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).

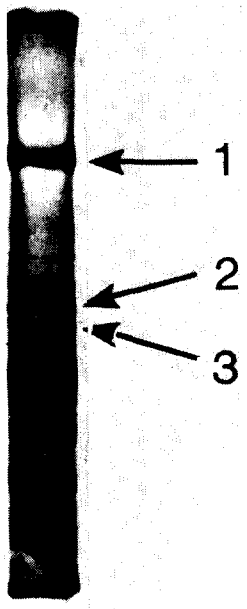

Fig. 1. Polyacrylamide gel electrophoresis of aqueous phase proteins of *M. lysodeikticus* membranes following *n*-butanol extraction as described in the text. Adenosine triphosphatase[16] (1) is by far the most prominent protein entering the separating gel. Bands (2) and (3) correspond to the "double-stranded" 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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ARE MYCOPLASMA MEMBRANE PROTEINS AFFECTED BY VARIATIONS IN MEMBRANE FATTY ACID COMPOSITION?

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SUMMARY

1. The influence of the fatty acid composition of membrane lipids on the quantitative distribution of membrane protein of *Mycoplasma laidlawii* has been investigated.

2. Cells were grown in media supplemented with either a saturated fatty acid (palmitic acid or stearic acid) or an unsaturated fatty acid (palmitoleic acid or oleic acid). Lipids isolated from membranes of these cells were markedly enriched in the supplemented fatty acid.

3. The proteins of the different types of membranes were analyzed by sodium dodecyl sulfate-acrylamide-gel electrophoresis of membranes from cells grown in the presence of radioactively labelled amino acids. In general, protein composition and relative abundance were unaffected by fatty acid variation. One high molecular weight protein fraction was present in higher concentration in membranes rich in saturated fatty acids than in those rich in unsaturated fatty acids.

4. The membranes rich in saturated fatty acids were also found to have a slightly higher density as determined by isopycnic centrifugation.

INTRODUCTION

Plasma membranes of *Mycoplasma laidlawii* cells contain lipid and protein as major components[1]. A substantial body of data indicates that the lipid molecules are predominantly if not exclusively organized as regions of bimolecular leaflets[2-4]. This tentative conclusion suggests that the structural properties of lipids in a biological membrane may be similar to their structural properties *in vitro*. No comparable conclusion can be drawn concerning the organization of proteins in the membrane. Although a majority of bulk membrane protein is probably localized on inner and outer membrane faces[5], there is very little information about the basic interactions by which different proteins bind to other proteins and/or lipids. It is not known, for example, whether functionally related proteins are organized in discrete regions or distributed throughout the membrane.

Considering the large number of different membrane polypeptides detected

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by gel electrophoresis[5,6] and the variety of protein-dependent functions in membrane-bound activities[7] there would appear to be little reason for assuming the existence of a single common pattern of protein structural organization. One can imagine that, in conjunction with different functional requirements, certain proteins might interact with specific fatty acyl chains, others with a particular lipid polar group, others with a particular set of proteins, *etc.* If such varieties of interactions occur, it may be more informative to consider the membrane experimentally as a mosaic of relatively unrelated proteins than to consider it as a bulk protein phase.

In the present investigation we have examined the effects of extensive variation in membrane fatty acid unsaturation and chain length on the quantitative distribution of *Mycoplasma laidlawii* membrane proteins detected by gel electrophoresis. We hoped to determine whether there were any classes of membrane proteins whose ability to bind to the membrane depended on the distribution of unsaturated bonds in fatty acids, or whether the protein composition of the membrane was entirely independent of the fatty acid content. We also hoped to gain some insight into the biochemical nature of the particles seen in freeze–fracture studies of these membranes, since significantly different particle distributions were reported for membranes containing predominantly saturated *versus* unsaturated fatty acids[4]. We found that one major protein band is altered in amount as a result of substantial changes in the degree of fatty acid unsaturation. This protein cannot be directly correlated with the freeze–fracture particles. All other membrane proteins showed no detectable changes in response to fatty acid variation.

