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Phospholipid composition of highly purified mitochondrial outer membranes of rat liver and *Neurospora crassa*. Is cardiolipin present in the mitochondrial outer membrane?

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Abstract

Isolated mitochondrial outer membrane vesicles (OMV) are a suitable system for studying various functions of the mitochondrial outer membrane. For studies on mitochondrial lipid import as well as for studies on the role of lipids in processes occurring in the outer membrane, knowledge of the phospholipid composition of the outer membrane is indispensable. Recently, a mild subfractionation procedure was described for the isolation of highly purified OMV from mitochondria of *Neurospora crassa* (Mayer, A., Lill, R. and Neupert, W. (1993) J. Cell Biol. 121, 1233–1243). This procedure, which consists of swelling and mechanical disruption of mitochondria followed by two steps of sucrose density gradient centrifugation, was adapted for the isolation of OMV from rat liver mitochondria. Using the appropriate enzyme markers it is shown that the resulting OMV are obtained in a yield of 25%, and that their purity is superior to that of previous OMV preparations. Analysis of the phospholipid composition of the OMV showed that phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol are the major phospholipid constituents, and that cardiolipin is only present in trace amounts. The phospholipid composition is very similar to that of the highly purified OMV from mitochondria of *Neurospora crassa*, although the latter still contain a small amount of cardiolipin.

Keywords: Mitochondrial outer membrane; Phospholipid composition; *Neurospora crassa*; Subfractionation; Cardiolipin; (Rat liver)

1. Introduction

The outer membrane of mitochondria harbours proteins with a diversity of functions: enzymes involved in the metabolism of amino acids and fatty acids, receptor complexes for protein import [1], pore forming proteins [2], and proteins controlling mitochondrial morphology [3]. Furthermore it is expected to contain systems for signal transduction, as well as a still unknown machinery for the import and export

Abbreviations: CL, cardiolipin; MAO, monoamine oxidase; OMV, outer membrane vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; (R)SA, (relative) specific activity; SD, swell–disruption; SSS, swell–shrink–sonicate

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of lipids. Mitochondrial outer membrane vesicles (OMV) with a right-side-out orientation can be easily isolated and have proven a suitable system for studying various aspects of outer membrane function [4–7]. The methods commonly used to prepare OMV include swelling of mitochondria [8], which is often followed by shrinking [4,9,10] and subsequent sonication [11–14]. The fraction enriched in outer membrane is isolated by sucrose density gradient centrifugation.

Knowledge of the lipid composition of the outer mitochondrial membrane is required if one wishes to study the lipid-dependence of the processes occurring in the outer membrane, e.g. by reconstituting outer membrane proteins in proteoliposomes or planar membranes, or by using model membrane systems mimicking the outer membrane [2,15–18]. The phospholipid composition and topology of the mitochondrial membranes are important parameters for research on transport of phospholipids in mitochondria. Furthermore, studies on mitochondrial lipid import require reliable methods of mitochondrial subfractionation which allow for an unambiguous localization of the imported lipid in the inner or outer membranes [19,20]. Moreover, for studying lipid transport into isolated outer membranes, the availability of pure OMV is essential to exclude interference by other membrane contaminants [7].

In the eukaryotic cell, cardiolipin (CL) is only present in mitochondria where it appears to be essential for respiration and oxidative phosphorylation [21], and where it may play a role in the import of precursor proteins [22,23]. The intramitochondrial localization of CL is controversial. Several authors suggest that CL found in the outer membrane is due to contamination with inner membranes which have a high content of CL (reviewed in Ref. [24]). Others consider it a true component of the outer membrane [14,25,26].

Recently, an improved procedure was reported for the isolation of mitochondrial OMV from the fungus *Neurospora crassa* [5,27]. In a swell–disruption (SD) procedure, the mitochondria were swollen and then mechanically disrupted in a glass-teflon homogenizer. Highly purified OMV virtually free of inner membrane were obtained after a sedimentation and a flotation sucrose gradient. This led us to adapt the SD procedure for the isolation of OMV from rat liver mitochondria, in order to reinvestigate the OMV phospholipid composition. Based on the analysis of marker enzymes the OMV are shown to be of excellent purity. Here we report that outer membranes of rat liver mitochondria are essentially devoid of cardiolipin. Highly purified OMV from mitochondria of *Neurospora crassa* [5] still contain a small amount of CL.

