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CHARACTERISTICS OF A LIPID-RICH NADH DEHYDROGENASE-CONTAINING PARTICULATE FRACTION OBTAINED FROM *MICROCOCCUS LYSODEIKTICUS* MEMBRANES

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SUMMARY

A fraction of homogenous particle size, enriched in lipid and NADH dehydrogenase has been obtained from *Micrococcus lysodeikticus* membrane by treatment with EDTA. Lipid activation of NADH dehydrogenase activity of a *n*-butanol-extracted EDTA wash has been demonstrated. The relationship of this fraction to other fractions obtained by mild procedures from this organism is discussed and a possible structure for the membrane is proposed.

INTRODUCTION

The biochemical approach to the study of the structure–function relationships of biological membranes faces many obstacles, not the least of which is the complexity and variety of functions in any one membrane[1–5]. Studies on a specialized subcellular organelle, the mitochondria, have suggested that enzymes performing sequentially related functions (*i.e.* electron transport) or linked functions (*i.e.* electron transport and oxidative phosphorylation) are to be found in distinct macromolecular complexes in the membrane[6]. Evidence from studies of other membranous organelles[7–9] and of bacterial membranes[5,10] also infer that the patterns of organization exemplified by the mitochondrial membranes may exist in many specialized membrane systems.

In our investigation into the structure–function relationships of the plasma membrane of the microorganism, *M. lysodeikticus*, various enzymatic markers of the membrane have been identified[5]. Selective release of some of these has been achieved by utilizing relatively mild methods to dissociate membrane components[5,11,12]. Analysis of the fractions, thus far isolated, has indicated their unique biochemical composition and the relative homogeneity of each as determined by morphological and physical-chemical criteria[5,11–13].

The results reported here describe the characteristics of a particulate fraction released from *M. lysodeikticus* membranes by EDTA, its relationship to other membrane fractions isolated from the same organism and its relationship to the architecture of the membrane. The structural pattern of the membrane suggested by

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

the data is consistent with a non-random, non-homogenous arrangement of functional macromolecular complexes.

METHODS AND MATERIALS

Growth, harvesting and preparation of membranes

M. lysodeikticus (NCTC 2665) was grown and harvested as previously described[14]. Membranes were prepared by the standard method[15,16] from lysozyme lysates, except that the buffer strength was reduced from 0.1 to 0.05 M Tris–HCl. Membranes were deposited at 30000 × *g* for 30 min at 0° and the pellets were suspended in 0.03 M Tris–HCl buffer containing 0.005 M EDTA and left overnight at 0–4°. They were centrifuged again (30000 × *g*, 30 min, 0°) and the supernatants (hereafter called the EDTA or W4 wash) were saved. The pellet was either suspended in 0.03 M Tris buffer (pH 7.5) and designated as the membrane fourth wash (MW4) or was subjected to "osmotic shock" as previously described[11]. Supernatants thus obtained were designated as W5 or shock wash and the pellets as membranes W5 or MW5, respectively.

In experiments utilizing ^{32}P approx. 0.1 mC was added to each flask containing 750 ml of peptone-water yeast extract medium.

Chemical composition

Protein was determined by the method of LOWRY *et al.*[17]. Total lipid contents were determined by extraction with chloroform–methanol (1:2, by vol.) according to the method of BLIGH AND DYER[18]. Protein content of the washes was adjusted to 15–20 mg/ml, two extractions were performed and the combined extracts dried under N_2 and weighed. Total lipid used for other studies was stored at −20°.

Qualitative and semiquantitative analyses of phospholipids were performed by chromatography on silica gel-loaded paper and the solvent system of chloroform–methanol–diisobutylketone–acetic acid–water (45:15:30:20:4, by vol.)[19]. Chromatograms were stained in 0.0012 % solution of Rhodamine 6-G in water, the spots located under ultraviolet light and identified by comparing with purified standards provided by Dr. August De Siervo of this laboratory. No ninhydrin staining material was found, indicating the absence of phosphatidyl ethanolamine and/or amino acyl lipids. For lipid from $^{32}P_i$-grown cells the spots were cut out and counted in a Nuclear Chicago gas-flow detector.

