BBA 75883

CHARACTERIZATION OF THE MYOPLASMA MEMBRANE PROTEINS

III. GEL FILTRATION AND IMMUNOLOGICAL CHARACTERIZATION OF *ACHOLEPLASMA LAIDLAWII* MEMBRANE PROTEINS

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(Received October 13th, 1971)

SUMMARY

Acholeplasma laidlawii (formerly *Mycoplasma laidlawii*) membranes solubilized by ionic and nonionic detergents were fractionated on Sephadex G-200 columns containing the detergent used for solubilization. When sodium dodecyl sulfate or sodium deoxycholate were present, membrane lipids were resolved as a single elution peak while membrane proteins formed several reproducible peaks. Gel filtration in the presence of the nonionic detergents Triton X-100, Brij 58 and Lubrol W was usually inferior. The proteins were eluted as two broad peaks, one of which included the void volume. Since the NADH oxidase, ATPase and *p*-nitrophenylphosphatase activities of the solubilized membranes could be detected in the first peak, containing little or no lipid, it appears that these enzymes do not depend on membrane lipids for activity. Serological activity could be demonstrated in membrane fractions isolated by gel filtration, even when sodium dodecyl sulfate was used. However, the use of deoxycholate enabled the separation of a membrane protein fraction highly enriched in antigens which elicited the production of antibodies inhibiting *A. laidlawii* metabolism and growth. A high content of antigens located on the outer membrane surface was evidenced by the high agglutination titer of *A. laidlawii* cells exhibited by antisera to this fraction.

INTRODUCTION

The solubilization of *Acholeplasma laidlawii* membrane proteins was dealt with in a previous report in this series[1]. Treatment of the membranes with EDTA in low ionic strength media released about 11 % of the membrane protein in a water-soluble form. However, the released protein fraction contained neither the NADH oxidase, ATPase, and *p*-nitrophenylphosphatase activities of the membrane[1] nor the antigens responsible for eliciting the antibodies inhibiting the metabolism and growth of *A. laidlawii*[2]. Since only a minor share of the mycoplasma membrane proteins was solubilized by EDTA, detergents had to be employed to effect a more complete solubilization. In this respect the strongly ionic detergents sodium dodecyl sulfate and cetyltrimethylammonium bromide proved more effective than the

nonionic detergents, Triton X-100, Lubrol W or Brij 58, with sodium deoxycholate occupying an intermediate position. However, in view of the rapid inactivation of the membrane enzymes by the strongly ionic detergents it seemed preferable to use deoxycholate and the nonionic detergents. The fractionation of the mycoplasma membrane proteins and their separation from membrane lipids by gel filtration, in the presence of detergents, forms the subject of the present communication.

MATERIALS AND METHODS

Membrane preparations

Acholeplasma laidlawii (oral strain, formerly *Mycoplasma laidlawii*) was grown in the presence of [^{3}H]oleic acid in order to label membrane lipids, and was harvested and lysed as described before[3]. The isolated membranes were washed three times alternately with deionized water and 0.05 M NaCl in 0.01 M phosphate buffer, pH 7.5. The washed membranes were suspended in dilute NaCl–Tris–β-mercaptoethanol buffer[3] and kept at $-70°$ until used.

Solubilization of membranes

Membrane suspensions (containing 3.6 mg protein per ml) were treated for 20–30 min at 37° with 16 mg/ml of sodium deoxycholate, Triton X-100, Lubrol W, Brij 58, or with 5.7 mg/ml of sodium dodecyl sulfate. The clear solutions obtained were centrifuged at 100000 $\times$ *g* for 1 h at 4° to separate the solubilized membrane material from the nonsoluble residue.

Solubilization of membrane lipids in Brij 58

Membrane lipids were extracted with chloroform–methanol (2:1, v/v). The solvent was evaporated under N_2 and the dried lipids (about 12 mg) were dissolved in 1.0 ml of chloroform–methanol (2:1, v/v). 4 ml of Brij 58 solution (16 mg/ml) were added and the resulting emulsion was heated under N_2 until all the chloroform evaporated and the emulsion cleared.

Gel filtration

Sephadex G-200 (Pharmacia, Uppsala, Sweden) columns (90 cm $\times$ 2.5 cm) were equilibrated with dilute NaCl–Tris–β-mercaptoethanol buffer containing 16 mg/ml of the detergent used for membrane solubilization. The void volume of the columns, as determined with Blue Dextran 2000, was found to be about 150 ml. About 4 ml of solubilized membrane material containing about 18–20 mg protein were applied to the column. Fractions of 4 ml were collected, the elution of the proteins being followed by measuring the absorbance at 280 nm, and that of the lipids by measuring radioactivity in 0.3-ml samples of the fractions. Each fraction was also tested for several enzymic activities as described under assay procedures. Gel filtration was carried out at room temperature since some of the detergents, especially sodium dodecyl sulfate came out of solution at a lower temperature. The flow rate varied between 10 and 50 ml/h, depending on the column and on the detergent used. For electrophoresis and immunological assay, pooled fractions from Sephadex G-200 columns containing sodium dodecyl sulfate or sodium deoxycholate were dialysed against dilute NaCl–Tris–β-mercaptoethanol buffer to remove as much of the detergent as possible. The fractions were then concentrated at room

temperature by ultrafiltration through a Diaflo PM-10 filter (Amicon N.V., Oosterhout, Holland).

