Structure and Metabolism of Rat Liver Heparan Sulphate

By ÅKE OLDBERG and MAGNUS HÖÖK

Department of Medical Chemistry, Royal Veterinary College, Uppsala, Sweden

and BJÖRN ÖBRINK, HÅKAN PERTOFT and KRISTOFER RUBIN

Department of Medical and Physiological Chemistry, University of Uppsala, Uppsala, Sweden

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Rat liver cells grown in primary cultures in the presence of [^{35}S]sulphate synthesize a labelled heparan sulphate-like glycosaminoglycan. The characterization of the polysaccharide as heparan sulphate is based on its resistance to digestion with chondroitinase ABC or hyaluronidase and its susceptibility to HNO_2 treatment. The sulphate groups (including sulphamino and ester sulphate groups) are distributed along the polymer in the characteristic block fashion. In ^3H -labelled heparan sulphate, isolated after incubation of the cells with [^3H]galactose, 40% of the radioactive uronic acid units are L-iduronic acid, the remainder being D-glucuronic acid. The location of heparan sulphate at the rat liver cell surface is demonstrated; part of the labelled polysaccharide can be removed from the cells by mild treatment with trypsin or heparitinase. Further, a purified plasma-membrane fraction isolated from rats previously injected with [^{35}S]sulphate contains radioactively labelled heparan sulphate. A proteoglycan macromolecule composed of heparan sulphate chains attached to a protein core can be solubilized from the membrane fraction by extraction with 6M-guanidinium chloride. The proteoglycan structure is degraded by treatment with papain, Pronase or alkali. The production of heparan [^{35}S]sulphate by rat liver cells incubated in the presence of [^{35}S]sulphate was followed. Initially the amount of labelled polysaccharide increased with increasing incubation time. However, after 10h of incubation a steady state was reached where biosynthetic and degradative processes were in balance.

Heparan sulphate is a polysaccharide, structurally related to heparin and composed of alternating D-glucosamine and uronic acid (L-iduronic acid or D-glucuronic acid) residues. Sulphate residues occur as sulphamino groups and in ester linkages to C-2 of the iduronic acid and C-6 of the glucosamine unit (for review see Lindahl, 1976). The term heparan sulphate has been ascribed to a variety of low-sulphated heparin-like polysaccharides ranging in average sulphate content from 0.5 to more than 1 sulphate residue per disaccharide.

The biosynthesis of heparan sulphate has been demonstrated in several cell lines (Dietrich & DeOca, 1970; Kraemer, 1971; Roblin *et al.*, 1975), including rat liver cells (Yamamoto & Terayama, 1973; Akasaki *et al.*, 1975) and rat hepatomas (Yamamoto *et al.*, 1973; Funakoshi *et al.*, 1974; Nakada *et al.*, 1975). The polysaccharide produced appears to be partly associated with the external surface of the cells (Kraemer, 1971); in addition, some cell lines also secrete heparan sulphate to the growth media (Roblin *et al.*, 1975). The amount and structure of the polysaccharide differ between various cell lines. Further, virus transformation of fibroblasts has been reported to provoke changes in the metabolism and properties of heparan sulphate (Roblin *et al.*, 1975; Underhill & Keller, 1975).

The physiological function of cell-surface heparan sulphate is unclear. Glycosaminoglycans have been suggested to contribute to the negative charge on the cell surface (Kojima & Yamagata, 1971), regulate the accessibility of cell-surface receptors to external factors (Kraemer, 1971), and participate in cell-to-cell communications (Roblin *et al.*, 1975) and the regulation of cell growth (Ohnishi *et al.*, 1975).

The present report concerns structural properties, location and metabolism of rat liver heparan sulphate.

Experimental

Materials

Samples of hyaluronic acid and chondroitin 4-sulphate isolated from rooster combs and bovine nasal septa respectively were kindly given by Dr. Å. Wasteson of this department. The heparin preparation used has been previously described (Höök *et al.*, 1974a). Mono- and di-sulphated uronosylanhydromannose disaccharides were isolated as described (Höök *et al.*, 1974a), after degradation of heparin with HNO_2 .

