A Comparison of Phosphorylase Isozymes in the Rabbit*

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ABSTRACT: Three "muscle-type" isozymes of glycogen phosphorylase *b*, which are active in the presence of 5′-adenylic acid (AMP), have been identified in rabbit tissues. Homogeneous preparations of the heart isozyme with the greatest electrophoretic mobility, I^b, and the crystalline isozyme from skeletal muscle and heart with the slowest mobility, III^b, are compared by physical, catalytic, and immunological techniques. Both isozymes have 2 moles of pyridoxal 5′-phosphate/mole of protein. Both have essentially identical values for temperature stability, temperature–activity dependency, pH optimum, and K_m for glycogen and glucose 1-phosphate. They are inhibited by adenosine triphosphate and glucose 6-phosphate competitively with respect to the activation by AMP. In the presence of an imidazole–citrate–L-cysteine buffer, pyridoxal 5′-phosphate can be removed from crystalline III^b and from I^b; the half-life periods for I^b and III^b in this buffer are, respectively, 2.4 and 2.9 min. This same buffer destabilizes isozyme I^a but does not affect the *a* form of the crystalline muscle isozyme III^a. I^b has an affinity for AMP that is 5–8-fold greater than that for III^b between pH 6 and 8. The Hill plot slope for AMP activation of III^b varies from 1.5 to 1.9 at a pH between 6 and 8 and at a temperature between 18 and 37°. The Hill plot slope for AMP activation of I^b is 1.0 at and above pH 7, but rises to 1.92 as the pH drops to 6; the slope does not show a significant variation between 18 and 37°. Rooster antibody to crystalline III^b inhibits the enzymatic activity of all of the rabbit phosphorylase isozymes but is most effective against the homologous antigen. The antibody gives a positive precipitin reaction with each of these isozymes.

Yunis *et al.* (1962) reported that glycogen phosphorylase *b* (EC 2.4.1.1, α-1,4-glucan:orthophosphate glucosyltransferase) of rabbit hearts could be separated by DEAE-cellulose chromatography into three separate activity peaks. Davis *et al.* (1967) were able to separate these isozymes by disc gel electrophoresis followed by staining for activity. The three heart isozymes in their dephospho forms were designated according to decreasing mobility on disc gel electrophoresis as I^b, II^b, and III^b; the corresponding phosphophosphorylases migrated in the same order and were designated I^a, II^a, and III^a. Isozyme III^b crystallized readily in the presence of Mg^{2+} and AMP and was found to be identical with crystalline skeletal muscle phosphorylase. Isozyme I^b was prepared in a homogeneous form from rabbit hearts and partially characterized. Isozyme II^b was found to be a hybrid molecule consisting of one subunit from I^b and one subunit from III^b. Sutherland and coworkers (Rall *et al.*, 1956; Sutherland and Wosilait, 1956) and Appleman *et al.* (1966) isolated from the livers of dogs, rabbits, and pigs a type of phosphorylase[1] that is different from I^b, II^b, and III^b. Bueding *et al.* (1964) showed that there were significant immunological differences between skeletal muscle and smooth muscle phosphorylases. The present work was undertaken to obtain additional comparative data on the phosphorylase isozymes with immunochemical techniques and also by studying their kinetic behavior. It was hoped that such information might aid in explaining their evolutionary development and the physiological implications of their tissue distribution. A portion of this work has been described in a preliminary report (Schliselfeld and Krebs, 1967).

Materials and Methods

Materials. Trizma grade Tris, pyridoxal-P,[2] and BSA were purchased from Sigma Chemical Co. AMP was purchased from both P-L Biochemicals, Inc., and Sigma Chemical Co. All other nucleotides were purchased from the former. Phosphoglucomutase from rabbit muscle was donated by Dr. Victor Najjar. Glucose-6-P and glucose-6-P dehydrogenase were purchased from Calbiochem. Shellfish glycogen from Krishell Laboratories, Inc., was purified according to the procedure of Somogyi (1957) to remove contaminating nucleotides. Freund's complete adjuvant was obtained from Difco Laboratories. Ionagar No. 2 was supplied by Consolidated Laboratories, Inc., Chicago Heights, Ill.

Phosphorylase Isozymes Preparations. Rabbit heart isozymes I^b, I^a, II^a, and III^a were prepared as described previously (Davis *et al.*, 1967). Rabbit heart III^b was prepared from the first phosphorylase *b* peak of the DEAE-cellulose chromatography of the *b* isozyme mixture. The peak was

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[1] Liver dephosphophosphorylase, though not activated by AMP in the absence of concentrated sulfate solution, is here considered as a *b* form and symbolized by LP^b; the liver phosphophosphorylase is considered as an *a* form and symbolized by LP^a.

