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Biochem. J. (1961) **79**, 648

The Composition of Collagen from Subcellular Fractions of Guinea-Pig Granuloma Tissue

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Investigations of the amino acid composition of vertebrate collagens have been largely confined to preparations directly derived from whole tissues of high collagen content (Eastoe & Leach, 1958). Most studies have been made on the insoluble fibrous protein remaining after treatment of the tissue with solvents which could remove other tissue components with minimum degradation of the collagen (Bowes & Kenten, 1948). Such preparations would be expected to consist largely of extracellular collagen fibres with characteristic 640 Å striations. Recently analyses have been made of collagen fractions extracted from tissues by neutral sodium chloride and slightly acid sodium citrate solutions (Bowes, Elliott & Moss, 1955; Jackson, Leach & Jacobs, 1958). These soluble collagens represent only a small percentage of the total collagen, and much evidence, based on the incorporation of radioactive amino acids, has accumulated which shows that the neutral-salt-soluble fraction is synthesized before the insoluble fibres, which are probably formed by aggregation of the smaller particles in the soluble fraction (Harkness, Marko, Muir & Neuberger, 1954; Jackson, 1957; Green & Lowther, 1959).

The object of the work described here was to determine the composition of the protein material extracted by sodium chloride solution from three

subcellular fractions of rapidly developing mesenchymal tissue from guinea-pig granuloma. It forms part of a more extensive investigation involving radioactive tracer studies of proline hydroxylation in the subcellular fractions and their examination by electron microscopy (Lowther, Green & Chapman, 1961; Chapman, 1961). Since the protein fractions had already been shown to contain hydroxyproline and to have solubility properties similar to neutral-salt-soluble collagen extracted from whole tissues, confirmation of the characteristic overall amino acid pattern would be important evidence of the intracellular synthesis of the collagen molecule. The very small quantities of protein, obtainable from a large bulk of granuloma tissue, required the adoption of a modified semi-micro method of amino acid analysis, based on resin chromatography, which is described in an Appendix to this paper.

MATERIAL AND METHODS

Subcellular collagen fractions. The collagen fractions were isolated and purified by Dr N. M. Green and Dr D. A. Lowther (see also Lowther *et al.* 1961) by the following methods. Granulomata, produced by subcutaneous injection of carrageenin solution into guinea pigs, were removed 7 days later, immediately after killing the animals. The tissue was chopped finely with two 6 in. razor blades and serum proteins were largely removed by centrifuging a

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suspension in 2.5 vol. of 0.25M-sucrose at 1000g for 30 sec. The sediment was homogenized in more sucrose soln. at 0° in a glass tube fitted with a Teflon pestle driven at 1400 rev./min. via a flexible shaft.

Fractions of different particle size were separated by differential centrifuging (Schneider & Hogeboom, 1950) with the following sedimentation conditions: (a) nuclear fraction, 10 min. at 1000g; (b) mitochondria, 10 min. at 10 000g; (c) microsomes, 35 min. at 105 000g. For each fraction the sediment was resuspended in sucrose soln. and again centrifuged. The washings were added to the bulk of the supernatant fluid.

Collagen in the three sedimented fractions from differential centrifuging was extracted by suspending them overnight at 4° in 0.2M-NaCl-6.7 mM-ethylenediaminetetraacetic acid (sodium salt), pH 7.4. Each suspension was centrifuged at 105 000g for 30 min., to remove insoluble matter, and the NaCl concn. of the supernatant solution was raised to 20% (w/v) to precipitate the collagen. The precipitate was centrifuged and the extraction and precipitation were repeated. Finally the precipitate was dissolved in 0.05M-acetic acid and dialysed overnight against 0.05M-acetic acid.

Analytical methods

Total N. This was determined by the micro-Kjeldahl method.

Hydrolysis. This was carried out under N₂ in sealed tubes with 6N-HCl at 100° for 48 hr.

