STUDIES OF L FORMS AND PROTOPLASTS OF
GROUP A STREPTOCOCCI*

II. CHEMICAL AND IMMUNOLOGICAL PROPERTIES OF THE CELL MEMBRANE

By EARL H. FREIMER, M.D.

(From The Rockefeller Institute)

Protoplasts are spherical structures obtained from living bacteria by enzymatic removal of the cell wall. These structures were first isolated from *Bacillus megaterium* by treatment with lysozyme in a hypertonic environment, a feature which proved essential for the preservation of intact protoplasts (1). Although the osmotically fragile protoplasts rupture when the hypertonicity is reduced, the limiting protoplast membrane remains as a well defined structure. The isolation, growth, and bacteriological characteristics of protoplasts prepared from Group A streptococci have been described, and chemical and immunological studies have demonstrated that these streptococcal protoplasts are essentially free of group-specific carbohydrate and M protein, two important antigens of the streptococcal cell wall (2). Membranes, isolated after osmotic rupture of streptococcal protoplasts, have been shown to be morphologically distinct from streptococcal cell walls.

In the present investigation, streptococcal protoplast membranes have been prepared essentially free of both cell wall and intracellular material, and the chemical composition and immunological properties of these membranes have been investigated. In addition, a method has been devised to separate from mechanically disrupted bacteria a membrane fraction similar in chemical composition and serological properties to those membranes isolated from enzymatically derived protoplasts.

Materials and Methods

Strains of Streptococci.—Group A streptococcal strains T1/119 (Type 1), D58X/11 (Type 3), S43/100 (Type 6), T12/36 (Type 12), T22/83 (Type 22), T25/41FP (Type 25), and A405 (Type 50); A-variant strain A305 (Type 14); Group B strain A537; Group C strains K64 and 26RP66; Group D strains C1, C3, and D76; Group G strains D166B and F68; two strains of *Streptococcus viridans*, SBE154 and B683; and *Staphylococcus aureus* strain NYH6 have been used in this investigation. These strains were all from The Rockefeller Institute Collection.

Serological Identification.—The serological identification of the strains used in these experiments was confirmed with the capillary tube precipitin test using streptococcal group- and type-specific rabbit antisera (3).

Phage-Associated Lysin.—The partially purified phage-associated lysin was prepared as described by Krause (4).

Analytical Methods.—Rhamnose was determined by the method of Dische and Shettles (5). Quantitative glucosamine determinations were done by a modification of the Elson and Morgan procedure (6). Quantitative glucose analyses were done by a modified method employing glucose oxidase (glucostat) available from the Worthington Biochemical Corporation, Freehold, New Jersey. Microdeterminations of phosphorus were performed by the method of Chen (7). Total nitrogen was determined by a modification of the micro-Kjeldahl technique involving the method of Koch and McMeekin (8). Desoxyribonucleic acid was determined by the Burton modification of the diphenylamine reaction (9). Ribonucleic acid was determined by the orcinol reaction. Total nucleic acid content was determined by the absorption of solutions at 260 mμ in the Beckman spectrophotometer.

Preparation of Cell Walls.—The cell walls were prepared by the method of Salton in which streptococci are disrupted in a Mickle disintegrator (10). The cell walls are separated from residual cellular material by centrifugation, washed with distilled water until free of debris, and then lyophilized.

Preparation of Immunodiffusion Slides.—Double diffusion slides were prepared by layering 3 ml of 1.4 per cent washed Noble agar (Difco Laboratories, Inc., Detroit) in M/30 phosphate-buffered saline (pH 7.3) on a standard microscope slide, and cutting cylindrical wells, 1 to 2 mm in diameter. In general, peripheral wells are arranged in a hexagonal pattern 5 to 8 mm from the central well, but by suitable placement of the wells, many combinations of antigens and antisera can be studied. Precipitin lines appear within several hours, and can be preserved on the slide as a permanent record. The slide is washed with saline to remove unprecipitated protein, with distilled water, and dried at 37°C under moistened filter paper. The precipitin reactions are preserved intact within a thin film of agar. For sharp photographic reproduction, the lines of antigen-antibody precipitate can be accentuated by stains such as amido-black which fix to protein.

Slides for immunoelectrophoresis were prepared by the Scheidegger technique (11).

EXPERIMENTAL

Isolation of Streptococcal Protoplast Membranes.—Protoplasts of Group A streptococci have been obtained by the muralytic action of a Group C phage-associated lysin which dissolves the Group A streptococcal cell wall (2, 12). A hypertonic environment is essential to preserve the osmotically fragile protoplasts after dissolution of the cell wall. Dilution of a suspension of protoplasts with water leads to rapid rupture of the protoplasts with release of intracellular contents. The ruptured cytoplasmic membranes persist as distinct structures containing some adherent cytoplasmic RNA and DNA. However, membranes, essentially free of these intracellular materials, have been obtained by the following modification of the original procedure (2).

Protoplasts are prepared from streptococci harvested during the logarithmic growth phase by treatment with phage-associated lysin in M/100 phosphate buffer (pH 6.5) containing 7 per cent sodium chloride. Following a 7 per cent sodium chloride wash, the protoplasts, free of cell wall carbohydrate as shown by chemical and serological analysis, are ruptured by

suspension in M/100 phosphate buffer (pH 7.3) containing 0.2 mg/ml of magnesium-activated DNase to reduce viscosity of released DNA, and the suspension is incubated at 37°C for 30 minutes. The protoplast membranes are collected by centrifugation at 8000 G, and washed in saline buffered with M/60 phosphate buffer (pH 7.5). These membranes are then incubated at 37°C for 30 minutes in phosphate buffer (pH 7.2) containing 0.02 mg/ml of both RNase and DNase, and washed twice with phosphate-buffered saline. After retreatment with RNase and DNase, the membranes are washed three times in phosphate-buffered saline, twice with distilled water at 4°C, lyophilized, and stored in a vacuum desiccator at 4°C.

The soluble supernatant that remains after the protoplasts have been osmotically ruptured and the protoplast membranes removed by centrifugation, is dialyzed against distilled water, and lyophilized. In subsequent studies, this cytoplasmic material has proved useful for comparison with the other cell fractions.

