*PARTIAL PURIFICATION OF A LIPOPROTEIN WITH 5′-NUCLEOTIDASE ACTIVITY FROM MEMBRANES OF RAT LIVER CELLS**

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During the course of studies in this laboratory on the turnover of the membranes of rat liver cells,[1, 2] it was found that a comparison between synthesis and turnover of a single protein present in the membranes of smooth and rough microsomes and in the plasma membrane would be desirable. To this purpose, we decided to isolate and purify a 5′-nucleotidase (E.C.3.1.3.5), since in our hands the total activity of this enzyme was distributed approximately equally between these three fractions. This communication reports the partial purification and some of the properties of this enzyme from isolated fractions of microsomes and plasma membranes. The purest fractions we have obtained show one major band on polyacrylamide gel electrophoresis; the enzyme is a lipoprotein with only one phospholipid, sphingomyelin, associated with it.

Materials and Analytical Procedures.—Reagents: Nucleotides were obtained from Calbiochem (Los Angeles, Calif.) or PL Biochemicals (Milwaukee, Wis.); adenosine deaminase was from Boehringer (New York, N.Y.); 6% Super Ago-gel (66–142 μ) and enzyme-grade sodium deoxycholate, tris(hydroxymethyl)aminomethane (Tris), ammonium sulfate, and urea were from Mann Research Laboratories (New York, N.Y.); 8-C^{14}-adenosine 5′-phosphate (AMP) (21.4 μc/μM) was from Nuclear-Chicago (Des Plaines, Ill.); Triton X-100 and sphingomyelin were generous gifts from Rohm and Haas (Philadelphia, Pa.) and General Biochemicals (Chagrin Falls, Ohio), respectively. All other chemicals were analytical-grade commercial products.

Cell fractionation: The techniques for the isolation of plasma membranes and total microsomes are described in detail elsewhere.[3] After gentle homogenization of the liver, plasma membranes were isolated by centrifugation at 1,000 *g*, followed by two flotation steps, the first in 1.6 *M* sucrose–1 mM $MgCl_2$ and the second in 1.45 *M* sucrose–1 mM ethylenediaminetetraacetate (EDTA). The 1,000 *g* supernate was centrifuged at 10,000 *g* for 10 min to eliminate mitochondria; the resulting supernate was further centrifuged at 100,000 *g* for 1 hr to obtain the microsome fraction.

Assay of 5′-nucleotidase: Three assays were used to measure the activity of the enzyme. The routine assay measured the release of inorganic phosphate from AMP and was carried out in a volume of 0.3 ml containing 100 mM Tris-HCl (pH 8.5), 10 mM AMP, and 10 mM $MgCl_2$; after incubation at 37° for 20 min, the reaction was stopped by the addition of 0.7 ml of a solution containing 1 part 10% ascorbic acid and 6 parts 0.42% ammonium molybdate in 1 *N* H_2SO_4.[4] After incubation at 45° for 20 min the color was read either at 820 mμ (directly) or at 795 mμ (after extraction into 1 ml of isoamyl alcohol).

The second assay measured the release of C^{14}-adenosine from C^{14}-AMP. The reaction was stopped by the addition of perchloric acid to a final concentration of 0.2 *N;* the denatured protein was removed by centrifugation and the perchlorate precipitated by neutralization with KOH at 0°. Ten μl of the supernatant was spotted on Whatman 3 MM paper, together with carrier adenosine and AMP; the adenosine was separated by descending chromatography for 6–8 hr in *n*-butanol/H_2O, 86:14, and the components were visualized under UV light. The spots containing adenosine and AMP were cut out and the radioactivity was determined by scintillation counting in Bray's solution[5] in a Nuclear-Chicago counter. The radioactivity on the chromatogram was recovered quantita-

tively in the adenosine and AMP spots, and the extent of reaction was determined by the ratio of radioactivity in adenosine to that in AMP.

In the third procedure, the 5'-nucleotidase reaction was carried out in the presence of excess adenosine deaminase and the adenosine formed was determined spectrophotometrically at 263 mμ in a Carey model 14 spectrophotometer.[6] The reaction was carried out in a final volume of 1.0 ml, containing 100 mM Tris-HCl (pH 8.5), 3 μg adenosine deaminase, and various concentrations of substrate, inhibitors, and metal ions.

