Acid Mucopolysaccharides of Chick-Embryo Cartilage in Osteolathyrism

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1. In view of the suggested association between collagen and acid mucopolysaccharides in the connective tissues, this study was designed to see whether the lathyrus factor, β -aminopropionitrile, affected the acid mucopolysaccharides as well as inhibiting the normal polymerization of collagen. The changing pattern of these two components of cartilage from normal and lathyrus chick embryos aged 14–20 days is described. The chondroitin sulphates and their protein complexes have been isolated from these cartilages, characterized and compared, particularly with respect to their sulphate content; no significant differences in quality or quantity were detectable. 2. Saline extracts of normal and lathyrus cartilages were also compared; whereas the collagen content of lathyrus extracts was increased to ten times that in the normal, the acid mucopolysaccharide content of both extracts was always the same. 3. It therefore appears that in the chick embryo β -aminopropionitrile does not affect the acid mucopolysaccharides of the developing cartilage.

Lathyrus agents such as BAPN† inhibit the normal polymerization of collagen at the intermolecular (Levene & Gross, 1959; Gross, 1963) and intramolecular (Martin, Gross, Piez & Lewis, 1961; Martin, Piez & Lewis, 1963) level but their possible effect on the mucopolysaccharides of connective tissue appears to be much less clearly defined. Bickley (1964) summarizes the available experimental data on the effects of lathyrus on the polysaccharides of connective tissue; for example, the response of the rat to lathyrus appears to vary with the tissue examined; in general the concentration of acid mucopolysaccharides is depressed in epiphyseal cartilage, unchanged in skin and elevated in aorta. The effect of lathyrus on sulphate metabolism also seems to vary with the system used (Bickley & Orbison, 1964; John, Sokol & Orbison, 1964; Peck, Mariz & Daughaday, 1963).

We therefore decided to ascertain whether the effect on collagen induced in the chick-embryo cartilage by BAPN was accompanied by an AMPS effect, particularly since it has been suggested that the AMPS may interact with collagen *in vivo* (Mathews, 1965). Our results show that the cartilage AMPS remain unaffected in osteolathyrism.

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† Abbreviations: BAPN, β -aminopropionitrile; AMPS, acid mucopolysaccharides.

EXPERIMENTAL

Production of lathyrism in the chick embryo (and dry-weight determination). White Leghorn chick embryos were injected at 14 days of incubation, through a pinhole in the shell, with 5mg. of BAPN (generous gift from Abbott Laboratories, N. Chicago, Ill., U.S.A.) in 0.1 ml. of sterile distilled water and the eggs were then reincubated. Survivors were harvested 2, 4 and 6 days later as follows: tibiae and femora were dissected in the cold, the cartilaginous ends were carefully freed of surrounding tissues, separated and pooled, and three samples were taken to measure the percentage dry weight after drying *in vacuo* for 48 hr. in an Abderhalden drying pistol at 105°. Control cartilage was harvested from normal 14-, 16-, 18- and 20-day-old embryos.

Histology. One set of cartilages was fixed in buffered formalin at neutral pH and embedded in wax. A series of sections were stained with haematoxylin and eosin, Van Gieson, periodate-Schiff, Alcian blue, toluidine blue and mucicarmine; similar stains were performed on specimens fixed with Lillie's lead acetate fixative.

Chemical analyses of total cartilage. Approx. 500 mg. of pooled dried cartilage from each age group was ground to a fine powder in an agate mortar and dried again. Portions were analysed for hydroxyproline content after hydrolysis in sealed tubes in an oil bath at 138° for 3 hr. in 6N-HCl, with a blank from which peroxide had been omitted to account for possible interference from other pyrroles (Neuman & Logan, 1950). Nitrogen was estimated by a colorimetric micro-Kjeldahl method and hexosamine by the method of Dische (1947).

