Changes in the Protein–Polysaccharides of Pig Articular Cartilage during Prenatal Life, Development and Old Age

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Analysis of the knee-joint cartilage of pigs at five ages (namely foetuses from the second half of pregnancy and animals 10 weeks, 25 weeks, 3 years and 5 years old) showed that the composition approached that of adult cartilage by 25 weeks of age, the most marked differences being between foetal and 10 week-old cartilage. Protein–polysaccharides were extracted sequentially, first by brief low-speed homogenization with iso-osmotic sodium acetate, then by two extractions with 2M-CaCl₂ for 24 h with gentle agitation interspersed with brief low-speed homogenization and agitation for another 24 h. About half of the protein–polysaccharides were removed from foetal cartilage by the first extraction and the remainder by the second. The proportion in the first extract declined sharply with the age of the animal, but that in the first CaCl₂ extract was similar at all ages other than 10 weeks. The amount left in the residue increased approximately with the collagen content from about one-fifth at 10 weeks of age to one-third in adult and old cartilage. The proportion of medium-sized protein–polysaccharides in the extracts changed little with age after birth, but the glucosamine content increased about fivefold and the protein content almost doubled between 10 weeks and 5 years of age. Other analytical values changed little. These results cannot be explained solely by changes in the proportion of 'link-glycoprotein' in the protein–polysaccharides. Since major changes in most parameters had taken place by 25 weeks of age, the first weeks after birth may be a critical period for cartilage development in the pig.

A varied population of protein–polysaccharides has been demonstrated in the laryngeal cartilage of pigs about 9 months old (Tsiganos & Muir, 1969*b*) and also in knee-joint cartilage of pigs 9 months and 5 weeks old (Brandt & Muir, 1969, 1971*a,b*). It has been shown (Tsiganos & Muir, 1970; Tsiganos *et al.*, 1971) that the heterogeneity in size and chemical composition is intrinsic to protein–polysaccharides and is not due solely to aggregation induced by association with a glycoprotein-like component (Hascall & Sajdera, 1969).

Whether the existence of a varied population of these macromolecules has any functional significance and whether there is a progressive change in molecular size with age resulting from aggregation or polymerization analogous to the progressive cross-linking of collagen was examined in articular cartilage of pigs at five ages, including foetuses from the second half of pregnancy to animals 5 years old. The physical state of the protein–polysaccharides, as shown by the ease of extraction by using a standard procedure, was also examined to ascertain how far entrapment in the collagen network (Schubert, 1964) and association with collagen changed with age, which might affect

the physical properties of the tissue. Some of these results have been reported in an abstract (Šimůnek *et al.*, 1970).

Materials and Methods

All reagents were of analytical grade, with the exception of 9-aminoacridine hydrochloride, glucosamine hydrochloride and acetylacetone, which was redistilled (b.p. 133°–134°C). For chemical analysis all samples were dried to constant weight at 80°C *in vacuo* over P₂O₅.

Analytical methods

The determination of hexuronic acid (Bitter & Muir, 1962), hexosamine (Cessi & Piliego, 1960), hexose (Trevelyan & Harrison, 1952), pentose (Tsiganos & Muir, 1966), protein (Lowry *et al.*, 1951) and glucosamine/galactosamine molar ratios (Tsiganos & Muir, 1969*b*) were performed exactly as described in detail by Brandt & Muir (1971*a*). For the determination of hydroxyproline, samples were hydrolysed in 6M-HCl for 24 h at 100°C and the hydroxyproline content was determined by a modification (Woessner, 1961) of Stegemann's (1958) method. Collagen contents were derived by multiplying by 7.

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Gel chromatography

Protein-polysaccharides. Gel chromatography of protein-polysaccharides was done by using 6% agarose (a gift from Dr. C. P. Tsiganos) and Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) in columns (2.5 cm × 100 cm) eluted with 0.5 M-sodium acetate buffer, pH 6.5, at a rate of 20 ml/h; fractions (6.5 ml) were collected and analysed for their uronic acid contents by an automated modification of the procedure of Bitter & Muir (1962). About 15 mg of protein-polysaccharide, dissolved in 4 ml of the sodium acetate buffer by stirring for 24 h at 4°C, was applied to the column. The fractions containing retarded and excluded material were each pooled and concentrated by ultrafiltration in an Amicon cell (Amicon Corp., Lexington, Mass., U.S.A.). Each fraction was chromatographed a second time to ensure that each was wholly retarded by or excluded from the gel, after which they were filtered through grade 4 sintered-glass funnels; the protein-polysaccharides were then precipitated by the addition of ethanol to 85% (v/v), washed with ethanol, acetone and dried *in vacuo* over P₂O₅.

