The glycosidic linkages of the type 3 capsular polysaccharide of *Streptococcus pneumoniae* ([(3)-β-D-GlcUA-(1→4)-β-D-Glc-(1→)]₄) are formed by the membrane-associated type 3 synthase (Cps3S), which is capable of synthesizing polymer from UDP sugar precursors. Using membrane preparations of *S. pneumoniae* in an *in vitro* assay, we observed type 3 synthesize activity in the presence of either Mn²⁺ or Mg²⁺ with maximal levels seen with 10–20 mM Mn²⁺. High molecular weight polymer synthesized in the assay was composed of Glc and glucuronic acid and could be degraded to a low molecular weight product by a type 3-specific depolymerase from *Bacillus circulans*. Additionally, the polymer bound specifically to an affinity column made with a type 3 polysaccharide-specific monoclonal antibody. The polysaccharide was rapidly synthesized from smaller chains and remained associated with the enzyme-containing membrane fraction throughout its synthesis, indicating a processive mechanism of synthesis. Release of the polysaccharide was observed, however, when the level of one of the substrates became limiting. Finally, addition of sugars to the growing type 3 polysaccharide was shown to occur at the nonreducing end of the polysaccharide chain.

The capsular polysaccharides of *Streptococcus pneumoniae* are essential components in virulence that are necessary to resist host phagocytic mechanisms. So far, ninety different capsular polysaccharides have been identified (1). The polymers are usually composed of repeating oligosaccharide subunits containing several different monosaccharides and, in many cases, are branched structures (2). In *S. pneumoniae*, the genes involved in the biosynthesis of any one capsular polysaccharide are contained within a single locus on the bacterial chromosome and are termed “type-specific.” Only two type 3-specific genes, *cps3D* and *cps3S*, which encode a UDP-Glc dehydrogenase and the type 3 synthase, respectively, are essential for synthesis of the type 3 polysaccharide (3). A linear structure composed of [(1→3)-β-D-GlcUA-(1→4)-β-D-Glc-(1→)]₄ repeating units (4). Two other genes, *cps3U*, encoding a Glc-1-P uridylyltransferase, and *cps3M*, encoding a homologue of phosphoglucomutases, are present in the type 3-specific locus. These sequences are not essential for type 3 capsule synthesis, however, because the functions they encode are duplicated by other genes in the pneumococcal chromosome (3, 5, 6). Flank-

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ride was synthesized in the *E. coli* strain, further confirming the assignment of cps3S as the type 3 synthesize. Because production of high levels of synthesize from cloned products has not been possible, we chose to use membranes isolated from *S. pneumoniae* as a source of synthesize activity for further biochemical studies. Using an assay that measures the incorporation of radiolabeled sugars into polysaccharide, we have characterized the mechanism of type 3 polysaccharide synthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mutanolysin, Type VIII-A β-glucuronidase from *E. coli*, β-glucosidase from *Caldococcus saccharolyticum*, Sepharose 2B, UDP-Glc, and UDP-GlcUA were obtained from Sigma. UDP-[14C]Glc (257 mCi/mmol) and UDP-[14C]GlcUA (287 mCi/mmol) were obtained from Andotek. Todd Hewitt Broth and yeast extract were from Difco. Type 1 and type 3 polysaccharides were from the American Type Culture Collection. Scinti Verse I was obtained from Fisher.

**Bacterial Strains, Growth Conditions, and Enzyme Preparation**—Membranes containing type 3 synthesize were isolated from *S. pneumoniae* based on a previously described procedure (25). The encapsulated type 3 strain WU2 and its nonencapsulated isogenic derivative JD908, which contains an insertion mutation in cps3S, have been described (7, 26). A 4-liter culture was grown at 37 °C in Todd Hewitt Broth supplemented with 0.5% yeast extract (THY) to a density of 3 × 10⁷ colony forming units/ml. The cells were collected by centrifugation at 10,000 × g for 20 min. The pellets were washed once in 2 liters of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 5.4 mM Na₂HPO₄·7H₂O, 1.8 mM KH₂PO₄, pH 7.4) and then suspended in 200 ml of protoplast buffer (20% sucrose, 5 mM Tris-HCl, pH 7.4, and 2.5 mM MgSO₄). Mutanolysin was added to a final concentration of 20 units/ml, and the mixture was incubated at room temperature overnight. Protoplast formation was checked by examining the cells using a phase contrast light microscope. The protoplasts were sedimented by centrifugation at 25,000 × g for 20 min, washed once in 200 ml of protoplast buffer, and then osmotically lysed by suspension in 100 ml of sterile water containing 10 mM EDTA. Lysis was confirmed by light microscopy. The membranes were collected by centrifugation at 100,000 × g for 30 min, washed three times in 400 ml of 100 mM Hepes (pH 8.0) buffer containing 10 mM sodium thiglycolate, and suspended in 15 ml of the same buffer. The final membrane preparation was adjusted to 3 mg protein/ml in 100 mM Hepes (pH 8.0) buffer containing 10 mM sodium thiglycolate and stored at −20 °C.

