[9] G. WEBER, personal communication to N. O. KAPLAN, quoted by S. SHIFRIN AND N. O. KAPLAN, *Advances in Enzymology*, 22 (1960) 353.
[10] B. L. VALLEE, in *The Enzymes*, Vol. 3, Ch. 15, Academic Press, Inc., New York, N.Y., 1960.
[11] A. WITTER, *Acta Chem. Scand.*, 14 (1960) 1717.
[12] D. D. ULMER AND B. L. VALLEE, *J. Biol. Chem.*, 236 (1961) 730.
[13] E. M. KOSOWER AND D. C. REMY, *Tetrahedron*, 5 (1959) 281.
[14] E. M. KOSOWER, *J. Am. Chem. Soc.*, 80 (1958) 3261.
[15] H. R. LEVY AND B. VENNESLAND, *J. Biol. Chem.*, 228 (1957) 85.
[16] T. S. SORENSEN, *Thesis, University of Wisconsin*, 1960.
[17] A. D. WINER AND H. THEORELL, *Acta Chem. Scand.*, 14 (1960) 1729.
[18] E. M. KOSOWER, *J. Am. Chem. Soc.*, 78 (1956) 3497.
[19] E. M. KOSOWER, *J. Am. Chem. Soc.*, 80 (1958) 3253.
[20] R. W. GURNEY, *Ionic Processes in Solution*, Mc Graw-Hill Book Co., Inc., New York, 1953, p. 50.
[21] E. LIPPERT, *Z. Elektrochem.*, 61 (1957) 962.
[22] J. CZEKALLA, *Z. Elektrochem.*, 64 (1960) 1221.

Biochim. Biophys. Acta, 56 (1962) 474–479

METABOLISM OF PHOSPHOLIPIDS

V. STUDIES OF PHOSPHATIDIC ACID PHOSPHATASE*

R. COLEMAN AND G. HÜBSCHER
Department of Medical Biochemistry and Pharmacology, The Medical School, Birmingham (Great Britain)

(Received June 19th, 1961)

SUMMARY

1. The distribution of phosphatidic acid phosphatase is studied in the liver, kidney, brain, and intestinal mucosa of the ox, pig, rabbit, guinea-pig, and rat. Cell fractionation of pig kidney revealed that 63% of the total activity is in the microsomal fraction.

2. The microsomal enzyme from pig kidney is particulate, shows a pH optimum at 6.0, has a K_m of $2.2 \cdot 10^{-4}$ for phosphatidic acid, and is inhibited by fluoride ions. SH groups of the enzyme seem to be essential for activity.

3. Using the microsomal enzyme, the effect of metal ions, chelating agents, detergents, some hydrolytic enzymes, autolysis, and of some higher alcohols is investigated.

4. Procedures for the partial purification and solubilisation of the enzyme are described. The purified enzyme is highly specific for phosphatidic acid and hydrolyses only the phosphoric acid ester bond.

INTRODUCTION

Phosphatidic acid occupies an important position in pathways of phospholipid biosynthesis. It has been shown to be the direct lipid precursor of phosphatidyl inositol[1]

Abbreviations: PA phosphatase, phosphatidic acid phosphatase; TCA, trichloracetic acid.
* Part IV, see ref. 13.

and possibly of diphosphatidyl glycerol[2]. D-α,β-Diglyceride, produced by the enzymic dephosphorylation of phosphatidic acid, may serve as the lipid precursor of phosphatidyl choline and phosphatidyl ethanolamine[3] and of triglycerides[4]. HOKIN AND HOKIN have postulated a phosphatidic acid cycle which serves to transport ions across membranes and involves PA phosphatase[5].

Although the biosynthetic route to phosphatidic acid has been known for some years[6,7], its occurrence in animal tissues was frequently questioned[8–10]. Using tracer techniques HOKIN AND HOKIN were able to demonstrate its presence in several mammalian tissues[11]. This observation was substantiated by the isolation of phosphatidic acid from the liver of the ox, pig, and rat[12,13].

PA phosphatase was first demonstrated in mitochondrial preparations of rat liver[14] and subsequently some if its properties were examined using a particulate chicken-liver preparation[15]. PA phosphatase was found to occur also in rat brain[16], in deoxycholate extracts of guinea pig brain[7], in particulate subcellular fractions of the albatross salt gland[5], and in erythrocyte membranes[17].

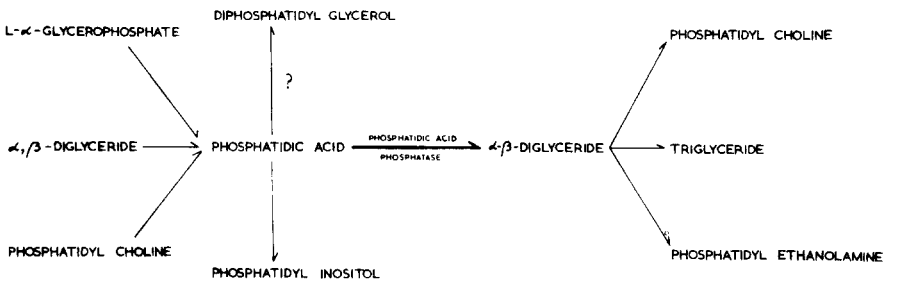

Fig. 1. Role of PA phosphatase in lipid metabolism.

