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SOLUBILITY CHARACTERISTICS OF *MICROCOCCUS LYSODEIKTICUS* MEMBRANE COMPONENTS IN DETERGENTS AND CHAOTROPIC SALTS ANALYZED BY IMMUNOELECTROPHORESIS

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Summary

In order to evaluate the effectiveness and selectivity of various reagents in the solubilization of bacterial membranes, membranes of *Micrococcus lysodeikticus* were treated with detergents and chaotropic agents. The composition of the extracts so obtained was analyzed by rocket and two-dimensional immunoelectrophoretic techniques. Recovery of succinate-, malate-, and reduced nicotinamide adenine dinucleotide- (NADH) dehydrogenases, ATPase, succinylated lipomannan and cytochromes in the extracts was measured. Treatment with a variety of non-denaturing detergents produced extracts that were generally qualitatively uniform although quantitative differences were observed. The degree of extraction of various components was correlated with the hydrophile-lipophile balance. Several chaotropic agents were also evaluated as reagents for membrane solubilization. These agents were less effective in extraction of bulk protein, but produced extracts enriched in some membrane components.

Introduction

Solubilization of biological membranes is a procedure which is fundamental to the resolution and purification of membrane components and their study at the molecular level. Disruption of membranes and release of their constituents has been achieved through treatment with a variety of agents such as surfactants [1—3], chaotropic salts [4—6], and nonpolar solvents [7—12]. The use of detergents to disrupt biological membranes has become increasingly important with the recent application of crossed (two-dimensional) immunoelectro-

phoresis to the analysis of solubilized membrane components [13—15]. The quantitative nature of immunoelectrophoretic techniques makes them suitable for a comparative study of membrane extracts prepared under different conditions. Investigations of this type should therefore aid in the selection of an agent which is optimal for particular experimental purposes such as membrane enzyme and/or antigen purification.

In the present communication, a qualitative and quantitative comparison is made of solubilized extracts prepared by treatment of bacterial membranes with a variety of detergents and chaotropic agents. In addition, the effects of detergent/protein ratio and the hydrophilic-lipophilic character of a series of detergents are evaluated.

Materials and Methods

Cultivation of organism. Micrococcus lysodeikticus (NCTC 2665) was grown on peptone-water-yeast extract medium [16] in 2800-ml Fernbach flasks filled to 750 ml and aerated by shaking. Cells were harvested after 17—18 h of growth at 30° C.

Preparation of membranes. Cells were washed once with distilled water and once with 50 mM Tris-HCl (pH 7.5). Washed cells were concentrated 15-fold in buffer and lysozyme (EC 3.2.1.17, hen's egg, A grade, Calbiochem, La Jolla, CA) was added at 200 μg/ml. This suspension was incubated at room temperature with stirring for approx. 45 min. A few crystals of deoxyribonuclease (EC 3.1.4.5, bovine pancreas, Sigma Chemical Co., St. Louis, MO) were added to the lysate. Membranes were sedimented by centrifugation at 18 000 rev./min (39 000 × *g*) for 30 min in a JA-20 rotor in a Beckman J-21C (Beckman Instruments, Palo Alto, CA) high speed centrifuge. The pelleted material was resuspended in buffer and centrifuged at 2500 rev./min for 10 min to sediment any debris. Membranes were collected at 18 000 rev./min and subjected to five washes in buffer and stored at —70° C until required for further use.

Solubilization of membranes. Membranes were suspended in 50 mM Tris-HCl, pH 7.5, to a final protein concentration of 20 mg/ml. The solubilizing agent was added from a concentrated stock solution and the mixture incubated with stirring for 30 min at room temperature. At the end of the incubation period, the insoluble residue was sedimented by centrifugation at 16 000 rev./min (30 000 × *g*) in the JA-20 rotor. The supernatant fluids containing the extracted membrane components were dialyzed against Tris-HCl buffer containing 4% Triton X-100.

Preparation of antiserum. Antiserum to unfractionated membrane was raised in New Zealand white rabbits by subcutaneous injection with membranes (1.0 mg protein/injection) dispersed in 50% Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI). Rabbits were inoculated on three consecutive weeks, rested 1 week, given a booster injection on the 5th week and bled 10 days later. Rabbits were bled through the marginal ear vein on three consecutive weeks; a booster injection was administered at the time of the last bleeding. After 7—10 days the animals were bled again and the schedule repeated.

