The Biosynthesis of Hyaluronic Acid by *Streptococcus*

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SUMMARY

The mechanism of hyaluronic acid chain growth in a particulate enzyme preparation obtained from Group A *Streptococcus* has been examined and additional characteristics of the enzyme detailed. Hyaluronic acid chain elongation occurs by the transfer of monosaccharide units from uridine nucleoside diphosphate derivatives to the nonreducing ends of endogenous oligosaccharide. This oligosaccharide which appeared to be chemically identical with hyaluronic acid was released from the enzyme by either acid hydrolysis or digestion with streptococcal hyaluronidase. The only detectable uridine nucleotide product of hyaluronic acid synthesis was UDP, and synthesis was not inhibited by bacitracin. There was no evidence for the participation of lipid-extractable intermediates during hyaluronic acid formation.

The pH optimum for polysaccharide formation is 7.1. The enzyme exhibits an absolute dependence upon divalent metal ion and is maximally activated by 10 mM Mg\(^{2+}\) or 1 mM Mn\(^{2+}\). Michaelis constants for UDP-GlcUA and UDP-GlcNAc are 5 × 10\(^{-5}\) M and 5 × 10\(^{-4}\) M, respectively. The enzyme is inactivated by treatment with 4% 1-butanol. Addition of Mg\(^{2+}\) and UDP, UDP-GlcUA, UDP-GlcNAc, or other uridine nucleoside diphosphate sugars prior to butanol treatment prevented inactivation. Fifty per cent stabilization of the enzyme during butanol treatment occurred at 7 × 10\(^{-5}\) M UDP.

A cell-free enzyme system obtained from Group A streptococci catalyzes the incorporation of radioactivity from either labeled UDP-GlcUA or UDP-GlcNAc into hyaluronic acid (1, 2). The particulate polymerizing enzyme is associated with the protoplast membrane and can be separated from soluble enzymes involved in nucleotide synthesis (2). Only Mg\(^{2+}\), UDP-GlcUA, and UDP-GlcNAc are required for hyaluronic acid synthesis (1). Although these studies on the biosynthesis of hyaluronic acid in streptococci were among the first concerning heteropolysaccharide biosynthesis, the mechanism by which hyaluronic acid is formed remains imperfectly understood. Detailed studies on the biosynthesis of chondroitin sulfate-protein complex carried out with a preparation from 13-day-old embryonic chick epiphysseal cartilage (3-5) clarified the mechanism of alternation of monosaccharides in chondroitin sulfate chains. These studies indicated that the mechanism of chondroitin sulfate formation involves the sequential alternate addition of glucuronic acid and N-acetylgalactosamine to the nonreducing ends of growing polysaccharide chains (5).

The knowledge and techniques evolved from the studies of chondroitin sulfate biosynthesis have made it desirable to re-investigate hyaluronic acid biosynthesis in streptococci. This report details such investigations together with additional properties of the hyaluronic acid-synthesizing system.

EXPERIMENTAL PROCEDURE

Growth and Collection of Cells

Group A streptococci (Type 18, Strain A111) were maintained as previously described (6). Large scale cultures were grown in 5-gallon bottles containing 2.5% veal infusion broth (Difco) and 0.5% n-glucose in 0.033 M Na\(_2\)HPO\(_4\)-KH\(_2\)PO\(_4\) buffer, pH 7.3, at 37°C. Such cultures were inoculated with a 5% volume of seed culture and growth was monitored by pH measurements. The pH decreased to 6.7, 6 to 8 hours after inoculation. The cultures were cooled in ice water, harvested with a Szent-Györgyi-Blum continuous flow system (Ivan Sorvall, Inc.) at 4-10°C, and stored at -20°C until used.

Preparation of Hyaluronic Acid-synthesizing System

Approximately 25 ml of frozen compacted cells were suspended in 0.05 M Na\(_2\)HPO\(_4\)-KH\(_2\)PO\(_4\) buffer, pH 7.4, containing 5 nm dithiothreitol in a volume of 75 ml. The cell suspension was placed in a 180-ml Rosett cooling cell (7) and treated with a Branson Sonifier (model W-140-C) for 12 min at an output of...
130 watts with a standard probe. The sonically disrupted suspension was centrifuged at $10,000 \times g$ for 10 min and the precipitate was discarded. Following centrifugation at $78,000 \times g$ for 60 min, the clear supernatant fluid was discarded and the pellet was suspended in fresh buffer and centrifuged at $229,000 \times g$ for 45 min. The washed pellet was suspended in approximately 5 ml of the phosphate buffer so that the final protein concentration was 5 mg per ml.

**Assay of Hyaluronic Acid-synthesizing System**

Unless otherwise stated the enzyme was assayed in a final volume of 1.5 ml containing 70 μmoles of Na$_2$HPO$_4$-KH$_2$PO$_4$ buffer, pH 7.1, 50 μmoles of MgCl$_2$, 1.0 μmole of UDP-Glc:UA-14C (1.42 × 10$^4$ cpmp), 1.0 μmole of UDP-GlcNAc, and 7.5 μmoles of dithiothreitol. Assays were initiated by addition of enzyme and incubations were performed at 37° for time intervals of 5 to 60 min depending upon the quantity and activity of the enzyme. Reactions were terminated by immersion of reaction tubes in boiling water for 5 min or by addition of 1.5 ml of cold 10% trichloroacetic acid. Zero time tubes which contained the complete reaction mixture were heated or acidified immediately after addition of the enzyme. The hyaluronic acid synthesized during an incubation was purified with carrier hyaluronic acid. After addition of 3.0 mg of carrier streptococcal hyaluronic acid (Procedure 1), 1.0 mg of hyaluronic acid (Procedure 2), or 100 μg of hyaluronic acid (Procedure 3) to the heated or acidified incubation mixture, the samples were centrifuged at 20,000 × g for 20 min. The hyaluronic acid in the supernatant solutions was assayed using one of these procedures.

