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Characterization of Streptococcus pneumoniae Type 3 capsular polysaccharide biosynthesis.

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CHARACTERIZATION OF STREPTOCOCCUS PNEUMONIAE TYPE 3 CAPSULAR POLYSACCHARIDE BIOSYNTHESIS

by

ROBERT T. CARTEE

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
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ABSTRACT OF DISSERTATION
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Title Characterization of *Streptococcus pneumoniae* Type 3 Capsular Polysaccharide Biosynthesis

Type 3 capsule of *Streptococcus pneumoniae* is composed of the repeating disaccharide (GlcA-ß1,4-Glc-ß1,3)$_n$. One of the genes essential for type 3 polysaccharide synthesis is *cps3S*, which encodes the type 3 polysaccharide synthase. This protein localizes to pneumococcal membranes and utilizes UDP sugar precursors to form both glycosidic linkages of the type 3 polysaccharide. In our initial work characterizing type 3 synthase activity, we established optimal conditions for synthase activity and determined that sugar addition occurred on the nonreducing end of the polysaccharide chain. The growing polysaccharide chain was shown to remain associated with the enzyme throughout polymerization, indicating a processive mechanism of synthesis. A portion of the polysaccharide, however, was released from the enzyme when the level of one of the substrates was depleted.

Further characterization of polysaccharide release showed that the process was actuated by the addition of a single nucleotide sugar substrate and was dependent on time, temperature, and the concentration of substrate. These data indicate that release is enzymatic and likely the result of the normal biosynthetic mechanism. Kinetic comparison between the release and the biosynthetic reactions suggest that there is positive and negative cooperative binding between the nucleotide sugar substrates. Incubation of membranes with a single substrate resulted in a loss of subsequent enzyme activity,

suggesting that once the polysaccharide is released the type 3 synthase is not capable of reinitiating synthesis. Characterization of the type 3 synthase expressed in *Escherichia coli*, however, indicated that the enzyme was capable of reinitiating synthesis after releasing its nascent chain. These data suggest that additional factors may be involved in controlling initiation of type 3 polysaccharide synthesis in *S. pneumoniae*.

DEDICATION

This dissertation is dedicated in memory of my mother Betty Carol Cartee.

ACKNOWLEDGMENTS

I first thank my committee Tom Forsee, John Baker, Ken Taylor, Bill Benjamin, Mark Jedrzejas, and Janet Yother for all their time and suggestions during the completion of this body of work. I especially thank Janet Yother for allowing me to work in her lab and having faith in my abilities. I would not be completing my doctoral work if it were not for her compassion, patience, and tutelage. Many thanks also go to Tom Forsee for not only his help in completing many of the experiments presented in this dissertation but also his daily companionship and advice on life and science. I will always consider Tom as both a mentor and a friend

I also thank the members of the Yother lab both present and past for all there help and friendship. They have made my graduate school experience much more rewarding and entertaining.

Many thanks also go out to my family, especially my father who has been a constant source of encouragement and support.

Lastly, I thank my wife Tara for not only her support and love but also giving me the most important thing in my life, my daughter Marissa.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ATP	adenosine triphosphate
BSA	bovine serum albumin
Cps3S	type 3 polysaccharide synthase
ELISA	enzyme-linked immunosorbent assay
Gal	galactose
GalNAc-1-P	N-acetylgalactosamine 1-phosphate
GalU	glucose-1-phoshpate uridylyltransferase
GBS	group B Streptococcus
Glc	glucose
GlcNAc-1-P	N-acetylglucosamine 1-phosphate
GlcUA	glucuronic acid
HCA	hydrophobic cluster analysis
IPTG	isopropyl-1-thio-ß-D-galactoside
KDO	phosphatidyl-2-keto-3-deoxyoctulosonic acid
ManNAc	N-acetylmannosamine
MES	2-(N-morpholino)ethanesulfonic acid
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween 20
PGM	Phosphoglucomutase

pmHAS	*Pasteurella multocida* hyaluronic acid synthase
PspA	Pneumococcal surface protein A
seHAS	*Streptococcus equisimilis* hyaluronic acid synthase
spHAS	*Streptococcus pyogenes* hyaluronic acid synthase
Tricine	N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine
UndP	undecaprenyl phosphate
UndPP	undecaprenyl pyrophosphate

INTRODUCTION

Pathogenic bacteria have devised many clever ways to survive and multiply within the hostile environment of their host. These means of survival can range from the secretion of antiphagocytic proteins to the scavenging of important metabolites. One common means of survival employed by bacteria is the production of a polysaccharide capsule. The capsule makes up the extreme outer surface of the bacterium and can serve many functions, such as adherence of the bacterium to a surface and the prevention of desiccation. Additionally, the capsule can protect the bacterium from the host's specific immune response by mimicking the host's naturally occurring polysaccharides and from the host's nonspecific immune response by preventing complement deposition and opsonophagocytosis (1).

The many different roles that polysaccharide capsules serve is due to their high degree of chemical and structural diversity. Chemically, they can be composed of a variety of common monosaccharides as well as highly modified and unique monosaccharides. Structurally, unlike proteins or nucleic acid where there is only one kind of linkage between the amino acid or nucleotide subunits, the monosaccharide residues of polysaccharides can be joined together in a number of different conformations, greatly adding to their diversity.

Capsule diversity is highly evident in the gram-positive pathogen *Streptococcus pneumoniae* where 90 different capsular serotypes have been identified (2). The capsule of *S. pneumoniae* has played an important role in several major scientific discoveries

since Pasteur originally described *S. pneumoniae* in 1881 (3). Griffith et. al. observed that an avirulent, unencapsulated strain of *S. pneumoniae* was transformed to a virulent, encapsulated phenotype when coinfected in mice with a heat-killed encapsulated strain (4). Avery and colleagues expanded on this study and demonstrated that DNA was the factor in this transformation and was the molecule of heredity (5). Although historically important, *S. pneumoniae* also poses a great threat to world health, despite the availability of antibiotics and a vaccine (6).

Streptococcus pneumoniae Capsule and Virulence

S. pneumoniae is a common inhabitant of the upper respiratory track of humans, with as much as 60% of the population carrying *S. pneumoniae* asymptomatically in the nasopharynx (7). *S. pneumoniae* can disseminate from the nasopharynx to several locations in the human body and cause a variety of diseases, including meningitis, otitis media, bacteremia, sinusitis, and, as its name implies, pneumonia. *S. pneumoniae* is the leading cause of bacterial pneumonia in the world, with over 500,000 cases reported each year in the United States alone (8). Approximately 10-25% of adult patients with pneumococcal pneumonia develop bacteremia. Additionally, *S. pneumoniae* is the leading cause of bacterial meningitis with 3,000 cases reported each year. *S. pneumoniae* is responsible for 30-50% of acute otitis media infections, resulting in 24 million pediatric visits per annum (8). Most cases of pneumococcal infection occur in infants and the elderly, with greater than normal infection rates seen in sickle cell patients, HIV-infected individuals, and patients with immunological deficiencies (9, 10). Overall pneumococcal infections cause an estimated 40,000 deaths a year in the United States (8). In *S. pneumoniae,* there is a growing level of antibiotic resistance, primarily to penicillin,

which is the drug of choice in treating *S. pneumoniae* infections. Certain areas of the world now report that more than 35% of pneumococcal isolates are resistant to penicillin. Many of these penicillin-resistant strains have also become resistant to other antibiotics as well (8). Due to the high degree of morbidity and mortality associated with pneumococcal infection and the growing level of antibiotic resistance, new therapies are needed to limit *S. pneumoniae* infections.

Virulence Factors

Several factors have been associated with *S. pneumoniae* virulence. These include cell surface proteins, such as pneumococcal surface protein A (PspA), as well as soluble proteins, such as pneumolysin, autolysin, and neuraminidase (11). However, none of these factors play as important a role in the pathogenesis of *S. pneumoniae* as the polysaccharide capsule. The capsule of *S. pneumoniae* is an essential virulence factor that is demonstrated by the fact that unencapsulated or partially encapsulated strains are avirulent in mice (12-14). Capsule protects the bacterium from phagocytosis by providing a barrier to opsonins, which recognize the surface of the pneumococcus and target the bacterium for destruction (15-17). Paradoxically, antibodies made to the capsular polysaccharide are protective (18). This is the basis of the current vaccine to *S. pneumoniae*, which is composed of the capsular polysaccharide of the 23 most common serotypes found in infections. The vaccine is relatively effective in preventing invasive pneumococcal disease but has poor efficacy in preventing pneumonia or upper respiratory tract infections (8). Although the use of capsular polysaccharides in a vaccine has not provided a means to limit all *S. pneumoniae* infections, understanding how capsules are synthesized may lead to a more efficacious therapy.

Capsule Genetics

Most of the work done on *S. pneumoniae* capsular polysaccharides in the past few years has been aimed at defining the genetics of polysaccharide biosynthesis. Several capsular types have been characterized genetically. These include types 1, 2, 3, 4, 14, 19, 23F, and 33F (19-34). All of the genes necessary for capsule production reside within a single locus that is defined by two different regions (Fig. 1). One region contains the type-specific genes which are required to produce the serotype-specific polysaccharide (19-21). The other region, which flanks the type-specific genes, contains conserved genes that are presumed to play a role in capsule transport, polymerization, and regulation of synthesis (19, 21). The cassette-like organization of the *S. pneumoniae* capsule locus facilitates the switching from one capsule type to another by allowing exchange of the capsule biosynthetic genes via homologous recombination through the common flanking regions (20, 35, 36).

Biochemistry of Capsule Synthesis

While there has been great progress in understanding the genetics behind capsule synthesis in *S. pneumoniae*, there is currently very little information regarding the biochemistry of capsule synthesis. Most of the information on capsule synthesis in *S. pneumoniae* has come from examining homology of the biosynthetic proteins to enzymes of known function in other organisms. Many of these proteins share homology with proteins involved in the synthesis of capsules and O-antigens from gram-negative bacteria. The mechanism of capsule and O-antigen synthesis in *Escherichia coli* has been well studied and provides an excellent paradigm for understanding synthesis of capsular polysaccharide in *S. pneumoniae*.

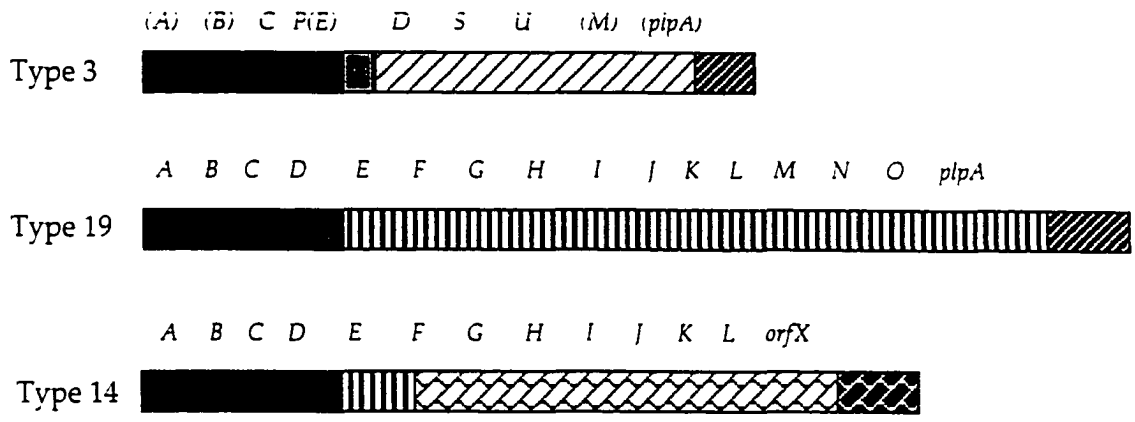


FIG. 1. Genetic organization of the capsule loci of *S. pneumoniae* types 3, 19, and 14. Regions of similar shading and pattern indicate conserved regions or genes. Regionswith a unique pattern indicate genes that are specific for the synthesis of that capsule type. Letters that are in parentheses indicate a partial or truncated gene.

The surface polysaccharides of *E. coli* are incredibly diverse with 173 distinct O-antigens and 80 different capsules or K antigens (37, 38). Synthesis of *E. coli* capsules and O-antigens can require up to 40 different genes, which are involved in the formation of precursors and the repeat unit, capsule polymerization, and transport to the bacterial surface (39). The basic steps in the synthesis of *E. coli* capsules and O-antigens are described below.

Precursor and Repeat Unit Formation

Bacterial polysaccharides are composed of repeated subunits that are linked together in long chains via glycosidic bonds. The repeated subunits can be as simple as a single monosaccharide or as complex as several different monosaccharides linked together into a branched structure. Production of capsule or O-antigen repeat units begins with the formation and activation of the monosaccharide precursors. In most cases activation takes the form of the addition of a nucleotide diphosphate to the sugar moiety. Once activated, the nucleotide sugars are utilized by a group of enzymes called glycosyltransferases to link the sugars into a repeat unit.

The repeat units of most *E. coli* capsules and O-antigens are formed on the lipid carrier, undecaprenyl phosphate (UndP) (40-42). Two enzymes have been identified in *E. coli* that are responsible for adding the first sugar to UndP and initiating repeat unit formation. The first enzyme, WecA (formerly Rfe), usually transfers either N-acetylglucosamine-1-phosphate (GlcNAc-1-P) or N-acetylgalactosamine-1-phosphate (GalNAc-1-P) from UDP-GlcNAc or UDP-GalNAc, respectively, to UndP (43-48). This glycosyltransferase has been shown to be involved in initiating not only capsule repeat unit formation but also formation of O-antigen repeat units.

The second enzyme is WbaP (formerly RfbP), an undecaprenylphosphate hexose-1-phosphate transferase. This enzyme initiates repeat unit formation by adding either a Glc-1-P or Gal-1-P to UndP (49). Homologues of this protein have also been identified in gram-positive and gram-negative bacteria whose capsules contain glucose (Glc) or galactose (Gal) (39). Cps19E of type 19 *S. pneumoniae* shares homology with WbaP of *Salmonella enterica* serovar typhimurium. This enzyme catalyzes the addition of Gal-1-P to UndP (21, 50). Although type 19 capsular polysaccharide does not contain Gal, mutation of *cps19E* results in a lack of incorporation of labeled Glc into a glycolipid fraction, suggesting that Cps19E catalyzes the addition of Glc-1-P to the lipid carrier (51). Characterization of Cps14E of type 14 *S. pneumoniae*, which is 95.8% identical to Cps19E, clearly demonstrated that this enzyme catalyzes the addition of Glc to a lipid carrier and initiates repeat unit formation (23, 24). The exact identity of the lipid carrier in *S. pneumoniae* is not known; however, it is presumed to be UndP.

The formation of the remainder of the repeat unit is catalyzed by additional glycosyltransferases that add sugars onto the nonreducing end of the repeat unit. Until recently, it was accepted that a single glycosyltransferase was responsible for forming each glycosidic linkage of the repeat unit (52). However, several glycosyltransferases have recently been isolated that are capable of forming more than one glycosidic linkage, some of which are discussed below (53-60). The polysaccharides or oligosaccharides formed by these multifunctional enzymes are usually homopolymers or polysaccharides composed of a simple disaccharide repeat.

Repeat Unit Polymerization

While the mechanisms of precursor and repeat unit formation is relatively conserved from polysaccharide to polysaccharide, there are two mechanisms of polysaccharide polymerization, Wzy-dependent and Wzy-independent, each of which is described in detail below.

Wzy-dependent polymerization. The polymerization of repeat units by a Wzy-dependent mechanism occurs by sequential addition of the individual repeat units to the reducing end of the nascent polysaccharide (61, 62). This reaction occurs in the periplasm and is performed by Wzy (formerly Rfc) (63-66). The mechanism of Wzy-dependent synthesis of polysaccharide is illustrated in Fig. 2. This method is usually employed in the synthesis of capsular polysaccharides and O-antigens where the repeat unit is complex and is composed of several monosaccharides.

Prior to polymerization by Wzy, the lipid-linked repeat unit is transported from the cytoplasmic to the periplasmic side of the inner membrane. The enzyme responsible for this "flipping" is Wzx (formerly RfbX) (67). Similar proteins have been identified in all sequenced *S. pneumoniae* types, except type 3 (19-34). Recent studies in *E. coli* have shown that translocation of O-antigen subunits by Wzx is independent of the chemical structure of the repeat unit even though the amino acid similarity between Wzx of different O-antigen strains is poor. These studies also demonstrated that a complete O-antigen repeat unit is not required for translocation by Wzx, suggesting that other proteins may interact with Wzx to allow complete synthesis of the O-antigen polymer (68).

Polymerization of O-antigen repeat units by Wzy is controlled by the chain length regulator Wzz (Cld or Rol) (69-71). This protein works in conjunction with Wzy and

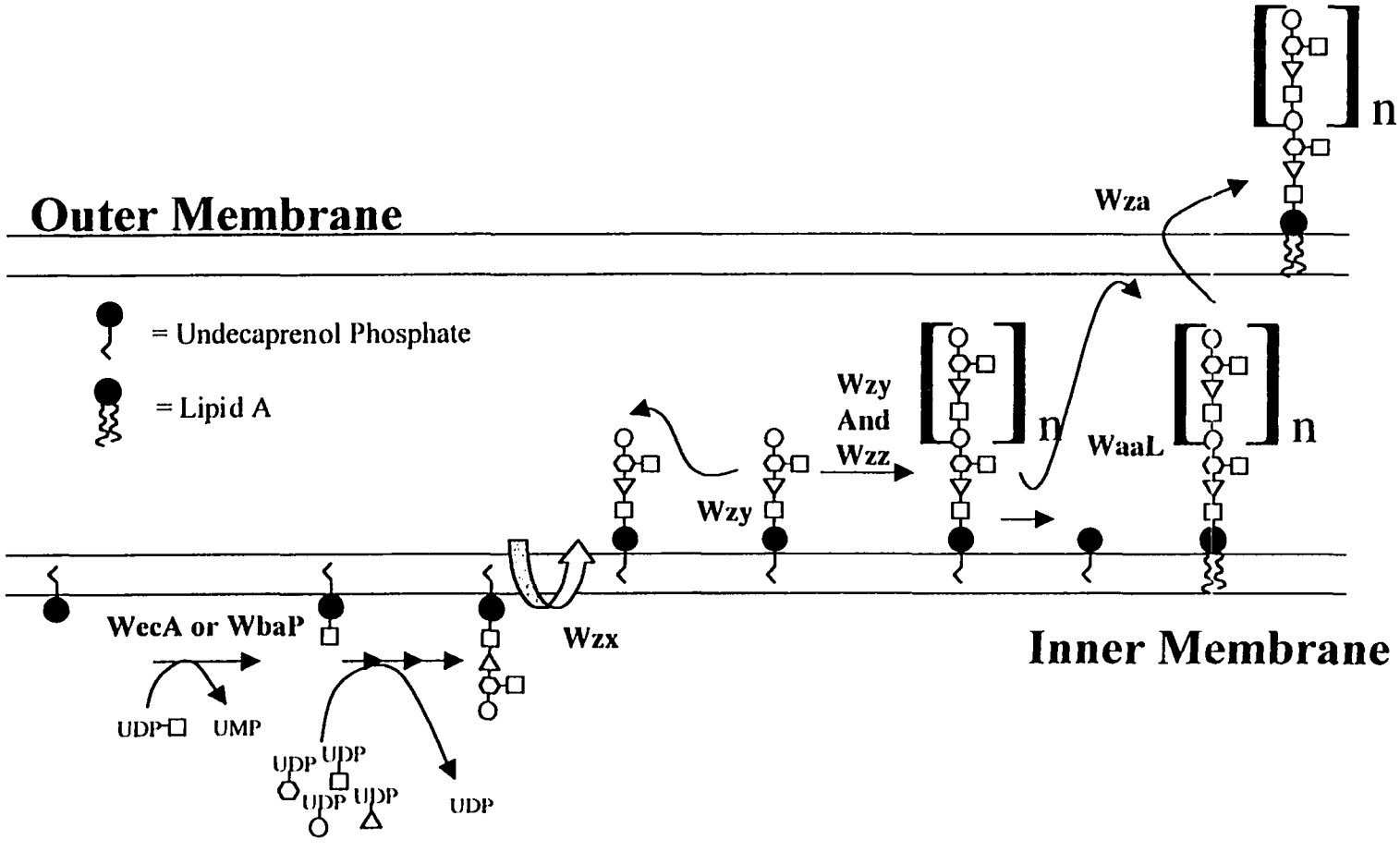


FIG. 2. **Wzy-dependent mechanism of polysaccharide synthesis.**

WaaL, a ligase that links polymerized O-antigen to lipid A (71). Wzz is an inner membrane protein with a periplasmic domain flanked by transmembrane domains. Several *E. coli* strains have been shown to lack the gene that encodes Wzz, suggesting that capsular chain length may be controlled by a different mechanism in these strains (72). One protein that may be involved in controlling capsule polysaccharide chain length in these strains is Wzc, a member of the cytoplasmic membrane periplasmic auxiliary proteins (73, 74). This structure of Wzc is similar to Wzz, except Wzc has a C-terminal adenosine triphosphate (ATP) binding site (75).

Wzc shares homology with a number of proteins that are involved in polysaccharide synthesis, including ExoP from *Rhizobium meliloti*, AmsA from *Erwinia amylovora*, EpsB from *Pseudomonas solanacearum*, and Orf6 from *Klebsiella pneumoniae* (76). ExoP, the best characterized of this group, is involved in the formation of succinoglycan, an exopolysaccharide important in alfalfa nodule invasion (77). Nodule invasion is critical for establishment of a nitrogen-fixing symbiosis between Rhizobium and the plant. Succinoglycan is produced as both a high molecular weight and a low molecular weight form, the latter consisting of one to three repeat units. The low molecular weight form seems to be important for successful nodule invasion (78, 79). Transposon mutagenesis of *exoP* results in the accumulation of the succinoglycan repeat unit, indicating an inability to polymerize (80-82). Based on these results, ExoP is presumed to function either as a polymerase or as a regulator of polymerase function (80). If the function of Wzc is similar to that of ExoP, it may be involved in regulating polymerization. Recently, Wzc has been shown to be an autophosphorylating protein-tyrosine kinase (76). Phosphorylation of ExoP has not been demonstrated, and the significance of Wzc phosphorylation remains to be determined. Following a common

locus design, the gene *wzb*, encoding a protein-tyrosine phosphatase whose function is dephosphorylating Wzc, has been found immediately upstream of Wzc (76). Genes encoding putative protein-phosphatases have also been found in close proximity to *amsA*, *espB*, and *orf5* (83-86).

The polymerization of the polysaccharide repeat units of all sequenced *S. pneumoniae* capsule types, except type 3, is presumed to occur on the outer surface of the plasma membrane by enzymes that share limited homology with Wzy. Although a mechanism of controlling chain length has not been identified in *S. pneumoniae*, the upstream common genes *cpsC* and *cpsD* encode proteins that together are homologous to the N-terminus and C-terminus of ExoP, respectively (21). Recent characterization of the upstream common genes, *cpsABCD*, has provided new evidence as to their role in capsule synthesis. Deletion of *cpsA* in *S. pneumoniae* type 19F resulted in reduced capsule production. Deletion of *cpsB*, *cpsC*, and *cpsD*, however, resulted in a non-encapsulated phenotype (87). Because CpsA is homologous (28% identity) to LytR, a transcriptional regulator of autolysin activity in *Bacillus subtilus*, it is believed that CpsA may function as a regulator of capsule transcription (21). CpsD has also now been shown to be a tyrosine kinase that autophosphorylates to regulate capsule (87). It is hypothesized that unphosphorylated CpsD associates with membrane-bound CpsC, where it can bind ATP. CpsD then autophosphorylates, using the bound ATP, and subsequently dissociates from CpsC, reducing capsule expression. The mechanism by which dissociation of CpsD from CpsC reduces capsule expression is not known, but based on data showing that mutations in *exoP* result in loss of polymerization of succinoglycan it might have some effect on either polysaccharide polymerization or transport of the repeat units. The exact role that CpsB plays in capsule regulation is unknown. Because of its up-

stream location relative to *cpsC* and cpsD, Morona *et. al.* hypothesized that it functions as a phosphatase that allows CpsD to reassociate with CpsC and upregulate capsule. CpsB, however, does not share homology with any known phosphatases nor has phosphatase activity been demonstrated (87).

Wzy-independent polymerization. The polymerization of capsules and O-antigens that do not involve Wzy occur on the cytoplasmic side of the inner membrane, as illustrated in Fig. 3 (39). Most of the capsular polysaccharides synthesized by this method are either homopolymers or polymers with a simple disaccharide repeat. Polymerization of these polysaccharides occurs by a processive mechanism from the nonreducing end of the polysaccharide chain and usually involves a single glycosyltransferase (57, 88-90). *E. coli* O-antigens O8 and O9 are also synthesized in the cytoplasm by a Wzy-independent mechanism but require the use of several glycosyl-transferases (91). None of the glycosyltransferases involved in synthesizing Wzy-independent polysaccharides are capable of initiating synthesis of their respective polysaccharides *de novo* from the nucleotide sugars. Almost all of these polysaccharides have been shown to require the formation of lipid-liked intermediates (48, 89, 92). Transport of these polysaccharides across the inner membrane utilizes an ATP-binding cassette (ABC) transporter (91, 93, 94). Studies on the K5 capsule of *E. coli* have indicated that the polymerization and transport of this polysaccharide involves a hetero-oligomeric membrane-bound complex (95). Bliss and Silver have hypothesized that polysaccharides synthesized by a Wzy-independent mechanism are polymerized and transported simultaneously via this hetero-oligomeric complex (96).

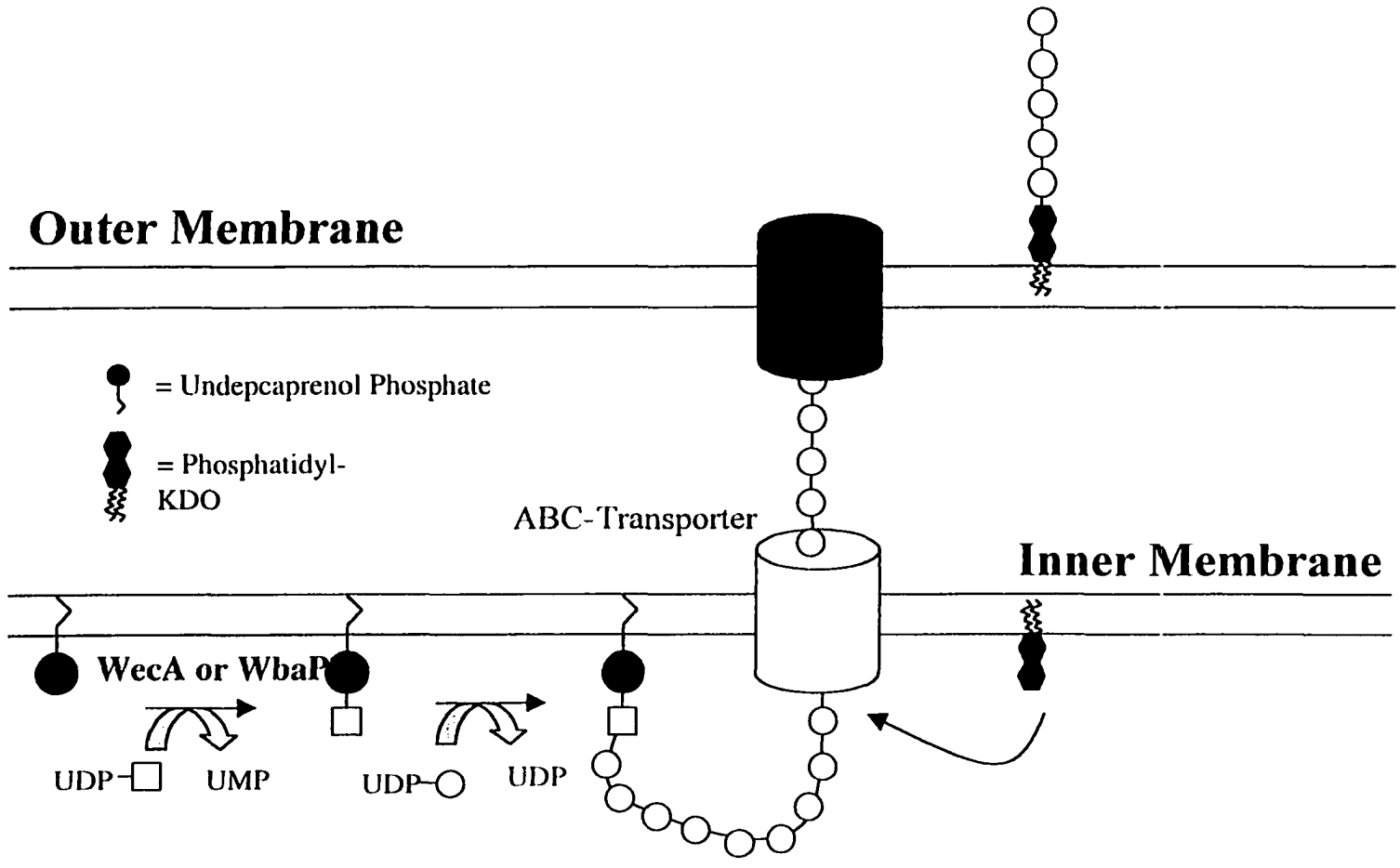


FIG. 3. **Wzy-independent mechanism of polysaccharide synthesis.**

Transport to Cell Surface

Transport of Wzy-dependent and Wzy-independent polymerized polysaccharides to the outer membrane of *E. coli* is poorly understood. Transport is believed, however, to occur at specific sites where the inner and outer membranes are in close proximity (97-99). In Wzy-dependent polymerized polysaccharides, Wza, an outer membrane lipoprotein, is required for the assembly of the capsule on the surface and has been shown to form high molecular weight complexes that are similar to secretins of type II and III protein secretion systems (Fig. 2). These complexes may form nonspecific gated channels through which the polysaccharide is transported for assembly on the cell surface (100). Currently, it is not known how Wzy-dependent polysaccharides are attached to the bacterial surface. Characterization of the attachment of the *E. coli* capsular polysaccharide K30 showed that a low molecular weight form of this polysaccharide can be attached to the surface of *E. coli* via lipid A (101). The higher molecular weight form, which is produced simultaneously, however, is attached to the bacterial surface via an alternative but unknown mechanism (101).

