ISOLATION OF RAT LIVER PLASMA MEMBRANES

Use of Nucleotide Pyrophosphatase and Phosphodiesterase I as Marker Enzymes

OSCAR TOUSTER, N. N. ARONSON, JR., JOHN T. DULANEY, and HERMAN HENDRICKSON

From the Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37203. Dr. Aronson's present address is the Department of Biochemistry, Frear Laboratories, Pennsylvania State University, University Park, Pennsylvania 16802, and Dr. Hendrickson's present address is the Department of Neurology, University of Miami Medical School, Miami, Florida 33124.

ABSTRACT

Nucleotide pyrophosphatase and phosphodiesterase I of rat liver have been found to be localized primarily in cell particulates highly enriched with respect to the most commonly accepted plasma membrane marker, 5′-nucleotidase, and therefore should themselves be assigned a plasma membrane localization. The observation that plasma membranes sediment in isotonic sucrose with both nuclear and microsomal fractions was exploited to obtain plasma membrane preparations from each fraction. Both preparations are similar in chemical and enzymic composition. Moreover, the preparative method developed in this study appears to give the best combination of yield, purity, and reproducibility available. The question of the possible identity of nucleotide pyrophosphatase and phosphodiesterase I is considered, and evidence is presented suggesting that these activities may be manifestations of the same enzyme.

INTRODUCTION

One of the more active areas of biochemical research, in which much progress recently has been made, is the study of membranes, including the surface membranes of cells. It is evident, from the number of papers which have appeared in the past few years on the isolation of plasma membranes from rat liver, that there is lacking a reproducible procedure for preparing this subcellular fraction in high yield and purity. We wish to offer such a procedure, which has heretofore been presented only in preliminary form (1).

Many preparations of plasma membranes employ the Neville procedure (2) as modified by Emmelot *et al.* (3), whereby the liver is homogenized in 0.001 M bicarbonate buffer (4–7), while other methods utilize isotonic sucrose for the homogenization medium (8–11). The difficulty in evaluating the quality and yield of the membrane product obtained in most of the reported procedures stems from their failure to give a complete analysis of the entire isolation scheme, including recoveries (see de Duve [12]), instead of only analyses of the final product. In general, 5′-nucleotidase is used as the indicator enzyme for the plasma membrane fraction. A wide range of relative specific activities and yields has been reported.

The present report stems from an examination, begun several years ago, of the subcellular distribution of nucleotide pyrophosphatase, an enzyme which has been reported to occur in nuclei (13), microsomes (14), and plasma membranes (3). When liver homogenates are fractionated in isotonic sucrose according to Hogeboom (15), both phosphodiesterase I (16) and nucleotide pyrophosphatase (17) are found mainly in the nuclear and microsomal fractions. We found, however, that when purified nuclei are prepared in 2.1 M sucrose according to Chauveau (18), less than 10% of these two activities are present in the nuclear fraction. Further investigation of the nuclei-free particulate fraction showed that the two activities followed 5′-nucleotidase in sucrose gradients and therefore should be assigned a plasma membrane localization. In connection with these enzymatic studies, an improved procedure was developed for the isolation of rat liver plasma membranes, based on the initial removal of nuclei followed by fractionation of the remaining subcellular particulates in a sucrose density gradient. Subsequently, a superior and more reproducible procedure was developed, based on the purification of the two plasma membrane fractions (nuclear and microsomal) obtained by differential centrifugation of liver homogenates in isotonic sucrose. In addition to its advantages as a preparative method for plasma membranes, this procedure employing isotonic sucrose permits a more complete characterization of all other subcellular organelles during the same experiment.

This paper therefore offers (*a*) a new procedure for the preparation of plasma membranes in high yield and purity, (*b*) the demonstration that liver plasma membrane fragments sedimenting with nuclei and microsomes in classical procedures, involving differential sedimentation of homogenates prepared in isotonic sucrose, are very similar in composition, (*c*) evidence that the major site of nucleotide pyrophosphatase and phosphodiesterase I is the plasma membrane, and (*d*) studies concerned with the question as to whether the latter two activities are attributable to a single enzyme.

MATERIALS AND METHODS

Analytical Procedures

Plasma membrane fractions (P_2 or N_2) were washed as follows before chemical analysis. Fractions containing 4 mg of protein were diluted with cold distilled H_2O to a volume of 30 ml and centrifuged at 30,000 rpm for 1 hr in the Spinco No. 30 rotor (Spinco Division, Beckman Instruments, Inc., Palo Alto, California). The pellets were resuspended in the same volume of water and recentrifuged at 30,000 rpm for 1 hr. Finally, the washed pellets were resuspended in distilled water at an approximate protein concentration of 1.4 mg/ml. Aliquots of this suspension were analyzed chemically in the following manner for protein, cholesterol, phospholipid, DNA, RNA, and sialic acid.

Protein was determined by the method of Miller (19), with bovine serum albumin as a standard. Lipids were extracted from approximately 0.7 mg of washed plasma membrane protein (fractions P_2 and N_2) by the method of Bligh and Dyer (20). The chloroform layer containing the lipids was dried with a stream of nitrogen and redissolved in a known volume of chloroform:methanol, 1:2 (v/v), and aliquots were removed, dried with nitrogen, and assayed for total cholesterol by the procedure of Searcy *et al.* (21) and for total organic phosphate by the procedure of Galliard *et al.* (22). N-acetylneuraminic acid was measured by the Warren procedure (23) after hydrolyzing washed membrane samples for 1 hr at 80°C in 0.1 M H_2SO_4. DNA and RNA were extracted according to Maggio *et al.* (24) and determined as described by Schneider (25).

Enzyme Assays

5′-Nucleotidase was assayed at 37°C in a total volume of 0.5 ml containing 0.005 M adenosine monophosphate (AMP), 0.01 M $MgCl_2$, and 0.05 M glycine buffer adjusted to pH 9.1 with NaOH. The reaction was stopped after 10–30 min by the addition of 2.5 ml of 8% trichloroacetic acid (TCA). The solution was filtered through Whatman No. 42 paper, and a 1.8 ml aliquot of the filtrate was assayed for inorganic phosphate by the method of Fiske and SubbaRow (26). Under the above conditions of assay, there was no phosphate released from β-glycerol phosphate by plasma membrane fractions, and *p*-nitrophenyl phosphate was hydrolyzed at only 1% of the rate of AMP hydrolysis.

