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PURIFICATION AND PROPERTIES OF A PHOSPHOPROTEIN PHOSPHATASE FROM RAT LIVER

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Summary

A phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16) has been partially purified from rat liver homogenates by $(NH_4)_2SO_4$ and ethanol precipitations followed by DEAE-cellulose and Sepharose 6B chromatography. The phosphoprotein phosphatase is capable of cleaving [^{32}P]phosphate from radiolabelled phosphopyruvate kinase (type L) (EC 2.7.1.40), phosphohistones, and phosphoprotamine. However, it did not detectably dephosphorylate ATP, ADP, DL-phosphorylserine or β-glycerophosphate.

Dephosphorylation of [^{32}P]phosphopyruvate kinase was stimulated by divalent cations and inhibited by ATP, ADP, Fru-1,6-P_2, and orthophosphate. Divalent cations could reverse inhibition induced by ADP or ATP. At least one function of the phosphoprotein phosphatase may be to remove phosphate groups from the phosphorylated form of pyruvate kinase in the liver.

Introduction

Phosphoprotein phosphatases (phosphoprotein phosphohydrolases, EC 3.1.3.16) are known to counteract the regulatory phosphorylation of enzymes [1,2]. For example, the phosphorylation of glycogen synthetase *a* (EC 2.4.1.11), or phosphorylase *b* kinase (EC 2.7.1.37), by a cyclic AMP-dependent protein kinase is reversed by a phosphoprotein phosphatase-catalysed dephosphorylation [2,3]. Phosphorylase *a* (EC 2.4.1.1) [4] and hormone-sensitive lipase [5] are inactivated by phosphoprotein phosphatase activity.

Recently it was shown that the inhibition of pyruvate kinase by phosphorylation with MgATP in the presence of a cyclic AMP-dependent protein kinase [6] is removed by the action of a histone phosphatase [7]. However, little is known about the factors governing the reaction of the phosphatase with phosphorylated pyruvate kinase. The aims of these investigations were to further

purify the phosphatase and to study the conditions optimal for the dephosphorylation of pyruvate kinase.

Experimental

[γ-^{32}P]ATP was synthesized according to the method of Engström [8] and purified as described Mårdh [9]. Whatman DEAE-cellulose (DE-52) was used. Sepharose 6B from Pharmacia, Uppsala, was washed with 20 vols. of deionized water to remove material which otherwise inactivated the phosphatase. Sephadex G-100 was from Pharmacia. ATP, ADP, dithiothreitol, mixed calf thymus histones (type IIA), myoglobin, human serum albumin and protamine sulphate were from Sigma. $MnCl_2$, $MgCl_2$, $CaCl_2$, 2-(*N*-morpholino)ethanesulphonic acid (MES), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) were reagent grade. Hen egg albumin and chymotrypsinogen were purchased from Boehringer-Mannheim. An Amicon ultrafiltration cell, Model 202, equipped with a Diaflo PM-10 membrane, or collodion bags from Sartorius GmbH, Gottingen, were used for concentration of enzymes. Dialysis tubing from Union Carbide Corp., Ill., U.S.A., was soaked in 10% acetic acid and rinsed with deionized water and buffer before use.

The catalytic subunit of the cyclic AMP-dependent protein kinase (peak I) from rabbit skeletal muscle was isolated as described under Method B by Beavo et al. [10].

Preparation of ^{32}P-labelled proteins. 20 mg of protamine or histones were incubated at 37° C for 9 h in a 5 ml reaction medium containing 20 mM MES/NaOH, pH 6.8, 10 mM $MgCl_2$, 1.2—1.4 mM [^{32}P]ATP (30—40 cpm/pmol), 5 mM β-mercaptoethanol and $3 \cdot 10^5$ units [10] of the catalytic subunit of the rabbit skeletal muscle cyclic AMP-dependent protein kinase. ^{32}P-labelled phosphoprotamine and ^{32}P-labelled phosphohistones were then isolated and their specific radioactivity determined [11]. ^{32}P-labelled phosphoprotamine contained 230—250 nmol of alkali-labile phosphate per mg of protein and the batch of ^{32}P-labelled phosphohistones used in this work contained 90 nmol of alkali-labile phosphate per mg of protein. Pyruvate kinase was phosphorylated using 750 units of rabbit muscle protein kinase per mg of pyruvate kinase at conditions described earlier [7]. The [^{32}P]phosphopyruvate kinase, purified by chromatography on a hydroxyapatite column, was concentrated and stored at —25° C. Before use portions of 1 ml were chromatographed on a Sephadex G-25 column (1.5 × 15 cm) equilibrated and eluted with 5 mM imidazole · HCl buffer, pH 7.5, containing 0.1 mM fructose 1,6-diphosphate, 10% glycerol and 1 mM dithiothreitol. 1 mol of phosphorylated pyruvate kinase contained about 4 mol [^{32}P]phosphate assuming the same specific radioactivity for the [^{32}P]phosphoenzyme as for the [γ-^{32}P]ATP used [7]. It was assumed that the molecular weight of pyruvate kinase tetramer is 250 000 [6].

