Biochem. J. (1973) **133**, 189–199
Printed in Great Britain

Properties of a 5′-Nucleotidase Purified from Mouse Liver Plasma Membranes

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(*Received 5 December* 1972)

1. Extraction of a mouse liver plasma-membrane fraction with a detergent buffer, *N*-dodecylsarcosinate–Tris buffer (sarcosyl–Tris buffer), solubilized 90% of the protein and 70% of the 5′-nucleotidase activity. 2. The proteins of the sarcosyl–Tris buffer extract were fractionated by a rate-zonal centrifugation in a sucrose–detergent gradient. The major protein peak sedimented ahead of phospholipids, which mainly remained in the overlay. Glycoproteins were separated ahead of the protein peak. 3. The 5′-nucleotidase activity peak was associated with 5% of the protein applied to the gradient, and contained relatively few protein bands. 4. The 5′-nucleotidase was purified further by gel filtration on Sepharose and Sephadex columns equilibrated with sarcosyl–Tris buffer, to give a single glycoprotein band on sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. The purified enzyme was lipid-free. 5. Electrophoresis in polyacrylamide gels in sarcosyl–Tris buffers showed that the enzymic activity was coincident with the protein band. 6. The molecular weight suggested for the enzyme activity by gel filtration or centrifugation in sucrose gradients was 140000–150000. Sometimes, a minor enzyme peak of lower molecular weight was obtained. 7. Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate indicated that as the polyacrylamide concentration was increased from 5 to 15%, the apparent molecular weight of the enzyme decreased from 130000 to 90000. 8. The evidence that 5′-nucleotidase is composed of two active and similar, if not identical, glycoprotein subunits and the role of detergent in effecting the separation of membrane proteins and glycoproteins are discussed. 9. Substrate requirements, pH optima and the nature of inhibition by an analogue of adenosine diphosphate are reported.

In most eukaryotic cells the enzyme 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) is located primarily in the plasma membranes, and on this basis is frequently used as a marker enzyme in cell-surface isolation studies. Some properties of the enzyme bound to liver membranes have been described (Song & Bodansky, 1966, 1967; Song *et al.*, 1968), and a 5′-nucleotidase partially purified from rat liver plasma membranes and a microsomal fraction by using a detergent mixture was shown to be a lipoprotein (Widnell & Unkeless, 1968). Although the 5′-nucleotidase and other alkaline phosphatase activities of liver and serum are used clinically to diagnose hepato-biliary malfunctioning (Sherlock, 1968), the physiological function of the enzyme on the cell surface is not known.

In the present study, a 5′-nucleotidase from mouse liver plasma membranes was solubilized by extraction with a *N*-dodecylsarcosinate–Tris (sarcosyl–Tris) medium (Evans & Gurd, 1972). Rate-zonal centrifugation of the detergent extract in a shallow sucrose–detergent gradient separated membrane glycoproteins, including the 5′-nucleotidase activity, from other proteins. The 5′-nucleotidase activity was purified further by gel filtration in sarcosyl–Tris buffer to give a single band on polyacrylamide-gel electrophoresis. The purified enzyme, a glycoprotein, was shown to be free of detectable amounts of lipid. Properties of the purified enzyme are described. A preliminary report has appeared (Gurd *et al.*, 1971).

Experimental

Methods

Preparation of membranes and extraction of 5′-nucleotidase. Plasma membranes were prepared from mouse liver homogenates by rate-zonal centrifugation in an MSE AXII rotor (Evans, 1970). The membranes were purified by centrifuging on to a 49% (w/v) sucrose 'cushion' (Gurd *et al.*, 1972*a*,*b*). In a typical enzyme preparation plasma membranes (100mg of protein) were stored at 4°C overnight as a pad and resuspended in 35ml of 4% (w/v) sarcosyl–Tris medium at 4°C by using a tight-fitting Dounce homogenizer (radial clearance 0.076mm). All sarcosyl–Tris solutions were prepared by dissolving 1g of *N*-dodecylsarcosinate and 0.545g of Tris base in water to give pH7.8. The partially clarified membrane suspension was centrifuged at 95000$g_{av.}$ for 40min and the supernatant collected. The pellet was then resuspended in 8ml of 3% (w/v) sarcosyl–Tris medium by using a Dounce homogenizer, and centrifuged at 140000*g* for 30min, and the supernatant collected. Pooled supernatants (43ml) were usually

used immediately for enzyme purification although in some cases the supernatant was kept at −20°C for up to 1 month. When samples were thawed, they were sometimes cloudy and were then recentrifuged at 100000*g* for 30min to give a clear solution for zonal centrifugation. The 5′-nucleotidase activity was present in the supernatant.

Enzyme purification. The sarcosyl–Tris extract, refractive index 1.342 (at 4°C), was introduced into the centre vein of an MSE BXIV Ti rotor containing a linear gradient made by mixing 200ml of 10% (w/v) and 200ml of 30% (w/v) sucrose (MSE automatic gradient former). Sucrose solutions were dissolved in 0.25% sarcosyl–Tris medium, except where stated. The rotor was filled from the outer edge with 225ml of 30% (w/v) sucrose, which was then displaced from the inner edge by the sarcosyl–Tris-medium extract of the plasma membranes (43ml) followed by a 180ml overlay of 0.25% sarcosyl–Tris medium. After centrifugation at 43000rev./min for 21–24h in an MSE 65 centrifuge the contents were unloaded by pumping 60% (w/v) sucrose into the outer edge of the rotor, and three 50ml fractions followed by 25ml fractions were collected manually. The refractive indices and other chemical and enzymic properties of the fraction were determined as described below.

Fractions containing the 5′-nucleotidase activity were pooled (volume 75–100ml) and concentrated to 6–8ml after dialysis against water by inserting the dialysis bag in Aquacide 11 (Calbiochem, London W.1, U.K.). In later experiments pressure filtration in an Amicon Ultrafiltration cell containing XM-50 filters was used. Pressure filtration was more rapid than Aquacide and the latter also imparted a coloured low-molecular-weight contaminant to the enzyme, which was later removed by gel filtration. Final purification of the enzyme activity was obtained by gel filtration through columns of Sepharose 6B followed by Sephadex G-100 or G-150 equilibrated with 0.25% sarcosyl–Tris medium. Peaks of 5′-nucleotidase activity were concentrated by using Aquacide or pressure filtration and stored at −20°C. Enzymic activity was stable for days at room temperature and −20°C.