**Assay of Synthesize Activity**—Type 3 synthesize activity was determined by the incorporation of 14C label from either UDP-[14C]Glc or UDP-[14C]GlcUA into polysaccharide. Synthesize assays were performed in a 100-μl reaction that contained 100 mM Hepes (pH 8), 10 mM sodium thiglycolate, 10 mM MnCl₂, UDP-Glc, UDP-GlcUA, and membranes isolated as described above. The concentrations of UDP-Glc, UDP-GlcUA, and membrane protein were indicated elsewhere in the text and in the figure legends. The reaction mixtures were incubated at 32 °C for 30 min. The reaction was terminated by addition of 10 μl of 12.5 M ammonium acetate, pH 7.0, or 1-propylthyl acetate/water 7:1 (v/v). The reaction components were separated by ascending paper chromatography on 3 MM Whatman paper in ethanol (95%)/1 mM ammonium acetate (pH 7.0), 7:3 (v/v) or 1-propylthyl acetate/water 7:1:2 (v/v/v). The chromatograms were cut into 1-cm strips, and radioactivity was measured by liquid scintillation counting. Monosaccharide standards were visualized using p-anisidine-phthale (27).

**Sepharose 2B Column Chromatography**—Gel filtration of polysaccharide samples was performed on a 1.4 × 37 cm Sepharose 2B column equilibrated with 0.2 mM NaCl and 5 mM Tris acetate (pH 7.4). SDS was added to the sample to a final concentration of 2% (v/v) prior to application to the column. The column flow rate was 16 ml/h, and 1-ml fractions were collected. The void and total volumes were determined using 0.788-μm diameter latex beads (Sigma) and [14C]Glc, respectively. Polysaccharide that eluted between 18 and 30 ml on Sepharose 2B is referred to as high molecular weight.

**Paper Chromatography**—The components of the hydrolysates were separated by ascending paper chromatography in ethyl acetate/glacial acetic acid/water 12:1:2 (v/v/v). The chromatograms were cut into 1-cm strips, and radioactivity was measured by liquid scintillation counting. Monosaccharide standards were visualized using p-anisidine-phthalate (27).

**Chromatography on a Monoclonal Antibody Sepharose Column**—Type 3 polysaccharide-specific monoclonal antibody 16.3 (28) was coupled to Sepharose 2B as described (29). The antibody-conjugated beads (0.5–1 ml) were packed into 2-ml columns and washed with 10 column volumes of 5 mM Tris acetate buffer (pH 7.4) containing 0.2 mM NaCl. Labeled polysaccharide was applied to the column, and the column was washed with the same buffer. Three 1.5-ml samples were collected, and 100 μl of each fraction was chromatographed on paper as described for the assay of synthesize activity. The percentage of radioactive polysaccharide that bound to the column was determined as 100 × [total counts applied − recovered counts]/total counts.

**Purification of Polysaccharide on a Monoclonal Antibody Sepharose Column**—Labeled polysaccharide that bound to the antibody affinity column described above was eluted with 0.05 M glycine (pH 2.5) buffer containing 0.1% Triton X-100 and 0.15 mM NaCl. Seven 1-ml fractions were collected in tubes containing 100 μl of Tris-HCl (pH 9.0). A portion (100 μl) of each fraction was chromatographed on paper as described for the synthase assay, and column fractions that contained labeled polysaccharide were pooled.

