The Synthesis of Type III Pneumococcal Capsular Polysaccharide from Uridine Nucleotides by a Cell-free Extract of Diplococcus pneumoniae Type III*†

Evelin E. B. Smith, George T. Mills, Harriet P. Bernheimer, and Robert Austrian

From the Department of Medicine, State University of New York College of Medicine at New York City, Brooklyn, New York

(Received for publication, February 1, 1960)

In 1953, Bernheimer (1) showed that suspensions of resting Type III pneumococci could synthesize capsular polysaccharide from glucose or from certain other monosaccharides in a medium containing magnesium, potassium, and phosphate ions. Although a considerable amount of polysaccharide was synthesized from glucose, little or none was formed when glucose was replaced by sodium glucuronate or by sodium cellobiuronate. More recently it has been shown that the glucuronic acid and acetylgalactosamine residues of the hyaluronide acid synthesized by Group A β-hemolytic streptococci are derived from glucose without cleavage of the glucose molecule (2) and that uridine diphosphoglucuronic acid and uridine diphospho-N-acetylgalactosamine are the immediate precursors of hyaluronic acid (3). These observations and those concerning the role of uridine pyrophosphoglycosyl compounds in the synthesis of chitin (4), cellulose (5), glycogen (6), and β-1,3-glucan (7) make it probable that the donation of glycosyl groups from uridine pyrophosphoglycosyl compounds represents a general mechanism for the biosynthesis of polysaccharides.

Previous investigations of the pneumococcus have revealed the presence of a number of uridine pyrophosphoglycosyl compounds in various types of this organism (8-10) and have shown that those uridine compounds are probably concerned in the synthesis of their polysaccharides. It has been found that, when the capsular polysaccharide of a particular type of pneumococcus contains uronic acid, the uridine pyrophospho-compound of that uronic acid is formed by cells of the pneumococcal type in question. More recent genetic and biochemical studies have shown that the synthesis of certain types of pneumococcal capsular polysaccharide is dependent upon an intact pathway for the biosynthesis of polysaccharides. Incorporation of radioactivity and net synthesis of polysaccharide were achieved with a partially fractionated, cell-free preparation from Type III pneumococcus.

**EXPERIMENTAL PROCEDURE**

The organism used was Diplococcus pneumoniae Type III (A60). The pneumococcal cells were grown in Difco brain-heart infusion medium (3.7% weight per volume; pH 7.4) containing 0.1% neopeptone. To reduce the large amount of preformed capsular polysaccharide which would otherwise have been present in the enzyme extracts, the cells were grown in the presence of the enzyme catalyzing the hydrolysis of Type III capsular polysaccharide. This enzyme was prepared by the method of Dubos (12) with the following modification: the autolysate of the organisms was centrifuged at high speed, the enzyme precipitated from the supernatant solution with NH₄SO₄ at half-saturation, collected by centrifugation at 16,000 x g for 30 minutes at 0°, dissolved in water, dialyzed overnight at 4° against distilled water, and sterilized by filtration. The sterile enzyme was added to a final concentration of 0.5 unit per ml of culture medium at the time of inoculation of the culture. One unit of enzyme is that amount which will catalyze the synthesis of 1 mg of polysaccharide in 1 ml of solution in 18 hours at 37°. After incubation of the culture overnight, glucose was added to a concentration of 1% (weight per volume) and the lactic acid formed on subsequent incubation was neutralized with 3 N NaOH.

All cultures were incubated at 30.5°. The encapsulated organisms were collected by centrifugation at 10,000 x g for 15 minutes at 0° after 70% of the added glucose had been utilized. The harvested cells were subjected to the procedure of disintegration and fractionation described by Smith et al. (13). Potassium thioglycollate (0.01 M) was present at all stages of fractionation (14) and the final enzyme extract used was that fraction eluted from the Celite filter cake between 3.0 M (NH₄)₂SO₄ and 1.0 M (NH₄)₂SO₄ as described previously (14). Glucose 1-phosphate uniformly labeled with C⁴ was prepared.
from C¹⁴-labeled starch (Radiochemical Centre, Amersham, England) by the method of McCready and Hassid (15) with rabbit muscle phosphorylase (16).