Assay of phosphoprotein phosphatase activity. The assay was based on the method described by Meisler and Langan [11]. The standard reaction mixture, 40 or 100 μl, contained 60 μM [^{32}P]phosphoprotamine, 2.5 mM $MnCl_2$, 1.0 mM dithiothreitol (or β-mercaptoethanol), and 25 mM Tris · HCl, pH 7.5 (at 30° C). After warming the partial incubation medium ($\frac{3}{4}$ vol.) at 30° C for 5 min, the reaction was started by the addition of the phosphoprotein phosphatase ($\frac{1}{4}$ vol.)

which was suitably diluted with 5 mM imidazole · HCl, pH 7.5, containing 0.1 mg bovine serum albumin per ml and 1 mM β-mercaptoethanol or 1 mM dithiothreitol. All tests were performed in duplicate. The incubation was carried out in 5 ml stopperd conical glass tubes for 5 min and stopped by the addition of 1 ml of 5 mM silicotungstic acid in 5 mM H_2SO_4. The [^{32}P]orthophosphate released was extracted as phosphomolybdate by a modified procedure of Martin and Doty [12]: 0.2 ml of a 5% solution of $(NH_4)_2MoO_4$ in 2 M H_2SO_4 and 1.5 ml isobutanol/benzene (1 : 1, v/v were added to each tube. The tubes were agitated for 20 s on a vortex mixer and centrifuged at 1000 × *g* for 5 min. 1 ml of the organic phase was mixed with 10 ml of 0.5 mM NaOH [9] in a polyethylene scintillation vial. The vials were swirled gently and their Cerenkov radiation measured in an Intertechnique SL liquid scintillation spectrometer. The amount of [^{32}P]orthophosphate released was calculated by comparison with the specific radioactivity of ^{32}P-labelled proteins (see above) samples of which were counted in parallel. In some experiments [^{32}P]phosphohistones, or [^{32}P]phosphorylated pyruvate kinase, were used instead of [^{32}P]-phosphoprotamine. The rate of the release of [^{32}P]orthophosphate was linear for at least 10 min at the enzyme concentrations used. 1 unit of phosphoprotein phosphatase is defined as that amount of enzyme which catalysed the release of 1 nmol of phosphate from [^{32}P]phosphoprotamine per min at the conditions of routine assay.

Miscellaneous. Protein was determined according to the method of Lowry et al. [13] with bovine serum albumin as a standard, or from the absorbance at 280 nm where 1 mg/ml of protein was taken to have an absorbance of 1.0 (light path = 1 cm). The K_m for the dephosphorylation of ^{32}P-labelled phosphoproteins was calculated from Lineweaver-Burk plots of the data of experiments conducted at the conditions of standard assay. In these experiments the concentrations of [^{32}P]phosphoprotamine and [^{32}P]phosphohistones varied from 5 to 60 μM whereas that of [^{32}P]phosphopyruvate kinase varied from 5 to 25 μM. The substrate was expressed as the concentration of the phosphate moiety in the phosphoproteins. Any endogenous phosphate present in pyruvate kinase before phosphorylation was not considered.

Purification of phosphoprotein phosphatase. The following buffers were used: 50 mM imidazole · HCl, pH 7.5, containing 15 mM β-mercaptoethanol (buffer A); 50 mM imidazole · HCl, pH 6.5, containing 2.5 mM $MgCl_2$, mM β-mercaptoethanol and 22% glycerol (buffer B). Except specified otherwise, centrifugations were done at 16 000 × *g* for 20 min in a Sorvall-RC-2B preparative centrifuge.