TABLE I

Distribution of Protoplast RNA during Isolation of Membranes

	Preparation	
	I	II
	mg	*mg*
Total in intact protoplasts.............................	134.5	300.0
After protoplast rupture:		
Supernatant......................................	86.3	177.4
Membrane pellet.................................	48.2	122.6
Removed from membrane pellet by enzymatic treatment...	45.4	118.3
Lyophilized membranes..............................	2.8	4.3

Evidence for removal of adherent intracellular material from the membranes was obtained by measuring the release of RNA into solution during each step of the isolation procedure. Table I summarizes the distribution of protoplast RNA in fractions obtained during isolation of membranes from two typical preparations of streptococcal protoplasts. After the protoplasts are ruptured, almost two-thirds of the original RNA is recovered in the supernatant. Most of the remaining RNA is removed from the membranes by enzymatic treatment, and the final lyophilized membrane preparation contains only 2 per cent of the original protoplast RNA. In similar studies, the DNA content of the lyophilized membranes represents less than 2 per cent of the total DNA in the protoplasts.

Electronmicroscopic examinations have demonstrated that protoplast membranes remain intact after enzymatic treatment. These thin structures are now free of the large electron-opaque granules associated with freshly isolated membranes. It should be emphasized that although membranes and ribosomes may be intimately associated in the intact bacterial cell and separation of these

structures may be artificial, these preparations of protoplast membranes, essentially free of intracellular components, have proved to be uniform material for chemical and immunological studies.

Preparation of Streptococcal Membranes by Mechanical Disruption.—After streptococci have been mechanically disrupted in a Mickle disintegrator, cell walls are recovered by centrifugation (13). Subsequent high speed centrifugation of the cell wall supernatant also yields a membrane fraction.

The cell walls of disrupted streptococci were collected by centrifugation at 3500 G for 60 minutes, washed several times with distilled water, and lyophilized. One gm of cell walls was obtained from 18 liters of overnight growth in beef heart infusion broth. To remove remaining cell wall particles, the cell wall supernatant was centrifuged for 2 hours at 4000 G. The resulting supernatant was centrifuged at 15,000 RPM (20,000 G) in a No. 30 rotor of the Spinco preparative ultracentrifuge, and the pellet which contained the membrane fraction was recovered. The pellet was washed several times with saline, and incubated at 37°C for 30 minutes in M/100 phosphate buffer (pH 7.4) that contained 0.04 M NaCl, 0.005 M MgCl₂, and 0.02 mg/ml of both DNase and RNase. The membrane fraction was collected by centrifugation, washed twice with saline, twice with distilled water at 0°C, and lyophilized. The final product, a white powder, weighed 600 mg.

Chemical analysis and immunological studies of the membrane fraction have demonstrated that it is essentially free of cell wall carbohydrate and cytoplasmic constituents. Although electronmicrographs reveal that the membrane material collected from mechanically disrupted cells is more fragmented than the membranes obtained by lysis of protoplasts, both are similar in chemical composition and antigenic properties.

Chemical Composition of Membranes Isolated from Protoplasts and from Mechanically Disrupted Streptococci.—The membranes isolated from protoplasts and the membrane fraction isolated by mechanical disruption of streptococci were analyzed for total lipid, carbohydrate, RNA, nitrogen, and phosphorus. The results of a typical analysis of membranes isolated from protoplasts and from disrupted cells of streptococcal strain T25/41FP are recorded in Table II. In general, cell membranes are largely lipoprotein, and it is apparent that a lipid-protein complex comprises more than 85 per cent of the streptococcal membrane. The membranes isolated by either technique are essentially similar in chemical composition, although some differences are noted. For example, protoplasts prepared with the cell wall dissolving enzyme have a smaller residue of the cell wall sugars, rhamnose, and hexosamine, than the membrane fraction of disrupted bacteria. On the other hand, since membranes obtained by mechanical disruption have been more efficiently washed free of intracellular RNA, they have a lower phosphorus content than protoplast membranes. Thus the slight differences between these two membrane preparations is probably a reflection of the method of isolation.

A comparison of the chemical composition of the membrane to that of the cell wall demonstrates essential differences which confirm earlier morphological

studies indicating that membranes were distinct from the cell wall (2). The results of analysis of streptococcal cell walls which were isolated during the preparation of the membrane fraction are included in Table II. The composition of these trypsin-treated cell walls is similar to that reported by McCarty (14). A major chemical difference between the membrane and the cell wall is the presence of a considerable amount of lipid in the membrane. In contrast, the lipid content of the cell wall preparation is less than 2 per cent, and there is evidence to indicate that even this small amount of lipid is due to membrane fragments which have remained in close association with the cell wall fraction. Another striking difference is the absence of more than traces of rhamnose and

TABLE II

Chemical Composition of Membranes and Cell Walls of Streptococcal Strain T25/41FP

	Membranes from		Cell walls
	Protoplasts	Disrupted cells	
	per cent	*per cent*	*per cent*
Total lipid	25.0	18.5	<2.0
Protein*	68.1	66.9	—
Rhamnose	<0.2	<1.1	27.0
Hexosamine	<0.2	<0.5	15.4
Glucose	2.1	2.2	<0.3
Total nitrogen	10.9	10.7	7.9
Total phosphorus	1.1	0.5	0.7
RNA	2.0	0.6	1.8

All analyses performed on lyophilized material containing approximately 5 per cent water.
* Amino nitrogen × 6.25.

hexosamine in the membranes. These two sugars which are combined in the group carbohydrate, comprise more than 42 per cent of the streptococcal cell wall. These analytical results are in agreement with quantitative precipitin analyses of extracts of protoplast membranes which have shown that cell wall carbohydrate comprises less than 0.05 per cent of the membrane (2). The membrane does contain 2 per cent glucose, a sugar detectable only in trace amounts in the cell wall.

The nitrogen-containing compounds of the cell wall are N-acetylglucosamine, N-acetylmuramic acid, and four amino acids. In contrast, the membranes, as shown by ascending paper chromatography of acid hydrolysates, using n-butanol:pyridine:water (6:4:3) as a solvent system, contain a wide variety of amino acids, thus suggesting a protein component. Quantitative amino acid analysis of the membranes was performed by the method of Moore and Stein (15), and the relative molar proportion of each amino acid with respect to alanine is recorded in Table III. For comparison, Table III also includes the

four major amino acids of the mucopeptide matrix of the Group A streptococcal cell wall as reported by Krause and McCarty (16).

