Biochimica et Biophysica Acta, 403 (1975) 393—411

BBA 67621

PURIFICATION AND CHARACTERIZATION OF A PHOSPHOPROTEIN PHOSPHATASE FROM BOVINE ADRENAL CORTEX

BUDDY ULLMAN and ROBERT L. PERLMAN

Department of Physiology and Program in Cell and Developmental Biology, Harvard Medical School, Boston, Mass. 02115 (U.S.A.)

(Received March 18th, 1975)
(Revised manuscript received July 7th, 1975)

Summary

A phosphoprotein phosphatase which is active against chemically phosphorylated protamine has been purified about 500-fold from bovine adrenal cortex. The enzyme has a pH optimum between 7.5 and 8.0, and has an apparent K_m for phosphoprotamine of about 50 μM. The hydrolysis of phosphoprotamine is stimulated by salt, and by Mn^{2+}. Hydrolysis of phosphoprotamine is inhibited by ATP, ADP, AMP, and P_i, but is not affected by AMP or cyclic GMP.

The purified phosphoprotein phosphatase preparation also dephosphorylates *p*-nitrophenyl phosphate and phosphohistone, and catalyzes the inactivation of liver phosphorylase, the inactivation of muscle phosphorylase *a* (and its conversion to phosphorylase *b*), and the inactivation of muscle phosphorylase *b* kinase. Phosphatase activities against phosphoprotamine and muscle phosphorylase a copurify over the last three stages of purification. Phosphoprotamine inhibits phosphorylase phosphatase activity, and muscle phosphorylase *a* inhibits the dephosphorylation of phosphoprotamine. These results suggest that one enzyme possesses both phosphoprotamine phosphatase and phosphorylase phosphatase activities. The stimulation of phosphorylase phosphatase activity, but not of phosphoprotamine phosphatase activity, by caffeine and by glucose, suggests that the different activities of this phosphoprotein phosphatase may be regulated separately.

Introduction

The reversible phosphorylation and dephosphorylation of enzymes is recognized as an important mechanism for the regulation of enzyme activity

Abbreviations: Buffer A, 50 mM Tris · HCl/1 mM EDTA/1 mM dithiothreitol, pH 7.4; Buffer B, 50 mM Tris · HCl/1 mM EDTA/1 mM dithiothreitol/5 mM $MnCl_2$/50 mM KCl/10% glycerol, pH 7.4.

[1,2]. Protein kinases, the enzymes which catalyze the phosphorylation of proteins, have been widely studied; protein kinases from many sources have been shown to be activated by cyclic AMP [3—7]. It has been proposed that all of the actions of cyclic AMP in eukaryotic cells result from the activation of protein kinases by the nucleotide [8]. Much less is known about the enzymes that dephosphorylate phosphoproteins.

The study of phosphoprotein phosphatases in the adrenal cortex is of considerable interest. Gill and Garren [6] have isolated a cyclic AMP-dependent protein kinase from adrenal cortex and have proposed that this enzyme mediates the effects of adrenocorticotropic hormone (ACTH) and of cyclic AMP on glucocorticoid synthesis [9]. In addition, both ACTH and cyclic AMP activate phosphorylase in the adrenal cortex [10]. Riley and Haynes [11] suggested that the activation of phosphorylase by cyclic AMP results from inhibition of phosphorylase phosphatase, rather than stimulation of phosphorylase kinase. Merlevede and Riley [12] proposed that the phosphorylase phosphatase exists in both active and inactive forms, and that cyclic AMP causes an inactivation of the enzyme.

We have recently developed methods for the chemical phosphorylation of seryl residues in protamine, and, using chemically phosphorylated protamine as a substrate, we have detected phosphoprotein phosphatase activity in a number of mammalian tissues [13]. We now report the purification and some properties of a phosphoprotein phosphatase from bovine adrenal cortex which is active against chemically phosphorylated protamine.

Materials and Methods

Buffers. Buffer A consists of 50 mM Tris · HCl/1 mM EDTA/1 mM dithiothreitol, pH 7.4. Buffer B is composed of 50 mM Tris · HCl/1 mM EDTA/1 mM dithiothreitol/5 mM $MnCl_2$/50 mM KCl/10% glycerol, pH 7.4.

Materials. Protamine chloride, skeletal muscle phosphorylase *b* (α-1,4-glucan: orthophosphate glucosyltransferase, EC 2.4.1.1, 25 units/mg protein), phosphorylase *b* kinase (ATP:phosphorylase phosphotransferase, EC 2.7.1.38, 114 units/mg protein), calf thymus histone II-A, and DEAE-Sephadex A50-120 were all obtained from Sigma. The molecular weight markers, bovine liver catalase, rabbit muscle lactic acid dehydrogenase and bovine pancreatic ribonuclease were also purchased from Sigma. Phosphorylase *a* (20 units/mg protein) from rabbit skeletal muscle and porcine heart malic acid dehydrogenase were purchased from Boehringer Mannheim. Sepharose 4B was obtained from Pharmacia. Enzyme-grade $(NH_4)_2SO_4$ was purchased from Schwarz/Mann. [γ-^{32}P]ATP and $H_3{}^{33}PO_4$ were supplied by the New England Nuclear Corp. Crystalline bovine serum albumin was a product of Miles Laboratories. All other chemicals were reagent grade. Glass distilled water was used throughout. Calcium phosphate gel was prepared according to the method of Keilin and Hartree [14], and was stored in the dark at 4° C for at least 6 weeks before use. Phosphoprotamine and [^{33}P]phosphoprotamine were synthesized as described previously, and contained approx. one residue of alkali-labile phosphate per mol [13].

Chemical assays. In most experiments, protein was determined by the

method of Lowry et al. [15]. During the initial steps in the purification of the phosphoprotein phosphatase the protein concentration was determined by a modification of the procedure of Page and Culver [16]. To a 0.4-ml sample containing 20—400 μg protein, 1.2 ml of 3% sulfosalicylic acid in water was added, and the mixture was agitated vigorously. The turbidity of the samples (absorbance at 600 nm) was measured after 10 min at room temperature. Crystalline bovine serum albumin was used as a protein standard for both assays. Inorganic phosphate was determined by the method of Ames and Dubin [17]. Acid-labile phosphate was estimated after incubation for 15 min at 100° C in 0.5 M H_2SO_4.

Preparation of ATP-Sepharose. To 80 ml of washed Sepharose 4B and 80 ml water were added 27 g of finely crushed CNBr. The pH of the solution was maintained at 11 with 8 M NaOH, and the temperature was maintained at 20° C by the addition of crushed ice. The mixture was stirred gently until cessation of proton release (about 10 min), at which time more crushed ice was added, and the activated gel was transferred to a Buchner funnel. The gel was washed with 800 ml of 0.1 M $NaHCO_3$ pH 10.0, the bottom of the funnel was stoppered with Parafilm, and 23.3 g of ε-amino caproic acid in 80 ml 0.1 M $NaHCO_3$ were added. The reaction mixture was then transferred to a 250-ml Erlenmeyer flask, and stirred slowly for 18 h at 4° C. The ε-amino caproic acid-substituted Sepharose was washed successively with 500 ml 0.1 M $NaHCO_3$, pH 10.0, 500 ml 0.01 M HCl, 500 ml 0.5 M NaCl, 2 l water, and 1 l pyridine/H_2O (4 : 1, v/v). To the washed gel were added 1.5 g ATP in 24 ml water, followed by 33 g dicyclohexylcarbodiimide in 96 ml pyridine. The mixture was stirred for 24—96 h at room temperature, and was then washed with water, 95% ethanol, *n*-butanol (at 37° C), 0.5 M NaCl, water, and Buffer B. The extent of coupling of ATP to Sepharose was estimated by measuring the acid-labile phosphate bound to the gel. Typical preparations of ATP-Sepharose contained 3—5 μmol ATP per ml of packed gel (assuming 2 mol of acid-labile phosphate per mol of ATP). In addition, aliquots of the substituted gel were treated with 0.5 M KOH for 2 h at 70° C, and the release of nucleotide from the gel was monitored by the ultraviolet absorbance of the supernatant. These measurements also indicated the presence of about 4 μmol of nucleotide per ml of packed gel.

