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The Degradation of Intravenously Injected Chondroitin 4-Sulphate in the Rat

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The degradation of chondroitin 4-[^{35}S]sulphate isolated from chick-embryo cartilage was studied in the rat by experiments on free-range animals, on wholly anaesthetized animals with ureter cannulae, by perfusion of isolated liver, by whole-body radioautography and by isolation of liver lysosomes. After injection into rats 68% of the radioactivity was recovered in the urine after 24h, approximately one-half of this being in the form of low-molecular-weight material, chiefly inorganic sulphate. Cannulation experiments demonstrated that the proportion of low-molecular-weight components excreted in the urine increased with time until, after 12h, virtually all was inorganic sulphate. Whole-body radioautography identified the liver as the major site of radioisotope accumulation after injection of labelled polysaccharide. Perfusion through isolated liver indicated that this organ has the ability to metabolize the polymer with the release of low-molecular-weight products, principally inorganic sulphate. Incubation of a lysosomal fraction prepared from rat liver after injection of chondroitin 4-[^{35}S]sulphate gave rise to degradation products of low molecular weight, and experiments *in vitro* with rat liver lysosomes confirmed that these organelles are capable of the entire degradative process from chondroitin sulphate to free inorganic sulphate.

It is thought that chondroitin sulphate chains attached to peptide are released from proteoglycans in the tissues by the proteolytic action of cathepsins (Dingle *et al.*, 1971; Wasteson *et al.*, 1972). Further degradation of the polysaccharide–peptides probably takes place at sites removed from their tissue of origin. Evidence for other sites of accumulation and possibly degradation stems from the work of Aronson & Davidson (1968), who showed that 1% of an injected dose of chondroitin [^{35}S]sulphate becomes concentrated in liver lysosomes after 15min and is subsequently removed within 4 days.

Various reports on the extent of degradation of circulating chondroitin sulphate (Dohlman, 1956; Dziewiatkowski, 1956) show that inorganic [^{35}S]-sulphate appears in the urine after the administration of ^{35}S-labelled polysaccharide. However, Revell & Muir (1972) have shown by bladder-irrigation techniques that chondroitin 4-sulphate can be excreted in an undegraded form after intravenous injection. Tudball & Davidson (1969) suggest that an enzyme system associated with rat liver lysosomes is in part responsible for the turnover *in vivo* of the ester sulphate group of chondroitin 4-sulphate, but no sulphatase activity was revealed by Dohlman (1956), who incubated various rat tissue homogenates with labelled chondroitin sulphate.

This study has been undertaken, by using a variety of physiological techniques to supplement studies *in vitro*, to establish the site and extent of degradation of chondroitin 4-sulphate in the rat.

Materials and Methods

General techniques

Analytical methods. Hexuronic acid was determined by the method of Bitter & Muir (1962) with glucuronolactone as standard. Amino acid composition was determined by using a Technicon amino acid analyser after hydrolysis in 6M-HCl at 110°C for 24h *in vacuo*, and hexosamines were similarly determined after hydrolysis in 4M-HCl at 100°C for 8h. Ester sulphate was assayed by the method of Wessler (1971).

Electrophoresis. Electrophoresis on cellulose acetate strips (Millipore Ltd., Bedford, Mass., U.S.A., 12cm×2.5cm) was performed at constant voltage of 250V for 40min in acetic acid–pyridine–water (20:1:179, by vol.) at pH3.1. The strips were stained with aq. 0.5% Toluidine Blue (BDH Chemicals Ltd., Poole, Dorset, U.K.) and scanned by using a Joyce–Loebl Strip-Scanner in reflectance operation.

Electrophoresis on paper (Whatman no. 1; 46cm×2.5cm) was performed at constant voltage of 250V for 2h in 0.1M-sodium acetate–acetic acid buffer, pH4.6.

Radioactive spots were detected by scanning cellulose acetate or paper strips with a model 7201 Packard

Radiochromatogram Scanner in 4π operation under gas flow. Radioactive zones were also detected by radioautography with X-ray film (Ilford Industrial grade B) for periods of up to 10 days.

