HYALURONIC ACID SYNTHESIS IN A CELL-FREE SYSTEM FROM RAT FIBROSARCOMA[1]

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Received October 7,1974

A transplantable rat fibrosarcoma (in both ascites and solid forms) has been shown to contain and synthesize large amounts of hyaluronic acid. A particulate hyaluronic acid synthesizing system has been isolated from the solid fibrosarcoma and some characteristics of the enzyme system are detailed. The enzyme complex transferred GlcUA or GlcNAc from UDP-GlcUA or UDP-GlcNAc at a rate of approximately 60 nmole/hr/mg protein to extend hyaluronic acid chains by approximately 45,000 daltons.

Hyaluronic acid has been implicated in such processes as cell aggregation (1, 2), mesenchyme cell mobility (3) and the specific aggregation of cartilage proteoglycan (4, 5). Although the mechanism of biosynthesis of hyaluronic acid in Group A streptococci has been extensively studied (6), little is known concerning hyaluronic acid biosynthesis and its control in eukaryotic cells. Enzyme preparations derived from mammalian systems demonstrated only low levels of hyaluronic acid synthesis (7, 8, 9). The investigation of hyaluronic acid synthesis is further complicated by the uncertainty as to the reducing end of the hyaluronic acid chain (the initiation site). Much of this difficulty is due to the unavailability of a suitable mammalian system for study of hyaluronic acid synthesis. This paper describes a rat fibrosarcoma that efficiently incorporates labeled precursors into high molecular weight hyaluronic acid in both tissue minces and particulate preparations.

MATERIALS AND METHODS

Animals and tumors. A rat fibrosarcoma developed in the ascites form (10), was maintained by intraperitoneal passage once weekly in adult Lewis x Brown Norway rats. Subcutaneous injection of the ascites form of the tumor

[1]This investigation is supported by USPHS Grant Nos. HD-04583, AM-05996 and a grant from the Chicago and Illinois Heart Association.

Abbreviations: diHA, 2-acetamido-2-deoxy-3-0-(-β-D-glucopyranosyluronic acid)-D-glucose; ΔdiHA, 2-acetamido-2-deoxy-3-0-(-β-D-gluco-4-enepyranosyluronic acid)-D-glucose; SDS, sodium dodecyl sulfate.

[2]Kennedy Fellow in the Neurosciences

produced in 10 days approximately 5g of solid tumor and 5 ml of viscous fluid per site.

Materials. Sodium [^{3}H]acetate (100 mCi/mmole), UDP-[^{14}C]GlcUA (233 mCi/mmole) and $H_2{}^{35}SO_4$ (43 Ci/mg) were obtained from New England Nuclear, Boston, Massachusetts. UDP-[^{14}C]GlcNAc (32 mCi/mmole) was purchased from Schwarz Bioresearch Inc., Orangeburg, New York. ATP, UDP-GlcUA and UDP-GlcNAc were obtained from Sigma Chemical Co., St. Louis, Missouri. Sodium dodecyl sulfate was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. Varidase was used as a source of streptococcal hyaluronidase since the preparation is rich in contaminating hyaluronidase. Commercial Varidase from Lederle Laboratories (Pearl River, New York), containing 100,000 unit of streptokinase, was dissolved in 3 ml of water.

ΔdiHA was isolated from a streptococcal hyaluronidase digest of pig skin hyaluronic acid (Miles Laboratories, Inc.) by ion-exchange chromatography on a column Dowex 1 X4, (formate form, 20 x 1 cm). A streptococcal hyaluronidase digest of a hyaluronic acid tetrasaccharide isolated from a testicular hyaluronidase digest of pig skin hyaluronic acid was used as a source of diHA. Pronase (B grade) was purchased from Calbiochem, Los Angeles, California. All other reagents were of analytical grade.

Analytical methods. Hexuronate content was determined by the method of Bitter and Muir (11) and protein content by the method of Lowry et al (12) with bovine serum albumin as standard. Amino acid and hexosamine analyses were performed after hydrolysis in 6M HCl for 20 hr at 100° in sealed tubes under vacuum by using a Technicon automatic amino acid analyser. Radioactivity was assayed as previously described (13).

