Purification and Properties of Glycogen Phosphorylase from Bovine Corpus Luteum. Kinetics of Salt Activation*

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ABSTRACT: The phosphorylated and unphosphorylated forms of bovine corpus luteum phosphorylase were purified by ammonium sulfate fractionation, DEAE-cellulose, and Sephadex G-200 column chromatography. The purified enzyme appeared to be homogeneous as determined by electrophoresis in polyacrylamide gel and antigen–antibody precipitation in agar. Molecular weight determination by sucrose density gradient ultracentrifugation gave a value of 185,000 for both forms of the enzyme. Kinetic experiments in the direction of glycogen synthesis showed substrate activation of the unphosphorylated enzyme by glucose 1-phosphate. This substrate activation was abolished at high (0.4 M) concentrations of sodium sulfate. No substrate activation was observed for the phosphorylated enzyme. The activation at high substrate concentration was attributed to an increase in the dissociation rate of (ES), the enzyme–substrate complex (attributed to a conformational change), to free enzyme + products. The substrate activation phenomenon observed here is consistent with the mechanism of negative cooperativity discussed by Conway and Koshland. Unphosphorylated corpus luteum phosphorylase was reversibly activated by sodium sulfate. Kinetic studies in the presence of sodium sulfate indicate that this salt acts as an allosteric activator. The mechanism of this activation is discussed. The phosphorylated enzyme was inhibited by sodium sulfate and the inhibition was competitive with respect to glucose 1-phosphate. Antibody prepared against phosphorylated corpus luteum phosphorylase gave a reaction of identity against bovine liver phosphorylase, a reaction of partial identity against human leukocyte phosphorylase, but showed no cross reactivity with bovine, rabbit, or human muscle phosphorylase. The similarity in the molecular weights of the phosphorylated and unphosphorylated enzyme, their behavior in the presence of high salt concentration, and the immunological findings indicate that glycogen phosphorylase from corpus luteum and liver are closely related.

The isolation of glycogen phosphorylase from many tissues has permitted extensive comparative studies on this enzyme. Although phosphorylases from various sources have many properties in common, certain distinct differences have been found in phosphorylase isolated from different tissues of the same species; rabbit heart (Yunis *et al.* 1962), rabbit liver (Appleman *et al.*, 1966), rabbit muscle (Cori *et al.*, 1943; Fischer and Krebs, 1958), and from similar tissues of different species; phosphorylase from rabbit muscle as compared with the enzyme from human (Yunis *et al.*, 1960), frog (Metzger *et al.*, 1968), rat (Sevilla and Fischer, 1969), and lobster (Assaf and Graves, 1969) and human muscle *vs.* shark muscle (Yunis and Assaf, 1970). The specific properties exhibited by the enzyme from a given tissue probably determine its role in the regulation of glycogen degradation in that tissue. Recent interest has centered on the role of cyclic AMP in hormone-stimulated steroidogenesis in the adrenals and ovaries. Marsh and Savard (1964) have suggested that the activation of corpus luteum phosphorylase may serve as one mechanism for the control of progesterone synthesis under the influence of luteinizing hormone of the anterior pituitary. Because little is known about the properties of phosphorylase from this source, we have undertaken the present study in which phosphorylase from bovine corpus luteum was purified to homogeneity. The molecular weight of the phosphorylated and unphosphorylated forms of the enzyme, its activation by salt and its immunologic and kinetic behavior were studied.

Materials and Methods

Yeast AMP crystalline sodium salt, ATP disodium salt, α-D-glucose 1-phosphate dipotassium salt, DEAE-cellulose, and glycogen (shell fish) were obtained from Sigma Chemical Co., St. Louis, Mo. Glycogen was freed of AMP by treatment with activated charcoal. Streptomycin sulfate was obtained from Squibb Company, New York, N. Y.

Phosphorylase Assay. Phosphorylase activity was assayed by a modification of the method of Sutherland and Wosilait (1956) as previously described (Yunis and Arimura, 1966a). The activity was expressed as enzyme units per ml or per mg of protein; a unit being defined as 1 μmole of inorganic phosphate liberated in a 30-min period, at 37°. Protein concentration was determined by the method of Lowry *et al.* (1951), using a crystalline bovine albumin as standard.

Preparation of Crude Phosphorylase Kinase. To obtain a crude preparation of phosphorylase kinase from corpus luteum, 50 ml of the crude extract (see Purification) was acidified to pH 5.4 with 1 N acetic acid. The precipitate was collected by centrifugation, dissolved in 2 ml of 0.05 M glycerophosphate–0.004 M EDTA, pH 8.5, bringing the final pH to 7.0. This kinase preparation was used to phosphorylate un-

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TABLE I: Phosphorylase Activities in Crude Bovine Corpus Luteum Extract as Assayed in Three Different Buffers with and without AMP before and after Conversion of the Enzyme into the Phosphorylated Form.

	Fluoride–EDTA Extract[b]			
	Activity (units/ml)			
	Before Phosphorylation		After Phosphorylation	
Assay Buffer[a]	−AMP	+AMP	−AMP	+AMP
Regular	26	78	338	367
Cysteine	25	76	335	372
Na_2SO_4	64	146	133	161

[a] *Regular* buffer consisted of 0.04 M glycerophosphate–0.02 M NaF–0.001 M EDTA, pH 6.3. *Cysteine* buffer is regular buffer containing 0.03 M cysteine. *Salt or* Na_2SO_4 buffer was prepared as for regular buffer but contained 1.2 M Na_2SO_4 to give a final concentration of 0.6 M in the reaction mixture. [b] Protein concentration, 12.5 mg/ml.

phosphorylated enzyme[1] to form phosphorylated enzyme using the procedure of Fischer and Krebs (1962).

