hyde, but the most serious errors arise when small quantities of amino sugar are estimated in the presence of large quantities of mixtures of amino acids with non-nitrogenous sugars (Palmer *et al.* 1937; Sideris, Young & Krauss, 1938; Lutwak-Mann, 1941; Yasuoka, 1944; Horowitz, Ikawa & Fling, 1950; Immers & Vasseur, 1950; Storey, Yensen, Lisie & Biliên, 1951). For this reason it is advisable to confirm determinations of 'total amino sugar' by alternative means such as that proposed by Gardell (1953). An account of a method whereby such a confirmation can be obtained will be given later.

SUMMARY

1. A modification of the method described by Elson & Morgan (1933) for the determination of amino sugars is given.

2. The 'best' range, the accuracy and the reproducibility of the determination are illustrated by results obtained by the use of authentic amino sugar samples, and the limitations of procedure, imposed by the instability of the chromophore produced, are given.

3. Factors of importance in the application of the method to naturally occurring substances are discussed.

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The Amino Acid Composition of Mammalian Collagen and Gelatin

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The name collagen is given to a group of related fibrous proteins, found in the mesodermal tissues of animals. The best-characterized forms of collagen occur in the higher animals, where they constitute the main organic components of skin, bone, tendon and loose connective tissue. Gelatin, a derived protein, can be prepared as a breakdown product of collagen by extracting these tissues with hot water (above 40°). Extraction with minimal degradation can normally be accomplished in neutral solutions after a prolonged pretreatment with cold alkali or by an acid extraction (Ward, 1954). Gelatin is the principal constituent of the commercial products 'gelatine' and glue and is responsible for their gel properties.

There are no rigid distinguishing features of proteins belonging to the collagen group. Bear (1952) used certain characteristics of the wide-angle X-ray diffraction pattern as the main criteria, supported, except in the collagen of the simplest organisms (e.g. spongin), by the existence of long spacings (approx. 600 Å), detectable either by small-angle X-ray diffraction or electron microscopy. Proteins falling within this classification are very widely distributed in many animal phyla, and for some purposes it appears useful to subdivide further this large group, on a chemical basis, distinguishing those members which contain significant numbers of hydroxyproline residues. Hydroxyproline and hydroxylysine are not known to occur in substantial quantities in proteins other than those of the collagen type. The restricted definition would include the characteristic mesodermal protein of vertebrates but might exclude many of the invertebrate types, since none of these has yet been shown to contain hydroxyproline. In addition, only vertebrate collagens have, so far, been shown to be capable of yielding a degradation product, a solution of which gels on cooling. This provides further support for distinguishing vertebrate collagens as special members of a wider group.

Complete amino acid analyses by chemical methods are available only for mammalian collagen and gelatin. Chibnall (1946), with his co-workers and later Bowes & Kenten (1948), have built up accurate data for gelatin and ox-hide collagen, using a wide variety of analytical techniques. These values, with recent minor adjustments, have been summarized by Tristram (1953). The amino acid composition of collagen and gelatin from a wider range of vertebrates has been studied by Neuman (1949), using microbiological methods. These results as a whole represent a higher order of accuracy than the earlier work, details of which have been summarized by Bowes & Kenten (1947). The present survey of the composition of mammalian collagens and gelatins has been made after using the ion-exchange chromatographic method of Moore & Stein (1951). Work is still in progress on the remaining classes of vertebrates.

EXPERIMENTAL

Materials

Bone collagen (ox and human). Collagen, substantially free from mucopolysaccharide–protein complex, was prepared from compact bone from the centre of the diaphyses of femora by the method of Eastoe & Eastoe (1954). (Moisture content: ox collagen, 17·0, human collagen, 15·7 %; ash: 0·08 and 0·04 % respectively.)

Wallaby tail tendon. The tail was skinned and the four massive tendon bundles were pulled from the attached vertebrae. Small fragments of bone and adhering muscle fibres were removed mechanically. The separated tendons were cut into 1 cm. lengths and washed for 48 hr. in four changes of 10 % aqueous NaCl at 0°. The tendon was washed with six changes of water, dehydrated with acetone, transferred to ether and finally air-dried. (Moisture, 17·7; ash, 0·09 %.)

Human Achilles tendon. The tendons were treated with ethanol and the fat was removed with changes of ether. Adhering muscle tissue was cut away, the tendon bundle opened out and as much as possible of the yellow sheath detached. Smaller fibre bundles were separated and cut into 1 cm. lengths. (Total N, 17·88; ash, 0·82 %.) The tendon was then treated successively with 10 % NaCl (two changes, 24 hr. each at 0°) and saturated aqueous Na_2HPO_4 (three changes, 24 hr. each at 0°). The material was washed thoroughly with distilled water, transferred successively to acetone and ether and air-dried. (Total N, 18·14; ash, 0·05 %.)

The phosphate-treated tendon was extracted for 1·5 hr. with four times its weight of 0·1 N-HCl at 100°. Most of the tendon went into solution. The solution was filtered through glass wool and evaporated to dryness *in vacuo*. The residue from evaporation was allowed to reach equilibrium with moisture in the atmosphere and used for the amino acid analysis. (Moisture content, 15·0; ash, 0·04 %.)

Table 1. *Moisture and ash contents, isoionic points and physical properties of gelatins*

Pretreatment	H_2O (%)	Ash (%)	Isoionic point (pH)	Kinematic viscosity* (cs at 40°)	'Jelly strength'*† (g. Bloom)
Ox hide I					
(*a*) First extraction; lime (3 months)	13·6	1·6	4·98	8·2	225
(*b*) Third extraction; lime (3 months)	14·3	1·7	4·92	8·2	140
Ox hide II					
Untreated, lime (4 months)	14·5	1·1	4·97	8·7	225
Purified, lime (4 months)	12·1	0·016	4·91	—	—
Bone, lime	15·3	1·4	5·13	5·5	195
Pig skin, acid	12·9	0·08	8·75	6·8	225
Whale skin, not known	17·5	1·0	5·70	7·5	180

* Determined according to B.S. 757:1944 at 6·67 % (w/w) concentration of gelatin with specified moisture and ash content and at pH 6·0–6·5.