**Preparation of Type 3 Polysaccharide-specific Depolymerase**—The type 3 polysaccharide-specific depolymerase was isolated from *Bacillus circulans* (ATCC 14175) using a modification of a previously described procedure (30). Briefly, a 5-ml culture of *B. circulans* was grown to mid
Synthase reactions were performed as described under “Experimental Procedures” with membranes containing 15 µg of total protein. The concentration of either UDP-Glc or UDP-GlcUA was held at a concentration of 100 µM, whereas the concentration of the other substrate was varied. The data are the results of duplicate samples.

| Metal ion
| Substrate | \( K_m \) µM |
|----------|-----------|---------|
| Mn\(^{2+}\) | UDP-GlcUA | 8.5 ± 1.2 |
| Mn\(^{2+}\) | UDP-Glc | 31.9 ± 0.7 |
| Mg\(^{2+}\) | UDP-GlcUA | 31.3 ± 2.0 |
| Mg\(^{2+}\) | UDP-Glc | 64.9 ± 7.0 |

* The concentration of metal ion in each reaction was 10 mM.

** The concentration of substrate ranged from 0 to 200 µM.

\( K_m \) values were determined using Lineweaver-Burk plots.

![FIG. 2. Acid hydrolysis of [\(^{14}\)C]Glc- and [\(^{14}\)C]GlcUA-labeled polysaccharides. Polysaccharide labeled with [\(^{14}\)C]Glc was prepared as described in the legend to Fig. 1 except that a 2-ml reaction mixture containing 3 mg of protein and 10 mM MnCl\(_2\) was used. The reaction was incubated for 10 min at 32 °C. UDP-Glc and UDP-GlcUA were added to a final concentration of 400 µM each, and incubation was continued for an additional 2 h. Polysaccharide was separated from unincorporated UDP sugars by Sepharose 2B column chromatography, and fractions 18–30 were pooled and concentrated 6-fold on Amicon YM10 ultrafiltration membranes. [\(^{14}\)C]GlcUA-labeled polysaccharide was prepared in the same manner except that 14 µM UDP-[\(^{14}\)C]GlcUA (287 mCi/mmol) and 100 µM UDP-Glc were used in the initial reaction. 100-µl aliquots of [\(^{14}\)C]Glc-labeled polysaccharide (5000 cpm) (A) and [\(^{14}\)C]GlcUA-labeled polysaccharide (9000 cpm) (B) were hydrolyzed with 4 (●), 1 (○), or 0 N HCl (□) at 100 °C for 2 h. The hydrolysis products were separated by paper chromatography in ethanol (95%)/1 M ammonium acetate (pH 7), 7.3, and the radioactivity present in 1-cm strips was determined by liquid scintillation counting and expressed as a percentage of the total cpm. C, a 400-µl sample of the [\(^{14}\)C]Glc-labeled polymer was hydrolyzed in 1 N HCl and chromatographed as described for A. The radioactivity present between 6 and 11 cm on the chromatogram was eluted in water. The eluted product was treated with 3475 units of \( \beta \)-glucuronidase for 2 days at 37 °C. An untreated sample (●) and the treated sample (○) were chromatographed as above. The locations of standard Glc, GlcUA, and GlcUA-lactone are indicated on the graph.

UDP-[\(^{14}\)C]Glc into polysaccharide. Membranes from the parent strain incorporated 15,301 cpm, whereas membranes from the mutant strain, under identical assay conditions, incorporated 31 cpm (data not shown). These data confirmed that CpsS5 is the critical enzyme responsible for the activity measured in this assay and that incorporation of label does not occur in the absence of type 3 polysaccharide synthesis. The activity of the parent type 3 synthase was characterized by measuring the incorporation of [\(^{14}\)C]Glc from UDP-[\(^{14}\)C]Glc and from UDP-[\(^{14}\)C]GlcUA into capsular polysaccharide. Formation of [\(^{14}\)C]Glc-labeled product using membranes isolated from the parent strain was linear with time for up to 30 min and was proportional to protein concentration. Incorporation of [\(^{14}\)C]Glc or [\(^{14}\)C]GlcUA in the absence of the other substrate was <5%
that observed when substrates were at equal concentrations. The synthase was active in the presence of Mn$^{2+}$ and Mg$^{2+}$, with the highest level of activity observed with 5–20 mM Mn$^{2+}$ (Fig. 1). The optimal pH for the synthase was between 8 and 8.5 in a reaction mixture containing either 10 mM Mn$^{2+}$ or 10 mM Mg$^{2+}$ (data not shown). The apparent $K_m$ values for both UDP-Glc and UDP-GlcUA were lower in the presence of Mn$^{2+}$ than with Mg$^{2+}$ (Table I).

