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THE EFFECT OF ALIPHATIC ALCOHOLS UPON THE DISSOCIATION OF *MICROCOCCUS LYSODEIKTICUS* MEMBRANE LIPIDS AND PROTEINS

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SUMMARY

The ability of aliphatic alcohols to extract ^{32}P-labeled phospholipid from aqueous dispersions of *Micrococcus lysodeikticus* membranes into the organic solvent phase has been investigated and the following order of efficiency of extraction observed: *tert*-amyl alcohol = *n*-butanol = *iso*-butanol = *sec*-butanol > *n*-amyl alcohol > *iso*-amyl alcohol > 3-pentanol. This order approximates that of the water solubility of these alcohols. Inclusion of urea (2 M or 6 M) or NaCl (0.1 M or 1.0 M) in the aqueous phase reduced the extractibility of ^{32}P-labeled lipid into the organic solvent phase. Residual ^{32}P-labeled material in the aqueous phase following *n*-butanol extraction could in part be accounted for as phospholipid. Almost maximal recovery of the phospholipid in the solvent phases occurred when aqueous dispersions of membrane lipid and bovine serum albumin (protein: lipid ratio, 2:1) were extracted.

The recovery of protein in the aqueous phase was about 45–50 % for all solvents and thus represents a much lower value for "soluble" protein than that observed with erythrocyte membranes. The lower recovery of soluble proteins in this bacterial membrane system is probably due to the presence of insoluble aggregates of the electron transport components of the membrane. Addition of 2 M urea increased solubilization of protein, whereas 6 M urea and 0.1 and 1.0 M NaCl decreased the aqueous phase protein. ATPase activity survived extraction with a number of the alcohols, being less sensitive to those with the hydroxyl group on carbon-1; it was largely inactivated by urea and NaCl additions to the aqueous phase. NADH-dehydrogenase showed good preservation following extraction with *tert*-amyl alcohol in the presence of 2 M urea.

INTRODUCTION

The resolution and isolation of membrane proteins is made difficult by the tendency of these proteins and membrane lipids to associate and form aggregates. Organic solvents, extremes of pH, detergents and chaotropic agents have all been utilized to dissociate membranes and "solubilize" membrane proteins or lipoprotein complexes[1]. Of the methods tried, only a few have been successful in achieving protein solubilization concurrently with retention of enzymatic functions. Early work by Morton[2] demonstrated the effectiveness of the alcohol, *n*-butanol, in releasing a

number of enzymes bound to tissue particles. Butanol extraction was further popularized by the highly successful dissolution of the erythrocyte membrane protein by Maddy[3,4]. Sporadic reports on the use of other alcohols to solubilize membranes have appeared[5,6], but apart from a recent study by Roelofsen *et al.*[7] few detailed comparisons of their effectiveness and selectivity have been made since the original work of Morton[2]. Investigations in van Deenen's laboratory[7,8] have shown that *n*-pentanol is effective in releasing lipoprotein complexes from membranes. This alcohol was, however, less effective than *n*-butanol in extracting lipids into the organic solvent phase.

During the course of attempts to isolate functional markers from *M. lysodeikticus* membranes, a number of four and five carbon aliphatic alcohols was investigated for their ability to (1) extract phospholipid, (2) solubilize protein and (3) preserve certain enzymatic functions. Conditions required for the optimal solubilization of proteins are obviously not necessarily the same as those needed for the preservation of enzymatic activities. We have thus limited the modification of the aqueous phase to systems which may be potentially useful for enzyme release and purification, rather than embarking on a more extensive study of "optimizing" total protein solubilization. The studies reported in this paper demonstrate the superiority of *tert*-amyl alcohol in extracting phospholipid from *M. lysodeikticus* membranes, and in solubilizing protein. Selectivity of protein denaturation among the various alcohols used in these investigations was also evident.

