THE CHEMICAL STRUCTURE OF EARTHWORM CUTICLE

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INTRODUCTION

The cuticle of the earthworm (*Allolobophora longa*) is composed of fibres arranged in layers parallel to the surface, with the fibre directions in successive layers at right angles to each other[1]. The fibres follow a spiral course for the full length of the worm.

REED AND RUDALL[1,2] classified the fibres as belonging to the collagen group on the basis of the wide-angle X-ray diffraction diagram, which was indistinguishable from that given by orthodox collagen fibres.

Collagen fibres in the electron microscope reveal a regularly banded structure, the period of which corresponds to the long spacing (640 A) of the small-angle diffraction diagram. Earthworm-cuticle fibrils show no sign of any cross-banded structure[1].

Cuticle collagen differs from mammalian collagen also in its stability towards hot water. Mammalian collagen contracts to about one-third of its length in water at a temperature of 63–64° C, and on prolonged treatment is converted into the water-soluble substance gelatin. Cuticles dissolve in water at a temperature in the region of 40–50° C[1].

The shrinkage temperature T_s of collagen is a measure of its hydrothermal stability, and is therefore probably related to the degree of hydrogen bonding[3]. Certain collagens, such as those of fish skins, have a low hydrothermal stability, with T_s of the order of 40–45° C, as compared with 60–65° C for mammalian skin[4]. Gelatin films also shrink at 45° C, and in view of the probable rupture of hydrogen bonds in the formation of this structure, it is considered that hydrogen bonds play a secondary part in the organisation of fish skin collagens.

TAKAHASHI[5] and GUSTAVSON[3] have correlated the hydroxyproline content of various collagens with T_s, and have found that low hydroxyproline content is accompanied by lower T_s. GREEN *et al.*[6] have pointed out that acetylation of the –OH groups of collagen results in a lowering of T_s by some 20° C, it being assumed that interchain bonds are broken by the process. Those –OH bonds which resist acetylation are assumed to form stronger bonds which may be responsible for the stabilisation of Teleost collagen[7]. GUSTAVSON[7] considers the interchain bonds to be hydrogen bonds between the –OH groups of side-chains and carbonyl groups of adjacent main-chains.

Certain features of the chemical structure of collagen are characteristic. Only in collagen is hydroxyproline found in comparatively large quantities, and the value is remarkably constant for mammalian collagen[8]. The other imino acid, proline, is

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also present in very substantial quantities, and the two together make up some 25% of the total residues. Recent analyses of a typical collagen[9] show high proportions of the non-polar residues glycine and alanine, with very low proportions of the aromatic residues. Sulphur-containing amino acids are virtually absent.

It is of considerable interest to examine the chemical composition of earthworm-cuticle collagen in relation to other collagens and to the structural features observed by small-angle X-ray analysis and electron microscopy.

EXPERIMENTAL

Preparation of material

The worms were collected in late summer and killed by soaking in boric acid (saturated aqueous solution) for 2 days. The cuticle was then eased off using a mounted needle, and most of the adhering blood and tissue was removed by squeezing between the side of the mounted needle and a glazed surface. Epidermal cells were removed by rinsing in cool saline (0.9% NaCl). The cuticles were then allowed to stand in saline at 2° C for 3 days, the saline being changed daily, and this was followed by a similar treatment in phosphate buffer (M/15 Na_2HPO_4). Salts were removed by dialysis in sausage skin against running tap water for 48 hours, followed by distilled water for 24 hours. Possible fatty substances were removed by treatment with acetone, the residual acetone being removed by means of iced distilled water. The cuticles were freeze-dried and stored in a stoppered flask at 0° C. Before use the freeze-dried material was dried in a hot-air oven for 2 hours at 105° C.

Total nitrogen

Determined by a micro-Kjeldahl procedure. The material was digested using a selenium catalyst, and distilled in the Quickfit and Quartz apparatus.

Hexosamine

The hexosamine content was determined by the method of ELSON AND MORGAN[10] as modified by JOHNSTON, OGSTON AND STANIER[11]. Sufficient cuticle was taken to provide half the hydrolysate for the blank determination, which was a convenient way of allowing for the trace of humin that was formed.

Total carbohydrate

Total carbohydrate was determined using the anthrone method devised by DREYWOOD[12] and modified by TREVELYAN AND HARRISON[13]. Suitable dilutions of a standard solution of glucose were taken for calibration of the Beckman U.V. spectrometer at 620 mμ.

Hydrolysis

The cuticles were hyordlysed by refluxing with HCl (5.7N) for 18 hours. Humin was removed by filtration through a fine sintered glass filter and aliquots were taken for total nitrogen determination.

