Biochimica et Biophysica Acta, 550 (1979) 1—15
© Elsevier/North-Holland Biomedical Press

BBA 78223

FRACTIONATION OF MEMBRANES FROM *ACHOLEPLASMA LAIDLAWII* A ON THE BASIS OF THEIR SURFACE PROPERTIES BY PARTITION IN TWO-POLYMER AQUEOUS PHASE SYSTEMS

ÅKE WIESLANDER [a], ANDERS CHRISTIANSSON [a], HARRY WALTER [b] and CLAES WEIBULL [a]

[a] *Department of Microbiology, University of Lund, Sölvegatan 21, S-223 62 Lund (Sweden) and* [b] *Laboratory of Chemical Biology, Veterans Administration Hospital, Long Beach, CA 90822 (U.S.A.)*

(Received June 9th, 1978)

Key words: Surface properties; Aqueous phase; Membrane fractionation; (Acholeplasma laidlawii)

Summary

Acholeplasma laidlawii A consists of pleomorphic cell clusters surrounded by a single membrane. When lysed, a cell gives rise to several membrane fragments which cannot be separated from each other by isopycnic sucrose gradient centrifugation. A heterogeneous lateral organization of the cell membranes was detected by countercurrent distribution of membrane fragments in a two-polymer aqueous phase system. It revealed that the membranes consist of at least two subpopulations with respect to surface properties. Changes in the fatty acid and cholesterol content of the membranes revealed that the resolution of different subpopulations was predominantly due to a critical ratio of monoglucosyldiglyceride to diglucosyldiglyceride. The heterogeneity of the membrane probably depends on lipid-lipid and lipid-protein steric interactions. Charged lipids, an apolar monoglucolipid and the ratio between lipids and proteins also affect membrane partition. The differences in the subpopulations were further reflected by different specific activities of NADH dehydrogenase, NADH oxidase and ATPase. These activities varied independently. Minor quantitative differences in the protein patterns of different subpopulations were apparent. The origin and the preservation of the membrane subpopulations are discussed in terms of lipid-lipid and lipid-protein interactions, their age and energy metabolism.

Introduction

Although biological membranes have been much studied, relatively little information is available on membrane organization in the lateral plane. Because of their physical properties, lipids are known to affect the localization and

sometimes the activity of the membrane proteins. The gel to liquid crystalline phase transition in *Acholeplasma laidlawii* membranes causes an aggregation of integral proteins as visualized by freeze-etch electron microscopy [1,2]. Surface proteins are affected as well [3]; and the interactions between a certain lipid (phosphatidylglycerol) and membrane proteins are changed (summarized in ref. 4).

In larger bacteria, e.g. *Escherichia coli*, a similar behaviour is observed with respect to protein aggregation [5]. Preparation of membranes from *E. coli* yields small vesicles of inner and outer membranes. Low temperature during these steps produce vesicles with large differences in protein-lipid ratio due to lateral phase separation [6,7]. Such vesicles can be separated by isopycnic centrifugation. The two or three membrane fractions obtained differ, in addition to lipid-protein ratio, with respect to protein composition, and ratio of saturated to unsaturated fatty acids. There is no change in lipid species [7].

In *A. laidlawii* we have found a regulatory system that varies the relative amounts of membrane polar lipid species in response to induced variations in membrane viscosity [8,9]. These metabolic changes proceed with different intralipid species and turnover rates [9]. The physical properties of the involved (glyco)lipids differ significantly [10]. Furthermore, loosely-bound molecules at the membrane surface in *A. laidlawii* affect the lateral packing properties of the lipids [11,12]. All this indicates the probability of a heterogeneous lateral membrane organization in this organism.

The co-ordination between DNA-replication and cell division in *A. laidlawii* is loose, causing the cells to normally grow in clusters surrounded by the same, single cell membrane [13]. If lateral heterogeneities exist, there will be differences in composition for different parts of a membrane enclosing these cell entities. Such an organization will cause different membrane regions to have different surface properties. Two-polymer, aqueous phase systems have proved to be very useful for the separation by partition of cells and membrane vesicles differing only slightly in surface properties [14—16]. Recently, partition studies have been successfully extended to include liposomes [17]. We report here on the existence of different membrane fractions from *A. laidlawii*, separated by countercurrent distribution in two-polymer aqueous phase systems.

Materials and Methods

Organism and growth conditions

A. laidlawii A, strain EF22 [8], was grown for 18 h at 37°C in a lipid-depleted bovine serum albumin-tryptose medium [8]. The medium was supplemented with palmitic and oleic acids in different amounts, the sum of which was always 0.150 mM. The ratios of palmitic to oleic acid used were 120/30, 90/60, 75/75, 30/120 and 0/150. The latter supplement was also given together with 0.020 mM cholesterol. Membrane lipids were radioactively labelled by adding 30 μCi/l [^{3}H]palmitic acid, and/or 10 μCi/l [^{14}C]oleic acid and, when needed, 40 μCi/l [^{3}H]cholesterol, (Radiochemical Centre, Amersham, England).

Cells were harvested by centrifugation at 11 000 $\times g$ for 20 min at 5°C and

washed once in cold β-buffer. Membranes were prepared by osmotic lysis at 22°C for 45 min during agitation and collected by centrifugation at 32 000 × *g* for 45 min at 5°C. They were washed twice in 0.010 M sodium phosphate buffer, pH 7.4, and kept at 2°C for not more than 1 h before continuing with the procedure (see below). Quantities of membranes were estimated by absorbance measurements at 500 nm in 15 ml 0.010 M sodium phosphate buffer and expressed as absorbance units, (i.e. one unit is equal to an absorbance of 1.0 for 15 ml membrane suspension read in a 1 cm cuvette).

