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Filamentous Capsulated Streptococci from the Human Respiratory Tract: Chemical and Immunochemical Characterization of a Glycoprotein Capsular Antigen of Provisional Binary Capsular Type 87

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A filamentous alpha-hemolytic streptococcus of provisional capsular type 87 isolated from the human respiratory tract has been shown to be binary capsulated. One of the capsular antigens appears to be a glycoprotein; the other appears to be a polysaccharide. Transformation reactions with deoxyribonucleic acid from streptococcus type 87 and a number of noncapsulated pneumococci yielded transformed pneumococci with either a glycoprotein capsule or a polysaccharide capsule, but not with both. Capsular precipitin (quellung) reactions were observed when streptococcus type 87 was treated with homologous antiserum or with antisera to either of the two distinct capsular transformants. Each of the transformed pneumococci gave a quellung reaction with its homologous antiserum or with antiserum to streptococcus type 87, but neither reacted with antiserum to the heterologous transformant. Chemical analysis showed the glycoprotein antigen of streptococcus type 87 to contain, in addition to amino acids, glucose, galactose, glucosamine, and phosphate. The amino acid composition of the glycoprotein capsular antigens from streptococcus type 87 and of those from transformed pneumococci were similar, showing only minor differences. The glycoprotein capsular antigen from streptococcus type 87 gave two closely associated precipitin bands with homologous antiserum or antisera to transformed pneumococci with the glycoprotein capsule. That the two precipitin bands represent two unrelated proteins is precluded largely on the basis of the unlikely probability of 100% cotransformation of the genes coding for both proteins in the pneumococcal transformants that were isolated. Chemical analyses of the various fractions of the glycoprotein indicate that the two precipitin bands may represent a glycoprotein and its corresponding apoprotein.

In previous reports (6, 17-19), some of the properties of several capsulated filamentous alpha- and nonhemolytic streptococci of the human respiratory tract have been described. These organisms are frequently found as components of the normal flora of the upper respiratory tract, but occasionally they give rise to infection of the middle ear or lower respiratory tract, functioning as opportunist pathogens. The present report concerns the filamentous streptococcal strain of provisional capsular type 87. This organism is unique among the 26 capsular types of filamentous alpha- and nonhemolytic streptococci identified; and, therefore, its properties will be described in some detail. Not only does it manifest binary capsulation, but one of the capsular antigens has been found to be a glycoprotein with some properties similar to those of group A streptococcal M proteins.

MATERIALS AND METHODS

Bacterial strains. The prototypic strain of the filamentous alpha-hemolytic streptococcus of provisional binary capsular type 87 was isolated from the sputum of a patient with an acute respiratory infection at the Philadelphia General Hospital in 1964. No other strain of the same capsular type was recovered in the ensuing 8 years. This organism will be referred to hereafter as streptococcus type 87.

The noncapsulated pneumococcal strains R36NC and A66R2 have been described previously (9, 19), and strain 472R is the strain designated S-III5 in reference 9.

Bacterial media. All strains were maintained in fresh beef heart infusion broth supplemented with

Neopeptone (Difco Laboratories, Inc., Detroit, Mich.) (4), and the same medium was used for bacterial transformations. To obtain large quantities of bacterial cells, the several organisms were grown in Todd-Hewitt broth (Difco) supplemented with 0.8% glucose, and the acid formed during growth was neutralized with 1 M Na_2CO_3.

Enzymes from the following sources were used in various procedures: deoxyribonuclease, ribonuclease, trypsin, and pepsin were obtained from Worthington Biochemicals Corp., Freehold, N. J., and Pronase was from Calbiochem, La Jolla, Calif.

Transformation of pneumococci with the deoxyribonucleic acids (DNAs) of streptococcus type 87. The methods used were those cited in an earlier report (19).

Preparation of rabbit antisera. Cells from 500 ml of late-exponential-phase cultures of streptococcus type 87 or of pneumococcal capsular transformants were treated with 1% Formalin for 1 h at room temperature and then heated at 56°C for 30 min. The cells were recovered by centrifugation and suspended in 25 ml of 0.15 M NaCl. Rabbits were immunized by daily intravenous injection of 1 ml of vaccine for 5 days and bled 7 days after the last injection. The schedule of immunization was repeated, and the production of antibodies was monitored by the quellung reaction and by double-diffusion precipitin reactions in agar with capsular antigens. Sera giving the strongest precipitin bands in agar gels were pooled and used for the quantitative assay of precipitins.

Serological techniques. Quantitative precipitin assays were performed by the procedure of McCarty and Lancefield (24); protein in the precipitate was determined by the method of Lowry et al. (23). Inhibition of precipitation with individual components of an antigen, i.e., sugars or amino acids, was carried out by addition of increasing concentrations of each hapten in phosphate-buffered saline, pH 7, to undiluted antiserum and by allowing the mixture to stand at 25°C for 30 min before the addition of antigen. The mixture was kept at 4°C for 18 h; then it was centrifuged, and the precipitate was washed and assayed for protein content.

Precipitin reactions in agar gels were performed in Immunoplates (Hyland Laboratories, Costa Mesa, Calif.), and immunoelectrophoresis was carried out by the method of Rosan (28).

The technique of the quellung reaction was that described earlier (12).

Electron microscopy. Visualization of capsular precipitin reactions by electron microscopy has been reported by Lai et al. (21), who termed the process "immunocoating." Electron micrographs of capsular precipitin reactions of streptococcus type 87 and of pneumococcal transformants producing one or the other of the capsular antigens of the former organism with antisera to each of the several variants have kindly been provided by C. H. Lai, the University of Pennsylvania School of Dental Medicine, Philadelphia. The following procedure was used. Cells grown to late exponential phase were harvested by centrifugation. They were then exposed to the appropriate antiserum at room temperature for 30 min and washed with 0.15 M NaCl. Controls were obtained by exposing cells either to normal serum or to 0.15 M NaCl alone. The cells were then prefixed in a mixure of 2% paraformaldehyde and 2.5% glutaraldehyde in sodium cacodylate buffer, pH 7.3. Postfixing of the cells in 2% osmic acid in *s*-collidine was followed by washing and staining in block with 0.5% uranyl acetate in Veronal buffer, pH 5.2. The cells were dehydrated in graded ethanol and embedded in Epon. Ultrathin sections (0.1 μm) were cut, stained with uranyl acetate and lead citrate, and examined in a Philips EM-300 microscope.

Analytical methods. Quantitative assay of neutral sugars was done by gas chromatography. The samples were hydrolyzed with 2 N HCl at 100°C for 8 h followed by removal of the HCl in vacuo and treatment of the sample with sodium borohydride to convert the sugars to alditols (1). The by-product, sodium borate, was removed by heating with methanol in the presence of acetic acid. The alditols were acetylated with a mixture of acetic anhydride and pyridine, and the alditol acetates were extracted with chloroform. Aliquots of the product were analyzed in a Hewlett-Packard model 5700 A gas chromatography unit on a column containing 3% OV 225 on Gas Chrom Q at 160 to 200°C.

Amino acid composition was determined in an automated amino acid analyzer (Honeywell M 7800) after hydrolysis of samples with 6 N HCl at 100°C for 16 h.

