THE LYSIS OF CELL WALLS OF GROUP A STREPTOCOCCI BY STREPTOMYCES ALBUS ENZYME TREATED WITH DIISOPROPYL FLUOROPHOSPHATE

Characteristics of the Lytic Reaction and the Soluble Cell Wall Fragments*

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Studies of the lytic action of enzymes on bacterial cell walls have provided considerable information about the chemistry of these structures (1–5). Enzymatic lysates have proved to be a useful source of soluble, immunologically active, microbial wall components (3, 5). The investigations of McCarty and Krause of the lysis of Group A streptococcal cell walls employing muralytic enzymes and formamide hydrolysis have furnished evidence of the immunochemical nature of A and A-variant polysaccharides, wall mucopeptide, and their structural relationships in the cell wall (6–8). Partially purified M protein of improved antigenicity has been obtained from phage-associated enzyme lysates (7, 9).

The initial studies of the lysis of streptococcal cell walls by McCarty demonstrated that the muralytic enzyme present in filtrates of *Streptomyces albus* dissolved these walls and released soluble, serologically active A and A-variant carbohydrates (6, 10). However, the difficulty in separating small amounts of proteolytic enzyme from partially purified preparations of the streptomyces glycolytic enzyme (6, 7) limited its use to experiments where concomitant proteolysis was unimportant. In subsequent investigations, the phage associated lysin, which appears to be specifically glycolytic, was employed to obtain detailed information about the hydrolysis of streptococcal walls and the immunologically active components released (3, 7, 8). If the lytic enzyme in

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partially purified preparations of *Streptomyces albus* filtrates could be refined by inactivation of contaminating proteolytic activity with diisopropyl fluorophosphate (11, 12), another useful source of specific streptococcal muralytic enzyme would be provided. This report describes experimental conditions for lysis of Group A streptococcal cell walls employing partially purified preparations of *Streptomyces albus* (S.A.) enzyme pretreated with diisopropyl fluorophosphate (DFP) to inhibit proteolytic activity. The chemical and immunological characteristics of lysates of whole cell walls prepared with DFP-treated *S. albus* enzyme are described and compared with lysates of trypsinized cell walls made with DFP-treated *S. albus* enzyme and *S. albus* enzyme unexposed to DFP. A number of similarities in the lytic action of DFP-treated *S. albus* enzyme and the phage-associated lysin are noted.

Materials and Methods

Strains of Streptococci.—The Group A streptococcus J 17D (Type 19) was obtained from The Rockefeller Institute Collection; SF 59 (Type 2) was furnished by W. R. Maxted; P20080 (Type 14) was isolated at the Streptococcal Disease Laboratory, Warren Air Force Base, Wyoming. Type 3 (strain Richards) came from the Streptococcal Disease Laboratory Collection, and large amounts of streptococcal cells of this strain were provided by Difco Laboratories, Inc., Detroit, through the courtesy of Mr. A. Lane. The A-variant strain, T 27A, was obtained from The Rockefeller Institute Collection.

Streptomyces albus Culture.—A strain of *Streptomyces albus* known to produce maximal amounts of streptolytic enzyme was kindly provided by Dr. M. McCarty. It was inoculated with a minimal number of serial transfers onto a large number of casamino acid agar slants which, after growth, were stored at 4°C and used as inocula for the preparation of lytic enzyme (6).

Antisera.—Streptococcal grouping and specifically absorbed typing sera were obtained from the Communicable Disease Center, United States Public Health Service, or were made in this laboratory according to directions furnished by Dr. R. C. Lancefield. A-variant antisera were prepared according to directions in refererence 10; two specimens were also generously provided by Dr. Lancefield. Streptococcal anticytoplasm and antimembrane sera were prepared by methods described by Freimer (13).

Preparation of Streptococcal Cell Walls.—Streptococci were grown in Todd-Hewitt broth sterilized by filtration (14). Cells were harvested after 18 hours' growth and washed 3 or 4 times with distilled water. Type 3 streptococcal cells obtained from Difco Laboratories, Inc. as frozen packed cells were thawed and washed three times with 0.15 M saline and three times with distilled water.

Cell walls were prepared by the method of Salton in the Mickle disintegrator (15) or in the Waring blendor. A charge of 100 to 120 ml of a 10 per cent suspension of streptococci and 300 gm of No. 13 Ballotini glass beads was put into a chilled standard glass blendor container and agitated for 5 minutes. This produced almost complete disruption of the cells. Some strains required two to four 5-minute periods of agitation to obtain nearly complete disruption. Minimal agitation is preferred to avoid fragmentation of cell walls which makes separation by differential centrifugation more difficult.

Separation of walls from other cell material was accomplished by differential centrifugation: first, at 2500 RPM for 15 minutes (No. 269 head) in the International PR 1 refrigerated centrifuge to sediment trace amounts of unbroken cells, then at 20,000 RPM for 1 hour (No. 21 head) in the Spinco model L preparative centrifuge to separate particulate material from the

supernatant cytoplasm. This sediment was then suspended homogeneously in 3 times the original volume and centrifuged in 50 ml amounts in the No. 823 angle head in the PR 1 centrifuge at 4300 RPM for 2 hours. The last step sedimented cell walls; wall fragments and cell membranes in the supernate were removed by aspiration. The cell walls were resuspended, washed in phosphate-buffered saline (pH 7.4, $\Gamma/2$ 0.03) three times followed by three distilled water washes, and stored lyophilized or as a frozen suspension at −20°C. Small, variable amounts of cell membranes were present in the cell wall preparations. Although poorly visualized by phase microscopy, they could be demonstrated by finding membrane-specific serological activity (13) in trypsin, pepsin or crude *S. albus* enzyme extracts of preparations of walls. We are indebted to Dr. Earl Freimer for the initial serological identification of membrane components in wall preparations. Serological testing with streptococcal cytoplasm antiserum demonstrated that preparations of walls did not contain cytoplasmic components.

Trypsin-Treated Cell Walls.—Cell walls prepared as described were suspended in phosphate buffer, pH 8, 0.06 M (300 mg walls per 100 ml), containing approximately 2.5 μg of dissolved crystalline trypsin per mg of cell walls and digested 20 hours at 37°C. Trypsinized walls were then washed 4 times with distilled water. Fifty to 60 per cent of the nitrogen of the wall is removed by this procedure.

Preparation of Streptomyces albus Lytic Enzyme.—*S. albus* enzyme was prepared by growing *S. albus* on a casamino acid medium (6). The streptolytic enzyme was purified by the McCarty method (6) with the exception that the lytic enzyme was precipitated from the dialyzed redissolved $(NH_4)_2SO_4$ precipitate by acidification with N acetic acid to pH 4.0. Assays of streptolytic and proteolytic activities of partially purified *S. albus* enzyme were modified slightly from directions in reference 6. Although the methods do not permit precise comparison, preparations used in this investigation contained similar amounts of lytic activity, but more proteolytic activity chiefly because less extraneous nitrogen and proteolytic activity were removed by precipitation with $CaCl_2$. Purified preparations of *S. albus* enzyme in pH 8, 0.006 M phosphate buffer were frozen quickly and stored at −20°C until used.

