THE EFFECT OF TEMPERATURE ON THE BIOSYNTHESIS OF CHONDROITIN 4-SULPHATE IN CARTILAGE SLICES *IN VITRO*

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1. Introduction

The incorporation of ^{35}S-sulphate into slices of cartilage *in vitro* has been used extensively in the study of the metabolism of acid glycosaminoglycans [1–4].

Measurement of radioactivity in the isolated glycosaminoglycan chains after fractionation with CPC on cellulose [3, 4] or ion exchange chromatography on Ecteola cellulose [1] gives evidence for a marked incorporation into the longest chains. The apparent existence of two populations of chains of different size with different rates of metabolism was further investigated in this study.

2. Experimental

Pig larynges were obtained fresh from the slaughterhouse. The thyroid cartilage was dissected free of perichondrium and other connective tissue, then cut into slices 200–300 μm thick and kept moist with 0.15 M sodium chloride at 4°. The slices were filtered rapidly on a sintered glass funnel and samples of 500 mg were taken for three kinds of incubation:

(a) for direct incubation with radioactive isotope, the slices were added to 2 ml of Krebs-Ringer bicarbonate pH 7.4, with $MgCl_2$ replacing $MgSO_4$, and containing 10 mM L-glutamine and, either 20 mM D-glucose and 20 μCi/ml of carrier free ^{35}S-sulphate or 8 μCi/ml U^{14}C-glucose (309 mCi/mmole) in a shaking water bath at 37°;

(b) in experiments involving preincubation, the slices were incubated in the buffer at 37° before the addition of radioactive isotope;

(c) in chase experiments, the slices were incubated in buffer containing ^{35}S-sulphate at 37° for 10 min, washed on a sintered glass funnel with Krebs buffer containing 1 mM sodium sulphate and transferred to fresh non-radioactive Krebs buffer at 37° containing 1 mM sodium sulphate. At the end of incubation, the tubes were immersed in a boiling water bath for 3 min and then cooled in ice. The slices were filtered from the supernatant on a 3 × 3 sintered glass funnel and washed with saline. About 5–8% of the total uronic acid of the cartilage appeared in the supernatant after incubation. When isolated, it contained very little radioactivity and was discarded.

The glycosaminoglycans were released by proteolytic digestion of the cartilage with papain. The cartilage was suspended in 5 ml of 0.1 M sodium acetate, pH 5.5, containing 50 mM EDTA and 10 mM L-cysteine. Activated crude papain (Koch-Light) (20 mg/g wet cartilage) was added to the suspension, and incubated for 4 hr at 60°. The small insoluble residue remaining after digestion contained no hexosamine and was removed by centrifugation and discarded. After dilution of the digest with an equal volume of distilled water, the glycosaminoglycans were precipitated by dropwise addition of 10% CPC (w/v), the precipitate was centrifuged, washed twice with 0.05% CPC (w/v) and then dissolved in 0.5 ml propan-1-ol. A few drops of saturated sodium acetate were added and then 5 volumes of absolute ethanol. The precipitate was collected by centrifugation at 4°, washed with ethanol and dried *in vacuo*.

Glycosaminoglycans comprise 6% of pig laryngeal cartilage (wet weight), 90% being chondroitin 4-sul-

phate and about 10% keratin sulphate [5]. The hexosamines present in chondroitin 4-sulphate and keratin sulphate are galactosamine and glucosamine, respectively. The CPC isolated preparation used in this study represents more than 95% of the chondroitin 4-sulphate (by uronic acid recovery) and very little of the keratin sulphate, as the molar ratio of galN:glcN was more than 100:1.

For gel chromatography, about 5–10 mg of glycosaminoglycan was dissolved in 0.1 M sodium acetate pH 6.8 and applied to a column (180 cm × 1.5 cm) of Sephadex G-200 which was eluted at room temperature with 0.2 M sodium acetate pH 6.8 at a hydrostatic pressure of 30 cm. 5 ml fractions were collected and their uronic acid contents determined [6] by an automated procedure [7]. 0.5 ml aliquots were added to 10 ml of scintillation liquid (toluene/2-methoxyethanol, (3:2 v/v) containing per litre, 4 g 2,5-bis-(5-tert-butyl benzoxal-2-yl) thiophen and 80 g of naphthalene) and counted in a Packard Tricarb liquid scintillation spectrometer. Counting rates were corrected for quenching using a channel ratio method with the external standard.

