The Presence of Glycopeptide Material in Cultured Mouse Mast-Cell Tumours

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1. Mouse mast-cell tumours P815 Y and HC were shown to contain glycoprotein material composed of glucosamine, galactosamine, sialic acid, galactose and mannose. 2. The major amino acids released after acid hydrolysis of Pronase-treated digests of the glycoprotein are aspartic acid, glutamic acid, serine, threonine, proline, glycine and alanine. The Pronase-digested material is not degraded in alkaline solution. 3. On incubation of mast cells with [^{35}S]sulphate, heparin is the major radioactive product. However, [^{14}C]glucosamine and D-[^{14}C]glucose are incorporated largely into the glycoprotein. 4. The fate of [^{35}S]sulphate-labelled and [^{14}C]glucosamine-labelled material was studied. In each case high-molecular-weight radioactive material is released from the cells into the culture medium. The $t_{\frac{1}{2}}$ of [^{35}S]sulphate-labelled material in cells is 70 hr. and that of [^{14}C]glucosamine-labelled material in cells is 40 hr. 5. About 60% of the [^{35}S]sulphate-labelled material is present in the mitochondrial and granular fraction. [^{14}C]glucosamine-labelled material is present in both microsomal and mitochondrial and granular fractions, [^{14}C]sialic acid being concentrated in the microsomal fraction.

Mouse mast-cell tumours possess a heterogeneous complement of sulphated glycosaminoglycans that have been separated on ECTEOLA-cellulose (epichlorohydrin triethanolamine cellulose) into fractions of different sulphate content and anticoagulant activity, the major fraction being heparin (Ringertz, 1965). It is reported that P815 Y mast cells, in culture, incorporate [^{35}S]sulphate, [^{14}C]glucosamine and D-[^{14}C]glucose into heparin (Green & Day, 1960). In the present paper it is confirmed that, when P815 Y or HC cells are cultured in the presence of [^{35}S]sulphate, heparin is the major ^{35}S -labelled glycosaminoglycan formed. However, [^{14}C]glucosamine and D-[^{14}C]glucose are incorporated predominantly into a non-sulphated fraction, which has been identified as a glycoprotein (Thomas & Pasternak, 1967).

MATERIALS AND METHODS

Chemicals. [^{14}C]Glucosamine was obtained from New England Nuclear Corp. (Boston, Mass., U.S.A.) or from The Radiochemical Centre (Amersham, Bucks.). D-[^{14}C]glucose and [^{35}S]sulphate were obtained from The Radiochemical Centre. High-molecular-weight blue dextran and

all grades of Sephadex were obtained from Pharmacia (Uppsala, Sweden). ECTEOLA-cellulose and *N*-acetylneuraminic acid were obtained from Sigma (London) Chemical Co. Ltd. (London, S.W. 6). Dowex 1 (X4; 200–400 mesh) and Dowex 50 (X8; 200–400 mesh) were obtained from V. A. Howe and Co. Ltd. (London, W. 11). All other chemicals were obtained from British Drug Houses Ltd. (Poole, Dorset) and were, wherever possible, of A.R. grade.

Cell culture. The cell line P815 strain Y derived from the Dunn–Potter mouse mastocytoma (Dunn & Potter, 1957) and the cell line HC derived from the Furth mouse mastocytoma (Furth, Hagen & Hirsch, 1957) were a gift from Dr G. A. Fischer. Both cell lines were maintained in the culture medium of Fischer & Sartorelli (1964). The methods of cell culture, cell counting and preparation of medium were as described by Wheldrake & Pasternak (1968).

Incorporation of radioactive metabolites. Cell cultures (250 ml. in 11. flasks) at an initial cell density of 1×10^5 – 2×10^5 cells/ml. were incubated with carrier-free [^{35}S]sulphate (500 μC), [^{14}C]glucosamine (40 μC ; 3–10 μmoles) or D-[^{14}C]glucose (10 μC ; 700 μmoles) and harvested in 3–5 days at a final cell density of 7×10^5 – 10×10^5 cells/ml. When D-[^{14}C]glucose was used, the concentration of glucose in the medium was decreased to one-half that normally present (i.e. to 0.5 g./l.), this being the minimum concentration for maximum growth.

