Studies on the Insoluble Residue of Human Costal Cartilage (35987)

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Recently we reported the isolation and partial characterization of three protein polysaccharide light (PPL) fractions from human costal cartilage (1). The PPL-2 and PPL-3 were single components and the PPL-3 was free of hydroxyproline. In contrast, the PPL-2 and PPL-4 contained, respectively, 2 and 10 residues of hydroxyproline/1000 residues of amino acid. Similar PPL fractions were also isolated from rat costal cartilage (2). The above fractions accounted for only about 10% of the dry weight of the cartilage samples. The composition and the structure of the cartilage residue after extraction of the PPL fractions has not been reported. The present study deals with: (A) solubilization of the residue, remaining after extraction of PPL fractions, by autoclaving or extracting with hot trichloroacetic acid (3); (B) physical and chemical studies on the components obtained; and (C) evidence for the involvement of both covalent and ionic bonds in the structure of the components.

Methods and Procedures. The procedures for solubilization and analysis of human costal cartilage residue (CRR) (1), obtained after extraction of the protein polysaccharide light (PPL) fractions, are shown in Fig. 1.

Five hundred milligrams of CRR were suspended in 10 ml of 0.01 *M* acetate buffer (pH 7.0), and autoclaved at 110° for 1 hr. The mixture was centrifuged at 284*g* for 10 min. The undissolved material was reautoclaved as above and centrifuged twice more. After the third centrifugation, a very small amount of undissolved material remained, which was soluble at pH 8.0 and above. This residue was less than 1% of the starting material and was discarded.

Another 500 mg sample of CRR was extracted (6 times) with 10 ml of 0.3 *M* TCA at 90° for 15 min (3). After extraction, the mixture was centrifuged at 1,770*g* for 10 min. TCA was removed from each of the extracts and the final residue by ether extraction. The final residue was soluble at pH 7.0 and above.

Sephadex G-200 chromatography (2.5 × 100 cm column): For the chromatography of the autoclaved and TCA extracts, the Sephadex column was equilibrated with water at 40°. A 50–100 mg sample in 5 ml was applied to the column. The column was eluted with water at a rate of 30 ml/hr and 10 ml fractions were collected. For the TCA insoluble residue, the column was equilibrated with the acetate buffer. Fifty milligrams of the residue were suspended in 5 ml of 0.06 *N* acetate buffer and 5–10 drops of 0.1 *N* NaOH were added to solubilize the sample. The final pH was 7.0. This solubilized residue was applied to the column and the column was eluted with 0.06 *N* acetate buffer, pH 7.0. Ten milliliter fractions were collected.

Paper electrophoresis was done in a Beckman Spinco Model R cell (Palo Alto, CA) with barbiturate buffer (pH 8.6), ionic strength 0.075, and a current of 2.5 mA for 16 hr as described previously (4). Each strip was cut lengthwise into three sections; and these were stained with light green (protein), toluidine blue (acid mucopolysaccharide[1]), or Schiff's reagent (hexose).

When there was more than one protein band in the electrophoretic pattern, the electrophoresis was repeated with another sample. In this case, 1/5 of the strip was stained with light green to locate the position of the proteins. The protein bands were cut from the remaining 4/5 of the wet strip and eluted with 0.1 *N* HCl and 0.05 *N* NaOH. The

[1] Chondroitin sulfate, heparin, and keratan sulfate are toluidine blue positive acid mucopolysaccharides.

