Incorporation of [^{35}S]sulphate into glycosaminoglycans by mineralized tissues *in vivo*

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To investigate the metabolism of proteoglycans in young growing rats, calvaria, incisors, femoral diaphysis and metaphysis were labelled *in vivo* for 0.5–72 h with [^{35}S]sulphate. At each time point the specific radioactivity, expressed as c.p.m. of [^{35}S]sulphate/ μg of uronic acid, of papain-resistant macromolecules in each tissue was determined. The identity of the glycosaminoglycans was established by the use of specific enzymic and chemical methods of degradation. Incorporation of the label into each tissue was maximal at 12 h; it then declined to 50–75% of that value by 72 h. Chondroitin sulphate was the predominant glycosaminoglycan in each tissue, representing 80–96% of the total; heparan sulphate comprised 2–14% of the total; in general, radioactive material sensitive to keratanase comprised less than 1% of the total. The relative amount of labelled chondroitin sulphate increased, whereas that of heparan sulphate decreased, with increasing time of incorporation. These data show that 25–50% of the newly synthesized glycosaminoglycans are lost from mineralizing tissues, during the time in which the newly secreted organic matrix becomes mineralized.

Bone and dentine are normally formed by the deposition of mineral on to a predominantly collagenous extracellular matrix. This process has been studied extensively during the past few years, yet the factors controlling the site and rate of initial mineral formation have not been elucidated. The search for matrix components capable of such a role has stimulated studies of several non-collagenous proteins, e.g. osteocalcin (Price *et al.*, 1981; Hauschka *et al.*, 1983), osteonectin (Termine *et al.*, 1981), bone sialoprotein (Fisher *et al.*, 1983b) and proteoglycans (Reddi *et al.*, 1978; Hjerpe *et al.*, 1979; Sugahara *et al.*, 1981; Fisher *et al.*, 1983a). Most of these studies describe the isolation and initial characterization of the various components, but present little or no information concerning their metabolism. The dearth of such information results from an inability to label these proteins specifically *in vivo*. However, bone and dentine proteoglycans can be labelled *in vivo* with [^{35}S]sulphate, thereby facilitating metabolic studies. Previous studies from our laboratory have demonstrated the utility of this radiolabel, not only for metabolic studies of rat calvaria proteoglycans (Prince *et al.*, 1983), but also for the isolation

and characterization of rat incisor proteoglycans (Rahemtulla *et al.*, 1981, 1984).

In our study of rat calvaria, we established that proteoglycans in this tissue are rapidly pulse-labelled by intraperitoneal injection of [^{35}S]sulphate, and that a maximum incorporation is reached 12 h after injection of the radiolabel. Thereafter the amount of radioactivity declined steadily until 36 h after injection, when a plateau value of 55–65% of maximum was reached. Also, the newly synthesized proteoglycans were largely susceptible to chondroitinase ABC (85–90% were digested). However, about 7–15% of the proteoglycans resisted digestion by this enzyme (Prince *et al.*, 1983).

The purpose of the present investigation was to determine the kinetic behaviour of [^{35}S]sulphate-labelled proteoglycans in mineralized tissues other than calvaria and to identify those newly synthesized proteoglycans resistant to chondroitinase ABC. Also, this study was designed so that the kinetics of incorporation of [^{35}S]sulphate into different mineralizing tissues could be compared on the basis of specific radioactivity, i.e. c.p.m. of [^{35}S]sulphate/ μg of uronic acid.

Fig. 1. Bio-Gel P-30 chromatography of intact and degraded [^{35}S]sulphate-labelled glycosaminoglycans. Mineralized-tissue glycosaminoglycans were radio-labelled, excised, digested and chromatographed as

Experimental

Radiolabelling of proteoglycans of mineralized tissues

To label newly synthesized proteoglycans, 4-week-old (105–135 g body wt.) male Sprague-Dawley-descended rats (Charles River Laboratories, Wilmington, MA, U.S.A.) were injected intraperitoneally with 0.5 mCi of carrier-free [^{35}S]sulphate (25–40 Ci/mg; Amersham, Arlington Heights, IL, U.S.A.) per 100 g body wt. At each time period (0.5, 2, 12, 36 and 72 h) after injection, four rats were killed. Calvaria, maxillary and mandibular incisors, both femurs and the xyphoid process were immediately excised and cleaned of adherent soft tissue. The frontal and parietal bones were trimmed free of calvarial sutures; pulp and soft pre-enamel were removed from the incisors; marrow was flushed out of the femurs and the articular cartilages were also removed. The femurs were then cut so that the middle one-third, the diaphysis, was freed from the distal and proximal one-third, here termed the metaphysis. After being freeze-dried and weighed, the tissues were digested as described below.

