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Extraction of mitochondrial membrane proteins into organic solvents in a functional state

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Protein-lipid complexes in organic solvents can be used as the starting material in the reassembly of functional planar and spherical bilayers (Montal, M., Darszon, A. and Schindler, H. (1981) Q. Rev. Biophys. 14, 1–79). The transfer of three enzymes of the inner mitochondrial membrane into organic solvents as protein-lipid complexes has been studied to understand better the extraction process. The enzymes studied were cytochrome *c* oxidase, ATPase and succinate dehydrogenase. These enzymes were transferred into hexane and diethyl ether in an active state, however, the activities extracted varied quantitatively, depending on the amount of protein of the starting preparation, the concentration of phospholipids and the cation employed. In all conditions cytochrome *c* oxidase was extracted with the highest yield and specific activity, and it was actually enriched in the organic extract. The values for succinate dehydrogenase and ATPase were lower, but their specific activities were similar to those of the starting material. This indicates that some membrane proteins are preferentially extracted into organic solvents in a functional state. The enzymes, as protein-lipid complexes, are fairly stable in organic solvents; in a month of storage at 4°C in hexane some enzymes loose less than 50% of their activity.

Introduction

In the last years a general procedure has been developed to transfer proteins directly, in their active form from biological membranes into organic solvents as protein-lipid complexes. These complexes in the hydrophobic media constitute the building blocks of several kinds of model membranes, i.e., monolayers, planar bilayers and liposomes of diverse sizes [1]. In addition, in mitochondrial membranes the technique has been used to determine the state of the ATPase which under physiological conditions can undergo transitions of its activity [2,3].

Previously, it had been shown that purified cytochrome *c* oxidase [4], reaction centers from photosynthetic bacteria [5] and rhodopsin, either purified or from rod outer segments [6,7], could be transferred into hexane as protein lipid complexes in high yields (50–90%). In contrast, we have found that the mitochondrial ATPase is less effi-

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ciently transferred from mitochondria or submitochondrial particles in this solvent (10–15%), although it maintains its specific activity [2]. These results raise the possibility that some membrane proteins have a higher probability of being transferred into the solvent as protein-lipid complexes in their active form.

To test this possibility, we have measured the transfer of the ATPase, cytochrome *c* oxidase and succinic dehydrogenase from submitochondrial particles into hexane. These different inner mitochondrial proteins partition into the solvent as protein-lipid complexes with different yields, but cytochrome *c* oxidase is transferred in better yields ($39 \pm 15\%$).

The overall results confirm the generality of the method used to transfer membrane proteins into organic solvents (for a review, see Ref. 1) and indicate that some proteins (i.e., cytochrome *c* oxidase) are preferentially extracted into the solvent as protein-lipid complexes in an active form.

Materials and Methods

Bovine heart mitochondria were prepared according to Löw and Vallin [8]. Submitochondrial particles were prepared by sonication of mitochondria (20 mg per ml) in 1 mM $MgSO_4$, 1 mM ATP and 250 mM sucrose (pH 7.2) for 35 s in a Branson sonifier. The sonicate was centrifuged at 10000 rpm in a Sorvall SS-34 rotor for 10 min. The supernatant was centrifuged at $105000 \times g$ for 45 min and the pellet washed and centrifuged again for 60 min. The washed pellet was suspended at a concentration of 50 mg per ml. The suspension was divided in aliquots which were stored at −40°C. The same preparation was used in all the experiments shown, however, essentially the same results have been obtained with many other preparations of inner mitochondrial membranes.

The general scheme for the extraction of submitochondrial particles was as follows: in most of the experiments 1 ml of hexane that contained 10 mg of soybean phospholipids was added to 3 mg of protein (suspended in 70 μl of sucrose 240 mM) in a 1.8 cm × 14.8 cm test tube. Immediately, the mixture was sonicated in a water bath sonicator (Bransonic 220, Heat Systems Ultrasonic, Plainview, New York) for 4 min. The suspension was transferred to 1 cm × 10 cm test tubes, and 100 μl of 1.0 M $CaCl_2$ were added (in some experiments Ca^{2+} was substituted with other cations) and the mixture stirred in a Vortex mixer for one min, and thereafter centrifuged for 4 min at 3/4 of the maximal speed of a clinical centrifuge with floating tubes. The upper hexane layer was withdrawn, avoiding the removal of the interphase and evaporated in an ice-bath under a current of nitrogen. To the dry residue 200 μl of 25 mM tris-acetate (pH 7.4) was added. The residue was allowed to hydrate and liposomes formed by stirring in a Vortex mixer for 4 min. Enzymic activities were assayed in the liposomes. Some of the steps of the extraction procedure were varied to test whether they affected the extraction. These changes are indicated in the Results section.

