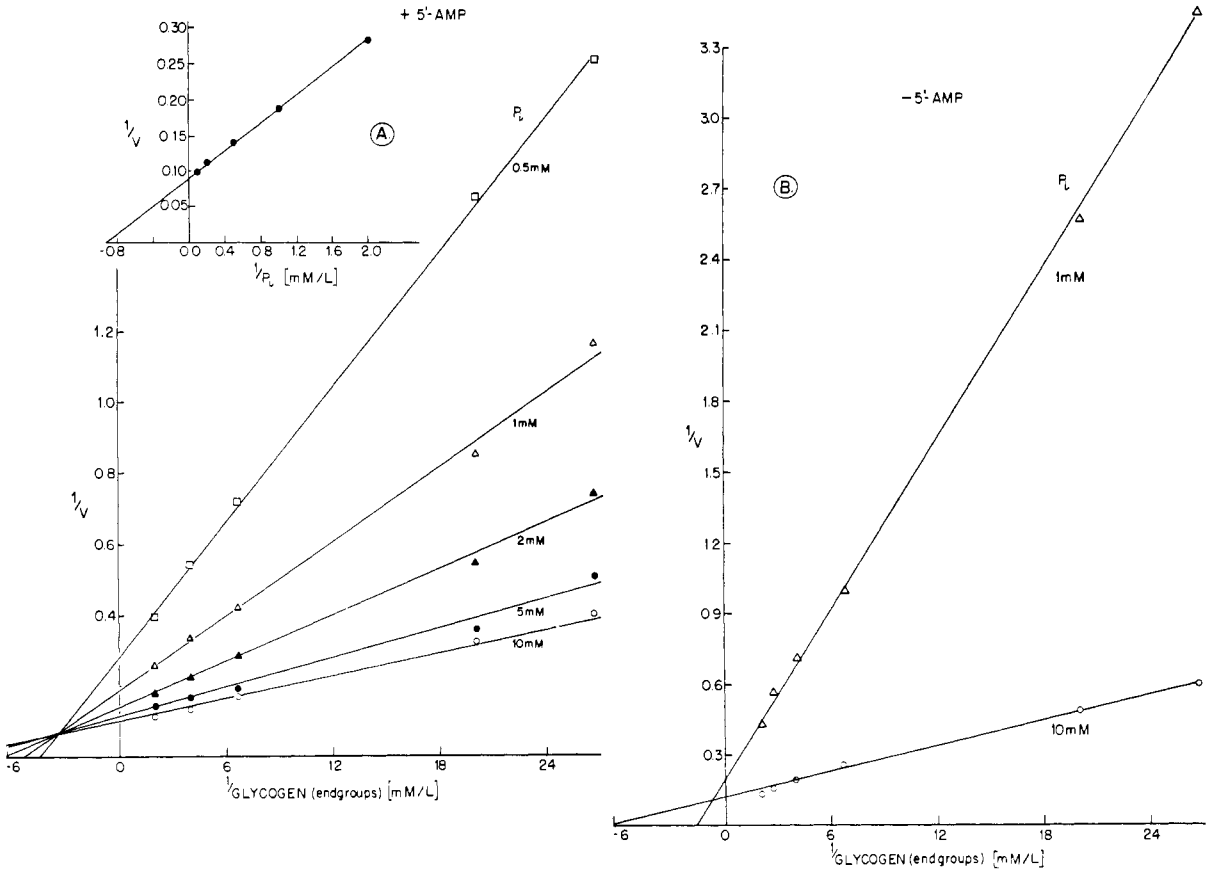

FIGURE 6: Dependence of initial velocity of frog muscle phosphorylase *a* on the concentrations of glycogen and P_i. Experiments were carried out using a coupled assay with phosphoglucomutase and glucose-6-P dehydrogenase as described by Helmreich and Cori (1964a). "*V*" (with the exception of the inserts) in this and subsequent graphs represents the initial velocity of the reaction expressed as micromoles of product formed per minute per milligram of enzyme at 25°. (A) The concentration of 5'-AMP was 1 mM. The concentrations of glycogen are expressed in terms of end groups at the nonreducing end of the chains. (1% glycogen corresponds to 5×10^{-3} M end groups). In the insert are plotted the values from the intercepts of the lines on the ordinate ($1/V_{max}$ *vs.* the reciprocal concentrations of P_i. (B) Data of experiments in the absence of 5'-AMP.

(Lee, 1960) are quite similar. Because of the ease with which frog muscle phosphorylase *a* dissociates to its dimeric form, no sedimentation equilibrium measurements were carried out with the phosphorylated form (*a*) of the frog enzyme.

Amino Acid Composition. Table II illustrates the results of preliminary amino acid analysis of frog skeletal muscle phosphorylase *a*. For comparison, data are also given in Table II on the amino acid composition of crystalline rabbit and human skeletal muscle phosphorylase *b* reported by Appleman *et al.* (1963). Although there are differences in the amino acid composition of the frog and the mammalian muscle phosphorylases, mainly with respect to the arginine, serine, and proline residues taken as a whole, the over-all amino acid composition is quite similar. Not withstanding the similarity in amino acid composition the immunologic cross-reactivity between the mammalian and the amphibian skeletal muscle enzymes was incomplete. Since it is likely that conformation recognition by an antibody plays a major role in determining the specific fit between antibody and the antigenic determinants of protein antigens (Sela, 1967), these findings suggest differences in the conformations of mammalian and amphibian muscle phosphorylases. The differences in the glycogen and temperature dependent dissociation reactions of the frog muscle and the rabbit muscle enzyme indicate differences in the subunit interactions of these two enzymes which might also be conformational in nature. These differences are described below.