Countercurrent distribution

In preliminary experiments an appropriate phase system for *A. laidlawii* was selected as described by Walter [18].

The phase system was composed of 5% (w/w) Dextran T500, M_r 500 000 (Pharmacia, Batch no. 5556), 4% (w/w) poly(ethylene-glycol), M_r 6000, trade name Carbowax 6000 (Union Carbide), 8.3 mmol sodium phosphate buffer, pH 7.4, and 3 mmol NaCl, per kg final mixture. After mixing the components, the phases were allowed to equilibrate overnight at 3°C. The top and bottom phases were then separated and stored at 3°C.

Washed membrane preparations corresponding to 0.50 absorbance units were suspended in 4.50 ml of top phase (load mix). An automatic thin-layer countercurrent distribution apparatus with 120 cavities was used [19]. Cavities 0—3, 40—43 and 80—83 each received 0.5 ml of bottom phase and 0.9 ml of one of the load mixes. All other cavities received 0.6 ml bottom phase and 0.8 ml top phase. 39 transfers were completed at 3°C using a settling time of 6.5 min and a shaking time of 25 s.

Analysis of membranes

After the countercurrent run, membranes from each cavity were collected directly into plastic tubes or, when chemical and enzymatic analyses were to follow, 1.6 ml 37 mM 2-mercaptoethanol was first added to each cavity to convert the phase system to a homogenous (one-phase) suspending medium.

Absorbance of membranes was measured at 500 nm. Protein content was estimated [20] after precipitation of the membranes with cold trichloroacetic acid according to Albertsson [21]. Radioactivity of the labelled membranes was determined by liquid scintillation counting [9] after digestion of the membranes in Protosol (New England Nuclear Co.) overnight. cpm were converted to dpm using known quenched ^3H and ^{14}C standards.

On the basis of the distribution patterns measured by absorbance, some adjacent tubes were pooled separately (see Figs. 1 and 4). These pooled membranes were freed from adhering polymers by three consecutive washes in β-buffer diluted 1 : 20 with deionized water. Pellets were dispersed in a small volume of the same buffer and stored at −70°C. Samples for enzyme assays were also made 20 mM with respect to 2-mercaptoethanol.

Lipids were extracted with the Bligh and Dyer method according to Kates [22]. Separation of lipids was performed by thin-layer chromatography (Silica gel H, Merck) on borate-buffered plates [8] in chloroform/methanol/water (65 : 25 : 4, v/v). Radioactivity was measured by liquid scintillation counting [9].

Protein patterns of different membrane fractions were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis according to Neville [23] as modified by Jergil and Ohlsson [24]. The gels were cast in 9.5 X 7.5 X 0.3 cm slab gel forms. Upper stacking gel contained 3.5% (w/w) acrylamide (6.5% crosslink) and the separation gel 11.8% (w/w) acrylamide (1% cross-link). Membrane samples were solubilized at 100°C for 3 min in double strength upper reservoir buffer [23] containing 3.8% (w/v) sodium dodecyl sulphate. Electrophoresis was carried out for 4 h at a constant current of 20 mA per gel. After the run, the gels were stained overnight with Coomassie Brilliant Blue R250. The following reference proteins were used for molecular weight determinations: lysozyme, DNAaseI, ovalbumin and bovine serum albumin.

Enzyme assays

NADH dehydrogenase (EC 1.6.99.3) activity. Membranes were treated with 2.5% (w/v) sodium deoxycholate in 6.36 mM tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.4, containing 1.27 mM 2-mercaptoethanol for 45 min at room temperature. $K_3[Fe(CN)_6]$ was used as artificial electron acceptor. Final concentrations were: 50—250 μg membrane protein, 1.00% (w/v) sodium deoxycholate, 2.50 mM Tris-HCl (pH 7.4), 0.50 mM 2-mercaptoethanol, 7.80 mM NaCl, 1.80 mM $K_3[Fe(CN)_6]$ and 0.120 mM NADH, in a volume of 1.20 ml. The reaction was measured continuously at 420 nm in a spectrophotometer at 37°C. Controls of any spontaneous reaction were also run. Enzyme activity was calculated from the initial decrease in absorbance for $K_3[Fe(CN)_6]$ and expressed as μmol NADH used per min per mg membrane protein.

NADH oxidase (EC 1.6.99.3) activity. The membranes were treated in the same manner as for the NADH dehydrogenase measurements. Final concentrations were the same except that $K_3[Fe(CN)_6]$ was omitted. The reaction was followed at 340 nm and 37°C. Activities were calculated from the absorbance decrease of NADH and expressed as μmol NADH oxidized/min per mg membrane protein.

Mg^{2+}-dependent ATPase (EC 3.6.1.3). The assay mixture contained 40—240 μg membrane protein, 50 mM Tris-HCl (pH 8.0), 1.25 mM $MgCl_2$, 1.1 mM NaCl, 0.6 mM 2-mercaptoethanol in a volume of 1.0 ml. The reaction was started by adding 10 μl 125 mM ATP and was permitted to proceed for 30 min at 37°C with agitation. Blanks and zero time samples were also run. The reaction was terminated by adding 1.0 ml 10% (w/v) trichloroacetic acid and cooling the test tubes. Precipitated protein was removed. Liberated inorganic phosphate (from ATP) was determined according to Chen et al. [25]. Enzyme activity was expressed as nmol phosphate liberated from ATP/min per mg membrane protein.