Preparation of a particulate hyaluronic acid-synthesizing system. Approximately 30g of solid tumor was minced in 200 ml of cold 60 mM Tris buffer, pH 7.05, containing 0.25M sucrose (Buffer A) and homogenized in 40 ml batches in a glass tissue grinder with a Teflon pestle using 3 to 4 passes at 800 rpm and 4°. The homogenate was centrifuged at 10,000 x g for 10 min and the pellet discarded. Following centrifugation at 100,000 x g for 60 min, the viscous supernatant solution was discarded and the pellet suspended in 30 ml of Buffer A. The final protein concentration was 5.0 mg/ml.

Assay of hyaluronic acid biosynthetic activity. Unless otherwise stated the enzyme system was assayed in a final volume of 0.25 ml containing 25 μmoles of Na_2HPO_4-KH_2PO_4 buffer, pH 7.05, 40 μmoles $MgCl_2$, 1.0 μmole dithiothreitol, 0.2 μmole UDP-GlcUA, 0.16 μCi of UDP-[^{14}C]GlcUA, 2.5 μmoles UDP-GlcNAc, 10 μmoles ATP, 0.5 mg of enzyme protein in 0.1 ml containing 16 μmoles Tris buffer, pH 7.05 and 37 μmole sucrose. Assays were initiated by the addition of enzyme and incubations were performed at 37° for a period of 40 mins. Reactions were terminated by immersion of reaction tubes in a freezing mixture and stored at -20°. Control incubation mixtures contained the complete reaction mixture except that the particulate enzyme preparation was boiled for 10 mins.

Incorporation of label into hyaluronic acid was estimated using one of the following procedures: Procedure 1. A 40 μl sample of the reaction mixture was chromatographed on Whatman 3MM paper in 1.0M ammonium acetate pH 5.0: ethanol (3.5:6.5 by volume; Solvent A), isobutyric acid:2.0M NH_3 (5:3 by volume; Solvent B) or 1-butanol: pyridine: acetic acid:water (15:10:3:12 by volume; Solvent

C). Sample channels were cut into 1 cm segments and the radioactivity measured on each segment after adding 0.5 ml of water. Procedure 2. A 50 µl aliquot of the reaction mixture was incubated with 50 µl 0.10M-Na_2HPO_4-KH_2PO_4 buffer, pH 6.1, 50 µl 0.30M NaCl and 50 µl of a Varidase solution for 2 hr at 37°. A 80 µl aliquot of this digest was chromatographed on paper in Solvent A and the distribution of radioactivity measured as described in Procedure 1.

RESULTS AND DISCUSSION

Identification of the polysaccharide components of the rat fibrosarcoma. When minced solid tumor or the ascites form of the tumor were incubated with sodium [^{3}H]acetate and $H_2{}^{35}SO_4$ there was considerable incorporation of label into glycosaminoglycans. Approximately 90%, 8% and 2% of the total ^{3}H-label associated with polysaccharides was identified as hyaluronic acid, chondroitin (or dermatan) sulfate and heparan sulfate respectively. The [^{35}S]-labeled polysaccharides were identified by their susceptibility to digestion with chondroitinase ABC (chondroitin or dermatan sulfate) or nitrous acid (heparan sulfate). The hyaluronic acid component was identified from its electrophoretic mobility (14) and by its specific susceptibility to digestion with streptococcal hyaluronidase. Hyaluronic acid was separated from other polysaccharides by ion exchange chromatography of an alkali digest of the solid tumor on ECTEOLA-cellulose (15). This preparation contained glucosamine as the sole amino sugar and glucuronic acid in a mole ratio of 0.93:1.00. The solid tumor contained approximately 2 mg of hyaluronic acid per g of tumor. A detailed presentation and discussion of these findings will be published elsewhere.

The 100,000 x g supernatant solution obtained during the isolation of the particulate hyaluronic acid synthesizing system was digested with Pronase (16) and chromatographed on a column (90 x 2.6 cm) of Sepharose 4B. All of the hexuronic acid-positive material in the digest was excluded from this gel. The void volume fractions contained glucosamine as the sole amino sugar and approximately 1% of the total fraction weight as amino acids. The molecular weight of this hyaluronic acid preparation, kindly estimated by Dr. Martin B. Mathews from its intrinsic viscosity was approximately 1.5×10^6.

Incorporation of radioactivity from UDP-derivatives into hyaluronic acid. Incubation of the particulate hyaluronic acid synthesizing system with UDP-[^{14}C]GlcUA produced a labeled component that was excluded from Sepharose 4B. More than 80% of the excluded labeled material was degraded with streptococcal hyaluronidase to produce a substance with the same mobility on paper as ΔdiHA in solvents A, B and C.