Protein determination. Approximate values for protein were obtained by the method of Lowry, Rosebrough, Farr & Randall (1951) with gelatin for calibration.

Amino acid analysis. This was carried out by using a semi-micro modification (Eastoe, 1960) of the method of Moore & Stein (1951) as described in the Appendix.

RESULTS

From 200 g. of granuloma, approximately 0.7 mg. of microsomal collagen was obtained, of which 0.55 mg. was available for amino acid analysis. After a preliminary determination of protein by the method of Lowry *et al.* (1951), the hydrolysate was divided between four hydroxyproline determinations and a single determination of the other amino acids. The larger quantities of the nuclear and mitochondrial fractions (Table 1) permitted, in addition, the determination of total nitrogen.

The values for the amino acid contents of the subcellular collagen fractions are given in Table 1. Since the total nitrogen content of the microsomal fraction was unknown, all results were calculated as numbers of amino acid residues per thousand total residues recovered. This basis for expressing analytical values facilitates comparison between protein samples with different proportions of non-protein impurities and is valid provided that recovery of amino acids is substantially complete (Eastoe & Leach, 1958). The recoveries of total nitrogen, 104.6 and 103.4% for nuclear and mitochondrial collagen respectively, were high. This tendency has been noticed previously and its

possible causes were discussed (Eastoe, 1955). The present recoveries are greater than those found previously but suggest complete recovery of total nitrogen within the limits of cumulative experimental error to be expected from the total nitrogen and amino acid determinations (Eastoe, 1960).

The appearance of the elution curves for all the collagen fractions was similar to that for microsomal collagen (see Appendix, Figs. 2 and 3). Good separations were obtained for all peaks except the pairs hydroxyproline-aspartic acid and tyrosine-phenylalanine. For this reason, hydroxyproline was determined separately. The presence of its yellow peak of low extinction was considered unlikely to cause substantial errors for aspartic acid. Unfortunately the hydroxylysine and histidine peaks from the column (17 cm. × 0.4 cm.) were not resolved when the mitochondrial hydrolysates was examined and none remained for further work. With the microsomal fraction, this difficulty was overcome by modifying the technique (see Appendix). Small amounts of ninhydrin colour were noticed near the hold-up volume for the 100 cm. column and may have been attributable to cysteic acid corresponding to traces of cystine in the protein. Tryptophan, which is considered to be absent from collagen (Bowes & Kenten, 1947), was not determined.

The quantities of ammonia, recovered from all three hydrolysates of subcellular fractions, greatly exceeded the amide nitrogen content of collagens from whole tissue. This was probably due to the presence of ammonium salts as impurities in the reagents used for preparation of the fractions.

DISCUSSION

The neutral-salt-soluble portions of the nuclear, mitochondrial and microsomal fractions, obtained by differential centrifuging, showed all the typical features of the amino acid pattern of vertebrate collagens. Thus all three contained as much or almost as much hydroxyproline as did the collagen and gelatin samples previously analysed (Table 1). Hydroxylysine was also present in the nuclear and microsomal material, and, although it was not satisfactorily demonstrated in the mitochondrial fraction, the size of the combined hydroxylysine-histidine peak indicated that both could be present in the same proportions as in insoluble collagen. Failure to resolve this pair, when using the same technique as for the mitochondrial fraction, has been subsequently confirmed for a gelatin hydrolysate, known to contain both amino acids. All the subcellular fractions contained large amounts of glycine, alanine and proline and small amounts of amino acids with hydrocarbon or aromatic side chains, both features typical of collagen.

Table 1. *Composition of neutral-salt-soluble collagens from subcellular fractions of guinea-pig granuloma and from whole rabbit skin, insoluble collagen from ox bone, and gelatin from ox skin*

Values are given as numbers of amino acid residues per 1000 total residues. For abbreviations of amino acids see *Biochem. J.* (1957), **66**, 6.

