Glycosaminoglycan Synthesis in Mouse Mastocytoma

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The glycosaminoglycan synthesis in Furth solid mastocytoma tissue has been studied. Approx. 10% of the polysaccharide isolated after incubation *in vitro* with [^{14}C]-glucosamine was digestible with chondroitinase ABC and the product of digestion was identified as 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-4-*O*-sulpho-D-galactose. Similarly, labelling of polysaccharide *in vivo* with $^{35}\text{SO}_4^{2-}$ followed by isolation of mast-cell fractions by density-gradient centrifugation on colloidal silica revealed the presence of a polysaccharide which migrated as did chondroitin sulphate on electrophoresis in barium acetate. Chondroitinase ABC produced the same digestion product as before. Finally, the presence of the UDP-*N*-acetylgalactosamine-chondroitin 6-sulphate hexasaccharide *N*-acetylgalactosaminyltransferase previously implicated in chondroitin sulphate biosynthesis was demonstrated in microsomal particles from fractions of purified mast cells.

Work in several laboratories has established the mast cell as the site of synthesis and storage of heparin (for references see Selye, 1965). The presence of other glycosaminoglycans in the mast cells is, however, subject to debate. Horsfield & Summerly (1966) fractionated labelled polysaccharide material from rat peritoneal cells by stepwise elution from anionic-exchange resin. By correlating the elution positions of the various fractions with those of standard glycosaminoglycans, these authors claimed that their mast-cell preparation contained all the known mucopolysaccharides. Since no further characterization of the polysaccharide material was undertaken, this conclusion must be viewed with caution, in particular when it is considered that labelled heparin of different degrees of sulphation, isolated from mast cells, would be similarly fractionated by anion-exchange chromatography into a number of subfractions (Ringertz, 1960a).

Although heparin was the only polysaccharide component found in mast cells from peritoneal washings of rats (Schiller & Dorfman, 1959; Bloom & Ringertz, 1960) and mice (Bloom & Ringertz, 1960), several reports have appeared on the presence in mast-cell tumours of galactosamine-containing polysaccharides with sulphate contents lower than that of heparin (Magnusson & Larsson, 1955; Korn, 1958; Ringertz, 1960b; Rodén, 1960).

In recent experiments, 30% of the total hexosamine content of a polysaccharide preparation from mouse mastocytoma tissue was found to be galactosamine (S. Ögren & U. Lindahl, unpublished work). Further, part of the preparation migrated like chondroitin sulphate on electrophoresis and was susceptible to treatment with chondroitinase ABC. However, the presence in the tumour tissue of significant amounts

of connective tissue would seem to present a severe obstacle in determining the cellular origin of any polysaccharides other than heparin.

The present investigation was undertaken to obtain more conclusive evidence about the nature of the polysaccharides present in a mast-cell tumour maintained in mice. The use of density-gradient centrifugation in colloidal silica and polyethylene glycol afforded fractions of mast cells that were utilized for experiments *in vitro*. Polysaccharide labelled *in vivo* could be isolated from the mast-cell fractions and characterized. Evidence is presented indicating that the newly synthesized polysaccharide isolated from the mast cells contains approx. 10% chondroitin 4-sulphate.

Experimental

Materials

The mast-cell tumour was that described by Furth *et al.* (1957). It has been maintained as a solid tumour in (A/Sn $\times$ Leaden) F_1 mice by subcutaneous and intramuscular transplantation in the hind legs every 10–14 days.

Pronase, chondroitinase ABC (EC 4.2), chondro-4-sulphatase (EC 3.1.6) and chondro-6-sulphatase (EC 3.1.6.4) were purchased from Miles Laboratories, Elkhart, Ind., U.S.A. Whereas the chondro-4-sulphatase hydrolysed only the standard Δ Di-4S,*

* Abbreviations: Δ Di-4S, 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-4-*O*-sulpho-D-galactose; Δ Di-6S, 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-6-*O*-sulpho-D-galactose; Δ Di-OS, 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-D-galactose.

the chondro-6-sulphatase preparation supplied was found to contain chondro-4-sulphatase activity as well as chondro-6-sulphatase activity. Testicular hyaluronidase (EC 3.2.1.35; 14000 units/mg) was a product of Leo, Hälsingborg, Sweden.

