Characterization of Membrane Components of the Flask-shaped Mycoplasma Mycoplasma mobile

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The cell membrane of Mycoplasma mobile was isolated by either ultrasonic or French press treatment of intact cells. The membrane fraction contained all of the cellular lipids, but only one-third of cellular proteins and had a density of 1.14 g ml⁻¹. The soluble fraction contained the NADH dehydrogenase activity of the cells, as well as a protein with an apparent molecular mass of 55 kDa that was phosphorylated in the presence of ATP. Lipid analyses of M. mobile membranes revealed that membrane lipid could be labelled by radioactive glycerol, oleate and to a much higher extent by palmitate but not by acetic acid. The membrane lipid fraction was composed of 54% neutral and 46% polar lipid. The major constituents of the neutral lipid fraction were free fatty acid, free cholesterol and cholesterol esters (45, 25 and 20%, respectively, of total neutral lipid fraction). The free cholesterol count was 13% (w/w) of total membrane lipids with a cholesterol:phospholipid molar ratio of about 0.9. Among the polar lipids, both phospho- and glycolipids were detected. The phospholipid fraction consisted of a major de novo-synthesized phosphatidylglycerol (~63% of total phospholipids), plus exogenous phosphatidylcholine and sphingomyelin incorporated in an unchanged form from the growth medium. The glycolipid fraction was dominated by a single glycolipid (~90% of total glycolipids) that was preferentially labelled by palmitic acid and showed a very high saturated:unsaturated fatty acids ratio.

INTRODUCTION

Mycoplasma sp. strain 163K was recently isolated from the gills of a tench (Tinca tinca L.) with red disease. On the basis of its metabolic, serological and biochemical characteristics, it was classified as a new species, Mycoplasma mobile (Kirchhoff et al., 1987). This organism has a peculiar flask-like shape with a distinct head-like structure and shares several features with other flask-shaped mycoplasmas (e.g. Mycoplasma gallisepticum and Mycoplasma pneumoniae), including the ability to adhere to inert surfaces and living cells and to show a gliding motion in the absence of locomotive organelles (Fischer et al., 1987; Kirchhoff & Rosengarten, 1984; Kirchhoff et al., 1984). Very little is known about the mechanisms of adherence and gliding motility, or of the components that determine the unique structural characteristics of these organisms. In the absence of a rigid cell wall (Razin, 1978), the interaction of mycoplasmas with the surrounding environment must occur via the cell membrane. Furthermore, it is reasonable to assume that membrane components are involved in both shape-determining and gliding mechanisms. Therefore, the characterization of the cell membrane of M. mobile is potentially of

Abbreviations: DCCD, dicyclohexylcarbodiimide; PC, phosphatidylcholine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; SPM, sphingomyelin.

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great interest. In the work reported in the present paper, the cell membrane of *M. mobile* was isolated and characterized. The results were compared with those previously reported for some other flask-shaped mycoplasmas.

**METHODS**

*Organisms and culture conditions.* *M. mobile* (strain 163K) and *M. gallisepticum* (A5969) were grown in 100–500 ml volumes of a modified Edward-Hayflick medium (Chanock et al., 1962) containing Difco PPLO broth, 16·8 g; 25% (w/v) fresh baker's yeast extract, 40 ml; glucose, 0·5 g; phenol red, 0·01 g; penicillin, 2000 IU ml⁻¹; deionized water added to 950 ml; the pH was adjusted to 7·5. The medium was supplemented with 5% (v/v) horse serum. To label membrane lipids, 0·002 pCi ml⁻¹ (74 [¹⁴C]palmitic acid (all from Amersham) were added to the growth medium. The organisms were grown for 2·3 d at 25 °C and harvested at the mid-exponential phase of growth by centrifugation at 12000 g for 30 min. The cells were washed once and resuspended in 0·25 M-NaCl containing 50 mM-Tris/HCl, pH 7·5 (to be referred to as NaCl/Tris solution).

