Biochem. J. (1968) **106**, 749
Printed in Great Britain

The Purification and Amino Acid Composition of Human Uterus Collagens, Rheumatoid-Arthritis-Nodule Collagen and Ox Tendon Collagen

By J. J. HARDING* and J. M. WESLEY
Gelatine and Glue Research Association, Birmingham, 12

(*Received* 9 *August* 1967)

Collagens and gelatins were isolated from human post-menopausal uterus, puerperal (post-partum) uterus, rheumatoid-arthritis-nodule and ox tendon. Different means of purifying collagen were studied and a method was devised that enables highly purified collagen to be obtained, even from the uterus. This method involves the use of a number of aqueous and organic extractants as well as digestion with elastase to eliminate elastin. The purity of the collagen preparations was assessed and they were used to study the amino acid composition of collagen. The amino acid compositions of all the collagens studied were similar to those of human bone and tendon collagen, but certain small differences were noted and are discussed. The soluble collagen extracted from some of the tissues was also studied.

The present work is a continuation of our studies on the amino acid compositions of human insoluble collagens (Harding, 1963). Soluble collagen is fairly easily purified by dissolution and reprecipitation, but insoluble collagen presents a much more difficult problem. The other components present vary considerably from tissue to tissue, but plasma-type proteins, mucoproteins and elastin are always present to a greater or smaller extent. In addition, there may be muscle proteins (e.g. in uterus), inorganic material (e.g. in bone), lipids and other non-collagenous matter. The method for isolation of the collagen therefore depends on the tissue taken. Collagen, like most proteins, is easily damaged by extremes of pH, elevated temperatures and certain non-aqueous solvents, and consequently such conditions must be avoided during the preparation of pure collagen. This severely limits the purification procedures that can be employed. Several techniques for the purification of insoluble collagens have been studied including (i) extraction of impurities into aqueous media, (ii) gelatinization and (iii) digestion with elastase. Complete purification could not be achieved by the first method only. Subsequent gelatinization eliminated non-collagenous impurities but involved structural changes, so that the resultant gelatin, though suitable for amino acid analysis, was of little value for structural studies. Purification with crude elastase has been reported, but no details of its efficiency or its effect on collagen were given (Klevens, 1963). The present purpose was to free the collagen from elastin. This has always been a most difficult problem as both collagen and elastin are insoluble fibrous proteins; it can be achieved only by the use of a proteolytic enzyme that attacks elastin. The obvious choice was elastase itself. It was, however, necessary to determine whether elastase attacks collagen, as this could invalidate its use in collagen purification.

Differences in the compositions of collagens from different tissues of one species have been established, e.g. the high hydroxylysine and low lysine contents of tendon collagen compared with skin and bone (Eastoe, 1955; Leach, 1957; Piez & Likins, 1960). However, collagens with greater metabolic activity, as in uterus, and those from rheumatic tissues have not yet been analysed completely for amino acids. Consden, Glynn & Stanier (1953) found no significant differences in the hydroxyproline and tyrosine contents of collagen extracted as a gelatin from rheumatic-fever nodules by autoclaving. As uterine collagen exhibits markedly different behaviour at different stages it was decided to study post-menopausal and puerperal uteri. After the menopause, the uterus decreases in weight and, although the collagen present never again undergoes the rapid changes induced by pregnancy, it still differs from non-uterine collagen in certain physical properties (Brown, Consden & Glynn, 1958). The wet weight of the uterus increases about 11-fold during pregnancy and decreases again during the puerperium, the period of time after parturition. The different collagens were studied in respect of

* Present address: Nuffield Laboratory of Ophthalmology, Oxford.

their amino acid composition and their extractability in several salt and buffer solutions.

METHODS

Preparation of collagens

Tissues were extracted with approx. 20 times their wet weight of each solution at 4° unless otherwise stated.

Post-menopausal uterus. The material U_1 described previously (Harding, 1963) was purified further for the present work. Preparation U_1 was a partially purified collagen from the uterus of a 59-year-old woman. Preparation U_1 (0·71g.) was soaked for 18hr. in 50ml. of 0·1M-$NaHCO_3$ buffer, pH8·8. The suspension was centrifuged and the supernatant liquid replaced by a further 50ml. of the same buffer. The supernatant gave no precipitate with tannic acid and no colour with ninhydrin and was therefore rejected. A 1mg. sample of elastase (Koch–Light Laboratories Ltd., Colnbrook, Bucks.) suspended in 5ml. of water was added to the suspension of preparation U_1 in a conical flask. The neck of the flask was then attached to the shaft of a slow motor (17rev./min.) so that it rotated about its own axis, which was inclined at about 60° to the vertical. This rotation caused a gentle movement of the fibres through the digest solution. Digestion was carried out at 24–25° and followed by ninhydrin determinations on 0·1ml. portions of the digest solution. After 28hr. the extinction at 570mμ after reaction of samples with ninhydrin had reached a constant value, so the supernatant was removed and replaced by a further 50ml. of the $NaHCO_3$ buffer, pH8·8, plus 5ml. of the elastase suspension (containing 1mg. of elastase). After a further 9hr. the ninhydrin colour had again levelled off and, as the increase of colour in this second stage of digestion was very much less than that in the first stage, digestion was assumed to be complete. The suspension was centrifuged and the supernatant added to the other digest solution. The fibres were washed four times with 50ml. lots of the $NaHCO_3$ buffer, pH8·8, to remove the elastase and protein-breakdown products. They were then left in saturated KCl for 60hr., both to inhibit and to remove any remaining elastase. The fibres were then washed free of chloride, washed with ethanol for 40min. (three changes) and then twice with ether before being spread out in a glass dish and left for 2 days to equilibrate with atmospheric moisture. This yielded 0·53g. of material referred to as preparation U_4.