Enzymatic activities

ATPase (EC 3.6.1.3) activities were measured as described by MUNOZ *et al.*[11]. NADH dehydrogenase activity (EC 1.6.99.3) was assayed using 2,6 dichlorophenolindophenol (DCIP) in a modification of the procedure of SAUGE[20]. Because of the crude nature of the preparation and in order to handle the large number of samples employed, units of activity were made arbitrary by recording absorbance (*A*) changes over 30 sec at 25° in a Bausch and Lomb Spectronic '20' spectrophotometer at 600 nm. The assay mixture contained 6.0 μmoles NADH, 100 μmoles sodium phosphate buffer (pH 7.5), 0.1 μmole DCIP in a final volume of 2.95 ml. The membrane or wash sample (0.05 ml) was added and the mixture read against a water blank at

30 sec. A change of 0.1 A unit in 30 sec was defined at 1 unit of activity. Samples were adjusted to give a final reading between 0.1–0.6 A. Corrections were made for decreasing absorbance of the indicator control (no sample).

Polyacrylamide gel electrophoresis

Electrophoresis of various fractions was performed in polyacrylamide gels as described by SALTON[15,16]. NADH dehydrogenase activities were visualized using the triphenyltetrazolium method as described by NACHBAR AND SALTON[5] or by decolorization of DCIP using the same mixture as for the regular enzymatic assay except that the amount of NADH was 20 μmoles and of DCIP 1.0 μmole in a volume of 3 ml. The gels were allowed to stand in the solution for 5 min at 25° and were then transferred to 0.05 M Tris–HCl buffer (pH 7.5). A very broad zone of decolorization was visible at 5 min and was frequently observed to be divided into several discrete zones upon standing in buffer.

Solvent extraction and lipid dispersion

Extraction with n-butanol was performed according to MADDY[21] but in the presence of 0.03 M Tris–HCl (pH 7.5) with or without 0.005 M EDTA. 3 ml of the EDTA wash, adjusted to contain 5 mg/ml of protein, was extracted 3 times with 2 ml of n-butanol at −4°. The butanol extracts (solvent phases) were evaporated under N_2 and taken up in chloroform for gravimetric analysis. The aqueous phase was dialyzed against 0.03 M Tris–HCl (pH 7.5) to remove butanol. To study the effect of added lipid on the restitution of the ability of aqueous phase protein to reduce DCIP, 6 mg of lipid from the butanol extract were dispersed by sonication in 3 ml of buffer (30–45 min of sonication using a Branson Sonifier, Model W140E at 350 W per inch^2) at 0°. Phospholipid dispersions were brought to room temperature and utilized the same day. Various amounts of lipid were added to depleted, "soluble" protein from the butanol-extracted EDTA wash and the mixture assayed for the ability to reduce DCIP after 30 min incubation at 37°.

Gel filtration

Conditions for gel filtration on Sephadex G-200 with 0.03 M Tris–HCl buffer (pH 7.5) were previously described[13]. The void volume (V_0) was determined with Blue Dextran 2000 and $V_0 + V_i$ volume was obtained from the elution of glucose, assayed with glucose oxidase. The values were $V_0 = 125$ ml, $V_0 + V_i = 270$ ml. Flow rate was 8–10 ml/h.

Sucrose gradient centrifugation

The EDTA and shock washes were subjected to centrifugation on a 27-ml discontinuous sucrose gradient[12]. The sucrose was dissolved in 0.03 M Tris–HCl (pH 7.5). 10 mg of protein of the appropriate wash in 1 ml of buffer was layered on the top and centrifuged in a Spinco Model L-2 ultracentrifuge in a SW-25 rotor at 25000 rev./min for 24 h at 0°. 1-ml fractions were collected and assayed for radioactivity, protein and enzymatic activity. Corrections due to the presence of sucrose were made in determinations of proteins.