MATERIALS AND METHODS

Organism and growth conditions

M. laidlawii B (recently renamed *Acholeplasma laidlawii*) was obtained from Dr H. J. Morowitz. Standard growth medium contained (per l); tryptose (Difco) 20 g; NaCl, 5 g; glucose, 7 g; penicillin G, 100000 units. The pH of the medium was 8.2–8.4 without adjustment. This medium was supplemented with sterile 10% defatted bovine serum albumin (Calbiochem) to a final concentration of 4 g/l, and generally also with a fatty acid (palmitic, palmitoleic, stearic, or oleic acid, obtained from Calbiochem and Sigma) in ethanol to a final concentration of 40 mg/l (ref. 8).

In order to minimize adaptive responses of cells to growth in a particular fatty acid, we stored stock cultures (grown in medium containing bovine serum albumin but without fatty acid) at −20 °C after adding glycerol to 20% final concentration. Stock cultures were transferred once before use as inocula for a particular experiment, and used for a maximum of four transfers. Growth was followed turbidimetrically at 500 nm in a Beckman DU spectrophotometer.

Radioisotope labelling

^{14}C- and ^{3}H-labelled amino acids (L-amino acid mixtures of 15 amino acids, from New England Nuclear Corp.) were added to growth medium at 100 μCi/l. In most experiments radioactivity was added at the time of inoculation although some experiments were carried out by labelling only for 2 h prior to harvesting. The level of incorporation for the longer labelling was approx. 1%. Final membrane preparations generally contained an activity of approx. 20000 dpm per mg protein. Gel patterns

produced from membranes labelled for 2 h were essentially identical to those of membranes labelled from the time of inoculation with cells.

Membrane isolation

Cells were harvested by centrifugation late in the logarithmic phase of growth, usually 30 h after inoculation. All subsequent operations were carried out at 4 °C. Cells were washed in B buffer (0.156 M NaCl, 0.05 M Tris, and 0.01 M 2-mercaptoethanol (pH 7.4) and lysed osmotically[9] by suspension in a 1:20 dilution of B buffer. Membranes were banded on discontinuous 25–50% (w/w) sucrose gradients in 1:20 B buffer, washed several times with deionized water, and then stored at −20 °C until further use.

Fatty acid analysis

Membrane suspensions were extracted with 10 vol. of chloroform–methanol (1:1, v/v). The organic solvent was evaporated and the residue subjected to a Folch extraction[10]. The isolated lipids were heated for 2 h at 80 °C with 0.5 M HCl in methanol to form fatty acid methyl esters. The esters were then analyzed by gas chromatography using an F and M Gas Chromatograph with an OV-1 column. Peak areas were automatically determined by an Infotronic automatic digital integrator. The column was calibrated with standard methyl esters of the different fatty acids (obtained from Applied Science, Pa.).

Gel electrophoresis and isotope counting

The sodium dodecyl sulfate–polyacrylamide-gel system of Maizel[11] was employed as previously described[5]. Membrane samples were solubilized in 1% sodium dodecyl sulfate with 0.01 M phosphate (pH 7) and 1% 2-mercaptoethanol by heating at 100 °C for 2 min. Electrophoresis was carried out for 18 h at 75 V in 20-cm gels. Immediately after the run, each gel was fractionated into 80–85 fractions of crushed particles on a Savant Autogel divider (Savant Instruments, N.Y.). Fractions were collected in scintillation vials with 0.1% sodium dodecyl sulfate as the eluting solvent, frozen and thawed to release protein from the gel particles, mixed with toluene–Triton X-100–Omnifluor (New England Nuclear Corp.) scintillation fluid, and counted in a Packard Tri-Carb liquid scintillation counter for 10 min. Molecular weight calibration of the gels was carried out as described by Shapiro *et al.*[12] using poliovirus proteins and myeloma proteins of known molecular weights as markers. Approximate molecular weights for some of the peaks seen in membrane gel profiles were obtained from this calibration.

Membrane density and protein content

Isotopically labelled membrane samples were layered over density gradients containing 25–50% (w/w) sucrose in 1:20 B buffer. Gradients were centrifuged at 4 °C for at least 2 h at 45000 rev/min in an SW-65 rotor Spinco L-2 ultracentrifuge. Longer centrifugations did not change the patterns observed. Gradients were unloaded by bottom puncture, collected in approximately 40 fractions, and analyzed for radioactivity and refractive index (using an Abbe 3-L refractometer). Densities of specific fractions were determined from standard tables after applying temperature corrections.

