The Membrane of the *Streptobacillus moniliformis* L-phase

By S. RAZIN AND CHASIA BOSCHWITZ

*Department of Clinical Microbiology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel*

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**SUMMARY**

Membranes of the stable L-phase of *Streptobacillus moniliformis* were isolated after lysis of the organisms by osmotic shock combined with ultrasonic treatment. The membranes were composed of about 53% protein and 40% lipid. About two-thirds of the lipids were polar; the rest consisted almost entirely of cholesterol. Radioactive oleic and palmitic acids were incorporated most efficiently from the growth medium into the membrane lipids. The L-phase membranes were solubilized by sodium dodecyl sulphate and sodium deoxycholate. Most of the solubilized membrane proteins could be separated from membrane lipid by ammonium sulphate precipitation. The precipitated proteins were very hydrophobic. The solubilized membrane proteins reassociated with membrane lipids after removal of the detergent by dialysis. The lipid:protein ratio in the resulting membrane re-aggregates depended on the magnesium concentration in the dialysis buffer. Polyacrylamide-gel electrophoresis showed that most of the protein species of the original membrane were incorporated into membrane re-aggregates. The protein composition of the re-aggregate was similar to, but not identical with, that of the hydrophobic protein fraction. The data obtained indicate that membranes of the stable L-phase of *S. moniliformis* resemble Mycoplasma membranes.

**INTRODUCTION**

The stable L-phase variants are distinguished from their parent bacteria by the absence of the rigid cell wall (Panos, 1967a). The loss of the cell wall might be spontaneous or induced, and is irreversible. The first stable L-phase was recovered from *Streptobacillus moniliformis* cultures by Klieneberger in 1935 and designated as L-I (Klieneberger, 1935). Since then stable L-phase variants have been obtained from other bacteria. Most of the biochemical studies made so far relate to L-phase variants of Proteus species and of *Streptococcus pyogenes* (Smith, 1964; Panos, 1967a) and information on the biochemical properties of the *S. moniliformis* L-phase is scanty.

Devoid of cell walls and osmotically fragile, the stable L-phase variants of bacteria are eminently suited for the investigation of the bacterial plasma membrane (Salton, 1967), although this membrane may have undergone certain modifications in composition and structure upon transition to the L-phase to compensate for the loss of the cell wall. Thus changes have been recorded in the composition of the plasma membrane lipids of *Streptococcus pyogenes* upon its transition to the L-phase (Panos, 1967b).

The stable L-phase variants morphologically resemble the organisms of the order Mycoplasmatales. Both types of organisms are bounded by a single lipoprotein
membrane, and are able to grow and multiply in the absence of a cell wall (Dienes & Bullivant, 1967). Since the major subject of our studies during recent years has been the biochemistry of the Mycoplasma membrane (Razin, 1967), it seemed worthwhile to use the techniques developed to compare some of the properties of the L-phase and the Mycoplasma membranes.

**METHODS**

**Organisms.** The stable L-phase of *Streptobacillus moniliformis* (L-1) was obtained from Dr E. Kleineberger-Nobel (The Lister Institute for Preventive Medicine, London). *Mycoplasma laidlawii* (oral strain), serologically related to *M. laidlawii* strain A, was isolated in our laboratory from the human oral cavity.

**Growth conditions.** The organisms were grown in 2 l. volumes, dispensed in 4 l. flasks, of a modified Edward medium (Razin, 1963) containing 3 % (v/v) of horse serum in place of Difco-PPLO serum fraction. For labelling membrane lipids, the medium was supplemented with 1 μc./l. of each [1-14C]-oleic acid (specific activity 32-5 mc./mmole), [1-14C]-palmitic acid (specific activity 36-6 mc./mmole) and uniformly labelled [14C]-sodium acetate (specific activity 15-7 mc./mmole) purchased from the Radiochemical Centre, Amersham, England. The organisms were harvested after 16–20 hr of static incubation at 37° by centrifugation at 12,000 g for 20 min., washed once and resuspended in 0.5 M-NaCl.

