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ANALYSIS OF MEMBRANE FRACTIONS FROM *MYCOPLASMA GALLISEPTICUM*

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Membrane fractions have been isolated from *Mycoplasma gallisepticum* following a procedure derived from that described by Maniloff, J. and Quinlan, D.C. (J. Bacteriol. (1974) 120, 495–501). A light fraction F_I was obtained which contained structures resembling the bleb-infrableb apparatus characteristic of *M. gallisepticum*. It was enriched in DNA and had an electrophoretic profile different from that of unfractionated membranes. Cholesterol-to-phospholipid ratios higher than two and elevated values of the ratio of saturated to unsaturated fatty acids were other characteristics of this fraction. The two other fractions isolated (F_{II} and F_{IV}) also differed from intact membranes by their cholesterol and phospholipid content as well as by their saturation ratios. The membrane fluidity of F_{II} and F_{IV}, estimated by fluorescence polarization, was similar to that of unfractionated membranes while a slight but significant difference was recorded for the light fraction. Possible relationships between the lateral heterogeneity of the *M. gallisepticum* membrane and the obtainment of fractions are discussed.

Introduction

Mycoplasmas are small prokaryotes that do not have cell walls, are unable to synthesize the cholesterol, and for most strains, the long-chain fatty acids necessary for their growth [1–3]. Among the different species the avian pathogen *Mycoplasma gallisepticum* shows unique properties which have focused interest. These cells are pear-shaped with polar bleb-infrableb structures which likely correspond to the site of DNA replication [4–6]. As compared to most other species they are more resistant to osmotic lysis [7]. The cell membrane has an usually high protein content, reaching 80% of the dry weight [8] with, in the native strain, a cholesterol-to-phospholipid ratio equal or higher than one [9–11].

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; β-buffer, 0.15 M NaCl/0.01 M 2-mercaptoethanol/0.05 M Tris-HCl, pH 7.4.

In their studies on DNA replication Maniloff and Quinlan have reported that the polar bleb of *M. gallisepticum* could be separated from the cell by freeze-thaw lysis and sonication [6]. They found that the fraction enriched in blebs was also enriched with newly replicated DNA and adenosine triphosphatase activity.

The present experiments were undertaken to give a more complete analysis of the lipids contained in the various membrane fractions as well as to estimate the physical state of membrane lipids, usually referred to as fluidity, in these fractions. Membrane physical state was estimated by the fluorescence polarization technique using 1,6-diphenyl-1,3,5-hexatriene (DPH) as the fluorescent probe [11,12].

Materials and Methods

Organism and growth conditions

M. gallisepticum strain A5969 was grown as

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previously described [11] in a modified Edward medium containing 0.5% fatty acid poor bovine serum albumin (Miles Laboratories Inc., Kankakee, IL) and 10 μg cholesterol per ml, 5 μl oleic acid per ml, and 5 μg palmitic acid per ml. Labeling of cholesterol in membranes was achieved by adding to each liter of growth medium either 30–60 μCi [^{3}H]cholesterol (15.6 mCi/mmol) or 10–20 μCi [^{14}C]cholesterol (50–60 mCi/mmol). Membrane fatty acids were labeled by adding either 10–20 μCi [^{14}C]oleic acid (59.7 mCi/mmol) or 30–60 μCi [^{3}H]palmitic acid (500 mCi/mmol) per liter of growth medium. Radioactive lipids were from Amersham Corporation, Arlington Heights, IL. Growth was determined by measuring the absorbance of cultures at 640 nm.

Harvesting and washing of cells

3–12 l of mid-log phase cultures were precooled to 2–5°C [5]. The procedure and buffers used for the harvesting, washing of cells, and for the isolation of membrane fractions were, in a first set of experiments, identical to those described by Maniloff and Quinlan [6]. This procedure was then modified as follows: precooled cultures were centrifuged at 11000 × *g* for 15 min at 5°C. Cell pellets were resuspended in a cold 0.25 M NaCl solution and centrifuged at 18000 × *g* for 20 min at 5°C. Washed organisms were resuspended (5–10 mg of cell protein/ml) in β-buffer (0.15 M NaCl/0.01 M 2-mercaptoethanol/0.05 M Tris-HCl, pH 7.4).