The disaccharides Δ Di-4S, Δ Di-6S and Δ Di-OS were obtained from Miles Laboratories. Chondroitin 6-sulphate was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Hexasaccharide from chondroitin 6-sulphate was prepared by digestion of the latter (200mg) with hyaluronidase (1mg; 24h at 37°C) in 200ml of 0.1M-acetate buffer, pH 5.0, containing 0.15M-NaCl, followed by gel chromatography in 0.2M-NaCl on a column (2cm $\times$ 180cm) of Sephadex G-50 (medium grade). The material emerging at an effluent volume of 300–320ml was pooled, desalted on a column (2cm $\times$ 40cm) of Sephadex G-10 and further purified by preparative paper chromatography in system (A) (see below). The hexasaccharide fraction (R_{GlcA} 0.12 in this solvent; cf. Telser *et al.*, 1966) contained equimolar amounts of glucuronic acid and galactosamine, whereas glucosamine was absent, as revealed by amino acid analysis.

[^{14}C]Glucosamine (56 $\mu\text{Ci}/\mu\text{mol}$) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. UDP-*N*-acetylhexosamine (UDP-HexNAc), a mixture of UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine (molar ratio, 2.2:1), of specific radioactivity 9.2×10^5 c.p.m./ μmol , was prepared from 100 μCi of [^{14}C]glucosamine as described by O'Brien (1966). The yield was 4.5 μmol of UDP-*N*-acetylhexosamine. Carrier-free $\text{Na}_2^{35}\text{SO}_4$ was supplied by The Radiochemical Centre.

Methods

Analytical methods. Uronic acid was determined by the method of Dische (1947) as modified by Bitter & Muir (1962). Hexosamine analysis was performed by an automatic amino acid analyser after hydrolysis in 6M-HCl at 100°C for 24h *in vacuo*. Protein was determined by the method of Lowry *et al.* (1951) with γ -globulin as standard.

Paper chromatography was performed on Whatman no. 3MM papers in the following systems: (A) butan-1-ol–pyridine–acetic acid–water (15:10:3:12, by vol.); (B) isobutyric acid–0.5M- NH_3 (5:3, v/v); (C) ethyl acetate–pyridine–water (2:1:2, by vol.; upper phase).

Paper electrophoresis was conducted in buffer (D), 0.080M-pyridine–0.046M-acetic acid, pH 5.3, at 80 V/cm, usually for 60min. Papers were stained with a silver dip reagent (Smith, 1960). Electrophoresis of polysaccharides was carried out on strips of cellulose acetate in 0.1M-barium acetate or in 0.1M-HCl as described by Wessler (1968, 1971). Paper strips were analysed for radioactivity by a Packard model 7201

strip scanner, kindly made available by Professor John Sjöquist. For quantitative results, the areas containing radioactive products were eluted with water and the radioactivity was determined in a Beckman model LS-250 liquid-scintillation spectrometer. Strips of cellulose acetate were cut into small segments which were dissolved in the scintillation fluid [containing 0.5% (w/v) 2,5-diphenyloxazole and 10% (w/v) naphthalene in dioxan] and counted for radioactivity directly.

Enzymic digestions. Digestion with Pronase (10mg) was carried out in 1ml of 0.1M-tris-HCl buffer, pH 7.6, containing 1 μmol of CaCl_2 and 7% (v/v) ethanol. The digestion was continued for 36h at 55°C, with a second addition of Pronase after 12h. Digestion of polysaccharide with chondroitinase ABC was performed in 0.5–1.0ml of 0.2M-tris-HCl buffer, pH 8.0, for 24h with 0.1–0.2 unit of enzyme. The buffer employed for digestion with chondro-4-sulphatase contained: 0.05M-tris-HCl–0.2M-sodium acetate, pH 8.0; bovine serum albumin (16 μg); substrate and enzyme (0.08 unit); total volume, 0.06ml. After 2h at 37°C, the reaction was stopped by heating for 2min and the products were analysed by paper electrophoresis in buffer (D).

Incorporation *in vitro* of [^{14}C]hexosamine into mastocytoma glycosaminoglycans. Tumours from two mice (approx. 2g of tissue) were strained through a wire mesh and the dispersed cells were incubated for 4h in 5ml of Krebs–Ringer phosphate buffer, pH 7.4 (Umbreit *et al.*, 1964), containing 20 μCi of [^{14}C]glucosamine (56 $\mu\text{Ci}/\mu\text{mol}$). Polysaccharide material was isolated by digestion of the cell suspension with Pronase followed by repeated precipitation with cetylpyridinium chloride from 0.4M-NaCl (Scott, 1960). The radioactive polysaccharide (8000 c.p.m.) was subsequently passed through a column of Sephadex G-25, as described in the legend to Fig. 1. The void-volume fractions, which contained all the radioactivity, were pooled, freeze-dried and digested with 0.2 unit of chondroitinase ABC. The digest was then analysed by chromatography on the Sephadex column.

