Preparation and Properties of Nexuses and Lipid-enriched Vesicles from Mouse Liver Plasma Membranes

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Extraction of mouse liver plasma membranes with 4% (w/v) *N*-laurylsarcosinate–tris buffer, pH 7.8, solubilized 80–90% of the protein and 60% of the 5′-nucleotidase activity. The membrane residue remaining after extraction was resolved on sucrose gradients into two fractions: a vesicular membrane fraction and a fraction characterized by the presence of large numbers of nexuses in an amorphous background. The vesicular fraction had a phospholipid/protein weight ratio of 7:1, it contained most of the plasma-membrane glycolipids, and polyacrylamide-gel electrophoresis indicated the presence of only five to eight proteins, including two or three glycoproteins. The 5′-nucleotidase and leucine naphthylamidase specific activities were 23- and 6-fold higher respectively than in the plasma membranes. Electron microscopy of thin sections and negatively stained preparations indicated that the nexuses present in the second fraction closely resembled gap junctions present in tissue sections and isolated plasma membranes. The nexus fraction contained a distinctive protein pattern, and of the 20 proteins present about four were identified as glycoproteins by Schiff–periodate staining. Examination of the lipid composition of the fractions by t.l.c. showed that in the nexus fraction, phospholipids and glycolipids were present in small amounts compared with triglycerides and cholesterol. Amino sugar analyses confirmed the t.l.c. results and amino acid analysis showed the fractions to have characteristic protein compositions. A ‘reconstituted’ membranous fraction prepared by dialysis against MgCl_2 of membrane components soluble in *N*-laurylsarcosinate–tris buffers, pH 7.8, lacked the trilaminar image characteristic of the two other membrane fractions isolated and was devoid of enzyme activities. The results indicate that proteins and glycoproteins play an important role in the structural maintenance of the nexuses isolated from the liver by the present procedure.

The plasma-membrane fraction prepared from liver homogenates consists of sheets of membranes interconnected by junctional complexes, and large numbers of vesicles (Neville, 1960; Evans, 1970). Junctional complexes present in this fraction are derived from the surfaces of parenchymal cells in apposition, and a study of their chemical and biochemical properties may be regarded as a necessary step towards understanding how aggregates of cells co-ordinate their activities. Hepatic junctional complexes have been extensively studied in tissue sections by optical techniques (Matter *et al.*, 1969; Chalcraft & Bullivant, 1970) and these studies have been extended to include isolated plasma membranes and subfractions containing tight or gap junctions (Benedetti & Emmelot, 1968; Goodenough & Revel, 1970). However, the low yield of fractions enriched in junctional complexes has precluded a more thorough examination of their chemical composition.

In the present study junctional complexes, identified as nexuses of the gap junction category, were prepared by sucrose-density-gradient centrifugation of the residue remaining after extraction of the mouse

liver plasma membranes with *N*-laurylsarcosinate–tris buffer, pH 7.8 (sarcosyl–tris medium). A fraction containing lipid-enriched vesicles of high 5′-nucleotidase specific activity was also obtained, and the chemical, enzymic and morphological properties of these two fractions, and also of ‘reconstituted’ membranes prepared by dialysis of the sarcosyl–tris medium-soluble components against Mg^{2+} are described.

Experimental

Preparation of subfractions

The plasma-membrane fraction was prepared from the livers of 100 fed Parkes’ white mice by using the MSE A XII zonal rotor (Evans, 1970). The pooled membranes from the zonal rotor were resuspended in 0.25M-sucrose in a tight-fitting Dounce homogenizer [clearance 0.076 mm (0.003 in)] and then were centrifuged at $97000g_{av}$ for 30 min on a ‘cushion’ of 47% (w/v) sucrose in a Beckman SW 27 rotor. The membranes at the interface were collected with a syringe, then they were diluted to

