Fractionation of rat liver plasma-membrane regions by two-phase partitioning

Peter GIEROW,* Marianne SOMMARIN,† Christer LARSSON† and Bengt JERGIL*

*Biochemistry, Chemical Centre, University of Lund, P.O.B. 124, S-221 00 Lund, Sweden, and †Department of Plant Physiology, University of Lund, P.O.B. 7007, S-220 07 Lund 7, Sweden

Rat liver plasma membranes, enriched in blood-sinusoidal or bile-canalicular regions by differential and sucrose-gradient centrifugation, were further purified by partitioning in an aqueous polymer two-phase system. This method separates membranes according to differences in surface properties rather than size and density. A several-fold increase in the ratio of leucine aminopeptidase (a bile-canalicular marker) and 5'-nucleotidase to asialo-orosomucoid binding (a blood-sinusoidal marker) was obtained in one fraction, whereas another fraction gave a 2–3-fold increase in ratio of blood-sinusoidal to bile-canalicular markers. Furthermore, the markers for both regions of the plasma membrane, as well as markers for Golgi membranes and lysosomes, showed a heterogeneous behaviour on counter-current distribution.

INTRODUCTION

Rat liver plasma membranes are usually purified by a combination of differential and density-gradient centrifugation techniques. By these means it has been possible to purify membrane fractions enriched either in the blood-sinusoidal region only (Aronson & Touster, 1974; Wisher & Evans, 1975; Taylor et al., 1983), in both blood-sinusoidal and lateral membranes (Meier et al., 1984), or in bile-canalicular and lateral membranes (Neville, 1960; Aronson & Touster, 1974; Wisher & Evans, 1975). More recently, a method was developed where all three plasma-membrane regions were obtained in the same final fraction (Hubbard et al., 1983). Since the procedures used are based on differences in size and density of the membrane material, an alternative approach, such as partitioning in aqueous polymer two-phase systems, which separates membranes according to differences in surface properties (Albertsson et al., 1982), might be useful both for purification of different regions of the plasma membrane and for studies of lateral-membrane heterogeneity.

Although partitioning in dextran/polyethylene glycol two-phase systems is well established in the purification and characterization of plant material, there are relatively few studies on animal membranes, including plasma membranes (reviewed by Albertsson et al., 1982). Components of rat liver homogenates have been partitioned microanalytically (Morris & Peters, 1982) and by a toroidal-coil centrifuge technique (Heywood-Waddington et al., 1984). Hino et al. (1978a,b) have subfractionated Golgi membranes by counter-current distribution. Preparation of plasma membranes by a two-phase batch procedure (Lesko et al., 1973), as well as fractionation of microsomal membranes (Ohlsson et al., 1978) and brain mitochondria and synaptosomes (López-Pérez et al., 1981) by counter-current distribution, have also been reported.

In this study we have examined the partitioning of rat liver plasma membranes and other membranes in dextran/polyethylene glycol two-phase systems of different compositions through analysis of established marker-enzyme activities. A more detailed picture of the partitioning of different markers was obtained by counter-current distribution. Conditions have been found which allow the separation of blood-sinusoidal and bile-canalicular membranes, and which reveal heterogeneous behaviour of several of the marker membranes investigated.

EXPERIMENTAL

Materials

Stock solutions of 20% (w/w) Dextran T500 (Pharmacia Fine Chemicals AB) and 40% (w/w) polyethylene glycol 3350 (Carbowax 3350; Union Carbide) were prepared as described by López-Pérez et al. (1981) [\(^{38}\)S]AMP and [\(^{14}\)C]galactose was from New England Nuclear. All other substrates and enzymes used for determination of marker enzyme activities were from Sigma Chemical Co.

Preparation of membranes

Male Sprague–Dawley rats weighing approx. 200 g were starved overnight and killed in the morning by decapitation. Livers were immediately transferred to ice-cold 0.25 m-sucrose in 5 mM-Tris/HCl buffer, pH 8.0, and processed by a modification (Wisher & Evans, 1975) of the method of Aronson & Touster (1974). A 1000 g supernatant used for the isolation of plasma membranes from the microsomal fraction, and a pellet sedimenting with the nuclear fraction, were obtained. The 1000 g supernatant was treated (Aronson & Touster, 1974) until a 105000 g pellet (the P-fraction) was obtained. Unless otherwise indicated, this pellet was further purified by sucrose-gradient centrifugation to yield the P fraction, enriched in blood-sinusoidal membranes. The 1000 g pellet was processed to yield a ‘fluffy’ pellet, followed by sucrose-density-gradient centrifugation to obtain the N fraction, enriched in bile-canalicular membranes. The plasma membrane fractions were sedimented and resuspended in 15 mM-Tris/H\(_2\)SO\(_4\) (pH 7.8)/0.25 m-sucrose.
Counter-current distribution

