REDUCED SYNTHESIS OF CHONDROITIN SULFATE BY CARTILAGE FROM THE MUTANT, NANOMELIA[1,2]

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Summary

Sternal cartilage from genetically micromelic chick embryos incorporated labelled glucose, glucosamine and sulfate at reduced rates compared to normal cartilage. There was no detectable breakdown of protein-linked ^{14}C-chondroitin sulfate by either mutant or normal cartilage. Chondroitin sulfate chains which were synthesized by the mutant cartilage appeared to have normal molecular weight and sulfate content.

Chick embryos homozygous for the autosomal recessive gene nanomelia (nm) develop an extreme form of micromelia (1). These embryos survive the incubation period but are unable to hatch. Mathews (2) reported that 17-day nanomelic cartilage contained about 1/10th the quantity of chondroitin sulfate (CS) found in normal sibs. The collagen content, as estimated from hydroxyproline determinations, appeared normal. Mathews proposed that the defect might be restricted to cartilage since skin from both mutant and normal embryos contained similar quantities of CS. The absence of CS breakdown products in the mutant allantoic fluid suggested to him that the reduced content of these macromolecules was due to a defect in their synthesis.

This report presents evidence that chondrocytes from nanomelic cartilage synthesize fewer chains of protein-linked chondroitin sulfate.

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Materials and Methods

Nanomelic embryos were among the progeny of matings between birds heterozygous for the recessive lethal gene nm. Phenotypically normal sibs served as controls. Sternal cartilage was used for in vitro culture since the early onset of ossification in the limb makes it difficult to obtain sufficient cartilaginous material from this source. Sternae were dissected from 14-day embryos and incubated at 37°C in one of the following: MEDIUM A - Waymouth's medium (Colorado Serum Co.), MEDIUM B - a mixture of equal parts Hank's balanced salts solution (minus the glucose) and Ham's F-10 nutrient medium (3) with 2X the vitamin and 4X the amino acid concentrations (Grand Is. Biol. Co.). Both media were supplemented with 50 units/ml. each of penicillin and streptomycin plus radioactive precursors to acid mucopolysaccharides. Two to four sternae were placed in 4-dram screw-cap vials containing 2.5 ml. of medium. The pH was adjusted in an atmosphere of 5% CO_2: 95% air, the vials were sealed and maintained at 37°C with constant shaking. At the end of the incubation period the sternae were rinsed with ice-cold Tyrode's solution, boiled 5 min. in fresh Tyrode's and stored at -20°C.

Acid insoluble radioactivity was determined by precipitating aliquots of the homogenized cartilage onto nitrocellulose filters (Matheson-Higgins Co.) with an equal volume of cold 10% trichloroacetic acid (TCA). One-half milligram of bovine serum albumen (Pentex) was added as carrier. The filters were washed with 15 volumes of cold 5% TCA and placed in counting vials with 1 ml. of 2N NH_4OH. Fifteen milliliters of Bray's solution (4) were added for counting in a Nuclear Chicago Mark I scintillation spectrometer equipped with external standardization.

Radioactive polysaccharide was isolated after pronase digestion by dialyzing first for 24 hrs. against 7 l. of 0.01M solutions of non-radioactive compounds and then for 48 hrs. against two changes of glass distilled water (DW) with thymol added as a preservative. The retentant was concentrated with Carbowax (Union Carbide) and lyophilized.

Digestion with testicular hyaluronidase, pronase (Calbiochem) and chondroitinase-ABC (Miles Labs) was accomplished by standard methods (5,6). The procedure of Nameroff and Holtzer (7) was used to determine the hexosamine content of labelled polysaccharides.

D-glucose-2-^{3}H (620 mc/mmole), D-glucose-U-^{14}C (138-207 mc/mmole), D-glucosamine-1-^{14}C (51 mc/mmole) and $Na_2{}^{35}SO_4$ (407 mc/mmole) were purchased from New England Nuclear Corp.