Electron microscopy

For negative contrasting 2% (w/v) potassium phosphotungstate (pH 7.0) was used. For thin sectioning a pellet of membranes was suspended in 3% (v/v) glutaraldehyde, pH 7.0, and left for 1 h at 20°C. The membranes were then washed with Michaelis buffer, pH 7.0, post-fixed with osmium tetroxide and uranyl acetate, as described by Ryter and Kellenberger [26], and embedded in Epon. Sections were stained with uranyl acetate for 1 h at 20°C.

Density gradient centrifugation

Linear 18 ml 30—60% (w/w) sucrose gradients in β-buffer diluted 1 : 20 were prepared. Membranes were centrifuged at 99 000 X *g* for 3.5 h at 3°C in a 3 X 20 ml swing-out rotor. The gradients were unloaded through a puncture in the bottom of the tubes. Absorbance at 280 nm was recorded.

For comparison membranes disintegrated by five consecutive passages through an X-press with intermittent freezing [27] were run on gradients under identical conditions.

Results

Countercurrent distribution

The phase system found suitable for the partition of *A. laidlawii* membranes (Materials and Methods) is one which has an electrostatic potential difference between the phases (top phase positive) [18]. Visual examination of membrane turbidity in the tubes of the countercurrent extraction train revealed that membranes with low partition coefficient were in the bottom phase, those with intermediate partition coefficients at the interphase, and those with high partition coefficients in the top phase. This visual inspection not only showed at once that membranes in different parts of the extraction were truly different one from another but also that factors in addition to charge are involved in determining the partition coefficient of these membranes since at least a portion of the negatively charged membranes are in the negatively charged bottom phase. Determination of membrane content in different tubes by measurement of absorbance at 500 nm indicated that the membranes were composed primarily of two subpopulations.

By selectively supplementing the growth media with various amounts of one saturated and one unsaturated fatty acid, i.e. palmitic and oleic acids, stepwise changes in the physical properties of the membranes were introduced.

In Fig. 1A the separation pattern of membranes from cells grown on 0.120 mM palmitic plus 0.030 mM oleic acid is shown.

Membranes from cells grown on 0.090 mM palmitic plus 0.060 mM oleic acids were also composed of two subpopulations (Fig. 1B). Here, the 'right-hand' subpopulation was the larger one.

A further increase of oleic acid in the growth medium at the expense of palmitic acid, i.e. 0.030 mM palmitic acid plus 0.120 mM oleic acid, caused the 'right-hand' subpopulation to further increase in quantity (Fig. 1C). The 'left-hand' subpopulation became smaller but was still distinguishable.

Ultrastructure

The morphology of *A. laidlawii* cells is very pleomorphic. Fig. 2, A, B and C shows the ultrastructure of unfractionated, 'left-hand' and 'right-hand' subpopulations, respectively, as seen in sectioned specimens. The size distribution of the unfractionated membranes indicates that a cell cluster gave rise to several individual membranes on osmotic lysis. Holes in some of the membranes were visible. These results were confirmed by observations on negatively-stained membranes. Membranes separated by countercurrent distribution seemed to consist of a larger number of small vesicles than did the unfrac-

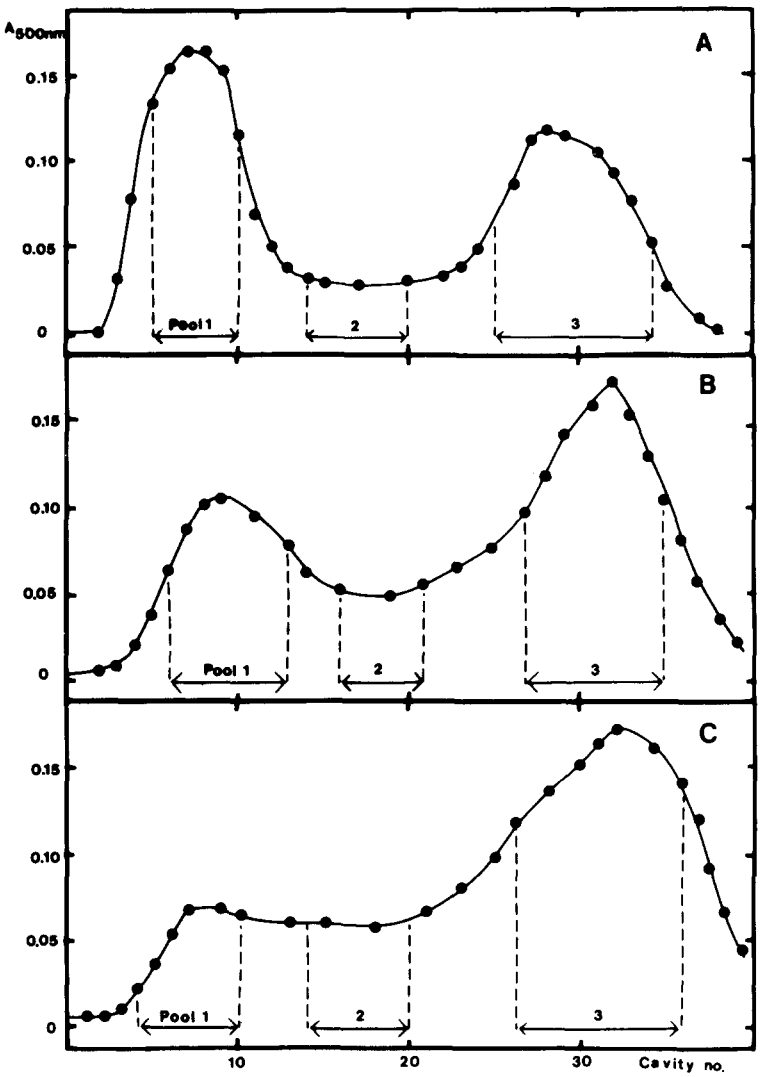

Fig. 1. Countercurrent distribution patterns of *A. laidlawii* membranes from cells grown with (A) 0.120 mM palmitic acid plus 0.030 mM oleic acid, (B) 0.090 mM palmitic acid plus 0.060 mM oleic acid and (C) 0.030 mM palmitic acid plus 0.120 mM oleic acid. Pools 1, 2 and 3: membranes within these fractions were pooled for chemical, enzymatic and ultrastructural analyses. Phase system contained 5% (w/w) dextran, 4% (w/w) poly(ethylene glycol), 8.3 mM sodium phosphate, pH 7.4, and 3 mM NaCl per kg final mixture. 39 Transfers were completed at 3°C.