Immunodiffusion techniques

Rabbit antisera to whole membrane were prepared according to FUKUI *et al.*[22]. Agar gel diffusion slides were prepared as described by CAMPBELL *et al.*[23].

Reagents

ATP was obtained from Pabst Brewing Company, Milwaukee, Wisc.; ^{32}P from Amersham/Searle Corp., Des Plaines, Ill.; NADH, nitro blue tetrazolium and triphenyltetrazolium chloride from Sigma Chemical Co., St. Louis, Mo.; and diisobutylketone from Matheson, Coleman and Bell, Cincinnati, Ohio.

RESULTS

Chemical composition of the EDTA wash

M. lysodeikticus membranes lose protein and lipid into the 30000 × *g* supernatant when exposed to EDTA (Table I). As can be calculated from the table the percentage of original membrane protein released by EDTA averaged 12.2%. If we assume that most of the protein released into the first and second washes represented cytoplasmic contamination[15,16] then this value becomes even greater. The lipid/protein ratios of the EDTA wash ranged from 1.2 to 1.5, with an average of 1.3. This is in sharp contrast to the ratios of 0.33–0.40 for extensively washed membrane[14] and 0.11 for the shock wash (Table I).

Chromatograms of lipid extracts of membrane and washes (Fig. 1) demonstrated little qualitative difference among the various samples tested. The main spots corresponded to phosphatidyl inositol, phosphatidyl glycerol and cardiolipin. A fourth spot traveling between cardiolipin and phosphatidyl glycerol is probably a glycolipid (A. De Siervo, personal communication). Spots assayed for radioactivity revealed a

TABLE I

PROTEIN, LIPID AND ENZYMATIC ACTIVITIES IN *M. lysodeikticus* CYTOPLASM, MEMBRANES AND MEMBRANE WASHES

Protein determined by method of LOWRY *et al.*[17]. Assay methods and units of NADH dehydrogenase and ATPase as given in text.

*Fraction**	*Protein (mg/ml)*		*NADH Dehydrogenase (units/ml)*		*ATPase (units/ml)*		*Lipid/protein ratio*	
	*Mean***	*Range*	*Mean*	*Range*	*Mean*	*Range*	*Mean*	*Range*
Cytoplasm	16.1	(14.2–18.3)	0.5	(0.3– 1.0)	0.51	(0.41–0.62)		
Membranes, no wash	19.5	(18.3–20.1)	13.3	(11.0–14.6)				
Wash 1	7.7	(6.0– 8.5)	4.2	(3.1– 5.0)	0.004	(0.002–0.004)		
Wash 2	3.7	(2.8– 4.5)	2.8	(1.9– 3.9)	0.003	(0.002–0.004)		
Wash 3	0.86	(0.65– 1.00)	1.6	(0.9– 2.1)	0.004	(0.003–0.004)		
EDTA wash (W 4)	2.4	(1.9– 3.0)	12.4	(10.6–14.4)	0.45	(0.41–0.62)	1.3	(1.2–1.5)
Shock wash (W5)	1.3	(1.1– 1.5)	2.9	(2.5– 3.6)	3.6	(2.8–4.0)	0.11	(0.10–0.12)

* Volume for each sample was 250 ml.
** Means of 5 experiments.

deviation from the normal 1/1 ratio of cardiolipin/phosphatidyl glycerol in the whole membrane and a ratio of 4.0–4.5/1 (Table II) was found for the EDTA wash. Thus, the EDTA wash differs from the membrane in both the lipid/protein ratio and in the relative amounts of the individual phospholipids present.

