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Preparation of Plasma-Membrane Subfractions from Isolated Rat Hepatocytes

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1. Rat livers were dissociated into their constituent cells by perfusion through the portal vein with a medium containing collagenase, and hepatocytes separated from non-parenchymal cells. 2. It is shown that the procedure described by Wisher & Evans [(1975) *Biochem. J.* **146**, 375–388] for preparation of plasma membranes from liver tissue when applied to isolated hepatocytes also yielded subfractions of similar morphology and marker-enzyme distribution. 3. Thus the distribution of alkaline phosphodiesterase, 5′-nucleotidase and the basal and glucagon-stimulated adenylate cyclase among two 'light' vesicular and one 'heavy' junction-containing plasma-membrane subfractions paralleled that reported for tissue-derived plasma-membrane subfractions. 4. Increased recoveries and specific activities of plasma-membrane marker enzymes were obtained when soya-bean trypsin inhibitor was included in the collagenase-containing perfusion media used to dissociate the liver. 5. Polyacrylamide-gel-electrophoretic analysis of the corresponding plasma-membrane subfractions prepared from liver tissue and isolated hepatocytes were generally similar. 6. The results indicate that the functional polarity of the hepatocyte's plasma membrane is retained after tissue dissociation. The damage occurring to plasma-membrane ectoenzymes by the collagenase-perfusion procedure is discussed.

Isolated hepatocytes, prepared by perfusion of the liver lobes with enzymes followed by cell separation, are widely used to study liver functions, e.g. hormone–receptor interactions, secretion of serum proteins and bile formation. Isolated hepatocytes are used to circumvent the problems caused by cellular heterogeneity of liver tissue, especially the metabolic contribution of the reticuloendothelial cells, since they allow specific hepatocyte functions to be studied under more controlled conditions than are possible in the perfused liver system. Similarly, although it is assumed that subcellular fractions prepared from liver tissue homogenates are derived mainly from hepatocytes, it would be advantageous to prepare purified subcellular fractions from isolated hepatocytes. The present paper describes the preparation and characterization of plasma membranes from isolated hepatocytes.

The functional heterogeneity of the hepatocyte's surface membrane was resolved biochemically by the preparation of plasma-membrane subfractions originating from each of the three major surface regions of the hepatocyte, i.e. the blood sinusoidal, bile canalicular and contiguous surfaces (Wisher & Evans, 1975). Although isolated hepatocytes secrete serum proteins (Weigand & Otto, 1974; Jeejeebhoy *et al.*, 1975) and bile salts (Anwer *et al.*, 1975), functions which in liver are performed at two topographically distinct hepatocyte surface regions, it is not clear to what extent these regions are maintained in isolated cells when the constraints imposed by tissue organization are removed. In the present paper we describe also the isolation and characterization of three highly purified plasma-membrane subfractions from isolated hepatocytes and show that they have similar properties to functionally defined subfractions prepared from liver tissue. Finally, the use of crude collagenases to dissociate liver decreases the recovery of hepatocyte plasma-membrane ectoenzymes, but this recovery can be improved by the addition of a trypsin inhibitor to the perfusing medium.

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Experimental

Preparation of isolated hepatocytes

Isolated hepatocytes were prepared from fed Sprague–Dawley rats (200–300g) by a modification of the method of Berry & Friend (1969). The livers were perfused through the portal vein with modified calcium-free Hanks solution, pH 7.4 (Jeejeebhoy *et al.*, 1975) at 37°C, equilibrated in O_2/CO_2 (19:1), by using a recirculating perfusion apparatus (Miller *et al.*, 1951). The liver was initially perfused with 50ml of Hanks' solution to flush blood from the organ,

and perfusion continued at a rate of 10–25ml/min with 100ml of Hanks solution containing (a) 50mg of collagenase and 100mg of hyaluronidase, (b) 50mg of collagenase (Ingebretsen & Wagle, 1972) or (c) 50mg of collagenase and 5mg of soya-bean trypsin inhibitor (Crane & Miller, 1974). After 15–25min, the liver was removed, transferred to a beaker containing 50ml of calcium-free Hanks medium at 4°C and the tissue agitated with a blunt spatula. The suspension was filtered through cheesecloth and nylon mesh (61 μm pore size), and the hepatocytes were separated from non-parenchymal cells by centrifugation at 50g for 2min. The supernatant was removed, the cells were resuspended in fresh medium at 4°C and the centrifugation was repeated twice more. Total cell counts on cell suspensions were made in a haemocytometer chamber after staining the cells in 0.2% Trypan Blue. Cells which excluded Trypan Blue were considered viable.

Isolation of plasma-membrane subfractions

Suspensions of isolated hepatocytes from two rats were pelleted by low-speed centrifugation (50g for 2min). The cells were suspended in 0.25M-sucrose at 4°C and homogenized in a Dounce homogenizer with a tight-fitting pestle [stated radial clearance 0.076mm (Blaessig Glass Co., Rochester, N.Y., U.S.A.)] until more than 90% of the cells were disrupted as observed by phase-contrast microscopy. Plasma-membrane fractions were then prepared from the homogenate as described previously for liver tissue (Touster *et al.*, 1970; Wisher & Evans, 1975).

