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Degradation of heparin proteoglycan in cultured mouse mastocytoma cells

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Pulse-labelling of mouse mastocytoma cell cultures, established from ascites fluid, with inorganic [^{35}S]sulphate for 1 h yielded labelled heparin proteoglycan containing polysaccharide chains of M_r 60000–100000. After chase incubation for 24 h most of the ^{35}S appeared in intracellular polysaccharide fragments similar in size to commercially available heparin, M_r 5000–25000, as indicated by gel chromatography. Products isolated from cultures after 6 h of chase incubation consisted of partially degraded free polysaccharide chains and, in addition, residual proteoglycans that were of smaller size than the proteoglycans initially pulse-labelled. The polysaccharide chains released by alkali treatment from the residual chase-incubated proteoglycans were of the same size as the chains derived from proteoglycans after 1 h of pulse labelling. These results suggest that the intracellular degradation of heparin proteoglycan to polysaccharide fragments is initiated by release of intact polysaccharide chains, probably by action of a peptidase, and is pursued through cleavage of these chains by an endoglycosidase. An endoglucuronidase with stringent substrate specificity [Thunberg, Bäckström, Wasteson, Robinson, Ögren & Lindahl (1982) J. Biol. Chem. **257**, 10278–10282] has previously been implicated in the latter step. Cultures of more purified mastocytoma cells (essentially devoid of macrophages) did not metabolize [^{35}S]heparin proteoglycan to polysaccharide fragments, but instead accumulated free intact polysaccharide chains, i.e. the postulated intermediate of the complete degradation pathway. When such purified cells were co-cultured with adherent mouse peritoneal cells, presumably macrophages, formation of polysaccharide fragments was observed. It is tentatively proposed that the expression of endoglucuronidase activity by the mast cells depends on collaboration between these cells and macrophages.

INTRODUCTION

The blood-anticoagulant activity of heparin depends largely on the occurrence of a specific pentasaccharide sequence, which is capable of interacting with the proteinase inhibitor antithrombin (Björk & Lindahl, 1982; Lindahl *et al.*, 1984; Atha *et al.*, 1985). Heparin isolated for clinical use from animal tissues such as pig intestinal mucosa or bovine lung is generally recovered as single polysaccharide chains of M_r 5000–25000 (see, e.g., Lindahl *et al.*, 1965). Only about one-third of such chains carries the antithrombin-binding region, the remaining molecules being essentially devoid of anticoagulant activity (Lam *et al.*, 1976; Höök *et al.*, 1976; Andersson *et al.*, 1976). In contrast, other sources, such as rat skin (Horner, 1971) or rat peritoneal mast cells (Yurt *et al.*, 1977), yield a macromolecular form of heparin composed of several polysaccharide chains. This macromolecule was identified as a proteoglycan in which 10–15 polysaccharide chains, M_r 60000–100000, are attached to a peptide core sequence of alternating serine and glycine residues (Robinson *et al.*, 1978). The linkage between the polysaccharide chains, composed of sulphated (hexuronosyl-glucosaminyl)$_n$ disaccharide units, and the serine residues of the peptide core is mediated by the same galactosyl-galactosyl-xylosyl trisaccharide sequence that has been identified in a variety of other proteoglycans (Rodén, 1980; Fransson, 1985). More recent findings indicate that the antithrombin-binding regions are accumulated in a limited fraction of the heparin proteoglycan molecules, and in a limited number of the polysaccharide chains in this proteoglycan fraction (Jacobsson *et al.*, 1986).

The polysaccharide chains bound in heparin proteoglycans are generally longer (M_r 60000–100000) than the free heparin chains (M_r 5000–25000) isolated from various tissues. Mammalian endo-β-D-glucuronidases acting on heparin-like polysaccharides have been identified (Oldberg *et al.*, 1980; Thunberg *et al.*, 1982; and references cited therein). One such enzyme, prepared from mouse mastocytoma tissue (Ögren & Lindahl, 1975), was found to catalyse the cleavage of the extended polysaccharide chains, released from heparin proteoglycan by alkali treatment, to fragments similar in size to commercially available heparin (Robinson *et al.*, 1978). Pulse labelling *in vivo* of mastocytomal heparin with inorganic [^{35}S]sulphate implicated this enzyme in the intracellular modification of newly synthesized heparin proteoglycan (Ögren & Lindahl, 1976).

In the present study the mechanism of this degradation process has been examined in more detail, by using a cell-culture system for pulse–chase-labelling of the heparin proteoglycan.

EXPERIMENTAL

Materials

The Furth mast-cell tumour used in this study was maintained in (A/Sn × Leaden) F_1 mice as described by Ögren & Lindahl (1971). Heparin from pig intestinal mucosa was purified and *N*-[^{3}H]acetyl-labelled as

reported by Jacobsson *et al.*, (1985). Inorganic [^{35}S]sulphate (carrier-free) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Pronase, chondroitin ABC lyase and guanidinium chloride were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; DEAE-cellulose (DE-52) was from Whatman, Maidstone, Kent, U.K.; Zwittergent 3-12 was from Calbiochem A.G., Luzern, Switzerland; Sephadex G-50 and Sepharose 4B-CL were from Pharmacia Fine Chemicals, Uppsala, Sweden.