UDP-glucose labeled with C¹⁴ in the glucose moiety was prepared by the method of Munch-Petersen et al. (17) with C¹⁴-labeled glucose 1-phosphate, UTP (Sigma Chemical Company), the uridylic transferase preparation of Munch-Petersen (18), and inorganic pyrophosphatase as prepared by Heppel and Hilmo (19). The UDP-glucose was adsorbed on Norit, eluted with 50% (volume per volume) ethanol (20), and separated by paper chromatography in the neutral ethanol-ammonium acetate solvent of Paladini and Leloir (21). Before use, the UDP-glucose was extracted from paper by descending elution with 0.1 M Tris buffer, pH 9.1. The preparation was entirely free from UDP-galactose as determined by spectrophotometric assay (22).

The specific activity of the UDP-glucose was 1.5 × 10⁵ c.p.m. per umole.

UDP-glucuronic acid labeled with C¹⁴ in the glucuronic acid portion of the molecule was prepared from C¹⁴-labeled UDP-glucose with the dehydrogenase preparation described by Strohminger et al. (23). This enzymic conversion of UDP-glucose to UDP-glucuronic acid was coupled with the DPN*-regenerating system described by Mills et al. (24). The DPN was removed from the reaction mixture by passage through a column of Dowex 50-H⁺ resin, the nucleotides adsorbed on Norit, eluted with 50% (volume per volume) ethanol (20), and the UDP-glucuronic acid so obtained was eluted from paper with 0.1 M Tris buffer, pH 9.1, and its specific activity was the same as that of the UDP-glucose.

The radioactivity of the samples was determined at infinite thinness in a microthin window gas flow counter operated in the proportional region. Radioactivity on chromatograms was located with an automatic windowless gas flow paper chromatogram scanner used in the Geiger region (Atomic Accessories Inc.).

Type III capsular polysaccharide was estimated photometrically by measurement of the turbidity of the specific antiserum-antibody precipitate formed in the presence of excess antibody (1). The uronic acid content of the polysaccharide was estimated by the carbazole method (25).

Chromatography of sugars was carried out on Whatman No. 1 paper in 2-butanol-acetic acid-acetone-water (26), n-butanol-ethanol-ammonium-water (27), n-butanol-pyridine-water (28), ethanol-ammonium acetate, pH 7.5 (8, 21), and in a solvent consisting of 3 parts of the neutral ethanol-ammonium acetate solvent (21) and 2 parts of paraaldehyde. Ascending chromatography in this latter solvent gave a good separation of glucuronic acid ($R_f = 0.11$), glucose ($R_f = 0.32$), and glucuronolactone ($R_f = 0.43$). The p-anisidine reagent of Hough et al. (29) was used for localizing sugar spots and the molybdate reagent (30) for localizing sugar phosphates.

Protein concentrations were determined spectrophotometrically by the method of Warburg and Christian (31).

The Type III capsular polysaccharide from each experiment was isolated in the following manner. After the specified incubation period, protein was removed from the enzyme digest by precipitation with perchloric acid added to a final concentration of 0.1 N, followed by centrifugation. Norit was added to the supernatant solution in an amount calculated to adsorb the nucleotides completely. After adsorption for 10 minutes at 0°C, the Norit was separated by centrifugation and the nucleotides were recovered for subsequent chromatography by elution with 50% (volume per volume) ethanol. The supernatant solution was adjusted to pH 7.0 with 3 N KOH, chilled at 0°C for 1 hour, and the potassium perchlorate formed removed by centrifugation. To the solution, substantially free of perchlorate, were added 3 volumes of 95% ethanol together with 1 drop of glacial acetic acid and 2 drops of sodium acetate to facilitate precipitation of the polysaccharide. The preparation was allowed to stand overnight at 0°C, the polysaccharide collected by centrifugation and washed with 95% ethanol, with ether, and dried under reduced pressure. The supernatant solution was retained.

