Glycerylphosphorylcholine Phosphodiesterase in Rat Liver

SUBCELLULAR DISTRIBUTION AND LOCALIZATION IN PLASMA MEMBRANES

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1. A simple new assay for glycerylphosphorylcholine phosphodiesterase is described, in which radioactive glycerylphosphorylcholine is used as substrate and the reaction products are separated by adsorption on an anion-exchange resin. 2. Rat liver subcellular fractions contained both particulate (58 %) and soluble (42 %) glycerylphosphorylcholine phosphodiesterase. Both activities released free choline from glycerylphosphorylcholine. 3. The particulate glycerylphosphorylcholine phosphodiesterase was recovered mainly in the nuclear and microsomal fractions and showed a distribution similar to those of 5'-nucleotidase and alkaline phosphodiesterase I, both of which are constituents of the liver plasma membrane. 4. During purification of plasma membranes glycerylphosphorylcholine phosphodiesterase, 5'-nucleotidase and alkaline phosphodiesterase I showed largely similar behaviour, indicating that glycerylphosphorylcholine phosphodiesterase is also localized in liver plasma membranes. Slight differences in the distributions of these three enzymes in density-gradient separations are discussed in relation to the possibility that they are unevenly distributed on different areas of the cell surface. 5. The differences between glycerylphosphorylcholine phosphodiesterase and alkaline phosphodiesterase I indicate that these two activities are not functions of a single enzyme. 6. The glycerylphosphorylcholine phosphodiesterase of liver plasma membranes has a pH optimum of 8.5 and a K_m for glycerylphosphorylcholine of 0.95 mM. It is inhibited by EDTA and fully reactivated by a variety of bivalent cations (and Fe^{3+}).

A recent study showed that many phospholipids are broken down by rat liver lysosomes (Fowler & de Duve, 1969). The phospholipids were deacylated to yield phosphodiester, but these were not hydrolysed further by the lysosomal preparations. However, these phosphodiester are broken down in liver at alkaline pH values by phosphodiesterase action (Dawson, 1956). After cell fractionation about half of the glycerylphosphorylcholine phosphodiesterase activity was found in the soluble fraction, with the remainder in the nuclear and microsomal fractions. Fowler & de Duve (1969) suggested that this distribution in the particulate fractions, which is similar to that previously described for enzymes known to be constituents of the cell surface membrane (see, e.g., Coleman *et al.*, 1967), might reflect localization of glycerylphosphorylcholine in the surface membrane.

We now report the subcellular distribution of the activity in more detail, including studies made with purified surface-membrane preparations. In addition, a convenient and specific radiochemical assay for

glycerylphosphorylcholine phosphodiesterase activity is described.

Materials and Methods

Reagents

All chemicals used were of analytical grade and, with the exception of those named below, were obtained either from BDH Chemicals Ltd. (Poole, Dorset, U.K.) or from Fisons Scientific Apparatus Ltd. (Loughborough, Leics., U.K.). Glycylglycine was obtained from Hopkin and Williams Ltd. (Chadwell Heath, Essex, U.K.); bovine serum albumin (fraction V), alkaline phosphatase (type I, from calf intestinal mucosa), AMP, NADP^+ , 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride, D-glucose 6-phosphate, thymidine 5'-monophospho-*p*-nitrophenyl ester (ammonium salt), phenolphthalein mono- β -glucuronic acid (sodium salt) and tris (Sigma 7-9) were from Sigma Chemical Co. Ltd. (London S.W.6, U.K.); carrier-free [^{32}P]P_i, type PBSI, from The Radiochemical Centre (Amersham, Bucks., U.K.) and Bio-Rad AG1

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(X10; 200–400 mesh) were from Micro-Bio Laboratories Ltd. (London W.11, U.K.).

Preparation of phosphatidylcholine and glycerylphosphorylcholine

Unlabelled phosphatidylcholine was isolated from the ethanol-soluble lipids of egg yolk or rat liver by chromatography on alumina and silicic acid (Ansell & Hawthorne, 1963). [^{32}P]Phosphatidylcholine was isolated similarly from the lipids of tissues (liver, kidneys, lungs, heart, spleen and skeletal muscle) taken from a rat (about 200 g) injected 18 h previously with 1–2 mCi of carrier-free $^{32}\text{P}_i$. The phosphatidylcholine was hydrolysed and glycerylphosphorylcholine isolated as described by Brockerhoff & Yurkowski (1965).

Subcellular fractionation

Animals. Male Wistar rats (6–8 weeks old) were used for all experiments.

Primary fractionation of liver. This was carried out on unperfused livers from exsanguinated rats by the method of Sedgwick & Hübscher (1965), based on that of de Duve *et al.* (1955), and with the omission of EDTA from the fractionation medium.