tionated ones (Fig. 2, B and C). Many, but not all, vesicles appeared to be closed. No significant enrichment of membranes of a given size could be dectected in either of the two subpopulations.

Lipid composition of different membrane fractions

Table I shows the lipid composition of the different pools as well as the saturation ratio of the lipids. Several features are evident. The quantity of each lipid is not the same in different pools from a separation series. The largest relative differences are in those lipids which comprise the minor components

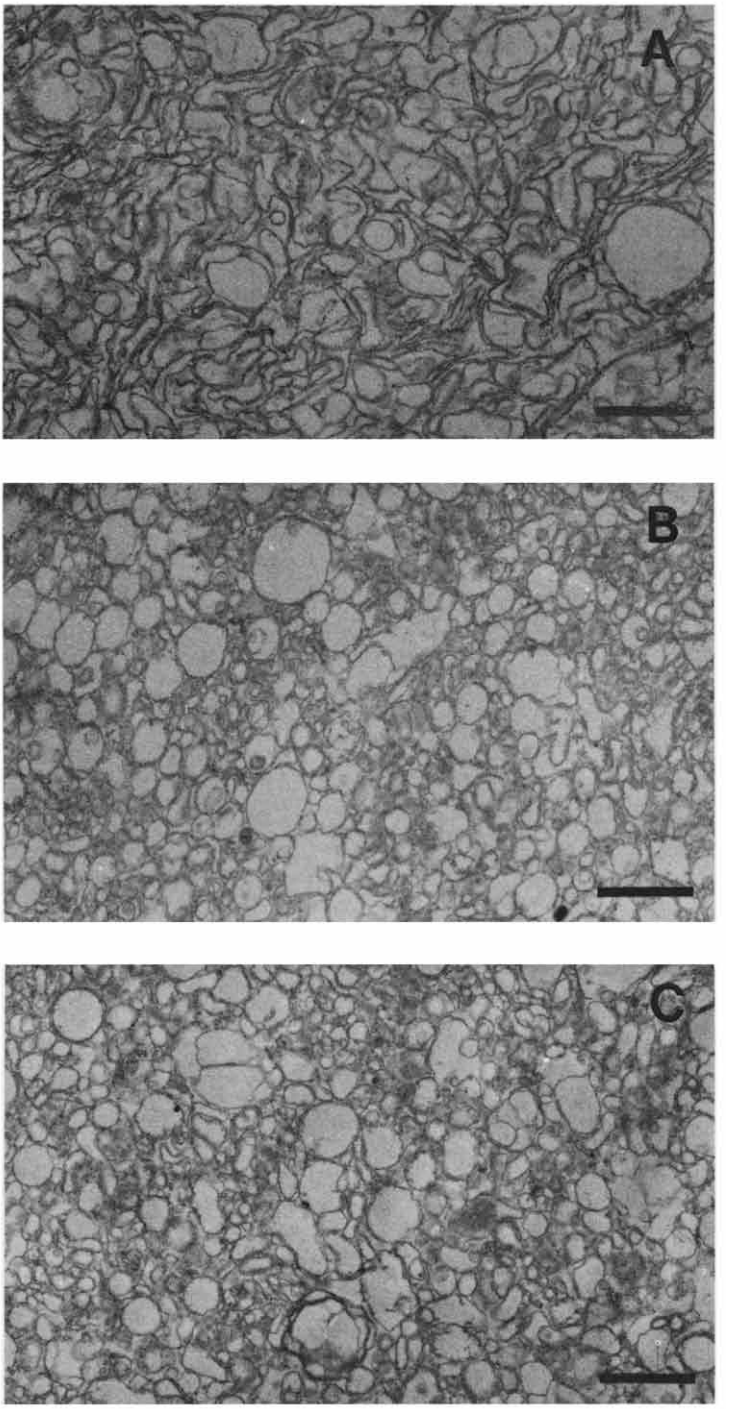

Fig. 2. Thin section electron micrographs of different membrane fractions obtained by countercurrent distribution. A, unfractionated membranes; B, membranes from a 'left-hand' population (see text); C, membranes from a 'right-hand' population. See Materials and Methods for technical data. The bars represent 1.0 μm.

TABLE I

LIPID COMPOSITION OF DIFFERENT MEMBRANE FRACTIONS FROM *A. LAIDLAWII* OBTAINED BY COUNTERCURRENT DISTRIBUTION

Pools 1, 2 and 3, see Figs. 1A, 1B and 1C for details. Lipid amounts in mol/100 mol.