Amino sugars were assayed by both methods cited. Deamination with nitrous acid of aminodeoxyhexoses to anhydrohexoses followed by reduction and acetylation yielded the corresponding alditol acetates, which were analyzed by gas chromatography as described by Porter (26). Amino sugars were analyzed also in the automated amino acid analyzer directly after hydrolysis of samples with 6 N HCl at 100°C for 16 h (27). Amino sugars served, therefore, as internal standards for unifying the results obtained by the two procedures for neutral sugars and for amino acids. Total carbohydrate was estimated by the phenol-sulfuric acid method (11), total protein by the procedure of Lowry et al. (23), total phosphate by the method of Chen et al. (10), and choline by the procedure of Hayashi et al. (16).

Polyacrylamide gel electrophoresis. Disc gel electrophoresis was carried out on 10% polyacrylamide gel in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.8, according to the procedure of Fairbanks et al. (13). Samples were dissolved in a solution containing 0.1 M Tris-hydrochloride, pH 8.8, 10% glycerol, and 40 mM dithiothreitol. The electrode buffer was 0.01 M Tris-hydrochloride, and a constant current of 6 mA/gel was used. Gels were stained with Coomassie brilliant blue for protein and with periodic acid-Schiff stain for carbohydrate (13).

RESULTS

Properties of streptococcus type 87. Streptococcus type 87 was isolated from the sputum of a patient with an acute respiratory infection at the Philadelphia General Hospital, although the infection was not attributed to this organism. It is unique among the strains of filamentous streptococcus isolated from humans at

this institution and is the only strain of this type to be isolated. The organism grows in long chains or filaments both in liquid and on solid media and is weakly alpha-hemolytic on human and on rabbit blood agar. Packed cells of streptococcus type 87 have a brownish pigmentation similar to that manifested by streptococcal cells of Lancefield's group B. No cross-reactivity between streptococcus type 87 and any of the known pneumococcal types was detected. Immunization of rabbits with a vaccine of streptococcus type 87 prepared as described in Materials and Methods resulted in an antiserum tightly agglutinating the organism in a liquid menstruum and giving rise to a positive quellung reaction under the light microscope. Attempts to isolate noncapsular variants of streptococcus type 87 after repeated passage of the organism in liquid cultures containing 20 to 50% anticapsular antiserum were unsuccessful. If noncapsulated mutants of the organism do arise spontaneously, their inclusion in filaments containing capsulated variants may have militated against their detection.

Demonstration of the binary capsulation of streptococcus type 87 by means of DNA-mediated heterospecific capsular transformations. A partially purified preparation of DNA was obtained from a streptomycin-resistant variant of streptococcus type 87 after lysis with a crude preparation of the enzymes of *Streptomyces albus* (25). It was used to determine whether interspecific genetic transfer of capsulation and of resistance to streptomycin to any of three noncapsulated strains of pneumococci, strains R36NC, 472R, and A66R2, could be effected by transformation. Resistance to streptomycin was conferred readily by transfor- to the first two strains but not to the last. In the first experiment in which transformation was attempted, two capsular transformants were obtained, one of strain R36NC and one of strain 472R, each of which gave positive quellung reactions with antiserum to streptococcus type 87. Vaccines were prepared from each of the two capsulated transformants and used to immunize rabbits. When antisera were obtained and used in capsular precipitin tests, it was found that the antiserum to each transformant quelled streptococcus type 87 and the transformant used as the source of vaccine, but neither antiserum reacted with the heterologous capsular transformant. These observations suggested that streptococcus type 87 was a binary capsulated organism. Additional transformations were carried out, and capsular transformants of each type were obtained with all three of the pneumococcal strains used as recipients of DNA. The serological reactions of each of these transformants and those of streptococcus type 87 in capsular precipitin reactions with homologous and heterologous anticapsular sera are shown in Table 1. Antiserum to streptococcus type 87 gave a positive capsular precipitin reaction with all the pneumococcal transformants, and antiserum to each of the latter strains gave a positive reaction with cells of streptococcus type 87.

Extracts of streptococcus type 87 and of the two capsular transformants of pneumococcus A66R2, strains A66R2T87S (having a polysaccharide capsule) and A66R2T87P (having a protein capsule), were prepared by heating the cells of each from a 500-ml culture in 10 ml of 0.1 M glycine hydrochloride, pH 2.0, at 90 to 92°C for 5 min. The crude extracts were clarified by centrifugation, and their precipitin reactions with antisera to each of three strains were tested by double diffusion in an agar gel. An extract of the noncapsulated pneumococcal strain A66R2 was included as a control. Antiserum to streptococcus type 87 gave three precipitin bands, two of which were closely associated and clearly separated from the third (Fig. 1). The single precipitin band formed by an extract of strain A66R2T87S formed a band of identity with the inner precipitin band of the extract of streptococcus type 87, whereas the two closely associated bands formed by the latter extract yielded lines of identity with the extract of strain A66R2T87P. No precipitate was formed by the extract of the untransformed pneumococcus, A66R2, indicating that neither precipitating an-

TABLE 1. *Capsular precipitin (quellung) reactions of streptococcus type 87 (Str 87) and of pneumococcal transformants thereof with antisera to the homologous and heterologous bacterial strains*

Strain	Antisera to:						
	Str 87	R36NCT87S	R36NCT87P	472RT87S	472RT87P	A66R2T87S	A66R2T87P
Str 87	+	+	+	+	+	+	+
R36NCT87S	+	+	−	+	−	+	−
R36NCT87P	+	−	+	−	+	−	+
472RT87S	+	+	−	+	−	+	−
472RT87P	+	−	+	−	+	−	+
A66R2T87S	+	+	−	+	−	+	−
A66R2T87P	+	−	+	−	+	−	+

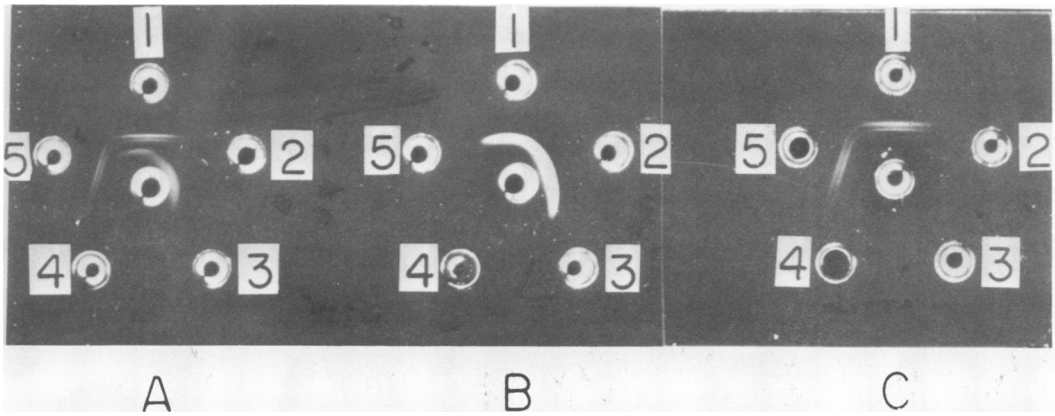

FIG. 1. *Precipitin reactions in agar of antigens extracted with hot glycine hydrochloride (pH 2.0). Outer wells have extracts from: (1) streptococcus type 87, (2) transformed pneumococcus A66R2T87S, (3) noncapsulated pneumococcus A66R2, (4) none, and (5) transformed pneumococcus A66R2T87P. Center wells contain antiserum to: (A) streptococcus type 87, (B) transformed pneumococcus A66R2T87S, and (C) transformed pneumococcus A66R2T87P.*

tigen was of pneumococcal origin. These findings, together with those of the quellung reactions, indicate that streptococcus type 87 is a binary capsulated organism.

