Protein Composition and Extractability of Lipid-Modified Membranes from *Acholeplasma laidlawii*†

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ABSTRACT: Membranes from *Acholeplasma laidlawii* have been extracted with neutral detergents, which solubilize the proteins and lipids *selectively*, or with sodium deoxycholate, which gives an almost total solubilization. The amounts of individual proteins present in the detergent extracts of membranes with induced variations in lipid compositions were determined by crossed immunoelectrophoresis. Extraction with the neutral detergent Tween 20 showed that ionic lipids and the glucolipid diglucosyl diglyceride were enriched in the Tween extracts whereas the glucolipid monoglucosyl diglyceride (which cannot easily be accommodated in micelles for geometrical reasons) was enriched in the membrane residue. The amount of monoglucosyl diglyceride in the Tween 20 extracts increased when the content of this lipid was increased in the membrane, and protein D_{12} was also more easily extracted from membranes rich in monoglucosyl diglyceride. This was not correlated with an increase in the *total* amounts of D_{12} in the membrane (as analyzed by crossed immunoelectrophoresis after sodium deoxycholate solubilization), indicating that monoglucosyl diglyceride is involved in the anchoring of protein D_{12} in the membrane. The *total* amount of the flavoprotein T_{4a} in the membrane was found to increase upon enhanced amounts of ionic membrane lipids. Furthermore, protein T_{4a} was found to be increasingly *extractable* upon an increase in the amounts of unsaturated fatty acyl chains in the lipids. Several other proteins also displayed lipid-dependent extractabilities. These results support the hypothesis that for membrane proteins the extractability with neutral detergents and thus interactions with lipids are partly dependent upon the molecular shapes of the membrane lipid molecules. Thus, by use of these selective extraction procedures, information about protein–lipid interactions in the membrane was gained.

Membrane proteins are dependent upon an interaction with the lipid matrix for optimal function. However, few membrane proteins have been shown to have requirement for specific lipids (Gennis & Jonas, 1977; Sandermann, 1978). Existence of different lipids in a membrane is probably necessary for a correct embedding of different proteins in the bilayer (Israelachvili, 1977) since the conformation of the hydrophobic parts of integral membrane proteins probably differ considerably between one protein and another. The shapes of these hydrophobic parts cause geometric packing constraints in the bilayer and may impose structural couplings between proteins and their lipid surroundings (Israelachvili, 1977). It is likely that no unperturbed lipid bilayer regions exist in biological membranes since they in general have a high protein/lipid ratio (Israelachvili et al., 1980). Geometrical factors are also considered to be very important for the assembly of amphiphilic molecules into membrane bilayers and other lipid structures (Israelachvili et al., 1976). They further set the limits to lipid variation in a membrane corresponding to a *stable* lamellar (bilayer) phase. The extensive variation in polar lipid composition that occurs in *Acholeplasma laidlawii* upon internal and environmental stimuli is a mechanism for securing a stable bilayer (Wieslander et al., 1980, 1981). The dominant changes occurred in the ratio of the main polar lipids monoglucosyl diglyceride (MGDG)[1] and diglucosyl diglyceride (DGDG)[1], which in pure state form a reversed hexagonal (H_{II})[1] and a lamellar phase, respectively, together with water (Wieslander et al., 1978).

By extensive induced variations in the lipid matrix, the structural couplings between proteins and lipid in membranes are likely to be changed (cf. Borochov & Shinitzky, 1976). Several investigations have indicated that variations in membrane protein composition can occur in *A. laidlawii* upon imposed changes in the acyl chain content of the membrane lipids (Morowitz & Terry, 1969; Pisetsky & Terry, 1972; Amar et al., 1979; Silvius et al., 1980). However, no individual proteins were identified.

The specificity and the quantitative nature of antigen–antibody reactions often make immunological methods superior to others in membrane studies (Bjerrum, 1977; Owen, 1981). Several individual integral membrane proteins of *A. laidlawii* have been purified, localized, and partially characterized by selective detergent solubilization and electroimmunochemical methods (Johansson & Hjertén, 1974; Johansson et al., 1979). The identities of the main precipitates obtained by crossed immunoelectrophoresis (CIE)[1] of the solubilized *A. laidlawii* membrane proteins have also been established (Johansson & Wróblewski, 1978).

When *A. laidlawii* membranes are extracted with a nonionic surfactant, e.g., Tween 20 (Tw 20)[1], a selective solubilization of integral proteins occurs (cf. above). The mechanism for solubilization with nonionic detergents can be understood on a geometrical basis. When inserted into a bilayer, the detergent molecules locally increase the curvature of the membrane by way of their pronounced wedge shape (▽, with polar head at top) and causes a (local) lamellar to micellar transition (Helenius & Simons, 1975).