Lipids	Fatty acids added in the growth medium								
	0.120 mM Palmitic acid 0.030 mM Oleic acid			0.090 mM Palmitic acid 0.060 mM Oleic acid			0.030 mM Palmitic acid 0.120 mM Oleic acid		
	Pool: 1	2	3	1	2	3	1	2	3
Charged lipids *	13.8	14.7	13.2	16.7	20.0	17.6	19.6	20.3	26.2
Diglucosyldiglyce	18.2	18.0	22.1	31.2	28.0	34.3	41.1	42.0	41.7
Monoglucosyldiglyceride	45.5	45.4	46.2	39.1	38.2	35.1	31.4	10.1	26.7
Glucolipid X	15.7	16.9	13.2	9.6	8.7	9.2	5.7	25.3	3.9
Ratio Monoglucosyldiglyceride to Diglucosyldiglyceride (mol/mol)	2.52	2.52	2.09	1.25	1.36	1.02	0.76	0.24	0.64
Ratio nmol lipids/μg membrane protein	0.28	0.50	0.46	0.85	0.90	0.70	0.85	0.86	0.75
Ratio palmitic acid/oleic acid in lipids (mol/mol)	2.22	2.26	2.62	1.17	1.30	1.31	0.75	0.84	0.84

* Charged lipids: glycerophosphorylmono- and diglucosyldiglyceride plus phosphatidylglycerol (see ref. 8).

(data not shown), but the dissimilarities between the dominating glycolipids are larger on a total basis. The monoglucosyldiglyceride/diglucosyldiglyceride ratio varies considerable from pool to pool (Table I). Furthermore, the amounts of charged lipids, i.e. phosphatidylglycerol and the glycerophosphoglycolipids, are not constant. The lipid saturation ratios (although differing between individual lipids) all increase with increasing partition coefficients (i.e. to the right). As noted earlier [8] increased amounts of a saturated fatty acid in the growth medium, i.e. palmitic acid, result in increased monoglucosyldiglyceride/diglucosyldiglyceride ratio and more vigorous synthesis of the apolar glucolipid X.

Membrane protein composition

Sodium dodecyl sulphate polyacrylamide gel electrophoresis showed more than 40 protein bands (Fig. 3) with a molecular weight range from 14 300 to

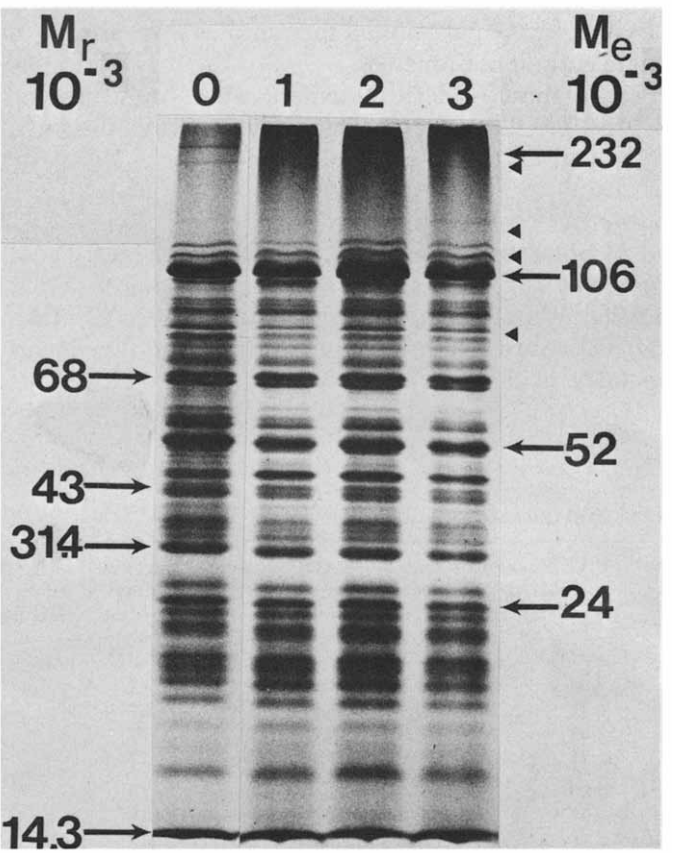

Fig. 3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of *A. laidlawii* proteins in different membrane fractions obtained by countercurrent distribution. The membranes were derived from cells grown on 0.120 mM palmitic acid plus 0.030 mM oleic acid. Sample 0, unfractionated membranes; samples 1, 2 and 3, membranes from countercurrent distribution pools 1, 2 and 3 (see Fig. 1). M_r, reference molecuar weights; M_e, estimated molecular weights of *A. lailawii* proteins; ▲, protein bands that differ between fractions.

230 000. The similarity between the unfractionated membranes and the pooled fractions was striking. Quantitative differences were noted in some bands in the high molecular weight range.

The protein patterns of unfractionated membranes showed quantitative variations in some bands probably as a consequent of differences in fatty acid supplementation in the growth medium (data not shown). The greatest difference was in protein having molecular weights in excess of 70 000.

Enzyme activities

The NADH dehydrogenase and oxidase activities were compared by digesting the membranes in an excess of sodium deoxycholate and the activities were estimated in the absence of polymers. Since the ATPase is very sensitive to detergent, this treatment was not used. Table II shows that both NADH dehydrogenase and oxidase activities differ significantly between unfractionated membranes and the different pools. The ratio between the two activities is also inconstant in the pools. NADH oxidase activities were always much lower in partitioned membranes than in unfractionated membranes. For NADH oxidase, highest specific activities in the partitioned membranes were always in fractions between the two main subpopulations.

In the case of the ATPase highest specific activities were found in the smallest subpopulations (cf. Figs. 1A, 1B and 1C). This enzyme also had significantly different activities in the various pools.