[^{35}S]Sulphate (carrier-free), D-[1- ^3H]galactose (5.2Ci/mmol) and D-[6- ^3H]glucosamine hydrochloride (29Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Crystalline papain was prepared from a crude preparation (obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.), by the procedure of Kimmel & Smith (1954). Pronase (lot no. 45550) was purchased from Calbiochem, Los Angeles, CA, U.S.A., chondroitinase ABC from Miles-Seravac, Maidenhead, Berks., U.K., and hyaluronidase from AB Leo, Helsingborg, Sweden. Bacterial heparitinase (heparan sulphate lyase, EC 4.2.2.8), partially purified, was a kind gift from Dr. A. Linker, Veterans Administration Hospital, Salt Lake City, UT, U.S.A. Sephadex and Sepharose gels were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Whatman DEAE-cellulose (DE-52) was a product of W. and R. Balston, Maidstone, Kent, U.K.

Sprague-Dawley rats (weight 200–250 g) were obtained from Anticimex, Stockholm, Sweden.

Methods

Methods for determination of protein and uronic acid were as described by Höök *et al.* (1975a).

Radioactivity was measured by a Packard model 2450 liquid-scintillation counter, with Insta-Gel (Packard Instrument Co., Downers Grove, IL, U.S.A.) as scintillation medium.

High-voltage paper electrophoresis was performed in 1.6M-formic acid, pH1.7 (40V/cm for 90min), on Whatman 3MM paper. Paper chromatography was carried out with the same paper developed with ethyl acetate/acetic acid/water (3:1:1, by vol.).

Carbohydrate standards were detected by a silver-dip procedure (Smith, 1960). Labelled components on paper were quantified by scintillation counting after elution with water, either directly or after localization with a Packard model 7201 strip scanner.

Ion-exchange chromatography of glycosaminoglycans was performed on DEAE-cellulose as described by Höök *et al.* (1975a). Radioactively labelled samples were mixed with standards (0.5mg of hyaluronic acid, 1 mg of chondroitin sulphate and 2mg of heparin), applied to a column (1cm×5cm) of DEAE-cellulose and eluted with a linear LiCl gradient (0.2–1.5M in 0.05M-sodium acetate buffer, pH4.0) obtained by means of a LKB model 11300 Ultrograd gradient mixer.

Deaminative degradation of glycosaminoglycans. Degradation of glycosaminoglycans with HNO₂ was carried out by treatment with 0.75ml of 0.24M-NaNO₂ in 1.8M-acetic acid for 80min at room temperature (20°C) (Lindahl *et al.*, 1973). This deamination procedure leads to cleavage of the glycosidic bonds of *N*-unsubstituted or *N*-sulphated glucosamine residues, with concomitant release of *N*-sulphate groups as inorganic sulphate.

Enzymic degradation of glycosaminoglycans. Digestion with chondroitinase ABC was carried out as follows. Samples of less than 0.5mg of polysaccharide

were dissolved in 0.5ml of 0.05M-Tris/HCl, pH8.0, containing 30μmol of sodium acetate, 0.1mg of bovine serum albumin and 0.1 unit of enzyme. Incubations were terminated after 15h at 37°C, by heating at 100°C for 3min.

Digestion with testicular hyaluronidase was performed in 0.5ml of 0.2M-NaCl/0.15M-sodium acetate, pH5.0, containing 25 units of enzyme. The mixture was incubated as described above.

Incubation of cells or plasma membranes with bacterial heparitinase was carried out in 0.1M-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid] buffer, pH6.8, at 30°C for 30min. The enzyme was used at a protein concentration of 0.1mg/ml.

Incubations with proteolytic enzymes. Cells were incubated with crystalline trypsin as described by Roblin *et al.* (1975).

