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# Purification and Properties of a Mouse Liver Plasma-Membrane Glycoprotein Hydrolysing Nucleotide Pyrophosphate and Phosphodiester Bonds

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1. A mouse liver plasma-membrane preparation was solubilized in an *N*-dodecylsarcosinate–Tris buffer, pH7.8, and the proteins and glycoproteins were separated by a rate-zonal centrifugation in sucrose–detergent gradients. 2. A peak of alkaline phosphodiesterase activity which sedimented ahead of the 5′-nucleotidase peak was associated with a major glycoprotein component of the plasma membrane. 3. The phosphodiesterase activity was then purified further by gel filtration and gave a single glycoprotein band after electrophoresis on polyacrylamide gels. The apparent molecular weight of the polypeptide at pH7.4 and 8.9 was 128000–130000 and was independent of the polyacrylamide concentration. Electrophoresis in gels containing deoxycholate showed that the protein band was coincident with phosphodiesterase activity. 4. After two-dimensional immunoelectrophoresis, with agarose containing rabbit anti-(mouse plasma-membrane) antiserum as second dimension, the enzyme showed one component which was also coincident with the phosphodiesterase activity. 5. An amino acid composition of the glycoprotein is presented. Carbohydrate analysis indicated the presence of glucosamine, neutral sugars and sialic acid. 6. The enzyme was also a nucleotide pyrophosphatase, as shown by a similar enrichment during purification of activity towards ATP, $NAD^+$, UDP-galactose and UDP-*N*-acetylglucosamine. The phosphodiesterase activity, measured by using dTMP *p*-nitrophenyl ester as substrate, was competitively inhibited by nucleotide pyrophosphate substrates. The enzyme showed little or no activity towards RNA, cyclic AMP, AMP, ADP and glycerylphosphorylcholine. 7. The significance of this enzyme activity in the plasma membrane is discussed.

The presence in liver membranes, especially the microsomal fraction, of enzymic activities that hydrolyse nucleotide pyrophosphate and alkaline phosphodiester bonds has been reported (Jacobson & Kaplan, 1957; Schliselfeld *et al.*, 1965; De Lamirande *et al.*, 1966; Futai & Mizuno, 1967; Bachorik & Dietrich, 1972). It has since been shown that these enzyme activities are mainly located in the plasma-membrane fraction, and there is evidence that the same enzyme is responsible for hydrolysing phosphodiester linkages and the pyrophosphate bonds of a wide range of substrates, including ATP, $NAD^+$ and sugar nucleotides, e.g. UDP-galactose (Lieberman *et al.*, 1967; Erecinska *et al.*, 1969; Touster *et al.*, 1970; Skidmore & Trams, 1970; Decker & Bischoff, 1972). The enzyme may play an important and central role in the nucleotide or carbohydrate metabolism of the cell surface, and a possible role in intercellular adhesion is suggested by the absence of the enzyme activity from transformed cells, in contrast with normal cells (Kalckar & Hakomori, 1972; Sela *et al.*, 1972).

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We now describe the composition and properties of this enzyme, which consists of a single glycoprotein component with dual specificity towards nucleotide pyrophosphate and phosphodiester bonds. The enzyme was extracted from mouse liver plasma membranes by a detergent, *N*-dodecylsarcosinate–Tris buffer, pH7.8, and a rate-zonal centrifugation was used to separate the membrane proteins and glycoproteins (Evans & Gurd, 1973*a*). When finally purified by gel filtration, the enzyme yielded a single band on polyacrylamide-gel electrophoresis or immunoelectrophoresis.

## Experimental

### *Enzyme purification by rate-zonal centrifugation and gel filtration*

Livers from Parkes white mice (6–8 weeks old; approx. 75 animals) were used to prepare a plasma-membrane fraction by using a rate-zonal centrifugation step, followed by isopycnic centrifugation as previously described (Evans, 1970; Evans & Gurd, 1972). The plasma membranes, in 0.25M-sucrose,

were pelleted by centrifugation and extracted twice with a stock solution of 4% (w/v) *N*-dodecylsarcosinate–2.1% (w/v) Tris base, pH7.8 (sarcosyl–Tris buffer) (Evans & Gurd, 1972). Other sarcosyl–Tris concentrations were prepared by dilution of this stock solution. The sarcosyl–Tris buffer extract (vol. 43ml) was centrifuged for 23h at 43000rev./min in an MSE B XIV Ti rotor containing a 10–40% (w/v) continuous sucrose gradient dissolved in 0.25% sarcosyl–Tris buffer; an overlay of 180ml of 0.25% sarcosyl–Tris buffer covered the sample (Evans & Gurd, 1973*a*). Sucrose concentrations were measured by refractometry. Fractions of 50 or 25ml were collected from the rotor, and those containing phosphodiesterase or pyrophosphatase activity were concentrated to approx. 4ml by pressure filtration in a Diaflow cell containing XM-50 filters (Amicon Ltd., High Wycombe, Bucks., U.K.). Final purification of the enzyme was obtained by gel filtration on Sephadex G-100 columns equilibrated with 0.25% sarcosyl–Tris buffer.

*Enzyme assays*

Protein was determined by the procedure of Lowry *et al.* (1951). Bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) was used as standard.