Phosphodiesterase I was assayed at 37°C in a total volume of 0.5 ml containing 0.001 M *p*-nitrophenyl 5′-thymidylate and 0.02 M tris(hydroxymethyl)amino methane–HCl buffer, pH 9.0. After 10–15 min the reaction was stopped by the addition of 1.5 ml of 8% TCA. The protein precipitate was removed by centrifugation and a 1.0 ml aliquot of the supernatant fluid was added to 3.0 ml of an alkaline buffer consisting of 0.133 M glycine, 0.083 M Na_2CO_3, and 0.067 M NaCl, adjusted to pH 10.7 with NaOH. The optical density at 400 nm was measured and compared with standard *p*-nitrophenol

in order to determine the amount of substrate hydrolyzed.

Glucose-6-phosphatase was assayed according to the method of de Duve *et al.* (27).

Nicotinamide adenosine dinucleotide phosphate (NADPH)–cytochrome *c* oxidoreductase and succinate–cytochrome *c* oxidoreductase were assayed according to the methods of Sottocasa *et al.* (28).

Cytochrome oxidase was assayed according to the spectrophotometric method of Appelmans *et al.* (29).

N-acetyl-β-D-glucosaminidase was assayed at 37°C in 0.5 ml of 0.1 M acetate buffer, pH 4.0, which was 0.005 M in *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide. The enzyme was preincubated for 5 min and then the substrate was added to begin the reaction. 1.5 ml of 8% TCA was added to stop the reaction after a 15–30 min incubation, and the precipitated protein was removed by centrifugation. The amount of *p*-nitrophenol released was determined on a 1 ml aliquot as described above (see phosphodiesterase I assay).

Monoamine oxidase was determined spectrophotometrically according to the procedure of Schnaitman *et al.* (30).

Nonspecific phosphodiesterase was determined according to Lansing *et al.* (6), with bis(*p*-nitrophenyl) phosphate as substrate.

Nucleotide pyrophosphatase was determined according to the procedures of Schliselfeld *et al.* (17) for various nucleotide pyrophosphates.

Aryl sulfatase was determined according to Horvat and Touster (31).

Electron Microscopy

Plasma membrane fractions P_2 or N_2 were diluted with cold 0.25 M sucrose to a final sucrose concentration of 8–10% and centrifuged at 40,000 rpm for 60 min. The pellets were fixed in 1% osmium tetroxide in Palade's buffer, pH 7.6 (32), dehydrated in graded alcohols and propylene oxide, and embedded in Epon 812 (Shell Chemical Company, N.Y.) (33) in flat embedding molds. Sections were made through the entire pellet on an LKB Ultrotome III (LKB Instruments, Inc., Rockville, Md.). After stirring with 7% uranyl magnesium acetate and lead hydroxide (34), the sections were examined in a Hitachi HU 11B electron microscope (Perkin-Elmer Corp. Norwalk, Conn.)

Materials

AMP, *p*-nitrophenyl phosphate, glucose-6-phosphate, and *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide were purchased from Sigma Chemical Co., St. Louis, Mo. *p*-Nitrophenyl 5′-thymidylate was obtained from Calbiochem, Los Angeles, Calif. Cytochrome *c* and cholesterol were purchased from Mann Research Labs. Inc., New York. All other chemicals were of reagent grade and were purchased from commercial sources.

RESULTS

Preliminary Studies Indicating That Nucleotide Pyrophosphatase and Phosphodiesterase I are Primarily Localized in Plasma Membranes

Our initial experiments involving the preparation of nuclei in 2.1 M sucrose (18) demonstrated that, contrary to previous reports, this fraction contains little nucleotide pyrophosphatase (35). In reinvestigating the question of the subcellular localization of this enzyme, the report of Emmelot *et al.* (3) commenting on the high specific activity of the enzyme in plasma membranes as compared with microsomes had limited value to us, since the absence of information on the yield of plasma membranes precluded reaching conclusions regarding the distribution of the major portion of nucleotide pyrophosphatase within the cell. In a complete and systematic fractionation of homogenates freed of nuclei, the remaining cell organelles were subjected to fractionation in a discontinuous sucrose density gradient buffered at pH 8.0 with 0.005 M Tris-HCl. After centrifugation, 5′-nucleotidase, nucleotide pyrophosphatase, nonspecific phosphodiesterase, and phosphodiesterase I activities were found to be highly concentrated in a peak fraction near density 1.14, well separated from marker enzymes for microsomes (glucose-6-phosphatase), mitochondria (succinic dehydrogenase), and lysosomes (aryl sulfatase). The over-all yield of the four enzymes was almost 40% of the homogenate activities, and their specific activities were 14- to 30-fold greater than found in the homogenate (35). However, as our work progressed, in the hands of different workers this method was found to lack reproducibility in regard to yield of the membrane preparation. Details of this procedure are available elsewhere (35). In those experiments important to the conclusions of this report in which this isolation procedure was successfully used, the relative specific activities of the membrane preparation employed will be given. Modification of this method has led to the new procedure for the preparation of plasma membranes described in detail below. It has proven to be reproducible by laboratory personnel other than the authors.

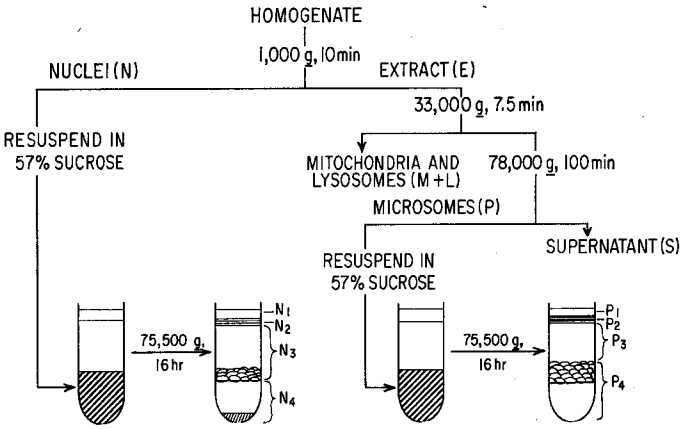

FIGURE 1 Isolation of plasma membranes (N_2 and P_2) from nuclear and microsomal fractions obtained from rat liver homogenate in isotonic sucrose. See Materials and Methods.

A New Procedure for the Preparation of Rat Liver Plasma Membranes

This method involves the preliminary isolation of the nuclear and microsomal fractions from a rat liver homogenate prepared in isotonic sucrose. Each of these fractions is then used for the preparation of plasma membranes by flotation through a discontinuous sucrose density gradient. The procedure is schematized in Fig. 1. It is described below rather fully because careful attention to detail is required for reproducibility.

