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The Solubilization of Collagen and Protein-Polysaccharides from the Developing Cartilage of Lathyritic Chicks

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1. The solubilization of collagen and protein–polysaccharides from the developing cartilage of normal and lathyritic chicks was studied by using mild extraction procedures. One-third of the protein–polysaccharides could be solubilized in salt solutions at neutral pH from normal cartilage, whereas 95–100% could be extracted from the cartilage of animals that were severely lathyritic. Likewise, whereas in normal animals the collagen of cartilage was essentially insoluble in salt solutions at neutral pH, in lathyritic animals it was almost completely soluble. 2. The increased solubility of the collagen of cartilage from lathyritic animals enabled sufficient material to be collected so that the pure $\alpha 1$ chains of the collagen were isolated by repeated reconstitution, precipitation and CM-cellulose column chromatography. The purified $\alpha 1$ component was characterized by its relatively high content of hydroxylysine (14 residues/1000 amino acids). 3. About 37% of the collagen from the cartilage of normal chick embryos could be extracted as the gelatin at pH 7·4 in lithium chloride solution. This was accompanied by the extraction of approx. 14% of the protein–polysaccharide content. 4. The protein–polysaccharides and the collagen from normal animals could be extracted from the cartilage relatively independently of one another under mild conditions. These same components obtained from lathyritic animals easily separated from one another after solubilization. This provided evidence that the two components are probably not covalently cross-linked. 5. The collagen of cartilage extracted as a gelatin from normal animals contained a high proportion of α chains compared with β dimers, similar to the lathyritic collagen of cartilage and other tissues, and similar to the gelatin extracted from normal chick bone.

The induction of lathyrism in animals increases the solubility of tissue collagen (Levene & Gross, 1959; Glimcher, Friberg, Orloff & Gross, 1965; Levene, Kranzler & Franco-Browder, 1966; Bickley, 1964; Gross, 1963), including the normally insoluble collagen of bone (Levene & Gross, 1959; Glimcher *et al.* 1965) and cartilage (Levene *et al.* 1966). The effect of the lathyrogens on the protein–polysaccharide components of connective tissues is, however, less clear. Levene *et al.* (1966) have reported that the solubility, or more precisely the amount, of protein–polysaccharides that could be extracted from the cartilage of developing chicks in cold salt solutions at neutral pH was unaltered in lathyrism, whereas about one-third of the collagen content of the lathyritic cartilage was extracted in the same salt solutions. However, since the amount of collagen that can be extracted from the bones of lathyritic chicks is increased five- to eight-fold (Glimcher *et al.* 1965) after multiple injections of lathyrogenic agents compared with the amount that can be extracted after a single injection of a lathyrogen, it was considered that a similar severe state of lathyrism might also alter the solubility properties of the protein–polysaccharide components of cartilage. This was indeed found to be the case. Moreover, the increased yield of soluble collagen from developing cartilage has also provided us with the opportunity to examine the amino acid composition and certain structural features of the collagen of cartilage, and certain of its interaction properties with the protein–polysaccharide components.

MATERIALS AND METHODS

Tissue source and induction of lathyrism. Groups of 300 White Leghorn eggs, incubated at 38° for 13 days, were injected with 5mg. of β-aminopropionitrile fumarate in 0·2ml. of sterile 0·9% NaCl on to the chorioallantoic

membrane through pin-holes in the shell. This procedure was repeated in the next 2 successive days, the dosage being increased on the last injection to 10 mg. of β-aminopropionitrile fumarate. The animals were killed 48 hr. later. Untreated chicks of the same age were used as controls.

Extraction of collagen and protein–polysaccharides. The cartilaginous ends of the femora were removed a sufficient distance from the bone–cartilage junction to ensure that no bone was included in the cartilage specimens. This was confirmed by histological examination of several samples. The bone, cleaned of residual cartilage, periosteum and marrow, was used to establish the presence of lathyrism, by measuring the solubility of the normally insoluble bone collagen (Levene & Gross, 1959; Glimcher *et al.* 1965). The cartilage was minced with a razor blade and extracted in 20 vol. (20 mg./g. wet wt. of the tissue) of 1 M-NaCl–0·05 M-tris–HCl buffer, pH 7·4, at 2° for 3 days. This procedure was repeated three times. The insoluble residues were briefly rinsed with water and extracted for an additional 10 days in 50 vol. of 0·5 M-acetic acid at 2°. The insoluble residues were then further extracted for 1·5 weeks in 5 M-LiCl (100 ml./g. wet wt.) followed by 1·5 weeks in 9 M-LiCl (100 ml./g. wet wt.), pH 7·4, at 2° (Glimcher *et al.* 1965). In addition, samples of normal cartilage were extracted directly in 9 M-LiCl, pH 7·4, for 5 days at 0° and the procedure was repeated once. The amount of collagen (or of gelatin in LiCl extracts) extracted was computed from the hydroxyproline content (Stegemann, 1958) of supernatants and residues.