Radioactivity counting. A small volume (25–250μl) of each sample was added to 10ml of a scintillation mixture (14g of 2,5-diphenyloxazole and 400ml of 2-methoxyethanol made to 2 litres with toluene) and counted for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer. The recorded counts were corrected for quenching and the background count eliminated by counting an appropriate portion of control urine, plasma, bile or eluent for radioactivity. When whole-blood samples or lysosomal fractions were counted for radioactivity the material was added to Hyamine hydroxide 10-X (Packard Instrument Co. Inc., Downers Grove, Ill., U.S.A.), digested at 37°C for 16h, neutralized with 2M-HCl, and a portion was counted in the usual way.

Gel chromatography. Samples (200μl) of the injected material, urine, plasma or lysosome supernatant solutions were loaded on to a column (550mm $\times$9mm) of Sephadex G-200 (Pharmacia Ltd., London W.5, U.K.) and eluted with 0.2M-NaCl at a flow rate of 4ml/h. Fractions (1ml) were collected and 100μl portions removed for measurement of radioactivity.

Other methods

Preparation of ^{35}S-labelled chondroitin 4-sulphate. Chondroitin 4-[^{35}S]sulphate was prepared (Aronson & Davidson, 1968) by injecting 10-day-fertilized hens' eggs with 440μCi of carrier-free $Na_2{}^{35}SO_4$ (The Radiochemical Centre, Amersham, Bucks., U.K.)/egg. After a further 4 days incubation the embryos were separated from the rest of the egg contents and the cartilage of the long bones and wings was dissected free and subjected to papain digestion (Scott, 1960). Glycosaminoglycans were isolated from the soluble digest by the use of mixture of Amberlite cation-exchange resins [IRC 50 and IR 120 (H^+ forms) (BDH Chemicals Ltd.), 1:1, w/w] to remove residual protein followed by chromatography on Dowex 1 (X2; Cl^- form; 200–400 mesh) (Mason & Wusteman, 1970). The chondroitin sulphate fraction was eluted from this resin by 2M-NaCl and then precipitated by 3.5 vol. of ethanol. The mixed chondroitin sulphates were further fractionated by precipitation with ethanol as calcium salts (Meyer *et al.*, 1956). The fraction precipitated by 30–40% (v/v) ethanol was converted into the sodium salt by passage through cation-exchange resin, reprecipitated with ethanol and freed from all traces of inorganic sulphate by passage through a column of Sephadex G-25 (fine grade; Pharmacia Ltd.). The unretarded peak, which was stored frozen at –20°C, had a specific radioactivity of 4.54μCi/μmol of uronic acid.

Two samples of chondroitin 4-[^{35}S]sulphate were prepared in this way. Per mol of uronic acid these contained 0.97mol of hexosamine (1.1% glucosamine, the rest galactosamine) and 0.09 residue of amino acid attached as peptide via serine residues (i.e. approx. 2% peptide by weight). The mean ester sulphate content was 0.81mol/mol of hexosamine. On cellulose acetate electrophoresis the preparations gave one band of relative mobility (with reference to chondroitin 4-sulphate from whale cartilage; Seikagaku Kogyo Ltd., Tokyo, Japan) of 0.88.

Experiments on free-range animals. Two male Medical Research Council hooded rats (166 and 186g), under light ether anaesthesia, were each injected via the penal vein with chondroitin 4-[^{35}S]-sulphate (0.2mg, 0.5μCi) in aqueous solution. After recovery the rats were placed in separate Metabowls (Jencons Scientific Co. Ltd., Hemel Hempstead, Herts., U.K.), which enabled the collection of urine to be made without contamination by faeces. The animals were allowed free access to food and water throughout the period. The urine was collected in containers cooled by ice to minimize any bacterial growth. Daily urine was collected, filtered through a glass-wool plug, and the volume was measured. After storage at –20°C urine samples were analysed by gel chromatography on Sephadex G-200 and also by paper electrophoresis and compared with control urine (from an uninjected animal) to which chondroitin 4-[^{35}S]sulphate was added subsequently.