All sucrose solutions were 0.005 M in Tris and were adjusted to pH 8.0 with 2 N HCl at room temperature. The solutions for the sucrose gradients were prepared on the basis of the percentage by weight of sucrose, and the final values were checked at 25°C in an Abbe-3L refractometer (Bausch and Lomb Incorporated, Rochester, N.Y.). All homogenizations were performed in a Potter-Elvehjem glass homogenizer (size C, A. H. Thomas, Philadelphia, Pa.) with a teflon pestle turning at a speed of 1,000 rpm. Calculation of the volumes of all solutions used for, and of all fractions obtained from, sucrose gradients were made from values for the weight and the density of the solution or fraction. The density of each sample at 5°C was obtained from the Handbook of Biochemistry (36) after measuring its sucrose concentration in the refractometer. All centrifugal forces are those at the center of the centrifuge tube. All centrifugations were performed at 2°C.

PREPARATION OF NUCLEAR (N) AND MICROSOMAL (P) FRACTIONS: Two male rats, of either the Sprague-Dawley or the Wistar strain, each weighing from 200 to 300 g, were fasted for 20 hr. After the animals were decapitated, their livers were removed and placed into cold 0.25 M sucrose. The livers were weighed and then perfused with cold 0.25 M sucrose via the hepatic artery until totally blanched. Normally about 20 g of liver were processed. The livers (kept cold throughout this processing) were minced on an ice-cold glass plate, and, in order to remove connective tissue, were then passed through a tissue press (Arbor tissue press, coarse sieve, Harvard Apparatus Co., Millis, Mass.). Three volumes of 0.25 M sucrose per g of liver were added to the pulverized tissue, and the suspension was then mixed with a spatula and homogenized with one up-and-down stroke of the pestle. The nuclear fraction (N) was separated by centrifugation at 2,000 rpm (1,000 *g*) for 10 min in the International rotor No. 269 (International Equipment Co., Needham Hts., Mass.). The supernatant fluid (E) was poured off and saved, while the pellet was resuspended in the same initial volume of 0.25 M sucrose and rehomogenized with one up-and-down stroke of the pestle. The nuclear fraction was again separated as before and the supernatant liquids were combined. Rehomogenization and recentrifugation of the nuclear pellet were performed once more. The separation of the plasma membranes (N_2) from this washed pellet is described later in this section.

The postnuclear extract (E) was used to prepare a combined heavy and light mitochondrial fraction (M + L), which contains both mitochondria and lysosomes (26), by centrifugation of this solution at 33,000 *g* for 7.5 min in the No. 30 rotor (Spinco Model L-2 centrifuge (Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.). The rotor was accelerated at its maximum rate until the speed reached 25,000 rpm, at which point the speed control was turned down to 25,000 rpm, and finally the rotor

was stopped at maximum deceleration with the brake on after a total centrifugation time of 7.5 min. The supernatant solution, including the "fluffy pink" layer loosely packed on top of the M + L pellet, was carefully removed by aspiration. This loose layer was most easily discerned by holding the centrifuge tube in such a way that the face of the M + L pellet was horizontal during the removal of the bulk of the supernatant liquid. When most of this liquid had been removed by aspiration, the tube was turned 90° in order to remove the pinkish material which slides off the M + L pellet. In order to wash the M + L pellet, it was resuspended in three volumes of 0.25 M sucrose per g liver and homogenized with one up-and-down stroke of the pestle. The centrifugation of, and the careful removal of, the supernatant liquid from the M + L pellet were repeated as described above. The washed M + L pellet was resuspended in 0.25 M sucrose and saved for enzyme assays.

In order to obtain a microsomal pellet (P), the combined M + L supernatant fractions were centrifuged in the No. 30 rotor at 30,000 rpm (78,000 *g*) for 100 min. The resulting final supernatant fraction (S) including the congealed fat floating on its surface was removed by aspiration, while care was taken to leave behind a whitish layer which was loosely packed on top of the microsomal pellet. The separation of the plasma membrane fraction (P_2) from the latter material is described later in this section.

Table I shows the intracellular distribution and over-all recoveries of all the marker enzymes assayed in preparing the four main subcellular fractions. Nucleotide pyrophosphatase was not used as a marker enzyme because we had already established that this activity always closely follows phosphodiesterase activity in separation procedures. The dual localization of 5′-nucleotidase and phosphodiesterase I in the microsomal and nuclear fractions is apparent. The isolation of the plasma membranes from each of these fractions is described below.

GRADIENT SEPARATION OF PLASMA MEMBRANES FROM (N): The nuclear pellet was resuspended in three volumes of 0.25 M sucrose per g liver with one up-and-down stroke of the homogenizer. In order to pack the nuclear pellet firmly, this homogenate was centrifuged at 25,000 rpm for 7.5 min in the No. 30 rotor, and the supernatant fluid was removed by aspiration and discarded. At the bottom of the packed nuclear pellet was a small pellicle of red cells and buff-colored material. The nuclei could be poured off of the latter, which was discarded. To the nuclei, 1.5 volumes of 57% sucrose (w/w) per g liver were added and the resulting suspension was mixed thoroughly with a spatula and homogenized with two up-and-down strokes of the homogenizer. The homogenizer was rinsed twice with 5 ml of 57% sucrose, and the washings were combined with the homogenate. The final volume was brought to three volumes per g liver with 57% sucrose. The weight percentage of sucrose in the thoroughly mixed homogenate was determined with the refractometer and should be at least 47.0%. From 20 to 25 ml of this nuclear extract in heavy sucrose were placed in the bottom of cellulose nitrate tubes for the Spinco S.W. No. 25.2 rotor (Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.). 25 ml of 37.2% sucrose were carefully layered above the sample. Finally, enough 0.25 M sucrose to make the total volume 58 ml was layered above the 37.2% sucrose. The discontinuous gradient was centrifuged at 25,000 rpm for 16 hours in the S.W. No. 25.2 rotor. After stopping the centrifuge with the brake on, the tubes were removed and placed in ice. They were then fractionated as shown in Fig. 1 into four fractions by carefully withdrawing material from the meniscus along the edge of the tube with a Buchler Polystaltic pump (Buchler Instruments, Inc., Fort Lee, N. J.).

The first (top) fraction (N_1) contained congealed fat and was free of all enzyme activities which were assayed. The plasma membrane fraction (N_2) was a band appearing at the interface between the 37.2% sucrose ($\rho_{5°C}$ = 1.17) and the 0.25 M sucrose overlayer. It consisted of small pieces of whitish material. The 37.2% sucrose layer was essentially clear, and it was combined with the material banding at the interface between the original sample and the 37.2% sucrose layer. This fraction (N_3) was mainly mitochondria and the other cell particulates including a considerable amount of plasma membrane, but excluding the nuclei, which formed a pellet (N_4) at the bottom of the centrifuge tube. If the sucrose concentration of the sample fraction is less than 47%, most of the mitochondria and other particulate matter in fraction N_3 will sediment to the bottom of the tube along with the nuclei.

