Fractionation of Microsomal Membranes on the Basis of their Surface Properties

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(Received 30 January 1978)

Partition in dextran–poly(ethylene glycol) aqueous-phase systems can be used for both membrane subfractionation and gaining information on membrane surface properties [H. Walter (1977) in *Methods of Cell Separation* (Catsimpoolas, N., ed.), vol. 1, pp. 307–354, Plenum, New York]. Smooth, light rough and heavy rough rat liver microsome (obtained by sucrose-density-gradient centrifugation) were subjected to countercurrent distribution in such a system. Smooth microsomal membranes had the highest, heavy rough microsomal membranes the lowest and light rough microsomal membranes an intermediate partition coefficient. The separation is based primarily on hydrophobic differences in the membrane surfaces of the three preparations and is thus due to microsomal properties not previously utilized in their fractionation. The method permits additional subfractionations of microsomes.

Microsomes isolated from liver cells represent a mixture of membrane vesicles which have, most commonly, been fractionated on the basis of size and density (DePierre & Dallner, 1975). Both smooth and rough microsomes appear biochemically heterogeneous when subfractionated by centrifugation (DePierre & Dallner, 1975), histochemical (Lewis & Tata, 1973) or immunochemical (Schulze *et al.*, 1972) methods. An interest in exploring microsomal-membrane surface properties prompted us to examine their partition behaviour in two-polymer aqueous-phase systems. The partition method has previously been used to subfractionate cell and membrane populations (many of which were not measurable by other currently available methods) on the basis of subtle differences in charge-associated or lipid-related surface properties (Walter, 1977; Walter & Krob, 1976, 1978).

Aqueous solutions of dextran and of poly(ethylene glycol), when mixed above certain concentrations, give rise to immiscible liquid two-phase systems with a poly(ethylene glycol)-rich top phase and a dextran-rich bottom phase (Albertsson, 1971). Although the two polymers are non-ionic, some salts (e.g. phosphate) have unequal affinity for the phases (Johansson, 1970), giving rise to an electrostatic potential difference between them (Reitherman *et al.*, 1973). The surface charge of cells or membranes added to such systems interacts with the phase charge, with the result that surface-charge-associated pro-

perties become a major determinant of the partition coefficient (Brooks *et al.*, 1971). [Partition coefficient is here defined as the quantity of microsomal membranes in the top phase plus interface (percentage of total microsomal membranes added). For erythrocytes, which partition between the top phase and interface, the partition coefficient is defined as the quantity of cells in the top phase (percentage of total cells added).] When salts with ions that have essentially equal affinity for both phases are chosen (e.g. NaCl), systems with virtually no potential difference between the phases are obtained. Cells or membranes added to the latter systems tend to be adsorbed in the interface. If the polymer concentrations are lowered, the partition coefficient of cells and membranes increases, even in systems without an electrostatic potential difference between the phases, and has been shown, at least for erythrocytes from different species, to be related to the ratio of their membrane poly-/mono-unsaturated fatty acids (Walter *et al.*, 1976). A correlation has also been found between the partition coefficient of erythrocytes and their membrane phosphatidylcholine and sphingomyelin, increasing with the former and decreasing with the latter (Walter *et al.*, 1976).

Experimental

Smooth, light rough and heavy rough microsomes were prepared from the livers of male Sprague-Dawley rats and separated by sucrose-density-gradient centrifugation as described earlier (Ohlsson & Jergil, 1977; Tata, 1972). The microsomal fractions were washed once in buffer [0.05 M-Tris/HCl (pH 7.4)/8 mM-MgCl₂/25 mM-KCl/0.1 mM-dithiothreitol] before suspension in the top phase of the two-polymer aqueous-phase system. This system was prepared as

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previously described (Albertsson, 1971; Walter, 1977). Its total composition was 5% (w/w) dextran T500 (lot no. 5556; Pharmacia Fine Chemicals, Uppsala, Sweden), 3.5% (w/w) poly(ethylene glycol) 6000 (Union Carbide, New York, NY, U.S.A.), 50mM-sodium phosphate buffer, pH6.8, and 3mM-MgSO₄.

