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ORIENTATION OF RAT-LIVER PLASMA MEMBRANE VESICLES

A BIOCHEMICAL AND ULTRASTRUCTURAL STUDY

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Using both biochemical and morphological methods, the membrane orientation of plasma membrane vesicles from rat liver which are capable of catalysing the active transport of amino acids was investigated. In intact vesicles, the plasma membrane enzyme (Na$^+$ + K$^+$)-ATPase displays only a minor portion of its total activity which is greatly increased upon vesicle disruption. The same intact vesicles show an almost maximal binding of ouabain, which binds only to the extracellular side of the plasma membrane. A freeze-fracture analysis of the vesicles shows that a distinct population of relatively large vesicles have predominantly the in vivo membrane orientation. These large vesicles are labelled with numerous filipin-sterol complexes following exposure to the cholesterol probe, filipin, and are therefore assumed to be plasma membrane vesicles. A population of smaller vesicles with mainly an inside-out orientation were not labelled with filipin and are probably microsomes. The data obtained with both biochemical and ultrastructural techniques indicate that the plasma membrane vesicles isolated from rat liver for transport studies are mostly (at least 70%) orientated as in vivo, i.e. inside-in.

Introduction

The plasma membrane of living cells constitutes a selective permeability barrier for a variety of solutes: carrier-mediated transport of amino acids is an essential function of plasma membranes. The study of transport processes across plasma membranes has greatly benefited from the use of isolated plasma membrane vesicles (for recent reviews, see Refs. 1–3). Such isolated vesicles offer the possibility of studying transport in an in vitro system, disconnected from intracellular solute conversions.

Recently, we isolated a plasma membrane preparation from rat liver [4] which enabled us to study the transport of various solutes [5–7]. An important unanswered question with regard to these studies concerns the orientation of the membrane in the vesicles. Generally speaking, there are two possibilities for the orientation of the vesicular membrane. (1) The vesicular membrane can have the same orientation as the cell membrane of the intact cell; in this situation, commonly referred to as 'inside-in', the inside of the vesicle corresponds to the cytosolic side of the cell membrane. (2) The opposite is the 'inside-out' situation, where the inside of the vesicles reflects the extracellular side of the plasma membrane, and is therefore opposite to the in vivo situation. Information on the orientation of the membrane vesicles used in transport

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experiments would enhance the relevance of the transport experiments by verifying that the direction of the solute flux observed in vitro is identical with the flux in vivo.

In the experiments described in this paper we used two approaches to determine the membrane orientation of amino acid transporting rat-liver plasma membrane vesicles. Firstly the activity of the plasma membrane enzyme $(Na^+ + K^+)$-ATPase has been measured under various conditions, in conjunction with binding studies of the cardiac glycoside [^{3}H]ouabain. Secondly, electron microscopic freeze-fracture experiments have been performed which take advantage of the difference in particle density on the two fracture faces of the plasma membrane. The latter method has been employed previously for similar purposes in bacterial membrane vesicles [8,9], erythrocyte ghosts [10] and rat renal and intestinal brush-border membrane vesicles [11].

From the combined biochemical and ultrastructural data we conclude that the membrane preparation from rat liver that catalyzes the active transport of amino acids consists of 40–50% plasma membranes, and 50–60% intracellular membranes, predominantly endoplasmic reticulum. The plasma membrane vesicles within this population appear to be mainly (70–90%) orientated 'inside-in'.

Materials and Methods

Isolation of plasma membrane vesicles. Plasma membrane vesicles were isolated from the livers of male Wistar rats (200–300 g), fed ad libitum. Tissue homogenization, differential and sucrose density centrifugation were carried out as described previously [4,5]. The final membrane preparation was stored in 0.3-ml aliquots (protein concentration about 10 mg/ml) in liquid nitrogen until use. Before an experiment, membranes were quickly thawed in a waterbath at 40°C.

Enzyme assays. The activity of glucose-6-phosphatase was measured according to Swanson [12]. $(Na^+ + K^+)$-ATPase activity was determined as described previously [4]; whenever the enzyme activity in intact vesicles was to be measured, 250 mM sucrose was present in the assay mixture.