[2] Abbreviations used are those listed in *Biochemistry 5*, 1446 (1966), and in addition: pyridoxal-P, pyridoxal 5′-phosphate; glucose-1-P, α-D-glucose 1-phosphate; glucose-6-P, glucose 6-phosphate; glycerol-P, β-glycerol phosphate; BSA, bovine serum albumin.

concentrated to at least 500 units/ml and then crystallized according to the procedure of Yunis *et al.* (1962). The enzyme was recrystallized once and stored at 0°. Rabbit LP^b and LP^a, purified as described by Appleman *et al.* (1966), were donated by Dr. D. P. Wolf. Skeletal muscle III^b was prepared according to Fischer and Krebs (1958) with modifications of Krebs *et al.* (1964). Skeletal muscle III^a was prepared from crystalline III^b with purified phosphorylase kinase and ATP (Krebs *et al.*, 1958).

Antibody Preparation. Roosters were immunized by several subcutaneous injections of 1 ml of an emulsion of 1 volume of fifth crystals of skeletal muscle III^b, 38 mg/ml, in 9 volumes of Freund's complete adjuvant. The serum was collected at or near the peak of the antibody response, and the antibody was precipitated by the addition of an equal volume of neutral saturated ammonium sulfate solution. The precipitate was dissolved in water and dialyzed against 0.85% NaCl. A nonimmune globulin fraction was prepared from the serum of nonimmune roosters exactly as described for the preparation of the antibody.

For part of the activity inhibition studies, the antibody was further purified by the procedure described by Yunis *et al.* (1962) for goat antiphosphorylase antibody. In a typical purification procedure, 2530 mg of $(NH_4)_2SO_4$-fractionated antibody protein (1 mg inhibited 0.328 unit of III^b) was dialyzed against 1.50 M NaCl and 0.001 M EDTA (pH 7) and mixed with 8.9 mg of skeletal muscle III^b. The mixture was incubated at 30° for 100 min and at 3° for 48 hr. The resulting precipitate was collected by centrifugation and washed once with cold 0.85% NaCl. The precipitate was dissolved at room temperature in 15 ml of 0.05 M glycine sulfate and 0.85% NaCl buffer at pH 2.4. After 15 min the mixture was neutralized with NaOH and cooled in an ice bath. A heavy precipitate of denatured phosphorylase formed and was removed by centrifugation. The supernatant contained 16.8 mg of antibody protein (1 mg inhibited 11.0 units of III^b).

Phosphorylase Activity Methods. Phosphorylase activity in the direction of glycogen synthesis was determined at 30° by the procedure of Hedrick and Fischer (1965) with 1% glycogen, 0.075 M glucose-1-P, and 1×10^{-3} M AMP (pH 6.5) except as these components were varied. When the activity in 0.075 M glucose-1-P was measured at varying values of pH, 0.10 M Tris was included to add buffering capacity to the 0.10 M maleate. When LP^b was assayed, 0.70 M Na_2SO_4 and 1×10^{-3} M AMP were included in the reaction mixture, except in the inhibition of LP^b by antibody where Na_2SO_4 concentration was reduced to 0.35 M. Activity in the direction of phosphorolysis at varying values of pH was carried out by a two-stage assay. The first reaction mixture at 20° with a final volume of 2.00 ml contained 0.04 M Tris, 0.04 M maleate, 0.018 M mercaptoethanol, 0.05 M P_i, 1×10^{-3} M AMP, 1% glycogen, and 0.04% BSA at varying values of pH. The reaction was stopped by placing the tubes in a boiling-water bath for 1 min. After cooling, 0.5-ml samples of each reaction and a zero-time sample were analyzed for glucose-1-P by the reduction of NADP catalyzed by phosphoglucomutase and glucose-6-P dehydrogenase (Larner *et al.*, 1960). One unit of enzyme is the amount that catalyzes the formation of 1.00 μmole/min of P_i in the direction of glycogen synthesis or glucose-1-P in the direction of the phosphorolysis of glycogen.

Inhibition of Phosphorylase Isozymes by Antibody. Enzyme–antibody reactions were carried out with a fixed amount of phosphorylase (0.2–0.5 unit) and increasing concentrations of antibody as in the procedure of Jókay and Tóth (1966) for measuring antibody potency. The tests were performed in 0.10 M maleate, 0.04 M mercaptoethanol, and 0.10% BSA buffer (pH 6.5) in a reaction mixture with a final volume of 0.2 ml. Incubations were carried out for 10 min at 30°. At the end of this period, 0.2 ml of substrate solution was added and phosphorylase activity was determined in the direction of glycogen synthesis. When necessary, reaction mixtures were deproteinized in 0.3 N perchloric acid before carrying out analyses for P_i. Controls were run with a nonimmune globulin fraction to correct for any nonspecific minor stabilization or activation effects. Units of phosphorylase activity remaining were plotted against antibody added, and the absolute value of the slope was used as a measure of antibody inhibition potency toward a given form of phosphorylase. Jókay and Tóth (1966) found the loss of phosphorylase units was proportional to the amount of antibody added up to 66% inhibition. In the present studies the amount of antibody added was proportional to the units of enzyme lost up to 80% inhibition.

