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MOLECULAR ORGANIZATION IN BACTERIAL CELL MEMBRANES

I. SODIUM DODECYL SULPHATE SOLUBILIZATION AND FRACTIONATION OF THE COMPONENTS OF A DEPLETED MEMBRANE FROM *Micrococcus lysodeikticus*

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SUMMARY

A method is presented for the solubilization and fractionation of *Micrococcus lysodeikticus* membrane after it has been subjected to mild washing procedures (*i.e.* depleted membrane). The method involves the solubilization of the membrane, treatment with iodoacetamide and sodium dithionite and the action of sodium dodecyl sulphate on the membrane extracted with *n*-butanol. Polyacrylamide electrophoresis and Sephadex G-200 filtration in the presence of dodecyl sulphate result in the separation of at least eleven protein components whose molecular weights range from 17000 to 75000. Residual lipid and carbohydrate are found as components of the depleted membrane, and fractionated as a glycolipoprotein complex. A component tentatively identified as ribonucleic acid is also found associated with the membrane although its association with other membrane components appears to be broken by the detergent.

INTRODUCTION

An understanding of the structural arrangements of the components of a biological membrane may help towards a rational explanation of its properties[1]. The establishment of the molecular organization of a natural membrane requires the study of the properties of its individual components and the nature of interactions which lead to the specific formation of that structure.

The cytoplasmic membrane of *Micrococcus lysodeikticus* has been thoroughly studied during the past few years[1]. The conditions for membrane isolation and the chemical composition have been reported[2,3]. About 24 % of the lipid content has been identified as neutral lipid[4] with carotenoids and menaquinones accounting for 0.1 and 4 %, respectively[5]. The fatty acid composition[6] indicated a high content of saturated C_{15}, branched-chain type (85.4 %) and may be related to the structural arrangement of the membrane components[7]. Cytochromes[8], succinate dehydrogenase (EC 1.3.99.1), NADH dehydrogenase (EC 1.6.99.3) and ATPase (EC 3.6.1.3) have been characterized as active membrane components[9]. The protein components appear to be orga-

nized in such a way that it is possible to obtain a gradual release of the material. Thus, ATPase has been released by subjecting the membranes to a low ionic strength environment[10] while NADH dehydrogenase was solubilized with EDTA buffers[3]. The removal of these detachable proteins was accompanied by a gradual reduction of the granular substructure of the membrane[11]. The smooth-surfaced residue contains 35 % of the standard membrane protein and 40–50 % of the membrane lipid content[9]. We tentatively designate this residual pellet as a membrane depleted of detachable proteins, with the understanding that different treatment may still selectively release other biological components from these structures. As a matter of fact, the isolation of a *M. lysodeikticus* membrane residue after deoxycholate extraction containing 15 % of the standard membrane protein and virtually no lipid has been reported.

We relate in this paper a method for the solubilization and isolation of the protein components of the smooth-surfaced residues of *M. lysodeikticus* membrane after lipid removal.

MATERIALS AND METHODS

Reagents

Sodium dodecyl sulphate (sodium lauryl sulphate) purchased from Fisher Scientific Company was used without further purification. Sodium dodecyl sulphate from other commercial sources (Sigma Chem. Co., Fluka and Buchs, Th. Schuchardt) was recrystallized once from boiling ethanol before use. Sephadex G-200 beads were supplied by Pharmacia Fine Chemicals Inc. Acrylamide, *N,N, N', N'*-tetramethylethylenediamine, *N, N'*-methylene bisacrylamide and ammonium persulphate were obtained from Fluka and Buchs. α-Iodoacetamide was bought from Calbiochem and *p*-chloromercuribenzoic acid (sodium salt) from Mann Research Labs. Glutathione, amido black 10B (naphtol blue black), basic fuchsin and Sudan III, were purchased from Merck. Toluidine blue and Nitroso-R salt were obtained from BDH Chemicals and Visking dialysis tubes from Scientific Instruments. All other chemical reagents were of analytical grade.

The following standard proteins were used: pepsin, 3 times crystallized, Nutritional Biochem., mol. wt. 35000; trypsin from Ferosa, mol. wt. 24000; bovine serum albumin, Sigma, mol. wt. 67000 for the monomer; cytochrome *c* (*Candida krusei*), 2 times crystallized from Sankyo Co., mol. wt. 12523; and human plasma γ-globulin from Hubber Laboratories.

Preparation of membranes

M. lysodeikticus (NCTC 2665) was grown and harvested as described previously[7]. Membranes were obtained by osmotic lysis of preformed protoplasts. Treatment with EDTA and low-ionic-strength buffers removed the detachable proteins, as described previously[13].

Solubilization and lipid extraction of the membrane depleted of detachable proteins

The resulting pellet was suspended in 50 mM sodium acetate–0.5 M urea buffer (pH 5.2). After determination of the protein content by the method of LOWRY *et al.*[14], 4–5 mg of sodium dodecyl sulphate were added per mg of protein. The suspension