MATERIALS

Preparations of membranes

M. lysodeikticus (NCTC 2665) was grown, harvested, and the membranes prepared as previously described[9,10]. In order to label the phospholipids of the membranes, 1 mCi of ^{32}P was usually added to 750 ml of PWYE medium (peptone–water–yeast extract) prior to growth of the organism with aeration in a New Brunswick incubator shaker. The membranes were washed 6 times (30000 × *g*, 30 min at 4 °C) in 0.03 M Tris–HCl buffer, pH 7.5, and the pellets held overnight at 0–4 °C. The following day the suspensions were adjusted to a final protein concentration of 5 mg/ml, allowances being made for the effects of Tris concentration on the assays. 3 ml of membrane suspension in the Tris buffer were then taken for extraction with a given alcohol.

Solvent extraction

2 ml of solvent were added to 3 ml of the aqueous suspension of membranes prepared as described above and vigorously agitated for 20 min. All reagents were cooled to −4 °C and all extractions were performed at this temperature.

The membrane–solvent mixtures were centrifuged at 30000 × *g*, 15 min at −4 °C to separate the phases. Two to four phases were obtained depending upon the solvent and conditions used. When all four phases were present, they were (1) an upper organic phase, (2) an interfacial layer, (3) a lower aqueous phase, and (4) an insoluble pellet. Insoluble proteins were generally in a highly aggregated state and were readily sedimented at 30000 × *g*, unlike lipid–protein complexes which require much higher gravitational fields for deposition. The insoluble pellets were pigmented

and on dissolution in 1 % sodium dodecyl sulphate, spectra analysis indicated the presence of the membrane cytochromes. The organic solvent and aqueous phases (*i.e.* 1 and 3) were, of course, invariably present, but Phases 2 and 4 were variable. The solvent phase up to, but not including, the interfacial material was carefully removed with a chilled Pasteur pipette. The aqueous phase was adjusted to 3 ml with distilled water and two further extractions performed. After a third extraction, a Pasteur pipette was introduced beneath the interface and the aqueous phase removed, taking care not to disturb the floating, interfacial layer or the pellet. The aqueous phase was dialyzed at 0 °C against several changes of buffer, its final volume measured and samples taken for protein, ^{32}P radioactivity and enzymatic determinations. It was imperative to keep the temperature well controlled during extraction, as marked variability in results was observed if temperature fluctuations were allowed to occur.

Radioactivity measurements

Organic solvent phase extracts were each dried under a stream of N_2 gas and taken up into 3 ml of chloroform. Samples of 0.05–0.1 ml were applied to Whatman glass fiber paper discs which were dried and placed on planchets and the ^{32}P determined in a Nuclear Chicago gas-flow detector. Radioactivity in the aqueous phases was similarly determined. Total phospholipid radioactivity was determined by extracting 3 ml of an aqueous suspension of membranes with chloroform–methanol (1:2, v/v) by the method of Bligh and Dyer[11]. Two extractions were performed, the two organic phases combined and dried under a stream of N_2 gas. The lipid was dissolved in 3 ml of chloroform and kept at −20 °C until required. Great difficulty was encountered in attempting to disperse the pellets and interfacial layers. The residual ^{32}P remaining in these combined phases was therefore calculated by substracting the ^{32}P in the aqueous and solvent phases from the total as determined by the chloroform–methanol extractions.

Chromatography

Paper chromatography of the lipid extracts was performed on silica-gel loaded paper (Whatman no. SG-81), using the solvent system of Wuthier[12] (chloroform–methanol–diisobutyl ketone–acetic acid–water (45:15:20:30:4, by vol.)). Lipids were located by staining with Rhodamine 6G (0.0012 % w/v) in distilled water, the spots cut out, placed on planchets and the radioactivity counted. Samples from the origin, solvent front and intermediate zones were also counted. Identification of the phospholipids was made by simultaneous chromatography of purified *M. lysodeikticus* phospholipids kindly supplied by Dr. August De Siervo.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out using standard procedures employed in this laboratory[13,14]. Samples were adjusted to 1–2 mg protein/ml and 0.15–0.18 ml applied to each column.

Chemical and enzymatic assays

Protein was determined by the method of Lowry *et al.*[15]. ATPase (EC 3.6.1.3) was assayed according to Muñoz *et al.*[16] and NADH dehydrogenase (EC 1.6.99.3) was assayed according to Nachbar and Salton[17].

