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Chemical Analysis of Changes in Membrane Composition During Growth of *Streptococcus pyogenes*

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Changes in the structural components of the *Streptococcus pyogenes* membrane between exponential and early stationary phases of growth are reported. The overall protein composition ranged from 70 to 73% of the dry weight of the membranes, irrespective of the phase of growth from which they were isolated. Amino acid analyses of membranes isolated from streptococci in either the exponential or stationary phase of growth demonstrated that two amino acids, cysteine and tryptophan, were absent. Further analysis of the membrane proteins by sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis demonstrated that there were proteins unique to a particular phase of growth as well as differences in the amount of specific proteins from the various growth phases. In addition, membranes isolated from exponential-phase cultures contained a higher percentage of peripheral protein than did stationary-phase membranes. There also appeared to be an increase in the amount of outer surface proteins during this growth phase. The phosphorus content of the membranes increased during the stationary phase of growth, whereas the sugar composition remained constant. The only sugar found under various conditions of growth in any of the strains was glucose. Total fatty acid content and the mole percent composition of various fatty acids did not change in the different phases of growth. However, the mole percent composition of fatty acids in the membranes of various group A streptococci did differ between strains. Therefore, these results provide evidence that the composition of membranes of *S. pyogenes* does not remain constant throughout the growth phases of the culture.

Streptococcus pyogenes (group A) is the human pathogen involved in the pathogenesis of the post-streptococcal sequelae acute rheumatic fever and glomerulonephritis (23). Various investigators, including ourselves, have demonstrated that contained among the antigens of the protoplast membrane are molecules which cross-react with organ-specific antigens. Included in these cross-reactions are those with the sarcolemmal sheath of the heart (27, 29), the caudate nucleus of the brain (9), and the basement membrane of the kidney (5, 15). In addition, the protoplast membrane also contains molecules which activate the alternate pathway of complement (4, 25).

With regard to these components of the membrane, the following questions can be asked. Under what conditions of growth are the molecules important in the pathogenesis of the disease state expressed? Are there unknown components of the membrane which are involved in the pathogenesis of the disease? Can specific molecules be induced by various environmental conditions? In other words, what is the qualitative and quantitative composition of the protoplast membrane during the infectious process? Previous experiments demonstrated that not all preparations of streptococcal membranes contained equal amounts of the antigens we were investigating and that under certain conditions these antigens were even absent (I. van de Rijn unpublished data). Therefore, it has become imperative that we understand more about the composition of the protoplast membrane of *S. pyogenes* under varied conditions.

For these reasons, we decided to examine the gross composition of the group A streptococcal membrane during the exponential and stationary phases of growth. In addition, we identified the outer surface and peripheral (guanidine-extractable) proteins and analyzed differences in these subclasses during the two phases of growth. In an accompanying paper, the immunology of the group A streptococcal membrane is examined in both exponential- and stationary-phase cultures (11).

MATERIALS AND METHODS

Bacteria and medium. Streptococcal strains were obtained from the Rockefeller University collection

and kindly supplied by R. C. Lancefield of our laboratory. The group A type 6 streptococcal strain S43/192/2 (mouse passed) and the group A type 49 streptococcal strain F301 (non-mouse passed) were both originally human isolates. The type 6 strain was isolated from a population with a streptococcal outbreak; the type 49 strain was isolated from an acute poststreptococcal glomerulonephritis outbreak.

Each culture was initially grown in dialyzed Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) for 18 h and concentrated by centrifugation, and samples were stored at −70°C. Cultures for these studies were always obtained from the initial frozen stock.

All organisms were grown in dialyzed Todd-Hewitt broth prepared as follows. Todd-Hewitt medium (1,800 g) and yeast extract (Difco) (180 g) were suspended in 1 liter of distilled water by heating at 80°C. Antifoam (Dow-Corning Corp., Midland, Mich.) was added to prevent excessive foaming. The solubilized medium was then placed in dialysis tubing (Fisher Scientific Co., Springfield, N.J.) and dialyzed against two changes of 30 liters of distilled water. The dialysate was sterilized by filtration through a 0.22-μm membrane filter (Millipore Corp., Bedford, Mass.) into a Biogen fermentor. The dialysate was then inoculated with 1.5 liters of a culture in the exponential phase of growth. Growth was followed by optical density measurements (650 nm), using a Coleman Junior spectrophotometer. Doubling times during exponential growth for the type 6 and type 49 strains were 37 to 42 min and 39 to 42 min, respectively. At midexponential and 2 h into the stationary phase of growth, a sample of the organisms was rapidly cooled with ice and then harvested by using a Sharples high-speed centrifuge (Sharples-Stokes Div., Pennwalt Corp., Warminster, Pa.). Membranes were immediately isolated from the collected cells in the following manner.