Glycosaminoglycans. Samples (about 5 mg) of protein-polysaccharide were dissolved in 3 ml of 0.2 M-sodium acetate buffer, pH 5.7, containing 0.02 M-cysteine and 0.004 M-EDTA (disodium salt), to which was added 3 mg of papain (Worthington Biochemical Corp., Freehold, N.J., U.S.A.; 2 × crystallized, 10 EC units/mg of protein). The digestion was done at 60°C under toluene for 24 h. The slight sediment that formed was removed by centrifugation. The molecular size of the chondroitin sulphate chains thus released from the protein-polysaccharides was assessed by gel chromatography as described by Wasteson (1969) on Sephadex G-200 (Pharmacia), by using a column (150 cm × 1.0 cm) eluted with 0.2 M-sodium acetate buffer, pH 6.5. Fractions (3 ml) were collected at a rate of 6 ml/h and their uronic acid contents determined. The samples from foetal and 10-week-old cartilage, which contained some excluded material, were re-digested with similar amounts of activated enzyme and the chromatography was repeated.

Preparation of protein-polysaccharides

Knee joints (stifle joints) from pigs of five different ages were taken as follows: nine foetuses from the second half of pregnancy, three animals 10 weeks old, six animals 25 weeks old, seven animals 3 years old and five animals 5 years old. The joints were obtained within a few minutes of death, and while still warm were frozen in solid CO₂. After being thawed to 4°C the joints were opened and the synovial fluid was wiped away. The cartilage was shaved from femoral condyles and femoral surfaces of the patellas and cut into small pieces with a scalpel.

The cartilage was suspended in 0.15 M-sodium acetate buffer, pH 6.8 (20 g in 150 ml), and cooled to 2°C. The suspension was homogenized three times for 2 min at setting 2 with a Sorvall Omni-Mixer. The container of the homogenizer was cooled in an ice-salt mixture during the homogenization. The homogenate was filtered through two layers of lint and the residue was washed three times with small portions of the sodium acetate buffer. The protein-polysaccharides were immediately precipitated from the combined filtrate and washings by 9-aminoacridine hydrochloride and purified as described by Tsiganos & Muir (1969a).

The remaining cartilage residue was suspended in 150 ml of 2 M-CaCl₂, pH 6.7 (Sajdera & Hascall, 1969), and agitated in a mechanical roller overnight and then homogenized as described above, before again being placed in a mechanical roller. The total time of extraction was 48 h. The homogenate was centrifuged and the supernatant, if not clear, was filtered through lint, before dialysis against water for 48 h. The protein-polysaccharides were isolated in the same way as those in the sodium acetate extract. The cartilage residue was extracted a second time with 2 M-CaCl₂, pH 6.7, in the same way and for the same length of time as described above and the protein-polysaccharides were isolated as before. All operations were performed in a cold-room at approx. 4°C.

To obtain the residual glycosaminoglycans the insoluble residue was digested with papain after the slices had been soaked in water overnight to remove excess of CaCl₂. For each g of cartilage residue 100 mg of crude papain was used, previously activated by incubation at 60°C for 30 min in 0.2 M-sodium acetate buffer, pH 5.7, containing 0.02 M-cysteine and 0.004 M-EDTA (disodium salt) (Kimmel & Smith, 1954). After 16 h at 60°C a further 50 mg of activated papain was added and the digestion was continued for a further 8 h. The very small amount of undigested material remaining was removed by centrifugation and the supernatant was dialysed against water for 48 h. The glycosaminoglycans were isolated and purified in the same way as the protein-polysaccharides.

Results

The gross chemical composition of cartilage from pig knee joints is shown in Table 1. There was a marked increase in dry weight and collagen content in early life, the proportion of collagen in 10-week-old cartilage being almost twice and the uronic acid content less than half that in foetal cartilage. By 25 weeks of age, however, when the animals were not fully grown, the gross chemical composition of the cartilage began to resemble that of the cartilage of adult animals 3 and 5 years old, except that the glucosamine/galactosamine molar ratio was much

Table 1. *Composition of pig knee-joint cartilage at five ages*

Experimental details are given in the text.

