The Glycosaminoglycans of Human Tracheobronchial Cartilage

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1. The glycosaminoglycans of human tracheobronchial cartilages from subjects of various ages were liberated by proteolysis of the tissue and purified by ion-exchange chromatography. Purified glycosaminoglycans were fractionated on Dowex 1 resin and cetylpyridinium chloride was used to separate chondroitin sulphates and keratan sulphates occurring in the same fraction. 2. The total chondroitin sulphate content of the cartilages decreased linearly with increasing age. Age-dependent changes in the chemical heterogeneity of chondroitin sulphate were observed, a low-sulphated compound making up 25% of the total glycosaminoglycan at birth but rapidly diminishing in content during the first 6 months of life. Of the total chondroitin sulphate the 6-isomer became rather more prominent than the 4-isomer with increasing age. 3. The total keratan sulphate content of the cartilages increased from trace amounts only at birth to a plateau value by the beginning of the fifth decade. Of the total keratan sulphate approx. 70% was due to a high-molecular-weight compound with a sulphate/hexosamine ratio of 1.5-1.8:1.0. The degree of sulphation varied between compounds isolated from different individuals. The remaining 30% of the keratan sulphate appeared to be intimately associated with chondroitin 6-sulphate and could only be separated from it after treatment with 0.45M-potassium hydroxide. The hybrid glycosaminoglycans were of lower molecular weight and had a lower sulphate/hexosamine ratio than the major keratan sulphate compound.

Changes in the galactosamine/glucosamine ratio in hydrolysates of human costal cartilage of different ages were first observed by Stidworthy, Masters & Shetlar (1958). Kaplan & Meyer (1959) and Mathews & Glagov (1966) confirmed that these changes were due to a decrease in the chondroitin sulphate content of the cartilage with increasing age and a corresponding increase of a second glycosaminoglycan component, keratan sulphate. The latter workers also investigated the cartilages of the larynx and the adjacent portion (upper) of the trachea and suggested that changes in the proportions of chondroitin sulphate and keratan sulphate with increasing age occurred more slowly in these tissues than in costal cartilage. Moreover, although early studies of articular-cartilage hydrolysates indicated an age-dependent fall in the galactosamine and a rise in the glucosamine contents of the tissue (von Kuhn & Leppelmann, 1958), more recent investigations of this tissue (Bollet & Nance, 1966; Greiling & Stuhlsatz, 1969; Maroudas, Muir & Wingham, 1969) suggest that the proportions of chondroitin sulphate and keratan sulphate hardly vary during adult life. Thus age-dependent changes

in hyaline-cartilage glycosaminoglycans may differ according to the anatomical site of the tissue.

In the present work the glycosaminoglycans from another group of hyaline cartilages, those of the bronchial tree, have been studied (cf. Saltzman, Sieker & Green, 1963). Glycosaminoglycans were extracted from the lower-trachea, primary-bronchus and secondary-bronchus cartilages of subjects ranging in age from birth to senescence. The fractionation and characterization of these compounds was undertaken and the results were related to the age of the tissue. A preliminary report of some of this work has previously been made (Mason, Dodgson & Wusteman, 1969).

EXPERIMENTAL

Preparation of tracheobronchial cartilage samples. Tissues were obtained at necropsy from subjects of various ages who had no known history or post-mortem findings associated with connective-tissue diseases. The interval between death and necropsy was always less than 36 h and usually less than 24 h. Cadavers were stored at 4°C during the interval between death and necropsy. After removal, tissues were either used immediately or stored at -20°C until required.

The bronchial tree was dissected free from the lung up

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to the level of and including the initial parts of the major subdivisions of the secondary bronchi. It was then divided at the branching points into trachea (lower four rings), primary bronchus, secondary bronchi and their major subdivisions. In some cases the secondary bronchi were kept separate as upper-, mid- and lower-lobe bronchi, but in others they were pooled.

The cartilages from each area were dissected from the walls of the air-ways. As much of the perichondrium as possible was removed from the cartilage, which was then cut into small pieces (approx. 5mm³), dried overnight in two changes of acetone and further dried to constant weight at 80°C over P₂O₅ *in vacuo*.

Extraction of glycosaminoglycans from cartilage samples.

Dry cartilage (40–60 mg) was suspended in 0.1 M-potassium phosphate buffer (50 ml), pH 6.3, containing 5 mM-EDTA and 5 mM-cysteine hydrochloride (Scott, 1961), and the whole was incubated for 30 min at 65°C. Activated papain (EC 3.4.4.10) [10.0 units of a twice-recrystallized suspension; Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] was added to the mixture, which was then incubated at 65°C for 24 h. After cooling, ficin (EC 3.4.4.12) [2.5 units of a twice-recrystallized suspension; Sigma (London) Chemical Co. Ltd.] was added and incubation continued for a further 24 h at 37°C to ensure complete proteolysis. Ethanol (3 vol.) was added and the mixture was left overnight at 0°C. The precipitate was collected by centrifuging, washed with 85% (v/v) ethanol and dissolved by adding 1.0 ml of 2.0 M-KOH and water to 50 ml. The pH of this solution was adjusted to 7.0.