Determination of [^{3}H]ouabain binding. Plasma membrane vesicles were centrifuged for 30 min at $100\,000 \times g$. The membrane pellet was resuspended to a concentration of approx. 1 mg protein/ml in binding medium. The binding medium consisted of 1 mM imidazole-EDTA, 1 mM dithiothreitol, 5 mM Tris-phosphate, 5 mM $MgCl_2$ and 30 mM imidazole-HCl, pH 7.4 [13–15]. In addition, 250 mM sucrose was present when ouabain binding to intact vesicles was to be measured.

Incubation in the presence of 5 μM [G-^{3}H]ouabain (spec. act. 600 000–700 000 cpm/nmol) was performed for 30 min at 25°C. Corrections for aspecific binding were made by comparison with parallel incubations in which an excess of 2.5 mM unlabelled ouabain was present.

The binding assays were terminated by Millipore filtration (HAWP, 0.45 μm), and the filters were analyzed for radioactivity by scintillation spectrometry after washing of the filter with 1 ml of the binding medium.

Protein. Protein concentrations were measured according to Lowry et al. [16] using bovine serum albumin (Sigma, fraction V) as a standard.

Freeze-fracture analysis. Membrane vesicles isolated as described above were fixed by resuspension in either 2% glutaraldehyde in 0.1 M phosphate buffer or in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, containing 0.2 mg/ml filipin (300 μM). Filipin (kindly provided by J.E. Grady, Upjohn) was dissolved in a drop of dimethyl sulphoxide (DMSO) before addition to the fixative. The final concentration of DMSO in the solution was 1%. Membranes were treated with the filipin solution for at least 1 h at room temperature before being recentrifuged into a pellet and shipped to Geneva, still in the glutaraldehyde/filipin mixture or in glutaraldehyde/buffer.

For freeze-fracture, membranes were cryoprotected for at least 30 min in 30% buffered glycerol, coated with polyvinyl-alcohol [17] and quenched in Freon 22 cooled with liquid nitrogen. They were then fractured at −100°C in a Balzers freeze-etching device [18]. Platinum-carbon replicas were cleaned with sodium hypochlorite and chloroform/methanol (2 : 1, v/v) rinsed in distilled water and picked up on copper grids prior to examination in a Philips EM 300 electron microscope.

For the quantification of the membrane vesicles,

six different preparations were used. Ten random photographs were taken from replicas of each sample, at a magnification of 22000 ×. The negatives were projected onto a graphic tablet (Tektronix type 4953) at a final magnification of 90000 ×. Vesicles were divided into 2 classes - inside-out and well orientated (see Results) and their surface area and number were measured by means of an electronic pen connected to an IMSAI 8080 microprocessor. A visual display of the vesicle outlines was simultaneously transmitted on a television monitor so that no vesicle could be counted more than once.

From the surface area of each circle, the mean vesicle diameter was obtained, assuming the vesicles to be perfect spheres. From this diameter, the total external membrane surface of each spherical vesicle was calculated. In this way the relative amount of membrane which was either well-orientated or inside-out was estimated for each of the six vesicle preparations.

Materials. Glutaraldehyde (E.M. grade) was from Pierce Chemical Company, Rockford, IL, U.S.A., filipin was a kind gift from J.E. Grady, Upjohn, and [G-^{3}H]ouabain (49 Ci/mmol) was from the Radiochemical Centre, Amersham, U.K.

Results and Discussion

The rat-liver plasma membrane preparation that is capable of catalyzing the active transport of amino acids appears to consist of both plasma membranes and intracellular membranes. Analysis of various marker enzymes [4,23] revealed that two membrane types are present in the vesicle preparations in significant amounts: plasma membrane and endoplasmic reticulum.

An approximation of the amount of endoplasmic reticulum present in our preparations is given in Table I, which shows the recoveries of two marker enzymes. $(Na^+ + K^+)$-ATPase was measured as a marker of the blood-sinusoidal region of the parenchymal cell membrane [19–21], whereas glucose-6-phosphatase represents the presence of endoplasmic reticulum. Our data were combined with those of Blouin et al. [22], who reported on the basis of extensive stereological morphometric studies that the surface areas of endoplasmic reticulum and blood-sinusoidal plasma membrane in rat-liver parenchymal cells differ by a factor of 7 to 9. Extrapolation of this factor to our data on enzyme recoveries indicates that 40–50% of the total amount of membranes in the final preparation is derived from the plasma membrane, whereas 50–60% originates from endoplasmic reticulum.