approx. 75 ml with water, and were pelleted by centrifugation at $97000g_{av}$ for 45 min in the SW 27 rotor. The enzymic, chemical and morphological properties of the membranes have been described (Evans, 1970; Evans & Gurd, 1971). The tubes containing the membrane pads (protein yield 110–150 mg) were covered with Parafilm and were kept at 4°C overnight. The membrane pads were suspended at 4°C by using the tight-fitting Dounce homogenizer in 34 ml of 4% (w/v) sarcosyl–tris medium (all sarcosyl–tris medium solutions were prepared by dissolving 1 g of *N*-lauryl-sarcosinate and 0.545 g of tris base in water to give pH 7.8). The suspension, which immediately showed partial clarification, was centrifuged at $97000g_{av}$ for 40 min (Beckman SW27 rotor). The yellowish supernatant was carefully removed with a Pasteur pipette, the pads were resuspended in 10 ml of 3% (w/v) sarcosyl–tris medium and the suspension was centrifuged at $100000g$ for 40 min in a Beckman 65 rotor. The supernatant was pooled with the previous supernatant to constitute the sarcosyl–tris medium extract, and the pellet, designated the sarcosyl–tris medium-insoluble membrane fraction, was resuspended in 8% (w/v) sucrose (approx. 10 ml) by using a tight-fitting Dounce homogenizer and was introduced on to a discontinuous sucrose gradient containing 10 ml of 64% (w/v), 10 ml of 54% (w/v) and 10 ml of 35% (w/v) sucrose. After centrifugation for 2 h (Beckman SW27 rotor) at $97000g_{av}$, a whitish fraction was present at the 8–35% interface (sarcosyl–tris medium-insoluble light) and aggregated membranous material (sarcosyl–tris medium-insoluble heavy) was present at the 54–64% interface. The sarcosyl–tris medium-insoluble light membranes were collected, diluted tenfold with water, centrifuged at $100000g$ for 2 h and suspended in a minimal volume of 0.25 M-sucrose. The sarcosyl–tris medium-insoluble heavy membranes were collected with a wide-bore Pasteur pipette, then they were diluted with water and washed twice by centrifugation at $3000g$ for 20 min (MSE Mistral). This fraction aggregated easily and was generally difficult to handle.

Injection of [^{32}P]phosphate

To label membrane phospholipids, a group of 10 of the 100 mice used for preparation of membrane fractions were injected with a total of 1 mCi of $\text{Na}_3^{32}\text{PO}_4$ in iso-osmotic saline containing phosphate buffer (The Radiochemical Centre, Amersham, Bucks., U.K.) 16 h before being killed. This procedure gives maximum labelling of phospholipids, especially sphingomyelin, in smooth microsomal fractions (Tata, 1970).

Chemical and enzymic determinations

Protein content of membrane samples was determined by the method of Lowry *et al.* (1951) with

bovine serum albumin as standard (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.). Amino sugar and amino acid contents were determined as previously described (Evans, 1970; Gurd *et al.*, 1972). Leucine naphthylamidase activity was determined by the method of Goldbarg & Rutenburg (1958), 5'-nucleotidase activity by the method of Michell & Hawthorne (1965) and adenosine triphosphatase activity by the method of Swanson *et al.* (1964). Membrane pads and extracts were extracted with chloroform-methanol (2:1, v/v) and the phospholipid content was determined as previously described (Evans, 1970; Gurd *et al.*, 1972). T.l.c. of extracts was done on silica gel G or H plates (E. Merck A.-G., Darmstadt, Germany) with the following systems: (a) chloroform-methanol-water (14:6:1, by vol.) to 12 cm; *n*-propanol-aq. 4.4% (w/v) ammonia (4:1, v/v) to 6 cm; light petroleum (b.p. $40\text{--}60^{\circ}\text{C}$)-diethyl ether-acetic acid (73:25:2, by vol.) to 18 cm; (b) chloroform-methanol-4M- NH_3 (9:7:2, by vol.). Plates were stained with H_2SO_4 and radioautograms were prepared with Ilford No-screen X-ray films.

Polyacrylamide-gel electrophoresis

Membrane samples were clarified by heating in 4M-urea-1% sodium lauryl sulphate-1% mercaptoethanol solution at 90°C for 3–5 min and then were dialysed against 0.1% sodium lauryl sulphate-10 mM-mercaptoethanol-0.03 M-sodium phosphate buffer, pH 7.2, for 16 h. Polyacrylamide gels (7.5%, w/v, acrylamide) were prepared as described by Weber & Osborne (1969) and samples were subjected to electrophoresis at 4 mA/tube for 16 h in gels (10 cm long $\times$ 4 mm diam.) and stained for protein with Amido Black or for carbohydrate by the Schiff-periodate procedure (Zacharias *et al.*, 1969). The specificity of the Schiff-periodate procedure was ascertained by using known proteins, including reoviral molecular-weight marker proteins (kindly given by Dr. J. Skehel of this Institute) known to be free of carbohydrate (Smith *et al.*, 1969). These proteins, trypsin, bovine serum albumin and lactate dehydrogenase were not stained by this procedure; further, the patterns obtained with membrane fractions differed greatly from those stained for protein, again arguing for specific staining of carbohydrate. Under the present conditions, therefore, it is unnecessary to resort to the more complex modified procedure devised by Glossmann & Neville (1971). Marker proteins were run under the same conditions, with the exception of immunoglobulin G, which was run in the unreduced form.