Diisopropyl Fluorophosphate-Treated S. albus Enzyme.—A 0.2 M stock solution of DFP (Aldrich Chemical Co., Milwaukee) was prepared in isopropanol. This was stable at refrigerator temperatures for months. DFP-treated *S. albus* enzyme solutions were prepared by adding an amount of 0.2 M DFP in isopropanol to a recently thawed portion of purified *S. albus* enzyme diluted in pH 8, 0.006 M phosphate buffer to make a final concentration of DFP of 0.0001 to 0.015 M. The DFP–*S. albus* enzyme mixture was then kept at room temperature for 25 minutes before addition of cell wall substrate.

A Enzyme Hydrolysis.—The β-*N*-acetylglucosaminidase or A enzyme which specifically removes the immunologically active determinant from A polysaccharide was generously provided by Dr. M. McCarty. Conditions for the use of this enzyme are detailed in reference 16.

Analytical Methods.—Total nitrogen was determined by direct nesslerization in the micro-Kjeldahl procedure (17). Protein or peptide concentration was measured by the Lowry modification of the Folin method (18). Methylpentose (rhamnose) determinations were made by the Dische-Shettles procedure (19). Hexosamines were estimated by the method of Rondle and Morgan (20) after hydrolysis with 2 N HCl at 100°C for 2 or 4 hours in sealed tubes. Glucosamine was quantified, after separation by chromatography on norit A-celite columns according to J. T. Park as cited by Perkins and Rogers (21), by reading optical density at 530 mμ within 1 hour after reaction with dimethylaminobenzaldehyde. Muramic acid (3-0-carboxyethylglucosamine) was determined after norit-celite chromatography by reading optical density at 505 mμ after 18 hours as described by Crumpton (22). Estimates of muramic acid in mixtures of hexosamines were made by calculation of the ratio of optical density values at 505 and 530 mμ (22). Analytically pure muramic acid, generously supplied by Dr. J. T. Park, was used as a standard as was purified muramic acid extracted from streptococci in this laboratory.

Electrophoresis.—Dialyzed lysates of streptococcal cell walls were fractionated by continu-

ous curtain electrophoresis in the Spinco model CP apparatus according to methods described by Halbert (23). Electrophoresis was performed at 4°C using constant currents of 20 to 40 ma at potential differences of 500 to 900 v. Samples collected from the curtain drip points were analyzed for protein or peptide by the Lowry method (18), for rhamnose (19), and for serological activity, the last by capillary precipitation or Ouchterlony agar migration methods. When necessary, samples were pooled or concentrated individually by lyophylization or dialysis against saturated $(NH_4)_2SO_4$ or 50 per cent polyvinylpyrolidone.

Immunoelectrophoresis.—Immunoelectrophoresis was performed according to methods described by Wilson and Wiley (24) on glass slides measuring 2 x 3 inches, employing veronal buffer, pH 8.6, $\Gamma/2$ 0.05 or phosphate buffer pH 7, $\Gamma/2$ 0.05. Runs were made at room temperature at a constant current of 21 ma per slide for 25 to 120 minutes. Slides were stained with amido-black or thiazine red.

Paper Chromatography.—Paper chromatographic studies (for methods see reference 25) of cell wall components to identify amino acids were made after hydrolysis of components in 5.7 or 6 N HCl for 18 to 22 hours in sealed tubes at 100° or 105°C. Acid was removed by evaporation *in vacuo* in a rotary evaporator and by sublimation over NaOH, P_2O_5 or drierite in a vacuum desiccator. Phenol, water containing 0.3 per cent NH_3; 3-butanol, formic acid, water; butanol, pyridine, water; and 2-butanol, acetone, water, diethylamine were employed as solvents. Ninhydrin and isatin were used as developing agents. Carbohydrate and amino sugar components after hydrolysis in 2 N HCl for 2 or 4 hours at 100°C were chromatographed in butanol, pyridine, water; aniline phosphate, *m*-phenylenediamine, ninhydrin and acetylacetone-dimethylaminobenzaldehyde were used for color development.

Column Chromatography.—Fractions of enzyme lysates of cell walls were chromatographed on diethylaminoethyl (DEAE) and carboxymethyl (CM) cellulose according to methods reported by Peterson and Sober (26). Columns 2 x 11 or 0.9 x 25 cm were made and elution was performed at 4° or 25°C with buffers of increasing ionic strength. Chromatography (27) with sephadex G-25 (medium grade) columns 0.9 × 30 cm was also employed for fractionation; distilled water was used for equilibration and elution of these columns. Column chromatography for quantification of amino acid components of acid hydrolysates of components of cell wall lysates was performed with the Spinco model MS amino acid analyzer according to the methods of Moore, Spackman, and Stein (28).[1] Hydrolysates were prepared by dissolving samples in 5.7 or 6 N HCl and heating to 105°C in sealed tubes for 20 to 24 hours. Acid was removed as described in paper chromatography methods. Unhydrolyzed samples of the dialyzable fraction of cell wall lysates were chromatographed on amberlite IR-120 in the Spinco apparatus using the buffer system recommended for amino acids (28).

Precipitin Reactions.—Qualitative precipitin tests for serological assay of lysate fractions were done by the capillary technique (29). Quantitative precipitin analyses were performed as previously reported (30). Gel precipitin studies were conducted according to techniques described by Ouchterlony (31).

RESULTS

Inhibition of Proteolytic Activity in S. albus Enzyme Preparations by DFP.—Experiments to determine if DFP would inhibit proteolysis in the *S. albus* enzyme system were conducted by treating purified preparations of albus enzyme with DFP and measuring the proteolytic activity of the DFP-enzyme complex on casein.

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[1] We are grateful to Dr. G. J. Gabuzda for assistance with the quantitative amino acid chromatography performed in his laboratory.

To 2.25 units of *S. albus* enzyme in phosphate buffer, varying amounts of DFP were added. DFP was permitted to react with enzyme solution for 25 minutes at room temperature; then 4.0 ml of a 1 per cent solution of casein were added. DFP-enzyme and substrate were mixed and incubated at 37°C. Samples of the digestion mixture were pipetted into 5 per cent trichloracetic acid (TCA) at 0 time, 20 and 45 minutes and the precipitate that formed removed by centrifugation. The optical density of the clear supernate was read at 280 mμ in the Beckman DU spectrophotometer. The optical density reading at 0 time was subtracted from the reading obtained at 20 and 45 minutes; the increase in optical density at 20 and 45 minutes is listed in Table I. The inhibitory action of DFP on 25 μg of crystalline trypsin is illustrated for comparison.