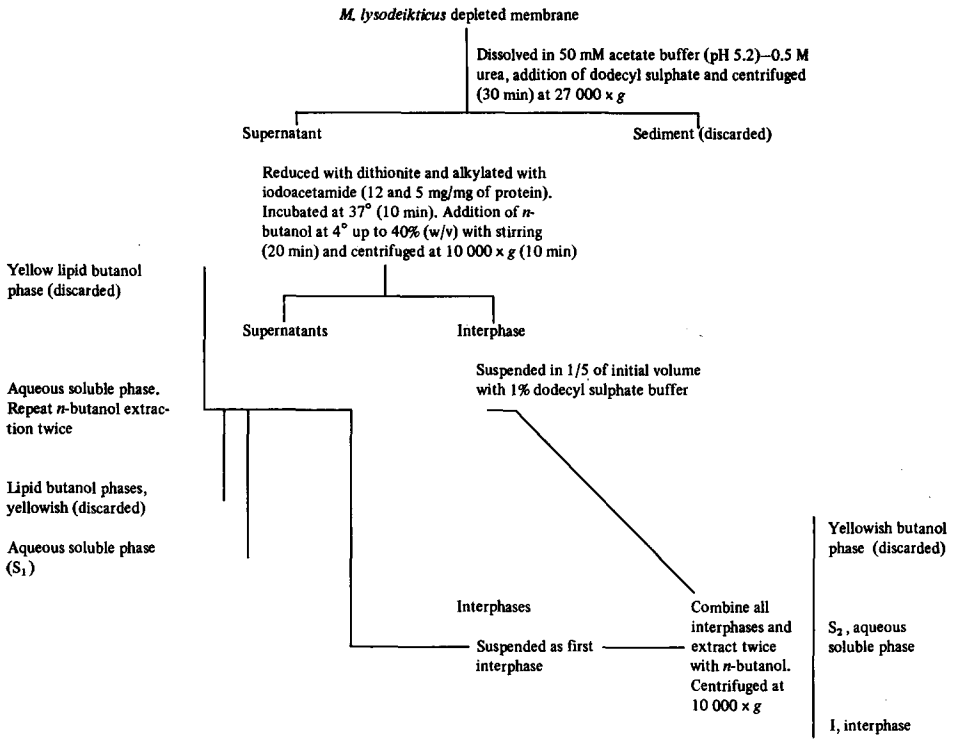
clarified instantaneously and was then centrifuged at 27000 × *g* for 30 min in a refrigerated Sorvall RC2-B. A black-coloured material which sedimented was discarded. Samples in which the pH was not adjusted to between 4.6 and 5.2 were not completely solubilized. All procedures were carried out at 4°. To this solution (25–50 ml) containing 0.5–5 mg of protein per ml was added sodium dithionite ($Na_2S_2O_4$) at a concentration of 1.2 mg/ml of membrane solution in order to reduce cytochromes[15]. After addition of iodoacetamide at variable concentrations, lipids were removed by extraction with *n*-butanol (40 %, w/v) at 4° (ref. 16). The resulting suspensions were centrifuged at approx. 10000 × *g* for 10 min. After centrifugation, three zones were observed: a yellow butanol phase containing most of the lipids, an interphase and an aqueous lower phase. Most of the upper phase was removed using a syringe and discarded. The tube was then slightly tilted to attach the interphase to the wall, and the aqueous phase was transferred with a syringe to another tube. The interphase was then suspended in 1/5 of the initial volume with 1 % dodecyl sulphate–acetate–urea buffer and kept until the complete removal of colour from the aqueous phase S_1 was achieved with *n*-butanol treatment (repeated twice). Each new interphase was suspended as described above. The three interphases so obtained were combined and their lipids were extracted with butanol as indicated for S_1. An aqueous phase S_2 was obtained after lipid extraction of the combined interphases. In previous experiments, the aqueous phase S_2 and the suspended interphase were dialyzed separately. As we will show later, the electrophoretic analysis indicated that both fractions were similar. Therefore, in subsequent experiments S_2 and I fractions were pooled and dialyzed against 1 % dodecyl sulphate buffer. Fraction S_1 was dialyzed against 0.1 % dodecyl sulphate buffer. After dialysis, all fractions appeared totally clear. If a slight turbidity was observed, it was removed by centrifugation at 27000 × *g* for 30 min at 4°. A general outline of the procedure is presented in Fig. 1. The yield of soluble proteins was determined in each solution after dialysis and centrifugation (if needed) using bovine serum albumin as standard.

Determination of sulphydryl groups in the solubilized depleted membrane

Estimation of SH groups[17] was carried out by measuring the immediate change of absorbance at 255 nm when the sample solution was added to 3 ml of titrated *p*-chloromercuribenzoic acid solution (pH 4.6) contained in a cuvette of a Gilford spectrophotometer. The *p*-chloromercuribenzoic acid solution was standardized with gluthathione.

Polyacrylamide gel electrophoresis

The proteins of the solubilized depleted membrane and fractions obtained after removing most of the lipid components were examined by gel electrophoresis in 7 % acrylamide gels[13], except that the samples (200 μl) in a dense solution (addition of a drop of glycerol) were layered on the top of the gels. The samples were applied without any other treatment. Tris–glycine buffer (pH 8.5 $\pm$ 0.2) was added to 10 % dodecyl sulphate solution to reach a final concentration of 0.1 % dodecyl sulphate in both gels and buffer. Electrophoresis was carried out on the Analytical Acrylophor apparatus, model 40 (Pleuger, Belgium). Gels of 4 cm were generally used. The electrophoreses were run at 2 mA per column for the first 30 min; after which the current was increased



I is resuspended in S_2 before dialysis against 1% dodecyl sulphate or resuspended in a small aliquot of buffer before dialysis

Fig. 1. General procedure for the solubilization and *n*-butanol extraction of *M. lysodeikticus* depleted membrane.

to 5–6 mA per gel until the tracking dye (bromophenol) blue reached 3.5–3.7 cm. This usually took an extra 30–40 min.

After electrophoresis the gels were withdrawn from their tubes and stained for of different components.

Proteins were visualized[18] with 2 % amido black in a mixture of methanol–acetic acid. The gels were destained electrophoretically. In some experiments, the gels were scanned at 535 nm in a Gilford 2400 spectrophotometer equipped with the 2410-S linear transport model running at a speed of 1 cm/min.

Carbohydrates were located with the periodic acid–Schiff base reagent[19]. Destaining was carried out as indicated by KEYSER[19], exept that the samples were finally washed in the NBS (New Brunswick) Gyrotory Shaker overnight in order to speed up the process. Gels were stored in 7 % acetic acid.

Iron containing proteins were detected with nitroso-R salt after fixing the gels for 2 h with a mixture of methanol–acetic acid–water (8:10:1, v/v/v)[20].

Nucleic acid, acidic mucopolysaccharides and other ionic polyelectrolytes were located[21] after immersion of the gels for 1 h in 1 M acetic acid. They were then transferred for 1 h into a 0.1 % toluidine blue solution in 1 % acetic acid. Residual dye was removed by running water, followed by overnight shaking in the Gyrotory NBS Shaker.

