Purification and Properties of Yeast Glycogen Phosphorylase *a* and *b**

Michel Fosset,† Larry W. Muir,‡ Larry D. Nielsen,§ and Edmond H. Fischer||

ABSTRACT: Glycogen phosphorylase (α-1,4-glucan:orthophosphate glucosyl transferase, EC 2.4.1.1) was purified from baker's yeast (*Saccharomyces cerevisiae*) and obtained in two molecular forms, homogeneous according to the criteria of gel electrophoresis in sodium dodecyl sulfate, isoelectric focusing in polyacrylamide gels, and sedimentation in the ultracentrifuge. Both have a subunit molecular weight of 103,000 ± 3000, identical amino acid compositions, optimal activity at pH 5.8, an absorbancy index, $A_{280}^{1\%}$, of 14.9, and contain *ca.* 1 mole of pyridoxal 5′-phosphate. One form contains *ca.* 1 mole of covalently bound phosphate per subunit and has a specific activity of 135 ± 10 units/mg; the other contains little or no phosphate and has a specific activity of 25 ± 5 units/mg. By analogy to the mammalian muscle system the two forms have been designated as phosphorylases *a* and *b*, respectively. Sedimentation experiments indicated that phosphorylase *a* exists as a slightly associated dimer with a molecular weight of 250,000 and an $s_{20,w}$ = 9.6 S, and phosphorylase *b*, as a slightly dissociated tetramer with a molecular weight of 390,000 and $s_{20,w}$ = 14.2 S. Kinetic analysis revealed similar Michaelis constants of 2.9 and 2.2 mM for glucose 1-phosphate and 0.51 and 0.65 mg/ml for glycogen for the *a* and *b* forms, respectively. However, these forms differed in their affinities for the first substrate in the absence of the second and in their inhibition by glucose 6-phosphate, with inhibition constants of 11 and 1 mM for *a* and *b*, respectively. Neither form was activated by 2′-, 3′-, or 5′-AMP. There was no immunological cross-reaction between antibodies prepared against yeast phosphorylase and phosphorylases from rabbit muscle, dogfish muscle, or rabbit liver. A protein kinase has been highly purified from yeast which transfers phosphate from ATP into the *b* form of the enzyme converting it into a species with properties identical with those of naturally isolated phosphorylase *a*. Neither rabbit muscle phosphorylase kinase nor phosphatase would interconvert the two forms of the yeast enzyme.

The control of glycogen breakdown and synthesis plays a central role in cellular metabolism. The two enzymes immediately responsible for the regulation of glycogen levels, glycogen synthetase and glycogen phosphorylase, have been studied extensively in a large number of organisms (for review, see Preiss, 1969, Helmreich, 1969, and Fischer *et al.*, 1970). Glycogen phosphorylases have been found in two general classes: those whose activity is regulated by covalent modification and specific effectors (of which the skeletal muscle enzyme has been the prototype) and those for which no covalent regulation has yet been found (of which the phosphorylases from bacteria (Chen and Segel, 1968) and from the potato (Kamogawa *et al.*, 1968) are most representative).

This study was undertaken for the following reasons. First, the regulation of phosphorylase activity by phosphorylation and dephosphorylation of the protein has not been described in either plants or bacteria. If such a control system could be demonstrated in a simple eukaryotic organism, it would be of considerable interest both from the point of view of comparative biochemistry and the evolution of those systems involved in the regulation of carbohydrate metabolism. Second, yeast cells can accumulate large quantities of glycogen when grown anaerobically on high carbohydrate media (Chester, 1963); when switched to minimal media under aerobic conditions, this polysaccharide is quickly depleted (Chester, 1964) suggesting a rapid increase in phosphorylase activity. Third, yeast is an organism amenable to genetic manipulation which could be of distinct advantage in this kind of investigation. This work was initiated on baker's yeast because of its availability in large amounts.

Although previous studies of yeast glycogen phosphorylase have been performed (Kiessling, 1939; Whelan, 1955; Sagardia *et al.*, 1968, 1971), the enzyme has never been obtained in pure form. The present article reports the isolation of baker's yeast phosphorylase in both a phosphorylated (active) and nonphosphorylated (partially active) form, referred to as *a* and *b*, respectively. Some physicochemical and enzymatic properties of the two enzyme species are described as well as the *b* to *a* conversion catalyzed by a purified yeast protein kinase. This paper constitutes the ninth publication on the comparative properties of glycogen phosphorylases (for previous publication, see Cohen and Fischer, 1971).

Materials and Methods

Fleischman baker's yeast was obtained in 1-lb pressed cakes as a generous gift of Standard Brands, Inc. Sephadex G-200 and DEAE-Sephadex were obtained from Pharmacia, Piscataway, N. J. Shellfish glycogen was obtained from Krishell Laboratories, Inc., Portland, Oreg., and further purified by the Somogyi (1957) procedure. Glucose 1-phosphate was obtained from General Biochemicals, Chagrin Falls, Ohio, and further purified as described in Sevilla and Fischer (1969).

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† On leave from CNRS, France. Supported by a North Atlantic Treaty Organization postdoctoral fellowship and the Fulbright-Hays Program. Present address: CNRS, CBM-31, Chemin Joseph-Aiguier, France.

‡ Supported by U. S. Public Health Service Postdoctoral Fellowship 1-F02-AM044,351-01.

§ Supported by U. S. Public Health Service Postdoctoral Fellowship F2-AM-28,039-02; present address: Division of Research, National Jewish Hospital and Research Center, Denver, Colo. 80206.

|| To whom correspondence should be addressed.

Phenylmethane sulfonylfluoride was purchased from Calbiochem and diisopropyl phosphorofluoridate from Pierce Chemical. Streptomycin sulfate was obtained in a 40% solution from Pfizer Laboratories, New York, N. Y. All other reagents were of the highest grade commercially obtainable.

Enzyme Assay. Phosphorylase activity was routinely determined in the direction of glycogen synthesis by the procedure of Hedrick and Fischer (1965) modified for the lower pH optimum of the yeast enzyme. The substrate solution contained 0.15 M glucose 1-phosphate, 2% glycogen, and 0.1 M sodium succinate adjusted to a final pH of 5.8. The enzyme was diluted in 0.1 M sodium succinate buffer (pH 5.8) containing 0.1% bovine serum albumin. Equal amounts of substrate and diluted enzyme were mixed and incubated at 30°. One unit of activity is that amount of enzyme releasing 1 μmole of P_i from glucose 1-phosphate/min at 30°.

