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Partial purification and characterization of the soluble phosphatidate phosphohydrolase of rat liver

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1. A method is described by which the Mg^{2+}-stimulated phosphatidate phosphohydrolase can be purified from the soluble fraction of liver from ethanol-treated rats. The increase in specific activity was about 416-fold. This involved purification by adsorption on calcium phosphate, chromatography on DE-52 DEAE-cellulose, separation on Ultrogel AcA-34 and chromatography on CM-Sepharose 6B. 2. The effects of phosphatidylcholine, phosphatidate and Mg^{2+}, Mn^{2+} and Zn^{2+} on the activity are described. 3. Inhibitor studies indicate that the phosphohydrolase contains functional thiol groups and arginine residues.

Evidence has accumulated that the Mg^{2+}-stimulated PAP has an important function in regulating the synthesis of glycerolipids in the liver (Brindley & Sturton, 1982). This is particularly the case when the supply of fatty acids to the liver is high. Two different levels of regulation have been observed. The synthesis of PAP is stimulated by glucocorticoids (Lehtonen *et al.*, 1979; Jennings *et al.*, 1981), and this action is antagonized by insulin (Lawson *et al.*, 1982*a*,*b*). The balance between these two hormones therefore participates in regulating PAP activity in the long-term. In addition, evidence for short-term regulation is provided from experiments with vasopressin, which stimulates hepatic PAP activity within 5min (Pollard & Brindley, 1984). Fatty acids also stimulate PAP activity and promote its association with membrane fractions from hepatocytes (Cascales *et al.*, 1984).

Further progress towards elucidating the mechanisms that control PAP activity and its distribution in different cell compartments depends on the ability to purify, characterize and raise antibodies against the Mg^{2+}-stimulated PAP of the liver. Progress in this respect has been slow, and in our experience very difficult.

A partial purification of PAP from rat liver mitochondria was reported by Sedgwick & Hübscher (1967). This enzyme was stimulated 1.5-fold by Mg^{2+} and the increase in specific activity was 16-fold. Hosaka *et al.* (1975) purified the Mg^{2+}-stimulated PAP from the soluble fraction of rat liver by 15–20-fold. Subsequently, an increase in specific activity of 52-fold for this enzyme was achieved (Sturton *et al.*, 1981). However, we were not successful in proceeding further with these techniques, because the enzyme became unstable and it remained associated with the bulk of the contaminating proteins when other separation methods were tested. In the present paper an increase of about 416-fold in the specific activity of the Mg^{2+}-stimulated PAP is reported. The purification was aided by addition of Tween 20 to the enzyme preparations. This detergent helped to stabilize the PAP.

Abbreviation used: PAP, phosphatidate phosphohydrolase (EC 3.1.3.4).

Materials and methods

Materials and animals

The sources of most of these have been described (Sturton & Brindley, 1977, 1978; Sturton *et al.*, 1981). Tween 20, butane-2,3-dione, *p*-chloromercuribenzoate, *N*-ethylmaleimide and iodoacetic acid were from Sigma Chemical Co., Poole, Dorset, U.K.; dithiothreitol, cyclohexane-1,2-dione and 1,10-phenanthroline hydrochloride were from Aldrich Chemical Co., Gillingham, Dorset, U.K.; PAGE Blue G-90 and calcium phosphate gel were from BDH Chemicals, Poole, Dorset, U.K.; CM-Sepharose 6B was from Pharmacia, London W.5, U.K.; Ultrogel AcA-34 was from LKB Instruments, S. Croydon, Surrey, U.K.

Rat liver phospholipids were prepared by extracting the liver lipids by the method of Bligh & Dyer (1959). The lipid residue was dissolved in chloroform (dried over $CaCl_2$ and used immediate-

ly) and loaded on to a silicic acid column. Neutral lipids were eluted with chloroform, and a phospholipid fraction was obtained by using chloroform/methanol (17:3, v/v). The solvent was removed by rotary evaporation under reduced pressure, and the residue was stored in chloroform at −20°C. A phospholipid emulsion was prepared by removing the solvent with a stream of N_2. The residue was then dispersed by sonicating twice for 30s at 22kHz with an amplitude of 8 μm peak to peak with appropriate buffer.