*Alkaline phosphodiesterase I* (*orthophosphoric diester phosphohydrolase, EC* 3.1.4.1). This was measured on a Unicam SP.800 recording spectrophotometer by following the release of *p*-nitrophenyl phosphate from thymidine 5′-monophosphate (dTMP) *p*-nitrophenyl ester (Razzel, 1963). Results were calculated by using $\epsilon_{405} = 1.20 \times 10^4$ litre·mol$^{-1}$·cm$^{-1}$.

*5′-Nucleotidase* (*5′-ribonucleotide phosphohydrolase, EC* 3.1.3.5). This was measured spectrophotometrically by the method of Ipata (1967). A value of $\epsilon_{265} = 0.22 \times 10^4$ litre·mol$^{-1}$·cm$^{-1}$ was used.

*Glycerylphosphorylcholine phosphodiesterase* (*sn-glyceryl-3-phosphorylcholine glycerylphosphohydrolase, EC* 3.1.4.2). This was assayed by the method of Fowler & de Duve (1969). Glycerylphosphorylcholine (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) was first freed of $Cd^{2+}$ by chromatography on Dowex 50 columns.

*ATP pyrophosphatase* (*ATP pyrophosphohydrolase, EC* 3.6.1.8). This was measured as described by Lieberman *et al.* (1967).

*NADH–nucleotide pyrophosphatase* (*dinucleotide nucleotidohydrolase, EC* 3.6.1.9). This was measured as described by Skidmore & Trams (1970).

*UDP-N-acetylglucosamine pyrophosphatase, UDP-galactose pyrophosphatase and ADP-diphosphatase* (*EC* 3.6.1.6). These activities were measured as described by Bachorik & Dietrich (1972). When hexose 1-phosphate was liberated it was acid hydrolysed after treatment of the incubation system with charcoal as described by Ogowa *et al.* (1966). Phosphate liberated was determined by the method of Bartlett (1959). The hydrolysis of UDP-*N*-acetylmuramylpentapeptide was also examined by this method.

*Alkaline phosphatase* (*p-nitrophenyl phosphatase, EC* 3.1.3.1). This was measured spectrophotometrically at 400nm by following the hydrolysis of *p*-nitrophenyl phosphate at pH8.9.

*Ribonuclease* (*EC* 2.7.7.16). This was measured by incubation of $^3$H-labelled RNA extracted from *Escherichia coli* ribosomes (given by Dr. R. Brimacombe of this Institute) with plasma membranes or enzyme in 0.05M-Tris–HCl buffer, pH7.6. After precipitation of the RNA in 12.5% (w/v) trichloroacetic acid, portions of the supernatant were used to determine radioactivity by scintillation spectrometry (Evans & Gurd, 1971).

*Leucine β-naphthylamidase* (*EC* 3.4.1.1). This was measured as described by Goldberg & Rutenburg (1958).

*Cyclic nucleotide phosphodiesterase* (*EC* 4.6.1.1). This was measured in plasma membranes and the purified enzyme solution as described by Thompson & Appleman (1971). We thank Dr. M. J. Crumpton for the cyclic AMP determinations.

*Gel electrophoresis and immunoelectrophoresis*

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate (0.1%) was carried out in (a) 5mM-sodium phosphate buffer, pH7.4, in 7, 10, 12.5 and 15% (w/v) polyacrylamide gels in tubes (10cm×8mm); gels were stained for protein with Amido Black or for carbohydrate by the periodate–Schiff procedure (Evans & Gurd, 1972), or (b) Tris (0.05M)–glycine (0.4M) buffer, pH8.9, in 10% (w/v) polyacrylamide gel in a flat-plate apparatus (E-L, Philadelphia, Pa., U.S.A.) as described by Maizel (1971). Proteins of known molecular weight were used to calibrate the gels as previously described (Evans & Gurd, 1973*a*). Gel electrophoresis of purified enzyme was also carried out in cylindrical gels equilibrated with 0.15% deoxycholate in 0.1M-sodium phosphate buffer, pH8.0. After electrophoresis at 25mA/tube for 4h, gels were cut longitudinally into four strips, of which two were fixed with 7% (w/v) acetic acid and stained for protein or carbohydrate. The remaining strips were used to determine the position of the alkaline phosphodiesterase activity, by immersing them immediately after electrophoresis in 5mM-dTMP *p*-nitrophenyl ester dissolved in 0.1M-Tris–HCl buffer, pH8.9. The development of a yellow band indicating the position of the enzyme occurred in about 5min, and the gel was photographed by using the appropriate filters.

Immunoelectrophoresis in two dimensions as

described by Laurell (1965) was carried out as previously described (Gurd *et al.*, 1973). The purified alkaline phosphodiesterase (5$\mu$g of protein in 0.25% sarcosyl–Tris buffer) was added to 1% (w/v) Triton X-100 and introduced into the well for electrophoresis in the first dimension. The agarose used for electrophoresis in the second dimension contained 0.2ml of mouse plasma-membrane antiserum (Gurd *et al.*, 1972). After electrophoresis, the gel was immersed in 5mM-dTMP *p*-nitrophenyl ester for activity staining. Another gel was stained for protein with Amido Black.