Protein was determined by the procedure of Lowry *et al.* (1951) or by absorbancy at 280 nm, using an absorbancy index of $A_{280}^{1\%}$ of 14.9 as determined refractometrically on the pure enzyme by means of the ultracentrifuge (Babul and Stellwagen, 1969). The value calculated from the amino acid composition (Hsiu *et al.*, 1964) was 14.8.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed in 5% gels with minor modifications of the procedure of Weber and Osborn (1969). Isoelectric focusing experiments were performed as described by Wrigley (1968); the gels were stained for protein according to Awdeh (1969). Phosphorylase activity was detected by the procedure of Davis *et al.* (1967), except that the buffer was 0.05 M sodium succinate at pH 5.8. Duplicate gels were sliced into 1-mm sections and soaked overnight in 0.2 ml of distilled water for determination of pH. The pH gradient was linear throughout the gels.

Sedimentation and Molecular Weight Studies. Sedimentation velocity and equilibrium experiments were performed on a Spinco Model E analytical ultracentrifuge with a titanium AN-H rotor and double-sector cells. High-speed equilibrium sedimentation runs were performed by the procedure of Yphantis (1964) using 3-mm columns and low-speed equilibrium runs by the procedure of van Holde and Baldwin (1958) using 2-mm columns. The weight-average molecular weight was determined from the Rayleigh patterns (Richards and Schachman, 1959), and the Z-average molecular weight from the schlieren patterns (van Holde and Baldwin, 1958). All runs were performed at 10° in 0.1 M sodium succinate buffer (pH 5.8) containing 1 mM EDTA. The density of this buffer at 10° was 1.008 g/ml and the relative viscosity 1.064.

Plates were measured with a Nikon microcomparator. The high-speed equilibrium data were processed on an IBM 7094 computer using the program of Teller *et al.* (1969). A partial specific volume of 0.736 ml/g at 20° was estimated from the amino acid composition and was corrected to 0.731 at 10° using the correction factor of $d\bar{v}/dT = 0.0005$ ml/g per deg (Svedberg and Pedersen, 1940).

Amino Acid Analyses. The amino acid compositions were analyzed by the procedure of Moore and Stein (1963) using norleucine as an internal standard (Walsh and Brown, 1962). Because the enzyme precipitated in water or in dilute NaCl solution, it was dialyzed extensively against 1 mM sodium succinate buffer (pH 5.8) before analysis. Duplicate hydrolyses were performed *in vacuo* (after repeated flushing with nitrogen) at 105 ± 1° for periods of 24, 48, 72, and 96 hr. The hydrolyzed samples were analyzed on a Beckman Model 120C automatic amino acid analyzer equipped with an Infotronics Model CRS-10AB2 integrator.

Supporting analyses (cysteine or cystine, tryptophan, or pyridoxal 5′-phosphate) were also performed. Half-cystine was determined as cysteic acid following performic acid oxidation as described by Moore (1963). Sulfhydryl groups were determined by the procedure of Ellman (1959) on sodium dodecyl sulfate denatured enzyme. Samples of phosphorylase *b* (0.5–1 mg) were incubated with 15 mM 2-mercaptoethanol for 30 min at 30°; mercaptoethanol was removed by passing the samples through a small column of Sephadex G-25. The enzyme was incubated in a cuvet containing 1% sodium dodecyl sulfate, 1 mM 5,5′-dithiobis(2-nitrobenzoic acid), and 0.1 M Tris·HCl at pH 8.0 and absorbancy readings were taken until the values remained constant.

Trytophan was determined by the spectrophotometric method of Bencze and Schmid (1957) and pyridoxal 5′-phosphate by the colorimetric procedure of Wada and Snell (1961). Neutral sugars were assayed by the phenolsulfuric acid procedure of Dubois *et al.* (1965).

Conversion of Phosphorylase b into a. Baker's yeast protein kinase was purified approximately 2000-fold (L. Muir and E. Fischer, manuscript in preparation) and γ-labeled [^{32}P]ATP was prepared by the procedure of Glynn and Chapelle (1964). At each step during the purification, fractions were assayed for phosphorylase activity directly and after 15-min preincubation with 20 mM $MgCl_2$, 4 mM ATP, 2 mM EDTA, and 5 μg of yeast protein kinase.

Fully purified yeast phosphorylase *b* was converted into the *a* form by incubating 1.2 mg of the enzyme with 20 μg of kinase in a solution of 0.05 Tris·HCl at pH 7.5, 6 mM $MgCl_2$, 0.75 mM [^{32}P]ATP (1.54 μCi/μmole), and 0.05% bovine serum albumin in a final volume of 1.0 ml. At given times, aliquots were removed and diluted into 0.1 M sodium succinate (pH 5.8) for the determination of phosphorylase activity; others were precipitated with trichloroacetic acid as described by Sevilla and Fischer (1969) for the determination of ^{32}P incorporation.

Immunological Procedures. Rabbits were immunized against yeast phosphorylase by injecting 3–5 mg of a purified mixture of the two forms of the enzyme in complete Freund's adjuvant (Difco) into the footpads. The sera, collected from the marginal ear vein, were precipitated with 40% ammonium sulfate; the pellets were dissolved in and dialyzed against 0.15 M NaCl. Ouchterlony plates were prepared using 1% Oxoid Ionagar No. 2 in 0.1 M sodium succinate buffer at pH 5.8.

Results

Purification of Yeast Phosphorylase. All steps were performed at 4°. Either phenylmethane sulfonylfluoride (0.1 mM) or diisopropyl phosphorofluoridate (1 mM) or both were routinely added at each step of the purification to inhibit proteases. Although up to 20 lb of yeast was sometimes used, the following procedure is described for 8 lb (3.7 kg).

Lots (2 lb) of pressed baker's yeast were soaked in 1 l. of water for 30 min. The yeast cells were dispersed by stirring and 1200 ml of prewashed glass beads (120-μ diameter, 3M Co.) were added. The yeast was ground for 25 min in an Eppenbach colloid mill, Model MV-6-3 which was cooled to 4° by circulating a water–alcohol mixture from a cooling bath. Microscopic examination revealed cell breakage of at least 80%. The extract was decanted and centrifuged at 12,000*g* for 1 hr to remove unbroken cells and debris. The supernatant solution was filtered through glass wool to remove lipids and the filtrate is referred to as the "crude extract" in Table I. A streptomycin sulfate solution was added to a final

TABLE I: Purification of Yeast Glycogen Phosphorylase.[a]

Step	Sp Act.[b] (U/mg)	Purification	Recov (%)
Crude extract	0.15		100
Streptomycin sulfate supernatant	0.20	1.3	94
DEAE-Sephadex eluate	1.8	12.0	55
55% Saturated ammonium sulfate pellet	2.2	14.7	47
First DEAE-Sephadex column	16	106	40
G-200 Sephadex column	45	300	31
Second DEAE-Sephadex column			
Total activity	63[c]	420	27
First peak (*a* form)	135		
Second peak (*b* form)	25		

[a] Average of four preparations. [b] Before activation with kinase. [c] The ratio of phosphorylase *b* to *a* was approximately 1.5.

