Highly purified bile-canalicular vesicles and lateral plasma membranes isolated from rat liver on Nycodenz gradients

Biochemical and immunolocalization studies

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INTRODUCTION

Hepatocytes generate and maintain a highly polarized cell surface, containing clear-cut basolateral and apical domains. The basolateral domain comprises a microvillar sinusoidal membrane facing the blood that merges into a flattened lateral region, whereas the apical surface, comprising 10–15% of the total surface area, consists of microvilli intruding into the bile-canalicular spaces. Subcellular-fractionation approaches have shown the non-homogeneous distribution at the hepatocyte's surface membrane of marker enzymes, proteins and lipids (Aronson & Touster, 1974; Toda et al., 1975; Evans, 1980; Hubbard et al., 1983; Taylor et al., 1983; Evans & Enrich, 1989).

The isolation and biochemical characterization of plasma-membrane fractions originating from the sinusoidal, lateral and canalicular domains of the hepatocyte have been based mainly on the distribution of enzyme markers. The demarcation of the microvillar sinusoidal and flat lateral surfaces of hepatocytes is not so clearly defined biochemically, but the tight junction provides a barrier effecting a clear segregation of the lateral and canalicular surface areas. High enrichments of marker enzymes in bile-canalicular vesicles, e.g. alkaline phosphatase and leucine aminopeptidase, attest to the major confinement of these constituents in this membrane domain, although considerations of transcytotic routes transferring metabolites between blood and bile may account for the observation that exclusivity of markers to one surface domain is difficult to demonstrate (Evans, 1980; Evans & Enrich, 1989).

An increasing number of components, many involved in signal transduction, are being shown to be present at high levels in bile-canalicular plasma membranes, e.g. inositol tris- and tetrakis-phosphatases (Shears et al., 1988), GTP-binding proteins (Ali et al., 1989a) and insulin-receptor tyrosine kinases (Margolis et al., 1988). Specific glycoproteins (Diamond et al., 1987) and bile acid (Meier et al., 1987) and drug (Kamimoto et al., 1989) carriers are also present. This has prompted a re-examination of the purity of bile-canalicular plasma-membrane vesicles relative to plasma membranes originating from other domains of the hepatocyte's surface. In the present work we describe a major improvement in the methodology for the preparation from rat liver homogenates of bile-canalicular vesicles and show that these vesicles are at least 2–3-fold higher in purity on the basis of five marker enzymes than those described hitherto. A by-product of the new procedure is lateral membranes isolated in vesicular and sheet-like form. We also demonstrate, using immunolocalization approaches, that antibodies to the highly purified bile-canalicular membranes locate around the bile-canalicular as well as to the sinusoidal aspect of hepatocytes. However, pre-absorption of this serum with sinusoidal plasma-membrane vesicles provided an antiserum that bound exclusively to the bile-canalicular plasma membrane of hepatocytes, suggesting that two subsets of antigens are present in bile-canalicular plasma membranes, with one confined mainly to the canalicular (apical) membrane domain and another shared with the sinusoidal (basolateral) plasma membrane.

MATERIALS AND METHODS

Materials

Nycodenz was purchased from Nycomed U.K., Sheldon, Birmingham, U.K. Antiserum SGI, recognizing the 41 kDa α-subunit of the inhibitory G-protein, was provided by Dr. G. Milligan, University of Glasgow (Ali et al., 1989a). The other chemicals were of analytical grade and of the highest purity available.

Abbreviation used: PBS, phosphate-buffered saline.

