Biosynthesis of Glycosaminoglycans by Cultured Mastocytoma Cells

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Biosynthesis of glycosaminoglycans by several lines of cultured neoplastic mouse mast cells was studied by incorporation of [^{35}S]sulphate (and in some cases [$6\text{-}^3\text{H}$]glucosamine) into macromolecular materials found in both the cells and their growth media. Such intracellular and extracellular radioactively labelled materials (shown to be glycosaminoglycans by susceptibility to digestion with heparinase) were further characterized by ion-exchange chromatography and by digestion with testicular hyaluronidase and chondroitinase. All but one cell line produced chondroitin sulphate as the major sulphated glycosaminoglycan; the remainder of the glycosaminoglycan was heparin-like material. No [^3H]hyaluronic acid was synthesized. Cells of a newly derived line, termed P815S, synthesized more glycosaminoglycan than the other lines. This glycosaminoglycan, found in both cells and growth medium, was almost entirely chondroitin 4-sulphate. No chondroitin 6-sulphate was found. The chondroitin 4-sulphate from the cells was shown by gel filtration to be smaller than the chondroitin 4-sulphate in the media of these cultures. This discovery of relatively high proportions of chondroitin 4-sulphate in these mastocytoma-derived cells is noteworthy, since mast cells have generally been considered to produce heparin as their major glycosaminoglycan.

Glycosaminoglycans are synthesized as a special function of differentiated connective-tissue cells for export to the extracellular matrix or for storage in intracellular granules. In addition, recent studies have shown that many cell types (not just highly differentiated ones of a connective-tissue origin) can synthesize smaller amounts of sulphated glycosaminoglycans (Kraemer, 1971*a,b*; Dietrich & Montes De Oca, 1970; Bates & Levene, 1971; Suzuki *et al.*, 1970). Some of these glycosaminoglycans have been reported to be found in the cell membranes (Kraemer, 1971*a*). The possible importance to the cell of such membrane-bound glycosaminoglycans is suggested by recent studies (Suzuki *et al.*, 1970) which have shown that the surface charge of certain types can be markedly altered by treatment with glycosaminoglycan-degrading enzymes.

This laboratory has initiated an investigation of the cellular metabolism of glycosaminoglycans and the possible role of these substances in cell structure. For such studies it was decided to use cell cultures *in vitro*. A search was begun for cell lines satisfying several criteria: (1) cultures should be homogeneous; (2) cells must have glycosaminoglycan-synthesizing activity great enough to be experimentally useful; (3) such cells should be able to be grown easily in quantities large enough to permit the preparation of subcellular fractions.

Several long-established cell lines derived from mouse mastocytomas were available and offered promise of satisfying the necessary criteria. Some of

these cell lines have previously been used for studies of heparin biosynthesis (Green & Day, 1960) as well as for other cellular studies including those of Green & Day (1963), Wheldrake & Pasternak (1968), Pasternak & Bergeron (1970) and Thomas (1968). We report here the results of a survey of several long-established and newly derived mouse mastocytoma cell lines. The biosynthesis of glycosaminoglycans by cells of these mastocytoma lines was analysed both quantitatively and qualitatively. The accompanying paper (Lewis *et al.*, 1973) extends this survey to an examination of glycosaminoglycan biosynthesis by subcellular preparations from the same mastocytoma cells. The results of these studies allow some comparison of cellular and subcellular glycosaminoglycan-synthesizing activity.

Experimental

Materials

Materials were obtained from the following sources: culture media formulations, serums and trypsin solutions, Grand Island Biological Co., Grand Island, N.Y., U.S.A.; culture flasks, Falcon Plastics, Los Angeles, Calif., U.S.A.; dimethyl sulphoxide, Fisher Chemical Co., Boston, Mass., U.S.A.; sodium [^{35}S]sulphate (0.34 Ci/mmol) and [$6\text{-}^3\text{H}$]glucosamine (1.3 Ci/mmol), New England Nuclear Corp., Boston, Mass., U.S.A.; pancreatin (grade VI), testicular hyaluronidase (type I), hyaluronic acid (grade I) and