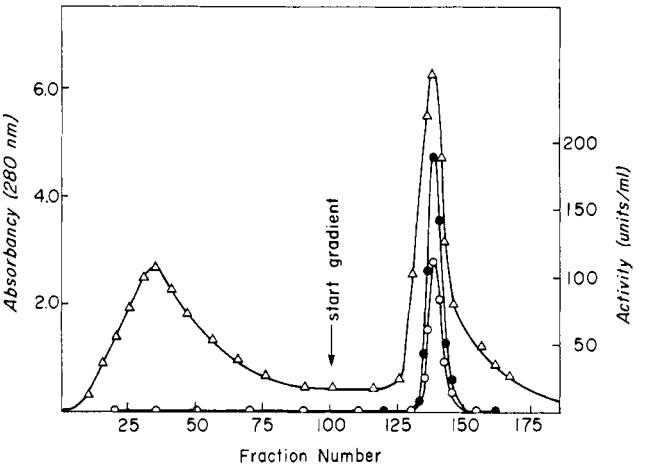

FIGURE 1: DEAE-Sephadex chromatography of yeast phosphorylase. The enzyme was applied to a 2.5 × 34 cm column of DEAE-Sephadex A-50 equilibrated with 0.1 M sodium succinate–1 mM EDTA (pH 5.8). The flow rate was 25 ml/hr and 5-ml fractions were collected. A linear gradient of 0.1–0.4 M sodium succinate–1 mM EDTA at pH 5.8 was run with 200 ml in each chamber. Absorbancy at 280 nm (△) and phosphorylase activity both prior to (○) and following activation (●) with yeast phosphorylase kinase as described in Methods are illustrated.

concentration of 0.7%. After a few minutes of stirring, the solution was centrifuged at 12,000*g* for 30 min, the large pellet was discarded and the supernatant solution was adjusted to pH 5.8 with 2 N NaOH.

This solution was passed through a large Büchner funnel containing approximately 100 g of DEAE-Sephadex A-50 equilibrated with 0.1 M sodium succinate and 1 mM EDTA (pH 5.8). The ion exchanger was washed with approximately 20 l. of the above buffer, phosphorylase activity was eluted with 0.5 M sodium succinate at pH 5.8 and this solution was precipitated by adding solid ammonium sulfate to 55% saturation. After 2 hr, the mixture was centrifuged at 10,000*g* for 30 min; the pellets were resuspended in a 55% saturated ammonium sulfate solution and recentrifuged to remove traces of proteolytic enzymes which remained in the first ammonium sulfate supernatant.[1] The second pellet was resuspended in a minimal volume of 0.1 M sodium succinate–1 mM EDTA (pH 5.8) and dialyzed overnight against this same buffer. The preceding steps were carried out in a single day to minimize proteolysis.

The dialysate was centrifuged at 100,000*g* for 1 hr and the clear supernatant solution was chromatographed on a column of DEAE-Sephadex A-50 as shown in Figure 1. The fractions containing phosphorylase acitivity were pooled and precipitated by addition of solid ammonium sulfate to 55% saturation. The precipitate was collected by centrifugation at 10,000*g* for 30 min and redissolved in 4 ml of 0.13 M sodium succinate–1 mM EDTA (pH 5.8). The solution was dialyzed for several hours in this buffer and centrifuged at 100,000*g* for 1 hr.

Phosphorylase was then chromatographed on Sephadex G-200 as shown in Figure 2; active fractions were pooled and concentrated by vacuum dialysis. Finally, the enzyme was chromatographed once more on DEAE-Sephadex A-50 as shown in Figure 3. The enzyme emerged in two symmetrical peaks which were pooled separately and concentrated by vacuum dialysis. Specific activities were constant at 135 ± 10 units/mg across the first peak and at 25 ± 5 units/mg across the second peak (as compared to 85 units/mg for crystalline rabbit muscle phosphorylase). By analogy with the rabbit muscle system, these two forms have been referred to as yeast phosphorylases *a* and *b*, respectively. Table I summarizes the average results obtained on four typical preparations.

Enzyme Purity. Both yeast phosphorylase fractions gave single bands on polyacrylamide gel electrophoresis in sodium dodecyl sulfate as shown in Figure 4A, 1 and 2. Mixtures of the two forms of the enzyme (Figure 4A, 3) gave a single band indicating that both have essentially identical subunit molecular weights, with an extrapolated value of 103,000 ± 3000 based on protein standards of known molecular weights (Figure 5). A mixture of the purified yeast and rabbit muscle phosphorylases gave two very close but distinct bands supporting the view that the former enzyme has a molecular weight

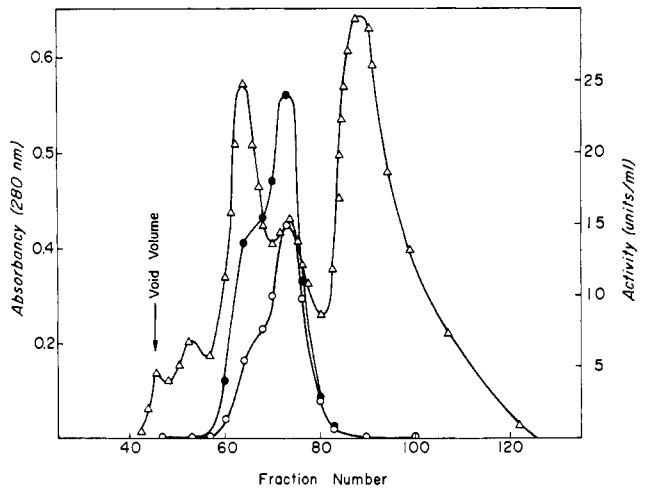

FIGURE 2: G-200 Sephadex gel filtration of yeast phosphorylase. A 5-ml sample was applied to a 2.5 × 120 cm column of Sephadex G-200 swollen in 0.13 M sodium succinate–1 mM EDTA (pH 5.8). The flow rate was 15 ml/hr and 6-ml fractions were collected. Absorbancy at 280 nm (△)and phosphorylase activity both before (○) and after (●) activation of individual fractions with yeast phosphorylase kinase are illustrated.

[1] R. Kuhn, personal communication.

