THE AMINO ACID COMPOSITION AND MORPHOLOGY OF SOME INVERTEBRATE AND VERTEBRATE COLLAGENS*

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SUMMARY

1. The complete amino acid analysis of collagens from a mammal (steer hide), an elasmobranch (shark elastoidin), an echinoderm (*Thyone* body wall), two coelenterates (*Metridium* body wall and *Physalia* float), and two structurally distinct collagens from the sponge are presented along with a description of some morphological characteristics of the tissues from which they were derived.

2. Structural observations include a typical collagen axial repeating period in fibrils of the body wall of *Thyone*, thin (200–300 Å) unbranched fibrils revealing periodic axial structure in the connective tissue of *Metridium* and the float of *Physalia*, and a reasonably well oriented collagen wide angle X-ray diffraction pattern obtained from water-washed *Physalia* float.

3. The most striking characteristic of these collagens is the uniformly high glycine content which in each case approaches one-third of the total amino acid residues. This is reflected in the corrected nitrogen values which are all close to 18.6 %. The data are consistent with the requirements of the most likely structure of the collagen molecule.

4. A high degree of variability of the other amino acids, including proline, hydroxyproline and hydroxylysine, occurs among the invertebrate phyla. In general the invertebrate collagens when compared to vertebrate collagens have a smaller proportion of imino acids, more total dicarboxylic and hydroxy amino acids, and fewer nonpolar amino acids.

5. *Thyone* collagen and spongin A have the lowest lysine values yet recorded for collagen, 7.5 and 9.0 residues per 1,000 residues as compared with 25 for steer hide. *Metridium*, *Physalia* collagen and spongin B have the highest recorded hydroxylysine content, 25, 30 and 24 residues per 1,000 residues as compared with 6.8 for mammalian collagen.

6. No relationship between amino acid composition and evolutionary development is obvious.

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INTRODUCTION

The numerous fibrous proteins classified as collagens are characterized by several common structural and chemical features[1,2]. These include X-ray diffraction patterns, structure as seen in the electron microscope, and amino acid composition. Some of these characteristics have been found in connective tissue proteins from many phyla in the animal kingdom. However, none of these features is apparently completely inviolate in its detail. It is important, therefore, to have available as much data as possible for collagens from different sources for an understanding of the basic molecular structure which appears to be common to all. There is an increasing number of complete amino acid analyses available on vertebrate collagens[3] and three complete analyses of earthworm cuticle[4,5], *Thyone* ejected threads[4], and a partial analysis of the byssus threads of the mollusc *Mytilus edulis*[5].

The carbohydrate content of five invertebrate and a number of vertebrate collagens, together with partial amino acid analyses have been previously reported[6,7]. The present study provides complete amino acid data for the same samples of invertebrate collagens obtained from members of the phyla Echinodermata, Coelenterata, and Porifera together with two vertebrate collagens from steer skin and shark fin (elastoidin). The histological appearance of the parent tissues, electron micrographs, and an X-ray diffraction pattern are also reported.

EXPERIMENTAL

The source of the invertebrate collagens and their preparation for analysis has been described[6,7]. The following samples were selected for complete amino acid analysis: shark elastoidin (Elasmobranch) from the fins of *C. glaucus*, *Thyone* body wall (Echinodermata), *Metridium* skin (Coelenterata), *Physalia* float (Coelenterata), and spongin A and spongin B from *S. graminae* (Porifera). Living *Thyone* and *Metridium* were obtained from the Marine Biological Laboratories, Woods Hole, Massachusetts; elastoidin fibers were kindly provided by Dr. E. FAURE FREMIET and fresh frozen marine sponge was furnished through the courtesy of the American Sponge and Chamois Co. These were all analyzed in the form of their gelatins except the spongins.

Dermis from a three-year-old steer was included as an example of mammalian collagen.

Histology

Fresh tissues were fixed in neutral formalin and also Zenker's acetic fixative. They were embedded in paraffin, sectioned, and stained with Mallory's aniline blue, Hematoxylin and eosin, and Gomori's reticulin stain. Sponge smears were also examined fresh with the polarizing microscope.

Electron microscopy

Mechanically isolated connective tissues were fragmented by freeze sectioning and homogenized in water. The dispersion of fibrils was deposited on collodion films and shadowed with chromium or stained with phosphotungstic acid.

X-ray diffraction

Only the *Physalia* float was examined in this study. The floats were washed free

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of slime, stretched and dried in sheets. These were cut into 1 mm wide strips and pressed together to form a dried laminated preparation 1 × 1 × 10 mm. Wide angle patterns were obtained using N_1-filtered Cu K_α radiation and a specimen to film distance of 5 cm. These patterns were obtained with equipment in the Department of Biology, Massachusetts Institute of Technology by Dr. CAROLYN COHEN. Low angle diffraction did not result in satisfactory patterns perhaps because of poor fibril orientation.

Preparation for analysis

Steer hide collagen was prepared by grinding the corium with dry ice in a Wiley mill, and extracting 5 times, each for 24 h, with pH 7.5, 0.2 *M* phosphate buffer at 5°. Gelatin was prepared by autoclaving the residue in water at 15 lb. pressure overnight, exhaustive dialysis of the supernate, and precipitation with ten volumes of acetone.

Elastoidin gelatin was obtained by autoclaving the powdered dried fiber in water at 15 lb. pressure for 5 h followed by centrifugation, dialysis of the supernatant fluid and lyophilization.

Thyone and *Metridium* body wall collagens were obtained by mechanically separating the epidermal and muscular layers from the layers of dense connective tissue; these were cut into small pieces and washed thoroughly with water. *Metridium* connective tissue was extracted with half saturated calcium hydroxide overnight, washed in water, and dialyzed free of calcium ions against dilute HCl. The tissues were then autoclaved in water at 15 lb. pressure for 16 h, centrifuged, and the supernatant fluids dialyzed against water and lyophilized.

Pieces of *Physalia* float were washed free of slime and the tough sheets dried in acetone without further treatment. Gelatin was prepared as for *Metridium*.