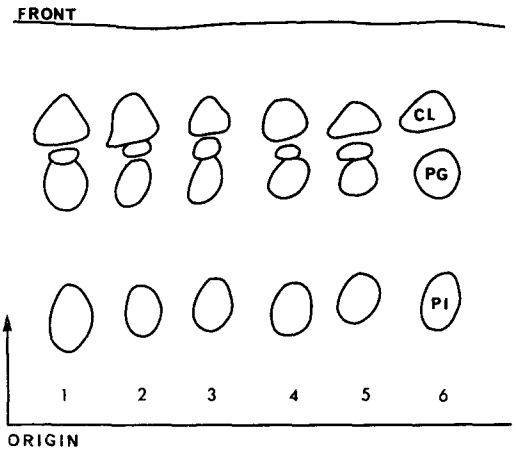


Fig. 1. Chromatography on Whatman silica gel-loaded paper of chloroform–methanol extracts of various membrane fractions and membrane washes from *M. lysodeikticus*. Conditions of chromatography, detection and solvent system as specified in METHODS AND MATERIALS. Preparations were as follows: 1, membrane prior to EDTA wash; 2, membrane residue after EDTA wash; 3, membrane residue after shock wash; 4, EDTA wash (30000 × *g* supernatant); 5, "shock" wash (30000 × *g* supernatant); 6, mixture of purified phospholipids characterized by Dr. August De Siervo, phosphatidyl inositol (PI), phosphatidyl glycerol (PG) and cardiolipin (CL).

TABLE II

RADIOACTIVITY IN VARIOUS PHOSPHOLIPIDS OF *M. lysodeikticus* MEMBRANES AND EDTA WASH

Preparation	*Cardiolipin*	^{32}P (*counts/min* × 10^{-3} *per spot*)		
		Phosphatidyl glycerol	*Phosphatidyl inositol*	*Glycolipid*
Membrane	6.3* (5.0–9.6)**	3.0 (2.0–3.9)	0.4 (0.3–0.5)	0.1
EDTA wash	7.0* (5.5–9.5)**	1.7 (1.0–2.5)	0.3 (0.3–0.4)	<0.1

* All numbers refer to the average of 5 experiments.
** All values in parentheses represent the ranges of ^{32}P determinations.

Electron microscopic appearance

EDTA wash and shock wash preparations were negatively stained with ammonium molybdate and Figs. 2*a* and 2*b* reveal the vesicular and particulate nature of the fractions, respectively. The smallest vesicles of the EDTA wash are of the order of 300 Å in diameter and the relative uniformity of the preparation is also apparent. (Fig. 2A). The small particles in the shock wash were approx. 100 Å in diameter.

Enzymatic composition of the EDTA wash

As can be seen from Table I, much of the NADH dehydrogenase was found in the EDTA wash. Nothing can be said of the specific activity of the preparations

