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The Glycosaminoglycans of Neonatal Rat Skin

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The acid glycosaminoglycans were extracted from the skins of young rats less than 1 day *post partum*. The isolated products were fractionated by a cetylpyridinium chloride–cellulose column technique and identified by chemical analysis, electrophoretic mobility and susceptibility to testicular hyaluronidase digestion. Hyaluronic acid (56%) dermatan sulphate (15.6%) and chondroitin 6-sulphate (9.1%) were the major components, but chondroitin 4-sulphate, heparan sulphate and heparin were also present, together with two further fractions tentatively suggested to be a heparan sulphate-like fraction and a dermatan sulphate fraction, both of short chain length or low degree of sulphation.

In a previous paper (Hardingham & Phelps, 1968) the pathway of biosynthesis from glucose through the nucleoside diphosphate intermediates of acid glycosaminoglycans was studied in neonatal rat skin. The present paper serves a complementary role in establishing the composition of the acidic polymers in the same tissue.

Since neonatal rat skin furnishes a highly active, tractable, system for the study of acid glycosaminoglycan biosynthesis the present paper sets out to characterize quantitatively these components in this tissue.

MATERIALS AND METHODS

Materials. Standard sugars were obtained from B.D.H. (Chemicals) Ltd., Poole, Dorset, U.K. Hyaluronic acid was prepared from human umbilical cords and purified by CPC† precipitation. Chondroitin sulphate from shark cartilage was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks, U.K. Heparin (94–95 i.u./mg) was obtained from B.D.H. (Chemicals) Ltd. Papain (EC 3.4.4.10) was obtained twice crystallized from Sigma Chemical Co., St Louis, Mo., U.S.A. Testicular hyaluronidase (EC 3.2.1.35; 300i.u./mg) was obtained from Koch–Light Laboratories Ltd. Cetylpyridinium chloride and microanalytical-grade H_2SO_4 were obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. Ethanol, A.R. grade, was obtained from James Burrough Ltd., London S.E.11, U.K. Gelatin ('Bacto') was obtained from Difco Laboratories, Detroit, Mich., U.S.A. Bovine serum albumin (fraction V) was obtained from Sigma Chemical Co. Silica gel G was obtained from E. Merck A.-G., Darmstadt, W. Germany. Whatman CF11 cellulose powder, CC41 microgranular cellulose and fat-extracted Soxhlet thimbles (80mm × 25mm) were supplied by H. Reeve Angel and Co. Ltd., London E.C.4, U.K. Oxoid cellulose acetate strips (36cm × 5cm) were obtained from Oxo Ltd., London S.E.1, U.K. Alcain Blue was obtained from G. T. Gurr Ltd., London S.W.6, U.K. Sephadex G-25 (fine grade) was obtained from Pharmacia, Uppsala, Sweden. Visking tubing was obtained from Scientific Instruments Centre, London W.C.1, U.K. It was boiled for 6h in several changes of water before use All other reagents were obtained from BDH (Chemicals) Ltd.

Centrifugation. Centrifugation was carried out in an MSE High-Speed 18 centrifuge.

Measurement of extinction. Extinctions were read on a Hilger Uvispek H700 instrument.

Extraction of acid glycosaminoglycans. Newborn Wistar rats less than 24h *post partum* were killed by decapitation and the skin was removed and finely chopped with scissors. About 20g of skin was placed in a Soxhlet apparatus and dried with acetone for 2h and then defatted with chloroform–methanol (2:1, v/v). The residue was freed of solvent by vacuum desiccation and then suspended in 0.1M-sodium acetate–5mM-cysteine–5mM-EDTA, pH5.5, containing 6mg of crystalline papain/g of dry skin (Scott, 1960). The papain was preactivated by incubation in the buffer for 30min at 60°C before addition of the dried skin. The digest was incubated overnight at 60°C, cooled to 4°C and any material left undigested was removed by centrifugation at 5000*g* for 10min. Further incubation after the addition of fresh enzyme did not significantly decrease the amount of undigested material. The undigested residue was washed twice in 0.1M-sodium acetate and kept for further treatment. The supernatant and washings were combined and 30% (w/v) trichloroacetic acid was added to give a final concentration of 5% (w/v). After 2h at 4°C the precipitate was removed by centrifugation at 5000*g* for 10min and washed in 5% (w/v) trichloroacetic acid. The combined supernatant and washings were adjusted to pH6.8 with 4M-NaOH and dialysed for approx. 12h against several