Cation-exchange chromatography. Unwanted cationic material was removed from the cartilage extract by passage through columns containing 30 ml of a 1:1 (w/w) mixture of Amberlite resins IRC-50 and IR-120 (H⁺ forms) (BDH Chemicals Ltd., Poole, Dorset, U.K.). After discarding the first 10 ml, the remaining effluent (40 ml) was collected. The extract was washed through the column with water (60 ml) and the pH of the total effluent (100 ml) was adjusted to 7.0.

Anion-exchange chromatography. Dowex 1 (X2; 200–400 mesh; Cl⁻ form) resin [Sigma (London) Chemical Co. Ltd.] was poured as a slurry into columns (12 mm diam.) to give a resin volume of 5.0 ml and then equilibrated with 0.2 M-NaCl. The effluent from the cation-exchange chromatography step was applied to the resin and washed in with 0.8 M-NaCl (75 ml). Only traces of hexosamine-containing material were recovered in the 0.8 M-NaCl effluent from any one cartilage extract. The column was then eluted in two steps with 2.5 M-NaCl containing 8.0 M-urea (this solution is referred to below as 2.5 M-NaCl+8.0 M-urea), the first of 15 ml and the second 12 h later of 10 ml. This procedure ensured equilibration of the resin with the eluent. The eluates from the two steps were pooled for desalting.

Desalting. The pooled solutions were applied to columns (2 cm diam., bed height 30 cm) of Sephadex G-25 (fine grade) [Pharmacia (G.B.) Ltd., London W.13, U.K.] and eluted with water. The salt-free crude glycosaminoglycan preparation was collected as a 40 ml effluent.

Fractionation of the crude glycosaminoglycan preparation. Fractionation was carried out on a second Dowex 1 (X2; 200–400 mesh; Cl⁻ form) resin column prepared as described above. The preparation was washed on with 0.8 M-NaCl and was eluted in steps of 1.2 M-, 1.4 M-, 1.6 M-

and 1.8 M-NaCl and 2.5 M-NaCl+8.0 M-urea. For each step a 20 ml fraction was collected in two 10 ml stages, 12 h apart.

The 1.2–1.8 M-NaCl fractions were assayed directly for uronic acid and the final fraction was assayed directly for hexose. Other analytical procedures were performed on desalted fractions.

The recovery of glycosaminoglycan after ion-exchange purification procedures and Dowex 1 fractionation was in the range 92–100% with a mean of 96.5%.

Purification of Dowex 1 fractions with cetylpyridinium chloride. Most Dowex 1 fractions contained both galactosamine and glucosamine in sufficient quantities to suggest that both chondroitin sulphate and keratan sulphate were present. When detailed analysis of a single glycosaminoglycan was required it was therefore necessary to purify the Dowex 1 fractions further. Columns (1 cm × 20 cm) containing 7.0 g of Chromedia CF11 cellulose powder (BDH Chemicals Ltd.) were prepared as described by Antonopoulos, Borelius, Gardell, Hamnström & Scott (1961) and were saturated with cetylpyridinium chloride (Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.) by washing with 50 ml of a 1.0% solution. Columns were run in a warm room at 28°C.

Dowex 1 fractions of the same salt concentration from cartilages of comparable ages were pooled, desalted, concentrated and applied (1.0 ml portions) to the column. The latter was then eluted with 1.0% cetylpyridinium chloride (3 bed volumes), the effluent being collected as the keratan sulphate fraction and then with 1.0 M-MgCl₂ (3 bed volumes), which was collected as the chondroitin sulphate fraction. Cetylpyridinium chloride was removed from the fractions as an insoluble salt at 4°C followed by extraction with pentan-1-ol as described by Scott (1961).

Hyaluronidase digestion of Dowex 1 fractions. Dowex 1 fractions were digested with hyaluronidase (EC 3.2.1.35) (from sheep testes, 1000 i.u./mg; Seravac Laboratories Ltd., Maidenhead, Berks., U.K.) as described by Mathews & Inouye (1961). Hyaluronidase-resistant glycosaminoglycans were precipitated from the digest by the addition of ethanol (3 vol.), taken up in water and purified by passage through Amberlite resin columns (as described above) before analysis.