The chemical determinations listed in Table II have been performed on twelve lyophilized preparations of streptococcal membranes. Calculated on the basis of dry weight, the membrane is composed of 26 per cent lipid, 71 per cent protein, and 2 per cent carbohydrate, largely as glucose. The initial lots have been utilized to establish suitable techniques and analytical procedures. In general, all preparations of protoplast membranes appeared similar when examined by

TABLE III

Relative Molar Proportion of Amino Acids in the Membrane and the Cell Wall of a Group A Streptococcus

	Membranes	Walls
Alanine	1.0	1.0
Arginine	0.37	
Aspartic acid	0.91	
Glutamic acid	0.95	0.28
Glycine	0.77	0.07
Histidine	0.15	
Isoleucine	0.61	
Leucine	0.83	
Lysine	0.68	0.28
Methionine	0.23	
Phenylalanine	0.37	
Proline	0.35	
Serine	0.46	
Threonine	0.53	
Tyrosine	0.24	
Valine	0.71	

The amino acid analysis of the protoplast membranes was performed by Dr. William Stein.

electronmicroscopy, and contained only trace amounts of cell wall carbohydrate. Analyses of four protoplast membrane preparations obtained from two streptococcal types are recorded in Table IV. As previously noted, the small amount of RNA detectable in each preparation suggests that the membranes are essentially free of intracellular material. The content of nitrogen, phosphorus, glucose, and lipid is similar for each lot of membranes indicating that the method of preparation is reproducible.

Membrane Lipids.—The semipermeable nature of the cell membrane is probably a function of layers of lipid and protein. Physicochemical studies which suggested that a similar selective osmotic barrier existed in bacteria had assumed the presence of a lipoprotein structure (17). Because the cell walls of Gram-positive bacteria contain only small amounts of lipid and protein, and

because the protoplasts, free of the rigid cell wall, are very sensitive to osmotic change, it appears likely that the membrane of the protoplast functions as the osmotic barrier in bacteria. Analyses of protoplast membranes from *B. megaterium* (18) and from *Micrococcus lysodeikticus* (19) have indicated that these structures are essentially a lipoprotein complex. The lipoprotein complex of the streptococcal membrane has been studied and its lipid components partially characterized by the procedures described below.

Most of the membrane lipid is easily extracted by such lipid solvents as a mixture of chloroform and methanol, while the remainder requires more vigorous extraction. The chloroform-methanol soluble lipid is extracted from membranes by a modification of the method devised by Folch for the isolation and purification of lipids from biological sources. In this procedure, the lipid extract is

TABLE IV

Chemical Analyses of Streptococcal Membranes

Strain	Lot No.	RNA	Phosphorus	Nitrogen	Total lipid	Glucose
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
T12	1	3.0	1.09	11.4	19.1	1.9
T25	4	1.9	1.00	10.8	23.7	2.1
	5	2.0	1.06	10.9	25.0	2.1
	6	1.8	1.08	10.7	24.3	2.3
Mean		2.2	1.06	10.9	23.0	2.1

washed with a dilute aqueous salt solution to remove non-lipid substances (20). Additional treatment of the membrane residue after Folch extraction has been employed to remove the remaining lipid.

Lyophilized membranes were extracted at 20°C with 20 volumes (w/w) of chloroform: absolute methanol 2:1 (v/v) in a glass-stoppered vessel. After stirring for 10 minutes, the solvent was removed, and the membrane residue was extracted three additional times with fresh solvent. The pooled chloroform-methanol extracts were filtered through ether-treated filter paper to remove particulate matter. After 0.2 volume of 0.04 per cent aqueous $CaCl_2$ was mixed with the filterate, the mixture separated into two phases without any interfacial precipitate. The upper phase was removed, the lower phase was washed, and brought to a known volume with absolute methanol. Aliquots of the lower phase were dried and weighed in tared glass shells by the method of Craig *et al.* (21). Each organic solvent used in these lipid studies has been of reagent grade, and each was redistilled in glass before use.

Additional lipid was extracted from the membrane residue by refluxing with a mixture of 6N HCl: absolute methanol 1:1 (v/v) in a Soxhlet extraction flask for 90 minutes. This mixture then was shaken with an equal volume of petroleum ether (60–70°), and the petroleum ether phase removed. Fresh petroleum ether was added three times, and the extracts were pooled, filtered through ether-washed paper, and dried under nitrogen in a rotary evaporator. The lipid was redissolved in a known volume of petroleum ether, and aliquots were weighed in

tared glass shells. The defatted membrane residue was dried *in vacuo*, and stored for further study.

The lipid composition of four membrane preparations is shown in Table V. Almost 90 per cent of the extractable lipid is removed by the Folch procedure, while the remainder is extractable only after partial acid hydrolysis. These two lipid extracts have been combined, and the content of phosphorus, nitrogen, and glucose in the total lipid has been determined. Roughly half of the total phosphorus of the membrane is found in the lipid, present largely as phosphoric acid in various phospholipids. Four per cent of the phospholipid molecule is phosphorus, and thus the lipid phosphorus content of 1.85 per cent indicates that phospholipids comprise almost 50 per cent of the total lipid. A similar

TABLE V

Composition of the Lipids in the Streptococcal Membrane

Strain	Lot No.	Folch-extractable lipid	Acid-extracted lipid	Content of combined fractions		
				Phosphorus	Nitrogen	Glucose
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
T12	1	85.0	15.0	1.9	1.0	2.5
T25	4	88.0	12.0	1.8	0.95	2.5
	5	88.3	11.2	1.9	1.0	2.4
	6	89.1	10.9	1.8	0.95	2.4

phosphatide content has been reported for the lipid fraction obtained from disrupted cells of *S. aureus* (22). In contrast, phospholipids represent almost 90 per cent of the total lipid isolated from membranes of *B. megaterium* and *M. lysodeikticus*. Only traces of nitrogen are detectable in the phospholipid fraction of these two species suggesting that a phosphatidic acid complex is the major phosphatide (18, 19). On the other hand, the presence of nitrogen in roughly equimolar proportion to phosphorus in the lipid of the streptococcal membrane indicates the presence of phosphatides that possess a nitrogenous base. The demonstration of choline in the lipid is further evidence for the presence of one of the common phosphatides, phosphatidylcholine (lecithin). In addition, the presence of glucose in the lipid fraction suggests the presence of glycolipids.

Partial separation and identification of the lipid substances present in streptococal membranes have been obtained by means of ascending chromatography using thin layers of silicic acid (23). Two solvent systems have been employed, a non-polar solvent composed of 30 per cent ethyl ether and 70 per cent petroleum ether (30–60°), as well as a polar solvent containing a 3:1 mixture of chloroform and water-saturated methanol. The chromatograms prepared with the non-polar solvent disclose the presence of free fatty acids, diglycerides (both

the 1,2, and the 1,3), and traces of triglyceride and an esterified hydroxy acid. It is of interest that sterols and sphingomyelin have not been detected in the membrane lipid. Use of the polar solvent system has revealed the presence of phospholipids and glycolipids. One large spot containing both phosphorus and choline has an R_f value very similar to that of phosphatidyl choline. In addition,