Monospecific antiserum was raised to ATPase (EC 3.6.1.3) which was

released from membranes by a low-ionic strength shock wash [17] and purified (Huberman, M. and Salton, M.R.J., unpublished data) by chromatography on DEAE-Sephadex (Pharmacia Fine Chemicals Inc., Piscataway, NJ). Rabbits were immunized by a single subcutaneous injection of 2.0 mg purified antigen dispersed in 30% Freund's incomplete adjuvant and bled weekly. The specificity of the antiserum was evaluated by crossed immunoelectrophoresis; only one precipitate was obtained when solubilized membrane preparations were used as antigen.

Pooled antiserum was fractionated by precipitation with $(NH_4)_2SO_4$ and dialysis against water and sodium acetate buffer (pH 5.0) [18]. After removal of precipitated lipoproteins [18], the immunoglobulin preparations were stored at 4°C in 0.1 M NaCl, 0.015 M NaN_3.

Immunoelectrophoretic techniques. Rocket immunoelectrophoresis and rocket affinoelectrophoresis were performed as previously described [19]. $MnCl_2$ (50 μM) and $CaCl_2$ (50 μM) were incorporated in the buffer and agarose for affinoelectrophoresis [20]. Electrophoresis at 2.5 V/cm was performed for 12—16 h. All measurements were based on duplicate or triplicate determinations made on separate plates. One extract was used as standard and its content arbitrarily set at 100 units/ml.

Two-dimensional, crossed immunoelectrophoresis [21] was performed essentially as previously described [22]. Electrophoresis of antigens at 7.9 V/cm for 1.25 h in the first dimension was followed by electrophoresis in the second dimension for 13—15 h at 2.9 V/cm. Tris-barbital buffer (4.48 g Tris, 8.86 g diethylbarbituric acid/l, pH 8.6) was used for all electrophoretic procedures. Immunoplates were processed and stained for protein and enzymatic activity [14]. Additional (2—5) washes of pressed immunoplates were required to reduce background staining. Phenazine methosulfate (25 μg/ml, Sigma Chemical Co.) was added to the reaction mixture for succinate dehydrogenase (EC 1.3.99.1) and immunoplates processed for ATPase were developed in 25% (w/v) $Na_2S \cdot 9H_2O$.

Analytical procedures. Protein was determined by the method of Lowry et al. [23] with bovine serum albumin as standard. Membrane preparations and extracts were incubated with 0.1% sodium dodecyl sulfate prior to addition of reagents for protein determination to solubilize the membranes and to eliminate interference by non-ionic detergents [24].

Cytochrome difference spectra were determined with a Cary 15 spectrophotometer. Membrane suspensions (250 μg protein/ml) in Tris-HCl buffer were placed in 1-cm quartz cuvettes and the material in the sample beam was reduced by the addition of a few crystals of sodium hydrosulfite. An estimate of total cytochrome content was made from the Soret peak [25,26].

Chemicals. Seakem HGT agarose (Marine Colloids, Inc., Rockland, ME) was used for all electrophoretic procedures. Concanavalin A was obtained as a lyophilized powder (Miles Laboratories, Elkhart, IN) and was used without further treatment. Triton X-100 was obtained from Research Products International (Elk Grove Village, IL); Nonidet P40 was a gift of Shell Chemicals U.K. Limited (London, England); Brij 58 was a gift of ICI Americas Inc. (Wilmington, DE); Berol 043 was a gift of Dr. F. Blomberg, The Rockefeller University (New York, NY); Tween 80 was obtained from Fisher Chemical Co. (Fairlawn, NJ)

and deoxycholate from Schwartz/Mann (Orangeburg, NY). All other surfactants were obtained from Sigma. Thiocyanate salts were obtained from ICN Pharmaceuticals Inc. (Plainview, NY). All other chemicals were obtained from standard commercial sources and used without further purification.

Results

In order to compare the effectiveness of various agents in the solubilization of bacterial membranes, washed membranes of *M. lysodeikticus* were treated with these reagents and the recovery of membrane components in the soluble fraction was measured. This fraction was operationally defined as that which failed to sediment at 30 000 × *g* in 30 min. Under these conditions a firm pellet and a 'fluffy layer' [27] were usually formed. The supernatant was decanted and analyzed, while the fluffy layer was retained with the remainder of the residue. The technical difficulties due to the presence of this layer were reflected in slight variations in recovery from one extract to the next. Centrifugation under greater force was avoided; this treatment may be used for further fractionation of detergent extracts [28—30].