Sucrose Density Gradient Centrifugation. A 0.2-ml sample of enzyme 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 at 35,000 rpm for 13 hr at 4° in a Spinco Model L centrifuge using an SW 39 rotor. A hole was pierced in the bottom of the centrifuge tube and 40 fractions, 9 drops each, were collected and assayed for phosphorylase activity.

Disc Gel Electrophoresis. Disc electrophoresis on polyacrylamide gel (Ornstein and Davis, 1963) was performed and the protein(s) and phosphorylase bands were identified as previously described (Yunis and Arimura, 1966b).

Kinetic Studies. Kinetic studies were carried out at 37° in the direction of glycogen synthesis. When sulfate was varied, glucose-1-P, AMP, and glycogen were fixed at 0.032 M, 1×10^{-3} M, and 1% final concentrations, respectively.

When glucose-1-P was the varied substrate, AMP and glycogen were fixed at 1×10^{-3} M and 1% final concentrations. To study the effect of Na_2SO_4, this salt was included in the assay buffer (0.04 M glycerophosphate–0.02 M NaF–0.001 M EDTA, pH 6.3) at double the desired final concentrations. The reaction was started by the addition of 0.2 ml of enzyme diluted in assay buffer at 37° to 0.2 ml of substrate preincubated at 37°.

Initial velocities were expressed in μmoles of P_i released per 30 min per ml.

Preparation of Antiserum. Antiserum to purified bovine corpus luteum phosphorylase was prepared in rabbits as follows: 1 ml of enzyme solution containing 5 mg of protein was mixed with 1 ml of complete Freunds adjuvant. This material (1 ml) was then injected into a rabbit giving two separate 0.2-ml injections subcutaneously and 0.5-ml intraperitoneally. This procedure was repeated after 4 weeks. Blood was collected from the rabbit 6 days after the last injection. Antigen–antibody precipitation in agar and enzyme inhibition by antibody were evaluated as previously described (Yunis and Krebs, 1962).

[1] The terms "phosphorylated" and "unphosphorylated" used herein denote "active" and "inactive" phosphorylase, terms used earlier by other workers.

Experimental Section and Results

Corpora lutea were obtained from ovaries of freshly killed cows in their first 6 months of pregnancy. Each corpus luteum was carefully shelled out of surrounding ovarian tissue and rapidly frozen (one corpus luteum per cow, 2–7 g). Phosphorylase purification was carried out at 0–4° as follows.

Step 1. PREPARATION OF CRUDE EXTRACT. Fresh frozen bovine corpora lutea (200–400 g) were partially thawed and homogenized in 2.5 volumes of 0.05 M NaF–0.001 M EDTA in a Waring Blendor for 1 min. The homogenate was centrifuged for 30 min at 15,000*g*. The supernatant solution was filtered through glass wool to remove fat particles.

Phosphorylase Activity in Crude Extracts. The phosphorylase activities in crude extracts ranged between 0.05 and 0.08 μmole per min per mg without AMP and 0.13–20 μmoles per min per mg with AMP (Table I). The activity was not further enhanced by the addition of cysteine. Since it is known that some inactive phosphorylases, *e.g.*, liver (Wosilait and Sutherland, 1956), rat chloroma (Yunis and Arimura, 1966a), and adrenal cortex (Riley and Hayes, 1963), exhibit little activity even in the presence of AMP, conversion of the enzyme into the phosphorylated form was necessary to determine total phosphorylase activity. This was accomplished by incubating the extract with 0.01 M Mg-acetate–0.01 M ATP, pH 7.0, for 30 min at 37°. This phosphorylation step resulted in approximately fivefold increase in activity shown in Table I. This indicates that most of the phosphorylase in crude corpus luteum extracts exists in the unphosphorylated form which is only slightly responsive to AMP. Unphosphorylated corpus luteum phosphorylase was found to be reversibly activated by high concentrations of sodium sulfate. Thus assay of the crude extract in the presence of 0.6 M Na_2SO_4 resulted in two- to threefold increase in activity. After phosphorylation of the enzyme, salt was highly inhibitory (see Table I). Because of these findings, unphosphorylated phosphorylase was routinely assayed in buffer containing 0.6 M Na_2SO_4 and 10^{-3} M AMP and the activity was expressed in "salt" units, to denote condition of assay.

Step 2. TREATMENT WITH STREPTOMYCIN SULFATE. To the crude extract one-sixth volume of a 5% aqueous solution of streptomycin sulfate was added slowly and the suspension allowed to stand in ice for 5 min. The precipitate was eliminated by centrifugation at 15,000*g* for 15 min.

Step 3. AMMONIUM SULFATE PRECIPITATION. Neutral, saturated (25°) ammonium sulfate solution was added to the extract to bring the salt concentration to 30%. The precipitate was eliminated by centrifugation. The supernatant solution was brought to 47% saturation by further addition of neutral saturated ammonium sulfate and the precipitate was collected and dissolved in 20–30 ml of 0.05 M NaF–0.001 M EDTA. It was then dialyzed against 4 l. of 0.005 M Tris–0.001 M EDTA–0.05 M NaF, pH 7.2, in preparation for column chromatography.