**Procedure 1**—The supernatant solutions were dialyzed exhaustively against distilled water in the cold. The samples were transferred to 12-ml conical centrifuge tubes and made 0.03 M with respect to NaCl. Sufficient 5% cetylpyridinium chloride was added to produce a flocculent precipitate. After standing for 1 hour at 37°, the precipitates were collected by centrifugation at 1000 × g for 10 min and washed twice with 0.02 M NaCl containing 0.1% cetylpyridinium chloride. The cetylpyridinium-hyaluronic acid complex was dissolved in 1.0 ml of 2 M CsCl, and the hyaluronic acid was precipitated with 9 volumes of ethanol-ether (2:1). After standing for 1 hour at -20°, the precipitated polysaccharide was centrifuged, washed twice with ethanol-ether (2:1), and once with ether. The purified hyaluronic acid was dissolved in 0.03 M NaCl and portions were taken for measurement of radioactivity and uronic acid. Samples were repurified to constant specific activity.

**Procedure 2**—The supernatant solutions were fractionated by gel filtration on columns (0.9 × 200 cm; void volume, 70 ml) of Sephadex G-25 which were equilibrated and eluted with 0.2 M NaCl in 15% ethanol. The hyaluronic acid emerged in the void volume while the sugar nucleotides emerged after elution with 135 ml of solvent. Compounds were located by uronic acid analysis (8) and the radioactivity of each fraction was measured.

**Procedure 3**—The supernatant solutions were dialyzed exhaustively against distilled water in the cold, and made 0.02 M with respect to NaCl. The hyaluronic acid was purified by a modification of the cetylpyridinium chloride-cellulose micro-column technique of Antonopoulos et al. (9). The samples were made 1% with respect to cetylpyridinium chloride and applied immediately to columns (0.6 x 3 cm) of Whatman cellulose powder (coarse) which had been previously equilibrated with 1% cetylpyridinium chloride. The nucleotide sugars were eluted with 5 ml of 1% cetylpyridinium chloride after which the hyaluronic acid was eluted with 3 ml of 1 M NaCl. Aliquots were taken for measurement of uronic acid and radioactivity. Isolation of hyaluronic acid by this method was rapid and quantitative for amounts of 30 to 150 μg.

**Preparation of Carrier Streptococcal Hyaluronic Acid**

Group A streptococci (Type 18, Strain A111) were grown in 4-liter flasks containing 3 liters of 2.5% yeast infusion broth (Difco) and 2% glucose. Cultures were grown at 37° for 18 hours with manual pH control, 6.8 to 7.1, during the last 6 hours. The cultures were inactivated by heat, the cells removed by centrifugation at 12,000 × g for 15 min, and the supernatant fluid was concentrated 4-fold on a rotary evaporator and exhaustively dialyzed against cold water. Two methods were used to purify the hyaluronic acid; one (Preparation A) did not involve the use of proteolytic enzymes; the other (Preparation B) involved a papain digestion. The dialyzed polysaccharide was treated with 75 mg of papain in 0.1 M sodium acetate buffer, pH 5.5, 4.3 mM cysteine-HCl, and 6.4 mM EDTA in a volume of 250 ml at 55° for 18 hours. The incubation was terminated by addition of trichloroacetic acid to a final concentration of 5%. The digest was clarified by centrifugation and the supernatant fluid was dialyzed exhaustively against distilled water. Subsequent procedures were identical for operations on both the untreated and papain-treated dialyzed solutions. Both solutions were made 0.02 M with respect to NaCl and the polysaccharide was precipitated by addition of 14 volumes of ethanol in the cold. The precipitated polysaccharide was collected by centrifugation, washed twice with methanol, washed with ether, and dried in air. This hyaluronic acid was dissolved in 0.02 M NaCl and reisolated as described above (Fraction 1). Yield was 500 mg of hyaluronic acid per liter of culture medium.

Further purification of 500 mg of the hyaluronic acid (Fraction 1) was achieved by chromatography on a column (4 × 30 cm) of Dowex 1-X2 (Cl- form, 200 to 400 mesh). The column was eluted with water, 0.15 M NaCl, and 0.5 M NaCl at a flow rate of 1 ml per min. The uronic acid-positive material which was eluted with 0.5 M NaCl was pooled, dialyzed, and concentrated to 50 ml. The hyaluronic acid was precipitated in 80% ethanol and isolated as described above (Fraction 2). Yield was 360 mg, 70%.

In the case of Preparation A, one additional purification step was employed. Fifty milligrams of hyaluronic acid (Fraction 2) were dissolved in 0.02 M NaCl, precipitated by addition of 5% cetylpyridinium chloride, and dissolved in 10 ml of methanol. The hyaluronic acid was reprecipitated by addition of 25 ml of acetic acid. The precipitated polysaccharide was collected by centrifugation, washed twice with methanol, washed with ether, and dried in air. This hyaluronic acid was dissolved in 0.02 M NaCl and reisolated as the calcium salt according to Procedure 1 described above (Fraction 3). The yield was 42 mg, 84%.

The amino acid content of Preparation A (Fractions 1 and 3) and Preparation B (Fractions 1 and 2) are shown in Table I. Fractions 1 and 3 of Preparation A had the following viscosities: $\eta_{sp} = 14.7$ and 11.3, respectively, which correspond to viscosity
average molecular weights of $7.9 \times 10^5$ and $5.8 \times 10^5$. Fraction 2 of Preparation B had $\eta_2$ = 5.02 which corresponds to a molecular weight of $2.1 \times 10^5$. Viscometry was performed according to the method of Matthews and Dorfman (10) and the viscosity average molecular weights ($M_v$) were calculated according to the equation: $\eta_2 / C = \eta_p = 3.6 \times 10^{-4} \times M_v^{0.78}$ (11).

