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Isolation, Composition, and Structure of Membrane of *Listeria monocytogenes*[1]

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The plasma membrane of *Listeria monocytogenes* strain 42 was prepared by osmotic lysis of protoplasts with tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 8.2, containing $MgCl_2$ and glucose, followed by washing with NaCl and $MgCl_2$ in Tris buffer. Electron microscopy showed that the preparation was not contaminated with cytoplasmic material. The membrane preparation was composed of 55 to 60% protein, 1.5% ribonucleic acid, 0.1% deoxyribonucleic acid, 1.3 to 2.3% carbohydrate, 0.17 to 0.38% amino sugar, 0.2 to 0.4% rhamnose, 3.5 to 4.0% phosphorus, 10.5 to 12.0% nitrogen, and 30 to 35% lipid. Amino acid composition of the washed membrane showed some variation from that of the whole cells. Sulfur-containing amino acids were not present in the membrane hydrolysate. The membrane carbohydrate contained glucose, galactose, ribose, and arabinose. The membrane lipid was 80 to 85% phospholipid and 15 to 20% neutral lipid. The lipid contained 2.3 to 3.0% phosphorus, 2.5 to 3.0% carbohydrate, and a very small amount of nitrogen (0.2 to 0.3%). The phospholipid was of the phosphatidyl glycerol type. Electron micrographs of the washed membrane showed three layers. The outer and inner layers varied in thickness from 25 to 37 A and the middle layer from 20 to 25 A. The total thickness varied between 85 and 100 A. These preparations contained many vesicles which stained heavily with lead citrate. Some vesicles were also attached to the protoplast ghosts in the form of extrusions or intrusions, or both. Membrane preparations obtained by lysis of protoplasts in the absence of $MgCl_2$ were fragmented and contained less lipid (20 to 22%) and ribonucleic acid (0.3 to 0.5%) than preparations prepared with $MgCl_2$.

The bacterial membrane has attracted the attention of many investigators since its isolation by Weibull (38, 39) as a ghost fraction of lysed protoplasts. Specialized subcellular membranous organelles, such as mitochondria, Golgi apparatus, and endoplasmic reticulum, have not been demonstrated in bacterial cells. However, mesosomes (intracytoplasmic membranous structures) of varying morphology have been demonstrated frequently in the gram-positive bacteria (23), the actinomycetes (5, 36), and rarely in the gram-negative bacteria (30). The role of these structures as subcellular organelles has not been demonstrated. The most widely accepted view is that the peripheral membrane of the bacterial cell, besides controlling permeability, carries out a wide range of metabolic work. Various investigators have studied the chemical composition (8, 17, 28, 33), the enzymatic makeup (7, 9, 13), the molecular anatomy (27), and the variation in composition resulting from changes in nutrition and growth phase (28, 33) of the membrane.

Listeria monocytogenes is a gram-positive bacterium, the cells of which have complex intracytoplasmic extensions of the plasma membrane (6, 10). The organism produces a lipid material which stimulates monocytosis in certain animals and in man (34). In gram-positive organisms, most of the lipid is concentrated in the plasma membrane (15). Thus, it seems possible that this pharmacologically interesting lipid may be localized in the plasma membrane. These considerations prompted us to study the plasma membrane of this organism more closely. We previously reported (10) that some strains of *L. monocytogenes*, including the one used for the present

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study (strain 42), are lysozyme-resistant and become sensitive to lysozyme only after lipase treatment. This report describes the procedure for isolation of the plasma membrane of *L. monocytogenes* (strain 42), as well as the chemical composition and structure of this membrane.

MATERIALS AND METHODS

Preparation and lysis of protoplasts. L. monocytogenes strain 42 was used throughout these experiments. Maintenance of the organism and conditions of cell cultivation were described in a previous communication (10).

Protoplasts were formed by lipase and lysozyme treatment (10). The protoplasts [10 to 15 mg (dry weight)/ml] were washed in a solution of 0.03 M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 6.8 to 7.0, 0.01 M $MgCl_2$, and 0.5 M sucrose (Tris-Mg-sucrose) at room temperature (28 to 30 C). The washed protoplasts were suspended in various lytic solutions and were shaken at 37 C for 30 min to effect complete lysis. After lysis, the optical densities of these solutions were measured against a blank containing only the suspending medium at 660 mμ. The lysate was centrifuged at 10,000 × *g* for 15 min, and the absorptions of the resulting clear supernatant fluid were measured at 260 and 280 mμ to assess release of intracellular soluble materials. This method was used to choose a suitable lysing solution. The lytic solutions tested were: (i) distilled water, (ii) 0.01 M $MgCl_2$, (iii) 0.01 M $MgCl_2$ in 0.01 M Tris buffer (*p*H 8.1), (iv) 1.0 M glycerol, and (v) 0.01 M Tris buffer (*p*H 8.1) containing 0.02 M $MgCl_2$ and 0.01 M glucose (Tris-Mg-glucose).

Preparation of the membrane. Lysis of the washed protoplasts was carried out in Tris-Mg-glucose solution as described above. The lysate was transferred into a Sorvall Omnimixer bottle (Ivan Sorvall, Inc., Norwalk, Conn.) and homogenized at 8,000 rev/min for 10 min under ice and was then frozen at −20 C overnight. The next morning, the lysate was thawed and homogenized as above. The homogenate was centrifuged at 41,000 × *g* for 30 min and the clear supernatant fluid was kept as soluble cytoplasm. The residue was suspended at room temperature (28 to 30 C) in a solution containing 0.01 M Tris buffer (*p*H 8.2), 0.05 M NaCl, and 0.02 M $MgCl_2$ (Tris-NaCl-Mg), and the mixture was centrifuged at 105,000 × *g* for 45 min. The process was repeated twice. Finally, the residue was washed and suspended in 0.01 M $MgCl_2$. A flow diagram of the preparation of washed membrane is shown in Fig. 1.