	Neutral-salt-soluble collagen				Rabbit skin (Jackson, Leach & Jacobs, 1958)	Insoluble collagen from ox bone	Gelatin from ox skin
	Guinea-pig granuloma (present study)						
	Nuclear	Mito- chondrial	Microsomal				
				(Eastoe, 1955)			
Ala	100.7	101.8	93.4	100.8	109.7	112.0	
Gly	324	300	263	296	314	333	
Val	20.5	24.1	29.1	26.9	21.2	20.1	
Leu	25.5	29.0	41.6	29.7	27.9	23.1	
Ileu	11.8	13.4	22.4	17.0	12.3	12.0	
Pro	115.6	109.8	101.5	111.5	118.8	129.0	
Phe	12.4	14.8	18.5	15.1	16.3	12.3	
Tyr	3.2	4.8	11.3	5.6	2.9	1.5	
Ser*	44.1	48.7	51.8	44.5	37.8	36.5	
Thr*	20.2	24.8	31.5	27.4	19.7	16.9	
Cy ₂ S ₂	Trace	Trace	Trace	Trace	Trace	Trace	
Met†	8.3	6.9	6.0	6.5	5.1	5.5	
Arg	51.6	55.8	59.0	47.6	49.0	46.2	
His	6.2	—	9.9	7.2	5.8	4.5	
Lys	25.8	28.3	36.1	31.9	26.2	27.8	
Asp	48.3	54.0	61.6	54.8	49.8	46.0	
Glu	68.2	71.7	76.0	75.7	75.8	70.7	
Hyp ^{ro}	104.4	100.0	79.6	97.1	100.8	97.6	
Hyl ^{ys}	8.8	‡	7.6	4.8	6.4	5.5	
Hydrolysate NH ₃ §	101	190	114	50.8	41.8	47.5	
Kjeldahl N (mg.)	0.42	0.215	—	—	—	—	
Total N recovered (mg.)	0.439	0.222	0.108	—	—	—	
Recovery of N (%)	104.6	103.4	—	105.2	101.4	100.9	
Total protein (Lowry <i>et al.</i> 1951) (mg.)	1.9	1.2	0.78	—	—	—	
Total protein from N value (mg.)¶	2.27	1.08	0.55	—	—	—	
Molar ratios	Gly/Hyp ^{ro}	3.10	3.00	3.30	3.05	3.12	3.41
	Ala/Hyp ^{ro}	0.97	1.02	1.17	1.04	1.09	1.15
	Pro/Hyp ^{ro}	1.11	1.10	1.28	1.15	1.18	1.32

* Corrected for decomposition during hydrolysis.

† Sum of methionine and methionine sulphoxide peaks.

‡ Sum of histidine and hydroxylysine 12.4 residues/10³.

§ Corrected for NH₃ formed by decomposition of serine and threonine.

|| Sum of amino acid and ammonia N.

¶ Calculated from N recovered as amino acids plus amide N (assumed value, 41.8/10³) assuming a total N content of 18.5%.

Small variations in the content of individual amino acids of vertebrate collagens (Eastoe & Leach, 1958) render difficult the selection of a standard set of values for comparison with the present series. Table 1 includes values for insoluble bone collagen from which certain impurities had been removed by extractive processes (Eastoe, 1955), collagen extracted from rabbit skin by neutral-salt solution (Jackson *et al.* 1958) and an alkali-processed gelatin. It has been suggested (Eastoe, 1955) that, with certain reservations concerning the arginine and amide contents, such gelatins may closely represent pure collagen in composition, since soluble and insoluble impurities have been removed in the

pretreatment and extraction stages of manufacture respectively.