Samples to be digested by papain were mixed with an equal volume of 0.1M-sodium acetate, pH5.5, containing 0.02M-EDTA, 0.2M-cysteine hydrochloride and 4M-NaCl. After addition of papain (2mg/ml) the mixtures were incubated at 65°C for 15h.

Incubations of cell cultures with Pronase were performed at 60°C after addition of 1mg of enzyme/ml of growth medium.

Preparation of plasma membranes. Rat liver plasma membranes were prepared from a nuclear fraction of a liver homogenate as described by Ray (1970).

Isolation and cultivation of rat liver cells. Cells were isolated from rat livers after perfusion with a collagenase solution by a modification of the procedure described by Seglen (1973). Rats were anaesthetized with diethyl ether. A catheter was inserted in the portal vein *in situ* and perfusion was carried out for 5min with oxygenated Buffer 1 [10mM-Hepes (pH7.4)/150mM-NaCl/5mM-KCl] at a flow rate of 50ml/min. During this perfusion the liver was gently excised as quickly as possible and put on a nylon net. Then 50ml of Buffer 2 [0.1M-Hepes (pH7.6)/65mM-NaCl/5mM-KCl/5mM-CaCl₂/1.5% (w/v) bovine serum albumin/0.05% (w/v) collagenase] was perfused for 10 min. This treatment was followed by perfusion with Buffer 3 [10mM-Hepes (pH7.4) / 140mM-NaCl / 5mM-KCl / 0.5mM-MgSO₄/1mM-CaCl₂] for 10min at a flow rate of 50ml/min. The liver capsule was then cut with scissors and the liver cells were gently washed out with Buffer 3 supplemented with 1.5% bovine serum albumin. The cell suspension was diluted to 75ml with the same buffer and filtered successively through nylon filters of pore sizes 200, 130 and 50μm respectively. The filtered cells were then purified by four consecutive centrifugations (2min at 70g in a swinging-bucket rotor) in the same buffer. The final cell pellet was suspended in Ham's F-10 medium (Ham, 1963) supplemented with 10% postnatal calf serum and 60μg of benzylpenicillin (AB KABI,

Stockholm, Sweden), 50 μ g of streptomycin (Novo Industri A/S, Copenhagen, Denmark) and 1.25 μ g of amphotericin B (E. R. Squibb and Son, London U.K.)/ml of medium. The Trypan Blue-exclusion test indicated 80–90% viable cells.

The cells were seeded on 60mm Petri dishes in 2ml of Ham's F-10 medium supplemented with 10% calf serum at a cell density of 1×10^6 cells/ml. They were incubated in a humidified incubation chamber at 37°C. Within 1h most of the cells were attached to the bottom of the dishes and had started to spread out.

Isolation of labelled glycosaminoglycans from rat liver cells. Rat liver cells, isolated and seeded as described above, were grown for 10h in the presence of 25 μ Ci of [35 S]sulphate, 25 μ Ci of [3 H]galactose or 50 μ Ci of [3 H]glucosamine/ml. The incorporation of labelled precursor into glycosaminoglycans was terminated by freezing, after the addition of 0.1 ml of 10% (v/v) Triton X-100/ml of cell suspension. Cells were released from the Petri dishes by Pronase treatment for 15min. In preparative experiments cultures, including the medium, were transferred to glass vials and digested with papain overnight; the digests were applied to a column (3cm $\times$ 80cm) of Sephadex G-50, equilibrated with 1M-NaCl. The labelled macromolecules emerging at the void volume were desalted by dialysis and freeze-dried.

In analytical experiments cultures grown in the presence of [35 S]sulphate were digested with papain overnight; 35 S-labelled glycosaminoglycans were quantified by scintillation counting after precipitation on filter paper with cetylpyridinium chloride (Wasteson *et al.*, 1973).