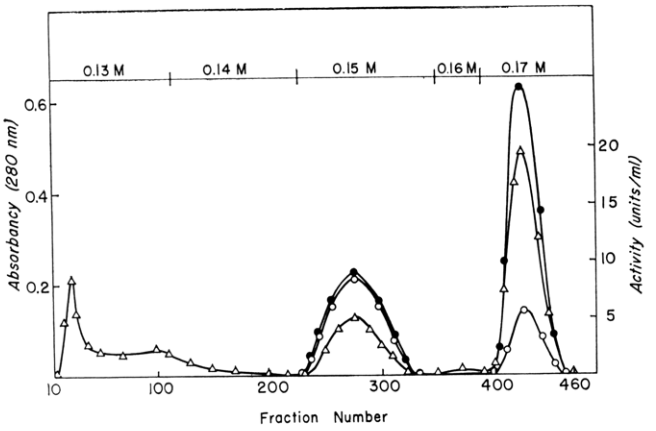

FIGURE 3: A 1.2 × 80 cm column of DEAE-Sephadex A-50 was equilibrated against 0.13 M sodium succinate–1 mM EDTA at pH 5.8. Stepwise elution as indicated in the figure was carried out at a flow rate of 35 ml/hr and 11-ml fractions were collected. Absorbancy at 280 nm (△) and phosphorylase activity both before (○) and after (●) kinase activation of individual fractions are illustrated.

slightly greater than the latter (mol wt 100,000 ± 2000; Cohen and Fischer, 1971).

Isoelectric focusing in polyacrylamide gels revealed single bands for the two phosphorylase species as visualized by both

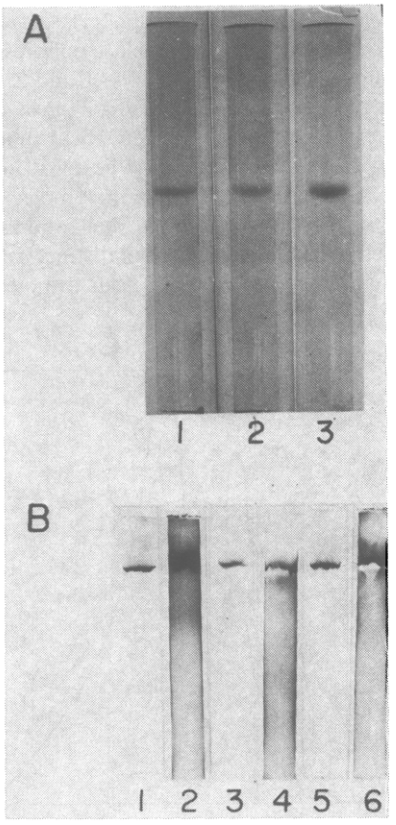

FIGURE 4: (A) Sodium dodecyl sulfate gel electrophoresis of yeast phosphorylase on 5% polyacrylamide gels. Five micrograms of each sample was applied per gel and these were stained with coomassie blue. (1) Phosphorylase *a*, (2) phosphorylase *b*, and (3) 1:1 mixture of both. (B) Polyacrylamide gel isoelectric focusing experiments. Protein and enzyme activity stains, respectively, are illustrated for phosphorylase *a* (1 and 2), phosphorylase *b* (3 and 4), and a mixture of both (5 and 6). Enzyme (20–50 μg) was applied in each case.

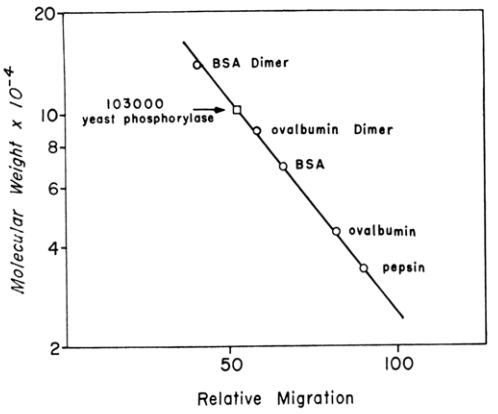

FIGURE 5: Calibration curve for sodium dodecyl sulfate gel electrophoresis. Approximately 5 μg of each protein was applied to the gels. BSA = bovine serum albumin.

protein and activity stains (Figure 4B). Measurement of the pH gradient in these gels indicated an isoelectric point of pH 5.1 ± 0.1.

Sedimentation Analysis. The molecular weights of the two forms of the enzyme were measured by low- and high-speed sedimentation equilibrium. The results are summarized in Table II. The agreement between the two sets of experiments is good. Considering the subunit molecular weight of *ca.* 103,000 suggested by sodium dodecyl sulfate gel electrophoresis, it must be assumed that the *b* form of the enzyme with a molecular weight of *ca.* 370,000 must exist in a partially dissociated tetrameric state. The large difference between the values for the number-, weight-, and Z-average molecular weights, together with the distribution of molecular weights of the species across the centrifuge cell supports this assumption.

Yeast phosphorylase *a* also showed a strong concentration dependence of molecular weight across the centrifuge cell

TABLE II: Sedimentation Constants and Distribution of the Average Molecular Weights for Yeast Phosphorylases *a* and *b*.

Form of the Enzyme	Condn for Sedimentation Equil	M_w	M_Z	$s_{20,w}$ (4.5 mg/ml)
a (phospho)	Low speed (5200 rpm, 5 mg/ml)	250,000	262,000	9.6 S
	High speed[a] (11,000 rpm, 1 mg/ml)	232,000	254,000	
b (dephospho)	Low speed (4400 rpm, 5 mg/ml)		380,000[1]	4.2 S
	High speed[b] (9000 rpm, 0.95 mg/ml)	373,000	390,000	

[a] An M_n value of 203,000 was calculated for phosphorylase *a*. [b] An M_n value of 350,000 was calculated for phosphorylase *b*.

TABLE III: Amino Acid Composition of Glycogen Phosphorylases (moles/100,000 g).