Lysis and isolation of fractions

Washed cell suspensions were transferred into siliconized glass bottles and alternatively frozen in liquid nitrogen and thawed at 37°C in a water bath. Three to five freeze-thaw cycles, each followed by a brief sonication in a disintegrator (20 s, 70 W, 20–25°C; Ultrasonics, Plainview, NY), were performed, reducing the absorbance of the suspension by more than 70% [11]. After lysis and sonic treatment the suspension was centrifuged at 5000 × *g* for 5 min and the supernatant obtained was centrifuged for 30 min at 49000 × *g* (5°C). The pellet obtained was resuspended (approx. 10 mg of membrane protein/ml) in diluted (1:20) β-buffer. The suspension was sonically treated in five 1 min bursts at 100–120 W (power) with the temperature maintained between 20 and 25°C. The resultant suspension was layered over a discontinuous gradient constituted of 4 ml of 15%, 4 ml of 20% and 5 ml of each 30, 45, 50, 55, and 60% sucrose (wt./vol) diluted in 1:20 β-buffer. Membranes were centrifuged for 12 h at 24000 rpm (5°C in an SW 27 rotor (Beckman L5-65). Fractions were collected, rediluted in 1:20 β-buffer and centrifuged for 2 h at 25000 rpm in the SW 27 rotor. Pellets obtained were washed with diluted β-buffer and kept at −70°C for further analysis.

Analysis of membranes

Protein was assayed according to the method of Lowry et al. [13] with bovine serum albumin as the standard. Lipids were extracted from membrane suspensions by the method of Bligh and Dyer [14]. Cholesterol and phosphorus contents in lipid samples were determined as previously described [11] by the methods of Wycoff-Parsons [15] and Ames [16], respectively. Separation of lipids was performed by thin-layer chromatography (Adsorbosil 5, Applied Science Laboratories, State College, PA) using benzene/diethyl ether/ethanol/acetic acid (50:40:2:0.2, v/v) for neutral lipids and chloroform/methanol/water/acetic acid (65:25:4:1, v/v) for phospholipids as the developing solvents. Lipid spots were detected by iodine vapor and identified by comparison with known standards. Methyl esters of fatty acids were prepared and subjected to gas-liquid chromatography using polar and non-polar columns as previously described [11]. Electrophoresis of membrane proteins was performed by the method of Weber and Osborn [17] on 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Protein bands were stained with Coomassie blue. Molecular weights of protein bands were estimated by comparison with the position of cross-linked hemoglobin and serum albumin (Sigma, St. Louis, MO). The DNA content of membrane fractions was determined as described by Schneider [18].

Fluorescence polarization

Fluorescence polarization studies were performed on an SLM 4000 apparatus (SLM Inc., Urbana, IL) as previously described [11]. Briefly, samples were resuspended (final concentration 150–250 μg membrane protein per ml) in 0.1 M

phosphate buffer (0.1 M phosphate/35 mM NaCl/1 mM KCl, pH 7.2). Labeling of membranes was achieved by incubating the suspensions with 2 μM DPH (Aldrich, Milwaukee, WI). DPH was excited at 362 nm while emission was measured at 430 nm. Results of steady-state depolarization experiments were expressed in terms of $((r_0/r)-1)^{-1}$, with r_0, the limiting anisotropy of DPH being set equal to 0.362 [12].

Electron microscopy

Samples were fixed by glutaraldehyde (2.5% in 0.2 M phosphate buffer), post fixed by OsO_4 1% and embedded in Araldit after dehydration by ethanol/propylene oxyde. Fine sections (300–400 Å) were stained by uranyl acetate (50% in ethanol)/lead citrate (1%) and examined under a Phillips EM 300 microscope. Freeze-fracture experiments were performed on organisms fixed at 37°C in a glutaraldehyde-isosmotic phosphate solution (final glutaraldehyde concentration 1%) as previously described [19].

