The Metabolic Fate of Intravenously Injected Peptide-Bound Chondroitin Sulphate in the Rat

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The degradation of intravenously administered chondroitin sulphate-peptide, obtained by trypsin digestion of rat cartilage preparations labelled in vitro with ^{35}S (and, in some cases, with ^{3}H), was studied in rats. As with free chains of chondroitin sulphate, the major site of accumulation and degradation in the body was the liver, although peptide-linked chains were taken up more rapidly than free chains. In the first 2h after intravenous injection of a chondroitin sulphate-peptide fraction, labelled macromolecular components were excreted in the urine. These were shown to be chondroitin sulphate-peptide of the same degree of sulphation but of smaller average size than the injected material. A similar observation was made when free chains of chondroitin sulphate from the same source were administered intravenously. An isolated perfused rat kidney failed to de-sulphate circulating chondroitin sulphate-peptide, but a component of lower average molecular weight was excreted in the urine. When a chondroitin sulphate-peptide fraction of relatively larger hydrodynamic volume was administered, very little chondroitin sulphate appeared in the urine in the first 2h. It was concluded that, depending on size and/or peptide content, the chondroitin sulphate-peptide released from connective tissues into the circulation would probably be subjected to one of two alternative fates. The smaller fragments are more likely to be excreted in the urine, whereas the larger ones are taken up by the liver and there degraded to inorganic sulphate and undefined carbohydrate components.

Previous work in these laboratories (Wood et al., 1973a,b) has shown that, when single chains of chondroitin 4-[^{35}S]sulphate are injected intravenously into rats, significant amounts are taken up by liver lysosomes and degraded with the release of inorganic [^{35}S]sulphate. The metabolism of intact [^{35}S]proteoglycan in the guinea-pig has been studied by Revell & Muir (1972), who found that more radioactive label was retained in the body than when free chains of chondroitin sulphate were administered, but that some was excreted in the urine in the form of free chondroitin sulphate, presumably after proteolytic degradation. By contrast, in the rat, excretion of chondroitin sulphate was not observed to any extent when proteoglycan from rat xiphoid cartilage was freed from components of lower molecular weight before injection (Wood et al., 1975).

The turnover of chondroitin sulphate in vivo is thought to involve, in addition to intracellular degradation in the connective tissue itself (Amadò et al., 1974), the release of partially degraded proteoglycan molecules into the circulation. These fragments contain more than one chondroitin sulphate chain attached to lengths of common peptide (Wasteson et al., 1972).

The purpose of the present investigation was to determine the sites of degradation and excretion in the rat of chondroitin sulphate-peptide prepared by trypsin digestion of rat cartilage. This form of proteolytic digestion was chosen so as to produce degraded proteoglycan similar to that released normally into the circulation after cathepsin action (Morrison et al., 1973; Woessner, 1973). To follow the fate of carbohydrate moieties, chondroitin sulphate preparations were labelled with ^{3}H (in the galactosamine residue) as well as ^{35}S (in the ester sulphate group).

Materials and Methods

The experimental and analytical techniques were those described by Wood et al. (1973a,b), except for the following:

Measurement of ^{35}S and ^{3}H in mixtures containing both isotopes

The amounts of ^{35}S and ^{3}H in the same sample were determined by liquid-scintillation counting in a Packard Tri-Carb liquid-scintillation spectrometer, model

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3375. The efficiency of counting each isotope in each channel was determined by adding known amounts of inorganic [^{35}S]sulphate and D-[6-^{3}H]glucosamine hydrochloride (The Radiochemical Centre, Amersham, Bucks., U.K.; dissolved in 0.5 ml of 0.2 M-NaCl) to 5 ml of PCS scintillation cocktail (Amersham/Searle Corp., Arlington Heights, IL, U.S.A.) in vials which had been counted previously for background radioactivity. The efficiencies so determined were used to compute the ^{3}H and ^{35}S content (expressed as d.p.m.) of 0.5 ml samples of urine, plasma, bile and column effluent.