Phosphoprotein phosphatase assay. Phosphoprotein phosphatase activity was routinely measured by the release of $^{33}P_i$ from [^{33}P]phosphoprotamine, according to the method described previously [13]. The enzyme was incubated with [^{33}P]phosphoprotamine, containing 0.1 mM alkali-labile phosphate (100—300 cpm/nmol), in a final volume of 380 μl. Unless otherwise stated, the incubation was performed for 30 min at 37° C, in a buffer composed of 50 mM Tris · HCl, 2 mM $MnCl_2$, 1 mM dithiothreitol and 400 mM NaCl, pH 7.4. The reaction was terminated by the addition of 25 μl of 0.1 M silicotungstic acid in 0.05 M H_2SO_4. 100 μl of 5% $(NH_4)_6Mb_7O_{24} \cdot 4H_2O$ in 2 M H_2SO_4, and 0.5 ml isobutanol/benzene (1 : 1, v/v) were added, and the mixture agitated vigorously for 30 s and then centrifuged. The radioactivity in duplicate 0.1 ml aliquots of the upper (organic) phase was measured in a gas-flow radiation counter. One unit of phosphoprotein phosphatase activity was defined as the amount of enzyme that catalyzes the release of 1 nmol of P_i from phosphoprotamine per min. In some experiments, phosphoprotein phosphatase was

assayed by a spectrophotometric determination of P_i. For this assay, the enzyme was incubated with non-radioactive phosphoprotamine containing 0.66 mM alkali-labile phosphate, under the conditions described above. The reaction was terminated by the addition of silicotungstic acid in H_2SO_4, the protein was removed by centrifugation, and the concentration of P_i in the supernatant measured.

Determination of subcellular distribution. For studies of the subcellular distribution of phosphoprotein phosphatase activity, two male Sprague-Dawley rats obtained from Charles River Breeding Laboratories were killed by decapitation, and the adrenal glands homogenized with 2.0 ml of Buffer B. A crude nuclear fraction was obtained by centrifugation at 800 × *g* for 10 min, and was resuspended in Buffer B and washed several times by centrifugation. A crude mitochondrial fraction was prepared by centrifugation of the 800 × *g* supernatant at 6500 × *g* for 20 min, and was washed similarly. The post-mitochondrial supernatant was separated into two portions. The first portion was centrifuged at 105 000 × *g* for 30 min to obtain a microsomal pellet. The second was treated with 1% sodium deoxycholate for 20 min at 0°C and was then centrifuged at 105 000 × *g* for 30 min to obtain a crude ribosomal fraction. The crude microsomal and ribosomal fractions were washed once, the former with Buffer B, the latter with Buffer B containing 1% deoxycholate. All pellets were resuspended in Buffer B, and along with the supernatants were assayed for phosphoprotein phosphatase activity and for protein.

Purification of phosphoprotein phosphatase. Bovine adrenal glands were obtained at a local slaughterhouse, and were either used immediately or else were stored at −70°C until use. The adrenal glands were dissected free of surrounding fat, and the medullae were scraped away from the cortices with a scalpel. The adrenal cortices were rinsed in Buffer A, drained and blotted, and then minced into small pieces with scissors and homogenized for 2 min in a Waring Blendor with 4 ml/g of cold Buffer A. All subsequent steps were carried out at 0—4°C. The homogenate was filtered through four layers of cotton gauze, and centrifuged for 50 min at 16 000 × *g*. The pellet was resuspended in Buffer A, rehomogenized, and centrifuged again for 50 min at 16 000 × *g*. The combined supernatants were filtered through glass wool, and then centrifuged for 3 h at 105 000 × *g*.

To the supernatant was added 0.164 g/ml of $(NH_4)_2SO_4$. The precipitate was allowed to settle, and was then collected by centrifugation for 30 min at 10 000 × *g*. To the supernatant was added an additional 0.127 g/ml of $(NH_4)_2SO_4$, to make the final solution 50% saturated. The precipitate was again collected by centrifugation, dissolved in Buffer A, and dialyzed against several liters of 5 mM Tris · HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4, until the dialysate was sulfate free (no precipitate formed upon the addition of 0.1 M $BaCl_2$).

2 mg (dry weight) of $Ca_3(PO_4)_2$ gel was added dropwise per ml of solution, and the resulting suspension was stirred occasionally for 30 min. The gel was collected by centrifugation, and washed thoroughly with 5 mM Tris · HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4. The gel was washed with Buffer A containing 0.3 M Na_2SO_4. The enzyme was eluted from the gel after vigorous shaking at 30°C with Buffer A containing 0.6 M Na_2SO_4. The 0.6 M Na_2SO_4

eluate was made 50% saturated with $(NH_4)_2SO_4$, the precipitated protein was collected by centrifugation, dissolved in Buffer B, and dialyzed against several changes of Buffer B until the dialysate was sulfate free.

The enzyme solution was applied to a column of DEAE-Sephadex (1.5 × 13 cm) that had previously been equilibrated with buffer B. The column was washed with three bed volumes of Buffer B containing 0.16 M KCl, and then the enzyme was eluted with Buffer B containing 0.4 M KCl. The KCl eluate was dialyzed against Buffer B.

4 ml of dialyzed eluate were incubated with 2 ml of ATP-Sepharose for 30 min, and the gel was removed by centrifugation. Unless otherwise indicated, the purified phosphoprotein phosphatase used for all of the experiments in this paper was that fraction of the DEAE-Sephadex eluate that did not bind to ATP-Sepharose.

Molecular weight estimation. The molecular weight of the phosphoprotein phosphatase was estimated by a modification of the method of Martin and Ames [18], using 5—30% sucrose density gradients containing 50 mM Tris · HCl, pH 7.4, and 5 mM $MnCl_2$. Two gradients one containing the phosphatase and the molecular weight standards, the other containing only the standards, were run simultaneously. The gradients were centrifuged for 17 h at 100 000 × *g* in a Beckman SW-41 rotor. 33 0.4-ml fractions were collected, and were assayed for enzyme activities. Catalase was assayed spectrophotometrically at 240 nm as described by Beers and Sizer [19]. Lactic acid dehydrogenase was assayed by decreased absorbance at 340 nm according to the method introduced by Kubowitz and Ott [20]. Malate dehydrogenase was also measured by decreased absorbance at 340 nm by the method of Mehler et al. [21]. Ribonuclease was assayed according to the method of Kalnitsky et al. [22]. Phosphoprotein phosphatase was assayed by measuring the release of *p*-nitrophenol (absorbance at 420 nm) from *p*-nitrophenyl phosphate. The molecular weight of the phosphatase was estimated from a graph of (molecular weight)$^{2/3}$ versus distance sedimented from the meniscus.

Preparation of active liver phosphorylase. Active liver phosphorylase was prepared from male Sprague-Dawley rats weighing about 300 g by a modification of the procedure of Sutherland and Wosilait [23]. The final $(NH_4)_2SO_4$ precipitate was dissolved in 1 ml of Buffer B per g of original tissue and dialyzed until the dialysate was sulfate free. The final preparation contained 2—3 units per mg protein.

