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The Characterization of a Protein–Polysaccharide Isolated from Kurloff Cells of the Guinea Pig

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Kurloff cells of guinea pigs increase in number and accumulate in the spleen on oestrogen treatment. Because they contain metachromatic inclusions and are considered to be lymphocytes they were examined as a possible model for mucopolysaccharidoses like Hurler's syndrome, where some lymphocytes are also metachromatic. Oestrogen treatment produced a large increase in a glycosaminoglycan resembling chondroitin 4-sulphate in chemical analysis, chromatographic behaviour and i.r. spectrum but with an additional strong band at 805 cm^{-1}. Material isolated without proteolysis behaved on gel chromatography as a multiple-chain protein–polysaccharide whose molecular size was decreased by proteolysis. It contained xylose and galactose in molar proportions with serine, compatible with the presence of the same linkage region as in cartilage chondroitin 4-sulphate proteins and which likewise underwent alkaline β-elimination. Kurloff glycosaminoglycan chains were significantly longer than chondroitin sulphate chains of cartilage protein–polysaccharides as assessed by gel chromatography and the molar ratios of galactosamine to xylose or to serine. Kurloff cells thus contain intact rather than partially degraded protein–polysaccharide and hence are not analogous to Hurler cells, and their electron micrographs were also different. The purified Kurloff protein–polysaccharide and glycosaminoglycan isolated here has been shown by Marshall, Swettenham, Vernon-Roberts & Revell (1970) to be toxic specifically to macrophages at extremely low concentrations *in vitro*, unlike chondroitin sulphate of protein–polysaccharides from cartilage. The toxic constituent may account for the i.r.-absorption band at 805 cm^{-1}. Although active incorporation of [^{35}S]sulphate occurs at early stages of Kurloff-cell induction (Marshall *et al.* 1970), the fully developed Kurloff cell studied here showed very low incorporation *in vitro* and *in vivo*, suggesting that the inclusions are specialized for the storage of the toxic material.

Kurloff-cell inclusion bodies in small numbers were identified by Ledingham (1940) in the lymphocyte population of male and female guinea pigs in blood samples taken immediately after birth and were also noted in impression preparations of foetal spleens. An increase in the number of these bodies was observed with increasing age, the increase being especially large in female animals during pregnancy and in both male and female animals on administration of oestrogen. On the basis of their histochemical findings, Marshall & Swettenham (1959) concluded that these inclusion bodies contained both glycoprotein and glycosaminoglycan.* The first chemical characterization of this GAG was effected by Muir & Marshall (1961), who found that apparently no linked amino acids were present, and that the electrophoretic mobility of the isolated GAG was the same as that of chondroitin sulphate. Like chondroitin sulphate it was susceptible to hyaluronidase and the principal amino sugar was galactosamine.

Metachromatic inclusions are also often found in a proportion of the circulating lymphocytes of patients suffering from mucopolysaccharidoses (McKusick, 1966) such as Hurler's syndrome (Muir, Mittwoch & Bitter, 1963). Knecht, Cifonelli & Dorfman (1967) have shown that the heparan sulphate isolated from the livers and urine of patients with Hurler's syndrome is heterogeneous, having a much lower molecular weight than that isolated from human aorta, and Fratantoni,

* Abbreviations: GAG, glycosaminoglycan; PP, protein–polysaccharide as defined by Tsiganos & Muir (1969*a*); CPC, cetylpyridinium chloride.

Hall & Neufeld (1968) demonstrated that patients' fibroblasts grown in culture in the presence of [^{35}S]sulphate stored labelled material that was more degraded than that secreted into the medium. These authors suggested that, on the basis of kinetic data, some newly synthesized material was diverted to an intracellular pool and subsequently partially degraded.

Further investigation of the material isolated from Kurloff-cell inclusion bodies was therefore undertaken to determine whether a similar mechanism occurs in the Kurloff cell under the influence of oestrogen, which leads to the storage of partially degraded PP. If so, the Kurloff cell might provide an experimental model for the mucopolysaccharidoses. Preliminary details of some of these findings have already been presented (Dean & Muir, 1969).

MATERIALS AND METHODS

All chemicals were of analytical grade with the exception of anthrone, Ehrlich's reagent, 9-aminoacridine–HCl, glucosamine–HCl, glucuronolactone, galactose, xylose, CPC and cetyltrimethylammonium bromide. Sterile [^{35}S]sulphate for injection was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Acetone and ethanol were RR grade (James Burroughs Ltd., London S.E.11, U.K.), acetylacetone was redistilled (b.p. 133–134°C) and water was glass-distilled.

Determination of hexuronic acid. Hexuronic acid was determined by the Bitter & Muir (1962) modification of the method described by Dische (1947) with glucuronolactone as a standard.

Determination of hexosamine. Total hexosamine was determined as described by Tsiganos & Muir (1969*a*), by using a modification of the distillation procedure of Cessi & Piliego (1960). Glucosamine hydrochloride was used as a standard.

Determination of hexose. The method of Trevelyan & Harrison (1952) was used with galactose as a standard. Control tubes containing equivalent amounts of hexosamine (glucosamine) and glucuronolactone to those of the sample were used to account for interference.

Determination of pentose. Pentose values were determined by using the anthrone method of Tsiganos & Muir (1966) with xylose as a standard. Interference from other sugars was allowed for by adding appropriate amounts of glucuronolactone, glucosamine and galactose to the control tubes.