Assay of radioactivity. Samples (0.05–0.5 ml.) were plated on aluminium planchets (4.5 cm.²) and dried at 100° for 60 min. to yield almost infinitely thin specimens. Acid or alkaline solutions were plated on glass coverslips mounted on aluminium planchets. The radioactivity was measured with an automatic sample changer (Isotope Developments

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Ltd., Beenham, Berks.); the counting efficiency was about 5%. A self-adsorption correction was necessary only for eluates from ECTEOLA-cellulose columns. This was done by measuring radioactivity of carrier-free [^{35}S]sulphate (20000 counts/100 sec./ml.) in solutions of increasing chloride concentration. Radioactivity on paper chromatogram strips was measured by using a lead castle with a G.E.C. Geiger-Müller end-window tube and registered on a type D 657 scaling unit (Panax Equipment Ltd., Redhill, Surrey). This apparatus gave a counting efficiency of about 5%.

Ion-exchange chromatography. Gradient elution was achieved by using a 250 ml. mixing vessel initially containing 0.05 M-sodium phosphate buffer, pH 7.0, into which the concentrated eluent was fed (20 ml./hr.) from a separating funnel; fractions (5 ml.) were collected with an LKB 3400 Radi-Rac automatic fraction collector (A. Gallenkamp and Co. Ltd., Widnes, Lancs.).

Gel filtration. This was carried out with columns (1.5 cm. $\times$ 30 cm.) of Sephadex of grades G-10, G-25, G-50 and G-75 with 0.1 N-acetic acid as the eluting solvent. The uniformity of flow, void volume and salt volume were determined by gel filtration of a mixture of high-molecular-weight (2×10^6) blue dextran (Pharmacia) and NaCl.

Isolation and fractionation of glycosaminoglycans. Cultures (250 ml.) of mast cells were harvested by centrifuging at 1500g for 10 min. in an MSE Mistral centrifuge with a 6 $\times$ 250 ml. angle head and the medium was removed as completely as possible. The cell pellet was resuspended in 0.1 M-sodium phosphate buffer, pH 7.5 (5–10 ml.), and homogenized with a cooled glass homogenizer (three to five strokes with a motor-driven pestle). The homogenate was boiled for 3 min. to coagulate protein and facilitate proteolytic digestion. The boiled homogenate was incubated with Nagarse (Teikoku Chemical Industry Co., Osaka, Japan) (0.1 mg./ml.) in 0.1 M-sodium phosphate buffer, pH 7.5, containing benzylpenicillin (200 i.u./ml.) and streptomycin sulphate (50 μg ./ml.) at 36° for 24 hr. The digestion was stopped by boiling for 3 min. and the homogenate was then digested with pancreatin (British Drug Houses Ltd.) (1 mg./ml.) in the presence of the above-mentioned antibiotics for a further 36 hr. The digest was boiled and centrifuged, and the supernatant plus washings were combined and desalted by gel filtration with a Sephadex G-50 column (1.5 cm. $\times$ 30 cm.) (step 1). The material eluted immediately after the void volume of the column was free of extraneous [^{35}S]sulphate, [^{14}C]glucosamine and the low-molecular-weight products of digestion. It was deproteinized by shaking with 0.2 vol. of chloroform-pentan-1-ol (5:1, v/v) for 5 min. on a vortex mixer and centrifuging; the upper aqueous layer was removed and the procedure repeated until no further protein appeared at the chloroform-water interface (step 2). The aqueous layer plus washings were concentrated (to 0.5–2.0 ml.) by rotary evaporation at 30° and made 0.05 M with respect to chloride by the addition of 0.5 N-HCl. The sample was applied to an ECTEOLA-cellulose column (0.8 cm. $\times$ 6 cm.), previously equilibrated with 0.05 N-HCl, and the column was washed with 3 column volumes of 0.05 N-HCl. The column was then eluted stepwise with 3 column volumes of solutions of 0.05 N-HCl of chloride concentration 0.1 M, 0.8 M, 1.0 M, 1.25 M, 1.6 M, 2.0 M and 2.5 M, and finally with 0.5 N-NaOH (step 3). The column chromatography was carried out at 3°.