Immunodiffusion. Immunodiffusion plates were prepared as described by Campbell *et al.* (1963) and contained 0.45% ionagar No. 2, 0.02 M Tris-maleate, 0.001 M EDTA, and 1.5 M NaCl (pH 7). The high concentration of salt was used in accordance with recommendations of Ouchterlony (1964) for using chicken antibody; Michaelides *et al.* (1964) have shown that in the presence of 1.5 M NaCl excess phosphorylase does not dissolve chicken antibody–phosphorylase precipitate. The plates were incubated at 3° for 2 weeks or more.

Quantitative Precipitin Test. Quantitative precipitin tests were carried out by the procedure described by Campbell *et al.* (1963). The supernatant solutions obtained after centrifuging down the antibody–phosphorylase precipitates were assayed for phosphorylase units remaining.

Pyridoxal-P Analysis. The pyridoxal-P of the isozymes was released in 0.30 M perchloric acid or 5% trichloroacetic acid at room temperature for 30 min. The supernatant solutions obtained after centrifuging down the denatured protein was assayed for pyriodoxal-P by the fluorometric procedure of Bonavita (1960).

Remaining Analytical Procedures. Glucose-6-P was determined by the glucose-6-P dehydrogenase catalyzed reduction of NADP (Larner *et al.*, 1960). The protein concentrations of III^b and III^a were determined from the absorbancy at 278 mμ, with an extinction coefficient of 1.19 ml mg^{-1} cm^{-1} (Appleman *et al.*, 1963). The antibody–phosphorylase precipitates in the quantitative precipitin tests were analyzed for protein by the procedure of Lowry *et al.* (1951) with BSA as the protein standard. All other protein analyses were carried out after a 5% trichloroacetic acid precipitation by a biuret method with BSA as the protein standard (Itzahaki and Gill, 1964).

Results

Spectrum of I^b. Figure 1 shows the spectrum of I^b. In addition to the typical absorbancy peak at 278 mμ there is a peak at 328 mμ. A similar spectrum with two absorbance peaks has been reported for skeletal muscle III^b (Kent *et al.*, 1958). The average extinction coefficient for I^b and I^a at

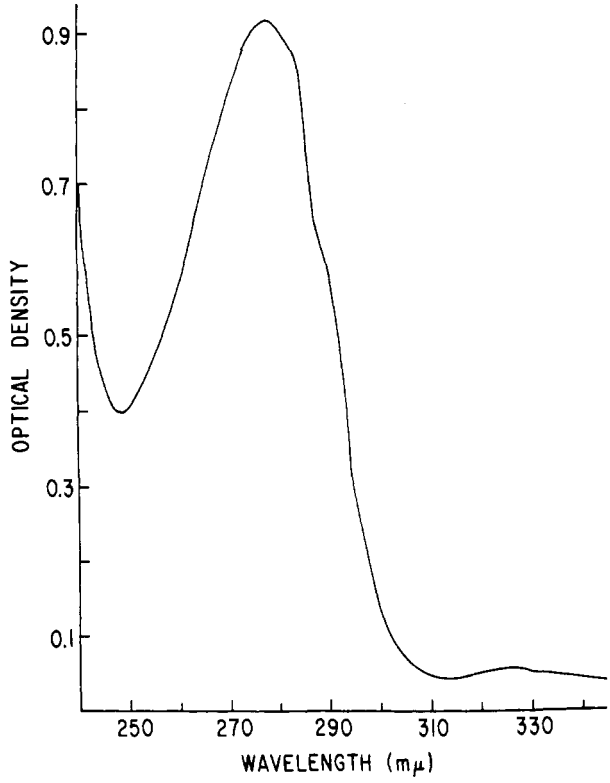

FIGURE 1: Absorption spectrum of I^b (0.625 mg/ml) in 0.005 M glycerol-P, 0.0005 M EDTA, and 0.0075 M mercaptoethanol (pH 6.8) was recorded against the above buffer utilizing the Cary 15 recording spectrophotometer.