Puerperal uterus. The uterus from a 29-year-old woman, who died 8hr. after giving birth to a child, was removed at post-mortem. The endometrium and the elastin-rich outer layers were scraped and cut away by Dr Glynn. The middle layer (myometrium) was frozen when received and had a wet weight of 297g. A sample of myometrium (40g. wet wt.) was extracted with 0·1M-KCl for 48hr. (one change) and 1·25M-KCl in 3% (w/v) Na_2HPO_4 (two changes) at pH8·4 for 4 days. After the residue had been washed free of chloride and phosphate ions (washings tested with $AgNO_3$ soln.) it was extracted with ether–ethanol (3:1, v/v) for 3hr., washed with deionized water and soaked in 300ml. of 0·1M-$NaHCO_3$ (adjusted to pH8·7) for 18hr. Elastase digestion was then carried out as described above except that a total of 10mg. of elastase, in two lots, was used. The subsequent washings and drying down were also performed as for preparation U_4 and yielded 2·2g. of material termed preparation P_1. Each aqueous extract was analysed for hydroxyproline, hexosamine and hexose content. The total nitrogen content of the elastase digest was also determined.

A sample of preparation P_1 (0·3g.) was extracted with three lots of 40ml. of citrate buffer, pH3·7 (containing 0·1M-citric acid and 0·05M-sodium citrate), for 24hr. each. The hydroxyproline content of the pooled extract was determined.

A further 22g. (wet wt.) of the myometrium was extracted with two lots of 500ml. of 0·9M-NaCl for 1 day each. The extract was dialysed against running tap water for 3 days. The fibrous precipitate formed was collected by centrifuging and washed with water, before being re-extracted with 80ml. of 0·9M-NaCl for 18hr. The residue was removed after centrifuging and the supernatant dialysed for 24hr. against distilled water. The precipitate was collected, redissolved in 0·9M-NaCl and reprecipitated by dialysis. This precipitate was washed with water, ethanol and ether, and then left to equilibrate with atmospheric moisture. This procedure was carried out to isolate any salt-soluble collagen present. An amino acid analysis was carried out on the isolated salt-soluble material.

Another sample of the myometrium (44·6g. wet wt.) was washed in 500ml. of distilled water for 3·5hr. at 25°. The hydroxyproline content of this extract was determined before and after dialysis.

Rheumatoid-arthritis nodule. A rheumatoid-arthritis nodule from a woman's elbow joint was supplied by Dr Consden and Dr Glynn (Canadian Red Cross Memorial Hospital, Taplow, Bucks.), who had cut away as much normal collagen as possible. The initial wet weight was 4·6g. The purification procedure employed for preparation P_1 was used, but the collagen obtained (0·54g.) was physically unlike the collagens described in the preceding sections. It was not so fibrous and did not cling together in long strands, and when wet had the appearance of aggregated rice particles.

The aqueous extracts were analysed for hydroxyproline, hexose, hexosamine and total nitrogen content (elastase digest only). There was not sufficient collagen for the determination of moisture and ash contents.

Ox tendon. Deep flexor tendons were cut from the hind feet of 4-year-old oxen and supplied by Mr Phillips of J. L. Henson Ltd., London, N. 7. Removal was carried out within a few minutes of the death of the animal. Extraneous material was removed, and the whole inner tendon was separated from the shank of the outer tendon. Each tendon was washed in running water, then left in 0·9M-NaCl for 2·5 days, cut into small pieces, frozen to −20° and macerated in a food disintegrator while frozen. After being thawed, samples were taken for moisture, ash, hexosamine and hexose determinations. Samples from the inner tendon and from the shank of the outer tendon (50g. wet wt. each) were subjected to the purification procedure described above for preparation P_1. This yielded 6·1g. of preparation T_1 and 8·0g. of preparation T_2 from the inner and outer tendons respectively. These were analysed for moisture, ash, hydroxyproline, hexose and hexosamine content. All the aqueous extracts were analysed for hydroxyproline, hexose and hexosamine.

Samples (0·3g. of preparations T_1 and T_2) were extracted with three 40ml. lots of the citrate buffer, pH3·7, as

described for preparation P_1. Hydroxyproline was determined in the supernatants.

