INTRASPECIES COMPOSITION DIFFERENCE IN COLLAGEN FROM CUTICLE AND BODY OF ASCARIS AND LUMBRICUS

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Collagens of diverse origin have a generally similar amino acid composition, the extreme differences recorded being for Lumbricus cuticle, in which there are 12-20 hydroxyproline residues per proline (Watson, 1958; Maser and Rice, 1962; Josse and Harrington, in press**) and for Ascaris cuticle, in which this ratio is reversed, with about 15 proline residues per hydroxyproline (Watson and Silvester, 1959; Josse and Harrington). In connection with studies on the synthesis and turnover of Lumbricus cuticle collagen, to be described elsewhere, we have made observations comparing the amino acid composition of cuticle collagen with that of collagen extracted from cuticle-stripped bodies in each species of worm. For both Ascaris and Lumbricus, these measurements indicate marked differences when body and cuticle collagen are compared with respect to hydroxyproline/proline ratios. To our knowledge, such large intraspecies tissue difference in collagen composition have not been reported, although only limited date are available comparing different tissues in the same species (Tristram and Smith, 1963).

Methods - Commercially obtained earthworms of 4 to 6 inches (Wholesale Bait Company, Hamilton, Ohio) were soaked in ether to obtain cuticles (Watson, 1958) and the residual bodies reserved for separate extraction. Ascaris suis were kindly provided by Dr. Ernest Bueding whom we thank also

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for guidance in the dissection of worms. Ascaris cuticle was obtained by peeling away the adherent muscle in longitudinally-bisected fresh worms; all tissue except cuticle was pooled as body. Forty individual Lumbricus and ten Ascaris worms were used for the studies reported.

Cuticles were washed by repeated rinsing in cold distilled water; collagen was extracted by treating the insoluble residue with 10 ml of 10% trichloroacetic acid (TCA) for 60 minutes at 90° (Fitch et al, 1955). After centrifugation the supernatant solutions were dialyzed against several changes of water (500 ml each) overnight. Residual bodies of either species were homogenized with 50 ml of water in a Servall Omnimixer for 1 minute. The pellets obtained from homogenates by 10-minute centrifugation at 10,000 x g were extracted with 100 ml of 10% TCA as above and centrifuged; the supernatant solutions were dialyzed against several changes of water (500 ml each) for 24 hours. A precipitate which appeared during dialysis was removed by centrifugation; the supernatants were concentrated to 5 ml in a flash evaporator (bath temperature below 35°), and freed of residual small molecules by passage through a Sephadex G-100 column (2x30 cm).

Collagen composition was defined primarily by the amino acids rendered dialyzable after treatment with highly purified Clostridium collagenase (Worthington Biochemical Corp., CLSP 6303-1). Absence of noncollagenase protease activity in this preparation was confirmed by failure to release counts from a highly-labeled C^{14}-proline-containing, water-soluble protein fraction in washings from Lumbricus cuticle.

The hot TCA extracts, estimated by hydroxyproline assays to contain 8-10 mg of collagen, were incubated at 37° for 18 hours with 2 mg of collagenase in 5 ml of 0.05 M Tris, pH 7.4, and 0.005 M $CaCl_2$; one drop of toluene was added to prevent bacterial contamination. After incubation, the reaction mixtures were chilled to 0° and dialyzed with stirring for 24 hours against 250 ml of cold water. The solution outside the bag was concentrated in a vacuum and hydrolyzed in 6 N HCl at 110° for 20 hours. Proline and hydroxyproline in the hydrolyzate were measured by the methods

of Piez et al (1956) and Neuman and Logan (1950) respectively, after removal of primary amino acids by the nitrous acid method (Hamilton and Ortiz, 1950). Another portion of the hydrolyzate was applied to a Beckman-Spinco amino acid analyzer. (We thank Dr. S.P. Bessman and Dr. E.C. Layne for the latter determinations.)

Results and Discussion - The date of Table 1 indicate a 10-fold difference in the hydroxyproline/proline ratio of collagenase-released amino acids when Lubricus cuticle and body are compared, and a 5-fold difference in this ratio when Ascaris cuticle and body are compared. Collagenase digests of each cuticle gave values for this ratio similar to that determined by Josse and Harrington for salt-soluble fractions of Lumbricus and Ascaris cuticle collagen.

Most of the hydroxyproline in the TCA extracts from each source was released by collagenase treatment (see Table 1). It is possible, however,

Table 1

Ratio of Hydroxyproline to Proline in Body and Cuticle Collagen of Ascaris and Lumbricus

The possible maximum value of the ratio was estimated by assuming that all the hydroxyproline remaining within the dialysis bag represented a collagenase-resistant core lacking in proline. Percent of total hydroxyproline left within the dialysis bag was: 10% for Lumbricus cuticle, 15% for Lumbricus body, 30% for Ascaris cuticle, and 13% for Ascaris body.