Results and Discussion

Incorporation data are expressed on a "per-sternum" basis since mutant and normal sternae contained similar amounts of DNA and protein (unpublished observations). Table 1 shows that normal cartilage incorporated glucosamine-^{14}C linearly over a 6 hr. period. The mutant tissue accumulated ^{14}C and ^{35}S at about 1/3 of the normal rates.

The above rates represent the net effects of macromolecule synthesis and degradation. Since either of these component processes could be abnormal in

Table 1

Incorporation of Precursors to Chondroitin Sulfate by Cartilage *In Vitro*

Treatment	DPM per Sternum		
	Normal	Mutant	Mutant / Normal
D-glucosamine-1-^{14}C			
2 hr.	5,300	—	—
4 hr.	13,700	5,030	0.36
6 hr.	19,300	—	—
$Na_2{}^{35}SO_4$			
3 hr.	545,000	160,000	0.29
6 hr.	813,000	224,000	0.28

Sternae were incubated in MEDIUM A containing 1 μc/ml of glucosamine-1-^{14}C. Homogenates were precipitated onto nitrocellulose filters and counted as described in Methods. For $^{35}SO_4$ incorporation, groups of four sternae were incubated in MEDIUM B supplemented with 50 μc/ml of $Na_2{}^{35}SO_4$ and 0.5 mg/ml of glucose. Labelled material was precipitated with TCA, digested with pronase and dialyzed. The retentant was assayed for radioactivity.

the mutant, the rate of accumulation and the stability of the products were studied using glucose-^{14}C as precursor (Table 2). The experiment also provided data on the ability of sternae to incorporate radioactivity throughout the culture period used. Normal sternae incorporated the isotope at a uniform rate between 3 and 9 hrs. of culture. With both types of cartilage, the amount of material lost to the medium was small and roughly proportional to the radioactivity recovered from the sternae. There was no detectable loss of TCA precipitable radioactivity from labelled sternae of either phenotype when these were cultured for an additional 12 hrs. in medium containing glucose-^{12}C. The ability of sternae to incorporate isotope did not decline noticeably during 12 hrs. of *in vitro* culture.

Table 2

Incorporation of Glucose-^{14}C into TCA Precipitable Material

Treatment		TCA Precipitable Radioactivity (DPM-^{14}C per Sternum)			
		Normal		Mutant	
		Sternae	Pulse Medium	Sternae	Pulse Medium
Pulse hr.					
3		50,000	2,630	34,300	2,510
7		165,000	11,600	—	—
9		243,000	18,100	168,000	13,400
Pulse hr. + Chase hr.					
3	4	51,000	2,920 (2,620)	—	—
3	6	50,600	2,240 (2,190)	30,300	3,740 (3,630)
3	8	67,700	3,430 (4,650)	—	—
3	12	46,600	1,460 (4,000)	35,600	3,010 (3,330)
Chase hr. + Pulse hr.					
4	3	57,600	5,350	—	—
6	3	65,400	2,310	19,000	1,030
8	3	68,400	1,720	—	—
12	3	56,500	3,300	20,000	997

Groups of three sternae were pulsed for the indicated times in MEDIUM B containing 5 μc/ml glucose-U-^{14}C. The chase medium contained 0.5 mg/ml glucose-^{12}C in place of the radioisotope. After incubation the sternae were homogenized and aliquots precipitated onto nitrocellulose filters. Figures in parentheses represent counts in the chase medium.