Materials

ATP was obtained from Pabst Brewing Company, Milwaukee, Wisc. NADH was obtained from Sigma Chemical Co., St. Louis, Mo. ^{32}P was obtained from Amersham/Searle Corp., Des Plaines, Ill. Diisobutylketone was obtained from Matheson, Coleman and Bell, Cincinnati, Ohio. Butanol, chloroform, methanol and acetic acid were all analytical grade reagents obtained from Fisher Chemical, Springfield, N. J.

RESULTS

Ability of organic solvents to extract phospholipids

Marked differences were found in the abilities of the various alcohols to extract phospholipids from aqueous dispersions of membranes into the organic solvent phase. The results summarized in Table I show that *n*-butanol, *sec*-butanol, *iso*-butanol and *tert*-amyl alcohol were about 3–4 times more effective than the other alcohols in removing phospholipids from membranes after three successive extractions of the aqueous suspensions in Tris-HCl buffer and *tert*-amyl alcohol was, by far, the most efficient solvent for a single extraction of the membranes (Table I). Apart from the nature of the aliphatic alcohol, the composition of the aqueous phase also had a marked influence on the efficiency of extraction of the phospholipids into the organic solvent phase. The addition of urea (2 M, 6 M) or NaCl (0.1 M, 1.0 M) to the aqueous phases reduced the amount of ^{32}P-labeled phospholipid extracted into the organic solvent phases (see Table I). Some changes in the order of effectiveness of the alcohols in removing lipid were also observed following the addition of urea or NaCl to the aqueous phases. It should be noted that proportionately more radioactivity was present in the interfacial and pellet materials of the membranes extracted in aqueous phases containing urea or NaCl than in the absence of these agents (see Tables I and II).

The residual ^{32}P radioactivity in the aqueous phases following three successive extractions with the solvents is summarized in Table II and reflects the efficiency of removal of the phospholipids into the organic solvent and the ^{32}P retained in the pellet and interfacial materials *i.e.*, total ^{32}P (alcohol-extracted ^{32}P *plus* aqueous phase ^{32}P). The total ^{32}P-labeled phospholipid extractable from the aqueous suspension of membranes by the chloroform–methanol procedure of Bligh and Dyer[11] accounted for 95–97 % of the total ^{32}P radioactivity. The residual 3–5 % ^{32}P content of the membrane could be accounted for largely by the presence of RNA in the membrane fractions as determined by the orcinol reaction for pentose[18]. When the residual ^{32}P contents of the aqueous phases, following three extractions with the most efficient aliphatic alcohols, were corrected for the RNA contents, some 4.3–10 % of the original ^{32}P of the membrane remained in this phase (see Table II). The nature of this residual labeled material can be partly accounted for as phospholipid and will be discussed further below.

In order to determine whether the results with the organic solvents were not due simply to the solubility properties and partitioning of phospholipids between the solvent and aqueous phases, the behaviour of aqueous dispersions of the lipid extracts was investigated in the presence and absence of bovine serum albumin (5 mg/ml). Combined butanol extracts of lipids from membranes were taken to dryness and aqueous suspensions of lipids were prepared by sonication to give preparations containing 2.5 mg lipid/ml. To study the effects of the presence of protein on the extrac-

TABLE I

EXTRACTIBILITY OF ^{32}P-LABELED LIPID FROM AQUEOUS MEMBRANE SUSPENSIONS INTO ORGANIC SOLVENT PHASES BY ALIPHATIC ALCOHOLS

Radioactivity in the organic solvent phase is expressed as a % of the original ^{32}P label in the membranes of *M. lysodeikticus* dispersed in the aqueous phases. Three successive extractions were performed and the results are given as the mean values and range of values for 5 individual experiments. Standard deviations are presented for only the 0.03 M Tris–HCl aqueous system.