With a few exceptions, all of the enzymes involved in the biogenesis of glycerides and phosphatides are closely linked to particulate subcellular structures (*cf.* HÜBSCHER[18]). Though it is tempting to assume that the enzymes handling lipid substrates are present as lipoproteins, no information is available about their actual composition. Supposing that they were lipoproteins, the lipid moiety might play a functional role. The enzymes participating in lipid biosynthesis may also be regarded as a multi enzyme unit. This unit may require a matrix and an ordered spacial arrangement of its components in view of its mainly water-insoluble, and thus not freely diffusible, substrates.

The present investigation is an attempt to study these problems by using PA phosphatase as a model enzyme. A preliminary report of this work has been published[19].

MATERIALS AND METHODS

The chemicals used were of "Analar" grade and the detergents and bile acids were commercial preparations. Wheat germ lipase and ribonuclease (4 × recrystallised) were obtained from Light and Co., and pancreatin from British Drug Houses Ltd.

Preparation of phospholipids

Phosphatidic acid was prepared from egg lipids as follows: phosphatidyl choline was separated from other phospholipids by chromatography on alumina[20] followed by

a second chromatography on silicic acid to remove lyso-phosphatidyl choline[21]. The phosphatidic acid formed from phosphatidyl choline by the action of phospholipase D[22] was purified by silicic acid chromatography[12]. The molar ratios of P:N and of P:ester of the final preparation were 1:0.09 and 1:2.02 respectively. The purity of phosphatidic acid was further checked by mild alkaline hydrolysis of the lipid[23] followed by chromatography of the resulting glycerophosphate on Nalcite SAR or by treatment with acid phosphomonoesterase[13]. These two methods indicated that the phosphatidic acid preparation was respectively 87 and 92% pure.

Diphosphatidyl glycerol was prepared according to MACFARLANE[10], phosphatidyl choline as indicated above, and "diphosphoinositide" as described by FOLCH[24]. These phospholipids and phosphatidic acid were emulsified in water as previously described[25,26]. The preparations were stored at —20° and adjusted to pH 6.0 prior to use.

Assay of enzymic activity

60 μmoles of maleate buffer, pH 6.0; 3 μmoles of phosphatidic acid; and the enzyme were incubated in a total volume of 1.0 ml for 40 min at 37°. A wide range of maleate concentrations was found to have no effect on the enzymic activity. The reaction was stopped by adding 0.1 ml of 50% TCA. After cooling to 0°, 1.0 ml of 5% TCA containing 12.5 g of Norit A charcoal/100 ml was added, followed by 1.9 ml of 5% TCA. The mixture was then filtered and a sample of the filtrate taken for determination of inorganic phosphate. The amount of inorganic phosphate released was corrected for the amount liberated from the enzyme preparation in the absence of substrate. In the absence of enzyme, no inorganic phosphate was liberated from phosphatidic acid.

One unit was defined as the amount of enzyme liberating 1 μmole of inorganic phosphate/h under the experimental conditions described. The specific activity was expressed as units/mg of protein.

Other methods used were previously described[25,26].

EXPERIMENTS AND RESULTS

Distribution of PA phosphatase

In Fig. 2, the PA phosphatase activity of various mammalian tissues is compared. 10% homogenates (w/v) of fresh material were prepared by mechanical blending in 0.25 *M* sucrose. The cortex and medulla of the kidney were taken. Samples of representative areas of the brain were pooled before homogenisation and mucosal scrapings of the small intestine were used. Since these experiments were carried out in the early stages of this work, the homogenates were incubated for 1 h at 37° using Tris buffer, pH 7.4 (final concentration $5 \cdot 10^{-2}$ *M*). The results indicate that all tissues had a considerable activity (see Fig. 2). Pig tissues had the highest specific activity, followed by those from the ox, guinea pig, rat, and rabbit in that order. With all animals, kidney and intestinal mucosa had a higher specific activity than brain or liver. The activity observed for rat tissues was similar to that reported by SMITH, WEISS AND KENNEDY[15].

A cell fractionation of pig kidney was carried out using the method of SCHNEIDER AND HOGEBOOM[27]. The results given in Table I show that over 60% of the activity of the homogenate was in the microsomal fraction and that the combined particulate

fractions contained over 85% of the total activity. The specific activity of the microsomal fraction was three times that of the homogenate. HOKIN AND HOKIN described a similar distribution of activity when deoxycholate extracts of subcellular fractions of the salt gland were assayed[5].