Because of the finite but very small capsular precipitin reactions observed with the capsular transformants of streptococcus type 87, we examined these reactions in thin sections of both the parent streptococcus and the two heterologous capsular transformants by electron microscopy. Figure 2 shows the results of the reactions of streptococcus type 87, of the two pneumococcal transformants, A66R2T87S and A66R2T87P, and of the noncapsulated recipient pneumococcal strain, A66R2, with antisera to each of the four strains as well as electron micrographs of the cells of each strain in the absence of antiserum. In the absence of antiserum, the cell walls were relatively clean, save for the presence of a few projections suggestive of fimbriae. Whereas the cells of streptococcus type 87 showed clearly antigen-antibody reactions with homologous antiserum and with antibody to each of the pneumococcal transformants, cells of A66R2T87S reacted significantly only with antisera to streptococcus type 87 and to pneumococcus A66R2T87S, and strain A66R2T87P reacted only with antisera to streptococcus type 87 and to pneumococcus A66R2T87P. These reactions agree with the reactions of the several strains in agglutination tests and when examined in capsular precipitin tests by light microscopy. Although it will be shown later that the capsules of the two transformed pneumococci are composed of macromolecules of different chemical classes, one a carbohydrate and the other a glycoprotein, it is not possible to distinguish the capsular precipitin reactions in the electron micrographs. The small amount of extracellular precipitate at the surface of cells of strain A66R2 and its capsular transformants when exposed to antiserum to strain A66R2 may reflect reaction of antibodies to one or more of the surface antigens, such as M protein (14) or C polysaccharide, of that strain.

Demonstration that the capsular antigen of the transformed pneumococcal strain A66R2T87P and one of the two capsular antigens of streptococcus type 87 are protein in nature. Previous studies of transformed pneumococci (5) and of filamentous alpha-hemolytic streptococci (17) have resulted in the identification of binary capsulated strains, each of the capsular components of which is a polysaccharide. To study the capsular components of streptococcus type 87, extracts of the two distinct capsular transformants, A66R2T87P and A66R2T87S, were prepared with glycine hydrochloride by the method described and treated sequentially with deoxyribonuclease (10 μg/ml), ribonuclease (5 μg/ml), and Pronase (25 μg/ml) for 2 h each at 37°C. The extracts were dialyzed against three changes of distilled water, and the nondialyzable fractions were tested against the homologous antisera in agar gels. Whereas no change was observed in the precipitin reactions of the crude or digested extracts of strain A66R2T87S, the precipitin reaction of the digested extract of strain A66R2T87P with homologous antiserum and with that to streptococcus type 87 was abolished after treatment with Pronase (Fig. 3), suggesting that its capsular antigen is a protein. Examination of a similar extract of streptococcus type 87 before and after

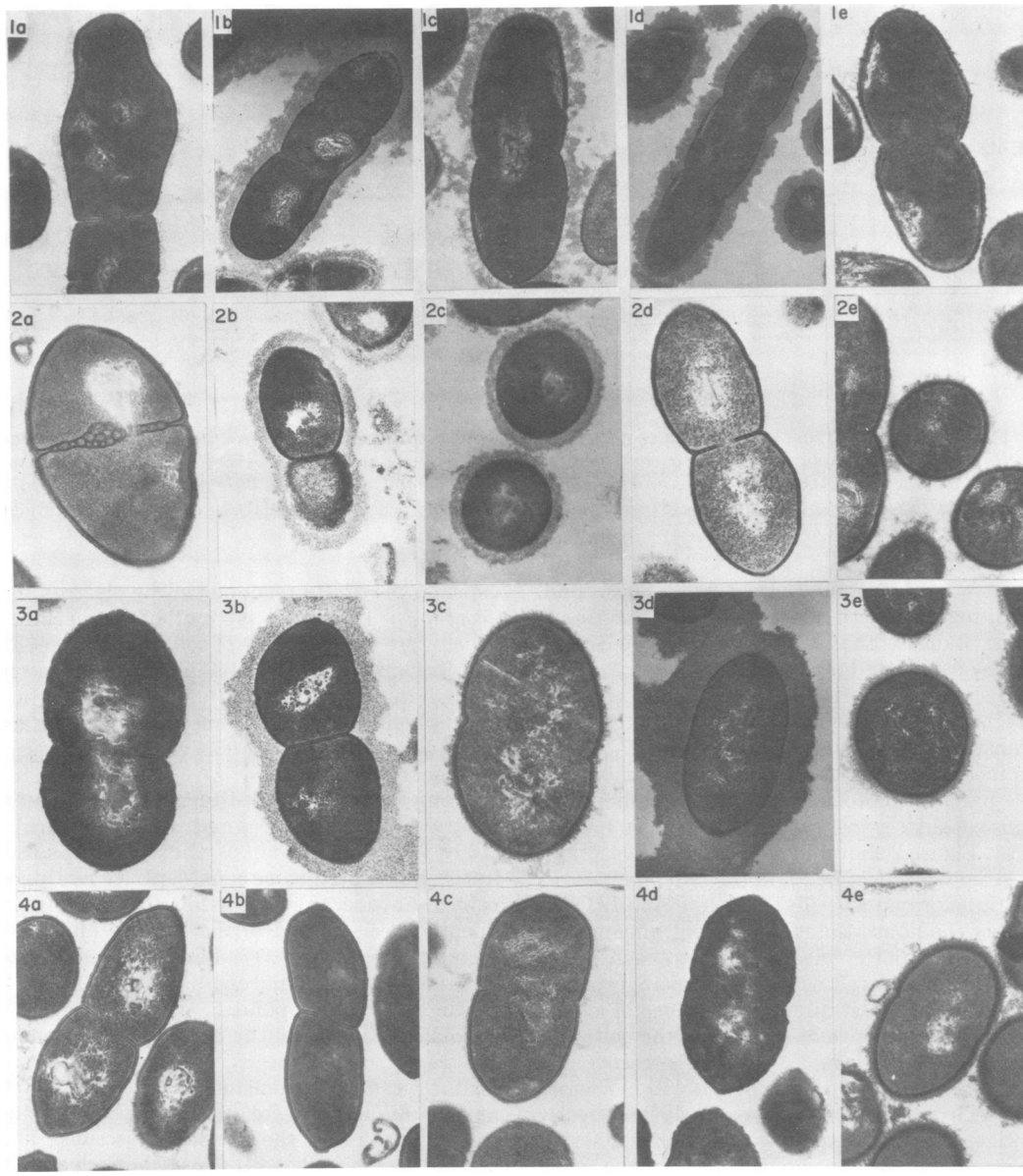

FIG. 2. *Electron micrographs of the immunocoating reactions of streptococcus type 87 (horizontal row 1), transformed pneumococcal strains A66R2T87P (horizontal row 2) and A66R2T87S (horizontal row 3), and the noncapsulated pneumococcus A66R2 (horizontal row 4) with antisera to each. Vertical row a, cells in the absence of antiserum; vertical row b, cells reacting with antiserum to streptococcus type 87; vertical row c, cells reacting with antiserum to strain A66R2T87P; vertical row d, cells reacting with antiserum to strain A66R2T87S; and vertical row e, cells reacting with antiserum to strain A66R2.*

