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MOLECULAR ORGANIZATION IN BACTERIAL CELL MEMBRANES

II. REEVALUATION AND IDENTIFICATION OF SOME CHEMICAL COMPONENTS OF *MICROCOCCUS LYSODEIKTICUS* MEMBRANES

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SUMMARY

Standard membranes of *Micrococcus lysodeikticus* were prepared by protoplast lysis in the presence of 50 mM Mg^{2+} and by repeated washings in the absence of this cation. Different washing procedures with EDTA–30 mM Tris (pH 7.5) and low-ionic-strength Tris buffers (pH 7.5) yielded three distinct depleted membranes. RNA and carbohydrates were reevaluated in all these membranes after extraction and/or partial fractionation of the membrane complexes. Values higher than 10% of membrane dry weight have been found for both components in the standard membranes. Figures between 6–12% for carbohydrates and 0.8–16% for RNA were found in the three depleted membranes. The protein: lipid ratio of depleted membranes is lower than that of the standard membrane.

The existence of RNA has been confirmed by polyacrylamide gel electrophoresis and by sensitivity to ribonuclease (EC 2.7.7.16). Different patterns of proteins, RNAs and carbohydrate-containing material were revealed by gel electrophoresis in sodium dodecyl sulphate of each type of *M. lysodeikticus* membrane. These results confirm and extend previous findings obtained with a depleted membrane of *M. lysodeikticus* (Estrugo, S. F., Larraga, V., Corrales, M. A., Duch, C. and Muñoz, E. (1972) *Biochim. Biophys. Acta* 255, 960–973). They suggest the existence of specific interactions between membrane components which are broken down and/or altered by different membrane treatments. They also suggest the likely significance in bacterial membranes of components other than lipids and proteins.

INTRODUCTION

The data so far available on the chemical composition of isolated bacterial membranes show very low contents of RNA and carbohydrate material provided that the membranes have been washed in the absence of divalent cations (for a review see ref. 1). Few exceptions have been found to this rule. In previous work, we isolated a standard membrane from *Micrococcus lysodeikticus* by osmotic lysis[2]. Nachbar and Salton[3] analyzed the chemical composition of this standard membrane. A depleted membrane was obtained by selective wash treatments of the standard membrane[2,4–6]. A complete

chemical analysis of this depleted membrane has not been reported. Data on its protein and lipid contents indicated an enrichment in lipid constituents[4].

In a previous study on the solubilization and fractionation of the components of *M. lysodeikticus* depleted membrane[6], we detected the presence of RNA-like material and glycoproteins. The present report describes the detailed evaluation and the partial identification of the chemical constituents of various types of *M. lysodeikticus* membranes, including the standard and three different types of depleted membranes.

MATERIALS AND METHODS

Reagents

Yeast RNA (lot No. 70 1137818) was purchased from Merck. Ribonuclease (RNAase) (EC 2.7.7.16) lot No. 12509, and deoxyribonuclease (DNAase) (EC 3.1.4.5), lot No. 1141818, were obtained from Koch and Light, and BDH Chemicals, respectively. Mannose, orcinol, L-cysteine·HCl and ribose were also from BDH Chemicals. $HClO_4$ and trichloroacetic acid were purchased from Merck. All other reagents were of analytical grade. Organic solvents such as acetone, chloroform and methanol were used without redistillation. The buffers and materials used besides those mentioned above were as previously described[6].

Preparation of membranes

Micrococcus lysodeikticus (NCTC 2665) was grown and harvested as described[2,7]. Cytoplasmic membranes were isolated by osmotic lysis of preformed protoplasts[2] in the presence of 50 mM $MgCl_2$ (ref. 8). Standard membrane and depleted membrane R1 were obtained, respectively, by washing the cytoplasmic membrane 4–6 times with 30 mM–100 mM Tris–HCl buffers (pH 7.5), or 3–4 times with 1 mM EDTA–30 mM Tris *plus* 2–3 times with 3 mM Tris (pH 7.5), as previously reported[2,6,8].

Two other types of depleted or residual membranes (membranes freed of detachable components by mild washing procedures) were prepared as follows: depleted membrane R10 was obtained as depleted membrane R1, except that 10 mM EDTA was used in the first 3–4 washes; depleted membrane RS10 was obtained from the standard membrane by three subsequent washes with 10 mM EDTA–30 mM Tris (pH 7.5), followed of two "shock washes" with 3 mM Tris (pH 7.5).

