Functional Polarity of the Rat Hepatocyte Surface Membrane

ISOLATION AND CHARACTERIZATION OF PLASMA-MEMBRANE SUBFRACTIONS FROM THE BLOOD-SINUSOIDAL, BILE-CANALICULAR AND CONTIGUOUS SURFACES OF THE HEPATOCYTE

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1. Six rat liver plasma-membrane subfractions of different density and morphological, enzymic and chemical properties were prepared from homogenates by a combination of differential, rate-zonal and density-gradient centrifugation. They consisted of three vesicular 'light' subfractions of density 1.12–1.13 and three 'heavy' subfractions of density 1.16–1.18 containing membrane strips and intercellular junctions. 2. All six subfractions contained a basal adenylate cyclase activity. One of the 'light' subfractions that showed the highest glucagon-stimulated adenylate cyclase activity was identified as deriving from the blood-sinusoidal face of the hepatocyte. This subfraction, unlike the others, was contaminated by Golgi components, as indicated by its morphological properties and the presence of galactosyl- and sialyl-transferase activities. 3. All the six subfractions showed high activities of the following plasma-membrane marker enzymes: 5'-nucleotidase, alkaline phosphodiesterase (nucleotide pyrophosphatase), alkaline phosphatase, leucine naphthylamidase and Mg^{2+} -activated adenosine triphosphatase. A 'light' subfraction that showed the highest specific activities of all the above marker enzymes, but lacked a glucagon-stimulated adenylate cyclase activity, was identified as deriving from the bile-canalicular face of the hepatocyte. 4. The 'heavy' subfractions, which showed generally the lowest activities of the above plasma-membrane enzyme markers, and were characterized by the presence of desmosomes and gap junctions, were taken to originate from the contiguous faces of the hepatocyte. 5. The protein composition of the six subfractions was generally similar, as shown by polyacrylamide-gel electrophoresis. Differences in the amounts of various protein and glycoprotein bands among the subfractions correlated with their morphology, enzymic composition and sialic acid content. 6. Hormonal and histochemical evidence supporting the identification of a bile-canalicular subfraction, a blood-sinusoidal subfraction and contiguous-face subfractions is discussed.

Most cells, especially those in tissues, exhibit varying degrees of polarity of structure and function that are reflected in the differentiation of the surface into areas or domains specialized for secretion, absorption, intercellular communication and attachment to neighbouring cells or a substratum. However, the majority of surface-membrane isolation studies are concerned mainly with the preparation of a single plasma-membrane fraction of acceptable yield and purity, and generally ignore the possibility that the presence of functional mosaics may result in heterogeneity of the plasma-membrane fragments. Although morphological specializations on the cell surface that are retained in subcellular fractions have enabled the isolation, for example of intercellular junctions (gap junctions) of epithelia, or synaptosomes of cerebral cortex, the microvilli involved in the secretion and absorption of metabolites yield populations of vesicular-membrane profiles that remain difficult to charac-

terize. Therefore, in subcellular-fractionation studies with cells displaying multiple or segregated secretory and absorptive functions, marker criteria related to the functions of the domains must be used to aid in their separation and identification.

The hepatocyte is a highly polarized cell, with a large microvillar surface area specialized for the exchange of metabolites with the blood, and a small microvillar surface area participating in bile formation. These two functionally distinct areas are geographically separated by a third contiguous face that interacts structurally and functionally with neighbouring cells. Biochemical heterogeneity that could correlate with the radically different functions occurring at the three major surface domains was suggested by the isolation of liver plasma-membrane subfractions with different densities, morphological and enzymic properties (Evans, 1969, 1970; Touster *et al.*, 1970; Evans & Gurd, 1971; House *et al.*, 1972). To prepare membranes corresponding to each of the

three major domains, and especially to distinguish between blood-sinusoidal and bile-canalicular membranes, the distribution of a hormone-sensitive adenylate cyclase and a range of enzymic, chemical and morphological properties were examined in six purified plasma-membrane subfractions. The results permit the identification of subfractions originating from the hepatocyte blood-sinusoidal, bile-canalicular and the intercellular faces. A short report has appeared (Wisher & Evans, 1974).

Experimental

Methods

Preparation of the parent liver plasma membranes. Plasma membranes were prepared from rat liver homogenates by two methods. The method of Evans (1970) was modified to increase the recovery of plasma membranes, by homogenizing rat livers (100–120 g) from 12 fed Sprague–Dawley rats (200 g) in 1 mM-NaHCO₃ (pH 7.6)–0.5 mM-CaCl₂ in a loose-fitting Dounce homogenizer. Addition of 0.5 mM-CaCl₂ (Ray, 1970) increased the amount of plasma membrane sedimented in the low-speed pellet, as indicated by measurement of 5'-nucleotidase activity. Plasma-membrane fragments were separated from nuclei and mitochondria by a rate-zonal centrifugation of the 'low-speed' pellet as described by Evans (1970), but the sucrose density gradient in the M.S.E. AXII rotor was modified to give a gradient of 6–60% (w/v) sucrose instead of 6–54% (w/v) sucrose. Centrifugation was carried out for 25–35 min at 3900 rev./min.

Plasma membranes were also prepared by a minor modification of the method of Touster *et al.* (1970). Unperfused livers (total wt. 25–40 g) from four starved Sprague–Dawley rats (200–300 g) were dispersed in 0.25 M-sucrose–5 mM-Tris–HCl, pH 8.0, by using two to three strokes of a loose-fitting Dounce homogenizer. Further homogenization was performed with one up-and-down stroke in a Potter–Elvehjem glass homogenizer with a Teflon pestle turning at a speed of 1000 rev./min. Clumps of connective tissue were removed manually at this stage of the procedure. Two plasma-membrane fractions were then prepared from the homogenate as described by Touster *et al.* (1970).

