# INCREASED FIXATION OF SULFUR$^{35}$ BY CARTILAGE IN VITRO FOLLOWING DEPLETION OF THE MATRIX BY INTRAVENOUS PAPAIN*

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In a recent study on the uptake of radioactive sulfur-35 ($S^{35}$) by human articular cartilage *in vitro*, it was found that the chondrocytes retained the ability to synthesize chondroitin sulfate, both in normal cartilage and in cartilage from joints affected by severe degenerative arthritis (osteoarthritis). Indeed, the findings indicated that the rate of synthesis was greater than normal in osteoarthritic cartilage (1). There is histological evidence of a loss of ground substance from the fibrillated areas of osteoarthritic cartilage (2–4), and increased fixation of $S^{35}$ by chondrocytes in these areas has been demonstrated by autoradiographic techniques (1, 5). That the histological changes do in fact represent a loss of chondroitin sulfate has been confirmed by chemical analysis (4, 6).

The foregoing observations may be interpreted in two ways. It is possible that a defect exists in the synthesis of chondroitin sulfate in osteoarthritis which results in a failure to fix the sulfated material in matrix. This would explain the simultaneous occurrence of a normal or increased fixation of $S^{35}$ with a diminished amount of chondroitin sulfate in matrix. Alternatively, the primary defect may be in the matrix itself, with a failure to retain normal chondroitin sulfate. It is conceivable that the consequent depletion could act as a stimulus for the synthesis of increased amounts of chondroitin sulfate by chondrocytes.

The demonstration by Thomas in 1956 (7) that widespread, reversible depletion of cartilage matrix could be produced in rabbits by the intravenous administration of crude papain provided an experimental situation in which to explore the hypothesis that depletion of matrix can stimulate increased syn-

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thesis of chondroitin sulfate. It has been demonstrated that the loss of basophilic staining of the matrix seen after papain represents an enzymatic breakdown of preformed chondromucoprotein (C.M.P.) with the release of large amounts of chondroitin sulfate into the circulation (8–10).

In the present investigation the functional activity of chondrocytes in normal and papain-depleted rabbit cartilage has been studied by measuring the fixation of $S^{35}$ as sulfate *in vitro*.

## *Material and Methods*

*Rabbits.*—Male hybrid albino rabbits of approximately 1 kilo were used.

*Papain.*—Crude, dried papaya latex was generously donated by Wallerstein Labs., Inc., New York. Solutions were prepared for injection by grinding the latex to a fine powder, and dissolving this in phosphate buffer, 0.1 M, pH 7.0, in a concentration of 10 mg. of the powder/ml. Insoluble components were removed by paper filtration, and the solutions contained approximately 4 mg. protein/ml.

*Sulfur*$^{35}$ was obtained as sodium sulfate in water and was added to the Tyrode's solution used for incubating cartilage in amounts sufficient to provide a level of radioactivity of approximately 0.5 microcuries/ml.

*Radiochemical Technique.*—The amount of sulfur$^{35}$ taken up by cartilage *in vitro* was measured using a modification of the method described by Layton (11, 12).

After incubation for 3 hours at 37°C. in the solution containing $S^{35}$, the cartilage slices, prepared as will be described, were rinsed with ice-cold isotonic (2.5 per cent) sodium sulfate. They were then immersed in four successive changes of isotonic sodium sulfate over a period of 36 hours at 4°C. The adequacy of this procedure to remove unfixed $S^{35}$ was confirmed by the complete removal of the isotope from cartilage heated to 100°C. prior to incubation.

Excessive moisture was removed from the washed slices of cartilage by blotting. Accurately weighed samples were digested by gentle heating in perchloric acid (70 per cent, 1 ml.) to which was added hydrogen peroxide (30 per cent, one drop). The solutions were then transferred to large (50 ml.) centrifuge tubes and distilled water added to a volume of 40 ml. After addition of bromphenol blue (0.1 per cent, one drop), sufficient NaOH was added to produce a definite blue color. Hydrochloric acid (0.1 M) was then added dropwise until the indicator showed the first change to a yellow color (approximately pH 3.0).