Molar ratios of glucosamine to galactosamine. The molar ratios of the two hexosamines were determined as described by Tsiganos & Muir (1969*b*).

Amino acid analysis. Analyses were carried out essentially as described by Tsiganos & Muir (1969*b*) on a Locarte amino acid analyser. Samples were dissolved in 6M-HCl (2ml/mg), evacuated and then flushed with O_2-free N_2 several times before the containers were sealed and heated for 24h at 105°C. Hydrolysates were then dried on a rotary evaporator, washed several times with water and clarified by centrifugation, before being dissolved in a known volume of starting buffer, pH2.8.

Alkaline β-carbonyl elimination. A known weight of PP from the spleens of four pregnant guinea pigs (about 1.5mg) was dissolved in 0.5ml of 0.5M-NaOH, sealed under N_2 and left for 24h at room temperature, after which 1.0ml of water and 1.5ml of conc. HCl were added. The sample was then hydrolysed as described above for amino acid analysis.

I.r. spectra. Samples (1mg) were dissolved in 1ml of a 0.1% solution of KCl and freeze-dried, and the resulting powder was pressed into a disc. A Unicam SP.200 recording spectrophotometer was used.

Gel chromatography. A column (0.9cm×45cm) was packed with 6% (w/v) agarose [a gift from Dr C. P. Tsiganos, prepared as described by Tsiganos & Muir (1969*b*)] and another (1.1cm×46cm) was packed with Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). Samples containing 3–4mg of PP were dissolved in 0.5M-sodium acetate, pH6.5, by stirring overnight at 4°C, and then applied to each column. The columns were eluted with the same solvent at a flow rate of 5ml/h. Fractions (0.8ml) were collected and their uronic acid contents determined. A column of Sephadex G-200 (0.9cm×100cm) was used for chromatography of free GAG chains. Amounts of 5mg dissolved in 1.0ml of water were applied, the column was eluted with water, and 0.8ml fractions were collected.

Ion-exchange chromatography. Fractionation of GAG chains was carried out as described by Knecht *et al.* (1967) on a column (50cm×2cm) of Dowex 1 (Cl^- form) (Sigma Chemical Co., St Louis, Mo., U.S.A.). Samples containing approximately 50mg of GAG in 5ml of water were applied to the column and were eluted with a stepwise gradient consisting of 3 column volumes each of water, followed by 0.5M, 1.0M, 1.3M, 1.5M, 1.7M and finally 2.0M-NaCl. Fractions (5ml) were collected and their uronic acid contents determined.

Micro CPC column profiles. Solubility profiles of the cetylpyridinium–protein–polysaccharide complexes were determined by using the method described by Antonopoulos, Gardell, Szirmai & De Tyssonsk (1964). Samples of PP containing 50–100μg in 50μl of water were applied to cellulose columns (6.0cm×0.5cm) that had been previously equilibrated with 1% (w/v) CPC containing 5mM-Na_2SO_4. The columns were eluted with 1ml each of 1% (w/v) CPC in 5mM-Na_2SO_4 followed by 0.3M-NaCl in 0.05% CPC and then with 0.05% CPC containing increasing concentrations of $MgCl_2$ from 0.2 to 0.6M in steps of 0.05M, and finally with 6M-HCl. Hydrolysis and determination of hexosamine content of each fraction was by the method described by Antonopoulos *et al.* (1964). The free GAG chains obtained by digesting the PP with crystalline papain for 3h at 37°C (5μg of crystalline papain per 0.5mg of PP in 250μl of the papain buffer described below) were examined in the same way.

Induction of Kurloff-cell formation. Adult male and female guinea pigs of mixed varieties were injected intramuscularly with 5mg (0.5ml) of oestradiol monobenzoate (CIBA Laboratories, Horsham, Sussex, U.K.), followed by a second injection of 2mg 21 days later. The animals were killed after 35 days, the spleens removed and either frozen immediately and stored at −20°C, or dehydrated and stored in acetone at room temperature. Those animals used for [^{35}S]sulphate incorporation experiments were given weekly doses of 0.5mg of oestra-

diol monobenzoate for a period ranging from 5 to 10 weeks.

Toluidine Blue staining. Spleens were removed, sliced and smears taken. The slides were air dried and then fixed in 0.1% CPC for 15min, stained by immersing in 1% (w/v) Toluidine Blue in 70% (v/v) ethanol for 10min, dehydrated and mounted.

