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Immunological and Chemical Studies of the Streptococcal Group O Protein Type Antigens

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Two antigens present in group O streptococci have been described and are designated as type antigens. No other antigens are present in extracts of group O cells in significant quantity. Antigen I is a cell wall protein, and II is a nucleoprotein of undetermined cell location. The immunological specificity of the latter resides only in the protein. Both antigens have been extracted from the cell with water or 0.2 N HCl (*p*H 2) at 100 C, and antigen I can be removed by trypsin. After purification, *p*H 2 was found to destroy the serological activity of each antigen. Antgens I and II and also the O polysaccharide antigen can be distinguished on a single agar-gel diffusion plate with group O whole-cell antiserum. N- and C-terminal amino acid analyses and total amino acid composition prove that I and II are separate molecular species. Each possesses properties which separate them from the streptococcal M, R, and T proteins. Antigen I separated into three fractions, each possessing serological specificity, on a diethylaminoethyl-Sephadex column at *p*H 8.6 during an increase in buffer molarity. The protein is considered to possess a multiple unit structure. The results indicate that the O streptococci are limited to two serological types. Group O whole-cell antisera, after adsorption with a strain not containing the O polysaccharide, can now be prepared for the positive identification of the types of group O streptococci.

The beta-hemolytic group O streptococci are the frequent cause of subacute endocarditis in man (3). Recent studies from our laboratory (19) have defined the chemical nature, immunological specificity, and location of the group O antigen in the streptococcal cell. These studies also revealed the presence of two additional principal antigens in the group O cell. This report identifies these two antigens as proteins which differ immunologically from all other known protein antigens of the streptococci. Their chemical and immunological properties are described. These data allow the preparation of specific sera for the identification of the two group O type antigens.

MATERIALS AND METHODS

Streptococcal strains. The cultures employed were the same as those used in the previous study (19). Each was reisolated on sheep blood agar and tested with group O antiserum by the capillary precipitin test (30). The cells for study were grown, harvested, and lyophilized as previously described (7). We thank Aaron Lane, Difco Laboratories, Detroit, Mich., for some of the B357 cells.

Extraction of type antigens. HCl (0.2 N) or 10% cold trichloroacetic acid extracts of antigens from dry, whole cells were prepared as described previously (19). Cold-water extracts (WCw) were obtained by stirring for 17 hr at 5 C 20 mg of dry cells in 2 ml of distilled water, followed by centrifugation at 10,400 × *g*. On agar-gel diffusion analysis, these extracts were found to contain other antigens as well as the group antigen (19).

Serological procedures. Each fraction obtained was tested for antigen by capillary precipitin test (30), agar-gel diffusion test (16), and immunoelectrophoresis (15). Streptococcal antisera were obtained as described (24).

Antibodies against the O group polysaccharide antigen were removed from whole-cell antiserum by adsorption. Serum (0.3 ml) was mixed with 30 μg of antigen preparation (Flt) (19), incubated for 1 hr at 37 C and for 17 hr at 5 C, and centrifuged. The adsorption was repeated. No antibodies were detected by the precipitin test.

Purification of type antigens. A 20-g amount of dry, whole cells (B357 strain) was suspended in 300 ml of distilled water, stirred at 5 C overnight, and centrifuged. The cells were washed once with 500 ml of distilled water and centrifuged. The supernatant fluids were combined and reduced by flash-evaporation to about 20 ml. The sediment was removed by centrifugation, and the supernatant fluid was equilibrated in

1 liter of 0.05 M $(NH_4)_2CO_3$, *p*H 8.5, buffer. The solution was chromatographed by the linear gradient concentration [140 ml of 0.1 M $(NH_4)_2CO_3$, *p*H 8.5, and 140 ml of 1 M $(NH_4)_2CO_3$, *p*H 8.6] on a diethylaminoethyl (DEAE) Sephadex A-25 column (1.5 by 55 cm). A 5.6-ml amount was collected in each case. Tubes (no. 32–37 and 42–45) which showed positive precipitin reaction against anti-O serum were collected, and both effluents were lyophilized and designated F2w° (40 mg) and F3w° (65 mg), respectively. The letter "F" stands for "fraction."

F2w° (25 mg) was further purified on a Sephadex G-200 column (1.1 by 55 cm) in 0.05 M $(NH_4)_2CO_3$, *p*H 8.5. Buffer (1.2 ml) was collected in each tube. The first, middle, and last peaks, which showed positive precipitin reactions, were designated F2w′, F2w″, and F2w fractions, respectively. After lyophilization, each was used for further analysis.