Determination of phosphatidate phosphohydrolase activity

Unless stated to the contrary, each incubation mixture contained, in 0.25ml, 100mM-Tris adjusted to pH 7.4 with HCl, 1 mM-dithiothreitol, 0.1 mM-EGTA, 5mM-$MgCl_2$, 2mg of fatty acid-poor bovine serum albumin/ml, 0.6mM-*sn*-diacyl[1,3-^{3}H]glycerol 3-phosphate (0.45Ci/mol), 0.3mM-phosphatidylcholine and an appropriate quantity of enzyme so that reaction rates were constant with time and proportional to the PAP added. These were re-established with the PAP eluted from calcium phosphate gel (Fig. 1). This was thought to be important, since the enzyme preparations contained Tween 20. The reaction was started by adding the mixed emulsion of phosphatidate and phosphatidylcholine (Sturton & Brindley, 1978). This is a better substrate than a pure phosphatidate emulsion (Fig. 1*a*), and it probably resembles the natural form of the substrate in a membrane. The reaction mixture was shaken at 37°C for 10–60min, and it was stopped with 1.88ml of chloroform/methanol (1:2, v/v). The *sn*-1,2-diacyl[^{3}H]-glycerol that was formed was separated from the phosphatidate by treatment with alumina and its concentration was determined by liquid-scintillation counting (Pollard & Brindley, 1984).

Determination of protein concentration

This was performed by the method of Bradford (1976), except that PAGE Blue G-90 was employed. The elution of protein from columns was assessed by measuring A_{280}.

Results

Step 1: treatment of rats and the preparation of the particle-free supernatant from the livers

Eight male or female rats were fed by stomach tube with 5g of ethanol per kg body wt, and they were killed by decapitation about 6h later. Blood was allowed to drain from the necks and the livers were removed. This produced about a 5-fold increase in the specific activity of PAP, as observed previously (Brindley *et al.*, 1979). Part of this increase is known to occur via the release of corticosterone into the circulation (Brindley *et al.*, 1979), which appears to stimulate the synthesis of PAP in the liver (Lehtonen *et al.*, 1979; Jennings *et al.*, 1981). The object of the ethanol treatment was therefore to increase the concentration of PAP in the livers.

The livers were washed in 0.25M-sucrose, adjusted to pH 7.4 with $KHCO_3$, and containing 0.2mM-dithiothreitol at 4°C. They were then homogenized in 3vol. of the same medium in a stainless-steel homogenizer with a Teflon pestle. The homogenate was adjusted to 2mM with phenylmethanesulphonyl fluoride to prevent the action of serine proteinases. The homogenate was then centrifuged

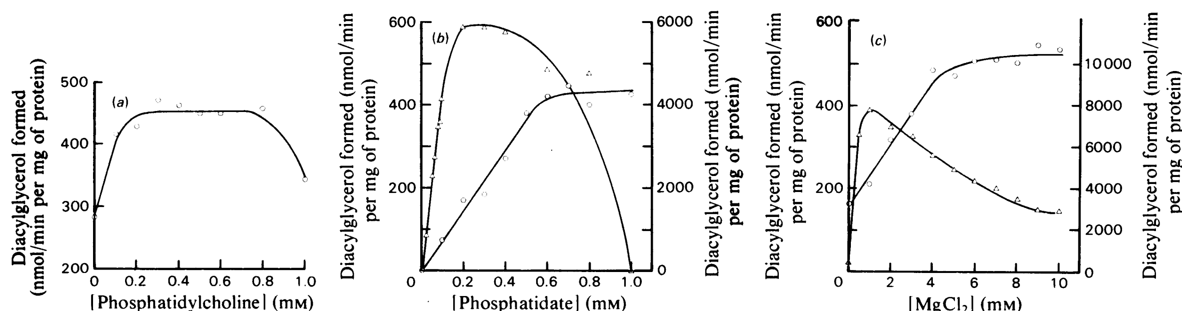

Fig. 1. *Optimization of the assay conditions for PAP*

The assays were performed with PAP that was eluted from calcium phosphate gel (○, left-hand scale) or from CM-Sepharose 6B (△, right-hand scale) as shown in Table 1. In (*a*) the concentration of phosphatidylcholine was varied and the concentrations of phosphatidate and $MgCl_2$ were 0.6mM and 5mM respectively. Phosphatidylcholine and phosphatidate were added together as mixed micelles. In (*b*) the concentration of phosphatidate was varied at a molar ratio of 2:1 relative to phosphatidylcholine. The molar ratio of phosphatidate to Mg^{2+} was kept constant at 1:8.3. The concentration of $MgCl_2$ was also varied in (*c*) in the presence of 0.6mM-phosphatidate and 0.3mM-phosphatidylcholine.

at 76000g ($r_{av.}$ = 7.62cm) at 4°C for 90min. After the floating lipid had been aspirated off, the particle-free supernatant was decanted.