The soluble fractions so obtained were subjected to quantitative analyses. Cytochrome content was determined spectroscopically and the succinylated lipomannan was measured by rocket affinoelectrophoresis [19]. Rocket immunoelectrophoresis with monospecific serum was employed to determine the amount of ATPase present in these extracts. The application of rocket immunoelectrophoresis to quantitative measurements was extended beyond situations where purified antigen or monospecific serum is available through the use of selective staining techniques. Extracts were electrophoresed into agarose prepared with rabbit anti-*M. lysodeikticus*-membrane serum and the precipitates formed between specific antibody and NADH- and malate dehydrogenases (EC 1.6.99.3, 1.1.1.37) and succinate dehydrogenase were identified by specific enzyme stains.

Results obtained by the quantitative rocket immunoelectrophoresis technique are illustrated in Fig. 1. Two immunologically distinct NADH dehydrogenases have been detected by crossed immunoelectrophoresis of extracts prepared from *M. lysodeikticus* membrane [31]. Quantitation by the rocket technique provides an accurate measure of the amounts of each enzyme present. Such a distinction could not be made on the basis of measurements of enzymatic activity. The two precipitates exhibiting NADH dehydrogenase activity were designated I and II (Fig. 1A) and correspond to immunoprecipitates in the crossed immunoelectrophoretic pattern which have been assigned numbers 13 and 10, respectively, in a previous study from this laboratory [31].

The extent of extraction of total protein, total cytochrome, mannan and several enzymes by Triton X-100 and chaotropic agents used alone and together is shown in Fig. 2. Triton X-100 was the most effective of these solubilizing agents as determined by the quantity of protein and individual membrane components recovered. The barium and lithium thiocyanate salts and lithium acetate were much less efficient in terms of quantitative yield. Extraction with 0.5 M lithium acetate was only slightly more effective than treatment with a concentration of 0.2 M of this salt. This same trend was ob-