F3w° (50 mg) was purified on a DEAE-Sephadex A-50 column (1.5 by 50 cm) by linear gradient concentration [140 ml of 0.5 M $(NH_4)_2CO_3$, *p*H 8.6, and 140 ml of 1 M $(NH_4)_2CO_3$]. A 5.3-ml amount was collected in each tube. The purified fraction, F3w, weighed 3.2 mg.

Following the same procedure, fractions F2a (25.2 mg) and F3a (6.5 mg) were obtained from the crude acid extract, WCa.

Whole cells (1 g) in 25 ml of water were treated in a Braun homogenizer for 5 min while CO_2 flowed over the flask. The cell wall fraction was collected, washed with 200 ml of water three times, and treated with 10 mg of ribonuclease (Schwarz/Mann, Orangeburg, N.Y.) at 37 C for 1 hr in 200 ml of 0.01 M phosphate buffer, *p*H 7.8. The crude extract, F2cw, was collected and lyophilized (51 mg).

Chemical analysis. Thin-layer chromatography for detection of amino acids and sugars was performed as described previously (15, 27). Total phosphorus (28) and total pentose (2) were estimated as reported.

Determination of amino acids was made on a Beckman model 116 amino acid analyzer (25, 29) and by liquid-gas chromatography (6), with some modifications. As for the latter method, 10 to 100 μg of dry sample was hydrolyzed with 6 N HCl at 110 C for 16 hr in a sealed tube. After vacuum drying, the amino acids were methanolized in 0.5 ml of 1.2 N HCl in absolute methanol at room temperature for 4 hr in a sealed tube, and dried under a vacuum. The derivatives were changed into butyl derivatives in 0.2 ml of 1.2 N HCl in absolute butanol at 75 C for 4 hr in a sealed tube. After drying, the residue was treated with 0.1 ml of 10% trifluoroacetic anhydride in methylene chloride at room temperature in a sealed tube for 2 hr. After evacuation, the residue was dissolved in 10 to 100 μliters of anhydrous chloroform, and 2 to 50 μliters was injected into a gas chromatograph (Varian Aerograph model 1200). The chromatograph was equipped with a glass column (180 by 0.31 cm) packed with 60- to 80-mesh acid-washed chromasorb W coated with 0.75% DEGS and 0.25% EGSS-X (6). The temperature of the column was increased at the rate of 8 C/min, from 60 C to 220 C. Butyl-trifluoroacetyl esters of known amino acids served as standards. The sensitivity of the recorder was 1 × 16, and the chart speed was 10 cm/min.

Analysis of nucleic acid components was done as follows. A 20- to 200-μg amount of sample was hydrolyzed by 50 μliters of N HCl at 100 C for 1 hr (26). The hydrolysate was chromatographed on a cellulose thin-layer plate in isopropanol-concentrated HCl-water (136:33:31) (32). Each nucleic acid base was located under a short-wave ultraviolet lamp, extracted with 0.1 N HCl, measured photometrically, and compared with standard bases.

Molecular weight estimation. Molecular weight of antigen was estimated by the gel-filtration method (1), using a Sephadex G-200 column (1.1 by 50 cm) and a flow rate of 0.05 ml/hr. Using blue dextran 2000 and phenylalanine, the void volume and the total bed volume of the column were 18.2 and 36.0 ml, respectively. Protein standards were bovine serum albumin (molecular weight, 67,000; Armour Pharmaceutical Co., Kankakee, Ill.) and ribonuclease (molecular weight, 12,640; Schwarz/Mann).

Terminal amino acid analysis. N-terminal amino acids were determined and measured by the dinitrophenylation method (13, 14), with the following modifications. For the purification of dinitrophenylation proteins, a Sephadex G-15 column (1.1 by 40 cm) in water was used, and for two-dimensional chromatography, cellulose-coated thin-layer plates were used instead of paper sheets. Lyophilized samples (300 μg) were used.

C-terminal amino acids of the antigens were analyzed by a carboxypeptidase A degradation method (12). A 2-mg amount of sample plus 0.1 mg of diisopropyl-fluorophosphate-treated carboxypeptidase A (Worthington Biochemical Corp., Freehold, N.J.) in 2 ml of 1% $NaHCO_3$ was incubated at 37 C with stirring for 10-, 30-, 60-, and 120-min intervals. A 30-μliter amount of the reaction mixture was removed at each interval, and the amino acids released by the enzyme were separated on a Sephadex G-15 column (1.1 by 40 cm) in water. The amino acids were lyophilized and analyzed by liquid-gas chromatography as described above.