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† Abbreviation: CPC, cetylpyridinium chloride.

changes of water. Then 4M-NaOH was added to give a final concentration of 0.5M and the digest was shaken for 8h at 4°C.

The residue left undigested by papain was added back to the extract before this stage, as it contained 2–6% of the total uronic acid extracted, and was made completely soluble by shaking with alkali. The extract was then adjusted to pH6–8 with 4M-HCl and dialysed for 48h against several changes of water. It was then passed through a column (10cm × 1.5cm) of Dowex 50 (H^+ form; 200–400 mesh). The water eluate and washings were collected. This removed 80% of the protein-reacting material without loss of uronic acid. The eluate was then adjusted to pH6.5 with 1M-NaOH and freeze-dried. This preparation was used for subsequent fractionations and analyses. Care was taken throughout to detect loss of uronic acid-reacting material during dialysis. It was found that, as indicated by Pearce & Mathieson (1967), a considerable amount of uronic acid-reacting material was not anionic, as it was not removed from the diffusate by passage through Dowex 1 (Cl^- form) resin. Losses at other stages were minimal.

Cetylpyridinium chloride fractionation. The cetylpyridinium complexes of the acid glycosaminoglycans were fractionated on a cellulose column according to their critical salt solubilities as described by Antonopoulos, Borelius, Gardell, Hamnstrom & Scott, (1961). For the extract of up to 20g wet wt of skin, a column (10cm × 1.5cm) of Whatman CF11 cellulose was prepared as described by Scott (1960). It was washed with several bed volumes of 1% CPC and the solution of acid glycosaminoglycans, in 2ml of 5mM-Na_2SO_4, was added dropwise to the top of the column. At the same time 1% CPC was also added dropwise. Each drop was allowed to soak into the column before the next was added. The column was then eluted with: 30ml of 1% CPC; 30ml of 0.3M-NaCl in 0.05% CPC; 30ml of 0.3M-$MgCl_2$ in 0.05% CPC; 30ml of 0.70M-$MgCl_2$ in 0.05% CPC; 30ml of 1.25M-$MgCl_2$ in 0.05% CPC; 20ml of 6M-HCl. Fractions (5ml) were collected. The column was run at room temperature. No CPC precipitated from solution at normal rates of elution (approx. 15ml/h).

The acid glycosaminoglycans were isolated from the eluates by the following methods (Scott, 1960). The 1% CPC fraction was concentrated by rotary evaporation at 40°C and 4–5 vol. of ethanol and several drops of saturated sodium acetate were added. The precipitate was collected by centrifugation after standing overnight at 4°C. It was washed in ethanol and diethyl ether and dried. Salt fractions were concentrated on a rotary evaporator and precipitated with 2–3 vol. of ethanol. The precipitates were collected, washed with ethanol and diethyl ether, and dried.

During the course of isolation of material from the 1% CPC fraction it was found possible to separate a fraction containing uronic acid, hexosamine and sulphate from one containing hexosamine and sulphate but very little uronic acid. The former was precipitated in the presence of sodium acetate by the addition of 2 vol. of ethanol, the latter was precipitated after 4–5 vol. of ethanol were added. These are designated, in Table 1, fractions 1% CPC *a* and *b* respectively.