Methods

Hexuronic acid was determined by the carbazole method of Bitter & Muir (1962). Radioactivity was determined by scintillation counting in a Beckman model 3800 apparatus. Treatment of saccharides with nitrous acid (pH 1.5 procedure) was performed as described by Shively & Conrad (1976). Proteoglycans were converted into single polysaccharide chains by treatment with 0.5 M-NaOH/0.025 M-$NaBH_4$ at 4 °C for 15 h, followed by neutralization with 4 M-HCl. Galactosaminoglycans were degraded by incubation at 37 °C for 24 h with 0.2 unit of chondroitin ABC lyase (Yamagata *et al.*, 1968) in 0.5 ml of 0.05 M-Tris/HCl buffer, pH 8.0, containing 0.03 M-sodium acetate and 0.1 mg of bovine serum albumin/ml. Gel chromatography and ion-exchange chromatography of glycosaminoglycans were carried out as specified in the Figure legends.

Cell-culture experiments. Mast-cell heparin was labelled *in vitro* with [^{35}S]sulphate in two different types of experiments. In one approach mastocytoma cells were established in culture after passage through an ascites stage as described in detail by Jacobsson *et al.* (1985). Briefly, solid tumour tissue from the hind leg of a (A/Sn × Leaden) F_1 mouse was dispersed and injected intraperitoneally into another mouse of the same strain. After 10–14 days cultures were established from ascites fluid and maintained in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY, U.S.A.) containing 17% (v/v) inactivated newborn-calf serum, 60 μg of penicillin/ml and 50 μg of streptomycin/ml. Cultures in 250 ml flasks (75 cm^2; Nunc, Roskilde, Denmark) reached near-confluency in 10–14 days (approx. 20×10^6 cells/culture) and were then used in labelling experiments. Histological examination of such cultures revealed more than 50% mast cells and, in addition, numerous phagocytic cells identified as macrophages (Jacobsson *et al.*, 1985). To each culture was added 5 ml of medium containing 100 μCi of inorganic [^{35}S]sulphate/ml. After 1 h of incubation at 37 °C the medium was removed, and the cell layers were gently washed three times with the same medium but without radioactive sulphate. Some cultures were frozen at this stage for subsequent isolation of pulse-labelled polysaccharide (see below). To other cultures were added 20 ml of fresh medium, and chase incubations were maintained for either 6 h or 24 h. Polysaccharide was isolated as described below.

The second type of experiment was designed to provide mast cells of a higher degree of purity and to study such cells in co-culture with macrophages. Cultures were established from ascites-cell suspensions, as outlined above, and were maintained for 2 weeks with daily changes of medium. Mast cells in mitosis were readily detached from the substratum by gentle agitation of the flasks, and 5 ml of culture medium containing such suspended cells was transferred to a suspension-culture flask containing 500 ml of the same medium. The suspension culture was incubated at 37 °C in an atmosphere of CO_2/air (1:19) with constant stirring. Every 2–3 days approx. 150 ml of the cell suspension was replaced with fresh medium. After 14 days of culture the medium withdrawn contained approx. 10^6 cells/ml, which were essentially pure mast cells, as judged from their metachromatic staining with Toluidine Blue (Jacobsson *et al.*, 1985). Portions (20 ml) of such suspensions were transferred to 60 mm × 15 mm (approx. 28 cm^2) Falcon 3002 cell-culture dishes (Falcon, Oxnard, CA, U.S.A.) to generate mast-cell cultures. Alternatively, mixed mast-cell/macrophage cultures were established by seeding the mast cells on to similar culture dishes containing preformed macrophage cultures. Macrophages were obtained as described by Cohn & Benson (1965), by peritoneal washing of normal (A/Sn × Leaden) F_1 mice with physiological saline (ACO, Solna, Sweden; 4 ml/mouse). Cells from three mice (approx. 18×10^6 cells) were pooled and diluted to 20 ml, and 10 ml was added to each of two Falcon 3002 dishes. After non-adherent cells had been washed away after 2 h, each dish contained approx. 4×10^6 cells, mainly macrophages, as verified by the ability of these cells to phagocytose IgG-coated sheep erythrocytes (Munthe-Kaas *et al.*, 1975). After addition of 20 ml of mast-cell suspension, the resulting mixed mast-cell/macrophage cultures were incubated as described above for 10 days with daily changes of medium, as were the analogous 'pure' mast-cell cultures, before ^{35}S labelling. The cells were pulse-labelled for 1 h with 3 ml of medium, containing 100 μCi of inorganic [^{35}S]sulphate/ml, and some of the cultures were then chase-incubated for an additional 24 h period with 10 ml of medium devoid of label.