Nuclear granuloma collagen closely resembles both insoluble collagen and gelatin in composition, many of its amino acid values lying between those of these two proteins (Table 1) and differences seldom exceeding the probable experimental errors (Eastoe, 1960). The mitochondrial fraction is similar in composition to the nuclear material but contains 4–7 % less of the typical collagen amino acids glycine, hydroxyproline and proline and correspondingly more of the less abundant residues. The microsomal fraction shows these departures to a greater degree, there being 15–20 % less glycine, proline, hydroxyproline and hydroxylysine. This is

consistent with the presence of 15–20 % of protein impurities in the microsomal fraction since the molar ratios of glycine, alanine and proline to hydroxyproline are within the range of other proteins in Table 1, whereas the amounts of some amino acids present in small proportions in collagen, e.g. tyrosine, leucine and isoleucine are significantly increased in the microsomal fraction. The neutral-salt-soluble fraction from whole rabbit skin is closely similar in composition to the present mitochondrial fraction extracted by that solution. Lowther *et al.* (1961) have shown that the microsomal fraction accounted for 7 % of the total nitrogen but only 0.3 % of the hydroxyproline of the granuloma, which indicates that this fraction contained only a small proportion of collagen and considerable amounts of other proteins. The contamination of the material extracted from the microsomal fraction with non-collagen material is likely to be due to the small proportion of collagen present in it.

Electron microscopy of thin sections of the entire fractions separated by centrifuging before extraction with salt solution has shown that considerable damage occurs to both nuclei and mitochondria owing to the severe treatment necessary to homogenize the tissue (Lowther *et al.* 1961). Large numbers of typical striated collagen fibres were present in the nuclear fraction and very few in the mitochondrial fraction. Both these fractions also contained large amounts of fine non-striated fibrils. The microsomal fraction was more homogeneous than the heavier fractions and consisted mainly of rough- and smooth-surfaced vesicles but it was apparently entirely free from both types of fibril.

This suggests that the neutral-salt-soluble collagen extracted from the microsomes may not occur as fibrils. Lowther *et al.* (1961) have shown that 6.5, 33 and 80 % of the hydroxyproline of the nuclear, mitochondrial and microsomal fractions respectively are soluble in the salt solution. Thus the protein analysed accounted for all of the microsomal collagen, except for a small amount not precipitated with 20 % sodium chloride solution and which was possibly water-soluble (Green & Lowther, 1959). Lowther *et al.* (1961) were able to co-precipitate neutral-salt-soluble collagens from nuclear and microsomal fractions, which indicates that the collagen of the microsomes is similar to and possibly identical with the neutral-salt-soluble collagen of the whole granuloma. If the collagen of the microsomes is non-fibrillar it would seem likely that this applies to the whole of the neutral-salt-soluble collagen of the cell.

Several workers have emphasized the importance of the neutral-salt-soluble fraction as representing an early stage in the formation of insoluble col-

lagen. The present investigation shows that the composition of this soluble collagen from three subcellular fractions is closely similar to that of insoluble collagen. Thus a protein having the characteristic composition of extracellular collagen fibres is found within the cell, being probably synthesized, like other proteins, by the microsomes.

SUMMARY

1. The amino acid composition of subcellular fractions of collagen, obtained by differential centrifuging of homogenized guinea-pig granuloma and purified by extraction with 0.2M-sodium chloride solution, has been determined by using a semi-micro modification of the ion-exchange chromatographic method of Moore & Stein.

2. The collagens from the nuclear and mitochondrial fractions were closely similar in composition to both insoluble collagen from ox bone and neutral-salt-soluble collagen from rabbit skin. These fractions had been shown by electron microscopy to be heterogeneous and to contain both striated and plain fibres. Collagen from the relatively homogeneous microsomal fraction had the same overall amino acid pattern as insoluble collagen but with quantitative differences, suggesting the presence of 15–20 % of protein impurities.

3. It is suggested that the non-fibrillar collagen, synthesized in the microsomes and which is almost completely soluble in 0.2M-sodium chloride solution, is similar to and possibly identical with the fibrils of insoluble collagen as regards amino acid composition.

I am grateful to Dr N. M. Green and Dr D. A. Lowther of the Department of Chemical Pathology, St Mary's Hospital, Paddington, for preparing the purified collagen fractions and for the opportunity to collaborate in this investigation. I should also like to thank Sir Wilfred Fish and Professor A. Neuberger, F.R.S., for their kind interest.