Preparation of streptococcal membranes. Phage-associated lysin was prepared and purified through the cellulose-phosphate chromatography step according to the procedure of Fischetti et al. (6) and then used to prepare group A streptococcal membranes as described by van de Rijn et al. (27) with the following modifications. All solutions and buffers contained 1 mM magnesium chloride. Once the organisms were separated from the growth medium, they were immediately washed with cold saline and suspended in 60 mM sodium phosphate buffer (pH 6.1) containing 30% raffinose and 0.5 mM dithiothreitol. The mixture, to which 1 mg of deoxyribonuclease (DNase) (Sigma Chemical Co., St. Louis, Mo.) was added, was then incubated at 37°C for 1 h. After what appeared to be 100% conversion of streptococci to protoplasts, the suspension was incubated for an additional 30 min. Protoplasts were then sedimented at 10,000 × *g* in a Sorvall RC2B centrifuge (Dupont Instruments, Newtown, Conn.). The protoplasts were resuspended in the protoplasting medium without enzymes and resedimented as above. The protoplasts were then lysed in hypotonic buffer (27) in the presence of ribonuclease (RNase) (1.5 Kunitz units/ml; Sigma Chemical Co.) and DNase (36 Kunitz units/ml; Sigma Chemical Co.) (DNase-RNase buffer). After the cytoplasmic constituents were removed, the protoplast membranes were re-treated with the enzymes in DNase-RNase buffer followed by extensive (five to seven) washings with 10 mM phosphate-buffered saline (pH 7.6) at 4°C.

Protoplast surface labeling. Protoplasts separated from soluble wall components were brought to an optical density of 0.35 at 540 nm in 50 mM phosphate-buffered saline (pH 7.5) containing 30% raffinose. The suspension (1.5 ml) of protoplasts was pelleted at 14,000 × *g* and labeled with ^{125}I by a slight modification of the method of Marchalonis et al. (14). All labeling solutions contained 30% raffinose in order to protect the protoplasts from lysis. Protoplasts were suspended in 25 μl of lactoperoxidase (1 mg/ml; Sigma Chemical Co.). To this was added 25 μl of [^{125}I]sodium (2 mCi/ml, carrier free; New England Nuclear Corp., Boston, Mass.) in 0.01 mM potassium iodine. The mixture was then incubated at room temperature for 20 min with addition (10 μl) of 0.03% hydrogen peroxide every 3 min. The protoplasts were then diluted (1.5 ml) with freshly made DNase-RNase buffer with 0.5 M cysteine and carried through the membrane preparation procedures in the absence of cysteine. This preparation of labeled protoplast membranes was then analyzed by sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis (SDS-PAGGE).

Polyacrylamide slab gel electrophoresis. Proteins were analyzed by SDS-PAGGE by the method of Laemmli (12), using a discontinuous tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer system. Electrophoresis was carried out on a 7 to 15% gradient slab polyacrylamide gel (25 by 30 cm) at 25 mA for 18 h.

Standards (bovine serum albumin, ovalbumin, carbonic anhydrase, and cytochrome *c*) were run on all gels for molecular weight determinations. Samples and standards with 2% SDS, 5% mercaptoethanol, and 10% glycerol were boiled (2 to 5 min) in a water bath before being loaded onto the gel. After electrophoresis, the gels were stained and destained as previously described (27).

Gels containing ^{125}I-labeled samples were run in a similar manner except that the gels were dried according to the procedure of Maizel (13) and exposed to Kodak X-ray film (Eastman Kodak Co., Rochester, N.Y., Blue Brand BB54) to determine the radiolabeled bands by radioautography.

Amino acid analysis. Membrane samples were prepared for amino acid analysis by treatment with 4 N methanesulfonic acid at 110°C for 22 h in evacuated tubes. After neutralization, the composition was analyzed on a Durrum D500 amino acid analyzer.

Carbohydrate analysis. The preparation of samples for gas chromatography was a modification of the method of Clamp et al. (2) whereby monosaccharides are released by methanolysis. Samples (100 μg) were initially dried under vacuum with phosphorus pentoxide overnight. To each sample was added 1.5 N methanolic HCl (0.5 ml), and the tubes were sealed under nitrogen and heated at 80°C for 18 h. The samples were neutralized by the addition of silver carbonate followed by 60 μl of acetic anhydride. The samples were then incubated for 6 h at room temperature. After centrifugation at 2,000 × *g* for 10 min to remove the silver carbonate, the silver carbonate was washed two to three times with methanol. The sample plus washes were pooled, and 40 μl of internal standard (ribitol, mannitol, and perseitol) was added to each sample. The samples were dried under vacuum and

stored until silylated for gas chromatography by the method of Sweeley and Walker (24).

