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SELECTIVE SOLUBILIZATION OF THE COMPONENTS OF THE MITOCHONDRIAL INNER MEMBRANE BY LYSOLECITHIN*

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SUMMARY

1. Of various phospholipids tested, lysolecithin was the most efficient in the solubilization of the components of beef heart submitochondrial particles. Lysolecithin solubilized selectively nicotinamide nucleotide transhydrogenase, succinate dehydrogenase, NADH dehydrogenase and oligomycin-sensitive ATPase. Various cytochromes other than cytochrome *c* were only slightly solubilized.

2. The effect of various parameters, e.g. ionic strength, pH, time of centrifugation, and concentrations of lysolecithin and protein was investigated. Increasing times of centrifugation led to a partial sedimentation of NADH dehydrogenase, and a complete sedimentation of oligomycin-sensitive ATPase and cytochrome oxidase.

3. Further fractionation of the lysolecithin extract by centrifugation in the presence of low concentrations of cholate gave a complete separation of NADH dehydrogenase and transhydrogenase, indicating that these enzymes are not related functionally.

4. With the lysolecithin fractionation procedure a more than 10-fold purification of transhydrogenase was achieved. Polyacrylamide gel electrophoresis of the partially purified transhydrogenase in the presence of sodium dodecyl sulphate showed major increases in protein-stained bands corresponding to between 70 000 and 54 000 daltons.

5. A possible mechanism for the detergent action of lysolecithin involving a specific exchange of bound phospholipids for lysolecithin is discussed.

INTRODUCTION

Solubilization and fractionation of the components of biological membranes is a major problem in the study of most membrane-bound proteins. The available

Abbreviation: F_1, ATPase (coupling factor 1).

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information on the solubilization of membrane-bound proteins has generally been obtained through relatively non-systematic attempts employing various detergents and assorted biological membranes. In these attempts the main problem has been to release particular proteins efficiently with preserved enzymatic activities. In selecting a suitable detergent for this purpose various physical and chemical properties of the detergent are generally considered to be crucial, e.g. its critical micellar concentration, charge and hydrophobic-lipophilic balance. For example, positively charged detergents or detergents with a combination of low molecular weight and a high critical micellar concentration appear to be less suitable (cf. ref. 1 for a review). However, there are several exceptions to this rule, indicating that the molecular basis for the interplay between the membrane-bound protein, its lipid environment and the detergent still is virtually unknown. This is particularly valid in the case of membrane-bound proteins that require lipids for their catalytic activity. The advantage of using so-called "natural" detergents, e.g. lysolecithin, which is formed in the degradation of lecithin by phospholipase A, was early recognized by several workers [2–4]. Lysolecithin is a component of most biological membranes [5] and was therefore assumed not to inactivate irreversibly membrane-bound enzymes.

In the search for a suitable agent for solubilizing the mitochondrial nicotinamide nucleotide transhydrogenase, several detergents were tried essentially without success (cf. ref. 6). However, lysolecithin solubilized the enzyme efficiently without causing inactivation at higher concentrations [6], suggesting that lysolecithin, in contrast to other detergents, acted not only as an efficient detergent but also as a phospholipid capable of satisfying a lipid requirement of the transhydrogenase. This was later demonstrated directly using a delipidated preparation of the enzyme [7, 8]. In the present communication, lysolecithin was used to resolve the mitochondrial inner membrane (i.e. submitochondrial particles) and the conditions for resolution were carefully investigated. It was found that lysolecithin extracted preferentially nicotinamide nucleotide transhydrogenase, succinate dehydrogenase, NADH dehydrogenase and oligomycin-sensitive ATPase, whereas cytochrome oxidase was solubilized only to a minor extent. ATPase, transhydrogenase, succinate dehydrogenase and NADH dehydrogenase were further fractionated by centrifugation in the absence and in the presence of low concentrations of cholate. The efficiency of the fractionation procedure was monitored by enzymatic, spectrophotometric and sodium dodecyl sulphate-polyacrylamide gel analysis.