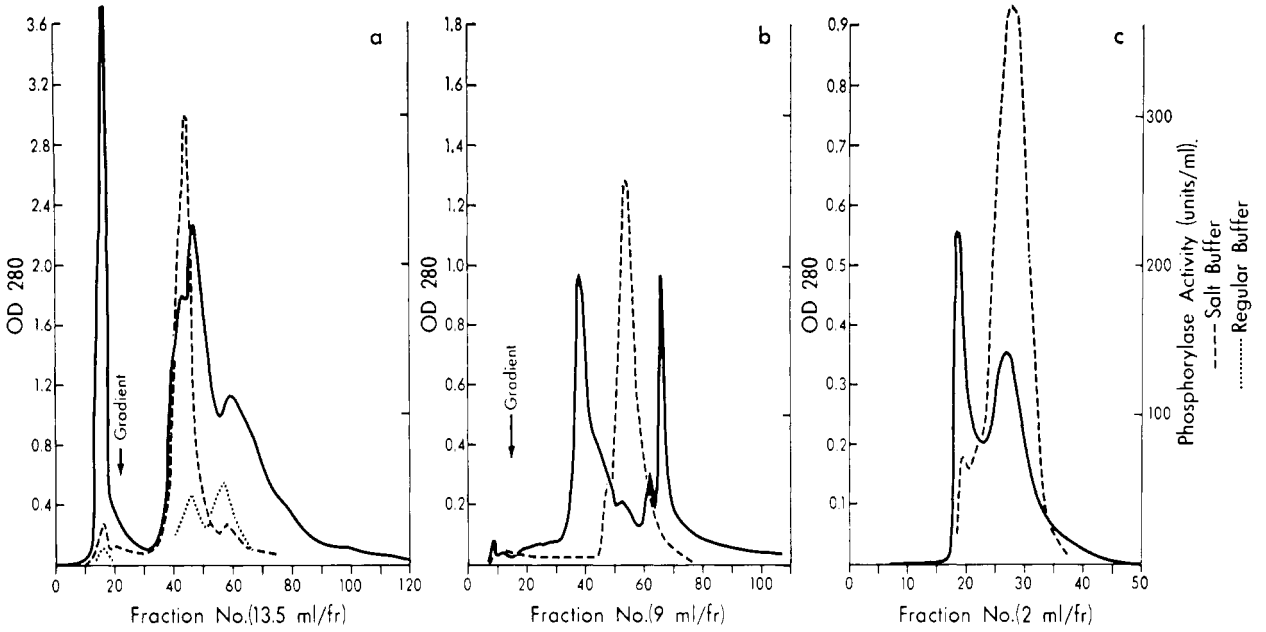

FIGURE 1: (a) Elution pattern of unphosphorylated bovine corpus luteum phosphorylase from DEAE-cellulose column chromatography. The dialyzed ammonium sulfate fraction (28 ml) from purification step 4 (see text) containing a total of 32,256 "salt" units of enzyme (total protein 800 mg) was applied to a 2.1 × 51 cm column and eluted as described in the text. Yield from this column amounted to 81%. (b) Rechromatography of corpus luteum phosphorylase after phosphorylation with MgATP and kinase. A 10-ml sample of concentrated phosphorylated enzyme from the column in (a) containing 29,280 units (total protein 103 mg) was applied to a 1.5 × 51 cm DEAE-cellulose column and eluted as described in the text. Total yield from the column was 71%. (c) Sephadex G-200 chromatography of concentrated enzyme from the DEAE-cellulose column in (b). A 1-ml sample containing a total of 7572 units of enzyme (7 mg of protein) was applied to a 1.1 × 108 cm column and eluted with 0.005 M Tris-HCl–0.001 M EDTA, pH 7.2. The minor activity peak could not be accounted for and was not studied further.

Step 4. COLUMN CHROMATOGRAPHY ON DEAE-CELLULOSE. Chromatography was carried out with a 2.1 × 51 cm DEAE-cellulose column equilibrated with initial buffer. A 15- to 20-ml sample of dialyzed fraction from step 3 containing 600–800 mg of protein and 25,000–35,000 "salt" units was applied to the column. Initial buffer was allowed to flow at a rate of 40–80 ml/hr until the appearance of a "breakthrough" peak. Elution was then performed with a linear gradient to 1.0 M NaCl–0.05 M NaF–0.005 M Tris–0.001 M EDTA, pH 7.2. Fractions were collected and their absorbance was measured at 280 mμ.

The results of a typical chromatography fractionation are shown in Figure 1 a. After a "breakthrough" peak, phosphorylase activity appeared in two peaks. One peak consisted of unphosphorylated phosphorylase and amounted to over 90% of the total activity and a minor peak of phosphorylated phosphorylase. Fractions of the major peak were pooled and concentrated by overnight dialysis against neutral saturated ammonium sulfate. The precipitate was collected by centrifugation, dissolved in 0.05 M NaF–0.001 M EDTA–0.005 M Tris, pH 7.2, and dialyzed against the same buffer.

For further purification of the enzyme, two alternate methods were followed. (1) Continuation of purification of the unphosphorylated enzyme. This was accomplished by repeating step 3 twice, rechromatography on DEAE-cellulose as in step 4, followed by Sephadex G-200 chromatography resulting an in overall 80-fold purification. (2) Purification after conversion into the phosphorylated form as described below.