In some experiments the deoxycholate-solubilized membranes were filtered through a Sephadex G-25 (fine) column (20 cm × 1.2 cm) to free the solubilized membrane material of the detergent[4]. In this case 0.01 M Tris–HCl buffer, pH 8.3, was used for the elution of the material which was then applied to three different columns with no detergent: a Sepharose 4B (Pharmacia) column (30 cm × 2.5 cm), a Sephadex G-200 (Pharmacia) column (90 cm × 2.5 cm), and a DEAE-cellulose DE-23 (Whatman, England) column (18 cm × 0.8 cm). All three columns were equilibrated with 0.01 M Tris–HCl buffer, pH 8.3. From the Sepharose and Sephadex columns the material was eluted with the same buffer, whereas from the DEAE-cellulose column the elution was done with a stepwise NaCl gradient (0.05–1.0 M) in the buffer.

Assay procedures

Protein was determined by the Folin–phenol method of Lowry *et al.*[5] using bovine plasma albumin as standard. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer using the scintillation mixture described previously[6]. NADH oxidase (EC 1.6.99.3) activity was measured spectrophotometrically by determining the decrease in absorbance at 340 nm on addition of NADH to the reaction mixture[7]. Data are expressed as decrease in absorbance at 340 nm per min per mg of protein. Adenosine triphosphatase (EC 3.6.1.3) activity was measured by the release of inorganic phosphate from ATP[7]. Data are expressed as μmoles of inorganic phosphate released per mg of protein in 30 min. *p*-Nitrophenylphosphatase activity was measured spectrophotometrically by determining the yellow color of the liberated *p*-nitrophenol, using the reaction mixture described previously[1]. Data are expressed as increase in absorbance at 420 nm per mg protein in 1 h.

Electrophoretic analysis of membrane proteins was carried out in polyacrylamide gels containing 35 % acetic acid and 5 M urea[8] or in gels containing 0.1 % sodium dodecyl sulfate[9].

Immunological techniques

Antisera to membrane fractions were prepared in rabbits as described by Kahane and Razin[10]. The antisera were tested for metabolism-inhibiting antibodies to *A. laidlawii* in a medium containing glucose, as described by Taylor-Robinson *et al.*[11], and for agglutination of *A. laidlawii* cells by the method of Bailey *et al.*[12]. Double-diffusion tests in agar were performed as described by Argaman and Razin[13]. Absorption of antisera with membrane protein fractions was carried out as described by Kahane and Razin[10]. Growth inhibition by antisera was tested by the method of Clyde[14], and complement fixation according to Lennette[15] using *A. laidlawii* membranes as antigen.

RESULTS

Fractionation of solubilized membrane components by column chromatography in the absence of detergents

Filtration of *A. laidlawii* membranes solubilized by deoxycholate through a Sephadex G-25 column, separated most of the detergent from the membrane

material. Membrane protein and lipid were excluded in the void volume of the column, forming a clear yellowish solution, while most of the detergent was eluted in subsequent fractions. A rough estimate of the amount of deoxycholate in the fractions was arrived at by its precipitation with 10 % acetic acid. The minimum concentration of deoxycholate detected by this method was 0.1 mg/ml.

In an attempt to fractionate the solubilized membrane material from which deoxycholate had been removed, gel filtration in the absence of detergent was used. About one half of the material was excluded in the void volume of a Sephadex G-200 column, but the other half could not be eluted. Filtration on Sepharose 4B (exclusion limit molecular weight of approx. 3000000) was more successful in that only a minor part of the solubilized membrane material was excluded in the void volume. However, the rest of the membrane material which was retained in the column was eluted as a single broad peak, the lipid, protein and NADH oxidase activity being shared about equally by all fractions. Fractionation of the solubilized membranes on a DEAE-cellulose column failed as all the material was retained on the column and could not be eluted even with 1 M NaCl.

It appears from the chromatographic results that the solubilized membrane material aggregates to complexes (probably lipoprotein in nature), some of them so big as to be excluded in the void volume of the Sepharose 4B column. This was also borne out by immunodiffusion tests in which solubilized membrane material (from which deoxycholate had been removed) produced only a faint precipitation line with an antiserum to *A. laidlawii* membranes, the same material before the removal of deoxycholate producing 4–5 precipitation lines.