Incorporation *in vivo* of [^{35}S]sulphate into mast-cell glycosaminoglycans. Four tumour-bearing mice were injected intraperitoneally with 0.8mCi of carrier-free $\text{Na}_2^{35}\text{SO}_4$ and killed 5h later. The tumours were removed and the cells dispersed as described by Pertoft (1970). Centrifugation on a continuous gradient of colloidal silica gave four bands of mast cells at different densities (Pertoft, 1970). The polysaccharides were isolated from each cell fraction by digestion with Pronase and precipitation with cetylpyridinium chloride as described above. The material was subsequently analysed by electrophoresis on strips of cellulose acetate and, after digestion with chondroitinase, by paper electrophoresis in buffer (D).

Transfer of N-acetylgalactosamine from UDP-N-acetylhexosamine to chondroitin 6-sulphate hexasaccharide. Five mast-cell tumours were dispersed and separated into four cell fractions by centrifugation on colloidal silica (Pertoft, 1970). The cells from each fraction were suspended in about 3 ml of a buffer containing 50 mM-tris-acetate buffer, pH 7.0, 70 mM-KCl, 1 mM-EDTA and 10 mM-MnCl₂, and ruptured in a bacteria press at -30°C (Edebo, 1960). After centrifugation at 10000g for 10 min, the supernatant was centrifuged at 100000g for 60 min. The resulting pellet was suspended in 0.5 ml of the above buffer and samples were incubated with hexasaccharide from chondroitin 6-sulphate and UDP-N-acetyl[¹⁴C]hexosamine. The incubation procedure and subsequent product analysis are described in detail in Table 1.

The radioactive hexosamine residue that had been transferred to the acceptor hexasaccharide was identified after hydrolysis of the product in 1 M-H₂SO₄ at 100°C for 3 h. The hydrolysate was neutralized by the addition of BaCO₃, reacylated (Wheat, 1966) and subjected to paper chromatography on borate-treated paper in system (C) (Spiro, 1966).

Results

Incorporation in vitro of [¹⁴C]hexosamine into chondroitinase-digestible polysaccharide

The polysaccharide isolated after incubation *in vitro* of dispersed tumour cells with [¹⁴C]glucosamine appeared with the void volume on chromatography on Sephadex G-25 (Fig. 1a). After digestion with chondroitinase ABC, a portion of the radioactivity (860 c.p.m.; 11%) emerged after the void-volume peak (Fig. 1b). On electrophoresis in buffer (D), the retarded material migrated at the rate of Δ Di-4S or Δ Di-6S, ahead of glucuronic acid. Further, paper chromatography in solvent (B) of this material showed that all the radioactivity migrated as did Δ Di-4S, clearly separated from the 6-sulphated isomer. In addition, chromatography on a column (1 cm $\times$ 95 cm) of Sephadex G-25 in 1 M-KCl showed that the radioactive compound co-chromatographed with Δ Di-4S standard. Finally, treatment with chondro-4-sulphatase converted the radioactive substance into Δ Di-OS, as evidenced by paper electrophoresis in buffer (D), thus establishing the identity of the radioactive product obtained after digestion with chondroitinase ABC as Δ Di-4S.

Sulphation in vivo of mast-cell glycosaminoglycans

To investigate whether the chondroitinase-digestible polysaccharide did indeed originate in the mast cells of the tumour it was necessary to separate these cells from connective-tissue elements that might

be present in crude homogenates of the tumour. After labelling of polysaccharides *in vivo* with Na₂³⁵SO₄, mast-cell fractions apparently devoid of other cell types were recovered from the tumours by gradient centrifugation on colloidal silica. Most of the cells appeared at densities 1.06 and 1.08. Figs. 2 and 3 show the patterns obtained when samples of polysaccharides isolated from either of these cell fractions were subjected to electrophoresis in barium acetate and in 0.1 M-HCl respectively. The separation obtained by electrophoresis in barium acetate depends primarily on the structure of the polysaccharide back-bone whereas the sulphate content is of minor importance (Wessler, 1968). In contrast, on electrophoresis in hydrochloric acid the migration rate of a polysaccharide reflects essentially its degree of sulphation (Wessler, 1971). With both media the stained electrophoretograms showed spots migrating like standard heparin and chondroitin sulphate. On analysis for radioactivity, about 10% of the label had migrated like chondroitin sulphate, whereas most of the remainder exhibited a pattern characteristic for heparin. Digestion with chondroitinase of ³⁵S-labelled polysaccharide from mast-cell bands released 8–9% of the radioactivity, which migrated