Perfusion of livers and preparation of plasma-membrane fractions. Animals were anaesthetized with ether and the abdomen and front of the rib cage were opened. As much blood as possible was withdrawn by cardiac puncture via the right ventricle. The right atrium was then opened and the animal perfused with 60–100 ml of 0.3 M-sucrose (pH 7.4) via a needle inserted through the wall of the left ventricle. At intervals during the perfusion the liver, which became engorged with the perfusion medium, was gently compressed. The liver was then removed from the animal and the perfusion continued via the hepatic vein until the tissue was a yellowish buff colour.

Plasma membranes were isolated by the method of Coleman *et al.* (1967). The principle of this method is first to homogenize the tissue gently (loose pestle, rotated slowly) and to isolate material in low gravitational fields, and then to homogenize this material vigorously (tight pestle, rotated rapidly) and to separate the resulting fragments by differential and density-gradient centrifugation. In the original report (Coleman *et al.*, 1967) the clearances of the homogenizers were quoted in error as radial clearances, whereas they were in fact differences in diameter between pestle and tube. The principle of the method is still valid, however, and further experience has suggested that the exact clearance and speeds of rotation may in fact be varied slightly without adverse effects. Recent membrane preparations in this laboratory have been of considerably greater purity

(5'-nucleotidase relative specific activities of 30–60; see, e.g., Fig. 5) than those originally reported and also have been of higher purity than many other preparations previously described from other laboratories.

As a routine a plasma-membrane fraction (the upper loose layer of pellet P_4) was obtained from the perfused livers by this method but without the final step in which the material is purified by density-gradient centrifugation. This fraction, which contains membrane fragments released from the crude nuclear fraction during vigorous homogenization, is referred to as partially purified plasma-membrane fraction; when the final discontinuous sucrose density-gradient stage of the preparation was included the product ($\rho < 1.13$) is referred to as a purified plasma-membrane fraction.

Storage of fractions. All fractions were suspended in 0.3 M-sucrose and stored at -20°C .

Chemical methods

Protein. In early work this was determined by a biuret method, modified to avoid interference by sucrose and by lipids (Hübscher *et al.*, 1965); for later experiments the method of Lowry *et al.* (1951), as modified by Price (1965), was adopted, with bovine serum albumin (fraction V) as standard.

Phosphate. P_i was measured by the method of Fiske & SubbaRow (1925), and total phosphate by the method of King (1932).

DNA. This was assayed as described by Michell *et al.* (1970), with purified calf thymus DNA as standard.

Enzyme assays

All assays gave rates of reaction that were constant throughout the specified periods of incubation. Enzyme activities were proportional to the protein concentrations used within the specified ranges, and were independent of small changes in substrate concentration.

5'-Nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5). This was measured by the method of Michell & Hawthorne (1965).

Succinate dehydrogenase [as succinate-2-(p-iodophenyl) - 3 - (p - nitrophenyl) - 5 - phenyltetrazolium chloride oxidoreductase, EC 1.3.99.1]. This was measured by the method of Pennington (1961), modified by the inclusion of 2 mM-EDTA in the assay system (Porteous & Clark, 1965) and by the addition of 0.1 vol. of methanol to the ethyl acetate extract (Michell *et al.*, 1970). Incubation was carried out for 15 min.

Glucose 6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9). This was determined by the method of Hübscher & West (1965), or by that of Shephard & Hübscher (1969).

6-Phosphogluconate dehydrogenase (decarboxylating) [6-phospho-D-gluconate-NADP oxidoreductase (decarboxylating), EC 1.1.1.44]. This was measured by the method of Glock & McLean (1953), the rate of NADPH production being measured on a Beckman DB-GT double-beam recording spectrophotometer.

β -Glucuronidase (β -D-glucuronide glucuronohydrolase, EC 3.2.1.31). This was measured by the method of Gianetto & de Duve (1955), and release of phenolphthalein was calculated by using $E_{550}^{1\text{cm}} 2.66 \times 10^4$ (Talalay *et al.*, 1946).

Alkaline phosphodiesterase I (orthophosphoric diester phosphohydrolase, EC 3.1.4.1). This was determined as described by Brightwell & Tappel (1968), except that the reactions were stopped by the addition of 0.1M-NaOH (Razzell, 1961) rather than trichloroacetic acid. Results were calculated by using $E_{410}^{1\text{cm}} 1.84 \times 10^4$ (Keszdy & Bender, 1962).

Glycerolphosphorylcholine phosphodiesterase (sn-glycerol-3-phosphorylcholine glycerophosphohydrolase, EC 3.1.4.2). Some of the early results were obtained by using the assay described by Fowler & de Duve (1969), but in most of the experiments described here the activity of glycerolphosphorylcholine phosphodiesterase was assayed by the measurement of the radioactive breakdown products of glyceryl[^{32}P]phosphorylcholine.

The assay system contained 0.1M-glycylglycine buffer, pH 8.5 at 20°C, 1.5mM-MgCl₂, 3.5mM-glyceryl[^{32}P]phosphorylcholine and 0.2–15mg of enzyme protein. The total volume was 1ml. After incubation for 2h at 37°C, the reaction was stopped by the addition of 4 μ mol of EDTA.