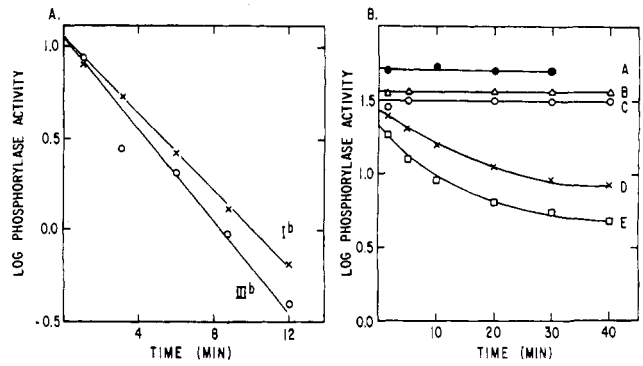

FIGURE 2: Action of imidazole–citrate–L-cysteine buffer on I^b, III^b, I^a, and III^a. Panel A shows the results of incubating 10.7 units of I^b (×——×) and skeletal muscle III^b (○——○) in 1.00 ml of 0.40 M imidazole and 0.10 M L-cysteine neutralized at 0° with citric acid to pH 6.5. The solutions were incubated at 0° and 0.10-ml samples were diluted to 2.50 ml with maleate dilution buffer (0.10 M maleate, 0.04 M mercaptoethanol, and 0.1% BSA at pH 6.5). The dilutions then were assayed for units of active enzyme remaining in the presence of 1×10^{-3} M AMP. The half-life periods are 2.4 min for I^b and 2.9 min for III^b. Panel B shows the results of incubating 46 units of I^a and 60 units of skeletal muscle III^a at 0° in 0.80 ml of the imidazole–citrate–L-cysteine buffer or in 0.80 ml of 0.40 M imidazole and 0.10 M mercaptoethanol neutralized with citric acid at 0° to pH 6.5. At intervals, 0.10-ml samples were diluted to 2.10 ml with maleate dilution buffer and assayed for units of active enzyme remaining in the presence and in the absence of 1×10^{-3} M AMP. Curve A: III^a incubated in the presence of L-cysteine and assayed with AMP. Curve B: I^a incubated in the presence of mercaptoethanol and assayed with AMP. Curve C: I^a incubated in the presence of mercaptoethanol and assayed without AMP. Curve D: I^a incubated in the presence of L-cysteine and assayed with AMP. Curve E: I^a incubated in the presence of L-cysteine and assayed without AMP.

278 mμ with the protein concentration determined by the biuret procedure is 1.48 ml mg^{-1} cm^{-1} (Itzahaki and Gill, 1964).

Pyridoxal-P and Its Removal from Isozymes I and III. The absorbancy peak for III^b at 330 mμ is due to protein-bound pyridoxal-P (Kent *et al.*, 1958). This suggests that the 328-mμ peak for I^b is due to the presence of the same coenzyme. Table I summarizes analyses for pyridoxal-P in III^b, I^b, and I^a, and it can be seen that each isozyme has 2 moles of pyridoxal-P/mole of protein.[3]

Shaltiel *et al.* (1966) have shown that pyridoxal-P can be removed from skeletal muscle III^b by incubation in 0.40 M imidazole and 0.10 M L-cysteine neutralized with citric acid at 0° to pH 6.5. Figure 2 (panel A) shows that this buffer causes a first-order loss of activity with essentially identical half-life periods for I^b and III^b, respectively, of 2.4 and 2.9 min. Figure 2 (panel B) shows that I^a also lost activity in this buffer when assayed in the presence or absence of AMP. When the 0.10 M L-cysteine is replaced by mercaptoethanol, the enzymatic activity is stable. Shaltiel *et al.* (1966) have shown that pyridoxal-P bound to III^a cannot be removed by this buffer so its activity is stable as is seen in Figure 2 (panel B). Therefore the *a* forms of these two isozymes can be distinguished by their stability in the presence of the pyridoxal-P-removing buffer.

Temperature Stability of I^b and III^b. Heart III^b, skeletal muscle III^b, and I^b have identical stabilities when incubated at varying temperatures for 5 min in 0.03 M glycerol-P, 0.001 M EDTA, and 0.015 M mercaptoethanol (pH 6.8) and then

TABLE I: Pyridoxal 5′-Phosphate Content of Phosphorylase Isozymes.

Isozyme Assayed	Pyridoxal-P	
	Moles/10^5 g of Protein	Moles/Mole of Protein
Skeletal muscle III^b	1.09	2.02[a]
Heart III^b	1.15	2.13[a]
I^b	0.98	1.98[b]
I^a	1.08	2.18[b]

[a] The molecular weight for skeletal muscle III^b and heart III^b is 185,000 (Seery *et al.*, 1967). [b] The molecular weight for I^b and I^a is 200,000 (Davis *et al.*, 1967).

[3] For this calculation the molecular weight for I^b and I^a of 200,000 determined by Davis *et al.* (1967) was used. This is 8.1% higher than the molecular weight for skeletal muscle III^b of 185,000 (Seery *et al.*, 1967). That I^b does have a slightly greater molecular weight than III^b is supported by the finding that the $s_{20,w}$ for I^b of 9.6 S is greater than the $s_{20,w}$ for III^b of 8.4 S. However, this small difference in molecular weight could be due to experimental error. Only further studies of the molecular weight of I^b can determine if this difference is real or not.