TABLE I

Inhibition of Proteolytic Activity in S. albus Enzyme by DFP

Enzyme	DFP	Increase in OD at 280 mμ	
		20 min.	45 min.
	M		
S. albus	0.006	0.02	0.03
S. albus	0.001	0.02	0.05
S. albus	0	1.07	1.36
Trypsin	0.004	0	0
Trypsin	0	0.37	0.57
No enzyme	0.004	0	0

The experiment presented in Table I demonstrated effective inhibition of proteolytic activity in *S. albus* enzyme preparations exposed to concentrations of 0.001 and 0.006 M DFP.

Action of DFP-Treated S. albus Enzyme on Streptococcal Cell Walls.—Experiments to examine the action of DFP-treated *S. albus* enzyme on streptococcal cell walls were designed to determine the concentrations of DFP necessary to inhibit proteolysis while permitting maximal lytic action. Lysis by *S. albus* enzyme after exposure to concentrations of DFP from 0.0001 to 0.015 was first studied by observing the decrease in optical density of suspensions of cell walls as illustrated in Fig. 1. Since almost all of the rhamnose in Group A streptococci occurs as a constituent of A polysaccharide in the cell wall (6), measurement of soluble rhamnose in wall lysates provided more accurate indication of the extent of lysis. Because of its susceptibility to proteolysis (32), the presence of serologically active M protein in lysates indicated absence of proteolytic enzyme activity. Lysis of cell walls by *S. albus* enzyme treated with different concentrations of DFP followed with these indicators is illustrated in Table II.

S. albus enzyme (0.3 lytic units per ml) was reacted with 0.0001, 0.001, 0.005, and 0.015 M DFP for 25 minutes at room temperature in phosphate buffer. Type 19 cell walls were added to make a final concentration of 108 μg of cell wall rhamnose per ml, and the mixture was incubated at 45°C for 18 to 22 hours. Samples of the suspension were removed at intervals and the

optical density measured at 600 mμ. They were then heated at 70°C for 3 minutes to inactivate the lytic enzyme, cooled, centrifuged at 20,000 RPM for 1 hour and the clear supernate pipetted from the sediment. Samples of supernates were added to 1 per cent casein solution to test for residual proteolytic activity. Dilutions were made for rhamnose determinations, and capillary precipitin tests were performed with undiluted supernates and Groups A, C, and Types 18 and 19 absorbed antisera.

Fig. 1 demonstrates that the lysis of streptococcal walls by *S. albus* enzyme treated with 0.0001 M DFP was not significantly different from the lytic action of untreated *S. albus* enzyme. Positive tests for casein proteolysis obtained with

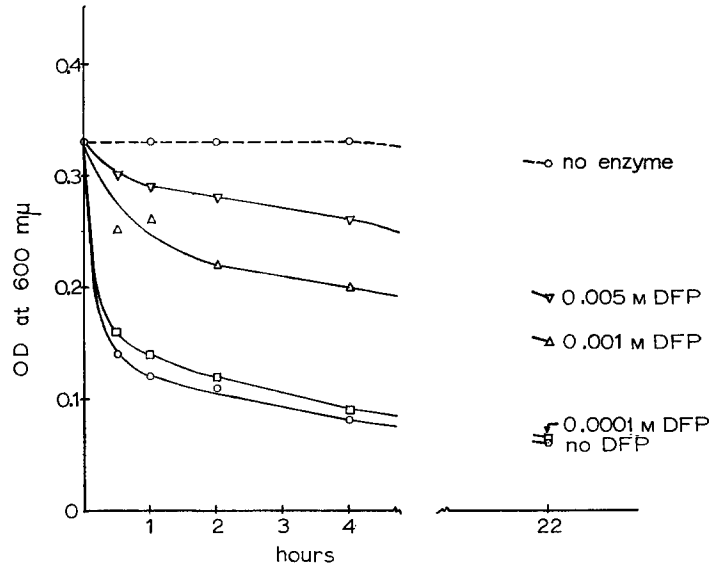

FIG. 1. Lysis of streptococcal cell walls by *S. albus* enzyme treated with various concentrations of DFP.

the supernate indicated that insufficient DFP was present at a concentration of 0.0001 M to inhibit proteolytic activity completely. *S. albus* enzyme treated with 0.001 and 0.005 M DFP produced, respectively, about 66 and 50 per cent of the decrease in optical density (Fig. 1) as the untreated enzyme. No proteolytic action on casein was detected in the supernates. *S. albus* enzyme treated with 0.015 M DFP (not plotted) produced only a minimal change in optical density in 22 hours. The extent of lysis of streptococcal walls by *S. albus* enzyme treated with 0, 0.001, 0.005, and 0.015 M DFP measured by release of rhamnose is presented in Table II. At 18 hours 89 per cent (96 μg) of the rhamnose present in the walls was released by *S. albus* enzyme treated with 0.001 M DFP; *S. albus* enzyme exposed to 0.005 M DFP solubilized 87 per cent (94 μg) of the cell wall rhamnose. Exposure of *S. albus* enzyme to 0.015 M DFP

resulted in considerable inhibition of cell wall lysis; only 36 per cent (39 μg) of the rhamnose in the wall was dissolved. The soluble rhamnose in these lysates was serologically active A carbohydrate as shown by the precipitin reactions listed in column 7, Table II. The precipitin reactions with Type 19 absorbed antiserum listed in the last column of Table II demonstrated that serologically active M protein was present in lysates when proteolysis was inhibited with DFP-treated *S. albus* enzyme.

Properties of S. albus Enzyme Lysates of Cell Walls.—After establishing optimal conditions for lysis of cell walls by *S. albus* enzyme complexed with DFP, large scale experiments were conducted to obtain quantities of lysates

TABLE II

Lysis of Streptococcal Cell Walls by DFP–S. albus Enzyme

DFP	Rhamnose solubilized					Precipitin reaction*	
	1 hr.	2 hrs.	4 hrs.	18 hrs.		Group A serum	Type 19 serum
M	μg	μg	μg	μg	*per cent of total*		
0	37.5		94	106	98	+++	−
0.001	21	27	40	96	89	+++	+
0.005	22	34	49	94	87	+++	++
0.015	11	12.5	19	39	36	++	+

* 18 hour sample.

suitable for separation and identification of components. Comparison of lysates of whole and trypsin-treated cell walls prepared with DFP-treated *S. albus* enzyme and untreated *S. albus* enzyme were made.

To prepare a typical DFP-treated *S. albus* enzyme lysate, 245 mg of Type 19 cell walls were added to a solution of 67 units of *S. albus* enzyme that had been reacted with 0.005 M DFP in phosphate buffer at pH 8. The mixture (240 ml) was incubated at 45°C; the pH was adjusted to 8.0 intermittently with dilute NaOH and the course of the digestion was followed for 40 hours by measuring the decrease in optical density. The digest was heated to 70°C for 3 minutes, chilled, and centrifuged at 20,000 RPM for 1 hour. The supernatant fluid was poured from the sediment, passed through a membrane filter (Millipore 0.45 μ) and concentrated *in vacuo* at low temperature. It was then dialyzed against several changes of distilled water for 3 days and the dialysand and dialysate concentrated *in vacuo*. Samples were taken for chemical analyses and the dialysand was divided into two fractions by addition of $(NH_4)_2SO_4$ to 0.6 saturation. The $(NH_4)_2SO_4$ precipitate after centrifugation was dissolved in water and this and the $(NH_4)_2SO_4$ supernate dialyzed until free of $(NH_4)_2SO_4$. Cell walls that had been digested with trypsin were lysed with DFP-treated *S. albus* enzyme or *S. albus* enzyme alone, and the lysates were processed as described above.

