Isolation and Characterization of Sulphated Mucopolysaccharides from Rat Leukaemic (RBL-1) Basophils

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Proteoglycans of 300000 mol.wt. were isolated from dispersed rat basophil tumour cells after labelling of the sulphated mucopolysaccharides with ^{35}S *in vitro*: 90% of the ^{35}S -labelled mucopolysaccharides were extracted at high salt concentration. Alkali degradation of the ^{35}S -labelled proteoglycans yielded glycosaminoglycan chains of 40000 mol.wt. The composition of the salt-extracted ^{35}S -labelled mucopolysaccharides, as defined by parallel or sequential degradation with chondroitinase AC, chondroitinase ABC and heparinase and resolution of the disaccharide-digestion products obtained with chondroitinase AC, was 48–61% chondroitin 4-sulphate, 20–30% dermatan sulphate, 10–15% heparin and 7–9% chondroitin 6-sulphate. Most of the salt-extracted ^{35}S -labelled mucopolysaccharides were highly charged, with heparin and chondroitin 6-sulphate being relatively uniform in this regard, whereas chondroitin 4-sulphate and dermatan sulphate exhibited a range of charge characteristics. The diversity of sulphated mucopolysaccharides present in the rat leukaemic basophil is in contrast with the predominance of heparin in the rat mast cell.

Mast cells and basophils of every species studied contain granules that stain metachromatically (Selye, 1965*a,b*; Parwaresch, 1976) and have receptors for species-specific immunoglobulin E (Ishizaka & Ishizaka, 1975; Lichtenstein *et al.*, 1978). The sulphated mucopolysaccharides of both rat (Yurt *et al.*, 1977*a*) and human (Metcalf *et al.*, 1978) mast cells are predominantly the proteoglycan heparin, as assessed by physicochemical and functional criteria. In the rat the proteoglycan heparin has been located in the secretory granule, on the basis of its release with histamine after immunological activation of the cell (Yurt *et al.*, 1977*b*).

Although the human neutrophil has been demonstrated to contain the proteoglycan chondroitin sulphate (Olsson, 1969), the sulphated mucopolysaccharides of the basophil have not previously been described as proteoglycans, because either physicochemical characterization was not carried out (Olsson *et al.*, 1970; Sue & Jaques, 1974) or the extraction procedure involved proteolytic treatment (Orenstein *et al.*, 1978), which would cleave a peptide core (Morrison, 1974). Rat leukaemic basophils represent a homogeneous cell source (Eccleston *et al.*, 1973) for isolation and characterization of cell-associated mucopolysaccharides. In addition, cultured cell lines derived from this tumour are being exten-

sively used for studies of the immunoglobulin E receptor (Kulczycki *et al.*, 1974; Conrad & Froese, 1978) and the release reaction (Siraganian & Metzger, 1978). Whereas rat mast-cell mucopolysaccharides are predominantly the proteoglycan heparin (Yurt *et al.*, 1977*a*), those of leukaemic basophils are predominantly a mixture of highly charged proteoglycan chondroitin sulphates.

Experimental

Materials

Whale cartilage chondroitin 4-sulphate (mol.wt. 25000–50000), shark cartilage chondroitin 6-sulphate (mol.wt. 40000–80000), porcine skin dermatan sulphate, porcine skin hyaluronic acid, chondroitin 4-sulphate disaccharide, chondroitin 6-sulphate disaccharide, chondroitin disaccharide, chondroitinase ABC from *Proteus vulgaris* and chondroitinase AC from *Arthrobacter aureus* were from Miles Laboratories, Elkhart, IN, U.S.A.; glucuronolactone and porcine intestinal heparin, (170 units/mg) were from Sigma Chemical Co., St. Louis, MO, U.S.A.; Sepharose 4B and Sephadex G-50 were from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.; Dowex AG 1-X2 (100–200 mesh; Cl^- form) and DEAE-cellulose were from Bio-Rad