From previous experiments [4,23] it is known that the endoplasmic reticulum fragments present do not contribute to the transport processes studied. Attempts to purify the plasma membrane preparation via lectin affinity chromatography (wheat germ agglutinin, concanavalin A) have not been successful to date (Sips, H.J., unpublished data). Also minor changes in the centrifugation procedure did not result in a significant improvement in membrane purity; in addition, rat-liver vesicles tend to lose their transport capacity upon prolonged centrifugation. The membrane isolation procedure we developed, therefore, does not aim at the highest purification or at maximal recoveries, but at preservation of the amino acid-

TABLE I

MARKER ENZYME ACTIVITIES IN A RAT-LIVER PLASMA MEMBRANE PREPARATION

The activities of the enzymes $(Na^+ + K^+)$-ATPase and glucose-6-phosphatase were determined as described in Methods. The recovery values refer to liver homogenates taken as 100%. Results are given as means ± standard error; *n*, number of experiments.

Organelle	Representative enzyme	Recovery	*n*	Factor [a]	% of vesicle preparation
Plasma membrane	$(Na^+ + K^+)$-ATPase	15.9 ± 1.1	5	1	38–44
Endoplasmic reticulum	Glucose-6-phosphatase	2.9 ± 0.1	3	7–9	56–62

[a] Factor derived from Blouin et al. [22]; see Results.

transport capacity of the plasma membrane.

The orientation of the plasma membrane vesicles within the mixed population of vesicles was investigated using two independent approaches, both directed towards highly plasma membrane-specific parameters.

Activity of ($Na^+ + K^+$)-ATPase in rat-liver plasma membrane vesicles

The enzyme ($Na^+ + K^+$)-ATPase has been used as a plasma membrane-specific marker enzyme in a variety of cells. Recent evidence indicates, that ($Na^+ + K^+$)-ATPase in the rat-liver parenchymal cell membrane is mainly, if not exclusively, located in the blood-sinusoidal region of this membrane [19–21].

Figure 1 shows the activity of ($Na^+ + K^+$)-ATPase in rat-liver plasma membrane vesicles under various conditions. Whereas the activity of this enzyme is largely latent in intact vesicles, a distinct increase in activity is seen upon disruption of the vesicular membrane. Comparable results are obtained by either osmotic lysis of the vesicles or by the addition of the detergent deoxycholate. However, the ($Na^+ + K^+$)-ATPase in rat-liver is apparently sensitive to higher concentrations of deoxycholate since both intact and osmotically lysed vesicles show a decrease in activity at high concentrations of the detergent. In our initial experiments we used the detergent Triton X-100, in analogy to Walter [24], who determined the ($Na^+ + K^+$)-ATPase latency in guinea pig kidney vesicles. This detergent, however, resulted in inactivation of the liver enzyme at all concentrations tested (data not shown), so care should be taken in the addition of detergents to the ($Na^+ + K^+$)-ATPase assay media and a detergent titration to determine the optimal concentration should be performed. For routine measurements of the total ($Na^+ + K^+$)-ATPase activity in membrane fractions where vesicular structures are present, a hypoosmotic assay medium might be more reliable.

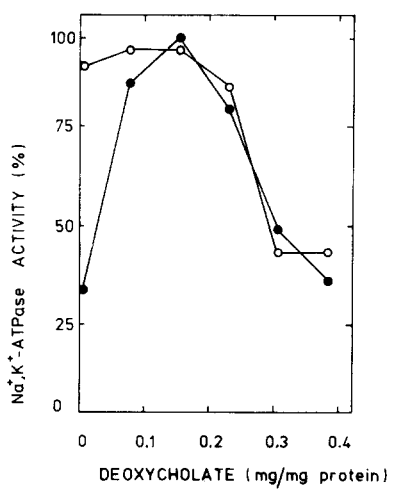


Fig. 1. The activity of the plasma membrane enzyme ($Na^+ + K^+$)-ATPase in rat-liver plasma membrane vesicles. Plasma membrane vesicles from liver tissue were diluted 10-fold either in water (○) or in homogenization buffer (●), and incubated for 10 min at 20°C in the presence of various concentrations of sodium deoxycholate. Subsequently, the activity of ($Na^+ + K^+$)-ATPase was assayed.