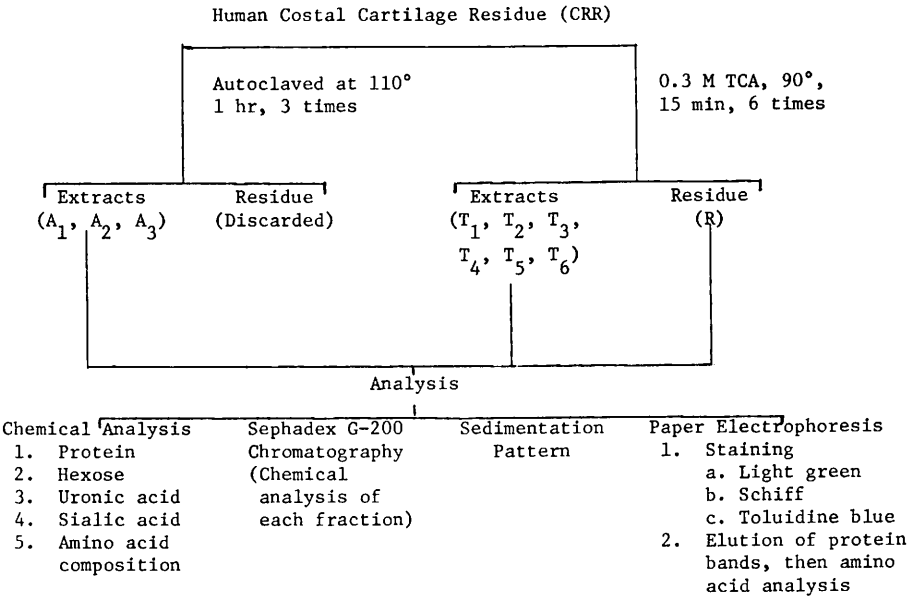

FIG. 1. Solubilization and analysis of human costal cartilage residue (CRR).

amino acid composition was determined with a Technicon amino acid analyzer (Tarrytown, NY).

Ultracentrifugation was done in a Beckman Spinco Model E ultracentrifuge (Palo Alto, CA).

Chemical analysis. Protein was determined by Lowry and co-workers' method (5); hydroxyproline by that of Neuman and Logan (6); sialic acid by Warren's thiobarbiturate technique (7); uronic acid, hexose, and hexosamine were determined by the methods of Dische (8), Weimer and Moshin (9), and Rondle and Morgan (10), respectively.

Results. The chemical composition of the various fractions obtained from CRR is shown in Table I. The three extracts obtained by autoclaving ranged from 30% collagen in A_1, to 60% collagen in A_3, as indicated by the hydroxyproline content. The carbohydrate content ranged from 20% in A_1 to 10% in A_3. Eighty percent of the carbohydrate component was made up of equal amounts of hexose and hexosamine. Uronic

TABLE I. Chemical Composition of Fractions Obtained from Human Costal Cartilage Residue (CRR).

	Method of extraction	Fractions	(mg) Protein	Uronic acid	Hexose	Hexosamine	Sialic acid	Hydroxyproline (%)
1.	Autoclaving (500 mg)	A_1	148	2.90	11.60	9.39	1.430	3.76
		A_2	128	1.37	6.20	6.55	0.660	4.10
		A_3	114	0.83	3.86	4.16	0.310	7.79
2.	TCA extraction (500 mg)	T_1	61	1.61	5.49	3.01	1.940	15.40
		T_2	50	1.13	4.03	3.30	0.540	16.60
		T_3	30	0.67	2.45	2.49	0.120	12.60
		T_4	11	0.41	1.56	2.14	0.012	11.80
		T_5	5	0.23	0.96	1.24	0.009	5.63
		T_6	4	0.19	0.82	1.03	0.002	
		Total	161	4.24	15.30	13.20	2.620	
		residue	160	1.39	6.96	8.51	0.170	0

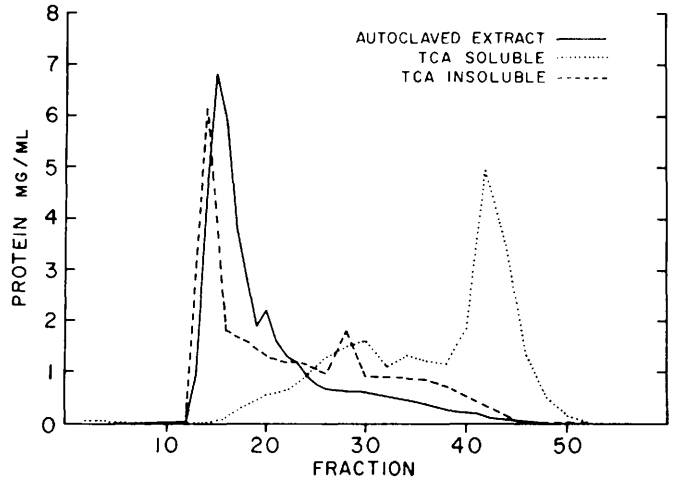

FIG. 2. Sephadex G-200 chromatography of human costal cartilage residue fractions. Approximately 50 mg samples of each were used (first autoclaved extract, first TCA-soluble extract, and TCA-insoluble residue).

acid and sialic acid were minor components; thus A_1 has a higher carbohydrate and lower collagen content than the later fractions. The three extracts contained 99% of the protein in the starting material.