Polyacrylamide gel electrophoresis: The system was based on that of Takayama *et al.*[7] Glass tubes, 6 mm internal diameter and 12.5 cm long, were filled with 1.9 ml of a solution containing 5% acrylamide, 35% acetic acid, 0.22% bis-acrylamide, 0.6% tetramethylene diamine, and 0.8 mg % riboflavin in 8 *M* urea. Then 75% acetic acid was layered over the solution, and the acrylamide polymerized by illuminating the gels with 400 ft-c from a fluorescent lamp. Protein samples were precipitated with 10% aqueous acetone at −20° and dissolved in phenol/acetic acid/water containing 2 *M* urea. Electrophoresis was carried out in a Canalco model 12 apparatus, at a constant current of 3 mA per gel for 3 hr.

Phospholipid characterization: Phospholipids were extracted from plasma membranes and total microsomes as described elsewhere.[1] The extraction of phospholipid from purified enzyme fractions was preceded by the removal of Triton; this was carried out by lyophilizing the samples and then extracting them with acetone saturated at −20° with $MgCl_2$. Three systems were used for the chromatographic separation of phospholipids, the routine method being that of Skipski *et al.*[8] The results from this procedure were confirmed by the technique of Rouser *et al.*[9] and also by a two-dimensional system using Kodak 301 R2 plates with chloroform/methanol/ammonia, 65/30/4, as the first solvent and chloroform/methanol/acetic acid/water, 70/25/25/6, as the second. The procedure for the recovery of phospholipids from thin-layer plates is described elsewhere.[3]

Analysis of protein and phospholipid: Protein was determined by the procedure of Lowry *et al.*,[10] with crystalline bovine serum albumin as a standard. For the analysis of fractions containing low concentrations of protein relative to detergent, the protein was first precipitated by 10% aqueous acetone at −20°. Inorganic phosphate was determined in phospholipid samples as described elsewhere.[3]

Results.—The initial studies on the purification of the enzyme were facilitated by the work of Song and Bodansky.[11] In preliminary experiments it was found that the activity was stable only in the presence of AMP and Mg^{2+}, and also that there was a marked tendency for the enzyme to form insoluble aggregates. The procedure described below results in the greatest purification with the minimum loss of activity. The purification is shown quantitatively in Table 1.

Purification from total microsomes: Unless stated otherwise, all operations were carried out at 0–4°. Microsomes were suspended to a final concentration of ~1 gm tissue equivalent/ml (~30 mg protein/ml) in a solution containing 0.1 *M* Tris-HCl, pH 7.5, 10 per cent saturated ammonium sulfate, 1 mM $MgCl_2$, 5 mM AMP, 1 per cent sodium deoxycholate (adjusted to pH 7.5 before addition), and 2 per cent Triton, the detergent mixture being added last (fraction I).

pH fractionation: After fraction I had been stirred magnetically for 10 minutes, the pH was adjusted to 5.2 by the addition of 0.2 *M* acetic acid in 10 per cent saturated ammonium sulfate. The suspension was stirred for 10 minutes and then centrifuged for 20 minutes at 40,000 rpm in the Spinco 40 rotor. The supernatant was discarded, and the loosely packed pellet, which contained nearly all the enzyme activity together with the deoxycholate, was suspended to the same final volume as fraction I in a solution containing 0.1 *M* Tris-HCl (pH 7.5), 10 per cent saturated ammonium sulfate, 1 mM $MgCl_2$, 5 mM AMP, and 1 per

TABLE 1. *Purification of 5'-nucleotidase from microsomes and plasma membranes.*

Fractionation and purification step			Volume (ml)	Total activity (units)	Specific activity (units/mg)
Microsomes	I	Total microsomes	100	190	0.09
	II	pH 5.2	100	174	0.24
	III	First ammonium sulfate	100	149	0.29
	IV	Heat	96	121	0.62
	V	Second ammonium sulfate	5	112	2.7
	VI	Agarose	2.5	43	29
Plasma membranes	I	Plasma membranes	20	77	0.46
	II	Supernatant	20	75	0.48
	III	First ammonium sulfate	25	61	0.72
	IV	Heat	24	58	1.21
	V	Second ammonium sulfate	2.0	46	4.2
	VI	Agarose	2.0	29	35

The material was obtained from 100 gm liver. The unit of activity is 1 μmole P_i released from AMP/min.

cent Triton. The pH of the suspension was adjusted to 7.5 with a few drops of *N* NaOH, after which the suspension was sonicated for 20 seconds at full power with a Branson LS75 sonifier (fraction II).