Step 5. PHOSPHORYLATION OF UNPHOSPHORYLATED PHOSPHORYLASE. Since phosphorylated phosphorylase appeared to elute distinctly after the unphosphorylated enzyme, advantage was taken of this observation for further purification. Unphosphorylated enzyme from step 4 was phosphorylated as described above. The incubation mixture was then dialyzed against initial buffer in preparation for rechromatography. This step resulted in tenfold increase in activity when compared to the unphosphorylated enzyme assayed without salt (Table II).

Step 6. RECHROMATOGRAPHY ON DEAE-CELLULOSE. A 10-ml dialyzed sample of concentrated phosphorylated enzyme from step 5 was applied to a 1.5 × 51 cm DEAE-cellulose column and eluted as described under step 4 collecting fractions of 9 ml. The elution profile is shown in Figure 1b. This step resulted in approximately fourfold purification.

Step 7. SEPHADEX G-200 CHROMATOGRAPHY. Concentrated dialyzed effluent (1 ml) from step 6 was chromatographed using a 1.1 × 108 cm Sephadex G-200 column equilibrated in 0.005 M Tris–0.05 M NaF–0.001 M EDTA, pH 7.2. Fractions (2 ml) were collected and their absorbance was measured at 280 mμ. The elution profile is shown in Figure 1c. This step resulted in approximately twofold purification.

The active fractions were pooled and concentrated as above and dialyzed against 4 l. of 0.005 M Tris–0.05 M NaF–0.001 M EDTA, pH 7.2.

Table II gives a summary of the purification steps in a typical preparation. The final yield of enzyme varied between 20 and 30% with approximately 94-fold purification.

Some Properties of Purified Corpus Luteum Phosphorylase Homogeneity. Purified unphosphorylated corpus luteum phosphorylase traveled essentially in a single band in poly-

TABLE II: Purification of Bovine Corpus Luteum Phosphorylase.

Preparation	Vol (ml)	Activity (units/ml)	Protein (mg/ml)	Sp Act.[c] (units/mg of Protein)	Total Activity (units)	Yield (%)	Purification (-fold)
Crude extract	465	132[a]	13.5	9.8	68,380	100	1
After streptomycin	500	115[a]	12.1	9.5	57,500	93	
Dialyzed ammonium sulfate fraction	49	1152[a]	29	40	56,448	91	4
1st DEAE-cellulose effluent	12	2822[a]	21.2	132	33,864	55	14
After phosphorylation	13.4	5856	20.6	284[b]	78,470	*b*	14[b]
2nd DEAE-cellulose effluent	6.5	7522	7.1	1060	48,958	35	52
Sephadex G-200 filtrate	11.4	2700	1.4	1900	30,780	22	94

[a] Because the unphosphorylated form is inactive, it was assayed in the presence of salt. After phosphorylation, salt was inhibitory and hence was omitted in the assay. [b] The doubling in specific activity in this step does not represent further purification but is a result of conversion of the enzyme into the phosphorylated form. [c] To obtain μmoles of P_i/min per mg of protein, divide given values by 30 (30-min assay time).

acrylamide gel (Figure 2a). Electrophoresis of the purified phosphorylated enzyme shown in Figure 2b revealed a single band for both protein and activity. Antiserum prepared against purified phosphorylated enzyme when reacted with crude corpus luteum extract in agar yielded a single precipitin line (Figure 2c).

Molecular Weight. Phosphorylated and unphosphorylated corpus luteum phosphorylase appear to have the same rate of sedimentation upon ultracentrifugation in sucrose gradient (Figure 3). Molecular weight determination from the sucrose gradient data according to the method of Martin and Ames (1960) using crystalline human placental alkaline phosphatase as a marker (mol wt 125,000, Harkness, 1968) gave a value of 185,000 for both forms of the enzyme. A similar molecular weight value was obtained using bovine liver catalase (mol wt 250,000, Sumner and Gralen, 1938) as a marker

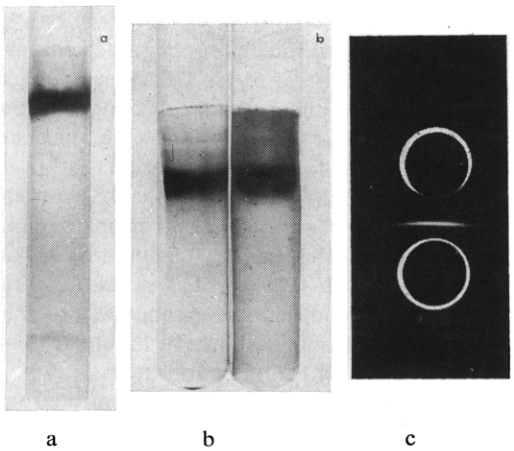

FIGURE 2: Electrophoresis of purified (a) unphosphorylated and (b) phosphorylated corpus luteum phosphorylase on polyacrylamide gel. A sample of 100 μg of protein was used in a gel column was stained with Amido Black. Amount of protein in sample was 80 μg in b; left, protein stain; right, enzyme stain. (c) Antigen–antibody precipitation in agar. Lower well contained rabbit antiserum prepared against purified phosphorylated corpus luteum phosphorylase. Upper well contained crude corpus luteum extract.

Rabbit muscle phosphorylase *b* gave a molecular weight value of 180,000 by this method.