Whole-body radioautography. Whole-body radioautography was carried out as described by Powell *et al.* (1967) on 1-month-old Medical Research Council hooded rats which had been injected with chondroitin 4-[^{35}S]sulphate (2.7μCi/animal) via the penal vein while under light ether anaesthesia. The rats were killed at time-intervals ranging from 30min to 24h after injection.

Perfusion of isolated liver. A liver (12.5g) from a Medical Research Council hooded rat (350g) was perfused in an apparatus similar to that described by Curtis *et al.* (1970) with whole blood (from Wistar rats) containing 33.4μCi of chondroitin 4-[^{35}S]-sulphate in 120ml of perfusate. Samples (2ml) of blood were removed from the apparatus at intervals and the plasma was assayed for total radioactivity and stored at –20°C until analysed by gel chromatography and by paper electrophoresis.

Cannulation of ureters and bile duct. A male Medical Research Council hooded rat (360g) was anaesthetized with ether and the trachea exposed and cannulated. The right jugular vein was then cannulated towards the heart and phenobarbitone sodium British Veterinary Codex (Nembutal; Abbot Laboratories Ltd., Queenborough, Kent, U.K.), dissolved in 0.95% NaCl, was administered via the jugular-vein cannula at a dose of 20mg/kg body wt. Similar doses of phenobarbitone were administered

as necessary to maintain anaesthesia throughout the experiment. The ureters and bile duct were cannulated through a midline abdominal incision and urine and bile samples were collected in tared containers (cooled by ice) at intervals of 2h. After the collection of urine and bile to act as controls, the jugular vein was used for administration of 3.3μCi of chondroitin 4-[^{35}S]sulphate. At 8h after administration of the labelled polymer, the rat was given small amounts (1ml/h) of a 5% solution of D-glucose in 0.95% NaCl. Each urine and bile sample was assayed for total radioactivity, then stored at −20°C until the urine samples were examined for degradation products by paper electrophoresis and by gel chromatography on Sephadex G-200.

Lysosomal experiments in vivo. Two male Medical Research Council hooded rats (350g) were injected via the penal vein, under light ether anaesthesia, with chondroitin 4-[^{35}S]sulphate (5.6μCi), and a lysosome-rich fraction was isolated from the liver, by the method of Davies *et al.* (1971) 1h after the animal was killed, and incubated in 20ml of 0.01M-Tris–acetate buffer, pH7.4, containing 0.25M-sucrose at 24°C. Samples (5ml) were removed at intervals from the incubation mixture and a portion (100μl) was assayed for total radioactivity. The remaining portion was centrifuged at 16500*g* for 20min, the supernatant was poured off, and the remaining pellet was resuspended in 0.01M-Tris–acetate buffer, pH7.4. The supernatant fractions were then assayed for radioactivity and a 7h supernatant fraction was freeze-dried and the resulting concentrated solution fractionated by gel filtration on Sephadex G-200.

Lysosomal experiments in vitro. Lysosomes were prepared from the livers of Medical Research Council hooded rats by a modification of the method of de Duve *et al.* (1955) and disrupted by freezing and thawing (eight cycles). This extract was incubated with chondroitin 4-[^{35}S]sulphate at 37°C in 22ml of 0.1M-sodium acetate buffer, pH4.55, and 3ml samples were removed at intervals and fractionated into polymer, a fraction assumed to be oligosaccharide and inorganic sulphate by precipitation with cetylpyridinium chloride and $BaCl_2$ (Wood *et al.*, 1973).