30—40 male Sprague-Dawley rats, weighing 300—350 g, were maintained on tap water and ordinary laboratory chow R3 (Astra-Ewos AB, Södertålje, Sweden) for at least 1 week before the experiments. The rats were killed by cervical fracture and bled. Their livers were quickly excised, rinsed with cold homogenising solution containing 250 mM sucrose, 1 mM EDTA, pH 7.5 (NaOH), and 15 mM β-mercaptoethanol and 70—90 g protions of the liver were homogenised in three volumes of the homogenising solution for 1 min in a Waring blender, set at maximum speed. Unless otherwise stated subsequent steps were performed at 0—4°C. The combined homogenates were centrifuged and the debris discarded. The supernatant was filtered through glass wool to remove fat.

Crystalline ammonium sulphate was added slowly (over a period of 15 min) to the enzyme solution (at 0°C) with constant stirring to a final concentration of 0.472 g per ml. The solution was maintained at pH 7.5 by intermittently titrating it with concentrated NH_4OH. After stirring for an additional 20 min, the precipitates were collected by centrifugation. The supernatant was carefully decanted and discarded. Any loosely packed precipitates were recentrifuged so that the supernatant could be decanted without loss of sedimented material. This precaution was necessary to obtain good yields of the phosphatase activity at the next step. The well packed precipitates were dissolved in 1 vol. of buffer A containing 5 mM EDTA and cooled to 0°C.

To 80-ml portions of the enzyme solution at 0°C, 400 ml of 95% ethanol (20°C) were added with constant stirring. The addition of the ethanol took about 20—30 s during which the temperature of the enzyme solution rose to about 15°C. The thick suspension was immediately poured into a 500 ml polyethylene centrifugation tube and plunged into an ice bath. After treatment of five portions the suspension was centrifuged in six tubes at 5000 × *g* and —10°C for 5 min. The precipitates were carefully drained and immediately suspended in a minimum volume of buffer A containing 5 mM EDTA. The time taken for the treatment of each 400 ml batch of the enzyme solution with ethanol, followed by centrifugation and suspension of the precipitates in buffer, was less than 30 min. The pooled suspensions were diluted with buffer A containing 5 mM EDTA such that a total volume of 1 ml of buffer A per g wet weight of liver was used. Portions of the pooled suspension were then homogenised at 100 rev./min with one stroke in a Potter-Elvehjem homogeniser fitted with a teflon pestle. The supernatant obtained after centrifugation was saved and the precipitate reextracted with 0.5 volume of buffer A. The combined extracts (about 1000 ml) were dialysed against 10 vols. of buffer A containing 5 mM EDTA, for 21 h. Three buffer changes were made at intervals of 5—7 h. During dialysis the temperature was maintained at close to 0°C with the aid of an ice bath.

The dialysed extract was clarified by centrifugation. DE-52 (0.33 ml per g of liver) equilibrated with buffer A was added and the mixture stirred for 2 h. More than 90% of the phosphoprotein phosphatase was bound to the ion-exchanger. The ion-exchanger with bound enzyme was poured into a column, 6.5 cm in diameter, layered with 100 ml of packed DE-52, also equilibrated with buffer A. After packing the column was washed with 1000 ml of buffer A, followed by 2000 ml of the same buffer containing 50 mM NaCl until the absorbance of the eluate at 280 nm was less than 0.2. Phosphoprotein phosphatase was then eluted with 2000 ml of buffer A containing 200 mM NaCl and 2.5 $MgCl_2$ at a flow rate of 160 ml per h. Fractions of 15 ml were collected. Phosphoprotein phosphatase activity was tested in undiluted aliquots (25 µl) of the fractions and those which contained more than 10 units/ml were pooled and cooled to 0°C. Solid $(NH_4)_2SO_4$ was added with constant stirring over a period of 15 min to a final concentration of 0.516 g/ml. After 30 min the precipitates were collected by centrifugation, dissolved in about 20 ml of buffer B and dialysed for 7—10 h at 0—2°C; against 100 vols. of the same buffer.