In contrast, the six TCA extracts (T_1—T_6) contained only 50% of the protein in the starting material. Of the protein in the TCA extracts, the first four extracts contained 95% of the total protein in the extracts; and this protein had a hydroxyproline content (11.8–16.6%) comparable to pure collagen. The remaining 5% of the total protein (extracts 5 and 6), calculated from the hydroxyproline content, was composed of approximately 42% collagen and 58% noncollagenous protein. Considering that the TCA-insoluble residue contained no collagen, the overall CRR contains approximately equal amounts of collagenous and noncollagenous protein. The TCA-soluble material contained approximately 20% carbohydrate while the insoluble residue contained 10% carbohydrate. The low uronic acid content (1–2%) of all fractions studied, compared to the 7–13% uronic acid content of the PPL of human costal cartilage (1), indicated that chondroitin sulfate composed only a small portion of the CRR.

Sephadex G-200 chromatography of the first autoclaved fraction (A_1), first TCA extract (T_1), and the TCA-insoluble fraction (R) are shown in Fig. 2. Most of the protein in A_1 and R were eluted immediately after the void volume. However, about 10% of the autoclaved protein was eluted after fraction 20. Approximately ⅓ of the TCA-insoluble protein eluted between fractions 20–40. On the other hand, the T_1 consisted mainly of smaller molecules eluted between fractions 40–50. Thus, 56% of the R and 90% of A_1 had molecular weights above 200,000 while the T_1 contained only substances with a molecular weight below 200,000, and predominantly low molecular weight substances. Uronic acid and hexose determinations on each fraction from all three samples were done but are not shown on the graph. The uronic and hexose curves were similar to their respective protein patterns. Therefore, acid mucopolysaccharide and hexose were associated with the proteins of all three preparations.

The sedimentation patterns of the first autoclaved fraction (A_1), the first TCA-soluble extract (T_1), and the TCA-insoluble protein (R) are shown in Fig. 3. The A_1 and R fraction gave single peaks, suggesting that these fractions were relatively homogenous in agreement with the data from the Sephadex G-200 chromatography. The T_1 fraction showed four or more peaks in the slow-

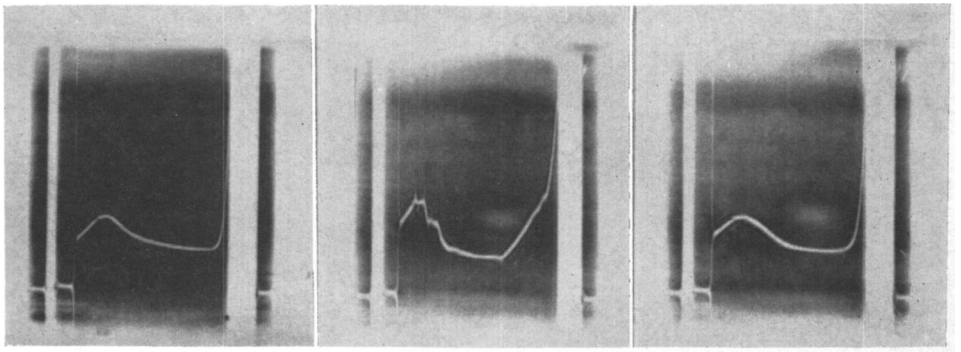

FIG. 3. Sedimentation patterns of fractions of human costal cartilage residue. The first autoclaved extract (left, 14 mg/ml 0.01 *M* acetate buffer, pH 7.0) and TCA-soluble extract (middle, 12.5 mg/ml H_2O) and TCA-insoluble residue (10 mg/ml 0.01 *N* phosphate buffer, pH 8.0) were centrifuged at 69,800 rpm in a Spinco Beckman Model E ultracentrifuge, 25°. The left picture was taken 60 min, and the middle and the right pictures were taken 90 min after reaching the speed. The phase angle was 60°.

moving part and considerable unresolved material in the fast-moving part of the sedimentation pattern. Again, this was in agreement with the Sephadex G-200 data on this fraction.

The paper elelectrophoretic patterns are shown in Fig. 4. In T_1 there was one slow-moving carbohydrate-containing protein (gelatin) and a fast-moving acid mucopolysaccharide. The TCA-insoluble residue (R) contained a fast-moving carbohydrate-containing protein and a fast-moving acid mucopolysaccharide. A_1 contained both slow(S)- and fast(F)-moving carbohydrate-containing proteins and the fast-moving acid mucopolysaccharide. The data indicated that the acid mucopolysaccharides were ionically bound to both the gelatin- and protein-polysaccharides since they could be separated from the protein components by electrophoresis.