Fractionation of solubilized membrane components by gel filtration in the presence of detergents

Since without detergents solubilized membrane components could not be fractionated, it seemed essential to include a detergent both in the column and in

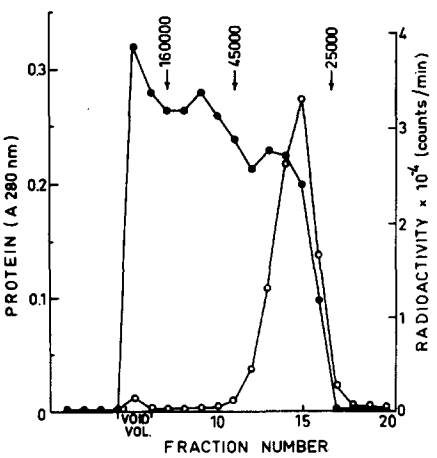

Fig. 1. Filtration of *A. laidlawii* membranes solubilized by 20 mM sodium dodecyl sulfate on a Sephadex G-200 column (30 cm × 2.5 cm) equilibrated with the same concentration of detergent. The column was calibrated by determination of the elution volumes of crystalline γ-globulin (mol. wt. 160000), ovalbumin (mol. wt. 45000) and chymotrypsinogen A (mol. wt. 25000); ●—●, membrane protein; ○—○, labeled membrane lipid.

the elution buffer so as to prevent the reaggregation of solubilized material. Fig. 1 shows the elution profile of membrane material solubilized by 20 mM sodium dodecyl sulfate and filtered through Sephadex G-200 containing the same concentration of sodium dodecyl sulfate. Filtration results in the separation of the protein into three overlapping peaks. The lipid was eluted as a single peak partially overlapping the

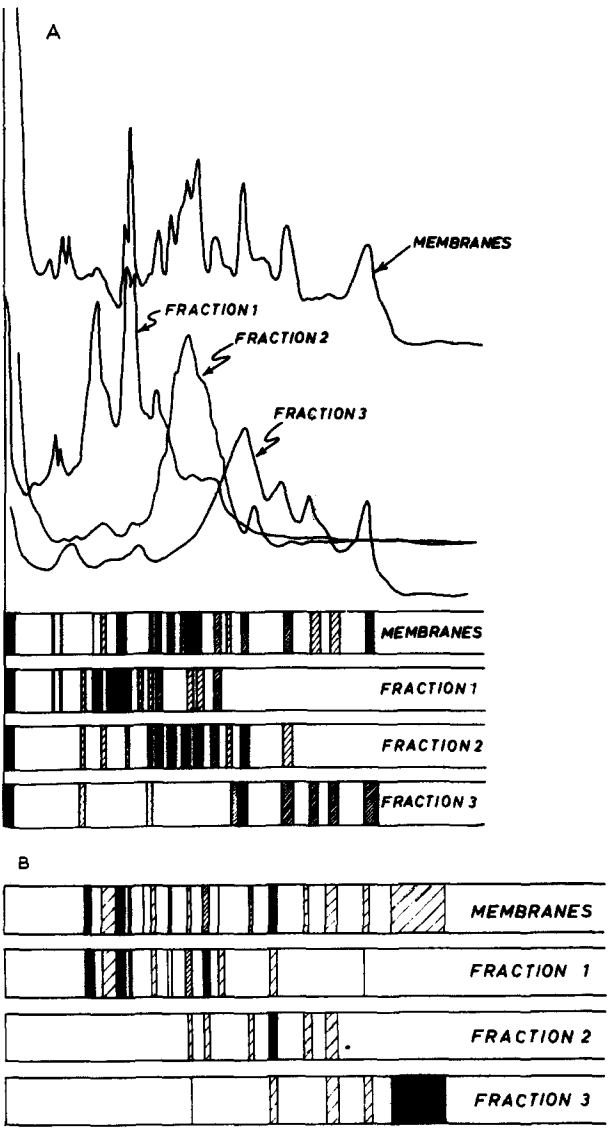

Fig. 2. Densitometer tracings and schematic representation of the electrophoretic patterns of three *A. laidlawii* membrane protein fractions separated by chromatography on a Sephadex G-200 column containing 20 mM sodium dodecyl sulfate. Fraction 1 consisted of pooled Fractions 4–6 eluted from the column shown in Fig. 1; Fraction 2 consisted of pooled Fractions 7–11 of the same column; Fraction 3 of pooled Fractions 12–18. A. Electrophoresis in polyacrylamide gels containing 35% acetic acid and 5 M urea. B. Electrophoresis in polyacrylamide gels containing 0.1% sodium dodecyl sulfate.

third protein peak. The molecular weights of the protein eluted in the various fractions could be roughly estimated by comparison with the elution profiles of several protein standards (Fig. 1), as well as by electrophoretic analyses of the fractions eluted from the sodium dodecyl sulfate containing Sephadex G-200 column (Fig. 2). The electrophoretic patterns obtained in polyacrylamide gels containing acetic acid and urea showed some resemblance to those obtained in gels containing sodium dodecyl sulfate. The linear relationship between the logarithm of the molecular weight of a protein and the distance it migrates in polyacrylamide gels containing sodium dodecyl sulfate is well established[16]. A similar relationship was recently also reported for gels containing 35% acetic acid and 5 M urea[3,17]. From a calibration curve obtained with proteins of known molecular weights, it appears that the molecular weight of the proteins in Fraction 1 (Fig. 2) is over $3 \cdot 10^4$; the proteins of Fraction 2 have molecular weights ranging between $1.3 \cdot 10^4$ and $7 \cdot 10^4$ and those of Fraction 3 have molecular weights ranging between $9 \cdot 10^3$ and $2.6 \cdot 10^4$.