† Gel matured at 10·0° for 17 hr.

Reticulin. Reticulin from the mid-cortical zone of human kidney, prepared by the method of Windrum, Kent & Eastoe (1955), was analysed.

Commercial gelatins. Six samples of commercial high-grade gelatin, manufactured from known raw materials, were selected for this study. The gelatins designated ox-hide gelatin I*a* and I*b* were from first- and third-extraction liquors respectively of a single batch of alkali-treated precursor. The bone gelatin had been prepared from decalcified 'Indian bone', which probably consisted mainly of cattle bones. The raw materials, methods of pretreatment, moisture and ash contents, isoionic points and characteristic physical properties of these gelatins are summarized in Table 1.

Purified gelatin. Ox-hide gelatin II was purified by fractionation with ethanol (Stainsby, 1955), followed by deionization with mixed-bed resins (Janus, Kenchington & Ward, 1951). Approximately 12% of the material from the high-viscosity end was removed from solution as a coacervate in three separations. The bulk of the material (83·5%) was then separated, leaving some 4·5% of low-viscosity material in solution. The middle fraction was dissolved in more water at 60° and deionized; the solution set to a gel, which was dried in a current of air at room temperature.

Methods

Moisture. The loss of weight on drying the finely divided collagen or a thin film of gelatin for 18 hr. at 105° was used to calculate the moisture content.

Ash. The sample was slowly incinerated, at a low temperature, in a platinum dish over a Bunsen flame and the residue heated for 2 hr. at 550° in an electric muffle furnace.

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

Isoionic point. The isoionic points of gelatins were determined by the deionization method of Janus *et al.* (1951).

Hydrolysis. The protein (0·2 g.) was hydrolysed for 24 hr. or 48 hr. in 20 ml. of 20% (w/w) HCl in a sealed resistance-glass tube immersed in water at 100°. The acid was removed by evaporation *in vacuo* at room temperature over P_2O_5 and NaOH. The dry residue could be stored at 0° over P_2O_5 for at least 2 months without change.

Chromatographic analysis. Separation of the amino acids was carried out on columns of Dowex 50 by the method of Moore & Stein (1951). The 1 ml. effluent fractions were analysed by the method of Moore & Stein (1948), 2 ml. of ninhydrin reagent being used per fraction. Details of minor alterations to the published procedures are described in the Appendix (p. 601).

Hydroxyproline. The hydroxyproline peak overlapped that of aspartic acid, the deep-blue colour of the latter preventing measurement of the pale yellow produced by the imino acid. Hydroxyproline was therefore determined independently, without preliminary chromatographic separation by the method of Neuman & Logan (1950), by means of the Martin & Axelrod's (1953) modification for removing hydrogen peroxide. To minimize errors due to lack of reproducibility between replicate samples or standards, determinations were carried out in duplicate in each of two separate experiments. The calibration was carried out on a chromatographically purified sample of hydroxyproline.

Corrections

Colour yields. The values for 'colour yield' used in the present calculations were those of Moore & Stein (1948, 1951), which were confirmed under the present experimental conditions, except for those of lysine and proline, which were found to be 1·08 (at 570 mμ.) and 3·52 (at 440 mμ.) respectively. A value of 1·08 was found for hydroxylysine, and the value 1·02 (Schram, Dustin, Moore & Bigwood, 1953) was assumed for methionine sulphoxide.

Methionine. The methionine content was calculated by combining values for the methionine peak and the small peak emerging immediately before hydroxyproline; this latter peak was assumed to be methionine sulphoxide, formed from methionine during the acid hydrolysis (Schram *et al.* 1953). No evidence was obtained for the identity of this peak, other than its reported position of emergence.

Glutamic acid. The values for glutamic acid have been corrected for an assumed 3% loss, probably resulting from pyrrolidonecarboxylic acid formation during passage through the column (Moore & Stein, 1951).

Serine, threonine and amide nitrogen. The values for serine and threonine were corrected for decomposition by 5 and 3% respectively with hydrolysis for 24 hr. and by 10 and 5% respectively with hydrolysis for 48 hr. at 100°. These corrections were adopted after experiments in which serine or threonine was heated with 20% (w/w) HCl at 100°. The results obtained were in agreement with the differences in the weights of these amino acids recovered from gelatin after 24 and 48 hr. hydrolysis at 100°. Losses of both serine and threonine after 24 hr. were almost exactly one-half of those found by Rees (1946), who used HCl of the same concentration, and heated for the same time but at a higher temperature, 108·5°. This indicates that the rate of decomposition increases by a factor of slightly more than 2 for a rise in temperature of 10°.

The value for amide nitrogen was calculated from the ammonia peak after correcting for ammonia formed by decomposition of serine and threonine during hydrolysis. Rees (1946) has shown that the decomposed hydroxy amino acids give a quantitative yield of ammonia.

Overlapping peaks. Where slight overlaps occurred between adjacent peaks, as in the pairs aspartic acid and threonine, threonine and serine, and isoleucine and leucine, the colour intensity of the fraction at the minimum between the peaks was divided equally between them, all other values being placed in their respective peaks. The considerable overlap between tyrosine and phenylalanine invalidated this method of calculation, and a procedure involving graphical reconstruction of the separate peaks was substituted (see Appendix). No attempt was made to estimate separately the small amount of ornithine occurring in the ox-hide gelatins.