Influence of membrane cholesterol

Although the distribution of polar lipid heads varied among pools, fatty acid properties are probably important for maintaining such heterogeneity. Lipid interactions can also be modified by the insertion of cholesterol [28]. To make the interpretation more straightforward, cholesterol was introduced into membranes containing only one fatty acid, i.e. oleic acid [8]. Here, endogenous

TABLE II

ENZYME ACTIVITIES IN DIFFERENT MEMBRANE FRACTIONS

Added fatty acids	Pools	NADH dehydrogenase (μmol NADH/min per mg membrane protein)	NADH oxidase (μmol NADH/ min per mg membrane protein)	ATPase (nmol ATP/min per mg membrane protein)
0.120 mM Palmitic acid + 0.030 mM oleic acid	0 *	1.02	2.53	81
	1	1.76	0.64	57
	2	1.08	1.21	73
	3	0.72	1.12	122
0.090 mM Palmitic acid + 0.060 mM oleic acid	0 *	0.84	2.05	70
	1	0.97	0.81	145
	2	0.79	1.06	67
	3	1.15	0.40	51
0.030 mM Palmitic acid + 0.120 mM oleic acid	0 *	0.71	1.38	38
	1	1.03	0.79	46
	2	1.22	0.85	37
	3	0.99	0.79	24

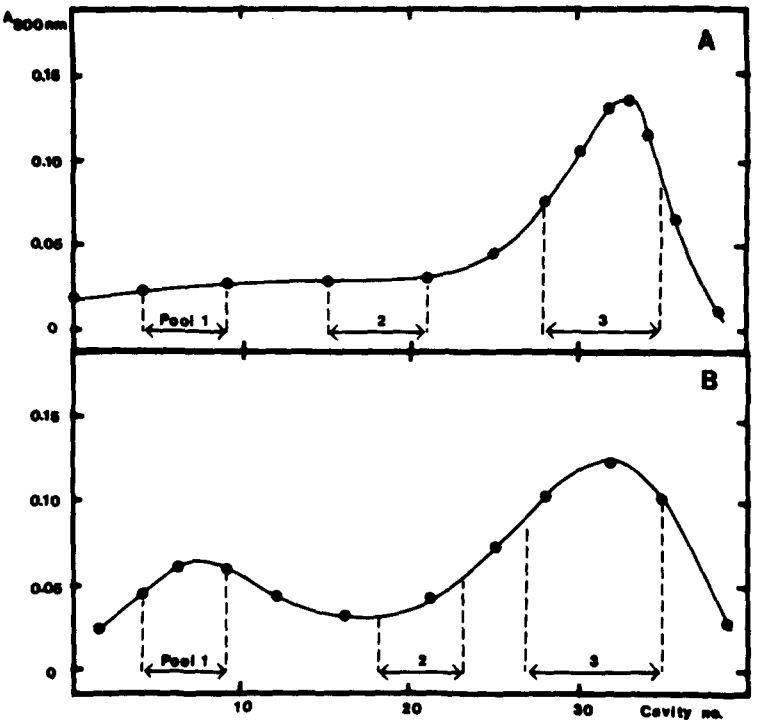

Fig. 4. Countercurrent distribution patterns of membranes from cells grown on (A) 0.150 mM oleic acid and (B) 0.150 mM oleic acid plus 0.020 mM cholesterol. See Fig. 1 for details.

TABLE III

LIPID AND CHOLESTEROL COMPOSITION OF DIFFERENT MEMBRANE FRACTIONS CONTAINING OLEIC ACID

Pools 1, 2 and 3, see Figs. 4A and 4B for details. Lipid amounts in mol/100 mol.

Lipids	Supplements					
	0.150 mM Oleic acid			0.150 mM Oleic acid plus 0.020 mM cholesterol		
	Pool: 1	2	3	1	2	3
Charged lipids *	34.6	35.3	36.1	43.0	49.1	43.3
Diglucosyldiglyceride	34.6	36.2	38.5	34.4	36.7	40.5
Monoglucosyldiglyceride	23.8	22.7	22.9	13.5	9.1	8.4
Ratio monoglucosyldiglyceride/ diglucosyldiglyceride (mol/mol)	0.69	0.63	0.59	0.39	0.25	0.21
Ratio nmol lipids/μg membrane protein	0.38	0.58	0.67	0.20	0.29	0.31
Ratio cholesterol/total lipids (mol/mol)	—	—	—	0.19 **	0.48	0.33

* Charged lipids: glycerophosphorylmono- and diglucosyldiglyceride plus phosphatidylglycerol (see ref. 8).

** Ratio in unfractionated membranes, 0.29.

saturated fatty acid synthesis is very low [8]. Fig. 4A shows the distribution pattern obtained from membranes containing oleic acid alone. In keeping with trends evident in Fig. 1 A, B and C, the 'left-hand' population completely vanished in the absence of a saturated fatty acid.

Incorporation of cholesterol (during growth) in these membranes caused the 'left-hand' population to reappear (Fig. 4B). Lipid analysis disclosed a more homogeneous distribution of lipids between pools when cholesterol was absent (Table III). The monoglucosyldiglyceride/diglucosyldiglyceride ratio also differed more in membrane pools containing cholesterol. The molar ratio of cholesterol to total lipids varied almost 2-fold between the two subpopulations compared to the average value which was 0.29 in unfractionated membranes (Table III). The 'left-hand' subpopulation contained more monoglucosyldiglyceride and less diglucosyldiglyceride, phosphatidylglycerol and cholesterol than unfractionated membranes. The opposite was true for the 'right-hand' subpopulation. Membranes from cavities between these two main subpopulations (i.e. pool 2) had significantly more charged lipids and cholesterol.

