Studies on the Skeletal Tissues

2. THE COLLAGEN CONTENT OF BONES FROM RABBITS, OXEN AND HUMANS

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It has been recognized for many years that collagen forms an important constituent of the organic matrix both of bones and of the dentine of teeth, but precise analytical information has been scanty. Lightfoot & Coolidge (1948) examined the collagen content of the tissues of the guinea pig and concluded that 68–70% of the total nitrogen in the whole skeleton was collagen. No examination was made of individual bones. Other results, however (Weidmann & Rogers, 1950), have shown that, even when thoroughly cleaned, there is a difference in the calcium to nitrogen ratio between cancellous and cortical bone taken from the femora of small laboratory animals, and variations in the nitrogen and ash content have been shown even in different parts of the shafts of long bones from rabbits (Dallemagne, 1943) and from oxen (Strobino & Farr, 1949). Changes also occur in the ash content of the skeleton of animals with increasing age (Hammett, 1925; Hess, Berliner & Weinstock, 1931; Strobino & Farr, 1949), and in the phosphorus to nitrogen ratio (Roche & Garcia, 1936); Strobino & Farr (1949) quote unpublished results in support of an increase with age in the ash content of human bone. It is difficult to interpret these differences and changes in the absence of more precise knowledge of the nature of the matrix, since the total nitrogen content of bone indicates only the amount of total protein present and the value obtained may be influenced by occluded plasma or by a layer of periosteum not removed during cleaning. When ash content is measured these uncertainties become even greater since the presence of non-nitrogenous organic substances and carbon dioxide can contribute to the result obtained for the amount of matrix. It is thus impossible to be certain that variations in either calcium to nitrogen ratio or in ash content correspond to degrees of calcification of a single substance.

In the present work a study has been made of the 'collagen' content of different types of bone, with various calcium to nitrogen ratios, obtained from rabbits and oxen and also of femora from human subjects of different ages. It is believed that the results obtained indicate a variability in the degree of calcification of a matrix which consists predominantly of a single protein, present in all types of bone at all ages.

METHODS

Rabbit bones from healthy adult animals were dissected immediately after death. Oxen femora were obtained freshly from the slaughter house. The human samples were of mid-femur taken at autopsies performed on children and adults dead as the result of accident or of such acute illnesses as were thought, by a competent pathologist, not to affect the skeleton. The cleaning, drying and defatting of all the samples was carried out as previously described (Weidmann & Rogers, 1950). Instead of grinding the bones with a dental drill as before, they were crushed between steel surfaces, a modification necessary to eliminate heat production which leads to the formation of considerable amounts of water-soluble nitrogen (Stack, 1950*a*).

The collagen content of the crushed, dried and defatted samples was estimated as follows. The powders were decalcified with N-HCl (15 ml./g. powder) for 18 hr. at 0–4°. The insoluble materials were removed by centrifugation, washed three times with water, each time adjusting the pH of the suspensions to 7·0, resuspended in 10 ml. of water and autoclaved for 2 hr. at 20 lb. pressure. The suspensions were then centrifuged whilst still hot, and the precipitates washed thrice with 10 ml. amounts of boiling distilled water. The original supernatants and the washings were cooled to 15° and made up to 50 ml. or to a suitable fraction of this volume if less than 1 g. of crushed bone had been available in the first instance. Samples (2·5 ml.) were taken from each of these solutions into centrifuge tubes, the pH adjusted to 4·6 with N-HCl, using bromocresol green as indicator, and 5·0 ml. amounts of 5·0% (w/v) tannic acid added. The mixtures were cooled for 1 hr. at 0–4°, centrifuged, and the precipitates dissolved in 5·0 ml. of 2 N-NaOH by breaking them up with a glass rod and warming (3–5 min.) in a boiling-water bath. Total nitrogen estimations (two from each sample) were made on the solutions by the micro-Kjeldahl technique using copper selenite and potassium sulphate as oxidation catalysts in the preliminary incineration which was continued for 18 hr. This method for collagen estimation is a necessary modification of that used by Lightfoot & Coolidge (1948) since, both in our experience and in that of Stack (1950*b*), attempts to extract gelatin from whole bone powder with the technique used by these authors lead to very low recoveries of nitrogen owing to adsorption of the proteins on the insoluble inorganic salts.