2. Materials and methods

2.1. Isolation of mitochondrial OMV from rat liver

Mitochondria were isolated from the livers of male Wistar rats by differential centrifugation and isotonic Percoll gradient centrifugation as described [14]. The SD method for the preparation of OMV is based on the method used to obtain mitochondrial OMV from *Neurospora crassa* [5,27]. The mitochondria were swollen in hypotonic buffer, 5 mM KP_i (pH 7.2), 5 mM EDTA, at a protein concentration of 5 mg/ml for 20 min on ice while being stirred. Next, the mitochondrial suspension was subjected to 20 strokes in a Potter-Elvehjem homogenizer with a tightly fitting teflon pestle operated at 650 rpm. The resulting homogenate (15 ml per SW28 tube) was applied to a sedimentation sucrose step gradient consisting of 9 ml 0.25 M sucrose and 12 ml 1.1 M (37.7% w/v) sucrose in 10 mM MOPS (pH 7.2), 2.5 mM EDTA (EM buffer). After ultracentrifugation for 1 h at $141\,000 \times g_{max}$ (SW28 rotor, Beckman), a fraction enriched in outer membrane was collected from the 0.25–1.1 M sucrose interface. The sucrose concentration of this fraction was adjusted to 1.1 M, and it was loaded (4 ml per SW41 tube) on the bottom of the second (flotation) sucrose gradient. A layer of 7 ml 1.065 M (36.5% w/v) sucrose was put on top, and the tube was filled up with EM buffer. After 16 h of ultracentrifugation at $141\,000 \times g_{max}$ (SW41 rotor), the purified OMV were collected from the 0–1.065 M sucrose interface, diluted in EM buffer, and pelleted for 1 h at $300\,000 \times g_{max}$ (Ti60 rotor). The pellet was resuspended in 220 mM mannitol, 70 mM sucrose, 2 mM Hepes (pH 7.4) at a protein concentration of 1–3 mg/ml. Aliquots of the OMV were frozen in liquid nitrogen and stored at -80°C until analysis.

Alternatively, OMV were prepared by the SSS procedure. After swelling, shrinking, and sonication as described [14], the mitochondrial suspension was diluted to 5 mg protein/ml with 10 mM KP_i (pH 7.4), and subjected to sedimentation and flotation sucrose density centrifugation as above.

2.2. Isolation of mitochondrial OMV from Neurospora crassa

Mitochondria were isolated from *Neurospora crassa* strain 74A by differential centrifugation as described [28]. Mitochondrial OMV were prepared by the SD procedure as published previously [5,27].

2.3. Enzyme assays

Unless stated otherwise, all assays were performed at 37°C on samples that had been frozen and thawed only once.

Monoamine oxidase was determined as described [29]. Succinate dehydrogenase and adenylate kinase were assayed according to Refs. [30,31] with the modifications introduced by Hovius et al. [14]. NADPH-cytochrome *c* reductase activity was measured according to Ref. [32] with the following modifications. The assay buffer contained 0.01 mg/ml antimycin A and the reaction was started by adding 0.2 mM NADPH and 60 μM cytochrome *c* after a 10 min pre-incubation of the sample (2–50 μg protein) in assay buffer. Alkaline phosphatase was measured essentially as described [33], by incubating samples (50–200 μg protein) for 15 min in a buffer containing 50 mM glycine (pH 10.4), 5 mM $MgCl_2$, and 5 mM sodium-*p*-nitrophenylphosphate as substrate. Acid phosphatase was assayed at 25°C as published [34] except that the assay buffer was at pH 5.6. 5′-Nucleotidase was determined as described [35], and glucose-6-phosphatase according to Ref. [36] in a buffer containing 14 mM histidine (pH 6.5), 2 mM EDTA, and 12 mM sodium potassium tartrate. Inorganic phosphate produced in the two latter assays was quantitated according to Rouser et al. [37].

In the range of protein concentrations used, all enzyme activities were linear with the protein concentration. Protein concentrations were determined by the BCA assay (Pierce) with 0.1% (w/v) SDS added and bovine serum albumin as standard. Specific activities are presented as nmol of substrate converted per min per mg of protein. Relative specific activities were calculated relative to the nuclei-free homogenate, i.e., the $600 \times g$ supernatant of the total rat liver homogenate [14].

2.4. Determination of OMV size

The *Z*-average particle size and polydispersity index of the OMV were determined by dynamic light scattering on a Malvern 4700 system equipped with a 25 mW He-Ne laser (NEC, Tokyo, Japan) using the automeasure version 3.2 software (Malvern, Malvern, UK). For viscosity and refractive index the values of pure water were used. The polydispersity is a measure of the width of the particle size distribution and ranges from 0.0 (for an entirely homogeneous size distribution) to 1.0 (for a completely heterogeneous one). Measurements were carried out in EM buffer at an OMV concentration of $\approx$ 0.15 mg protein/ml.