Additions to 0.03 M Tris buffer phase	*Extract No.* *	^{32}P *in solvent as % initial total* ^{32}P *in membrane*						
		n-Butanol	*sec-Butanol*	*iso-Butanol*	*n-Amyl alcohol*	*iso-Amyl alcohol*	*3-Pentanol*	*tert-Amyl alcohol*
None	1	42.8 (39.0–47.2) S.D. ± 2.5	44.1 (29.2–50.0) ± 8.7	41.5 (36.4–45.6) ± 3.2	2.7 (2.4–3.0) ± 0.8	2.5 (2.0–3.1) ± 0.45	4.8 (4.1–6.0) ± 0.8	63.8 (60.0–68.1) ± 3.6
	2	28.0 (15.4–35.0) S.D. ± 7.5	31.8 (22.4–36.2) ± 4.4	33.2 (15.3–39.4) ± 9.9	13.0 (7.0–23.4) ± 9.6	11.4 (10.0–12.9) ± 1.0	6.8 (4.8–9.6) ± 1.9	13.2 (4.6–18.1) ± 5.0
	3	7.2 (1.6–9.8) S.D. ± 3.2	3.4 (1.9–7.0) ± 1.9	4.9 (2.8–8.4) ± 2.2	15.8 (9.3–20.2) ± 4.0	8.2 (5.9–11.5) ± 2.6	9.0 (5.5–12.4) ± 2.8	2.3 (1.5–3.6) ± 0.8
	Total	78.0	79.3	79.6	31.5	22.1	20.6	79.3
2 M Urea	1	45.2 (38.6–49.0)	35.1 (33.1–39.5)				2.4 (1.4–5.1)	52.0 (46.2–55.1)
	2	12.4 (9.1–14.6)	18.6 (16.0–20.4)				6.8 (3.9–9.3)	6.4 (5.0–8.5)
	3	1.9 (0.7–2.5)	1.5 (0.8–2.1)				4.9 (3.2–8.8)	0.7 (0.5–1.2)
	Total	59.5	55.2				14.1	59.1
6 M Urea	1	58.8 (50.1–61.9)	35.1 (32.0–38.1)				6.9 (5.5–8.9)	55.1 (50.1–61.4)
	2	9.5 (3.6–14.2)	15.6 (11.8–19.2)				5.6 (3.6–8.2)	4.0 (2.8–8.4)
	3	1.0 (0.5–2.0)	0.8 (0.4–1.6)				3.8 (2.7–4.3)	1.8 (0.7–2.6)
	Total	69.3	51.5				16.3	60.9
0.1 M NaCl	1	42.2 (33.0–52.2)	44.5 (34.0–55.0)				4.8 (2.9–8.4)	50.4 (39.0–62.0)
	2	15.6 (8.5–18.5)	17.7 (16.5–19.3)				14.6 (11.4–16.3)	11.8 (7.6–14.9)
	3	8.6 (4.3–16.0)	10.6 (2.8–14.4)				4.5 (3.8–5.5)	9.6 (5.1–14.6)
	Total	66.4	72.8				23.9	71.8
1.0 M NaCl	1	38.0 (31.9–40.2)	40.1 (34.5–48.3)				6.2 (3.9–8.2)	44.4 (39.6–52.4)
	2	12.6 (9.5–14.8)	14.2 (10.0–18.3)				9.6 (8.0–12.6)	6.0 (4.1–8.6)
	3	8.1 (6.9–8.8)	8.3 (3.6–12.1)				4.0 (2.9–5.5)	5.0 (4.1–7.3)
	Total	58.7	62.6				19.8	55.4

* Indicates 1st, 2nd, and 3rd extractions.

TABLE II

PERCENTAGE ORIGINAL ^{32}P REMAINING IN THE AQUEOUS PHASE OF MEMBRANE SUSPENSIONS FOLLOWING THREE SUCCESSIVE EXTRACTIONS WITH ALIPHATIC ALCOHOLS

Results are given as means and range of values for 5 experiments; values have not been corrected for ^{32}P in residual RNA of membrane as discussed in text.