3. Results and discussion

In the initial experiments, the cartilage slices were incubated directly in radioactive buffer at 37°. The incorporation of ^{35}S-sulphate into the isolated chondroitin 4-sulphate was linear with time for up to 6 hr, with a slightly slower rate of incorporation over the first hour (fig. 1). The labelled chondroitin 4-sulphate was fractionated by gel chromatography on Sephadex G-200 according to its chain length, as shown by Wasteson [8]. The peak of radioactivity shifted with time from the region of longest chains towards that of shorter chains, and this was most marked for incubations of from 2 to 30 min (fig. 2). The appearance of radioactivity primarily in the region on long chains was in agreement with the results of Kleine and Hilz [1], using Ecteola-cellulose chromatography of labelled chondroitin sulphate from calf costal cartilage, and Rokosová-Čmuchalová and Bentley [3], using CPC-cellulose chromatography of labelled chondroitin sulphate from puppy epiphyseal cartilage. A similar result was also obtained with CPC-cellulose chromatography of our labelled chondroitin sulphate.

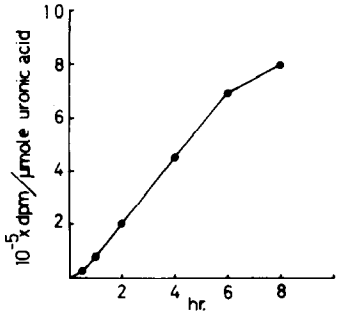

Fig. 1. The incorporation of ^{35}S-sulphate into chondroitin 4-sulphate after various times of incubation.

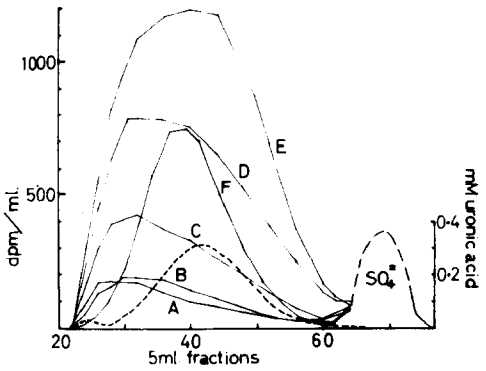

Fig. 2. Gel chromatography on Sephadex G-200 of chondroitin 4-sulphate from cartilage incubated for various times with ^{35}S-sulphate at 37° without preincubation. A) 2 min; B) 5 min; C) 10 min; D) 20 min; E) 30 min; F) 4 hr, scale of radioactivity shown to be muliplied by 20. - - - - -, Uronic acid content of fractions.

A possible explanation was that long chains, labelled initially, were subsequently rapidly reduced in length; however, when the cartilage was incubated for 10 min with radioactive sulphate and then transferred to a non-radioactive medium containing 1 mM sodium and incubated for up to 5 hr, there was no change in the G-200 radioactive profile. This implied that, initially, radioactivity was incorporated into apparently much larger chains than subsequently. This was further demonstrated by incubating the cartilage at 37° for various times prior to the addition of radioactive sulphate (fig. 3). Preincubation for 30 min was sufficient for subsequent radioactive incorporation to be

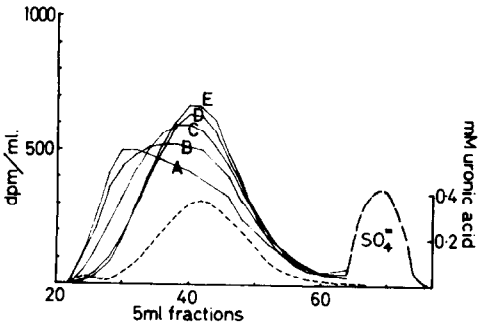

Fig. 3. Gel chromatography on Sephadex G-200 of chondroitin 4-sulphate from cartilage incubated with ^{35}S-sulphate for 10 min at 37°. A) 0 min; B) 5 min; C) 15 min; D) 30 min; E) 110 min. - - - - -, Uronic acid content of fractions.

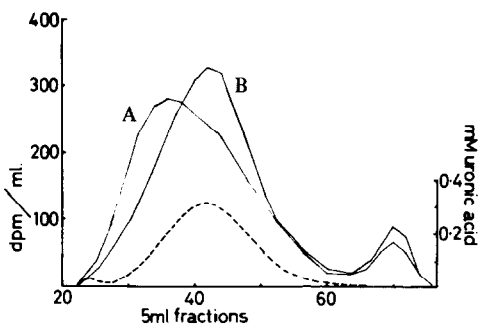

Fig. 4. Gel chromatography on Sephadex G-200 of chondroitin 4-sulphate from cartilage incubated with $U^{14}C$-glucose for 2 hr at 37°. A) with no preincubation; B) with 30 min preincubation at 37°. - - - -, Uronic acid content of fractions.

