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# FOSSIL PROTEINS IN VERTEBRATE CALCIFIED TISSUES

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[Plate 1]

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Selected fossil vertebrates and the enclosing sediments dating from 1300 years B.C. to approximately 400 million years ago were subjected to amino acid assay. The amino acid analyses revealed little evidence of intact collagen in fossils of Tertiary, Mesozoic

or Palaeozoic age. There was, however, evidence of contemporary proteinaceous material which may have been derived from bacteria. In Palaeozoic material the analyses detected a general background of amino acids common to both fossils and sediments. The degree of racemization was routinely determined as a means of measuring modern contamination of geologically older samples. An electron microscope study of Quaternary (Pleistocene) collagen revealed a significant reduction of the 64 nm banding to about 50 nm. The same Pleistocene material gave amino acid compositional profiles typical of collagen. However, when this material was subjected to digestion by the proteolytic enzymes collagenase, pronase and subtilisin, the resulting peptide fingerprints showed small but significant differences from those obtained from modern collagen digests, indicating the possibility of changes having occurred during fossilization affecting susceptible cleavage sites in the molecule.

## 1. INTRODUCTION

Extraction and qualitative identification of amino acids from various fossil vertebrates of different geological ages by Abelson (1954a, b, 1955, 1956, 1957, 1963) opened up a new field of research in biogeochemistry. The detection by Erdman et al. (1956) of the same amino acids in sediments emphasized the importance of obtaining quantitative analyses before meaningful conclusions could be made. The first detailed quantitative analyses, on Pleistocene bone from Rancho la Brea tar pits, were published by Ho (1965, 1966, 1967), working in Wyckoff's laboratory. Subsequent analyses of Pleistocene bones and teeth have also suggested that collagen is a protein capable of surviving the vicissitudes of fossilization and geological time (Bucci et al. 1969; Wyckoff 1972; Dungworth et al. 1975). Analyses of more ancient fossils have been published by various authors, for example Armstrong (1966), Armstrong & Halstead Tarlo (1966), Halstead Tarlo (1967), Kjellstrom (1971), Matter & Miller (1972), Wyckoff (1972), Ferdinand et al. (1973), Jope (1976) and Jope & Jope (1979), and have shown significant differences from modern collagen in their amino acid compositional profiles. Recent related reviews are by Jope (1980) and Wyckoff (1980). In much the same way, the amino acid analyses of Pleistocene woolly mammoth hair by Gillespie (1970) showed it to be similar to modern keratin, whereas the analyses of 35 million year old fossil material by Halstead & Wood (1973) revealed a number of significant differences.

The interest in the existence of fossil protein is that detailed studies may contribute eventually to our knowledge of protein evolution. A number of significant studies have been made in this area, such as the pioneering theoretical work of Zuckerkandl (1965) and Zuckerkandl & Pauling (1965), who compared the sequence of amino acids along the haemoglobin polypeptide chains from one species to another, and also the different chain types within a single species. From such studies relationships can be drawn up and the numbers of effective amino acid substitutions can be related to base changes in the genetic code of the DNA molecule. The relation of these hypotheses to the fossil record has been discussed briefly by Halstead (1974). A more extensive study of the primary structure of a well known protein in numerous species, in this case myoglobin, has recently been published by Romero-Herrera et al. (1978).

Similar studies have not been extensively undertaken yet for collagen, whose tropocollagen subunit is triple-stranded, with each strand over 1000 amino acid residues long; however, the primary structure has been determined for a few samples (Fietzek et al. 1972; Hulmes et al. 1973). There is every likelihood that it will be possible to construct comparable evolutionary schemes for collagens, with the use of automated amino acid sequencing techniques. The work of

Pikkarainen (1968) showed that collagens differ between groups of organisms, and hence useful comparisons could be made between primary structures of different collagens.

The existence of fossil collagens raises the possibility of testing the evolutionary hypotheses derived from comparative studies of modern proteins, by observations on proteins still preserved in fossil bones and teeth. Any attempt to investigate possible evolutionary changes through studies on fossil proteins requires that a number of fundamental problems first be resolved To recognize any changes due to evolution, it is necessary to eliminate all possible sources of error, such as might be due to, for example, contamination or the differential breakdown of particular amino acids. Until these have been eliminated, or allowed for, there is little of value to be gained from attempting interpretation of possible evolutionary changes. The discovery that ancient sediments contained amino acids made it necessary, as a control and reference point, to analyse not only the fossil but also its enclosing sediment, as previously emphasized by Armstrong & Halstead Tarlo (1966).

In the present investigation both soluble and insoluble amino acid residue components were extracted from the fossils and the enclosing sediments, and their hydrolysate components were compared. With such routine controls, it was possible to determine the possible levels of organic matter contributed from the depositional environments. As in the work of Wyckoff (1972), many amino acid compositional profiles showed similarities to bacterial amino acid hydrolysates and it is tempting to suggest that the predominant components of fossil bones and teeth are derived in many cases from ancient bacterial contamination, consequent upon the normal processes of disintegration and breakdown following the death of the vertebrate.

Because of the minute amounts of organic matter that occur in either fossils or enclosing sediments, one of the most difficult aspects is the determination of modern contamination. Fortunately, a method is available for determination of contamination of geologically older material. Apart from glycine, all amino acids have asymetrically substituted carbon-2 atoms and can exist as two stereoisomers, D and L. All living things tend to consist of amino acids of the L configuration, although some D forms are found, for example in some bacterial cell walls. Hare and coworkers (Hare & Mitterer 1967, 1969; Hare & Abelson 1968) established that in fossil shells the amino acids had become partially racemized. As a result, instead of the amino acids being entirely in the L configuration, a proportion are in the D configuration form. They discovered that there was an increase with time after death and during fossilization of the proportion of D amino acids until an equilibrium of both D and L forms was attained at between 20 and 30 million years. It follows that all ancient materials that are older than this and yet still possess a preponderance of the L amino acids are likely to have been contaminated by modern organic matter: hence analyses of such specimens become automatically suspect. In the present study the degree of racemization has been monitored, wherever possible, for the samples. This serves as an index of the reliability of the analyses, because it measures the extent to which the samples have been subjected to modern contamination.

Identification and the quantification of the amino acid components in fossil material requires the complete breakdown of the intact protein or peptide fragment into its constituent amino acids. The presence of amino acids in fossil material having been established, the next step is to examine the characteristic features of the intact protein.

The first hint of the survival of a remnant of organic matrix in fossil teeth and bones was Bennett's report (included in Humphrey (1908)) on the decalcification of fossil fish material at the British Museum (Natural History) in London. Subsequently, work on sectioned decalcified

fossils was published by Moss (1961), Halstead Tarlo & Mercer (1961, 1966), Little *et al.* (1962) and Isaacs *et al.* (1963). It was, however, Wyckoff *et al.* (1963) and Shackleford & Wyckoff (1964) who, for their examination of decalcified fossil bone and dentine, inferred the existence of intact collagen molecules by demonstrating the characteristic banding under the electron microscope. They observed that the 64 nm banding of contemporary native collagen was reduced to about 60 nm in Pleistocene material and to 50 nm in the Miocene fossils. In 1966 Doberenz & Lund, again from electron microscopical examination, claimed the survival of intact collagen from fossil fish of Lower Jurassic age, approximately 180 million years. This was a remarkable result, since analyses of vertebrate fossils from the same sediments did not give a result suggestive of collagen. By far the most exciting electron microscopical study is that of Towe & Urbanek (1972), in which collagen-like structures were identified from the periderm of Ordovician (500 million year old) graptolites. The fibrillar structure was perfectly preserved and was examined in great detail under the electron microscope. The physical form of the protein had been perfectly replicated during the process of fossilization but there were no surviving amino acids. Hence, a detailed electron microscopical study portraying the characteristic electron micrographic structure of collagen may not in fact be evidence of its presence as an actual organic component. For this to be ascertained, biochemical analysis is required.

The recent development, by Lowenstein (1980), of a radioimmunoassay technique for an immunological investigation of fossil collagens perhaps offers promise as a means of investigating possible phylogenetic relationships between fossil specimens.

Further advances in the study of fossil proteins and their evolutionary significance should also come from enzymic degradation studies on intact fossil proteins. The preparation of peptide digests by using proteolytic enzymes such as collagenase, pronase and subtilisin enables a form of peptide 'fingerprinting' of fossil collagen to be achieved. Detailed peptide analyses have now been undertaken by us on Pleistocene fossil bone for which both amino acid compositional profiles and electron micrographs of the identical bone were available. In spite of the close identity of the amino acid compositional profile with collagen, the fossil peptide fingerprinting revealed a number of significant differences, which were possibly a consequence of a geological ageing or fossilization process. The results here reported suggest that a most fruitful line for further investigation of fossil proteins would be in this area of peptide fingerprinting, coupled with amino acid analysis and sequencing studies on those peptides of special interest.

## 2. Amino acid assay

### (a) Materials and methods

The materials subjected to amino acid assay are listed in table 1.

### (i) Preparation of fossil material

Whenever possible both the fossil and its adjacent sediment were analysed. In all handling of the samples, precautions were taken, routinely, to avoid direct manual contact with the specimens. Polyethylene gloves were worn, and the exterior surface of specimens was cut away before analysis, in an attempt to minimize errors from contamination. Where possible, at least 5 g of fossil material were processed, though in some instances less than 1 g was available, and this was used.