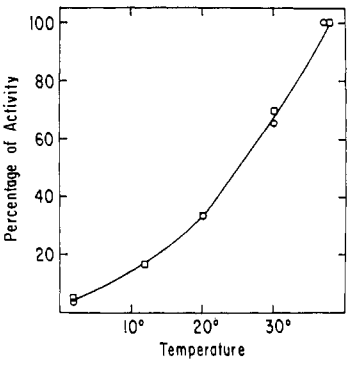

FIGURE 3: Effect of temperature on the enzymatic activity of I^b and III^b. (□——□) I^b and (○——○) skeletal muscle III^b. Activity at 37° is arbitrarily taken as 100%.

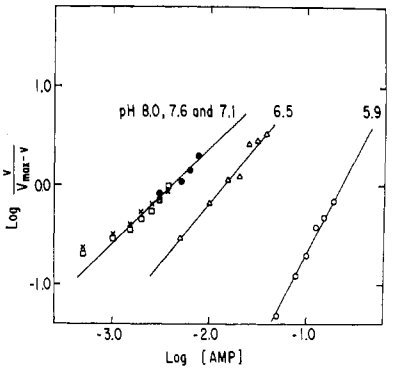

FIGURE 5: Effect of pH on the Hill plots of the activation of I^b by AMP. (□——□) pH 8.0; (×——×) pH 7.6; and (●——●) pH 7.1 with a slope of 1.00 and [AMP] at 0.5 V_{max} of 0.43 × 10^{-5} M; (△——△) pH 6.5 with a slope of 1.23 and [AMP] at 0.5 V_{max} of 1.4 × 10^{-5} M; (○——○) pH 5.9 with a slope of 1.92 and [AMP] at 0.5 V_{max} of 2.3 × 10^{-4} M.

assayed for the activity remaining. The isozymes are stable up to 51°, lose activity as the temperature rises above 51°, and are inactivated completely at 60°.

Effect of Temperature and pH on the Activities of I^b and III^b. Figure 3 shows that I^b and III^b have similar activity–temperature dependencies between 1.5 and 38°. Figure 4 shows that both isozymes have identical pH optima between pH 6.5 and 6.8, when assayed in the direction of glycogen synthesis. The only significant difference occurs at pH 6 where I^b has 65% of its pH 6.5 activity, whereas III^b has only 34% of its pH 6.5 activity. In the direction of phosphorolysis of glycogen catalyzed by I^b the pH optima is 6.8–6.9.

Activation of Isozymes I and III by AMP. Sealock and Graves (1967) have shown that there is a homotropic effect for the allosteric activation of skeletal muscle phosphorylase *b* by AMP. Figures 5 and 6, respectively, show Hill plots (log $v/V_{max}-v$ *vs.* log [AMP]) for the activation of I^b and III^b by AMP. The slope for I^b shows a marked pH dependency, going from 1.0 at or above pH 7 to 1.92 at pH 5.9. The slope for III^b varies slightly but randomly between 1.5 and 1.8 at the different pH values. Plots of the negative logarithm of the AMP concentration required for 50% activation *vs.* pH, as recommended by Dixon (1953) are seen in Figure 7. Both isozymes yield parallel plots of essentially two lines with a concave zone of intersection at pH 6.8–6.9. This suggests that either the protein or the AMP has an ionizable group essential for binding AMP with a pK'_a in this region. The nearest pK'_a for AMP is 6.1 (Pabst Laboratories, 1956), which is too low to be the probable group. The ionizable group may be on protein-bound glucose-1-P or the imidazole ring of a histidine residue. The concentration of AMP required for 50% activation of III^b is five to eight times that required for 50% activation of I^b at all values of pH studied. Thus at pH 6.5 the concentrations of AMP required for 50% activation are 9.2 × 10^{-5} M for III^b and 1.4 × 10^{-5} M for I^b.

Table II summarizes the effect of temperature on the Hill plots of the AMP activation of I^b and III^b. The slope for each isozyme does not vary significantly with temperature. The concentration of AMP required for 50% activation of III^b increases as the temperature rises from 18 to 38°. The concentration of AMP required for 50% activation of I^b is constant at 19 and 30° but at 38° it is 2.5 times larger. The paradox is that as the reaction temperatures rises, the maxi-

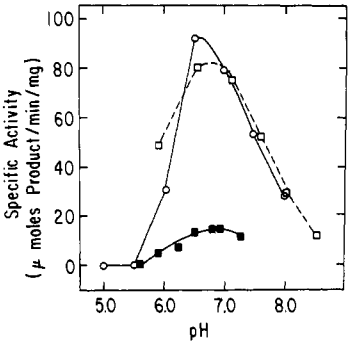

FIGURE 4: Effect of pH on the enzymatic activity of I^b and III^b (□——□) I^b assayed by the formation of P_i from glucose-1-P in the presence of 1 × 10^{-3} M AMP; (○——○) skeletal muscle III^b assayed by the formation of P_i from glucose-1-P in the presence of 1 × 10^{-3} M AMP; (■——■) I^b assayed by the formation of glucose-1-P from glycogen and P_i in the presence of 1 × 10^{-3} M AMP.