Amino Acid	Yeast *a*[a]	Yeast *b*[b]	Rabbit Muscle[c]	Rabbit Liver[d]	Potato[e]
Lysine	55.7 + 1.1	59.0 ± 2.0	46.9	59.4	65.7
Histidine	16.1 ± 0.6	16.6 ± 0.7	20.9	23.7	15.8
NH_3		102	77.2		38.0
Arginine	37.9 ± 0.6	37.3 ± 0.9	63.8	47.5	39.1
Aspartic acid	114.9 ± 1.7	114.8 ± 2.0	98.2	99.4	89.8
Threonine	41.9 ± 2.3	40.8 ● 0.7	34.3	35.6	44.0
Serine	51.7 ± 1.1	51.4 ± 1.0	29.5	42.1	45.2
Glutamic acid	104.6 ± 1.7	102.9 ± 1.1	102.0	90.8	109.3
Proline	39.6 ± 1.7	38.8 ± 1.8	42.5	38.9	36.8
Glycine	49.4 ● 1.1	48.3 ± 0.9	49.6	52.9	59.4
Alanine	55.7 ± 0.6	56.4 ± 1.4	65.2	67.0	74.4
Valine	58.6 ± 1.1	59.5 ± 1.0	61.3	62.6	55.5
Methionine	14.9 ± 0.6	16.6 ± 0.5	21.8	24.8	13.3
Isoleucine	57.4 ± 1.1	57.0 ± 0.8	48.5	51.8	53.8
Leucine	84.9 ± 1.1	82.6 ± 1.3	81.4	86.1	67.1
Tyrosine	33.9 ± 0.6	34.8 ± 0.8	36.6	27.0	30.3
Phenylalanine	40.8 ± 0.6	41.8 ± 0.5	38.5	42.1	38.0
Half-cystine	5.1 ± 0.3	5.0 ± 0.3	8.5	12.9	
Tryptophan	17.8 ± 0.3	18.1 ± 0.5	13.0	12.9	20.8
Total residues	880.9	881.7			

[a] Average of two times of hydrolysis (24 and 48 hr) on duplicate samples. [b] Average of four times of hydrolysis on duplicate samples. [c] Recalculated from data of Sevilla and Fischer (1969). [d] Recalculated from data of Wolf *et al.* (1970). [e] Recalculated from data of Kamogawa *et al.* (1968).

in the high-speed sedimentation experiment (Table II). Using higher concentrations of enzyme, low-speed sedimentation equilibrium gave an M_w value of 250,000, significantly greater than the molecular weight of *ca.* 200,000 expected for the dimeric form of the enzyme, and suggested about 20% association to higher forms. This assumption is supported by the sedimentation velocity pattern in Figure 6, which shows a slight forward skewing of the *a* form indicative of an association–dissociation equilibrium. Calculation of the sedimentation coefficient from the second moment gave an $s_{20,w} = 9.6$ S. The tetrameric form of the enzyme gave a single symmetrical peak (Figure 6) with an $s_{20,w} = 14.2$ S. Since both forms were homogeneous by the criteria of disc gel electrophoresis and isoelectric focusing, it would seem unlikely that the slight polydispersity seen in the ultracentrifuge could be attributed to protein contaminants.

Amino Acid Analyses. Values presented in Table III are the averages obtained from four times of hydrolysis on duplicate samples of phosphorylase *b*. Values for serine, threonine, and ammonia were extrapolated to zero time of hydrolysis and those for valine and isoleucine were calculated from the maximum values obtained upon prolonged hydrolysis. Yeast phosphorylase *a* was also analyzed but only after 24- and 48-hr hydrolysis. Also listed in Table III are the compositions of three other phosphorylases, each representative of a different type of regulation, namely, enzymes that are activated by both phosphorylation and AMP (rabbit muscle), by phosphorylation alone (rabbit liver), and by neither one nor the other (potato).

Sulfhydryl groups were titrated by the Ellman procedure; 5.9 residues were detected per 103,000 g of yeast phosphorylase *b* following denaturation in sodium dodecyl sulfate. Since performic acid oxidation yielded 5.5 cysteic acid residues/103,000 g of enzyme, a value of 6 half-cystines per enzym monomer appears most reasonable.

Identification and Determination of Pyridoxal 5'-Phosphate. Yeast phosphorylase (5 mg) was precipitated with 0.3 N perchloric acid; after centrifugation of the denatured protein,

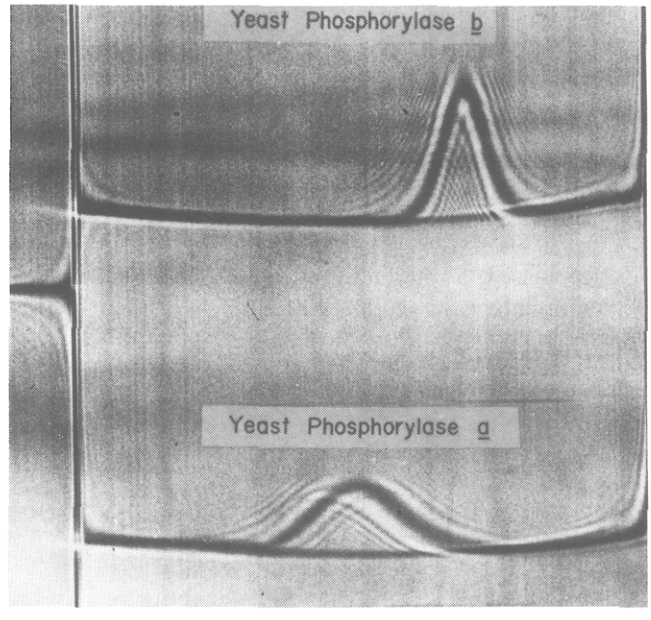

FIGURE 6: Sedimentation velocity ultracentrifugation of yeast phosphorylase *b* (upper pattern) and *a* (lower pattern). Conditions were 5 mg/ml in 0.1 M sodium succinate (pH 5.8) with 1 mM EDTA and 7 mM 2-mercaptoethanol at 48,000 rpm, 64 min. Sedimentation from left to right.

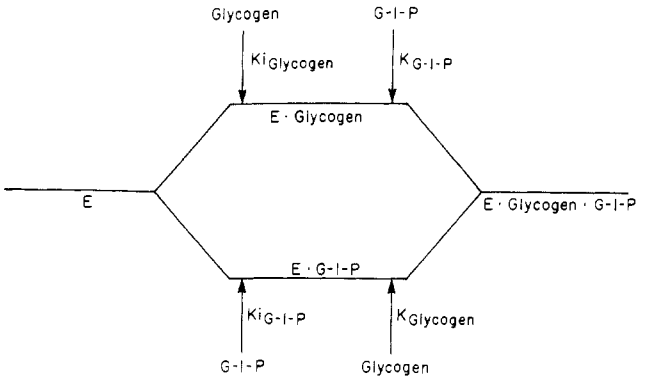

FIGURE 7: Schematic representation of a possible reaction mechanism for yeast phosphorylase according to Cleland (1967, 1970).

