THE OCCURRENCE OF INTRACELLULAR CHONDROITIN SULFATE

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ABSTRACT

Suspensions of chondrocytes were prepared by treatment with trypsin of the epiphyses of tibias and femurs of 13-day-old chick embryos. After washing to remove the matrix, such suspensions readily incorporate radioactive sulfate into both intracellular and extracellular chondroitin sulfate. Following disruption of the cells, the cell constituents were fractionated by centrifugation. Fractions obtained from cells incubated for 10 minutes showed a concentration of radioactivity in the material which sediments at 10,000 to 20,000 *g*. At this time the radioactivity of the extracellular chondroitin sulfate is low, but at 1 hour the radioactivity of the intracellular material is relatively unchanged, while that of the extracellular polysaccharide is markedly increased. Following incubation of the chondrocyte suspensions in a tissue culture medium, the intracellular chondroitin sulfate was isolated. This was compared with chondroitin sulfate isolated from the cartilage matrix. Chemical analysis and infrared spectroscopy indicated that both the intracellular and extracellular polysaccharides consist of a mixture of chondroitin sulfuric acids A and C. A portion of the chondroitin sulfate is not sulfated.

Previous investigations have demonstrated that following parenteral administration of S^{35}-sulfate to animals, radiosulfate may be demonstrated within the cells of cartilage by radioautography (3, 10, 17, 18). Similar observations have been made following the incubation of cartilage slices in a medium containing radioactive sulfate. This localization of radiosulfate has been taken as a measure of intracellular synthesis of chondroitin sulfate or some precursor of this substance. However, radioautographic or histochemical studies do not permit definitive identification of the S^{35}-containing material. To overcome this objection, Dziewiatkowski (7) has compared intracellular radioactivity with that of chondroitin sulfate isolated from cartilage slices previously incubated for 4 hours in the presence of S^{35}-sulfate. Since radioautography indicated that 85 per cent of activity was localized in the chondrocytes, and since 64 to 83 per cent of the radioactivity was recovered in the isolated chondroitin sulfate, it was concluded that chondroitin sulfate was synthesized intracellularly.

Despite the indirect nature of the evidence, this conclusion is reasonable in view of the extant knowledge of the biosynthesis of acid mucopolysaccharides. It is unlikely that the enzymes for the complex series of steps essential for polysaccharide synthesis are present in the matrix. The energy requirements of this biosynthetic system militate against the reactions proceeding far removed from the energy-generating systems of the cell.

The pathway of biosynthesis of hyaluronic acid in Group A streptococci has been elucidated (11). The enzyme responsible for the final polymeriza-

tion of the requisite uridine nucleotides has been localized in the protoplast membranes (12). A similar enzyme has also been demonstrated in an extract of embryonic rat skin (20).

Although it has been assumed that biosynthesis of other acid mucopolysaccharides follows similar pathways, formation of the chondroitin sulfates in a cell-free system has not yet been demonstrated. Since the report by D'Abramo and Lipmann (5), a number of investigators have demonstrated the transfer of sulfate from 3′-phosphoadenosine-5′-phosphosulfate to various polysaccharide acceptors (1, 21–23). Whether sulfation always occurs after polysaccharide formation within the cell remains an unsettled question.

In order to obtain a better appreciation of the synthesis of matrix by cartilage cells, a method was devised for the study of matrix formation by cartilage cell suspensions. It is the purpose of this first communication to describe the preparation of such suspensions and the characterization of the intracellular and extracellular chondroitin sulfate of such suspensions.

