MACROMOLECULAR ASSEMBLY OF CHONDROITIN*

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Summary

A microsomal preparation from chick embryo epiphyseal cartilage has been used to study the assembly of the heteropolysaccharide, chondroitin. Endogenous chondroitin sulfate in the microsomal preparation was found to act as a primer for the addition of relatively short non-sulfated polysaccharide chains to form large molecules with a large sulfated portion and a small non-sulfated portion. A smaller primer was found to act as an acceptor for the polymerization of long non-sulfated chains to form large, predominantly non-sulfated chondroitin. Although the incorporation of sugars was time dependent, there was no detectable accumulation of short-chain molecules at the earliest times examined. The results suggest that the mechanism of polysaccharide formation is one of rapid assembly of most of the heteropolysaccharide chain, with subsequent addition taking place at a much slower rate. This would indicate a tightly organized mechanism of synthesis which may represent a general pattern for synthesis of heteropolysaccharides.

Biosynthesis of the heteropolysaccharide, chondroitin, has been shown to proceed by addition of sugars onto a primer of endogenous protein-bound material (1). The sugar nucleotides UDP-N-acetylgalactosamine and UDP-glucuronic acid were shown to be the precursors, and a microsomal preparation from chick embryo epiphyses was utilized as the source for both enzyme and primer. Although oligosaccharides also have been utilized as primers (2) only a few sugar units can be added rather than a long chain of repeating units as is the case when the endogenous protein-bound primer is utilized. The relative size of the endogenous primer and of the final polysaccharide chains has not been established, nor is it known whether or not the sequential addition of sugar units results in a distribution of chain sizes or in chains of a relatively uniform size.

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The present report describes the use of microsomal preparations from chick embryo epiphyses to study the method of assembly of the polysaccharide chain on the endogenous primer.

Experimental Procedure

UDP-glucuronic acid-^{14}C, uniformly labeled in the glucuronic acid moiety and UDP-N-acetylgalactosamine were prepared as previously described (1,3). Chondroitin 4-sulfate (M.W. 25,000) and chondroitin 6-sulfate (M.W. 40,000) were gifts from Seikagaku Kogyo Ltd. Hyaluronic acid and heparin (167 units/mg) were purchased from Calbiochem. Chondroitinase ABC and Δ-Di-0S { 2-acetamido-2-deoxy-3-0-(β-D-gluco-4-enepyranosyl uronic acid)-D-galactose} was purchased from Miles Laboratories, and pancreatin from Viobin. Frozen 14 day old chick embryos were purchased from Pel-Freez Biologicals.

Twice-washed microsomal preparations sedimenting between 10,000 x g and 105,000 x g were prepared from chick embryo epiphyses as previously described (1,4). A microsomal preparation (0.040 ml) was incubated at 37° with 0.05 M MES, pH 6.5; 0.01 M $MnCl_2$; UDP-glucuronic acid-^{14}C, 62 mμmoles (1.5×10^6 cpm); and UDP-N-acetylgalactosamine, 160 mμmoles, in a total volume of 0.1 ml. Aliquots were removed at varying times, boiled and spotted on Whatman No. 1 paper. The glucuronic acid-^{14}C-labeled glycosaminoglycans were then isolated as previously described (1,4) by chromatography in ethanol - 1M ammonium acetate, pH 7.8 (5:3). The origins of the chromatograms (containing all the glycosaminoglycans) were incubated overnight with 2 ml of 1% pancreatin in 0.05 M Tris, pH 8.5. The suspensions were boiled, centrifuged, and the pellets washed with 1M NaCl as previously described (5). The 1M NaCl washing was added to the initial supernatant from the boiled pancreatin incubation, and the total taken directly (without dialysis) for chromatography.

Polysaccharide standards were assayed by the method of Bitter and Muir (6).