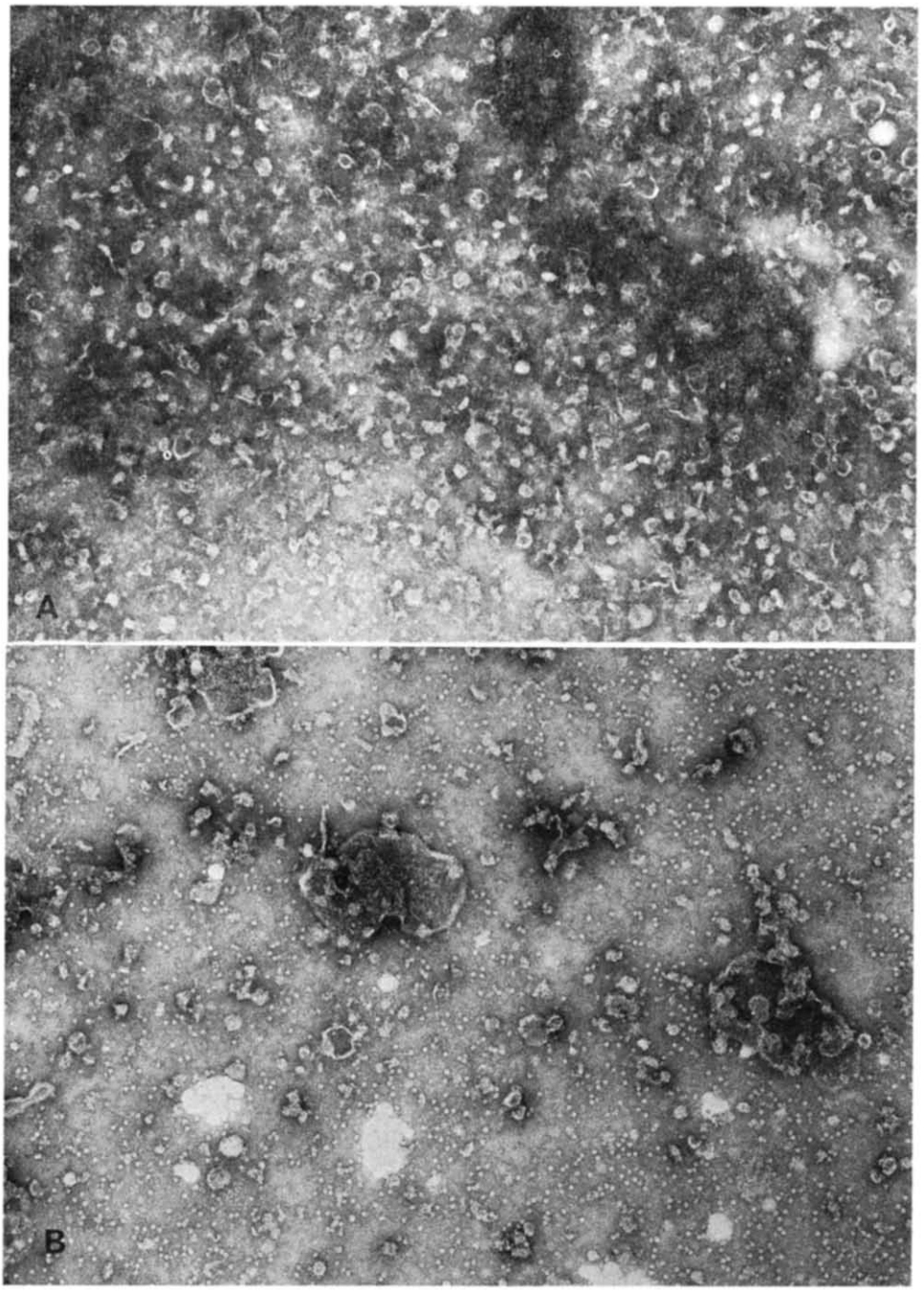


Fig. 2. Preparations from *M. lysodeikticus* membranes negatively stained with 2% ammonium molybdate. A. EDTA wash showing the relatively uniform nature of the vesicles in this wash fraction; the smallest vesicles are approximately 300 Å in diameter (67600 ×). B. "Shock" or low ionic strength wash fraction showing 100-Å particles bearing ATPase activity[13], together with membrane fragments of varying sizes (67600 ×). Electron micrograph kindly taken by Dr. Kwang S. Kim.

since the total number of units recovered in the washes exceeds that measured in the untreated membrane, *i.e.* apparent "activation" upon release. Some ATPase (Table I) is also present but the total activity is small, especially when compared to the shock wash (W5).

Sucrose gradient centrifugation

The differences in sedimentation properties between the EDTA and shock washes are illustrated graphically in Fig. 3. Two bands (one major and one minor band) are noted for the EDTA wash and only one band for the shock wash.

The greater density of the shock wash fragments is not surprising since they contain much less lipid than the EDTA wash. Analysis of the protein and ^{32}P radioactivity present in the sucrose gradient of the EDTA wash (Fig. 4) demonstrates that the bulk of the protein, radioactivity and enzymatic activity were present in one large peak corresponding to the major band observed. Some phospholipid free of protein is found at the top of the gradient. Thus the fraction sediments in a relatively homogeneous manner. Less than 50% of the original NADH dehydrogenase activity was recovered. To determine if the bulk of the enzyme activity was in particulate form, the EDTA wash was centrifuged at 100000 × *g* for 2 h at 0° and assays demonstrated that 10% of the activity remained in the supernatant while 90% was recovered in the pellet which was redispersed in buffer with 1% deoxycholate. NADH dehydrogenase was thus shown to be localized in a particulate fraction.