Fig. 1 indicates that the majority (about 70%) of the total ($Na^+ + K^+$)-ATPase molecules is incapable of hydrolyzing ATP, probably as a result of the inaccessibility of the enzyme to its substrate. A tentative conclusion, therefore, is that the latent part of the enzyme activity represents closed and inside-in orientated plasma membrane vesicles. However, the observed latency of ($Na^+ + K^+$)-ATPase could also be the result of the fact that the inhibitor ouabain is not able to interact with the enzyme in intact vesicles. In that case, Fig. 1 would be compatible with the presence of a large amount of inside-out plasma membrane vesicles. Experiments on the binding of ouabain exclude this alternative explanation.

Binding of [^{3}H]ouabain to rat-liver plasma membrane vesicles

Additional evidence for the inside-in orientation of the majority of rat-liver plasma membrane vesicles was derived from ouabain binding experiments. The cardioactive steroid ouabain is a specific inhibitor of the plasma membrane enzyme ($Na^+ + K^+$)-ATPase. Moreover, ouabain binds to the enzyme at the extracellular side of the cell membrane only [24,25]. Knowing this, the binding of [^{3}H]ouabain to intact and osmotically shocked rat-liver plasma membrane vesicles was determined.

Intact rat-liver plasma membrane vesicles were found to have a specific ouabain binding capacity of 0.59 ± 0.09 pmol ouabain/mg protein (mean of three experiments ± S.E.). Only a small increase in

TABLE II

Sample	Vesicle orientation	No. of vesicles	Mean vesicle diameter (μm)	Mean vesicle surface (μm^2)	Total vesicle surface (μm^2)	% of total surface	% of total number
1	Inv.	606	0.15 ± 0.002	0.069 ± 0.002	41.1	71.6	89.8
	Corr.	69	0.26 ± 0.02	0.23 ± 0.04	16.3	28.4	10.2
2	Inv.	574	0.14 ± 0.002	0.057 ± 0.002	32.4	66.3	89.7
	Corr.	66	0.29 ± 0.02	0.026 ± 0.03	16.5	33.7	10.3
3	Inv.	504	0.14 ± 0.003	0.058 ± 0.002	28.7	64.5	87.2
	Corr.	74	0.26 ± 0.02	0.23 ± 0.03	15.8	35.5	12.8
4	Inv.	620	0.13 ± 0.002	0.055 ± 0.002	33.2	65.4	87.6
	Corr.	88	0.25 ± 0.007	0.20 ± 0.012	17.6	34.6	12.4
5	Inv.	521	0.15 ± 0.004	0.073 ± 0.004	36.9	62.0	88.0
	Corr.	71	0.32 ± 0.013	0.32 ± 0.026	22.6	38.0	12.0
6	Inv.	738	0.14 ± 0.003	0.054 ± 0.006	43.7	75.5	92.8
	Corr.	57	0.28 ± 0.012	0.25 ± 0.023	14.2	24.5	7.2
All samples	Inv.	3563	0.14 ± 0.003	0.061 ± 0.003	216	67.7	89.3
	Corr	425	0.28 ± 0.011	0.25 ± 0.02	103	32.3	10.7

ouabain binding was observed upon osmotic disruption of the vesicles: a specific ouabain binding of 0.65 ± 0.11 was observed when lysed vesicles were used.

Apparently almost the total ouabain binding capacity of the plasma membrane vesicles is available in intact vesicles. It can thus be concluded that the majority of the ouabain binding sites is present on the outside of intact vesicles. If the vesicles were predominantly inside-out, a significant increase in ouabain binding would be expected upon osmotic lysis of the vesicles.