Isolation of acid mucopolysaccharides of cartilage. The isolation and purification of the AMPS was carried out by a

modification of a previously described method (Scott, 1960; Schiller, Slover & Dorfman, 1961); fresh samples of cartilage were digested with papain for 4 hr., the subsequent NaOH and trypsin treatment being omitted. The AMPS were then precipitated, with cetylpyridinium chloride, and Celite (Johns-Manville, New York, N.Y., U.S.A.) was added; the various fractions of AMPS were eluted from this complex by increasing concentrations of NaCl. The cetylpyridinium chloride was then removed from the eluates by precipitation as the thiocyanate and the AMPS subsequently purified. Chemical analyses were performed on these uronic acid-containing fractions for nitrogen (by a colorimetric micro-Kjeldahl method), uronic acid (Dische, 1947), hexosamine (Boas, 1953), carbohydrate (Dubois, Gilles, Hamilton, Rebers & Smith, 1951) and sulphate (Muir, 1958).

Identification of the AMPS. (a) Effect of testicular hyaluronidase. Selected fractions were treated with testicular hyaluronidase and the effect was measured turbidimetrically (Dorfman & Ott, 1948) to differentiate chondroitin 4-sulphate and chondroitin 6-sulphate, which are both digested by testicular hyaluronidase, from dermatan sulphate, which is not.

(b) Infrared spectroscopy. Selected samples were examined in a Perkin-Elmer model 21 spectrophotometer with an NaCl prism. The presence of chondroitin 6-sulphate is indicated by a peak at 815 cm^{-1} and that of chondroitin 4-sulphate by a peak at 855 cm^{-1} ; both peaks are found in a mixture of the two compounds (Orr, 1954; Mathews, 1958).

(c) Electrophoresis. All the purified fractions were examined by electrophoresis in phosphate buffer, pH 6.9, with cellulose acetate paper (Sepraphore; Gelman Instrument Co., Chelsea, Mich., U.S.A.) and 10 v/cm. for 30 min. under Varso; the strips were stained with acridine orange and washed with running tap water (Von Berlepsch, 1962). Known samples of chondroitin 4-sulphate and chondroitin 6-sulphate were used as controls (generous gift from Dr J. A. Cifonelli). In this system, chondroitin 6-sulphate moves slightly slower than chondroitin 4-sulphate; the best distinctions obtainable were not, however, very good.

Isolation of AMPS-protein complex of cartilage. The AMPS-protein complex was extracted from normal and lathyrctic cartilage by using Mathews's (1965) modification of the method of Malawista & Schubert (1958); fresh samples of cartilage were homogenized in a VirTis homogenizer at high speeds in cold distilled water. The collagen in the homogenate was converted into gelatin by heating at 80°

for 1 min.; after cooling to 45° the mixture was rapidly filtered through Celite. The AMPS-protein complex was precipitated from the filtrate by cetylpyridinium chloride and dissolved in a small volume of methanol-5 M-NaCl-water (1:1:1, by vol.), from which it was precipitated by adding 6 vol. of 80% (v/v) ethanol, followed by washing in ethanol and then ether and drying.

Hydroxyproline estimation. Each sample of AMPS-protein complex was analysed for hydroxyproline content to exclude any collagenous impurity (Martin & Axelrod, 1953).

Other analyses. Nitrogen, hexosamine, uronic acid, carbohydrate and sulphate were determined by the methods mentioned above on a 0.1% solution of the complex dissolved in 0.05 M-NaCl.

Measurements of intrinsic viscosity. These were performed (under the supervision of Dr M. B. Mathews) on several samples of the purified complex dissolved in Miller no. 1 phosphate buffer [0.01 M-phosphate buffer (pH 7.0)-0.38 M-NaCl].

Amino acid analysis. Analyses were performed on several samples of the complex with the Technicon amino acid analyser.

Examination of m-sodium chloride extracts of cartilage. Fresh samples of normal and lathyrctic cartilage were minced in the cold with fine scissors and extracted for 24 hr. at 5° with constant shaking in 3 vol. (v/w) of cold m-NaCl buffered with phosphate, pH 7.6 and 1.0. Extracts were then separated from residues by centrifugation in the Spinco model L preparative ultracentrifuge at 25000 rev./min. for 30 min. and the cleared supernatant fluid was passed through a fine sintered-glass filter in the cold. The relative viscosity of each extract was measured at 5° in an Ostwald viscometer (buffer time 59 sec.), this reading giving a direct measure in this system of the amount of collagen present in solution (Gross, 1958). Samples of the extracts were also chemically analysed for hydroxyproline, uronic acid, hexosamine and nitrogen content by the methods mentioned above.