Results

Experiments on free-range animals

Of the radioactivity injected into free-range animals, 71% was recovered in the urine collected over 72h but the majority (68%) was excreted in 24h. The results of gel chromatography of the 24h urine sample on Sephadex G-200 are shown in Fig. 1. Analysis of the elution profiles indicated there was little or no difference between that of chondroitin 4-[^{35}S]sulphate and of the radioactive component of higher molecular weight in the urine. The peak of lower molecular weight (52% of total radioactivity) was studied further by freeze-drying pooled fractions and subjecting them to paper electrophoresis.

The retarded peak had the same electrophoretic mobility as that of inorganic [^{35}S]sulphate added to control urine and was found to remain unchanged on addition of inorganic [^{35}S]sulphate. It was hence concluded that the labelled material of low molecular weight was predominantly, if not entirely, inorganic sulphate. Traces (less than 7% of total radioactivity) of inorganic sulphate were detected in the control urine (Fig. 1), presumably owing to slight degradation of the type reported by Revell & Muir (1972).

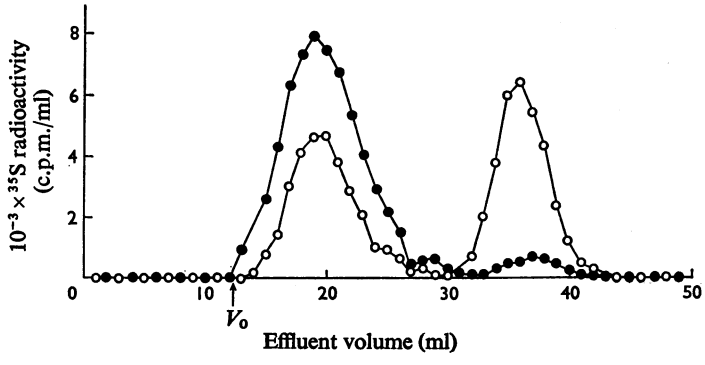


Fig. 1. *Gel chromatography on Sephadex G-200 of urine from rats after injection of ^{35}S-labelled chondroitin 4-sulphate*

The column was eluted with 0.2M-NaCl. ○, Radioactivity of 24h urine sample; ●, radioactivity in control urine with added chondroitin 4-[^{35}S]sulphate equal to the injected dose. Void volume of the column (550mm×9mm) is indicated by V_0.

Whole-body radioautography

At 30min after injection a general distribution of radioactivity throughout the body was seen and a considerable amount was found in blood (Plate 1). The liver showed the most striking uptake of radioactivity, with the large hepatic veins and other blood spaces being virtually devoid of label; this suggests rapid uptake by the parenchyma and consequent fall in the blood concentrations 1h after injection (Plate 1*b*). At this time accumulation in the small intestine was observed, suggesting some biliary excretion of the label. At 2h after administration the amount of radioactivity in the body, and particularly in the liver, had fallen simultaneously with the appearance of radioactivity in the bladder. After 3 and 4h the radioactive content of the body was even lower and the major area of accumulation was the small intestine. A similar pattern was seen after 6.5h, but the accumulation in the gastrointestinal tract was found in the large intestine. The longest time-interval between injection and killing was 24h. At this stage faint areas of accumulation of radioactivity were seen in the epiphyseal plates of long bones, an indication of inorganic [^{35}S]sulphate in the circulation.

Liver perfusion

The progressive degradation of perfused chondroitin 4-sulphate was studied by gel filtration of samples of plasma removed from the liver at intervals of up to 6h. Fig. 2 shows that 3.3mg of the polymer was 25% desulphated after 6h perfusion. Further incubation of blood samples removed from the liver gave no further release of inorganic sulphate, indicating that the degradation depended on the presence of the liver.

Cannulation of ureters and bile duct

Of the injected radioactivity 72% was recovered in the urine and 3% in the bile after 12 h. Fig. 2 also shows that 0.33mg of the polymer yielded only inorganic sulphate in the urine after 6h had elapsed. The peak of labelled material of higher molecular weight was again indistinguishable from that of the starting material.