Laboratories, Richmond, CA, U.S.A.; Azure A and Toluidine Blue O were from Fisher Scientific Co., Fair Lawn, NJ, U.S.A.; carbazole was from Eastman Kodak Co., Rochester, NY, U.S.A.; carrier-free $\text{H}_2^{35}\text{SO}_4$ (10–1000 mCi/mmol) was from New England Nuclear, Boston, MA, U.S.A.; Whatman chromatography paper no. 1 was from Whatman, Kent, U.K.; Hanks' balanced salt solution and Eagle's sulphate-free minimal essential medium with Earle's salts were from Microbiological Associates, Walkersville, MD, U.S.A.; Phenol Red was from Matheson, Coleman and Bell, Norwood, OH, U.S.A. Purified heparinase prepared from *Flavobacterium heparinum* was obtained from Dr. A. Linker (University of Utah, Salt Lake City, UT, U.S.A.).

Assays

The uronic acid content of rat leukaemic basophils was determined by the modified carbazole reaction (Bitter & Muir, 1962) and compared with a known glucuronolactone standard. Histamine was quantified by bioassay on atropine-treated guinea-pig ileum (Brocklehurst, 1960).

Isolation, labelling and extraction of basophils

Pooled rat basophil leukaemia tumour (Eccleston *et al.*, 1973; Wasserman & Austen, 1977) (20–30 g) was finely minced in Hanks' balanced salt solution to obtain a cell suspension for radiolabelling. After nylon-wool filtration of the minced fragments, 60–70% of the cell suspensions were well-differentiated basophils, the remaining 30–40% being poorly differentiated basophils, as recognized by phase-contrast microscopy, with and without vital staining with Toluidine Blue, or by light-microscopy of Giemsa-stained ethanol-fixed smears. Dispersed cells were sedimented at 400 g for 10 min at 22°C and washed twice with Hanks' balanced salt solution. For radiolabelling, 3×10^8 – 8×10^8 cells were resuspended in 300–800 ml of sulphate-free Eagle's minimum essential medium with Earle's salts containing 2 mmol of L-glutamine and 1 mCi of $\text{H}_2^{35}\text{SO}_4$ /100 ml. After incubation for 18 h at 37°C under O_2/CO_2 (19:1) (Hotpack Carbon Dioxide Incubator, Philadelphia, PA, U.S.A.), the cells were washed five times in Hanks' balanced salt solution, resuspended in 1.0 M-NaCl at a concentration of 1×10^8 cells/ml, and disrupted by freezing and thawing six times. After cell disruption, cell debris was sedimented by centrifugation at 400 g for 10 min at 22°C. The supernatant containing the salt-solubilized extract was stored at 4°C. The residual cell debris was suspended in 3–8 ml of 0.5 M-NaOH at 22°C for 18 h to solubilize the remaining ^{35}S -labelled mucopolysaccharides by degradation to their glycosaminoglycans. After sedimentation at 400 g for 10 min at 22°C, the supernatant containing the alkali-solubilized extract was stored at 4°C.

Characterization of extracted ^{35}S -labelled mucopolysaccharides

The salt-solubilized and alkali-solubilized cell extracts were each dialysed against 40 vol. of 1.0 M-NaCl for 18 h at 4°C, and separately applied to a column (1 cm $\times$ 5 cm) of Dowex 1 equilibrated in 1.0 M-NaCl. The columns were washed with 20 ml of 1.0 M-NaCl, and the ^{35}S -labelled mucopolysaccharides eluted in stepwise fashion with 20 ml of each of 3.0 M- and 4.0 M-NaCl (Schiller *et al.*, 1961; Slorach, 1971). The eluates were then dialysed against 40 vol. of distilled water for 18 h at 4°C, freeze-dried and resuspended in 0.5 ml of distilled water. The combined 3.0 M-NaCl and 4.0 M-NaCl eluates from Dowex 1 were desalted further by filtration over Sephadex G-50 columns (1 cm $\times$ 60 cm) equilibrated in water. All the radioactivity was in the excluded fractions, which were pooled, freeze-dried and stored at 4°C as the starting material for physicochemical characterization or determination of susceptibility to enzyme degradation.