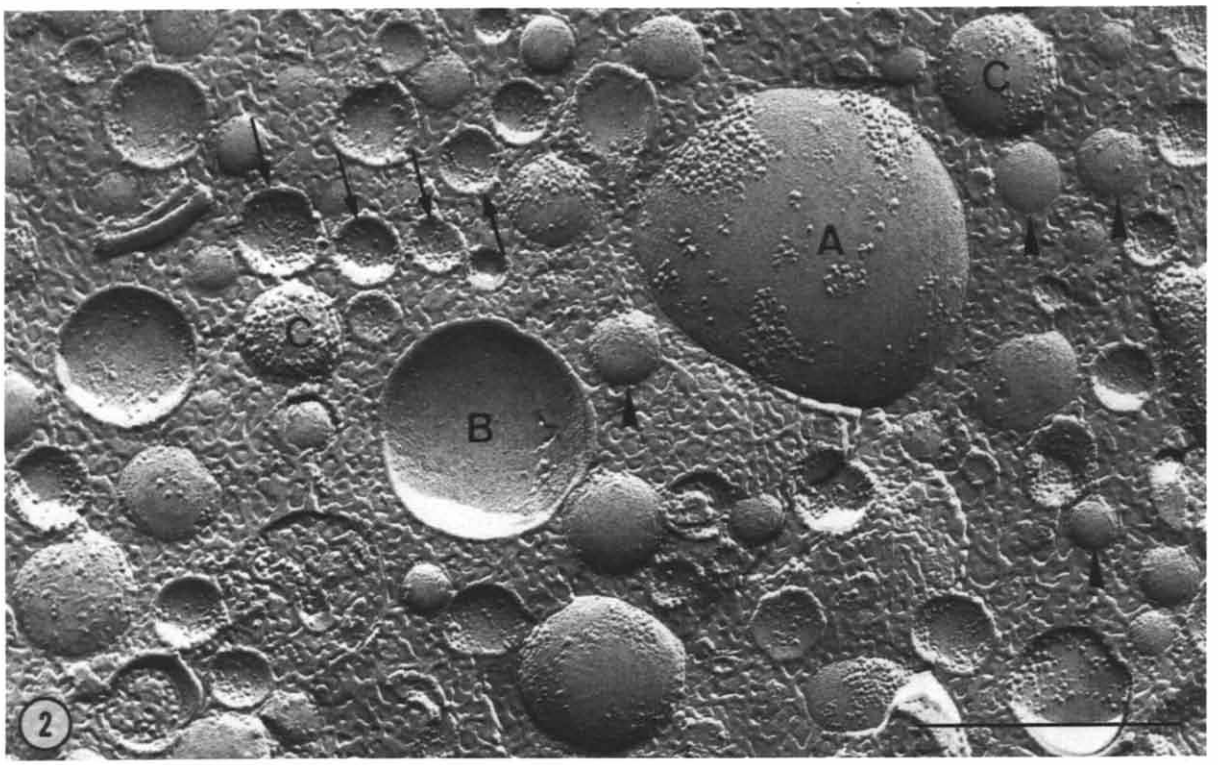
Ultrastructural analysis of rat-liver plasma membrane vesicles

In addition to the biochemical evidence on the vesicular membrane orientation, we performed an ultrastructural analysis of our preparations using freeze-fracture electron microscopy.

The determination of the vesicle orientation was based on the distribution of intramembrane particles on the exposed membrane leaflets. In a membrane vesicle preparation, fracture faces appear either as convex or concave profiles (Fig. 2). In the case of the plasma membrane vesicles, convex faces correspond to the membrane P-face (or cytoplasmic leaflet) whereas concave images show the membrane E-face (or extracellular leaflet; for terminology, see Ref. 26). Since, in situ, the plasma membrane P-face of liver parenchymal cells is highly particulate compared to the E-face, all vesicles with a large number of particles on their convex (P-)face were considered as well-orientated. In the same way, vesicles having a sparse population of particles on their concave (E-)face were also taken to be well-orientated. Conversely, vesicles having a highly-particulate concave face and a poorly-particulate convex face were scored as inside-out vesicles.