RESULTS

The values obtained for the amino acid contents of mammalian collagen, gelatin and reticulin samples are given in Tables 2–4. Results have been calculated on the weight of dry, ash-free protein (Tables 2 and 3) and on the total nitrogen content (Table 4). The amino acid composition of human renal reticulin (Windrum *et al.* 1955) has been

Table 2. *Amino acid composition of mammalian collagens and gelatins*

Values are given as g. of amino acid per 100 g. of dry ash-free protein.

	Ox-hide gelatin I				Ox-hide gelatin II		Mean for ox-hide gelatin	Com-mercial bone gelatin	Pig-skin gelatin	Whale-skin gelatin	Ox-bone collagen	Human bone collagen	Human tendon acid extract	Wallaby tendon
	a, First extrac-tion	b, Third extraction			Un-treated	Puri-fied								
Duration of hydrolysis (hr.) ...	24	24		48	48	48	—	24	24	24	24	24	24	24
Alanine	11·0	10·70	10·90	10·9	11·1	11·3	11·0	11·3	10·7	10·8	10·5	10·9	10·3	10·7
Glycine	27·6	27·57	27·41	27·6	27·2	27·6	27·5	27·2	26·4	26·7	25·3	25·8	25·4	25·7
Valine	2·57	2·53	2·45	2·56	2·82	2·63	2·59	2·77	2·77	2·64	2·65	2·97	3·10	2·90
Leucine	3·41	3·22	3·24	3·35	3·41	3·36	3·33	3·45	3·34	3·56	3·93	3·60	3·57	3·69
Isoleucine	1·72	1·72	1·73	1·78	1·68	1·70	1·72	1·54	1·36	1·57	1·73	1·88	1·53	1·25
Proline	16·5	16·25	16·38	16·2	16·3	16·5	16·35	15·5	16·2	16·2	14·7	15·3	15·2	14·7
Phenylalanine	2·29	2·13	2·21	2·26	2·24	2·23	2·23	2·49	2·56	2·32	2·88	2·49	2·46	2·83
Tyrosine	0·27	0·35	0·29	0·37	0·29	0·18	0·29	0·23	0·60	0·72	0·56	0·86	0·69	0·78
Serine*	4·08	4·30	4·28	4·32	4·11	4·15	4·21	3·73	4·13	4·71	4·24	4·06	4·05	4·37
Threonine*	2·20	2·25	2·18	2·20	2·23	2·27	2·22	2·36	2·19	3·12	2·52	2·35	2·30	2·56
Cystine†	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Methionine‡	0·86	1·00	0·96	0·88	0·81	0·82	0·89	0·63	0·88	0·79	0·80	0·84	0·90	1·05
Arginine	8·7	8·8		8·7	9·1	8·9	8·8	9·0	9·1	9·5	9·2	8·8	8·9	9·5
Histidine	0·82	0·80		0·76	0·74	0·76	0·78	0·70	1·01	0·96	0·96	0·96	0·87	0·84
Lysine	4·26	4·38		4·44	4·70	4·70	4·50	4·36	4·14	4·14	4·11	4·40	3·29	3·84
Ornithine	§	§		§	§	§	§	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Aspartic acid	6·6	6·94	6·91	6·8	6·6	6·6	6·7	6·7	6·7	6·7	7·1	6·7	6·7	7·0
Glutamic acid	11·6	11·39	11·45	11·5	11·1	11·4	11·4	11·6	11·3	11·2	11·9	11·4	11·1	11·5
Hydroxyproline	13·4	13·9		14·3	14·4	14·4	14·1	13·3	13·5	12·8	14·1	14·1	12·6	13·0
Hydroxylysine	0·91	0·95		0·87	1·07	1·04	0·97	0·76	1·04	1·02	1·12	0·62	1·50	1·39
Total	118·8	119·2		119·8	119·9	120·5	119·6	117·6	117·9	119·5	118·3	118·0	114·5	117·6
Total N	18·19	18·15		18·15	18·11	18·12	18·14	18·10	18·30	18·57	18·26	18·45	17·94	18·32
Amide N‖	0·12	0·11		0·10	0·16	0·08	0·11	0·24	0·62	0·39	0·63	0·56	0·65	0·66
Mean wt. of residue	90·4	90·7		90·7	91·0	90·7	90·7	90·6	91·3	91·1	92·4	91·4	91·6	92·1
Z¶	119·9	119·9		119·9	119·8	119·9	119·9	119·9	119·7	119·8	119·5	119·7	119·7	119·6
Recovery by wt. (%)	99·1	99·4		99·9	100·1	100·5	99·8	98·1	98·5	99·7	99·0	98·6	95·6	98·3
Recovery of N (%)	100·2	100·6		100·7	101·7	101·8	100·9	100·6	100·6	100·5	101·4	100·1	100·4	101·4

* Corrected for decomposition during hydrolysis (see text).
† Trace (see text).
‡ Sum of methionine and methionine sulphoxide peaks.
§ The small amount of ornithine present is included in the lysine value.
‖ Corrected for ammonia formed by decomposition of serine and threonine.
¶ Chibnall (1942).

Table 3. *Amino acid composition of mammalian collagens and gelatins*

Values are given as moles/10^5 g. of dry ash-free protein.

