Biosynthesis of Hyaluronic Acid by *Streptococcus*

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Synthesis of hyaluronic acid was investigated in a cell-free system derived from a strain of Group A streptococci. Preparative procedures were improved so that an enzyme system 70 times more active than that previously reported was obtained. The hyaluronic acid synthesized could be separated into trichloroacetic acid-soluble and -insoluble fractions. On the basis of pulse-chase experiments, it was shown that the trichloroacetic acid-insoluble fraction is a precursor of the soluble fraction. The release of the trichloroacetic acid-insoluble hyaluronic acid is specifically blocked with p-chloromercuribenzoate, without inhibition of chain elongation. The addition of butanol to trichloroacetic acid resulted in solubilization of all of the hyaluronic acid. No detectable difference in molecular size was observed between the two hyaluronic acid fractions, both of which were estimated to be more than one million daltons in size. Testicular hyaluronidase digestion of either one of the two types of hyaluronic acid yielded no high molecular weight fragments, indicating that hyaluronic acid is not bound covalently to protein. However, following incubation of enzyme assay mixtures with UDP-[14C]GlcUA, even in the absence of UDP-GlcNAc, radioactive high molecular weight hyaluronic acid was obtained which suggests that the enzyme system elongates rather than initiates hyaluronic acid chains. Tunicamycin did not inhibit hyaluronic acid synthesis, indicating lack of participation of an intermediate of pyrophosphorylpolyisoprenol type. The results obtained are consistent with the hypothesis that chain elongation of hyaluronic acid proceeds by alternate addition of monosaccharides from UDP-sugars by a membrane-bound synthesizing system followed by release of completed hyaluronic acid chains.

Hyaluronic acid, a ubiquitous constituent of connective tissues, has been implicated in such diverse biological phenomena as lubrication (1), cell adhesion (2-4), cell mobility (5), and formation of aggregates with chondroitin sulfate proteoglycan (6, 7). However, the mechanism of hyaluronic acid biosynthesis is imperfectly understood. Among the reported hyaluronic acid-synthesizing systems (8-11), the *Streptococcus* system has been studied most extensively (12-17) and has several advantages including the exclusive synthesis of one glycosaminoglycan (hyaluronic acid) and the high specific activity of the enzyme preparations. Previous studies led to the speculation that hyaluronic acid is synthesized by alternate addition of monosaccharides (15). However, recently Turco and Heath reported the isolation of a dolichyl-phosphatidylglucosamine-GlcNAc-GlcUA from SV40-transformed human lung fibroblasts (18). Hopwood and Dorfman also reported the isolation of glycolipids containing glucuronic acid and N-acetylgalactosamine from a rat fibrosarcoma (19). Although the role of these lipid intermediates is unknown, it is possible that they are involved in either heparin (or heparan sulfate) or hyaluronic acid synthesis. Takateaki and Tamura reported the inhibition by tunicamycin of the total glycosaminoglycan synthesis in cultured embryonic chick fibroblasts (20). In the present study, the *Streptococcus* system was used to reinvestigate the mechanism of hyaluronic acid synthesis including the possible involvement of a lipid intermediate.

**EXPERIMENTAL PROCEDURES**

Materials—UDP-[U-14C]GlcUA (290 mCi/mmol) and UDP-[6-3H]GlcNAc (6.6 Ci/mmol) were purchased from New England Nuclear. Other materials were obtained from the following sources: p-nitrophenyl-p-n-glucuronoside and p-nitrophenyl-2-acetamido-2-deoxy-p-glucopyranoside from Koch-Light Laboratories; cetlypyridinium chloride from K & K Laboratories; trilauric acid from J. T. Baker Chemical Co.; N-ethylmaleimide from Schwarz BioResearch Inc.; UDP-GlcUA, UDP-GlcNAc, GlcUA-1-P, xylose-1-P, p-nitrophenyl-p-n-glucuronoside, p-nitrophenyl-2-acetamido-2-deoxy-p-galactopyranoside, p-chloromercuribenzoic acid, and phenylmethylsulfonyl fluoride from Sigma. Nonidet P-40 from Shell Oil Co.; Triton X 100 from Rohm & Haas; Tween 80 from E. H. Sargent & Co.

Beef lung heparin, streptococcal hyaluronic acid, and GlcNAc-1-P were the gifts of Dr. J. Anthony Cifonelli, University of Chicago. Hyaluronic acid from human umbilical cord was generously supplied by Dr. Martin B. Mathews, University of Chicago. Hyaluronic acid tetra- and hexa-saccharide fragments were prepared according to the method of Underhill and Dorfman (4). Adi-HA was prepared by streptococcal hyaluronidase digestion of streptococcal hyaluronic acid. Hyaluronic acid trisaccharide fragment (GlcNAc-GlcUA-GlcNAc) was prepared by β-glucuronidase digestion of tetrascaccharide and was a gift from Dr. Alan Appel, University of Chicago. A mixture of Adi-HA and di-HA was prepared by streptococcal hyaluronidase digestion of hyaluronic acid tetrasaccharide and was used as a standard for paper chromatography.