Analytical methods

Moisture and ash contents were determined by methods already described (Harding, 1963). Hydrolysis and amino acid analysis were also as described by Harding (1963) except for the rheumatoid-arthritis-nodule collagen and the salt-soluble material from the puerperal uterus; these preparations (32mg. in each case) were hydrolysed in 10ml. of 6N-HCl at 100° for 24hr. The hydrolysate was made up to 25ml. with deionized water and 2ml. portions of this solution were evaporated *in vacuo* over P_2O_5 and NaOH; one-tenth of one of the dried portions was used for analysis with an automatic amino acid analyser (Technicon Instruments Co. Ltd., Chertsey, Surrey). This method, which was based largely on that of Piez & Morris (1960), employed the peristaltic pump and other modules of the automatic analysis equipment described by Skeggs (1957), and was also used for desmosine and isodesmosine determinations. Calculation of the results was by direct comparison of the sample chromatogram with that of a synthetic mixture of amino acids separated under the same conditions and within a few days of the sample run. Desmosine and isodesmosine were determined by comparison with samples of these two amino acids kindly supplied by Dr S. M. Partridge (Low Temperature Research Station, Cambridge).

Determination of total nitrogen. Total nitrogen was determined by the micro-Kjeldahl method of Chibnall, Rees & Williams (1943) as modified by Eastoe & Eastoe (1954).

Determination of hexose. To 1ml. of sample solution was added 1ml. of orcinol soln. (1·6g. of orcinol in 100ml. of water) and 7·5ml. of 60% (v/v) H_2SO_4. After heating at 80° for 1hr. the mixture was cooled and its extinction was read at 505mμ in 2cm. cells. This was a slight modification of the method used by François, Marshall & Neuberger (1962). Solutions containing equal amounts of glucose and galactose (0·03–0·7mM with respect to each) were used as standards.

Determination of hexosamine. To 1ml. of sample was added 1ml. of freshly-prepared acetylacetone reagent (1ml. of redistilled acetylacetone in 50ml. of 0·25M-Na_2CO_3). The mixture was heated at 100° for 15min. in stoppered tubes. After cooling, 5ml. of 95% (v/v) ethanol was added and mixed, followed by 1ml. of Ehrlich's reagent [0·8g. of *p*-dimethylaminobenzaldehyde in 60ml. of methanol–conc. HCl (1:1, v/v)]. After being shaken the mixture was allowed to stand for 30min. before the extinction was read at 530mμ in 2cm. cells (Winzler, 1955). Glucosamine was used as a standard (0·03–0·3mM solns.).

Collagen (0·1g.) was hydrolysed, before analysis, with 2ml. of 3N-HCl for 4hr. at 100° in a sealed tube. The hydrolysate was neutralized with 2ml. of 3N-NaOH and made up to 10ml. with water; 1ml. samples were analysed. For analysis of extracts 5ml. of 6N-HCl was added to 5ml.

Table 1. *Amino acid compositions and estimated elastin contents of human uterus, rheumatoid-arthritis-nodule and ox tendon collagens*

The amino acid compositions are expressed as residues/1000 residues. Results for preparations U_1 and U_2 were taken from Harding (1963).

	Post-menopausal uterus			Puerperal uterus	Rheumatoid nodule	Ox tendon	
	U_1	U_2	U_4	P_1		T_1	T_2
Ala	103·2	95·9	106·3	97·2	107·0	111·6	106·6
Gly	301·8	337·1	327·5	326·5	326·8	334·0	337·3
Val	36·5	21·6	19·9	21·0	25·7	19·4	18·9
Leu	37·5	24·5	29·1	29·2	27·2	25·5	26·7
Ile	17·4	10·8	12·7	13·8	11·9	10·7	11·8
Pro	109·6	108·2	111·8	111·1	126·5	128·0	127·2
Phe	18·5	12·3	13·5	13·9	14·0	12·5	13·0
Tyr	10·4	2·2	4·2	5·6	3·0	3·7	4·1
Ser	40·2	41·9	39·8	40·7	35·3	33·7	33·9
Thr	22·7	20·6	19·9	20·2	17·8	16·7	17·3
Cysteic acid	2·8	Trace	Trace	Trace	Trace	Trace	Trace
Met	7·2	5·8	6·1	7·3	5·1	3·7	4·7
Arg	43·7	49·0	45·8	47·3	47·9	44·5	44·4
His	7·3	5·4	5·2	7·6	5·1	4·2	5·3
Lys	28·1	24·9	23·3	24·9	24·3	21·8	17·2
Asp	53·7	52·5	52·0	54·7	48·8	48·8	49·9
Glu	75·8	73·6	74·2	75·5	73·3	72·5	75·8
Hyp	80·4	108·6	103·9	96·6	91·2	102·6	98·3
Hyl	3·2	5·1	4·8	6·9	9·2	6·1	7·6
Amide N	45·8	41·9	45·8	46·5		39·9	39·6
Total hydroxy amino acids		178·4	172·7	170·0	156·5	162·8	161·2
Elastin content	—	—	—	1·6%	0·36%	—	—

Table 2. *Composition of aqueous extracts of human puerperal uterus (40g. wet wt.), human rheumatoid-arthritis nodule (4·6g. wet wt.) and two samples of ox tendons (50g. wet wt. of each)*

Extractions were performed at 4° except the elastase digestion, which was carried out at 24–25°. Tissues were extracted with about 20 times their wet weight of each solution in succession.