Step 2: adsorption of PAP on calcium phosphate gel

Potassium phosphate buffer, pH 7.4, and Tween 20 were added to the particle-free supernatant to give a final concentration of 70mM and 0.15% (w/v) respectively. To this was then added a suspension of calcium phosphate gel (0.5mg of calcium phosphate/mg of protein) and the mixture was stirred at 4°C for 1 h. The gel was collected by centrifugation at 2075g and the supernatant was discarded. The calcium phosphate was then washed repeatedly with 70mM-potassium phosphate buffer, pH 7.4, containing 0.2mM-dithiothreitol and 0.05% Tween 20 until no further protein was eluted. PAP was then recovered by washing the gel with 5vol. of 300mM-potassium phosphate buffer, pH 7.4, containing 0.2mM-dithiothreitol and 0.05% Tween 20 until the A_{280} was less than 0.1. This increased the specific activity of PAP by about 24-fold (Table 1). The reproducible increase in the yield of PAP resulted from the stimulatory effect of both the phosphate buffer and the Tween 20 that were used to elute it from the calcium phosphate gel (Fig. 2).

Step 3: purification of PAP by ion-exchange chromatography on DE-52 DEAE-cellulose

The eluate from the calcium phosphate gel was diluted with 0.2mM-dithiothreitol containing 0.05% Tween 20 until the potassium phosphate concentration was 30mM. This was loaded on to a column (internal diam. 7cm × 8cm) of DE-52 DEAE-cellulose. The column was washed with loading buffer until negligible quantities of protein were recovered. PAP was normally eluted by increasing the concentration of potassium phosphate buffer, pH 7.4, to 260mM. This yielded fairly quickly a further purification of about 2.6-fold. This purification can be increased by using a linear gradient of phosphate for the elution (Fig. 3), but this has to be balanced against the longer time required and the loss of PAP activity that occurs.

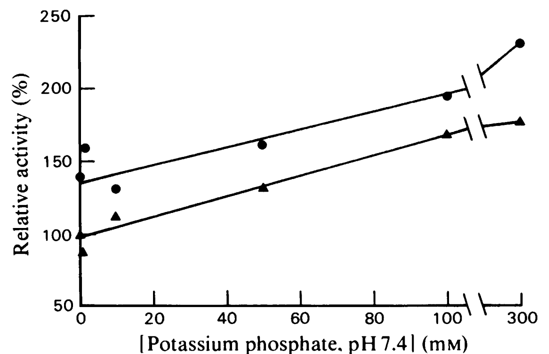

Fig. 2. *Effects of phosphate and Tween 20 on PAP activity*
Samples of the particle-free supernatant of rat liver were diluted with 0.25M-sucrose, adjusted to pH 7.4 with $KHCO_3$, and containing 0.2mM-dithiothreitol in the absence (▲) or in the presence of a final concentration of 0.05% Tween 20 (●). Potassium phosphate buffer, pH 7.4, was added as indicated, and the enzyme preparation was left for 15min at 4°C. PAP activity was then determined. The phosphate concentration in the preincubation is shown, whereas that in the assay was one-quarter of that given in the Figure.

Table 1. *Purification of the soluble PAP from the livers of ethanol-treated rats*
The purification scheme is described in the Results section.

Step	Protein (mg)	PAP activity (μmol/min)	PAP specific activity (nmol/min per mg of protein)	Yield (%)	Increase in specific activity (fold)
1. Particle-free supernatant	3360	83.3	24.8	100	1
2. Calcium phosphate	315.5	189	600	227	24
3. DE-52 DEAE-cellulose	92.2	143	1551	172	63
4. Concentration	51.2	91.4	1785	110	72
5. Ultrogel AcA-34	14.2	36.5	2570	44	104
6. CM-Sepharose 6B					
Peak A	3.6	7.6	2111	9	85
Peak B (assayed with 5mM-$MgCl_2$ and 0.6mM-phosphatidate)	3.2	14.3	4469	17	180
Peak B (assayed with 1mM-$MgCl_2$ and 0.4mM-phosphatidate)	3.2	33.0	10323	40	416