A Varian 3700 gas chromatograph with a flame ionization detector interfaced to a Varian CDS 111 computer (Varian Instrument Div., Palo Alto, Calif.) was used to analyze the methylglycosides. The gas chromatograph was equipped with a glass column (10 ft [ca. 3 m] by 2 mm inner diameter) packed with 3% G.C. GE-SE-30 by weight on 100/120 Gas-Chrom Q (Applied Science Laboratories Inc., State College, Pa.) and a carrier gas (N_2) flow of 30 ml/min. The injector and detector temperatures were kept constant at 250°C. The silylated methylglycosides were eluted with the following temperature program: 115°C for 5 min followed by a temperature increase (1°C/min) until 220°C was reached.

Fatty acid analyses. The fatty acids of the *S. pyogenes* membrane were analyzed by two different methods.

(i) Membranes (5 mg) were resuspended in 2 ml of a freshly made 6% hydrochloric acid in methanol solution and 0.5 ml of benzene. The tubes were flushed with nitrogen, sealed, and heated at 80°C for 16 h. After the samples were cooled, 1 ml of water and 2 ml of hexane were added to each sample. The layers were separated and the hexane phase was removed. The aqueous layer was washed with an additional 2.0 ml of hexane. Next the hexane layers were combined, and a mixture (1 g) containing 4 parts sodium sulfate and 1 part ammonium bicarbonate was added to each sample and allowed to incubate at room temperature for 1 h. After removal of the salts, the samples were allowed to dry under nitrogen and were resuspended in carbon disulfide for analysis by gas chromatography.

(ii) The second method used was that described by McCreary et al. (16), using (*m*-trifluoromethylphenyl)trimethylammonium hydroxide. Samples (5 mg) were solubilized in 250 μl of benzene, to which was added 100 μl of Meth-Prep II (Applied Science Laboratories, Inc.). Reaction mixtures were allowed to stand at room temperature for 30 min. After removal of the sediment, the sample was ready for injection into the gas chromatograph.

The methyl esters of the fatty acids prepared by both procedures were analyzed by using the same Varian 3700 gas chromatograph equipped with a glass column (10 ft [ca. 3 m] by 2 mm inner diameter) containing GP 3% SP-2100 DOH on 100/120 Supelcoport (Supelco Inc., Supelco Park, Belfonte, Pa.). A 30-min program was used, starting at 150°C with an 8°C/min rise and finally holding at 240°C. The injector and detector temperatures were maintained at 250°C, and a carrier gas (N_2) flow rate of 20 ml/min with a make-up gas flow rate of 10 ml/min was used. An internal standard ($C_{15:0}$) was used with both methods.

Solubilization of membranes with 6 M guanidine. Membranes (~10 mg) were solubilized in 6 M guanidine (2 ml) and placed at 4°C for 15 min with constant stirring. The insoluble residues were then sedimented at 150,000 × *g* for 60 min. The top half of the supernatant was removed and dialyzed against 10 mM Tris-hydrochloride buffer (pH 8.0). The remainder of the supernatant was aspirated, and the pellet was resuspended in 2 ml of 6 M guanidine at 4°C for an additional 15 min. After the membranes were sedimented, the supernatant was removed for analysis and the pellet was resuspended in 10 mM Tris-hydrochloride buffer (pH 8.0) and dialyzed to remove any residual guanidine.

Assay of ATPase activity. Adenosine 5′-triphosphatase (ATPase; EC 3.6.1.3) was assayed by the method of Abrams (1) on freshly isolated membranes. The released inorganic phosphate from adenosine 3′,5′-triphosphate was measured by the procedures of Taussky and Shorr (26).

RESULTS

In all the experiments described below, microorganisms were harvested from the same culture during midexponential growth and 2 h into the stationary phase of growth. We have previously established that the membrane and phage-associated lysin do not contain proteolytic activity (I. van de Rijn, *in* S. E. Read and J. B. Zabriskie, ed., *Streptococcal Diseases and the Immune Response*, in press). To dismiss the possibility that proteolytic enzymes present in either the cytoplasmic or cell wall fraction were acting on the intact protoplast membrane, the two protease inhibitors Trasylol (500 U/ml; FBA Pharmaceuticals, New York, N.Y.) and phenylmethylsulfonyl fluoride (1 mM) were added to all isolation and purification buffers. Membranes prepared with and without the protease inhibitors were assayed by SDS-PAGGE. The only difference detected between the two preparations was the addition of a single polypeptide in the high-molecular-weight region. There were no signs of proteolytic breakdown of any of the stained protein bands as determined by SDS-PAGGE when the protease inhibitors were present or absent.