Spongin A and B were obtained from water-washed pieces of fresh sponge which were treated with trypsin and then extracted with cold water[6]. Spongin A could be readily separated from B by light centrifugation, the former being found in the supernatant and the latter in the sediment. The heavy B fibers could be almost completely cleared of A by repeated washing in water. The viscous water suspension of A fibrils was then sedimented at 105,000 × *g* for 1 h as a loose pellet. This was repeatedly suspended in water, washed free of amorphous contaminants in the centrifuge, and lyophilized. The residual spongin B was washed and air dried.

All samples were kept for several days at 43 % relative humidity and 25° prior to analysis. The water content of the samples was determined by drying at 100° under high vacuum for 16 h. Nitrogen was determined on the dry sample by micro-Kjeldahl. Ash was measured by heating at 800° to constant weight.

All samples were hydrolyzed in sealed tubes at 105° for 24 h using 1 ml of 6 *N* HCl per 10 mg of protein. The hydrolysates were taken to a syrup once *in vacuo*, diluted to a known volume and kept frozen until analyzed.

Ion Exchange Chromatography

The neutral and acidic amino acids were separated by use of a 100 × 0.9-cm column of Dowex 50-X12 beads, 15–70 μ size (wet)[8] eluted at 7 ml/h. The basic amino acids were separated by use of a 30 × 0.9-cm column of the same resin. The methods were modified from those developed by MOORE AND STEIN[9] and have been described[10,11]

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Samples containing approximately 1 mg of nitrogen were analyzed. One ml fractions from the columns were analyzed with the ninhydrin reagent of MOORE AND STEIN[12]. Color values were determined by analyzing a mixture of known amino acids made to simulate a collagen hydrolysate.

Proline and hydroxyproline

Since these imino acids give a poor ninhydrin color by the usual procedure[12] separate samples containing about 1 mg of protein were analyzed[9] by elution of a 50 × 0.9 cm column of the resin described above with pH 3.30 citrate buffer (0.25 *N* Na^+). A ninhydrin–glacial acetic acid reagent was used to develop a color in the effluent fractions. The procedure previously described [13,14] was somewhat modified as follows: The color was allowed to develop in a constant temperature bath at 30° (rather than at room temperature) using 7 ml of 0.150% ninhydrin in glacial acetic acid (accurately prepared and equilibrated at 30°) for each 1 ml fraction. Hydroxyproline reached a maximum color in 80 min and proline in 180 min. The color was read at these times in a Beckman DU spectrophotometer at 350 mμ using a tungsten light source (with filter), photomultiplier tube, 0.2 mm slit, and silica cells with a 1 cm light path. Under these conditions 0.1 μmole of hydroxyproline in a 1-ml fraction from the column produced an absorbance of 0.142. The value for proline was 0.150. The calibration curve was linear to an absorbance of at least 1.0. Replicate analyses, done at different times, of known mixtures carried through the entire procedure had a standard deviation of 1.0%.

RESULTS

Morphology

The histology of elastoidin has been described in detail by FAURÉ-FREMIET and colleagues[15]. Electron micrographs obtained of fragmented fibers revealed ribbons with the typical 650 Å axial period (Fig. 1). Only three intraperiod bands were observed. Similar electron micrographs have been published previously by others[16,17].

Histological examination of the body wall of *Thyone* reveals it to be composed mainly of massive densely packed bundles of typical collagen (Fig. 2). These bundles are often well ordered in what appears to be successive waves of connective tissue. They stain in the same manner as collagen fibers of mammalian tissues with the classical methods used. No black silver-staining fibers were observed. Relatively few cells are seen in the connective tissue. The free surfaces are covered with a columnar type of epithelium while a less well ordered layer of cells and large longitudinal bands of muscle line the inner layer of the body wall.

Electron microscopy of fragmented *Thyone* connective tissue revealed non-branching fibrils ranging in diameter from 150 Å to 2,000 Å (Fig. 3). Most fibrils have the characteristic 650 Å axial period and in favorable cases phosphotungstic acid staining has revealed six intraperiod bands in an asymmetric pattern very similar to that of mammalian collagen. There are also present very thin "beaded" fibrils with a periodicity of 300–400 Å. Both wide angle and small angle X-ray diffraction patterns of *Thyone* body wall collagen have been reported by MARKS, BEAR AND BLAKE[18].

At least a portion of the body wall of *Metridium* contains two large layers of connective tissue connected by a series of transverse bands of similar appearance

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(Fig. 4). These layers consist of roughly parallel, densely packed fibers which have the typical staining characteristics of collagen. They are also sparsely populated with cells. CHAPMAN[19] discusses the histological organization of this tissue in detail. Electron micrographs (Fig. 5a) of *Metridium* connective tissue revealed thin (200–300 Å) non-branching fibrils very uniform in width with barely perceptible axial periodicity too indistinct for accurate measurement. After extracting in half saturated $Ca(OH)_2$ a fine periodic banding of about 200 Å was clearly visible in phosphotungstic acid stained preparations (Fig. 5b). However there are suggestions of a superimposed larger periodicity.

Histological sections of the water-washed float of *Physalia* revealed a nearly homogeneous appearing tissue superficially resembling cartilage in that the cells are large and encapsulated and there is voluminous intercellular material (Fig. 6). The latter stains uniformly like vertebrate collagen. This intercellular substance is seen to consist of a mass of widely ramifying bundles of very fine fibers seemingly embedded in an amorphous material. The serrated edges of the tissue apparently have lost their cellular covering and the large encapsulated cells are obviously degenerated. (These floats were obtained from dead animals on the beach and stored in the frozen state.) Electromicrographs of the fragmented tissue revealed fibrils very similar in size to those observed in *Metridium*. Here, again, they were difficult to free from an amorphous envelopment and only a suggestion of a periodicity was observed.

Wide angle X-ray diffraction patterns of dried stretched *Physalia* float revealed a moderately well oriented collagen pattern (Fig. 7). Low angle diffraction produced a single meridional line and was considered unsatisfactory.