Composition	Age of pigs				
	Foetus	10 weeks	25 weeks	3 years	5 years
Dry wt. (% of wet wt.)	14.6	25.5	29.9	34.7	36.3
Uronic acid (% of dry wt.)	7.6	3.05	3.04	3.1	2.85
Collagen (% of dry wt.)	19.6	38.2	49.0	52.7	54.6
Glucosamine/galactosamine molar ratio	1:27	1:30	1:17	1:4	1:3.9

Table 2. *Composition of protein-polysaccharides extracted sequentially from pig knee-joint cartilage at five ages*

The extraction was with: (1) 0.15M-sodium acetate, pH6.8; (2) 2M-CaCl₂, pH6.7; (3) 2M-CaCl₂, pH6.7. For further details see the text.

Age of pigs	Extract	Composition (% of dry wt.)						Glucosamine/galactosamine molar ratio
		Uronic acid	Hexosamine	Hexose	Pentose	Protein	Hydroxyproline	
Foetus	1	29.9	28.0	4.6	1.3	4.0	0	1:11.7
	2	29.0	28.3	5.0	1.2	6.3	0	1:13.0
	3	—	—	—	—	—	—	—
10 weeks	1	28.4	28.3	5.4	1.3	4.5	0	1:29.5
	2	28.1	27.9	5.5	1.2	7.5	0.03	1:12.5
	3	27.0	27.5	5.9	1.3	8.0	0.04	1:12.4
25 weeks	1	27.2	26.6	4.9	1.2	6.7	0.05	1:19.2
	2	27.5	26.2	5.7	1.1	10.4	0.08	1: 8.7
	3	26.9	25.8	5.4	1.2	10.7	0.10	1: 6.5
3 years	1	25.2	26.3	5.8	1.2	8.3	0.05	1: 6.5
	2	24.3	26.3	5.4	1.4	12.8	0.10	1: 3.3
	3	24.7	25.2	6.6	1.3	14.0	0.15	1: 4.7
5 years	1	26.3	26.7	7.7	1.3	8.4	0.07	1: 3.0
	2	24.6	25.9	7.9	1.3	12.0	0.10	1: 2.5
	3	25.0	25.6	7.3	1.3	13.8	0.14	1: 2.4

lower. This ratio was even lower in foetal and 10-week-old cartilage (Table 1). There was a considerable difference between the glucosamine/galactosamine molar ratios of the whole cartilage (Table 1) and that of the extracted protein-polysaccharides at younger ages (Table 2), indicating that some material containing galactosamine was present in addition to the chondroitin sulphate of protein-polysaccharides. This was most evident in foetal cartilage, whereas in 3- and 5-year-old cartilage there was little difference between the molar ratios of the cartilage and of the protein-polysaccharides, and there was little change between 3 and 5 years of age (Tables 1 and 2).

The proportion of the dry weight due to collagen increased with age, at the expense partly of protein-polysaccharides but also of other constituents not measured by the analyses (Table 1). These con-

stituents are presumably proteins, some of which may contain galactosamine, since the discrepancy between the dry weight and the analyses was greatest (almost 60%) in foetal cartilage, where the difference in glucosamine/galactosamine molar ratios of the tissue and of the extracted protein-polysaccharides was also greatest. Even in old cartilage, however, about one-third of the dry weight was unaccounted for by collagen and protein-polysaccharide. In these calculations it was assumed that about one-third to one-quarter of the weight of protein-polysaccharides is due to uronic acid, depending on their keratan sulphate and protein contents (Brandt & Muir, 1971*a,b*).

The results in Table 3 show how much of the total uronic acid in the cartilage was extracted by the three successive extractions, starting with one extraction

Table 3. *Amount of uronic acid in protein-polysaccharide extracts and residue*

The protein-polysaccharide was extracted sequentially with 0.15M-sodium acetate, pH 6.8, and twice with 2M-CaCl₂, pH 6.7, from knee-joint cartilage of pigs at five ages. That remaining in the residue was obtained as glycosaminoglycan. For further details see the text.