The strongly ionic detergent sodium dodecyl sulfate has the disadvantage of inactivating membrane enzymes very rapidly and at low concentrations[1]. Sodium dexoycholate was shown to be less harmful to membrane enzymes[1,7]; about 75% of the initial NADH oxidase activity of *A. laidlawii* membranes was found in the soluble membrane fraction obtained with 16 mg/ml of this detergent[1]. Consequently, Sephadex G-200 columns containing deoxycholate were used for fractionation of membrane material. As may be seen in Fig. 3 these columns were about equally effective as the columns containing sodium dodecyl sulfate in separating membrane lipid from most of the membrane proteins. The elution profile of the proteins was characterized by 5–6 peaks. The NADH oxidase activity was associated with the proteins excluded in the void volume.

Nonionic detergents have been shown to be still less harmful to the enzymic

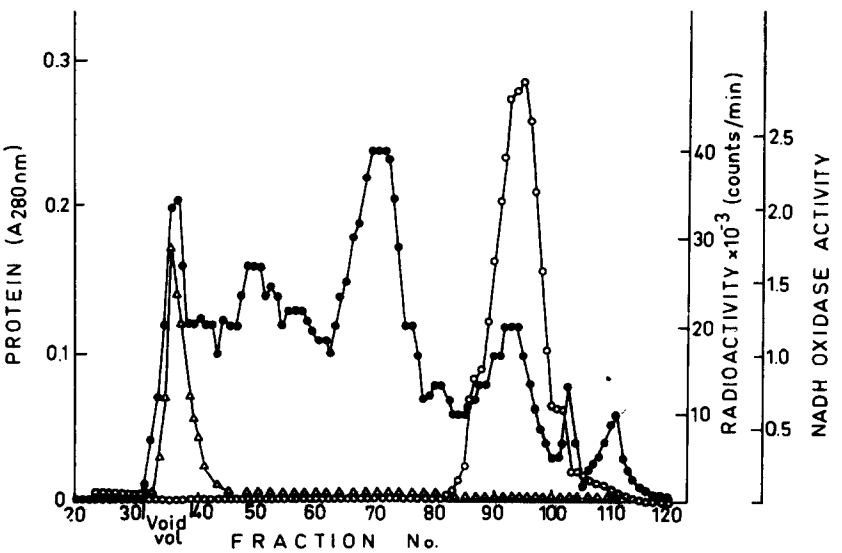

Fig. 3. Filtration of *A. laidlawii* membranes solubilized by 16 mg/ml of sodium deoxycholate on a Sephadex G-200 column (90 cm × 2.5 cm) equilibrated with the same concentration of detergent. ●—●, membrane protein; ○—○, labeled membrane lipid; △—△, NADH oxidase activity.

activities of *A. laidlawii* membranes than deoxycholate. Thus membranes solubilized by Brij 58 retained a large portion of their *p*-nitrophenylphosphatase and ATPase in addition to their NADH oxidase activity[1]. However, the fractionation of *A. laidlawii* membrane material on Sephadex G-200 columns containing nonionic detergents was usually inferior to that obtained with ionic detergents, as the proteins were essentially eluted in two broad peaks, in one of which the void volume was also included (Figs 4, 5). Inferior protein separation was associated with a much smaller total elution volume of solubilized membrane material than on the column containing deoxycholate. Thus the total elution volume of membrane material from a Brij 58-containing column was 160 ml as against 360 ml in the deoxycholate-containing column.

Fig. 4 shows that all three enzymic activities tested were localized in the first elution peak which also contained a minor share of the membrane lipids. When Triton X-100 was used, there was almost no lipid in the first elution peak which contained all the NADH oxidase activity (Fig. 5). Similar results were obtained with Lubrol W. The presence of lipid in the protein peak eluted from the Brij 58-containing column (Fig. 4) raised the question of whether it is an expression of the existence of lipoprotein complexes, or whether it reflects the presence of two different populations of lipid–detergent micelles. The second possibility seemed to be ruled out when membrane lipids alone solubilized in Brij 58 were eluted from the Sephadex G-200 column containing Brij 58 as a single symmetrical peak corresponding to the major lipid peak of Fig. 4.