Extinction Coefficient. The absorbing index, $A_{280\,m\mu}$ (1% × cm^{-1}), of frog skeletal muscle phosphorylase is 12.8. This value is based on repeated nitrogen determinations of the most highly purified preparations of frog skeletal muscle phosphorylase *a*. The enzyme was free of 5'-AMP and exhaustively dialyzed as described in the Methods section on amino acid analysis. The nitrogen content of frog muscle phosphorylase was 16.23% (see Table II). A similar value (*A* 13.5) which is higher than the values reported by Velick and Wicks (1951) and Appleman *et al.* (1963) (11.7 and 11.9, respectively) was recently reported by Buc and Buc (1967) for crystalline rabbit skeletal muscle phosphorylase *b*. This was confirmed recently by L. Kastenschmidt who found *A* 13.2 for rabbit skeletal muscle phosphorylase *b* (based on a nitrogen content of 16.9% (*cf.* Appleman *et al.*, 1963)) for the rabbit

muscle enzyme). Details are given in a forthcoming publication (L. L. Kastenschmidt, J. Kastenschmidt, and E. Helmreich, in preparation).

Total Phosphate and Pyridoxal 5-Phosphate Content. Using the extinction coefficient of 12.8 ($1\% \times cm^{-1}$) for calculating the concentration of protein, it was found in three separate determinations that frog skeletal muscle phosphorylase *b* (3.5 mg) contained 0.0408, 0.0413, and 0.0403 μmole of pyridoxal-5-P. This is equivalent to a content of 1 mole of pyridoxal-5-P/86,000 g of protein or of 2.18 moles/188,000 g of protein. Analyses of the total phosphate content of frog skeletal muscle phosphorylase *a* yielded 0.0208, 0.0196, and 0.0215 μmole of orthophosphate/mg of protein. Thus 1 mole (376,000 g) of frog skeletal muscle phosphorylase *a* contained 7.86 moles of P_i, about 4 of which can be accounted for as pyridoxal-5-P. The remaining phosphates are assumed to be serine phosphate groups in analogy with rabbit skeletal muscle phosphorylase *a* (*cf.* Brown and Cori, 1961).

Kinetic Properties. Several kinetic parameters of frog muscle phosphorylase *a* have been determined. The dependence of the initial velocity of the enzyme on the concentrations of glycogen and P_i in the presence and absence of 5′-AMP at 25° is shown in Figure 6A,B. Similar data for glycogen and arsenate are shown in Figure 7A,B and for glycogen and glucose-1-P in Figure 8A–D. The kinetic constants derived from the initial rate measurements are given in Table III. The concentrations of substrates, P_i and glucose-1-P, required for half-maximal activity of the frog muscle phosphorylase *a* are somewhat higher than those for rabbit muscle phosphorylase *a* (Helmreich *et al.*, 1967). This difference is greatest (about tenfold) in the case of glycogen. The response of frog muscle phosphorylase *a* and the rabbit muscle enzyme to the allosteric modifier 5′-AMP is similar. With both the rabbit and the frog muscle enzyme 5′-AMP decreases the K_m values for substrates with little or no change in V_{max} (*cf.* Figure 8C). At saturating concentrations of glycogen and inorganic P or glucose-1-P the ratio of activities of −5′-AMP/+5′-AMP is about 83%. However, the response of frog muscle phosphorylase *a* to adenylic acid is strongly modified by substrates. Similar observations were recently reported by Lowry *et al.* (1967) with mammalian muscle and brain phosphorylases under somewhat different assay conditions with imidazolium as buffer cation. The data in Table III indicate that the modifier, 5′-AMP, and any one of the substrates, glycogen, P_i, or glucose-1-P, act in a similar manner. They tighten the binding of whichever ligand is present at nonsaturating concentrations. Because both the effects of substrates and of 5′-AMP are indirect, either substrate or 5′-AMP can act as a positive allosteric modifier. For example, increasing the concentration of P_i or glucose-1-P results in a decrease in the K_m values for glycogen and conversely increasing the concentration of glycogen lowers the K_m values for P_i or glucose-1-P for frog muscle phosphorylase *a*. It appears that substrate or modifier can be substituted for each other at least to some extent, since at low concentrations of one substrate the effect of 5′-AMP on the