The second TCA extract (T_2) gave the same electrophoretic pattern as T_1. In the

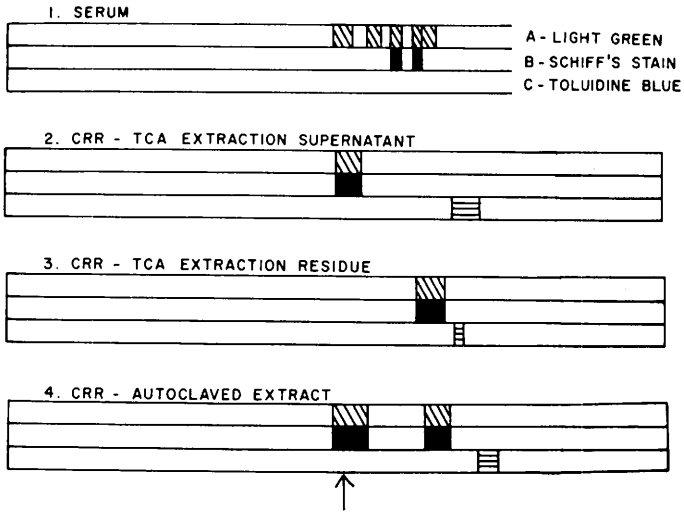

FIG. 4. Schematic electrophoretic patterns of human serum and fractions obtained from human costal cartilage residue (first TCA extract, TCA-insoluble residue, and first autoclaved extract). Arrow indicates positions where samples were applied. Bars with slanting and horizontal lines represent bands of protein and acid mucopolysaccharides, respectively. Solid bars represent hexose.

TABLE II. Amino Acid Composition of Fractions from Human Costal Cartilage Residue (CRR) (residues/1000 residues).[a]

Amino acid	Autoclaved			TCA	
	A_1	S	F	T_1	R
1/2 Cystine	10.4	1.4	17.2	1.8	10.6
Methionine	8.9	3.9	11.0	2.3	11.5
Hydroxyproline	49.8	82.3	8.5	91.7	0
Aspartic acid	74.6	45.3	104.0	49.5	100.0
Threonine	43.5	21.2	69.1	23.1	67.5
Serine	47.9	30.2	72.1	29.7	68.0
Glutamic acid	104.0	91.4	117.0	92.5	121.0
Proline	94.6	119.0	64.9	120.0	66.8
Glycine	207.0	336.0	111.0	323.0	84.7
Alanine	79.9	100.0	57.1	98.5	62.1
Valine	50.2	23.1	72.3	22.2	82.6
Isoleucine	27.7	12.4	40.5	11.8	43.7
Leucine	55.6	29.1	76.7	28.7	83.5
Tyrosine	19.7	1.6	30.5	1.7	45.8
Phenylalanine	24.7	14.2	33.4	15.0	37.5
Hydroxylysine	13.2	17.7	9.9	20.1	9.2
Lysine	29.0	18.2	38.2	17.5	40.6
Histidine	8.1	2.4	13.6	2.2	13.1
Arginine	50.8	50.5	53.7	48.8	51.3

[a] Samples were hydrolyzed in 6 *N* HCl, 110° for 16 hr. Corrections have been made on the destruction during hydrolysis (11). S, electrophoretic slow-moving protein polysaccharide; F, electrophoretic fast-moving protein polysaccharide.

third (T_3) and the fourth (T_4) TCA extracts, the acid mucopolysaccharide was bound firmly to the protein and the complexes had slow mobility, similar to that of T_1. Therefore, in the TCA extracts, 60% of the acid mucopolysaccharides were bound to the gelatin with ionic bonds and 30% with covalent bonds. The electrophoretic patterns of A_2 and A_3 were similar to that of A_1 except there was more of the slow-moving and less of the fast-moving components than in the first extract, A_1. The acid mucopolysaccharides were present as fast-moving bands in A_2 and A_3.