Sediments were always collected by centrifugation in a Sorvall RC2-B centrifuge (30 min at $27000 \times g$). Membranes were dialyzed against distilled water for 24 h, and lyophilized.

Determination of proteins, lipids, RNA and hexoses

Protein content was determined by the method of Lowry *et al.*[9] after dissolving the membranes in 0.1 M NaOH.

Lipid content of the bacterial membranes was determined by the method of Folch *et al.*[10] as modified by Salton and Schmitt[11]. Lyophilized membranes (50 mg) were extracted first with hot methanol (37 °C) several times, followed by chloroform–methanol (2:1, v/v) extraction. After centrifugation, the residue was dried under vacuum until constant weight (delipidated residue). The organic phases were pooled

and evaporated to dryness. They were then taken up with chloroform–methanol (2:1, v/v) and left standing overnight. The residue that appeared was separated by decantation. Part of this residue was solubilized with water (water-soluble material). The water-insoluble fraction was not further analyzed. The chloroform–methanol phase was washed according to Folch *et al.*[10]. The washing fluids gave an aqueous upper phase. The lower phase was evaporated to dryness under vacuum to determine the lipids by weighing. Corrections for dry weight contaminants of the solvents were made by evaporating equal volumes of solvents treated as the samples.

Carbohydrates were estimated in the delipidated residue, in the aqueous upper phase obtained from the Folch's washing procedure and in the water-soluble material obtained as indicated above. The determination was carried out with the H_2SO_4–cysteine reaction[12]. The results are expressed using mannose as standard.

To determine nucleic acids the membranes (10–20 mg) were extracted as follows: (1) following the method of Ogur and Rosen[13]; (2) according to the procedure of Ghosh and Murray[14], except that lipids were extracted with methanol and acetone–methanol (7:2, v/v)[11]; (3) lipids were removed by extraction with methanol and chloroform–methanol[10], and then nucleic acids were extracted as in 1; (4) lipids were first removed by reflux with 95% methanol for 4 h, the sediment was further extracted with chloroform–methanol, and then nucleic acids were extracted as indicated in 1.

The concentration of RNA was estimated by the orcinol reaction[15] using as standards yeast RNA and ribose, which, in the experimental conditions, gave the following relationship:

$$\frac{\text{(mg/absorbance) RNA}}{\text{(mg/absorbance)ribose}} = 5.2 \text{ (average of 10 determinations).}$$

Deoxyribonucleic acids were assayed by the diphenylamine reaction[15] and gave negative results in all membrane preparations.

Polyacrylamide gel electrophoresis

The electrophoresis procedures were published in detail previously[6]. Membrane preparations were dissolved for electrophoresis in buffers containing 1% sodium dodecyl sulphate. Electrophoresis for the identification of RNA was carried out in 10% acrylamide gels, prepared as indicated by De Wachter and Fiers[17] but in Tris–glycine–0.1% dodecyl sulphate buffers. RNA in the electrophoretograms of *M. lysodeikticus* membranes was visualized by staining with toluidine blue. To confirm that the toluidine-positive materials were RNA, the different membrane preparations were also treated with RNAase prior to solubilization with dodecyl sulphate. Aliquots of each membrane preparation (approx. 2 mg dry wt) were suspended in 1 ml of 0.2 M Tris buffer (pH 7.5) containing $2 \cdot 10^{-3}$ M EDTA and RNAase (0.1 ml of a solution at 5 mg/ml). The suspensions were incubated for 18 h at 4 °C. Sodium dodecyl sulphate was then added to obtain clear solutions that were applied to the gels for electrophoresis.

Electron microscopy

Membranes were examined in a Philips EM300 electron microscope after negative staining with 2% phosphotungstic acid neutralized (pH 7.5) with NaOH.

RESULTS

The RNA content of different types of *M. lysodeikticus* membrane preparations using extraction Methods 1–4 is illustrated in Table I. The concentration of RNA estimated after Methods 1 and 2 gave in nearly all cases the highest value. Direct extraction of *M. lysodeikticus* standard membranes with 0.5 M $HClO_4$ gave identical figures as those obtained after extracting lipids with reflux methanol. It is worth noting that when lipids were removed with methanol and chloroform–methanol without reflux (Method 3) we obtained the lowest value.