Preparation of glycosaminoglycans. Glycosaminoglycans were prepared from acetone-dried spleens by using the procedure of Bitter & Muir (1966). The dried spleens were suspended in 0.2M-sodium acetate, pH5.7, containing 4mM-EDTA and 20mM-cysteine–HCl (50ml/g dry wt.) and incubated at 60°C with a solution of crude papain (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) that had been previously activated by incubating for 30min at 60°C in the above buffer. Incubation was continued overnight under toluene, as a preservative, with 100mg of papain/g of tissue, followed by addition of fresh batches of enzyme (50mg/g dry wt.) after 15h and again after 36h. Digestion was completed after about 48h, the suspension was then dialysed for a further 48h at 4°C against several changes of water and clarified by filtration through Celite 545. Polyanions were then precipitated with 9-aminoacridine–HCl (Muir & Jacobs, 1967) and converted into soluble sodium salts by shaking with a suspension of Dowex 50W (8% cross-linked, Na^+ form). Finally, GAG chains were precipitated in 80% (v/v) ethanol, that contained a few ml of a solution consisting of 30g of anhydrous sodium acetate and 15ml of acetic acid made up to 100ml with water. The precipitated GAG chains were washed with ethanol and acetone and then dried. To remove as much of the residual amino acid as possible the GAG chains were redissolved in 0.1M-sodium borate buffer, pH8.2, containing 20mM-$CaCl_2$ and digested with pronase from *Streptomyces griseus* (Nomoto & Narahashi, 1959) for 24h at 55°C, by using 10mg of enzyme in 50ml of buffer for each gram of original tissue. The GAG chains were then isolated and purified as described above for the digestion with papain. The sodium salts were dried to constant weight at 80°C *in vacuo* over P_2O_5.

Preparation of protein–polysaccharides. Spleens that had been stored at −20°C were frozen in liquid N_2 and pulverized in a steel die cooled in the same manner. They were then homogenized (in 10g samples) in 75ml of 0.15M-sodium acetate, pH6.8, at 4°C with a blade homogenizer. The batches were centrifuged, the supernatant solution decanted and the pooled residues subjected to a further extraction as above. This process was repeated a further three times, making a total of five extracts in all. The extracts were pooled, filtered through Celite 545 and the polyanions were precipitated with 9-aminoacridine–HCl. Conversion into the sodium salt was then carried out as described above and the supernatant material was centrifuged to remove any denatured protein that had co-precipitated. The clarified solution was reprecipitated with 9-aminoacridine–HCl and the whole procedure was repeated, followed by a final precipitation with ethanol.

Contaminating nucleic acids were removed as described by Olsson & Gardell (1967). Crude material weighing 42mg was dissolved in 2ml of 0.05M-sodium phosphate, pH7.4, containing 5mM-$MgCl_2$ and 1mg of both ribonuclease and deoxyribonuclease (Koch–Light Laboratories Ltd.) were added. After 3h at 37°C the mixture was centrifuged, the supernatant solution was added to a cellulose column (Whatman CF11, 38cm×2cm) and washed in with 0.5ml of water. The column had been previously washed with 3 column volumes of 1% (w/v) cetyltrimethylammonium bromide containing 5mM-Na_2SO_4. Protein and digested nucleic acids were removed by washing with 3 column volumes of the cetyltrimethylammonium bromide solution and the PP was finally eluted with 1.0M-$MgCl_2$ containing 0.05% cetyltrimethylammonium bromide. A few drops of 5.0M-$MgCl_2$ were added to the eluate to ensure complete solubilization of the cetyltrimethylammonium protein–polysaccharide complex and the PP was then precipitated with 4 volumes of ethanol. Conversion into the sodium salt was effected by redissolving in a few ml of 0.15M-sodium acetate and reprecipitating with 5 volumes of ethanol. The precipitate was washed with ethanol and acetone and then dried. Samples for analysis were dried to constant weight at 80°C *in vacuo* over P_2O_5. No phosphate could be detected in the purified material by using the method of Chen, Toribara & Warner (1956) and solutions containing 0.5mg/ml had no significant absorption at 260nm.

Incorporation of [^{35}S]*sulphate.* (*a*) *In vitro.* Control spleens and spleens containing Kurloff cells were removed 35 days after the first oestrogen injection, diced and three samples of 1g were taken from each group. Each sample was incubated with 10μCi of [^{35}S]sulphate in 10ml of the following buffer: 59mM-NaCl, 0.5mM-$CaCl_2$, 2.25mM-KCl, 0.6mM-NaH_2PO_4, 0.25mM-$MgCl_2$, 12.5mM-$NaHCO_3$, glucose (9mg/l) and glutamine (44mg/l), adjusted to pH7.4 by bubbling in a mixture of N_2+CO_2 (95:5) just before use. After incubation for 90 or 180min at 37°C in a shaking water bath the medium was decanted, centrifuged to remove tissue debris, and the polyanions in the supernatant solution were precipitated with 9-aminoacridine–HCl. The tissue was washed with a further 10ml of buffer, each sample was homogenized in 10ml of 0.2M-sodium acetate, pH6.8, centrifuged and polyanions in the supernatant solution were precipitated with 9-aminoacridine–HCl. Both sets of precipitates were collected by centrifugation, washed twice with dilute 9-aminoacridine–HCl and converted into soluble sodium salts as described above, after which they were again centrifuged. The supernatant solution was made 10mM with respect to $MgSO_4$ and dialysed against several changes of water to remove any remaining traces of inorganic [^{35}S]sulphate. The non-diffusible sample was finally precipitated with 4 volumes of ethanol containing a few ml of the sodium acetate solution as previously described, washed and dried in acetone.