Acid and enzyme stability of antigens. Purified antigens (1 μg/μliter) were treated with various concentrations of HCl at several temperatures. Trypsin, pepsin, β-glucosidase, and alkaline phosphatase were used as described (19). Ribonuclease (5%, w/w) was used in 0.01 M phosphate buffer (*p*H 7.8), and deoxyribonuclease (5%, w/w, plus 10^{-2} M $MgSO_4$) was used in 0.01 M phosphate (*p*H 6.5). After 0.5, 1, 2, and 4 hr, the reaction mixture was ajdusted to *p*H 7.0 to 7.5 with NaOH and tested for antigenity by the capillary precipitin reaction.

In the case of the nucleoprotein antigen, the reaction mixture after treatment with ribonuclease was chromatographed on a Sephadex G-25 column (1.1 by 50 cm) in cold water. The nucleic acids were analyzed as described above. The unhydrolyzed protein fraction was lyophilized and used for the measurement of molecular weight, antigen specificity by agar-gel diffusion, and for amino acid content.

Preparation of cell walls and release of antigens by trypsin or HCl. Whole, dry cells were broken by either

Mickle shaking (23) or Braun homogenation. After ribonuclease treatment (23) and three water washes, the material was lyophilized (yield, 37.8%). The dry wall preparation from 50 mg of cells was then held at 65 C for 90 min in 5 ml of 1% sodium dodecyl sulfate, washed three times, and lyophilized. The sodium dodecyl sulfate treatment was used to remove contaminating protoplast membrane. The final yield of wall material was 19.0%. A 20-mg amount was treated with 0.4 mg of trypsin in 0.4 ml of 0.01 M phosphate (*p*H 7.8) at 37 C for 60 min, or with 0.02 N HCl at 100 C for 10 min. After centrifugation, each extract was adjusted to *p*H 7.2 and used in agar-gel diffusion and capillary precipitin tests.

Whole-cell agglutination in capillary tubes. After adsorption with F2 or F3, 10 μliters of whole-cell B357 antiserum was drawn into a capillary precipitin tube, followed by an equal amount of a smooth cell suspension (10 mg of cells/5 ml of saline), and held for 60 min at 37 C and overnight at 4 C.

RESULTS

Extraction of protein antigens. The detection of antigens other than the polysaccharide group O antigen in HCl extracts was reported earlier (19). The initial studies to define these antigens were concerned with an effective means of extraction. Washing lyophilized group O cells removed two antigens (WCw) which reacted with group O whole-cell antiserum that had been adsorbed with the O polysaccharide (Fig. 1).

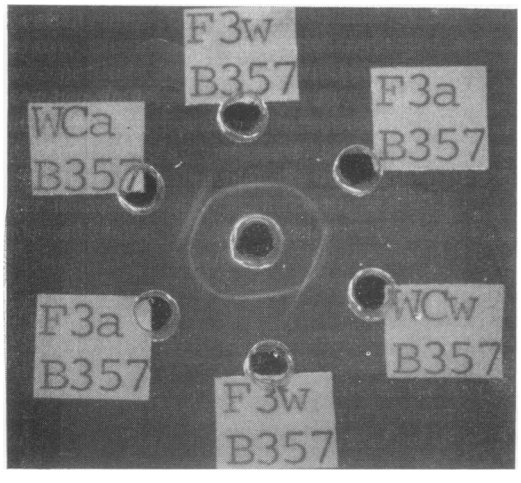

FIG. 1. *Type antigens from B357 (group O) dry, whole cells. Center well, 20 μliters of anti-B357 whole-cell serum after adsorption with group O polysaccharide antigen. Each outer well contains 20 μliters of crude extract or 20 μg of purified antigen. WCw, Water extract of whole cells; WCa, HCl extract of whole cells; F3w (Fig. 3), purified from water extract of whole cells; F3a, purified from WCa by chromatography.*

The second wash contained very little of each antigen, and the third wash was negative. The extraction of these cells with trypsin removed one of the two antigens, later designated as F2. Also, Fig. 1 shows that the same two antigens were removed by either 0.2 N HCl at 100 C (WCa) or water at 4 C (WCw). Neither of these antigens were extracted with trichloroacetic acid, indicating the presence of protein.

Purification of antigens. A water extract of B537 dry, whole cells was chromatographed on DEAE-Sephadex A25 (Fig. 2). Two separate fractions were obtained. The first, F2w°, contained protein but no appreciable phosphorus; the second, F3w°, contained significant quantities of protein and phosphorus. The serological reaction of both with B357 serum from which the group antibodies had been adsorbed was strong. High concentrations of $(NH_4)_2CO_3$ were required (0.55 M and 0.75 M, respectively) to elute these antigens from the column. This behavior, plus the adsorption and phosphorus values, indicated that F2w° was a protein and F3w° was a nucleoprotein. Fraction F2w° was purified further on Sephadex G-200, and three fractions were obtained (Fig. 3). A separate protein peak and a strong precipitin reaction were characteristic of each fraction.