Partial purification of the (0.05 M-chloride) fraction. The

material, extracted from [^{14}C]glucosamine-labelled cells, that was not retained by ECTEOLA-cellulose at 0.05 M-chloride was neutralized with 0.5 N-NaOH, desalted on a column of Sephadex G-50 and freeze-dried. This material is referred to as the (0.05 M-chloride) fraction.

Pronase digestion of the (0.05 M-chloride) fraction. The (0.05 M-chloride fraction) (2–6 mg. of protein) was incubated with Pronase (California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.) (10 μg ./mg. of protein) in 0.1 M-sodium phosphate buffer, pH 7.0 (2 ml./mg. of protein), containing NaN_3 (1 mM) at 40° for 24 hr. Pronase equal to one-half that previously used was added and the incubation continued for a further 24 hr. After centrifuging, the digest was applied to and eluted from a column of Sephadex G-50.

Alkali treatment of glycopeptides. A sample of fraction B (Fig. 3) (2–4 mg. of protein; 10000–20000 counts/100 sec.) was kept in 4 ml. of 0.5 N-NaOH for 24 hr. at 3°, neutralized with 0.5 N-HCl and reisolated by gel filtration with Sephadex G-25.

Subcellular fractionation. Cultures of HC cells, previously incubated with [^{14}C]glucosamine or [^{35}S]sulphate, were harvested as described above. The cell pellet (3×10^7 – 4×10^7 cells) was washed twice by resuspending in 100 ml. of isotope-free medium containing glucosamine (2 mM) or Na_2SO_4 (10 mM) respectively and centrifuging. The washed cell pellet was resuspended in 8 ml. of ice-cold 0.25 M-sucrose and combined with a pellet of non-radioactive cells (equivalent to 10^8 cells). The cell suspension was treated ultrasonically (Mullard magnetostrictor oscillator operating at 25 kcyc./sec.) for 2 min. at 0° and centrifuged at 900g for 10 min. at 0° in an MSE Mistral centrifuge to give the nuclear fraction. All further centrifugations were carried out in a Spinco model L centrifuge at 0°. The mitochondrial and granular fraction (Korn, 1959) was obtained by centrifuging at 19000g for 10 min., and the microsomal fraction by centrifuging at 100000g for 1 hr. All sediments were washed once with 8 ml. of 0.25 M-sucrose and resuspended in 4 ml. of 0.25 M-sucrose. A sample (0.5 ml.) of each subcellular fraction and wash was removed for the determination of total radioactivity and protein.

Hydrolysis of subcellular fractions. An equal volume of 20% (w/v) trichloroacetic acid was added to each of the [^{14}C]glucosamine-labelled subcellular fractions and the suspensions were centrifuged. The sediments were washed once with 5% (w/v) trichloroacetic acid, resuspended in 4 ml. of 5% (w/v) trichloroacetic acid, heated at 80° for 1 hr. and centrifuged, and the radioactivity of the supernatants was determined.

Preparation of neutral sugar and amino sugar fractions. For qualitative and quantitative analyses of neutral sugar and amino sugar, the (0.05 M-chloride) fraction was hydrolysed by N- H_2SO_4 at 110° for 4 hr., neutralized with saturated $\text{Ba}(\text{OH})_2$ solution and the precipitate removed by centrifuging. All radioactivity in a sample of the supernatant was retarded on Sephadex G-10, indicating complete hydrolysis. The recovery of radioactivity after hydrolysis and neutralization was 60–80%.

The hydrolysate was applied to a column (0.8 cm. $\times$ 10 cm.) of Dowex 50 (X8; H^+ form); the column was washed with three 10 ml. lots of distilled water and the eluates were combined and concentrated by rotary evaporation to give the neutral sugar fraction. The column was next eluted with 0.3 N-HCl, each fraction (2 ml.) being assayed for amino sugar. The appropriate fractions were combined and

evaporated to dryness *in vacuo* at 0° in the presence of H₂SO₄ and solid KOH to give the amino sugar fraction.

