Eur. J. Biochem. 55, 487–495 (1975)

# Solubilization and Characterization of Phosphoprotein Phosphatase(s) from Bovine Corpus-Luteum Plasma Membranes

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(Received January 28 / March 24, 1975)

Plasma membrane fractions I and II isolated from bovine corpus luteum contain phosphoprotein phosphatases. Enzyme activities associated with both membrane fractions showed pH optima in the neutral range and were most active with phosphoprotamine as the exogenous substrate. The enzyme activity was partially inhibited by $Co^{2+}$, $Zn^{2+}$ and $Fe^{2+}$. Dithioerythritol, glutathione (reduced) and 2-mercaptoethanol stimulated the enzyme activity, whereas *N*-ethylmaleimide and *N*-phenylmaleimide were inhibitory. Similarly, various cyclic nucleotides and nucleoside triphosphates also inhibited phosphoprotein phosphatase activities. The phosphatase activity was also observed with endogenous phosphorylated membrane proteins as substrate. The endogenous phosphorylation of membranes was rapid and attained a maximal level after 15–20 min of incubation. Initially endogenous dephosphorylation was also very rapid, but did not reach completion. In addition to phosphoprotein phosphatase, membrane preparations also possessed very active cyclic-AMP-dependent protein kinase activity. Phosphoprotein phosphatase activity from plasma membranes was solubilized by ionic and nonionic detergents. Optimal solubilization was achieved with 0.1% sodium deoxycholate. Sucrose density gradient centrifugation of deoxycholate-solubilized fraction I and fraction II membranes resolved phosphoprotein phosphatase activity into two species with apparent sedimentation coefficients of 6.7 S ($M_r$ 130000) and 4.8 S ($M_r$ 90000). Cyclic-AMP-stimulated protein kinase activity sedimented as a broad peak with a sedimentation coefficient of 5.5 S ($M_r$ 110000).

Adenosine cyclic 3′:5′-monophosphate (cyclic AMP) acts as an intracellular mediator of actions of several protein hormones [1]. It is now believed that various cellular effects elicited by cyclic nucleotides are mediated through regulation of the activity of cyclic-AMP-dependent protein kinases [2]. These protein kinases have been found both in the soluble and particulate fractions of the cell [3,4]. More recently, the cyclic-AMP-dependent phosphorylation of plasma membranes by membrane-associated kinases has been reported in a variety of systems [5–13]. A considerable number of polypeptide constituents of the membranes have been shown to be phosphorylated by membrane-associated kinases. A rapid turnover of protein-bound phosphorylserine has also been shown in several systems, suggesting that the kinase and phosphatase activities associated with membranes may regulate the turnover of protein-bound phosphate [14–22]. In addition to these protein kinases, an enzymatic system causing dephosphorylation of phosphorylated membrane proteins has also been reported [14,15]. However, relatively little is known about the nature and properties of particulate-bound and more specifically about plasma-membrane-associated phosphoprotein phosphatase(s). In our continuing effort on the study of the mechanism of action of gonadotropin in bovine corpus luteum we have studied the binding of the labelled gonadotropin

*Abbreviations.* Fraction I, plasma membrane fraction sedimenting at the interface of sucrose densities, *d*: 1.14 and 1.16; fraction II, plasma membrane fraction sedimenting at the interface of sucrose densities, *d*: 1.16 and 1.18; cyclic AMP, adenosine cyclic 3′:5′-monophosphate; cyclic GMP, guanosine cyclic 3′:5′-monophosphate; cyclic IMP, inosine cyclic 3′:5′-monophosphate; EGTA, ethyleneglycol bis-(2-aminoethyl)-*N*,*N*′-tetraacetic acid.

*Enzymes.* Alkaline phosphatase (EC 3.1.3.1); 5′-nucleotidase (EC 3.1.3.5); cytochrome *c* oxidase (EC 1.9.3.1); (NADH) cytochrome *c* reductase (EC 1.6.99.3); NAD(P)H dehydrogenase (EC 1.6.99.2); succinate dehydrogenase (EC 1.3.99.1); glucose-6-phosphate dehydrogenase (EC 1.1.1.49); glucose-6-phosphatase (EC 3.1.3.9); catalase (EC 1.11.1.6); glyceraldehyde-phosphate dehydrogenase (EC 1.2.1.12); alcohol dehydrogenase (EC 1.1.1.1).

to plasma membrane [23] and the phosphorylation of membrane proteins by membrane-associated protein kinase(s) [13]. In this communication we describe the solubilization and characterization of the plasma-membrane-associated phosphoprotein phosphatases which dephosphorylate the phosphorylated membrane proteins as well as exogenously added substrates, such as phosphoprotamine, phosphohistone and phosphocasein.

## MATERIALS AND METHODS

### *Materials*

Casein, mixed histone, protamine, cyclic AMP, cyclic CMP, cyclic GMP, cyclic UMP, cyclic IMP, ATP, GTP, CTP, Lubrol WX, Lubrol-PX, Triton X-100, 2-mercaptoethanol, cysteine and ouabain were purchased from Sigma Chemical Co. *N*-Ethylmaleimide, *N*-phenylmaleimide, iodoacetic acid and glutathione (reduced) were obtained from Nutritional Biochemical Corp. Carrier-free [$^{32}$P]orthophosphate was supplied by ICN. Other chemicals used were of reagent grade.