Two-phase polymer systems containing 6.0% Dextran T500, 6.0% polyethylene glycol 3350, 15 mM-Tris/H₂SO₄, pH 7.8, 3 mM-KCl and 0.25 M-sucrose were prepared in advance and stored at 4 °C until use. The subsequent steps were performed at this temperature with solutions kept at 4 °C at least overnight. Separate top and bottom phases were obtained by adding the components to a separatory funnel, mixing thoroughly, and allowing the phase system to settle overnight before collecting the two phases. Sample systems with a total weight of 8.00 g were prepared containing 1 ml of sample (20 mg of protein of the P₂- or N₂-fraction) and the solutes as above.

A thin-layer counter-current-distribution apparatus (Albertsson, 1965, 1970; Albertsson et al., 1982) equipped with a partition block with 60 cavities (numbered 0–59) was used. The bottom-phase capacity of each cavity was 0.73 ml. Since the partition of membranes took place between the top phase and the interface plus bottom phase, the cavities were loaded so that only the top phase would be transferred, leaving the interface plus the bottom phase stationary. This was achieved by using a bottom-phase volume of 0.60 ml. Cavities 0–4 were each loaded with 1.40 ml of thoroughly mixed sample system. Each of the other cavities received 0.60 ml of bottom phase and 0.80 ml of top phase. A mixing time of 20 s and a settling time of 12 min were used, and the cycle was terminated after 55 transfers. The phase system was broken by the addition of 1.40 ml of 10 mM-Tris/HCl (pH 7.4)/0.25 M-sucrose to each cavity. The block was shaken for 1 min before unloading.

The fractions obtained were analysed directly for protein and marker enzymes. In some experiments fractions were combined as indicated in each case, diluted 5-fold with 10 mM-Tris/HCl, pH 7.4, also containing 10 mM-MgCl₂ and centrifuged at 105000 g for 2 h. Membrane pellets were suspended in 10 mM-Tris/HCl, pH 7.4, before analysis.

Effect of KCl and polymer concentrations

The effect of varying the concentrations of KCl or phase polymers on the distribution of membranes was studied by using a series of 4.00 g sample systems prepared in centrifuge tubes as above with 0.5 ml of membrane sample (the P₂- or P₃-fraction; 10 mg of protein). The systems were mixed by vortexing, turned upside down 15 times, and vortexed again. The settling was speeded up by centrifugation at 1500 g for 5 min in a swing-out rotor, and the phases (top phase and bottom phase plus interface, respectively) were collected.

Assays

Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as standard with appropriate amounts of phase polymers added. Since polyethylene glycol interferes with the determination, the protein was first precipitated with 5% (w/v) trichloroacetic acid in presence of 0.012% deoxycholate. The following plasma membrane markers were measured: S'-nucleotidase (EC 3.1.3.5; Auvruch & Wallach, 1971), leucine aminopeptidase (EC 3.4.11.1; Roman & Hubbard, 1983), and 125I-asialo-orosomucoid binding (Hubbard et al., 1983). Orosomucoid was desialylated as described by Doyle et al. (1979), and the amount of sialic acid released (over 80%) was measured by the thiobarbituric acid assay (Warren, 1959). The protein was labelled with [125I]iodine by using immobilized lactoperoxidase/glucose oxidase (Bio-Rad) as described by the manufacturer. 125I-Asialo-orosomucoid was separated from free iodine by gel filtration. The Golgi-membrane marker UDP-galactose:N-acetylgalactosamine galactosyltransferase (EC 2.4.1.38) was measured by the method of Fleischer & Smigel (1978), lysosomal acid phosphatase (EC 3.1.3.2) as described by Bergmeyer (1974), mitochondrial cytochrome c oxidase (EC 1.9.3.1) as described by Shore & Tata (1977), and the endoplasmic-reticulum markers NADPH-cytochrome c reductase (EC 1.6.2.4) and glucose-6-phosphatase (EC 3.1.3.9) were assayed as described by Hrycay & O'Brien (1974) and Gierow & Jergil (1980) respectively.