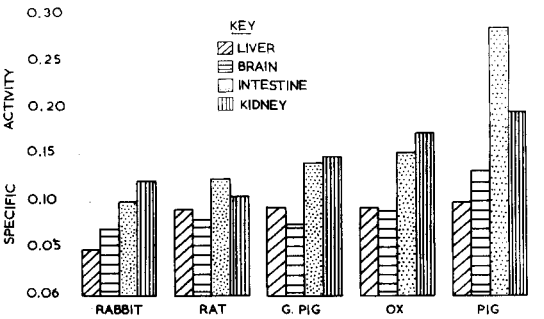

Fig. 2. PA phosphatase activity in mammalian tissue homogenates. For details see text.

TABLE I

DISTRIBUTION OF PA PHOSPHATASE IN CELL FRACTIONS OF PIG KIDNEY

The assay system was that described under METHODS, except that incubation was carried out for 1 h at pH 7.4 using 0.04 *M* Tris buffer.

Cell fraction	*Specific activity*	*Activity per cell fraction (%)*
Homogenate	0.182	100
Cell debris + nuclei	0.176	4.7
Mitochondria	0.197	21.2
Microsomes	0.585	63.0
Supernatant	0.104	14.6

Properties of the microsomal enzyme from pig kidney

The microsomal preparation used for the following experiments had been kept in $5 \cdot 10^{-3}$ *M* Tris buffer, pH 7.4, for varying periods at —20°. A decrease in activity of about 15% was noted after 3 to 4 months.

A time study indicated that zero-order conditions lasted for 40 min of incubation when 25–30% of the substrate had been hydrolysed.

For the determination of the optimal pH of the reaction, a universal buffer consisting of ethanolamine–Tris–acetate was employed. The pH optimum appeared to be near 6.0 but there was only a slow decrease in apparent activity at the higher pH values (see Fig. 3A). Very low activities were found at pH 5 and pH 10 indicating that the acid and alkaline phosphomonoesterases of pig kidney had little if any activity towards phosphatidic acid at their optimal pH values. The same pH optimum and the same activity at the optimal pH value were observed when maleate buffer was tested over a smaller range of pH values.

Fig. 3B shows the effect of increasing concentrations of substrate on the reaction velocity. When these values were treated according to LINEWEAVER AND BURK[28], a K_m of $2.2 \cdot 10^{-4}$ was found for phosphatidic acid prepared from egg lecithin.

The microsomal enzyme was inhibited by fluoride ions, a $1 \cdot 10^{-2}$ *M* concentration

giving 50% inhibition. There was an apparent lag in the inhibition of PA phosphatase at low concentrations of fluoride. The same degree of inhibition at low concentrations of fluoride was obtained in the presence of EDTA and thus a complexing of fluoride ions with divalent metal ions present in the enzyme preparation is unlikely.

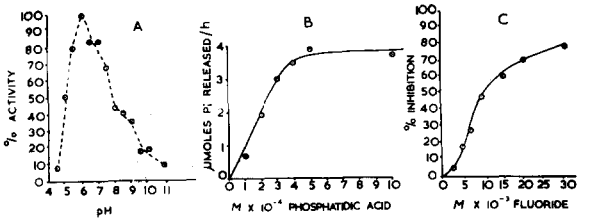

Fig. 3. Some factors affecting PA phosphatase activity. A, effect of pH; B, substrate concentration; C, fluoride ions.

The enzyme was unaffected by cyanide, glutathione, or cysteine at concentrations which would inhibit alkaline phosphatase. The inhibition brought about by $1 \cdot 10^{-3}$ *M* *p*-chloromercuribenzoate was reversed by excess glutathione (see Table II). This would suggest that thiol groups are essential for enzymic activity, though inhibitors with different modes of action such as iodoacetate or cystine were without effect on the enzyme.

TABLE II

THE EFFECT OF SULPHYDRYL COMPOUNDS AND OF INHIBITORS OF SULPHYDRYL GROUPS

The crude microsomal enzyme was used and all compounds were added to give a final concentration of $5 \cdot 10^{-3}$ *M*, with the exception of *p*-chloromercuribenzoate ($1 \cdot 10^{-3}$ *M*). The reaction rate of 100% refers tot 0.68 μmole of inorganic phosphate released.

Additions	*Rate of reaction, in %*
None	100
Glutathione	99
Cysteine	97
Cystine	93
Iodoacetic acid	105
p-Chloromercuribenzoic acid	38
p-Chloromercuribenzoic acid + glutathione	104

There appears to be no requirement for metal ions, since chelating agents had no significant effect on the activity at $2 \cdot 10^{-2}$ *M* concentration. Some divalent cations, particularly Hg^{2+}, Zn^{2+} and Fe^{2+} (all $5 \cdot 10^{-3}$ *M*) brought about a marked inhibition (see Table III). Unlike the chicken liver enzyme[15], the kidney preparation was unaffected by Ca^{2+} or Ba^{2+} ions, and Mg^{2+} ions gave only a 25% inhibition at a higher concentration than that required for a complete inhibition of the chicken liver enzyme.