Isolation of polysaccharide. Two different procedures were employed to isolate labelled polysaccharide. One protocol involved solubilization of the cells by treatment with Zwittergent 3-12/guanidinium chloride, essentially as described by Kimura *et al.* (1981). To the frozen cell layers was added 3 ml of 8% (w/v) Zwittergent 3-12 in 0.05 M-sodium acetate buffer, pH 5.8, containing 10 mM-EDTA, 5 mM-benzamidine and 0.5 mM-phenylmethanesulphonyl fluoride. After 30 min at 4 °C, 3 ml of 8 M-guanidinium chloride in the same buffer (with proteinase inhibitors) was added, and incubation at 4 °C was continued for another 30 min. The solubilized material was applied to a column (3 cm × 18 cm) of Sephadex G-50, which was then eluted with 4 M-guanidinium chloride at a rate of 12 ml/h. Labelled polysaccharide emerged as a distinct peak at the void volume of the column and was concentrated by ultrafiltration before further analysis.

Alternatively, labelled polysaccharide was solubilized by digestion with Pronase, and was then isolated by ion-exchange chromatography on DEAE-cellulose. The procedures were as described by Jacobsson *et al.* (1985), except that alkali treatment of the labelled material was omitted. The yields of labelled polysaccharide did not differ significantly between the two isolation protocols adopted (results not shown). Further, the products were of approximately similar molecular size, as shown by gel chromatography (see the Results section), in agreement with the notion that the heparin proteoglycan is

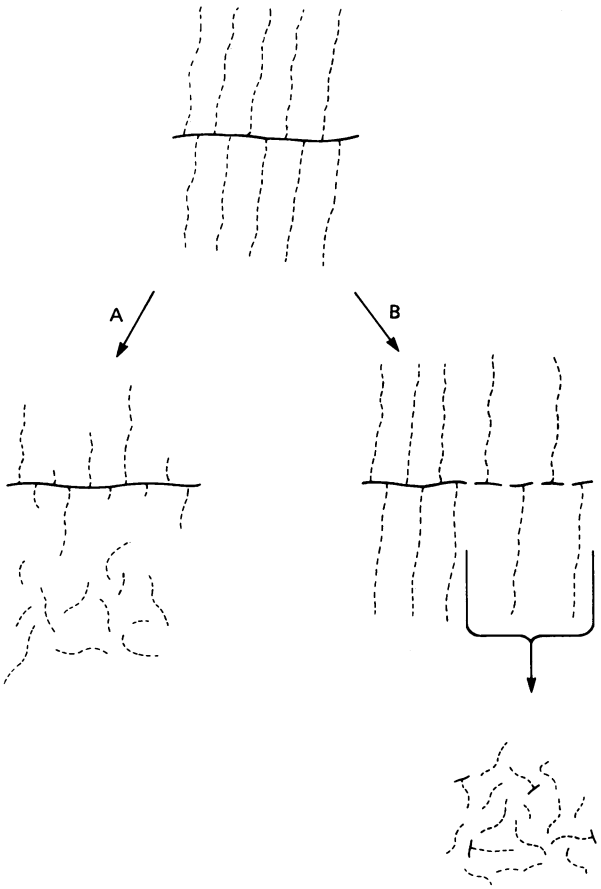

Fig. 1. Alternative mechanisms for the degradation of the heparin proteoglycan

In pathway A the polysaccharide chains are cleaved by an endoglucuronidase while still joined by the peptide core. Pathway B implies initial scission of the core (by an enzyme here arbitrarily assumed to operate from one end of the molecule towards the other), followed by degradation of the resulting free polysaccharide chains. Only the polysaccharide-substituted portion of the protein core (continuous line) is shown. The polysaccharide chains are depicted as broken lines. For additional information see the text.

essentially resistant to proteolytic cleavage (Yurt *et al.*, 1977; Robinson *et al.*, 1978).

RESULTS

The study was designed to differentiate between the two alternative modes of proteoglycan degradation outlined in Fig. 1. In pathway A the heparin proteoglycan is directly attacked by the endoglucuronidase, which cleaves the polysaccharide chains while they are still joined by the polypeptide core. Products formed halfway through the degradation process would presumably include partially degraded proteoglycans with truncated polysaccharide chains. Pathway B is initiated by release of intact polysaccharide chains from the proteoglycan; in a subsequent step these chains are fragmented by the endoglucuronidase. Partial degradation by this latter pathway would yield proteoglycan fragments with a decreased number of full-length polysaccharide chains.