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Subcellular fractionation

Livers from 12 male Sprague-Dawley rats (150–200 g) were removed and perfused via the portal vein with ice-cold 0.15 M NaCl. The livers (total weight approx. 120 g) were minced and then homogenized at 4 °C in 1 mm-NaHCO₃/0.5 mm-CaCl₂ (50 ml/5 g of liver) with eight strokes of a loose-fitting Dounce homogenizer. The homogenate (1200 ml), after filtration through two layers of muslin cloth, was centrifuged at 1000 g for 15 min in 6 × 250 ml polycarbonate pots. The supernatant was collected and used to prepare a sinusoidal plasma-membrane fraction as described by Wisher & Evans (1975), and the pellets, gently resuspended with the loose-fitting homogenizer, were washed a further two times each with 400 ml of 1 mm-NaHCO₃ by centrifugation at 1000 g for 10 min and at 4000 g for 10 min respectively. The pellets were resuspended in sucrose (> 70 %, w/v) and plasma membranes prepared by flotation through layers of 54, 48, 43 and 8 %, (w/v) sucrose by centrifugation (100000 g for 3 h; Beckman SW28 rotor). Membranes located at the 8/43 % interphase were collected and pelleted (100000 g for 30 min). They were resuspended in 8 % sucrose and homogenized in a tight-fitting Dounce homogenizer. After centrifugation for 2 h at 100000 g (Beckman SW28 rotor), canalicular, lateral₁ and lateral₉ plasma-membrane fractions were collected at 8/37 %, 37/43 % and 43/49 %-sucrose interphases respectively, as described previously (Wisher & Evans, 1975). In the modified procedure, plasma membranes collected from the 8/37 % interphase were pelleted (100000 g for 30 min) and resuspended in approx. 30 ml of 8 % sucrose. They were sonicated (6 × 15 s bursts interspersed with 30 s cooling periods in ice, 2 μm peak-to-peak value, M.S.E. Soniprep with micro-tip probe) and loaded on to continuous gradients formed by mixing 12 ml each of 10 % (w/v) and 30 % (w/v) Nycodenz dissolved in 0.25 m-sucrose. A 4 ml 60 %-sucrose cushion supported the gradients. After centrifugation at 100000 g for 2 h (Beckman SW28 rotor), gradients were unloaded and 30 × 1.2 ml fractions collected for analysis. New canalicular and lateral₁ plasma membranes were collected from these gradients (see Fig. 1a). The plasma membranes collected at the 37/43 %-sucrose interphase (see above) were also analysed on Nycodenz gradients in a similar way, yielding canalicular and lateral₁ plasma membranes as well as a lateral₉ plasma-membrane fraction (Fig. 1b); the mean buoyant density of these fractions in Nycodenz gradients was 1.055, 1.135 and 1.175 g/cm³ respectively.

### Fig. 1. Analysis of liver plasma membranes on Nycodenz gradients

Plasma-membrane subfractions collected at the 8/37 %-sucrose (a) or 37/43 %-sucrose (b) interfaces were collected, sonicated, and analysed in continuous iso-osmotic Nycodenz gradients. The distribution of protein and three marker enzyme activities is shown. Bars indicate fractions pooled routinely for further studies: CAN, canalicular plasma membrane; LAT₁ and LAT₉, lateral plasma-membrane subfractions.
Purified bile-canalicular membranes

Table 1. Enzymic activities and their relative enrichment in various plasma-membrane fractions of rat liver

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>L-Leucyl naphthalamidase</th>
<th>5'-Nucleotidase</th>
<th>Alkaline phosphodiesterase</th>
<th>Alkaline phosphatase</th>
<th>Ca²⁺-ATPase</th>
<th>Na⁺ + K⁺-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>14000</td>
<td>0.52±0.09</td>
<td>0.57</td>
<td>3</td>
<td>0.18</td>
<td>0.75</td>
<td>0.36±0.02</td>
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<tr>
<td>(100)</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Parent plasma membrane</td>
<td>33.04</td>
<td>8.05±1.71</td>
<td>13.38</td>
<td>75</td>
<td>2.10</td>
<td>26.9</td>
<td>3.39±0.13</td>
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<tr>
<td>(0.24)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Sinusoidal plasma membrane</td>
<td>12.20</td>
<td>5.75±1.45</td>
<td>3.82</td>
<td>10.1</td>
<td></td>
<td>10.12±2.28</td>
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<tr>
<td>(0.09)</td>
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<tr>
<td>Lateral, plasma membrane</td>
<td>2.22</td>
<td>6.16±1.81</td>
<td>16.67</td>
<td>81</td>
<td>6.00</td>
<td>15.9</td>
<td>13.46±4.18</td>
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<td>(0.02)</td>
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<tr>
<td>Lateral, plasma membrane</td>
<td>2.98</td>
<td>2.60±1.20</td>
<td>11.38</td>
<td>68</td>
<td>3.60</td>
<td>11.8</td>
<td>13.46±4.18</td>
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<td>(0.02)</td>
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<tr>
<td>Bile-canalicular plasma membrane (sucrose gradient)</td>
<td>8.24</td>
<td>16.40±3.29</td>
<td>18.46</td>
<td>102</td>
<td>10.35</td>
<td>50.7</td>
<td>1.48±0.40</td>
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<td>(0.06)</td>
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<tr>
<td>Bile-canalicular plasma membrane (Nycodenz gradient)</td>
<td>0.97</td>
<td>59.80±6.05</td>
<td>78.90</td>
<td>374±131</td>
<td>27.60</td>
<td>90.4</td>
<td>Not detectable</td>
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<td>(0.01)</td>
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Enzyme determinations