Particulate nature of the enzyme

The enzyme was found to be tightly bound to some particulate matter of the microsomal fraction. Several experimental approaches gave evidence for this:

Physical means and autolysis: At a concentration of 10 mg/ml in $5 \cdot 10^{-3}$ *M* Tris buffer, pH 7.4, the preparation was 9 times frozen and thawed in the course of several

TABLE III

THE EFFECT OF DIVALENT METAL IONS

All metal ions were added to give a final concentration of $5 \cdot 10^{-3}$ *M* whereas the concentrations of EDTA, citrate and tartrate were $2 \cdot 10^{-2}$ *M*. The crude microsomal enzyme was used. The reaction rate of 100 % refers to a release of 0.68 μmole of inorganic phosphate.

Additions	*Rate of reaction in %*	*Additions*	*Rate of reaction in %*
None	100	Mg^{2+}	76
EDTA	102	Co^{2+}	57
Citrate	91	Cu^{2+}	55
Tartrate	98	Mn^{2+}	43
Cyanide	103	Fe^{2+}	24
Ba^{2+}	95	Zn^{2+}	12
Ca^{2+}	93	Hg^{2+}	9
Ni^{2+}	91		

days or subjected to high speed mechanical blending for 6 min. No significant loss of enzymic activity occurred and, after centrifugation, no activity was found in the supernatant fluid. Solubilisation was judged in these and in the following experiments by centrifuging the preparation (suspended in $5 \cdot 10^{-3}$ *M* Tris buffer, pH 7.4) at 3300000 *g* × min.

The preparation may undergo autolysis at room temperature for several days without loss of enzymic activity when protected against bacterial contamination by a thin layer of toluene. A higher proportion of protein was released under these conditions compared with the above two methods. Thirty per cent of the protein was solubilised after 3 days and thereafter, release of protein occurred only slowly though PA phosphatase was stable up to 7 days of autolysis. Again, all of the enzymic activity remained in the pellet obtained after centrifugation.

The effect of detergents: PA phosphatase was markedly inhibited by certain detergents. All of the detergents used were made up in 0.2 *M* maleate buffer, pH 6.0, and the final pH of the solution was, if necessary, again adjusted to 6.0 by adding a few drops of HCl. Cholate and deoxycholate were incompletely soluble at this pH and were added as a suspension. As can be seen in Table IV, all detergents caused inhibition though of varying degree. It is unlikely that these compounds act on the active centre of the enzyme or on the phospholipid substrate by a single mechanism, since some are anionic while others are cationic.

Using the sediment obtained after centrifugation of a 3-day autolysate, partial solubilisation of enzymic activity was observed with some detergents at pH 7.4. The detergents were tested over a range of concentrations up to $2.5 \cdot 10^{-2}$ *M* (this was about 3 mg of detergent/mg of protein). In control experiments, the degree of inhibition was determined at the various concentrations of the detergent. Solubilisation of PA phosphatase was expressed in per cent of the inhibited enzyme. In general, solubilisation of protein preceded that of PA phosphatase. About 50 % of the enzyme was solubilised by cholate and deoxycholate at concentrations of $2 \cdot 10^{-2}$ *M*. A change of pH value from 7.4 to 9.0 did not increase solubilisation using the two bile acids. The effect of bile acids observed here is in agreement with the results of HOKIN AND HOKIN[7] but in view of an only partial solubilisation accompanied by a considerable inhibition, bile acids seem to be of limited use for the solubilisation of PA phosphatase.

TABLE IV

THE EFFECT OF SURFACE-ACTIVE COMPOUNDS

Crude microsomal enzyme from pig kidney was used. All compounds were added to give a final concentration of 5 mg/ml. The reaction rate of 100% refers to 0.66 μmole of inorganic phosphate liberated.

Additions	*Rate of reaction in %*
None	100
Cholate	80
Deoxycholate	75
Tween 20	75
Teepol	56
Cetavlon	30
Dodecyl sulphate	30

The effect of hydrolytic enzymes: Pancreatin, wheat germ lipase, and ribonuclease were incubated in $6.6 \cdot 10^{-2}$ *M* Tris buffer, pH 7.3, for 2 h at 37° at a ratio of 1 mg of enzyme/10 mg of autolysed microsomal protein.

Pancreatin caused a 40% loss of enzymic activity and the corresponding values for wheat germ lipase and ribonuclease were 25 and 0% respectively. Only with wheat germ lipase was a slight solubilisation (10%) of PA phosphatase obtained. The hydrolysis of ribonucleic acid was accompanied by a considerable release of protein into solution. After 6 h of incubation, over 30% of the microsomal protein was solubilised but even the prolonged treatment with ribonuclease did not bring about a release of PA phosphatase.