Total nitrogen estimations were also made on the samples of the whole bone powder, the decalcifying fluid and on the material not rendered soluble by autoclaving. Two estimations were made for each sample, and the results throughout this paper are given as the mean of these duplicates. The overall recovery of nitrogen for several of the samples recorded in this paper is slightly greater than 100%. It is presumed that this is partly due to the sampling error involved in determinations of total nitrogen in the bone powders and partly to the normal distribution curve of the experimental error. The average recovery of nitrogen throughout all the experiments performed is 99%.

Phenolic compounds, presumably tyrosine, in the material rendered soluble by autoclaving were estimated by use of the Folin & Ciocalteu phenol reagent. A preliminary treatment of the solutions for 15 min. with 0·5 N-NaOH (equal volumes of the solutions and N-NaOH were mixed) at 100° was found to be necessary in order to prevent the precipitation which otherwise occurred when the phenol reagent was added.

RESULTS

Collagen content of rabbit bones

The results expressed in Table 1 show that from 89 to 97% of the total nitrogen in all the rabbit bones examined is present as collagen, or exists in such a form in the decalcified matrix that it can be removed by autoclaving at neutral pH and subsequently precipitated by tannic acid. There is certainly no suggestion that there is less collagen in bones with a relatively high nitrogen content. For example, the cancellous bones of the femurs examined contain 4·5 and 4·1% nitrogen, whereas the shafts from this bone contain only 3·0 and 2·8%, yet the proportion of collagen in the cancellous structures is higher than that in the shaft. The differences are small and a large group of animals would have to be examined to verify any general statement, but it appears that there is a tendency for there to be a slightly higher proportion of collagen in the cancellous than in the shaft bone.

A further indication of this tendency is given by the amount of nitrogen from the matrix which is not brought into solution by autoclaving. The cancellous tissues from the long bones contain only 0·6–1·1%, the hard shaft bone 2–8%, of the nitrogen in this form. A preliminary examination (Rogers & Hall, 1951) of the amino-acid composition of the hydrolysed insoluble material, which will be reported at greater length in a later publication, suggests that it may contain a high proportion of elastin. If this is so, then the presence of larger amounts in the shaft bone is understandable, since the walls of the numerous blood vessels will contribute elastin, whereas all except the largest trabeculae are probably free of vessels. The material rendered soluble by autoclaving shows an amino-acid pattern similar to that for skin collagen (Bowes & Kenten, 1949).

There are no regular differences in the small amounts of nitrogen which are dissolved from different types of bone during decalcification. Fractionation and examination of this latter material shows that it contains some collagen and some nitrogen volatile when the solution is made alkaline. The latter is presumed to be ammonia. It may be noted that vigorous washing with water will remove an amount of total nitrogen similar to that removed by the acid during decalcification.

An examination of the amount of phenolic substances present in the crude gelatin solutions was made because it is known that collagen prepared in a highly purified state from various sources has a low content of aromatic amino-acids. Neumann (1949), for example, quotes values of 2·3–2·6% for phenylalanine, 0·86–1·1% for tyrosine and $<0·01$% for tryptophan in samples of collagen prepared from eleven different sources. Moreover, a low content of substances absorbing ultraviolet light at a wavelength of 2575–2875 A. has been suggested (Loofbourow, Gould & Sizer, 1949) as a criterion for the purity of collagen. The figures in column 10 of Table 1 are the amounts of phenolic substances (expressed as μg. tyrosine/mg. total nitrogen) present in the solution of the materials removed

Table 1. *Collagen content of samples of rabbit bones*

(The results recorded under I are for bones from a single animal, whilst those under II are for powders prepared as pools from bones of three animals.)