The dried polysaccharide was suspended in 2 ml of distilled water, 0.5 N KOH added to pH 7.0 to effect complete solution, and the preparation dialyzed against several changes of distilled water at 0°C for 2 days. The polysaccharide was precipitated and dried by the method described previously. This procedure was repeated twice more and, after the final dialysis, the material was dried under reduced pressure and dissolved in a standard volume of distilled water for analysis. During the last two manipulations, the nondialyzable radioactivity remained constant.

**EXPERIMENTAL**

**Incorporation of C¹⁴-UDP-Glucose and C¹⁴-UDP-Glucuronic acid into Type III Capsular Polysaccharide**—Numerous preliminary experiments showed that glucose and glucuronic acid from C¹⁴-labeled UDP-glucose and C¹⁴-labeled UDP-glucuronic acid could be incorporated into Type III capsular polysaccharide in the presence of the pneumococcal extracts described previously. The results reported here represent a quantitative analysis of these reactions. In order to study the distribution of labeling in the polysaccharide, DPN⁺ was omitted from the reaction mixtures to eliminate the conversion of UDP-glucose to UDP-glucuronic acid by the UDP-glucose dehydrogenase present in such extracts.

The results presented in Table I show the incorporation of

**Table I**

<table>
<thead>
<tr>
<th>Substrate used</th>
<th>Incubation time</th>
<th>Amount of Type III polysaccharide by precipitin reaction (μg)</th>
<th>Radioactivity of Type III polysaccharide (c.p.m./mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP-glucose</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UDP-galactose</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UDP-glucose</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UDP-galactose</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The enzyme extract for the following experiments was obtained from an 800-ml culture of Type III pneumococcus grown in the presence of capsular depolymerase in the manner described. The volume of extract was 4 ml and the protein concentration 11.8 mg per ml. Each reaction contained 12 ml of 0.1 M Tris buffer, pH 0.1, containing 0.005 M MgSO₄, the amount of substrates specified, and 1 ml of the pneumococcal extract. Incubation was carried out at 25°C for the time shown.
hydrolysates of Type III pneumococcal capsular polysaccharides with ethanol-ammonium acetate solvent for 16 hours. The volume of the extract was 2.4 ml and the protein concentration 8.3 mg per ml. Each reaction contained 12 ml of 0.1 M Tris buffer, pH 9.1, containing 0.005 M MgS04, the amounts of substrates specified, and 0.8 ml of the pneumococcal extract. Incubation was carried out at 25°C for 30 minutes.

The enzyme extract for the following experiments was obtained from a 300-ml culture of Type III pneumococcus grown in the presence of capsular depolymerase. The volume of the extract was 2.4 ml and the protein concentration 8.3 mg per ml. Each reaction contained 12 ml of 0.1 M Tris buffer, pH 9.1, containing 0.005 M MgS04, the amounts of substrates specified, and 0.8 ml of the pneumococcal extract. Incubation was carried out at 25°C for 30 minutes.

The several experiments show that hydrolysates of the Type III capsular polysaccharide contained labeled glucose (Experi-

![Table II]

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Substrate used</th>
<th>Amount of Type III polysaccharide by precipitin reaction</th>
<th>Radioactivity of Type III polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>C&quot;-UDP-glucose, UDP-glucuronic acid, C&quot;-glucose-1-P</td>
<td>µg</td>
<td>c.p.m./mg</td>
</tr>
<tr>
<td>1</td>
<td>2.5, 2.5, 2.5</td>
<td>96</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2.5, 2.5, 2.5</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2.5, 2.5, 2.5</td>
<td>90</td>
<td>0</td>
</tr>
</tbody>
</table>