Discussion

The conditions used cause *A. laidlawii* to grow in large filamentous cell clusters surrounded by one common cell membrane. Comparisons of normal [29] and thick [30] electron microscopic sections of *A. laidlawii* cell clusters with the membranes obtained here (Fig. 2A) indicate that, on the basis of size, each cell cluster gives rise to several membranes on lysis. These membranes yield a homogeneous peak when centrifuged on a sucrose gradient (data not shown). When membranes were broken in an X-press several subpopulations were obtained. These cannot be considered natural since drastic pressure-collapse treatments are needed to produce them. Furthermore, a significant amount of protein is lost (data not shown). Several enzymes lost their activities and such membranes could not be properly separated by countercurrent distribution due to clumping.

It is thus evident that membranes from *A. laidlawii*, which are homogeneous with respect to buoyant density, can be separated on the basis of their surface properties into (at least) two subpopulations (Figs. 1A, B and C and 4B). Analysis of membrane proteins in different subpopulations reveals very small quantitative differences. Since these occur among the higher molecular weight proteins the total number of proteins differing is small. In contrast to other observed differences (see below), we consider these specific proteins to be of minor importance in determining the membrane partition coefficient.

Since the two-polymer aqueous phase system reflects membrane surface properties [18,21], the ratio between protein and lipids may be of importance. This ratio will, to some extent, determine the exposure of charged and/or hydrophobic surface groups. Examination of ratios obtained with different *A. laidlawii* membranes (Tables I and III) shows large fluctuations which appear not directly related to the partition coefficients of the two subpopulations (Figs. 1A, B and C and 4B).

Lipid composition and saturation ratios (i.e. palmitic/oleic acid) have a direct bearing on both the appearance and relative sizes of membrane subpopu-

lations. In *A. laidlawii* A membranes the physiological ratio between the two dominating glycolipids, monoglucosyldiglyceride and diglucosyldiglyceride, undergoes profound variations in response to changes in membrane viscosity caused by changes in fatty acid content [8], temperature and cholesterol content [9]. NMR and X-ray studies revealed great variations in physical properties between these lipids [10], the most important being that monoglucosyldiglyceride has a small upright polar head while the polar head of diglucosyldiglyceride has an alignment parallel to the bilayer plane and is only capable of binding a limited amount of water [10]. The exposure of the hydrophilic parts of the membrane polar heads is, thus, probably greater in those membranes which are rich in monoglucosyldiglyceride. In an analogous argument, this exposure will probably be less when diglucosyldiglyceride occurs in large amounts because of interference by its polar head. Some recent findings substantiate these structural differences, e.g. that monoglucosyldiglyceride in monolayers is less sensitive to increases in surface pressures when reacting with concanavalin A than is diglucosyldiglyceride [31]. Furthermore, a certain excess of monoglucosyldiglyceride over diglucosyldiglyceride makes the cell membrane of an *A. laidlawii* mutant resistant to reactive complement lysis [32].

With decreasing amounts of palmitic acid in the membrane lipids during growth, decreasing amounts of monoglucosyldiglyceride are synthesized (Tables I and III) in agreement with an earlier proposed mechanism [8]. A large ratio of monoglucosyldiglyceride to diglucosyldiglyceride (Table I) correlates positively with a large 'left-hand' subpopulation (Figs. 1A, B and C). Below certain lipid ratios (Table III) the 'left-hand' population disappears (Fig. 4A). Since cholesterol, being able to interact sterically with the lipid acyl chains, restores the 'left-hand' population at a lipid ratio below the critical one, lipid steric interactions are most probably involved. The phase system used appears to separate *A. laidlawii* membranes both on the basis of charge and hydrophobic/hydrophilic properties. The positively charged top phase should interact with negatively charged *A. laidlawii* membranes. However, in membranes in which hydrophilic surface groups are pronounced, these may predominate in determining the partition coefficient obtained. The 'left-hand' population was primarily in the bottom phase. A large 'left-hand' population thus means that a substantial part of the membranes partitioned due to surface properties other than charge. These (probably hydrophilic) properties manifest themselves because of the polar head configuration of the dominating monoglycosylglyceride. Other aspects that may influence the membrane partition behaviour include the contribution of glucolipid X [8] and the consistently larger fraction of oleic acid in the lipids in a 'left-hand' population than in the corresponding 'right-hand' peak (Table I).

In addition to decreased glycolipid ratios, membranes having only oleic acid contain a larger fraction of the negatively charged lipids. Both of these properties will increase the 'right-hand' population, which is thus separated primarily because of the affinity between negatively-charged membranes and positively-charged top phase. That cholesterol can 'induce' the appearance of a 'left-hand' population (Figs. 4A and 4B), despite the very high amounts of charged lipids in these membranes (Table III), is noteworthy. Cholesterol content in the 'left-

hand' population is substantially lower, and the concentration in the 'right-hand' population higher, than the mean (Table III). Membranes partitioned between these main populations contain more charged lipids and cholesterol than average. These results indicate that specific lipid-cholesterol interactions occur. Furthermore, exposure of charged groups may also be influenced by the presence of cholesterol.

The difference in specific activities of enzymes in the subpopulations further strengthens the concepts of an heterogeneous membrane organisation. Since the molecular weights for the enzymes tested are not knonw, it is not possible to relate the different activities with differences in the membrane protein patterns. The use of deoxycholate in the NADH dehydrogenase and oxidase assays rules out the possibility that different sidedness of the membranes is responsible for the observed differences. Neither of these enzymes is dependent on lipid, but several workers have pointed to the importance of microenvirnment on them [33,34]. Clearly, different lipid organization may alter their functions. The obtained activities may also be a result of a difference in membrane age since the enzymes are known to vary with age in growing cells [34]. ATPase has recently been shown to depend on phosphatidylglycerol for maximum activity [4]. Although a small amount of the total phosphatidylglycerol molecules was needed, different organization of phosphatidylglycerol in the membrane subpopulations could affect the activity of the enzyme.