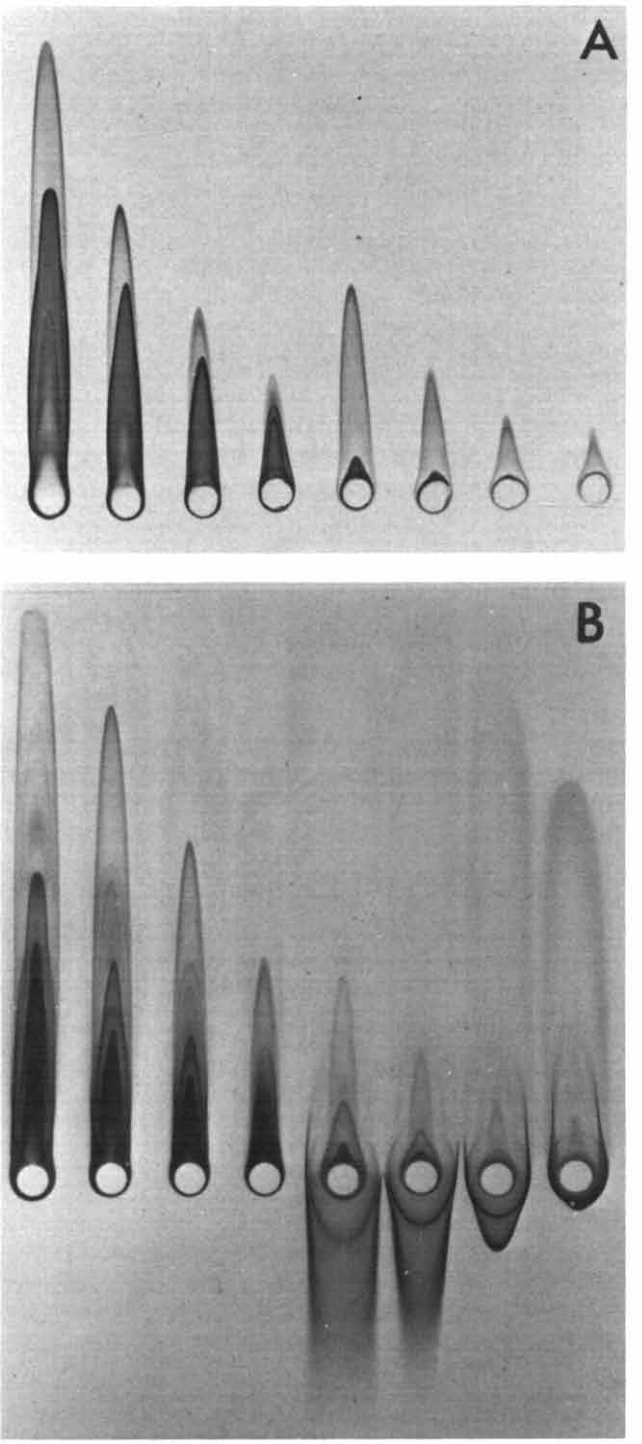

Fig. 1. Rocket immunoelectrophoresis of *M. lysodeikticus* membrane extracts into agarose containing anti-membrane serum. Wells contained from left to right 5 μl of 1% Triton X-100 extract, undiluted, diluted 1 : 2, 1 : 4, and 1 : 8; 0.25 M barium thiocyanate extract, undiluted, diluted 1 : 2, 1 : 4, and 1 : 8. (A) Stained for NADH dehydrogenase activity. (B) Stained with Coomassie brilliant blue. NADH dehydrogenase I is dense precipitate. NADH dehydrogenase II is lighter, taller precipitate.

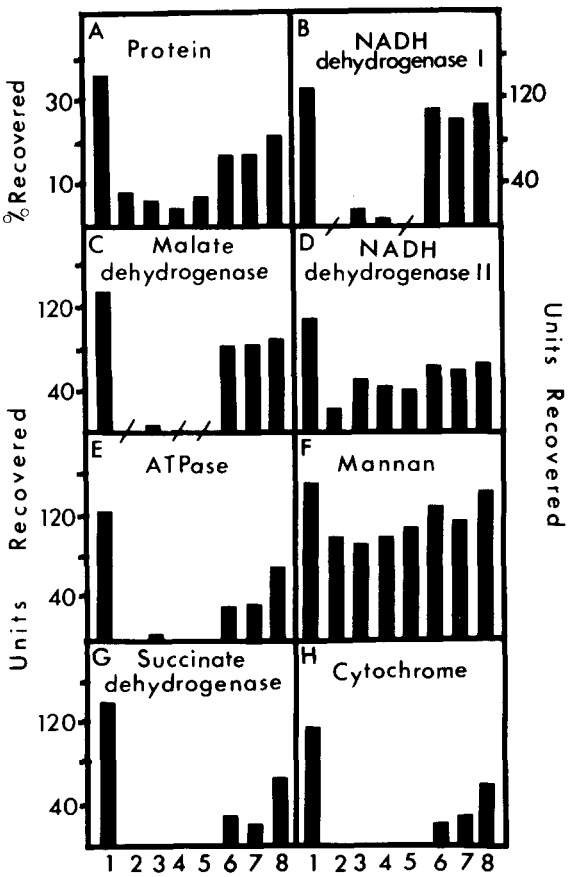

Fig. 2. Recovery of membrane components in extracts prepared by treatment of *M. lysodeikticus* membranes with (1) 1% Triton X-100; (2) 0.10 M $Ba(SCN)_2$; (3) 0.20 M LiSCN; (4) 0.20 M lithium acetate; (5) 0.50 M lithium acetate; (6) 1% Triton X-100 and 0.10 M $Ba(SCN)_2$; (7) 1% Triton X-100 and 0.20 M LiSCN, (8) 1% Triton X-100 and 0.20 M lithium acetate. Protein expressed as per cent total recovered; other quantities expressed in arbitrary units. Slash marks indicate that component was present, but in amounts insufficient to quantitate by this technique.

served with extracts prepared with the thiocyanate salts (not shown). When Triton X-100 was used in combination with any of these chaotropic agents, the degree of solubilization was less than that obtained with detergent alone. This may reflect stabilization of the membrane by cations. It is interesting to note the similarity in the profiles of Figs. 2A and 2C as contrasted to Figs. 2D and 2F. Malate dehydrogenase may be said to be 'typical' of the bulk membrane protein in terms of its solubility properties since these characteristics correspond closely to those of the extractable protein.