The dialysed extract was applied to a DE-52 column (2.5 × 20 cm) equilib-

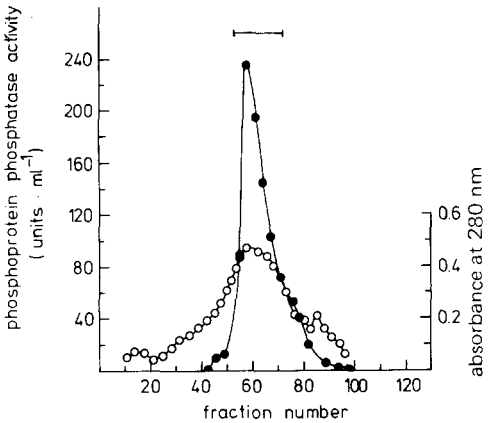

Fig. 1. Chromatography of phosphoprotein phosphatase on DEAE-cellulose. The column (2.5 X 20 cm) was equilibrated and eluted at pH 6.5 as described under Experimental. Samples from every third fraction were diluted and tested for phosphoprotein phosphatase activity (●———●). Open circles (○———○) refer to absorbance at 280 nm. The horizontal bar indicates the fractions which were used for further purification.

rated with buffer B. After washing the column with 1 l of buffer B or until the absorbance at 280 nm was less than 0.1, the enzyme was eluted with a 500 + 500 ml linear gradient of 0—0.35 M NaCl in buffer B at a flow rate of 48 ml per h. Fractions of 8 ml were collected. Phosphoprotein phosphatase activity usually eluted between 30 and 60% of the gradinet volume (Fig. 1). A third chromatography was performed on a similar column if the specific activity

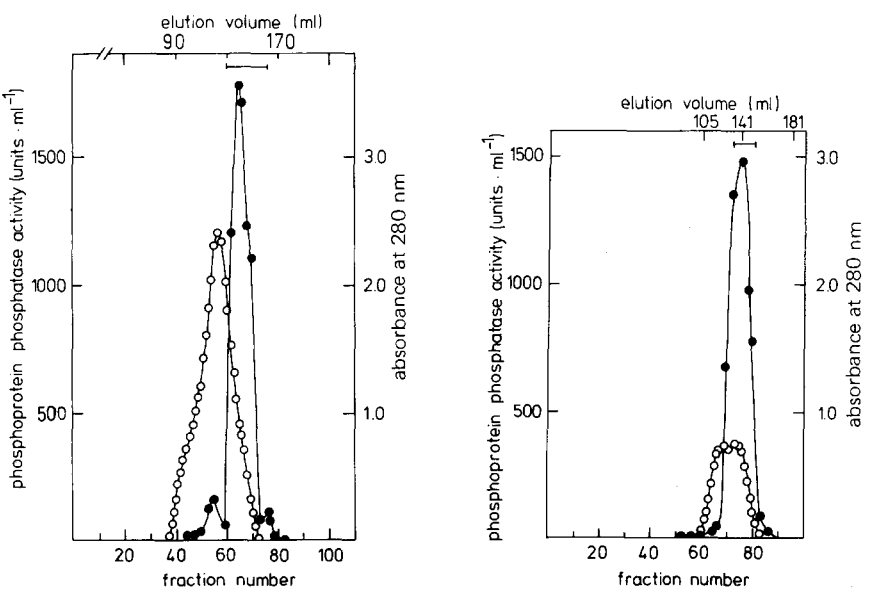

Fig. 2. Chromatography of phosphoprotein phosphatase on Sepharose 6B. The column (2.0 X 46.5 cm) was equilibrated and eluted as described under Experimental. ●———●, phosphoprotein phosphatase activity; ○———○, absorbance at 280 nm. (A) The material loaded to the column was from a DE-52 II chromatogram (cf. Fig. 1). (B) The material loaded to this column was that purified in the experiment shown on A. The horizontal bars indicate the fractions which were used for rechromatography (A) or in the experiments (B).

of the enzyme was less than 150 units per mg protein (A_{280nm}). The pooled fractions (from the second DE-52 chromatogram) with phosphoprotein phosphatase activity were concentrated to about 2 ml by successive ultrafiltration in an Amicon cell model 202 and a collodion bag. The phosphoprotein phosphatase preparation was then chromatographed on a Sepharose 6B column (2.0 × 46.5 cm), equilibrated and eluted with buffer B containing 200 mM NaCl at a flow rate of 6 ml per h. Fractions of about 2 ml were collected. The phosphoprotein phosphatase activity which was partially separated from the main protein peak (Fig. 2A) was concentrated by ultrafiltration in a collodion bag to about 1 ml and rechromatographed on the Sepharose 6B column (Fig. 2B).

The pooled enzyme from the second Sepharose 6B column was concentrated to about 1—2 ml and stored at 4°C where phosphoprotein phosphatase activity remained stable for at least 4 weeks. Attempts to store the enzyme at —25°C met with variable results. One out of a total of four preparations from the Sepharose 6B step lost 75% of its activity when stored at —25°C for 4 weeks, the other three preparations were stable for at least 4 weeks at —25°C.