heparin (grade IV), Sigma Chemical Co., St. Louis, Mo., U.S.A.; *Flavobacterium* heparinase, courtesy of Dr. A. Linker, Veterans Administration Hospital, Salt Lake City, Utah, U.S.A.; chondroitinase ABC, chondroitin 4-sulphate, chondroitin 6-sulphate, Δ Di-OS [2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-D-galactose], 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, Miles Laboratory, Kankakee, Ill., U.S.A.; Di-4S (acetylchondrosine-4S), prepared by chondroitinase digestion of tetrasaccharides; Blue Dextran 2000, Sephadex G-50 and Sepharose 4B, Pharmacia Fine Chemicals, Piscataway, N.J., U.S.A.; DEAE-cellulose (Cellex-D), Bio-Rad Laboratories, Richmond, Calif., U.S.A.; crystallized bovine serum albumin, Armour Pharmaceutical Co., Kankakee, Ill., U.S.A.

Methods

Culture techniques. Cells were cultured in 250 ml tissue-culture flasks containing 12 ml of medium supplemented with 10% (v/v) serum. As a routine, Fischer's medium (Fischer & Sartorelli, 1964) supplemented with horse serum was used. For special studies, the F-12 medium of Ham (1965) and MEM medium of Eagle (1959) were used with supplements of horse serum or foetal calf serum. No antibiotics were added to the cultures at any time. The flasks were maintained in an environment of CO₂+air (5:95) at 37°C. Mastocytoma cells do not adhere to the flask surface; therefore the cells could be subcultured twice weekly by agitating the culture and transferring portions of the resulting suspension by using an approx. 1:200 (v/v) transfer ratio. Mastocytoma cells grown in this manner had a mean generation time of 10–12 h. This technique of culturing mastocytoma cells in tissue-culture flasks, rather than in culture tubes, allowed for more efficient gas exchange between culture liquid and culture gas phases.

Cell sources. The following established cell lines were used: P815Y (X-2) and X163 (subline of X-1) clonally derived by Schindler *et al.* (1959) from the Dunn–Potter mouse mastocytoma (Dunn & Potter, 1957), and HC73 (Mannaioni *et al.*, 1965) clonally derived from the Furth–Hagen–Hirsch mouse mastocytoma (Furth *et al.*, 1957). These cell lines have been kept in a frozen cell bank by Dr. G. A. Fischer, who kindly provided the starting cultures.

A new cell line, termed P815S, was derived from a solid Dunn–Potter mastocytoma line which has been carried *in vivo* in this laboratory for more than 10 years. Free cells were obtained by mincing the tissue followed by brief trypsin treatment [0.25% (w/v) trypsin solution]. The trypsin-treated mince was washed several times by centrifugation (400g) in

growth medium and the cells were then grown under routine culture conditions.

The Dunn–Potter ascites-tumour cells, source of a new line designated P815AS, were obtained from ascites-tumour-bearing mice provided by Arthur D. Little Inc., Cambridge, Mass., U.S.A. Ascites fluid from these mice was centrifuged (400g) and the resulting sedimented cells were cultured under routine conditions.

Neither the P815S nor the P815AS lines were cloned. However, the cells of both lines appeared uniform and grew in a free-floating fashion (not adhering to the flask surface). This is a typical characteristic of mastocytoma cell cultures and a characteristic not shared by fibroblasts. Cells of the two newly derived lines reinitiated tumours when inoculated into DBA/2 mice.

A frozen bank (–195°C) of all cell lines was established, by using the slow-freezing technique of Hauschka *et al.* (1959) with a freezing medium consisting of growth medium–horse serum–dimethyl sulphoxide (8:1:1, by vol.). Frozen stocks were periodically used to reinitiate cultures.

Determination of glycosaminoglycan production. Sodium [³⁵S]sulphate (116×10⁶ c.p.m.) was added to flasks containing an initial culture density of approx. 10⁴ cells/ml. For double-labelling experiments, [6-³H]glucosamine (20×10⁶ c.p.m.) was also added. After 3 days' growth, the cells were separated from the growth medium by centrifugation (400g).

