CAPSULATION OF PNEUMOCOCCUS WITH SOLUBLE CELL WALL-LIKE POLYSACCHARIDE

II. NONIDENTITY OF CELL WALL AND SOLUBLE CELL WALL-LIKE POLYSACCHARIDES DERIVED FROM THE SAME AND FROM DIFFERENT PNEUMOCOCCAL STRAINS

BY GERALD SCHIFFMAN, Ph.D., DONALD L. BORNSTEIN, M.D., AND ROBERT AUSTRIAN, M.D.

(From the John Herr Musser Department of Research Medicine, The University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104)

(Received for publication 21 May 1971)

In a previous paper (1), it was shown that strains of pneumococci producing a capsule of soluble polysaccharide \( C_s \) related chemically and immunologically to the \( C \) or cell-wall polysaccharide of pneumococcus arise by spontaneous mutation and can be isolated readily from several noncapsulated variants of this organism. Vaccines of the \( C_s \) mutants are highly antigenic in rabbits, giving rise to antisera of high titer which serve as useful tools for the study of the properties not only of the \( C_s \) capsular polysaccharides, but also of the closely related \( C \) polysaccharides.

Although pneumococcal \( C \) polysaccharide was first described by Tillett et al. as early as 1930 (2, 3), detailed knowledge of the cell wall of pneumococcus has lagged behind that of many other Gram-positive organisms. Liu and Gotschlich (4-6) proposed that the \( C \) polysaccharide of strain R36A, a noncapsulated variant of pneumococcus Type II, is a polymer with a repeating unit composed of beta-\( N \)-acetyl-\( D \)-galactosamine-1-phosphate joined through its phosphate group to the 6-hydroxyl group of the next unit, and that this teichoic acid is cross-linked to a mucopeptide by phosphodiester bonds with muramic acid. Exception to this structure was taken by Brundish and Baddiley (7) because it did not contain ribitol, diaminotrideoxyhexose, or choline (8). In addition, the proposed structure did not account for the well-known lability of \( C \) polysaccharide to nitrous acid. Brundish and Baddiley extracted noncapsulated pneumococci with 10% trichloroacetic acid; after purification they isolated a substance, the structure of which is consistent with a repeating unit, \( N \)-acetyl-
galactosaminyl-glucosyl-\(N\)-acetyldiaminotrideoxyhexosyl-ribitol-5-phosphate, joined through its phosphate group to either position 3 or 4 of \(N\)-acetylgalactosamine of an adjacent repeating unit. To this backbone 1 mole of choline phosphate is added to the ribitol to complete the rather complex teichoic acid. The structure proposed by Brundish and Baddiley was concurred in by Mosser and Tomasz (9), who developed a model for the partial structure of the pneumococcal cell wall. Their model consists of a teichoic acid similar to that described by Brundish and Baddiley to which is attached a peptidoglycan similar to that commonly found in other Gram-positive bacteria as suggested by Liu and Gotschlich (4). The peptidoglycan appears to be cross-linked to form dimers (or trimers); this property places it among the Type III cell wall mucopolptides according to the classification of Ghuysen (10).

This report describes the isolation of the \(C_s\) capsular polymers and of the \(C\) polysaccharides from \(C_s\) capsulated and from noncapsulated strains of pneumococcus and demonstrates that, despite marked similarities, differences exist not only between the \(C_s\) and \(C\) polysaccharides of a single strain, but also among the \(C_s\) polysaccharides and the \(C\) polysaccharides of different strains. The results suggest that these differences are attributable to variations in the mucopolptide portions of the molecules, and that the extensive cross-reactions are related largely to the teichoic acid moieties thereof in agreement with the findings of Brundish and Baddiley, who showed that the teichoic acids from several strains of pneumococcus were chemically identical.

Materials and Methods

Nomenclature of Pneumococcal Variants and Strains of Pneumococcus Employed.---The nomenclature of pneumococcal variants and of the strains used are described in the preceding paper (1) except as noted below:

\(S_{11},\) a noncapsulated mutant of pneumococcus derived from the Type I strain ID.

\(S_{111},\) a noncapsulated mutant of the Type II strain, D39S, described previously as strain R36NC.

\(S_{111},\) a noncapsulated mutant of pneumococcus Type II derived from strain R36NC and described previously as strain R630.