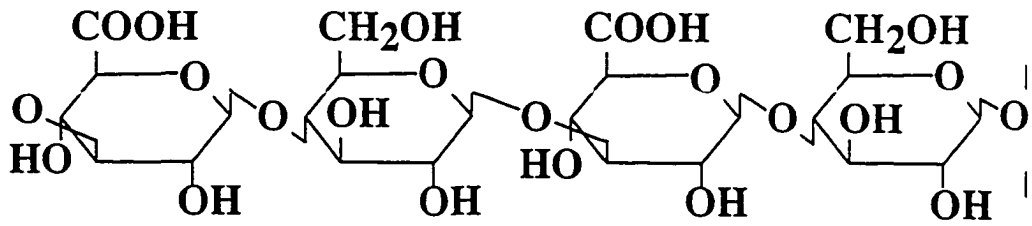
Polysaccharide polymerized by a Wzy-independent method is hypothesized to be transported across both membranes simultaneously (96). Translocation of K1 capsular polysaccharide across the outer membrane has been shown to require both proteins encoded in the capsule locus and outer membrane porin proteins (96). *E. coli* capsular polysaccharides that are synthesized by a Wyz-independent method are attached to the bacterial cell surface via a phosphatidic acid or a phosphotidyl-2-keto-3-deoxyoctulosonic acid (KDO) at the reducing end of the polysaccharide chain (102, 103). The stage at which this lipid moiety is added to the polysaccharide chain, however, is in

question. Some evidence suggests that it occurs after polysaccharide polymerization, while other evidence suggests it occurs before polymerization begins (88).

Most *S. pneumoniae* capsules, except type 3, are covalently attached to the cell surface (104, 105). Immunological examination of capsule attachment in several *S. pneumoniae* serotypes indicates that it is tethered to the cell wall peptidoglycan through an unknown linkage (105). Recent characterization of the linkage between type III capsular polysaccharide from Group B streptococcus (GBS) and the cell wall demonstrated that the attachment is via a phosphodiester bond and an oligosaccharide linker to the GlcNAc residues of peptidoglycan (106). The enzyme responsible for linking these polysaccharides to the cell surface has not been identified but potentially represents an excellent target for drug development.

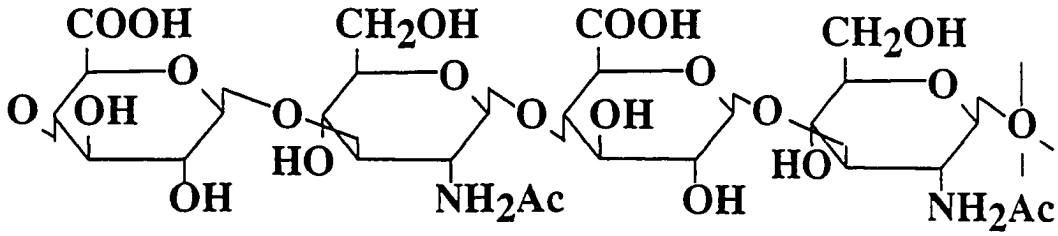
Type 3 Capsule

As mentioned previously, the type 3 capsule of *S. pneumoniae* is not synthesized or displayed on the cell surface in a manner similar to that of the other characterized *S. pneumoniae* capsules. Of the pneumococcal capsule types that have been characterized, type 3 is probably the simplest both structurally and genetically. Type 3 capsule is composed of the repeating subunit 3)-ß-D-GlcUA-(1->4)-ß-D-Glc-(1->, as illustrated in Fig. 4 (107). Type 3 polysaccharide is produced by the four type-specific genes located in the type 3 locus (19, 20, 55). Because almost all of the genes in the upstream common region of the type 3 locus have been truncated and are not required for capsule synthesis, regulation of type 3 polysaccharide production probably occurs by a different mechanism than that proposed for the other capsule types (55, 108). Recent studies on regulation of hyaluronic acid synthesis in *Streptococcus pyogenes* have shown that the two-



Type 3 Polysaccharide:

[β-D-glucuronic acid (1→4)-β-D-glucose (1→3)-]n



Hyaluronan:

[β-D-glucuronic acid (1→3)-β-D-glucose (1→4)-]n

FIG. 4. **Structure of type 3 polysaccharide and hyaluronan.**

component regulatory system *csrR/csrS* negatively regulates hyaluronic acid synthesis during stationary phase growth (109, 110). The genetic organization of the HA synthesis locus in *S. pyogenes* and the type 3 locus is similar (19, 20, 111), and, although transcription of the type 3 capsule locus is reduced during stationary phase growth, a two-component regulatory system has not been identified in the regulation of type 3 polysaccharide synthesis (104).

Based on protein homology of the four proteins encoded in the type 3 capsule locus and biochemical data for three of these four proteins, a biosynthetic pathway has been proposed for type 3 polysaccharide synthesis (Fig. 5) (55). The first two steps of this pathway involve the gene products of *cps3M*, a putative phosphoglucomutase, and *cps3U*, a Glc-1-P uridylyltransferase. Cps3M is proposed to catalyze the formation of Glc-1-P from Glc-6-P while Cps3U catalyzes the conversion of Glc-1-P to UDP-Glc, a precursor of type 3 polysaccharide biosynthesis (55). Neither of these genes is necessary for capsule production, as shown by insertional mutagenesis and deletion of the genes (55 and unpublished observation). An additional phosphoglucomutase (PGM) and a Glc-1-P uridylyltransferase (GalU) that lie outside the type 3 capsule locus have been identified and shown to be necessary for normal type 3 capsule formation (104, 112). The two other type 3 capsule locus gene products, Cps3D and Cps3S, are essential for capsule production and encode a UDP-Glc dehydrogenase and a polysaccharide synthase, respectively (55). Cps3D is responsible for forming the second precursor of type 3 polysaccharide synthesis by oxidizing UDP-Glc to UDP-GlcUA. Cps3S, as the polysaccharide synthase, forms the glycosidic linkages between the sugars utilizing UDP-Glc and UDP-GlcUA as substrates. Activities for both Cps3D and Cps3S have been demonstrated *in vitro* (53, 55, 113).

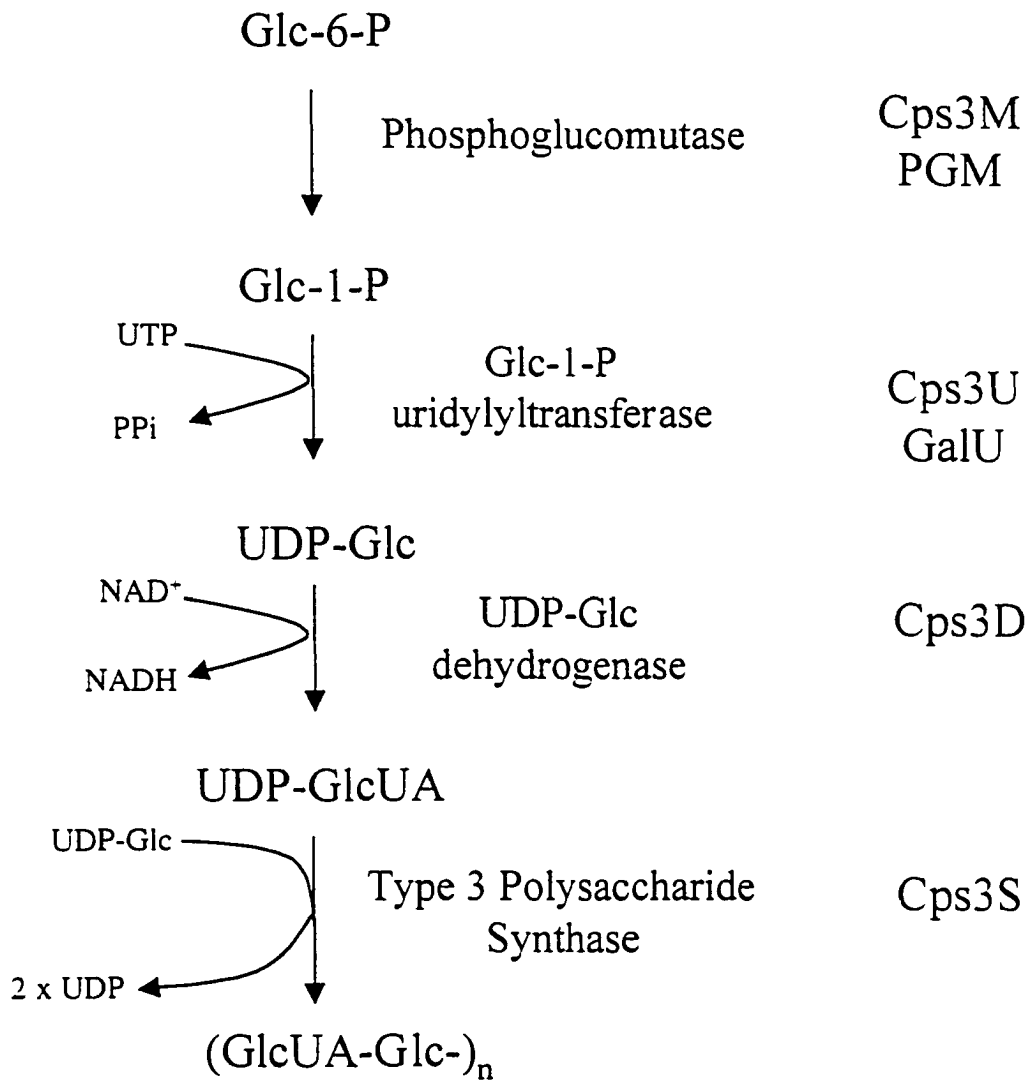


FIG. 5. **Biochemical pathway for *S. pneumoniae* type 3 polysaccharide synthesis.**

Type 3 Polysaccharide Synthase

The type 3 synthase is a 417 amino acid protein that contains three hydrophobic domains (55). Type 3 synthase activity localizes to *S. pneumoniae* membranes, utilizes UDP sugars as substrates, and requires a divalent metal ion (114). Using an antibody to type 3 polysaccharide to quantitate polysaccharide production in an *in vitro* assay, Smith *et. al.* determined optimal temperature, pH, and substrate concentrations for type 3 synthase activity (115). Several groups have attempted to clone and overexpress the type 3 synthase in *E. coli* with only limited success due to the toxicity of the overexpressed enzyme to *E. coli* (53, 55). Arrecubieta *et. al.* have shown limited expression of Cps3S in *E. coli*. The type 3 synthase expressed in *E. coli* was active, and type 3 polysaccharide was detected in both the cytoplasm and periplasm of *E. coli* (53). These data indicate that Cps3S is capable of forming both glycosidic linkages of type 3 polysaccharide (53). Additionally, the presence of type 3 polysaccharide in the periplasm of *E. coli* suggests that Cps3S may be capable of transporting type 3 polysaccharide across membranes. Because a protein has not been identified in association with type 3 capsule production that has transport function or has homology with proteins with transport function, it is feasible to suggest that Cps3S may be acting in this role. This ability of Cps3S to form two glycosidic linkages places it in a newly emerging class of processive ß-glycosyl-transferases that include enzymes from both prokaryotes and eukaryotes (45, 116). Some of the more notable members of this class of glycosyltransferase include the cellulose synthases from plants and bacteria, the nodulation factor synthase from *Rhizobium* sp, RfbBO:54 from *Salmonella enterica*, hyaluronic acid synthases from both prokaryotes and eukaryotes, and chitin synthases from *Saccharomyces cerevisiae* and *Candida albicans*. It has been proposed that all these enzymes function via a similar mechanism

(45, 116). The current knowledge about the mechanism of synthesis for a few of the better characterized members of this family is discussed below.

Cellulose synthase. Cellulose is the most abundant biopolymer on the earth and is produced by both plants and bacteria. Cellulose is composed of long linear repeats of ß1-4 linked glucose that form paracrystalline cables composed of several dozen polysaccharide chains (117). Many attempts have been made to purify and reconstitute the cellulose synthesizing machinery from plants; however, none of these attempts have been successful. Even with the discovery of the cellulose synthase gene (*celA*) from cotton, *in vitro* synthesis was still not observed (118). Most of the information about cellulose synthesis has come from studies on the cellulose synthesizing bacteria *Acetobacter xylinum*. The genes required for the synthesis of cellulose are found in a single operon. While a single protein, AcsAB, is responsible for synthesizing the chains of cellulose, the other genes in the operon have been shown to play a role in the export and crystallization of the cellulose chains (60, 119, 120). Characterization of the mechanism of synthesis of the bacterial cellulose has recently demonstrated that growth of cellulose occurs from the nonreducing end (121).

O-polysaccharide (O:54) synthase. O-polysaccharide O:54 of *S. enterica* serovar borreze is a homopolymer of N-acetylmannosamine (ManNAc) with the repeating unit 4)-ß-D-ManNAc-(1-3)-ß-D-ManNAc-(1-. Synthesis of this polysaccharide requires two glycosyltransferases, RfbAO:54 and RfbBO:54, which are encoded on the small plasmid pWQ799 (122). RfbAO:54 encodes a ManNAc transferase that catalyzes the first addition of ManNAc from UDP-ManNAc to GlcNAc- pyrophosphorylundecaprenol

(UndPP). GlcNAc-UndPP is formed by the activity of WecA described above in repeat unit formation (45, 122). RfbBO:54 is responsible for polymerizing the O-polysaccharide from UDP-ManAc after the addition of the first ManNAc by RfbAO:54. RfbBO:54, like the other members of the family of processive ß-glycosyltransferases, localizes to the membrane and is predicted to have four transmembrane domains (45). Polymerization of O:54 polysaccharide is believed to occur in the cytoplasm, and, unlike other O-polysaccharides, O:54 polysaccharide does not seem to require an ABC transporter or a Wzx O-unit flippase for transport across the inner membrane (45).

Nodulation factor synthase. *Rhizobium*, *Azorhizobium*, and *Bradyrhizobium* species form a symbiotic relationship with legumes that result in the formation of nitrogen-fixing nodules (123). The formation of these nodules results from the induction of expression of the bacterial nod genes by plant flavonoids (124, 125). The result of induction of the *nod* genes is the formation of a chitin oligosaccharide backbone containing three to five ß1-4-linked GlcNAc residues. This chitooligosaccharide backbone is modified at the nonreducing end by an acyl group and along the length of the oligosaccharide by additional modifications (126, 127). The enzyme responsible for forming the chitin backbone is the inner membrane protein NodC (56, 128). Analysis of the structure of NodC shows three hydrophobic domains, one on the N-terminus and two on the C-terminus. The hydrophobic domains flank a large hydrophilic region, which contains the catalytic sites for the enzyme (129). Topological analysis of NodC using *phoA* and *lacZ* fusions revealed that there is one transmembrane domain in the N-terminus in an N_{out}-C_{in} orientation adjacent to the catalytic domain which lies within the cytoplasm. The cytoplasmic localization of the catalytic domain is dependent on the

presence of the C-terminal hydrophobic domains. These data indicate that synthesis of the chitooligosaccharide occurs in the cytoplasm (129). How NodC controls the length of the chitooligosaccharide is not clear. Expression of the *Azorhizobium caulinodans* NodC in *E. coli* resulted in the production of a normal sized chitooligosaccharide, suggesting that control of chain length is inherent in the protein (56). The mechanism of transport of Nod factor into the legume rhizosphere has not been explored, but it is believed to occur via an ABC transporter encoded by *nodI* and *nodJ* (130).

Hyaluronic acid synthase. The best characterized of the processive ß-glycosyltransferases are the hyaluronic acid synthases. Hyaluronic acid or hyaluronan is a ubiquitous polysaccharide that is one of the few polysaccharides to be produced by both mammals and prokaryotes. Hyaluronic acid was originally isolated from the vitreous of the eye and shown to be composed of long repeats of 4)-ß-D-GlcUA-(1->3)-ß-D-Glc-(1-> (131, 132). Hyaluronan is identical to type 3 polysaccharide except the 1-4 linked sugar, rather than the 1-3, contains the carboxyl group and there is an acetoamido group (Fig. 4). Structural analysis of hyaluronic acid has shown that it forms a two-fold helix with rigid, rod like properties (133). Hyaluronic acid has the ability to associate with large quantities of water to form a jelly-like matrix that is necessary for the precise shape of the eyeball and serves as a lubricant in the synovial fluid of joints (133). Hyaluronan produced by bacteria is in the form of a capsule that prevents recognition of the bacteria by the immune system and therefore enhances virulence of the organism (134-137). The activity of hyaluronic acid synthases has been characterized in both bacteria and eukaryotic cells.

Most of the work in determining the mechanism of synthesis of hyaluronate in eukaryotes has been performed in differentiated teratocarcinoma cells. In these studies, Prehm demonstrated that hylauronate is synthesized at the plasma membrane and that it grows at the reducing end of the polysaccharide by alternating transfer of the growing polymer to the enzyme-bound nucleotide sugar (138-140). Studies examining hyaluronic acid synthesis in a human glioma cell line also indicated growth of hyaluronic acid at the reducing end (141). Hyaluronan chain growth occurs independent of any endogenous primers or lipid intermediates, suggesting the enzyme can initiate synthesis from the nucleotide sugar precursors (138, 142, 143). Expression of the hyaluronic acid synthase DG42 from *Xenopus laevis* in yeast, which does not make UDP-GlcUA, demonstrate that DG42 is capable of initiating hyaluronic acid synthesis in the absence of any nascent hyaluronic acid acceptor. These data suggests that DG42 is also capable of initiating synthesis from the nucleotide sugar precursors *in vitro* (144, 145). Three different isoforms of the hyaluronic acid synthase (HAS1, HAS2, and HAS3) have been cloned and characterized from mice and humans (146-151). These three isoforms have been shown to synthesize hyaluronan of different sizes *in vitro*. The differing ability to synthesize high molecular weight hyaluronan was attributed to differences in the apparent K_m values for UDP-GlcUA and UDP-GlcNAc (152).

Hyaluronic acid synthases have been cloned and characterized from *Streptococcus pyogenes* (spHAS) and *Pasteurella multocida* (pmHAS) (54, 153). Studies on the mechanism of hyaluronic acid synthesis in *S. pyogenes* and *P. multocida* demonstrated that growth of the polymer occurs from the nonreducing end, in contrast to what has been reported for the eukaryotic enzyme (154, 155). However, consistent with the eukaryotic enzymes, the synthesis of hyaluronic acid in *S. pyogenes* occurs

independent of a lipid primer (155, 156). Recently, the pmHAS was shown to be capable of adding single sugars to a hyaluronic acid tetrasaccharide (154). Attempts to use hyaluronic acid oligosaccharides as an acceptor in *S. pyogenes*, however, were unsuccessful (154, 155). The pmHAS is structurally different from the other hyaluronic acid synthases, with this enzyme being about twice the size of spHAS and containing only two predicted transmembrane domains (153). All other hyaluronic acid synthases, including the eukaryotic enzymes, are predicted to have four or more transmembrane domains that flank the large cytoplasmic catalytic domain (157). Solubilization and reconstitution of the hyaluronic acid synthase from *Streptococcus equilimilis* (seHAS) membranes have shown that the enzyme requires the phospholipid cardiolipin for optimal activity (158). Supporting this finding are recent studies examining the phospholipid requirement of both spHAS and seHAS that was expressed, solublized, and purified from *E. coli* (159). Inactivation of the spHAS and seHAS by increasing doses of radiation in combination with mass spectrometry indicates that these enzymes function as monomers but complex with 16 molecules of cardiolipin (160). It has been proposed that the protein-lipid interactions help form a pore through which the elongating hyaluronic acid chain is extruded (160, 161). Further enzymatic characterizations of both enzymes, purified from *E. coli* membranes, have shown kinetic patterns consistent with cooperative binding between the two substrates (161).

Proposed Mechanism of Synthesis for Processive ß-glycosyltransferases

Although the overall primary amino acid homology between the members of this family of glycosyltransferases is not high, hydrophobic cluster analysis (HCA) of the large hydrophilic regions of these proteins indicates a conserved architecture (45, 116).

HCA is a method of predicting the tertiary structure of proteins by aligning the primary amino acid sequence in an α-helical two dimensional pattern (162). HCA has proven useful in showing conserved features among highly divergent groups of proteins (162). This method has been used with the highly diverse glycosyl hydrolases, allowing them to be grouped into categories based on conserved structural features (163, 164).

HCA of the globular portions of glycosyltransferases has shown two conserved domains, domain A, which is present in all the glycosyltransferases analyzed, and domain B, which is present only in processive glycosyltransferases (116). All the glycosyl-transferases discussed above are processive and contain both domain A and domain B (45, 116). Each of these domains is believed to be able to bind a nucleotide sugar and catalyze the formation of a glycosidic linkage (116). Based on analogy to the glycosyl hydrolases, there are two catalytic mechanisms for glycosyltransferases, either by retention or inversion of the anomeric configuration of the reaction center (165, 166). Because the polymers synthesized by enzymes like the type 3 synthase contain alternating ß-linkages but the UDP sugars are linked in an α-configuration, it is thought that these enzymes provide for an inverting mechanism. It is possible then that α-linkages are formed by a retaining mechanism (116). Both mechanisms require the use of two carboxylic acids, usually in the form of aspartate or glutamate residues (167). Examination of conserved amino acid residues in the conserved domains of the processive ß-glycosyltransferases has shown that domain A contains two conserved aspartate residues while domain B contains only one. Domain B also contains the conserved sequence QXXRW (116). Although mutation of this sequence in HAS1 from mice results in loss of activity, the role it plays in synthesis is not known. Mutation of the conserved aspartate residues in domain A and domain B of the hyaluronic acid synthases

from mice also eliminated hyaluronic acid synthase activity (168). Additionally, mutation of these conserved aspartate residues in chitin synthase 2 of *S. cerevisiae* eliminated activity (169).

To form polymers that contain alternating ß-glycosidic linkages, these glycosyltransferases must overcome the steric problem that each sugar must be flipped nearly 180° relative to the adjacent sugar (133, 170, 171). Taking this into account, Saxena *et. al.* have proposed a model in which the two conserved binding sites are oriented 180° from one another. This orientation allows polymers that contain a two-fold symmetry to be formed without having to turn the polymer or the enzyme after the addition of each sugar (116). Saxena *et. al.* predicted that the sugars are added in tandem either sequentially or simultaneously to the reducing end of the growing polysaccharide chain (116). The resulting release of two UDPs after addition was proposed to provide the necessary energy to translocate the polymer and open up the binding sites for two new nucleotide sugars (45). The enzymes in this class have been shown to be processive enzymes, whereby the elongating polymer remains associated with the enzyme during synthesis. Consequently, this model predicts that there must also be a binding site for the growing polysaccharide chain (116). Although this model provides a plausible mechanism for the function of processive ß-glycosyltransferases, there is little to no data supporting this model.

Most of the early work on the type 3 synthase involved defining its basic biochemical features. These experiments took advantage of the availability of an antibody to the type 3 polysaccharide and determined the optimal temperature, optimal pH, divalent metal ion requirements, and the effect of substrate concentrations on polysaccharide production (115). These experiments were limited, however, by the

versatility of the assay and the lack of genetic data. By utilizing a more versatile assay that involves the incorporation of radiolabeled nucleotide sugars into polysaccharide, we have expanded on these initial experiments to gain a more complete understanding of the mechanism of polysaccharide synthesis in type 3 *S. pneumoniae* and of polysaccharides synthesized by other members of the family of processive ß-glycosyltransferases. The first paper of this dissertation describes the characterization of type 3 synthase activity using the new assay, as well as new information regarding the direction of growth and a possible mechanism of polysaccharide release. In the second paper, polysaccharide release is further characterized and a model for polysaccharide release and substrate binding is proposed. The last paper addresses differences in type 3 synthase activity observed when the type 3 synthase is expressed heterologously in *E. coli*.

MECHANISM OF TYPE 3 CAPSULAR POLYSACCHARIDE SYNTHESIS IN *STREPTOCOCCUS PNUEMONIAE*

by

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SUMMARY

The glycosidic linkages of the type 3 capsular polysaccharide of *Streptococcus pneumoniae* ([3)-ß-D-GlcUA-(1->4)-ß-D-Glc-(1->]$_n$) 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 synthase activity in the presence of either Mn^{2+} or Mg^{2+} with maximal levels seen with 10-20 mM Mn^{2+}. High molecular weight polymer syn-thesized 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.

INTRODUCTION

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 [->3)-ß-D-GlcUA-(1->4)-ß-D-Glc-(1->] 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). Flanking either side of the type-specific genes are sequences common to all capsule types (7-11). These common genes have been proposed to play a role in polysaccharide transport and in determination of polysaccharide chain length in some capsular polysaccharides. In type 3 strains, however, most of these sequences are mutated, and are not required for polysaccharide synthesis or transport (3, 5, 12). No other sequences likely to be involved in transport of the type 3 polysaccharide have been identified, and this function may be performed by the type 3 synthase.

The type 3 polysaccharide synthase (Cps3S) [1] shares significant protein homology with several other glycosyltransferases from both prokaryotes and eukaryotes, including the hyaluronan synthase from *S. pyogenes*, the Nod factor oligosaccharide synthase (NodC) from *Rhizobium meliloti*, FbfA of *Stigmatella aurantiaca*, pDG42 of *Xenopus laeveis*, and chitin synthases from both *Saccharomyces cerevisiae* and *Candida albicans* (3). All of these enzymes produce homopolymers or polysaccharides with a simple disaccharide repeat, and current evidence suggests that each is capable of forming all of the glycosidic linkages present in their respective polysaccharides (3, 13-21). Hydrophobic cluster analysis of the deduced sequences of these proteins has re-vealed

two conserved domains that are believed to be responsible for binding the nucleo-tide sugars and catalyzing the formation of the glycosidic linkages (22, 23). Based on this analysis, it has been proposed that these glycosyltransferases synthesize poly-saccharide via a common mechanism that involves dual addition of sugars to the growing saccharide chain (23).