Marker enzymes were measured by standard techniques: L-leucyl β-naphthalamidase (EC 3.4.11.1) (Goldberg & Rutenberg, 1958); 5'-nucleotidase (EC 3.1.3.5) (Michell & Hawthorne, 1965); alkaline phosphodiesterase 1 (EC 3.1.4.1) (Razzell, 1963); alkaline phosphatase (EC 3.1.3.1) (Pekarth et al., 1972); Na⁺ + K⁺-dependent ATPase (EC 3.6.1.37) (Poupon & Evans, 1979). A high-affinity Ca²⁺-ATPase activity was determined as described by Birch-Machin & Dawson (1986). Free Ca²⁺ and Mg²⁺ concentrations were determined to be 0.5 μM and 0.2 μM respectively, by using the iterative algorithm COMICS (concentration of metal ion and complexed species) devised by Epping & Bygrave (1984). To establish whether enzyme activities were latent, subcellular fractions were also subjected to enzyme measurements in the presence of 0.2 mg of saponin/ml.

Electrophoresis and Western blotting

Membrane fractions (50 μg per channel) were solubilized in 75 mM-Tris/HCl, pH 6.8, 2 % (w/v) SDS, 0.55 M-β-mercaptoethanol, 0.003 % Bromophenol Blue and 10 % (w/v) glycerol, boiled for 2–3 min and resolved in 10 % acrylamide gels containing SDS buffer (Laemmli, 1970). Proteins were either stained with Coomassie Brilliant Blue or transferred to nitrocellulose papers (0.1 μm pore size; Schleicher and Schuell, Dassel, Germany) for Western blotting followed by autoradiography as described by Ali et al. (1989a).

Preparation of antiserum

Antibodies to the bile-canalicular vesicles were raised in rabbits injected intramuscularly with approx. 400 μg of protein in Freund’s complete adjuvant. After 7 weeks, rabbits were boosted subcutaneously with 200 μg of protein in Freund’s incomplete adjuvant, and serum was collected twice a week after 10 days. For absorption of the antiserum, 500 μl of sinusoidal plasma membranes (4–5 mg of protein) was incubated at 37 °C for 1 h with 500 μl of the serum, centrifuged for 10 min at 12000 g, and the clear supernatant was used for immunomicroscopy as described below.

Immunofluorescent microscopy

Small pieces of fresh rat liver were rapidly frozen in solid CO₂ and stored at −70 °C. Frozen sections (6–10 μm) were cut and applied to gelatin-coated microscope slides, dipped in acetone at −20 °C for 5 min or immersed in chloroform acetone (1:1, v/v) for 3 min (both procedures gave the same results), and then stored at −20 °C. To stain the sections, the slides were first equilibrated at room temperature and then immersed in a phosphate-buffered saline, pH 7.4 (PBS; Shears et al., 1988) followed by incubation with the first antibody diluted in 1 % BSA/PBS (1:100) for 40 min at room temperature. After several washes with PBS, the second antibody was applied for 1 h, and finally the sections were washed as described above.

Immunoelectron microscopy

For immunolabelling, livers were perfused in 2 % paraformaldehyde/0.1 % glutaraldehyde in 0.1 M-cacodylate (pH 7.2) and 3 % polyvinylpyrrolidone. The tissue was left in fixative overnight at 4 °C and then washed sequentially for 60 min in PBS, and then in 0.5 M-NH₄Cl/PBS, dehydrated, and embedded in Lowicryl K4M. Thin sections of liver were prepared on Formvar/carbon-coated nickel grids. They were incubated with the rabbit anti-(bile-canalicular plasma membrane) antiserum, washed, treated with Protein A conjugated to 10 nm-diameter gold (Janssen Pharmaceutica, Beerse, Belgium), and stained with uranyl acetate for 5 min and lead citrate. Sections were viewed in a Philips EM300 microscope at 60 kV.