Purification of collagen. The extracts were dialysed against water and freeze-dried (crude collagen preparations). The salt- and acid-extractable collagens were purified by acidification or addition of concentrated NaCl (three times), and by dialysis of acetic acid solutions of collagen against 0·02 M-tris buffer–HCl, pH 7·4 (twice) (Glimcher, Francois, Richards & Krane, 1964). Throughout the extraction and purification procedures great care was taken to avoid bacterial contamination.

Further purification of the collagen was achieved by a modification (Glimcher *et al.* 1964) of the method described by Gross (1958) with trichloroacetic acid and by chromatography on CM-cellulose columns (Piez, Eigner & Lewis, 1963). The effluent from the CM-cellulose columns was monitored by polyacrylamide disc gel electrophoresis in urea (Nagai, Gross & Piez, 1964; Francois & Glimcher, 1965). The relative proportions of the α and β components in the extracts was determined after the gels were stained with Amido Schwartz by densitometry of the gels in a Gilford model 2400 spectro-photometer equipped with a special cell holder and drive.

Amino acid analyses were performed on samples hydrolysed for 24 hr. in triple-distilled 6 M-HCl at 108° with a commercial automatic amino acid analyser (Phoenix Precision Instrument Co., Philadelphia, Pa., U.S.A.). The extracts, the purified collagen preparations and the tissue residues after extraction were analysed for neutral sugars (Winzler, 1955), uronic acid (Dische, 1947) and hexosamines (Boas, 1953). The individual sugar components were qualitatively estimated by paper chromatography of partial acid hydrolysates (Eylar & Jeanloz, 1962).

RESULTS

The marked increase in the amount of collagen and protein–polysaccharide extracted from the cartilage of lathyritic chicks, as compared with normal animals, is evident from the results of a typical experiment shown in Table 1. Optical-rotatory-dispersion and 'melting' curves demonstrated that the collagen was undenatured, as did experiments showing that the purified lathyritic collagen from cartilage was readily precipitated into native-type fibrils by the heat gelation of neutral salt solutions of the collagen, and to segment long spacing aggregates by the addition of ATP to acetic acid solutions of collagen.

The amino acid compositions of the five-times-reconstituted lathyritic collagen, the trichloroacetic acid-purified collagen and the α1 component (obtained by chromatography on CM-cellulose) which migrated as a single band on disc gel electrophoresis, are shown in Table 2. Despite repeated chromatography it was not possible to obtain sufficient amounts for amino acid analysis of an α2 fraction that was free of α1 component as demonstrated by disc gel electrophoresis.

Analytical disc gel electrophoresis of the collagen extracted from the cartilage of lathyritic animals demonstrated that there was a marked preponderance of single-stranded α components as compared with the covalently cross-linked β dimers (Fig. 1), similar to the findings reported for the lathyritic collagens of other tissues (Martin, Gross, Piez & Lewis, 1961).

Paper chromatography of acid hydrolysates of the neutral sugars extracted in neutral pH salt solutions and in acetic acid from the lathyritic cartilage revealed galactose : glucose : mannose molar

Table 1. *Extraction of collagen and protein–polysaccharide from normal and lathyritic chick cartilage*

Sample	Collagen sequentially extracted (expressed as % of original collagen content)				% of the total carbohydrate extracted in	Collagen and protein–polysaccharides extracted in 9 M-LiCl (% of original content)	
	In 1 M-NaCl, pH 7·4	In 0·5 M-acetic acid	In 5 M-LiCl, pH 7·4	In 9 M-LiCl, pH 7·4	1 M-NaCl at pH 7·4	Collagen	Carbohydrate
Normal	0·16	1·2	10·4	27·1	37·1	37·0	13·8
Lathyritic	91·0	~9	—	—	96·4	—	—

Table 2. *Amino acid composition of the collagen of developing limb cartilage from lathyritic chicks*

Amino acids	Amino acid composition (residues/1000 total amino acids) Reconstituted collagen fibrils	Additional purification by trichloroacetic acid	α1 component from CM-cellulose
4-Hyp	82	100	105
Asp	60	52	44
Ser	35	31	25
Thr	28	26	21
Glu	85	84	79
Pro	101	111	119
Gly	264	307	328
Ala	108	107	126
Val	30	22	14
Met	5	5	8
Ile	20	10	7
Leu	42	21	20
Tyr	44	4	6
Phe	21	15	14
Hyl	11	14	14
Lys	37	37	25
His	11	8	4
Arg	52	48	51

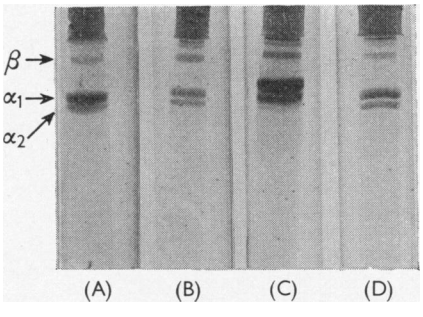

Fig. 1. Analytical disc polyacrylamide-gel electrophoresis of collagens in 6·5% acrylamide at pH 5·15. (A) Neutral salt extract of lathyritic cartilage collagen; (B) neutral salt extract of lathyritic tendon collagen; (C) LiCl extract of normal cartilage; (D) neutral salt extract of lathyritic skin collagen. The marked preponderance of the α components compared with the β components in all samples should be noted.

proportions 5:1:1 in the crude lathyritic extracts, and a galactose:glucose molar ratio 1:1 in the purified lathyritic collagens. The hexosamines of the crude lathyritic extract had a galactosamine: glucosamine molar ratio 10:1.