In this work, the influence of membrane lipid composition on total amounts and extractability of membrane proteins from *A. laidlawii* B(ju) have been studied by sensitive electroim-

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[1] Abbreviations used: CFU, colony-forming units; CIE, crossed immunoelectrophoresis; DGDG, diglucosyl diglyceride; H_{II}, reversed hexagonal phase; MGDG, monoglucosyl diglyceride; $NaDodSO_4$, sodium dodecyl sulfate; NaDOC, sodium deoxycholate; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; Tw 20, Tween 20; TX-100, Triton X-100; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Table I: Detergent Systems Used in Crossed Immunoelectrophoresis

detergent	concn of detergent in the gel: first dimension	concn of detergent in the gel: second dimension	buffer	field strength, first dimension[c] (V/cm)	duration of the run, first dimension[c] (min)	antiserum
Tw 20 or TX-100	1% (w/v)	1% (w/v)	80 mM Tris-acetic acid (pH 8.6)	15	50	polyspecific[d]
NaDOC[a]	12 mM	0 mM	0.1 M glycine-NaOH[b] (pH 9.1)	10	20	monospecific

[a] Cf. Wróblewski et al. (1977). [b] Gives sharper immunoprecipitates in the presence of NaDOC than the Tris buffer. [c] The field strength and the duration of the run for the second-dimensional electrophoresis were 2 V/cm and 15–20 h, respectively. [d] In some experiments, intermediate gels containing a monospecific antiserum were used.

munochemical methods. It was found that both the total amounts of certain individual proteins in the membrane and their detergent extractability are partly dependent upon the molecular shapes of the membrane lipid molecules. The most pronounced effect was observed for a membrane-bound flavoprotein.

Experimental Procedures

Organism and Growth Conditions. Acholeplasma laidlawii, strain B(ju), was grown statically at 30 °C in thoroughly lipid-depleted bovine serum albumin/tryptose media (Christiansson & Wieslander, 1980) supplemented with palmitic and oleic acids in the following proportions (μM/μM): 0/150, 30/120, 75/75, and 120/30. A mixture of oleic acid, linoleic acid, and cholesterol was obtained by supplementing the medium (containing no bovine serum albumin) with 1% (v/v) PPLO serum fraction (Difco Laboratories). The cells were well adapted to the different supplements by at least ten consecutive passages. Membrane lipids were labeled by adding 10 μCi/L of [^{14}C]oleic acid (56 Ci/mol) to *all* media. The PPLO serum fraction medium was also supplemented with 10 μCi/L of [^{14}C]linoleic acid (51 Ci/mol) and 40 μCi/L of [^{3}H]cholesterol (43 Ci/mmol). The palmitic acid containing media were supplemented with 30 μCi/L of [^{3}H]palmitic acid (57.9 Ci/mol).

Growth was followed by light microscopy, measurement of pH and the optical density at 400 nm, and determination of colony-forming units (CFU).[1]

Preparation of Membranes. Membranes were prepared by osmotic lysis (Johansson et al., 1975) and stored in diluted (1:20) β buffer (Razin et al., 1965) at −70 °C until used.

Materials. Agarose A was obtained from Pharmacia Fine Chemicals. Sodium dodecyl sulfate ($NaDodSO_4$)[1] was purchased from Kebo AB, Stockholm, Sweden. Sodium deoxycholate (NaDOC)[1] and Triton X-100 (TX-100)[1] were from Sigma, and Tween 20 (Tw 20)[1] was from Atlas Chemie GmbH, Essen, Germany.

Solubilization of Membranes with Different Detergents. The following four detergent solutions were used: $NaDodSO_4$ (0.2 M), TX-100 (5% w/v), Tw 20 (5% w/v) in 0.1 M Tris–HCl buffer (pH 8.0), and NaDOC (0.12 M) in 0.1 M glycine–NaOH buffer (pH 9.1). A volume of 50 μL of a membrane suspension containing 40 mg/mL protein and lipid was mixed with 50 μL of the detergent solution. With respect to Tw 20, the final concentration in the membrane-detergent mixture, i.e., 2.5% (w/v), corresponds to approximately 1.2 mol of Tw 20 per mol of membrane lipids (cf. Simons et al., 1973). The mixtures were left for 1 h at room temperature, and insoluble material was then sedimented by centrifugation at 130000*g* for 10 min in a Beckman Airfuge$^{T.M.}$.

The supernatants, which will be referred to as, e.g., the NaDOC supernatant, were stored at −70 °C until used. Total protein content was estimated according to Hartree (1972) and by measuring the absorbance at 280 nm. Tw 20 does not contribute significantly to the absorbance at 280 nm.

Polyacrylamide Gel Electrophoresis. Protein composition of membranes and extracts were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis in a discontinuous buffer system as described (Wieslander et al., 1979). Resolution was enhanced by preparing a 7–22.5% (w/v) linear gradient of acrylamide with 1% cross-link.