Before measuring radioactivity each sample was redissolved in 0.5ml of water, and centrifuged. The supernatant solution was decreased in volume to 100μl and applied to micro CPC-cellulose columns of larger size (7cm×0.8cm) than those used to examine solubility profiles. The columns were eluted with 2ml of 1% (w/v) CPC in 5mM-Na_2SO_4, followed by 2ml of 0.3M-NaCl in 0.05% CPC and finally with 2ml of 6M-HCl. All fractions were then hydrolysed for 8h in 6M-HCl, the acid was removed *in vacuo* over KOH and samples were dissolved in 1.0ml of water. One half was taken for the determination of the hexosamine content by using the method of Antonopoulos *et al.* (1964) and the other half was used to measure radioactivity. Each sample was added to 10ml

of scintillation fluid consisting of 20g of naphthalene, 1g of 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen and 130ml of toluene made up to 250ml with 2-methoxyethanol (Hardingham & Phelps, 1968) and the radioactivity was measured in a Packard Tri-Carb model 3320 liquid-scintillation spectrometer. The efficiency of counting was calculated by counting each sample in the presence and absence of the automatic external standard to determine the degree of quenching and all counts were corrected to d.p.m. Efficiency was normally between 60% and 70%.

(*b*) *In vivo*. Ten control guinea pigs and ten oestrogen-treated animals were each injected intraperitoneally with 500μCi of [^{35}S]sulphate. Five from each group were killed 24h later and the other five 96h later. The spleens from each group were homogenized in 50ml of 0.2M-sodium acetate, pH6.8, centrifuged and the sedimented material rehomogenized in a further 50ml of buffer. The polyanions in the pooled supernatant solutions were then precipitated with 9-aminoacridine–HCl, converted into soluble sodium salts and finally precipitated with ethanol as described above. Each precipitate was redissolved in 250μl of water and 50μl amounts were applied to the smaller micro CPC-cellulose columns (6cm×0.5cm) and the procedure of Antonopoulos *et al.* (1964) was followed, except that samples were redissolved in 1.0ml of water after hydrolysis. Half was taken for hexosamine determination and half for scintillation counting.

RESULTS

Spleen smears taken 3 weeks after administration of the first dose of oestrogen and stained with Toluidine Blue contained large numbers of cells with metachromatic inclusion bodies. These inclusion bodies were still readily distinguishable in those animals that had been maintained on a weekly dose of oestrogen for periods of up to 10 weeks. Administration of oestrogen was not continued beyond this point.

The amount of uronic acid-positive material isolated from acetone-dried Kurloff spleens after digestion with papain and pronase was four times that obtained from the same weight of control spleens. The GAG chains isolated from spleens of untreated animals were separated into two components by ion-exchange chromatography on Dowex 1 (Fig. 1*a*). The fraction eluted in 1.0M-sodium chloride contained only glucosamine and had an electrophoretic mobility on cellulose acetate similar to a heparan sulphate from human aorta (Muir, 1965). The second, smaller, fraction which was eluted with 1.3M-sodium chloride, accounted for 28% of the total GAG obtained from the spleen. The galactosamine/glucosamine molar ratio in this fraction was 1:1.5. On the other hand, GAG isolated from Kurloff spleens separated into three components (Fig. 1*b*) which were eluted with 1.0M-, 1.3M- and 1.5M-sodium chloride and comprised 50%, 41% and 9% respectively of the total uronic acid in the eluate. The predominant hexosamine in each

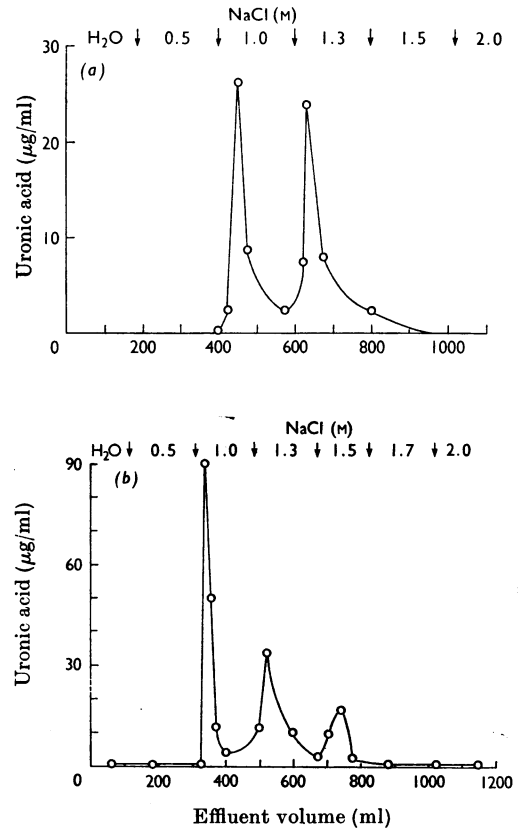


Fig. 1. Fractionation on Dowex-1 of the GAGs isolated from guinea-pig spleens. (*a*) 39mg of GAG from control spleen tissue; (*b*) 53mg of GAG from Kurloff spleen tissue. Both samples were applied and eluted as described in the text.

component was galactosamine, the galactosamine/glucosamine molar ratios being 40:1, 150:1 and 80:1 respectively. An authentic sample of chondroitin sulphate prepared from pig laryngeal cartilage by the method of Muir & Jacobs (1967) separated into three similar components on Dowex 1, although the relative proportions of each fraction differed from those of the Kurloff material, constituting 18%, 75% and 7% of the total respectively. The Kurloff-cell GAG is thus very similar to chondroitin sulphate. The i.r. spectrum showed distinct absorption bands at 720cm^{-1}, 850cm^{-1} and 928cm^{-1} characteristic of chondroitin 4-sulphate (Mathews, 1958), but there was in addition a strong unidentified band at 805cm^{-1} (Fig. 4).

