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MEMBRANE LIPIDS OF RAT LIVER LYSOSOMES PREPARED BY FREE-FLOW ELECTROPHORESIS

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SUMMARY

Rat liver lysosomes were isolated by free-flow electrophoresis and were examined morphologically and enzymatically for purity. Their membrane fraction was prepared by osmotic shock and analyzed for cholesterol, phospholipids and fatty acids. The results were compared with the membrane fraction of Triton WR 1339-filled lysosomes and with mitochondria. The cholesterol content (0.269 M cholesterol per M lipid phosphorus), the sphingomyelin concentration (7.9% of total lipid phosphorus) and the degree of unsaturation of fatty acids (38–45%) were found to be intermediate between those of membranes of Triton WR 1339-filled lysosomes ("plasma membrane-like") and mitochondria ("endoplasmic reticulum-like"). The similarity of these results with corresponding data for the Golgi apparatus support the present view concerning the formation of primary lysosomes via the Golgi apparatus. The drastic changes in the lipid composition found after overloading with Triton WR 1339 confirm that the plasma membrane participates in the formation of the secondary lysosomal membrane. The data presented here underline the significance of the analysis of membrane lipids in evaluating correlations between morphologically different but functionally closely related membrane types.

INTRODUCTION

Knowledge of the chemical composition of the lysosomal membrane is based mainly on analyses of membrane fractions prepared from lysosomes which were isolated after overloading with the nonionic detergent Triton WR 1339 [1–5]. This procedure, which changes the density of rat liver lysosomes drastically, has usually been applied because the common methods such as differential centrifugation and density equilibrium centrifugation failed to isolate lysosomal fractions pure enough for a detailed membrane component analysis.

Triton WR 1339-filled lysosomes (tritosomes) have to be regarded as special types of lysosomes since they arise as a result of excessive heterophagy and autophagy

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which apparently cause extreme changes in the chemical composition of the resulting membrane [6–10]. The influence of the plasma membrane by means of the endocytosis process could be demonstrated mainly in the lipid sector [2]. On the other hand, the participation of autophagy could be shown on electron micrographs [8] and through the detection of high concentrations of ubiquinone [9] and of cytochromes [8, 10] in the tritosomal membrane fraction.

The development of free-flow electrophoresis as a new principle for the isolation of subcellular organelles [11, 12] and its successful application to the isolation of a highly purified lysosome fraction [13] offers an excellent opportunity to answer unclarified questions concerning the composition of native rat liver lysosomal membranes. In the first approach, we analyzed the cholesterol content and the distribution of the phospholipids with their fatty acid composition. Lipids have been found to be one class of the general bio-membrane constituents which show distinctive features suitable for a membrane classification parallel to morphological methods [14]. Furthermore, these lipid compounds have been selected because they cover approximately 80% of the total lipids in the tritosomal membrane [15] and even more in other types of subcellular membranes.

MATERIALS AND METHODS

Preparation of subcellular fractions

Lysosomes were prepared as described by Stahn et al. [13] with the following modifications. Both the isolation and the electrophoresis media contained 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA and 0.306 M sucrose, pH 7.4 (2 M NaOH). The heavy mitochondrial fraction was obtained at $4100 \times g$ for 10 min, the light mitochondrial fraction containing the bulk of the lysosomes at $19\,600 \times g$ for 20 min. The latter fraction was washed at least six times at $19\,600 \times g$ for 10 min. Prior to electrophoresis, the sample was spun at $1100 \times g$ for 10 min and the supernatant was used for the electrophoresis run. The conditions in the electrophoresis apparatus were: 110 mA, $110 \pm 10\%$ V/cm, buffer flow 1.9 ml per fraction per h. Pure lysosome-containing fractions were pooled according to the acid phosphatase and cytochrome *c* oxidase distribution as indicated in Fig. 1 and spun down at $19\,600 \times g$ for 10 min.

The pellet was resuspended in 20 ml bidistilled water and after 10 min in ice was centrifuged at $55\,000 \times g$ for 15 min.

Triton WR 1339-filled lysosomes were prepared by slight modifications [2] of the procedure originally introduced by Wattiaux et al. [6].

Highly purified mitochondria were prepared by free-flow electrophoresis in order to remove contaminations of endoplasmatic reticulum according to Heidrich et al. [16].