Chemical and enzymic determination. Protein was determined against appropriate blanks containing Tris buffer. In column eluates E_{280} was used as a measure of protein concentration. 5′-Nucleotidase was determined either by measuring liberated P (Michell & Hawthorne, 1965) or spectrophotometrically in the presence of an excess of adenosine deaminase by coupling the reaction of 5′-nucleotidase to the deamination of adenosine as described by Ipata (1967, 1968). Phosphodiesterase 1 was determined spectrophotometrically as described by Razzel (1963). Refractive indices were measured on a Hilger and Watts refractometer. Phospholipid phosphorus contents of chloroform–methanol extracts were determined as previously described (Gurd *et al.*, 1972*a*). Membrane phospholipids were labelled by injection of mice with 1mCi of $Na_3{}^{32}PO_4$ and radioactivity was determined by scintillation spectrometry (Evans & Gurd, 1972).

Polyacrylamide-gel electrophoresis. Samples were heated in 4M-urea–1% sodium dodecyl sulphate–1% mercaptoethanol solution at 90°C for 3–5min. Polyacrylamide gels [5, 7.5, 10, 12.5 and 15% (w/v) polyacrylamide] were prepared as described by Weber & Osborn (1969) and electrophoresis was carried out at 4mA/tube for 16h in gels 10cm long × 4mm diam. For accurate determination of molecular weights, the gel length and the distance migrated by the dye front were measured before cutting the gel longitudinally and staining separate lengths for protein with Amido Black and for carbohydrate by the periodate–Schiff procedure (Zacharias *et al.*, 1969; Evans & Gurd, 1972). Standard proteins (see Fig. 5 and Plate 2) were run and stained under the same conditions. Purified enzyme samples were also run on polyacrylamide gels in sarcosyl–Tris medium to correlate enzyme activity with the stained protein band. Fractions containing enzyme activity in 0.25% sarcosyl–Tris buffer (0.1–0.2ml) were added to Bromophenol Blue (5mg/ml) dissolved in 50% (v/v) glycerol and layered on 7.5% (w/v) polyacrylamide gels in 30mM-Tris–30mM-glycine–0.25% sarcosyl adjusted to pH7.4 with HCl. The electrolysis buffer, containing 5mM-Tris–5mM-glycine–0.25% sarcosyl, was adjusted to pH7.4. Electrophoresis was carried out at 1mA/tube for 3h, and the tubes were kept cool by circulating water. Gels were frozen at −70°C and cut longitudinally. One half was stained for protein as described above and the other half cut transversely into 1–2mm sections on a bed of equally spaced razor blades. Gel portions were dispersed in media used for the 5′-nucleotidase assay during passage into test-tubes by using a thin needle and syringe.

Materials

These were generally obtained from either British Drug Houses, Poole, Dorset, U.K. or Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K. and were of the highest purity available. *N*-Dodecylsarcosinate was obtained as a gift from Geigy Ltd., Simonsway, Manchester, U.K., as Sarkosyl-L. Glass-distilled water was used throughout. $\alpha\beta$-Methylene-ADP was obtained from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A. Proteins used as molecular-weight-calibration markers were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. Immunoglobin G (porcine) was a gift from Dr. M. J. Crumpton of this Institute. Reoviral proteins, known to be free of carbohydrate (Smith *et al.*, 1969), were obtained from Dr. J. Skehel of this Institute.

Results

Rate-zonal separation

Extraction of mouse liver plasma membranes with sarcosyl–Tris buffer under the specified conditions gave a soluble fraction that contained approx. 70% of the 5'-nucleotidase activity of the intact membranes, 80–90% of the membrane protein, and about 60% of the membrane phospholipids. Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate buffers showed that the majority of the plasma-membrane protein and glycoprotein constituents were extracted by sarcosyl–Tris medium and t.l.c. of chloroform–methanol extracts indicated the presence of a similar phospholipid and neutral-lipid composition to that of plasma membranes (Evans & Gurd, 1972).

The feasibility of separating the 5'-nucleotidase activity present in sarcosyl–Tris-medium extract from the bulk of the membrane proteins was first investigated by using shallow sucrose gradients dissolved in 0.8% sarcosyl–Tris medium in a swing-out rotor. The enzyme activity sedimented ahead of the major protein peak and marker proteins run concurrently under the same conditions indicated a sedimentation value for the enzyme activity similar to that of immunoglobulin G, whereas the major protein peak migrated slowly into the gradient with a sedimentation value less than that of bovine serum albumin. For larger-scale separations with extracts of up to 100mg of plasma-membrane protein the BXIV zonal rotor was used. A similar separation of the 5'-nucleotidase from the bulk of the membrane protein was obtained, resulting in a fourfold purification of the enzyme, which was associated with 3–5% of the total protein applied (Fig. 1, Table 1).

Distribution of phospholipids

An examination of a possible association of lipids and 5'-nucleotidase activity was prompted by the report of Widnell & Unkeless (1968), who showed