2.5. Phospholipid determination

Phospholipids were extracted according to the method of Bligh and Dyer [38] from samples of mitochondria and OMV corresponding to 1.5 mg and 0.2 mg protein, respectively. Quantitation of the phosphorus content of the organic phase [39] yielded the phospholipid phosphorus/protein ratio. Phospholipid analysis of the extracts was performed by two-dimensional HPTLC on silica gel 60 plates (Merck, FRG) impregnated with 2.4% (w/v) boric acid (modified from Ref. [40]). The solvent systems used were chloroform/methanol/water/ammonia, 120:75:6:2 (v/v) for the first dimension, and chloroform/methanol/acetic acid, 65:25:10 (v/v) for the second dimension. Spots were visualized with I_2 vapour, and scraped off. Phospholipid phosphorus was quantitated by the method of Fiske and Subbarow [39] after destruction for 3 h at 180°C in 70% perchloric acid.

3. Results and discussion

3.1. Purity and yield of the OMV

Percoll gradient-purified mitochondria from rat liver were processed according to the SD method,

which was used previously to prepare highly purified OMV from mitochondria of *Neurospora crassa* [5]. The released OMV were purified from the mitochondrial homogenate in two consecutive steps by sedimentation and flotation centrifugation through sucrose step gradients. The purity and yield of OMV were optimal upon flotation onto a layer of 36.5% (w/v) sucrose. The OMV-enriched fractions obtained from the sedimentation and the flotation gradient were analyzed for protein content and for marker enzymes of the outer and the inner mitochondrial membrane, monoamine oxidase (MAO) and succinate dehydrogenase (SDH), respectively, as summarized in Table 1. Both the enrichment of MAO and the depletion of SDH exceed values reported in the literature for OMV from rat liver mitochondria which were prepared either by a swell–shrink method [4,10] or by a swell–shrink–sonicate procedure [14], and purified by sedimentation sucrose gradient centrifugation on 37.7% (w/v) sucrose. The purity of the OMV preparation clearly gains from the flotation gradient while the yield of total MAO activity is only slightly decreased (Table 1). The final OMV preparation contains around 25% of the MAO activity originally present in the mitochondria. Combining this number with the protein recovery of approximately 1%, it can be calculated that the outer membrane contains about 4% of total mitochondrial protein in agreement with Ref. [4]. The enrichment of outer membrane marker and depletion of inner membrane marker (Table 1) are comparable to the values achieved for OMV from *Neurospora crassa* [5].

OMV from mitochondria subjected to the SSS procedure [14] were also purified by sedimentation and flotation gradient centrifugation. In this case, the OMV-enriched fraction obtained from the sedimentation gradient contained more inner membrane, probably due to fragmentation of the inner membrane by the sonication step (Table 1). As a result, the final OMV preparation was slightly more contaminated with inner membrane. Moreover, a much lower yield of OMV was obtained compared to that of the SD procedure (Table 1). Adherence of the OMV to remnants of the inner membrane induced at some stage of the SSS treatment, may prevent the OMV from flotation on 36.5% (w/v) sucrose, and thus account for the low yield obtained from the second gradient.

Rat liver mitochondrial OMV were found to contain adenylate kinase (data not shown), which is generally believed to be a soluble enzyme of the intermembrane space in rat liver [41]. The OMV prepared by the SD method contain less adenylate kinase activity (RSA relative to mitochondria: 0.12 ± 0.04, $n = 5$) than OMV originating from mitochondria subjected to the SSS procedure (RSA: 0.25 [14]). Over 80% of the OMV-associated adenylate kinase

Table 1
Purification of OMV from rat liver mitochondria by the swell–disruption (SD) and the swell–shrink–sonicate (SSS) method

		Specific activity (nmol mg^{-1} min^{-1})		MAO enrichment	SDH depletion	MAO/SDH	Yield (mg protein)	Yield based on MAO
		MAO	SDH	(%)				
Mitochondria		7.7 ± 1.6	320 ± 80	1	1	1	100	100
OMV (SD)	1st gradient	120 ± 20	33 ± 20	16 ± 3	0.10 ± 0.06	160	2.5 ± 0.5	39 ± 6
	2nd gradient	200 ± 30	9 ± 6	26 ± 4	0.03 ± 0.02	870	0.9 ± 0.2	25 ± 5
OMV (SSS) [a]	1st gradient	86	78	10	0.23	40	4.2	37
	2nd gradient	208	29	23	0.08	290	0.45	8