MATERIALS AND METHODS

Freshly laid eggs from white Leghorn chickens were obtained commercially and incubated at 38°C for 13 days. Usually 20 to 30 per cent of the eggs were sterile or contained dead embryos. Femurs and tibias, dissected free of adherent connective tissue and muscle, were immediately immersed in ice-cold Puck's solution. This solution is a synthetic protein-free tissue culture medium consisting of Puck's medium N_{10} and 56 per cent Puck's saline F_4, sulfate-free (19). It consists of a mixture of amino acids, inorganic salts, vitamins, cofactors, carbohydrate intermediates, and antibiotics. Serum and embryo extract were omitted to avoid contamination with polysaccharides and to prevent multiplication of cells. In order to obtain suspensions of cartilage cells, trypsin was used to remove matrix, as described by Moscona and Moscona (15). The cartilaginous portions of the bones were separated from the bony shafts and the cartilage diced into 1 mm segments. The segments of cartilage from 60 embryos (in some experiments, a larger number of embryos were used) were placed in 1 per cent trypsin (Pentex, Inc., Kankakee, Illinois, one time recrystallized) dissolved in the tissue culture medium, and the mixture was manually disrupted in a loose fitting Potter-Elvehjem homogenizer. The suspension of segments was incubated at 37° for 90 minutes in a Dubnoff shaker with continuous gassing with 95 per cent oxygen–5 per cent carbon dioxide. Three additional disruptions of the cartilage were carried out during the incubation period. The cell suspension was centrifuged in a table model clinical centrifuge at top speed for 5 minutes and the supernatant solution was discarded. The cell pellet was washed 3 times with freshly gassed culture medium. The second and third washings were free of uronic acid, indicating that the matrix had been thoroughly removed. Contaminating bone fragments were removed from the cell suspension by centrifugation at very low speed in the clinical centrifuge for 1 minute.

In certain experiments, 100 mg of crystalline soy bean trypsin inhibitor (Worthington Biochemicals Corporation, Freehold, New Jersey) were added to the cell suspension during the washing procedure and allowed to react for 5 minutes. One mg of inhibitor was also added to the contents of each flask prior to incubation.

Uronic acid was determined by the carbazole method of Dische (6). Hexosamine was determined by the Boas modification of the Elson-Morgan reaction omitting the resin treatment (2). Nitrogen was determined by a colorimetric micro-Kjeldahl method. Sulfate analyses were carried out by the Muir (16) modification of the method of Dodgson and Spencer. Hyaluronidase digestion assay was performed using the method of Mathews *et al.* (13). Infrared spectra were obtained by the use of a Perkin-Elmer spectrophotometer, model 21, with a sodium chloride prism. Pressed discs of KBr containing sample were used. Purified samples of chondroitin sulfate were prepared for infrared analysis by precipitation of the samples from a water-ethanol (1:3) solution by the addition of a few drops of 1 M NaCl. The flocculent precipitate was washed twice with 95 per cent ethanol, absolute ethanol, and ether, and dried in a desiccator.

For metabolic experiments, appropriate aliquots of the chondrocyte suspensions were incubated at 38°C in an atmosphere of 95 per cent O_2 and 5 per cent CO_2. Carrier-free S^{35}-sulfate was used.[1] The amount of S^{35}-sulfate varied in different experiments from 15 to 50 μc. All experiments were internally controlled. No cell counts were performed.

Counting was carried out in a Nuclear-Chicago Corporation (Des Plaines, Illinois) gas flow counter set for 1280 counts.

In experiments designed to localize the radioactivity in cell constituents, the cells were obtained by centrifugation following the incubation period and suspended in 0.25 M sucrose. Cells were disrupted in a Potter-Elvehjem teflon homogenizer at 0°C. Centrifugations were carried out in a Servall automatic superspeed centrifuge and a Spinco preparative ultracentrifuge, Model L, using a size 40 rotor. The cell particulate fractions were washed once (the 600 *g*

[1] S^{35}-sulfate was obtained from the Union Carbon and Carbide Company, Oak Ridge, Tennessee.

pellet was washed three times) with isotonic sucrose to remove contaminating isotope.

For the isolation of chondroitin sulfate from the incubation medium, the supernatant fluid obtained after removal of cells by centrifugation was dialyzed for 24 hours against two changes of 7 liters of 0.07 M Na_2SO_4 and for 48 hours against five changes of distilled water. After concentration of the dialyzed samples, the NaCl concentration was brought to 0.05 M and the chondroitin sulfate was precipitated by the addition of aqueous cetylpyridinium chloride (CPC). The resultant precipitate was washed with 0.1 per cent CPC in 0.03 M NaCl and then dissolved in a minimal volume of 2.0 M NaCl. Occasionally, when not all of the precipitate dissolved in the 2.0 M NaCl, the addition of methanol to 20 per cent resulted in complete dissolution. Preparations were reprecipitated to constant specific activity.