### *Carbohydrate and amino acid analyses*

Membranes dialysed against running tap water or purified enzyme were hydrolysed in 2M-HCl at 100°C for 2h and after acetylation the neutral sugar fraction was analysed by g.l.c. An amino sugar determination on the purified enzyme was made on a Beckman 120C analyser after hydrolysis for 4h in 4M-HCl at 110°C *in vacuo* and then treatment as described by Fanger & Smyth (1970). Amino acid analysis was carried out after hydrolysis for 24h in 6M-HCl at 110°C *in vacuo* (Evans, 1970; Evans & Gurd, 1971). Neutral sugar was determined by the anthrone method (Mokrasch, 1954) and sialic acid by the method of Aminoff (1961).

### *Materials*

All biochemicals were of analytical grade. *N*-Dodecylsarcosinate (Sarkosyl) was obtained from Geigy Ltd., Simonsway, Manchester, U.K. Deoxycholic acid was obtained from E. Merck, Darmstadt, Germany, and agarose (Indubiose A37) from L'Industrie Biologique Francaise, 35 Quai du Moulin de Cage, 92 Gennevilliers, France. Glass-distilled water was used throughout. UDP-*N*-acetylmuramyl-pentapeptide was supplied by Dr. H. R. Perkins of this Institute.

## Results

### *Solubilization and purification of a phosphodiesterase–nucleotide pyrophosphatase*

Previous work (Evans & Gurd, 1972, 1973*a*) showed that addition of the anionic detergent *N*-dodecylsarcosinate to mouse liver plasma membrane solubilized most of the proteins while preserving at least two enzymic activities, 5′-nucleotidase and leucine naphthylamidase. An alkaline phosphodiesterase (assayed by using dTMP *p*-nitrophenyl ester) was also extracted by the same sarcosyl–Tris buffer (Table 1).

Centrifugation of the sarcosyl–Tris buffer extract into a shallow sucrose gradient dissolved in 0.25% *N*-dodecylsarcosinate–Tris buffer, pH7.8, was effective in fractionating the proteins and glycoproteins

Table 1. *Distribution of enzyme activities in mouse liver plasma membranes and the various steps leading to the isolation of the purified glycoprotein*

Fraction nos. refer to Figs. 1 and 2. Values quoted represent specific activities expressed as $\mu$mol of substrate hydrolysed/h per mg of protein except for ribonuclease activity which is expressed as c.p.m./mg of protein liberated in 15min. Values in parentheses indicate the enrichment or loss in activity relative to that present in the plasma-membrane fraction.

| Fraction | Protein (mg) | Alkaline phosphodiesterase | ATP pyrophosphatase | $NAD^+$ pyrophosphatase | UDP-galactose pyrophosphatase | UDP-*N*-acetylglucosamine pyrophosphatase | ADP diphosphatase | Glyceryl-phosphorylcholine phosphodiesterase | Cyclic AMP phosphodiesterase | 5′-Nucleotidase | Ribonuclease |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Liver plasma membranes | 100 | 4.4 | 2.20 | 440 | 30.5 | 21* | 24.5 | 1.5 | 1.6 | 16.4 | $1.65\times10^6$ |
| Sarcosyl–Tris buffer extract | 88 | 2.4 (0.55) | 0.15 (0.68) | — | — | — | — | — | — | 15.8 (0.97) | $0.84\times10^6$ (0.3) |
| B XIV rotor fractions | | | | | | | | | | | |
| No. 14 | — | 5.2 (1.2) | 0.85 (0.39) | — | — | — | — | — | — | 70 (4.3) | — |
| Nos. 15 and 16 | — | 7.7 (1.75) | 1.24 (0.56) | — | 37.5 (1.2) | — | 34.2 (1.4) | — | — | 136 (8.3) | — |
| No. 17 | 1.0 | 14.6 (3.3) | 1.92 (0.87) | 220 (0.5) | — | 58.5 (2.8) | — | — | — | 96 (5.8) | — |
| No. 18 | 1.4 | 43.2 (9.8) | 7.50 (3.4) | — | — | — | — | 3.0 (2.0) | — | 21.6 (1.3) | — |
| No. 19 | 2.1 | 25.0 (5.7) | 6.70 (3.0) | — | 97 (3.2) | 83 (4.0) | — | 0 | — | 3.7 (0.23) | — |
| Sephadex fractions from fractions 18 and 19 from B XIV rotor | 0.81 | 45.1 (10.3) | 14.80 (6.7) | 1824 (4.2) | 250 (8.2) | 314 (15) | 6.5 (0.27) | 1.1 (0.7) | 0 | 29.1 (1.7) | $0.86\times10^6$ (0.5) |

* A different plasma-membrane preparation was used to assay this substrate.