Vandase used as a source of streptococcal hyaluronidase, purchased from Lederle Laboratories, was partially purified by ammonium sulfate precipitation by Dr. Allen L. Horwitz, University of Chicago. Testicular hyaluronidase (EC 3.2.1.35) (20,000 IU/mg) was purchased from Leo Helsinborg Laboratories, Sweden, and pronase from Calbiochem. Bovine liver β-glucuronidase type B-10 (10,000 units/mg) was obtained from Sigma. Tunicamycin was kindly provided by Dr. G. Tamura, Tokyo University, and was dissolved at a concentration of 100 μg/ml in 0.01 N NaOH for use.

* The abbreviations used are: GlcUA, glucuronic acid; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; Adi-HA, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-D-glucose; di-HA, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-D-glucose; PMSF, phenylmethylsulfonyl fluoride; p-CMD, p-chloromercuribenzoate.

‡ Established Investigator of the American Heart Association.

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Growth and Harvest of Cells—Group A streptococci (type 18, Strain A111) were maintained as previously described (12). Cultures were grown and harvested according to the method of Stoolmiller and Dorfman (15).

Preparation of Hyaluronic Acid-synthesizing System—The enzyme system was prepared according to the method of Stoolmiller and Dorfman (15), except that during sonication of the cells, the probe was cooled with ice water at 1-min intervals after every three cycles of sonication. The disrupted suspension was centrifuged at 10,000 × g for 10 min and the supernatant fluid withdrawn. The pellet was sonicated six times and the supernatant fluid removed after each sonication. Following centrifugation of the combined 10,000 × g supernatant solutions at 70,000 × g for 90 min, the resultant pellet was suspended in fresh buffer and centrifuged at 229,000 × g for 45 min. The washed pellet was suspended in 0.033 M NaHPO4/KH2PO4 buffer, pH 7.4, containing 5 mM dithiothreitol yielding a final protein concentration of 8 mg/ml. Approximately 72.5 mg of the enzyme protein was obtained from about 80 ml of frozen compacted cells.

Assay of Hyaluronic Acid-synthesizing System—A standard incubation mixture of 100 ml contained 3.33 mM of MgCl2, 0.5 mM of dithiothreitol, 0.6 mM of UDP-[14C]GlcUA (1.6 to 16 × 10^6 cpm), and 0.1,900 mg of enzyme protein depending on the experiment. Reactions at 37°C were initiated by the addition of enzyme and terminated by addition of 0.9 ml of cold 5.6% trichloroacetic acid solution for liquid scintillation counting. After addition of 500 µg of carrier hyaluronic acid, soluble hyaluronic acid synthesized was purified from the combined trichloroacetic acid supernatant fluid by cetylpyridinium chloride precipitation followed by ethanol precipitation according to "Procedure 1" described in the previous paper (15). The radioactivity of aliquots was determined by liquid scintillation counting.

Extraction of Trichloroacetic Acid-insoluble Hyaluronic Acid—For solubilization of trichloroacetic acid-insoluble hyaluronic acid, the precipitate from a standard incubation mixture was extracted by either one of the following two procedures: Procedure A, the precipitate was extracted three times with 1 ml of 0.3% sodium deoxycholate solution (15); Procedure B, following extraction of the precipitate with 1 ml of acetone three times, the residue was treated with 1.0 ml cold methanol, dissolved in water, and subjected to descending paper chromatography on Whatman No. 1 in the Solvent System R using di-HA, glucuronic acid, glucuronolactone, xylose, and glucose as standards. The radioactive material was located by liquid scintillation counting of the paper strips. Sugars were detected by silver nitrate method.

Incorporation of Radioactivity from Substances A and B—Substance A or B containing approximately 20,000 cpm was incubated with 200 µg of enzyme protein in the absence or presence of 0.1% Tween 80 in a final volume of 80 µl of 0.059 M NaHPO4/KH2PO4 buffer, pH 7.0, containing 3.33 µmol of MgCl2 and 0.6 µmol of dithiothreitol. After 15 min of incubation at 37°C, 10 µl each of 60 mM UDP-[14C]GlcUA and 60 mM UDP-GlcNAc were added to the incubation mixture and incubation was continued for an additional 75 min. The reaction was terminated by addition of trichloroacetic acid and the supernatant fluid was subjected to gel filtration on a column (1.2 × 108 cm) of Sephadex G-50 (superfine). The trichloroacetic acid precipitate was assayed for radioactivity as described under "Experimental Procedures."

RESULTS

Trichloroacetic Acid-soluble and -insoluble Hyaluronic Acid

As previously demonstrated (15), the incorporation of radioactivity from UDP-[14C]GlcUA into both trichloroacetic acid-soluble and -insoluble hyaluronic acid fractions was observed. Fig. 1A shows the level of incorporation of radioactivity into the trichloroacetic acid-insoluble fraction reached a plateau after 30 min, whereas incorporation into the tricho-
Hyaluronic Acid Biosynthesis

Roacetic acid-soluble hyaluronic acid fraction increased linearly with time up to 70 min. In the experiments shown in Fig. 1B, the incubation conditions were changed so that the amount of radioactive substrate was rate-limiting. As a result, more complete utilization of the radioactive UDP-GlcUA was achieved. Of the total counts added to the incubation mixtures, 75% were incorporated into the trichloroacetic acid precipitates, and 23% into the trichloroacetic acid-soluble hyaluronic acid fraction. The incorporation of radioactivity into both fractions reached a plateau due to the consumption of UDP-GlcUA rather than enzyme inactivation. As shown in Fig. 2, the enzyme remains stable after 60 min of incubation. Based on the results shown in Fig. 1A, the enzyme system was calculated to have the capacity to transfer GlcUA from UDP-GlcUA at a rate of approximately 510 nmol/h/mg of protein, which is approximately 70 and 8% times more active than the same enzyme system previously prepared from streptococci (15) or from rat fibrosarcoma (11), respectively.