Additions to 0.03 M Tris buffer phase	*n-Butanol*	*sec-Butanol*	*iso-Butanol*	*n-Amyl alcohol*	*iso-Amyl alcohol*	*3-Pentanol*	*tert-Amyl alcohol*
None	15.1 (6.9–21.0)	9.3 (6.0–15.0)	14.9 (10.8–21.8)	23.8 (18.4–30.2)	28.0 (24.6–36.5)	23.2 (19.0–27.0)	11.2 (5.0–15.6)
2 M Urea	8.1 (3.4–12.6)	9.1 (4.5–13.2)				14.2 (11.9–21.0)	12.6 (10.8–15.4)
6 M Urea	6.2 (5.8–8.4)	6.0 (5.6–7.3)				19.3 (16.2–22.8)	6.4 (6.0–7.6)
0.1 M NaCl	18.5 (16.2–21.0)	10.2 (6.5–11.0)				52.8 (41.2–69.0)	15.2 (12.1–18.6)
1.0 M NaCl	21.0 (20.0–24.0)	12.4 (9.0–14.1)				60.9 (52.8–70.1)	18.6 (14.1–20.6)

TABLE III

ABILITY OF SELECTED ALCOHOLS TO EXTRACT ^{32}P-LABELED PHOSPHOLIPID FROM AN AQUEOUS SUSPENSION OF LIPID MICELLES IN THE ABSENCE AND PRESENCE OF BOVINE SERUM ALBUMIN

Extraction of ^{32}P-labeled lipid determined in the presence and absence of bovine serum albumin (protein : lipid ratio, 2 : 1, by weight) as indicated in Materials and Methods. Residual ^{32}P in aqueous phase determined directly. Values expressed as % original ^{32}P-labeled lipid added and represent means of duplicate experiments.

Solvent	*System*	% *Extracted by 3 successive extractions*			% *Remaining in aqueous phase*
		1st	*2nd*	*3rd*	
n-Butanol	—	85.6	9.6	0.5	3.2
	plus bovine serum albumin	87.8	7.4	0.6	1.0
3-Pentanol	—	80.5	14.2	2.1	2.0
	plus bovine serum albumin	81.6	12.7	2.5	2.3
tert-Amyl alcohol	—	89.9	5.1	1.1	1.5
	plus bovine serum albumin	85.4	8.1	0.1	2.0

tion procedure, the lipid dispersions were mixed with solutions of bovine serum albumin in 0.03 M Tris–HCl buffer, pH 7.5 at a final protein concentration of 5 mg/ml, thus giving a protein to lipid ratio of 2:1, by weight. Extraction of these aqueous lipid dispersions in the presence or absence of protein gave no interfacial or pellet fractions and 93–96 % of the initial ^{32}P-labeled phospholipid was recovered in the usual three successive extractions with the organic solvents. The residual ^{32}P-labeled lipid in the aqueous phase ranged from 1.0 to 3.2 % and the results are summarized in Table III. A comparison of these results, which demonstrate almost maximal recovery in the solvent phases, with those obtained with the membranes indicates that a significant amount of residual ^{32}P-labeled material was not extracted from the aqueous phase under these conditions (Table II). The origin and nature of this residual material has not been fully elucidated and could represent phospholipid which is tightly bound to membrane protein and possibly other phosphorus-containing macromolecules (*e.g.* phosphoprotein, or membrane teichoic acid[19]). About one-half of the residual ^{32}P of the *n*-butanol extracted membranes can be extracted with chloroform–methanol and identified as phospholipid as indicated below and this could therefore represent the more firmly bound phospholipid.

Chromatography of extracted phospholipids

Paper chromatography of the solvent extractions demonstrates that 95–97 % of the extractable radioactivity travelled with known phospholipids (54 % cardiolipin 38–40 % phosphatidyl-glycerol and 3–5 % phosphatidylinositol in accord with published data from this laboratory[20]). 3.0–4.0 % travelled ahead of the fastest moving cardiolipin, near the front and was not identified.

Analysis of the residual chloroform–methanol extractable material from the aqueous phase following *n*-butanol extraction, revealed essentially the same chromatographic appearance and phospholipid composition as for whole membranes, thus confirming the presence of residual phospholipids in this phase. There was thus no evidence of preferential solubility of certain phospholipids in *n*-butanol under these extraction conditions.