Lysosomal experiments in vivo

The supernatant from the lysosomal fraction obtained from the liver of animals injected with labelled chondroitin sulphate contained radioactivity that was released from the insoluble fractions over the course of 6h incubation (Fig. 3). Gel filtration again indicated radioactive component(s) of low molecular weight eluted in the position of inorganic [^{35}S]-sulphate and no polymer could be detected.

Lysosomal experiments in vitro

Disrupted lysosomes from the liver of one rat were found to degrade 1mg of chondroitin 4-[^{35}S]sulphate added *in vitro* and incubated, with agitation, at 37°C over a period of 3h (Fig. 4). When incubated for a further 4h at 37°C, degradation had decreased the amount of polymer to 14%, oligosaccharide to 51% and inorganic sulphate to 36% of the original dose.

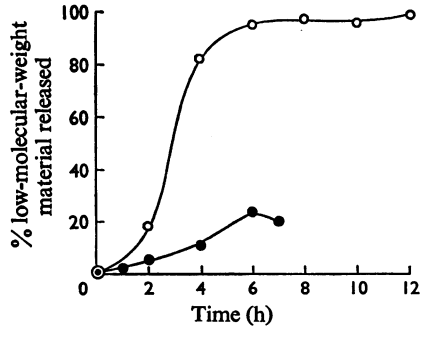


Fig. 2. *Release of ^{35}S-labelled fragments of chondroitin 4-sulphate during liver perfusion and cannulation*

For details see the text. The fraction of total radioactivity in the retarded peak on Sephadex G-200 (see Fig. 1) in plasma from liver perfusion (●) and in urine from the cannulation experiment (○) is shown.

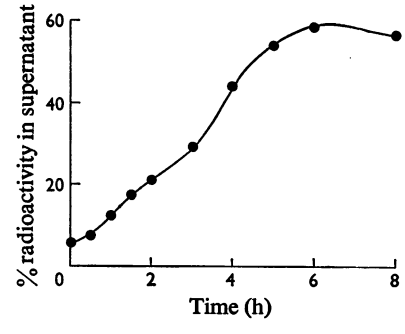
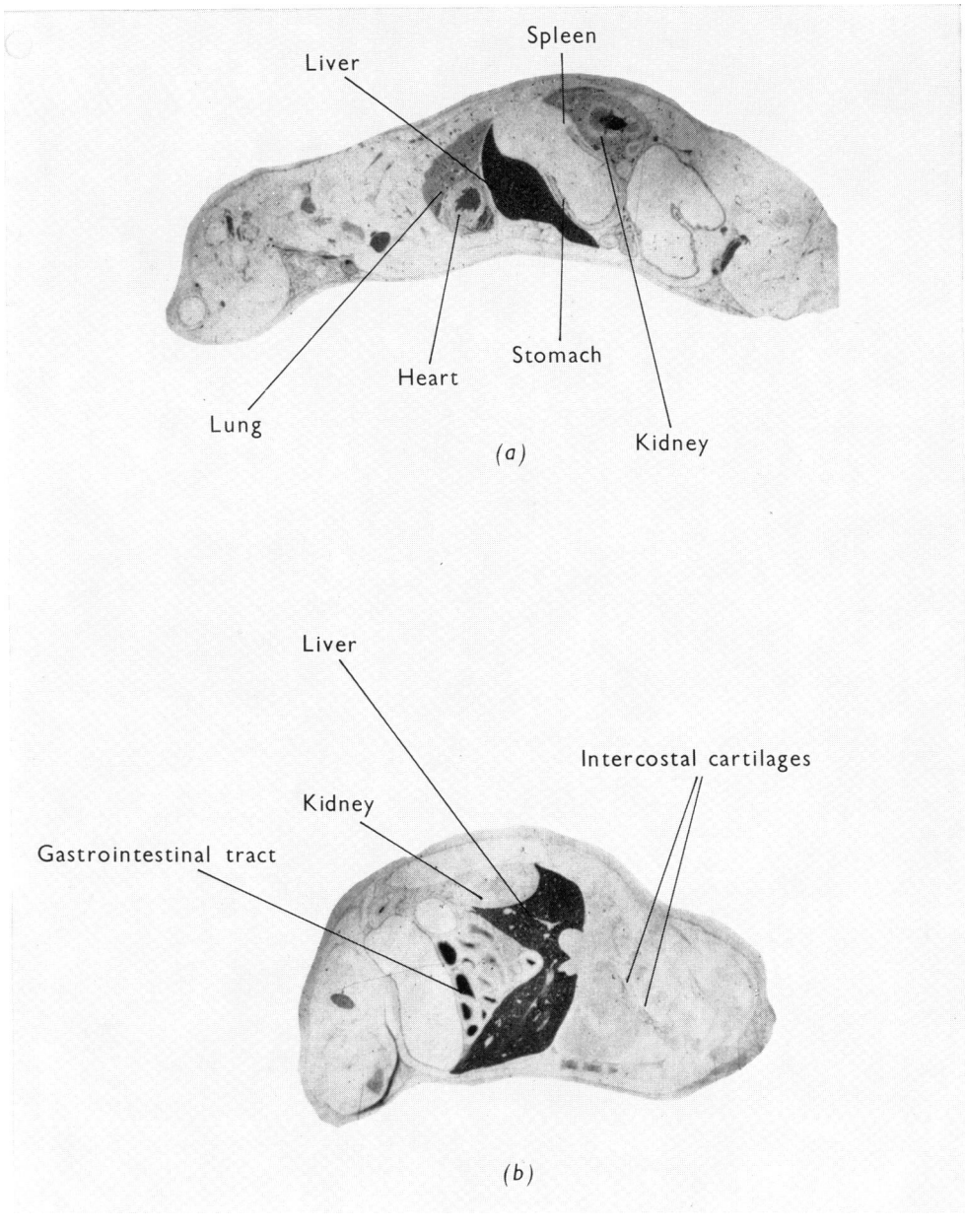