digestion with the same enzyme resulted in elimination of two of the three precipitin bands arising from interaction with homologous antiserum (Fig. 3) and of the bands resulting from interaction with antiserum to strain A66R2T87P. Treatment of the extracts with either pepsin or trypsin gave similar findings, indicating that one of the capsular components of streptococcus type 87 and that of the transformant A66R2T87P are protein. Treatment of intact cells of the latter strain with the same enzymes resulted also in abolition of the capsular precipitin reaction both with homologous antiserum and with antiserum to streptococcus type

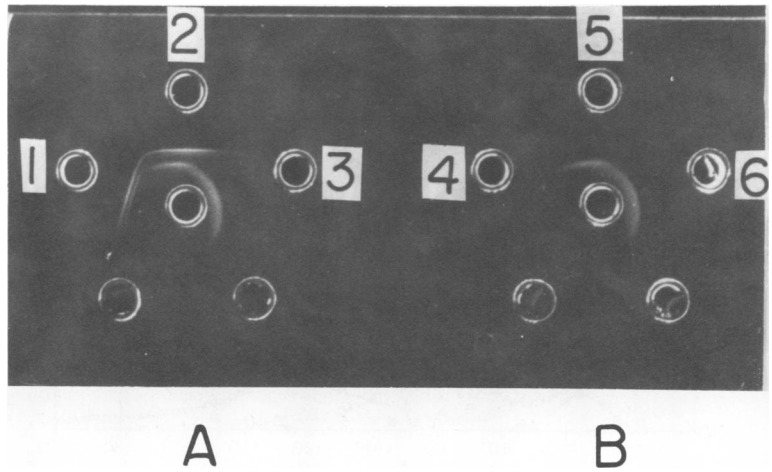

FIG. 3. *Precipitin reactions in agar. Antigens from pneumococcus A66R2T87P, streptococcus type 87, and pneumococcus A66R2T87S before digestion with Pronase are in wells 1, 2, and 3, respectively; after digestion with Pronase, they are in wells 4, 5, and 6. Center wells A and B contain antiserum to streptococcus type 87.*

87. By way of contrast, exposure of the extract of strain A66R2T87S to proteolytic enzymes had no detectable effect on its precipitability by homologous antiserum or by antiserum to streptococcus type 87, and treatment of its cells did not alter their reactivity in capsular precipitin reactions. The properties of the capsular component of streptococcus type 87 resistant to proteolytic enzymes and those of the transformant A66R2T87S are considered in the accompanying report (30).

Isolation and purification of the protein capsular antigen of streptococcus type 87 and of the pneumococcal transformant A66R2T87P. Streptococcus type 87 possesses two capsular antigens, one of which is degraded by several proteolytic enzymes and appears to be a protein. Extraction of the cells of streptococcus type 87 with dilute HCl, pH 2.0, at 90 to 92°C by the technique of McCarty and Lancefield (24) or with dimethylhydrazine formate by the procedure of Anderson et al. (2) resulted in solutions containing both capsular antigens. Extraction with cold 10% trichloroacetic acid yielded only the nonprotein capsular antigen, the protein antigen apparently being inactivated by this agent. Hot distilled water, 90 to 95°C, extracted most of the nonprotein antigen and a portion of the protein antigen, but attempted separation of the two capsular moieties in the mixture by ethanolic precipitation or by chromatography on diethylaminoethyl (DEAE)-cellulose was unsuccessful. The procedure of treating the cells with hot distilled water, which extracted the nonprotein antigen and a portion of the protein antigen, followed by reextraction of the cells with 0.1 M glycine hydrochloride, pH 2.0, at 90 to 95°C for 10 min yielded the protein capsular antigen free of contamination with the nonprotein capsular moiety. The glycine hydrochloride extract obtained in this fashion was dialyzed, lyophilized, and dissolved in 0.15 M NaCl in 0.1 M phosphate buffer (pH 7.0). In agar gels, this extract gave two precipitin bands with antisera to streptococcus type 87 and to pneumococcal strain A66R2T87P but not to pneumococcal strain A66R2T87S. The protein antigen was purified further by chromatography on DEAE-cellulose, and aliquots of the fractions eluted with $(NH_4)_2CO_3$ were assayed for their content of protein, carbohydrate, and phosphate; then the fractions were pooled as shown in Fig. 4. The first three pools contained relatively large amounts of carbohydrate, its ratio to protein being approximately 2:1 in all three, but none reacted with antiserum to streptococcus type 87. In contrast, pools IV through VII, all of which reacted with this antiserum, had carbohydrate-to-protein ratios decreasing progressively from 1:1 to 1:20. Their phosphate content was less than 5%. Although pools VIII and IX were serologically reactive, the amount of material in each was too small to assay. In an agar gel (Fig. 5), pool IV gave a single precipitin band with antiserum to streptococcus type 87, whereas pools V, VI, and VII each gave two, the outer of which, closer to the well containing antigen, was continuous with that of pool IV. With pool VI, the outer precipitin band was somewhat more prominent, whereas the reverse was true with pool VII. The crude glycine hydrochloride extract of streptococcus type 87 is included in Fig. 5 for reference, showing the outer precipitin bands due to protein capsule, a

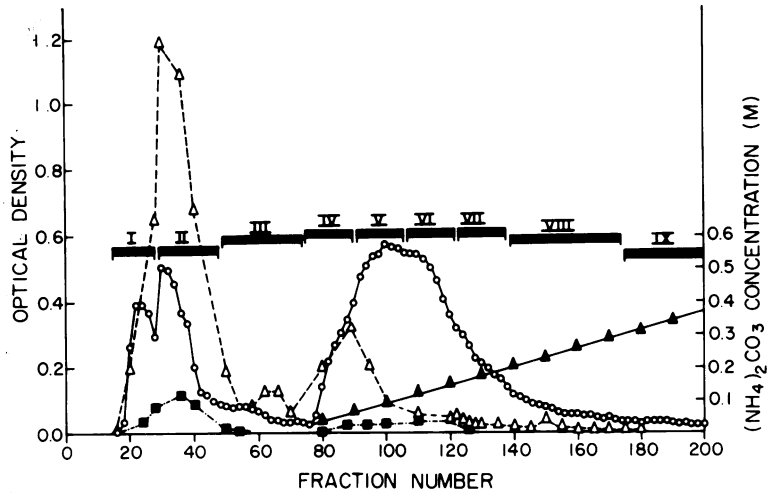

FIG. 4. *Purification of the glycoprotein capsular antigen of streptococcus type 87 by chromatography on a DEAE-cellulose column. $(NH_4)_2CO_3$ gradient (▲); protein estimated by the method of Lowry et al. (23) (optical density at 650 nm) (△); carbohydrate estimated by the phenol-sulfuric acid assay (optical density at 490 nm) (○); phosphate assay (optical density at 820 nm) (■). Roman numerals represent pooled fraction numbers.*