For determination of protein content, membrane samples of approx. 10 mg dry wt were lyophilized in tared flasks and weighed on an analytical balance (Mettler B6). The samples were then dissolved in 0.1 M NaOH and 1% sodium dodecyl sulfate and assayed for protein content at different dilutions by the method of Lowry *et al.*[13]. Different standards (bovine serum albumin, lysozyme, trypsin) gave calibration curves of different slopes; thus it was not possible to obtain reliable absolute values of membrane protein content, although bovine serum albumin gave values most comparable to previously reported values[1]. In order to compare the protein content of saturated and unsaturated fatty acid-supplemented membranes, every effort was made to treat parallel samples identically as they were carried through the procedures.

RESULTS

Fatty acid incorporation

Each of the fatty acids added to the growth medium was incorporated substantially by the cells (Table I). Fatty acid analyses of membrane lipids showed concentrations of 65–70% palmitate, oleate, and palmitoleate after growth in these respective supplements. Cells grown in stearic acid yielded membranes with 42% stearate and 32% palmitate. These results are comparable with those reported by other investigators[2,4,8]. Measured in terms of degree of fatty acid saturation, cells grown in the presence of saturated fatty acids contained 15–20% unsaturated acids in membrane lipids, whereas cells grown in unsaturated media produced membranes with 65–70% unsaturated fatty acids (Table I).

Noticeable morphological differences between these cell types were detected by phase microscopy. Unsaturated-supplemented cells typically grew as long beaded filaments, whereas saturated-supplemented cells grew as round spheres of variable size.

TABLE I

FATTY ACID COMPOSITION OF MEMBRANES FROM MYCOPLASMA CELLS GROWN IN THE PRESENCE OF DIFFERENT FATTY ACIDS

Total membrane lipids were extracted from purified membranes and analyzed for fatty acid content by gas chromatography. Fatty acids are designated by the number of carbon atoms followed by the number of double bonds. The fatty acid used to supplement the growth medium is indicated on the left.

Supplement	*Fatty acid composition of polar lipids (moles/100 moles)*					
	14:0	*16:0*	*16:1**	*18:0*	*18:1**	*Others*
Palmitate (16:0)	7.0	71.5	2.8	1.5	12.5	5.7
Palmitoleate (16:1)	3.9	23.8	63.6	1.5	3.5	4.7
Stearate (18:0)	3.5	31.9	Trace	41.8	20.6	2.2
Oleate (18:1)	1.6	22.5	Trace	6.6	68.2	1.3

* In these experiments, the column used did not distinguish monounsaturates from other unsaturates of the same chain length; however, other experiments with a different column indicate only trace levels of the other unsaturated fatty acids.

Acrylamide-gel analysis

The sodium dodecyl sulfate–polyacrylamide-gel system has been shown previously to separate *Mycoplasma* membranes into their maximally dispersed and disaggregated protein components, presumably single polypeptide chains[5]. In all the present experiments, relatively complex but highly repeatable gel profiles of membrane protein distribution were obtained, with 10–15 major peaks (Figs 1 and 2). Patterns were reproducible both with respect to the position of each major peak and to the relative distribution of radioactivity between different regions of the gel. At least four independent membrane samples were prepared from independent inocula for each fatty acid supplement investigated, and each of these preparations gave essentially identical gel patterns.