Lipoproteins were detected[22] after fixing the samples with 20 % trichloroacetic acid. The gels were stained with Sudan III (oil red) solution[23]. After staining overnight at 37°, the gels were removed and placed in methanol–20 % trichloracetic solution (40:60, v/v) to remove the excess dye. The Sudan III brown band was fixed with 0.2 % iodine solution in 7 % acetic acid.

Molecular weight mobility profiles were obtained as described[24] except that the samples were dissolved in 1 % dodecyl sulphate buffer and no mercaptoethanol was added either in the gels or in the buffers. Bovine serum albumin, γ-globulin, pepsin, trypsin and cytochrome *c* were used as markers. The molecular weights of polymers of albumin were calculated as described by DUNKER AND RUECKT[25]. The faster moving band of each standard protein was regarded as the monomer.

Gel filtration

Sephadex G-200 beads were equilibrated with 50 mM NH_4HCO_3–5 mM EDTA solution containing 1 % dodecyl sulphate. The material fractionated by the gel was the combined S_2 and I fractions obtained as described above and dialyzed against the same buffer prior to being applied to the column.

RESULTS

The sulphydryl content of the depleted membrane of *M. lysodeikticus* is illustrated in Table I. As shown, 25 equiv. of SH groups are titrated per 10^5 g of proteins. This figure is almost doubled after the solubilized membrane has been reduced with dithionite and titrated immediately after reduction. The original value for titrable SH groups is, however, recovered after storage of the dithionite-treated preparation for one week in the cold. The titration curves are shown in Figs. 2–2c.

The yields of soluble proteins in the different fractions after removal of the lipids by butanol extraction are illustrated in Table II. The results indicate that the addition of iodoacetamide produced higher yields of solubilized proteins.

The electrophoretic analysis of the depleted membrane and the fractions obtained by the procedure described is illustrated in Fig. 3. The electrophoretic profile of fraction S_1 shows a predominance of a fast moving protein (Fig. 3b). Separate anal-

TABLE I

SULPHYDRYL CONTENT OF *M. lysodeikticus* DEPLETED MEMBRANE

The depleted membrane (5 mg protein per ml) was solubilized in 33 mM acetate buffer (pH 4.6) containing 0.5 M urea and 2.5 % dodecyl sulphate. SH content was estimated as indicated in MATERIALS AND METHODS.

Preparation	*SH content (moles/10^5 g of proteins)*
Depleted membrane	25*
Depleted membrane reduced with $Na_2S_2O_4$	45
Depleted membrane reduced and kept 1 week at 4°.	29

* Average of three preparations.

yses of fractions S_2 and I (Figs. 3c and 3d) reveal that both fractions possess a similar pattern of protein bands. Based on these results, subsequent work was done with the pooled fractions. Since the predominant protein band of fraction S_1 is also seen in the other fraction, it has not been studied further. The densitometric tracings of the electrophoretograms of these fractions are shown in Fig. 4. Nine to eleven bands are seen in the tracings of the solubilized membrane (Fig. 4a), and fractions S_2 and I (Figs. 4b and 4c). Their mobilities were calculated relative to the mobility of the fast moving band. The total protein content of each peak was estimated by weighing the

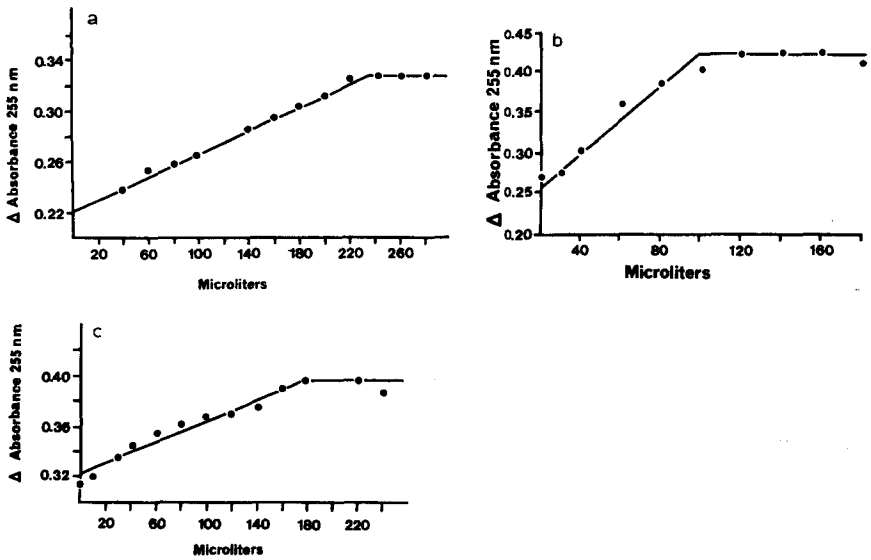


Fig. 2. Titration of SH groups in the depleted membrane of *M. lysodeikticus*. (a) Aliquots of a membrane solution containing 5.1 mg protein per ml of 2.5% dodecyl sulphate buffer were added to 3 ml of $8.5 \cdot 10^{-5}$ M *p*-chloromercuribenzoate solution. (b) The same protein solution as in (a) but reduced with dithionite was added to 3 ml of $7.7 \cdot 10^{-5}$ M *p*-chloromercuribenzoate solution. (c) The reduced membrane solution was kept at 4° for 1 week and added to 3 ml of $9.03 \cdot 10^{-5}$ M solution of *p*-chloromercuribenzoate. Points represent the average of duplicate experiments and are corrected for dilution. Readings at 255 nm were taken immediately after addition of samples. Inflection points are at 231, 98 and 180 μl for (a), (b) and (c), respectively.

TABLE II

EFFECT OF IODOACETAMIDE ON THE YIELD OF SOLUBLE PROTEINS FROM *M. lysodeikticus* DEPLETED MEMBRANE

Proteins were determined by the method of LOWRY *et al.*[14]. S_1, S_2, and I were obtained as indicated in Fig. 1.

