Fractionation of Liver Plasma Membranes Prepared by Zonal Centrifugation

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1. Plasma membranes were isolated from crude nuclear sediments from mouse and rat liver by a rate-dependent centrifugation through a sucrose density gradient contained in the 'A' type zonal rotor. 2. The membranes were further purified by isopycnic centrifugation, and characterized enzymically, chemically and morphologically. 3. When the plasma-membrane fraction of sucrose density 1.17 g/cm^3 was dispersed in a tight-fitting homogenizer, two subfractions of densities 1.12 and 1.18 were obtained by isopycnic centrifugation. 4. The light subfraction contained 5'-nucleotidase, nucleoside diphosphatase, leucine naphthylamidase and Mg^{2+} -stimulated adenosine triphosphatase activities at higher specific activities than unfractionated membranes. The heavy subfraction was deficient in the above enzymes but contained higher $\text{Na}^+ + \text{K}^+$ -stimulated adenosine triphosphatase activity. 5. The light subfraction contained twice as much phospholipid and cholesterol, and three times as much *N*-acetylneuraminic acid relative to unit protein weight as the heavy subfraction. Polyacrylamide-gel electrophoresis indicated differences in protein composition. 6. Electron microscopy showed the light subfraction to be vesicular. The heavy subfraction contained membrane strips with junctional complexes in addition to vesicles.

The plasma-membrane fraction of liver, first prepared by Neville (1960), has been extensively characterized, morphologically and biochemically, by several studies (see for example Benedetti & Emmelot, 1968). Electron microscopy showed the isolated plasma membrane fraction to contain a number of heterogeneous structures, mainly long strips of membranes interconnected by junctional complexes and bile canaliculi intermingled with large numbers of vesicles. The fraction exhibits a number of enzyme activities, some of which are used as specific markers for this cell fraction (Emmelot, Bos, Benedetti & Rumke, 1964).

Plasma membranes are generally isolated by gentle disruption of livers in a slightly alkaline hypo-osmotic solution with a loose-fitting homogenizer. The large surface membrane fragments sedimenting at low speed as an overlay on nuclei and cell debris are collected, washed repeatedly by low-speed centrifugation to eliminate mitochondria, and finally isolated free of nuclear fragments by isopycnic banding in a sucrose gradient (Neville, 1960). Variations of this method utilizing iso-osmotic sucrose as the medium for disruption, and a number of flotation stages in sucrose in addition to low-speed differential centrifugation, have also been described (Takeuchi & Terayama, 1965; Neville, 1968; Stein,

Widnell & Stein, 1968; Berman, Gram & Spirtes, 1969). By intensive homogenization of the 'nuclear' pellet, Coleman, Michell, Finean & Hawthorne (1967) prepared a plasma-membrane fraction that sedimented at higher centrifugal forces and was of a lower sucrose density than membranes obtained by other methods. Plasma membranes prepared by using zonal rotors have not been characterized in such detail although accounts have emphasized the advantages of this method for large-scale preparation (El Aaser *et al.* 1966; Anderson, Lansing, Lieberman, Rankin & Elrod, 1968; Weaver & Boyle, 1969).

This paper describes the preparation of plasma membranes in good yield from low-speed sediments of liver gently dispersed in 1 mM-NaHCO_3 , by using a rate separation through a sucrose density gradient contained in the 'A' type zonal rotor. Homogenization of the isolated plasma-membrane fraction in a tight-fitting Dounce homogenizer produces two major subfractions, which can be separated by sucrose-density-gradient centrifugation. The enzymic, chemical and morphological differences between the two subfractions are investigated relative to those of unfractionated plasma membranes. This account deals mainly with plasma membranes from mouse liver; a short account of the

subfractionation of plasma membranes from rat liver was published previously (Evans, 1969).

EXPERIMENTAL

Preparation and subfractionation of plasma membranes. Fed unperfused Sprague-Dawley rats (150g) or Parkes' White mice (25g) were killed by cervical fracture, and the livers were collected in ice-cold 1mM-NaHCO₃, pH 7.6. Livers were cut into small pieces, which were repeatedly washed with NaHCO₃ until the supernatant appeared free of blood. Tissue was dispersed in a 35 ml loose-fitting Dounce homogenizer (Blaessig Glass Co., Rochester, N.Y., U.S.A.; stated radial clearance 0.006in) by using four to six strokes with mouse liver and about 15 strokes with rat liver in 80 ml of medium/10g of liver. Approx. 80–120g of livers from 14 rats or 75–100 mice were used in a preparation. The tissue dispersion was filtered through two layers of muslin cloth to remove connective tissue and clumps of unhomogenized tissue, and centrifuged at 1000g for 10 min in 250 ml polycarbonate pots. The supernatants were carefully removed by aspiration and the gelatinous pellets were gently resuspended in a minimum volume of NaHCO₃, usually 40–50 ml, with two to four strokes of the loose Dounce homogenizer.

