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# DEFECTIVE PAPS-SYNTHESIS IN EPIPHYSEAL CARTILAGE FROM BRACHYMORPHIC MICE

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## SUMMARY

Activity levels of sulfotransferases, requisite for the sulfation of chondroitin sulfate proteoglycan, were measured in cell-free homogenates prepared from neonatal epiphyseal cartilage of normal C57Bl/6J or homozygous brachymorphic mice. In the presence of [$^{35}$S]-PAPS only or [$^{35}$S]-PAPS plus an exogenous sulfate acceptor, comparable amounts of $^{35}SO_4^{2-}$ were incorporated into chondroitin sulfate by the normal and mutant types of cartilage. In contrast, the mutant cartilage catalyzed the conversion of only 30% of the $^{35}SO_4^{2-}$ into chondroitin sulfate as compared to normal mouse cartilage when synthesis was initiated from ATP and $H_2{}^{35}SO_4$. These results suggest that the production of an undersulfated proteoglycan which has previously been reported in brachymorphic mice (Orkin, R.W. et al. (1976) Devel. Biol. 50, 82-94) may result from a defect in the synthesis of the sulfate donor PAPS.

Brachymorphism in mice is characterized by the gross morphological aberration of shortened limbs (1). Histological and ultrastructural studies on homozygous recessive brachymorphic mice (bm/bm) suggest that a defect in cartilage matrix is associated with their shortened limbs (2). Upon biochemical analysis, the mutant cartilage was shown to contain normal levels and types of collagen as well as normal levels of glycos-

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aminoglycans (3). However, both the level of inorganic sulfate and incorporation of $^{35}SO_4^{2-}$ were lower in mutant than in normal cartilage. On further chemical analysis, considerable amounts of unsulfated glycosaminoglycan disaccharide were obtained from the mutant cartilage only (3). A recent autoradiographic study showed that the differences in $^{35}SO_4^{2-}$ incorporation between normal and mutant cartilage were most pronounced, but not exclusive to, the proliferative zone, which displays the largest increase relative to the other zones in normal cartilage (4). In the present study, several possible caused of production of under-sulfated proteoglycan were investigated.

## MATERIALS AND METHODS

3'-Phosphoadenylyl [$^{35}$S]sulfate ([$^{35}$S]-PAPS) (2.2 Ci/mmol) and carrier-free $H_2{}^{35}SO_4$ (43 Ci/mg) were purchased from New England Nuclear. Chondroitinase ABC, AC, Δ-di-4S (2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-)-sulfo-D-galactose); Δ-di-6S, (2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose) were obtained from Miles Laboratories.

Homozygous brachymorphic mice (bm/bm) were obtained from the Jackson Laboratory (Bar Harbor, Maine) and a breeding colony established. Cartilage tissue was obtained from 4-day-old homozygous brachymorphic and normal C57Bl/6J mice by dissection of cartilagenous knee joint (distal femoral head and proximal tibial head) as described (4). Cartilage tissue was homogenized at 4° in 0.05M Mes buffer, pH 6.5, containing 0.25M KCl, 0.003M $MnCl_2$ and 0.01M $MgCl_2$ (5). Enzyme assays were performed directly on crude cell homogenates or on 10,000 x g pellet and supernatant fractions of the homogenates. The concentration of protein in various enzyme preparations was measured by the method of Lowry et al. (6).

Transfer of sulfate from [$^{35}$S]-PAPS to the N-acetyl-galactosaminyl residues of chondroitin sulfate were assayed in a total volume of 0.135 ml by incubating 1.2 nmol of [$^{35}$S]-PAPS (0.01 ml), 0.1 μmol of cysteine hydrochloride (0.01 ml), 0.1 μmol $MnCl_2$ (0.005 ml), 0.3 mg of a sulfate acceptor (0.06 ml) or Mes buffer (0.06 ml) and varying amounts of enzyme protein in 0.05 ml of Mes buffer. The sulfate acceptor was prepared from chondroitin 4-sulfate by desulfation with methanolic-HCl according to Kantor and Shubert (7). After 60 min at 37°, the was terminated by heating the tubes at 100° for 2 min. 1.5 mg of carrier chondroitin sulfate (0.15 ml), 1.0 mg of papain suspension (0.05 ml) and 1 ml of 0.05M cysteine hydrochloride were added and incubated overnight at 60°. Following extensive dialysis, sulfated polysaccharides were isolated as previously described (8).

In order to determine the concentration of chondroitin 4-sulfate and chondroitin 6-sulfate synthesized by the cell-

free systems, an alternate procedure was performed following papain digestion and dialysis. Incubation mixtures (0.10 ml) were digested with 0.2 units of chondroitinase-ABC (reconstituted in enriched Tris-acetate buffer, pH 8.0 (9); 0.20 ml) for 16 hr at 37°. Reactions were terminated by boiling for 3 min, centrifuged and the supernatants applied to Whatman 1MM. Standard Δ-di-4S and Δ-di-6S were added directly to applied samples. Disaccharides were separated by chromatography in an acetic acid:butanol: 1M ammonia (3:2:1) solvent system for 30 hr. After drying, the disaccharides were visualized under UV light. Regions containing disaccharides were cut into 1-cm segments and the radioactivity determined by liquid scintillation counting (10).

The synthesis of [$^{35}$S]-PAPS and $^{35}SO_4^{2-}$ transfer to chondroitin sulfate was assayed by a modification of the procedure described by Meezan and Davidson (10). Reaction mixtures of 0.275 ml contained 20 μmol ATP (0.025) ml, 5μCi $H_2{}^{35}SO_4$ (0.005 ml), 0.05μmol MgCl (0.005 ml), 0.5μmol $NAD^+$ (0.005 ml), 0.1μmol of cysteine hydrochloride (0.01 ml), 0.125mg of a sulfate acceptor (0.025 ml) and varying amounts of enzyme protein in 0.10 ml of 0.05M Tris-HCl, pH 8.0 buffer. Following incubation for 1 hr at 37°, the labeled chondroitin sulfate was isolated exactly as described when synthesis was initiated from [$^{35}$S]-PAPS.