Growth of Organisms and Isolation of Polysaccharides.---Pneumococci were grown overnight in fresh beef heart infusion broth supplemented with 1% Neopeptone (Difco Laboratories, Inc., Detroit, Mich.). Glucose was then added to a final concentration of 1%, and incubation of the culture was continued with periodic neutralization with 3 N \(\text{NaOH}\) of the lactic acid formed. At the end of the exponential phase of growth, the bacterial cells were harvested by centrifugation and allowed to autolyze in 30 ml of 5% sodium acetate at 37°C for 18 hr. The insoluble cellular debris was removed by centrifugation and the supernatant fluid was either dialyzed and lyophilized, or the polysaccharides were precipitated by the addition of 3 volumes of ethanol. Protein was removed from the soluble nondialyzable material by shaking with chloroform-octanol (Sevag method), after which procedure the supernatant was fractionated with ethanol. The insoluble cellular debris was removed by centrifugation and the supernatant fluid was either dialyzed and lyophilized, or the polysaccharides were precipitated by the addition of 3 volumes of ethanol. Protein was removed from the soluble nondialyzable material by shaking with chloroform-octanol (Sevag method), after which procedure the supernatant was fractionated with ethanol. After the addition of 0.8 volume of ethanol, a fraction is precipitated which is usually of low specific activity when measured by its precipitability with appropriate antisera. The fraction precipitating between 44 and 55% ethanol (0.8-1.2 volumes) is mainly \(C_s\) capsular polysaccharide, whereas that precipitating between 55 and 75% ethanol (1.2-3.0 volumes) is mainly \(C\) polysaccharide.

The \(C\) and \(C_s\) polysaccharides were purified further by gel filtration on Sephadex G-200
in 0.2 N acetic acid (6). Effluent fractions were assayed by measuring their weights after lyophilization and their reactivity with antiserum. Preparations of teichoic acid were made according to the procedure of Brundish and Baddiley (7). Teichoic acid is extracted from whole pneumococcal cells with 10% trichloroacetic acid (TCA). When wild-type capsulated cells are treated in this fashion, the teichoic acid obtained is presumably that portion of the C polysaccharide which is attached to the mucopeptide of the intact cell wall. When C\textsubscript{s} capsulated strains are employed, the material derived from treatment with TCA represents the teichoic acid moieties of both the C and C\textsubscript{s} polysaccharides of such strains.

Preparation of Antisera and Immunologic Techniques.—Antisera were prepared in rabbits. 50 μg bacterial nitrogen in the form of formalin-treated log-phase pneumococci which had been heated at 60°C for 30 min was injected intravenously three to four times weekly for 2 wk, and the animals were bled by cardiac puncture 6 days after the last injection. This schedule was repeated for eight or more courses, antisera of high titer being obtained usually after two to four such courses of immunization.

Ouchterlony analysis of the various polysaccharides was performed in 1% Difco purified agar dissolved in Gelman barbital buffer (Gelman Instrument Co., Ann Arbor, Mich.), 0.05 ionic strength, pH 8.6. Concentrations of antigen were 1.0 mg/ml. Wells were made with an LKB die (LKB Instrument Co., Washington, D.C.) and were of approximately 8 μl volumes.

Quantitative precipitin analysis was performed in the manner described previously (1) with the ninhydrin technique (11).

Preparation of DNA and Techniques of Transformation.—The methods used were those described by MacLeod and Krauss (12) and by Bernheimer, Wermundsen, and Austrian (13).

RESULTS

Isolation of C\textsubscript{s} and C Polysaccharides from C\textsubscript{s} Capsular Mutants.—Supernatant fluids from neutralized cultures of C\textsubscript{s} pneumococcal mutants contain C\textsubscript{s} polysaccharide precipitable by 50% ethanol, whereas similar fluids from cultures in which noncapsulated pneumococci have been grown do not contain appreciable quantities of such polymers. This difference provides a method, therefore, for obtaining C\textsubscript{s} polysaccharide which is relatively free from C polysaccharide. Because many other substances are also precipitated from such complex culture fluids in the presence of 50% ethanol, the final purification of the C\textsubscript{s} polysaccharide is cumbersome. On the other hand, autolysates of bacterial cells after deproteinization contain 25-70% of C\textsubscript{s} and C polysaccharides. The problem of purification, therefore, is largely one of separating C\textsubscript{s} from C polysaccharide. Because capsular polysaccharides are, in general, polymers of high molecular weight, whereas C polysaccharide from pneumococcal autolysates has been reported to have a molecular weight as low as 26,400 (14), separation of the two by fractionation with alcohol was attempted. As can be seen in Fig. 1, the fraction of a deproteinized, dialyzed autolysate of the C\textsubscript{s} pneumococcal strain S\textsubscript{IIr}, C\textsubscript{s} precipitated with 33% ethanol (0.5 volume), produces material of low specific activity in precipitating rabbit antibody to the same strain. The fraction precipitated by 33-44% ethanol (0.5-0.8 volume) is somewhat more active but still much less so than the fraction precipitated by concentrations of ethanol between 44 and 55% (0.8-1.2 volumes). The high specific activity
of the fraction precipitated by 55-67% (1.2–2.0 volumes) ethanol was anticipated because this fraction is the one that yielded C polysaccharide in earlier studies. As an initial step in purification, therefore, deproteinized, dialyzed autolysates of Cₙ pneumococcal strains were fractionated with ethanol by employing the following concentrations: 0–44% (0.8 volume), 44–55% (0.8–1.2 volumes), and 55–67% (1.2–2.0 volumes), the last two fractions containing the Cₙ and C polysaccharides respectively.