Zonal centrifugation was carried out at 4°C in a type 'A' zonal rotor (Measuring and Scientific Equipment Ltd., Buckingham Gate, London S.W.1, U.K.) run in an MSE 'Mistral' centrifuge. The sucrose density gradient (1300 ml) was generated by using a simple MSE fixed-profile gradient former and introduced at a speed of 50 ml/min into the rotor spinning at 800 rev./min. Solutions of sucrose in 1mM-NaHCO₃ were introduced into the rotor in the following order: 300 ml of 6% (w/v); 100 ml of 24% (w/v); a gradient of 100 ml of 24% (w/v) mixed with 100 ml of 36% (w/v); 300 ml of 36% (w/v) mixed with 300 ml of 54% (w/v); 100 ml of 54% (w/v). The sample was introduced into the centre of the rotor and was overlaid with 40 ml of 1mM-NaHCO₃. Centrifugation was carried out for 40–50 min at 3900 rev./min. After the rotor speed decreased to 800 rev./min, 50 ml fractions were collected by pumping 60% (w/v) sucrose at 50 ml/min into the outer edge of the rotor. The sucrose concentration of each fraction was determined by measurement of refractive index and it was examined by phase-contrast microscopy. The fractions were also assayed for protein content and enzymic activities. Fractions containing plasma-membrane fragments were pooled, diluted with approx. $\frac{1}{3}$ vol. of water and centrifuged at 40000g for 20 min to give a pellet and a clear supernatant. Pellets were resuspended in a minimum volume of 8% (w/v) sucrose in 5mM-tris-HCl buffer, pH 7.6.

Density-gradient centrifugation was generally carried out in Beckman SW 25.1 cellulose nitrate tubes containing a cushion of 55% (w/v) sucrose (2 ml) and the following continuous gradient of sucrose dissolved in 5mM-tris-HCl buffer, pH 7.6: 12 ml of 55% (w/v) against 12 ml of 35% (w/v) sucrose. Unfractionated membranes were prepared by gentle resuspension of pellets in a loose-fitting Dounce homogenizer. For fractionation studies the membranes were resuspended with 20 strokes of a tight-fitting Dounce homogenizer, stated radial clearance 0.003in. Membranes were layered on to the gradients and centrifuged for

4–16 h at 60000g_{av} in a Beckman or MSE ultracentrifuge. After centrifugation, gradients were resolved by using an Isco density-gradient fractionator (Instrumentation Specialists Co. Inc., Lincoln, Nebr., U.S.A.) with simultaneous recording of E_{280} . Fractions (1 ml) were collected and analysed and the membrane peaks were pooled.

In one experiment, membranes collected from the 'A' zonal rotor were gently suspended in 55% (w/v) sucrose and introduced into the outer edge of an MSE B XIV aluminium rotor containing a gradient formed by mixing 250 ml of 35% (w/v) sucrose and 250 ml of 50% (w/v) sucrose. An underlay of 200 ml of 55% (w/v) sucrose was introduced to fill the rotor. Membranes were centrifuged for 3 h at 42000 rev./min, and after deceleration fractions (25 ml) were collected for analysis as described above.

Measurement of enzymic activities. 5'-Nucleotidase activity was measured as described by Michell & Hawthorne (1965). Nucleoside diphosphatase activity was measured as described by Plaut (1962); with ADP and IDP, the monophosphate product of the reaction becomes a substrate for the 5'-nucleotidase, with liberation of another equal amount of orthophosphate. Consequently, observed values for adenosine diphosphatase and inosine diphosphatase activities have been halved. Glucose 6-phosphatase activity was assayed by the method of Swanson (1955), and acid phosphatase by the method of Gianetto & de Duve (1955), with β -glycerophosphate as substrate. Adenosine triphosphatase activity, and the increment in activity in the presence of 100 mM-Na⁺, was assayed as described by Swanson, Bradford & McIlwain (1964). Na⁺-stimulated adenosine triphosphatase activity was measured immediately after preparation of membrane fractions, because prolonged standing at 0–4°C or freezing caused rapid loss of activity. Inorganic phosphate liberated at 37°C in the above assays was determined by the method of Martin & Doty (1949). Leucine β -naphthylamidase activity was measured as described by Goldberg & Rutenburg (1958). Succinate dehydrogenase activity was assayed at 37°C by the method of Green, Mii & Kohout (1955) as modified by Earl & Korner (1965).