Electron microscopy

Membrane pellets were fixed for 1 h at 4°C in a mixture (2:1, v/v) of 1% (w/v) osmium tetroxide and

2.5% glutaraldehyde in 0.1M-sodium cacodylate buffer, pH7.4 (Hirsch & Fedorko, 1968). The pellets were 'post-fixed' for 15min in 0.25% (w/v) uranyl acetate in 0.1M-veronal-acetate buffer, pH6.2, and embedded in Epikote 812. Thin sections were stained with uranyl acetate and lead citrate. For negative staining, membrane suspensions in water were stained on carbon-coated Parlodion films with either 3% ammonium molybdate, pH6.5, or 1% uranyl acetate, pH4.4. Observations were made with a Philips EM-300 electron microscope.

Materials

Chemicals of AnalaR grade were obtained from British Drug Houses (Poole, Dorset, U.K.) and biochemicals were from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Immunoglobulin G was a gift from Dr. M. J. Crumpton of this Institute. The *N*-laurylsarcosinate was a gift from Geigy (U.K.) Ltd., Manchester, U.K. Glass-distilled water was used throughout.

Results

Solubility of membranes in detergents

The present studies were begun in an attempt to achieve complete solubilization in detergents of the liver plasma-membrane fraction before fractionation and separation of the protein components. The plasma membranes were completely soluble in 1% sodium lauryl sulphate-mercaptoethanol mixtures, but with concomitant loss of activity of such enzymes as 5'-nucleotidase and β -leucine naphthylamidases and probably most, if not all, other enzymes. However, sarcosyl-tris medium solubilized most of the membrane protein (>80%), with retention of 5'-nucleotidase activity. In these studies, soluble material is defined as being non-sedimentable after centrifugation at 100000g for 30min in aqueous media of density 1.10 or less.

The ability of various concentrations of sarcosyl-tris medium to solubilize membrane proteins is shown in Fig. 1. Although in 1% (w/v) sarcosyl-tris medium, over 80% of the membrane proteins were extracted, higher concentrations of detergent were required to release maximum amounts of the 5'-nucleotidase (Fig. 1). The fractionation procedure finally used entailed extraction of membranes in 3-4% sarcosyl-tris medium, pH7.8, since this ensured maximal solubilization of the protein and 5'-nucleotidase activity and was reproducible.

Fractionation of detergent-insoluble residue

After sarcosyl-tris-medium extraction, approx. 10-15% of the membrane protein was recovered in the pad. This residue was resuspended in 8% (w/v)

Fig. 1. Solubility in sarcosyl-tris medium solutions (pH7.8) of liver plasma membrane protein and 5'-nucleotidase

Plasma-membrane pellets (approx. 4mg) were suspended by using a Dounce homogenizer in 2ml of sarcosyl-tris medium solutions at 4°C and then were centrifuged at 100000g for 1h. The pellet was re-extracted with the same concentration of sarcosyl-tris medium and the final pellet and pooled supernatants were analysed for protein and 5'-nucleotidase activity as described in the Experimental section. Protein in supernatant, ○; in pellet, ●; 5'-nucleotidase in supernatant, △.

sucrose and was resolved into two components by centrifugation on discontinuous sucrose gradients. In describing and discussing these fractions, they are referred to as the sarcosyl-tris-medium-insoluble light or vesicular fraction [found at the 8-35% (w/v) sucrose interface] and the sarcosyl-tris-medium-insoluble heavy or nexus fraction, found at the 54-64% sucrose interface. The properties of these two membrane fractions are also compared with the sarcosyl-tris-medium-soluble fraction and a 'reconstituted' membrane fraction.

Morphological properties of the fractions

The isolated plasma-membrane fraction is morphologically heterogeneous, consisting of strips of membranes interconnected by junctional complexes, and large numbers of vesicles (Evans, 1970). Plate 1(a) shows that the sarcosyl-tris-medium-insoluble light fraction is vesicular. Many of the vesicles were multi-layered, and the insert indicates that the trilaminar unit membrane (width 7.0-7.5 nm) was well preserved in this fraction. Negative staining of these preparations (Plate 1b) showed that the vesicles were smooth-surfaced, and a number of surveys confirmed the

absence of the projections that are seen on negatively stained plasma-membrane preparations (Benedetti & Emmelot, 1965).