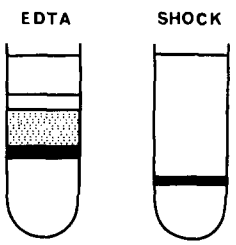


Fig. 3. Behavior of EDTA wash and shock wash fractions from *M.lysodeikticus* membranes upon centrifugation on a discontinuous sucrose gradient as described in METHODS AND MATERIALS. As indicated in the diagram, one major band was found in each fraction.

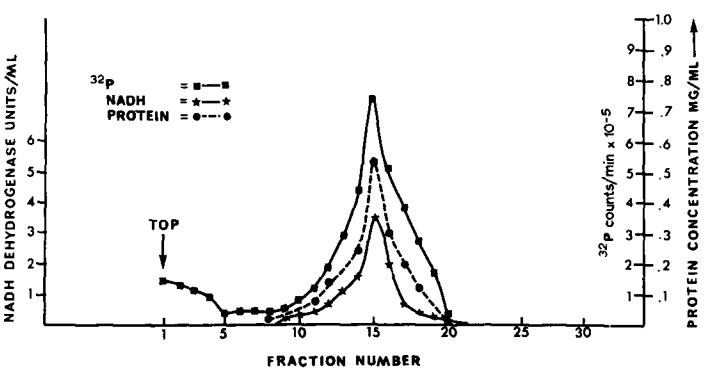


Fig. 4. Distribution of protein, NADH dehydrogenase and ^{32}P following sucrose gradient centrifugation of the EDTA wash fraction obtained from the membranes isolated from *M. lysodeikticus* grown in peptone-water yeast extract medium containing ^{32}P. The peak containing the bulk of the activity, protein and ^{32}P corresponded to the major band in Fig. 3.

Behavior on Sephadex G-200 column

Almost all of the NADH dehydrogenase present in the EDTA wash was excluded from the Sepahdex G-200 column (Fig. 5). The enzyme was unstable during the elution process and less than 50% of the original activity was usually recovered.

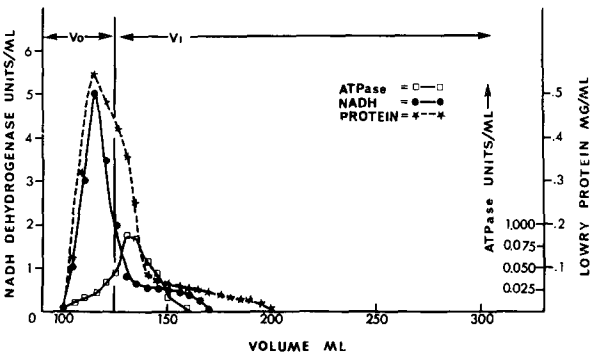


Fig. 5. Gel filtration of the EDTA wash (20 mg total protein) on a Sephadex G-200 column shows the exclusion of much of the NADH dehydrogenase activity in the void volume. The excluded peak of NADH dehydrogenase consisted of uniform vesicular particles; the shoulder of activity is probably due to the free form detectable in acrylamide gels. This is contrasted with the filtration pattern for the ATPase.

Polyacrylamide gel electrophoresis

Two major protein bands were observed in polyacrylamide gels after electrophoresis (Fig. 6a). Several minor bands migrating faster and slower than the two major bands are seen. Triphenyltetrazolium staining of the gels (Fig. 6b) demonstrated the presence of NADH dehydrogenase activity in the double-band region. DCIP decolorization verified the presence of dehydrogenase activity in this area (Fig. 6c). At earlier times complete decolorization from the double-band region to the junction of the stacking and separating gels was observed. As the gel was allowed to stand in buffer alone, the upper portion gradually assumed a blue color leaving a sharp zone of decolorization in the double band region. In some washes, a faster moving activity was detectable with tetrazolium. Its relationship to the major bands is unknown. ATPase activity was located enzymatically[24] and was noted in its usual place[11].