Characterization of the Polysaccharide Product—Polymer synthesized in a 2-h incubation with a high concentration (400 $\mu$m) of both UDP sugars eluted in the excluded volume of a Sepharose 2B column (data not shown). Polysaccharide labeled with UDP-[14C]Glc was completely hydrolyzed by 4 N HCl to [14C]Glc in 2 h at 100 °C (Fig. 2A). Polysaccharide labeled with UDP-[14C]GlcUA was completely hydrolyzed to [14C]GlcUA and GlcUA-lactone under the same conditions (Fig. 2B). The presence of Glc and GlcUA in the polymer was confirmed by chromatography in a second solvent containing 1-propanol/ethyl acetate/water, 7:1:2 (data not shown). Hydrolysis in 1 N HCl of both [14C]Glc- and [14C]GlcUA-labeled polysaccharide resulted in the liberation of both monosaccharides, as well as a product that migrated between 6 and 11 cm from the origin, suggestive of a disaccharide (Fig. 2, A and B). Mild hydrolysis of polysaccharides containing GlcUA readily yields aldobiouronic acids because of the strong resistance of the uronidic linkage to hydrolysis with acids (31). When the putative disaccharide was digested with exo-$\beta$-glucuronidase, 75% of the radioactivity was liberated as free glucose (Fig. 2C). These results confirmed the presence of a $\beta$-glucuronidic linkage and are consistent with the slower migrating product being the disaccharide GlcUA-$\beta$-Glc.

Additional evidence for the identity of the polysaccharide was obtained by digestion of the high molecular weight polysaccharide with type 3 polysaccharide-specific depolymerase from B. circulans. This enzyme is highly specific for type 3 polysaccharide and has been shown to hydrolyze the 6,1,4 linkages of this polymer to yield oligosaccharides with an average length of a tetrasaccharide (30). Following treatment with the depolymerase for 48 h, the high molecular weight [14C]Glc-labeled polysaccharide was degraded to a lower molecular weight product as determined by chromatography on Sepharose 2B (Fig. 3). Identical results were obtained using [14C]GlcUA-labeled polysaccharide (data not shown).

A monoclonal antibody specific for type 3 polysaccharide was coupled to Sepharose 2B and shown to specifically bind the high molecular weight polymer. Columns packed with 0.5 ml of

![Figure 3](image)

**FIG. 3. Degradation of high molecular weight product by a type 3 polysaccharide-specific depolymerase.** High molecular weight [14C]Glc-labeled polysaccharide (8400 cpm), synthesized as in Fig. 2, was incubated in 20 mM MES buffer (pH 6.0) with 100 $\mu$L of the depolymerase preparation for 48 h at 37 °C. Untreated (●) and treated (○) samples were chromatographed on Sepharose 2B. The amount of radioactivity present in the even-numbered fractions was determined. A background of 20 cpm was subtracted from each fraction.

The antibody-conjugated beads bound 99% of both the [14C]Glc-labeled and the [14C]GlcUA-labeled high molecular weight products (data shown in Fig. 4 for the Glc-labeled product). The addition of unlabeled type 3 polysaccharide added along with the labeled polysaccharide resulted in a concentration-depend-ent inhibition of binding of the [14C]Glc-labeled polymer (Fig. 4). No inhibition was observed when unlabeled type 1 polysaccharide was added. All of these results confirmed that the isolated product had the expected properties of authentic type 3 polysaccharide.

*Polysaccharide Chain Elongation and Release—*Pulse-chase experiments with the type 3 synthase showed that low molecular weight polysaccharide labeled in a 3-min pulse could be chased after 20 min into high molecular weight chains that eluted near the excluded volume of a Sepharose 2B column (Fig. 5). A polysaccharide of intermediate length was observed...
after a 5-min chase. These results are suggestive of a processive biosynthetic mechanism, whereby the elongating polysaccharide chain remains associated with the enzyme-membrane complex.