Analysis of membranes for purity and biological activity. Each membrane preparation was analyzed for contamination by molecules from either the cell wall or the cytoplasmic fraction. Rhamnose and *N*-acetylglucosamine were used as marker sugars for cell wall contamination. Neither sugar was present in excess of 0.01% in the various membrane preparations. Ribose and deoxyribose served as cytoplasmic markers, and neither sugar was detected at levels of 0.1%. The various sugars were detected by gas-liquid chromatography and amino acid analysis where applicable.

Since ATPase appears to be a peripheral enzyme in other organisms (20), the ATPase activity of the streptococcal membranes was analyzed to determine whether the various procedures used in purification of the membranes released peripheral enzymes into the buffers (Table 1). Preparations of type 6 membranes contained 19.7 U of ATPase activity (specific activity, 0.5

U/mg per min). No ATPase activity was found in the soluble wall fraction, whereas 7.8 U was found in the cytoplasmic fraction. The specific activity of the membrane-bound enzyme was five- to sixfold greater than that of the cytoplasmic enzyme. Only a total of 2.1 U was found in the various washings of the membrane, indicating that only minor losses of enzyme occurred during these procedures. Similar amounts of enzyme were present in type 49 membranes, and there was no difference in specific activity of the enzyme in either exponential- or stationary-phase membrane preparations. Schnebli and Abrams (21) have demonstrated that membrane-bound ATPase activity in the group D streptococci are cold sensitive. Less than 1 U of ATPase activity remained in group A streptococcal membrane preparations after storage at −20°C overnight, indicating that this ATPase is also cold sensitive.

TABLE 1. *ATPase activity during membrane purification*

Fraction analyzed	Total activity (U)[a]
Soluble wall	0
Cytoplasm	7.8
Washes	2.1
Membrane	19.7
Membrane (−20°C)	0.9

[a] The reaction mixture (1 ml) contained: 50 mM Tris-hydrochloride (pH 7.5), 5 mM $MgCl_2$, 5 mM adenosine 5′-triphosphate, and the fraction to be tested for activity. Controls for inorganic phosphorus in the sample and release of phosphorus from adenosine 5′-triphosphate nonenzymatically were run simultaneously. The total ATPase activity of lysed streptococci was 32.3 U. Membranes (72 mg of protein/5 ml) were stored at −20°C for the cold-sensitive experiment, and amounts equal to those used in the initial experiments were assayed. All reactions were carried out in triplicate, and the values listed are the means of three experiments.

Chemical analysis of the streptococcal membranes. To determine the total protein content and distribution of amino acids, the membrane preparations were analyzed for their amino acid content (Table 2). None of the membrane preparations contained the amino acids cysteine and tryptophan. There appeared to be no difference in the mole ratio of amino acid content between various types of membrane preparations. The total protein content, as calculated from amino acid analysis, from type 6 exponential- and stationary-phase cells was 72 and 70%, respectively, whereas the type 49 membrane contained 73% protein in both preparations. Thus, no significant differences were seen in total amount of protein in the various prepa-

TABLE 2. *Amino acid composition of S. pyogenes membranes*[a]

Amino acid	Type 6 membrane				Type 49 membrane			
	Exponential		Stationary		Exponential		Stationary	
	Residue (%)	Concn (μg/mg of membrane)	Residue (%)	Concn (μg/mg of membrane)	Residue (%)	Concn (μg/mg of membrane)	Residue (%)	Concn (μg/mg of membrane)
Asp	10	78.39	10	75.68	10	79.14	10	78.75
Thr	6	39.20	6	38.03	6	40.01	6	39.81
Ser	7	38.55	6	36.35	7	40.50	7	39.99
Glu	11	95.54	11	92.64	11	95.67	11	95.25
Pro	4	23.60	4	22.84	4	28.87	4	28.54
Gly	8	29.85	8	28.65	8	30.07	8	29.81
Ala	10	47.44	10	45.87	10	47.83	10	47.58
Cys	0	0	0	0	0	0	0	0
Val	6	40.54	6	30.09	6	40.99	6	40.53
Met	3	25.75	3	25.03	3	26.13	3	25.89
Ile	6	41.87	6	40.49	6	42.11	6	41.94
Leu	8	66.57	9	64.32	9	66.51	9	66.34
Tyr	4	42.33	4	40.97	4	42.64	4	42.38
Phe	4	38.30	4	37.03	4	38.50	4	38.29
His	2	16.37	2	15.78	2	16.81	2	16.75
Lys	7	57.68	7	55.81	7	58.00	7	57.73
Arg	4	40.12	4	39.25	4	40.18	4	40.00
Trp	0	0	0	0	0	0	0	0
Total	100	722.10 (72%)	100	697.93 (70%)	101	733.96 (73%)	101	729.58 (73%)