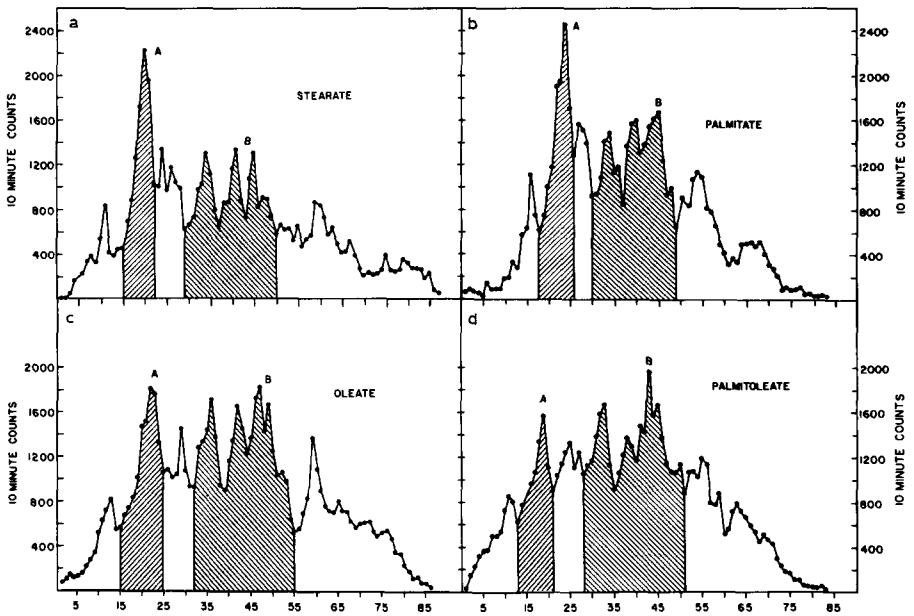

Fig. 1. Acrylamide-gel profiles of [^{14}C]amino acid-labelled membrane proteins from cells supplemented with: (a) stearic acid (C_{18}, saturated); (b) palmitic acid (C_{16}, saturated); (c) oleic acid (C_{18}, monosaturated); and (d) palmitoleic acid (C_{16}, monosaturated). Samples were applied at the left and migrated towards the anode at right. The gels were then crushed, collected in scintillation vials, and counted. Molecular weights of the different protein bands decrease from left to right.

Fig. 1 shows typical membrane protein gel patterns obtained from the four different types of fatty acid supplements investigated. Three results are evident:

(1) There are no gross dissimilarities between the location and spacing of different peaks, indicating that most, if not all, protein species are present in each type of membrane preparation. This conclusion is more obvious from the double-label experiments described below.

(2) Both stearate- and palmitate-supplemented membrane preparations show a greater percentage of total radioactivity in the first major high molecular weight peak (labelled A in Fig. 1) than is contained in the corresponding peak from oleate- and palmitoleate-supplemented membranes.

(3) Stearate and palmitate membranes are similar, as are oleate and palmitoleate membranes. Differences of two carbon atoms in chain length of the fatty acids appear to have little if any effect on membrane protein composition.