RESULTS

Histology. The structural disorganization commonly found in the epiphyseal cartilage of the lathyrctic rat was not found in the lathyrctic chick-embryo cartilage. Moreover, it was not possible to

Table 1. *Analyses of normal and lathyrctic chick-embryo cartilage*

Each value is from a single determination with pooled powdered cartilage.

Embryo age (days)	Collagen (g./100 g. dry wt.)		Nitrogen (g./100 g. dry wt.)		Hexosamine (g./100 g dry wt.)	
	Normal	BAPN-treated	Normal	BAPN-treated	Normal	BAPN-treated
14	15.82	—	9.67	—	4.61	—
	15.60	—	9.04	—	4.27	—
16	21.88	17.90	11.68	9.91	3.92	4.27
	19.10	19.78	10.91	10.53	4.21	3.89
18	25.50	20.52	10.32	10.40	4.36	5.39
	22.20	19.60	10.30	10.63	4.53	5.37
20	29.60	27.16	10.00	9.67	5.95	5.22
	27.80	24.20	10.34	9.03	5.51	5.30

distinguish between normal and lathyrctic chick-embryo cartilage with any of the stains employed at any of the ages examined.

Chemical analyses of total cartilage. The results suggest a fall in total collagen content of the lathyrctic cartilage but the hexosamine and nitrogen values showed no consistent changes (Table 1).

Isolation of AMPS. (a) Qualitative. The AMPS of normal and lathyrctic cartilages were isolated and purified after papain digestion of fresh cartilage. Both samples showed peaks in the infrared spectrophotometer at approx. 815 and 855cm.⁻¹, indicating a mixture of chondroitin 4-sulphate and chondroitin 6-sulphate. On digestion with testicular hyaluronidase they were identical and produced no turbidity on the addition of acid albumin. This suggests that dermatan sulphate was absent. There were no differences in electrophoretic mobility of

normal or lathyrctic samples at 16, 18 and 20 days, which were the same as standard chondroitin 4-sulphate and chondroitin 6-sulphate. As an index of the polysaccharide chain lengths, measurements of reduced viscosity were made on the normal and lathyrctic 16-day samples; the normal value was 0.816 (concentration 0.2020%) and the lathyrctic sample 0.899 (concentration 0.1748%) (the difference not being considered greatly significant).

(b) Quantitative. No significant differences were

Table 2. *AMPS and sulphate contents of normal and lathyrctic chick-embryo cartilage after papain digestion, AMPS isolation and purification*

These results are from one complete set of analyses performed in duplicate.

Embryo age (days)	AMPS (g./100g. dry wt.)		Sulphate (g./100g. dry wt.)	
	BAPN-		BAPN-	
	Normal	treated	Normal	treated
14	11.66	—	1.78	—
16	8.24	8.67	1.21	1.16
18	11.27	9.89	1.55	1.70
20	12.98	12.74	1.86	1.99

Fig. 1. Percentage dry weight of AMPS from normal and lathyrctic chick-embryo cartilage. Lower-sulphated fraction soluble in 0.4M-NaCl: ○, normal; ●, lathyrctic; higher-sulphated fraction soluble in 1.2M-NaCl: △, normal; ▲, lathyrctic. Each value represents the average of three separate estimations.

Table 3. *Composition of purified AMPS isolated from normal and lathyrctic chick-embryo cartilage after papain digestion*

These results are from one complete set of analyses performed in duplicate.