![Graph showing precipitin curves of fractional ethanolic precipitates of an autolysate of pneumococcal strain S-II₄Cₜ with homologous rabbit antiserum. Fraction precipitated by 0–0.5 volume ethanol, □; by 0.5–0.8 volume ethanol, ●; by 0.8–1.2 volumes ethanol, ○; by 1.2–2 volumes ethanol, x.

AbN, antibody Nitrogen; Ag, antigen.

As can be seen in Fig. 2, right and left, unFractioned lysates obtained from noncapsulated organisms give single bands of precipitate in agar gels with antibody to a Cₙ capsular mutant, whereas similar preparations from Cₙ mutants yield two bands with the same antiserum. Fractional precipitation with ethanol by the procedure cited above separates the faster moving inner band of C polysaccharide from the slower moving outer band of Cₙ polysaccharide.

A study of the quantitative distribution of the C polysaccharides from pneumococcal strains S-II₄, S-II₅, and S-II₄₅ and of the Cₙ and C polysaccharides from their respective spontaneous Cₙ mutants was made. After isolation of the Cₙ
mutants, it was noted that the sizes of the capsules of strains S-\textsubscript{III}\textsubscript{C}, and S-\textsubscript{VIII}\textsubscript{C}, as observed by the capsular precipitin reaction, were significantly larger than those of strains S-\textsubscript{I2}\textsubscript{C}, and S-\textsubscript{II}\textsubscript{C}. It was expected, therefore, that the 0.8–1.2 volumes ethanol fractions would represent a larger percentage of the total polysaccharide isolated from the strains with the larger capsules than from the strains with smaller ones. The realization of this expectation is shown in the results recorded in Table I. The C\textsubscript{s} polysaccharide precipitable by 0.8–1.2 volumes ethanol represents 30 and 50% of the total 0.8–2.0 volumes of precipitable polysaccharides of strains S-\textsubscript{VIII}\textsubscript{C}, and S-\textsubscript{III}\textsubscript{C}, respectively, whereas only 6–13% of the total is found in the corresponding fraction from strains S-\textsubscript{I2}\textsubscript{C}, and S-\textsubscript{II}\textsubscript{C}. It should be noted, however, that the relative amount of serologically reactive polysaccharide precipitated from the 0.8–1.2 volumes ethanol fraction of lysates of strain S-\textsubscript{I2}, was significantly higher than that from comparable fractions of lysates of other noncapsulated strains, although fractions of lysates of strain S-\textsubscript{I2}, showed only a single band of precipitate in precipitin reactions in agar gels (Fig. 2 right).

\textit{Purification of C\textsubscript{s} and C Polysaccharides by Gel Filtration.}—Preparations of polysaccharides obtained by fractionation with ethanol were purified further by filtration through Sephadex G-200 or, in some cases, through Sephadex G-150. Fig. 3 shows the results of such a fractionation. Material precipitated by the addition of 0.8 volume ethanol to an autolysate of strain S-\textsubscript{III}\textsubscript{C}, (Fig. 3 \textit{a}) contains an extremely heterogeneous population of molecular sizes extend-
ing from that of the excluded material (peak about 100 ml) to those of very small size. On the other hand, material precipitating between 0.8–1.2 volumes ethanol (Fig. 3 b) consists primarily of material of large molecular weight (peak at 130–165 ml, 57 mg) with only small amounts of material (22 mg) being eluted from the column in the effluent fraction from 165–330 ml. The material precipitating between 1.2 and 3.0 volumes ethanol (Fig. 3 c) eluted in a skewed peak, but one, the maximum of which was at 300 ml of effluent, and clearly indicative of material of smaller molecular size than that in the 0.8–1.2 volumes ethanol precipitate. Even very small columns of Sephadex G-200, 44 X 0.9 cm, can be used to purify small amounts (5–50 mg) of material from pneumococcal autolysates.