Subfractionation of plasma membranes. Subfractions were prepared (see Scheme 1) by resuspending the three parent plasma-membrane pellets in 0.25 M-sucrose–5 mM-Tris–HCl, pH 7.6, by using 20 strokes of a tight-fitting Dounce homogenizer [stated radial clearance 0.076 mm (Blaessig Glass Co., Rochester, N.Y., U.S.A.)]. The subfractions were separated on discontinuous sucrose gradients by centrifugation for 3–16 h at 95000 *g*_{av} in a Beckman SW27 rotor. Zonal-light (Z-L) (density 1.13), zonal-

heavy A (Z-H_A) (density 1.16) and zonal-heavy B (Z-H_B) (density 1.18) subfractions prepared from plasma membranes isolated by using the zonal rotor were collected at 8–37%, 37–43% and 43–49% (w/v) sucrose interfaces respectively (Evans *et al.*, 1973a). Two plasma-membrane subfractions derived from the 'nuclear' and one from the 'microsomal' pellet of the Touster *et al.* (1970) procedure were collected at 8–37% [nuclear-light (N-L), microsomal-light (M-L)] and 37–49% [nuclear-heavy (N-H)] sucrose interfaces. Membranes were stored in 0.25 M-sucrose–5 mM-Tris–HCl, pH 7.6, at –20°C.

In two experiments, freshly prepared samples of the microsomal-light (M-L) subfractions were centrifuged on continuous sucrose density gradients. To make the latter, 1.5 ml of 40% (w/v) sucrose was placed in the bottom of cellulose nitrate tubes and a continuous gradient prepared by mixing 6 ml of 20% (w/v) with 6 ml of 40% (w/v) sucrose was then introduced. A further 1 ml of 20% (w/v) sucrose was layered on top of the gradient, after which the plasma membranes resuspended in 0.25 M-sucrose (6.4 mg of protein) were layered. Sucrose solutions used in the gradient were buffered with 5 mM-Tris–HCl, pH 7.6. After centrifugation for 5 h at 94000 *g*_{av} in a Beckman SW27 rotor, 0.5 ml fractions were collected by withdrawing the sucrose from the bottom of the tube through a thin needle. The density of each fraction was determined by measurement of refractive index. Fractions were stored at –20°C.

Determination of enzyme activities. 5'-Nucleotidase (EC 3.1.3.5) was determined spectrophotometrically as described by Ipata (1967) and alkaline phosphodiesterase I (EC 3.1.4.1) was determined as described by Razzel (1963). Leucine β -naphthylamidase activity (EC 3.4.11.1) was measured by the method of Goldberg & Rutenberg (1958). Alkaline phosphatase activity (EC 3.1.3.1) was measured spectrophotometrically, as described by Pekarthy *et al.* (1972), by using *p*-nitrophenyl phosphate as substrate. Mg²⁺+K⁺-stimulated adenosine triphosphatase activity (EC 3.6.1.3) was measured as described by Swanson *et al.* (1964). Glucose 6-phosphatase activity (EC 3.1.3.9) was assayed by the method of Swanson (1955), and acid phosphatase (EC 3.1.3.2) by the method of Gianetto & de Duve (1955), with β -glycerophosphate as substrate. P_i liberated in the above assays was determined as described by Martin & Doty (1949). NADPH-cytochrome *c* oxidoreductase activity (EC 1.6.2.4) was assayed by the method of Sottocasa *et al.* (1967). Succinate dehydrogenase activity (EC 1.3.99.1) was assayed by the procedure of Earl & Korner (1965). Monoamine oxidase activity (EC 1.4.3.4) was determined spectrophotometrically as described by Schnaitman *et al.* (1967).

Adenylate cyclase activity (EC 4.6.1.1) was determined in an incubation mixture containing in

0.2 ml the following: 3.2 mM-ATP, 5.0 mM-MgCl₂, 25 mM-Tris-HCl, pH 7.6, 0.1 % bovine serum albumin, 20 mM-creatine phosphate, 0.5 mg of creatine kinase/ml (135 EC units/mg), 1 mM-EDTA, and between 200 and 250 µg of plasma membranes. The reaction was started by the addition of the membrane sample. After incubation for 10 min at 30°C the reaction was stopped by boiling for 3 min and particulate material was then removed by bench centrifugation. A portion of the supernatant was taken and the cyclic AMP formed was determined by addition of cyclic [³H]AMP to compete with enzymically formed unlabelled cyclic AMP for binding to a cyclic AMP-binding protein (Gilman, 1970). The nucleotide-protein complex formed was absorbed on Millipore cellulose ester filters. Dry filters were placed into scintillation vials and dissolved in 1 ml of 2-methoxyethanol (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.). Scintillation fluid (10 ml) was added and the vials were shaken vigorously. The scintillation fluid consisted of 1:4 (v/v) 2-methoxyethanol-toluene mixture containing 6.4 g of 2,5-diphenyloxazole in a final volume of 1 litre. Radioactivity was measured in a Packard model 2420 scintillation counter. Duplicate determinations were performed on each sample. Galactosyltransferase activity (EC 2.4.1.38) was measured by the paper-chromatographic method of Fleischer *et al.* (1969) as modified by Bergeron *et al.* (1973a). The determination, using 50–75 µg of plasma-membrane protein, was carried out in the presence of 0.6 % Triton X-100. Sialyltransferase activity (EC 2.4.99.1) was measured by the paper-electrophoretic method of Carlson *et al.* (1973). The incubation mixture contained the following components (in µmol) in a final volume of 0.05 ml: CMP-[¹⁴C]sialic acid, 0.04 (specific radioactivity 0.4×10^6 – 1.3×10^6 c.p.m./µmol); lactose, 2.0; sodium phosphate buffer, pH 6.9, 5.0; 0.6 % Triton X-100; 100–450 µg of plasma membranes. After 30 min at 37°C, the reaction was stopped by freezing. The thawed samples were transferred to Whatman no. 3MM paper and electrophoresed as described by Carlson *et al.* (1973).