The very low amino acid content of the more highly purified fractions (Fraction 3 of Preparation A and Fraction 2 of Preparation B) indicate protein contents of 0.13 and 0.21%, respectively. In view of the small quantities of galactosamine present, it is possible that these preparations are contaminated with small glycopeptides derived from the culture medium. However, in view of the high molecular weight of the hyaluronic acid from these preparations, it is conceivable that these amino acids may result from an actual linkage to protein.

**Preparation of Oligosaccharides**

Oligosaccharides of hyaluronic acid and chondroitin sulfate were separated and purified following hydrolysis of 500 mg of polysaccharide for 18 hours in 100 ml of 0.15 M NaCl and 0.1 M sodium acetate buffer, pH 5.0, with 30,000 i.u. of testicular hyaluronidase as previously described (5). Digestion of the polysaccharide by hyaluronidase, a β-hexosaminidase, results predominately in the formation of hexa- and tetrasaccharides having a terminal reducing hexosamine moiety and a nonreducing glucuronic acid moiety. The pentasaccharides derived from hyaluronic acid and chondroitin sulfate were formed by treatment of the appropriate hexa- and tetrasaccharide with β-glucuronidase (5).

The resultant pentasaccharides have a hexosamine moiety at both their reducing and nonreducing termini. The hexa- and pentasaccharides were tested as exogenous substrates in the hyaluronic acid-synthesizing system. Hyaluronic acid tetrasaccharide furnished a convenient source for preparation of saturated and unsaturated disaccharides as described below.

**Digestion of Hyaluronic Acid Tetrasaccharide with Streptococcal Hyaluronidase**

Hyaluronic acid tetrasaccharide, 10 mg, and streptococcal hyaluronidase (Lederle Laboratories), 2 mg, were dissolved in 5 ml of 0.1 M NaHPO$_4$, containing 0.15 M NaCl adjusted to pH 5.3 with 0.1 M KH$_2$PO$_4$. Streptococcal hyaluronidase is an endo-β-N-acetylhexosaminidase which catalyzes the cleavage of the hyaluronic acid-tetrasaccharide with elimination of water to produce the disaccharides, di-HA and Adi-HA. The reaction was monitored by the absorbance at 232 nm and was complete after 2 hours. The disaccharides were purified by gel filtration on a column (0.9 x 200 cm) of Sephadex G-15 equilibrated with 0.2 N NaCl in 15% ethanol. The two disaccharides appear after approximately 125 ml of 0.2 N NaCl in 15% ethanol had passed through the column. The purity of the disaccharides was confirmed by paper chromatography on Whatman No. 3MM paper in Solvent A; di-HA and Adi-HA had $R_f$ values of 0.21 and 0.40, respectively. These compounds were used without further resolution as carrier disaccharides in experiments to be described.

**Methods**

Protein was determined by the biuret method (12) or the method of Lowry et al. (13) with bovine serum albumin as standard. Uronic acid was determined by the carbazole method of Dische (8) and hexosamines by the Boas modification (14) of the Elson-Morgan reaction, omitting the Dowex treatment. Samples of hyaluronic acid (approximately 20 mg) were prepared for amino acid analysis by hydrolysis in 6 M HCl for 20 hours at 100°C and repeated evaporation in a rotary evaporator. Amino acids were estimated quantitatively with an automatic amino acid analyzer (15, 16).

Descending paper chromatography was performed on Whatman No. 1 or No. 3MM paper in the following solvents: A, 1-butanol-pyridine-acetic acid-water (15:10:3:12); B, 95% ethanol-1 M ammonium acetate buffer, pH 5.0 (7:3); C, t-pentanol-isopropanol-water (8:2:2). Compounds were visualized with an ultraviolet lamp or silver nitrate dip reagent (17).

Where possible radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer (model 3375). Aqueous solutions were counted in a mixture which consisted of 0.6 ml of sample, 6.0 ml of ethanol, and 5.5 ml of toluene containing 0.5%, 2.5-diphenyloxazole and 0.01% 1,4-bis[2-(5-phenyloxazoyl)]benzene. Radioactive compounds resolved by paper chromatography were measured in a Packard radiochromatogram scanner (model 7901).

**Materials**

Ribonucleotides, uridine, cytidine, and UMP-3H (2.4 mC per mmole) were purchased from Schwartz BioResearch, Inc. UDP-GlcUA and UDP-GlcNAc were obtained from Sigma. UDP-GlcUA-14C (125 mC per mmole) were purchased from New England Nuclear Corporation. Bovine serum albumin was obtained from Sigma and bacitracin was a product of Upjohn. Oligosaccharides of hyaluronic acid and chondroitin sulfate, generous
The pH activity profile of the hyaluronic acid-synthesizing system. The activity of the hyaluronic acid-synthesizing system was measured between pH 6.1 and 7.7 in phosphate buffer. The incubation mixtures contained 0.24 mg of enzyme protein. The reactions were terminated after 60 min by immersion of the reaction tubes in boiling water for 3 min, and the hyaluronic acid was purified by Procedure 3. Radioactivity of the hyaluronic acid fraction is plotted against pH.