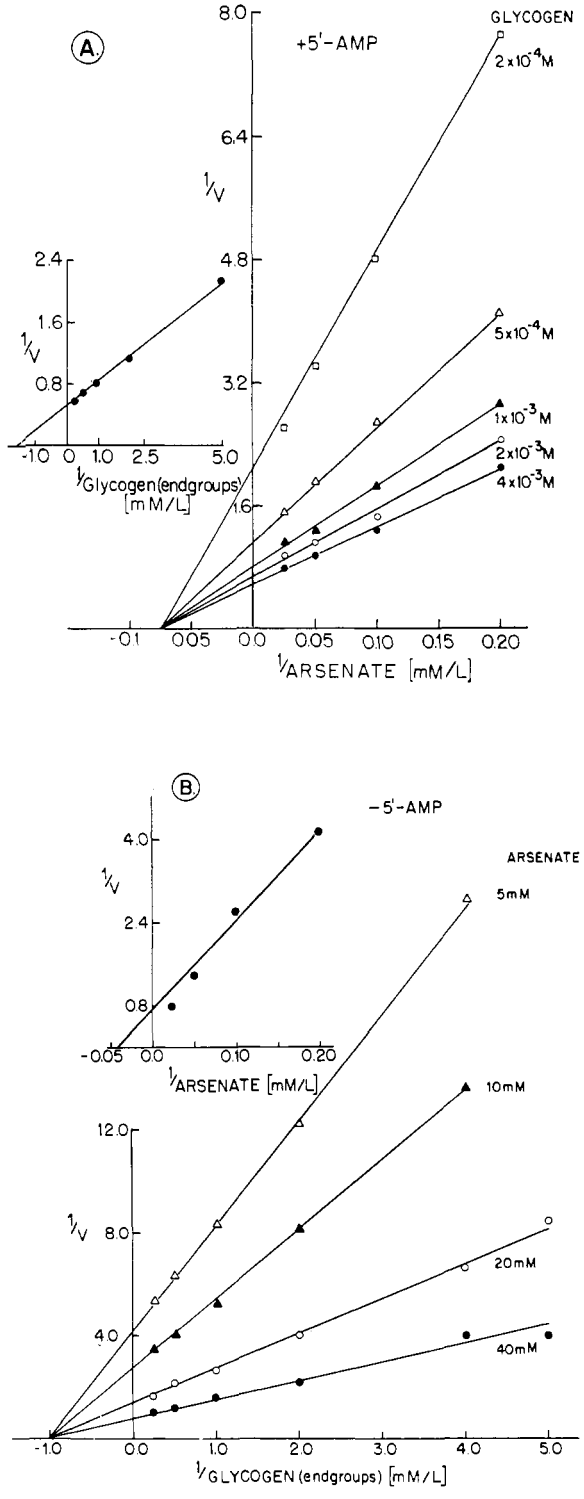

FIGURE 7: Dependence of initial velocity of frog muscle phosphorylase *a* on the concentrations of glycogen and arsenate. The assay conditions are described in Methods. The concentration of enzyme was 45 μg/ml. (A) The concentration of 5′-AMP was 1 mM. In the insert are plotted the values from the intercepts of the lines on the ordinate ($1/V_{max}$) *vs.* the reciprocal concentrations of glycogen. The concentrations of glycogen are expressed in terms of end groups at the nonreducing end of the chains. (B) Data of experiments in the absence of 5′-AMP. In the insert are plotted the values from the intercepts of the lines on the ordinate ($1/V_{max}$) *vs.* the reciprocal concentrations of arsenate.