Analytical. A sample of the washed membrane suspensions was transferred to a cleaned and weighed aluminum dish, then heated at 70 to 80 C for 2 to 3 hr, followed by overnight drying in vacuo over P_2O_5. The difference in weight after subtracting the equivalent blank of $MgCl_2$ gave the dry weight of the membrane preparation. Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were extracted according to the method of Ogur and Rosen (25), with the exception that the initial precipitation was carried out with 0.2 N perchloric acid (PCA) rather than with ethyl alcohol. The amounts of separated RNA and DNA were estimated by the determination of phosphorus (7), ribose (25), and deoxyribose (32). These results were checked by ultraviolet absorption measurements. Protein was estimated in 10% cold trichloroacetic acid precipitate by Lowry's method (20). Total amino acid was determined in 6 N HCl hydrolysate (for 18 hr) by Rosen's method (27). Individual amino acids were analyzed in a Beckman Spinco model 120 amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.). Carbohydrate was estimated by the anthrone method (31) in the acid hydrolysate of the membrane (2 N HCl for 2 hr at 100 to 105 C), after removing the acid by treating with Dowex 50 ion-exchange resin. The reducing sugar was determined according to the method of Nelson (24) and individual sugars were identified by paper chromatography (14). The rhamnose content was estimated by the spectrophotometric cysteine-sulfuric acid method (4), whereas glucosamine was determined by the use of a modified Elson and Morgan reaction (26). Hydrolysis of the membrane for this determination was conducted in a sealed ampoule in 6 N HCl for 2 hr at 100 to 105 C. The HCl was removed by repeated evacuation over P_2O_5 and KOH pellets. Nitrogen was assayed by a modified Nessler's reaction (19). For lipid analysis, a membrane preparation was lyophilized, after which the lipid was extracted from the lyophilized membrane powder by shaking at room temperature with chloroform-methanol (2:1) for 12 to 18 hr. The extract was filtered and evaporated in a flash evaporator. The resulting residue was dissolved in chloroform, and the quantity of residue was determined by weighing a sample of the solvent after evaporation under nitrogen. The lipid was analyzed for nitrogen, phosphorus, carbohydrate, and amino acid. Phospholipid and neutral lipid were determined by Florisil column chromatography, and the phospholipids were further characterized by thin-layer chromatography. (2).

Electron microscopy. Membrane structure was examined by use of a Phillips EM 100 electron microscope (Phillips Electronics and Pharmaceutical Industries Corp., St. Joseph, Mo.). Membrane preparations in different stages of purification were prefixed in 3% glutaraldehyde and then fixed and embedded according to the method used by Ryter and Kellenberger (16). Details of the thin sectioning and staining methods were described in an earlier communication (10). Membrane preparations were negatively stained with 1% phosphotungstic acid (neutralized to *p*H 6.1 with KOH) or were shadowed lightly (3:1 slope) with tungsten oxide in an Edward's Evaporator (model 13 E6; Philips Electronic Instruments, Mt. Vernon, N.Y.

RESULTS

Changes during lysis. Protoplasts suspended in Tris-Mg-sucrose gradually swelled and liberated small amounts of protein and low molecular weight material absorbing at 260 mμ.

The membranes of protoplasts suspended in distilled water broke up into fragments. Fragmentation could be prevented by the addition of 0.01 M $MgCl_2$. This was not a suitable washing solution, however, as it prevented the release of

cytoplasmic protein and nucleic acid. The lysis in Tris-Mg-glucose was gradual, and there was very little fragmentation of the membrane material. At an alkaline *p*H (8.1), Tris buffer prevented agglutination of the protoplast lysate and allowed greater solubilization of the cytoplasmic material.

The supernatant fluids of the lysozyme incubation mixture, the disintegrated protoplasts, and the respective washes were collected separately and assayed for protein and nucleic acid (Fig. 1). Figure 2 illustrates the release of protein and nucleic acids on successive washings. Only a small amount of protein was released by the lysozyme treatment, but there was no liberation of RNA and DNA, indicating no gross lysis of protoplasts in the lysozyme incubation medium. The suspension of the protoplasts in Tris-Mg-glucose caused the liberation of 53% of the total protein, 63% of the total RNA of the cell, but only 6% of the total DNA. The wash in Tris-NaCl-Mg removed about 90% of the total DNA, but less RNA and protein were released.

After the fourth wash, successive washes removed a small but constant amount of RNA, DNA, and protein. This may be an indication of partial fragmentation of the membrane rather than removal of unbound or weakly bound nonmembrane components (Fig. 2). Analysis of the ratio of 260-mμ absorption to 280-mμ absorption indicated that the main release of material absorbing at 260 mμ occurred during the second wash.

During the successive washes of the membrane, it was noted that about 20% of the protein and 10% of the RNA could not be recovered, whereas nearly all of the DNA was recovered. This may be due to ribonuclease and proteolytic activity in the degraded cell. Results of these balance experiments (Table 1) show that the membrane contained 8 to 10% of the total cell protein. Practically all of the cellular lipid was present in the membrane, and the membrane also retained a significant amount of the cell's carbohydrate.