MATERIALS AND METHODS

EDTA-submitochondrial particles were prepared as described by Lee and Ernster [9]. Particle protein was determined according to the biuret method. In the case of highly diluted samples or samples containing high concentrations of lipids, protein was determined after trichloroacetic acid precipitation by staining with Amido black [10]. NADH dehydrogenase was assayed as NADH-ferricyanide reductase as described by Minakami et al. [11]. Succinate dehydrogenase was assayed as succinate-ferricyanide reductase in the presence of 1 μg antimycin per ml, according to King [12]. Cytochrome oxidase was estimated spectrophotometrically using reduced cytochrome *c* according to Sottocasa et al. [13], and ATPase activity (in the absence and in the presence of 1 μg oligomycin) was determined as described by

Pullman et al. [14]. Non-energy-linked transhydrogenase activity was measured as described earlier [15]. Absorbance spectra of various preparations (0.8 mg protein) were taken using 3-ml standard cuvettes (with 1 cm light path) and an Aminco-Chance DW-2 spectrophotometer; reduction of cytochromes were achieved by the addition of a few grains of $Na_2S_2O_4$. Analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis was carried out essentially as described by Poyton and Schatz [16] using 12 % gels and 0.5 % sodium dodecyl sulphate. Samples containing 20 μg protein were pretreated by heating at 100 °C for 5 min in the presence of 2.5 % sodium dodecyl sulphate, 2 % β-mercaptoethanol, 10 % glycerol and 10 mM sodium phosphate (pH 7.0). Extracts were concentrated using an Amicon ultrafiltration cell (Amicon Corp., Lexington, Mass., U.S.A.), equipped with a PM 10 filter. Cholic acid was purified according to Schneider et al. [17]. Chromatographically pure lysolecithin (egg), composed of 60 % palmitic acid and 30 % stearic acid, was obtained from Koch-Light Laboratories, Ltd. (Colnbrook, Buchs., England). Cardiolipin (beef heart), lecithin (egg), phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol were obtained from General Biochemicals (Chagrin Falls, Ohio, U.S.A.). Sonication of phospholipids was carried out as described by Kagawa et al. [18]. Other biochemicals were purchased from Sigma Chem. Co. (St. Louis, Mo., U.S.A.) or from Boehringer, Mannheim GmbH (Mannheim, Germany).

RESULTS

Table I shows a comparison of the solubilization of various activities in submitochondrial particles by different phospholipids, expressed as percent activity recovered in the supernatant as compared to the sum of the activities recovered in the supernatant plus the pellet. Lysolecithin solubilized* 100 % of transhydrogenase, succinate dehydrogenase, NADH dehydrogenase and about 63 % of the oligomycin-sensitive ATPase; at 25 °C the latter enzyme was solubilized to an extent of about 73 %. Cardiolipin solubilized about 40 % of the oligomycin-insensitive ATPase at 25 °C, whereas no ATPase activity was recovered in the supernatant after cardiolipin treatment at 0 °C. The total recovery at 0 °C was only 8 %, indicating that cold-labile F_1 was solubilized. Cardiolipin also solubilized about 28 % of NADH dehydrogenase, but no transhydrogenase or succinate dehydrogenase. Phosphatidylserine and phosphatidylinositol were relatively ineffective and only partially solubilized ATPase. Lecithin, phosphatidylethanolamine and lysophosphatidylethanolamine were without effect with respect to solubilization, but stimulated transhydrogenase slightly (not shown). Recoveries of activities (not shown), although variable, were generally higher than 100 %, in particular in the case of NADH dehydrogenase and cytochrome oxidase. However, treatment with either cardiolipin or lysolecithin was found to be slightly inhibitory in the case of transhydrogenase and succinate dehydrogenase, possibly reflecting a poor activating effect of these phospholipids (cf. ref. 8). As expected, only lysolecithin extracted a major amount of protein (Table I). It may be concluded from the data in Table I that, of the phospholipids tested, lysolecithin was the most efficient solubilizing agent.

* In this paper the term solubilization is used to denote displacement of membrane-bound proteins from the bulk of the membrane into mixed phospholipid micelles of lower density, rather than release of lipid-free protein (cf. Discussion).

TABLE I

EFFECTS OF PHOSPHOLIPIDS ON THE SOLUBILIZATION OF VARIOUS ACTIVITIES IN SUBMITOCHONDRIAL PARTICLES

The chilled extraction medium contained 8 mg phospholipid sonicated under nitrogen to clarity in 0.1 M Tris/acetate (pH 7.4), 3 mM EDTA and 0.25 M sucrose. After addition of 8 mg submitochondrial particles the mixture was centrifuged for 30 min at $15000 \times g$. The supernatant was decanted, the pellet suspended in 1 ml 0.1 M Tris/acetate (pH 7.4) and 3 mM EDTA, and the various activities assayed immediately. During the extraction procedure temperature was kept between 0 and 4 °C, except for the extraction of cold-labile F_1 in which case temperature was 25 °C. Solubilization is expressed as percent activity recovered in the supernatant as compared to the total activity in the supernatant plus the pellet.

