

INTRACELLULAR SYNTHESIS OF CHONDROITIN SULFATE

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ABSTRACT

In autoradiograms of slices of costal cartilage, incubated for 4 hours in a salt solution containing S^{35} -sulfate and then washed extensively and dehydrated, about 85 per cent of the radioactivity was assignable to the chondrocytes. From alkaline extracts of similarly prepared slices of cartilage, 64 to 83 per cent of the total sulfur-35 in the slices was isolated as chondroitin sulfate by chromatography on an anion-exchange resin. In view of the estimate that only about 15 per cent of the radioactivity was in the matrix, the isolation of 64 to 83 per cent of the total sulfur-35 as chondroitin sulfate is a strong argument that the chondrocytes are the loci in which chondroitin sulfate(s) is synthesized.

It has been reported that shortly after the administration of S^{35} -sulfate to animals sulfur-35 is demonstrable autoradiographically in the cells of cartilages, the matrix giving a very much weaker reaction (1-3). A similar intracellular localization of sulfur-35 has also been noted after the incubation of cartilage slices in salt solutions containing S^{35} -sulfate (2, 4, 5). For the most part, the sulfur-35 thus visualized does not react as inorganic sulfate (6, 7). Indeed, in the *in vivo* and in the *in vitro* experiments, up to 24 hours, progressively increasing amounts of the isotope have been isolated in association with chondroitin sulfate (8, 9).

The studies cited above strongly suggest that chondrocytes are capable of synthesizing chondroitin sulfate(s). Definitive proof, however, is needed; in none of the studies was an attempt made to compare the amount of sulfur-35 associated with chondroitin sulfate and the amount of the isotope in the cells. As a consequence, the possibility exists that the chondroitin sulfate- S^{35} was present in the matrix and the S^{35} -labeled materials visualized in the cells were precursors, which were eliminated during the isolation and purification of the chondroitin sulfate. Even the observation (1) that sulfur-35 is removed from sections of cartilage during incubation with hyaluronidase, though

strongly suggestive of the presence of chondroitin sulfate in the chondrocytes, might be interpreted otherwise. Again the alternative possibility arises that sulfated oligosaccharides of low molecular weight are removed, for, although the major end product is a tetrasaccharide, a series of oligosaccharides is also demonstrable on incubation of chondroitin sulfate with hyaluronidase (10) and these too can serve as substrates.

MATERIALS AND METHODS

In each of twenty 25 ml Erlenmeyer flasks 200 mg of slices of costal cartilage from calves were incubated for 4 hours in 5 ml of a solution of salts, buffered to pH 7 with phosphate (5) and containing 100 μ c of S^{35} -sulfate.¹

A. Eight of the flasks were then immersed in a boiling water bath for 30 minutes. After cooling, to each of these lots of cartilage slices, still in the medium in which they had been incubated, 10 mg of papain² were added. The flasks were placed in a water bath and were shaken therein slowly for 16 hours at 37°C. Following an immersion for 30 minutes in a boiling

¹ The sulfur-35 was supplied by the Oak Ridge National Laboratory, Tennessee.

² The papain was a suspension of a crystalline preparation in 0.03 M cysteine from Worthington Biochemical Corporation, Freehold, New Jersey.

water bath, the contents of each flask were reincubated for 24 hours with 20 mg of trypsin.³ The cartilage slices were thereby solubilized, except for a few thin flecks of tissue. The latter were removed by filtration, using sintered glass funnels, after the flasks had been heated again for 30 minutes in a boiling water bath and cooled. The filtrates and washes were brought to a volume of 10 ml with water.