DODGSON, ROSE AND SPENCER[29] reported a solubilisation of aryl sulphatase C which had resisted treatments similar to those applied to the microsomal PA phosphatase. By combining the hydrolytic activities of pancreatin with the surface active properties of Lissapol N, they achieved a 90% extraction of soluble aryl sulphatase. Lissapol N was thought to increase the lipolytic and to inhibit the proteolytic activities of pancreatin[29]. With PA phosphatase, however, a combination of pancreatin and detergents gave up to 40% loss and less than 50% solubilisation of the enzyme.

The effect of higher alcohols: In these experiments, microsomal preparations were used which had been autolysed for 3 days followed by a treatment with ribonuclease for 6 h under conditions described above. Control experiments showed that the action of higher alcohols on untreated microsomal preparations was far less pronounced and gave unsatisfactory results.

The effect of iso-, *tert.*-, and *n*-butanol was studied by varying the concentration of the butanols at a constant pH value or by varying the pH value at a given alcohol concentration. The concentration of protein was adjusted to 3.5 mg/ml and the ionic strength was kept at $2.5 \cdot 10^{-2}$ *M*. The addition of solvent was carried out at 0° over a period of 15 min under continuous stirring. The mixture was left for 1 h at 0° and then centrifuged at 3300000 *g* × min.

It was found that *n*-butanol was the most effective solvent. If the solubilisation of PA phosphatase, brought about by *n*-butanol at the optimal pH value and alcohol concentration, is arbitrarily set at 100, the corresponding figures for iso- and *tert.*-butanol at their optimal conditions were 70 and 33% respectively. Details of the results with *n*-butanol are shown in Fig. 4. There was an increasing solubilisation of

PA phosphatase with increasing butanol concentration up to an optimum at 8%. This amount of butanol still gives a one phase system. Two phase systems gave very poor yields of soluble enzyme. Increasing the pH value at a given concentration of butanol also increased solubilisation of PA phosphatase (see Fig. 4B).

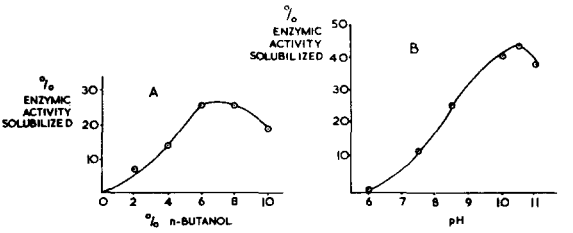

Fig. 4. Effect of pH and of *n*-butanol concentration on the solubilization of PA phosphatase.

At pH 10.5 and 8% of *n*-butanol, all of the PA phosphatase had been solubilised as judged by an assay of the pellet obtained after high speed centrifugation. However, the supernatant contained usually only 45–50% of the original activity indicating that *n*-butanol had an inhibitory effect. After dialysis of the supernatant against 100 volumes of $5 \cdot 10^{-2}$ *M* maleate buffer, pH 6.0, between 85 and 95% of the original activity was recovered.

Partial purification of PA phosphatase

All operations were carried out at 0° unless otherwise stated.

Preparation of the microsomal fraction: Pig kidneys were obtained as soon as possible after slaughtering and transported in ice. Fat and connective tissue were removed and cortex and medulla were cut into small pieces. They were homogenised in 0.25 *M* sucrose using a Waring blendor at full speed for 2 min. Cell debris, nuclei and mitochondria were removed by centrifugation at 65000 *g* × min. The supernatant was centrifuged at 3300000 *g* × min and the sediment suspended in $5 \cdot 10^{-3}$ *M* Tris buffer, pH 7.4 (specific activity 1.1).

Autolysis and treatment with ribonuclease: The microsomal preparation (10 mg protein/ml) was left for 3 to 4 days at room temperature under a thin layer of toluene. The pH usually fell to 4 to 5 during this period. The digest was centrifuged at 140000 *g* × min and the sediment suspended in $6.6 \cdot 10^{-2}$ *M* Tris buffer, pH 7.4 (specific activity 1.5–1.6; recovery 94–97%).

The autolysed suspension (10 mg protein/ml) was incubated for 6 h at 37° in the presence of highly purified ribonuclease (1 mg of ribonuclease/10 mg of microsomal protein) and centrifuged at 140000 *g* × min. The sediment was suspended in a small volume of water (specific activity 2.1–2.3, recovery 90–94%).

Extraction with n-butanol and dialysis: The suspension was diluted with water and ethanolamine buffer pH 10.5, to give a final concentration of $2.5 \cdot 10^{-2}$ *M* for the buffer and a protein concentration of 3.5 mg/ml. Ice-cold *n*-butanol was added dropwise under constant stirring over a period of 20–30 min to give a final concentration of 8%. The mixture was left for 1 h and centrifuged at 140000 *g* × min.