Properties of Hyaluronic Acid-synthesizing System—Before undertaking studies on the mechanism of hyaluronic acid biosynthesis, we wished to further characterize the enzyme system. The effect of pH on the hyaluronic acid-synthesizing system is shown in Fig. 1. The enzyme preparation exhibits maximum activity at pH 7.1. Enzymic activity decreases abruptly above pH 7.3, with virtually no activity present above pH 7.4. To test the effect of various buffers, the enzyme preparation was dialyzed exhaustively against each buffer. Standard assay mixtures containing 0.18 mg of enzyme protein were incubated for 60 min after which the reactions were terminated by addition of trichloroacetic acid and the hyaluronic acid isolated by Procedure 3. The enzymic activity was not significantly different in 0.05 M Na₂HPO₄-KH₂PO₄ buffer, 0.1 M Tris buffer, 0.05 M HEPES buffer, or 0.05 M HEPES-0.05 M KCl buffer. The enzyme was inactive in 0.1 M succinate buffer. It appeared that enzyme activity may be favored by low ionic strength since there was slightly more activity in HEPES and Tris buffers than in either phosphate or in HEPES-KCl buffers.

The hyaluronic acid-synthesizing system exhibits an absolute requirement for divalent metal ion with optimal Mg²⁺ concentration of 50 mM in phosphate buffer (1). In order to determine the optimal concentration of free metal ion, assays were performed in Tris buffer to avoid complexing of Mg²⁺ by phosphate. Following dialysis of the enzyme preparation against 0.1 M Tris buffer, pH 7.1, in the cold, the hyaluronic acid-synthesizing system was completely inactive in the absence of divalent metal ion. Fig. 2 indicates that the optimal concentration for Mg²⁺, 10 mM, was 10-fold greater than that for Mn²⁺, 1 mM; however, the optimal activation by Mn²⁺ was only 73% of that achieved with Mg²⁺. Mg²⁺ was slightly inhibitory at levels above the optimal concentration, but Mn²⁺ inhibited the enzymic activity markedly at concentrations greater than its optimal concentration.

Fig. 3 illustrates the effect of varying the UDP-GlcUA concentration from 0.005 mM to 0.6 mM while maintaining the concentration of UDP-GlcNAC at 1.0 mM. A Lineweaver-Burk plot (20) of these data gave a Kₘ of 5 × 10⁻⁵ M with respect to UDP-GlcUA. Similarly, the effect of varying the concentration of UDP-GlcNAC between 0.015 mM and 1.2 mM in the presence of 0.005 mM UDP-GlcUA is shown in Fig. 4. Although the reciprocal relationship between velocity and UDP-GlcNAC concentration was not strictly linear, the Kₘ of UDP-GlcNAC was estimated to be 5 × 10⁻⁴ M. The Kₘ for UDP-GlcNAC is approximately 10-fold greater than that for UDP-GlcUA. The relationship between velocity and the concentration of UDP-GlcNAC appears similar to situations in which the substrate functions as an activator of the enzyme. The Michaelis constants must of course be considered approximations in view of the particulate nature of the enzyme.

Studies from these laboratories on the biosynthesis of chondroitin sulfate demonstrated that the cell-free enzyme preparation from 13-day-old embryonic chicken cartilage catalyzes the transfer of specific monosaccharides to oligosaccharide acceptors. Therefore, the hyaluronic acid-synthesizing system was examined to determine whether glucuronic acid or N-acetylglucosamine moieties could be transferred from their uridine nucleoside diphos
phosphate derivatives to exogenous oligosaccharide acceptors. There was no detectable transfer of radioactivity from UDP-GlcUA to the pentasaccharides derived from either hyaluronic acid or chondroitin sulfate when tested alone or in the presence of UDP-GlcNAc. Likewise, there was no detectable transfer of radioactivity from UDP-GlcNAc to the hexasaccharides derived from either hyaluronic acid or chondroitin sulfate when tested alone or in the presence of UDP-GlcUA. In these experiments, hyaluronic acid, the oligosaccharides of hyaluronic acid or chondroitin sulfate, and uridine nucleotide sugars were separated by Procedure 2 which resolved these three components completely.

The effect of certain other compounds on the enzymic activity of the hyaluronic acid-synthesizing system was tested. Glucuronic acid and galacturonic acid in concentrations of 1 mM inhibited approximately 15% while N-acetylglucosamine appeared to stimulate the enzymic activity slightly, 10%. No substantial effect was observed when the following compounds were tested in the standard enzyme assay at concentrations of 1.0 mM: UDP-glucose, glucosamine, galactosamine, UTP, UMP, or ATP. The pentas- and hexasaccharides of hyaluronic acid at concentrations of 100 µg per ml neither stimulated nor inhibited the formation of polysaccharide in the enzymic reaction.

Treatment of Hyaluronic Acid-synthesizing System with I-Butanol—Since Telser, Robinson, and Dorfman (5) showed that the particular enzyme system from chicken cartilage is stimulated by treatment with 4% 1-butanol, the effect of butanol treatment on the hyaluronic acid-synthesizing system was studied. Such treatment at 0° for 10 min caused complete inactivation of the enzymic activity (Table II). However, if UDP-GlcUA, UDP-GlcNAc, and Mg++ were present during the butanol treatment; i.e., the constituents required for hyaluronic acid biosynthesis, the enzyme was completely stabilized. Neither the nucleotide sugars nor Mg++ alone prevented inactivation.