CPC fractionations of isomeric chondroitin sulphates and dermatan sulphate. The acid glycosaminoglycans of the 0.70M-$MgCl_2$ fraction were subfractionated as described by Antonopoulos & Gardell (1963), by using the preferential solubility of chondroitin 4-sulphate in organic solvents and of chondroitin 6-sulphate in acid solvents compared with dermatan sulphate. A column (10cm × 1.5cm) of Whatman CF11 cellulose was prepared as described above. The sample was applied and eluted with: 30ml of 0.3M-$MgCl_2$ in 0.05% CPC; 30ml of 0.05% CPC; 30ml of propan-1-ol–methanol–acetic acid–water (80:40:3:77, by vol.) containing 0.4g of CPC/100ml; 30ml of 0.05% CPC; 30ml of 0.75M-$MgCl_2$ in 0.1M-acetic acid–0.05% CPC; 30ml of 0.05% CPC; 30ml of 0.75M-$MgCl_2$ in 0.05% CPC, 20ml of 6M-HCl.

Fractions (5ml) were collected and samples were assayed for uronic acid (Bitter & Muir, 1962). Samples from those fractions containing organic solvents were evaporated to dryness at 90°C, dissolved in water and then assayed. Fractions were isolated as described above. The organic-solvent fraction was concentrated by rotary evaporation and 4–5 vol. of ethanol and a few drops of saturated sodium acetate were added to precipitate the acid glycosaminoglycan. They were collected and dried as described above.

Electrophoresis. Electrophoresis of acid glycosaminoglycans was carried out on cellulose acetate strips (12cm × 5cm) in an EEL horizontal-electrophoresis apparatus, with short bridge adaptors. The strips were carefully wetted on the under surface only. Excess of moisture was then removed by blotting before they were placed in position in the electrophoresis tank. Double thicknesses of Whatman 3MM chromatography paper were used as conducting wicks.

After 1h equilibration in the chosen buffer, the solution of acid glycosaminoglycans (approx. 1–4mM-uronic acid) was applied with a 10μl syringe. The strips were then run at 10–15V/cm for 2–3h. After electrophoresis the strips were dried in a 100°C oven for 2min then stained with 1% (w/v) Alcian Blue in 10% (v/v) acetic acid for 5min. The strips were washed in 10% (v/v) acetic acid for 10min followed by running tap water for several hours.

For quantitative results the stained strips were cleared in glycerol and scanned with an EEL scanner. The slit was set as narrow as possible (0.5mm) within the sensitivity of the instrument in order to obtain maximum resolution of the components.

Hyaluronidase digestion of acid glycosaminoglycans. Acid glycosaminoglycans were digested with hyaluronidase from sheep testes (300i.u./mg), as described by Mathews & Inouye (1961), in 0.1M-sodium citrate–phosphate buffer, pH5.5. The digests were incubated in stoppered tubes at 37°C for 24h and then chromatographed on a column (40cm × 1cm) of Sephadex G-25 in 0.4M-NaCl–50mM-sodium phosphate, pH6.5.

Hexosamines. Acid glycosaminoglycans (1–2mg/ml) were hydrolysed in 3.8M-HCl at 100°C for 6h in sealed tubes. Hydrolysates were dried by vacuum desiccation over KOH. The hexosamines were isolated for chromatography by applying the sample in water to a column (3cm × 0.5cm) of Dowex 50 (H^+ form; 200–400 mesh). The column was washed in 3ml of water and the hexosamines were then eluted with 2ml of 0.5M-HCl. The eluate was freeze-dried and assayed by the method of Blix (1948). The water eluate and washings contained no hexosamine. Samples were chromatographed on thin layers of silica gel G, eluted with propan-1-ol–ammonia

(sp. gr. 0.89) (13:7, v/v). This system was used quantitatively as previously described (Hardingham & Phelps, 1968). The water eluate of the column containing neutral sugars was freeze-dried and chromatographed on thin layers of cellulose phosphate eluated with acetone–butan-1-ol–water (5:4:1, by vol.) as described by Hardingham & Phelps (1968). Protein was assayed by the Miller (1959) modification of the Folin method. Bovine serum albumin was used as standard.