The reaction products were separated as follows. The assay system was made up to 10ml with water; approx. 1ml of 50% (w/v) suspension of Bio-Rad AG1 anion-exchange resin (X10; 200–400 mesh; formate form) was then added and the system mixed thoroughly. At this ionic strength, the products formed by diesterase hydrolysis of glycerolphosphorylcholine (3-glycerophosphate and P_i; see the Results section) are adsorbed to the resin whereas glycerolphosphorylcholine itself is not. Residual glycerolphosphorylcholine was removed by washing the resin with two 5ml portions of water. The reaction products were eluted from the resin with two 5ml portions of 0.5M-ammonium formate containing a little ethanol, combined, and the radioactivity of the eluate was measured by using a liquid Geiger-Müller counter (type M6H, Twentieth Century Electronics).

Results

pH and K_m of glycerolphosphorylcholine phosphodiesterase

The activity of a partially purified plasma membrane fraction from rat liver was determined at a

Fig. 1. pH-dependence of glycerolphosphorylcholine phosphodiesterase in partially purified liver plasma membranes

Glycylglycine (○) and glycine (□) buffers were used. Points are percentages of maximum observed activity and are means for two experiments.

series of pH values in two buffers, namely 0.1M-glycylglycine-NaOH (pH 6.5–9.0) and 0.1M-glycine-NaOH (pH 8.0–10.0). For these experiments the assay of Fowler & de Duve (1969) was used. Fig. 1 shows the results, expressed as a percentage of the maximum observed activity. The pH optimum was in the range 8.5–9.0, and the pH chosen for the assay was 8.5. A K_m for glycerolphosphorylcholine of 0.95mM was derived from a Lineweaver-Burk plot.

Characterization of reaction products

The hydrolysis products of glyceryl[^{32}P]phosphorylcholine were studied with both a partially purified plasma-membrane fraction and a particle-free supernatant fraction (prepared by centrifuging a 10%, w/v, homogenate of liver at 100000g for 60min) as sources of enzyme protein. With the plasma-membrane fraction, the products were characterized at three pH values, 7.3, 8.5 (glycylglycine buffer) and 9.25 (glycine buffer); the activity of the supernatant fraction was studied only at pH 8.5.

The assays were set up as described above; blanks containing no enzyme were also incubated, enzyme

being added to these at the end of the incubation period.

Of the possible reaction products, *sn*-glycero-3-phosphoric acid and P_i had been found to be adsorbed completely to Dowex 1 (X10; formate form) under the conditions used. Phosphorylcholine, however, was only partially retained and its recovery was checked by the use of an unlabelled internal standard. Glycerylphosphorylcholine itself was not adsorbed.

After incubation, each assay mixture was diluted to 50ml with water, and $5\mu\text{mol}$ of phosphorylcholine chloride (Ca^{2+} salt) was added. The diluted sample was then applied to a resin column (Bio-Rad AG 1: X10; 200–400 mesh; formate form; $6\text{cm} \times 1\text{cm}$) and the eluates from loading the column and from washing it with 20ml of water were collected ('glycerylphosphorylcholine fraction'). The column was then eluted with 20ml of 0.05M-ammonium formate ('phosphorylcholine fraction'), followed by 20ml of 0.25M-ammonium formate ('glycerophosphate + P_i fraction').

The radioactivity of a 10ml portion of each fraction was determined. Total phosphate was determined for the phosphorylcholine fraction, to calculate the recovery of phosphorylcholine. To an 8ml portion of the glycerophosphate + P_i fraction 10ml of 2-methylpropan-1-ol – benzene (1:1, v/v) and 2ml of ammonium molybdate (5%, w/v, in 2M- H_2SO_4) were added and the mixture was shaken. This procedure, which is based on the method of Martin & Doty (1949), gives a biphasic system with P_i in the upper (organic) and glycerophosphate in the lower (aqueous) phase. The radioactivity of the separated phases was then determined. Table 1 shows that under all conditions studied glycerophosphate was the main product, with no appreciable phosphorylcholine.

Confirmation of the identity of the major radioactive product as *sn*-glycero-3-phosphoric acid was obtained by ion-exchange chromatography (Galliard, 1963). The products from the hydrolysis of glycerylphosphorylcholine by a crude plasma-membrane

fraction were mixed with *sn*-glycero-3-phosphoric acid and P_i (1mg of P of each), and applied to a column ($0.9\text{cm} \times 17\text{cm}$) of Bio-Rad AG 1 (X10; 200–400 mesh; formate form). The column was washed with 50ml of water and then eluted with a gradient of formic acid, by using a constant-volume mixing chamber (70ml) initially containing 0.5M-formic acid and a reservoir of 4M-formic acid. Fractions (5ml) were collected and analysed for radioactivity, P_i and organic (total minus inorganic) phosphate. Approximately two-thirds of the radioactivity was recovered in a peak that co-chromatographed with *sn*-glycerol-3-phosphoric acid. The remaining radioactivity behaved as P_i (Fig. 2). The presence of appreciable P_i in the products of glycerylphosphorylcholine hydrolysis by plasma membranes, but not by the soluble fraction, is probably due to the hydrolysis of *sn*-glycerol-3-phosphoric acid either by alkaline phosphatase, which has been reported in liver plasma-membrane preparations (House & Wiedemann, 1970; Börning *et al.*, 1967; Ray, 1970), or by lysosomal acid phosphatase, which is present to a small extent in the partially purified plasma-membrane fraction.