The sarcosyl-tris-medium-insoluble heavy fraction (Plate 2a) was isolated as a pellet in a preliminary experiment in which 57% (w/v) sucrose was substituted for 64% sucrose and the 54% sucrose was omitted. Large numbers of intracellular junctions in addition to some vesicles similar to those described in Plate 1(a) are present, and these structures closely resemble nexuses found in intact tissue and the isolated plasma-membrane fraction (Goodenough & Revel, 1970; Evans, 1970). The pentalaminar structure is well preserved and the central lamina is sometimes seen to contain structures resembling globular units. The pentalaminar structure is visible if the correct plane of sectioning is achieved; the membranous continuations of these structures appeared diffuse. Introduction of a 54% (w/v) sucrose step minimized the number of vesicular structures recovered in the sarcosyl-tris-medium-extracted heavy fraction, and Plate 2(b) shows this fraction as prepared for chemical and enzymic analysis. Densely packed junctional complexes were found in an amorphous background. Examination of this fraction by negative staining (Plate 3a) showed the frequent presence of polygonal lattices, previously shown to be typical of gap junctions, which were especially obvious after brief treatment of membranes with deoxycholate solutions (Benedetti & Emmelot, 1968; Goodenough & Revel, 1970). Therefore, the morphological evidence indicates the preponderance of nexuses, mostly of the gap-junction variety, in the sarcosyl-tris-medium-extracted heavy fraction, whereas the sarcosyl-tris-medium-insoluble light

fraction consists of vesicular structures with well-preserved unit membranes.

Enzymic and chemical properties

The 5'-nucleotidase and leucine naphthylamidase enzymes were active in both sarcosyl-tris-medium-insoluble membrane fractions and in the sarcosyl-tris medium-soluble fraction. However, Mg^{2+} -stimulated adenosine triphosphatase activity was absent in the sarcosyl-tris medium extract and the vesicular fraction, and a low activity was detected in the nexus fraction. The sarcosyl-tris medium extraction solubilized 61% of the plasma-membrane 5'-nucleotidase activity, and 33% of the leucine naphthylamidase activity (Table 1). The two enzymic activities remaining membrane bound were almost entirely located in the vesicular fraction; on a specific activity basis, the 5'-nucleotidase activity in the vesicles was 23-fold higher than in the plasma membranes, and the leucynaphthylamidase activity was also enhanced but to a lesser extent.

Although 80–90% of the plasma-membrane protein was soluble in sarcosyl-tris medium, extraction of phospholipid was less complete (75%) (Table 1). Almost all of the unextracted phospholipids were associated with the vesicles, which had six to eight times as much phospholipid as protein on a weight basis. The lipids of the sarcosyl-tris-medium-soluble and the sarcosyl-tris-medium-insoluble membrane fractions and the plasma-membrane fraction were compared by using t.l.c. (Plate 4a). The sarcosyl-tris-medium-soluble fraction closely resembled the plasma-membrane fraction in lipid content. Quantitative analyses of the lipids of liver plasma membranes

Table 1. *Distribution of protein, phospholipid and enzymes in fractions of sarcosyl-tris-medium-treated liver plasma membranes*

The extraction of the liver plasma membranes was carried out as described in the Experimental section. Enzymic activities are expressed as μmol of product liberated/h per mg of protein at 37°C. N.D., Not determined.

Fraction	Protein (mg)	Phospholipid (mg/mg of protein)	5'-Nucleotidase activity	Leucine naphthylamidase activity
Homogenate	16000	N.D.	0.60	0.33
Plasma membranes	148	0.65	13.5	1.65
Sarcosyl-tris-medium-soluble	130	0.56	9.1	0.61
Sarcosyl-tris-medium-insoluble (total)	15	N.D.	51.2	N.D.
Sarcosyl-tris-medium-insoluble light (vesicles)	1.5	6.5	313	7.4
Sarcosyl-tris-medium-insoluble heavy (nexuses)*	5	0.05	11.6	1.4

* The membranes in this fraction adhered to glass and cellulose nitrate and this accounts for the low recovery.

EXPLANATION OF PLATE I

(a) *Electron micrograph of the sarcosyl-tris-medium-insoluble light fraction*

Large numbers of vesicles preponderate, many of which somewhat resemble myelin figures. The insert indicates that the membranes are trilaminar. The bar represents $0.1\ \mu\text{m}$.

(b) *Sarcosyl-tris-medium-insoluble light fraction negatively stained with ammonium molybdate*

The vesicles are smooth-surfaced. The bar represents $0.1\ \mu\text{m}$.

