Structural and Kinetic Properties of Crystalline Shark Muscle Glycogen Phosphorylase†

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ABSTRACT: Glycogen phosphorylase (EC 2.4.1.1) *a* and *b* forms of silky shark (*Carcharhinus falciformis*) were crystallized and characterized. Unique in the purification of phosphorylase from the muscle of this shark is the fractionation with ammonium sulfate at a temperature of 40°. The ease of crystallization from dilute buffer and the remarkable stability of shark phosphorylase *a* and *b* in the absence of AMP and thiol reducing compounds allowed an extensive study of their kinetic and association–dissociation properties. Shark phosphorylase *a* was found to exist in a dimeric form (mol wt = 1.7–2.0 × 10^5 g/mol; $s_{20,w}$ = 9 S) under conditions where rabbit phosphorylase *a* remains a tetramer. However, shark phosphorylase *a* can be tetramerized at high protein concentration ($\geq$6 mg/ml) and cold temperature ($\leq$12°). This latter property of the shark enzyme contrasts sharply with lobster phosphorylase *a*, a dimer (Cowgill, R. W. (1959), *J. Biol. Chem. 234*, 3146) which has been shown to resist tetramerization (Assaf, S. A., and Graves, D. J. (1969), *J. Biol. Chem. 244*, 5544). Gel electrophoresis of shark, rabbit, and human muscle phosphorylase *b* in the presence of sodium dodecyl sulfate and 2-mercaptoethanol showed that these enzymes are made up of subunits of identical molecular weight (9.5 × 10^4). Shark phosphorylase *b*, which exists as a dimer when dissolved in buffer and examined at a wide range of temperature and protein concentration, was found to undergo association in the presence of AMP, P_i, and AMP, or AMP and NaF. But when compared to rabbit phosphorylase *b* which tetramerizes easily, the shark *b* enzyme like the *a* form seems to prefer a dimeric quaternary structure. Phosphorylase from silky shark was found to be immunologically similar to that crystallized from lemon shark (*Negaprion brevirostris*) but immunologically different from that of rabbit or human. The amino acid compositions of silky and lemon shark phosphorylases were essentially the same except for a minor difference in isoleucine. The content of arginine, leucine, and proline in phosphorylase from the sharks was significantly lower than that found for rabbit. However, the isoleucine content was significantly less in the latter. Considering all the published amino acid compositions for muscle phosphorylase of homoiotherms and poikilotherms, one finds that the enzyme from the latter contains much less arginine. The kinetic behavior of shark phosphorylase *a* and *b* at 30 and 0° was investigated. Although both enzyme forms have a similar maximal velocity at 30°, at 0° the maximal velocity of the *a* form was double that of the *b*. Shark phosphorylase *b*, whose activity is dependent on AMP when studied in the direction of glycogen synthesis, was found to be partially active when examined in the direction of glycogen degradation at high P_i and in the absence of AMP as reported for rabbit and lobster phosphorylase *b*. The shark *b* enzyme also exhibited a deviation in its kinetics when studied at a wide range of concentrations of AMP; it showed a greater activator affinity at the low micromolar AMP levels. Except for the high K_m value of shark phosphorylase *a* and *b* toward P_i as found in lobster phosphorylase *b*, the K_m values for substrates and activator are more in keeping with those reported by many workers for rabbit phosphorylase *a* and *b*.

Literature on the comparative aspects of muscle glycogen phosphorylase (α-1,4-glucan, orthophosphate glucosyltransferase, EC 2.4.1.1) was reviewed recently by Cori (1969) and Fischer *et al.* (1970). Phosphorylase isolated from rabbit muscle (Green and Cori, 1943; Fischer and Krebs, 1958) has been used extensively as a model for studying the structure–function relationship of the muscle phosphorylases. However, the use of rabbit phosphorylase as a model is not without limitations. *E.g.*, (1) it is highly unstable to freezing and thawing or to prolonged storage in the cold. (2) The need of this enzyme for AMP and/or reducing sulfhydryl compounds for its crystallization and stability requires subsequent removal of these cofactors for most studies. (3) Our observation and that of others (DeVincenzi and Hedrick, 1967; Seery *et al.*, 1967) show that structural heterogeneity is induced after stripping the enzyme of added sulfhydryl compounds. Shark muscle phosphorylase, as will be seen, is free of these problems. In view of some differences found among the phosphorylases purified from muscles of three cold-blooded animals, lobster (Cowgill, 1959; Assaf and Graves, 1969), frog (Metzger *et al.*, 1968), and dogfish (Cohen *et al.*, 1971), a study of phosphorylase crystallized from muscle of silky shark, a poikilotherm caught in the Gulf Stream off Florida's Biscayne Bay,[1] was undertaken to determine how its structural and kinetic properties differ from those of phosphorylase isolated from warm- and other cold-blooded animals.

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[1] A study by Strasburg (1958) on shark distribution in the Pacific Ocean indicates that the silky shark is never found at temperatures below 15°. The temperature of the Gulf Stream off Key Biscayne, Fla., varies according to depth, being 5° at the bottom (a depth of 800 m) to 26–28° (depending on the season) near the surface homogeneous layer of the first 50 m (Stommel, 1965). The silky sharks used in this work were caught in the Gulf Stream with the guidance of Dr. Gordon Hubbell of the Crandon Park Zoo of Miami and Philip Heemstra of the Department of Marine Science, University of Miami. Unpublished observations by Dr. S. H. Gruber (shark research section of the University of Miami School of Marine and Atmospheric Sciences) on the distribution of the silky shark suggest that the range of temperature in the Gulf Stream where this animal was found abundantly is between 15 and 28°.

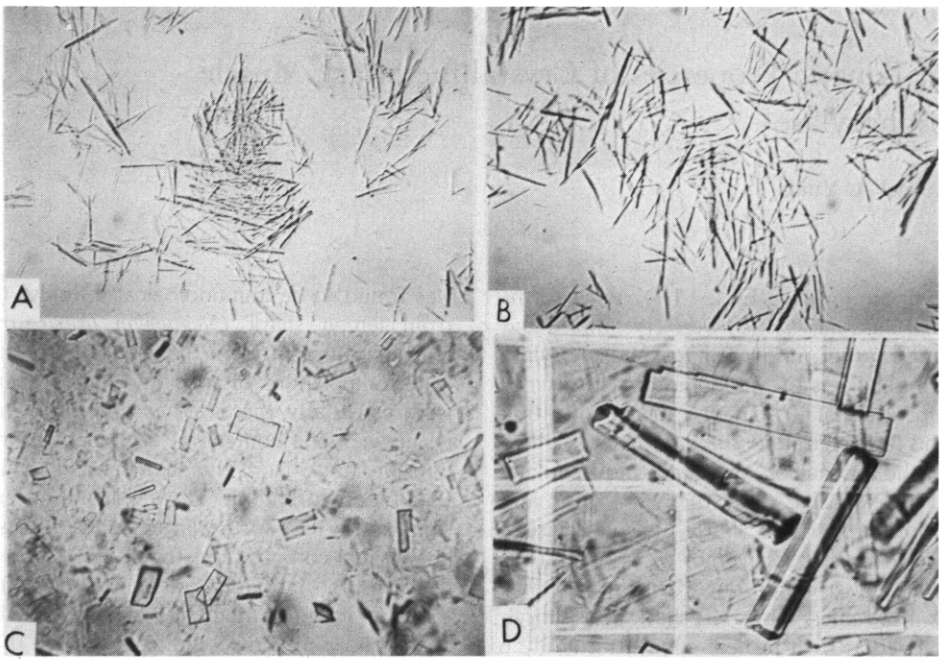


FIGURE 1: Crystals of silky shark phosphorylase *a* and *b* in buffer (0.05 M Tris–0.01 M EDTA, pH 7.5). Crystallization was performed as described under the section on enzyme purification. All photographs were taken at room temperature in an ordinary microscope at a magnification of 430×. The upper panels A and B represent photographs of freshly recrystallized shark phosphorylase *a* and *b*, respectively. The C panel represents 2-months aged crystals of shark phosphorylase *a* in buffer containing 0.05 M glucose (see Table III, pattern E). The crystallization of the *a* form of a muscle phosphorylase dimer in the presence of the dissociating agent, glucose, has never been reported. The D photograph represent crystals of shark phosphorylase *b* which were aged for 4 months. The distance across the grid is 0.05 mm. The dimension of the aged crystals is approximately 0.1 × 0.02 mm.