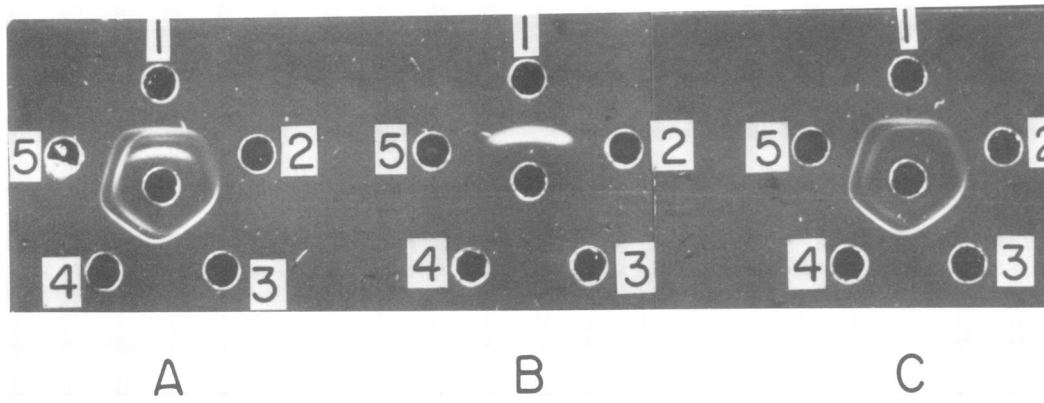

FIG. 5. *Precipitin reactions of pooled fractions IV through VII of the glycoprotein capsular antigen from streptococcus type 87 in an agar gel. Outer wells contain antigens: (1) hot glycine hydrochloride extract of whole cells of streptococcus type 87, (2) pool IV, (3) pool V, (4) pool VI, and (5) pool VII. Center wells contain antisera to: (A) streptococcus type 87, (B) transformed pneumococcus A66R2T87S, and (C) transformed pneumococcus A66R2T87P.*

faint middle band due to an undefined antigen, and a brighter band due to the polysaccharide antigen. The faint middle band was observed only with antiserum to streptococcus type 87 and not with antisera to either of the transformed pneumococcal types.

The protein capsular antigen of transformed pneumococcus A66R2T87P was isolated after autolysis of its cells at 37°C for 2 h in 0.1 M ammonium acetate buffer, pH 7.2, containing 0.1% sodium deoxycholate. The lysate was centrifuged to remove cellular debris, and the supernatant fluid was shown to react with antisera to streptococcus type 87 and to pneumococcus A66R2T87P. The protein in the supernatant fluid was precipitated with 100% ammonium sulfate, and the precipitate was redissolved sequentially in 80, 60, 40, 20, and 0% ammonium sulfate in 0.1 M Tris-hydrochloride, pH 7.0. Each fraction was dialyzed against 0.02 M Tris-hydrochloride, pH 7.0, and examined for precipitation with the same two antisera. Only the fraction extracted with 20% ammonium sulfate yielded a positive reaction and was purified further by fractionation on DEAE-cellulose (Fig. 6). Pooled fractions were examined for precipitin reactions in an agar gel with antiserum to transformed pneumococcus A66R2T87P. As was true of the

antigen extracted from streptococcus type 87 and as shown in Fig. 7, the earlier fractions gave a single precipitin band, whereas the later ones gave two lines of precipitate; the inner band nearer the well containing antiserum increased progressively in intensity. A similar pattern of precipitates was also obtained with antiserum to streptococcus type 87.

The protein capsular antigen of transformed pneumococcus R36NCT87P was extracted with dimethylhydrazine formate by the procedure of Anderson et al. (2) and purified by column chromatography on DEAE-cellulose. The pooled fractions gave a sequence of single and double bands of precipitate similar to that depicted in Fig. 7.

Chemical analysis of the purified protein capsular antigens of streptococcus type 87 and of pneumococcus A66R2T87P. To determine the composition of the protein capsular antigen of streptococcus type 87, pools IV, V, VI, and VII (Fig. 5) were hydrolyzed as described in Materials and Methods and chromatographed in the automated amino acid analyzer (Table 2). All four pools had a similar composition of all amino acids except serine, but the content of carbohydrate, as reflected by the amount of glucosamine, decreased progressively with the increasing number of the pool. The ratios of methionine to glucosamine in pools IV, V, VI, and VII were 1:21, 1:3, 1:0.6, and 1:0.5, respectively. As determined by assay for neutral sugars, a similar decrease in the content of glucose and of galactose was observed. It is noteworthy that a 20- to 40-fold decrease in the carbohydrate content of the glycoprotein did not

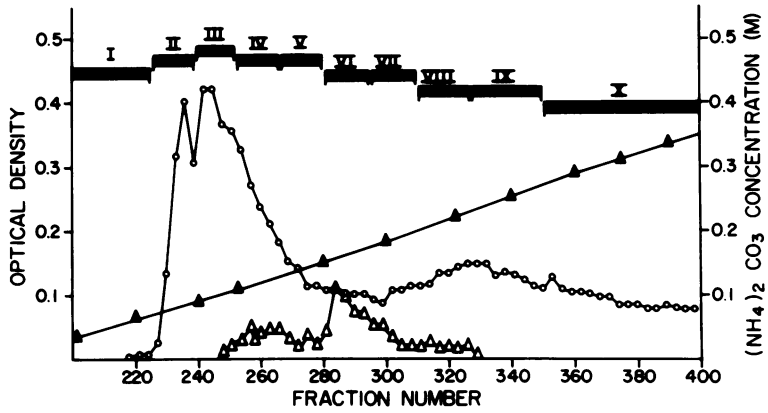

FIG. 6. *Fractionation of the glycoprotein capsular antigen from transformed pneumococcus A66R2T87P on a DEAE-cellulose column. $(NH_4)_2CO_3$ gradient (▲); total protein content (Lowry assay read at 650 nm) (○); total carbohydrate (phenol-sulfuric acid assay read at 490 nm) (△). Roman numerals indicate the pooled fractions.*