RESULTS

Effects of polymer concentrations and KCl on the partitioning of membrane markers

In order to establish optimum conditions for the fractionation of rat liver plasma membranes, the influence of polymer concentrations and KCl on their partitioning was examined. Since ions have different affinities for the two phases (Johansson, 1970), the addition of a salt, such as KCl, creates an electrical potential difference between the phases, strongly affecting the partitioning of charged particles (Albertsson et al., 1982). This factor may predominate and give a separation of membrane particles correlating with their electrophoretic mobilities (Walter et al., 1965). A change in polymer concentrations may also influence the partitioning of membranes (Albertsson et al., 1982). In this case, however, there is no correlation with any easily measurable surface property. Therefore, other characteristics than charge, such as hydrophilic/hydrophobic properties, may then determine the partitioning (Westrin et al., 1983).

The P₂-fraction (see the Experimental section) containing appropriate marker populations for plasma membranes and Golgi was used to study the effect of a parallel increase of the phase polymers from 5.3 to 6.4% (Fig. 1a). At the lowest polymer concentrations, more than 90% of the plasma-membrane markers partitioned into the top phase. An increase in the polymer concentrations resulted in a decrease in the amount of markers in the top phase, and this decrease was more rapid for asialo-orosomucoid-binding components (blood–sinusoidal marker) than for leucine aminopeptidase (bile-canalicular marker). Thus, with both polymers at 6.0% approx. 30% of the asialo-orosomucoid binding partitioned into the top phase, compared with 75% of the leucine aminopeptidase activity. The partitioning of S'-nucleotidase, an enzyme preferentially located in bile-canalicular membranes (Evans, 1977), followed that of leucine aminopeptidase closely. The amount of the Golgi-membrane marker galactosyltransferase in the top phase decreased less rapidly than asialo-orosomucoid binding components when the concentrations of the phase polymers were increased (Fig. 1a). Thus markers for different regions of the plasma membrane were affected differently by an increase in polymer concentrations.

Plasma membranes prepared by sucrose-gradient centrifugation, besides Golgi, also contained some contamination by mitochondria, endoplasmic reticulum
Partitioning of plasma membranes

and lysosomes. Therefore the partitioning of marker enzymes for these membranes was followed in a parallel experiment (Fig. 1b) using a crude membrane preparation (the P-fraction; see the Experimental section) to get well-measurable activities. An increase in the polymer concentrations from 5.4 to 6.0% decreased the endoplasmic-reticulum markers NADPH-cytochrome c reductase and glucose-6-phosphatase in the top phase from approx. 90% to 2–3% of the total activity recovered, and mitochondrial cytochrome c oxidase from 45% to 10%; acid phosphatase, a lysosomal marker, decreased from 65 to 35%.

The effect of KCl on the partitioning of the plasma- and Golgi-membrane markers was examined by using the P2-fraction and a phase system containing 6.0% of each polymer. Increasing concentrations of KCl progressively suppressed the marker components from the top phase (Fig. 2). At 3 mM-KCl less than 10% of asialoorosomucoid-binding components were recovered in the top phase, compared with 20% of galactosyltransferase and 45–50% of 5'-nucleotidase and leucine aminopeptidase activities.

Counter-current distribution

To study further the separation of plasma-membrane markers, the P2- and N2-fractions (Aronson & Touster, 1974) were examined by counter-current distribution with 6.0% of each phase polymer and 3 mM-KCl. The P2-fraction, mainly containing membranes of the blood-sinusoidal domain, together with Golgi membranes (Wisher & Evans, 1975; Table 1), separated into two peaks (Fig. 3). The left-hand peak (i.e. material partitioning into the bottom phase and at the interface) contained a large part of the asialo-orosomucoid-binding components. This marker decreased from left to right, leaving little activity under the right-hand protein peak (i.e. material partitioning into the top phase). Leucine aminopeptidase preferentially distributed to the right, together with 5'-nucleotidase, although a small distinct peak of these markers was found to the left. Galactosyltransferase activity decreased steadily from left to right, whereas acid phosphatase distributed equally between left and right in two distinct peaks of activity.

The specific activities and recoveries of markers were determined after sedimenting the membrane material fractionated by counter-current distribution (Table 1). The specific asialoorosomucoid binding in the left-hand peak remained similar to that of the P2-fraction (approx.

