The Resolution of *Ascaris* Cuticle Collagen into Three Chain Types†

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ABSTRACT: Reduced and methylated collagen from *Ascaris lumbricoides* cuticle was resolved into three major components by chromatography on phosphocellulose. The components have similar molecular weights of about 52 000 by sedimentation equilibrium and molecular sieve chromatography, but they have different amino acid compositions. Since they do not appear to be stoichiometrically related, they apparently represent chains from collagens of more than one type. All three chains contain about 27 residue % glycine, 36 residues of proline, and 17 residues of methylcysteine, suggesting that the collagens can be maximally about 80% triple helical and are extensively disulfide cross-linked in the native state. Two minor components from the cuticle are apparently derived from one of the major chains by cleavage in a single region to give two-third and one-third fragments.

Collagen obtained from the cuticle of *Ascaris lumbricoides* has been shown by McBride and Harrington (1967a) to consist of chains joined by disulfide bonds. Essentially the entire cuticle is soluble in reducing solutions. They obtained two fractions by elution of reduced, carboxymethylated cuticle collagen from CM-Sephadex. Amino acid analyses gave significantly different compositions. Sedimentation equilibrium experiments indicated that the components had molecular weights of approximately 62 000.

Since this collagen has several unusual properties, the present investigation was undertaken to further characterize it with the eventual aim of showing its relationship to the vertebrate collagens. CM-cellulose[1] chromatography of reduced, methylated *Ascaris* (RMe-*Ascaris*) cuticle collagen gave results similar to those of McBride and Harrington (1967a), but further separation was achieved by phospho-cellulose and molecular sieve chromatography and by gel electrophoresis. These results are reported here.

Experimental Section

Preparation of RMe-Ascaris Cuticle Collagen. Live *Ascaris lumbricoides* were obtained from hog intestines immediately after slaughter and were frozen on dry ice. The procedures used were essentially those of Josse and Harrington (1964). The frozen worms were slit lengthwise and the cuticles were peeled from the worms after thawing in deionized water at 5 °C. The cuticles were cut into lengths of approximately 1 cm and washed four times by suspension in deionized water at 5 °C. Reduction and methylation were done by established procedures (Heinrikson, 1970). Typically, 2 g (wet weight) of cuticle were reduced with 1% 2-mercaptoethanol in 8 ml of the Tris–urea–EDTA solvent described by Heinrikson. After reduction for 3 h at 25 °C, the small amount of insoluble material remaining was removed by passage of the sample through a 5-μm Millipore filter and the filtered solution was methylated at 40 °C. After methylation, the product was desalted at 25 °C by passage through a 3 × 45 cm column of Bio-Gel P-2 (100–200 mesh) equilibrated and eluted with 0.1% acetic acid. If storage was required, the solution was lyophilized and later redissolved. Complete conversion of cystine to methylcysteine was confirmed by amino acid analysis. The product gave the same melting curve by optical rotation as that previously reported for reduced, carboxymethylated cuticle collagen (McBride and Harrington, 1967b).

Chromatography. The RMe-*Ascaris* collagen was applied to phospho-cellulose (Whatman P1) in a jacketed column 18 × 25 mm maintained at 40 °C by a circulating water bath and equilibrated with 0.01 N sodium formate adjusted to pH 3.5 with formic acid. After application of the protein in 0.1% acetic acid, the column was washed with the same buffer containing 0.05 N sodium chloride (the starting buffer). The protein was eluted with a linear gradient prepared from 400 ml of the starting buffer and 400 ml of the starting buffer containing 0.45 N NaCl as the limit buffer. The column effluent was continuously monitored at a wavelength between 228 and 234 nm in a Beckman DB-G spectrophotometer containing a flow cell.

Molecular sieve chromatography was performed at room temperature on columns of 10% (Bio-Gel A-0.5m, 200–400 mesh) or 8% (Bio-Gel A-1.5m, 200–400 mesh) agarose with dimensions of 2.1 × 120 cm. The columns were equilibrated and eluted with 1.0 M calcium chloride, 0.05 M Tris-HCl, pH 7.5, as described by Piez (1968). The column effluent was monitored at a wavelength between 224 and 240 nm.