Labelling and isolation of chondroitin sulphate-peptide from cartilage

Sliced sections from rat xiphoid cartilage (approx. 1 g) were incubated for 23 h at 37°C in 4 ml of Krebs–Ringer bicarbonate solution, pH 7.3 (Umbreit *et al.*, 1964), containing carrier-free Na$_2$^{35}SO$_4$ (80 μCi/ml) as described by Hardingham & Muir (1972). Sections from rat rib cartilage (approx. 2 g) were similarly incubated in 4 ml of solution containing both carrier-free Na$_2$^{35}SO$_4$ (25 μCi/ml) and D-[6-^{3}H]glucosamine hydrochloride (20 μCi/ml). The washed cartilage sections were extracted with 4 M-guanidinium chloride and intact proteoglycan was isolated as reported by Wood *et al.* (1975). The residue, after washing to remove guanidinium chloride, was homogenized in 0.05 M-Tris/HCl buffer, pH 8.3 (10 ml), in a Virtis 45 homogenizer (Virtis Co., Gardiner, NY, U.S.A.) at 45000 rev./min for 3 min. This homogenate was added to 20 ml of the Tris/HCl buffer containing trypsin [8 mg; crystalline from ox pancreas; 7500–8000 BzArgOEt (benzoylarginine ethyl ester) units/mg (1 unit causes an increase in E_{253} of 0.001/min at 25°C when incubated in phosphate buffer, pH 7.5, with BzArgOEt); BDH Chemicals Ltd., Poole, Dorset, U.K.], a crystal of thymol and 0.002 M-CaCl$_2$. No attempts were made to remove contaminating proteolytic activities from this trypsin preparation. After incubation for 24 h at 37°C, the digest was filtered through Whatman no. 1 filter paper. Ethanol (3.5 vol.) was added to the filtrate, followed by a saturated solution of sodium acetate in ethanol (0.5 vol.). After storage at 4°C for 16 h, the precipitate was collected by centrifuging and dissolved in a minimum volume of 1 M-NaCl. This solution was applied to a column (100 mm × 12 mm) of Dowex 1 (X2; Cl$^-$ form; 200–400 mesh; Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.), which had been previously equilibrated with 1 M-NaCl. The column was washed with 1 M-NaCl (the effluent was discarded) and eluted with 2 M-NaCl in separate portions making a total of 3 bed volumes. The labelled chondroitin sulphate-peptide was precipitated from the combined 2 M-NaCl effluent by addition of 4 vol. of ethanol, and was collected by centri-

fuging after storage at 4°C to allow the precipitate to flocculate.

Chondroitin sulphate-peptide preparations from rat xiphoid and rib cartilage contained 0.98–0.99 mol of uronic acid, 0.01 mol of glucosamine, 0.34 mol of amino acid residues and 0.92–0.94 mol of ester sulphate/mol of galactosamine. Chondroitin 4-sulphate [estimated as a fraction of total chondroitin sulphate by method III of Saito *et al.* (1968)] was 93 % of the product from xiphoid cartilage residues and 91 % of that from rib. Osmotic-pressure and equilibrium-ultracentrifuge measurements (Wusteman & Davidson, 1975) indicated that it contained 2.6 times the average number of uronic acid residues in single chains of chondroitin sulphate isolated from the same cartilage source by papain digestion, and that it was predominantly a mixture of two-chain and four-chain units. The preparation had a number-average mol.wt. of 24750 (determined by osmometry) and a weight-average mol.wt. of 28200 (determined by sedimentation-equilibrium centrifugation).

The specific radioactivity of the ^{35}S-labelled product from xiphoid cartilage was in the range 2.4–9.1 μCi/μmol of uronic acid. From rib cartilage the double-labelled product contained 1.42 μCi of ^{35}S and 0.31 μCi of ^{3}H radioactivity/μmol of uronic acid. Hexosamine analysis showed that over 98 % of the ^{3}H was associated with galactosamine residues.

Fractionation of double-labelled chondroitin sulphate-peptide by gel chromatography

A preparation of ^{3}H- and ^{35}S-labelled chondroitin sulphate-peptide obtained from 0.5 g of rib cartilage dissolved in 3 ml of 0.2 M-NaCl, was applied to a column (580 mm × 9 mm) of Sephadex G-200 [Pharmacia (G.B.) Ltd., London W.5, U.K.] and eluted with 0.2 M-NaCl at a flow rate of 4 ml/h. The effluent from 14–17 ml (fraction A) and 18–30 ml (fraction B) (see Fig. 3) were pooled and precipitated by addition of ethanol (4 vol.).

