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X-Ray Ultramicroanalysis of Sulfur in Calf Costochondral Plates*

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1. X-Ray absorption spectrophotometry has been applied to ultramicroanalysis of sulfur. Model systems with S*** concentrations in the range 0.7–2.5 % were used to test the method.

2. Regional distribution of S concentration was determined in cartilage sections subsequent to extraction by various techniques.

3. Evidence was obtained that formalin fixation and incubation of histologic slices in 0.5 molar EDTA*** for 16 h resulted in a significant loss of S compounds from the distal hypertrophic cell zone of calf epiphyseal plates.

Dentine, bone and cartilage have been intensively studied by a variety of histologic and histochemical techniques, which have provided qualitative data supporting a role of acid mucopolysaccharides in the development of calcifying matrix[1–3]. Biochemical studies of demineralized bones, fracture callus[4,5], and epiphyseal plates reveal significant concentrations of acid mucopolysaccharides. In studies of calf bones, chondroitin 4-sulfate, chondroitin 6-sulfate, as well as unidentified sulfate fractions have been isolated[5]. Slices of calf epiphyseal cartilage incubated *in vitro* have shown that 90 % or more of labeled S when provided as $Na_2{}^{35}SO_4$ appears within SMPS*** fractions as ester sulfate[6,7], and similar results occur if the isotope is given *in vivo*. Based on biochemical evidence ^{35}S radioautography has been employed extensively to examine SMPS metabolism of hard tissues[8]. However, information yielded, even with grain counts, is only of partially quantitative value. In those tissues such as calf rib plate, in which less than 10 % total S has been isolated in fractions other than SMPS,

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*** Abbreviations: EDTA, ethylenediamine tetraacetic acid; SMPS, sulfated mucopolysaccharides; S, sulfur.

micro-regional variations in total S would provide additional information on the fate of these compounds.

The present report utilizes a method of monochromatic X-ray absorption spectrophotometry for analysis of total S in tissue in amounts of 10^{-11} g and demonstrates its use in respect to extractive procedures for SMPS. The theoretical foundation of this method of microanalysis has been described in detail by Engström[9] and Lindström[10].

MATERIALS AND METHODS

The spectrophotometric analysis for S was principally performed according to the procedure described by Lindström[10]. Some modifications of apparatus and measuring technique were introduced and will be published elsewhere. The densitometric evaluation of the microradiograms was performed with an evenly illuminated measuring spot 20 μ in diameter. The total dry mass per unit area was determined from one of the microradiograms using a value of the mass absorption coefficient calculated for the elementary composition of collagen. This simplified calculation of the mass absorption coefficient may introduce an error if the elements Na, Mg, P, K and Ca are present in appreciable amounts. An estimate of this error has been calculated for the case of cartilage using the concentration values of these elements given by Howell *et al.*[11]. The error is about 15 % for the region of provisional calcification and adjacent hypertrophic cells and 6 % for the region of resting cells, giving too high values of total mass. Another possible source of error is the effect of re-radiation[12] but this will introduce an error of only circa 2 %. The data of Table 2 are presented uncorrected because of the approximative nature of these corrections.

Models for use as testing objects were prepared as follows: To aqueous solutions containing 3–5 % gelatin (Difco) at 90°C, crude chondroitin sulfate (Sigma) was added in varying proportions with constant stirring for ½ h. Portions were saved for gravimetric analysis of dried ashed samples for S by the method of Andersen[13]. The remainder of each gelatin preparation was spread as a thin film on glass slides. After cooling, microscopic strips were sliced from these films, placed between two parlodion foils, and pressed on grids for X-ray analysis.

Tissue analysis. Samples were dissected from costochondral junctions of normal calves within ½ h of sacrifice and were frozen in isopentane surrounded by liquid nitrogen. Materials were sectioned in a cryostat at 10–40 μ thickness and freeze-dried. Appropriate regions were removed under a dissecting microscope and placed on grids for X-ray exposures. Eight samples could be analyzed simultaneously on a single grid. A standard gelatin model was included on each grid.

For the investigation of extractability of S compounds (Table 2) adjacent slices of cartilage were treated as follows: Group 1, no treatment. Group 2, incubation at 37°C for 24 h in unbuffered baths of distilled water to which was added testicular hyaluronidase (20 000 international units/ml) and penicillin (250 units/ml)[14]. Group 3, methylation and desulfation by the method of Kantor and Schubert[15] with incubation at 58°C for 24 h[16]. Group 4, fixation in neutral formalin an extraction with 0.5 molar EDTA at pH 7.4 at 20°C for 16 h. Group 5, extraction with water after heating briefly to 100°C. Adjacent slices of some of the tissues were appraised visually for region and intensity of staining by aqueous toluidine blue[17] and Astrablau[18].

RESULTS

Data obtained by X-ray elemental analysis of gelatin models (Table 1) reveal good agreement between X-ray and chemical determinations on samples containing S in concentrations ranging from 0.7 to 2.5 %. The discrepancy between mean values by the two methods did not exceed 6 % of the gravimetric value of S. The accuracy of the chemical determination is about $\pm$ 5 %.

The X-ray analysis results for untreated cartilage can be compared with previously published data gravimetrically measured in pooled slices of 4 to 6 weeks

Table 1. Results of X-ray S analysis upon models varied in respect to S concentration.