Digestion of tissues and determination of specific radioactivity

The dry tissues were crushed manually and then solubilized at 65°C with 2–4 ml of a solution containing 0.15 mg of papain (twice-crystallized; Sigma Chemical Co., St. Louis, MO, U.S.A.)/ml of 0.1 M-sodium acetate buffer, pH 5.6, containing 0.2 M-EDTA and 5 mM-cysteine hydrochloride. Each digest was adjusted to neutral pH by the addition of NaOH and was then centrifuged at 2000 g for 20 min to remove undigested material. To remove 'free' (unincorporated) [^{35}S]sulphate, all samples were chromatographed on columns (0.7 cm $\times$ 50 cm) of Bio-Gel P-30 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) eluted in 1 ml fractions with 0.2 M- NH_4HCO_3 , pH 8.0, at a flow rate of 4–5 ml/h. Fractions were analysed for radioactivity by using ScintiVerse I (Fisher Scientific Co., Fair Lawn, NJ, U.S.A.) in a Beckman LS 8000 liquid-scintillation spectrophotometer programmed for automatic quench compensation. A typical chromatogram is shown in Fig. 1(a). The sample represented is the metaphysis after 12 h of [^{35}S]sulphate incorporation. Fractions at the void

described in the text. Elution profiles shown represent intact (a), chondroitinase-ABC-digested (b), keratanase-digested (c) and HNO_2 -degraded (d) glycosaminoglycans from the femoral metaphysis after 12 h of incorporation. These profiles are similar to those obtained for other tissues and other periods of incorporation.

volume were pooled, freeze-dried and then assayed for radioactivity and uronic acid (Blumenkrantz & Asboe-Hansen, 1973).

Enzymic and chemical degradation of glycosaminoglycans

To verify that the [^{35}S]sulphate was labelling glycosaminoglycans, the identity of the papain-resistant macromolecules was determined. Samples were pooled with respect to tissue and length of incorporation and then freeze-dried. The dry material was dissolved in 0.1 M-Tris/HCl, pH 7.90, and digested overnight at 37°C with 0.1 unit (1 $\mu\text{mol}/\text{min}$; 37°C; pH 8) of chondroitinase ABC (Miles Laboratories, Elkhart, IN, U.S.A.). Each digest was chromatographed on a column (0.7 cm $\times$ 45 cm) of Bio-Gel P-30 as described above. Radioactive material eluted near the total volume of the column was susceptible to the enzyme, and therefore represents chondroitin sulphate or dermatan sulphate. Radioactive material still eluted at the void volume (fractions 2–8; see Fig. 1b) was resistant to the enzyme and was pooled and freeze-dried.

The chondroitinase-ABC-resistant material was dissolved in water. One-half of each sample was digested overnight at 37°C with 0.1 unit of keratanase (Miles Laboratories) in 0.02 M-Tris/HCl, pH 7.40. The remaining samples were degraded with HNO_2 by the low-pH procedure of Shively & Conrad (1976). After each digestion, samples were chromatographed on Bio-Gel P-30 as described above (see Figs. 1c and 1d respectively). Radioactive material eluted after the void volume of the column was susceptible to the above treatments, and thus represents keratan sulphate or heparan sulphate respectively.

Recovery of radioactivity from all gel-filtration runs was over 80% and usually over 90%. Similar results were obtained when the entire experiment was repeated.

Results and discussion

The injection protocol followed in these studies resulted in a pulse-labelling of about 6–12 h in duration (Prince *et al.*, 1983). The papain/EDTA digestion completely solubilized the calvaria, diaphysis, metaphysis and xyphoid process, resulting in the release of [^{35}S]sulphate-labelled glycosaminoglycans. Small amounts of the incisors, presumably enamel, resisted digestion. This material contained negligible amounts of radioactivity and was discarded.

The time course of incorporation of [^{35}S]sulphate into newly synthesized macromolecules is presented in Table 1, which clearly shows that each of the mineralized tissues attained a maximum incorporation by 12 h. With longer times of incorporation, the amount of radioactivity began to decline, so that by 72 h each mineralized tissue contained 50–75% of the maximal amounts, values consistent with those obtained in previous experiments with rat calvaria (Prince *et al.*, 1983). During this experiment the levels of uronic acid in each tissue did not change significantly. Mean values $\pm$ S.D., as μg of uronic acid/mg of freeze-dried tissue, for calvaria, incisors, diaphysis, metaphysis and the xyphoid process were 0.63 ± 0.07 , 0.57 ± 0.12 , 0.72 ± 0.13 , 2.13 ± 0.13 and 13.20 ± 3.14 respectively. Therefore such decreases in specific radioactivity result from loss of radioactivity itself, and not from a later accumulation of unlabelled proteoglycans in the tissues during the course of the experiment. Also, the degree of loss of proteoglycans observed during this experiment is generally in good agreement with other studies of the proteoglycan content of the non-mineralized and mineralized proteins of bone (Engfeldt & Hjerpe, 1976). Furthermore, a substantially different pattern was observed in the xyphoid process, a non-calcifying cartilagenous tissue, where incorporation was maximal at the earliest time point,

Table 1. Incorporation of [^{35}S]sulphate into papain-resistant macromolecules

Four rats per time period were injected with 0.5 mCi of [^{35}S]sulphate/100 g body wt. After the indicated period of incorporation the tissues listed below were dissected out, digested with papain and assayed as described in the text. Values shown are means $\pm$ S.D. ($n=4$).