Chemical determinations. Protein content of membrane suspensions was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, U.K.) as standard. Membranes collected from gradients were diluted sufficiently to obviate interference by sucrose in colour development. The amount of membrane protein soluble in saline was determined on supernatants after centrifugation at 40000g for 15 min of membrane fractions dispersed in a tight-fitting Dounce homogenizer and stirred for 2 h at room temperature in 0.15M-NaCl in 5mM-tris-HCl buffer, pH 7.6. RNA content of membranes was determined by the Schmidt-Thannhauser method as described by Munro & Fleck (1966) and DNA by the diphenylamine method of Giles & Myers (1965). For the determination of phospholipids and cholesterol, membranes were washed with water by low-speed centrifugation, and the pellets were extracted three times with chloroform-methanol (2:1, v/v) at room temperature over a period of 24 h. The extract was partitioned against KCl as described by Folch, Lees & Sloane-Stanley (1957). A sample of the extract was digested with perchloric acid (70%, w/v) and used for phosphorus estimation by the

method of Bartlett (1959). Phospholipid was calculated by assuming $25\mu\text{g}$ of phospholipid/ μg of lipid phosphorus (Kopaczky, Perdue & Green, 1966). Cholesterol was determined as described by King & Wootton (1956). Portions of the chloroform-methanol extract were examined by t.l.c. Silica gel G plates (E. Merck A.-G., Darmstadt, Germany) were developed in the solvent systems of Jatzkewitz & Mehl (1960) and stained with H_2SO_4 . *N*-Acetylneuraminic acid content of membrane fractions that had been washed twice with water by centrifugation was determined by the method of Aminoff (1961) after hydrolysis in 50 mM- H_2SO_4 at 80°C for 60 min; the correction factor of Warren (1963) was applied. For amino acid analyses, freeze-dried membrane fractions were hydrolysed in 6 M-HCl at 110°C *in vacuo* for 24 h and 72 h, and analysed by using a Beckman 120C amino acid analyser. Cysteine content was determined independently as cysteic acid after performic acid oxidation (Moore, 1963).

Polyacrylamide-gel electrophoresis. This was carried out in a vertical flat-sheet electrophoresis apparatus similar to that described by Reid & Bielecki (1968) by a modification of the method of Summers, Maizel & Darnell (1965) in 7% (w/v) acrylamide gel containing 0.1% (w/v) sodium dodecyl sulphate. The electrolyte (pH 8.4) contained 5 mM-tris, 40 mM-glycine and 0.01% (w/v) sodium dodecyl sulphate. The polyacrylamide gel was polymerized in 0.37 M-tris-HCl buffer, pH 8.9, containing 0.1% (w/v) sodium dodecyl sulphate. The gel was formed by dissolving in this buffer the following: 7% (w/v) acrylamide, 0.19% (w/v) bisacrylamide, 0.07% (w/v) ammonium persulphate and 0.03% (w/v) *NNN'*-tetramethylethylenediamine. A 2 cm layer of large-pore gel, polymerized in 0.125 M-tris-HCl buffer, pH 6.7, containing 0.1% (w/v) sodium dodecyl sulphate and of the following composition was poured over the gel: 2.5% (w/v) acrylamide, 0.6% (w/v) bisacrylamide, 0.001% (w/v) riboflavin. Membrane fractions were sedimented by low-speed centrifugation, and dissolved at a concentration of 5 mg/ml in 5% (w/v) sodium dodecyl sulphate in 1 M-sucrose. Samples (0.2 ml) were applied with a syringe to slots 1.5 cm deep and 0.8 cm wide at the top of the gel, which was 2 mm thick. Electrophoresis was carried out for 16 h at a constant current of 11 mA. Under these conditions, the tracking dye [1% (w/v) Bromophenol Blue in 1 M-sucrose] was observed to have moved 7 cm through the small-pore gel. Gels were stained for 4 h in 2% (w/v) Procion Blue Michrome no. 1132 (E. Gurr Ltd., London, U.K.) in methanol-water-acetic acid (8:11:1, by vol.), and destained in the same solution.

Electron microscopy. Membrane pellets were fixed for 1 h at 0°C in a 2:1 (v/v) mixture of 1% (w/v) osmium tetroxide and 2.5% glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 7.4 (Hirsch & Fedorko, 1968). The pellets were then dislodged carefully from the tubes, rinsed twice with ice-cold 0.9% NaCl and 'post-fixed' for 15 min in 0.25% (w/v) uranyl acetate in 0.1 M-veronal acetate buffer, pH 6.2. Specimens were dehydrated with ethanol and embedded in Epikote 812 epoxy resin. Thin sections were stained with uranyl acetate and lead citrate, and observed with a Philips EM.300 electron microscope.

Materials. Glass-distilled water was used in all experiments. Chemicals of AnalaR standard were obtained from British Drug Houses (Poole, Dorset, U.K.) and

biochemicals from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.

RESULTS

Preparation of plasma membranes. The initial fractionation was closely similar to the method of Neville (1960). Up to 120 g of livers was used in the preparation of the plasma membrane fraction with the zonal rotor. Centrifugation of the filtered liver homogenate suspended in 1 mM- NaHCO_3 , pH 7.6, gave a pellet accounting for 8–12% of the protein and 30–40% of the total 5'-nucleotidase activity of the homogenate. To increase the proportion of 5'-nucleotidase activity recovered in the low-speed pellet we used higher gravitational forces, but although more enzyme was recovered more mitochondria were also sedimented, resulting in poorer separation of mitochondria from plasma membranes in the subsequent centrifugation. The use of 1 mM- NaHCO_3 –1 mM- MgCl_2 or 0.25 M-sucrose as medium for initial fractionation was also examined, but the appearance of a large number of bands during the zonal separation stage discouraged their use.