Electron microscopy

Fractions were centrifuged at $37\,000 \times g$ for 10 min at 4 °C. The pellets were fixed with glutaraldehyde (2% in isolation medium pH 7.4) and OsO_4, dehydrated, and embedded in Epon 812. Thin sections were stained with uranylacetate and lead citrate and examined with a JEM 100B electron microscope (80 kV).

Protein and enzyme determinations

Protein determination was carried out in an automated Technicon AutoAnalyzer as has been described in ref. 17. Acid phosphatase (EC 3.1.3.1) activity was determined according to Bergmeyer [18] using *p*-nitrophenylphosphate as substrate. Cytochrome *c* oxidase (EC 1.9.3.1) was measured as described in ref. 19, succinate dehydrogenase (EC 1.3.99.1) activity as in ref. 20, glucose-6-phosphatase (EC 3.1.3.9) as in ref. 16, and NADPH–cytochrome *c* reductase (EC 1.6.2.3) as in ref. 21.

Lipid analysis

Total lipids were extracted from lyophylized membrane fractions by a slightly modified [3] Folch procedure [22] with two chloroform–methanol (2 : 1, v/v) extractions (10 and 6 h at 22 °C). The residue was reextracted with chloroform– methanol (1 : 2, v/v) for 15 min under reflux conditions. The combined extracts were filtered and evaporated under a stream of nitrogen. The lipids were redissolved in chloroform–methanol (2 : 1, v/v) and washed with 0.2 vol. of 0.1 M KCl and twice with Folch's theoretical upper phase (without KCl). The lower phase was evaporated under nitrogen, redissolved in chloroform and stored at −20 °C.

The cholesterol content was determined after thin-layer chromatographic separation of the neutral lipids on silica gel H plates (solvent system: petroleum ether–diethyl ether–acetic acid (70 : 30 : 1, v/v/v). Cholesterol and esterified cholesterol-containing spots were visualized by a short exposure to iodine vapour, scraped off and eluted with ether. The combined extracts were evaporated and total cholesterol was determined according to Zlatkis et al. [23]. The recovery of total cholesterol in test runs using standard mixtures of synthetic cholesterol samples was found to be in the range of 95–98%.

Phospholipids were separated either by one-dimensional thin-layer chromatography according to Skipski et al. [24] or, for quantitative analyses, by two-dimensional thin-layer chromatography as described by Debuch et al. [25] (Silica gel N-HR, Machery und Nagel, Düren, Germany; solvent systems: first direction, chloroform–methanol–water (65 : 25 : 4, by vol.); second direction, chloroform–methanol–acetic acid (60 : 25 : 8, by vol.)). For better reproducibility, the separations were run at constant temperature (10 °C). Phosphorus determinations were performed in triplicate after two-dimensional thin-layer chromatographic separations according to a modified Bartlett procedure [26, 27].

The fatty acids of isolated phospholipids were analyzed by gas–liquid chromatography after direct transesterification to the fatty acid methyl esters with 5% HCl in methanol (w/v) according to Stoffel et al. [28]. The analyses were carried out on a Perkin Elmer F 20 gas chromatograph equipped with a flame ionization detector using a 2.5% diethylene glycol succinate polyester on chromosorb W/AW - DMCS, 80–100 mesh, column (2 m, Serva, Heidelberg) operated isothermally at 185 °C. The relative fatty acid concentrations were determined by the triangulation method. The unsaturated fatty acids were analyzed before and after catalytic hydrogenation (PtO_2).

RESULTS AND DISCUSSION

Lysosomal membranes

Modifications of the isolation procedure described in ref. 13 resulted in a good

yield of a highly purified preparation of rat liver lysosomes, which is a precondition for the study presented here. The use of EDTA in the isolation as well as the electrophoresis buffer had as a consequence that all the mitochondria in the preparation were present in the condensed form. Mitochondria in this state run as a very sharp band in the electrophoresis system used and do not have the tendency to smear to the anode (and therefore into the lysosomal fraction) as do mitochondria in the orthodox state (Heidrich, H. G. and Näthke, H., unpublished). Thus, the lysosomes could be observed in the electrophoresis apparatus as a separate band closer to the anode. The occurrence of inner mitochondrial membranes, which also can contaminate the lysosomal fraction due to its similar electrophoretic mobility, was avoided by very careful homogenization during the preparation procedure. Microsomes were completely removed by using at least six washing steps prior to electrophoresis.