	mg. N/g. dry defatted bone powder								Recovery of N (%)		Tyrosine (μg./ml.) / Total N (mg./ml.)
	Total*		Soluble†		Collagen		Insoluble‡				
Type of bone	I	II	I	II	I	II	I	II	I	II	II
Femur shaft	30·2	28·3	1·25	0·95	26·9	26·4	2·41	1·72	101	102	91·5
Femur cancellous	45·3	41·3	2·03	0·93	43·8	—	0·28	0·46	101	—	73·2
Tibia shaft	36·3	33·4	1·07	0·99	32·8	29·7	1·92	0·68	93	94	84·0
Tibia cancellous	47·2	42·9	—	1·16	—	42·0	—	0·28	—	101	74·2
Scapula compact	44·2	38·7	1·0	0·79	41·8	36·1	1·67	1·96	100	100	65·5
Scapula blade	50·9	45·6	0·84	0·82	49·2	—	0·35	0·44	99	—	77·1
Vertebrae cancellous	41·9	46·7	1·50	1·11	40·3	44·8	0·31	0·50	100	99	81·9

* The total N content of 1 g. of dry defatted bone powder in mg.
† Found in the decalcifying fluid.
‡ Not rendered soluble during autoclaving.

from the decalcified bone by autoclaving. It will be seen that there is a considerable degree of variation in the values obtained. Two explanations are possible: either the amino-acid composition of collagen may vary according to its source, or, more probably, small amounts of proteins, polypeptides or amino-acids other than collagen and its derivatives may be brought into solution when the matrix is autoclaved. It is of some interest that the solutions prepared from the cancellous bone contain less phenolic substances than those from shaft bone. Thus it seems likely that the matrix of the cancellous bones not only contains more material rendered soluble by autoclaving, but also less of the contaminating substances, containing a higher proportion of aromatic amino-acids, which can also be brought into solution during the process. This matter will be discussed at greater length below.

The collagen content of oxen bones

According to Dallemagne (1943) and to Strobino & Farr (1949) there is a maximum in ash and minimum in nitrogen content at a characteristic point in each shaft bone from rabbits and oxen. It seems therefore of importance to know the proportion of collagen in various parts of such bones. In order to do this disks of bone from ox femora were cut as indicated in Fig. 1*a*. Three samples were then chipped from each of the disks (Fig. 1*b*) except from that of the epiphyseal bone which was sampled from the centre of the disk and well away from its cartilagenous edge. Thus, altogether, thirteen samples were taken, each of which was crushed, defatted and analysed as previously described.

The results obtained from a typical experiment are shown in Table 2. It will be seen that, as Strobino & Farr (1949) reported, there is considerable variation in nitrogen content. The values for collagen are similar to those shown in Table 1 for the rabbit bones, with the exception that there is less evidence for a higher proportion of collagen in the samples with a high nitrogen content. In all the samples the collagen nitrogen makes up about 91–96 % of the total. There is again some fluctuation in the amount of material not rendered soluble by autoclaving, but the relationship between this and the total nitrogen in the bone is not so clear as it is in the rabbit skeleton. For example, despite the high nitrogen in the epiphyseal sample, the 'autoclave insoluble' material makes up about 1 % of the total as compared with 2–4 % for the samples of shaft bone with the lowest total nitrogen.

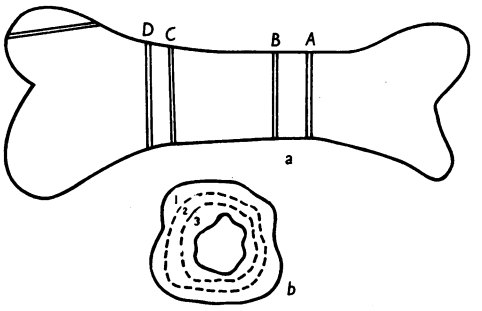

Fig. 1. Method of sampling ox femora.

Consideration of the amount of phenolic substances present in the gelatin solutions prepared from the oxen bones together with the results obtained using rabbit bones leads to an interesting conclusion graphically represented in Fig. 2. It will be seen that there is indication of an inverse correlation between the amount of total nitrogen and the proportion of phenolic substances. Thus the pro-

Table 2. *Collagen content of samples of bone from different parts of an oxen femur*

(See Fig. 1*a* and *b* for the position of the samples.)