Added iodoacetamide (mg/mg of proteins)	*Depleted membrane proteins (mg)*	*Fractions S_1 proteins*		*Fractions $S_2 + I$ proteins*		*Total recovery*	
		mg	%	*mg*	%	*mg*	%
None	18.3	0.8	4.3	1.2	6.5	2.0	10.8
12	12.7	3.4	26.7	10.0	78.7	13.4	105.4
5	14.4	3.2	22.2	9.0	62.5	12.2	84.7

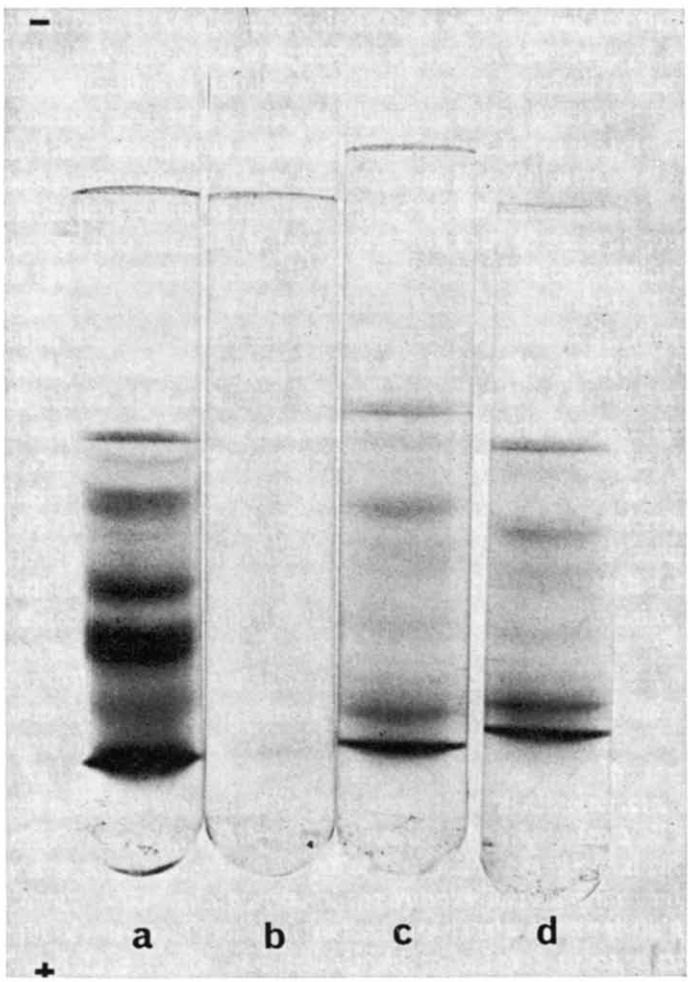
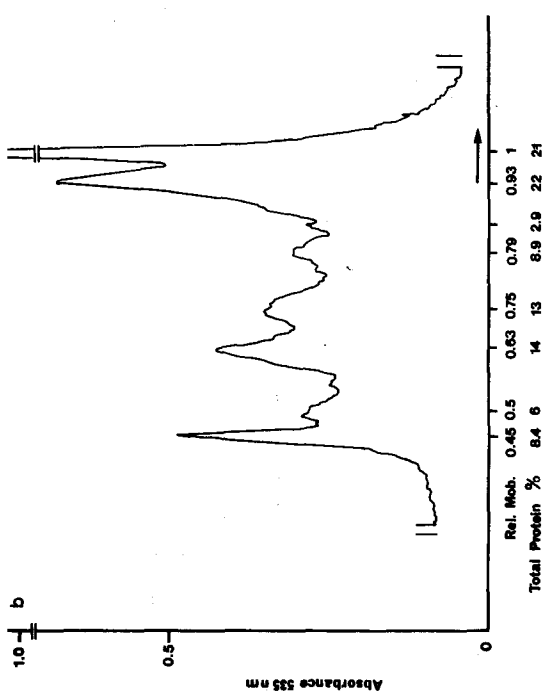
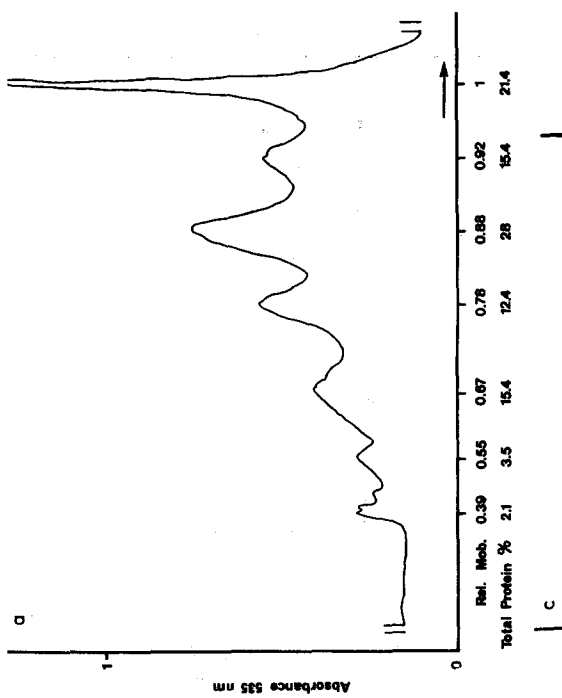


Fig. 3. Polyacrylamide electrophoresis of the depleted membrane of *M. lysodeikticus* and of the fractions obtained after membrane extraction with *n*-butanol. Membrane solution was prepared as described in the text. (a) The solubilized membrane before extraction (1.25 mg protein per ml). (b) S_2 fraction (0.13 mg protein per ml). (c) and (d) represent S_2 and I fractions at a protein concentration of 0.52 mg/ml.

respective cut cut area. Due to the limitation of the method and the close proximity of the bands, these values are given tentatively. No protein content higher than 30 % was observed.