Tissue	Extractant	Hyp (mg.)	Collagen (mg.)	Hexosamine (mg.)	Hexose (mg.)	$\frac{\text{Hyp N}}{\text{Total N}}$ (%)
Puerperal uterus	0·1 M-KCl	2·3	17	17·6	78	
	1·25 M-KCl in 3% Na_2HPO_4	0·68	4·8	0	36	
	0·1 M-$NaHCO_3$	0·21	1·5	1·3	3·0	
	Elastase digest	—	—	10·5	63	0·17
	Saturated KCl	0·34	2·4	0	16	
Rheumatoid-arthritis nodule	Water	1·5	10·6	1·0	8·1	
	0·1 M-KCl	0·37	2·6	1·9	3·1	
	1·25 M-KCl in 3% Na_2HPO_4	0·33	2·3	0·4	2·7	
	0·1 M-$NaHCO_3$	0·13	0·9	1·6	1·3	
	Elastase digest	0·54	—	1·7	3·1	0·43
	Saturated KCl	—	—	0·8	1·7	
Ox tendon (inner tendon)	0·9 M-NaCl	1·35	9·7	8·4	34	
	0·1 M-KCl	0·68	4·9	8·9	10·5	
	1·25 M-KCl in 3% Na_2HPO_4	0·40	2·9	0·7	25·0	
	0·1 M-$NaHCO_3$	0·07	0·53	1·7	<3	
	Elastase digest	1·0	—	7·5	5·1	0·81
	Saturated KCl	0·08	0·54	0	9·4	
Ox tendon (outer tendon)	0·9 M-NaCl	1·35	9·7	8·4	34	
	0·1 M-KCl	0·37	2·6	8·4	7·4	
	1·25 MKCl in 3% Na_2HPO_4	0·42	3·0	0·1	22·1	
	0·1 M-$NaHCO_3$	0·09	0·64	2·7	<3	
	Elastase digest	1·9	—	9·7	11·1	0·56
	Saturated KCl	0·35	2·5	0	8·8	

of the extract. Hydrolysis took place in a sealed tube at 100° for 4hr. and the hydrolysate was evaporated to dryness before being made up to 5ml.

RESULTS

The amino acid contents of preparations U_1 and U_2 (Harding, 1963) are given in Table 1, for comparison with the elastase-purified collagen (preparation U_4) from the same uterus. The compositions of preparations P_1, T_1 and T_2 and the rheumatoid-nodule collagen are also given in Table 1. Presentation as residues/1000 residues eliminates differences produced by non-protein impurities or by slight errors in the standard amino acid solutions. It was apparent that preparation U_1 was impure from the low nitrogen value (17·3%) and the amino acid composition (Table 1). In previous work (Harding, 1963) the collagen was converted into gelatin (preparation U_2) to eliminate the major contaminants, notably elastin. In the present work elastin was removed with pancreatic elastase. Digestion with elastase does, however, take out some mucoprotein by breaking it down. This breakdown was probably aided by other enzymes contaminating the commercial elastase preparation. Only 0·16% of preparation P_1, 0·1% of preparation T_1 and 0·03% of preparation T_2 were extracted by the citrate buffer, pH3·7. The hydroxyproline nitrogen to total nitrogen ratios of the elastase digests are given in Table 2.

Attempts to estimate gravimetrically the elastin content of elastase-treated collagen preparations were unsuccessful. An alternative method was established, in which the content of desmosine and isodesmosine, amino acids unique to elastin, were determined. These amino acids were determined by greatly overloading the automatic amino acid analyser column; a column loading of 8·8mg. was required to recover 1·2μg. of desmosine, and for reasons given in the Discussion section this method may not give accurate results at the relatively low concentrations involved, especially with respect to isodesmosine. For puerperal-uterus and rheumatoid-arthritis-nodule collagen respectively the elastin contents were 1·6% and 0·36% considering desmosine only, or 2·1% and 1·0% if based on the combined contents of desmosine plus isodesmosine (Table 1).

The moisture, ash and hydroxyproline contents of the puerperal-uterus myometrium showed that of the 55g. dry weight present 24% was collagen. This dry weight was more than ten times that of a

similarly prepared myometrium from a non-pregnant pre-menopausal uterus (J. J. Harding, unpublished work).

It was decided to ascertain how much collagen and how much mucopolysaccharide was removed by each of the aqueous extractants from each tissue. The results of these analyses are given in Table 2. The 0·1M-potassium chloride removes readily soluble proteins, blood proteins and some muscle protein (Perry, 1956; Mommaerts, 1958; Kronman, Weinberger & Winterbottom, 1960; Laszt & Hamoir, 1961; Giles, 1962; Hartshorne & Perry, 1962; Hamoir & Konosu, 1965) as well as some mucoprotein and a little collagen. Subsequent extracts contained a negligible amount of collagen. The 1·25M-potassium chloride in 3% (w/v) disodium hydrogen phosphate solution removes further hexose-containing contaminants. Most of the mucoprotein has been eliminated at this stage and little further extraction is brought about by the 0·1M-sodium hydrogen carbonate and saturated potassium chloride solutions used immediately before and after the elastase digestion.

The amino acid composition of the salt-soluble material isolated from the puerperal uterus showed that it included some collagen (0·04% hydroxylysine was present). The soluble material is however mainly another protein as shown by the very high tyrosine (0·58%) and low glycine (4·7%) contents; this protein is probably largely actomyosin.