EXPLANATION OF PLATE 2

(a) *Electron micrograph of the sarcosyl-tris-medium-insoluble heavy fraction isolated in a preliminary experiment*
Nexuses of the gap junction category are present in addition to some vesicles. The bar represents 0.1 μm .

(b) *Nexus fraction used for chemical and enzymic estimations*
Large numbers of nexuses are present in an amorphous background. The bar represents 0.1 μm .

EXPLANATION OF PLATE 3

(a) *Electron micrograph of the nexus fraction negatively stained with uranyl acetate*
Polygonal lattice in the substructure is evident. The bar represents 0.1 μm .

(b) *'Reconstituted' membranes*

These membranes have a granular appearance. The bar represents 0.1 μm .

EXPLANATION OF PLATE 4

T.l.c. of the fractions

(a) Silica-gel plate (G) run in solvent (a) (see Experimental section). (1) Plasma membranes; (2) sarcosyl-tris-medium-soluble; (3) nexus fraction; (4) vesicular fraction. (b) Silica-gel plate (H) run in solvent (b). (1) Plasma membranes; (2) vesicular fraction; (3) total sarcosyl-tris-medium-insoluble residue; (4) nexus fraction; (5) sarcosyl-tris-medium-soluble. The modified pattern observed with the sarcosyl-tris-medium-soluble fraction is due to the persistence of sarcosyl-tris medium.

Table 2. *Amino acid and amino sugar content of liver fractions*

For details see the text. Amino acid values are expressed as mol/100 mol. Amino sugar values are expressed as $\mu\text{mol/g}$ of protein.

Amino acid	Plasma membranes	Sarcosyl-tris-medium-soluble	Light sarcosyl-tris-medium-insoluble membranes	Nexus fraction	'Reconstituted' membranes
Lys	7.5	6.8	7.5	6.0	7.1
His	2.3	1.7	2.5	2.8	2.1
Arg	5.0	4.5	2.9	5.8	4.8
Asp	8.4	8.3	9.4	8.0	9.4
Thr	4.5	4.3	4.7	4.6	5.1
Ser	6.1	4.8	12.1	6.2	6.0
Glu	11.5	11.3	9.0	13.3	11.9
Pro	4.6	5.0	5.1	3.0	5.9
Gly	8.5	7.5	10.0	14.4	7.7
Ala	9.0	8.8	10.8	7.9	7.7
Val	6.2	8.8	7.3	7.0	6.6
Met	1.6	2.0	0.5	1.4	2.4
Ile	5.6	6.6	5.1	4.6	5.5
Leu	10.5	12.1	9.4	9.0	10.9
Tyr	3.1	2.1	0.9	2.5	2.8
Phe	4.6	5.0	3.4	3.3	4.3
GlcN	85†	76	1	5.2*	
GalN	34†	14	82	1*	

* Expressed as a ratio of glucosamine to galactosamine.

† Values quoted from Gurd *et al.* (1972).

have been reported (Dod & Gray, 1968; Ray *et al.*, 1969).

A comparison of the two sarcosyl-tris-medium-insoluble fractions showed large differences in the distribution of various lipid components. The vesicular fraction contained two components that were absent in the sarcosyl-tris-medium extract and the nexus fraction, and these have been identified as glycolipids by measuring the incorporation of [^{14}C]-glucosamine into liver membranes (Evans & Gurd, 1971). The identity and distribution among the fractions of the glycolipids was also confirmed by using another solvent system (Plate 4b), which gave greater separation of the hydrophilic lipids. By using extracts of membrane fractions prepared from mice injected with [^{32}P]phosphate to label phospholipids (Tata, 1970), it was confirmed by radioautography that the three spots identified as glycolipid contained no radioactivity. These glycolipids have similar chromatographic properties to haematosides and gangliosides (Payne, 1964). The nexus fraction was deficient in phospholipids, but triglycerides and cholesterol (especially its esterified form) were present. Therefore, although sarcosyl-tris medium extracts phospholipids from liver plasma membranes, glycolipids are resistant to extraction and are recovered in the vesicular fraction. The nexus fraction is

deficient in phospholipids relative to the neutral-lipid content.