In freeze-fracture replicas of rat-liver plasma membrane vesicles, 89% of the vesicles are inside-out according to the criteria described above (Table II). However, the mean diameter of these vesicles is only 0.14 μm, compared to 0.28 μm for the 12% of the vesicles which are well-orientated. When the total surface area of the two types of vesicles is calculated, this size difference means that the population of well-orientated large vesicles makes up about 32% of the membrane in the sample (Table II). It should be noted that, possibly as a result of the isolation procedure, the intramembrane particles were not distributed at random within the plane of the membrane of many large vesicles. Instead, large aggregates of particles formed islands in the smooth lipidic domain of the membrane (Fig. 2). Such vesicles were still included in the quantification.

Two points should be borne in mind, however;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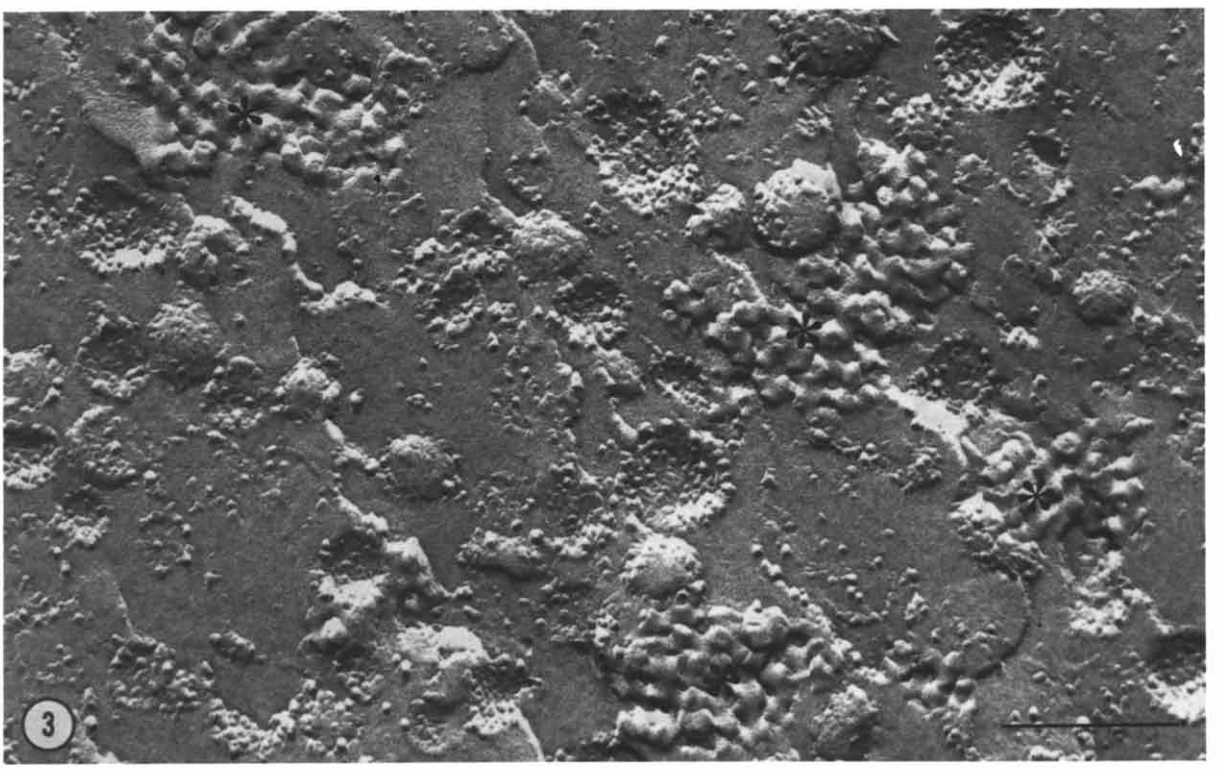
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firstly, the vesicles were not subdivided into size categories so that some large vesicles which were inside-out were included in the general population of smaller inside-out vesicles despite the fact that they may have been derived from the plasma membrane. Thus the 32% figure for total well-orientated vesicle surface does not reflect the total percentage of plasma membrane in the sample. In fact, Fig. 1 and the data on ouabain binding show that up to 30% of the plasma membrane may be orientated inside-out. Thus this amount of membrane would have been included in our calculations along with the inside-out population. If the 32% figure is corrected (i.e. increased by 30%) for this potential loss, it approaches the biochemical estimate that the vesicle population contains 40–50% plasma membrane (Table I).