TABLE VI

Distribution of Fatty Acids in Membrane Lipids

Fatty acids		Silicic acid fractions	
Shorthand designation	Familiar name	Ether	Methanol
		per cent	*per cent*
12:0	Lauric	0.1	0.5
12:Br		—	0.1
13:0		0.1	0.1
13:Br		—	0.1
14:0	Myristic	1.2	2.2
14:Br		0.6	0.8
15:0		0.8	0.6
15:Br		0.5	0.4
16:0	Palmitic	25.8	29.6
16:1	Palmitoleic	19.7	16.4
17:0		2.2	1.5
17:Br		2.7	1.9
18:0	Stearic	14.4	11.8
18:1	Oleic	27.9	21.0
18:2	Linoleic	1.5	2.8
18:4		—	0.4
19:0		1.8	1.2
19:Un		—	1.1
19:Br		—	1.8
20:0	Arachidic	0.9	1.3
20:1		—	0.6
21:0		—	1.9
22:0		—	1.6

The fatty acids were separated by gas-liquid chromatography in conjunction with Dr. Hans Meinertz.

several ninhydrin-positive spots which contain phosphorus correspond to those formed by phosphatidyl serine and phosphatidyl ethanolamine. Finally, two large glucose-containing spots have no phosphorus and are ninhydrin-negative. These have the same R_f value as the common cerebrosides.

Additional studies have been concerned with the fatty acid composition of the total membrane lipid. Partial separation of this lipid into two fractions has been accomplished by means of silicic acid chromatography. One fraction is

eluted with a non-polar solvent, a 1:1 mixture of petroleum ether (60–70°) and ethyl ether. This fraction, composed largely of fatty acids represents 20 per cent of the total lipid. The other fraction, containing the remainder of the lipid, is then eluted with methanol. Fatty acids comprise half of this fraction. After conversion to their respective methyl esters by the method of Stoffel (24), the fatty acids of each fraction are separated by gas-liquid chromatography. The distribution of fatty acids in each fraction is recorded in Table VI. Although sixteen and eighteen carbon fatty acids predominate, a wide variety of saturated and unsaturated fatty acids are present. It is clear that the pattern of fatty acids found in the lipid of streptococcal membranes is quite similar to that described for human adipose tissues. In contrast, there is no evidence for the presence of branched fatty acids which have been demonstrated in membrane lipids of other bacterial species.

These chemical studies have disclosed the lipoprotein nature of the streptococcal membrane, and demonstrated the similarity of membranes isolated either from mechanically disrupted cells or from protoplasts. The following immunological studies indicate that membranes of Group A streptococci possess a common antigen which is unrelated to the antigens of the cell wall or the cytoplasm.

Immunological Studies of Streptococcal Membranes.—In the following experiments, the antigenic nature of the streptococcal membrane is compared to that of other components of the streptococcus. Antisera were prepared to each streptococcal fraction, the cell wall, the membrane, and cytoplasmic material. Soluble antigens extracted by various techniques from these fractions were employed with the antisera in precipitin tests, in double diffusion studies in agar gel, and in immunoelectrophoresis.

Sera with antibodies directed against the three streptococcal fractions were prepared in the following manner. A saline suspension of 5 mg of lyophilized membranes was injected intravenously into New Zealand Red rabbits three times a week for 5 weeks. The rabbits were bled after a 5 day rest period, and the sera were tested in capillary precipitin tubes with extracts of membranes. In general, antisera which gave strong precipitin reactions were obtained after 4 to 6 weeks of immunization.

Group A streptococcal antisera, containing antibodies to the group-specific carbohydrate of the cell wall were prepared in the usual manner (3).

Antisera to cytoplasmic material were obtained by a weekly subcutaneous injection of 20 mg of lyophilized cytoplasmic material suspended in 1 ml of incomplete Freund's adjuvant.

Soluble antigen preparations suitable for precipitin analysis with these antisera were obtained by treating the membranes with proteolytic enzymes. Antigen-containing extracts of cell fractions were prepared with the enzymes produced by *Streptomyces albus* which have proteolytic activity as well as the capacity to dissolve the Group A streptococcal cell wall (14). Similar immunologically reactive extracts were prepared by treating the membranes with other proteolytic enzymes such as trypsin, chymotrypsin, or papain. In addition,

preliminary experiments indicate that extracts of membranes prepared by boiling for 10 minutes at pH 2 also contain antigenic material. The precise relationship of this material to that obtained with proteolytic enzymes has not yet been determined.

In most of the following experiments, extracts have been prepared with trypsin or the enzymes of *Streptomyces albus*. Lyophilized membranes, suspended in M/15 phosphate buffer (pH 7.4), are mixed with an equal volume of

TABLE VII

Serological Specificity of Streptococcal Cell Fractions

Soluble antigens from	Rabbit antisera to				
	Killed Group A streptococci	Protoplast membrane			Cytoplasmic material
		Type 6	Type 12	Type 25	
Cell wall					
Type 6	4+*	+	tr	tr	tr
Type 12	4+*	tr	+	tr	tr
Type 25	4+*	tr	tr	+	tr
Protoplast membrane					
Type 6	tr	4+	4+	4+	tr
Type 12	tr	4+	4+	4+	tr
Type 25	tr	4+	4+	4+	tr
Cytoplasmic material					
Type 6	+	+	+	+	4+
Type 12	+	+	+	+	4+
Type 25	+	+	+	+	4+

tr, trace reaction.

* This precipitate represents the interaction of Lancefield Group A carbohydrate and group-specific antiserum.

saline containing 20 μg trypsin/ml, and incubated at 37°C for 6 hours. When extracts are prepared with the enzymes of *Streptomyces albus*, the suspension is buffered at pH 7.85, and incubated at 45°C. The insoluble residue is removed by centrifugation, and the supernatant is employed in precipitin tests.

Capillary precipitin tests employing soluble antigens of the cell wall, the membrane, and cytoplasmic material, and antisera to each of these fractions indicate that the membrane contains an antigen distinguishable from those in the other fractions. The results of some of these precipitin tests are recorded in Table VII. Cell walls and protoplast membranes from three Group A streptococcal Types, 6, 12, and 25, were extracted with the *Streptomyces albus* enzymes. Each of these extracts as well as phosphate-buffered solutions of cyto-

plasmic material from each strain was tested with antiserum to the group carbo-
hydrate of the cell wall, antisera to each of the three membrane preparations, and
antiserum to cytoplasmic material.

It is clear that each membrane extract produced a strong precipitin reaction
with each of the three membrane antisera, while only trace reactions appeared
with either the cell wall antiserum or the antiserum to cytoplasmic antigens.
Extracts of freshly isolated membranes which have been insufficiently washed,
and therefore contain adherent nucleoprotein, react strongly with cytoplasmic
antisera. However, during treatment of the membranes with nucleases, the
RNA-protein substances that react with cytoplasmic antisera are released into
solution. Extracts of the final membrane preparation, therefore, give only trace
reactions with cytoplasmic antisera. The soluble antigens present in the other
two fractions reacted strongly only with sera directed against the specific
fraction, and produced only faint reactions with membrane antisera. Thus, the
precipitin reaction that develops between membrane antigen and membrane
antiserum is specific, and supports the chemical evidence which suggests that
the streptococcal membrane preparations are essentially free of other cellular
components.