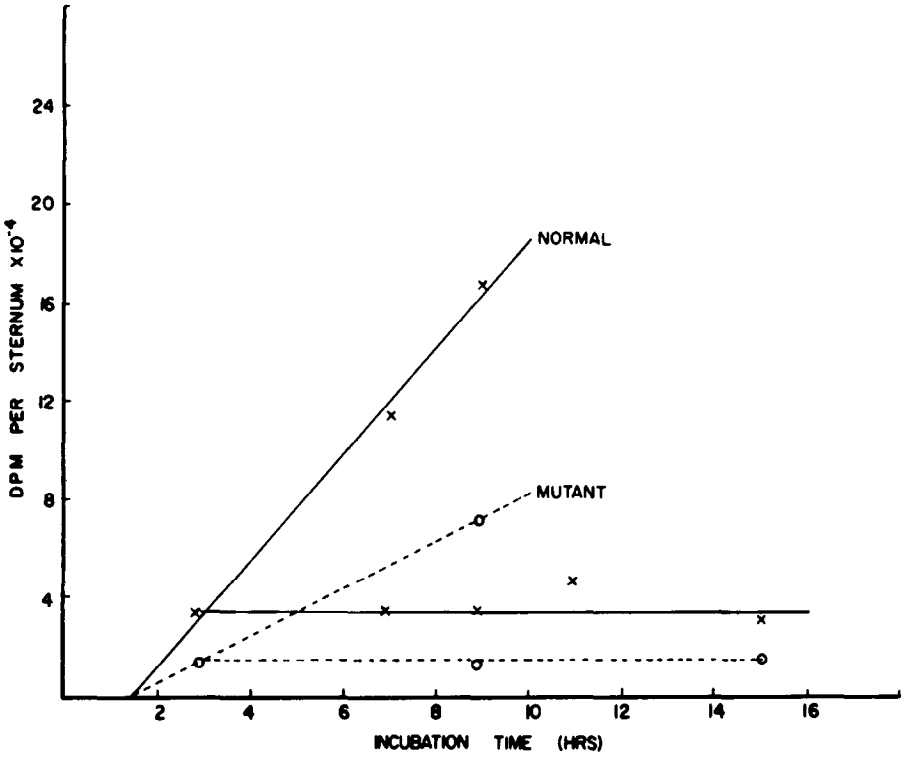


FIG. 1 - Time course of glucose-^{14}C incorporation into TCA precipitable, chondroitinase-ABC sensitive material. Homogenized sternae from the experiment described in Table 2 were suspended in Tris buffer (6) and aliquots of 0.45 ml. were taken for enzyme treatment and control. After incubation for 2 hrs. at 37°C the mixtures were precipitated onto nitrocellulose filters and counted (see Methods). The upper curves are for sternae exposed to isotope continuously, the lower are for sternae pulsed for 3 hrs. and then transferred to the chase medium.

The CS content of the glucose labelled material was estimated from its sensitivity to a specific mucopolysaccharidase and the accumulation of ^{14}C-CS with time is plotted in Fig. 1. Chondroitinase-ABC removed an average of 69.8% of the counts from normal sternae and 43.3% from the mutant. Nanomelic cartilage accumulated TCA precipitable (i.e. protein-linked) CS at 43% of the normal rate and there was no loss of this material during a 12 hr. incubation in the chase medium. The amounts of chondroitinase-ABC insensitive material were similar in both types of sternae, indicating that the mutant defect is specific for CS metabolism and that the reduced rate of ^{14}C incorporation is not the result of a larger precursor pool in the mutant.