First ammonium sulfate fractionation: Fraction II was stirred magnetically and solid ammonium sulfate (15 gm/100 ml) added slowly to give a final concentration of about 35 per cent saturated; the suspension was stirred for 10 minutes and then centrifuged at 20,000 rpm for 10 minutes in the Lourdes 9RA rotor. After centrifugation, the insoluble material, which contained all the enzyme activity together with both the Triton and deoxycholate, formed a plug at the surface; the solution beneath was removed with a syringe. The material was suspended to the same final volume as fraction I in a solution containing 0.1 *M* Tris-HCl (pH 7.5), 1 mM $MgCl_2$, and 5 mM AMP; assuming that the volume of the plug was 35 per cent saturated in ammonium sulfate, we adjusted the suspension to a final concentration of 10 per cent saturated ammonium sulfate. The suspension was stirred for 10 minutes and then sonicated for 20 seconds as described above (fraction III).

Heat treatment: Fraction III was heated at 50° for 5 minutes, cooled to 0°, sonicated for 20 seconds, and then centrifuged at 40,000 rpm for 10 minutes in the Spinco 40 rotor. The enzyme activity was recovered in the supernate (fraction IV).

Second ammonium sulfate fractionation: Fraction IV was stirred magnetically and saturated ammonium sulfate added dropwise. At a concentration of about 27 per cent saturation (this could not be determined precisely because of the difficulty in knowing the exact concentration in fraction III), the solution became opaque due to the precipitation of Triton; when the concentration was increased to an estimated 30–31 per cent, a heavy precipitate formed which contained the deoxycholate and the bulk of the protein. After this point was reached, the

suspension was stirred for 10 minutes, and then centrifuged for 10 minutes at 20,000 rpm. The soluble fraction, which contained the enzyme activity, was removed with a syringe as described above, solid ammonium sulfate (20 gm/100 ml) was added to give a final concentration of 60 per cent saturated, and the enzyme activity was recovered in the surface plug whcih was obtained after centrifugation for 10 minutes at 20,000 rpm. The material was dissolved in a minimum volume of a solution containing 0.1 *M* Tris-HCl (pH 7.5), 0.1 mM $MgCl_2$, and 1 mM AMP (fraction V).

Agarose chromatography: A column (50 × 1.8 cm) of 6 per cent agarose was equilibrated with a solution containing 1 per cent Triton, 0.05 *M* Tris-HCl (pH 7.5), 0.05 M KCl, 0.1 mM $MgCl_2$, and 1 mM AMP. Fraction V was sonicated for 10 seconds and loaded on the column; the column was run in the cold room under a hydrostatic pressure of 30 cm at a flow rate of 10 ml/hr. Fractions of 2 ml were collected automatically with an LKB UltroRac fraction collector. The major part of the enzyme activity was eluted as a peak with essentially constant specific activity shortly after the dead volume (Fig. 1*A*). The second minor peak was probably a degradation product of the enzyme, since the proportion of the activity in this peak was increased when fraction I was left overnight at 0° before fractionation. The enzyme was freed from the bulk of the Triton in