Age of pigs	% of total uronic acid in the cartilage			Cartilage residue
	Extracting solutions			
	(1) 0.15M-Sodium acetate	(2) 2M-CaCl ₂	(3) 2M-CaCl ₂	
Foetus	54.7	41.5	0	0
10 weeks	27.8	23.6	18.2	20.3
25 weeks	15.8	50.2	6.8	24.6
3 years	6.2	45.0	14.6	32.3
5 years	5.2	39.0	18.8	31.5

with iso-osmotic neutral sodium acetate followed by two with neutral 2M-CaCl₂. The procedure was standardized so that the ease with which the protein-polysaccharides were extracted could be compared at different ages. They were most readily extracted from foetal cartilage, where over half was extracted with sodium acetate and the remainder was brought into solution by the first CaCl₂ extraction. By 10 weeks of age, however, the proportion of the total uronic acid extracted with sodium acetate had fallen to about one-quarter, the remainder being distributed approximately equally between the two CaCl₂ extracts and the residue. By 25 weeks of age, only about 16% of the total uronic acid was obtained in the sodium acetate extract, half was in the first CaCl₂ extract and a quarter remained in the residue. This approached the distribution found in adult and old cartilage, where almost one-third remained in the residue and 39–45% of the total uronic acid was in the first CaCl₂ extract (Table 3). Despite the increasing glucosamine/galactosamine molar ratios of the protein-polysaccharides (Table 2), the proportion remaining in the residue increased between the ages of 10 weeks and 5 years roughly in relation to the increase in collagen content of the cartilage (Table 1).

Each successive extraction of cartilage at a given age, including that from adult and old animals, brought into solution an increasing proportion of larger molecules, as shown by gel chromatography (Fig. 1 and Tables 4 and 5), that contained more glucosamine, protein and hexose, although other analytical values were very similar (Table 2). The small contamination with collagen indicated by the presence of some hydroxyproline would not account for the differences in protein content.

At any one age there was an approximate relationship between molecular size and the content of protein and keratan sulphate, in accord with previous results (Tsiganos & Muir, 1969b; Brandt & Muir, 1969, 1971a). However, this generalization did not

apply when protein-polysaccharides from cartilage of different ages were compared. Thus the higher glucosamine, hexose and protein contents of protein-polysaccharides from older cartilage were not accompanied by proportionate increases in molecular size (Fig. 1 and Tables 4 and 5), as would be expected had the protein-polysaccharides come from cartilage at one age. This was particularly evident in protein-polysaccharides in the second CaCl₂ extract, which showed minimal changes in elution profiles (Fig. 1), but a marked increase in glucosamine/galactosamine molar ratios between 10 weeks and 5 years of age.

As foetal cartilage was exceptional in several respects it will be considered separately below. Cartilage at 10 weeks of age contained the most varied population of protein-polysaccharides, and old cartilage the least (Fig. 1). The proportion of molecules of intermediate size remained constant at about 30–40% at all ages after birth (Table 4, column 'B–A'). These proportions were calculated by subtracting the proportions retarded by 6% agarose (Table 4, column A) from the proportions retarded by Sepharose 4B (Table 4, column B). The proportion of small molecules diminished from about 23 to 6% between 25 weeks and 5 years of age, and the proportion of larger molecules excluded from Sepharose 4B increased from 21 to 29% (Table 5); amongst these are some very large molecules, since about half the protein-polysaccharides from articular cartilage of 9-month-old pigs that were excluded from Sepharose 4B were also excluded from Sepharose 2B (Brandt & Muir, 1971a).

The elution profiles of the protein-polysaccharides extracted with sodium acetate varied the most with age, whereas the profiles of those in the second CaCl₂ extract varied the least (Fig. 1). Because sodium acetate preferentially extracts smaller molecules (Tsiganos & Muir, 1969b; Brandt & Muir, 1969, 1971a), differences in the population of small molecules will be most evident in this fraction.

Fig. 1. Elution of protein-polysaccharides from Sepharose 4B

A column (2.5cm×100cm) of Sepharose 4B was eluted with 0.5M-sodium acetate, pH6.5. The uronic acid content of each 6.5ml fraction was determined. V_0 indicates the void volume of the column. (1) Sodium acetate, (2) CaCl_2 and (3) CaCl_2 indicate the protein-polysaccharides in three sequential extracts of pig-knee joint cartilage that were applied to the column.