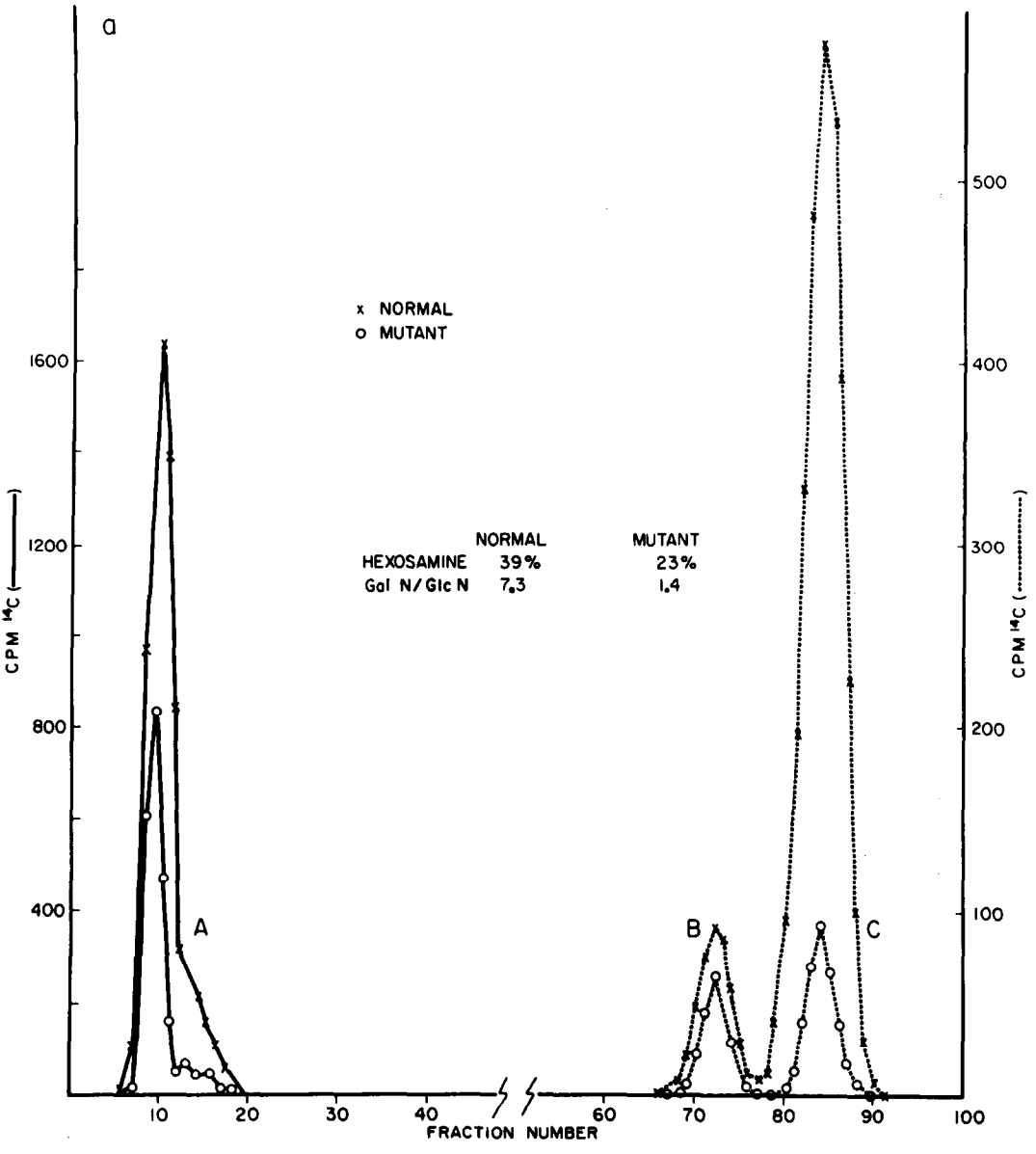
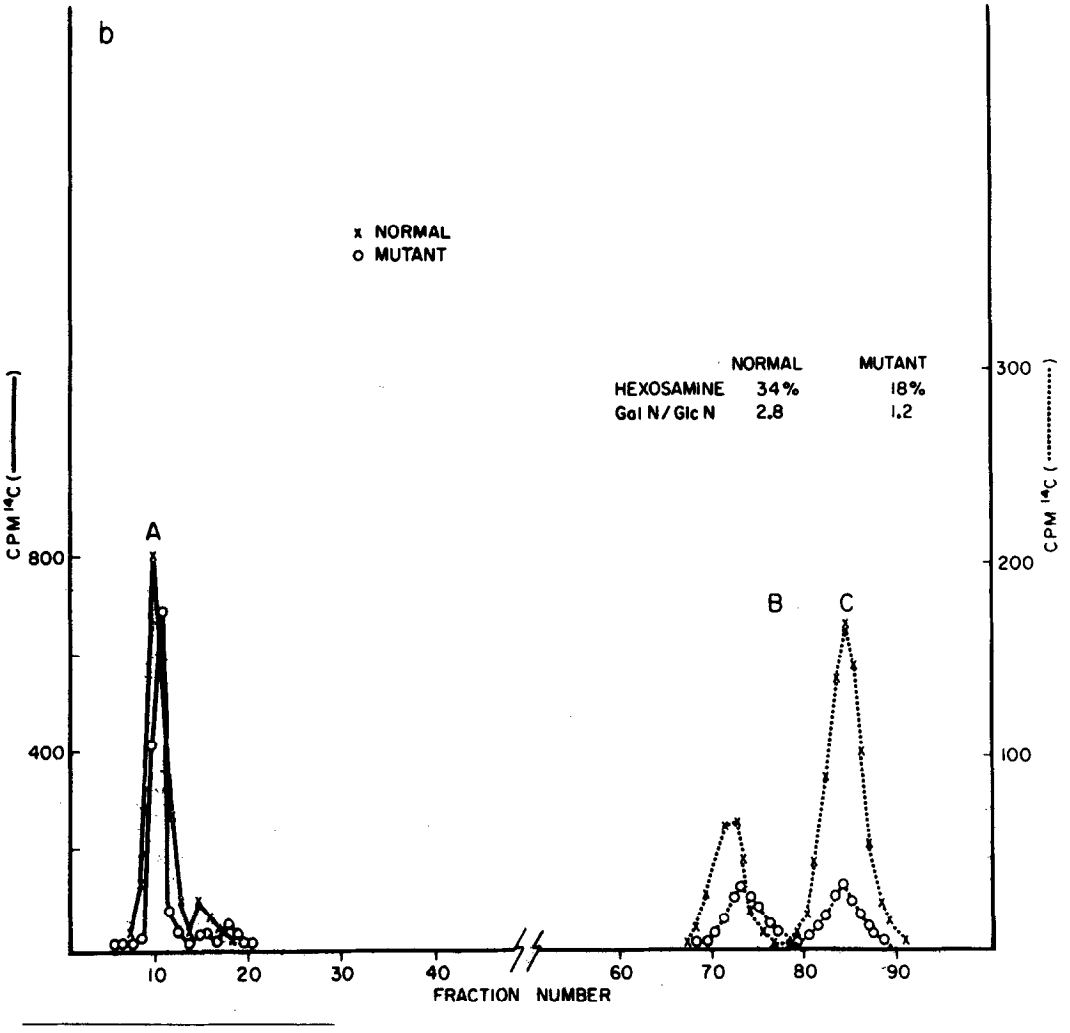