Polyacrylamide Gel Electrophoresis. The method of Panyim and Chalkley (1969), which uses 6 M urea at pH 3.2 in gels containing 5% acrylamide, was employed.

Amino Acid Analysis. The single-column system was essentially that of Piez and Morris (1960) and Miller and Piez (1966), which was based on the original method of Spackman et al. (1958), but using a step gradient of four buffers to resolve all the amino acids in collagen hydrolysates. The column, 0.9 × 55 cm, contained Beckman M82 resin and was mounted on a Beckman Model 117 analyzer with a sample injector. The column was jacketed at 50 °C and the buffers were pumped at 70 ml/h. The ninhydrin re-

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[1] Abbreviations used: CM-cellulose, carboxymethyl-cellulose; RMe-*Ascaris* cuticle collagen, reduced, methylated *Ascaris* cuticle collagen.

agent (Spackman et al., 1958) was pumped at 35 ml/h. The starting buffer, pH 2.91, was prepared and deammoniated as previously described (Piez and Morris, 1960) but was 0.20 N rather than 0.25 N in Na^+. The second and third buffers were prepared by addition of 155 and 310 ml of 0.2 N sodium citrate to the pH 2.91 buffer to give total volumes of 1l. and pH 3.32 and 4.17, respectively. The fourth buffer was 0.3 N sodium citrate, 0.9 N NaCl containing 0.1 g/l. of EDTA adjusted to pH 6.20 with HCl. The buffers were pumped for 42, 46, 50, and 120 min, respectively. The column was regenerated by pumping the starting buffer for 30 min before the next sample application.

To resolve methylylcysteine, the above procedure was slightly altered. The column temperature was kept at 40 °C for the first 63 min and at 50 °C for the remaining time. The first buffer was adjusted to pH 2.94 with a small amount of 0.2 N sodium citrate. Buffers were pumped for 44, 56, 43, and 142 min, respectively.

Protein samples were hydrolyzed in redistilled 6 N HCl in a nitrogen atmosphere for 24 h at 110 °C. The hydrolysates were taken to dryness on a rotary evaporator and the residues were taken up in a small volume of water to give a concentration of 0.05–0.25 mg/250 μl, the volume applied to the column.

Analyses for tryptophan were performed on hydrolysates which contained 2% thioglycolic acid (Matsubara and Sasaki, 1969). Tryptophan and the basic amino acids were resolved by elution from a 0.9 × 5 cm column of Beckman AA 15 resin at 50 °C with a 0.34 N (Na^+) sodium citrate, pH 5.28 buffer.

Protein Determination. Protein concentrations were determined by a microbiuret procedure (Zamenhof, 1957), using bovine serum albumin to prepare the standard curve.

Sedimentation Equilibrium. Sedimentation equilibrium measurements of molecular weight were made by Dr. Marc Lewis on a Beckman Model E analytical ultracentrifuge equipped with a multiplexed He–Ne laser light source for the interference optical system. The interference patterns were recorded on 70-mm Kodak Spectroscopic IV-F film and measured on a Nikon Model 6 comparator equipped with digital readout. Samples were run at 20 °C in 12-mm path length cells with carbon-filled epoxy centerpieces filled to give approximately 6-mm liquid column heights.

Results

Ion-Exchange Chromatography. Chromatography on phospho-cellulose produced the typical elution pattern shown in Figure 1. Three major peaks, designated A, B, and C, were observed. As will be shown, these are chemically different components and probably represent distinct collagen chains. As will also be shown, the material chromatographing between the B and C chains contained fragments of the C chain; these are designated C1 and C2. The tail on the first major peak contained noncollagenous protein, which can be nearly eliminated by careful washing of the cuticle. The combined weight of the protein isolated after desalting and lyophilization accounted for >90% of the material applied to the column.