Fig. 8 is a photomicrograph of a smear of the sponge, *S. graminae*, taken with the polarizing microscope using the first order red plate. The large branching fibers showing positive axial birefringence are spongin B and the finely fibrous darker mass is a tangle of spongin A fibrils which appear to be poorly birefringent here because of the loose random orientation. Electron micrographs reported previously[6] revealed the typical collagen repeating period in both types of fibers and the typical wide angle diffraction diagrams were also obtained from both plus a good low angle pattern from spongin A[6].

Chemical analysis

Analytical values for ash, water, and nitrogen are presented in Table I. Nitrogen values, corrected for ash and water, and further corrected for nonprotein material (not accounted for as amino acids, see Tables III and IV) are also presented. A value close to 18.6 % was obtained for all samples.

The amino acid composition of the collagens is presented in Tables II, III, and IV. The data appear in three forms: residues of amino acid per 1,000 total residues (Table II), residue weight as % of total weight (Table III), and nitrogen as % of total nitrogen (Table IV). The first listed, residues per 1,000 has recently been used by EASTOE AND LEACH[3]. As pointed out by them, this allows a direct comparison of frequency of occurrence to be made which cannot always be done with the other common methods of calculating the data. This is particularly true of the invertebrate collagens since they contain large and varying amounts of carbohydrate. The other two methods of presentation allow a direct calculation of recovery as weight and as nitrogen. All of the values are averages of two complete determinations except for the *Physalia*

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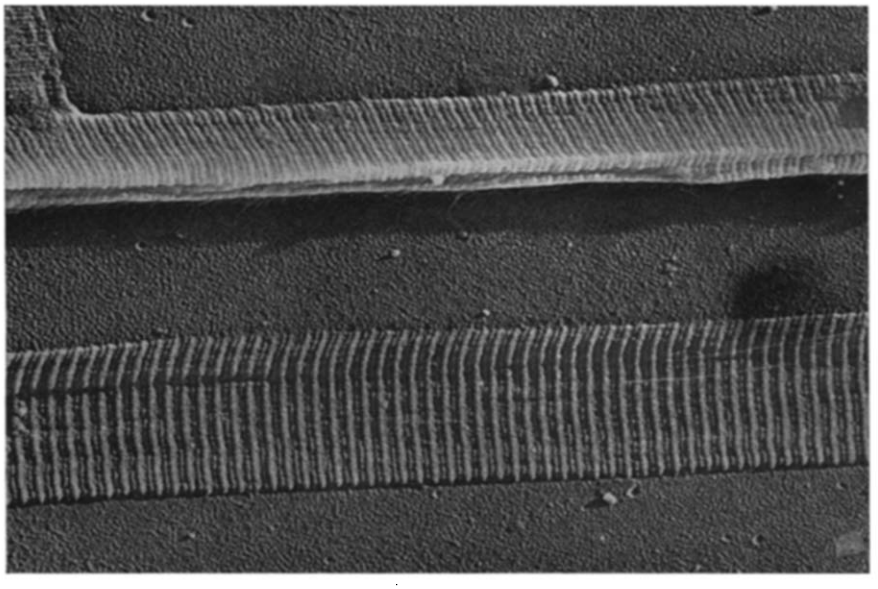

Fig. 1. Electron micrograph of fragmented elastoidin fiber. Shadowed with chromium. Mag. 31,300 ×.

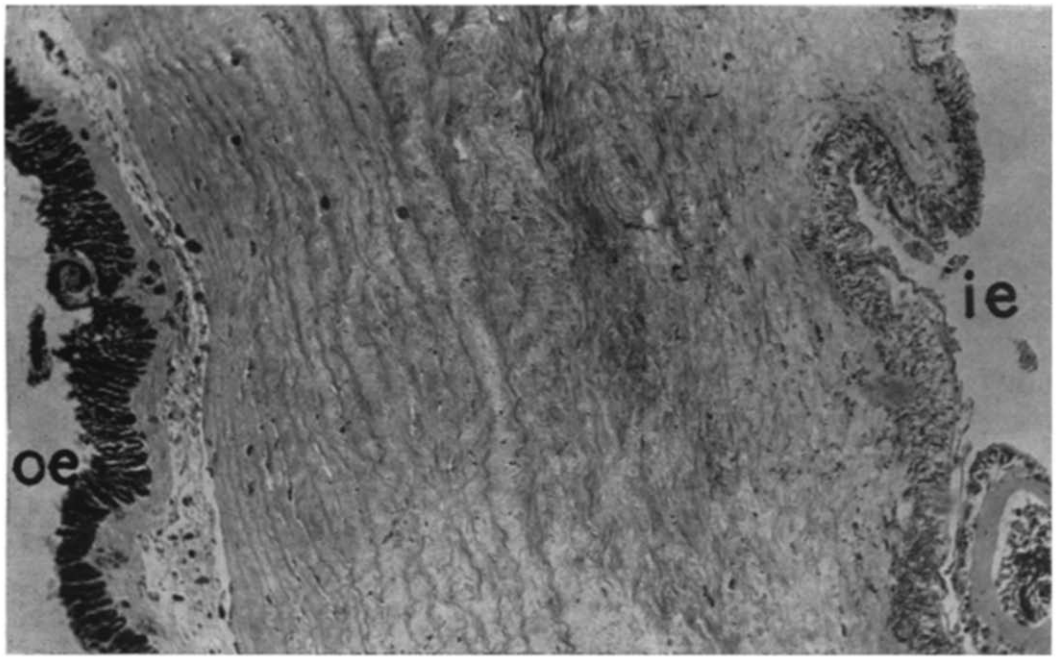

Fig. 2. Photomicrograph of body wall of *Thyone*. Stained with Mallory's aniline blue connective tissue stain. The entire region between the two layers of epithelia is densely packed collagenous connective tissue which stains blue. oe, outer epithelium; ie, inner epithelium.