Results

Isolation of membrane fractions

A first set of experiments using a identical medium and procedure to that previously described in literature [6] did not allow us to obtain any significant amounts of membrane subfractions from A5969 cells. We then turned to organisms growth in a modified Edward medium supplemented with cholesterol and oleic plus palmitic acids. These organisms showed the characteristics pear-shape and bleb-infrableb structures of the A5959 strain (Fig. 1A). These structures were still present (Fig. 1B) in the membrane fraction obtained after the freeze-thaw cycles performed either in β-buffer or in the buffer used by Maniloff and Quinlan ('Na buffer', Ref. 6). Changing the temperature from 20–25°C to 0–5°C for the brief sonication steps had no effect on the morphological appearance of membrane preparations. On the other hand, high-power sonication had to be performed at 20–25°C to produce sufficient amounts of material in the light fraction after centrifugation on discontinuous sucrose gradients. Light fraction (F_I) material was found over the 20% shelf ($d=$ 1.071 g/ml) (Fig. 2). A second fraction (F_{II}) was recovered at a mean density of 1.141 g/ml. The third fraction (F_{III}) had the same density as the unfractionated membrane (1.193 g/ml). Below this fraction a rather diffuse band comprised in the lower part of the 45% sucrose shelf and within the entire 50% sucrose layer was obtained. This diffuse fraction was also collected and will be referred to as F_{IV}. Recovery of proteins from fractions I–IV was about 80% of the amount layered, distributed as follows: F_I, 1–5%; F_{II}, 10–20%; F_{III}, 45–65%; F_{IV}, 5–15%. Fig. 1 (C–F) illustrates the morphological appearance of the different fractions. Fraction I (Fig. 1C) contained structures similar to those described by Maniloff and Quinlan (Fig. 4, Ref. 6), which are assumed to correspond to blebs of *M. gallisepticum*. It was also enriched in non-membranous material probably constituted of the infrableb contents. In fraction II large sheets of membranes coexisted with small vesicles, some resembling blebs (Fig. 1D). Fraction III contained larger vesicles and resembled the unfractionated membrane preparations (Fig. 1E). Finally, F_{IV} was constituted by large filamentous membrane sheets, often aggregated, and also contained an unidentified electron-dense material (Fig. 1F).

Analysis of membrane constituents

Unfractionated membranes of mid-log phase A5969 organisms had a low level of lipid phosphorus and contained 0.2–0.25 μmol cholesterol/mg membrane protein, which resulted in cholesterol-to-phospholid ratios slightly higher than unity (Table I). When the cells were grown on [^{14}C]oleate, 10 to 20% of the label was found in the neutral lipid fraction, incorporated mainly in diacylglycerols. Values up to 25–30% were obtained when [^{3}H]palmitate was used instead of oleate. A rough calculation based on the fatty acid composition of neutral lipids (Table II) indicated that this fraction may represent, cholesterol included, 40–45% of total lipids in A5969 membranes. Phosphatidylglycerol accounted for 75–80% (mol/mol) of phospholipids, the remaining part being constituted of phosphatidylcholine and sphingomyelin. While using [^{14}C]oleate no radioactivity could be recovered in these last two phospholipids, but labeling by [^{3}H]palmitate resulted