The apparent molecular weights of both salt-extracted and alkali-extracted ^{35}S -labelled mucopolysaccharides were estimated by filtration over a previously standardized column (1 cm $\times$ 60 cm) of Sepharose 4B equilibrated in 2 M-NaCl at 22°C. To define the charge characteristics, salt-extracted ^{35}S -labelled mucopolysaccharides were applied to a DEAE-cellulose column (1 cm $\times$ 5 cm) equilibrated in 0.01 M-sodium acetate (pH 5.5)/0.1 M-LiCl, and eluted with sequential LiCl logarithmic gradients from 0.1 to 1.0 M- and 1.0 to 2.0 M-LiCl (Lewis *et al.*, 1973; Yurt *et al.*, 1977a). The column was eluted at a rate of 10 ml/h, and fractions of volume 2 ml were collected.

To determine susceptibility to degradation by chondroitinase AC or ABC, samples of salt-extracted and alkali-extracted ^{35}S -labelled mucopolysaccharides were suspended in 1 ml of 0.05 M-Tris/HCl buffer, pH 7.6, containing 0.1 M-NaCl and 0.1% bovine serum albumin, with or without 1 unit of either chondroitinase AC or chondroitinase ABC (Yamagata *et al.*, 1968). Chondroitin 4-sulphate (250 μg) and chondroitin 6-sulphate (250 μg) were added to the mixture containing chondroitinase AC, and these standards plus 250 μg of dermatan sulphate were added to the enzyme-free reaction mixture or that containing chondroitinase ABC. After 90 min incubation at 37°C, the entire reaction mixture or a sample was subjected to Sephadex G-50 gel filtration as described for desalting the eluates from Dowex 1. ^{35}S -labelled-mucopolysaccharide degradation was quantified by the change in elution of ^{35}S , and degradation of the internal standard was established by the change in the filtration pattern of the uronic acid.

The relative amounts of chondroitin 4-sulphate and chondroitin 6-sulphate in one-half of the

reaction mixture that resulted from chondroitinase AC degradation were determined by analysis of the disaccharide-digestion products. Portions of this mixture and known disaccharide standards were applied to Whatman no. 1 paper and subjected to descending chromatography in butanol/acetic acid/aq. 1M-NH₃ (2:3:1, by vol.) for 12 h at 22°C (Saito *et al.*, 1968). After chromatography, the paper was cut into 1 cm squares, and ³⁵S was assessed in a low-beta planchette counter (Beckman Instruments, Fullerton, CA, U.S.A.).

To determine the relative content of heparin, the chondroitinase ABC-resistant ³⁵S-labelled mucopolysaccharides were pooled, freeze-dried and re-suspended in 0.1M-sodium acetate buffer, pH 7.0, containing 250 µg of commercial porcine heparin, with or without 250 µg of purified heparinase (Hovingh & Linker, 1970). After incubation for 90 min at 30°C, the samples were subjected to Sephadex G-50 gel filtration to determine the extent of degradation by heparinase.

Results

Identification of the ³⁵S-labelled mucopolysaccharides

The salt-solubilized and alkali-solubilized extracts of 3×10^8 and 8×10^8 leukaemic basophils obtained from two separate minced tumour suspensions, designated 1 and 2, and containing 2 µg of histamine/ 10^8 cells were individually chromatographed on Dowex 1. The fractions eluted with 1.0M-NaCl, which are known to be free of sulphated mucopolysaccharides (Schiller *et al.*, 1961), contained more than 99% of the residual unincorporated precursor ³⁵S and total protein (Yurt *et al.*, 1977a; Slorach, 1971). The relative amounts of ³⁵S-labelled mucopolysaccharides in a portion of the salt-solubilized cell extracts in the combined 3.0M- and 4.0M-NaCl eluates were assessed by degradation with chondroitinase AC, followed by paper chromatography of the resulting disaccharides and by digestion with chondroitinase ABC followed by treatment with heparinase. Before enzyme treatment (Fig. 1a), ³⁵S-labelled mucopolysaccharides were excluded, as were internal chondroitin sulphate standards. Treatment with chondroitinase AC (Fig. 1b) degraded all of the internal standards and 55% of the ³⁵S-labelled mucopolysaccharides, and the latter consisted of 87% chondroitin 4-sulphate and 13% chondroitin 6-sulphate, as determined by paper chromatography. Chondroitinase ABC (Fig. 1c) degraded 85%, indicating that 30% of the ³⁵S-labelled mucopolysaccharide was composed of dermatan sulphate. The chondroitinase ABC-resistant ³⁵S-labelled mucopolysaccharides were degraded by heparinase (Fig. 2). Table 1 summarizes the results of the identification of salt- and alkali-extracted ³⁵S-labelled mucopolysaccharides in the combined 3.0M- and

4.0M-NaCl eluates from Dowex 1 in preparation 1, as shown in Figs. 1 and 2 and for preparation 2.