A sample selected for analysis was ground to a fine powder, usually in a vertically oscillating

TABLE 1. MATERIALS SUBJECTED TO AMINO ACID ASSAY

| specimen no. | organism | sediment provenance | geological age |
|---|---|---|---|
| | | *Caenozoic specimens* | |
| 1 | ungulate (*Equus*) | Thames gravel, U.K. (from Mr P. F. Whitehead, Pershore) | Upper Pleistocene |
| 2 | *Homo sapiens* | Megalithic burial, U.K. | 1300–1700 B.C. |
| 3 | woolly mammoth (*Mammuthus primigenius*) | Thames gravel, U.K. | Upper Pleistocene |
| 4 | woolly rhinoceros (*Coelodonta*) | Kent's Cavern, Devon; cave breccia (from Dr R. M. C. Eagar, Manchester Museum) | Upper Pleistocene |
| 5 | woolly rhinoceros (*Coelodonta*) | Kent's Cavern, Devon; cave breccia (from Dr A. J. Sutcliffe, British Museum (Natural History), London) | Upper Pleistocene |
| 6 | *Australopithecus* | Swartkrans, South Africa; cave breccia | Lower Pleistocene |
| 7 | turtle carapace (*Emys*) | Bembridge Marls, Isle of Wight | Oligocene |
| | | *Mesozoic specimens* | |
| 8 | dinosaur | Mongolia (from Prof. Zofia Kielan-Jaworowska, Warsaw) | Upper Cretaceous |
| 9 | siluroid fish | U.S.S.R. (from Prof. D. Obruchev, Moscow) | Upper Cretaceous |
| 10 | teleost fish (*Pachythrissops*) | Wealden sandstone, Sussex (from Prof. P. Allen, Reading) | Lower Cretaceous |
| 11 | dinosaur (*Cetiosaurus*) | Inferior Oolite limestone, Oxfordshire (from Dr W. S. McKerrow, Oxford) | Middle Jurassic |
| 12 | ichthyosaur | Liassic shales, Dorset (from Mr R. A. Langham, Reading) | Lower Jurassic |
| 13 | holostean fish | U.S.S.R. mudstone (from Prof. D. Obruchev, Moscow) | Lower Triassic |
| | | *Palaeozoic specimens* | |
| 14 | placoderm fish (*Plourdosteus*) | U.S.S.R. sandstone (from Prof. D. Obruchev, Moscow) | Upper Devonian |
| 15 | placoderm fish (*Plourdosteus*) | limestone (from Prof. D. Obruchev, Moscow) | Upper Devonian |
| 16 | placoderm fish (*Plourdosteus*) | sandstone/clay (from Prof. D. Obruchev, Moscow) | Upper Devonian |
| 17 | placoderm (*Wijdeaspis*) | Spitzbergen, calcareous shales (from Prof. D. Obruchev, Moscow) | Middle Devonian |
| 18 | conodonts | (from Prof. F. G. E. Pautard, Leeds) | Devonian |
| | | *'Living fossils'* | |
| 19 | coelacanth (*Latimeria*) | | Recent |
| 20 | coelacanth (*Macropoma*) | | Cretaceous |
| 21 | lungfish (*Neoceratodus*) | | Recent |
| 22 | lungfish (Protopterus) | | Recent |
| 23 | lungfish (Lepidosiren) | | Recent |
| 24 | lungfish (*Chirodipterus*) | | Upper Devonian |
| 25 | rhynchocephalian lizard (*Sphenodon*) | | Recent |

Specimens 19–25 were supplied by Dr M. Meredith-Smith, London.

steel ball mill (Glen Greston Micropulverisor). The powdered material was placed overnight in 1 M HCl (Aristar) at 4 °C, 10 ml of acid being used per gram of sample. The mixture was centrifuged, the insoluble residue being designated as fraction I in the subsequent analyses. The 1 M HCl supernatant, which contained a fine suspension, was divided into two equal portions, fractions II and III. Fractions I and II were evaporated to dryness, dissolved in 6 M HCl (Aristar) and hydrolysed in a sealed ampoule, under nitrogen, at 110 °C for 18 h. They were then evaporated to dryness in a vacuum desiccator. Thus for each fossil sample three analyses were made wherever possible, i.e.:

fraction I, insoluble residue (hydrolysed);
fraction II, supernatant (hydrolysed);
fraction III, supernatant (not hydrolysed).

Fractions I–III were each subjected to amino acid analysis after an initial desalting stage described below. This removal of cations was essential, as their presence severely distorts the elution profile of the amino acids from the ion exchange chromatography column.

### (ii) *Desalting*

Several methods were investigated for the removal of cations from amino acid mixtures. None of these proved entirely satisfactory as some loss of amino acids always occurred. An 'ion retardation resin' method (Biograd AG11A8) was first tried, but found to be not reliably reproducible. Next an ion exchange resin (Amberlite IR120) was used, followed by a regeneration–elution step. Initially, promising results were obtained, though recoveries of the basic amino acids in particular decreased after the resin had been used a number of times. Eventually it was found that the best results were obtained by using an ion exchange resin only once, and then discarding it. The resin, Zerolit 255 (S.R.C. 13, B.D.H., U.K.), was found to be the most suitable, and acceptably reproducible results were obtained with the following technique.

*Preparation of resin.* Resin (100 g) was stirred with 1 l of 1 M NaOH for 1 h on a boiling water bath. After decanting, the resin was next stirred in a beaker over a boiling water bath, with 1 l of 0.2 M EDTA, adjusted to pH 9. The resin was then washed and then stirred with 1 l of distilled deionized water and then stirred with five portions of 5 M HCl, over a boiling water bath. Finally, the resin was washed free of acid with distilled, deionized water. The resin was stored at 4 °C.

*Desalting of fossil sample.* (1) Glass-distilled deionized water was passed through the column until the eluate was near neutral pH.

(2) The sample (volume 1 ml or less) was then loaded on top of the resin bed and the flow rate was adjusted to 1 ml min$^{-1}$ ($\pm 0.1$ ml min$^{-1}$). Distilled deionized water was then added to the top of the column and the pH of the eluate was tested. When the eluate again approached neutral pH, the flow was stopped, and a 0.2 M piperidine solution was placed in the column reservoir. It was necessary to re-distil piperidine before use, to remove ninhydrin-positive breakdown products, which interfered with interpretation of the amino acid analyses. Piperidine was allowed to flow at 1.0 ml min$^{-1}$ ($\pm 0.1$ ml min$^{-1}$) until the colour that it induced in the resin bed approached the bottom of the column. At this stage the eluant was changed to NH$_4$OH, and collection of eluate was begun. Elution was continued at the same rate, and 200 ml of eluate were collected. The combined technique of using piperidine, followed by concentrated

ammonia, for elution of amino acids from the column gave better results than using either eluant exclusively. It gave reliable removal of metallic cations, and the amino acid recoveries as shown in table 2 were reproducible.

TABLE 2. AMINO ACID RECOVERY FROM DESALTING COLUMN (ZEROLIT 255)

| | recovery (%) | | recovery (%) |
|---|---|---|---|
| aspartic acid (Asp) | 85.9 | isoleucine (Ile) | 79.5 |
| threonine (Thr) | 85.8 | leucine (Leu) | 86.2 |
| serine (Ser) | 92.9 | tyrosine (Tyr) | 78.5 |
| glutamic acid (Glu) | 84.4 | phenylalanine (Phe) | 77.8 |
| proline (Pro) | 90.4 | ornithine (Orn) | 80.0 |
| glycine (Gly) | 85.7 | lysine (Lys) | 70.2 |
| alanine (Ala) | 86.1 | histidine (His) | 80.1 |
| valine (Val) | 87.1 | arginine (Arg) | 79.9 |

Other amino acid symbols used are as follows: hydroxyproline (Hyp); cysteine (Cys); hydroxylysine (Hyl); methionine (Met); glutamine (Gln); asparagine (Asn); unknown (uk).

TABLE 3. BUFFER SYSTEM FOR DUAL COLUMN SYSTEM AMINO ACID ANALYSIS

| buffer | pH | time/min |
|---|---|---|
| *Acidic and neutral column* | | |
| (a) elution | | |
| (1) 0.2 M Na citrate | 3.05 | 235 |
| (2) 0.2 M Na citrate | 4.40 | 165 |
| (b) regeneration | | |
| (1) 0.2 M NaOH | — | 40 |
| (2) 0.2 M Na citrate | 3.05 | 225 |
| *Basic column elutions* | | |
| 0.35 M Na citrate | 5.00 | 265 |
| (no regeneration) | | |

Temperature: 45 °C for 50 mins, 55 °C for rest of run.

TABLE 4. BUFFER SYSTEM FOR SINGLE COLUMN SYSTEM AMINO ACID ANALYSIS

| buffer | pH | time/min |
|---|---|---|
| (a) elution | | |
| (1) 0.2 M Na citrate | 3.05 | 215 |
| (2) 0.2 M Na citrate | 4.25 | 115 |
| (3) 0.2 M Na citrate | 5.25 | 225 |
| (in 0.6 M NaCl) | | |
| (b) regeneration | | |
| (1) 0.2 M NaOH | — | 40 |
| (2) 0.2 M Na citrate | 3.05 | 140 |

Temperature: 45 °C for 50 min; 60 °C for rest of run.

The buffer elution systems for the amino acid analysis are given above (table 3, dual column system; table 4, single column system). The single column system was the preferred choice in terms of convenience and speed of operation. However, with this system integration of the basic amino acid elution peaks was sometimes made obscure or inaccurate by the presence of a high ammonia plateau. This effect was circumvented by use of the two column system with separate elution of the basic amino acids from a short column. The two column system also provided greater resolution of certain peaks and confirmed their identification when the single column system proved equivocal.

(iii) *Processing of fractions*

(1) *Fraction* I (*insoluble residue*). This was hydrolysed as described. After hydrolysis any insoluble material remaining was washed, the washings were pooled with the hydrolysate, and the residue was discarded. It was assumed that any proteinaceous material would have been solubilized by this procedure. The hydrolysate plus pooled washings was evaporated to dryness in a vacuum desiccator over NaOH and $P_2O_5$, then redissolved in water and again evaporated to dryness to remove residual HCl. The sample was then desalted as described above. After desalting the 200 ml of eluate containing the amino acids was evaporated to dryness on a rotary evaporator at 40 °C, methyl cellosolve twice being added to assist removal of ammonia from the sample. The sample was taken up in a small volume of distilled water, and subjected to automated amino acid analysis.