A portion of the clear filtrate, equivalent to 40 mg of the wet cartilage slices, was delivered onto a 1 cm by 25 cm column of Amberlite IRA 401 \times 2 resin in the chloride form. The outlet of the chromatographic tube was connected to a jacketed G-M (Geiger-Mueller) tube, Fig. 1, which in its turn was connected to a ratemeter and a recorder. The sulfur-35 was quantitatively eluted in two well separated peaks by the use of a 0.3 N and then a 4 N

per cent solution of monoiodoacetic acid was added. The fluid was then removed and the slices were washed three times with 10 ml of water and soaked overnight in 10 ml of a saturated solution of sodium sulfate. Following a threefold wash, each of 10 ml of water, the cartilage slices were fixed for 48 hours at 25°C in 15 ml of a 10 per cent solution of formalin. The tissue was next dehydrated in graded concentrations of ethanol, initially in 30 per cent ethanol and finally in absolute ethanol.

a. Each of two lots of the cartilage slices was then transferred to xylol. The xylol was subsequently checked for radioactivity. Only a few counts above background were found. The slices of cartilage were embedded in paraffin and sectioned at 7 μ . Coated autoradiograms (12) of deparaffinized sections were prepared.

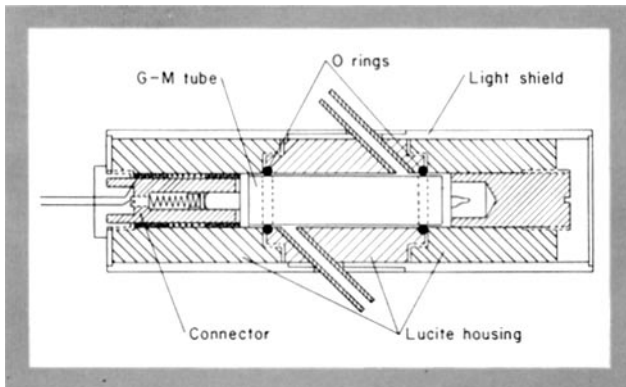


FIGURE 1

Sketch of the Geiger-Mueller tube, in its Lucite housing and light shield, used to detect radioactivity in the effluent from a chromatographic column. Note that the Lucite housing and G-M tube are combined to act as a condenser. The G-M tube was constructed of a perforated cylinder of copper and this was overlaid with a sheet of mica, 1.43 mg/cm². It was constructed by the Nucleonic Corporation of America, Brooklyn, New York.

solution of potassium chloride. These eluting solutions were chosen on the basis of preliminary experiments, which showed that inorganic sulfate could be removed from the column of resin with a 0.3 N solution of potassium chloride, whereas solutions containing much higher concentrations of this salt were required for the elution of chondroitin sulfate. The higher the concentration of potassium chloride the sharper was the peak of radioactivity or of glucuronic acid, the latter determined according to Dische (11), when a well characterized sample of chondroitin sulfate-S³⁵ was used.

Additionally, successive 50 ml fractions of the eluate were collected. The radioactivity in these was determined on aliquots after acid hydrolysis (5). An aliquot of the solution applied to the column was similarly and simultaneously analyzed.

B. To each of twelve of the flasks, after the cartilage slices had been incubated for 4 hours, 1 ml of a 2

³ Trypsin, 2 \times crystallized was purchased from Worthington Biochemical Corp.

b. Each of two lots of cartilage was hydrolyzed in a sealed tube with 20 ml of 6 N hydrochloric acid for 24 hours at 110°C. Aliquots of the filtered hydrolysates were used for a determination of sulfur-35 as barium sulfate (5).

c. Each of eight lots of cartilage slices was homogenized into 5 ml of a 2 per cent solution of sodium hydroxide. The homogenates were set aside at 4°C for 16 hours. Insoluble material was removed by centrifugation and was washed once with 2 ml of water in the centrifuge. The supernatant solution and the water wash were combined and adjusted to pH 7 with glacial acetic acid. After recentrifugation, each supernatant solution was diluted to 10 ml with water. Aliquots equivalent to 80 mg of cartilage were analyzed chromatographically as described under *A.* above.