Phospholipid	Solubilization (%)						Protein solubilized (%)
	Trans-hydrogenase	Succinate dehydrogenase	NADH dehydrogenase	ATPase		Cytochrome oxidase	
				0 °C	25 °C		
Disphosphatidylglycerol	0	0	28	0	40.5	0	6.3
Lysolecithin	100	100	100	63.5	73	8.2	35.6
Phosphatidylserine	0	0	0	1.2	11	0	2.0
Phosphatidylinositol	0	0	0	22	46.5	0	2.7

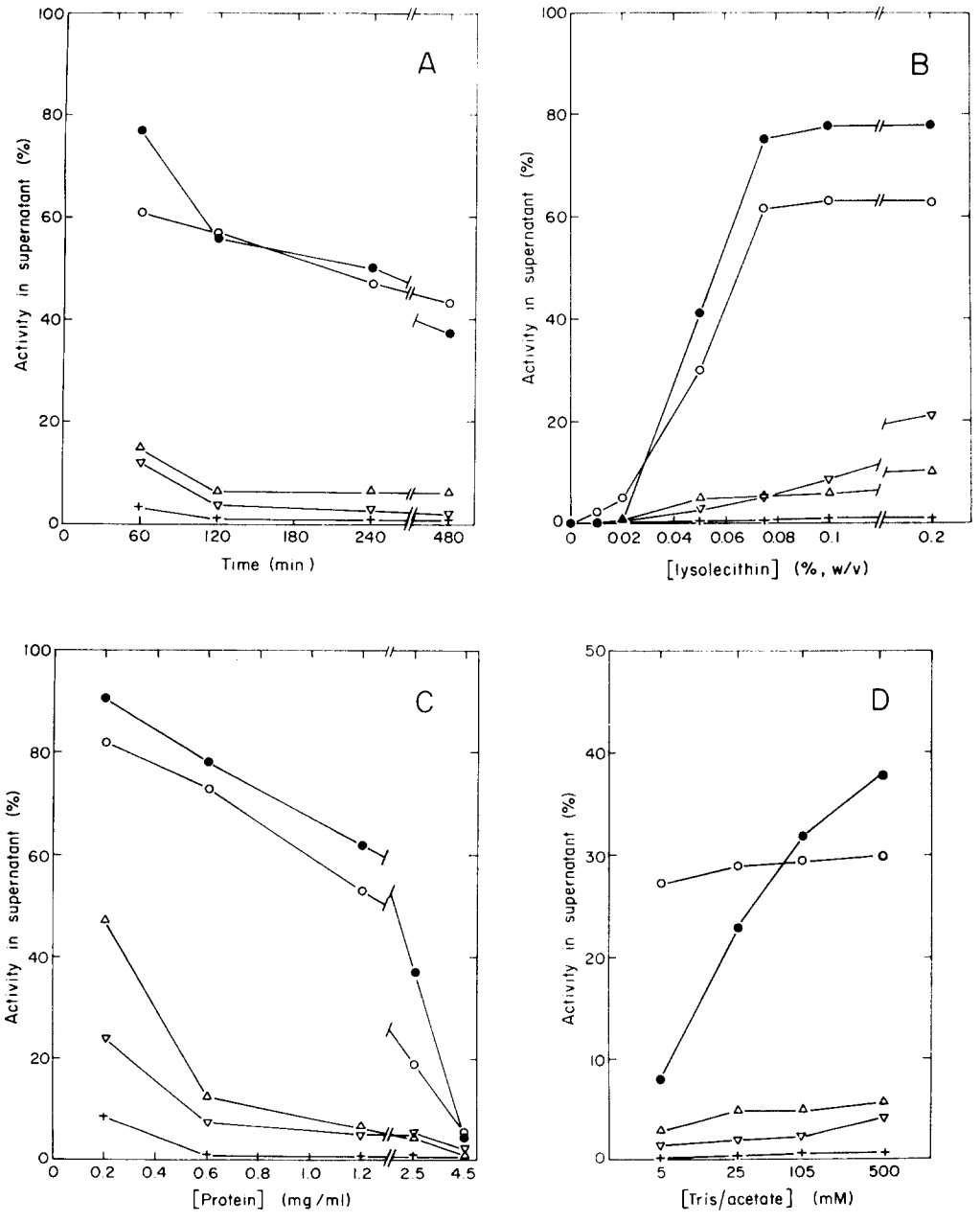

Fig. 1. Effects of time of centrifugation (A), concentration of lysolecithin (B), concentration of protein (C) and ionic strength (D) on the solubilization of various activities from submitochondrial particles by lysolecithin. Activities were: ●, transhydrogenase; ○, succinate dehydrogenase; △, NADH dehydrogenase; ▽, ATPase, and +, cytochrome oxidase. Except for each parameter that was varied in the separate experiments in A–D, the replacement of 0.25 M sucrose for 1 mM dithiothreitol, and a protein concentration of 1.5 mg/ml in the experiment of D, the conditions for extraction were as described in Table I. In B, C and D, the extracts were centrifuged for 4 h at 200000 $\times g$. Concentrations of Tris/acetate in D are plotted in a logarithmic scale.

Fig. 1 shows the effect of various parameters on the solubilization of enzymatic activities in submitochondrial particles by lysolecithin. Increasing the time of centrifugation of the lysolecithin extract led to a precipitation of mainly oligomycin-sensitive ATPase, NADH dehydrogenase and cytochrome oxidase (Fig. 1A), whereas the bulk of the transhydrogenase and succinate dehydrogenase remained in the supernatant. Treatment with various concentrations of lysolecithin at a protein concentration of 1 mg/ml revealed that solubilization occurs within a concentration range of 0.02–0.08 % (Fig. 1B). Protein concentration affected the solubilization markedly (Fig. 1C). Low concentrations of protein (0.2 mg/ml) resulted in about 91 and 82 % solubilization of transhydrogenase and succinate dehydrogenase, respectively, and these values decreased with increasing protein concentration. Similar solubilization patterns, although more pronounced, were seen with NADH dehydrogenase, oligomycin-sensitive ATPase and cytochrome oxidase (Fig. 1C). Increasing ionic strength, in the form of increasing concentrations of