Bovine corpora lutea were collected from a local slaughterhouse and kept in ice-cold saline. They were processed within 2–3 h or kept frozen at −80 °C until further use.

### *Preparation of Plasma Membrane*

Corpus luteum plasma membrane fractions I and II were prepared by the method of Gospodorowicz [24,25]. The purified membrane fractions I (*d*: 1.14, 1.16) and II (*d*: 1.16, 1.18) were not pooled, but used separately for the present studies owing to their differences in chemical composition [25]. Purity of plasma membrane was assessed by assaying for various marker enzymes [13,25].

### *Isotopically Labelled ATP*

[$\gamma$-$^{32}$P]ATP was prepared as Tris salt by the procedure described by Glynn and Chappell [26].

### *Preparation of [$^{32}$P]Phosphoproteins*

Phosphorylation of protamine, histones or casein was carried out by incubating with protein kinase II, which was isolated by a previously published procedure [27]. A typical reaction mixture in a final volume of 1 ml contained 100 µg protein kinase II; 50 µmol sodium glycerophosphate buffer, pH 6.0, 1 mg protamine, casein or mixed histone, 10 µmol [$\gamma$-$^{32}$P]ATP ($1 \times 10^7$ counts/min); 10 µmol magnesium acetate; 10 µmol sodium fluoride; 2.0 µmol theophylline; 0.3 µmol EGTA, and 5 nmol cyclic AMP. The phosphorylation reaction was terminated by the addition of 0.25 ml of 100% (w/v) trichloroacetic acid. The resulting precipitate was centrifuged, washed twice by dissolving in water and reprecipitated with 40% (w/v) trichloroacetic acid, and then dialyzed against two changes of distilled water for 24 h. The [$^{32}$P]phosphoprotein thus obtained was used for the phosphoprotein phosphatase assay. Alkali-labile radioactivity of labelled proteins was carried out by incubating the samples in 1 N NaOH at 37 °C for 1 h. The samples were then neutralized with 1 N HCl and proteins were again precipitated by trichloroacetic acid. Bovine serum albumin was added as carrier protein. The [$^{32}$P]orthophosphate from deproteinized supernatant was then extracted as phosphomolybdate complex by the procedure described under phosphoprotein phosphatase assay and the radioactivity was determined. All the [$^{32}$P]phosphate incorporated into proteins was released by this treatment. Contamination of substrates by [$\gamma$-$^{32}$P]ATP was checked by determination of acid-labile phosphate after boiling samples for 15 min in a mixture containing 0.8 N HCl and 0.2 N $H_2SO_4$. The phosphate released by this procedure was negligible.

### *Enzyme Assay*

*Protein Kinase.* Protein kinase activity in the plasma membranes was assayed by a slight modification of the published procedures [27,28]. The incubation mixture in a final volume of 0.2 ml contained 10 µmol glycerol 1-phosphate, pH 6.0, 1 nmol [$\gamma$-$^{32}$P]-ATP ($1.2 \times 10^6$ counts/min), 2 µmol KF, 3 µmol $MgCl_2$, 0.5 µmol theophylline, 0.4 mg calf thymus mixed histone (type IIA), 1 nmol cyclic AMP and 20–30 µg membrane protein. The mixture was incubated at 30 °C for 10 min and then the reaction was stopped by the addition of 2 ml 10% trichloroacetic acid (w/v). The tubes were then processed for radioactivity determination. One unit of protein kinase activity is defined as 1 pmol [$^{32}$P]phosphate incorporated into histone under the above assay conditions.

*Phosphoprotein Phosphatase.* Phosphoprotein phosphatase activity was assayed according to the procedure described by Maeno and Greengard [15]. [$^{32}$P]-Phosphate from the deproteinized supernatant was extracted by the modified method of Plaut [29]. To 0.4 ml of supernatant were added 0.05 ml of 0.01 M $KH_2PO_4$ and 0.15 ml of 5% ammonium molybdate (w/v). The resulting phosphomolybdate complex was extracted with 1.0 ml of isobutyl alcohol and after centrifugation the radioactivity of the isobutyl alcohol

phase was determined. The 0.5-ml aliquot extract was transferred to scintillation vials and to this 0.5 ml of NCS tissue solubilizer (Amersham/Searle) and 10 ml Herberg's fluid [30] were added and radioactivity was determined in a Beckman LS230 liquid-scintillation spectrometer. One unit of phosphoprotein phosphatase activity is defined as 1 pmol of [$^{32}$P]phosphate released from [$^{32}$P]phosphoproteins under the above experimental conditions.

*$Mg^{2+}$, $K^{+}$-Dependent p-Nitrophenyl Phosphatase (Alkaline Phosphatase).* The activity was measured by a modified procedure of Emmelot and Bos [31] using *p*-nitrophenyl phosphate as substrate. One unit of enzyme activity is defined as that amount of enzyme which caused a change in absorbance of 1.0 under above experimental conditions.