A comparison of the distribution of rhamnose and nitrogen in the supernates, dialysates, and residues of trypsinized and whole Type 19 cell walls lysed with

S. albus enzyme and DFP-treated *S. albus* enzyme is presented in Table III. Approximately the same amount of rhamnose was released by *S. albus* enzyme or DFP-treated *S. albus* enzyme from trypsinized or whole cell walls. Less than 1 per cent of the wall carbohydrate was hydrolyzed to small molecules that passed through dialysis membrane. Fifty-three per cent of the nitrogen of whole cell walls was dissolved by DFP-treated *S. albus* enzyme; 12 per cent of this was dialyzable. DFP–*S. albus* enzyme released 88 per cent of the nitrogen that remained in cell walls after trypsin treatment; 28 per cent of this was dialyzable.

TABLE III

Composition of Fractions of S. albus Enzyme Lysates of Cell Walls

Fraction	Cell walls				Trypsin-treated cell walls							
	DFP-treated *S. albus* enzyme				DFP-treated *S. Albus* enzyme				*S. albus* enzyme			
			Precipitin reaction				Precipitin reaction				Precipitin reaction	
	Rhamnose	Nitrogen	Group A serum	Type 19 serum	Rhamnose	Nitrogen	Group A serum	Type 19 serum	Rhamnose	Nitrogen	Group A serum	Type 19 serum
	per cent	*per cent*			*per cent*	*per cent*			*per cent*	*per cent*		
Supernatant	97	41	++++	++	98.5	60	++++	−	98	63	++++	−
$(NH_4)_2SO_4$ precipitate	3*	14*	+	++	Trace of precipitate				No precipitate			
$(NH_4)_2SO_4$ supernatant	90*	25*	++++	−								
Dialysate	0.9	12	±	−	0.5	28	±	−	0.8	32.5	±	−
Residue	2.1	47			1.0	12			1.2	4.5		

* Small amounts of rhamnose and nitrogen are lost during dialysis to remove $(NH_4)_2SO_4$.

Nearly complete hydrolysis of trypsin-treated walls was accomplished by *S. albus* enzyme; 95 per cent of the nitrogen of the wall was dissolved of which 32.5 per cent was dialyzable. Similar data were obtained in experiments with Types 2, 3, and 14 cell walls. Usually the variation in distribution of rhamnose and nitrogen in the various fractions in duplicate experiments was 5 per cent or less. Although the figures for nitrogen release indicated that lysis of trypsinized cell walls was more extensive than lysis of whole cell walls by DFP-treated *S. albus* enzyme, this was more likely a relative rather than an absolute difference because of the considerable simplification of the wall obtained by removal of 50 to 60 per cent of the total nitrogen by trypsinization before treatment with DFP–*S. albus* enzyme or *S. albus* enzyme. Evidence of loss of macromolecular and serologically active components of the wall by trypsin

digestion was provided by the finding that only a trace of precipitate and no type-specific antigen were obtained by the addition of $(NH_4)_2SO_4$ to 0.6 saturation to the DFP-treated *S. albus* enzyme lysate of trypsinized walls, whereas considerable precipitate and Type 19 antigen were present in the comparable fraction from lysates of whole cell walls. Therefore, to avoid loss or degradation of immunologically active wall components, DFP-treated *S. albus* enzyme lysates were usually prepared from whole cell walls.

Experiments to characterize the portion of the cell wall present in the insoluble residue were not feasible because of the variable contamination of this fraction with cell membranes not removed by differential centrifugation.

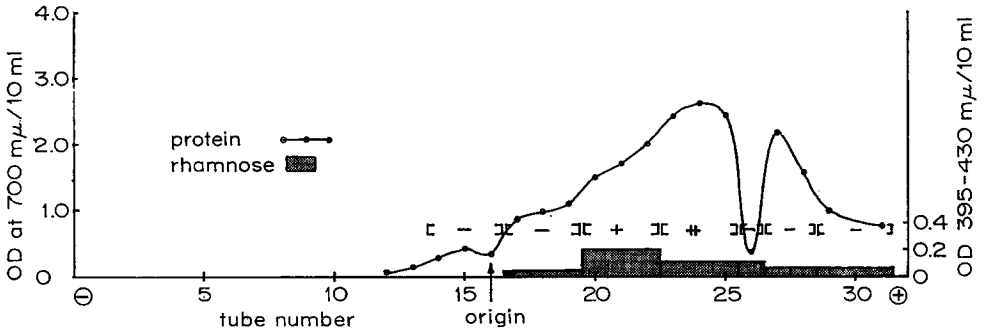

FIG. 2. Curtain electrophoresis of the 0.6 saturated $(NH_4)_2SO_4$ precipitate of DFP-*S. albus* enzyme lysate of Type 19 cell walls. Electrophoresis in borate buffer, pH 8.9, Γ/2 0.025, at 40 ma, 550 v. for 18 hours, at 4°C. Fractions from the curtain were pooled according to protein content (OD at 700 mμ), concentrated, and rhamnose (OD 395 − 430 mμ) and serological analyses made. Precipitin reactions with absorbed Type 19 antiserum are shown in brackets.

Serological analysis of lysates showed that membrane components contaminating preparations of walls were not solubilized by DFP-treated *S. albus* enzyme.

Separation and Properties of M Protein.—The ammonium sulfate precipitate obtained from DFP-*S. albus* enzyme lysates of Types 2, 3, 14, and 19 cell walls gave a positive precipitin reaction with homologous, type-specific absorbed antisera, indicating the presence of M antigen. Further separation of the M antigen from the $(NH_4)_2SO_4$ precipitate fraction of lysates of Type 19 cell walls was performed by continuous curtain electrophoresis. Fig. 2 illustrates the results of one of these electrophoresis experiments.

Two electrophoresis fractions comprising concentrates of tubes 20 through 22 and 23 through 25 reacted by capillary precipitation with absorbed Type 19 antiserum but not with Type 3 serum. Both of these fractions produced a single, fused line of precipitate with Type 19 absorbed antiserum in Ouchterlony preparations; this line gave the reaction of identity with the major of two bands

of precipitate produced by a partially purified preparation of Type 19 M protein prepared by the Lancefield method (32). None of the other curtain electrophoresis fractions reacted with type-specific antiserum.