Composition. In common with all biological membranes, the plasma membrane of *L. monocytogenes* is composed mainly of protein and lipid. The results of the general analysis of the plasma membrane, prepared both in the presence and absence of $MgCl_2$, are given in Table 2. Data from the analysis of the whole cell were included for comparison. Removal of cell wall material was indicated by the absence of rhamnose and amino sugar, both of which are typical cell wall components. Table 1 illustrates the fraction of cell components found in the cell membrane. Membrane preparations made in the presence of $MgCl_2$ contained a considerable amount of RNA with respect to dry weight, but this represented only about 2 to 3% of the total cellular RNA. Despite the small amount of RNA present, it seems to be a significant membrane component. RNA was very firmly bound to the membrane and could only be removed by ribonuclease treatment. However, the amount of RNA dropped to a very low value when the membrane was prepared in the absence of $MgCl_2$. A very small amount of DNA (0.8% of total DNA) was detected in the membrane prepared both in presence and absence of $MgCl_2$, but its significance was not apparent from the present experiments. The phosphorus content of the membrane was lower than that of whole cells. The latter contained more nucleic acid phosphorus than did the membrane. The membrane, on the other hand, contained almost all of the phospholipid phosphorus. The total amount of phosphorus determined in the membrane could not be accounted for by the lipid and nucleic acid phosphorus. Therefore, other phosphorus-containing materials must remain bound to the membrane. It is not unlikely that components like teichoic acid exist partly as membrane-bound materials. A large amount of phosphoserine was present in the protein hydrolysate of the membrane.

The general composition of the lipid is shown in Table 3. The nitrogen content of this lipid was very low, and amino acids and choline were absent in this preparation. The major phospholipid fraction, obtained from an acid-treated Florisil column, was further fractionated by thin-layer chromatography. A chloroform solution of the phospholipid fraction was applied to the thin-layer plate as a band, and this plate was then developed in a chloroform-methanol-water (60:20:3) solvent system. Three opaque bands were obtained after spraying with water: (i) lower (near origin), (ii) middle, and (iii) upper (near the running front). The nitrogen, phosphorus, and carbohydrate analyses of these fractions are presented in Table 4. The results show that these fractions have a low nitrogen content. The middle fraction is low in phosphorus but high in carbohydrate. Paper chromatography of the hydrolysate of the middle fraction showed the presence of glucose and galactose. The phosphorus concentration was highest in the upper fraction. Preliminary characterization showed that the major phospholipid may be of a phosphatidyl glycerol type. Studies on the detailed characterization of this lipid are in progress (K. K. Carroll, H. J Cutts, and E. G. D. Murray, *unpublished data*).

The amino acid composition of the membrane is presented in Table 5. Membrane and whole