From 44·6g. (wet weight) of the puerperal uterus 2·2mg. of hydroxyproline (equivalent to 15·7mg. of collagen) was extracted by water. This represents 0·63% of the collagen (or the hydroxyproline) present. Dialysis of the water extract established that 0·39% of the total hydroxyproline present in the myometrium was in the form of water-soluble low-molecular-weight material. Determination of the hydroxyproline content of the water extract without hydrolysis should give the content of free hydroxyproline, as peptide-bound hydroxyproline would be unlikely to react. In this way it appeared that the extract contained 2·5p.p.m. of free hydroxyproline. This compares closely with the decrease in total hydroxyproline found after dialysis (2·7p.p.m.). It appears therefore that the diffusible hydroxyproline of the water extract is probably free (Table 3).

The characterization of the unpurified and purified tendon preparations appears in Table 4.

DISCUSSION

The problems to be faced when attempting to purify insoluble collagen were mentioned in the introduction. It is almost as difficult to establish the purity of the collagen once prepared. Criteria of purity used in the present work were largely those that are provided by amino acid analysis.

The amino acid composition of post-menopausal uterus collagen was established previously (Harding, 1963) by converting the collagen into gelatin, but purification, as a collagen, had not been achieved. The only way to remove elastin from collagen appeared to be by specific enzymic digestion. This was achieved with pancreatic elastase. A major concern was that the collagen must not be attacked by the enzyme. Purification of post-menopausal uterus collagen by using elastase digestion yielded preparation U_4, whose amino acid composition (Table 1) was closely similar to that of the corresponding gelatin U_2 (Harding, 1963), indicating a high degree of purity. A similar method was applied to a puerperal uterus, a rheumatoid-arthritis nodule and ox tendon. The citrate extraction at pH 3·7 seemed unwise (Consden &

Table 4. *Characterization of ox tendon collagens*

	Unpurified (wet)		Purified	
	Inner tendon (%)	Outer tendon shank (%)	T_1 (%)	T_2 (%)
Moisture	86·9	87·2	17·0	17·1
Ash	0·35	0·43	0·35	0·36
Hydroxyproline	—	—	14·1	13·7
Hexose	1·3*	1·5*	0·61	0·69
Hexosamine	0·42*	0·38*	0·04	0·04

* Based on dry weight.

Table 3. *Free hydroxyproline and diffusible bound hydroxyproline from puerperal uterus*

	Woessner & Brewer (1963)	Present work
Part of uterus used	All of uterus excluding cervix	Myometrium
Period post partum	9–11 days	8hr.
Original extractant	0·9M-NaCl	Water
Total diffusible hydroxyproline (mg./g. wet wt.)	50	30
Free hydroxyproline (mg./g. wet wt.)	44	28
Diffusible bound hydroxyproline (mg./g. wet wt.)	6	2·3

Kirrane, 1967) and was not used again; the other modifications have led towards simplification. The initial aqueous extracts were altered insofar as 0·1M-potassium chloride was used in place of 0·9M-sodium chloride to remove soluble proteins, sarcoplasmic proteins and some mucoproteins. Also, the separate potassium chloride and disodium hydrogen phosphate extractants were combined. The 1·25M-potassium chloride in 3% disodium hydrogen phosphate should remove mucopolysaccharide as well as actomyosin. The removal of mucopolysaccharide into these solutions was demonstrated by the hexose and hexosamine contents of the extracts (Table 2). Further loss of hexose and hexosamine-containing material occurred during elastase digestion. This was to be expected, as elastase is known to attack mucoproteins. The 0·1M-potassium chloride extracted some soluble collagen from the puerperal uterus.

The absence of elastin from the collagens is indicated by the low contents of valine, leucine and isoleucine. The total residues of these three amino acids/1000 total residues in preparation U_4 (61·7), preparation P_1 (64·0), and rheumatoid-nodule collagen (64·8) were similar to those for dura-mater collagen (64·0) and gelatin U_2 (56·9) (Harding, 1963), and were significantly lower than the value for the impure material U_1 (91·4). Elastin contents were also assessed from the desmosine and isodesmosine contents, which gave values of 1·6% and 0·36% (calculated from the content of desmosine) and 2·1% and 1·0% (calculated from the content of desmosine plus isodesmosine) for preparation P_1 and the rheumatoid-nodule collagen respectively. Estimation of elastin in this way cannot be very accurate but, with so much collagen present, it was not possible to determine elastin gravimetrically or by an orcein-binding method (J. M. Wesley, unpublished work). The desmosine and isodesmosine procedure suffers from certain distinct disadvantages. The two peaks are small shallow humps and therefore difficult to calculate. Other difficulties are caused by the assumption that the desmosine and isodesmosine content of the contaminating puerperal-uterine elastin was the same as that of the elastins for which the content of these amino acids has been determined. Published values vary, as shown in Table 5. All these elastins contain more desmosine than isodesmosine, whereas the reverse seemed to be true of the elastin contaminant in collagen. It is therefore possible that at the high column loadings used the isodesmosine peak included a contribution from another unknown ninhydrin-positive compound. For this reason the values deduced from desmosine alone are probably more reliable. The values taken for the estimation of elastin were those of Franzblau, Sinex & Faris (1965). The method is clearly not accurate, but gives an approximate estimate of elastin content in purified collagens.