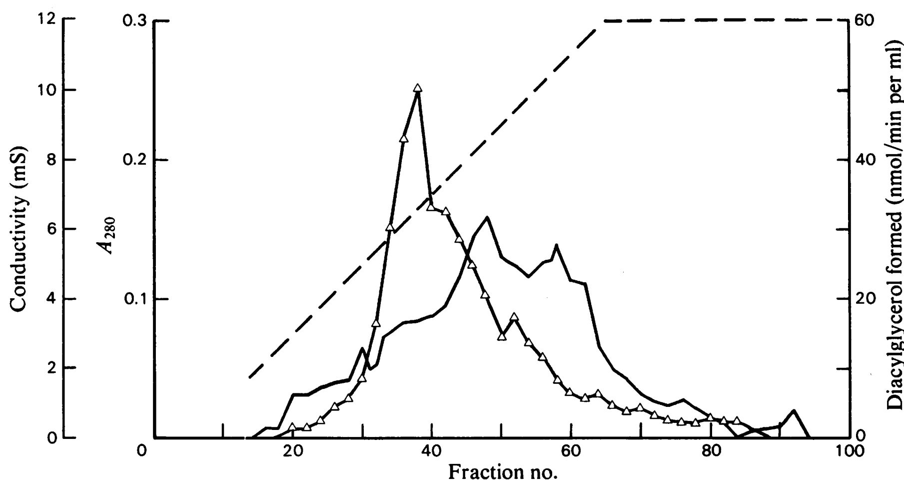

Fig. 3. *Purification of PAP by chromatography on DE-52 DEAE-cellulose*
PAP activity (△) and protein as measured by A_{280} (——) were eluted from DE-52 DEAE-cellulose with a linear gradient of potassium phosphate buffer, pH 7.4, from 30mM to 300mM, as indicated by the change in conductivity (--).

Steps 4 and 5: purification of PAP on Ultrogel AcA-34

The PAP activity that was eluted from the DE-52 DEAE-cellulose column was diluted with 0.2mM-dithiothreitol containing 0.05% Tween 20 until the phosphate concentration was about 0.1 M. Calcium phosphate gel (5mg of gel/mg of protein) was added and the solution stirred for 2h. The gel was collected by centrifuging at 2287g ($r_{av.}$ = 8.19cm) for 5min and the supernatant was discarded. PAP was eluted by stirring the gel with four 5ml portions of 400mM-potassium phosphate buffer, pH 7.4, containing 0.2mM-dithiothreitol and 0.05% Tween 20 and then removing the gel by centrifugation. This first extract was applied directly to a column (1.6cm × 90cm) of Ultrogel AcA-34 that had been equilibrated with 150mM-potassium phosphate buffer, pH 7.4, containing 0.2mM-dithiothreitol and 0.05% Tween 20 (Fig. 4*a*). The column was eluted with this buffer at a flow rate of 10ml/h. Alternatively, 100μg of total rat liver phospholipid was added to the extract in 100μl of emulsion (see the Materials and methods section) and the extract was then incubated at 4°C for 2–4h before chromatography on Ultrogel AcA-34 (Fig. 4*b*).

Treatment of PAP with the phospholipid produced a clearer separation on the column, and this was the procedure that was routinely employed. In this system, the PAP separates into two discrete peaks of activity. A variable amount of activity always appeared at the void volume of the column even with gel filtration on Sepharose 6B. This material has an apparent M_r of more than 2×10^6, and it is probably an aggregated form of PAP. The

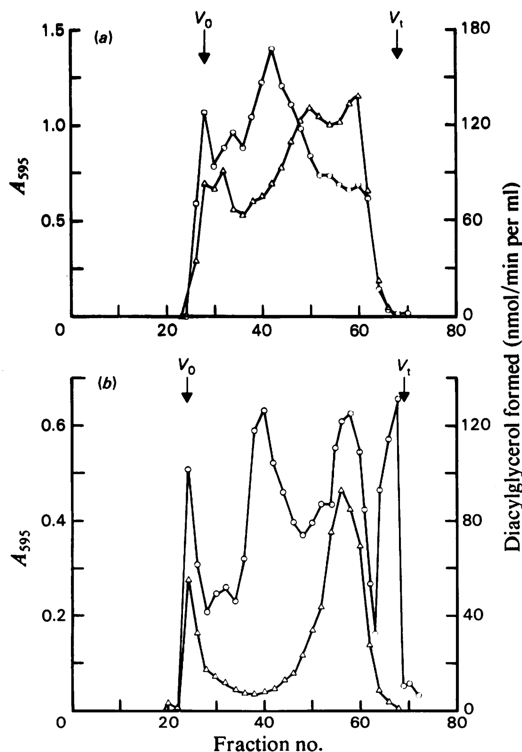

Fig. 4. *Purification of PAP by gel filtration on Ultrogel AcA-34*
The PAP activity that was eluted from DE-52 DEAE-cellulose was concentrated and applied to the Ultrogel column after incubation at 4°C in the absence (*a*) or in the presence (*b*) of rat liver phospholipids (see the Results section). The PAP activity (△) and protein as detected with PAGE Blue G-90 (A_{595}) (○) are shown.

other peak of activity occurred in the region expected for proteins with M_r values in the range 12000–68000. Fractions 50–60 (Fig. 4*b*) were combined. The total procedure gave an increase of 1.7-fold in the specific activity of PAP (Table 1).