Sialic acid analysis. For the qualitative identification of sialic acid, the (0.05 M-chloride) fraction was hydrolysed by 0.1 N-H₂SO₄ at 80° for 1 hr. The hydrolysate was neutralized with saturated Ba(OH)₂ solution and the precipitate removed by centrifuging. The supernatant was fractionated by one of the following methods. (A) The hydrolysate was applied to a column (0.8 cm. × 6 cm.) of Dowex 1 (X4; Cl⁻ form) that was washed with 3 column volumes of distilled water. The original eluates and washings were combined and the total radioactivity was determined. The column was treated with 3 column volumes of 0.1 N-HCl; the combined eluates were evaporated to dryness *in vacuo* at 0° in the presence of H₂SO₄ and solid KOH and the total radioactivity was determined. (B) The hydrolysate was applied to, and eluted from, a column of Sephadex G-50. Fractions corresponding to retarded material were combined and freeze-dried.

Paper chromatography of sialic acid was carried out on Whatman no. 1 paper with butan-2-ol-acetone-acetic acid-water (6:6:3:5, by vol.). Sialic acids were identified with the thiobarbituric acid reagent (Warren, 1960).

For quantitative analysis of sialic acid, samples of the acid hydrolysate were analysed directly by the Warren (1959) method, with *N*-acetylneuraminic acid as standard. The first empirical method (Warren, 1959) of correction for interfering substances was applied.

Amino sugar analysis. Amino sugars were determined by the method of Levvy & McAllan (1959) with D-glucosamine hydrochloride as standard.

Qualitative paper chromatography of amino sugars was carried out on Whatman no. 1 paper with pyridine-ethyl acetate-acetic acid-water (5:5:1:3, by vol.) (Fischer & Nebel, 1955). Amino sugars were detected with 0.2% (w/v) ninhydrin in 95% (v/v) ethanol. The coincidence of radioactive and stained areas was confirmed by radioautography by using Kodirex X-ray film (Kodak Ltd., Kirkby, Liverpool).

The glucosamine/galactosamine ratio in hydrolysates was determined with the amino acid analyser (see below).

Hexose analysis. For the quantitative determination of neutral hexose, the orcinol method (François, Marshall & Neuberger, 1962) was used directly on unhydrolysed samples of the (0.05 M-chloride) fraction, and the Nelson-Somogyi method (Hodge & Hofreiter, 1962) after hydrolysis and separation of neutral sugar. D-Galactose was used as a standard.

D-Galactose in a final volume of 0.5 ml. of 0.1 M-sodium phosphate buffer, pH 7.0, was determined with the Galactostat kit (Worthington Biochemical Corp., Freehold, N.J., U.S.A.). D-Glucose in a final volume of 3.5 ml. of 0.1 M-tris, adjusted to pH 7.0 with 2 N-HCl, was determined with the Glucostat kit (Worthington Biochemical Corp.).

Qualitative paper chromatography of neutral sugars was carried out on Whatman no. 1 paper with ethyl acetate-pyridine-water (8:2:1, by vol.) (Leopold, 1962) for 16–24 hr. Neutral sugars were detected by the alkaline-AgNO₃ method (Trevelyan, Procter & Harrison, 1950). The *R*_{Glc} values were 0.78 for galactose, 1.22 for mannose and 1.70 for fucose. The neutral sugars were also identified initially by chromatography with standards in butan-1-ol-pyridine-0.1 N-HCl (5:3:3, by vol.) (Bourrillon, Michon & Got, 1959).

Amino acid analyses. These were carried out with an automatic amino acid analyser (Evans Electroselenium Ltd., Halstead, Essex), kindly operated by Dr D. B. Hope. Samples (2–7 mg. of protein) were dissolved in constant-boiling HCl (5.7 N; three-times glass-distilled) and hydrolysed at 110° for 17 hr. by the method of Crestfield, Moore & Stein (1963). No correction was made for amino acid destruction during the acid hydrolysis.

Protein. This was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) as standard.