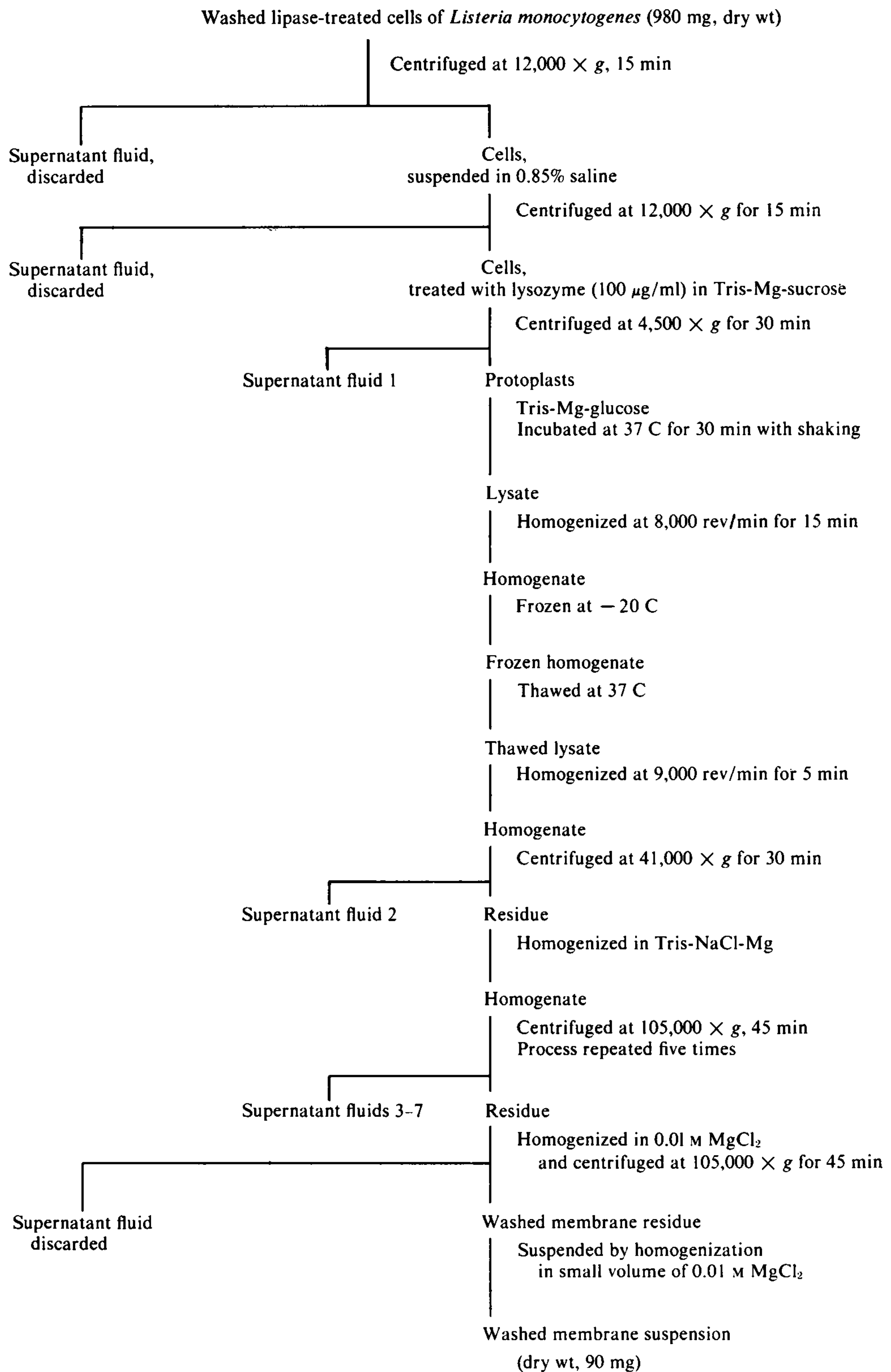


FIG. 1. *Procedure for isolation of pure Listeria monocytogenes membrane.*

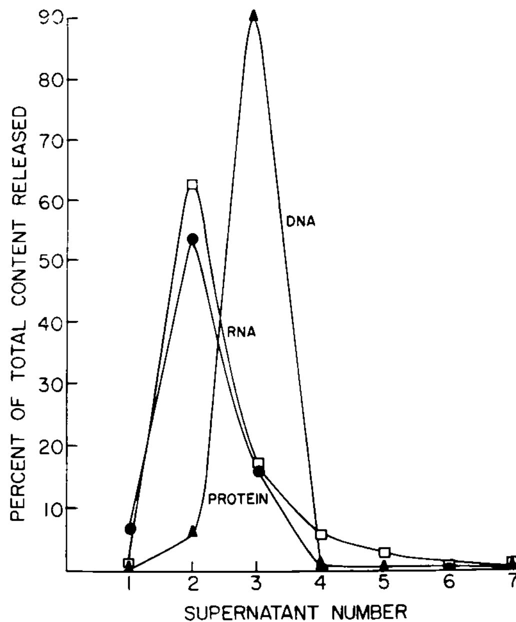


FIG. 2. *Release of protein and nucleic acid during lysozyme incubation, lysis of protoplasts, and washing of membrane. Supernatant fluid number represents number of supernatant fluids designated in Fig. 1.*

cell preparations contained a very large amount of aspartic acid, glutamic acid, alanine, glycine, lysine, and leucine. Ornithine was present only in trace amounts in the whole-cell preparation, but the membrane preparation contained a considerably greater amount of this substance. Hydroxyproline was present in very small amounts, and there was no peak for half cystine. Methionine could not be detected in the membrane preparation, but the whole cell preparation contained about 0.8%. In the whole cell preparation, there were two peaks in the acidic region, one of which was identified as methionine sulfoxide. There were no such peaks in the membrane preparation. In both whole cell and membrane preparations, there were peaks of phosphoserine and glycerylphosphoryl ethanolamine.

The carbohydrate composition of the membrane is shown in Table 6. There was very little glucosamine or rhamnose present. Qualitative paper chromatography revealed that, in addition to hexoses, there were two pentoses, ribose and arabinose, in the membrane. The hexoses were identified as mainly glucose and galactose, in approximately equimolar amounts.

The membrane generally constituted 7 to 10% of the dry weight of the whole cell. The yield of the membrane material prepared in the absence of $MgCl_2$ was reduced to 4 to 5%. In the latter

TABLE 1. *Proportion of whole cellular material retained by the membrane*[a]

Cellular material	Content as percentage of whole cellular material
Protein	8–10
Lipid	88–91
Carbohydrate	6–8
RNA	2–3
DNA	0.8–1.0

[a] Figures represent the range of the values obtained in five different experiments.

preparation, the RNA and lipid content were much smaller than in the preparation made in the presence of $MgCl_2$ (Table 2). But the compositions of lipid, protein, and carbohydrate were comparable in both cases.

The membrane preparation made without $MgCl_2$ fragmented into smaller parts which reformed into small vesicles (Fig. 7 and 8). In addition, many particles which could not be separated by centrifugation at 105,000 × *g* were also released. Both $CaCl_2$ and $SrCl_2$ could partially protect the membrane against fragmentation.

Structure. A negatively stained preparation of a typical protoplast used for the preparation of plasma membrane is shown in Fig. 3. The preparation shows three flagella still attached to the membrane. Cells of *L. monocytogenes* grown at room temperature (28 C) showed the presence of flagella (1 to 3 in number), but the cells grown at 37 C contained no flagella.

An enlarged picture of the plasma membrane is presented in Fig. 4. The plasma membrane, with some amorphous cytoplasmic material and internal membranous structures still attached can be seen. In such preparations, a specialized region can be noted frequently. The outer component of the three-layered membrane can branch and surround an inner membrane, which then takes the form of a small vesicle.

Figure 5 shows a thin section of a membrane preparation after the third wash. A substantial amount of cytoplasmic material is still retained by the membrane material. There are many concentric vesicular structures of various sizes. These may be reformed structures from the fragmented plasma membrane and do not have any special significance. There are some smaller vesicular structures, very heavily stained with lead citrate, which may be different in character from the reformed vesicles. There are also many small vesicles attached to the membrane which could be derived from the small invaginated structures seen in the membrane of the whole cell. A membrane preparation washed eight times is illustrated

TABLE 2. *Chemical composition of membrane of Listeria monocytogenes strain 42*[a]

Membrane	Protein	Total amino acid	RNA	DNA	Total nitrogen	Phosphorus	Carbohydrate (anthrome)	Rhamnose	Amino sugar	Lipid
Whole cell (5 sets)	54–61	47–52	5.0–5.4	1.7–2.1	10.1–11.4	5.1–5.5	6.0–6.2	1.4–2.0	4.4–4.5	4–5
Membrane washed in presence of $MgCl_2$ (7 sets)	55–60	51–52	1.5–1.6	0.1–0.2	10.5–12.0	3.5–4.0	1.3–2.3	0.2–0.4	0.17–0.38	30–35
Membrane washed in absence of $MgCl_2$ (3 sets)	61–62	53–55	0.3–0.5	0.1–0.2	—	3.7–4.1	1.7–2.0	.06–0.1	0.15–0.2	20–22

[a] Results expressed as percentage dry weight, whereas figures represent the range of variation in different sets of experiments.