Effect of AMP and Salt. Unphosphorylated corpus luteum phosphorylase was reversibly activated by high concentrations of sodium sulfate (Figure 4a), the optimum salt concentration being 0.6 M. The specific activity of purified unphosphorylated

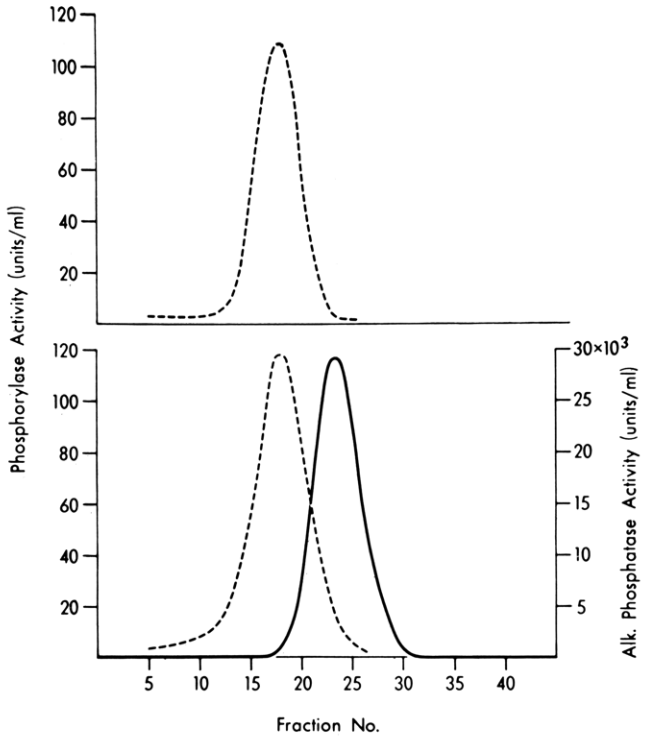

FIGURE 3: Sucrose density gradient ultracentrifugation of phosphorylated and unphosphorylated bovine corpus luteum phosphorylase. Phosphorylated and unphosphorylated enzyme are shown in the upper and lower dashed curves, respectively. The unphosphorylated enzyme was assayed in 0.6 M Na_2SO_4. The solid lower curve represents human placental alkaline phosphatase (mol wt 125,000) used as a marker.

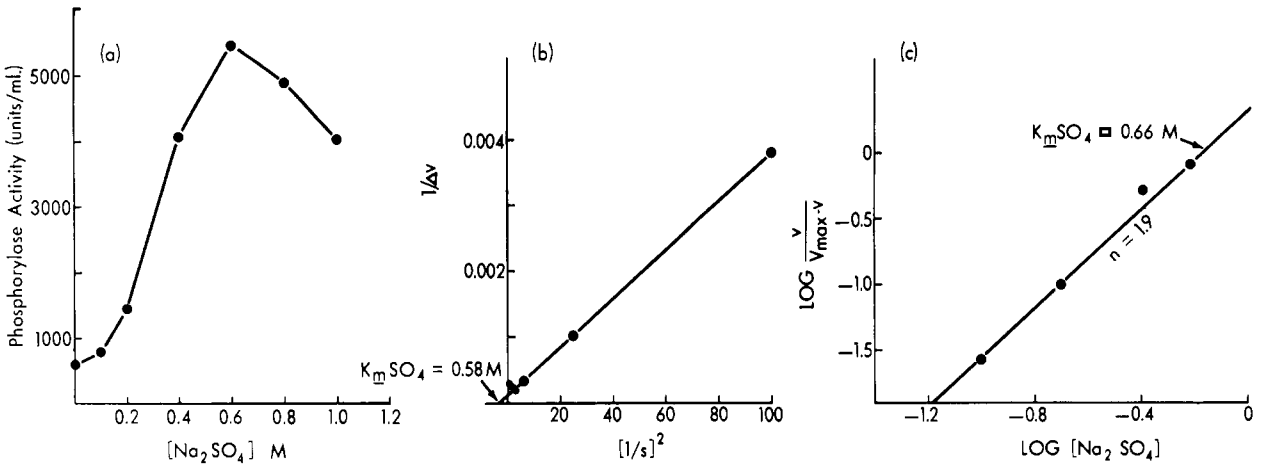

FIGURE 4: (a) Plot of velocity against sodium sulfate concentration. The unphosphorylated enzyme solution used for the assay contained 6 mg of protein/ml. Other conditions are as described under Methods. (b) Plot of $1/(\Delta v)$ *vs.* $(1/Na_2SO_4)^2$ for the data in a. Δv represents the increment in velocity in the presence of salt. (c) Hill plot of the data used in 4a and b. The K_m shown here represent the Na_2SO_4 concentration on the X axis which makes 90° with the zero on the Y axis.

corpus luteum phosphorylase when assayed in regular buffer was 88 units/mg (2.9 μmoles/min per mg) without AMP and 220 units/mg (7.3 μmoles/min per mg) with AMP (10^{-3} M). In the presence of 0.6 M sodium sulfate the specific activity was 495 units/mg (16.5 μmoles/min per mg) without AMP and 1100 units/mg (36 μmoles/min per mg) with the nucleotide. For comparison the purified phosphorylated corpus luteum phosphorylase has a specific activity of 1900 units/mg (63 μmoles/min per mg) and exhibited 85–90% of its maximum activity without AMP. It is strongly inhibited by high concentrations of sodium sulfate (60% inhibition at 0.6 M Na_2SO_4). Neither phosphorylated nor unphosphorylated phosphorylase activity was stimulated by cysteine. The pH optimum for both forms of the enzyme was 6.3, significantly lower than the value of 6.7–6.8 reported for muscle phosphorylases.