The two parent plasma-membrane fractions were then subfractionated, and two plasma-membrane subfractions derived from the nuclear and one from the microsomal pellet were collected at 8–37% (w/v) [nuclear–light (N–L), microsomal–light (M–L)] and 37–49% (w/v) [nuclear–heavy (N–H)] sucrose interfaces. Membranes were stored in 0.25M-sucrose/5mM-Tris/HCl, pH7.6, at −20°C.

Determination of enzyme activities

Adenylate cyclase (EC 4.6.1.1) activity was measured as described by Wisher & Evans (1975) by using the procedure of Gilman (1970) to determine the amount of cyclic AMP formed. Alkaline phosphodiesterase I (EC 3.1.4.1) and 5′-nucleotidase (EC 3.1.3.5) were determined spectrophotometrically as described by Razzel (1963) and Ipata (1967) respectively. Acid phosphatase (EC 3.1.3.2) activity was measured by the method of Gianetto & de Duve (1955) with β-glycerophosphate as substrate, and glucose 6-phosphatase (EC 3.1.3.9) by the method of Swanson (1955). Phosphate liberated in the above assays was measured by the procedure of Martin & Doty (1949). Galactosyltransferase activity (EC 2.4.1.38) was measured in the presence of 0.6% Triton X-100 by the paper-chromatographic method of Fleischer *et al.* (1969) as modified by Bergeron *et al.* (1973). Monoamine oxidase activity (EC 1.4.3.4) was determined spectrophotometrically as described by Schnaitman *et al.* (1967) and succinate dehydrogenase (EC 1.3.99.1) was measured by the method of Earl & Korner (1965).

Chemical determinations

Protein content of membrane suspensions was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.) as standard.

Polyacrylamide-gel electrophoresis

Polyacrylamide-slab-gel electrophoresis of plasma-membrane samples, washed in saline and hypo-osmotic media and dissolved in 4M-urea/1% sodium dodecyl sulphate/1% mercaptoethanol solution, was carried out as described by Wisher & Evans (1975).

Electron microscopy

Samples were fixed for 1h at 4°C in a mixture (2:1, v/v) of 1% (w/v) OsO_4 and 2.5% (v/v) glutaraldehyde in 0.1M-sodium cacodylate buffer, pH7.4 (Hirsh & Fedorko, 1968). The pellets were 'postfixed' in 0.25% (w/v) uranyl acetate in 0.1M-veronal acetate buffer, pH6.2, for 15min and embedded in Epikote 812. Thin sections were stained with uranyl acetate and lead citrate and observed with a Philips EM-300 electron microscope.