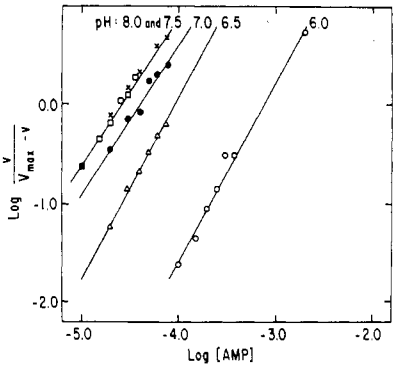

FIGURE 6: Effect of pH on the Hill plots of the activation of skeletal muscle III^b by AMP. (□——□) pH 8.0 and (×——×) pH 7.5 with a slope of 1.51 and [AMP] at 0.5 V_{max} of 2.7 × 10^{-5} M; (●——●) pH 7.0 with a slope of 1.52 and [AMP] at 0.5 V_{max} of 4.0 × 10^{-5} M; (△——△) pH 6.5 with a slope of 1.85 and [AMP] at 0.5 V_{max} of 8.9 × 10^{-5} M; (○——○) pH 6.0 with a slope of 1.82 and [AMP] at 0.5 V_{max} of 7.4 × 10^{-4} M.

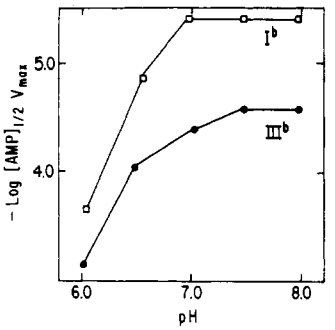

FIGURE 7: Effect of pH on the affinity of AMP for I^b and skeletal muscle III^b. (□——□) I^b and (●——●) III^b.

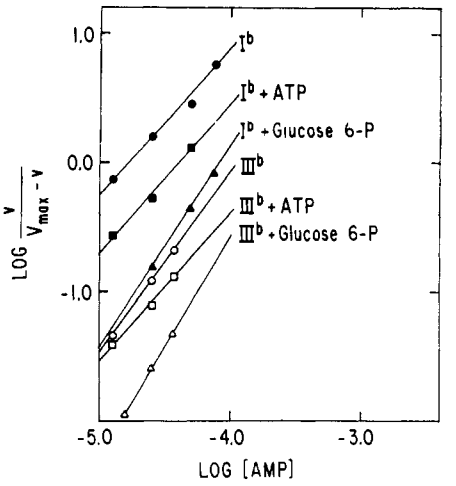

FIGURE 8: Competitive inhibition of the AMP activation of I^b and skeletal muscle III^b by ATP and glucose-6-P. (●——●) I^b, with a slope of 1.04 and [AMP] at 0.5 V_{max} of 1.7 × 10^{-5} M; (■——■) I^b in the presence of 7.6 × 10^{-3} M ATP, with a slope of 1.16 and [AMP] at 0.5 V_{max} of 4.0 × 10^{-5} M; (▲——▲) I^b in the presence of 6.4 × 10^{-4} M glucose-6-P, with a slope of 1.56 and [AMP] at 0.5 V_{max} of 8.3 × 10^{-5} M; (○——○) III^b, with a slope of 1.40 and [AMP] at 0.5 V_{max} of 1.1 × 10^{-4} M; (□——□) III^b in the presence of 6.8 × 10^{-3} M ATP, with a slope of 1.15 and [AMP] at 0.5 V_{max} of 2.1 × 10^{-4} M; (△——△) III^b in the presence of 6.4 × 10^{-4} M glucose-6-P, with a slope of 1.37 and [AMP] at 0.5 V_{max} of 2.2 × 10^{-4} M.

mum obtainable velocity rises, but affinity of the isozymes for AMP (especially with respect to III^b) decreases.

Isozymes I^a and III^a are partially active in the absence of AMP, but addition of AMP causes a stimulation in their activity. The AMP concentration required for 50% of maximum increase in activity is 6 × 10^{-6} M for III^a and 2.5 × 10^{-7} M for I^a.

Inhibition of Activities of I^b and III^b by ATP and Glucose-6-P. Morgan and Parmeggiani (1964) have shown that ATP and glucose-6-P are competitive inhibitors of the AMP activation of III^b. Figure 8 shows the effect of ATP and glucose-6-P on the Hill Plots for the AMP activation of I^b and III^b. The inhibitions are overcome for both isozymes by 3.75 × 10^{-3} M AMP. Both are much more sensitive to inhibition by glucose-6-P than to inhibition by ATP. Furthermore, at identical concentrations of inhibitor and AMP, I^b is much more active than III^b.