TABLE I

RNA CONTENTS IN *M. LYSODEIKTICUS* MEMBRANES

Membranes were extracted according to Methods 1–4 as described under Materials and Methods. RNA concentrations were calculated from the orcinol reaction (see also Materials and Methods) and are expressed as % of membrane dry weight. Results are the mean values of different preparations as indicated by the figures between parentheses. The different preparations of each type of membrane gave deviations of the mean not higher than 15%. Variation in duplicate experiments of a given preparation was 10%.

Material	*Method of membrane extraction*			
	1	*2*	*3*	*4*
Standard membrane	15.4(3)	17.7(2)	11.8(3)	15.6(1)
Depleted membrane RS10	0.8(2)	2.1(2)	—	—
Depleted membrane R1	11.1(2)	8.2(1)	—	—
Depleted membrane R10	16.4(2)	12.5(1)	—	—

The visualization of RNA bands after polyacrylamide gel electrophoresis is shown in Fig. 1. The results indicate the presence of slowly migrating bands that disappear as an effect of preincubation of the membranes with RNAase. This disappearance is paralleled by an increase in the toluidine-positive material of faster mobility. It is interesting to note the presence of slowly migrating material sensitive to RNAase in all membrane preparations. The electrophoretic migration of RNA material suggests that they are from various types and, probably, from different origins (*i.e.* ribosomal, messenger, and/or transfer RNA). We previously suggested the presence of tRNAs in a butanol-extracted depleted membrane of Type R1 (ref. 6). In the present work we have not attempted to identify the nature and function of the several species of RNA, actually found in all types of membrane. But, these results provide a good confirmation of the existence of RNA material in the membrane preparations irrespective of the degree of cation depletion reached by washing treatments.

The electrophoretic patterns of proteins and carbohydrate-containing proteins are illustrated in Fig. 2. Some slowly migrating proteins present in the standard membrane are missing in all the other preparations, particularly in depleted membranes R10 (Gel c) and RS10 (Gel g). The glycoprotein constituents behave in the same manner. Several bands (minimum 5) are detected in the standard membrane, whereas only three rapidly moving components are seen in the other preparations.

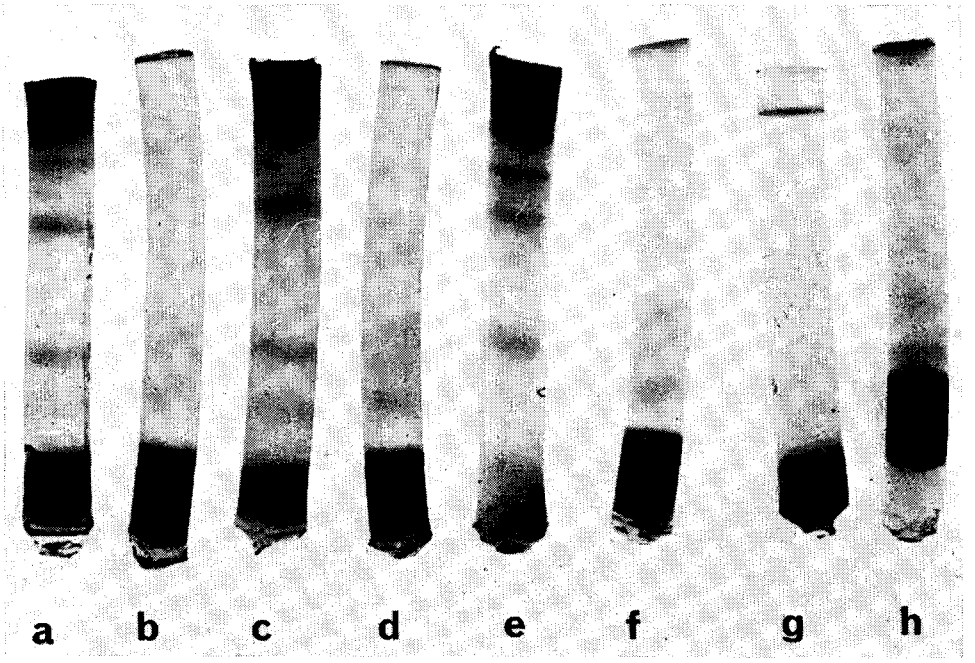

Fig. 1. Identification of RNA in *M. lysodeikticus* membranes by polyacrylamide electrophoresis in sodium dodecyl sulphate. Samples were treated as indicated in Materials and Methods. Their concentrations are given in dry weight. The electrophoreses were run in Tris–glycine–0.1% dodecyl sulphate (see the text). (a) 0.300 mg standard membrane; (c) 0.460 mg depleted membrane R10; (e) 0.240 mg depleted membrane R1; (g) 0.310 mg depleted membrane RS10. Without RNAase treatment. (b) 0.320 mg standard membrane; (d) 0.280 mg depleted membrane R10; (f) 0.200 mg depleted membrane R1; (h) 0.280 mg depleted membrane RS10. After preincubation with RNAase.