To further evaluate the mechanism of the synthase reaction, we investigated the association of polysaccharide with the enzyme complex during the course of synthesis. Reaction mixtures containing 100 μM UDP-Glc and 200 μM UDP-GlcUA were sampled during a 60-min incubation and sedimented by centrifugation to separate the soluble polysaccharide from the membrane-associated polysaccharide. There was a steady increase in the incorporation of [14C]Glc into membrane-associated polysaccharide during the first 30 min of incubation, whereas only a small fraction (12.4%) was incorporated into soluble polysaccharide (Fig. 6A). By 60 min, the fraction of radioactivity found as soluble polysaccharide had increased to 35%, whereas the amount found as membrane-associated polysaccharide had begun to decrease, suggesting that the membrane-associated product might be a precursor to the soluble polymer.

At lower concentrations of UDP-Glc and UDP-GlcUA (2 and 20 μM, respectively), the incorporation of Glc into membrane-associated polysaccharide reached a maximum by 5 min and then declined rapidly so that by 30 min approximately equal amounts of label were found in the soluble and membrane-associated fractions. Again, Glc was initially incorporated into membrane-associated polymer, which was subsequently released as a soluble form. The soluble and membrane-associated polysaccharides obtained under a variety of conditions were analyzed by Sepharose 2B chromatography. Similar size profiles were observed for both fractions, indicating that release of the polysaccharide from the membrane was independent of the size of the polymer (data not shown).

Although size did not appear to be a factor in polysaccharide release, the increase in the soluble form of polysaccharide in Fig. 6 coincided with the depletion of UDP-Glc. To further examine the effect of substrate concentration on polysaccharide release, a series of reactions were performed as in Fig. 6B above, except that additional substrate was added 5 min after initiating the reaction. As shown in Fig. 7, the simultaneous

**Fig. 5.** Pulse-chase analysis of type 3 polysaccharide synthesis. Membranes containing 300 μg of total protein were incubated in 100 mM Hepes (pH 8), 10 mM sodium thioglycollate, 10 mM MnCl₂, 2 μM UDP-Glc (257 mCi/mmol), and 20 μM UDP-GlcUA at 32 °C in a 200-μl reaction. After 3 min, a 30-μl sample was removed, UDP-Glc and UDP-GlcUA (400 μM each) were added, and incubation was continued. 30-μl samples were then removed after 5 and 20 min of chase. The samples were brought to 500 μl in 100 mM Hepes buffer (pH 8.0), 10 mM sodium thioglycollate, and 2% SDS. The pulse (O), 5 min chase (○), and 20 min chase (□) samples were then applied to a Sepharose 2B column, and the amount of radioactivity present in the even numbered fractions was determined. The values for each fraction are reported as the percentages of the total radioactivity present in the sample. The void and total volumes are indicated as V₁ and Vₜ, respectively.

**Fig. 6.** Formation of soluble and membrane-associated polysaccharide. Reactions containing 100 μM UDP-Glc (5 mCi/mmol) and 200 μM UDP-GlcUA (A) or 2 μM UDP-Glc (257 mCi/mmol) and 20 μM UDP-GlcUA (B) were prepared as described under "Experimental Procedures." Samples (50 μl) were taken after 0, 5, 10, 30, and 60 min of incubation, and the soluble and membrane-associated polysaccharides were separated as described under "Experimental Procedures." The amount of radioactivity present as soluble polysaccharide (●), membrane-associated polysaccharide (○), and unincorporated UDP-[14C]Glc (□) was determined after ascending paper chromatography in ethanol/1 M ammonium acetate (pH 7.0), 7.3 as a percentage of the total radioactivity.
addition of both substrates prolonged the association of the polysaccharide with the membrane (Fig. 7A), whereas the separate addition of either UDP-Glc or UDP-GlcUA markedly stimulated the appearance of soluble polymer (Fig. 7, B and C). These data suggest that polysaccharide release may be enhanced when one substrate is limiting. Polysaccharide release was not due to the generation of free UDP during polysaccharide synthesis, because the addition of 1 mM UDP did not stimulate release (Fig. 7D).