Amino acid and amino sugar analyses showed differences between the fractions (Table 2). The results for the sarcosyl-tris medium-soluble fraction, which accounts for the bulk of the membrane protein, corresponded closely to those for the plasma membranes. The vesicular fraction contained a relatively high serine content and a low tyrosine content; the proportion of basic amino acids was also low. Because of the high lipid content of this fraction, chloroform-methanol-extracted membranes were used for analysis. The nexus fraction contained a higher glutamic acid and glycine content than the other fractions. A high content of glycine is a characteristic feature of most structural proteins (Seifter & Gallop, 1966) and also glomerular basement membranes (Westberg & Michael, 1970; Spiro, 1967). However, not surprisingly, the overall amino acid composition of the nexus fraction is similar to that of the total plasma membrane. These differences contrast with the results obtained by analysis of the liver plasma-membrane fractions soluble or insoluble in EDTA-bicarbonate and pyridine solutions (Gurd *et al.*, 1972). The low galactosamine content of the sarcosyl-tris-medium-soluble and nexus fractions is in agreement with the t.l.c. results indicating the absence of glycolipid

components in these fractions. It has been shown (Evans & Gurd, 1971) that in mouse liver plasma-membrane fractions most of the galactosamine is lipid-bound, whereas glucosamine is protein bound.

The plasma-membrane proteins are resolved into 20 or more components by polyacrylamide-gel electrophoresis in sodium lauryl sulphate at pH 7.4 (Fig. 2a). A major portion of the liver plasma-mem-

Fig. 2. Polyacrylamide-gel tracings of (a) liver plasma membranes; (b) sarcosyl-tris-medium-soluble fraction; (c) nexus fraction; (d) sarcosyl-tris-medium-insoluble light fraction

Gels were run and stained as described in the Experimental section. Corresponding bands in the fraction can be identified by referring to the numbers on each peak. Shaded bands indicate glycoproteins as identified by Schiff-periodate staining. Arrows indicate position of molecular-weight markers; I, immunoglobulin G (150 000); B, bovine serum albumin (69 000); L, lactate dehydrogenase (33 000); T, trypsin inhibitor (20 000); M, myoglobin (17 000). Gel (d) does not correspond closely to the other gels because of the high lipid concentration of this fraction. Tracings were made from gels with the gel-scanning accessory of the Unicam SP.1800 spectrophotometer.

brane protein is found in the 40000–80000 mol.wt. range; these proteins are not stained by the Schiff–periodate procedure, and therefore have little or no attached carbohydrate. There are proportionately few low-molecular-weight components, and therefore the presence in liver plasma membranes of large amounts of low-molecular-weight polypeptides or glycopeptides (Laico *et al.*, 1970) can be discounted. Many of the higher-molecular-weight components (80000–200000) are glycoproteins, and normally about six of these components stain with the Schiff–periodate procedure. The sarcosyl-soluble proteins gave a similar gel profile to the total plasma membranes (Fig. 2*b*). However, modified patterns were obtained for the two sarcosyl–tris-medium-insoluble fractions. The nexus fraction contained a large number of proteins including glycoproteins of high molecular weight and a low-molecular-weight component (component 20) was also prominent (Fig. 2*c*). The vesicular fraction contained few proteins. In different preparations, from five to eight proteins were separated, of which two or three of the highest-molecular-weight bands were glycoproteins (Fig. 2*d*). It was difficult to resolve the proteins of this fraction, presumably because of the large amount of lipid present, and delipidation before electrophoresis did not prove satisfactory. Staining with the Schiff–periodate procedure also confirmed the t.l.c. and amino sugar analyses on the glycolipid distribution. Plasma membranes, and especially the vesicular membranes, gave intense staining near the front of the gel where glycolipids migrate, whereas the nexus fraction showed very little staining.

‘Reconstituted’ membranes

The vesicular fraction, described above, possesses a number of unusual morphological, enzymic and chemical features. It is possible that it could have arisen by reconstitution of some of the more hydrophobic components present in the sarcosyl–tris-medium extract. The formation of membranous material from the sarcosyl–tris-medium extract was therefore briefly investigated. The procedure of Engelman & Morowitz (1968) for reconstituting *Acholeplasma laidlawii* membranes dissociated in sodium lauryl sulphate into lipid and protein components was used and the sarcosyl–tris-medium extract was dialysed against 1 mM-MgCl₂ at 4°C for 16 h. The cloudy material that appeared was collected by centrifuging at 100000*g* for 30 min and sections were examined in the electron microscope. Although at lower magnifications the material appeared membranous, at higher magnification (Plate 3*b*) a granular substructure was evident, and the trilaminar image observed in the other fractions was almost entirely absent. Amino acid analysis of the ‘reconstituted’

membranes (Table 2) showed a composition similar to plasma membranes. Several attempts to analyse these membranes by polyacrylamide-gel electrophoresis were unsuccessful. No 5′-nucleotidase activity or leucine naphthylamidase activity could be measured.