Smith *et al.* (24) demonstrated that type 3 synthase activity resides within the particulate fraction of *S. pneumoniae* lysates. Using a type 3-specific antibody to precipitate and quantitate polysaccharide, they observed synthesis of type 3 polysacc-haride using cell-free lysates, uridine nucleotide sugars, and a divalent metal cation (24). Consistent with the observed membrane location of type 3 synthase activity, the deduced amino acid sequence of Cps3S contains four hydrophobic domains potentially capable of spanning the membrane (3). The role of Cps3S as the type 3 synthase was demonstrated using isogenic strains containing insertion mutations either in or immediately down-stream of *cps3S*. Type 3 polysaccharide could be synthesized in the *in vitro* system using crude membrane preparations from the latter but not from the former, thus esta-blishing *cps3S* as the critical gene (3). Expression of the synthase in *Escherichia coli* has proven difficult, apparently due to toxicity of the membrane protein (3, 13). The only reported clone expressing the enzyme produced low levels of protein, despite being contained in a high copy number expression vector (13). The possibility of mutations in the clone was not eliminated, but a small amount of type 3 polysaccharide was syn-thesized in the *E. coli* strain, further confirming the assignment of *cps3S* as the type 3 synthase. Because production of high levels of synthase from cloned products has not been possible, we chose to use membranes isolated from *S. pneumoniae* as a source of synthase activity for further biochemical studies. Using an assay that measures the in-corporation of

radiolabeled sugars into polysaccharide, we have characterized the mech-anism of type 3 polysaccharide synthesis.

EXPERIMENTAL PROCEDURES

Materials. Mutanolysin, Type VIII-A ß-glucuronidase from *E. coli*, ß-glucosidase from *Caldocellum sachrolyticum*, Sepharose 2B, UDP-Glc, and UDP-GlcUA were obtained from Sigma. UDP-[^{14}C] Glc (257 mCi/mmol) and UDP-[^{14}C] 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 synthase 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 x 10^8 colony forming units/ml. The cells were collected by centrifugation at 10,000 x *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 $Na_2HPO_4 \cdot 7H_20$, 1.8 mM KH_2PO_4, 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 Mg_2S0_4). 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 x *g* for 20 min, washed once in 200 ml protoplast buffer, and then osmotically lysed by suspension in 100 ml sterile water containing 10 mM EDTA. Lysis was confirmed by light microscopy. The membranes were collected by centrifugation at 100.000 x *g* for 30 min, washed three times in 40 ml of 100 mM Hepes (pH 8.0) buffer containing 10 mM sodium thioglycolate, 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 thioglycolate and stored at -20 °C.

Assay of Synthase Activity. Type 3 synthase activity was determined by the incorporation of ^{14}C label from either UDP-[^{14}C] Glc or UDP-[^{14}C] GlcUA into polysaccharide. Synthase assays were performed in a 100-µl reaction that contained 100 mM Hepes (pH 8), 10 mM sodium thioglycolate, 10 mM $MnCl_2$, UDP-Glc, UDP-GlcUA, and membranes isolated as described above. The concentrations of UDP-Glc, UDP-GlcUA, and membrane protein are indicated elsewhere in the text and in the figure legends. The reaction mixtures were incubated at 32 °C for 20 min. The reaction was terminated by addition of 10 µl of 12.5 M glacial acetic acid, and the reaction components were separated by ascending paper chromatography on 3 MM Whatman paper in ethanol (95%):1 M ammonium acetate (pH 7.0), 7:3 (v/v) for 16-18 hr. The chromatograms were dried, and the product retained at the origin (1 inch strip) was determined by liquid scintillation counting in Scinti Verse I.

Assay of Soluble and Membrane-associated Polysaccharide Products. Isolated membranes containing 300 µg total protein were added to reactions containing 100 mM

Hepes (pH 8), 10 mM sodium thioglycolate, 10 mM $MnCl_2$, UDP-Glc, and UDP-GlcUA in a 600-µl volume. The reactions were incubated at 32 °C and, at the times indicated in the figure legends, 50-µl samples were taken. These aliquots were submitted to the following protocol to determine the amount of soluble and membrane-associated polysaccharide formed under various experimental conditions. EDTA was added to the aliquots to a final concentration of 10 mM, and the samples were stored on ice. The volume was adjusted to 200 µl with 100 mM Hepes (pH 8) buffer containing 10 mM sodium thioglycolate. The membranes were collected by centrifugation at 100,000 x *g* for 30 min. The supernatants were saved, and the pellets were suspended in 200 µl of the same buffer. The membranes were sedimented again by centrifugation at 100,000 x *g* for 30 min. The resulting supernatants were combined with the previous supernatants, and the pellets were suspended in 400 µl of the same buffer. Aliquots of the supernatant and pellet fractions were chromatographed on paper as described above for the synthase assay. The radioactivity at the origin, as well as that corresponding to authentic UDP-Glc, was determined by liquid scintillation counting.

Sepharose 2B Column Chromatography. Gel filtration of polysaccharide samples was performed on a 1.4 cm x 37 cm Sepharose 2B column equilibrated with 0.2 M 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 0.798-µm diameter latex beads (Sigma) and [^{14}C]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 ethanol (95%)/1 M ammonium acetate (pH 7), 7:3 (v/v) or 1-propanol/ethyl 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-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 M 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 synthase activity. The percent of radioactive polysaccharide that bound to the column was determined as 100 x [(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 M 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 exponential phase in THY, diluted 1/5 in fresh THY, and incubated for 20 min at 37 °C . Expression of the depolymerase by *B. circulans* was induced by the addition of 50 µl of heat killed type 3 *S. pneumoniae* strain WU2, grown to late exponential phase, centrifuged, and concentrated 30-fold in THY. A second inoculum (50 µl) of WU2 was added after 60 min, and the *B. circulans* culture was incubated at 37 °C for an additional 60 min. The cells were then heat killed at 65 °C for 20 min and sedimented for 2 min at 13,000 x *g*, and the supernatant was collected and stored at -70°C.

Preparation of Labeled Polysaccharide for Direction of Growth Experiments. For preparation A, uniformly labeled polysaccharide membranes (300 µg of total protein) were incubated in a 200-µl reaction containing 100 mM Hepes (pH 8), 10 mM sodium thioglycolate, 10 mM $MnCl_2$, 400 µM UDP-GlcUA, and 400 µM UDP-Glc (7 mCi/mmol) for 30 min at 32 °C. The reaction was terminated by the addition of 10% SDS to a final concentration of 2% and chromatographed on Sepharose 2B as described above. For preparation B, terminally labeled polysaccharide membranes (1.5 mg of total protein) were incubated in a 1-ml reaction containing 100 mM Hepes (pH 8), 10 mM sodium thioglycolate, 10 mM $MnCl_2$, 400 µM UDP-GlcUA, and 400 µM UDP-Glc for 30 min at 32 °C. The reaction mixture was placed on ice, and 1 ml of ice-cold 100 mM Hepes (pH 7.5) containing 10% glycerol was added. The membranes in the reaction mixture were sedimented by centrifugation at 100,000 x *g* for 30 min. The pelleted

membranes were suspended in 2 ml of 100 mM Hepes (pH 7.5) containing 10% glycerol and sedimented as above. The membranes were washed four more times using this procedure. The washed membranes were incubated in a 1-ml reaction containing 100 mM Hepes (pH 8), 10 mM sodium thioglycolate, 10 mM $MnCl_2$, and 2 μM UDP-Glc (287 mCi/mmol) for 30 min at 32 °C. The reaction was terminated by the addition of 10% SDS to a final concentration of 2% and then chromatographed on Sepharose 2B as described above. For preparation C, low molecular weight terminally labeled polysaccharide membranes (1.5 mg of total protein) were incubated in a 1-ml reaction containing 100 mM Hepes (pH 8), 10 mM sodium thioglycolate, 10 mM $MnCl_2$, and 2 μM UDP-Glc (287 mCi/mmol) at 32 °C for 10 min. The reaction was sonicated for 1 min in a sonicating water bath at 80 watts (Ultrasonics, Plainview, NY) to release the polysaccharide from the membrane. The insoluble material was removed by centrifugation at 100,000 x *g* for 30 min. The soluble polysaccharide was purified by chromatography on antibody-Sepharose 2B as described above.

Enzyme Treatments. Disaccharide produced by partial acid hydrolysis was treated with Type VIII-A ß-glucuronidase (27,800 units/ml) at 37 °C for the times indicated. Digestion of polysaccharide with ß-glucosidase was performed in a 100-μl reaction containing 0.1 M sodium acetate (pH 5.0) and ß-glucosidase (3 units/ml) at 37 °C for the times specified. Experiments using the type 3 polysaccharide-specific depolymerase from *B. circulans* (purification described above) were performed in 20 mM MES buffer (pH 6.5) containing 0.2% sodium azide at 37 °C for the length of time specified.

Protein Determinations. Protein concentrations were determined using the micro protein determination kit from Sigma.

RESULTS

Characterization of Type 3 Synthase Activity. Membranes were isolated from the type 3 *S. pneumoniae* strain WU2 and from an isogenic derivative (JD908) that is non-encapsulated due to an insertion mutation in *cps3S*. Synthase activity was determined by measuring the incorporation of ^{14}C from 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 Cps3S 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 from both UDP-[^{14}C]Glc and from UDP-[^{14}C] GlcUA into capsular polysaccharide. Formation of ^{14}C-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% than 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).

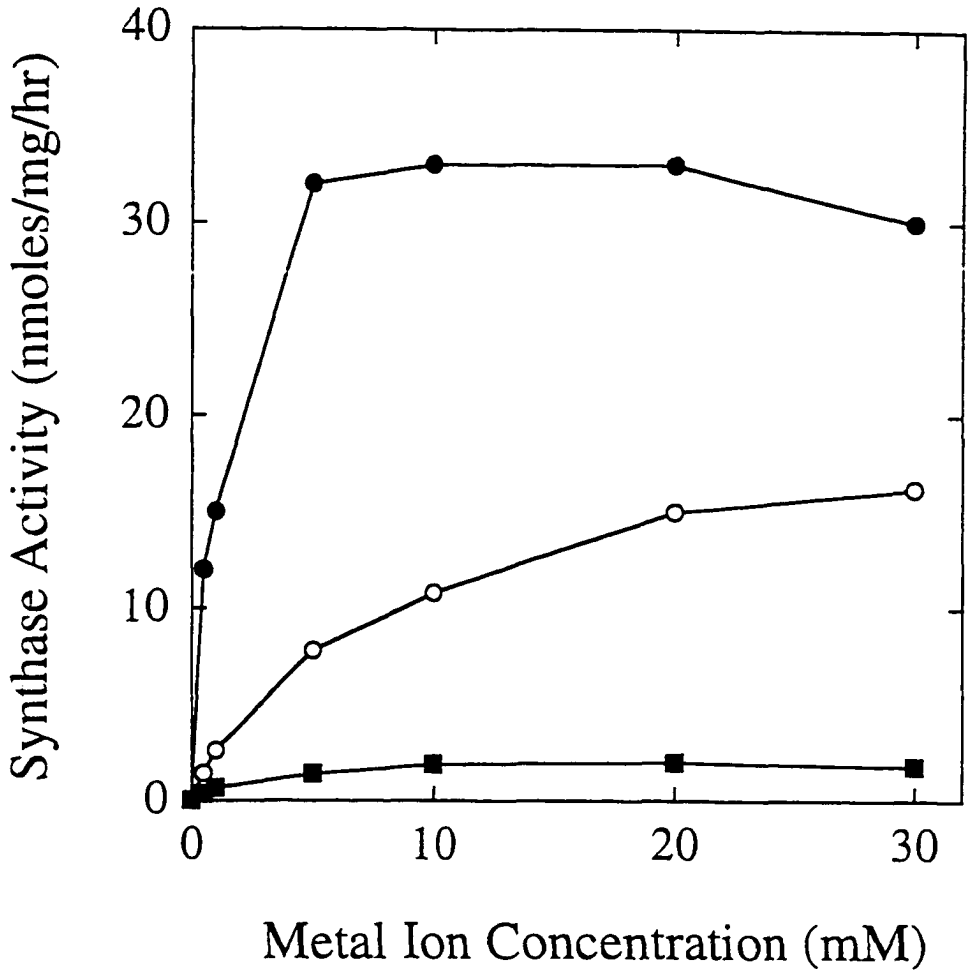


FIG. 1. **The effect of metal ion concentration on Cps3S activity**. Membranes (3 μg total protein) from type 3 *S. pneumoniae* were incubated at 32 °C for 20 min in a 100-μl reaction containing 100 mM Hepes (pH 8), 10 mM sodium thioglycolate, 2 μM UDP-Glc (257 mCi/mmol), 20 μM UDP-GlcUA, and increasing amounts of either $MnCl_2$ (●), $MgCl_2$ (○), or $CaCl_2$ (■). The product was separated by paper chromatography as described under "Experimental Procedures," and the radioactivity was determined by liquid scintillation counting. The amount of radioactivity in a sample that contained no metal ion was subtracted as background. Each point is the average of duplicate samples. Activity was determined as nmol of ^{14}C/mg of total protein/h incorporated into polysaccharide.

TABLE I

The effect of metal ion on Michaelis-Menton constants

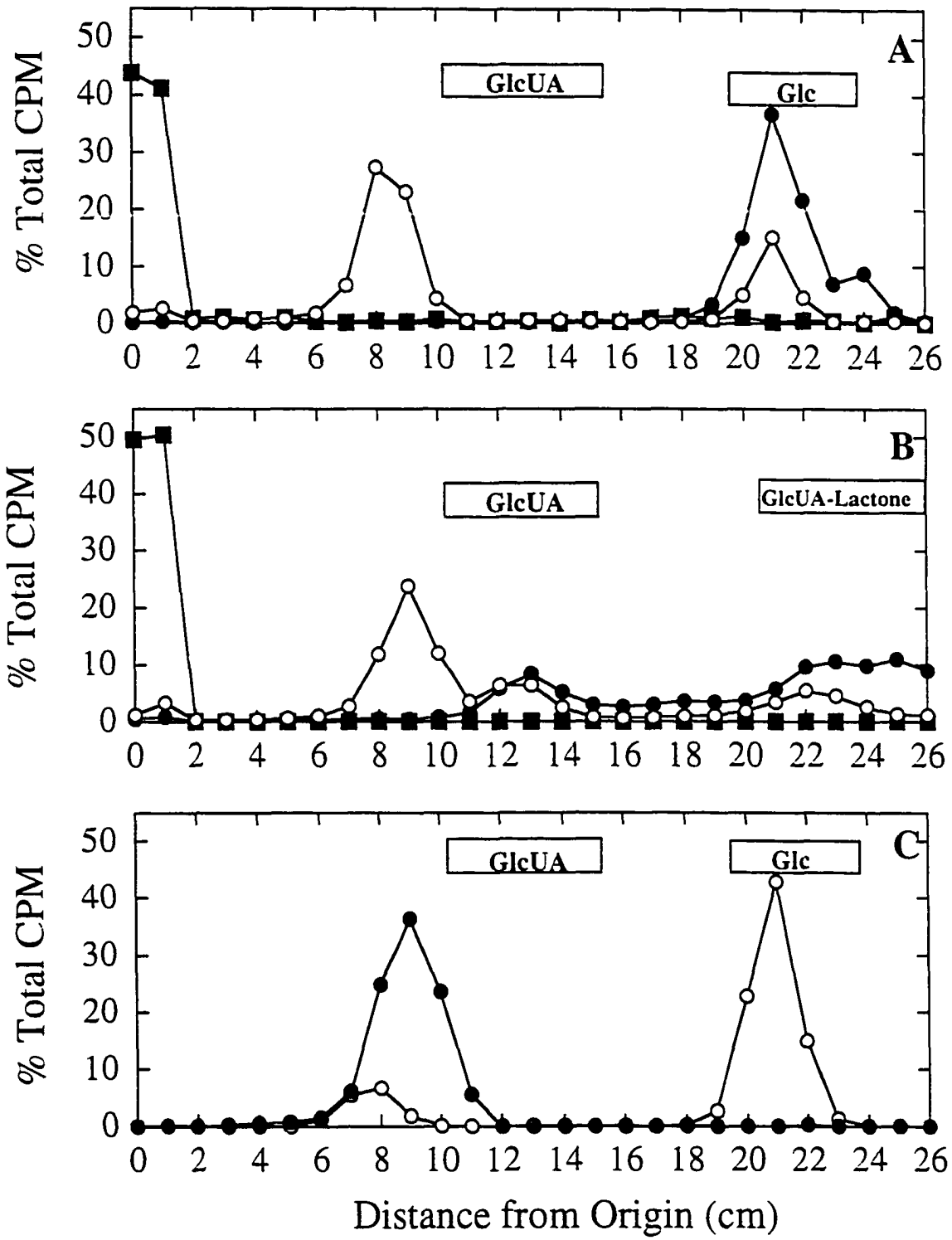
Synthase reactions were performed as described under "Experimental Procedures" with membranes containing 15 μg total protein. The concentration of either UDP-Glc or UDP-GlcUA was held at a concentration of 100 μM, whereas the concentration of the other substrate was varied. The data are the results of duplicate samples.

Metal ion[a]	Substrate[b]	*Km*[c]
		μM
Mn^{2+}	UDP-GlcUA	8.5 ± 1.2
	UDP-Glc	11.9 ± 0.7
Mg^{2+}	UDP-GlcUA	31.3 ± 2.0
	UDP-Glc	64.9 ± 7.0

[a] The concentration of metal ion in each reaction was 10 mM
[b] The concentration of substrate ranged from 0 to 200 μM
[c] K_m values were determined using Lineweaver-Burk plots

Characterization of the Polysaccharide Product. Polymer synthesized in a 2-h incubation with a high concentration (400 μM) of both UDP sugars eluted in the excluded volume of a Sepharose 2B column (data not shown). Polysaccharide labeled with UDP-[^{14}C] Glc was completely hydrolyzed by 4 N HCl to [^{14}C] Glc in 2 h at 100 °C (Fig. 2*A*). Polysaccharide labeled with UDP-[^{14}C] GlcUA-labeled product was completely hydrolyzed to [^{14}C] GlcUA and GlcUA-lactone under the same conditions (Fig 2*B*). 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 [^{14}C] Glc- and [^{14}C] 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 2*B*). Mild hydrolysis of

FIG. 2. **Acid hydrolysis of [^{14}C] Glc- and [^{14}C] GlcUA-labeled Polysaccharide**. Polysaccharide labeled with [^{14}C] Glc was prepared as described in 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 ml 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 N (●), 1 N (○), or 0 N HCl (■) at 100 °C for 2 hr. 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 percent of the total cpm. *C*, a 400-μl sample of the [^{14}C] Glc-labeled polymer was hydrolyzed in 1 N HCl 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 ß-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.

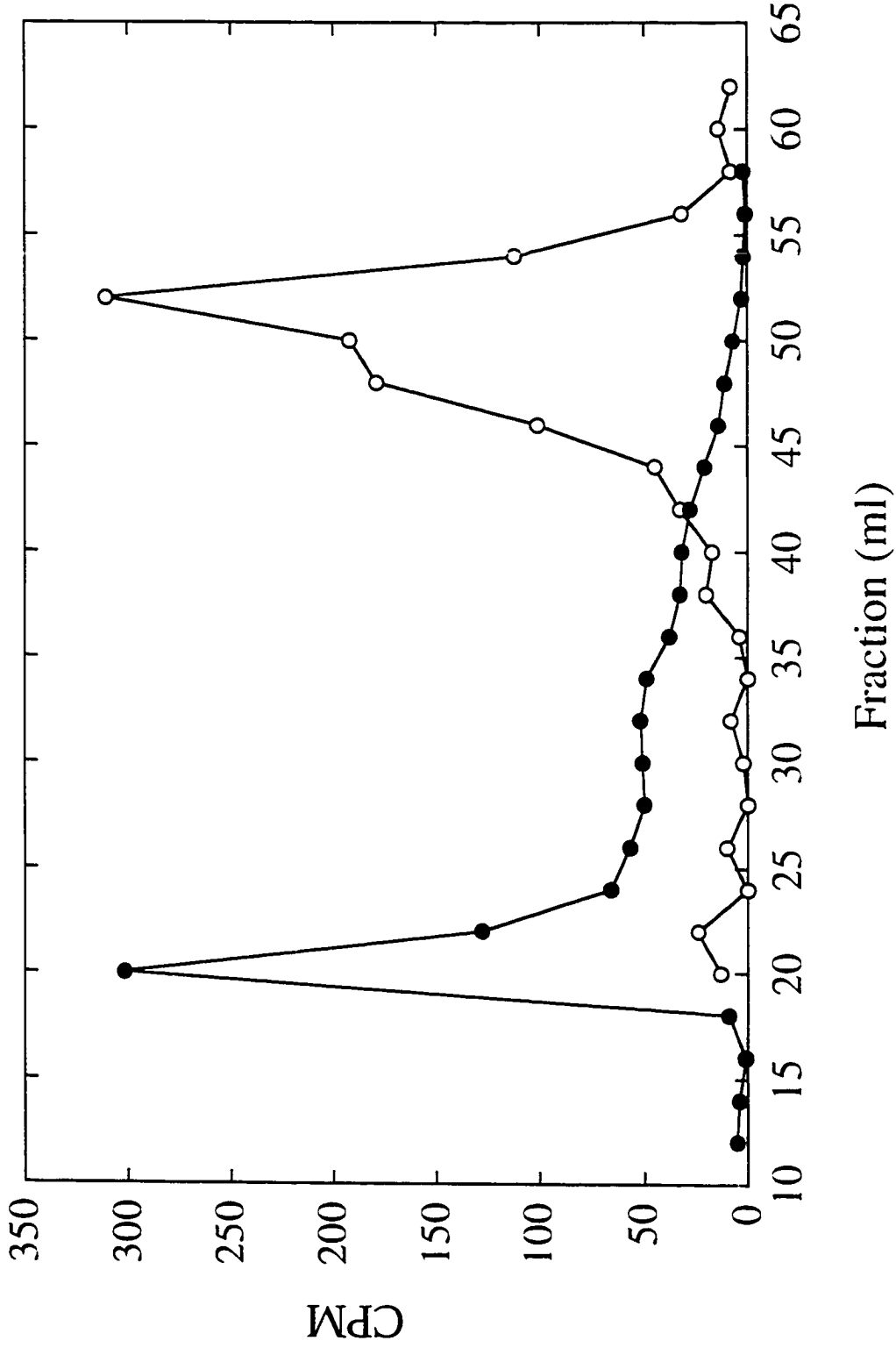


polysaccharides containing GlcUA readily yields aldobiouronic acids because of the strong resistance of the uronidic linkage to hydrolysis with acids (31). When the putative disaccharide was digested with exo-ß-glucuronidase, 75% of the radioactivity was liberated as free glucose (Fig. 2*C*). These results confirmed the presence of a ß-glucuronidic linkage and are consistent with the slower migrating product being the disaccharide GlcUA-ß-Glc.

Additional evidence for the identity of the polysaccharide was obtained by digestion of the high molecular weight polysaccharide with a type 3 polysaccharide-specific depolymerase from *B. circulans*. This enzyme is highly specific for type 3 polysaccharide and has been shown to hydrolyze the ß1,4 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 [^{14}C] 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 [^{14}C] 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 the antibody-conjugated beads bound 99% of both the [^{14}C] Glc-labeled and the [^{14}C] GlcUA-labeled high molecular weight products (data is 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-dependent inhibition of binding of the [^{14}C] Glc-labeled polymer (Fig. 4). No inhibition was observed when unlabeled type 1 polysaccharide was added. All of these results

FIG. 3. **Degradation of high molecular weight product by a type 3 polysaccharide-specific depolymerase.** High molecular weight [^{14}C] Glc-labeled polysaccharide (8400 cpm), synthesized as in Fig. 2, was incubated in 20 mM MES buffer (pH 6.0) with 100 μl 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.



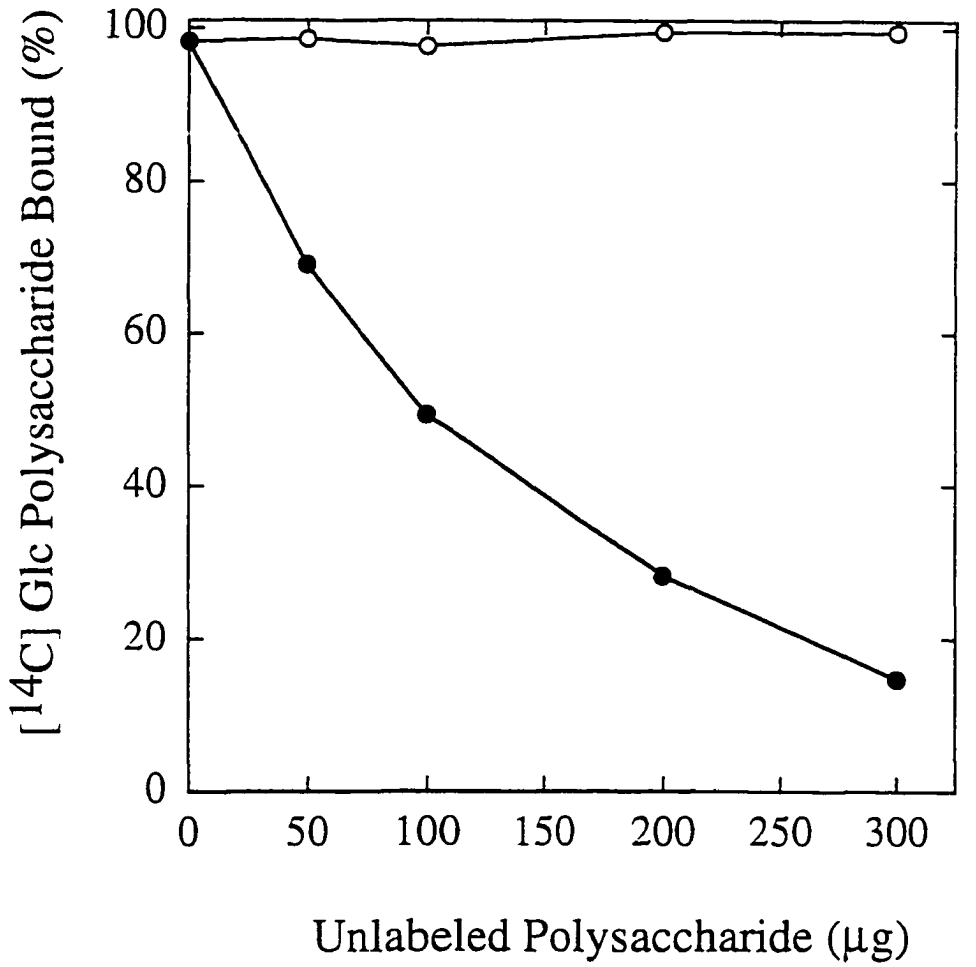


FIG. 4. **Specific binding of product to a type 3 polysaccharide-specific monoclonal antibody column.** High molecular weight [^{14}C] Glc labeled-polysaccharide (2300 cpm), synthesized as in Fig. 2, was mixed with the indicated amounts of either unlabeled type 3 polysaccharide (●) or unlabeled type 1 polysaccharide (○). The samples were applied to affinity columns made with a monoclonal antibody to type 3 polysaccharide, and the percentage of radioactivity that bound to the column was determined as described under "Experimental Procedures."

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 [^{14}C] 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. 6*A*). 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

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 thioglycolate, 10 mM $MnCl_2$, 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, and 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 thioglycolate, and 2% SDS. The pulse (●), 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 percent of the total radioactivity present in the sample. The void and total volumes are indicated as V_i and V_t, respectively.