The sucrose density gradient in the zonal rotor was designed to obtain optimum separation between fractions containing succinate dehydrogenase activity and 5'-nucleotidase activity. One advantage of the 'A' zonal rotor over the 'B' rotors for the separation of heavy subcellular particles is that the rate and extent of separation of bands through the sucrose density can be seen during centrifugation. The time required for separation of the plasma-membrane band from the band containing mitochondria can first be roughly determined visually. The profile across the sucrose gradient of a typical run with mouse liver tissue is shown in Fig. 1. Centrifugation for 50 min separated the low-speed pellet into four major bands; three were visible in the rotor and the remaining one was located against the outside edge. Peak A, comprising fractions 1–6, contained most of the protein added to the rotor, and usually contained a small peak of 5'-nucleotidase activity. Phase-contrast microscopy of these fractions showed extremely small particles most of which are probably derived from the endoplasmic reticulum. Peak B, comprising fractions 9–14, was never completely separated from peak A and contained the succinate dehydrogenase activity and mitochondria observed by phase-contrast microscopy. Peak C (fractions 18–24) contained a large triphasic peak of 5'-nucleotidase activity. Observation of these fractions by phase-contrast microscopy showed large numbers of membrane fragments of the characteristic shapes described by Neville (1960). Peak D (fractions 26–28) was shown by microscopic examination to contain mainly nuclei and cell debris.

Fig. 1. Profile of 'crude' nuclear suspension from mouse liver, centrifuged at 3900 rev./min for 50 min in 'A' type zonal rotor. The suspension contained 2.1 g of protein derived from 21.4 g of homogenate protein (70 livers). ○, protein; ●, 5'-nucleotidase; □, succinate dehydrogenase; △, sucrose concentration. Fractions 19–24 were pooled and centrifuged to give 127 mg of plasma membranes, which were subsequently subjected to density-gradient centrifugation to give 105 mg of membranes, or subfractionated to give 17.2 mg of light subfraction and 52 mg of heavy subfraction. 5'-Nucleotidase activities (μmol of P_i liberated/h per mg of protein) were: tissue homogenate, 0.96; 'crude' nuclear suspension, 3.2; plasma membranes, 17.4; purified plasma membranes, 18.9; light subfraction, 37.8; heavy subfraction, 15.0.

As a routine, fractions were monitored by phase-contrast microscopy and refractometry and those fractions of sucrose density 39% to 45% (w/v) were pooled for collection of plasma membranes. From 100 g of wet liver, pooled membranes from the zonal rotor usually contained approx. 100–120 mg of protein for mice, or 60–80 mg for rats.

Plasma membranes were collected from the 'A' zonal rotor (see Fig. 1) and were further purified by centrifugation in a sucrose density gradient. The 5'-nucleotidase activity increased and 20–40% of the protein was removed in soluble form. From the legend to Fig. 1 it can be calculated that about 10% of the total 5'-nucleotidase activity present in the tissue homogenate was recovered in the plasma-membrane fraction. When the membranes collected from the 'A' zonal rotor were resuspended gently by using the loose Dounce homogenizer, plasma membranes were found at sucrose density 1.17 with a minor whitish band at density 1.12. As mentioned above, slight overlap of 5'-nucleotidase and succinate dehydrogenase activities occurred in the 'A' zonal rotor, and an attempt to increase further the yield of plasma membranes caused greater contamination with mitochondria. Fractions containing higher than average succinate dehydrogenase activity were centrifuged in a continuous sucrose density gradient in a B XIV zonal rotor (Fig. 2). There was still an overlap of 5'-nucleotidase activity and succinate dehydrogenase activity, indicating that marked decrease of mitochondrial contamination by density-gradient centrifugation can only be accomplished at the expense of a big decrease in yield.

Subfractionation studies on plasma membranes.

When mouse plasma membranes collected from the 'A' zonal rotor were resuspended by using the tight-fitting Dounce homogenizer, and centrifuged for 16 h to equilibrium on a continuous sucrose gradient, two major membrane peaks of density 1.12 and 1.18 were obtained. This was in agreement with the results obtained with rat plasma membranes [the density of the heavy membrane subfraction from rat (Evans, 1969) has been revised from 1.21 to 1.18]. The membrane bands were collected, diluted with water and centrifuged at 40000g for 20 min. Resuspended membrane subfractions were used for enzymic, chemical and morphological study.