The combined cells from two flasks were washed four times by centrifugation (400g) with phosphate-buffered saline (Dulbecco & Vogt, 1954) and then homogenized in 5 ml of water with a Teflon Potter–Elvehjem homogenizer. Complete cell disruption was observed after approximately 30 s at a speed of 1–2000 rev./min. Samples were analysed for cell protein. The homogenates were then boiled and incubated in 0.05 M-Tris buffer, pH 9.0, at 37°C with 1 mg of pancreatin. Several drops of toluene were added to retard bacterial growth. After the first day of incubation, the pancreatin and toluene were replenished. Total incubation period was 2–3 days. The combined growth medium from two flasks was boiled and incubated with pancreatin (10 mg) in a manner similar to that used with the cell homogenates.

After incubation with pancreatin, samples were boiled and centrifuged at 20000g for 30 min. The supernatants resulting from the centrifugation of both the cellular and medium materials were then dialysed against water to remove radioactive sulphate. In some experiments, the supernatant preparations were chromatographed on a column (3 cm×25 cm) of Sephadex G-25. Removal of sulphate and recovery of macromolecular material were essentially the same whether gel filtration or dialysis was used.

In all cases, ³⁵S-labelled macromolecular material was shown to be exclusively glycosaminoglycan. This

was demonstrated by its total digestion with heparinase preparations, as described below.

Glycosaminoglycan characterization. Radioactively labelled glycosaminoglycans were incubated with testicular hyaluronidase, *Flavobacterium* heparinase and chondroitinase ABC. The hyaluronidase reaction mixtures in a volume of 0.2 ml contained 0.5 mg of hyaluronidase, 0.1 M-sodium acetate buffer (pH 5.0), 0.05 M-NaCl and 5 mg of chondroitin 4-sulphate and were incubated overnight at 37°C. The heparinase reaction mixtures in a volume of 0.2 ml contained 0.5 mg of heparinase, 0.1 M-sodium acetate buffer (pH 6.0) and 1 mg of chondroitin sulphate or heparin and were incubated overnight at 22°C. The chondroitinase reaction mixtures in a volume of 0.2 ml contained 0.02 ml of 'Tris mixture' (Saito *et al.*, 1968), 0.12 mg of standard chondroitin 4-sulphate, 0.12 mg of standard chondroitin 6-sulphate and 0.5 unit (Yamagata *et al.*, 1968) of chondroitinase ABC and were incubated for 1 h at 37°C.

After incubation, the hyaluronidase and heparinase reaction mixtures were boiled for 10 min and NaCl was added to a final concentration of 1 M. Samples were placed on columns (1 cm × 15 cm) of Sephadex G-50 and eluted with 1 M-NaCl. The radioactivity and uronic acid content of samples of the fractions were determined. The amount of material appearing in the included fractions was considered to represent the degraded material.

After incubation with chondroitinase, entire reaction mixtures were spotted on Whatman no. 1 paper and chromatographed overnight with butan-1-ol-acetic acid-1 M-aq. NH₃ (2:3:1, by vol.). Chromatograms were cut into strips and eluted with water. Portions of the eluate were assayed for radioactivity and uronic acid. Alternatively, the positions of the carrier disaccharides were determined by scanning the chromatograms with u.v. light.

Labelled glycosaminoglycans were separated on the basis of charge densities by ion-exchange chromatography. The resin (DEAE-cellulose) was pre-cycled and equilibrated with water. Samples were placed on columns (1 cm × 3 cm) with 2 mg of hyaluronic acid, 5 mg of chondroitin sulphate and 5 mg of heparin as standards. Materials were eluted with a logarithmic gradient by using a 125 ml mixing chamber and a reservoir of 1 M-LiCl, followed by a reservoir of 2 M-LiCl solution, as previously described (Silbert & DeLuca, 1969). Fractions (2.5 ml) were collected and analysed for radioactivity, uronic acid content and (in some cases) hexosamine content.

Molecular size was estimated by chromatography on columns (1 cm × 60 cm) of Sepharose 4B by a method similar to that of Wasteson (1971), by using chondroitin 6-sulphate (mol. wt. 40 000, as determined by the manufacturer) as the standard. Blue Dextran 2000 and Phenol Red solutions were used to determine the void (V_0) and included (V_i) volumes re-

spectively. The eluent was 0.1 M-LiCl and fractions (1 ml) were collected. Portions of the fractions were assayed for radioactivity and uronic acid content.