	Ox-hide gelatin I			Ox-hide gelatin II		Mean for ox-hide gelatin	Commercial bone gelatin	Pig-skin gelatin	Whale-skin gelatin	Ox-bone collagen	Human bone collagen	Human tendon acid extract	Wallaby tendon
	a, First extraction	*b*, Third extraction		Un-treated	Puri-fied								
Duration of hydrolysis (hr.) ...	24	24	48	48	48	—	24	24	24	24	24	24	24
Alanine	123·0	121·0	122·4	124·6	126·6	123·2	126·2	119·5	121·0	117·6	122·4	115·6	120·2
Glycine	368	366	368	362	368	366	363	352	357	337	344	338	342
Valine	22·0	21·2	21·8	24·1	22·4	22·1	23·7	23·6	22·6	22·7	25·4	26·5	24·8
Leucine	26·0	24·6	25·5	26·0	25·7	25·4	26·3	25·5	27·2	29·9	27·5	27·2	28·1
Isoleucine	13·1	13·3	13·6	12·8	13·0	13·2	11·7	10·3	12·0	13·2	14·3	11·6	9·5
Proline	143·3	141·6	140·6	141·0	143·0	141·9	134·4	140·6	140·3	127·2	133·0	132·0	127·2
Phenylalanine	13·8	13·2	13·7	13·5	13·5	13·5	15·1	15·5	14·2	17·5	15·0	14·8	17·1
Tyrosine	1·5	1·7	2·0	1·7	1·0	1·6	1·3	3·4	3·9	3·1	4·8	3·8	4·4
Serine*	38·9	41·2	41·2	39·1	39·5	40·2	35·5	39·3	44·9	40·5	38·7	38·5	41·7
Threonine*	18·5	18·6	18·3	18·8	19·1	18·6	19·8	18·4	26·3	21·1	19·8	19·3	21·5
Cystine†	—	—	—	—	—	—	—	—	—	—	—	—	—
Methionine‡	5·8	6·7	5·9	5·4	5·6	6·0	4·2	5·8	5·2	5·5	5·7	6·0	7·0
Arginine	50·2	50·5	50·0	52·3	51·2	50·8	51·9	52·0	54·8	52·5	50·8	51·2	54·6
Histidine	5·2	5·1	4·9	4·8	4·9	5·0	4·5	6·5	6·2	6·2	6·2	5·6	5·4
Lysine	29·0	30·0	30·4	32·1	32·1	30·6	29·9	28·2	28·4	28·1	30·2	22·5	26·3
Ornithine	§	§	§	§	§	§	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Aspartic acid	49·7	52·1	51·1	49·3	49·4	50·6	50·5	50·5	50·7	53·4	50·6	50·5	52·7
Glutamic acid	79·4	77·7	78·4	75·6	77·7	77·8	78·6	77·6	76·2	81·2	77·8	75·5	78·0
Hydroxyproline	102·3	106·0	109·0	109·9	109·9	107·4	101·0	103·0	97·6	108·0	108·0	96·2	99·1
Hydroxylysine	5·6	5·8	5·4	6·6	6·4	6·0	4·7	6·4	6·3	6·9	3·8	9·3	8·6
Total	1095·3	1096·3	1102·2	1099·6	1109·0	1099·9	1082·3	1078·1	1094·8	1071·6	1078·0	1044·1	1068·2
Amide‖	8·5	8·1	7·1	11·7	5·6	8·2	17·0	44·0	28·0	44·8	40·2	46·0	47·4

* Corrected for decomposition during hydrolysis (see text).
† Trace (see text).
‡ Sum of methionine and methionine sulphoxide peaks.
§ The small amount of ornithine present is included in the lysine value.
‖ Corrected for ammonia formed by decomposition of serine and threonine.

included in Table 4 to facilitate comparison with values for human collagen, which were not previously available. The reticulin figures are based solely on nitrogen content owing to the substantial proportion of fatty acid and carbohydrate present.

Table 4. *Amino acid composition of human collagen and reticulin*

Results are expressed as g. of amino acid nitrogen/100 g. of total nitrogen.

	Bone collagen	Tendon collagen, acid extract	Renal reticulin
Duration of hydrolysis (hr.) ...	24	24	24
Alanine	9·3	9·0	7·7
Glycine	26·2	26·4	24·6
Valine	1·93	2·07	2·13
Leucine	2·09	2·12	2·85
Isoleucine	1·09	0·91	1·43
Proline	10·1	10·3	7·7
Phenylalanine	1·15	1·15	1·44
Tyrosine	0·36	0·30	0·24
Serine*	2·94	3·00	3·40
Threonine*	1·50	1·51	1·74
Cystine	†	†	0·04
Methionine‡	0·43	0·47	0·68
Arginine	15·4	16·0	14·4
Histidine	1·41	1·31	1·27
Lysine	4·59	3·51	3·44
Ornithine	0·0	0·0	0·0
Aspartic acid	3·84	3·94	4·21
Glutamic acid	5·9	5·9	6·1
Amide§	3·06	3·59	3·42
Hydroxyproline	8·2	7·5	8·6
Hydroxylysine	0·58	1·45	1·93
Total	100·1	100·4	97·4‖

* Corrected for decomposition during hydrolysis (see text).

† Trace (see text).

‡ Sum of methionine and methionine sulphoxide peaks.

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

‖ Including 0·07 % of glucosamine nitrogen.

Complete recovery of nitrogen (100·1–101·8 %), within the experimental error, was obtained for all collagen and gelatin samples (Table 2). The tendency for this figure to be persistently high has also been reported for other proteins (Smith & Stockell, 1954; Smith, Stockell & Kimmel, 1954). The effect in the present study is probably attributable to small errors in the colorimetric factors, and possibly a slight underestimation of the total nitrogen content. The recovery of weight ranged from 95·6 % for human Achilles tendon to 100·5 % for ox-hide gelatin II. The difference between the weight and nitrogen recovery figures represents the non-nitrogenous constituents, but it can only be regarded as approximate since it reflects, in addition, the combined experimental errors of the moisture, ash and total nitrogen determinations.

The chromatograms of all samples qualitatively resembled those for ox-hide gelatin II on both 100 and 15 cm. columns of Dowex 50 (Figs. 1 and 2). Good separations of peaks were obtained except for the pairs of hydroxyproline, aspartic acid and tyrosine, phenylalanine. The optical densities of the test solutions, measured against water, are shown plotted against eluent volume to illustrate the extent of variations in the ninhydrin blank during the course of a chromatogram.