Enzymic studies on membrane subfractions. The profile of 5'-nucleotidase specific activity across the sucrose density gradient is shown in Fig. 3. Highest 5'-nucleotidase activity is associated with peak A, isopycnic with 30% (w/v) sucrose, whereas peak B, corresponding to 45% (w/v) sucrose, contained low irregular enzyme activity. Pooled subfractions from mouse were examined for enzyme markers considered specific for the plasma membrane fraction. As shown previously for plasma-membrane sub-fractions from rat (Evans, 1969), the light subfraction contains three recognized plasma-membrane markers: 5'-nucleotidase, inosine diphosphatase and leucine naphthylamidase, all at appreciably higher specific activities than the heavy subfraction (Table 1). Adenosine triphosphatase activity measured in the presence of Mg^{2+} and K^+ is mainly concentrated in the light subfraction, whereas additional activity elicited in the presence of 100 mM- Na^+ is present at higher specific activities

Fig. 2. Profile of plasma-membrane fraction from mouse liver and contaminated by mitochondria, centrifuged at 42000 rev./min for 3 h in the BXIV zonal rotor. $\circ$, protein; $\bullet$, 5'-nucleotidase; $\square$, succinate dehydrogenase, $\triangle$, sucrose concentration. Plasma membranes (about 50 mg), collected from the 'A' zonal rotor were gently resuspended in sucrose (55%, w/v) and introduced into the outer edge of the 'B' zonal rotor, which contained a gradient made by mixing 250 ml of 35% (w/v) sucrose with 250 ml of 50% (w/v) sucrose. The rotor was then filled with 55% (w/v) sucrose. Fractions 4–12 were not assayed for 5'-nucleotidase activity.

Fig. 3. Preparation of plasma-membrane subfractions from mouse liver by sucrose-density-gradient centrifugation. $\circ$, protein; $\bullet$, 5-nucleotidase; $\triangle$, sucrose concentration. About 20 mg of membranes from the 'A' zonal rotor were homogenized in the tight-fitting homogenizer and loaded on to the gradient. After centrifugation at 60000 g_{av} for 4 h, the gradient was resolved into 1 ml fractions by using the Isco fractionator. Fractions 3–9 and 18–21 were pooled and designated light and heavy subfractions respectively.

in the heavy subfraction, although the extent of enrichment for this enzyme was found to be variable. The orthophosphate liberated in the presence of Mg^{2+} and K^+ cannot be taken as a stoichiometric indication of adenosine triphosphatase activity because nucleoside diphosphatases and 5'-nucleotidases are present, especially in the light subfraction. The presence, in plasma-membrane subfractions from rat, of nucleoside diphosphatase activity with

ADP or IDP as substrate is shown in Table 2. Although in both rat and mouse inosine diphosphatase activity always exceeded adenosine diphosphatase activity in subfraction A, the substrates are probably mainly hydrolysed by a non-specific nucleoside diphosphatase enzyme, and in addition this enzyme was unaffected by the presence of 0.03% (w/v) deoxycholate. The presence in plasma membranes of nucleoside diphosphatase activity with IDP or

Table 1. *Enzyme activities in plasma-membrane fractions from mouse liver*

Plasma membranes obtained from the 'A' zonal rotor were resuspended in 8% (w/v) sucrose, 5 mM-tris-HCl, pH 7.6, by using the loose or tight-fitting Dounce homogenizer, and subjected to sucrose-density-gradient centrifugation as described in the Experimental section to give respectively unfractionated membranes (density 1.17) or light (1.12) and heavy (1.18) subfractions. Preparations (a), (b) and (c) denote three similar preparations. Enzymic activities are expressed as μmol of product liberated/h per mg of protein at 37°C. The value given under each fraction is the weight of protein recovered in that fraction in each preparation. The 5'-nucleotidase activity of the mouse homogenates was 0.87 ± 0.25 (7). N.d., not determined.

Enzyme activity	Protein ...	Preparation (a)			Preparation (b)		Preparation (c)	
		Unfrac- tionated membranes 46 mg	Light sub- fraction 8.8 mg	Heavy sub- fraction 23.8 mg	Light sub- fraction 8.4 mg	Heavy sub- fraction 31.0 mg	Light sub- fraction 3.8 mg	Heavy sub- fraction 20.8 mg
5'-Nucleotidase		26.5	75.4	15.2	34.8	8.4	46.0	11.8
Inosine diphosphatase		6.0	24.0	3.5	12.1	0.6	58.0	2.7
Adenosine diphosphatase		5.4	19.8	3.5	N.d.	N.d.	N.d.	N.d.
Mg ²⁺ + K ⁺ -stimulated adenosine triphosphatase		79.0	270.0	59.0	N.d.	N.d.	20.0	4.2
Na ⁺ - (Mg ²⁺ + K ⁺)-stimulated adenosine triphosphatase		33.0	0	42.0	N.d.	N.d.	0.8	18.2
Leucine naphthylamidase		3.4	9.6	2.8	2.3	0.6	6.4	1.6
Acid phosphatase		N.d.	N.d.	N.d.	0.5	1.4	2.6	0.7
Succinate dehydrogenase		0.03	0	0.05	0	0.06	0	0.22
Glucose 6-phosphatase		0.13	0.12	0.32	0.6	0.1	0	0.9

Table 2. *Distribution of nucleoside diphosphatases and 5'-nucleotidase in rat-liver subfractions*

Plasma membranes were subfractionated as described in the Experimental section, and enzymic activities were measured. The 5'-nucleotidase activity of the liver homogenate was $2.2 \mu\text{mol/h}$ per mg.