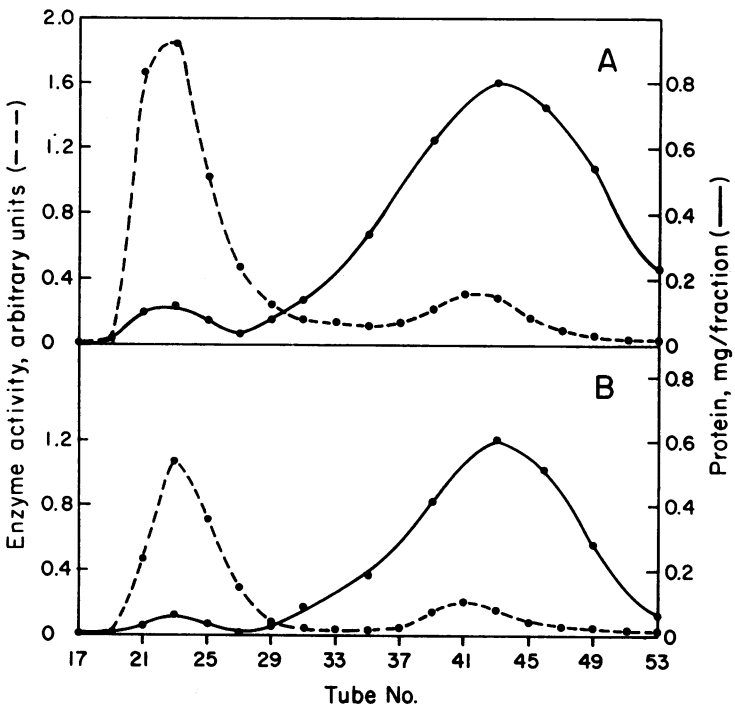

FIG. 1.—The elution pattern of fraction V from agarose. Enzyme activity was determined directly with 0.01-ml samples of the fractions (this gave a final Triton concentration in the assay which was slightly inhibitory), and protein was determined in suitable samples after acetone precipitation.

(*A*) Microsome fraction V. (*B*) Plasma membrane fraction V.

the same manner as described for the second ammonium sulfate fractionation (fraction VI).

Purification from plasma membranes: Plasma membranes were suspended in the same medium as the microsomes, sonicated for 20 seconds (fraction I), and centrifuged at 40,000 rpm for 20 minutes in the Spinco 40 rotor. The enzyme was purified from the supernatant (fraction II) exactly as described for the microsomes, except that the pH fractionation was omitted. The pattern of the elution of enzyme activity and protein from the agarose column is shown in Figure 1*B*.

It was found desirable to carry the purification of the enzyme from the microsomes through to the agarose chromatography in one day; the plasma membrane fraction could be left overnight after the heat treatment. If these precautions were not taken, the agarose chromatography was not reproducible.

Characterization of the purified fractions: The purified fractions from the microsomes and plasma membranes had essentially the same specific activity (Table 1). When the protein in the fractions was examined by polyacrylamide gel electrophoresis, both fractions were found to contain one major component (Fig. 2); for purposes of comparison, the pattern obtained after electrophoresis of the total protein of microsomes and plasma membranes is also shown. It has not yet been possible to determine whether the major protein component is responsible for the enzymatic activity, since further attempts at purification have not been successful. However, earlier work[1] has indicated that it is possible to

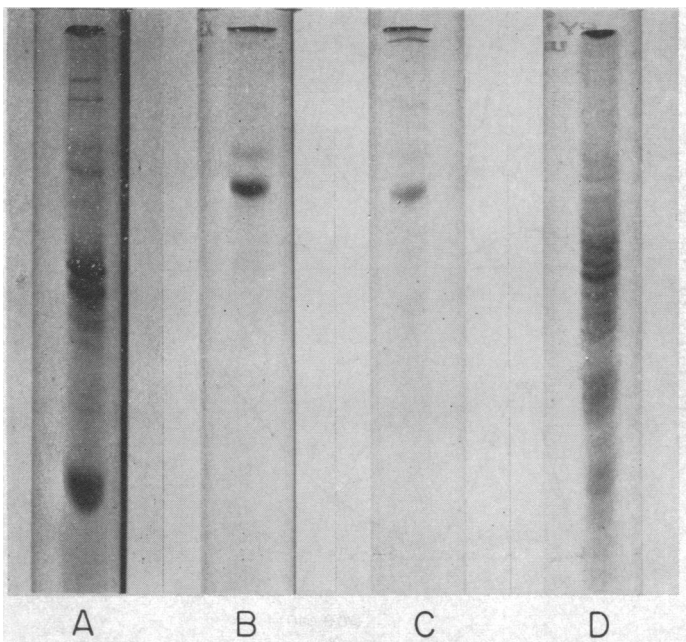

FIG. 2.—Polyacrylamide gel electrophoresis of proteins from the fractions. Experimental conditions were as described in the text. (*A*) Total plasma membrane protein; (*B*) plasma membrane fraction VI protein; (*C*) microsome fraction VI protein; (*D*) total microsomal protein.