Gel filtration was used to separate individual bands and the results are shown in Fig. 5. The elution profile does not indicate a good resolution. However, the electrophoretic analysis of individual fractions shows a good fractionation into slow, intermediary and fast moving components (see insert of Fig. 5). Retardation of the eluted fractions is consistent with their electrophoretic migration. Similar results in human erythrocyte membrane have been reported[26]. The last peak detected in the chromato-





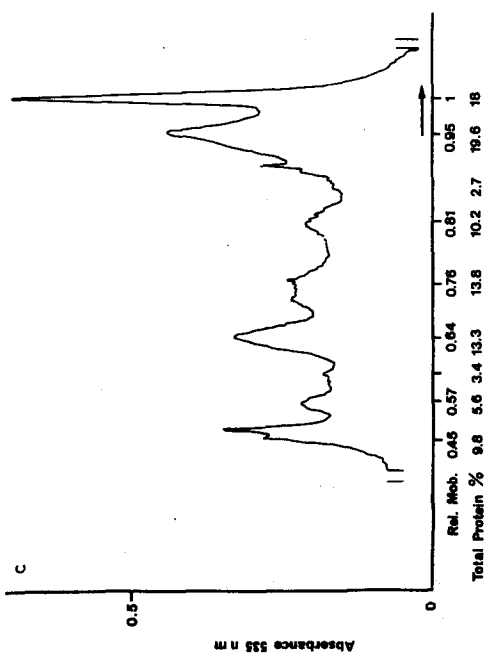


Fig. 4. Densitometric tracings of the electrophoretograms. After electrophoresis and protein staining, the gels were scanned as described in the text. (a) The membrane before *n*-butanol extraction (see (a) in Fig. 3). (b) and (c) show the electrophoretic profile of S_2 and I fractions (see (c) and (d) in Fig. 3).

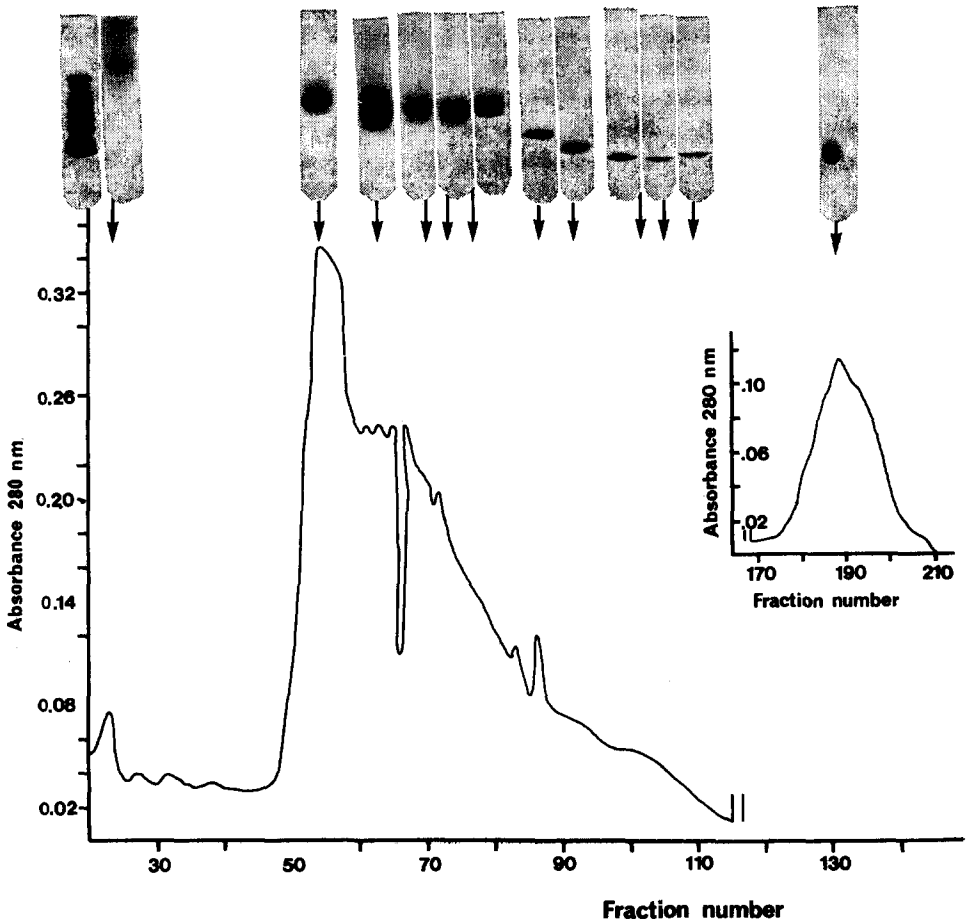


Fig. 5. Sephadex G-200 gel filtration of combined S_2 and I fractions. Proteins (11 mg) were dialyzed against 50 mM NH_4HCO_3 containing 5 mM EDTA and 1 % dodecyl sulphate before being applied to the column (2.5 cm × 70 cm). Elution was carried out with the same buffer. Fractions of 2.5 ml collected with a Beckman fraction collector. Protein elution was followed by measuring the absorbance at 280 nm. The electrophoretic migration of individual fractions is shown at the positions indicated by the arrows. The insert represents the last eluted peak and its toluidine staining after gel electrophoresis.

gram (insert of Fig. 5) did not show protein staining but was identified by the toluidine reaction. Moreover, it had an absorbance ratio $A_{260\text{ nm}}/A_{280\text{ nm}} = 1.42$.