Assay for phosphorylase and phosphorylase phosphatase activity. Liver phosphorylase phosphatase was assayed by the method of Wosilait and Sutherland [24]. Rat liver phosphorylase was incubated with phosphoprotein phosphatase for 30 min at 37°C, in 0.05 M Tris · HCl, pH 7.4, containing 0.7 mM caffeine. Following this incubation, residual phosphorylase activity was assayed by the method of Wosilait and Sutherland [24]. P_i released from glucose-1-*P* was assayed by the method of Post and Sen [25]. One unit of phosphorylase is defined as the amount of enzyme that releases 1 μmol of P_i from glucose-1-*P* per min. One unit of phosphorylase phosphatase is defined as the amount of enzyme required to inactivate one unit of phosphorylase per min. The assay of muscle phosphorylase phosphatase activity was identical, except that phos-

phorylase was incubated with the phosphatase for 10 min, in the absence of caffeine. Phosphorylase *a* was assayed as described above. Phosphorylase *b* was assayed by the addition of 1.4 mM AMP to the assay mixture, and subtraction of the phosphorylase *a* activity.

Assay for phosphorylase b kinase phosphatase activity. Phosphorylase *b* kinase phosphatase activity was measured by a decrease in phosphorylase *b* kinase activity, by a modification of the method of Riley et al. [26]. 15 units of phosphorylase *b* kinase (1 unit of kinase converts 1 unit of phosphorylase *b* to phosphorylase *a* per min at pH 6.8) were incubated with the phosphoprotein phosphatase for zero to 20 min at 30°C in 200 µl of Buffer B containing 8 mM $MgCl_2$. A control without phosphatase was incubated simultaneously. The reactions were terminated by the addition of 1 ml of ice-cold 15 mM cysteine, pH 7.0, and the phosphorylase *b* kinase activity at pH 6.8 was measured, as described [26].

Preparation of phosphohistone. ^{32}P-labeled phosphohistone was synthesized according to the procedure of Langan [5], using rat liver cyclic AMP dependent protein kinase prepared by the method of Kuo et al. [27]. Phosphohistone phosphatase activity was measured by the release of $^{32}P_i$ from ^{32}P-labeled phosphohistone. The enzyme was incubated at 37°C for 30 min with ^{32}P-labeled phosphohistone containing 0.3 µM alkali-labile phosphate (15—25 cpm/pmol), in a buffer consisting of 50 mM Tris · HCl, 2 mM $MnCl_2$, 1 mM dithiothreitol, and 0.16 M NaCl, pH 7.4 (final volume 380 µl). The reaction was terminated by the addition of 25 µl of 0.1 M silicotungstic acid in 0.05 M H_2SO_4. Released P_i was measured as described previously [13].

Results

Subcellular distribution of phosphoprotein phosphatase activity. We have previously detected phosphoprotein phosphatase activity in crude extracts and in soluble fractions of rat adrenal glands. Table I shows the results of a more detailed study of the subcellular distribution of phosphoprotein phosphatase activity in

TABLE I

SUB-CELLULAR DISTRIBUTION OF PHOSPHOPROTEIN PHOSPHATASE ACTIVITY IN RAT ADRENAL CORTEX

Adrenal glands from four male rats were pooled and treated as described in the text. One-half of the post-mitochondrial supernatant was treated with sodium deoxycholate to prepare ribosomes, and the other half was centrifuged at 105000 × *g* without deoxycholate treatment to prepare a microsomal fraction. The supernatant fraction represents the pooled post-ribosomal and post-microsomal supernatants.

Fraction	Phosphoprotein phosphatase (units)	Protein (mg)	Specific activity (units/mg)
Crude homogenate	5.12	11.50	0.45
Nuclei	2.44	6.88	0.35
Mitochondria	0.01	0.25	0.04
Microsomes	0.18	0.54	0.33
Ribosomes	0.41	0.34	1.21
Supernatant	2.48	3.35	0.74

TABLE II

PURIFICATION OF ADRENAL PHOSPHOPROTEIN PHOSPHATASE

Phosphoprotein phosphatase was purified according to the procedure in the text, and was assayed for activity against phosphoprotamine, muscle phosphorylase *a*, and *p*-nitrophenyl phosphate. The numbers described represent the results of one experiment. In three experiments, the increases in the specific activities of phosphorylase phosphatase and phosphoprotamine phosphatase in the ATP-Sepharose purification step correlated within 10% of each other. Numbers in parentheses refer to percentage of activity recovered. n.d., not determined.

Purification stage	Phosphatase activity against		
	Phosphoprotamine (units/mg)	Muscle phosphorylase *a* (units/mg)	*p*-Nitrophenyl phosphate (units/mg)
16000 × *g* supernatant	1.3 (100)	n.d.	n.d.
105000 × *g* supernatant	1.5 (118)	n.d.	n.d.
$(NH_4)_2SO_4$ precipitate	9.4 (78)	0.80	5.1
$Ca_3(PO_4)_2$ gel eluate	20.9 (85)	2.1	8.9
DEAE-Sephadex eluate	59.5 (76)	5.7	13.4
ATP-Sepharose supernatant	625 (27)	65	112

these glands. Almost all of the enzyme activity is located in the crude nuclear and the soluble fractions of the adrenal gland; these two fractions contain approximately equal amounts of enzyme activity. Although the ribosomal fraction contains less than 10% of the total phosphoprotein phosphatase activity in the adrenal, the highest specific activity is found in this fraction.

Purification of adrenal phosphoprotein phosphatase. After determining the subcellular localization of phosphoprotein phosphatase activity in the adrenal gland, we chose to purify the soluble enzyme from bovine adrenal cortex. Table II summarizes a typical purification of this enzyme. Conventional techniques for protein purification resulted in a 40-fold increase in specific activity with a 76% recovery of phosphatase activity. Ion-exchange chromatography and gel filtration on Sephadex G-100 and G-200 resulted in no further purification (data not shown). However, incubation of the DEAE-Sephadex eluate with ATP-Sepharose gels resulted in an additional 11-fold purification. About one-third of the phosphoprotein phosphatase activity and only about 3% of the protein are found in the supernatant after incubation of the DEAE-Sephadex eluate with the ATP-Sepharose gel. Incubation of the DEAE-Sephadex eluate with either caproic acid Sepharose or unsubstituted Sepharose did not result in purification of phosphoprotein phosphatase activity. All preparations of phosphatase used in subsequent experiments were purified at least 200-fold. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified enzyme shows several protein bands, indicating that the purified enzyme is not homogeneous (data not shown).

Characteristics of the phosphoprotein phosphatase asssay. The dephosphorylation of phosphoprotamine by the purified phosphoprotein phosphatase was approximately proportional to the incubation time up to 30 min and to the enzyme concentration up to 10 μg per assay tube (data not shown). Upon prolonged incubation (24 h at 37°C), 32—35% of the alkali-labile phosphate in phosphoprotamine is released by the phosphatase. Fig. 1 indicates that the pH

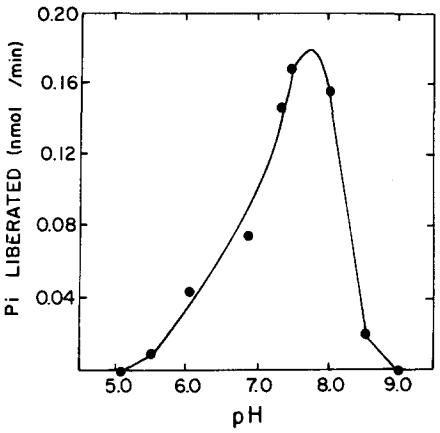


Fig. 1. Effect of pH on phosphoprotein phosphatase activity. Incubation conditions were as described in the text, except for the variation in the pH of the assay. At pH values below 6.9, 50 mM histidine · HCl was used instead of 50 mM Tris · HCl as buffer. The enzyme activities observed in the presence of histidine buffer were multiplied by 1.3, to correct for the observation that, at pH 6.9, enzyme activity in Tris buffer was 1.3 times greater than that in histidine buffer.