As shown in Table III, all uridine nucleoside diphosphate sugars tested at a concentration of 1 nmoI afforded significant protection of enzymic activity in the presence of Mg++. Free uronic acids and amino sugars provided no measurable protection when tested at a concentration of 1 mM. Since those compounds which prevented inactivation by butanol each contained a uridine nucleoside diphosphate moiety, the enzyme was treated with butanol in the presence of 10 mM Mg++ and ribonucleotides, uridine, and cytidine to determine whether these compounds could prevent inactivation. Only UDP and UTP protected the

Table II
Effect of Mg++ and substrates on hyaluronic acid-synthesizing system during I-butanol treatment

For the butanol treatment 125 µmoles of Na₂HPO₄-KH₂PO₄ buffer, pH 7.1, 12 µmoles of cysteine, 1.5 mg of enzyme protein, and 0.24 ml of 1-butanol were mixed at 0° in a volume of 6 ml. Other additions are shown in the table. After 10 min the tubes were centrifuged at 105,000 × g for 60 min, and the supernatant liquid was discarded. The pellets were suspended in 3 ml of 50 mM sodium phosphate-potassium phosphate buffer, pH 7.2, containing 5 mM cysteine, and dialyzed exhaustively against the same buffer. Aliquots of 0.4 ml were removed and assayed; the hyaluronic acid was isolated by Procedure 3 and its radioactivity was measured.

Table II

<table>
<thead>
<tr>
<th>Experiment and additions</th>
<th>Enzyme activity (cpm/tube)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None*</td>
<td>3085</td>
</tr>
<tr>
<td>2. None</td>
<td>265</td>
</tr>
<tr>
<td>3. 10 mM MgCl₂</td>
<td>340</td>
</tr>
<tr>
<td>4. 1 mM UDP-GlcUA + 1 mM UDP-GlcNAc</td>
<td>128</td>
</tr>
<tr>
<td>5. 10 mM MgCl₂ + 1 mM UDP-GlcUA + 1 mM UDP-GlcNAc</td>
<td>3110</td>
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</tbody>
</table>

* In the control, butanol treatment was omitted.
disaccharide intermediates were involved in hyaluronic acid synthesis. Butanol treatment in the presence of UDP actually increased the enzyme activity. Fig. 5 shows that the concentration of UDP which provided 50% stabilization of the enzyme activity was approximately $7 \times 10^{-5}$ M.

Possible Mechanisms for Synthesis of Heteropolysaccharides—Much information has accumulated concerning the synthesis of heteropolysaccharides. In each study where the monosaccharide precursors have been identified, they are nucleoside diphosphate-derivatives (21), and the assembly of the nucleoside diphosphate sugars to form polysaccharide is known to occur by at least two pathways. In one pathway, polysaccharide chain growth occurs by the sequential addition of monosaccharide units to the nonreducing terminus of a growing chain (5). In contrast, polysaccharide synthesis also proceeds by polymerization of lipid-linked oligosaccharide units (22, 23). The lipid has been identified as a C15-isopreny alcohol derivative and is known to occur in several microorganisms (24-26). It was important to determine whether a similar lipid participated in the synthesis of hyaluronic acid, so three types of experiments were performed for this purpose. Following incubation of enzyme assay mixtures with UDP-GlcUA-14C or UDP-GlcNAc-14C, no radioactivity appeared in extracts prepared with butanol, chloroform-methanol (2:1), ethanol-ether (2:1), ethyl acetate, or 95% ethanol-1 M ammonium acetate, pH 5.0 (7:3). In the formation of lipid-linked oligosaccharide intermediates, the initial reaction between lipid and a UDP-sugar substrate can result in the formation of UMP (27). Thus, if lipid-linked disaccharide intermediates were involved in hyaluronic acid synthesis, UMP might be a reaction product. The identity of the uridine nucleotide products formed during hyaluronic acid synthesis was established as follows. A standard enzyme incubation mixture containing 1 mg of enzyme, 1 \mu mole of UDP-GlcUA, and 1 \mu mole of UDP-GlcNAc was incubated for 0, 30, and 60 min. In control tubes, 1 \mu g of enzyme was incubated with either 2 \mu moles of UDP or 2 \mu moles of UMP. Reactions were terminated by heating at 100°C for 5 min and removal of the denatured protein by centrifugation at 20,000 x g for 10 min. A 0.5-ml aliquot of each tube was chromatographed on a column (0.2 x 0.5 cm) of acid-washed charcoal (Darco G-60). After washing the column with 1 ml of water, the uridine nucleotide derivatives were eluted with 4 ml of 50% ethanol, pH 8.0. The eluent was evaporated to dryness on a rotary evaporator, dissolved in 20 \mu l of water, and the entire sample chromatographed on Whatman No. 1 paper in Solvent B. UDP, UMP, and uridine are clearly resolved from the substrates, UDP-GlcUA and UDP-GlcNAc, in this chromatographic system. Qualitative examination of the chromatogram with an ultraviolet lamp revealed that UDP was slowly hydrolyzed to uridine by the hyaluronic acid-synthesizing system, approximately 10% and 20%, during the 30- and 60-min incubations, respectively. UMP was hydrolyzed completely to uridine within the first 30 min of incubation. Chromatographic analysis of portions of the complete reaction mixture revealed that almost all, 70 to 90%, of the nucleotide product chromatographed as UDP. Small quantities of uridine were visualized but these amounts could have arisen by degradation of UDP. Thus, proposed mechanisms in which equimolar quantities of UDP and UMP would be generated are eliminated. Bacitracin specifically blocks peptidoglycan synthesis by inhibition of the dephosphorylation of lipid pyrophosphate to form lipid phosphate (28), an enzymic reaction requisite for lipid participation. If indeed an identical lipid were involved in the formation of hyaluronic acid in streptococci, then bacitracin should inhibit polysaccharide synthesis. No inhibition of hyaluronic acid formation was observed in assay mixtures which contained from 0.66 \mu g per ml to 133 \mu g per ml of bacitracin. These experiments furnished no evidence for the participation of lipid-linked intermediates of the type which participate in the synthesis of O-antigen (26) and peptidoglycan (28).