*Other Enzyme Assays.* $Na^{+}$, $K^{+}$, $Mg^{2+}$-dependent adenosinetriphosphatase and 5′-nucleotidase were assayed according to Solyom and Trams [32], cytochrome *c* oxidase was assayed according to Cooperstein and Lazarow [33] and (NADH) cytochrome *c* reductase and NAD(P)H dehydrogenase were assayed according to Phillips and Langdon [34]. Succinate dehydrogenase was assayed according to Veeger *et al.* [35] and glucose-6-phosphate dehydrogenase according to Langdon [36]. Glucose-6-phosphatase was assayed according to Nordle and Arion [37]. Catalase was assayed by the method of Beers and Sizer [38], glyceraldehyde-3-phosphate dehydrogenase by the procedures of Velick [39] and alcohol dehydrogenase as described by Vallee and Hock [40].

*Protein Measurement.* Protein was determined by the method of Lowry *et al.* [41] using bovine serum albumin as standard.

### *Solubilization of Plasma-Membrane-Associated Phosphoprotein Phosphatase Activity by Ionic and Nonionic Detergents*

Plasma membrane fractions I and II were suspended in buffer A (50 mM Tris-HCl pH 7.4, 100 mM KCl, 1 mM 2-mercaptoethanol) containing different concentrations of various detergents, and suspensions were allowed to stand for 60 min at 4 °C and then centrifuged at 150000 × *g* for 60 min. The supernatants were carefully aspirated off and pellets were washed with the same volume of respective buffers containing the detergents. The pellets in each case after second centrifugation were resuspended in buffer A containing detergents. The addition of detergent to the sediments was necessary, since detergents unmasked the latent phosphoprotein phosphatase activity. The individual supernatant and sediment were then assayed for phosphoprotein phosphatase activity.

Table 1. *Cyclic-AMP-stimulated and plasma-membrane-associated protein kinase from bovine corpus luteum*
The incubation mixture in a final volume of 0.2 ml contained 10 µmol glycerol 1-phosphate, pH 6.0, 1 nmol [$\gamma$-$^{32}$P]ATP ($1.2 \times 10^{6}$ counts/min), 2 µmol KF; 3 µmol $MgCl_2$, 0.5 µmol theophylline, 0.4 mg protein substrate, 1 nmol cyclic AMP and 20 µg membranes (protein). After incubation at 30 °C for 10 min, the reaction was stopped by the addition of 2 ml of 10% trichloroacetic acid. Other details were similar to those described under Methods. The values have been corrected for endogenous phosphorylation, which was observed in the absence of exogenous protein substrate

| Protein substrate | $10^{-2}$ × Enzyme activity ([$^{32}$P]phosphate incorporated in 10 min) | | | |
|---|---|---|---|---|
| | plasma membrane fraction I | | plasma membrane fraction II | |
| | control | 5 µM cyclic AMP | control | 5 µM cyclic AMP |
| | pmol/mg protein | | | |
| Mixed histone (type IIA) | 6.4 | 12.9 | 4.4 | 12.3 |
| Casein | 2.0 | 2.1 | 1.5 | 1.6 |
| Protamine | 2.4 | 3.7 | 1.5 | 2.9 |

### *Sucrose Density Gradient Centrifugation*

Sucrose density gradient centrifugation of detergent-solubilized phosphoprotein phosphatase and protein kinase activities were carried out by a modified procedure of Kuczenski [42]. Samples (200 µl) containing 50 – 100 µg proteins were layered over 4.8 ml of a 5 – 20% linear sucrose density gradient in buffer B (20 mM Tris-HCl pH 7.4, 0.1 mM EGTA and 1 mM dithioerythritol) and centrifugations were performed at 38000 rev./min in a Beckman SW 50.1 rotor for 15 h at 4 °C. At the end of centrifugation the bottom of each tube was punctured and fractions of 11 drops were collected. Phosphoprotein phosphatase and protein kinase activities were then assayed in these fractions. Sedimentation coefficients and molecular weight determinations were carried out according to the method of Martin and Ames [43] with catalase (11.6 S, $M_r$ 232000), glyceraldehyde-3-phosphate dehydrogenase (7.7 S, $M_r$ 140000) and horse liver alcohol dehydrogenase (5.4 S, $M_r$ 84000) as internal markers [44].

## RESULTS

Plasma membrane fractions I and II from bovine corpus luteum, isolated by discontinuous sucrose density gradient centrifugation, contained both phosphoprotein phosphatase and cyclic-AMP-dependent protein kinase activities (Tables 1, 2). Results presented in Table 1 show that protein kinase was most active

Table 2. *Plasma-membrane-associated phosphoprotein phosphatase activity from bovine corpus luteum*
The reaction mixture in a final volume of 0.1 ml contained 10 μmol of Tris-HCl buffer, pH 7.5 (I) or 7.0 (II), 0.1 μmol of dithioerythritol, 100 μg $^{32}$P-labelled phosphoprotein ($5 \times 10^5$ counts/min) and 60 μg membrane protein. After incubation at 30 °C for 20 min the reaction was stopped by adding 0.4 ml of 25% trichloroacetic acid and 0.1 ml of 0.63% bovine serum albumin. The tubes were then processed for radioactivity measurements as described under Methods

| [$^{32}$P]Phosphoprotein | Enzyme activity ([$^{32}$P]phosphate released) | |
|---|---|---|
| | plasma membrane fraction I | plasma membrane fraction II |
| | pmol/mg membrane protein | |
| Mixed histone | 36.1 | 31.1 |
| Protamine | 63.1 | 38.6 |
| Casein | 17.8 | 18.4 |