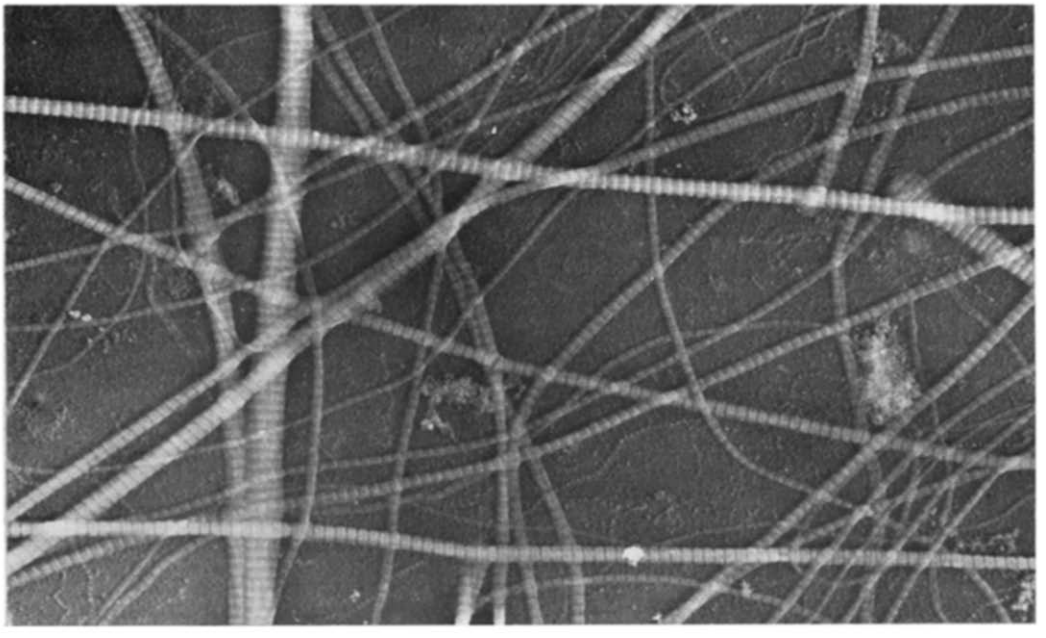

Fig. 3. Electron micrograph of fragmented *Thyone* collagen. Shadowed with chromium. Mag. 20,300 ×.

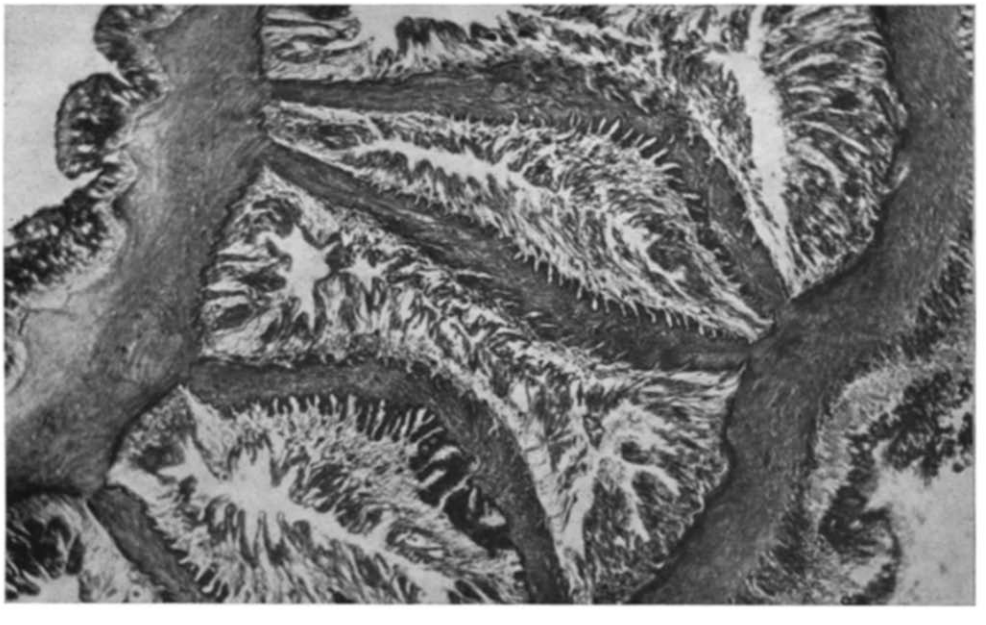

Fig. 4. Photomicrograph of *Metridium* body wall. Stained with Mallory's aniline blue connective tissue stain. The fibrous laminae and crossbands are composed of densely packed collagen fibrils and stain intensely blue.

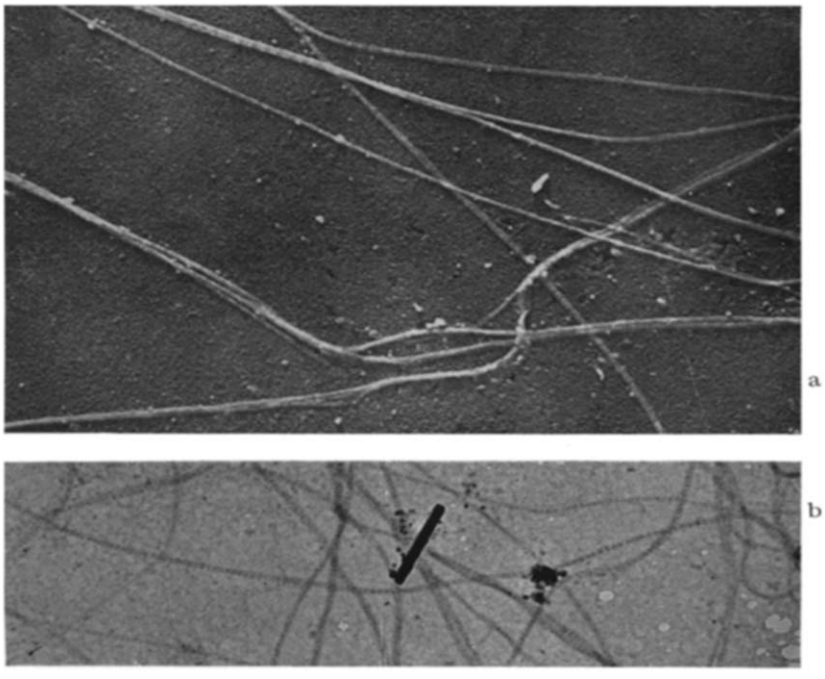

Fig. 5a. Electron micrograph of *Metridium* fibrils. Shadowed with chromium. Mag. 15,700 ×.
Fig. 5b. Electron micrograph of $Ca(OH)_2$ extracted *Metridium* fibrils stained with 1% aqueous PTA. Mag. 28,000 ×.