(2) *Fraction* II (*supernatant*). After hydrolysis, fraction II was subjected to amino acid analysis.

(3) *Fraction* III (*supernatant*). This was vacuum desiccated, to remove the HCl, redissolved in water and desalted. After removal of ammonia from the desalting column eluate, a sample was run on the amino acid analyser, to detect any peptide fragments that might still be present, but such peptides were never found.

(iv) *Amino acid analysis*

A Locarte Amino Acid Analyser, model AAA/4/6/D, was used for the investigations. Details of the buffer and reagent preparation are given in Appendix 1, and buffer loading times are given in tables 3 and 4. The resin used was an 8 % DVB (divinylbenzene) crosslinked polystyrene bead resin (13 µm diameter) supplied by the Locarte Company.

### (b) Results

The complete quantitative results of the amino acid analyses obtained are given in Appendix 2, tables 11–14. Selected results are illustrated in the form of histograms as this facilitates comparisons (figures 1–7). At the same time histograms of collagen and bacterial proteins are included as a guide to the quantitative identifications of the amino acid data obtained from the fossil bone and sediment analyses.

### (c) Discussion

The amino acid analyses on Pleistocene fossil bones were the only ones that gave compositional profiles at all similar to that of collagen. Four showed significant levels of hydroxyproline although it is noteworthy that this amino acid was found in reduced amounts in the analysis from the insoluble residue of sample 1. Hydroxylysine is an indicator of the presence of collagen; yet ninhydrin-positive peaks appeared in the hydroxylysine position in chromatograms of only two specimens. Its presence was recorded in the residue of the second woolly rhinoceros *Coelodonta* (specimen 5), even though in this instance no hydroxyproline was actually found. Nevertheless the first woolly rhinoceros (specimen 4) gave an amino acid profile characteristic of collagen, even though hydroxylysine was absent. Specimen 5 gave a profile that was similar to that of bacterial protein (Wyckoff 1972), except for the apparent presence of an hydroxylysine peak, perhaps indicating the former presence of collagen, but probably artefactual. As stated above, hydroxylysine peak in hydrolysate chromatograms is usually suggestive of some collagenous components: however, an examination of the complete amino acid composition profile provides a necessary further and often more informative assessment of this possibility, and in addition indicates other events such as bacterial contamination and the occasional
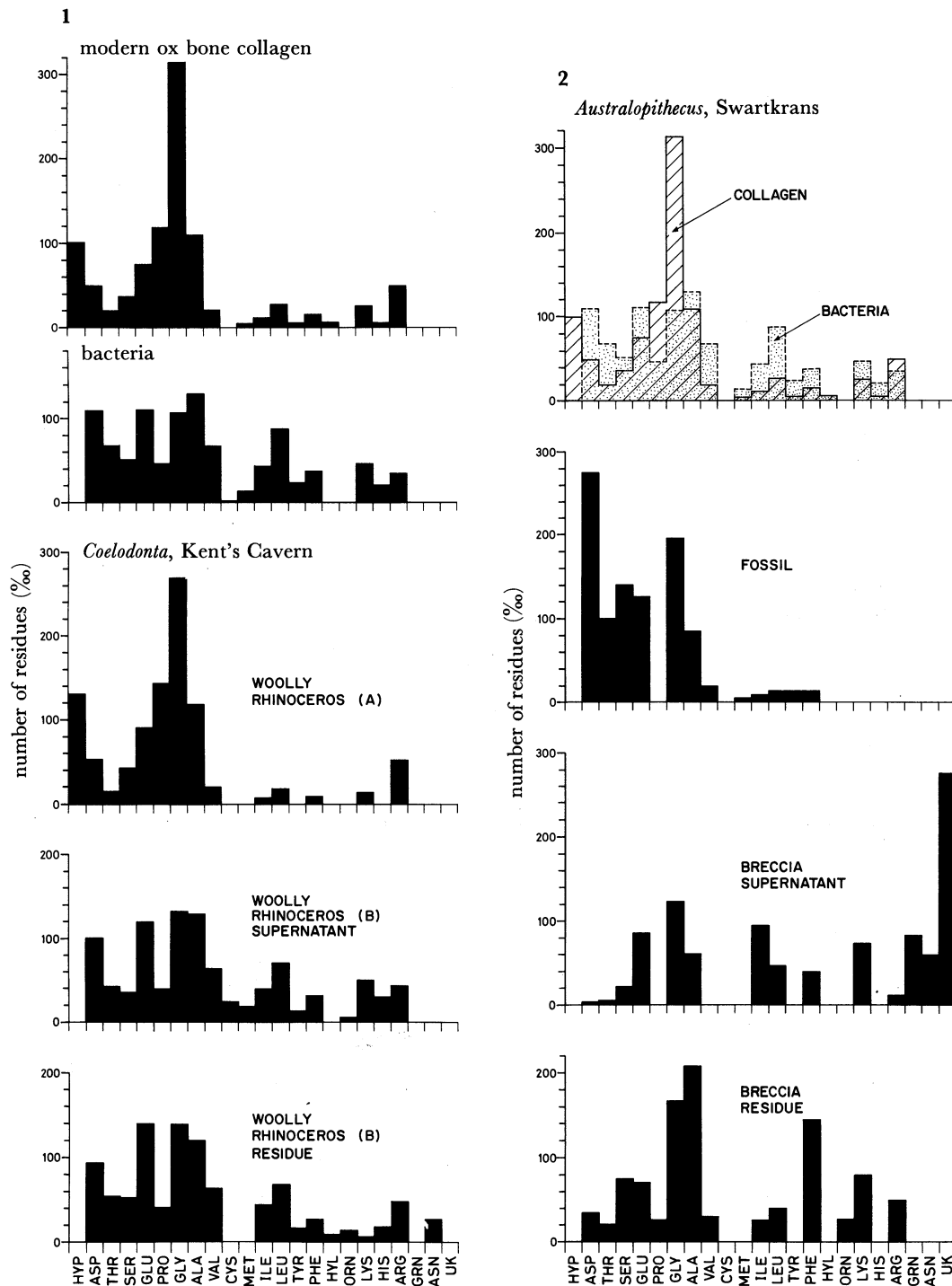
FIGURE 1. Amino acid analyses of two woolly rhinoceros fossil samples, compared with modern ox-bone collagen (our analyses) and bacteria (from Wyckoff 1972) hydrolysates. (Results are expressed as number of residues of each type of amino acid per 1000 total residues.) See table 11 (specimens 4, 5).

FIGURE 2. Amino acid analyses of preparations from *Australopithecus*, from Swartkrans, compared with modern collagen and bacteria hydrolysates. (Results are expressed as number of residues of each type of amino acid per 1000 total residues.) See table 11 (specimen 6).

FIGURE 3. Amino acid analyses of *Emys* fossil bone and surrounding sediment samples, compared with modern collagen and bacteria hydrolysates. (Results are expressed as number of residues of each type of amino acid per 1000 total residues.) See table 11.

chromatogram aberration. Observations on the early disappearance of hydroxyproline and hydroxylysine from the vertebrate fossil record (Wyckoff 1972) are pertinent in this context. The amino acid methionine is only recorded among the most modern material and most likely was rapidly oxidized to the corresponding sulphoxide or sulphone.

The analyses (figure 2) from the early Pleistocene Swartkrans cave breccia showed a clear distinction between the fossil and the surrounding sediment. The enormous increase in aspartic acid content, coupled with the complete absence not only of hydroxyproline but of proline also, does not suggest the survival of any collagenous material. In both fossil and sediment there is some suggestion of possible contemporaneous bacterial contamination, especially in the increase of the aspartic acid level. The amino acid analyses of fragments of the turtle bony carapace from
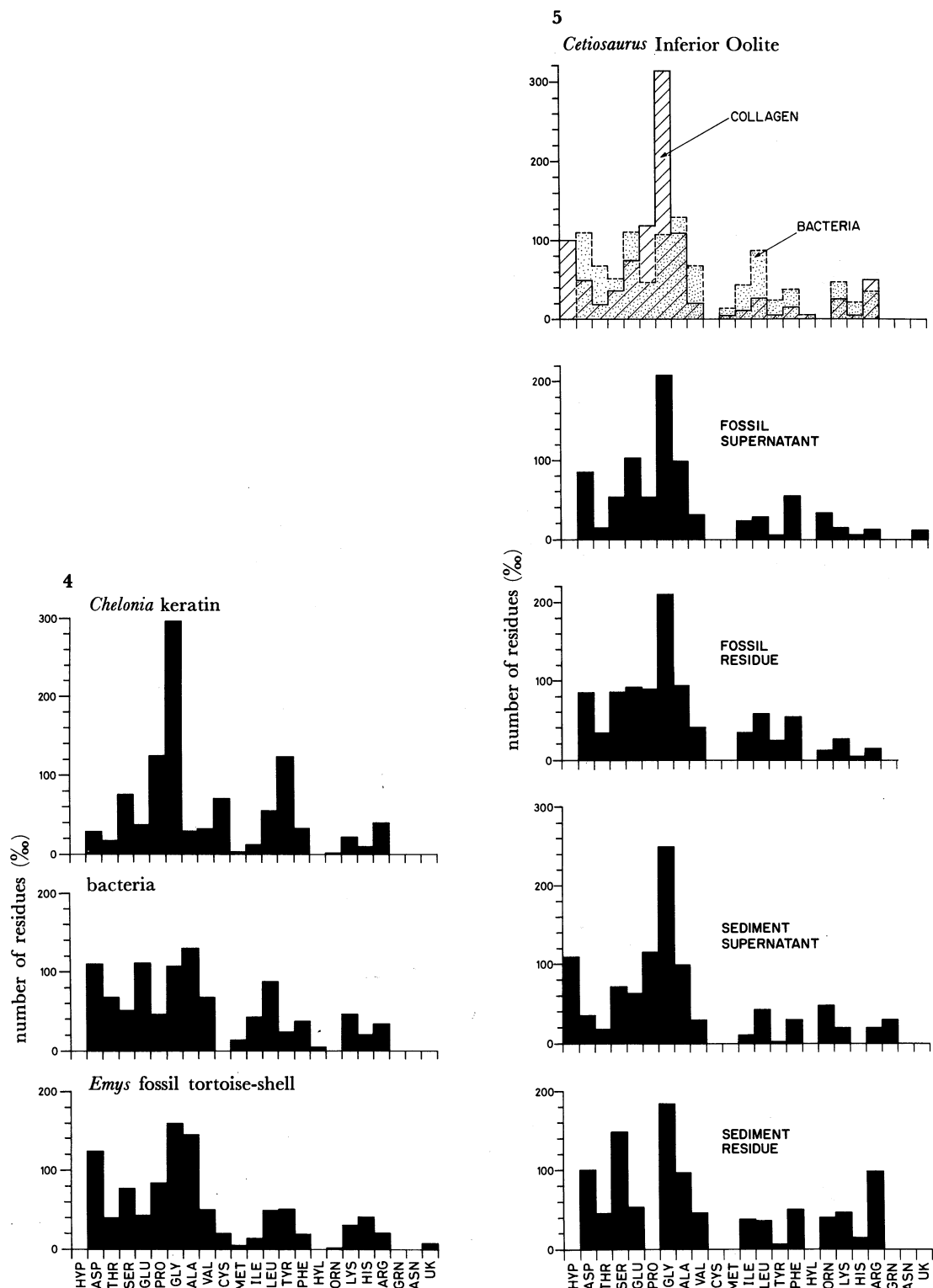
FIGURE 4. Amino acid analyses of *Emys* fossil tortoiseshell samples, *Chelonia* tortoiseshell and bacteria hydrolysates. (Results are expressed as number of residues of each type of amino acid per 1000 total residues.) (Halstead & Wood 1973)