After the removal of small aliquots for the determination of radioactivity, the remainders of those fractions in which chondroitin sulfate was expected to be present were combined. Such pools from a number of chromatographic runs were in turn pooled

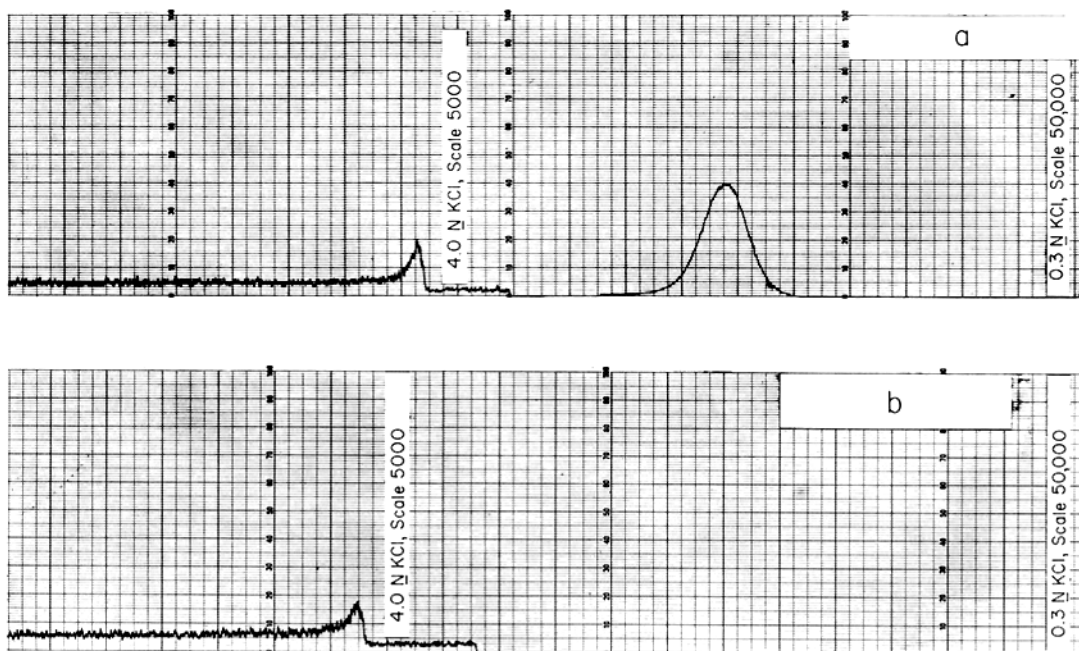


FIGURE 2

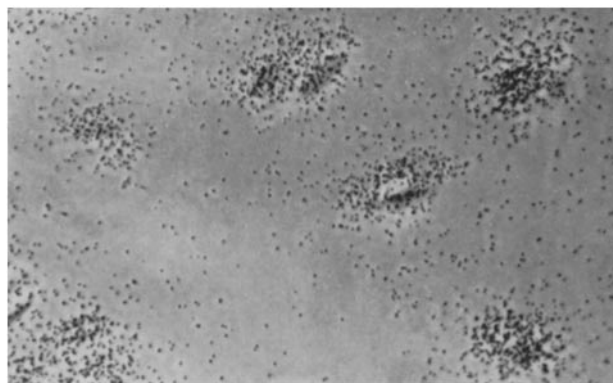
Records of radioactivity in the effluent from a column of Amberlite IRA 401 \times 2, 200 to 400 mesh, 1 cm by 25 cm, in the chloride form.

Record *a* was obtained when an aliquot of an enzymatic digest of slices from a calf's costal cartilage, still in the S^{35} -labeled medium in which they had been originally incubated for 4 hours, was placed on the column. An aliquot equivalent to 40 mg of the slices was used. The larger of the two peaks represents inorganic sulfate, recorded at a setting of the instruments so that full scale corresponds to 50,000 cpm. The smaller peak, chondroitin sulfate, was recorded at a setting of 5000 cpm for full scale.

Record *b* was obtained when an alkaline extract of similarly and simultaneously incubated slices of cartilage was placed on the column of resin. The slices were exhaustively washed, fixed in formalin, and dehydrated with ethanol before extraction. An aliquot of the extract equivalent to 80 mg of the slices was used. The single peak of activity observed represents chondroitin sulfate.