Inhibition by EDTA and reactivation by cations

In previous assays of glycerylphosphorylcholine phosphodiesterase, 1.5mM- MgCl_2 was included (e.g. Fowler & de Duve, 1969). The requirement for Mg^{2+} was checked by using a partially purified plasma-membrane fraction; no change in activity was detected at Mg^{2+} concentrations of 0–3.0mM.

The effect of EDTA on the enzyme activity was studied: 1mM-EDTA produced 99.5% inhibition when included in assays from which external Mg^{2+} had been omitted. Ethanedioxybis(ethylamine)tetraacetic acid, a specific chelator for Ca^{2+} that does not bind Mg^{2+} at physiological pH values, also gave essentially complete inhibition at this concentration.

An attempt was therefore made to reactivate the EDTA-inactivated enzyme by the addition of a range

Table 1. *Products of glycerylphosphorylcholine hydrolysis*

Details are given in the text.

Fraction	pH of incubation mixture	Glycerylphosphorylcholine hydrolysis ($\mu\text{mol}/\text{mg}$ of protein)	Products (mol/100mol)		
			Choline phosphate	Glycero-phosphate	P_i
Partially purified plasma membrane	7.3	0.141	1.0	67.5	31.6
	8.5	0.37	0.4	79.8	19.7
	9.25	0.293	0.3	76.8	23.0
		($\mu\text{mol}/\text{ml}$ of particle-free supernatant)			
Particle-free supernatant	8.5	3.21	0.5	97.8	1.7

Fig. 2. Anion-exchange chromatography (AG1; X10; formate form) of the products of glycerylphosphorylcholine hydrolysis by partially purified plasma membranes

Radioactivity (○) and internal standards of α -glycerophosphate (■) and P_i (□). The broken curve denotes the formic acid concentration. Further details are given in the text.

of cations. The membranes were incubated for 15 min at room temperature in 0.1 M-glycylglycine buffer, pH 8.5, with 1 mM-EDTA; the appropriate cation was then added (to a final concentration of 5 mM), followed by 3.5 mM-glycerylphosphorylcholine, and incubation continued at 37°C for 2 h. The observed reactivations, expressed as a percentage of the activity of the enzyme in the absence of both EDTA and Mg^{2+} , ranged from 69 to 108% (Table 2).

Distribution of glycerylphosphorylcholine phosphodiesterase activity in primary subcellular fractions from liver

The activities of glycerylphosphorylcholine phosphodiesterase and of known markers for several subcellular components were studied in the primary fractions obtained from liver. The markers chosen were: DNA (nuclei), 5'-nucleotidase and alkaline phosphodiesterase I (plasma membranes), succinate dehydrogenase (mitochondria), β -glucuronidase (lysosomes), glucose 6-phosphatase (endoplasmic reticulum) and 6-phosphogluconate dehydrogenase (cytosol) (Coleman *et al.*, 1967; Erecińska *et al.*, 1969).

Table 3 shows the distributions of glycerylphosphorylcholine phosphodiesterase and of the

Table 2. Dependence of glycerylphosphorylcholine phosphodiesterase activity on cations

Plasma membranes were incubated with EDTA and then with the specified ions. Activities are percentages of the activity in the absence of EDTA. See the text for details.

Added cation	% of activity in control
None	0.5
Ni^{2+}	108.0
Cu^{2+}	102.7
Co^{2+}	99.1
Ca^{2+}	98.0
Mg^{2+}	93.3
Fe^{3+}	88.9
Zn^{2+}	85.9
Fe^{2+}	83.7
Mn^{2+}	69.2

marker enzymes, expressed as percentages of the recovered activities, and the specific activities of the homogenate. The markers for different subcellular components showed distributions that differed clearly from each other and conformed to previous studies (Fig. 3; see, e.g., de Duve *et al.*, 1955). The

Table 3. *Primary subcellular fractionation of rat liver*

All values are given as means $\pm$ s.d. Protein content is expressed in mg/ml of homogenate, DNA in μ g/mg of protein and all enzymic activities in μ mol of product formed/h per mg of protein. The percentages are based on total recovered activity; recovery and the number of fractionations (in parentheses) are given in the final column. N.D., not detected.