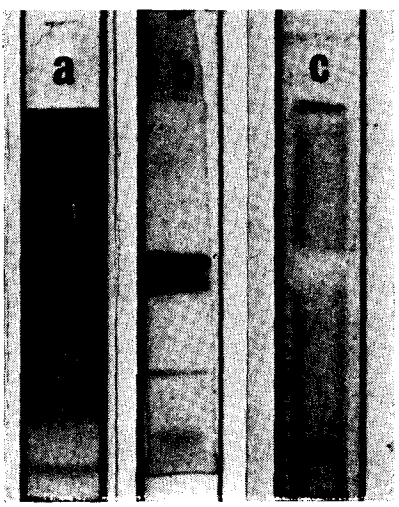
The gels did not stain for malate dehydrogenase, succinate dehydrogenase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase or non-specific esterase. No stain with tetrazolium or decolorization with DCIP was seen if NADPH was substituted for NADH. An unknown amount of protein either did not enter the stacking gel or was trapped between the stacking gel and the separating gel. The addition of sodium deoxycholate or Triton X-100 to samples in a final concentration of 1 and 0.5%, respectively, markedly reduced the protein staining in these regions but did not alter the pattern observed in the gels. We therefore concluded that the patterns seen are truly representative of the protein present in the particulate fraction and that NADH dehydrogenase is the main protein entering the gels.

TABLE III

NADH DEHYDROGENASE ACTIVITY AND SOLUBLE PROTEIN REMAINING IN THE AQUEOUS PHASE FOLLOWING BUTANOL EXTRACTION OF EDTA WASH

Initial protein concentration in all experiments was 5 mg/ml.

Expt.	*Initial NADH dehydrogenase (units/ml)*	*NADH dehydrogenase after butanol extraction*	*Protein remaining in aqueous phase (mg/ml)*
1	12.6	4.4	2.5
2	15.2	3.1	2.0
3	14.6	2.2	1.8



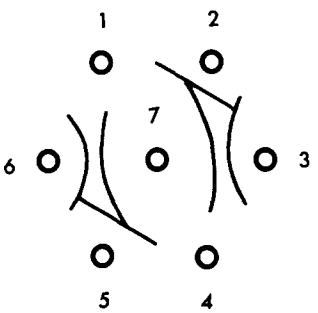


Fig. 6. Polyacrylamide gel electrophoresis of EDTA wash from *M. lysodeikticus* membranes. a. Gel of EDTA wash stained with coomassie blue. b. Gel stained for NADH dehydrogenase activity with triphenyltetrazolium chloride as described by NACHBAR AND SALTON[5]. c. Activity in gel following electrophoresis, detected by decolorization of DCIP. Note that the double-band region stains the heaviest for both protein and NADH dehydrogenase activity.

Fig. 7. Diagrammatic representation of precipitation lines in an agar gel diffusion reaction between the EDTA wash (wells 2 and 5), sonicated membrane (wells 3 and 6) and membrane antiserum (well 7) and antiserum against cytoplasm (wells 1 and 4).

Lipids and NADH dehydrogenase activity

NADH dehydrogenase activity was drastically decreased following *n*-butanol extraction and exceeded the losses of protein (Table III). This suggested the possibility that the addition of *n*-butanol-extracted lipids to the remaining soluble protein might lead to a greater recovery of activity. This, indeed, did occur (Table IV) and the best activity was obtained at a lipid/protein ratio of about 1/1. These results suggested an important role of lipid in the activity which this protein demonstrated towards an artificial acceptor and it offers a possible explanation of its presence in a lipid-rich matrix.