GRADIENT SEPARATION OF PLASMA MEMBRANES FROM (P): The microsomal pellet was placed in a homogenizer together with 1.5 volumes of 57% sucrose, thoroughly mixed with a spatula, and homogenized with 2 up-and-down strokes of the pestle. The homogenizer was rinsed twice with 5 ml of 57% sucrose, and the washings were combined with the homogenate. The final volume was brought to 2.5 volumes per g liver with 57% sucrose. The weight percentage of sucrose in the thoroughly mixed

TABLE I

Intracellular Distribution of Enzymes in Rat Liver

One unit of enzyme activity equals 1 μmole of substrate changed per min, except for cytochrome oxidase which is given in units per min as described by Appelmans *et al.* (29). Protein units are in milligrams. The difference between the molar extinction coefficients of reduced and oxidized cytochrome *c* was assumed to be 18.5×10^6 cm² per mole. The molar extinction coefficient of benzaldehyde formed in the monoamine oxidase reaction was assumed to be 13.3×10^6 cm² per mole. Statistics, presented as in de Duve *et al.* (27), refer to the means ± the standard deviations, with the number of experiments indicated in parentheses. E = extracts after removal of nuclear fraction, N = nuclear fraction, M + L = combined mitochondrial and lysosomal fraction, P = microsomal fraction, S = final supernatant. For each fraction, % is based on E + N representing 100%.

Enzyme	Liver homogenate (E + N)	Fraction				
		N	M + L	P	S	Recovery
	units/g liver	%	%	%	%	
Protein (5)	181. ± 3.	11.8 ± 3.6	33.5 ± 2.3	24.1 ± 4.0	28.7 ± 3.0	98.1 ± 5.5
5′-Nucleotidase (5)	13.6 ± 2.4	22.8 ± 4.0	12.9 ± 2.6	54.5 ± 2.1	10.9 ± 4.5	101.1 ± 6.2
Phosphodiesterase I (4)	4.43 ± 0.1	17.4 ± 3.4	13.5 ± 4.8	50.9 ± 6.0	4.5 ± 1.6	86.3 ± 6.5
Glucose-6-phosphatase (5)	15.5 ± 1.2	7.0 ± 2.6	22.0 ± 2.2	68.1 ± 2.5	5.3 ± 1.9	102.4 ± 6.8
NADPH-cytochrome *c* reductase (2)	3.17 ± 0.1	4.6 ± 0.6	14.9 ± 4.1	68.4 ± 3.0	3.3 ± 0.6	92.2 ± 1.9
Cytochrome oxidase (1)	17.9	7.2	79.8	1.2	0	88.2
Succinate-cytochrome *c* reductase (2)	14.6 ± 2.5	6.6 ± 0.9	96.0 ± 4.7	5.3 ± 5.6	0	107.9 ± 4.2
N-acetyl-β-D-glucosaminidase (3)	2.2 ± 0.4	6.3 ± 1.1	83.7 ± 1.6	5.4 ± 1.1	1.5 ± 1.3	96.9 ± 0.8
Monoamine oxidase (1)	0.38	10.1	78.2	24.2	0	112.5

homogenate was checked by use of the refractometer and should be at least 49.0%. 15 ml of this microsomal extract in heavy sucrose were placed in the bottom of a cellulose nitrate tube for the Spinco S.W. No. 25.2 rotor. 30 ml of 34% sucrose was layered above the sample, and finally 13 ml of 0.25 M sucrose was placed on top of the 34.0% sucrose. Centrifugation and fractionation of the discontinuous gradients were performed exactly as for the isolation of plasma membranes (N_2) from the nuclear fraction as described above.

The uppermost fraction (P_1) contained a small amount of congealed fat and was free of all enzymes which were measured. The plasma membrane fraction (P_2) was a thick band of white material appearing between the 34% ($\rho_{5°C}$ = 1.15) and the 0.25 M sucrose overlayer. Fraction P_3 was cloudy and pale yellow and contained both plasma membrane and endoplasmic reticulum enzymes, while P_4 contained, by enzymatic estimation, the bulk of the internal membranes placed in the gradient with a considerable amount of plasma membrane also present. (When fraction P_4 was subdivided into the particulate matter at the top of the fraction and the clear, yellow portion at the bottom, lacking any particulate matter, there was very low activity of plasma membrane enzymes in the latter clear solution.) One should avoid adding any of fraction P_3 to fraction P_2 during fractionation of the gradient, since the former fraction contains a considerable amount of internal membrane which will lower the specific activity of the isolated plasma membranes (P_2).

Characterization of the Two Isolated Plasma Membrane Fractions N_2 and P_2

Table II shows the distribution of the marker enzymes in subfractions obtained from the nuclear (N) and microsomal (P) fractions. Several conclusions may be drawn from the table. (*a*) 5′-Nucleotidase and phosphodiesterase I follow each other very closely. (*b*) The enzymic yields (32.4 and 25.3%) and relative specific activities (21.5–28.5) of these two activities in the isolated plasma membranes P_2 and N_2 are high compared with those previously published, as is the total yield of membranes (2.9 mg of membrane protein per g liver). (*c*) The contamination by other marker enzymes appears to be very low. Estimates of contamination of the plasma membrane fractions were made in several experiments by first calculating the specific activities of the endoplasmic reticulum marker enzymes, glucose 6-phosphatase and NADPH-cytochrome *c* reductase (37), and of those for mitochondria, cytochrome oxidase and succinate-cytochrome *c* reductase, in their respective organelles, with corrections being made for cross-contamination of fractions. Calculations indicated that P_2 is composed of endoplasmic reticulum to the extent of 20% and mitochondria to the extent of 2%, while the corresponding values for N_2 are 10 and 4%. (These estimates may be high because of the assumption that the marker enzymes are not normal constituents of the plasma membrane and are present exclusively as contaminants.)

Electron microscopic examination of the plasma membrane fractions indicated that they contained primarily vesicular elements and little nonmembranous material or organelles (Fig. 2). In Table III are given the results of analyses for cholesterol, phospholipid, sialic acid, DNA, and RNA. The value for the lipid content is among the highest reported for rat liver plasma membranes (see reference 35 for review), and that for the sialic acid is higher than the value reported by Emmelot *et al.* (3). The two fractions were apparently remarkably similar in content of DNA and RNA, but the low readings obtained make the DNA results very tentative.

We do not know whether there are as yet undetermined differences in the plasma membrane fractions which influence their affinity for the nuclei, or whether the adherence of membrane fragments to nuclei is a purely random matter.

Enzymatic Studies on Nucleotide Pyrophosphatase and Phosphodiesterase I

In order to obtain insight into the question of the identity of nucleotide pyrophosphatase with phosphodiesterase I, several additional experiments were carried out. In this work, a soluble fraction was obtained by treatment of a plasma membrane preparation with deoxycholate (as described in the legend of Fig. 3).