Material isolated from Kurloff spleens without proteolysis and freed of nucleic acids (Olsson & Gardell, 1967) on gel chromatography was eluted with the void volume from Sephadex G-200 and

from 6% (w/v) agarose but was almost wholly included in Sepharose 4B. When, after digestion with papain and reisolation, the material was again chromatographed on Sephadex G-200, the uronic acid-positive material was almost completely included (Fig. 2). At the position of maximum uronic acid content the molecular weight of the free GAG chains was calculated to be greater than 20000, by using the data obtained by Wasteson (1969).

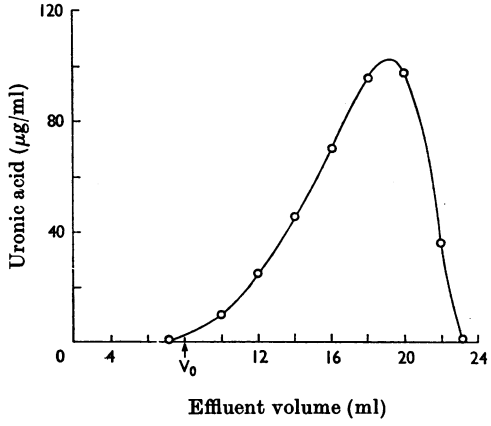


Fig. 2. Gel chromatography of Kurloff-cell GAG on Sephadex G-200. PP (10mg) was digested with papain and then applied and eluted as described in the text.

The solubility profile on elution from micro CPC-cellulose columns of the Kurloff-cell material obtained without proteolysis is shown in Fig. 3(*a*). The bulk of the material was eluted by magnesium chloride of relatively high molarity. After digestion of part of this preparation with crystalline papain there was a marked shift in the profile, most of the material being eluted by salts of lower concentration (Fig. 3*b*). The CPC solubility profile of the PP isolated from the spleens of naturally pregnant animals that had not been treated with oestrogens, was essentially the same in all major respects to that obtained from oestrogen-treated guinea pigs.

The PP of Kurloff spleens had the following analysis: hexosamine, 37.5; hexuronic acid, 33.8; hexose, 1.5; pentose, 0.6%. The precise galactosamine/glucosamine molar ratio was not determined because of the small amount of material available and the low proportion of glucosamine, which amounted to less than 1% of the total hexosamine present.

The molar ratios of serine, pentose and hexose were 1:1.07:2.2, and the hexosamine/hexuronic acid molar ratio was 1.09:1 and that of hexose/pentose 2.095:1.

The amino acid analysis of the PP is given in Table 1. Serine was the major constituent, closely followed by glycine and glutamic acid in almost equimolar amounts. Total protein was calculated to be 2.8% by weight of the PP. A similar analysis of a sample of GAG–peptide showed that serine was again the major amino acid together with

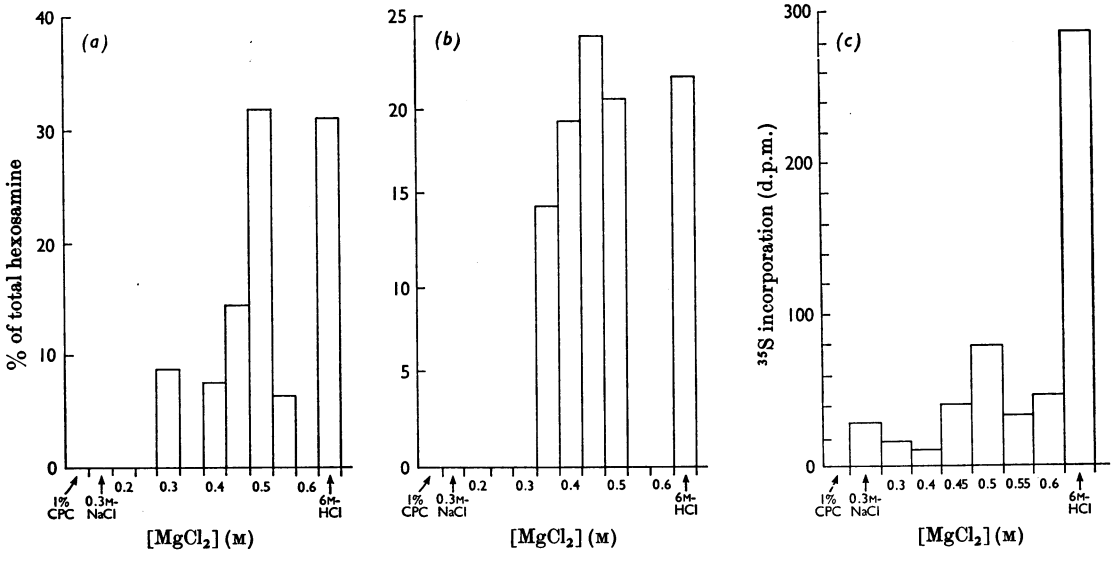


Fig. 3. Solubility profiles of cetylpyridinium complexes of (*a*) protein–polysaccharide; (*b*) glycosaminoglycan after digestion with papain; 100μg of samples were applied to each column. (*c*) Radioactivity (d.p.m./g wet wt.) observed in protein–polysaccharide fractions 96h after injection of [^{35}S]sulphate (see the text).