The centrifuge tubes were placed in a water bath maintained at 95°C. and after addition of sodium sulfate (5.0 ml., 0.025 M), barium chloride (3 ml., 0.5 M) was added, this being sufficient to precipitate all of the sulfate as the barium salt. The solutions were maintained at 95°C. for 2 hours, and then were cooled to room temperature. After centrifugation at 2,000 R.P.M., the precipitates of barium sulfate crystals were washed once with distilled water and then resuspended in 70 per cent ethanol. The precipitates were deposited uniformly on Millipore filter discs (diameter of disc 22 mm., diameter of filter area 18 mm.), by forced filtration. The discs were dried for 12 hours at 37°C.

The beta activity of the precipitates was counted using a Geiger-Muller tube (Tracerlab TGC-2, mica end window, 1.3 mg./cm.$^2$), and the uptake of $S^{35}$ was calculated as C.P.M./mg. wet cartilage.

*Histology.*—Pieces of costal cartilage 1 to 2 mm. in length were incubated as above in Tyrode's solution containing $S^{35}$, and were fixed in 10 per cent buffered formalin and imbedded in paraffin. Sections were cut at 5 $\mu$, and were either stained with hematoxylin and eosin, or used to prepare autoradiographs by the stripping film technique of Doniach and Pelc (13), with Kodak AR 10 stripping film.

*Chondroitin Sulfate.*—In the period 24 to 48 hours after the administration of crude papain to rabbits, there is a substantial increase in the amount of chondroitin sulfate in blood, representing material released from cartilage matrix (8). In order to confirm that this effect had subsided prior to removal of cartilage for measurement of $S^{35}$ uptake, sera from the papain-treated rabbits were tested by a method recently described (10) using hexamminecobaltic chloride.

TABLE I

*Uptake of $S^{35}$ by Normal and Depleted Cartilage*

| Rabbit No. | Uptake of $S^{35}$ expressed as counts/minute/mg. of wet costal cartilage | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 11 days after i.v. crude papain | | | | Controls | | | |
| | Sample of right cartilage | | Sample of left cartilage | | Sample of right cartilage | | Sample of left cartilage | |
| | A | B | A | B | A | B | A | B |
| 1 | 180 | 191 | 155 | 169 | 105 | 102 | 198 | * |
| 2 | 196 | 235 | 114 | 111 | 69 | * | 65 | 75 |
| 3 | 106 | 142 | 157 | 148 | 74 | 106 | 69 | 103 |
| 4 | 98 | 133 | 130 | 197 | 59 | 47 | 72 | 49 |
| 5 | 117 | 137 | 104 | 101 | 110 | 108 | 65 | 77 |
| 6 | 137 | 121 | * | 155 | 117 | 167 | 106 | 140 |
| 7 | 143 | 162 | 154 | * | 113 | 143 | 119 | 100 |
| 8 | 189 | * | 110 | 174 | 70 | 73 | 52 | 54 |
| 9 | 144 | 157 | 143 | 129 | | | | |
| Total No. of rabbits | 9 | | | | 8 | | | |
| No. of samples | 33 | | | | 30 | | | |
| Mean uptake | 146 | | | | 94 | | | |
| Pilot study: Ear cartilage | | | | | | | | |
| No. of rabbits | 5 | | | | 5 | | | |
| No. of samples | 9 | | | | 10 | | | |
| Mean uptake | 49 | | | | 36 | | | |

* Samples discarded for technical reasons.

## EXPERIMENTAL AND RESULTS

Ten rabbits received an injection of 1.4 ml. of a solution of crude papain freshly prepared as described, into the marginal ear vein. Ten control rabbits received no injection. All of the ten papain-treated rabbits subsequently developed characteristic collapse of the ears indicating depletion of the matrix. One treated rabbit and two controls succumbed to intercurrent infection. Eleven days after the injection the survivors were killed by intracardiac nembutal. None of the dialyzed sera obtained from the papain-treated animals at this point

showed an increase of more than 0.035 in O.D. at 615 mμ after addition of hexamminecobaltic chloride, this being well below the upper limit of normal previously reported (10).