The purity of preparations U_4, P_1 and the rheumatoid-nodule collagen was also demonstrated by their high contents of hydroxyproline, proline, hydroxylysine and glycine and by the low contents of tyrosine, phenylalanine and cystine (Table 1). Isolation of pure collagens from these sources has not previously been reported.

Sufficient material was not available for hexose and hexosamine determinations of the highly purified human collagens, but these were assessed to be low by the appearance of the hydrolysates and the absence of hexosamine peaks during amino acid analysis. The same isolation method was, however, applied to ox tendon collagens and decreased the hexosamine content from 0·40% to 0·04%, decreased the hexose content from 1·4% to 0·65% and resulted in a product with 13·9% hydroxyproline (Table 4). The hexose content is that of purified collagens (Harding, 1965).

Less than 1·5% of the total puerperal-uterine hydroxyproline was extracted by the aqueous extractants; of this just under 1% was extractable in dilute salt solutions. Woessner (1962) found that 2·5% of collagen in puerperal rat uterus was extractable by aqueous sodium chloride and 0·5N-acetic acid. In view of the rapid loss of collagen from the uterus during this post-partum period these findings were a little unexpected (Harkness & Harkness, 1954; Harkness & Moralee, 1956; Montfort & Perez-Tamayo, 1961; Woessner, 1962; Morrione & Seifter, 1962; Grant, 1965). However, 0·4% of the total myometrium hydroxyproline was present in water-soluble low-molecular-weight material. Further, it appeared that most of the low-molecular-weight material was free hydroxyproline itself. Similar results, reported by Woessner & Brewer (1963), are summarized in Table 3 together with the results of the present work. The

Table 5. *Published values for the desmosine and isodesmosine contents of elastin (residues/1000 residues)*

Source	Desmosine	Isodesmosine	Reference
Bovine	1·7	1·1	Franzblau *et al.* (1965)
Bovine ligamentum nuchae	2·1	1·7	Anwar (1966)
Human aorta	1·5	1·3	Anwar (1966)
Rabbit ear	1·43	1·38	Anwar (1966)

most noteworthy feature of these results is that the amount of diffusible peptide-bound hydroxyproline is small compared with the free hydroxyproline. It therefore appears that the collagen being removed from the uterus during the puerperium is broken down completely to amino acids, at least as far as hydroxyproline is concerned. The mechanism of this degradation remains largely unknown. Woessner & Brewer (1963) studied the catheptic activity of human uteri during the puerperium; the cathepsins had optimum activity at pH 3·5 and a negligible activity at neutral pH. As firm evidence of low pH regions in involuting uteri remains to be produced, the mechanism of involution is still a matter for conjecture. Enzymes capable of attacking collagen at pH 2·7–3·5 have also been found in rat uteri (Schaub, 1963, 1964*a*,*b*, 1965; Woessner, 1965). No uterine enzyme able to attack collagen at neutral pH could be found (Woessner, 1965).

Almost 4% of the hydroxyproline content of the rheumatoid-arthritis nodule was extractable into the mild aqueous solutions used; approx. 2·6% was extractable in water. These values are greater than those found for the post-partum (puerperal) uterus and may be significant in relation to the diseased condition of the collagen. The collagen itself was physically unlike the normal collagens studied previously.

The elastase was shown not to have attacked the collagens to any extent by estimation of the hydroxyproline and total nitrogen contents of the digest supernatants. Collagen and elastin are the only mammalian proteins that contain hydroxyproline and the ratio of hydroxyproline nitrogen to total nitrogen (expressed as a percentage) is approx. 8·2% for collagen and 0·8–0·9% for elastin. Consequently a ratio greater than 0·9% would indicate that collagen had been attacked. With the materials described in this paper the ratio fell between 0·17 and 0·81% (Table 2), demonstrating the stability of a variety of collagens to elastase digestion under our conditions. The lower values (less than 0·8%) indicate that elastase is removing proteins other than collagen or elastin from these materials. It is possible that a ratio of less than 0·8% could be obtained even if some collagen had been attacked owing to simultaneous attack on another protein. It is, however, clear that to keep the ratio low a much greater amount (more than ten times as much) of extraneous protein would have gone into solution. After the other aqueous extractions the residue in each case consists predominantly of collagen and elastin, and these ratios demonstrate therefore that no massive attack on collagen has occurred. They also demonstrate that the collagens studied, including the rheumatoid-nodule collagen, are native collagens.

It was also necessary to show that the cross-links of collagen were not broken, as occurs with certain other proteolytic enzymes (Barkin & Oneson, 1961; Highberger, 1961; Kühn, Hannig & Hörmann, 1961; Nishihara, 1962; Schmitt, 1963; Kühn, Fietzek & Kühn, 1963; Rubin, Pfahl, Speakman, Davison & Schmitt, 1963). The resultant collagen in these cases is soluble in acid buffers. Only 0·16% of preparation P_1, 0·1% of preparation T_1 and 0·03% of preparation T_2 were extractable by citrate buffer, pH 3·7, demonstrating that the cross-links of these collagens were not attacked by elastase under the conditions described.