Enzyme activity	Light subfraction	Heavy subfraction
5'-Nucleotidase	167.0	29.4
Inosine diphosphatase	36.0	6.1
Adenosine diphosphatase	21.0	3.8

ADP as substrate contrasts with the results of Emmelot *et al.* (1964), who detected very low activities, and agrees with the results of Wattiaux-de Coninck & Wattiaux (1969). The low activities of glucose 6-phosphatase, acid phosphatase and succinate dehydrogenase (Table 1) indicated little contamination of the plasma-membrane fractions by elements of the endoplasmic reticulum, lysosomes and mitochondria respectively.

Chemical analysis of subfractions. The ratios of phospholipid and cholesterol contents to protein weight in unfractionated and fractionated membranes are shown in Table 3. The dominant feature is the presence of about twice as much phospholipid and cholesterol in the light subfraction as in the heavy subfraction, as observed for rat membranes (Evans, 1969). However, rat liver subfractions appear to contain a lower ratio of cholesterol to

phospholipid than in the mouse, but in both animals the molar ratios are similar in the subfractions. The published molar ratios for plasma membranes from rat liver vary from 0.26 (Ashworth & Green, 1966) to 0.7 (Coleman, 1968); 0.80 has been quoted for plasma membranes from mouse liver (Benedetti & Emmelot, 1968).

Studies on chloroform-methanol extracts of the subfractions indicated no great visual differences in the classes of phospholipids stained by sulphuric acid. Saline-soluble protein of fractionated and unfractionated membranes of rat and mouse did not account for more than 10% of the total protein, in contrast with the value of 20-35% reported by Emmelot *et al.* (1964). Values for RNA and DNA content reflect an extremely low contamination of the fraction by rough endoplasmic reticulum, nuclear elements and/or mitochondria.

Unfractionated plasma membranes from rat and mouse contain similar amounts of *N*-acetylneuraminic acid, in agreement with the values reported by Benedetti & Emmelot (1968). However, the light subfraction contained about three times as much *N*-acetylneuraminic acid as the heavy subfraction.

Amino acid analysis of the two subfractions derived from plasma membranes from rat and mouse (Table 4) indicate that both subfractions are of a generally similar composition. Acidic, basic and non-polar amino acids account respectively for approx. 20%, 14% and 54% of the total, values that are in close agreement with analyses of erythrocyte membranes (Bakerman & Wasemiller, 1967;

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate of plasma-membrane fractions from rat liver. A, unfractionated plasma membranes; B, light subfraction; C, heavy subfraction. Single arrows point to bands present in the light subfraction that are absent from the heavy subfraction; double arrow points to a band absent from the light subfraction that is present in other fractions.

EXPLANATION OF PLATE 2

Plasma-membrane fraction (A) and light subfraction (B) from mouse liver. Arrow points to a tight junction. Magnification: $\times 42000$.

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EXPLANATION OF PLATE 3

Heavy subfraction of plasma membranes from mouse liver. Arrow points to a tight junction with a 'free-end' of a membrane nearby. b, Bile canaliculus; d, desmosome. Magnification: $\times 41000$.

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Table 3. *Chemical composition of plasma-membrane fractions*

Values are given $\pm$ s.d. with the numbers of determinations in parentheses. Phospholipid and cholesterol are expressed as mg and RNA and DNA as μ g/mg of membrane protein. *N*-Acetylneuraminic acid is expressed as nmol/mg of membrane protein. Phospholipid weight was obtained by multiplying the phosphorus weight by 25. In calculating molar ratios, the mol.wt. of phospholipid was taken as 780. N.d., not determined.

	Unfractionated membranes	Light subfraction	Heavy subfraction
Phospholipid (mouse)	0.95 ± 0.16 (5)	1.61 ± 0.18 (6)	0.85 ± 0.15 (6)
Cholesterol (mouse)	0.20 ± 0.03 (6)	0.37 ± 0.07 (6)	0.18 ± 0.02 (6)
Cholesterol/phospholipid molar ratio (mouse)	0.43 ± 0.06 (5)	0.46 ± 0.06 (6)	0.43 ± 0.06 (6)
RNA (mouse)	1.5 (1)	3.1 (2)	2.6 (2)
RNA (rat)	N.d.	0.6 (1)	2.2 (1)
DNA (mouse)	4.1 (1)	4.5 (2)	3.0 (1)
DNA (rat)	N.d.	0	0
<i>N</i> -Acetylneuraminic acid (mouse)	32.1 ± 2 (5)	65 ± 9 (5)	25.5 ± 2 (3)
<i>N</i> -Acetylneuraminic acid (rat)	35 (1)	73 (1)	22 (1)

Table 4. *Amino acid composition of plasma-membrane subfractions from rat and mouse liver*

Values are expressed as mol/100 mol. Results are corrected for destruction of serine and threonine. Cysteine was determined as cysteic acid. Tryptophan was not determined.