Serological studies using capillary precipitin tests have shown that soluble
antigens can be extracted from membranes by several proteolytic enzymes.
Immunodiffusion experiments suggest that the membrane extracts prepared
with these enzymes contain a common antigen. In the experiment illustrated in
Fig. 1, extracts of T25 membranes prepared with trypsin, chymotrypsin,
papain, and the *Streptomyces albus* enzymes have been placed in four of the
peripheral cylinders. A precipitin line has developed between each extract and
the T25 membrane antiserum in the central cylinder. Although each extract was
prepared with a different proteolytic enzyme, these precipitin lines have fused
into a common band of identity which intersects the faint line of precipitate
that has formed between the membrane antiserum and cytoplasmic material in
cylinder No. 3. No precipitate is visible between the membrane antiserum and
the *Streptomyces albus* extract of cell walls in cylinder No. 2. The membrane
band continues into the cylinder that contains cytoplasm and into the cylinder
that contains the cell wall extract. This experiment demonstrates that the same
antigen is released from the membrane by a variety of proteolytic enzymes, and
that this antigen is distinct from antigens present in the cell wall and in the
cytoplasm.

Chemical studies have indicated that membranes isolated from protoplasts
and from disrupted streptococci are identical. The antigenic properties of both
membrane preparations are also similar when compared by various immuno-
logic techniques. Precipitin tests have shown that extracts of these membrane
preparations react strongly with antisera prepared with membranes obtained
by either technique, and that only trace reactions occur with either cell wall or

cytoplasmic antisera. The antigenic identity of these membrane preparations has been confirmed by microdiffusion experiments such as the one seen in Fig. 2. Membranes were prepared from strain T25 by each method. A trypsin extract of each preparation was placed in one of a pair of wells, and antisera prepared with these membranes were placed in the other two wells. It is evident that both membrane extracts have reacted with both antisera. Lines of antigen-
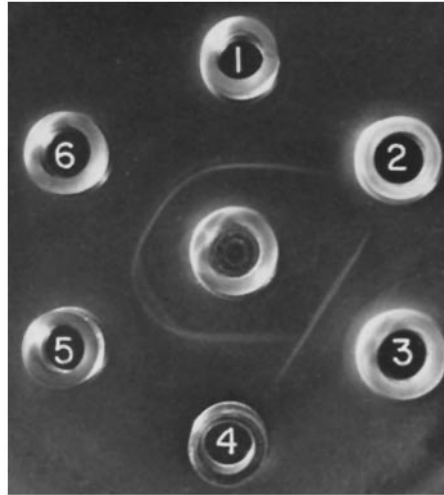

FIG. 1. Ouchterlony plate showing the serological specificity of Group A streptococcal membranes when compared to the other cell fractions, and the formation of a band of identity by membrane extracts prepared with different proteolytic enzymes. The central cylinder contains antiserum to membranes of strain T25/41. The peripheral cylinders contain cell fractions of this strain. No. 1 and Nos. 4 to 6 contain extracts of membranes prepared with trypsin (No. 1), papain (No. 4), enzymes of *Streptomyces albus* (No. 5), and chymotrypsin (No. 6); No. 2 contains a *Streptomyces albus* extract of the cell wall; and No. 3 contains untreated cytoplasmic material.

antibody precipitate have formed, and antigenic identity of the membrane preparations is clearly indicated by the fusion of these precipitin lines.

Both of these preparations of membranes were obtained from the same serological type of Group A streptococci. Proteolytic extracts of membranes isolated from many different serological types produce strong precipitin re-reactions with different membrane antisera in capillary precipitin tubes. Diffusion experiments designed to further investigate the antigenic relationships of membranes from different types of Group A streptococci demonstrate that these membranes contain an antigen in common. Two representative experiments are pictured in Fig. 3. In Fig. 3 *a*, the central well contained antiserum prepared with membrane from Type 25 streptococci.

Trypsin extracts of five membrane preparations, each from a different serological type of Group A streptococcus, were placed in five peripheral wells. The sixth well contained a trypsin extract of membranes isolated from an A-variant Type 14 streptococcus. The precipitin lines which have appeared between the antiserum and each of the six extracts have formed a circular band of identity.

In the experiment illustrated in Fig. 3 b, the positions of the antigen and antiserum were reversed. Antisera to membranes isolated from many different serological types of Group A streptococci have been prepared, and each of the five peripheral wells contained a different membrane antiserum. The central
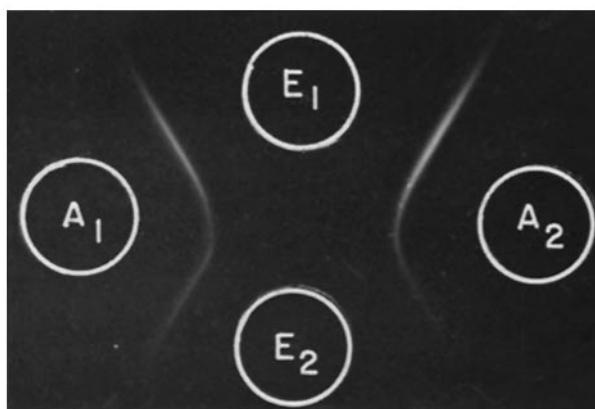

FIG. 2. Microimmunodiffusion demonstrating the identity of membranes from protoplasts and from disrupted cells of streptococcal strain T25/41. Membrane extracts: E_1, from protoplasts; E_2, from disrupted cells. Antisera to membranes: A_1, from disrupted cells; A_2, from protoplasts.

well was filled with a trypsin extract of T25 membranes. As expected, precipitin lines developed between the membrane extract and each serum, and these lines have merged to form a continuous pentagonal figure. Similar patterns appear when extracts of membranes isolated from a wide variety of serological types of Group A and Group A-variant streptococci are studied. The formation of these bands of identity is evidence for the presence of an antigen common to the membranes of all Group A streptococci.