buffer (Fig. 1D), showed negligible effects on the solubilization of the activities measured, except for transhydrogenase, in which case solubilization increased with increasing ionic strength. Replacement of high buffer concentrations with high concentrations of salts (e.g. KCl) did not alter this ionic strength dependence. pH (between 6.0 and 8.0), prolonged incubation times and temperature (incubation at 30 °C instead of 0 °C), had little if any effect on the solubilization patterns (not shown). A more extensive extraction of mainly transhydrogenase, succinate dehydrogenase and NADH dehydrogenase was accomplished by

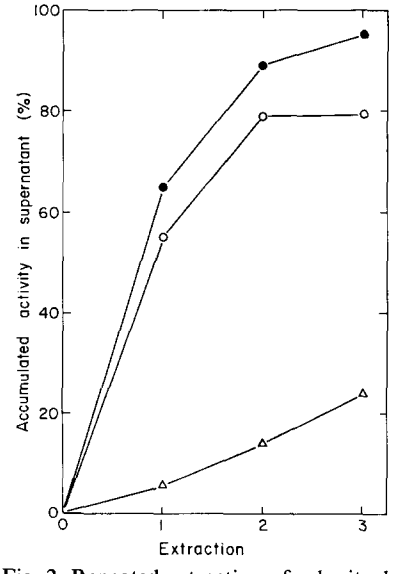

Fig. 2. Repeated extraction of submitochondrial particles by lysolecithin. Activities were: ●, transhydrogenase; ○, succinate dehydrogenase, and △, NADH dehydrogenase. The composition of the extraction medium was as described in Table I, except that the concentration of protein was 1.5 mg/ml. Volume was 28 ml. After incubation for 30 min at 0° C, the mixture was centrifuged for 4 h at 200000 × *g*, the supernatant decanted and the pellet suspended as described in Table I. A small aliquot (0.1 ml) of the pellet was withdrawn for assay and the remainder was suspended in 28 ml of a new lysolecithin mixture, incubated and centrifuged as above. This extraction was then repeated once more.

repeated extraction with lysolecithin (Fig. 2). However, ATPase and cytochrome oxidase were still not solubilized by this procedure. Attempts at increasing protein and lysolecithin concentrations above 2 mg/ml in parallel, to achieve a better absolute yield of solubilized components, were less successful and resulted in a lower extent of relative solubilization. A lysolecithin extract obtained with 0.1 M Tris/acetate and 1 mg/ml of lysolecithin and protein contained after centrifugation for 4 h at 200 000 × *g* about 15 % of the original protein and was virtually devoid of cytochromes *b* and *a* as evident from a dithionite-reduced minus oxidized absorbance spectrum (Fig. 3B). The absorption peaks appearing at 550, 520 and 420 nm correspond to the α, β and γ bands, respectively, of cytochrome *c*. For a comparison, the spectrum of submitochondrial particles is shown in Fig. 3A.