TABLE IV: Kinetic Parameters of Yeast Phosphorylase.[a]

	Phosphorylase *a*	Phosphorylase *b*
K_{G-1-P} (mM)	2.9	2.2
$K_{glycogen}$ (mg/ml)	0.51	0.65
$K_{i,G-1-P}$ (mM)	0.7	1.5
$K_{i,glycogen}$ (mg/ml)	0.04	0.54
V_I [b]	135 ± 10	25 ± 5
V_I/E_t [c]	1.35×10^4	2.5×10^3

[a] The nomenclature of Cleland (1967) has been used. [b] Expressed as micromoles of inorganic phosphate released per minute per milligram of enzyme at 30°. [c] Expressed as moles of G-1-P consumed per mole of enzyme subunit per minute at 30°.

pyridoxal 5′-phosphate was identified in the clear supernatant solution by its uv absorption spectrum at various pH's and its ability to reactivate rabbit muscle apophosphorylase *b* prepared by the procedure of Shaltiel *et al.* (1966). Quantitative analysis by the procedure of Wada and Snell (1961) indicated the presence of 0.98 ± 0.05 mole of pyridoxal 5′-phosphate/103,000 g of protein.

Carbohydrates. Phenol–sulfuric analysis revealed less than 1% carbohydrate (expressed as glucose) for the isolated *a* form of the enzyme but less than 0.1% for the *b* form. This low value which represents less than 1 molecule of hexose/enzyme subunit can probably be ascribed to contamination rather than covalently bound carbohydrate.

pH and Enzyme Activity. Yeast phosphorylase has optimal activity at pH 5.8. Exposure to pH values lower than pH 5.0 results in precipitation of the enzyme; attempts to make use of this property during purification of the enzyme were abandoned because of poor recoveries. This was attributed to the presence of an acid protease (Hata *et al.*, 1967a) which is inhibited neither by diisopropyl phosphorofluoridate nor phenylmethane sulfonylfluoride.

Activity is rapidly and irreversibly lost by exposure of the enzyme to pH values greater than 7.0. Incubation of the enzyme at pH 7.5 in the presence of a variety of compounds including 0.1 mM pyridoxal phosphate, 7 mM AMP, 0.1% glycogen, 0.1 M NaCl, 0.1 M NaF, 30 mM 2-mercaptoethanol, and 30 mM L-cysteine did not prevent this inactivation. On the other hand, 10 mM divalent metal ions including Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Ba^{2+}, and Ni^{2+}, and 10 mM glucose 6-phosphate completely stabilized the enzyme. Enzyme activity could not be restored once it had been lost. Preliminary sedimentation velocity runs with phosphorylase *a* indicated that inactivation of the enzyme was accompanied by a decrease in the sedimentation constant; inclusion of as little as 1 mM Mg^{2+} partially prevented this decrease. These results suggested that the loss of enzyme activity was related to a monomerization of the enzyme and that divalent metal ions and glucose 6-phosphate were effective in preventing this dissociation.

Kinetics. The reaction rate in the standard assay was linear with respect to time and enzyme concentration. Concentrations of glucose 1-phosphate greater than 25 mM caused a slight inhibition of phosphorylase *a* noted by an upward curvature of the Lineweaver–Burk plot; it did not exceed 10% in the standard test carried out at 75 mM glucose 1-phosphate.

Kinetic parameters for glucose 1-phosphate and glycogen were determined in the direction of glycogen synthesis according to Cleland (1967, 1970). This procedure for bisubstrate reactions gives both the Michaelis constant, K_a, for one substrate (A) in the presence of saturating levels of the other (B) and the dissociation constant, K_{ia}, for substrate A in the absence of substrate B (Figure 7). As shown in Table IV, the Michaelis constants for both enzyme forms are quite similar whereas phosphorylase *a* has considerably higher affinity for a single substrate in the absence of the other. These results suggest that at low levels of glucose 1-phosphate the *a* form of the enzyme would be more readily bound to glycogen.

A rapid equilibrium random bi-bi mechanism has been proposed for rabbit muscle phosphorylase (Lowry *et al.*, 1964, 1967; Engers *et al.*, 1969; Gold *et al.*, 1970) and *E. coli*, maltodextrin phosphorylase (Chao *et al.*, 1969). The possibility of a Ping-Pong mechanism for yeast phosphorylase has been excluded in the present study; although the data are consistent with a random bi-bi mechanism, further work is required to rule out other possibilities.

Effectors. In contrast to most animal phosphorylases, the yeast enzyme was not affected by AMP over a concentration range of 5×10^{-5} to 5×10^{-3} M and at various substrate concentrations. Likewise UMP, CMP, IMP, UDPG, and fructose 6-phosphate (all at 2 mM), 2′- and 3′-AMP (10 mM), and glucose and sodium fluoride (20 mM) had no effect on either form of the enzyme under standard assay conditions.

As with the glycogen phosphorylases from rabbit liver (Appleman *et al.*, 1966) and from corpus luteum (Yunis and Assaf, 1970), Na_2SO_4 activated the nonphosphorylated *b* form of the enzyme almost twofold (maximum activation at 0.4 M). In contrast to the inhibitory effects of this salt noted with the phosphorylated forms of liver and corpus luteum phosphorylases, yeast phosphorylase *a* was stimulated 20% at 0.4 M Na_2SO_4.

Glucose 6-phosphate was an effective inhibitor of both forms of the enzyme; the inhibition was noncompetitive with respect to both glucose 1-phosphate and glycogen. Reciprocal plots yielded an apparent inhibition constant, K_I, of 11 mM for phosphorylase *a* and 1 mM for phosphorylase *b*. By replotting the slopes and intercepts of these primary plots according to Cleland (1970), a linear relationship was obtained with respect to glucose 1-phosphate, and parabolic with respect to glycogen. Further interpretation of these data will have to await a more detailed kinetic analysis of the system, which is beyond the scope of this communication.

Since AMP and glucose 6-phosphate act in opposite and competing ways on the activity of muscle phosphorylase *b*

(Parmeggiani and Morgan, 1962) an attempt was made to determine whether this nucleotide affected glucose 6-phosphate inhibition of yeast phosphorylase, even though it does not affect the activity of the enzyme itself. Inclusion of 2.5 mM AMP did not alter the glucose 6-phosphate inhibition of either form of the enzyme.