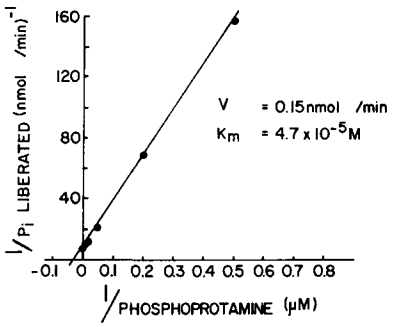


Fig. 2. Lineweaver-Burk analysis of phosphoprotamine phosphatase; Phosphoprotamine phosphatase activity was assayed according to the procedure described in the text, except for the variation in phosphoprotamine concentration. The data is plotted according to the method of Lineweaver and Burk.

optimum for phosphoprotamine hydrolysis is between 7.5 and 8.0. The enzyme activity decreases gradually at acid pH, but decreases rapidly on the alkaline side of the pH optimum. Enzyme activity is only 10% of maximal at pH 8.5.

Kinetic constants for phosphoprotamine dephosphorylation. Phosphoprotein phosphatase activity varies with phosphoprotamine concentration. Phosphoprotein phosphatase activity can be observed at phosphoprotamine concentrations as low as 1 μM (alkali-labile phosphate), and activity increases up to 200 μM phosphoprotamine. A Lineweaver-Burk analysis results in an estimate of the apparent K_m for phosphoprotamine of 50 μM (Fig. 2).

Effect of divalent cations on enzyme activity. Phosphoprotein phosphatase activity is dependent upon the presence of divalent cations. Dialysis of a DEAE-Sephadex purified enzyme preparation against divalent cation-free buffer containing 1 mM EDTA results in almost complete loss of enzyme activity. This dialyzed, inactivated enzyme can be partially reactivated by the addition of $MnCl_2$. Fig. 3 shows the activity of the dialyzed enzyme as a function of increasing $MnCl_2$ concentrations. Phosphoprotein phosphatase activity is stimulated 5-fold by $MnCl_2$ concentrations above 1 mM; half-maximal activation of the phosphatase occurs at 0.4 mM $MnCl_2$. 10 mM $ZnCl_2$ abolishes residual phosphatase activity, and prevents the reactivation of the enzyme by Mn^{2+} (Fig. 3). Phosphatase activity is also abolished by 10 mM Co^{2+}, Cu^{2+}, and Fe^{2+}, whereas Mg^{2+} and Ca^{2+} at this concentration have no effect on enzyme activity in the presence or absence of Mn^{2+} (not shown).

Substrate specificity. The substrate specificity of the purified phosphoprotein phosphatase was studied by testing its ability to hydrolyze a variety of small phosphorylated compounds. Under conditions in which the purified

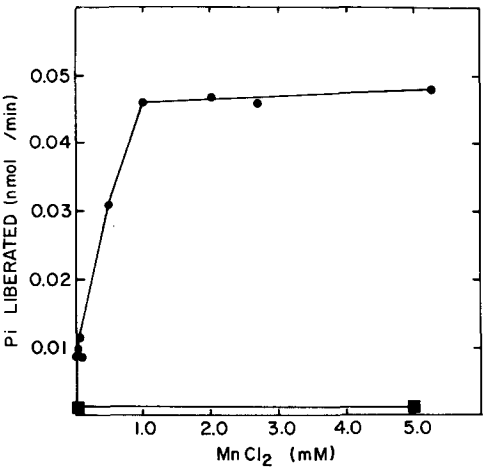


Fig. 3. Effect of $MnCl_2$ on phosphoprotein phosphatase activity. A sample of phosphoprotein phosphatase purified through the stage of DEAE-Sephadex chromatography was dialyzed against Mn^{2+}-free Buffer B. The activity of the dialyzed enzyme was then assayed according to the method described in the text, except for variations in the concentrations of divalent cations. $MnCl_2$ was added at the concentrations indicated in the figure. ●———●, $MnCl_2$ alone, no $ZnCl_2$; ■———■, $MnCl_2$ plus 10 mM $ZnCl_2$.

enzyme hydrolyzes phosphoprotamine, the enzyme shows no activity against a large number of low molecular weight phosphorylated compounds. The enzyme causes no detectable hydrolysis of ATP, ADP, AMP, GTP, glucose-6-*P*, β-glycerophosphate, phosphoserine, or phosphothreonine. The only low molecular weight substrate found so far for this enzyme is *p*-nitrophenyl phosphate; at a concentration of 1.0 mM, *p*-nitrophenyl phosphate is hydrolyzed at about 30% the rate of phosphoprotamine hydrolysis.

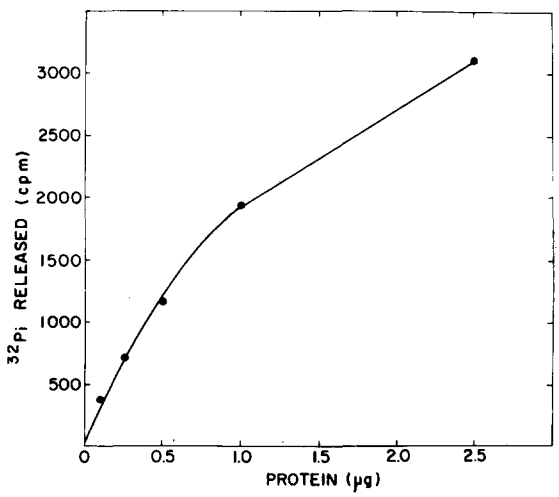


Fig. 4. Effect of enzyme concentration of phosphohistone phosphatase. The assay for phosphohistone dephosphorylation was described in the text.

However, the phosphoprotein phosphatase is active against other phosphoproteins. As shown in Fig. 4, the enzyme catalyzes the release of $^{32}P_i$ from ^{32}P-labeled phosphohistone. Phosphohistone dephosphorylation is also dependent on divalent cations. Dialysis of a partially purified DEAE-Sephadex eluate against divalent cation-free, EDTA-containing buffer causes a 50% loss in enzyme activity. This activity can be restored by the addition of 10 mM Mn^{2+}. Similar concentrations of Mg^{2+} and Ca^{2+} have no effect on the phosphatase activity in the absence or presence of Mn^{2+}, while Zn^{2+} abolishes residual phosphatase activity and prevents its reactivation by Mn^{2+}. The addition of 50 μM phosphoprotamine inhibits the release of $^{32}P_i$ from phosphohistone by 70%; identical concentrations of free protamine inhibit histone dephosphorylation by less than 20% (data not shown). In addition, the enzyme inactivates both liver and muscle phosphorylase, and promotes the conversion of muscle phosphorylase *a* to the *b* form.