Identification of Hyaluronic Acid

Trichloroacetic acid-soluble and -insoluble hyaluronic acid were isolated, respectively, from the incubation mixture described in Fig. 1B by cetylpyridinium chloride precipitation and ethanol precipitation, and by acetone/phosphate buffer as described under "Experimental Procedures." Both radioactive preparations were digested with streptococcal hyaluronidase and then subjected to paper chromatography on Whatman No. 3MM in Solvent A. Each digest yielded only one radioactive spot upon scanning, which had the same RF value (0.68) as that of authentic Δdi-HA, confirming that the radioactive compounds isolated from both trichloroacetic acid-soluble and -insoluble fractions were hyaluronic acid.

The Relationship between Trichloroacetic Acid-soluble Hyaluronic Acid and Trichloroacetic Acid-insoluble Hyaluronic Acid

A chase experiment was performed to examine the relationship between the soluble hyaluronic acid and the insoluble hyaluronic acid. The incubation was fast carried out with an amount of UDP-[14C]GlcUA that was consumed, and then an excess amount of nonradioactive UDP-GlcUA was added. As shown in Fig. 2, after 20 min of incubation, radioactive UDP-[14C]GlcUA was almost depleted, and following addition of nonradioactive UDP-GlcUA, the bound hyaluronic acid was released from the particulate enzyme fraction. The results indicate that the bound hyaluronic acid is nascent and that it is released from the particulate enzyme after elongation or completion of the chain.

Properties of the Soluble and Bound Hyaluronic Acid

The trichloroacetic acid-insoluble radioactive material is known to be extracted with 50% pyridine or 0.5% sodium bicarbonate.
deoxycholate (15). Extraction also can be achieved by acetone/phosphate buffer as described under "Experimental Procedures." In contrast, 4% butanol, 4% butanol containing 0.01 M EDTA, 95% ethanol, chloroform/methanol (2:1), ethanol/ether (2:1), 2 to 8 M urea, 2 M NaCl, 1 M sodium acetate, or 80% saturated (NH₄)₂SO₄ did not solubilize the radioactive material (15). The mechanism by which the radioactive material is rendered soluble is not known. Attempts to extract the trichloroacetic acid-precipitable radioactive material with absolute pyridine, absolute butanol, chloroform/methanol/water (1:6:4) or (1:1:0:3) were unsuccessful. However, extraction was accomplished with an unique solvent system, which is a mixture of a small amount of butanol and 5% trichloroacetic acid. As shown in Fig. 3, more than 80% of the insoluble radioactive material was extracted at butanol concentrations above 5%, while below 5% only 15 to 20% was extracted. It should be noted that aqueous butanol without trichloroacetic acid extracted less than 30% of the radioactive material even at butanol concentrations greater than 6%. The results suggest that there is some hydrophobic interaction resulting in binding of hyaluronic acid.

On gel filtration there was no detectable difference in size between soluble and bound hyaluronic acid. Since both hyaluronic acid preparations were almost totally excluded from a Sepharose 2B column as shown in Fig. 4, their average molecular size was estimated to be at least 1 × 10⁵ daltons. The peak eluted in the column volume is mainly due to the unreacted UDP-[¹⁴C]GlcUA. Further investigation of the retarded peaks are described below.

p-Chloromercuribenzoate in a concentration greater than 1.0 mM inhibited hyaluronic acid biosynthesis (Table I). There is a marked difference in the extent of the inhibition of synthesis of soluble and bound hyaluronic acid. A time course experiment using 1.5 mM p-chloromercuribenzoate demonstrated (data not shown) that even after 100 min, inhibition of synthesis of insoluble hyaluronic acid was only 30%, but inhibition of incorporation of radioactivity into the soluble fraction was almost complete. These results as well as those illustrated in Fig. 2 indicated that bound hyaluronic acid chains are nascent or elongating, and soluble hyaluronic acid chains are mature and have been released from the membrane.

p-Chloromercuribenzoate appears to inhibit the release of completed hyaluronic acid chains.

**Binding of Insoluble Hyaluronic Acid**

It was of interest to determine whether hyaluronic acid in the trichloroacetic acid-insoluble fraction is bound to some nonpolysaccharide component. In order to determine the possible nature of such a compound, the enzyme was incubated with UDP-[¹⁴C]GlcUA in the absence of UDP-GlcNAc. A phosphate buffer extract of the trichloroacetic acid precipitate from such an incubation mixture gave two radioactive peaks on Sephadex G-100 as shown in Fig. 5. One was eluted in the void volume and the other which was included had the same molecular size as authentic UDP-GlcUA on Sephadex G-50 (superfine) (data not shown). The results suggest that the acceptor for the glucuronic acid transfer from UDP-[¹⁴C]-GlcUA has a size of greater than 1 × 10⁵ daltons. This could either be a nonpolysaccharide acceptor or a growing hyaluronic acid chain.