Conversion of Yeast Phosphorylase b into a by Phosphorylation of the Protein. Early in this study it was noted that incubation of a crude phosphorylase preparation with Mg^{2+} and ATP caused increases in enzyme activity. Later, by using γ-labeled [^{32}P]ATP and more purified enzyme solutions, it could be shown that activation of phosphorylase was accompanied by an incorporation of phosphate into the protein. This reaction obviously required an additional enzyme since the most purified fractions were no longer activated by addition of Mg^{2+}·ATP alone; purified rabbit muscle phosphorylase kinase would not substitute for the assumed endogenous yeast phosphorylase kinase. A search was therefore undertaken for the enzyme that would convert phosphorylase *b* into *a*; a yeast protein kinase was identified and eventually isolated in highly purified form (L. Muir and E. Fischer, manuscript in preparation). The action of this enzyme on phosphorylase at various stages of purification is illustrated in Figures 1–3: individual fractions across all phosphorylase peaks were tested for activity before and after conversion in the presence of Mg^{2+} and ATP. In Figure 1 activated phosphorylase emerges at the same position as the unactivated phosphorylase indicating that this chromatographic step does not separate the two forms of the enzyme. The Sephadex G-200 elution profile (Figure 2) shows maximum activation at the leading edge of the phosphorylase peak with essentially no activation at the trailing edge; this result agrees with sedimentation experiments which showed that the unactivated *b* form of the enzyme has a higher molecular weight than the activated one. Figure 3 illustrates the complete separation of the two yeast phosphorylases; phosphorylase *a* in the first peak is not activated by the protein kinase whereas phosphorylase *b* in the second peak can be activated to essentially the same specific activity as phosphorylase *a*.

A more detailed study of the time course of phosphate incorporation and activation of phosphorylase *b* was performed as described under Methods (Figure 8A). Phosphorylase *b* incorporated 0.83 mole of phosphate/100,000 g of protein. Activation occurred more rapidly than phosphorylation as observed for the conversion of rabbit muscle phosphorylase b into *a* by rabbit kinase (Krebs *et al.*, 1958; Sevilla and Fischer, 1969) possibly indicating the formation of partially phosphorylated hybrids (Hurd *et al.*, 1966; Fischer *et al.*, 1968). Preliminary sedimentation velocity data indicated that phosphorylation was accompanied by a change in quaternary structure of the enzyme in which the tetrameric form was partially converted into dimeric species. Yeast phosphorylase *a* neither incorporated phosphate nor was it further activated under the same conditions. Figure 8B shows that the bound phosphate is acid stable and base labile suggesting that phosphorylation had occurred at a seryl or threonyl residue.

Immunodiffusion. Antibodies to a mixture of both forms of yeast phosphorylase were prepared as described under Methods. These reacted equally well with either pure yeast phosphorylase *a* or *b* by the Ouchterlony double-diffusion procedure. A continuous precipitin band formed with no spurs. Pure rabbit muscle phosphorylase *b*, dogfish muscle phosphorylase *b*, or rabbit liver phosphorylase did not cross-react with the antibodies to yeast phosphorylase. Furthermore, antibodies to glycogen phosphorylase from bovine corpus luteum (provided by Dr. A. Yunis) gave no cross-reaction with either form of the yeast enzyme.

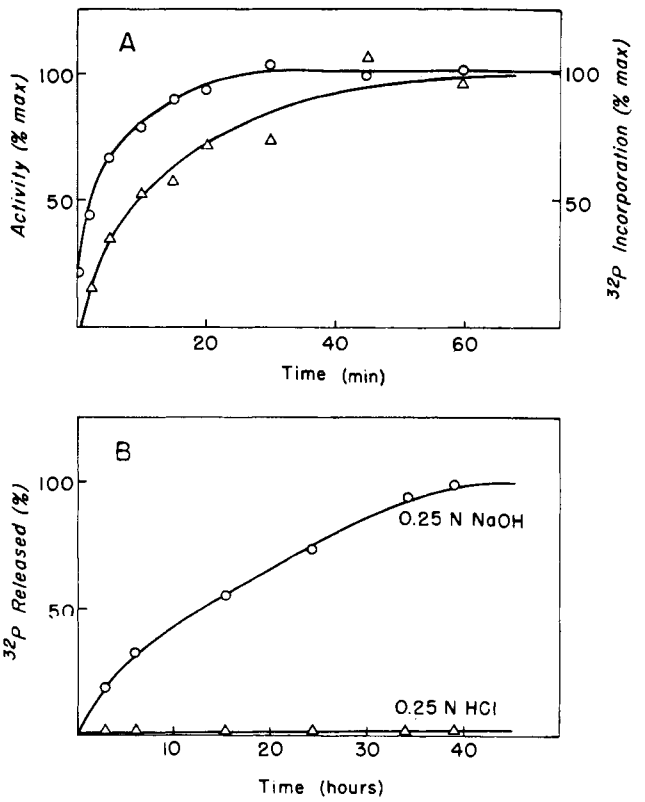

FIGURE 8: (A) Action of the yeast protein kinase on yeast phosphorylase *b*. Increases in activity (○) and incorporation of ^{32}P into the protein (△) are plotted as per cent of maximum values obtained. Conditions are indicated under Methods. (B) Release of $^{32}P_i$ from ^{32}P-labeled phosphorylase *a* in 0.25 N HCl and 0.25 N NaOH at 25°.

Discussion

By analogy with the mammalian muscle system, the phosphorylated and nonphosphorylated forms of yeast glycogen phosphorylase have been referred to as *a* and *b*, respectively. The choice of this nomenclature, originally introduced by Cori and Green (1943) to differentiate the forms of rabbit muscle phosphorylase that are active and inactive in the absence of AMP, was dictated by the following considerations. First, their designation as "phospho" and "dephospho" phosphorylases as used for the rabbit liver enzyme (Sutherland and Rall, 1960) is misleading since all glycogen phosphorylases contain bound phosphate in the form of pyridoxal 5′-phosphate. It has also been demonstrated that during interconversions of the two forms of the rabbit muscle enzyme, hybrids made up of phosphorylated and nonphosphorylated subunits can be produced whose state of activity varies with the concentration of substrate and effectors such as glucose 6-phosphate (Hurd *et al.*, 1966; Fischer *et al.*, 1968). Second, their labeling as "dimeric" and "tetrameric" species should be avoided since one is dealing with an associating–dissociating system whose state of aggregation also varies considerably with experimental conditions (dilution, temperature, salts, etc). Moreover, there is no consistent relationship between the quaternary structure and state of activity of various phosphorylases (for review, see Fischer *et al.*, 1970). Finally, a

physiologically appealing designation such as "active" and "inactive" is generally ambiguous because, once more, activity can be elicited or suppressed by appropriate effectors; it is erroneous in this case since the *b* form of the yeast enzyme appears to possess some activity of its own.