The effects of alkali on the pancreatin-solubilized glycosaminoglycans were determined by incubation of the preparations with 0.1 M-NaOH overnight at 4°C. After neutralization, the alkali-treated samples were chromatographed on Sepharose columns as described above.

Analytical methods

Uronic acid content of chromatographic fractions was determined by the Bitter & Muir (1962) technique. The sulphate content of the growth medium was determined by using the procedure of Letonoff & Reinhold (1936). The protein content of the cell homogenates was determined by the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard. The procedure used for amino sugar characterization was essentially the chromatographic technique of Gardell (1953), the colorimetric method of Levvy & McAllan (1959) being used for hexosamine assay.

For most experiments, an Ansitron liquid-scintillation spectrometer was used to determine radioactivity by using the scintillation fluid of Bray (1960). Appropriate corrections were made for quench and overlap when necessary. With doubly labelled materials, additional corrections were made for the efficiencies of counting for ³⁵S and ³H. A Beckman low-background (0.5 c.p.m.) planchet counter was used to determine the radioactivity of eluted materials from the paper chromatograms of chondroitinase degradation products, as well as the radioactivity of samples of the fractions obtained from Sepharose 4B gel chromatography.

Results

Production of ³⁵S-labelled glycosaminoglycans

Synthesis of sulphated glycosaminoglycans was determined by incorporation of [³⁵S]sulphate into macromolecular products found in both cells and growth medium (Table 1). The long-established cell lines P815Y, X163 and HC73 were found to synthesize labelled materials in similar small amounts. In all three cases, more ³⁵S-labelled glycosaminoglycan was found within the cells than in the growth medium.

The newly derived P815S cell line had a higher incorporation than any of the established lines, and seemed to offer a good system for detailed study. In the P815S cultures, more labelled material was found in the medium than in the cells. Newly derived P815AS cells incorporated label at a low amount similar to the established lines and were not studied further.

Table 1. *Incorporation of [^{35}S]sulphate into macromolecular glycosaminoglycans by mastocytoma cells cultured in vitro*

Details of the procedure are in the Experimental section. Estimated glycosaminoglycan production was calculated by determining the sulphate content of the medium as 0.107mm, assuming 1 sulphate group/glycosaminoglycan disaccharide unit, and assuming 6×10^6 cells/mg of cell protein.

Established lines	$10^{-5} \times ^{35}\text{S}$ incorporated (c.p.m.)		Total cell protein (mg)	$10^{-5} \times$ Total ^{35}S incorporated (c.p.m./mg of cell protein) (cells + medium)	Estimated glycosaminoglycan production ($\mu\text{g}/10^6$ cells) (cells + medium)
	Cells	Medium			
Dunn-Potter tumour					
P815Y	1.90	0.85	1.0	2.75	0.26
X163	0.97	0.42	0.6	2.32	0.22
Furth tumour HC73	2.46	0.85	1.2	2.75	0.26
New lines					
Dunn-Potter tumour					
P815S	6.80	9.90	0.9	18.6	1.70
P815AS	2.28	9.70	3.9*	3.07	0.28

* A larger initial cell inoculum was used in these cultures, yielding a higher final density.

Characterization of labelled products

To assist in characterization studies, cells of the established P815Y, X163 and HC73 lines were incubated with [^3H]glucosamine and [^{35}S]sulphate. The resulting radioactive products were chromatographed on DEAE-cellulose, along with standard glycosaminoglycans as positional references (Fig. 1). In all cases, doubly labelled product was eluted from the column in the fractions that contained glycosaminoglycan standards. The doubly labelled materials from both P815Y and HC73 cells appeared as mixtures having some material (peak A) with a charge density similar to that of chondroitin sulphate and some material (peak B) with a charge density similar to that of heparin. The doubly labelled material (peak B) from X163 cells had a charge density similar to that of heparin. In addition, all three cell lines produced large amounts of ^3H -labelled products, which were eluted from the DEAE-cellulose columns before standard hyaluronic acid. No [^{35}S]sulphate was present in these fractions.