Isolation of double-labelled single chains of chondroitin sulphate

A portion (1 g) of the rib cartilage residue was added to 10 ml of 0.1 M-sodium acetate buffer, pH 5.5, containing 0.01 M-L-cysteine hydrochloride, 0.05 M-EDTA and a crystal of thymol, and the suspension was homogenized in a Virtis 45 homogenizer at 45000 rev./min for 3 min. A further 10 ml portion of buffer was added, the mixture was warmed to 60°C and papain [6 mg; twice crystallized; 4 units/mg (1 unit will liberate 1 μmol of NH$_3$/min from *N*-α-benzoyl-L-arginine amide at 37°C in citrate buffer, pH 6); BDH] was added. Digestion at 60°C was continued for 24 h with a similar addition of papain after 16 h. The solution was allowed to cool, filtered through Whatman no. 1 paper, then precipitated and

fractionated on Dowex 1 (X2) as described for the chondroitin sulphate-peptide. The product was further fractionated by gel chromatography on Sephadex G-200, as described for the chondroitin sulphate-peptide, but one pooled fraction only (elution vol. 15–22ml) was precipitated for use in the present study.

Papain digestion of urine

Portions of urine (100 μl) were added to an equal volume of 0.1 M-sodium acetate buffer, pH 6.3, containing 0.002 M-L-cysteine hydrochloride and 0.005 M-EDTA, and papain (1 mg; BDH) was added. The mixture was incubated at 60°C for 12h and, as a control, chondroitin [^{35}S]sulphate-peptide was dissolved in a control urine and subjected to the same digestion. These digests were stored at −20°C until samples (100 μl) were analysed by gel chromatography on Sephadex G-200.

Isolated perfused rat kidney preparation

The apparatus was essentially that for perfusing the isolated liver (Wood et al., 1973b), except that an additional peristaltic pump was included in the system. This pump supplied perfusate directly to the kidney and the flow was adjusted to maintain a pressure of 8–16kPa (60–120mmHg). The rat donating the kidney was anaesthetized with Nembutal, and the right kidney was isolated in situ as described by Curtis et al. (1969). Chondroitin 4-[^{35}S]sulphate-peptide (14 μCi; 3.4mg dissolved in 3.3ml of 0.95% NaCl) was added to the perfusate (whole blood, 100ml) and the kidney was perfused at an average rate of 1.2ml/min (range 1.0–1.5ml/min) for 4h. Samples (2ml) of the perfusate were removed every 30min and the plasma was separated by centrifuging for 20min at 2000g at 4°C. Urine was collected over the 4h period, and continuous flow was maintained by the addition of mannitol [0.5ml of 5% (w/v) mannitol in 0.95% NaCl] 2.5h after the start of perfusion.

Results

The distribution and metabolic fate of chondroitin [^{35}S]sulphate-peptide, derived from rat xiphoid cartilage by trypsin digestion, in the plasma and liver was compared with the results already reported for single chains of chondroitin 4-[^{35}S]sulphate (Wood et al., 1973a). Chondroitin [^{35}S]sulphate-peptide was administered intravenously at a dose of 5.4mg/kg body wt. to a group of rats (340–360g body wt.) which were killed at intervals ranging from 30min to 6h. Blood plasma and liver tissue were isolated, digested with papain, and radioactivity was measured before and after precipitation of intact chondroitin sulphate with cetylpyridinium chloride and of inorganic sulphate with BaCl$_2$. The remaining supernatant solu-