Production of Antisera. Polyspecific antisera directed against the membrane proteins were raised in rabbits by inoculation with 0.4 mg of membranes per immunization (Harboe & Ingild, 1973). Monospecific antisera were produced against the proteins T_2, T_{4a}, and D_{12} (Johansson et al., 1979; Johansson & Wróblewski, 1978) by immunizing rabbits with excised immunoprecipitates (Watson & Wildly, 1969) obtained by preparative line immunoelectrophoresis (Krøll, 1973) of the purified proteins.

Crossed Immunoelectrophoresis. The amounts of individual membrane proteins in the detergent supernatants were determined by crossed immunoelectrophoresis (CIE)[1] (Laurell, 1965) in the presence of detergent (Johansson & Hjertén, 1974) with the modifications given in Table I. Peak areas subtended by individual immunoprecipitates were estimated by weighing the excised drawings obtained after magnification (100×) in a standard photographic enlarger. The identities of some immunoprecipitates were established by CIE with intermediate gels containing monospecific antisera (Axelsen, 1973). In the CIE–NaDOC system, only monospecific antisera were used. The concentration of the antisera was in general 10 μL/cm^2, and the thickness of the antibody-containing gel was 1.2 mm. The titer of the antiserum against T_{4a} was lower than the titers of the other monospecific antisera. Thus, the concentration of this antiserum in the CIE experiments was 20 μL/cm^2.

Lipid Analysis. Lipids were extracted from membranes and residues with a modified Bligh and Dyer method according to Kates (1972). Excess of unlabeled carrier lipids (from *A. laidlawii*) was added to the extraction mixtures. Separation by thin-layer chromatography, identification, purification, and quantification by gas-liquid chromatography and liquid scintillation counting were performed as described (Christiansson & Wieslander, 1978, 1980) with the modification that 6% (v/v) Biosolv (Beckman) was included in the liquid scintillation cocktail.

Results

Solubilization Conditions. The impact of several experimental factors were established for membranes from cells grown with 75 μM each of palmitic and oleic acids. As judged from CIE, all detectable proteins were more efficiently solubilized with Tw 20 than with TX-100. This difference was

Table II: Lipid Composition in Membranes from *A. laidlawii* B(ju) Grown with Different Amounts of Saturated and Unsaturated Fatty Acids (24 h, 30 °C)[a]

sample	I	II	III	IV	V
supplementation of palmitic and oleic acids (μM/μM) to the growth medium	PPLO serum fraction[b]	0/150	30/120	75/75	120/30
% oleic acid in lipids (mol/mol)	60 (+25% 18:2c)[c]	95	84	57	30
ratio MGDG/DGDG[d] (mol/mol)	0.28 (1.07)[e]	0.35	0.41	0.51	0.62
% ionic lipids[f] of total polar lipids (mol/mol)	48	40	36	35	23
μmol of total lipids per μg of membrane protein	0.60	1.44	0.86	0.89	0.82

[a] The variability of the values, expressed as ±SE (3), never exceeded 5%. The number of experiments is given within parentheses. [b] PPLO serum fraction (Difco Laboratories), commercial serum for mycoplasmas, acting here as a source of oleic (18:1c) and linoleic (18:2c) acid and cholesterol. Palmitic acid was not added. [c] Membrane lipids contained approximately 25% (mol/mol) 18:2c and 60% 18:1c (mol/mol), as determined by gas-liquid chromatography and liquid scintillation counting. [d] Ratio monoglucosyl diglyceride/diglucosyl diglyceride. [e] Ratio (MGDG + cholesterol)/DGDG. These membranes contained 24% (mol/mol of total lipids) cholesterol. [f] Ionic lipids: phosphatidylglycerol, glycerophosphorylmonoglucosyl diglyceride, and glycerophosphoryldiglucosyl diglyceride.

observed independently of the presence of Tw 20 or TX-100 in the gels. Extraction of membranes with Tw 20 at different pHs (between pH 8.0 and 9.1) did not affect the immunoprecipitation pattern in the Tw 20 system (see Table I) significantly. No drastic changes in relative concentrations of the proteins T_2, T_{4a}, and D_{12} were observed by CIE–NaDOC of NaDOC supernatants of membranes from cells harvested at different phases of the growth cycle.