It is worthy to note that preparations of depleted membrane R1 (Gel e) show a similar pattern to that of depleted membranes of *M. lysodeikticus* previously reported[6]. However, certain differences are found between these preparations and a depleted membrane as examined before by Muñoz *et al.*[2]. The dissimilarities could be explained by the different treatments or the samples: (a), preheating overnight at 37 °C in sodium dodecyl sulphate or (b), immediate solubilization with dodecyl sulphate[6]. It is nevertheless interesting to point out that each type of depleted membrane possesses a distinct protein profile, as judged from, at least, the upper part of the gels (compare Gels a, c, e and g in Fig. 2).

Table II illustrates the percentages of protein, lipid, carbohydrate and RNA in the membrane preparations. The figures indicate that all types of membrane are complexes of these components in different proportions. A puzzling question is that the sum of the percentages of the constituents in depleted membranes R10 and RS10 accounts for only 57 and 56% of their dry weight, respectively. While in standard and depleted membranes R1, these sums amount to 85 and 80% of their respective dry weights. There is, as yet no, explanation for these facts. One could argue the likely enrichment in components that were less sensitive or not accounted for by the analytical procedures used by us. In fact, the presence of teichoic acid in our membrane preparations is a possibility, as recently suggested by Hughes *et al.*[17].

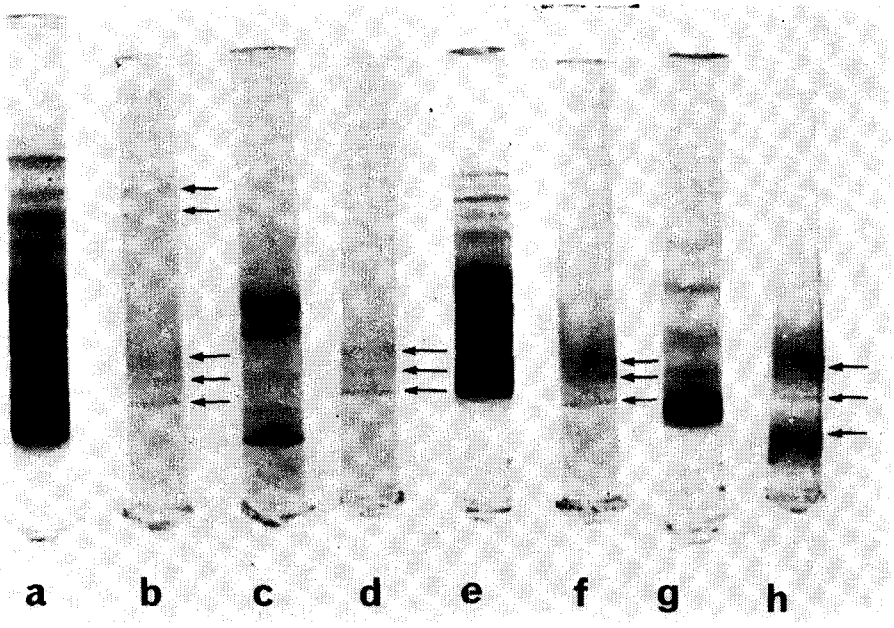

Fig. 2. Identification of proteins and glycoproteins of *M. lysodeikticus* membranes by polyacrylamide electrophoresis in sodium dodecyl sulphate. Sample concentrations are given in dry weight. (a) 0.210 mg standard membrane; (c) 0.400 mg depleted membrane R10; (e) 0.240 mg depleted membrane R1; (g) 0.310 mg depleted membrane RS10. All stained with Amido Black. (b) 0.420 mg standard membrane; (d) 0.400 mg depleted membrane R10; (f) 0.240 mg depleted membrane R1; (h) 0.620 mg depleted membrane RS10. All stained with the periodic acid–Schiff reaction. The positive-Schiff material is indicated by arrows to make its detection easier, but this does not imply its identity from gel to gel.