Table 2. *Amino acid and amino sugar composition of the purified glycoprotein*

Residues/mol are calculated on the basis of a subunit molecular weight of 130000. The enzyme (approx. 1 mg) was hydrolysed in 6M-HCl at 110°C for 24h and the amino acids were determined on a Beckman 120C analyser. Serine and threonine values are corrected for an assumed 10 and 5% loss during hydrolysis. No cysteine or galactosamine were detected. Tryptophan was not measured. Amino sugars were determined on the analyser after hydrolysis in 4M-HCl at 110°C for 4h. Sialic acid content was determined colorimetrically after hydrolysis in 0.05M-$H_2SO_4$ at 80°C for 1h. Amino sugar and sialic acid values are not corrected for destruction occurring during hydrolysis.

| | Content (mol of residues/mol) |
|---|---|
| Lys | 96 |
| His | 33 |
| Arg | 85 |
| Asp | 110 |
| Thr | 62 |
| Ser | 69 |
| Glu | 167 |
| Pro | 61 |
| Gly | 59 |
| Ala | 75 |
| Val | 70 |
| Met | 24 |
| Ile | 69 |
| Leu | 130 |
| Tyr | 48 |
| Phe | 70 |
| GlcN | 51 |
| Sialic acid | 22 |

of the liver plasma membrane (Evans & Gurd, 1973*a*). Whereas the major protein peak containing components of molecular weight 20000–60000 entered the sucrose gradient slowly, higher-molecular-weight proteins, many of which were glycoproteins, migrated further into the gradient. A peak of 5′-nucleotidase activity was present in some of the fractions, and this enzyme activity was subsequently purified and shown to be a glycoprotein (Evans & Gurd, 1973*a*). Analysis of the higher-molecular-weight components present in fractions collected from the outer edge of the sucrose gradient showed a major protein band, distinct from the band identified as 5′-nucleotidase, which was also periodate–Schiff-positive (Plate 1). These fractions also contained alkaline phosphodiesterase activity, which migrated into the gradients ahead of the 5′-nucleotidase peak (Fig. 1). After concentration of the fractions containing the phosphodiesterase activity, fraction 19 from the zonal rotor gave by gel filtration on a Sephadex G-100 column a protein peak coincident with enzyme activity (Fig. 2). This enzyme was further characterized. The enzyme was also purified from fraction 18, since it was possible to separate the phosphodiesterase and 5′-nucleotidase activities by gel filtration (Evans & Gurd, 1973*b*).

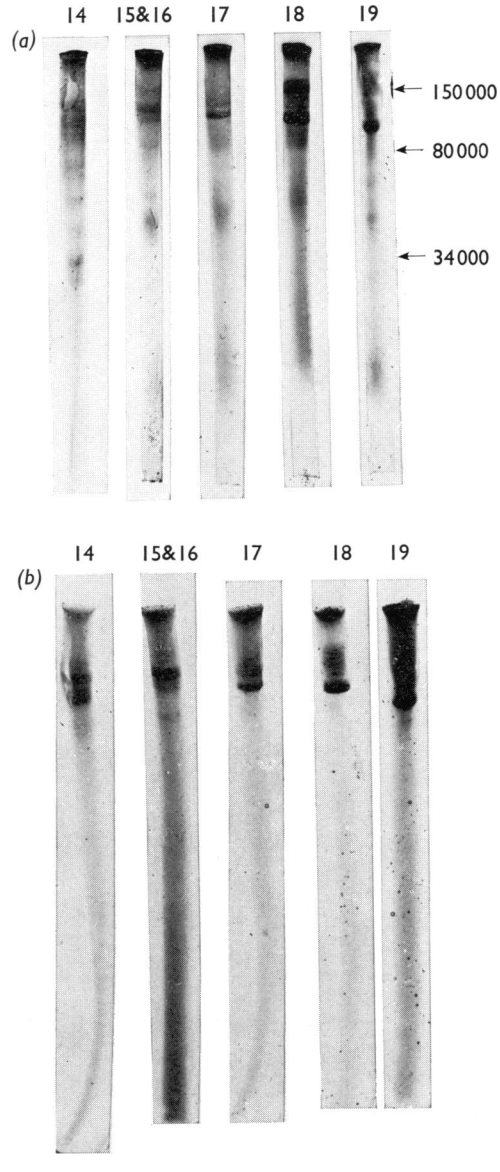
*Characterization of the alkaline phosphodiesterase*

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate–phosphate buffer showed that the enzyme recovered from the Sephadex G-100 column gave a single band when stained for protein or carbohydrate (Plate 2*a*). An exact correspondence between the alkaline phosphodiesterase activity and the protein stained with Amido Black or periodate–Schiff reagent was obtained when the electrophoresis was carried out in polyacrylamide gels in the presence of deoxycholate (Plate 2*a*). The purified enzyme activity therefore appeared to be associated with one glycoprotein. Further evidence of its homogeneity and the correspondence of protein and phosphodiesterase activity was obtained by two-dimensional immunoelectrophoresis in agarose gels containing rabbit anti-(mouse plasma-membrane) serum. One protein band was stained with Amido Black and the enzyme activity as detected by hydrolysis of dTMP *p*-nitrophenyl ester corresponded exactly (Plate 2*b*).

The apparent molecular weight of the polypeptide determined by electrophoresis in sodium dodecyl sulphate–sodium phosphate, pH7.4, gels containing various polyacrylamide concentrations (w/v) was 7.5%, 126000; 10%, 128000; 12.5%, 128000; 15%, 130000. An apparent molecular weight of 130000 was also obtained by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate–Tris–glycine, pH8.9. A slightly lower molecular weight was obtained for the enzyme activity during purification of the enzyme by gel filtration on calibrated Sephadex G-100 columns equilibrated with 0.25% sarcosyl–Tris buffer. However, owing to such unknown factors as the amount of sarcosyl bound and the shape of the sarcosyl–glycoprotein complex, molecular-weight estimations under these conditions are unreliable.