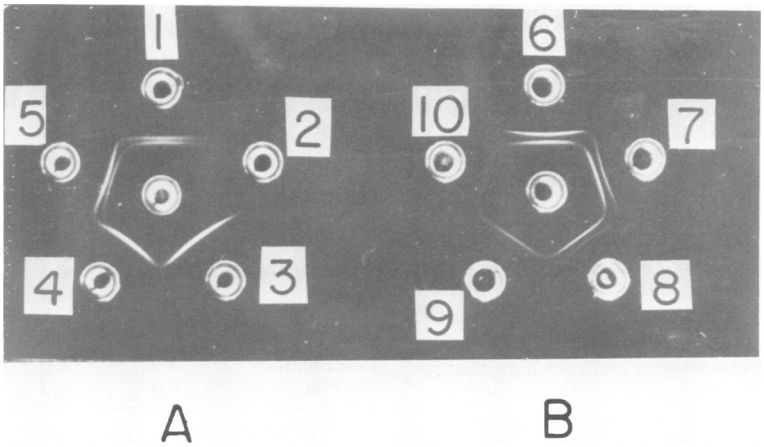

FIG. 7. *Precipitin reactions of capsular glycoprotein fractions of transformed pneumococcus A66R2T87P in an agar gel. Outer wells contain antigens: (1) crude antigen before chromatography and (2-10) pools I through IX. Center wells contain antiserum to pneumococcus A66R2T87P.*

TABLE 2. *Chemical composition of pools IV through VII obtained from DEAE-cellulose column fractionation of the glycoprotein capsular antigen of streptococcus type 87*[a]

Chemical	IV	V	VI	VII
Aspartic acid	13.2	14.8	13.7	10.3
Threonine	12.5	12.3	11.3	7.2
Serine	26.3	9.8	7.3	5.8
Muramic acid	—	—	—	—
Glutamic acid	16.3	14.1	14.2	11.7
Glycine	6.4	7.9	8.4	6.3
Alanine	17.7	19.9	18.5	15.1
Valine	9.4	8.7	8.4	6.5
Methionine	1.0	1.0	1.0	1.0
Isoleucine	2.7	3.9	5.1	4.4
Leucine	5.4	6.6	5.8	5.2
Tyrosine	1.9	2.9	2.1	1.3
Phenylalanine	1.8	1.8	1.5	1.3
Lysine	10.8	14.8	15.8	11.8
Histidine	0.0	0.6	0.9	1.0
Arginine	6.4	4.0	3.5	2.6
Glucosamine	21.2	3.1	0.6	0.5
Glucose	27.2	8.3	2.7	2.4
Galactose	7.4	2.4	0.8	1.0
PO_4	17.3	6.6	5.8	4.8
Choline	0.0	0.0	0.0	0.0

[a] Values represent number of residues per unit of methionine.

abolish its serological reactivity. The fact that fractions V, VI, and VII yielded two precipitin bands with antiserum to streptococcus type 87 suggests that they may represent mixtures of two components, a glycoprotein and its apoprotein, the latter of which may have lost its carbohydrate moiety during the process of purifying the capsular material.

It was noted earlier that pool IV of the glycoprotein capsular antigen of streptococcus type 87 formed a single precipitin band, whereas pools V, VI, and VII formed two precipitin bands with homologous antiserum (Fig. 5) or with antiserum to transformed pneumococcus A66R2T87P. Absorption of each antiserum with pooled fraction IV resulted in the abolition of both precipitin bands when pool VII of streptococcus type 87 or pool VI of transformed pneumococcus A66R2T87P was allowed to react with either absorbed antiserum in an agar gel. The elimination of both precipitin bands by an antigen giving rise only to one band suggests that the antigens giving rise to each band are immunologically similar. The antigen responsible for the second band could not be isolated in sufficiently pure form to permit similar immunological studies.

The glycoprotein capsules of transformed pneumococcal strains A66R2T87P and R36NCT87P were extracted respectively by autolysis or with aqueous dimethylhydrazine formate as described earlier. Representative fractions were analyzed for their amino acid composition on the amino acid analyzer and for their content of sugars, and the findings were compared with those of pool VII of streptococcus type 87. The similarity of the amino acid composition of the three capsular proteins is shown in Table 3. Contamination of the glycoprotein antigen of strain R36NCT87P with pneumococcal C polysaccharide is evidenced by the precipitin reaction with antibody to this pneumococcal cell wall constituent, by the presence of choline and muramic acid, and by the high proportion of phosphate, alanine, and lysine in the hydrolysate of the preparation.

Polyacrylamide disc gel electrophoresis of the glycoprotein capsular antigen of streptococcus type 87 pool VII, which is a mixture of both the glycoprotein and the apoprotein, in two identical gels followed by staining of one gel (no. 1) with Coomassie brilliant blue and the other (no. 2) with periodic acid-Schiff stain is shown in Fig. 8. Two precipitin bands, a and b, seen in gel no. 1 were stained with Coomassie blue, whereas only one, a, was stained with the periodic acid-Schiff stain (gel 2). These findings indicate that band a, with slower mobility and apparently higher molecular weight, was also the one carrying the

TABLE 3. *Amino acid composition of the capsular glycoprotein fractions of streptococcus type 87 and of transformed pneumococci A66R2T87P and R36NCT87P*[a]

Amino acid	Streptococcus type 87 pool VII	Pneumococcus A66R2T87P pool II	Pneumococcus R36NCT87P pool VI
Aspartic acid	10.3	8.8	9.7
Threonine	7.2	6.1	6.2
Serine	5.8	2.9	5.3
Muramic acid	—	—	1.5[b]
Glutamic acid	11.7	12.3	9.9
Glycine	6.3	3.0	8.5
Alanine	15.1	17.2	31.3[b]
Valine	6.5	4.0	7.9
Methionine	1.0	1.0	1.0
Isoleucine	4.4	2.1	2.8
Leucine	5.2	2.9	5.7
Tyrosine	1.3	1.7	2.4
Phenylalanine	1.3	0.3	0.8
Lysine	11.8	11.2	18.4[b]
Histidine	1.0	—	0.3
Arginine	2.6	0.9	3.4
Phosphate	4.8	1.3	45[b]
Choline	0.0	0.0	9.4[b]

[a] See Table 2.

[b] The presence of muramic acid and choline and the excess of phosphate, alanine, and lysine are due to contaminating C polysaccharide.

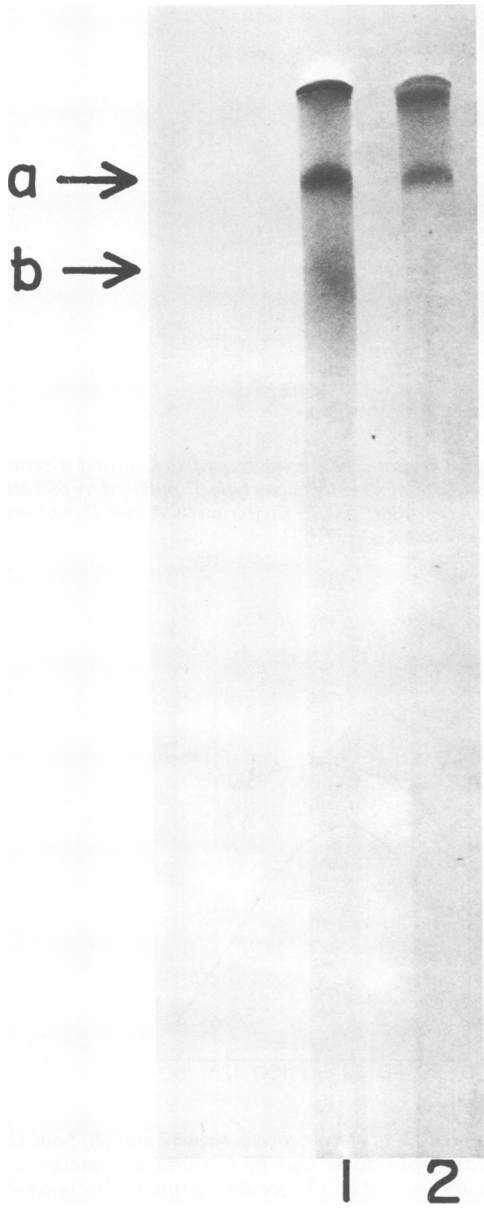

FIG. 8. *Polyacrylamide gel electrophoresis of pool VI of the glycoprotein capsular antigen of streptococcus type 87. Gel no. 1 was stained with Coomassie brilliant blue, and gel no. 2 was stained with periodic acid-Schiff stain.*

carbohydrate moiety and that band b in gel 1 was caused by the corresponding apoprotein.