The results for identification of non-protein components in the lipid-extracted depleted membrane of *M. lysodeikticus* are illustrated in Fig. 6. The protein pattern is shown in Fig. 6a. Two bands can be seen in the toluidine blue-stained material, one close to the origin and a broad and diffuse band nearer to the end of the gel, neither of them apparently correlate with protein bands. (Fig. 6b). The location of lipid component (Fig. 6c) indicates that only one band can be identified. Two bands are detected by the periodic–Schiff reaction (Fig. 6d), one as a weak band, and the other as a broad and diffuse band which most likely corresponds to the spread out band stained with the toluidine. When higher concentrations of protein were used, a slight protein stain was

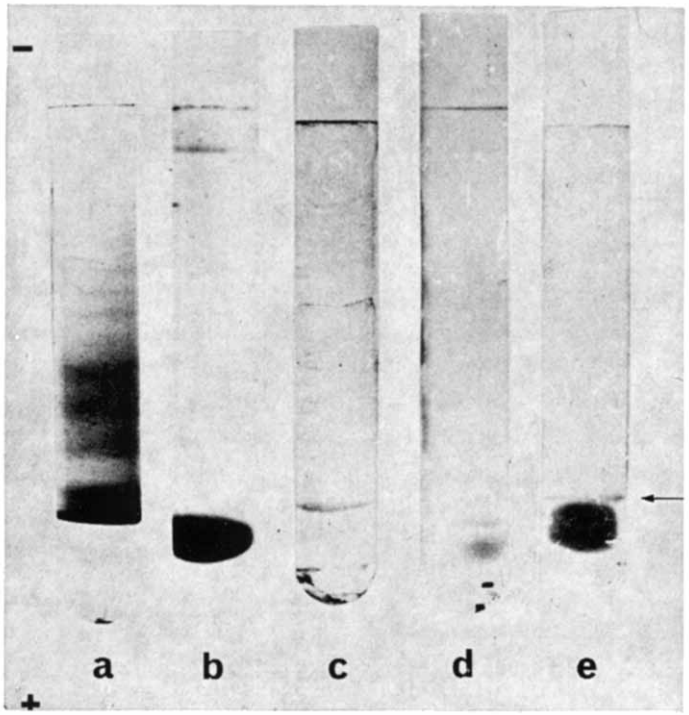


Fig. 6. Identification of membrane components after polyacrylamide electrophoresis. (a) Amido black stain. (b) Toluidine blue reaction. (c) Stain with Sudan III. (d) Periodic acid–Schiff stain. (e) Nitroso-R salt stain. (For details see MATERIALS AND METHODS.)

also seen in this region. The failure to clearly identify proteins in the zone beneath the sharp faster band could be attributed to several reasons; (i) the amido black may be a poor stain for very acidic proteins, (ii) the fast migrating material in the gels is a glycopeptide bearing a short peptide chain with low affinity for the protein dye, (iii) the material is mainly of a nucleotidic nature since it is also stained with toluidine blue (see above). The iron-containing band (indicated with an arrow in Fig. 6e) is also located in the lower part of the electrophoretogram. The colourless area seen in the picture beneath the indicated band is not nitroso-R salt stain but a high density area.

All these chemical reactions gave positive results in the lower part of the electrophoretograms where the precision of fractionation with 7% acrylamide is poor. To gain some insight into the complexity of this material, preliminary experiments were carried out on fractions from the gel filtration. They indicated that the iron-containing protein was present in Fraction 83 but not after it, whereas lipids were detected in Fractions 83–106. Carbohydrates were detected in Fractions 86–99 and also in concentrated pools of subsequent fractions. The toluidine reaction gave the same two bands previously observed in the unfractionated sample in Fractions 83–106. However, a concentrated pool of late fractions (184–196) gave a very strong toluidine reaction with only the fast moving component, whereas it stained lightly for proteins and periodic acid–oxidized carbohydrates. These results suggest that the depleted membrane of *M. lysodeikticus* contained a firmly bound material that we tentatively refer as ribonucleic acid. We base our assumption on its absorbance ratio A 260 nm/A 280

nm (see above) and on its staining properties described previously. We are aware that these staining procedures are not very specific since other ionic polyelectrolytes would also give those reactions. However, we do not think that many other polymers would show the afore mentioned spectrophotometric properties. Moreover, current investigation in our laboratory has revealed the presence of ribose not only in the depleted membrane but in S and I fractions after selective fractionation (manuscript in preparation). Therefore, we think that the material staining with toluidine is probably ribonucleic acid. The band with slow migration might be high molecular weight RNA while the faster band which also gave a positive reaction with decolourated fuchsin could be related to low molecular weight ribonucleic acids (*i.e.* tRNAs). Since tRNAs have free hydroxyl groups in the ribose moiety of the last nucleotides, one would expect that they gave a strong reaction with toluidine and a faint one with fuchsin after periodic oxidation; the second reaction being dependent on the concentration of end groups. The glycolipoprotein eluted as a single peak in the 86–99 fractions seems to be contaminated by the low molecular weight RNA materials whose peak appears

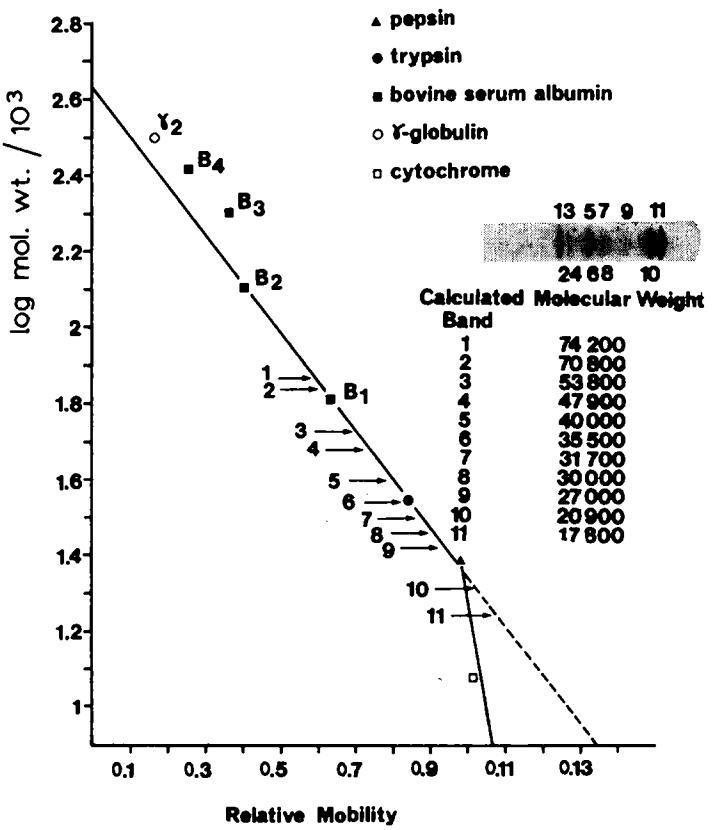


Fig. 7. Plot of logarithms of the molecular weight of a series of proteins *versus* their relative mobility on 7% polyacrylamide gels containing 0.1% dodecyl sulphate. Arrows indicate the relative mobility of the protein bands of S_2 + I fractions (see the text). Inserts show the electrophoretogram and the calculated values of molecular weights.