Step 6: chromatography on CM-Sepharose 6B

The PAP activity from fractions 50–60 of Fig. 4(*b*) were combined and diluted to a conductivity of about 0.8mS with 0.2mM-dithiothreitol containing 0.05% Tween 20. This solution was then adjusted to pH6.5 with 16M-H_3PO_4 and applied to a column (1.0cm × 5.5cm) of CM-Sepharose 6B. The column was washed with 2 vol. of 25mM-potassium phosphate buffer, pH6.5, containing 0.2mM-dithiothreitol and 0.05% Tween 20, followed by two linear gradients of 25–100mM and 100–300mM-potassium phosphate buffer, pH6.5, containing 0.2mM-dithiothreitol and 0.05% Tween 20 (Fig. 5). Two peaks of PAP activity were obtained. The smaller peak A (fractions 26–50; Fig. 5) corresponded to an apparent increase in specific activity of 85-fold (Table 1). Peak B (fractions 56–74; Fig. 5) gave an apparent increase in specific activity of 180-fold. However, when the assay system was re-characterized, it became evident that the requirement for $MgCl_2$ and phosphatidate had changed compared with that observed when the system was originally established with PAP eluted from calcium phosphate gel (Figs. 1*b* and 1*c*). The PAP activity in peak B was therefore subsequently determined with 1mM-$MgCl_2$ and 0.4mM-phosphatidate. It then showed an increase in specific activity of 416-fold. This must be considered as a minimum value, since magnesium phosphatidate produces a strong substrate inhibition (Fig. 1*b*). At 1mM-phosphatidate, and at 1.3, 1.5 and 2.0mM (results not shown), no PAP activity was detected. The kinetics with this lipid substrate do not follow the Michaelis–Menten pattern. It was therefore impossible to extrapolate to $V_{max.}$ with a double-reciprocal plot (results not shown).

Effects of phospholipids and bivalent cations on PAP activity

The experiments were performed to see if PAP activity could be stimulated at various stages of purification by phospholipids or bivalent cations. Coleman & Hübscher (1962) have described a phospholipid requirement for microsomal PAP, and the results in Fig. 3(*b*) indicate that there is an interaction between PAP and rat liver phospholipids. Samples of PAP from Steps 3 and 6 (Table 1) of the purification were incubated at 4°C with 0.1–0.3mM-phospholipids for 0, 4, 22 and 48h and then assayed for PAP activity. No significant changes were detected.

Many phospholipases need bivalent cations for activity, and PAP is stimulated by Mg^{2+} (Fig. 1*b*). However, this appears to be caused by an effect of Mg^{2+} on the phosphatidate substrate rather than an absolute requirement for Mg^{2+}. PAP activity is retained in the presence of high concentrations of EDTA provided that an amphiphilic cation, e.g. chlorpromazine, is added (Bowley *et al.*, 1977). The chlorpromazine and Mg^{2+} probably enable the

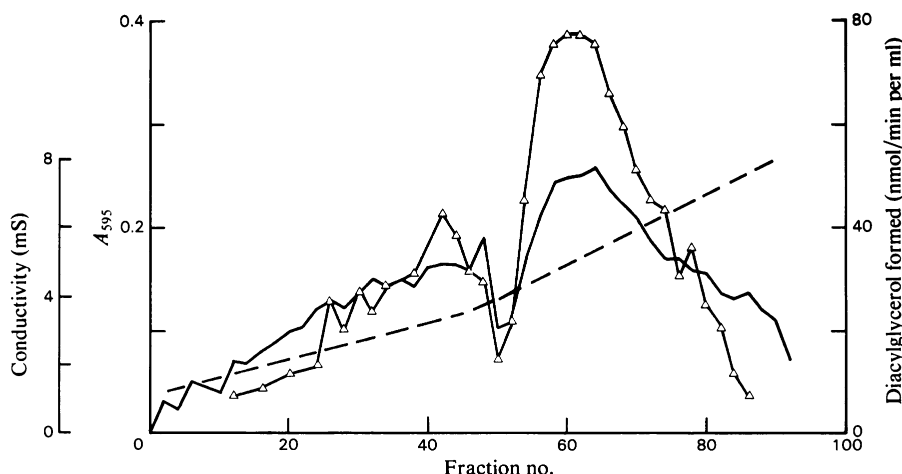

Fig. 5. *Purification of PAP by chromatography on CM-Sepharose 6B*
Fractions containing PAP activity from Fig. 4(*b*) were applied to a CM-Sepharose 6B column (see the Results section), and eluted with two linear gradients of 25–100mM- and 100–300mM-potassium phosphate buffer, pH6.5, containing 0.2mM-dithiothreitol and 0.05% Tween 20. PAP activity (Δ), protein as detected with PAGE Blue G-90 (A_{595}) (——) and the conductivity (– – –) are shown.