Additional experiments were devised to investigate the nature of the serologically active substances present in the streptococcal membrane. These studies were directed initially towards the release of the antigenic material into solution. Lipids were extracted from several membrane preparations at $-5°C$ with a 3:1 mixture of ethyl ether and ethanol. Extracts of this defatted membrane residue were prepared at various temperatures using water, saline, or neutral phosphate buffers. Although these procedures did not release any
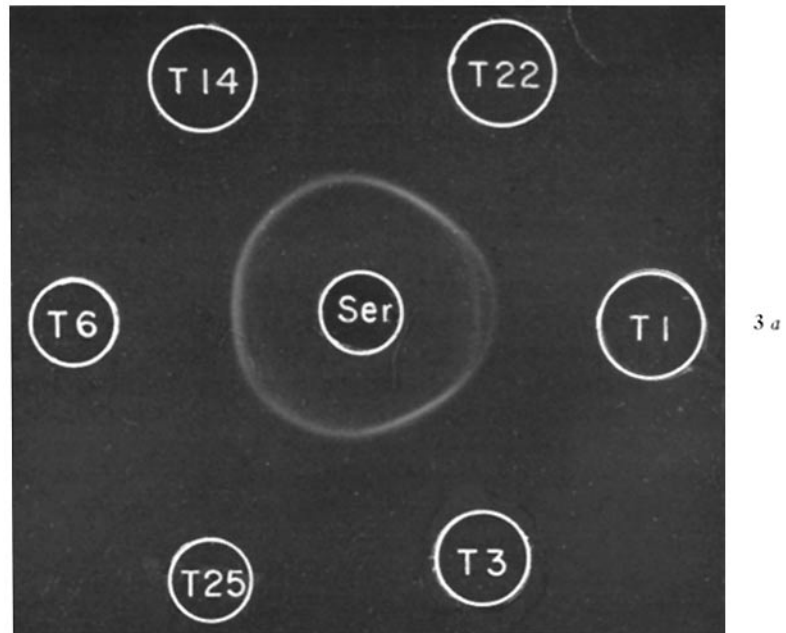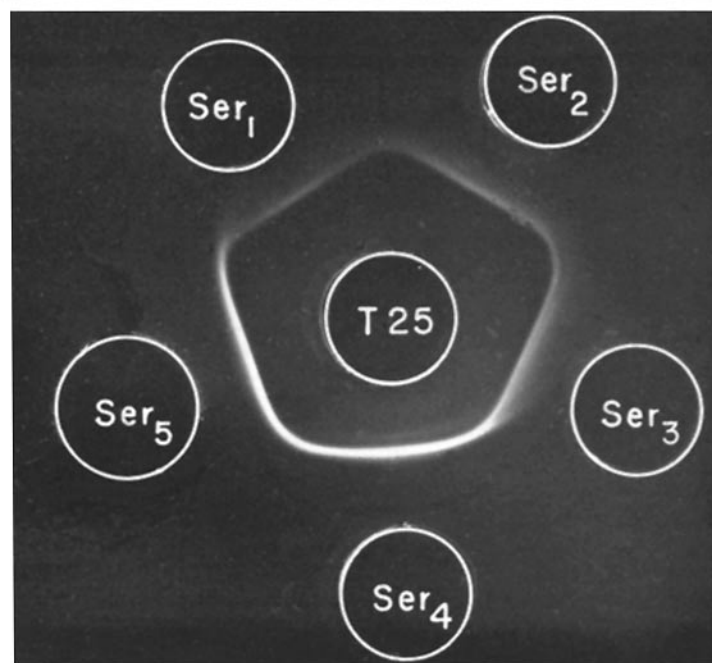
Fig. 3 a. Serological identity of membranes from various types of Group A streptococci. Central well: antiserum to Type 25 membranes. Peripheral wells: trypsin extracts of membranes from Types 1, 3, 6, 22, and 25 streptococci. Well designated T14 contains extract of membranes from Type 14, Group A-variant streptococci.

Fig. 3 b. The formation of a band of identity between a membrane extract and antisera to membranes from different types of Group A streptococci. The center well contains a trypsin extract of T25 membranes. Ser 1 to 5: Antisera prepared with membranes from Types 6, 12, 14, 25, and 50 streptococci.

antigenic substances into solution, soluble antigens were obtained by subsequent proteolytic extraction of the defatted membranes. In the studies that follow, extracts have been prepared by trypsin digestion of membranes without prior defatting. During digestion with trypsin, almost 50 per cent of the membrane preparation is released into solution. Lipid containing substances are not released by this process, but can be quantitatively isolated from the residue by the usual lipid solvents. The soluble antigenic material from the membranes is sensitive to changes in ionic strength, and is insoluble in sodium chloride concentrations below 0.05 N, or in solutions of ammonium sulfate above 0.6 saturation. Additional studies of the physical properties of membrane antigens are now in progress.

Immunoelectrophoretic analyses of proteolytic extracts of membranes have been performed on ammonium sulfate-fractionated material by the usual methods. These studies have indicated the presence of antigenic substances that migrate towards the cathode, and development with membrane-specific antisera has revealed these antigens as a pair of overlapping precipitin arcs as shown in Fig. 4 a. Membranes isolated from different serological types of Group A streptococci form similar precipitin arcs when extracts are prepared under standard conditions. In the modified immunoelectrophoretic experiment drawn in Fig. 4 b, extracts of T12 and T25 membranes have been electrophoresed. Subsequent development with antisera to each of these membrane preparations has produced typical precipitin arcs, and the antigenic identity of these membranes is clearly demonstrated by the fusion of the precipitin lines.

Membranes of Other Bacterial Species.—With the demonstration of common antigens in the membranes of Group A streptococci, it seemed of interest to compare the serological relationship of these membranes to those from other Gram-positive cocci. In general, cell wall–dissolving enzymes suitable for the preparation of protoplasts have not been available for most bacterial species. However, by means of mechanical disruption of the bacterial cells, membranes have been isolated from strains of hemolytic streptococci, *Streptococcus viridans*, and *Staphylococcus aureus*, and rabbit serum has been prepared against each of these membranes. When these antisera were compared in capillary precipitin tubes with trypsin extracts of membranes, each serum reacted strongly with its homologous membrane extract. The results of these studies are recorded in Table VIII.