TABLE 3. *Gross composition of membrane lipid of Listeria monocytogenes strain 42*[a]

Lipid	As percentage of whole lipid
Phospholipid	80–85
Neutral Lipid	15–20
Phosphorus	2.3–3
Nitrogen	0.2–0.3
Carbohydrate	2.5–3.0

[a] Figures represent the range of variation in five different experiments.

TABLE 4. *Gross composition of the three phospholipid fractions of the membrane lipid of Listeria monocytogenes separated on thin layer*

Fraction	Nitrogen	Carbohydrate (anthrone method)	Phosphorus
Upper	0.45[a]	0.67	2.40
Middle	0.16	12.00	0.65
Lower	0.37	1.00	1.10

[a] Results expressed as a percentage.

in Fig. 6. Here we see the complete disappearance of cytoplasmic material, although the heavily stained vesicular structurs are still present. Clean preparations like this were used for chemical analysis.

Figures 7 and 8 present shadowed preparations of membrane material, prepared with and without $MgCl_2$, respectively. This clearly demonstrates the fragmentation of large protoplast ghosts into smaller vesicles in the absence of $MgCl_2$.

Thin sections presented in the above illustrations show that the membrane of *L. monocyto-*

TABLE 5. *Amino acid composition of the cytoplasm membrane and whole cells of Listeria monocytogenes strain 42*

Amino acid	Concn [as % (dry wt) of membrane]	Molar ratio[a]	Concn [as % (dry wt) of wholecell]	Molar ratio
Alanine	3.33	12.5	4.6	9.3
Arginine	2.50	4.3	2.37	2.5
Aspartic acid	5.88	13.7	3.54	4.8
Glutamic acid	5.94	13.4	5.1	6.2
Glycine	2.50	14.0	1.87	4.5
Histidine	0.73	1.7	0.86	1.0
Hydroxyproline	trace	—	trace	—
Isoleucine	1.99	6.6	1.67	2.3
Leucine	3.82	10.8	2.88	4.0
Lysine	6.23	9.8	5.15	6.3
Methionine	—	—	0.81	1.0
Ornithine	1.55	1.0	trace	—
Phenylalanine	1.80	4.5	1.31	1.5
Proline	1.80	6.5	1.57	2.5
Serine	2.24	9.6	1.62	2.8
Threonine	2.90	8.2	1.71	2.6
Tyrosine	1.22	2.3	1.16	1.1
Valine	1.93	6.9	2.1	3.2

[a] Determined by dividing all the amino acid concentrations (in micromoles) by the smallest amount.

genes has three layers (outer and inner, electron-dense; middle, electron-lucid) similar to other biological membranes. The total thickness of the three layers varied from 85 to 100 A. The thicknesses of individual components were: outer and inner, 25 to 37 A; middle, 20 to 25 A.

DISCUSSION

Estimates of the nucleic acid content of bacterial membranes made by various investiga-

TABLE 6. *Carbohydrate composition of membrane of Listeria monocytogenes strain 42*

Component	Amt as percentage of membrane (dry wt)
Total sugar by anthrone method (as glucose)	2.3
Reducing sugar by Nelson Somyogi method (as hexose)	1.2
Rhamnose by cysteine sulfuric acid method (as methyl pentose)	0.4
Total pentose by orcinol method (as Ribose)	0.82
Hexosamine (as glucosamine HCl)	0.15

tors (11, 37, 40, 41) differ widely. The reasons for this arise from the variety of preparative procedures, particularly in washing procedures and in the use of hydrolytic enzymes (such as deoxyribonuclease or ribonuclease). The chief criterion of purity used in the present study was the appearance of electron micrographs of membranes after each treatment. We accepted the possibility that membranes may have nucleic acid and carbohydrate bound to them in a special way, and therefore we desisted in the use of injurious techniques, such as deoxyribonuclease or ribonuclease treatment.

Our electron micrographs clearly indicate that Mg is required to preserve membranes during osmotic lysis. However, the slower lysis obtained in Tris-Mg-glucose gives a superior membrane to those made with Tris-Mg.

Successive washing of the isolated membrane with various media indicated that the cytoplasmic material contained in the membrane can be removed. The extreme viscosity of DNA in protoplast lysate was overcome by two cycles of freezing and thawing. Residual free DNA was removed most effectively in media containing NaCl. By use of an alkaline *p*H, aggregation of the membrane fraction was substantially reduced.

The requirement for $MgCl_2$ in the maintenance of the structural and functional integrity of the membrane was reported by various workers. Lukoyanova (21) reported that succinic oxidase activity of *Bacillus megaterium* membrane depended upon optimal $MgCl_2$ concentration. Abrams (1) reported that membrane-bound adenosine triphosphatase was released from *Streptococcus faecalis* membrane if washed in the absence of $MgCl_2$. Lampen (18) reported that the release of membrane-bound penicillinase from *B. licheniformis* depended upon the integrity of membrane structure, and this structural stability could be retained by an optimal concentration of $MgSO_4$. The role of Mg^{++} in relation to RNA content of the membranes has been discussed by Mizushima (22). Our results also demonstrated that Mg^{++} must be present to maintain the chemical and physical integrity of the membrane. It is quite possible that the divalent cations act as cross-linking components between protein and various other constituents of membrane.