Sample	mg. N/g. dry defatted bone powder				Recovery of N (%)	Tyrosine (μg./ml.) / Total N (mg./ml.)
	Total*	Soluble†	Collagen	Insoluble‡		
A 1	36·9	0·88	33·4	1·57	97	69·7
A 2	36·2	0·67	33·0	1·54	97	73·2
A 3	38·2	0·73	36·4	0·98	100	83·8
B 1	35·8	0·88	34·2	1·49	102	82·9
B 2	35·1	0·71	33·7	0·78	100	76·2
B 3	37·3	0·97	34·8	0·69	98	86·8
C 1	35·8	0·98	33·9	0·70	99	70·0
C 2	39·1	0·91	35·8	1·54	98	55·7
C 3	42·2	0·86	39·5	1·10	98	53·2
D 1	38·5	0·99	35·8	0·78	99	58·1
D 2	40·2	0·88	38·6	0·90	100	59·6
D 3	41·9	1·06	40·3	0·96	100	69·7
Epiphysis	46·2	0·85	44·2	0·64	99	85·7

* Total N content of 1 g. of dry defatted bone powder in mg.
† Found in the decalcifying fluid.
‡ Not rendered soluble by autoclaving.

visional conclusions drawn from the results for the rabbit skeleton are confirmed and it seems probable that in these two species of animal the higher the nitrogen content of the bone the lower will be the degree of contamination of the collagen matrix with small amounts of other proteins, relatively rich in aromatic amino-acids.

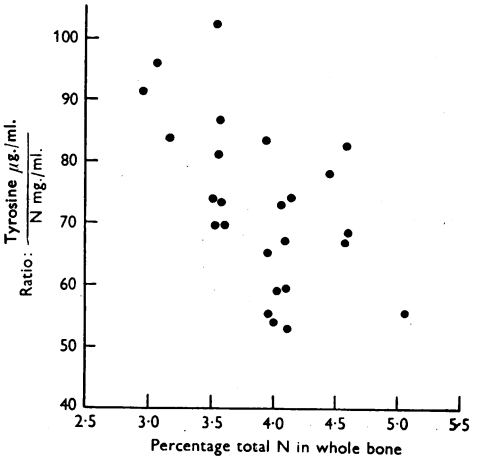

Fig. 2. Scatter diagram showing the relationship between total N in the bone and the proportion of phenolic substances in the autoclave-soluble material.

Changes in the calcium to nitrogen ratio and the collagen content of samples of human femur

Preliminary results (Rogers, 1949), with a few samples, suggested that there was an increase in the calcium to nitrogen ratio with the age of the subject from which femur samples had been taken. In the present series some forty samples have been taken from subjects ranging in age from 9 months to 90 years, and in Fig. 3 the results for calcium to nitrogen ratios are plotted against age. It will be seen that there is a steep rise in this ratio up to an age of about 20–30 years which corresponds to the time of closure of the epiphyses. Later in life there may still be a very slow rise, though the spread of the figures is too great to allow any definite conclusion to be drawn. If, however, the figures are plotted semi-logarithmically they fall on an approximately straight line which suggests that the increase is continuous throughout life. No evidence was found for a decalcification of the bone in advanced age, and thus the reduction in radiographic density which is sometimes referred to as decalcification is probably due largely to a reduction in the amount of bone and not to any change in its degree of calcification. These results agree with the statement made by Strobino & Farr (1949) and are paralleled by their results for oxen bones. They are, however, in conflict with Vogt's (1949) findings. The latter author examined

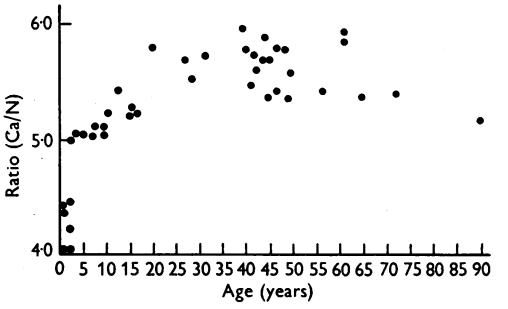

Fig. 3. Scatter diagram showing the relationship between Ca/N ratio and the age of the human subject from which the bone was taken.

the ash content of samples of iliac crest taken by biopsy from normal subjects of from 2 days to 73 years of age. He could detect no significant differences between the samples. It may be that not all the bones in the skeleton alter in their content of ash with age. These latter results would nevertheless be more convincing if some other constituent, such as total nitrogen which varies inversely with the ash content, had been measured at the same time, since one parameter alone is liable to be influenced by a number of factors such as the completeness of drying and cleaning of the samples.