Thermal inactivation studies with this fraction (Fig. 3) yielded, with five different substrates, almost identical curves for nucleotide pyrophosphatase and phosphodiesterase I. Moreover, nucleotide pyrophosphates were found to inhibit competitively the hydrolysis of *p*-nitrophenyl 5′-thymidylate, the substrate of phosphodiesterase I, and of bis(*p*-nitrophenyl) phosphate, the substrate

TABLE II

Distribution of Enzymes in Rat Liver Plasma Membrane Fractions

Statistics are presented as in Table I. % indicates yield based on the original homogenate (E + N). Relative specific activity is the ratio of the specific activity of the fraction to that in the original homogenate (E + N).

Enzyme	Yield						Relative specific activity	
	P_2	P_3	P_4	N_2	N_3	N_4	P_2	N_2
	%	%	%	%	%	%		
Protein (4)	1.2 ± 0.0	2.1 ± 0.6	19.8 ± 3.1	0.4 ± 0.2	3.4 ± 1.8	10.1 ± 1.5		
5′-Nucleotidase (5)	23.4 ± 2.0	10.0 ± 2.8	21.0 ± 2.6	9.0 ± 4.0	3.2 ± 1.1	10.2 ± 3.1	21.5 ± 2.1 (9)*	28.5 ± 8.1 (8)*
Phosphodiesterase I (4)	19.1 ± 3.5	10.2 ± 2.9	21.4 ± 4.8	6.2 ± 1.7	3.3 ± 1.6	7.9 ± 2.8	23.9 ± 4.3 (7)*	23.7 ± 6.8 (7)*
Glucose 6-phosphatase (4)	1.2 ± 0.5	3.9 ± 0.6	62.6 ± 1.3	0.3 ± 0.2	1.6 ± 1.1	6.1 ± 1.0	1.1 ± 0.1	0.7 ± 0.2
NADPH-cytochrome *c* reductase (2)	1.2 ± 0.1	10.0 ± 1.4	58.1 ± 1.5	0.1 ± 0.0	1.5 ± 0.7	3.0 ± 0.2	1.1 ± 0.2	0.5 ± 0.1
Cytochrome oxidase (1)	0.05	0	1.2	0.2	5.5	1.8	0.04	0.08
Succinate-cytochrome *c* reductase (1)	0.3	0.27	4.7	0.06	0.9	5.6	0.05	0.16
N-acetyl-β-D-glucosaminidase (3)	0.2 ± 0.2	0.2 ± 0.2	5.0 ± 0.8	0.1 ± 0.1	1.4 ± 1.7	4.9 ± 0.7	0.2 ± 0.3	0.2 ± 0.3
Monoamine oxidase (1)	0			0			0	0

* Numbers in parentheses indicate the number of experiments in which 5′-nucleotidase and phosphodiesterase activities were measured.

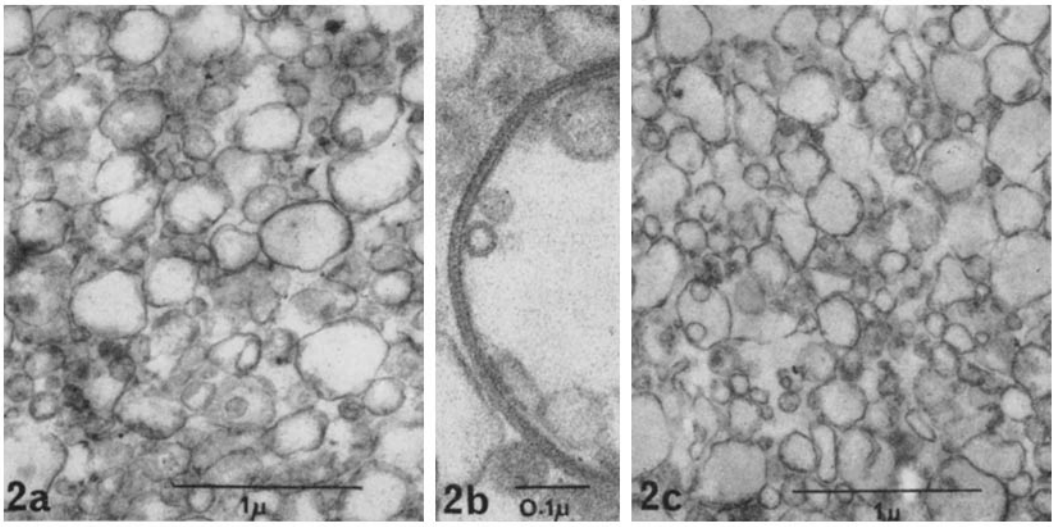

FIGURE 2 Electron micrographs of rat liver plasma membranes isolated from nuclear (N_2) (2 *a*) and microsomal (P_2) (2 *c*) fractions. Both fractions consist primarily of membranous vesicles. A few tight junctions are visible, but no desmosomes have been observed in any of several preparations. One tight junction in P_2 is shown (2 *b*). Magnifications: 2 *a* and 2 *c*, 25,000; 2 *b*, 98,000.

TABLE III

Chemical Analyses of Plasma Membrane Fractions

Analyses were performed as described under Materials and Methods. Statistics are presented as in Table I.

Component	P_2	N_2
Total cholesterol, *μmole/mg protein*	0.78 ± 0.08 (6)	0.76 ± 0.03 (5)
Phospholipid phosphorus, *μmole P/mg protein*	0.99 ± 0.3 (5)	0.92 ± 0.12 (5)
Cholesterol/phospholipid, *mole/mole*	0.79	0.83
Sialic acid, *mμmoles/mg protein*	46.4 ± 10 (7)	48.0 ± 4.7 (5)
DNA, *μg/mg protein*	12.2 ± 12. (3)*	11.2 ± 4.6 (3)*
RNA, *μg/mg protein*	71.3 ± 5.0 (3)	71.7 ± 4.7 (3)

* Uncertain because of low readings.

of nonspecific phosphodiesterase (Fig. 4). The inactivation and competition data provide strong, but not conclusive, evidence for the identity of the phosphodiesterase and nucleotide pyrophosphatase activities. In addition, nucleotide pyrophosphatase, phosphodiesterase I, and nonspecific phosphodiesterase activities in the soluble extract sedimented together in a linear sucrose density gradient and were retarded similarly on Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) and on Sepharose 4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) (35).