Experimental Procedure

Methods. The methods employed by Assaf and Graves (1969) for lobster phosphorylase were followed in the determination of protein concentration, disc gel electrophoresis, and enzyme assay except for using the substrate concentrations of Illingworth and Cori (1953). Gel electrophoresis in sodium dodecyl sulfate was carried out according to Shapiro *et al.* (1967) using the procedure of Weber and Osborn (1969) for determining the molecular weight. Immunological studies were carried out as previously described (Yunis and Assaf, 1970).

Sedimentation velocity and equilibrium determinations (using the meniscus depletion method at 20°) which were carried out at 40,000 and 13,000 rpm, respectively, were done according to Assaf and Graves (1969) except for using the computer program of Small and Resnik (1965) in the calculations. See also Assaf and Yunis (1971a). The sucrose density gradient ultracentrifugation experiments were performed as described by Yunis and Assaf (1970), using bovine liver catalase as a molecular weight standard (mol wt = 250,000) according to Martin and Ames (1961) using their formula in which the $\bar{v}$ value for the catalase standard and the unknown is considered to be identical. In our sedimentation equilibrium studies, however, a $\bar{v}$ value of of 0.74 ml/g was used in the calculations.

Amino Acid Analyses. Phosphorylase *b* samples were first dialyzed extensively against 1×10^{-3} M Tris–5×10^{-4} M EDTA, pH 7.0, and then for 2 days against deionized water prior to hydrolysis. An appropriate aliquot of each enzyme solution was taken in duplicate and analyzed for nitrogen according to Lani *et al.* (1950) The nitrogen values were converted to protein by multiplying by 6.25. Using this protein concentration for an identical silky shark phosphorylase *b* sample dialyzed against 0.15 M Tris–0.01 M EDTA, pH 7.5, an absorbancy index value (at 280 mμ) of 1.30 was calculated for a 0.1% (1 mg/ml) enzyme solution. Except for cysteine and tryptophan which were determined according to Spencer and Wold (1969) and Matsubara and Sasaki (1969), respectively, the amino acid analysis was performed according to standard procedures on a Spinco 120 amino acid analyzer. Norleucine was used as an internal standard and proper extrapolations were employed for the various hydrolysis periods. The few alloisoleucine residues found in some of the analyses were added to its isomer isoleucine as was also done by Assaf and Graves (1969) for lobster phosphorylase.

Kinetic Studies. The kinetics in the direction of glycogen synthesis at 30 and 0° were performed at pH 6.8 (the enzyme's pH optimum) using 0.01 M Tris–0.01 M maleic acid–1.5×10^{-3} M dithiothreitol–1×10^{-3} M EDTA. The final concentrations of glycogen, AMP, and glucose 1-phosphate when fixed were 2%, 1×10^{-3} M, and 2.4×10^{-2} M, respectively. When varied, the final concentration of glycogen was between 1 and 0.02%, glucose 1-phosphate between 0.024 and 0.002 M, and AMP between 2.5×10^{-4} and 5×10^{-7} M. In each instance a minimum of five different concentrations was employed. Initial rates were expressed in micromoles of P_i released per minute per milligram of phosphorylase.

Kinetic experiments were also carried out in the direction of glycogen degradation according to Helmreich and Cori (1964) as previously described by Assaf and Graves (1969), except for making the changes indicated here in the buffer system and increasing the concentration of magnesium and the auxiliary enzymes. The final concentrations in the reaction mixture were 7×10^{-4} M NADP, 1×10^{-2} M Tris, 1×10^{-2} M maleic acid, 5×10^{-4} M EDTA, 1.5×10^{-3} M dithiothreitol, 5×10^{-8} M glucose 1,6-diphosphate, 0.45% glycogen, and

TABLE I: Purification Profile of Shark Muscle Glycogen Phosphorylase.

Step No.	Fraction	Volume (ml)	Concn (mg/ml)	Total Protein (mg)	Sp Act. (Units)[a]	Total Act. (Units)[a]	Yield (%)	Purification (Fold)
1	Crude extract	2500	8	20,000	4	80,000	100	1
2	Precipitation with $(NH_4)_2SO_4$	250	28	7,000	10	70,000	88	2.5
3	Ultracentrifugation	230	20	4,600	14	64,400	81	3.5
4a	30% $(NH_4)_2SO_4$ pptn at cold temp (0°)	280	15	4,200	15	63,000	79	3.8
4b	30% $(NH_4)_2SO_4$ pptn at warm temp (40°)	150	5	750	60	45,000	56	15.0
5a	Crystalln[b] in $(NH_4)_2SO_4$	25	18	450	70	31,500	39	17.5
5b	First crystalln in buffer	8	27	216	80	17,280	22	21.5
5c	Second crystalln in buffer	7	21	147	85	12,495	16	22.5
5d	Third crystalln in buffer	10	11	110	85	9,250	12	22.5

[a] Specific activity is expressed in international units, *i.e.*, micromoles of product formed per minute per milligram of enzyme at specified assay conditions. See Methods. [b] The yield from crystallization depended on the time allowed for crystallization to take place. These data are for a crystallization period of approximately 36 hr.

1.5×10^{-2} M $MgCl_2$ at pH 6.8. Shark phosphorylase *a* and *b* were used at 2 μg/ml. Freshly crystallized phosphoglucomutase and glucose-6-phosphate dehydrogenase were employed in great excess, at 90 and 60 μg/ml, respectively, to compensate for their inhibition by P_i and AMP. Under these experimental conditions, phosphorylase was the only limiting enzyme in the reaction and initial rates were properly measured. When P_i was used at concentrations higher than 0.05 M, the concentration of the auxiliary enzymes was further increased by approximately another tenfold. NADPH[2] formation was measured at 340 nm using a Zeiss DMR-21 double beam recording spectrophotometer water jacketed at 30°. A molar extinction coefficient of 6.2×10^3 for NADPH was used in calculating the equivalent micromoles of the glucose 1-phosphate formed per minute per milligram of shark phosphorylase.

Enzyme Purification. The purification of glycogen phosphorylase from muscle of silky shark (*Carcharhinus falciformis*) does not differ from that of lobster muscle except in the step preceding enzyme crystallization. The ion exchange column chromatography step employed by Assaf and Graves (1969) in the purification of lobster phosphorylase is replaced by the following step.

To a crude cold solution of shark phosphorylase *b* (in 0.05 M Tris–0.01 M EDTA, pH 7.0) saturated neutralized ammonium sulfate was added to 30% saturation. The small amount of precipitate which formed after standing 4 hr at 0–4° was removed by centrifugation. The concentration of EDTA in the supernatant was brought up to 0.01 M and then the crude enzyme was incubated at 40° for 20 min. The resulting viscous suspension[3] was eliminated by centrifugation for 15 min at room temperature at 18,000*g*. The phosphorylase supernatant solution obtained was then concentrated by dialysis against neutralized saturated ammonium sulfate and the precipitate was collected, dissolved in 0.05 M Tris–0.01 M EDTA, pH 7.5, and dialyzed overnight against the same in preparation for crystallization.

Crystallization. Crystallization was achieved by dialyzing enzyme solution (20–50 mg/ml) overnight against water or dilute buffer (0.05 M Tris or 0.05 M glycerophosphate, pH 6.8–7.5) with or without 10^{-2}–10^{-3} M EDTA. In general, the yield was better when crystallization was first done by the addition of saturated ammonium sulfate to 32% saturation. Therefore, the first enzyme crystals were routinely obtained from ammonium sulfate. Because of their high salt content, these crystals dissolved readily in buffer and recrystallization was done by simply dialyzing against 0.004 M Tris–0.002 M EDTA buffer, pH 7.5. The needle-shaped crystals thus obtained (Figure 1) were dissolved in a minimal volume of 1 M Tris and, when dialyzed or diluted with buffer or water to 10^{-2}–10^{-3} M Tris final concentration, recrystallization readily occurred even at 25°, provided the enzyme concentration was kept above 6 mg/ml. Shark phosphorylase *b*, which was converted to the *a* form using rabbit phosphorylase kinase according to Fischer and Krebs (1962), was also crystallized by dialysis against dilute buffer. It also crystallized in the presence of the dissociating agent, glucose, as illustrated in Figure 1.