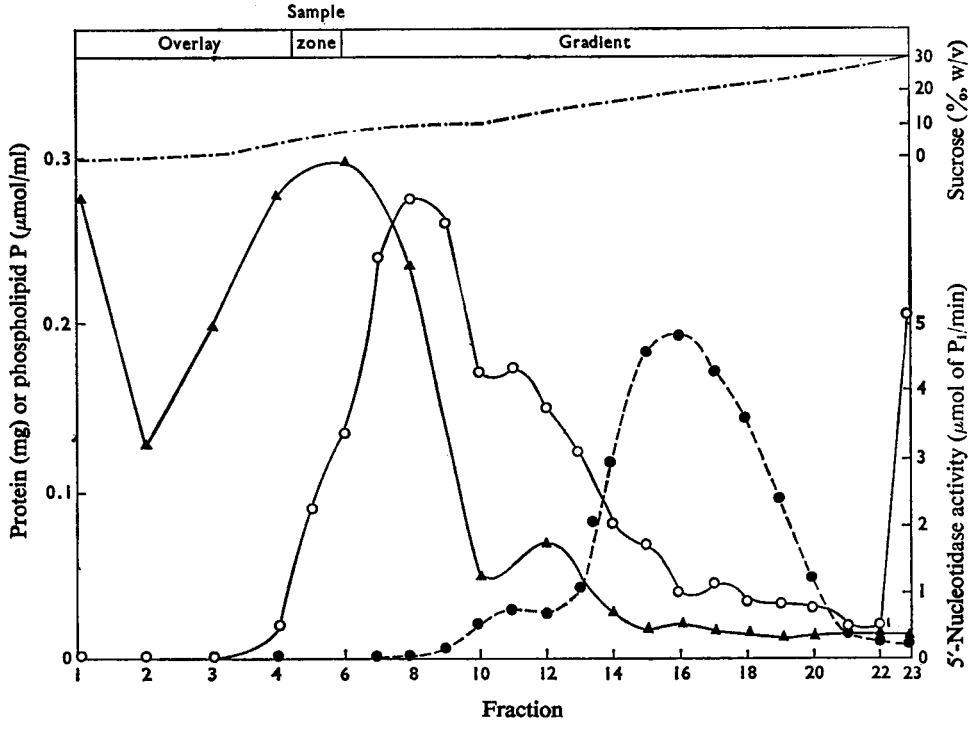


Fig. 1. *Rate-zonal centrifugation of sarcosyl–Tris-medium extract*

Centrifugation at 43000rev./min for 24h of a sarcosyl–Tris-medium extract of approx. 100mg of plasma membranes into a sucrose–detergent gradient contained in an MSE BXIV zonal rotor. Details of the gradient construction, and determination of protein (○), phospholipid (▲), 5'-nucleotidase (●) and amount of sucrose (–·–) are reported in the Experimental section.

Table 1. *Purification of 5′-nucleotidase from mouse liver plasma membranes*

For details see the text.

Fraction	Protein recovered (mg)	Enzyme activity recovered (units)	Specific activity (μmol of substrate liberated/h per mg of protein)	Yield (%)	Purification ratio
Plasma membranes	88	1443	16.4	100	
Sarcosyl–Tris-medium extract	81	1037	12.8	72	0.8
Enzyme peak from BXIV zonal rotor	4.2	223	53	15.5	3.2
Sepharose 6B peak	0.71	157	221	10.9	13.5
Sephadex G-100	0.22	64	290	4.5	17.8

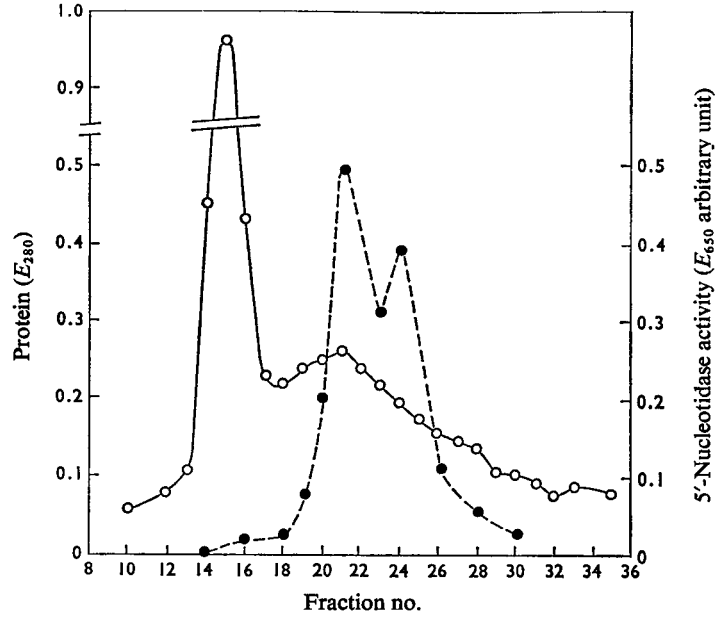
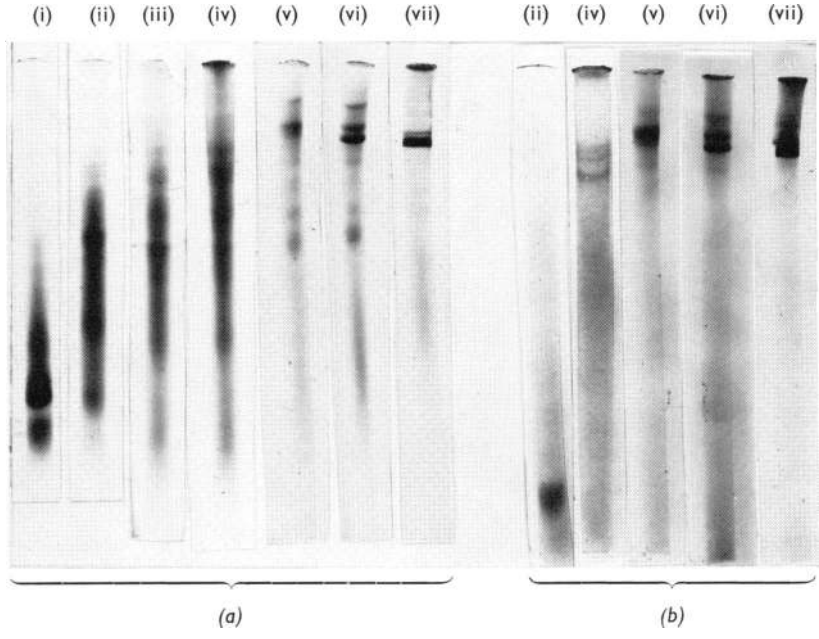


Fig. 2. *Gel filtration of enzyme purified from a zonal rotor*

Gel filtration on columns (100cm×1.6cm) of Sepharose 6B equilibrated with 0.25% sarcosyl–Tris buffer of the 5′-nucleotidase peak concentrated by Diaflow filtration from the BXIV zonal rotor. ○, Protein at E_{280}; ●, 5′-nucleotidase, E_{650} (arbitrary units). Fractions of volume 2.4ml were collected.