### TABLE I

| Organism | 0.0–0.8 vol. | 0.8–1.2 vol. | 1.2–2.0 vol. | 0.8–1.2/0.8–2.0 |
|----------|--------------|--------------|--------------|-----------------|
| S-I      | 11           | 16           | 36           | 0.31            |
| S-II     | 14           | 7            | 48           | 0.13            |
| S-IIIa   | 13           | 5            | 38           | 0.12            |
| S-VIIa   | 24           | 7            | 69           | 0.09            |
| S-IIICa-1* | 53         | 3            | 48           | 0.06            |
| S-IIICa-2 | 37         | 6            | 56           | 0.10            |
| S-IIICa-3 | 77         | 7            | 66           | 0.10            |
| S-IIICa-4 | 32         | 4            | 50           | 0.07            |
| S-IIICa  | 11           | 10           | 69           | 0.13            |
| S-IIIaCa | 25           | 47           | 48           | 0.50            |
| S-VIIaCa | 26           | 41           | 78           | 0.30            |

* 1, 2, 3, and 4 represent analyses of independently isolated Ca capsulated mutants of strain S-Ia.

**Immunological Characterization of Cα and C Polysaccharides.**

(a) Analysis by the quantitative precipitin reaction with antisera to Cα capsular mutants: In the preceding paper (1), it was reported that absorption of an antiserum prepared against a noncapsulated pneumococcus with the capsular polysaccharide of a Cα mutant removed 95% or more of the antibody precipitable by C polysaccharide. This finding demonstrated the close immunological relationship between C and Cα polysaccharides. With certain antisera, however, significant antigenic differences between these polysaccharides were noticed by precipitin reactions in agar gels (Fig. 4). Differences were found to exist between the C and Cα polysaccharide of a single pneumococcal strain and between the C polysaccharide of the Cα mutant and that of the parent pneumococcal strain from which that mutant was derived. Fig. 5 presents the results...
Fig. 3. Chromatographic separation on Sephadex G-200 of C and C₆ polysaccharides in ethanolic fractional precipitates of an autolysate of pneumococcal strain S₁₁₆₇C₆. (a) Elution pattern of C and C₆ polysaccharides from the 0-0.8 volume ethanolic fraction. (b) Elution pattern of the 0.8-1.2 volumes ethanolic fraction showing predominately C₆ polysaccharide. (c) [facing page] Elution pattern of the 1.2-3 volumes ethanolic fraction showing predominately C polysaccharide. In each graph, the limits of the column, defined by blue dextran (left) and by NaCl (right) are indicated by arrows.
obtained with an antiserum produced with a vaccine of strain S-VIII C. The homologous C polysaccharide precipitates 10 μg antibody nitrogen from 12.5 μl of antiserum, whereas the C polysaccharide from the same strain precipitates only 6 μg nitrogen from the same amount of this reagent. The C polysaccharide from the parent noncapsulated strain, S-VIII C, precipitates only 4 μg antibody nitrogen from the antiserum to the C mutant. Teichoic acid preparations from both the C mutant, S-VIII C, and from the parent strain, S-VIII C, precipitated 4.5-5 μg antibody nitrogen. After complete absorption of antiserum to strain S-VIII C, serum 1416, with the C polysaccharide of the homologous strain, the addition of C polysaccharide from strain S-VIII C, or of the teichoic acids of strains S-VIII C, or S-VIII C, caused no additional precipitation. The homologous C polysaccharide, however, was capable of precipitating the remaining 4 μg of antibody nitrogen. With antiserum 1416, the reactions with the following pairs of noncapsulated pneumococcal strains and their respective C mutants were examined: S-12 and S-12 C, S-112 and S-112 C, and S-1114 and S-1114 C. Similar results were obtained, i.e., the C polysaccharides precipitated about 9 μg antibody nitrogen and the C polysaccharides 3-4 μg. After absorption with the autologous C polysaccharide of strain S-VIII C, the serum no longer reacted.

---

Fig. 3c
with the C polysaccharides of any of the noncapsulated strains, whereas the three heterologous C₅ polysaccharides were capable of precipitating the residual 3–4 µg of antibody nitrogen. The results indicate that this serum is able to distinguish between C₅ and C polysaccharides. The common antigenic determinants of C and C₅ polysaccharides appear to reside in the teichoic acid moiety of the molecules since absorption of the serum with C polysaccharide results in the complete loss of reactivity with autologous and heterologous preparations of teichoic acid.