The labelled products found in the media of P815Y, X163, and HC73 cultures appeared to have elution patterns from DEAE-cellulose similar to those for the intracellular products. However, amounts were too small for accurate representation and these materials were not analysed further.

In a similar manner, [^{35}S]sulphate-labelled materials from both the cells and medium of P815S cultures were chromatographed on DEAE-cellulose (Fig. 2). Labelled materials from the cells had an elution pattern that was different from that of the medium,

indicating significant charge-density differences between the intracellular and extracellular substances.

The chromatographically separated ^3H , ^{35}S -labelled glycosaminoglycans from the P815Y, X163 and HC73 cells were identified by enzymic degradation and hexosamine analysis (Table 2). Peak A material from P815Y and HC73 cells was degradable by testicular hyaluronidase and had a high percentage of [^3H]galactosamine relative to [^3H]glucosamine. Most of the peak B material from all these lines was resistant to degradation by testicular hyaluronidase. Peak B materials had a low percentage of [^3H]galactosamine relative to [^3H]glucosamine. These findings support the identification of peak A material as largely chondroitin sulphate-like glycosaminoglycan and peak B material as largely heparin-like glycosaminoglycan.

None of the ^3H -labelled products eluted from DEAE-cellulose before standard hyaluronic acid was degradable by testicular hyaluronidase or heparinase. No further identification was attempted. These products may be glycoprotein, as described in these cells by Thomas (1968).

All of the sulphate-labelled materials from the cultures of newly derived P815S cells were degradable by chondroitinase ABC (Fig. 3). The ^{35}S -labelled glycosaminoglycan from the medium was degraded to a disaccharide product which co-chromatographed with $\Delta\text{Di-4S}$, identifying the ^{35}S -labelled glycosaminoglycan as chondroitin 4-sulphate. With the cellular chondroitin sulphate, a small percentage of the chondroitinase degradation product chromato-

Fig. 1. Charge densities of ^3H , ^{35}S -labelled glycosaminoglycans from cells of established mastocytoma lines

Macromolecular materials were chromatographed on DEAE-cellulose columns as described in the Experimental section. Fractions of 2.5 ml were collected. The reservoir was changed from 1 M- to 2 M-LiCl after fraction 80 and the LiCl concentration gradient is shown by the solid line in (a). The positions of standard glycosaminoglycan were determined by assay for uronic acid. ●, Radioactivity as ^{35}S ; ○, radioactivity as ^3H . Fractions were pooled as indicated for further characterization. (a) Standards; (b) P815Y cells; (c) X163 cells; (d) HC73 cells. The numbers in parentheses are the d.p.m. for that peak fraction.

graphed in the position of acetylchondrosine-4S (Di-4S), whereas the remainder co-chromatographed with $\Delta\text{Di-4S}$.

In a similar study, 86% of the doubly labelled peak material from HC73 cells was degraded to the $\Delta\text{Di-4S}$ disaccharide by chondroitinase ABC, but the re-

maining 14% was not degraded. These results indicate that most of this labelled peak A glycosaminoglycan was chondroitin 4-sulphate.

Size estimates were made of the P815S chondroitin 4- ^{35}S sulphate by gel filtration with Sepharose 4B (Fig. 4). The extracellular chondroitin 4- ^{35}S sulphate

Fig. 2. Charge densities of glycosaminoglycans from P815S cells and medium

³⁵S-labelled glycosaminoglycan was chromatographed on DEAE-cellulose columns as described in the Experimental section and in the legend of Fig. 1. (a) Standards; (b) medium; (c) cells.

Table 2. Characterization of DEAE-cellulose separated glycosaminoglycans of P815Y, X163 and HC73 cells

The techniques used in the characterization are described in the Experimental section.