**Direction of Chain Growth**—To determine whether sugar addition occurs at the reducing or nonreducing end of the type 3 polysaccharide, polymer was labeled with [14C]Glc either uniformly (preparation A) or on the terminus of the growing end (preparation B). Both methods of preparing polysaccharide resulted in a radioactive product that eluted near the void volume of a Sepharose 2B column as well as a peak of radioactivity that corresponded to UDP-Glc (Fig. 8A). The polysaccharide product obtained by both of the methods was degraded by the type 3 polysaccharide-specific depolymerase (Fig. 8B). Digestion with the depolymerase confirmed that both labeled products were indeed type 3 polysaccharide. Digestion of the terminally labeled polysaccharide with an exo-β-glucosidase for 24 h liberated 72.8% of the counts as [14C]Glc, whereas uniformly labeled polysaccharide yielded undetectable levels of [14C]Glc after digestion with exo-β-glucosidase (Fig. 8C). The degradation of the terminally labeled polysaccharide with exo-β-glucosidase was time-dependent (Fig. 8D). Because exo-β-glucosidase removes only the terminal Glc residues from the nonreducing end of the polysaccharide, these data demonstrate that type 3 polysaccharide growth occurs from the nonreducing end.

Terminally labeled polysaccharide was also made by incubating membranes with UDP-[14C]Glc in the absence of UDP-GlcUA and without any initial elongation with unlabeled substrates (preparation C). This procedure yielded labeled polysaccharide that bound to an affinity column made with monoclonal antibodies to type 3 polysaccharide. Exo-β-glucosidase treatment of polysaccharide eluted off the affinity column released 75.4% of the counts as [14C]Glc (data not shown). This result further supports growth of type 3 polysaccharide from the nonreducing end. Furthermore, the incorporation of Glc in the absence of UDP-GlcUA into a product that bound to a type 3 polysaccharide-specific antibody, supports the presence of preformed type 3 polysaccharide acceptor in *S. pneumoniae* membranes.

**DISCUSSION**

The type 3 synthase of *S. pneumoniae* belongs to a family of processive β-glycosyltransferases whose members include the hyaluronic acid synthases from *S. pyogenes* and *X. laevis*, the Nod factor synthase from *Rhizobium sp.*, chitin synthases from *C. albicans* and *S. cerevisiae*, RfbB-0:54 from *Salmonella enterica*, and the cellulose synthases from bacteria and plants. All of these enzymes catalyze the formation of all the glycosidic linkages of their respective polysaccharides and are thought to function via a similar mechanism (22, 23). The β-glycosidic linkages of the polysaccharides synthesized by these enzymes are derived from UDP sugar precursors, which are linked in the α configuration. By analogy with the extensively characterized glycosyl hydrolase systems, these enzymes would be expected to provide for an inverting mechanism during polymer formation (32). A model has recently been proposed for a mechanism of polymerization for the family of processive β-glycosyltransferases that would allow for the simultaneous or consecutive formation of two β-glycosidic linkages (23). Hydrophobic clustering analysis of the enzymes in this family showed two conserved domains, each of which is believed to be capable of binding a nucleotide sugar and catalyzing the formation of a glycosidic linkage (22, 23). Because polysaccharides like chitin, cellulose, and hyaluronic acid adopt a 2-fold screw axis (33–35), this model proposed that the binding sites for each of the nucleotide sugars are oriented 180° in regard to one another, thus allowing such polysaccharides to be generated without rotating either the enzyme or the polysaccharide (23). The loss of two UDP molecules from the catalytic site after addition of the two monosaccharides has been proposed to provide the necessary energy to translocate the polymer and allow two more nucleotide sugars to bind (23).

We have shown here that the type 3 synthase in *S. pneumoniae* membrane preparations is optimally active in the presence of Mn2+. Furthermore we have shown that the apparent $K_m$ values for both UDP-Glc and UDP-GlcUA are lower in the presence of Mn2+ than in Mg2+. In our standard assay, no significant incorporation of Glc from UDP-Glc occurred in the
Glc and UDP-GlcUA into high molecular weight polysaccharide. We have shown that the polysaccharide remained associated with the membrane-enzyme complex during elongation, indicating a processive mechanism. The addition of Glc and GlcUA to type 3 polysaccharide could occur from either the reducing or nonreducing end. The direction of chain elongation catalyzed by hyaluronan synthases has been debated for a number of years. Stoolmiller and Dorfman (36) demonstrated convincingly in 1969 that the direction of hyaluronic acid chain growth in S. pyogenes occurs by the addition of monosaccharide units to the nonreducing end of endogenous polysaccharide. The results of this investigation were, however, largely obscured by the subsequent finding of dolichol-linked disaccharides and oligosaccharides containing GlcUA and GlcNAc (37, 38) and particularly by the report that hyaluronate chain growth occurs at the reducing end in teratocarcinoma cells (39, 40). Correspondingly, the model of Saxena et al. (23) indicated that growth of hyaluronic acid, as well as several other β-glycans, occurred from the reducing end. We have shown, however, that approximately 75% of labeled Glc added to the growing end of type 3 polysaccharide chains can be removed by an exo-β-glucosidase, indicating that type 3 polysaccharide grows from the nonreducing end. Recent reports on the direction of chain growth for cellulose in Cladophora and Acetobacter (41) and hyaluronate in P. multocida (42) have also indicated that growth occurs from the nonreducing end. Whether all the members of the family of processive β-glycosyltransferases synthesize their polysaccharides from the nonreducing end, as suggested by Koyama et al. (41), remains to be determined.