The supernatant obtained after high speed centrifugation, i.e. at 200 000 × *g* for 4 h, was further fractionated by recentrifugation in the presence of cholate (Table II). After 4 h at 200 000 × *g*, in the presence of 0.06 % cholate, about 18 % of transhydrogenase, 44 % of succinate dehydrogenase and close to 100 % of NADH dehydrogenase remained in the supernatant. Part of the sedimented succinate dehydrogenase was apparently inactivated since the total recovery for this enzyme was only about 60 %; reactivation by added phospholipids was not successful. About 15 % of total protein was precipitated during the centrifugation, indicating a major purification of mainly transhydrogenase. In a similar experiment the different steps resulted in a more

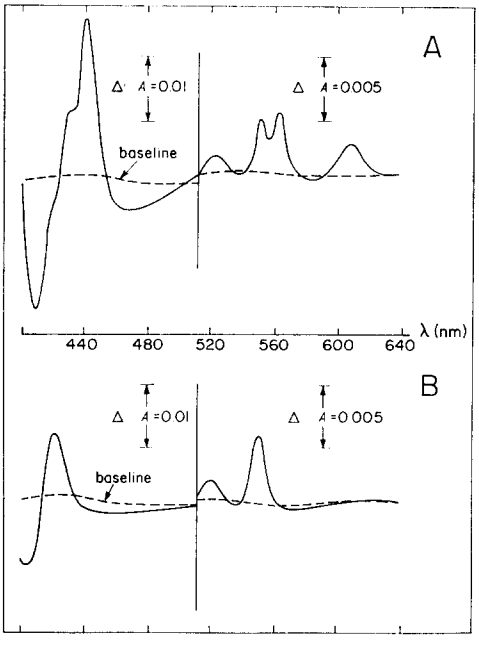

Fig. 3. Dithionite-reduced minus oxidized spectra of submitochondrial particles (A) and lysolecithin extract (B). The lysolecithin extract was obtained using 1 mg lysolecithin and 1 mg protein per ml, a medium containing 0.1 M Tris/acetate (pH 7.4), 3 mM EDTA and 1 mM dithiothreitol, and centrifugation for 4 h at 200000 × *g*. Spectra were recorded as described in Materials and Methods.

TABLE II

FRACTIONATION OF LYSOLECITHIN EXTRACT BY CENTRIFUGATION IN THE PRESENCE OF CHOLATE

The lysolecithin extract was obtained as described in Fig. 3. 0.06 % cholate was added and the extract was recentrifuged for 4 h at 200000 × *g*. The supernatant was decanted and the pellet was suspended as described in Table I.

Enzyme	Activity remaining in supernatant (%)	Activity precipitated (%)	Total recovery (%)
Transhydrogenase	18	82	100
Succinate dehydrogenase	44	14	58
NADH dehydrogenase	98	2	100

than 10-fold purification of the transhydrogenase as shown in Table III. Analysis of the various fractions by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate showed that lysolecithin treatment preferentially solubilized proteins from the submitochondrial particles with molecular weights of 70 000, 58 000–54 000 (three close bands), 34 000 and 29 000 (Figs. 4A and 4B); lysolecithin and other phospholipids gave rise to stained bands close to the front of the gels. The largest band corresponding to a protein of a molecular weight of 29 000 may be identified as the so called hydrophobic protein described earlier [19]. Similarly, the precipitate obtained after high speed centrifugation in the presence of cholate appeared to contain large amounts of the same protein. A conclusive identification of the bands corresponding to molecular weights between 70 000 and 54 000 (Figs. 4B and 4C) is difficult although the size of these bands correlate clearly to the relative specific activities of transhydrogenase and succinate dehydrogenase in the various fractions. One of the high molecular weight bands may be accounted for by the larger 67 000 molecular weight subunit of succinate dehydrogenase [20]. A less clear correlation was found with the 34 000 molecular weight band which may be the smaller subunit of succinate dehydrogenase [20]. The possibility that the 70 000–54 000 molecular weight bands are due to NADH dehydrogenase and/or ATPase is less likely since the bulk of these enzymes were not extracted in the first fractionation step (Fig. 1). In addition,

TABLE III

PURIFICATION OF NICOTINAMIDE NUCLEOTIDE TRANSHYDROGENASE BY LYSOLECITHIN

Conditions were as described in Fig. 3 and Table II except that the protein concentration was 1.4 mg/ml in the first extraction step.