Chemical determinations. Protein content of membrane suspensions was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.). RNA content of membranes was determined by the Schmidt-Thannhauser method as described by Fleck & Monro (1962) and DNA by the diphenylamine method of Giles & Myers (1965). The sialic acid content of membrane fractions that had been washed three times with water by centrifugation was determined by the method of Aminoff (1961) after hydrolysis in 0.05 M-H₂SO₄ at 80°C for 60 min; the correction factor of Warren (1963) was applied.

Polyacrylamide-gel electrophoresis. To release adsorbed or occluded proteins the plasma-membrane samples were first washed once in iso-osmotic saline (0.9 % NaCl, 5 mM-Tris-HCl, pH 7.6) by centrifugation. The pellets were resuspended in water and then an equal volume of 1.8 % NaCl–10 mM-Tris-HCl (pH 7.6) was added before re-centrifugation. The washed membrane samples were heated in 4 M-urea–1 % sodium dodecyl sulphate–1 % mercaptoethanol solution at 90°C for 3–5 min. Polyacrylamide-slab-gel electrophoresis was carried out in sodium dodecyl sulphate–Tris–glycine buffers by using the E.C. apparatus (Philadelphia, Pa., U.S.A.) essentially as described by Maizel (1971). The gels were discontinuous, with a 3.6 % (w/v) acrylamide spacer gel, pH 6.7, and an 8.5 % (w/v) acrylamide resolving gel, pH 8.9 (12 cm × 0.4 cm). Electrophoresis was carried out at 30 mA for 16 h and the bands were stained for protein with Coomassie Blue (Maizel, 1971) and for carbohydrate by the Schiff-periodate procedure (Zacharias *et al.*, 1969; Evans & Gurd, 1972). Destained gels were scanned at 595 nm for Coomassie Blue or 560 nm for Schiff-periodate stain in a Unicam SP.1809 scanning densitometer. The molecular weights were calculated from the position of the following reo-viral polypeptides: λ₁, mol.wt. 155 000; λ₂, mol.wt. 140 000; μ₂, mol.wt. 72 000; ε₂, mol.wt. 38 000; ε₃, mol.wt. 34 000 (Smith *et al.*, 1969).

Electron microscopy. Membrane pellets were fixed for 1 h at 4°C in a mixture (2:1, v/v) of 1 % (w/v) OsO₄ and 2.5 % (v/v) glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 7.4 (Hirsch & Fedorko, 1968). The pellets were 'post-fixed' for 15 min in 0.25 % (w/v) uranyl acetate in 0.1 M-veronal acetate buffer, pH 6.2, and embedded in Epikote 812. Thin sections were stained with uranyl acetate and lead citrate, and observed 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., Kingston-upon-Thames, Surrey KT2 7BH, U.K. The ammonium salt of UDP-[¹⁴C]galactose was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. CMP-[4-¹⁴C]sialic acid (9 mCi/mmol) was obtained from New England Nuclear Corp., Boston, Mass., U.S.A. The cyclic AMP assay kit was obtained from Boehringer Corp. (London) Ltd., Ealing, London W5 2TZ, U.K.

Results

Purity and yield of plasma membranes

Two methods were used to prepare the parent plasma membranes from which the six subfractions

Scheme 1. Origin of plasma-membrane subfractions

For details see the text.

were then isolated (Scheme 1). Although some of the properties of the parent plasma membranes and sub-fractions have been described (Evans, 1969, 1970; Touster *et al.*, 1970) these were assessed more comprehensively under the same experimental conditions, since the purity and representativeness of the fractions, reflected in the yield of protein and enzymes, are critical parameters in assessing the origin of the subfractions.

The plasma membranes prepared from a nuclear pellet by rate zonal centrifugation, followed by density-gradient centrifugation (Evans, 1970; Evans *et al.*, 1973a), accounted for 0.28 mg of protein/g wet wt. of liver and 7–15% of the 5'-nucleotidase or alkaline phosphodiesterase activities of the homogenate. The plasma membranes prepared from nuclear and microsomal pellets by density-gradient centrifugation (Touster *et al.*, 1970) accounted for 0.7 mg of protein/g wet wt. of liver and 21–25% of the 5'-nucleotidase or alkaline phosphodiesterase activities of the homogenate. Tables 1 and 2 show that the fractions were of satisfactory purity as assessed by determination of the specific activities, relative to that in the tissue homogenate, of markers for mitochondria (succinate dehydrogenase), outer mitochondrial membranes (monoamine oxidase), lysosomes (acid phosphatase), endoplasmic-reticulum membranes (glucose 6-phosphatase, NADPH-cyto-

chrome *c* reductase and RNA). Markers aiding the identification of nuclear membranes are more uncertain; however, the low DNA content, indicating the absence of attached chromatin (Franke *et al.*, 1970), combined with the low glucose 6-phosphatase activity [which was two to three times higher in nuclear membranes than in microsomal fractions (Bergeron & Lanoix, 1974)], suggests that the contribution of nuclear membranes to all the subfractions was low.

Properties of the six plasma-membrane subfractions

Three 'light' subfractions (Z-L, M-L and N-L) of density 1.13 and three 'heavy' subfractions (Z-H_A, density 1.16; Z-H_B, density 1.18; and N-H, density 1.18) were prepared by density-gradient centrifugation after vigorous Dounce homogenization of the plasma membranes prepared as shown in Scheme 1. No attempt was made to subfractionate the nuclear-heavy (N-H) subfraction further. The 'microsomal' plasma membranes were subfractionated into a major light (M-L) component and a very minor heavy subfraction of density 1.18 that was not examined.

