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Triton X-100 Solubilization of Mitochondrial Inner and Outer Membranes

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Abstract

Rat liver mitochondrial inner and outer membranes were subjected to the solubilizing effect of the nonionic detergent Triton X-100 under various conditions. After centrifugation, the supernatants (containing the solubilized fraction) and pellets were characterized chemically and/or ultrastructurally. The detergent seems to act by inducing a phase transition from membrane lamellae to mixed protein–lipid–detergent micelles. Different electron-microscopy patterns are shown by the inner membranes after treatment with different amounts of surfactant, whereas the corresponding images from outer membranes vary but slightly. Selective solubilization of various components is observed, especially in the case of the inner membrane. Some membrane lipids (e.g., cardiolipin) are totally solubilized at detergent concentrations when others, such as sphyngomyelin, remain in the membrane. Other inner-membrane components (flavins, cytochromes, coenzyme *Q*) show different solubilization patterns. This allows the selection of conditions for optimal solubilization of a given membrane component with some degree of selectivity. The influence of Triton X-100 on various mitochondrial inner-membrane enzyme activities was studied. The detergent seems to act especially through disruption of the topology of the functional complexes, although the activity of the individual enzymes appears to be preserved. Relatively simple enzyme activities, such as ATPase, are more or less solubilized according to the detergent concentration, whereas the more complex succinate-cytochrome *c* reductase activity practically disappears even at low Triton X-100 concentrations.

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Introduction

Although the overall structure and compartmentation of mitochondria are already well known, the details of the topology of the inner and outer membranes are still incompletely resolved. This is especially true of the molecular organization of the different respiratory complexes of the inner membrane. However, detailed knowledge of their architecture is essential for a better understanding of their functional characteristics. The present study is aimed at the resolution of some of these problems by the use of the nonionic detergent Triton X-100.

Triton X-100 (4-(1,1,3,3-tetramethylbutyl)-phenoxypolyethyleneglycol (n = 9 – 10), Rohm & Haas) has been extensively used in membrane research because of its effective solubilization power and relatively mild effects on membrane-bound enzyme activities [1]. Triton X-100 effects on erythrocyte membrane ghosts have been studied in detail [2, 3], and selective solubilization of some membrane components has been shown. A systematic approach to the ability of several members of the Triton series to disrupt mitochondrial membranes has been described [4]. Because of its "mild" character, Triton X-100 has also been extensively used in reconstitution experiments. Although its low critical micellar concentration (CMC) precludes its use in such experiments when the excess detergent has to be removed by dialysis, this inconvenience has been overcome by the use of hydrophobic resins which selectively absorb the surfactant, thus removing it from the aqueous phase [5].

Among the most interesting questions arising from the use of detergents in membrane studies are the mechanisms by which the different membrane components are solubilized, the possibility of selective solubilization of some components, and the effect of the detergent on membrane-bound enzyme activities, and hence its possible application for reconstitution studies. The data reported in the present paper give some insight into these important problems. A preliminary account of this work has been given elsewhere [6].

Materials and Methods

Membrane Preparations

Rat liver mitochondria were obtained from male Wistar rats weighing approximately 200 g. The animals were starved for 18 hr, with water *ad libitum,* and killed by decapitation. Livers were homogenized in 0.25 M sucrose, and mitochondria were isolated according to Hogeboom [7]. Inner and outer mitochondrial membranes were obtained essentially as described by Parsons et al. [8]. The isolated mitochondria were resuspended in a

hypotonic buffer, 0.02 M phosphate, pH 7.4, containing 0.02% bovine serum albumin, and incubated for 20 min at 4°C. This suspension was then centrifuged at 35000 × *g* for 20 min at 4°C so that inner and outer membranes were sedimented while most of the matrix and inter-membrane space proteins remained in solution. The sediment was resuspended in the same hypotonic buffer and centrifuged at 1900 × *g* for 15 min at 4°C. The resulting pellet contained mainly inner membranes, while the outer remained in the supernatant. The crude preparation of inner membranes was washed in hypotonic buffer and centrifuged under the same conditions. A second washing stage was performed in 0.25 M sucrose, with centrifuging at 8500 × *g* for 10 min at 4°C.

The inner mitochondrial membranes were then purified by sucrose-density-gradient centrifugation in order to eliminate contamination from matrix and outer membranes. The purification was carried out in a Beckman "swing out" rotor, model SW-25-2. A discontinuous gradient was formed in each tube, containing 30 ml 1.32 M sucrose, 15 ml 0.76 M sucrose, and 5 ml of the inner membrane suspension in 0.25 M sucrose. The gradient tubes were then centrifuged at 76.000 × *g* for 90 min at 4°C, and the purified inner membranes were recovered from the bottom of the tube. They were washed in 0.25 M sucrose, 0.02 M Tris·HCl, pH 7.4 ("sucrose buffer"), and sedimented at 150,000 × *g* for 60 min at 4°C.

The outer membranes, on the other hand, were purified as follows. The supernatant from the first centrifugation at 1900 × *g*, containing the outer membranes, was centrifuged at 35,000 × *g* for 20 min. The sediment was resuspended in the hypotonic phosphate buffer, and layered on top of a discontinuous gradient, formed by equal volumes of 51.3, 37.7 and 25.2 aqueous sucrose. The tubes were centrifuged at 100,000 × *g* for 60 min at 4°C, and the purified outer membranes remained between the 37.7 and 25.2% sucrose solutions. The membranes were then washed in sucrose buffer and sedimented at 150,000 × *g* for 60 min at 4°C. The purity of membrane preparations was checked in all cases by the assay of marker enzymes (see *Analytical Techniques*).

Detergent Treatments

The pellets of purified mitochondrial membranes were resuspended in the same sucrose buffer to a final concentration of 1 or 4 mg protein/ml, as required. Aliquots (1 ml) of these suspensions were treated with different amounts of Triton X-100 in order to obtain final detergent concentrations ranging from 0.1 to 2% (w/v). Sodium or calcium chloride, or EDTA, were also added at this stage when required. The membrane suspensions were then incubated for 30 min at 20°C, unless otherwise stated, and centrifuged at

150,000 $\times g$ for 60 min at 4°C. The lipid and protein present in the supernatant were assumed to be the solubilized membrane fraction.