tion was assumed to contain any oligosaccharide produced during the degradation of chondroitin sulphate. The total recovery of injected radioactivity in plasma and liver was 49% at 30min, 22% at 1h and falling thereafter to 7% at 6h. At 30min to 1h after injection, the concentration of radioactivity in the plasma was 12–17% of that in the liver [by contrast, when single chains of chondroitin 4-[^{35}S]sulphate were administered (Wood et al., 1973a), the plasma radioactivity was 89–130% of that in the liver between 30min and 1h after injection]. After 1h had elapsed, the radioactivity in the plasma was predominantly inorganic sulphate (Fig. 1a) and at no time did any ^{35}S-labelled oligosaccharide appear in
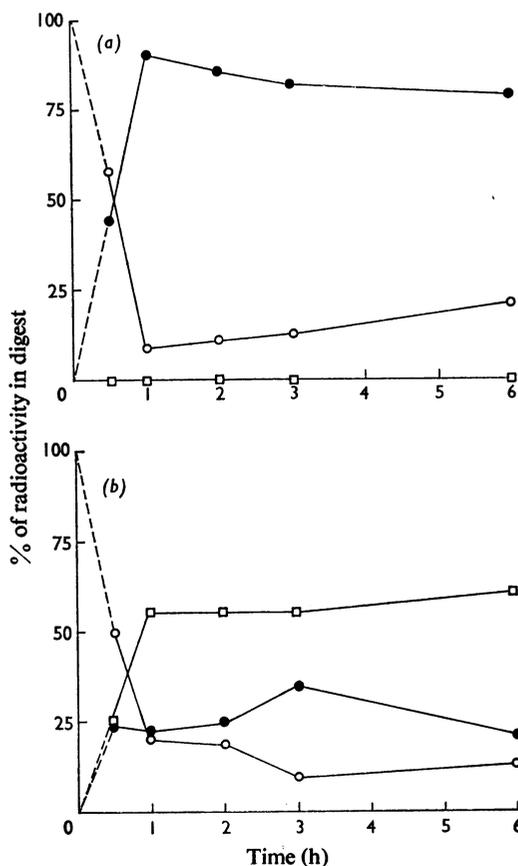
Fig. 1. Time-course of uptake of label from injected chondroitin [^{35}S]sulphate-peptide into rat liver components and plasma

The percentages of total radioactivity of (a) plasma and (b) liver in polymer (○), oligosaccharide (□) and inorganic sulphate (●) are shown. Extrapolation to zero time (dashed lines) is based on assumption that at zero time, chondroitin sulphate is present in plasma and liver as the undegraded polymer.
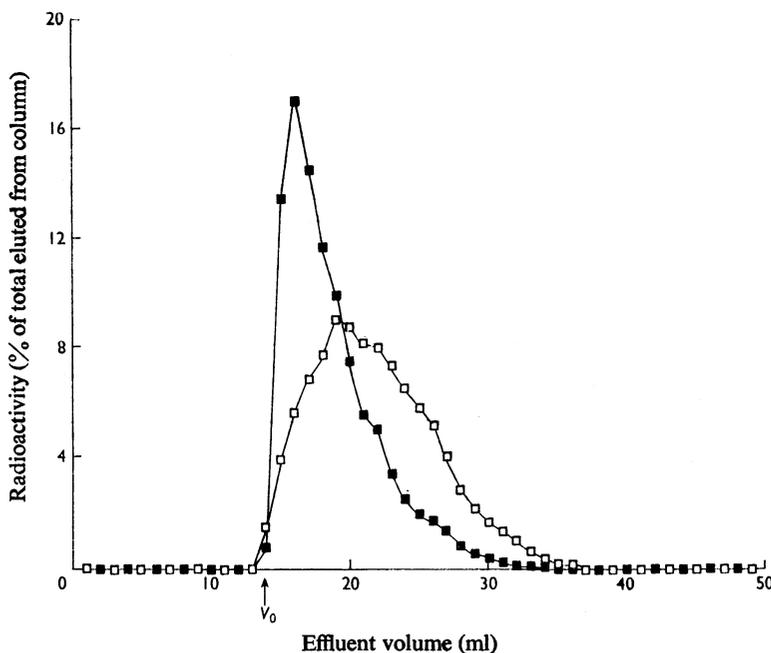
Fig. 2. *Gel chromatography on Sephadex G-200 of plasma obtained from the perfusate and urine from a rat kidney preparation after the addition of chondroitin [^{35}S]sulphate-peptide to the perfusate*

The column was eluted with 0.2M-NaCl. Distribution of radioactivity in 4h plasma sample (■) and in 4h urine sample (□). Void volume of the column (580mm×9mm) is indicated by V_0.

the plasma. By contrast, the liver contained both oligosaccharide and inorganic sulphate, whereas the amount of polymeric chondroitin sulphate decreased to 10–16% after 3h had elapsed (Fig. 1b).