Electron-microscopic examination of subfractions. All three light subfractions were seen under the electron microscope to be mainly vesicular, smooth-membrane fractions from which strips of membranes

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

Unfractionated plasma membranes prepared from a nuclear pellet and from a microsomal pellet were isolated from homogenate A by the procedure of Touster *et al.* (1970) as described in the Experimental section. Zonal-light (Z-L), zonal-heavy A (Z-H_A) and zonal-heavy B (Z-H_B) subfractions were prepared from homogenate B by the modified method of Evans (1970) as described in the Experimental section. (a), (b), (c) and (d) are separate plasma-membrane preparations. Enzymic activities are expressed as μmol of product liberated/h per mg of protein. N.D., Not determined.

Enzyme activity	Homogenate A		Nuclear plasma membrane		Microsomal plasma membrane		Homogenate B		Z-L		Z-H _A		Z-H _B	
	(a)	(b)	(a)	(b)	(a)	(b)	(c)	(d)	(c)	(d)	(c)	(d)	(c)	(d)
	(a)	(b)	(a)	(b)	(a)	(b)	(c)	(d)	(c)	(d)	(c)	(d)	(c)	(d)
Succinate dehydrogenase	4.80	6.00	0	0	0	0	2.48	2.54	0	0	0.87	0.91	0.97	1.03
Monoamine oxidase	0.10	0.16	0.47	0.55	0.26	0.32	0.11	0.12	0.11	0.33	0.12	0.14	0.15	0.04
Acid phosphatase	0.73	0.81	0.62	0.63	1.84	1.15	0.46	0.58	0.56	0.68	0.50	0.75	0.18	0.44
Glucose 6-phosphatase	0.86	1.06	0.35	0.36	1.46	0.83	0.81	0.90	0	0.13	0.12	0.24	0.26	0.27
NADPH-cytochrome <i>c</i> reductase	7.80	10.20	5.20	7.00	4.00	4.90	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Table 2. Chemical composition of plasma-membrane subfractions of rat liver

Plasma-membrane subfractions were prepared as described in the Experimental sections. The abbreviations used are defined in Scheme 1. (a) and (b) are separate plasma-membrane preparations. Sialic acid is expressed as nmol/mg of protein and RNA and DNA as μg/mg of protein. RNA and DNA content was determined on only one plasma-membrane preparation.

Subfraction ...	Z-L		N-L		M-L		N-H		Z-H _A		Z-H _B	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Sialic acid	96	99	94	92	91	91	38	38	56	53	42	31
RNA	0.1		3.3		1.3		5.1		5.6		4.5	
DNA	3.5		1.1		3.0		1.5		3.7		7.3

Fig. 1. Enzyme activities in plasma-membrane subfractions from rat liver

Plasma-membrane subfractions were prepared as described in the Experimental section and as summarized in Scheme 1. The six subfractions isolated were as follows: Z-L, zonal-light; N-L, nuclear-light; M-L, microsomal-light; N-H, nuclear-heavy; Z-H_A, zonal-heavy A; Z-H_B, zonal-heavy B. Histogram (a) records the protein recovered in each subfraction, expressed as mg of protein/g wet wt. of liver. The following enzyme activities were measured in the subfractions: (b) 5'-nucleotidase; (c) alkaline phosphodiesterase; (d) leucine naphthylamidase; (e) alkaline phosphatase; (f) Mg^{2+} -stimulated adenosine triphosphatase; (g) adenylate cyclase; (h) galactosyltransferase; (i) sialyltransferase. Enzyme relative specific activities were calculated as the ratio of the specific activity of the subfraction to that of the homogenate. Basal adenylate cyclase activity (hatched bars) and glucagon ($2 \mu M$)-stimulated activity (open bars) are both expressed relative to the homogenate basal adenylate cyclase activity. The values above the bars are the recoveries of the enzyme in each subfraction expressed as % of total homogenate enzyme activity. Recovery of glucagon-stimulated adenylate cyclase was calculated by first subtracting the basal activity from the glucagon-stimulated activity. The enzyme activities of the homogenate, expressed as μmol of product liberated $\pm s.e.(n)$ /h per mg of protein unless otherwise stated, were as follows: 5'-nucleotidase (b), 2.52 ± 0.73 (4); alkaline phosphodiesterase (c), 0.77 ± 0.12 (4); leucine naphthylamidase (d), 0.34 ± 0.07 (2); alkaline phosphatase (e), 1.20 ± 0.0 (2); Mg^{2+} -stimulated adenosine triphosphatase (f), 3.82 ± 0.22 (2); basal adenylate cyclase (g), 0.078 ± 0.012 (2) nmol of cyclic AMP formed/h per mg of protein [glucagon-stimulated activity was 0.132 ± 0.012 (2) nmol of cyclic AMP formed/h per mg of protein]; galactosyltransferase (h), 2.52 ± 0.16 (2) nmol of galactose transferred/h per mg of protein; sialyltransferase (i), 1.46 ± 0.04 (2) nmol of sialic acid transferred/h per mg of protein; protein (a), 148 ± 29 (13) mg/g wet wt. of liver. The values were obtained from two to four separate plasma-membrane preparations.

EXPLANATION OF PLATE I

Electron micrographs of plasma-membrane subfractions from rat liver

(a) Nuclear-light (N-L) subfraction. Filamentous material (arrows) was associated with the inside of some of the smooth-membrane vesicles. The bar represents $0.1\ \mu\text{m}$. (b) Nuclear-heavy (N-H) subfraction. Filamentous material (arrows) was associated with some of the membrane vesicles and membrane strips. The arrowheads point to gap junctions. The insert indicates the structure of a gap junction. The bars represent $0.1\ \mu\text{m}$.