The amino acid composition of the first autoclaved extract (A_1), its electrophoretic components (S and F), first TCA extract (T_1), and TCA-insoluble protein (R) are shown in Table II. T_1 had the composition of pure collagen. No hydroxyproline was present in R. A_1 had an amino acid composition comparable to the average composition of T_1 and R. The slow-moving component (S) and A_1 had the amino acid composition of pure collagen. The fast-moving component (F) had an amino acid composition similar to R, except that 8.5 residues of hydroxyproline and a relatively higher content of glycine were present in this fraction. Therefore, the fast-moving component (F) in A_1 is a covalent bound protein polysaccharide–gelatin complex.

Discussion. Recently, Steven *et al.* (12–14) extracted and fractionated the proteins from human articular, costal, and intervertebral cartilages. From the insoluble cartilage residues they obtained two so-called NGF fractions. After collagenase digestion, the NGF-1 could be separated into gelatin and core protein fractions on a cetyltrimethylammonium bromide-cellulose column. With an LKB ampholine electrofocusing column, they demonstrated the formation of tropocollagen–PPL complexes from its components, tropocollagen and PPL. Therefore, they suggested that the collagen–protein polysaccharide complexes are stabilized by purely ionic interactions. Likewise, previous *in vitro* work by Mathews

(15) and Mathews and Decker (16) with acid mucopolysaccharide, its protein complexes and soluble collagen suggested that the complexes found between them were stabilized by only ionic linkages. On the other hand, Mashburn and Hoffman (17) isolated from the cartilage of blue shark and stillborn human, homogenous electrophoretic fractions having acid mucopolysaccharide and collagen weight ratios of approximately 10. From this, they suggested that covalent bonds were present between the carbohydrate and collagen in these complexes.

The present data obtained by Sephadex G-200 chromatography show an association of acid mucopolysaccharide and hexose with the proteins in all three fractions (A_1, T_1, and R) since there is a close similarity of the curves for protein, uronic acid, and hexose in each sample. Under our electrophoretic conditions, the separation of the toluidine blue positive acid mucopolysaccharides from the proteins indicated that the bonds between the acid mucopolysaccharides and the proteins are due to ionic interactions in most of the fractions that we obtained (*i.e.*, A_1, A_2, A_3, T_1, T_2, and R) exceptions being T_3 and T_4. In T_3 and T_4, the acid mucopolysaccharide appeared to be covalently bound to the collagen and could not be separated by electrophoresis. Therefore, our data are in agreement with those of Steven *et al.* (14), Mathews (15), and Mashburn and Hoffman (17) in that there are both ionic and covalent bonds between collagen and acid mucopolysaccharide. The association of the acid mucopolysaccharides with the proteins in R and in A_1, A_2, and A_3 were all ionic interaction.

Under our electrophoretic condition, there were two protein components in the autoclaved extracts (A_1, A_2, and A_3). The slow-moving protein (S) had an amino acid composition comparable to pure collagen. The fast-moving component (F) had an amino acid composition similar to the noncollagenous protein in the TCA-insoluble residue (R), except that it contained 8.5 residues of hydroxproline and a higher content of glycine (111 residues) than the R (85 residues). By calculation, this protein-polysaccharide–collagen complex contained approximately 90% noncollagenous and 10% collagenous protein and there is no acid mucopolysaccharide present. This fast-moving component (F) must be a covalently bound protein–polysaccharide–collagen complex. It is different from the one reported by Mashburn and Hoffman (17), and the PPL–collagen complex reported by us (1, 2). The structure of the protein–polysaccharide–collagen complex cannot fit the model proposed by Mathews (15). The complex does not contain mucopolysaccharide which is required in his model for the binding of the collagen and the noncollagenous protein. Therefore, our conclusion from the present data is that the human cartilage residue is composed of carbohydrate-rich collagen fraction and a noncollagenous protein carbohydrate complex fraction linked to a relatively small amount of acid mucopolysaccharide. The acid mucopolysaccharides are bound to the complexes mainly through ionic bonds, but one or more minor complexes are bound through covalent bonds. Furthermore, the present data also demonstrate the presence of a covalently bound protein–polysaccharide–collagen complex in the human costal cartilage residue.

Summary. Human costal cartilage residue, remaining after the extraction of the protein polysaccharide light portion, was solubilized either by autoclaving in acetate buffer at pH 7.0 or by hot trichloroacetic acid extraction. The human costal cartilage residue was composed of a carbohydrate-rich collagen, a noncollagenous protein polysaccharide, and a protein polysaccharide–collagen complex. All three components were ionically bound to the acid mucopolysaccharides.

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