The isolated lysosomes were examined for contaminations with mitochondrial membranes or with membranes of the endoplasmatic reticulum (Fig. 1). For reasons discussed above, the mitochondrial markers such as cytochrome *c* oxidase and succinate dehydrogenase appeared as a sharp peak in the electrophoresis fraction closer than the lysosomes to the cathode. Very little inner mitochondrial membrane material could be found in the fractions closer to the anode. The endoplasmatic reticulum marker glucose-6-phosphatase (in some control experiments NADPH cytochrome *c* reductase) could not be found at all. Starting with 20–25 g rat liver the procedure presented here results in 15 mg lysosomal protein.

The purity of this lysosomal fraction was also tested by difference spectroscopy [8]. Mitochondrial cytochromes such as cytochrome *c*, b_5 and aa_3 could not be detected. This is strong evidence for the absence of, or presence of only very little, contamination with mitochondria. The occurrence of other cytochromes in the lysosomal fraction in comparison with tritosomes is discussed elsewhere [8].

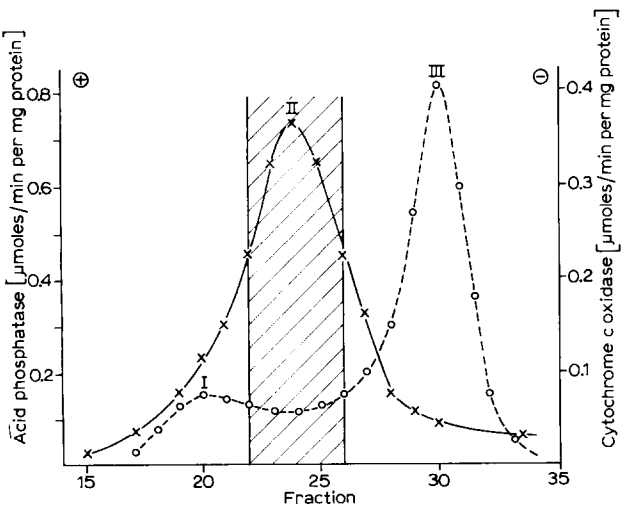

Fig. 1. Distribution of specific activities (μmoles/mg protein per min) of acid phosphatase (×–×) and cytochrome *c* oxidase (○- - -○) after free-flow electrophoresis. Data represent mean values calculated from 5 typical runs. Peak I consists of inner mitochondrial membranes [16], Peak II of lysosomes, and Peak III of mitochondria. The fractions were pooled as indicated for the preparation of the lysosomal membranes used for lipid analyses. Injection port was above fraction 65.