![Table III]

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Incubation time (min)</th>
<th>Radioactivity added (c.p.m.)</th>
<th>Counts recovered from nucleotide fractions (c.p.m.)</th>
<th>Total counts in supernatant solvents from polysaccharide precipitation (c.p.m.)</th>
<th>Total counts in purified polysaccharide (c.p.m.)</th>
<th>Counts rejected during purification of polysaccharide (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>300,000</td>
<td>150,000</td>
<td>261,000</td>
<td>133,000</td>
<td>1,500</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>37,000</td>
<td>9,000</td>
<td>250,000</td>
<td>128,000</td>
<td>7,022</td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>150,000</td>
<td>20,800</td>
<td>128,000</td>
<td>27,200</td>
<td>7,056</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td>300,000</td>
<td>150,000</td>
<td>368,000</td>
<td>14,766</td>
<td>10,500</td>
</tr>
</tbody>
</table>

![Fig. 1]

Fig. 1. Location of radioactivity in chromatograms of the acid hydrolysates of Type III pneumococcal capsular polysaccharides from Experiments 2, 3, and 4 of Table I. Chromatograms were developed by ascending chromatography in the paraldehyde-ethanol-ammonium acetate solvent for 16 hours.

Lack of Incorporation of C14-Glucose and C14-Glucose-1-Phosphate into Type III Polysaccharide—It has been shown that pneumococcal extracts prepared by the Celite-(Na2SO4)4 fractionation technique are substantially free from phosphatases and pyrophosphatases which produce glucose and glucose 1-phosphate from UDP-glucose (13). It was considered essential, however, to determine whether or not free glucose or glucose 1-phosphate could act as glucose donors for the synthesis of Type III polysaccharide in the system employed. The results of these experiments are shown in Table II.

The data presented in Tables I and II show that the glucose and glucuronic acid incorporated into the Type III polysaccharide by the preparations used were derived only from the uridine pyrophosphoglucosyl compounds and that, in the case of glucose, the free sugar or its 1-phospho-derivative are not utilized directly.

Characterization of Labeled Polysaccharide

Precipitin Reaction—A preparation of Type III capsular polysaccharide obtained by use of 2 µmoles of C14-UDP-glucuronic acid and 2 µmoles of nonradioactive UDP-glucose as donor substrates and with a specific activity of 118,000 c.p.m. per mg was precipitated with rabbit anti-Type III capsular serum in the region of antibody excess to ensure maximum precipitation. The reaction was carried out with 11.8 µg of polysaccharide, the precipitate removed by centrifugation, washed twice with cold 0.9% (weight per volume) NaCl, and finally dissolved in 0.1 N NH4OH. The supernatant solutions and washings were combined and retained for measurement of radioactivity. The antigen-antibody complex in 0.1 N NH4OH was plated at infinite thinness and the total activity was found to be 1260 c.p.m. The radioactivity of the initial material was 1400 c.p.m., the difference in counts being located in the supernatant solution.

Hydrolysis of Labeled Polysaccharide—One-half of each of the samples of polysaccharide from Experiments 2, 3, and 4 listed in Table I was hydrolyzed individually with 1 ml of 0.6 N H2SO4 at 120°C for 16 hours in a sealed tube. Each hydrolysate was neutralized with an excess of solid BaCO3, the BaSO4 removed by centrifugation, washed twice with 1 ml of distilled water, and the combined supernatant solutions and washings from each hydrolysate dried under reduced pressure. The hydrolysates were chromatographed in the paraldehyde-ethanol-ammonium acetate solvent with glucuronic acid, glucose, and glucuronolactone as markers. The chromatograms were scanned for radioactivity with the automatic chromatogram scanner. The results are shown in Fig. 1.

Under the conditions of hydrolysis, the Type III polysaccharide was degraded to glucose and glucuronic acid. No cellobiuronic acid or higher oligosaccharides were found. Part of the glucuronic acid was converted to glucuronolactone and identified as such chromatographically.