PAP to interact with its substrate by modifying its physical form, e.g. by neutralizing the negative charge (Brindley & Sturton, 1982). Phospholipase C activity from *Bacillus cereus* can also be measured in the presence of high concentrations of EDTA (Ottolenghi, 1965; Johnson & Bonventre, 1967), but it was inactivated by preincubation with this chelating agent, or with 1,10-phenanthroline (Ottolenghi, 1965; Otnæss *et al.*, 1972). Full reactivation occurred on adding back Zn^{2+}. Since PAP is also a phospholipase C, the effect of various cations, including Zn^{2+}, was tested.

The first objective was to determine their effects in the assay system. This was done by using the particle-free supernatant as a source of PAP, since this is less likely to be depleted of Zn^{2+} or other bivalent cations. Concentrations of Zn^{2+} and Mn^{2+} greater than about 0.1 mM inhibited PAP activity when it was determined in the presence of 5 mM-$MgCl_2$ (Fig. 6). The further addition of Mg^{2+} had no effect on PAP activity (Figs. 1*c* and 6). Samples of PAP from steps 3 and 6 of the purification (Table 1) were then incubated with 1 mM-$ZnCl_2$, -$MnCl_2$ or -$MgCl_2$ for 5 min at 23°C. The samples were then diluted in the assay system so that the final concentration of these cations from the preincubation was less than 0.1 mM. No stimulation of PAP activity was observed.

In order to investigate further whether PAP might be a Zn^{2+}-containing enzyme, samples of PAP from step 6 of the purification (Table 1) were incubated for 30 min at 4°C with 0.5 mM-1,10-phenanthroline. Some of the samples were then incubated for 5 min at 23°C with 1 mM-$ZnCl_2$ or -$MgCl_2$. The enzyme samples were then diluted in the assay system so that the final concentration of Zn^{2+} was less than 0.1 mM. Incubation with 1,10-phenanthroline decreased the PAP activity by an average of 17% with two separate preparations. PAP activity was regained after incubating with either Mg^{2+} or Zn^{2+}.

Effects of thiol-blocking reagents on PAP activity

The effects of three thiol-blocking reagents on the PAP activity were initially tested with enzyme from step 2 of the purification (Table 1). Iodoacetate had no significant effect on PAP activity (Fig. 7). However, *p*-chloromercuribenzoate and particularly *N*-ethylmaleimide produced marked inhibitions. The effects of these latter reagents were tested by incubating 1 mM of each with the PAP obtained from step 6 of the purification. In

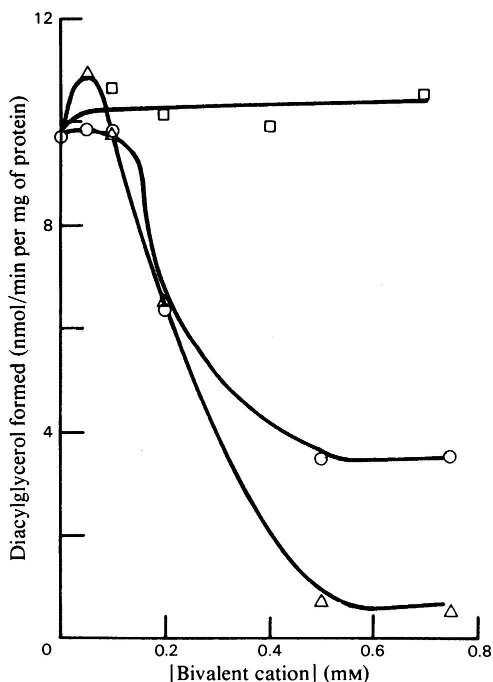

Fig. 6. *Effects of Zn^{2+}, Mn^{2+} and Mg^{2+} on PAP activity*
The particle-free supernatant of rat liver was incubated under the standard assay conditions in which 5 mM-$MgCl_2$ was present. The effects of adding $ZnCl_2$ (△), $MnCl_2$ (○) and more $MgCl_2$ (□) are shown.