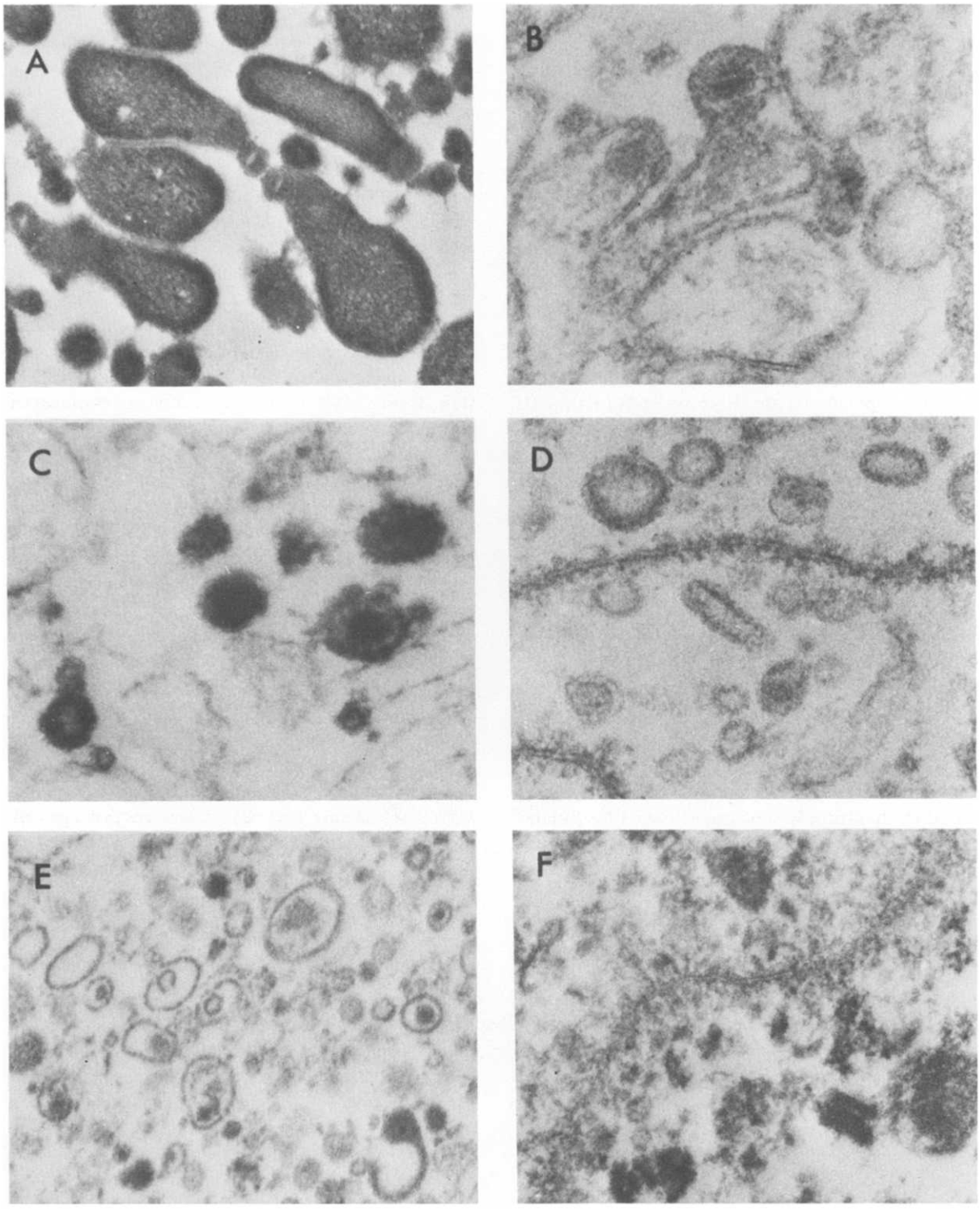

Fig. 1. Thin section electron micrographs of intact cells (A, ×45000); unfractionated membranes (B, ×113000); fraction I (C, ×108000); fraction II (D, ×103000); fraction III (E, ×50000), and fraction IV (F, ×50000). Magnifications were chosen to allow comparison with data of Ref. 6.

TABLE I

PHOSPHOLIPID AND CHOLESTEROL CONTENTS OF UNFRACTIONATED MEMBRANES AND MEMBRANE FRACTIONS OF *M. GALLISEPTICUM* A5969

Membranes of A5969 cells grown in presence of cholesterol (10 μg/ml) and oleate (5 μg/ml) plus palmitate (5 μg/ml) were isolated by freeze-thaw lysis and sonication. Fractions were obtained following high power sonication and centrifugation on discontinuous sucrose gradient. Membranes, unfractionated membranes. F_I, F_{II}, F_{III}, and F_{IV}, membrane fractions.

Fractions	Contents of membranes		
	Lipid-P (μmol P_i/mg membrane protein)	Cholesterol	
		μg/mg of membrane protein	μmol/μmol of lipid P_i
Membranes	0.19	92	1.25
F_I	0.90	1024	2.95
F_{II}	0.38	198	1.35
F_{III}	0.17	82	1.24
F_{IV}	0.11	39	0.91

in a low (≃ 5%) but significant incorporation of tritium in the phosphatidylcholine species. The fatty acid analysis of the pooled sphingomyelin-plus-phosphatidylcholine fractions demonstrated that they contained a high percentage of saturated fatty acids, namely palmitate and stearate (Table III). Pooling of sphingomyelin and phosphatidylcholine was made because of the low amount of material recovered from some fractions. The lipid composition of F_I was markedly different from that of unfractionated membranes. It contained about 10-times more cholesterol and 5-times more lipid phosphorus, resulting in cholesterol-to-phospholipid ratios significantly higher than 2. The percentage of sphingomyelin plus phosphatidylcholine increased from 20 to 30–40% while the distribution of [^{14}C]oleate was unchanged. The percentage of [^{3}H]palmitate incorporated in neutral lipids (40–50%) was almost doubled. Fatty acid analysis of both neutral lipids and phosphatidylglycerol showed a large increase in their saturation ratios (saturated/unsaturated). The same calculation as before indicated that neutral lipids may represent 60–65% of total lipid of F_I.