Some extraction conditions, while resulting in poor yields may be useful for their selectivity. Examination of Fig. 3 reveals that extracts obtained with the lithium salts have a high specific content of NADH dehydrogenase II, despite a poor recovery (Fig. 2). Likewise, the chaotropic agents produced extracts relatively high in mannan (Fig. 3E) content. Extracts prepared with Triton in concert with salts exhibited the highest specific content of NADH dehydrogenase I (Fig. 3A).

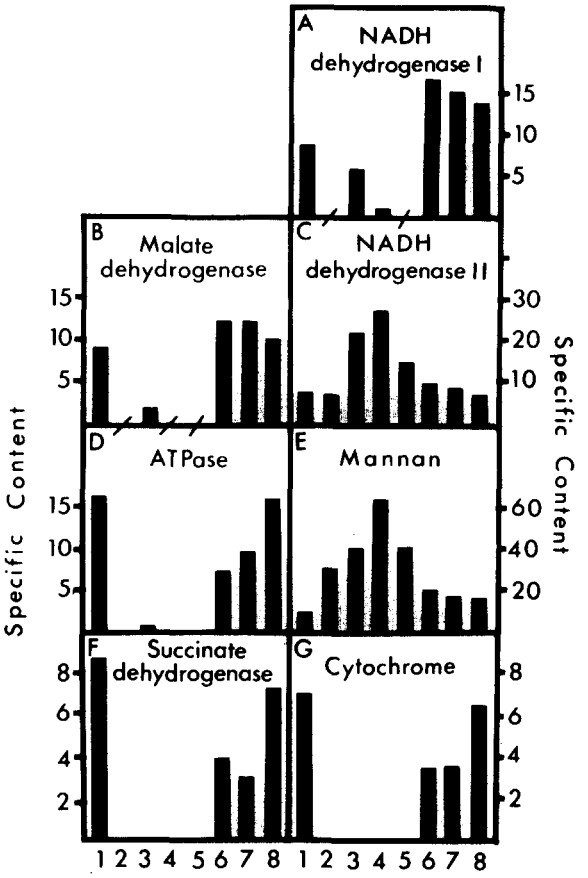

Fig. 3. Specific content of individual components in extracts prepared from *M. lysodeikticus* membranes under conditions described for Fig. 2. Specific content is ratio of units membrane component/total protein in extract.

The degree of solubility of these membrane components as a function of Triton X-100 concentration was investigated (Fig. 4). Total protein and individual components are solubilized in parallel. Under the conditions of this experiment, a threshold concentration of 1—2% Triton X-100 (detergent/protein ratio, 0.5—1 : 1) resulted in approximately maximal solubilization of all membrane constituents measured. Additional detergent did not significantly enhance recovery.

An apparent exception to this finding, is the ATPase. Lower total recovery of this enzyme molecule was observed at 4 and 6% Triton. This may, however, be a reflection of an artifact of measurement rather than of solubility properties. Crossed immunoelectrophoretic analysis indicated that treatment of the ATPase under these conditions reduced the electrophoretic mobility of the enzyme. Consequently, valid quantitation of extracts prepared with 4 and 6% Triton X-100 cannot be made on the basis of rocket immunoelectrophoresis [32].