Enzymic activities

The activity of succinate dehydrogenase was measured spectroscopically following the reduction of dichlorophenolindophenol [9]. ATPase was assayed in a coupled enzyme system, following NADH oxidation spectroscopically [10]. Cytochrome *c* oxidase was recorded with an oxygen electrode using cytochrome *c* as substrate in the presence of ascorbate and *N*,*N*,*N*′,*N*′-tetramethyl-1,4 phenylenediamine dihydrochloride [11]. Apparently, the activities obtained were maximal, since disruption of the liposomes by 2% Triton X-100 did not increase the activity rates. However, Triton X-100 induced a decrease in the rate of cytochrome *c* oxidase in liposomes, thus whether this activity is completely expressed in the liposomes cannot be strictly ascertained.

Other procedures

Protein of the particles was assayed by the Biuret method, while that of the liposomes was determined according to Lowry et al. [12]. Soybean phospholipids (asolectin) were purified according to Kagawa and Racker [13]. Polyacrylamide gel electrophoresis was carried out as follows: samples that contained a final concentration of 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS) and 3% mercaptoethanol were electrophoresed in 0.1% sodium dodecyl sulfate, 10%

polyacrylamide slab gels (9 cm) according to Laemmli [14]. The gels were fixed and stained for protein with 0.1% coomassie blue in 30% methanol/10% acetic acid and destained in 30% methanol/10% acetic acid.

Spectral determination of cytochromes aa_3 and b was performed in the presence of 50% glycerol in an SLM-Aminco Midrian II spectrophotometer at room temperature (1.0 cm light-path cuvettes). The concentration of the cytochromes was estimated from the difference spectra (dithionite-reduced minus persulfate-oxidized) of liposomes or submitochondrial particles, using the following wavelength pairs and extinction coefficients: cytochrome aa_3, extinction coefficient at 630 to 603 nm $E_{603-630} = 24\ \text{mM}^{-1} \cdot \text{cm}^{-1}$, cytochrome b, $E_{563-575} = 22\ \text{mM}^{-1} \cdot \text{cm}^{-1}$ [15].

Electron microscopy

For freeze fracture, liposomes formed from submitochondrial particle organic extracts were centrifuged at $100\,000 \times g$ for 2 h, and the pellets fixed for 15 min in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at room temperature. Thereafter, the pellets were washed twice with 0.1 M cacodylate buffer and were gradually impregnated with increasing concentrations of glycerol in cacodylate buffer for 30 min, up to a concentration of 20% glycerol and allowed to stand for 30 min. The fixed, glycerol-impregnated pellets were cut and rapidly frozen in the liquid phase of partially solidified Freon 22, cooled and stored in liquid nitrogen for a few days. Freeze fracture was carried out at -115°C in a Balzers 300 apparatus (Balzers Co., Liechtenstein) equipped with a turbo molecular pump.

Replicas were produced by evaporation from a carbon platinum source. The specimens were shadowed at $2 \cdot 10^{-6}$ mm Hg with 2–4 s of fracturing. After cleaning in sodium hypochlorite, replicas were washed with destilled water, mounted on Formvar-coated grids, and observed with a Zeiss EM 10 (Zeiss Co., Ober-Kochen, F.R.G.) electron microscope.

Results

The results in Fig. 1A show that more protein was extracted into the solvent from which liposomes were formed, as the protein in the starting preparation was increased up to 4 mg. Fig. 1B illustrates the dependence of the extraction on added lipid. When no lipid was added, protein was not extracted into the solvent. Fig. 1C illustrates that the presence of cations enhanced the amount of protein extracted into the solvent.