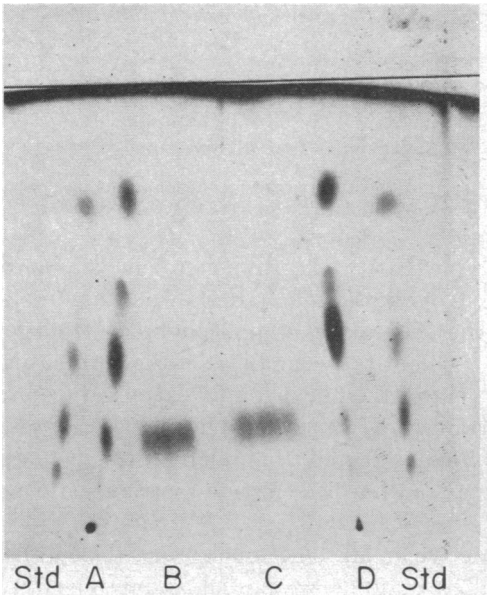

FIG. 3.—Thin-layer chromatography of the phospholipids from the fractions. Experimental conditions were as described in the text. The four standard (*Std*) phospholipids are, from the top down: phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine. (*A*) Total plasma membrane phospholipid; (*B*) plasma membrane fraction VI phospholipid; (*C*) microsome fraction VI phospholipid; (*D*) total microsomal phospholipid.

obtain essentially pure enzymes from microsomes after a 100–150-fold purification; hence the 300-fold purification obtained here (Table 1) would indicate that it is likely that the major component on the gel is responsible for the enzyme activity.

The two fractions contained phospholipid in addition to protein (Table 2). The phospholipid-to-protein ratio was similar in each fraction and was threefold greater than that observed in microsomes or plasma membranes. The phospholipids were extracted and chromatographed as described above; in each of the three systems studied, the phospholipid showed one major spot which cochromatographed with a sphingomyelin standard. Further evidence that the phospholipid was indeed sphingomyelin was obtained after alkaline hydrolysis (0.4 *N* KOH in 90% methanol for 2 hr at 37°); after this treatment, which hydrolyzes all other phosphatides found in these membranes except sphingomyelin, the material

TABLE 2. *Protein and phospholipid content of the fractions.*

Fraction	Mg protein/μmole lipid phosphorus*
Microsomes	1.8
Microsome fraction VI	0.57
Plasma membranes	2.05
Plasma membrane fraction VI	0.66

* These values may be converted to the approximate value of mg protein/mg phospholipid by multiplying by 1.3.

TABLE 3. *Activity of the purified enzyme with various substrates.*

AMP	100%	2′-AMP	5%
UMP	106%	3′-AMP	<1%
CMP	85%	3′5′-AMP	<1%
GMP	33%	Glucose-6-phosphate	<1%
IMP	32%	β-Glycerophosphate	<1%

The activities were determined by the release of phosphate as described in the text. The substrate concentration was 5 mM. Similar results were obtained at other substrate concentrations.

again cochromatographed with the sphingomyelin standard. The results obtained with the system of Skipski *et al.*[8] are shown in Figure 3; this chromatogram also demonstrates the separation of phospholipids from the microsomes and plasma membranes. Quantitative analysis of the chromatograms demonstrated that at least 90 per cent of the phospholipid extracted from the purified enzyme fractions was sphingomyelin. Since this phosphatide represents only 5 per cent of the total phospholipid extracted from microsomes and 25 per cent of that from the plasma membranes, our purified enzyme represents a concentration of sphingomyelin.