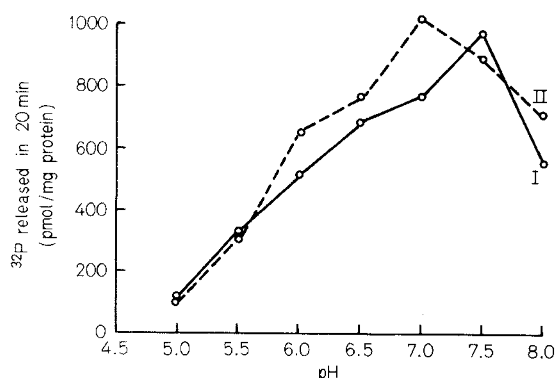


Fig. 1. *Effect of pH on plasma-membrane-associated phosphoprotein phosphatase activity.* The incubation mixture in a final volume of 0.1 ml contained 10 μmol acetate or Tris buffer, 0.1 μmol dithiothreitol, 100 μg $^{32}$P-labelled protamine ($5 \times 10^5$ counts/min) and 40 μg membrane proteins. The incubations were carried out at 30 °C for 20 min and then tubes were processed for radioactivity measurement as described under Materials and Methods. Acetate and Tris buffers were used for pH range 5.0–6.5 and 7.0–8.0 respectively

with mixed histone as substrate. Both casein and protamine were less effective. In addition, maximal cyclic AMP stimulation of kinase activity was also observed with histones. The substrate specificity of plasma-membrane-associated phosphoprotein phosphatase is given in Table 2. The fraction I plasma membrane enzyme was more active with [$^{32}$P]phosphoprotamine than [$^{32}$P]phosphohistone or [$^{32}$P]phosphocasein as substrate. In contrast, both [$^{32}$P]phosphoprotamine and [$^{32}$P]phosphohistone were equally effective for fraction II plasma membrane enzyme.

*Enzyme Kinetics*

Plasma-membrane-associated phosphoprotein phosphatases in fractions I and II showed pH optima of 7.5 and 7.0 respectively (Fig. 1). The enzyme activities were linear up to 90 μg of membrane protein, the maximum amount of enzyme used in the present studies. A linear relationship of fractions I and II enzyme with incubation time was also observed. Unlike protein kinase, phosphoprotein phosphatases did not require divalent metal ions for maximal activity (Table 3). Cations such as $Mg^{2+}$, $Mn^{2+}$ and $Ca^{2+}$ had no effect on enzyme activity but $Co^{2+}$, $Zn^{2+}$ and $Fe^{2+}$ inhibited the enzyme between 30–80%. Sulfhydryl-group activators, such as dithiothreitol, glutathione and 2-mercaptoethanol, stimulated both enzymes, while higher concentrations of *N*-ethylmaleimide and *N*-phenylmaleimide were inhibitory (Table 4). All the anions tested inhibited the membrane phosphoprotein phosphatases. Pyrophosphate was the most potent inhibitor, followed by phosphate and fluoride ions (Table 5). Protein phosphatases from both plasma membranes were also inhibited to approximately 30–40% by various cyclic nucleotides and nucleoside triphosphates. There was no apparent specificity for this inhibition, since all nucleotides were equally effective. Inhibition of fraction II plasma membrane enzyme by these compounds was less pronounced (Table 5).

Table 3. *Effect of divalent metal ions on plasma-membrane-associated phosphoprotein phosphatases from bovine corpus luteum*
Incubation conditions were similar to those described in Table 1 except that the indicated concentrations of different metal ions were also included. 100 μg [$^{32}$P]phosphoprotamine ($4.0 \times 10^5$ counts/min) and 60 μg membrane protein were used in each assay

| Divalent metal ion | Concn | Enzyme activity ([$^{32}$P]phosphate released) | |
|---|---|---|---|
| | | plasma membrane fraction I | plasma membrane fraction II |
| | mM | pmol/mg membrane protein | |
| None | – | 50.5 | 27.8 |
| $Mn^{2+}$ | 0.1 | 50.6 | 27.9 |
| $Mn^{2+}$ | 2 | 51.0 | 27.7 |
| $Mg^{2+}$ | 0.1 | 50.8 | 27.6 |
| $Mg^{2+}$ | 2 | 50.9 | 27.8 |
| $Co^{2+}$ | 0.1 | 35.7 | 21.0 |
| $Co^{2+}$ | 2 | 18.3 | 11.3 |
| $Ca^{2+}$ | 0.1 | 50.4 | 27.3 |
| $Ca^{2+}$ | 2 | 50.6 | 27.8 |
| $Zn^{2+}$ | 0.1 | 30.5 | 19.5 |
| $Zn^{2+}$ | 2 | 15.7 | 6.8 |
| $Fe^{2+}$ | 0.1 | 34.8 | 21.8 |
| $Fe^{2+}$ | 2 | 16.5 | 8.3 |