The antigenicity of the Type 19 M protein of tubes 20 to 22 was tested by immunizing rabbits. An aliquot of this fraction of approximately 25 μg of protein nitrogen in complete Freund adjuvant was injected into 4 subcutaneous sites in each rabbit. Subsequently, at intervals of about 1 week, 3 courses of intravenous injections totaling approximately 175 μg of protein nitrogen were given. Precipitin reactions with purified Type 19 M protein, lysate fractions and serum from the first bleeding after this course of immunization from rabbit 3-0 are shown in Table IV. Further evidence identifying the type-specific lysate antigen as M protein was obtained by treating the 0.6 saturated

TABLE IV

Precipitin Reactions of Lysate Protein Fractions

Antigen	Serum		
	Type 19	Type 3	Rabbit 3-0
0.6 saturated $(NH_4)_2SO_4$ precipitate fraction	++	−	++
Trypsin-treated 0.6 saturated $(NH_4)_2SO_4$ precipitate fraction	−	−	−
Pepsin-treated 0.6 saturated $(NH_4)_2SO_4$ precipitate fraction	−	−	
0.6 saturated $(NH_4)_2SO_4$ fraction: pH 2, 98°C	++	−	
Curtain electrophoresis fraction 20-22	+	−	
Curtain electrophoresis fraction 23-25	++	−	
Purified Type 19 M protein	++	−	++

$(NH_4)_2SO_4$ precipitate with trypsin and pepsin and by heating to 98°C at pH 2 for 10 minutes. Precipitin tests with type-specific, absorbed antisera were performed after these procedures, and the results are listed in Table IV. Resistance to heat and acid and susceptibility to proteolytic action demonstrated by these electrophoresis fractions are features which characterize M protein (32).

Polysaccharide Components of Lysate Fractions.—Nearly all of the streptococcal cell wall polysaccharide was found in the portion of DFP–*S. albus* enzyme lysates that was soluble in 0.6 saturated $(NH_4)_2SO_4$. Three per cent or less of the polysaccharide measured as rhamnose was present in the $(NH_4)_2SO_4$ precipitate (Table III). This carbohydrate migrated with M protein in curtain electrophoresis (Fig. 2) and therefore is probably chemically bound to M protein. Krause demonstrated that phage-associated enzyme lysates of Type 6 streptococcal cell walls contained a small amount of protein-bound A polysaccharide (7).

The polysaccharide soluble in 0.6 saturated $(NH_4)_2SO_4$ was studied extensively to determine its chemical and immunological nature. Like the carbohydrate studied by Krause (7) and Krause and McCarty (8) obtained by *S. albus* enzyme or phage-associated enzyme lysis of trypsinized streptococcal walls (7), carbohydrate released with DFP-treated *S. albus* enzyme from whole

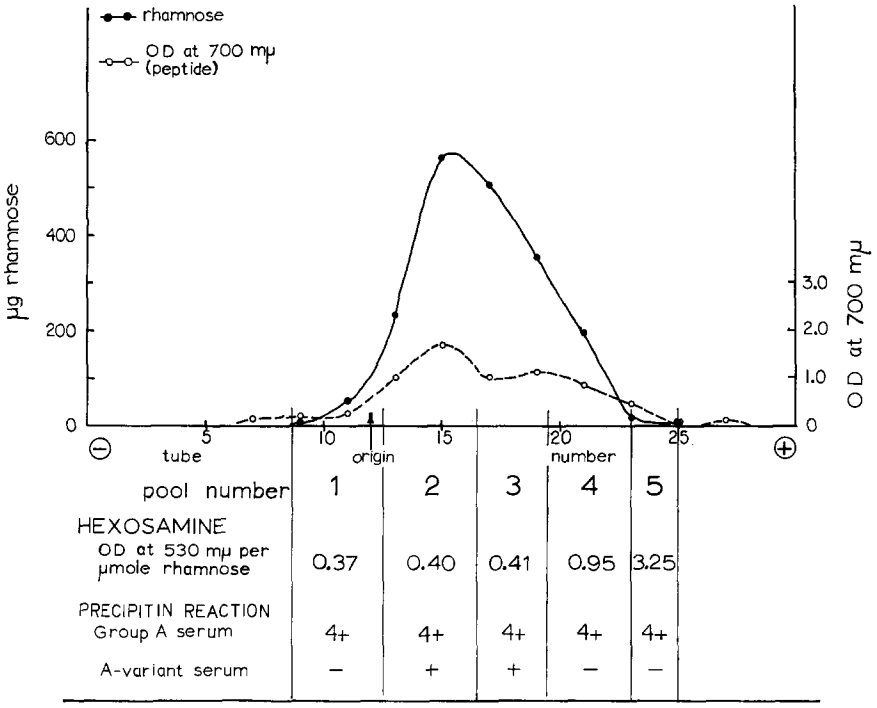

FIG. 3. Curtain electrophoresis of the $(NH_4)_2SO_4$ supernatant polysaccharide fraction of DFP-*S. albus* enzyme lysate of Type 19 cell walls. Chemical and serological analyses of electrophoresis fractions. Electrophoresis in tris-HCl buffer, pH 8.8, Γ/2 0.03, at 23 ma, 700 v., for 24 hours, at 4°C. Precipitin tests with A and A-variant sera were performed with concentrations of 10 μg of polysaccharide rhamnose per ml.

cell walls was found to be electrophoretically heterogeneous and to give precipitin reactions with A and A-variant antisera indicating the presence of at least two immunologic determinants. Several chemical and immunological characteristics of the $(NH_4)_2SO_4$-soluble polysaccharide from a lysate of Type 19 walls are summarized in Fig. 3. On continuous curtain electrophoresis in tris buffer pH 8.8, this polysaccharide formed a broad peak migrating toward the anode as depicted in Fig. 3 by analysis of curtain fractions for rhamnose.

However, electrophoretic migration of polysaccharide under the same experimental conditions varied according to the method of preparation and

the strain of streptococcus from which it was obtained as shown in Fig. 4. Here, polysaccharide preparations obtained from whole and from trypsinized Type 2 and Type 19 cell walls by DFP-treated *S. albus* enzyme lysis are compared. The carbohydrate from trypsinized J 17D (Type 19) walls migrated to