Period of incorporation (h)	Incorporation (c.p.m. of [^{35}S]sulphate/ μg of uronic acid)				
	Calvaria	Incisors	Diaphysis	Metaphysis	Xyphoid
0.5	167 $\pm$ 108	246 $\pm$ 100	574 $\pm$ 310	741*	2371*
2	508 $\pm$ 130	606 $\pm$ 157	728 $\pm$ 30	729 $\pm$ 334	1748 $\pm$ 825
12	625 $\pm$ 80	687 $\pm$ 198	1111 $\pm$ 343	984 $\pm$ 267	757 $\pm$ 340
36	515 $\pm$ 78	478 $\pm$ 48	778 $\pm$ 40	951 $\pm$ 80	1142 $\pm$ 170
72	445 $\pm$ 32	365 $\pm$ 22	540 $\pm$ 36	728 $\pm$ 30	952 $\pm$ 211

* $n=2$.

Table 2. *Susceptibility of mineralized-tissue glycosaminoglycans to various treatments*

[³⁵S]Sulphate-labelled glycosaminoglycans were isolated and degraded as described in the text. Abbreviations: N.T., not treated; N.D., none detected; –, not determined.

Period of incorporation (h)	Tissue	Percentage of total recovered radioactivity susceptible to:			
		Chondroitinase ABC	Keratanase	HNO ₂	Resistant
0.5	Calvaria	80.8	N.T.	N.T.	–
	Incisors	86.5	N.T.	N.T.	–
	Metaphysis	88.8	0.5	7.1	3.6
	Diaphysis	87.8	0.6	8.0	3.6
2	Calvaria	84.8	2.7	12.4	0.1
	Incisors	84.8	N.D.	13.7	1.5
	Metaphysis	88.1	0.4	8.7	2.8
	Diaphysis	89.3	0.3	7.1	3.3
12	Calvaria	86.8	2.3	9.7	1.2
	Incisors	88.9	N.D.	10.0	1.1
	Metaphysis	92.2	0.2	5.7	1.9
	Diaphysis	90.4	0.4	6.1	3.1
36	Calvaria	86.5	5.9	6.2	1.4
	Incisors	93.2	N.D.	5.1	1.7
	Metaphysis	94.5	0.2	3.5	1.8
	Diaphysis	92.1	0.6	5.1	2.2
72	Calvaria	90.9	N.T.	N.T.	–
	Incisors	95.8	N.T.	N.T.	–
	Metaphysis	95.7	0.3	2.3	1.7
	Diaphysis	93.4	0.6	3.8	2.2

½h, and then rapidly decreased to a plateau that was about 40% of the maximum value.

Additionally, this study presents new data concerning the sulphated glycosaminoglycan content of mineralized tissues (Table 2). It has been known for some time that chondroitin 4-sulphate is the major glycosaminoglycan in bones and teeth (Engfeldt & Hjerpe, 1972, 1976; Hjertquist & Vejlens, 1968). More recently, chondroitin 6-sulphate and dermatan sulphate have been reported as constituents of normal mineralized tissues (Hunter *et al.*, 1984; Hjerpe *et al.*, 1979, 1983; Prince & Navia, 1983; Prince *et al.*, 1983). In the present study we found, as expected, that most (81–95%) of the newly synthesized glycosaminoglycans was susceptible to chondroitinase ABC, and therefore represents chondroitin sulphate and dermatan sulphate. However, a novel finding was that, in each mineralized tissue examined, the proportion of this material increased with time after injection (Table 2).

The presence of small amounts of radiolabelled material susceptible to HNO₂ (i.e. heparan sulphate) accounts for most of the chondroitinase-ABC-resistant material. The presence of heparan sulphate in human dentine has been reported previously (Branford White, 1978), although in a more recent study Hjerpe *et al.* (1983) were unable to detect this glycosaminoglycan in dog dentine. This disparity probably arises from differences in tissue preparation and extraction. In the latter

study dentine was prepared by ultracentrifugation of finely ground tooth powder, thus producing a sample presumably devoid of cells. The former study, as well as the present one, used intact tissue samples. Thus it is possible that heparan sulphate in bones and teeth is cell-associated or basement-membrane-associated, rather than being a component of the extracellular matrix. Data supporting this view have been obtained recently by the immunohistochemical examination of rat incisors with antibodies to heparan sulphate proteoglycans (Laurie *et al.*, 1982). These authors found significant reactivity in the enamel organ and in capillaries and arteries of the tooth.

Finally, although keratan sulphate has been reported in pig incisors (Linde, 1973) and in medullary bone of quail (Fisher & Schraer, 1980), in the present study the amount of radiolabelled material sensitive to keratanase was so small that it is difficult to judge whether or not keratan sulphate is a component of the sulphated glycosaminoglycans of rat bones and teeth.

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