Table 1: Synthesis of Hyaluronic Acid by the Particulate Enzyme Preparation

The reaction mixture from Experiment 1 was centrifuged at 12,000 x g for 30 mins at 4°, the pellet washed with 4M LiCl and re-centrifuged. Both supernatant solutions were separately fractionated on a column of Sephadex G-50. The pellet fraction was treated with 0.5M-KOH for 4 days at 4°, neutralized with acetic acid and centrifuged at 12,000 x g for 30 mins at 4° and the supernatant fraction chromatographed on a column of Sephadex G-50.

The reaction mixture from Experiment 2 was centrifuged at 12,000 x g for 30 mins, the pellet washed with 0.2M pyridine-acetic acid buffer pH 5.0 and re-centrifuged. The supernatant solutions were combined and applied to a column of Sepharose 4B (2.5 cm x 90 cm) in 0.2M pyridine-acetic acid buffer pH 5.0 containing 0.058% SDS and eluted with that buffer. The pellet fraction was suspended in 0.12M Tris buffer, pH 7.05, containing 0.058% SDS incubated for 30 mins at 4°, centrifuged at 12,000 x g for 30 mins at 4°. The supernatant solution was fractionated on Sepharose 4B.

Fraction	Void (HA-product)	Retarded (GlcUA-substrate)
	Percent of Total Counts	
Experiment 1		
Soluble	0.48	85.24
4M LiCl-Solubilized	0.54	9.85
0.5M KOH-Solubilized	2.93	0.62
Pellet	0.34	-
Experiment 2		
Soluble	1.02	92.36
SDS-Solubilized	3.92	2.42
Pellet	0.28	-

Only 20 to 25% of the total label incorporated into hyaluronic acid remained in the supernatant solution after centrifugation of the incubation mixtures described in Table 1. The remaining counts bound to the pellet could be solubilized by treating the pellet with SDS or dilute alkali (Table 1). In each case the label associated with the supernatant solution, the SDS and the alkali-solubilized hyaluronic acid fractions was excluded from gels of Sepharose

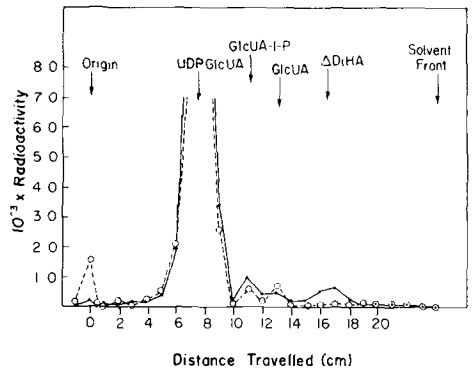

Fig. 1: Profile of radioactivity of standard incubation mixture before – – – – and after ——— digestion with streptococcal hyaluronidase (Procedure 1 and 2 respectively - see Methods). Samples were chromatographed on paper in Solvent A.

4B. This result indicates that the labeled hyaluronic acid in each fraction (Table I) has a molecular weight consistent with that found by viscometery. Paper chromatography in solvents A and B of streptococcal hyaluronidase digests of these hyaluronic acid fractions showed that more than 90% of the radioactivity co-chromatographed with ΔdiHA.

The relative levels of diHA and ΔdiHA in streptococcal hyaluronidase digests of hyaluronic acid provide a convenient measure of average molecular weight (6). Ratios of labeled ΔdiHA/diHA of 130 and 156 respectively were obtained for the soluble and SDS-solubilized hyaluronic acid fractions from the incubation described in the second experiment reported in Table 1. These values indicate that approximately 130-156 disaccharide units have been added to hyaluronic acid chains present in the particulate enzyme preparation to extend their average length by a molecular weight of approximately 45,000.

Approximately 30 to 60 nmoles of GlcUA/hr/mg protein were incorporated into hyaluronic acid by the rat fibrosarcoma particulate system. For incubations mixtures in which UDP-[^{14}C]GlcNAc replaced UDP-[^{14}C]GlcUA, the concentration of UDP-GlcUA was increased to 2.5 μmole and the UDP-GlcNAc omitted. Under these conditions hyaluronic acid chains are extended at a rate of approximately 60 nmole GlcNAc/hr/mg protein. This level of biosynthetic activity is similar to that reported for particulate systems isolated from Group A streptococci (6) and considerably higher than that described for particulate fractions isolated from other mammalian tissues (7, 8, 9). Fig. 1 shows the

Table 2: Requirements of the Hyaluronic Acid Synthesizing System

Particulate enzyme was incubated with UDP-[C^{14}]GlcUA as described under Methods. An aliquot from each incubation mixture was fractionated by chromatography on paper solvent A as described in Procedure 1 under Methods.