	Value for homogenate	Distribution (%)				Particle-free supernatant	Recovery
		Nuclear	Mitochondrial	Lysosomal	Microsomal		
Protein	15.1 $\pm$ 2.06	14.0 $\pm$ 0.80	18.1 $\pm$ 1.60	14.3 $\pm$ 1.59	17.4 $\pm$ 1.66	36.2 $\pm$ 1.17	85.0 $\pm$ 2.56 (4)
Glycerylphosphorylcholine phosphodiesterase	0.0095 $\pm$ 0.0047	22.8 $\pm$ 3.20	3.0 $\pm$ 1.64	11.2 $\pm$ 4.38	21.2 $\pm$ 1.16	42.0 $\pm$ 2.31	104.1 $\pm$ 8.67 (4)
5'-Nucleotidase	3.04 $\pm$ 0.34	44.1 $\pm$ 6.19	4.7 $\pm$ 0.97	16.8 $\pm$ 3.34	27.3 $\pm$ 4.06	7.1 $\pm$ 5.44	91.7 $\pm$ 3.69 (4)
Alkaline phosphodiesterase I	4.21 $\pm$ 0.74	46.0 $\pm$ 5.30	3.2 $\pm$ 1.26	18.7 $\pm$ 8.02	30.2 $\pm$ 10.69	1.9 $\pm$ 3.85	99.3 $\pm$ 8.87 (4)
Glucose 6-phosphatase	3.73 $\pm$ 0.22	2.0 $\pm$ 1.86	5.7 $\pm$ 1.89	34.7 $\pm$ 4.47	57.7 $\pm$ 1.93	N.D.	79.6 $\pm$ 7.87 (4)
DNA	5.58 $\pm$ 0.84	98.0 $\pm$ 1.39	1.4 $\pm$ 0.88	0.1 $\pm$ 0.30	0.4 $\pm$ 0.49	N.D.	113.1 $\pm$ 4.39 (4)
Succinate dehydrogenase	1.15 $\pm$ 0.11	11.0 $\pm$ 1.44	72.2 $\pm$ 1.91	14.9 $\pm$ 1.71	1.9 $\pm$ 0.94	0.1 $\pm$ 0.20	86.9 $\pm$ 17.59 (4)
β -Glucuronidase	0.12 $\pm$ 0.05	4.7 $\pm$ 3.34	11.5 $\pm$ 5.92	53.5 $\pm$ 6.14	30.2 $\pm$ 3.09	N.D.	75.5 $\pm$ 22.30 (3)
6-Phosphogluconate dehydrogenase	0.45 $\pm$ 0.07	2.4 $\pm$ 0.22	0.9 $\pm$ 0.35	1.5 $\pm$ 0.45	4.8 $\pm$ 5.35	90.5 $\pm$ 4.99	145.8 $\pm$ 15.33 (4)

Fig. 3. Distribution patterns of glycerylphosphorylcholine phosphodiesterase, marker enzymes and DNA

(a) Glycerylphosphorylcholine phosphodiesterase; (b) 5'-nucleotidase; (c) alkaline phosphodiesterase I; (d) glucose 6-phosphatase; (e) DNA; (f) succinate dehydrogenase; (g) β -glucuronidase; (h) 6-phosphogluconate dehydrogenase. Fractions are represented, in the order of their isolation, from left to right: Nuclear (N); mitochondrial (Mt); lysosomal (L); microsomal (Mc); particle-free supernatant (PFS). Each fraction is represented separately on the ordinate scale by its relative specific activity (percentage of total recovered activity/percentage of total recovered protein). On the abscissa scale each fraction is represented (cumulatively from left to right) by its protein content, expressed as a percentage of the total recovered protein. The results are the means from three (β -glucuronidase) or four (all other assays) experiments.

two enzymes known to be characteristic of the plasma membrane showed a nuclear-microsomal distribution and were closely parallel; their activities in the particulate fractions had almost identical distribution patterns.

About three-fifths of the glycerylphosphorylcholine phosphodiesterase activity was in the particulate fractions, and showed a distribution pattern that was much closer to those of the plasma-membrane markers than to any other marker activity examined. The only other marker showing any similarity in distribution to these three enzymic activities was the glucose 6-phosphatase of the lysosomal and microsomal fractions. Thus these results do not exclude the possibility of a minor contribution to glycerylphosphorylcholine phosphodiesterase activity from the endoplasmic reticulum.

Fig. 4 shows a comparison of the distribution of glycerylphosphorylcholine phosphodiesterase activity with that of 5'-nucleotidase, alkaline phosphodiesterase I, glucose 6-phosphatase and β -glucuronidase in the particulate fractions. The alkaline phosphodiesterase I/5'-nucleotidase activity ratio is included, showing that these two enzymes, which are

known to occur in the same subcellular site, were recovered in a constant ratio in each of the isolated fractions. Glycerylphosphorylcholine phosphodiesterase activity increased slightly in later fractions compared with either 5'-nucleotidase or alkaline phosphodiesterase I, reflecting the slightly greater activity in the microsomal fraction. The ratios of glycerylphosphorylcholine phosphodiesterase to glucose 6-phosphatase and to β -glucuronidase, however, were different in each fraction and indicated that most of the glycerylphosphorylcholine phosphodiesterase could not be located either in lysosomes or in the endoplasmic reticulum.