In our experiments determining the direction of growth, we observed that S. pneumoniae membranes contain nascent polysaccharide that can be labeled with Glc from UDP-Glc without the addition of UDP-GlcUA. The presence of type 3 polysaccharide in membrane preparations of S. pneumoniae (3) and our results showing that polysaccharide terminally labeled with Glc specifically binds to an affinity column made with monoclonal antibodies to type 3 polysaccharide indicate that pre-existing type 3 polysaccharide serves as an acceptor in these experiments. Because our membrane preparations contain preformed polysaccharide acceptor, we are unable to determine whether the type 3 synthase is capable of de novo synthesis from the nucleotide sugars or whether some form of primer is required. However, the recent expression of a related glycosyltransferase in S. cerevisiae (20) could provide a means to answer this question because this organism does not make UDP-GlcUA.

The growth of type 3 polysaccharide at the nonreducing end and the synthesis by a processive mechanism suggests that the growing polymer is bound to the enzyme via a site that recognizes the terminal sugar(s) of the nonreducing end. Examination of the association of type 3 polysaccharide with the membrane-enzyme complex indicated that the interaction of the polysaccharide chain with the enzyme is affected by the UDP sugar concentrations. We found that when both substrates are in excess, the polysaccharide chain remained associated with the membrane-enzyme complex. However, a portion of the polysaccharide was released from the membrane-enzyme complex when the level of one of the substrates became limiting and was stimulated when one UDP substrate was added in excess. That this effect occurred at the point of substrate depletion suggests that the presence of a single substrate may stimulate release. Prehm (40) also observed a slow but distinct shedding of pulse-labeled hyaluronate when one obligatory component, such as MgCl₂, UDP-GlcNAc, or UDP-GlcUA, was omitted during the chase period. This release of hyaluronate was shown to be

![Graph](image-url)
S. pneumoniae Type 3 Capsule Synthesis

independent of size (40), an observation that we have also made for type 3 polysaccharide. The selective inhibition of hyaluronic chain release, but not elongation, by p-chloromercuribenzoate prompted the suggestion that the release process might be an enzymatic mechanism (43). Current experiments examining the effect of a single substrate on type 3 polysaccharide release have shown that the release is dependent on time, temperature, and the concentration of the UDP-monomer, also suggesting an enzymatic mechanism.

In summary, the data presented here provide an in-depth characterization of the type 3 synthase involved in the synthesis of pneumococcal capsule. The demonstration of growth of type 3 polysaccharide from the nonreducing end, as well as a potential mechanism of polysaccharide release, provides new information on the mechanism of type 3 polysaccharide synthesis as well as other β-glycans synthesized by related enzymes.