FIGURE 5. Amino acid analyses of *Cetiosaurus* fossil samples and surrounding sediment. (Results expressed as number of residues of each type of amino acid per 1000 total residues.) See table 12 (specimen 11).

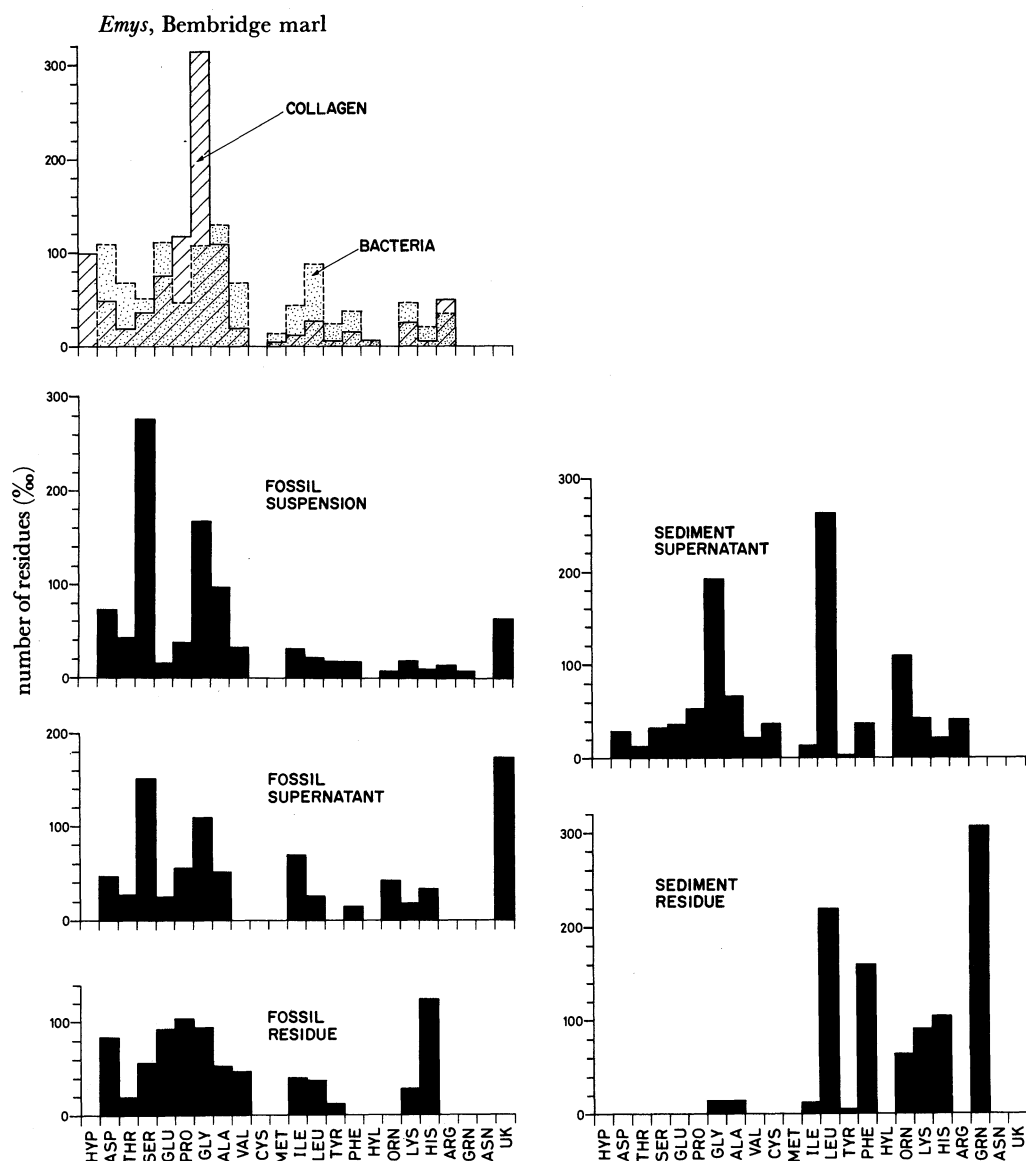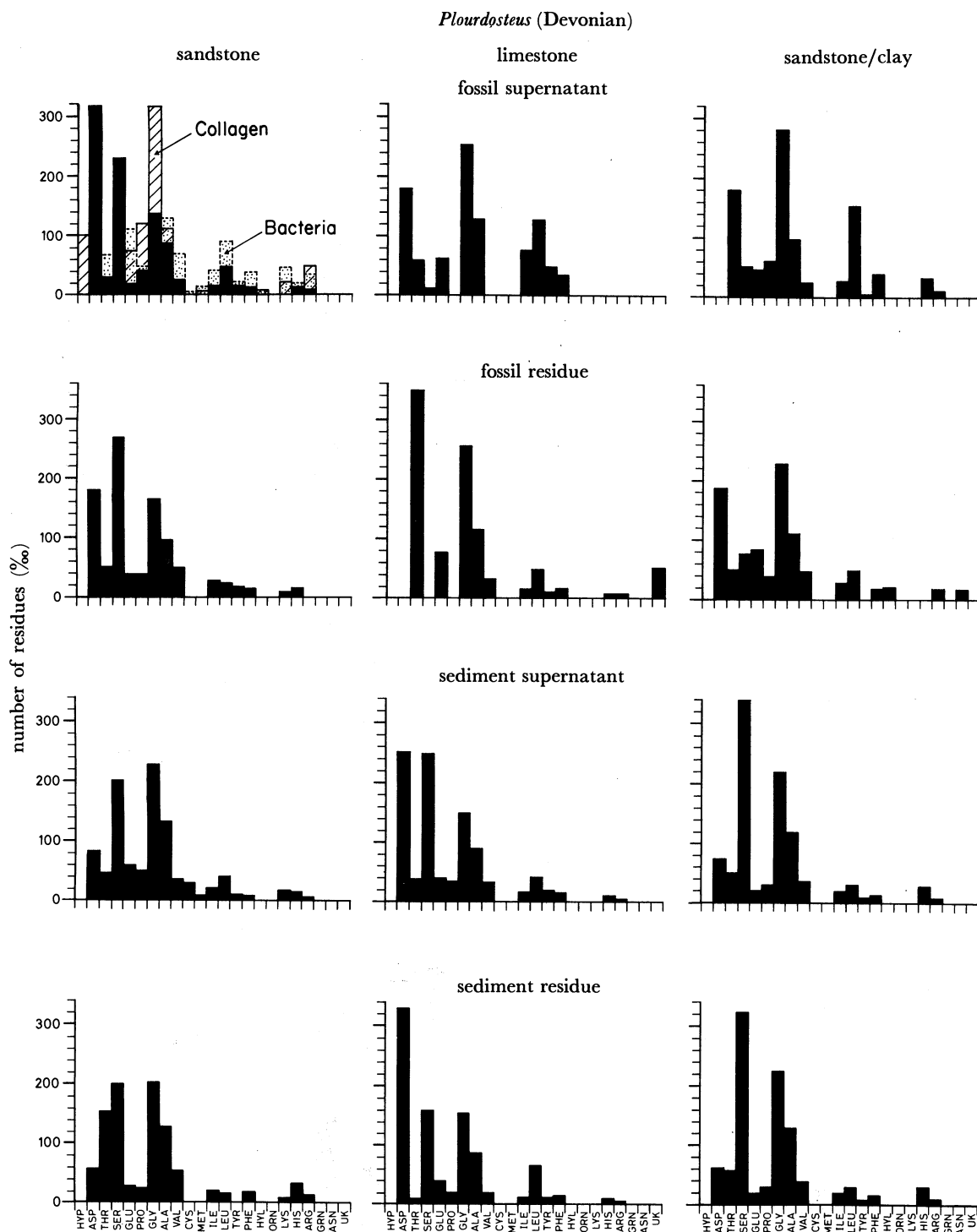FIGURE 6. Amino acid analyses of a series of *Plourdosteus* specimens and surrounding sediments, from various sites. (Results are expressed as number of residues of each type of amino acid per 1000 total residues.) See table 13.
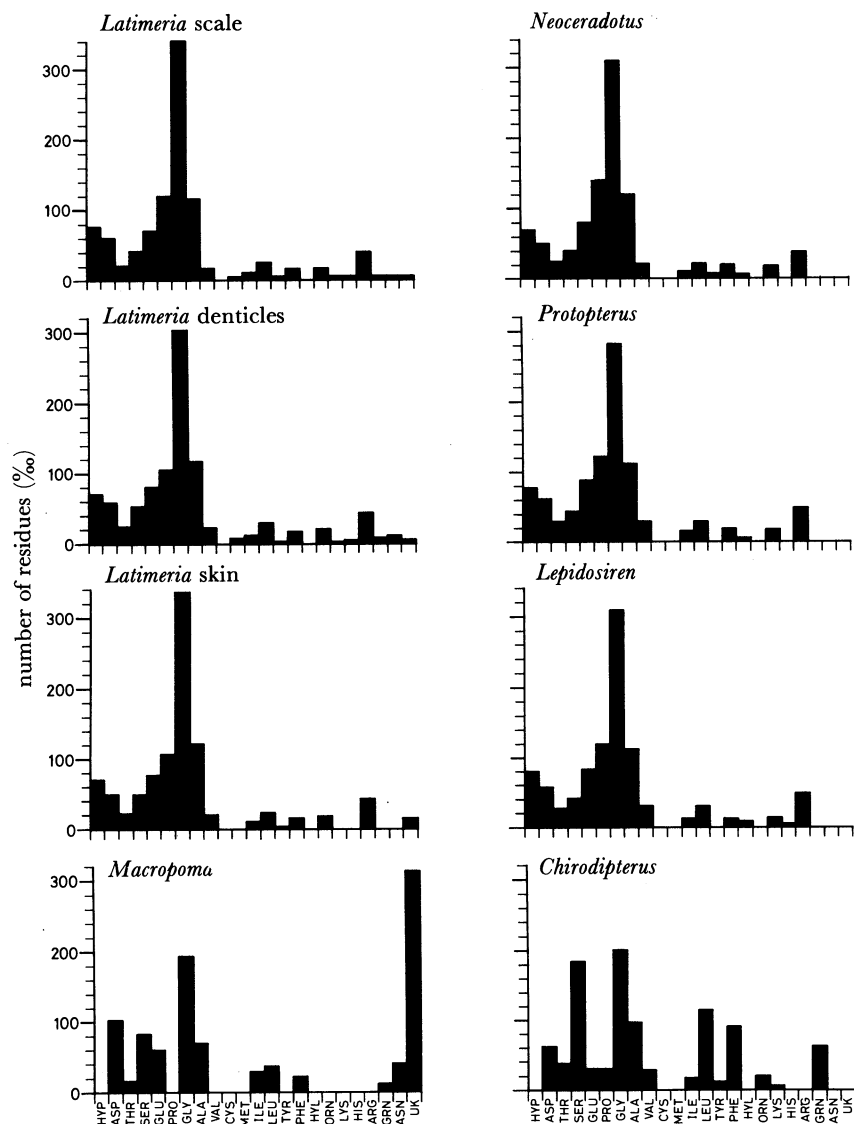
FIGURE 7. Amino acid analyses of collagen preparations from various 'living fossil' specimens. (Results are expressed as number of residues of each type of amino acid per 1000 total residues.) See table 14.