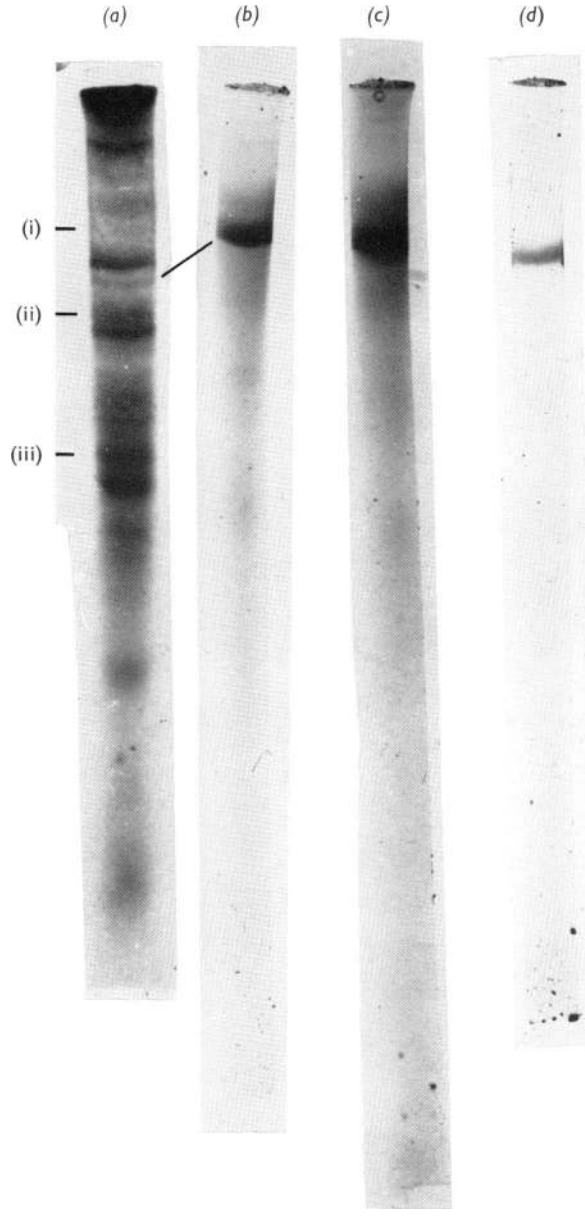
that the enzyme purified from rat liver plasma membranes and microsomal fractions was exclusively associated with sphingomyelin. Analysis of the distribution of phospholipids in chloroform–methanol extracts of samples across the gradient by chemical determination (Fig. 1), or radiochemically by using membranes labelled with $Na_3{}^{32}PO_4$ (not shown), showed that phospholipids remained mainly in the overlay region and sedimented into the sucrose gradient only to a slight extent. The 5′-nucleotidase activity peak was associated with extremely small amounts of phospholipids. A complete separation of 5′-nucleotidase activity and membrane proteins from phospholipid phosphorus was also achieved by gel filtration of the sarcosyl–Tris-medium extract on columns of Sepharose 6B equilibrated with 0.25% sarcosyl–Tris medium (W. H. Evans & J. W. Gurd, unpublished work).



EXPLANATION OF PLATE 1

Distribution of proteins and glycoproteins across a BXIV zonal-rotor gradient

Gel electrophoresis in 7.5% (w/v) polyacrylamide gels in sodium dodecyl sulphate of samples obtained from across a BXIV gradient and concentrated by Diaflow filtration. The gels correspond to the following fractions in Fig. 1: (i) 5+6; (ii) 8; (iii) 10; (iv) 12; (v) 15–17; (vi) 20–21; (vii) 23. (*a*) Stained for protein by Amido Black; (*b*) stained for glycoprotein by the periodate–Schiff procedure.



EXPLANATION OF PLATE 2

Gel electrophoresis of plasma membrane and purified 5′-nucleotidase

Electrophoresis in 7.5% (w/v) polyacrylamide gels in sodium dodecyl sulphate of (*a*) total plasma-membrane fraction, (*b*) and (*c*) the 5′-nucleotidase peak from a Sepharose 6B column stained for protein and glycoprotein respectively, and (*d*) the peak-activity tube from the Sephadex G-100 column stained for glycoprotein. The position of three viral major protein markers are also shown and correspond to the following molecular weights: (i) 150000, (ii) 72000 and (iii) 38000 (Smith *et al.*, 1969).

Distribution of proteins and glycoproteins

The distribution of proteins, glycoproteins and glycolipids across the gradient was examined by polyacrylamide-gel electrophoresis (Plate 1). The major protein peak contained a large number of polypeptide chains of molecular-weight range 20000–70000. The protein bands were diffuse and indistinct, possibly owing to proteolysis occurring in the sarcosyl–Tris medium. Staining by the periodate–Schiff procedure indicated the absence of carbohydrates attached to protein, and fractions obtained from the lighter end of the gradient stained intensely at the front of the gel, indicating the possible presence of glycolipids. The 5'-nucleotidase peak contained relatively few bands of a higher molecular weight, and many of these were glycoproteins. Samples from the outer edge of the rotor showed many bands of higher-molecular-weight material, with one predominant glycoprotein band. The polyacrylamide-gel patterns indicated that membrane proteins were separated from glycoproteins on the gradient and this separation was broadly related to molecular weight, thus confirming previous findings showing that the majority of the liver plasma membrane proteins are of molecular-weight range 20000–70000, and that glycoproteins predominate among the higher-molecular-weight components (Evans & Gurd, 1972).

Gel filtration of enzyme recovered from the zonal rotor

Fractions containing high 5'-nucleotidase activities (50–100ml) were concentrated to 3–7ml and applied to a Sepharose 6B column equilibrated with sarcosyl–Tris medium. Higher-molecular-weight components were eluted at the void volume and a major peak of 5'-nucleotidase activity appeared at a point coincident with the molecular-weight-marker immunoglobulin G (Fig. 2). In general, the activity peaks on Sepharose 6B were broad and sometimes two or three shoulders of enzymic activity were observed. Examination of the enzyme peak on polyacrylamide gels (Plate 2) indicated a further simplification of the protein pattern. The pooled enzymic-activity peak from the Sepharose 6B was concentrated, and applied to a Sephadex G-100 or G-150 column. Enzyme activity and the protein peak were coincident and a molecular weight of 140000–150000 was calculated (Fig. 3). During some experiments, a double peak was observed on Sephadex columns (Fig. 4), both of which exhibited 5'-nucleotidase activity, the peak of minor activity having a molecular weight of approximately one-half that of the major peak. Electrophoresis of both peaks on 7.5% (w/v) polyacrylamide gel in sodium dodecyl sulphate buffers indicated the presence of one component of apparent molecular weight 120000.