Immediately following sacrifice, the anterior thoracic cage of each rabbit was removed. While being prepared for incubation in the medium containing $S^{35}$, the specimens were kept at 4°C. under gauze moistened with Tyrode's solution. The perichondrium and surrounding tissues were removed from the 5th and 6th costal cartilages on each side, and these were then cut transversely into slices of approximately 0.5 mm. The slices of cartilage from each side of the animals were pooled separately for incubation. From the 4th costal cartilages slices of 1 to 2 mm. were obtained for histological and autoradiographic purposes.

Following incubation in the radioactive medium the pools of cartilage were washed and from each pool two samples of approximately 20 mg. were taken. After these were weighed, barium sulfate precipitates were prepared as described above.

TABLE II

*Uptake of $S^{35}$ by Cartilage in Vitro: Analysis of Variance in 63 Samples*

| Source of variation | D.F.* | Sum of squares | Mean squares | F. |
|---|---|---|---|---|
| Between papain-treated group and control group | 1 | 41,039 | 41,039 | 19.5‡ |
| Between rabbits in same group | 16 | 33,567 | 2,098 | 2.4§ |
| Between right and left cartilage in same animal | 1 | 1,650 | 1,650 | 1.9 |
| Between successive samples of same pool of cartilage | 1 | 1,591 | 1,591 | 1.8 |
| Residual | 43 | 37,620 | 875 | |
| Total | 62 | 115,475 | | |

* D.F., degrees of freedom.

‡ Significant at 1 per cent level.

§ Significant at 5 per cent level.

Of the 68 precipitates thus obtained for counting, 5 were technically unsatisfactory and were discarded. The uptake observed in 33 samples of cartilage from papain-treated rabbits and 30 samples from controls is presented in Table I as C.P.M./mg. The average uptake of $S^{35}$ by cartilage obtained from rabbits 11 days after an injection of crude papain was 50 per cent greater than the uptake by control cartilage. Analysis of the variance (Table II) showed that this difference is highly significant. Differences between individual animals in the uptake of $S^{35}$, irrespective of previous treatment, were also significant, but the differences between samples of cartilage from the same side of the same animals, and from opposite sides of the same animal, were small and non-significant.

Histologic examination of the hematoxylin and eosin sections of cartilage from the papain-treated animals showed, in comparison with the control sections, well marked loss of basophilic staining of the matrix (Fig. 1, *a* and *b*). At

this stage after the injection of crude papain, a basophilic rim around the chondrocytes confirmed that replacement of the matrix was taking place. The ears had regained in the gross normal rigidity approximately 96 hours after the injection of papain.

Autoradiographs prepared from normal and depleted cartilage following incubation in $S^{35}$ showed that the retained radioactive material was present mainly within the chondrocytes (Fig. 1 *c*).

## DISCUSSION

From the data presented in this report, it is evident that, 11 days after *in vivo* administration of crude papain, rabbit costal cartilage fixed increased amounts of $S^{35}$ *in vitro*. In pilot experiments which are not described in detail it was found that ear cartilage also fixed increased amounts of $S^{35}$ after depletion (Table I). Previous studies have shown that, following depletion, the normal rigidity of the ears returns within a few days in rabbits which have received crude papain. However, normal histologic appearances of this and other cartilage may not be restored for as long as 3 weeks after depletion by this agent (14). On this basis it is likely that the increased uptake of $S^{35}$ measured 11 days after papain coincided with active regeneration of the ground substance of matrix, and this was confirmed by the appearance of basophilic material surrounding the chondrocytes. Moreover, the cessation of enzymatic breakdown of the matrix was confirmed by the absence of increased amount of cobalt-precipitable material in the sera.

There is much evidence that the *in vitro* fixation of $S^{35}$ by cartilage depends upon vital processes directly involved in the synthesis of chondroitin sulfate. For example, autoradiographic studies have shown that the uptake of sulfate by cartilage *in vivo* and *in vitro* is a function of intact chondrocytes which can be used as a test for viability of the cells (15, 16).