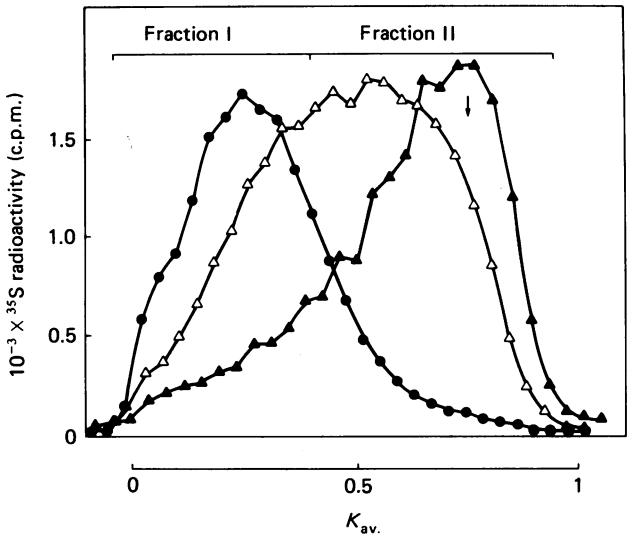

Fig. 2. Pulse–chase labelling of mastocytoma heparin

Three mastocytoma-cell cultures were pulse-labelled for 1 h (●) with inorganic [^{35}S]sulphate, and two of the cultures were then chase-incubated for 6 h (△) or 24 h (▲), as described in the Experimental section. Labelled polysaccharide was isolated by gel chromatography on Sephadex G-50 after solubilization of the cells with Zwittergent/guanidinium chloride (see the Experimental section), and the macromolecular fractions (approx. 0.7×10^6 c.p.m.) were separated further by gel chromatography on a column (1.2 cm × 140 cm) of Sepharose 4B-CL, as shown in the Figure. The column was eluted with 4 M-guanidinium chloride at a rate of approx. 10.5 ml/h. Effluent fractions (3.5 ml) were collected, analysed for radioactivity, and pooled into two fractions (I and II) as shown by the horizontal bars. The arrow indicates the peak elution position of pig mucosal heparin standard.

We attempted to discriminate between these alternatives by pulse–chase labelling of heparin proteoglycans in cultured mouse mastocytoma cells.

Experiments with mixed peritoneal-cell populations

Cultures established directly from ascites fluid contained mainly mast cells and macrophages (see the Experimental section). After labelling of such cultures for 1 h with inorganic [^{35}S]sulphate, solubilization of the cell layer with Zwittergent/guanidinium chloride yielded approx. 50×10^3 c.p.m./10^6 cells (approx. 1×10^6 c.p.m./culture) of macromolecular labelled material (excluded from Sephadex G-50). The pulse medium contained a total of 50×10^3 c.p.m. of ^{35}S-labelled macromolecular material, corresponding to approx. 5% of the cellular pool. After 24 h of chase the amounts of cellular labelled macromolecules had generally decreased, and ranged in separate experiments between 40 and 90% of that present after the 1 h pulse period (results not shown). At no time during the chase did the labelled macromolecular material in the medium exceed 6% of that in the cellular fraction. It thus appears probable that part of the labelled proteoglycan formed during the pulse period was degraded during chase incubation to products no longer excluded from Sephadex G-50. Such products appeared mainly in the culture medium and had

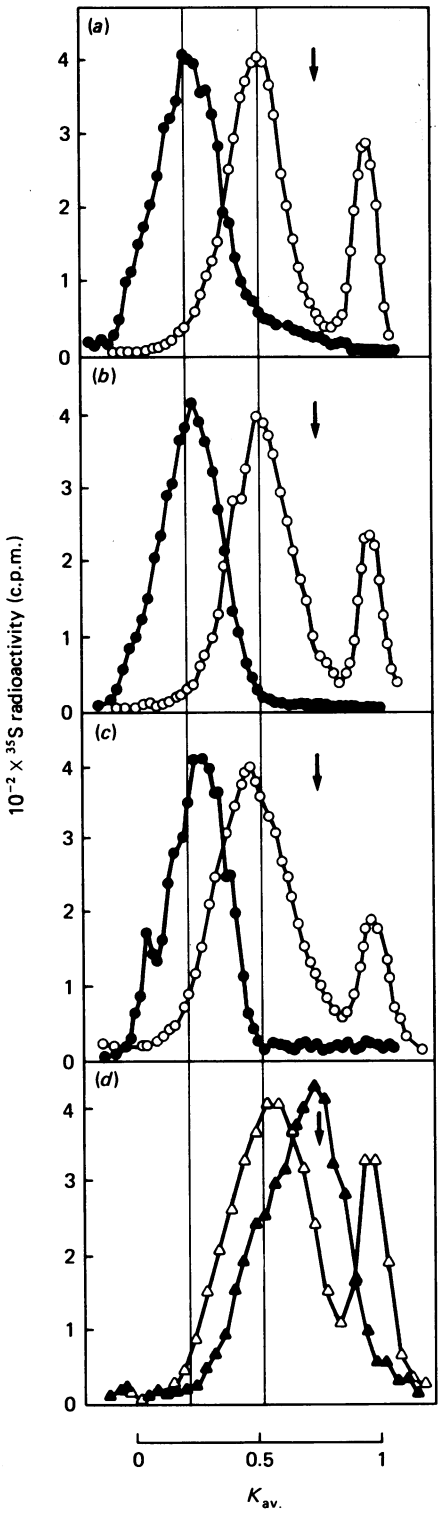

Fig. 3. Gel chromatography on Sepharose 4B of proteoglycan and polysaccharide chain fractions obtained after pulse–chase labelling of mastocytoma cultures