With most samples, a trace of ninhydrin-positive material emerged from the 100 cm. column at a point corresponding to zero retention volume (22 ml.); this may have been cysteic acid (Moore & Stein, 1951) but it was not definitely identified. The ninhydrin blank in the region between alanine and valine was sometimes slightly erratic, but in no instance was a clear indication of a much-flattened cystine peak obtained. These results suggest that the cystine content of all samples examined was less than 0·05 %.

The position of emergence of hydroxylysine was checked, using a synthetic mixture of the diastereoisomers kindly supplied by Dr J. R. Weisiger of the Rockefeller Institute for Medical Research, New York. Asymmetry of the leading edge of the lysine peak with hydrolysates of lime-processed gelatin, but not with acid-processed gelatin or collagen, was attributed to the presence of ornithine, which emerges at this point (Moore & Stein, 1951). No separate identification of ornithine was made. No peak occurred in the position reported for tryptophan, although this cannot be regarded as evidence for the complete absence of this amino acid owing to the extensive destruction of tryptophan that is frequently reported as occurring during acid hydrolysis. A large body of evidence suggests, however, that tryptophan is absent from gelatin (Bowes & Kenten, 1947), and no attempt was made to determine it separately.

DISCUSSION

Values for duplicate determinations on 24 hr. hydrolysates of ox-hide gelatin I*b* (Table 2) show a reproducibility of better than ± 4 % for all amino acids other than tyrosine and better than ± 2 % for amino acids present in amounts greater than 3 % of the gelatin.

Values for amino acids present in amounts less than 5 % have been given to two decimal places in Table 2. This is intended to imply not that the second place is accurately known but that it may be taken as an indication for comparative purposes. It has also been used in computing totals.

Hydrolysis

Recently 6 N [20 % (w/w)] hydrochloric acid has been used by many workers at temperatures of 105° (Smith & Stockell, 1954), 108·5° (Macpherson, 1946) and 110° (Harfenist, 1953; Hirs, Stein & Moore, 1954) for times varying from 20 to 140 hr. for hydrolysis of proteins before amino acid analysis. Close control of temperature appeared important for obtaining reproducible hydrolysis, a point that has been recently emphasized by Hirs *et al.* (1954). To avoid the inaccuracies of control and the long time required for the sample to reach the operating temperature of an electric oven, a temperature of

Fig. 1. Elution curve of the hydrolysate from 5·56 mg. of untreated ox-hide gelatin II, chromatographed on a 100 cm. × 0·9 cm. column of Dowex 50. ——, Optical density/cm. optical path at 570 mμ.; ....., optical density/cm. for imino acids at 440 mμ.; ----, the tops of the aspartic acid, glycine and alanine peaks at one-quarter vertical scale. The buffer was changed from pH 3·42 to 4·25 and the temperature from 37·5° to 60° at the point marked **X**.

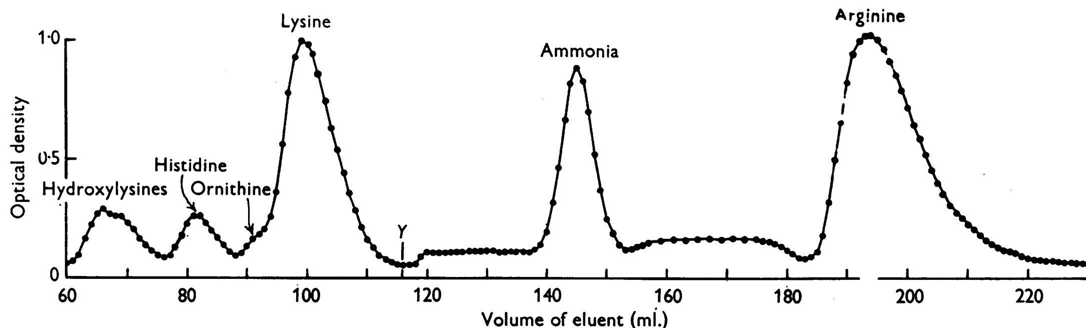

Fig. 2. Elution curve of the hydrolysate from 11·13 mg. of untreated ox-hide gelatin II, chromatographed on a 15 cm. × 0·9 cm. column of Dowex 50 to separate the basic amino acids. The buffer was changed from pH 6·75, 0·1 M sodium phosphate, to pH 6·5, 0·2 M sodium citrate, at the point marked **Y**.

100°, maintained by immersion in boiling water, was employed. Superheating and exposure of the hydrolysate to atmospheric oxidation, such as may occur when the hydrolysate is boiled directly (Macpherson, 1946), were also avoided by this procedure.

The decreased hydrolysis rate at this lower temperature did not result in the need for an inconveniently long period for the complete hydrolysis of collagen and gelatin. Synge (1953) considered that gelatin does not contain significant proportions of those amino acid sequences which are extremely stable to acid hydrolysis. The release of valine and isoleucine by hydrolysis with 6 N hydrochloric acid has been shown to be incomplete after 20–24 hr. for certain other proteins such as insulin (Harfenist, 1953), carboxypeptidase (Smith & Stockell, 1954), papain (Smith *et al.* 1954) and ribonuclease (Hirs *et al.* 1954) at temperatures of 105–110°. However, despite the decreased hydrolysis rate at 100°, release of amino acids from oxhide gelatin was found to be substantially complete after 24 hr. (Tables 2 and 3), the increase in valine and the leucines on extending the heating period to 48 hr. being of the same order as the experimental error. Collagen, like gelatin, may be supposed to be completely hydrolysed after 24 hr. at 100°, since the peptide bonds broken during the conversion of collagen into gelatin are labile under very mild conditions. Protein impurities in collagen may contain sequences of amino acids which require more prolonged hydrolysis, but the proportion left in peptide combination after 24 hr. will be very small compared with those already released from both collagen and impurity.