It has been established that *in vitro* uptake of $S^{35}$ is inhibited by homogenization of the cartilage, or by freezing and thawing, or heating to temperatures over 43°C. Uptake is also inhibited by incubation in an oxygen-free atmosphere, and by chemical agents such as cyanide, malonate, mersalyl, cortisone, and many others (17, 18). A small uptake of $S^{35}$ occurs at 4°C. (18); at higher temperatures the amount taken up increases progressively to a maximum at 37 to 43°C. (17). The uptake is also increased by the addition of glucose to the medium (18).

The foregoing clearly shows that the uptake of $S^{35}$ may be influenced by a number of agents. In the present study, the increased uptake of $S^{35}$ by depleted cartilage could represent either an increased uptake by a normal cell population, or else a normal uptake by a relatively increased cell population. In depleted cartilage any loss of volume is small, and is insufficient to account for the observed increase in uptake. Moreover, histological examination of the sections revealed no obvious increase in the number of cells per unit area of

cartilage. This is in agreement with the findings of Thomas (7). For these reasons it is believed that the increased fixation of sulfate by cartilage represents an absolute increase in the incorporation of the isotope by individual cells.

The results of the present study strongly suggest that depletion of cartilage matrix by papain can act as a stimulus for the production of increased amounts of chondroitin sulfate by chondrocytes.

In relation to the increased incorporation of sulfate in depleted areas of osteoarthritic cartilage (1, 5) the findings in the rabbit experiments support the interpretation that the changes in human articular cartilage represent a failure of the matrix to retain normal chondroitin sulfate, with a consequent stimulus to increased activity of the chondrocytes. This interpretation is in keeping with the hypothesis proposed by Bennet, Waine, and Bauer (3) that the proliferative features of osteoarthritis represent an attempt at regeneration. The recent observation of a significant elevation of sulfate in synovial fluid in osteoarthritis supports the concept that there is increased synthesis of sulfur-containing material in this disease with a failure of retention in matrix (19, 20).

The precise mechanisms which regulate the functional activity of the chondrocytes remain unidentified. It is possible that activity may be controlled simply by the influence of the matrix on the diffusion of metabolites to and from the cells. Undoubtedly, the metabolism of the chondrocytes is influenced by corticosteroids (21, 22) growth hormone (23, 24), and other hormones (25). The concept that the matrix itself may exercise a restraining influence upon growth has been recently discussed (26).

On this basis, it is likely that the changes seen in articular cartilage in osteoarthritis could arise from the continuing influence of factors which impair the retention of normal chondroitin sulfate in the matrix. While enzymes present in blood and tissue can cause a breakdown of the mucoprotein of cartilage (27, 28), it is also possible that failure to retain chondroitin sulfate in osteoarthritic cartilage could be due to a disorganization of the fibrillary framework of matrix. As previously reported, one of the earliest histologic features of the disease is a change occurring in relation to the superficial tangential segments of fibers of Benninghoff as described in reference 20. The role which trauma could play in the production of this change is obvious. The demonstration that depletion of the matrix results in an increased metabolic activity of chondrocytes supports the view that hyperactivity of the chondrocytes in damaged cartilage is an attempt at regeneration which fails only because of a continuing defect in the matrix.

## SUMMARY

The uptake *in vitro* of sulfur-35 by costal cartilage obtained from nine rabbits 11 days after an intravenous injection of crude papain solution was compared

with that in costal cartilage from eight normal untreated rabbits. An increased fixation of the isotope was found in treated animals compared with controls.

The depletion of cartilage matrix by papain provided an experimental situation to test the hypothesis that the depletion of matrix which occurs in osteoarthritic cartilage can stimulate increased synthesis of chondroitin sulfate.

The results give further support to the view that the primary lesion in osteoarthritis occurs in the matrix rather than in the chondrocyte of articular cartilage.

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## EXPLANATION OF PLATE 71

FIG. 1, *a* to *c*. × 385.

(*a*) Rabbit costal cartilage showing depletion 11 days after intravenous papain. Basophilic material is present in the areas immediately surrounding the chondrocytes. Hematoxylin and eosin.

(*b*) Normal basophilic cartilage from a control animal. Hematoxylin and eosin.