The following experiments were performed to determine the possible nature of any component attached covalently to bound hyaluronic acid.

**Gel Filtration of Insoluble Hyaluronic Acid under Dissociating Conditions**—Insoluble hyaluronic acid was extracted by acetone/phosphate buffer and subjected to gel filtration on a G-100 column (1.2 × 10⁷ cm) in the presence of 2 M NaCl, 2 M urea, or 0.5% sodium dodecyl sulfate. In the last case, the

![Fig. 4. Molecular size of soluble and insoluble hyaluronic acid.](image)

**Table I**

The effect of p-chloromercuribenzoate on the incorporation of radioactivity from UDP-[¹⁴C]GlcUA into hyaluronic acid

<table>
<thead>
<tr>
<th>Concentration</th>
<th>TCA-soluble</th>
<th>TCA-insoluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>cpm x 10⁵</td>
<td>cpm x 10⁵</td>
</tr>
<tr>
<td>0</td>
<td>27.4 (100)</td>
<td>12.0 (100)</td>
</tr>
<tr>
<td>0.05</td>
<td>23.8 (94)</td>
<td>13.1 (109)</td>
</tr>
<tr>
<td>0.20</td>
<td>26.4 (96)</td>
<td>11.8 (98)</td>
</tr>
<tr>
<td>0.50</td>
<td>18.8 (69)</td>
<td>10.7 (89)</td>
</tr>
<tr>
<td>1.0</td>
<td>0.1 (0.4)</td>
<td>9.6 (86)</td>
</tr>
<tr>
<td>1.5</td>
<td>0.05 (0.2)</td>
<td>6.6 (55)</td>
</tr>
</tbody>
</table>
sample was pretreated with 4% sodium dodecyl sulfate at 100°C for 10 min before application to the column. In all cases, the radioactivity emerged in the void volume (data not shown). No smaller molecular weight peak was revealed under any of those dissociating conditions, indicating that insoluble hyaluronic acid is not an aggregated form.

**Pronase Digestion and Alkaline Treatment of Insoluble Hyaluronic Acid**—Experiments were undertaken to determine whether the insoluble hyaluronic acid is bound covalently to a protein. A radioactive insoluble hyaluronic acid preparation obtained by acetone/phosphate buffer extraction was treated with pronase or dilute alkali under conditions which cleave O-glycosidic bonds (29). After each treatment, the sample was subjected to gel filtration on a Sepharose 4B column. In both cases, no significant change in gel filtration pattern was observed since the radioactivity emerged in the void volume (data not shown). The results suggest that insoluble hyaluronic acid is not attached to a protein sufficiently large to affect the gel filtration pattern on Sepharose 4B.

**Testicular Hyaluronidase Digestion**—Since hyaluronic acid is a large molecule, gel filtration on Sepharose 4B may not have detected the possible slight decrease in molecular size in the experiments described above. Therefore, both soluble and insoluble hyaluronic acid fractions were digested with highly purified testicular hyaluronidase and subjected to gel filtration on a column of Sephadex G-100 (Fig. 6A). Sufficient radioactivity was used so that if any oligosaccharide remained attached covalently to a protein, and if the addition of radioactive sugar takes place not only in the distal region but also in the proximal region of the polysaccharide it would be expected to be detected and separated from the free oligosaccharides produced by enzymic digestions. However, both digests gave only one peak which was eluted in the column volume, suggesting the absence of a protein core. Similar results were obtained by streptococcal hyaluronidase digestion followed by Sephadex G-50 chromatography (data not shown).

Each radioactive peak in the column volume was further analyzed on a column of Sephadex G-50 (superfine). Both preparations showed the same chromatographic pattern, giving two major radioactive peaks, which are probably hexa- and tetrasaccharide, respectively, and two minor radioactive peaks, presumably octa- and disaccharide (Fig. 6B). It seems unlikely that an additional constituent is attached covalently to only insoluble hyaluronic acid. If any, it would be very small in molecular size.

**Effect of Sugars and Glycosides on Hyaluronic Acid Synthesis**

Much information has been accumulating concerning the synthesis of glycosaminoglycans. Chondroitin sulfate synthesis is known to be initiated by addition of xylose to serine residues onto an acceptor protein (26). However, nothing is known concerning chain initiation of hyaluronic acid. No substance with acceptor activity, natural or synthetic, has been found. Although Stoolmiller and Dorfman (16) tested several radioactive UDP-sugars, such as UDP-Glc, UDP-Gal, UDP-xylose, and UDP-arabinose in a streptococcal microsomal system, no measurable radioactivity was incorporated from any except from UDP-[4C]Glc. In the case of UDP-[4C]Glc, radioactivity was incorporated into the particulate enzyme fraction. Although the nature of radioactive product was not determined, the addition of UDP-Glc did not stimulate the hyaluronic acid synthesis. We tested a variety of sugars and glycosides including hyaluronic acid hexa- and hyaluronic acid itself to determine whether they stimulate hyaluronic acid synthesis (Table II). No compound tested was found to stimulate the incorporation of radioactivity from UDP-[4C]GlcUA into either soluble or insoluble hyaluronic acid. Neither hyaluronic acid trisaccharide (GlcNAc-GlcUA-GlcNAc) nor pentasaccharide (GlcNAc-GlcUA-GlcNAc-GlcUA-GlcNAc) stimulated the incorporation of radioactivity from UDP-[4C]GlcUA into hyaluronic acid in a cell-free system from rat fibrosarcoma. These experiments again furnished no evidence for capacity of any small molecular weight acceptor to initiate hyaluronic acid synthesis.