*Substrate specificity of the enzyme*

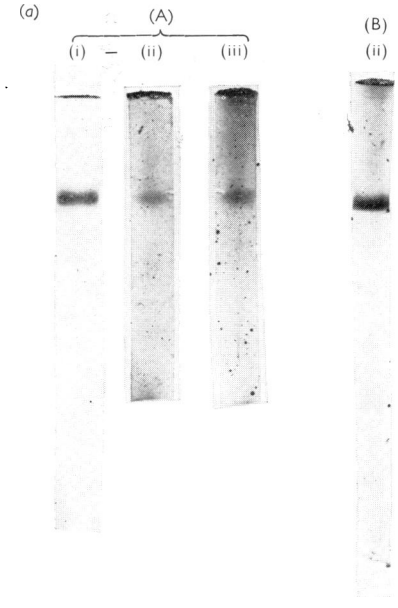
The phosphodiesterase activity approximately doubled when 10mM-$Mg^{2+}$ and $Ca^{2+}$ were added to the incubation medium. The enzyme was strongly inhibited by 1mM-EDTA or -EGTA [ethanedioxy bis(ethylamine)tetra-acetic acid]; the subsequent addition of 10mM-$Mg^{2+}$ only partially restored activity, but 10mM-$Ca^{2+}$ restored the activity to 120%




EXPLANATION OF PLATE 1

*Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate–phosphate buffer, pH* 7.4, *of concentrated samples obtained from the rate-zonal separation in the B XIV rotor*

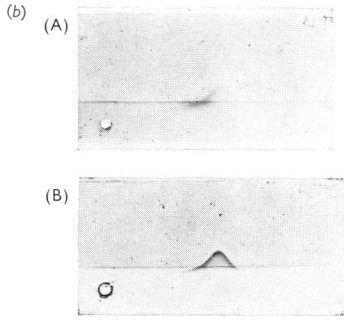
Values correspond to the fractions in Fig. 1. Gels were stained (*a*) for protein with Amido Black and (*b*) for carbohydrate by the periodate–Schiff reagent. The relative position of reoviral molecular-weight markers is indicated.




EXPLANATION OF PLATE 2(*a*)

*Polyacrylamide-gel electrophoresis of the purified enzyme obtained from a Sephadex G-100 column run in (A) deoxycholate–phosphate buffer, pH 8.0, and (B) sodium dodecyl sulphate–phosphate buffer, pH 7.4*

Gels were stained for (i) activity by immersion in a solution of dTMP *p*-nitrophenyl ester, (ii) protein with Amido Black, (iii) carbohydrate by the periodate–Schiff procedure. Further details are included in the Experimental Section.




EXPLANATION OF PLATE 2(*b*)

*Two-dimensional immunoelectrophoresis of the purified enzyme*

Gels were stained (A) for activity by immersion in dTMP *p*-nitrophenol ester and (B) for protein with Amido Black.

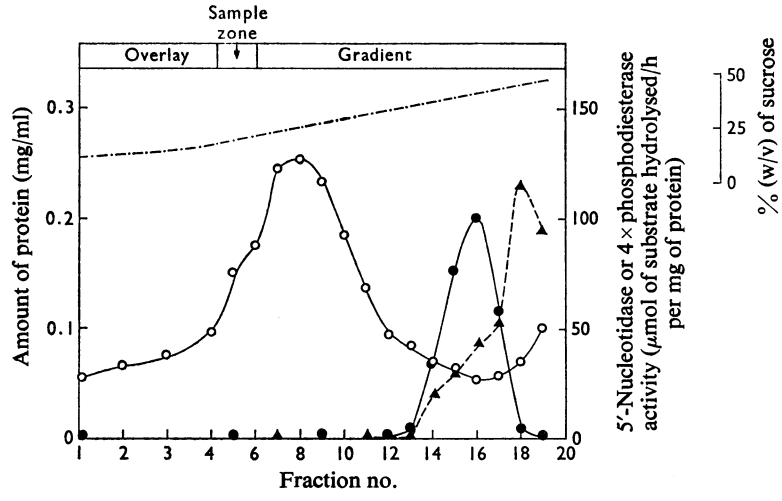



Fig. 1. *Rate-zonal centrifugation profile of a sarcosyl–Tris buffer extract of mouse liver plasma membranes*

Approx. 100mg of plasma-membrane sarcosyl–Tris buffer-extract protein was applied to the gradient. Details of the gradient, sample and overlay volumes are given in the Experimental section. The sample was centrifuged at 43000rev./min for 24h. ○, Protein; ●, 5′-nucleotidase activity; ▲, alkaline phosphodiesterase activity; –·–·–, sucrose.