**Examination of lysis of organisms.** Lysis of washed organisms by osmotic shock, or by alternate freezing and thawing was measured by the decrease in turbidity of suspensions in place of Diisco-PPLO serum fraction. For labelling membrane lipids, the medium was supplemented with 1 μc./l. of each [1-14C]-oleic acid (specific activity 32-5 mc./mmole), [1-14C]-palmitic acid (specific activity 36-6 mc./mmole) and uniformly labelled [14C]-sodium acetate (specific activity 15-7 mc./mmole) purchased from the Radiochemical Centre, Amersham, England. The organisms were harvested after 16–20 hr of static incubation at 37° by centrifugation at 12,000 g for 20 min., washed once and resuspended in 0.5 M-NaCl.

**Isolation of cell membranes.** L-phase organisms harvested from 2 to 4 l. medium were resuspended in 10 to 20 ml. 0.5 M-NaCl. The black sulphide precipitate which accompanied the centrifuged deposit was dissolved by adding dilute HCl to pH 2.5. The organisms were then collected by centrifugation, washed twice in 500 volumes of 0.5 M-NaCl and resuspended in 20 ml. de-ionized water. The suspension was treated in the ultrasonic disintegrator for 30 sec. and centrifuged at 6000 g for 5 min. The supernatant fluid was collected and the sediment resuspended in de-ionized water and treated in the disintegrator for another 30 sec. The treated suspension was centrifuged as above and the supernatant fluid combined with the first one. Phase-contrast microscopy showed the pooled supernatant fluids to consist of membraneous material. Pancreatic deoxyribonuclease (10 μg./ml.) was added to the membrane suspension, and after incubation at room temperature for 15 min. the membranes were collected by centrifugation at 34,000 g for 40 min. The membranes were washed 8 times alternately with 0.05 M-NaCl and 0.05 M-phosphate buffer pH 7-0 (Panos, Cohen & Fagan, 1966) and finally with de-ionized water. For chemical analysis the membranes were resuspended in a small volume of de-ionized water and freeze-dried. For solubilization and re-aggregation experiments, the membranes were resuspended in β-buffer diluted 1/20 in de-ionized water (Razin, Morowitz & Terry, 1965) and kept at −20° until used.
Solubilization and reaggregation of membrane material. Solubilization of membranes by detergents was tested by adding 0.6 ml. membrane suspension (equiv. 20 mg. protein/ml.) in 1/20 β-buffer to 3 ml. solutions containing various concentrations of sodium dodecyl sulphate (SDS) or sodium deoxycholate (DOC) in the same dilute buffer. Changes in the extinction of the suspensions at 500 mp were measured after 15 min. incubation at 37°. For re-aggregation experiments the membranes were solubilized in SDS (0.5 mg. detergent/mg. membrane protein). The non-solubilized material was removed by centrifugation at 34,000 g for 40 min. and the clear solution dialysed in the cold for 2 to 3 days against 1/20 β-buffer containing various magnesium concentrations (Razin et al. 1965). The re-aggregated membrane material formed in the dialysis bag was collected by centrifugation at 34,000 g for 40 min.

Isolation of the hydrophobic membrane proteins. The method used was that of Cridde, Bock, Green & Tisdale (1962) as modified by Rodwell, Razin, Rottem & Argaman (1967). Membranes were dissolved in a mixture of SDS (1 mg. detergent/mg. membrane protein) and sodium deoxycholate (DOC, 2 mg. detergent/mg. membrane protein). Ammonium sulphate was added to 12 % saturation. The heavy precipitate formed was collected by centrifugation at 10,000 g for 10 min. and dissolved in the detergent mixture. The procedure of protein precipitation and resolution was repeated twice more and the final protein precipitate washed twice and resuspended in 1/20 β-buffer. The protein suspension was kept at 4° until used, with 1/10,000 thiomersalate to prevent bacterial growth.

Polyacrylamide-gel electrophoresis of membrane proteins. The procedure described by Rottem & Razin (1967) was used. The gels contained 7.5 % (w/v) acrylamide, 35 % (v/v) acetic acid and 5 M-urea; the electrophoresis buffer was 10 % (v/v) acetic acid.