**Investigation of the Involvement of Lipid in Hyaluronic Acid Synthesis**

Although no lipid intermediate was found in previous studies (15), the improvements in preparation of the enzyme system suggested that reinvestigation of lipid extraction might be worthwhile. Following incubation of enzyme assay mixtures with UDP-[4C]GlcUA, no measurable radioactivity appeared in butanol extracts of the total reaction mixtures, with or without trichloroacetic acid treatment, irrespective of the time.
Fig. 6. Gel filtration of testicular hyaluronidase digests of both soluble and insoluble hyaluronic acid. A standard incubation was increased three times and was carried out with 600 µg of enzyme protein and 27 nmol of UDP-[14C]GlcUA (1.60 x 10^5 cpm). After incubation for 1 h, the reaction was terminated by addition of trichloroacetic acid as usual. Trichloroacetic acid supernatant fluid was applied to a column (1.2 x 10^7 cm) of Sephadex G-50 (superfine) after neutralization with 2 N NaOH to isolate the soluble hyaluronic acid (2.54 x 10^5 cpm). The insoluble hyaluronic acid (1.29 x 10^7 cpm) was isolated by gel filtration on the same column following extraction of the trichloroacetic acid precipitate with 0.5% sodium deoxycholate. The column was eluted with 0.1 M pyridine acetate, pH 5.5, and the radioactive peak in the void volume was freeze-dried. After testicular hyaluronidase digestion, each digest was analyzed on a column (1.2 x 10^7 cm) of Sephadex G-100 (A). Each radioactive peak that emerged in the column volume of the Sephadex G-100 column was further analyzed on a column (1.2 x 10^7 cm) of Sephadex G-50 (superfine) (B). The columns were eluted with 0.1 M pyridine acetate, pH 5.5, at a flow rate of 7 ml/h and fractions of 1.55 ml were collected. Aliquots (0.5 ml) were assayed for radioactivity. The elution profiles for soluble (Δ----Δ) and insoluble (○------○) hyaluronic acid are shown.

### Table II

The effect of sugars or glycosides upon hyaluronic acid synthesis

The incubation conditions were as used for the experiments described in Fig. 1A except that 1 mM of the indicated compounds was present. Incubation was conducted for 30 min, and incorporation into trichloroacetic acid-soluble and -insoluble hyaluronic acid was determined, respectively, as described under "Experimental Procedures." Percentage of control value is indicated in parentheses.

<table>
<thead>
<tr>
<th>Additions</th>
<th>TCA-soluble*</th>
<th>TCA-insoluble*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>14.2 (100)</td>
<td>14.0 (100)</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>14.2 (100)</td>
<td>13.5 (96)</td>
</tr>
<tr>
<td>GalNAc</td>
<td>13.8 (97)</td>
<td>14.0 (100)</td>
</tr>
<tr>
<td>GlcUA</td>
<td>13.4 (95)</td>
<td>11.6 (83)</td>
</tr>
<tr>
<td>Galactose</td>
<td>12.8 (90)</td>
<td>10.2 (73)</td>
</tr>
<tr>
<td>Glucose</td>
<td>14.3 (101)</td>
<td>13.3 (95)</td>
</tr>
<tr>
<td>Mannose</td>
<td>13.3 (95)</td>
<td>13.2 (94)</td>
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<tr>
<td>Xylose</td>
<td>13.2 (93)</td>
<td>13.2 (94)</td>
</tr>
<tr>
<td>PNP-β-GlcUA</td>
<td>13.4 (94)</td>
<td>11.6 (83)</td>
</tr>
<tr>
<td>PNP-β-GlcNAc</td>
<td>13.4 (94)</td>
<td>13.3 (95)</td>
</tr>
<tr>
<td>PNP-β-xylose</td>
<td>12.3 (87)</td>
<td>12.6 (90)</td>
</tr>
<tr>
<td>HA hexasaccharide</td>
<td>14.5 (102)</td>
<td>14.6 (104)</td>
</tr>
<tr>
<td>HA</td>
<td>14.1 (99)</td>
<td>13.6 (97)</td>
</tr>
<tr>
<td>None (control)</td>
<td>14.1 (99)</td>
<td>10.3 (85)</td>
</tr>
<tr>
<td>GlcNAc-1-Pβd</td>
<td>15.0 (100)</td>
<td>34.2 (120)</td>
</tr>
<tr>
<td>Glc1NAc-1-Pβd</td>
<td>13.5 (105)</td>
<td>36.2 (105)</td>
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<tr>
<td>Glc11A-1-Pβd</td>
<td>14.9 (99)</td>
<td>36.8 (107)</td>
</tr>
<tr>
<td>Xylose-1-Pβd</td>
<td>13.3 (89)</td>
<td>35.7 (104)</td>
</tr>
</tbody>
</table>

* The abbreviations used are: TCA, trichloroacetic acid; PNP, p-nitrophenol; HA, hyaluronic acid.