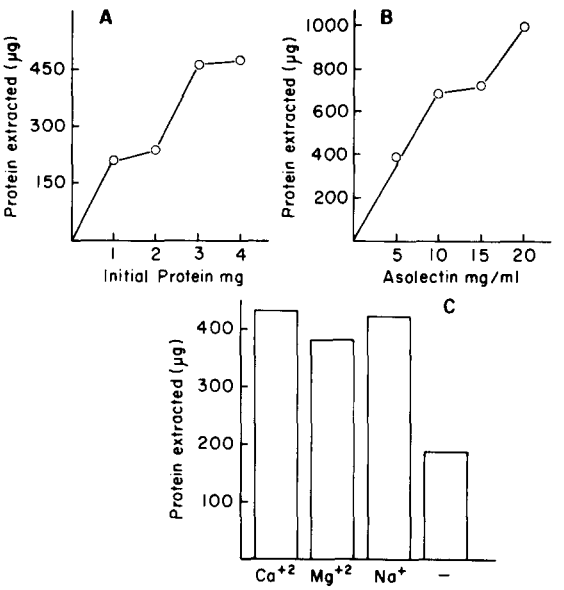

Fig. 1. Extraction of protein from submitochondrial particles into hexane. The extraction of protein from submitochondrial particles is detailed under Methods, except that in (A) the amount of starting protein was varied. The results represent the average of three experiments. In (B) the amount of asolectin per ml of hexane was as shown. In (C) 100 µl of 3 M NaCl, 1 M $MgCl_2$ or 1 M $CaCl_2$ were added; the right bar shows the amount of protein extracted with no added cation. The overall standard deviation in these type of experiments was $\pm 35\%$, $n = 10$.

Electrophoretic analysis in SDS gels of hexane and diethyl ether extracts from submitochondrial particles showed that many of the polypeptides present in the original membrane were transferred into the solvent (data not shown). Apparently, a few bands were enriched, while others were in a lower concentration. This suggested the possibility that some proteins were preferentially being extracted into the solvent.

According to these data it was decided to explore whether the proteins extracted into the organic phase possessed some enzymic activity and whether during the procedure some enzymes were preferentially extracted. To this purpose three

membrane enzymes were assayed: succinate dehydrogenase, cytochrome *c* oxidase and ATPase. These are three enzymes with different characteristics and variable degrees of complexity (for reviews see Refs. 16, 17 and 18). The specific activities of succinate dehydrogenase and ATPase in submitochondrial particles were 0.1 ± 0.01 and 0.38 ± 0.1 μmol/min per mg protein, respectively. It has been shown that the activity of cytochrome *c* oxidase depends on the phospholipid milieu [19,20]. Accordingly, particles were sonicated in the presence of soybean phospholipids, to mimic the conditions of the liposomes, and their cytochrome *c* oxidase specific activity measured and used to estimate the enrichment and recovery of the extracted enzyme. Interestingly, the specific activity of submitochondrial particles sonicated with lipids (3946 ± 576 natoms O/min per mg) was approx. 4-times higher than in the untreated particles.

Figs. 2 and 3 summarize the results of experiments in which the specific activities and recoveries of the three enzymes were measured after the extraction of the particles into hexane under various conditions and subsequent incorporation of the protein-lipid complexes into liposomes. A parallelism was found; in general, conditions which favor higher protein extraction yield higher recoveries and specific activities for the three

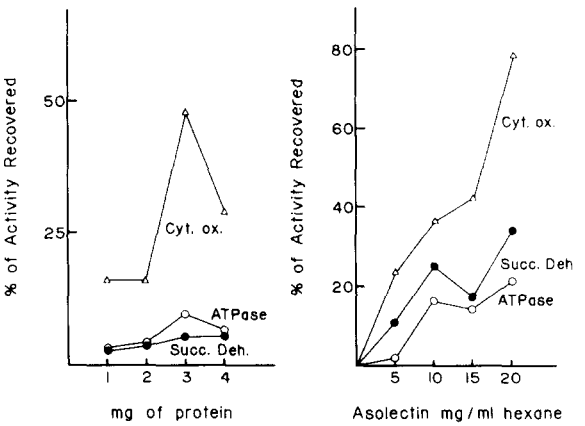

Fig. 3. Percent recovery of succinate dehydrogenase, cytochrome *c* oxidase and ATPase activities in the liposomes. The experimental conditions were as in Fig. 2. The total activity of these enzymes in submitochondrial particles was taken as 100%. In the case of cytochrome *c* oxidase the value after sonicating the particles with lipid was considered as 100%.