Solubilization of Membranes with Different Lipid Compositions. When the fatty acid composition of the growth medium is changed, extensive variation of membrane polar lipid composition can be introduced. These variations are much more pronounced than variations occurring within a growth cycle (Wieslander & Rilfors, 1977; Wieslander et al., 1980). Table II shows the lipid composition in membranes from *A. laidlawii* B(ju) grown to the late log phase with different amounts of saturated and unsaturated fatty acids. Great differences in lipid composition were revealed. When small amounts of unsaturated fatty acids (i.e., oleic acid) were incorporated, MGDG synthesis increased, and the amounts of DGDG and ionic lipids decreased (Table II). This B strain thus possesses the same regulating mechanisms for polar lipid composition as strain A of *A. laidlawii* (Wieslander et al., 1980). Although significant variations occurred in the ratio lipid/protein (Table II), $NaDodSO_4$–polyacrylamide gel electrophoresis patterns of membrane protein were almost identical (Figure 1). These membranes were also extracted with Tw 20 and NaDOC (see Experimental Procedures). Table III shows that 50–90% of the membrane was solubilized with Tw 20 and 70–100% with NaDOC, as judged from absorbance measurements at 280 nm. The membrane preparations, which were most soluble in NaDOC (corresponding to samples III and IV), were also solubilized to a great extent with Tw 20. The protein compositon in NaDOC supernatants

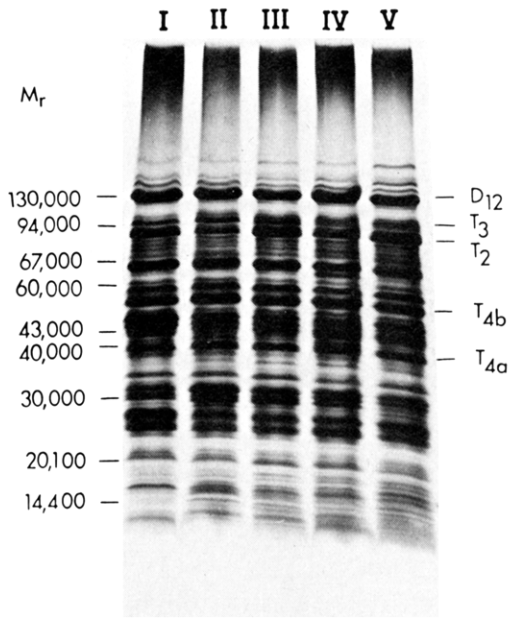

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis [7–22.5% (w/v) linear acrylamide gradient] of membrane proteins from *A. laidlawii* B(ju) grown with different fatty acid supplements. See Table II for growth medium supplements and membrane lipid composition. The positions shown for individual *A. laidlawii* proteins are those obtained by coelectrophoresis of the purified, single proteins. M_r, molecular weights of reference proteins.

Table III: Solubilization of Membranes with Different Lipid Composition[a]

sample	supplementation of palmitic and oleic acids (μM/μM) to the growth medium[b]	% in Tw 20 supernatant	% in NaDOC supernatant
I	PPLO serum fraction	52	89
II	0/150	60	73
III	30/120	88	100
IV	75/75	76	99
V	120/30	81	87

[a] SE (5) never exceeded ±10%. Percentages refer to concentration of membrane constituents as determined by absorbance measurements at 280 nm in the two detergent supernatants, respectively, as compared to the total concentration in $NaDodSO_4$ (see Experimental Procedures). [b] See Table II for lipid composition of the corresponding membrane.

of membranes I–V was almost indistinguishable from that in the corresponding membranes (Figure 1) as analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (data not shown).

$NaDodSO_4$–polyacrylamide gel electrophoresis of proteins in the Tw 20 insoluble residues obtained after Tw 20 extraction revealed a more individual pattern (data not shown) than that shown for intact membranes, i.e., Figure 1, or NaDOC supernatants. The differences in protein composition after Tw 20 extraction are more easy to quantify by CIE analysis of Tw 20 supernatants; see Figure 2. Note that the relative proportions of several proteins vary significantly, much more than what can be seen in Figure 1. Most likely, this is caused by differences in affinity of the proteins for the Tw 20/lipid mixed micelles or the membrane residues, respectively. It should be noted that the relative electrophoretic rates of migration in the first dimension on the CIE plates were the same for proteins T_{4a}, T_3, T_2, t_{1a} (=D_{12}), and t_{1b} (Figure 2), which was not always the case when Tw 20 supernatants of membranes from different strains of *A. laidlawii* were analyzed