Extraction of *M. lysodeikticus* membrane was performed with a homologous

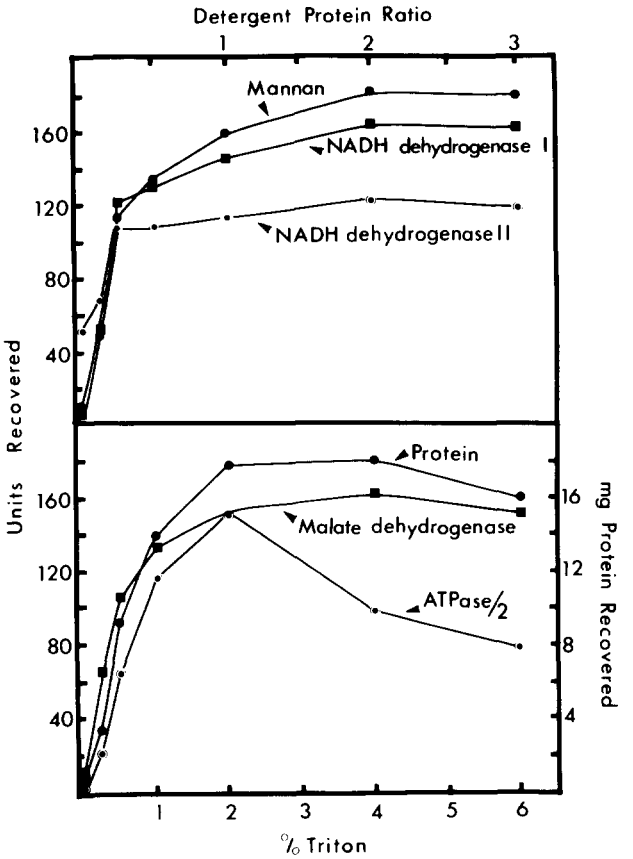

Fig. 4. Recovery of total protein and individual membrane components in extracts prepared by treatment of *M. lysodeikticus* membranes (40 mg protein) with various concentrations of Triton X-100.

series of Triton X detergents. Recovery of the solubilized membrane components was measured and evaluated as a function of the degree of hydrophobicity of the surfactant used (Fig. 5). This is expressed by the hydrophile-lipophile balance. On this arbitrary scale high numbers characterize hydrophilic detergents; low numbers characterize more hydrophobic detergents [33].

Triton X-100 with a hydrophile-lipophile balance number of 13.5 was found to be the most effective of this series of detergents in causing the solubilization of total protein and all individual components measured. When the degree of solubilization produced by detergents not of the Triton X series was assessed, the relationship between hydrophile-lipophile balance and degree of solubilization did not always correspond well to that exhibited by the series of Triton X detergents. Brij 56, for example, with a value of 12.9 solubilized only 22% of the protein whereas Triton X-114 (12.4) and Triton X-100 (13.5) solubilized 34 and 37% of the protein, respectively (Table I). Extraction of membranes with Brij 58 (15.7) resulted in the solubilization of 24% of the protein. Tween 20 (16.7) and Tween 80 (15.0) released 16 and 11% of the membrane protein, respectively. Comparison with Fig. 5A indicates that these values deviate from those obtained with detergents of the Triton X series.

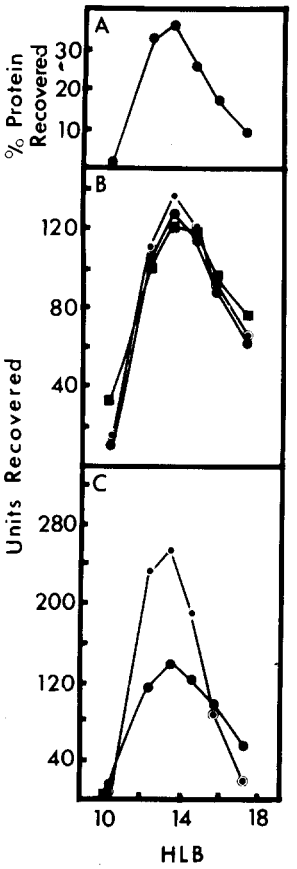

Fig. 5. Evaluation of extraction of membrane components by detergents of the Triton X series as a function of hydrophile-lipophile balance (HLB). Points represent average recovery from two extractions. (A) Protein; (B) •, NADH I dehydrogenase; ▪, NADH II dehydrogenase; ⊙, malate dehydrogenase; ○, C-ATPase; ●, mannan.

When the degree of detergent-mediated solubilization of a particular membrane component was examined as a function of total protein solubilized, a linear relationship was observed. The data for Fig. 6 were generated by treating *M. lysodeikticus* membrane with four detergents of the Triton X series. Variation in the extent of solubilization resulted from variation in the hydrophile-lipophile balance of the detergent or the concentration used. These data indicate that the specific content of the extracts obtained under these conditions is relatively constant. When this analysis was expanded to consider additional detergents (Table I), more variation occurred in the values. The Tween detergents showed a selectivity in the membrane components extracted. This selectivity has been observed previously [34] and exploited for preparative purposes [35]. Treatment with Triton X-45 and-305 similarly produces extracts which vary considerably in composition from the norm. These deviations follow the same trend as do those of extracts prepared with the Tweens. It is possible that this variation in composition characterizes extracts which are obtained under conditions suboptimal for total protein recovery.