Analytical methods. Protein, carbohydrate and nucleic acids were determined in defatted organisms or membranes. Protein was determined according to Lowry et al. (1951) with crystalline bovine plasma albumin as standard. Nucleic acids were extracted according to Schneider (1945). RNA was determined by the method of Drury (1948) and DNA according to Burton (1956). Total carbohydrate was estimated according to Dubois et al. (1956) after removal of the nucleic acids and lipids. Lipids were extracted with chloroform + methanol (2 + 1, by vol.) as described by Razin, Argaman & Avigan (1963). Polar lipids were separated from the non-polar lipids by chromatography on silicic acid columns (Ansell & Hawthorne, 1964). The non-polar lipids were eluted from the column with chloroform and the polar lipids with methanol (Razin et al. 1966). The two fractions were weighed after evaporation of the solvents under a stream of nitrogen. Cholesterol in the non-polar lipid fraction was separated by thin-layer chromatography and estimated by the FeCl₃ reaction (Argaman & Razin, 1965). Radioactivity in lipid fractions was determined in a Packard Tri-Carb liquid scintillation spectrometer. The scintillation mixture consisted of 800 ml. di-oxane, 150 ml. toluene, 50 g. naphthalene, 10 g. 2,5-diphenyloxazole (PPO) and 150 mg. 1,4-bis-2-(5-phenyl-oxazolyl)-benzene (POPOP).

Membranes or membrane protein solutions in 1/20 β-buffer containing 0.02 M-SDS (7 mg. protein/ml.) were centrifuged at 59,780 rev./min. in a Spinco model E analytical ultracentrifuge at 20°. Sedimentation coefficients were calculated without correction for medium viscosity or density.
RESULTS

Susceptibility of the L-phase organisms to lysis

Osmotic lysis. The L-phase organisms lysed in de-ionized water and dilute NaCl solutions. Lysis manifested itself in a decrease in colony counts, in the extinction of the suspensions, and a greater amount of material absorbing at 260 mp released from the organisms (Fig. 1).

![Graph](image)

Fig. 1. Osmotic lysis of the L-phase of *Streptobacillus moniliformis* and *Mycoplasma laidlawii*. The extent of lysis in the various NaCl solutions was measured after 15 min. incubation at 37°. Extinction readings at 600 mp of suspensions (●); extinction readings at 260 mp of the supernatant fluids of the corresponding suspensions (△); number of viable particles/ml. suspension (○).

<table>
<thead>
<tr>
<th>Time of ultrasonic treatment (sec.)</th>
<th>L-phase</th>
<th>Mycoplasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.0 x 10^8</td>
<td>8.2 x 10^8</td>
</tr>
<tr>
<td>3</td>
<td>3.5 x 10^9</td>
<td>2.1 x 10^9</td>
</tr>
<tr>
<td>5</td>
<td>5.9 x 10^9</td>
<td>1.4 x 10^9</td>
</tr>
<tr>
<td>10</td>
<td>3.6 x 10^9</td>
<td>1.1 x 10^9</td>
</tr>
<tr>
<td>30</td>
<td>2.2 x 10^9</td>
<td>9.0 x 10^8</td>
</tr>
<tr>
<td>180</td>
<td>3.8 x 10^8</td>
<td>1.8 x 10^7</td>
</tr>
</tbody>
</table>

*Mycoplasma laidlawii* organisms were included in these experiments for purposes of comparison. The extinction readings at 600 mp of the L-phase suspension in de-ionized water were not as low as those of *M. laidlawii*. This was largely due to the presence of sulphide crystals in the L-phase suspensions. Large quantities of these crystals formed during growth, sedimented with the organisms at harvest and could
L-phase membrane

not be completely separated from them by differential centrifugation. Phase-contrast microscopy of the L-phase culture in Edward medium showed the spherical organisms to differ very much in diameter: some had a diameter of several microns while the diameter of others was near the limit of the microscope resolution. The larger forms were the first to lyse when the tonicity of the medium was decreased, leaving behind the smaller forms, some of which resisted lysis even in de-ionized water (Fig. 1).