These results indicated that, as with free chains of chondroitin sulphate, the liver is the major site for depolymerization and desulphation of chondroitin sulphate-peptide. Evidence in support of this hypothesis was obtained by intravenous administration of chondroitin [^{35}S]sulphate-peptide at 1.8mg/kg body wt. to hepatectomized rats (Wood *et al.*, 1973a). As with the free chains of chondroitin sulphate, removal of the liver prevented the desulphation of chondroitin sulphate-peptide, which was unchanged in the plasma (as estimated by gel chromatography on Sephadex G-200). It still accounted for 35% of the injected radioactivity after 5h had elapsed.

Some (22.5%) of the chondroitin sulphate-peptide administered to the hepatectomized rats was excreted in the urine within 5h, and gel chromatography indicated that this was of slightly lower molecular weight than the injected material. To investigate any possible role of the kidneys in producing this component, an isolated kidney from a rat was perfused with blood containing chondroitin [^{35}S]sulphate-peptide (14µCi; 3.4mg). Elution profiles obtained by gel chroma-

tography of the urine and plasma recovered after 4h perfusion (Fig. 2) showed no peak of inorganic [^{35}S]sulphate, which, when present, was eluted at $V_e = 41$ ml. The elution profile of the labelled material in the plasma was similar to that of the unchanged polymer, whereas the labelled material in the urine, though still macromolecular, emerges at a larger elution volume. This implies either that a portion had been slightly degraded in passage through the kidneys or that a subfraction of lower molecular weight in the plasma had been preferentially filtered by the kidneys.

To resolve these two possibilities, the ^{35}S and ^{3}H-labelled chondroitin sulphate-peptide from rat rib was fractionated by gel chromatography on a column of Sephadex G-200 and arbitrarily divided into fractions A and B (see the Materials and Methods section). These differed in hydrodynamic size (Fig. 3a) and, on papain digestion, yielded chondroitin sulphate chains, which gave elution profiles (Fig. 3b) suggesting that fraction A contains chondroitin sulphate chains which are longer than those of fraction B.

Chondroitin sulphate-peptide fractions A and B, together with doubly labelled single chains of chondroitin sulphate from the same source, were each administered intravenously, at a dosage of 0.9mg/kg body wt., to rats with ureter and bile-duct fistulae.
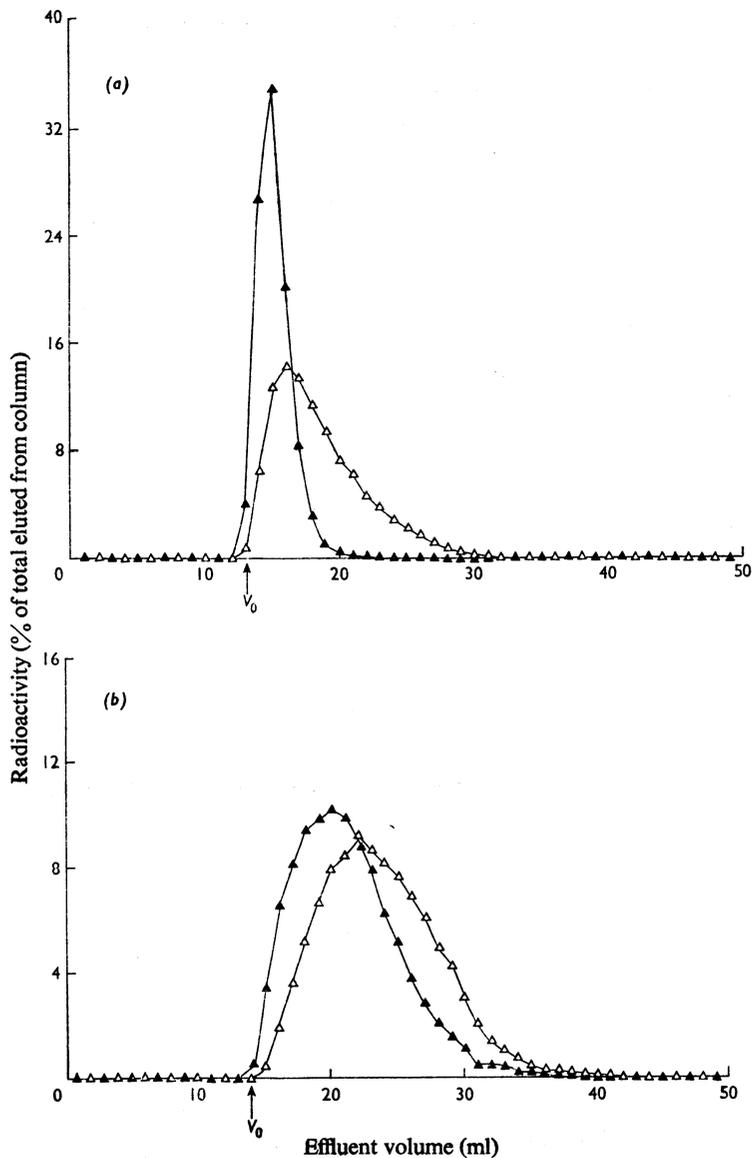
Fig. 3. *Gel chromatography on Sephadex G-200 of double-labelled chondroitin sulphate-peptide, fractions A and B, before and after papain digestion*