	Total activity (nmol/min)	Specific activity (nmol/min per mg protein)	Recovery (%)
Submitochondrial particles ± lysolecithin	9600	120	100
Lysolecithin extract	3300	480	35
Precipitate obtained after centrifugation in the presence of 0.06 % cholate	2250	1400	23.5

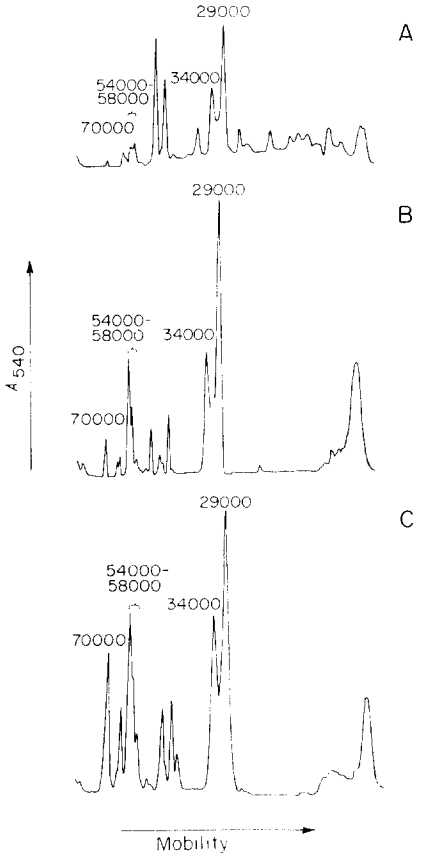

Fig. 4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis patterns of submitochondrial particles (A), lysolecithin extract (B) and precipitate obtained after centrifugation in the presence of cholate (C). Lysolecithin extract and precipitate was obtained as described in Table II. Electrophoresis was carried out as described in Materials and Methods.

the small amount of NADH dehydrogenase that was extracted remained in the supernatant after the last fractionation step. ATPase that was extracted was not fully recovered throughout the fractionation procedure and it is therefore possible that the major subunits of cold-inactivated ATPase contributed to the 58 000–54 000 and 34 000 molecular weight bands in the gel shown in Fig. 4C.

DISCUSSION

The data presented here show that various lipids may act as detergents in the solubilization of proteins from the mitochondrial inner membrane. Of the lipids tested lysolecithin was found to be the most efficient detergent for bringing about the selective release of transhydrogenase, succinate dehydrogenase, NADH dehydrogenase and oligomycin-sensitive ATPase. Cytochromes *a* and *b* were solubilized only to a minor extent. Solubilization by lysolecithin was strongly influenced by the concentration of protein as well as by the concentration of detergent, although pH and

temperature had little if any effect. Ionic strength influenced selectively the release of transhydrogenase. Lysolecithin was previously shown to have detergent properties [31]. When added to membranes such as erythrocyte membranes [2], myelin [3] or sarcoplasmic reticulum [4], lysolecithin binds strongly to the membrane and causes a dispersion or "solubilization" of the membrane components, i.e. lipids and proteins, at concentrations of the order of 2–10 mol lysolecithin per mol lipid [22]. Dispersion occurs as a transfer of the membrane components into highly asymmetric and thermodynamically stable mixed aggregates or micelles of molecular weights of approx. 10^6 [22–24]. In the absence of additional lipids lysolecithin forms micelles of molecular weights of approx. 92 000 [24].