Results and Discussion

Enzyme Purification and Crystallization. The results of a typical purification from 1000 g of silky shark muscle are summarized in Table I.

The unique and simple purification and crystallization procedure described above for this muscle phosphorylase gave an enzyme which was homogeneous by the criteria of disc gel electrophoresis, sedimentation velocity, and immunoprecipitation in agar.

Microscopic examination of shark phosphorylase crystals revealed no differences in shape between those of the *a* and *b* forms (Figure 1). The needle-shaped crystals transform to plates or prism forms upon aging for 4 months as shown in Figure 1. An estimate of the dimensions of the aged crystals is 0.1×0.02 mm, a size probably suitable for X-ray diffraction work (Fasold *et al.*, 1972). In contrast to the crystals of rabbit phosphorylase *b* (Kent *et al.*, 1958) which dissolve upon warming, crystals of shark phosphorylase *b* remained insoluble at room temperature and dissolved upon increasing the salt concentration of the crystalline suspension to 0.2 M. The shark enzyme retained its full activity after 4 months in the

[2] Abbreviations used are: NADPH, reduced nicotinamide adenine dinucleotide.

[3] Examination of this suspension revealed small needle-shaped crystals. These crystals possessed no phosphorylase activity and dissolved instantly upon cooling and therefore were removed by centrifugation while warm. The nature of this crystalline protein is yet to be determined.

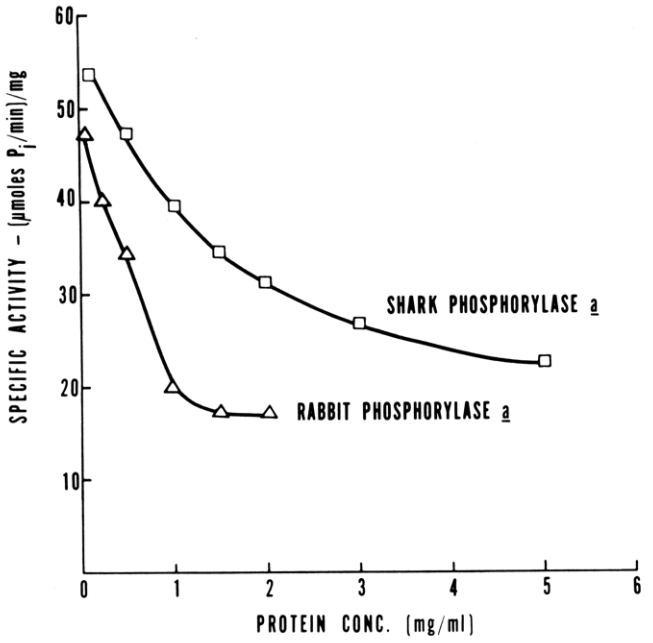
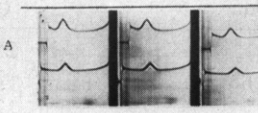
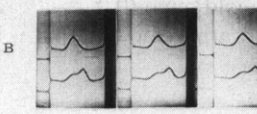
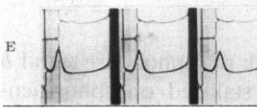


FIGURE 2: Variation of specific activity with concentration for shark and rabbit phosphorylase *a*. The rapid dilution in 0.05 M Tris–0.01 M EDTA, pH 6.8, and enzyme assay were performed at 22° according to Huang and Graves (1970).

TABLE II: Effect of Temperature on the Sedimentation Velocity of Shark and Rabbit Phosphorylase *a*.[a]

	Sedimentation Pattern	Enzyme	Temp.	$S_{20,w}$
A		Shark Phos. b	11°	9.3S
		Shark Phos. a	"	14.3S
B		Shark Phos. b	25°	9.5S
		Shark Phos. a	"	9.4S 35% 14.1S 65%
C		Rabbit Phos. a	9°	13.9S
D		Rabbit Phos. a	22°	14.4S
E		Rabbit Phos. a	22° in 0.05 M glucose	9.2S
		Shark Phos. a	"	9.2S

[a] Sedimentation velocity experiments were performed as indicated under Methods. All photographs shown were taken at 16-min intervals. $s_{20,w}$ values were calculated using ten photographs at 8-min intervals.

absence of sulfhydryl compounds whether stored in crystalline form or as a refrigerated or frozen solution. Under these conditions the enzyme also remained homogeneous as determined by disc gel electrophoresis and analytical ultracentrifugation. Freezing for 1 year resulted in less than 15% loss in activity. Shark phosphorylase *b* was stable for 1 hr at 45°; however, it lost 90% of its activity in 2 min when heated to 60°.

Enzyme Association–Dissociation Studies. SEDIMENTATION VELOCITY EXPERIMENTS ON PHOSPHORYLASE *a*. Silky shark phosphorylase *a* sedimented as a tetramer with an $s_{20,w}$ of 14.3 S (Table II, pattern A) at temperatures below 12° under these conditions: enzyme concentration of at least 6 mg/ml in a buffer of 0.10 M Tris–0.005 M EDTA, pH 6.8–7.5, or in 0.10 M glycerophosphate–0.005 M EDTA, pH 6.8. At 25°, Table II, pattern B, it formed two peaks, one with an $s_{20,w}$ of 14.1 S (65%) and a second peak with an $s_{20,w}$ of 9.4 S (35%). This temperature-induced dissociation of phosphorylase *a* was reversible, since upon cooling to 9° after the ultracentrifuge cell contents were well mixed, the sedimentation pattern (not illustrated) showed only one peak with $s_{20,w}$ of 13.6 S (see also the experiment in Table II, pattern A). Shark phosphorylase *a* which was treated with 0.05 M glucose sedimented as a dimer ($s_{20,w}$ = 9.2 S, Table II, pattern E) even at cold temperature, a finding similar to that reported by Wang *et al.* (1965) for rabbit phosphorylase *a* and repeated here for comparison.

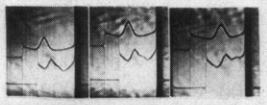
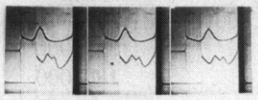
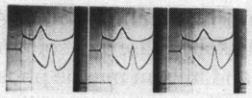
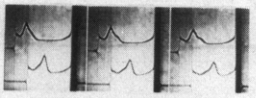
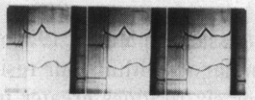
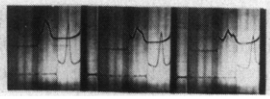
In contrast to the temperature-dependent dissociation of shark phosphorylase *a*, rabbit phosphorylase *a* (at 1.8 mg/ml and 9 or 20°) sedimented in a single peak as a tetramer ($s_{20,w}$ = 13.9–14.4 S, Table II, patterns C and D) in agreement with data reported by others (Kent *et al.*, 1958; Wang and Graves, 1964).

Wang and Graves (1964), Metzger *et al.* (1967), and Huang and Graves (1970) have demonstrated that tetrameric rabbit phosphorylase *a* becomes more active as it dissociates to its dimeric form upon dilution. Hence the method of Huang and Graves (1970) was used to determine the activity concentration dependence of shark phosphorylase *a*. The results shown in Figure 2 confirm the analytical ultracentrifuge data presented in Table II in that shark phosphorylase *a* begins dissociation to the dimeric form at warm temperatures above 22° at a protein concentration as high as 5 mg/ml. Under these conditions it can also be seen from Figure 2 that rabbit phosphorylase *a* remains a tetramer and does not dissociate until the protein concentration is decreased to less than 1 mg/ml in agreement with results reported by the above mentioned workers.