TABLE II

CHEMICAL COMPOSITION OF DIFFERENT TYPES OF *M. LYSODEIKTICUS* MEMBRANE

Details of protein, lipid and carbohydrate estimations are given in Materials and Methods. RNA values were obtained from the orcinol reaction after extraction according to Procedure 1 (see the text). Figures between parentheses indicate the number of preparations of each type of membrane. Results are the mean values of these different preparations. Deviations were not larger than 26% for lipids, proteins and carbohydrates, and 15% for RNA. Duplicate experiments with a given preparation gave 8% variation for lipids and proteins and 10% for carbohydrates and RNA. Results are expressed as % dry weight.

Material	*Proteins*	*Lipids*	*Carbohydrates**	*RNA*
Standard membrane	40.2(3)	18.3(4)	11.9(2)	15.4(3)
Depleted membrane RS10	26.5(3)	15.3(3)	12.0(2)	0.8(2)
Depleted membrane R1	32.5(2)	30.5(2)	5.5(2)	11.1(2)
Depleted membrane R10	21.4(1)	14.1(2)	6.0(2)	16.4(2)

* Similar values of carbohydrate contents have been obtained with the anthrone reaction[15].

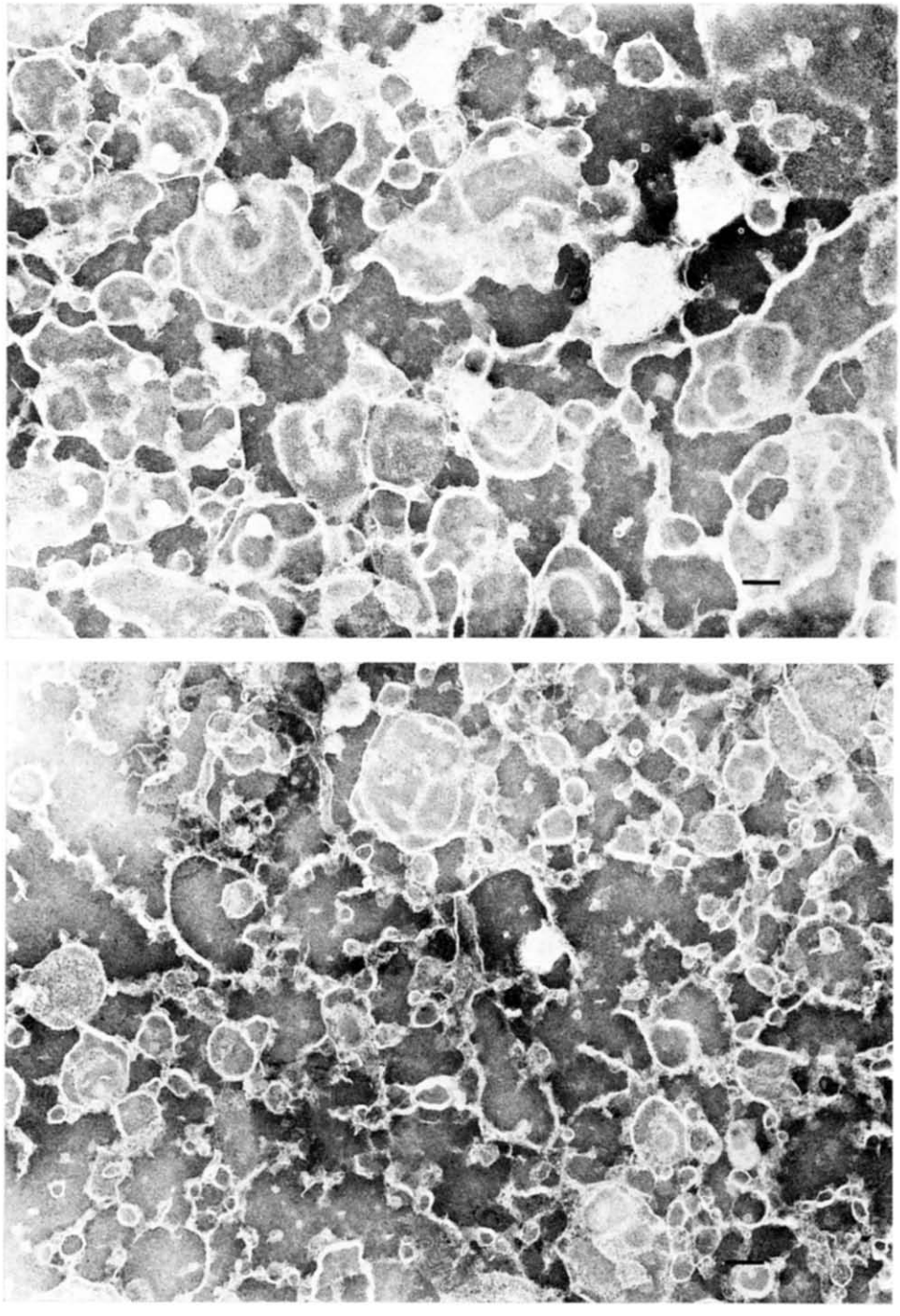

Fig. 3. Electron micrographs of *M. lysodeikticus* membrane preparations. (a) Depleted membrane R10 and (b) depleted membrane RS10, corresponding to 72000 amplifications. Bars represent 0.1 μm.

The carbohydrate contents shown in Table II are compatible with the presence of polysaccharides (*e.g.* mannan[18]) and glycolipids, as well as glycoproteins tentatively identified by us. Our current work has revealed the presence of glucosamine and a complex pattern of sugars. These results cannot be interpreted by the sole presence of a membrane-associated mannan. From the data reported in Table II it is also reasonable to conclude that the washing with EDTA buffers has removed a major proportion of the protein components. In fact, the protein to lipid ratio of the depleted membranes is considerably lower than that of the standard membrane. The data for the depleted membrane RS10 preparation indicate an enrichment in carbohydrate and a loss of RNA. Opposite results are observed for the R1 and R10 depleted membranes. These quantitative results agree with the electrophoretic patterns previously illustrated (see Figs 1 and 2).

The morphology by negative staining of the R10 and RS10 depleted membranes is shown in Figs 3a and 3b. They appear to be formed by vesicles and membrane fragments of smooth aspect and different size. In spite of the different negative staining procedure, the depleted membrane R10 resembles morphologically the depleted membrane obtained by 1 mM EDTA–30 mM Tris and 3 mM Tris–washes as examined before by Ellar *et al.*[5]. It must also be noted that the depleted membrane RS10 preparation consists of smaller vesicles and membrane fragments.

DISCUSSION