Fraction F_{II} was still enriched in cholesterol and lipid phosphorus but the cholesterol/phospholipid ratios, the saturation ratios of neutral lipids and phosphatidylglycerol, and the percentage of sphingomyelin plus phosphatidylcholine in total phospholipids (20–25%) were only slightly higher than those of unfractionated membranes. Fraction III showed a lipid composition similar to that of intact membranes. In accordance with its higher density, F_{IV} had a low phospholipid content. The absolute amount of cholesterol was even more decreased resulting in cholesterol/phospholipid ratios equal to or slightly below unity. Neutral lipid content decreased in the same proportion as phospholipid content and the fatty acid analysis revealed only minor differences from unfractionated membranes. The percentage of sphingomyelin plus phosphatidylcholine found in that fraction was also comparable to that of unfractionated membranes.

TABLE II

FATTY ACID COMPOSITION OF NEUTRAL LIPIDS IN UNFRACTIONATED MEMBRANES AND MEMBRANE FRACTIONS FROM A5969 ORGANISMS

Fractions	Concn. of fatty acids (mol/100 mol)							
	12:0	14:0	16:0	16:1	18:0	18:1	18:2	Saturated/ Unsaturated
Membranes	1.4	0.6	58.3	1.5	8.3	28.2	1.1	2.2
F_I	2.4	–	72.3	3.3	5.5	16.5	–	4.1
F_{II}	1.8	–	64.9	1.0	6.1	25.8	1.0	2.6
F_{III}	2.0	0.4	57.5	–	9.5	28.7	1.8	2.3
F_{IV}	0.9	–	60.4	1.6	7.1	29.5	1.4	2.1

TABLE III

FATTY ACID COMPOSITION OF PHOSPHOLIPIDS IN UNFRACTIONATED MEMBRANES AND MEMBRANE FRACTIONS FROM A5969 CELLS

PG, phosphatidylglycerol; Sph, sphingomyelin; PC, phosphatidylcholine.

Fractions	Concn. of fatty acids (mol/100 mol)							
	12:0	14:0	16:0	16:1	18:0	18:1	18:2	Saturated/ Unsaturated
(A) PG								
Membranes	–	0.4	54.9	0.4	4.5	38.5	1.1	1.5
F_I	3.6	3.2	61.6	–	10.1	21.5	–	3.7
F_{II}	0.2	0.4	58.5	1.2	3.9	35.0	0.5	1.7
F_{III}	0.2	1.0	53.5	0.3	5.0	40.0	0.8	1.4
F_{IV}	–	1.1	53.0	0.2	4.6	40.7	1.0	1.4
(B) Sph+PC *								
Membranes	0.7	0.6	48.3	–	40.2	8.0	1.7	9.3
F_I	1.1	0.3	50.9	–	34.5	13.6	–	6.4
F_{II}	0.2	1.1	53.0	–	39.0	6.0	–	15.6
F_{III}	–	–	55.2	–	37.6	4.3	0.5	19.3
F_{IV}	0.3	1.2	51.6	–	39.1	7.0	1.2	11.2

* Because of the reduced amounts of material recovered in some fractions, sphingomyelin and phosphatidylcholine were pooled to allow determinations of P_i contents and analysis of corresponding fatty acids.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of intact cells, unfractionated membranes and fractions I–IV are presented in Fig. 3A. It was observed that F_I contained a fewer number of bands. Protein bands corresponding to molecular weights of 47000, 68000, 77000, 91000, and 116000 were significantly enriched in the different F_I preparations. A similar pattern was also observed in a light fraction obtained from the PG31 strain of *M. gallisepticum* (Fig. 3B). The electrophoretic profiles of F_{II} and F_{IV} presented only minor differences compared with those of unfractionated membranes or F_{III}. The DNA content of F_I was 33 μg/mg membrane protein compared to 13, 7, and 12 μg/mg membrane protein for F_{II}, F_{III}, and F_{IV}, respectively.