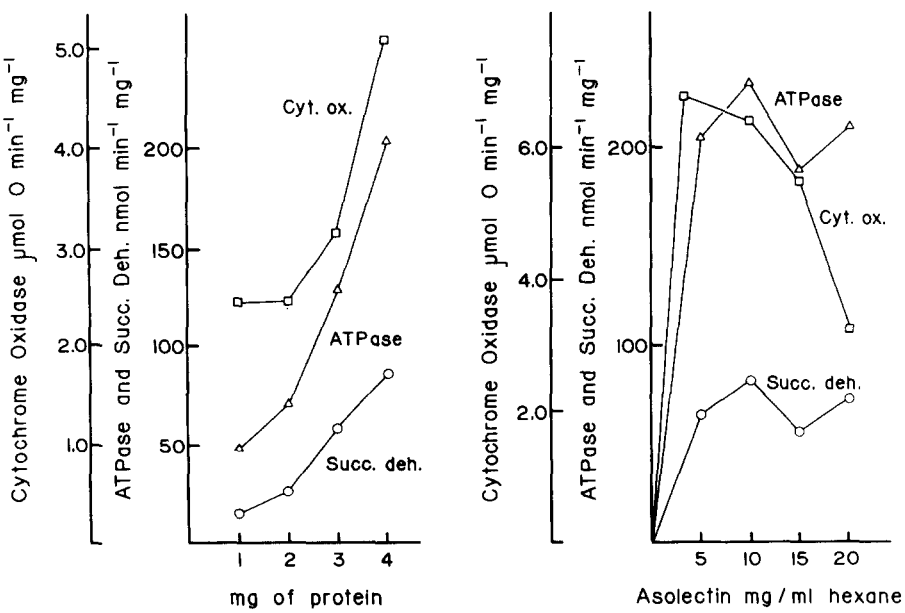

Fig. 2. Specific activities of succinate dehydrogenase (Succ. Deh.), cytochrome oxidase (Cyt. ox.) and ATPase in liposomes. The experimental conditions were as in Fig. 1; the indicated activities were assayed in liposomes formed from the respective hexane extracts.

enzymes. Under all conditions studied (amount of membrane protein and phospholipid concentration) cytochrome *c* oxidase had the highest recovery and specific activity. When 10 mg of soybean lipids and 3 mg of mitochondrial protein were used, this enzyme was transferred into the solvent with a yield higher than 30%, and in the liposomes, it could reach specific-activity values (6232 ± 643 natoms O/min per mg) that are close to twice those found in submitochondrial particles sonicated in the presence of lipid. Since cytochrome *c* oxidase was enriched in the liposomes formed from the organic extracts of submitochondrial particles with respect to its specific activity (factor of 1.6), we decided to explore the possibility that the solvent itself was activating this enzyme. Therefore, cytochromes aa_3 and *b* were determined spectroscopically in an assay which is independent of enzymatic activity (see Ref. 15 and Methods). The content of cytochromes aa_3 and *b* in nmol/mg protein were: in submitochondrial particles 0.68 and 0.72 and in the liposomes 1.21 and 0.81, respectively (average of two experiments). These results indicate that cytochrome *c* oxidase is enriched in the liposomes by a factor of 1.8 similar to what is found with respect to specific activity (1.6). On the other hand, it is clearly seen that cytochrome *b* is not enriched in the liposomes and that the ratio cytochrome aa_3/cytochrome *b* is higher in the liposomes than in submitochondrial particles.

The recovery of succinate dehydrogenase (around 20%) and of the ATPase (about 15%) was lower than that of cytochrome *c* oxidase, and their specific activities were similar to those observed in the particles. This indicates that these latter enzymes are transferred without being damaged, and in the same proportions as they are found in particles, or that although they are damaged other proteins are less efficiently transferred into the solvent.

In agreement with Figs. 2 and 3, the results in Fig. 4 indicate that the extraction of the functional enzymes is favored by various cations in a nonspecific manner. We would like to point out that although the methodology is very simple, the variability is significant (±35%, $n = 10$) and care must be taken to perform all the steps in a systematic manner. Special precautions must be taken in the sonication step, in the sense that complete dispersion of the membrane material must be achieved, otherwise the yield of the extraction will be low.