The relative amount of protein in each fraction from various preparations of RMe-*Ascaris* collagen was highly variable. The areas under the peaks on chromatograms of five different preparations were estimated by planimetry after drawing approximate lines to separate overlapping peaks. These values were corrected for slightly different absorption coefficients measured on known amounts of the purified

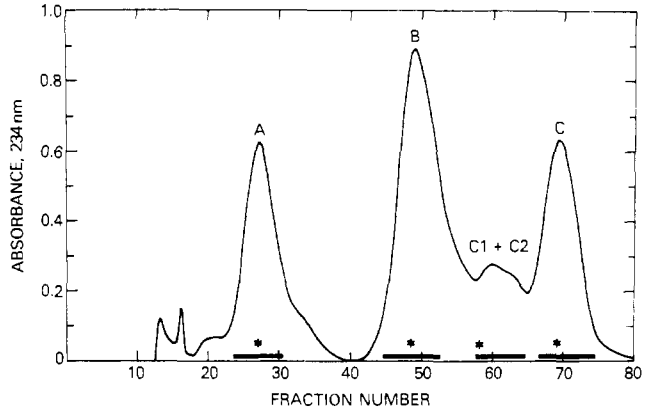

FIGURE 1: Elution pattern of approximately 80 mg of RMe-*Ascaris* collagen chromatographed on phospho-cellulose. Fractions of 10 ml were collected at a flow rate of approximately 200 ml per hour. Fractions indicated by * were taken for polyacrylamide gel electrophoresis (Figure 2). Bars indicate fractions pooled for further characterization.

Table I: Relative Amounts of the Different Chains in RMe *Ascaris* Collagen Preparations.[a]

Preparation	A Chain	B Chain	C Chain	C Chain + C1 + C2
1	1.0	1.5	1.0	1.8
2	1.0	2.3	1.2	2.0
3	1.0	2.8	1.8	3.0
4	1.0	2.9	2.5	5.1
5	1.0	3.9	3.1	6.1

[a] As determined by phospho-cellulose chromatography; normalized to a value of 1.0 for the A chain.

components. The results are reported in Table I as relative amounts of the three chains; the C1 and C2 fragments are also included as a total with the C chain. The different preparations were taken from progressively smaller specimens of *Ascaris*, suggesting that the progressive increase in the amounts of the B and C chain relative to the A chain may be related to age or developmental changes.

Polyacrylamide gel electrophoresis was performed on selected tubes from the phospho-cellulose chromatogram shown in Figure 1 as well as the original RMe-*Ascaris* collagen sample. The results are shown in Figure 2. Each of the major chromatographic peaks contained a single major component, and each corresponds to a major band in the original sample. Fraction 58 shows the C1 and C2 fragments together with traces of the B and C chains. The C2 fragment is stained only faintly because of its small size. The sharp band beneath C in the gel pattern of the original sample was not present in the purified components and has not been investigated further. The three bands near the bottom of the gel pattern of the original sample were not always seen and are presumably contaminants not removed by the washing procedure.

Molecular Sieve Chromatography. When RMe-*Ascaris* collagen was chromatographed on 10% agarose, the pattern seen in Figure 3 was observed. For comparison with the phospho-cellulose column separation, samples corresponding to pools 1 and 2 (Figure 3) from a larger collagen sample were isolated and then chromatographed on phospho-cellulose. These results are shown in Figure 4. It is evident that the three major components, the A, B, and C chains, are approximately the same size, judging by the large sym-

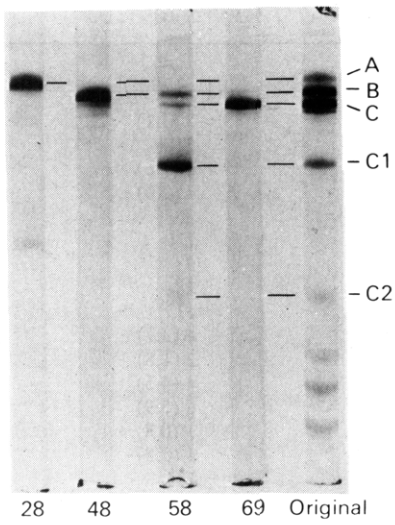

FIGURE 2: Polyacrylamide gel electrophoresis of the indicated fractions from Figure 1 and the original RMe-*Ascaris* cuticle collagen. Approximately 20 μg of protein from the indicated tubes and 70 μg of the starting material were placed on the top of the gels. Electrophoresis was performed for 2 h with a constant current of 3 mA per gel. The cathode was at the bottom and migration was from top to bottom.