RESULTS

Incorporation of [³⁵S]sulphate, [1-¹⁴C]glucosamine and D-[¹⁴C]glucose. Initial studies showed that when the [³⁵S]sulphate-labelled material extracted from P815 Y or HC cells was fractionated on

Table 1. Fractionation of [³⁵S]sulphate-labelled material from P815 Y and HC cells

Fractionation step	10 ⁻³ × Radioactivity (counts/100 sec./10 ⁸ cells)	
	P815 Y cells	HC cells
1. Radioactivity in proteolytic digests excluded by Sephadex G-50	145	1160
2. Radioactivity after deproteinization of fraction from step 1	120	980
3. Elution from ECTEOLA-cellulose		
0.05 M-Cl ⁻	< 0.5	< 0.5
0.1 M-Cl ⁻	3	16
0.8 M-Cl ⁻	6	21
1.0 M-Cl ⁻	8	29
1.25 M-Cl ⁻	18	61
1.60 M-Cl ⁻	21	150
2.0 M-Cl ⁻	37	344
2.5 M-Cl ⁻	4	164
0.5 N-NaOH	3	18

Table 2. Fractionation of [1-¹⁴C]glucosamine-labelled and D-[¹⁴C]glucose-labelled material from P815 Y and HC cells

Radioactive precursor	10 ⁻³ × Radioactivity (counts/100 sec./10 ⁸ cells)		
	P815 Y cells	HC cells	P815 Y cells
	[1- ¹⁴ C]Glucosamine	[1- ¹⁴ C]Glucosamine	D-[¹⁴ C]Glucose
Fractionation step			
1. Radioactivity in proteolytic digests excluded by Sephadex G-50	180	170	9
2. Radioactivity after deproteinization of fraction from step 1	143	132	6
3. Elution from ECTEOLA-cellulose			
0.05 M-Cl ⁻	98	50	5
0.1 M-Cl ⁻	11	9	< 0.5
0.8 M-Cl ⁻	2	4	
1.0 M-Cl ⁻	1	2	
1.25 M-Cl ⁻	3	7	
1.6 M-Cl ⁻	2	4	
2.0 M-Cl ⁻	5	18	
2.5 M-Cl ⁻	< 0.5	4	
0.5 N-NaOH	8	6

Fig. 1. Fate of [³⁵S]sulphate-labelled material (a) and [1-¹⁴C]glucosamine-labelled material (b) in HC cells. Cultures (250ml.) of HC cells, previously incubated with [³⁵S]sulphate (500μc) or [1-¹⁴C]glucosamine (25μc; 8.3μmoles) for 6 days, were harvested at a cell density of 8 × 10⁵–10 × 10⁵ cells/ml., washed with non-radioactive medium and reinoculated into non-radioactive medium. At each of the times indicated a 20ml. sample was removed, the cell density determined and the remainder centrifuged. The cell pellet was digested with Nagarase (see the text), and the digest applied to and eluted from a column of Sephadex G-50. A 10ml. sample of the respective culture medium was also analysed by gel filtration on Sephadex G-50. Corrections were applied for increase in cell density. ○, Radioactivity in cell digests excluded by Sephadex G-50; ●, radioactivity in culture medium excluded by Sephadex G-50; ▲, radioactivity in culture medium retarded by Sephadex G-50; △, total radioactivity of cell digests and culture medium.

ECTEOLA-cellulose most of the radioactivity (50–70%) was eluted at 1.6–2.5 M-chloride (Table 1). This was characterized as heparin by (a) paper

chromatography with commercial heparin on Whatman no. 1 paper in 0.04 M-ammonium formate (pH 4.3)–propan-2-ol (13:7, v/v) (Spolter & Marx,

1960) and (b) precipitation with cetylpyridinium bromide according to the procedure of Korn (1959).

Similar elution profiles were obtained by both stepwise and gradient elution from ECTEOLA-cellulose. The heterogeneity of the [^{35}S]sulphate-labelled material on column chromatography (Table 1) is thought not to be an artifact of the isolation procedure. Thus, when the 1.6M- and 2.0M-chloride radioactive fractions were combined with a homogenate of non-radioactive cells and the glycosaminoglycans thereof re-extracted and fractionated on ECTEOLA-cellulose, the radioactivity was again eluted at 1.6M- and 2.0M-chloride. Also, no difference was observed in the elution profile of material extracted from [^{35}S]sulphate-labelled cells that had been incubated previously in non-radioactive medium (for 3 cell divisions). This indicates that the heterogeneity is not due to the presence of incompletely sulphated glycosaminoglycans.