The enzyme was active on all the 5′-nucleotides tested (Table 3); it was found in addition that uridine, cytidine, guanosine, and inosine 5′-phosphates inhibited the hydrolysis of AMP competitively (Fig. 4), which suggested that the same

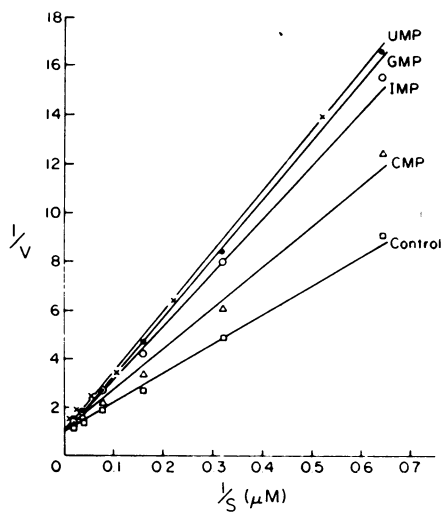

FIG. 4.—Kinetics of the inhibition of the hydrolysis of AMP by other 5′-nucleotides. The activity was measured by the adenosine deaminase assay. The *Km* for AMP was 12 μM; the *Ki*'s for the other 5′-nucleotides were: UMP, 9 μM; GMP, 10 μM; IMP, 12 μM; and CMP, 51 μM.

active site was responsible for the hydrolysis of all the nucleotides. Our analysis of the characteristics of the purified enzyme is in general agreement with reports from workers in other laboratories[11–13] who have studied the activity either in cell fractions or in partially purified fractions thereof. These results will be presented in detail elsewhere.

Discussion.—Our results show that the 5′-nucleotidase of rat liver microsomes and plasma membranes may be obtained in a highly purified form as a lipoprotein containing essentially only one phospholipid, sphingomyelin. The possibility of a relationship between this phosphatide and the enzyme activity is

being investigated. Although there is already evidence associating specific phospholipids with the activity of membrane-bound enzymes (see, for example, refs. 14–16), this is, as far as we are aware, the first report of the purification of a membrane enzyme in association with a specific phospholipid.

It is possible that the purification techniques described here, which are simply the procedures of classical enzymology carried out in the presence of detergent, may be applicable to the purification of other membrane lipoproteins.

The enzyme that we have isolated from the microsomes and plasma membranes differs markedly in activity, substrate specificity, and *Km* from a 5′-nucleotidase purified from a rat liver acetone powder[17] or from sheep brain.[6] The physiological significance of these activities in the liver remains to be determined.

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[1] Omura, T., P. Siekevitz, and G. E. Palade, *J. Biol. Chem.*, **242**, 2389 (1967).

[2] Widnell, C. C., and P. Siekevitz, *J. Cell Biol.*, **35**, 142A (1967).

[3] Stein, Y., C. C. Widnell, and O. Stein, *J. Cell Biol.*, in press.

[4] Chen, P. S., T. Y. Toribara, and H. Warner, *Anal. Chem.*, **28**, 1756 (1956).

[5] Bray, G. A., *Anal. Chem.*, **32**, 238 (1960).

[6] Ipata, P. L., *Biochemistry*, **7**, 507 (1968).

[7] Takayama, K., D. H. MacLennan, A. Tzagoloff, and C. D. Stoner, *Arch. Biochem. Biophys.*, **114**, 223 (1966).

[8] Skipski, V. P., R. F. Peterson, and M. Barclay, *Biochem. J.*, **90**, 374 (1964).

[9] Rouser, G., G. Kritchevsky, and D. Heller, *J. Am. Oil Chem. Soc.*, **42**, 215 (1965).

[10] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[11] Song, C. S., and O. Bodansky, *Biochem. J.*, **101**, 5C (1966).

[12] Segal, H. L., and B. M. Brenner, *J. Biol. Chem.*, **235**, 471 (1960).

[13] Song, C. S., and O. Bodansky, *J. Biol. Chem.*, **242**, 694 (1967).

[14] Fleischer, S., G. Brierly, H. Klouwen, and D. G. Slautterback, *J. Biol. Chem.*, **237**, 3264 (1962).

[15] Jones, P. D., and S. J. Wakil, *J. Biol. Chem.*, **242**, 5267 (1967).

[16] Duttera, S. M., W. L. Bryne, and M. C. Ganoza, *J. Biol. Chem.*, **243**, 2216 (1968).

[17] Itoh, R., A. Mitsui, and K. Tsushima, *J. Biochem.*, **63**, 165 (1968).