EXPLANATION OF PLATE 2

Electron micrographs of plasma-membrane sub-fractions from rat liver

(a) Electron micrograph of microsomal-light (M-L) subfraction. Arrowheads point to vesicles enclosing dense-staining particles (diameter 60–100 nm). The insert shows an occasionally observed cisternal element also enclosing dense-staining particles. The bars represent 0.1 μ m. (b) Electron micrograph of zonal-light (Z-L) subfraction. The bar represents 0.1 μ m. (c) Electron micrograph of zonal-heavy (Z-H) subfractions. Filamentous material (arrows) is associated with desmosomes (arrowhead) and membrane strips. The bar represents 0.1 μ m.

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and junctional complexes were absent (Plates 1a, 2a, 2b). In the microsomal-light (M-L) subfraction (Plate 2a), small vesicles and flattened cisternal elements enclosing dense-staining particles (diameter 60–100nm) were occasionally observed, suggesting that this subfraction contained elements of the Golgi apparatus (Ehrenreich *et al.*, 1973).

The heavy subfractions (Plates 1b, 2c) also contained vesicles but differed from the light subfractions by the presence of membrane strips and junctional complexes. The nuclear-heavy (N-H) subfraction (Plate 1b) contained larger vesicles and fewer membrane strips and junctional complexes than the zonal-heavy (Z-H) subfractions (Plate 2c). The Z-H_A and Z-H_B subfractions were generally similar, but the Z-H_B subfraction contained more desmosomes. All three heavy subfractions, but especially nuclear-heavy (N-H), contained filamentous material associated with the plasma membranes (Plate 1b). Similar filamentous material, always associated with the inner side of membrane vesicles, was occasionally observed in the light subfractions (Plate 1a).

Distribution of enzyme among the subfractions. Figs. 1(b)–1(f) show the distribution among the six subfractions of typical liver plasma-membrane markers. Highest activities of five plasma-membrane marker enzymes (5'-nucleotidase, Fig. 1b; alkaline phosphodiesterase, Fig. 1c; leucine naphthylamidase, Fig. 1d; alkaline phosphatase, Fig. 1e; Mg²⁺-acti-

vated adenosine triphosphatase, Fig. 1f) were found in the light subfractions, with the zonal-light (Z-L) showing the highest, microsomal-light (M-L) the lowest, and the nuclear-light (N-L) showing intermediate specific activities relative to the homogenate. As indicated in the Discussion section, histochemical results indicate that these enzymes stain most intensely at the bile canaliculus. The three heavy subfractions showed much lower enzyme activities. Fig. 1 also indicates the recoveries of the various enzymes in all the subfractions. An overall recovery of 28–42% of the homogenate 5'-nucleotidase (Fig. 1b) and alkaline phosphodiesterase (Fig. 1c) activities indicated that representative portions of the hepatocyte surface membrane were probably recovered.

Adenylate cyclase activity of the subfractions was examined because the enzyme was shown to be stimulated after interaction of hormones with receptors that are located mainly at the blood sinusoidal and possibly the contiguous faces of the hepatocyte surface (Rodbell *et al.*, 1969). Fig. 1(g) shows that all subfractions contained adenylate cyclase activity, and in five of them this activity was stimulated manyfold by 2 μ M-glucagon. The effects of various glucagon concentrations on the adenylate cyclase activity of four subfractions were examined in a separate experiment (Fig. 2). The highest stimulation of enzyme activity by glucagon was found in the microsomal-light (M-L) subfraction (Fig. 2a),

Fig. 2. Effect of glucagon on adenylate cyclase activity

Adenylate cyclase activity in the plasma membrane subfractions was measured as described in the Experimental section: ●, glucagon-stimulated activity; ○, activity when 15 mM-NaF was present. The results are expressed as the ratio of stimulated adenylate cyclase activity relative to the basal adenylate cyclase activity. The subfractions assayed were as follows, with basal activity expressed as nmol of cyclic AMP liberated $\pm$ s.e. (n)/h per mg of protein: (a) microsomal-light subfraction, 0.384 ± 0.204 (5) (b) zonal-light subfraction, 0.582 ± 0.162 (6); (c) zonal-heavy A subfraction, 0.654 ± 0.192 (4); (d) zonal-heavy B subfraction, 0.492 ± 0.210 (4).

with lowest glucagon-stimulated values being recorded in the heavy subfractions (Figs. 2c and 2d). The zonal-light (Z-L) subfraction enzyme (Fig. 2b) was inhibited as the glucagon concentration was increased. However, NaF stimulated the adenylate cyclase activities of the zonal-light (Z-L) subfraction (Fig. 2b) and microsomal-light (M-L) subfraction (Fig. 2a). It is noteworthy that these two light subfractions of similar morphology (Plate 2) differ remarkably in the response of the adenylate cyclase to glucagon, and this is an important observation used in the assignment of these fractions to the blood-sinusoidal or bile-canalicular faces of the hepatocyte. The recovery of glucagon-stimulated adenylate cyclase activity (Fig. 1g) in the three subfractions prepared by using the zonal-centrifugation procedure (Z-L, Z-H_A and Z-H_B) was 3.7% of the homogenate activity. However, a much higher recovery of 18% of the homogenate activity was obtained in the microsomal-light (M-L) subfraction. Altogether 20% of the homogenate adenylate cyclase activity was recovered in the three subfractions prepared by the Touster *et al.* (1970) procedure (Scheme 1). Stimulation of the adenylate cyclase activity by glucagon in the heavy subfractions (N-H, Z-H_A, Z-H_B) and nuclear-light (N-L) subfraction (Fig. 1g) was similar to those observed in the plasma-membrane subfractions isolated by House *et al.* (1972). Pohl *et al.* (1971) found that the stimulation produced by glucagon was lower in fully purified than in partially purified plasma membrane, which corresponded to the parent fraction of subfractions Z-L, Z-H_A and Z-H_B. The lower extents of glucagon stimulation now observed in the plasma-membrane subfractions may therefore reflect a partial loss of activity during the extensive subfractionation procedure.