These results, together with the behavior of the different enzyme activities during the fractionation of liver homogenates, would ordinarily be rather strong evidence that the pyrophosphatase and phosphodiesterase activities were due to the same enzyme. Futai and Mizuno (38), in fact, reported a 200-fold purification of phosphodiesterase from rat liver particulates treated with butanol and found that the chromatographically purified enzyme hydrolyzed nicotinamide adenine dinucleotide (NAD) as well as phosphodiesters. Nonetheless, these authors suggested, on the basis of certain differences in pH optima and effects of inhibitors, that their enzyme was different from the nucleotide pyrophosphatase of Schliselfeld *et al.* (17). In an attempt to resolve this problem, we carried out

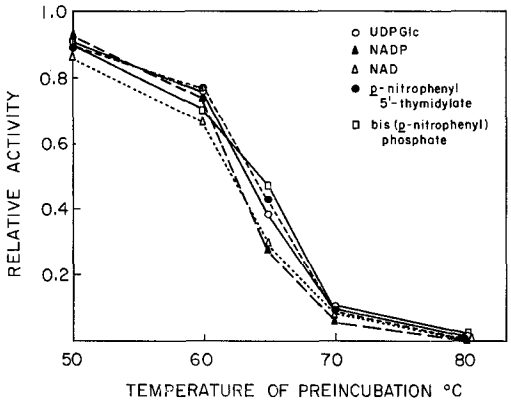

FIGURE 3 Thermal inactivation of solubilized plasma membrane nucleotide pyrophosphatase and phosphodiesterase activities. The early preparation of plasma membranes, from sucrose gradient fractionation of the nuclei-free particulates, was used (35). The nucleotide pyrophosphatase-containing fraction (density 1.14) was collected and, after preliminary centrifugations to obtain the membrane pellet, the latter was suspended in 0.25 M sucrose-0.005 M Tris-HCl, pH 8.5 (1.7 ml per g of original tissue). The relative specific activities (based on homogenates) were as follows: 5′-nucleotidase, 30; UDPGlc pyrophosphatase, 15; NAD^+ pyrophosphatase, 14; $NADP^+$ pyrophosphatase, 14; phosphodiesterase I, 14; nonspecific phosphodiesterase, 15. An aliquot (10 ml) of the membrane suspension was centrifuged at 45,000 rpm (Spinco rotor No. 50 titanium [Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.]) for 1 hr. The pellet was then suspended in 10 ml of 2.5% sodium deoxycholate–0.01 M Tris-acetate, pH 8.0, and stirred overnight, all at 2°C. The suspension was then centrifuged at 45,000 rpm (Spinco rotor No. 50, Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.) for 60 min. An aliquot of the supernatant was placed on a Sephadex G-75 column (22 × 48 cm) (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) equilibrated with 0.01 M Tris-HCl, pH 8.0, and elutriated with the same buffer. This step was to effect the removal of the sodium deoxycholate. The fractions containing the activity were pooled and concentrated by either ultrafiltration or partial lyophilization.

Aliquots were heated in a water bath at each temperature for exactly 5 min. with brief agitation to effect rapid temperature equilibrium. After the heating period, the sample was placed in an ice bath, again with agitation, to effect rapid cooling. Assays were performed as described under Materials and Methods. Relative activity refers to the activity compared with a sample held at 0°C in ice, with no heat treatment.

two key experiments: the preparation of the Futai and Mizuno enzyme as described in their report (through step 4), and the preparation of butanol-solubilized enzyme (according to Futai and Mizuno) from plasma membranes P_2, prepared as described in the present paper. Both enzyme preparations had pH optima near 9 (for NAD hydrolysis) and were inhibited by glycine and nicotinamide as previously described (17). These results are in accord with the data indicating identity of the enzymes.

DISCUSSION

The Preparation and Properties of Rat Liver Plasma Membranes

Since a rather large number of papers have appeared recently describing modifications of published procedures for preparing rat liver plasma membranes, it is pertinent that the various procedures and products be discussed briefly in the context of our own report. Frequently these reports deal insufficiently, or not at all, with questions of purity and yield of the product, or reproducibility of the method. In addition, there is the ever present problem that the starting material is composed of multiple cell types. In the present study we, like others, have assumed that 5′-nucleotidase is the most specific marker for the plasma membrane, although Widnell and Unkeless (39) have recently claimed that this enzyme is present in both the plasma membranes and endoplasmic reticulum. We have, in fact, found that most sucrose gradient fractions other than those containing plasma membranes release phosphate from AMP. Whether or not this low activity is due to the 5′-nucleotidase characteristic of plasma membranes, or to unspecific phosphatases (e.g. alkaline phosphatase), is unclear at present. On the other hand, glucose-6-phosphatase is generally accepted as an indicator of contamination by the endoplasmic reticulum, although there is no definitive evidence to indicate that this enzyme or others which catalyze hydrolysis of glucose-6-phosphate are indeed absent from plasma membranes. To our knowledge, there is only one report on the preparation of plasma membranes essentially free of this enzyme activity (11). It is difficult to assess the significance of the report, which utilizes preparative methods which are reported by others to yield membranes that do contain some glucose-6-phosphatase. Our results, which show a very close correlation in the amount

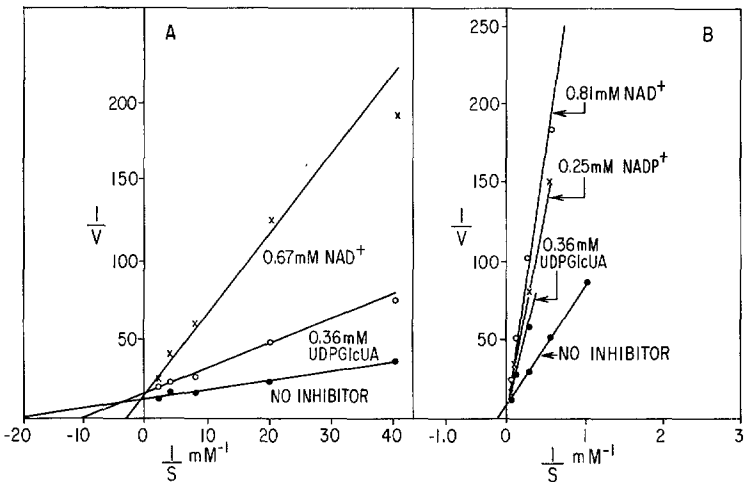

FIGURE 4 Competitive inhibition of phosphodiesterases by nucleotide pyrophosphates. A. Inhibition of phosphodiesterase I (*p*-nitrophenyl 5′-thymidylate as substrate) by NAD^+ and by UDPGlcUA. B. Inhibition of non-specific phosphodiesterase (bis[*p*-nitrophenyl] phosphate as substrate) by NAD^+, $NADP^+$, and UDPGlcUA. The solubilized membrane preparation was the same as described in Fig. 3. The assays were performed as described under Materials and Methods.

of both glucose-6-phosphatase and NADPH–cytochrome *c* reductase in the purified membranes, imply that glucose-6-phosphatase activity here is indeed due to the presence of microsomal contamination. On the basis of the low relative specific activities of 5′-nucleotidase in membranes isolated by some of the other procedures, a few of which claim extremely low glucose-6-phosphatase content, it would appear that contaminants other than the endoplasmic reticulum are present in the preparations. Likely candidates could be nuclear envelopes (40) and mitochondrial outer membranes (41), especially in those procedures using 0.001 M bicarbonate for homogenizing the liver. The densities of both of these membranes (40, 41) have been reported to be very close to that of plasma membranes as isolated by the Neville procedure (2). In the present work, since no monoamine oxidase was found in the purified P_2 and N_2 fractions, they appear to be free of contamination by outer mitochondrial membranes.