Table 1. *Amino acid composition of the protein–polysaccharide and corresponding GAG–peptide isolated from Kurloff-cell inclusion bodies*

Analyses were not corrected for losses during hydrolysis.

	Protein–polysaccharide		
(/)	(μmol/g)	(residues/1000 residues)	GAG–peptide (μmol/g)
Asp	19.2	76.2	5.1
Thr	18.1	72.0	3.2
Ser	37.1	147.0	12.3
Glu	33.5	132.8	4.4
Pro	14.0	55.5	—
Gly	34.0	134.4	—*
Ala	18.7	74.0	4.4
Cys†	—	—	—
Val	11.6‡	46.1	2.3
Met	1.1§	4.3	—
Ile	6.9	27.2	1.3
Leu	15.7	62.3	2.1
Tyr	4.9	19.3	—
Phe	7.3	29.0	—
Lys	9.3‖	36.9	7.6
His	14.5	57.6	2.4
Arg	6.3	24.9	1.3

* The glycine content could not be accurately determined because of a contaminant with the same R_F value.

† Cystine was not separated or confirmed.

‡ The value for valine was previously misquoted as being that for cystine (Dean & Muir, 1969).

§ Methionine previously omitted was found on re-examination.

‖ Lysine chromatographed as a double peak.

relatively large amounts of lysine, aspartic acid and glutamic acid (Table 1). A considerable amount of threonine was found in both the intact PP and the GAG–peptide. During alkaline β-carbonyl elimination 62% of serine and 72% of threonine were destroyed in the PP isolated from the spleens of normally pregnant animals.

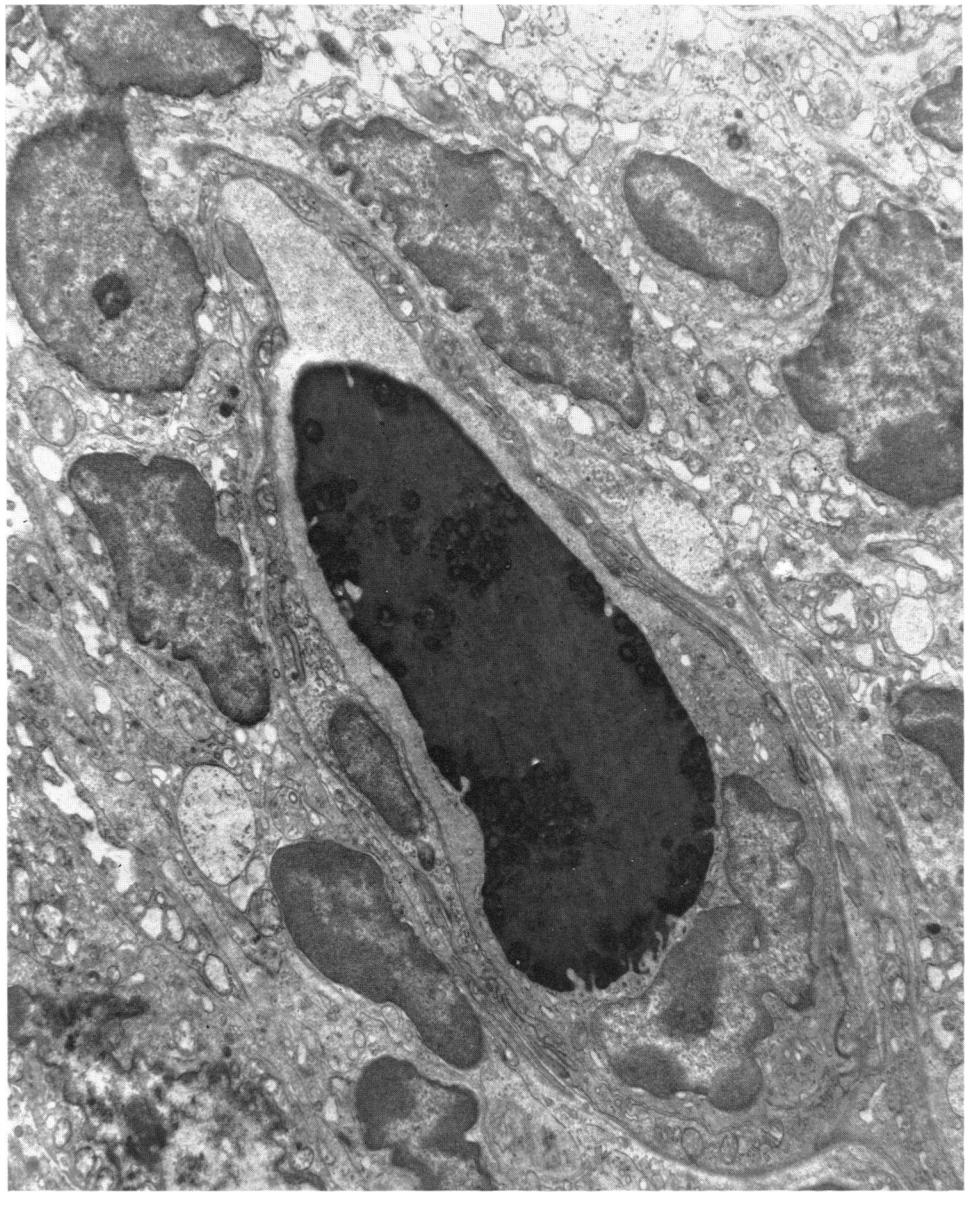
There was very little incorporation of [^{35}S]-sulphate into the GAG of Kurloff spleens *in vitro*. After incubation for 90min, the radioactivity varied between 130 and 340 d.p.m./g wet wt. of spleen, the average total radioactivity of those fractions eluted from CPC-cellulose columns with 6M-hydrochloric acid amounting to only 260 d.p.m./g wet wt. In comparison control spleens had an average total radioactivity of 54 d.p.m./g wet wt. for the corresponding fraction. On incubation of the spleens with [^{35}S]sulphate for 180min there was a very similar amount of incorporation ranging from 120 to 260 d.p.m./g wet wt. with an average total radioactivity of 238 d.p.m./g wet wt. The corresponding values for control spleens averaged 40 d.p.m./g wet wt. In both sets of experiments approximately half the radioactivity of the GAG was present in the incubation medium, and the remainder in the spleen tissue itself. The total amount of hexosamine, however, was too low to calculate specific radioactivities with any degree of accuracy.