The mild hydrolytic conditions employed minimized the breakdown of amino acids. Decomposition of serine and threonine with formation of ammonia was observed, but there was no indication of decomposition of aspartic and glutamic acids in periods up to 48 hr., as was observed by Smith *et al.* (1954) and Hirs *et al.* (1954) at higher temperatures.

The asymmetry of the hydroxylysine peak (Fig. 2) has been shown by Piez (1954) to be due to the presence of diastereoisomers, which he separated on a 30 cm. Dowex 50 column. Recently, Hamilton & Anderson (1955) have obtained evidence that only hydroxy-L-lysine residues are present in collagen, *allo*hydroxy-D-lysine being formed during hydrolysis by racemization at the α-carbon atom.

Purity of collagen

It is difficult to prepare collagen free from impurities (Bowes & Kenten, 1948; Kendrew, 1954). No methods are available for the removal of elastin and other less well-defined proteins (Eastoe & Eastoe, 1954) which resist solution in hot water, without risk of simultaneous modification of collagen. Further, it is not easy to find valid criteria of purity for collagen, nor to define whether a given minor component is strictly part of the collagen structure or adventitious. Chemical composition appears, at present, to be the most sensitive tool for examining the purity of collagen systems.

Two approaches have been used for isolating samples of collagen. The first, reviewed by Baker, Lampitt & Brown (1954), was aimed at dissolving impurities with suitable reagents, leaving intact collagen fibres. Treatments include the use of: 10% sodium chloride to dissolve albumins and globulins; disodium phosphate, which removes other connective tissue proteins, possibly collagen precursors (see Harkness, Marko, Muir & Neuberger, 1954); alkaline trypsin, which breaks down muscle; lime water, to dissolve mucopolysaccharides and mucoproteins; and fat solvents. The chief difficulty, already mentioned, is the presence of proteins more resistant than collagen itself.

The second type of method involves dissolving the collagen in a slightly acid solution, possibly with intermediate purification (Salo, 1950), before precipitating the collagen, in a form that still shows structure in the electron microscope (Schmitt, Hall & Jakus, 1942), by dialysis or addition of sodium chloride. The main objection to this method is that only some 10% of collagen (Nageotte & Guyon, 1934) dissolves, leaving a residue of the bulk of the collagen, whose chemical composition is close to that of the soluble collagen (Bowes, Elliott & Moss, 1953) but which clearly differs from it in solubility. It appears that fractionation of a heterogeneous collagen population occurs, the basis of which is not understood. Orekhovitch (1952) has supposed acid-soluble collagen, termed 'procollagen', to be the metabolic precursor of relatively insoluble collagen, but more recent work casts doubt on this (Harkness *et al.* 1954).

Commercially, collagen can be converted into gelatin in 80–90% yield, so that gelatin, unlike acid-soluble collagen, is derived from the bulk of the collagen.

In the present study, native collagenous tissue has been analysed after comparatively mild treatment with salt solutions. Use of enzymes has been avoided, owing to risk of alteration of the collagen (Bowes & Kenten, 1948).

Differences in composition between collagen and gelatin

The amino acid data for collagen and gelatin in the recent literature (Tristram, 1953) show close similarities (Table 5). This has been confirmed in the present study for collagens and gelatins from a

number of mammalian sources (Tables 2 and 3). Certain differences have, however, been noted:

(1) The amide-nitrogen content of gelatin prepared from limed collagen is less than that of the original collagen and falls to a low value after prolonged liming (Table 2). There is a resulting fall in the isoionic point of the gelatin to a limiting value of approximately pH 4·8 for complete removal of amide groups (Ames, 1944). Gelatin extracted after short acid pretreatment, however, may contain substantially all the amide nitrogen of the original collagen (Table 2) and have an isoionic point in the region pH 8·5–9·5, which presumably approximates to that of collagen, although the isoelectric point of acid-soluble collagen measured in the presence of sodium acetate appears to be in the region of pH 5·8 (Brown & Kelly, 1953). A more prolonged acid treatment of the precursor results in the removal of a proportion of the amide groups, indicated by a fall in the isoionic point to pH 6–8 (Rousselot, 1944).

(2) Treatment of collagen with lime results in a slow conversion of arginine residues into ornithine. In the present study an accurate determination of ornithine was not possible, since the ornithine peak was superimposed on the leading edge of the lysine peak. The presence of a small quantity of ornithine could be detected, however, by the shape of the curve in this region, positive indications being obtained only for the two ox-hide gelatins, the precursors of which had been limed for 3–4 months. The upper limit for the ornithine content of these gelatins was estimated to be about 0·2% of amino acid/100 g. of gelatin, corresponding to about 3% conversion of arginine.

(3) The two differences set out above are well defined; in addition, there is a less definite tendency for gelatin to contain very slightly more of those amino acids, e.g. glycine and proline, that are present in large amounts in both gelatin and collagen, and the collagen samples tend to have slightly larger amounts of amino acids such as tyrosine, leucine and phenylalanine, which are present in both proteins in small proportion only. These differences could be explained by assuming the presence, in the collagen preparations, of small proportions of protein impurities that are not present, or present only in smaller proportion, in gelatin, with amino acid compositions markedly different from collagen and gelatin. Bowes *et al.* (1953) considered that conversion of citrate-soluble collagen into insoluble collagen fibres *in vivo* must involve the addition of a protein fraction rich in tyrosine, histidine, lysine, proline and amide nitrogen and low in hydroxyproline, alanine and serine.

Relationship of gelatin and collagen

The possibility that gelatin is relatively free from protein-degradation products, derived from contaminants of native collagen and not otherwise easily removed, suggests that gelatin may represent highly purified collagen in amino acid composition. This applies especially to lime-processed gelatin, since removal of mucopolysaccharide and mucoprotein constituents (Eastoe & Eastoe, 1954) would be expected to take place during the pretreatment of raw material and subsequent washing.