A larger-scale preparation of hyaluronidase-resistant glycosaminoglycans was obtained from 4 g of dry pooled tracheal cartilage from subjects aged 45–75 years. This was digested with papain and ficin as described above but on an enlarged scale. After elution from the Amberlite resin bed the solution was immersed in a boiling-water bath for 5 min, cooled, concentrated to 10 ml and then digested with a total of 40 mg of hyaluronidase over a period of 72 h. After the addition of ethanol to the digest it was left overnight at 0°C, the precipitate was separated by centrifugation, dissolved in water and desalted on Sephadex G-25. Portions of this solution were fractionated on Dowex 1 in the usual way.

Analytical methods. (a) Hexuronic acid and hexose. The method of Bitter & Muir (1962) was used for hexuronic acid with D-glucuronolactone as standard. Hexose was assayed by the anthrone reaction (Dische, 1962), with D-galactose as standard.

(b) Hexosamine. Glucosamine and galactosamine were assayed with the Technicon amino acid analyser as described by Wusteman & Johnson (1967). The colour yield

of both hexosamines was 92.0% of that of L-norleucine with ninhydrin and hydrindantin from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., U.K.). Hexosamine values were corrected (glucosamine 5%, galactosamine 10%) for degradation during hydrolysis (100°C for 8 h in 4M-HCl in lightly capped tubes). The optimum hydrolysis time and corrections were deduced from time-hydrolysis studies of pure samples of chondroitin sulphate and keratan sulphate.

(c) Sulphate. The ester sulphate content of glycosaminoglycans was determined after hydrolysis by an automated version (*Methodology Manual*; Technicon Instrument Co., Chertsey, Surrey, U.K.) of the BaCl₂-gelatin technique (Dodgson, 1961). The hydrolysis conditions (110°C for 5 h in 1.0M-HCl in sealed tubes) were those recommended by Dodgson & Price (1962).

(d) Hydroxyproline. The method of Stegman (1958) was employed, with L-hydroxyproline as standard. Cartilage samples were hydrolysed in 6M-HCl at 110°C for 24 h before analysis.

(e) Ash. The method of Eastoe & Courts (1963) was employed to determine the ash content of cartilage samples.

(f) Chondroitin 6-sulphate. This was determined in the presence of other glycosaminoglycans by the method of Mathews & Inouye (1961).

Electrophoresis. This was carried out on cellulose acetate strips (12.0 cm) for either 50 or 80 min at a constant voltage of 260 V in acetic acid-pyridine buffer (100 ml of acetic acid, 5 ml of pyridine and 895 ml of water) at pH 3.0. The strips were stained with aq. 0.5% Toluidine Blue.

Infrared spectra. These were measured on the Perkin-Elmer model 257 spectrophotometer. Discs were prepared from glycosaminoglycan (2.5–3.0 mg) mixed with KBr (200 mg).

Determination of molecular weight by osmotic-pressure measurement. Measurements were made with a Hewlett Packard model 503 osmometer, an instrument designed to measure dynamic osmotic pressure. The advantages of this approach over measurements made at equilibrium have been described by Bruss & Stross (1961). Measurements were made with a Bac-T-Flex B.19 membrane (Carl Schleicher and Schuell Co., Keene, N.H., U.S.A.) at 25°C with 0.5M-NaCl as solvent.

RESULTS

Changes in uronic acid content of cartilages with age. The uronic acid-containing material from proteolytically digested infant cartilage was eluted in the 1.2–1.6M-sodium chloride fractions from Dowex 1 and that from adult cartilage was recovered in the 1.2–1.8M-sodium chloride fractions. The total uronic acid recovered in the fractions is an indication of the total chondroitin sulphate content of the cartilage. Values for the total uronic acid content of primary bronchial cartilages from a number of individuals are shown in Fig. 1 and indicate a marked decrease with increasing age. The result obtained through regression analysis of these limited data and of those obtained for tracheal and secondary-bronchial cartilages may suggest that the

Fig. 1. Regression line y (uronic acid as % of tissue dry wt.) on x (age) for tracheobronchial cartilages from subjects ($n = 32$) in the age range birth–65 years. —, $y = 4.436 - 0.021x$. The broken lines give the limits to the predictions that can be made from the line at the 95% confidence level. Δ , Experimental values for 14 primary-bronchus cartilages within the group.

relationship between decreasing uronic acid content and increasing age is a linear one (Table 1). Histological examination (R. M. Mason, unpublished work) of cartilages from subjects in the eighth decade showed degenerative changes ('asbestotic degeneration'; see Silberberg & Silberberg, 1961) and results from this age group were therefore not included in the analysis. The regression coefficients for the tracheal, primary-bronchus and secondary-bronchus cartilages indicated that the age-related decrease in uronic acid content of the tissue proceeded at similar rates in the three sites. Bartlett's test (Snedecor & Cochran, 1967) indicated that there was no significant heterogeneity between the residual variance about the regression lines for the three sites. The regression coefficients were compared by an analysis of variance technique (Snedecor & Cochran, 1967) and no significant difference was found between them. A regression line can therefore be drawn for the combined uronic acid data (Fig. 1).