Amino acid	Light subfraction		Heavy subfraction	
	Rat	Mouse	Rat	Mouse
Lys	7.2	7.0	6.0	8.1
His	2.2	2.3	2.0	2.3
Arg	5.0	4.6	4.6	5.4
Asp	8.6	9.3	9.7	7.5
Thr	5.7	4.5	5.8	4.5
Ser	7.0	6.2	7.3	6.0
Glu	11.1	11.8	10.9	11.2
Pro	4.2	3.7	4.2	5.2
Gly	9.2	8.3	8.1	8.7
Ala	8.6	9.6	7.9	8.5
Cys	0.5	0.8	0.9	1.3
Val	6.9	5.6	7.1	6.9
Met	1.5	1.3	1.2	1.9
Ile	5.6	5.7	5.9	5.5
Leu	10.4	11.7	11.3	9.4
Tyr	2.7	3.1	2.6	3.1
Phe	4.7	4.6	4.6	4.7

fractions were examined side by side. However, differences in the intensities of staining of a number of bands are apparent. Further, four bands are present in the light subfraction that are virtually absent in the heavy subfraction; one of these bands also appears to be absent in the unfractionated membrane pattern. Additionally, one band present in the heavy subfraction is absent from the light subfraction. The polyacrylamide-gel-electrophoretic studies clearly indicate that the heavy and the light membrane subfractions isolated differ in their protein composition, a finding that is in agreement with the differences found in enzymic activities displayed.

Electron microscopy of membrane subfractions. Electron microscopy on sections of isolated plasma-membrane fraction from mouse (Plate 2A) indicates the presence of a population of membrane strips and vesicular components. The membrane strips can be seen in section to form, at intervals, structures believed to correspond to the tight junctions that extend into oval profiles of the bile canaliculi found *in situ* (Farquhar & Palade, 1963). Occasionally localized thickenings corresponding to the desmosomes found *in situ* were observed. Morphologically, therefore, the plasma-membrane fraction isolated from mouse resembles closely that from rat (Benedetti & Emmelot, 1968; Evans, 1969).

The light subfraction is shown (Plate 2B) to consist of a population of vesicular structures, and membrane sheets with junctional complexes are absent. A section of the heavy subfraction (Plate 3) shows the predominance of long membrane strips with junctional complexes especially in evidence. The presence of these junctional complexes is normally accepted as an important criterion in attributing their derivation from the surfaces of apposed liver parenchyma cells. However, vesicles are also present in this subfraction, although their number

Mazia & Ruby, 1968) and mitochondrial 'structural' protein (Lenaz, Haard, Silman & Green, 1968). Surface membranes isolated from HeLa cells differ in containing a higher content (19.2%) of basic amino acids (Bosmann, Hagopian & Eylar, 1968).

Protein components. Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate resolved the proteins of plasma-membrane fractions from the rat into about 20 bands (Plate 1). Similar numbers of bands appeared when both heavy and light sub-

appeared to be less than in unfractionated membranes. The two major subfractions of mouse plasma correspond closely to those isolated from rat livers (Evans, 1969).

DISCUSSION

Preparation of plasma membranes. The preparative method described, utilizing the low-speed zonal rotor, can be regarded as a development of the methods of Neville (1960) and Emmelot *et al.* (1964). The isolated membranes resembled closely in chemical, morphological and enzymic detail those prepared by variations of this method. By using the zonal rotor, large quantities of tissue homogenate can be fractionated at the same time. Further, preparation is rapid, thus affording within 4 h a plasma-membrane preparation relatively free of mitochondria and nuclei, which should prove useful for biosynthetic studies when early time points are often required. A major source of loss of plasma membranes, as judged by recoveries of 5'-nucleotidase, appears to be the initial low-speed centrifugation. The possibility that the 5'-nucleotidase activity remaining in the supernatant is located in low-density vesicles of high specific activity similar to those isolated as the light subfraction cannot be ignored. Attempts to isolate such vesicular structures from the low-speed supernatant were not successful.

The low-speed zonal rotor profile (Fig. 1) indicates that liver plasma membranes can be sedimented through a sucrose density gradient as a band occupying a position intermediate between mitochondria and nuclei. This procedure can be regarded as an improvement, especially from the viewpoint of yield of purified membranes, on methods that depend upon repeated low-speed differential centrifugation. Liver mitochondria and plasma-membrane fractions contain populations of structures which differ in size and it is to be expected that the fractions will be heterogeneous with respect to their sedimentation properties, resulting in the slight overlap seen in the 'A' zonal rotor. Clearly the extent of overlap will depend, among other reasons, critically on the method of tissue dispersion and the extent of fragmentation of the surface membrane.