Isolation of labelled glycosaminoglycans from purified plasma membranes. Rats were injected intraperitoneally with 1mCi of [35 S]sulphate, and 2h later they were decapitated and a plasma-membrane fraction was prepared from the livers. 35 S-labelled polysaccharides were isolated from the membranes, after papain digestion, by gel chromatography on Sephadex G-50 as described above. Alternatively, 35 S-labelled proteoglycans were extracted by stirring the membranes (corresponding to 2mg of protein) with 5ml of 6M-guanidinium chloride/0.05M-sodium acetate buffer, pH4.6, overnight at 4°C. After centrifugation at 100000g for 1h the supernatant was dialysed against several changes of water and was then stored at -20°C until used.

Results

Identification of glycosaminoglycans synthesized by rat liver cells

After incubation of rat liver cells in the presence of [35 S]sulphate for 10h, papain-resistant labelled macromolecules were isolated and chromatographed

on a column of DEAE-cellulose. The 35 S-labelled material emerged as a single peak, with an elution position intermediate between those of standard chondroitin sulphate and heparin (Fig. 1a). The 35 S-labelled polysaccharide was resistant to digestion with either chondroitinase ABC or hyaluronidase, as shown by subsequent gel chromatography on Sephadex G-50. In contrast, it was quantitatively degraded to oligosaccharides by HNO_2 , indicating the presence of sulphamino groups (Lindahl *et al.*, 1973). These findings suggest that rat liver cells synthesize a polysaccharide related to heparan sulphate, but no significant amounts of any other sulphated glycosaminoglycan.

The 35 S-labelled polysaccharide isolated from purified rat liver plasma membranes was similar to that obtained from whole cells with regard to polyanion character (Fig. 1b) as well as susceptibility to HNO_2 , chondroitinase ABC and hyaluronidase.

^3H -labelled polysaccharides isolated after incubation of liver cells with [^3H]glucosamine yielded two peaks on anion-exchange chromatography

Fig. 1. Chromatography on DEAE-cellulose of radioactively labelled polysaccharides isolated from rat liver cells after incubations with [35 S]sulphate (a) or [^3H]glucosamine (c) or from purified rat liver plasma membranes prepared from animals previously injected with [35 S]sulphate (b)

Before application to the DEAE-cellulose column the sample was mixed with standard preparations of hyaluronic acid (HA), chondroitin sulphate (CS) and heparin (Hep). Effluent fractions of volume about 2.5ml were collected and analysed for radioactivity (○) and uronic acid (●). For further experimental details, see the Experimental section.

(Fig. 1c). The more-retarded material was identified as heparan sulphate by the specific degradation procedures outlined above. The less-anionic material (elution position coinciding with that of standard hyaluronic acid) was not further characterized

Composition of rat liver heparan sulphate

Judged from the elution properties on ion-exchange chromatography (Fig. 1), the rat liver heparan [^{35}S]sulphate seemed to have a sulphate content intermediate between that of chondroitin sulphate (one sulphate group/dissaccharide) and that of heparin (>two sulphate groups/disaccharide). The distribution of label between sulphamino groups and ester sulphate groups was determined after paper electrophoresis (Fig. 2) of the products obtained by deaminative degradation of the polysaccharide (Jansson *et al.*, 1975). The distribution of ^{35}S radioactivity between inorganic [^{35}S]sulphate (derived from sulphamino groups) and ^{35}S -labelled oligosaccharides (containing the ester sulphate groups of the parent polysaccharide) was approx. 2:3 (estimated after elution of the paper with water).

The uronic acid composition of rat liver heparan sulphate was determined by using labelled polysaccharide isolated from rat liver cells incubated

with [^3H]galactose. The polysaccharide was degraded by a combination of HNO_2 deamination and acid hydrolysis (Höök *et al.*, 1974a). The released uronic acid monosaccharides were separated by paper chromatography and quantified by scintillation counting. The ratio of [^3H]iduronic acid to total [^3H]uronic acid was 0.4. The specific radioactivities of [^3H]iduronic acid and [^3H]glucuronic acid in the ^3H -labelled heparan sulphate are probably the same because (a) both uronic acids are synthesized from the same precursor (Höök *et al.*, 1974b) and (b) labelled heparan sulphate was obtained after incubations long enough to saturate all pools involved in the metabolic turnover of the polysaccharide. The ratio of radioactive [^3H]iduronic acid to [^3H]glucuronic acid thus probably represents the chemical composition of uronic acids in rat liver heparan sulphate.