The incorporation of [^{35}S]sulphate into the GAG of spleens *in vivo* was also low. Thus, 24h after injection of [^{35}S]sulphate, complete micro CPC-column fractionation showed that only the fraction eluted in 6M-hydrochloric acid was radioactive, the activity being 215 d.p.m./g wet wt. Even 96h after the injection of [^{35}S]sulphate, the radioactivity of the fraction eluted in 6M-hydrochloric acid had increased to only 298 d.p.m./g wet wt., although there was now radioactivity in the fraction eluted with 0.3M-sodium chloride and in some magnesium chloride fractions (Fig. 3*c*). There was no detectable radioactivity in any CPC fraction of GAG from control spleens 96h after the injection of [^{35}S]-sulphate.

DISCUSSION

The fourfold increase in GAG content/g dry wt. of spleen after treatment with oestrogen was confined to GAG chains containing galactosamine, as assessed from the galactosamine/glucosamine molar ratios of GAG fractionated on Dowex 1. The large increase in the relative proportion of galactosamine is explained by the fact that the spleens of oestrogen-treated animals enlarge several times due to infiltration by Kurloff cells, so that the proportion of cells to structural material of the spleen increases considerably (Ledingham, 1940; Marshall & Swettenham, 1959). The GAG due to connective tissue/g dry wt. will thus be diluted by the GAG of Kurloff cells. Authentic chondroitin sulphate behaved like the Kurloff material on Dowex 1 chromatography; and since the GAG from Kurloff spleens is susceptible to hyaluronidase (Muir & Marshall, 1961) the increase was attributed largely to a GAG closely resembling chondroitin sulphate. Further the i.r. spectrum showed bands characteristic of chondroitin 4-sulphate (Fig. 4) (Mathews, 1958).

As the PP isolated from Kurloff spleens without proteolysis was excluded from Sephadex G-200, it contained more than one chondroitin sulphate chain, since single chains can penetrate this gel (Wasteson, 1969). After proteolysis, however, the size was decreased sufficiently to penetrate Sephadex G-200 (Fig. 2). Likewise a decrease in molecular size after proteolysis was indicated by the lower concentrations of magnesium chloride that would elute the GAG chains from micro CPC-cellulose columns, compared with the intact PP (Figs. 3*a* and 3*b*).



EXPLANATION OF PLATE 1

Electron micrograph of typical Kurloff cell stained with lead citrate. Magnification ×9040. The electron-dense body in the centre is a typical inclusion.

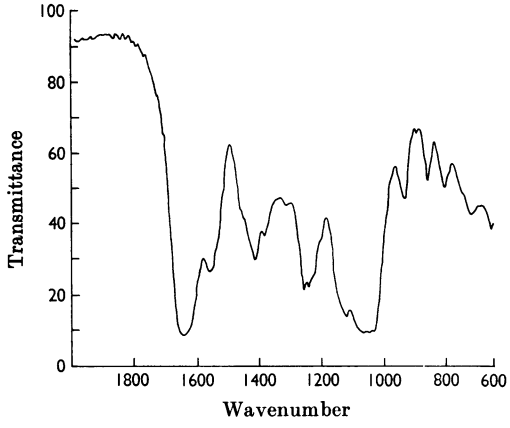


Fig. 4. I.r.-absorption spectrum of GAG from Kurloff spleens.

The PP from Kurloff cells was excluded from 6% (w/v) agarose columns and was therefore larger than the smallest PP of cartilage (Tsiganos & Muir, 1969*b*; Brandt & Muir, 1969), and considerably larger than the intracellular material of cultured Hurler fibroblasts, which was mainly retarded on Sephadex G-200 (Fratantoni *et al.* 1968). Further, as discussed below, the average length of the chondroitin sulphate chains was high, so that intracellular PP of Kurloff cells was not partially degraded, unlike the intracellular material of cultured Hurler fibroblasts (Fratantoni *et al.* 1968) or Hurler organs (Knecht *et al.* 1967). Thus Kurloff-cell inclusions do not appear to be enlarged lysosomes filled with partially degraded material, as has been suggested is the origin of the metachromatic inclusions of cells in the organs of patients with mucopolysaccharidoses (Van Hoof & Hers, 1968). Indeed, electron micrographs of the inclusions of Kurloff cells stained with lead citrate (Plate 1) appear very different from those of cells from Hurler spleen and liver similarly stained (Van Hoof & Hers, 1964).