	Origin	UDP-GlcUA
	Percent of Total Counts	
Control	14.1	82.2
Boiled Blank	0.1	92.2
-UDP-GlcNAc	1.8	88.0
-ATP and PO_4	0.2	0

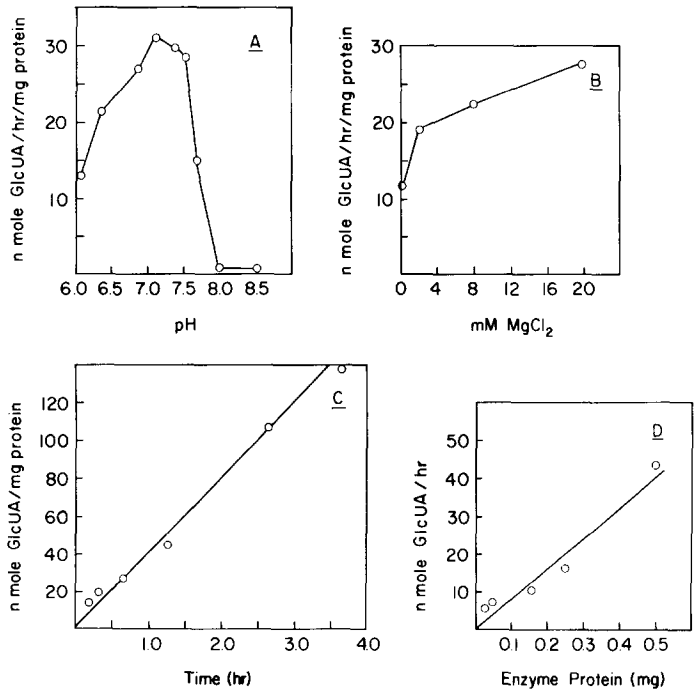

Fig. 2: The effect of pH (A), Mg^{++} concentration (B), incubation time (C) and protein concentration (D) on hyaluronic acid synthetic activity. The hyaluronic acid synthetic activity in (A) was measured between pH 6.1 and 8.0 in phosphate buffer and at pH 8.5 in Tris buffer.

profile of radioactivity obtained when an incubation mixture was chromatographed on paper in solvent A before and after digestion with streptococcal hyaluronidase. At least 80% of the radioactivity that remained at the origin was degraded by streptococcal hyaluronidase to produce a labeled component that co-chromatographed with ΔdiHA. The other peaks of radioactivity in Fig. 1 correspond with authentic standards of UDP-GlcUA, GlcUA-1-PO_4 and GlcUA. Incubation with the particulate enzyme system in the absence of ATP and phosphate buffer resulted in rapid degradation of UDP-[^{14}C]GlcUA with a considerable reduction in hyaluronic acid synthesis (Table 2). When UDP-GlcNAc was omitted from the incubation mixture the transfer [^{14}C]GlcUA to hyaluronic acid was inhibited by 88% (Table 2). This result is consistent with the extension of hyaluronic acid chains by the alternate transfer of GlcNAc and GlcUA from UDP intermediates.

The hyaluronic acid synthesizing system present in the particulate preparation was further characterized using the paper chromatographic method, depicted in Fig. 1, to estimate both the level of hyaluronic acid synthesis and the level of residual UDP-[^{14}C]GlcUA in a series of incubations.

The effect of pH on the level of hyaluronic acid synthesis is shown in Fig. 2A. The enzyme preparation exhibited a maximum hyaluronic acid biosynthetic activity at a pH between 7.0 and 7.5. Activity decreased abruptly above pH 7.6, with virtually no activity above 8.0. The hyaluronic acid synthesizing system required Mg^{++} for maximum hyaluronic acid biosynthetic activity (Fig. 2B). The incorporation of radioactivity from UDP-[^{14}C]GlcUA into hyaluronic acid by the particulate hyaluronic acid synthesizing system was linear with both time of incubation and enzyme protein concentration (Figs. 2C and D).

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