Amino acids

The method used[14] was based on the Sanger technique of end-group assay. The protein hydrolysate is allowed to react with 1-fluoro-2,4-dinitrobenzene in alcoholic sodium carbonate solution, followed by separation on buffered silica[15,16] or Kieselguhr[17] columns. The resulting DNP-amino acids are determined in sodium bicarbonate solution by the optical density at 385 mμ for DNP-hydroxyproline, 380 mμ for DNP-proline, and 360 mμ for the remainder. Some difficulty was experienced in separating DNP-alanine and DNP-proline. Since it was known that both acids reacted quantitatively when treated separately with the reagent, an attempt was made to estimate them by difference by decomposing the DNP-proline with 5.7N HCl.[14]

Using a control solution made up from synthetic amino acids, the following mean percentage recoveries were obtained: glycine 100.2 $\pm$ 1.7; alanine 97.5 $\pm$ 1.1; leucine 97.5 $\pm$ 7.0; serine 96.8 $\pm$ 2.5; threonine 98.5 $\pm$ 2.0; proline 97.6 $\pm$ 2.3; hydroxyproline 101.0 $\pm$ 1.2; lysine 80.9 $\pm$ 5.7; glutamic acid 96.2 $\pm$ 2.8; aspartic acid 92.2 $\pm$ 2.5. Correction factors were applied in the case of lysine and aspartic acid.

Shrinkage temperature

Beakers containing large volumes of water were maintained at constant temperatures covering the range 30° C to 42° C in steps of 2° C. The original length of each cuticle was found by laying

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it out on a plastic rule. It was then immersed in one of the water-baths for 5 minutes, since preliminary experiments had shown that, at any temperature within the above range, shrinkage is complete after 4 minutes. The cuticle was then dipped in iced water and the new length measured as before. Fresh cuticle was used for each determination.

RESULTS

Total nitrogen content

Replicate determinations of the total nitrogen content of earthworm cuticle gave a mean value of 15.3 g/100 g. This is considerably lower than for mammalian collagen (18.6)[9] and citrate soluble collagen (17.7)[9].

Hexosamine

The mean value of 0.37 g/100 g which was obtained showed that the proportion of hyaluronic acid or chondroitin sulphate in the earthworm cuticle is small.

Total carbohydrate

The cuticles were found to have a carbohydrate content of 4.9 g/100 g as glucose. This is not sufficiently high to account completely for the low nitrogen content; but TREVELYAN AND HARRISON[13] have pointed out that the colour intensity produced with the anthrone reagent depends on the carbohydrate and SEIFTER *et al.*[18] have indicated that some substances interfere with the course of reaction; hence the value is probably on the low side.

TABLE I

AMINO ACID ANALYSIS OF EARTHWORM CUTICLE

Amino acid	*N as % total nitrogen*
Glycine	30.8
Alanine	6.8
Phenylalanine + Valine	2.9
Leucines	2.8
Proline	3.1
Hydroxyproline	14.6
Glutamic acid	7.8
Aspartic acid	4.4
Lysine	2.8
Serine	8.4
Threonine	4.4
Tyrosine	0.8

Amino acid composition

The amino acid composition determined by the FDNB method is given in Table I. These results are in agreement with those recently published by WATSON AND SMITH[19] for the analysis of cuticles of *Lumbricus* sp.

The value for hydroxyproline is the highest yet reported[20] for any member of the collagen group. On the other hand only a small proportion of proline was found, but the two together give 17.7% of the total N as compared with 16.9% in collagen.

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Higher proportions of the hydroxylic amino acids serine and threonine than is usually associated with collagen were also found in earthworm cuticle.

The high glycine and alanine content is typical for this class of protein, the glycine constituting approximately one-third of the total residues. It is also characteristic to find a paucity of the aromatic amino acids, and a small but definite proportion of tyrosine.

Using the FDNB method of estimation, no trace was found of the sulphur-containing amino acids, but a paper chromatogram of the hydrolysate showed a very faint spot in the position normally occupied by cystine.

The proportions of the acidic amino acids, aspartic and glutamic, were higher than values reported for collagen, but the basic amino acid lysine was found to be less abundant in earthworm cuticle than in collagen.

The values obtained for proline and alanine are probably subject to a larger error than in the case of the remaining amino acids, as follows from the method of determination.

Several amino acids remain to be determined, but in view of the nitrogen already accounted for, it seems unlikely that the proportions of the remainder will be high.

TABLE II

EFFECT OF TEMPERATURE ON THE SHRINKAGE OF EARTHWORM CUTICLE

Expt.	*% shrinkage*				
	32° C	*34° C*	*36° C*	*38° C*	*42° C*
1	5.0	21.7	33.3	45.0	55.0
2	5.3	21.0	36.8	47.3	57.9
3	5.3	21.0	34.0	48.7	56.0

Shrinkage temperature

Earthworm cuticle was found to have a very low shrinkage temperature (32–33° C). The results in Table II show that shrinkage takes place over a range of temperature, but the onset occurs at close to 32° C. Cuticles immersed in water at 42° C were found to break into smaller fragments, thus confirming the observation of REED AND RUDALL that the structure dissolves at a temperature in the region of 40° C.