When the products of [^{14}C]glucosamine or D- ^{14}C]glucose incorporation were fractionated on ECTEOLA-cellulose (Table 2), most of the radioactivity (50–70%) was not retained by the column. This material is referred to as the (0.05M-chloride) fraction.

Fate of [^{35}S]sulphate-labelled and [^{14}C]glucosamine-labelled material. The loss of radioactivity from [^{35}S]sulphate-labelled cells (Fig. 1a) and [^{14}C]glucosamine-labelled cells (Fig. 1b) on incubation in non-radioactive medium was associated with the appearance of high-molecular-weight ($>1 \times 10^4$; Sephadex G-50 exclusion limit) radioactive material in the medium. Low-molecular-weight material, as defined by retention of radioactivity on Sephadex G-50, was detected only in samples of the culture medium from [^{35}S]sulphate-labelled cells. No attempt was made to characterize further the radioactive material in the medium. The total radioactivity present in [^{14}C]glucos-

amine-labelled cells and the medium was constant throughout the experimental period, indicating no loss as $^{14}\text{CO}_2$. The t_4 of [^{35}S]sulphate-labelled material in the cells was 70hr. and that of [^{14}C]glucosamine-labelled material was 40hr. The presence of [^{35}S]sulphate-labelled material in the medium (Fig. 1a) at zero time may be due to initial cell damage.

Subcellular fractionation. By subcellular fractionation of HC cells (Table 3), [^{35}S]sulphate-labelled material was shown to be present mainly (60%) in the mitochondrial and granular fraction (Korn, 1959) and [^{14}C]glucosamine-labelled material was distributed equally between mitochondrial and granular (40%) and microsomal fractions (40%). About 30% of the radioactivity in the mitochondrial and granular fraction and 50% of that in the microsomal fraction of [^{14}C]glucosamine-labelled cells was released by acid hydrolysis (5% trichloroacetic acid at 80° for 1hr.). In each case 30–35% of the released radioactivity co-chromatographed with *N*-acetylneuraminic acid; residual radioactivity was retained at the origin.

Properties of the (0.05M-chloride) fraction. The (0.05M-chloride) fraction was eluted immediately after the void volume from Sephadex G-50 and was slightly retarded on elution from Sephadex G-75 ($V_e/V_0=1.5$, where V_e is the elution volume and V_0 the void volume). The (0.05M-chloride) fraction also showed no apparent heterogeneity in a gradient elution from DEAE-cellulose (Fig. 2). It resembled other glycoprotein preparations, such as the seromucoid fraction (Winzler, 1955), in being soluble in strong acids. Thus in the presence of bovine serum albumin (0.2%, w/v) 80–90% of the radioactivity was soluble in 0.3M-trichloroacetic acid or 0.5M-perchloric acid.

Carbohydrate constituents of the (0.05M-chloride) fraction. When the (0.05M-chloride) fraction from

Table 3. *Subcellular fractionation of [^{35}S]sulphate-labelled and [^{14}C]glucosamine-labelled HC cells*

Radioactive precursor	...	...	10 $\times$ Radioactivity (counts/100sec./mg. of protein)		
			[^{35}S]Sulphate	[^{14}C]Glucosamine	
				Total radioactivity	Radioactivity of trichloroacetic acid hydrolysates
Subcellular fraction			Total radioactivity	Total radioactivity	
Nuclear			1.8	2.5	1.3
Mitochondrial			8.8	8.0	3.0*
Microsomal			3.4	7.9	5.3*
Supernatant			1.9	2.2	0.7

* After heating subcellular fractions in 5% (w/v) trichloroacetic acid at 80° for 1hr. and centrifuging, the final supernatants of microsomal and mitochondrial-granular fractions were extracted three times with an equal volume of diethyl ether and concentrated by rotary evaporation. Samples were then chromatographed on Whatman no. 1 paper with butan-2-ol-acetone-acetic acid-water (6:6:3:5, by vol.).