The release of proteins from membranes can be visualized to involve several parameters, e.g. localization of the protein in the membrane, protein-protein interactions, lipid composition of the membrane and the presence of tightly bound lipids. Obviously, an evaluation of the extraction pattern for a membrane-bound protein obtained with a detergent is therefore complicated. The extents of solubilization of cytochrome oxidase, oligomycin-sensitive ATPase, NADH dehydrogenase, succinate dehydrogenase and transhydrogenase obtained after lysolecithin treatment may be assumed to reflect partially the localization of these proteins in the membrane. Cytochrome oxidase [25] and presumably also oligomycin-sensitive ATPase [26] are transmembraneous proteins which were less efficiently solubilized, in contrast to NADH dehydrogenase, succinate-dehydrogenase and transhydrogenase which were completely solubilized and probably are more superficially located on the outside of the submitochondrial particle membrane (cf. ref. 27). Protein-protein interactions would lead to a decreased solubilization of a particular protein and simultaneous release of intact and functional complexes as has been found with mitochondrial proteins employing synthetic detergents [28]. An influence of the lipid composition of the membrane on the solubilization is an interesting possibility. It may be noticed that, apart from a partial solubilization of ATPase (oligomycin sensitive as well as oligomycin insensitive) by phosphatidylinositol, cardiolipin solubilized not only oligomycin-insensitive ATPase (F_1) as demonstrated earlier [29, 30], but also some NADH dehydrogenase. Release of F_1 appears to be related to a charge interaction between cardiolipin and the membrane components rather than a detergent effect since the critical micelle concentration value for cardiolipin is extremely low [31] and the release restricted to acidic phospholipids [29, 30]. Such a charge-dependent effect may involve a transfer of F_1 from membrane-associated cardiolipin, or another negatively charged component, to free cardiolipin micelles. A similar mechanism may be responsible for the release of NADH dehydrogenase by cardiolipin since it was shown earlier that cardiolipin is essential for the binding of this enzyme to the membrane [32, 33]. In the case of transhydrogenase the energy-linked reaction requires lecithin [34] which may indicate that in the intact membrane the lipid environment of the enzyme is composed preferentially of lecithin. In sarcoplasmic reticulum lysolecithin was shown to extract and replace lecithin rather than other phospholipids, e.g. phosphatidylethanolamine [4]. Thus, a similar exchange of lecithin for lysolecithin in the mitochondrial membrane would lead to a local increase in fluidity of the lipid environment [35] of the transhydrogenase and eventually to a release of the protein from the membrane in the form of a mixed micelle. Likewise, release [36] and purification [37] of oligomycin-sensitive ATPase by lysolecithin demonstrated earlier

may occur as a consequence of a specific release of the 29 000 molecular weight protein that appears to constitute one of the major membrane-associated components of the ATPase complex. Once released from the membrane the mixed micelles, which are assumed to be of relatively uniform sizes, are separated according to their density that appears to be determined mainly by the included proteins. In the presence of an additional detergent with a high critical micelle concentration value, e.g. cholate, the size of the micelles are markedly decreased and the difference in their relative weights increased, thus allowing a further separation by differential centrifugation.

Selective release of membrane components was earlier demonstrated employing various synthetic detergents, e.g. Triton X-100, sodium dodecyl sulphate and sodium deoxycholate [38, 39]. In these cases no enzymatic activities were estimated.

The extraction of transhydrogenase with lysolecithin followed by differential centrifugation in the absence and in the presence of cholate described in this paper is a simple procedure for the preparation of small amounts of partially purified enzyme. Preparation of larger amounts of somewhat less pure enzyme is more easily accomplished with cholate-ammonium sulphate fractionation of submitochondrial particles [34]. Activity measurements and analyses of the various fractions obtained after lysolecithin fractionation by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate indicated that transhydrogenase was completely separated from NADH dehydrogenase by differential centrifugation in the presence of cholate. A functional relationship between transhydrogenase and NADH dehydrogenase as has been proposed by Hatefi and coworkers [40, 41] seems therefore unlikely. By correlating the specific activities of the enzymes tested in the various fractions with the densitometric traces of the corresponding gels, it appears that transhydrogenase, or the larger subunit of the enzyme, may have a molecular weight of between 70 000 and 54 000, although at the present time a possible contribution to these bands of subunits cold-inactivated F_1 cannot be ruled out.

It appears likely that due to its chemical and physical similarities with native phospholipids lysolecithin may solubilize most biological membranes into their active components provided that the solubilization is carried out under proper conditions.