Protein solubilization and polyacrylamide gel electrophoresis

No marked differences could be detected in the abilities of the various alcohols to "solubilize" the membrane proteins as determined by aqueous phase protein after removal of lipid into the organic solvent. However, the recovery of protein in the aqueous phase was affected by the addition of urea or sodium chloride as shown in Table IV. Thus, in the presence of 2 M urea, *sec*-butanol and *tert*-amyl alcohol gave superior yields of protein in the aqueous phases. On the other hand, the presence of sodium chloride resulted in greater denaturation and less soluble, aqueous phase protein for all the alcohols tested. Although Maddy[3,4] has reported that optimum erythrocyte protein solubilization occurred at low ionic strengths, extraction of the *M. lysodeikticus* membranes suspended in distilled water or molarities of Tris buffer below 0.003 M did not substantially alter the amount of protein recovered in the aqueous phase (unpublished observations).

The profile of the aqueous phase proteins following extraction with *n*-butanol is illustrated in Fig. 1, similar results having been obtained with *tert*-amyl alcohol. The ability of alcohols to cause denaturation of the membrane proteins was also reflected

TABLE IV

PROTEIN REMAINING IN AQUEOUS PHASE FOLLOWING ALCOHOL EXTRACTION OF *M. lysodeikticus* MEMBRANES

Results are given as the mg protein recovered in the aqueous phases which contained 15 mg initial membrane protein prior to extraction as described in Materials and Methods. The means and range of values (in parentheses) are from 5 experiments.

Additions to 0.03 M Tris buffer phase	*mg Protein remaining from initial 15 mg*						
	n-Butanol	*sec-Butanol*	*iso-Butanol*	*n-Amyl alcohol*	*iso-Amyl alcohol*	*3-Pentanol*	*tert-Amyl alcohol*
None	6.6 (4.5–7.9)	6.6 (5.0–7.5)	6.6 (4.9–7.9)	6.8 (5.5–8.1)	7.0 (6.0–7.8)	6.5 (5.5–7.3)	7.1 (5.0–9.2)
2 M Urea	6.5 (5.2–7.8)	9.6 (7.6–12.9)				6.9 (6.4–9.9)	8.9 (6.8–10.9)
6 M Urea	5.9 (4.7–7.0)	6.2 (4.3–8.5)				5.4 (4.6–6.2)	6.9 (5.8–7.9)
0.1 M NaCl	3.6 (2.6–4.4)	4.1 (3.1–4.7)				4.2 (3.4–4.8)	3.8 (2.9–4.8)
1.0 M NaCl	2.5 (2.0–2.9)	2.9 (2.6–3.1)				4.1 (3.2–5.4)	3.6 (2.9–4.0)

TABLE V

MEMBRANE ADENOSINE TRIPHOSPHATASE IN AQUEOUS PHASES FOLLOWING EXTRACTION WITH ALIPHATIC ALCOHOLS

Results are expressed as units of ATPase (as assayed and defined by Muñoz *et al.*[16])/ml aqueous phase following extraction with the alcohols as described in text. All aqueous phases were dialyzed against 0.03 M Tris–HCl buffer to remove solvent and additives prior to assay. Means and range of values for 5 experiments are given; "0" indicated no detectable activity, "—" not tested.

Additions to 0.03 M Tris buffer phase	*ATPase (units/ml)*						
	n-Butanol	*sec-Butanol*	*iso-Butanol*	*n-Amyl alcohol*	*iso-Amyl alcohol*	*3-Pentanol*	*tert-Amyl alcohol*
None	(2.5–4.0)	(0–0.9)	(2.2–4.0)	(0.9–3.5)	(0.5–4.2)	(0–1.1)	(1–4.6)
	3.8	0.3	3.5	2.8	3.0	0.5	2.5
2 M Urea	0	0	—	—	—	0	(0–1.1)
6 M Urea	0	0	0	0	0	0	0
0.1 M NaCl	(0–1.6)	(0–0.2)	—	—	—	(0–1.1)	0
	0.5	0.1	—	—	—		
1.0 M NaCl	0	0	—	—	—	(0–1.1)	0
		0	—	—	—		