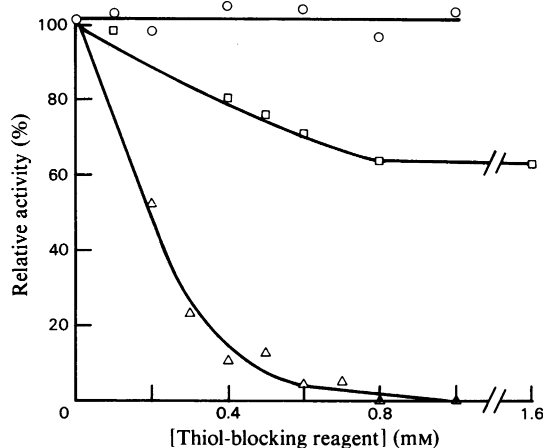

Fig. 7. *Effects of some thiol-blocking reagents on PAP activity*
PAP from step 2 of the purification (Table 1) was incubated for 15 min at 37°C in 25 mM-potassium phosphate buffer, pH 7.4, containing 0.2 mM-dithiothreitol, 0.05% Tween 20 and various concentrations of iodoacetate (○), *p*-chloromercuribenzoate (□) or *N*-ethylmaleimide (△). These samples were then assayed for PAP activity, and the results are expressed relative to the activity when no thiol-blocking reagent was added.

two separate experiments the mean inhibition with *N*-ethylmaleimide and *p*-chloromercuribenzoate was 82% and 48% respectively. These inhibitions were not seen if 2mM-dithiothreitol was added together with the thiol-blocking reagents.

Effects of butane-2,3-dione and cyclohexane-1,2-dione on PAP activity

Several enzymes that act on anionic substrates are known to have arginine residues at their active sites (Hjelmgren *et al.*, 1976; Kantrowitz & Lipscomb, 1976; Kazarinoff & Snell, 1976). These include alkaline phosphatase (Daemen & Riordan, 1974) and mitochondrial ATPase (Marcus *et al.*, 1976). Phospholipase C from *B. cereus* has also been shown to have an arginine residue at its active site (Aurebekk & Little, 1977). Initial experiments were therefore performed to establish whether the arginine reagents, butane-2,3-dione and cyclohexane-1,2-dione inhibit PAP activity from step 2 of the purification. This proved to be the case (Fig. 8), and the effects of these reagents on the PAP from step 6 of the purification (Table 1) were tested.

The average inhibition of this purified PAP activity by 120mM-butane-2,3-dione was 83% in two independent experiments. The final concentration of this reagent in the assay system was 4.8mM, and this in fact stimulated PAP activity by 39%. Incubation with butane-2,3-dione at pH 7.4 is also known to modify α- and ε-amino groups on proteins, and therefore PAP was also incubated with 120mM-cyclohexane-1,2-dione at pH 8.1 containing 0.2M-borate. This procedure is thought to be more specific for arginine residues (Patthy & Smith, 1975*a*,*b*). This again produced an inhibition of 83%. The final concentration of 28mM-cyclohexane-1,2-dione that occurred in the assay did not in itself significantly alter PAP activity.

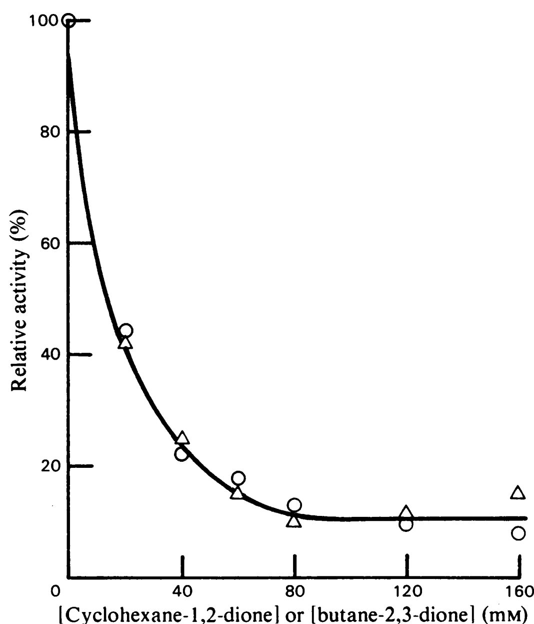

Fig. 8. *Effects of butane-2,3-dione and cyclohexane-1,2-dione on PAP activity*

The effects of butane-2,3-dione were determined (△) by incubating it with PAP obtained from step 2 of the purification (Table 1). The incubation was for 15min at 37°C, and the medium contained 25mM-potassium phosphate buffer, pH 7.4, 0.2mM-dithiothreitol, 0.05% Tween 20, 83μg of enzyme protein/ml and the concentration of butane-2,3-dione that is indicated. The activity of PAP was then determined. The effects of cyclohexane-1,2-dione (○) were determined after preincubating 375μg of enzyme protein/ml in the same incubation medium that had been adjusted to pH 8.1 with 200mM-sodium borate buffer, The results are expressed relative to the activity in incubations that did not contain the inhibitors.