OMV were prepared from rat liver mitochondria either by the SD or by the SSS method, and purified on two successive sucrose step gradients: sedimentation (1st gradient) and flotation (2nd gradient). Mitochondria and OMV fractions from the first and second gradient were assayed for the enzyme markers monoamine oxidase (MAO) and succinate dehydrogenase (SDH). The average values ($\pm$S.D., $n = 12$ for OMV (SD)) of the specific activities and of the enrichment/depletion factors relative to the corresponding mitochondrial fraction are shown. The enrichment of MAO over SDH (MAO/SDH) is the quotient of the enrichment and depletion of the two enzyme markers, respectively. The yield is expressed both as mg protein obtained per 100 mg mitochondrial protein, and as the recovery of MAO activity ($\pm$S.D., $n = 7$).

[a] Average values from two batches of OMV prepared by the SSS method are shown (the maximum deviation from the average values is 20%).

activity is resistant to digestion by trypsin (not shown), reflecting the sealed nature of the majority of the OMV [5,7,26].

3.2. Contamination with other organelles

In Table 2 the specific activities of MAO and several non-mitochondrial marker enzymes present in the OMV prepared by the SD method, are compared to those of the homogenate. The contaminating organelles present in the mitochondria tend to co-purify with the OMV. The flotation sucrose gradient considerably reduces the contamination with ER, plasma membrane, and lysosomes. Relative to the homogenate the lysosomal enzyme acid phosphatase is slightly enriched in the OMV preparation (Table 2). In studies employing purified lysosomes, an enrichment of acid phosphatase with respect to the homogenate up to a factor of 70 was obtained [42]. This would imply that the contamination of the OMV with lysosomes does not exceed 4% (protein-based). Since acid phosphatase is able to use both glucose-6-phosphate and AMP as substrate, it was essential to include 12 mM tartrate in the assay buffers for glucose-6-phosphatase and 5′-nucleotidase to inhibit the lysosomal enzyme [42].

The relative specific activities of the two ER markers tested, glucose-6-phosphatase and NADPH-cytochrome *c* reductase, behave similarly throughout the purification of OMV (Table 2). The ER contaminating the OMV apparently differs from the mitochondria-associated membrane (MAM), which exhibits a higher specific activity of glucose-6-phosphatase and a lower specific activity of NADPH-cytochrome *c* reductase as compared to microsomes [43]. Based on the specific activity of glucose-6-phosphatase in microsomes (isolated as the $160\,000 \times g_{max}$ pellet of the $27\,000 \times g_{max}$ postmitochondrial supernatant [14]), the microsomal contamination of the OMV is calculated to be less than 9.4% based on protein.

Based on the relative specific activity of 5′-nucleotidase in the OMV preparation, and the enrichment of this plasma membrane marker obtained for purified rat liver plasma membranes [44], the contamination of the OMV with plasma membrane is less than 3.3% (protein-based). However, this number is most likely an overestimation as part of the 5′-nucleotidase of rat liver is present in lysosomes [45,46]. Accordingly, a much lower contamination of the OMV with plasma membrane was found using alkaline phosphatase as marker (Table 2). The contamination of OMV with peroxisomes determined from the specific activity of catalase, was negligible (relative specific activity of 0.05 with respect to the homogenate, data not shown).

Data on the contamination of OMV with other organelles are scarce. Niot et al. [10] prepared OMV from rat liver by swelling and shrinking, and reported a similar contamination with ER as found here. Compared to OMV prepared by the SSS procedure [14],

Table 2
Relative specific activity of monoamine oxidase and several non-mitochondrial marker enzymes in mitochondria and purified OMV fractions from rat liver

Organelle	Marker enzyme	Homogenate specific activity (nmol mg^{-1} min^{-1})	Mitochondria RSA	OMV 1st gradient RSA	OMV 2nd gradient RSA	*n*
Mitochondrial outer membrane	monoamine oxidase	2.8 ± 0.7	2.8 ± 0.5	44 ± 8	70 ± 14	13
Endoplasmic reticulum	NADPH-cytochrome *c* reductase	65 ± 5	0.30 ± 0.05	1.3 ± 0.3	0.6 ± 0.1	6
	glucose-6-phosphatase	149 ± 15	0.23 ± 0.06	1.1 ± 0.3	0.5 ± 0.2	4
Plasma membrane	alkaline phosphatase	2.2 ± 0.3	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	4
	5′-nucleotidase	12.5 ± 4.4	0.3 ± 0.05	1.6 ± 0.5	1.3 ± 0.4	4
Lysosome	acid phosphatase	18 ± 3	1.3 ± 0.5	4.7 ± 1.1	2.8 ± 0.5	6