![Graph](image-url)

**Fig. 1. Effect of polymer concentrations on partitioning of rat liver membranes**

Each point represents the amount recovered in the top phase (% of total) after partitioning in two-phase sample systems (4.00 g) of increasing phase-polymer concentrations containing 0.5 ml (10 mg of protein) of the P2-fraction (a) or the P-fraction (b) and 15 mM-Tris/H2SO4, pH 7.8. Protein content (●—●), 5'-nucleotidase (■—■), leucine aminopeptidase (△—△), asialoorosomucoid binding (○—○), galactosyltransferase (▲—▲), acid phosphatase (□—□), cytochrome c oxidase (O—O), glucose-6-phosphatase (□—□) and NADPH-cytochrome c reductase (△—△) were measured in top and bottom phases as described in the Experimental section. The recovery of protein and enzyme activities was 85–105%.
40 times higher than in the liver homogenate), but it had diminished 6-fold in the right-hand peak. Contrarily, the specific activities of the leucine aminopeptidase and 5'-nucleotidase decreased to the left, and increased approx. 2-fold in the right hand peak. The specific activity of galactosyltransferase remained similar to that in P2-fraction in both peaks, whereas NADPH-cytochrome c reductase and cytochrome c oxidase decreased in both peaks. The last two enzymes could not be analysed accurately, however, since the activities were close to the detection limit. Judging from the partitioning of these markers in single tube experiments (Fig. 1b), they should both be enriched in the left-hand peak. This was also the case when a crude membrane preparation (the P-fraction) was subjected to counter-current distribution (results not shown).

Upon counter-current distribution of the N2-fraction, mainly containing bile-canalicular membranes, again two protein peaks were found (Fig. 4). The markers leucine aminopeptidase and 5'-nucleotidase preferentially distributed to the right, whereas asialo-orosomucoid-binding components were found both to the left and in the middle. In contrast with the P2-material, the right-hand peak of the N2-fraction had a distinct peak of galactosyltransferase activity (although close to the detection limit). Sedimented material from this peak (Table 1) showed a more than 2-fold increase in the specific activity of 5'-nucleotidase and a 50% increase of leucine aminopeptidase over the N2-fraction, whereas the specific asialo-orosomucoid binding decreased to approximately half.

The recoveries of the various activities analysed require some comments. Usually a recovery of about 75% of the enzyme activities applied was obtained on analysis of the counter-current-distribution fractions directly. However, an inhibition of plasma-membrane and Golgi markers by up to 50% was observed with some batches of the phase polymers (presumably polyethylene glycol). This inhibition was reversed when the phase polymers were removed by sedimenting the membranes. To avoid inhibition, and to concentrate the material, membranes were often sedimented before analysis. The recovery then was 50–70% of the enzyme activities applied. The addition of 10 mM-MgCl2 (or 1 mM-CaCl2) before centrifugation was necessary to avoid loose pellets, resulting in still lower yields.

**DISCUSSION**

The partitioning experiments showed that rat liver membrane fractions at low polymer concentrations have an increasing affinity for the polyethylene glycol-rich top phase in the order: endoplasmic reticulum, mitochondria, lysosomes, Golgi membranes and plasma membranes. A similar order has been observed for plant membranes.
Table 1. Enzyme activities in plasma-membrane fractions before and after counter-current distribution