HC cells or P815 Y cells was hydrolysed by 0.1N-sulphuric acid at 80° for 1 hr., 30–40% of the radioactivity was released as low-molecular-weight material that was retarded by Sephadex G-50 and retained by Dowex 1 (Cl⁻ form). Two radioactive areas were obtained on paper chromatography of this material, one of which was at the origin; the other co-chromatographed with *N*-acetylneuraminic acid. In both cell types sialic acid accounted for 10% of the radioactivity of the (0.05M-chloride) fraction. No distinction would have been made between *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid in this chromatographic procedure.

The neutral sugar fraction was examined qualitatively by paper chromatography; three

components were identified on the basis of coincidence with galactose, mannose and fucose markers.

Glucosamine and galactosamine were identified by paper chromatography of the amino sugar fraction.

The results of quantitative analysis of the (0.05M-chloride) fraction are given in Table 4. No glucose was detected by the D-glucose oxidase method (see the Materials and Methods section). No correction was made for loss (20–40%) of material during strong-acid hydrolysis and neutralization and therefore values are minimal. Fucose could not be detected by the Dische & Shettles (1948) procedure,

Fig. 2. Ion-exchange chromatography of (0.05M-chloride) fraction on DEAE-cellulose. The (0.05M-chloride) fraction (53000 counts/100 sec.) was applied to a column (1.5 cm. × 18 cm.) of DEAE-cellulose, washed with 100 ml. of 0.05M-sodium phosphate buffer, pH 7.0, and eluted with a gradient of NaCl in phosphate buffer; the mixing vessel contained 250 ml. of 0.05M-sodium phosphate buffer, pH 7.0, to which 0.3M-NaCl in the same buffer was added continuously.

Fig. 3. Gel filtration of (0.05M-chloride) fraction after Pronase digestion. A Pronase digest of the (0.05M-chloride) fraction (10000–40000 counts/100 sec.) was applied to a column (1.5 cm. × 30 cm.) of Sephadex G-50 and eluted with 0.1N-acetic acid. ●, HC cells; ○, P815 Y cells.

Table 4. Carbohydrate constituents of (0.05M-chloride) fraction

The means of two determinations are given.

Carbohydrate constituents (μmoles/10⁹ cells)

Cell type	Glucosamine	Galactosamine	Total reducing sugar*	Galactose	Mannose†	Fucose	Sialic acid	Glucose
P815 Y	2.14	0.16	4.10	1.12	2.98	<0.30	0.64	<0.10
HC	1.34	0.06	2.87	0.93	1.94	<0.30	0.85	<0.10

* Estimated by the Nelson-Somogyi method on the neutral sugar fraction (see the Materials and Methods section).

† Values obtained by difference from the total reducing sugar and galactose estimations.

Table 5. *Composition of Pronase digests of (0.05 M-chloride) fraction*

Fraction A refers to material excluded by Sephadex G-50; fraction B is the material retarded by Sephadex G-50; fraction C refers to total Pronase digest after gel filtration (see Fig. 3); fraction B (OH) refers to sample B treated with alkali. Values for amino acids are in molar terms in relation to aspartic acid. Trace represents a value of 0.04 or less.

Fraction	Amino acid composition (molar proportions)				HC cells C
	P815 Y cells				
	C	A	B	B(OH)	
$10^{-9} \times$ No. of cells	2	4.2	4.2		(3.5)
Hexose content (mg.) before Pronase digestion ...	0.8	—	—	—	0.60
Protein content (mg.) before Pronase digestion ...	2.0	6.8	6.8	—	2.2
Sum of amino acids recovered from analyser (mg.) ...	1.0	0.87	1.77		0.81
Constituents					
Ammonia	1.36	2.20	1.20	1.54	4.30
Aspartic acid	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)
Glutamic acid	0.95	0.84	1.10	1.14	1.05
Threonine	0.37	0.68	0.30	0.30	0.51
Serine	0.39	0.58	0.33	0.31	0.42
Proline	0.47	0.37	0.64	0.89	0.47
Glycine	0.42	0.45	0.36	0.45	0.40
Alanine	0.19	0.52	0.24	0.32	0.34
Leucine	0.15	0.72	0.15	0.19	0.40
Lysine	0.15	0.49	0.17	0.06	0.33
Valine	0.17	0.49	0.17	0.26	0.27
Isoleucine	0.09	0.34	0.09	0.14	0.18
Histidine	0.09	0.27	0.06	0.06	0.16
Arginine	0.08	0.21	Trace	0.09	0.13
Cystine (half)	0.13	Trace	0.20	0.07	0.09
Phenylalanine	0.08	0.42	Trace	Trace	0.20
Tyrosine	Trace	0.17	Trace	Trace	0.09
Methionine	Trace	Trace	Trace	Trace	Trace