[a] Values are the means of analyses of two preparations of membranes. All amino acids were corrected where necessary for breakdown under the hydrolysis conditions. Cysteine was assayed for by looking for the presence of cysteic acid after performic acid oxidation (8).

rations. Analysis of the membrane protein content by a modified Lowry procedure (3) demonstrated a mean percentage of 65% protein for the membrane preparations, using bovine serum albumin as standard.

Analysis of membranes from six different serotypes of group A streptococci demonstrated that glucose was the only sugar present in any preparation (26). Membranes isolated from type 6 exponential- and stationary-phase cells contained 3.3 and 3.0% glucose, respectively, whereas membranes from type 49 exponential- and stationary-phase cells contained 2.8 and 3.3% glucose (Table 3). No other sugar could be detected in our purified membrane preparations even when the organisms were grown in different media or various sources of serum were added (unpublished observation). In these studies, there was no significant difference between the amount of glucose present in exponential- and stationary-phase membranes.

A significant change between membranes from exponential- and stationary-phase cells occurred in the phosphorus fraction of the streptococcal membrane (Table 3). The phosphorus content of type 6 membranes increased from 5 to 7 μg/mg, and that of type 49 membranes increased from 4 to 7 μg/mg. Phosphorus content increased further from membranes which were isolated from streptococci harvested 4 h later in the stationary phase (9 μg/mg).

Analysis of the membrane preparations for fatty acids demonstrated that they comprised 15% of the dry weight of the membrane, which agrees with the work of others (18). The data from the two methods for preparation of samples showed no significant differences in either the composition or quantitation of fatty acids in the membrane. Analysis of the fatty acids by gas-liquid chromatography demonstrated that four major fatty acids were present in both types of membranes: $C_{16:U}$, $C_{16:O}$, $C_{18:U}$, and $C_{18:O}$ (Table 4). There were no significant differences between membranes from exponential- or stationary-phase cells. There was, however, a difference in the ratio of the four major fatty acids between type 6 and type 49 membranes. The type 49 membranes also contained significantly greater amounts of $C_{12:O}$ and $C_{14:O}$ fatty acids.

TABLE 3. *Glucose and phosphorus composition of S. pyogenes membranes*[a]

Membrane	Concn (μg/mg of membrane)	
	Glucose	Phosphorus
Type 6		
Exponential	33	5
Stationary	30	7
Type 49		
Exponential	28	4
Stationary	33	7

[a] Values are the means of analyses of two preparations of membranes.

TABLE 4. *Fatty acid composition of S. pyogenes membranes*[a]

Fatty acid	Composition (mol%)	
	Exponential	Stationary
Type 6 membrane		
12:0	—[b]	—
14:0	0.55 ± 0.06	0.59 ± 0.05
15:0	—	—
16:U[c]	6.74 ± 0.17	7.78 ± 0.16
16:0	22.73 ± 0.15	22.29 ± 0.12
17:0	—	0.22 ± 0.01
18:U	49.89 ± 0.22	50.25 ± 0.24
18:0	15.48 ± 0.05	14.47 ± 0.17
19:0	0.26 ± 0.02	0.20 ± 0.01
20:0	0.96 ± 0.03	0.89 ± 0.08
X[d]	3.39 ± 0.50	3.31 ± 0.45
Type 49 membrane		
12:0	1.03 ± 0.05	0.97 ± 0.22
14:0	2.10 ± 0.02	2.47 ± 0.02
15:0	—	0.36 ± 0.02
16:U	18.51 ± 0.18	20.51 ± 0.53
16:0	29.56 ± 0.76	27.93 ± 0.55
17:0	0.37 ± 0.03	0.24 ± 0.01
18:U	39.50 ± 0.20	39.69 ± 0.52
18:0	6.47 ± 0.53	5.49 ± 0.15
19:0	0.20 ± 0.01	0.27 ± 0.07
20:0	0.57 ± 0.02	0.57 ± 0.21
X	2.43 ± 0.22	2.51 ± 1.32

[a] Results are presented as mean mole percent composition ± standard error of the mean of at least four analyses. Since both procedures of preparation (see Materials and Methods) gave similar results, the values shown are compilations of the two types of sample preparation.

[b] —, Not detected.