*Membrane preparation.* This was done by disrupting the washed *M. mobile* cells by either passage through a French press or ultrasonic treatment. For the French press procedure, 20 ml washed cell suspension (0·4 mg protein ml⁻¹) were disrupted by a single passage through a French press at 1000 p.s.i. at a rate of 4 ml min⁻¹. For ultrasonic treatment, the cell suspension was treated for 15 min at 0 °C in a W-350 Heat Systems sonicator operated at 50%, duty cycles at 200 W. Unbroken cells were removed by centrifugation at 5000 g for 10 min and membranes were then collected by centrifugation at 34000 g for 30 min, washed once and resuspended in the NaCl/Tris solution. The density of the membrane preparations was determined by sucrose density gradient analysis using 13 ml of a linear 5–40% (w/v) sucrose gradient (Rottem et al., 1968). The gradients were centrifuged at 100000 g for 2·5 h and fractions of 600 μl were collected and analysed (Rottem et al., 1968). Initial swelling rates of the isolated membrane preparations and of intact cells were determined in 0·5 M-erythritol as previously described (Shirvan et al., 1982). Sealed membrane vesicles were prepared by fusing *M. mobile* membranes with lipid vesicles utilizing the freeze–thaw–sonication procedure (Cirillo et al., 1987). Proton retention by the membranes was determined as previously described (Cirillo et al., 1987).

*Protein phosphorylation.* This was assayed in *vitro* in a reaction mixture containing 10–20 μg cell protein, 1 mM-dithiothreitol; 10 mM-sodium fluoride; 1 mM-MgCl₂; 20 mM-Tris/maleate buffer, pH 6·0; and 1 mM-[γ-³²P]ATP (5 μCi, Amersham). The reaction was done for 1–2 min at 37 °C, terminated and analysed as described before (Platt et al., 1988).

*Analytical methods.* Protein was determined by the Lowry method. Total lipid phosphorus was determined by the method of Ames (1966) after digestion of the sample with an ethanolic solution of magnesium nitrate (10%, w/v). In order to determine phosphorus in phospholipid spots resolved by thin-layer chromatography (TLC), the spots were scraped off the plate into acid-washed test tubes and digested with 0·4 ml ethanolic magnesium nitrate solution in the presence of silica gel. Total cholesterol, free cholesterol and cholesterol esters in the lipid extracts were measured colorimetrically (Rudell & Morris, 1973). Free cholesterol was separated from cholesterol esters by TLC. Membrane proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970); protein bands were either identified by staining with Coomassie brilliant blue or electrophoretically transferred to nitrocellulose for the detection of immunological cross-reactivity (see below). ATPase activity in membranes was determined according to Rottem & Razin (1966) and results were expressed as nmol Pi released min⁻¹ (mg protein⁻¹). NADH dehydrogenase activity in whole cells, supernatant fluids or isolated membranes was determined spectrophotometrically (Ne'eman & Razin, 1975) in a reaction mixture containing 1 mg sodium deoxycholate ml⁻¹. Results were expressed as the decrease in absorbance at 340 nm min⁻¹ (mg protein⁻¹).

*Lipid analysis.* Lipids were extracted by the method of Bligh & Dyer (1959), for 1 h at room temperature, then at 4 °C overnight with constant shaking. Neutral lipids were separated from polar lipids by silicic acid chromatography. Neutral lipids were eluted with 20 bed vols chloroform. The column was then eluted with 10 bed vols chloroform/methanol (1:1, v/v) for the polar lipids. In some cases, after neutral lipids were eluted, the column was eluted with 10 bed vols acetone (to elute glycolipids) followed by 10 bed vols chloroform/methanol (2:1, v/v) to elute the phospholipids. The lipid fractions were evaporated to dryness under a stream of nitrogen and redissolved in chloroform. Neutral lipids were chromatographed on silica gel G plates (20 cm × 20 cm), developed at room temperature in benzene/diethyl ether/ethanol/acetic acid (50:40:2:0·2, by vol.), allowing the solvent front to move 14 cm from the bottom and followed by hexane/diethyl ether (94:6, v/v) to 19 cm from the bottom of the plate. Polar lipids were separated on silica gel plates (DC Fertigplatte, Kieselgel 60; Merck) developed at 4 °C in chloroform/methanol/water (65:25:4, by vol.). Lipid spots were detected by iodine vapour, phospholipid spots by the molybdate spray reagent (Dittmer & Lester, 1964), glycolipids by the anthrone reagent (van Just et al., 1968) and the glycol-containing lipids by the periodate-Schiff reagent (Shaw, 1968). The lipid spots were scraped off the plate and analysed for radioactivity and phosphorus.
RESULTS AND DISCUSSION