SEDIMENTATION VELOCITY EXPERIMENTS ON PHOSPHORYLASE *b*. Association of rabbit muscle phosphorylase *b* to the tetrameric form is known to be favored at cold temperature in the presence of certain associating agents (Kent *et al.*, 1958; Sealock and Graves, 1967; Kastenschmidt *et al.*, 1968). Shark phosphorylase *b*, like rabbit phosphorylase *b* when examined in buffer (0.05 M Tris–0.01 M EDTA, pH 6.8), sedimented in homogeneous form as a dimer (Table III, patterns A–C). In the presence of 0.5 M P_i known to induce tetramer formation in rabbit muscle phosphorylase *b* (Assaf and Graves, 1969), shark phosphorylase *b* underwent 20% tetramerization at 21° (Table III, pattern A), increasing to 40% at 8° (Table III, pattern B), and becoming completely associated when AMP is included with 0.05 M P_i (Table III, pattern C). It may be noted that this sedimentation behavior of shark and rabbit phosphorylase *b* in the presence of 0.5 M P_i is in sharp contrast to lobster muscle phosphorylase *b* (Assaf and Graves, 1969) which dissociated to a monomeric form with a sedimentation coefficient of 5.3–5.6 under identical conditions.

Distinct differences in the association–dissociation behavior of shark and rabbit phosphorylase *b* were found using AMP and mercaptoethanol (Table III, patterns E and F); while shark phosphorylase *b* remained largely a dimer under these

TABLE III: Sedimentation Velocity Patterns of Shark and Rabbit Muscle Phosphorylase *b*.[a]

	Sedimentation Pattern	Source	Temp.	Associating Agent	$S_{20,w}$	% of Total
A		Shark	21°	-	9.1S	100
		"	"	0.5 M P_i	8.4S	80
					12.6S	20
B		Shark	8°	-	9.2S	100
		"	"	0.5 M P_i	8.2S	60
					12.6S	40
C		Shark	8°	-	9.0S	100
		"	"	0.5 M P_i + 0.01 M AMP	13.0S	100
D		Rabbit	9°	-	8.3S	100
E		Shark	8°	0.04M SH* + 0.005 M AMP	9.0S	80
					13.2S	20
		Rabbit	"	"	8.7S	15
					13.6S	85
F		Shark	15°	0.04 M SH* + 0.005 M AMP	8.6S	90
					13.1S	10
		Rabbit	"	"	9.0S	40
					13.4S	60
G		Shark	8°	0.04 M SH* + 0.005 M AMP + 0.2 M NaF	8.9S	60
					14.0	40
		Rabbit	"	"	13.7	100

[a] Sedimentation velocity experiments were performed as indicated under Methods. The photographs shown were taken at 16-min intervals except those in panel G which were taken at 8-min intervals. $s_{20,w}$ values were calculated using ten photographs at 8-min intervals. An asterisk indicates 2-mercaptoethanol.

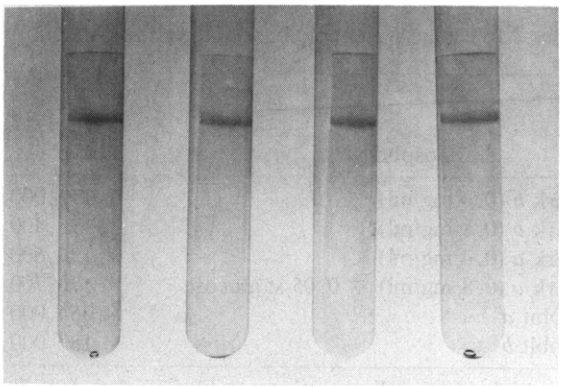


FIGURE 3: Polyacrylamide disc gel electrophoresis of silky shark, rabbit, and human muscle phosphorylase *b* in the presence of sodium dodecyl sulfate and 2-mercaptoethanol. Purified phosphorylase *b* from shark, rabbit, and human muscle was treated with sodium dodecyl sulfate as indicated uner Methods. When run separately, 12 μg of each enzyme was placed on the gel. When run as mixtures, the sodium dodecyl sulfate treated sample contained 4 μg of each enzyme. From left to right: phosphorylase from shark, rabbit, and human, and the three enzymes mixed together.

conditions, rabbit phosphorylase *b* exhibited 85% tetramer formation at 8° (Table III, pattern E) and 60% at 15° (Table III, pattern F). When sodium fluoride was added in the presence of mercaptoethanol and AMP (Table III, pattern G), rabbit phosphorylase *b* sedimented in a single peak with an $s_{20,w}$ of 13.7 S corresponding to that of a tetramer, whereas shark phosphorylase *b* sedimented in two peaks, one corresponding to a tetramer with an $s_{20,w}$ of 14.0 S (40%), and another corresponding to a dimer with an $s_{20,w}$ of 8.9 S (60%). The complete tetramerization of rabbit phosphorylase *b* in the presence of fluoride and AMP was recently communicated by Assaf and Yunis (1971a).

Molecular Weight of Shark Phosphorylase a and b. Figure 3 shows that sodium dodecyl sulfate gel electrophoresis of phosphorylase *b* crystallized from shark, rabbit (Fischer and Krebs, 1958), and human muscle (Yunis *et al.*, 1960) applied singly or in combination gave a single band indicating that the subunits from these enzymes have an identical molecular weight. Hedrick *et al.* (1969) showed that rabbit phosphorylase *b* dimer when subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate yielded a single band corresponding to a mol wt of 92,000 g/mol. When this method was used for the electrophoresis of shark phosphorylase *b* against five other protein standards according to Weber and Osborn (1969) a similar molecular weight value (94,500 ∓ 5%) was found. Dogfish and rabbit muscle phosphorylase examined by Cohen *et al.* (1971) by this method also gave a similar value for phosphorylase *b* monomer (99,000–100,000). Molecular weight determination of rabbit phosphorylase *b* monomer using 6 M guanidine-HCl by Ullman *et al.* (1968) yielded a value of 98,000 g/mol.

The data in Table IV indicate that the number and weight average molecular weight values (171,000–205,000 g/mol) of shark phosphorylase *a* and *b* studied at 0.4 and 0.8 mg/ml at 20° using a $\bar{V}$ value of 0.74 ml/g are nearly similar and represent that of a phosphorylase dimer. The value of 198,000 for the whole cell weight average molecular weight ($\bar{M}_w$) of shark phosphorylase *b* is interestingly identical with that reported for dogfish phosphorylase *b* by Cohen *et al.* (1971). The molecular weight of the *a* form in the presence of the dissociating agent, glucose, is similar to that in its absence and is more in keeping with values reported for phosphorylase dimer.

In contrast, however, rabbit phosphorylase *a* exists primarily as a tetramer when studied in the absence of glucose as may be seen in Table IV from our sucrose density gradient ultracentrifugal data (mol wt = 335,000) and the high speed sedimentation equilibrium data ($\bar{M}_w$ = 363,000) of Fischer *et al.* (1968).

Of further interest in Table IV is the finding that untreated phosphorylase *b*, which exists as a dimer under a wide range of concentrations and temperatures, seems to undergo some dissociation to monomer when diluted in buffer to concentration approaching zero as may be observed for the low values of the number average molecular weight ($\bar{M}_n$) at *c* 0. Such dissociation to monomer was also observed with lobster phosphorylase *b* (Assaf and Graves, 1969). The value of $\bar{M}_n$ at *c* 0 for the *a* form also suggest that it too probably undergoes dissociation to monomer at concentrations approaching zero. De Vincenzi and Hedrick (1970) and Cohen *et al.* (1971) presented gel filtration data which indicate monomer formation in rabbit phosphorylase.