the Oligocene Bembridge Marls (figure 3) could be interpreted as being essentially made up of bacterial protein with perhaps an admixture of some collagenous remnants. The fossil tortoise-shell, originally identified as keratin by Halstead & Wood (1973), seems to have been contaminated by contemporaneous bacterial protein (figure 4). Among the analyses from the Mesozoic era (225–64 million years ago) a number of curious results emerged. In the supernatant of a Cretaceous dinosaur bone (specimen 8), hydroxyproline suddenly reappears but the rest of the profile is quite unlike that of collagen, with remarkably high levels of aspartic acid and threonine and low levels of glycine and serine, with glutamic acid, proline and valine absent. With the insoluble residue there is, as expected, no hydroxyproline but substantial levels of glycine, alanine and valine. Although aspartic acid and threonine remain high, serine, glutamic acid, proline and hydroxylysine are present even though absent from the supernatant. This analysis may indicate the presence of some remnant of the original collagenous matrix.

The results from the Jurassic dinosaur (specimen 11) indicate some denatured collagenous material contaminated with bacterial protein. However, the supernatant of the sediment produces an amino acid profile that is almost identical to that of collagen even though hydroxy-lysine is absent. This result emphasizes the necessity of analysing the sediment as well as the fossil. In this instance the analysis is most likely due to present day contamination of the sediment. As the analyses are pursued back in geological time, it becomes increasingly difficult to offer any meaningful interpretations of the results. By the time that Devonian fossils and sediments are examined, the results seem to be totally devoid of any real significance. It certainly appears that what is being recovered is simply the background organic content of both fossils and sediment, which cannot be used to throw light on the nature of the original protein.

A number of analyses were made on so-called 'living fossils' such as the coelacanth, lung-fishes and the tuatara lizard of New Zealand. These all clearly gave typical collagen profiles although a number of minor differences could be observed among the different lungfishes for example, as well as among the collagens from different parts of the coelacanth. The amino acid profiles from the Cretaceous coelacanth and the Devonian lungfish bore little relation to either collagen or even bacterial protein, again confirming the need for caution when investigating such fossil material.

### 3. RACEMIZATION

#### (a) Introduction

Amino acids, with the exception of glycine, have chirality, or handedness, in the form of L and D stereoisomers. Living organisms contain mainly L-configuration amino acids, but on death these gradually racemize, to form equilibrium mixtures containing equal amounts of the D and L enantiomers:

$$\text{L amino acid} \rightleftharpoons \text{D amino acid.}$$
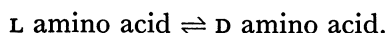
When amino acids contain more than one asymmetric carbon atom, and epimerization occurs at one centre only, the equilibrium state may not be a mixture of equal amounts of two stereoisomers. For example in the racemization L-isoleucine $\rightleftharpoons$ D-*allo*-isoleucine, Bada & Schroeder (1972) have estimated that the ratio of D-*allo*-isoleucine to L-isoleucine is 1.4:1 at equilibrium.

The mechanism of racemization involves formation of negatively charged carbanion inter-mediates. This process occurs whether the amino acids are free or incorporated in fossil poly-peptides and proteins (Evans 1972).

Three approaches have been used in the study of racemization kinetics:

(1) theoretical considerations, such as the effect of substitution of different groups on the amino and/or carboxyl groups upon racemization (Smith & Evans 1980);

(2) the use of elevated temperatures in the laboratory to simulate the effects of geological time on fossils;

(3) direct measurements of the extent of racemization in fossil material.

These studies have led to the awareness that rates of racemization are influenced by many variables. Among these the more important factors that have been implicated as affecting observed D:L ratios for amino acids in fossils (in addition to geological time) are: (i) temperature; (ii) pH; (iii) ionic concentration of salts and buffers; (iv) concentration of amino acids and proteins in fossil; (v) presence of water; (vi) type of fossil and its geometry; (vii) state of the amino acid being considered; (viii) preparation techniques used in fossil analysis; (ix) presence

of other organic ions or molecules; (x) presence of D amino acids in contaminating proteins; (xi) bacterial racemases.

Variables (i)–(ix) are considered briefly below, but for more detailed information reference should be made to Hare *et al.* (1980).

### (i) *Temperature*

Rates of racemization are critically dependent upon temperature. Thus a ratio of 0.2 for D-*allo*-isoleucine:L-isoleucine would be reached after four million years at 0 °C, but after only two million years at 3 °C.

Also, since the relation of racemization rate to temperature is exponential, it is not sufficient merely to know the average palaeo-temperature in order to estimate fossil age from a measurement of the extent of amino acid racemization. Such considerations and observations led Miller & Hare (1980) to conclude (for molluscs at least) that, because of the general inability to define the integrated thermal history of fossil sample, assessments of absolute ages based on mollusc racemization data were unlikely to be reliable.

That factors other than thermal history affect racemization rates has recently been substantiated experimentally by Bischoff & Rosenbauer (1981). Using a uranium dating method, these workers obtained ages of 8300 years ($^{230}$Th) and 9000 years ($^{231}$Pa) for the Sunnyvale skeleton, previously dated at 70000 years by Bada & Helfman (1975) on the basis of aspartic acid racemization.

### (ii) pH

Bada (1980) investigated the effect of pH on racemization rates both for free amino acids in aqueous solution and for amino acids in bone by means of controlled temperature experiments. The conclusion was that, in spite of the fact that considerable variations in racemization rates with pH change are found with free amino acids in solution, in intact bone the protein amino acid racemization rates are unaffected over the pH range 3–9.

Three caveats have to be borne in mind when considering the application of these findings to fossil dating.

(1) The assumption is made that measurements of racemization kinetics at elevated temperatures can be extrapolated to much lower temperatures. Steinberg & Bada (1981) have reported that formation of diketopiperazines can occur when dipeptides are subjected to heating, so that geochemical conclusions extrapolated from such experiments will require revision.

(2) It is also assumed that either the fossil had not been exposed at any time to pH levels below 3 or above 9, or if this had occurred, then the bone/fossil mineral would have effectively buffered against extreme pH changes.

(3) During sample preparation for analysis the hydrolysis in strong acid or base used will itself induce a small degree of racemization.

### (iii) *Ionic concentration of salts and buffers*

Smith *et al.* (1978) found that increasing the concentration of phosphate buffer resulted in a corresponding increase in rate of racemization of alanine. Because it is virtually impossible to assess the various salt concentrations that fossils may have been exposed to throughout their

history, the possibility that such effects occur also underline the need for a cautionary approach to using racemization data for fossil dating.

### (iv) *Concentration of amino acids and protein in fossil*

Kessels & Dungworth (1980) have questioned whether the Arrhenius parameters determined for racemization kinetics in fresh bone (30–40 % protein) are applicable to fossil bone where the amino acid content is often considerably less than 1 % (see our tables 11–14).