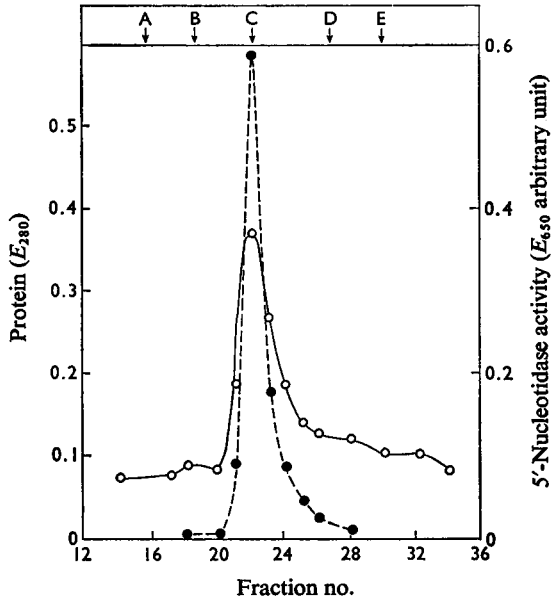


Fig. 3. *Gel filtration of enzyme purified from a Sepharose column*

Gel filtration on a Sephadex G-100 column equilibrated with 0.25% sarcosyl–Tris buffer of the 5'-nucleotidase peak concentrated from a Sepharose 6B column. ○, Protein at E_{280}; ●, 5'-nucleotidase, E_{650} (arbitrary units). Fractions of 1.8ml were collected. Marker proteins used were: A, Blue Dextran; B, catalase (mol.wt. 240000); C, aldolase (mol.wt. 147000); D, ovalbumin (mol.wt. 45000); E, cytochrome *c* (mol.wt. 13500). The arrows indicate the peak positions of these proteins.

Polyacrylamide-gel studies

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate buffers in 7.5% (w/v) polyacrylamide gels (Plate 2) showed that purification was accompanied by a decrease in the number of bands, only one protein band being present in the purified enzyme product recovered from the protein peak obtained on Sephadex columns. This band was also strongly stained by the periodate–Schiff procedure, suggesting that it was a glycoprotein. A molecular weight of 110000–120000, determined in 7.5% (w/v) polyacrylamide gels, was inconsistent with a molecular weight of 140000–150000 for the enzyme-activity peak obtained by zonal centrifugation and Sephadex gel filtration. Since the electrophoretic mobility of glycoproteins may not be proportional to their molecular weights (Segrest *et al.*, 1971), the enzyme was run on gels containing between 5 and

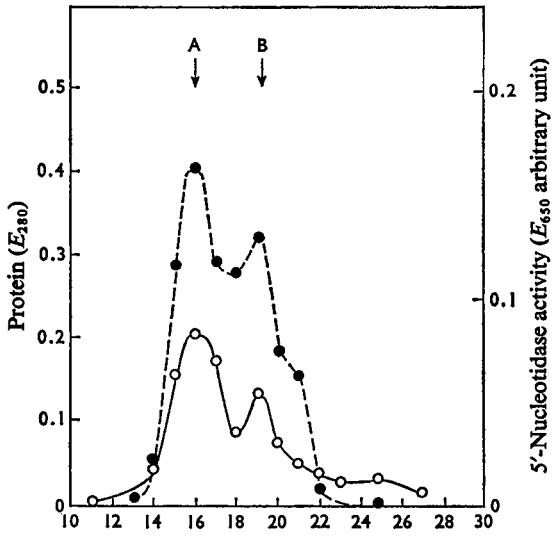


Fig. 4. *Dissociation of purified enzyme on a Sephadex column*

Gel-filtration profile occasionally obtained on a Sephadex G-100 column equilibrated with 0.25% sarcosyl–Tris buffer of a 5′-nucleotidase peak concentrated from a Sepharose 6B column. ○, Protein at E_{280}; ●, 5′-nucleotidase, E_{650} (arbitrary units); A and B represent the positions of the markers immunoglobulin G and bovine serum albumin respectively.

15% (w/v) polyacrylamide. As the percentage of polyacrylamide in the gel increased, the molecular weight calculated relative to protein markers decreased (Fig. 5). The molecular-weight curve obtained by varying the polyacrylamide concentration decreased towards a value approximately one-half that obtained for the activity by gel filtration. The molecular weight obtained in the sodium dodecyl sulphate gels by using samples prepared under strongly dissociating and reducing conditions is that of a polypeptide subunit, and the results suggest that the enzyme 5′-nucleotidase, molecular weight 140000–150000, is made up of two similar, if not identical, polypeptide subunits with attached carbohydrate. During certain purification procedures, the appearance of a minor protein peak with corresponding 5′-nucleotidase activity in addition to the major peak of molecular weight 150000 would suggest that dissociation into monomer can also occur in sarcosyl–Tris buffer, and that the monomer is also active. Polyacrylamide gels in sarcosyl–Tris buffers were used to show that the protein band corresponded to the major enzymic activity, and these gels also indicated activity associated with a lower-molecular-weight component that may correspond to the active monomer described above (Fig. 6). The linear relationship between the distance migrated by proteins and the logarithm of their molecular weights (Weber & Osborn, 1969) did not hold in sarcosyl–Tris-buffered polyacrylamide gels.

Properties of the isolated enzyme

Analyses of chloroform–methanol extracts of the purified enzyme for the presence of phospholipids or phospholipid phosphorus gave negative results. For example, extraction of up to 200 μg of purified enzyme protein with chloroform–methanol and an examination of the total extract on t.l.c. plates did not show any lipid components when stained with H_2SO_4 or ammonium molybdate, under conditions that made visible 10 μg of phospholipid standards. Further, with enzyme purified from mice injected with $Na_3{}^{32}PO_4$ (Evans & Gurd, 1972), long-term exposure of lipid extracts to radioautographic plates did not show 'fogging', thus confirming the above result. Therefore the 5′-nucleotidase purified by the current procedure is active in the absence of detectable amounts of phospholipids.

Although there was an overall increase in the specific activity of 5′-nucleotidase during the purification scheme described, there was an appreciable loss of overall enzyme activity (Table 1). Indeed, the 5′-nucleotidase specific activity of a vesicular membrane fraction prepared from the sarcosyl–Tris medium-insoluble residue, which was associated with a full complement of lipids, was 30-fold higher than that of total plasma membranes, compared with the 18-fold increase achieved by the present purification procedure (Evans & Gurd, 1972). However, during the extraction and purification of the high-activity 5′-nucleotidase from the sarcosyl–Tris medium-insoluble residue with non-ionic detergents, a similar loss of activity was also observed, and the properties of this enzyme showed close correspondence to those described in the present work (W. H. Evans, unpublished work).