The method of isolation and purification used to prepare the puerperal-uterus and the rheumatoid-arthritis-nodule collagens could probably be applied to the preparation of collagen from most non-calcified tissues. The successful isolation of collagen from uterus demonstrates that muscle proteins, blood proteins, mucoproteins, lipids and elastin have been removed. The stability of the uterine collagens to the purification procedure indicates that no damage should occur to any native collagen being treated similarly.

Study of the amino acid compositions of the five collagens and one gelatin considered to be pure preparations (U_2, U_4, P_1, T_1 and T_2 and rheumatoid-arthritis-nodule collagen) show that all are typical of collagens examined previously (see also Harding, 1967). The amino acid compositions of the gelatin and the collagens isolated from uterus were very similar. The glycine contents are close to the values invariably found for collagen (about 333). The total hydroxy amino acid contents for preparations U_2, U_4 and P_1 are significantly greater than the values for dura-mater collagen (Harding, 1963) or for the ox tendon collagens or human bone and human tendon collagens (Eastoe, 1955). In this respect preparations U_2, U_4 and P_1 are similar to reticulin, although the total hydroxy amino acid content of reticulin is still higher (Windrum, Kent & Eastoe, 1955). Also, like reticulin, preparations U_2, U_4 and P_1 have low proline contents. The hydroxylysine and lysine contents of preparations U_2, U_4 and P_1 are intermediate between those of bone and tendon collagen. The high hydroxyproline content of preparations U_2 and U_4 is of interest in view of the low shrinkage temperature of uterine collagen (Brown *et al.* 1958). It had been thought that shrinkage temperature and hydroxyproline content were closely related. The more recent suggestion that the total imino acid content was more important for thermal stability (Piez & Gross, 1960), seems more appropriate for post-menopausal uterus, where the sum of proline and hydroxyproline contents is slightly less than for dura mater. The difference of total imino acid content of preparations U_2 and U_4 compared with the non-uterine collagens is almost within the experimental error (Table 1). The total

imino acid content of puerperal uterus is even lower than that for post-menopausal uterus. The amino acid composition of collagen from the puerperal uterus gives little indication of differences from other collagens that could explain its rapid disappearance from the uterus during post-partum involution. Similarly the small compositional differences between the uterus collagens and other mammalian collagens give no revelations pertinent to the high metabolic activity of uterus collagen. The low shrinkage temperatures of uterus collagens are related to the low imino acid content, but these differences are barely outside experimental error.

The amino acid composition of the rheumatic-nodule collagen, like uterus collagens, had not been determined until the present work. The composition is again typical of collagen (Table 1). When compared with human dura-mater, bone and tendon collagens (Table 1; see also Eastoe, 1955), the only significant difference is the low tyrosine content. This can easily be explained in terms of greater purity. The hydroxyproline content is rather low and the hydroxylysine content high, but even these values are, within experimental error, the same as for human tendon. The hydroxylysine content is higher than in the great majority of mammalian collagens (see Eastoe & Leach, 1958), but not as high as values reported by Piez & Likins (1960) for ox tendon, rat bone and dentin from both cattle and rats. In terms of total hydroxy amino acid content, imino acid content and amino acid composition generally the rheumatoid-arthritis-nodule collagen is very similar to other human collagens, especially human tendon collagen. Its high hydroxylysine content is an interesting feature, but is by no means outstanding. The amino acid composition certainly does not indicate that this collagen is different from any other human collagen, nor is this collagen denatured. It was more extractable than normal collagens and this indicates that more subtle features of collagen structure such as the cross-links may be altered during rheumatoid arthritis and similar diseases.

The amino acid compositions of the ox tendon collagens (Table 1) are similar to that reported by Steven & Jackson (1967) for ox tendon purified by using bacterial α-amylase. The hydroxyproline contents of preparations T_1 and T_2 were greater than that of the α-amylase-purified collagen, but the hydroxylysine contents were lower. These differences are probably due to difficulties in calculating the area of the hydroxyproline and hydroxylysine peaks from the autoanalyser chromatograms. Methionine and cystine coincided in the method of analysis used by Steven & Jackson (1967) because of the addition of 5ml. of methanol to the first two chambers of the Autograd; however, these amino acids emerge separately with the manual method, or when the automatic method is used with 4ml. of methanol in the first chamber of the Autograd (as used in the present paper for the rheumatoid-arthritis nodule). Consequently we could estimate the significant amounts of methionine present. The presence of ornithine reported by Steven & Jackson (1967) is more difficult to explain. Ornithine can be formed by the breakdown of arginine under alkaline conditions and has been found in alkali-pretreated gelatins (Eastoe, 1960). It does, however, also appear on autoanalyser chromatograms, irrespective of what sample or mixture of amino acids is being analysed, owing to finger contamination. In general the analyses indicate that the ox tendon collagens T_1 and T_2 and the α-amylase-treated tendon collagens are of similar purity. The α-amylase method is applicable to tendon, skin, synovial tissue and cornea, but has been found unsuitable for decalcified bone and dura mater (F. S. Steven, personal communication).