Electron micrographs suggested that the microsomal-light subfraction contained elements of the Golgi apparatus. To ascertain the extent of this contamination by Golgi membranes, the plasma-membrane subfractions were assayed for galactosyl- and sialyl-transferase activities, enzymes shown to be localized in Golgi membranes (Fleischer & Fleischer, 1970; Schachter *et al.*, 1970; Bergeron *et al.*, 1973a; Bennet *et al.*, 1974). A similar distribution of the two enzyme activities in the subfractions was observed (Figs. 1h and 1i). Very low activities were found in five of the subfractions, especially the zonal-light (Z-L) and the heavy subfractions (Z-H_A, Z-H_B) (Fig. 1), but high activities of both enzymes were found in the microsomal-light (M-L) subfraction, confirming the morphological evidence that this subfraction was contaminated by Golgi membranes.

A freshly prepared microsomal-light (M-L) subfraction was subjected to density-gradient centrifugation in continuous sucrose gradients in an

Fig. 3. Distribution of microsomal-light subfraction enzymes in a sucrose gradient

Density-gradient centrifugation was carried out as described in the Experimental section. Fractions from the gradient were assayed for the following: (a) protein; (b) alkaline phosphodiesterase; (c) 5'-nucleotidase; (d) leucine naphthylamidase; (e) glucagon-stimulated adenylate cyclase (1 μ M-glucagon); (f) galactosyltransferase. Results in (a)–(d) are expressed as relative concentrations, i.e. the ratio of the concentration of the protein or enzyme in the fraction relative to the concentration of the protein or enzyme if it were uniformly distributed in the whole gradient. Adenylate cyclase activity is expressed as pmol of cyclic AMP formed/h per ml, and galactosyltransferase activity as μ mol of [14 C]galactose transferred/h per ml.

attempt to separate vesicles derived from plasma membrane and from the Golgi apparatus. The distribution in the gradient of the following plasma-membrane marker enzymes was determined (Fig. 3): 5'-nucleotidase, alkaline phosphodiesterase, leucine naphthylamidase, glucagon-stimulated adenylate cyclase and the Golgi marker enzyme galactosyltransferase. The results showed that the five enzymes were present on membranes of similar density. A small separation of the alkaline phosphodiesterase (Fig. 3b) and 5'-nucleotidase peaks (Fig. 3c) was observed, but little separation of Golgi and plasma membranes was obtained by this method.

Chemical composition of the subfractions. Table 2 shows that the light plasma-membrane subfractions contained more sialic acid on a protein basis than did the heavy subfractions. The zonal-heavy A (Z-H_A) subfraction contained more sialic acid than zonal-

Fig. 4. *Polypeptide composition of plasma-membrane subfractions*

Polyacrylamide-gel electrophoresis of plasma-membrane subfractions in sodium dodecyl sulphate was carried out as described in the Experimental section. The gels were stained for protein by using Coomassie Blue stain. The following subfractions were analysed: (a) nuclear-heavy (N-H); (b) zonal-heavy A (Z-H_A); (c) zonal-heavy B (Z-H_B); (d), zonal-light (Z-L); (e) nuclear-light (N-L); (f) microsomal-light (M-L). For details of numbered bands see the text.

heavy B (Z-H_B) subfraction, indicating further differences between these subfractions.

Polyacrylamide-gel electrophoresis resolved the proteins of the plasma-membrane subfractions into about 20 major bands (Fig. 4). The apparent molecular weights of these polypeptides ranged between 200000 and 14000. The staining patterns of the light and heavy subfractions were similar, but differences in the intensities of a number of bands were apparent. For example, band 7 was present in the light subfractions in higher amounts than in the heavy subfractions. Similarly, bands 13, 17, 18 and 20 in the heavy subfractions were only found in small amounts in the light subfractions. Band 17 was present in large amounts, especially in the zonal-heavy A (Z-H_A) and B (Z-H_B) subfractions, and may be the major protein present in gap junctions (Evans & Gurd, 1972; W. H. Evans, unpublished work). The staining patterns for zonal-heavy A (Z-H_A) and B (Z-H_B) subfractions were very similar. A number of bands in the nuclear-heavy (N-H) subfraction (2, 3, 8 and 15) showed increased intensity of staining compared with the other heavy subfractions. The light subfractions were also of very similar composition, with the staining intensity of many

bands in the nuclear-light (N-L) subfraction being intermediate between the zonal-light (Z-L) and the microsomal-light (M-L) subfractions.

The plasma-membrane subfractions contained about ten glycoproteins, all of molecular weights greater than 70000 (Fig. 5). Unfractionated plasma membranes were previously shown by Glossman & Neville (1971) to contain between 6 and 11 glycoprotein subunits. The staining patterns in the light and heavy subfractions were similar, but higher staining intensities of a number of bands in the light subfractions were observed. The increased concentration of glycoproteins in the light subfractions agreed with their higher sialic acid content and higher activities of glycoprotein enzymes, e.g. 5'-nucleotidase and alkaline phosphodiesterase (Evans & Gurd, 1973; Evans *et al.*, 1973b). The similar protein and glycoprotein staining patterns indicated that similar populations of protein and glycoprotein subunits were present in each of the subfractions.