The second point is that the vesicle population was compared to the plasma membrane as a reference for vesicle orientation. However, the orientation of intracellular vesicles and endoplasmic reticulum cisternae in situ is the reverse of the plasma membrane since their concave P-faces are adjacent to the cytoplasm. Thus, if the smaller so-called inside-out vesicles are, in fact, derived from endoplasmic reticulum, then they are really well-orientated vesicles when their cellular origin is considered. For the purposes of simplicity, however, they were referred to as inside-out in this study to distinguish them from plasma membrane vesicles.

In summary, the freeze-fracture analysis shows that over 30% of the membrane in the samples is in the form of well-orientated predominantly large vesicles. The inside-out population consisted mostly of smaller vesicles, probably microsomes, and a few larger vesicles which may represent inside-out plasma membrane, as also detected by biochemical methods.

Filipin labeling

Since it is known that endoplasmic reticulum membranes are poor in cholesterol compared to plasma membranes [27] we exposed isolated vesicles to filipin, an antibiotic which selectively binds to cholesterol and other 3 β-OH sterols [28]. This binding results in the formation of visible filipin/sterol complexes in freeze-fractured membranes [29]. These complexes appear either as stud-like protuberances or as pits in the membrane, about 25 nm in diameter and they can therefore be easily distinguished from the much smaller, protein-containing [30,31] intramembrane particles.

As shown in Fig. 3, the membrane preparation consisted of a mixture of vesicles both labeled and unlabeled with characteristic filipin/sterol complexes. In general, the labeled vesicles were much larger than the unlabeled vesicles, although some large structures were also unlabeled. This observation supports the contention that most of the larger, correctly-orientated vesicles are derived from the (cholesterol-rich) plasma membrane. A quantification of these structures was not performed for two main reasons. Firstly, following filipin treatment the labeled vesicles tended to collapse (even after fixation) and they usually appear as flattened sheets of membrane. Since such membranes no longer resemble spheres, determination of their surface area would be quite inaccurate. Secondly, intramembrane particles which allow the determination of vesicle orientation were usually sparse on labeled membranes for reasons which are, at present, unknown.

Summarizing, the ultrastructural analysis of rat-liver plasma membrane vesicles reveals a distinct heterogeneity in size of the vesicles. Filipin labeling experiments indicate that the large vesicles are most probably derived from the plasma mem-

Fig. 2. Freeze-fracture of a glutaraldehyde-fixed membrane preparation as used for the quantitative analysis. Most of the more numerous small vesicles have smooth convex faces (arrowheads) and particulate concave faces (arrows). Such vesicles were scored as inside-out, but probably represent well-orientated microsomes. On the other hand, large vesicles with particulate convex (A) and smooth concave (B) membrane faces were scored as well-orientated, i.e. inside-in, and probably represent plasma membrane vesicles. In addition, some smaller vesicular profiles were also well-orientated (C) and some large profiles were inside-out. See text and Table II for details of the quantification. Magnification: ×70000 (bar = 0.5 μm).

Fig. 3. Freeze-fracture of a membrane vesicle preparation fixed in the presence of the polyene antibiotic filipin. Filipin-sterol complexes can be recognised as large (25 nm diameter) pits and protuberances. While large membrane sheets are deformed by filipin-sterol complexes, the smaller vesicles appear devoid of such complexes. Magnification ×90000 (bar = 0.25 μm).

brane and that these large vesicles are mostly orientated inside-in.

From the combined biochemical and morphological data we conclude that the rat-liver plasma membrane vesicles isolated for transport studies are predominantly orientated inside-in. The question arises whether inside-out plasma membrane vesicles contribute to the amino acid transport processes as studied previously [4–7]. Amino acid uptake in vesicles is catalyzed via an artificial Na^+ gradient so the possibility exists that inside-out vesicles can also accumulate amino acids. The condition for this would require a symmetric carrier, that performs amino acid translocation down the Na^+ gradient.