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APPENDIX

A Semi-Micro Method for the Determination of Amino Acids by Ion-Exchange Chromatography

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The method of protein analysis, introduced by Moore & Stein (1951) and based on separation of amino acids on ion-exchange columns eluted with buffer solutions of accurately controlled pH, has become widely adopted. Collaborative tests have shown that, provided sufficient care is taken with all details of technique, the accuracy of $\pm 3\%$, originally claimed, can be attained for most amino acids (Bender, Palgrave & Doell, 1959). In some fields of investigation, e.g. purified enzymes and microbial toxins, enamel matrix (Eastoe, 1960*a*) and subcellular protein fractions, it is difficult or tedious to obtain even the 2–4 mg. of protein required for analysis by the standard method. A semi-micro method suitable for 0.3–0.6 mg. samples of protein was developed and has been described in a preliminary communication (Eastoe, 1960*b*). It is based on the original method of Moore & Stein (1951) with the minor changes suggested by Eastoe (1955). The modifications necessary for the semi-micro method, together with an indication of the accuracy and resolution obtainable, are described briefly below. It is not considered that this modification represents the ultimate in small-scale protein analysis, but is an exploratory step in that direction.

MATERIALS AND APPARATUS

Resin, buffers etc. The same batch of Dowex 50 resin (200–400 mesh; nominal 12% cross-linking) was used as previously (Eastoe, 1955). Citrate and phosphate buffers, thymol, BRIJ 35, thiodiglycol, ninhydrin and ethylene glycol monomethyl ether were prepared or purified as before. Industrial spirit (74 over proof) has been found to be a satisfactory substitute for absolute ethanol in the preparation of the diluent solution. The semi-micro method is considerably more economical in ninhydrin and buffer

solutions than the original method and it is therefore unnecessary to store large volumes of solutions.

Columns. The borosilicate glass tubes for the 15 and 100 cm. resin columns were 28 and 112 cm. in overall length respectively. They were selected for straightness and for an internal diameter of 4.0 ± 0.1 mm. (external diam. approx. 6.8 mm.). Each tube had a fused-in sintered-glass disk (porosity 1 or 2), 2.0 cm. from its lower end, which was cut off at 45° and its tip fused to a small blob of glass. Care was necessary to ensure that the disk was fused to the wall all round but not fused solid itself. The portion of tube below the sinter did not normally remain filled with solutions which contained detergent. The tubes were fitted by means of rubber bungs into side-armed glass jackets of overall length 18 and 102 cm. respectively. The columns were filled with resin as described by Moore & Stein (1951) with additional precautions to prevent trapping of air bubbles. The tubes were freed from grease with nitric acid–chromic acid and well rinsed with water and the walls left wet. The resin suspension was poured into the accurately vertical tubes very carefully to avoid air being trapped. When this happened, the bubbles were immediately shifted by prodding with a piece of 20-gauge copper wire.

A small column, for preliminary separation of basic amino acids, had an overall length of 8 cm. and an internal diameter of 4.0 mm. It was cut off straight and a sintered disk (porosity 1) was sealed in flush with the bottom of the tube. The top had a B7 socket with hooks. The tube was packed with resin, the total height of the resin being 2.2 ± 0.1 cm.

Fraction collector. A photoelectric drop-counting fraction collector (Locarte Co., 24 Emperors Gate, London, S.W. 7), capable of collecting 160 fractions in 6 in. $\times$ $\frac{1}{2}$ in. test tubes, was used. Drops formed at the square-cut, lower end (external diam. 3.2 mm.) of a small glass funnel, attached by a Terry clip to a rigid brass plate, which itself was attached by similar clips to the two rods supporting the photocell. The position of the bottom of the funnel relative to the photocell was adjusted in two directions by sliding the clips, until the growth of the small drops reduced the