We are grateful to Dr W. Gerrard, Dr A. A. Leach, Dr I. Oneson, Dr S. M. Partridge, Dr R. Consden and Dr L. E. Glynn for helpful discussions, to Dr Partridge for the desmosine and isodesmosine, to Dr Consden and Dr Glynn for providing the human tissues, to Mr Phillips of J. L. Henson Ltd. for the cattle tendons and to Mr D. Ashton for technical assistance during part of this work. The work was carried out under a grant from the Medical Research Council. Some of this work formed part of a Thesis approved by the University of London for the award of the degree of Ph.D. to J.J.H.

REFERENCES

Anwar, R. A. (1966). *Canad. J. Biochem.* **44**, 725.
Barkin, S. M. & Oneson, I. (1961). *J. Polym. Sci.* **55**, 113.
Brown, P. C., Consden, R. & Glynn, L. E. (1958). *Ann. rheum. Dis.* **17**, 196.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Consden, R., Glynn, L. E. & Stanier, W. M. (1953). *Biochem. J.* **55**, 248.
Consden, R. & Kirrane, J. A. (1967). *Nature, Lond.*, **215**, 165.
Eastoe, J. E. (1955). *Biochem. J.* **61**, 589.
Eastoe, J. E. (1960). *J. appl. Chem.* **10**, 393.
Eastoe, J. E. & Eastoe, B. (1954). *Biochem. J.* **57**, 453.
Eastoe, J. E. & Leach, A. A. (1958). In *Recent Advances in Gelatine and Glue Research*, p. 173. Ed. by Stainsby, G. London: Pergamon Press Ltd.
François, C., Marshall, R. D. & Neuberger, A. (1962). *Biochem. J.* **83**, 335.
Franzblau, C., Sinex, F. M. & Faris, B. (1965). *Nature, Lond.*, **205**, 802.
Giles, B. G. (1962). *J. Sci. Fd Agric.* **13**, 264.
Grant, R. A. (1965). *J. Reprod. Fertil.* **9**, 285.
Hamoir, G. & Konosu, S. (1965). *Biochem. J.* **96**, 85.
Harding, J. J. (1963). *Biochem. J.* **86**, 574.
Harding, J. J. (1965). *Advanc. Protein Chem.* **20**, 109.
Harding, J. J. (1967). *J. Soc. Leath. Tr. Chem.* (in the Press).

Harkness, M. L. R. & Harkness, R. D. (1954). *J. Physiol.* **123**, 492.
Harkness, R. D. & Moralee, B. E. (1956). *J. Physiol.* **132**, 502.
Hartshorne, D. J. & Perry, S. V. (1962). *Biochem. J.* **85**, 171.
Highberger, J. H. (1961). *U.S. Patent* 2979438.
Klevens, H. B. (1963). *U.S. Patent* 3071477.
Kronman, M. J., Weinberger, L. E. & Winterbottom, R. J. (1960). *Arch. Biochem. Biophys.* **86**, 238.
Kühn, K., Fietzek, P. & Kühn, J. (1963). *Naturwissenschaften*, **50**, 444.
Kühn, K., Hannig, K. & Hörmann, H. (1961). *Leder*, **12**, 237.
Laszt, L. & Hamoir, G. (1961). *Biochim. biophys. Acta*, **50**, 430.
Leach, A. A. (1957). *Biochem. J.* **67**, 83.
Mommaerts, W. F. H. M. (1958). *Meth. med. Res.* **7**, 3.
Montfort, I. & Perez-Tamayo, R. (1961). *Lab. Invest.* **10**, 1240.
Morrione, T. G. & Seifter, S. (1962). *J. exp. Med.* **115**, 357.
Nishihara, T. (1962). *U.S. Patent* 3034852.
Perry, S. V. (1956). *Physiol. Rev.* **36**, 1.
Piez, K. A. & Gross, J. (1960). *J. biol. Chem.* **235**, 995.
Piez, K. A. & Likins, R. C. (1960). *Calcification in Biological Systems*, p. 411. Washington: American Association for the Advancement of Science.
Piez, K. A. & Morris, L. (1960). *Analyt. Biochem.* **1**, 187.
Rubin, A. L., Pfahl, D., Speakman, P. T., Davison, P. F. & Schmitt, F. O. (1963). *Science*, **139**, 37.
Schaub, M. C. (1963). *Helv. physiol. Acta*, **21**, 227.
Schaub, M. C. (1964*a*). *Helv. physiol. Acta*, **22**, 271.
Schaub, M. C. (1964*b*). *Gerontologia*, **9**, 52.
Schaub, M. C. (1965). *Hoppe-Seyl. Z.* **343**, 130.
Schmitt, F. O. (1963). *Leder*, **14**, 21.
Skeggs, L. T. (1957). *Amer. J. clin. Path.* **28**, 311.
Steven, F. S. & Jackson, D. S. (1967). *Biochem. J.* **104**, 534.
Windrum, G. M., Kent, P. W. & Eastoe, J. E. (1955). *Brit. J. exp. Path.* **36**, 49.
Winzler, R. J. (1955). *Meth. biochem. Anal.* **2**, 279.
Woessner, J. F. (1962). *Biochem. J.* **83**, 304.
Woessner, J. F. (1965). *Biochem. J.* **97**, 855.
Woessner, J. F. & Brewer, T. H. (1963). *Biochem. J.* **89**, 75.