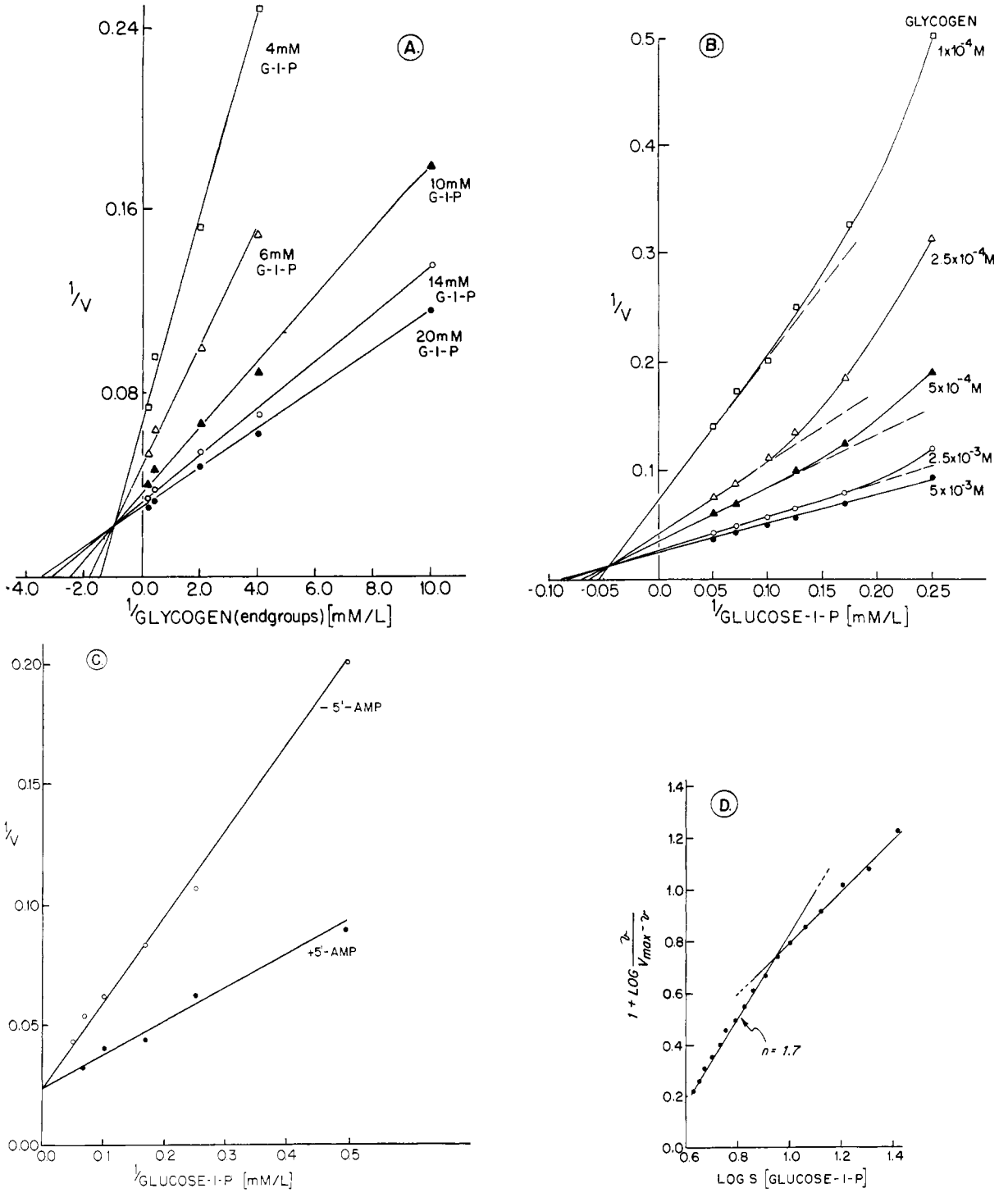

FIGURE 8: Dependence of initial velocity of frog muscle phosphorylase *a* on the concentrations of glycogen and glucose-1-P. The assay conditions are described in Methods. The concentrations of enzyme ranged from 10 to 30 μg/ml. Initial velocities were calculated from the linear part of the rate curves. (A) The effect of glucose-1-P on the K_m values of glycogen in the absence of 5′-AMP. (B) The effect of glycogen on the K_m values of glucose-1-P in the absence of 5′-AMP. (C) The effect of 5′-AMP (1 mM) on the K_m values of glucose-1-P. The concentration of glycogen was 1% (5×10^{-3} M end groups). (D) Hill plot. The values were taken from the curve for experiments with 2.5×10^{-4} M glycogen (end groups) in B and were replotted as shown. The best-fit straight line was drawn according to the method of least squares.

affinity of the frog muscle enzyme for the other substrate is much stronger than at high concentrations. Moreover, the K_m values for a substrate at a low but constant concentration of the other substrate approach the same apparently limiting value in the presence of 5′-AMP that is reached in the absence of 5′-AMP at saturating levels of the other substrate.

The kinetic behavior is readily interpreted on the basis of the two-state model of Monod *et al.* (1965) if one assumes that all substrates and 5′-AMP bind

TABLE III: Kinetic Constants of Frog Muscle Phosphorylase *a* at 25°.[a]

Variable Substrate	Constant Substrate (mM)	Apparent "K_m" Values for Variable Substrates (mM)		V_{max} (μmoles mg^{-1} min^{-1})[b]		Hill's Interaction Coefficient
		−5′-AMP	+5′-AMP[c]	−5′-AMP	+5′-AMP[c]	
Glycogen	Inorganic P (infinite concentration)	0.15	0.11	8.3	11	
Glycogen	Inorganic P (0.5)		0.22			
Glycogen	Inorganic P (1.0)	0.67				
Glycogen	Arsenate ($\infty \rightarrow 5.0$)	1.00	0.60	1.4	1.8	
Glycogen	Glucose-1-P (20)	0.33		33.0		
Glycogen	Glucose-1-P (4)	0.80				
Inorganic P	Glycogen (infinite concentration)	4.00[d]	1.06			
Inorganic P	Glycogen (0.038)	>10.00[d]	4.00[d]			
Arsenate	Glycogen ($\infty \rightarrow 0.2$)	22.00	13.00			
Glucose-1-P	Glycogen (5.0)	11.00	5.40		40.0	
Glucose-1-P	Glycogen (0.1)	>20.00				1.5[e]

[a] The assay conditions are described in Methods and in the legends to Figures 6–8. The temperature was 25°. The concentration of glycogen is expressed in terms of glucose residues at the nonreducing end of the chains. [b] V_{max} is the velocity at saturating levels of both substrates. [c] 1×10^{-3} M. [d] Separate experiments not shown in Figure 7A,B. The saturation curves for P_i at low glycogen concentrations and in the absence of 5′-AMP were sigmoidal. [e] A Hill plot for glucose-1-P at 2.5×10^{-4} M glycogen (end groups) in the absence of 5′-AMP is shown in Figure 8D.

preferentially to the R or active state (Helmreich *et al.*, 1967). In this case, high concentrations of substrates alone or low concentrations of substrates combined with a saturating amount of 5′-AMP should be equally effective in shifting the T $\rightleftharpoons$ R equilibrium toward the R state. Quantitative differences which exist between 5′-AMP and substrates and among the substrates themselves might then be thought to reflect differences in the binding ratios of the various ligands to the T (inactive) and R (active) state.

In keeping with this proposal is the observation that at low concentrations of glycogen, the saturation curves for the anionic substrates glucose-1-P (Figure 8B) and P_i (Table III) become distinctly sigmoidal or concave upwards in double-reciprocal plots. The interaction coefficients "*n*" were calculated for the experiments with glucose-1-P (Figure 8B) from Hill plots (Brown and Hill, 1922). A typical Hill plot is shown in Figure 8D. As expected from theory the Hill equation cannot apply at the extreme ends of the curve where *n* must approach unity (Wyman, 1964). For the steep part of the curve, where interaction is maximal, $n = 1.7$ for glucose-1-P in the range from 4 to 9 mM concentrations. The substrate site–site interactions observed with frog muscle phosphorylase *a* are reciprocal since increasing the concentrations of either one of the anionic substrates also lowers the apparent K_m values for glycogen (Figures 6A,B and 8A). However, since a falling off of the rate at low glycogen concentrations may have other than allosteric reasons, rate measurements at very low glycogen concentrations must be interpreted with caution.

The results of kinetic experiments are summarized in Table III. The K_m value for arsenate and frog muscle phosphorylase *a* is very much larger than the K_m value for phosphate and rabbit muscle phosphorylase *a* (*cf.* Helmreich and Cori, 1964a,b).[4] The K_m value for glycogen and frog muscle phosphorylase *a* is independent of the concentrations of arsenate both in 25 mM sodium glycerol-P buffer (Figure 7A,B) at pH 6.8 and (not shown here) in 45 mM Tris-acetate buffer containing 10 mM $MgCl_2$ at pH 7.4.[5] We have recently suggested on the basis of other evidence that for rabbit skeletal muscle phosphorylase *a* the order of addition of substrates is compulsory with glycogen as the first substrate, but so far this has not been proved (*cf.* Michaelides and Helmreich, 1966; Helmreich *et al.*, 1967). Although a compulsory order of addition of substrates is a possibility, the kinetic data with frog muscle phosphorylase *a* presented here do not give

[4] The maximal velocities of frog and rabbit muscle phosphorylase *a* measured by arsenolysis are considerably smaller (five to six times) (see Table III) than the maximal velocities measured by phosphorolysis (*cf.* also Helmreich and Cori, 1964b; Metzger *et al.*, 1967).