Distribution of glycerylphosphorylcholine phosphodiesterase activity within a discontinuous sucrose-density gradient

A crude plasma-membrane fraction was sub-fractionated on a discontinuous sucrose-density gradient of the type described by Coleman *et al.* (1967) and the distribution of activities of glycerylphosphorylcholine phosphodiesterase, 5'-nucleotidase, alkaline phosphodiesterase I and glucose 6-phospha-

tase within the gradient was studied (Table 4). Four fractions were taken from the gradient and classified according to density ($\rho < 1.13$, $\rho > 1.13 < 1.16$, $\rho > 1.16 < 1.18$ and $\rho > 1.18$), the last being the resuspended pellet from the bottom of the gradient. The results are shown in Fig. 5, in which the enzymic activities are expressed relative to those of the total particulate activities, assuming a distribution of activity between soluble and particulate material identical with that in Table 3.

5'-Nucleotidase and alkaline phosphodiesterase I showed closely similar distributions throughout the gradient, and both showed about a fourfold enrichment of activity between the partially purified material applied to the gradient and the most active fraction from the gradient ($\rho < 1.13$; purified plasma-membrane fraction). The overall enrichment of 5'-nucleotidase activity was about two-thirds that of alkaline phosphodiesterase I. This appears to be due to the recovery of a larger proportion of the alkaline phosphodiesterase I of the nuclear pellet in the partially purified plasma-membrane fraction, rather than to a difference at a later stage in the purification procedure. The specific activity of each enzyme decreased as the density of the membranes isolated from the gradient increased.

As it seemed that some of the glycerylphosphorylcholine phosphodiesterase activity might be located in the endoplasmic reticulum, the distribution of glucose 6-phosphatase activity within the gradient was examined. Its enrichment pattern differed very markedly from the patterns for the plasma-membrane enzymes. The relative specific activities of both plasma-membrane enzymes in the partially purified plasma membrane fraction and in the combined lower layers of pellet P₄ differed by about 11-fold. For glucose 6-phosphatase these fractions had almost identical specific activities, and the distribution of specific activities within the gradient showed an exactly reversed pattern compared with that observed for the plasma-membrane enzymes. The overall enrichment in the most active ($\rho > 1.18$) fraction from the gradient was less than fourfold, which is one-tenth of the overall enrichment of 5'-nucleotidase and one-fifteenth of that of alkaline phosphodiesterase I.

Glycerylphosphorylcholine phosphodiesterase behaved similarly to the plasma-membrane enzymes but not to glucose 6-phosphatase. There were, however, some differences, the chief of which were: (i) a 2.7-fold enrichment of activity from partially purified to the purified ($\rho < 1.13$) plasma membrane fraction, as opposed to a fourfold increase for 5'-nucleotidase and alkaline phosphodiesterase I; (ii) a rather higher proportion of the activity in the more dense membrane fractions isolated from the gradient. The possible significance of these differences is discussed below.

Fig. 4. Ratios of enzyme activities in the particulate fractions

Experimental details are given in the text. 5'-Nucleotidase/alkaline phosphodiesterase I (●), glycerylphosphorylcholine phosphodiesterase/5'-nucleotidase (▲), glycerylphosphorylcholine phosphodiesterase/alkaline phosphodiesterase I (■), glycerylphosphorylcholine phosphodiesterase/glucose 6-phosphatase (△) and glycerylphosphorylcholine phosphodiesterase/β-glucuronidase (○) ratios were derived from the percentages of the total recovered particulate activity present in the fractions being considered. The points for the mitochondrial fraction may be somewhat inaccurate, owing to the small proportion of the surface membrane that is isolated in this fraction. Abbreviations are as described for Fig. 3.

Table 4. *Behaviour of glycerylphosphorylcholine phosphodiesterase and other enzymes during isolation of plasma membranes*

Results, except for the recoveries, are given as percentages of the total activity of the original homogenate, and are the means of two experiments.

	Protein	Glycerylphosphorylcholine phosphodiesterase	5'-Nucleotidase	Alkaline phosphodiesterase I	Glucose 6-phosphatase
Homogenate	(100)	(100)	(100)	(100)	(100)
Nuclear fraction	14.1	16.7	34.9	34.0	17.9
Fractions derived from nuclear fraction					
P ₄ (upper)	2.23	10.0	23.8	30.3	6.31
P ₄ (lower)	2.02	0.54	1.81	2.6	4.66
Subfractions from density-gradient separation of fraction P ₄ (upper)					
$\rho < 1.13$	0.196	1.58	7.67	11.3	0.26
$\rho > 1.13 < 1.16$	0.23	1.33	3.90	6.28	0.45
$\rho > 1.16 < 1.18$	0.125	0.44	1.16	1.76	0.31
$\rho > 1.18$	1.25	4.85	8.69	10.2	4.43
Recovery of activity from density-gradient separation of fraction P ₄ (upper)					
	83.3%	81.9%	89.5%	97.6%	91.1%