Copurification of muscle phosphorylase and protamine phosphatase. The activities of the phosphatase against phosphoprotamine, muscle phosphorylase *a* and *p*-nitrophenyl phosphate were monitored over several stages of purification. Table II indicates that phosphorylase phosphatase and phosphoprotamine phosphatase activities copurified over the last three purification steps. It was not possbile to monitor the activities of phosphorylase phosphatase in crude adrenal cortical homogenates due to the presence of phosphorylase and phosphorylase kinase, which interfered with the assay of phosphorylase phosphatase activity. *p*-Nitrophenyl phosphatase activity also copurified with the other phosphatase activities, although the purification of this activity did not correlate precisely with the purification of the other phosphatase activities.

Competition experiments. A series of experiments was performed in order to determine whether phosphorylase and phosphoprotamine competed for hydrolysis by the same phosphoprotein phosphatase. The inactivation of liver and muscle phosphorylase by the phosphoprotein phosphatase was measured in the presence and absence of phosphoprotamine (Table III). Liver phosphorylase inactivation was inhibited 48% by 45 μM phosphoprotamine, while muscle phosphorylase phosphatase activity was virtually abolished (90% inhibition) by

TABLE III

INHIBITION OF PHOSPHORYLASE INACTIVATION BY PHOSPHOPROTAMINE

Phosphorylase phosphatase assays in the presence and absence of phosphoprotamine and free protamine were carried out according to the procedure described in the text. Phosphorylase phosphatase activity is expressed in terms of percentage of activity obtained in the absence of protamine. n.d., not determined.

Addition	Protein (μg/ml)	Alkali-Labile phosphate (μM)	Phosphorylase inactivation (percent control activity)	
			Liver	Muscle
None			100	100
Phosphoprotamine	150	45	52	n.d.
Protamine	150		120	n.d.
Phosphoprotamine	30	9	n.d.	10
Protamine	30		n.d.	85

TABLE IV

INHIBITION OF PHOSPHOPROTAMINE PHOSPHATASE BY MUSCLE PHOSPHORYLASE

Phosphoprotamine phosphatase activity was assayed as described in the text, in the presence and absence of muscle phosphorylase *a*. Enzyme activity is expressed in terms of percentage of activity obtained in the absence of phosphorylase.

Concentration of muscle phosphorylase (nM)	Phosphoprotamine phosphatase (percent control activity)
None	100
60	83
300	54

9 μM phosphoprotamine. Identical concentrations of unphosphorylated protamine did not interfere significantly with phosphorylase inactivation. Phosphoprotamine at this concentration had no effect on phosphorylase activity. Under conditions of the phosphorylase assay no liberation of P_i from the phosphoprotamine could be detected. Table IV shows the effects of purified muscle phosphorylase *a* on the hydrolysis of phosphoprotamine. 60 and 300 nM muscle phosphorylase *a* inhibited the dephosphorylation of phosphoprotamine by 17 and 46%, respectively.

Effect of salt. The optimal assay conditions for phosphorylase phosphatase and phosphoprotamine phosphatase are similar; however, phosphorylase phosphatase activity is maximal at lower ionic strength. Fig. 5 shows the effect of NaCl on phosphorylase phosphatase and phosphoprotamine phosphatase activities. While 1.0 M NaCl stimulates protamine dephosphorylation 3-fold, it inhibits inactivation of liver phosphorylase by 90%. In other experiments, we found that KCl effects enzyme activity in a manner similar to NaCl (data not shown). In subsequent experiments, protamine dephosphorylation was measured in the presence of 0.4 M NaCl, and phosphorylase phosphatase was assayed in the absence of NaCl.

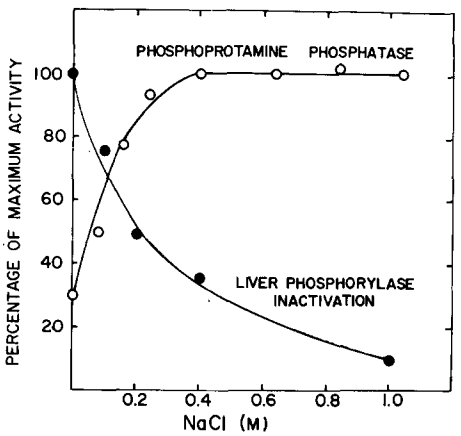


Fig. 5. Effect of NaCl on phosphoprotein phosphatase activities. Incubation conditions for phosphoprotamine phosphatase and phosphorylase phosphatase activities were the same as described in the text, except for the variation in NaCl concentration.

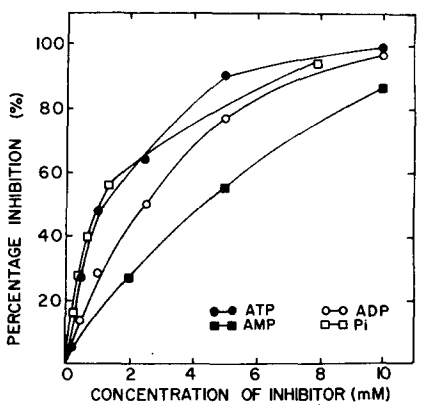
TABLE V

EFFECTORS OF PHOSPHATASE ACTIVITY

Phosphoprotamine phosphatase and phosphorylase phosphatase assays were carried out as described in the text, except for the addition of the compounds listed. Enzyme activity is expressed in terms of percentage of activity obtained in the absence of the modifier. n.d., not determined.

Effector	Concentration (mM)	Phosphatase activity (%)		
		Phosphoprotamine	Liver phosphorylase	Muscle phosphorylase
No addition		100	100	100
Glucose	3	101	149	131
Glucose-1-*P*	3	102	31	44
Glucose-6-*P*	3	97	55	65
ATP	0.5	67	10	55
ATP	0.05	100	50	n.d.
ADP	0.5	86	56	n.d.
AMP	0.5	100	34	n.d.
P_i	0.1	82	57	n.d.
NaF	75	n.d.	35	n.d.
NaF	32	23	n.d.	n.d.

Effectors of phosphorylase and phosphoprotamine phosphatase activities. A variety of possible effectors were tested for their effects on phosphorylase phosphatase and phosphoprotamine phosphatase activities. The results of these studies are summarized in Table V and Fig. 6. The adenine nucleotides are inhibitors of phosphoprotamine phosphatase activity (Fig. 6). In these experiments a half-maximal inhibition effect is produced by 1 mM ATP, 2.5 mM



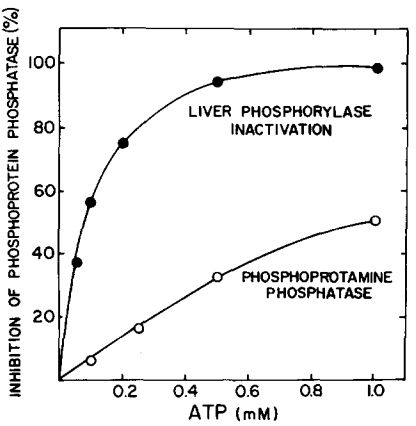


Fig. 6. Inhibition of phosphoprotamine phosphatase activity by adenine nucleotides and by P_i. Phosphoprotamine phosphatase activity was assayed according to the method described in the text, with the addition of various inhibitors at the concentrations indicated in the figure. The figure presents the percentage inhibition of phosphoprotamine phosphatase activity by ATP (●———●), ADP (○———○), AMP (■———■), and P_i (□———□).

Fig. 7. Effect of ATP on phosphoprotein phosphatase activities. Phosphoprotamine phosphatase and liver phosphorylase phosphatase were assayed as described in the text, with the addition of ATP at the concentrations indicated in the figure. The figure presents the percentage inhibition of phosphoprotein phosphatase activities by ATP.