Electron Microscopy

The pellets containing the nonsolubilized material were imbibed in 2.5% glutaraldehyde in sucrose buffer. After 2 hr the pellets were separated from the bottoms of the tubes and transferred to a new portion of identical glutaraldehyde solution, where they were left for another 2 hr. The sediments were then cut in fragments about 1 mm^3 and washed in sucrose buffer for 24 hr; they were then treated with 1% osmium tetroxide in sucrose buffer for 2 hr. The fragments were then dehydrated in aqueous acetone through the following steps: 30%, 15 min; 50%, 30 min; 70% + 1% uranyl acetate (as counterstain), 30 min; 90%, 30 min; pure acetone, 30 min (2 steps). The samples were treated with propylene oxide for 30 min and included in the following resin mixture: Araldite CY-212 (10 ml), hardener HY-964 (10 ml), catalyst DY-064 (0.4 ml), and plastifier (dibutylphthalate, 0.4 ml). Inclusion was allowed to take place for 12 hr at room temperature, and then for 48 hr at 60°C. Thin sections (700–900 Å) of the polymerized resin blocks were obtained in an LKB Ultratom III and contrasted with lead citrate. The sections were observed in a EM-300 Philips electron microscope at 80 kV.

Analytical Techniques

Proteins were determined by the method of Lowry et al [9]; determinations in the presence of Triton X-100 were made following the modification of Wang and Smith [10]. Total iron was measured by atomic absorption spectrophotometry, using a Zeiss model FMD-4 spectrophotometer. Polypeptides were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE), according to Fairbanks et al. [11]. The gel cylinders were stained with Coomassie brilliant blue and scanned at 550 nm with a SP 1800 Unicam spectrophotometer equipped with a densitometry accesory.

Flavins [12] and cytochromes [13] were determined by differential spectrophotometry of their oxidized and reduced forms. Measurements were carried out in a double-beam Beckman DB-GT spectrophotometer. Coenzyme Q was also measured from its difference spectrum, after light petroleum extraction according to Redfearn [14]. The following enzyme activities were determied at 37°C according to established techniques: ATPase [15], succinate:coenzyme Q oxidoreductase [16], succinate:cytochrome c reductase [17], rotenone-sensitive NADH:cytochrome c reductase [18], cytochrome c oxidase [19], and monoamine oxidase [20].

Lipids were extracted from membrane suspensions, or from the pellets of non-solubilized material according to Santiago et al. [21]. Supernatants of

the centrifugation after detergent treatment were extracted essentially according to Folch et al. [22], washing repeatedly the organic phase with the "theoretical upper phase" in order to eliminate the detergent. Lipid phosphorus was analyzed in the extracts according to Bartlett [23]. Phospholipid classes were separated by thin-layer chromatography following the method of Neskovic and Kostic [24] and quantified as lipid phosphorus. Fatty acids were examined by gas-liquid chromatography as their methyl esters. Transesterification was carried out in boron trifluoride–methanol (14%, w/v) according to Morrison and Smith [25]. Fatty acid methyl esters were then separated in a Hewlett-Packard 5120A gas chromatograph, equipped with a dual-flame ionization detector. Stainless steel columns packed with polyethyleneglycol adipate were used.

Chemicals

Organic solvents were purchased from Merck, Darmstadt; GLC reagents were from Xpectrix, Barcelona; phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase were from Boehringer, Mannheim. The resins for electron microscopy were of the Durcupan-ACM series (Fluka), and the electron micrographs were taken on Agfa-Gevaert Scientia plates. All the other reagents were from Sigma, London.

Results

Overall Solubilization Patterns

Solubilization patterns of protein and phospholipid from the inner and outer mitochondrial membranes are shown as a function of detergent and membrane concentration in Fig. 1. Proteins follow in most cases a similar solubilization pattern: Protein solubilization depends on detergent concentration at low values of the latter (<0.25%, w/v), but tends to reach a plateau above that Triton concentration. However, outer membranes at 4 mg protein/ml differ slightly, because a clear plateau is not observed at the studied surfactant concentrations. In all cases, the maximum solubilized protein is between 40–60% of the total; slightly higher proportions of protein are solubilized from outer- than from inner-membrane preparations.

Inner and outer membranes differ with respect to the solubilization of lipid phosphorus (Fig. 1). Higher proportions of phosphorus than of protein are solubilized in the case of the inner membranes; the opposite is true for the outer membranes. The solubilization of lipid phosphorus is also considerably affected by membrane concentration, especially in the inner membrane case, where practically 100% of the lipid P is solubilized when membrane protein concentration is 1 mg/ml, but considerably less at 4 mg/ml. In the case of the

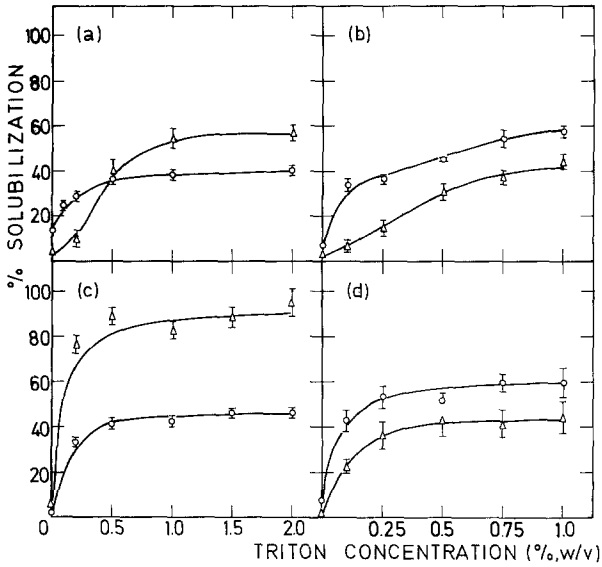


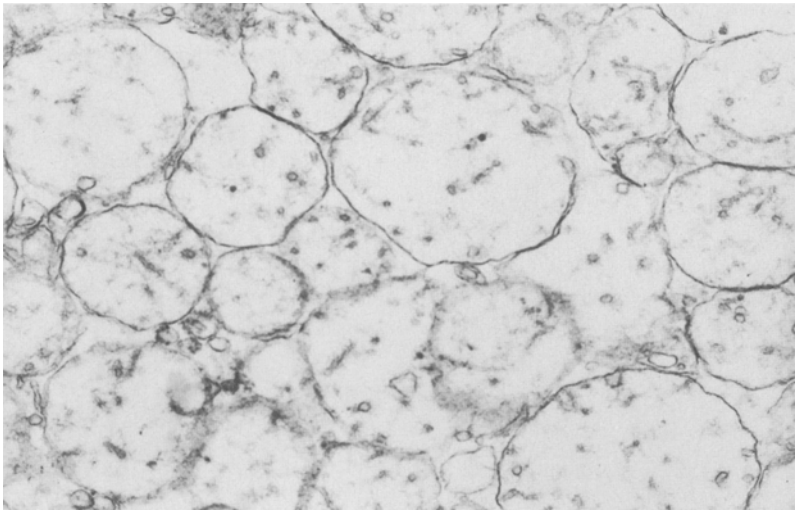
Fig. 1. Solubilization patterns of protein (○) and phospholipid (△) from the inner and outer mitochondrial membranes as a function of detergent concentration. (a) Inner membranes, 4 mg protein/ml; (b) outer membranes, 4 mg protein/ml; (c) inner membranes, 1 mg protein/ml; (d) outer membranes, 1 mg protein/ml. Bars denote ±1 S.E.M. of at least six experiments.

outer membrane, the phospholipid solubilization plot is about linear in the high, but clearly parabolic in the low, membrane protein concentration. These differences reflect the different detergent:membrane ratios; the detergent:protein ratio is higher in Fig. 1c, 1d, and the detergent:lipid ratio is higher in Fig. 1a, 1c.