Osmotic behaviour of M. mobile cells in isoosmotic salt solution

Fig. 1 shows the osmotic behaviour of the flask-shaped fish mycoplasma, M. mobile, in comparison with that of M. gallisepticum. Whereas M. gallisepticum cells swell when suspended in isoosmotic NaCl solution for several hours, M. mobile cells did not swell. The swelling of M. gallisepticum cells in the absence of an energy source was suggested to be due to the inward diffusion of Na⁺ into the cells as a result of Gibbs–Donnan forces generated by the intracellular anionic macromolecules (Rottem et al., 1981). The Na⁺ diffusion is followed by water uptake, which results in cell swelling. However, in the presence of an energy source (e.g. glucose) M. gallisepticum cells do not swell, apparently due to the extrusion of Na⁺ by a dicyclohexylcarbodiimide (DCCD)-sensitive ATPase (Shirvan et al., 1987). Therefore, treating M. gallisepticum cells with DCCD induces swelling even in the presence of glucose. Such treatment did not induce cell swelling of M. mobile (Fig. 1). It has been suggested that the permeation of Na⁺ through the cell membrane of M. gallisepticum is due to the existence of segregated fluid and solid lipid domains in the membrane (Rottem & Verkleij, 1982). Such domains are the result of the accumulation of disaturated phosphatidylcholine (PC), formed by modifying exogenous PC incorporated from the growth medium (Rottem & Markowitz, 1979), and provide the sites for increased permeation of small solutes (Blok et al., 1975). The absence of detectable cell swelling of M. mobile in isoosmotic NaCl solution suggests, therefore, that the cell membrane of M. mobile is much less permeable to Na⁺ and that this might be related to a lack of disaturated PC. Indeed, whereas all the flask-shaped mycoplasmas analysed so far, including M. pneumoniae, M. pulmonis and M. gallisepticum, share the unique biochemical property of an extensive incorporation of PC from the growth media and its modification into a disaturated phospholipid species (Rottem et al., 1986), the PC is incorporated by M. mobile from the growth medium in an unchanged form and to a very low extent (about 10% of total phospholipids, see Table 3).

Isolation and characterization of M. mobile membranes

Due to the osmotic stability of M. mobile, the study of the cell membrane has been hampered by the lack of an efficient method to isolate purified membrane preparations. Table 1 shows basic biochemical characteristics of two membrane preparations obtained by ultrasonic and French press treatments of washed M. mobile cells. The isolated membranes contained about 33% of total cell protein and practically all lipids present in intact cells. This resulted in an increase in the labelling intensities of the membrane preparations (Table 1) and a density of 1.14 g ml⁻¹. Membranes isolated from low-passage M. mobile cells (passage no. 11) were practically identical to membranes isolated from high-passage cells (passage no. 252). The extremely low initial swelling rate of the membrane preparations in isoosmotic erythritol solution (Table 1) and the inability of these membranes to retain protons (not shown) strongly suggest that M. mobile membranes are unsealed. Sealed M. mobile vesicles were, however, obtained by fusing membrane fragments with azolectin cholesterol vesicles. This was done by freezing and thawing followed by brief sonication treatment as previously described (Cirillo et al., 1987). The membrane preparations totally lacked the soluble NADH dehydrogenase activity found in intact M. mobile cells (Table 1). The NADH dehydrogenase activity of M. mobile was
Fig. 1. Swelling of *M. mobile* and *M. gallisepticum* cells in isoosmotic NaCl solution. Washed mid-exponential-phase cells were suspended in isoosmotic NaCl solution. The cells were incubated at 25 °C (*M. mobile*) or 37 °C (*M. gallisepticum*) for various periods of time. Cell swelling was measured spectrophotometrically (OD500). Open symbols refer to *M. gallisepticum*; closed symbols refer to *M. mobile.* ○, 0.25 M-NaCl alone; □, 10 mM-glucose in 0.25 M-NaCl; △, 100 µM-DCCD and 10 mM-glucose in 0.25 M-NaCl.