later on in the column. This last peak is still contaminated with proteins judging from protein staining of the electrophoretograms of the concentrated pool fractions.

The standard curve of molecular weight *versus* mobility under our experimental conditions is represented in Fig. 7. The mobility of each standard-sample is relative to the bromophenol blue used as tracer. The apparent molecular weight of each protein band of the S_2 + I-pooled fractions was determined by plotting the respective relative mobility in the standard curve. The poor resolution of 7 % acrylamide gel for proteins with molecular weight ranges between cytochrome *c* and trypsin, makes the estimation for Bands 10 and 11 only tentative. The calculated molecular weights are inserted in Fig. 7. It is worth noting that the standard proteins were run without alkylation whereas membrane proteins were pre-treated with iodoacetamide. Since alkylation does not introduce charge differences in the proteins, it is very unlikely that this different treatment would affect the molecular weight estimation.

DISCUSSION

Selective treatments for the detachment of some proteins of *M. lysodeikticus* membranes resulted in a smooth-surfaced residue[11]. It was interesting to learn about the compostion of that residual structure. We described a method for the solubilization of these membranous structures. The method is based on the action of sodium dodecyl sulphate on membrane components. The effect of dodecyl sulphate in proteins and lipoproteins is under current investigation in several laboratories[27–29]. Recently, SIMON AND HELENIUS[30] have reported that dodecyl sulphate separates lipids and proteins from light density plasma proteins by gel filtration. Gel filtrates of erythrocyte membranes dialyzed against mercaptoethanol and dissolved with dodecyl sulphate also separate lipids and proteins[26]. Similar results were reported for bacterial membranes by use of detergents[31, 32]. We extracted the lipid components with butanol before attempting the separation of proteins by gel chromatography. In our initial experiments, we observed that the proteins aggregated in insoluble residues[33] after *n*-butanol treatment, but could be redissolved partially by treatment with mercaptoethanol–dodecyl sulphate buffer. This indicated that at least some of the aggregate formation was due to the oxidation of thiol groups in our experimental conditions. Since thiol groups were effectively titrated in the depleted membrane, iodoacetamide was added as alkylating agent. Alkylation of thiol groups is usually conducted at alkaline pH[34, 35]. In short reaction times and at slightly acid or neutral pH, histidine residues are also alkylated[36] whereas methionine is alkylated at acid or neutral pH in long reaction times[37]. Since the addition of iodoacetamide in our experimental conditions (pH 4.6–5.2) avoided the formation of insoluble aggregates, our results suggest that alkylation did occur and that the dissociation constant of thiol groups was probably low[38, 39]. Identical procedure applied in this laboratory for the solubilization of proteins of Streptomyces membrane at alkaline pH also improved the yield of soluble proteins[40]. It must be noted that the aggregation of proteins did occur readily when lipids were removed. It is not clear at the present time what reasons are responsible for this fact.

We have detected only one band of iron containing protein. This too simple result may be due to the poor resolution of the fractionation methods. Despite the drastic treatment to remove lipids, lipid material could still be detected in the solubiliz-

ed membrane. Moreover, the dodecyl sulphate appeared to be unable to break the association between the lipid and some protein and carbohydrate components even after Sephadex filtration, suggesting, therefore, the existence of a covalent bond. It is noteworthy that the ribonucleic acid material unlike the residual lipid in the "depleted" membrane after lipid extraction was eluted separately from proteins. The significance of this RNA associated with the membrane can not be discussed at the present time, although it is interesting in connection with the recent review by SHAPOT AND DAVIDOVA on the existence of liporibonucleoprotein complexes as an integral part of animal cell membranes[41].

Electrophoretic analyses were conducted in the absence of mercaptoethanol and without pre-heating the samples. We are aware that the maximal dodecyl sulphate binding capacity of the proteins might be restricted by these mild conditions (*e.g.* presence of previously existing dithiol bridges)[42]. Consequently the degree of dissociation of polymers and the unfolding of proteins might introduce errors in the molecular weight estimation[43]. In any case, the results presented in this paper show that there is no protein component among those contributing to the intrinsic organization of this bacterial membrane with a molecular weight higher than 75000. This is interesting in relationship to the results reported by ROSENBERG AND GUIDOTTI[44], GWYNNE AND TANFORD[45], LENARD[26] on the erythrocyte membrane. Since our fractionation procedures are based on a molecular weight basis, a more detailed characterization of each individual component is needed to determine if each one is a protein species or a mixture of different polypeptide chains. Amino acid analyses of the alkylated proteins will indicate which amino acids are responsible for the solubilization reported here.