Fig. 6. Photomicrograph of cross section of *Physalia* float, water-washed. Mallory's aniline blue stain.

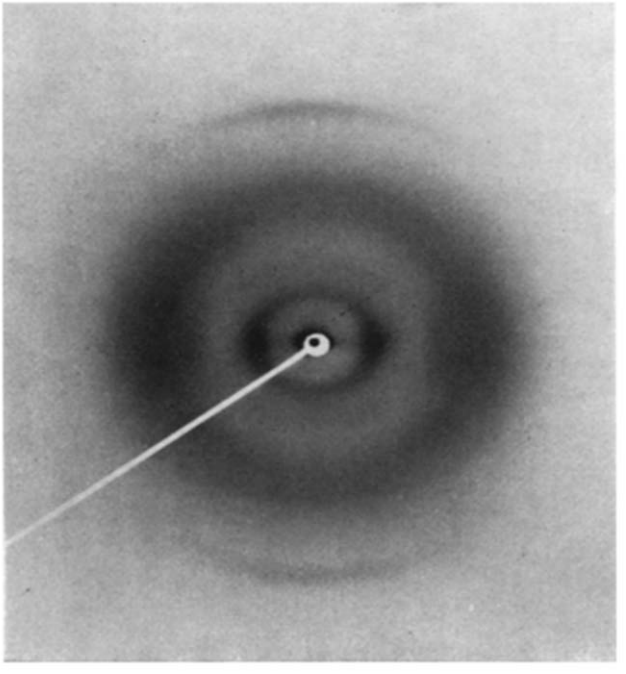

Fig. 7. Wide angle X-ray diffraction pattern of water-washed and dried *Physalia* float.

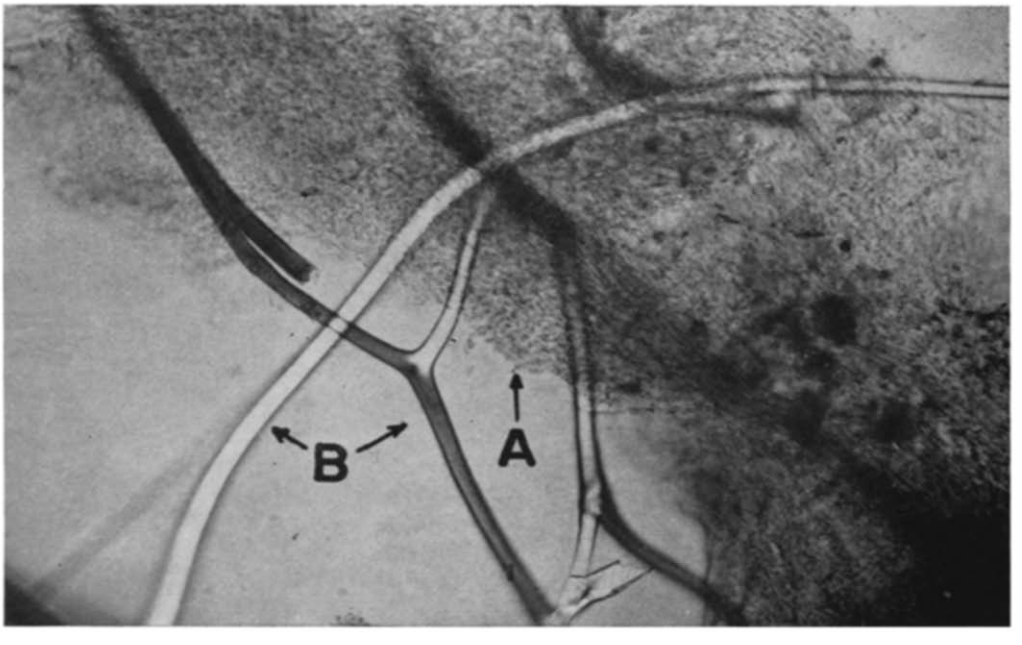

Fig. 8. Photomicrograph of smear of fresh sponge body, *S. graminae*. Polarized light with first order red plate. A, mass of spongin A fibrils; B, branched spongin B fibers.

TABLE I

ANALYTICAL VALUES OF COLLAGENS

	Steer skin	*Elastoidin*	*Thyone*	*Metridium*	*Physalia*	*Spongin A*	*Spongin B*
% ash	0	1.0	0.8	1.1	1.0*	3.0*	8.5
% water	11.0	8.0	10.1	9.5	10.3	9.5	10.7
% N, corr. for ash and water	18.2	18.2	16.1	16.7	16.4	14.0	16.7
% of weight accounted for as amino acids**	97.6	95.3	90.4	87.4	85.0	67.5	78.1
% of N accounted for as amino acids***	100.4	96.7	103.7	98.7	96.3	88.9	87.2
% N, further corr. for weight and N not accounted for as amino acids§	18.7	18.5	15.6	18.9	18.6	18.4	18.6

* Ash was not determined on these samples. These values were taken from similar samples.
** From Table III.
*** From Table IV.
§ % N (corr. for ash and water) × % of N accounted for as amino acids ÷ % of weight accounted for as amino acids.

TABLE II

THE AMINO ACID COMPOSITION OF SOME INVERTEBRATE AND VERTEBRATE COLLAGENS

Residues of amino acid/1000 total residues.