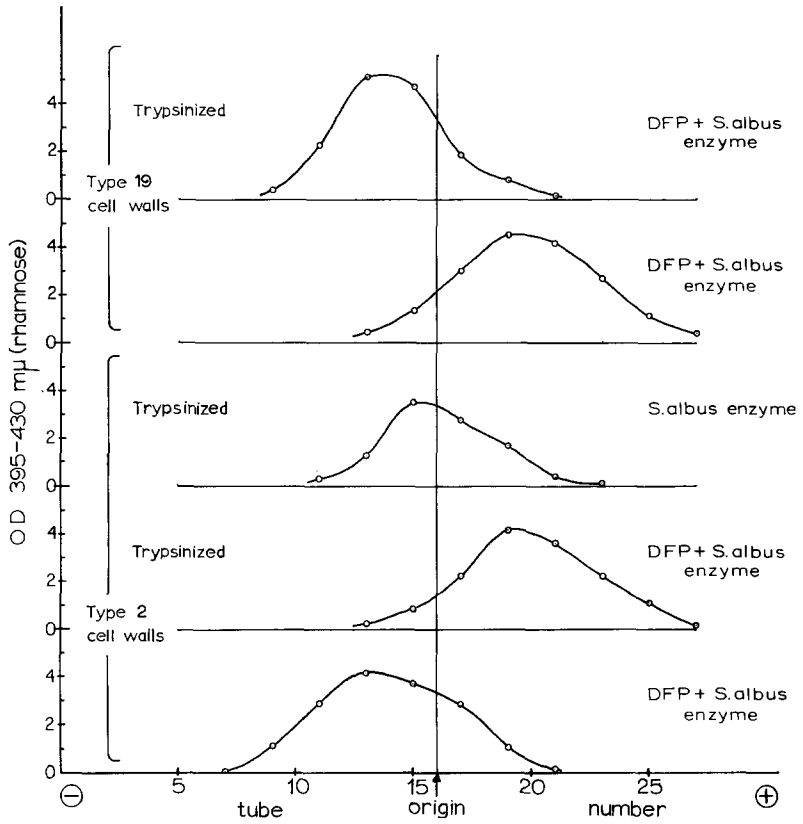

FIG. 4. Curtain electrophoresis of polysaccharide released from cell walls by *S. albus* lysis. Comparison of electrophoretic mobilities of carbohydrate obtained from whole and trypsinized Type 2 and Type 19 walls with DFP treated and untreated *S. albus* enzyme. Electrophoresis in tris-HCl buffer pH 8.8, Γ/2 0.03, at 23 ma, 780 v., for 15 hours, at 4°C.

the cathode whereas that from whole cell walls migrated toward the anode. Polysaccharide from whole cell walls of the SF 59 (Type 2) strain showed cathodal migration and that from trypsinized SF 59 walls migrated to the positive electrode. Polysaccharide obtained from trypsinized SF 59 walls by *S. albus* enzyme, without DFP treatment, showed only minimal migration toward the cathode. Curtain electrophoresis under other conditions of pH and ionic strength did not result in qualitatively different migration of polysaccharide.

Further examination of cell wall carbohydrate subjected to continuous curtain electrophoresis revealed differences in the peptide and hexosamine content of electrophoresis fractions. Polysaccharide fractions collected near the origin and the anode contained relatively more peptide as indicated by the broken line in Fig. 3. After combination of carbohydrate fractions into five pools based on relative differences in peptide content, analysis of samples for hexosamine were made following acid hydrolysis. The relative hexosamine content (Fig. 3) of pool 1 polysaccharide was slightly less than that of pools 2 and 3. Polysaccharide in pools 4 and 5 contained, respectively, 2 and 8 times as much hexosamine as the carbohydrate in pool 2. It was also found that the carbohydrate of pools 4 and 5 contained relatively more muramic acid than the other fractions.

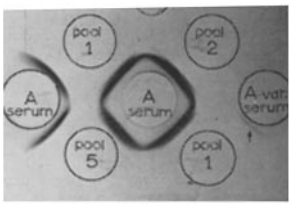

FIG. 5. Immunodiffusion reactions (Ouchterlony) of polysaccharide released from Type 19 cell walls by DFP-treated *S. albus* enzyme. Pooled electrophoresis fractions identified in Fig. 3 were reacted with two group A antistreptococcal rabbit sera and an A-variant serum.

The serological reactivity of the curtain electrophoresis fractions was measured by performing capillary precipitin and immunodiffusion tests with samples of the pools adjusted to the same rhamnose concentrations. Several concentrations were tested. Each pool gave a strong precipitin reaction with Group A sera; reactions at antigen concentrations of 10 μg of polysaccharide rhamnose per ml are listed in Fig. 3. Pools 2 and 3 reacted with A-variant sera (Fig. 3). Pools 1 and 4 did not react but produced small precipitates with A variant serum at concentrations of 20 to 40 μg of rhamnose per ml. None of these preparations reacted with sera containing antibodies to M protein, T protein, streptococcal membranes, or cytoplasm. A number of experiments attempting to separate A and A-variant polysaccharide utilizing curtain electrophoresis and column chromatography at various conditions of pH and ionic strength were unsuccessful.

Examination of the five pools of curtain electrophoresis fractions (Fig. 3) by immunodiffusion confirmed the results obtained by capillary precipitin tests that A and A-variant carbohydrates were the only serologically active components in these preparations. Fig. 5 illustrates typical precipitation reactions in Ouchterlony plates between pools 1, 2, and 5 polysaccharide and Group A and A-variant rabbit antisera. The lines of precipitate near the A serum wells

have joined showing the serologic identity of the A polysaccharide in these pools. Polysaccharide in pools 3 and 4 (not shown) formed similar bands of identity with carbohydrate of pools 1, 2, and 5. The presence of a small amount of A-variant carbohydrate in pool 1 is shown by the faint band of precipitate (see arrow) at the edge of the well containing A-variant serum. A band had also been formed near this well by carbohydrate of pool 2 but had dissolved before the immunodiffusion experiment illustrated was photographed. Rapid migration and insolubility of antigen-antibody precipitates over a relatively narrow range were found to be characteristic of A-variant polysaccharide. The lines of precipitate formed by A carbohydrate appeared as broad bands which may be indicative of the polydisperse nature of this substance. With several strong A antisera, a band of precipitate was noted to split off from the main band after 24 to 48 hours. This is evident in Fig. 5 in the precipitates between pools 1 and 2 and the center well. Polysaccharide of electrophoresis pools 2, 3, and 4 more readily formed this second band. Samples from these pools were treated with A enzyme which removes the β-*N*-acetylglucosamine determinant from A polysaccharide thus destroying A serologic activity (16). This completely abolished the precipitating activity with group A antisera and identified the two bands in each instance as A carbohydrate–A antibody precipitate. This demonstration of immunological homogeneity of preparations of A polysaccharide, when considered with the evidence of chemical heterogeneity (Fig. 3) and variation in rate of diffusion in agar (Fig. 5), suggest the possibility that A carbohydrate in enzymic lysates consists of a spectrum of molecules having the same immunological determinant but differing qualitatively and quantitatively in conjugated mucopeptide content.

Further experiments were performed to attempt to separate A polysaccharide into the fractions forming the two bands of precipitate in immunodiffusion. This was accomplished by fractional precipitation of 0.6 saturated $(NH_4)_2SO_4$ supernates of DFP–*S. albus* enzyme wall lysates with ethanol and acetone.

The $(NH_4)_2SO_4$ supernate was dialyzed to remove salt and concentrated by lyophilization to one-half or one-third volume. Cold ethanol was added to 75 per cent concentration in the presence of sodium acetate to facilitate precipitation. The precipitate that formed was removed by centrifugation. Then 5 volumes of cold acetone were added, and after 20 to 60 minutes at 0°C, another precipitate formed. This was removed by centrifugation, and both precipitates were dissolved in distilled water and lyophilized. About 55 per cent of the polysaccharide measured as rhamnose was found in the ethanol fraction; less than 0.3 per cent was unprecipitable by either reagent.