Embryo age (days)	AMPS fraction	Molar ratios to hexosamine							
		Nitrogen		Uronic acid		Carbohydrate*		Sulphate	
		Normal	BAPN- treated	Normal	BAPN- treated	Normal	BAPN- treated	Normal	BAPN- treated
14	0.4M-NaCl	2.00	—	1.09	—	0.50	—	0.88	—
	1.2M-NaCl	1.85	—	1.17	—	0.53	—	0.93	—
16	0.4M-NaCl	1.43	1.37	1.08	1.13	0.56	0.51	0.80	0.75
	1.2M-NaCl	1.73	1.73	1.18	1.06	0.62	0.57	1.01	0.81
18	0.4M-NaCl	2.60	2.20	1.30	0.90	0.70	0.60	0.88	0.80
	1.2M-NaCl	2.10	2.20	1.20	1.10	0.50	0.60	0.94	1.00
20	0.4M-NaCl	2.09	2.70	1.10	1.15	0.58	0.62	0.72	0.80
	1.2M-NaCl	1.43	2.04	1.00	1.21	0.42	0.77	0.79	1.06

* 'Total' carbohydrate is estimated as galactose by the phenol-H₂SO₄ method of Dubois *et al.* (1951); this reagent does not react with hexosamines.

observed between the AMPS and sulphate contents of normal and lathyrctic cartilage (Table 2). The purified AMPS were fractionated further into a 0.4M-sodium chloride-soluble fraction and a 1.2M-sodium chloride-soluble fraction; this method has

been shown to separate lower-sulphated AMPS fractions from higher-sulphated fractions (Schiller *et al.* 1961). The results showed that, although there was a constant difference in the sulphate contents of these fractions, it was a very small difference (Table 3). As the cartilage aged, the proportion of the lower-sulphated fraction fell and that of the higher-sulphated fraction rose. Lathyrism made no difference (Fig. 1). Nevertheless, the AMPS-protein complex may have been altered in some way in lathyrism.

Table 4. *Summary of analyses of AMPS-protein complexes as determined with a Technicon amino acid analyser, after isolation from cartilage of 16-day-old normal and lathyrctic chick embryos*

Each value represents the mean $\pm$ S.D. of eight separate determinations.

(a) Total AMPS-protein complex analysis

	Values (μ moles/100 μ moles of amino compounds)	
	Normal	Lathyrctic
Total amino acids	26.03 $\pm$ 5.17	24.17 $\pm$ 3.75
Ammonia	16.74 $\pm$ 3.21	16.58 $\pm$ 3.97
Glucosamine	5.12 $\pm$ 1.10	5.25 $\pm$ 1.57
Galactosamine	52.15 $\pm$ 3.97	53.11 $\pm$ 2.51

(b) Amino acid analysis

	Values (μ moles/100 μ moles of amino acids)	
	Normal	Lathyrctic
Aspartic acid	7.57 $\pm$ 1.03	7.70 $\pm$ 0.90
Threonine	9.20 $\pm$ 2.46	8.20 $\pm$ 1.82
Serine	13.57 $\pm$ 2.99	12.10 $\pm$ 1.73
Glutamic acid	14.07 $\pm$ 1.55	13.90 $\pm$ 0.60
Proline	7.96 $\pm$ 1.58	8.02 $\pm$ 1.15
Glycine	12.51 $\pm$ 2.36	12.02 $\pm$ 1.42
Alanine	8.20 $\pm$ 1.03	8.70 $\pm$ 1.43
Valine	6.24 $\pm$ 1.04	6.00 $\pm$ 0.20
Cystine	Nil	Nil
Methionine	Nil	Nil
Isoleucine	4.50 $\pm$ 1.00	4.80 $\pm$ 0.30
Leucine	6.55 $\pm$ 0.90	6.78 $\pm$ 0.50
Tyrosine	1.04 $\pm$ 0.02	1.08 $\pm$ 0.78
Phenylalanine	3.73 $\pm$ 0.68	3.32 $\pm$ 1.25
Lysine	2.46 $\pm$ 0.48	2.52 $\pm$ 0.60
Histidine	1.89 $\pm$ 0.33	1.83 $\pm$ 0.29
Arginine	3.53 $\pm$ 0.41	3.75 $\pm$ 0.46

Isolation of AMPS-protein complex. Chemical analyses of the purified normal and lathyrctic AMPS-protein complexes showed no significant differences between the protein moieties (Table 4) or the AMPS moieties (Table 5). The intrinsic viscosities of the complexes were measured; they were found to be higher than those of the AMPS isolated by proteolysis owing to the macromolecular structure of the AMPS-protein complex, but the differences between the normal and lathyrctic complexes were not enough to indicate significant differences in shape or molecular weight (Table 6).