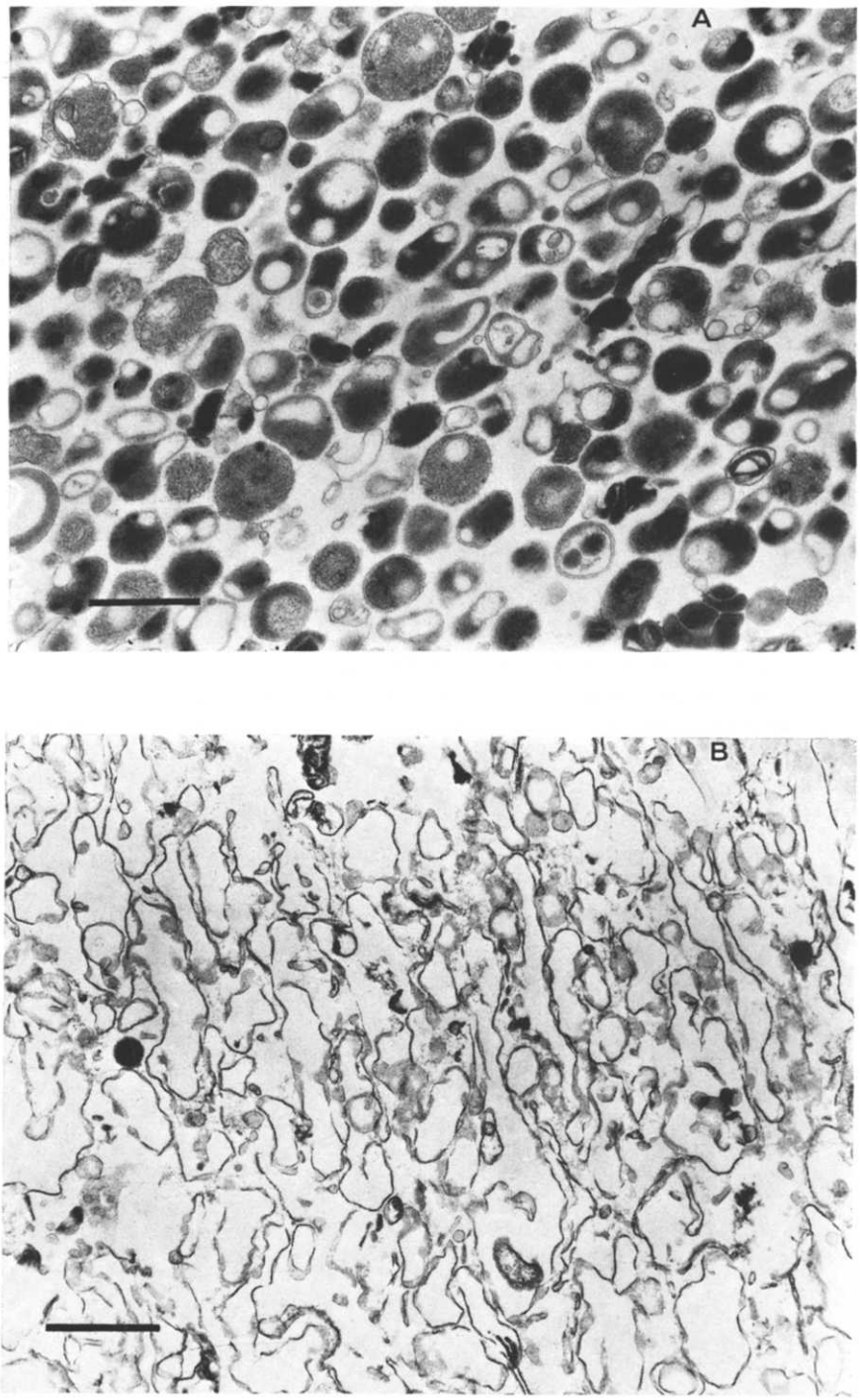

Fig. 2. Electron micrographs of rat liver lysosomes (A) used for preparing lysosomal membrane: and lysosomal membranes (B). Magnification: 15 000 ×; bars are 1 μm.

Electron microscopy (Fig. 2A) demonstrates that not more than 5% mitochondrial contaminations are present. No endoplasmatic reticulum vesicles and no peroxisomes can be detected. Peroxisomal catalase runs with mitochondria or even slightly slower while free catalase migrating very slowly together with other free enzyme activities is found near to the cathode [13]. The modifications of the original preparation procedure [13] introduced here have no influence on the migration behaviour of peroxisomes (or other subcellular material) but warrant a homogeneous status of mitochondria as is described above. The lysosomes are morphologically in perfect shape. Fig. 2B shows the lysosomal membrane fraction obtained upon osmotic shock. The membranes have no characteristic morphological appearance; dense material which is seen in intact lysosomes, occasionally appears to adhere to the membranes. 15 mg of lysosomal protein yields approximately 4–5 mg protein of the lysosomal membrane fraction which was then used for the analysis of the lipids.

Lipids

The quantity and purity of the lysosomal membrane preparation presented here allows an analysis of the lipid composition and its comparison with membranes of tritosomes and with mitochondria. Differences between various subcellular membranes are well established by morphological observations [29] which have been found to correspond to distinctive differences in the chemical composition [14]. High concentrations of sphingomyelin and cholesterol as well as a low content in unsaturated fatty acids have been recognized as a characteristic feature of the plasma membrane. These observations led to a differentiation between "plasma membrane like" (high sphingomyelin concentration) and "endoplasmic reticulum like" (low sphingomyelin concentration) membranes [14]. Consequently, the lipid composition of tritosomal membranes found to be "plasma membrane like" was thought to be strongly influenced by the contribution of the plasma membrane during the pinocytotic uptake of Triton WR 1339 [2].