Electron microscopy of subcellular fractions

Freshly prepared membrane fractions were pelleted, fixed overnight at 4 °C in 2 % glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 7.4, and post-fixed first in 1 % OsO₄ in cacodylate buffer and then in aq. 1 % uranyl acetate, for 1 h each. Samples were dehydrated through a graded ethanol series and embedded in Araldite. Grid staining of thin sections was performed in uranyl acetate saturated in 75 % (v/v) ethanol for 20 min and in Reynolds’s lead citrate for 10 min, and they were viewed in a Philips EM 300 electron microscope.
RESULTS

In previous work, plasma-membrane fragments recovered by flotation from a low-speed centrifugal pellet of rat liver homogenates (Neville, 1960) were subfractionated in sucrose gradients after resuspension in a tight-fitting Dounce homogenizer (Evans, 1969, 1970). Three plasma-membrane subfractions designated as originating from the canalicular and lateral (contiguous) surface areas of the hepatocyte, as well as a further vesicular fraction derived from the sinusoidal surface area and recovered from microsomal pellets, were extensively characterized (Wisher & Evans, 1975; Kremmer et al., 1976). In the present studies, it is shown that a substantial improvement in the purity of liver plasma membranes was achieved by fractionation in Nycodenz gradients, and immunocytochemical evidence is provided to confirm independently the domain origin of the canalicular plasma-membrane vesicles.

Separations in Nycodenz gradients

The further analysis of the plasma membranes collected at the 8/37% sucrose interphase (designated bile-canalicular plasma membranes) is shown in Fig. 1(a). In the continuous iso-osmotic Nycodenz gradients, two major protein peaks were obtained and categorized mainly on the basis of enzymic content as canalicular (CAN) (density 1.040–1.070 g/ml) or lateral (LATA) (density 1.110–1.150 g/ml). The new canalicular plasma membranes contained high specific activities of 5'-nucleotidase, alkaline phosphodiesterase and leucyl naphthylamidase. Most of the protein applied to the gradient, however, was recovered in a second peak (LATA), containing approximately one-third the specific-activity levels of the three marker enzymes examined. Analysis of the membranes collected at the 37/43% sucrose interphase in the same continuous Nycodenz gradients (Fig. 1b) showed a further...
Comparison of the polypeptide compositions of the fractions

Fig. 2(a) shows an analysis of the Coomassie-Blue-stained polypeptides in the fractions prepared by using the Nycodenz gradients. A major difference observed between the parent and new canalicular plasma membranes (Fig. 2, lanes 1, 2 and 5) was the diminution in polypeptides of 40–55 kDa, and the greater prominence of polypeptides of 100–170 kDa, in the bile-canonical plasma membranes. Lateral plasma membranes (Fig. 2, lanes 4 and 5) were characterized by the presence of major polypeptides of 40–55 kDa. Lateral fractions (lane 3) had a polypeptide composition that was intermediate between those of canalicular and lateral, in agreement with the enzyme comparison (Table 1).

It was also shown by immunoblotting of the fractions resolved by polyacrylamide-gel electrophoresis that the highest relative level of the α-subunit of the inhibitory G-protein was in bile-canonical plasma membranes (Fig. 2b). The G-protein α-subunit was still associated with the canalicular plasma membranes recovered from the Nycodenz gradients. The slightly lower immunostaining intensity relative to that in the parent canalicular plasma membranes was shown, in a separate experiment (Fig. 2c), to be caused by the loss of the α-subunit during the sonication step. This loss was confined mainly to the bile-canonical vesicle fraction, for little release of the subunits occurred in the lateral plasma-membrane fraction.

Morphology of the fractions

The canalicular plasma-membrane fraction collected from the Nycodenz gradients consisted of closed vesicles (Fig. 3). The lateral fraction consisted of slightly larger vesicles with occasional membrane strips (not shown). The lateral fraction contained a far higher number of membrane strips punctuated with intercellular junctions, with the overall morphology being similar to that described previously for lateral plasma membranes (Wisher & Evans, 1975; Poupon & Evans, 1979).

Immunolocalization studies

Antibodies were raised in rabbits to the canalicular plasma membranes collected from the Nycodenz gradients and used to stain liver sections. Fig. 4(a) shows, in frozen liver sections, that the antibodies stained, by immunofluorescence, the blood-sinusoidal as well as the bile-canonical-facing surfaces of trabecular arrangements of hepatocytes; the staining was low or absent at the lateral plasma membrane. In contrast, antisera that had been pre-absorbed with a sinusoidal plasma membrane (Fig. 4b) gave a staining that was confined mainly to the bile canaliculi.

Immunoelectron microscopy of thin liver sections confirmed the location of the antigens stained by the pre-absorbed serum. Fig. 5 shows that the immuno-gold particles were attached predominantly to microvilli projecting into the bile-canonical spaces, and that little or no staining was present in the lateral and sinusoidal plasma-membrane regions.