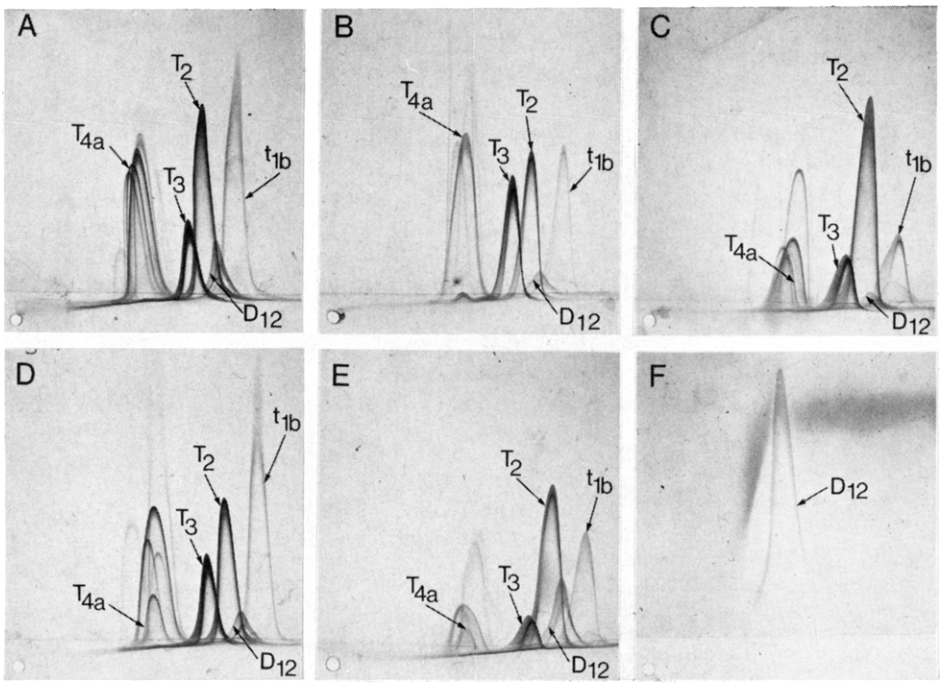

FIGURE 2: CIE of detergent supernatants of membranes from *A. laidlawii* grown with different fatty acid supplements. (A–E) Tw 20 supernatants; (F) NaDOC supernatant. (A) Supplement I (see Table II); (B) supplement II; (C) supplement III; (D) supplement IV; (E) supplement V. The identities of individual immunoprecipitates in (A–E) were established by CIE with an intermediate gel containing a monospecific antiserum. (F) CIE of a NaDOC supernatant against a monospecific antiserum (anti-D_{12}) in the NaDOC–CIE system.

Table IV: Lipid Composition in Membranes after Extraction with 2.5% (w/v) Tween 20 (1 h, 20 °C)[a]

sample	I	II	III	IV	V
supplementation of palmitic and oleic acids (μM/μM) to the growth medium[b]	PPLO serum fraction	0/150	30/120	75/75	120/30
% oleic acid in lipids (mol/mol)	*c*	95	84	54	20
ratio MGDG/DGDG (mol/mol)	0.89 (1.45[d])	0.59	0.86	1.44	1.93
ratio of MGDG after/before extraction	2.52	1.34	1.71	1.66	1.34
% ionic lipids of total polar lipids (mol/mol)	40	35	31	25	14
μmol of total lipids per μg of membrane protein	0.75	1.40	0.77	0.90	0.88

[a] See Table II for symbols and explanations. SE (3) never exceeded ±5%. [b] Cf. Table II. [c] Not determined; cf. Table II. [d] Ratio (MGDG + cholesterol)/DGDG. Membrane residue contains 14% (mol/mol of total lipids) cholesterol.

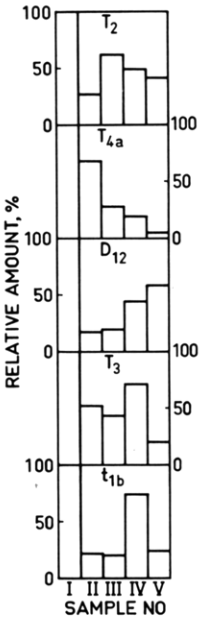

FIGURE 3: Relative amounts of the *A. laidlawii* membrane proteins T_2, T_{4a}, D_{12}, T_3, and t_{1b} in Tw 20 supernatants of the membrane as determined by CIE with a polyspecific antiserum. The sample numbers refer to the fatty acid supplements given in Table II, where the corresponding lipid compositions of the membranes are also given. Identification and quantification of individual proteins were done as described under Experimental Procedures. SE (3) never exceeded ±8%.

by CIE (Steinick et al., 1980).

Selective solubilization was also observed for membrane lipids. Table IV shows the lipid composition in membrane residues after Tw 20 extraction. A comparison with the compositions in intact membranes (Table II) reveals that unsaturated lipids are slightly enriched in the Tw 20 supernatant, which also applied for ionic lipids, DGDG, and cholesterol. However, MGDG was highly enriched in the Tw 20 insoluble membrane residue (Table IV). The amount of total proteins or lipids transferred into Tw 20 micelles seems to depend on the lipid/protein ratio in intact membranes; i.e., a low ratio yields a protein-enriched Tw 20 supernatant (cf. Tables II and IV).