In order to distinguish these phosphatases from nonspecific phosphatases acting at a neutral pH range, the conditions for the assay of *p*-nitrophenyl phosphatase activity from bovine corpus luteum plasma

Table 4. *Effect of sulfhydryl group inhibitors and activators and EDTA on plasma-membrane-associated phosphoprotein phosphatase*
The incubation mixture in a final volume of 0.1 ml contained 10 µmol Tris-HCl, pH 7.5 (I) or 7.0 (II), 100 µg [$^{32}$P]phosphoprotamine ($5 \times 10^6$ counts/min), 60 µg membrane protein and indicated concentrations of various test substances. The tubes were incubated at 30 °C for 20 min and then processed for radioactivity measurements as described under Materials and Methods. The numbers in parentheses represent enzyme activity expressed as percentage of the control assuming the control value as 100% activity

| Addition | Concn | Enzyme activity ([$^{32}$P]phosphate released in 20 min) | | | |
|---|---|---|---|---|---|
| | | plasma membrane fraction I | | plasma membrane fraction II | |
| | mM | pmol/mg protein | (%) | pmol/mg protein | (%) |
| None | – | 114 | (100) | 73 | (100) |
| EDTA | 0.5 | 111 | (98) | 84 | (115) |
| EDTA | 1 | 131 | (113) | 89 | (122) |
| Cysteine | 10 | 138 | (122) | 84 | (115) |
| Glutathione (Reduced) | 10 | 127 | (112) | 79 | (108) |
| Dithioerythritol | 1 | – | – | 85 | (116) |
| Dithioerythritol | 10 | 182 | (160) | 143 | (200) |
| 2-Mercapto-ethanol | 10 | 151 | (133) | 99 | (135) |
| Iodoacetic acid | 0.1 | 109 | (96) | – | – |
| Iodoacetic acid | 1 | 112 | (98) | – | – |
| N-Ethyl-maleimide | 0.1 | 87 | (76) | 71 | (97) |
| N-Ethyl-maleimide | 1 | 73 | (64) | 60 | (82) |
| N-Phenyl-maleimide | 0.1 | 91 | (80) | 66 | (90) |
| N-Phenyl-maleimide | 1 | 86 | (75) | 57 | (78) |

membranes was determined (Table 6). Although *p*-nitrophenyl phosphatase was more active at neutral pH, it required $Mg^{2+}$ ions for maximal activity. In addition, this hydrolase was also distinguishable from phosphoprotein phosphatase by its sensitivity towards EDTA (Table 6). Phosphoprotein phosphatase activity under identical experimental conditions was not affected by EDTA (Table 5). Similarly, this enzyme activity was also insensitive to ouabain.

### *Phosphorylation and Dephosphorylation of Plasma Membranes*

As shown in Fig. 2, the plasma membrane fractions I and II have the necessary enzymes for phosphorylation and dephosphorylation. The initial rate of phosphorylation was rapid, especially in plasma membrane fraction II, and attained a maximum level after

Table 5. *Effect of certain substances on plasma-membrane-associated phosphoprotein phosphatase activity from bovine corpus luteum*
The incubation mixture in a final volume of 0.1 ml contained 10 µmol of Tris-HCl, pH 7.5 (I) or 7.0 (II), 100 µg [$^{32}$P]phosphoprotamine ($5 \times 10^6$ counts/min), 1 µmol dithioerythritol, 60 µg membranes (protein) and indicated concentrations of various test substances. Other details are similar to those described under Materials and Methods. The number in parentheses represent relative activities taking the activity in the absence of test substances as 100%

| Addition | Conc | Enzyme activity ([$^{32}$P]phosphate released in 20 min) | | | |
|---|---|---|---|---|---|
| | | plasma membrane fraction I | | plasma membrane fraction II | |
| | mM | pmol/mg protein | (%) | pmol/mg protein | (%) |
| None | – | 21.6 | (100) | 14.8 | (100) |
| NaF | 1 | 21.2 | (98) | 14.3 | (96) |
| NaF | 10 | 13.1 | (61) | 9.1 | (62) |
| $NaH_2PO_4$ | 1 | 16.6 | (77) | 10.6 | (71) |
| $NaH_2PO_4$ | 10 | 11.9 | (55) | 7.5 | (50) |
| Sodium pyro-phosphate | 1 | 5.4 | (25) | 4.8 | (32) |
| Sodium pyrophosphate | 10 | 4.7 | (22) | 2.9 | (19) |
| Cyclic AMP | 0.005 | 19.9 | (92) | 11.9 | (80) |
| Cyclic AMP | 0.1 | – | – | 12.0 | (80) |
| Cyclic IMP | 0.005 | 17.3 | (80) | 13.3 | (90) |
| Cyclic IMP | 0.1 | 14.1 | (65) | 11.1 | (75) |
| Cyclic GMP | 0.005 | 16.0 | (74) | 12.4 | (83) |
| Cyclic GMP | 0.1 | 16.0 | (74) | 12.3 | (83) |
| ATP | 0.1 | 14.7 | (68) | 12.3 | (83) |
| GTP | 0.1 | 13.2 | (61) | 13.4 | (90) |
| CTP | 0.1 | 15.1 | (69) | 13.2 | (90) |
| UTP | 0.1 | 13.5 | (62) | 11.8 | (80) |