The several experiments show that hydrolysates of the Type III polysaccharide contained labeled glucose (Experi-
ment 2), labeled glucuronic acid and glucuronolactone (Experiment 3), and labeled glucose, glucuronic acid, and glucuronolactone (Experiment 4), in accordance with the C\textsuperscript{14}-labeled substrates used.

Degradation of Labeled Polysaccharide by Enzyme Depolymerizing Type III Capsular Polysaccharide—The remaining 50% of the polysaccharide from Experiment 4 in Table I was dissolved in 1 ml of 0.01 m phosphate buffer, pH 7.0, containing 0.1% (weight per volume) NaCl. To this solution were added 15 units of the Type III capsular depolymerase, and the mixture was incubated at 37\degree C for 5 hours. At the end of this period, the material was dialyzed against several changes of distilled water for 24 hours at 3\degree C. The total counts in the polysaccharide before incubation with the depolymerase were 7350. After incubation and dialysis the total counts were reduced to a value of 986.

Fractional Distribution of Total Added Radioactivity—Table III shows the distribution of total radioactivity present in Experiments 1 through 4 of Table I. The values for radioactivity of the UDP-glucose and UDP-glucuronic acid were obtained by elution of the Norit-adsorbed nucleotides with 50% (volume per volume) ethanol followed by chromatography in the neutral ethanol-ammonium acetate solvent of Paladini and Leloir (21). In each reaction, the ultraviolet-absorbing bands corresponding to UDP-glucose and UDP-glucuronic acid were cut from the chromatograms and eluted quantitatively with distilled water by descending chromatography. Aliquots of the eluates were then plated and counted at infinite thinness. Scanning of the chromatograms with the automatic scanner before elution showed no areas of radioactivity other than those associated with the nucleotides under consideration.

It will be noted that a very considerable portion of the added radioactivity was located as material which was not adsorbed with Norit but was present in the supernatant solutions from the first polysaccharide precipitation. When the supernatant solutions were concentrated under reduced pressure, no Type III polysaccharide could be detected by the precipitin reaction. Chromatography of suitable aliquots of the concentrated materials in ethanol-ammonium acetate (21), 2-butanol-acetone-acetic acid water (26), \(\alpha\)-butanol-ethanol-ammonia-water (27), and \(\alpha\)-butanol-pyridine-water (28) showed that the radioactivity in each experiment was not associated with glucose, glucose 1-phosphate, glucuronic acid, glucuronic acid 1-phosphate, or a uridine nucleotide. The nature of this material is under investigation.

**DISCUSSION**

The role of UDP-glucose as a donor of glucose in the synthesis of various polysaccharides containing only glucose has been demonstrated by a number of investigators (5-7) and UDP-N-acetylglucosamine has been established as the precursor of chitin (4). In the synthesis of heteropolysaccharides such as hyaluronic acid (3), it has been shown that both of the constituent glycosyl components must be present initially as uridine pyrophosphoryl compounds.

Type III pneumococcal polysaccharide is a polymer of cello-biuronic acid (\(\beta-1,4\)-glucuronosido-glucose), and it seemed probable that UDP-glucose and UDP-glucuronic acid would act as glycosyl donors for its synthesis. Earlier work (11) had shown that synthesis of capsular polysaccharide by certain strains of pneumococci is dependent on an intact pathway of uridine pyrophosphoglycosyl metabolism, and that the lack of only one enzyme therein leads to a block in the synthesis of such polysaccharide.

