FORMATION OF CHONDROITIN SULFATE BY A MICROSOMAL PREPARATION FROM CHICK EMBRYO EPIPHYSEAL CARTILAGE*

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It has been shown that radioactively labeled glucuronic acid and N-acetylgalactosamine are incorporated into a glycosaminoglycan (mucopolysaccharide) when labeled uridine diphosphate glucuronic acid and uridine diphosphate N-acetylgalactosamine are incubated with a microsomal preparation from chick embryo epiphyseal cartilage (Silbert, 1964; Perlman et al., 1964). This glycosaminoglycan has been identified by sugar analysis as the nonsulfated compound chondroitin (Silbert, 1964). The same microsomal preparation has also been shown to contain particle-bound chondroitin sulfate, and to be able to catalyze the incorporation of labeled sulfate from PAPS** into this particle-bound chondroitin sulfate (DeLuca and Silbert, 1968). This suggests that the synthesis of the polysaccharide chain of chondroitin and sulfation to form the complete chondroitin sulfate product might take place with the same microsomal preparation. The present report describes the use of this microsomal preparation from chick embryo epiphyseal cartilage to form chondroitin sulfate.

Experimental Procedure

UDP-glucuronic acid-^{14}C, uniformly labeled in the glucuronic acid moiety was prepared as previously described (Silbert, 1962). UDP-N-acetylgalactosamine

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**Abbreviation: PAPS, 3'-phosphoadenosine 5'-phosphosulfate

was prepared as previously described (Silbert, 1964). PAPS was synthesized with yeast enzyme (Robbins, 1962), chromatographed on Dowex 1-X8 (Wilson et al., 1961), and finally obtained as previously described (Silbert, 1967). Chondroitin-4-sulfate was a gift from Seikagaku Kogyo Ltd. Hyaluronic acid was purchased from Sigma. Heparinase from Flavobacterium heparinum (adapted to heparin) was a generous gift of Dr. A. Linker, and streptococcal hyaluronidase was generously provided by Dr. P. Bell of Lederle Laboratories. Testicular hyaluronidase was purchased from Sigma 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 (Silbert, 1964; Silbert, 1966). Two identical microsomal preparations were incubated at 37° with 0.05 M Tris, pH 7.5; 0.01 M $MgCl_2$; UDP-glucuronic acid-^{14}C, 4 mμmoles (6 x 10^5 cpm); and UDP-N-acetylgalactosamine, 12 mμmoles, in a total volume of 0.025 ml. In addition 23 mμmoles of PAPS were added to one incubation mixture at 0 time and additional amounts of 23 mμmoles were added at 15, 30, 60, 90, and 120 minutes. No PAPS was added to the other incubation mixture. Both mixtures were incubated for 3 hours. The sequential addition of PAPS in relatively large amounts was required due to the rapid degradation of this substrate by the microsomal preparation (DeLuca and Silbert, 1968). The glucuronic acid-^{14}C-labeled glycosaminoglycans were isolated from these reaction mixtures as previously described (Silbert, 1964; Silbert, 1966) by chromatography on Whatman No. 1 paper in ethanol - 1 M ammonium acetate (7.5:3), pH 7.8. The origins of the chromatograms (containing all the glycosaminoglycans) were then 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 1 M NaCl as previously described (DeLuca and Silbert, 1968). The 1 M NaCl washing was added to the initial supernatant from the boiled pancreatin incubation and dialyzed overnight against 0.01 M K_2SO_4 and then against several changes of water. The material within the dialysis

tubing was assayed for radioactivity. The glucuronic acid-^{14}C-labeled material that had been at the origins of the chromatograms was always recovered quantitatively within the dialysis tubing. Incubations (with or without PAPS) yielded 8,000 - 10,000 c.p.m. of glucuronic acid-^{14}C-labeled glycosaminoglycan per reaction mixture.

Results and Discussion

Samples of ^{14}C-labeled glycosaminoglycan were precipitated with 0.1% cetyltrimethylammonium bromide in the presence of Celite and extracted with varying concentrations of NaCl (Korn, 1959; Schiller et al., 1961). Results are shown in Table I. In this system the non-sulfated glycosaminoglycan, hyaluronic acid, is mainly extractable with 0.4 M NaCl, while chondroitin sulfate requires 1.2 M NaCl for extraction. Compounds that are more highly sulfated require higher concentrations of NaCl for extraction (2.1 M NaCl for heparin). Glycosaminoglycan formed in the reaction mixture not containing PAPS showed a pattern of extraction which indicated a high proportion of material that was non-sulfated. In comparison, the glycosaminoglycan-^{14}C formed in the reaction mixture containing PAPS indicated a higher proportion of sulfated material.