FIGURE 3

A coated autoradiogram of a section from a slice of costal cartilage which had been incubated in a solution of salts containing S^{35} -sulfate. The slice had been exhaustively washed before it was fixed in slightly acidic formalin. Unstained. \times 595.



and dialyzed against running tap water for 3 days. The dialyzed solutions were evaporated to dryness *in vacuo* at 50°C and the residues were dissolved in a minimum volume of water. On the addition of two volumes of 95 per cent ethanol saturated with sodium chloride a white, flocculent precipitate formed. This was isolated by centrifugation, washed twice with 95 per cent ethanol and twice with anhydrous ether. After drying for 48 hours *in vacuo* over calcium chloride at room temperature, the samples were analyzed for their content of hexuronic acid (11), hexosamines (13), nitrogen by a semimicro Kjeldahl

TABLE I
Summary of Radioactivities Found in Slices of Costal Cartilage Incubated with S³⁵-sulfate

Preparation	cpm/100 mg*	Recovered %
Enzymatic digest, total activity (8)	6.50×10^6	100.00
Enzymatic digest, CSA fraction (8)	6.03×10^4	0.93
Dehydrated slices, total activity (2)	3.77×10^4	0.58
Extract of dehydrated slices, CSA fraction (8)	3.41×10^4	0.52

The sulfur-35 in the CSA fractions was determined by analysis of the effluent from a column of Amberlite IRA 401 resin. The number of determinations from which these averaged values and those of total activity are derived are given in parenthesis in column 1. The range of values in no case was greater than ± 6 per cent of the average value. CSA is abbreviation for chondroitin sulfate.

* The radioactivity is given per 100 mg of wet cartilage, weighed before incubation.

method, sulfate-sulfur by a modification of Paulson's method (14), and S³⁵-sulfate (5). Solutions of the samples were also subjected to electrophoretic analysis on paper; autoradiograms were prepared before the papers were stained with an 0.1 per cent solution of toluidine blue in 30 per cent ethanol.

RESULTS

The separation of inorganic sulfate from chondroitin sulfate on a column of Amberlite IRA 401 X 2 resin is shown in Fig. 2 *a*, when an enzymatic digest of slices still in the original incubation medium was used. Similar results were obtained if a mixture of inorganic sulfate-S³⁵ and chondroitin

sulfate-S³⁵ in water was used. On the other hand, in extracts of cartilage slices, washed and dehydrated subsequent to incubation in a medium labeled with S³⁵-sulfate, sulfur-35 was detected only as chondroitin sulfate, Fig. 2 *b*. It is noteworthy that in the extracts about 50 per cent as much sulfur-35 was recovered as chondroitin sulfate as in the enzymatic digests. This is not surprising: the extensive washing with water and the soaking in a saturated solution of sodium sulfate undoubtedly removed some chondroitin sulfate from the slices. It has been shown that water will extract protein-polysaccharide complexes from comminuted cartilage (15, 16).

TABLE II
Analyses of Chondroitin Sulfate Samples
Values are given as per cent of dry weight.

	From enzymatic digest of cartilage slices	From NaOH extract of dehydrated cartilage slices
Nitrogen	3.08	2.92
Sulfate-sulfur	4.46	4.49
Hexuronic acid	35.00	34.70
Hexosamine	30.50	28.80

Autoradiograms of slices after washing and dehydration showed that the sulfur-35 was primarily localized in the chondrocytes, Fig. 3. As estimated by grain counting, about 85 per cent of the sulfur-35 in the slices was present in the cells.

It is pertinent then that of the total activity (3.77×10^4 cpm per 100 mg of wet cartilage) present in the washed, formalin-fixed, and dehydrated slices about 90 per cent (3.4×10^4 cpm per 100 mg wet cartilage) was recovered from the column of resin in the effluent fractions which were expected to contain chondroitin sulfate, Table I. Moreover, of the total radioactivity placed on the column of resin, 64 to 83 per cent of the radioactivity was isolated as chondroitin sulfate from such effluent fractions. The analytical data on one such sample are given in Table II. On electrophoretic analysis these samples did migrate like chondroitin sulfate. Furthermore, the autoradiograms of the electrophoretograms showed no other radioactivity to be present but that directly assignable to chondroitin sulfate, Fig. 4.