The recovery of enzymic activities in the first protein peak eluted from the detergent-containing Sephadex columns is shown in Table I. With Brij 58 the value of total enzyme units of NADH oxidase and *p*-nitrophenylphosphatase recovered

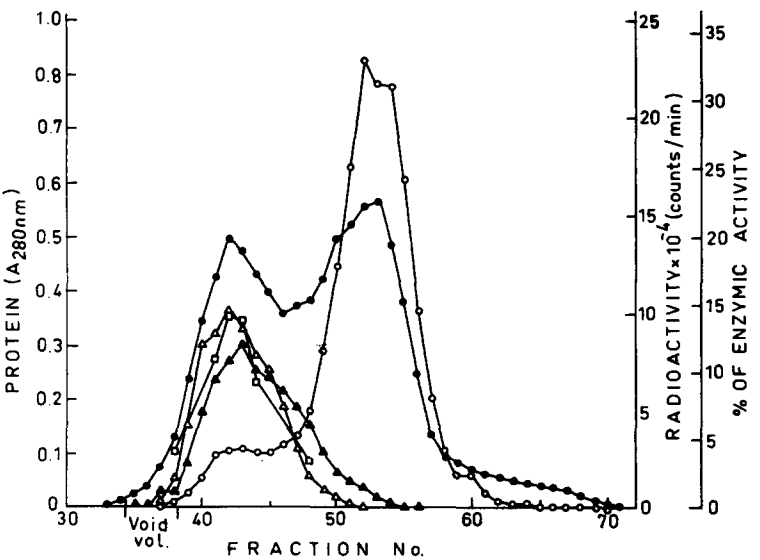

Fig. 4. Filtration of *A. laidlawii* membranes solubilized by 16 mg/ml of Brij 58 on a Sephadex G-200 column (90 cm × 2.5 cm) equilibrated with the same concentration of detergent. ●—●, membrane protein; ○—○, labeled membrane lipid; △—△, NADH oxidase activity; ▲—▲, *p*-nitrophenylphosphatase activity; □—□, ATPase activity.

in the fraction exceeded that of the native membranes used for fractionation. The marked activation of membrane enzymes occurring on membrane solubilization may explain this finding[1]. A marked increase in the specific activity of some of the enzymes in the eluted protein fraction was noticed (Table I).

Immunological properties of membrane fractions

In spite of the intensive denaturation effect of sodium dodecyl sulfate, the membrane protein fractions obtained by gel filtration in the presence of this detergent

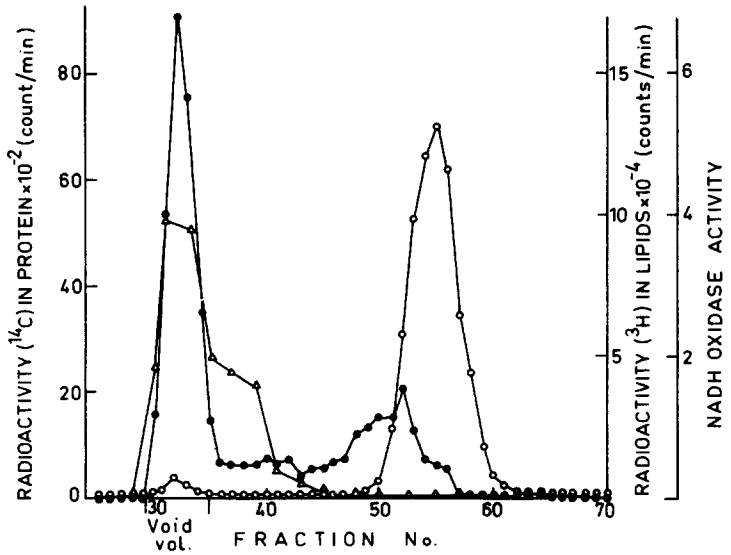

Fig. 5. Filtration of *A. laidlawii* membranes solubilized by 16 mg/ml of Triton X-100 on a Sephadex G-200 column (90 cm × 2.5 cm) equilibrated with the same concentration of detergent. Membranes were derived from cells grown with L-[^{14}C]phenylalanine to label membrane proteins and with [^{3}H]oleic acid to label membrane lipids[6]. Labeling was necessary because of the difficulty of determining the protein with the Folin reagent in the presence of Triton X-100 (ref. 1). ●—●, labeled membrane protein; ○—○, labeled membrane lipid; △—△, NADH oxidase activity.

TABLE I

ENZYMIC ACTIVITIES IN *A. laidlawii* MEMBRANE PROTEIN FRACTIONS SEPARATED ON SEPHADEX G-200 COLUMNS CONTAINING DETERGENTS

Detergent in column (16 mg/ml)	*Protein in fraction* (% total membrane protein)*	*Enzymic activity*	*Recovery of enzymic activity in fraction***	*Specific activity (enzyme units)*	
				Native membranes	*Fractic*
Deoxycholate	25	NADH oxidase	59	3.00	7.10
Triton X-100	76	NADH oxidase	62	2.65	2.16
Brij 58	38	NADH oxidase	126	4.43	14.60
		p-Nitrophenylphosphatase	152	5.42	21.70
		ATPase	63	1.84	3.05

* Consisting of the first elution peak.

** Percentage of total activity in enzyme units of the native membranes used for fractionation.

still exhibited serological activity. Thus Fractions 2 and 3 (Fig. 2) produced a distinct precipitation line with an antiserum to the native membranes, corresponding to one of the two precipitation lines produced with native membranes (Fig. 6). Fraction 1, which consisted mostly of high molecular weight proteins, produced only a faint and diffuse precipitation band with the antiserum to membranes. Some of the membrane protein fractions also exhibited considerable immunogenic activity (Table II). Fractions containing the proteins of medium molecular weight were at least as effective as the native membranes in eliciting metabolism-inhibiting antibodies in rabbits.