The polysaccharide samples shown in panels (*a*)–(*c*) were analysed before (●) and after (○) alkali treatment and represent: (*a*) fraction I of polysaccharide pulse-labelled for 1 h; (*b*) fraction I of polysaccharide isolated after a 6 h chase period; (*c*) fraction I of polysaccharide isolated after a 24 h chase period. Panel (*d*) shows fraction II of material obtained after chase incubation for 6 h (△) or 24 h (▲). The isolation of the various polysaccharide fractions is shown in Fig. 2. The samples were applied to a column (0.5 cm × 140 cm) of Sepharose 4B-CL that was eluted with 4 M-guanidinium chloride at a rate of 2 ml/h. Effluent fractions (approx. 0.65 ml) were collected and analysed for radioactivity. Before analysis the various alkali-treated fraction I samples (panels *a*–*c*) as well as fraction II from the 6 h chase (panel *d*) were dialysed, concentrated and digested with chondroitin ABC lyase (see the Experimental section), yielding the retarded peaks of ^{35}S radioactivity that emerged after the elution position (arrow) of the pig mucosal heparin standard.

an elution position similar to that of inorganic sulphate. This material, which corresponded in quantity roughly to the loss of macromolecular material, might represent a specific degradative pathway, akin to that described by Yanagishita & Hascall (1984) for heparan sulphate in rat ovarian granulosa cells.

Gel chromatography of the pulse-labelled material on Sepharose 4B-CL showed a fairly homogeneous distribution of activity with a peak $K_{av.}$ value of approx. 0.25 (Fig. 2). In contrast, the material recovered after 24 h of chase incubation was largely retarded, the major portion emerging at the same elution position as commercial heparin. The 6 h-chase product was of intermediate molecular size, broadly distributed over the chromatogram. These elution patterns were highly reproducible in separate experiments. Effluent fractions of the three samples were combined into two pools as indicated in Fig. 2, fraction I corresponding essentially to proteoglycans and fraction II to degradation products. On repeated gel chromatography fraction I showed an appreciable shift towards larger elution volume with increasing periods of chase incubation (Figs. 3*a*–3*c*). Treatment of the same fraction with alkali before gel chromatography resulted in the appearance of a distinct single peak with a $K_{av.}$ value of approx. 0.5, i.e. a value similar to that of intact polysaccharide chains released from rat skin heparin proteoglycan (M_r 60000–100000; Robinson *et al.*, 1978). It is significant that the elution positions of these polysaccharide chains were the same when the parent fraction I was derived from just pulse-labelled proteoglycan (Fig. 3*a*) or from material isolated after chase for 6 h (Fig. 3*b*) or 24 h (Fig. 3*c*). Digestion of the alkali-treated (neutralized, dialysed and concentrated) samples with chondroitin ABC lyase resulted in the formation of small-molecular-size components, presumably disaccharides, that emerged close to the V_t of the Sepharose 4B column (Fig. 3). The less-retarded peaks, corresponding to polysaccharide chains, were eliminated when the samples had been treated with nitrous acid at pH 1.5 before gel chromatography (results not shown). Thus a minor portion, approx. 20%, of the pulse-labelled proteoglycan consisted of chondroitin sulphate, whereas the major portion was heparin. These results clearly suggest that the residual polysaccharide chains in partially degraded proteoglycan molecules remain intact even through extended chase incubation.

Fraction II of the 6 h-chase material showed an elution position intermediate between that of the single polysaccharide chains released by alkali treatment of fraction I and that of fraction II from the 24 h-chase material (Fig. 3*d*). This elution profile did not change on alkali treatment of the polysaccharide (results not shown). It is concluded that fraction II of the 6 h-chase sample consists largely of partially degraded single

polysaccharide chains. Taken together, the data shown in Fig. 3 indicate that endoglycosidic cleavage of polysaccharide chains occurs only after the release of these chains from the proteoglycan structure, and thus strongly favour pathway B in Fig. 1.