A separation of the bulk of the protein and phosphorus from the immunologically active material in F3w° was tried on Sephadex G-200; however, no separation was achieved. DEAE-Sephadex A25 produced an excellent separation (Fig. 4), although a significant quantity of phosphorus remained in the antigen material.

Acid extracts containing the same two antigens,

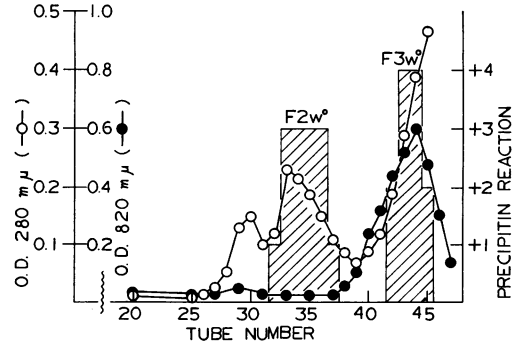

FIG. 2. *DEAE-Sephadex chromatography of water extract from B357 (group O) whole cells. Extract applied to DEAE-Sephadex column A-25 (1.5 by 55 cm). Initial buffer, 140 ml of 0.1 M $(NH_4)_2CO_3$, pH 8.5. Final buffer, 140 ml of M $(NH_4)_2CO_3$, pH 8.6. Each tube, 5.6 ml. Symbols: ○, adsorbance at 280 nm; ●, total phosphorus; shaded area, capillary precipitin reaction against anti-type B357 serum.*

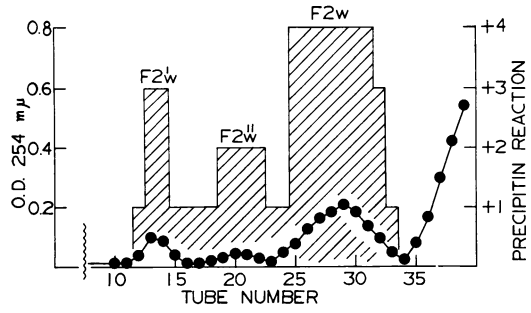

FIG. 3. *Purification of F2w° fraction. F2w° (see Fig. 2) was passed through Sephadex G-200 column (1.1 by 55 cm) in 0.05 M $(NH_4)_2CO_3$, pH 8.5, buffer. Each tube, 1.2 ml. Symbols: ●, adsorbancy at 254 nm; shaded area, precipitin reaction against anti-O serum.*

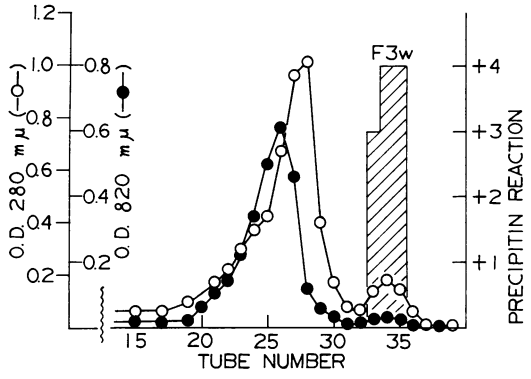

FIG. 4. *Purification of F3w° fraction. F3w° (see Fig. 2) was chromatographed on DEAE-Sephadex A-50 column (1.5 by 50 cm). Initial buffer, 140 ml of 0.5 M $(NH_4)_2CO_3$, pH 8.6. Final buffer, 140 ml of 1 M $(NH_4)_2CO_3$, pH 8.6. Each tube, 5.3 ml. Symbols: ○, adsorbance at 280 nm; ●, total phosphorus; shaded area, positive precipitin reaction against anti-O serum.*

F2a and F3a, were purified by the same procedures.

Immunological properties of the fractions. F2w and F3w are separate and distinct antigens (Fig. 5). No cross-reaction is evident between the two fractions.

F2w′, F2w″, and F2w possess a single common antigen (Fig. 6), although a separate molecular size for each is indicated (Fig. 3). The strongest precipitin reaction is possessed by the fraction with the largest quantity of protein, F2w.

Earlier studies described the group polysaccharide antigen of the O streptococci (19). This antigen, Flt, can be distinguished from F3w due to the marked difference in mobility (Fig. 7). As expected, the polysaccharide has very little mobility. F2w also shows little movement. The disappearance of the precipitin band due to Flt (group O polysaccharide) when adsorbed serum is used is further evidence of the nonidentity of the two protein antigens and the O antigen.