Physicochemical characterization of salt-solubilized ³⁵S-labelled mucopolysaccharides

Approximately two-thirds of the salt-solubilized ³⁵S-labelled mucopolysaccharides appeared in a broad peak with an average mol.wt. of 300 000, estimated by Sepharose 4B gel filtration, and the remainder was eluted in a second peak coincident with and just after a commercial heparin marker of 12 000 mol.wt. When fractions were pooled, subjected to alkali hydrolysis to break xylosyl-serine bonds (Muir, 1958; Lindahl & Rodén, 1966), dialysed and refiltered on the same Sepharose 4B column, the ³⁵S-labelled mucopolysaccharides

Fig. 1. Sephadex G-50 gel filtration of salt-solubilized ³⁵S-labelled mucopolysaccharides from rat leukaemic basophils after Dowex 1 chromatography before (a) and after degradation with chondroitinase AC (b) or chondroitinase ABC (c)

Uronic acid content represents internal standards. The data in (b) are corrected for the fact that half the sample was not applied. The Blue Dextran and Phenol Red markers were filtered separately. ●, Radioactivity; ○, uronic acid.

exhibited an average mol.wt. of 40 000, as did the ^{35}S -labelled mucopolysaccharides in the alkali extracts.

Fig. 2. *Sephadex G-50* gel filtration of salt-solubilized chondroitinase ABC-resistant ^{35}S -labelled mucopolysaccharides from rat leukaemic basophils before (a) and after (b) treatment with purified heparinase

Uronic acid content represents the internal heparin standard. The Blue Dextran and Phenol Red markers were filtered separately. ●, Radioactivity; ○, uronic acid.

DEAE-cellulose chromatography of the salt-solubilized ^{35}S -labelled mucopolysaccharides gave a peak of highly charged fractions (85–120) and a shoulder with less charged fractions (40–84) (Fig. 3). Selective enzyme degradation revealed that the highly charged ^{35}S -labelled mucopolysaccharides in the peak consisted of 40% chondroitin 4-sulphate, 15% chondroitin 6-sulphate, 10% dermatan sulphate and 35% heparin, whereas the less highly charged ^{35}S -labelled mucopolysaccharides were composed of 50% chondroitin 4-sulphate and 50% dermatan sulphate. Sepharose 4B gel filtration of the highly charged ^{35}S -labelled mucopolysaccharides revealed two peaks of radioactivity eluted with molecular weights similar to that of the starting material (Fig. 4).

Discussion

Proteoglycans were isolated by salt extraction from a homogeneous cell source composed of dispersed rat leukaemic basophils. More than 90% of the ^{35}S -labelled mucopolysaccharides extracted were solubilized by high salt concentrations, and the remainder was solubilized by degradation in alkali. The salt-solubilized ^{35}S -labelled mucopolysaccharides contained 300 000-mol.wt. proteoglycans, with glycosaminoglycan side chains of 40 000 mol.wt. as assessed by Sepharose 4B gel filtration before and after hydrolysis with alkali. The sulphated mucopolysaccharides from leukaemic basophils, considering both salt-extracted and alkali-solubilized material, were comprised of 49–63% chondroitin 4-sulphate, 19–30% dermatan sulphate, 9–15% heparin and 7–9% chondroitin 6-sulphate (Figs. 1 and 2 and Table 1).