Concentration of Individual Proteins in Membranes and Detergent Extracts. The solubility of both membrane proteins and lipids by detergents was affected by membrane lipid composition (see above). Variation of protein composition in Tw 20 supernatants can be caused by a variation in amounts of individual proteins in the membrane and/or by a difference in extractability of a certain protein, caused by variations in

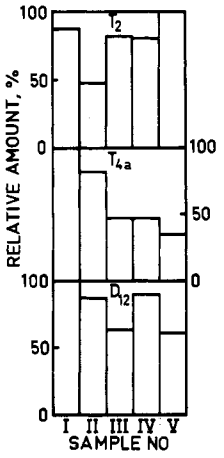

FIGURE 4: Relative amounts of the proteins T_2, T_{4a}, and D_{12} in NaDOC supernatants of *A. laidlawii* membranes as determined by CIE with monospecific antisera. The sample numbers refer to the fatty acid supplements given in Table II. SE (3) never exceeded ±8%.

lipid organization around that protein. Therefore, the content of some of the major membrane proteins was determined by CIE of Tw 20 supernatants and NaDOC supernatants. Figure 3 shows the relative amounts of these proteins in Tw 20 supernatants expressed as percentage of the amount in sample I. The sample numbers refer to those used in Table II. All proteins investigated were most easily extracted from sample I, i.e., membranes from cells grown in the presence of PPLO serum fraction (Figure 3). The content of the proteins T_2, T_{4a}, and D_{12} in NaDOC supernatants was determined by CIE against the corresponding monospecific antiserum. Figure 2F illustrates one representative experiment. Figure 4 shows the relative amounts of these proteins expressed as percentage of the amount in the sample containing the highest concentration. Since protein composition in NaDOC extracts was very similar to that in membranes, Figure 4 illustrates total variations of these proteins in the membrane preparations with different lipid compositions. However, the amounts given in Figure 3 represent the solubility of the particular protein in Tw 20. Comparison with Table II reveals that the extractability (Figure 3) of protein T_{4a} is proportional to the amount of unsaturated acyl chains *or* the amount of ionic lipids in the membranes. For protein D_{12}, large amounts of H_{II} lipids (MGDG and cholesterol) in the membranes correlate with a large extractability (Figure 3 and Table II). The total amount of protein T_{4a} in the membranes (Figure 4) correlates with the amounts of ionic lipids present in the membranes (Table II). This variation of T_{4a} in the membrane is representative for whole cells since analysis of cytoplasmic fractions by CIE revealed no T_{4a} (data not shown).

The concentration of protein T_2 in the Tw 20 supernatant (Figure 3) was *not* proportional to its relative amount in the membrane (Figure 4), indicating that the extractability of this protein is also dependent upon its lipid surroundings.

Discussion

Solubilization of Lipids. The rules governing the selective extraction of membrane protein with detergents are probably very complex. Protein–protein interactions may be important for this selectivity when mild detergents are used. To some extent, however, the selectivity can also be rationalized on the basis of the interactions between the proteins and the lipid matrix (Helenius & Simons, 1975). These interactions are limited by packing constraints that are dependent upon the molecular geometry of the surrounding lipid molecules [cf. Figures 1 and 2 in Israelachvili (1977)]. Furthermore, the action of neutral detergents can be explained by their pronounced wedge-shape geometry.

When the polar lipid composition in the *A. laidlawii* membranes is changed, the sizes of lipid domains and their interactions with membrane proteins are likely to vary. Many membrane proteins are selectively solubilized with Tw 20 [cf. Figures 1 and 2; see also Johansson et al. (1979) and Steinick et al. (1980)]. This selectivity must depend on the distribution of a certain protein (and its lipid surroundings) between the lamellar (membrane) and micellar phase. MGDG (molecular shape ▲, with polar head at top) and neutral detergents have geometrical properties which are complementary. However, the shape of MGDG is probably energetically very unfavorable in a Tw 20 micelle due to the high curvature of the mixed aggregate. Therefore, MGDG is less likely to take part in the lamellar to micellar transition. Table IV reveals a great enrichment of MGDG in membrane residues after Tw 20 extraction. A high degree of unsaturation results in a more accentuated wedge shape of MGDG [see Wieslander et al. (1980, 1981)]. The most unsaturated supplement (sample I) yields the highest proportional enrichment of MGDG in the Tw 20 residue (Table IV). Furthermore, this residue is depleted of cholesterol, probably as a consequence of the packing mismatch between MGDG and cholesterol (cf. Khan et al., 1981; Israelachvili et al., 1980). Similar observations have been made with other lipids; e.g., phosphatidylethanolamine (PE)[1] is enriched in the TX-100 insoluble residue of Semliki Forest virus (Simons et al., 1974).