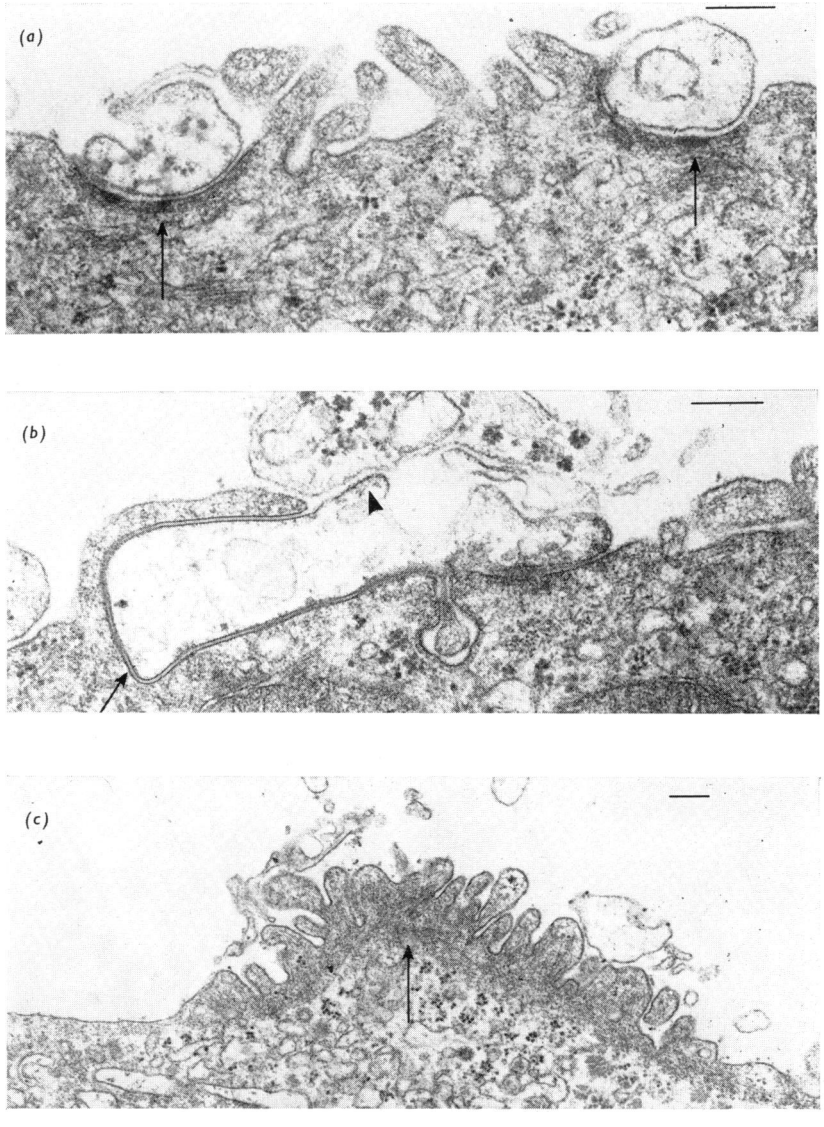
Materials

Biochemicals were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K., and chemicals of AnalaR grade were obtained from British Drug Houses, Poole, Dorset, U.K. The cyclic AMP assay kit was obtained from Boehringer Corp. (London), Ealing, London W5 2TZ, U.K. The ammonium salt of UDP-[^{14}C]galactose was supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Collagenase used was Sigma type I prepared from *Clostridium histolyticum*. Nylon mesh was obtained from Henry Simon Ltd., Cheadle Heath, Stockport, Cheshire, U.K.

Results

Surface properties of isolated hepatocytes

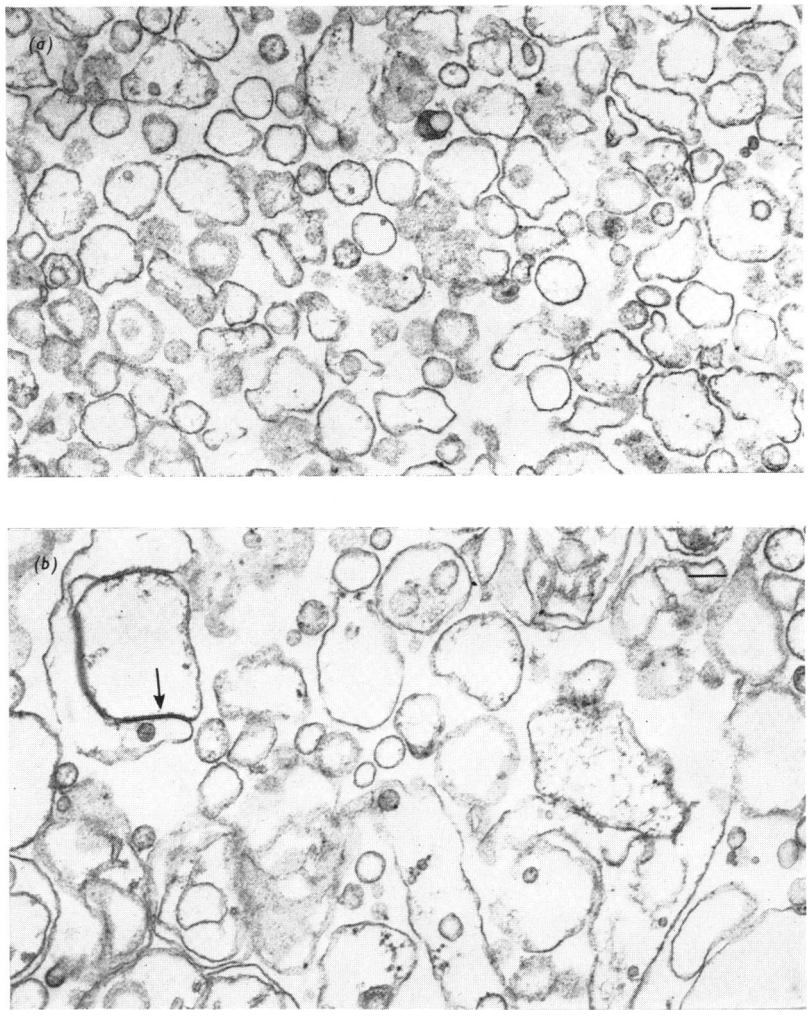
To obtain sufficient material from which to prepare plasma membranes, isolated hepatocytes were prepared from two rat livers. The yield of isolated hepatocytes from two livers was between 6.3×10^8 and 1.3×10^9 cells and was similar to that reported previously (Ingebretsen & Wagle, 1972; Zahlten & Stratman, 1974). Examination of isolated hepato-



EXPLANATION OF PLATE 1

Electron micrographs of plasma-membrane specializations on isolated rat hepatocytes