15–20 min of incubation, and cyclic AMP had little effect on this endogenous phosphorylation (data not shown). To study the kinetics of dephosphorylation, the plasma membranes were first allowed to attain a maximum level of [$^{32}$P]phosphate incorporation, after which the phosphorylation reaction was stopped by the addition of ATP–EDTA mixture and samples were withdrawn at different times and tested for the bound radioactivity. In both membrane fractions a rapid initial decrease in the level of [$^{32}$P]phosphate incorporation was observed (Fig. 2). The dephosphorylation, however, did not reach completion, and generally 60–70% of the phosphate was still linked to protein even after prolonged incubations (40 min) in the presence of EDTA–ATP.

### *Extraction and Solubilization of Plasma-Membrane-Associated Phosphoprotein Phosphatase by Ionic and Nonionic Detergents*

Phosphatases activities associated with plasma membrane fractions I and II could not be solubilized

Table 6. *$K^+$,$Mg^{2+}$ -dependent p-nitrophenyl phosphatase activity from bovine corpus luteum plasma membrane*
The complete incubation mixture in a final volume of 0.5 ml contained 40 µmol Tris-HCl, pH 7.2, 7.4 or 7.8; 5 µmol $MgCl_2$, 10 µmol KCl, 5 µmol *p*-nitrophenyl phosphate, and 60 µg enzyme protein. After incubation at 37 °C for 15 min the reaction was stopped by the addition of 1 ml of 0.1 N NaOH. Other details were the same as described under Materials and Methods

| Experimental condition | | Enzyme activity | |
|---|---|---|---|
| pH | incubation mixture | plasma membrane fraction I | plasma membrane fraction II |
| | | U/mg protein | |
| 7.2 | Complete | 1.95 | 1.75 |
| | Complete − KCl, $MgCl_2$ | 1.25 | 0.63 |
| | Complete + 10 mM EDTA | 1.60 | 0.95 |
| 7.4 | Complete | 1.30 | 0.85 |
| 7.8 | Complete | 1.01 | 0.65 |
| | Complete − KCl, $MgCl_2$ | 0.66 | 0.41 |
| | Complete + 10 mM EDTA | 0.70 | 0.55 |

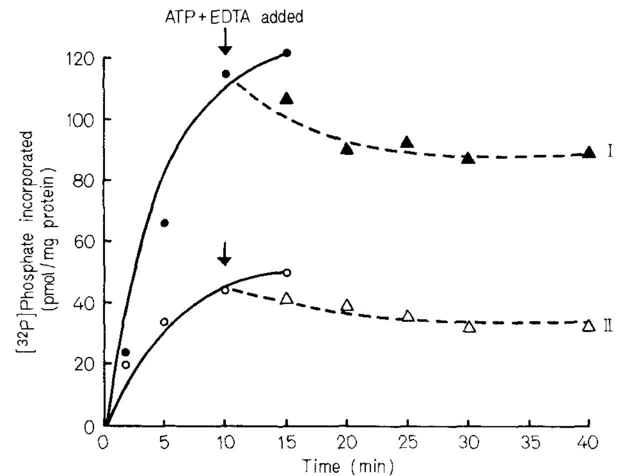


Fig. 2. *Self-phosphorylation and dephosphorylation of bovine corpus luteum plasma membranes.* The incubation medium in a final volume of 0.1 ml contained 5 µmol of glycerol 1-phosphate buffer, pH 6.0, 0.03 µmol EGTA, 1.5 µmol magnesium acetate, 2 nmol of [$\gamma$-$^{32}$P]-ATP and 40 µg plasma membrane proteins. The reaction was started by the addition of [$\gamma$-$^{32}$P]ATP and carried out at 30 °C for the indicated time. At the point indicated by arrow, 0.2 ml of a solution containing 0.8 mM ATP and 7.5 mM EDTA were added. The reaction was terminated by the addition of 0.2 ml of 0.63% bovine serum albumin. The tubes were then processed for radioactivity measurement as described under protein kinase assay in the text