Cartilage extracts. Normal and lathyrctic cartilages contain approximately the same total amounts of collagen and AMPS; it was decided to compare the M-sodium chloride extracts of these cartilages. It was evident that, whereas the collagen content of the lathyrctic extract was ten times as great as that of the normal, the AMPS contents of both extracts were identical (Fig. 2 and Table 7).

Table 6. *Measurements of intrinsic viscosity of purified AMPS-protein complexes isolated from normal and lathyrctic chick-embryo cartilage*

Embryo age (days)	Intrinsic viscosity of complex	
	Normal	BAPN-treated
14	1.94	—
16	1.80	1.85
18	1.62	1.90
20	1.85	2.20

Table 5. *Composition of purified AMPS-protein complex isolated from normal and lathyrctic chick-embryo cartilage*

Composition of AMPS-protein complex (% of dry wt.)

Embryo age (days)	Nitrogen		Uronic acid		Hexosamine		Carbohydrate		Sulphate	
	Normal	BAPN-treated	Normal	BAPN-treated	Normal	BAPN-treated	Normal	BAPN-treated	Normal	BAPN-treated
14	4.52	—	27.35	—	24.88	—	15.12	—	10.08	—
16	4.84	5.17	27.93	27.16	24.88	23.63	19.44	18.18	8.64	8.35
18	5.18	4.76	27.16	29.87	23.43	23.81	16.20	15.48	7.97	9.12
20	4.48	4.62	30.65	33.37	24.35	26.50	16.02	16.20	11.04	10.37

Fig. 2. Comparative analyses of m-NaCl extracts of normal (a) and lathyritic (b) chick-embryo cartilage: O, collagen; Δ, nitrogen; ●, hexosamine; ▲, uronic acid.

Table 7. Partial composition of m-sodium chloride extracts of 16-day-old normal and lathyritic cartilage

	Composition of m-NaCl extract of cartilage (g./100 g.)	
	Normal	BAPN-treated
Proportion of dry wt. extracted	23.5	34.0
Proportion of total collagen extracted	2.9	32.0
Proportion of total AMPS extracted	33.0	31.0

DISCUSSION

This study indicates that normal growth is accompanied by an increase in the collagen content of chick-embryo cartilage, and in the AMPS content as well, with the exception of the constantly observed fall in value at 16 days. There also appears to be a distinct change in the pattern of sulphation with age, namely an increase in the proportion of higher-sulphated AMPS and a fall in the proportion of lower-sulphated AMPS.

The effect of lathyrisms on cartilage seems to be confined to an increase in the salt-solubility of the collagen, whereas the AMPS and its protein complex appear to be normal.

Analyses of the saline extracts of normal and lathyritic cartilages confirm that lathyrogens act on collagen but not on the AMPS, at least in the chick embryo. In a system in which both are being synthesized over a period of 6 days, lathyrisms profoundly affects the extractability of collagen but

not that of AMPS. Hence there is probably no close association, such as a covalent link, between the two substances.

Pedrini-Mille & Pedrini (1965) found that rats treated with a different lathyrogen, aminoacetonitrile, showed a 50% decrease in the hexosamine content of fracture-bone callus as compared with controls. However, no change in the isolated AMPS of the skin was found (Lenzi & Pedrini-Mille, 1965); they therefore concluded that this lathyrogen affects only chondroitin 4-sulphate and chondroitin 6-sulphate but no others. This suggestion appears at present to conflict with our results in which these chondroitin sulphates, albeit of chick cartilage, remain unaffected by BAPN.

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