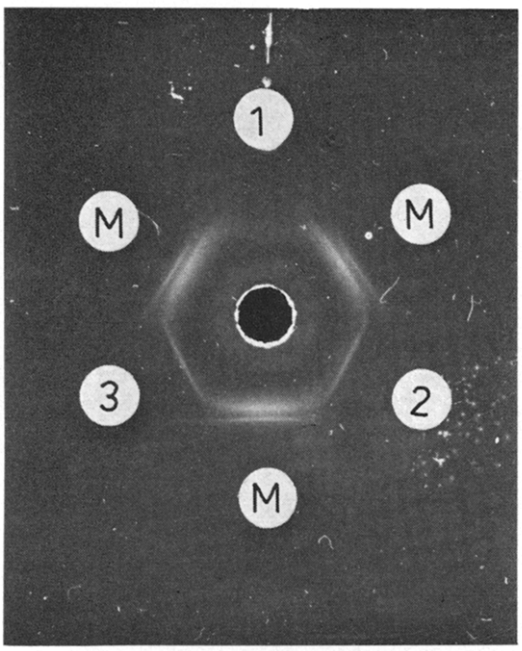

Fig. 6. Precipitin reactions of *A. laidlawii* membranes and membrane fractions separated on a Sephadex G-200 column containing 20 mM sodium dodecyl sulfate. Central well contains antiserum to *A. laidlawii* membranes; (M) *A. laidlawii* membranes; 1, 2, 3 correspond to the three membrane protein fractions shown in Fig. 2. All antigens were solubilized by sodium dodecyl sulfate (1 mg detergent per mg protein).

The membrane protein fractions obtained by gel filtration in the presence of sodium deoxycholate showed a higher serological activity than the fractions obtained with sodium dodecyl sulfate. Table III shows that one of the fractions (Fraction 3) had a much higher immunogenic activity than the native membranes, while the other three fractions were less immunogenic. Table IV shows that the antiserum to Fraction 3 agglutinated *A. laidlawii* cells to a much higher titer than the antisera to native membranes or to the other protein fractions. Similarly, the antiserum to Fraction 3 showed the highest complement-fixing activity with *A. laidlawii* membranes, though the antiserum to Fraction 4 (which contained the membrane lipids, see Fig. 3) was about as active. The antiserum to Fraction 3 was as effective as the antiserum to native membranes in inhibiting growth of *A. laidlawii*, while the

TABLE II

IMMUNOGENICITY OF *A. laidlawii* MEMBRANE PROTEIN FRACTIONS SEPARATED ON A SEPHADEX G-200 COLUMN CONTAINING 20 mM SODIUM DODECYL SULFATE

Each fraction was injected into 3 rabbits. The total amount of protein injected per rabbit was 0.75 mg. Fraction 1 consisted of pooled Fractions 4–6 eluted from the column shown in Fig. 1; Fraction 2 consisted of pooled Fractions 7–11 of the same column, Fraction 3 of pooled Fractions 12–15, and Fraction 4 of pooled Fractions 15–18.

Material used for immunization	*Range of reciprocal of metabolism-inhibiting antibody titer to A. laidlawii*	
	Before immunization	*After immunization (4 weeks)*
Fraction 1	8	80–160
Fraction 2	8–16	2560–5120
Fraction 3	2–16	2560–5120
Fraction 4	8–16	80
Native membranes	16	1280–2560

TABLE III

IMMUNOGENICITY OF *A. laidlawii* MEMBRANE PROTEIN FRACTIONS SEPARATED ON A SEPHADEX G-200 COLUMN CONTAINING 16 mg/ml OF SODIUM DEOXYCHOLATE

Each rabbit received a total of 1.6 mg of membrane protein. Fraction 1 consisted of pooled Fractions 32–40 eluted from the column shown in Fig. 3; Fraction 2 consisted of pooled Fractions 41–63 eluted from the same column; Fraction 3 consisted of pooled Fractions 64–80, and Fraction 4 of pooled Fractions 81–120.

Material used for immunization	*No. of rabbits*	*Geometric mean and range of reciprocal of metabolism-inhibiting antibody titer*			
		Immunization period (weeks):			
		*P**	*3*	*4*	*5*
Fraction 1	6	10 (2–32)	23 (20–40)	40 (40)	20 (20)
Fraction 2	6	9 (8–16)	56 (40–80)	100 (80–160)	56 (40–80)
Fraction 3	7	9 (8–16)	3450 (320–20480)	1900 (640–5120)	1720 (640–2560)
Fraction 4	5	12 (8–16)	18 (10–40)	30 (20–80)	35 (20–80)
Native membranes	6	14 (8–16)	360 (160–1280)	320 (160–1280)	200 (80–1280)

P*, pre-immunization serum.

antisera to the other protein fractions failed to inhibit growth (Table IV). The Fraction 3 proteins were also more effective than the proteins of the other fractions in absorbing metabolism-inhibiting antibodies from the antiserum to the native membranes. Complete absorption of these antibodies could, however, be achieved only with membranes. Antigenic differences between the protein fractions could also be detected by immunodiffusion tests with an antiserum to *A. laidlawii* membranes (Fig. 7).