Fig. 1. Gel chromatography on Sephadex G-25 of ¹⁴C-labelled polysaccharide from mouse mastocytoma

(a) The column (2 cm $\times$ 120 cm) was eluted with 10% (v/v) ethanol at a rate of 10 ml/h. Effluent fractions were analysed for uronic acid (●) and for radioactivity (▲). The material from peak V₀ (8000 c.p.m.) was concentrated and digested with chondroitinase ABC. (b) Subsequent analysis on the Sephadex column yielded the retarded peak Δ Di-4S, which was subjected to further characterization.

Fig. 2. Electrophoresis in barium acetate buffer of polysaccharides isolated from mast-cell fractions after labelling *in vivo* with $^{35}\text{SO}_4^{2-}$.

The stained strip was cut into small segments, which were dissolved in scintillation fluid (see the Methods section) for determination of radioactivity. It should be noted that on electrophoresis in this medium (Wessler, 1968) as well as in 0.1M-HCl (cf. Fig. 3), standard heparin yields two spots, one of which represents polysaccharide which precipitates at the site of application. (I), Standard heparin; (II), $^{35}\text{SO}_4^{2-}$ -labelled polysaccharides from mast cells; (III), standard chondroitin sulphate (fast component) and standard dermatan sulphate (slow component).

at the rate of a sulphated disaccharide on electrophoresis in buffer (D). Again, paper chromatography in solvent (B) showed the presence of Δ Di-4S. These results establish the presence in the mast cells of a polysaccharide resembling chondroitin 4-sulphate both in electrophoretic migration rate and by its conversion into Δ Di-4S on treatment with chondroitinase ABC.

Fig. 3. Electrophoresis in 0.1M-hydrochloric acid of mast-cell polysaccharides

(I), Standard heparin; (II), $^{35}\text{SO}_4^{2-}$ -labelled polysaccharides from mast cells; (III), standard chondroitin sulphate (fast component) and standard hyaluronic acid (slow component). For other details see Fig. 2.

Transfer of N-acetyl[^{14}C]galactosamine from UDP-N-acetyl[^{14}C]hexosamine to chondroitin 6-sulphate hexasaccharide in a cell-free system from purified mast cells

Evidence was also obtained for the presence in the mast-cell fractions of the *N*-acetylgalactosaminyl-transferase previously described (Telser *et al.*, 1966), which has been implicated in the polymerization of chondroitin sulphate chains from the requisite nucleotide sugars. This enzyme was first studied in embryonic chick cartilage, where it was shown to catalyse the transfer of *N*-acetylgalactosamine to a hexasaccharide from chondroitin 6-sulphate.

Table 1 summarizes the amounts of product

Table 1. *Transfer of N-acetylgalactosamine from UDP-N-acetylgalactosamine to chondroitin 6-sulphate hexasaccharide*

Each tube contained the 100000g pellet fraction from one mast-cell fraction, UDP-*N*-acetyl[¹⁴C]hexosamine (5×10⁴ c.p.m.; molar ratio of UDP-*N*-acetylglucosamine to UDP-*N*-acetylgalactosamine, 2.2:1) and hexasaccharide from chondroitin 6-sulphate (0.6 μmol), in a total volume of 0.06 ml. After 2 h at 37°C, the reaction mixtures were heated for 3 min in a boiling-water bath, spotted on paper and subjected to electrophoresis in buffer (D) for 75 min. The product migrated faster than UDP-*N*-acetylhexosamine or degradation products thereof, and slightly slower than the hexasaccharide acceptor. In the absence of acceptor, no product formation was observed. The distribution of radioactivity on the electrophoretogram was similar to that observed when the hexasaccharide was incubated together with UDP-*N*-acetylhexosamine and a particulate fraction from chick embryo epiphyse (cf. Telser *et al.*, 1966).