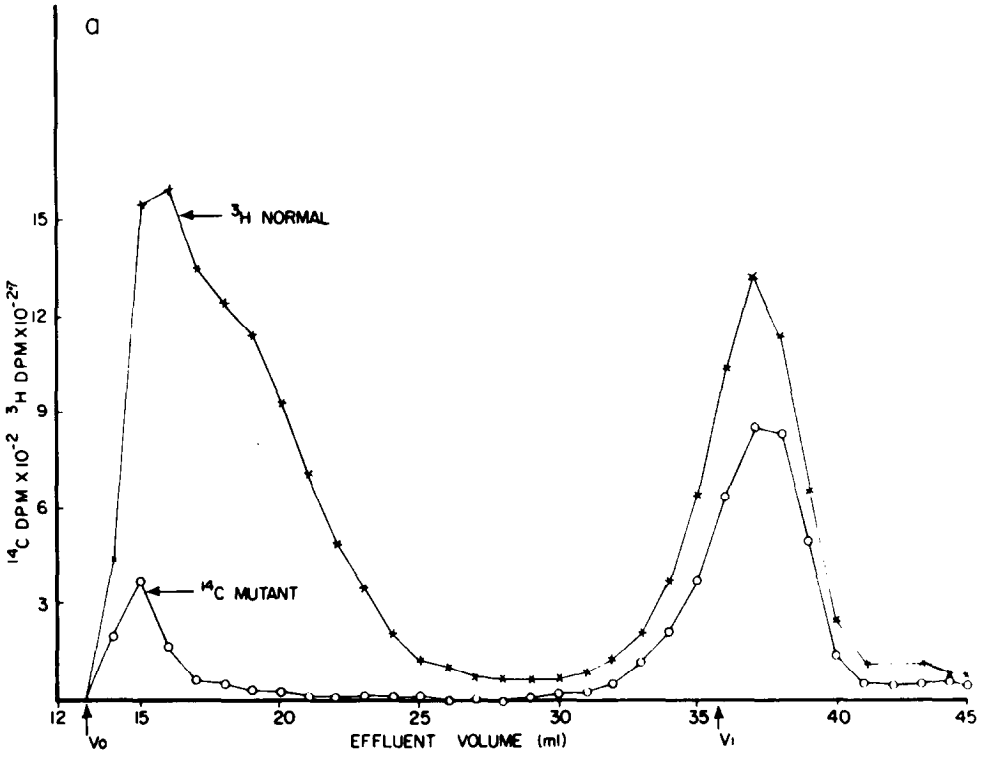