Other assays. Sulphate was assayed by the turbidimetric method of Dodgson (1961) after release from acid glycosaminoglycans by hydrolysis in 3M-HCl at 100°C for 4h.

Sulphamate in acid polymers was assayed by the method of Luganoff & Warren (1962) with glucosamine as standard.

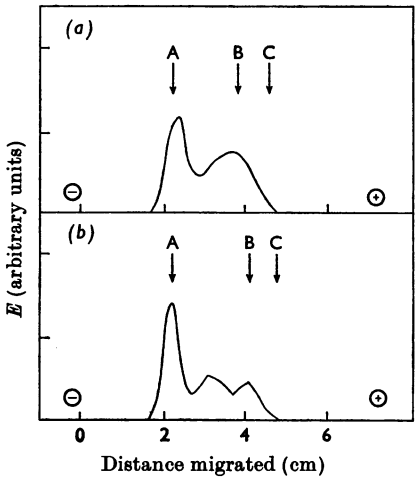

Fig. 1. Electrophoresis on cellulose acetate of acid glycosaminoglycans extracted from neonatal rat skin. (*a*) Electrophoresis in 0.1M-sodium formate, pH3.0, 12V/cm for 40min. (*b*) Electrophoresis in 0.2M-$ZnSO_4$, pH4.8, 8V/cm for 120min. Both were stained with Alcian Blue, cleared and scanned as described in the text. A, mobility of standard hyaluronic acid; B, mobility of chondroitin sulphate from shark cartilage; C, mobility of heparin.

N-Acetylhexosamine was assayed by the Good & Bessman (1964) modification of the Morgan–Elson reaction. *N*-Acetylglucosamine was used as standard.

Hexuronic acid was assayed by the method of Bitter & Muir (1962) and Khym & Doherty (1952) by using glucuronolactone as standard.

RESULTS

The extraction procedure described above released 1.75 ± 0.09 μmol of hexosamine/g wet tissue, of acid glycosaminoglycans. This was similar to the results reported by Pearce & Mathieson (1967) for adult rat skin (approx. 1.6μmol/g wet wt.) and Prodi (1964), for skins of rats 1–5 days old (1.8μmol/g wet wt.). The fat-free dry weight of the skin was $11.45 \pm 0.25\%$ of the wet weight.

Electrophoresis of the whole extracts in 0.2M-zinc sulphate (Fig. 1) showed three major components, the fastest component corresponding in mobility to standard chondroitin 6-sulphate and the slowest to hyaluronic acid. The intermediate component has a mobility similar to that reported for dermatan sulphate (Haruki & Kirk, 1967). Electrophoresis in 0.1M-sodium formate, pH3.0, that does not separate dermatan sulphate from the chondroitin sulphates, showed only two major components; one with the mobility of hyaluronic acid and the other a rather broad band with the mobility of the chondroitin sulphates and dermatan sulphate.

Fractionation with CPC (Table 1) enabled five main groups of compounds of different characteristics to be separated.

The presence of uronic acid in the 1% CPC fraction was notable. The 1% CPC elutes those compounds not forming CPC complexes, e.g. glycoproteins, and those complexes soluble in excess of CPC, e.g. keratan sulphate (Scott, 1960). The uronic acid present in the fraction formed a fairly constant proportion of the total extracted uronic acid and was unaffected by the amount put on to the column or the volume in which it was applied. Standard acid glycosaminoglycans and

Table 1. *CPC fractionation of acid glycosaminoglycans from* 10*g of neonatal rat skin on a column of cellulose* (10*cm* × 1.5*cm*)