Results

Purification and properties of a rat liver phosphoprotein phosphatase

The purification of a phosphoprotein phosphatase from rat liver is given in Table I. An early step in the procedure was based on the observation [14] that rat liver phosphoprotein phosphatase could withstand an ethanol precipitation step at 20°C without appreciable loss of enzymatic activity. Upon further purification on DEAE-cellulose the phosphoprotein phosphatase activity eluted

TABLE I

PURIFICATION OF RAT LIVER PHOSPHOPROTEIN PHOSPHATASE

For a typical preparation from 678 g of rat liver 187 000 units of ^{32}P-labelled phosphoprotamine phosphatase activity were obtained in the crude homogenate. Protein was determined by the method of Lowry et al. [13] for all fractions preceding the first DEAE-cellulose step, and subsequently from the absorbance at 280 nm.

Fraction	Specific activity (units/mg protein)	Yield (%)	Activity ratio ** [^{32}P]protamine / [^{32}P]pyruvate kinase
Homogenate	2.2	100	n.d.
16 000 × *g* supernatant	2.7	65	n.d.
0—70% $(NH_4)_2SO_4$ fraction	3.7	86	n.d.
Extract of ethanol precipitate *	2.4 *	20 *	17.0
DEAE-cellulose I	23.4	34	n.d.
0—75% $(NH_4)_2SO_4$ fraction	32.7	37	21.8
DEAE-cellulose II	260	14.8	29.9
Second Sepharose 6B	2277	7.7	22.1

* This yield probably reflects an inactivation of the enzyme during storage overnight at 4°C, since recoveries of 50—60% were obtained in experiments where the phosphatase was assayed immediately after dialysis.

** The pooled fractions from the steps indicated were stored at —25°C until thawed once and assayed in parallel with either [^{32}P] phosphoprotamine (60 μM) or [^{32}P] phosphopyruvate kinase (1.5 μM) as a substrate

n.d., not determined.

as a single peak (Fig. 1). It was verified that no additional activity was eluted from the DEAE-cellulose column when the NaCl concentration in the gradient buffer was increased from 0.35 to 0.5 M (not shown).

After chromatography on a Sepharose 6B column (Fig. 2) the phosphoprotein phosphatase was purified 1200-fold, with a yield of 7.7%; the specific activity of the purified enzyme averaged 3000 units/mg protein and ranged from 2000 to 4000 units. The studies reported below were performed with phosphoprotein phosphatases which had been purified through the stage of Sepharose 6B chromatography.

The molecular weight of the enzyme as estimated by gel chromatography on a calibrated Sephadex G-100 was approx. 32 000 (with or without 22% glycerol in the elution buffer (Fig. 3.). A similar molecular weight of 32 000 was also determined for rat liver phosphorylase *a* phosphatase after purification through an ethanol precipitation step at 20° C [14].

With [^{32}P]phosphoprotamine or [^{32}P]phosphopyruvate kinase as a substrate, phosphoprotein phosphatase activity was optimal around pH 7.5—8.0 (results not illustrated). The phosphoprotein phosphatase also catalysed the dephosphorylation of [^{32}P]phosphohistones. However, it did not detectably dephosphorylate ATP, ADP, β-glycerophosphate or DL-phosphorylserine in the presence or absence of 5 mM $MgCl_2$ at the conditions of standard assay. As can be seen in Table I phosphoprotamine and phosphopyruvate kinase activities nearly co-purified during the last steps of the purification procedure, indicating that these activities had similar properties. It should be noted that the concentration of [^{32}P]phosphopyruvate kinase in these tests (Table I) is far below the K_m for its dephosphorylation (see below). When present at a concentration of