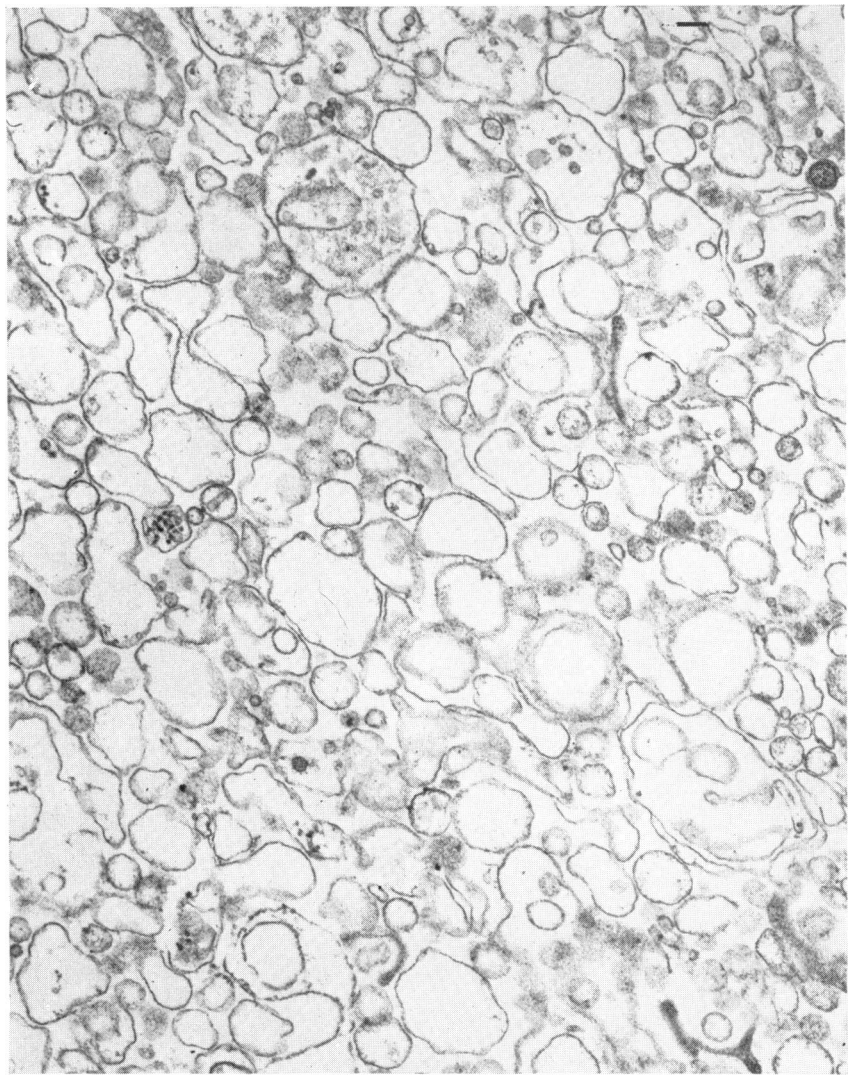
(*a*) Hemidesmosome structures (arrows); (*b*) an intact gap junction (arrow), and membrane and associated cytoplasm with ribosomes torn from an adjacent hepatocyte (arrowhead); (*c*) microvilli with an organelle-free cytoplasm underneath (arrow). Bars represent 0.2 μm.



EXPLANATION OF PLATE 2

Electron micrograph of plasma-membrane subfractions prepared from isolated hepatocytes

(*a*) Nuclear–light (N–L) plasma-membrane subfraction; (*b*) nuclear–heavy (N–H) plasma-membrane subfraction. Arrow identifies gap junction. Bars represent 0.1 μm.



EXPLANATION OF PLATE 3

Electron micrograph of the plasma-membrane microsomal–light (M–L) subfraction prepared from isolated hepatocytes
Bar represents 0.1 μm.

cytes by phase-contrast microscopy showed less than 3% non-parenchymal cells and that the hepatocytes were present either as single cells or as clumps of two or three cells. Electron-microscopic examination of thin sections showed that morphologically recognizable cell-surface structures were preserved in isolated hepatocytes maintained at 4°C for up to 2h after preparation. Microvillar areas (Plates 1*a*, 1*b* and 1*c*) were observed on extensive areas of the surfaces of the isolated hepatocytes, and some of these areas had an underlying organelle-free zone (Plate 1*c*), suggesting that they corresponded to the bile-canalicular surface area (Biava, 1964) as also observed by Phillips *et al.* (1974). The remains of dissociated desmosomes (hemidesmosomes) were observed on the cell surfaces (Plate 1*a*), but no evidence for separation of membrane comprising the gap-junctional complex was obtained; indeed these junctional complexes were always observed as complete structures (Plate 1*b*), often associated with small pieces of plasma membrane and attached cytoplasm, indicating that they had been torn from the adjacent cell. Although the hepatocytes often adhered together to form small clumps, no junctional complexes were observed at the point of contact.

Properties of plasma-membrane subfractions

The distribution of enzyme activities in the crude subcellular fractions isolated by differential centrifugation and in the purified parent plasma-membrane fractions was similar to that described by Wisher & Evans (1975) for plasma-membrane fractions from whole liver. Table 1 shows the specific activities of marker enzymes for endoplasmic reticular, lysosomal and mitochondrial inner and outer membranes in the plasma membranes derived from the parent nuclear fraction and the microsomal fraction before further subfractionation.

Vigorous Dounce homogenization of the two parent plasma-membrane fractions followed by sucrose-density-gradient centrifugation yielded three major subfractions: two 'light' subfractions [nuclear–light (N–L) and microsomal–light (M–L)] of density 1.13g/μl and one 'heavy' subfraction [nuclear–heavy (N–H)] of density 1.18g/ml. A minor heavy subfraction of density 1.18g/ml was isolated after subfractionation of the 'microsomal' plasma membranes, but this was not studied further. Thus the subfractionation characteristics of the plasma membranes of isolated hepatocytes was similar to that obtained with plasma membranes from liver tissue (Wisher & Evans, 1975).