Fig. 4. Precipitin reaction in an agar gel of the C and C₅ polysaccharides of pneumococcal strain S-I₄C₅ with antiserum to Strain S-I₄C₅. The nonidentity of the two polysaccharides is indicated by the spurring of the two bands of precipitate. Well 3, C₅ polysaccharide; well 4, C polysaccharide.

A somewhat different picture is presented by antiserum to another C₅ mutant. Serum 668₁₉ prepared against strain S-II₂C₅ reacted equally well with the C₅ polysaccharide of the autologous strain and with the C polysaccharide of the parental strain S-II₂, each precipitating 16–18 µg of antibody nitrogen from an equal volume of antiserum (Fig. 6). Preparations of teichoic acid from each of strains S-II₂C₅, S-VIII₂C₅, and S-VIII₃ precipitate over 80% of the precipitable antibody. This result indicates that serum 668₁₉ contains antibody directed chiefly against a component common to all these polysaccharides, i.e., teichoic acid. Consistent differences in the antibody responses to vaccines of these two strains, S-VIII₂C₅ and S-II₂C₅, were observed in several rabbits and appear to
reflect configurational and immunological differences in their Cₐ polysaccharides.

(b) Analysis by quantitative precipitin reactions with antisera to noncapsulated pneumococci: Antisera of high titer to pneumococcal C polysaccharide were difficult to obtain. Prolonged immunization according to a protocol which resulted in high titered antisera when vaccines of Cₐ capsular mutants were employed failed to produce potent antisera when vaccines of noncapsulated strains were used. Resumption of immunization with noncapsulated strains after a rest period of 3 months, however, was followed by good responses.

Table II presents the data from quantitative precipitin reactions obtained with sera from sequential bleedings of rabbit 18 immunized with strain S₉III. After 4 months of immunization, the titer of anti-C antibody in bleeding 12 was high enough to analyze by the precipitin technique. In the next month, the titer rose to 430 μg antibody nitrogen/ml, but, thereafter, it fell progressively despite continuous immunization. Immunization was discontinued for 3 months, and when it was resumed, the titer of antibody rose rapidly again, remained high for 2 months, and then fell progressively.
Antiserum from rabbit 18 was of unusual interest because it was capable of discriminating among the C polysaccharides of several pneumococcal strains. Fig. 7 shows the precipitin curves obtained when C polysaccharides from four noncapsulated strains, S-15, S-H2, S-vm, and S-vm1, react with bleeding 15 from this rabbit, (serum 18). C polysaccharide from strain S-15 in amounts ranging up to 15 µg failed to precipitate any antibody nitrogen from 50 µl of serum 18, whereas 2.5-5 µg of C polysaccharide from strains S-H, S-H1, and S-vm, precipitated 9-10 µg of antibody nitrogen. C polysaccharide from strain S-15 was shown to be an effective antigen, however, precipitating the same amount of antibody from antiserum to strain S-15 as the C polysaccharide of strain S-15.

Fig. 8 depicts the data from quantitative precipitin tests obtained when the C polysaccharides prepared from the corresponding Cs strains, S-15Cs, S-H1Cs, S-H11Cs, and S-vm1Cs, react with antiserum 18 to strain S-vm1. It can be seen that the C polysaccharides from strains S-15Cs and S-H11Cs react with antiserum 18 to precipitate between 9 and 10 µg antibody nitrogen from 10 µl of serum. In sharp contrast, C polysaccharides from strains S-15Cs and S-vm1Cs precipitate only 1-2 µg antibody nitrogen. These latter preparations precipi-
rated larger amounts of antibody nitrogen from other antisera, showing thereby that their poor reactivity with serum 18h resulted from its low reactivity with the specific determinants of these polysaccharides. Analogous results were obtained when serum 18h reacted with C₄ polysaccharides of these four strains.

The teichoic acid prepared from strain Sᵥ₁₁₁, precipitated less than 2 μg antibody nitrogen from 10 μl of serum 18₂₀ from which 10 μg antibody nitrogen is precipitable by the autologous C polysaccharide (Fig. 9). This antiserum appears to be directed, therefore, against determinants other than the teichoic acid portion of C polysaccharide.