Quantitative precipitin curves. F2w and F3w precipitin curves are shown in Fig. 8. The equiv-

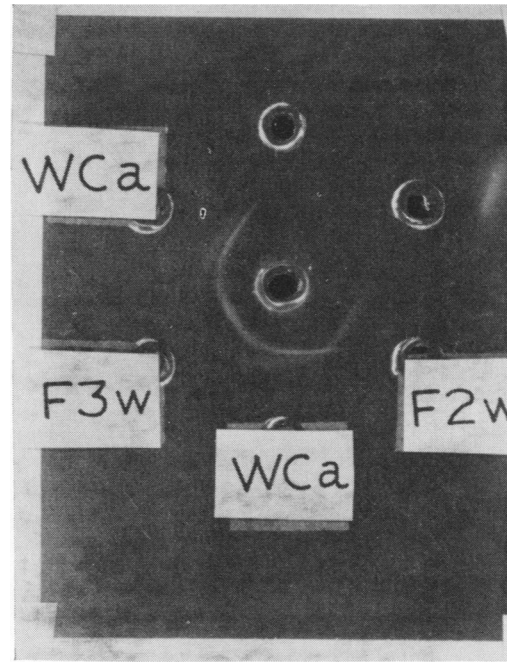

FIG. 5. *Reaction of nonidentity between F2w and F3w (Fig. 3, 4). WCa, Acid extract from whole cells. Center well contained anti-B357 serum adsorbed with the group O polysaccharide antigen (Flt). Procedure same as in Fig. 1.*

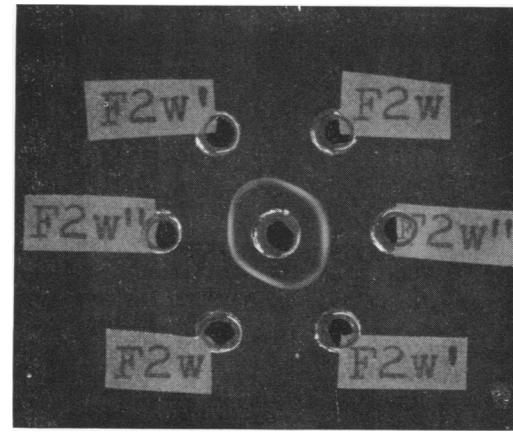

FIG. 6. *Reaction of identity between fractions F2w′, F2w″, and F2w. Analytical conditions same as in Fig. 1.*

alence point of both was about the same (8–10 μg). The shape of the upper curve (F2w) may be an indication of a multiple-unit structure (see Table 3), whereas the lower curve is more commonly seen with single proteins.

Chemical composition. Table 1 lists the composition of the two antigens. The total amino acid analysis of F2w was 89.9%; however, only 53.4% of F3w was recovered. Gas chromatographic analysis showed that no sugars were present in either, but that an unknown substance corresponding to ribose was present in F3w. Chemical analysis showed the presence of ribonucleic acid. Analysis for adenine, guanine, cytosine, uracil, phosphorus, and pentose, established the presence of nucleic acid (37.5%). The total recovery of F3w was 90.9%.

Physicochemical properties. Molecular weight estimation by gel filtration showed that F2w′ was greater than 200,000, and F2w″ and F2w were considerably smaller (Table 2). The molecu-

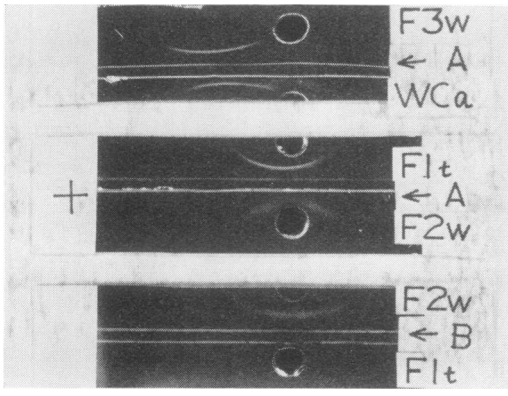

FIG. 7. *Immunoelectrophoresis of B357 antigens. Each well, 10 μg of antigen or 10 μliter of extract; trough A, anti-B357 whole-cell serum; trough B, anti-B357 whole-cell serum adsorbed with the group O antigen (Flt); WCa, whole acid extract; F2w and F3w (Fig. 3, 4).*