In order to make more quantitative comparisons of these similarities and

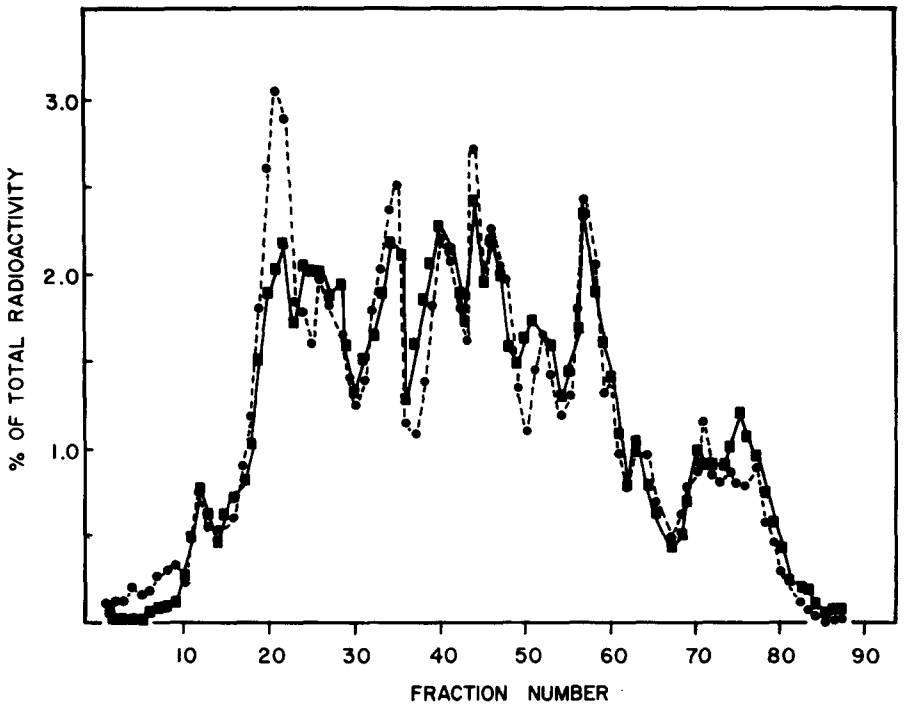

Fig. 2. Double-label acrylamide-gel profile of ^{3}H-labelled membrane proteins from cells supplemented with oleic acid (■—■); and ^{14}C-labelled membrane proteins from cells supplemented with stearic acid (●---●). Both samples were run on the same gel; procedures were as described in the legend to Fig. 1. The spillover of ^{14}C counts into the ^{3}H channel was 16%. The final data for each isotope are expressed as percent of total isotope counts on the gel associated with each fraction.

TABLE II

PERCENTAGE OF TOTAL PROTEIN LABEL FOUND IN REGIONS DESIGNATED A AND B IN GEL ELECTROPHEROGRAMS SHOWN IN Fig. 1

The boundaries for regions designated A and B were selected on the basis of curve shape for each of many different gel patterns. The percentage of total counts found in each region was determined and expressed as Peak A (%) and Peak B (%), along with the standard error for each such calculation. The number of different gel profiles examined is indicated below.

Fatty acid supplement	*Number of gels examined*	*Peak A* (%)	*S.E.*	*Peak B* (%)	*S.E.*
Oleate	6	14.8	0.6	41.7	0.5
Stearate	4	18.9	1.0	36.9	1.1
Palmitate	4	18.4	1.7	36.8	1.0
Palmitoleate	4	11.5	1.4	43.2	1.2

differences, we have arbitrarily labelled Regions A and B (Fig. 1) according to the location of certain peaks. Region A contains a major peak corresponding to a molecular weight of approx. 80000, while Region B encompasses several peaks in a molecular weight region of approx. 30000–50000. A total of 18 different gel patterns were analyzed for the distribution of radioactivity between these two regions, with the data presented in Table II. Despite slight differences in the positioning of boundaries from pattern to pattern, the results are extremely consistent. Protein A accounts for 18–19% of total radioactivity in both stearate- and palmitate-supplemented membrane preparations, but only 12 and 15% in palmitoleate- and oleate-rich membranes, respectively.

The double-label experiment shown in Fig. 2 allows a more careful comparison between the proteins present in oleate-enriched membranes and those in stearate-enriched membranes. Both membrane samples were run together on the same gel and their respective isotopes discriminated by scintillation counting. It is apparent that the only prominent difference between the two preparations is the greater amount of protein present in the first major high molecular weight peak in stearate-enriched membranes. Several other double-label comparisons were run including the reciprocal experiment to the one just described (oleate-enriched membranes labelled with ^{14}C *versus* stearate-enriched membranes labelled with ^{3}H). In each case the two patterns were similar except for the difference in the first major peak.

Although a detailed comparison of the different patterns in Fig. 1 might suggest that there are other peaks which differ slightly in amount with respect to variations in fatty acid supplement we believe these differences are largely artifactual and arise because of slight variations in the gel crushing process from gel to gel. Space does not permit publishing all the different gel profiles we have obtained, but there are minor variations even in the pattern of a single sample when run on two parallel gels. However, we have consistently observed the difference in Peak A protein between saturated and unsaturated-fatty acid supplemented membranes in both single label and double label comparisons in over 30 gel runs.

Membrane density and protein content

A sample of membranes derived from oleate-supplemented [^{14}C]amino acid-labelled cells was mixed with a comparable amount of membranes from stearate-supplemented [^{3}H]amino acid-labelled cells. This mixture was centrifuged to isopycnic equilibrium on a density gradient with the resulting distribution of material shown in Fig. 3. The stearate membranes were about 1.3% denser than the oleate membranes (peak densities were approx. 1.182 gm/cm^3 for stearate, 1.166 gm/cm^3 for oleate). A nearly identical result was obtained in a comparable gradient analysis of palmitate-supplemented, [^{14}C]amino acid-labelled membranes *versus* palmitoleate-supplemented, [^{3}H]amino acid-labelled membranes (graph not shown). A measurement of protein content of lyophilized, weighed samples of stearate- and oleate-enriched membranes by Lowry assay[13] gave values of 54 and 52% protein, respectively, but this measurement is of questionable sensitivity. We conclude that the protein content of saturated- and unsaturated-fatty acid rich membrane differs only slightly, assuming that density is a function of protein: lipid ratio[5]. The slight increase in protein content of stearate membranes could be correlated with the increase in Peak A protein observed above.