The specific activities of the marker enzymes were determined in the fractions indicated, and are shown for the nuclei-free rat liver homogenate. Relative specific activities (RSA) were calculated relative to the corresponding nuclei-free homogenate. Data are presented as average values (± S.D.) from *n* preparations.

the OMV from the SD method contain less contaminating lysosomes and plasma membrane.

3.3. OMV size

The *Z*-average particle size of the OMV prepared by the SD method is about twice that of OMV prepared by the SSS method, which is probably due to the omission of sonication in the first procedure (Table 3). OMV prepared by the SD method originating from rat liver and from *Neurospora crassa* are of a similar size. The particle sizes as determined by dynamic light scattering are in agreement with the sizes estimated from electron micrographs [14,27]. Also the relatively large heterogeneity in OMV size apparent from the polydispersity index in Table 3, is not unexpected based on the electron micrographs.

3.4. Phospholipid composition

The Percoll gradient-purified mitochondria from rat liver and derived OMV were analyzed for their phospholipid content (Table 4). The phospholipid-to-protein ratio and the phospholipid composition determined for mitochondria are in accordance with published data [14,24]. Compared to mitochondria, OMV are enriched in PC and PI while they have a slightly lower content of PE and are virtually free of CL. The other phospholipid classes are present in trace amounts, with sphingomyelin originating from the contaminating lysosomes and plasma membrane (Table 2). The OMV exhibit a phospholipid-to-protein ratio of 1220 nmol phospholipid phosphorus per mg protein (Table 4), which is higher than values reported previously [14,24], confirming the greater purity of the present OMV. After correcting for the phospholipid contents of the contaminating organelles which account for at most 6.5% of the total phospholipid present in the OMV preparation [24], a phospholipid phosphorus/protein ratio of 1370 nmol/mg is obtained.

To our knowledge, this is the first report on an OMV preparation from rat liver mitochondria that is virtually devoid of CL. The OMV-enriched fraction obtained from the sedimentation sucrose gradient already had a negligible content of CL (data not shown), indicating that at least 40% of the mitochondrial outer membrane is essentially free of this phospholipid. Previously, CL was considered a true component of the rat liver mitochondrial outer membrane based on three observations [14,26]: (i) OMV obtained by the SSS method contained more CL than could be accounted for by contamination with inner membrane, (ii) CL was released when the outer membrane of intact mitochondria was selectively solubilized with digitonin, and (iii) treatment of intact mitochondria with phospholipase A_2 resulted in a preferential hydrolysis of CL while mitochondrial intactness was maintained. The present results negate

Table 3
Particle size of the OMV isolated from mitochondria from rat liver and *Neurospora crassa*, as determined by dynamic light scattering

	Z-average particle size (nm)	Polydispersity index
Rat liver OMV (SD)	470	0.48
Rat liver OMV (SSS)	270	0.37
Neurospora crassa OMV (SD)	560	0.47

Data are average values obtained for at least two batches of OMV, the variation between different batches of OMV is less than 5%.

Table 4
Phospholipid composition of rat liver mitochondria and mitochondrial outer membrane vesicles prepared by the SD method

	Mitochondria	OMV
Phospholipid/protein	220 ± 20	1220 ± 150
Phosphatidylcholine	44.3 ± 2.2	54.7 ± 3.1
Phosphatidylethanolamine	34.4 ± 2.0	27.5 ± 1.7
Cardiolipin	13.5 ± 2.1	0.3 ± 0.3
Phosphatidylinositol	5.4 ± 0.8	13.4 ± 2.3
Phosphatidylserine	0.5 ± 0.2	2.1 ± 0.5
Phosphatidic acid	nd	0.4 ± 0.5
Phosphatidylglycerol	0.1 ± 0.1	0.2 ± 0.3
Lyso-phospholipids	0.4 ± 0.4	0.4 ± 0.6
Sphingomyelin	0.9 ± 0.4	0.7 ± 0.6

The numbers represent phospholipid phosphorus expressed as percentages of total lipid phosphorus, and are presented as mean values ± S.D., which derive from six batches of mitochondria and 11 batches of OMV; the phospholipid-to-protein ratios are expressed as nmol of phospholipid phosphorus per mg of protein. nd, not detectable.