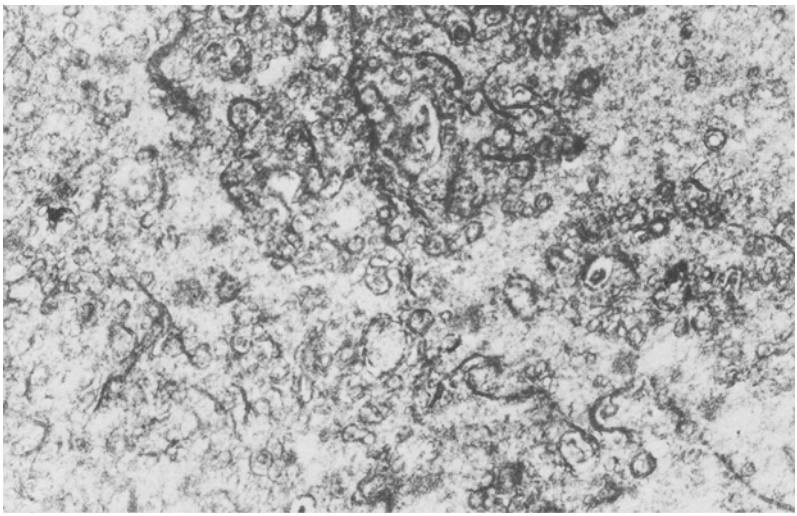
Systematically altering the ionic strength with NaCl or $CaCl_2$ up to 100 mM, or changing the temperature of extraction from 4 to 37°C, exerted only minor effects upon the solubilization of membrane components (data not shown).

Ultrastructural Studies

Thin sections of the pellets containing the nonsolubilized material were observed by electron microscopy. Even low concentrations of the detergent cause major alterations of membrane architecture. However, inner and outer mitochondrial membranes are affected differently. The appearance of inner membranes changes as Triton X-100 concentration is increased (Fig. 2). Considerable structural deterioration is already observed at detergent concentrations as low as 0.05%, which cause very little membrane solubiliza-

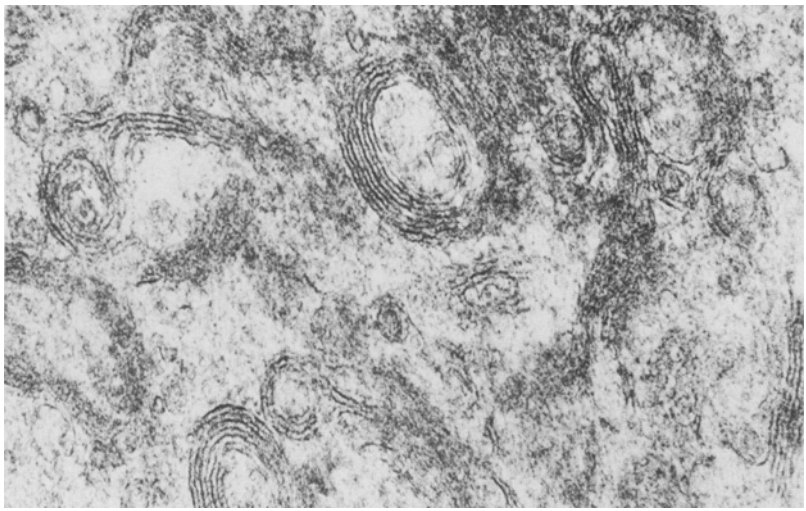


(a)

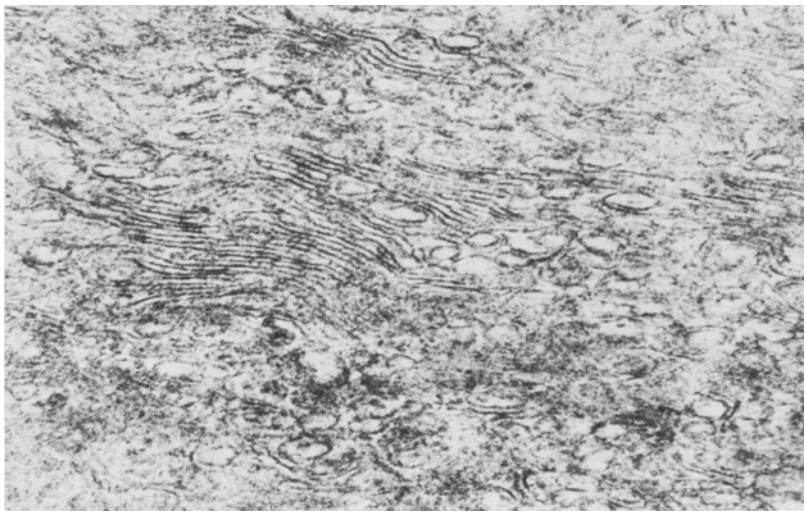


(b)

Fig. 2. Electron micrographs of thin sections of the pellets containing the nonsolubilized fraction of the mitochondrial inner membranes, after detergent treatment. (a) Control inner membranes (20,000 × 1.5); (b) after treatment with 0.05% Triton X-100 (w/v) (10,000 × 1.5); (c) same sample, higher magnification (33,000 × 1.5); (d) after treatment with 0.1% Triton X-100 (w/v) (42,000 × 1.5); (e) after treatment with 0.5% Triton X-100 (w/v) (33,000 × 1.5); (f) after treatment with 1.0% Triton X-100 (w/v) (114,000 × 1.5).