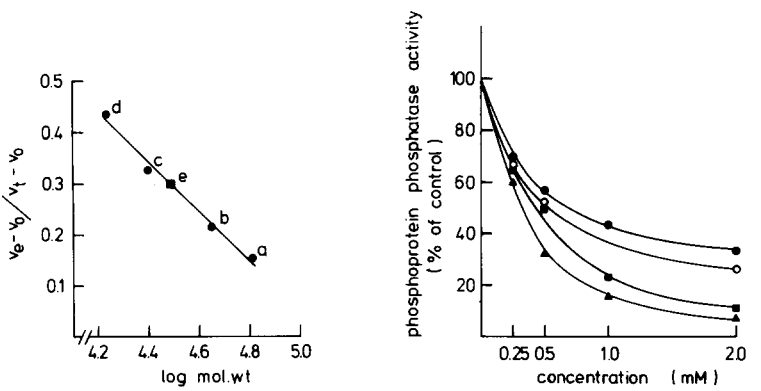

Fig. 3. Molecular weight estimation of protein phosphatase. The Sephadex G-100 column (2.5 X 47 cm) was calibrated with (a) human serum albumin (67 000), (b) hen egg albumin (45 000), (c) chymotrypsinogen (25 000), (d) myogobin (17 500). Elution position of chymotrypsinogen was determined in a separate experiment on the same column. The void volume was determined with blue dextran. The proteins (a)—(d) were identified from the absorbance at 280 nm and phosphoprotein phosphatase (e) by activity measurements. The column was eluted with 50 mM imidaole · HCl, pH 6.5, containing 15 mM β-mercaptoethanol, 2.5 mM $MgCl_2$ and 100 mM NaCl.

Fig. 4. Inhibition of the dephosphorylation of [^{32}]phosphopyruvate kinase by phosphoesters. The incubation medium, 40 μl, contained 25 μg bovine serum albumin, 0.043 unit phosphoprotein phosphatase and 5 μM [^{32}P]phosphopyruvate kinase in the absence of divalent cations. Other conditions are given under Experimental. 100% was the activity measured in the absence of the phosphoesters, (●——●) phospho*enol*pyruvate, (○——○) Fru-1,6-P_2, (■——■) ADP and (▲——▲) ATP.

20 μM, [^{32}P]phosphopyruvate kinase was dephosphorylated at a rate nearly equal to 50% of the rate of dephosphorylation of 60 μM [^{32}P]phosphoprotamine.

Dephosphorylation of [^{32}P]phosphopyruvate kinase

Previous experiments had indicated that [^{32}P]phosphorylated pyruvate kinase is a substrate of the phosphoprotein phosphatase [7]. When the substrate was expressed as the concentration of the [^{32}P]phosphate moiety, the apparent K_m for the dephosphorylation of [^{32}P]phosphopyruvate kinase was 27 ± 4 μM (mean ± S.D. of five determinations). Assuming that 1 mol of [^{32}P]-phosphopyruvate kinase contains 4 mol of [^{32}P]phosphate (see Experimental), the apparent K_m of 27 μM corresponds to about 7 μM pyruvate kinase, which is of the same order as 4—10 μM, the physiological concentration of pyruvate kinase in livers of rats given food rich in carbohydrates [7,15].

Fructose 1,6-diphosphate (Fru-1,6-P_2) up to 25 μM stimulates the activity of the phosphorylated form of pyruvate kinase [6]. However, when present at concentrations of 25 μM (not shown), Fru-1,6-P_2 had little effect (less than 5%) on the dephosphorylation of [^{32}P]phosphopyruvate kinase; 1 mM Fru-1,6-P_2 inhibited the dephosphorylation of [^{32}P]phosphopyruvate kinase by 60% (Fig. 4). The substrates of pyruvate kinase, ADP and phospho*enol*pyruvate, as well as ATP, also inhibited the phosphoprotein phosphatase (Fig. 4). The concentrations which caused half maximal inhibition were 0.25 mM for ATP and about 0.5 mM for ADP, phospho*enol*pyruvate and Fru-1,6-P_2.

The concentration of alanine, an inhibitor of pyruvate kinase activity, is increased in the livers of rats during gluconeogenesis when pyruvate kinase (type L) is postulated to be in the phosphorylated form [6]. Thus the possible effects of alanine and pyruvate on the dephosphorylation of [^{32}P]phosphopyruvate kinase were studied in vitro. In Fig. 5, it is shown that 1 mM alanine or pyruvate inhibited the phosphatase activity by less than 20%.