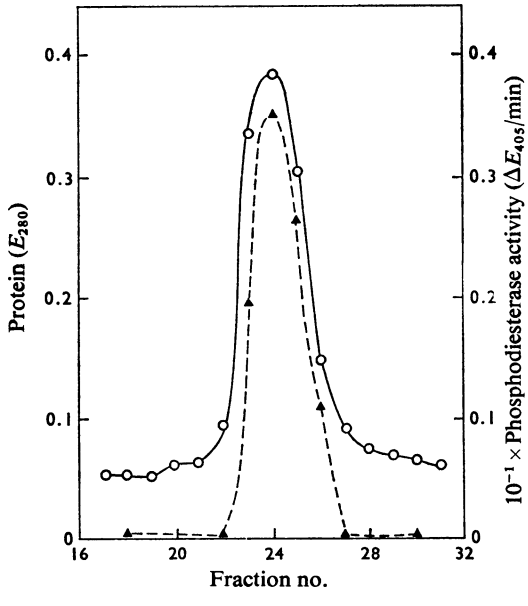



Fig. 2. *Gel filtration on a Sephadex G-100 column of phosphodiesterase peak from zonal rotor (Fig. 1, Fraction 19)*

The column was equilibrated with 0.25% sarcosyl–Tris buffer. Fractions (2ml) were collected. ○, $E_{280}$; ▲, phosphodiesterase activity.

of the initial value. It is concluded that the phosphodiesterase activity is $Ca^{2+}$-dependent, and can be stimulated by $Mg^{2+}$.

The pH–activity profile of the purified phosphodiesterase activity indicated maximum activity at pH9.6 (Fig. 3), in close agreement with the result obtained by Skidmore & Trams (1970) with the $NAD^+$ nucleotide pyrophosphatase of rat liver plasma membranes and with the UDP-galactose pyrophosphatase purified in Triton X-100 from rat liver microsomal fractions by Bachorik & Dietrich (1972).

Comparison of the activity of the isolated glycoproteins towards various substrates indicated that the protein now purified was a nucleotide pyrophosphatase that hydrolysed a number of substrates containing pyrophosphate bonds (Table 1). Variation in the degree of enrichment of specific activities can be attributed to the slight loss of activity resulting from successive freezing and thawing. The purified enzyme was thus active in hydrolysing ATP, NADH, UDP-*N*-acetylglucosamine and UDP-galactose. Surprisingly, no activity was exhibited towards UDP-*N*-acetylmuramylpentapeptide from *Staphylococcus aureus*. The purified enzyme showed low or no activity against RNA, cyclic AMP, ADP, AMP and glycerylphosphorylcholine. The enzyme also showed no alkaline phosphatase or leucine naphthylamidase activity.

To establish whether the same active site on the purified enzyme was reponsible for the hydrolysis

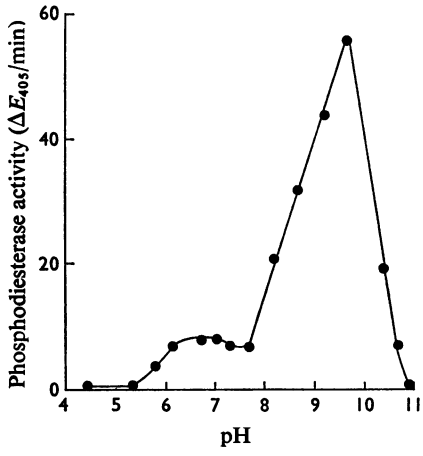



Fig. 3. *pH–activity profile of the purified enzyme*

Alkaline phosphodiesterase activity was investigated at 37°C by using 5mM-dTMP *p*-nitrophenyl ester as substrate. The buffers (0.2M) used were: pH4.0–5.0, sodium acetate–acetic acid; pH5.0–6.7, maleic acid–NaOH; pH6.8–8.7, Tris–HCl; pH8.9–10.0, $Na_2CO_3$–$NaHCO_3$; pH10.0–11.0, $Na_2CO_3$–NaOH.

of phosphodiester and nucleotide pyrophosphate bonds, the effect of the presence of UDP-galactose on the hydrolysis of dTMP *p*-nitrophenyl ester was investigated. The results showed the purified phosphodiesterase had a $K_m$ value of $2.1 \times 10^{-4}$M and was competitively inhibited by UDP-galactose: a $K_i$ of $2.9 \times 10^{-4}$M was calculated (Dixon, 1953).

*Composition of the enzyme*

Amino acid analysis of the enzyme (Table 2) showed a composition similar to that of the total plasma-membrane fractions (Evans, 1970; Evans & Gurd, 1972). Polar amino acids (aspartic acid, glutamic acid, lysine, serine, arginine, threonine, histidine) accounted for 49% of the total composition of the glycoprotein (Table 2), and therefore the isolated enzyme shows higher polarity than some other membrane proteins (Capaldi & Vanderkooi, 1972). Although no cysteine or cystine were detected in the acid hydrolysate, the presence of thiol groups cannot be excluded at this stage, and a definitive answer must await the analysis of the reduced and alkylated product. Quantitative analysis of the glycoprotein by the anthrone method showed it to contain 5% by weight of neutral sugars. Amino sugar analysis showed the presence of glucosamine and the absence of galactosamine. A sialic acid assay indicated the presence of sialic acids. The presence of glucose, mannose, galactose and fucose was shown qualitatively by g.l.c.