For the isolation of chondroitin sulfate of the matrix, the supernatant solutions following trypsin treatment from several experiments were pooled and exhaustively dialyzed. The solution was made 0.5 M with respect to NaOH by the careful addition of 4 M NaOH. After exposure to 0.5 M NaOH for 6 hours at 0°C, the solutions were redialyzed to remove the NaOH. This material was treated with 3.0 mg of papain (twice recrystallized, Worthington Biochemicals Corporation) for 48 hours at 60°C in 0.1 M sodium acetate buffer, pH 5.5, containing 0.00428 M cysteine-HCl and 0.00636 M ethylenediaminetetraacetic acid. Following digestion, trichloracetic acid was added to a concentration of 10 per cent, the samples were allowed to stand for 30 minutes at 0°C, and the protein precipitate was removed by centrifugation. After exhaustive dialysis against distilled water, the solutions were filtered through a pad of Celite and concentrated *in vacuo* at room temperature.

For identification of the intracellular chondroitin sulfate, cartilage cells were isolated as described above from 525 chick embryos. Following incubation in Puck's solution for 2 hours at 37°C, the cells were disrupted by homogenization in the cold in a Potter-Elvehjem homogenizer. The larger particles were removed by centrifugation in a clinical centrifuge and the supernatant solution was treated with NaOH, papain, and trichloracetic acid. The polysaccharide was twice precipitated with CPC and ethanol. Finally, an aqueous solution was further purified by passage over a column of Dowex 50 W-X8 (200–400 mesh) in the hydrogen form. This treatment removes contaminating nitrogenous materials.

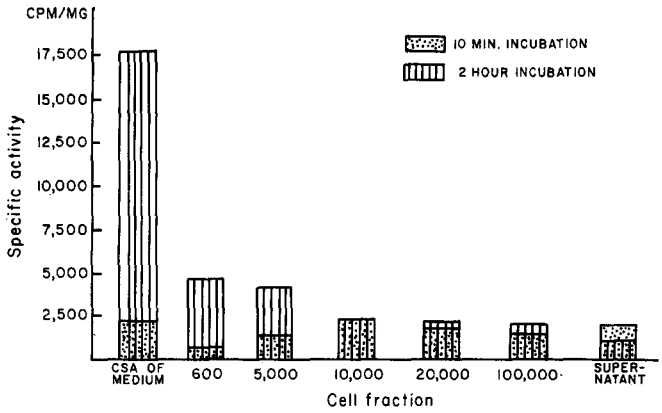

FIGURE 1

Distribution of radioactivity in cell fractions: Fractions are designated by number of *g* at which pellet was obtained. 5,000 *g* and 10,000 *g* centrifugations were carried out for 30 minutes, while 20,000 *g* and 100,000 *g* centrifugations were carried out for 1 hour.

RESULTS

Cell suspensions prepared in the fashion indicated have been used for a number of metabolic studies in this laboratory, and details of the results of these experiments will be published elsewhere. It has been demonstrated that such suspensions readily incorporate radioactive sulfate into chondroitin sulfate and that such incorporation can be demonstrated in the absence of evidence of synthesis of the polysaccharide chain. These experiments suggested that sulfation of a preformed pool of polysaccharide may occur. For this reason an attempt was made to localize the intracellular polysaccharide by cell fractionation following incubation with radiosulfate.

Cartilage cell suspensions were incubated with radiosulfate for 10 minutes and 2 hours. Controls

in which the cell suspension was incubated with S^{35}-sulfate at 0°C showed no significant incorporation of S^{35}. The specific activities (counts per milligram of fraction) of the various fractions are indicated in Fig. 1. These data indicate that, at 10 minutes, a maximum labeling is observed in the 10,000 and 20,000 *g* pellets. It is to be noted that these fractions, although crude and undoubtedly containing many extraneous substances, had a higher specific activity (activity per milligram of solids) than did the purified chondroitin sulfate which had been secreted into the medium. With longer incubation, the intracellular fractions do not increase in radioactivity, in contrast to the marked increase of radioactivity of the extracellular chondroitin sulfate.