Madsen and Shechosky (1967) have shown that ATP is also a competitive inhibitor of III^b activity with respect to glucose-1-P. An attempt was made to detect such an inhibition for I^b with no success. At 6.7 × 10^{-3} M glucose-1-P (in the presence of 1% glycogen and 1 × 10^{-3} M AMP) I^b is inhibited no more than 6.8% by 2.38 × 10^{-3} M ATP. This suggests that I^b is not inhibited competitively with respect to glucose-1-P as has been established for III^b.

K_m Values of I^b and III^b for Glycogen and Glucose-1-P. The K_m values of I^b and III^b for glycogen and glucose-1-P were determined in the presence of saturating concentrations of the other constituents. No differences were observed for the two isozymes. The K_m for glycogen is 1 × 10^{-4} M in terms of nonreducing end groups; the K_m for glucose-1-P is 6 × 10^{-3} M.

Inhibition of Activities of the Isozymes by Antibody. Figure 9 illustrates the inhibition of the activities of isozymes I^b and III^b by antibody to skeletal muscle III^b. For both isozymes, the units lost are proportional to the amount of antibody added in the region plotted in this figure. The absolute value of the slope of the linear inhibition, the inhibition potency, is useful for comparing the interaction of the antibody with the isozymes (Jókay and Tóth, 1966). Table III summarizes the inhibition potencies of the antibody against the phosphorylase isozymes. In expt 1 and 2 the less pure ammonium sulfate fractionated antibody is used. The inhibition potencies with

TABLE II: Summary of the Temperature Dependency of Hill Plots for Isozyme I^b and III^b with Respect to Activation by AMP.

Isozyme	Assay Temp (°C)	[AMP] at 0.5 V_{max} (M)	Slope
I^b	18.8	2.5 × 10^{-5}	1.14
	30.0	2.1 × 10^{-5}	1.16
	38.0	5.1 × 10^{-5}	1.38
III^b	19.8	6.5 × 10^{-5}	1.64
	30.0	9.3 × 10^{-5}	1.86
	36.5	22.0 × 10^{-5}	1.50

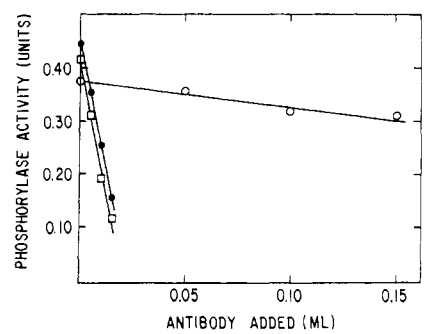

FIGURE 9: Inhibition of the activity of I^b and III^b by antibody. (□——□) Skeletal muscle III^b, 1.0 ml of antibody inhibits 19.4 ± 0.2 unit (mean value ± standard error); (●——●) heart III^b, 1.0 ml of antibody inhibits 20.3 ± 1.3 units; (○——○) I^b, 1.0 ml of antibody inhibits 0.483 ± 0.100 unit.

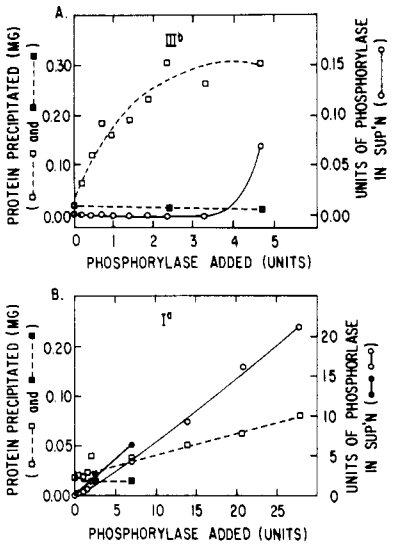

FIGURE 10: Quantitative precipitin tests on I^a and III^b. The following components were mixed: 0.30 ml of antibody (1.0 ml inhibited 26 units of III^b) or nonimmune globulin, increasing amounts of heart III^b (41 units per mg of protein) or I^a (63 units/mg of protein) in 0.05 M glycerol-P and 0.002 M EDTA (pH 7.0), 0.70 ml of 0.85% NaCl, and sufficient 0.05 M glycerol-P and 0.002 M EDTA (pH 7.0) to give a final volume of 2.0 ml. The resulting antibody–phosphorylase precipitates were assayed for protein by the procedure of Lowry *et al.* (1951). The supernatant solutions, after removing the precipitates, were assayed for the phosphorylase units remaining. Panel A shows the results with heart III^b: (□——□) protein precipitate formed with antibody; (■——■) protein precipitate formed with nonimmune globulin; and (○——○) phosphorylase units remaining with antibody. Panel B shows the results with I^a: (□——□) protein precipitate formed with antibody; (■——■) protein precipitate formed with nonimmune globulin; (○——○) phosphorylase units remaining with antibody; and (●——●) phosphorylase units remaining with nonimmune globulin.

heart III^b and III^a are identical with the inhibition potency of skeletal muscle III^b. With isozyme II^a it is 50% of the value for skeletal muscle III^b. This is the value expected if II^a consists of an equal number of subunits of I^a and III^a as previously shown (Davis *et al.*, 1967). In expt 3 of the table, the antibody employed has been purified by precipitation with III^b followed by acid denaturation of the III^b in the precipitate. This purified antibody inhibits I^b, I^a, rabbit LP^b, and rabbit LP^a with an inhibition potency of 6–11% of the value given with III^b.