'normal', i.e. to be distributed more evenly throughout chains of all sizes; longer preincubation had no further effect.

To investigate the cause of the abnormal incorporation the effects of temperature, the type of medium and the availability of substrates were examined (table 1). Only temperature effected the 'abnormal' incorporation since preincubation for 30 min at 37° in all the combinations of medium and substrates tried was sufficient to eliminate the effect, whereas incorporation at 37° immediately after transfer from medium at 4° was always 'abnormal' and was independent of the presence of substrates. Even when the cartilage was transferred from medium at 23° rather than at 4°, the incorporation was 'abnormal', although less markedly so.

The effect of temperature was confirmed by incubating the cartilage with radioactive sulphate for 5 hr at 23° after 1-hr equilibration at this temperature. The distribution of radioactivity was again distorted towards the region of long chains implying that abnormal incorporation was continuous at this temperature.

The incorporation of radioactivity from $U^{14}C$-glucose was also examined by incubating cartilage for 2 hr with and without a preincubation period of 30 min at 37° (fig. 4). The results were similar to those using ^{35}S-sulphate. Without preincubation, the label was incorporated preferentially into the region of long chains, while preincubation altered the distribution of radioactivity towards the region of shorter chain length.

The similarity between the results using ^{35}S and ^{14}C showed that synthesis of apparently abnormally large molecules and not just their selective sulphation had taken place.

The appearance of both ^{14}C and ^{35}S radioactivity in the region of long chains is most readily interpreted as representing the synthesis of long chondroitin 4-sulphate chains, but the amounts synthesised during the incubations are small and the physical and chemical verification of their size has not been shown. It is possible that below physiological temperatures, the attachment of some fragment other than polysaccharide to chondroitin 4-sulphate chains produces the observed results, but the agreement between ion-exchange chromatography on Ecteola cellulose [1], as well as CPC-cellulose chromatography [3, 4] and the present gel filtration results support the former explanation; extended proteolytic digestion did not alter the radioactive chain profile on G-200.

If abnormally long chondroitin 4-sulphate chains are synthesised at below physiological temperatures it may be because factors that govern the termination of chains are particularly sensitive to temperature.

The incorporation profile of ^{35}S observed after preincubation is still somewhat distorted toward longer chains (fig. 3), but there is no indication of two distinct populations of chains in this particular cartilage. The small difference that does exist may result from the radioactive profile representing newly synthesised chains and the uronic acid profile the average

Table
The effect of the buffer and saline, and of temperature and substrates on the incorporation of ^{35}S-sulphate into chondroitin 4-sulphate.

Preincubation				Incubation				Gel chromatography. Profile of radioactivity
Medium	Time (min)	Temp. (°C)	Substrates	Medium	Time (min)	Temp. (°C)	Substrates	
NaCl	30	4	–	Krebs	10	37	+	Abnormal
Krebs	30	37	+	Krebs	10	37	+	Normal
NaCl	30	37	–	NaCl	10	37	–	Normal
NaCl	30	37	+	Krebs	10	37	+	Normal
Krebs	30	37	–	Krebs	10	37	+	Normal
Krebs	60	4	+	Krebs	10	37	+	Abnormal
Krebs	60	4	–	Krebs	10	37	+	Abnormal
Krebs	60	23	+	Krebs	10	37	+	Abnormal
Krebs	60	23	+	Krebs	240	37	+	Abnormal
Krebs	60	37	+ and 1 mM sulphate	Krebs	90	37	+ and 1 mM sulphate	Normal

The medium was Krebs-Ringer bicarbonate as described in the text or unbuffered isotonic saline. Substrates were: 20 mM D-glucose and 10 mM L-glutamine. Profiles of radioactivity were clearly distinguishable into 'normal' as observed after 30 min preincubation (fig. 3D) and 'abnormal', that is distorted towards long chain length as observed for the 10 min incubation (fig. 2C).

of chains of all ages. That new chains may be slightly longer than older chains implies that chain-shortening occurs during their extracellular life. From the molecular weight range reported for chondroitin sulphate on Sephadex G-200 [8], the degree of shortening would require only the removal of on average 2 disaccharide units per chain to explain the observed results.

This study has centred on only one type of cartilage but the marked effect of temperature on the radioactive profile observed on gel chromatography would commend caution in the interpretation of results with tissue from other sources unless no effect of temperature has been demonstrated.

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