Demonstration that the capsular glycoprotein of streptococcus type 87 forms an alcohol-soluble picrate. Because of the localization of the glycoprotein capsular antigen of streptococcus type 87, it seemed of interest to determine whether it shared any of the properties of streptococcal M proteins. It had been shown previously that it is possible to partially purify pneumococcal M protein by formation of a picrate (7). The glycoprotein antigen of streptococcus type 87, obtained by extraction with glycine hydrochloride, was precipitated from 1 volume of aqueous solution with 2 volumes of saturated picric acid. The precipitate was collected by centrifugation, extracted with 2 volumes of 70% acetone, and precipitated by the addition of equal volumes of water and saturated picric acid. The precipitate was collected and redissolved in 2 volumes of a mixture of 95% ethanol and 3 N HCl in a ratio of 3:1. Addition of 20 volumes of acetone to the solution yielded a flocculent precipitate after several hours at 4°C which was collected by centrifugtion, redissolved in water, and dialyzed against three changes of 100 volumes of distilled water. The nondialyzed portion was examined for precipitin reactions with antiserum to streptococcus type 87 and to pneumococcus A66R2T87P. It gave a single precipitin band continuous with the outer precipitin band of the glycine hydrochloride extract of streptococcus type 87 and with that of pneumococcus A66R2T87P (Fig. 9).

Serological studies with the glycoprotein capsular antigen of streptococcus type 87 and of transformed pneumococcus A66R2T87P. To investigate the potential immunological role of the carbohydrate moiety of the glycoprotein capsule of streptococcus type 87, quantitative precipitin curves were obtained with pool IV of the fractionated extract of streptococcus type 87 (Fig. 10A, 11A) and with pool II of the fractionated extract of transformed pneumococcus A66R2T87P (Fig. 10B, 11B) and their respective antisera. The titrations of glycoprotein and antiserum were performed with a fixed amount of antiserum and increasing quantities of antigen (Fig. 10). From these curves, the amounts of antigen and of antibody at equivalence were obtained. Increasing amounts of the component sugars of the glycoprotein, glucose, galactose, *N*-acetylglucosamine, and glucosamine, in amounts up to 25 mg/ml, were added to the antisera, followed by addition of the equivalent amount of antigen, and the precipitated antigen-antibody complexes were assayed for total protein. No inhibition of precipitation was observed by any of the component sugars or by alanine in comparable amounts. The results indicate that the carbohydrate moiety of the glycoprotein plays a negligible role, if any, in its immunological reactivity.

Immunoelectrophoretic analysis of pool VII of the protein capsular antigen from streptococcus type 87 and of pool VII of the protein capsular

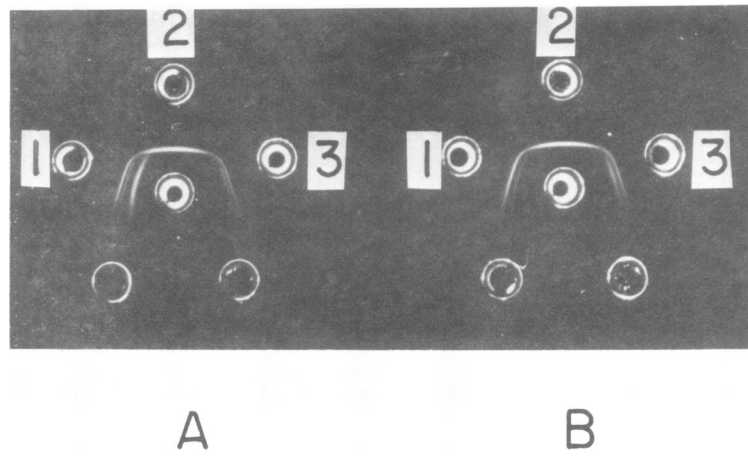

FIG. 9. *Precipitin reactions in agar. Outer wells 1, 2, and 3 contain, respectively, unfractionated glycine hydrochloride extract of streptococcus type 87, glycoprotein antigen of streptococcus type 87 purified by picrate precipitation, and glycine hydrochloride extract of pneumococcus A66R2T87P. Center wells A and B contain, respectively, antisera to streptococcus type 87 and to pneumococcus A66R2T87P.*

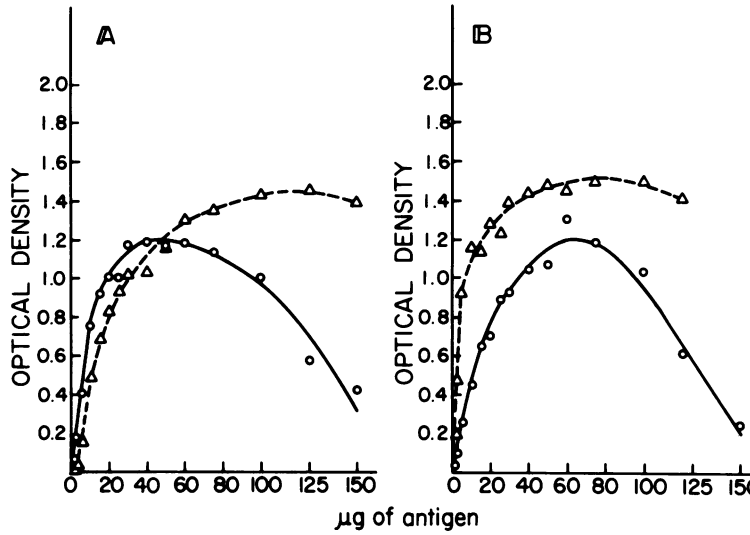

FIG. 10. *Titration curves of the glycoprotein antigens, (A) pool IV of streptococcus type 87 and (B) pool II of transformed pneumococcus A66R2T87P, with antisera to streptococcus type 87* (○) *and pneumococcus A66R2T87P* (△). *The precipitate was assayed by the method of Lowry et al. (23) for total protein. The optical density was read at 650 nm.*

antigen from transformed pneumococcus A66R2T87P with antiserum to the former (Fig. 11A) and with that of the latter (Fig. 11B) showed that the two precipitin bands had identical electrophoretic mobility. A third precipitin band observed with the antigen from transformed pneumococcus A66R2T87P with homologous antiserum was caused by contaminating C polysaccharide.