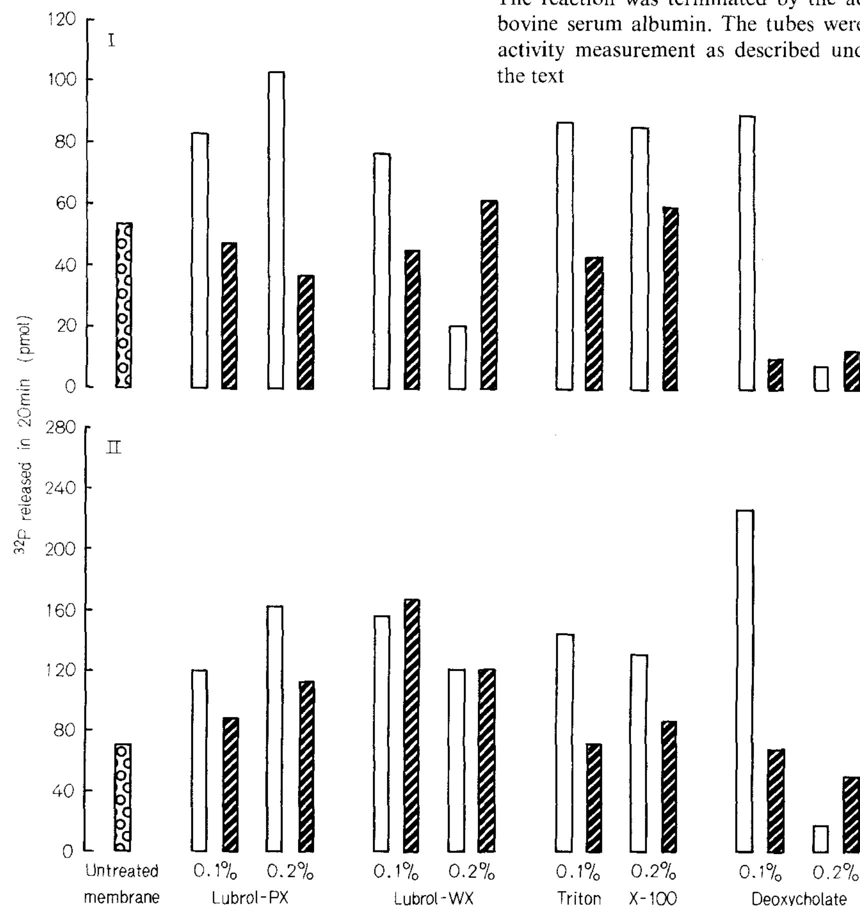


Fig. 3. *Solubilization of plasma-membrane-associated phosphoprotein phosphatase activity by ionic and nonionic detergents.* Plasma membrane fractions I and II were suspended in buffer A (50 mM Tris-HCl pH 7.4, 100 mM KCl, 1 mM 2-mercaptoethanol) containing indicated concentrations of various detergents. The suspensions were allowed to stand at 4 °C for 60 min. The tubes were centrifuged at 150000 × *g* for separation of sediment and supernatant as described under Materials and Methods. Plain bars represent 150000 × *g* supernatant; hatched bars represent sediment

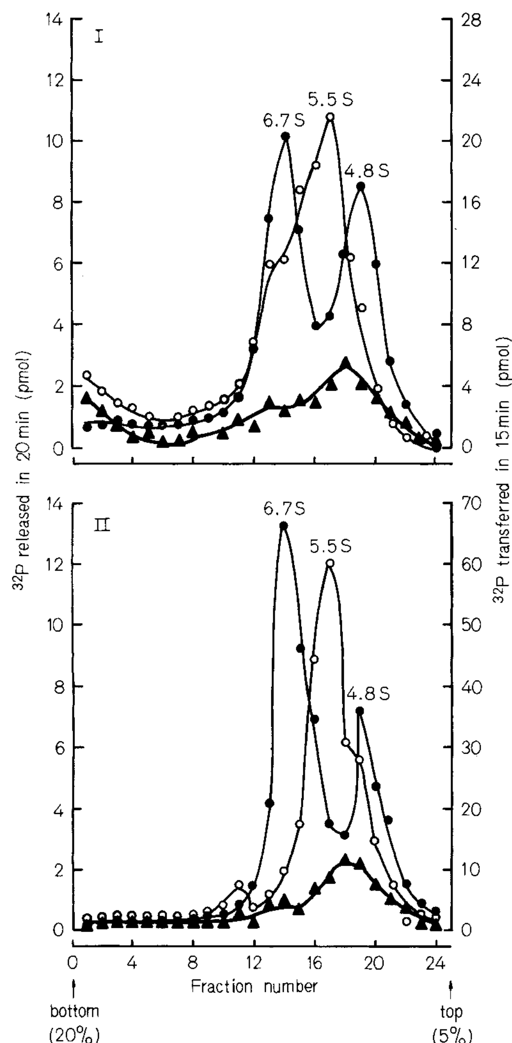


Fig. 4. *Sucrose density gradient centrifugation of deoxycholate-solubilized phosphoprotein phosphatase and protein kinase activities of plasma membranes.* Aliquots of solubilized fractions I and II (100 µg each) in 0.2 ml of 20 mM Tris-HCl, pH 7.4, 0.1 mM EGTA and 1 mM dithioerythritol were layered in separate centrifuge tubes over 4.8 ml of 5 – 20% sucrose density gradient containing the same concentrations of Tris, EGTA and dithioerythritol. After centrifugation fractions from each tube were assayed for phosphoprotein phosphatase and protein kinase activities. (●) $^{32}$P released; (▲) $^{32}$P transferred in absence of cyclic AMP; (○) $^{32}$P transferred in presence of cyclic AMP

by treatment with salt solutions of low and/or high ionic strength (data not shown). However, these activities were solubilized by treating the plasma membranes with ionic and nonionic detergents (Fig. 3). Maximal solubilization was achieved with sodium deoxycholate at a final concentration of 0.1%. Considerable solubilization was also achieved using Lubrol-PX (0.2%) or Triton (0.1%) as solubilizing agents (Fig. 3). In addition all these detergents, with the exception of 0.2% deoxycholate, also increased the phosphoprotein phosphatase activity, possibly by partially unmasking of the latent activity (Fig. 3).