Streptococci of Groups A, C, and G have a number of biological characteristics in common, and therefore it is not surprising to find cross-reactions among these strains. Extracts of Group C and Group G membranes react with antisera to Group A membranes, and extracts of Group A and Group G membranes react with serum prepared against Group C membranes. On the other hand, immunodiffusion studies have shown that the antigenic substances in the membranes of these groups are only partially related. In the microdiffusion
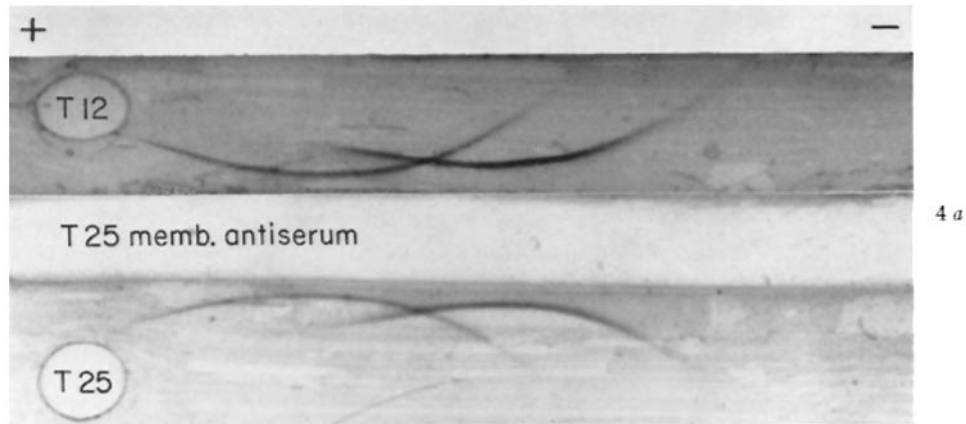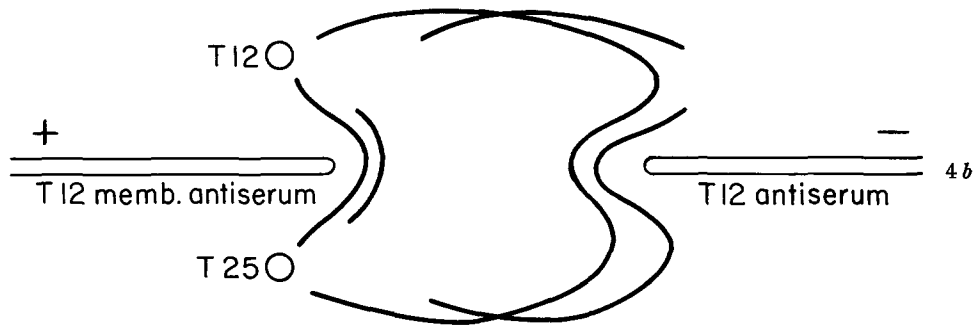
FIG. 4 a. Immunoelectrophoretic comparison of trypsin extracts of membranes from Type 12 and Type 25 streptococci. The patterns have been developed with antiserum to Type 25 membranes.

FIG. 4 b. Drawing of a modified immunoelectrophoretic experiment showing the precipitin arcs which develop with extracts of Type 12 and Type 25 membranes, and antisera to each of these membrane preparations. The antigenic identity of the membranes is demonstrated by the fusion of the precipitin lines.

experiment shown in Fig. 5 a, trypsin extracts of membranes from Groups A, C, and G have been compared by means of antisera to Group A membranes. Each membrane extract has formed a precipitin line with each antiserum, and these lines have partially fused into bands of identity. However, the precipitin lines

of the Group C and the Group G extracts have formed spurs with the lines of the Group A extract suggesting that the antigens of these three groups possess only partial identity. In other experiments using antisera to Group C membranes some of the precipitin lines have intersected indicating non-identity of the antigens. Thus, although the membranes of these three groups are related, they contain antigens which are similar but not identical.

Although extracts of Group D membranes react with antisera to membranes of Group A and Group C, it is evident that this is a one-way cross since antisera to Group D membranes do not react with membrane preparations of other groups. In addition, double diffusion studies have clearly demonstrated that the

TABLE VIII

Species Specificity of Bacterial Membranes

Soluble antigens from membranes of	Rabbit antisera to membranes of					
	Group A	Group C	Group D	S. viridans		Staph. aureus
				SBE 154	B 683	
Group A	4+	2+	0	0	0	0
Group B	0	tr	0	tr	0	0
Group C	2+	4+	0	0	0	0
Group D	2+	2+	4+	0	0	0
Group G	+	+	0	tr	0	0
S. viridans SBE 154	0	0	0	4+	tr	0
B 683	0	0	0	+	4+	0
Staph. aureus	0	0	0	0	0	4+

antigens of Group D membranes are unrelated to those present in Group A, C, or G membranes. In the experiment illustrated in Fig. 5 *b*, extracts of two Group A membranes, T12 and T25, have been analyzed with two Group A membrane antisera. The precipitin lines which have developed, have fused into a common band of identity that enters the well containing the extract of Group D membranes. Although Group D extracts form a precipitin line with some antisera to Group A membranes, no reaction has developed with this Group A membrane antiserum. In those experiments in which a precipitin line forms between Group D membranes and Group A membrane antiserum, the line intersects that formed by Group A membranes.

The experiment illustrated in Fig. 6 clearly demonstrates the non-identity of membranes isolated from two serologically unrelated species, Group A streptococci and *S. aureus*. The lines of precipitate which have formed between each of these membrane preparations and their respective antisera have intersected
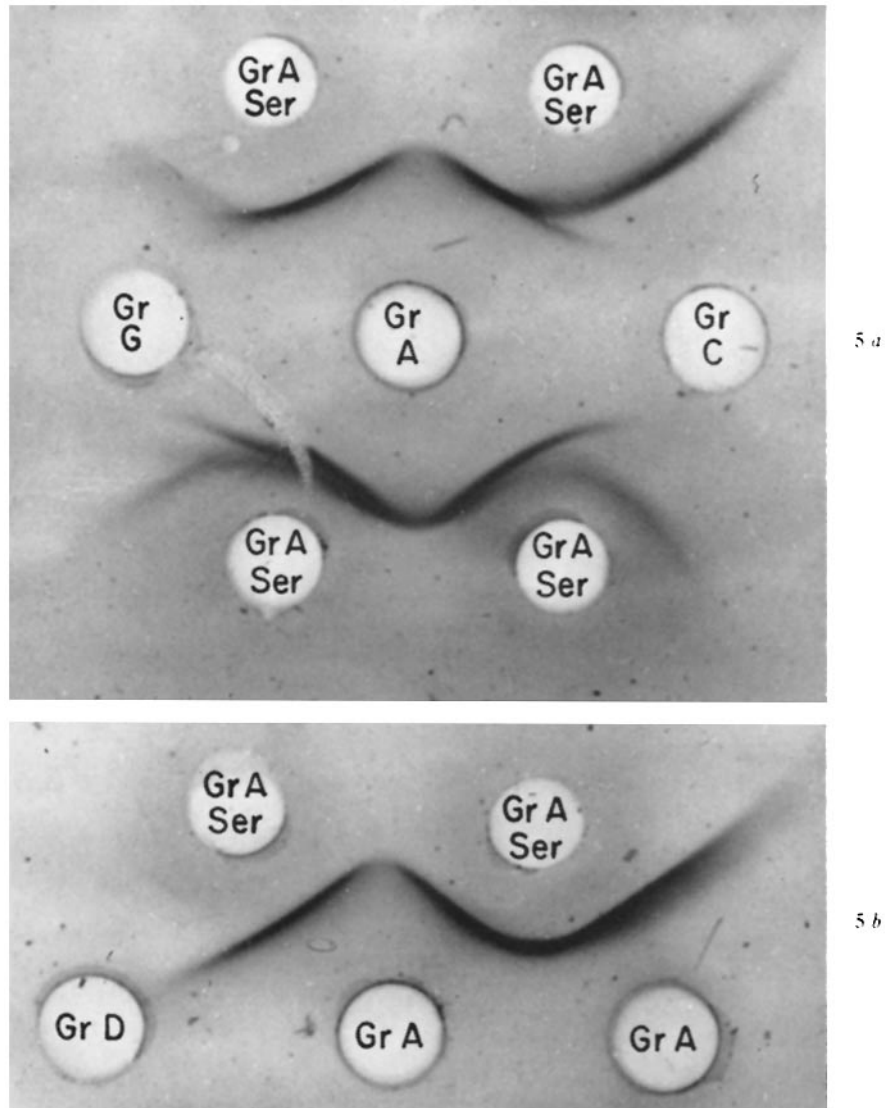
FIGS. 5 *a* and 5 *b*. Wells labeled *Gr A Ser* contain antisera to membranes from two Group A strains. Wells labeled Gr A, Gr C, Gr D, and Gr G contain trypsin extracts of membranes from each of these groups.