Use was made of the fact that C polysaccharide from strain Sᵥ₁₁₁ precipitated antibody from serum 18h, but that C polysaccharides from strains S₁₃C₄ and Sᵥ₁₁₁C₄ did not. DNA from each of the latter two strains of pneumococcus was used to transform strain Sᵥ₁₁₁ to the C₄ capsulated state. After isolation of the C₄ capsular transformants, C and C₄ polysaccharides were recovered and tested for their ability to react with serum 18. It was found in all cases examined that their ability to react with serum 18 was similar to that of the recipient strain, Sᵥ₁₁₁, i.e., the C polysaccharide reacted with serum 18 indicating that transformation to the C₄ capsular state did not modify the mucopeptide portion of the C polysaccharide of the recipient strain in such a way as to change its

### TABLE II
*Response of Rabbit 18 to Immunization with Pneumococcal Strain Sᵥ₁₁₁*

| Bleeding No. | Milligrams of antibody N/mL precipitable with C polysaccharide (Sᵥ₁₁₁) |
|-------------|--------------------------------------------------------------------------|
| 1–11        | †                                                                         |
| 12          | 124                                                                       |
| 13          | 122                                                                       |
| 14          | 430                                                                       |
| 15          | 216                                                                       |
| 16          | 180                                                                       |
| 17§         | 150                                                                       |
| 18          | 106                                                                       |
| 19          | 1000                                                                      |
| 20          | 1030                                                                      |
| 21          | 840                                                                       |
| 22          | 325                                                                       |
| 23          | 106                                                                       |

* 40 μg bacterial N/injection; 3-4 injections/wk; bleeding every 2 wk.
† Bleedings 1–11 all contained less than 100 μg antibody N/mL by quantitative precipitin test.
§ After bleeding No. 17, rabbit 18 was rested for 3 months.
reactivity with this antiserum. Data listing the reactions of the C polysaccharides of these strains before and after transformation are shown in Table III.

**DISCUSSION**

In an earlier publication, some of the properties of mutant strains of pneumococcus producing a capsule of C-like (C₆) polysaccharide were described (1). In the present report, the separation of the C₆ capsular polysaccharide from the C or cell wall polysaccharide has been presented in greater detail. C₆ polysaccharide can be recovered from the supernatant fluid of cultures of suitable strains relatively free of C polysaccharide by precipitation with 1 volume of ethanol. It can be separated also from the C polysaccharide in autolysates of the same organisms by appropriate chemical or physical techniques. Purification of C₆ polysaccharides from cell lysates entails deproteinization, fractional precipitation, and gel filtration. The precipitation of C₆ polysaccharide with lower concentrations of ethanol than are required to precipitate C polysac-
charide, the earlier elution of the former from mixtures filtered through gels, and the relative position of the two polysaccharides in immunoprecipitates in agar gels all point to the higher molecular weight of the C₃ polysaccharide.

Immunologic analysis of the C and C₃ polysaccharides from a variety of pneumococcal strains has brought to light a number of findings regarding their properties. Contrary to the expectation that the C polysaccharides of diverse pneumococcal strains would be identical, differences among them have been found. Such differences have been discovered to exist between the C polysaccharides of wild-type capsulated strains by means of selected sera and appear to reside in the mucopeptide moiety of the C polysaccharide (Table IV). The C polysaccharide of a Type I strain, ID, reacts with an antiserum to the C polysaccharide of the noncapsulated strain S⁻VIII, whereas that of the Type II strain, II D39S, and that of the Type VIII strain, VIII H, fail to do so. Analogous differences exist among noncapsulated variants isolated from each of these three strains. Of additional interest is the nonidentity of the C polysaccharides of independently isolated noncapsulated mutants of a single capsulated strain. Each member of pairs of such noncapsulated variants isolated from cultures of pneumococci of three capsular types has been shown to have
a C polysaccharide differing in immunologic behavior from that of the other. It would appear, therefore, that mutations affecting the structure of the cell wall polysaccharide of pneumococcus are not uncommon. It is of additional interest that no correlation between the competence of noncapsulated strains of pneumococcus in transformation reactions and the immunologic reactivity of their C polysaccharides has been observed.

When strains of noncapsulated pneumococci of diverse origins mutate to form capsules of C polysaccharide, the C polysaccharides of five such C capsule mutants have been found to retain the parental pattern of reactivity with antiserum to the C polysaccharide of strain S-VIII. The single exception to this observation has been noted in C capsule mutants of strain S-VIII itself. The C polysaccharides of several independently isolated C variants of strain S-VIII have been shown to differ consistently in their reactivity with antiserum to strain S-VIII from that of the parental noncapsulated strain. It would appear, therefore, that mutation(s) of strain S-VIII to the C capsule state are complex, affecting not only the production of C capsule material but also the structure of the cell wall polysaccharide.