Countercurrent distribution was carried out in a thin-layer apparatus with 120 concentric cavities as described by Albertsson (1970). Bottom-phase capacity on our unit is 0.7ml. Since the partition of the microsomal membranes takes place between the bottom phase and the interface, the unit was loaded in a manner such that the transfer at each step separated the top phase plus interface from the bottom phase. Approx. 6mg of protein of smooth, light rough, heavy rough or a mixture of all three microsomal preparations (containing equal quantities of each) was suspended in 2.7ml of top phase. Cavities 0-2, 30-32, 60-62 and 90-92 each received 0.8ml of bottom phase and 0.8ml of the 'load mix' containing one of the membrane preparations suspended in top phase. All other cavities were loaded with 0.8ml of bottom phase and 0.8ml of top phase. In all, 29 transfers were completed; a shaking time of 25s and a settling time of 8min were used. In this manner all four preparations could be run simultaneously for comparison without overlap. The entire procedure was carried out at 2-4°C. At the end of a countercurrent run the

samples from the different cavities were collected into tubes. Buffer (0.5ml) was added to each tube (to convert the two-phase system into a single phase), and the absorbance of each suspension was measured at 280nm against a similarly diluted phase system. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and RNA by the Fleck & Munro (1962) procedure, both after precipitation with 5% (w/v) trichloroacetic acid (10% trichloroacetic acid also precipitates the phase polymers).

Results and Discussion

In Fig. 1 we depict the countercurrent distribution curves of smooth, light rough and heavy rough microsomes, as well as the distribution of a mixture of all three. The distribution of smooth microsomes (Fig. 1a) is to the right and heavy rough microsomes (Fig. 1c) to the left. The light rough membranes (Fig. 1b) are highly heterogeneous with the far left of their distribution partly overlapping the heavy rough microsomes and the right peak overlapping in position the distribution of smooth microsomes. The distribution of the mixture (Fig. 1d) reflects the separation of the smooth- and heavy-rough-microsomal membranes with the light rough microsomes partly hidden between the two peaks and partly overlapping the smooth microsomes.

Fig. 1. Countercurrent-distribution patterns of different microsomal preparations

Countercurrent-distribution patterns are shown of smooth (a), light rough (b) and heavy rough (c) microsomal membranes, and of an equal mixture of smooth, light rough and heavy rough microsomes (d). A_{280} (●) and the RNA/protein ratio (○) are plotted. The distributions were carried out in a phase system containing 5% (w/w) dextran, 3.5% poly(ethylene glycol) 6000, 50mM-sodium phosphate buffer, pH6.8, and 3mM-MgCl₂; 29 transfers were completed at 2-4°C. RNA/protein ratios (w/w) of the applied material were: for smooth microsomes, 0.06; light rough; 0.20; heavy rough, 0.46; the mixture, 0.23. For additional details see the text.

The RNA/protein ratios (w/w) are rather constant under the distribution curves of each of the three microsomal fractions and the ratios are similar to those of the respective unfractionated membranes. Thus the left- and right-hand peaks obtained with light rough microsomes appear to consist not of contaminating heavy rough and smooth membranes respectively, but rather reflect heterogeneity of the light rough microsomal fraction. This heterogeneity is also suggested by the RNA/protein ratios of the total microsomes (Fig. 1*d*), which approach those of heavy rough microsomes under the left-hand peak and decrease to values higher than those of smooth microsomes under the right-hand peak.

The distribution to the left of heavy rough microsomes and the distribution to the right of smooth microsomes is highly reproducible and is obtained in every experiment. The distribution of the bulk of light rough microsomes is always to the right of heavy rough microsomes and to the left of smooth microsomes, but the extent of overlap of the ends of the distribution with heavy-rough- or smooth-microsomal membranes varies. This may reflect differences resulting from slight variations in rat populations and membrane preparations. Similarly the distribution of the mixture sometimes breaks into the clear two peaks depicted in Fig. 1(*d*), but at other times results in a broad distribution curve stretching from end to end. However, even in such a case, RNA/protein ratios are always high to the left and low to the right, indicating a resolution similar to that obtained in Fig. 1(*d*).

The microsomes (primarily the smooth membranes) have a tendency to aggregate partially in the phase system. Although separation by partition of different microsomal fractions can be effected even under these conditions, it is clear that subfractionations can be improved if aggregation is altogether avoided. Ways of diminishing or abolishing the partial microsomal aggregation need to be investigated.

Let us now consider the probable basis for the differential-partition behaviour of the three microsomal-membrane preparations. The phase system used contains phosphate buffer [i.e. giving an electrostatic potential difference between the phases, with the top phase positive (Reitherman *et al.*, 1973)] and polymer concentrations low enough to reflect lipid-related membrane properties (Walter *et al.*, 1976). We have found that changes in the concentration of phosphate in the phases, which greatly alter the magnitude of the potential difference (Walter *et al.*, 1976), have virtually no effect on the partition behaviour of the microsomes. In addition, the partition of the negatively charged membranes takes place between the bottom phase and the interface: rough microsomes, which have a higher negative surface charge than smooth microsomes [as determined by electrophoresis (Blad *et al.*,

1977)], are found primarily in the negative bottom phase, whereas the smooth membranes are adsorbed in the interface. We conclude that the partition coefficient is primarily a reflection of hydrophobic (i.e. probably lipid) membrane properties (Walter, 1977; Walter *et al.*, 1976).