Discussion

Assay of glycerylphosphorylcholine phosphodiesterase activity

The new assay method developed in the present studies has major advantages over the linked glycerylphosphorylcholine phosphodiesterase-alkaline phosphomonoesterase assay described by Fowler & de Duve (1969). Their assay had the convenience of being a simple measurement of P_i release. In some assays, however, particularly with lysosomal fractions and with the relatively highly diluted particle-free supernatants, the very high blank values (sometimes more than 80% of the P_i release in the complete assay), combined with the very low absolute activity of glycerylphosphorylcholine phosphodiesterase, made accurate assays extremely difficult. A more specific assay of choline release was used by several other workers. We have used the radiochemical assay at about the same sensitivity as is possible with the assay of choline release, but if necessary the sensitivity of the radiochemical method can easily be increased by raising the specific radioactivity of the radioactive glycerylphosphorylcholine used as substrate.

Products of glycerylphosphorylcholine hydrolysis by plasma-membrane and soluble fractions

sn-Glycero-3-phosphoric acid and choline have been identified previously as the principal products of the enzymic hydrolysis of glycerylphosphoryl-

choline by preparations from liver (Dawson, 1956), brain (Webster *et al.*, 1957) and *Serratia plymuthicum* (Hayaishi & Kornberg, 1954; Prasad & Benson, 1969). However, the multiple localization of this enzyme in liver subcellular fractions (Fowler & de Duve, 1969) raised the possibility that there might be two distinct species, one in particulate form and one in the cytosol. The assay used by Fowler & de Duve (1969) would not have distinguished which phosphodiester bond was attacked, and we therefore separately characterized the products of glycerylphosphorylcholine hydrolysis by partially purified plasma membrane and by a particle-free supernatant. In both cases the bond split was that between choline and the phosphate group (Table 1).

Properties of glycerylphosphorylcholine phosphodiesterase in partially purified plasma membranes

Our results were in general agreement with those of other workers with regard to pH optimum, *K_m* and inhibition by EDTA. pH optima previously reported for liver are 7.5 (Dawson, 1956) and 8.5 (Fowler & de Duve, 1969). The *K_m* for glycerylphosphorylcholine calculated from the results of Dawson (1956) was approx. 1 mM, which agrees fairly closely with our result.

Unlike Dawson (1956), who found an appreciable increase in activity on the addition of 1 mM-Mg²⁺ to a soluble enzyme preparation from acetone-treated rat liver, we found no change in activity in the presence of 0–3.0 mM-Mg²⁺.

Fig. 5. *Relative specific activities of particle-bound enzymes at key stages during purification of liver plasma membranes*

Relative specific activities are expressed relative to the original homogenate (5'-nucleotidase, alkaline phosphodiesterase I and glucose 6-phosphatase), or relative to 58% of the activity (the particulate activity; Table 3) of the original homogenate (glycerylphosphorylcholine phosphodiesterase). Fractions are designated: N, nuclear; P₄ (upper), partially purified plasma membrane; P₄ (lower), lower layers of pellet P₄ in the procedure of Coleman *et al.* (1967). Layers from the sucrose density gradient: $\rho < 1.13$ ($\longrightarrow \circ$); $\rho > 1.13 < 1.16$ ($\longrightarrow \blacksquare$); $\rho > 1.16 < 1.18$ ($\dashrightarrow \bullet$); $\rho > 1.18$ ($\cdots \longrightarrow \blacktriangle$).

Virtually complete inhibition of the enzyme by EDTA has, however, now been reported in liver (Dawson, 1956, and results in the present paper), brain (Webster *et al.*, 1957) and *Serratia plymuthicum* (Hayaishi & Kornberg, 1954). Complete reactivation by added metal ions has not previously been reported, although some reactivation was reported by Webster *et al.* (1957). None was achieved with *Serratia* (Hayaishi & Kornberg, 1954). We have observed almost complete reactivation with several different ions (Table 1), but from our studies with cations and with EDTA and ethanedioxybis(ethylamine)tetra-acetic acid it is not clear which cation is physiologically important.

Are glycerylphosphorylcholine phosphodiesterase (particulate) and alkaline phosphodiesterase I the same?

Fowler & de Duve (1969) suggested that these two activities might be properties of the same enzyme. This now seems unlikely, because in our experiments they were not distributed identically either during primary subcellular fractionation or during isolation of plasma membrane (see below).

Subcellular distribution

Particulate and soluble activity. Early studies of glycerylphosphorylcholine phosphodiesterase activity in preparations from various sources gave no information on its intracellular localization. The only previous results of this type were reported by Fowler & de Duve (1969), who found that in liver one-third to one-half of this activity was particulate, with the remainder in the soluble fraction (calculated from their Figs. 7 and 8). This general pattern has been confirmed, except that we have found a smaller (42%) and more constant proportion of the total activity in the particle-free supernatant fractions: this may be due to the more accurate measurements made possible by the radiochemical assay procedure.

