Utilization of S^{35}-Labelled Sodium Sulphate in the Synthesis of Chondroitin Sulphuric Acid, Taurine, Methionine and Cystine

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The high incorporation of S^{35}-labelled sodium sulphate in sulpho-mucopolysaccharides of mammalian tissues has been demonstrated by different workers [1–4]. In several autoradiographic studies [5–8] on the sulphate fixation in different mammalian organs, it was shown that the main uptake of sulphate took place in those tissues, such as cartilage, the aorta, sclera, cornea and intestinal mucosa, which are known to contain large amounts of sulpho-mucopolysaccharides. On the other hand, it is known that S^{35} fed to rats as sulphate does not appear in cystine [9]. These facts thus speak in favour of the opinion that S^{35} administered as sulphate is mainly incorporated as ester sulphate in mucopolysaccharides.

In the present investigation a comparative study was made of the utilization of sodium sulphate in the synthesis of chondroitin sulphuric acid, taurine, methionine and cystine.

Four groups of adult white rats *, each group comprising 20 rats weighing 250–300 g, were given intraperitoneal injections of 1.5 mg of S^{35}-labelled Na_2SO_4 ** in 0.5 ml of distilled water (3.5×10^6 counts per minute per cm^2 measured as $BaSO_4$ at infinite thickness). The different groups of animals were sacrificed 2, 4, 16, and 24 hours, respectively, after the injection. The livers of all the animals in a single group were pooled and likewise the ribs with the intercostal muscles. The material thus obtained was handled in the following way.

* The same animals as those used for previous experiments [3].

** Obtained from *A.E.R.E.*, Harwell, England.

Chondroitin sulphuric acid was prepared from the cartilage of the ribs by applying the method of Jorpes [10] as modified by Strandberg [11]. The details of the method used and the analytical figures of the preparations obtained are given in a previous paper [3].

Isolation of taurine. The livers from each group of animals were minced into alcohol and the pooled livers ground in a Turmix blender (Swiss), filtered, and dried with more alcohol. The material was then extracted for two hours with boiling 3 : 1 alcohol : ether. The alcohol and alcohol-ether extracts were pooled and evaporated to dryness at reduced pressure. The residue was dissolved in a minimum of chloroform; this was poured into a large volume of acetone, and the precipitate filtered off. The material was twice more dissolved in chloroform and precipitated with acetone. The precipitate was then hydrolyzed in boiling 6 *N* HCl for 24 hours. After cooling, the solidified fatty acids were filtered off. The filtrate was evaporated to dryness, and the excess hydrochloric acid removed by repeated evaporations *in vacuo*. The final residue was dissolved in *N* HCl and applied to a column of the cation exchange resin Dowex 50 in the H^+ form. The length of the column was 20 cm and the diameter 4 cm. The separation was carried out with *N* HCl as the eluting solvent. The taurine emerges as a sharp peak very early in the chromatogram. This peak occurs between the solvent front and the peak of the first amino acid (aspartic acid). The fractions containing taurine were pooled and evaporated to dryness. Excess acid was removed by repeated evaporations *in vacuo*. The taurine was then chromatographed on starch, using the solvent system 2 : 1 propanol : 0.5 *N* HCl to check the identity and purity [12]. The taurine was then put through a small Dowex 50 column (20 cm × 0.9 cm) from which it was eluted in *N* HCl in a highly pure form. The identity and purity were further verified by paper chromatography.

Isolation of methionine and cystine. The liver material remaining after extracting the lipid components was extracted twice with 5% trichloroacetic acid (TCA) at 90° C for 15 minutes according to Schneider [13]. The residue was dried with alcohol and ether. About 5 g of this TCA-protein was hydrolyzed by boiling for 24 hours in a solution 6 *N* with respect to HCl and 50 per cent with respect to formic acid. The solution was freed from excess acid by repeated evaporations *in vacuo* and applied to Dowex 50 for the separation of the amino acids [14]. The column was 60 cm × 5 cm. To obtain a better resolution of methionine and

Substance	Radioactivity in counts/min./cm² at different times after injection			
	2 h	8 h	24 h	48 h
Chondroitin sulphuric acid isolated from the costal cartilage	1392	2136	2953	2933
Taurine isolated from the liver	32	68	53	35
Methionine isolated from the liver	—	—	3	—
Cystine isolated from the liver	—	—	4	—

isoleucine, the column was run at 70° C, which resulted in a complete separation of these two amino acids. The fractions corresponding to methionine and cystine, respectively, were pooled and evaporated to dryness. Both were then re-run on small Dowex 50 columns (20 cm × 0.9 cm) from which they were eluted in a few milliliters of 2.5 *N* HCl. No further purification of methionine and cystine was undertaken. As in the case of taurine, the identity and purity were checked by paper chromatography.