We previously suggested the presence of RNA and carbohydrates in a depleted membrane preparation that corresponds to the depleted membrane R1 of this paper. The present work gives additional proof of such a presence, as well as the quantitative evaluation of its content. The amount of RNA in the standard membrane preparations is 5–10 times higher than the values previously reported[3,7]. The discrepancies might be due to the fact that RNA determinations were carried out in different conditions. As a matter of fact, we have found that lipids interfere with the orcinol reaction. When a solution of yeast RNA was added with butanol-extracted lipids from *M. lysodeikticus* membranes, the orcinol reaction gave values 5 times lower than the control. Furthermore, we do not discard the possibility that the figures given in this paper are not the actual content of RNA. Preliminary experiments indicate the presence of ultraviolet-absorbing material (maximum at 260 nm) in the organic phases obtained during extraction.

Carbohydrate contents in *M. lysodeikticus* membrane preparations were previously reported by Gilby *et al.*[19] and by Nachbar and Salton[3]. Our results are intermediate values between those reported. The lower results of this communication with respect to those by Gilby *et al.*[19] may be due to unaccounted losses in our extractions with methanol and chloroform–methanol as compared with ether reflux and methanol[19]. Similar considerations as for RNA may tentatively explain the lower values obtained by other authors.

We do not rule out the possibility that the high content of RNA on our membrane preparations may be the result of an artifact by the presence of Mg^{2+} in the medium of protoplast lysis. However, it has been recently observed that divalent cations and polyamines appear to be physiologically significant for *M. lysodeikticus*[8,20,21]. In this regard, the lysis in absence of Mg^{2+} may have damaged the native

structures and produced losses of some membrane-associated material (RNA and carbohydrates), which might be involved in membrane functions not taken into account before (*e.g.* membrane biogenesis and biosynthesis of recognition sites).

Another interesting fact emerges from the results described herein. Different profiles of membrane components are observed depending upon the method of membrane preparation used. This suggests the existence of specific interactions between membrane components. These interactions may be broken down and/or altered by differences in the methods of membrane isolation by the different washing procedures.

The early membrane models[22,23] suggested that membranes were composed of lipid molecules in a bilayer arrangement. More recent models have been reviewed and evaluated[24]. In all cases, the models were based fundamentally on spatial arrangements of the major components, lipids and proteins. The presence of other molecules in concentrations higher than traces should also be taken into consideration when proposing membrane models.

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