TABLE I

COMPOSITION OF DETERGENT EXTRACTS

Final concentration of detergent, 1% (w/w). Hydrophile-lipophile balance number is from Refs. 27 and 33. Values for the specific content and percent total protein recovered represent averages from two extractions rounded to the nearest integral value.

Detergent	Hydrophile-lipophile balance number	Specific content					% total protein recovered
		Malate dehydrogenase	NADH dehydrogenase I	NADH dehydrogenase II	ATPase	Mannan	
Triton X-45	10.4	13	13	35	—	19	2
Triton X-114	12.4	8	8	7	17	9	34
Triton X-100	13.5	9	9	8	17	10	37
Triton X-102	14.6	11	11	11	18	12	27
Triton X-165	15.8	13	13	13	12	13	18
Triton X-305	17.3	19	18	21	5	15	9
Brij 56	12.9	11	11	11	14	11	22
Brij 58	15.7	9	9	9	14	10	24
Tween 20	16.7	—	18	—	*	18	16
Tween 80	15.0	—	17	—	*	23	11
Nonidet P40	13.1	—	8	—	14	11	43
Sodium deoxycholate		—	8	—	14	12	44
Berol EMU-043		—	9	—	10	11	33

* ATPase was present, but at levels too low to quantitate by this technique.

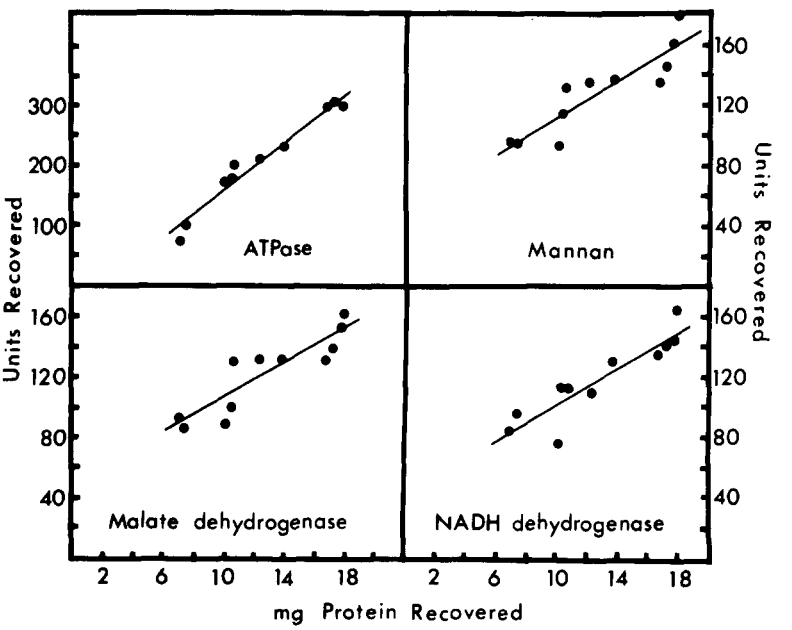

Fig. 6. Recovery of individual membrane components assessed as a function of total protein recovery. Extracts were prepared with 1—4% Triton X-100 and 1% Triton X-114, -102, -165. Each point expresses the relationship between recovery of total protein and of an individual membrane component in a given extract.

In light of these findings, it was not surprising that the crossed immunoelectrophoretic profiles of these extracts were generally quite similar to one another and to those detailed in previous studies [14,31]. Qualitatively, few differences were observed. However, the area subtended by the various precipitates differed, reflecting the slightly different quantities of the individual components recovered in the extracts obtained under different conditions. The patterns seen upon crossed immunoelectrophoresis of extracts obtained by treating membranes with chaotropic agents differed more substantially from those obtained with detergents. This is predictable on the basis of the data presented in Figs. 2 and 3. Additionally, some of these agents solubilized antigens which migrated toward the cathode (Fig. 1A). Nothing with this electrophoretic mobility was found in detergent extracts.

Discussion

Solubilization of membranes is an important step, both for preparative and analytical purposes, in the study of the structure-function relationships which exist in biological membranes. This study was undertaken to evaluate the efficiency and selectivity of membrane extraction under various conditions in order to provide guidelines for a rational choice of solubilizing agent. By investigating the solubility characteristics of a diverse group of membrane components under a variety of conditions, it was hoped that the general principles of membrane solubilization by these treatments would be elucidated. The enzymes which were examined for this investigation were quantified by rocket immunoelectrophoresis. This technique determines the amount of a given molecular species present rather than the total enzymatic activity; measurements of the latter are subject to possible error due to apparent detergent activation [36,37] or inactivation [37—39] of membrane enzymes. The survival of enzymatic activity is demonstrated in the present study by the zymogram staining techniques.