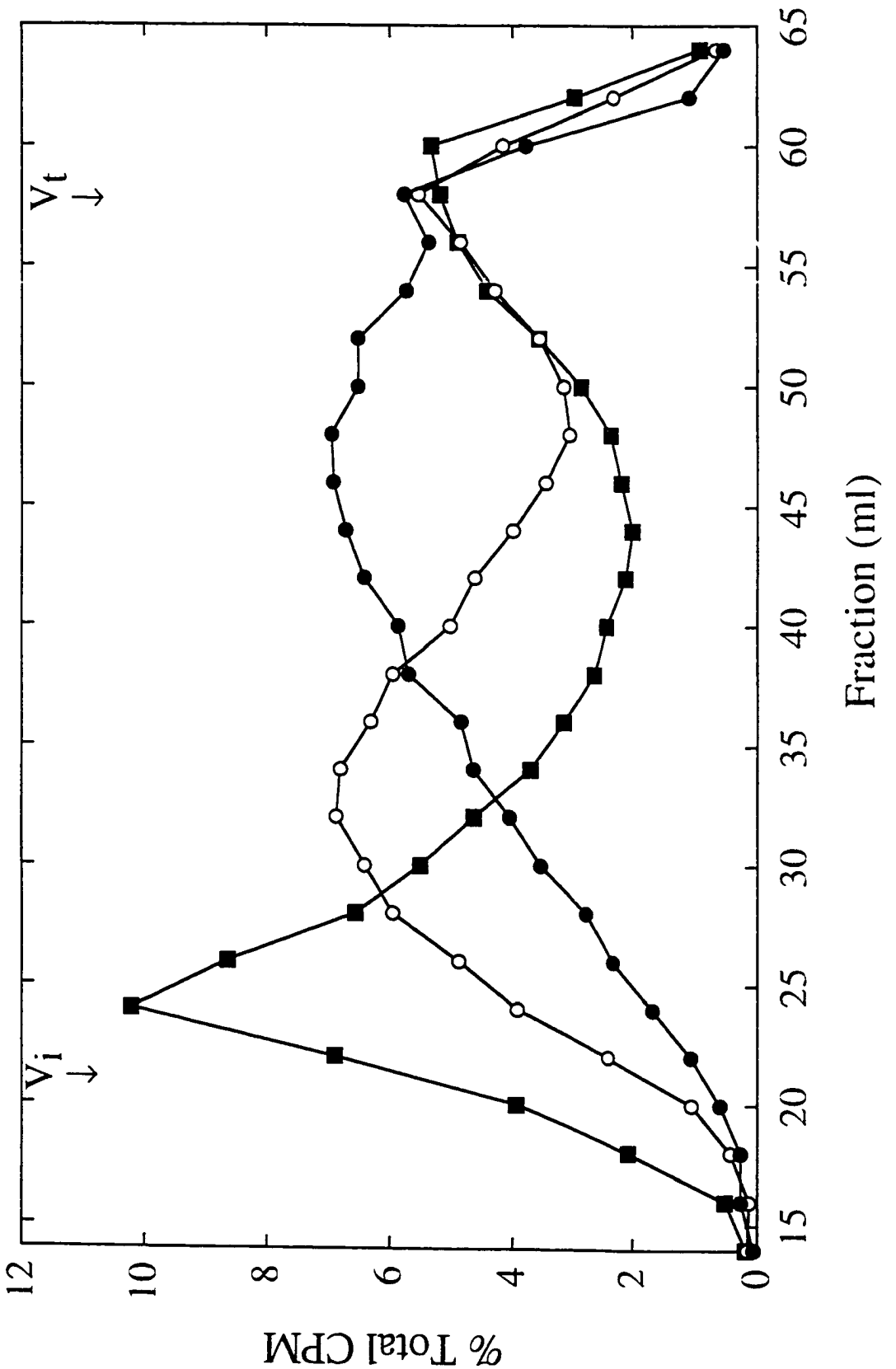
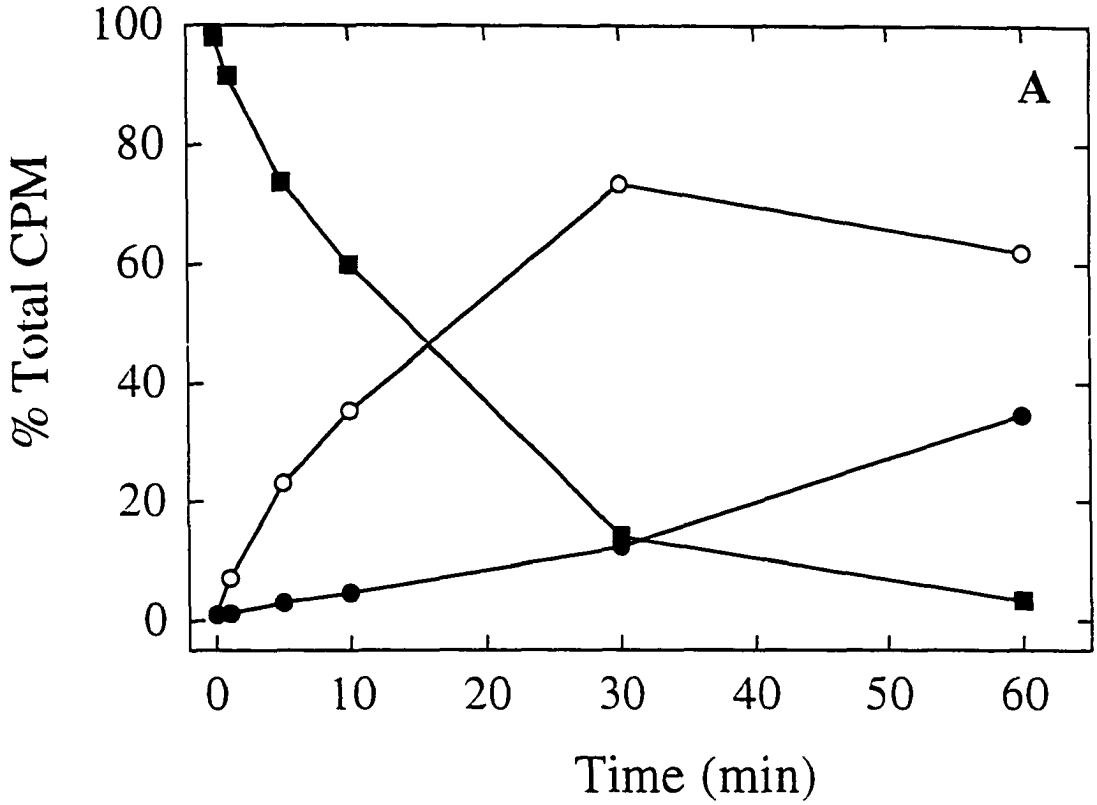
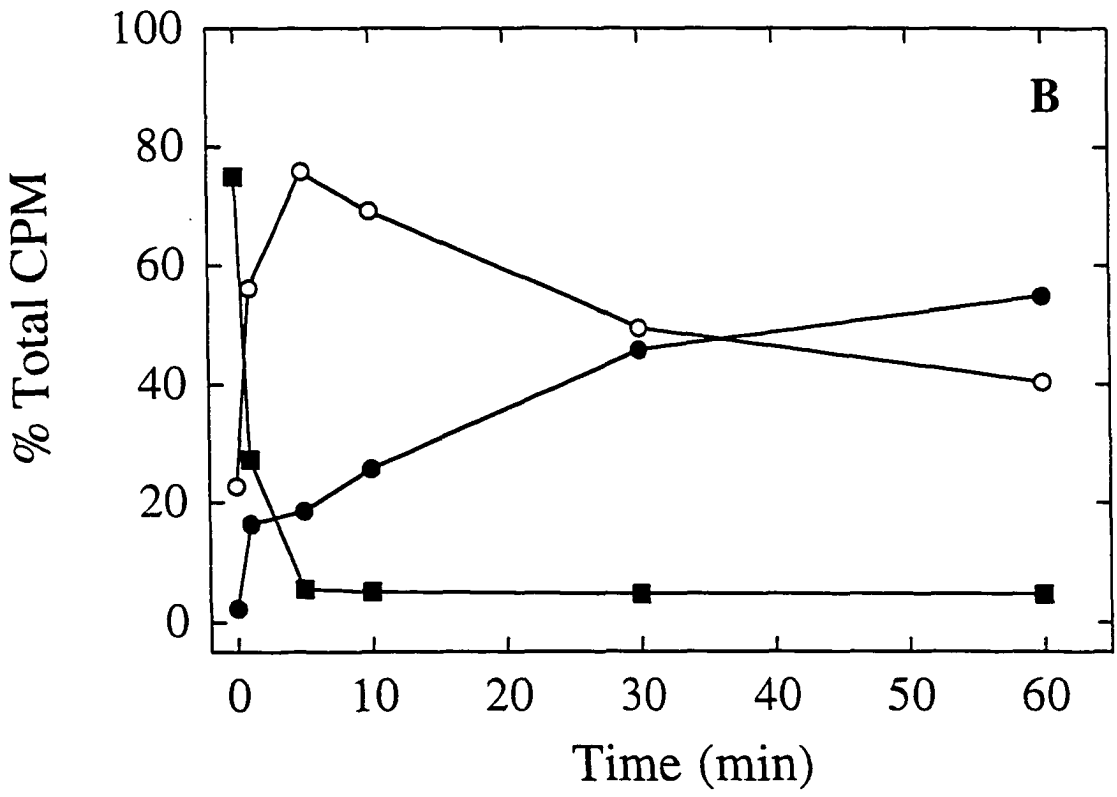


FIG. 6. **Formation of soluble and membrane-associated polysaccharide.** Reactions containing 100 μM UDP-Glc (5 mCi/mmol) and 200 μM UDP-GlcUA (*A*) or 2 μM UDP-Glc (257 mCi/mmol) and 20 μM UDP-GlcUA (*B*) were prepared as described under "Experimental Procedures." Samples (50 μl) were taken after 0, 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-[^{14}C] Glc (■) was determined after ascending paper chromatography in ethanol/1M ammonium acetate (pH 7.0), 7:3 as a percentage of the total radioactivity.



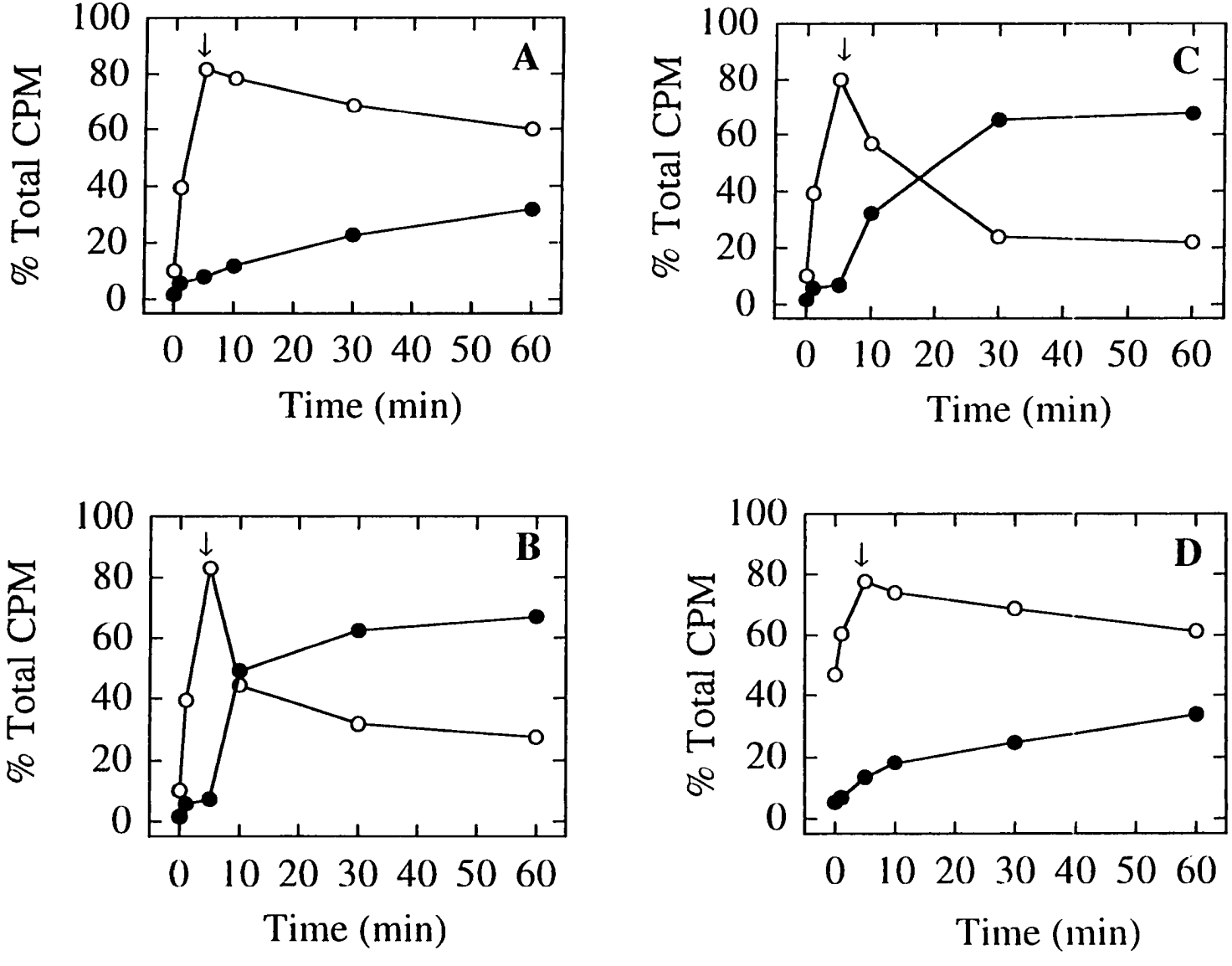


amounts of label were found in the soluble and membrane-associated fractions. Again, Glc was initially incorporated into membrane-associated polymer, which was subsequently released as a soluble form. The soluble and membrane-associated polysaccharides obtained under a variety of conditions were analyzed 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. 6*B* above, except that additional substrate was added 5 min after initiating the reaction. As shown in Fig. 7, the simultaneous addition of both substrates prolonged the association of the polysaccharide with the membrane (Fig 7*A*), whereas the separate addition of either UDP-Glc or UDP-GlcUA markedly stimulated the appearance of soluble polymer (Fig 7*B* and 7*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. 7*D*).

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 [^{14}C] 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

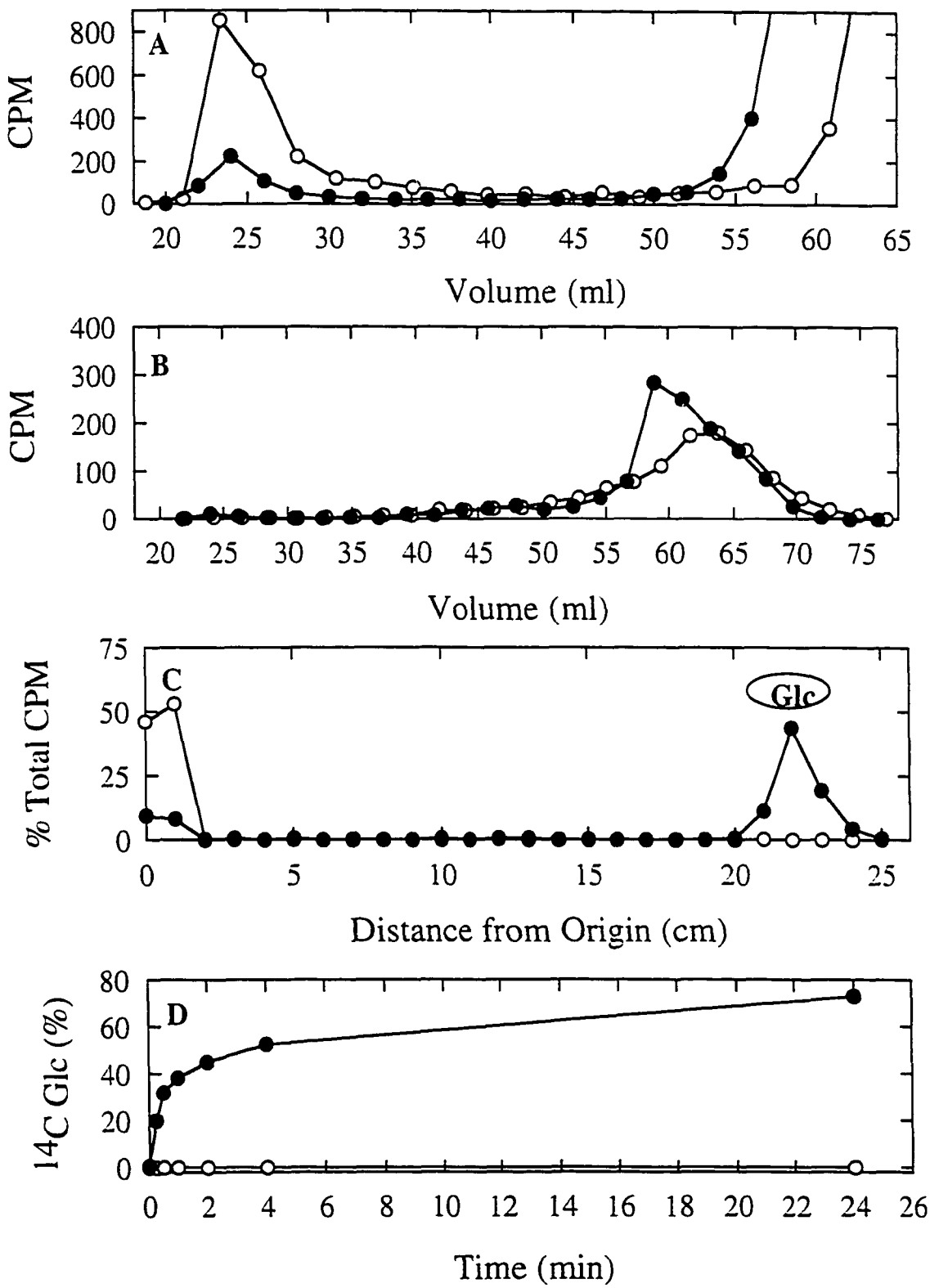
FIG. 7. **The effect of substrate on polysaccharide release**. Reactions were performed as described in Fig. 6 panel *B*, except the following additions were made after 5 min of incubation (see *arrow*): *A*, 400 μM of both UDP-Glc and UDP-GlcUA; *B*, 400 μM UDP-GlcUA; *C*, 400 μM UDP-Glc; or *D*, 1 mM UDP. The radioactivity present as soluble (●) and membrane-associated (○) polysaccharide was determined as described under "Experimental Procedures." The rate of UDP-Glc depletion was similar to that shown in Fig. 6*B*.



radioactivity that corresponded to UDP-Glc (Fig. 8*A*). The polysaccharide product obtained by both of the methods was degraded by the type 3 polysaccharide-specific depolymerase (Fig. 8*B*). Digestion with the depolymerase confirmed that both labeled products were indeed type 3 polysaccharide. Digestion of the terminally-labeled polysaccharide with an exo-ß-glucosidase for 24 h liberated 72.8% of the counts as [^{14}C] Glc. whereas uniformly labeled polysaccharide yielded undectable levels of [^{14}C] Glc after digestion with exo-ß-glucosidase (Fig. 8*C*). The degradation of the terminally labeled polysaccharide with exo-ß-glucosidase was time-dependent (Fig. 8*D*). Because exo-ß-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 non-reducing end.

Terminally labeled polysaccharide was also made by incubating membranes with UDP-[^{14}C] 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-ß-glucosidase treatment of polysaccharide eluted off the affinity column released 75.4% of the counts as [^{14}C] 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.

FIG. 8. **Direction of type 3 polysaccharide growth.** Polysaccharide was labeled either uniformly (○, preparation A) or terminally (●, preparation B) with [^{14}C] Glc as described under "Experimental Procedures." *A*, the labeled polysaccharides were chromatographed on Sepharose 2B, and the amount of radioactivity in the even-numbered fractions was determined. *B*, fractions (18-30 ml) containing the high molecular weight polysaccharide were pooled and concentrated 10-fold by filtration on Amicon YM10 ultrafiltration membranes followed by ethanol precipitation. An aliqout of each polysaccharide was treated with 100 µl of depolymerase for 72 h and then chromatographed on Sepharose 2B. The amount of radioactivity found in the even numbered fractions was determined. *C*, samples of both polysaccharides were treated for 24 h with 1.5 units of exo-ß-glucosidase. The digestions were chromatographed in ethanol (95%)/1 M ammonium acetate (pH 7.0), 7:3, and the amount of radioactivity present in successive 1 cm strips was determined as a percent of the total radioactivity present in all the strips. A background of 20 cpm was subtracted from each strip. The location of Glc on the chromatogram is indicated on the graph. *D*, samples of both polysaccharides were treated with 1.5 units of exo-ß-glucosidase for the times indicated on the graph. The digestions were chromatographed as in *C*, and the amount of radioactivity present as Glc was determined as a percent of the total radioactivity found at the origin and as Glc.



DISCUSSION

The type 3 synthase of *S. pneumoniae* belongs to a family of processive ß-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. cervisiae*, 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 ß-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 ß-glycosyltransferases that would allow for the simultaneous or consecutive formation of two ß-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 Mn^{2+}. Furthermore we have shown that the apparent K_m values for both UDP-Glc and UDP-GlcUA are lower in the presence of Mn^{2+} than in Mg^{2+}. In our standard assay, no significant incorporation of Glc from UDP-Glc occurred in the absence of UDP-GlcUA, and no incorporation of GlcUA from UDP-GlcUA occurred in the absence of UDP-Glc. These data are in agreement with the expected catalytic properties for the formation of a polymer containing alternating glucosyl and glucuronosyl residues. The product was shown to be composed of Glc and GlcUA, and could be degraded by a type 3 polysaccharide specific-depolymerase. Furthermore, the polymer bound specifically to an affinity column made with a monoclonal antibody to type 3 polysaccharide, and no activity was observed with membranes from strains containing an insertion mutation in *cps3S*. We confirmed the presence of ß-linkages by demonstrating sensitivity to both an exo-ß-glucosidase and an exo-ß-glucuronidase. All of these properties are consistent with the product being type 3 polysaccharide.

The type 3 synthase is capable of rapidly forming both glycosidic linkages of type 3 polysaccharide to polymerize UDP-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. Theoretically 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 hyaluronic acid 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 ß-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-ß-glucosidase, indicating that type 3 polysaccharide grows from the nonreducing end. Recent reports on the direction of chain growth for cellulose in *Cladaphora* 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 ß-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 of 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 $MgCl_2$, UDP-GlcNAc, or UDP-GlcUA, was omitted during the chase period. This release of hyaluronic acid was shown to be independent of size (40), an observation that we have also made for type 3 polysaccharide. The selective inhibition of hyaluronate 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,[2] 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 ß-glycans synthesized by related enzymes.

ACKNOWLEDGMENTS

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FOOTNOTES

[1]The abbreviations used are: Cps3S, type 3 synthase; GlcUA, glucuronic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

[2]W. T. Forsee, R. T. Cartee, and J. Yother, manuscript in preparation

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BIOSYNTHESIS OF TYPE 3 CAPSULAR POLYSACCHARIDE IN *STREPTOCOCCUS PNEUMONIAE*: ENZYMATIC CHAIN RELEASE BY AN ABORTIVE TRANSLOCATION PROCESS

by

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SUMMARY

The type 3 polysaccharide synthase from *Streptococcus pneumoniae* catalyzes sugar transfer from UDP-Glc and UDP-GlcUA to a polymer with the repeating disaccharide unit of [3)-ß-D-GlcUA-(1→4)-ß-D-Glc-(1→]. Evidence is presented that release of the polysaccharide chains from *S. pneumoniae* membranes is time-, temperature-, and pH- dependent and saturable with respect to specific catalytic metabolites. In these studies, the membrane-bound synthase was shown to catalyze a rapid release of enzyme-bound polysaccharide when either UDP-Glc or UDP-GlcUA alone was present in the reaction. Only a slow release of polysaccharide occurred when both UDP sugars were present or when both UDP sugars were absent. Chain size was not a specific determinant in polymer release. The release reaction was saturable with increasing concentrations of UDP-Glc or UDP-GlcUA, with respective apparent K_m values of 880 and 0.004 μM. The apparent V_{max} was 48-fold greater with UDP-Glc compared to UDP-GlcUA. The UDP-Glc-actuated reaction was inhibited by UDP-GlcUA with an approximate K_i of 2 μm, and UDP-GlcUA-actuated release was inhibited by UDP-Glc with an approximate K_i of 5 μm. In conjunction with kinetic data regarding the polymerization reaction, these data indicate that UDP-Glc and UDP-GlcUA bind to the same synthase sites in both the biosynthetic reaction and the chain release reaction and that polymer release is catalyzed when one binding site is filled and the concentration of the conjugate UDP-precursor is insufficient to fill the other binding site. The approximate energy of activation values of the biosynthetic and release reactions indicate that release of the polysaccharide occurs by an abortive translocation process. These results are the first to demonstrate a specific enzymatic mechanism for the termination and release of a polysaccharide.

INTRODUCTION

The type 3 capsular polysaccharide is composed of the repeating cellobiuronic acid disaccharide unit [3)-ß-D-GlcUA-(1→4)-ß-D-Glc-(1→] (1), and the antiphagocytic property of this polymer is essential in the virulence of *Streptococcus pneumoniae*. The glucan backbone of type 3 polysaccharide is identical to that for hyaluronic acid; however, the overall structure differs in having the carboxyl groups on the 1-4 linked rather than the 1-3 linked glucosyl units, and there are no acetamido constituents. *In vitro* studies have shown that the biosynthesis of type 3 polysaccharide occurs by the alternate addition of Glc and GlcUA[1] from UDP-Glc and UDP-GlcUA (2), and work in our laboratory has recently demonstrated that growth of the polymer occurs at the non-reducing end (3). Genetic analysis has established that a single open reading frame (*cps3S*) encodes the protein that catalyzes the formation of the glycosidic linkages in the type 3 polysaccharide, and that the predicted Cps3S protein has significant homology to a number of polysaccharide synthases which form polymers composed of β(1-4)linked repeating disaccharide units (4). On the basis of hydrophobic cluster analysis (5), Cps3S has been included as a member of a family of closely related, processive, β-glycosyl-transferases (6). By analogy to hyaluronate synthase from *Streptococcus pyogenes* (7), Cps3S is presumed to have two separate binding sites for the two UDP sugars, with each site catalyzing a distinct glycosidic linkage. Because no export system has been found to exist for type 3 polysaccharide or for most of the other polysaccharide members synthesized by this family of enzymes, it has been suggested that these enzymes catalyze the vectorial extrusion of the growing polysaccharide chain, possibly through a pore or channel (6, 8). Although some evidence has been presented for a role for phospholipids in creating a pore in the hyaluronate synthase complex (9, 10), very little is known about

the binding of the polysaccharide to the enzyme complex or about the mechanism of translocation during polymer formation.

Models of polysaccharide biosynthesis that can explain the mechanism of chain termination and release have not been extensively investigated. Genetic analyses in Gram-negative bacteria have indicated the existence of molecular determinants for the length of the chains attached to the lipid A-core component of the lipopolysaccharide molecule (11). Recent evidence has suggested that the size of mammalian hyaluronate chains may be isozyme-dependent (12). In the synthesis of hyaluronic acid in *S. pyogenes*, Sugahara *et al.* (13) observed that a trichloroacetic acid-insoluble fraction was a precursor to a soluble fraction The selective inhibition by *p*-chloromercuribenzoate of the soluble fraction prompted the suggestion that its release might be an enzymic process. However, there was no detectable difference in the molecular size of these two fractions. Prehm (14) noted that the omission of one obligatory assay component, such as $MgCl_2$, UDP-GlcNAc, or UDP-GlcUA, resulted in a low but distinct shedding of pulse-labeled, mammalian hyaluronate chains. We previously showed that the release of type 3 polysaccharide chains from the membrane-enzyme complex was markedly stimulated when an excess of one UDP substrate was added at the point of depletion of the corresponding UDP-sugar (3). Here, we describe the catalytic nature of this polysaccharide release reaction.

EXPERIMENTAL PROCEDURES

Materials and Analytical Methods. UDP-[^{14}C]Glc (257 mCi/mmol) was obtained from Andotek, and UDP-[^{14}C]GlcUA (338 mCi/mmol) was from ICN. Econo-Safe scintillation cocktail was from Research Products International, Corp. Mutanolysin,

Sephacryl S-500-HR, UDP-Glc, and UDP-GlcUA were purchased from Sigma, Nonidet P-40 was from Calbiochem, and Todd Hewitt Broth and yeast extract were from Difco. Protein was determined with the micro protein determination kit from Sigma. Paper chromatography was carried out on Whatman 3MM paper, and sugar standards were visualized on paper using *p*-anisidine-phthalate (15).

Preparation and Assay of Type 3 Synthase. Membranes containing type 3 synthase were isolated from the encapsulated WU2 *S. pneumoniae* strain (16) as previously described (3). Membranes were stored at -80 °C in a solution containing 100 mM Hepes (pH 8.0), 10% glycerol, and 10 mM sodium thioglycolate, at a protein concentration of 3 mg/ml. Type 3 synthase activity was determined as previously described (3). Reaction mixtures containing 100 mM Hepes (pH 8.0), 10 mM $MnCl_2$, 100 μM UDP-GlcUA, and 100 μM UDP-[^{14}C]Glc (0.02 μCi) were incubated for 10 min at 35 °C with membranes containing 15 μg of protein. The products were separated by paper chromatography with a solvent containing ethanol (95%)/1 M ammonium acetate (pH 5.5), 65:35 (v/v), and quantified by liquid scintillation counting.