Eluant	Molar ratios: Uronic acid / Hexosamine	Molar ratios: Carbazole / Orcinol	Molar ratios: Sulphate / Hexosamine	Molar ratios: Sulphamate / Hexosamine	% of total hexosamine	Identification of hexosamine by t.l.c.	% degraded by testicular hyaluronidase measured by gel chromatography
1% CPC (*a*)*	0.13	0.12	—	0.10	3.8±0.7	Glucosamine	0
(*b*)*	0.83	0.89	0.80	0.03	4.8±1.0	Glucosamine	0
0.3M-NaCl	1.05	1.11	0.04	<0.01	56.0±3.9	Glucosamine	100
0.3M-$MgCl_2$	0.97	0.85	0.90	0.11	5.0±0.8	Glucosamine + galactosamine	0
0.7M-$MgCl_2$	0.93	0.84	0.98	0.07	26.0±2.1	Galactosamine	39
1.25M-$MgCl_2$	1.09	1.41	1.45	0.91	3.9±1.6	Glucosamine	0

* (*a*) precipitated with 2vol. of ethanol and sodium acetate; (*b*) precipitated with 4–5vol. of ethanol and sodium acetate.

tissue extracts, e.g. from human umbilical cords, did not produce a comparable fraction; it was not, therefore, a result of bad technique.

The ability to isolate two fractions with different analyses by fractional ethanol precipitation (Table 1) showed that more than one component was present. However, electrophoresis in both solvent systems showed only a single band with a mobility just greater than that of hyaluronic acid (Fig. 2). T.l.c. of the acid hydrolysate showed glucosamine as the only hexosamine, and galactose in the neutral sugar fraction. The large excess of glucosamine over uronic acid in the analysis of fraction *a* and the presence of a considerable amount of galactose in the mixture suggested that keratan sulphate was one component. The analysis indicated that the other component was of the heparan sulphate type, showing some degree of *N*-sulphation (Table 1), but presumably differing from true heparan sulphate, which is eluted by 0.3 M-magnesium chloride (Svejcar & Robertson, 1967), by having a lower degree of sulphation or a shorter chain length. The complete resistance of both components to hyaluronidase digestion was in agreement with this identification.

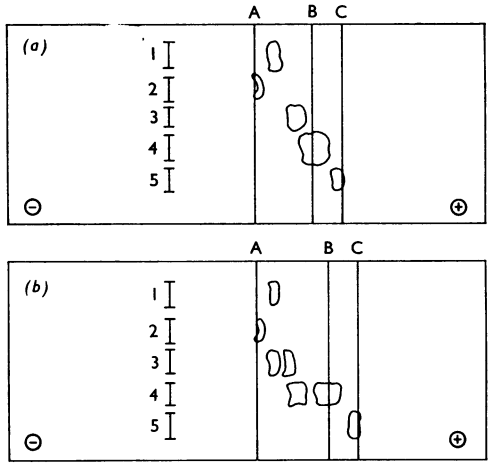

Fig. 2. Electrophoresis on cellulose acetate of the CPC fractions of the acid glycosaminoglycans extracted from neonatal rat skin. (*a*) 0.1 M-sodium formate, pH 3.0; conditions as in Fig. 1. (*b*) 0.2 M-$ZnSO_4$ pH 4.8, conditions as in Fig. 1. Both stained with Alcian Blue. A, B, and C as for Fig. 1. 1, 2, 3, 4, 5 represent the fractions eluted from the CPC–cellulose column with 1% CPC, 0.3 M-NaCl, 0.3 M-$MgCl_2$, 0.7 M-$MgCl_2$ and 1.25 M-$MgCl_2$ respectively.

The 0.3 M-sodium chloride fraction, by its analysis, electrophoresis and digestion by testicular hyaluronidase, was identified as hyaluronic acid.

The 0.3 M-magnesium chloride fraction contained two components in equal amounts, resolved by electrophoresis in 0.2 M-zinc sulphate, but having equal mobilities in 0.1 M-sodium formate, pH 3.0. Neither component was degraded by testicular hyaluronidase. Analysis showed equal amounts of glucosamine and galactosamine and some *N*-sulphate (Table 1). The glucosamine and *N*-sulphate suggested heparan sulphate to be present, which would be in agreement with the position of elution of heparan sulphate shown by Svejcar & Robertson (1967). The galactosamine-containing component, undigested by hyaluronidase, was possibly dermatan sulphate of short chain length or low degree of sulphation.