Experiments with purified and with reconstituted mixed-cell populations

Pathway B implies the release of free intact polysaccharide chains as an intermediate of the degradation process (Fig. 1). Preliminary experiments with purified mast cells suggested, unexpectedly, an accumulation of such chains within the cells, and this observation formed the basis for the following experiment. A pulse–chase protocol, essentially similar to that described above, was designed with a 1 h pulse and a 24 h chase period. However, two types of cell cultures were employed, one involving purified mastocytoma cells only, the other a mixture of such cells and peritoneal macrophages (see the Experimental section). The latter cultures were designed to provide a cell population reasonably similar to that established in the experiments with ascites-cell cultures. Both types of cultures incorporated approx. 50×10^3 c.p.m. of ^{35}S into polysaccharide/10^6 cells during 1 h of incubation with inorganic [^{35}S]sulphate; approx. 90% of this radioactivity was recovered with the

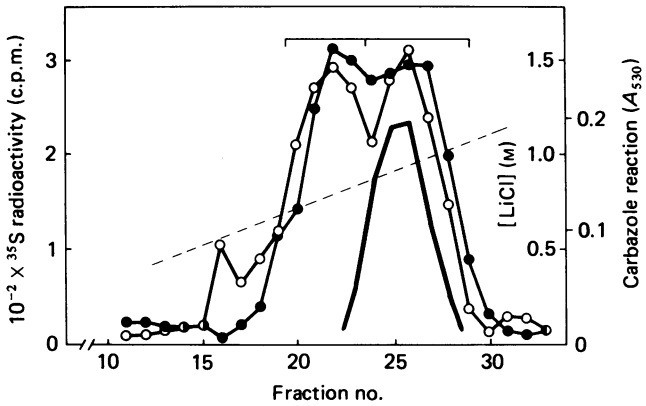

Fig. 4. Ion-exchange chromatography of [^{35}S]polysaccharide recovered from pulse-labelled cell cultures

Cell cultures were established from purified mastocytoma cells with (●) or without (○) added peritoneal cells as described in the Experimental section. Cultures were pulse-labelled with inorganic [^{35}S]sulphate for 1 h, and labelled polysaccharide was isolated either directly after the pulse (as shown in the Figure) or after a 24 h chase period (results not shown). For isolation of polysaccharide the cell layers were digested with Pronase and the digests were applied to columns (1 cm × 4 cm) of DEAE-cellulose, along with internal standards (2 mg) of pig mucosal heparin. The columns were washed with 30 ml of 0.05 M-LiCl in 0.05 M-acetate buffer, pH 4.0, and were then eluted at a rate of approx. 6 ml/h with a gradient (total volume 150 ml) of 0.05–1.2 M-LiCl in the same buffer (----). Fractions (3.5 ml) were collected and analysed for radioactivity (● and ○) and for hexuronic acid (carbazole reaction; —— indicates distribution of heparin standard, shown for one sample only but similar in the other). Fractions were pooled as indicated by the horizontal bars, and were dialysed and concentrated before further analysis. For further experimental details see the text.

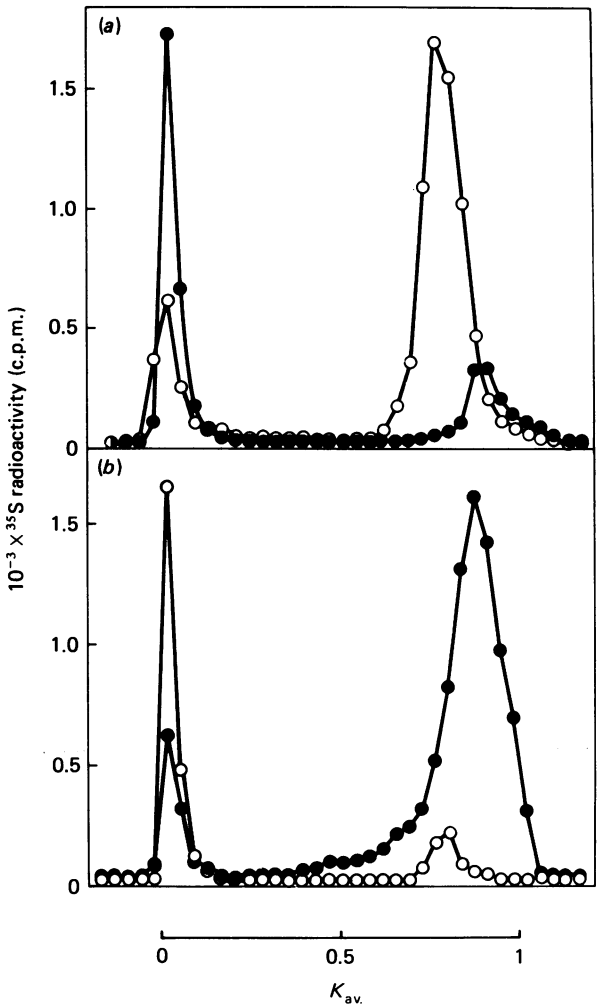

Fig. 5. Identification of [^{35}S]polysaccharide fractions by selective chemical or enzymic degradation