An attempt was therefore made to increase the enzymic activity of the purified enzyme by addition of phospholipids. The addition of dispersions of purified sphingomyelin, phosphatidylcholine, phosphatidic acid and a total lipid extract of brain to the purified enzyme in sarcosyl–Tris medium produced no significant increase in enzyme activity. Also, treatment of the enzyme in sarcosyl–Tris medium with phospholipase C (2mg/ml) under conditions where the phospholipase is known to be active (W. H. Evans, unpublished work) had no effect on the 5′-nucleotidase activity.

The pH curve (Fig. 7) indicated a pH–activity optimum at pH7.4 with a second pH optimum at pH10 in the presence of added Mg^{2+}. This is in

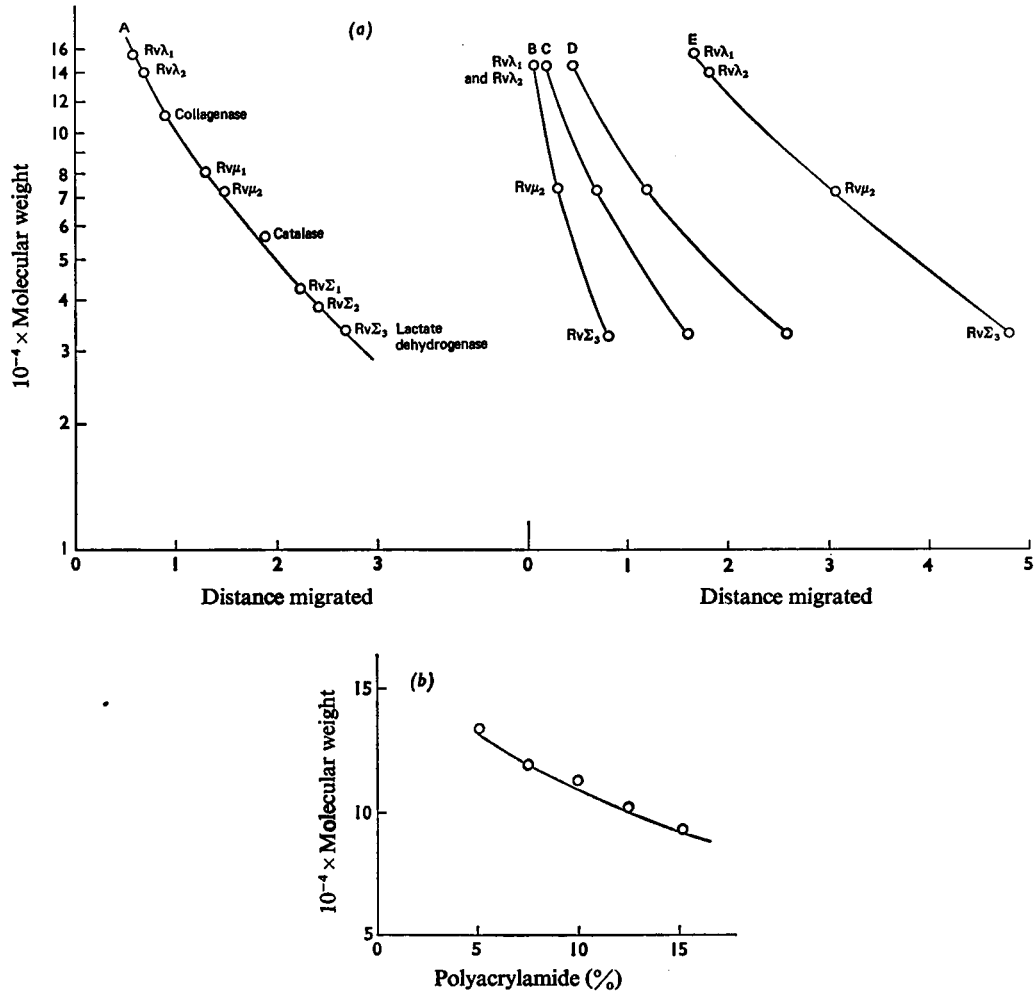


Fig. 5. *Determination of the apparent molecular weight of the purified 5′-nucleotidase*

Electrophoresis of the enzyme and various marker proteins was carried out in sodium dodecyl sulphate gels made with different concentrations of polyacrylamide. (*a*) Calibration curves obtained with the following polyacrylamide concentrations: A, 7.5%; B, 5%; C, 10%; D, 12.5%; E, 15%. Reoviral proteins of known molecular weight were used as markers: $Rv\lambda_1$, $Rv\lambda_2$, $Rv\mu_1$, $Rv\mu_2$, $Rv\Sigma_1$, $Rv\Sigma_2$ and $Rv\Sigma_3$ (Smith *et al.*, 1969), and catalase, collagenase and lactate dehydrogenase were also used. (*b*) Variation of the apparent molecular weight of 5′-nucleotidase with the % of polyacrylamide in the gel.

agreement with results obtained with human or rat liver plasma or microsomal membranes (Song & Bodansky, 1966, 1967), bull seminal plasma (Levin & Bodansky, 1966) and a partially purified 5′-nucleotidase obtained from the smooth muscle of small intestine (Burger & Lowenstein, 1970) and heart (Edwards & Maguire, 1970). The 5′-nucleotidase isolated from liver membranes differs in a number of properties, including pH optimum and substrate specificity (see below), from a 5′-nucleotidase obtained from a supernatant fraction of rat liver homogenates (Itoh *et al.*, 1968; Fritzson, 1969; Fritzson & Smith, 1971).

The specificity of the enzyme towards a range of nucleotides and other substrates is shown in Table 2. Generally, the nucleotide specificity resembled closely

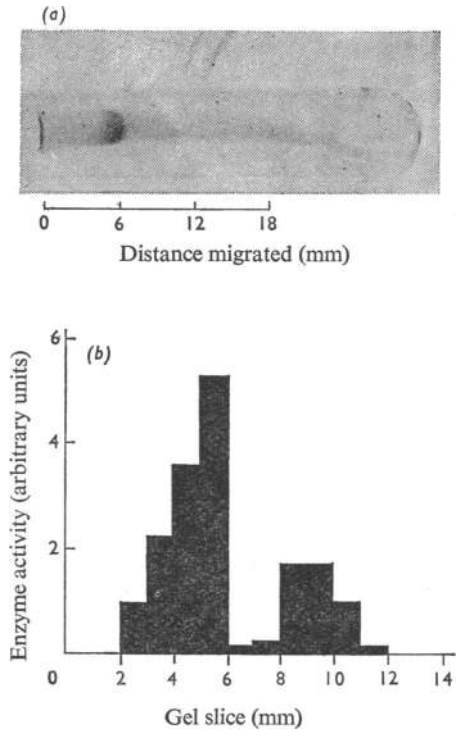


Fig. 6. *Distribution of protein staining and enzymic activity in polyacrylamide gel*

Polyacrylamide-gel electrophoresis of the purified 5′-nucleotidase activity on sarcosyl–Tris-buffer gels. See the Experimental section for details. One half of the 7.5% polyacrylamide gel was stained for protein with Amido Black (*a*), and the other half was cut into 1–2mm sections for assay of 5′-nucleotidase activity (*b*). No activity was detected beyond 12mm.