Kinetic Properties. The kinetics of corpus luteum phosphorylase were studied with special emphasis on the effect of salt. Although salt activation has been described for phosphorylase from liver (Appleman *et al.*, 1966) and adrenal cortex (Riley and Haynes, 1963), an analysis of the kinetics of this activation has not been reported.

Activation of the unphosphorylated form of corpus luteum phosphorylase was observed at salt concentrations between 0.1 and 0.6 M. A plot of initial velocity *vs.* Na_2SO_4 concentration gave a sigmoidal curve shown in Figure 4a. Since a Lineweaver–Burk plot of these data was nonlinear, the reciprocal of velocity was plotted against $(1/Na_2SO_4)^2$ (Figure 4b). Values for the apparent maximal velocity and apparent K_m were obtained by extrapolating the straight line obtained to the Y and the negative X axis, respectively. A V_{max} of 10,000 units (55 μmoles/min per mg) and K_m of 0.58 were obtained.

A replot of the data in the form of a Hill plot, *i.e.*, log $v/(V_{max} - v)$ *vs.* log sodium sulfate concentration yielded a straight line with a slope (interaction coefficient n) of 1.9 (Figure 4c). The Michaelis–Menten constant (K_m) for sulfate obtained from the Hill plot was found to be 0.66 M in close agreement with that obtained from plot of $1/(\Delta v)$ *vs.* $(1/s)^2$. These data are comparable to those of Appleman *et al.* (1966), on liver phosphorylase when similarly analyzed. Hill plot slopes determined for muscle phosphorylase *b* under conditions different from those used here have been reported by others (Madsen and Scheckosky, 1967; Assaf, 1969; Assaf and Graves, 1969) with an interaction coefficient approaching 2.0 when interaction is maximum between the binding sites of the phosphorylase *b* dimer.

The data presented in Figure 4 and the information obtained from the Hill plot are consistent with two molecules of of SO_4^{2-} binding per molecule of enzyme and are suggestive of a homotropic cooperative binding, *i.e.*, the binding of one molecule of sulfate facilitates the binding of an additional sulfate ion as shown below

$$E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{–}SO_4 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\begin{matrix}\diagup SO_4 \\ \diagdown SO_4\end{matrix}$$

where k_2, the constant for the rate of formation of $E(SO_4)_2$ is larger than k_1 the constant for the formation of $E\text{–}SO_4$. This difference may be ascribed to alterations of corpus luteum

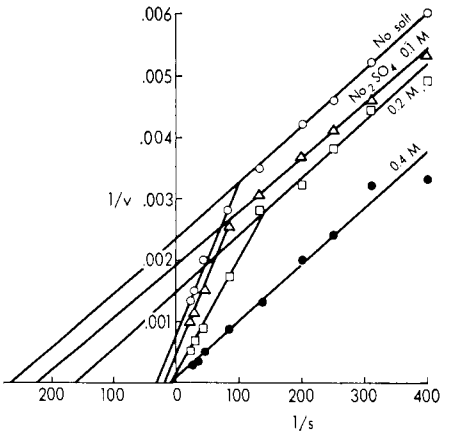

FIGURE 5: Double-reciprocal plot of unphosphorylated corpus luteum phosphorylase with respect to glucose-1-P at three fixed levels of sodium sulfate. Conditions are as described under Methods.

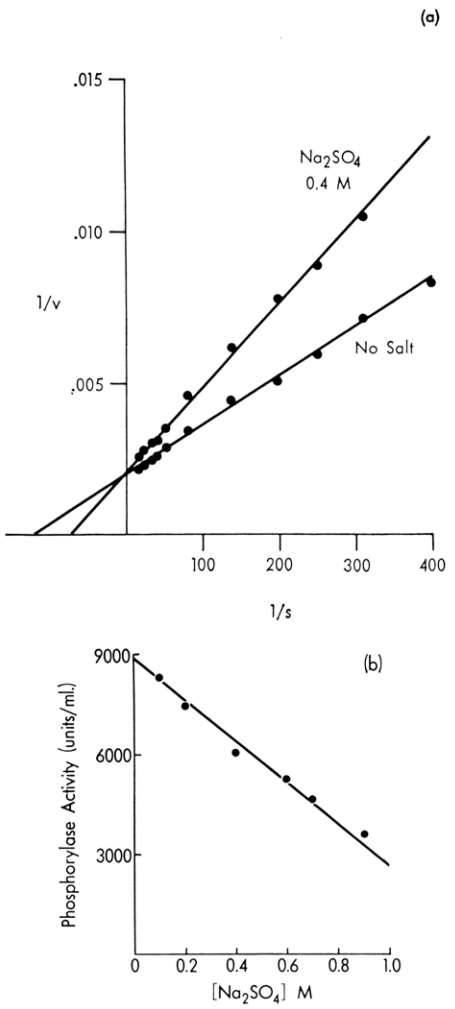

FIGURE 6: (a) Double-reciprocal plot of phosphorylated corpus luteum phosphorylase with respect to glucose-1-P in the presence and absence of sodium sulfate. Conditions are as described under Methods. (b) The effect of increasing concentrations of salt on the initial velocity of phosphorylated corpus luteum phosphorylase. The enzyme solution used for the assay contained 4.7 mg of protein/ml.