Fig. 2. *In vitro* phosphorylation of *M. mobile* whole-cell lysate. Whole-cell lysate was phosphorylated and analysed by SDS-PAGE as described in Methods. A, Coomassie blue-stained gel; B, autoradiogram of A after 12 h exposure.

Table 1. Characterization of *M. mobile* membranes

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protein (mg)</th>
<th>Labelling intensity [c.p.m. (mg protein)⁻¹]</th>
<th>Initial swelling rate [ΔOD500 min⁻¹ (mg protein)⁻¹]</th>
<th>NADH dehydrogenase [ΔA₃₄₀ min⁻¹ (mg protein)⁻¹]</th>
<th>ATPase [nmol Pi released min⁻¹ (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells</td>
<td>2·50</td>
<td>28 400</td>
<td>0·058</td>
<td>0·17</td>
<td>16·8</td>
</tr>
<tr>
<td>Isolated membranes (French press method)</td>
<td>0·81</td>
<td>88 000</td>
<td>&lt;0·002</td>
<td>&lt;0·03</td>
<td>&lt;0·01</td>
</tr>
<tr>
<td>Isolated membranes (ultrasonication method)</td>
<td>0·87</td>
<td>82 800</td>
<td>&lt;0·002</td>
<td>&lt;0·01</td>
<td>34·5</td>
</tr>
</tbody>
</table>
Table 2. Incorporation of radioactive lipid precursors into M. mobile cells

Cells were grown in 100 ml volumes of a modified Edward-Hayflick medium (Chanock et al., 1962) supplemented with 5% horse serum and 2 μCi radioactive lipid precursor. Lipid extraction was done as described in Methods. The results are representative of three experiments done on different batches of M. mobile cells.

<table>
<thead>
<tr>
<th>Lipid precursor</th>
<th>Labelling intensity [c.p.m. (mg cell protein)]</th>
<th>Percentage label in total lipid fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>&lt;200</td>
<td>–</td>
</tr>
<tr>
<td>Glycerol</td>
<td>4300</td>
<td>65</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>201700</td>
<td>90</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>674500</td>
<td>97</td>
</tr>
</tbody>
</table>

exceedingly low, reaching only about 5% of the activity of M. gallisepticum cells determined simultaneously. This activity was qualitatively recovered in the supernatant fluid (results not shown). The membrane preparations, however, retained most of the ATPase activity (Table 1). This activity was better retained in membrane preparations obtained by the French press procedure (92% of total), as compared with recovery from membranes isolated following ultrasonic treatment (71% of total). The residual ATPase activity could not be detected in the soluble fraction and was apparently lost during membrane isolation. Monospecific polyclonal antibody against the β subunit of E. coli ATPase reacted with a single polypeptide, with an apparent molecular mass of 52 kDa, in the cell membrane of M. mobile (results not shown). This observation suggests that, as with M. gallisepticum (Zilberstein et al., 1986), M. mobile possesses an F1F0-proton-translocating ATPase. As the membrane yields obtained by the French press procedure were high, with very little variation between batches, and both the labelling intensity and the ATPase activity of the French press membrane preparation were higher than those of membrane preparations obtained by ultrasonic treatment, the French press procedure is the more gentle and therefore was used subsequently throughout this study.

Protein phosphorylation by M. mobile

Incubation of M. mobile whole-cell lysate with [γ-32P]ATP resulted in the phosphorylation of a single polypeptide band with an apparent molecular mass of 55 kDa (Fig. 2). The extent of phosphorylation was almost the same whether whole-cell lysate or the soluble fraction, obtained after the removal of the cell membranes, was utilized (results not shown). However, phosphorylation was not observed in reaction mixtures containing isolated membrane preparation instead of the soluble fraction. A similar soluble 55 kDa protein that undergoes reversible phosphorylation has recently been described in M. gallisepticum (Platt et al., 1988) and other flask-shaped Mycoplasma and Spiroplasma species (M. W. Platt & S. Rottem, unpublished results). This protein, in its dephosphorylated form, was attached preferentially to membrane fragments and it was therefore proposed that the reversed phosphorylation may be involved in establishing and/or controlling the unique morphology of the flask-shaped Mycoplasma and Spiroplasma species (Platt et al., 1988).