In the present work, it is apparent that UDP-glucose and UDP-glucuronic acid act as glycosyl donors in the formation of Type III capsular polysaccharide by a cell-free system. When UDP-glucuronic acid is used as the donor of glucuronic acid, neither glucose nor glucose 1-phosphate will act as acceptors for the formation of polysaccharide. Although glucuronic acid and glucuronic acid 1-phosphate have not been examined as donors of glucuronic acid in the presence of UDP-glucose, UDP-glucuronic acid would appear to be the most likely donor of uronic acid to the system, as the initial formation of uronic acid in the pneumococcus occurs through the oxidation of UDP-glucose to UDP-glucuronic acid.

It is interesting to note that the incorporations of glucose and of glucuronic acid from C\textsuperscript{14}-UDP-glucose and from C\textsuperscript{14}-UDP-glucuronic acid, respectively, occur in a ratio of 1:1. When C\textsuperscript{14}-UDP-glucuronic acid is used as the limiting substrate, it is apparent from Table I that any increase in the concentration of UDP-glucose above equimolarity with UDP-glucuronic acid produces no further increase in the incorporation of isotope. When both substrates are labeled, the incorporation of isotope is doubled.

The pathway for the formation of Type III pneumococcal capsular polysaccharide may then be summarized as follows:

\[
\text{UDP + Glucose-1-P} \rightarrow \text{PP} \downarrow + \text{UDP-glucose} \rightarrow \text{UDP-glucuronic acid} \rightarrow \text{UDP-glucuronic acid 1-phosphate, glucuronic acid, or a uridine nucleotide. The nature of this material is under investigation.}
\]

Acid hydrolysis of the Type III polysaccharide to glucose and glucuronic acid shows that C\textsuperscript{14}-UDP-glucose contributes only to labeling of the glucose residues of the molecule and C\textsuperscript{14}-UDP-glucuronic acid to labeling of the glucuronic acid residues. When both labeled substrates are used simultaneously, both residues of the Type III polysaccharide are labeled.

The radioactive material which is not associated with Type III capsular polysaccharide, nucleotides, free sugars, or sugar phosphates, but which is freely dialyzable may consist of compounds intermediate in the synthesis of Type III polysaccharide.

Although the present data are insufficient to suggest a precise mechanism for the formation of Type III pneumococcal capsular polysaccharide, any proposed mechanism must take cognizance of the fact that, in the uridine nucleotides, the glycosyl groups

\[\text{1 A. Torriani, personal communication.}\]
are probably in the α form, whereas, in Type III polysaccharide, all linkages are of the β variety. Transfer of these glycosyl units, therefore, must involve an inversion at C-1, as in the formation of β-glucuronides and as in the synthesis of hyaluronic acid.

SUMMARY

An enzyme system catalyzing the synthesis of Type III pneumococcal capsular polysaccharide from uridine diphosphoglucose and uridine diphosphoglucuronic acid has been obtained by fractionation of disrupted Type III capsulated pneumococci with ammonium sulfate.

The synthesis of polysaccharide was demonstrated by the incorporation of C14-labeled precursors, and the radioactive polysaccharide was shown to be precipitable by Type III pneumococcal capsular antiserum. Hydrolysis of the polysaccharide followed by chromatography showed the specificity of uridine diphosphoglucose and uridine diphosphoglucuronic acid as donors of glucose and of glucuronic acid, respectively. Glucose and glucose 1-phosphate were not incorporated into the polysaccharide in the presence of uridine diphosphoglucuronic acid. Net synthesis of polysaccharide has been demonstrated.

REFERENCES

31. Warburg, O., and Christian, W., Biochem. Z., 310, 384 (1941).
The Synthesis of Type III Pneumococcal Capsular Polysaccharide from Uridine Nucleotides by a Cell-free Extract of *Diplococcus pneumoniae* Type III
Evelyn E. B. Smith, George T. Mills, Harriet P. Bernheimer and Robert Austrian


Access the most updated version of this article at [http://www.jbc.org/content/235/7/1876.citation](http://www.jbc.org/content/235/7/1876.citation)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/235/7/1876.citation.full.html#ref-list-1](http://www.jbc.org/content/235/7/1876.citation.full.html#ref-list-1)