* The range of ATPase units in the unextracted aqueous phase was of the order of 1.2–2.3 units/ml. However, ATPase is a nascent enzyme in this bacterial system and the activity reported for the unextracted state represents only a part of the total "releasable" enzyme activity[16].

in polyacrylamide-gel-electrophoretic protein profiles. Denaturation leading to protein insolubility resulted in exclusion of proteins from the separating gel. The marked reduction of the major protein band, identifiable as the ATPase[16], was a conspicuous feature of the *sec*-butanol and 3-pentanol extracted membranes. The ATPase reduction in the acrylamide gels was paralleled by the almost complete lack of ATPase activity in the aqueous phase preparations (see Table V).

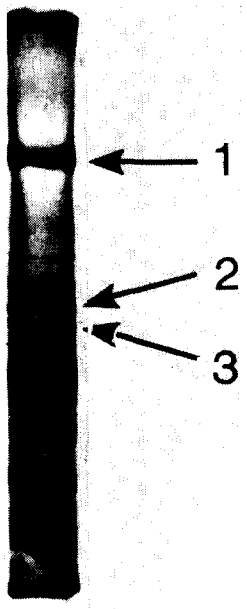

Fig. 1. Polyacrylamide gel electrophoresis of aqueous phase proteins of *M. lysodeikticus* membranes following *n*-butanol extraction as described in the text. Adenosine triphosphatase[16] (1) is by far the most prominent protein entering the separating gel. Bands (2) and (3) correspond to the "double-stranded" region exhibiting NADH dehydrogenase activity as described by Nachbar and Salton[17].

Enzymatic functions

Aqueous phase preparations were assayed for the two enzymes, ATPase and NADH dehydrogenase and the results are presented in Tables V and VI.

ATPase activity was recovered in good yield only under buffered aqueous conditions. The selective denaturing effects of *sec*-butanol and 3-pentanol were also apparent.

NADH dehydrogenase was detectable under all but the 6 M urea conditions. Of great interest was the preservation of activity in the presence of 2 M urea and the higher activities with *tert*-amyl alcohol and *sec*-butanol, thus again emphasizing the selectivity which the various alcohols exhibit in protein denaturation and/or preservation.

DISCUSSION

From the results reported in this paper, it is evident that it is not possible to predict the effectiveness of a particular aliphatic alcohol in the differential solubiliza-

TABLE VI

MEMBRANE NADH DEHYDROGENASE IN AQUEOUS PHASES FOLLOWING EXTRACTION WITH ALIPHATIC ALCOHOLS

Results are expressed as units of NADH dehydrogenase/ml, as assayed and defined by Nachbar and Salton[17], following extraction with the alcohols as described in text. All aqueous phases were dialyzed against 0.03 M Tris–HCl buffer to remove solvent and additives prior to assay. Means and range of values for 5 experiments are given; "0" indicated no detectable activity, "—" not tested.

Additions to 0.03 M Tris buffer phase	*NADH dehydrogenase (units/ml)*							
	Unextracted	*n-Butanol*	*sec-Butanol*	*iso-Butanol*	*n-Amyl alcohol*	*iso-Amyl alcohol*	*3-Pentanol*	*tert-Amyl alcohol*
None	(10.8–13.4) 12.2	(2.0–3.5) 2.8	(5.3–8.8) 7.4	(3.3–4.2) 3.6	(3.1–6.8) 6.0	(5.8–8.3) 7.2	(4.4–7.1) 7.0	(7.6–11.8) 9.4
2 M Urea	(8.7–15.0) 10.9	(7.7–8.7) 8.0	(9.2–11.1) 10.0	— —	— —	— —	(2.8–5.9) 4.4	(10.2–16.2) 12.4
6 M Urea	(2.0–3.6) 2.5	0	0	—	—	—	0	0
0.1 M NaCl	(11.0–27.0) 17.6	(3.0–10.4) 5.4	(7.0–10.4) 9.0	— —	— —	— —	(2.8–8.6) 5.1	(6.8–10.2) 7.9
1.0 M NaCl	(13.0–24.0) 17.0	(5.2–8.5) 7.8	(9.6–10.4) 9.8	— —	— —	— —	(2.6–9.4) 5.4	(7.8–9.8) 8.7