though material migrated with the mobility of fucose on paper chromatography of the neutral sugar fraction (see above).

Pronase digestion of the (0.05 M-chloride) fraction. When the (0.05 M-chloride) fraction was digested with Pronase and the digest applied to a column of Sephadex G-50, two radioactive species were eluted (Fig. 3). Fractions were pooled as indicated and freeze-dried; fraction B was desalted on a column of Sephadex G-25 before freeze-drying. Amino acid analyses of hydrolysed glycopeptides showed that in each fraction aspartic acid, glutamic acid, serine, threonine, proline, glycine and alanine were the principal amino acids (Table 5). After alkali treatment (see above) fraction B was eluted as a single radioactive peak from a column of Sephadex G-25 and showed no change in elution volume or amino acid composition (Table 5).

DISCUSSION

In contrast with an earlier report of the absence of sialic acid from P815 mast cells (Green & Robinson, 1962) both P815 Y and HC cells have

now been shown to contain a glycoprotein, the (0.05 M-chloride) fraction, containing glucosamine, galactosamine, sialic acid, mannose, galactose and trace quantities of fucose.

Aspartic acid and glutamic acid are the major amino acids present in the material obtained after Pronase digestion of the glycoprotein (Table 5). The alkali-stability of fraction B (Table 5) suggests that the carbohydrate-peptide link in the glycoprotein is, not a glycosidic bond to serine or threonine residues, but an *N*-acylglycosylamine bond. This link is known to be present in many glycoproteins (Grant & Simkin, 1964) and has been unequivocally shown for ovalbumin (Marshall & Neuberger, 1964).

The loss of [³⁵S]sulphate from [³⁵S]sulphate-labelled cells in non-radioactive medium is accompanied by the appearance of two radioactive species in the medium (Fig. 1a) as defined by their behaviour on Sephadex G-50. The turnover value, *t*₁, is 70 hr. for [³⁵S]sulphate-labelled cells and is in agreement with a previous estimate of 2.5 days for both [³⁵S]sulphate-labelled and [¹⁻¹⁴C]glucosamine-labelled heparin in P815 cells (Green & Day, 1960). The release of radioactive material into the culture

medium is thought not to be a result of cell destruction, since the t_i values for $[1-^{14}\text{C}]$ glucosamine-labelled cells (40 hr.) and $[^{35}\text{S}]$ sulphate-labelled cells (70 hr.) are different (Fig. 1b). Only high-molecular-weight ($> 1 \times 10^4$; Sephadex G-50 exclusion limit) radioactive material could be detected in the culture medium of $[1-^{14}\text{C}]$ glucosamine-labelled cells (Fig. 1b). In a study of $[1-^{14}\text{C}]$ glucosamine incorporation by Ehrlich ascites cells *in vivo* and *in vitro*, Molnar, Teegarden & Winzler (1965) also have found that high-molecular-weight radioactive material appeared outside the cells. Similarly, on incubation of HeLa cells with $[1-^{14}\text{C}]$ glucosamine (Kornfeld & Ginsburg, 1966), high-molecular-weight radioactive material containing $[^{14}\text{C}]$ glucosamine, $[^{14}\text{C}]$ galactosamine and $[^{14}\text{C}]$ sialic acid was released into the medium.

In a study of heparin biosynthesis *in vitro* by the P815 mast-cell tumour (Silbert & Brown, 1961) both UDP- α -D-glucosamine and UDP- α -D-galactosamine were detected; the significance of the latter compound was not appreciated. The occurrence of a glycoprotein containing galactosamine would account for this observation.

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