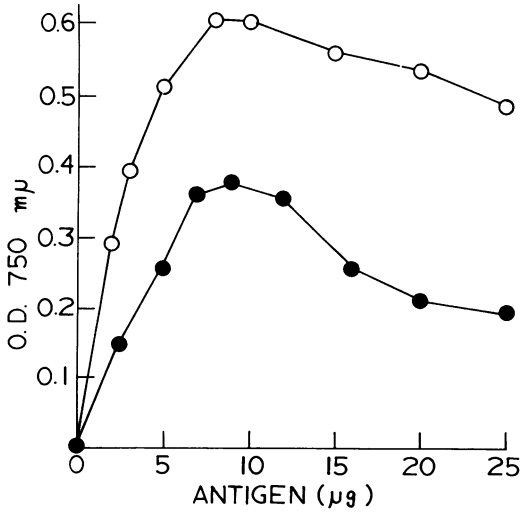

FIG. 8. *Quantitative precipitin curves of F2w and F3w (Fig. 3, 4). Anti-B357 serum (25 μliters) was used. Symbols: ○, F2w; ●, F3w.*

TABLE 1. *Chemical composition of F2w and F3w antigens*[a]

Components	Amt (μmoles/mg × 100)		Ratio F3w/F2w	Percentage F3w[c]
	F2w	F3w[b]		
Amino acid				
Lysine	5.6	9.6	1.71	
Histidine	3.2	2.2	0.69	
Arginine	1.5	4.9	3.27	
Aspartic acid	10.5	4.8	0.47	
Threonine	6.0	1.6	0.27	
Serine	5.0	2.7	0.54	
Glutamic acid	8.3	2.6	0.78	
Proline	3.2	6.5	2.03	
Glycine	8.3	2.6	0.31	
Alanine	4.6	6.4	1.39	
Methionine	1.3	1.2	0.92	
Valine	4.3	4.3	1.00	
Isoleucine	1.2	3.8	3.17	
Leucine	2.3	4.7	2.04	
Tyrosine	4.2	5.2	1.24	
Phenylalanine	3.5	0.9	0.26	
Nucleic acid				
Adenine				2.75
Guanine				4.19
Cytosine				4.50
Uracil				4.78
Pentose				14.40
Phosphorus				6.90

[a] Recovery of protein as isolated: F2w, 89.9%; F3w, 53.4%. Recovery of nucleic acid component: F3w, 37.5%.
[b] Calculated on the protein part of the molecule.
[c] No nucleic acid present in F2w.

TABLE 2. *Molecular weight of F2w and F3w antigens*

Antigen	Molecular weight[a]
F2w′	$>2.0 \times 10^5$
F2w″	1.4×10^5
F2w	4.7×10^4
F3w[b]	4.5×10^4

[a] Estimated by gel-filtration method.
[b] Calculations based on protein portion of antigen.

lar weight of F3w (1.0×10^5) was reduced to about one-half by treatment with ribonuclease.

N-terminal amino acids of F2w were phenylalanine, a small amount of glycine, and aspartic acid; the C-terminal amino acids were leucine and glutamic acid (Table 3). These results suggest that F2w is either a mixture of two or three proteins or a single protein composed of subunits. N- and C-terminal amino acids of F3w were tyrosine and phenylalanine. The relatively low yield of the terminal amino acids might be due to an inhibition of their release by the nucleic acid moiety of the complex.

Acid and enzyme stability. Table 4 shows that 0.05 N HCl at 60 C for as long as 60 min did not destroy the immunological activity of either antigen. An increase in temperature or time of exposure resulted in a partial loss of activity, with a complete loss after 1 hr at 100 C in 0.1 N HCl. Both antigens were resistant to trypsin but destroyed by pepsin, and ribonuclease and deoxyribonuclease did not destroy the serological activity. The protein released from F3w with ribonuclease was shown to be free of ribonucleic acid (RNA) and gave a reaction of identity in agar gel with untreated F3w. After digestion with pepsin, F3w did not react with group O serum. These data demonstrate that the activity of F3w was due to the protein portion of the molecule.

TABLE 3. *Terminal amino acids of F2w and F3w antigens*

Amino acids	F2w		F3w	
	μg/1 mg	mole/mole of protein	μg/1 mg	mole/mole of protein
N-Terminal[a]				
Phe	8.2	2.3		
Gly	0.9	0.6		
Asp	1.0	0.3		
Try			2.6	0.65
C-Terminal[b]				
Leu	6.7	2.4		
Glu	6.3	2.1		
Phe			2.8[c]	0.76[d]

[a] Estimated by dinitrophenol method. Abbreviations: Phe, phenylalanine; Gly, glycine; Asp, aspartic acid; Try, tyrosine.