[5] In the case of rabbit muscle phosphorylase *b* it was shown that arsenate and P_i compete for the same site (Helmreich and Cori, 1964a). Therefore if one assumes that arsenate and P_i also bind to the same site on frog muscle phosphorylase *a*, the lack of interactions between arsenate (in contrast to P_i) and glycogen is puzzling. It is not readily explained by the argument developed above which is based on the two-state model of Monod *et al.* (1965). Some of the unusual and as yet unexplained features of the P_i binding site of rabbit muscle phosphorylase *a* have recently been discussed (Helmreich *et al.*, 1967).

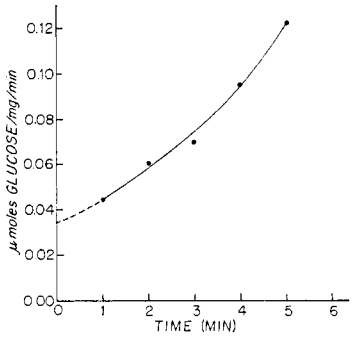

FIGURE 9: Arsenolysis of glycogen by high concentrations of enzyme. The concentration of frog muscle phosphorylase *a* was 3.1 mg/ml. Incubations were carried out at 10° with glycogen and arsenate under the conditions described in Methods. Aliquots were then removed at the times indicated and the amount of glucose formed during the period of incubation was determined as described.

direct evidence for or against this possibility. If one assumes however that the order of addition of substrates is compulsory with glycogen as the first substrate then the K_m value for glycogen in the presence of arsenate should represent the enzyme–substrate dissociation constant, as does the value derived from the intersection point of the lines in reciprocal plots at various levels of glucose-1-P or P_i (Frieden, 1957). It may be noted that the values agree reasonably well. They are 1.4 mM for experiments with P_i and glucose-1-P and 1.0 mM for experiments with arsenate in the absence of 5′-AMP (*cf.* Figures 6B, 8A, and 7B). For experiments with P_i and arsenate in the presence of 5′-AMP the values are 0.3 and 0.6 mM, respectively (*cf.* Figures 6A and 7A).

The kinetic and allosteric properties of mammalian phosphorylases are very sensitive to a variety of factors, among others, aging of the enzyme, changes in ionic strength, pH, and temperature and the nature of the buffer ion (glycerol-P *vs.* imidazolium ions) (Helmreich *et al.*, 1967; Lowry *et al.*, 1967). The influence of some of these factors might have to be taken into account in kinetic experiments with frog muscle phosphorylase *a*. A more detailed study of the kinetic and allosteric

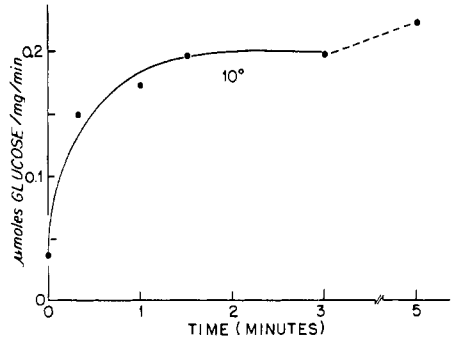

FIGURE 10: Rate of activation of frog muscle phosphorylase *a* by glycogen. Enzyme (3.5 mg/ml) was preincubated with 1.25% glycogen at 10° for the periods of time indicated (see Methods). Activity was then assayed for 30 sec by arsenolysis as described in Methods.

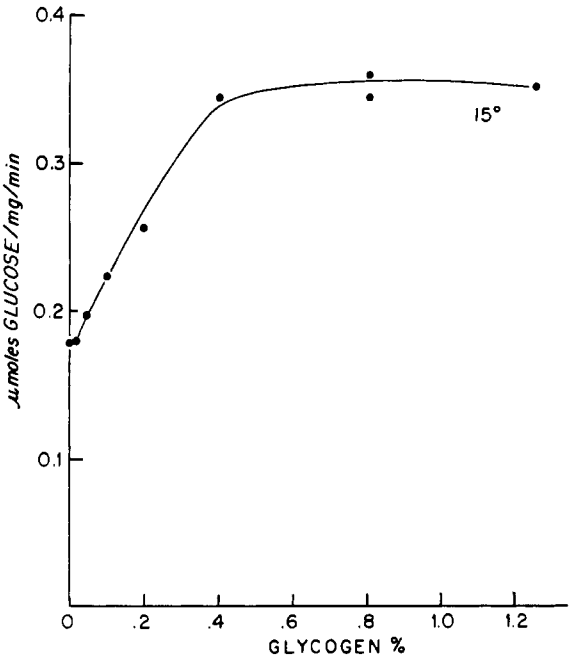

FIGURE 11: Extent of activation of frog muscle phosphorylase *a* as a function of glycogen concentration. Enzyme (3.4 mg/ml) was preincubated for 35 min at 15° with glycogen at the concentrations indicated (see Methods). Activity was assayed for 30 sec by arsenolysis as described in Methods.

properties of frog muscle phosphorylase should be of considerable interest.