When the C polysaccharides of the spontaneous mutants of the noncapsu-
lated strains of pneumococci included in Table IV are examined with antiserum to the noncapsulated strain S\textsubscript{VIII}, it is observed that the immunologic reactions of the C\textsubscript{a} polysaccharides are similar to those of the C polysaccharides of the same strains and, with the exception of strain S\textsubscript{VIII}C\textsubscript{a} noted above, similar

### TABLE III

**Serologic Reactivity of C Polysaccharides of C. Capsular Transformants and Recipient Noncapsulated Pneumococcal Strains with Antisera to the Teichoic Acid (Anti-S\textsubscript{II}C\textsubscript{a}) and to the Mucopeptide (Anti-S\textsubscript{VIII}C\textsubscript{a}) Moieties of C and C\textsubscript{a} Polysaccharides**

| Recipient strain | Reaction with Anti-teichoic acid serum | Reaction with Anti-teichoic acid serum (Source of DNA) | Transformants | Reaction with Anti-teichoic acid serum | Reaction with Anti-teichoic acid serum (Source of DNA) |
|------------------|---------------------------------------|------------------------------------------------------|---------------|---------------------------------------|------------------------------------------------------|
| S\textsubscript{II} | + -                                    | S\textsubscript{II}C\textsubscript{a} + +             | S\textsubscript{II} TC\textsubscript{a} + +           | -                                                     |
| S\textsubscript{VIII} | + +                                   | S\textsubscript{VIII}C\textsubscript{a} + +           | S\textsubscript{VIII} TC\textsubscript{a} + +         | -                                                     |
| S\textsubscript{VIII}C\textsubscript{a} | + -                                   | S\textsubscript{VIII}C\textsubscript{a} + +           | S\textsubscript{VIII} TC\textsubscript{a} + +         | -                                                     |

### TABLE IV

**Serologic Reactivity of C and C\textsubscript{a} Polysaccharides of Wild-Type Capsulated Pneumococci and of Related Noncapsulated and C\textsubscript{a} Capsular Mutants with Antisera to the Teichoic Acid and Mucopeptide Moieties of C and C\textsubscript{a} Polysaccharides. Isolated C and C\textsubscript{a} Polysaccharides of C\textsubscript{a} Capsulated Strains React in Similar Fashion with Each Antiserum**

| Pneumococcal strain | Anti-teichoic acid serum (Anti-S\textsubscript{II}C\textsubscript{a}) | Anti-mucopeptide serum (Anti-S\textsubscript{VIII}C\textsubscript{a}) |
|---------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| S\textsubscript{I} D  | +                                                             | +                                                             |
| S\textsubscript{II}  | +                                                             | -                                                             |
| S\textsubscript{II}C\textsubscript{a} | +                                                           | -                                                             |
| S\textsubscript{III} | +                                                             | +                                                             |
| S\textsubscript{D39S}  | +                                                             | -                                                             |
| S\textsubscript{II}C\textsubscript{a} | +                                                           | -                                                             |
| S\textsubscript{III}C\textsubscript{a} | +                                                           | +                                                             |
| S\textsubscript{VIII} | +                                                             | +                                                             |
| S\textsubscript{III}A66 | +                                                          | +                                                             |
| S\textsubscript{III}A66 | +                                                          | +                                                             |
| S\textsubscript{VIII}C\textsubscript{a} | +                                                          | -                                                             |
| S\textsubscript{VIII}C\textsubscript{a} | +                                                          | -                                                             |
| S\textsubscript{VIII}C\textsubscript{a} | +                                                          | -                                                             |
| S\textsubscript{VIII}C\textsubscript{a} | +                                                          | -                                                             |
to those of the C polysaccharides of the noncapsulated strains from which they were derived. It should be borne in mind, however, that serologic similarity of reactivity with a single antiserum does not imply chemical identity. As noted earlier, immunologic nonidentity can be demonstrated between the C and C₅ polysaccharides of a single pneumococcal strain if the appropriate antiserum is used (Fig. 4).

When the C and C₅ polysaccharides of pneumococcal transformants are examined with the antiserum to strain S₁₅, the results of such studies indicate that both the isolated C and C₅ polysaccharides of the transformant reflect the structural properties of the C polysaccharide of the recipient strain rather than those of the polysaccharides of the donor strain from which the DNA was obtained. Although this observation does not establish the chemical identity of the C and C₅ polysaccharides of the transformant or the identity of its C polysaccharide with that of the strain employed as the recipient in the transformation, it is consistent with the concept that transformation affects especially the capacity to produce a capsule of C₅ polysaccharide. The final significance of inferences such as these must await detailed chemical study of the structure of the several polysaccharides involved. It would appear from the immunologic data available, however, that the several differences among C and C₅ polysaccharides described will reside in the mucoprotein moiety of the molecules rather than in the teichoic acid portions thereof. All preparations of teichoic acids from pneumococcal C and C₅ polysaccharides examined have proved to be immunologically identical.