Mast-cell band density	Enzymic activity	
	Radioactivity of product (c.p.m.)	Radioactivity of product (c.p.m./mg of protein)
1.06	4050	900
1.08	1600	1160
1.12	550	1100
1.15	75	195

Fig. 4. [¹⁴C]Hexosamine analysis of product formed from chondroitin 6-sulphate hexasaccharide and UDP-*N*-acetyl[¹⁴C]hexosamine

After hydrolysis of the presumed heptasaccharide product (1 M-H₂SO₄; 3 h at 100°C), the material was reacylated and subjected to paper chromatography in solvent (C). The radioscan pattern should be correlated with that of the authentic standards shown below the tracing: (I), *N*-acetylglucosamine; (II), *N*-acetylgalactosamine.

formed when the 100000g pellets from mast-cell fractions appearing at increasing densities were incubated with a mixture of UDP-*N*-acetyl[¹⁴C]-glucosamine, UDP-*N*-acetyl[¹⁴C]galactosamine and chondroitin 6-sulphate hexasaccharide. The assay, described in Table 1, differs somewhat from the procedure previously described (Telser *et al.*, 1966). The highest total enzymic activity was found in the fractions appearing at densities 1.06 and 1.08 where most of the cells had banded. The specific enzymic activity was more evenly distributed.

Acid hydrolysis of the product followed by reacylation and chromatography in solvent (C) revealed that all the radioactivity migrated similarly to *N*-acetylgalactosamine, well behind the *N*-acetylglucosamine standard (Fig. 4). This would indicate that UDP-*N*-acetylglucosamine was inactive as a substrate for the UDP-*N*-acetylgalactosamine-chondroitin 6-sulphate hexasaccharide *N*-acetylgalactosaminyltransferase present in the mast-cell preparation, in agreement with earlier observations on the enzyme from embryonic chick cartilage (Telser, 1968).

Discussion

Although the presence of galactosamine-containing polysaccharides in mast-cell tumours is well established, it was considered necessary to obtain more definite information about the nature of these polysaccharides. Also, it has been unclear whether they derive from the mast cell or from the connective-tissue elements of the tumour. The occurrence in mouse mastocytoma of galactosaminoglycans would seem to be of particular relevance with regard to the use of such tumours in studies on heparin biosynthesis. In several studies the incorporation of radioactive precursors into mast-cell glycosaminoglycans has been considered equivalent to the formation of heparin.

The results obtained here establish the presence in the mast cell of newly sulphated chondroitin 4-sulphate corresponding to approx. 10% of the total [³⁵S]sulphate incorporated into the glycosaminoglycans. Since chondroitin sulphate usually contains less sulphate per disaccharide than heparin, the

former polysaccharide would be expected to make up a larger percentage of the total glycosaminoglycan fraction when estimated after labelling with [^{14}C]-glucosamine. However, the ^{14}C -labelled fraction susceptible to chondroitinase only slightly surpassed the corresponding value for ^{35}S -labelled polysaccharides. The reason for this is unknown but the high sulphate value may possibly be due to the presence of over-sulphated chondroitin sulphate.

The presence in the mast-cell fractions of an *N*-acetylgalactosaminyltransferase previously implicated in the biosynthesis of chondroitin sulphate supports the conclusion that this polysaccharide is indeed synthesized in the neoplastic mast cell. In contrast, Silbert (1963) found no evidence for the incorporation of galactosamine into ^{14}C -labelled polysaccharide in the mouse mastocytoma (DBA) described by Dunn & Potter (1957). In this case, however, the polysaccharide was isolated after incubation with UDP-*N*-acetyl[^{14}C]glucosamine, and the result may be explained by the absence of UDP-*N*-acetylglucosamine epimerase activity. It is noteworthy that the polysaccharide isolated from DBA mast-cell tumours after labelling with [^{14}C]glucuronic acid was claimed to be resistant to digestion with testicular hyaluronidase (Silbert, 1963). It is thus conceivable that the DBA tumour may differ from the tumour studied here in its capacity to synthesize glycosaminoglycans other than heparin.

The peak in Fig. 2, indicated by an arrow, supposedly representing the portion of the heparin that migrates on barium acetate electrophoresis, includes radioactivity with a mobility similar to that of dermatan sulphate. The stained electrophoretogram also showed the presence of a spot, which was poorly resolved from heparin as well as from the dermatan sulphate standard. Further work is needed to establish whether this polysaccharide may also be present in small quantities in the mastocytoma studied here.

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