Polysaccharide isolated from purified mastocytoma cells after 1 h of labelling with inorganic [^{35}S]sulphate was separated into two fractions by ion-exchange chromatography, as shown in Fig. 4. The less-retarded fraction (panel *a*) and the more-retarded fraction (panel *b*) were each treated with nitrous acid at pH 1.5 (●) and digested with chondroitin ABC lyase (○). The products were separated on a column (1 cm × 90 cm) of Sephadex G-50, which was eluted with 1 M-NaCl at a rate of 4 ml/h. Effluent fractions (2 ml) were analysed for radioactivity. The corresponding polysaccharide fractions derived from a mixed mastocytoma-cell/macrophage culture (displayed in Fig. 4) and from 24 h-chase cultures (purified mastocytoma-cell and mixed cultures) yielded essentially the same degradation patterns as shown in the Figure (results not shown).

polysaccharide fraction after 24 h of chase incubation. Owing to a relatively high content of chondroitin [^{35}S]sulphate, the polysaccharide solubilized by Pronase digestion was fractionated by ion-exchange chromatography before further analysis (Fig. 4; shown for the 1 h-pulse samples only). Two poorly resolved peaks of ^{35}S radioactivity were obtained, the more retarded of which appeared at the same elution position as pig mucosal heparin. Fractions were pooled as indicated in Fig. 4 and analysed by selective enzymic and chemical

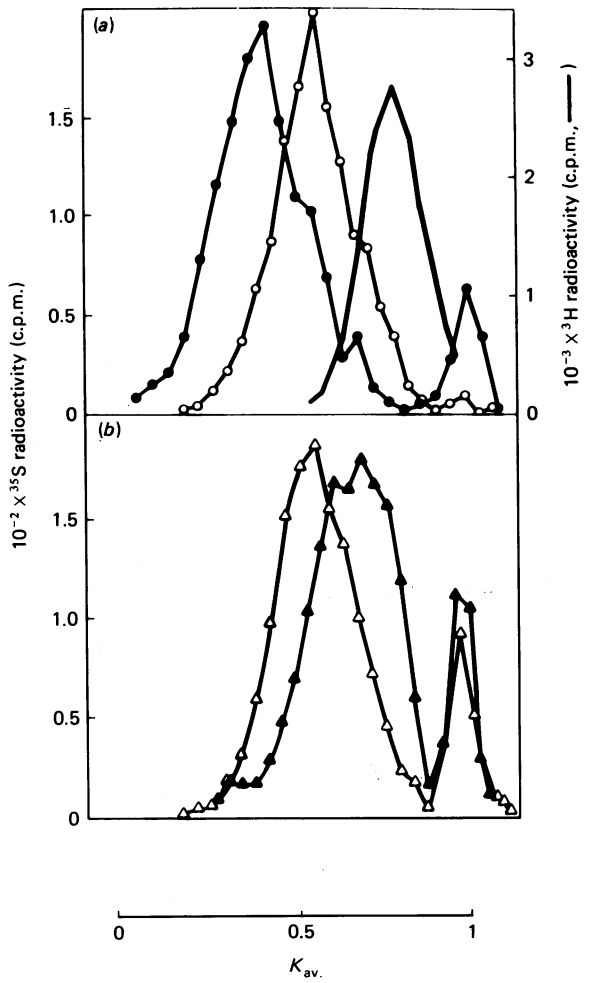

Fig. 6. Effect of macrophages on the degradation of heparin proteoglycan in mastocytoma cells

Purified mastocytoma cells were incubated for 1 h with inorganic [^{35}S]sulphate, and labelled heparin (most-retarded fraction in Fig. 4) was isolated by ion-exchange chromatography. The product was subjected to gel chromatography on a column (0.5 cm × 140 cm) of Sepharose 4B-CL, before (●) or after (○) alkali treatment (panel *a*). A sample of *N*-[^{3}H]acetyl-labelled pig mucosal heparin was added as internal standard (——). The minor peak of small-molecular-size components is due to digestion of the proteoglycan fraction with chondroitin ABC lyase before gel chromatography. The corresponding heparin proteoglycan isolated from similarly pulse-labelled mixed mastocytoma-cell/macrophage cultures yielded similar elution patterns to those in panel (*a*), both before and after alkali treatment (results not shown). Panel (*b*) shows the elution patterns of heparin fractions isolated from cultures of purified mastocytoma cells (△) or from mixed mastocytoma-cell/macrophage cultures (▲) that had been pulse-labelled with inorganic [^{35}S]sulphate for 1 h and then chase-incubated for 24 h. The minor retarded peaks are due to degradation of chondroitin sulphate contaminants by chondroitin ABC lyase. Alkali treatment of the samples before chromatography did not affect the elution patterns (results not shown). The column was eluted with 4 M-guanidinium chloride at a rate of 2 ml/h. Effluent fractions (approx. 0.65 ml) were analysed for radioactivity. For additional experimental details see the text.