The extraction itself results in the conversion of insoluble precursor into soluble gelatin, by a process whose mechanism is not understood, but which probably involves, among other factors, thermal shrinkage of the precursor. Much of the original inter- and possibly intra-chain structure, dependent on hydrogen bonding, is lost or altered. In addition, occasional peptide bonds are probably broken, since the molecular weight of collagen, as determined by end-group methods, exceeds $1{\cdot}5 \times 10^6$ (Bowes & Moss, 1953), and gelatins have a number-average molecular weight of $5\text{–}7 \times 10^4$ (Pouradier & Venet, 1950; Courts, 1954). After extraction of gelatin an insoluble protein residue, which is clearly a contaminant of the original collagen, remains.

This theoretical picture of the collagen–gelatin conversion does not indicate any changes in amino acid composition except those resulting from removal of impurities. In assuming the amino acid composition of a gelatin as representative of a purified collagen, amide-nitrogen content should clearly be determined on native collagen, and ornithine present in gelatin which has been subjected to alkaline pretreatment should be interpreted as an equivalent proportion of arginine.

Composition of serially extracted gelatin

Differences in amino acid composition of gelatin extracted in the first and third liquors from limed ox hide did not greatly exceed the experimental error of the method (Table 2). The average molecular weights of these were identical as judged from the viscosity data (Table 1), although the molecular-weight distributions could be expected to be different (Stainsby, private communication); but the mechanical rigidity of gels of the same concentration, matured under identical conditions, were markedly different, as indicated by the Bloom jelly strength value, which is an arbitrary measure of gel rigidity. Unexplained anomalies of this type often occur in successively extracted gelatin samples. It is possible that, though there are no large differences in amino acid composition, there may be an overall difference in sequence with respect to the ends of the polypeptide chains resulting from a shift in the site of bond-breaking.

Composition of a purified gelatin

Ox-hide gelatins I and II, prepared from different batches of hide, do not differ markedly in composition. Gelatin II was purified by a process designed to remove (1) high- and low-viscosity fractions known to have abnormally low nitrogen contents and slight brown colours, (2) small ions and certain neutral molecules, e.g. glucose (Eastoe, unpublished work), absorbed on a mixture of strongly acid and strongly basic resins. After purification, only a slight change in the amino acid composition, of the same order as the experimental error, was observed, indicating a further slight trend, in the direction already described, on passing from collagen to gelatin.

The mean value for five to six determinations of the amino acid composition of ox-hide gelatin has been calculated (Tables 2 and 3) by ignoring differences in raw material, extraction, time of hydrolysis and purification treatment, all of which were small.

Comparison with previous results

The mean values, obtained in the present study, for the amino acid composition of ox-hide gelatin are compared in Table 5 with those published values which have appeared more recently than the earlier summary of Bowes & Kenten (1947). The values for ox-hide collagen compiled by Tristram (1953) are also included.

Agreement between the various methods is good both in the amino acid composition as a whole and in many of the individual values. The most marked discrepancies between the present values and previous ones occur for alanine and serine. The present value for alanine exceeds that of Tristram (1946) by 18% and those of Neuman (1949) by 24%. It is unlikely that all the present series of determinations are in error by such a large amount unless an unknown ninhydrin-positive substance coincides almost exactly with the alanine peak, which shows no abnormality in shape. A similar comment holds for serine, where the present value exceeds by some 32% that of Rees (1946), who used periodate oxidation. The present values are in good agreement with those of Bowes (private communication) for ox-hide collagen, obtained by using ion-exchange chromatography.

The tyrosine content of different gelatins varies a great deal. The present results, which indicate a distinctly lower tyrosine content for gelatin than for collagen preparations, are lower than most of the literature values, but are in good agreement with

Table 5. *Comparison of the mean of present values for the amino acid composition of ox-hide gelatin with recent literature values*

Values are expressed as g. of amino acid/100 g. of protein.

Reference	Present study	Tristram (1953)*	Tristram (1953)*	Neuman (1949)	Neuman (1949)	Graham, Waitkoff & Hiers (1949)	Robson & Selim (1953)
Material	Ox-hide gelatin	Gelatin	Ox-hide collagen	Calf-skin gelatin	'Difco Bacto' gelatin	Pig-skin gelatin	'Coignet' gelatin
Analytical method	Resin chromatography	Various chemical	Various chemical	Microbiological	Microbiological	Microbiological	Various chemical
Alanine	11·0	9·3	9·5	8·7	8·6	—	—
Glycine	27·5	26·9	27·2	26·9	25·7	—	—
Valine	2·59	3·3	3·4	2·6	2·8	2·5	—
Leucine	3·33	3·4	5·6 (Leucine + Isoleucine)	3·1	3·1	3·2	—
Isoleucine	1·72	1·8		1·9	1·5	1·4	—
Proline	16·35	14·8	15·1	14·0	16·3	18·0	—
Phenylalanine	2·23	2·55	2·5	1·9	2·3	2·2	—
Tyrosine	0·29	1·0	1·0	0·14	0·91	0·44	—
Serine	4·21	3·18	3·37	2·9	3·2	—	—
Threonine	2·22	2·2	2·28	2·2	2·0	1·9	—
Cystine	Trace	0·0	0·0	0·05	0·09	0·07	—
Methionine	0·89	0·9	0·8	0·85	0·92	1·0	—
Arginine	8·8	8·55	8·59	6·4	8·3	8·0	8·6
Histidine	0·78	0·73	0·74	0·63	0·85	0·79	0·69
Lysine	4·50	4·60	4·47	5·2	5·2	4·1	4·33
Aspartic acid	6·7	6·7	6·3	6·9	6·4	6·7	—
Glutamic acid	11·4	11·2	11·3	12·1	11·5	11·5	—
Hydroxyproline	14·1	14·5	14·0	14·4†	13·6†	—	—
Hydroxylysine	0·97	1·2	1·1	—	—	—	0·92

* Based mainly on Macpherson (1946) for basic amino acids, Rees (1946) for serine and threonine, Tristram (1946) for alanine, valine, leucines, proline, phenylalanine and tyrosine, and Bowes & Kenten (1948) for methionine and acidic amino acids, with later revisions.