[b] Carboxypeptidase method followed by gas chromatography.

[c] Maximal release of the amino acid was obtained after 30 min, under conditions described above in Methods. Abbreviations: Leu, leucine; Glu, glutamic acid; Phe, phenylalanine.

[d] Maximal release of the amino acid was obtained after 15 min, under conditions described above in Methods.

Distribution of antigens in O streptococci. Nine strains of group O streptococci were extracted with 0.2 N HCl at 100 C for 10 min. The extracts were analyzed by agar-gel diffusion with whole-cell antiserum. Five strains contained both protein antigens, two contained either F2w or F3w, and two contained neither (Table 5).

F2w and F3w were tested with specific antiserum to streptococcal groups A to H, K to N, and P to T. No reaction was obtained.

DISCUSSION

Significant differences exist between the F2 and F3 antigens and those protein antigens of the streptococci which have been described (the M, R, and T antigens of group A and an undesignated antigen of the group B streptococci). The properties of each are summarized in Table 6. The F antigens can be separated from M

TABLE 4. *Acid and enzyme stability of F2w and F3w antigens*[a]

Treatment	Capillary precipitin reaction					
	F2w			F3w		
	10 min	30 min	60 min	10 min	30 min	60 min
0.05 N HCl, 60 C	+4	+4	+4	+3	+3	+3
0.05 N HCl, 100 C	+3	+2	+1	+2	+2	+1
0.1 N HCl, 100 C	+2	+1	0	+1	+1	0
0.4 N HCl, 80 C	+1	+1	0	+1	+1	0
1% Trypsin	+4	+4	+3	+3	+2	+2
1% Pepsin	+1	+1	0	+2	+2	0

[a] β-glucosidase, lysozyme, ribonuclease, deoxyribonuclease, and alkaline phosphatase did not change the serological activity of these antigens.

TABLE 5. *Antigens present in nine strains of group O streptococci*[a]

Strains	Antigen		
	Group polysaccharide	Protein type	
	Flt	F2w	F3w
B361	+	−	−
SS533	+	−	−
SS808	+	+	−
B357	+	+	+
11019	+	+	+
1360	+	+	+
SS809	+	+	+
SS669	−	+	+
DS1234	−	−	+

[a] Agar-gel diffusion was used for assay.

TABLE 6. *Properties of streptococcal protein antigens*[a]

Treatment	Antigen					
	F2w[b]	F3w[c]	M	R[b]	T[b, c]	B
Trypsin	r	r	d(9)	r(10)	r(11)	v(22)
Pepsin	d	d	d(9)	d(10)	r(11)	d(22)
*p*H 2, 100 C	d	d	r(10)	d(10)	d(11)	r(22)

[a] Abbreviations: r, resistant; d, destroyed; and v, variable. Numbers in parenthesis refer to literature references.

[b] Extracted from the cell with trypsin.

[c] Present in the cell as nucleoprotein; serological specificity due only to the protein portion.

protein on resistance to trypsin and destruction at *p*H 2. In addition, saline solutions (1 mg/ml) of purified F2 and F3 preparations were negative to sera which contained antibodies for the following M protein types: 1 to 6, 8, 11 to 15, 17 to 19, 22 to 27, 29 to 33, 36 to 43, 46, 47, 49, and 51 to 55. F3 was also tested against types 56, 57, and 58. All gave negative results. The presence of T protein in addition to M in many of these strains (10) appears to eliminate the possibility that F2 and F3 possess any serological relationship to the T proteins. Separation from the T proteins is also evident on the basis of the action of pepsin, and from the group B protein by the effect of *p*H 2 at 100 C (Table 6). Separation from the R protein is shown by a negative precipitin reaction with group A, type 28 serum. Type 28 is defined on the basis of the R protein (11). The B protein is resistant to *p*H 2, in contrast to F2 and F3. The latter are distinct from these other streptococcal antigens on the basis of these characteristics.

The F2 and F3 antigens should be considered as the type antigens of the O streptococci. F2 and F3 are designated as types I and II, respectively. The results show that various methods of extraction remove these two antigens without any appreciable contamination by other proteins or nucleoproteins, and they are present in the extracts in sufficient quantity to be readily identified by the capillary precipitin test. Ease of extraction and assay satisfy two important requirements of a type antigen. In a group of nine strains received as O streptococci, seven were found to contain either the F2 or the F3 antigens, or both (Table 5). The absence of the group polysaccharide antigen from SS669 and DS1234, and the presence of the F2 and F3 antigens is proof that the type antigens are not restricted to the group O cell. Similar examples with both polysaccharide (8, 20) and protein (17, 18) antigens of other streptococci are known. The isolation and identification of the group O polysaccharide (19) and the F2 and F3 proteins make possible the preparation of specific antisera by adsorption for use in the taxonomic description and classification of the O streptococci. Agar-diffusion assays have shown that the three antigens can be readily distinguished in HCl extracts with whole-cell antiserum by a single determination. Strains SS669 and DS1234 do not belong to any of the presently recognized serological groups, and their characterization must await further study.