The selective lipid enrichment observed here after extraction of *A. laidlawii* membranes with Tw 20 is much more pronounced than what has been observed after erythrocyte membranes were extracted with similar amounts of TX-100 (i.e., molar ratio detergent:membrane lipid 1:1) (MacDonald, 1980). However, different neutral detergents (and lipids) differ considerably in physical and solubilizing properties (Egan et al., 1976).

Solubilization of Proteins. Protein D_{12} is proportionally extracted by Tw 20 from the *A. laidlawii* membranes as a function of their H_{II} lipid content but *not* more abundant in such membranes (cf. Table II and Figures 3 and 4). It should be pointed out that most of D_{12} is enriched in the Tw 20 insoluble membrane residue [see Results and Johansson & Wróblewski (1978)], which also holds for MGDG. However, more MGDG in the membranes yields more MGDG and D_{12} in the Tw 20 supernatants (Tables II and IV). This indicates that MGDG might be important for the anchoring of D_{12} in the membrane.

It is known that ionic amphiphilic molecules like anionic polar lipids (e.g., PG) more easily form mixed micelles together with neutral detergents than zwitterionic or nonionic polar lipids (Tanford, 1973; Israelachvili et al., 1976). Tables II and IV reveal an enrichment of ionic lipids in Tw 20 micelles. A comparison of Figure 4 and Table II also shows a positive correlation between the amounts of ionic lipids in the membranes and the amounts of protein T_{4a}. However, for T_{4a}, the extractability diminishes faster with decreased amounts of unsaturated acyl chains in membrane lipids than does the actual amounts of T_{4a} and ionic lipids in the membranes (see Tables II and IV and Figures 3 and 4). The lipids in the least unsaturated sample (i.e., sample V) are probably in the gel state during the extraction procedure (cf. Wieslander et al., 1978). A high degree of molecular ordering thus seems to make T_{4a} less extractable.

The specificity of protein solubilization can depend on several factors. It may occur during the primary micellari-

zation event (Helenius & Simons, 1975) and be dependent upon protein–lipid interactions in the membrane. Alternatively, the specificity is caused by a secondary equilibrium, dependent upon the precise proportions of detergents and (previously) solubilized lipids present in micellar form. In the latter case, the extractability would be correlated with the presence of certain extramembrane lipids in vitro. No transfer of proteins occurs from *A. laidlawii* membranes to various lipid aggregates (containing no detergent) (Kahane & Razin, 1977; Slutzky et al., 1977; Razin et al., 1980). Furthermore, variation of the Tw 20 concentration between 0.5% and 2.5% (w/v) in the extraction mixtures (cf. Experimental Procedures) caused no detectable differences in the proportions between the proteins in the extracts, as judged from CIE (data not shown).

Variation of Protein Amounts in Membranes. When synthesis of ionic lipids is increased, *A. laidlawii* compensates for increased amounts of unsaturated acyl chains in the lipids, probably in order to maintain the surface charge density and the stability of the lamellar (bilayer) phase (Wieslander et al., 1980). The variation of T_{4a} amounts in the membranes (Figure 4) probably does not involve any incorrect insertion of this integral protein (cf. Tyhach et al., 1979) or a limited number of binding sites (cf. Kung & Henning, 1972) since we could not detect T_{4a} (with CIE) in the cytoplasmic fractions of the cells. This major membrane flavoprotein T_{4a} (Johansson et al., 1979) is probably a very important protein in the truncated electron transport chain of *A. laidlawii* (Razin, 1979). It has been shown that the passive permeability of *A. laidlawii* membranes and derived lipids increases dramatically with increased amounts of unsaturated fatty acyl chains in the lipids [summarized by Razin (1975)]. Thus, the increase in T_{4a} amounts in unsaturated ("permeable") membranes might be a mechanism to maintain a certain membrane potential at conditions with a larger leakiness. Variation in membrane proteins and electron transport chain constituents upon changes in environmental conditions, e.g., reduction/oxidation potential and metabolic status, is very common in other bacteria, [cf. Gel'man et al. (1975) and Mallick & Herrlich (1979)]. It is known that phage infection of *Escherichia coli* causes a disturbance of membrane energetization (Braun & Oldmixon, 1979). Infection of *A. laidlawii* with mycoplasma virus group 2 (MVL 2) greatly enhances the amounts of protein T_{4a} found in a Tween 20 extract of membranes (Steinick et al., 1980), supporting a regulation of T_{4a} amounts in the membrane.