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REFERENCES

1 M. R. J. SALTON, in F. SNELL, J. WOLKEN, G. J. IVERSON AND J. LAM, *Physical Principles of Biological Membranes*, Gordon and Breach Science Publishers, New York, 1970, p. 259.
2 M. R. J. SALTON AND J. H. FREER, *Biochim. Biophys. Acta*, 107 (1965) 531.
3 M. S. NACHBAR AND M. R. J. SALTON, in M. BLANK, *Surface Chemistry of Biological Fluids*, Plenum Press, New York, 1970, p. 175.
4 M. G. MACFARLANE, *Biochem. J.*, 80 (1961) 45 P.
5 M. R. J. SALTON AND M. SCHMITT, *Biochim. Biophys. Acta*, 135 (1967) 196.
6 K. Y. CHO AND M. R. J. SALTON, *Biochim. Biophys. Acta*, 116 (1966) 73.
7 E. KODICEK, *Recent Progress in Microbiology, Intern. 8th Congr. Microbiol. Montreal, 1962*, University of Toronto Press, Montreal, 1965, p. 23.
8 M. R. J. SALTON AND A. F. M. EHTISHAM-UD-DIN, *Aust. J. Exp. Biol. Med. Sci.*, 43 (1965) 255.
9 E. MUÑOZ, M. R. J. SALTON AND D. J. ELLAR, in J. R. VILLANUEVA AND F. PONZ, *Membranes: Structure and Function, FEBS Proc. of the 6th Meeting, Madrid, 1969*, Academic Press, London and New York, 1970, p. 51.
10 E. MUÑOZ, M. S. NACHBAR, M. T. SCHOR AND M. R. J. SALTON, *Biochem. Biophys. Res. Commun.*, 32 (1968) 539.
11 D. J. ELLAR, E. MUÑOZ AND M. R. J. SALTON, *Biochim. Biophys. Acta*, 225 (1971) 140.
12 M. R. J. SALTON, J. H. FREER AND D. J. ELLAR, *Biochem. Biophys. Res. Commun.*, 33 (1968) 909.

13 E. MUÑOZ, M. R. J. SALTON, M. H. NG AND M. T. SCHOR, *Eur. J. Biochem.*, 7 (1969) 490.
14 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
15 D. E. GREEN, H. D. TISDALE, R. S. CRIDDLE, P. Y. CHEN AND R. M. BOCK, *Biochem. Biophys. Res. Commun.*, 5 (1961) 109.
16 R. K. MORTON, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1955, p. 25.
17 P. D. BOYER, *J. Am. Chem. Soc.*, 76 (1954) 4331.
18 E. DE VITO AND J. A. SANTOME, *Experientia*, 22 (1966) 124.
19 J. W. KEYSER, *Anal. Biochem.*, 9 (1964) 249.
20 G. H. BEATON, A. E. SILBY AND A. M. WRIGHT, *J. Biol. Chem.*, 236 (1961) 2001.
21 J. T. CLARKE, *Ann. N.Y. Acad. Sci.*, 121 (1964) 428.
22 K. A. NARAYAN AND F. A. KUMMERON, *Clin. Chim. Acta*, 13 (1966) 428.
23 O. SMITHIES, in C. B. ANFINSEN, JR., M. L. ANSON, K. BAILEY, J. T. EDSALL, *Advances in Protein Chemistry, Vol.* 14, Academic Press, New York, 1959, p. 65.
24 A. L. SHAPIRO, E. VIÑUELA AND J. V. MAIZEL, *Biochem. Biophys. Res. Commun.*, 28 (1967) 815.
25 A. K. DUNKER AND R. R. RUECKERT, *J. Biol. Chem.*, 244 (1969) 5074.
26 J. LENARD, *Biochemistry*, 9 (1970) 1129.
27 C. TANFORD, in C. B. ANFINSEN, JR., M. L. ANSON, J. T. EDSALL, F. M. RICHARDS, *Advances in Protein Chemistry*, Vol. 23, Academic Press, New York and London, 1968, p. 122.
28 J. A. REYNOLDS AND C. TANFORD, *Proc. Natl. Acad. Sci. U.S.*, 66 (1970) 1002.
29 B. JIRGENSONS AND S. CAPETILLO, *Biochim. Biophys. Acta*, 214 (1970) 1.
30 K. SIMONS AND A. HELENIUS, *Febs. Lett.*, 7 (1970) 59.
31 M. R. J. SALTON AND M. D. SCHMITT, *Biochem. Biophys. Res. Commun.*, 27 (1967) 529.
32 T. H. JONES AND E. KENNEDY, *J. Biol., Chem.*, 244 (1969) 598.
33 C. HUGGINS, D. F. TAPLEY AND E. V. JENSEN, *Nature*, 167 (1951) 592.
34 R. CECIL AND J. R. MCPHEE, in C. B. ANFINSEN, JR., M. L. ANSON, K. BAILEY, AND J. T. EDSALL, *Advances in Protein Chemistry*, Vol. 14, Academic Press, New York, 1959, p. 255.
35 R. CECIL, in H. NEURATH, *The Proteins*, Vol. 1, Academic Press, New York and London, 2nd ed., 1963, p. 379.
36 H. G. GUNDLACH, W. H. STEIN AND S. MOORE, *J. Biol. Chem.*, 234 (1959) 1754.
37 H. G. GUNDLACH, S. MOORE AND W. H. STEIN., *J. Biol. Chem.*, 234 (1959) 1761.
38 H. LINDLEY, *Biochem. J.*, 74 (1960) 577.
39 H. LINDLEY, *Biochem. J.*, 82 (1962) 418.
40 V. LARRAGA AND S. F. ESTRUGO, *Proc. 5° Congr. Nacional de Bioquímica, Barcelona, 1971*, Abstract No. 39.
41 V. S. SHAPOT AND S. YA. DAVIDOVA, in J. N. DAVIDSON AND W. E. COHN, *Progress in Nucleic Acid Research and Molecular Biology*, Vol. 11, Academic Press, New York and London, 1971, p. 81.
42 R. PITT-RIVERS AND F. S. A. IMPIOMBATO, *Biochem. J.*, 109 (1968) 825.
43 A. L. SHAPIRO AND J. V. MAIZEL, *Anal. Biochem.*, 29 (1969) 505.
44 S. A. ROSENBERG AND G. GUIDOTTI, *J. Biol. Chem.*, 244 (1969) 5118.
45 J. T. GWYNNE AND C. TANFORD, *J. Biol. Chem.*, 245 (1970) 3269.