FIG. 2 - Dowex-50 chromatography of hydrolyzed glucose-^{14}C labelled cartilage. Sternae which had been exposed to isotope for 6 hrs. were homogenized, digested with pronase and dialyzed. The retentants were freeze-dried and hydrolyzed in 0.05 N HCl with Dowex-50 resin. Internal standards of 1 mg. each of glucuronic acid, glucosamine and galactosamine were carried through the hydrolysis and chromatography. The peaks of radioactivity coincided with the elution profiles of the internal standards (A-glucuronic acid; B-glucosamine; C-galactosamine). From 74-90% of the radioactivity placed in the hydrolysis tubes was recovered from the columns. (a) Not digested with hyaluronidase. (b) Digested with hyaluronidase and dialyzed before hydrolysis.



The hexosamine content of glucose-^{14}C labelled cartilage was determined by Dowex-50 chromatography of hydrolyzed polysaccharide (Fig. 2). Mutant sternae contained 23% hexosamine whereas normal material contained 39%. This difference may be accounted for by the reduced quantity of hyaluronidase-sensitive, galactosamine containing polysaccharide (i.e. chondroitin or chondroitin sulfate) in the mutant cartilage. Both nanomelic and normal sternae incorporated similar amounts of ^{14}C-glucose as glucosamine, providing further evidence for the specificity of the mutant defect.

The above results indicate that nanomelic cartilage synthesizes less CS than does tissue from normal sibs. There are two possible explanations for


a
^{3}H NORMAL
^{14}C MUTANT
^{14}C DPM X10^{-2} ^{3}H DPM X10$^{-2?}$
15
12
9
6
3
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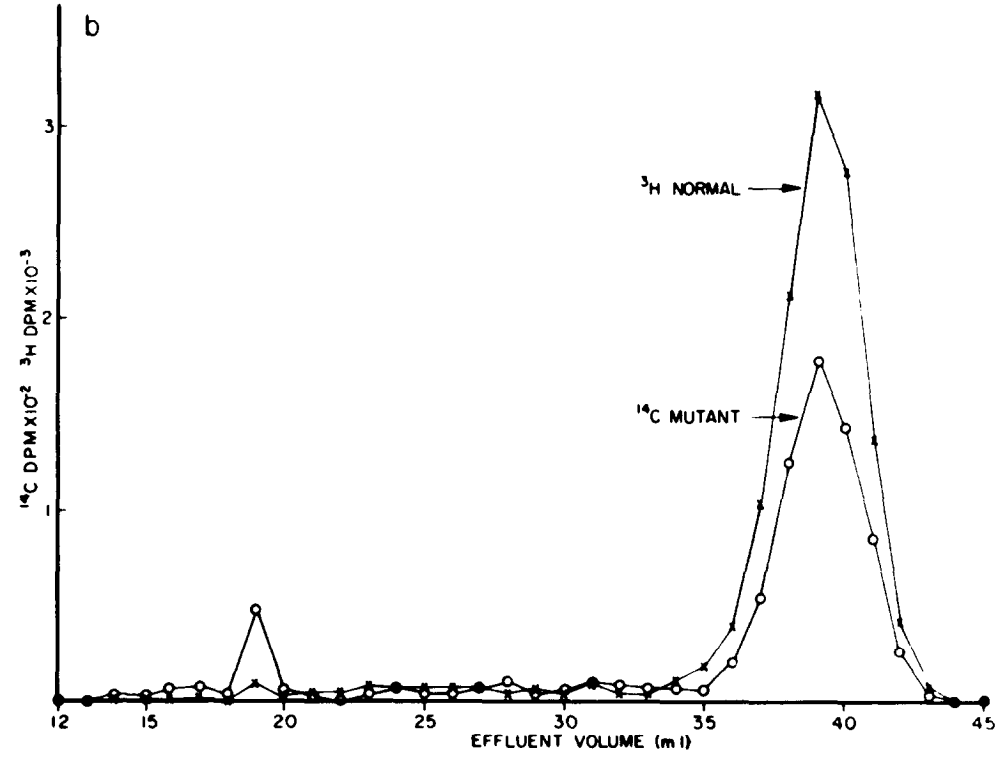
EFFLUENT VOLUME (ml)