### (v) *Presence of water*

In fossils found in anhydrous conditions in the La Brea tar pits (Kvenvolden & Peterson 1974) racemization was less extensive than in fossils deposited in more typical sediments. According to Hare (1974), although water assists in racemization, excessive amounts can actually decrease the rate, and the same caution as in §(iv) above must apply.

### (vi) *Type of fossil and its geometry*

Wehmiller (1980) has demonstrated that intergeneric differences in racemization kinetics for molluscs and foraminiferans are apparently related to initial rates of hydrolysis of long-chain polypeptides. Furthermore certain genera of molluscs could be categorized as either 'fast racemizers' or 'slow racemizers'.

In addition to inter- and intrageneric differences in rates of racemization, the possibility that the size or geometry of a fossil may influence kinetics of the reaction has been investigated by Von Endt (1980). He showed that hydrolysis of bone protein at 120 °C proceeded at a rate inversely proportional to the size of the bone, and proposed that a 'racemization gradient' existed, with less-racemized material found in the fossil bone interior.

### (vii) *State of amino acid*

The rates of racemization for the free amino acids differ from those found for acids bound in peptide form. The N- and C-terminal amino acids also racemize at different rates from their corresponding amino acid residues contained within a peptide or protein. There is a suggestion that the adjacent amino acids in a protein's primary structure may influence racemization rates. Juxtaposition of amino acids arising from secondary, tertiary and quaternary structures may also influence the rates; this cannot be directly tested by controlled thermal experiments because of protein denaturation on heating (Smith & Evans 1980).

Kriausakul & Mitterer (1980) have produced evidence indicating that epimerization of isoleucine varies with molecular mass for protein extracted from a late Pleistocene *Mercenaria* shell.

### (viii) *Preparation techniques used in fossil analysis*

The possible effect on racemization of acid or alkaline hydrolysis (an inescapable procedure prior to measurement of the extent of racemization in all but free amino acids) has been mentioned above under pH effects.

Reliability of measurements improved when repeated measurements of multiple analyses of D:L ratios were made for several different amino acids from several different samples derived from the same outcrop. Using this approach, Belknap & Wehmiller (1980) made estimates of 'kinetic model ages' for Quaternary molluscs which were calculated to have a precision of approximately ± 20 %.

(ix) *Presence of other ions, molecules or surfaces*

Bada (1975) found that epimerization of isoleucine *in vitro* at pH 7.6 and 100 °C was catalysed by the presence of cupric ions. Catalysis of amino acid racemization by trace elements has been investigated more fully by O'Brien (1978 *a*, *b*), who found that a number of metallic ions (and also pyruvate) catalysed amino acid racemization, particularly at higher pH.

An additional complication arises from the observation that montmorillonite clay surfaces catalyse racemization (Frenkel & Heller-Kallai 1977).

### (b) Results and discussion

Measurement of the percentage of D-*allo*-isoleucine in total isoleucine shows a general trend of increasing levels of epimerization in the older fossil samples (tables 5, 11–14).

Considerable variation in the extent of epimerization, however, was found between fossils of a similar age. Some fossils of Jurassic age showed an epimerization range of 15–29 % (for *Cetiosaurus*), which compares markedly with the 12 % epimerization found for an ichthyosaur fossil from the same period.

Variations were also observed between analyses made on individual fossil specimens, with the soluble supernatant fraction sometimes showing a greater degree of epimerization. For example, a Cretaceous siluroid fish showed 40 % epimerization in its supernatant fraction compared with only 15.5 % in the sediment. Conversely, in the Triassic fish fossil analysed, the supernatant only showed 25 % epimerization in contrast to 33 % in the residue sediment fraction. Such observations therefore lend support to the view that racemization studies are to be viewed with extreme caution in terms of their value in dating fossil specimens in any absolute sense. In this context there appear to be two current views concerning the extent to which data on D:L amino acid ratios in fossil bones can contribute reliable information concerning absolute fossil ages. On the one hand, Bada and coworkers tend towards the view that, once a particular region has been 'calibrated' by a fossil bone, a 'calibration constant' can be used to date other samples 'from the general region as long as the site to be dated has the same temperature of the calibration site' (Bada & Shou 1980). On the other hand, the more cautious workers tend towards the view that, as racemization in bone is controlled by such a complex and poorly understood combination of factors, 'much more work must be done before D:L values in bone can be used independently to date geological deposits and archaeological remains' (Lajoie *et al.* 1980). The measurements made of the extent of racemization in our fossil studies (see table 5) support the latter viewpoint, if the fact that the various specimens came from different and 'uncalibrated' sites is allowed for.

The current view of the present authors is that the main and unequivocal value of racemization data is in its use in detecting the presence of any modern contamination, as evidenced by any appearance of an anomalously high proportion of L amino acids in the older fossil specimens. An example of this in the present study is in the low figure of 6–27 % D-*allo*-isoleucine measured for Devonian *Plourdosteus*. This result fosters suspicion that the fossil must have been contaminated with recent L-isoleucine at some stage (table 5).

In spite of the limited value of racemization studies for absolute dating, the determination of D:L ratios from fossil shells in a restricted time sequence, as for example in the Quaternary of Great Britain (Andrews *et al.* 1979; Miller *et al.* 1979), is of very great practical value in establishing relative ages within a given region, as shown by many recent studies (Miller & Hare 1975; Blake 1980; Kvenvolden & Blunt 1980).

TABLE 5. RACEMIZATION RESULTS

| | D-*allo*-Ile (%) | | |
|---|---|---|---|
| *Elephas primigenius* | 3 | | |
| woolly rhinoceros | 5 | | |
| woolly rhinoceros | | late Pleistocene | contamination not |
|    supernatant | 2.5 | | detectable |
|    residue | 8 | | |
| Swartkrans | 5 | early Pleistocene | |
| *Emys* | | | |
|    suspension | 28 | Oligocene | |
|    residue | 47 | | |
| dinosaur | 27 | | |
| siluroid fish | | Cretaceous | |
|    supernatant | 40 | | |
|    residue | 15.5 | | |
| *Cetiosaurus* | | | |
|    supernatant | 29 | Jurassic | |
|    residue | 15 | | contaminated |
| ichthyosaur | 12 | | |
| fish peptide | 43 | | |
|    supernatant | 25 | Triassic | |
|    residue | 33 | | |
| *Plourdosteus* + sediment | 6–27 | | |
| conodonts | 40 | Devonian | |
| Lower Devonian sediment | 58.8–36 | | |

## 4. ELECTRON MICROSCOPY

The demonstration of intact collagen fibres from fossil teeth and bones under the electron microscope was first done by Wyckoff *et al.* (1963) and Shackleford & Wyckoff (1964). These authors observed that there was a change in the characteristic 64 nm banding, which appeared to become reduced as one went back through time. By the Miocene, 25 million years ago, it was down to 50 nm.

One of the problems encountered in studying electron micrographs of fossil material is that they are calibrated on the assumption that the modern collagen micrographs show a true 64 nm banding. However, various factors argue against adoption of this value as an absolute yardstick for comparison purposes.

First, to determine the actual magnification the instrument itself must be calibrated, as the nominal magnification is seldom correct. Furthermore, if it is intended to compare material from different sources it is important that the specimens should have been similarly prepared and viewed under identical conditions in the same instrument, so that it may be seen whether apparent differences are valid. In addition distortion can occur during fixing, dehydration, embedding and cutting procedures. Comparison of material prepared by different techniques or comparison of published figures alone, therefore, is of little value, especially since magnifications are often worked out on the basis of the repeat banding of collagen being 64 nm.

Ideally, the instrument should be calibrated statistically, by examining different specimens, believed to be the same, on different days at the same magnification. Many instruments, especially the older models, have 5–10 % error in reproducibility. The repeat distance of collagen banding is very frequently assumed to be a constant of 64 nm but a sampling of thin

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FIGURE 8. Transmission electron micrograph of collagen preparation from demineralized woolly rhinoceros specimen (magn. × 121 000).

section micrographs will usually show a standard deviation of about $\pm 60$ nm. These variations are due to the preparation method, the height of the specimen in the microscope, and distortion due to the sectioning itself.

Modern human dentine, ox-bone and fossil woolly rhinoceros bone was demineralized in 15 % EDTA at room temperature, then immersed in 2 % solution of glutaraldehyde and 2 % paraformaladehyde in a cacodylate buffer, post-fixed in osmium tetroxide solution, dehydrated in methanol and embedded in Araldite. Rat cementum and rat periodontal ligament were fixed *in situ* by perfusion with a similar aldehyde solution, then demineralized in 15 % EDTA at 40 °C, post-fixed in osmium tetroxide solution and subsequently processed in the same way as the previous specimens. The five preparations were viewed on the same Philips 201C instrument under the same conditions at a nominal magnification of $\times 28\,500$, a series of twelve micrographs of each were taken and three to six spacings in negative were measured with a measuring eyepiece (with an accuracy of $\pm 0.05$ mm). The following results were obtained:

modern human dentine, 64.2 nm $\pm$ s.d. 5.2 nm;
modern ox-bone, 63.1 nm $\pm$ s.d. 3.5 nm;
fossil woolly rhinoceros bone, 50.1 nm $\pm$ s.d. 2.2 nm (see figure 8);
rat cementum, 57.1 nm $\pm$ s.d. 3.0 nm;
rat periodontal ligament, 60.3 nm $\pm$ s.d. 3.8 nm.