DISCUSSION

The major conclusion to be drawn from these data is that the protein composition of *Mycoplasma* membranes is generally insensitive to variations in (1) the degree of unsaturation of incorporated fatty acids over a range from 15–70 % unsaturation and (2) the length of fatty acid chains in the range 16–18 carbon atoms. With one exception (a consistent difference in the relative amount of protein in one electrophoretic band, discussed below) we did not detect significant changes in the number of different membrane proteins present or in their relative abundance as a function of gross changes in fatty acid composition. In terms of membrane architecture, this result suggests that protein–lipid interactions do not depend significantly on the binding of membrane proteins to specific fatty acids.

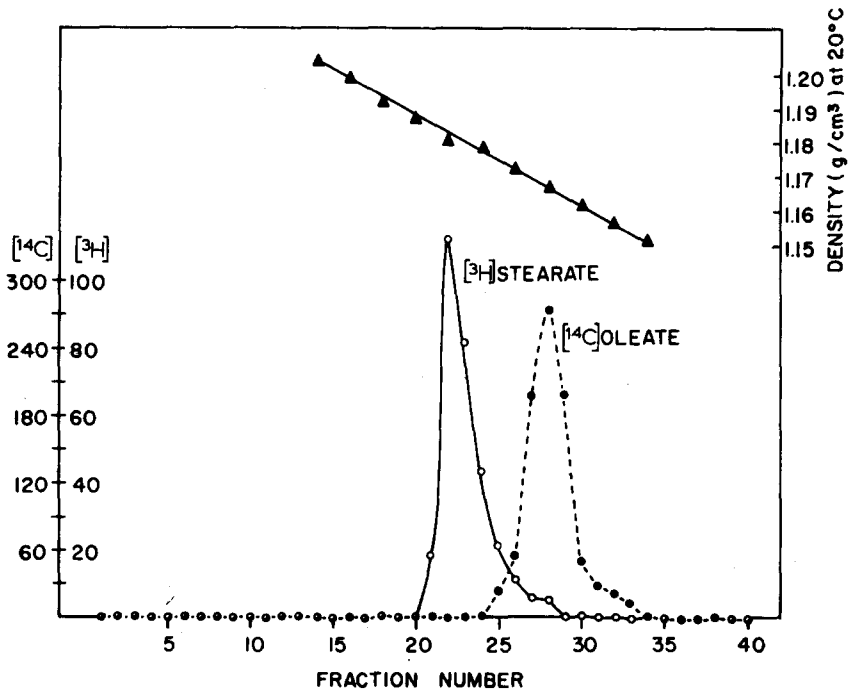

Fig. 3. Density gradient analysis of isopycnically banded membranes from stearate-supplemented cells labelled with ^{3}H-labelled amino acids (○—○) and oleate-supplemented cells labelled with ^{14}C-labelled amino acids (●---●). Membrane samples were mixed applied to a 25–50% (w/w) sucrose gradient, and centrifuged at 4 °C for 2 h at 45000 rev./min. After fractionation, samples were analyzed for radioactivity and refractive index (▲—▲).

This conclusion is consistent with other data concerning the interaction of protein and lipid in *Mycoplasma* membranes. (1) Kahane and Razin[14] demonstrated that membranes in chloramphenicol-treated cells grew progressively lighter in density with continued cell growth, suggesting that the incorporation of lipids into the membrane could be substantially uncoupled from protein incorporation. (2) Evidence from X-ray scattering[2], differential scanning calorimetry[3,15] and freeze–fracture electron microscopy[4] suggests that lipids are organized in regions of bimolecular leaflets,

irrespective of differences in fatty acid composition. (3) Studies correlating morphological and biochemical changes of membranes exposed to proteolytic digestion[5] suggest that membrane proteins are localized predominantly on inner and outer membrane surfaces. These data are consistent with the general model of Danielli and Davson[16]; however, they do not provide any understanding of the details of protein–protein or protein–lipid interactions.