b
^{3}H NORMAL
^{14}C MUTANT
^{14}C DPM X10^{2} ^{3}H DPM X10^{-3}
3
2
1
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EFFLUENT VOLUME (ml)

FIG. 3 - Sephadex G-200 chromatography of glucose labelled sternae. (a) Normal sternae were labelled for 6 hrs. with glucose-2-^{3}H; mutant sternae with glucose-U-^{14}C. The sternae were mixed, homogenized and precipitated with TCA. After digestion with pronase, the mixture was again precipitated with TCA. The supernatant was extracted twice with 2 vols. of ether, concentrated and applied to a 58 x 0.9 cm. column of Sephadex G-200. Labelled material was eluted with 0.025 M NaCl at 8 ml/hr. Aliquots from each fraction were counted by liquid scintillation. (b) The high molecular weight peak from the above column was desalted on Sephadex G-10, concentrated and digested with chondroitinase-ABC. The digest was rechromatographed as in (a).

this reduction. Either the mutant synthesized fewer CS chains of normal length, or else the chains synthesized have about 1/3 of the normal length. To distinguish between these possibilities, the size distribution of radioactive polysaccharides was examined by gel-filtration (8) on Sephadex (Fig. 3a). The elution profiles of both phenotypes showed two peaks of radioactivity. There was no difference between the mutant and the normal with respect to the uncharacterized low molecular-weight peak. Quantitatively, the mutant cartilage contained less of the high molecular weight material than did normal. Judging from its sensitivity to chondroitinase-ABC (Fig. 3b) this material is chondroitin-sulfate. The molecular weight of these labelled CS chains was calculated from their respective average elution volume (8) to be 27,600 for the mutant and 22,300 for the normal (Table 3). These figures

Table 3

Calculation of Average Molecular Weight for CS from Two Sets of Data

	Gel Filtration		Chemical Analysis[b]	
	Av. Elution Vol. (ml)	Av. M[a]	Galactosamine: Serine	Av. M
Nanomelic	16.1	27,600	50	23,350
Normal	18.1	22,300	38.5	18,105
Hypothetical	28.4	7,450	—	—

(a) Estimated from gel-filtration data using the relationship: AV. ELUTION VOLUME (ml) = 112.74 - 21.77 log M (modified from ref. 8 for differences in column size). The weight of the hypothetical chains was taken as 1/3 of normal. (b) Data from Mathews (2) for material isolated by cetylpyridinium chloride precipitation. Molecular weights were calculated assuming a periodic weight of 457 for each galactosamine containing disaccharide and assuming one xylose plus two galactose residue per chain.

are somewhat higher than estimates made from Mathew's data (2), but it is clear that the mutant CS chains are at least as long as those from normal sibs. If the mutant chains had 1/3 of the normal length they would have eluted at an average volume of 28 ml.

We conclude that chondrocytes from namomelic cartilage produce fewer chains of chondroitin sulfate than those from normal sibs. The similarity in the rates of glucosamine-^{14}C and $^{35}SO_4$ incorporation by the mutant tissue suggests that the CS chains which are made have a normal sulfate content.

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