**Enzyme-bound Hyaluronic Acid**—The incorporation of radioactivity from UDP-GlcUA-14C into both soluble hyaluronic acid and the particulate enzyme was examined. Fig. 6 shows that

### Table III

**Stabilization of hyaluronic acid-synthesizing system by various compounds during 1-butanol treatment in presence of Mg**

The conditions of the butanol treatment and assays were identical with those described in Table II. The concentration of each compound was 1 mM.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Enzyme activity</th>
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<tr>
<td></td>
<td>cpm/tube</td>
</tr>
<tr>
<td>None</td>
<td>AMP 265</td>
</tr>
<tr>
<td>None</td>
<td>CMP 2540</td>
</tr>
<tr>
<td>UDP-GlcUA</td>
<td>UMP 550</td>
</tr>
<tr>
<td>UDP-GlcUA</td>
<td>ATP 272</td>
</tr>
<tr>
<td>UDP-GlcNAc</td>
<td>ADP 440</td>
</tr>
<tr>
<td>UDP-GalNAc</td>
<td>GTP 361</td>
</tr>
<tr>
<td>UDP-glucose</td>
<td>CTP 231</td>
</tr>
<tr>
<td>UDP-xylose</td>
<td>GMP 316</td>
</tr>
<tr>
<td>Glucuronic acid</td>
<td>UTP 2220</td>
</tr>
<tr>
<td>Galacturonic acid</td>
<td>ATP 284</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>CTP 440</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>UMP 272</td>
</tr>
</tbody>
</table>

*a* in the control, butanol treatment was omitted.
Fig. 6. The incorporation of radioactivity from UDP-GlcUA-\(^{14}C\) into soluble and particulate hyaluronic acid. Incubation mixtures contained 0.1 \(\mu\) mole of UDP-GlcUA-\(^{14}C\) (2.8 \(\times\) 10\(^3\) cpm) and 1.5 mg of enzyme protein. Incubations were performed at 37\(^\circ\)C and terminated by addition of 1.5 ml of cold 10\% trichloracetic acid at the times indicated. Hyaluronic acid was isolated from the trichloracetic acid supernatant fractions by Procedure 3. The precipitated enzyme was washed three times with cold 5\% trichloracetic acid, the residues were dissolved in 1 ml of formic acid, and the radioactivity of aliquots was determined. Radioactivity in hyaluronic acid isolated from the trichloracetic acid soluble (\(\bullet\)) and precipitable fractions (\(\oplus\)) of incubation mixtures are plotted against time.

The specific activity of hyaluronic acid increased linearly with time (carrier hyaluronic acid was used in the isolation of hyaluronic acid according to Procedure 3). The incorporation of radioactivity into the particulate enzyme reached a maximum after approximately 10 min and remained unchanged.

A variety of conditions were employed to remove the bound radioactivity from the particulate enzyme. For this purpose the enzyme was precipitated from incubation mixtures with trichloracetic acid and washed three times with cold 5\% trichloracetic acid, the residues were dissolved in 1 ml of formic acid, and the radioactivity of aliquots was determined. Radioactivity in hyaluronic acid isolated from the trichloracetic acid soluble (\(\bullet\)) and precipitable fractions (\(\oplus\)) of incubation mixtures are plotted against time.

The presence of free radioactive glucuronic acid is explained by the fact that the hyaluronidase preparation contains a small amount of \(\beta\)-glucuronidase. These results indicate that the

### Table IV

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time*</th>
<th>Radioactivity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>di-HA</td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
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<tr>
<td>2</td>
<td>10</td>
<td>38</td>
</tr>
<tr>
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* Time of incubations following the addition of UDP-GlcNAc.

Previously equilibrated and eluted with 0.2 M NaCl in 15% ethanol, and all of the detectable radioactivity appeared in the void volume. This material was digested with streptococcal hyaluronidase which splits the glycosaminidic linkages of hyaluronic acid through an intramolecular hydrolysis and elimination of water. As a result of this reaction, digestion of this acid leads to the formation primarily of \(\Delta\)di-HA while a much smaller quantity of di-HA is formed from the nonreducing terminal disaccharide of the hyaluronic acid chain. After digestion of the radioactive void volume material with 1 mg of streptococcal hyaluronidase at 37\(^\circ\)C for 18 hours, the radioactive material was retarded on chromatography on Sephadex G-25. All of the measurable radioactivity appeared in a region characteristic of \(\Delta\)- and monosaccharides. The major radioactive product of the hyaluronidase digestion was identified as \(\Delta\)di-HA, while much lesser quantities of both radioactive di-HA and glucuronic acid were identified by paper chromatography in Solvents A and C. The presence of free radioactive glucuronic acid is explained by the fact that the hyaluronidase preparation contains a small amount of \(\beta\)-glucuronidase. These results indicate that the
nature of hyaluronic acid chain growth—Experiments were undertaken to determine the mechanism of hyaluronic acid chain growth. Incubation of the enzyme with only UDP-GlNAc resulted in the incorporation of glucuronic acid into the trichloroacetic acid-precipitable enzyme. Digestion of the precipitated labeled enzyme (Table IV, Experiment 1) with streptococcal hyaluronidase released 25% of the bound radioactivity. Following addition of carrier disaccharides, di-HA and Adi-HA were resolved by chromatography on Dowex 1. The predominance of radioactivity in di-HA formed by hyaluronidase digestion indicated that most of the radioactive glucuronic acid (88%) was at the nonreducing terminus of the hyaluronic acid chains. In another experiment, glucuronic acid digestion of precipitated enzyme, which had been labeled by incubation with UDP-GlNAc alone, resulted in release of 38% of the radioactivity. Glucuronic acid was the sole radioactive product of the β-glucuronidase digestion as established by paper chromatography in Solvents A and C. Thus, it appeared that single monosaccharide units can be transferred to the nonreducing ends of the nascent hyaluronic acid chains. Presumably steric hindrance prevents the streptococcal hyaluronidase and β-glucuronidase from removing more of the radioactive glucuronic acid which was incorporated into nascent chains on the particulate enzyme.