Preparation of [^{14}C] Polysaccharide-Synthase Complex. Membranes (1.2 mg of protein) were labeled with [^{14}C]Glc in a reaction mixture consisting of 100 mM Hepes (pH 8.0), 10 mM $MnCl_2$, 25 μM UDP-[^{14}C]Glc (0.8 uCi), and 25 μM UDP-GlcUA in a total volume of 0.8 ml. The mixture was incubated for 5 min at 35 °C, and the reaction was terminated by the addition of 6.0 ml of ice-cold buffer containing 100 mM Hepes (pH 7.5) and 10% glycerol. The membranes were sedimented by centrifugation at

100,000 x *g* for 30 min. The pellets were gently suspended with a loose-fitting teflon pestle in 1.0 ml of wash buffer for 2-3 s on a vortex mixer. The suspension was diluted to 6 ml with wash buffer, and the membranes were collected by centrifugataion at 100,000 x *g* for 30 min. The wash step was repeated a total of five times. Prior to the final centrifugation, the membranes were suspended in 1.0 ml of wash buffer, frozen, thawed, and then diluted to 6.0 ml with wash buffer. The final pellet was suspended in 100 mM Hepes (pH 7.5) and 10% glycerol at a protein concentration of 3.0 mg/ml. The extensive washing was required to remove all of the UDP-GlcUA, which apparently binds with high affinity to the membranes. After washing the membrane pellet five times, the recoveries of synthase activity and enzyme-bound labeled polysaccharide were between 85-90% of the yields obtained prior to these washes. In addition, the chain length distribution of polysaccharides was not changed by the washing procedure. The labeled polysaccharide-synthase complex could be maintained in an active state for up to 2 months by storage at -80 °C.

Polysaccharide substrate of different size chains was synthesized by controlling the nucleotide sugar concentration and the time of incorporation of [^{14}C]Glc or [^{14}C]GlcUA in the biosynthetic reaction. A lower molecular weight polysaccharide substrate complex was prepared as described above, except the reaction mixture contained 1.0 μM UDP-[^{14}C]GlcUA and 25 μM UDP-Glc, and the time of incubation was 3 min. A higher molecular weight polysaccharide substrate complex was prepared as above except the reaction mixture contained 250 μM UDP-[^{14}C]GlcUA and 250 μM UDP-Glc, and the time of incubation was 25 min.

Assay of Polysaccharide Release. [^{14}C] Polysaccharide-synthase complex (20,000 cpm) was incubated at 35 °C in a 100-µl reaction mixture consisting of 100 mM Hepes (pH 8.0), 10 mM $MnCl_2$, and either UDP-Glc or UDP-GlcUA as indicated. The reaction was terminated by placing the reaction tubes on ice and adding 2 volumes of ice-cold buffer containing 100 mM Hepes (pH 7.5) and 10% glycerol. The membranes were sedimented by centrifugation at 100,000 x *g* for 30 min at 4 °C. The supernatants were saved, and the pellets were solubilized in 300 µl of a solution containing 0.1 M NaCl and 0.5% Nonidet P-40. The radioactivity in both the supernatant and pellet fractions was determined by scintillation counting, and the activity was expressed as the percent of the total radioactivity found in the supernatant.

Type 3 Polysaccharide-specific Depolymerase. Depolymerase was partially purified by ammonium sulfate precipitation from the culture medium of *Bacillus circulans* (American Type Culture Collection 14175), as previously described (17). The ammonium sulfate precipitate was dissolved in 0.033 M sodium phosphate (pH 6.0) at a protein concentration of 1.9 mg/ml. Labeled polysaccharide (10,000 cpm) was digested for 1 h at 34 °C with depolymerase (2 µg of protein) in a 200-µl reaction mixture containing 40 mM Mes (pH 6.0) and 0.1% Nonidet P-40. The digest was then heated at 95 °C for 3 min, and the precipitate was removed by centrifugation at 10,000 x *g* for 5 min. The products were analyzed by chromatography on Sephacryl S-500 as described below and by paper chromatography in a solvent of butanol/pyridine/H_2O (44:16:40 v/v/v), and ethanol (95%)/1 M ammonium acetate (pH 7.0) (7:3 v/v). The radioactivity present in 1-cm strips was determined by liquid scintillation counting.

Depolymerase Digestion of Intact Polysaccharide-Synthase Complex. Both unlabeled- and [^{14}C]-labeled polysaccharide-membranes (600 μg) were suspended in 200 μl of 0.1 M Hepes (pH 6.0) and incubated with 2 μg of depolymerase at 34 °C for 10 min. The reaction was terminated by the addition of 3.4 ml of an ice-cold solution containing 0.1 M Hepes (pH 7.5) and 10% glycerol, and the membranes were collected by sedimentation at 100,000 x *g* for 30 min. The membranes were washed two more times in the same manner. The labeled membranes were suspended in 100 μl of the wash buffer and stored at -80 °C. The unlabeled membranes were suspended in a standard reaction mixture, and the polysaccharides were then labeled as described under "Preparation of [^{14}C]Polysaccharide-Synthase Complex."

Sephacryl S-500 Column Chromatography. Gel filtration chromatography was carried out on a 1.4 x 37-cm column of Sephacryl S-500 eluted with a solution consisting of 5 mM Tris (pH 7.5), 200 mM NaCl, 0.1% Nonidet P-40, and 0.02% sodium azide at a flow rate of 20 ml/h. Prior to application to the column, polysaccharide samples were solubilized with 0.2% Nonidet P-40, heated to 100 °C for 3 min, and clarified by centrifugation at 10,000 x *g* for 5 min. Column fractions of 1.0 ml were collected, and 0.33 ml aliquots were mixed with 3.0 ml of scintillation fluid for determination of radioactivity.

RESULTS

Characterization of Polysaccharide-Synthase Complex. To explore the mechanism of chain termination and release, we first prepared a membrane fraction containing labeled polysaccharide-synthase complex by incorporating [^{14}C]Glc from

UDP-[^{14}C]Glc in the presence of UDP-GlcUA, as described under "Experimental Procedures." As shown in Fig. 1, the labeled membranes were free of contaminating radioactive nucleotide, and the product was degraded by digestion with type 3 polysaccharide-specific depolymerase from *B. circulans*, thus confirming the identity of the polysaccharide (17). Incubation of the labeled polysaccharide-synthase membranes in a biosynthetic reaction mixture containing 250 μM UDP-Glc and 250 μM UDP-GlcUA chased the radioactivity into a higher molecular weight product that eluted near the void volume of a Sephacryl S-500 column (Fig. 1). Elongation of the polysaccharide demonstrated that the ^{14}C-labeled chain was still engaged with the active site of the enzyme and that the synthase was still active and able to extend the polymer.

Enzymatic Characteristics of Polysaccharide Release. Incubation of the [^{14}C]polysaccharide-synthase membranes in the presence of either UDP-Glc or UDP-GlcUA alone resulted in rapid release of the polysaccharide from the membranes into the supernatant (Fig. 2). The rate of release was more rapid in the presence of UDP-Glc alone and was linear for 5 min. Release actuated by UDP-GlcUA was linear for up to 10 min. Only a very slow release occurred in the absence of both nucleotide sugars. A slow rate of polymer release was also observed when both nucleotide sugars were present, a condition that supports rapid synthesis of the polysaccharide (3).

Release of polysaccharide in the presence of UDP-Glc was dependent on $MnCl_2$ or $MgCl_2$, whereas release by UDP-GlcUA was not (shown in Table I for $MnCl_2$). In the absence of divalent cation, similar levels of release were observed when both UDP sugars were present or with UDP-GlcUA alone, again indicating a metal ion requirement for UDP-Glc (Table I). UDP-Xyl also actuated polysaccharide release in the presence of

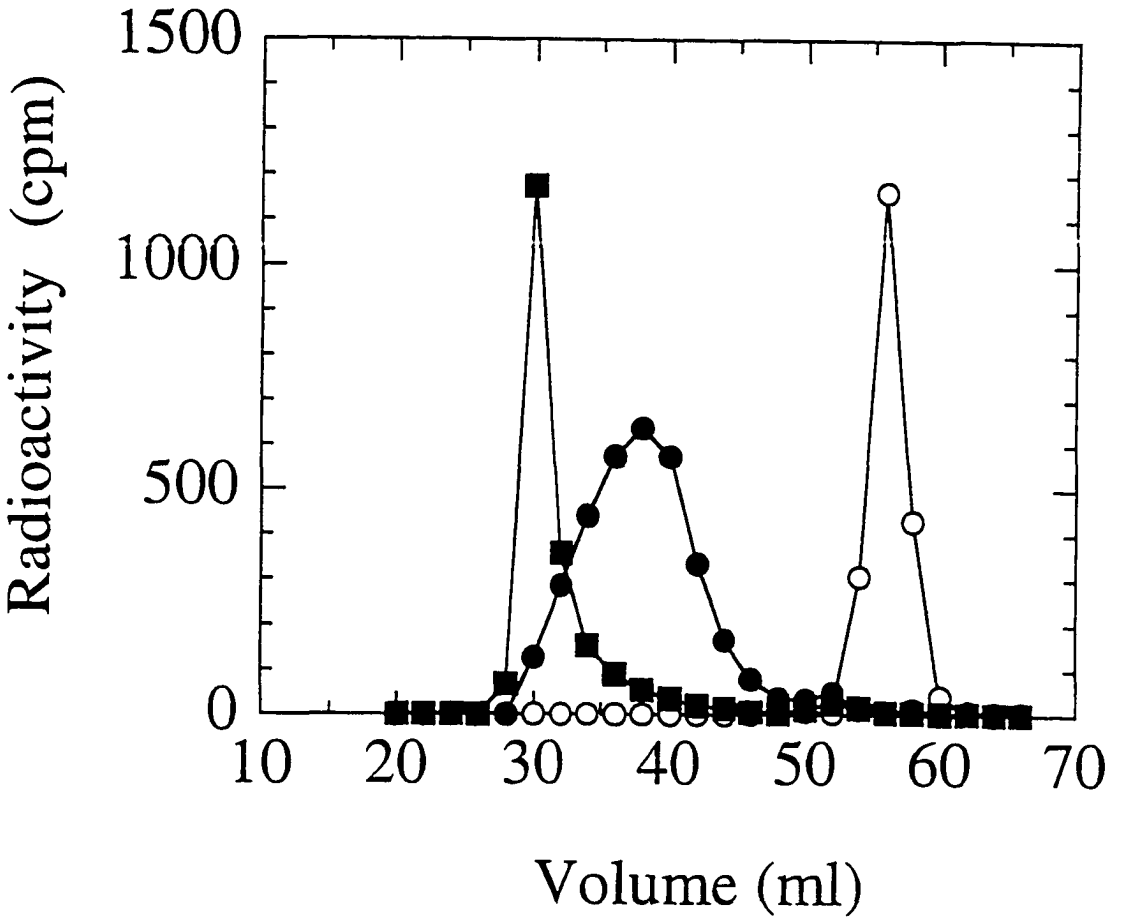


FIG. 1. **Characterization of the [^{14}C]polysaccharide-synthase complex.** Labeled polysaccharide membranes (15,000 cpm), prepared as described for the standard release assay, were solubilized with 0.1% Nonidet P-40 in 300 μl of 40 mM MES (pH 6.0). One half of the mixture was chromatographed on a column of Sephacryl S-500 (●). The remainder was treated with type 3-specific depolymerase and analyzed by Sephacryl S-500 (○). Labeled polysaccharide membranes (15,000 cpm) were also incubated under optimal conditions for synthase activity in a reaction mixture containing 250 μM UDP-Glc and 250 μM UDP-GlcUA. Following incubation for 25 min at 35 °C, the resulting polysaccharide was solubilized with 0.2% Nonidet P-40 and chromatographed on Sephacryl S-500 (■). The total and void volumes are as indicated. Paper chromatography of the depolymerase-digested polysaccharide in butanol/acetic acid/H_2O (44:16:40) confirmed that 75-80% of the radioactivity migrated like a tetrasaccharide.

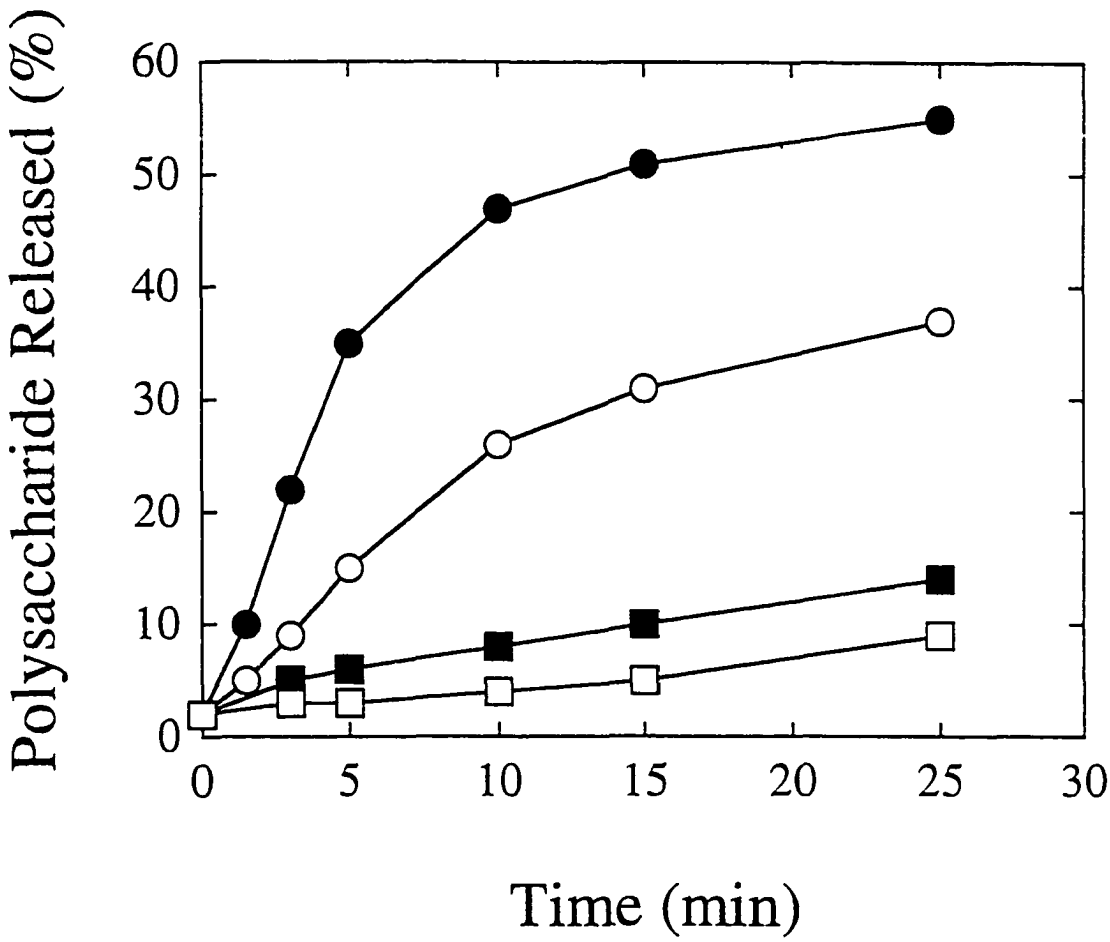


FIG. 2. **Time course of polysaccharide release from membrane-synthase complex**. Washed membranes (225 μg of protein) containing 160,000 cpm of labeled polysaccharide were incubated at 35 °C in a 800-μl reaction mixture containing 0.1 M Hepes (pH 7.5), 10% glycerol, 10 mM $MnCl_2$, and either 20 μM UDP-Glc (●), 20 μM UDP-GlcUA (○), both UDP sugars (20 μM of each) added at 0 min and again at 12 min (■), or no addition (□). Aliquots (100 μl) were withdrawn at the indicated times, placed on ice, and diluted with 200 μl of ice cold buffer containing 0.1 M Hepes (pH 7.5) and 10% glycerol. After centrifugation, the radioactivity was in the membrane and supernatant fractions determined as described under "Experimental Procedures."

TABLE I

Nucleotide specificity and metal ion requirement of polysaccharide release

Labeled polysaccharide-synthase complex (20,000 cpm) was incubated for 10 min at 35 °C in a reaction mixture containing a 50 μM concentration of the indicated nucleotide, 0.1 M Hepes (pH 7.5), 10% glycerol, and 10 mM $MnCl_2$, as indicated. The polysaccharide released from the membranes was separated by centrifugation and quantified as described under "Experimental Procedures."

	Polysaccharide released			
	No $MnCl_2$		+ $MnCl_2$	
Addition	*cpm*	%	*cpm*	%
None	1016	5	623	4
UDP-Glc	921	5	10,107	53
UDP-GlcUA	6165	30	5240	25
UDP-Glc + UDP-GlcUA	4367	23	1594	8
UDP	1197	6	1448	8
UDP-Xyl	952	5	10,560	55

divalent cation. UDP-Xyl did not, however, support synthase activity in combination with UDP-GlcUA, and it inhibited the biosynthetic reaction when both UDP-Glc and UDP-GlcUA were present (data not shown). The presence of UDP did not result in significant release, and the addition of 50 μM UDP-GlcNAc, UDP-Gal, or ADP-Glc resulted in no more release than with UDP (data not shown).

The effect of pH on the release reaction is shown in Fig. 3. Polysaccharide release actuated by UDP-Glc was optimal at pH 7.0 in Hepes buffer. UDP-GlcUA-actuated release was inhibited in Hepes buffer when the pH was less than 8.0, and the optimal pH for this reaction was 7.5 in imidazole buffer as shown. The pH profile for the UDP-GlcUA reaction in Tricine buffer was similar to that for imidazole (data not shown). Both imidazole and Tricine buffers inhibited the UDP-Glc-actuated reaction when the pH

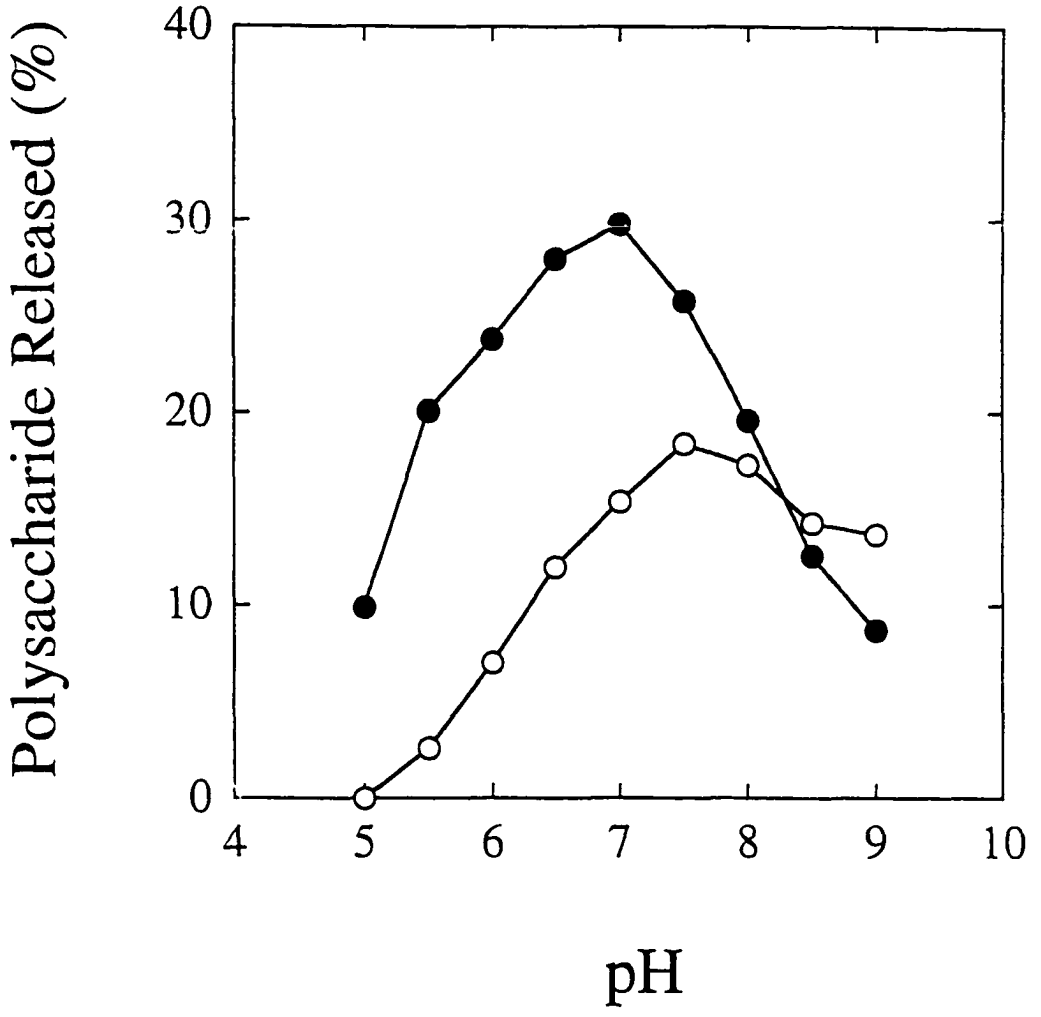


FIG. 3. **Effect of pH on polysaccharide release by UDP-Glc and UDP-GlcUA.** Polysaccharide release was conducted as described under "Experimental Procedures." Reaction mixtures either contained 50 μM UDP-Glc (●) and were incubated at 35 °C for 5 min, or contained50 μM UDP-GlcUA (○) and were incubated for 10 min. Both sets of reaction mixtures contained 0.1 M imidazole/acetate from pH 5.0 to 6.0, 0.1 M Hepes from pH 8.0 to 8.5, and 0.1 M glycyl-glycine from pH 8.5 to 9.0. At pH 6.5 to 7.5, the UDP-Glc and UDP-GlcUA reactions employed 0.1 M Hepes and 0.1 M imidazole/acetate, respectively.

was above 6.0. A number of different buffers were tested, and none provided optimum conditions for both reactions at their optimal pH. The determinations of all catalytic constants for a direct comparison of both the release reaction and the polymerization reaction were therefore carried out at pH 8.0 in Hepes buffer.

Effect of Polysaccharide Length and Other Factors on Release. To assess the effect of chain size on polysaccharide release, synthase complex consisting of either high or low molecular weight polysaccharide was synthesized as described under "Experimental Procedures." The size of the chains was analyzed by chromatography on Sephacryl S-500, following solubilization of the synthase complex with detergent. The low molecular weight chains were included in the column bed and gave a broad profile (Fig. 4*A*), whereas the high molecular weight sample eluted near the excluded column volume (Fig. 4*B*). The gel filtration profiles of the polymer released by UDP-Glc or UDP-GlcUA were similar to the initial polysaccharide profiles, indicating that the release reaction shows no specificity for polysaccharide size. Although both low and high molecular weight chains were readily released, the initial release of the smaller size polymer was more rapid (Fig. 5). To further assess the significance of the chain size in the release reaction, the activation energies of release were determined with low and high molecular weight polysaccharide chains. The Arrhenius plots of the release activity with UDP-GlcUA were linear between 15 and 35 °C for the low and between 20 and 40 °C for the high molecular weight chains. The essentially identical approximate activation energies of 29 and 28 kcal/mol, respectively, indicate that the synthase is nonspecific with regard to polysaccharide chain size. However, at any given temperature, the rate of release was

FIG. 4. **Effect of chain size on polysaccharide release.** Membrane-synthase complex containing [^{14}C]polysaccharide of either low (*A*) or high (*B*) molecular weight was prepared as described under "Experimental Procedures." A sample (20,000 cpm) from each preparation was solubilized with detergent and chromatographed on a column of Sephacryl S-500 (●). Another sample (20,000 cpm) was incubated in a polysaccharide release reaction mixture consisting of either 100 mM Hepes (pH 7.0), 10 mM $MnCl_2$, and 50 μM UDP-Glc for 20 min (○) or 100 mM imidazole (pH 7.5), 10 mM $MnCl_2$, and 50 μM UDP-GlcUA for 30 min (■). The released polysaccharide was separated from the membranes by sedimentation, and the supernatants were chromatographed on Sephacryl S-500.