DISCUSSION

The plasma membrane of the hepatocyte, as in most polarized epithelial cells (Simons & Fuller, 1985), is structurally and functionally differentiated into three major domains (Evans, 1980; Maurice et al., 1985). In the present work we describe the preparation and properties of bile-canonical plasma-membrane vesicles of substantially higher purity than described hitherto, and utilize antibodies raised to these membranes to demonstrate directly, in rat liver sections, their origin from the microvilli projecting into the bile spaces.

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The present work shows that sonication of two plasma-membrane fractions isolated on sucrose gradients (Evans, 1970; Wisher & Evans, 1975; Meier et al., 1984, 1987) resulted in the isolation of a vesicular population of canalicular vesicles and two lateral plasma-membrane fractions of higher density that were separated in the Nyocenoz gradients. Enzymic and morphological studies indicated that the lighter of these two lateral fractions (LAT) consisted of membrane strips interspersed with canalicular membranes, whereas the heavier fraction (LAT) consisted mainly of membrane strips containing intercellular junctions originating from the lateral surface of hepatocytes.

The identification of bile-canalicular vesicles is attested by analysis of marker enzymes, shown to be present in this hepatocyte domain by enzyme histochemistry (Wachstein, 1959; Sierakowska et al., 1963). Of the enzymes examined (see Table 1), leucine aminopeptidase and 5'-nucleotidase have also been immunolocalized to bile-canalicular structures by using subcellular fractions and sections respectively (Roman & Hubbard, 1984a; Geuze et al., 1984). Five enzymes were shown to be enriched, relative to the homogenate, 115-153-fold in the bile-canalicular vesicles. Since our initial isolation and biochemical characterization of bile-canalicular vesicles (Evans, 1970; Wisher & Evans, 1975), many reports have appeared which describe the isolation of canalicular vesicles (examples are summarized in Table 2). It is clear that the method now described provides a major improvement to those utilizing sucrose gradients, Ca²⁺ precipitation, counter-current distribution in two-phase systems and free-flow electrophoresis. Immunoabsorption procedures using antibodies to leucine aminopeptidase attached to bacterial cells have been used to absorb specifically canalicular vesicles, and, although a 154-fold increase in enzyme activity was estimated (Roman & Hubbard, 1984b), these bound vesicles appear to be of limited practical use. Petell et al. (1987) also used antibodies to a 110 kDa plasma-membrane antigen to immunoabsorb bile-canalicular vesicles, but enzymic monitoring was not carried out. Analysis by free-flow electrophoresis of bile-canalicular vesicles collected from the sucrose gradient stage showed one major peak of membranes (Ali et al., 1989a), and these results, taken with those of Evers et al. (1989), indicate that electrophoretic methods afford only marginal improvement over the use of sucrose gradients. The present density-gradient method utilizing iso-osmotic Nyocenoz gradients has the additional utility of not requiring specialized or expensive equipment.

A Ca²⁺-ATPase activity was enriched 133-fold relative to the liver homogenate in the purified canalicular vesicles. Controversy exists regarding the precise location on the hepatocyte surface of Ca²⁺-ATPases and Ca²⁺ transport (Epping & Bygrave, 1984; Lester et al., 1986; Blitzer et al., 1988). For example, Evers et al. (1988) demonstrated ATP-dependent Ca²⁺ uptake in liver basolateral plasma membranes, whereas Bachs et al. (1985) showed ATP-dependent Ca²⁺ transport to be highly enriched in bile-canalicular vesicles. In the present work, the Ca²⁺-ATPase measured in the bile-canalicular vesicles functioned in the presence of extremely low (endogenous) Ca²⁺ and Mg²⁺ levels. It is likely that this activity probably corresponds to the ectoenzyme purified and characterized from rat liver plasma membranes by Lin & Fain (1984) and Lin (1989). The function of this enzyme is unclear, but, in view of its external location on the bile-canalicular vesicles, it is unlikely to be involved directly in Ca²⁺ transport. Na⁺+K⁺-ATPase activity was not detected in the canalicular vesicles, but was enriched in the lateral plasma membranes recovered from the Nyocenoz gradients, confirming its baso-