It is noteworthy that the partition of erythrocytes from different species in phase systems that measure lipid-related membrane properties takes place between the top phase and the interface when the ratio of their membrane poly-/mono-unsaturated fatty acids is high (Walter *et al.*, 1976). Thus the partition of microsomes between the interface and bottom phase may reflect a low ratio. The high degree of saturation of acyl chains and the *trans* double bond and amide bond of sphingomyelin induce high rigidity within membrane lipid layers, whereas phosphatidylcholine forms highly fluid lipid regions (Borochov *et al.*, 1977). The partition coefficient of erythrocytes has, therefore, also been found to increase with increasing membrane phosphatidylcholine and to decrease with increasing sphingomyelin (Walter *et al.*, 1976). What correlations between partition behaviour and surface structures of microsomes pertain will have to await resolution of the nature of the phospholipid distribution in microsomal membranes. The question of whether phospholipids are symmetrically or asymmetrically arranged is currently under active investigation and has given rise to considerable controversy (DePierre & Dallner, 1975; Sundler *et al.*, 1977; Higgins & Dawson, 1977).

Conclusion

Partition in two-polymer aqueous phases permits the segregation of smooth, light rough and heavy rough microsomes on the basis of membrane surface properties not previously utilized in their fractionation. The distribution curves of the microsomal preparations clearly reveal sub-heterogeneities, since they are broader than theoretical. Furthermore preliminary experiments on microsomal marker enzymes under the curve of smooth microsomes also indicate heterogeneity by this criterion (R. Ohlsson, B. Jergil & H. Walter, unpublished work). Partition in two-polymer aqueous systems holds out the prospect, therefore, not only for the separation of microsomal membranes but also for their further subfractionation.

We thank Professor Per-Åke Albertsson for stimulating discussions and encouragement during the course of this work. Adine Lundberg provided technical assistance. H. W. was on sabbatical leave from the Veterans Administration Hospital, Long Beach, CA, U.S.A. This investigation was supported in part by the Swedish Natural Science Research Council.

References

- Albertsson, P.-Å. (1970) *Sci. Tools* **17**, 53–57
- Albertsson, P.-Å. (1971) *Partition of Cell Particles and Macromolecules*, Almqvist and Wiksell, Stockholm, Wiley-Interscience, New York
- Blad, D., Winqvist, L. & Dallner, G. (1977) *J. Cell Sci.* **23**, 285–297
- Borochoy, H., Zahler, P., Wilbrandt, W. & Shinitzky, M. (1977) *Biochim. Biophys. Acta* **470**, 382–388
- Brooks, D. E., Seaman, G. V. F. & Walter, H. (1971) *Nature (London) New Biol.* **234**, 61–62
- DePierre, J. W. & Dallner, G. (1975) *Biochim. Biophys. Acta* **415**, 411–472
- Fleck, A. & Munro, H. N. (1962) *Biochim. Biophys. Acta* **55**, 571–583
- Higgins, J. A. & Dawson, R. M. C. (1977) *Biochim. Biophys. Acta* **470**, 342–356
- Johansson, G. (1970) *Biochim. Biophys. Acta* **221**, 387–390
- Lewis, J. A. & Tata, J. R. (1973) *Biochem. J.* **134**, 69–78
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Ohlsson, R. & Jergil, B. (1977) *Eur. J. Biochem.* **72**, 595–603
- Reitherman, R., Flanagan, S. D. & Barondes, S. H. (1973) *Biochim. Biophys. Acta* **297**, 193–202
- Schulze, H. V., Pönnighaus, J. M. & Standinger, H. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 1195–1203
- Sundler, R., Sarcione, S. L., Alberts, A. W. & Vagelos, P. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3350–3354
- Tata, J. R. (1972) in *Subcellular Components* (Birnie, G. D., ed.), pp. 185–213, Butterworths, London
- Walter, H. (1977) in *Methods of Cell Separation* (Catsimpoilas, N., ed.), vol. 1, pp. 307–354, Plenum, New York
- Walter, H. & Krob, E. J. (1976) *Biochim. Biophys. Acta* **455**, 8–23
- Walter, H. & Krob, E. J. (1978) *Br. J. Haematol.* **38**, in the press
- Walter, H., Krob, E. J. & Brooks, D. E. (1976) *Biochemistry* **15**, 2959–2964