tion of membrane lipid and protein into organic solvent and aqueous phases respectively. In a simple system of an aqueous dispersion of phospholipid and soluble protein such as bovine serum albumin, *n*-butanol, *iso*-butanol, *sec*-butanol and *tert*-amyl alcohol were equally effective in extracting the lipid. However, when membranes of *M. lysodeikticus* were extracted under identical conditions, marked differences in the effectiveness of lipid removal were observed (see Table I). The water solubility of the alcohols seemed to give the closest approximation to their effectiveness in lipid extraction from the membranes. Thus the order of water solubility for the series used in this study was: *tert*-amyl alcohol = *sec*-butanol > *iso*-butanol > *n*-butanol > *n*-amyl alcohol > *iso*-amyl alcohol > 3-pentanol, compared with the experimentally observed order of: *tert*-amyl alcohol = *n*-butanol = *iso*-butanol = *sec*-butanol > *n*-amyl alcohol > *iso*-amyl alcohol > 3-pentanol for the extraction efficiency based on the total amount of lipid removed in three successive extractions. Differences between the two series must be due to other factors involved in the intermolecular interactions and environments in the membrane structure. Until more precise information is available on these factors, it will not be possible to fully understand and predict the mechanism involved in lipid displacement from protein in a particular membrane system. In this connection, it will be recalled that Morton[2] stated that the straight chain alcohol favored the solubilization of enzymes and that the branched chain type exhibited greater toxicity to enzymatic function. Herskovits *et al.*[21] concluded that the effectiveness of alcohols in denaturing proteins increased with chain length and diminished with branching, but noted deviations with certain proteins. Although protein solubilization in the present study (Table IV) exhibited small differences only with the various alcohols, there were marked differences in the response of the two enzymes, ATPase and NADH dehydrogenase to release and/or survival in the aqueous phases. Moreover, there appeared to be no systematic relationship between the structure of the alcohols and their ability to inactivate or denature the two enzymes.

Considering the results of Morton[2], the denaturation studies of Herskovits *et al.*[21] and the present investigation, there appears to be no obvious way of predicting the suitability of these alcohols for enzyme release and recovery from membranes. The individuality in the response of enzymes is perhaps not surprising and it is worthy of note that in this bacterial membrane system, the ATPase is essentially water soluble and devoid of lipid requirement for its hydrolytic activity[16] in contrast to the NADH dehydrogenase which is in a lipid rich particle and exhibits a degree of lipid stimulation after partial extraction of the lipids[17]. The degree of dependency of a particular membrane enzyme upon lipid for its stability and function may thus have an important bearing on the response of the enzyme to lipid displacement and survival in such organic solvent systems. This may account, for example, for the inactivation of erythrocyte ATPase[7] on the one hand and its survival in the bacterial system following *n*-butanol extraction.

The recovery of protein in the aqueous phase after *n*-butanol extraction of *M. lysodeikticus* membranes is considerably lower than that observed by Maddy[3,4] and by Rega *et al.*[22] for erythrocyte membranes (*e.g.* 44 % compared to values ranging from about 80–95 % for erythrocyte membranes). There are undoubtedly a number of reasons which could account for such differences. This bacterial membrane structure is a multifunctional system bearing electron transport components, many of which are strongly hydrophobic and are exceedingly difficult or impossible to obtain in

soluble form except by dissociation with detergents[23]. Moreover, bacterial membranes appear to lack the glycoproteins which may have greater intrinsic solubility in aqueous systems.

From our experience in this study, we feel that as yet it is not possible to predict the effectiveness of an aliphatic alcohol to dissociate lipid and protein of a particular membrane, especially when the preservation of specific enzymes is also sought. However, it is clear that the systematic evaluation of several alcohols, together with the manipulation of the aqueous environment (*e.g.* 2 M urea for the preservation of NADH dehydrogenase) may yield information of value in the study and purification of membrane enzymes.

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