Model	Sulfur concentration		Number of densitometric points	Range of sulfur mass per unit area 10^{-5} g cm^{-2}
	Gravimetric analysis %	X-ray analysis %		
A	0.69	0.73 ± 0.04[a]	25	1–4
B	0.88	0.91 ± 0.04	15	1–5
C	1.09	1.15 ± 0.07	10	1–2
D	1.44	1.49 ± 0.06	14	2–8
E	2.45	2.60 ± 0.04	25	5–8

[a] Standard error of the mean.

old calves in the costal epiphyseal plate[11]. The mean value of X-ray determinations at 6 mm from the bone marrow surface (Group 1, Table 2) 1.33 %, agreed well with the mean value at this site in similar samples estimated by the gravimetric procedure, 1.26 %. The 5-fold reduction in S content caused by hyaluronidase treatment (Group 2) or methylation and desulfation of the current samples (Group 3) measured by X-ray analysis was in accord with histologic observations of Fisher and Lillie[14]. Loss of metachromasia from methylated sections was observed by them for cartilage stained with toluidine blue. Also, methylation of potassium chondroitin sulfate by Kantor and Schubert[15] gave a high yield of potassium methyl sulfate, soluble in methanol hydrochloric acid mixtures which bathed the current samples. A finding not anticipated from previous studies[16]

Table 2. Effect of various extractive procedures upon S content of normal calf costal plates.

Group number	Treatment	Distal hypertrophic zone and provisional calcification			Resting cell zone		
		Sulfur concentration %	No. densitometric points	Range of S per unit area 10^{-5} g cm^{-2}	Sulfur concentration %	No. densitometric points	Range of S per unit area 10^{-5} g cm^{-2}
1	None	2.48 ± 0.15[a]	26	1 –4	1.33 ± 0.10[a]	15	0.2–2
2	Hyaluronidase	0.52 ± 0.07	18	0.2–3	0.41 ± 0.05	12	0.3–1
3	Methylation and desulfation	0.52 ± 0.09	17	0.3–1	0.35 ± 0.07	14	0.2–1
4	EDTA extraction	1.68 ± 0.24	14	1 –4	1.24 ± 0.15	14	1 –3
5	Aqueous extraction	1.17 ± 0.19	14	1 –4	0.90 ± 0.10	8	0.4–1

[a] Standard error of the mean.

was the reduction of S by about one third in the distal hypertrophic zone after formalin fixation and treatment with EDTA for 16 h (Group 4). Less than 10 % of total S was extracted from points in the resting cell layer. Approximately one half the S at points within the distal hypertrophic zone was removed by 96 h water extraction of fresh untreated freezedried cartilage, and approximately one third was removed from the resting cell zone (Group 5). Large standard errors in S analysis of the distal hypertrophic zone corresponded to increased microheterogeneity of dye distribution judged from toluidine blue and Astrablau staining of regions in adjacent slices. The effect was the natural consequence of a large cell size and steep gradient of S concentration between matrix and cell columns. This problem may be overcome by use of differently shaped densitometer beams, by which cells and matrix regions can be separately analyzed.

Visual inspection of stained models showed increasing intensity of staining by Astrablau and greater metachromasia by toluidine blue with increasing S content. Similarly, staining of untreated cartilage by the former dye was shown to increase in intensity progressing from the resting cell zone to the zone of provisional calcification. A weak reaction to Astrablau and slight metachromasia remained in the distal hypertrophic zone of both hyaluronidase-treated and methylated cartilages, while remainder of the tissue registered no stain. Considerable positive staining by Astrablau, heterogeneously located, remained in the tissues after water extraction.

DISCUSSION

Ultramicroanalysis by the present method is particularly advantageous for study of essentially unaltered tissues, using frozen sectioned, freeze-dried material. Demonstrated here (Table 1) is the fact that sulfur concentration can be accurately assessed in homogeneous models and that it is possible to measure wide distributional differences of S content among various histologic areas of cartilage. This was performed in a region representing 3000 μ^3 of tissue containing from 1 to 8×10^{-11} g of S.

In spite of the bulk extraction of S following treatment of cartilage slices with hyaluronidase or the methylating mixture, residual levels of S were high. At a theoretical concentration of 0.15 – 0.20 % S in mammalian collagen[19] only 0.05 – – 0.10 % S would be expected in the current tissues based on levels of hydroxyproline measured in calf costal cartilage slices pooled according to histologic zone[11]. However, in the desulfated and hyaluronidase-treated tissues an average residual of 0.3 – 0.4 % of S was obtained. Since previous studies of calf costal cartilage indicate that at least 90 % of total S in the pooled tissue can be accounted for by SMPS[5], the present discrepancy between theoretical and actual S content following extraction is most likely explained by incomplete extraction of SMPS as well as reduction of total mass by extraction of components lacking S. Loss of metachromasia invoked by the procedures could result from the presence of a variety of degradative products and, of course, provide no index of the total sulfated compounds remaining in the tissue. Astrablau staining at pH 0.2 should theoretically react only with anionic groups which remain dissociated at that hydrogen ion concentration, primarily sulfate[18]. The results of the persistent

staining reaction with Astrablau after water extraction indicate partial desulfation of the matrix and incomplete extraction of sulfated compounds.

Previous absence of quantitative ultramicro methods have left in doubt the integrity of SMPS within histologic slices of cartilage fixed and demineralized by various techniques[20]. The present results prove conclusively that with the use of EDTA under the conditions of the present experiments there were losses of S compounds, probably including SMPS, from certain microscopic areas. These losses could only be detected to a slight and variable degree with the staining procedures and visual inspection. S extraction might be minimized with shorter periods of incubation and reduced pH.

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