Chemical and Immunological Analysis of Ethanol andc Aoetne Precipitated Polysaccharide Fractions.—The rhamnose, nitrogen, glucosamine, and muramic acid content of the ethanol and acetone-precipitated A polysaccharide fractions was determined as described in the Materials and Methods section. Paper chromatography of HCl hydrolysates was performed to identify the amino

acid and carbohydrate constituents. Quantitative estimates of the amino acids were made by eluting ninhydrin-stained spots from chromatograms and determining the optical density of the eluates at 540 mμ (25). Amino acids were more precisely quantified by column chromatography. The constituents of the polysaccharide fractions found in a typical lysate of Type 19 cell walls are listed in Table V. Like the polysaccharide described by Krause and McCarty which was obtained by phage-associated enzymatic lysis of trypsin-treated Type 12 streptococcal walls (8), a significant portion of the polysaccharide obtained by DFP–*S. albus* enzyme action consisted of mucopeptide fragments

TABLE V

Components of Streptococcal Polysaccharide Obtained by DFP–S. albus Enzyme Lysis of Type 19 Cell Walls

	Ethanol precipitate		Acetone precipitate	
	μmole	*mole ratio*	*μmole*	*mole ratio*
Rhamnose	84.0	23.7	64.0	23.2
Glucosamine	35.4	(10)	27.5	(10)
Muramic acid	5.0	1.4	4.9	1.8
Alanine	18.0	5.1	12.1	4.4
Glutamic acid	7.85	2.2	4.85	1.7
Glycine	1.8	0.5	1.1	0.4
Lysine	7.45	2.1	4.1	1.5
Aspartic acid	2.0	0.6	1.2	0.45
Threonine	0.7	0.2	0.65	0.2
Serine	0.8	0.2	0.65	0.2
Valine	0.7	0.2	0.8	0.3
Proline	1.2	0.35	Trace	
Leucine	2.2	0.65	0.6	0.2
Isoleucine	Trace		0.3	0.1

bound to carbohydrate. If it is assumed that 1 μmole of glucosamine is associated with each μmole of muramic acid in the mucopeptide bound to A carbohydrate, then 23 per cent of the acetone-precipitated polysaccharide and 29 per cent of the ethanol polysaccharide is mucopeptide. In addition to the amino acids alanine, glutamic acid, glycine, and lysine which Krause and McCarty found in the mucopeptide linked to polysaccharide obtained from trypsinized walls (8), the mucopeptide portion of polysaccharide from whole cell walls contained aspartic acid, threonine, serine, valine, proline, leucine, and isoleucine. The latter group represented 16 to 18 per cent of the total amino acids in carbohydrate-linked peptide. Comparison of the ethanol-and acetone-precipitated polysaccharide fractions can best be made by examining the mole ratios of their respective constituents. These are presented in Table V and are based on a value of 10 for glucosamine. The two fractions are quite similar, differing

only in the presence of slightly more alanine, glutamic acid, lysine, leucine, and proline in the ethanol fraction, whereas more muramic acid and isoleucine were found in the acetone-precipitated material.

Analysis of the ethanol-and acetone-precipitated A polysaccharide fractions by immunodiffusion demonstrated their serologic identity and different rates of diffusion. Fig. 6 illustrates the reaction of equal amounts of acetone-precipitated polysaccharide, ethanol-precipitated polysaccharide, A enzyme-treated acetone-precipitated A carbohydrate and A carbohydrate extracted with formamide, with Group A antiserum. The lines of precipitate formed by A antibody and each of the A carbohydrate preparations are joined indicating their immunological homogeneity. Acetone–A polysaccharide subjected to specific enzymatic hydrolysis did not form a precipitate with group A antibody; A enzyme also destroyed the reactivity of ethanol-precipitated A polysaccharide

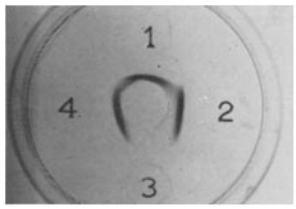

FIG. 6. Ouchterlony plate showing immunodiffusion reactions of Group A antiserum (lot 64) in the central well with equal amounts (15 μg of polysaccharide rhamnose) of acetone-precipitated (well 1) and ethanol-precipitated (well 2) A carbohydrate from DFP-treated *S. albus* enzyme lysates. Well 3 contained an equivalent amount of acetone–A carbohydrate treated with A enzyme. Well 4 contained the same amount of formamide-extracted A carbohydrate.

(not shown). The acetone-precipitated and formamide-extracted A polysaccharides diffused more rapidly as demonstrated by the position of their precipitate bands at the edge of the serum well. The precipitate line of antibody and ethanol–A polysaccharide is located 4 mm from the center well and has formed a very slight spur with relation to the acetone-precipitated carbohydrate.

A second immunodiffusion experiment employing ethanol and acetone fractions of another preparation of A carbohydrate is shown in Fig. 7. Equal amounts of ethanol-and acetone-precipitated A polysaccharide were placed in wells 1 and 2 respectively; well 3 contained another A antiserum (lot 43). Again the line of precipitate formed by the ethanol fraction is farther from the serum well and forms a very short, rounded spur where it fused with the acetone polysaccharide-antibody precipitate. Examination of the ethanol-and acetone-precipitated carbohydrate fractions by immunoelectrophoresis did not reveal clear-cut differences in their electrophoretic mobilities.

Dialyzable Constituents of DFP-Treated S. albus Enzyme Lysates.—Krause

and McCarty reported that the dialyzable portion of lysates of trypsinized Type 12 cell walls prepared with phage muralytic enzyme (8) contained a hexosamine-peptide complex composed of glucosamine, muramic acid, alanine, glutamic acid, lysine, and glycine in approximate mole ratios of 1.2, 1.0, 7.8, 2.3, 2.4, and 0.9. The comparable dialysate fraction of DFP-treated *S. albus* enzyme lysates of whole cell walls was found by paper and column chromatography of acid hydrolysates to consist of glucosamine, muramic acid, alanine, lysine, glycine, and, in addition, aspartic acid, valine, serine, threonine, leucine, isoleucine, and proline. Arginine was present in dialysates of lysates of Type 19 cell walls. A small amount of rhamnose was present and was also found in dialysates of lysates prepared with phage muralytic enzyme (8).

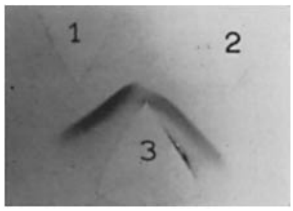

FIG. 7. Immunodiffusion reactions of ethanol (well 1)-and acetone (well 2)-precipitated A polysaccharide (15 μg polysaccharide rhammose) with Group A antiserum-lot 43 (well 3). The narrow line at the edge of well 3 is a staining artifact.