Amino Acid Composition. Amino acid analyses were performed on two preparations of both rabbit and silky shark phosphorylase and one preparation of lemon shark phosphorylase. These analyses were conducted utilizing five hy-

TABLE IV: Molecular Weight of Shark and Rabbit Phosphorylase.[a]

Phosphorylase	Whole Cell Mol Wts			No. Av. at c 0	By Sucrose Density Gradient[b]
	No. Av	Wt Av	Z Av		
Shark *b* (0.4 mg/ml)	171,000	198,200	214,000	148,700	175,000
Shark *b* (0.8 mg/ml)	196,400	202,300	220,600	194,000	179,000
Shark *a* (0.4 mg/ml)	190,600	205,500	231,200	160,000	180,000
Shark *a* (0.8 mg/ml) + 0.05 M glucose	176,700	198,500	201,100	157,300	177,000
Rabbit *a*	354,000	363,000	373,000		335,000
Rabbit *b*	187,000	190,000	196,000		180,000

[a] The high-speed sedimentation equilibrium data for rabbit phosphorylases *a* and *b* were taken from Fischer *et al.* (1968). A value of 0.74 ml/g for $\bar{V}$ was used in our molecular weight calculations. The molecular weight of shark phosphorylase *b* in sodium dodecyl sulfate using the method of Weber and Osborn (1969) was 94,500, identical with that of rabbit and human muscle phosphorylase *b*. [b] A 0.2-ml sample containing 0.5–3 mg of shark or rabbit phosphorylase was layered on 4.6-ml linear sucrose gradient (5–20% sucrose in 0.005 M Tris–0.001 M EDTA, pH 7.2). Gradients were centrifuged and fractions collected as previously described (Yunis and Assaf, 1970). The enzyme concentrations in the extreme left column do not represent those used in the sucrose density gradient ultracentrifugation experiments.

drolysis times and resulted in a 97–99% recovery. The difference in the results of the two analyses performed on the same enzyme was not significantly different according to a Chi Square test ($p < 0.01$) and therefore an average is reported in Table V. A Chi Square analysis of our amino acid composition data for rabbit phosphorylase against that of Sevilla and Fischer (1969) indicated no significant difference between the two analyses ($p < 0.01$).

Inspection of the data in Table V for phosphorylase composition from silky and lemon shark (Assaf and Yunis, 1971b) indicates that both enzymes were similar to within four residues except for isoleucine (which was six residues greater in the lemon shark). However, a comparison of the data for phosphorylase *b* from silky and lemon shark against that of rabbit revealed differences in more than half of the amino acids. Five of the basic and bulky side-chain amino acids shown in Table V differ by 8–11 residues. It should be noted that phosphorylase from the poikilotherms, silky and lemon shark and lobster, has significantly less arginine, leucine, and proline than that of rabbit or rat whose content of isoleucine is greater than that of the poikilotherms. The rabbit phosphorylase value for half-cystine, which was determined separately in triplicate, is greater than that reported by Sevilla and Fischer (1969). The reason for this discrepancy is not yet clear.

TABLE V: Amino Acid Composition of Muscle Phosphorylases.[a]

Amino Acid	Silky Shark[b]	Lemon Shark[b]	Rabbit[b]	Rabbit[c]
Lysine	59	62	51	47
Histidine	22	20	21	21
Arginine	57	56	68	64
Aspartic acid	97	101	99	98
Threonine	34	34	34	34
Serine	36	32	30	30
Glutamic acid	98	99	101	102
Proline	36	34	45	43
Glycine	50	52	50	50
Alanine	63	64	66	65
Valine	59	55	62	61
Methionine	19	21	20	22
Isoleucine	61	67	51	49
Leucine	75	73	85	81
Tyrosine	34	35	37	37
Phenylalanine	39	39	40	39
Half-Cystine	12	14	12	9
Tryptophan	14	14	13	13
Total Residues	865	872	885	865
Ammonia	70	72	75	77

[a] The amino acid composition is expressed in residues per 100,000 grams protein weight. [b] Data from this study. [c] Data from Sevilla and Fischer (1969).

Immunological Studies. Antiserum prepared against silky shark muscle phosphorylase *b* in roosters did not cross-react with rabbit or human muscle phosphorylase *b* as shown by precipitation in agar (Figure 4a). Antisilky shark phosphorylase serum, however, cross-reacted with crystalline lemon shark phosphorylase yielding a single line in an agar ouchterlony diffusion test (Assaf and Yunis, 1971b). Phosphorylase crystallized from yet another shark, the nurse shark, also yielded a reaction of immunological identity with the enzyme from lemon and silky shark. The effect of rooster anti-silky shark and rooster anti-rabbit phosphorylase on the activity of rabbit and shark phosphorylase *b* is shown in Figure 4b. In each case the antibody inhibited the specific antigen with little or no effect on the other phosphorylase.

Kinetic Studies on Shark Phosphorylase a and b. KINETICS IN THE DIRECTION OF GLYCOGEN SYNTHESIS. In their comparative studies, Cowgill (1959) and Assaf and Graves (1969) found that the K_m values for substrates and activator of lobster phosphorylase *b* were much higher than those of rabbit phosphorylase. We have employed similar conditions as those of Assaf and Graves (1969) for studying the kinetic behavior of the *a* and *b* forms of the shark enzyme; see Methods. Shark phosphorylase *a* or *b* gave straight lines for the double reciprocal plots of the substrates glucose 1-phosphate and glycogen (Figures 5a and 5b) indicating lack of homotropic cooperativity between glucose 1-phosphate or glycogen binding sites at saturating levels of activator, a finding consistent with

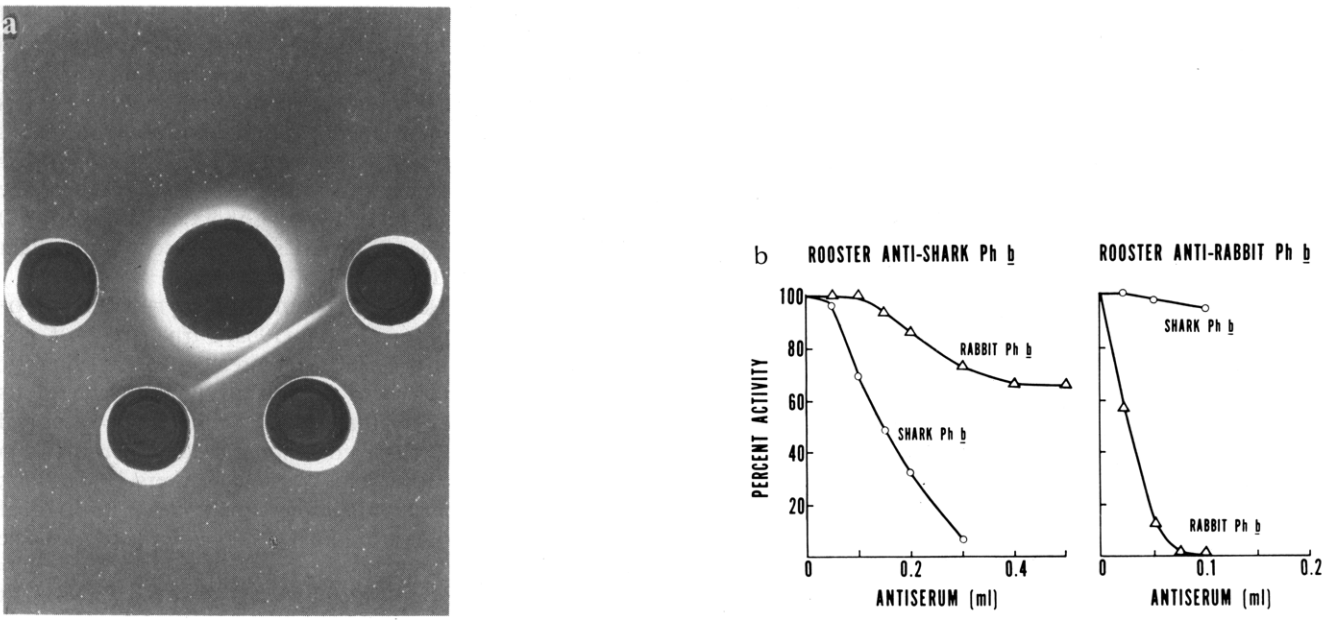


FIGURE 4: (a) Antigen–antibody precipitation in agar for phosphorylase *b* from various sources. Center well contained rooster anti-silky shark muscle phosphorylase serum. Wells from left to right contained: purified phosphorylase *b* from rabbit muscle, human muscle, shark muscle, and partially purified phosphorylase *b* from rooster muscle. (b) Effect of rooster anti-shark muscle phosphorylase *b* on the activity of rabbit and shark muscle phosphorylase *b*. Solutions of rabbit and shark phosphorylase *b* were adjusted to the same activity prior to performance of experiments on the effect of various amounts of phosphorylase antibody on enzymatic activity. Control tubes contained equivalent amounts of normal rooster serum.

that reported for rabbit phosphorylase by other workers (for a recent review see Cori, 1969). One of the major objectives in this phase of the project, however, was the evaluation of changes in kinetic parameters of shark phosphorylase with changes from warm to cold temperature. The synthesis of glycogen at 30° *vs.* 0° by phosphorylase *a* and *b* of this poikilotherm is shown in Figures 5a and 5b. The data in Figures 5a clearly show that the affinity of shark phosphorylase *a* for glucose 1-phosphate is greater than that for the *b* form and is the same at 30 and 0°. This is not so for the *b* form of this enzyme whose affinity was increased by twofold at the cold temperature.