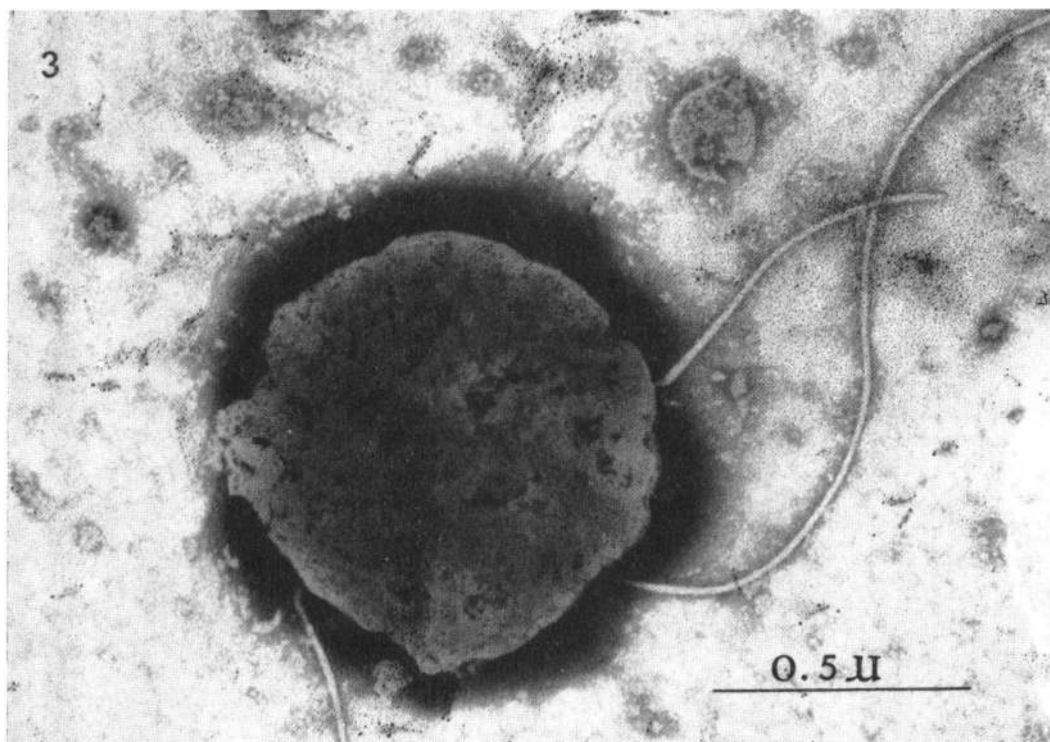
There was very little DNA in the washed membrane, and it is not known whether this small amount of membrane-associated DNA (about 0.8% of whole DNA content) had any significance. It is clear, however, that it is quite strongly bound.

Although most of the RNA is free in the cytoplasm, 1.5 to 1.6% of the total is very strongly bound to the membrane. This binding, however, is markedly dependent on the presence of Mg, and in its absence the binding drops to 20 to 30% of this figure.

The membrane of *L. monocytogenes*, prepared by the method described, did not contain cell wall material since the typical cell wall components like hexosamine or rhamnose were absent. Absence of cell wall material was also demonstrated by electron microscopy. Therefore, the carbohydrate material of the membrane appears to be a significant membrane component. There are many conflicting reports concerning the presence of carbohydrate in the bacterial membrane. Weibull and Bergstrom (39, 40) reported 1 to 10% carbohydrate in a *B. megaterium* membrane. In the same organism, however, Godson et al. (12) reported about 40% carbohydrate. Gilby (11) showed the presence of 20% mannose in *Micrococcus lysodeikticus* membrane.

The protoplast membrane contained about 90% of the lipid of *L. monocytogenes*. The lipid was very poor in nitrogen and contained glucose and galactose. Phospholipid was the major fraction of the total lipid. The neutral lipid content increased when the lipid was stored for some time before analysis. Presumably, this was due to spontaneous degradation of the phospholipids. Analysis of the lipid immediately after extraction showed a 15 to 20% neutral lipid content. However, the possibility of degradation and formation of neutral lipid during the extraction procedure cannot be excluded. The presence of considerable amounts of phosphorus and nitrogen in the chloroform-insoluble, but methanol-soluble, fraction indicates that proteolipid may be a component.

No evidence is yet available concerning the significance of glycolipid in the membrane. It was recently reported that membranes of L forms of bacteria contain very large amounts of glycolipid when compared with the original protoplast. Elevated glycolipid and decreased phospholipid