In the presence of UDP-GlNAc the incorporation of radioactivity from UDP-GlNAc into the enzyme was stimulated 50- to 100-fold and labeled hyaluronic acid began to appear in the medium. The enzyme is specific with regard to the monosaccharide unit transferred as evidenced by the fact that the presence of UDP GalNAc stimulated neither incorporation of radioactivity from UDP-GlNAc nor formation of hyaluronic acid. Following trichloroacetic acid precipitation of enzyme from an incubation mixture which contained both UDP-GlNAc and UDP-GlNAc, streptococcal hyaluronidase digestion released approximately 90% of the incorporated radioactivity. From such digests the radioactive di-HA and Adi-HA were isolated with carrier as above. The predominance of labeled Adi-HA indicated that under conditions of chain growth a large proportion of the incorporated glucuronic acid (98%) is no longer terminal. Table IV indicates the effect of time of incubation on the ratio of radioactivity of Adi-HA to that of di-HA (Experiments 2 to 8). This ratio is a measure of the terminal labeled glucuronic acid residues compared to labeled internal glucuronic acid residues. After 1 min, the ratio becomes constant representing equilibrium between relative labeling of terminal and internal positions. The maximum ratio is a measure of average molecular weight of bound chains; this was calculated to be 24,000. The reason for the discrepancy between the times required for maximal labeling of the particulate enzyme and that for producing a constant ratio of radioactivity of Adi-HA to that of di-HA is not clear. This may be explained by the fact that streptococcal hyaluronidase does not appear to completely digest short chains, thus the disaccharide ratio does not adequately measure the degree of labeling of the internal portion of the chain. In contrast, the data in Fig. 6 reflect the rate of labeling of the total chain. In any case it is clear that chain growth proceeds by addition to the nonreducing terminus of the chain.
serine residues on an acceptor protein (4), and is inhibited by puromycin (3). In view of the demonstrable similarity between chondroitin sulfate and hyaluronic acid chain growth, the effect of inhibitors of protein synthesis on hyaluronic acid formation in streptococcal was tested. Fig. 7 illustrates the effect of puromycin and L-chloramphenicol upon the incorporation of $^{14}$C-leucine into total cell protein in suspensions of streptococci. Both compounds slightly stimulate the incorporation of $^{14}$C-leucine into protein at low concentrations (20 µg per ml or less), sharply inhibit the incorporation of amino acid at higher concentrations (>20 µg per ml), and nearly completely inhibit $^{14}$C-leucine incorporation at 500 µg per ml. Table V shows that hyaluronic acid synthesis was not affected by puromycin or L-chloramphenicol (500 µg per ml) as measured by the incorporation of $^{3}$H-acetate (30). Thus hyaluronic acid synthesis in streptococci does not appear to be dependent on concomitant protein synthesis.

**DISCUSSION**

Knowledge has accumulated rapidly concerning the enzymic synthesis of heteropolysaccharides. Three major questions regarding polysaccharide synthesis may be asked. How are polysaccharide chains initiated? How do polysaccharide chains grow? How is polysaccharide chain growth terminated?

An understanding of polysaccharide chain initiation exists for the glycosaminoglycans for which structural studies have established the relationship between the polysaccharide and protein (31). Synthesis of chondroitin sulfate proper is preceded by synthesis of a neutral sugar linkage region, galactosyl-galactosyl-xylose (31), in which xylose is linked by an O-glycosidic bond to the hydroxyl group of serine residues of a preformed protein acceptor. Transfer of a glucuronic acid moiety from UDP-GlcUA to the second galactose moiety initiates synthesis of the chondroitin sulfate chain (32). As a consequence of these ordered reactions, it was possible to demonstrate a dependence of polysaccharide synthesis on protein synthesis (3), thus puromycin treatment of tissue minces inhibited both protein and polysaccharide synthesis. Similarly, chondroitin sulfate and hyaluronic acid synthesis in normal and Hurler human fibroblasts grown in tissue culture are inhibited by puromycin or cycloheximide (33) which suggests a dependence upon protein synthesis in these systems also. In contrast, hyaluronic acid synthesis in streptococci was not affected by puromycin or chloramphenicol and indicated that this system may differ with respect to a requirement for concomitant protein synthesis.

It is not yet clear whether hyaluronic acid in streptococci is bound covalently to protein. Table I showed that our most highly purified streptococcal hyaluronic acid (Preparation A, Fraction 3) contains a limited number of amino acids (0.13% protein) and galactosamine. The amino acid content was not reduced by proteolysis with papain during purification. In fact, the papain-treated hyaluronic acid (Preparation B) contained a slightly greater quantity of amino acids. Sandson and Hamerman (34) reported that streptococcal hyaluronic acid contains galactose and a limited number of amino acids, and these studies suggested to them that these features are in common with chondromucoprotein of cartilage and hyaluronic acid-protein complex from synovial fluid. In attempts to determine the nature of the reducing terminus of hyaluronic acid chains, preliminary evidence for the presence of galactose has also been found in this laboratory. Although the presence and quantity of amino acids in our preparations would be consistent with participation of these compounds in covalent linkage with hyaluronic acid, the insensitivity of hyaluronic acid synthesis in *Streptococcus* to puromycin and chloramphenicol does not appear to support that conclusion. It is possible that the presence of amino acids and galactosamine in these streptococcal hyaluronic acid preparations results from contamination with glycopeptides derived from the culture medium. If hyaluronic acid were covalently linked to protein during synthesis, the insensitivity of hyaluronic acid formation to inhibition of protein synthesis could be explained in several ways. Hyaluronic acid could be covalently linked to protein during synthesis, but released enzymically from its site of synthesis. The release of each hyaluronic acid chain would free an acceptor site and new synthesis could be initiated at that site without additional protein synthesis. Another explanation may be that the hyaluronic acid chain is attached to a component whose synthesis is insensitive to these protein synthesis inhibitors.