Apart from the general decrease in the chondroitin sulphate content with increasing age, changes in the relative amounts of this material appearing in each Dowex 1 fraction occurred during early life. Table 2 indicates that about 25% of the total chondroitin sulphate of neonatal cartilage is eluted in the 1.2M-sodium chloride fraction. During the first few months of life there is a rapid decline in the amount of chondroitin sulphate eluted in this fraction and a corresponding increase in the amount of material recovered in the 1.6M-sodium chloride fraction. With increasing age approx. 95.0% of the chondroitin sulphate is recovered in the 1.4–1.8M-sodium

chloride fractions and this elution pattern remains the same throughout adult life.

The chondroitin sulphate present in pooled

Table 1. *Regression analysis of the uronic acid contents of tracheal, primary-bronchial and secondary-bronchial cartilages from birth to 65 years of age*

In the regression line $y = a + bx$, where y is the uronic acid content as a percentage of the dry cartilage weight, x is the age (years) and a and b are the regression coefficients. n is the number of samples tested. A test of the significance of b is given by $t = b/s_b$ when s_b^2 is the variance of b . P is the level of significance of t .

	Trachea	Primary bronchus	Secondary bronchus
n	11	14	7
a	4.494	4.472	4.128
b	-0.026	-0.026	-0.022
t	5.344	5.625	3.104
P	<0.001	<0.001	<0.025

Table 2. *Percentage of the total uronic acid content of tracheobronchial cartilages eluted in different Dowex 1 fractions at different ages*

Uronic acid-containing material from neonatal cartilages was completely eluted in the 1.2-1.6M-NaCl fraction. In cartilages from older subjects up to 10% of the total was eluted in the 1.8M-NaCl fraction and this has been added to the 1.6M-fraction in this table for comparative purposes.

Age (years)	1.2M-NaCl fraction	1.4M-NaCl fraction	1.6M-NaCl+ 1.8M-NaCl fraction
Birth	24.7	59.3	16.0
Birth	25.7	57.0	17.3
0.08	17.5	63.5	19.0
0.21	15.0	65.0	20.0
0.42	10.3	53.0	36.7
2.0	8.5	54.5	37.0
4.5	7.5	52.5	40.0
7.0	4.5	50.2	45.3
22.0	4.0	39.0	57.0
41.0	4.2	37.9	57.9
56.0	6.0	42.0	52.0

neonatal 1.2M-sodium chloride fractions was further purified on a cellulose-cetylpyridinium chloride column and its chemical composition was then compared with those of similarly purified chondroitin sulphates from adult cartilage fractions (Table 3). Sulphate/hexosamine ratios just below 1.0 and uronic acid/galactosamine ratios in excess of 1.1 were observed for the chondroitin sulphate of the pooled 1.2M-sodium chloride fractions, and, although not recorded in Table 3, very similar results were obtained with material isolated from three individual early postnatal cartilages. The analysis of the 1.4M- and 1.6M-sodium chloride fractions from neonatal cartilage were similar to those recorded in Table 3 for chondroitin sulphates from adult cartilage eluted in the same Dowex 1 fractions.

The small amount of chondroitin sulphate recovered in the 1.2M-sodium chloride fraction from adult cartilages differed from the 1.4M- and 1.6M-sodium chloride fraction material in having a relative electrophoretic mobility of only 0.86. Similarly, chondroitin sulphate from adult 1.8M-sodium chloride fractions differed slightly from the bulk of the chondroitin sulphate; in particular it had a relatively large amount of glucosamine associated with it even after further purification on the cellulose-cetylpyridinium chloride column.

In almost all the samples investigated rather more of the total chondroitin sulphate was determined to be due to the 6-sulphate than to the 4-isomer. A mean contribution of 54.8% (s.d. 5.5, $n = 16$) of 6-isomer to the total chondroitin sulphate was found for one group of cartilages (aged from birth to the beginning of the fifth decade) whereas for an older group (sixth to eighth decade) a value of 61.5% (s.d. 3.8, $n = 12$) was recorded. The difference between the groups is significant (t test, $P = 0.01$).

Changes in the keratan sulphate content of cartilages with age. Hyaluronidase-resistant material that moved as a single band on electrophoresis was present in all the Dowex 1 fractions from adult cartilage. Traces of such material were also present in the final Dowex 1 fraction (2.5M-sodium chloride

Table 3. *Chemical composition of low-sulphated chondroitin sulphate from neonatal tracheobronchial cartilage and the chondroitin sulphates from adult cartilage*

Results are presented as molar ratios to hexosamine. Each compound was purified on a cellulose-cetylpyridinium chloride column before analysis (see the text). Relative electrophoretic mobility values (M_x) are with respect to a commercial sample of chondroitin 6-sulphate.