The column was eluted with 0.2 M-NaCl. Distribution of ^{35}S-radioactivity in fraction A (▲) and fraction B (△) (*a*) before papain digestion and (*b*) after papain digestion. The void volume V_0 of the column (580 mm × 9 mm) is indicated by the arrow.

Of the total radioactivity injected in the form of ^{35}S, approximately half was retained in the body after 6 h. The bulk was excreted in the urine and less than 4 % in the bile. In all cases the overall excretion of ^{3}H in the urine was lower than that of ^{35}S (Table 1), but the proportion of ^{3}H excreted was much greater with

fraction B and with the single chains of chondroitin sulphate than with fraction A. Gel chromatography of the radioactive material excreted in the urine indicated that inorganic [^{35}S]sulphate (that is, the peak of ^{35}S radioactivity emerging at $V_e = 41$ ml) appeared more rapidly with fraction A (Figs. 4*a* and 4*c*) than

Table 1. *Recovery of ³H and ³⁵S radioactivity in the urine collected from rats with cannulated ureters after injection of double-labelled chondroitin sulphate-peptide and of single chains of chondroitin sulphate*

The results are expressed as percentage of injected dose.

| | Chondroitin sulphate-peptide | | | | Single chains of chondroitin sulphate | |
| | Fraction A | | Fraction B | | | |
Urine sample (h)	³⁵S	³H	³⁵S	³H	³⁵S	³H
1	0.5	3.8	20.6	19.3	35.9	32.5
2	7.2	6.1	12.1	9.9	8.4	2.1
3	13.8	0.8	5.6	1.0	6.4	1.0
4	11.8	0.8	5.6	0.6	—	—
5	8.9	0.7	5.5	0.4	—	—
6	5.8	0.6	4.2	1.1	—	—
Recovery after 3 h	21.5	10.7	38.3	30.2	50.7	35.6
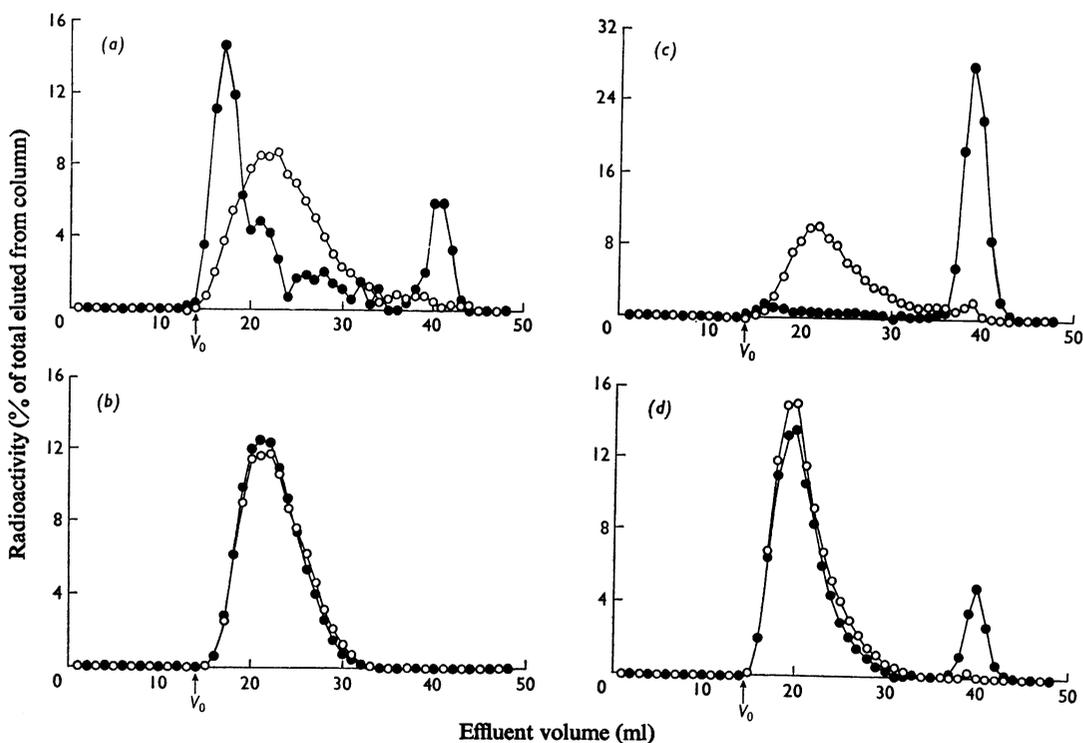
Fig. 4. *Gel chromatography on Sephadex G-200 of urine from rats with cannulated ureters collected after injection of double labelled chondroitin sulphate-peptide subfractions*