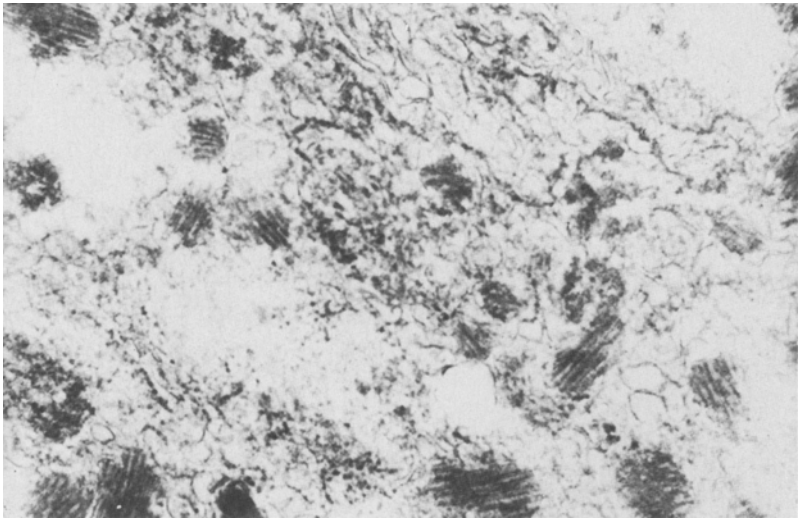


(c)



(d)

Fig. 2. Continued.



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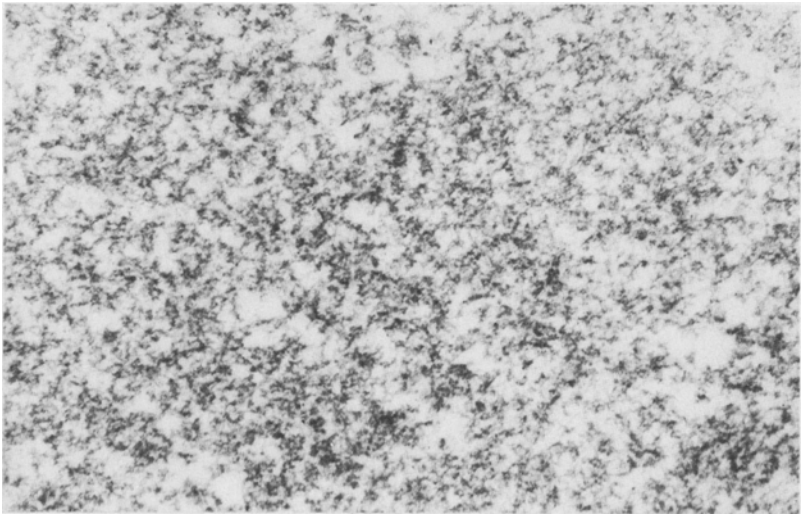


(f)

Fig. 2. Continued.

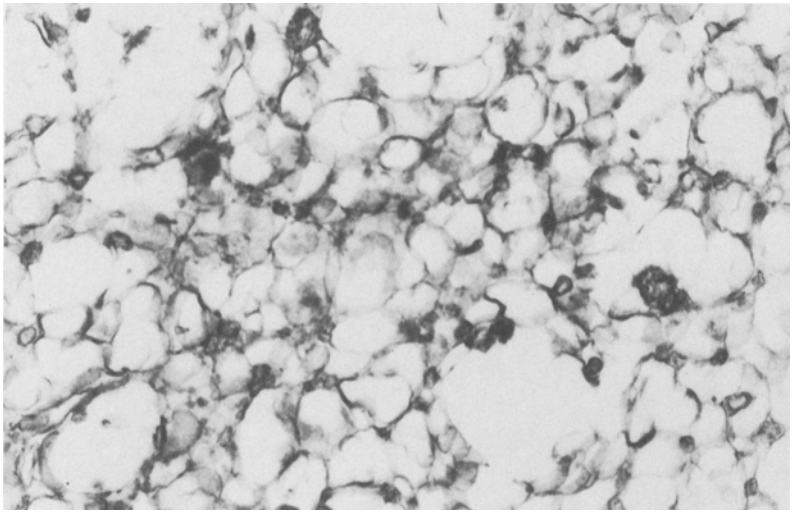
tion (Fig. 2b). These pellets are not completely homogeneous, and, in their cores, myelinlike figures can be seen (Fig. 2c). In the case of the membranes treated with 0.10% Triton X-100 (w/v), (Fig. 2d), the pellets contain no myelinlike bodies, but rather "open" lamellar structures, which retain the membranous trilaminar appearance. These lammellae appear in the pellet perpendicularly to the gravitational field, interspersed with amorphous material. The proportion of amorphous, granular material is higher in the pellets originated from inner membranes treated with 0.5% Triton X-100 (Fig. 2e). Spherical or ellipsoidal bodies are typically seen in these pellets, containing a structured, laminar material, which shows formal similarities with the nucleoids of some peroxisomes. Finally, the nonsolubilized material from membranes treated with detergent concentrations of 1% and higher (Fig. 2f) is constituted exclusively of amorphous, granular, somewhat aggregated material.

The ghostlike appearance of the outer mitochondrial membranes (Fig. 3a) is also lost after detergent treatment, but unlike the case for the inner membranes, different detergent concentrations give rise to essentially identical images of the nonsolubilzed material (Fig. 3b–d). The membranous bags are disintegrated, and laminar bodies, as well as dense granules, appear now together with electron-transparent areas. Two types of granules can be distinguished: some of them are smaller, not so dense, and with some tendency to aggregation, while others are very dense and remain isolated in the middle of optically empty areas. These granules, especially the bigger ones, tend to decrease in number at higher detergent concentrations.

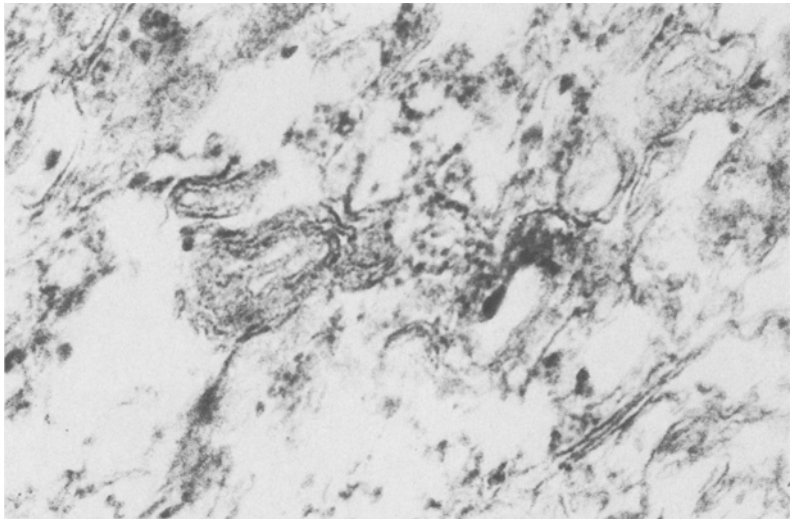
Study of the Solubilized Polypeptides and Lipids