### Table III

The effect of tunicamycin upon incorporation of radioactivity from UDP-[14C]GlcUA into hyaluronic acid

Enzyme protein (80 µg) was preincubated with varying concentrations of tunicamycin ranging from 1.0 to 15 µg/ml under the conditions described in Fig. 1A, except that 68 nmol of nonradioactive UDP-GlcUA were added instead of UDP-[14C]GlcUA. After 30 min of incubation, 0.34 nmol (160,000 cpm) of UDP-[14C]GlcUA was added and incubation was continued for additional 70 min. Incorporation into trichloroacetic acid-soluble and -insoluble hyaluronic acid was determined, respectively, as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Tunicamycin µg/ml</th>
<th>TCA-soluble cpm x 10^{-3}</th>
<th>TCA-insoluble cpm x 10^{-2} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>28.8 (100)</td>
<td>42.0 (100)</td>
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<td>1</td>
<td>31.6 (110)</td>
<td>48.7 (116)</td>
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<tr>
<td>5</td>
<td>31.7 (110)</td>
<td>47.5 (113)</td>
</tr>
<tr>
<td>10</td>
<td>29.9 (104)</td>
<td>46.6 (111)</td>
</tr>
<tr>
<td>15</td>
<td>29.0 (101)</td>
<td>43.3 (103)</td>
</tr>
</tbody>
</table>

Of incubation. Addition of butanol extracts of the enzyme preparation to assay mixtures did not result in an increase in incorporation of radioactivity from UDP-[14C]GlcUA.

Tunicamycin specifically blocks glycoprotein synthesis by inhibition of the formation of dolichyl-pyrophosphate-GlcNAc (27-29). If indeed such a lipid intermediate were involved in hyaluronic acid synthesis, tunicamycin should inhibit such synthesis. No inhibition was observed in either soluble or insoluble fractions with tunicamycin concentrations ranging from 1 to 15 µg/ml (Table III). These experiments furnished...
no evidence for the participation of lipid-linked intermediates of at least pyrophosphoryl-polyprenol type.

**Investigation of the Trichloroacetic Acid-soluble Fraction**

When the trichloroacetic acid-soluble fraction was examined in detail by gel filtration, low molecular weight substances were found which possibly could be involved in hyaluronic acid biosynthesis. The trichloroacetic acid-soluble fraction from a standard incubation was analyzed by gel filtration on several gels. Two peaks were obtained on Sepharose 2B, one in the void volume (containing hyaluronic acid) and the other in the column volume (Fig. 4). However, when the trichloroacetic acid-soluble fraction was analyzed on Sephadex G-50 (superfine), the retarded peak observed on the Sepharose 2B was successfully separated into two peaks (Fig. 7), one of which is UDP-GlcUA with a small shoulder of presumptive free glucuronic acid and the other is an unidentified radioactive peak, designated as Substance A. The radioactivity incorporated into Substance A accounted for approximately 1.0% of the total radioactivity added to the incubation mixture.

**Properties of Substance A**—Substance A also was observed in preparations not treated with trichloroacetic acid. The high speed supernatant solution (78,000 $\times g$ for 60 min) from a standard incubation or a sodium deoxycholate extract of a standard incubation mixture gave a radioactive peak on a Sephadex G-50 column which was similar in elution position and quantity, suggesting that Substance A does not result from degradation by trichloroacetic acid. Rather, it seems to be produced by an enzymatic reaction as indicated in time course experiments (Fig. 8), the rate of production is parallel to the synthesis of hyaluronic acid. On paper chromatography using Solvent B on Whatman No. 1, the hydrolysate of Substance A had the same $R_F$ value (0.20) as the hydrolysate of [14C]GlcUA-labeled hyaluronic acid, which is the $R_F$ of glucuronic acid. The results suggest that the radioactivity is not due to a contaminant present in the UDP-[14C]GlcUA. On paper electrophoresis at pH 5.3, Substance A migrates as a single spot with a $R_{UDP}$ value of 0.83. A single radioactive spot was observed on paper chromatography in Solvent A. The molecular weight was estimated to be approximately 1,100 on a Bio-Gel P-2 column (Fig. 9). Interestingly, Substance A was not observed on a Sephadex G-50 column when UDP-GlcNAc was omitted from an incubation mixture, suggesting that Substance A contains both GlcUA and GlcNAc. However, it was not possible to label Substance A with UDP-[3H]GlcNAc.

Although a similar peak (Substance B) was observed on a Sephadex G-50 (superfine) column when UDP-[3H]GlcNAc was used in the presence of unlabeled UDP-GlcUA, Substance B differed from Substance A in that it had a $R_{UDP}$ value of 0.63 on paper electrophoresis and had a molecular weight of 3 $R_{UDP}$ refers to as the rate of flow on paper relative to UDP.
Bio-Gel P-2 (under 400 mesh) was eluted with 0.1 M buffer, pH 5.5, at a flow rate of 10 ml/h at room temperature. The radioactivity was recovered in the void volume. We, therefore, when the trichloroacetic acid supernatant solution was subjected to gel filtration on a column of Sephadex G-50, no radioactive substance (as determined by Sephadex G-50 gel filtration) found in the trichloroacetic acid-soluble fraction. As previously indicated, p-chloromercuribenzoate inhibits the release of hyaluronic acid from insoluble fractions. Tween 80 at a concentration of 0.1% resulted in extraction of the insoluble hyaluronic acid by trichloroacetic acid. Inhibition of hyaluronic acid synthesis was observed; one soluble and the other insoluble in trichloroacetic acid. An average molecular size was estimated to be more than 1 × 10^6 daltons for both types. On the basis of the substrate, hyaluronic acid is still in the process of elongation and is released only after completion. p-Chloromercuribenzoate appears to block release of completed chains preferentially. The concurrent reduced inhibition of synthesis of soluble chains may result secondarily from the prevention of new chain initiation. The effects of p-chloromercuribenzoate may indicate an enzymatic mechanism of chain release. The action of p-chloromercuribenzoate in the presence of dithiothreitol may have resulted from partial oxidation of the latter in the buffer.