## Discussion

*Purification of the enzyme*

The results show that mouse liver plasma membranes contain a major glycoprotein component of apparent molecular weight 130000 which has alkaline phosphodiesterase and nucleotide pyrophosphatase activity. The enzyme was purified from a sarcosyl–Tris buffer extract of liver plasma membranes by taking advantage of its high sedimentation rate in shallow sucrose–detergent gradients. It was previously shown that rate-zonal centrifugation of the sarcosyl–Tris buffer extract of liver plasma membranes separated membrane proteins from glycoproteins (Evans & Gurd, 1973*a*). Although the distribution of proteins and glycoproteins across the sucrose–detergent gradient was shown by polyacrylamide-gel electrophoresis to be related to the molecular weights of the components, with glycoproteins possessing higher molecular weights than the proteins, bound detergent was also implicated in effecting the separation (Evans & Gurd, 1972, 1973*a*). No studies were made of the amount of sarcosyl bound to the purified glycoprotein enzyme, but it is already established from other work that glycoproteins bind less sodium dodecyl sulphate than do proteins (Pitt-Rivers & Ambesi-Impiombato, 1968). A similar rate-zonal procedure also resulted in the purification of the 5′-nucleotidase, which sedimented into the gradient less rapidly than the alkaline phosphodiesterase–nucleotide pyrophosphatase (Evans & Gurd, 1973*a*). Although no direct studies were made to investigate the possible association of membrane lipids with the phosphodiesterase–nucleotide pyrophosphatase, this would appear unlikely, since previous studies on the distribution of phospholipid phosphorus across sucrose–sarcosyl gradients indicated that phospholipids only partially enter the gradient (Evans & Gurd, 1973*a*). No phospholipids were associated with the purified 5′-nucleotidase, and it therefore seems likely that the present enzyme, which migrated further into the sucrose gradient than the 5′-nucleotidase, and is also eluted early on Sephadex columns, is free of membrane lipids.

During the purification of the alkaline phosphodiesterase activity (Table 1) over 90% of the total plasma-membrane enzyme activity and approx. 50% of the enzyme activity present in the sarcosyl–Tris buffer extract was lost. The tendency of many membrane-bound enzymes to lose some or all activity when separated from membrane lipids, as occurs during solubilization with anionic detergents, is well established (see for example Razin, 1972). In this respect, the present enzyme resembles the 5′-nucleo-

tidase in that purification in sarcosyl–Tris buffer resulted in the retention of some of the enzyme activity although phospholipids were unlikely to be associated with the enzyme. This can be regarded as evidence that specific phospholipids are not required for enzyme activity, and that bound detergent can function as a substitute in stabilizing the enzyme when it is removed from its normal membrane hydrophobic environment. The two enzymes, phosphodiesterase–nucleotide pyrophosphatase and 5′-nucleotidase, purified by the procedures described, thus differ from other hepatic membrane enzymes, e.g. UDP-glucuronyltransferase (Vessey & Zakim, 1972) and glucose 6-phosphatase (Garland & Cori, 1972), which cannot function when phospholipids are perturbed by phospholipases or removed by gel filtration of detergent extracts of the membranes. Therefore *N*-dodecylsarcosinate functions as a detergent which solubilizes the majority of the liver plasma-membrane proteins and glycoproteins, but unlike, e.g., sodium dodecyl sulphate at similar concentrations does not denature those membrane enzymes that appear to require amphipathic stabilizers to maintain solubility and activity when removed from the membrane. The maintenance of enzymic activity, although at a lower value, allowed the enzymes to be purified by monitoring activity in parallel with the analytical techniques of polyacrylamide-gel electrophoresis, gel filtration and immunoelectrophoresis. No attempt was made to investigate the stability of the enzyme in the absence of detergent. However, during the purification of a nucleotide pyrophosphatase from Triton-solubilized rat liver microsomal fraction, it was shown by Bachorik & Dietrich (1972) that removal of the detergent resulted in the precipitation of protein and the loss of enzymic activity.

*Substrate specificity*

The polyacrylamide-gel evidence presented suggests that the glycoprotein enzyme now isolated consists of a single polypeptide chain possessing dual specificity towards phosphodiester and nucleotide pyrophosphate substrates. Simultaneous purification of activity towards dTMP *p*-nitrophenyl ester, ATP, $NAD^+$, UDP-galactose and UDP-*N*-acetylglucosamine was obtained. Further, evidence that the same enzyme was active against phosphodiesterase and nucleotide pyrophosphate bonds was indicated by the competitive-inhibition kinetics obtained by using the purified enzyme and plasma membranes. A phosphodiesterase purified from rat liver plasma membranes was also shown to be competitively inhibited by nucleotide pyrophosphate substrates (Decker & Bischoff, 1972). The present results also show that the supposition expressed by Prospero *et al.* (1972) that rat liver plasma-membrane alkaline phosphodiesterase is closely related to, if not identical with, an alkaline ribonuclease and a glycerylphosphorylcholine phosphohydrolase described by Lloyd-Davies *et al.* (1972) is incorrect. The glycoprotein enzyme now purified showed little or no activity towards these substrates. The failure to hydrolyse cyclic AMP is to be expected from the substrate-specificity studies; in addition it is known (Bastomsky *et al.*, 1971) that cyclic phosphodiesterases are soluble intracellular enzymes whereas the present enzyme is membrane bound, and is also located on the outer perimeter of the plasma membrane as demonstrated by chemical-labelling experiments (W. H. Evans, unpublished work).