Determination by Coleman (42) of the molar ratio of cholesterol to phospholipid in surface and various cytoplasmic membranes emphasizes one special characteristic of plasma membranes; rat liver plasma membranes were shown to have a ratio of 0.70, whereas other types of membranes studied had a ratio of one-third to one-fourth this value. Other reported values for plasma membranes have ranged from 0.26 to 0.8 (3, 7, 8, 42–45), which may indicate a range of contamination by intracellular membranes. The values of 0.79 (P_2) and 0.83 (N_2) reported in the present study are near the highest values found by others and provide a basis for considering these preparations to be of considerable purity.

Nucleic acid analyses of plasma membranes have only occasionally been reported. RNA in plasma membranes has been found to vary from 10 to 33 μg per mg of protein (8, 9, 46). DNA has been reported both to be undetectable (9) or to be present to the surprising extent of about 300–1400 μg per mg of protein (calculated from the data in reference 11). Rat liver microsomes have been reported to contain DNA (47), and a new form of informational DNA ("I-DNA") has recently been reported to occur in cell cytoplasm of eukaryotic cells (48). It is impossible at this time to assess the significance of our values of about 12 and 11 μg DNA, and 71 and 72 μg RNA, per mg protein in P_2 and N_2, respectively, other than to point out the apparent similarity of the two present preparations with regard to these substances. Weiss and Mayhew (49) have recently made the pertinent observation that there are ribonuclease–susceptible charged groups at the surface of Ehrlich ascites cells.

Emmelot and his coworkers (3, 46) have reported that rat liver plasma membranes have a sialic acid content of 33 mμmoles per mg of mem-

brane protein, a value that is higher than values reported for mitochondria, nuclei, and microsomes (50), and higher than that for Ehrlich tumor cell plasma membranes (51). The values reported here, 46 mμmoles per mg for P_2, and 48 mμmoles per mg for N_2, are therefore in the range expected.

The variation in density of rat liver plasma membrane vesicles obtained by various investigators is of interest. It should be pointed out that, since most of the gradients employed in the literature are discontinuous, one cannot give a value to the equilibrium density of each plasma membrane fraction. Most procedures yield preparations with apparent densities between 1.16 and 1.18 (2, 3, 8, 9, 10, 45, 52), whereas the densities of our preparations and those of Coleman *et al.* (11) are lower. The density may be influenced by the technique of homogenization and by the media employed in various steps of the isolation, as well as by dietary and other factors. In addition, the higher densities found by others may reflect the presence of desmosomes, which have been commonly seen in electron micrographs of membranes prepared in other laboratories (2, 3, 5), but rarely in our own. In support of this suggestion is the fact that Coleman *et al.* (11) similarly obtained vesicles of lower density and containing few desmosomes. In addition, a rough correlation may be seen between the density at which plasma membranes band in a density gradient and their lipid content. However, because of the uncertainties already mentioned regarding the equilibrium densities of various preparations, it may be premature to assess the evidence on this point. Fractions P_2 and N_2 banded above sucrose of densities 1.15 and 1.17, respectively. The equilibrium median density of P_2 as determined in the B XIV zonal rotor (Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.) was 1.14. Thus, our product showed one of the lower densities reported, a relatively high lipid content, and the absence of desmosomes. After the present manuscript was completed, Evans (53, 54) reported on the subfractionation of rat liver plasma membranes prepared by the Neville procedure and then subjected to further homogenization and fractionation by sucrose density gradient centrifugation. His light fraction, consisting of vesicles relatively high in lipid and *N*-acetylneuraminic acid, and free of junctional complexes, resembles the fractions obtained by our procedure. The paper by Evans (54) contains pertinent information and discussion on the question of membrane heterogeneity.

The question of yields in various procedures is of obvious importance, but of course must be considered in the light of the purity of the preparation obtained. Yields of rat liver plasma membrane in terms of milligrams of membrane protein per gram of liver have been variously reported to be 0.23 (52), 0.41 (3), 0.44 (9), 0.46 (4), 0.88 (7), 1–2 (11, 55), and 1.5 (39). Reported relative specific activities of 5′-nucleotidase (ratio of the specific activity of the plasma membrane preparation to the specific activity of the starting liver homogenate) vary about 2-fold, though it must be taken into consideration that not all enzymic assays were performed under identical conditions. Values reported include 11.6 (9), 15 (10), approximately 13–19 (55), 13–24 (11), 20 (52), and 24 (4). The yield in the present work was 1.6% ($P_2 + N_2$) of liver protein as plasma membrane, or 2.9 mg of membrane protein per g of liver. The relative specific activity of 5′-nucleotidase was 21.5 in P_2 and 28.5 in N_2; that for phosphodiesterase I was about 24 in both P_2 and N_2. Assays for marker enzymes indicate that the purity of the plasma membranes was approximately 80%. The yield of 5′-nucleotidase in the combined P_2 and N_2 fractions was 32% of the activity in the homgenate, a value higher than any thus far reported (4, 5, 11, 52). It is evident that the values for both the yield and the apparent purity are similar to the highest values for each of these criteria. If both yield and purity are considered together in evaluating preparative methods for plasma membranes, the present method appears to be the one of choice. It is of interest that a calculation based on the yield of membrane protein and on enzyme markers indicates that approximately 5% of the cell protein is plasma membrane. The same calculation applied to the data of other workers (4, 11) gives quite similar estimates.

It is worth noting that an enzyme activity (e.g. phosphodiesterase I and nucleotide pyrophosphatase) exhibiting a bimodal distribution between nuclei and microsomes after homogenization and differential centrifugation in isotonic sucrose is likely to be present in plasma membranes sedimenting in the two fractions. In recent reports, the specific activities of two enzymes, phosphatidylinositol kinase (56) and nucleoside diphosphatase (4), were shown to be highest in the nuclear and microsomal fractions, as was the specific activity of 5′-nucleotidase. Further purification of plasma

membranes according to the method of Coleman *et al.* (11) in the case of the kinase, and of Neville (2) in the case of the phosphatase, led to the conclusion that the two enzymes are indeed plasma membrane constituents.