ADP, and 5 mM AMP. These compounds also inhibit phosphorylase inactivation (Table V). Fig. 7 shows the effects of varying concentrations of ATP on liver phosphorylase inactivation and phosphoprotamine dephosphorylation. Under our assay conditions, phosphorylase phosphatase activity was inhibited 50% by 50 μM ATP, while phosphoprotamine phosphatase was inhibited 50% by 1 mM ATP. 0.5 mM ATP inhibited muscle phosphorylase inactivation by 45%.

The effects of cyclic AMP and cyclic GMP on phosphorylase phosphatase and phosphoprotamine phosphatase were also studied. Cyclic AMP at 10 μM had no effect on phosphorylase phosphatase in the presence or absence of Mg^{2+}-ATP, and with or without the addition of cyclic AMP-dependent protein kinase; neither cyclic AMP nor cyclic GMP at concentrations as high as 500 μM have any effect on phosphoprotamine dephosphorylation (not shown). Phosphoserine, phosphothreonine, pyridoxal phosphate, and phenformin also had no effect on phosphoprotamine phosphatase activity. The two end products of phosphoprotamine dephosphorylation were also studied. P_i at a concentration of 1 mM inhibits the phosphatase activity 50% (Fig. 6), while free protamine at a concentration of 0.1 mM has no effect on the enzyme (not shown). P_i also inhibited liver phosphorylase inactivation, a concentration of 100 μM causing a 50% inhibition of phosphorylase phosphatase activity. NaF inhibits both phosphoprotamine and phosphorylase phosphatase activities.

Glucose and its phosphorylated derivatives had differential effects on the hydrolysis of phosphoprotamine and phosphorylase. At a concentration of 3 mM, glucose stimulated liver and muscle phosphorylase phosphatase activity by 49 and by 31%, respectively. Glucose-1-*P* and glucose-6-*P* are inhibitors of phosphorylase phosphatase. Glucose-1-*P* (3 mM) inhibited liver phosphorylase inactivation by 69% and muscle phosphorylase inactivation by 56%, while glucose-6-*P* (3 mM) inhibited the inactivation of liver phosphorylase by 45% and of muscle phosphorylase by 35%. These compounds had no effect on phosphoprotamine dephosphorylation.

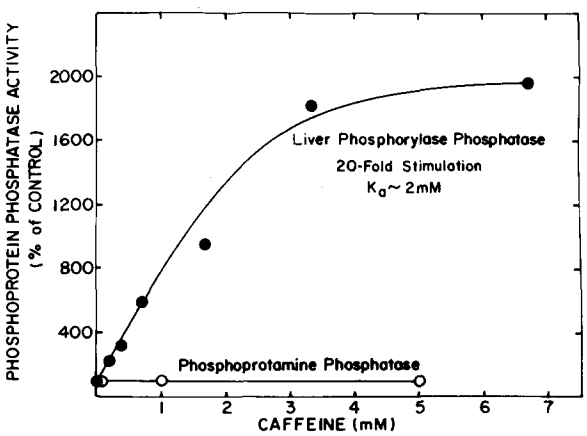


Fig. 8. Effect of caffeine on phosphoprotein phosphatase activities. Phosphoprotamine phosphatase and liver phosphorylase phosphatase activities were assayed as described in the text, with the addition of caffeine at the concentrations indicated in the figure. The figure presents the phosphatase activities in the presence of caffeine as a percentage of the activities found in the absence of caffeine.

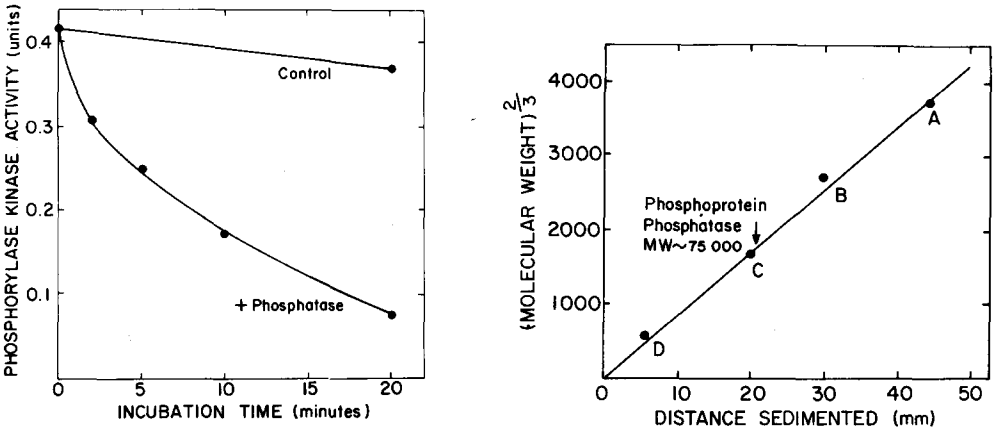


Fig. 9. Time course for phosphorylase *b* kinase inactivation. Phosphorylase *b* kinase inactivation was assayed according to the procedure in the text.

Fig. 10. Estimation of the molecular weight of the phosphoprotein phosphatase. The molecular weight of the phosphoprotein phosphatase was estimated by sucrose gradient centrifugation, according to the method described in the text. The activities of the phosphatase (using *p*-nitrophenyl phosphate as substrate) and of the marker enzymes were measured, and the migration of these enzymes was plotted as a function of (molecular weight)$^{2/3}$. The molecular weight standards used were: A, bovine liver catalase (mol. wt 232 000); B, rabbit muscle lactic acid dehydrogenase (mol. wt 142 000); C, porcine heart malic acid dehydrogenase (mol. wt 70 000); and D, bovine pancreatic ribonuclease A (mol. wt 13 700). The slope of the line was determined by least squares analysis.

Caffeine markedly stimulated the inactivation of liver phosphorylase. Fig. 8 indicates that caffeine at a concentration of 10 mM stimulated the inactivation of liver phosphorylase 20-fold; 2 mM caffeine caused a half-maximal stimulation of phosphorylase inactivation. 5 mM caffeine had no effect on phosphoprotamine phosphatase activity.

Inactivation of muscle phosphorylase b kinase. The purified phosphoprotein phosphatase preparation also catalyzed a time-dependent inactivation of phosphorylase *b* kinase (Fig. 9).

Molecular weight estimation. The molecular weight of the phosphoprotein phosphatase was estimated by centrifugation in a sucrose gradient. The migration of the marker enzymes in this gradient was proportional to their (molecular weight)$^{2/3}$, and was not affected by phosphatase. The molecular weight of the phosphoprotein phosphatase (using *p*-nitrophenyl phosphate as substrate) was estimated by this method to be about 75 000 (Fig. 10).

Discussion

Phosphoprotein phosphatases catalyze the release of P_i from phosphoseryl or phosphothreonyl residues in phosphoproteins. Thus, they might be expected to antagonize any actions of cyclic AMP that are mediated by protein kinases. The study of phosphoprotein phosphatase activity might add to our understanding of any system in which cyclic AMP is involved. The role of phosphoprotein phosphatases in the regulation of enzyme activity has only recently been investigated. Phosphoprotein phosphatases have been purified and charac-

terized from skeletal muscle [28], heart [29], liver [30], and brain [31]. We have purified and characterized a soluble phosphoprotein phosphatase from bovine adrenal cortex.