Physical state of membrane preparations

Fluorescence polarization experiments on unfractionated membranes confirmed the previous findings [11] that, for the native A5969 cells grown in the presence of oleate plus palmitate, the membrane is highly ordered $((r_0/r)-1)^{-1}=2.77$, at the growth temperature and does not undergo lipid thermotropic transitions between 40 and 7°C (Fig. 4). Examination of the different fractions also failed to reveal any lipid phase transition in that temperature range. The fluidity properties of F_{II}, F_{III}, and F_{IV} were identical to those of unfractionated membranes. On the other hand, F_I was found to be slightly but significantly more rigid at 37°C and more fluid at low temperatures than unfractionated membranes. Although questionable [20,21] absolute values of microviscosities were calculated [12] for a comparison basis with data published on other biological membrane systems. Values of 6.7 and 7.0 poises at 37°C, and of 11 and 10 poises at 23°C, significantly higher than those reported for most mammalian membranes [22], were obtained for unfractionated membranes and F_I, respectively.

In spite of the absence of a detectable lipid phase transition, control experiments using freeze fracture technique showed that, in our growth conditions, the distribution of membrane particles at 37°C was not homogeneous, a lower density of particles frequently being observed at the level of blebs (Fig. 5).

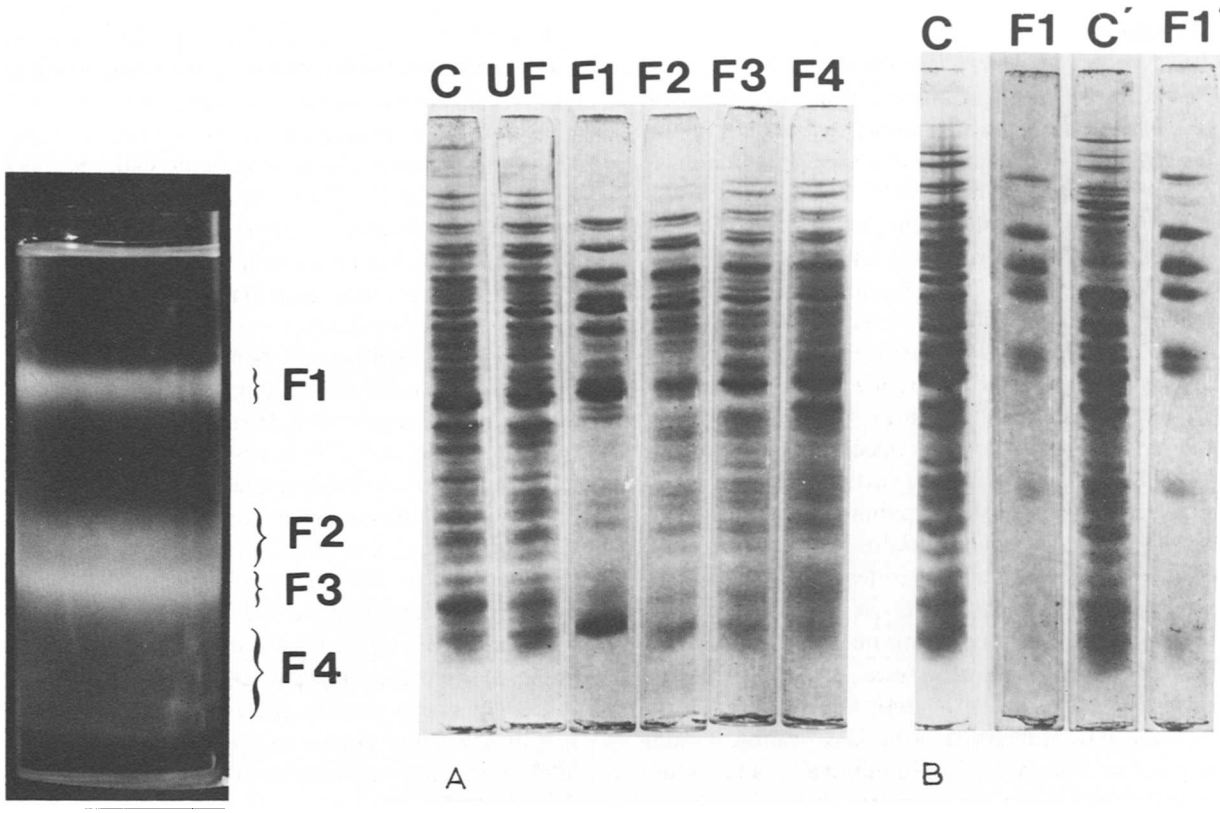

Fig. 2. (Left) Photograph of the distribution of the different fractions after centrifugation on discontinuous sucrose gradient.