Tables I and II show the cholesterol content, total lipid phosphorus, the phospholipid distribution and the fatty acid composition of a normal rat liver lysosomal membrane fraction, of membranes of Triton WR 1339-filled lysosomes and of mitochondria. The sphingomyelin, phosphatidylcholine and cholesterol contents as well as the concentrations in polyunsaturated fatty acids are found to be in an intermediate position between tritosomal membrane ("plasma membrane like") and mitochondria ("endoplasmic reticulum like"). The comparatively high content in lipid phosphorus in the solvent front might be due to the presence of bis-(monoacylglyceryl)phosphate in both types of lysosomal membranes, since this component has been described to be enriched in membrane fractions of tritosomes [30]. Running even higher than cardiolipin, this component is expected to run together with the solvent front in the solvent systems used. In the fatty acids of sphingomyelin in lysosomal membranes no polyunsaturated fatty acids could be found. The pattern is similar to that of other sphingolipids in tritosomal membranes [3]. According to their electron microscopic appearance, the lysosome population of rat liver isolated by free-flow electrophoresis consists mainly of peribiliary dense bodies derived from hepatocytes. They have to be regarded as secondary lysosomes, i.e. those being already engaged in hetero- and/or autophagy processes as shown by the uptake of colloidal

TABLE I

CHOLESTEROL CONTENT AND PHOSPHOLIPID COMPOSITION OF MEMBRANE FRACTIONS OF NORMAL RAT LIVER LYSOSOMES, OF TRITON WR 1339 FILLED LYSOSOMES AND OF MITOCHONDRIA

Cholesterol concentrations are given in molar cholesterol/phospholipid phosphorus ratios. Data of phospholipid distribution are presented in percentage of total lipid phosphorus. Phosphatidylglycerol, lysophosphatidyl inositol and -serine could not be detected.

	Tritosomal membranes ($n = 3$)	Lysosomal membranes ($n = 5$)	Mitochondria
Cholesterol	0.520	0.269±0.031	0.055±0.006
Total lipid P (μM/mg protein)	0.256	0.438±0.059	0.091±0.041
Lysophosphatidylcholine	1.2	0.9±0.23	N.D.
Sphingomyelin	28.4	7.6±1.3	< 0.5
Phosphatidylcholine	36.2	41.4±7.9	48.5±8.2
Lysophosphatidylethanolamine	1.7	2.9±0.6	< 0.5
Phosphatidylinositol	4.5	9.7±2.3	8.2±1.9
Phosphatidylserine	3.1	3.0±0.8	3.1±0.7
Phosphatidylethanolamine	19.7	26.3±6.9	27.9±7.2
Cardiolipin	1.9	4.0±1.0	11.8±3.8
Solvent front	3.3	4.2±2.0	< 0.5

gold particles [8,43] or horse radish peroxidase after intravenous administration of these materials [8]. All evidence available suggests that the enormous enlargement of peribiliary dense bodies during the uptake of Triton WR 1339 is due to a pinocytosis process, as has also been shown by electron optical studies [7, 8]. Therefore, the changes seen in the lipid composition between the normal lysosomal membrane and the tritosomal membrane (Tables I and II) are in agreement with the electron microscopic observations supporting strongly the pinocytotic uptake of a polymer fraction of Triton WR 1339 [7].

Besides the influence of the plasma membrane the participation of the Golgi apparatus in the biogenesis of lysosomes should be emphasized. According to present knowledge [31, 32] several lines of evidence suggest that the lysosomal enzymes are synthesized in the rough endoplasmic reticulum or even in a special endoplasmic reticulum fraction [33] and are then transported via the cisternae of the smooth endoplasmic reticulum to the Golgi apparatus [33, 34]. The latter has been regarded as the site of endomembrane differentiation. The Golgi apparatus is concerned either with completing (by glycosylation reactions, for references see ref. 35) and packing materials to be secreted by exocytosis (zymogen [36, 37], lipoproteins [38, 39], albumin [40, 41]) or with packing of lysosomal enzymes into small Golgi vesicles. Since, at this time the latter have to be considered as primary lysosomes this mechanism consequently implies that the membrane of primary lysosomes is at least partially derived from the Golgi membrane system.