[c] Total unsaturated fatty acids, using X:1 as standard.

[d] Unknown.

SDS-PAGGE analysis of the streptococcal membrane. In an attempt to determine whether the individual proteins of the membranes varied between exponential- and stationary-phase membranes, membrane preparations (150 μg) were solubilized in 2% SDS and resolved by using 7 to 15% gradient polyacrylamide slab gels (Fig. 1). On initial observation, 60 to 70 polypeptides could readily be discerned with this technique. The majority of the polypeptides ranged from 80,000 down to 10,000 daltons, with two large polypeptides just entering the running gel. By inspection, the polypeptides from exponential- and stationary-phase membranes appeared to differ in quantity or the presence of additional polypeptides, or both. In addition,

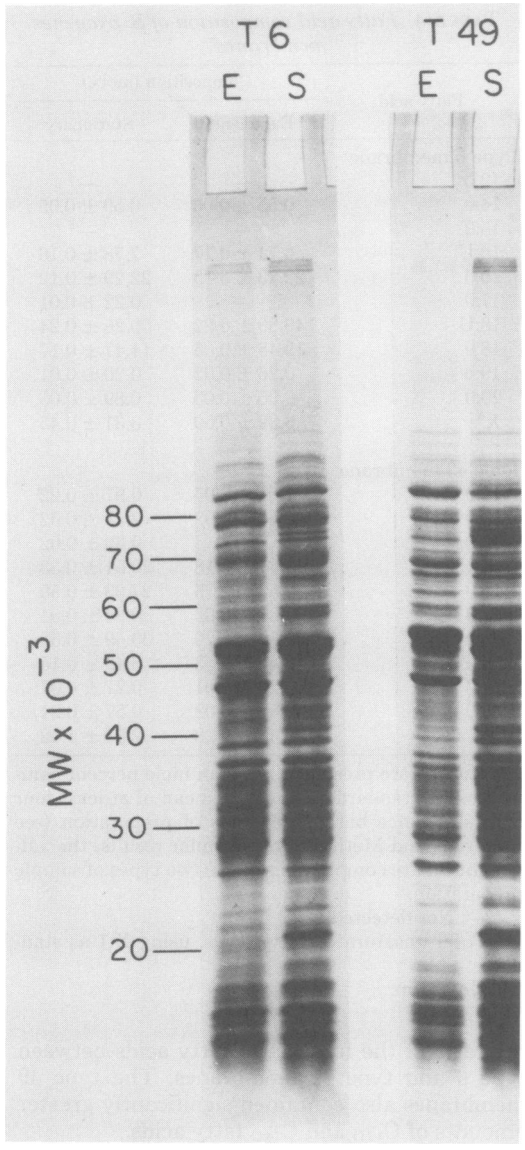

FIG. 1. *SDS-PAGGE (7 to 15%) of S. pyogenes membrane proteins. Samples (150 μg) were solubilized, electrophoresed, and stained as described in Materials and Methods. The loads were: type 6 (T6) and type 49 (T49) membranes, exponential (E) and stationary (S).*

there seemed to be a similar difference in polypeptides between the membranes from the two strains.

To further delineate the differences in the *S. pyogenes* membrane during the midexponential and early stationary phases of growth, membrane preparations were treated with 6 M guanidine at 4°C. Under the conditions of extraction, the proteins solubilized by the treatment were termed peripheral, whereas those that remained insoluble were termed integral membrane proteins (22). Type 6 exponential- and stationary-phase membranes released 53.3 and 46.3% of their total protein content under these procedures, respectively, whereas type 49 exponential- and stationary-phase membranes released 56.7 and 37.9% (Table 5). In addition, using both the soluble and insoluble fractions and analyzing them by SDS-PAGGE, we were able to further resolve the integral and peripheral proteins of the membranes (Fig. 2). Samples (150 μg) were loaded onto each slot and separated on a 7 to 15% gradient gel. The membranes that could initially be separated into 60 to 70 polypeptides now were shown to contain nearly double that number, with approximately one-half in each fraction. When the peripheral proteins of the type 6 exponential- and stationary-phase membranes were analyzed, the exponential-phase membranes contained the following polypeptides in greater amounts or as additional polypeptides: 5, 18, 23, 37, 39, 56, and 64. The stationary-phase membranes contained the following in increased amounts or as additional polypeptides: 10, 12, 49, 55, and 68. In addition, when the integral proteins of the type 6 membrane were analyzed, the stationary-phase membranes contained polypeptides 32 and 51 in additional amounts or as extra proteins. Next, upon analysis of peripheral and integral proteins from the membrane of the type 49 streptococci, polypeptides 9, 23, and 25 were found to be present in greater quantities or as additional proteins, and polypeptides 35, 36, 43, and 56 were found in membranes from exponential-phase cells as compared with stationary-phase cells. Peripheral and integral proteins from type 49 stationary-phase membranes contained bands (12, 17, 21, 43, 57, and 62) and (29, 30, 55, and 61), respectively, as compared with the exponential-phase membranes. Similar results were obtained when 8 M urea was used (gel not shown). These studies therefore provide further evidence that there are both quantitative and qualitative differences between exponential- and stationary-