Lysis by ultrasonic treatment. The L-phase and Mycoplasma organisms rapidly lysed by ultrasonic treatment. Most organisms lysed within the first 10 sec. of treatment (Fig. 2; Table 1). Again, the first to lyse were the larger forms. The sulphide crystals in the L-phase suspensions were responsible for most of the residual turbidity after prolonged ultrasonic treatment.

Lysis by alternate freezing and thawing. The *Streptobacillus moniliformis* L-phase organisms and *Mycoplasma laidlawii* organisms were relatively resistant to lysis by alternate freezing and thawing when suspended in 0.5 M-NaCl. Alternate freezing and thawing of the L-phase suspension in de-ionized water added little to the lytic effect of the de-ionized water.

**Fig. 2.** Lysis of the L-phase of *Streptobacillus moniliformis* and *Mycoplasma laidlawii* by ultrasonic treatment. Extinction readings at 600 mµ of suspensions (●); extinction readings at 260 mµ of the supernatant fluids of the corresponding suspensions (Δ). ——, L-phase; ——, mycoplasma.

**Fig. 3.** Effect of magnesium on the re-aggregation of protein and lipid of L-phase membranes solubilized by sodium dodecyl sulphate. Percentage of membrane protein re-aggregated (●); percentage of membrane lipid re-aggregated (○); ratio of lipid (expressed as radioactivity) to protein in re-aggregates (Δ).

Isolation and chemical composition of the L-phase membranes

The results of the lysis experiments indicated that osmotic lysis could be used for the isolation of the L-phase membranes. However, several problems had to be solved before this method could be used for the isolation of large quantities of membranes.
First, the accompanying sulphide crystals had to be removed. The addition of dilute HCl to pH 2.5 to the suspension dissolved the crystals, producing the characteristic H₂S odour. The acid treatment did not cause lysis, not did it affect the gross chemical composition of the cell membranes, as determined by comparative chemical analysis of membranes isolated without acid treatment. Since the lysis experiments indicated that some of the minute L-phase forms did not lyse by osmotic shock, ultrasonic treatment was used with osmotic shock to assure maximal lysis. The yield of membranes obtained by the above method from 1 l. of culture was about 80–100 mg. dry weight.

Table 2. Chemical composition of organisms and membranes of *Streptobacillus moniliformis* L-phase

<table>
<thead>
<tr>
<th></th>
<th>Organisms</th>
<th>Membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean *</td>
<td>Range</td>
</tr>
<tr>
<td></td>
<td>% dry weight</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>48·7</td>
<td>39·4–52·0</td>
</tr>
<tr>
<td>Lipid</td>
<td>31·2</td>
<td>28·7–36·4</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>2·1</td>
<td>1·2–2·7</td>
</tr>
<tr>
<td>RNA</td>
<td>11·6</td>
<td>9·2–12·2</td>
</tr>
<tr>
<td>DNA</td>
<td>5·1</td>
<td>4·6–6·0</td>
</tr>
</tbody>
</table>

* The mean of determinations performed on five different batches of cells.
† The mean of determinations performed on eight different batches of membranes.
‡ Determined on a single batch of membranes, all other batches were treated with pancreatic deoxyribonuclease and were essentially free of DNA.

Table 3. Incorporation of radioactive fatty acids into lipids of *Streptobacillus moniliformis* L-phase

Organisms were grown in 100 ml. Edward medium containing 3% (v/v) horse serum and 0·1 μc of radioactive fatty acid.