Distribution of sulphate residues in the heparan sulphate polymer

Heparan sulphate isolated after incubation of rat liver cells with [^3H]glucosamine or with [^{35}S]sulphate was treated with HNO_2 . The degradation products were fractionated by gel chromatography on Sephadex G-25 (Fig. 3). The elution pattern of ^{35}S radioactivity included a peak at the position of inorganic [^{35}S]sulphate, presumably representing [^{35}S]sulphamino groups in the intact polymer. ^{35}S -labelled (*O*-[^{35}S]sulphated) as well as ^3H -labelled oligosaccharides were observed. The ratio of ^3H to ^{35}S radioactivity decreased with increasing elution volume, indicating that *O*-sulphate groups are preferentially located in relatively small deamination products. This conclusion was supported by electrophoretic analysis of the deaminated [^3H]oligosaccharide (Fig. 4).

Enzymic removal of cell-associated ^{35}S -labelled polysaccharide

^{35}S -labelled whole liver cells were incubated with bacterial heparitinase or with trypsin, under the conditions described under 'Methods'. After centrifugation of the cell suspensions, 20 and 40% respectively of the initial cell-associated ^{35}S -labelled polysaccharide were recovered in the supernatants.

Proteoglycan character of cell-surface heparan sulphate

Rat liver plasma membranes isolated from animals previously injected with [^{35}S]sulphate were extracted with 6M-guanidinium chloride. More than 90% of the ^{35}S -labelled macromolecules in the extract was heparan sulphate, as shown by its resistance to digestion by chondroitinase ABC and susceptibility to deamination by HNO_2 .

The macromolecular properties of the ^{35}S -labelled material were investigated by gel chromatography

Fig. 2. High-voltage electrophoresis of degradation products obtained by HNO_2 deamination of heparan [^{35}S]sulphate. Heparan [^{35}S]sulphate isolated from cultured rat liver cells was degraded by HNO_2 treatment. After desalting of the products by passage over a column (0.8cm $\times$ 135cm) of Sephadex G-15 eluted with 10% (v/v) ethanol the deamination products were subjected to high-voltage paper electrophoresis at pH 1.7. Radioactive components were detected with a radiochromatogram scanner. (a) Sample; (b) inorganic [^{35}S]sulphate. Additional standards: I, monosulphated uronosylanhidromannose; II, disulphated uronosylanhidromannose.

Fig. 3. Gel chromatography of degradation products obtained by HNO_2 deamination of (●) ^3H - or (○) ^{35}S -labelled rat liver heparan sulphate

Heparan sulphate isolated after incubation of cultured rat liver cells with ^3H glucosamine or with ^{35}S -sulphate was degraded by HNO_2 treatment and applied to a column (1 cm $\times$ 195 cm) of Sephadex G-25 eluted with 0.2 M-NaCl at 4–5 ml/h. Fractions (about 2.5 ml) were collected and analysed for radioactivity. Fractions A, B and C were pooled as indicated by the vertical lines. The arrow (Di) indicates the peak elution position of standard uronosyl-anhydromannose disaccharides.

Fig. 4. High-voltage electrophoresis of ^3H -labelled oligosaccharides obtained after gel chromatography of rat liver ^3H heparan sulphate after deamination with HNO_2 . Oligosaccharide fractions A, B and C obtained as described in the legend to Fig. 3 were desalted by passage through a column (0.8 cm $\times$ 135 cm) of Sephadex G-15 eluted with 10% ethanol and analysed by high-voltage paper electrophoresis at pH 1.7. Standards: I, monosulphated uronosyl-anhydromannose; II, disulphated uronosyl-anhydromannose.

before and after treatment with Pronase, papain or alkali (Anderson *et al.*, 1965). Appreciable degradation was observed in each case (Fig. 5), although degradation by Pronase digestion was less extensive than that observed after treatment with papain or alkali.