FIG. 5 *a*. Comparison of membrances from Groups A, C, and G streptococci.

FIG. 5 *b*. Comparison of membranes from Groups A and D streptococci.

to form an X. Similar experimental results have been obtained when extracts of membranes from strains of *Streptococcus viridans* are compared with those from the Group A streptococcus. These serological studies strongly suggest that the membranes of Group A streptococci have no serological relationship to the membranes of other species of Gram-positive cocci.
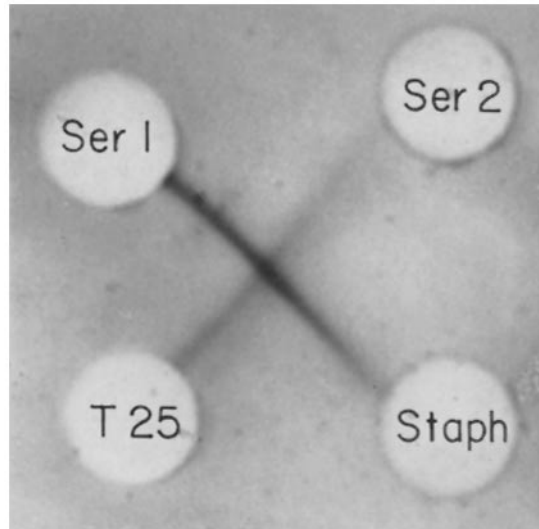

FIG. 6 shows the serological non-identity of membranes from Group A streptococci and *S. aureus.* Well labeled *Staph* contains a trypsin extract of membranes from *S. aureus* and well labeled Ser 1 contains antiserum to these membranes. Similarly, T25 designates an extract of Group A membranes and Ser 2 antiserum to membranes of this strain.

DISCUSSION

The identification and classification of a bacterial species is usually determined by the specific nature of its cell surface. It is evident that such distinguishing characteristics as morphology and the capacity to take the Gram stain are dependent upon the presence of the rigid cell wall. The immunochemical properties of the bacterial cell wall have been extensively studied, and the evidence strongly suggests that the serological specificity of a bacterial species is determined by the chemical nature of its cell wall (13, 25–27). Recent investigations have shown that this rigid cell wall is absent in the mechanically and osmotically fragile derivative forms of bacteria, protoplasts, and L forms. These bacterial variants, which are capable of many of the biological functions of the intact bacterial cell, are subcellular units enclosed in a distinct cytoplasmic membrane.

The rigid cell wall also is absent in another group of microorganisms, the "pleuropneumonia-like organisms" (PPLO), which are morphologically similar to L forms. PPLO are fragile

organisms found as parasites or saprophytes in the respiratory or urinary tracts of a wide variety of species. Interest in PPLO has increased in recent years as a result of the growing awareness of their role as disease producing organisms, as well as the presence of these organisms as contaminants in tissue cultures (28, 29). As an example, Eaton agent, a major etiologic agent of primary atypical pneumonia in man has been characterized recently as a strain of PPLO (30).

The investigation of these microorganisms has been hindered by the failure to establish any consistent basis for identification. In addition to colonial morphology, the persistence of viable material after passage through filters which retain bacteria, and the capability of growth in cell-free media have remained as the major criteria for classification as a PPLO. Detailed biological information about the relationship of PPLO species to each other and to bacteria has not been available. The bacterial origin of these organisms is obscure, and although reversion of PPLO to bacteria have been reported, the experimental evidence has not been convincing.

Although properties of bacterial membranes are not as well established as those of the cell wall, bacterial membranes also may have a specific chemical composition. It is evident that the membrane of the streptococcus is distinct from those isolated from other bacterial species. In addition, the serological studies which have demonstrated the specificity of the Group A membrane, suggest that specific antigens may be present in membranes of other bacterial species. A serological basis for identification of bacterial membranes might prove of value in studies of the relationships among bacterial species as well as in determining the specific bacterial origin of those cell wall–free derivatives which have been isolated free in nature.

Investigations of the nature of bacterial membranes have been limited to those few bacterial species for which cell wall–dissolving enzymes suitable for the preparation of protoplasts have been available. The isolation from mechanically disrupted bacteria of a membrane fraction with chemical and serological properties of the protoplast membrane has removed this restriction, and membrane material can now be obtained from those bacteria for which there have been no known muralytic enzymes.

SUMMARY

Intact bacterial membranes have been isolated from protoplasts prepared from Group A streptococci by a cell wall–dissolving enzyme. A membrane fraction with identical chemical and serological properties has been obtained by differential centrifugation of mechanically disrupted streptococci. The membrane is chemically distinct from the cell wall and is composed of 72 per cent protein, 26 per cent lipid, and 2 per cent carbohydrate. Capillary precipitin tests and analysis by microdiffusion have demonstrated that the membrane contains antigens distinct from those of the cell wall and from those of the cytoplasm which it envelops. Evidence is presented which demonstrates that this antigenic material is common to the membranes of Group A streptococci, and that it can be distinguished by immunodiffusion from related antigenic sub-

stances present in membranes of several other serological groups of hemolytic streptococci. This antigenic material does not cross-react with the membrane antigens of other Gram-positive cocci.

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