Aliquots of the supernatants containing the solubilized fraction of inner and outer membranes were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. Densitograms of some Coomassie brilliant blue stained gels are shown in Fig. 4. In the case of the outer membrane (a–c), it is apparent that virtually the same polypeptides, and in the same proportions, are solubilized at 0.25 and 1.0% detergent. Further increases in Triton X-100 concentration, up to 2%, do not lead to any changes in the pattern of solubilized polypeptides. This pattern differs from the polypeptide spectrum of the whole membrane especially because one of the main bands of the total membranes is not solubilized at all. For the inner membrane (d–f), increasing concentrations of the surfactant bring into solution new polypeptides. Thus, the gel densitograms corresponding to Triton X-100 concentrations of 0.25 and 1.0% are in this case quite different. The electrophoretic patterns corresponding to high detergent concentrations differ also from that of the intact inner membrane.

Different aliquots of the supernatants were analyzed for phospholipids and fatty acids as described under Materials and Methods. The quantitative

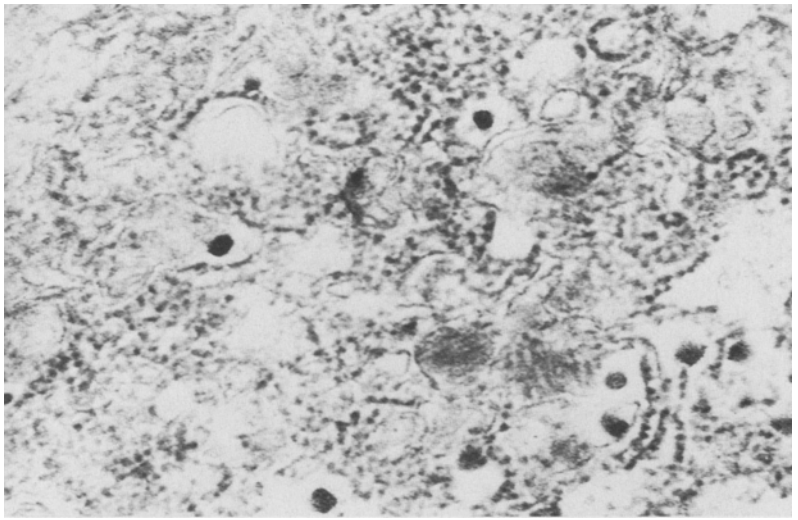


(a)

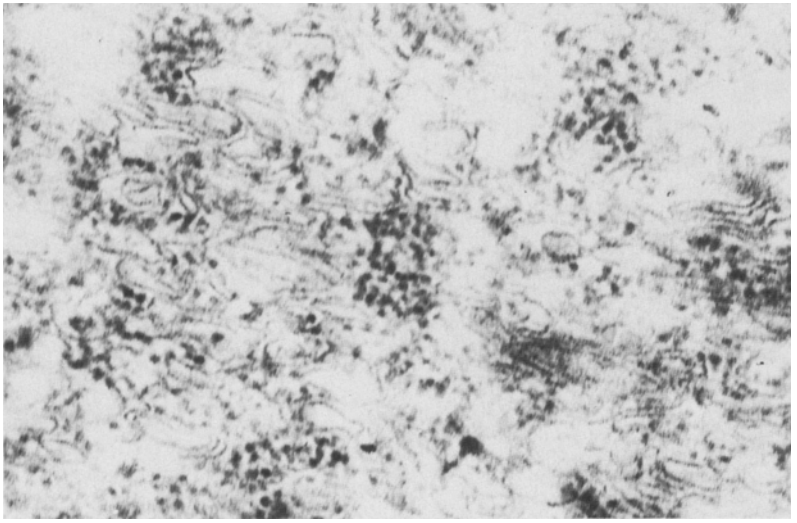


(b)

Fig. 3. Electron micrographs of thin sections of the pellets containing the nonsolubilized fraction of the mitochondrial outer membranes, after detergent treatment. (a) Control outer membranes (16,000 × 1.5); (b) after treatment with 0.1% Triton X-100 (w/v) (55,000 × 1.5); (c) after treatment with 0.25% Triton X-100 (w/v) (55,000 × 1.5); (d) after treatment with 1% Triton X-100 (w/v) (55,000 × 1.5).



(c)



(d)

Fig. 3. Continued.

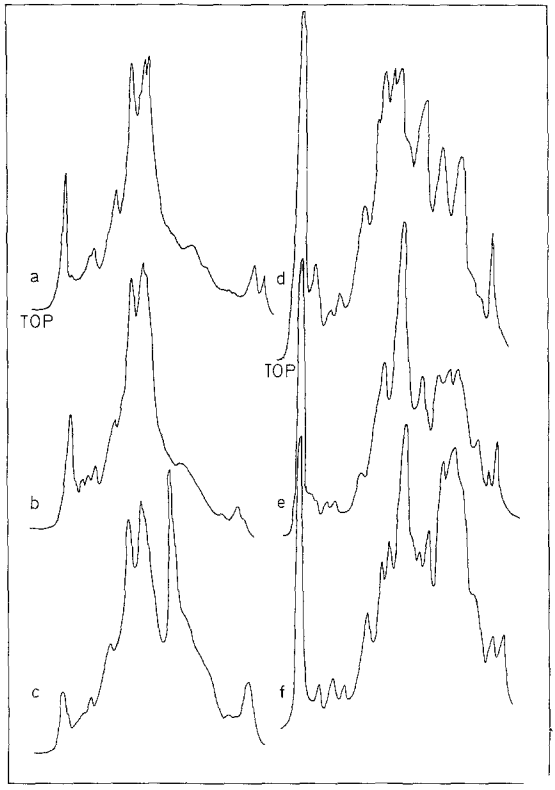


Fig. 4. Polypeptide constituents of mitochondrial inner and outer membranes, and fractions solubilized thereof. (Densitograms of Coomassie brilliant blue stained gels.) Outer membranes: (a) fraction solubilized by 0.25% Triton X-100; (b) fraction solubilized by 1.0% Triton X-100; (c) intact outer membranes. Inner membranes: (d) fraction solubilized by 0.25% Triton X-100; (e) fraction solubilized by 1.0% Triton X-100; (f) intact inner membranes.

analysis of the different phospholipid classes both in the intact membrane preparation and in the supernatant after detergent treatment allows us to make an estimation of the extent of the solubilization of each phospholipid class. This is expressed as a percentage of each phospholipid present in the intact membrane that has been found solubilized in the supernatant. The results are summarized in Table I. Again a difference in found in the behavior of inner and outer membranes. Triton X-100 at a concentration of 0.25% (w/v) solubilized approximately equal proportions of each of the different