Discussion

The present results, assessed in conjunction with other plasma-membrane hormone-binding and histochemical studies, permit the identification of plasma-

Fig. 5. Glycoprotein composition of plasma-membrane subfractions

Polyacrylamide-gel electrophoresis of plasma-membrane subfractions in sodium dodecyl sulphate was carried out as described in the Experimental section. The gels were stained by the Schiff-periodate procedure. Apparent molecular weights were estimated by using gels calibrated by proteins of known molecular weight. The following subfractions were analysed: (a) nuclear-heavy (N-H); (b) zonal-heavy A (Z-H_A); (c) zonal-heavy B (Z-H_B); (d) zonal-light (Z-L); (e) nuclear-light (N-L); (f) microsomal-light (M-L). For details of numbered bands see the text.

membrane subfractions originating primarily from each of the three major functional domains of the hepatocyte surface.

Blood-sinusoidal face

A number of polypeptide hormones, catecholamines and prostaglandins are known to influence liver metabolism. Several characteristics of the binding of glucagon to a liver plasma-membrane fraction were found to be similar to or identical with those of the glucagon-stimulated adenylate cyclase (Pohl *et al.*, 1971; Rodbell *et al.*, 1971; Avruch & Pohl, 1973). To be effective, these receptors should be mainly located at the outer surface of the microvilli of the blood-sinusoidal face of the hepatocyte so as to be accessible to circulating hormones. The contiguous face of the hepatocyte may also be to some extent accessible, since low-molecular-weight compounds were shown to diffuse into the inter-

hepatocytic space (Goodenough & Revel, 1970). Plasma membranes derived from the blood-sinusoidal face should therefore be primarily vesicular, since microvilli usually form vesicles on tissue disruption, and show a high degree of glucagon-stimulated adenylate cyclase activity. Since the blood-sinusoidal face accounts for nearly one-half of the total surface area of the hepatocyte (Weibel *et al.*, 1969) a subfraction derived from it should also be recovered in higher amounts than a subfraction derived from the small surface area involved in bile secretion. The present studies identify the vesicular microsomal-light (M-L) subfraction as fulfilling these criteria. Although all six liver plasma-membrane subfractions possessed basal adenylate cyclase activities, the microsomal-light (M-L) subfraction recovered in the greatest protein yield showed the greatest activation in the presence of glucagon. The adenylate cyclase activity of three 'heavy' subfractions prepared from 'nuclear' pellets was also stimulated by glucagon, but to a lesser degree.

Morphological evidence indicated that the microsomal-light (M-L) subfraction contained low amounts of Golgi components, since cisternal elements and vesicles containing electron-dense material probably corresponding to very-low-density lipoproteins were recognized. The quantification of the Golgi contamination of this plasma-membrane subfraction is difficult, since sugar transferase enzymes may not be exclusive to the Golgi apparatus in secretory tissues. A further complication is that plasma membrane and Golgi vesicles were of similar density, and centrifugation into sucrose gradients of the microsomal-light subfraction (Fig. 3) could not distinguish between marker enzymes of plasma and Golgi membranes. The similarity of the polypeptide and glycoprotein patterns of all six plasma-membrane subfractions (Figs. 4 and 5) indicated that contamination of the microsomal-light (M-L) fraction was low, since a comparison of the Golgi and plasma-membrane fractions indicated large differences between their polypeptide compositions (Bergeron *et al.*, 1973b). Sugar transferases function mainly, if not exclusively, in the Golgi apparatus (Neutra & LeBlond, 1966; Fleischer & Fleischer, 1970; Bergeron *et al.*, 1973a; Bennet *et al.*, 1974). It was therefore surprising to find that although the microsomal-light (M-L) subfraction contained high specific activities of plasma-membrane marker enzymes, relatively high activities of galactosyl- and sialyl-transferases were also present, contrasting with the very low activities in the other subfractions (Fig. 1). The zonal-light (Z-L) and -heavy subfractions (Z-H_A, Z-H_B) also contained low specific activities of glycosyltransferase (Evans & Gurd, 1971). The high sugar transferase activities recorded in the microsomal-light subfraction agree with the activities found by Dewald & Touster (1973) in the parent microsomal plasma-membrane fraction, and the presence of Triton X-100 in the reaction mixture, shown to stimulate enzyme activity (Schachter *et al.*, 1970), may account, in part, for high activities. A further possibility is that sugar transferases, shown to be glycoproteins (Trayer & Hill, 1971; Podolsky *et al.*, 1974), may be selectively inserted, in common with other glycoproteins (e.g. 5'-nucleotidase, insulin receptor), by the Golgi components into the sinusoidal plasma membrane during secretion (Bergeron *et al.*, 1973b), thus contributing to the selectively high activity at this area of the hepatocyte surface.

Sugar transferases claimed to be present on the cell surface have been credited with an important role in intercellular adhesion (Roth *et al.*, 1971) and in the metabolism by liver of asialoglycoproteins (Aronson *et al.*, 1973). The present results suggest that sugar transferase activities present in the microsomal-light plasma-membrane subfraction (M-L) are due mainly to contamination by Golgi elements, and if small amounts of sugar transferases truly

belong to the cell surface, they are confined to the blood-sinusoidal area, since the other plasma-membrane subfractions showed low activities. The identification of an externally located hepatocyte surface-membrane nucleotide pyrophosphatase that could hydrolyse sugar nucleotides (Evans, 1974) also makes it unlikely that galactosyl- and sialyl-transferases on the hepatocyte play a major functional role in addition to their well-established role in the biosynthesis of glycoprotein in the Golgi apparatus.

Bile-canicular face

The bile canaliculus comprises a small microvillar surface area of the hepatocyte that is primarily concerned in secretion of bile and excretion of products detoxicated in the hepatocyte. Histochemical studies (Wachstein & Meisel, 1957; Essner *et al.*, 1958; Novikoff *et al.*, 1962; Sierakowska *et al.*, 1963) showed that a variety of enzymes (e.g. phosphatases, aminopeptidases) are not uniformly distributed around the hepatocyte surface, but are concentrated at the bile canaliculus. It would be expected therefore that a subfraction from the bile canaliculus would show the highest activities of these enzymes, and would be vesicular. The zonal-light subfraction, as previously suggested (Evans, 1970), fulfils this requirement, since this subfraction, which was low in protein content relative to the other subfractions, contained the highest specific activities of the five plasma-membrane marker enzymes (Figs. 1b-1f).