Discussion

Anatomically, the liver parenchymal-cell surface has long been considered to consist of three major areas corresponding to the microvillar membranes facing the sinusoidal lumen on the one hand and the bile canaliculus on the other, and the membranes closely apposed to and forming junctions with neighbouring cells (Bruni & Porter, 1965; Heath & Wissig, 1966). After the development of procedures for isolation of surface- (plasma-) membrane fractions, the subfractionation and resolution into membranes corresponding to the above functional areas becomes a major objective (Evans, 1970). Intercellular junctional complexes can be investigated because of the well-characterized morphological structures, observed in sections of intact tissues and isolated fractions, that can be followed during the isolation procedure. In the present studies, a fraction enriched in junctional complexes involved in the attachment of adjacent parenchymal cells has been isolated and characterized.

Epithelial-tissue junctional complexes were classified into three major types by Farquhar & Palade (1963): *a*, tight junctions (*zona occludens*) in which the intercellular space is completely obliterated; *b*, intermediate junctions (*zona adherens*) characterized by the presence of a 20 nm intercellular space; *c*, the desmosomes (*macula adherens*), which possess a central disc of dense material. A further division of the tight junction was introduced by Goodenough & Revel (1970), who showed that in some tight junctional complexes of mouse liver, the intercellular space is not completely obliterated, and a 2 nm gap, permeable to colloidal lanthanum, remained between the closely apposed membranes. This is termed a gap junction. Gap junctions also contain the polygonal substructure which is seen in negative-stained preparations (Benedetti & Emmelot, 1968), in sections after the application of colloidal lanthanum (Revel & Karnovsky, 1967) and by freeze-etching techniques (Goodenough & Revel, 1970). The term nexus, first introduced by Dewey & Barr (1962), is now often used to cover both tight and gap junctions (McNutt & Weinstein, 1970). The intercellular junctional complexes of the heavy sarcosyl–tris-medium-insoluble fraction corresponded most closely to gap junctions, since a gap is apparent in most sections and the junctional diameter (20 nm) is 2.5 times that of plasma membranes (approx. 8 nm), whereas in

tight junctions the overall diameter is less than twice that of single membranes (Brightman & Reese, 1969; McNutt & Weinstein, 1970). In addition, a polygonal lattice is shown by negative staining. Although the presence of a gap should be demonstrated by lanthanum ion penetration, it is noteworthy in this context that freeze-etch techniques have thus far failed to demonstrate the presence of a gap (McNutt & Weinstein, 1970). In view of the possible modification of the junctional fraction by prolonged exposure to sarcosyl-tris medium, the term nexus has been applied in the present paper to the junctional complexes isolated, because both tight and gap junctions may originally have been present.

The chemical studies described show that the nexus-enriched fraction has a very low phospholipid content, and the t.l.c. studies indicate the preponderance of the neutral lipids, free and esterified cholesterol and triglyceride. This is surprising, since it is recognized that cholesterol is the most loosely bound and easily exchangeable lipid in membranes (Roelofsen *et al.*, 1964; Higgins & Green, 1966). The retention of these neutral lipids in these junctional complexes points to an important function. In spite of the relative absence of phospholipids, the morphological appearance in thin sections or by negative staining corresponds closely to nexuses of isolated plasma-membrane fractions and also to those exposed briefly to deoxycholate (Benedetti & Emmelot, 1968). These results, therefore, contrast with those of Goodenough & Revel (1970, 1971) who, on the basis of acetone extractions and phospholipase treatment of mouse liver plasma membranes, attributed a major role to phospholipids in the preservation of morphological integrity and especially the 2nm gap and associated polygonal lattice of the gap junction. The present results therefore indicate that phospholipids play only a minor role in the maintenance of the structural features of the nexus of liver parenchymal cells. Morphological intactness of the nexus in sections and negatively stained preparations is maintained in the fraction that is depleted of phospholipids. Indeed, it appears that protein and glycoprotein components play a far more important role in the structure of the nexus. The nexus has been suggested as the site that mediates in cell-cell communication by providing a route for the passage of small molecules (Dewey & Barr, 1962, 1964), and models of nexal subunits incorporate hydrophilic channels for this function (McNutt & Weinstein, 1970). In view of the low 5'-nucleotidase specific activity associated with the nexus fraction compared with the vesicular fraction, it would seem that this enzyme does not play any major role in intercellular communication. It has also been shown that the 5'-nucleotidase activity of plasmacytoma cells decreased when these cells come into contact (Lelievre *et al.*, 1971). Conclusions about the role of the adenosinetriphosphatase activity

in the nexus must be reserved in view of the lipid-dependence of this enzyme (Wheeler & Whittam, 1970), although Lelievre *et al.* (1971) showed that the enzyme activity also decreases on contact inhibition of plasmacytoma cells.