Fig. 3. *Release of ^{35}S-labelled fragments of chondroitin 4-sulphate from rat liver lysosomes labelled in vivo*

The lysosome-rich fraction was isolated from rats 1h after intravenous injection of 5.6μCi of chondroitin 4-[^{35}S]sulphate and samples were removed during incubation at 24°C in 0.01M-Tris–acetate buffer, pH7.4, containing 0.25M-sucrose. These samples were centrifuged and the supernatant was examined for degradation products (retarded peak in Fig. 1) by gel chromatography.



EXPLANATION OF PLATE 1

Whole-body radioautograms of rats after injection of ^{35}S-labelled chondroitin 4-sulphate

Rats aged 1 month were injected via the penal vein with 2.7μCi of chondroitin 4-[^{35}S]sulphate and killed at intervals of (*a*) 30min and (*b*) 1h after injection.

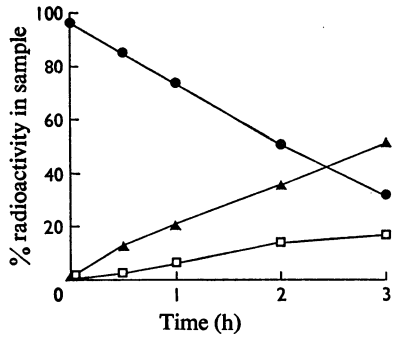


Fig. 4. *Time-course of degradation of ^{35}S-labelled chondroitin 4-sulphate when incubated with a rat liver lysosome preparation in vitro*

Samples were removed at intervals and fractionated into intact polymer (●) (precipitated by cetylpyridinium chloride), inorganic sulphate (▲) (precipitated by $BaCl_2$) and oligosaccharide (□) (precipitated by neither).