Electron-microscopic examination of the subfractions

The light subfractions [nuclear–light (N–L) and microsomal–light (M–L)] were seen under the electron microscope to consist of smooth membrane-vesicular profiles from which strips of membranes and junctional complexes were generally absent (Plates 2*a* and 3). The nuclear–heavy (N–H) subfraction (Plate 2*b*) was composed predominantly of smooth-membrane vesicles of varying dimensions, but strips of membranes with 'free ends' and gap junctions were also observed. Closer examination of the microsomal–light (M–L) subfraction indicated that very few recognizable Golgi elements were observed. Filamentous material associated with membrane strips and the inner surface of vesicles was frequently seen in all three subfractions.

Distribution of enzymes among the subfractions

The distribution of three plasma-membrane marker enzymes in subfractions from isolated hepatocytes, prepared by dissociating tissue in the presence or absence of trypsin inhibitor, was compared with plasma-membrane fractions isolated from whole liver homogenates (Fig. 1). No difference in enzyme activities was observed when hyaluronidase was included in addition to collagenase in the perfusion medium, so these results are not tabulated separately. Similar distribution patterns of 5′-nucleotidase (Fig. 1*b*) and alkaline phosphodiesterase (Fig. 1*c*) to those observed in whole liver subfractions were found in isolated hepatocyte subfractions prepared when the perfusing mixture also contained a trypsin

Table 1. *Enzyme activities in plasma-membrane fractions prepared from isolated rat hepatocytes*

Rat livers were enzymically dissociated and plasma-membrane fractions were prepared as described in the Experimental section. Enzymic activities are expressed as μmol of product formed/h per mg of protein, ±S.E.M. for the numbers of experiments in parentheses. The activities of galactosyltransferase (a Golgi-membrane marker) in plasma-membrane subfractions are shown in Fig. 1.

Enzyme activity	Homogenate	Plasma membranes prepared from nuclear fraction	Plasma membranes prepared from microsomal fraction
Glucose 6-phosphatase	3.48 ± 1.04 (3)	1.68 ± 0.27 (4)	1.34 ± 0.50 (3)
Acid phosphatase	1.47 ± 0.46 (3)	1.27 ± 0.46 (3)	1.69 ± 0.40 (3)
Succinate dehydrogenase	0.082 (2)	0.045 (2)	0.019 (2)
Monoamine oxidase	0.359 (2)	0.40 ± 0.03 (3)	0.183 (2)
5′-Nucleotidase	2.60 (2)	68.9 (2)	83.0 (2)
Alkaline phosphodiesterase	1.84 (2)	40.0 (2)	60.0 (2)