The PP of the Kurloff cell, which was large enough to be excluded from 6% (w/v) agarose, contained only 2.8% protein. Tsiganos & Muir (1969*b*) and Brandt & Muir (1969) observed that the extracellular PP complexes of cartilage contained more protein the larger they were. However, even the smallest, which penetrated 6% (w/v) agarose, contained about twice as much protein (Tsiganos & Muir, 1969*b*) as the Kurloff-cell PP, and the cartilage PP excluded from this gel contained more than 7% protein.

On the other hand, a PP fraction of cartilage separated by electrophoresis (Muir & Jacobs, 1967), resembles the Kurloff-cell PP in containing only about 2% protein, and less than 1% of the total hexosamine as glucosamine. Other than this minor fraction, however, the extracellular PP complexes of cartilage all contain appreciable amounts of glucosamine, attributable to keratan sulphate and hence they contain considerably more hexose (Tsiganos & Muir, 1969*b*; Brandt & Muir, 1969) than the Kurloff-cell PP, which had only 1.5% hexose.

The amino acid analysis of the GAG–peptide from Kurloff cells and of the original protein suggests that the majority of serine residues were linked to carbohydrate chains, since after proteolysis the proportion of serine increased from 12% by weight of the total amino acid residues in the PP to 20% by weight of the GAG–peptide. This was confirmed by the destruction of 62% of the serine on β-carbonyl elimination in alkali, by using the PP from Kurloff cells of pregnant animals. This is similar to the destruction in alkali of 64% of serine in an intracellular PP of human leucocytes, which similarly contained little glucosamine (Olsson, 1969). Since the majority of serine residues in the cartilage PP of high electrophoretic mobility (Muir & Jacobs, 1967) appeared also to be linked to chondroitin sulphate chains, a high proportion of serine in glycosidic linkage seems to be a feature of PP lacking keratan sulphate. About half as much threonine as serine was present in the PP and the concomitant destruction of 72% of threonine is as yet unexplained. However, since the molar proportion of threonine to serine decreased from 1:2 in the PP to 1:4 in the GAG–peptide after exhaustive proteolysis, it is unlikely that much of the threonine is bound to carbohydrate chains. Glycosaminoglycan–protein linkages involving xylose have so far been found to be confined to serine.

The serine/pentose/hexose molar ratio in the Kurloff cell PP was 1:1.07:2.2, which closely agreed with the theoretical ratio of 1:1:2 if each serine residue of the protein core was linked to chondroitin sulphate via xylose and two galactose residues, as in PP of bovine nasal cartilage (Rodén & Smith, 1966). From the hexosamine/xylose and hexosamine/serine molar ratios the average lengths of GAG chains was between 51 and 57 disaccharide units, corresponding to a number average molecular weight of 26000–29000. This is significantly greater than the lengths of chondroitin sulphate chains in PP of pig laryngeal and articular cartilage, calculated in the same way, which were 28 (Tsiganos & Muir, 1969*b*) and 20–26 (Brandt & Muir, 1969) disaccharide units respectively. The elution profile on Sephadex G-200 (Fig. 2) shows that the majority of GAG chains have molecular weights greater than 20000 (Wasteson, 1969) but that there is polydispersity particularly towards chains of higher

molecular weight. The relatively longer chains would explain why the cetylpyridinium–GAG complexes of Kurloff cells required rather higher electrolyte concentrations for elution (Fig. 3*b*) than cartilage cetylpyridinium–chondroitin sulphate complexes.

The incorporation *in vitro* of [^{35}S]sulphate into the GAG of spleens containing Kurloff cells was slight, although somewhat greater than in the controls. Incorporation *in vivo* was also low even after 4 days (Fig. 3*c*). Thus in the fully developed Kurloff cell studied here there is little synthesis of sulphated GAG. On the other hand, by using [^{35}S]sulphate Marshall, Swettenham, Vernon-Roberts & Revell (1970) have shown that towards the beginning of Kurloff-cell induction there is active synthesis in these cells *in vivo*. In contrast administration of isotope before induction showed that Kurloff cells did not take up labelled material derived from other sites in the body. The inclusion bodies appear to be specialized storage organelles (Plate 1) for the material synthesized by these cells. Marshall *et al.* (1970) showed that both the crude and the purified GAG and PP isolated were toxic to macrophages *in vitro* at concentrations as low as 1pg/ml whereas other cells remained unaffected. Chondroitin 4-sulphate and PP from cartilage passed through each step of the purification were without effect. Marshall *et al.* (1970) have evidence that Kurloff cells migrate towards the placenta and release their contents in the placental labyrinth. No release was found elsewhere. These authors suggest, therefore, that the PP or GAG of Kurloff cells, by killing macrophages, may interfere with cell-mediated immunity and so prevent rejection of the foetus. The Kurloff cell GAG differs from cartilage chondroitin 4-sulphate in the greater length of the chains and in having a strong i.r.-absorption band at 805cm^{-1} (Fig. 4). It is possible that this band may be due to the constituent that is toxic to macrophages.

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