Mutation affecting the structure of cell wall polysaccharides has been observed previously. McCarty and Lancefield (15), and McCarty (16), have described variations in the chemical structure of the C polysaccharide of Group A streptococci which were related to alterations in the carbohydrate constituents of the molecule. Data from the immunologic study of the C and C₅ polysaccharides of pneumococcus suggest that, in this organism, variations in the serologic reactivity of these compounds are more likely to result from differences residing in the mucoprotein portion of the molecule or in the region of its attachment to the teichoic acid moiety.

**SUMMARY**

Methods are described for the separation of the C or cell wall polysaccharide from the C₅ or soluble C-like capsular polysaccharide of C₅ pneumococcal strains. Immunologic analysis has shown that both the C and C₅ polysaccharides of a variety of pneumococcal strains are heterogeneous and that the dissimilarities appear to reside in the mucoprotein portion of the molecule or in the region of its attachment to the teichoic acid moiety of the molecule rather than in the teichoic acid fraction. Differences of the type described have been observed in the C polysaccharides of wild-type capsulated strains of several types, in those of independently isolated noncapsulated
variants derived from a single strain of a given capsular type, and in the C
and C₆ polysaccharides of spontaneous mutant or transformed strains of pneu-
mococci producing capsules of C₆ polysaccharide.

BIBLIOGRAPHY
1. Bornstein, D. L., G. Schiffman, H. P. Bernheimer, and R. Austrian. 1968. Cap-
sulation of pneumococcus with soluble C-like (C₆) polysaccharide. I. Biological
and genetic properties of C₆ pneumococcal strains. J. Exp. Med. 128:1385.
2. Tillett, W. S., and T. Francis, Jr. 1930. Serological reactions in pneumonia with a
non-protein somatic fraction of pneumococcus. J. Exp. Med. 52:561.
3. Tillett, W. S., W. F. Goebel, and O. T. Avery. 1930. Chemical and immunological
properties of a species-specific carbohydrate of pneumococci. J. Exp. Med. 52:
895.
4. Liu, T-Y, and E. C. Gotschlich. 1963. The chemical composition of pneumococcal
C polysaccharide. J. Biol. Chem. 238:1928.
5. Liu, T-Y, and E. C. Gotschlich. 1967. Muramic acid phosphate as a component
of the mucoprotein of gram-positive bacteria. J. Biol. Chem. 242:471.
6. Gotschlich, E. C., and T-Y. Liu. 1967. Structural and immunological studies on
the pneumococcal C polysaccharide. J. Biol. Chem. 242:463.
7. Brundish, D. E., and J. Baddiley. 1968. Pneumococcal C-substance, a ribitol
tetrahydrofolic acid containing choline phosphate. Biochem. J. 110:573.
8. Tomasz, A. 1967. Choline in the cell wall of a bacterium: novel type of polymer-
linked choline in pneumococcus. Science (Washington). 157:694.
9. Mosser, J. L., and A. Tomasz. 1970. Choline containing teichoic acid as a struc-
tural component of pneumococcal cell wall and its role in sensitivity to lysis by
an autolytic enzyme. J. Biol. Chem. 245:287.
10. Guysen, J. M. 1968. Use of bacteriolytic enzymes in determination of wall struc-
ture and their role in cell metabolism. Bacteriol. Rev. 32:425.
11. Schiffman, G. 1966. Immunological methods for characterizing polysaccharides.
Methods Enzymol. 8:79.
12. MacLeod, C. M., and M. R. Krauss. 1947. Stepwise intratype transformation of
pneumococcus from R to S by way of a variant intermediate in capsular poly-
saccharide production. J. Exp. Med. 86:439.
13. Bernheimer, H. P., I. E. Wermundsen, and R. Austrian. 1967. Quantitative differ-
ces in the behavior of pneumococcal deoxyribonucleic acids transforming to
the same capsular type. J. Bacteriol. 93:320.
14. Koenig, V. L., and J. D. Perrings. 1955. Sedimentation and viscosity studies on
the capsular and somatic polysaccharides of pneumococcus type III. J. Biophys.
Biochem. Cytol. 1:93.
15. McCarty, M., and R. C. Lancefield. 1955. Variation in the group-specific carbo-
hydrate of group A streptococci. I. Immunochemical studies on the carbohydrates
of variant strains. J. Exp. Med. 102:11.
16. McCarty, M. 1956. Variation in the group-specific carbohydrate of group A streptococci. II. Studies on the chemical basis for serological specificity of the carbohydrates. J. Exp. Med. 104:629.