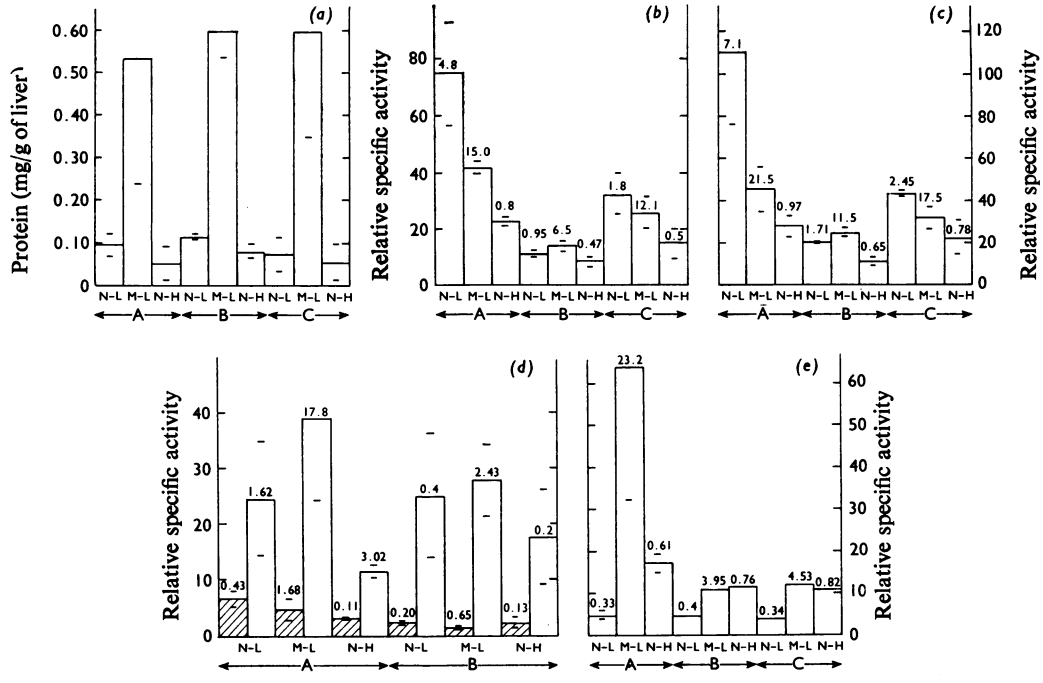


Fig. 1. *Distribution of enzyme activities in plasma-membrane subfractions from isolated hepatocytes and undissociated liver*
Plasma-membrane subfractions were prepared as described in the Experimental section. Nuclear-light (N–L), microsomal-light (M–L) and nuclear-heavy (N–H) subfractions were prepared from undissociated liver tissue (A) or from isolated hepatocytes prepared in the absence (B) or presence (C) of trypsin inhibitor. Histogram (*a*) records the protein recovered in each subfraction expressed as mg of protein/g wet wt. of liver, with the weight of liver calculated on the basis of 9.8×10^7 cells/g wet wt. of liver (Zahlten & Stratman, 1974). The protein contents of the homogenates (±S.E.M.) were: (A) 148±29(13); (B), 135(2); (C), 132±22(5) mg/g wet wt. of liver. The following enzyme activities were measured in the subfractions: (*b*) 5′-nucleotidase; (*c*) alkaline phosphodiesterase; (*d*) adenylate cyclase; (*e*) galactosyltransferase. Enzyme relative specific activities were calculated as the ratio of the specific activity of the subfraction to that of the homogenate (±S.E.M.). Basal adenylate cyclase activity (hatched bars) and glucagon (2 μM)-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 the total homogenate 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 (±S.E.M.; nos. of expts. in parentheses) were as follows: 5′-nucleotidase (*b*), A, 2.52±0.73(4), B, 4.63(2), C, 3.33±1.19(5) μmol of adenosine liberated/h per mg of protein; alkaline phosphodiesterase (*c*), A, 0.77±0.12(4), B, 2.04(2), C, 1.73±0.19(5) μmol of *p*-nitrophenol liberated/h per mg of protein; basal adenylate cyclase (*d*), A, 0.078(2), B, 0.159(2) nmol of cyclic AMP formed/h per mg of protein [glucagon-stimulated activity was as follows: A, 0.132(2); B, 0.936(2) nmol of cyclic AMP formed/h per mg of protein]; galactosyltransferase (*e*), A, 2.52(2), B, 8.32, C, 4.92 nmol of galactose transferred/h per mg of protein. The values were obtained from two to five separate plasma-membrane preparations, except for galactosyltransferase activity determinations, where only one plasma-membrane preparation was measured.

inhibitor. Further, a similar distribution pattern of the glucagon-stimulated adenylate cyclase activity (Fig. 1*d*) was observed in whole liver and isolated hepatocyte subfractions. This uneven distribution of marker enzymes in plasma-membrane subfractions from liver tissue was one of several criteria used to assign these subfractions to the different functional surfaces of the hepatocyte. As discussed below, a similar uneven distribution of marker enzymes found in plasma membranes prepared from isolated hepatocytes suggests that these subfractions are derived from different functional surfaces of the hepatocyte.

Fig. 1 also indicates an overall recovery of 0.74–0.79 mg of protein/g of liver in the plasma-membrane subfractions from isolated hepatocytes, which compares favourably with the recovery of 0.68 mg of protein/g of liver obtained in whole liver subfractions. The protein recovery from isolated hepatocytes was calculated on the basis of a recovery of 9.8×10^7 cells/g wet wt. of liver (Zahlten & Stratman, 1974). When

hepatocytes were prepared by using collagenase, the overall recovery of the plasma-membrane markers 5′-nucleotidase and alkaline phosphodiesterase in all the subfractions was between 8 and 14% of the homogenate activity. This compared with recoveries of 20–30% obtained in plasma-membrane subfractions prepared from whole-liver homogenates. Since the lower recoveries and specific activities of these plasma-membrane marker enzymes in isolated hepatocyte subfractions may have resulted from proteolytic activity of the crude collagenase, hepatocytes were also prepared with a trypsin inhibitor included in the perfusing medium when increased recoveries (14–21% of the homogenate 5′-nucleotidase and alkaline phosphodiesterase activities) were obtained.

In agreement with the morphological observations, the microsomal–light (M–L) plasma-membrane subfractions from the isolated cells contained a much lower activity of the Golgi-marker enzyme galactosyltransferase than did the corresponding subfractions from the whole tissue (Fig. 1). Moreover, similar activities of this enzyme were obtained in isolated hepatocyte subfractions prepared in the presence or absence of trypsin inhibitor.

The viability of the hepatocyte suspensions, determined by their ability to exclude Trypan Blue, did not affect the enzymic composition of the plasma-membrane subfractions. Similar specific activities relative to the homogenate and similar recoveries of 5′-nucleotidase, alkaline phosphodiesterase, basal and glucagon-stimulated adenylate cyclase were obtained from freshly prepared cell populations of which 30% or 90% excluded Trypan Blue.