Analytical differences between protein-polysaccharides in the three successive extracts diminished with age, so that by 5 years of age the analyses of protein-polysaccharides in all three extracts were very similar (Table 2).

Foetal cartilage did not fit this pattern of change. It contained a high proportion of large molecules, more than half being excluded from Sepharose 4B (Table 5), and almost none of the smallest ones (Table 4), although glucosamine/galactosamine molar ratios and protein and hexose contents of the protein-polysaccharides were relatively low (Table 2). Moreover, despite the size of the protein-polysaccharides, more than half the tissue uronic acid was extracted with the sodium acetate (Table 3), in which

nearly half the protein-polysaccharides were large enough to be excluded from Sepharose 4B (Table 5), the remainder being of intermediate size with almost none small enough to be retarded on 6% agarose (Table 4). Protein-polysaccharides were thus much more easily extracted from foetal cartilage, irrespective of their size, and only two extractions were needed to remove all the tissue uronic acid (Table 3). This may be caused by the lower collagen content of foetal cartilage (Table 1) and perhaps also by a difference in the association or binding of protein-polysaccharides with collagen.

Fig. 2 shows the elution profiles from Sephadex G-200 of the chondroitin sulphate chains obtained after proteolysis of the protein-polysaccharides in

Table 4. *Protein-polysaccharides in three sequential extracts (see Table 3) retarded on gel-chromatography in 6% agarose and Sepharose 4B*

For further details see the text.

% of total uronic acid in the cartilage					
Age of pigs	6% Agarose				
	(1) Sodium acetate	(2) CaCl ₂	(3) CaCl ₂	Total (A)	
Foetus	1.64	1.25	—	2.89	
10 weeks	13.6	7.55	2.18	23.33	
25 weeks	4.27	8.03	0.81	13.11	
3 years	1.73	9.45	1.75	12.93	
5 years	0.94	3.51	1.31	5.76	
Age of pigs	Sepharose 4B				
	(1) Sodium acetate	(2) CaCl ₂	(3) CaCl ₂	Total (B)	(B)–(A)
Foetus	30.6	13.7	—	44.3	41.41
10 weeks	22.5	16.3	9.46	48.26	24.93
25 weeks	11.53	30.6	3.13	45.26	32.15
3 years	4.46	31.0	7.6	43.06	30.13
5 years	2.81	23.45	7.71	33.97	28.21

sodium acetate extracts that were more varied in size than those in subsequent extracts. There was some excluded material in the chondroitin sulphate from foetal and 10-week-old cartilage, with traces at 25 weeks of age (Fig. 2), but as these elution profiles did not change on repeating the papain digestion, proteolysis appeared to have been complete. Apart from this minor fraction, Fig. 2 shows that at all five ages the distribution of chain lengths was the same. Differences in size of the protein-polysaccharides cannot therefore be attributed to differences in the length of their constituent chondroitin sulphate chains. The small amount of longer chains in the protein-polysaccharides in extract 1 from foetal, 10- and 25 week-old cartilage (Fig. 2) was not enough to account for differences in size of the original protein-polysaccharides, especially as those in extract 1 from foetal and from 10-week-old cartilage had the most dissimilar distribution in sizes (Fig. 1).

Discussion

During adult life, little change in composition of pig (Table 1) or human (Maroudas *et al.*, 1969; Muir *et al.*, 1970) knee-joint cartilage has been observed, nor was there any appreciable change in dry weight in human cartilage between 10 and 26 years of age (Miles & Eichelberger, 1964). Similarly in pigs that were not fully grown at 25 weeks of age, there was less difference in the composition of their cartilage and that of old animals than of pigs only 15 weeks younger (Table 1). On the other hand, considerable changes took place in the first few weeks after birth.

Thus the dry weight and collagen content increased from 14.6 and 19.6% respectively in foetal cartilage (Table 1), to 20 and 26% at 5 weeks (Brandt & Muir, 1969) and to 25.5 and 38.2% at 10 weeks of age (Table 1). The distribution of sizes among the protein-polysaccharides also changed considerably during this time, for although there was almost none of the smallest protein-polysaccharides retarded by 6% agarose in foetal cartilage, and little at 5 weeks of age (Brandt & Muir, 1969), they amounted to almost one-quarter of the total at 10 weeks of age (Table 4), whereas from 25 weeks onwards the range of sizes approached that of protein-polysaccharides from adult cartilage (Tables 4 and 5), and also the extractability of protein-polysaccharides at these three later ages was rather similar (Table 3). The proportions of very large molecules that would have been excluded from Sepharose 2B (Brandt & Muir, 1971a) were not estimated, however. Most of the uronic acid in the tissue was accounted for, which was satisfactory in view of the number of manipulations that had been done.