Inhibition Studies

Although all the experiments described above failed to detect an intermediate involved in hyaluronic acid synthesis in this system, we could not rule out the possibility of the existence of an intermediate with a high turnover rate. Such an intermediate might accumulate and be detected under certain conditions which inhibit hyaluronic acid synthesis. Accordingly the effects of various possible inhibitors on synthesis of soluble and insoluble hyaluronic acid was investigated. These results are summarized in Table IV. However, under none of these conditions used, was an intermediate radioactive substance (as determined by Sephadex G-50 gel filtration) found in the trichloroacetic acid-soluble fraction. As previously indicated, p-chloromercuribenzoate inhibits the release of hyaluronic acid from insoluble fractions. Tween 80 at a concentration of 0.1% resulted in extraction of the insoluble hyaluronic acid by trichloroacetic acid. Inhibition of hyaluronic acid synthesis paralleled inhibition of synthesis of Substance A, possibly suggesting that Substance A is associated with hyaluronic acid synthesis in some unknown manner.

**Table IV**

<table>
<thead>
<tr>
<th>Additions</th>
<th>TCA-insoluble HA</th>
<th>TCA-soluble HA</th>
<th>Substance A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>258,000 (100)</td>
<td>66,000 (100)</td>
<td>3,100 (100)</td>
</tr>
<tr>
<td>PMSF (1.0 mM)</td>
<td>204,000 (78)</td>
<td>93,300 (140)</td>
<td>3,170 (102)</td>
</tr>
<tr>
<td>p-CMB (1.5 mM)</td>
<td>150,000 (57)</td>
<td>Trace</td>
<td>1,540 (50)</td>
</tr>
<tr>
<td>Minus MgCl₂</td>
<td>Trace</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>7,740 (3)</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>Hg(NO₃)₂ (10 mM)</td>
<td>Trace</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>Heparin (0.05%)</td>
<td>149,000 (57)</td>
<td>44,300 (67)</td>
<td>2,080 (67)</td>
</tr>
<tr>
<td>Tween 80 (0.1%)</td>
<td>16,800 (6)</td>
<td>190,000 (287)</td>
<td>4,710 (152)</td>
</tr>
<tr>
<td>Triton X-100 (0.5%)</td>
<td>Trace</td>
<td>4,630 (1)</td>
<td>Trace</td>
</tr>
<tr>
<td>Nonidet P-40 (0.5%)</td>
<td>Trace</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>Butanol (4.0%)</td>
<td>Trace</td>
<td>Trace</td>
<td>Trace</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The aim of this study was to further characterize hyaluronic acid synthesis and to determine whether hyaluronic acid synthesis proceeds via a lipid intermediate.

As previously demonstrated (15), two types of newly synthesized hyaluronic acid were observed; one soluble and the other insoluble in trichloroacetic acid. An average molecular size was estimated to be more than 1 × 10^6 daltons for both types. On the basis of the substrate, hyaluronic acid preparations, Stoolmiller and Dorfman (15) suggested that the latter contains growing chains. Our results from the chase experiments more clearly indicate that insoluble hyaluronic acid is still in the process of elongation and is released only after completion. p-Chloromercuribenzoate appears to block release of completed chains preferentially. The concurrent reduced inhibition of synthesis of soluble chains may result secondarily from the prevention of new chain initiation.

As previously demonstrated (15), incubation of the enzyme with only UDP-[³⁵]GlcUA resulted in incorporation of [³⁵]GlcUA into insoluble hyaluronic acid. The molecular size of the [³⁵]GlcUA-labeled material was large enough to be excluded from a column of Sephadex G-100. It seems reasonable, therefore, to assume that there should be either a protein acceptor or a primer of high molecular weight on the membrane. It is not yet certain whether hyaluronic acid is bound covalently to protein. Even after exhaustive purification of hyaluronic acid, small amounts of amino acids have been...
found in preparations from various sources such as synovial fluid (30-32), rooster comb (33, 34), brain (35), human umbilical cord (32), bovine vitreous (32), and streptococcus (36). No linkage region, however, has been isolated. Stoolmiller and Dorfman (15) observed no inhibition by puromycin or chloramphenicol of hyaluronic acid synthesis in Streptococcus. In our studies, no evidence was obtained for the covalent linkage of hyaluronic acid to protein. Neither testicular hyaluronidase nor streptococcal hyaluronidase treatment resulted in the association of any hyaluronate fragment with a high molecular weight compound as determined by gel filtration. If, however, the enzyme system is elongating the chain rather than initiating, as presumed from the experiments described in Fig. 5, this approach might not be sensitive enough to detect an initiator. Therefore, alkali treatment and pronase digestion also were used. Neither of these treatments of insoluble hyaluronic acid decreased the molecular size when examined on Sepharose 4B. Furthermore, none of the monosaccharides or glycosides tested for acceptor activity stimulated hyaluronic acid synthesis. On the basis of these negative results, it seems most likely that hyaluronic acid synthesis, unlike that of chondroitin sulfate, is not initiated on a protein core. It seems possible that the chain initiation takes place on a membrane-bound enzyme system.