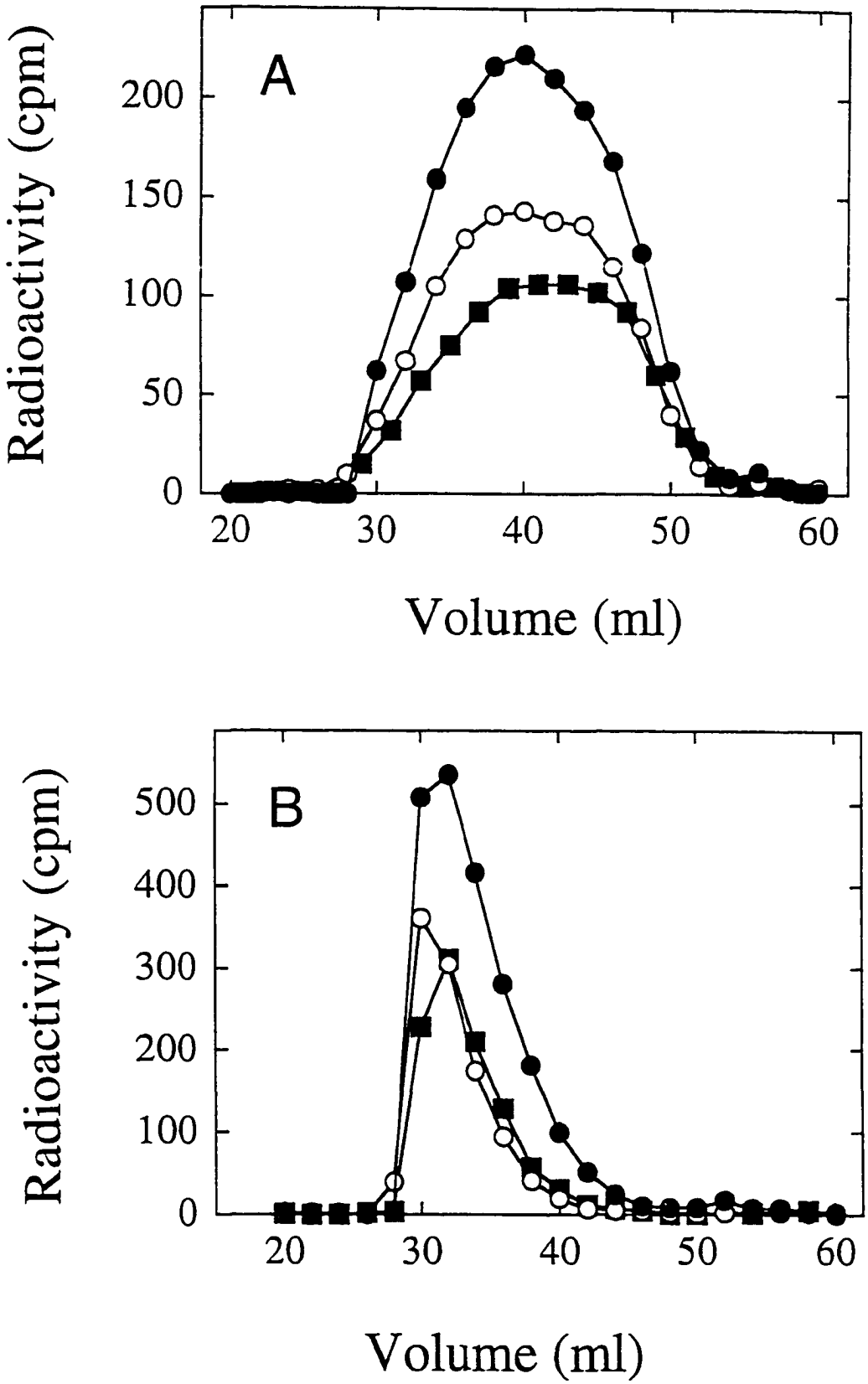
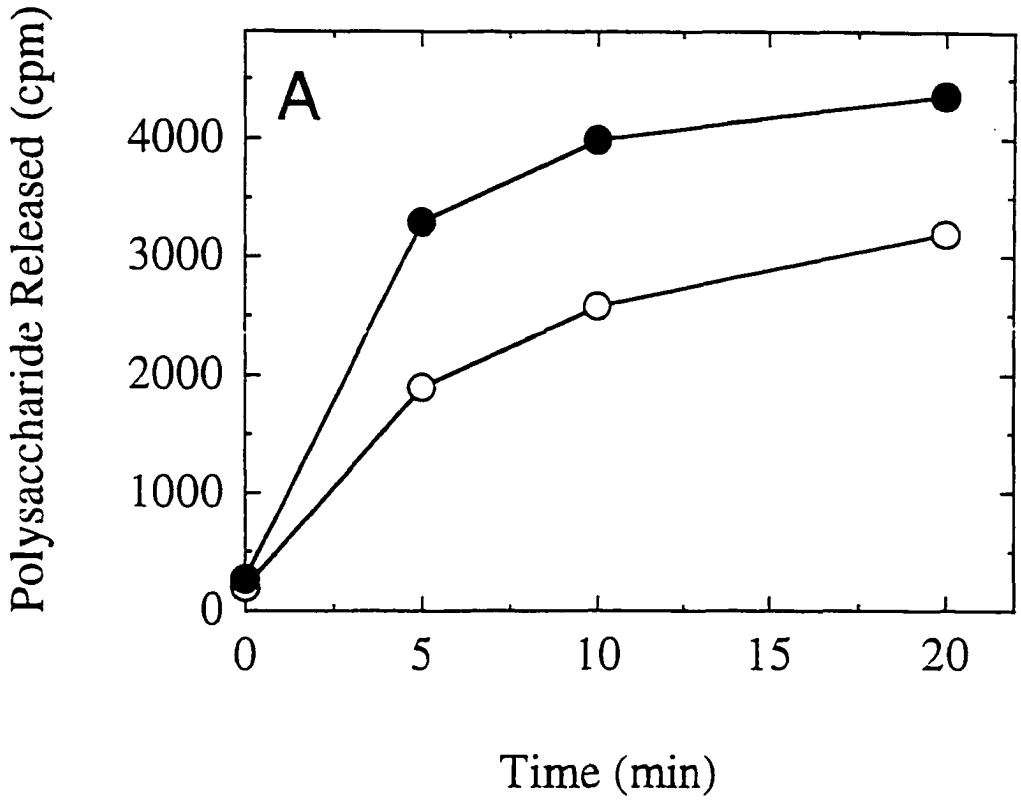
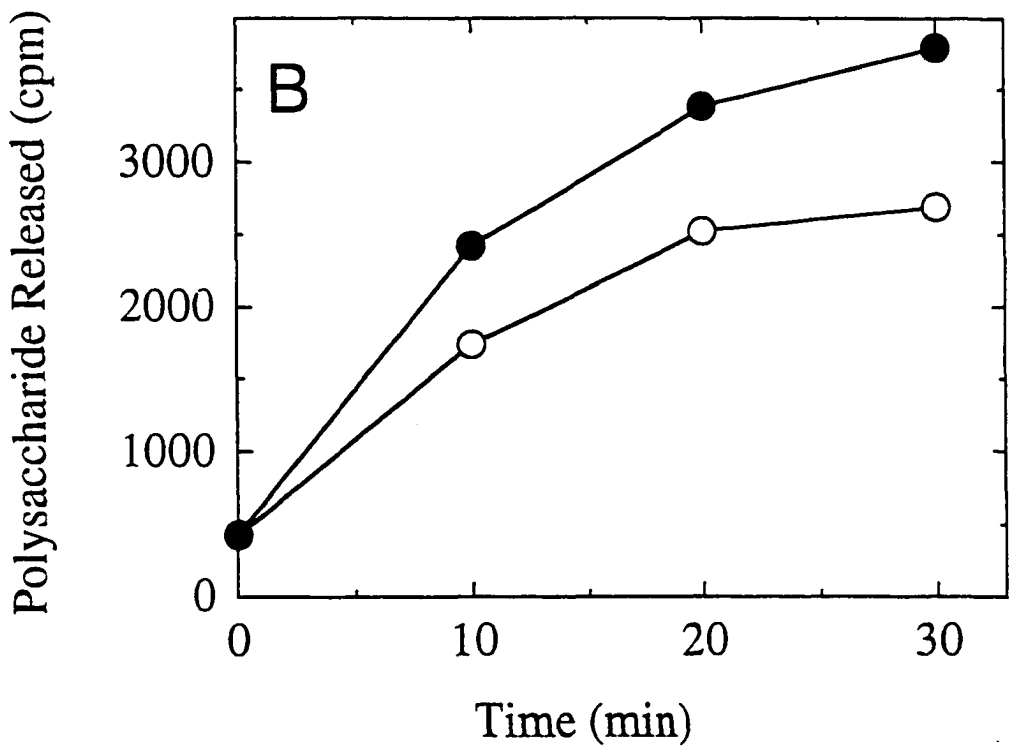


FIG. 5. **Effect of chain size on the rate of polysaccharide release.** Low (●) and high (○) molecular weight polysaccharide-synthase preparations were incubated with either UDP-Glc (*A*) or UDP-GlcUA (*B*), as described in the legend to Fig. 4. At the indicated times, the reactions were terminated, and the amount of released polysaccharide was determined as described under "Experimental Procedures."





more rapid with smaller polymer, demonstrating that the more rapid release with the smaller chains was a thermal property of the reaction.

In the above experiments, complete release of the polysaccharide was not observed, even at higher concentrations of nucleotide sugar (data not shown). Therefore, a possible additive release by the sequential addition of UDP sugars was investigated. Polysaccharide-enzyme complex was first incubated with either UDP-Glc or UDP-GlcUA for 20 min as in Fig. 2, resulting in 54 and 40% release, respectively, of the polysaccharide into the supernatant. After washing to remove the first nucleotide sugar, incubation of the membranes with the same or the alternative UDP sugar resulted in no more than 8% additional release over that observed with the control membranes (data not shown).

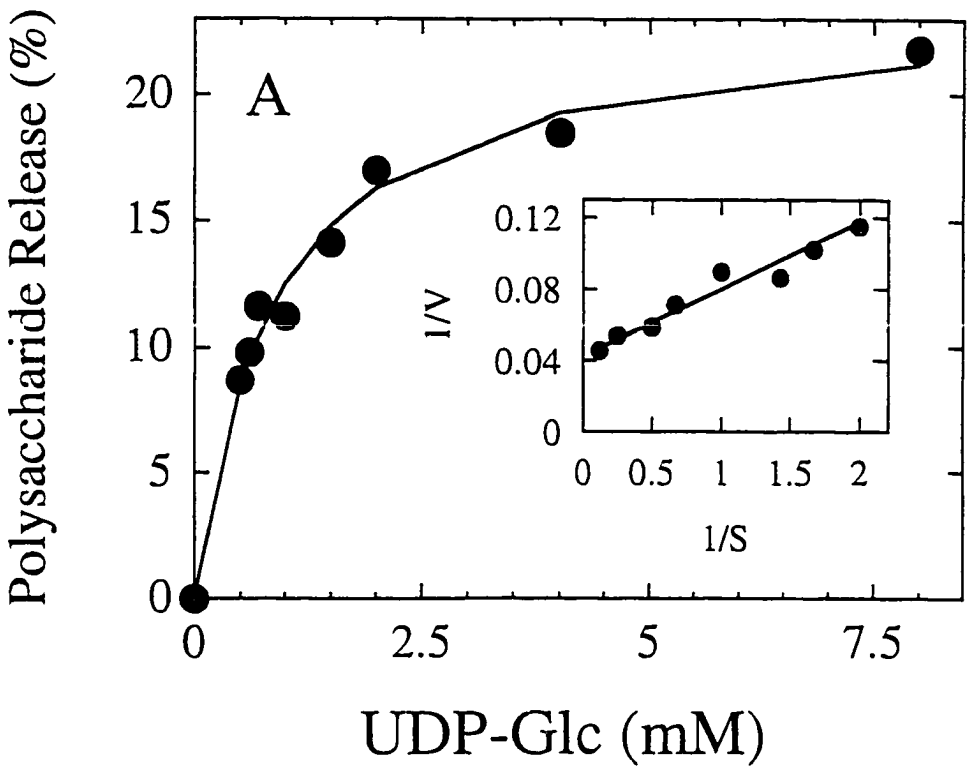
Depolymerase digestion of the synthase complex was employed to explore the possibility that some of the polysaccharide chains might be entangled at their reducing ends or anchored to the membranes, thereby preventing their free release. Labeled polysaccharide membranes were prepared by (*a*) the standard protocol; (*b*) the standard protocol followed by depolymerase digestion; or (*c*) depolymerase digestion followed by the standard labeling protocol. The depolymerase digestion removed 67% of the radioactivity from the labeled polysaccharide chains. Longer digestions did not significantly reduce the amount of membrane-bound radioactivity, indicating that all the accessible length of the chains had been cleaved. Incubation of the membrane preparations for 20 min with 100 μM UDP-Glc resulted in 34, 56, and 34% release, respectively, for preparations *a*, *b*, and *c*. Thus, depolymerase treatment still did not allow complete release of the polysaccharide.

Reaction Constants of Polysaccharide Release. The effect of the concentration of UDP-Glc on the initial rate of polysaccharide release is shown in Fig. 6*A*. The plot exhibited typical hyperbolic kinetics, the double reciprocal plot was linear, and the apparent K_m value was 880 μm. Release of polysaccharide as a function of the concentration of UDP-GlcUA also exhibited hyperbolic kinetics (Fig. 6*B*), and the apparent K_m value was 0.004 μM. The apparent V_{max} value for UDP-Glc release was 48-fold greater than that for UDP-GlcUA, indicating that the release mechanism is much more readily facilitated by saturating levels of UDP-Glc.

Polysaccharide release was inhibited by increasing the concentration of one UDP sugar in the presence of a fixed level of the conjugate UDP sugar (Fig. 7). UDP-Glc inhibited release actuated by UDP-GlcUA, with an approximate K_i of 5 μm. UDP-GlcUA inhibited release actuated by UDP-Glc, with an approximate K_i of 2 μm. It is noteworthy that these values are very similar to the respective apparent K_m values of 12 and 9 μM that we previously reported for UDP-Glc and UDP-GlcUA in the biosynthesis of type 3 polysaccharide (3).

The polysaccharide release reaction was markedly temperature dependent. Below 15 °C there was no significant activity, but above this temperature the activity increased rapidly up to 40 °C. Arrhenius plots of the release activity were linear between 20 and 40 °C, and approximate activation energies of 32 and 28 kcal/mol were calculated for the release reactions actuated by UDP-Glc and UDP-GlcUA, respectively (Fig. 8). The Arrhenius plot for the biosynthetic reaction was linear from 15 to 40 °C, and the approximate activation energy was 17 kcal/mol.

FIG. 6. **Effect of UDP sugar concentrations on the rate of polysaccharide release.** *A*, polysaccharide release by UDP-Glc was measured in a 0.5-min reaction at pH 8.0 as described under "Experimental Procedures." *B*, polysaccharide release by UDP-GlcUA was measured in a 15-min reaction at pH 8.0. Double reciprocal plots are shown in the *insets*. Kinetic parameters for K_m and curve fitting were established with the kinetic program ENZFIT (Elsevier-Biosoft) or by manual graphical methods.



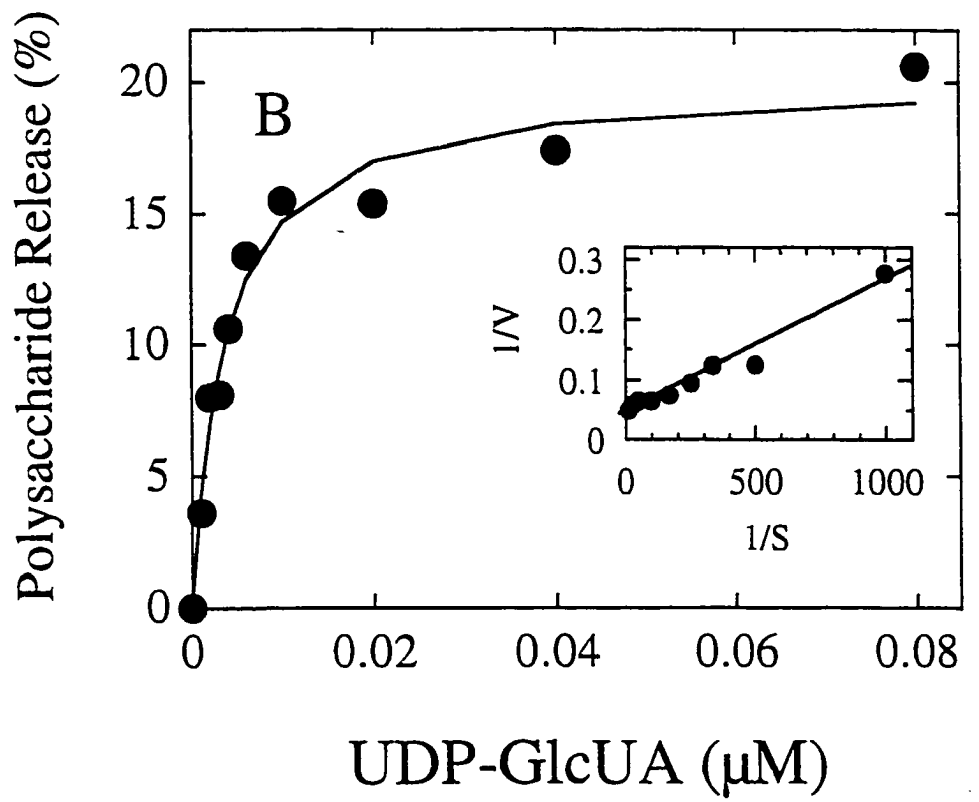
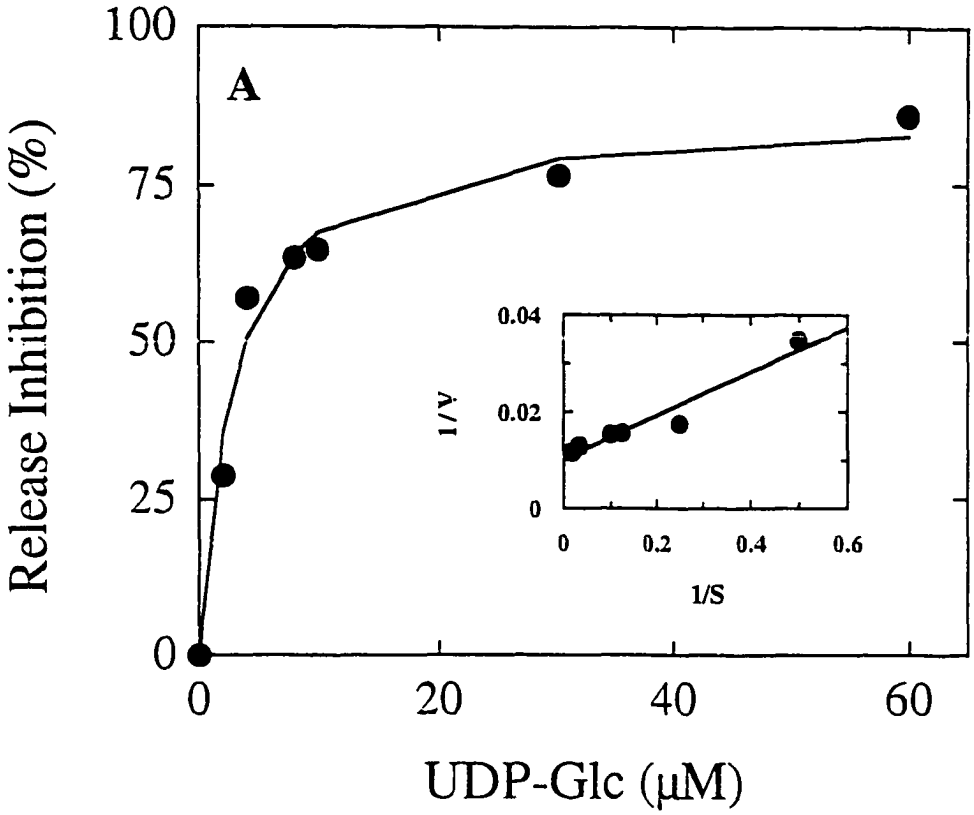


FIG. 7. **Inhibition of polysaccharide release by increasing concentrations of the conjugate UDP sugar.** *A*, polysaccharide release by 100 μM UDP-GlcUA was determined in 10-min reactions at pH 8.0 as described under "Experimental Procedures." Initial rates of release were measured in the presence of the indicated concentrations of UDP-Glc. *B*, polysaccharide release by 100 μM UDP-Glc was determined in 3-min reactions at pH 8.0. Initial rates of release were measured in the presence of the indicated concentrations of UDP-GlcUA. Double reciprocal plots are shown in the *inset*.



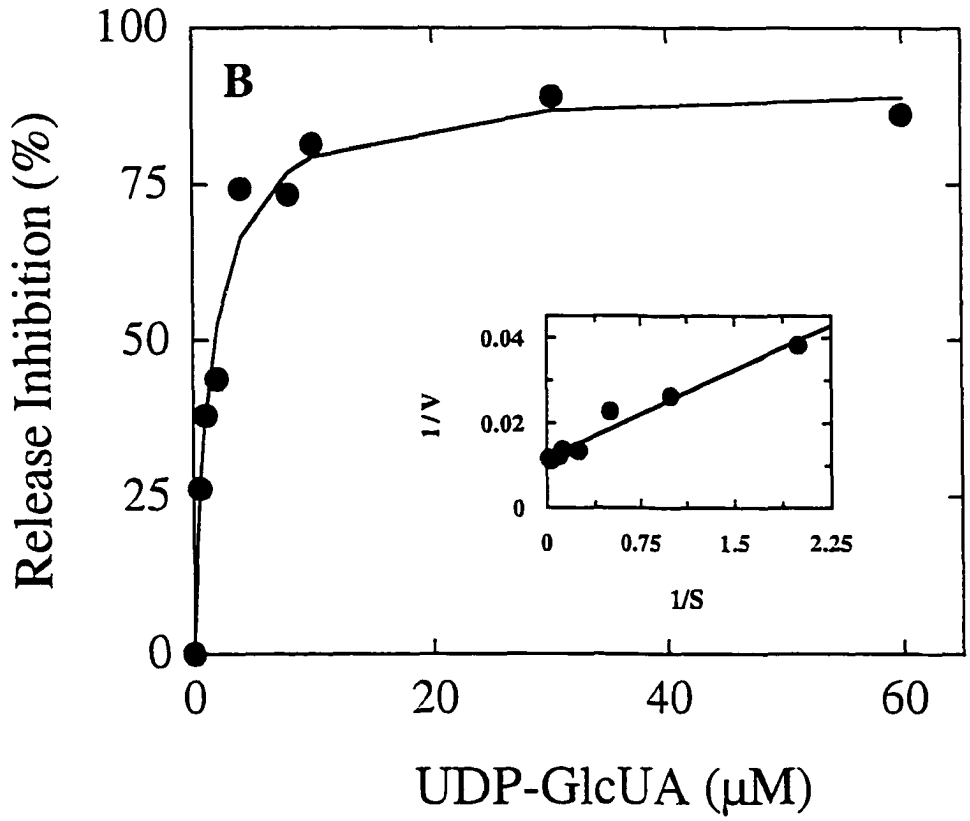
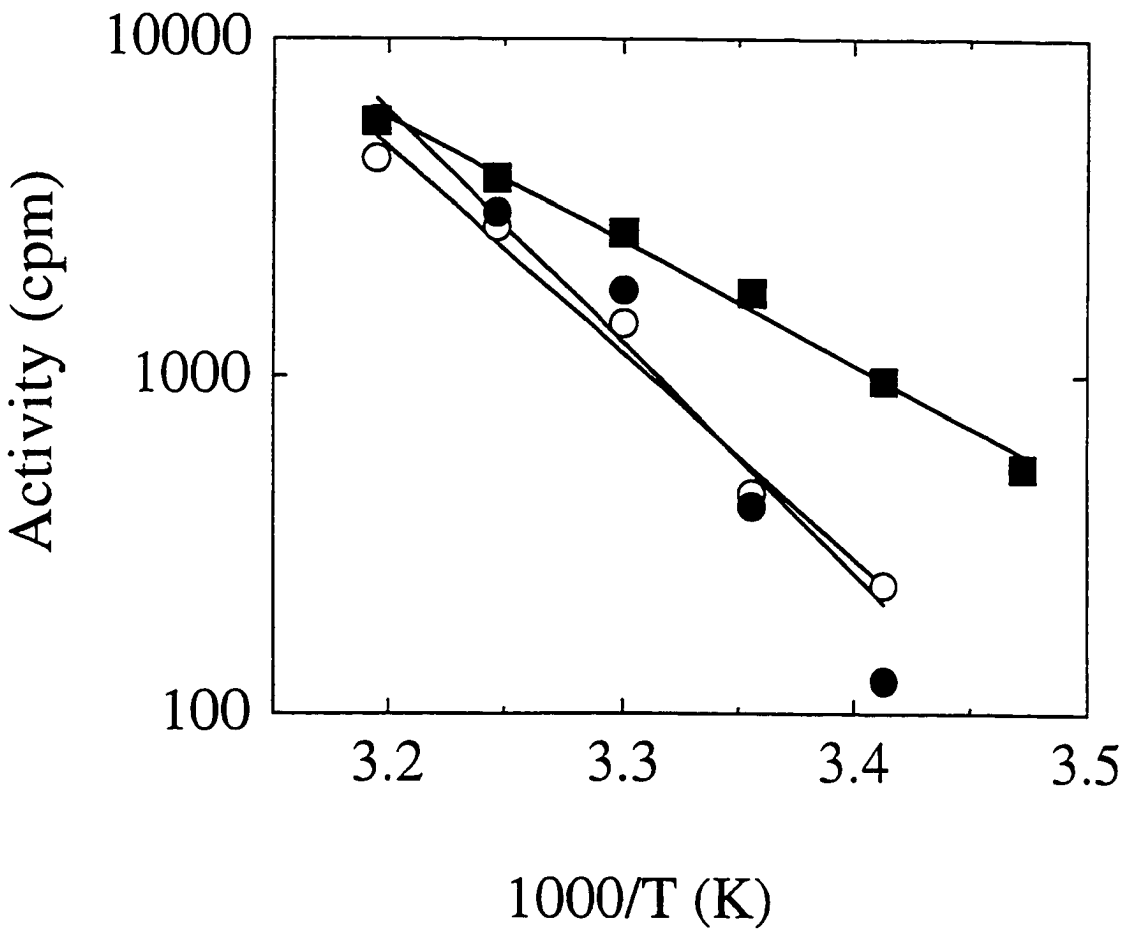


FIG. 8. **Arrhenius plots of the type 3 synthase polymer formation and polysaccharide release activities**. Polysaccharide release in the presence of 50 μM UDP-Glc for 3 min (●), 50 μM UDP-GlcUA for 8 min (○), and type 3 synthase activity (■) was determined by the standard protocols at the indicated temperatures. A control release in the absence of UDP sugar was conducted at each temperature and subtracted from the experimental release values. The lines were fitted by linear regression analysis.



Nascent Chain Release. Release of low molecular weight polysaccharide chains by either UDP-Glc or UDP-GlcUA (Fig. 5), suggested that release of endogenous, nascent polysaccharide chains might also be possible. Incubation of the membranes containing type 3 synthase with either 50 μM UDP-Glc or UDP-GlcUA prior to initiating chain elongation, resulted in a rapid time-dependent inhibition of the synthase activity (Fig. 9). When both UDP sugars were added or when neither was added, the time-dependent inhibition was much slower. The maximum reduction in synthase activity was 66% when the membranes were incubated with UDP-Glc and 63% with UDP-GlcUA, consistent with the comparable maximum release of polysaccharide observed in Fig. 5 and in other similar reactions. These data suggested that endogenous nascent polysaccharide chains are released by the same mechanism. Further, release of nascent polysaccharide chains was concentration dependent, and plots of the inhibition of the polymerization reaction as a function of the concentration of UDP-Glc or UDP-GlcUA were hyperbolic, and the double reciprocal plots were linear (data not shown), yielding respective apparent K_m values of 230 μM for UDP-Glc and 0.015 μM for UDP-GlcUA (Table II). Nascent chain release, as determined by synthase inhibition, also demonstrated the same specificity for other UDP nucleotides as reported above for polysaccharide release (data not shown). These results further confirm the similarity of the release reaction for polysaccharide product and nascent polysaccharide chains.

TABLE II

Reaction constants

The apparent K_m values for polysaccharide release were determined as described in Fig. 6. The approximate K_i values for polysaccharide release were determined as described in Fig. 7. The approximate activation energies (ΔE) were determined as described in Fig. 8. The apparent K_m values for nascent chain release were determined as described in Fig. 9. Labeled UDP-GlcUA was incorporated in the synthase assay when the concentration of UDP-Glc was varied to actuate release. Conversely, labeled UDP-Glc was incorporated when UDP-GlcUA was varied.

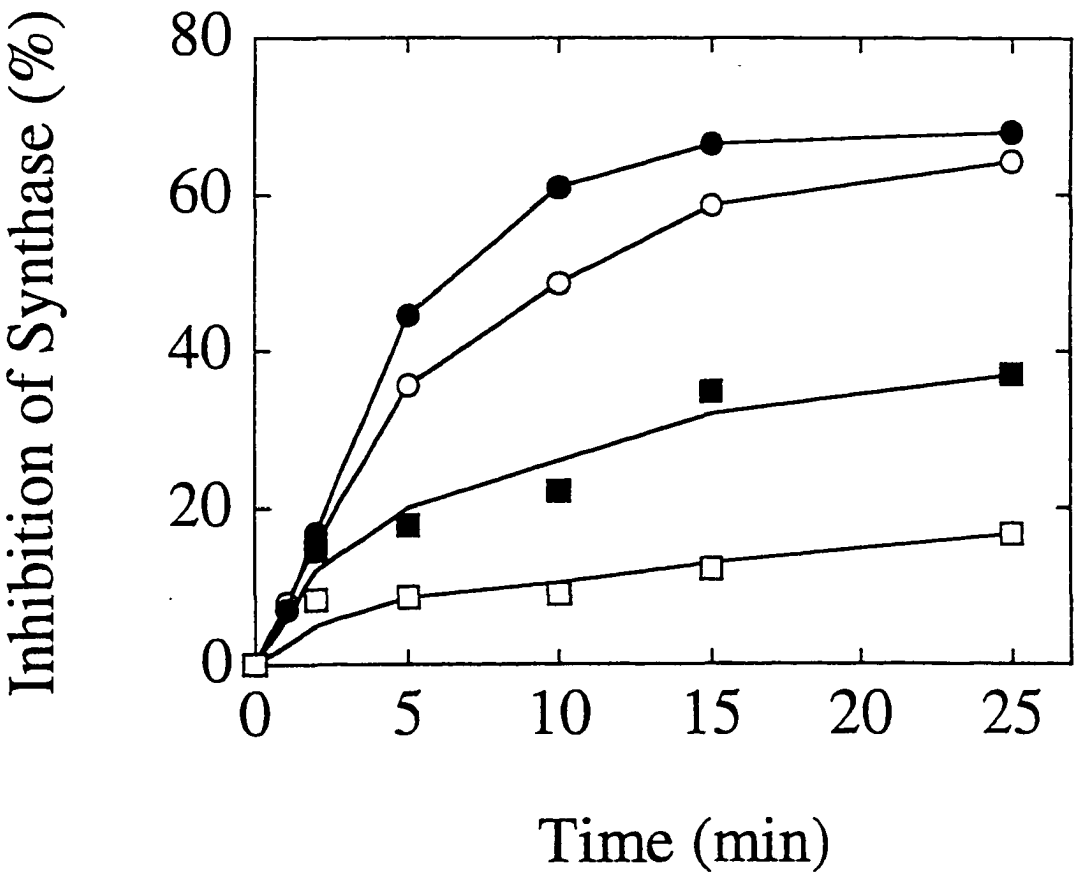
Reaction	Nucleotide sugar	K_m	K_i	ΔE
		μM	μM	Kcal/mol
Polysaccharide release	UDP-Glc	880	5	32
Polysaccharide release	UDP-GlcUA	0.004	2	28
Nascent chain release	UDP-Glc	230		
Nascent chain release	UDP-GlcUA	0.015		
Polymerization	UDP-Glc	12[a]		17
	+			
	UDP-GlcUA	9[a]		

[a] Reported in Ref. 3.