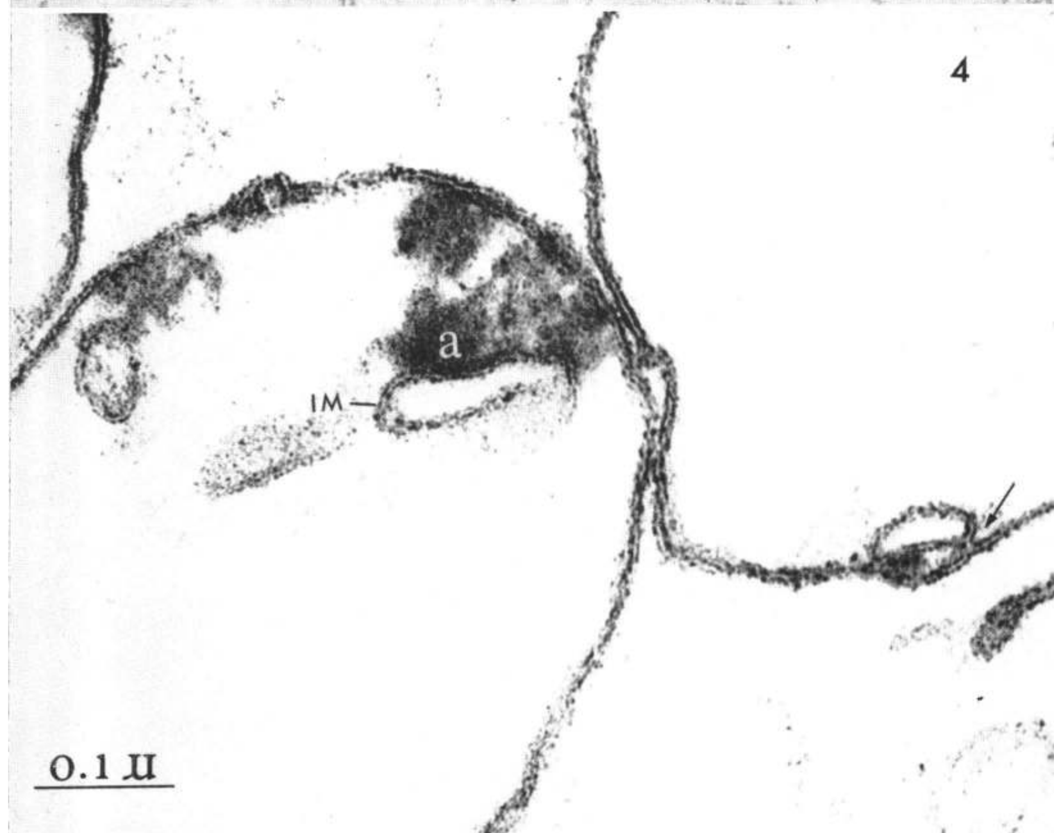


FIG. 3. *Negatively stained preparations of protoplast with PTA.*

FIG. 4. *Thin section of plasma membrane stained with lead citrate. Note the branching of plasma membrane (arrow) and internal membrane (IM) in the amorphous cytoplasmic material retained by the ghost.*

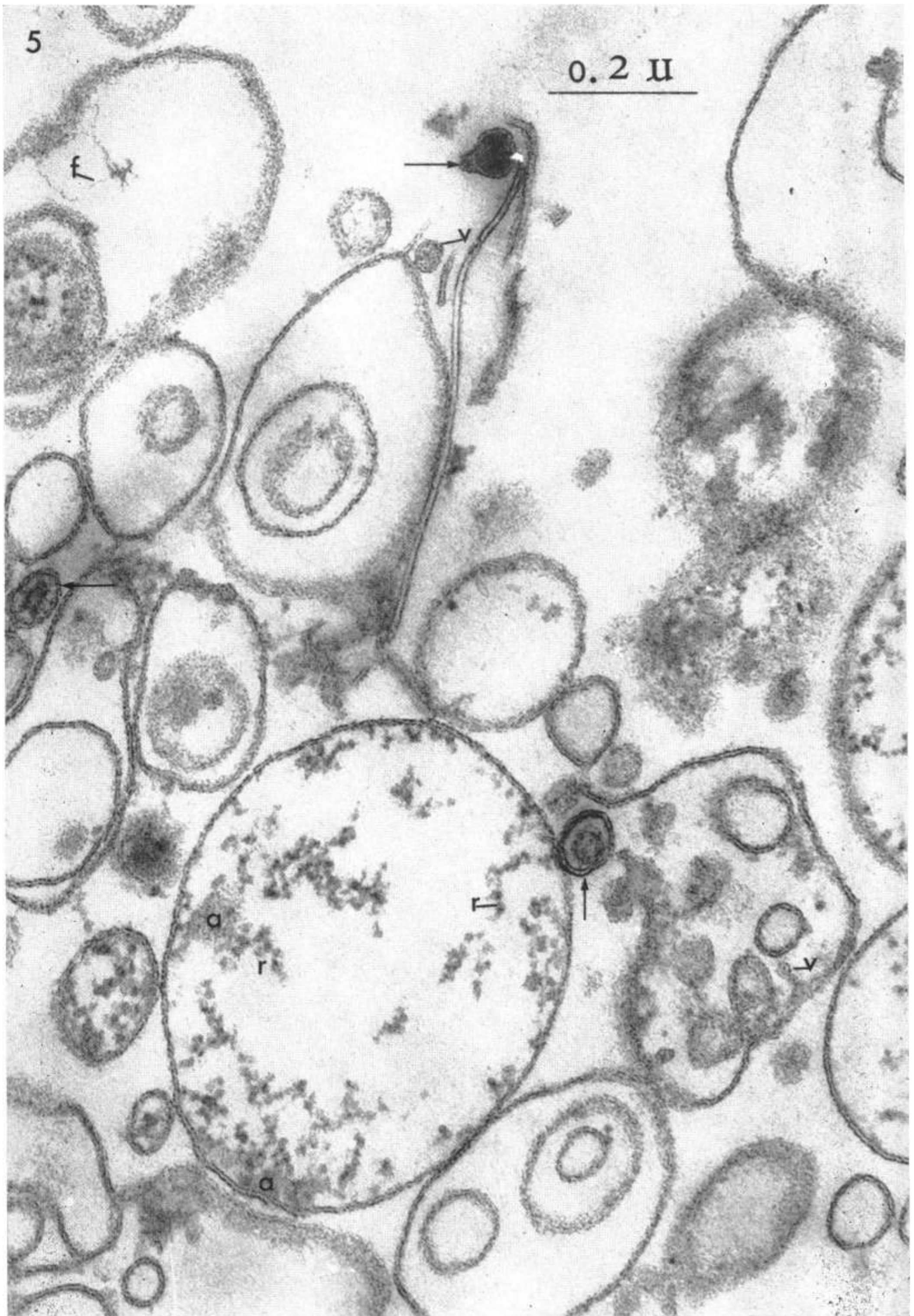


FIG. 5. *Thin section of plasma membrane preparation, after the third wash, stained with lead citrate. Note the contamination with ribosomes (r), amorphous cytoplasmic material (a), and the fibrilar material (f). There are some heavily stained vesicular structures (arrow) and some small vesicular structures (v) originating from plasma membrane.*