TABLE IV

SEROLOGICAL ACTIVITIES OF ANTISERA TO *A. laidlawii* MEMBRANE PROTEIN FRACTIONS SEPARATED BY GEL FILTRATION IN THE PRESENCE OF SODIUM DEOXYCHOLATE

The antisera used were those obtained after 3 weeks of immunization (Table III). *A. laidlawii* membranes exhibited considerable anticomplementary activity. The antigen (4 mg membrane protein per ml) was, therefore, used at a 1:2560 dilution.

Antiserum	*Agglutination of A. laidlawii cells (reciprocal of titer)*	*Complement fixation with A. laidlawii membranes (reciprocal of titer)*	*Growth inhibition of A. laidlawii (inhibition zone in mm from disc)*
Fraction 1	128	640	0
Fraction 2	64	160	0
Fraction 3	256	1280	3
Fraction 4	64	1280	0
Native membranes	32	160	3

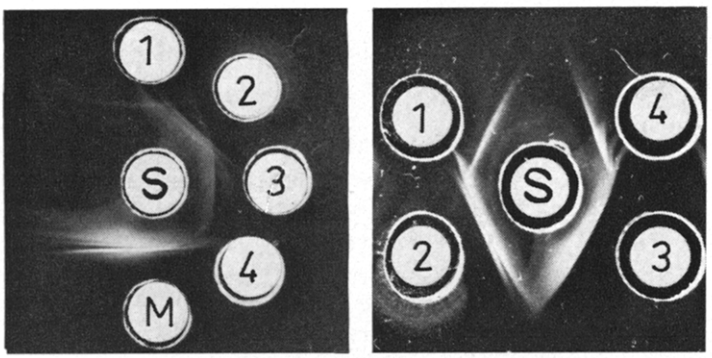

Fig. 7. Precipitin reactions of *A. laidlawii* membranes and membrane fractions separated on a Sephadex G-200 column containing 16 mg/ml sodium deoxycholate. (S) Antiserum to *A. laidlawii* membranes; 1, 2, 3, 4 correspond to the four protein fractions defined in Table III; (M) *A. laidlawii* membranes solubilized by deoxycholate. Spur formation indicates antigenic differences.

A. laidlawii membranes were shown to contain a significant amount of hexosamine, probably in a polymeric form[9]. The possibility that hexosamine residues play a role in the immunological activity of the membranes led us to analyze the four membrane fractions, isolated in the presence of deoxycholate, for their hexosamine content according to the method described by Morowitz and Terry[9]. The hydrolysates of all the four fractions were found to contain considerable amounts of hexosamine, the values ranging between 120 and 140 μg hexosamine per mg protein for Fractions 1, 2 and 4 and 60–65 μg hexosamine per mg protein for Fraction 3. The value for native membranes ranged between 70 and 80 μg hexosamine per mg protein, in accordance with the values obtained by Morowitz and Terry[9].

DISCUSSION

The failure to fractionate membrane material solubilized by detergents on Sephadex or Sepharose columns devoid of detergents is apparently due to reaggrega-

tion of membrane proteins and lipids upon dilution or removal of the detergent during filtration. The tendency of solubilized membrane components to reaggregate upon dilution or removal of the detergent is well documented[4,8,18]. Detergents herefore had to be included throughout the fractionation procedure. In spite of the prolonged exposure to detergents, a considerable portion of the biological activity of the mycoplasma membrane proteins was preserved. Of the detergents used sodium deoxycholate appears to be the most favorable. Its inclusion in the Sephadex G-200 column resulted in the separation of membrane proteins into several reproducible peaks most of which were devoid of membrane lipids (see also refs. 19 and 20). Fractionation of membrane proteins was at least as good as with sodium dodecyl sulfate, while the fractions retained more of their biological activity. Sodium deoxycholate does not seem to cause gross conformational changes in membrane proteins[21] or form complexes with them[19,20], whereas sodium dodecyl sulfate binds to membrane proteins in considerable quantities and unfolds the polypeptide chains[22]. Bile salts have, therefore, been used extensively in the solubilization and fractionation of active enzyme complexes from mitochondrial and other membranes[23-25]. On the other hand, the use of sodium dodecyl sulfate in gel filtration has the advantage of separating the membrane proteins according to their molecular weights, as shown in this and in other studies[26].

Although the nonionic detergents are less destructive to the biological activities of the solubilized membrane proteins than deoxycholate[1], their use in column chromatography has several disadvantages: (1) Fractionation of membrane proteins is inferior to that achieved with the ionic detergents. Nonionic detergents do not seem to disaggregate the membrane completely and leave protein aggregates and sometimes even lipoprotein particles intact (Fig. 4). A considerable part of the solubilized membrane material is thus excluded in the void volume of the column. (2) It is extremely difficult to remove the nonionic detergents from the fractions by such simple methods as dialysis[27,28] or ultrafiltration (Z. Ne'eman, unpublished data). (3) The nonionic detergents used by us interfered with protein determination by the Folin reagent; Triton X-100 also interfered with the determination of proteins by absorbance at 280 nm. This difficulty could be overcome by using membranes containing labeled proteins (Fig. 5).