Immunochemical studies

NADH dehydrogenase was identified as one of the major antigens of the membrane (Fig. 7). The single precipitin line of the EDTA wash and the outer precipitin

TABLE IV

EFFECT OF ADDITION OF EXTRACTED LIPID ON NADH DEHYDROGENASE ACTIVITY OF BUTANOL-EXTRACTED PROTEIN OF EDTA WASH

Protein concentration for each experiment adjusted to 100 μg per assay, sample of 0.05 ml.

	NADH dehydrogenase (units/ml)			
Expt.	*Initial activity after butanol extraction (No phospholipid added)*	*Following incubation with various amounts of lipid Lipid/protein ratio*		
		1/2	*1/1*	*2/1*
1	2.0	3.0	4.2	4.0
2	2.4	3.2	4.6	4.5
3	2.5	4.0	5.1	5.1

line of the sonicated membrane were decolorized on staining for DCIP activity and were stained with nitro blue tetrazolium in the presence of NADH. The inner precipitin line of the sonicated membranes has recently been identified as ATPase[22]. From this it would appear that NADH dehydrogenase and ATPase have some common antigenic determinants and peptide mapping of highly purified ATPase and NADH dehydrogenase demonstrated the presence of several major common peptides[22].

DISCUSSION

In these experiments we have utilized a mild procedure to "dissect" away a functional component of *M. lysodeikticus* membranes. This fraction contained 12–30 % of the original or "true" membrane protein, respectively, and 30–50 % of the lipid. It is thus enriched with lipid. The predominant protein species detectable in acrylamide gels is that of NADH dehydrogenase which has also been identified as a major membrane antigen. It is likely that the NADH dehydrogenase protein entering the polyacrylamide gel accounts for only a small fraction of the total activity of the preparations. These results and those of the Sephadex filtration suggest that the dehydrogenase exists in a free and lipid-associated form and that the equilibrium between these two states favors the direction of bonding to the membrane phospholipids. The particles are present in the form of vesicles of relatively uniform size and they behave as though possessing a uniform buoyant density when studied by sucrose density centrifugation. It is noteworthy that a similar lipid-rich NADH dehydrogenase-containing fraction has been obtained from a liver microsomal preparation.[9]

The results of the addition of lipid to *n*-butanol-extracted NADH dehydrogenase indicate some role for lipid in the enzyme's activity. Moreover, the observed optimum ratio of lipid/protein of 1/1 closely parallels the observed ratio for the whole particulate preparation. If the butanol-extracted protein still had tightly bound lipid to the extent of 5–10 % of the original lipid content, as we have recently shown for whole membrane extracted with butanol[25], then the actual optimum lipid/protein ratio for activity very nearly approximates that of the whole fraction.

Evidence that the particulate fraction released by the EDTA wash procedure exists as such in the original membrane is circumstantial and further studies will be needed to establish this possibility. However, the rather selective release of the NADH dehydrogenase activity, its dependence on lipid for optimal activity at approximately the same ratios as that found in the released fraction and the fact that EDTA would not be expected to disrupt hydrophobic associations of proteins and lipids thereby releasing these components individually, make it unlikely that the fraction is an artifact resulting from recombination.

The finding of a homogenous, sedimenting band in sucrose gradients of the "osmotic shock" wash and similar findings for a deoxycholate-insoluble fraction obtained from the membranes[12], both of which have a unique chemical composition and protein individuality and are different from the EDTA wash, may reflect the existence in the membrane of a high degree of non-random organization. It is worthy of note that together, these three fractions comprise approx. 35–50 % of the total membrane protein and about 50 % of the lipid. Each fraction could represent a discrete macromolecular complex joined to each other by weak cohesive forces and would be consistent with a mosaic pattern of membrane architecture. A similar suggestion has recently been proposed for the erythrocyte membrane[26]. Greater confidence in the existence of a mosaic organization of the membrane structure with a regional distribution of specific functions would be forthcoming if reconstitution experiments using these fractions should prove successful. Because each fraction is related to electron transport and/or oxidative phosphorylation, the restitution of these activities after recombination is being attempted.

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