Quantitative Precipitin Tests. These tests were carried out on heart III^b and on heart I^a (Figure 10). The curves obtained for heart III^b are identical with those for skeletal muscle III^b, the latter are not shown here, but are identical with those reported by Jókay (1967). Significant loss of enzymatic activity occurs when additions of I^a are low, but no rise in precipitate formation takes place. A rise in precipitate formation occurs only after a significant amount of I^a units is recovered in the final supernatant solution. Because of the limited amount of I^a available, no point of maximum precipitation is reached. Substitution of nonimmune globulin fraction for the antibody prevented the loss of enzyme activity and the formation of precipitate for both I^a and III^b.

Immunodiffusion tests utilizing antibody to III^b yielded precipitin lines with LP^b and I^b as well as with III^b.

TABLE III: Inhibition Potencies of Antibody with the Isozymes of Phosphorylase.

Expt	Isozyme Tested	Inhibition Potency ± Std Error (Units of Phosphorylase Inhibited by 1.0 ml of Antibody)	Ratio
1[a]	Skeletal muscle III^b	19.4 ± 0.24	1.00
	Heart III^b	20.3 ± 1.24	1.05
2[a]	Skeletal muscle III^b	22.5 ± 0.81	1.00
	Heart III^a	21.7 ± 1.4	0.96
	Heart II^a	11.3 ± 2.0	0.50
3[b]	Skeletal muscle III^b	11.6 ± 2.5	1.00
	I^b	0.695 ± 0.066	0.060
	I^a	1.25 ± 0.19	0.11
	Rabbit LP^b	0.787 ± 0.040[c,d]	0.067
	Rabbit LP^a	0.809 ± 0.12[d]	0.070

[a] The antibody was purified through the ammonium sulfate fractionation step. [b] The antibody was purified through the phosphorylase precipitation followed by acid treatment. [c] Assayed for LP^b in 0.35 M Na_2SO_4 and 1×10^{-3} M AMP. [d] The rabbit LP^b and LP^a were purified through the DEAE-cellulose chromatography step of Appleman *et al.* (1966).

Discussion

Homogeneous preparations of phosphorylase I^b and I^a have been compared immunologically, physically, and catalytically to phosphorylases III^b and III^a. They have similar ultraviolet light spectra, and they contain 2 moles of pyridoxal-P/mole of protein. The pyridoxal-P can be removed from the *b* forms of both isozymes by the procedure of Shaltiel *et al.* (1966) with essentially identical half-life periods. The isozymes have similar temperature stabilities, temperature–activity dependencies, pH–activity optima, and K_m values for glycogen and glucose-1-P. Both are inhibited by antibody to skeletal muscle phosphorylase, though there are significant quantitative differences. The *b* forms are inhibited by both ATP and glucose-6-P competitively with respect to AMP.

Five important differences have been found between isozymes I and III. (i) As reported earlier, I^b and I^a have identical molecular weights (Davis *et al.*, 1967), whereas III^a has twice the molecular weight of III^b (Keller and Cori, 1957). (ii) There is a significant difference in the electrophoretic mobility and in the DEAE-cellulose chromatography of I^b compared to III^b and of I^a compared to III^a (Davis *et al.*, 1967). These differences have been employed to identify and separate the isozymes. (iii) Antibody to skeletal muscle phosphorylase shows only 6–11% of its inhibition potency against I^b and I^a compared to its inhibition potency against III^b. Though the isozymes cross react in the precipitin test, there are significant quantitative differences in this reaction. (iv) I^a loses activity in the presence of the imidazole–citrate–L-cysteine buffer for removing pyridoxal-P from phosphorylase *b*, but III^a is stable in this buffer. (v) The most important differences were the smaller dissociation constant for the binding of AMP to I^b than for the binding of AMP to III^b, and the difference in

the Hill plot slopes for the *b* forms with respect to AMP. The concentration of AMP required for the 50% activation of III^a is five to eight times the AMP concentration required for 50% activation of I^b.

Morgan and Parmeggiani (1964) have shown that the activity of phosphorylase is regulated by the ATP and glucose-6-P inhibition of the AMP activation of the enzyme. Since I^b has a greater affinity for AMP than does III^b, enzymatically it would be active under conditions where III^b would show only slight activity or no activity at all.

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