The supernatant was dialysed against 100 volumes of $5 \cdot 10^{-2}$ *M* Tris buffer, pH 7.4. For dialysis, maleate buffer, pH 6.0 and Tris buffers, pH 7.8 or 8.7 (all

$5 \cdot 10^{-2}$ *M*) were shown to be equally effective but with deionised water, no reactivation of the enzyme was observed. After dialysis, the preparation had a specific activity of 4.5 to 4.9 and the overall recovery was 85–90%. Thus a 4 to 4.5 fold purification was achieved with good yields.

Specificity of the purified preparation

The purified preparation was a whitish, turbid solution. Enzymic activity was not sedimented in 0.25 *M* sucrose under conditions which were sufficient for a complete sedimentation of microsomal preparations. However, in $5 \cdot 10^{-2}$ *M* maleate buffer, pH 6.0, about half of the enzyme was sedimented at 3300000 *g* × min.

The purified preparation still contained a considerable amount of lipid. The amount of lipid present was determined by measuring the esterified long chain fatty acids as hydroxamic acids. The ester values were then related to either protein or to units of enzyme. If related to protein, relatively less lipid than protein had been removed during the course of isolation of PA phosphatase. When related to units of enzyme, more lipid than units of enzyme had been lost. The original microsomal preparation contained 0.6 μmole of carboxylic acid ester bond per unit of enzyme whereas the purified preparation had 0.3 μmole/unit. The possibility that PA phosphatase may be a lipoprotein can, therefore, not be excluded. The molar ratio of P: ester of the final preparation was 1:1.42.

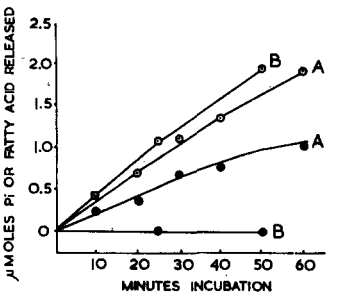

Fig. 5. Bond splitting in phosphatidic acid by microsomes and by purified enzyme. A, microsomal enzyme; B, purified enzyme. ○—○, phosphate liberated; ●—●, fatty acid liberated.

Since the routine assay used in this investigation determines only the release of inorganic phosphate, it was essential to show that the enzyme attacks primarily the phosphoric acid ester bond. An alternative release of inorganic phosphate from phosphatidic acid could be achieved by hydrolysis of the fatty acid ester bonds by phospholipases A and B. This would give glycerophosphate which might then be hydrolysed by an unspecific phosphomonoesterase. This possibility was excluded by measuring simultaneously the release of phosphoric acid and the decrease in carboxylic acid ester bonds during enzymic hydrolysis (see Fig. 5). The original microsomal preparation had a considerable activity of the phospholipases A and B type or possibly of a lipase (see Fig. 5, curves A). However, the amount of fatty acid ester hydrolysed was too small to assume that the formation of inorganic phosphate via glycerophosphate was the predominant sequence of reactions. The purified enzyme did not hydrolyse fatty acid ester bonds (see curves B) and thus represents a true PA phosphatase activity.

The ability of the enzyme to hydrolyse phosphatidic acid was compared with the activities towards other phospholipids (see Table V). Diphosphatidyl glycerol which is structurally related to phosphatidic acid but contains no monoesterified phosphoric acid was hydrolysed at a negligible rate. Another related phospholipid, "Diphosphoinositide", is a mixture of phosphatidyl inositol, phosphatidyl inositol monophosphate, and phosphatidyl inositol diphosphate[30] and contains monoesters of phosphoric acid but linked to inositol. It was not hydrolysed.

TABLE V

SPECIFICITY OF PA PHOSPHATASE

Purified microsomal enzyme was used. The final concentration of the compounds added was: phosphatidic acid, "diphosphoinositide", α-, and β-glycerophosphate, $3 \cdot 10^{-3}$ *M*; diphosphatidyl glycerol, $2.5 \cdot 10^{-4}$ *M*. The release of 100% of P_i or $P_{org.}$ refers to 0.9 μmole.

Compound	*Rate of Release of P_i in* %	*Rate of Release of $P_{org.}$ in* %
Phosphatidic acid	100	100
Diphosphatidyl glycerol	< 1	3
"Diphosphoinositide"	< 1	0
α-Glycerophosphate	2	—
β-Glycerophosphate	10	—

α-Glycerophosphate was hydrolysed at a negligible rate and β-glycerophosphate at 10% of the rate of phosphatidic acid. This is a further evidence for the true PA phosphatase activity of the enzyme but also indicates that acid phosphomonoesterase may be a contaminant. This latter enzyme is known to hydrolyse β-glycerophosphate at a faster rate than the α-isomer[31]. The original microsomal preparation hydrolysed α- and β-glycerophosphate. Phenyl phosphate (with pH optima at 5.0 and 10.0) and glucose 6-phosphate were hydrolysed by the crude microsomes at rates which exceeded that for phosphatidic acid. Glucose 6-phosphatase was destroyed during autolysis.