Plasma-membrane fractions are often purified further by sucrose-density-gradient centrifugation. The extent of separation of 5'-nucleotidase activity and succinate dehydrogenase activity was investigated by using a sucrose density gradient contained in the high-speed BXIV zonal rotor. Although the mean densities of unfractionated membranes and mitochondria differ, there is an overlap, indicating that discarding of fractions containing succinate dehydrogenase activity also resulted in

appreciable losses of plasma membranes. This finding is in agreement with the extensive studies by Leighton *et al.* (1968) on the isopycnic distribution of various liver subcellular fractions. The present experiments indicate that liver plasma membranes are best prepared to the above criteria by a rate-dependent zonal centrifugation followed by density-gradient centrifugation in sucrose.

Subfractionation studies. The results indicate that the liver plasma-membrane fraction is composed of at least two types of membrane components, of widely different densities, which can be separated on sucrose density gradients after mechanical disruption in a tight-fitting homogenizer. The liver plasma-membrane fraction of sucrose density 1.13 isolated by Coleman *et al.* (1967) that differed from the plasma-membrane fractions found by others at densities of 1.16–1.19 (Emmelot *et al.* 1964; Berman *et al.* 1969) was probably similar to the light subfraction isolated in the present paper. Coleman *et al.* (1967) did not investigate other heavier bands appearing in the discontinuous sucrose density gradient. Song, Rubin, Rifkind & Kappas (1969) isolated from rat liver homogenates two plasma-membrane fractions of sucrose density 1.16 and 1.18, the former possessing higher 5'-nucleotidase activity than the latter. These two fractions, which were subsequently combined to give a purified plasma-membrane fraction rich in bile canaliculi and junctional complexes, probably correspond to the unfractionated and heavy subfraction described in the present paper.

The light subfraction, which accounts for approx. 30% by weight of the total plasma-membrane fraction of rat and mouse liver, is rich in 5'-nucleotidase, nucleoside diphosphatases, Mg^{2+} -stimulated adenosine triphosphatase and leucine β -naphthylamidase. In contrast, Na^{+} -stimulated adenosine triphosphatase activity is located at higher specific activities in the heavy subfraction. Attempts to produce more complete separation of these enzyme activities by repeated homogenization of the heavy subfraction were not successful.

Chemical analyses of the two subfractions provide further points of difference. The presence of twice as much phospholipid and cholesterol in the light subfraction as in the heavy subfraction explains their widely differing densities in sucrose. *N*-Acetylneuraminic acid, usually accepted from studies on erythrocytes, as a mammalian cell-surface marker (Eylar, Madoff, Brody & Oncley, 1962) is shown to be located at a threefold higher concentration in the light subfraction. Although the total amino acid compositions of the subfractions are similar, definite differences in protein composition are evident when the number and intensity of the bands resolved by polyacrylamide-gel electrophoresis are examined.

Electron microscopy showed the liver plasma membrane fraction to be complex (Benedetti & Emmelot, 1968). Although investigators always emphasized the presence of junctional complexes and bile canaliculi in their isolated fractions as an important factor in attributing the derivation of this subcellular fraction from the cell surface of liver parenchyma cells, large numbers of vesicular components of unknown origin and of various sizes are always present in sections observed in the electron microscope (Korn, 1969). Therefore the isolation of a subfraction that is entirely vesicular can be regarded as a contribution to the partial resolution of the plasma-membrane fraction into its components. In the present paper, the vesicles of the light subfraction are characterized by their chemical and enzymic composition. The heavy subfraction retains the characteristic membrane strips with their junctional complexes, but many vesicles are still present.

The plasma-membrane fraction and subfractions of rat and mouse livers correspond closely in their biochemical, chemical and morphological composition. The liver parenchyma-cell membrane, as seen in sections of tissue, must be regarded as complex, since it has three physiologically specialized surface areas. Certain parts of the plasma membrane are in contact with lacunae and neighbouring parenchyma cells, whereas other parts form microvilli that surround the bile canaliculi (Elias & Sherrick, 1969). It is likely that these liver-cell surface areas have different compositions. The two subfractions characterized in the present paper probably originate from different areas of the cell surface and thus have different enzymic and chemical properties. The junctional complexes present in the heavy subfractions indicate that they are derived from areas in contact with neighbouring parenchyma cells. Histochemical studies on the distribution of nucleoside tri-, di- and mono-phosphatases in liver tissue indicated the greatest concentration of these enzyme activities where the plasma membrane folds to form the microvilli of the bile canaliculi (Novikoff, Essner, Goldfischer & Heus, 1962). The vesicular light subfraction isolated in the present investigation is enriched in 5'-nucleotidase, nucleoside diphosphatase and adenosine triphosphatase activities, and this fact, coupled with the histochemical evidence, points to the derivation of these vesicles from the microvillar surfaces of the bile canaliculi. The origin of the vesicles remaining with the junctional complexes in the heavy subfraction was not determined.

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