Dowex 1 fraction	Age (years)	Total hexosamine	Uronic acid/ galactosamine ratio	Sulphate/ hexosamine ratio	Galactosamine/ glucosamine ratio	M_x
1.2M-NaCl	Neonatal	1.0	1.26	0.92	66	0.81
1.4M-NaCl	22-43	1.0	1.04	1.03	43	0.98
1.6M-NaCl	22-43	1.0	0.90	1.02	52	0.99
1.8M-NaCl	56-76	1.0	1.06	1.17	6	0.99

Table 4. Composition of hyaluronidase-resistant compounds from adult tracheobronchial cartilages separated on Dowex 1 resin by a stepwise procedure with increasing sodium chloride concentration (see the text)

Hexose is expressed as D-galactose. M_x is electrophoretic mobility relative to a commercial sample of chondroitin 6-sulphate.

Dowex 1 fraction	Total hexosamine	Glucosamine/galactosamine ratio	Hexose/hexosamine ratio	Sulphate/hexosamine ratio	M_x	M_n
1.2M-NaCl	1.0	1.5	1.06	1.40	0.65	<14000
1.4M-NaCl	1.0	3.6	1.08	1.43	0.69	<14000
1.6M-NaCl	1.0	4.5	1.00	1.49	0.69	<14000
1.8M-NaCl	1.0	6.1	1.27	1.52	0.76	14900
2.5M-NaCl	1.0	8.4	1.18	1.55	0.93	19400

+ 8.0M-urea) from neonatal cartilages. This material was assumed to be keratan sulphate and analysis of samples separated from older cartilages confirmed this. In particular, the final Dowex 1 fraction from adult tracheobronchial cartilages contained about 70% of the total glucosamine initially present in the glycosaminoglycan preparation and analysis of the hyaluronidase-resistant component of this fraction suggested it to be a keratan sulphate of high molecular weight (Table 4). The hexose content (as D-galactose) of the 2.5M-sodium chloride + 8.0M-urea fraction, expressed as a percentage of the dry cartilage weight, is a measure of the high-molecular-weight keratan sulphate content of the cartilage and clearly showed the increasing content of this material in the tissue with increasing age. This is shown in Fig. 2 for a series of primary-bronchus cartilage samples from subjects ranging in age from birth to 65 years. Similar results were obtained with corresponding fractions from tracheal and secondary-bronchial cartilage. From trace amounts only at birth, the proportion of keratan sulphate increases rapidly in tracheobronchial cartilages during the first two decades and then more slowly to reach a plateau at the beginning of the fifth decade that is maintained up to the seventh decade.

Heterogeneity of high-molecular-weight keratan sulphate. The major keratan sulphate fraction (2.5M-sodium chloride + 8.0M-urea) was separated from cartilages of a number of individual subjects and detailed analysis showed all samples to be oversulphated. However, the degree of oversulphation varied between different individuals (sulphate/hexosamine ratios varying from 1.5:1.0 to 1.8:1.0) although there was no correlation with increasing age.

Hybrid glycosaminoglycans containing both keratan sulphate and chondroitin sulphate. Of the total glucosamine initially present in the glycosaminoglycan preparation from adult cartilages 30% was recovered in the 1.2–1.8M-sodium chloride fractions. On electrophoresis each of these fractions gave two

Fig. 2. Age-dependent changes in the content of high-molecular-weight keratan sulphate in primary-bronchus cartilage (0–65 years). Δ , 2.5M-NaCl + 8.0M-urea-fraction hexose (D-galactose) expressed as a percentage of the dry cartilage weight; this is an index of the keratan sulphate content (see the text).

bands, the slower of which was hyaluronidase-resistant and contained glucosamine as its major hexosamine. Detailed analysis of these glucosamine-containing compounds prepared from pooled adult tracheal cartilage after hyaluronidase digestion is shown in Table 4. Compounds having very similar glucosamine/galactosamine ratios to those shown in Table 4 were also isolated from non-hyaluronidase-digested Dowex 1 fractions by the cellulose-cetylpyridinium chloride column technique.