Time course of ^{35}S sulphate incorporation into rat liver heparan sulphate

The time course of ^{35}S sulphate incorporation into heparan sulphate, synthesized in primary cultures of rat liver cells, is illustrated in Fig. 6(a). The formation of labelled polysaccharide was linear with time during the initial incubation period. However, incubations prolonged beyond 10 h resulted in only a limited increase in synthesis of heparan ^{35}S sulphate.

Cells retained the capability of heparan ^{35}S -sulphate biosynthesis after preincubation for 10 h before the addition of radioisotope (Fig. 6b). Further, degradation of heparan sulphate could be demonstrated in a pulse-chase experiment where the incorporation of ^{35}S sulphate into heparan sulphate was stopped after 10 h of incubation by addition of

Fig. 5. Gel chromatography of plasma-membrane-associated heparan ^{35}S sulphate macromolecules before (●) and after treatment with papain (▲) and Pronase (○)

Heparan ^{35}S sulphate macromolecules were obtained after solubilization of a purified rat liver plasma-membrane fraction with 6 M-guanidinium chloride (see the Experimental section). Samples were applied to a column (2 cm $\times$ 120 cm) of Sepharose 4B, which was eluted with 6 M-guanidinium chloride/50 mM-acetate buffer, pH 4.6, at 1.5 ml/h. Fractions (1 ml) were collected and analysed for radioactivity. The elution pattern obtained by alkali degradation of heparan ^{35}S sulphate macromolecules (not shown in the Figure) was virtually identical with that obtained after papain treatment.

Fig. 6. Time course of incorporation of [^{35}S]sulphate into heparan sulphate

Isolated rat liver cells were incubated in the presence of [^{35}S]sulphate, and synthesized labelled polysaccharide was quantified by cetylpyridinium chloride precipitation on filter paper. The [^{35}S]sulphate was added at the start of the incubation (a) or after cells had been preincubated for 10h (b; ----). In a pulse-chase experiment the incorporation of [^{35}S]sulphate into polysaccharides was terminated after a 10h incubation by addition of $50\mu\text{mol}$ of unlabelled inorganic sulphate (b; —).

excess of unlabelled sulphate (for details see legend to Fig. 6). The decrease in ^{35}S -labelled polysaccharide during the chase period indicates degradation of previously labelled heparan sulphate (Fig. 6b). During the chase period all ^{35}S -labelled polysaccharide was not degraded, and further, after preincubation the amount of labelled polysaccharide synthesized by the cells was decreased. These findings could be explained by an observed decrease in cell viability during long incubations.

Discussion

The only [^{35}S]sulphated polysaccharide synthesized during incubation of rat liver cells with [^{35}S]sulphate belongs to the family of heparin-related polysaccharides. This finding is in agreement with a previous report (Yamamoto & Terayama, 1973). When cells were incubated in the presence of [^3H]glucosamine, a non-sulphated ^3H -labelled polysaccharide was synthesized in addition to the heparin-related [^3H]glycosaminoglycan.

The elution position on anion-exchange chromatography of the ^{35}S -labelled rat liver polysaccharide is between that normally found for heparan sulphate (or chondroitin sulphate) and heparin (see Fig. 1). On the basis of its polyanionic pro-

perties, the rat liver ^{35}S -labelled polysaccharide can thus be classified either as a high-sulphated heparan sulphate or as a low-sulphated heparin. The heparin-related polysaccharide from normal rat liver appears to have a higher sulphate content than the corresponding polysaccharide isolated from an ascites hepatoma (the latter contains about one sulphate group/disaccharide unit; Funakoshi *et al.*, 1974). This difference in sulphate content can possibly be related to the amount of *O*-sulphate, which contributes about 60% and 40% of the total sulphate contents respectively in rat liver and hepatoma (Funakoshi *et al.*, 1974) polysaccharides. A similar difference in sulphate content of heparan sulphate isolated from normal and virus-transformed fibroblasts has been reported (Underhill & Keller, 1975).