<table>
<thead>
<tr>
<th>Radioactive fatty acid in medium</th>
<th>Whole organisms</th>
<th>Cell lipids</th>
<th>Percentage of radioactivity incorporated of total radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1-14C]-Palmitic acid</td>
<td>70,600</td>
<td>65,000</td>
<td>59·0</td>
</tr>
<tr>
<td>[1-14C]-Oleic acid</td>
<td>55,800</td>
<td>53,000</td>
<td>44·2</td>
</tr>
<tr>
<td>[14C]-Sodium acetate</td>
<td>2,580</td>
<td>2,180</td>
<td>2·2</td>
</tr>
</tbody>
</table>

The chemical composition of organisms and membranes of the L-phase organism is compared in Table 2. The membranes were essentially composed of protein and lipid, and their low RNA content indicated that they were relatively free from cytoplasmic contaminants. Silicic acid chromatography showed that polar lipids consisted about two-thirds (range 56·7 to 75·2 %) of the total membrane lipids. The non-polar lipid fraction consisted almost entirely of non-esterified cholesterol.

Membrane lipids were selectively labelled by adding radioactive oleic or palmitic acids to the growth medium of the L-phase organism (Table 3). The long-chain fatty acids were most efficiently incorporated into cell lipids, as shown by the high percentage of the total radioactivity in the growth medium which was incorporated. Sodium acetate, the common precursor for fatty acid synthesis, was poorly incorporated by the
L-phase membrane

L-phase organisms (Table 3). Since the long-chain fatty acids were selectively incorporated into membrane lipids, radioactivity determinations could be used for the estimation of the relative lipid content of various membrane fractions, as described in the following sections.

Solubilization and re-aggregation of membrane material

The L-phase membranes were solubilized by detergents. Sodium dodecyl sulphate (SDS) was more effective in membrane solubilization than sodium deoxycholate (DOC). The clear solution obtained by solubilization of the membranes with SDS became turbid after removal of the detergent by dialysis against dilute β-buffer containing magnesium ions. The re-aggregated membrane material was collected by centrifugation, and chemical analysis showed it to consist of protein and lipid. The Mg²⁺ concentration of the dialysis buffer determined both the degree of re-aggregation of membrane material and its composition (Fig. 3). Since membrane lipids re-aggregated at a lower Mg²⁺ concentration than membrane proteins, re-aggregates obtained at low Mg²⁺ concentration contained a higher percentage of lipid. The ratio of polar to non-polar lipids in re-aggregates obtained at the various Mg²⁺ concentrations was about the same.

Table 4. *Streptobacillus moniliformis* L-phase: separation of membrane protein from lipid by ammonium sulphate fractionation

The L-phase membranes containing labelled lipid were solubilized by SDS+DOC, and membrane proteins were precipitated by ammonium sulphate at 12% saturation as described under Methods.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protein</th>
<th>Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg.</td>
<td>% Total</td>
</tr>
<tr>
<td>Solubilized membranes</td>
<td>26·1</td>
<td>100·0</td>
</tr>
<tr>
<td>Supernatant fluid obtained after</td>
<td>6·0</td>
<td>23·0</td>
</tr>
<tr>
<td>first (NH₄)₂SO₄ precipitation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined supernatant fluids obtained after second and third (NH₄)₂SO₄ precipitations</td>
<td>3·7</td>
<td>11·7</td>
</tr>
<tr>
<td>Combined washing fluids of</td>
<td>2·8</td>
<td>10·7</td>
</tr>
<tr>
<td>precipitate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Washed precipitate</td>
<td>12·9</td>
<td>49·5</td>
</tr>
</tbody>
</table>

Characterization of membrane proteins of *Streptobacillus moniliformis* L-phase

A large portion of L-phase membrane protein could be separated from membrane lipid by a method based on the procedure of Criddle *et al.* (1962) for the isolation of the mitochondrial structural protein. The method, described in detail under Methods, consisted of the solubilization of the membranes by a mixture of SDS+DOC and precipitation of the protein by ammonium sulphate. Table 4 summarizes the results of a typical experiment and shows that the major part of the solubilized L-phase membrane protein precipitated at 12% saturation of ammonium sulphate, while most of the membrane lipid remained in the supernatant fluid. The small amount of lipid that precipitated with the protein could be almost completely removed by repeated dissolution of the precipitate in the detergent mixture and by reprecipitation.
with ammonium sulphate. Since the precipitated protein was highly hydrophobic it could be washed in dilute \( \beta \)-buffer. The hydrophobic protein could be dissolved in 67% acetic acid, dilute NaOH or 0.02 M-SDS. The solution of the hydrophobic membrane protein in SDS exhibited a single symmetrical schlieren peak in the analytical ultracentrifuge. This peak had an uncorrected sedimentation coefficient of 2.4 S, similar to the single peak shown by whole membranes solubilized in SDS.