Source	Peak	Fractions combined	% of total radioactivity (³⁵ S)	% degradable by testicular hyaluronidase	Hexosamine content (GalN/GlcN ratio)
P815Y	A	60–100	64	95	97:3
	B	102–125	27	24	46:54
X163	B	100–126	73	36	22:78
HC73	A	54–94	60	91	78:22
	B	96–120	30	20	29:71

Fig. 3. Identification of ^{35}S -labelled glycosaminoglycans from P815S cells by chondroitinase degradation

Chondroitinase degradation and paper chromatography were performed as described in the Experimental section. Radioactivity of the materials eluted from 0.5 cm sections of the strips was determined. The positions of the unsaturated disaccharides were indicated by their u.v. absorbance and by uronic acid determination. (a) Cells; (b) medium.

was similar in size to the standard chondroitin 6-sulphate (mol.wt. approx. 40000), whereas the cellular material was smaller relative to this standard. Treatment of these materials with alkali did not alter their gel-filtration patterns. The apparent size difference between the chondroitin 4-sulphate of cells and the chondroitin 4-sulphate of the media would explain their different charge densities as shown by DEAE-cellulose chromatography (Fig. 2).

Culture conditions and glycosaminoglycan synthesis

Culture conditions and techniques were modified to ascertain the effects of several variables on glycosaminoglycan synthesis. These modifications included use of different growth media (MEM and F-12 rather than Fischer's) and different serum supplement (foetal calf instead of horse serum). Cells were also studied for glycosaminoglycan synthesis before and after

long periods of culture *in vitro*. None of these variations in culture technique significantly modified glycosaminoglycan biosynthesis by these cells. The cells continued to synthesize small amounts of glycosaminoglycan and, in the case of HC73 and P815Y cells, continued to contain a relatively large proportion of chondroitin sulphate.

An experiment was devised to determine if the cells had been altered by our culture or cell-freezing techniques during the course of these studies. A new culture of P815Y cells was obtained from the original source. These new cells were cultured on arrival by our usual techniques and also by the technique of Wheldrake & Pasternak (1968), in culture tubes. Cells cultured in both ways were then tested for production of sulphated glycosaminoglycans. The results obtained with cells from the two types of cultures were compared with each other and with the results of our previous studies. These comparisons indicated no significant differences.

Fig. 4. Sepharose 4B gel chromatography of chondroitin 4- $[^{35}\text{S}]$ sulphate from P815S cells and medium

Material was chromatographed with standard chondroitin 6-sulphate (mol.wt. 40000) as described in the Experimental section. Fractions of 1.0ml were collected, and samples assayed for radioactivity (●) and uronic acid content (E_{530} , Δ). (a) Cells; (b) medium. The void (V_0) and included (V_i) volumes are shown.

Discussion

Differentiated normal mast cells are generally thought to synthesize only heparin as their glycosaminoglycan product. In contrast, it has been reported that neoplastic mast-cell tumours also contain significant amounts of chondroitin sulphate (Rodén & Dorfman, 1959; Ringertz, 1963). It has been suggested that this chondroitin sulphate might not arise from the mastocytoma cells themselves, but from other connective-tissue elements pervading the tumours. Helting *et al.* (1972) reported that purified mastocytoma cells produced chondroitin sulphate as 10% of their total sulphated glycosaminoglycans. The studies reported here show that mastocytoma cells of several lines cultured *in vitro* can synthesize even larger percentages of chondroitin 4-sulphate.

A surprisingly small amount of total glycosaminoglycans was produced by the mastocytoma cells of the long-established P815Y, X163, and HC73 lines,

as well as the newly established P815AS line. This amount is similar to that reported for numerous other cell types where biosynthesis of glycosaminoglycan is not an expected differentiated function. It is also considerably lower than the amounts previously reported for the cells of the P815Y (X-2) and X163 (X-1) lines (Green & Day, 1960). Therefore, it seems likely that glycosaminoglycan production may no longer be a primary differentiated function of these lines of mastocytoma cells.

The newly derived P815S cells synthesized sulphated glycosaminoglycans in amounts considerably greater than the cells of the other tested lines. Unexpectedly, this glycosaminoglycan was found to be almost entirely chondroitin 4-sulphate. This apparently also represents a change in the differentiated function (heparin production) of this line. The chondroitin 4-sulphate found in the medium of the P815S cell cultures appeared to be larger than

that found in the cells. These differences in size might be an indication of separate function for intracellular and extracellular glycosaminoglycans.

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