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Alkaline Hydrolysis of Phospholipids in Model Membranes and the Dependence on Their State of Aggregation†

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ABSTRACT: The rate of alkaline hydrolysis of phospholipids in different model membranes was studied as a probe of the phospholipid conformation and packing and for a better understanding of the effect of phospholipid aggregation on hydrolysis by phospholipase A_2. The products of hydroxide attack on phosphatidylcholine were free fatty acids and glycerophosphorylcholine, with lysophosphatidylcholine as an intermediate. The kinetics of phospholipid hydrolysis could be analyzed as a pseudo-first-order reaction by having the hydroxide concentration in large excess. Egg phosphatidylcholine dispersed in Triton X-100 mixed micelles at a mole ratio of 8:1 detergent:phospholipid was hydrolyzed with a second-order rate constant of 14.7×10^{-3} s^{-1} M^{-1}. Egg phosphatidylcholine dispersed in single bilayer vesicles and multibilayers was hydrolyzed at rates 7–11- and 3-fold lower, respectively. The calculation of these rates had to take into account the extent of inaccessibility of the inner layers to hydroxide which was measured by the fluorescence of a pH-sensitive probe (pyranine) trapped inside the vesicles and multibilayers. The monomeric phospholipid dihexanoylphosphatidylcholine was hydrolyzed at a rate 7-fold higher than when this lipid was incorporated into Triton X-100 micelles. The activation energy for alkaline hydrolysis of egg phosphatidylcholine in Triton X-100 micelles was about 10 kcal/mol whereas it was about 19 kcal/mol in vesicles. Monomeric dihexanoylphosphatidylcholine was hydrolyzed with an activation energy of about 13 kcal/mol. When hydrolysis at the *sn*-1 and *sn*-2 positions of dipalmitoylphosphatidylcholine was followed independently, the rates were similar within experimental error at each position for both mixed micelles and vesicles over the temperature range of 15–60 °C. An Arrhenius plot of the hydrolysis of dipalmitoylphosphatidylcholine vesicles indicated that the phospholipid phase transition exerted a small but detectable effect on the rate of hydrolysis. As with phospholipase A_2, hydroxide-catalyzed hydrolysis rates depend critically on the aggregation state of the phospholipid.

Information about the conformation and packing of phospholipids at the lipid/water interface of model membranes has generally been obtained by physical techniques such as ^1H NMR (Roberts et al., 1978a), ^2H NMR (Seelig & Seelig, 1974), Raman spectroscopy (Gaber & Peticolas, 1977), and X-ray crystallography (Hitchcock et al., 1974). However, the relative rates of reactions on the phospholipid in various model membranes, such as hydrolysis of the fatty acyl bonds, may also be a useful probe of the properties of the phospholipid in the interfacial region. For example, the activity of the enzyme phospholipase A_2 (which specifically catalyzes hydrolysis of the *sn*-2 fatty acid ester bond) has been shown to be sensitive to the nature of the phospholipid/water interface (Verger, 1980) and thermotropic phase transitions of the phospholipid (Kensil & Dennis, 1979). Certain aggregation states, particularly phospholipid–detergent mixed micelles of the nonionic detergent Triton X-100, appear to be favored as substrates for the enzyme from cobra venom (Deems et al., 1975; Kensil & Dennis, 1979). Nonenzymatic reactions may also be sensitive to phospholipid conformation and packing. For example, Wells (1974) reported that alkaline hydrolysis of monomeric dibutyrylphosphatidylcholine is faster than that of dioctanoylphosphatidylcholine micelles, suggesting that the attack of hydroxide on phospholipid is affected by the aggregation state although an effect of fatty acyl chain length on the rate was not ruled out. In order to better understand phospholipase A_2 specificity (Adamich et al., 1979; Roberts et al., 1979) and phospholipid conformation and packing and to further probe the phospholipid/water interface, we have determined hydrolysis rates and activation energies for hydroxide ion attack on phospholipids in various aggregation states (mixed micelles, multibilayers, vesicles, monomers) and found them to depend critically on the lipid aggregation state.

Experimental Procedures

Lipids and Detergents. Egg phosphatidylcholine (PC)[1] was purified from egg yolks (Singleton et al., 1965). L-α-Dimyristoyl-, L-α-dipalmitoyl-, and L-α-dilauroyl-PC were purchased from Calbiochem. If more than a single spot was apparent after thin-layer chromatography in $CHCl_3$–CH_3OH–H_2O (65:25:4 v/v), the phospholipids were purified by column chromatography on Unisil silicic acid (100–200 mesh) obtained from Clarkson Chemicals. Egg PE (transesterified from egg PC) was purchased from Avanti Biochemicals. 1-Palmitoyl-2-[1-^{14}C]palmitoyl-PC (specific activity 59

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[1] Abbreviations used: PC, 1,2-diacyl-*sn*-glycero-3-phosphorylcholine; LPC, monoacyl-*sn*-glycero-3-phosphorylcholine; GPC, *sn*-glycero-3-phosphorylcholine; PE, 1,2-diacyl-*sn*-glycero-3-phosphorylethanolamine; LPE, monoacyl-*sn*-glycero-3-phosphorylethanolamine; DHPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphorylcholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine; T_c, thermotropic phase transition.

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