Since the enzymes tested are all important mediators of the energy-yielding mechanisms in *A. laidlawii*, their different activities in the subpopulations might mirror the status of energy metabolism in these membranes.

In summary, we conclude that *A. laidlawii* membranes, considered homogeneous by standard criteria, consist of at least two subpopulations under normal conditions with regard to surface properties. These subpopulations differ in their polar lipid content, lipid/protein ratios, enzyme activities and, to some extent, in fatty acid content. The latter components seem to be of importance in maintaining the heterogeneity. Such variations in surface properties will most likely affect the relationship between the parasite *A. laidlawii* and its hosts.

Acknowledgements

We thank Professor P.-Å. Albertsson and co-workers at the Department of Biochemistry, Chemical Centre, Lund, for generous help; B.A. L.-E. Steinick and B.A. K. Forslund for optimizing the enzyme assays; U. Wulf and T. Magnusson for excellent technical assistance and Drs. E.M. Bevers and J.A.P. Op den Kamp, Utrecht, The Netherlands for making results available to us prior to publication. H. Walter was on leave from Veterans Administration Hospital, Long Beach, California while working in the Department of Biochemistry, University of Lund. This investigation was supported by the Swedish Natural Science Research Council.

References

1 Verkleij, A.J., Ververgaert, P.H.J.Th., van Deenen, L.L.M. and Elbers, P.F. (1972) Biochim. Biophys. Acta 288, 326–332

2 James, R. and Branton, D. (1973) Biochim. Biophys. Acta 323, 378—390
3 Wallace, B.A., Richards, F.M. and Engelman, D.M. (1976) J. Mol. Biol. 107, 255—269
4 Bevers, E.M. (1978) Ph.D. Thesis, State University of Utrecht
5 Haest, C.W.M., Verkleij, A.J., de Gier, J., Scheek, R., Ververgaert. P.H.J.Th. and van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 356, 17—26
6 Van Heerikhuizen, H., Kwak, E., van Bruggen, E.F.J. and Witholt, B. (1975) Biochim. Biophys. Acta 413, 177—191
7 Letellier, L., Moudden, H. and Shechter, E. (1977) Proc. Natl. Acad. Sci. U.S. 74, 452—456
8 Wieslander, Å. and Rilfors, L. (1977) Biochim. Biophys. Acta 466, 336—346
9 Christiansson, A. and Wieslander, Å. (1978) Eur. J. Biochem. 85, 65—76
10 Wieslander, Å., Ulmius, J., Lindblom, G. and Fontell, K. (1978) Biochim. Biophys. Acta 512, 241—253
11 Carlemalm, E. and Wieslander, Å. (1975) Nature 254, 537—538
12 Carlemalm, E. (1976) Ph.D. Thesis, University of Lund
13 Razin, S. (1973) in Advances in Microbial Physiology (Rose, A.H. and Tempest, D.W., eds.), Vol. 10, pp. 1—80, Academic Press, New York
14 Walter, H., Krob, E.J. and Brooks, D.E. (1976) Biochemistry 15, 2959—2964
15 Walter, H. and Krob, E.J. (1976) Biochim. Biophys. Acta 455, 8—23
16 Andersson, B., Åkerlund, H.-E. and Albertsson, P.-Å. (1976) Biochim. Biophys. Acta 423, 122—132
17 Eriksson, E. and Albertsson, P.-Å. (1978) Biochim. Biophys. Acta 507, 425—432
18 Walter, H. (1977) in Methods of Cell Separation (Catsimpoolas, N., ed.), Vol. 1, pp. 307—354, Plenum Press, New York
19 Albertsson, P.-Å. (1970) Sci. Tools 17, 53—57
20 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
21 Albertsson, P.-Å. (1971) Partition of Cell Particles and Macromolecules, 2nd edn., Almqvist and Wiksell, Uppsala, Sweden
22 Kates, M. (1972) in Laboratory Techniques in Biochemistry and Molecular Biology (Work, T.S. and Work, E., eds.), Techniques of Lipidology, Vol. 111, pp. 269—610, North Holland, Amsterdam
23 Neville, Jr., D.M. (1971) J. Biol. Chem. 246, 6328—6334
24 Jergill, B. and Ohlsson, R. (1974) Eur. J. Biochem. 46, 13—25
25 Chen, Jr., P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756—1758
26 Ryter, A. and Kellenberger, E. (1958) Z. Naturforsch. 13b, 597—605
27 Hughes, D.E., Wimpenny, J.W.T. and Lloyd, D. (1971) in Methods in Microbiology (Norris, J.R. and Ribbons, D.W., eds.), Vol. 5b, pp. 1—54, Academic Press, New York
28 Demel, R.A. and de Kruijff, B. (1976) Biochim. Biophys. Acta 457, 109—132
29 Maniloff, J. (1970) J. Bacteriol. 102, 561—572
30 Carlemalm, E. (1973) J. Ultrastruct. Res. 42, 412
31 Read, B.D., Demel, R.A., Wiegant, H. and van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 470, 325—330
32 Dahl, J.S., Hellewell, S.B. and Levine, R.P. (1977) J. Immonol. 119, 1419—1426
33 Jinks, D.C. and Matz, L.L. (1976) Biochim. Biophys. Acta 452, 30—41
34 Larraga, V. and Razin, S. (1976) J. Bacteriol. 128, 827—833