degradation (Fig. 5). The least-retarded fraction was essentially resistant to degradation by nitrous acid, but was largely susceptible to digestion with chondroitinase ABC (Fig. 5*a*), whereas the more-retarded fraction reacted in the reverse fashion (Fig. 5*b*). The latter fraction, thus identified as heparin, was analysed further by gel chromatography on Sepharose 4B-CL. The pulse-labelled material emerged at the elution position of a proteoglycan, and was converted into single extended polysaccharide chains by treatment with alkali (Fig. 6*a*). The same results were obtained regardless of whether macrophages had been present during the incubation or not; moreover, these results did not differ significantly from those obtained with the corresponding samples from ascites-cell cultures (Fig. 3*a*). After 24 h of chase incubation, the labelled polysaccharide in the mastocytoma-cell cultures lacking macrophages had been apparently quantitatively converted into single undegraded chains (Fig. 6*b*). The elution position of this material was unaffected by previous alkali treatment (results not shown), and was similar to that of the alkali-treated pulse-labelled proteoglycan (Fig. 6*a*). With macrophages present in the cultures during the chase, the released polysaccharide chains were degraded further and approached the elution position of commercial pig mucosal heparin on subsequent gel chromatography (Fig. 6*b*). While the liberation of single, essentially undegraded, polysaccharide chains during chase incubation of purified mastocytoma cells was a consistent finding in repeated experiments, the presence of macrophages invariably induced the formation of polysaccharide fragments. However, the extent of such degradation varied from one experiment to another. The reason for this variability is unknown.

DISCUSSION

The results of the present study suggest that the intracellular degradation of the heparin proteoglycan to polysaccharide fragments in the mast cell occurs in two stages. In the first step intact polysaccharide chains are released from the proteoglycan, and these chains are subsequently degraded by an endoglycosidase (pathway B in Fig. 1). Major evidence in support of this pathway was the observation that partially degraded polysaccharide chains occurred in the free state only, and not as part of proteoglycans. Moreover, the formation of the postulated intermediate, i.e. free undegraded polysaccharide chains, was actually demonstrated in mastocytoma cultures devoid of macrophages.

The mechanism of chain release is unknown, and could involve cleavage of either the peptide core or the polysaccharide-protein linkage region. The former alternative appears more likely, considering the presence of residual, covalently bound, amino acids in low-M_r heparin preparations (Lindahl *et al.*, 1965). Pathways for the intracellular (Yanagishita & Hascall, 1984) or extracellular (Matzner *et al.*, 1985; Bar-Ner *et al.*, 1985) degradation of heparan sulphate proteoglycans have been proposed by which cleavage of the protein core precedes, and is possibly a prerequisite for, endoglycosidic attack of the polysaccharide chains. Indeed, work by Horner (1983) suggested a similar sequential mechanism for the degradation of rat skin heparin proteoglycan by platelet enzymes. The reason for the sequential order of proteolysis and glycan cleavage may

be that the polysaccharide chains are sterically inaccessible to the endoglycosidase as long as they are crowded in a proteoglycan structure. Intracellularly, the process may be further influenced by compartmentalization of the enzymes and their substrates. It may be noted that Yurt *et al.* (1977) did not observe degradation of ^{35}S-labelled heparin proteoglycan during extended culturing of rat peritoneal mast cells. Finally, we observe that previous studies on the mastocytoma endoglucuronidase involved either crude enzyme preparations [possibly containing peptidase(s) along with the glucuronidase] degrading a heparin proteoglycan substrate (Ögren & Lindahl, 1975) or partially purified glucuronidase acting on single polysaccharide chains (Robinson *et al.*, 1978; Thunberg *et al.*, 1982); the results were thus inconclusive in terms of any requirements of the enzyme with regard to the macromolecular properties of its substrate.

An intriguing observation of the present study was that the second step of the degradative process, i.e. the cleavage of the polysaccharide chains, appeared to depend on collaboration between the mast cells and other cells, tentatively identified as macrophages. The nature of this collaboration has not been investigated and at present remains obscure. The macrophages may either induce the synthesis of the endoglucuronidase in the mast cells, or somehow promote the intracellular interaction between this enzyme and its substrate, the free polysaccharide chains. Alternatively, the enzyme may actually have been synthesized by the macrophages, and then transferred to and taken up by the mast cells. Mast cells and macrophages often occur together in the tissues. Indeed, McNamara *et al.* (1985) have proposed that macrophages can transfer β-glucuronidase to co-cultured fibroblasts, either by direct cell-to-cell contact or indirectly via receptor mediated endocytosis. The endoglucuronidase implicated in the intracellular degradation of the heparin proteoglycan acts in a highly specific fashion, cleaving the polysaccharide chains only at glucuronidic linkages located between (rather than within) the antithrombin-binding regions (Thunberg *et al.*, 1982). It remains to be seen whether this enzyme is in fact derived from macrophages.

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