DISCUSSION

There has been considerable progress in the understanding of the biosynthesis of a variety of carbohydrate polymers, however, most investigations have provided relatively little information with regard to possible mechanisms of termination and release of The results obtained here indicate that chain release can occur by an enzymatic mechanism that may provide insight into the translocation process of the polymerization reaction.

FIG. 9. **Nascent polysaccharide chain release as determined by the inhibition of polymer formation.** Membranes were incubated at 35 °C in a 100-μl reaction mixture consisting of 100 mM Hepes (pH 8.0), 10 mM $MnCl_2$, and 50 μM UDP-Glc (●), 50 μM UDP-GlcUA (○), both UDP-sugars at 50 μM of each (■), or no addition (□). At the indicated times, 10-μl samples were removed and assayed for synthase activity as described under "Experimental Procedures."



Indeed, the release reaction appears to be a futile attempt to translocate the growing polysaccharide chain when only a single substrate binding site is occupied.

Inhibition of the release reaction occurred at K_i values similar to the biosynthetic K_m values, consistent with the hypothesis that both polymerization and release are catalyzed by interaction of the UDP sugars with the same set of binding sites on the synthase. These results indicate that polysaccharide release occurs when one UDP sugar is present at a concentration sufficient to bind to its active site, and the concentration of the conjugate UDP-precursor is insufficient to bind to the other substrate binding site. As the concentration of the conjugate UDP-nucleotide is increased above its K_i, the rate of polysaccharide release is rapidly reduced, apparently as the normal polymerization cycle is re-established. Because the type 3 synthase has not been purified, it is conceivable that the release reaction involves either an additional protein interacting with the synthase or that the synthase contains additional UDP-precursor binding sites. However, all of the data are readily explained by a single peptide with a single set of binding sites for UDP-Glc and UDP-GlcUA, whereby binding of either UDP-sugar would trigger release of the polysaccharide chain by either: (*a*) constrainment of the enzyme upon binding of the nucleotide sugar, (*b*) hydrolysis of the nucleotide sugar, or (*c*) transfer of the sugar to the polysaccharide.

The mechanism of polymerization for type 3 polysaccharide is not known in any detail, but a polymer translocation step, as has been postulated in the formation of other ß-glycans (5, 18) is a likely candidate as the common mechanistic step that would incrementally move the polymer between disaccharide additions during the biosynthetic reaction or, alternatively, eject the polymer in the release reaction. If detachment of the polymer from the carbohydrate binding site were to occur following the binding of a

single UDP-precursor or alternatively as a consequence of the addition of a single monosaccharide, the chain may be unable to reattach if it either now lacked the appropriate recognition sequence or had been shifted too far out of alignment with the enzyme binding site. It has been suggested that the driving force for polymer translocation could be generated by the hydrolysis of the pyrophosphoryl linkage of the nucleotide sugar. However, no evidence has been presented for this hypothesis, and it is possible that the energy could be derived from constrainment of the enzyme upon binding of the nucleotide sugar. The experimental activation energy for the polymerization reaction was about half that observed for the release reaction, suggesting that the rate-limiting process is greatly facilitated when two nucleotide sugars, rather than one, are bound to the synthase. These data are then consistent with the binding of each of the nucleotide sugars contributing approximately equally to the activation energy postulated to drive the translocation step in the polymerization sequence.

We have so far been unable to demonstrate that the abortive translocation of polysaccharide chains is accompanied by the addition of either Glc or GlcUA (unpublished data). However, considering the low level of isotope that would be incorporated by the addition of a single monosaccharide and the increased background incorporation that occurs when the level of protein is necessarily scaled up, we can not exclude the possibility that a low level of transfer to the released chain does take place. Nevertheless, release of the polysaccharide accompanied by the transfer of a single sugar seems unlikely for two reasons. First, the triggering of translocation due to the formation of either a single glucosidic or a single glucuronidic linkage, although not inconceivable, does not readily fit the current model of growth of ß-glycans by repetitive disaccharide

addition (5, 18). Second, the observed release of polysaccharide by UDP-Xyl does not support this mechanism, because xylose is not incorporated during polymer formation.

In contrast to the K_i values, the respective apparent K_m values for UDP-Glc and UDP-GlcUA were dramatically different in the abortive translocation reaction and the polymerization reaction. It would appear to be significant that the K_m value for UDP-Glc is 2 orders of magnitude higher and that for UDP-GlcUA is three orders of magnitude lower. To the extent that the apparent K_m values reflect binding affinities, these data suggest that the binding of UDP-Glc greatly decreases the affinity of the synthase for UDP-GlcUA and that the binding of the latter greatly enhances the affinity of the former. Although the chemistry of the release reaction is still undefined, the translocation step would presumably be slow in comparison to the association and dissociation of the nucleotide sugars. Hence, the K_m values would be predicted to be similar to the dissociation constants and should reflect the affinities of the synthase for the nucleotide sugars.

Previous investigations of *S. pyogenes* hyaluronate biosynthesis have noted a sigmoidal response to increasing concentrations of UDP-GlcNAc (19, 20), and the latter have interpreted these results as evidence of cooperativity and "cross-talk." The possibility of a cooperative interaction of the two UDP sugar precursors has also been suggested in view of the enhanced photoaffinity labeling of *S. pyogenes* hyaluronate synthase with [^{32}P]5-azido-UDP-GlcNAc in the presence of UDP-GlcUA (21). We have also observed sigmoidal kinetics in our system when the concentration of one UDP-precursor is varied in the presence of the conjugate UDP sugar. However, under some conditions this effect could readily be the consequence of a decrease in abortive trans-

location as the concentration of the nucleotide sugar is increased. This added complication precludes any simple interpretation of those data.

Nascent polysaccharide chains, which are present in our membrane preparations and which serve as primer in the polysaccharide elongation assay, were also released under the identical conditions and by the same apparent mechanism as the elongated [^{14}C]polymer. We have no evidence to indicate that *S. pneumoniae* membrane preparations are able to self-prime under the conditions of our assay, and following the release of some of the chains, all of the remaining polymerization activity appeared to occur by the elongation of pre-existing chains, without any evidence of the initiation of new low molecular weight chains. Taken together, the similarity of the conditions which actuate release of the nascent polysaccharide and also of the elongated [^{14}C]polymer strengthen the hypothesis that both polymerization and release are catalyzed by the interaction of the UDP sugars with the same set of binding sites on the synthase. The failure to achieve complete release of polysaccharide from the membranes, even after depolymerase treatment to free potentially tethered chains, is at present an unexplained observation. It is perhaps significant that clinical type 3 isolates produce both cell-bound and released forms of the polysaccharide and the fraction of each varies with the strain under study (22, and unpublished data). An understanding of the physiological relevance of the *in vitro* release will thus be an important step in understanding type 3 polysaccharide biosynthesis.

ACKNOWLEDGMENTS

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FOOTNOTES

[1]The abbreviations used are: GlcUA, glucuronic acid; Cps3S, type 3 synthase; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tricine, N-[2-hydroxy-1, 1-bis(hydroxymethyl)ethyl]glycine.

[2]W. T. Forsee, R. T. Cartee, and J. Yother unpublished data.

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CHARACTERIZATION OF THE *STREPTOCOCCUS PNEUMONIAE* TYPE 3 SYNTHASE EXPRESSED IN *ESCHERICHIA COLI*: EVIDENCE FOR *IN VITRO DE NOVO* SYNTHESIS OF TYPE 3 POLYSACCHARIDE

by

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In preparation for *Journal of Biological Chemistry*

Format adapted for dissertation

SUMMARY

Type 3 polysaccharide from *Streptococcus pneumoniae* is composed of the repeating subunit [3)-β-D-GlcUA-(1->4)-β-D-Glc-(1->]. Activity of the enzyme responsible for its synthesis, the type 3 polysaccharide synthase, has been well characterized in its native membrane. Here, we describe characterization of the synthase in *Escherichia coli* and note distinct differences from the *S. pneumoniae* system. Antibodies directed to the C-terminus of the synthase recognized a 40 kDa band in both wild-type *S. pneumoniae* and in *Escherichia coli*, where low levels of type 3 polysaccharide were detectable. Membrane-localized activity of the *E. coli*-expressed synthase was essentially the same as that reported for *S. pneumoniae* with regard to metal ion, pH, and apparent K_m values for UDP-Glc and UDP-GlcUA. In contrast to the *S. pneumoniae* membrane system, where incubation of membranes with a single substrate results in release of approximately 60% of the polysaccharide and inhibition of synthase activity, all of the polysaccharide could be released from the *E. coli* membrane-enzyme complex, and activity was not inhibited. When *E. coli* membranes containing ^{14}C-labeled polysaccharide of intermediate size were incubated with either UDP-Glc or UDP-GlcUA, followed by incubation with UDP-[^{3}H]Glc, the resulting high molecular weight product was labeled with ^{3}H only. These results indicate that incubation with a single substrate disengages all the polysaccharide from the *E. coli*-expressed enzyme, and synthesis can reinitiate in the absence of preformed polysaccharide. The ability to release all of the polysaccharide and to synthesize *de novo* are in distinct contrast to the observations with *S. pneumoniae* membranes. These results may suggest differences in modification or processing of the enzyme in the two systems, in the availability of a primer, or in the control of *de novo* synthesis by *S. pneumoniae*.

INTRODUCTION

The type 3 capsular polysaccharide of *Streptococcus pneumoniae* is an essential virulence factor that serves to protect the bacterium from opsonophagocytosis. Synthesis of this polymer, which is composed of the repeating subunit [3)-β-D-GlcUA-(1->4)-β-D-Glc-(1->] (1), requires only a single glycosyltransferase. This enzyme, termed the type 3 synthase, utilizes UDP-Glc and UDP-GlcUA[1] to form both glycosidic linkages of type 3 polysaccharide (2, 3). The type 3 synthase shares significant homology with a number of ß-glycosyltransferases, including the hyaluronic acid synthases from prokaryotes and eukaryotes, the cellulose synthases from plants and bacteria, the chitin synthases from yeast, and the Nod factor synthases from *Rhizobium* (2). Synthesis of polymers by this group of glycosyltransferases is believed to occur by a common mechanism that involves dual addition of the sugars to the growing polysaccharide chain (4). Following addition, the resulting release of two molecules of uridine diphosphate (UDP) is postulated to provide the necessary energy to translocate the growing polysaccharide and allow two more nucleotide sugars to bind (5).

The mechanism of type 3 polysaccharide synthesis has been well characterized in pneumococcal membranes. These studies have demonstrated that growth of the polymer occurs by alternate addition of Glc and GlcUA to the nonreducing end of the polysaccharide chain. The growing polysaccharide was shown to remain associated with the enzyme during its synthesis, indicating a processive mechanism of synthesis (6). Release of the polysaccharide from the enzyme occurred upon depletion of one of the substrates. Further characterization of polysaccharide release demonstrated that the process was actuated upon incubation with a single substrate and was dependent on time, temperature, and substrate concentration. These data indicated that the process was

enzymatic and possibly the result of an abortive attempt to translocate the polymer. These studies also demonstrated that, once the polysaccharide chain was released, the synthase was not capable of reinitiating synthesis, suggesting that some unknown factor is required to initiate polysaccharide synthesis (7). Although polysaccharide release has not been examined in any detail with any of the homologous glycosyltransferases, expression of the hyaluronic acid synthase from *S. pyogenes* and DG42 from *Xenopus laevis* in yeast indicates that these enzymes are capable of *de novo* synthesis (8). Additionally, hyaluronic acid synthesis in both *S. pyogenes* and teratocarcinoma cells has been shown to occur independent of a lipid or endogenous oligosaccharide primer (9-13).

High level expression of the type 3 synthase in *E. coli* has proven difficult due to the toxicity of the protein. Recently, Arrecubieta *et al.* cloned and expressed the type 3 synthase in *E. coli* but were able to produce low levels of the protein and did not characterize type 3 synthase activity (14). Here, we report the expression and characterization of the type 3 synthase in *E. coli* and describe new findings regarding the mechanism of synthesis.

EXPERIMENTAL PROCEDURES

Materials and Analytical Methods. UDP-[^{14}C]Glc (257 mCi/mmol) was obtained from Andotek, UDP-[^{14}C]GlcUA (338 mCi/mmol) was from ICN, and UDP-[^{3}H]Glc (1 Ci/mmol) was from Amersham Pharmacia Biotech. Econo-Safe scintillation cocktail was from Research Products International Corporation. Goat antirabbit IgG-biotin and Streptavidin-alkaline phosphatase were from Southern Biotechnology Associates, Inc. Rabbit polyclonal antiserum to the C-terminal 14 amino acids of the type 3 synthase linked to KLH was obtained from Research Genetics, Inc. (Huntsville, AL).

Mutanolysin, Sephacryl S-500HR, UDP-Glc, and UDP-GlcUA were obtained from Sigma. Nonidet P-40 was from Calbiochem, and Todd Hewitt Broth, yeast extract, and tryptone were from Digco. Protein was determined by flourography as previously described (15).

Growth Conditions and Membrane Preparations. *S. pneumoniae* membranes from strains WU2 (16) and its unencapsulated derivative JD908 (17), were prepared as previously described (6). Membranes were stored at –80 °C in a solution containing 100 mM Hepes (pH 8.0), 10% Glycerol, and 10 mM sodium thioglycolate. Membranes from *E. coli* strains JD422 and JD424 (2) were prepared by modification of the procedure described by Tlappak-Simmons *et al.* (18). *The E. coli* strains were grown in Luria broth containing 50 mM glucose and 100 μg/ml ampicillin at 37 °C to an A_{600} of 1.2. The cells were induced with 1 mM isopropyl-β-D-thiogalactoside and grown for an additional 2 hr at 37 °C. The cells were harvested by centrifugation at 4 °C for 10 min at 10,000 x *g*. The cells were washed twice with phosphate buffered saline (PBS) containing 10% glycerol and frozen at –80 °C. Cell pellets were thawed and resuspended to 1% of the original culture volume in 20% sucrose, 30 mM Tris-HCl (pH 8.2), 10 mM $MgCl_2$, 1mM DTT, 1 mM EDTA (pH 8.0), 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin. Lysozyme (4 μg/ ml of culture volume) was added, and the suspension was incubated at 4 °C for 40 min with constant mixing. The cells were centrifuged at 4 °C for 10 min at 12,000 x *g* to pellet the cells. The cells were suspended in sterile deionized water at 1% the original culture volume. Phenylmethanesulfonyl fluoride, $MgCl_2$, DNase, and RNase were added to the suspension to a final concentration of 1 mM, 60 mM, 1 μg/ml, and 1 μg/ml, respectively. The suspension was incubated at 4 °C for 20 min with constant mixing.

Lysis of the cells was confirmed by phase contrast light microscopy. Membranes were harvested by centrifugation at 100,000 x *g* for 1 h. The membranes were washed once in 100 mM Hepes, pH 7.5, containing 10% glycerol and 10 mM EDTA, and twice in 100 mM Hepes, pH 7.5 containing 10% glycerol. The final pellet was suspended to 1% the original culture volume in 100 mM Hepes, pH 7.5, containing 10% glycerol and stored at –80°C.

Immunoblots and Detection of Type 3 Polysaccharide. Proteins from *E. coli* and *S. pneumoniae* were separated on 10% SDS-PAGE gels and transferred to nitrocellulose. The blots were blocked for 45 min in phosphate buffered saline with 0.5% Tween 20 (PBST) containing 5% dried milk and 1% bovine serum albumin (BSA). Primary antibody diluted in PBST was incubated on blots overnight. Blots were washed with PBST and incubated for 30 min with secondary antibody (goat antirabbit IgG-biotin) and steptavidin-alkaline phosphatase. After washing in PBST and Tris-HCl (pH 8.0), immunoblots were visualized using *p*-nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt. For detection of type 3 polysaccharide, enzyme-linked immunosorbent assays were performed as previously described (19).

Assay of Synthase Activity. Type 3 synthase activity was determined by the incorporation of ^{14}C label from either UDP-[^{14}C]Glc or UDP-[^{14}C]GlcUA into polysaccharide. Assays were carried out in 100-µl reactions containing 100 mM imidazole (pH 7.0), 10 mM $MnCl_2$, UDP-Glc, and UDP-GlcUA, and membranes were isolated as described above. The reactions were incubated at 35 °C for 10 min and stopped by addition of 10 µl of 12.5 M glacial acetic acid. Reaction components were

separated by ascending paper chromatrography on 3MM Whatman paper in ethanol (95%): 1 M ammonium acetate (pH 5.5), 7:3 (v/v) for 16-18 h.

Sephacryl S-500 Column Chromatography. Gel filtration chromatography was carried out on a 1.4 x 37 cm column of Sephacryl S-500 eluted with a solution consisting of 5 mM Tris (pH 7.5), 200 mM NaCl, 0.1% Nonidet P-40, and 0.02% sodium azide. Prior to application to the column, the sample was solubilized with 0.5% sodium dodecyl sulfate, heated to 100 °C for 5 min, and clarified by centrifugation at 10,000 x g for 5 min. The column flow rate was 20 ml/h, and 1-ml fractions were collected.

Preparation of [^{14}C]-Polysaccharide-Synthase Complex and Assay of Polysaccharide Release. Type 3 polysaccharide was synthesized in a 800 µl reaction containing 100 mM imidazole (pH 7.0), 10 mM $MnCl_2$, 20 µM UDP-Glc, 20 µM UDP-[^{14}C]GlcUA (135 mCi/mmol), and 2 mg of *E. coli* membranes. The reaction was incubated for 30 s at 35 °C and stopped by placing on ice. The volume of the reaction was brought up to 2 ml with 100 mM Hepes (pH 7.5) containing 10% glycerol. The membranes were washed as previosly described (7). Release assays were also performed as previously described (7), except reaction mixtures consisted of 100 mM imidazole (pH 7.0), 10 mM $MnCl_2$, and either UDP-Glc or UDP-GlcUA as indicated.

Preparation of Type 3 Polysaccharide Depolymerase and Depolymerase Digestion of Polysaccharide. Type 3 polysaccharide depolymerase was prepared from *Bacillus circulans* (American Type Culture Collection 14175) as previously described (7). Digestion of solubilized polysaccharide was performed as previously described (7).

Digestion of [^{14}C] labeled polysaccharide-synthase complexes (20,000 cpm) prepared as described above were performed in 100 µl reactions containing 100 mM Hepes (pH 6.0) and 2 µg of depolymerase. The reaction was terminated by placing on ice and adding 200 µl of ice-cold 100 mM Hepes (pH 7.5) and 10% glycerol. The membranes were sedimented by centrifugation at 100.000 x g for 30 min. The amount of radioactivity released into the supernatant was quantitated by liquid scintillation counting. The pellet was solubilized in 300 µl of a solution containing 0.1 M NaCl and 0.5% Nonidet P-40, and the amount of radioactivity was quantitated.

RESULTS

Expression of the Type 3 Synthase. For detection of the synthase protein, we obtained rabbit polyclonal antiserum specific for the C-terminal 14 residues of the protein. The antibody reacted with extracts of the parent type 3 strain but not with a mutant containing an insertion that inactivates *cps3S* (Fig. 1). In *E. coli*, *cps3S* was cloned into pKK223-3 on a fragment containing the 3' end of *cps3D* and the complete *cps3S* (2). Expression was induced using Isopropyl ß-D-thiogalactopyranoside (IPTG), and samples were tested for expression at 2 h following induction. As we have observed before (2), growth of induced cultures containing the cloned synthase ceased, but no new proteins were detectable by Coomassie or silver staining of samples separated on SDS-PAGE gels. Using the polyclonal antiserum, however, a protein of the same size as that observed in *S. pneumoniae* was detected in both the induced and uninduced cultures but not in *E. coli* strains carrying vector alone (Fig. 1). The apparent molecular size of the protein (40 kDa) in both *S. pneumoniae* and *E. coli* is smaller than the predicted size (48

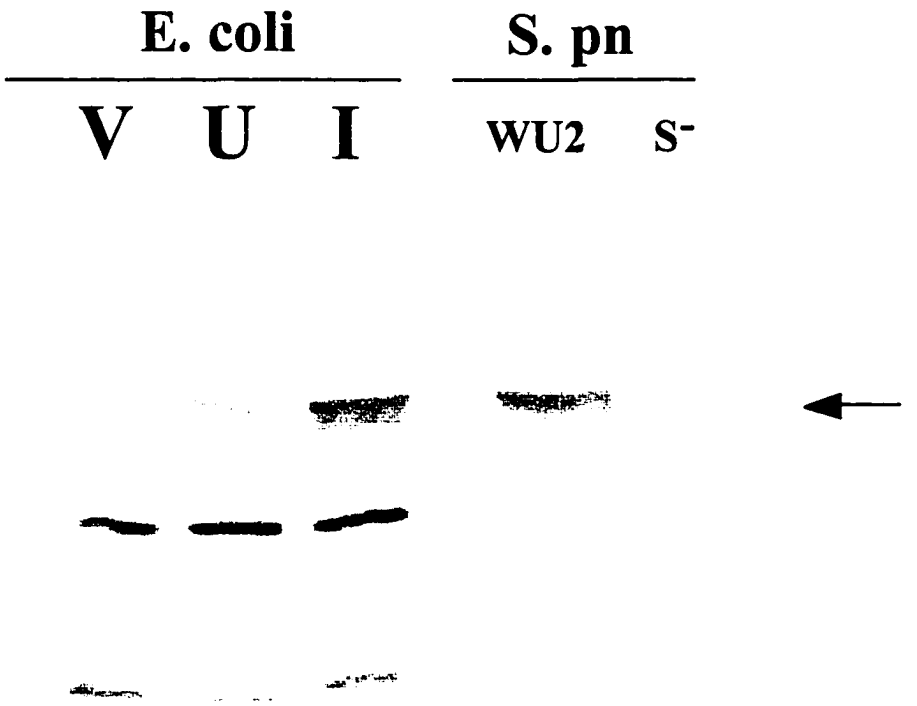


FIG 1. **Western analysis of *S. pneumoniae* and *E. coli* expressing *cps3S*.** *E. coli* strains JD422 (vector control) and JD424 (Cps3S+) were grown to an OD_{600} of 0.6 and induced for 2 h with IPTG. Equivalent cell volumes of JD422 (V), uninduced JD424 (U), and induced JD424 (I) were analyzed along with membrane preparations of type 3 *S. pneumoniae* strain WU2 and JD908 (S^-) on a 10% SDS-PAGE gel and then transferred to nitrocellulose. Type 3 synthase was detected with polyclonal antiserum directed to the C-terminal 14 amino acids of the type 3 synthase.

kDa). Low levels of type 3 polysaccharide were detected in *E. coli* membranes expressing the synthase, indicating the enzyme was active.

Characterization of Synthase Activity. In order to characterize synthase activity in *E. coli*, membranes from strain JD422 (plasmid vector only) and JD424 were prepared and used in an assay that measures the incorporation of ^{14}C from either UDP-[^{14}C]Glc or UDP-[^{14}C]GlcUA into polysaccharide. Membranes from JD424 incorporated 5618 cpm. whereas membranes from JD422 only incorporated 172 cpm under identical assay conditions. The incorporation of label in JD424 was dependent on the presence of both UDP-Glc and UDP-GlcUA and was proportional to protein concentrations and linear with time for up to 1 h. Synthase activity was observed in the presence of both Mn^{2+} and Mg^{2+} and had an optimal pH between 7 to 7.5. Apparent K_m values for UDP-Glc and UDP-GlcUA were 26 and 20 μM, respectively.

Characterization of the Polysaccharide Product. High molecular weight product, confirmed to be the type 3 polysaccharide by digestion with the type 3-specific depolymerase, could be generated during reactions in which the polymer was uniformly labeled using UDP-[^{14}C]Glc. In contrast to both this experiment and what occurs with *S. pneumoniae* membranes, pulse-labeling with UDP-[^{14}C]Glc resulted in only a low molecular weight product that could not be chased to a higher molecular weight with unlabeled substrate. Neither product was produced by the *E. coli* control strain containing the plasmid vector only, indicating that both were the result of synthase activity. The two methods of labeling polysaccharide are distinguished by the fact that the former uses high concentrations (500 μM) of both substrates during the entire

reaction, whereas the latter uses low and unequal concentrations of both substrates (2 μM UDP-[^{14}C]Glc and 20 μM UDP-GlcUA) during the pulse reaction. If the substrates are depleted rapidly in the *E. coli* system, the inability to pulse-label high molecular weight product could be due to release of the polysaccharide during the pulse reaction and the generation of only a low molecular weight labeled product.

Polysaccharide Release. To determine if release occurs with the type 3 synthase expressed in *E. coli*, soluble and membrane associated polysaccharide were separated by centrifugation over a 60-min reaction. There was steady increase in the incorporation of [^{14}C]Glc into membrane-associated polysaccharide during the first 30 min of incubation (Fig. 2). Unlike what occurred in *S. pneumoniae*, however, there was no increase in the level of radioactivity in the soluble fraction, even after the UDP-Glc was depleted (6). Attempts to measure polysaccharide release by synthesizing membrane-enzyme-polysaccharide complexes and incubating them with high concentrations of each substrate also showed no release of the polysaccharide into a soluble form (data not shown). When these complexes were first incubated with a single substrate followed by incubation with high levels of both substrates, the polysaccharide was not extended into a higher molecular weight form. In contrast, if high levels of both precursors were added without prior incubation with an excess of one precursor, the size of the labeled product increased, indicating that the synthase was still active and the polysaccharide was actively engaged with the synthase (data not shown). These results suggest that not only can polysaccharide release be actuated upon incubation with a single substrate, but also all the polysaccharide chains are releasable. Entrapment or entanglement of the released polysaccharide could readily explain why the chains are not released from the membrane.

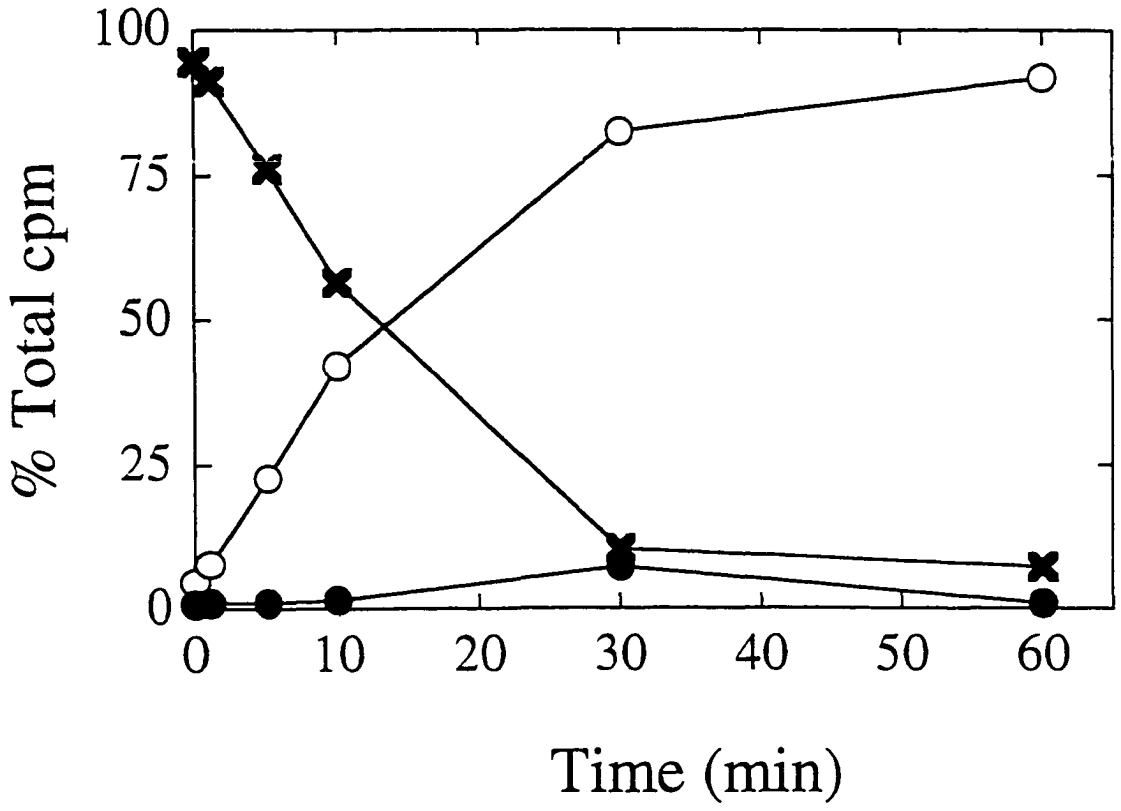


FIG 2. **Formation of soluble and membrane-associated type 3 polysaccharide.** A 300 µl reaction containing 100 µM UDP-Glc (5 mCi/mmol) and 200 µM UDP-GlcUA was prepared as described under "Experimental Procedures." Samples (50 µl) were taken after 0, 5, 10, 30, and 60 min of incubation at 35 °C, and the soluble and membrane-associated polysaccharide were separated as described under "Experimental Procedures." The amount of polysaccharide present as soluble polysaccharide (●), membrane-associated polysaccharide (o), and unincorporated [^{14}C] UDP-Glc (x) was determined after ascending paper chromatography in ethanol/1 M ammonium acetate (pH 5.0), 65:35, as a percentage of the total radioactivity.