A selection of the samples of human femur has been examined for collagen content by the usual technique. Table 3 shows the results obtained; as

Table 3. *Collagen content of samples of human femur taken from subjects of various ages*

Age of subject (years)	Sex	mg. N/g. dry defatted bone powder				Recovery of N (%)	Tyrosine (μg./ml.) / Total N (mg./ml.)
		Total*	Soluble†	Collagen	Insoluble‡		
2·5	M.	48·3	1·01	46·4	2·02	102	65·6
7	M.	49·7	0·94	47·7	0·42	99	69·0
10	M.	49·3	1·03	44·2	1·99	96	69·6
15	M.	47·8	0·68	44·3	1·38	97	72·6
27	M.	47·3	0·87	45·2	0·68	99	73·3
41	F.	46·5	0·95	43·6	1·49	99	75·9
65	M.	46·7	1·02	41·3	3·11	97	70·2

* Total N content of 1 g. of dry defatted bone powder in mg.
† Found in the decalcifying fluid.
‡ Not rendered soluble during autoclaving.

with the bones from the other species 89–96 % of the total nitrogen behaves as collagen. There is wide fluctuation in the values for the small amounts of material not rendered soluble by autoclaving, but there is no correlation with the age of the subjects from which the samples were obtained. No evidence can be found for the presence of a lower proportion of collagen in the bones from younger subjects having a lower ratio of calcium to nitrogen, and there is no trend in the reverse direction as there is with the animal bones. There is also no significant variation in the concentration of phenolic substances in the autoclave extracts; with one exception, the bone from the infant 2·5 years of age, the ratios of tyrosine in μg.: total N in mg. agree with the average value for the series to within $\pm 2{\cdot}1$ %. Thus there is no evidence for the presence of more contaminating protein of any kind in the collagen matrix from bones of young subjects than in that from the older ones.

DISCUSSION

Such a survey as this might clearly be extended almost indefinitely through the skeletons of any number of species. It is thought, however, that sufficient has been done to justify the statement that the organic matrix of bones from skeletons of mammals including *Homo sapiens* consists almost exclusively of a protein which is rendered soluble by autoclaving at neutral reaction and is subsequently precipitable by tannic acid. Such a protein can be considered to be a collagen, and this supposition is supported by preliminary examinations of its amino-acid content. Variations in the ratio of calcium to nitrogen, whether due to type of bone or to age of subject, can be safely regarded as differences in degree of calcification of this collagen. Thus the differences observed by Dallemagne (1943), Strobino & Farr (1949) and Weidmann & Rogers (1950) can be said to reflect differences in degree of calcification. Hitherto the criticisms made by Baker, Butterworth & Langley (1946) of claims for such variations were difficult to refute. The cleaning of intricate structures such as the epiphysis and metaphysis is difficult and the inclusion of minute amounts of extraneous protein would be sufficient to cause a marked alteration in the calcium to nitrogen ratio. The latter authors' own results for cancellous and cortical human bone gave point to these criticisms since they found that if the cleaning were adequate then the calcium to nitrogen values for different types of bone showed no statistically significant difference.

The question naturally arises as to how far collagens from the different bones are identical both with one another and with this type of protein isolated from other sites in the body such as the skin. We have obtained no results which will give guidance in this matter since it seems unlikely that the variations in the concentration of phenolic substances in the crude autoclaved extracts from the bone matrix indicate differences in the amino-acid composition of the collagens. It seems more likely that they are due to lack of specificity in the technique for collagen estimation and to consequent inclusion of small amounts of other proteins rich in aromatic amino-acids together with the collagen. As far as the relation between skeletal collagen and that from other sites in the body is concerned, Neumann's (1949) thorough examination of this protein from a variety of sources including bone shows that, although there are small variations, these are of dubious significance and that the amino-acid composition is probably constant.