Protein composition of subfractions

The polypeptides of plasma-membrane subfractions prepared from liver tissue and isolated hepato-

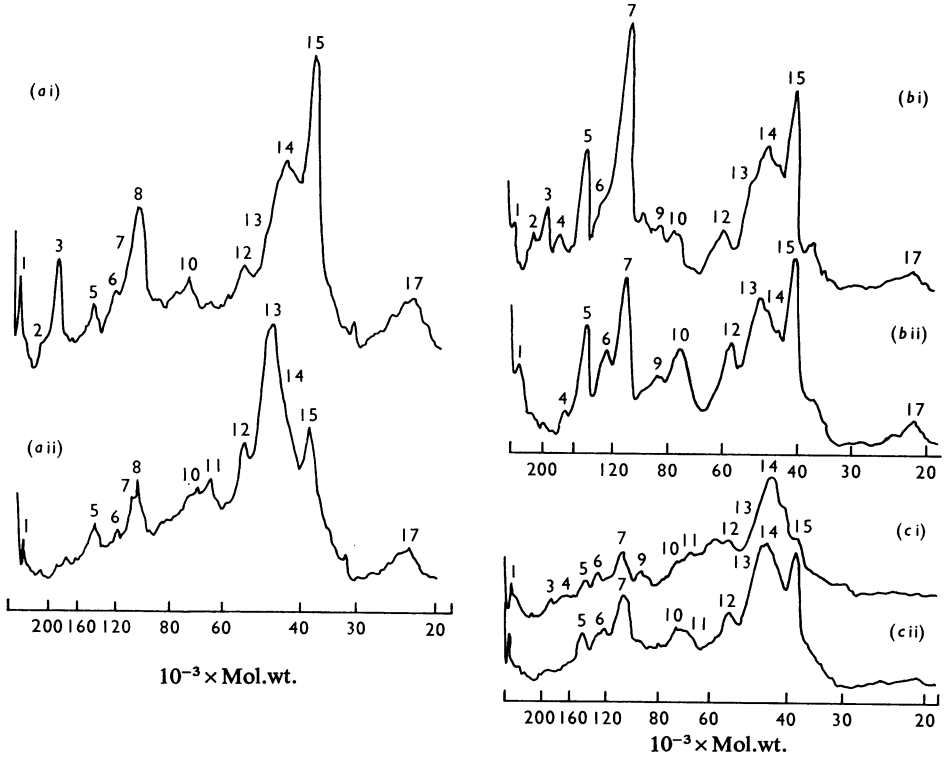


Fig. 2. *Comparison of the polypeptide profiles of the plasma-membrane subfractions prepared from liver tissue and isolated hepatocyte homogenates*

The plasma-membrane subfractions were prepared and polyacrylamide-gel electrophoresis was carried out as described in the Experimental section. (*a*) Nuclear–heavy (N–H), (*b*) nuclear–light (N–L) and (*c*) microsomal–light (M–L) plasma-membrane subfractions prepared from either liver tissue (i) or isolated hepatocyte (ii) homogenates. Numbers indicate corresponding polypeptides that co-electrophorese at positions indicating the same apparent molecular weight.

cytes were compared by polyacrylamide-slab-gel electrophoresis (Fig. 2). The number of major co-electrophoresing polypeptide bands in the isolated-hepatocyte and whole-tissue subfractions was similar, but differences were observed between the intensities of a number of bands. For example, one of the major differences between tissue- and hepatocyte-derived plasma membranes appeared in some of the polypeptides of higher molecular weight (e.g. bands 2, 3 and 7) that are glycoproteins (Wisher & Evans, 1975), this being especially apparent in the plasma-membrane subfraction prepared from the nuclear fraction.

Discussion

Plasma membranes and cellular heterogeneity of liver tissue

Subcellular-fractionation approaches to liver function underestimate the problem of tissue cellular heterogeneity. Although hepatocytes account for 85–95% of tissue weight, in terms of cell numbers they account for only approx. 50% (Iype *et al.*, 1965; Greengard *et al.*, 1972). Little is known about the subcellular fractionation of the non-parenchymal cells, i.e. Kupffer, reticuloendothelial, fibroblastic and bile-ductule cells, the enzymic properties of their constituent membranes, and hence their contribution to 'purified' membrane fractions prepared from tissue homogenates. It was shown that most of the glucagon-stimulated adenylate cyclase activity is present in hepatocytes (Sweat & Hupka, 1971; Wincek *et al.*, 1975), but that both Kupffer cells and hepatocytes bind insulin to a similar extent, although a higher degradation of insulin occurred in Kupffer cells (Wagle *et al.*, 1976). Hence, it is important to demonstrate that at least the liver plasma-membrane fractions prepared from liver tissue homogenates are derived predominantly from hepatocytes. Previous attempts to prepare a plasma-membrane fraction from isolated hepatocytes gave very low yields or damaged membranes (Wright & Green, 1971; Solyom *et al.*, 1972). The present paper describes a procedure for the preparation from isolated hepatocytes of plasma membranes in good yield (i.e. a recovery of approx. 20% of marker enzyme activities) and such membranes are thus likely to be representative of the total surface area. Further, the recovery of the plasma membranes distributed among three subfractions that corresponded enzymically, morphologically and chemically to similar subfractions prepared from liver tissue allows inferences to be made about the maintenance of the functionally differentiated topographical areas of the hepatocyte surface.