The column was eluted with 0.2 M-NaCl. Elution profiles of ³H- (○) and ³⁵S- (●) labelled metabolites in urine collected in the first hour after injection of (a) fraction A and (b) fraction B and in the second hour after injection of (c) fraction A and (d) fraction B, and applied to Sephadex G-200. The void volume V_0 of the column (580 mm × 9 mm) is indicated by the arrow.

fraction B (Figs. 4b and 4d), and it was the sole form in which the ³⁵S label of fraction A emerged after the first hour.

It was during the first hour that the most note-

worthy differences appeared in the excretion pattern of radioactivity from fraction A and from the two preparations of lower molecular weight. Very little ³⁵S label was detected in the urine collected in the first

Table 2. *Comparison of $^{35}S/^{3}H$ ratio (d.p.m.) of the injected polymers and the corresponding metabolites in urine collected during the first hour after injection*

The macromolecular fraction in the urine was isolated by gel chromatography on Sephadex G-200 as shown in Fig. 4(b).

Sample	Elution volume	Average $^{35}S/^{3}H$ (d.p.m.) ratio	
		Injected polymer	Metabolites in urine
Chondroitin sulphate-peptide (Fraction B)	13–28 ml	0.8	0.7
Single chains of chondroitin sulphate	16–30 ml	1.4	1.4
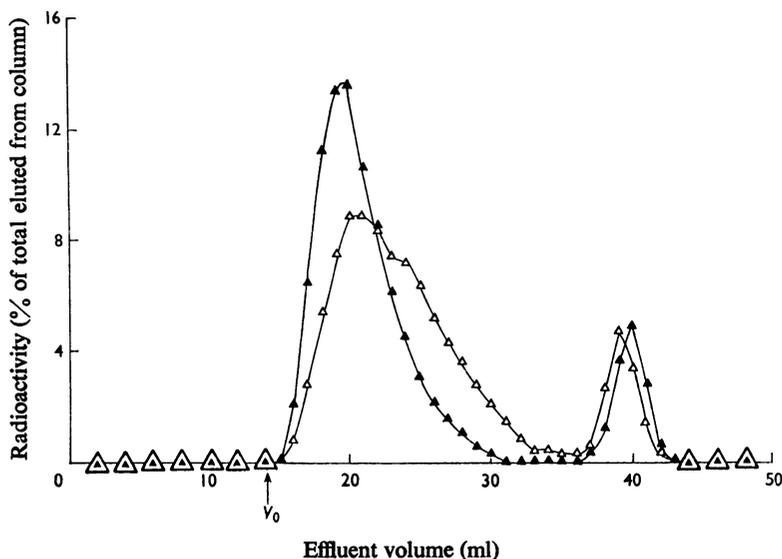
Fig. 5. *Effect of papain digestion on the elution profile of the ^{35}S-labelled metabolites in urine collected in the second hour after injection of chondroitin sulphate-peptide Fraction B*

The column was eluted with 0.2M-NaCl. Elution profiles of the labelled metabolites in the urine before (▲) and after (△) digestion by papain. The void volume, V_0, of the column (580 mm × 9 mm) is indicated by the arrow.