A somewhat similar pattern was also observed with respect to glycogen at 30 and 0° (Figure 5b), where it could be seen that the affinity of the *b* form for this substrate was greatly increased at cold temperature. The effect of temperature on maximal velocity of the two enzyme forms was also different. Whereas both enzyme forms gave a similar V_m [60–77 (μmol min^{-1})/mg] at 30° with respect to either glycogen or glucose 1-phosphate, at 0° the *a* form was twice as active as the *b* form as can be seen in Figures 5a and 5b. The affinity of lobster phosphorylase *b* (Assaf and Graves, 1969) for its substrates, glucose 1-phosphate and glycogen, was also increased by decreasing the temperature especially with respect to glycogen as also found here for the shark enzyme. In the case of rabbit phosphorylase *b*, affinity to glycogen (Lowry *et al.*, 1967) but not glucose 1-phosphate (Assaf and Graves, 1969) was increased by decreasing the temperature from 30 to 0°.

While simple Michaelis–Menten kinetics were obeyed when the shark phosphorylase data were plotted with respect to either substrate, nonlinearity was observed with respect to the activator AMP. Kinetics of shark phosphorylase *b* with varying AMP showed deviation from linearity with a break in the line of the double reciprocal plot at AMP concentrations $>10^{-5}$ M (Figure 5c). The downward curvature at high AMP levels represents a decrease in this enzyme activator affinity ($K_{m,AMP} = 3 \times 10^{-2}$ mM) from that found at micromolar AMP concentrations where a $K_{m,AMP}$ of 2×10^{-4} mM was found. Wang *et al.* (1970) theorized the presence of two AMP sites with different affinities for similar data on rabbit phosphorylase *b* obtained using calometric measurements. A similar kinetic behavior was found by Assaf and Yunis (1971c) for chloroma glycogen synthetase with respect to its activator, glucose 6-phosphate.

To overcome heterotropic cooperative effects between the allosteric substrate P_i and the activator AMP which give nonlinear (curved upward) reciprocal plots, we carried out our experiments (Figure 6) at relatively high substrate concentrations. Allosteric effects were also abolished in lobster phosphorylase (Assaf and Graves, 1969) when the concentration of the fixed substrate was greatly increased, a finding consistent with the model of Monod *et al.* (1965) for allosteric enzymes. Figures 6 and 7 illustrate double reciprocal plots for shark phosphorylase *b* and *a* with varying substrate (P_i) under various fixed levels of activator, magnesium (0.015 M), and glycogen (0.45%). Under these experimental conditions, the K_{m,P_i} for shark phosphorylase *b* (Figure 6B) was found to be 20–30 mM which is four- to sixfold greater than that found for shark phosphorylase *a* (Figure 7B) with a K_{m,P_i} of 5 mM. It should also be noted from the shark phosphorylase *b* data in Figure 6B that although the K_{m,P_i} was the same (20 mM) at AMP concentrations between 5×10^{-4} and 2×10^{-5} M, the K_{m,P_i} increased to 26–30 mM at an AMP concentration $\leq 5 \times 10^{-6}$ M. Additionally, when a secondary reciprocal plot of the phosphorylase *b* data of Figure 6B is made with respect to AMP (Figure 6C), a deviation from linearity was observed indicating a sharp increase in enzyme affinity to the activator at concentrations $< 5 \times 10^{-6}$ M. This kinetic behavior is similar to that pointed out in Figure 5c for varying AMP concentrations in the direction of glycogen synthesis. Working with rabbit

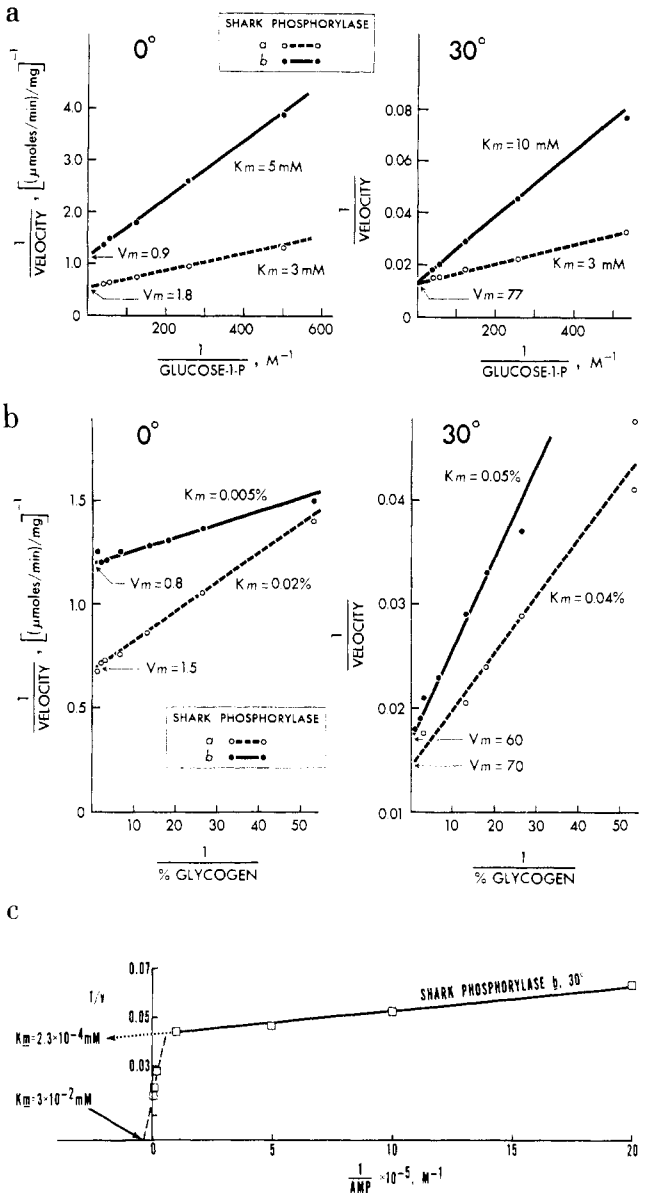


FIGURE 5: Kinetics of glycogen synthesis by shark phosphorylase. (a) Double reciprocal plot for the initial velocity of shark phosphorylase *a* and *b* at 30 and 0° with respect to varied concentration of glucose 1-phosphate at fixed concentrations of glycogen and AMP. Details of the experimental conditions are described under Methods. (b) Double reciprocal plot for the initial velocity of shark phosphorylase *a* and *b* at 30 and 0° with respect to varied concentration of glycogen at fixed concentrations of AMP and glucose 1-phosphate. (c) Double reciprocal plot for the initial velocity of shark phosphorylase *b* at 30° with respect to varied concentration of AMP at fixed concentrations of glycogen and glucose 1-phosphate.

phosphorylase *b*, Kastenschmidt *et al.* (1968) also found deviation in their velocity data at AMP concentrations below 5×10^{-6} M. Madsen and Schechosky (1967) observed velocities at low AMP levels which they indicated did not fit the major portion of their data.