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References

1 Murer, H. and Kinne, R. (1980) J. Membrane Biol. 55, 81–95
2 Sachs, G., Jackson, R.J. and Rabon, E.C. (1980) Am. J. Physiol. 238, G 151–G164
3 Lever, J.E. (1980) CRC Crit. Rev. Biochem. 7, 187–246
4 Van Amelsvoort, J.M.M., Sips, H.J. and Van Dam, K. (1978) Biochem. J. 174, 1083–1086
5 Sips, H.J., Van Amelsvoort, J.M.M. and Van Dam, K. (1980) Eur. J. Biochem. 105, 217–224
6 Sips, H.J. and Van Dam, K. (1981) J. Membrane Biol. 62, 231–237
7 Sips, H.J., De Graaf, P.A. and Van Dam, K. (1982) Eur. J. Biochem. 122, 259–264
8 Konings, W.N., Bisschop, A., Veenhuis, M. and Vermeulen, G.A. (1973) J. Bacteriol. 116, 1456–1465
9 Altendorf, K.H. and Staehelin, L.A. (1974) J. Bacteriol. 117, 888–899
10 Steck, T.L., Weinstein, R.S., Strauss, J.H. and Wallach, D.F.H. (1970) Science 168, 255–257
11 Haase, W., Schäfer, A., Murer, H. and Kinne, R. (1978) Biochem. J. 172, 57–62
12 Swanson, M.A. (1955) Methods Enzymol. 2, 541–543
13 Matsui, H. and Schwartz, A. (1968) Biochim. Biophys. Acta 151, 655–663
14 Schuurmans Stekhoven, F.M.A.H., De Pont, J.J.H.H.M. and Bonting, S.L. (1976) Biochim. Biophys. Acta 419, 137–149
15 Rogers, T.B., Lapalus, P. and Lazdunski, M. (1979) in: ($Na^+ + K^+$)-ATPase: Structure and Function (Skou, J.C. and Nørby, J.G., eds.), pp. 431–441, Academic Press, New York
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
17 Brown, D. (1981) J. Microscopy (Lond.) 121, 283–287
18 Moor, H., Mühlethaler, K., Waldner, H. and Frey-Wyssling, A. (1961) J. Biophys. Biochem. Cytol. 10, 1–13
19 Blitzer, B.L. and Boyer, J.L. (1978) J. Clin. Invest. 62, 1104–1108
20 Poupon, R. and Evans, W.H. (1979) FEBS Lett. 108, 374–378
21 Latham, P.S. and Kashgarian, M. (1979) Gastroenterology 76, 988–996
22 Blouin, A., Bolender, R.P. and Weibel, E.K. (1977) J. Cell Biol. 72, 441–455
23 Van Amelsvoort, J.M.M., Sips, H.J., Apitule, M.E.A. and Van Dam, K. (1980) Biochim. Biophys. Acta 600, 950–960
24 Walter, H. (1975) Eur. J. Biochem. 58, 595–601
25 Perrone, T.R. and Blostein, R. (1973) Biochim. Biophys. Acta 291, 680–689
26 Branton, D., Bullivant, S., Gilula, N.B., Karnovsky, M.J., Moor, H., Mühlethaler, K., Northcote, D.H., Packer, L., Satir, B., Satir, P., Speth, V., Staehelin, L.A., Steere, R.L. and Weinstein, R.S. (1975) Science 190, 54–56
27 Jain, M.K. (1975) Curr. Topics Membrane Transp. 6, 1–57
28 Norman, A.V., Demel, R.A., De Kruijff, B. and Van Deenen, L.L.M. (1972) J. Biol. Chem. 247, 1918–1929
29 Elias, P.M., Friend, D.S. and Goerke, J. (1979) J. Histochem. Cytochem. 27, 1247–1260
30 Vail, W.J., Papahadjopoulos, D. and Moscarello, M.A. (1974) Biochim. Biophys. Acta 345, 465–467
31 Yu, J. and Branton, D. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3891–3895