Quantitative analyses of acid-hydrolyzed dialysates from Type 2 and Type 19 cell wall lysates were made by chromatography on norit-celite columns (21) to determine hexosamines and by chromatography on amberlite IR-120 to measure amino acids (28). The average micromole amounts of the various constituents and the mole ratios of these components based on a value of 10 for alanine are presented in Table VI. The composition of dialysates of the Type 2 and Type 19 strains was very similar. The Type 19 fraction had less glutamic acid, slightly less glycine, aspartic acid, valine, serine, threonine and very little isoleucine; however, arginine was present. The Type 2 fraction lacked arginine but contained more isoleucine. The dialyzable fraction from DFP–*S. albus* enzyme lysates of trypsin-treated Type 19 cell walls resembled the dialysate from whole Type 19 walls but contained less glycine, proline, threonine, valine and no aspartic acid, leucine, isoleucine, or serine. The mole ratios of glucosamine, muramic acid, alanine, glutamic acid, lysine, and glycine in these dialysate fractions are comparable to those reported for lysates of trypsinized Type 12 cell walls (8).

Chemical analyses and chromatographic studies indicated that only small amounts of free hexosamines and amino acids were present in unhydrolyzed dialysates. Chromatography of unhydrolyzed dialysates on sephadex G-25

columns produced two overlapping peaks. The very small first peak was composed of rhamnose-linked material. This reacted as A carbohydrate in the hemagglutination inhibition test described in the following paper (34). The second large peak contained conjugated hexosamine and peptide and was serologically inactive by precipitation, hemagglutination, and hemagglutination inhibition. Other chromatographic experiments were performed using columns of amberlite IR-120, 0.9 x 150 and 0.9 x 15 cm, and citrate buffers at pH 3.25, 4.25, and 5.28 at 50°C for elution (28). The dialysate fraction was separated into one large peak, 5 small peaks, and trace amounts of several amino acids.

TABLE VI

*Constituents of the Dialyzable Fraction of DFP–S. albus Enzyme Lysates of Type 2 and Type 19 Cell Walls**

	Type 2		Type 19	
	μmole	*mole ratio*	*μmole*	*mole ratio*
Rhamnose	0.6		2.3	
Glucosamine	7.5	1.1	7.7	1.2
Muramic acid	9.7	1.45	8.5	1.35
Alanine	66.2	(10.0)	62.7	(10.0)
Glutamic acid	23.4	3.5	13.3	2.1
Lysine	23.6	3.5	22.0	3.5
Glycine	8.6	1.3	5.6	0.9
Aspartic acid	8.8	1.3	4.4	0.7
Valine	8.8	1.3	5.9	0.95
Serine	5.6	0.85	3.7	0.6
Threonine	5.9	0.9	3.7	0.6
Leucine	4.9	0.75	4.4	0.7
Isoleucine	3.4	0.5	0.5	
Proline	3.0	0.45	2.2	0.35
Arginine			6.1	1.0

* After hydrolysis with HCl except rhamnose.

Further analyses of the dialysate components were not made since they were serologically inactive. Characterization of these components as a means of elucidating the specificity of the enzyme responsible for lysis of streptococcal cell walls was not done because of the complexity of the whole cell wall as substrate, the relatively small amounts of material recoverable after chromatography and the possibility that preparations of DFP-treated, partially purified *S. albus* enzyme represented a complex of non-proteolytic enzymes rather than a monospecific system.

DISCUSSION

Investigation of the reaction of DFP-treated, partially purified *Streptomyces albus* enzyme with casein demonstrated effective inhibition of the proteolytic

activity in the enzyme preparation by diisopropyl fluorophosphate. The presence of serologically active M protein, which is readily susceptible to hydrolysis by proteolytic enzymes, in DFP-treated *S. albus* enzyme lysates of cell walls and the absence of significant amounts of free amino acids and small peptides in lysate dialysates indicated that proteolysis does not contribute to the streptolytic activity of DFP–*S. albus* enzyme.

The similarity of *S. albus* and phage muralytic enzymes based on chemical and serological characteristics of the carbohydrate released from streptococcal cell walls has been demonstrated by Krause (7). The mucopeptide-conjugated ethanol- and acetone-precipitated A polysaccharide and the dialyzable fraction of DFP–*S. albus* enzyme lysates of Type 2 and Type 19 cell walls, described in the present study, closely resemble the polysaccharide and dialysate fractions produced by phage muralytic enzyme lysis of trypsinized Type 12 cell walls (8). This provides further evidence of a remarkable degree of similarity of these enzymes which, at present, are the only streptolytic agents known to exist. The evidence that the streptococcal cell wall substrate has a particular configuration susceptible to hydrolysis by enzyme(s) of highly selective specificity in contrast to lysozyme or the "F_1" and "32" enzymes described by Ghuysen (4, 5) suggests that enzyme(s) of the specificity of the streptococcal lysins should exist in mammalian tissues to participate in the disposal of non-viable streptococci. The occurrence of antibody formation to the M protein (9, 32, 33) and A polysaccharide (34) components of enzymic lysates of streptococcal cell walls is an implication that these antigens are enzymatically released *in vivo*.

Non-trypsinized cell walls were used in the investigation of the components released from streptococcal cell walls by DFP-treated *S. albus* enzyme to avoid the possibility of removing constituents from these components which might alter their immunologic behavior. A-polysaccharide from whole cell walls was found to contain more amino acids in conjugated mucopeptide than polysaccharide from trypsinized walls. Evidence is presented in the following paper that mucopeptide conjugated to A polysaccharide functions in the fixation of the A antigen to tannic acid–treated erythrocytes employed in hemagglutination to measure A carbohydrate antibody (34). The ability of this preparation of A carbohydrate to attach to erythrocytes is destroyed by trypsin (34). It has also been found that mucopeptide conjugated to cell wall polysaccharide is necessary for the production of an *in vivo* immunologic reaction to A carbohydrate in man (35). Polysaccharide prepared by enzymic lysis of streptococcal cell walls produces a skin reaction after intradermal injection into persons who have experienced streptococcal infection. Formamide-extracted A carbohydrate which does not contain conjugated mucopeptide does not produce this reaction. The potentially charged amino acid residues of mucopeptide conjugated to polysaccharide, by virtue of their greater affinity for tissue components, may generally enhance the immunologic activity of cell wall carbohydrate solubilized by enzymatic lysis.

SUMMARY

Diisopropyl fluorophosphate (DFP) effectively inhibited proteolytic activity in preparations of partially purified *Streptomyces albus* enzyme used to lyse cell walls of Group A streptococci.

Lysis of non-trypsinized Group A cell walls with DFP-treated *S. albus* enzyme released a soluble protein fraction containing antigenic type-specific M protein, a carbohydrate fraction consisting of Group A and a small amount of A-variant polysaccharides, and a dialyzable fraction. The similarities of the products of DFP-treated *S. albus* enzyme lysis of streptococcal cell walls to those released by phage muralytic enzyme furnish additional evidence of the close relationship of these wall lysins.

In view of small differences in electrophoretic mobility, immunodiffusion, and chemical composition, it is suggested that Group A streptococcal cell wall polysaccharide dissolved by DFP–*S. albus* enzyme consists of a spectrum of molecules having the same immunological determinants but differing in content of conjugated mucopeptide.

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