Fig. 3. (A) Electrophoresis profiles of cells, unfractionated membranes and fractions I–IV from A5969 organisms. C, intact cells; UF, unfractionated membranes. (B) Electrophoresis profiles of unfractionated membranes and F_1 obtained from A5969 (left, C) and PG31 (right, C') *M. gallisepticum* strains.

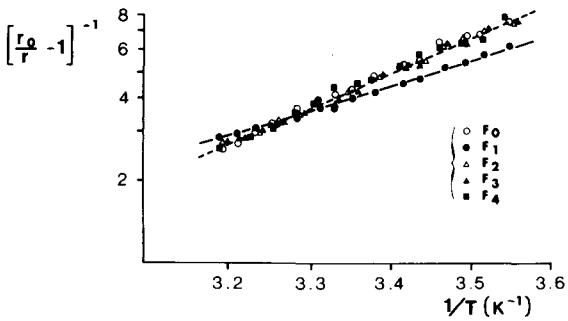

Fig. 4. Temperature dependence of the fluorescence polarization of diphenylhexatriene in A5969 membranes and fractions; F_0, unfractionated membranes.

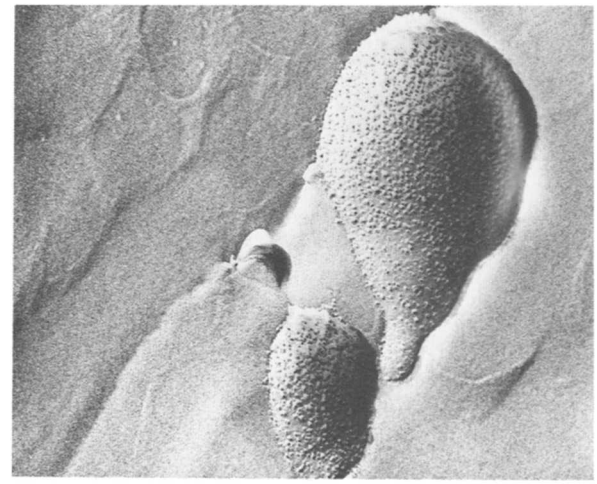

Fig. 5. Freeze-fracture photograph of A5969 fixed at 37°C by glutaraldehyde (magnification: ×72000).

Discussion

Except for a few minor quantitative differences the lipid composition of our preparations of unfractionated membranes from *M. gallisepticum* concords with literature [8–11] and more particularly with the most recent results reported by Rottem and Markowitz [10]. Thus cholesterol constituted more than 75–80% of neutral lipids, the remainder being principally accounted for by diglycerides. Beside phosphatidylglycerol, sphingomyelin and phosphatidylcholine were the two other major phospholipids found in A5969 membranes. These two phospholipids were not labeled by [^{14}C]oleate added to the growth medium while a significant amount of [^{3}H]palmitate was recovered in phosphatidylcholine species. This observation confirmed the existence of a deacylation-acylation enzymatic process for PC in *M. gallisepticum* membranes, probably acting on the ester bond at position 2 [10], but which was however less efficient in our organisms than in those used by Rottem and Markowitz [10]. The finding of non negligible amounts of sphingomyelin and phosphatidylcholine in our membrane preparations in the absence of horse serum in the growth medium can be explained by the fact that the serum albumin fatty acid poor although depleted in free fatty acids still contained large amounts of both phospholipids.