Plasma membranes were purified to yield the P$_r$- and N$_r$-fractions. These were subjected to counter-current distribution (CCD) as described in the Experimental section. Left- and right-hand peaks were combined as indicated (Figs. 3 and 4), diluted 5-fold with 10 mM-Tris/HCl (pH 7.4)/10 mM-MgCl$_2$, and sedimented by centrifugation (105000 g, 2 h). The pellets were suspended in 10 mM-Tris/HCl, pH 7.4, before analysis. Enzyme activities are expressed (± s.d.) as nmol/min per mg of protein, except for galactosyltransferase (nmol/h per mg of protein). Asialo- orosomucoid binding is given as ng/mg of protein. The numbers of preparations analysed (n) are indicated in parentheses. The specific activity of each marker in the homogenate was set to 1.0 and used to calculate the relative specific activity (RSA), shown in brackets. Protein is expressed as mg/g wet wt. of liver, and the yield as % of the total activity in the homogenate.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Homogenate</th>
<th>P$_r$-fraction</th>
<th>N$_r$-fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before CCD</td>
<td>After CCD, left peak</td>
<td>After CCD, right peak</td>
</tr>
<tr>
<td></td>
<td>Sp. activity (n)</td>
<td>Sp. activity (n)</td>
<td>Yield (%)</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>29.9 ± 6.1 (10)</td>
<td>464 ± 158 (10)</td>
<td>12.8</td>
</tr>
<tr>
<td>Asialo- orosomucoid binding</td>
<td>7.63 ± 1.35 (5)</td>
<td>321 ± 123 (5)</td>
<td>34.7</td>
</tr>
<tr>
<td>Leucine aminopeptidase</td>
<td>11.2 ± 1.8 (10)</td>
<td>75.7 ± 22.5 (10)</td>
<td>5.3</td>
</tr>
<tr>
<td>N-Acetylglucosamine galactosyltransferase</td>
<td>43.7 ± 3.2 (10)</td>
<td>488 ± 131 (10)</td>
<td>9.3</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>51.3 ± 4.2 (10)</td>
<td>141 ± 29 (10)</td>
<td>2.3</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase</td>
<td>26.7 ± 5.5 (5)</td>
<td>27.9 ± 18.8 (5)</td>
<td>0.86</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>162 ± 67 (5)</td>
<td>11.9 ± 7.5 (5)</td>
<td>0.06</td>
</tr>
<tr>
<td>Protein</td>
<td>145 ± 17 (10)</td>
<td>1.20 ± 0.30 (10)</td>
<td>0.83</td>
</tr>
</tbody>
</table>
fractionations
centrifuge
homogenates

Fig. 4. Counter-current
distribution.

Membranes containing asialo-orosomucoid-binding components, a blood-sinusoidal membrane marker, behaved differently from membranes containing bile-canicular or Golgi markers in that they were suppressed more rapidly from the top phase with increasing concentrations of the phase polymers (Fig. 1). This property allowed a phase system to be devised for the separation of membranes derived from the two plasma-membrane regions, yielding blood-sinusoidal markers to the left and bile-canicular markers to the right on counter-current distribution. Thus the specific activity of leucine aminopeptidase, and also that of 5'-nucleotidase, increased, whereas the specific binding of asialo-
orosomucoid decreased in the right-hand peak obtained with both the N2- and the P2-fractions (Figs. 3 and 4), yielding a several-fold increase in the ratio of bile-canicular to blood-sinusoidal markers. However, the specific activities of 5'-nucleotidase and leucine aminopeptidase of the right-hand peak derived from the P2-fraction remained less than half of those derived from N2-fraction, suggesting that the former membranes were less pure.

In a similar way asialo-orosomucoid binding in the left-hand peak of the P2-fraction increased approx. 3- and 2-fold relative to leucine aminopeptidase and 5'-nucleotidase, indicating an enrichment of blood-sinusoidal membranes at the expense of bile-canicular membranes. In this case, however, a further increase in the relative specific asialo-orosomucoid binding compared with that in the P2-fraction was not attained.

Plasma-membrane fractions enriched in blood-sinusoidal or bile-canicular membranes purified according to differences in size and density thus can be further purified by utilizing differences in membrane surface properties. Furthermore, counter-current distribution revealed a heterogeneous behaviour of both the plasma-membrane markers and the markers for Golgi membranes and lysosomes. Whether this reflects a lateral heterogeneity within each type of membrane, or whether these markers occur in more than one membrane, e.g. as a result of membrane flow, is not possible to conclude from our present data. However, a lateral heterogeneity of a membrane should, on fragmentation, result in vesicles having different surface properties, but they may not differ in size or density. Therefore, phase partitioning, being a surface-dependent separation method, should be a useful method for further studies on these problems.

This work was supported by grants from the Swedish Natural Science Research Council and by a personal grant from Bengt Lundquist’s Memorial Fund to P.G.

REFERENCES
Albertsson, P.-Å. (1965) Anal. Biochem. 11, 121–125

(Larsson, 1983), and therefore seems to be consistent for material from widely different sources. The earlier microanalytical partitioning experiments on rat liver homogenates (Morris & Peters, 1982), and the toroidal-coil centrifuge fractionations (Heywood-Waddington et al., 1984), and partitioning studies on Golgi membranes (Hino et al., 1978a,b) and rat liver microsomal fractions (P. Gierow, unpublished work) also indicate this order of distribution. It has been suggested (Larsson, 1983) that this general order of partitioning might be explained by similarities in the lipid composition of corresponding membranes from widely different sources. Model experiments with liposomes have shown that the polar head group of phospholipids plays a dominant role in determining the partitioning of such particles (Eriksson & Albertsson, 1978).
Partitioning of plasma membranes