As has been previously reported for the salt- and acetic acid-insoluble collagen of chicken bone (Glimcher & Katz, 1965), approx. 35–40% of the total collagen content of normal developing cartilage can be extracted as the gelatin in denaturing reagents such as lithium chloride (Table 1). Like chicken bone collagen (Glimcher & Katz, 1965), the α components are the major species present in the gelatin of the normal cartilage extracted in lithium chloride (Fig. 1). Less of the protein–polysaccharides of normal developing cartilage were extracted in lithium chloride than in sodium chloride (Table 1).

Table 3. *Concentration of carbohydrates in the pooled neutral-salt-soluble and lithium chloride extracts of normal and lathyritic chick cartilage and of purified reconstituted lathyritic collagen fibrils*

Sample	Concentration (g./100g. dry wt.) Neutral sugars	Uronic acid	Hexosamines
Normal cartilage (neutral salt extract)	3·6	12·4	12·9
Normal cartilage* (LiCl extract)	5·4	12·0	9·1
Lathyritic cartilage (neutral salt extract)	1·3	5·6	4·5
Normal cartilage (LiCl extract after previous NaCl extraction)	0·5	0·0	0·0
Purified lathyritic collagen (neutral salt extract)	0·5	~0	~0

* Extracted directly with LiCl.

Analyses of the supernatants and precipitates before and after reconstitution of the lathyritic cartilage collagen revealed that essentially all of the protein–polysaccharides could be readily separated from the collagen under very mild conditions (Table 3), resulting in a hexose content of the reconstituted cartilage collagen similar to that reported for other purified collagens (Blumenfeld, Paz, Gallop & Seifter, 1963; Francois & Glimcher, 1966, 1967). The collagen extracted from normal cartilage in lithium chloride after previous extraction with sodium chloride also had a neutral sugar content of approx. 0·5% and no uronic acid or hexosamines were detected (Table 3). Because the protein–polysaccharides that are insoluble in sodium chloride and acetic acid are also insoluble in 9 M-lithium chloride, the collagen extracted in this fashion as the gelatin is virtually free of protein–polysaccharide.

DISCUSSION

Contrary to the results reported by Levene *et al.* (1966), the present experiments show that the solubility characteristics of the protein–polysaccharides of developing chick cartilage are

markedly affected by lathyrism. The difference in the experimental results probably arise from the fact that a much more severe degree of lathyrism was induced in our experimental animals. The increased solubility of the protein–polysaccharides of lathyritic cartilage could result from changes in either the interaction properties of the protein–polysaccharides with collagen in the tissue and/or from changes in the structure, composition and covalent structure of the protein–polysaccharide moieties themselves.

A number of findings from these experiments indicate that the collagen and protein–polysaccharide components in developing chick cartilage are not covalently linked. Extraction of normal cartilage in neutral salt solutions dissolves about one-third of the protein–polysaccharide components with virtually no concomitant dissolution of the collagen. Moreover, after this fraction of the protein–polysaccharides is extracted, approx. 35–40% of the collagen can be extracted as the gelatin in lithium chloride and this fraction of the collagen is virtually free of protein–polysaccharide. In severe lathyrism, where both components are almost completely solubilized in neutral salt solutions, the two can be readily separated under very mild conditions where covalent bonds are unlikely to have been broken. Although the protein–polysaccharides and collagen of connective tissues are intimately related, the evidence from these experiments is therefore consistent with the conclusions of others that, even in a tissue like cartilage where the concentration of protein–polysaccharide relative to collagen is quite high, the interaction between the two components is most probably non-covalent (Mathews, 1965; Mathews & Decker, 1968; Toole, 1968).

As has been previously noted for the normal neutral-salt-soluble collagens of a variety of tissues (Francois & Glimcher, 1966) a considerable amount of non-collagenous protein remains associated with the cartilage collagen even after multiple reconstitutions. Most of this material can be removed by the addition of trichloroacetic acid to acetic acid solutions of the collagen (Francois & Glimcher, 1966). The most highly purified material is obtained by column chromatography on CM-cellulose. The most striking difference in the amino acid composition of the cartilage collagen as compared with other collagens is the very high content of hydroxylysine, similar to that reported for young chick bone collagen (Miller, Martin, Piez & Powers, 1967). As has been demonstrated in other lathyritic collagens, the lathyritic collagen of cartilage showed a very marked preponderance of the α components (Fig. 1), indicating a paucity of covalent cross-linkages. This was also true, however, of the normal cartilage collagen extracted as a gelatin in lithium chloride similar to that reported for normal chick bone collagen (Glimcher & Katz, 1965).

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