Attempts to establish the nature of the bond between nascent hyaluronic acid chains and the particulate enzyme have yielded little substantial information. The release of nascent chains by acid or base treatment might be consistent with hydrolysis of a covalent bond between the hyaluronic acid chain and membrane, and solubilization of the nascent chains by pyridine or sodium deoxycholate may only be the result of disruption of membranes. Although the nature of chain binding is not yet known, it is clear that in the enzyme system in *vitro* nascent chains must grow to a molecular weight exceeding 24,000 since that is the minimal average molecular weight of the bound chains and subsequently appear in the medium.

Polysaccharide chain growth has been studied extensively and Robbins et al. (35) have reviewed the current knowledge. There is experimental evidence which supports the existence of at least two pathways of polysaccharide chain growth. One mechanism involves the polymerization of pre-formed oligosaccharide intermediates; the second mechanism involves the sequential transfer of monosaccharide units from nucleoside diphosphate derivatives to the nonreducing terminus of a growing polysaccharide chain. It seemed reasonable that hyaluronic acid synthesis in streptococci proceeds via the second mechanism as originally suggested by Markovitz, Cifonelli, and Dorfman (1), however, a decision between this and other mechanisms has awaited further experimental evidence.

The first mechanism involves the polymerization of lipid-linked pre-formed oligosaccharide intermediates such as in O-antigen synthesis (22) and peptidoglycan synthesis (23, 24). The following evidence fails to support the participation of a lipid intermediate in streptococcal hyaluronic acid synthesis. (a) No radioactive intermediates were detected following extraction of the enzyme with a variety of lipid solvents: butanol, chloroform-methanol (2:1), ethanol-ether (2:1), or ethyl acetate. (b) UMP is not a major reaction product of hyaluronic acid synthesis (36). The presence of phosphatases in this system complicates the stoichiometric determination of UDP and UMP such that UMP formation cannot be excluded completely, but proposed mechanisms in which equimolar amounts of UDP and UMP would be produced are eliminated. (c) Bacitracin does not inhibit hyaluronic acid biosynthesis.

Experiments were undertaken which subsequently demonstrated that hyaluronic acid chain growth occurs by the transfer of single monosaccharide units to the nonreducing ends of nascent hyaluronic acid chains. The process of chain growth in this
system appears analogous with the process of chondroitin sulfate oligosaccharide elongation in embryonic chicken cartilage (3).

Sufficient information is not yet available to formulate a detailed mechanism for hyaluronic acid chain growth, but any proposed mechanism should recognize certain characteristics of the enzyme system. Hyaluronic acid synthesis requires UDP-GlcUA, UDP-GlcNAC, Mg++, and a particulate enzyme. Oligosaccharide chains of hyaluronic acid are bound to the enzyme, and these serve as a primer for polysaccharide synthesis. These nascent chains cannot be completely removed by enzymic digestion with hyaluronidase. Under no conditions tested could monosaccharides be transferred from UDP-GlcUA or UDP-GlcNAC to exogenous oligosaccharides of either hyaluronic acid or chondroitin sulfate. Since butanol treatment often activates or solubilizes particulate enzymes (37), the irreversible inactivation of the hyaluronic acid-synthesizing system by butanol treatment was unexpected, but the enzymic activity was stabilized by addition of Mg++ and substrates (conditions of hyaluronic acid synthesis) prior to treatment with butanol. This observation is not totally unlike the observation that the hyaluronate synthesis system appears analogous with the process of chondroitin sulfate oligosaccharide elongation in embryonic chicken cartilage (3).

These considerations would be consistent with the mechanism previously proposed by Markovitz et al. (1) providing the model is extended to account for binding of nascent chains and participation of Mg++. The irreversible inactivation of the enzyme system by mild perturbants such as temperature (37°) and butanol treatment suggests the importance of the integrity of the active surface and geometry of the various binding sites. It is probable that nascent chains are bound to the membrane via the reducing ends in a manner not yet explained. The presence of bound nascent chains may account for the failure of exogenous oligosaccharides to serve as acceptors.

The regulation of hyaluronic acid synthesis in streptococci could occur at several levels. It has been observed that the Km values for UDP-GlcUA and UDP-GlcNAC are 5 x 10^-4 M and 5 x 10^-4 M, respectively. Synthesis of hyaluronic acid is the only known synthetic route of UDP-GlcUA in streptococci, while UDP-GlcNAC is required for many additional vital cellular activities such as synthesis of Group A specific polysaccharide, cell wall, and glycopeptide. It seems appropriate that UDP-GlcUA should have the lower Km for cessation of UDP-GlcUA synthesis could prevent hyaluronic acid formation without simultaneously requiring a change in the intracellular concentration UDP-GlcNAC. Because UDP, a reaction product, inhibits polysaccharide synthesis, it has been postulated that UDP may be a possible regulator (21). It would seem possible that both substrates and product could exert control of hyaluronic acid synthesis. Hyaluronic acid synthesis appears dependent upon Mg++ and the uridine nucleoside diphosphate moiety. Therefore changes in the surface configuration may regulate the rate of synthesis of hyaluronic acid. The activity or state of the enzyme thus may be affected by the binding of substrates, products, or cofactors to the particles.

Little is known concerning the mechanism of hyaluronic acid chain termination in this system. Having demonstrated the turnover of nascent chains, it seems possible that the release of mature hyaluronic acid chains to the medium may be catalyzed enzymically.

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