Although glucosamine was the major hexosamine component of the hyaluronidase-resistant compounds in the 1.2–1.8M-sodium chloride fractions, galactosamine was present in amounts suggesting that both chondroitin sulphate and keratan sulphate may be covalently linked to a common peptide. Accordingly the hyaluronidase-resistant compounds

were re-examined electrophoretically after exposure to 0.45M-potassium hydroxide under nitrogen for 18h at room temperature. The latter procedure usually brings about a β -carbonyl elimination reaction at the glycosidic linkage between glycosaminoglycans and peptide-bound hydroxyamino acids (see Seno, Meyer, Anderson & Hoffman, 1965). After the procedure the 1.2M- and 1.4M-sodium chloride fraction material separated into two adjacent but clearly different electrophoretic bands, the slower being predominant in each case. The 1.6M-sodium chloride fraction compound also separated into two bands after alkali treatment, but the distinction between them was less clear-cut than in the earlier fractions. The 1.8M-sodium chloride hyaluronidase-resistant fraction gave only a single band after treatment, but it was, however, much wider than that given by the untreated material in simultaneous runs, suggesting that two overlapping compounds may be present. Treatment of the 2.5 M-sodium chloride+8.0M-urea fraction keratan sulphate with potassium chloride did not affect its subsequent electrophoretic behaviour.

Infrared analysis of all the hyaluronidase-resistant compounds showed an absorption band at 820 cm^{-1} (characteristic of 6-sulphate substitution) but none at 855 cm^{-1} or 920 cm^{-1} (characteristic of 4-sulphate substitution).

Traces of material with glucosamine/galactosamine ratios 1.3–2.0:1.0 were also recovered in the 1.0%-cetylpyridinium chloride fraction of the 1.2–1.6M-sodium chloride Dowex 1 fractions from pooled neonatal cartilage samples.

Glycosaminoglycan contents in aged cartilage. The age-related changes in chondroitin sulphate and keratan sulphate contents of tracheobronchial cartilage described above extend from birth to the seventh decade. Cartilages beyond this age were considered unreliable for quantitative work. Histochemical examination of bronchial cartilages from subjects over 65 years of age indicated the presence of 'asbestotic' degenerative foci; some cartilages from very old subjects were grossly calcified and even in those that were not the ash content was found to be about 8.0% of the dry tissue weight as opposed to 6.0% in young adult cartilages. In those cartilages from subjects over 65 years old that were examined the uronic acid content consistently fell below the regression line shown in Fig. 1 and the 2.5M-sodium chloride+8.0M-urea fraction hexose fell below the plateau value shown in Fig. 2 to about 1.5% of the dry cartilage weight.

DISCUSSION

The glycosaminoglycans found in tracheobronchial cartilage, as in other human hyaline cartilages, comprise two main groups of compounds, chondroi-

tin sulphates and keratan sulphates, the tissue content of each group showing age-dependent changes. Within each group there are small variations of structure, best described by the term microheterogeneity (Meyer, 1966), and these also are dependent on the age of the cartilage.

The finding that the chondroitin sulphate content of cartilages from the trachea, primary bronchus and secondary bronchus decreases linearly with increasing age is in keeping with similar observations made on human costal cartilage by Kaplan & Meyer (1959). Quantitative comparisons between the chondroitin sulphate contents of the two tissues at different ages (uronic acid content $\times 2.46$ = chondroitin sulphate content; Shetlar & Masters, 1955) indicate that values for tracheobronchial cartilages tend to be higher throughout life than those for costal cartilage. However, since different procedures were used to isolate the glycosaminoglycans from the cartilage in this investigation and in that of Kaplan & Meyer (1959) the basis of quantitative comparisons is uncertain. The slope of the line drawn by the latter workers for the decrease in the chondroitin sulphate content of costal cartilage with increasing age is similar to that of the regression line drawn for the uronic acid content of aging bronchial cartilage.

Mathews & Glagov (1966) showed that the decrease in the chondroitin sulphate content of costal cartilage was mainly attributable to a decrease in chondroitin 4-sulphate, the content of the 6-isomer remaining fairly constant during adult life. In tracheobronchial cartilage a greater proportion of the total chondroitin sulphate was also accounted for by the 6-isomer in old age than in earlier years. The decrease in the proportion of 4-isomer in old age was not as dramatic as that described for costal cartilage. Mathews & Glagov (1966) also showed that changes in chondroitin sulphate isomer ratios in cricoid, thyroid and upper-tracheal cartilages were less pronounced than in costal cartilage.

The significance of the changing proportions of the two chondroitin sulphate isomers in aging cartilage remains obscure. Until recently most determinations of the relative proportions of the two compounds in a mixture have been based on the method of Mathews & Inouye (1961). One drawback of this method is the assumption that hyaluronidase degrades chondroitin sulphates completely to tetrasaccharides, and this has been shown not to be so (Inoue & Yosizawa, 1966). In the present work, however, a pure sample of chondroitin 6-sulphate (Seikagaku Kogyo Co. Ltd., Tokyo, Japan) was included in the analysis and gave a satisfactory result. Thus although results of investigations employing the method should be interpreted with caution there seems to be little doubt that chondroi-

tin 6-sulphate is the predominant isomer in aged hyaline cartilage. A possible reason for this may be the association of the 6-isomer with keratan sulphate, which is a major glycosaminoglycan component in adult cartilage.