	Steer skin gelatin	*Elastoidin gelatin*	*Thyone gelatin*	*Metridium gelatin*	*Physalia gelatin*	*Spongin A*	*Spongin B*
Glycine	334	319	306	311	307	315	323
Alanine	105	123	113	70	66	56	94
Leucine	25	19	22	37	31	28	24
Isoleucine	11	18	13	23	22	24	17
Valine	19	17	30	34	26	29	24
Proline	131	119	100	63	83	78	73
Hydroxyproline	91	72	66	49	61	108	94
Phenylalanine	13	15	8.9	12	11	0.3	10
Tyrosine	4.7	9.2	7.0	7.9	5.6	4.7	4.0
Serine	38	42	43	54	47	38	24
Threonine	17	23	35	39	33	43	27
Methionine	6.6	13	2.2	8.8	5.8	4.7	3.1
Cystine	0	0.9	2.5	3.2	1.6	3.3	6.0
Hydroxylysine	6.8	7.0	11	25	30	12	24
Lysine	25	24	7.5	27	27	9.0	24
Arginine	48	50	54	57	54	47	43
Histidine	4.6	4.4	2.8	5.1	1.9	3.9	3.2
Aspartic acid	48	45	62	80	83	92	97
Glutamic acid	72	80	110	94	104	95	86
Amide N	(41)	(32)	(75)	(71)	(60)	(102)	(90)
Totals	1000	1000	1000	1000	1000	1000	1000
Glucosamine	0	0	2.4	4.0	2.5	10	1.6
Galactosamine	0	0	0	0	3.2	7.7	0

TABLE III

THE AMINO ACID COMPOSITION OF SOME INVERTEBRATE AND VERTEBRATE COLLAGENS

Weight (residue) of amino acid as % of total weight.

	Steer skin gelatin	*Elastoidin gelatin*	*Thyone gelatin*	*Metridium gelatin*	*Physalia gelatin*	*Spongin A*	*Spongin B*
Glycine	20.3	18.8	16.4	16.0	15.3	12.5	15.0
Alanine	7.91	9.09	7.61	4.52	4.08	2.78	5.41
Leucine	3.02	2.16	2.35	3.77	3.09	2.24	2.20
Isoleucine	1.33	2.12	1.38	2.30	2.17	1.90	1.57
Valine	2.01	1.71	2.83	3.05	2.28	2.02	1.91
Proline	13.6	11.9	9.97	5.50	7.04	5.27	5.78
Hydroxyproline	10.9	8.35	6.37	4.98	6.05	8.45	8.60
Phenylalanine	1.96	2.18	1.23	1.53	1.36	0.96	1.24
Tyrosine	0.83	1.55	1.51	1.15	0.81	0.53	0.56
Serine	3.53	3.67	3.56	4.24	3.57	2.31	1.73
Threonine	1.85	2.44	3.38	3.55	2.94	3.01	2.22
Methionine	0.92	1.80	0.28	1.03	0.66	0.38	0.31
Cystine	0	0.19	0.49	0.62	0.30	0.48	1.08
Hydroxylysine	1.04	1.04	1.48	3.20	3.75	1.21	2.81
Lysine	3.44	3.15	0.91	3.04	3.04	0.80	2.51
Arginine	7.95	8.09	7.97	7.93	7.33	5.13	5.43
Histidine	0.67	0.62	0.36	0.63	0.23	0.37	0.35
Aspartic acid	5.78	5.30	6.68	8.30	8.32	7.40	9.29
Glutamic acid	9.88	10.6	13.5	11.02	11.7	8.56	8.92
Amide N	0.70	0.53	1.13	1.02	0.93	1.13	1.18
Totals	97.6	95.3	89.4	87.4	85.0	67.5	78.1
Glucosamine	0	0	0.38	0.60	0.38	1.14	0.22
Galactosamine	0	0	0	0	0.45	0.87	0

TABLE IV

THE AMINO ACID COMPOSITION OF SOME INVERTEBRATE AND VERTEBRATE COLLAGENS

Weight of N as % of total N.

	Steer skin gelatin	*Elastoidin gelatin*	*Thyone gelatin*	*Metridium gelatin*	*Physalia gelatin*	*Spongin A*	*Spongin B*
Glycine	27.4	25.3	25.0	23.6	22.9	21.9	22.0
Alanine	8.57	9.82	9.30	5.35	4.90	3.92	6.37
Leucine	2.05	1.46	1.81	2.80	2.33	1.98	1.62
Isoleucine	0.91	1.43	1.06	1.71	1.64	1.68	1.16
Valine	1.56	1.33	2.49	2.59	1.97	2.04	1.61
Proline	10.8	9.43	8.90	4.75	6.19	5.43	4.99
Hydroxyproline	7.45	5.66	4.91	3.69	4.59	7.47	6.37
Phenylalanine	1.03	1.15	0.72	0.88	0.79	0.65	0.70
Tyrosine	0.39	0.72	0.66	0.60	0.43	0.33	0.29
Serine	3.12	3.23	3.56	4.08	3.50	2.64	1.67
Threonine	1.40	1.85	2.90	2.95	2.50	3.00	1.83
Methionine	0.54	1.05	0.18	0.66	0.44	0.33	0.21
Cystine	0	0.15	0.42	0.48	0.24	0.46	0.81
Hydroxylysine	1.10	1.09	1.78	3.72	4.44	1.68	3.27
Lysine	4.13	3.78	1.24	3.98	4.04	1.25	3.29
Arginine	15.7	15.8	17.8	17.1	16.1	13.2	11.6
Histidine	1.12	1.05	0.69	1.16	0.44	0.81	0.63
Aspartic acid	3.85	3.54	5.05	6.07	6.19	6.43	6.79
Glutamic acid	5.89	6.29	9.05	7.18	7.76	6.63	5.79
Amide N	3.36	2.53	6.15	5.34	4.95	7.08	6.18
Totals	100.4	96.7	103.7	98.7	96.3	88.9	87.2
Glucosamine	0	0	0.20	0.31	0.20	0.70	0.12
Galactosamine	0	0	0	0	0.24	0.54	0

gelatin and spongin A which are single analyses. The values listed for proline and hydroxyproline are single values obtained by a separate determination on a 50-cm column (see experimental) which is highly reproducible. The less precise figures for these amino acids provided by analysis of the effluent from the 100-cm column by the MOORE AND STEIN ninhydrin reagent[12] agreed within experimental error. The values obtained for hydroxyproline have been increased by 2% to correct for the conversion to allohydroxy-D-proline during hydrolysis[14]. The results given for serine and threonine have been corrected for losses of 5% and 3% as recommended by EASTOE[20]. The amide nitrogen was proportionately decreased. None of the samples were analyzed for tryptophan: it is assumed to be absent.