According to the pinocytosis rate of the hepatocyte, which under normal circumstances is very low [42], the lipid composition of membranes of the peribiliary dense body should be expected to resemble that of the Golgi membranes rather than that of the plasma membrane. In fact, a comparison of lipid analyses from rat liver

TABLE II

FATTY ACID COMPOSITION OF PHOSPHOLIPIDS IN MEMBRANE FRACTIONS OF NORMAL RAT LIVER LYSOSOMES, TRITON WR 1339-FILLED LYSOSOMES AND IN MITOCHONDRIA

Data given in percent of total.

	Lysosomal membranes					Tritosomal membranes*			Mitochondria			
Chain length/ number of double bonds	PE[a]	PI[b]	PS[c]	PC[d]	SPHM[e]	PE[a]	PI[b]/PS[c]	PC[d]	PE[a]	PI[b]	PS[c]	PC[d]
12:0	1.0	0.6	0.7	<0.5	1.1	–	–	–	<0.5	<0.5	<0.5	<0.5
14:0	0.8	0.8	3.9	0.9	3.2	<0.5	<0.5	–	<0.5	<0.5	<0.5	<0.5
16:0	21.5	8.6	12.2	17.7	21.0	22.1	14.8	38.3	17.2	8.5	14.1	18.1
16:1	0.9	3.0	5.3	1.5	8.5	3.3	3.1	3.8	0.9	2.4	1.2	0.7
18:0	26.2	39.0	35.5	28.3	8.3	21.1	44.8	20.0	28.2	42.5	23.2	25.0
18:1	3.1	1.1	2.3	5.3	5.3	16.1	15.4	10.5	3.0	3.5	6.5	5.5
18:2	6.1	2.5	5.6	11.5	–	13.5	4.4	10.5	6.5	3.1	14.0	15.8
18:3	–	2.6	3.3	–	–	–	–	–	<0.5	<0.5	<0.5	<0.5
20:0	–	0.5	1.9	–	4.0	–	–	–	–	–	–	–
20:4	23.4	26.7	16.2	21.6	<0.5	13.5	12.9	10.9	28.3	30.5	17.6	24.5
20:5	<0.5	–	–	–	–	3.6	4.6	1.9	1.9	1.5	–	–
21:0	–	–	–	–	2.0	–	–	–	–	–	–	–
22:0	–	2.4	–	–	6.9	–	–	–	–	–	–	–
22:5	2.4	2.5	3.0	2.9	–	2.1	<0.5	–	1.9	1.8	7.2	2.9
22:6	14.4	9.9	9.9	9.4	–	3.6	<0.5	2.2	11.7	3.9	13.8	6.9
23:0	–	–	–	–	4.6	–	–	–	–	–	–	–
24:0	–	–	–	–	19.0	–	–	–	–	–	–	–
24:1	–	–	–	–	15.6	–	–	–	–	–	–	–
Sum of poly-unsaturated	46.8	44.2	38.0	45.4	–	36.9	21.9	25.5	50.8	41.3	53.1	50.6

★ Data from earlier work [2].

[a]Phosphatidylethanolamine; [b]Phosphatidylinositol; [c]Phosphatidylserine; [d]Phosphatidylcholine; [e]Sphingomyelin.

Golgi apparatus with that of the plasma membranes and the endoplasmic reticulum by Keenan and Morré [14] revealed a sphingomyelin content of 12% of total lipid phosphorus, a molar cholesterol/phospholipid ratio of 0.37 (calculated from Keenan and Morré's data) and a degree of fatty acid unsaturation (33–55%) being as well intermediate between the plasma membrane like and the endoplasmic reticulum like membrane type as the data which we obtained from normal rat liver lysosomal membranes.

Although this comparison with the data presented in this report should be considered with careful reserve since the results have been taken from different laboratories the conclusions thus reached fit well into the present view of the formation of primary lysosomes. According to this view, the analysis of the lipid composition of subcellular membranes shows promise of becoming a useful tool in the demonstration of correlations between morphologically and topographically different but functionally related membranes such as those belonging to the "vacuolar apparatus".

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