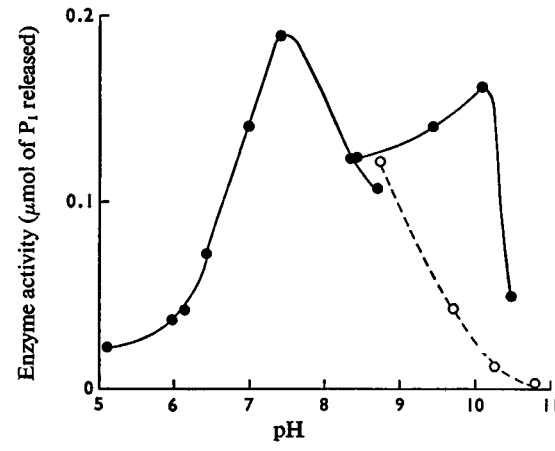


Fig. 7. *pH–activity profile of the purified 5′-nucleotidase*

Effect of pH on the activity of 5′-nucleotidase was investigated in the presence (●) or in the absence (○) of Mg^{2+}. Below pH7.8, Mg^{2+} was not required for activity. Buffers used in the pH range examined were: pH5.0–6.5, Tris–acetate; pH7.0–9.0, Tris–glycine; pH8.7–10.8, Na_2CO_3–$NaHCO_3$. The indicated pH was that measured at the end of a 15min incubation and enzymic activity was determined by measuring P_i release as described in the Experimental section.

Table 2. *Substrate specificity of the purified 5′-nucleotidase*

For details see the text. Results are expressed as a percentage of the specific activity obtained with adenosine 5′-monophosphate as substrate at the appropriate pH. N.D., not determined.

	Activity (%)	
Substrate	pH7.6	pH10.0
Adenosine 5′-monophosphate	100	100
Uridine 5′-monophosphate	105	116.5
Cytidine 5′-monophosphate	69.5	60.0
Guanosine 5′-monophosphate	88	49
Adenosine 3′-monophosphate	0	N.D.
Adenosine 2′-monophosphate	0	N.D.
Thymidine *p*-nitrophenyl phosphate	0	13
p-Nitrophenyl phosphate	0	0
Adenosine triphosphate (5mM)	0	N.D.
Adenosine triphosphate (2.5mM)	0	N.D.

that determined by Widnell & Unkeless (1968) for the rat liver enzyme. The enzyme failed to hydrolyse *p*-nitrophenyl phosphate and was therefore free of alkaline phosphatase activity. A low phosphodiesterase activity with thymidine *p*-nitrophenyl phosphate as substrate was present at pH10. This phosphodiesterase activity is due to contamination of the 5′-nucleotidase by a highly reactive phosphodiesterase which is also extracted by sarcosyl–Tris medium, but which can be separated from 5′-nucleotidase during zonal centrifugation (W. H. Evans & J. W. Gurd, unpublished work).

The 5′-nucleotidase was inhibited by ATP in agreement with the enzyme activity studied in other organs (Burger & Lowenstein, 1970; Ipata & Cercignani, 1970; Sullivan & Alpers, 1971). The inhibition of 5′-nucleotidase by the artificial substrate $\alpha\beta$-methylene-ADP, an analogue of ADP (Burger & Lowenstein, 1970), was investigated in greater detail in view of its potential use in the purification of the 5′-nucleotidase

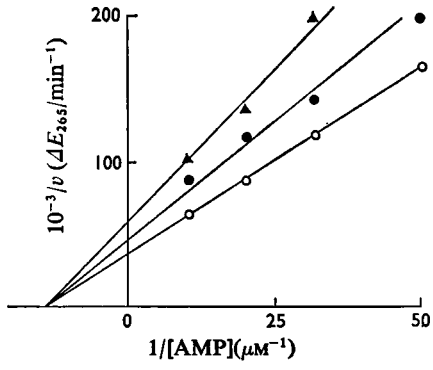


Fig. 8. *Inhibition of purified 5'-nucleotidase by αβ-methylene-ADP*

Concentration of inhibitor used was: ▲, 0.1M; ●, 0.05M; ○, no inhibitor. A K_m value of 63μM was obtained. Enzyme activity was determined spectrophotometrically as described in the Experimental section.

by affinity chromatography. However, kinetic studies showed that the inhibition was probably non-competitive (Fig. 8), and this inhibitor is therefore unsuitable for purification of the enzyme by affinity chromatography.

Discussion

The rate-zonal-centrifugation technique described resolved the liver plasma-membrane protein components extracted into *N*-dodecylsarcosinate–Tris buffer according to their apparent molecular weights. The method separated membrane glycoproteins from proteins, and also gave a partial resolution of the glycoproteins. The separation obtained could be due either to the different hydrodynamic behaviour (shape) of the components or to different binding of detergent by the proteins. The second possibility is favoured for the following reasons. Sedimentation coefficients determined for detergent-solubilized membrane components are of doubtful validity, since it would be expected that the amount of detergent bound would depend on the hydrophobicity of the proteins, and this would be grossly affected by the presence of carbohydrate residues. It is known, for example, that glycoproteins bind less sodium dodecyl sulphate, which is a similar detergent to sarcosyl, than do proteins (Pitt-Rivers & Impiombata, 1968). A decrease in density, and therefore sedimentation coefficient, caused by a higher degree of sarcosyl binding to proteins compared with glycoproteins is regarded as a most likely explanation for the separation obtained in the present work. A similar explanation, supported by experimental evidence, has been proposed to explain unusual hydrodynamic properties of a cholinergic receptor protein of *Electrophorus electricus*, solubilized with Triton X-100 (Meunier *et al.*, 1972).