A second conclusion to be drawn from these data is that one protein band does change in amount as a result of fatty acid variation. We consistently observed an increase of 30–60 % in the amount of protein found in the highest molecular weight peak (labelled Peak A; see Fig. 1) when comparing membranes supplemented with saturated fatty acids to those supplemented with unsaturated fatty acids. We have not yet identified the function of this protein (or class of proteins). We can summarize its properties as follows:

(1) Peak A protein has a molecular weight of roughly 80000 (calibrated by gel electrophoresis of known molecular weight standards).

(2) Peak A protein accounts for approx. 18–19 % of the total protein in stearate- and palmitate-supplemented membranes, 15 % in oleate-supplemented membranes, and 12 % in palmitoleate-supplemented membranes.

(3) Peak A protein is the highest molecular weight protein fraction detected as a major membrane constituent. Gels which have been stained to localize protein rather than assayed for isotope distribution show a prominent protein band (sometimes resolved as two bands) in the same region (see Figs. 11 and 14 in ref. 5) and we assume they correspond to the same protein fraction. In earlier work[5] we demonstrated two further properties of this high molecular weight protein: (a) it was the protein fraction most susceptible to solubilization by detergent at low concentrations and (b) it was the protein fraction most susceptible to digestion by pronase at low concentrations. These properties suggest that at least a substantial portion of this protein is localized on the membrane surface.

Conceivably the difference in protein patterns observed between saturated and unsaturated fatty acid-supplemented cells could reflect a decrease in the amount of other major proteins in the membrane rather than an increase in Peak A protein. Measurements of membrane density argue against the latter explanation. Membranes rich in saturated fatty acids are 1.3 % denser than membranes rich in unsaturated fatty acids, and both contain slightly more than 50 % protein as measured by Lowry assay. These data are consistent with the interpretation that cells growing in media supplemented with saturated fatty acids incorporate a larger quantity of Peak A protein into their membranes than when growing in media supplemented with unsaturated fatty acids. Our data suggest that this is the only major protein species which undergoes any significant change in concentration as a result of changes in fatty acid composition. It is not clear which mechanisms of cellular control or membrane synthesis generate this change.

Tourtellotte *et al.*[4] reported a significant difference in the distribution of membrane-bound particles visualized by freeze–fracture electron microscopy between saturated *versus* unsaturated fatty acid-supplemented membranes. Their pictures show extended regions of smooth fracture faces interrupted by patches of small particles. In stearate-supplemented cells these particles occupy approx. 10 % of the fracture-exposed membrane surfaces; in oleate-supplemented cells they occupy

approx. 20 % of the surface area. The chemical identity and function of the particles is not yet known; however, it is tempting to speculate that they might be proteins. Our data do not offer any evidence to support this speculation. (We assume here that we can compare our gel results with the electron microscopy results since the same cells and growth conditions were used in both studies.)

If the particles were protein we should expect the gels to show a difference of substantial proportions in the amounts of one or more protein bands corresponding to the particles in comparisons of stearate- *versus* oleate-supplemented cells. We observe an increase in Peak A protein in comparison of membranes from stearate cells to membranes from oleate cells, while freeze–fracture shows a decrease in particle frequency in the same comparison. Thus it seems unlikely that Peak A protein corresponds to the particles. The fact that no other bands can be seen to change in amount with respect to this comparison raises the possibility that, if the particles are proteinaceous, then their absence from the fracture plane does not necessarily imply their absence from the membrane.

Two limitations of the present study deserve mention. (1) Our conclusions regarding comparisons of proteins from membranes enriched with different fatty acids are necessarily limited to those proteins present in substantial amounts. There are a number of very sharp acrylamide-gel bands visible by staining which contain minute amounts of protein per band; we have not been able to resolve these as discrete peaks by gel analysis of labelled proteins. (2) Our study has been limited to the analysis of membrane proteins as biochemical components. It is possible that the functional properties of some membrane proteins may be found to change as a result of changes in fatty acid composition.

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