Fig. 5. Immunelectron microscopy of rat liver thin sections

The sections were stained with antibodies to bile-canalicular plasma membranes pre-absorbed with sinusoidal plasma membranes and Protein A-gold (10 nm). Arrows point to positions of tight junctions. Abbreviations: can, canalculus; sin, sinusoidal surface area interfacing with space of Disse. Bar represents 0.5 μm.
Table 2. Relative enrichment of marker enzymes in bile-canalicular plasma membranes prepared by various methods

<table>
<thead>
<tr>
<th>Methods</th>
<th>Leucyl naphthylamidase</th>
<th>5'-Nucleotidase</th>
<th>Alkaline phosphodiesterase</th>
<th>Alkaline phosphatase</th>
<th>References</th>
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<tr>
<td>Sucrose-density-gradient</td>
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<tr>
<td>1</td>
<td>40</td>
<td>70</td>
<td>100</td>
<td>40</td>
<td>Wisher &amp; Evans (1975)</td>
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<td></td>
<td>38</td>
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<td>Hubbard et al. (1983);</td>
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<td></td>
<td>48</td>
<td>64</td>
<td>116</td>
<td>71</td>
<td>Roman &amp; Hubbard (1984b)</td>
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<td>N₂ cavitation and Ca²⁺ precipitation</td>
<td>57</td>
<td>–</td>
<td>–</td>
<td>55</td>
<td>Inoue et al. (1982)</td>
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<td>Countercurrent distribution in dextran/</td>
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<td>ethylene glycol</td>
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<tr>
<td>1</td>
<td>29</td>
<td>94</td>
<td>–</td>
<td>–</td>
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<td>2</td>
<td>70</td>
<td>50</td>
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<td>Gierow et al. (1988)</td>
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<td>Free-flow electrophoresis</td>
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<td>138</td>
<td>125</td>
<td>153</td>
<td>Present paper</td>
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Lateral topographical location on hepatocytes by biochemical (Poupoun & Evans, 1979) and immunolocalization (Szul et al., 1987) studies. However, the relative distribution between the basolateral and apical domains of one of the enzyme subunits is still in dispute (Sutherland et al., 1988).

A number of glycoproteins have recently been shown to be present in hepatic canalicular plasma membranes, including pp₁₀⁶, an endogenous substrate for insulin-receptor kinase (Margarolakis et al., 1988), a 110 kDa glycoprotein (Diamond et al., 1987), cell-CAM 105 (Odin & Obrink, 1988; Tingstrom & Obrink, 1989) and Gp 170, postulated to be involved in drug excretion into bile (Thiebaut et al., 1987; Kamimoto et al., 1989; Yoshimura et al., 1989). A high enrichment at the canalicular relative to the sinusoidal plasma membrane domain of inositol tris- and tetrakis-phosphatase (Shears et al., 1988) and G-protein subunits (Ali et al., 1989a,b) was shown. Comparison of the polypeptide composition of the plasma membrane of high purity prepared on Nycodenz gradients indicated a relative enrichment of polypeptides in the 100–170 kDa range in the canalicular plasma membranes. The opportunity was also taken to re-examine the association of the α-subunit of G-proteins in these fractions in view of their enhanced purity. The results confirm the specific association of a G-protein subunit with canalicular plasma membranes (Ali et al., 1989a) and show that this subunit was partially released during the sonication step used in the preparation of the membranes. In contrast, the G-protein subunit in lateral membranes was more tightly associated with the membranes. The cause of this discrepancy between the canalicular and lateral plasma-membrane domains is unclear.

Finally, the results show directly by immunocytocchemical staining of liver sections that the highly purified canalicular vesicles originate from the tight-junction-enclosed microvillar plasma membrane surrounding the bile sinusoids. The antibodies generated to the canalicular vesicles stained both canalicular and sinusoidal surfaces of hepatocytes. However, after pre-absorption with a sinusoidal plasma-membrane fraction, the antibodies immunolabelled the bile canaliculi, thus identifying a further subset of antigens confined to this surface region. One interpretation of this result is that certain proteins are transferred directly from the Golgi apparatus to the bile-canalicular plasma membrane domain, thereby avoiding a detour to the sinusoidal plasma membrane. The demonstration that at least six glycosylphosphatidylinositol-anchored proteins were routed directly to the bile-canalicular surface, two of these via a 'late' endosomal compartment (Ali & Evans, 1990), is in agreement with such an interpretation. The preparation from liver of domain-defined plasma membranes of the highest purity are essential tools in clarifying by the subcellular-fractionation approach the various mechanisms that generate a functionally differentiated plasma membrane.

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REFERENCES