DISCUSSION

The previous investigations on PA phosphatase[5,7,14–17] and the present results show that the enzyme is widely distributed in animal tissues. The different levels of PA phosphatase in the organs and animals examined may, at least partly, be a reflection of the turnover of some phospholipids and glycerides in the respective tissues.

Of all subcellular fractions of pig kidney, over 60% of the PA phosphatase was in the microsomal fraction. A similar distribution was reported for the albatross salt gland[5]. Since phospholipids and triglycerides are roughly equally distributed between the mitochondrial and microsomal fractions, it seems unlikely that the high level of PA phosphatase in the microsomal fraction is merely a reflection of the lipid metabolism but might indicate that in the endoplasmic reticulum, this enzyme has an additional function.

Over 85% of the total activity of the kidney homogenate was recovered in the particulate subcellular fractions. The small percentage of PA phosphatase in the cell sap may also be particulate since centrifugal forces of up to 6000000 $g \times$ min are needed to completely free the cell sap from microsomal contamination[32]. The parti-

culate nature of the enzyme was indicated by various experimental techniques using physical means, autolysis, hydrolytic enzymes, detergents and higher alcohols. Deoxycholate and higher alcohols, particularly *n*-butanol, have been used to either "solubilise" lipoproteins or to bring the protein moiety of lipoproteins into solution[33]. The failure to obtain soluble PA phosphatase by freezing and thawing, autolysis, or digestion with ribonuclease and the partial or complete success in this respect given by deoxycholate or butanols suggests that PA phosphatase might be a lipoprotein. The purified preparation still contained significant amounts of lipid though it remains to be shown whether the lipid is associated with PA phosphatase. It should be mentioned in this connection that the crude microsomal enzyme was extremely stable, whereas the purified preparations were less stable.

PA phosphatase has properties reminiscent of acid phosphomonoesterase but is highly specific. α-Glycerophosphate (I) was not hydrolysed by the purified preparation, indicating that the free hydroxyl groups of glycerol have to be esterified if the phosphoric acid ester bond is to be cleaved. The latter bond is hydrolysed at a negligible rate when the phosphoric acid is further substituted (III). A glycerophosphatide with monoesters of phosphoric acid linked to inositol (IV) was not hydrolysed, suggesting that the monoester bond of phosphoric acid must be near the diglyceride moiety of the molecule and probably directly esterified with it if hydrolysis is to take place. The compounds shown in Fig. 6 give, however, no indication whether lyso-phosphatidic acids or a plasmalogen type of phosphatidic acid would be attacked. Furthermore, the chain length and degree of saturation of the substituting fatty acids may influence the rate of reaction.

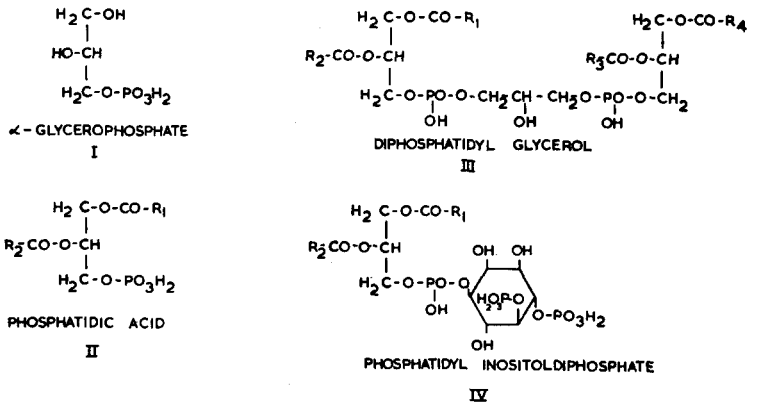

Fig. 6. Phosphatidic acid and structurally related compounds.

The inhibitory effect of surface-active compounds may be caused either by affecting the enzyme or by altering the physical state or charge of the substrate. The work of BANGHAM AND DAWSON on the action of phospholipase B using monomolecular films of lecithin showed that the zeta-potential of the lipid surface is a determining factor in the rate of enzymic hydrolysis[34]. The addition of bivalent cations or of anionic amphipathic compounds was shown to alter the surface potential of the lipid and at the same time the rate of reaction[34]. A similar effect might have been caused by some of the detergents used in the present investigation.

The reports on the requirement of divalent cations in PA phosphatase action are contradictory. The deoxycholate-solubilised PA phosphatase from brain[7] and the enzyme from erythrocytes[7] were stimulated by Mg^{2+} ions. The chicken liver enzyme[15] was inhibited by Mg^{2+}, Ca^{2+}, and Ba^{2+} ions while the PA phosphatase from pig kidney was slightly inhibited by Mg^{2+} ions but unaffected by Ba^{2+} and Ca^{2+} ions. Since EDTA and similarly acting compounds did not influence the rate of reaction, it seems likely that the chicken liver and pig kidney enzymes have no requirement for divalent cations though a tightly bound metal cannot be excluded, particularly in view of the particulate nature of the enzymes. It is also possible that there are organ specific PA phosphatases with different properties and requirements.