On the basis of the data presented in this paper, it appears that nucleotide pyrophosphatase and phosphodiesterase I activities have utility at least equal to that of 5′-nucleotidase as enzymic markers in the purification of rat liver plasma membranes. Very recently, after completion of the present work, Erecinska *et al.* (57), utilizing cell fractionation procedures different from those in the present paper, offered evidence that phosphodiesterase I may be used as a plasma membrane marker. Finally, it may be mentioned that Thines-Sempoux *et al.* (58) reported that, on treatment of microsomes with a low concentration (0.25%) of digitonin, a class of microsomal particles high in cholesterol, 5′-nucleotidase, and alkaline phosphodiesterase I are altered in median equilibrium density, whereas fractions enriched in other enzymes assayed do not so shift. In harmony with the conclusions in the present paper, the authors concluded that the phosphodiesterase and nucleotidase are components of the same membrane fraction, which were considered to be plasma membranes, or precursors or products thereof.

Relationship of Rat Liver Nucleotide Pyrophosphatase to Phosphodiesterase

Nucleotide pyrophosphatase activity in rat liver was first reported by Kornberg and Lindberg (13), who employed NAD as substrate. Subsequently, Jacobson and Kaplan (14) found NAD pyrophosphatase in both the nuclear and microsomal fractions. Later Schliselfeld *et al.* (17) commented on the similar properties of the nuclear and microsomal enzymes, while Emmelot *et al.* (3) made the highly significant observation that the specific activity of NAD pyrophosphatase is higher in plasma membranes than in microsomes. It is important to note, however, that the plasma membrane fraction obtained by the latter workers represented a very low yield of these membranes and that their report therefore left open the question as to the intracellular distribution of most of the nucleotide pyrophosphatase activity of liver. Since the role of the enzyme(s) is unknown, both localization and specificity studies are crucial to the further consideration of its biological significance.

Analysis of the fractions from the discontinuous sucrose gradient centrifugation of the nuclei-free particulate fraction (35) gave similar relative specific activities towards various nucleotide pyrophosphates, (uridine 5′-diphosphate glucose [UDPGlc], NAD, NADP), a result suggesting that all of the activity had the same subcellular localization. Substrate competition experiments in the present and previous (17) reports are consistent with the presence of a single nucleotide pyrophosphatase with broad specificity.

The present study is also concerned with the possible identity of nucleotide pyrophosphatase and phosphodiesterase I. De Lamirande *et al.* (16) found the latter activity in nuclear and microsomal fractions prepared by the procedure of Hogeboom (15), an observation which suggested to us that, like nucleotide pyrophosphatase, it may also be a plasma membrane enzyme. Moreover, W. E. Razzell, in a personal communication to us, raised the question of the possible identity of the two enzymes. Nucleotide pyrophosphates are chemically related to phosphodiesters in that, in the structure R—O—P(O) (OH)—O—R′, R′ is derived from a nucleotide in the former and from an alcohol in the latter. The two activities have the same subcellular localization, and the various studies reported, including heat inactivation and substrate competition experiments, are consistent with the idea that the two enzymes are identical.

The suggestion of Futai and Mizuno (38) that their "new phosphodiesterase" of rat liver is different from the nucleotide pyrophosphatase studied by Schliselfeld, van Eys, and Touster (17) is not supported by the studies reported in the present paper, namely, experiments on pH optima and on inhibition by glycine and nicotinamide employing various types of preparations (see Results). Except for a possible difference in the strain of rats used for these experiments, there is no apparent explanation for the difference in results. The purified preparation of Ogawa *et al.* (59) probably is the same nucleotide pyrophosphatase described in the present paper. It is possible also that the nucleoside triphosphate pyrophosphohydrolase of rat liver plasma membranes (60) is identical with nucleotide pyrophosphatase. However, in the one relevant experiment reported thus far, ATP was found to be a noncompetitive inhibitor of the hydrolysis of uridine 5′-diphosphate glucuronic acid (UDPGlcUA) (17).

All nucleotide pyrophosphates and phosphodi-

esters tested by us, by Futai and Mizuno (38), and by Ogawa *et al.* (59) are substrates of the respective rat liver enzyme preparations. The preparations from the Japanese laboratories appear to constitute the major portion of the pyrophosphatase or phosphodiesterase activities in their tissue fractions. The results included in the present report strongly suggest that these activities are concentrated in plasma membranes and are consistent with the assumption that the activities are properties of a single enzyme. Definitive evidence on this question must obviously be obtained in studies of very highly purified enzyme, which is not at present available.

It may be noted that an enzyme recently purified by Razzell (61) from hog liver microsomes by a procedure employing a *t*-amyl alcohol treatment as well as a trypsin digestion clearly possessed the properties of both a phosphodiesterase I and a nucleotide pyrophosphatase. Although the facts that the yield was relatively low, that the source was different from ours, and that isolation procedure was relatively complex preclude a very direct application to the question involving the rat liver plasma membrane enzyme activities, our results and conclusions are clearly consistent with those of Razzell.

The question of the possible identity of 5′-nucleotidase with nucleotide pyrophosphatase requires comment. Glaser *et al.* (62) and Neu (63) have each reported *Escherichia coli* enzymes which possess these two types of activity, and a yeast nucleotide pyrophosphatase has been reported to have 5′-nucleotidase activity (64). This situation seems not to hold for the activities in rat liver plasma membranes. Schliselfeld *et al.* (17) found UMP as the only uracil-containing product of UDPGlc hydrolysis, and we have observed (in an experiment not described here) that butanol treatment of plasma membranes inactivates 5′-nucleotidase but not phosphodiesterase I.

It is evident that we have little understanding regarding the function of the enzymatic activity in plasma membranes towards nucleotide pyrophosphates and phosphodiesters. The normal biological substrate(s) may not yet have been tested. Perhaps some understanding will be gained from studies on the nucleic acid components of the membranes, or from changes in activity observed under differing physiological or pathological conditions. Drug-induced changes in UDPGlcUA pyrophosphatase activity have in fact been reported for rat liver (65) and skin (66).

We are grateful to Dr. Jane Baxandall Bibring for the electron micrographs and to Mrs. Vera Coleman for technical assistance in many ways. Mrs. Jane P. Griffin and Mr. Maurice Bondurant assisted by checking the reproducibility of preparative methods.

This work was supported in part by Grant GB-7425X from the National Science Foundation and Grant 2-R01-CA-07489 from the National Cancer Institute, U. S. Public Health Service. Part of this report was taken from the dissertation submitted by H. H. to the Graduate School of Vanderbilt University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Molecular Biology. Dr. Aronson was a Fellow of the Helen Hay Whitney Foundation.

Received for publication 8 April 1970, and in revised form 22 June 1970.

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