Extraction of the liver plasma membrane with sarcosyl-tris medium results in the selective solubilization of membrane protein or lipid components. The junctional complexes, unlike most of the surface membranes, are resistant to sarcosyl-tris-medium extraction as well as to mechanical disruption. The results also show that sarcosyl-tris medium is more effective in the overall solubilization of protein components than phospholipids. However, the differential solubility in sarcosyl-tris medium of different areas of cell surface is illustrated by the two fractions obtained in the present studies. The nexus fraction is deficient in phospholipids, whereas the vesicular fraction is deficient in protein. Solubilization of membranes by detergents is a complex process, and the effects of temperature, ions and pH on the kinetics of solubilization by ionic and non-ionic detergents have been investigated (Philippot, 1971; Allan & Crumpton, 1971; Miller, 1970). The greater solubility in detergents of non-junctional surface-membrane areas has enabled the preparation of fractions enriched in junctional complexes from rat liver by Emmelot *et al.* (1970), who used deoxycholate, and from brain synaptosomes by Cotman *et al.* (1971), who used Triton X-100. The origin of the lipid-enriched sarcosyl-tris-medium-insoluble light vesicles is unknown. The possibility that they are derived from intracytoplasmic membranes, e.g. endoplasmic-reticular vesicles, is unlikely in view of the purity of the plasma membranes used (Evans, 1970; Evans & Gurd, 1971), and the higher solubility of mouse liver microsomal fractions in sarcosyl-tris medium and deoxycholate solutions (W. H. Evans, unpublished work). Although it is possible that these membranes are artifacts resulting from detergent extractions, their further insolubility in deoxycholate-Triton X-100 mixtures (W. H. Evans, unpublished work) may point to an important structural function. These membranes could provide a framework in the bile-canalicular region of the liver cell, where insolubility in the presence of bile salts would be obligatory. The high 5'-nucleotidase activity associated with this fraction supports this contention, as histochemical studies indicate intense nucleotidase activity in this area of the liver-cell surface (Essner *et al.*, 1958).

Both the sarcosyl-tris-medium-insoluble fractions examined in the present work have a well-preserved unit-membrane structure, despite the findings that the vesicular fraction is mainly lipid and the nexus fraction is mainly protein. The vesicular membranes show some similarities to liposomes made from phospholipid mixtures (Bangham & Haydon, 1968), and it is noteworthy that the typical trilaminar image

of the unit membrane can be obtained with mitochondrial membranes depleted of 95% of their lipid (Fleischer *et al.*, 1967) and with myelin from which all lipids had been extracted (Napolitano *et al.*, 1967). The molecules or groups responsible for the unit-membrane appearance in electron microscopy remain to be defined (Korn, 1968). Negative staining of the vesicular fraction showed it to consist of smooth vesicles, and the projections 5–6 nm in diameter present on the intact plasma membranes (Benedetti & Emmelot, 1968; W. H. Evans, unpublished work) were absent. Emmelot & Visser (1971) have claimed that these globular projections consist exclusively of aminopeptidase molecules that are released after exposure of plasma membranes to papain. The vesicular fraction is enriched in leucine naphthylamidase activity, and these results, like those made on rat hepatoma plasma membranes (Emmelot & Visser, 1971), indicate that the measured enzymic activity is probably not associated with the projections observed in negatively stained preparations.

Glycolipids present in the vesicular fraction, and glycoproteins present in the nexus fraction, indicate that carbohydrate-containing membrane components not extracted by sarcosyl–tris medium are tightly embedded in the membrane, and are not merely localized at the cell surface. The strong degree of attachment of glycoproteins to liver plasma membranes is also emphasized by their poor solubility in alkaline EDTA solutions, which extract 25% of the total membrane protein (Gurd *et al.*, 1972). A glycoprotein that extends across the erythrocyte membrane has been demonstrated by Bretscher (1971). In the construction of membrane models incorporating carbohydrate components, these facts should be recognized.

The present results represent an advance towards unravelling the functional specialization associated with the three major surfaces of the liver parenchymal cell and provide a chemical and biochemical description of the plasma-membrane region associated with the nexus. The preparation of a functional vesicular membrane of simplified protein composition opens the possibility of using freeze-etch techniques to give greater insight to the contribution of protein, lipid and carbohydrate in membrane structure.

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