The Effect of Enzyme Association on Activity. As with rabbit skeletal muscle phosphorylase *a* (Wang *et al.*, 1965; Metzger *et al.*, 1967) the specific activity of the frog enzyme decreases at high protein concentrations, but increases following preincubation with glycogen. With rabbit muscle phosphorylase *a*, the activity at high protein concentration could be measured by arsenolysis in 200 mM arsenate with very little further activation during assay (Metzger *et al.*, 1967). The reason is that high ionic strength decreases the rate of interconversion of rabbit muscle phosphorylase tetramer *a* $\rightleftharpoons$ dimer *a*. With frog muscle phosphorylase *a*, however, dissociation of the tetramer *a* to the dimeric form proceeds fairly rapidly upon dilution even at high ionic strength. Arsenolysis was therefore carried out by adding the reagents in concentrated solutions in order to minimize dilution during the assay (see Methods); and the assay periods were shortened to 30 sec. Activation of frog muscle phosphorylase *a* during assay is shown in Figure 9. It can be seen (Figure 9) that even with these precautions some activation occurred during the assay period.

Figure 10 illustrates the rate of activation of frog muscle phosphorylase *a* during preincubation with 1.25% glycogen (6.45×10^{-3} M end groups) at 10°. The rate of glycogen activation of frog muscle phosphorylase *a* is very much faster than that of the rabbit muscle enzyme. In fact, it was too fast to be measured accurately by this technique at temperatures above 15°. Figure 11 shows the dependence of the final extent of activation (at equilibrium) on glycogen concentra-

tion at 15°. Since it was shown that only the dimeric form of rabbit muscle phosphorylase *a* can bind significantly to glycogen (Metzger *et al.*, 1967), it was assumed that this also applies to frog muscle phosphorylase *a*. Taking this into consideration and assuming then that the specific activity of the enzyme prior to incubation with glycogen represents the amount of dimer *a* present, one can calculate from the data in Figure 11 an apparent dissociation constant for the dimer *a*–glycogen complex. This value is 1.1×10^{-3} M in terms of end groups. The K_m value for glycogen at 15° determined by arsenolysis was 0.79×10^{-3} M. It was independent of the concentration of arsenate, and, therefore, it most likely represents the enzyme–glycogen dissociation constant (*cf.* Frieden, 1957). The two values agree reasonably well.

Table IV presents data on the activity of frog muscle phosphorylase *a* before and after maximal activation by glycogen in the temperature range from 5 to 35°. In Figure 12 are shown the concentrations of frog muscle phosphorylase dimer *a* calculated from light-scattering experiments at different temperatures. These values are compared with the amount of frog muscle phosphorylase dimer *a* predicted from the data on activity in Table IV. It was assumed that the activity observed before preincubation with glycogen is that of dimer *a*. The agreement between the two sets of data is fairly good, although it cannot be excluded on the basis of these observations alone, that the tetrameric form (*i.e.*, tetramer (R)) of frog muscle phosphorylase *a* has some activity with glycogen as substrate. The small differences between the values for the concentration of dimer *a* calculated from light-scattering experiments and the values for the predicted activity on the basis of the values in Table IV probably result from some activation during assay (*cf.* Figure 9). It would have been desirable to correlate the activity of the frog muscle enzyme with its ability to bind to glycogen. Binding experiments were done previously with rabbit muscle phosphorylase *a* (Metzger *et al.*, 1967). They indicated that the tetramer *a* cannot bind to glycogen. However, the rapid rate of activation of the frog muscle enzyme by glycogen precluded binding studies by the centrifugal separation technique (Madsen and Cori, 1958).

TABLE IV: Activity of Frog Muscle Phosphorylase *a* Following Preincubation with and without Glycogen.[a]

Temp (°C)	No Addn a	Preincubated with 1.25% Glycogen b	a/b
5	0.018	0.085	0.214
10	0.078	0.213	0.366
15	0.188	0.392	0.480
20	0.414	0.656	0.631
25	0.944[b]	1.20[b]	0.787
30	1.62[b]	1.81[b]	0.896
35	2.56[b]	2.72[b]	0.942

[a] The concentration of enzyme was 3.5 mg/ml. The data are given as micromoles of glucose per minute per milligram of enzyme formed during the arsenolysis of glycogen under the conditions described in Methods. The concentration of arsenate was 200 mM. [b] These values are corrected for the change in the K_m values with increasing temperature (see text).

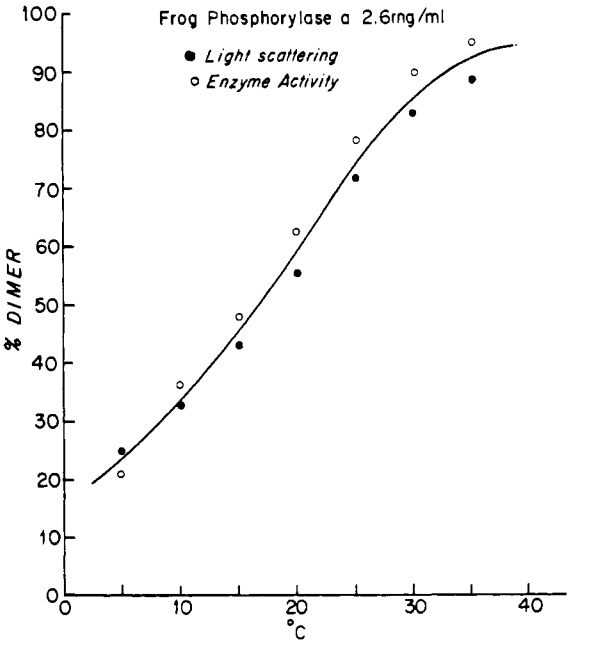

FIGURE 12: Correlation of frog muscle phosphorylase dimer *a* concentrations with activities. The concentrations of frog muscle phosphorylase dimer *a* were determined from light-scattering experiments at different temperatures (*cf.* Metzger *et al.*, 1967). It was assumed in the calculations that dimer *a* and tetramer *a* represent the only forms of frog muscle phosphorylase *a* present in solution. The concentration of protein in the light-scattering experiments was 2.6 mg/ml. The buffer system is described in Methods (Activation of Enzyme by Glycogen). The changes in molecular weight as a function of temperature observed by light scattering were completely reversible. The concentrations of dimer *a* which were expected on the basis of activity measurements at different temperatures were calculated from the data in Table IV, assuming that the only active form of the enzyme is dimer *a*, and that the equilibrium between dimer and tetramer is represented by the equilibrium constant $K_{eq} = (\text{dimer } a)^2/(\text{tetramer } a)$.