It would appear from these results that in the pig, a species in which the young are born fully mobile, the first few weeks after birth may be a critical period for the correct development of their joints, and hence unsuitable conditions of rearing at this time may produce abnormalities that lead to lameness, which often occurs in pigs reared under intensive conditions at an early age. In other species that develop more slowly, such as man, the critical period may be less acute but more prolonged.

Collagen and protein-polysaccharides did not

Table 5. Proportion of protein-polysaccharides in three sequential extracts (see Table 3) excluded from Sepharose 4B on gel-chromatography

For further details see the text.

Age of pigs	% of total uronic acid in the cartilage			
	(1) Sodium acetate	(2) CaCl ₂	(3) CaCl ₂	Total
Foetus	24.05	27.8	—	51.85
10 weeks	5.28	7.08	8.73	21.09
25 weeks	4.26	19.6	3.67	27.53
3 years	1.74	13.95	7.01	22.70
5 years	2.39	15.64	11.1	29.13

account for all the dry weight, however, presumably owing to the presence of other proteins. A similar discrepancy was noted in adult human knee-joint cartilage, where it was as much as 50% in some regions (Muir *et al.*, 1970).

In general, at all ages, protein-polysaccharides of smaller size were extracted more readily than larger ones, the latter being therefore more prevalent in later extracts (Tables 4 and 5). With the exception of foetal cartilage, the efficiency of iso-osmotic sodium acetate in extracting protein-polysaccharides was approximately related to the proportion of small molecules in the cartilage, so that this solvent was most effective in extracting protein-polysaccharides from 10-week-old cartilage (Table 3), which contained the greatest proportion of small molecules (Table 4). It had been shown previously that iso-osmotic sodium acetate preferentially removed small protein-polysaccharides with low glucosamine/galactosamine molar ratios, and it was noted that they were unlikely to have arisen from degradation during the extraction procedure (Brandt & Muir, 1969, 1971a; Tsiganos & Muir, 1969b). In contrast with sodium acetate, 2M-CaCl₂ extracted much more protein-polysaccharide (Table 3). Sajdera & Hascall (1969), in examining the efficiency of various solvents, showed that 2M-CaCl₂ was one of the most effective solvents for extracting protein-polysaccharides from bovine nasal cartilage, although even in that case 10–15% remained in the tissue and could not be dissolved by repeated extraction. A similar proportion resisted extraction from pig laryngeal cartilage (Tsiganos *et al.*, 1971). The amount of uronic acid left in the residue was even greater in pig knee-joint cartilage, and increased to almost one-third in adult and old cartilage (Table 3). Pig laryngeal cartilage contains less collagen than does articular cartilage (Brandt & Muir, 1969) and as all the protein-polysaccharides were extracted from foetal cartilage, which contained the least collagen (Table 1), collagen appears to impair extraction of protein-polysaccharides, presumably because of

Fig. 2. Elution of glycosaminoglycans from Sephadex G-200

A column (1.0cm × 150cm) of Sephadex G-200 was eluted with 0.2M-sodium acetate buffer, pH6.5. The uronic acid content of each 6ml fraction was determined. V₀ indicates the void volume of the column. The glycosaminoglycans were obtained by papain digestion of protein-polysaccharides in sodium acetate extracts of cartilage (first extract, see the text).

mechanical entrapment in the collagen network (Schubert, 1964). However, there may also be some rather specific association of the residual protein-polysaccharide with collagen, since repeated extraction was without effect. These protein-polysac-

charides differed from those that were extractable in containing a lower proportion of glucosamine relative to galactosamine (Brandt & Muir, 1971*b*).