The primer or bound hyaluronic acid appears to be associated with the enzyme particle through a noncovalent bond. It has been shown that insoluble hyaluronic acid is extracted with 50% pyridine, 0.5% sodium deoxycholate, or acetone/phosphate buffer (16). It was also shown that hyaluronic acid could be extracted with butanol-containing trichloroacetic acid solution. These findings suggest that a lipid may be involved in the formation of the enzyme particle or in its arrangement in the membrane. We cannot eliminate the possibility that there is a very small aglycone for hyaluronic acid molecule, which acts as a chain initiator or as an anchor inserted in the membrane.

Although little is known concerning the mechanism of chain elongation of hyaluronic acid in mammalian systems, previous studies of the Streptococcus system furnished no evidence of involvement of a lipid intermediate and suggested alternative addition of monosaccharide units (15). The following evidence has been presented: (a) no radioactive intermediates were detected by organic solvent extraction; (b) a major reaction product from UDP-sugars in hyaluronic acid synthesis is not UMP, but UDP; (c) bacitracin, an inhibitor for dephosphorylation of lipid pyrophosphate in peptidoglycan synthesis, does not inhibit hyaluronic acid synthesis.

Recently, Turco and Heath (18) reported the isolation of GlcUA-(1 → 4)-GlcNAc-P-P-dulcitol from SV40-transformed human lung fibroblasts, and suggested that it might be involved in the biosynthesis of heparin, heparan sulfate, or both. Hopwood and Dorfman (19) also reported the isolation of lipid-linked oligosaccharides containing both glucuronic acid and N-acetylglucosamine, which might be lipid intermediates for heparin (heparan sulfate) or hyaluronic acid biosynthesis. Although it has not been demonstrated that those lipid components can be transferred to glycosaminoglycans, these findings induced a reinvestigation of the involvement of a lipid intermediate. We repeated the lipid extraction with butanol, which was used to isolate the lipid components from a rat fibrosarcoma (19). However, no measurable radioactivity appeared in the extracts.

Tunicamycin is known to specifically inhibit the formation of N-acetylglucosaminyl pyrophosphorylpolysaccharide (27-29), the lipid intermediate for biosynthesis of N-glycosidically linked glycoproteins (57). If indeed such a lipid intermediate is involved in hyaluronic acid biosynthesis, tunicamycin would be expected to inhibit hyaluronic acid synthesis. Recently, Takatsuki and Tamura (20) have reported that the drug inhibits the biosynthesis of total glycosaminoglycans in cultures of chick embryo fibroblasts. No inhibition, however, by tunicamycin of hyaluronic acid synthesis in Streptococcus was found by us. A similar observation in this laboratory using cultured rat glial cells was made. We concluded that a pyrophosphorylpolysaccharide type of lipid intermediate was not involved in hyaluronic acid synthesis. In the course of preparation of this manuscript, Hart and Lennarz (38) reported no inhibition of hyaluronic acid synthesis by tunicamycin in embryonic chick cornea. It has been recently reported by Yamamori et al. (39) that a tunicamycin-like antibiotic 2410 inhibits the formation of N-acetylglucosaminyl pyrophosphoryldecaprenol, but not of some N-acetylglucosaminyl phosphoryldecaprenol. Therefore, the possibility of the involvement of phosphorylpolysaccharide can not be ruled out.

On examination of the trichloroacetic acid-soluble fraction from an incubation, Substance A was found which appeared to be enzymically synthesized from UDP-[14C]GlcUA. Since it was not produced when UDP-GlcNAC was omitted from the incubation mixture, it seems to contain both glucuronic acid and N-acetylglucosamine. Although the Substance A was not labeled with UDP-[3H]GlcNAC, it is presumably because of the fact that the Michaelis constant for UDP-GlcNAC of the enzyme system is 10 times greater than that for UDP-GlcUA (15), and that as a result hyaluronic acid is not synthesized effectively at a very low concentration of UDP-[3H]GlcNAC.

The addition of Substance A to an incubation mixture did not result in its transfer to hyaluronic acid. Furthermore, under none of the inhibitory conditions used for hyaluronic acid synthesis, Substance A accumulated. Those findings do not support its role as an intermediate for hyaluronic acid synthesis. Interestingly, the production of Substance A seems to parallel hyaluronic acid synthesis as observed in the time course experiments and the inhibition studies. Although it is not feasible to elucidate the structure of Substance A due to the very small amounts of the material, it could be a UDP-glucosaccharide. UDP-glucosaccharides have been isolated from Group A streptococci (40) and from several other sources, such as milk (41, 42) and hen oviduct (43-46), although their role is not yet known. On the basis of the results presented here, we can conclude with a fairly high degree of certainty that the chain elongation of hyaluronic acid synthesis in Streptococcus proceeds by alternative addition of monosaccharide units, but not via a lipid intermediate.

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