	Lumbricus		Ascaris	
	cuticle	body	cuticle	body
Dialyzable fraction of collagenase digest	23	2.1(2.4)[1]	0.10	0.52
Possible maximum value	25	2.5(2.8)[1]	0.14	0.60
Possible minimum value (Hot TCA extract)	23	0.64	0.057	0.19
Salt-soluble purified collagen (Josse and Harrington (1964))	21	-	0.065	-

[1]Values in parentheses represent a correction based on the relatively high 3-hydroxyproline content determined with the amino acid analyzer, since 3-hydroxyproline gives color in the proline assay but not in the hydroxyproline assay.

that the small fraction of hydroxyproline remaining within the dialysis bag after collagenase treatment represents a core which is resistant to collagenase and/or dialysis. This possibility is considered in Table 1 by calculating the hydroxyproline/proline ratios at their maximum extreme, considering that all the hydroxyproline remaining within the bag should be added to the dialyzable hydroxyproline ("Possible maximum value", Table 1).

The other extreme possibility, that all the proline of each initial TCA extract (as well as the hydroxyproline) is collagen-derived, is also considered in Table 1 ("Possible minimal value"). In either extreme case it is clear that the hydroxyproline/proline ratio differs markedly for the body as compared with the cuticle of each species.

Table 2

Amino Acid Composition of Collagenase-Digests: Residues per Thousand

Automatic amino acid analysis was performed using the method of Spackman et al (1958). No corrections were made for losses on hydrolysis. Quantities of each sample run in total μmoles of amino acids were as follows: Lumbricus cuticle, 4.5; Lumbricus body, 16.9; Ascaris cuticle, 4.2; Ascaris body, 3.4. Amino acids indicated as "not measured" were present at a low level, but appeared as flat peaks that prevented reliable estimation.

	Lumbricus		Ascaris	
Amino Acid	cuticle[1]	body	cuticle	body
3-Hypro	0.9	13	not detected	not detected
4-Hypro	155	120	28	70
Asp	53	51	50	70
Thr	53	31	54	50
Ser	121	86	52	41
Pro	6.6	41	243	149
Glu	62	64	17	26
Gly	333[1]	} 432[2]	332	326
Ala	97		48	73
Val	20	22	23	not detected
1/2 Cys	not detected	not detected	10	not measured
Met	0.5	2	6	8
Ileu	13	7	7	18
Leu	24	34	17	62
Tyr	6	4	4	12
Phe	9	10	12	6
Lys	not measured	12	54	73
His	" "	3	not detected	not detected
Arg	" "	84	48	not measured
Total		1,000	1,000	1,000

[1]Calculated assuming Gly residues = 333; [2]Sum of glycine and alanine, since these two peaks were not sufficiently resolved.

A more detailed summary of the composition of each collagenase-digest is shown in Table 2 which presents data from the amino acid analyzer. Several points of interest may be noted here: (1) Body collagen of Lumbricus yields a rather high value for 3-hydroxyproline in contrast to its low value in Lumbricus cuticle and its virtual absence from Ascaris cuticle or body. Since 3-hydroxyproline is approximately 10% of 4-hydroxyproline, the ratios for Lumbricus body collagen shown in Table 1 are corrected as indicated there. (2) For each source there is good agreement in the hydroxyproline/proline ratio as determined by direct analysis on the deaminated collagenase-digests or by the record from the amino acid analyzer. (3) There is generally good agreement between the data of Table 2 for collagenase-digests of Lumbricus cuticle and the data of Josse and Harrington derived from carefully purified salt-soluble collagen from Lumbricus cuticle. Our data on collagenase digests of Ascaris cuticle, however, yield somewhat different values from the data of Josse and Harrington on purified salt-soluble fractions and from those of Watson and Silvester on gelatinized preparations. In particular our value for glycine (332 residues per 1000) is substantially higher than the respective values of 261 and 286 in the other analyses cited. It is also notable that our values for glutamate, alanine, serine and threonine differ substantially from the data presented by the other two groups. This may indicate heterogeneity of composition with respect to collagenase-resistant portions, yielding fragments too large to dialyze under our conditions. In any case, the analytic differences merit further study.

Collagen extracted from the entire cuticle-free bodies of Ascaris or Lumbricus must be heterogeneous in tissue origin, so that each set of data represents a composite of collagens of perhaps widely different composition. In both Ascaris and Lumbricus, body-collagen ratios of hydroxyproline to proline are much closer to the range for other invertebrate and vertebrate collagens (approximately 0.5 to 1.5 from the table compiled by Tristram and Smith (1963)) than are the respective cuticle collagens. However, values

for this ratio in the body collagen of these two species still represent abnormally high hydroxyproline in the case of Lumbricus (ratio of 2 to 3) and abnormally high proline in the case of Ascaris (ratio of 0.2 to 0.6).

The large intraspecies difference in proline and hydroxyproline content indicated here for anatomically different collagens in worms suggests the possibility that in vertebrates as well, collagens of specific tissue origin may show unusual pyrrolidine ratios when compared with the collagens of commonly studied sources such as tendon, skin, bone, and swim-bladder. Certain collagens which might show such aberrant ratios because of anatomical distinctness (e.g., "reticulin" or basement-membrane collagen) are not readily accessible for direct study. It is of interest to note, however, that a study of collagen derived from human kidney cortex (presumably rich in basement-membranes because of the high content of tubular and glomerular epithelium) showed a relatively high (for vertebrate collagen) hydroxyproline/proline ratio of 1.1 (Windrum et al, 1955).

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