The iduronic acid content of the ^3H -labelled rat liver polymer (40% of the total [^3H]uronic acid) is intermediate between that generally found in heparin (80–90%) and in heparan sulphate (20–30%) (Höök *et al.*, 1974a; Cifonelli, 1974).

A characteristic of a heparan sulphate molecule is that the *N*-acetylglucosamine-containing disaccharides are to a large extent accumulated in extended block structures; in heparin *N*-acetylglucosamine-containing disaccharides occur preferentially as isolated units (Cifonelli & King, 1972; Lindahl, 1976).

As HNO_2 cleaves heparin-related polysaccharides at *N*-sulphated but not at *N*-acetylated glucosamine residues, deamination of heparan sulphate is expected to yield degradation products with *O*-sulphate groups preferentially located in oligosaccharides of relatively small size (Höök *et al.*, 1974a). Gel-chromatographic analysis of the deamination products obtained after HNO_2 degradation of the rat liver polysaccharide (see Fig. 3) shows that [^{35}S]sulphate residues are distributed in a non-random fashion along the polymer. The rat liver polysaccharide should therefore be designated a heparan sulphate. In agreement with this conclusion, the polysaccharide was found to be depolymerized by rat liver heparitinase, an endoglycosidase with little or no activity towards heparin (Höök *et al.*, 1975b).

Several workers have reported that heparan sulphate is associated with the external surface of cells (see the introduction). Such statements are usually based on the fact that cell-associated heparan sulphate can be released by mild trypsin treatment; in some cases the occurrence of heparan sulphate was demonstrated in a purified plasma-membrane fraction (Yamamoto *et al.*, 1973; Funakoshi *et al.*, 1974; Akasaki *et al.*, 1975; Nakada *et al.*, 1975).

The use of trypsin in this context is questionable, as proteolytic enzymes might disrupt or damage some

cells, leading to the release of intracellular material. In the present study the association of heparan sulphate with the surface of cells was also indicated by additional techniques, namely release of heparan sulphate from whole cells by treatment with bacterial heparitinase and isolation of a plasma-membrane fraction containing heparan sulphate. Heparitinase treatment of the cells did not release more than half of the trypsin-sensitive ^{35}S -labelled polysaccharide. This difference could reflect the release of intracellular material by trypsin, but it could also be due to restricted susceptibility of cell-surface heparan sulphate to the heparitinase. The latter possibility is perhaps favoured by the finding that digestion of purified plasma membranes with heparitinase did not release more than 50% of the heparan [^{35}S]sulphate, whereas all labelled macromolecules were released by digestion with trypsin.

The heparan sulphate associated with rat liver plasma membranes is at least partly of proteoglycan nature, from which single polysaccharide chains may be liberated by β -elimination under alkaline conditions (Anderson *et al.*, 1965) or by proteolytic cleavage of the protein component. Similar properties for the heparan sulphate component associated with Chinese-hamster cells have previously been reported (Kraemer & Smith, 1974).

The incorporation of [^{35}S]sulphate into heparan sulphate synthesized by rat liver cells *in vitro* increased with incubation time during the initial 10 h, after which a plateau was observed, which may reflect either a blocking of the biosynthetic process or a steady state with biosynthetic and degradative processes in balance. Pulse-chase experiments favour the steady state alternative in which heparan sulphate is involved in continuous metabolic turnover with an observed half-life of less than 5 h.

Several studies have shown that the rat liver plasma-membrane components experience a differentiated but generally rapid turnover (Dehlinger & Schimke, 1971; Landry & Marceau, 1975). However, in comparison with previously studied rat liver membrane glycoproteins (Kawasaki & Yamashina, 1971), the half-life of heparan sulphate is exceptionally short.

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