† Neuman & Logan (1950).

determinations on the same samples made in this Laboratory (Kenchington, private communication) by the colorimetric method of Udenfriend & Cooper (1952). Harkness *et al.* (1954) found that the tyrosine contents of the various collagenous components of skin differ appreciably from one another; gelatin prepared from insoluble collagen contained more tyrosine than either alkali- or acid-soluble collagen. Fractionation of this gelatin by precipitation with trichloroacetic acid, however, reduced the tyrosine content from 1 to 0·5%. A corresponding reduction from 0·29 to 0·18% for ox-hide gelatin, after fractionation with ethanol, was found in the present study. This last value for tyrosine is close to that of 0·14% recorded by Neuman (1949) for a carefully purified calf-skin gelatin made by Eastman Kodak Ltd. It is possible that the main protein constituent of gelatin and collagen contains no tryosine, but the question cannot yet be regarded as settled.

The present values for valine are in agreement with those obtained by microbiological methods (Table 5), but are significantly lower than those obtained by Tristram (1946). The proline values obtained in this study are somewhat higher than those of Tristram (1946). The sample of proline used for the present calibration gave a 4% higher intensity of colour than that reported by Moore & Stein (1951) and was therefore considered to be substantially pure. The values for lysine in gelatin reported by Tristram (1953) may possibly be augmented by the presence of a small amount of ornithine, as is the present value. The high values reported by Neuman (1949) may result from the inability of the microbiological method to distinguish hydroxylysine from lysine. The present values for hydroxylysine in gelatin are slightly lower than most of those in the literature, obtained by chemical methods (Ramachandran, 1953).

Reticulin

Human renal reticulin, which contained myristic acid, carbohydrates and inorganic matter (Windrum *et al.* 1955), was hydrolysed without preliminary separation of the protein. The general picture of the amino acid composition of reticulin is quite similar to that of collagen; there are, however, some notable differences. The low values for alanine, glycine and proline, together with the high values for the leucines, phenylalanine and methionine, suggest that reticulin contains collagen accompanied by other proteins in smaller amounts. This is contradicted, however, by high values for hydroxyproline, hydroxylysine, serine and threonine. Examination of reticulin prepared from other sites in the body appears to be necessary for a full appraisal of its range of chemical composition.

Variation of collagen composition in mammals

The mammalian gelatins and collagens form a compact group as regards their amino acid composition. The placental land mammals man, ox and pig show few differences in composition, except for the low value for isoleucine in pig-skin gelatin.

The whale, on the other hand, shows striking increases in the hydroxyamino acids serine and threonine, compared with the land mammals. Even higher levels of these two amino acids are found in fish collagens (Neuman, 1949; Gustavson, 1955), combined with decreased levels of hydroxyproline and proline. Whales, which are descendants of land mammals, have probably lived in the sea since Eocene times or earlier (Young, 1950). The high content of hydroxyamino acids in whale collagen may perhaps therefore be attributed to the long-term effect of a marine diet.

The wallaby also differs from the other land mammals in having increased levels of serine and threonine. The effect is only slight compared with the whale, but may be of evolutionary significance, since the wallaby belongs to the marsupials, a group which has been separated from the placentals for a long period.

Achilles tendon is not a satisfactory material for the preparation of human collagen that is sufficiently pure for determinations of amino acid composition. It appears to contain substances which render it difficult to convert into gelatin. The strong acid extraction finally employed evidently dissolved considerable quantities of non-nitrogenous constituents, shown by the low recovery of material by weight, despite the complete recovery of nitrogen (Table 2). The increased hydroxylysine content and correspondingly diminished lysine value of this material is difficult to explain. The same effect is shown, but to a smaller degree, in wallaby tendon.

The specialized variations in composition of collagen from widely different mammalian species, superimposed on an otherwise fixed composition, suggests that, although collagen is built up according to a definite pattern, its structure is not absolutely fixed and amino acids in certain positions may be replaceable within limits by others.

Examination of the amino acid composition of collagen from a much wider range of animals may indicate that a proportion of the residues form an invariable framework throughout the series. Such information might be an important guide to the interpretation of X-ray-diffraction data and thus assist in resolving the complete picture of collagen structure.

SUMMARY

1. The amino acid composition of collagen or gelatin from five species of mammals (man, ox, pig, whale and wallaby) has been determined by ion-exchange chromatography.

2. The results obtained for ox-hide gelatin are in good agreement with previously published values, except for alanine and serine.

3. The composition of gelatin is closely similar to that of the collagen preparations, suggesting that gelatin is representative of the main protein constituent of collagenous tissues in amino acid composition.

4. The collagen of the land placentals studied did not show any marked variation between species. The content of serine and threonine in whale gelatin exceeded that in land mammals, but was not so high as that from other classes of marine vertebrates. Wallaby collagen also contained very slightly higher amounts of serine and threonine than the other land mammals.

5. There were no significant differences in amino acid composition between two gelatins, serially extracted from limed ox hide. These gelatins differed in rigidity properties, although viscosity measurements indicated that they had the same average molecular weight.

6. Gelatin contained less tyrosine than the collagenous precursors. Fractionation of gelatin with ethanol to remove impurities led to a further reduction in tyrosine content. It has not yet been proved that tyrosine is a true constituent of gelatin.

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