The salt-solubilized ^{35}S -labelled mucopolysaccharides exhibited a wide range of charge heterogeneity, with two-thirds of the material being highly charged and being eluted from DEAE-cellulose just before and with commercial porcine heparin (Fig. 3). The highly charged ^{35}S -labelled mucopolysaccharides were eluted with an average mol.wt. of 300 000, which is compatible with a proteoglycan structure

Table 1. *Composition of ^{35}S -labelled mucopolysaccharides from rat leukaemic basophils*

All calculations are rounded off to the nearest integer. The total percentage of each ^{35}S -labelled mucopolysaccharide class takes into account the relative contribution of each extraction procedure; 99 and 91% of the ^{35}S -labelled mucopolysaccharides were extracted by salt in preparations 1 and 2 respectively.

Preparation	Type of extraction	Mucopolysaccharide class (%)			
		Chondroitin 4-sulphate	Dermatan sulphate	Chondroitin 6-sulphate	Heparin
1	NaCl	48	30	7	15
	NaOH	77	10	13	0
	Total	49	30	7	15
2	NaCl	61	20	9	10
	NaOH	86	5	9	0
	Total	63	19	9	9

Fig. 3. DEAE-cellulose chromatography of salt-solubilized ^{35}S -labelled mucopolysaccharides from rat leukaemic basophils after Dowex 1 chromatography

Metachromasia by Azure A ($\square$) represents commercial porcine heparin (3 mg) added as an internal standard; the column was previously standardized with porcine hyaluronic acid (2 mg), whale chondroitin 4-sulphate (3 mg), porcine dermatan sulphate (3 mg), shark chondroitin 6-sulphate (3 mg) and porcine commercial heparin (3 mg).
 $\bullet$, Radioactivity.

(Fig. 4). Degradation studies revealed that the more highly charged ^{35}S -labelled mucopolysaccharides contained all the heparin and chondroitin 6-sulphate, whereas chondroitin 4-sulphate and dermatan sulphate exhibited a wider range of charge heterogeneity.

Rat leukaemic basophil sulphated mucopolysaccharides, in contrast with those of the rat mast cell (Yurt *et al.*, 1977a), are not composed predominantly of heparin, or of any single proteoglycan species. Rat leukaemic basophil proteoglycan (mol.wt. 300 000) is not as large as the 750 000-mol.wt. mast-cell proteoglycan heparin, although both are composed of glycosaminoglycans of average mol.wt. 40 000 (Yurt *et al.*, 1977a). Rat leukaemic basophils contain an average of $21.7\mu\text{g}$ of uronic acid/ 10^8 cells, as compared with $1800\mu\text{g}$ of uronic acid/ 10^8 rat mast cells (Yurt *et al.*, 1977a; Lynch *et al.*, 1978).

The presence of heparin among the sulphated mucopolysaccharides of human basophils was inferred from cytochemical observations (Lennert & Parwaresch, 1968) and the demonstration of anticoagulant activity in extracts of peripheral-blood leucocytes (Martin & Roka, 1953; Amann & Martin, 1961). A human leukaemic basophil preparation of

Fig. 4. Sephacrose 4B gel filtration of salt-solubilized ^{35}S -labelled mucopolysaccharides from rat leukaemic basophils eluted in DEAE-cellulose fractions 85-120. The markers Blue Dextran, chondroitin 6-sulphate, commercial heparin, and Phenol Red were filtered separately.

25% purity resembled the rat leukaemic basophil (Table 1) in containing about 18% heparin, with the remainder of the sulphated mucopolysaccharides being chondroitin sulphates of unspecified types (Olsson *et al.*, 1970). This conclusion was based upon the degradation by heparinase of both [^{35}S]sulphate- and [^{14}C]glucosamine-labelled chondroitinase ABC-resistant mucopolysaccharides. In contrast, the ^{35}S -labelled glycosaminoglycans of basophil-enriched preparations of guinea-pig peripheral blood leucocytes were devoid of heparin but contained 15% heparan (Orenstein *et al.*, 1978). Further, although both guinea-pig basophils and rat leukaemic basophils contained 30% dermatan sulphate, the ratio of chondroitin 4-sulphate to chondroitin 6-sulphate was reversed, being 1:6 in the guinea pig and 6.7:1 in the rat. The absence of heparan from the rat leukaemic basophil is consistent with previous observations, in which permanent cell lines were shown to possess chondroitin sulphates rather than heparan (Mutoh *et al.*, 1976; Dietrich *et al.*, 1977).

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