The *radioactivity* in the different samples was measured in the following manner. 80 mg of each of the preparations of chondroitin sulphuric acid were hydrolyzed with 6 *N* HCl for 4 hours on a boiling water bath and the sulphate precipitated as $BaSO_4$. In the case of taurine, methionine and cystine, $BaSO_4$ was precipitated after oxidation of about 40 mg of the substance with sodium peroxide according to Bailey. The different samples of $BaSO_4$ were then plated as described elsewhere[3] and measured by means of a Geiger-Müller counter with a 2.1 mg per cm² mica end-window tube. The radioactivity of the samples was expressed as counts/min./cm² at infinite thickness.

The results are given in the table. In agreement with earlier investigations [1,3] the incorporation of S^{35} in the sulphate group of chondroitin sulphuric acid of cartilage was very high after a single injection of labelled sulphate. A very low although significant uptake of S^{35} was demonstrated in taurine, isolated from the liver. The maximum uptake in chondroitin sulphuric acid and taurine was reached in 24 and 8 hours, respectively. As also shown by Tarver and Smith [9], S^{35} administered as sulphate was not utilized in the synthesis of cystine, nor did any incorporation of S^{35} take place in methionine.

The present investigation seems to afford further support to the opinion that the main uptake of administered sulphates occurs in mucopolysaccharides containing ester sulphates.

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Exchange of the Acetyl Group of Chondroitin Sulphuric Acid in Slices of Cartilage

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In an earlier paper[1], we have reported on the exchange of the sulphate group of chondroitin sulphuric acid in slices of cartilage. The object of the present study was to investigate the exchange of the acetyl group by means of the previously described slicing technique, after addition of sodium acetate labelled with C^{14} in the methyl group. A relatively low but definite uptake of C^{14} was demonstrated for both the methyl and the carboxyl groups of the acetic acid.

Fresh costal cartilage from 1 to 3-day-old calves was freed from muscles, connective tissue and perichondrium, and sliced to a thickness of approximately 0.2 mm. The slices were incubated in portions of 10 g with shaking in an oxygen-carbon dioxide atmosphere (93.5 % O_2 plus 6.5% CO_2) at 37° C in Erlenmeyer flasks, each containing 50 ml of Krebs' solution [3], sodium acetate labelled with C^{14} in the methyl group *, and 50 000 I.U. of penicillin. The slices were taken at different times and boiled for five minutes. Sodium salts of chondroitin sulphuric acid were then prepared from all the samples, according to a method described elsewhere [2]. The yield was 200 to 300 mg from each portion of 10 g. The figures obtained on analyzing the different preparations are given in the table.

150 mg of the preparations obtained were hydrolyzed under reflux with 10% by volume of sulphuric acid for three hours at 100° C. The acetic acid was then isolated by distillation. 0.05 *N* NaOH was added to pH 7 (glass electrode control), and the solution evaporated to dryness. The sodium acetate was then degraded according to the method of Phares [4]. After plating of the barium carbonate obtained, employing a filtering technique, the radioactivity of each of the carboxyl and methyl carbons was measured in a Geiger-Müller counter with a 2.1 mg per cm^2 mica end-window tube.

Three different experiments, A, B, and C, were carried out in which 1.6 mg, 0.5 mg, and 1.0 mg, respectively, of sodium acetate labelled with C^{14} in the methyl group and with an activity of 2.5×10^6 c.p.m. per cm^2 (measured as $BaCO_3$ at infinite thickness) were added. The rate of uptake of C^{14} in the methyl and carboxyl carbon of the acetyl group of the chondroitin sulphuric acid was determined. The periods of incubation varied from 0 to 30 hours. In two control experiments the incubation was performed with cartilage slices which had been boiled for five minutes prior to incubation with 1.0 mg of labelled acetate.

The results of the experiments are shown in the figure. Curves A, B, and C represent the uptake of C^{14} in the methyl group of the acetyl group in the chondroitin sulphuric acid as a function of time. Curves a, b, and c denote the corresponding uptake in the carboxyl group. The points D mark the retention in the methyl carbon in the acetyl group of the chondroitin sulphuric acid prepared from slices that had been boiled prior to incubation with the isotope.

It is seen from curves A, B, C, and a, b, c that in slices of calves' cartilage some methyl-labelled sodium acetate is built into the acetyl group of the chondroitin sulphuric acid, the labelled carbon being found both in the methyl group and, although to a lesser extent, in the carboxyl group. If the slices were boiled prior to addition of the isotope, there was no uptake (experiment D).

* Obtained from *The Radiochemical Centre Amersham, England.*