Purification of the phosphoprotein phosphatase by conventional methods of enzyme purification has been difficult; we have been able to achieve only a 40-fold enrichment in enzyme activity by these methods. Other workers have apparently had similar problems in the purification of phosphoprotein phosphatases [28,29,32]. We found that fractional adsorption onto ATP-Sepharose resins was a useful method for purifying the enzyme. Under appropriate conditions, up to 98% of the protein in partially purified phosphoprotein phosphatase preparations will bind to this resin. The small fraction of protein that does not bind to the resin contains about 30% of the phosphoprotein phosphatase activity. Only a small percentage of the protein in adrenal extracts binds to unsubstituted Sepharose or to caproic acid-Sepharose resins. It appears, therefore, that most of the protein in the DEAE-Sephadex eluate has an affinity for ATP, and that the phosphoprotein phosphatase has a somewhat lower affinity for this nucleotide than does the bulk of the other protein.

The adrenal phosphoprotein phosphatase is stabilized and stimulated by Mn^{2+}. Mg^{2+} and Ca^{2+} have no effect on the phosphatase, while Zn^{2+}, Cu^{2+}, Co^{2+}, and Fe^{2+} abolish phosphatase activity regardless of the presence of Mn^{2+}. Maeno and Greengard [31] reported that a soluble phosphoprotein phosphatase from brain was stimulated by Mn^{2+} and inhibited by Zn^{2+}, Co^{2+}, and Cu^{2+}. Kato and Bishop [28] found that glycogen synthase phosphatase from skeletal muscle was also activated by Mn^{2+}. Mn^{2+} activation and inhibition by other divalent cations may turn out to be a common property of phosphoprotein phosphatases.

The adrenal phosphoprotein phosphatase has an apparent molecular weight of about 75 000, as estimated by sucrose gradient centrifugation. Using the same methods, Nakai and Thomas [29] have estimated the molecular weight of glycogen synthase phosphatase from bovine heart to be 65 000—70 000. In contrast, the molecular weight of liver phosphoprotein phosphatase was estimated by gel exclusion chromatography to be approx. 190 000 [30].

We have focused our attention on the substrate specificity of the adrenal cortical phosphoprotein phosphatase. This enzyme hydrolyzes *p*-nitrophenyl phosphate, but the rate of this reaction is small compared to that of phosphoprotamine dephosphorylation. Other low molecular weight compounds, including nucleotides, sugar phosphates, and phosphorylated amino acids are not substrates for the phosphatase. It is noteworthy that the phosphatase does not hydrolyze phosphoserine, since it catalyzes the release of P_i from phosphoseryl residues in phosphoprotamine. Apparently, the enzyme is only active against protein-bound phosphoseryl residues. The phosphoprotein phosphatase preparation catalyzes the release of P_i from phosphoseryl residues in phosphorylated calf thymus histone fIIb. The properties of histone and protamine dephosphorylation are similar. Phosphoprotamine inhibits the hydrolysis of phosphohistone. It is likely that the same enzyme dephosphorylates both phosphoprotamine and phosphohistone.

The adrenal cortex contains the enzymes necessary for glycogen synthesis

and breakdown. Haynes found that ACTH increased cyclic AMP levels in adrenal slices [10] and activated adrenal phosphorylase [33]. The elevation of cyclic AMP levels presumably leads to the activation of phosphorylase, either by the stimulation of phosphorylase kinase or the inhibition of phosphorylase phosphatase [11]. If the purified phosphoprotein phosphatase possessed phosphorylase phosphatase activity, it should catalyze the inactivation of phosphorylase. We found that the adrenal phosphoprotein phosphatase inactivated both the adrenal-like liver phosphorylase and the less similar muscle phosphorylase. We chose to study the inactivation of liver and muscle phosphorylase, since they are both well-characterized enzymes. Riley and Haynes [11] have shown that phosphorylase kinase and phosphorylase phosphatase from liver, respectively, activated and inactivated adrenal phosphorylase, and that the adrenal modifying enzymes also modified liver phosphorylase.

Although the phosphoprotein phosphatase preparation that possessed both phosphorylase phosphatase activity and phosphoprotamine phosphatase activity had been purified several hundred-fold, the preparation was not homogeneous. It was important to determine whether or not the same phosphatase catalyzed the dephosphorylation of phosphoprotamine and the inactivation of phosphorylase. Two types of experiments provided evidence that one enzyme catalyzed both reactions. First, we observed a similar increase in specific activity of both muscle phosphorylase phosphatase and phosphoprotamine phosphatase activities through the last three purification procedures. The second type of evidence for the one enzyme-multi-substrate hypothesis was provided by a series of complementary competition experiments in which phosphorylase inhibited phosphoprotamine hydrolysis and phosphoprotamine inhibited phosphorylase phosphatase. A quantitative comparison of the apparent K_i values for phosphoprotamine and for phosphorylase *a* with the apparent K_m values for the hydrolysis of these compounds is difficult, since the assays are performed under somewhat different conditions. Although we have not eliminated the possibility that the purified phosphoprotein phosphatase preparation contains several separate phosphatase enzymes, the copurification of phosphoprotamine and muscle phosphorylase phosphatase activities, together with the competition experiments, suggests that one enzyme possesses both of these activities.

The adrenal phosphoprotein phosphatase preparation also catalyzes the inactivation of skeletal muscle phosphorylase *b* kinase. Phosphorylase *b* kinase activity has been detected in adrenal cortex, but it is not known whether this enzyme is distinct from the cyclic AMP-dependent protein kinase, as it is in skeletal muscle. Since the active forms of phosphorylase and phosphorylase *b* kinase both promote glycogen breakdown, the activity of the two enzymes might be regulated coordinately. We have been unable to demonstrate an activation of glycogen synthase from the D to I form by the adrenal phosphoprotein phosphatase. This phosphatase inactivates both synthase D and synthase I from liver and muscle (not shown). We do not understand the action of phosphoprotein phosphatase on glycogen synthase. It is possible that the phosphoprotein phosphatase converts glycogen synthase D to a partially phosphorylated inactive form, of the kind proposed by Rosell-Perez [34].

Several laboratories have reported that phosphoprotein phosphatases from other tissues have a broad substrate specificity. Zieve and Glinsmann [35] have

evidence that a single skeletal muscle enzyme dephosphorylates glycogen synthase and phosphorylase *b* kinase [35]. The glycogen synthase phosphatase isolated from skeletal muscle by Kato and Bishop [28] also dephosphorylates histone. A phosphoprotein phosphatase preparation from cardiac muscle catalyzes the activation of glycogen synthase, the inactivation of phosphorylase, and the dephosphorylation of glycogen synthase, phosphorylase *a*, phosphorylase *b* kinase, casein, and histone [29,36].

We have attempted to determine whether the several activities of the adrenal phosphoprotein phosphatase are regulated separately or together. The adenine nucleotides inhibit both phosphoprotamine dephosphorylation and phosphorylase inactivation. Significant inhibition of phosphorylase inactivation occurs at a much lower nucleotide concentration than found for protamine dephosphorylation. A detailed comparison of the effects of nucleotides on phosphorylase inactivation and phosphoprotamine hydrolysis is not warranted, since the assays of these two activities are performed under somewhat different conditions. Other workers have also reported an inhibition of phosphorylase phosphatase from several tissues by adenine nucleotides [36—40]. However, these workers have concluded that the inhibition of phosphorylase phosphatase activity by AMP is due to an effect of the nucleotide on phosphorylase, and not on the phosphatase [38—40]. It is possible that the phosphoprotein phosphatases from the adrenal and muscle differ in their sensitivity to inhibition by adenine nucleotides. P_i, like the adenine nucleotides, inhibits both phosphorylase phosphatase and phosphoprotamine phosphatase activities of the adrenal phosphoprotein phosphatase. P_i also inhibits phosphoprotein phosphatase activity in skeletal and cardiac muscle [28,34,38].