The results of the collagen from human dentine and ox-bone showed a good correlation and the results from the fossil which differ by about 20 % are statistically highly significant. The difference in the banding between the fossil and modern material may not indicate a decrease in the size of the banding but may well represent a change in the protein that alters its dimensional response to the processing involved in preparing the material for electron microscopical examination. This hypothesis receives some support in the figures obtained from rat collagen in which the initial fixing technique differed and there was a significant reduction in the spacing. The photomicrograph (figure 8, plate 1) illustrates the intact nature of the fossil collagen. It should be noted, however, that the appearance of collagen fibrils under the electron microscope cannot always be taken as evidence of the survival of proteinaceous material. Towe & Urbanek (1972) and Towe (1980) demonstrated the electron microscopical appearance of collagen in fossil graptolites in the absence of organic matter. This situation must involve the replacement of the protein by mineral so that the apparent architecture is perfectly replicated. (Data for this section were provided by Dr A. F. Hayward.)

## 5. Peptide studies

### (a) Introduction

Analyses of the first woolly rhinoceros specimen (*Coelodonta*) examined showed that its amino acid composition (table 11) and histogram profile (figure 1) was consistent with the presence of a collagen structure, albeit in small amounts. The subsequent electron microscope examination of the demineralized fossil confirmed the presence of a characteristic collagen structure (§4, figure 8). The fact that even small amounts had survived fossilization and post-fossilization events over several hundred thousand years suggests that it may have been modified to a form resistant, in part at least, to such associated changes. An attempt to detect whether such modification to the collagen molecule had occurred during fossilization and/or post-fossilization

events was made by using enzymic degradation of the fossil collagen. Bacterial collagenase (*Clostridium histolyticum*), pronase (*Streptomyces griseus*) and subtilisin (*Bacillus subtilis*) are enzymes capable of digesting collagen to smaller peptide fragments. Each enzyme possesses a different specificity in its mode of attack and the bonds hydrolysed, with the result that each enzyme forms a different pattern of peptide components ('fingerprint') in its breakdown of collagen. It was considered possible that the residual collagen component(s) locked up in the woolly rhinoceros fossil femur may have undergone changes that modified susceptibility to attack by collagenolytic enzymes. These modifications could range from quite specific susceptible bond locations in the molecules to a more general restricted accessibility to enzyme action over many residue lengths of the collagen molecular structure. Such postulated modifications would be likely to be detected as altered peptide fingerprint maps from the fossil collagen residues when compared with identical enzymic digests of comparable modern collagen preparations. It is possible too that species-specific primary structure sequence differences could also account for different peptide fingerprint maps, but it seems unlikely that such genetic variants would account for major map differences.

Tuross *et al.* (1980) attempted peptide analysis using cyanogen bromide which cleaves the tropocollagen molecule at methionine. Since this amino acid is one of the most unstable it is not surprising that their results were not encouraging. Indeed they concluded that 'most fossil bones a thousand years old or more will not be likely to yield much information by conventional means in the way of intact discrete peptide units' (Tuross *et al.* 1980). Use of proteolytic enzymes as detailed below has demonstrated that such a counsel of despair is unwarranted.

### (b) Materials and methods

#### (i) Collagen preparations

(1) *Modern ox-bone collagen.* Ox-bone femora were collected from the local butcher's cold room and adhering soft tissue and the periosteum were completely removed. The central shafts were then removed and the marrow was cleaned out: the bones were then sawn up with a hacksaw into discs 1 cm thick. Cancellous bone extensions attaching to the inner aspects of the femur discs were removed by chiselling and drilling. The discs were then sawn into blocks about 1 cm³ in volume and soaked in several changes of 0.9% saline, followed by repeated soakings in distilled water. They were next broken down to small fragments by smashing with hammer blows delivered through a thick polyethylene sheet. These fragments were reduced to fine powder form in a Glen Creston Micropulverisor (a stainless steel, vertically oscillating, ball mill) and demineralized in a 20% EDTA solution in 0.15 M phosphate buffer pH 7.4. The point of total demineralization was established by burning off samples of powder in a crucible until no calcined residue deposit was observed. The demineralized ox-bone powder was then repeatedly washed in water to remove the EDTA, then acetone washed and air dried.

(2) *Fossil bone collagen.* Fragments of a fossil femur of a woolly rhinoceros specimen (*Coelodonta*) were prepared similarly by delivery of hammer blows through polyethylene sheeting followed by powdering in the Micropulverisor, and demineralization was effected by using 20% EDTA solution. The point of maximum demineralization took longer to reach and even at this stage a small residue remained in the crucible test. Nevertheless this usually represented only a very small proportion of the original material (*ca.* 2%) and the demineralized product obtained was of light fluffy appearance and consistency. A small fraction of it was examined by transmission electron microscopy, the result of which is illustrated in figure 8. As with the

modern ox-bone collagen, the demineralized fossil collagen preparations were washed in repeated changes of water, then acetone washed and air-dried.

### (ii) *Enzyme preparations*

(1) *Collagenase.* This was a partially purified, freeze-dried, $(NH_4)_2SO_4$-precipitated preparation supplied as a gift by the Burroughs Wellcome Company. Preliminary assays with use of a collagen substrate breakdown procedure (Armstrong 1958) confirmed its collagenolytic potency.

(2) *Pronase.* This was a commercial preparation, supplied by the Sigma Company, obtained from a *Streptomyces griseus* strain. The batch used represented the highest purity grade of the enzyme available at the time (fungal protease VI, batch no. 22B-773).

(3) *Subtilisin.* This was a commercial preparation supplied by B.D.H. and was then the highest grade of material available commercially (batch no. 487220/640428).

### (iii) *Peptide separations and analysis*

The existing ion exchange column of the Locarte amino acid analyser which consisted of an $8\%$ DVB polystyrene bead resin (beads 13 μm in diameter) was used. The elution procedure employed at 38 °C is summarized in table 6. A total elution–regeneration time of 12 h was necessary to elute all the peptides produced and prepare the column for the next sample. Peak areas were integrated manually from the recorder charts and form the basis of the comparisons made.

### (iv) *Enzyme studies*

Preliminary investigations established that the following enzyme concentrations (dry mass per millilitre of solution) gave substantial breakdown of the collagen substrate in the times indicated:

> collagenase, 0.2 mg ml$^{-1}$ in 0.01 M phosphate buffer pH 7.4 (1–3 h);
> pronase, 0.2 mg ml$^{-1}$ in 0.01 M phosphate buffer pH 7.4 (24 h);
> subtilisin, 0.2 mg ml$^{-1}$ in 0.01 M phosphate buffer pH 7.4 (1–3 h).

Of each collagen preparation (modern ox-bone or fossil woolly rhinoceros) 10 mg were weighed into 25 ml conical flasks secured to the platform of a temperature-controlled shaking water bath at 37 °C; 5 ml portions of the pre-equilibrated enzyme solution were then added to each flask and the shaking rate was adjusted to give a gentle agitation of the collagen particles. At appropriate times samples of the incubate were removed and rapidly centrifuged in a bench centrifuge, and the supernatant was heated in a boiling water bath for 10 min to destroy enzyme activity. Measured volumes of this supernatant were then taken for analysis. Ninhydrin assay of these showed that a sample of 400 μl usually contained sufficient peptide material to give reasonable peptide levels and separations on the column. Initially incubation was continued for 24–48 h to determine (*a*) when maximum breakdown had been reached and/or (*b*) whether prolonged incubation indicated that further breakdown of the first-formed peptide by contaminant proteases was occurring to any extent. In all experiments comparable control incubations of enzyme solutions alone were run concurrently and any peptide components detected in these enzyme incubates were allowed for in the subsequent fingerprint comparisons. It was also established that incubations of either substrate alone over the experimental periods

employed did not show any breakdown or peptide release. The experimental conditions finally adopted for optimum breakdown in reasonable time were the enzyme substrate concentrations detailed above with 3 h incubation periods for collagenase and 1 h for subtilisin; pronase was rather slower in action and required overnight incubation to produce workable levels of peptide material. After the stated incubation times a 400 µl sample of the supernatant loaded on to the ion exchange column gave peptide levels suitable for separation and assay.

TABLE 6. BUFFER SYSTEM FOR PEPTIDE ELUTION

| buffer | pH | time/min |
|---|---|---|
| (a) elution (11 h approx.) | | |
| (1) 0.2 M Na citrate | 3.05 | 275 |
| (2) 0.2 M Na citrate | 4.25 | 175 |
| (3) 0.2 M Na citrate (in 0.6 M NaCl) | 5.25 | 225 |
| (b) regeneration (3 h approx.) | | |
| (1) 0.2 M NaOH | — | 40 |
| (2) 0.2 M Na citrate | 3.05 | 140 |

### (v) Peptide fingerprint chromatograms

The buffer elution system used on the amino acid analyser ion exchange column at 38 °C (table 6) is based in part on earlier experience with a Technicon Peptide Analyser. This system produced a sequential separation of numerous peptide peaks from the different enzyme–collagen incubates investigated. Unlike the Technicon system employed many years ago in our laboratories (Armstrong 1967), a time-synchronized split-stream hydrolysed peptide analytical line was not included. The advantage claimed for its inclusion was that, theoretically, it gave indications of peptide size: thus a decapeptide should give some ten times the peak area colour yield of its unhydrolysed form (with only one free α-amino group). However, our earlier investigations showed this to be quantitatively arbitrary. With the many technical difficulties inherent in running a system with concurrent split-stream time-delayed alkali-hydrolysed peptide lines (plus neutralization and dilution factor corrections), the advantages accruing were debatable to say the least. Hence we used the single-stream unhydrolysed peptide elution approach as the more reliable technique, with the disadvantage that the first eluted peaks give low colour yields relative to their molar concentration. The nature of the chromatographic procedure employed is such that the larger peptides elute first, with peptide size decreasing as the elution proceeds and the smallest peptides eluting last. Thus the relatively small peak areas at the beginning of the chromatogram will in fact contain quite significant amounts of the larger peptides. This is because, though large in terms of molecular mass, each is detected by the ninhydrin colour level given by reaction of its *one* terminal α-amino group with the reagent. As discussed above a decapeptide will in theory yield the α-amino N colour value equivalent to that given by a single amino acid. In practice, larger peptides often give less than this theoretical maximum value, presumably because steric hindrance effects modify their reaction with ninhydrin. Three different enzyme–substrate incubations were examined for each system, with incubation time the only variable.