That the NADH oxidase, ATPase and *p*-nitrophenylphosphatase activities were detected chiefly in the fraction excluded in the void volume of the detergent-containing Sephadex columns might indicate the association of these enzymes with minute nonsolubilized membrane fragments. However, the absence of membrane lipid from these fractions speaks against this interpretation. Our results tend to show that the three tested enzymic activities of *A. laidlawii* membranes do not require the presence of membrane lipids. However, analogous enzymic activities in other biomembranes do appear to require membrane lipids[29,30]. Since our fractions contained detergents, some of which may be capable of replacing the lipids required for enzymic activities of membranes[31,32], no final conclusions should be drawn. Nevertheless, the removal of deoxycholate from the enzymatically active fraction by filtration through Sephadex G-25 did not affect the NADH oxidase activity.

Of the three enzymic activities tested, NADH oxidase appears to be the most resistant to detergent action. It even resisted a short exposure to sodium dodecyl sulfate and could, therefore, be detected in reconstituted *A. laidlawii*

membranes[8]. The NADH oxidase of *A. laidlawii* is thus a favorable candidate for further purification and characterization. Preliminary experiments indicate that by combined $(NH_4)_2SO_4$ fractionation and gel filtration the NADH oxidase activity may be considerably purified. The apparent absence of cytochromes from *A. laidlawii* membranes (Z. Ne'eman, unpublished data) points to a simple electron transport system probably consisting of one or two enzymes, which might explain its unusual resistance to detergent action.

It is still a moot point whether or not the ATPase and *p*-nitrophenylphosphatase activities of *A. laidlawii* membranes are the expression of a single enzyme. Although they differ in their sensitivity to detergents[1] and to organic solvents (L. Gottfried and S. Razin, unpublished data), the present study failed to separate between them by gel filtration (Fig. 4) thus leaving the question open.

The immunological activities of membrane proteins resist inactivation by detergents far better than the enzymic activities. Membrane protein fractions exposed to sodium dodecyl sulfate for 24 h or more still retained a considerable portion of their serological and immunogenic activities. The ability of membrane material solubilized by sodium dodecyl sulfate to react with antibodies in immunodiffusion tests has been noted before[10, 13, 33], however, Helenius and Simons[34] claim that the serological activities of an apo-protein of human plasma lipoproteins are altered (but not destroyed) by this detergent. Our results show that the antigens eliciting metabolism-inhibiting antibodies to *A. laidlawii* are apparently membrane proteins and not lipids, contrary to the findings with *Mycoplasma pneumoniae*[35]. However, *A. laidlawii* lipids were found capable of fixing complement with antisera to *A. laidlawii* cells[36]. The fractionation of solubilized *A. laidlawii* membranes on a deoxycholate-containing Sephadex G-200 column was found to be superior to that obtained with sodium dodecyl sulfate, allowing as it did for the separation of a protein fraction highly enriched with the antigens responsible for production of metabolism- and growth-inhibiting antibodies. Since the titer of these antibodies was directly correlated with resistance to mycoplasma infections[37], the study of these antigens is of great importance for the development of effective vaccines. That the antiserum to the enriched fraction also exhibited the highest titer of antibodies agglutinating *A. laidlawii* cells may point to the antigens, being at least in part exposed on the outer membrane surface. Although mostly contained in Fraction 3 the antigens were also found in small quantities in the remaining membrane fractions collected with deoxycholate. As Fraction 3 could not adsorb all the metabolism-hinibiting antibodies from an antiserum prepared to membranes, it appears that more than one protein antigen is responsible for their production.

The possibility that hexosamine residues play a role in the immunological activity of *A. laidlawii* membranes cannot be ruled out. All the membrane fractions separated in the presence of deoxycholate contained substantial amounts of hexosamine. However, the finding that the fraction exhibiting the highest immunological activity (Fraction 3) had the lowest hexosamine content and the thermolability of the membrane antigens capable of absorbing metabolism-inhibiting antibodies to *A. laidlawii*[10] speak against the notion that hexosamine residues play a major role in the immunological activity of the membrane. Moreover, recent experiments (Kahane, unpublished data) have shown that the ability of *A. laidlawii* membranes to absorb the metabolism-inhibiting antibodies to this organism is

virtually lost when 45 % or more of the proteins are digested by pronase. This strongly indicates that membrane proteins are responsible for eliciting the production of the metabolism-inhibiting antibodies. The possibility that hexosamine residues bound to membrane proteins function as antigenic determinants cannot be excluded, though the findings of Morowitz and Terry[9] do not support the attachment of hexosamine to membrane protein in *A. laidlawii*.

ACKNOWLEDGMENTS

We thank Mr. M. Wormser for his able assistance. This work was supported by grant FG-Is-286 from the U.S. Department of Agriculture under Public Law 480.

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