The 0.7 M-magnesium chloride fraction contained galactosamine as the sole hexosamine. It separated into two components on electrophoresis in 0.2 M-zinc sulphate, their mobilities corresponding to that of chondroitin 6-sulphate and that reported for dermatan sulphate (Haruki & Kirk, 1967). It moved as a single wide band during electrophoresis in 0.1 M-sodium formate, pH 3.0. Treatment of the 0.7 M-magnesium chloride fraction as described by Antonopoulos & Gardell (1963) indicated that only 6% of the fraction was chondroitin 4-sulphate, compared with 35% as chondroitin 6-sulphate and 59% as dermatan sulphate (Table 2).

The fraction was tested for susceptibility to testicular hyaluronidase. Of the three possible components only dermatan sulphate was not degraded by hyaluronidase and its presence in the

Table 2. *Fractionation of isomeric chondroitin sulphates and dermatan sulphate*

The fraction of acid glycosaminoglycan containing the chondroitin sulphates and dermatan sulphate was applied to a cellulose column (10 cm × 1.5 cm) prewashed with 1% CPC. The column was eluted with the solvents described in the text. Uronic acid was measured by the orcinol method; values are means of three determinations.

Solvent	Uronic acid (μmol)	Carbazole / orcinol molar ratio	Isomer	% of sample	% of tissue glycosaminoglycans
Organic	0.50	1.05	Chondroitin 4-sulphate	5.6	1.5
Acid	3.13	0.96	Chondroitin 6-sulphate	35.0	9.1
Neutral	5.34	0.80	Dermatan sulphate	59.5	15.6

digest was revealed by gel chromatography on Sephadex G-25 (Schmidt & Dmochowski, 1964) (Fig. 3). Of the mixture 61% was undegraded and assumed to be dermatan sulphate. This result was in good agreement with the CPC method. The proportion of chondroitin 6-sulphate and chondroitin 4-sulphate was determined by analysing the mixture for Morgan–Elson-positive groups before and after digestion. It was assumed that chondroitin 6-sulphate produced one equivalent of Morgan–Elson-positive groups per tetrasaccharide after complete digestion and chondroitin 4-sulphate no positive groups (Mathews & Inouye, 1961). The increase in Morgan–Elson-positive groups after digestion (Table 3) thus showed 25% of the mixture to be chondroitin 6-sulphate, and by difference, the remaining 14% was chondroitin 4-sulphate. The lower proportion of chondroitin 6-sulphate indicated by this method may have resulted from incomplete digestion to tetrasaccharides, for on gel chromatography (Fig. 3) the digested material did not appear homogeneous, although there was little increase in the Morgan–Elson-positive groups in the second 24h of digestion. For this reason more reliance is placed on the CPC determination.

The 1.25M-magnesium chloride fraction was shown to be heparin by analysis, electrophoresis and the resistance to testicular hyaluronidase digestion.

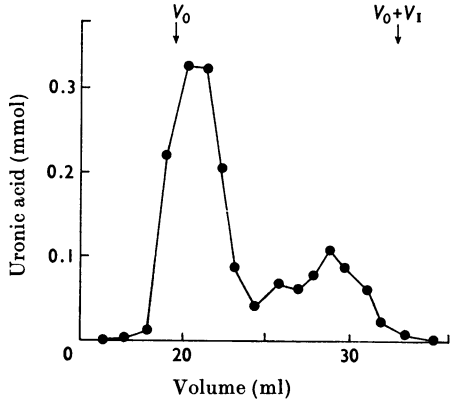

Fig. 3. Gel chromatography of the 0.7M-$MgCl_2$ fraction (containing 1.8μmol of uronic acid) after digestion with testicular hyaluronidase, on a column (60cm × 1cm) of Sephadex G-25. Fractions (25 drops; 1.05ml) were collected and assayed for uronic acid by the orcinol method. Abbreviations: V_0, void volume; V_0+V_I, total internal volume.