Further experiments indicated that 2.5 mM $MgCl_2$, $MnCl_2$ or $CaCl_2$ stimulated the dephosphorylation of pyruvate kinase 40—60% (Table II). Mg^{2+} is

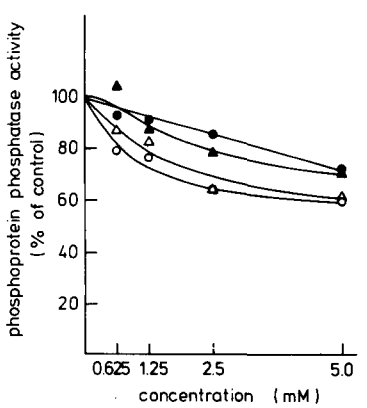

Fig. 5. The effects of alanine and pyruvate on the dephosphorylation of [^{32}P]phosphopyruvate kinase. The conditions were described in the legend of Fig. 4. △———△, ▲———▲, alanine; ○———○, ●———●, pyruvate. Open symbols refer to experiments performed in the absence of Fru-1,6-P_2. Filled symbols represent experiments performed in the presence of 25 μM Fru-1,6-P_2.

TABLE II

ACTIVATORS AND INHIBITORS OF THE DEPHOSPHORYLATION OF [^{32}P]PHOSPHOPYRUVATE KINASE

The reaction medium contained 5 μM [^{32}P]phosphopyruvate kinase and 0.025 mM Fru-1,6-P_2 in a final volume of 40 μl. 100% represents the phosphoprotein phosphatase activity in the absence of the compounds indicated. For each experiment the values used for calculating 100% agreed within 5%. The mean ± S.D. of data from separate experiments are given. The number of determinations are shown in parentheses. Other conditions were given in Experimental.

Additions	Phosphoprotein phosphatase activity (% of control)
Control	100
2.5 mM $MgCl_2$	142 ± 8 (4)
2.5 mM $MnCl_2$	177 ± 24 (4)
2.5 mM $CaCl_2$	139 ± 5 (4)
100 mM KCl	83 ± 14 (4)
1 mM β-glycerophosphate	71 ± 32 (4)
1 mM β-glycerophosphate + 2.5 mM $MgCl_2$	58 ± 2 (2)
1 mM orthophosphate (pH 7.5)	52 ± 9 (4)

required for pyruvate kinase activity and Mn^{2+} is an activator of phosphoprotein phosphatases from other sources [16]. Therefore, the inhibition by the phosphoesters (Fig. 4) was reexamined in the presence of Mg^{2+} and Mn^{2+}. As can be seen in Table III, Mg^{2+} and Mn^{2+} removed the inhibition induced by the phosphoesters. Since the effective concentration of Mg^{2+} in liver (5—10 mM) is greater than those of the inhibitory phosphoesters [17], it is possible to speculate that Mg^{2+} probably promotes the dephosphorylation of pyruvate kinase in the liver.

The dephosphorylation of [^{32}P]phosphoprotamine

Some kinetic experiments were performed with [^{32}P]phosphoprotamine as substrate of the phosphoprotein phosphatase. The apparent K_m for the dephos-

TABLE III

EFFECTS OF Mg^{2+} AND Mn^{2+} ON THE INHIBITION OF THE DEPHOSPHORYLATION OF [^{32}]-PHOSPHOPYRUVATE KINASE

The concentration of [^{32}P]phosphopyruvate kinase was 5 μM in a final reaction volume of 40 μl. The results are expressed as percent of control in which the ligands tested were omitted. The mean ± S.D. of data from separate experiments are given. The number of determinations are shown in parentheses. Other details see the legend to Fig. 2 and Experimental.

Inhibitor (1 mM)	Activity of protein phosphatase in the presence of effectors		
	Inhibitor alone	Inhibitor + 2.5 mM $MgCl_2$	Inhibitor + 2.5 mM $MnCl_2$
ATP	19 ± 4(4)	115 ± 9(4)	94 ± 9(4)
ADP	22 ± 5(6)	91 ± 16(4)	100 ± 43(4)
Fru-1,6-P_2	36 ± 17(6)	65 ± 13(4)	95 ± 0(2)
Phospho*enol*pyruvate	36 ± 16(6)	71 ± 6(4)	80 ± 30(4)

TABLE IV

THE EFFECTS OF SELECTED COMPOUNDS ON THE DEPHOSPHORYLATION OF [^{32}P]PHOSPHOPROTAMINE

Reactions were run in duplicate in a final volume of 100 μl. The concentration of [^{32}P]phosphoprotamine was 60 μM. 100% was the activity measured at the conditions of standard assay in the absence of $MnCl_2$. The mean ± S.D. of data of experiments with three separate enzyme preparations are given. The figure in parentheses indicate the number of experiments.