Incorporation of lipid precursors into M. mobile cells

Table 2 shows that labelled fatty acids as well as labelled glycerol from the growth medium were incorporated into lipids of M. mobile cells. However, [14C]acetate was not incorporated, suggesting that, like other sterol-requiring mycoplasmas and unlike the Acholeplasma species (Rottem, 1980; Smith, 1979), M. mobile is unable to utilize acetate for lipid biosynthesis. Table 2 also shows that [14C]palmitate was incorporated much better than [14C]oleate. Preferential incorporation of palmitate into membrane lipids has been described previously in a variety of Mycoplasma species (Rottem, 1980).

The neutral lipid fraction of M. mobile membranes

Quantitative analysis of M. mobile membrane lipids revealed that about 54% (w/w) of the lipids were eluted from a silicic acid column by chloroform and hence comprised the neutral
Table 3. The major polar lipids of M. mobile

Cells were grown in a medium containing 5% horse serum supplemented with either \(^{14}C\)oleate or \(^{14}C\)palmitate. Membrane isolation and lipid analysis were done as described in Methods. The results are representative of three experiments done on different batches of M. mobile membranes.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Radioactivity (% of total) when grown with:</th>
<th>Lipid (P_i) (% of total)</th>
<th>Tentative identification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[^{14}C]Oleate</td>
<td>[^{14}C]Palmitate</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.04</td>
<td>&lt;1.0</td>
<td>7.3</td>
</tr>
<tr>
<td>B</td>
<td>0.08</td>
<td>&lt;1.0</td>
<td>11.4</td>
</tr>
<tr>
<td>C</td>
<td>0.20</td>
<td>70.2</td>
<td>63.2</td>
</tr>
<tr>
<td>D</td>
<td>0.34</td>
<td>15.2</td>
<td>46.8</td>
</tr>
<tr>
<td>E</td>
<td>0.48</td>
<td>4.4</td>
<td>2.1</td>
</tr>
<tr>
<td>F</td>
<td>0.53</td>
<td>9.0</td>
<td>0</td>
</tr>
</tbody>
</table>

Lipids. This fraction contained 25% free cholesterol, 20% esterified cholesterol, 45% free fatty acids and 10% glycerides (mainly diglycerides). The free cholesterol:phospholipid molar ratio in M. mobile was about 0.9, similar to that observed in other Mycoplasma species (Rottem, 1980; Smith, 1971). Nevertheless, as phospholipids comprise only about 55% (w/w) of the polar lipid fraction of M. mobile, the free cholesterol level found in this organism was only about 13% of total membrane lipids. In other sterol-requiring mycoplasmas, cholesterol levels of 25–30% were described (Rottem, 1980). The high content of free fatty acids initially suggested a potent endogenous phospholipase activity. However, their undiminished presence under conditions minimizing endogenous phospholipase activity, such as extraction of the lipids directly from whole cells instead of membranes, renders it more likely that most of the fatty acids are incorporated as such from the growth medium. Furthermore, incubating \(^{14}C\)oleate-labelled intact cells, or cells disrupted by sonication, at 37 °C for up to 2 h failed to show the release of radioactive fatty acids into the free fatty acid fraction. These cell preparations were incubated in a mixture previously utilized for the detection of endogenous phospholipases in mycoplasmas (Rottem et al., 1986). When cells were grown with radioactive fatty acid, the label in the neutral lipid fraction was found exclusively in the free fatty acid fraction, reaching 60–80% of the total fatty acids incorporated. Since the addition of the radioactive fatty acids failed to label the cholesterol esters, it is apparent that the esters are not synthesized by the organism but are incorporated as such from the growth medium.