Indeed, depolymerase treatment of intact *E. coli* membranes did not degrade the labeled polysaccharide, in contrast to the result noted above when the membranes were solubilized. Additionally, *E. coli* membranes required solubilization before type 3 polysaccharide could be detected by antibody. Although preincubation of membranes with a single UDP sugar precursor prevented further elongation of labeled polysaccharide, it did not inhibit synthase activity, as measured by the subsequent ability to synthesize labeled polymer (Fig. 3). This result suggests that the enzyme is capable of reinitiating synthesis *de novo* following release.

De novo synthesis of type 3 polysaccharide. To determine if *de novo* synthesis occurs in *E. coli* membranes, ^{14}C GlcUA-labeled product of intermediate size was synthesized and washed extensively to remove any unincorporated UDP sugars (Fig. 4*A*). Preincubation of the membrane-associated product with either UDP-Glc (Fig. 4*B*) or UDP-GlcUA (Fig. 4*C*) for 10 min followed by incubation with a reaction mixture containing UDP-[^{3}H]Glc and UDP-GlcUA for an additional 20 min resulted in a high molecular weight ^{3}H-labeled product. The ^{14}C-labeled product, however, remained the same size. If the [^{14}C] labeled polysaccharide-synthase complexes were preincubated with no substrate, approximately half the ^{14}C-labeled product was extended into a higher molecular weight form that coeluted with ^{3}H labeled product following incubation with additional substrate (Fig. 4*D*). These data indicate that preincubation with a single substrate will actuate release of all the ^{14}C labeled polysaccharide from the enzyme and that the synthase is then capable of reinitiating synthesis of a new polysaccharide chain. The high molecular weight ^{14}C- and ^{3}H-labeled products were both degraded by the type

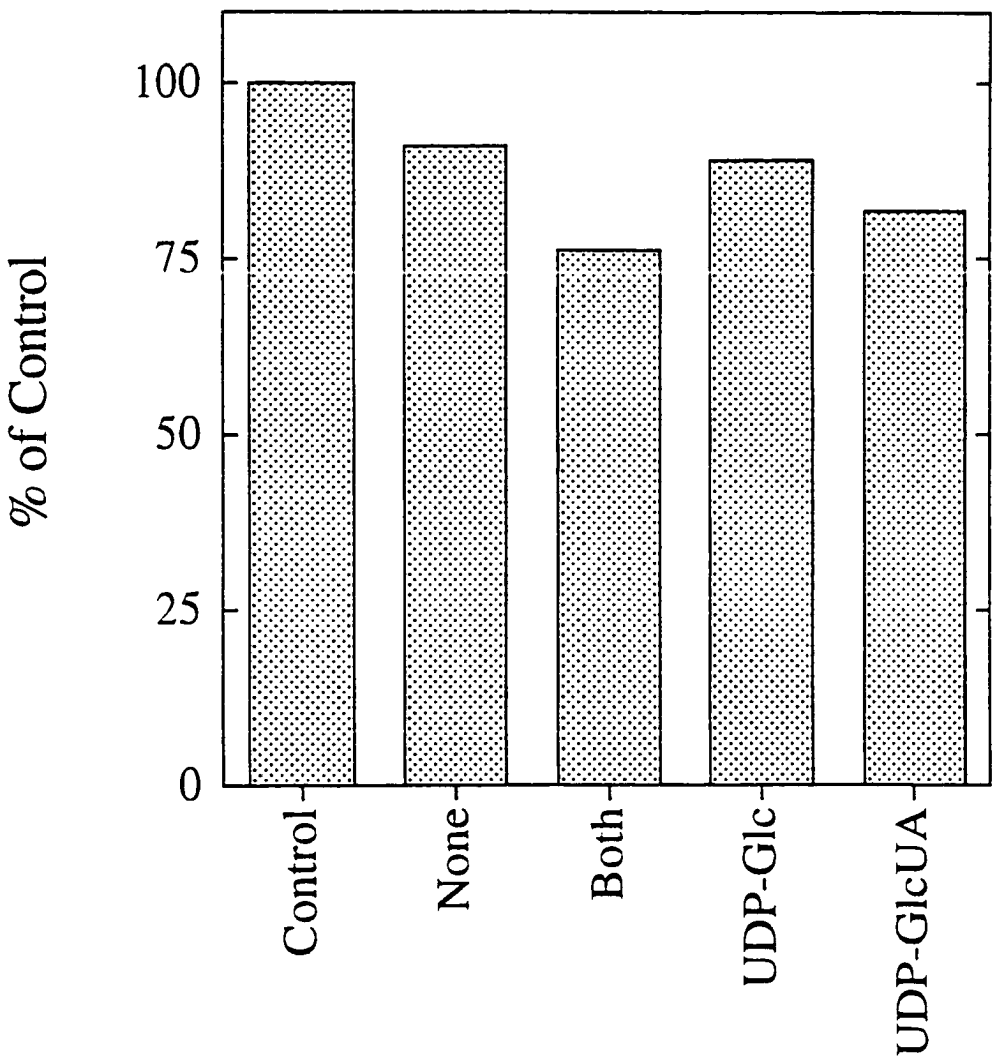
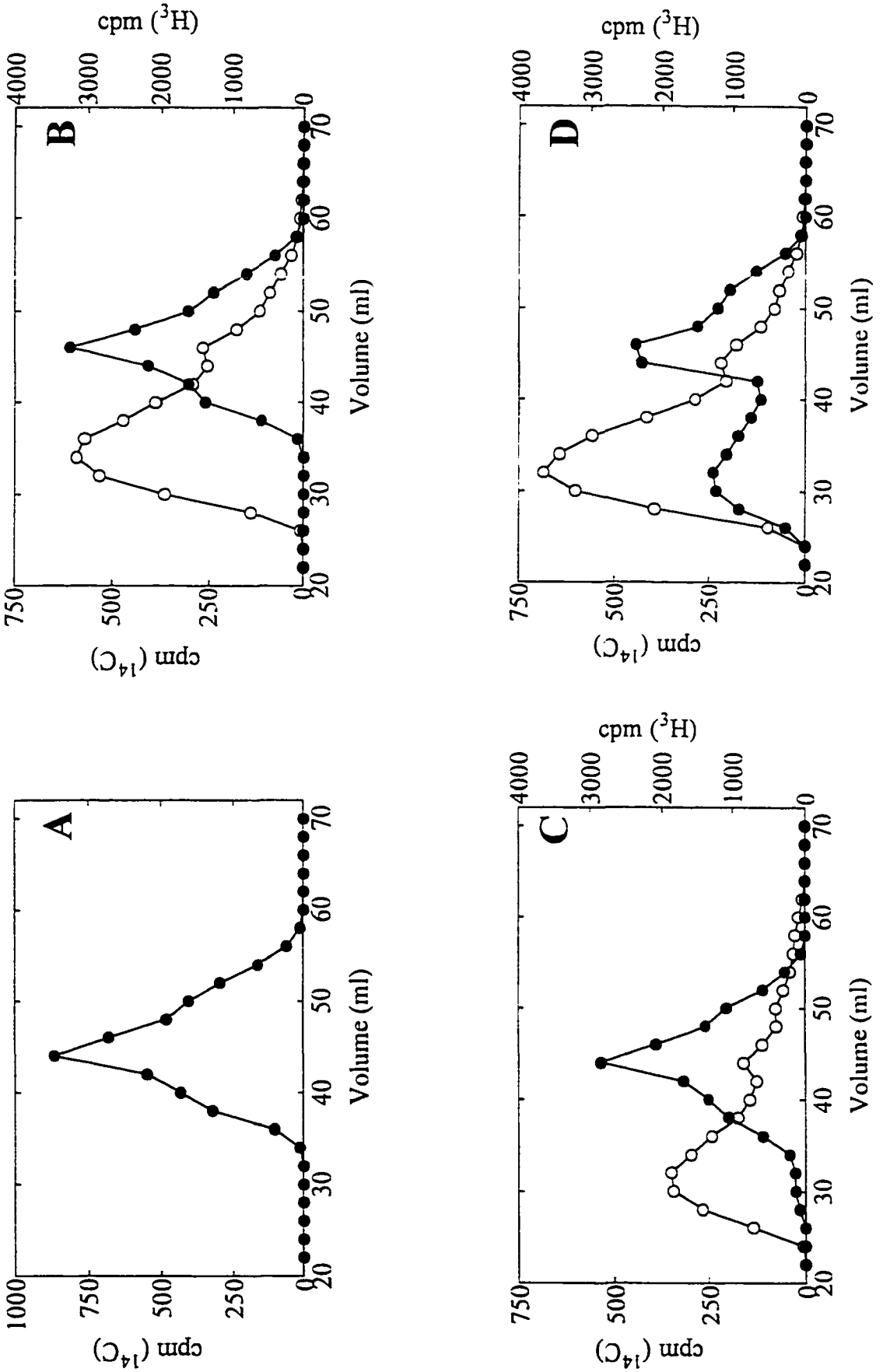


FIG 3. **Effect of preincubation of *E. coli* membranes on type 3 synthase activity**. *E. coli* membranes were incubated in a 50-μl reaction containing 100 mM imidazole (pH 7.0), 10 mM $MnCl_2$, and either 100 μM UDP-Glc, 100 μM UDP-GlcUA, 100 μM of both UDP sugars, or no UDP sugars for 10 min at 35 °C. The reactions were brought up to 100 μl so that the final reaction mixtures contained 100 mM imidazole (pH 7.0), 10 mM $MnCl_2$, 100 μM UDP-Glc (5 mCi/mmol), and 100 μM UDP-GlcUA. These reactions were incubated for 20 min at 35 °C and stopped by the addition of 12.5 M acetic acid. The amount of radiolabeled polysaccharide produced in the assays was determined by ascending paper chromatography in ethanol/1M ammonium acetate (pH 5.0), 65:35, as a percentage of radiolabeled polysaccharide produced from membranes that were not incubated in the first reaction.

FIG 4. ***De novo* synthesis of type 3 polysaccharide.** *A*, [^{14}C]GlcUA-labeled polysaccharide-enzyme complexes were prepared as described in "Experimental Procedures," and an aliguot was chromotagraphed on a column of Sepharcryl S-500. Labeled complexes from *A* were incubated for 10 at 35 °C in reactions containing 100 mM imidazole (pH 7.0), 10 mM $MnCl_2$, and either *B*, 200 μM UDP-Glc; *C*, 200 μM UDP-GlcUA; or *D*, 200 μM of both UDP sugars. Reactions were brought up to 100 μl so that the final reaction contained 100 mM imidazole (pH 7.0), 10 mM $MnCl_2$, 200 μM UDP-[^{3}H]Glc, and 200 μM UDP-GlcUA. The reactions were incubated for 20 min at 35 °C and terminated by placing on ice and adding 200 μl of ice-cold wash buffer (100 mM Hepes [pH 7.5] containing 10% glycerol). The membranes were collected by centrifugation at 100,000 x *g* for 30 min, suspended in wash buffer, and chromotagraphed on Sephacryl S-500. The amount of ^{14}C (●) and ^{3}H (o) present in the even numbered fractions was determined.



3 polysaccharide depolymerase, indicating that both labels were incorporated into type 3 polysaccharide.

DISCUSSION

The type 3 synthase of *S. pneumoniae* is a processive glycoslytransferase that is capable of forming both glycosidic linkages of type 3 polysaccharide (6). Recent studies on type 3 polysaccharide synthesis in *S. pneumoniae* have provided some key information regarding the mechanism of synthesis as well as a mechanism of termination and release (5, 6). These studies demonstrated that, once a polysaccharide chain is released from the synthase, synthesis does not reinitiate (7). The results presented here now show that the type 3 synthase, when expressed heterologously in *E. coli*, is able to reinitiate synthesis after releasing its nascent polysaccharide chain.

Antibody to the C-terminus of the synthase recognized a 40 kDa protein in both *S. pneumoniae* and *E. coli*. This size is smaller than that predicted size for the synthase (48 kDa) but consistent with observations reported for the hyaluronic acid synthase (20). Synthase activity in *E. coli* membranes was very similar to that observed in *S. pneumoniae* membranes in regard to metal ion specificity, pH, and apparent K_m values for both substrates. Additionally, membranes from both bacteria were able to polymerize high molecular weight polysaccharide that was readily degradable by the type 3 polysaccharide-specific depolymerase (6).

E. coli membranes, however, were unable to label high molecular weight polysaccharide in a pulse-chase reaction. One explanation for this result is that the polysaccharide was being released due to the low and uneven levels of substrate present

in the pulse reaction. However, we were unable to show polysaccharide release from the membrane during either a 60-min time course where substrate levels were uneven or when labeled enzyme-polysaccharide complexes were incubated with either UDP-Glc or UDP-GlcUA. Although these data indicated that polysaccharide release does not occur in *E. coli* membranes, incubation of enzyme-polysaccharide complexes with single substrate did prevent further elongation of the polysaccharide. These data argue the presence of a single substrate will actuate polysaccharide release from the enzyme just as it does in *S. pneumoniae* membranes (7). The fact that the polysaccharide is not physically released from the membranes can be explained by entrapment of the polysaccharide within the membranes. This hypothesis is supported by our data showing that the polysaccharide is not susceptible to degradation by the depolymerase or detectable with antibody to type 3 polysaccharide unless the membranes are solubilized. While only approximately 50% of the polysaccharide is releasable in *S. pneumoniae* membranes, our data here demonstrate that nearly all the polysaccharide in *E. coli* membranes can be released (7). The reason for the difference in the amount of release seen in *E. coli* and *S. pneumoniae* membranes is not clear. One possibility could be that there are additional factors in *S. pneumoniae* that are responsible for controlling the amount of release or what population of synthase is capable of release.

E. coli membranes like those of *S. pneumoniae* have type 3 polysaccharide present when used in an *in vitro* synthesis assay (2). In *S. pneumoniae*, this polysaccharide is actively engaged with the enzyme and is used as an acceptor in synthesis. If this nascent polysaccharide is released via incubation with a single substrate, the synthase is not capable of reinitiating synthesis, resulting in a decrease in synthase activity (7). When *E. coli* membranes, however, were incubated with a single substrate, no inhibition

of synthase activity was observed. Because we demonstrated that the polysaccharide can be released from the synthase under these conditions, these data suggested that the *E. coli*-expressed synthase may be capable of reinitiating synthesis. By stimulating release of a [^{14}C]GlcUA labeled nascent polysaccharide chain by incubation with a single substrate, we demonstrated that the synthase in *E. coli* was capable of reinitiating synthesis of a new polysaccharide chain labeled with [^{3}H]Glc. Not all the [^{14}C]GlcUA-labeled polysaccharide, however, was associated with the enzyme as only 50% of this polysaccharide was extended into a higher molecular weight form when it preincubated with no UDP sugars. This nonextendable form is likely polysaccharide that was released from the enzyme during the initial synthesis, but was not removed during the extensive washing because the polysaccharide is not released from the membranes.

Whether the ability of the type 3 synthase to reinitiate polysaccharide synthesis occurs *de novo* from the UDP sugar precursors or occurs because *E. coli* membranes contain the necessary primer to reinitiate synthesis is not known. Studies on the hyaluronic acid synthase from *S. pyogenes* and teratocarcinoma cells indicate that synthesis of hyaluronic acid does not require the use of a primer and likely occurs *de novo* from the UDP sugars (9-13). Expression of the hyaluronic acid synthases from *S. pyogenes* and DG42 from *Xenopus laevis* in yeast was able to synthesize high molecular weight hyaluronic acid in an *in vitro* assay (8). Since yeast do not make UDP-GlcUA, these enzymes do not have nascent hyaluronic acid to use as an acceptor. While this information argues that synthesis occurs *de novo*, it does not eliminate the possibility that yeast produce a primer that initiates the *in vitro* reaction.

It is possible that the type 3 synthase is capable of *de novo* synthesis in *S. pneumoniae* but that there are factors that prevent reinitiation of synthesis as a means to

control type 3 polysaccharide synthesis. The synthesis of capsule in *S. pneumoniae* draws from a pool of precursors that are also used for the synthesis of essential polysaccharides such as teichoic acid, lipoteichoic acid, and peptidoglycan. In order to prevent depletion of the pool of precursors by the synthesis of any one of these polysaccharides, *S. pneumoniae* must somehow control their synthesis. For most *S. pneumoniae* capsules, there are common genes upstream of the biosynthetic genes that are thought to play a role in regulating transcription of the biosynthetic genes and controlling polymerization (16, 20-22). These genes in the type 3 capsule locus, however, are mutated and are not functional in type 3 polysaccharide synthesis (2, 18, 23). *S. pneumoniae* may control type 3 polysaccharide synthesis by preventing reinitiation once the enzyme has released its nascent chain. Further characterization of type 3 synthase purified from *S. pneumoniae* membranes may provide an answer to whether or not there are additional factors involved in regulating type 3 synthase activity. There is already growing evidence that the synthesis of most capsules require proteins outside those encoded in the capsule locus (25, 26).

ACKNOWLEDGMENTS

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FOOTNOTES

[1]The abbreviations used are: GlcUA, glucuronic acid; Cps3S, type 3 synthase; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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CONCLUSION

S. pneumoniae infections still result in a great deal of morbidity and mortality throughout the world despite the use of antibiotics and a vaccine. Increasing levels of penicillin- and multidrug-resistant strains of *S. pneumoniae* have prompted studies into novel ways of limiting pneumococcal infection. The polysaccharide capsule, which is essential for *S. pneumoniae* virulence, is a prime target for novel therapies. An understanding of capsule synthesis, however, is paramount in designing new therapies. Considerable progress has been made in understanding the genetics of capsule synthesis, but very little information regarding the biochemistry of capsule synthesis has been generated.

Type 3 polysaccharide is one of the simplest capsule types in both the structure of its repeat unit as well as in number of proteins necessary for its synthesis. For these reasons it was one of the first capsule types characterized on a biochemical level. Activities of three of the four genes found in the type 3 locus have been described (53, 55, 113). Type 3 polysaccharide synthesis required only a single glycosyltransferase, the type 3 synthase (53, 55). The type 3 synthase shares significant amino acid homology with several glycosyltransferases that synthesize some of the most important and abundant biopolymers (55). All of these glycosyltransferases are believed to have a common mechanism of synthesis (45, 116). Although a model has been proposed for the synthesis of these polymers, there is very little data to support or refute it (45, 116). The

information presented in this dissertation on the synthesis of type 3 polysaccharide provides some new insights into this model of synthesis.

In the first paper, we described the use of a new, more versatile assay that measures type 3 polysaccharide production by the incorporation of radiolabeled sugars. Using this new assay we provided the first evidence for the mechanism of type 3 polysaccharide synthesis. Previous characterization of the type 3 synthase provided little description of the mechanism by which type 3 polysaccharide is synthesized. These experiments used a polyclonal or monoclonal antibody to type 3 polysaccharide to quantitate polysaccharide production from crude membrane preparations of type 3 *S. pneumoniae* (55, 114, 115). They were unable to measure initial rates of synthesis due to the limitations in the size of polysaccharide detectable by the antibody. As with the previous characterization of type 3 synthase activity, crude membrane preparations of a type 3 *S. pneumoniae* strain were used (55, 114, 115). Using the radioactive assay, we initially established optimal conditions for synthase activity. Characterization of the polysaccharide produced in the assay by several methods demonstrated that it contained all the properties of authentic type 3 polysaccharide. Most importantly, work performed on determining the mechanism of type 3 polysaccharide synthesis demonstrated that the polymer grows from the nonreducing end of the growing polysaccharide. This was in contrast to what was generally accepted for the direction of growth of processive ß-glycosyltransferases (116). Recent characterization of the direction of growth of hyaluronic acid in *P. multocida* and cellulose in *A. aceti*, however, indicates that these polysaccharides grow from the nonreducing end as well (121, 154). Determination of whether there is truly a difference in the direction of growth between members of this

family of ß-glycosyltransferases awaits further characterization of the additional members of this family.

The polysaccharide produced by our enzyme preparation was rapidly polymerized from small polysaccharide chains into high molecular weight polysaccharide, suggesting a processive mechanism of synthesis. Analysis of the quantity of soluble and membrane-associated polysaccharide during a 60-min incubation under varying substrate concentrations indicated that the polysaccharide remained associated with the membrane-bound enzyme as long as there was sufficient substrate present to support synthesis. This data demonstrated that the type 3 synthase is indeed a processive enzyme. Depletion of either UDP-Glc or UDP-GlcUA from the reaction, however, resulted in release of the polysaccharide into a soluble form. Similar findings were observed when one obligatory component, such as a substrate or metal ion, was omitted during the synthesis of hyaluronic acid in differentiated teratocarcinoma cells (139). Release of polysaccharide chains was also observed during the synthesis of hyaluronic acid in *S. pyogenes* and was thought be enzyamatic (156).

The mechanism of release was further characterized in work described in the second paper. Our first observation in these studies indicated that type 3 polysaccharide release from the membranes was stimulated when the enzyme was incubated with a single nucleotide sugar, specifically UDP-Glc or UDP-GlcUA. The release stimulated by either sugar was concentration, time, and temperature dependent, indicating that the process was enzymatic and likely involved at the same sites as biosynthesis. Apparent Michaelis-Menton constants for release with UDP-Glc and UDP-GlcUA, however, were 880 μM and 0.004 μM, respectively. These values are strikingly different from the K_m values reported for the biosynthesis (11 μM for UDP-Glc and 8 μM for UDP-GlcUA).

We believe the differences in K_m values arise from both positive and negative cooperative binding when both substrates are present in the biosynthesis reaction. This hypothesis is supported by our data showing that the addition of UDP-GlcUA inhibited the release with UDP-Glc and that UDP-Glc inhibited the release observed with UDP-GlcUA. This inhibition is concentration dependent, and apparent K_i values for UDP-Glc and UDP-GlcUA were 5 μM and 2 μM, respectively. Similar conclusions on cooperative binding between substrates have also been made for the hyaluronic acid synthases in *S. pyogenes* and *S. equisimilis*. These conclusions were based on possible allosteric effects observed during kinetic characterization of the purified enzymes (161).

The characterization of release in type 3 polysaccharide synthesis has provided the first mechanism to explain how polysaccharide synthesis is terminated by processive ß-glycosyltransferases. Synthesis of processive ß-glycosyltransferases is believed to occur by tandem addition of the two nucleotide sugars, resulting in the release of two molecules of UDP (116). The release of UDP is believed to provide the energy to translocate the polymer and allow two more UDP sugars to bind (45). Comparison of the experimental activation energy for release with a single substrate and the biosynthetic reaction suggests that the binding of each sugar contributes equally to the energy postulated to drive translocation. We believe that the addition of a single nucleotide sugar triggers translocation of the polysaccharide either by simply binding to the enzyme, by hydrolysis of the UDP sugar, or by hydrolysis of the UDP sugar followed by addition of the sugar. Whether adding a single UDP sugar results in addition or not, the subsequent translocation of the polysaccharide would result in incorrect alignment of the polysaccharide in its binding site and thus release of the polysaccharide. This mechanism of release may provide a model by which bacteria control the level of surface-bound

polysaccharide and released polysaccharide by varying substrate levels. Currently, there is no data demonstrating that release of polysaccharide by this mechanism occurs *in vivo*.

Because the type 3 synthase isolated from *S. pneumoniae* contains preformed type 3 polysaccharide, it is difficult to determine whether polysaccharide synthesis occurs *de novo* from the nucleotide sugars or whether some form of primer is involved. Preincubation of *S. pneumoniae* membranes with either UDP-Glc or UDP-GlcUA, however, resulted in a loss of subsequent synthase activity, indicating that the type 3 synthase is not capable of reinitiating synthesis once its nascent chain has been released. This result suggests that initiation of type 3 polysaccharide synthesis requires some form of primer. Most polysaccharide synthesized by bacteria requires the use of a lipid intermediate (52). Synthesis of type 3 polysaccharide is assumed to not utilize a lipid intermediate based on studies of the homologous hyaluronic acid synthases and that there are no other genes present in the type 3 capsule locus with homology to known lipid-sugar transferases (19, 20, 138, 142, 143, 155, 156). It is possible, however, that type 3 polysaccharide synthesis is initiated on a lipid intermediate that is produced by enzymes that are not encoded in the type 3 capsule locus. Recently, two genes (*pgm* and *galU*) that lie outside the type 3 capsule locus have been shown to be essential for the production of type 3 polysaccharide precursors (104, 112). Synthesis of O:54 polysaccharide by the homologous glycosyltransferase RfbBO:54 requires the use of a lipid intermediate formed in part by WecA, an enzyme that is involved in the synthesis of other polysaccharides in the bacterium and which is not linked genetically to RfbBO:54 (45, 122). If a lipid intermediate is used in type 3 polysaccharide synthesis, then a portion of the polysaccharide could be tethered to the membrane via the lipid. This

hypothesis could explain why only approximately 50% of the polysaccharide is releasable upon incubation with a single substrate.

Further evidence for additional factors playing a role in type 3 polysaccharide biosynthesis was obtained upon expression and characterization of the type 3 synthase in *E. coli*. While the basic activity of the type 3 synthase was the same when isolated from *E. coli* and *S. pneumoniae* membranes, differences were noted in the ability to synthesize and release polysaccharide. We were not able to measure release of polysaccharide from *E. coli* membranes, but we were able to demonstrate that almost all the polysaccharide could be disengaged from the enzyme upon incubation of the membranes with a single nucleotide sugar. One explanation for why all the polysaccharide can be released from the synthase in *E. coli* but not in *S. pneumoniae* is that *E. coli* lack a factor that allows a portion of the enzyme to retain its polysaccharide. There may be a biological function associated with retaining or releasing the polysaccharide from the cell surface. This hypothesized factor could take the form of an additional protein that is present only in *S. pneumoniae* or could be due to modification of the synthase that only occurs in *S. pneumoniae*. Answers to these questions could come from the purification of the type 3 synthase from *S. pneumoniae* membranes.

Another observation made during the characterization of the type 3 synthase in *E. coli* membranes that suggests that an additional factor may be involved in the synthesis of type 3 polysaccharide in *S. pneumoniae* comes from examining the ability of the synthase to reinitiate synthesis after stimulating polysaccharide release. In *E. coli* membranes, preincubation with a single substrate did not inhibit synthase activity as it did in *S. pneumoniae* membranes. Because we had demonstrated previously that under these conditions the polysaccharide was released from the enzyme, this suggested that the

synthase may be reinitiating synthesis after polysaccharide release. Experiments that examined the incorporation of both ^{14}C and ^{3}H into type 3 polysaccharide clearly demonstrated that the type 3 synthase expressed in *E. coli* was capable of releasing its nascent polysaccharide and initiating synthesis of a new polysaccharide chain. Although this data suggests that there may be factors present in *S. pneumoniae* that prevent *de novo* synthesis, we cannot rule out the possibility that *E. coli* contains a primer that allows the type 3 synthase to reinitiate polysaccharide synthesis even after release. Characterization of the reducing end of type 3 polysaccharide produced in both *E. coli* and *S. pneumoniae* would provide insights into possible mechanisms of initiation of type 3 polysaccharide synthesis.

In summary the data presented here provide the first in-depth characterization of an enzyme involved in the synthesis of *S. pneumoniae* capsule. Although type 3 polysaccharide synthesis is the exception rather than the rule for how *S. pneumoniae* capsules are assembled, the information learned about the type 3 synthase is directly applicable to the activity of processive ß-glycosyltransferases. The studies presented here provide new information regarding the direction of growth as well as a possible mechanism for polysaccharide translocation and termination of synthesis. Additionally, these studies indicate that additional factors other than the type 3 synthase may be involved in controlling polymerization of type 3 polysaccharide in *S. pneumoniae*.

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I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that he may be recommended for the degree of Doctor of Philosophy.

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