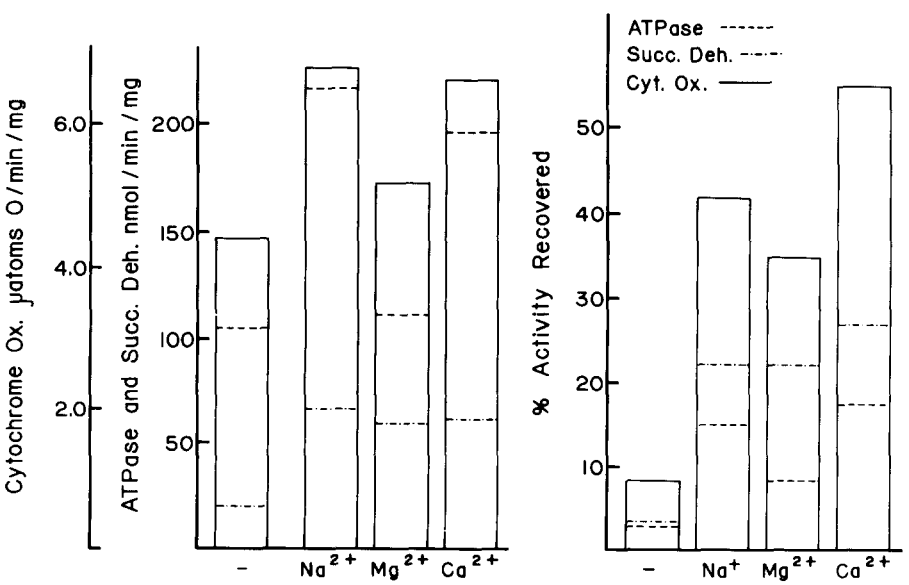

Fig. 4. Effect of different cations on the extraction of succinate dehydrogenase (·–·–·) cytochrome *c* oxidase (————), and ATPase (— — —) into hexane. The experimental protocol was as described under Methods, except that 100 µl of 3 M NaCl, 1 M $MgCl_2$ or 1 M $CaCl_2$ were added. Where shown, no cation was added. The enzyme recovery was normalized as in Fig. 3.

Another important source of variability is in the formation of the liposomes: the protein-lipid residue after solvent evaporation must be carefully resuspended in the desired buffer, since otherwise the residue may remain adhered to the wall of the test tube.

Also it was of interest to explore whether these membrane proteins could be extracted into organic solvents other than hexane, and whether this change affected the pattern of extraction of the enzymes. Thus, the same general procedure was used, except that hexane was substituted for diethylether. Diethylether was chosen because it was used previously in the extraction of vertebrate rhodopsin [6] and the ATPase [2]. The results (not shown) indicate that this change of solvent did not result in a significant variation of the amount of protein that was extracted, nor in the characteristics of the three enzymes studied.

It is the impression of the authors that this methodology has a potential use in the purification of membrane-bound enzymes that is worth exploring, therefore it was of importance to determine the stability of the enzymes in hexane. The results are shown in Fig. 5. The enzymes seem to be fairly stable in hexane, since after 10 days of storage at 4°C the organic extracts lost less than 50% of the activity of the three enzymes, and after 30 days the activities of succinate dehydrogenase and cytochrome *c* oxidase were preserved by more than 50%.

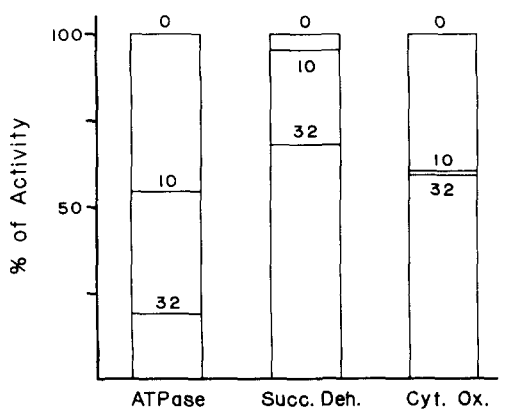

Fig. 5. Stability of succinate dehydrogenase, cytochrome *c* oxidase and ATPase in hexane. Hexane extracts were formed as described under Methods. Immediately after preparation, an aliquot was used to form liposomes and the enzymic activities assayed. The rest of the organic extract was kept at 4°C for 10 and 32 days, at this time aliquots were used to form liposomes to assay the activities. The initial activity was taken as a 100%.

Discussion

The present study shows that the three mitochondrial membrane enzymes that were assayed are functionally present in varying amounts in liposomes formed from the protein-lipid complexes obtained after extracting submitochondrial particles with organic solvents. All conditions tested in the extraction procedure (amount of protein, phospholipid concentration in hexane and different cations) influence the yield of the protein extracted, and differentially affect the resulting activity of the enzymes. It is therefore possible to take advantage of this behavior, and by selecting appropriate conditions (amount of protein, etc.) modify the transfer of some enzymes.