Glucose and its phosphorylated derivatives affect phosphorylase phosphatase activity, but do not affect the phosphoprotamine phosphatase activity of the adrenal phosphoprotein phosphatase. The activation of phosphorylase phosphatase by glucose, and its inactivation by glucose-1-*P* and glucose-6-*P*, may result from changes in the conformation of the substrate (phosphorylase). De Barsy et al. [39] and Bailey and Wehlan [40] have reported a stimulation of phosphorylase inactivation by glucose, and have concluded that the sugar exerts its effect by altering the conformation of phosphorylase. A similar conclusion was also reached by Martensen et al. [41], although in their experiments glucose-6-*P* appeared to stimulate muscle phosphorylase phosphatase activity.

Previous experiments have indicated a stimulatory effect of methylxanthines on liver phosphorylase phosphatase in vitro [42]. The phosphoprotein phosphatase from bovine adrenal cortex is also markedly stimulated by caffeine with liver phosphorylase as substrate. Methylxanthines may also stimulate phosphorylase phosphatase activity by binding to phosphorylase, since they have no effect on the hydrolysis of phosphoprotamine. If the hydrolysis of phosphoprotamine and the inactivation of phosphorylase are catalyzed by the same enzyme, then, in the presence of glucose derivatives and methylxanthines, the different activities of this enzyme appear to be regulated independently. The methylxanthines mimic and potentiate the effects of cyclic AMP in most tissues. However, theophylline antagonizes the effects of ACTH and of exogenous cyclic AMP on steroidogenesis [43]. Haynes and Berthet [33] proposed

that phosphorylase activation played a prominent role in the control of steroidogenesis by ACTH. It is possible that the methylxanthines exert their anti-cyclic AMP effects on steroidogenesis by promoting the inactivation of phosphorylase.

Merlevede and Riley [12] reported a reversible inactivation of adrenal phosphorylase phosphatase in the presence of ATP, Mg^{2+}, and cyclic AMP. Using chemically phosphorylated protamine as a substrate for the phosphoprotein phosphatase, we have been unable to duplicate their results. However, our enzyme may be distinct from the enzyme studied by these workers. Neither phosphoprotein phosphatase preparation is homogeneous, although our phosphatase preparation is considerably more purified. The cyclic AMP-dependent inactivation of phosphorylase phosphatase they observed may reflect an interaction of cyclic AMP with other components n their partially purified extracts.

Acknowledgments

This investigation was supported by Research Grant AM 15135 from the National Institutes of Health. R.L.P. is the recipient of a Research Career Development Award, AM 70648, from the National Institutes of Health. We thank Ms Michele Carvotta for expert technical assistance.

References

1 Segal, H.L. (1973) Science 180, 25—32
2 Holzer, H. and Duntze, W. (1971) Annu. Rev. Biochem. 40, 345—374
3 Walsh, D.A., Perkins, J.P. and Krebs, E.G. (1968) J. Biol. Chem. 243, 3763—3765
4 Miyamot, E., Kuo, J.F. and Greengard, P. (1969) J. Biol. Chem. 244, 6395—6402
5 Langan, T.A. (1968) Science 162, 579—580
6 Gill, G.N. and Garren, L.D. (1970) Biochem. Biophys. Res. Commun. 39, 335—343
7 Corbin, J.D. and Krebs, E.G. (1969) Biochem. Biophys. Res. Commun. 36, 328—336
8 Kuo, J.F. and Greengard, P. (1969) Proc. Natl. Acad. Sci. U.S. 64, 1349—1355
9 Walton, G.M., Gill, G.N., Abross, I.B. and Garren, L.D. (1971) Proc. Natl. Acad. Sci. U.S. 68, 880—884
10 Haynes, R.C. (1958) J. Biol. Chem. 233, 1220—1222
11 Riley, G.A. and Haynes, R.C. (1963) J. Biol. Chem. 238, 1563—1570
12 Merlevede, W. and Riley, G.A. (1966) J. Biol. Chem. 241, 3517—3524
13 Ullman, B. and Perlman, R.L. (1975) Biochem. Biophys. Res. Commun. 63, 424—431
14 Keilin, D. and Hartree, E.G. (1938) Proc. R. Soc. London, Ser. B, 124, 397—405
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
16 Page, L.B. and Culver, P.J. (1966) A Syllabus of Laboratory Examination in Clinical Diagnosis, revised edn, p. 300, Harvard University Press, Cambridge
17 Ames, B.N. and Dubin, D.T. (1960) J. Biol. Chem. 235, 769—775
18 Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372—1379
19 Beers, Jr, R.F. and Sizer, I.W. (1952) J. Biol. Chem. 195, 133—140
20 Kubowitz, F. and Ott, P. (1943) Biochem. Z. 314, 94—117
21 Mehler, A.H., Kornberg, A., Grisolia, S. and Ochoa, S. (1948) J. Biol. Chem. 174, 961—977
22 Kalnitsky, G., Hummel, J.P. and Dierks, C. (1959) J. Biol. Chem. 234, 1512—1516
23 Sutherland, E.W. and Wosilait, W.D. (1956) J. Biol. Chem. 218, 459—468
24 Wosilait, W.D. and Sutherland, E.W. (1956) J. Biol. Chem. 218, 469—481
25 Post, R.L. and Sen, A.K. (1967) Methods Enzymol. 10, 762—768
26 Riley, W.D., DeLange, R.J., Bratvold, G.E. and Krebs, E.G. (1968) J. Biol. Chem. 243, 2209—2215
27 Kuo, J.F., Krueger, B.K., Sanes, J.R. and Greengard, P. (1970) Biochim. Biophys. Acta 212, 79—91
28 Kato, K. and Bishop, J.S. (1972) J. Biol. Chem. 247, 7420—7428
29 Nakai, C. and Thomas, J.A. (1973) Biochem. Biophys. Res. Commun. 52, 530—536
30 Meisler, M.H. and Langan, T.A. (1969) J. Biol. Chem. 244, 4961—4968

31 Maeno, H. and Greengard, P. (1972) J. Biol. Chem. 247, 3269–3277
32 Villar-Palasi, C. (1969) Ann. N.Y. Acad. Sci. 166, 719–730
33 Haynes, R.C. and Berthet, L. (1957) J. Biol. Chem. 225, 115–124
34 Rosell-Perez, M. (1972) Ital. J. Biochem. 21, 34–69
35 Zieve, F.J. and Glinsmann, W.H. (1973) Biochem. Biophys. Res. Commun. 50, 872–878
36 Nakai, C. and Thomas, J.A. (1974) J. Biol. Chem. 249, 6459–6467
37 Gilboe, D.P. and Nuttall, F.Q. (1973) Biochem. Biophys. Res. Commun. 53, 164–171
38 Martensen, T.M., Brotherton, J.E. and Graves, D.J. (1973) J. Biol. Chem. 248, 8323–8328
39 De Barsy, T., Stalmans, W., Laloux, M., De Wulf, H. and Hers, H.G. (1972) Biochem. Biophys. Res. Commun. 46, 183–190
40 Bailey, J.M. and Whelan, W.J. (1972) Biochem. Biophys. Res. Commun. 46, 191–197
41 Martensen, T.M., Brotherton, J.E. and Graves, D.J. (1973) J. Biol. Chem. 248, 8329–8333
42 Sutherland, E.W. (1951) Ann. N.Y. Acad. Sci. 54, 693–706
43 Halkerston, I.D.K., Feinstein, M. and Hechter, O. (1966) Proc. Soc. Exp. Biol. Med. 122, 896–900