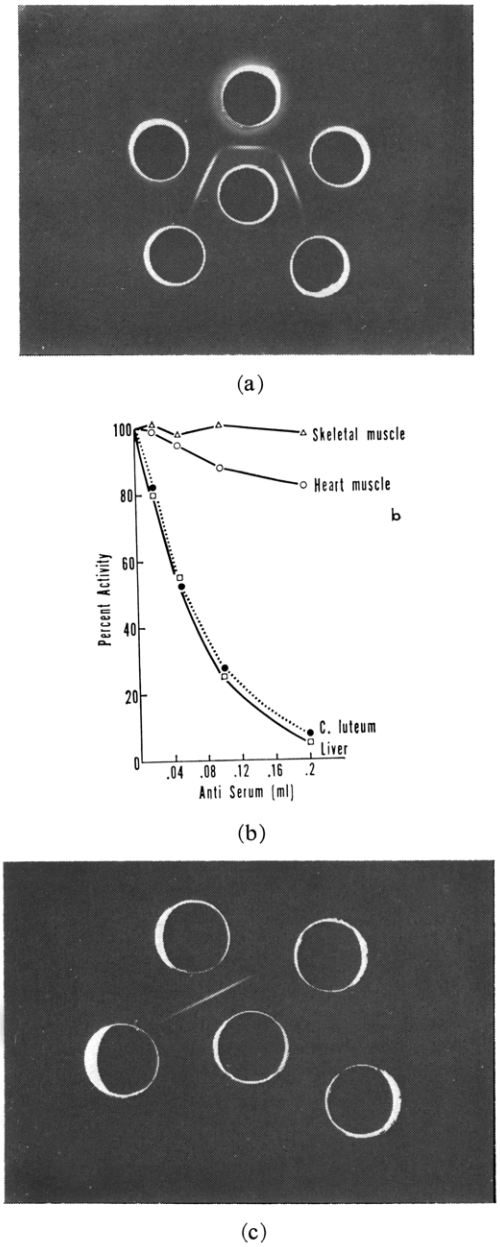

FIGURE 7: (a) Antigen–antibody precipitation in agar showing a reaction of identity with bovine liver phosphorylase. Center well contained rabbit antibovine corpus luteum phosphorylase serum. Wells from left to right contained partially purified phosphorylase from bovine skeletal muscle, bovine corpus luteum, bovine liver, bovine corpus luteum, and bovine cardiac muscle. (b) Effect of rabbit antibovine corpus luteum phosphorylase serum on the activity of phosphorylase from bovine corpus luteum, skeletal muscle, and heart muscle. (c) Antigen–antibody precipitation in agar showing a cross reaction with human leukocyte phosphorylase but absence of cross reactivity with either rabbit or human muscle phosphorylase. Center well contained rabbit antibovine corpus luteum phosphorylase serum. Wells from left to right contained partially purified phosphorylase from: rabbit muscle, bovine corpus luteum, human leukocytes, and human muscle.

phosphorylase to a more favorable conformational state designated "R" state in the concerted transition model of Monod *et al.* (1965).

To better understand the mechanism of salt activation, kinetic experiments were carried out in the direction of glycogen synthesis at pH 6.3 at 37° (Figure 5).

Sodium sulfate appeared to increase the maximal velocity without increasing enzyme–subsrate affinity; in fact there was a slight increase in the apparent K_m for glucose-1-P in the presence of salt. Thus the mechanism of salt activation of corpus luteum phosphorylase appears to differ from that described for lobster muscle phosphorylase where salt has been shown to increase enzyme–substrate affinity (Cowgill, 1959). In the case of corpus luteum phosphorylase a decrease rather than an increase in the enzyme affinity for its substrate glucose-1-P was observed when sulfate was added. Activation by sodium sulfate has also been observed for rabbit phosphorylase by Engers and Madsen (1968). Direct comparison to that study, however, is not possible, since their experiments were carried out in the absence of AMP. Their data show a maximum activation by sodium sulfate of about 20% of the V_{max} obtained in presence of AMP. In contrast unphosphorylated

corpus luteum phosphorylase, when assayed in the presence of sodium sulfate and AMP, had a V_{max} 60% of that of the phosphorylated enzyme, similar to what has been found for liver phosphorylase (Appleman *et al.*, 1966).

An interesting aspect of our kinetic study is the nonlinear double-reciprocal plots with respect to glucose-1-P showing a definite substrate activation (Figure 5). In their study of rabbit muscle phosphorylase *b* prepared by limited tryptic attack, Graves *et al.* (1968) observed a bimodal reciprocal plot (similar to substrate activation) which they attributed to having more than one form of the enzyme, a prediction they supported by gel electrophoresis. There was no evidence for enzyme heterogeneity in corpus luteum phosphorylase as shown above.

Two K_m and two V_{max} values were evaluated from the bimodal plot in Figure 5. With glucose-1-P concentrations less than 0.01 M, K_m values for glucose-1-P were 4.0, 4.6, 6, and 120 mM at 0, 0.1, 0.2, and 0.4 M Na_2SO_4, respectively. The corresponding K_m values at glucose-1-P concentration greater than 0.01 M were 34, 40, 130, and 120 mM. Substrate activation disappeared at 0.4 M Na_2SO_4 and linear Michaelis–Menten kinetics were obeyed. Also to be emphasized from Figure 5 is the great increase in maximal velocity at glucose-1-P concentration range higher than 0.01 M.