REFERENCES

1 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79
2 Chapman, D. (1968) in Membrane Models and the Formation of Biological Membranes (Bolis, L. and Pethica, B. A., eds.), North Holland Publ. Co., Amsterdam
3 Gent, W. L. G., Gregson, N. A., Gammack, D. B. and Raper, J. H. (1964) Nature 204, 553–555
4 Deamer, D. W. (1973) J. Biol. Chem. 248, 5477–5485
5 Crane, F. L. and Sun, F. F. (1972) in Electron and Coupled Energy Transfer in Biological Systems (King, T. E. and Klingenberg, M., eds.), Vol. 1B, pp. 477–587, Marcel Dekker, Inc., New York.
6 Rydström, J., Hoek, J. B. and Hundal, T. (1974) Biochem. Biophys. Res. Commun. 60, 448–455
7 Rydström, J. (1975) Fed. Proc. 34, 577
8 Rydström, J., Hoek, J. B., Ericsson, B. and Hundal, T. (1976) Biochim. Biophys. Acta 430, 419–426
9 Lee, C. P. and Ernster, L. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds.), Vol. 10, pp. 543–548, Academic Press, New York
10 Schaffner, W. and Weissman, C. (1973) Anal. Biochem. 56, 502–514
11 Minakami, S., Ringler, R. L. and Singer, T. P. (1962) J. Biol. Chem. 237, 569–576
12 King, T. E. (1963) J. Biol. Chem. 238, 4032–4036

13 Sottocasa, G. L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) J. Cell Biol. 32, 415–438
14 Pullman, M. E., Penefsky, H. S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322–3329
15 Teixeira da Cruz, A., Rydström, J. and Ernster, L. (1971) Eur. J. Biochem. 23, 212–219
16 Poyton, R. O. and Schatz, G. (1975) J. Biol. Chem. 250, 752–761
17 Schneider, D. L., Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 247, 000–000
18 Kagawa, Y., Kandrach, A. and Racker, E. (1973) J. Biol. Chem. 248, 676–684
19 Capaldi, R. A., Komai, H. and Hunter, D. R. (1973) Biochem. Biophys. Res. Commun. 55, 655–659
20 Davis, K. A. and Hatefi, Y. (1971) Biochemistry 10, 2509–2516
21 Bangham, A. D. and Horne, R. W. (1964) J. Mol. Biol. 8, 660–668
22 Saunders, L. (1957) J. Pharm. Pharmacol. 9, 834–840
23 Robinson, N. (1961) J. Pharm. Pharmacol. 13, 53–57
24 Saunders, L. (1966) Biochim. Biophys. Acta 125, 70–74
25 Eytan, G. D., Carrol, R. C., Schatz, G. and Racker, E. (1975) J. Biol. Chem. 250, 8598–8603
26 Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477–5487
27 Ernster, L. and Kuylenstierna, L. (1969) in Mitochondria Structure and Function (Ernster, L. and Drahota, Z., eds.), Vol. 17, pp. 5–32, Proc. Eur. Biochem. Soc. Fifth Meet. Prague 1968, Academic Press, London
28 Hatefi, Y. (1976) in the Enzymes of Biological Membranes (Martonosi, A. N., ed.), Vol. 4, Plenum Publishing Corp., London, in the press
29 Toson, G., Contessa, A. R. and Bruni, A. (1972) Biochem. Biophys. Res. Commun. 48, 341–347
30 Lee, C. P., Huang, C. H. and Cierkosz, B. I. T. (1974) in Membrane Protein in Transport and Phosphorylation (Azzone, G. F., Klingenberg, M. E., Quagliariello, E. and Siliprandi, N., eds.), Proc. Int. Symp., Bressanone 1974, North-Holland Publ., pp. 161–170, Co., Amsterdam
31 Tanford, C. (1973) in The Hydrophobic Effect, John Wiley and Sons, Inc., New York
32 Awasthi, Y. C., Berezney, R., Ruzicka, F. J. and Crane, F. L. (1969) Biochim. Biophys. Acta 189, 457–460
33 Awasthi, Y. C., Ruzicka, F. J. and Crane, F. L. (1970) Biochim. Biophys. Acta 203, 233–248
34 Rydström, J., Kanner, N. and Racker, E. (1975) Biochem. Biophys. Res. Commun. 67, 831–839
35 Inoue, K. and Kitagawa, T. (1974) Biochim. Biophys. Acta 363, 361–372
36 Sadler, M. H., Hunter, D. R. and Haworth, R. A. (1974) Biochem. Biophys. Res. Commun. 59, 804–812
37 Serrano, R., Kanner, B. I. and Racker, E. (1976) J. Biol. Chem. 251, 2453–2461
38 Kirkpatrick, F. M., Gordesky, S. E. and Marinetti, G. V. (1974) Biochim. Biophys. Acta 345, 154–161
39 Ehrhart, J. C. and Chauveau, J. (1975) Biochim. Biophys. Acta 375, 434–445
40 Hatefi, Y. (1973) Biochem. Biophys. Res. Commun. 50, 978–984
41 Hatefi, Y. and Hanstein, W. G. (1973) Biochemistry 12, 3515–3522