Two main factors, which depend to a large extent on the extraction conditions, determine the specific activity and recovery of a particular enzyme in the liposomes formed from the mitochondrial organic extracts: (a) the efficiency with which the specific protein is transferred into the solvent and (b) how well the enzyme withstands the treatment. An enzyme can partition very efficiently into the solvent, but its transfer results in denaturation; therefore, its specific activity would be low. On the other hand, it can partition poorly, but retain its functional capacity and have the same or higher specific activity, depending on how well other proteins are transferred. With the measurements performed here it is not possible to detect a specific denatured enzyme in the liposomes; however, this would be possible using antibodies, or other suitable markers of the enzyme. Therefore, to evaluate if an enzyme has been purified or enriched it is necessary to consider: (1) the total yield of extracted protein; (2) the total activity of the particular enzyme recovered; (3) its specific activity in the starting material and in the liposomes; and (4) if the enzyme has been activated during treatment.

This analysis can be done for cytochrome *c* oxidase and several conclusions may be derived: (a) on the average this activity is enriched by a factor of 1.6 in the liposomes formed from the

particle extracts considering the specific activity of submitochondrial particles sonicated in the presence of soybean lipid vesicles to mimic the extraction conditions; (b) the recovery of cytochrome *c* oxidase activity is higher (30%) than the total protein yield of extraction (10–15%). The possibility that solvent activation could explain the enrichment factor obtained for cytochrome *c* oxidase considering its specific activity was ruled out by finding that the content of cytochrome aa_3 increased in the liposomes proportionally to their specific activity. In addition, it was observed that cytochrome *b* was not enriched in the liposomes and that the ratio cytochrome aa_3/cytochrome *b* was higher in liposomes than in submitochondrial particles. Therefore, it is clear that cytochrome *c* oxidase preferentially partitioned into the solvent retaining its function. This case exemplifies the potential of the technique in the purification of some membrane proteins.

Considering the cytochrome *c* oxidase results, it does not seem likely that whole membrane fragments of submitochondrial particles are being transferred into the solvent. If this was the case the specific activity of this enzyme in the extracts should not be enriched and its recovery should reflect its natural abundance (approx. 10%) in the inner mitochondrial membrane. In addition, if whole membrane fragments were being transferred into the lipid containing solvent, freeze fracture replicas of the liposomes formed from the organic extracts should show single-layered submitochondrial fragments with membrane particles and multilayered lipid vesicles devoid of particles. In contrast, the replicas of the liposomes (see Fig. 6) reveal an apparently homogeneous distribution of membrane particles within mostly multilamellar vesicles.

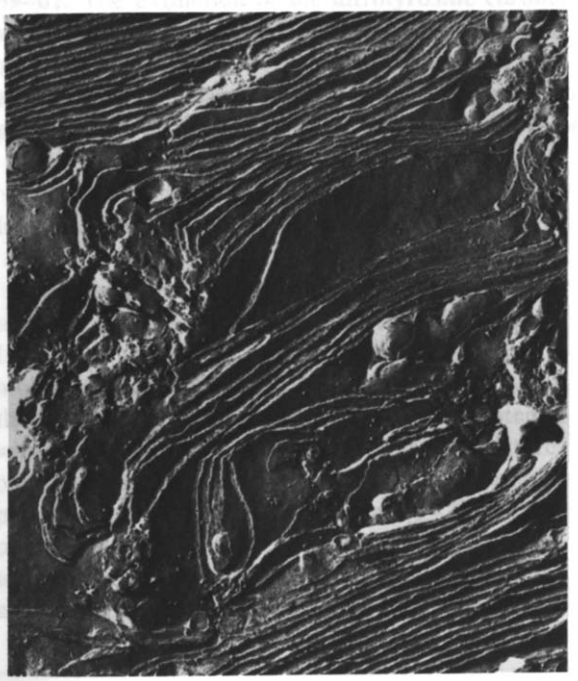

Fig. 6. Freeze fracture appearance of liposomes formed from hexane extracts of submitochondrial particles (see Methods). Magnification, 31 000×.

In principle, the role of the cation in the transfer of proteins as protein-lipid complexes into apolar solvents is to neutralize the overall charge of the complex. However, it has been found that there is a small dependence on Ca^{2+} in the extraction of rhodopsin from purified bovine rod outer segments [21], which indicates that the role of cations is not entirely defined. The data described here (Fig. 4) show that cations besides enhancing the partition of protein into the solvent may play a more specific role in allowing the transfer of functional complexes.

Protein-lipid complexes in hydrophobic media besides being the starting material of several kinds of model systems [1] are interesting preparations from which new methods for protein purification could be developed. The protein-lipid complexes are reasonably stable in the organic solvent, and thus they could be used to further purify a specific enzyme in a hydrophobic environment without the use of detergents.

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