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REFERENCES

1. Henrichsen, J. (1995) J. Clin. Microbiol. 33, 2759–2762
2. Van Dam, J. E., Fleer, A., and Snippe, H. (1990) Antonie Van Leeuwenhoek 58, 1–47
3. Dillard, J., Vandersea, M., and Yother, J. (1995) J. Exp. Med. 181, 973–983
4. Reeves, R., and Goebel, W. (1941) J. Biol. Chem. 139, 511–517
5. Caimano, M. J., Hardy, G. G., and Yother, J. (1998) Microb. Drug Resist. 4, 11–23
6. Mollerach, M., Lopez, R., and Garcia, E. (1999) J. Exp. Med. 188, 2047–2056
7. Dillard, J., and Yother, J. (1994) Mol. Microbiol. 12, 959–972
8. Guidolin, A., Morona, J., Morona, R., Hansman, D., and Paton, J. (1994) Inf. Immun. 62, 5384–5396
9. Munoz, R., Mollerach, M., Lopez, R., and Garcia, E. (1997) Mol. Microbiol. 25, 79–92
10. Kolkman, M., Morrison, D., van der Zeijst, B., and Nuijten, P. (1996) J. Bacteriol. 178, 3736–3741
11. Kolkman, M. A. R., van der Zeijst, B., and Nuijten, P. J. M. (1997) J. Biol. Chem. 272, 19502–19508
12. Arrecubieta, C., Garcia, E., and Lopez, R. (1995) Gene (Amst.) 167, 1–7
13. Arrecubieta, C., Lopez, R., and Garcia, E. (1996) J. Exp. Med. 184, 449–455
14. DeAngelis, P. L., Papaconstantinou, J., and Weigel, P. H. (1993) J. Biol. Chem. 268, 14568–14571
15. DeAngelis, P. L., Papaconstantinou, J., and Weigel, P. H. (1993) J. Biol. Chem. 268, 19181–19184
16. Bulawa, C., Slater, M., Cabib, E., Au-Young, J., Sburlati, A., Adair, W., and Robbins, P. (1986) Cell 46, 213–225
17. Geremia, R. A., Merpertz, P., Geelen, D., Montagu, M. V., and Holsters, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2669–2673
18. Chen-Wu, J. L., Zwicker, J., Bowen, A. R., and Robbins, P. W. (1992) Mol. Microbiol. 6, 497–502
19. Meyer, M., and Krell, G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4543–4547
20. DeAngelis, P. L., and Achyuthan, A. M. (1996) J. Biol. Chem. 271, 23657–23660
21. Silakowski, B., Pospiech, A., Neumann, B., and Schairer, H. U. (1996) J. Bacteriol. 178, 6706–6713
22. Keenleyside, W. J., and Whitfield, C. (1997) J. Biol. Chem. 271, 28581–28592
23. Saxena, I., R. Brown, J., Fevere, M., Geremia, R., and Henrissat, B. (1995) J. Bacteriol. 177, 1419–1424
24. Smith, E., Mills, G., Bernheimer, H., and Austrian, R. (1960) J. Biol. Chem. 235, 1876–1880
25. Yother, J., and White, J. (1994) J. Bacteriol. 176, 2976–2985
26. Briles, D. E., Claflin, J. L., Schroer, K., Davie, J., Baker, P., Kearney, J., and Barletta, R. (1981) J. Exp. Med. 153, 694–705
27. Zweig, G., and Whitaker, J. R. (1967) Paper Chromatography and Electrophoresis, Academic Press, New York
28. Briles, D. E., Claflin, J. L., Schroer, K., and Forman, C. (1981) Nature 294, 88–90
29. Springer, T. A. (1990) Current Protocols in Molecular Biology (Janssen, K., ed.) John Wiley & Sons, Inc., New York
30. Torriani, A., and Pappenheimer, A. (1962) J. Biol. Chem. 237, 3–13
31. Pigman, W. W., and R. M. Goepp, J. (1948) Chemistry of Carbohydrates, Academic Press, New York
32. Sinnott, M. L. (1990) Chem. Rev. 90, 1171–1202
33. Heatley, F., and Scott, J. E. (1988) Biochem. J. 254, 489–493
34. Gardner, K. H., and Blackwell, J. (1974) Biopolymers 13, 1975–2001
35. Minke, R., and Blackwell, J. (1975–1981) The Structure of Carbohydrate Polymers, John Wiley & Sons, New York
36. Stoolmiller, A., and Dorfman, A. (1969) J. Biol. Chem. 244, 236–246
37. Turco, S. J., and Heath, E. C. (1977) J. Biol. Chem. 252, 2918–2928
38. Hopwood, J. J., and Dorfman, A. (1977) Biochem. Biophys. Res. Commun. 75, 472–479
39. Oprem, P. (1983) Biochem. J. 211, 191–198
40. Oprem, P. (1983) Biochem. J. 211, 181–189
41. Kogawa, K., Helbert, W., Imai, T., Sugiyama, J., and Henrissat, B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9091–9095
42. DeAngelis, P. L. (1999) J. Biol. Chem. 274(37), 26557–26562
43. Sugahara, K., Schwartz, N. B., and Dorfman, A. (1979) J. Biol. Chem. 254, 6252–6261