![Diagram](image)

**Fig. 4.** Electrophoretic patterns of the *Streptobacillus moniliformis* L-phase membrane proteins. (A) Proteins of whole membranes; (B) proteins of membrane re-aggregate formed at 0.01 M-MgCl\(_2\); (C) hydrophobic membrane proteins precipitated at 12% saturation of \((NH_4)_2SO_4\); (D) membrane proteins in the supernatant fluid obtained after removal of the re-aggregate formed at 0.01 M-MgCl\(_2\).

Electrophoretic analysis of the L-phase membranes in polyacrylamide gels containing 35% acetic acid and 5 M-urea revealed about 20 different protein bands (Fig. 4). Membrane re-aggregates formed in the presence of 0.01 M-MgCl\(_2\) contained most of the protein bands characterizing the original membrane. However, several proteins were not incorporated into the re-aggregate and were detected in the supernatant fluid obtained after its sedimentation. The electrophoretic pattern of the hydrophobic protein fraction was similar to, but not identical with, that of the re-aggregate.
DISCUSSION

Over 99% of the *Streptobacillus moniliformis* L-phase organisms were lysed by osmotic shock, or by ultrasonic treatment. However, in all experiments a small portion of the forms did not lyse and remained viable. Moreover, the L-phase organisms resisted lysis by alternate freezing and thawing in 0.5 M-NaCl. In these respects the L-phase organisms resembled the Mycoplasma organisms rather than the bacterial protoplasts which are extremely sensitive to lysis by these methods (Razin & Argaman, 1963; Razin, 1964). Phase-contrast microscopy revealed that the smaller forms in the L-phase culture were the most resistant to lysis. Apparently, the larger surface area of the minute forms allows a rapid efflux of intracellular solutes during the osmotic shock so that the stretching and tearing effects on the cell membrane are less. Thorsson & Weibull (1958) also noticed that the small bodies in a Proteus L-phase culture were relatively resistant to osmotic lysis. The higher resistance to osmotic lysis of the L-phase than of bacterial protoplasts may also be due to variations in the chemical composition of the plasma membrane. That the L-phase membrane is highly elastic is evident from the fact that in hypotonic solutions the forms are able to swell markedly without bursting, as can be seen in the phase-contrast microscope.

The composition of the membrane lipids which, according to the model of Danielli & Davison (1935), form the backbone of the biological membrane, may have a marked influence on its physiological properties, as was recently shown in Mycoplasma (Razin et al. 1966). Cohen & Panos (1966) found the membranes of a *Streptococcus pyogenes* L-phase to contain 35.6% lipid, while membranes of protoplasts prepared from the same bacterium contained only 15.3% lipid. Similar results were obtained by Ward & Perkins (1968) on comparing the lipid content of membranes of the L-phase with protoplasts of *Staphylococcus aureus*. In both cases the percentage of glycolipids was much higher in the L-phase membranes than in the corresponding protoplast membranes. While oleic acid was the predominant C18 monoenoic acid in the L-phase membrane of *S. pyogenes*, cis-vaccenic acid predominated in the *S. pyogenes* protoplast membrane (Panos et al. 1966). Smith & Rothblat (1962) showed that the lipid content of the L-phase of *Streptococcus moniliformis* was about 5 times that of the bacterial form; L-phase variants of other bacteria contained 2–3 times as much lipid as their parent bacteria. The high lipid content (about 40%) found in the present study in membranes of the *S. moniliformis* L-phase is in line with these observations. The data obtained so far seem to indicate that when a bacterium is transformed into its stable L-phase the percentage of lipid in the plasma membrane increases, and changes occur in its composition. The high percentage of lipid in the L-phase membrane resembles that found in Mycoplasma membranes (Razin, 1967).