As chondroitin sulphate contents decrease in tracheobronchial cartilage with increasing age the contents of keratan sulphate rise to reach a plateau value before the beginning of the fifth decade. Saltzman *et al.* (1963) studied the galactosamine/glucosamine ratio in hydrolysates of bronchial cartilage and found that it decreased significantly with increasing age. This is compatible with continued decreases in the chondroitin sulphate content of the tissue while keratan sulphate content remains constant. Kaplan & Meyer (1959) reported a similar situation in human costal cartilage, but their results suggested that the plateau value was reached during the third decade. On the other hand Mathews & Glagov (1966) found increases in the keratan sulphate content of costal cartilage during the first three decades of life and plateau values were reached during the fifth decade. The general trends in the changes in the glycosaminoglycan composition of both human costal and tracheobronchial cartilages follow a similar pattern but differ in detail. Tracheobronchial cartilages are relatively richer in chondroitin sulphate and the isomeric ratio undergoes less change than in costal cartilage. Adult costal cartilage is probably relatively richer in keratan sulphate (Mathews & Glagov, 1966) and maximum contents of this glycosaminoglycan may be attained at an earlier age in life than in tracheobronchial cartilage.

Comparison of the glycosaminoglycan changes in aging tracheobronchial and costal cartilage with those occurring in articular cartilage indicates that the latter tissue ages rather differently to the other hyaline cartilages. Bollet & Nance (1966) were unable to detect any decrease in the uronic acid content of articular cartilage throughout adult life. Their results were based on wet cartilage samples but have been supported by the findings of other workers. Maroudas *et al.* (1969) did not find any correlation between the uronic acid content of femoral condyle cartilage and its age and Greiling & Stuhlsatz (1969) reported only very small decreases in the chondroitin sulphate of human 'knee-joint' cartilage between the ages of 25 and 85 years. The latter authors also report small decreases in the keratan sulphate content of the same cartilage during adult life.

Thus there appear to be at least three different classes of human hyaline cartilage with respect to the nature of the age changes in the matrix glycosaminoglycans: costal, tracheobronchial and laryngeal, and articular cartilage. Mathews & Glagov (1966) proposed that differences between the rates

of change in cartilages from different anatomical locations may be due to differences in the physical and nutritional environment at any one site. In the case of articular cartilage, subjected to load-bearing and probably deriving its nutrition from the synovial fluid, the local environment is different from that of other hyaline cartilage. However, it is more difficult to appreciate why, say, costal and tracheal cartilages should differ since both are subjected to little mechanical stress and derive their nutrition by diffusion from the vessels of the perichondrium. One difference in local environment in costal cartilage may be the result of the greater distance over which diffusion has to occur compared with the radially smaller cartilage rings of the trachea. On the other hand no differences were observed between the rate of age changes in tracheal cartilage and the thin cartilage plates of the major secondary bronchi. Histochemical examination (R. M. Mason, unpublished work) of the much smaller cartilages of the peripheral secondary bronchi indicated that they do exhibit somewhat delayed changes compared with the larger cartilages of the bronchial tree.

Microheterogeneity. The marked change in the elution pattern of chondroitin sulphate in different Dowex 1 fractions during early life illustrates the variation in the type of molecule synthesized at different ages. The undersulphated chondroitin sulphate that is prominent in neonatal tracheobronchial cartilage has previously been isolated from human embryonic costal cartilage, but was not found in postnatal tissue from that site. Thus costal cartilage already differs from tracheobronchial cartilage in its glycosaminoglycan composition by the beginning of postnatal life.

In adult tracheobronchial cartilage microheterogeneity was apparent in the high-molecular-weight keratan sulphate fraction from the tissue. Variation in the degree of oversulphation of this fraction obtained from the cartilage of different individuals was marked, but was not related to age. This finding confirms similar observations made by Mathews & Glagov (1966) on keratan sulphate from costal cartilage. The molecular weight of the major keratan sulphate from adult tracheobronchial cartilage was much higher than that recorded by Mathews & Cifonelli (1965) for human cartilage keratan sulphate. This difference may be attributed to the static-equilibrium osmotic-pressure measurements from which earlier molecular-weight estimates have been derived. The dynamic osmotic-pressure measurements from which the present value was calculated probably lead to a better estimate of molecular weight.