Discussion

This paper describes a procedure by which the specific activity of PAP can be increased by about 416-fold compared with that in the particle-free supernatant. It is difficult to be certain about the increase in purity of the PAP, especially when related to normal rat liver, since: (*a*) the specific activity in the original supernatant was increased by about 5-fold by feeding the rats with ethanol; this increase results in part from an increase in the synthesis of PAP; (*b*) the phosphate buffer and Tween 20 used in the purification stimulate PAP (Fig. 2); (*c*) the enzyme was unstable in the latter stages of purification; (*d*) the purified PAP was strongly inhibited by excess of Mg^{2+} and phosphatidate, and it was impossible to estimate true $V_{max.}$ values.

The present method is a significant advance over the procedures previously used to purify PAP from liver (see the introduction) and lung (Casola & Possmayer, 1981*a*). The inclusion of Tween 20 in combination with a phosphate buffer helped to stabilize PAP and enable it to be separated from some of the contaminating proteins. However, fraction B of step 6 in the purification (Table 1) still contains at least ten bands after electrophoresis on polyacrylamide in the presence of sodium dodecyl sulphate (results not shown). It is not yet known which of these bands are derived from PAP or whether the PAP activity in peaks A and B (Fig. 5) represents different forms of the enzyme. We also do not think that it is yet possible to estimate the molecular mass of PAP from gel filtration. In our

experience PAP can aggregate and appear to have a relative molecular mass of many millions (Fig. 4; Hosaka *et al.*, 1975). In this respect we have avoided purification steps that involve precipitating PAP. The elution profile of PAP from gel-filtration columns is further modified when phospholipids are added to the preparation (Fig. 4). This is probably caused by the interaction of PAP with phospholipids, but this was not accompanied by a change in the enzyme's activity. Finally, we have also observed binding of PAP to gel-filtration columns, thus delaying its elution.

The PAP activity that was partially purified in this work was that from the soluble fraction of rat liver. It appears that the same PAP activity associates with the particulate fractions of rat liver, since this translocation is observed when oleate is incubated with isolated hepatocytes (Cascales *et al.*, 1984). We do not yet know whether this translocation involves a structural modification of the PAP. The PAP activity that was obtained after step 6 of purification (Table 1) was stimulated by about 20-fold when Mg^{2+} was added to the incubation medium (Fig. 1*c*). This is likely to result from the interaction of Mg^{2+} with the negatively charged phosphatidate (Brindley & Sturton, 1982). The present work does not give clear-cut evidence as to whether PAP is a metalloenzyme, and in particular whether it contains Zn^{2+} as do other phospholipases of the C type. Previous work with a phospholipase C showed that it was completely inhibited after incubation for 30 min at 4°C with 0.1 mM-1,10-phenanthroline, whereas PAP activity in the present study was only inhibited by 17% with 0.5 mM of this reagent. Mn^{2+} (Fig. 6) and Ca^{2+} (Sturton & Brindley, 1980) were also ineffective in stimulating PAP activity.

PAP activity appears to depend on functional thiol groups, since *p*-chloromercuribenzoate and particularly *N*-ethylmaleimide inhibit strongly (Fig. 7). However, iodoacetate was ineffective under the conditions employed. PAP activity from rat lung was also not inhibited by iodoacetamide, although *p*-chloromercuribenzoate was effective (Casola & Possmayer, 1981*b*). Hosaka *et al.* (1975) also demonstrated an inhibition of the soluble PAP of rat liver by *p*-chloromercuribenzoate. Furthermore, the microsomal PAP of rat liver is inhibited by *p*-chloromercuribenzoate and not by iodoacetic acid (Coleman & Hübscher, 1962). It is also possible that the inhibition by *p*-chloromercuribenzoate could involve a binding of the mercury to a metal-binding site on PAP. $HgCl_2$ is also known to inhibit PAP activity from lung (Johnston *et al.*, 1978; Casola & Possmayer, 1981*b*).

The inhibition of PAP by butane-2,3-dione and cyclohexane-1,2-dione (Fig. 8) indicates that it requires a functional arginine residue. This group is also required for a number of other enzymes that act on anionic substrates, and a similar effect has been shown with the phospholipase C activity of *B. cereus* (Aurebekk & Little, 1977).

It is hoped that the present results will facilitate further work concerning the mechanisms by which PAP is involved in regulating glycerolipid synthesis.

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