The process of estimating the physical state of membrane lipids from changes in steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene is well documented [12]. Although as mentioned before the absolute values of 'microviscosity' must be interpreted with extreme caution, it has been demonstrated [23–25] that steady-state measurements of fluorescent polarization with this probe allow to determine the degree of molecular packing (order), and by extension 'lipid fluidity' [25] in the apolar regions of membranes. Obtention of membrane subfractions from *M. gallisepticum* therefore differs from what has been described for *Escherichia coli* [26–28] in the sense that no lipid phase transition was necessary to achieve it. In spite of this difference in the preparation procedures striking similarities were found in the evolution of the membrane composition from light to heavy fractions of *E. coli* and *M. gallisepticum*. Thus, aside from the large increase in the phospholipid content of the light fraction, this fraction was enriched in saturated fatty acids and particular protein species suggesting a preferential association of these proteins with the lipid components of F_I. These similarities suggest that even in the absence of a lipid phase transition, the ratio of saturated to unsaturated fatty acids may take different values in different parts of the *M. gallisepticum* membrane and play an important role in the obtention of membrane subfractions. This hypothesis is strengthened by the results obtained in different experimental conditions by Wieslander et al. [29] in *Acholeplasma laidlawii*. Using partition in two-polymer aqueous phase systems, these authors have demonstrated that the ratio of saturated to unsaturated fatty acids had a direct effect on both the appearance and the relative size of membrane subfractions obtained from a membrane preparation homogeneous with respect to density. In addition they demonstrated that cholesterol would induce the appearance of membrane subpopulations. The large decrease in cholesterol and cholesterol-to-phospholipid ratios from F_I to F_{IV} observed in the present experiments may therefore have also contributed to the obtention of the different fractions. The lipid composition of F_I deserves further comments. First, ratios of cholesterol-to-phospholipid significantly higher than two were reproducibly obtained for that fraction. This gives rise to the possibility that cholesterol may be present in the form of microaggregates in F_I. The large increase in neutral lipids demonstrated by [^{3}H]palmitate data may also partly explain the high cholesterol content of F_I. As also noticed, the percentage of sphingomyelin plus phosphatidylcholine was also significantly increased in F_I. It is possible that this increase may be related to a preferential association of cholesterol with these two phospholipids as described for model systems [30]. Finally, one cannot totally exclude the possibility that part of the cholesterol may be bound to some protein in the electron dense material, which likely corresponds to the infrableb apparatus.

The position of fractions on sucrose gradient clearly differed from what was described by Maniloff and Quinlan [6] who obtained only two bands. Their bottom band was enriched in blebs

and represented about 45% of the material layered on the gradient. According to electron microscopy pictures and DNA analysis, our fraction enriched in bleb-infrableb material corresponded to F_I, i.e., the light fraction, which represented at most 5% of membrane protein, a value more in accordance with the size of blebs in our A5969 cells. It is difficult to explain the reason for such differences. They are possibly related to differences in the membrane composition of the A5969 organisms used in the two studies. This possibility is supported by the high densities of both their membrane fractions, which were much higher than those we and other investigators [8,31] have obtained for unfractionated membranes.

Although one cannot exclude that the small but significant difference in fluidity between F_I and other fractions may have played some role in the obtainment of the light fraction, identity in the fluidity properties of F_{II}, F_{IV} and unfractionated membranes demonstrates that differences in membrane fluidity are not a prerequisite for obtention of membranes subfractions. Several lines of arguments support the view that obtainment of F_I corresponds to a lateral heterogeneity of A5969 membranes. Existence of a marked polarity of A5969 membranes 'in vivo' suggested a non-random distribution of membrane constituents between the bleb and the remaining parts of the membrane. Such non-random distribution in intact cells was already demonstrated several years ago for the activity of phosphatase [32]. The morphological appearance of F_I and the DNA content strongly suggests it corresponds to this bleb-infrableb part of the membrane. Accordingly this would mean that the bleb 'in vivo' must have a lower protein content that the remaining part of the membrane, which agrees with our freeze-fracture experiments. Finally, the proposal that F_I originated from a lateral heterogeneity of intact membranes is reinforced by the similarities between the electrophoretic profiles of the light fractions from two different strains of *M. gallisepticum*.

With regard to F_{II} and F_{IV} it is more difficult to draw a conclusion concerning the origin of the fractions. While biochemical and biophysical analyses suggest they may correspond to special parts of the A5969 membranes, electron microscopy pictures of these fractions reveal the presence of large sheets of membranous material. Such membranous structures imply that a membrane reassociation phenomenon has taken place during the preparation. Therefore it cannot be ascertained, from the present experiments, if these large membranous sheets were formed by fusion from the population of surrounding vesicles corresponding to a special part of the A5969 membranes or, if they represent a preferential association of some membrane constituents during the high sonication step. Significant reorganizations during membrane preparation have already been demonstrated in other systems even when 'mild' treatments such as osmotic lysis were used [22].

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