Plasma membranes related to function

Three topographically defined functional areas are present on the hepatocyte surface, i.e. blood-sinusoidal, lateral and bile-canalicular areas. Wisher & Evans (1975) prepared six plasma-membrane subfractions from liver tissue homogenates and identified those subfractions that were derived predominantly from the three functional areas. Since isolated hepatocytes secrete fibrinogen and albumin (Crane & Miller, 1974; Jeejeebhoy *et al.*, 1975) as well as bile acids (Anwer *et al.*, 1975), processes that occur at geographically distinct areas of the hepatocyte surface in the intact liver, the present work also allows conclusions to be drawn about the question of whether these areas remain distinct in the isolated cells. Morphological examination of isolated hepatocytes provided clues about the retention of microvillar and junctional areas on the hepatocyte surface. For example, bile-canalicular-like microvillar areas on the hepatocyte surface similar to those described by Phillips *et al.* (1974) were seen. Gap junctions were not cleaved into the constituent membranes contributed by the adjacent hepatocytes, and intact gap junctions were seen frequently in one of the plasma membrane subfractions. The relative distribution of marker enzymes among the three plasma-membrane subfractions corresponded to that of the subfractions prepared from liver tissue. Thus the microsomal–light (M–L) subfraction identified as being derived predominantly from the blood-sinusoidal plasma membrane on the basis of indirect histochemical and enzymic evidence (Wisher & Evans, 1975) and directly by surface-domain-specific ligand-labelling experiments (Carey & Evans, 1977), showed similar properties relative to the other subfractions when prepared from tissue and cell homogenates. The nuclear–heavy (N–H) subfraction that contained low activities of plasma-membrane marker enzymes relative to the other fractions was, like its counterpart prepared from tissue homogenates, shown to contain membrane strips and junctional elements, that were derived from the lateral area, which in isolated cells had once been involved in interhepatocytic adhesion and communication. The identification of a bile-canalicular plasma-membrane fraction from isolated hepatocytes was not possible, since in liver tissue the preparation of these membranes was dependent on first centrifuging at low speed the bile-canalicular complexes attached to long membrane strips and junctional complexes in a hypo-osmotic medium. However, it is likely that the bile-canalicular membranes of isolated cells were present to an unknown extent in the 'nuclear–light' subfraction, since this fraction showed the highest 5′-nucleotidase and alkaline phosphodiesterase activities. The high glucagon-stimulated adenylate cyclase activity present in this fraction suggests that vesicles derived from the blood-sinusoidal area that were of a similar density were also present.

Thus the morphological and subcellular-fractiona-

tion results argue that isolated hepatocytes, at least when maintained at 4°C for periods of up to 2h, were able to maintain functionally and biochemically distinct regions of the surface membrane. It is concluded that the maintenance of a functionally differentiated surface membrane by the isolated hepatocyte is not so much related to factors governing tissue organization at the level of cell–cell interactions, but probably reflects the high order of intracellular metabolic compartmentation of the hepatocyte. It may well be that this cell-surface functional polarity is retained when cells are kept at 37°C for some time, but this needs to be tested by the subcellular-fractionation approaches now described.

Enzymic damage to the plasma membrane during tissue dissociation

Crude collagenase preparations used to dissociate tissues contain proteolytic activity, and this may explain the lower recovery in isolated hepatocytes, compared with liver tissue, of plasma-membrane enzymes. The two plasma-membrane marker enzymes used, 5′-nucleotidase and alkaline phosphodiesterase, were shown to be glycoproteins whose active sites face the external medium (Evans & Gurd, 1973; Evans *et al.*, 1973; Gurd & Evans, 1974; Evans, 1974; Bischoff *et al.*, 1976) and are thus positioned for being lost or modified during perfusion. Inclusion of a soya-bean trypsin inhibitor decreased the loss of activity of plasma-membrane marker enzymes, as indicated by their increased recovery in the plasma-membrane subfractions. However, it is not clear whether peptidases present in *Clostridium* extracts would be inhibited by soya-bean trypsin inhibitor.

A further possible reason for the decreased activities of ectoenzymes (plasma-membrane enzymes whose active sites face the external medium rather than the cytoplasm) in the plasma-membrane subfractions is that they are inserted from Golgi membranes into the sinusoidal plasma membrane during secretion of hepatic products into the blood (Evans, 1976). The decreased secretory activity of isolated hepatocytes, relative to liver (East *et al.*, 1973), can thus result in a lowering of the activities of these enzymes on the hepatocyte surface.

Comparisons of the polypeptides present in the plasma-membrane subfractions prepared from liver tissue and hepatocyte homogenates indicated that polypeptide bands, corresponding to those already identified as glycoproteins, were decreased in intensity, especially in the nuclear–heavy subfraction that originated from the hepatocyte's lateral surface area at which enzymic treatment has to be effective to dissociate the liver.

Functional heterogeneity between hepatocytes

The present paper considers the heterogeneity of the liver plasma-membrane fraction at the level of cells of disparate function and also at the level of the functional polarity of individual hepatocytes. However, it still leaves unresolved the problem of biochemical heterogeneity among hepatocytes. Immunochemical studies have indicated that albumin and fibrinogen synthesis are not carried out equally by hepatocytes (Hamishina *et al.*, 1964). Further, Drochmans *et al.* (1975) were able, after liver dissociation, to separate two types of hepatocytes that contained different amounts of glycogen. It remains to be examined to what extent, if any, such heterogeneity between hepatocytes contributes to the divergent properties of the plasma-membrane subfractions prepared.

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