Keratan sulphate-like molecules of lower molecular weight were isolated from tracheobronchial cartilage in the earlier Dowex 1 fractions. These,

however, were almost certainly 'hybrid doublets' composed of short chains of both keratan sulphate and chondroitin sulphate attached to the same peptide fragment of a core protein, and they are therefore comparable with a similar fraction isolated from costal cartilage by Seno *et al.* (1965). The compound described by these authors had been exhaustively digested with hyaluronidase and gave a single band on electrophoresis, but from its analysis appeared to contain both chondroitin sulphate and keratan sulphate. In the present study a range of molecules with similar properties was isolated from tracheobronchial cartilage. Their glucosamine/galactosamine ratios suggested that different fractions contained different proportions of chondroitin sulphate and keratan sulphate attached to the same peptide. This variation between molecules is unlikely to be a result of the method of preparation, since similar compounds could be isolated on the basis of their solubility in cetylpyridinium chloride and without exposure to hyaluronidase. Indeed, since the glucosamine/galactosamine ratios of the compounds isolated by the two different methods were so similar it is possible that the presence of keratan sulphate protects neighbouring chondroitin sulphate chains attached to the same peptide from degradation by hyaluronidase.

Exposure of the hyaluronidase-resistant 1.2–1.8M-sodium chloride fractions from tracheobronchial cartilage to 0.45M-potassium hydroxide effected their separation into two different components as judged by subsequent electrophoresis. Seno *et al.* (1965) have shown that *O*-glycosidic bonds attaching costal cartilage keratan sulphate to peptide-bound serine and threonine are cleaved under these conditions. Similarly the *O*-glycosidic bond linking chondroitin sulphate to peptide-bound serine undergoes a β -carbonyl elimination reaction in alkaline conditions (Anderson, Hoffman & Meyer, 1965). Mathews & Cifonelli (1965) also exposed keratan sulphate from human costal cartilage to alkaline conditions and reported that this had no apparent effect on its electrophoretic properties. However, their preparation had a glucosamine/galactosamine ratio 10:1 and was therefore comparable with the high-molecular-weight keratan sulphate fraction from tracheobronchial cartilage, which was also unaffected by alkali.

Assuming that the galactosamine content of each of the hyaluronidase-resistant compounds in the 1.2–1.8M-sodium chloride fractions is mainly contributed by chondroitin sulphate, then infrared analysis indicates that, as with the fraction described by Seno *et al.* (1965), this must be the 6-isomer (Mathews, 1958). The implication of this finding may be that keratan sulphate and chondroitin 6-sulphate are synthesized together as a distinct

proteoglycan. Tsiganos & Muir (1967) isolated a proteoglycan from pig laryngeal cartilage that contained both keratan sulphate and chondroitin sulphate but that was immunologically distinct from the major chondroitin sulphate proteoglycan. The suggestion that chondroitin 6-sulphate is associated with keratan sulphate proteoglycans does not exclude it as a component of other proteoglycans. If the synthesis and turnover of chondroitin 6-sulphate were, however, partly linked to that of keratan sulphate proteoglycans it would explain the smaller decrease in the cartilage content of this isomer with aging than that of the 4-isomer.

Shulman & Meyer (1968) have suggested three stages of chondrocyte differentiation in tissue-culture systems, 'undifferentiated' cells synthesizing non-sulphated glycosaminoglycans, 'young-differentiated' synthesizing chondroitin sulphate and 'old-differentiated' synthesizing both chondroitin 6-sulphate and keratan sulphate. It is not known whether these glycosaminoglycans are attached to a single distinct protein.

The type of differentiation achieved in cultured cells seems to depend on factors acting on the cell membrane such as the degree of pericellular charge (Shulman & Meyer, 1968). It seems possible therefore that changes in the microenvironment of the chondrocyte *in vivo* may induce the cell to change from the synthesis of one type of proteoglycan (with chondroitin sulphate as its predominant glycosaminoglycan) to the synthesis of another having keratan sulphate as its major glycosaminoglycan. Shulman & Meyer (1968) suggested that the decreasing matrix concentration of chondroitin sulphate may decrease the pericellular negative charge sufficiently to induce this effect. However, this seems to be an unattractive possibility in view of the very slow decrease in chondroitin sulphate with aging, especially in some cartilages, and the falling contents may be the result of a switch in the synthetic abilities of the chondrocytes rather than its cause. Moreover, traces of keratan sulphate-like compounds (including tentative evidence for the presence of hybrid doublets) are found in tracheobronchial cartilage and probably in other human hyaline cartilages even at birth when the matrix chondroitin sulphate content is at its highest.

An alternative factor that may influence the degree of response of chondrocytes *in vivo* to external control factors is the amount of diffusion possible through the surrounding matrix. Although Stockwell & Scott (1965) have suggested that cells receive poor nutrition as a result of decreased diffusion with a consequent change in their synthetic abilities, other effects may also be envisaged in this situation. For example, growth hormone, which is known to stimulate the incorporation of ^{14}C from [^{14}C]glucose into the galactosamine of

rabbit cartilage (Davidson & Small, 1963), may exert progressively less influence on cells as diffusion through the matrix becomes more difficult with increasing age.

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