TABLE 5. *Solubilization of peripheral proteins of S. pyogenes membranes by 6 M guanidine*[a]

Membrane	Fraction		Protein released (%)
	Integral (mg)	Peripheral (mg)	
Type 6			
Exponential	4.3	4.9	53.3
Stationary	4.4	3.8	46.3
Type 49			
Exponential	2.9	3.8	56.7
Stationary	7.7	4.7	37.9

[a] Values are the means of analyses of two preparations of membranes.

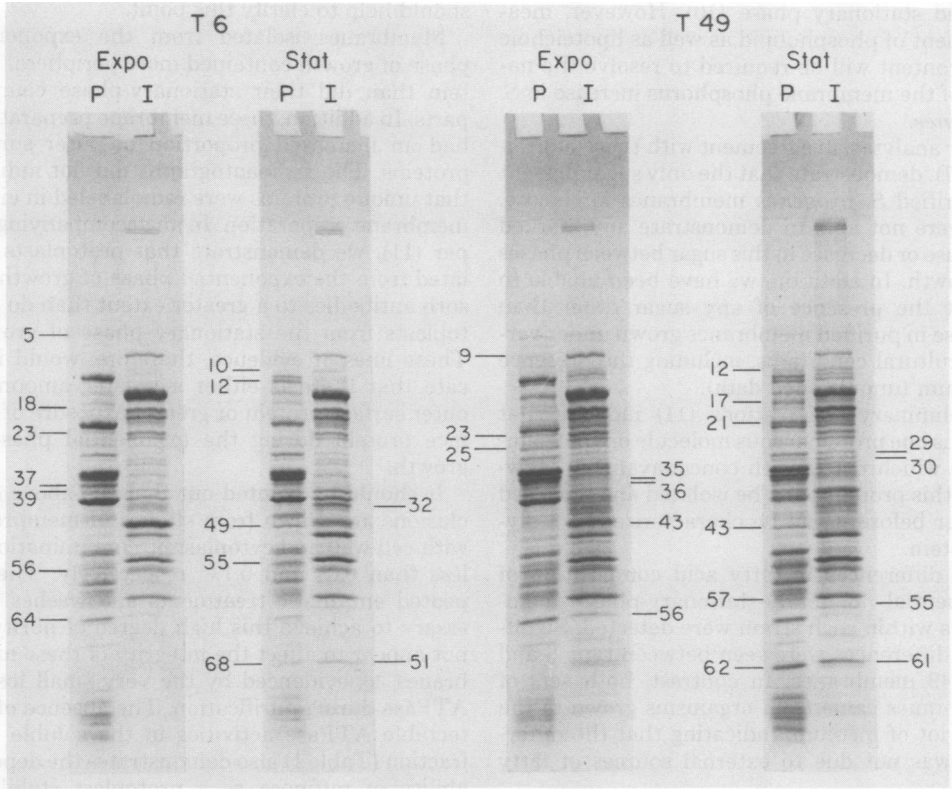

FIG. 2. *SDS-PAGGE (7 to 15%) of peripheral and integral proteins of S. pyogenes membranes. Samples (150 μg) were solubilized, electrophoresed, and stained as described in Materials and Methods. The polypeptides were numbered by analyzing membrane preparations separated on six separate gels starting at the top of each slot. The loads were: type 6 (T6) and type 49 (T49) membranes, exponential (Expo), peripheral (P), and integral (I); stationary (Stat), peripheral (P), and integral (I).*

phase membranes. These differences appear to reside mainly in the peripheral rather than the integral proteins.