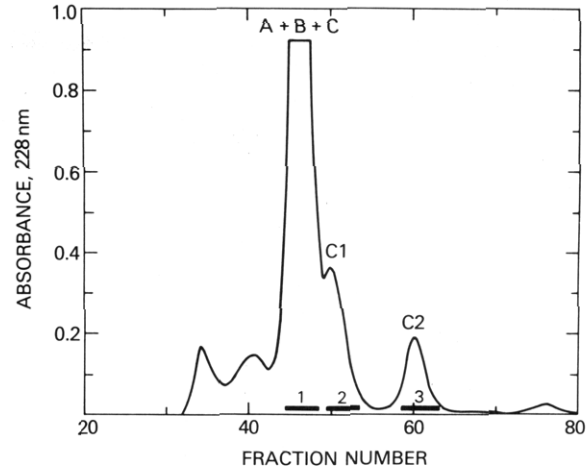

FIGURE 3: Molecular sieve chromatography of approximately 15 mg of RMe-*Ascaris* collagen on 10% agarose. Fractions of 5 ml were collected at a flow rate of 25 ml per hour. Bars indicate pooled fractions used for further analysis.

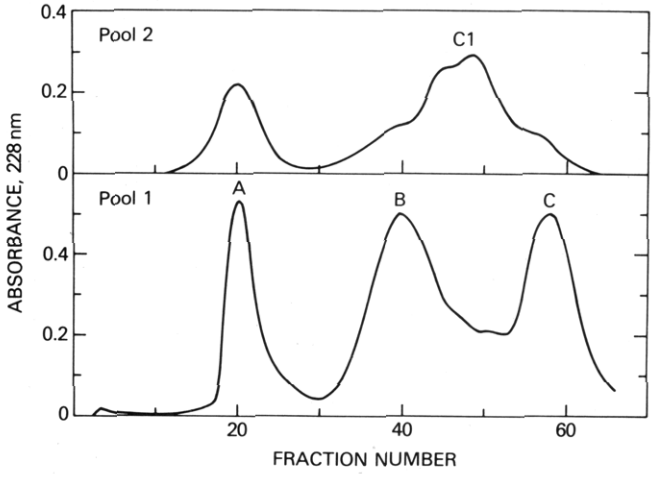

FIGURE 4: Rechromatography on phospho-cellulose of pools obtained by molecular sieve chromatography on agarose (Figure 3). Pools from 96 mg of RMe-*Ascaris* collagen chromatographed as in Figure 3 were desalted, lyophilized, and chromatographed on phospho-cellulose. Fractions of 10 ml were collected at a flow rate of 180 ml per hour. Top: Elution pattern of pool 2 protein. Bottom: Elution pattern of pool 1 protein.

metrical peak seen in Figure 3. The C1 + C2 fragments are smaller, as indicated by their later elution from the agarose column. These results were confirmed by chromatography on agarose of samples of the A, B, and C chains obtained by phospho-cellulose chromatography. They all eluted from agarose at the same volume. The C1 + C2 fragments isolated as a mixture by phospho-cellulose chromatography gave two peaks on agarose, as seen in Figure 5, which correspond to the smaller and later eluting peaks seen in the original molecular sieve chromatogram (Figure 3).

Molecular Weight. An 8% agarose column was standardized for molecular weight determination with rat skin collagen α1, α1-CB7, and α1-CB3 (Piez, 1968). The data expressed as log molecular weight vs. the ratio of elution volume to included column volume could be accurately described by a straight line. By this method, the A, B, and C chains have an apparent molecular weight of 52 000; the C1 and C2 fragments have apparent molecular weights of about 35 000 and 17 000, respectively.

To be sure that RMe-*Ascaris* collagen components do not behave anomalously on molecular sieve columns under these conditions, the molecular weights of the B and C chains were determined by high-speed sedimentation equilibrium. Portions were taken from peak tubes of samples rechromatographed on agarose. The C chain was centrifuged in the column buffer, 1.0 M $CaCl_2$, 0.05 M Tris, pH 7.5, for 45 h at 20 000 rpm; the B chain sample was dialyzed against 0.2 N NaCl, pH 7.0, and centrifuged for 45 h at 16 000 rpm. The concentration in both cases was approximately 0.18 mg/ml. The partial specific volumes were calculated from the amino acid compositions using the partial specific volumes reported by Zamyatnin (1972).