The use of 0.2 N HCl at 100 C for 10 min is recommended for extraction of both antigens from whole cells in a short time period. The purified antigens, however, are destroyed under the above conditions (Table 4). Water extraction, although effective, requires many hours.

The location of the protein antigens in the cell is of interest. The removal of a part of each antigen by a water wash indicates that a fraction is free in the cell and the remainder is bound. A difference in location of each is also indicated by the removal of F2 by trypsin. F2 has been found in cell walls treated with ribonuclease and sodium dodecyl sulfate and was removed by trypsin. The action of pepsin on these walls destroyed the serological activity of F2. A cell wall location is also indicated by a strong agglutination of B357 whole cells with F2-specific antiserum.

Similar cell walls contained a small quantity of F3, as judged by a very weak reaction in agar diffusion with F3-specific antiserum. Whole-cell agglutination was also weak. The low level of nucleoprotein in the cell walls of gram-positive bacteria would indicate another location for F3 in the cell; however, it is possible that the serologically active protein, normally present in the wall, became bound to the nucleic acid in the water extract. Approximately one-half of the total F3 (by weight) is removed by the single, water wash. RNA was removed from group A, E, and T cells by a water wash. The values were 35.2, 16.2, and 25.0 μg/5 mg of dry cells, respectively, and for group O, 38.8. Free RNA is available in the cell for the formation of a complex with protein. Such a complex would have been eluted from the DEAE-Sephadex as a single substance (Fig. 2). A difference in charge would have separated the protein and nucleic acid on the column if they had been present in the wash as single substances. On the other hand, lysis of the cell in washing would probably have resulted in a release of nucleoprotein which previously had been a part of the mesosome or associated units. However, F3 was not present in the second or third water washes of whole cells and, consequently, lysis of the cells, if present, was not a continuing process.

Also, the optical density of the cell suspension did not change during the washing. No conclusion is possible at this time as to the location of F3 as a nucleoprotein in the cell. The T nucleoprotein is extracted from the cell by trypsin (10, 21), and whole cells are agglutinated with T antiserum (10).

The data (Fig. 3, Table 2) indicate that the F2w° antigen possesses a multiple structure. The complete antigen was separated into fractions of molecular weight 47,000, 140,000, and >200,000. Each of these fractions showed by electrophoresis that they possessed a weak charge and thus very little mobility. Fraction F2w (Fig. 7) illustrates the behavior which is common to all three. Two possibilities exist as to the structure of the complete protein: (i) a multiple unit structure in the cell composed of a number of each of the fractions, or (ii) a structure composed of a number of units of the same size (represented by one of the fractions). The first seems most likely because the weight of the smaller unit appears to be a numerical fraction of the larger ones. Two fractions of streptococcal M protein isolated after alkaline (*p*H 10) hydrolysis, which possess an identical immunological specificity and a fivefold difference in molecular weight, are considered to be units which are combined in the native protein (4, 5). N- and C-terminal analysis (31) also indicate that M protein may contain multiple units, although the absence of N-terminal quantitative data prevents a definite conclusion.

F2w° fractionated into three components as the ionic strength of the carbonate buffer (*p*H 8.5) was changed from 0.55 M to 0.05 M. The lack of solubility of F2w° in distilled water, similar to those of some beta and gamma globulins, and ready solubility in 0.85% NaCl, indicates that the native protein fractionates easily in solutions of weak ionic strength.

N- and C-terminal amino acid analysis (Table 3), and total amino acid analysis (Table 1) further illustrate that F2w and F3w are different proteins. Valine, tyrosine, and methionine are present in both antigens in approximately the same quantities, whereas the remaining amino acids vary from 8 to 10 times in each antigen. These data, plus data from electrophoresis and immunological assay, show that the two antigens are separate and distinct. The N- and C-terminal analyses of an acid-extracted M protein fraction (31) also support the serological difference between F2w and F3w and M protein.

These studies, in addition to previous work from our laboratory (19), establish the chemical nature of the group O antigen and the two principal type antigens of the O streptococci and methods for their preparation. Antisera specific for each of these antigens can now be prepared, and an in vivo determination can be made of the protective effect of each in group O infections.

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