Effects of Temperature. Figure 13 illustrates the effect of temperature on V_{max} of rabbit muscle and frog muscle phosphorylase *a*. The data are plotted in the form of Arrhenius plots. V_{max} was measured by arsenolysis. The sharp break in the Arrhenius plot for the rabbit muscle enzyme is not seen with the frog muscle enzyme. The decrease in affinity of the frog muscle enzyme for arsenate with increasing temperature required separate determinations of the K_m values for arsenate at temperatures above 20°. In contrast to the behavior of the frog muscle enzyme, the rabbit muscle phosphorylase *a* remained saturated at 0.2 M arsenate

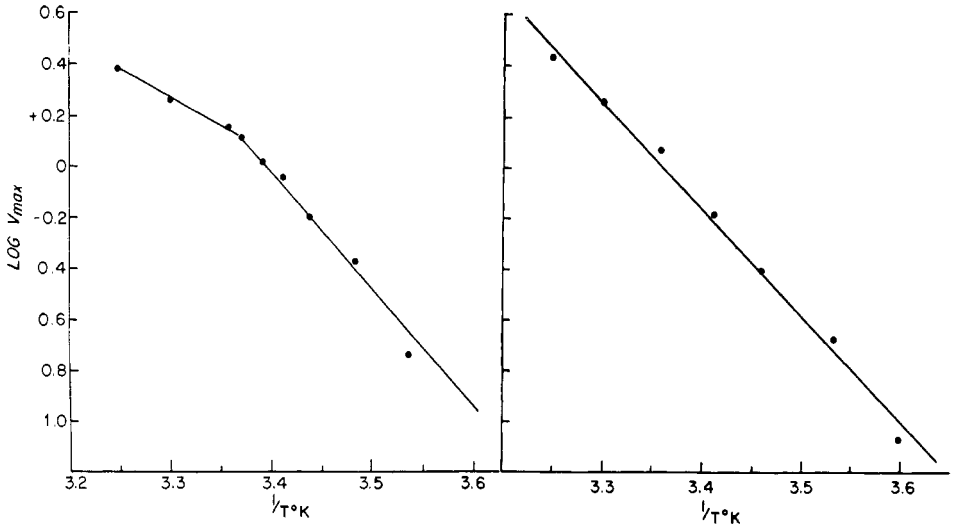

FIGURE 13: Arrhenius plots for rabbit and frog muscle phosphorylase *a*. In the panel on the right side the data for frog muscle phosphorylase *a* in column b of Table IV are plotted. In the panel on the left side the data for activity of rabbit muscle phosphorylase *a* after preincubation with 2% glycogen shown in Table I in the previous publication of Metzger *et al.* (1967) (in the absence of 5′-AMP) have been used. These data were replotted in the form of an Arrhenius plot.

concentrations at temperatures up to 50° (*cf.* Helmreich and Cori, 1964b). V_{max} values for frog muscle phosphorylase *a* corrected for the increase in K_m with temperature fell on a straight line (see Figure 13). In the experiments with rabbit muscle phosphorylase *a*, shown in Figure 13, the inflection point was at 23° and the value for $-\Delta H$ was 22 kcal/mole for the lower slope. In a previous smaller series of experiments with rabbit muscle phosphorylase *a* in the absence of 5′-AMP the inflection point was at 31° and the value for $-\Delta H$ was estimated to be about 30 kcal for the lower slope (Helmreich and Cori, 1964b). The use of different preparations of enzyme and the fact that in the newer experiments the enzyme (3 mg/ml) was fully activated by preincubation with glycogen (2%) could account for these differences.

Various explanations have been given for this type of temperature behavior of some enzymes (Kistiakowsky and Lumry, 1949; Levy *et al.*, 1959; Helmreich and Cori, 1964b; Graves *et al.*, 1965; Massey *et al.*, 1966). It is now generally agreed that the abrupt change in the slope of an Arrhenius plot at a critical transition temperature represents some kind of a conformational rearrangement. Since all the temperature experiments in the present study were done with enzymes fully activated by glycogen, the conformational transition observed in Figure 13 involves the two monomeric subunits of dimer *a*. It was observed recently (M. C. Michaelides and E. Helmreich, unpublished results) that in the case of rabbit muscle phosphorylase *a* a similar sharp break occurs at 23° when the true bimolecular association constant for 5′-AMP, determined from equilibrium dialysis binding measurements (see Helmreich *et al.*, 1967) is plotted against the reciprocal of the absolute temperature in a range from 5 to 38° (van't Hoff plots). The inflection point of the lines in these plots was the same whether glycogen was present or not. This makes it unlikely that the break in the Arrhenius plots with rabbit muscle phosphorylase *a* actually represents a change in the energy of activation of the catalytic system of one and the same conformational state of the enzyme. This discontinuity more likely is due to a transition from one active form (R) of the enzyme to another active form (R) with somewhat different kinetic and ligand binding properties. This aspect will be dealt with in more detail in a forthcoming publication (L. L. Kastenschmidt, J. Kastenschmidt, and E. Helmreich, in preparation). Whatever the molecular basis of this transition may be, the differences in the temperature behavior of the rabbit and the frog muscle phosphorylase *a* which are manifest in the Arrhenius plots deserve further consideration. They may point to differences in the thermodynamic parameters of subunit interactions between mammalian and amphibian phosphorylases.