The molecular weights were computed by means of a nonlinear least-square analysis of the concentration distributions using standard equations. This method of data analysis readily permits the analysis of multicomponent systems and since, when fitting concentration as a function of radial position, the variance of each of the experimental points is the same, and since no transformation of variables is involved, the standard errors obtained for the fitting parameters have reasonable significance. They do not, however, include errors that might arise from sources other than the closeness of the fit. For the B chain, a molecular weight of 51 850 ± 200 was obtained; for the C chain, a molecular weight of 50 850 ± 800 was obtained, but a small amount of lighter material was present. It could be satisfactorily accounted for in the calculations as a second component of molecular weight 10 200 ± 6100 comprising 8% of the mass. This result suggests that the C chain is somewhat unstable, consistent with the finding of fragments of the C chain in the original sample.

Amino Acid Compositions. Samples purified by successive phospho-cellulose and molecular sieve chromatography were hydrolyzed and analyzed. The results are given in Table II expressed as residues per molecule. The compositions of the A, B, and C chains are similar but differ significantly in several respects. The sum of the compositions of the C1 and C2 fragments is very similar to that of the C chain. This finding, together with the molecular weight

Table II: Amino Acid Composition of *Ascaris* Cuticle Collagen Chains.[a]

Amino Acid	A Chain	B Chain	C Chain	C1	C2
4-Hydroxyproline[b]	13	12	10	7 (7.1)	4 (3.6)
Aspartic acid	28	32	34	21	12
Threonine	12	8 (8.4)	7 (7.4)	6 (5.6)	3 (2.8)
Serine	15	9 (9.2)	8 (7.9)	7 (7.0)	2 (2.4)
Glutamic acid	25	29	38	26	12
Proline	210	209	194	130	60
Glycine	152	149	153	98	47
Alanine	34	37	24	18	8 (7.8)
Methylcysteine	17	17	17	11	5 (5.1)
Valine	7 (7.1)	7 (7.3)	6 (6.4)	4 (3.7)	2 (2.1)
Methionine	7 (6.9)	2 (1.8)	4 (4.4)	2 (1.8)	3 (2.8)
Isoleucine	7 (7.0)	4 (4.1)	6 (6.0)	4 (4.5)	2 (1.7)
Leucine	9 (9.2)	9 (9.2)	6 (6.5)	6 (5.9)	2 (2.0)
Tyrosine	1 (1.3)	1 (1.2)	1 (0.8)	1 (0.8)	0
Phenylalanine	4 (3.5)	4 (4.2)	3 (2.9)	1 (1.3)	1 (0.9)
Lysine[b]	10	21	20	13	7 (6.9)
Histidine	3 (3.4)	4 (3.7)	5 (5.0)	3 (3.3)	2 (2.0)
Arginine	14	13	22	15	8 (8.4)
Tryptophan	1 (1.0)	1 (0.9)	1 (0.6)	Trace	Trace

[a] Reduced and methylated. Values are expressed as residues per molecule using molecular weights of 52 000 for the A, B, and C chains and 35 000 and 17 000 for the C1 and C2 fragments, respectively. Where less than 10 residues were present, actual values are given in parentheses. The average of analyses of two samples is given except for the tryptophan value which was determined on one sample. [b] 3-Hydroxyproline and hydroxylysine are absent.

studies, justifies their probable identification as fragments of the C chain.

As previously reported for *Ascaris* cuticle collagen (McBride and Harrington, 1967a), the proline content, about 36 residue % in our studies, is exceptionally high, while the hydroxyproline content, about 2 residue %, is exceptionally low compared with vertebrate collagens where both are present in similar amounts and total 16–23 residue %. The glycine content of the *Ascaris* collagen chains is about 27 residue % compared with 33 residue % for interstitial vertebrate collagens. All three chains are similar in these respects. The presence of 17 residues of methylcysteine in each chain is consistent with extensive disulfide cross-linking in the native cuticle.