Results

After 2 minutes incubation of the sugar nucleotides with the microsomal preparation, an aliquot (0.075 ml) was removed and the glycosaminoglycan-^{14}C was isolated. A sample of this was chromatographed on a DEAE cellulose column using a logarithmic gradient of LiCl as previously described (7). Results are shown in Table I. Glycosaminoglycan-^{14}C appeared in two distinct peaks which did not overlap. (There was a small amount of some other labeled material which was eluted before the first peak. This material did not appear to be a glycosaminoglycan but amounts were too small for further identification). The first peak (I) appeared in the fractions near those where a standard of the nonsulfated glycosaminoglycan, hyaluronic acid, appeared, and the second peak (II) appeared just before, but overlapping, the area where a standard of chondroitin 4-sulfate appeared.

Table I

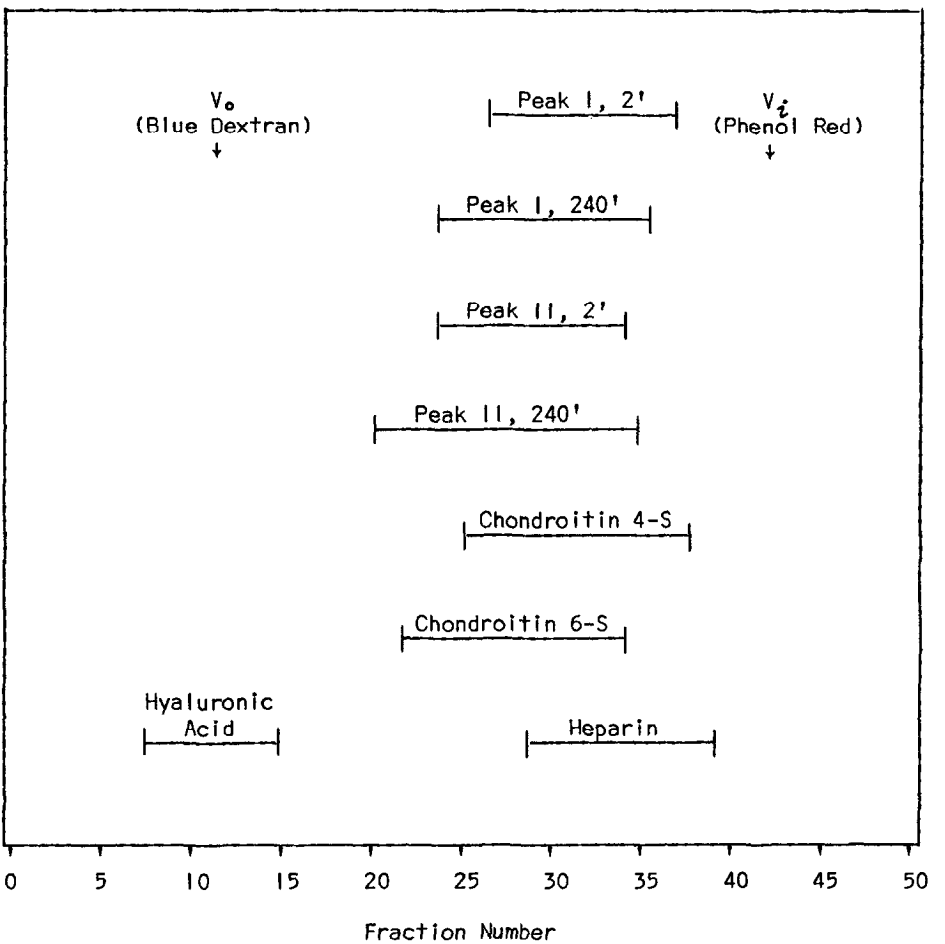
Incubation		DEAE Cellulose Chromatography			
Time	cpm Incorporated	Fraction Numbers	cpm	cpm Adjusted to Equal Volume	% of Total
2 min.	1725 (23/µl)	25-38 (Peak I)	445	445	26
		44-60 (Peak II)	775	775	45
240 min.	4125 (273/µl)	31-39 (Peak I)	700	3500	17
		43-60 (Peak II)	2750	13750	67

After 240 minutes incubation of the sugar nucleotides with the microsomal preparation, another aliquot (0.015 ml) was removed. The amount of ^{14}C-labeled polysaccharide in this aliquot was approximately 2.5 times that found in the larger aliquot removed after 2 minutes incubation. (This represents 12 times as much glycosaminoglycan-^{14}C when amounts are represented

on the basis of equal aliquots.) The pattern of this glycosaminoglycan-^{14}C on DEAE cellulose chromatography (Table I) was similar to that of the 2 minute sample. Chromatography of similar material has been previously reported (7).