Discussion

The frog skeletal muscle phosphorylase *a* described in this communication has certain features in common with the well studied mammalian skeletal muscle phosphorylase *a*, but differs in other aspects. Mammalian and amphibian phosphorylases have similar molecular weight, the same number of subunits and binding sites for specific ligands as judged from their pyridoxal-5-P and total P_i content (*cf.* Brown and Cori, 1961). The two enzymes also have a similar amino acid composition. They differ with respect to some of their antigenic determinants and with respect to certain kinetic properties. The most notable difference between frog and rabbit skeletal muscle phosphorylase *a*, however, is in their association–dissociation behavior. The activity of frog muscle phosphorylase *a* was studied at high concentrations approaching those existing in the living muscle (about 3 mg/ml of intracellular water, as calcu-

lated on the basis of the data in Table I). It was found that at high concentrations (3 mg/ml) the frog muscle enzyme is more dissociated than the rabbit muscle enzyme. Moreover, the rate of tetramer *a*–dimer *a* dissociation is very much faster in the case of the frog muscle enzyme. Thus, as shown in Figure 10, the frog muscle enzyme is maximally activated in 1.5 min at 10° while at 15° several hours are required for complete activation of the rabbit muscle enzyme (*cf.* Metzger *et al.*, 1967). Since the association–dissociation reaction is temperature dependent complete activation of frog muscle phosphorylase *a* at 10° would correspond to the formation of active dimer *b* to the extent of about 35% of total (see Figure 12).

We have suggested previously that the association–dissociation behavior of phosphorylase *a* may be important for the regulation of glycogen breakdown "*in vivo*" (Metzger *et al.*, 1967). This led to the prediction that poikilothermic animals should have a phosphorylase *a* which dissociates more readily since at low temperatures activation of the mammalian enzyme was too slow to allow it to function. This prediction is now borne out by the data presented in this paper. Certain properties of the frog muscle phosphorylase *a* make it uniquely suited to function at low temperatures. For example, at 10° both the extent and the rate of dissociation of the frog enzyme are comparable to that of the rabbit enzyme at 37°. However it should be emphasized again (Metzger *et al.*, 1967) that the actual rate and extent of dissociation are greatly dependent on a variety of other factors in addition to concentrations of enzyme and glycogen and temperature (for example, pH, ionic strength, accessibility of substrates, and cofactors to the enzyme, etc). It is, therefore, not possible to predict at present what the rate and the extent of dissociation of the enzyme may be in the living cell. There exist types of phosphorylase *a* which are dimers and do not associate to tetramers; for example, the mammalian heart muscle phosphorylase *a* Type I (Davis *et al.*, 1967) and the mammalian liver phosphophosphorylase (Sutherland and Wosilait, 1956). These phosphorylases differ, therefore, from frog and rabbit skeletal muscle phosphorylase *a*. It is tempting to speculate that there is a casual relationship between the ability of skeletal muscle to retain its glycogen and the ability of skeletal muscle phosphorylase *a* to readily reassociate to an inactive tetrameric form. In the case of skeletal muscle, it was suggested by Cori (1945) that the unresponsiveness of the phosphorylase system in a fatigued muscle may be part of a regulatory system that prevents complete exhaustion of skeletal muscle glycogen. Recent evidence indicates that changes in cellular glycogen concentration represent changes in the number of glycogen molecules rather than in the size of the molecules (Parodi, 1967). Therefore control over the rate of the phosphorylase reaction would depend on changes in the total concentration of tissue glycogen. If this is the case, then the very slow rate (which is measured in minutes) of tetramer *a*–dimer *a* interconversion gains special significance. This rate should be compared with the very much faster rates for the partial steps of the over-all catalytic reaction of an enzyme which are in the millisecond to microsecond range (Eigen and Hammes, 1963). Thus, control by a substrate over the slow interconversion between an active and an inactive form of an enzyme and control over the enzymatic rate by means of substrate saturation are different with respect to time. Furthermore, the former mechanism results, in essence, in changes in V_{max}, whereas in the latter case, effective control requires changes in K_m. The slowness of the interconversion reaction allows the enzyme to escape from its substrate long before the substrate is actually depleted. The pause in phosphorylase activity might then allow resynthesis of glycogen through the action of glycogen synthetase. This is exactly the type of mechanism which would be required to prevent exhaustion of tissue glycogen stores.

Whether the association–dissociation reaction of muscle phosphorylase *a* described in this and in previous reports (Wang *et al.*, 1965; Metzger *et al.*, 1967) is actually the "on" and "off" mechanism for glycogen breakdown (*cf.* Helmreich and Cori, 1965) is largely a matter of speculation at present. However it was demonstrated recently that reversible association of an enzyme (bovine liver glutamic dehydrogenase) can change its control behavior (*cf.* Frieden and Colman, 1967), and attention was directed in more general terms to the possible regulatory significance of fast and slow allosteric transitions of enzymes (Frieden, 1967). In a similar way, reversible association of phosphorylase which is responsive to levels of cellular glycogen, may serve to modulate the rate of glycogenolysis. Experiments are needed to determine whether this hypothesis has merit.

Considerable efforts have recently been made in several laboratories (Helmreich *et al.*, 1967; Lowry *et al.*, 1967; Madsen and Shechosky, 1967; Buc, 1967; Buc and Buc, 1967) to understand the allosteric properties of mammalian skeletal muscle phosphorylases *b* and *a*. Because of the differences between mammalian and amphibian skeletal muscle phosphorylase which have been reported and discussed in this paper, it might be profitable to include this enzyme in these studies and more specifically to try to hybridize amphibian and mammalian skeletal muscle phosphorylase subunits. These and other experiments might aid in the understanding of subunit interactions of muscle phosphorylase.

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