Table I. Solubilized Phospholipids from Mitochondrial Membranes After Treatment with 0.5% Triton X-100[a]

Phospholipid	Intact outer membrane		Outer membrane supernatants		% Solubilized outer membrane phospholipids
	% Phospholipid composition	Amount of lipid P (μg)	% Phospholipid composition	Amount of lipid P (μg)	
Phosphatidylserine + sphyngomyelin	3.3	32	3.2	11	33.2 ± 1.05
Phosphatidylinositol	3.6	35	3.2	10	29.8 ± 0.86
Phosphatidylcholine	56.3	554	59.1	196	35.3 ± 3.94
Phosphatidylethanolamine	32.4	319	30.8	102	31.9 ± 2.20
Cardiolipin	4.4	43	3.6	12	27.7 ± 2.63
Total					31.2 ± 2.63
	Intact inner membrane		Inner membrane supernatants		
Phospholipid	% Phospholipid composition	Amount of lipid P (μg)	% Phospholipid composition	Amount of lipid P (μg)	% Solubilized inner membrane phospholipids
Phosphatidylserine + sphyngomyelin	5.5	61	4.5	23	38.5 ± 0.79
Phosphatidylinositol	4.3	48	5.2	27	55.3 ± 2.82
Phosphatidylcholine	39.1	434	35.8	183	42.3 ± 2.01
Phosphatidylethanolamine	39.0	432	26.0	133	30.8 ± 0.48
Cardiolipin	12.2	136	28.3	145	102.0 ± 2.10
Total					55.2 ± 3.46

[a]The values are expressed as mean ± S.E.M. of four independent experiments.

phospholipid classes from the outer membranes. This is not the case for the inner membranes, where a selective solubilization of some phospholipids takes place. The differences are more clearly shown at 0.5% detergent concentration, when the overall solubilization of lipid phosphorus is about 55%. At this concentration, only 31% of the phosphatidylethanolamine is solubilized, while cardiolipin is solubilized completely. Interestingly a small but constant excess of cardiolipin is seen, indicating that more than 100% is in fact solubilized. This could mean that some cardiolipin molecules which were originally "hidden" in the intact membrane have been made accesible by the surfactant to the organic solvents used in the lipid extraction procedure (see Discussion).

Triton X-100 does not appear to selectively solubilize lipids with a particular fatty acid composition, though there tends to be less linoleic (C18:2) and linolenic (C18:3) acids in the solubilized material than in the original membranes (data not shown).

Respiratory Chain Components

Several components of the mitochondrial electron transport chain were investigated both in the solubilized and nonsolubilized subfractions of membranes after detergent treatment. The pattern but not the extent of

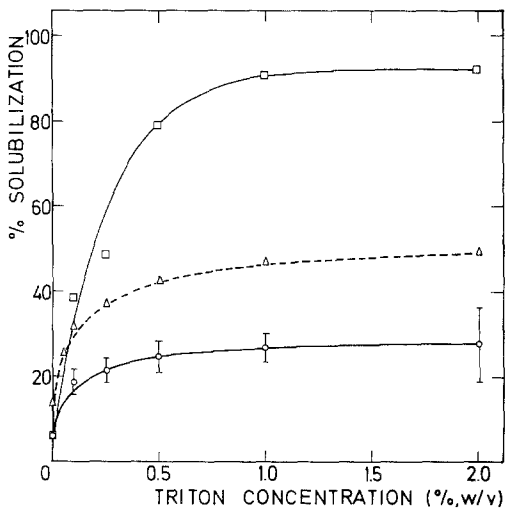


Fig. 5. Pattern of total flavin (○) and coenzyme *Q* (□) solubilization as a function of Triton X-100 concentration. Data for overall protein solubilization (△) are included for comparison. In the case of flavins, bars denote ± S.E.M. of at least five experiments. For coenzyme *Q*, points reflect mean values of two experiments.

solubilization of flavins is very similar to the overall protein solubilization, which is also included in the figure for comparative purposes (Fig. 5). However, only a relatively small proportion of the total flavins (~27%) was solubilized, even at 2% Triton X-100. The curve of coenzyme *Q* solubilization (Fig. 5) follows again a parabolic pattern, but, at Triton X-100 concentrations higher than 0.5% (w/v), most of the ubiquinone present in the membrane was solubilized.

The solubilization of cytochromes *a, b,* c_1, and *c* was investigated by differential spectrophotometry, at various Triton X-100 concentrations (Fig. 6). Figure 6a contains, in addition, the solubilization curve for total iron, which should account for the solubilization of the different iron-sulfur proteins, in addition of the cytochromes. The different cytochromes, or cytochrome chromophores, are solubilized following different patterns: Cytochrome *a* is solubilized following a nearly linear relationship to the concentration of surfactant; the other three follow parabolic patterns. A plateau is reached for cytochrome c_1 solubilization at Triton concentrations higher than 1%, but this does not happen for cytochromes *b* and *c*.

Inner-Membrane Enzyme Activities

The influence of Triton X-100 at different concentrations on various membrane-bound enzyme activities was tested. The results are expressed in Fig. 7 as a function of detergent concentration. The enzyme activities under consideration are ATPase, succinate:coenzyme *Q* oxidoreductase, and succinate:cytochrome *c* reductase. In each case, assays were performed on the

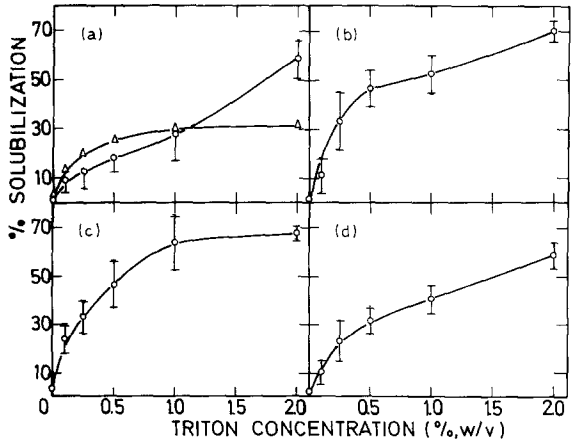


Fig. 6. Solubilization of cytochrome chromophores (○) and total iron (△) from the inner mitochondrial membranes at various Triton X-100 concentrations. (a) Cytochrome *a* ; (b) cytochrome *b;* (c) cytochrome c_1; (d) cytochrome *c*. Bars denote ±1 S.E.M. of five independent experiments.