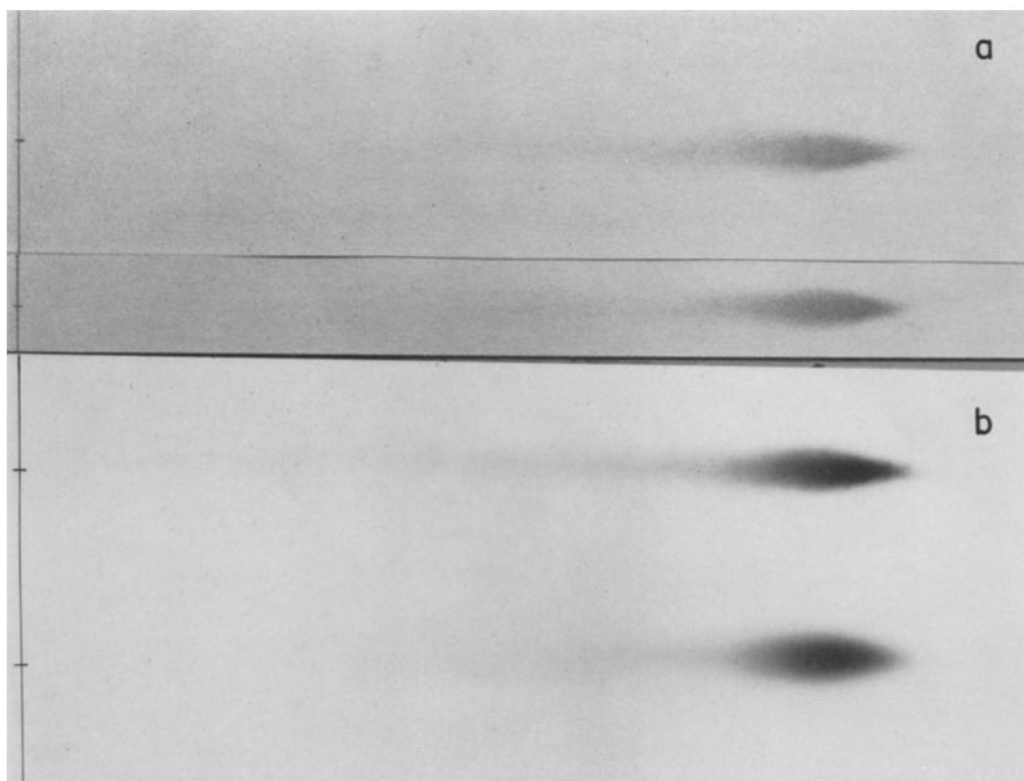


FIGURE 4

An electrophoretogram *a* and its autoradiogram *b*. The sample of chondroitin sulfate was one isolated by chromatography from an alkaline extract of slices of cartilage incubated for 4 hours in a solution of salts containing S^{35} -sulfate. Electrophoresis was in 0.03 M sodium citrate buffer, pH 5.9, on Whatman 3 MM paper at 600 v for 4 hours. The paper was stained with toluidine blue in 30 per cent ethanol after the autoradiogram had been prepared.

DISCUSSION

From separate studies on the turnover of S^{35} -sulfate in cartilages as determined by autoradiography and as determined by assay of S^{35} in isolated samples of chondroitin sulfate it had been inferred that chondroitin sulfate is synthesized intracellularly. But, as pointed out in the introduction, such an inference might not be warranted; no direct correlation between concentrations of intracellularly localized sulfur-35 and isolated chondroitin sulfate- S^{35} had been made. In the experiments described herein this has been done. It was found, by grain counting of autoradiograms, that of the

total sulfur-35 present in slices of cartilage, following extensive washing and dehydration, about 85 per cent was associated with chondrocytes. Furthermore, of the total radioactivity present in such slices about 90 per cent was characterized chromatographically as in chondroitin sulfate. Indeed, 63 to 84 per cent of the total sulfur-35 in the slices was isolated in reasonably well characterized samples of chondroitin sulfate.

On the basis of these data it is reasonable to conclude that chondroitin sulfate is indeed synthesized in chondrocytes.

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