DISCUSSION

The results described indicate that the filamentous capsulated alpha-hemolytic streptococcus of provisional capsular type 87, isolated from human respiratory secretions, is an unusual organism. Only one isolate of this capsular type was identified among more than 450 isolates of filamentous capsulated streptococci recovered from the human respiratory tract, and it is the sole strain with pigmented cells. Like other filamentous streptococci from humans, streptococcus type 87 lacks virulence for white mice.

In a systematic study of the genetic relatedness of filamentous streptococci of different capsular types to pneumococci, pneumococcal capsular transformants of streptococcus type 87

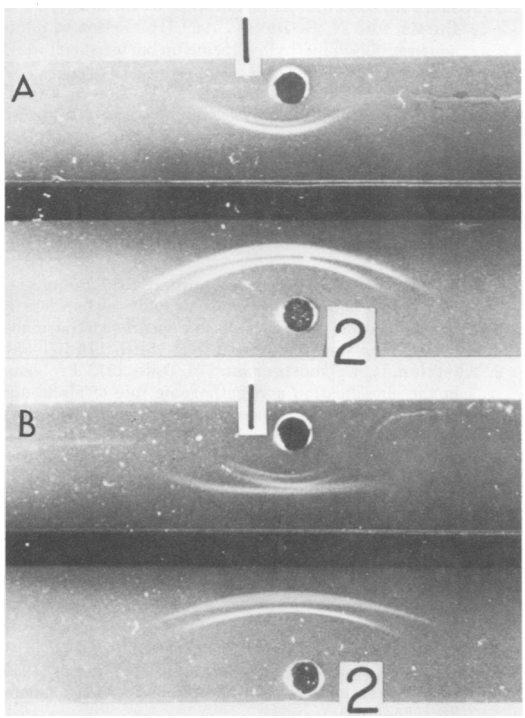

FIG. 11. *Immunoelectrophoresis of the glycoprotein capsular antigens: (well 1) fraction pool VII of the glycoprotein capsular antigen of transformed pneumococcus A66R2T87P and (well 2) fraction pool VII of the glycoprotein capsular antigen of streptococcus type 87. Troughs A and B contain antisera to streptococcus type 87 and pneumococcus A66R2T87P, respectively.*

were obtained, and this obtainment led to the unexpected discovery that streptococcus type 87 was a binary capsulated organism. This finding in itself was not altogether surprising, for binary capsulated pneumococci had been obtained earlier in the laboratory by means of DNA-mediated transformation (5), and a binary capsulated streptococcus producing the polysaccharide capsular antigens of provisional types 83 and 89 had been described previously (17). The unusual finding was that one of the capsular components of streptococcus type 87 proved to be a glycoprotein and represents, so far as is known, a new chemical class for a capsular antigen giving a positive capsular precipitin reaction. Earlier studies by other investigators have demonstrated that some bacteria of the genus *Bacillus,* including *B. anthracis,* produce capsules of polyglutamic acid that give a positive capsular precipitin reaction (29), but a true protein capsule behaving in similar fashion has not been described. The presence of phosphoglycoprotein has been reported in such organisms as *Bacillus licheniformis* (8), *Penicillium charlesii* (15), *Hansenula hostii* (20), *Cladosporium* (22), and *Trichophyton mentagrophytes* var. *granulosum* (3), but their role as capsular antigens has not been suggested.

The protein capsular antigen of streptococcus type 87 and of the two pneumococcal transformants, A66R2T87P and R36NCT87P, gave two precipitin bands with antisera to these organisms. If the two protein bands represented two unrelated proteins, it would be difficult, on the basis of probability, to account for the 100% cotransformation of the genes coding for the production of both proteins in the pneumococcal transformants that were isolated. A more likely explanation for the findings is that the two proteins, demonstrable in agar gels, represent two forms of the same antigen, monomeric and/or polymeric forms of the protein composed of multiple chains or a glycoprotein and its deglycosylated apoprotein. Examination of the glycoprotein by precipitation with homologous antiserum in agar gels both in the presence and in the absence of dithiothreitol revealed no differences in the precipitin reactions. It is unlikely, therefore, that the two bands represent a monomer and polymer of the glycoprotein, the latter made up of units connected by one or more disulfide bonds. Analysis of the four pools of fractions of the glycoprotein capsule of streptococcus type 87 shows that the carbohydrate content decreases progressively from pool IV to pool VII, the ratio of glucosamine to methionine decreasing from 21:1 to 0.5:1. Whereas pool IV gives a single precipitin band with antiserum to the glycoprotein, pool VII gives two bands, the outer band closer to the well containing antigen being the one giving a line of continuity with that formed by pool IV. The appearance of the inner band closer to the well containing antiserum formed by pools V, VI, and VII is associated with a corresponding decrease in the content of glucose, galactose, and glucosamine in these pools.

The fractions appear to represent a mixture of glycoprotein and its apoprotein, and the fact that the percentage of the latter increases with the fraction number of the pools is consistent with this interpretation. Immunoelectrophoretic analysis reveals that the two antigenic species of protein capsular antigen of streptococcus type 87 as well as those of transformed pneumococcus A66R2T87P have identical electrophoretic mobility, indicating that they are similarly charged species. The most convincing evidence in support of the view that the capsular antigen of streptococcus type 87 is a glycoprotein is the constancy of the ratio of amino acids in pools IV through VII with the exception of that of serine;

the amount of this amino acid decreases progressively with that of the carbohydrate. This finding also suggests that the carbohydrate moiety of the molecule may be linked to the apoprotein through the hydroxyl group of serine by a glycosidic or phosphodiester linkage that is split during the process of extraction with hot acid with the loss of both serine and carbohydrate.

Polyacrylamide disc gel electrophoresis of the pool VII of the glycoprotein capsular antigen of streptococcus type 87 shows that, of the two bands, a and b (Fig. 8), observed in the Coomassie blue-stained gel, only one, the slower-moving, higher-molecular-weight fraction is stained by the periodic acid-Schiff stain, indicating the presence of carbohydrate in fraction a. The faster-moving, lower-molecular-weight fraction responsible for protein band b appears to be the corresponding apoprotein. The immunological similarity of the antigen in pool IV with the two antigens present in pools V through VII is borne out by the absorption experiments described, the results of which are consistent with the hypothesis that the two bands formed by the latter pools in precipitin reactions result from the presence of a glycoprotein and its apoprotein.

The formation of two precipitin bands by the glycoprotein capsular antigen of streptococcus type 87 is similar to the behavior of some preparations of group A streptococcal M proteins described by Vosti et al. (31), who reported the isolation of two fractions of M protein, "a" (large) and "b" (small), with similar chemical composition. The amino acid analyses and the peptide maps of the large and small M protein fractions were very similar, suggesting a repeating molecular structure in M protein; however, unlike the capsular antigen reported here, no carbohydrate residue was found in any of the fractions of M protein. Although the analogies observed between the glycoprotein of streptococcus type 87 and the M proteins of group A streptococci and of pneumococci suggest similarities in certain of their properties, the glycoprotein described would appear to be distinct from the other streptococcal antigens characterized previously.

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