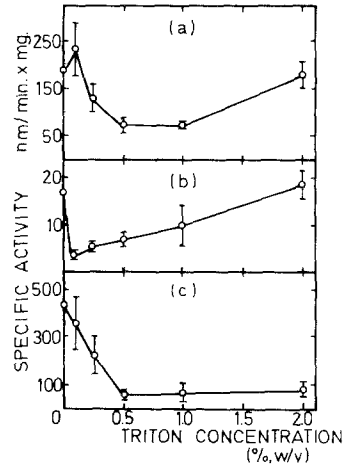


Fig. 7. Influence of Triton X-100 at different concentrations on various membrane-bound enzyme activities. (a) ATPase; (b) succinate:coenzyme *Q* oxidoreductase; (c) succinate:cytochrome *c* reductase. Bars denote ±1 S.E.M. of five independent experiments.

membrane suspension, after incubation with the detergent, and on the corresponding supernatant, containing the solubilized membrane fraction, after centrifugation. (Fig. 8)

After an initial short increase the specific activity of ATPase in membrane suspensions decreases gradually as the Triton concentrations increase, up to 1% (w/v); higher detergent concentrations tend to restore the activity, such that the native value is exceeded at a Triton X-100 concentration of 2% (Fig. 7a). In the supernatants, the ATPase specific activity nearly follows a linear relationship to the detergent concentration, exceeding that of the membrane suspension above 0.5% Triton X-100. (Fig. 8a). A representation of the percent solubilization of this enzyme activity as a function of detergent concentration is shown in Fig. 9.

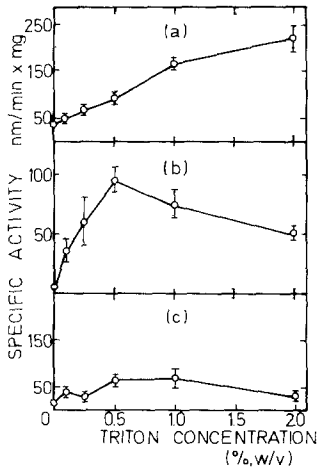


Fig. 8. Specific activities of several membrane-bound enzyme activities present in the solubilized fraction of mitochondrial inner membranes after treatment with Triton X-100 at various concentrations. (a) ATPase; (b) succinate:coenzyme *Q* oxidoreductase; (c) succinate:cytochrome *c* reductase. Bars denote ±1 S.E.M. of five independent experiments.

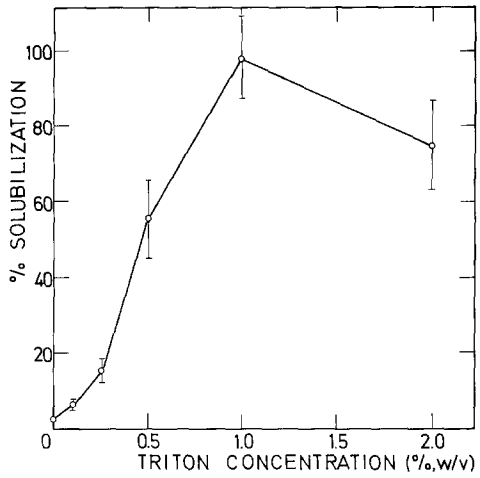


Fig. 9. Percent solubilization of mitochondrial ATPase activity as a function of Triton X-100 concentration. Bars denote ± S.E.M. of six independent experiments.

Succinate:coenzyme *Q* oxidoreductase (Fig. 7b) in membrane suspensions appears to be very sensitive to small detergent concentrations, and about 80% of its specific activity is lost after treatment with 0.1% Triton X-100. However, high detergent concentrations lead to a restoration of the specific activity, this restoration being complete at 2% Triton X-100. In this respect this enzyme is similar to ATPase, but the supernatants differ considerably, since their specific activities are much higher (up to 14-fold) than in the corresponding detergent-treated membrane suspensions. The activities in the supernatants increase with increasing detergent concentrations up to 0.5% Triton, and decrease with further additions of surfactant (Fig. 8b).

Finally, as could be expected from its very complex nature, the specific activity of succinate:cytochrome *c* reductase (Fig. 7c) was markedly inhibited by small amounts of detergent, and it could not be restored by further increases in Triton X-100 concentration. Furthermore, only negligible amounts of this enzyme activity were solubilized. (Fig. 8c)

Discussion

Characterization of Membrane Preparations

The membrane preparations under study were of great purity to the usual marker enzyme assays (monoamine oxidase, malate dehydrogenase, rotenone-sensitive and insensitive NADH:cytochrome *c* reductase). Their

contents in specific components are in accord with published data [26–28]. Slightly high values are found for some specific enzyme activities, and for some other components, when expressed per milligram of total protein. This is probably due to the fact that our membranes seem to contain only small amounts of extrinsic or peripheral proteins. This is confirmed by their low contents of cytochrome *c* (0.213 nmoles/mg protein) and by the fact that washing with 100 mM NaCl or $CaCl_2$ only solubilized 14–15% of the membrane protein.

Some of the main features of the behavior of mitochondrial inner and outer membranes towards Triton X-100 will now be discussed separately.

Mechanism of Action of Triton X-100

The mechanism of detergent solubilization of pure lipid bilayers has been studied in detail (see [29] and [30] for a review). Studies on the binding of some widely used surfactants to membranes have also been carried out [31, 32]. According to those studies, our chemical and ultrastructural data can be interpreted in terms of a stepwise dissociation of mitochondrial membranes with increasing Triton X-100 concentrations. This generalization is based mainly on experiments carried out at a membrane protein concentration of 4 mg/ml, when all the detergent in solution will be in the monomer form [1].

In the present study, membrane lysis is already clearly visible at 0.05% Triton X-100, and it appears to increase at higher detergent concentrations (Figs. 2–3). Initially, however, membrane solubilization is only modest, and membranes only begin to disintegrate and solubilize above 0.1% Triton X-100. As increasing amounts of Triton are added, further lysis occurs, together with membrane component solubilization. It has been shown that it is the monomer form of the detergent that is bound to membranes, and not the micellar [31, 33]. Binding of micelles to membranes when these are mixed with micellar solutions of surfactants is also possible [34]. The free equilibrium concentration of Triton X-100 remains below or equal to the CMC in the presence of membranes, the excess being bound to the membrane components [31, 35].

Solubilization, i.e., the loss of membrane structure, could be interpreted as a phase transition from a lamellar to a micellar phase. When the membranes are saturated with detergent, mixed micelles of lipids, detergent, and proteins are formed, resulting eventually in complete phase transition.