Hascall & Sajdera (1969) have shown that protein-polysaccharides in extracts of bovine nasal cartilage consist of aggregates and monomers, aggregation being effected by a 'glycoprotein-link' fraction that is rich in protein and contains more glucosamine than galactosamine. Part of the differences in molecular size revealed by gel chromatography were probably due to the simultaneous presence of monomers and aggregates, the latter being very large and wholly excluded from the gel, and also perhaps to the presence of dimers, as suggested by Rosenberg *et al.* (1970). The observed differences in size and composition would be difficult to explain, however, if a single species of monomer was present at all ages, because then differences in composition would be due solely to combination with the 'link-glycoprotein' in the formation of aggregates, which would thereby contain more glucosamine and protein than monomers. Although the glucosamine and protein contents of protein-polysaccharides increased with size when they came from articular cartilage at one age (Brandt & Muir, 1971*a*), this was not so when protein-polysaccharides from cartilage of different ages were compared. Thus, although the proportion of medium-sized molecules remained unchanged (Table 4) between the ages of 10 weeks and 5 years, the glucosamine content of the protein-polysaccharides increased about five times whilst their protein content nearly doubled. On the other hand, the proportion of larger molecules increased by only 8%, which would hardly account for the observed differences in analysis, particularly as the 'glycoprotein-link fraction' accounted for only 3% of the complex from bovine nasal cartilage (Hascall & Sajdera, 1969). Moreover, if interaction with 'link-glycoprotein' were solely responsible for differences in analysis, there should have been greater changes in protein content than in glucosamine content, because the 'glycoprotein-link' fraction accounted for only a small percentage of the glucosamine in protein-polysaccharides extracted from pig laryngeal cartilage but for about one-third of the protein (Tsiganos *et al.*, 1971). The 'glycoprotein-link' fraction from bovine nasal cartilage was of similar composition (Hascall & Sajdera, 1969) so that it is likely to be similar in different types of cartilage. The small contamination with collagen does not account for the differences in protein content, nor is it likely that hyaluronic acid, which contains glucosamine, was present in significant amounts, because it was not found in protein-polysaccharides prepared in the same way from pig articular (Brandt & Muir, 1969, 1971*a*) or laryngeal cartilage (Tsiganos & Muir, 1969*b*), in which the absence of serum proteins was demonstrated by immunological methods.

It therefore appears that, as cartilage matures, different protein-polysaccharides are formed that contain increasing proportions of keratan sulphate. As keratan sulphate contains glucosamine and galactose it would account for the increase in hexose as well as glucosamine in protein-polysaccharides of older cartilage (Table 2). Some of the hexose, however, will be the galactose of the region linking the chondroitin sulphate chains to the protein core, while the pentose is the xylose of this region (Rodén & Armand, 1966; Lindahl & Rodén, 1966).

The observed differences in size or composition cannot be attributed to differences in the length of chondroitin sulphate chains themselves. In sodium acetate extracts, where differences in the size and composition of protein-polysaccharides were most evident, the chondroitin sulphate had the same distribution of chain length at all five ages (Fig. 2), apart from small amounts of longer chains in protein-polysaccharides from foetal and 10-week-old cartilage. Longer chains might perhaps be a feature of a 'foetal' form of protein-polysaccharide that persists for the first few weeks after birth. Although protein-polysaccharides of different size were not separated in the present investigation, when this was done the average lengths of chondroitin sulphate chains were essentially similar in protein-polysaccharides of different size from pig articular (Brandt & Muir, 1971*a*) or laryngeal cartilage (Tsiganos & Muir, 1969*b*).

The changes in the extractability of protein-polysaccharides with age (Table 2), although partly due to changes in the population of molecules of different size and to the greater collagen content of older cartilage, may also reflect changes in the association with collagen of the protein-polysaccharides of older cartilage that contain more keratan sulphate. Cartilage collagen is more resistant to solubilization than that in other tissues (Miller *et al.*, 1969) and there is a form of collagen that is unique to cartilage, which has certain differences in amino acid composition (Miller & Matukas, 1969; Miller *et al.*, 1971). In lathyrctic cartilage this collagen was not as readily extracted with neutral 1M-NaCl as was normal collagen (Miller, 1971), possibly because it has a greater affinity for protein-polysaccharides. If this were so and the proportion of unique to normal collagen changed with age, the proportion of protein-polysaccharides associated with collagen might also change. The collagen of adult rabbit articular cartilage has an appreciable turnover (Repo & Mitchell, 1971) so that it is conceivable that the mixture of collagens in cartilage might slowly change with time during adult life.

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