(*c*) Autoradiograph of depleted cartilage following incubation in medium containing radioactive sulfate. The distribution of the isotope is predominantly cellular.

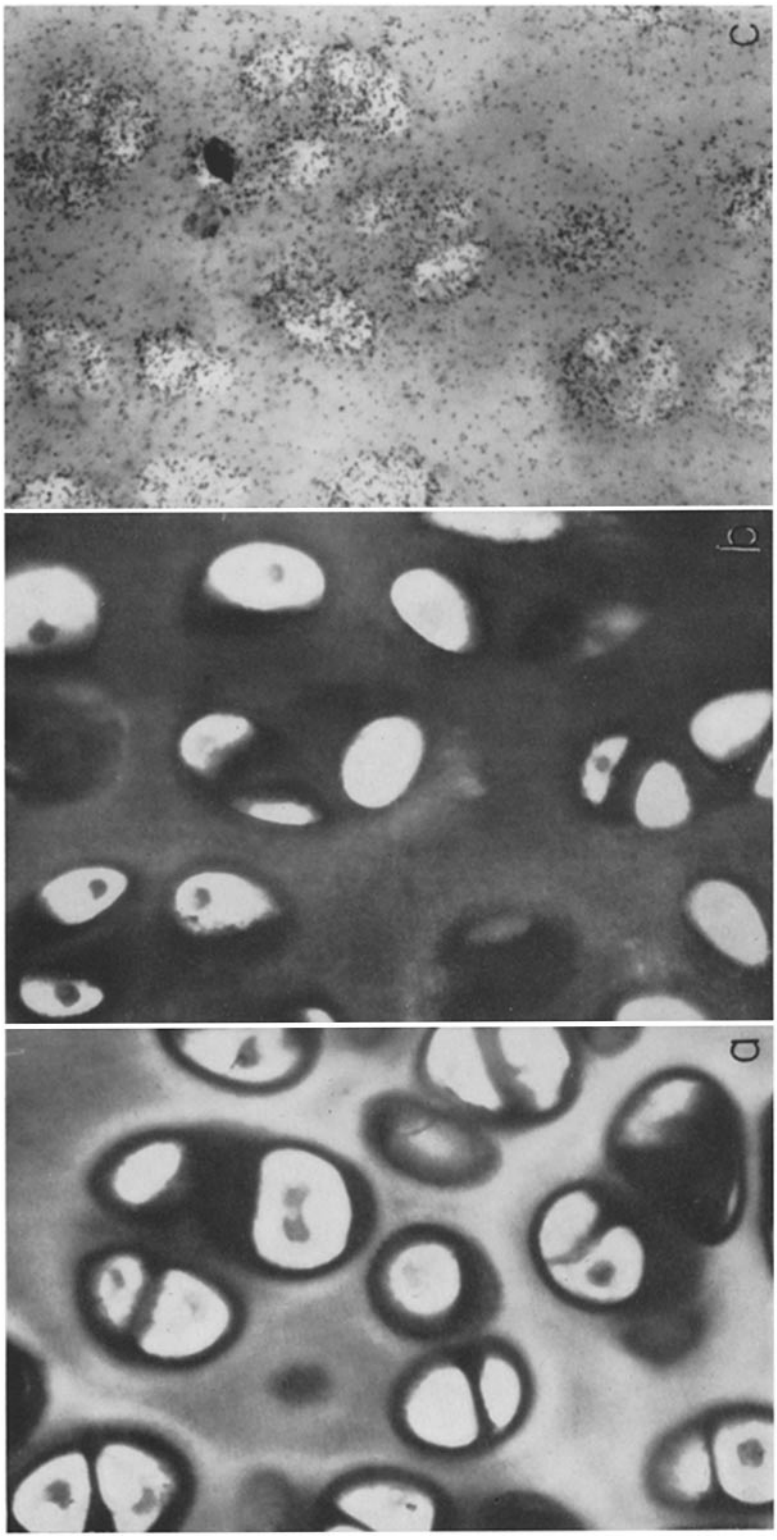


FIG. 1
(McElligott and Potter: Matrix loss and increased chondrocyte activity)