There is evidence that at least part of the membrane proteins are originally released as lipoprotein–detergent complexes [31, 35]. The relative proportions of the different micelle components depend on the amount of detergent bound.

Although it has been suggested for erythrocytes that the "trilaminar image" of the membrane was still observed in highly delipidated preparations

[2], this structure is lost in our inner-membrane sediments as soon as about 60% phospholipid is solubilized. It persists, however, in very small areas of the more damaged outer-membrane sediments, but in those cases the proportion of solubilized lipid P is not bigger than 40%.

When higher concentrations of detergent are added to membranes than is needed to induce the lamellar–micellar phase transition, separation of lipids from proteins takes place. The granular material which is found in the inner membrane pellets when the Triton concentration exceeds 0.5% appears to correspond mostly to protein-rich aggregates. The morphological appearance of such complexes is characteristic and is similar to that described by Helenius and Soderlund [35] with Semliki–Forest virus membranes.

Different Behavior of Inner and Outer Membranes. Selectivity

Mitochondrial inner and outer membranes differ in their biogenesis, ultrastructure, chemical composition, and functional complexity. Their behavior toward Triton X-100 is also considerably different. First, a relatively higher proportion of protein than of lipid is solubilized by Triton X-100 in the outer membrane, while the opposite holds for the inner membrane. The effect is more clearly observed for membrane suspensions containing 1 mg protein/ml. This is probably due to the fact that the actual amount of phospholipid is smaller in the inner- than in the outer-membrane suspensions, because of the higher protein:lipid ratio of the former. Another point that may help to explain their different behavior is the higher content of cholesterol in the outer membrane. It has been observed in model systems [37] as well as in biological membranes [36] that cholesterol confers to lipid bilayers some degree of resistance towards Triton X-100 solubilization.

The discussion of the differences between both types of mitochondrial membranes leads us to discuss the selective solubilization of some membrane components. The expression "selective solubilization" has, at least, two different meanings: It can refer to the ability or inability of the detergent to solubilize a given membrane component, or else it can denote the more or less sequential release of different membrane components. Both types of selective solubilization are observed when mitochondrial membranes are treated with Triton X-100. An example of the first type of selectivity is that, over the concentration range studied, Triton X-100 selectively fails to solubilize a very prominent band of the outer-membrane polypeptides (Fig. 4). Similarly, flavins are only poorly solubilized from the inner membranes. The second type of selectivity is observed in the case of ATPase solubilization (Fig. 9). A considerable proportion of this enzyme activity is solubilized at detergent concentrations between 0.50 and 1%, when the overall increase in protein solubilization is virtually zero.

Membrane lipids can also be selectively extracted with detergents [3, 38]. In our case, for Triton X-100 concentrations of 0.25 and 0.50%, this selectivity was only observed in the inner membrane. Cordiolipin was highly solubilized, while most of the phosphatidylethanolamine remained with the nonsolubilized material at the 0.50% detergent concentration, when 55% lipid phosphorus was solubilized on average. Although the spots corresponding to sphyngomyelin and phosphatidylserine were usually unresolved, and were pooled for phosphorus assay, some preliminary experiments with a different solvent system [39] showed that practically none of the sphyngomyelin could be solubilized by Triton X-100. The small amounts of cholesterol of the inner membrane were not solubilized either (data not shown). This is in accordance with the observations of other workers on erythrocyte ghosts [3] or sarcoplasmic reticulum membranes (G. Sarzała, personal comunication). The poor solubilization by Triton X-100 of phosphatidylethanolamine has also been observed in Semliki-Forest virus membranes [40]. Different explanations for this selective extraction of membrane components have been proposed, as reviewed by Helenius and Simons [1]. However, there is no strong experimental evidence in favor of any of them.

Selective solubilization of membrane proteins is best exemplified in the case of membrane-bound enzyme activities. In our case, electron-transport-chain components can also be conveniently studied. It is already known that treatment of mitochondria with various detergents under the appropriate conditions leads to the solubilization of cytochromes *b*, *c*, and c_1, while cytochromes *a* and a_3 remain insoluble [27]. Our data confirm this observation and show that, by careful control of detergent:membrane ratios, subtle differences in the solubilization of the different cytochromes may occur (Fig. 6), of which advantage can be taken for preparative purposes.

The three enzyme activities of the inner metochondrial membrane that have been considered represent three degrees of structural complexity: The ATP phosphohydrolase, located in Racker's *F*1 fraction, can be readily separated from the membrane and assayed in a lipid-free, soluble form [41]. This activity is not seriously affected by the detergent. Moreover, the enzyme activities of the supernatants and membrane suspensions are of about the same order of magnitude. It is remarkable that an important fraction of this enzyme activity is solubilized at detergent concentrations between 0.25 and 0.50%. In this range of concentrations, most of the cardiolipin of the mitochondrial inner membrane is solubilized (Table I). In fact, some "excess" cardiolipin molecules appear upon the detergent action. This would be in accord with the proposed specific interaction of cardiolipin and mitochondrial ATPase [42, 43]. The opposite extreme with respect to enzyme structural complexity is represented by succinate:cytochrome *c* reductase, whose activity requires the concerted operation of Green's "complex II" and

"complex III." Such structural complexity explains the nearly total loss of activity even at low concentrations of Triton X-100. Such alteration is probably due to disintegration of the complexes into their constituent elements, rather than to enzyme inactivation.

Succinate:coenzyme *Q* oxidoreductase represents a special case. Small concentrations of detergent cause its activity to be lost in membrane suspensions. However, when these suspensions are centrifuged, the supernatants show a specific activity one order of magnitude higher than the corresponding suspensions. This implies an appreciable increase in actual enzyme activity. This phenomenon is difficult to explain: A hypothesis would be that, while in the membrane suspension, some catalytic subunit(s) of the complex would remain loosely bound to an inhibitory subunit, the latter being firmly linked to the bulk of nonsolubilized material. The centrifugation would be able to break the weak bond between the catalytic and the inhibitory subunits. This would imply the specific solubilization of some components of the mitochondrial inner membrane by Triton X-100.

Previous studies by Egan et al. [4] have shown that optimum extraction of mitochondrial proteins and lipids occurs with detergents showing a hydrophile–lipophile balance of about 13; the corresponding value for Triton X-100 is 13.5. Our data for protein, lipid, and enzyme solubilization, obtained with purified mitochondrial membranes, are in good agreement with the previous ones, obtained with whole mitochondria. Our work also points to the possibility that a portion of the solubilized membrane protein may be in the form of lipid–protein complexes or very small membrane fragments. Work in grogress in our laboratory intend to test this hypothesis through the solubilization and reconstitution of some mitochondrial functional subunits.

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