Fig. 9 shows a typical effluent chromatogram. The fractions were read against a water blank. Cystine, when present, appeared immediately following alanine. It was usually partially or completely oxidized to cysteic acid during the various preparative steps. Cysteic acid, appearing at the solvent front, was calculated as cystine. Similarly, methionine was partially or completely oxidized to the sulfoxide. In the case of spongin A (Fig. 9) the oxidation was complete. When present, methionine appeared between galactosamine and isoleucine. The sulfoxide was calculated as the parent compound.

The lower limit of sensitivity of the methods is approximately 1 residue per 1,000 residues. A value of zero indicates less than this amount and values close to 1 contain a large error.

Some of the samples contained hexosamine as previously reported[6,7]. Glucosamine and galactosamine were present in spongin A and appear in Fig. 1 as well resolved peaks in the effluent from the 100-cm column. Galactosamine moved with

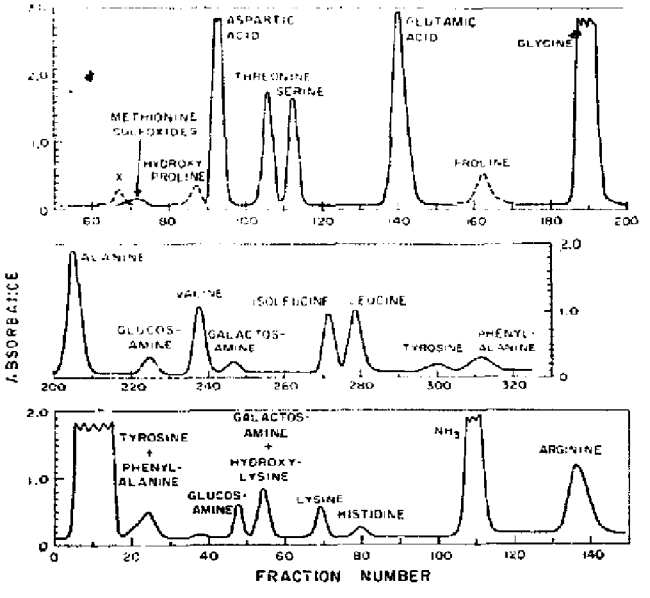

Fig. 9. An effluent chromatogram showing the separation of amino acids in an acid hydrolysate of 3.5 mg of spongin A. The neutral and acidic amino acids were separated on a 100 × 0.9 cm column of Dowex 50 (first part of figure). The basic amino acids were separated on a 30 × 0.9 cm column (second part of figure). The absorbances were read, after development with ninhydrin[8], at 570 mμ (solid line) or 440 mμ (dash line). "X" is unidentified.

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hydroxylysine on the 30-cm column. If it was present, as determined by the 100-cm column, its absorbance was subtracted from the combined peak to yield a value for hydroxylysine. It was not observed in the column eluate of the spongin B hydrolysate nor was it definitely identified previously by paper chromatography[6].

The analytical data for the hexosamines are included in Tables II, III and IV although the hydrolysis conditions were not necessarily optimum for their determination. The peak labeled "X" was found in both spongin A and B. Traces may be present in the other invertebrate collagens. It is unidentified. This material reacts with the ninhydrin reagent of MOORE AND STEIN in a manner similar to hydroxyproline and proline and may, therefore, be related to the cyclic imino acids. However, it is not known whether it is associated with the protein or carbohydrate portion of the samples.

DISCUSSION

Histologically and in the electron microscope it is difficult to distinguish *Thyone* body wall collagen from its mammalian counterpart. Whether or not the thin "beaded" fibrils found associated with the larger typical cross striated fibrils are collagen is yet to be determined.

The connective tissue layers of *Metridium* and *Physalia* stain characteristically for collagen. The fibrils obtained by fragmentation and viewed in the electron microscope resemble superficially those seen in the sponges[6], *Haliclona* and *Microciona*, spongin A from *S. graminae* and the fine collagen fibrils of mammalian vitreous humor [21] and cartilage (GROSS, unpublished). A definite axial repeating period was observed in both the coelenterate collagens which are of the order of 200 Å but there is evidence of a larger macroperiod not adequately resolved of which the smaller repeat may be a subunit. This type of subdivision is well known for collagen fibrils. As discussed elsewhere spongin B is very unusual with regard to the large size and branching of its fibers. Both spongins have the typical collagen axial periodicity[6].

The recovery of the invertebrate collagen samples in terms of weight is not complete (Table III). The difference has previously[6,7] been shown, at least in part, to be a complex mixture of carbohydrates which are firmly bound to the protein. Essentially all of the nitrogen is present as amino acids, except for the two spongins (Table IV). These samples contain unidentified compounds which account for about 10% of the total nitrogen (such as "X" in Fig. 1). Therefore, expressing the results for the spongins as residues of amino acid per 1,000 total residues (Table II) is not completely accurate if it is shown that the unidentified, nitrogen-containing compounds are amino acids and a part of the collagen polypeptide chains. Pending further information it will be assumed that this is not the case.

Steer skin collagen is very similar to other mammalian collagens[3]. The hydroxyproline content is on the low side of the observed range while proline is somewhat higher than the average. The value for tyrosine is higher than reported for some mammalian collagens and may reflect an impurity (see the discussion of elastoidin).