Samples of glycosaminoglycan-^{14}C were incubated together with carrier hyaluronic acid or chondroitin sulfate in the presence of bacterial heparinase or bacterial hyaluronidase. Aliquots of the reaction mixtures were chromatographed on Whatman No. 1 paper for 44 hr. with butanol - acetic acid - water, 50:15:35 in a descending system. The chromatograms were cut into 1/2 cm strips which were eluted with water. The eluates were assayed for radioactivity and uronic acid content (Bitter and Muir, 1962).

Glycosaminoglycan-^{14}C formed in reaction mixtures without PAPS was degraded by bacterial heparinase to a mixture of radioactive products. Less than 4% of these products corresponded in mobility to the major degradation product of carrier chondroitin-4-sulfate. (The major product is the

TABLE I

Extraction of Glycosaminoglycan-^{14}C from Cetyltrimethylammonium Precipitate

Sample Analyzed	Total Radioactivity	
	Glycosaminoglycan-^{14}C formed in absence of PAPS	Glycosaminoglycan-^{14}C formed in presence of PAPS
	c.p.m.	c.p.m.
Supernatant after precipitation with cetyltrimethylammonium and 0.03 M NaCl	3	8
Extraction with 0.03 M NaCl	0	0
Extraction with 0.4 M NaCl		
First	395	245
Second	65	55
Third	23	18
Fourth	12	7
Extraction with 1.2 M NaCl		
First	180	265
Second	32	35
Third	12	8
Fourth	3	5
Extraction with 2.1 M NaCl		
First	0	50
Second	0	5
Total	725	701

Samples of glycosaminoglycan-^{14}C (800 c.p.m.) were added to 2.5 ml solutions containing 0.1% cetyltrimethylammonium bromide, 0.03 M NaCl, and 50 mg of Celite. After centrifugation for 10 minutes at 2000 x g, aliquots of the supernatants were assayed for radioactivity. The sediments were then extracted repeatedly with NaCl as shown. Each supernatant was collected by centrifugation at 2000 x g for 5 minutes and assayed for radioactivity.

disaccharide-4-sulfate (A. Linker, personal communication)). However, approximately 24% of the degradation products of glycosaminoglycan-^{14}C formed in reaction mixtures containing PAPS appeared to be identical with this disaccharide sulfate from chondroitin-4-sulfate.

Almost all (98%) of the glycosaminoglycan-^{14}C formed in the reaction mixture without PAPS was degraded by bacterial hyaluronidase to material which moved 22 - 24 cm from the origin, in the vicinity of the disaccharide

degradation product of carrier hyaluronic acid. The chromatogram of the degradation products of glycosaminoglycan-^{14}C formed in reaction mixtures with PAPS differed. Approximately 28% of the radioactivity remained within 4 cm of the origin corresponding to larger oligosaccharides or polysaccharide, while the remainder of the material was degraded to disaccharide.

Almost all (95%) of the degradation products of glycosaminoglycan-^{14}C formed in the absence of PAPS was retarded when filtered on a column of Sephadex G-25. Less than 5% was excluded from the gel, confirming that the radioactive material was essentially totally degraded to molecules of low molecular weight. However, 27% of the bacterial hyaluronidase degradation products of glycosaminoglycan-^{14}C formed in the presence of PAPS was excluded by the gel. These results agree with the paper chromatograms of the digestion products.

Bacterial hyaluronidase is known to degrade chondroitin (non-sulfated), the chief product being a disaccharide (Davidson and Meyer, 1954). However it is inactive towards either chondroitin-4-sulfate or chondroitin-6-sulfate. The glycosaminoglycan-^{14}C (chondroitin-^{14}C) formed in the absence of PAPS was readily degraded, confirming its non-sulfated state. However, approximately 25% of the glycosaminoglycan-^{14}C formed in the presence of PAPS was resistant to degradation. In addition, approximately 25% of this ^{14}C-labeled glycosaminoglycan was degraded by bacterial heparinase to material which appeared to be identical to the disaccharide-4-sulfate product of chondroitin-4-sulfate degradation. (The disaccharide-6-sulfate from chondroitin-6-sulfate has a similar mobility and is not differentiated from the disaccharide-4-sulfate in this system.) The pattern of extraction from the cetyltrimethylammonium complex also indicates that a more polyanionic product was formed when PAPS was present in the reaction mixture.

The results indicate that approximately 25% of the glycosaminoglycan-^{14}C formed when PAPS is present in the reaction mixture with UDP-glucuronic acid-^{14}C and UDP-N-acetylgalactosamine appears to be chondroitin sulfate.

The location of the sulfate (-4-sulfate or -6-sulfate) cannot be determined by these experiments. This formation of chondroitin sulfate is similar to the previously described formation of the sulfated glycosaminoglycan, heparin (Silbert, 1967a). All the reactions take place with a particulate preparation and the product remains bound to this preparation. This suggests that polymerization and sulfation may take place in close proximity in the cell.

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