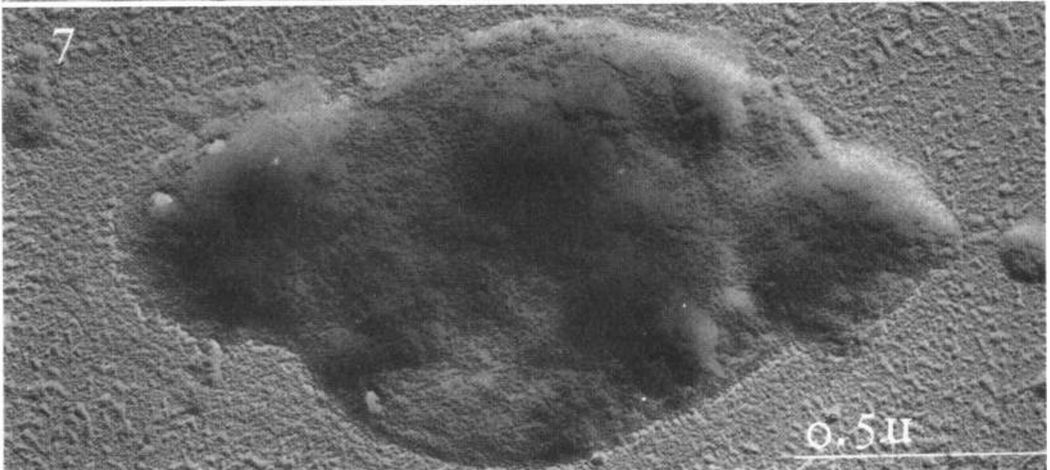


FIG. 6. *Thin section of plasma membrane preparation, after seventh wash, stained with lead citrate. Cytoplasmic material is completely removed. Note the heavily stained vesicular structures (arrow).*

FIG. 7. *Membrane prepared with $MgCl_2$. Preparation shadowed with tungsten oxide.*

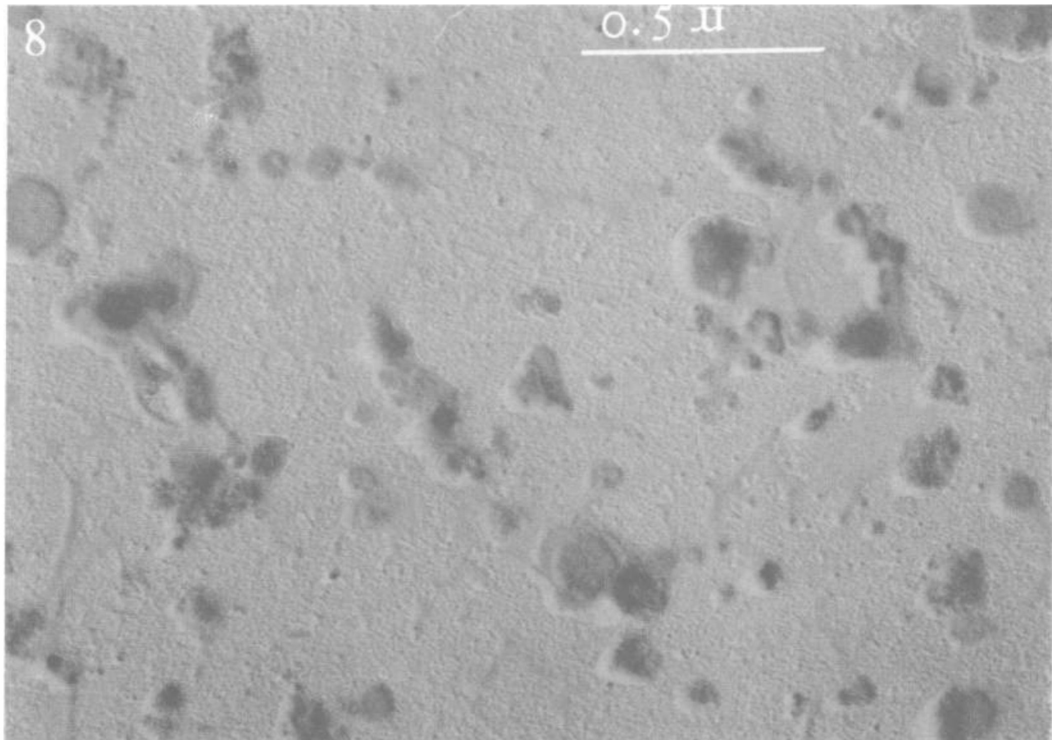


FIG. 8. *Membrane prepared in absence of $MgCl_2$. Preparation shadowed with tungsten oxide.*

were thought to explain the inability of L-form cells to synthesize cell wall material (3). A phospholipid factor is required in a penicillin-insensitive but vancomycin-sensitive, step of cell-wall biosynthesis (35). There is evidence which supports the view that the lipids of membranes are passive components of the permeability barrier, as well as components of diverse biosynthetic processes of the cell.

Electron microscopic observations indicate the importance of extensive washing to obtain a clean membrane preparation. However, since a small amount of firmly bound cytoplasmic material may be present even after extensive washing, minor components, such as nucleic acids, should be viewed, with reservations, as membrane components.

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