These findings suggested that a sufficient pool of intracellular chondroitin sulfate was probably present to permit the direct isolation of this material and its chemical identification. Such an isolation was carried out by the procedure already detailed. In Table I the analyses of the material isolated from cartilage cells are compared with those of a sample isolated from the matrix. Neither preparation was free of protein as indicated by some excess of nitrogen. In both cases the low sulfate content indicated the presence of material containing less than one sulfate per disaccharide unit. The infrared spectra indicated that these samples were mixtures of chondroitin sulfate A(CS-A) and chondroitin sulfate C(CS-C). The A_{800}/A_{850} for CS-A is 0.70 and for CS-C is 1.80.

The method of Mathews and Inouye (14) was applied to both samples to quantitate the amounts of the two chondroitin sulfate isomers present. This method depends upon the fact that *N*-acetyl hexosamines substituted in the 4 position do not give the Morgan-Elson color reaction in contrast to 6 substituted compounds. Since CS-A contains a 4-sulfate and CS-C contains a 6-sulfate, the oligosaccharides obtained by digestion of the two polysaccharides with testicular hyaluronidase may be distinguished by this method. Non-sulfated polysaccharide (chondroitin) will react as CS-C in this method.

TABLE I

Analyses of Chondroitin Sulfates of Embryonic Chick Cartilage

	Hexosamine	Uronic	Sulfate	N	Infrared spectrum
	*molar ratio**	*molar ratio**	*molar ratio**	*molar ratio**	A_{800}: A_{850}‡
Intracellular	1.00	1.09	0.87	1.75	1.08
Extracellular	1.00	0.90	0.78	1.70	1.38

* All analyses are expressed as molar ratios with hexosamine taken as 1.00.

‡ A_{800}/A_{850} for CS-A and CS-B is 0.70 and for CS-C it is 1.80.

TABLE II

Chondroitin Sulfates of 13 Day Chick Epiphyseal Cartilage

	CS	CS-A	CS-C
	per cent	*per cent*	*per cent*
Intracellular	13	36	51
Extracellular	22	30	48

Table II indicates the relative amounts of chondroitin sulfates present in the intra- and extracellular material. The CS-C content was calculated by subtraction of the amount of non-sulfated material which was determined by sulfate analysis. The differences between the intra- and extracellular material are probably within the limits of precision of the methods employed.

DISCUSSION

The pathway of biosynthesis of hyaluronic acid in Group A streptococci is now well delineated, and it has been shown that the presence of an enzyme involved in the polymerization of the uridine nucleotides is likely, but has not been isolated in soluble form (11). A number of investigators, however, have demonstrated the presence of an enzyme responsible for the transfer of sulfate from 3′-phosphoadenosine-5′-phosphosulfate to chondroitin sulfates. The weight of evidence indicates that preformed polysaccharide may be sulfated, but there is yet no conclusive evidence to rule out sulfation of a smaller intermediate. A non-sulfated polysaccharide might

serve as precursor of either CS-C or CS-A. The data presented in this paper indicate that cartilage cells of 13-day-old chick embryos contain a mixture of both CS-A and CS-C, as well as non-sulfated chondroitin. These data do not permit a decision as to whether the low sulfate content is due to contamination with non-sulfated chondroitin or the presence of unsulfated positions in some of the polysaccharide molecules. Further fractionation studies are in progress to decide between these possibilities. The incorporation of radioactivity into sedimentable cell fractions is in keeping with the electron microscope studies of Godman and Porter (8), showing the accumulation of large vesicles of material which are discharged from the cell. It seems likely the vesicles may contain the chondroitin-protein complex. Further studies are necessary to more clearly define the interrelationships of the synthesis of the polysaccharide and protein components. Gross *et al.* (9) showed that turnover rates of the protein-polysaccharide complex of rat costal cartilage are similar when measured with $S^{35}O_4$ and C^{14}-labeled lysine. More recently, Campo and Dziewiatkowski (4) have shown the parallel in incorporation of $S^{35}O_4$ and radioactive amino acids in slices of calf cartilage.

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