Additions	Phosphoprotein phosphatase activity (% of control)
No additions	100
2.5 mM $MgCl_2$ + 1 mM ATP	139 ± 37(7)
2.5 mM $MnCl_2$ + 1 mM ATP	162 ± 54(5)
2.3 mM $MgCl_2$	103 ± 11(7)
2.3 mM $MnCl_2$	216 ± 62(4)
2.3 mM $CaCl_2$	98 ± 8(5)
1 mM ADP	88 ± 8(5)
5 mM KH_2PO_4, pH 7.5	35 ± 10(5)
0.1 mM Fru-1,6-P_2	92 ± 9(5)
2.5 mM β-glycerophosphate	83 ± 14(5)
50 mM KCl	90 ± 5(5)
2.5 mM alanine	106 ± 18(5)

phorylation of [^{32}P]phosphoprotamine was 34 ± 14 μM (mean ± S.D. of five determinations) as compared with 22 μM for phosphohistones. A similar K_m was obtained for the dephosphorylation of [^{32}P]phosphohistones by rabbit liver phosphoprotein phosphatase [16].

The pattern of regulation of the phosphoprotein phosphatase activity on [^{32}P]phosphoprotamine was similar to that observed with [^{32}P]phosphopyruvate kinase as substrate. ATP, orthophosphate were inhibitory; $MnCl_2$ stimulated the reaction, but KCl, Fru-1,6-P_2, ADP, $CaCl_2$ and alanine had small effects, less than 20% (Table IV, Fig. 6). In the absence of $MgCl_2$, or $MnCl_2$, ATP precipitated phosphoprotamine. This precipitate could be solubilised with 0.25 M KCl. Therefore, the effects of ATP and orthophosphate were studied in the

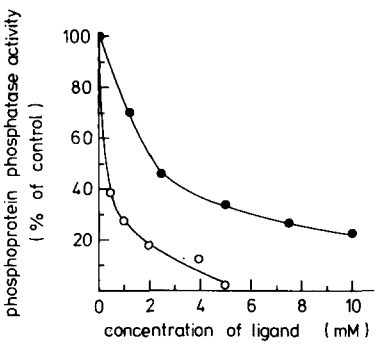

Fig. 6. The effect of ATP and orthophosphate on the dephosphorylation of [^{32}P]phosphoprotamine. The reaction mixture (100 μl) contained 60 μM [^{32}P]phosphoprotamine and 0.25 M KCl. 100% was the activity determined in the absence of ATP and orthophosphate. Explanations are given in the text and under Experimental. ●———●, orthophosphate; ○———○, ATP.

presence of 0.25 M KCl (Fig. 6). The concentrations of ATP and orhtophosphate which caused half maximal inhibition of the dephosphorylation of [^{32}P]phosphoprotamine were about 0.5 and 2 mM, respectively (Fig. 6).

Discussion

The partial purification and characterisation of phosphoprotamine and phosphohistone phosphatase(s) from rat liver have been described earlier [7,11]. However, the present procedure gives material with a higher specific activity (Table I) than that determined for previous preparations [7,11]. The activity of the phosphoprotein phosphatase on [^{32}P]phosphopyruvate kinase, [^{32}P]-phosphoprotamine and [^{32}P]phosphohistones is in agreement with the broad specificity observed for other phosphoprotein phosphatase preparations [1,2]. The molecular weight of 32 000 determined for the phosphoprotein phosphatase (Fig. 3) was in agreement with previous reports [14,18] in which the treatment of liver extracts with 80% ethanol was shown to release the catalytic subunit from a phosphoprotein phosphatase complex. But it remains to be determined if the phosphoprotein phosphatase activity, described in this report, was released from the same complex as the catalytic subunit of phosphorylase *a* phosphatase [14].

Previously, it was shown that the activity of pyruvate kinase (type L) can be regulated in vitro by a phosphoprotein phosphatase [7]. The kinetic studies reported on Fig. 4 showed that 1 mM ATP, ADP, Fru-1,6-P_2 and phospho*enol*-pyruvate all inhibited the dephosphorylation of [^{32}P]phosphopyruvate kinase by at least 60% relative to the control. However, the inhibition, induced by these phosphoesters was removed by 2.5 mM $MgCl_2$ and 2.5 mM $MnCl_2$ (Table III). Besides, the apparent K_m of 27 μM, determined for the dephosphorylation of [^{32}P]phosphopyruvate kinase, approached the concentration of pyruvate kinase subunits in rat liver [15]. These results are consistent with the hypothesis [7], that a phosphoprotein phosphatase may act on the phosphorylated form of pyruvate kinase in the liver.

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