*Sucrose Density Gradient Centrifugation*

In an effort to determine whether the deoxycholate-solubilized enzyme consist of single or multiple species, aliquots of the solubilized membrane fractions I and II were applied to a linear sucrose density gradient and centrifuged in the presence of internal markers. The results are presented in Fig. 4. Phosphoprotein phosphatase activities associated with membrane fractions I and II sedimented as two distinct species with an apparent sedimentation coefficient of 6.7 S ($M_r$ 130000) and 4.8 S ($M_r$ 90000) respectively. Protein kinase activities assayed in the presence or absence of cyclic AMP (5 µM) sedimented as a broad peak with a sedimentation coefficient of 5.5 S ($M_r$ 110000). The phosphoprotein phosphatase and protein kinase activities were separated from one another by sucrose density gradient centrifugation (Fig. 4).

## DISCUSSION

The results presented in the paper show that plasma membrane fractions I and II from bovine corpus luteum contain phosphoprotein-specific phosphatase activity, which could dephosphorylate membrane proteins as well as exogenously added phosphoproteins like histone, protamine or casein. The pH optimum (7.0 – 7.5) differed from that found for soluble phosphoprotein phosphatases from other sources [45 – 47]. The results reported here clearly indicate that the membrane-associated phosphoprotein phosphatases are sulfhydryl dependent but do not require divalent metal ions for maximal activity. The requirement for reduced –SH groups is in accord with the observations of Sundararajan and Sarma [45] and Paigen [46] but not with Rose and Heald [47]. The present system also differs from cytosol phosphoprotein phosphatases of rat brain [15] and mouse mitochondrial enzyme [46] in its inability to be stimulated by divalent metal ions. The inhibitory effects of pyrophosphate, orthophosphate and fluoride ions on plasma-membrane-associated phosphoprotein phosphatases is consistant with soluble brain [15], skeletal muscle [48] and heart histone phosphatases [49].

These membrane-associated phosphoprotein phosphatase activities seem to be specific for phosphoproteins. This enzyme can be differentiated from neutral *p*-nitrophenyl phosphatase activity which is $Mg^{2+}$ dependent and inhibited by EDTA. Furthermore, these membrane phosphoprotein phosphatases also differ from nonspecific alkaline phosphatases, which require divalent metal ions and higher pH

values for their activity [32]. Additionally, phosphoproteins are known to be poor substrates for non-specific phosphatases [50].

The initial endogenous phosphorylation and dephosphorylation of plasma membranes was very rapid suggesting an active turnover of protein bound phosphate. This appears to arise from coupled action of kinase and phosphatase activities [14]. In the present system, however, more than half of the incorporated phosphate was not released even after a prolonged incubation period under dephosphorylation conditions, and thus it appears that not all of the phosphorylated proteins were available for dephosphorylation reactions. It is possible that phosphoprotein phosphatase may regulate the selective turnover of protein-bound phosphate in plasma membranes. However, the possibility of slow turnover of such stable phosphate in intact cells cannot be ruled out. Alternatively, specific organization of phosphoprotein phosphatases, protein kinase and phosphoproteins within the membrane matrix could also explain this observation [22].

Sucrose density gradient centrifugation of solubilized fraction I and fraction II enzymes resolved them into two catalytically active components with a sedimentation coefficient of 6.7 S ($M_r$ 130000) and 4.8 S ($M_r$ 90000) respectively. Protein kinase activity also sedimented as a single peak in a position corresponding to a sedimentation coefficient of 5.5 S ($M_r$ 110000). These results indicate that bovine corpus luteum plasma membranes contain catalytically and physically distinct phosphoprotein phosphatase and protein kinase activities. The demonstration in the present study of multiple forms of phosphoprotein phosphatases raises the possibility that different phosphatases may regulate the turnover of different phosphoproteins in the plasma membranes. Our recent finding that 8–10 plasma membrane polypeptides could be phosphorylated by associated protein kinase(s) further supports this possibility (Azhar and Menon, unpublished observations).

The possible physiological function of membrane-associated phosphoprotein phosphatases, protein kinases and active turnover of phosphoprotein in bovine corpus luteum plasma membrane is not understood at the present time. It is possible that changes in the extent of membrane phosphorylation could have a significant effect on the net charge of plasma membranes and changes in its quaternary structure which, in turn, could lead to parallel changes in the rate of hormone binding or activation of adenylate cyclase. The presence of gonadotropin-binding [23,24] and adenyl cyclase activities [23], along with membrane-associated phosphoprotein phosphatase and protein kinase, might suggest that these two activities may be involved in the regulation of adenyl cyclase by hormones [51–53].

The excellent technical assistance of Ms Kathie Nosal is gratefully acknowledged. We also wish to thank Ms Joanne Robinson and Ms Cindy Giraud for the secretarial help and the Standard Beef Company, Detroit, Michigan for their help and cooperation in obtaining corpora lutea. This work was supported in part by Grant HD06656 from NIH. S.A. is a Population Council postdoctoral scholar.

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