The *Streptobacillus moniliformis* L-phase membrane also resembles Mycoplasma membranes, and differs from protoplast membranes, in its high cholesterol content. Almost all the non-polar lipid fraction of the L-phase membranes (about one-third of the total lipid) consisted of cholesterol in the non-esterified form. Cholesterol is apparently incorporated from the growth medium, as shown by Rebel, Bader-Hirsch & Mandel (1963) for the L-phase of Proteus sp. strain P18. The presence of large amounts of cholesterol in the L-phase membranes may be associated with the fairly high osmotic resistance of the organisms, since cholesterol was found to increase the osmotic stability of parasitic mycoplasmas (Razin, 1967).
The question whether the bacterial L-phase variants require cholesterol for growth is of great importance for their differentiation from mycoplasmas which do require it. The few stable L-phase variants of Proteus species (Medill & O'Kane, 1954; Abrams, 1955) and of Streptococcus pyogenes (van Boven, Kastelein & Hijmans, 1967) so far grown in chemically defined media did not show a cholesterol requirement. Edward (1953) when using a complex medium was unable to demonstrate that the Streptobacillus moniliformis L-phase required cholesterol. Since no defined medium has so far been devised for the growth of this L-phase, the question of its cholesterol requirement does not seem to be definitely settled.

Solubilization of the L-phase membranes by detergents enabled the major part of membrane protein to be separated from lipid. The protein fraction isolated was very hydrophobic, resembling the 'structural' protein fractions isolated by detergent action from a variety of biological membranes including those of Mycoplasma (Green & Tzagoloff, 1966; Rodwell et al. 1967). Polyacrylamide-gel electrophoresis showed this fraction to consist of most of the protein species found in the membrane. Polyacrylamide-gel electrophoresis has recently shown that the mitochondrial structural protein, formerly regarded as a homogeneous protein species also consists of several different proteins (Beattie, Basford & Koritz, 1967).

Most of the membrane proteins which were separated from membrane lipids by the detergent action re-associated with membrane lipid on removal of the detergent by dialysis against a magnesium solution. The membrane re-aggregates consisted mostly of typical triple-layered membranes (S. Rottem, O. Stein & S. Razin, unpublished). As with Mycoplasma and bacterial protoplast membranes (Razin et al. 1965; Terry, Engelman & Morowitz, 1967; Butler, Smith & Grula, 1967; Rottem, Stein & Razin, 1968) a divalent cation, e.g. Mg\(^{2+}\), was essential for the re-aggregation of the solubilized L-phase membrane material. The ratio of lipid to protein in the re-aggregate was greater at a low Mg\(^{2+}\) concentration, in accord with the results obtained with Mycoplasma membranes. The varying lipid:protein ratio in membrane re-aggregates was regarded as a contra-indication for the presence of homogeneous lipoprotein subunits in the solubilized membrane material (Rottem et al. 1968).

Comparison of the proteins incorporated into membrane re-aggregates with those found in the hydrophobic protein fraction and in the original membranes is of interest (Fig. 4). The incorporation of solubilized membrane proteins into the re-aggregates was selective. While the proportion of the majority of the membrane proteins was the same in the re-aggregate as in the original membranes, some proteins did not re-associate with the membrane lipids and remained in the soluble supernatant fluid fraction. Comparison of the electrophoretic patterns of the hydrophobic protein fraction and of the re-aggregate shows the marked similarity of the two. But again, some proteins found in the re-aggregate were not present in the hydrophobic protein fraction, and vice versa. It seems, therefore, that membrane re-aggregates are not formed only of the hydrophobic membrane proteins. This fits with the discovery of the protein(s) responsible for NADH oxidation in membrane re-aggregates of Mycoplasma laidlawii, which was absent from the hydrophobic protein fraction (Razin et al. 1965; Rodwell et al. 1967). Polyacrylamide-gel electrophoresis seems to be a most efficient tool for further studies on membrane reassembly.
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S. RAZIN AND C. BOSCHWITZ