The extent of solubilization with Triton X-100 was found to increase with the increasing ratio of detergent/protein until a plateau is reached at a value of 0.5—1. This observed saturation effect appears to be a common phenomenon; it has been demonstrated in the Triton X-100 treatment of bacterial membranes [28,40] as well as with many other detergent-membrane systems [27,29,41—47].

The relationship between the hydrophile-lipophile balance of a surfactant and its membrane-solubilzing potential has been evaluated by others [27,36, 48]. The results presented here confirm and extend their findings. A study of the specific contents of extracts obtained by treatment of membranes with a variety of detergents (Fig. 6, Table I) reveals that there is a general tendency toward uniform composition. This is compatible with previous observations [49]. Deviations from this norm are not uncommon, and may be attributable to chemical differences between detergents, but such deviations do not obliterate the overall trend. The bulky structure of the Tween head group may be responsible for the anomalous behavior [34,50] of this group of surfactants. Similarly, charge differences between detergents may result in extraction of preparations of different composition. The discrepancies observed in the

present study and by others [36] in the correlation of the hydrophile-lipophile balance with solubilization efficiency may also reflect differences in chemical structure or charge of the detergents being compared. Moreover, the polydispersity and variations between batches of detergents [36] must be considered.

Despite these anomalies, a pattern is recognizable. The solubilization of membrane by surfactants, measured by the liberation of protein or individual components, increases with detergent concentration until a plateau is reached. Detergents with a hydrophile-lipophile balance of approximately 13—13.5 are optimal not only for the extraction of bulk protein, but also for solubilization of several unrelated individual components. The composition of these detergent extracts of membranes is relatively uniform and selectivity of extraction by different detergents is to a large extent the exception rather than the rule. The exceptions are generally related to chemical structure or to an unsuitable hydrophile-lipophile balance. When treated under such conditions the membrane is less completely disrupted (see last column, Table I) and a non-representative fraction of membrane components is released. Such extracts are enriched in peripheral proteins such as NADH dehydrogenase II, which may be released from membranes of *M. lysodeikticus* by washing with ethylenediaminetetraacetate [51].

Taken together, these data suggest a model for the solubilization of membranes by non-denaturing detergents. These surfactants bind to the membrane and at low concentrations may selectively release some components (Fig. 3) [45]. At a sufficiently high concentration of detergent, the membrane becomes saturated with surfactant, the disintegration of the bilayer commences and membrane components are liberated [52]. The preparation obtained under such conditions may be fractionated by centrifugation to obtain an insoluble residue and a soluble extract composed of mixed micelles, which may include protein-detergent, lipid-detergent, lipoprotein-detergent micelles [33,52,53]. This mechanism for membrane disruption probably applies in general to the interaction of non-denaturing detergents with membrane. Consequently, the class of membrane components solubilized by different surfactants is generally qualitatively uniform in composition.

The content [1,2,53] of detergent extracts may differ from that of the lipid-depleted [1,2,54,55] residue, but the composition of these two fractions is probably not entirely unique. Conditions may be chosen to optimize this difference [45]. Re-extraction of the residue under the same or harsher conditions may solubilize some of the same components as those obtained in the initial treatment [30].

While surfactants solubilize membranes through disruption of the lipid bilayer and the formation of mixed micelles with membrane components, the mechanism of action of chaotropic agents is different. These reagents reduce hydrophobic interactions by increasing the solubility in water of apolar moieties [4]. Not unexpectedly, extracts prepared with chaotropic agents differ quantitatively and qualitatively from those prepared with detergents. Although some components are common to extracts prepared with detergent or chaotropic agents (Fig. 2), some unique antigens have been identified in immunoelectrophoresis in this study (Fig. 1A) and others performed in this

laboratory [56]. Despite the low yield obtained when these agents are used to solubilize membrane protein, their selectivity can make them useful for preparative purposes. Conversely, Triton X-100 is the reagent of choice to qualitatively and quantitatively maximize the variety and yield of soluble membrane components recovered under non-denaturing conditions.

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