The livers from which the primary fractions were obtained were from exsanguinated rats, but were not perfused. Approximately one-tenth of the protein of homogenate from such material is derived from blood (Castanga, 1965). We therefore assayed samples of rat blood for glycerylphosphorylcholine phosphodiesterase activity and found very little, only sufficient

to account for less than 1 % of the total activity of our homogenates. Thus both the particulate and soluble activities seem to be intrinsic to liver tissue, rather than being derived from contaminating blood.

Activity in the particulate material from the primary fractionation. Fowler & de Duve (1969) found that particulate glycerylphosphorylcholine phosphodiesterase was mainly located in the nuclear and microsomal fractions from liver and suggested that this might be due to localization in plasma membrane. They did not, however, study the distribution of known plasma membrane enzymes in their fractions. We have confirmed their observations and also find close similarity between the distribution of glycerylphosphorylcholine phosphodiesterase and of 5'-nucleotidase and alkaline phosphodiesterase I (Figs. 3 and 4), both of which are localized largely in the plasma membranes of liver cells (Coleman *et al.*, 1967; Erecińska *et al.*, 1969; Touster *et al.*, 1970).

Glycerylphosphorylcholine phosphodiesterase, plasma-membrane and endoplasmic-reticulum activity during isolation of a purified plasma-membrane fraction. More direct confirmation of the localization of glycerylphosphorylcholine phosphodiesterase in the plasma membranes comes from the similar distribution of its activity and that of 5'-nucleotidase and alkaline phosphodiesterase I throughout the preparation of purified plasma membranes (Fig. 5). These three activities showed distribution patterns very different from that of glucose 6-phosphatase.

Differences between distributions of glycerylphosphorylcholine phosphodiesterase, alkaline phosphodiesterase I and 5'-nucleotidase. The distributions of these enzymes throughout our fractionations were similar, and clearly differed from those of all the other marker enzymes that were assayed. However, glycerylphosphorylcholine phosphodiesterase was slightly more concentrated in microsomal fractions and less concentrated in nuclear fractions than were the other two activities (Figs. 3 and 4). In addition, the extent to which each of the three enzymes became purified during isolation of plasma membranes was not identical: there was an approximately threefold lower final purification of particulate glycerylphosphorylcholine phosphodiesterase than of alkaline phosphodiesterase I, with 5'-nucleotidase showing an intermediate pattern (Fig. 5).

Alternative explanations for these differences include: (i) the three activities are not equally distributed in the different types of cell present in liver; (ii) the activities may be present on surface and cytoplasmic membranes, which are of two or more different types and have only slightly different physical properties; (iii) the activities are all properties of the liver cell plasma membrane, but are not distributed in exactly the same way over the functional areas on different faces of the cells.

Although our information does not fully exclude any of these explanations the last-named seems likely for several reasons. First, after the initial gentle homogenization of the tissue, large sheets of membrane sedimented in the nuclear fraction and the small fragments were recovered in the microsomal fractions. Under these conditions there were only small differences in the distributions of the three activities. The larger differences were not observed until the membrane sheets had been disrupted by vigorous homogenization: the resulting fragments were then separated into enzymically distinct classes. The result is analogous to that of Evans (1970), who found that purified liver plasma could be disrupted and that the fragments separated similarly. Two fractions equivalent to those reported by Evans (1970) have also been reported by House & Wiedemann (1970), who found a further, but less well characterized, fraction that they considered was also produced during fragmentation of the plasma membrane. Secondly, if the three enzymes were distributed on three distinct membranes, it is unlikely that they would show such similar separation characteristics throughout an isolation procedure that includes a step involving fragmentation of the membranes. Finally, 5'-nucleotidase and alkaline phosphodiesterase I are both activities for which there is strong previous evidence for localization in the plasma membrane (see, e.g., Coleman *et al.*, 1967; Touster *et al.*, 1970).

Contribution of glycerylphosphorylcholine phosphodiesterase(s) to turnover of liver phospholipids

Turnover studies on the glycerophosphate backbone of liver phospholipids in which [^{14}C]glycerol was used as the precursor indicate turnover of approx. 1.5 % of the tissue phospholipids/h (Siekevitz *et al.*, 1967). Approximate rates of phospholipid breakdown by rat liver lysosomes can be calculated from the initial rates of hydrolysis reported by Fowler & de Duve (1969). Such calculations suggest that in 1 h the lysosomal enzymes could deacylate at least 5–10 % of the tissue phospholipids.

When phospholipids have been deacylated the released phosphodiesterases can be further degraded by specific phosphodiesterase(s) studied both previously (Dawson, 1956; Fowler & de Duve, 1969) and in the present paper. The rate of this breakdown is approx. 1.2 $\mu\text{mol/h}$ per g of fresh tissue (Table 3), which is equivalent to breakdown of phosphodiesterases derived from about 3 % of the tissue phospholipids/h.

From these rough calculations it seems that the activities of the lysosomal phospholipases and of the glycerylphosphocholine phosphodiesterase(s) are probably sufficient to account for the observed turnover of liver phospholipids, especially when it is remembered that the reported turnover rates must

be somewhat overestimated owing to the export in plasma and bile of phospholipids from liver.

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