Two sets of correlations have been observed in examining the results for animal bones. First, the higher the total nitrogen in the bone the lower is the content of phenolic substances in the autoclave extracts. Secondly the higher the proportion of collagen in the bone the lower are the amounts of substances not rendered soluble by autoclaving. Both these relationships can be adequately explained by the presence of a greater number of blood vessels in some samples than in others. The walls of the vessels might be expected to contribute proteins not rendered soluble by autoclaving. The plasma in the vessels will contribute polypeptides and proteins containing higher concentrations of aromatic amino-acids. This hypothesis is consistent with the presence of larger numbers of vessels in the shaft bone than in the epiphysis, and is supported by the larger content of phenolic substances and of 'autoclave-insoluble' nitrogen present in the former type of bone from rabbits.

SUMMARY

1. The organic matrix in the bones of rabbits, oxen and humans consists of from 90 to 96 % collagen, or collagen-like proteins.

2. Variations in the amount of total nitrogen relative to calcium in bones correspond to true differences in degree of calcification, and not to the inclusion of proteins other than collagen.

3. The degree of calcification of mid-femur samples of human bone increases with increasing age of the subject. This process is rapid up to the age of 20–25 years, but probably continues much more slowly throughout life.

4. The crude gelatin extracts from those rabbit and oxen bones which have a low calcium to nitrogen ratio are less contaminated by small amounts of other proteins than are those from the more highly calcified structures. This may possibly be correlated with the presence of blood vessels in the latter.

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A Search for Specific Chemical Methods for Fission of Peptide Bonds

1. THE *N*-ACYL TO *O*-ACYL TRANSFORMATION IN THE DEGRADATION OF SILK FIBROIN

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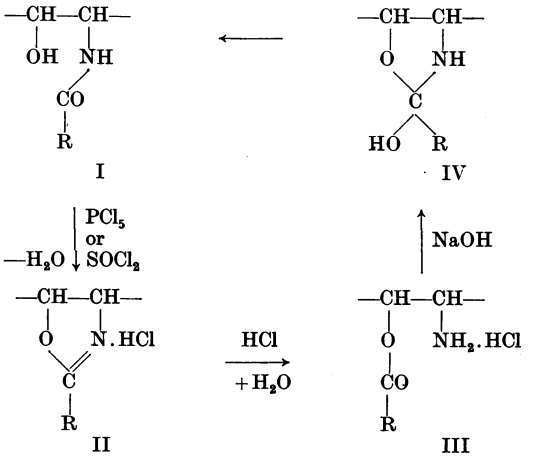
Partial degradation of proteins or polypeptides by chemical or enzymic hydrolysis is lacking in specificity and results obtained in this way have generally been very complex and difficult to interpret. Interest in the chemistry of the natural amino-acids has quickened considerably in recent years, the more complex amino-acids receiving particular attention. In the light of this recent knowledge it seems possible that the polypeptide chain could be broken in a predictable fashion by using a reagent showing selective reactivity with one amino-acid. It would be expected that only those amino-acids possessing functional groups in addition to the α-amino and carboxyl groups could be specifically attacked. The results described below show that serine displays distinctive chemical reactivity of the required type.

The migration of acyl groups in acyl derivatives of substances having a primary amino group and a hydroxyl group on adjacent carbon atoms was first observed in an aliphatic system by Bergmann, Brand & Dreyer (1921). Later, Bergmann & Miekeley (1924) demonstrated similar transformations in acyl derivatives of serine. The transformation of an *N*-acyl derivative (I) to an *O*-acyl derivative (III) and reconversion of the latter to the *N*-acyl derivative involved a number of steps which Bergmann, Brand & Weinmann (1923) showed to be as follows:



Compounds of types II and III were capable of independent existence and several examples were described by Bergmann and his collaborators, but compounds of type IV were assumed to have merely a transitory existence. Bergmann *et al.* (1923) were aware of the implications of their work in the protein field and put forward the suggestion that linkages such as those represented in II and IV might be found in proteins. Bergmann & Miekeley (1924) tried to substantiate this view by preparing an