DISCUSSION

Earthworm cuticle would appear to consist of approximately 80% protein. The nature of the remainder has not been fully established, but the experiments with the anthrone reagent indicate that a considerable proportion consists of non-nitrogenous carbohydrate. It is curious that the carbohydrate survived the purification procedure; treatment with the alkaline phosphate buffer would have removed the usual mucopolysaccharide.

The high proportion of hydroxyproline indicates that the protein is a collagen, in accordance with the X-ray findings. This is further confirmed by other features typical of collagen, *viz.* the high proportion of glycine and alanine and the low proportions of aromatic residues. The finding of a faint spot corresponding with cystine

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is interesting since it has been pointed out by BEAR[21] that relatively primitive collagens often contain more sulphur than do mammalian collagens.

The proportion of hydroxyproline found is the highest yet reported, but it is balanced by a low proportion of proline. NEUMAN AND LOGAN[8] have pointed out that the hydroxyproline content of mammalian collagen is remarkably constant, but fish skin collagen has a lower hydroxyproline content (9.1 g/100 g), and certain other collagens show lower values than mammalian collagen. Earthworm cuticle is unique in having a hydroxyproline content higher than that of mammalian collagen.

Values for the basic amino acids, histidine and arginine, have recently been published. These are lower than for mammalian collagen and, since a higher proportion of the acidic amino acids is found in earthworm collagen, it might be that some of the acidic side-chains are present as amides in order to maintain the balance of charge.

In view of the observations of TAKAHASHI that the shrinkage temperature varies with the hydroxyproline content, it is surprising to find that earthworm cuticle has such an extremely low shrinkage temperature. For instance, from TAKAHASHI's list, the lowest hydroxyproline value (7%) reported was obtained for the skin of the rockfish (*Sebastodes*), corresponding with the lowest T_s ever recorded (33–34° C).

It is interesting to note, however, that the sum of the pyrrolidine residues in the earthworm cuticle (17%) compares with the sum obtained for calf-skin collagen (*Bos taurus*) by GUSTAVSON (16.9%). Calf skin collagen has, of course, a high T_s (65° C).

It is not easy to reconcile these findings with the hypothesis of GUSTAVSON that the collagen molecule is stabilised by cross-linkages involving the –OH groups of hydroxyproline and the CO groups of adjacent chains. The shrinkage properties and solubility of earthworm collagen would indicate a loosely bound structure, presumably stabilised by few hydrogen bonds.

JACKSON has obtained evidence that collagen is stabilised by mucopolysaccharide, his criterion of stability being again the shrinkage temperature. In this connection it is tempting to speculate on the function of the carbohydrate in earthworm cuticle.

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SUMMARY

The chemical composition of earthworm cuticle has been examined. Although the protein moiety has features characteristic of collagen, certain important differences have been observed, *e.g.* high proportions of the hydroxylic amino acids, the value for hydroxyproline being the highest yet reported. The acidic amino acids also are present in higher proportions than in collagen, but the basic amino acid lysine is present in lower proportion.

The shrinkage temperature of earthworm cuticle has been determined and appears to be unrelated to the hydroxyproline content, assuming that collagen is stabilised by cross-links involving the hydroxyl group of hydroxyproline.

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THE MOLECULAR WEIGHT OF α-CHYMOTRYPSINOGEN

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Recent estimates of the molecular weight of crystalline α-chymotrypsinogen have varied within the range of 22,000–25,000[1], which is considerably lower than the original value of approximately 36,000 reported by KUNITZ AND NORTHROP[2]. In view of the widespread interest in this protein as a precursor of a family of proteolytic enzymes[1,3,4] and as a highly purified and relatively homogeneous product[5,6,7], it appeared of importance to define the molecular weight of α-chymotrypsinogen within narrow limits of experimental error. To this end, a combination of chemical and physical methods of measurement has been employed, including amino acid analysis, light scattering, sedimentation rate, and diffusion. Molecular weight calculations deduced from X-ray diffraction data of salt-free crystals, already reported by BLUHM AND KENDREW[8], have formed part of the present, cooperative project. All of these data converge toward 25,000 as the most probable value for the molecular weight of α-chymotrypsinogen.

EXPERIMENTAL

Material. α-Chymotrypsinogen was obtained from Worthington Biochemical Laboratory, Freehold, New Jersey, as a once-crystallized filter cake and was recrystallized seven times with am-

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