Further support for this identification comes as a by-product of the adenylate cyclase studies initiated to identify a blood-sinusoidal plasma-membrane subfraction. The zonal-light subfraction contained a basal adenylate cyclase activity that was slightly higher than in four of the other subfractions, but it was inhibited by a range of glucagon concentrations (Fig. 2). This subfraction was derived from plasma membranes prepared from a 'nuclear' pellet similar to that extensively used in a wide variety of plasma-membrane studies. Therefore the inhibition by glucagon of adenylate cyclase activity, combined with the lower insulin-binding activity observed in this subfraction (Evans *et al.*, 1973a), raises questions concerning the interpretation of studies that identify a number of categories of binding sites in heterogeneous parent plasma-membrane fractions (Giorgio *et al.*, 1974; Kahn *et al.*, 1974). The physiological significance of the inhibition of adenylate cyclase by glucagon in this subfraction is unclear. Glucagon and insulin are found in bile at a concentration similar to that in the portal vein (Buchanan *et al.*, 1968) and insulin introduced into blood is eliminated 17 min later in bile (Henderson, 1974). The inhibition of the adenylate cyclase by a polypeptide hormone, and the decreased insulin

binding observed at the bile canaliculus, may therefore form part of a control mechanism operating to prevent a delayed hormone response occurring.

The nuclear-light (N-L) subfraction contained relatively high specific activities of plasma-membrane marker enzymes (Figs. 1b-1f) and glucagon-stimulated adenylate cyclase activity (Fig. 1g), indicating that it is probably composed of a mixture of smooth vesicles derived from the blood-sinusoidal and bile-canalicular surfaces.

Contiguous face

It has long been recognized on the basis of morphological criteria that the sheets of membranes interconnected by desmosomes and gap junctions present in plasma membranes prepared from 'nuclear' pellets by Neville (1960, 1968), and by variants of this method, originated from the contiguous face of the hepatocyte. Sheets of membranes and intercellular junctions characterized the three heavy subfractions, and the higher buoyant density of these subfractions probably results from the presence of protein-rich junctional areas. The greater number of desmosomes present in the zonal-heavy B subfraction may explain why its buoyant density is higher than that of the other two heavy subfractions. Vesicular profiles were also present in the heavy subfraction, and therefore it is difficult to assign the glucagon-stimulated activity present to the sheets or attached vesicles, or to a balance between the high activities associated with blood-sinusoidal-derived vesicles and low or inhibitory activity of the bile-canalicular components. It would be expected that the vigorous homogenization in the tight-fitting Dounce homogenizer used to prepare the three heavy subfractions would have removed most of the lower-density vesicles, but a residual number of attached vesicles remained. This is an important question in view of the selection of an appropriate plasma-membrane fraction for studies on the hormonal activation of adenylate cyclase (Pohl *et al.*, 1971).

The filamentous material found attached to the plasma membranes (especially in the heavy subfractions) may correspond to microfilaments, constructed of actin-like subunits that were found attached to the cytoplasmic side of surface membrane of many non-muscular cells (Ishikawa *et al.*, 1969; Pollard & Korn, 1972).

Cell-surface topography

The present studies with liver tissue show that functional differences at the cell-surface membrane that are geographically separated can be resolved into discrete populations of membranes by subcellular fractionation. Two 'light' vesicular plasma-membrane subfractions of similar density, but greatly differing in their enzymic properties, were

prepared. The bile-canalicular vesicles were amenable to isolation, owing to attachment of the bile-canalicular complex to the large membrane sheets and junctions occurring during the initial loose-fitting Dounce-homogenization step; they were subsequently released by the tight-fitting Dounce-homogenization step that preceded the subfractionation procedure. The blood-sinusoidal membranes were vesicularized during the initial loose-fitting Dounce- and Potter-Elvehjem-homogenization steps, and were recovered from the microsomal fraction; the final subfractionation procedure served to give added purification by the release of a minor heavier subfraction that was not characterized. The contiguous-face membranes that initially sedimented in the nuclear fraction were separated from the vesicular membranes by virtue of their higher density on sucrose gradients.

The heterogeneous distribution of cell-surface components now shown in hepatocytes occurs also in other cells. Cytochemical studies have indicated enzymic polarity on the cell surface of intestinal epithelial cells (Nachlas *et al.*, 1960), kidney, pancreas and mammary-gland cells (de-Thé, 1968). Plasma-membrane subfractions that represent the resolution of this polarity to varying degrees have been prepared from intestinal epithelium (Fujita *et al.*, 1973), kidney proximal tubule (Heidrich *et al.*, 1972), the electroplax organ (Duguid & Raftery, 1973), blood platelets (Barber & Jamieson, 1970) and cultured hepatocytes (Perdue *et al.*, 1971).

Heterogeneity of subcellular fractions can also be ascribed in tissues to the presence of populations of cells with different functions. In liver, hepatocytes account for 70-95% of the cellular weight (Iype *et al.*, 1965; Greengard *et al.*, 1972) and the contribution of reticulo-endothelial cell membranes to the subfractions now characterized is likely to be minor. The glucagon-stimulated adenylate cyclase activity was shown to be exclusively located in hepatocytes (Sweat & Hupka, 1971). The enzymic characteristics of the plasma membrane of non-parenchymal cells of liver have not been investigated in detail. Further conclusions on the contribution of non-parenchymal cells to the heterogeneity of liver plasma-membrane subfractions must await the preparation of plasma membranes from separated cells.

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