DISCUSSION

In a previous paper (Hardingham & Phelps, 1968), the pathways in the biosynthesis of acid glycosaminoglycans in young rat skin had been studied. The analysis of the composition of the acid glycosaminoglycans in this tissue, as reported above, was a necessary prerequisite for studying the flow of radioactivity from precursors into the final polymers (T. E. Hardingham & C. F. Phelps, unpublished work).

The general composition of these polymers in rat skin was reported by Schiller, Slover & Dorfman (1961). Three fractions, hyaluronic acid, chondroitin sulphate and heparin, were identified and their relative proportions at the youngest age studied (21 days) were 55:22:23 respectively. The 'chondroitin sulphate' fraction of adult rat skin was later shown to contain heparan sulphate (Schiller, 1966), though the same fraction from the skins of young rats was not examined. Prodi (1964), using a similar fractionation scheme, determined the composition from the age of 1–5 days to 30–36 months. At 1–5 days hyaluronic acid accounted for 77% of the total and chondroitin sulphate for 21.7%. However, in comparison with the work of Schiller *et al.* (1961), a much lower proportion of heparin was found at all ages. No explanation of this discrepancy was apparent. The total acid glycosaminoglycans extracted (800μg of hexosamine/g of dry skin at 2–9 months) was similar in both reports.

Table 3. *Hyaluronidase digestion of the* 0.7M-*magnesium chloride fraction*

A sample of the chondroitin sulphate and dermatan sulphate mixture was digested with hyaluronidase (300 units) in 0.1M-sodium citrate–sodium phosphate buffer, pH5.5. The mixture was assayed for *N*-acetylhexosamine before and after digestion and the digested product was freeze-dried, dissolved and chromatographed on a Sephadex G-25 column. Standard chondroitin 4-sulphate and 6-sulphate were similarly treated.

	Uronic acid (μmol)	*N*-Acetylhexosamine (μmol) 0h	24h	48h	*N*-Acetylhexosamine / uronic acid molar ratio	Composition (%) Chondroitin 6-sulphate	Chondroitin 4-sulphate	Dermatan sulphate (by gel chromatography)
Skin fraction	0.83	0.004	0.102	0.108	0.125	25	14	61
Chondroitin 6-sulphate	0.48	0.002	—	0.208	0.429	86	14	—
Chondroitin 4-sulphate	0.50	0.002	—	0.020	0.036		94	—

Barker, Cruickshank & Webb (1965) reported the identification of keratan sulphate, hyaluronic acid, chondroitin 4-sulphate, chondroitin 6-sulphate, dermatan sulphate and heparin in a digest of newborn rat skin. However, neither the amounts present nor their relative proportions were stated.

The number of apparently distinct glycosaminoglycans poses problems for any analytical technique aimed at resolving each component. Two methods have been used by the majority of workers. Anion-exchange chromatography of the acid polymers in rat skin has been used by Schiller *et al.* (1961) and by Barker *et al.* (1965). In our experience this method was neither as reproducible nor as discriminating as the CPC–cellulose column method of Antonopoulos *et al.* (1961). Even this method, however, was incapable of resolving some mixtures of acid glycosaminoglycans, but the use of a later modification (Antonopoulos & Gardell, 1963) enabled the separation of the isomeric chondroitin sulphates and dermatan sulphates with results that compared well with those obtained by the procedure described by Mathews & Inouye (1961).

The advantages of electrophoresis as an adjunct to the identification of mixed components is shown by the analysis of the composition of the 0.3M-magnesium chloride fraction, which enabled its tentative identification as a mixture of heparan sulphate and dermatan sulphate. The reason for the appearance of dermatan sulphate in this fraction was unclear. It may differ from that in the 0.7M-magnesium chloride fraction in being of smaller size, of lower degree of sulphation, or both. This indicates the possible heterogeneity of some acid glycosaminoglycans present in tissue extracts and emphasizes the difficulty of securing integral uncontaminated fractions of each glycosaminoglycan present in this tissue.

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