The 5'-nucleotidase was isolated as a single protein component. The molecular weight determined for the enzyme by gel filtration (140000–150000) corresponds closely to the value of 140000 obtained in the absence of detergent with sheep brain 5'-nucleotidase (Ipata, 1968). Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate buffers showed a single band, with an apparent molecular weight that was dependent upon the percentage of polyacrylamide in the gel. The molecular weight on 5% (w/v) polyacrylamide gels was 130000, decreasing to 90000 on 15% (w/v) polyacrylamide gels, with the curve decreasing asymptotically to a molecular weight in the region of 70000–75000. A minor enzymically active peak was also obtained in some instances on gel filtration at an apparent molecular weight of 70000. The purity of the plasma membranes used in the current work (Evans, 1970; Evans & Gurd, 1971) argues against the possibility that the minor lower-molecular-weight enzymic-activity peak sometimes observed could be attributable to a 5'-nucleotidase present in contaminating intracellular membranes. The conversion of the 5'-nucleotidase into a lower-molecular-weight active subunit by the proteolytic enzymes of plasma membranes is also a possibility, which was not tested, although no indication of proteolytic activity was observed. Hence, we favour the interpretation that mouse liver plasma-membrane 5'-nucleotidase is composed of two glycoproteins of identical molecular weight (70000–75000), and enzymic activity is also associated with the monomers. The conditions which result in dissociation into active monomers remain to be determined.

The properties of many membrane-associated enzymes appear to be linked to the intactness of membrane phospholipids, as shown by the effect on these enzymes of treatments which remove or perturb lipids. For example, the adenosine triphosphatase of muscle sarcoplasmic reticulum (Martonosi *et al.*, 1968), hepatic microsomal uridine diphosphate glucuronyl transferase (Attwood *et al.*, 1971; Vessey & Zakim, 1972), acyl-CoA–L-glycerol 3-phosphate acyl transferase (Abou-Issa & Cleland, 1969) and glucose 6-phosphatase (Duttera *et al.*, 1968; Cater *et al.*, 1970) were all inactivated by treatment with phospholipases, and the loss of activity was restored in many cases by addition of phospholipids. However, Emmelot & Bos (1968) showed that pretreatment of rat liver plasma membranes with phospholipase C or extraction with butanol–ether solvents did not affect 5'-nucleotidase activity, whereas other membrane phosphohydrolases, notably adenosine triphosphatase activity, were completely inhibited, thus suggesting that membrane 5'-nucleotidase was not a phospholipid-dependent enzyme.

The present results show unequivocally that the purified 5'-nucleotidase of liver plasma membranes was active in the absence of detectable amounts of phospholipids. The 5'-nucleotidase, isolated in Triton X-100–deoxycholate mixtures as a lipoprotein by Widnell & Unkeless (1968), contained approx. 1.3 mg of phospholipid (identified as sphingomyelin)/mg of protein, representing about 100 sphingomyelin molecules for each molecule of protein of molecular weight 75000–80000. This is a large amount of lipid compared, for instance, with adenosine triphosphatase purified from sarcoplasmic reticulum, which was associated with only one-third of its weight of lipid (MacLennan, 1970). By using identical solubilization and separation procedures to Widnell & Unkeless (1968), Huang & Keenan (1972) obtained from bovine milk-fat globule membranes a partially purified 5'-nucleotidase that was associated with sphingomyelin and phosphatidylcholine. Results such as these demonstrating the association of 5'-nucleotidase with large amounts of phospholipids can be explained in terms of the properties of detergent solutions and mixed phospholipid–detergent micelles. Thus solubilization with high concentrations of deoxycholate solutions disrupts membrane protein–lipid interactions, permitting the almost complete separation of protein and lipid components (Philippot, 1970; Allan & Crumpton, 1971; Helenius & Simons, 1972). Triton X-100 solutions, at concentrations used for membrane solubilization, contain specific micellar complexes of molecular weight 160000 (Helenius & Simons, 1972). Shankland (1970) has prepared phosphatidylcholine–cholate micelles and shown that a range of higher-molecular-weight complexes are present and Philippot (1970) has also shown that erythrocyte phospholipids form specific micelles with deoxycholate solutions. The simultaneous use by Widnell & Unkeless (1968) of two detergents with divergent properties for the purification of 5'-nucleotidase makes their system extremely complex. However, we suggest that the phospholipids reported associated with the partially purified 5'-nucleotidase could be mainly associated with (mixed) detergent micelles, so that their presence would bear little or no relationship to any specific lipid–protein interactions that may occur in the intact membrane.

Although the specific activity of the 5'-nucleotidase increased during purification, there was a loss in total enzymic activity. This probably resulted from the replacement of the membrane hydrophobic environment by one less favourable to the enzyme in which the detergent is acting as a lipid substitute. It has been shown for other enzymes, e.g. sarcoplasmic reticular adenosine triphosphatase (Martonosi *et al.*, 1968) and glucuronyl transferase (Attwood *et al.*, 1971), that activity lost after treatment with phospholipases can be restored by addition of low concentration of detergents. The *N*-dodecylsarcosinate present in the medium may have replaced, albeit poorly, the specific lipids which interact with the enzyme in the membrane. However, the addition of pure phospholipids to 5'-nucleotidase solutions failed to increase specific activity, possibly owing to their inability to replace or exchange with the detergent.

The 5'-nucleotidase of liver plasma membranes is a glycoprotein. Although a number of soluble enzymes have attached carbohydrate (Jutisz & De la Llosa, 1972), there are only a few reports of enzymically active membrane-bound glycoproteins (Sottocasa *et al.*, 1972; Guidotti, 1972). It appears likely that the 5'-nucleotidase of pig lymphocyte plasma membranes is also a glycoprotein, since the glycoprotein fraction absorbed from deoxycholate extracts of membranes by *Lens culinaris* phytohaemagglutinin attached to Sepharose columns and subsequently released by methyl D-mannopyranoside accounted for 85% of the deoxycholate-soluble enzyme (Hayman & Crumpton, 1972). The 5'-nucleotidase of vesicular liver plasma-membrane subfractions (Evans, 1970) is inhibited by antisera raised against unfractionated plasma membranes (J. W. Gurd & W. H. Evans, unpublished work), indicating, in agreement with histochemical studies (Widnell, 1972), that the enzyme is probably located at the outer surface of the plasma membrane. Its physiological function, however, remains to be determined.

We thank Dr. H. R. Perkins for his advice and encouragement and Mrs. D. Hood for technical assistance. J. W. G. was supported by a Canadian Medical Research Council Fellowship.

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