An early approach to the purification of yeast phosphorylase involved the adsorption of the partially purified enzyme to glycogen molecules and separation of the enzyme–glycogen complex by high-speed centrifugation. The pellet was dissolved with crystalline human salivary α-amylase and the digest was concentrated and applied to a column of Sephadex G-200. Although this step gave a 10- to 15-fold purification, and yielded material with a specific activity of 40–50 units/mg, problems with reproducibility and low yields dictated a different approach.

There is ample evidence that yeast cells contain different proteases which can modify enzymes during their purification. One well-documented case is that of hexokinase in which Schulze and Colowick (1969) used phenylmethane sulfonylfluoride to minimize proteolysis: even highly purified preparations still contained small amounts of proteases whose presence became apparent during sodium dodecyl sulfate gel electrophoresis (Pringle, 1970a,b). A negative response in standard assays cannot be taken as proof for the absence of proteolytic enzymes since wide variations in substrate specificities and experimental conditions exist and certain proteases are released upon cell breakage in an inactive form that becomes activated upon standing (Lenney and Dalbec, 1969). Therefore, inhibitors such as diisopropyl phosphorofluoridate and phenylmethane sulfonylfluoride should be added through the purification even though some proteases are resistant to these inhibitors (Hata *et al.*, 1967a,b; Maddox and Hough, 1970).

Problems with proteases were unusually acute during the early stages of purification since several of these were eluted from DEAE-Sephadex together with phosphorylase. On the other hand, most of them could be eliminated by precipitation with 55% saturated ammonium sulfate as they do not appear to precipitate before 90% saturation.[1] Although no evidence could be obtained indicating that the purified enzyme had sustained any proteolysis, this possibility cannot be completely excluded.

The estimated subunit molecular weight of 103,000 ± 3000 obtained from sodium dodecyl sulfate gel electrophoresis is consistent with the extent of ^{32}P incorporation and pyridoxal 5′-phosphate content. Peptide maps following tryptic digestion have indicated the presence of approximately 70 ninhydrin-positive spots while the maximum theoretical number would be 95.

The active conformation of all mammallian muscle phosphorylases (except rabbit heart isoenzyme III, have a strong tendency to tetramerize, even though the phosphorylated dimeric species were shown to be the most active (Wang and Graves, 1964). Yeast phosphorylase behaves more logically in this respect since phosphorylation of the protein converts the less active tetramer into an active dimer. Phosphorylase *a* prepared from purified yeast phosphorylase *b* by conversion with the yeast protein kinase had properties indistinguishable from those of the most active species isolated directly from the cell.

Throughout much of this study, it was not known whether the low activity present in phosphorylase *b* preparations was an intrinsic property of this form of the enzyme, or whether it was due to contamination by some active *a* form. This latter possibility cannot be easily confirmed by direct phosphate determination because of the amount of pure material that would be required and the presence of pyridoxal 5′-phosphate which must be quantitatively eliminated prior to the analysis. In its favor is the fact that both forms of the enzymes have essentially identical K_m's for glycogen and glucose 1-phosphate, the two substrates for the enzyme (Table IV). Against it is the observation that the two enzyme forms have distinctly different binding constants for the first substrate in the absence of the second; they show different inhibition by glucose 6-phosphate and activation by Na_2SO_4. Furthermore, they are well resolved by DEAE-Sephadex chromatography and contamination of the tetrameric *b* form by *ca.* 20% of dimeric phosphorylase *a* should have been readily detected during sedimentation analysis. There remains however the possibility that the activity displayed by phosphorylase *b* is due to phosphorylated subunits present not in the form of typical homologous phosphorylase *a* molecules (fully phosphorylated dimers) but in that of partially phosphorylated hybrids. These hybrids might be expected to display different quaternary structures, kinetic properties, and ion-exchange elution patterns. If they exist, they would have to be considerably more stable than those produced from rabbit muscle phosphorylase (Fischer *et al.*, 1968).

When the amino acid composition of yeast phosphorylase (Table III) was compared to that of other phosphorylases in terms of the composition divergence factor of Harris *et al.* (1969), this enzyme appeared to be as distantly related to rabbit muscle phosphorylases (0.049) as to potato phosphorylase (0.047). Least divergence was obtained with dogfish muscle (0.034) or rabbit liver (0.038) phosphorylases. By contrast, divergence among all vertebrate muscle phosphorylases does not exceed 0.027 whereas the difference between mammalian muscle phosphorylases and the potato enzyme is *ca.* 0.06; totally unrelated proteins would have divergence factors between 0.1 and 0.2.

Animal phosphorylase kinases and phosphatases have low degrees of species specificities: the rabbit muscle enzymes will catalyze the interconversions of vertebrate and crustacean phosphorylases *a* and *b*. As expected, the amino acid sequence of the phosphorylated site of all enzymes so far examined are remarkably homologous (for review, see Fischer *et al.*, 1970). By contrast, no interconversion of yeast phosphorylase *b* and *a* could be catalyzed by purified rabbit muscle phosphorylase kinase or phosphatase and, conversely, the purified yeast protein kinase described herein would not phosphorylate either dogfish or rabbit muscle phosphorylase *b*. No fraction has yet been isolated from yeast that will catalyze the dephosphorylation of yeast phosphorylase *a* though this reaction could be carried out by a crude rabbit muscle extract. The factor responsible for this conversion is presently being investigated in both yeast and rabbit muscle. The factors controlling glycogen breakdown in yeast are not fully understood. As will be discussed in more detail in a subsequent publication, the yeast protein kinase which catalyzes the phosphorylase *b* to *a* conversion has been found only in an active form. In growing yeast cells, the intracellular concentration of ATP varies between 1 and 4 mM, *i.e.*, more than enough to bring about the complete conversion of phosphorylase *b* into *a*. In the baker's yeast used in this study, approximately 60% of the enzyme was found in the nonactivated *b* form.

Rothman and Cabib (1969) have measured the levels of several metabolites during glycogen accumulation in growing cells. During periods of maximal glycogen synthesis, glucose 6-phosphate levels rose as high as 4–8 mM; such concentrations would virtually eliminate phosphorylase *b* activity and fully activate yeast glycogen synthetase. Conversion of phos-

phorylase *b* into *a* may provide a means of overcoming this inhibition when glycogen breakdown is required even in the presence of high levels of glucose 6-phosphate. The role of glucose 6-phosphate in yeast metabolism is probably not limited to this dual effect on glycogen phosphorylase and synthetase; recent reports suggest that this compound also serves as an "inducer" for the synthesis of the glycolytic enzymes (Maitra and Lobo, 1971a,b). A detailed understanding of the regulation of glycogen levels in yeast will require further study on both glycogen phosphorylase and synthetase and the factors involved in the interconversion of the two forms of these enzymes.

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