Radioiodination of surface membrane proteins. To determine which proteins were expressed on the outer surface of intact protoplasts, osmotically stabilized protoplasts were radiolabeled with ^{125}I. In two experiments with type 6 exponential- and stationary-phase membranes, the ratios of labeling of exponential- to stationary-phase protoplasts were 1.51:1 and 1.48:1. Similar results were obtained with type 49 protoplasts, where the ratio was 1.55:1, suggesting that exponential protoplasts bear a greater amount of surface protein on their membrane. Isolated cytoplasmic proteins contained less than 1% incorporation of the label as compared with the isolated protoplast membrane, apparently indicating that only the proteins of the outer surface of the intact protoplast membrane were labeled. To further delineate which outer proteins were enzymatically labeled with ^{125}I, the intact protoplasts were osmotically lysed, purified, and then analyzed by SDS-PAGGE. Initial observations suggested that there were only five to six highly radioiodinated polypeptides on the outer surface of the protoplast membrane. In addition, there were 10 to 15 polypeptides with low amounts of iodination (radioautograph not shown). No major differences were determined between exponential- and stationary-phase membranes except for the presence of label in polypeptide 68 (Fig. 2).

DISCUSSION

Although the gross composition of the *S. pyogenes* membrane appeared to remain relatively constant between the exponential and stationary phases of growth, changes in individual components were found. Of the major categories of protein, carbohydrate, fatty acid, and phosphorus, only the latter appeared to change significantly. The phosphorus content was greater in stationary-phase than exponential-phase membranes. This increase is consistent with the observation that the lipoteichoic acid of *S. faecium* increased during valine starvation, which is a

defined stationary phase (10). However, measurement of phospholipid as well as lipoteichoic acid content will be required to resolve the nature of the membrane phosphorus increase in *S. pyogenes*.

Our analyses, in agreement with those of Freimer (7), demonstrate that the only sugar present in purified *S. pyogenes* membranes is glucose. We were not able to demonstrate any marked increase or decrease in this sugar between phases of growth. In addition, we have been unable to detect the presence of any sugar other than glucose in purified membranes grown under various cultural conditions, including the presence of serum (unpublished data).

Preliminary observations (11) indicate that there is one proteinaceous molecule on the membrane which reacts with concanavalin A. However, this protein must be isolated and analyzed further before it can be characterized as a glycoprotein.

No differences in fatty acid composition of exponential- or early-stationary-phase membranes within each strain were detected. Significant differences were seen between type 6 and type 49 membranes. In contrast, both sets of membranes came from organisms grown in the same lot of medium, indicating that the difference was not due to external sources of fatty acids.

The total protein and amino acid composition of the membranes from both type 6 and type 49 streptococci did not vary between phases of growth, and the mole ratios of amino acids compared well with those previously published (7, 19). Cysteine and tryptophan were undetectable in our membrane preparations. However, it is possible that a minority of proteins contain these amino acids and that the amount present is below our level of detection.

Clear differences were demonstrated in the polypeptide pattern of the membrane by SDS-PAGGE. The addition of protease inhibitors did not alter the profile of polypeptides by SDS-PAGGE or crossed immunoelectrophoresis (unpublished observation), indicating the absence of active proteases during the purification procedures. The integral protein pattern remained fairly constant, whereas the peripheral membrane protein pattern varied both between strains and between growth phases. There appeared to be changes in the concentration of individual proteins between growth phases as well as proteins unique to a particular growth phase. It is still possible that the differences we see in the concentration of individual proteins are due to an overlap of distinct proteins. The O'Farrell two-dimensional electrophoresis system (17) or crossed immunoelectrophoresis (28) should help to clarify this point.

Membranes isolated from the exponential phase of growth contained more peripheral protein than did their stationary-phase counterparts. In addition, these membrane preparations had an increased proportion of outer surface proteins. The radioautographs did not indicate that unique proteins were radiolabeled in either membrane preparation. In an accompanying paper (11), we demonstrate that protoplasts isolated from the exponential phase of growth absorb antibodies to a greater extent than do protoplasts from the stationary phase of growth. These lines of evidence, therefore, would indicate that there is either a greater amount of outer surface protein or greater exposure of surface protein during the exponential phase of growth.

It should be pointed out that the above conclusions are drawn from studies of membranes with cell wall and cytoplasmic contamination at less than 0.01 and 0.1%, respectively. The repeated enzymatic treatments and washes necessary to achieve this high degree of purity do not appear to affect the integrity of these membranes, as evidenced by the very small loss of ATPase during purification. The absence of detectable ATPase activities in the soluble wall fraction (Table 1) also demonstrates the dependability of raffinose as a protoplast stabilizer. Further evidence of maintenance of integrity during purification is the presence of two additional enzyme activities, reduced nicotinamide adenine dinucleotide dehydrogenase and polynucleotide phosphorylase, as shown in the accompanying paper (11).

These studies demonstrate that the composition of the group A streptococcal membrane may be different depending upon the conditions of growth. The implications of these differences and perhaps those occurring under other cultural or environmental conditions should be borne in mind in studies related to the pathogenesis of streptococcal or any other bacterial diseases.

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