Discussion

As reported by Revell & Muir (1972) in studies on guinea pig, a substantial proportion of ^{35}S-labelled chondroitin 4-sulphate is eliminated from the circulation of the rat in a largely undegraded form. In the present paper the site of degradation of the remaining chondroitin sulphate has been pinpointed, hence accounting for the inorganic ^{35}S-labelled sulphate also found in the urine after injection of labelled polymer. No other site of accumulation apart from the liver was detected by radioautography. Perfusion and cannulation of this organ indicated that the liver of one rat could theoretically degrade 0.8 mg of chondroitin sulphate in 6h. Thus over a period of 10 days (the approximate half-life of chondroitin sulphate proteoglycan; Wasteson *et al.*, 1972) the rat liver is capable of handling 32mg of chondroitin sulphate. Together with urinary excretion this should account for the turnover of all rat chondroitin sulphate. Confirmation of this suggestion has been provided by experiments with hepatectomized rats (Wood *et al.*, 1973), in which it has been shown that removal of the liver decreases degradation to control values.

Once in the liver lysosomes the chondroitin sulphate is presumably subjected to the degradation sequence of hyaluronidase, β-glucuronidase and β-*N*-acetylhexosaminidase with lysosomal sulphatase, as described by Tudball & Davidson (1969), acting at the level of oligosaccharide sulphate to release inorganic sulphate, which is subsequently reused or excreted via the kidneys.

Though the present study clearly establishes the fate of slightly undersulphated chondroitin sulphate chains that are virtually free of peptide and still polymeric, the situation *in vivo* may be more complex. Intracellular degradation of the polysaccharide chains together with extensive desulphation within the cartilage has been reported by Wasteson *et al.* (1972) for a portion of chondroitin sulphate of cartilage labelled in tissue culture. The bulk of chondroitin sulphate released by normal turnover from cartilage and other connective tissues is probably in the form of proteoglycan partially degraded by cathepsin D (Dingle *et al.*, 1971) and so includes substantial amounts of peptide. This may affect uptake by liver lysosomes, but Calatroni *et al.* (1969) have detected free polysaccharide chains in the human plasma, so a mechanism clearly exists for further proteolytic digestion to yield chondroitin sulphate chains similar to those used in this study.

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References

Aronson, N. N. & Davidson, E. A. (1968) *J. Biol. Chem.* **243**, 4494–4499
Bitter, T. & Muir, H. (1962) *Anal. Biochem.* **4**, 330–334
Calatroni, A., Donnelly, P. V. & Di Ferrante, N. (1969) *J. Clin. Invest.* **48**, 332–343
Curtis, C. G., Powell, G. M. & Stone, S. L. (1970) *J. Physiol.* **213**, 14P–15P
Davies, M., Lloyd, J. B. & Beck, F. (1971) *Biochem. J.* **121**, 21–26
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–617
Dingle, J. T., Barrett, A. J. & Weston, P. D. (1971) *Biochem. J.* **123**, 1–13
Dohlman, C. H. (1956) *Acta Physiol. Scand.* **37**, 220–234
Dziewiatkowski, D. D. (1956) *J. Biol. Chem.* **223**, 239–249
Mason, R. M. & Wusteman, F. S. (1970) *Biochem. J.* **120**, 777–785
Meyer, K., Davidson, E., Linker, A. & Hoffman, P. (1956) *Biochim. Biophys. Acta* **21**, 506–518
Powell, G. M., Curtis, C. G. & Dodgson, K. S. (1967) *Biochem. Pharmacol.* **16**, 1997–2001
Revell, P. A. & Muir, H. (1972) *Biochem. J.* **130**, 597–606
Scott, J. E. (1960) in *Methods of Biochemical Analysis* (Glick, D., ed.), vol. 8, pp. 145–197, Interscience Publishers Inc., New York
Tudball, N. & Davidson, E. A. (1969) *Biochim. Biophys. Acta* **171**, 113–120
Wasteson, Å., Lindahl, U. & Hallén, A. (1972) *Biochem. J.* **130**, 729–738
Wessler, E. (1971) *Anal. Biochem.* **41**, 67–69
Wood, K. M., Curtis, C. G., Powell, G. M. & Wusteman, F. S. (1973) *Biochem. Soc. Trans.* **1**, 840–842