The fractions containing each of the peaks obtained from the DEAE cellulose chromatography were pooled, dialyzed to remove LiCl, and lyophilized. Aliquots from each of these pooled peaks were then applied to

Figure I



Samples were applied to a 1 x 50 cm column of Sepharose 4B and eluted with 0.1 M LiCl. Fractions of 1 ml were obtained, and were assayed for radioactivity and for standards (uronic acid). The lines represent the range of fractions containing each substance.

a column of Sepharose 4B for estimation of molecular size. Results are shown in Figure 1 together with standards of hyaluronic acid, chondroitin 6-sulfate, chondroitin 4-sulfate, and heparin.

The glycosaminoglycan-^{14}C (peaks I and II from 2 minute and 240 minute incubations) appeared to range in size from approximately 20,000 to 40,000 M.W. The peak I material from the 2 minute incubation appeared to be more uniform in molecular size and smaller than peak I material from the 4 hour incubation, but still appeared to be almost the size of the chondroitin 4-sulfate standard, and larger than the heparin standard.

Samples of each of the peaks were incubated with chondroitinase ABC and chromatographed on Whatman No. 1 paper with 1-butanol - acetic acid - 1N NH_4OH, 2:3:1 (8). All radioactivity moved together with a standard of Δ-DI-OS.

Discussion

The glycosaminoglycan-^{14}C appeared as two discrete peaks rather than as a continuum on DEAE cellulose chromatography. Furthermore, the ^{14}C-labeled material was all in the size range of chondroitin sulfate. This suggests that there are two types of glycosaminoglycan-^{14}C. Since the two types appear to be in the same size range, it would seem that the primer upon which each type is built determines the relative position of each in DEAE cellulose chromatography. The two types of primer in the microsomal preparation are suggested to be: (1) a short chain primer which accepts a long chain of non-sulfated chondroitin (peak I); (2) a long chondroitin sulfate chain which accepts a short chain of non-sulfated chondroitin (peak II). There would appear to be little chondroitin sulfate primer of intermediate size, since there was no significant formation of glycosaminoglycan-^{14}C consisting of intermediate proportions of primer and

newly formed chondroitin-^{14}C.*

The peak I material formed in 2 minutes was almost as large in molecular size as that formed in 240 minutes, even though there was only 1/8 as much peak I material formed. This would indicate that synthesis occurs rapidly on a small primer molecule to form a large, nearly complete, polysaccharide chain. Thus the increased incorporation over a 240 minute period might, in large part, represent formation of more chains, rather than a growth of individual chains throughout this period. Following initial polymerization, smaller increments of chain growth occur more slowly. Addition of sugars to the large chondroitin sulfate chains (peak II) is of this type.

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* An alternative possibility that the peak I material represents a small increment of chain growth on a large, non-sulfated primer has been ruled out as follows: Newly formed peak I material can readily be sulfated with 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to produce fully sulfated chondroitin sulfate (7, unpublished observations). On DEAE cellulose chromatography this material appears as a third peak, requiring a higher concentration of LiCl for elution than standard chondroitin 4-sulfate or peak II. If peak I were to represent a small increment of polymerization on a large non-sulfated primer, preincubation of the microsomal preparation with PAPS should change this to a large sulfated primer. Subsequent addition of sugars would result in material similar to the material appearing in peak II, and a disappearance of material appearing in peak I. Experimentally, however, preincubation of the microsomal preparation with PAPS did not affect the amount of material appearing in the peak I area after polymerization. It can be concluded, therefore, that there is no significant amount of large non-sulfated primer present.