ACKNOWLEDGEMENTS

We wish to thank Professor A. C. FRAZER for his encouraging interest in this work. Dr. J. N. HAWTHORNE kindly supplied a sample of purified brain diphosphoinositide. The competent technical help of Miss S. PERRY and Miss D. WILLETTS is gratefully acknowledged.

This work was supported by a grant from the Medical Research Council to one of us (R.C.).

REFERENCES

1 H. PAULUS AND E. P. KENNEDY, *J. Biol. Chem.*, 235 (1960) 1303.
2 J. KIYASU, H. PAULUS AND E. P. KENNEDY, *Federation Proc.*, 19 (1960) 233.
3 E. P. KENNEDY AND S. B. WEISS, *J. Biol. Chem.*, 222 (1956) 193.
4 S. B. WEISS, E. P. KENNEDY AND J. Y. KIYASU, *J. Biol. Chem.*, 235 (1960) 40.
5 L. E. HOKIN AND M. R. HOKIN, *J. Gen. Physiol.*, 44 (1960) 61.
6 A. KORNBERG AND W. E. PRICER JR., *J. Biol. Chem.*, 204 (1953) 345.
7 M. R. HOKIN AND L. E. HOKIN, *J. Biol. Chem.*, 234 (1959) 1381.
8 R. M. C. DAWSON, *Biochim. Biophys. Acta*, 14 (1954) 374.
9 G. V. MARINETTI AND E. STOTZ, *Biochim. Biophys. Acta*, 21 (1956) 168.
10 M. G. MACFARLANE, *Biochem. J.*, 78 (1961) 44.
11 L. E. HOKIN AND M. R. HOKIN, *J. Biol. Chem.*, 233 (1958) 800.
12 G. HÜBSCHER AND B. CLARK, *Biochim. Biophys. Acta*, 41 (1960) 45.
13 G. HÜBSCHER, *Biochim. Biophys. Acta*, 52 (1961) 571.
14 S. B. WEISS, S. WAGNER SMITH AND E. P. KENNEDY, *Nature*, 178 (1956) 594.
15 S. WAGNER SMITH, S. B. WEISS AND E. P. KENNEDY, *J. Biol. Chem.*, 228 (1957) 915.
16 R. J. ROSSITER AND K. P. STRICKLAND, *Ann. N.Y. Acad. Sci.*, 72 (1959) 790.
17 L. E. HOKIN AND M. R. HOKIN, *Nature*, 189 (1961) 836.
18 G. HÜBSCHER, *Symposium No. 7, 5th Intern. Congr. Biochem., Moscow, 1961.*
19 R. COLEMAN AND G. HÜBSCHER, *Biochem. J.*, 80 (1961) 11 P.
20 D. J. HANAHAN, M. B. TURNER AND M. E. JAYKO, *J. Biol. Chem.*, 192 (1951) 623.
21 D. N. RHODES AND C. H. LEA, *Biochem. J.*, 65 (1957) 526.
22 F. M. DAVIDSON AND C. LONG, *Biochem. J.*, 69 (1958) 458.
23 G. HÜBSCHER, J. N. HAWTHORNE AND P. KEMP, *J. Lipid Research*, 1 (1960) 433.
24 J. FOLCH, *J. Biol. Chem.*, 177 (1949) 497.
25 B. CLARK AND G. HÜBSCHER, *Biochim. Biophys. Acta*, 46 (1961) 479.
26 P. KEMP, G. HÜBSCHER AND J. N. HAWTHORNE, *Biochem. J.*, 79 (1961) 193.
27 W. C. SCHNEIDER AND G. H. HOGEBOOM, *J. Biol. Chem.*, 183 (1950) 123.
28 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
29 K. S. DODGSON, F. A. ROSE AND B. SPENCER, *Biochem. J.*, 66 (1957) 357.
30 R. B. ELLIS AND J. N. HAWTHORNE, *Biochim. Biophys. Acta*, 51 (1961) 385.
31 J. ROCHE, in J. B. SUMNER AND K. MYRBÄCK, *The Enzymes*, Vol. 1, Academic Press, Inc., New York, p. 473.
32 G. POPJÁK, L. GOSSELIN, I. YOUHOTSKY GORE AND R. G. GOULD, *Biochem. J.*, 69 (1958) 238.
33 R. K. MORTON, in S. P. COLOWICK AND N. A. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, Inc., New York, p. 25.
34 A. D. BANGHAM AND R. M. C. DAWSON, *Biochem. J.*, 75 (1960) 133.