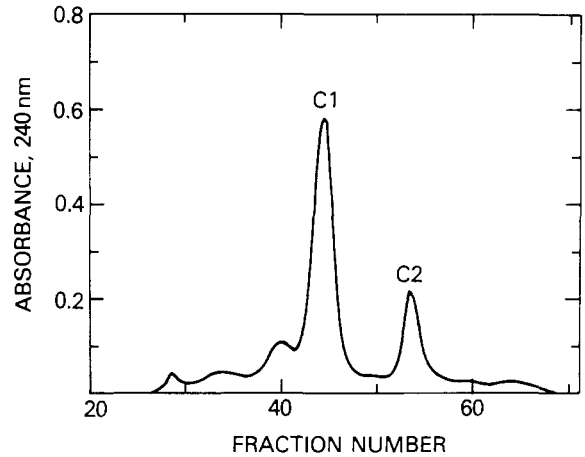

FIGURE 5: Separation of the C1 and C2 fragments by molecular sieve chromatography on 10% agarose. Fractions of 5 ml were collected at a flow rate of 20 ml per hour. The original material was obtained by chromatography on phospho-cellulose as in Figure 1 (fractions 58–64).

Discussion

Since the cuticle of *Ascaris* is essentially all soluble in reducing solutions, and since recoveries from the phospho-cellulose chromatograms are better than 90%, our results provide a detailed picture of the chain composition of the cuticle collagen. The collagen is made up of three major components, which by the tests applied here seem to be single polypeptide chains. It is likely that these chains are genetically distinct, as judged by their amino acid compositions, and represent different collagen types in the same manner as has been found for mammalian species (Miller, 1973; Miller et al., 1971). More complete primary structure studies are required to confirm this. The lack of stoichiometry between the A, B, and C chains is consistent with each representing a different collagen. This would also be expected since it is believed that these chains form the triple-helical collagen structure by some type of *intra*chain folding (McBride and Harrington, 1967a,b; Harrington and Karr, 1970) rather than by association of three separate chains as occurs in mammalian collagens. The reason for the variability in relative amounts of the three chains is not known but may be related to the complex layered structure of the cuticle (Bird and Deutsch, 1956), which may change with growth or age.

The molecular weight of 52 000 found by us for the A, B, and C chains differs from the earlier value of about 62 000 (McBride and Harrington, 1967a). It is likely that the lower value is correct since more highly purified samples were used in our studies and agreement was obtained by two independent methods.

The C1 and C2 fragments, identified as portions of the C chain, have a molecular weight ratio of 2:1. The amounts found by molecular sieve chromatography (Figures 3 and 5) suggest that they are present in about a 2:1 weight ratio, and therefore a 1:1 mole ratio. This suggests that they arise by cleavage of the C chain in a single region. This apparently is not an artifact of isolation since immediate freezing of live *Ascaris* did not prevent their occurrence. Preliminary experiments with enzyme inhibitors also failed to prevent their formation. The cleavage is presumably proteolytic, but we do not have direct evidence to support this. We also cannot tell whether a single bond is cleaved, or one or more different bonds are cleaved in a susceptible region.

The finding that the C1 and C2 fragments represent respectively two-thirds and one-third pieces of the C chain may be related to the molecular structure of the *Ascaris* collagen. Since the molecular structure of all collagens requires three strands, McBride and Harrington (1967a,b) have suggested that *intra*chain folding of *Ascaris* collagen is accomplished by backfolding of chain thirds separated, in the sequence, by noncollagenous regions, giving rise to antiparallel strands. These regions, or one of them, may be the site of cleavage that gives rise to the C1 and C2 fragments. An alternate, but possible, molecular structure of three parallel strands joined at one end by some as yet unidentified link, would also require a division of the sequence in some way into thirds again giving rise to a potential cleavage site.

That *Ascaris* cuticle collagen chains are partly noncollagenous was evident from earlier analyses showing less than the one-third glycine content required for the collagen triple helix (McBride and Harrington, 1967a). Our value of 27 residue % is consistent with a maximum of 80% triple helix. This may be contrasted to greater than 95% triple helix in the major mammalian collagens, but is reminiscent of the much lower triple-helical content of procollagen (see Martin et al., 1975) and possibly of basement membrane collagen (see Kefalides, 1973).

Although these results provide a reasonably complete description of the chain composition of *Ascaris* cuticle collagen, there is as yet only suggestive evidence as to how the chains are organized into molecules, and there is essentially no evidence as to how the molecules are organized in the cuticle.

Acknowledgments

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