The analysis of elastoidin gelatin can be compared with the values reported by DAMADORAN *et al.*[16]. Since it has previously been shown[22] that elastoidin contains a non-collagenous residue that has a high tyrosine content which is difficult to solubilize by autoclaving, it would be expected that the gelatin, reported here, would differ

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from the whole fiber analyzed by DAMADORAN *et al.*[16]. The tyrosine content of the gelatin is only about one-fourth the value for the unfractionated fiber. All of the other hydroxy amino acids are higher in the gelatin, by about 10 to 40%, while histidine is less than one-half as large. The only large difference between elastoidin gelatin and mammalian gelatins is a lower value for hydroxyproline in the former.

Thyone gelatin shows two large differences relative to mammalian gelatins. Hydroxyproline is lower by about a third, in a manner similar to many nonmammalian vertebrate collagens. Lysine is very much lower than in any collagen thus far known. The value is one-third to one-fifth as large as that found for vertebrate collagens, with the result that the lysine:hydroxylysine ratio is less than one. Arginine is only slightly elevated. Recently KUHN *et al*[23] have claimed that arginine binds phosphotungstic acid most tightly whereas that bound by lysine and hydroxylysine may be washed out. They imply that the intraperiod band pattern as seen in the electron microscope in mammalian collagen fibrils is mostly attributable to arginine. This argument fits well with our observation of a detailed six banded intraperiod pattern in *Thyone* fibrils stained with PTA (to be described elsewhere) in the face of a comparatively low lysine content. The amount of acidic amino acids in the hydrolysate, particularly glutamic acid, is high. In terms of the acidity of the protein, this is partially offset by a larger amount of amide nitrogen. To some extent this feature is common to all the invertebrate collagens reported here. Galactosamine was not found in the column eluates but was definitely identified previously by paper chromatography in a hydrolysate of the same material[22].

The hydroxyproline content of *Metridium* gelatin is very low, one of the lowest yet reported for a collagen. Proline is also decreased approximately in proportion. The largest quantitative difference is hydroxylysine. It is several times as large as is usually seen and is approximately twice the highest reported value for a mammalian collagen, namely that for rat dentin (16 residues/1,000 total residues)[14].

Physalia gelatin is very similar to *Metridium* gelatin as might be expected since they are derived from members of the same phylum. The proline and hydroxyproline contents are not quite as low for *Physalia* as for *Metridium*; the hydroxylysine value is even higher, 30 residues per 1,000.

Spongin A presents a quantitative pattern unlike any of the above. The hydroxyproline content is slightly higher than any collagen known except for the earthworm cuticle[4], which is very much higher. The proline content is somewhat low. The value for lysine is very low, about the same as for *Thyone* gelatin. Spongin B, although from the same animal as spongin A, is quite different. Hydroxyproline and proline values are somewhat lower than in spongin A, with the former about the same as in steer hide and proline considerably lower. Lysine and hydroxylysine are much higher. In the latter respect spongin B is very similar to *Metridium* and *Physalia* gelatins while spongin A, as stated, resembles *Thyone*. Examining the values for alanine, the situation is reversed. Spongin A, like *Metridium* and *Physalia* gelatins, has less alanine than vertebrate collagens while spongin B has nearly as much. Structurally, as seen in the electron microscope, spongin A and the two coelenterate collagens are very similar; they are all thin, non-branching fibrils, while spongin B is a relatively huge branched fiber[6].

Thus far, the differences among collagens have been stressed, but it is as important to look for similarities. The qualitative ones are readily apparent and allow the

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chemical classification of these diverse proteins as collagen. Quantitatively, only one amino acid, glycine, occurs with nearly constant frequency. For the collagens examined in this study the range is 306 to 334 residues per 1,000. It is possible that agreement would be even closer if purer samples were available. Thus, the requirement of the collagen model proposed by RICH AND CRICK[24] that every third residue be glycine is apparently fulfilled by invertebrate collagens. WATSON[4] has shown that this is also true for the earthworm cuticle. On the basis of these data it is likely that the glycine content of a collagen is the best chemical measure of purity. The high glycine content is reflected in the corrected nitrogen values which are remarkably constant, ranging from 18.4 to 18.9 %. The arginine content of the various collagens is also relatively constant. However, the differences appear to be real from the present evidence.

The glycine analyses previously performed by a colorimetric method[7] agree very closely with the chromatographic values presented here. There is a less close agreement between the colorimetric and chromatographic values for proline and hydroxyproline. It is likely that the chromatographic values are more reliable, particularly in view of the fact that the invertebrate collagens contain non-protein material which is incompletely defined.

It can be concluded that there are no striking features in the amino acid composition of invertebrate collagens which distinguish them from vertebrate collagens, unless it be a high degree of variability. There are several less obvious differences between the two groups which appear to be consistent. For example, the invertebrate collagens have a smaller proportion of imino acids (particularly proline), a larger content of hydroxyl groups (owing largely to threonine and serine, except for spongin B), uniformly higher amounts of hydroxylysine, a greater proportion of dicarboxylic acids (but not of acidic groups), and fewer nonpolar amino acids.

It can be further concluded that there is no obvious relationship between amino acid composition and evolutionary level.

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AMINO ACID ANALOG INCORPORATION INTO BACTERIAL PROTEINS

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SUMMARY

The amino acid analogs norleucine and para-fluorophenylalanine are shown to be incorporated into the proteins of *E. coli*. Analysis of proteins by an ion-exchange column showed that the proteins formed in the presence of the analogs are not radically different molecular species but are physicochemically similar to the proteins normally synthesized. The substitution of norleucine for methionine in the bacterial proteins was shown to occur in the same proportions in all of the "protein classes" resolved by the ion-exchange column.

INTRODUCTION

Considerable quantities of certain amino acid analogs may be incorporated into the proteins of *Escherichia coli*[1-6]. The analogs substitute for corresponding naturally occurring amino acids and cause various biological effects. In general, cellular growth becomes linear with time and specific enzymic activities may be lost, depressed, or remain unaffected. Such effects depend upon the degree and kind of substitution produced. Since the degree and kind of substitution can be controlled, the use of analogs provides a method for the quantitative examination of the relationship between altered molecular structure and enzymic activity. Evidence can also be adduced concerning susceptibility of bacterial protein types to analog substitution.

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