*Biological role of surface membrane glycoproteins*

Recently it was reported that two hamster cell lines transformed by Rous sarcoma or Simian virus were blocked in their ability to hydrolyse sugar nucleotide pyrophosphate bonds (Sela *et al.*, 1972). The present results characterizing the phosphodiesterase–nucleotide pyrophosphatase as a glycoprotein enzyme located on the plasma membrane support the view (Kalckar & Hakomori, 1972; Sela *et al.*, 1972) that some transformed cells may differ from normal cell lines with respect to the properties of a surface-orientated glycoprotein which can modify the carbohydrate architecture or hydrolyse substrates of sugar transferases implicated in cell adhesion (Roseman, 1970).

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## References

Aminoff, D. (1961) *Biochem. J.* **81**, 384–392
Bachorik, P. S. & Dietrich, L. S. (1972) *J. Biol. Chem.* **247**, 5071–5078
Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
Bastomsky, C. H., Zaharija, M. & McKenzie, J. M. (1971) *Biochim. Biophys. Acta* **230**, 286–295
Capaldi, R. A. & Vanderkooi, G. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 930–932
Decker, K. & Bischoff, E. (1972) *FEBS Lett.* **21**, 95–98
De Lamirande, G., Boileau, S. & Morais, R. (1966) *Can. J. Biochem.* **44**, 273–279
Dixon, M. (1953) *Biochem. J.* **55**, 170–171
Erecinska, M., Sierakowski, H. & Shugar, D. (1969) *Eur. J. Biochem.* **11**, 465–471
Evans, W. H. (1970) *Biochem. J.* **116**, 833–842
Evans, W. H. & Gurd, J. W. (1971) *Biochem. J.* **125**, 615–624
Evans, W. H. & Gurd, J. W. (1972) *Biochem. J.* **128**, 691–700
Evans, W. H. & Gurd, J. W. (1973*a*) *Biochem. J.* **133**, 189–199
Evans, W. H. & Gurd, J. W. (1973*b*) in *Methodological Developments in Biochemistry* (Reid, E., ed.), vol. 3, pp. 121–126, Longman, London

Fanger, M. W. & Smyth, D. G. (1970) *Anal. Biochem.* **34**, 491–499
Fowler, S. & de Duve, C. (1969) *J. Biol. Chem.* **244**, 471–481
Futai, M. & Mizuno, D. (1967) *J. Biol. Chem.* **242**, 5301–5307
Garland, R. C. & Cori, C. F. (1972) *Biochemistry* **11**, 4712–4781
Goldberg, J. A. & Rutenburg, A. M. (1958) *Cancer (Philadelphia)* **11**, 283–291
Gurd, J. W., Evans, W. H. & Perkins, H. R. (1972) *Biochem. J.* **130**, 271–280
Gurd, J. W., Evans, W. H. & Perkins, H. R. (1973) *Biochem. J.* **135**, 827–832
Ipata, P. L. (1967) *Anal. Biochem.* **20**, 30–36
Jacobson, K. B. & Kaplan, N. O. (1957) *J. Biophys. Biochem. Cytol.* **3**, 31–43
Kalckar, H. M. & Hakomori, S. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 1647a
Laurell, C. B. (1965) *Anal. Biochem.* **10**, 358–361
Lieberman, I., Lansing, A. I. & Lynch, W. E. (1967) *J. Biol. Chem.* **242**, 736–739
Lloyd-Davies, K., Michell, R. H. & Coleman, R. (1972) *Biochem. J.* **127**, 357–368
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Maizel, J. V. (1971) in *Methods in Virology* (Maromorosch, K. & Koprowski, H., eds.), vol. 5, pp. 180–246, Academic Press, New York
Mokrasch, L. C. (1954) *J. Biol. Chem.* **208**, 55–59
Ogowa, H., Sawada, M. & Kawada, M. (1966) *J. Biochem. (Tokyo)* **59**, 126–134
Pitt-Rivers, R. & Ambesi-Impiombato, S. (1968) *Biochem. J.* **109**, 825–830
Prospero, T. D., Burge, M. L. E., Norris, K. A., Hinton, R. H. & Reid, E. (1972) *Biochem. J.* **132**, 449–458
Razin, S. (1972) *Biochim. Biophys. Acta* **265**, 241–296
Razzel, W. E. (1963) *Methods Enzymol.* **6**, 236–238
Roseman, S. (1970) *Chem. Phys. Lipids* **5**, 270–297
Schliselfeld, L. H., van Eys, J. & Touster, O. (1965) *J. Biol. Chem.* **240**, 811–818
Sela, B., Lis, H. & Sachs, L. (1972) *J. Biol. Chem.* **247**, 7575–7590
Skidmore, J. R. & Trams, E. G. (1970) *Biochim. Biophys. Acta* **219**, 93–103
Thompson, W. J. & Appleman, M. M. (1971) *Biochemistry* **10**, 311–316
Touster, O., Aronson, N. N., Dulaney, J. T. & Hendrickson, H. (1970) *J. Cell Biol.* **47**, 604–618
Vessey, D. A. & Zakim, D. (1972) *J. Biol. Chem.* **247**, 3023–3028