hour after administration of fraction A, whereas the largest portion of both ^{35}S and ^{3}H radioactivity were excreted immediately after administration of Fraction B and of the single chains of chondroitin sulphate. The elution profiles of ^{35}S and ^{3}H radioactivities excreted from fraction B during the first hour correspond closely (Fig. 4b; a similar result was obtained after administration of single chains of chondroitin sulphate), so it seemed likely that some labelled chondroitin sulphate was being excreted without desulphation. The ratio of $^{35}S/^{3}H$ in the polymeric material excreted in the 1 h urine was the same as that of the injected material (Table 2), which confirmed that the polymeric material excreted immediately consists of chains of chondroitin sulphate that have not been desulphated.

Though not desulphated, these polymeric excretion products emerged at a larger elution volume than the injected material [compare Figs. 3a (△) and 4b (●)], indicating a decrease in molecular weight. Portions of urine collected during the second hour after administration of fraction B were digested by papain and compared, by gel chromatography on Sephadex G-200, with the untreated urine. Comparison of the elution profiles (Fig. 5) shows that the macromolecular ^{35}S-labelled components in the urine are still susceptible to papain digestion and thus are unlikely to have resulted from proteolytic degradation of the circulating chondroitin sulphate-peptide.

Discussion

The initial observations with ^{35}S-labelled chondroitin sulphate-peptide suggest that linking two to four chains of chondroitin sulphate via a common peptide core does not greatly affect its fate in the liver.

As shown in Fig. 1, the relative amount of polymer in liver decreased soon after administration and was replaced by inorganic sulphate and an ester sulphate fraction presumed to be oligosaccharide. This latter component was not seen in the plasma, where inorganic sulphate rapidly replaced the polymer as the major radioactive material. In this respect, chondroitin sulphate-peptide preparations share the same fate as free chondroitin sulphate chains (Wood et al., 1973a,b), with the one notable difference that they disappear more rapidly from the circulation.

In contrast with the liver, where rapid uptake and degradation take place, the kidneys seem to function chiefly in filtering the smaller components, whether peptide-bound or not, and excreting them in the urine. Whereas macromolecular proteoglycan (Wood et al., 1975) and the larger of the chondroitin sulphate-peptide subfractions are retained in the circulation, the smaller peptide-bound chondroitin sulphate chains and single chains are likely to be excreted unchanged, and so avoid uptake by the liver. The possibility of proteolytic digestion in the kidneys cannot be excluded entirely, but it is unnecessary to invoke this to account for the observed size distribution of urinary chondroitin sulphate.

Greater size, or the presence of a larger peptide component, seems also to favour uptake by the liver. Radioactive components were more rapidly taken up by the liver from the plasma during the time-course experiments after injection of chondroitin sulphate-peptide than in those reported earlier (Wood et al., 1973a) in which free chains of chondroitin sulphate were used, even allowing for the filtration of smaller components by the kidneys. The uptake of circulating chondroitin sulphate by liver cells presumably involves absorptive pinocytosis and Kresse et al. (1975) have reported that absorptive pinocytosis of proteoglycans by cultured fibroblasts is markedly decreased by degradation of the protein core.

In addition to the release of inorganic sulphate by the liver after desulphation of the ingested chondroitin sulphate, a small amount of ^{3}H-labelled material derived from the carbohydrate chain is excreted in the urine. This ^{3}H-material was not chemically characterized. Gel filtration of this on Sephadex G-200 gives an elution profile in a similar position to that of free chondroitin sulphate (compare Figs. 3b and 4a), implying a polymeric yet desulphated product. One

possible explanation for the origin of this ^{3}H-labelled polymeric material stems from the work of Liau & Horowitz (1974) who have reported that extracts of rat stomach and small intestine can desulphate chondroitin 4-sulphate and that desulphation seems to precede depolymerization. Another possibility is that this polymeric material arises as a result of complete degradation of the doubly labelled chondroitin sulphate and re-utilization of the liberated N-[^{3}H]acetylgalactosamine in the biosynthesis of high-molecular-weight polymers. This latter view is supported by data obtained in this department (A. D. MacNicoll, unpublished work) which showed that N-acetyl-[^{14}C]galactosamine is utilized by the isolated perfused rat liver for the biosynthesis of ^{14}C-labelled polymers which are secreted into the blood.

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