The polar lipid fraction of M. mobile membranes

About 46% (w/w) of the total membrane lipids of M. mobile were eluted from the silicic acid column with chloroform/methanol (1:1, v/v) and hence constitute the polar lipids. TLC of the polar lipid fraction on silica gel plates revealed that the polar lipid fraction consisted of six major spots designated A–F (Table 3). When the cells were grown with a radioactive fatty acid, only four spots were labelled, with over 95% of the radioactivity recovered in three of these spots (spots C, D and F). Of the three labelled spots, spots C and D reacted with the molybdate reagent used to detect phosphorus-containing lipids, had a positive periodate/Schiff reaction and comigrated with standards of phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG). Accordingly, these compounds were tentatively identified as PG (spot C) and DPG (spot D). Compound F did not react with the molybdate reagent but reacted with the anthrone reagent which detects glycolipids. PC and sphingomyelin (SPM), incorporated in significant amounts by many Mycoplasma species (Rottem, 1980), were taken up by M. mobile to a very low extent, reaching levels of 7.3 and 11-4%, respectively, of total lipid phosphorus. Table 3 also shows that whereas the phospholipids, PG and DPG, incorporated both \(^{14}C\)palmitate and \(^{14}C\)oleate, the glycolipid (spot F) was intensely labelled by radioactive palmitate but very slightly by radioactive oleate. This was also reflected in the fatty acid profile of the phospho- and glycolipid fractions (Table 4). The phospho- and glycolipids were obtained by silicic acid chromatography of the polar lipid fraction. About 45% (w/w) of the polar lipid fraction was eluted from the silicic
The cell membrane of Mycoplasma mobile

membranes.

Table 4. Fatty acid composition of M. mobile polar lipid fractions

<table>
<thead>
<tr>
<th>Lipid fraction</th>
<th>10:0</th>
<th>12:0</th>
<th>14:0</th>
<th>16:0</th>
<th>18:1</th>
<th>18:2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolipids</td>
<td>4-6</td>
<td>8-6</td>
<td>19-3</td>
<td>54-0</td>
<td>8-7</td>
<td>&lt;0-5</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>&lt;0-5</td>
<td>&lt;0-5</td>
<td>1-0</td>
<td>29-0</td>
<td>64-5</td>
<td>1-9</td>
</tr>
</tbody>
</table>

* In the fatty acid designations, the first number indicates chain length and the second indicates the number of double bonds. The results are expressed as percentages of total peak area.

acid column with acetone and hence constituted the glycolipid fraction. The residual 55% was eluted with chloroform/methanol (1:1, v/v) and hence consisted of phospholipids. The dominant lipid in the phospholipid fraction of M. mobile was PG. It appears that the synthesis of PG as the major membrane phospholipid is a property shared by many mycoplasmas (Rottem, 1980; Smith, 1979). The glycolipid fraction contained a single glycolipid (Table 3, spot F). Glycolipids are major components of Acholeplasma species and have been detected in many fermentative Mycoplasma species (Smith, 1979). The radical difference in the fatty acid composition of the phospho- and glycolipid fractions of M. mobile may be regarded as our most salient finding. While the phospholipid fraction contained both saturated and unsaturated fatty acids, the fatty acids predominating in the glycolipid fraction were saturated fatty acids (Table 4). The preferential use of saturated fatty acids for glycolipid biosynthesis accounts for the much higher labelling intensity of the cells when grown with [14C]palmitate than with [14C]oleate. It was previously suggested that the flask-shaped mycoplasmas share a unique biochemical property, the presence of disaturated lipid components (Rottem, et al., 1986). In M. gallisepticum, M. pulmonis and M. pneumoniae, the major disaturated lipid component is a PC, incorporated from the growth medium and modified by a decylation–reacylation enzymic sequence to give a disaturated species (Rottem & Markowitz, 1979). It seems that in M. mobile the disaturated lipid component is a glycolipid synthesized de novo rather than PC. The highly saturated glycolipid may affect the physical state of M. mobile membranes and, if preferentially accumulated in one half of the lipid bilayer, may have a pronounced effect on cell morphology (Rottem et al., 1986).

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