## RESULTS

The sulfation of endogenous or exogenously added chondroitin sulfate proteoglycan was assessed in extracts obtained from normal C57Bl/6J or brachymorphic mice. The results (Table 1) indicate that both 4- and 6-sulfotransferase activities are present in the mutant cartilage and capable of catalyzing the transfer of sulfate from phosphodenylysulfate (PAPS) to either the native intracellular acceptor or a chemically desulfated acceptor. Slightly higher than normal sulfotransferase activity levels were measured in bm/bm cartilage extracts; however, the ratio of 4:6 activity are similar for both types of cartilage (Table 1).

When sulfation was initiated from ATP and $^{35}SO_4^{2-}$ by the cell-free cartilage preparations, a significant difference in incorporation of $^{35}SO_4^{2-}$ into chondroitin sulfate was observed. As shown in Table II, total $^{35}SO_4^{2-}$ incorporated into purified chondroitin sulfate was decreased approximately 70% in the brachymorphic cartilage.

TABLE I

SULFOTRANSFERASE ACTIVITY IN NORMAL AND BRACHYMORPHIC CARTILAGE

| COMPONENT | $^{35}SO_4^{2-}$ INCORPORATION INTO PRODUCT | | |
|---|---|---|---|
| | Δ-di-6S | Δ-di-4S | TOTAL |
| | cpm/mg protein x $10^{-5}$ | | |
| C57Bl/6J(+ACCEPTOR) | 1.46 | 2.59 | 4.05 |
| C57Bl/6J(ENDOGENOUS) | 0.07 | 0.13 | 0.20 |
| bm/bm-1(+ACCEPTOR) | 2.61 | 3.44 | 6.05 |
| bm/bm-1(ENDOGENOUS) | 0.20 | 0.21 | 0.40 |
| bm/bm-2(+ACCEPTOR) | 2.21 | 3.90 | 6.11 |
| bm/bm-2(ENDOGENOUS) | 0.15 | 0.35 | 0.50 |

## DISCUSSION

Since the integration of inorganic sulfate into chondroitin sulfate proteoglycan involves several steps, a number of sites exist at which a defect might result in an undersulfated molecule. In the present study, the low-sulfated proteoglycan produced by brachymorphic cartilage (2,3) was shown not to be caused by reduced levels of or inactivation of sulfotransferase enzymes. Furthermore, the intracellular acceptor in the mutant cartilage can be readily sulfated, and in the same relative positions as the endogenous acceptor from normal cartilage or an exogenous desulfated acceptor.

A possible defect in the sulfation pathway during synthesis of phosphoadenylylsulfate from ATP and $^{35}SO_4^{2-}$ in bm/bm cartilage is suggested from differences in the amount of $^{35}SO_4^{2-}$ incorporated into chondroitin sulfate proteoglycan by the two types of cartilage.

TABLE II

PAPS-SYNTHESIZING ACTIVITY IN NORMAL AND BRACHYMORPHIC CARTILAGE

| EXPERIMENT | PAIRS OF EPIPHYSES | $^{35}SO_4^{2-}$ INCORPORATION (cpm/mg protein) C57Bl/6J | bm/bm |
|---|---|---|---|
| | | cpm/mg protein x $10^{-3}$ | |
| 1 | 8 | 12.10 | 3.94 |
| 2 | 10 | 18.70 | 4.37 |
| 3 | 9 | 7.95 | 2.18 |

It is realized however, that several steps are involved in this pathway, anyone of which could alter levels of ATP or subsequent adenylsulfate or phosphoadenylsulfate formation in brachymorphic cartilage.

It also may be inferred from these studies that the availability of the sulfate donor-PAPS may be the rate limiting step in the sulfation process of glycosaminoglycans. A similar suggestion recently was made based upon the observations that the corneal glycosaminoglycan sulfotransferases changed too little during development to account for the increase in degree of sulfation of corneal glycosaminoglycans that occurs(12). Therefore, the degree of sulfation of glycosaminoglycans may not be regulated by changes in the specific activities of the sulfotransferases, but rather regulation may occur at the level of the availabiltiy of the sulfate donor.

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## REFERENCES


1. Lane, P.W., and Dickie, M.M. (1968) J. Hered. 59, 300-308.
2. Orkin, R.W., Williams, B.R., Cranley, R.E., Poppke, D.C., and Brown, K.S. (1977) J. Cell Biol. 73, 287-299.
3. Orkin, R.W., Pratt, R.M., and Martin, G.R. (1976) Devel. Biol. 50, 82-94.
4. Greene, R.M., Brown, K.S., and Pratt, R.M. (1978) Anat. Record, in press.
5. Schwartz, N.B. and Rodén, L. (1974) Carbohydr. Res. 37, 167-180.
6. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
7. Kantor, T.G. and Shubert, M. (1957) J. Amer. Chem. Soc. 79, 152-156.
8. Dorfman, A. and Ho, P.-L. (1970) Proc. Natl. Acad. Sci. USA 66, 495-499.
9. Saito, H., Yamagata, T., and Suzuki, S. (1968) J. Biol. Chem. 243, 1536-1542.
10. Schwartz, N.B. (1976) J. Biol. Chem. 251, 3346-3351.
11. Meezan, E. and Davidson, E.A. (1967) J. Biol. Chem. 242, 1685-1689.
12. Hart, G.W. (1978) J. Biol. Chem. 253, 347-353.