The possibility of product activation in these experiments was ruled out by the results of short-term assays (3–10 min) showing no change in initial rates from the 30-min assays. This also indicates that initial rate was being properly measured in our experiments.

No substrate activation was observed with the phosphorylated form of corpus luteum phosphorylase (Figure 6) even in the absence of salt where the greatest substrate activation was observed for the unphosphorylated enzyme. In addition the phosphorylated enzyme was competitively inhibited by salt. The inhibition of this form of the enzyme with sodium sulfate appears to be a linear function of sulfate concentration (Figure 6b).

Immunologic Studies. As shown in Figure 2c, reaction of rabbit antibovine corpus luteum phosphorylase serum with corpus luteum phosphorylase in agar yielded a single precipitin line. The activity of corpus luteum phosphorylase was completely inhibited by the antibody. Comparative immunologic study revealed a reaction of identity between bovine corpus luteum phosphorylase and bovine liver phosphorylase as determined by precipitation in agar (Figure 7a) and by enzyme inhibition test (Figure 7b). There was slight reactivity of the antibody with bovine heart muscle phosphorylase (Figure 7b) but no cross reaction with bovine, rabbit, or human skeletal muscle phosphorylase (Figure 7a,b,c). A reaction of partial identity was obtained against human leukocyte phosphorylase (Figure 7c).

Discussion

Purified corpus luteum phosphorylase was found to have many similarities to liver phosphorylase. Thus the enzyme exists in two forms: an inactive unphosphorylated form which is activated only slightly by AMP but is reversibly activated by high salt concentration and an active phosphorylated form which exhibits 85% of its maximal activity in the absence of AMP and is inhibited by high salt concentration. The molecular weight for both forms of the enzyme is 185,000. Perhaps most significant is the demonstration of immunologic identity between the two enzymes. The reaction of partial immunologic identity with human leukocyte phosphorylase is also of interest since this enzyme was also shown to be closely related to liver phosphorylase (Yunis and Arimura, 1966a,b, 1968).

The activation of unphosphorylated corpus luteum phosphorylase by high sodium sulfate concentration allows identification of this form of the enzyme and estimation of the ratio of unphosphorylated to total phosphorylase. However, the highest activity attained for unphosphorylated phosphorylase in the presence of salt is only 60% of the maximal activity of the phosphorylated form of the enzyme. Because of this and the inhibition of the phosphorylated enzyme by salt, it is not possible to determine total phosphorylase activity in a given preparation in the presence of high salt concentration. This can be done only by total conversion of the enzyme into the phosphorylated form with MgATP and phosphorylase kinase. The data on salt activation indicate that sodium sulfate acts an allosteric effector, *i.e.*, the sigmoidal substrate saturation curve, the linearity of both the Hill plot and $1/(\Delta v)$ *vs.* $(1/Na_2SO_4)^2$.

Perhaps most unique about the kinetics of corpus luteum phosphorylase was the activation by high substrate (glucose-1-P) concentration. This substrate activation was most marked in the absence of salt, decreasing in magnitude with increasing salt concentration. The highest apparent maximal velocity was obtained in the presence of 0.4 M Na_2SO_4. At this sulfate concentration, it appeared that the enzyme reached its most favorable catalytic conformation overshadowing the substrate activation effect. This effect by sulfate seemed to be specific, since high concentrations of glycerophosphate, acetate, or chloride could not substitute for this ion.

The great sulfate effect observed here could be attributed to a change in enzyme conformation. The decreased affinity of the unphosphorylated corpus luteum enzyme for the substrate glucose-1-P could be due to overloading of the glucose-1-P binding sites by sulfate ions. That sulfate may be binding at the substrate site and not acting through a salting out effect (Robinson and Jencks, 1965) is also suggested by the kinetic data for the phosphorylated enzyme. This enzyme is inhibited by salt and the inhibition is a linear function of sodium sulfate concentration and is competitive with respect to glucose-1-P.

One possible interpretation of substrate activation is that the binding of additional glucose 1-phosphate ions to the enzyme increases initial velocity by accelerating dissociation of ES complex to free enzyme and product. This in turn would be reflected by an increase in K_m and V_{max} due to an increase in the velocity constant (k) of $ES \xrightarrow{k} E + P$.

This interpretation is consistent with the model of negative cooperativity proposed and discussed by Conway and Koshland (1968), where, as stated by these authors, "the kinetic data can be fitted with two different K_m values where the K_m at the higher substrate concentration is 5–12 times the K_M at the lower concentration. Such effects have been called "activation at high substrate concentration."

Also Frieden (1964) pointed out that nonlinear double-reciprocal plots due to substrate activation may be interpreted to mean that there are two active sites for substrate which have different kinetic parameters.

The nonlinear kinetics presented here for corpus luteum phosphorylase are complex and deviate from the recently proposed mechanism [rapid equilibrium Random Bi Bi in Cleland's terminology (1963)] for glycogen phosphorylase

from rabbit muscle (Lowry *et al.*, 1964, 1967; Engers *et al.*, 1969; Gold *et al.*, 1970) and from *E. coli* (Chao *et al.*, 1969). This mechanism has also been proposed by Maddiah and Madsen (1966) for phosphorylated liver phosphorylase. While negative cooperativity may be a plausible interpretation for the kinetics of the corpus luteum enzyme, other alternatives are possible, including a steady-state random mechanism.

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