the argument based on the first observation, unless the two methods of OMV preparation yield two mutually exclusive populations of OMV deriving from complementary parts of the outer membrane, which we consider a remote possibility. More likely, the presence of CL in the OMV prepared by the SSS method is due to the sonication step which leads to fragmentation of the inner membrane, and possibly even to fusion of inner and outer membrane (as discussed above), finally resulting in the cofractionation of CL-containing membranes with the OMV on sucrose gradients. The question remains why OMV prepared by the SSS method contain more CL than expected from the contamination by inner membranes [14]. One possibility is that the inner membrane protein markers are not reliable indicators for contamination with inner membrane lipids due to different behaviour of proteins and lipids during subfractionation of mitochondrial membranes. Alternatively, cofractionation of contact sites between inner and outer membranes with OMV during the SSS procedure might be responsible for the apparent discrepancy in fractionation behaviour, as contact site fractions reportedly are enriched in CL [47,48]. OMV devoid of CL have been isolated before from plant mitochondria by a mild swell–shrink procedure [49,50]. Subsequent sonication was found to increase the contamination of OMV with inner membrane [49] in accordance with the present findings.

In Table 5 the phospholipid compositions of mitochondria and highly purified OMV from *Neurospora crassa* are compared. The phospholipid composition of the mitochondria resembles that of rat liver mitochondria (Table 4) with PE constituting the major class of phospholipids as was reported previously [12,51]. The phospholipid composition of OMV from *Neurospora crassa* is similar to that of OMV from rat liver, with PC, PE, and PI being the major phospholipid constituents. The phospholipid-to-protein ratio of OMV from *Neurospora crassa* is much higher than that of OMV from rat liver (Tables 4 and 5), which is consistent with the difference in density. OMV from *Neurospora* already float on a 0.72 M sucrose layer [5], whereas it takes 1.065 M sucrose to float the OMV from rat liver. The phospholipid-to-protein ratio determined for the OMV from *Neurospora* is twofold higher than the value reported previously for OMV prepared by the SSS procedure [12], reflecting the greater purity of the present preparation. Consistent with the enhanced purity, the content of CL in the OMV is lower than was published previously [12]. In contrast to material from rat liver, OMV from *Neurospora* do contain a small amount of CL. Based on the virtual absence in the OMV preparation of inner membrane protein markers [5], this is unlikely to be due to contamination with inner membranes.

Table 5
Phospholipid composition of mitochondria and mitochondrial outer membrane vesicles from *Neurospora crassa*

	Mitochondria	OMV
Phospholipid/protein	490 ± 50	2580 ± 240
Phosphatidylcholine	36.7 ± 3.0	50.2 ± 1.6
Phosphatidylethanolamine	39.9 ± 1.0	32.7 ± 2.6
Cardiolipin	11.8 ± 0.9	3.1 ± 0.5
Phosphatidylinositol	4.5 ± 0.3	9.2 ± 1.1
Phosphatidylserine	0.7 ± 0.2	0.6 ± 0.2
Phosphatidic acid	0.6 ± 0.2	0.7 ± 0.4
Phosphatidylglycerol	1.0 ± 0.4	0.4 ± 0.3
Lyso-phospholipids	2.3 ± 1.7	1.2 ± 1.1
PX [a]	1.5 ± 0.3	1.7 ± 0.3

The numbers represent phospholipid phosphorus expressed as percentages of total lipid phosphorus, and are presented as mean values ± S.D., which derive from four batches of mitochondria and six batches of OMV. The phospholipid-to-protein ratios are expressed as nmol of phospholipid phosphorus per mg of protein.
[a] PX denotes an unidentified phosphorus-containing lipid.

4. Concluding remarks

The present study shows that the SD method previously used to isolate highly pure OMV from *Neurospora crassa* [5], can be applied to purify OMV from rat liver mitochondria. Thus, the method may be generally applicable for obtaining a good yield of mitochondrial OMV of excellent purity. Analysis of the phospholipid content of the OMV demonstrated that the outer mitochondrial membrane from rat liver is essentially devoid of CL. On the other hand, the phospholipid composition of OMV isolated from *Neurospora crassa* suggests the presence of a small amount of CL. From the results presented here and data published previously [14,26], the picture emerges that CL, which is primarily an inner membrane phos-

pholipid, has easy access to the outer membrane. The big challenge now will be to establish the biological function and the regulation of the proposed dynamic behaviour of CL.

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