Shark phosphorylase *a* like rabbit phosphorylase *a* (Cori, 1969) has only a very small requirement for AMP even when it was aged for nearly 6 weeks. The substrate saturation curve was hyperbolic at all the AMP levels used (Figure 7A) and the double reciprocal plot with respect to P_i (Figure 7B) gave one intersection point for all the fixed AMP levels between 10^{-6} and 10^{-4} M. In the absence of AMP, the K_{m,P_i} of shark phosphorylase *a* was 10 mM, exactly twice that obtained in the presence of AMP. It is interesting to find that even in the absence of AMP, the affinity of shark phosphorylase *a* for its substrate, P_i, is so much greater than that for the *b* form but is considerably lower than that known for rabbit phosphorylase *a*. Furthermore, different from shark phosphorylase *b*, the AMP secondary plot for shark phosphorylase *a* (Figure 7C) was linear for all the AMP levels used (except for a slight deviation near 1×10^{-6} M).

ACTIVITY OF SHARK PHOSPHORYLASE *b* IN THE ABSENCE OF AMP. According to Monod *et al.* (1965) a true enzyme *K* system should show a decreasing requirement for its allosteric activator or modifier with increasing concentration of its allosteric substrate and theoretically (if it can be saturated with its substrates), the enzyme should become fully active in the absence of its activator. Various workers (Buc, 1967; Engers and Madsen, 1968; Kastenschmidt *et al.*, 1968; Assaf and Graves,[4] 1969) measured phosphorylase *b* activity in the absence of AMP at high P_i concentrations. Their results differed in the extent to which phosphorylase *b* is activated at high P_i concentrations with Buc and Buc (1968) reporting a full activation in complete compliance with the model of Monod *et al.* (1965). While Assaf and Graves (1969) presented a modification to the model of Monod *et al.* (1965) (assuming a different catalytic rate constant at zero AMP) to account for their data, Kastenschmidt *et al.* (1968) attributed the discrepancy from that of Buc (1967) and Buc and Buc (1968) to the use of different buffer systems. In the present study we found rabbit phosphorylase *b* activity in the direction of glycogen degradation in the presence or absence of AMP to be less in glycylglycine, pH 7.5, used by Buc and Buc (1968) than that in Tris used by Assaf and Graves (1969), and thus, the full activity observed by Buc (1967) for rabbit phosphorylase *b* with high P_i in the absence of AMP cannot be explained on the basis of differences in buffer.

These variations in the literature prompted us to examine shark phosphorylase *b* activity in the absence of AMP. It could be seen from Figure 8 that the maximal velocity in the absence of AMP, 8 (μmol min^{-1})/mg, obtained from data at 0.1–0.5 M P_i is only 31% of 26, the V_m obtained in the presence of AMP (Figure 8) at the same fixed concentration of glycogen. The maximal velocity obtained here for shark phosphorylase *b* in the absence of AMP is interestingly similar to that published by Engers and Madsen (1968) for rabbit phosphorylase *b* studied at high P_i concentrations. The downward curvature in Figure 8 at high P_i concentrations could be due to the decrease in the enzyme affinity for its substrate at such extremely high salt concentrations.

Discussion

Muscle glycogen phosphorylases purified from various animals share many important properties. These include the activation of the *b* form by AMP, the independence of phosphorylase *a* from significant activation by AMP, and the presence of pyridoxal phosphate as a prosthetic group without which phosphorylases *a* and *b* are inactive. From detailed studies on the activity–subunit structure relationship of rabbit phosphorylase by Wang and Graves (1964), Metzger *et al.* (1967), Huang and Graves (1970), and De Vincenzi and Hed-

[4] The velocities in the direction of glycogen degradation in the paper of Assaf and Graves (1969) printed in OD/minute should be in OD/second as stated in their Methods section.

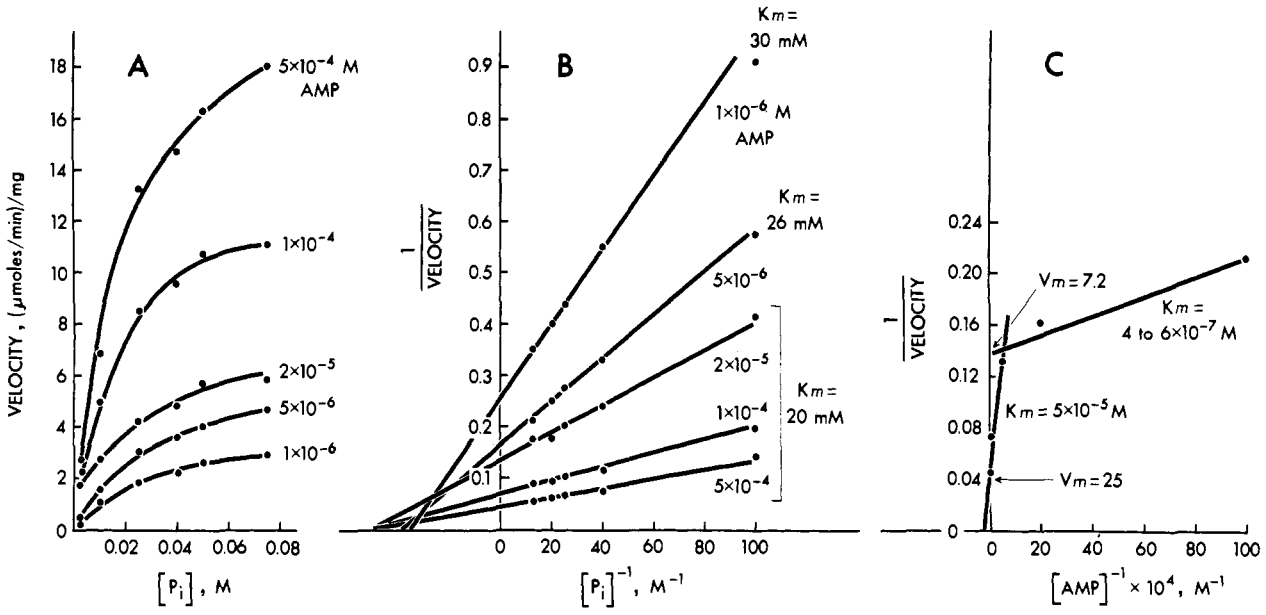


FIGURE 6: Kinetics of glycogen phosphorolysis by shark phosphorylase *b* with respect to varying concentrations of P_i and AMP at fixed concentration of glycogen. For experimental details see Methods: (A) phosphorylase *b* saturation curve with respect to varied P_i at various fixed levels of AMP; (B) double reciprocal plot of the data in part A; (C) secondary plot of the primary plot data in part B.

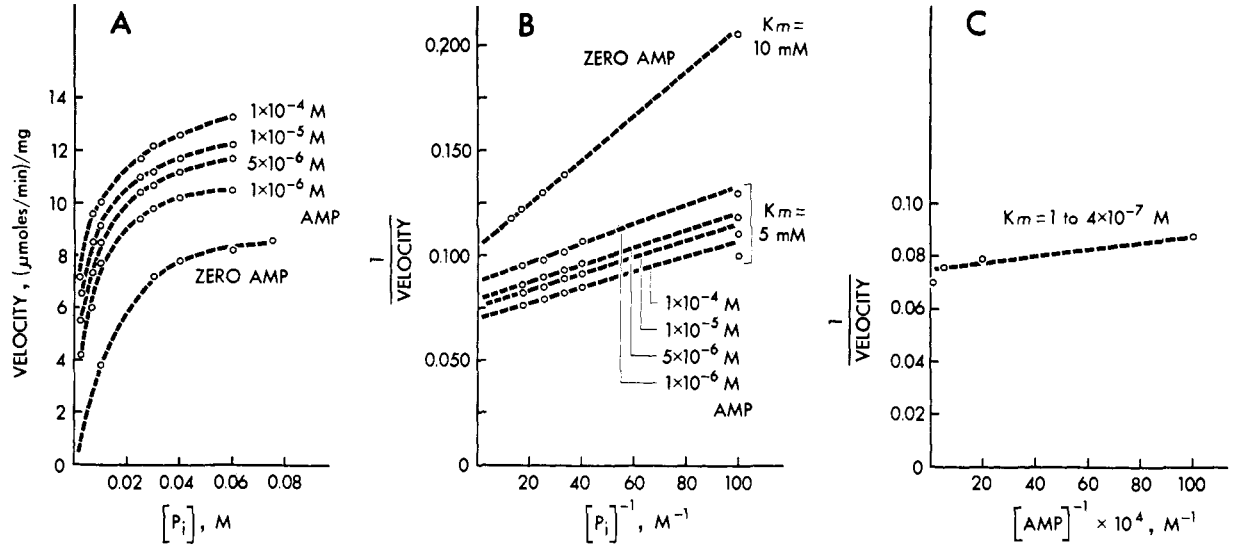


FIGURE 7: Kinetics of glycogen phosphorolysis by shark phosphorylase *a* with respect to varying concentrations of P_i and AMP at a fixed concentration of glycogen. For experimental details see Methods: (A–C) as described in Figure 6.

rick (1970), a picture emerged suggesting that phosphorylase *a* activity may be regulated by a mechanism involving the association of phosphorylase *a* dimer to its less active tetrameric form. The presence of high enzyme concentration in muscle (1–2 mg/ml of cell water, see Table I) seems to support such a mechanism *in vivo*. This type of regulation of enzymatic activity may very well be operative *in vivo* for phosphorylases of rabbit and rat, and, perhaps, for that of frog (Metzger *et al.*, 1968) and dogfish (Cohen *et al.*, 1971) where the enzyme appears to exist in a rapid equilibrium between dimer and tetramer. However, our data showing the virtual absence of tetramerization of shark phosphorylase *a* at protein concentrations approaching physiological levels and temperatures of 20° suggest that dissociation $\rightleftarrows$ association is not involved in regulating shark phosphorylase *a* activity. Instead, the activity of this enzyme in shark muscle is probably controlled by its dephosphorylation to the highly AMP-dependent *b* form.

The phosphorylation of shark phosphorylase *b* to the *a* form results in an increase in the enzyme's maximal velocity at 0° but not at 30°. The physiological significance of this observation remains uncertain. The true maximal velocity at 0° of phosphorylase *b* from another poikilotherm, that of lobster, was found to be greater than that of rabbit phosphorylase *b*. No initial velocity studies were made at 0° on the *a* form from these animals. As in rabbit phosphorylase *a*, the affinity of shark phosphorylase *a* for its substrates glycogen, glucose 1-phosphate, and P_i at 30° is greater than that of the *b* form.

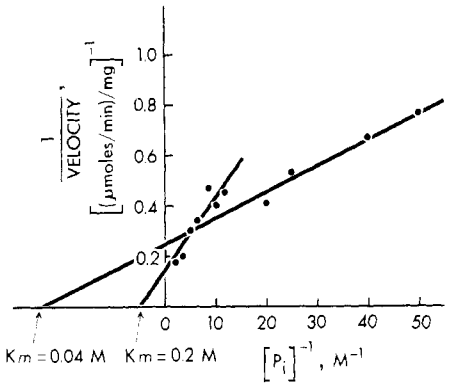


FIGURE 8: Double reciprocal plot of the initial velocity of shark phosphorylase *b* at high varied P_i concentrations and zero AMP. Details are in the text.

Also, while the shark *a* enzyme is nearly fully active at zero AMP, the *b* form is only partially active at the low micromolar cellular AMP levels present in shark muscle.

It is interesting to find that shark phosphorylase *b* like rabbit and lobster phosphorylase *b* under some experimental conditions (for example, at high P_i concentration, >0.1 M) has some activity in the absence of AMP. Such activity of phosphorylase *b* is small when compared to shark phosphorylase *a* or to that of phosphorylase *b* in the presence of AMP. However, this level of activity may not be negligible when one considers the large amount of this enzyme present in muscle. Furthermore, during rest the animals' requirement to degrade glycogen is so small that the little activity of phosphorylase *b* at low AMP concentration is sufficient; during emergency or excessive muscular activity conversion to phosphorylase *a* may be required. Such a physiological interpretation of our kinetic data seems reasonable as Danforth *et al.* (1962) have demonstrated a conversion from phosphorylase *b* → *a* in contracting frog muscle. A physiological significance for phosphorylase *b* activity in the absence of AMP is also suggested from studies on the "I" strain of mice; these mice possess only the *b* form of phosphorylase but are able to degrade glycogen (Lyon, 1970).

The importance of some properties of shark phosphorylase *b* which differ from that of other muscle phosphorylases such as association tendency of the *b* form, amino acid composition, immunological reactivity, crystallization, and stability in the absence of AMP and thiol reducing compounds remains to be elucidated. It is possible that examining these and other properties in other phosphorylases could lead to determining how some structural parameters are important to the enzyme function.

Acknowledgment

The generous technical assistance of Mary Ann Gross in various stages of this project is gratefully appreciated.

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Influence of Electron Transport on the Interaction between Membrane Lipids and Triton X-100 in *Halobacterium cutirubrum*†

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ABSTRACT: Earlier results (Lanyi, J. K. (1972), *Biochim. Biophys. Acta 282*, 439) showed that respiring *Halobacterium cutirubrum* cells were resistant to Triton X-100 treatment but respiration-inhibited cells could be lysed readily. In this study, the kinetics of (1) turbidity decrease, (2) the appearance of menadione reductase activity, an enzyme that marks the interior surface of the cell membrane, (3) the release of various intracellular constituents, and (4) the shift in the bacteriorubberin absorption spectrum, which indicates the association of this pigment and presumably other membrane lipids with Triton X-100, were followed after adding the detergent to potassium cyanate inhibited and respiring cells. The results indicate that respiring cells lose the integrity of their cell envelope, but, unlike the inhibited cells, resist the penetration of Triton into the membrane lipid phase. The maintenance of respiring cell envelopes in the presence of the detergent is apparently a consequence of this resistance to perturbation.

Numerous enzyme systems have been described which show increased resistance to denaturation in the presence of substrates (Sulkowski and Laskowski, 1968; Reshef and Heller, 1969; Linn *et al.*, 1969; Sudi, 1970), presumably because of structural changes on binding. In biological membranes, which are complex lipoprotein entities, the metabolic state of respiratory enzymes has been found in some cases to influence the behavior of the lipid phase as well. Thus the solubilization of complex III and succinic dehydrogenase in mitochondria by hydrophobic bond-breaking agents is diminished when substrates are present (Rieske *et al.*, 1967; Baginsky and Hatefi, 1969). Respiratory inhibitors, which prevent the resolution of membrane proteins by chaotropes (which increase the lipophilicity of water), also inhibit spontaneous lipid oxidation (Hatefi and Hanstein, 1970). In submitochondrial particles, hydrolysis of lipids by cobra venom phospholipase was found to be less extensive during active electron transport (Luzikov and Romashina, 1972). Another example is the observation that the extraction of lipoproteins from *Escherichia coli* cells by sodium dodecyl sulfate and lysis is dramatically enhanced when the respiratory system is inhibited with potassium cyanide (Bolle and Kellenberger, 1958; Woldringh and van Iterson, 1972). Recently, we found that the *Halobacterium cutirubrum* cell envelope, which contains the electron transport mechanism (Cheah, 1969; Lanyi, 1971), was nearly completely resistant to the nonionic detergent Triton X-100, but when any one of a number of respiratory inhibitors was added the cells rapidly disintegrated (Lanyi, 1972a). Because the halophilic cells, unlike *E. coli*, do not possess a rigid carbohydrate cell wall (Kushner *et al.*, 1964), we expected that the dissolution of the cell envelope in these cells was primarily dependent on disrupting protein–lipid interactions. In the present study, we have investigated the mechanism of the dispersing effect of Triton X-100 on the cell envelope and the membrane lipids, and the possibility that the metabolic state of membrane enzymes has an influence on these processes. While halophilic membranes exhibit some unique characteristics, most notably disintegration upon removal of salt (Kushner, 1964; Stoeckenius and Rowen, 1967; Lanyi, 1971), the results presented in this paper have little obvious relation to such properties. On the contrary, it is our hope that the conclusions drawn may be of general interest in the study of the structure of bacterial membranes.

Materials and Methods

H. cutirubrum cells were grown as described by Hochstein and Dalton (1968), but the medium also contained 0.05 M Tris adjusted to pH 7.0 using a Corning triple-purpose glass electrode (with low Na error). As before (Lanyi, 1972a), late logarithmic phase cells were used, at 3–6 × 10^9 cells/ml, de-

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