These general observations apart, it was found that the peptide elution pattern recorded for a particular enzyme incubate was sufficiently characteristic to justify the appelation fingerprint. Although this term is more usually applied to two-dimensional chromatogram–electrophoresis
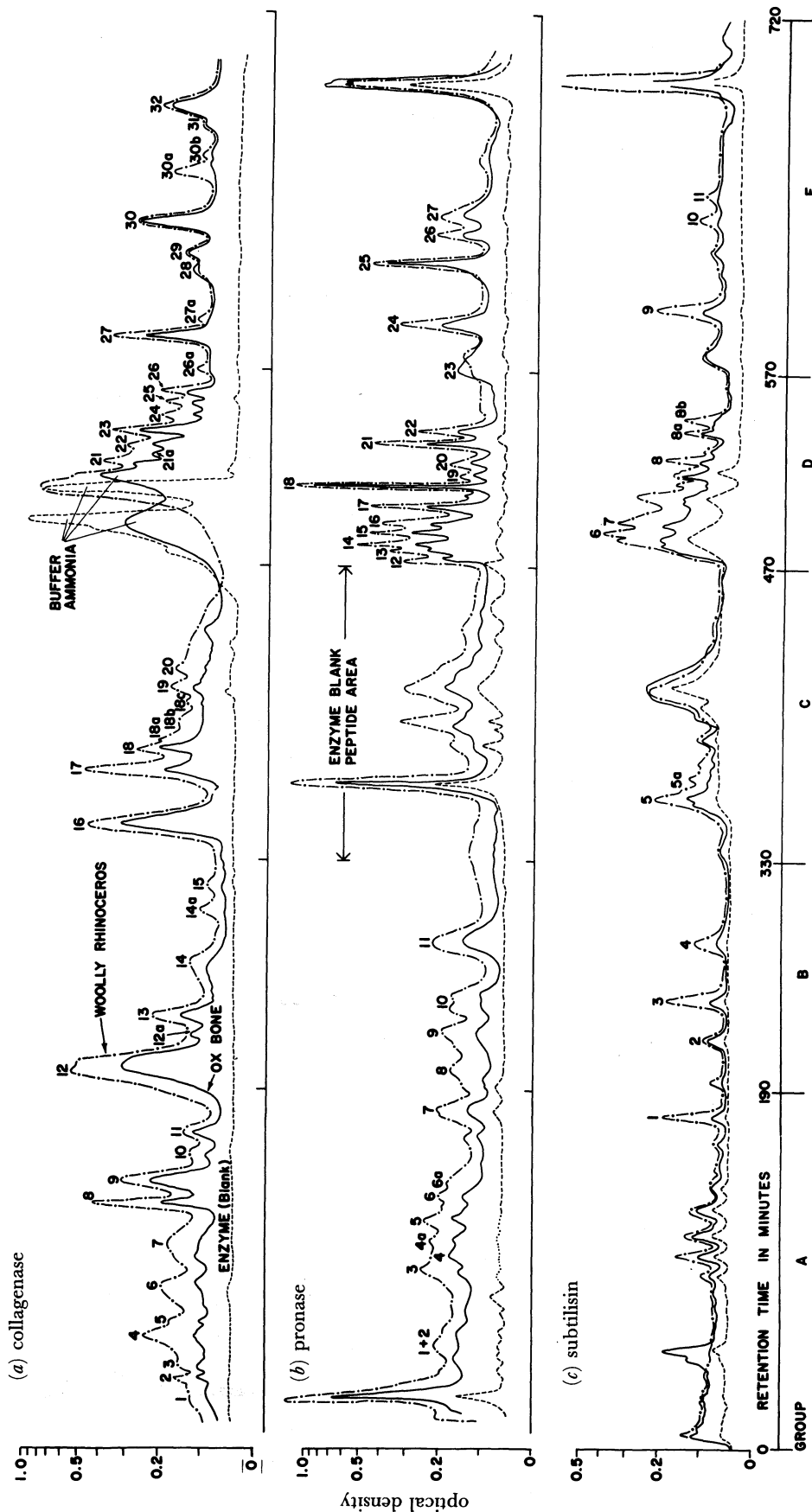
FIGURE 9. Ion-exchange column chromatogram patterns of peptide products formed by digestion of woolly rhinoceros collagen with three collagenolytic enzymes (collagenase, pronase, subtilisin), compared with similar digests of modern ox-bone collagen. See tables 8–10.

maps it is also applicable to the column chromatogram elution patterns obtained with the system described. In addition the column chromatogram fingerprints have the distinct advantage that their form allows quantification on an area basis. Direct area comparisons between fossil and modern ox-bone collagen incubates are made with each peptide component for each specific enzyme digest. Peptides were located by their individual *retention times* on the column, the time in minutes required for the peak to elute under specifically defined conditions of buffer strength, pH and temperature.

### (c) Results

*Comparisons between fossil and modern collagen enzyme digests*

(i) *Collagenase–collagen incubations.* The results of a typical chromatogram obtained from 3 h enzyme–substrate incubations are shown in figure 9 *a*. Because of the length of the run, and the numerous complex peaks (single, multiple and overlapping), the chromatograms have been arbitrarily divided into five chronological stages: A (when the larger peptides elute); B, C (when the medium-sized peptides are found); and D, E (when the smallest peptides are eluted from the column). Chromatograms obtained with 24–48 h incubates compared with 3 h ones showed overall somewhat larger total amounts for each peak in the collagenase–substrate incubate: these reflected marginally higher degrees of overall rate of attack but no significant qualitative or quantitative ratio differences were observed, indicating the absence of the effect of further non-collagenase type protease breakdown. The enzyme control incubate showed, apart from the expected buffer rise and ammonia peaks, only a few minor peaks due to contaminants in the enzyme preparation. These areas were taken into account in subsequent quantitative and qualitative comparisons and evaluations.

A general visual inspection of the chromatograms shows that a complex pattern of peaks, with characteristic shapes, bunching and elution profiles, can be discerned. It will be observed that the collagenase–collagen pattern in figure 9 *a* is quite distinctive from, say, the pronase–collagen digest fingerprint map shown in figure 9 *b*. The enzyme blank incubate contained relatively few contaminant peaks to obscure the interpretation of the chromatograms. The ox-bone and fossil woolly rhinoceros collagen digests show an overall similarity in pattern. However, closer detailed inspection reveals that there appear to be present in the fossil digests peptide peaks that are absent in the ox-bone digests, as at positions 14 a, 18 a, b, 27 a, 30 a and 30 b. Conversely, there appear to be absent from the fossil digests but present in the bone digests peptide peaks in positions 12 a and possibly 21 a also. Quantitative comparisons, on a numerical basis, are considerably more informative. The amounts of peptide in each peak are calculated as peak area units per 1000 total area units (‰) (analogous to expressing amino acids as number of residues per 1000 residues). This method of quantitation is certainly not ideal but, in the absence of any standard conversion factor that could be applied to all peaks, it enables direct comparisons to be made. Direct comparisons between the 'units per 1000' of corresponding peaks from each digest would only be allowable if the rate of breakdown (in terms of $\alpha$-NH$_2$ release) had been approximately the same. This was not so, as comparisons (table 7) between the *total* peak areas for the respective digests showed, with approximately twice the rate of breakdown occurring in the fossil collagen (261 total $\alpha$-amino N ninhydrin 'units') as with the modern ox-bone sample (130 $\alpha$-NH$_2$ 'units'). Hence the adoption of the 'peptide area units per 1000' method.

In the context of this peak area quantitation it is important to emphasize that it is highly

unlikely that most of the peaks observed represented homogeneous peptide species. The broader peaks, and peaks with shoulders, will certainly contain more than one component. Nevertheless, since the peak elution sequence is related to decreasing peptide size, direct quantitative comparisons on a retention time basis will measure gross differences in the relative amounts of the peptides present in the narrow size-range band present in the peak areas compared.

TABLE 7. COMPARATIVE RATES OF α-AMINO N PRODUCTION
IN EACH ENZYME DIGEST

| enzyme | collagenase | pronase | subtilisin |
|---|---|---|---|
| (incubation time) | (3 h) | (20 h) | (1 h) |
| collagen sample | | | |
| fossil woolly rhinoceros | 261 | 197 | 53 |
| modern ox-bone | 130 | 121 | 19 |

The results of such direct quantitative comparison between the fossil and ox-bone collagen digest peptide levels in each of the 44 peak area zones delineated are summarized in table 8. As stated, the peptide peaks have been divided into the five groups, A–E. In table 8 (also tables 9 and 10), column 2 lists the individual peak area units per 1000 for the ox-bone (OB) and woolly rhinoceros (WR), column 3 lists the values as gains ($+$) or losses ($-$) for woolly rhinoceros (i.e. WR $-$ OB), column 4 expresses the same data as percentage differences (100 WR/OB $-$ 100), also listed as gains ($+$) or losses ($-$) relative to a baseline of 100. These results are shown in figure 10 in histogram form.

Although relative gains and losses for the two incubates have been indicated for each peak, it is doubtful if the smaller shifts can be individually assessed as significant. For this purpose it was judged that differences of less than $\pm 5$ peak area units between comparable peaks (column 3) should be treated as uncertain even when, with the smaller peak shifts, they might represent apparently quite large overall percentage changes (column 4). Similarly with the larger peaks (over 20 peak area units each), percentage differences between comparable peak areas of low retention time were only considered significant if they exceeded $\pm 15 \%$. Despite these reservations, all absolute peak area differences are included in table 8 as the overall pattern of changes does indicate important trends which will be examined in the Discussion section.

Group A (peaks 1–11). Inspection of the calculated data in table 8 shows that for group A (the early eluting and therefore larger peptides) 5 out of 11 peak areas showed quite significant relative increases in the fossil digests, peaks 1, 5 and 10 showed more limited increases, and relative areas of peaks 3, 9 and 11 were smaller for the fossil.

Group B (peaks 12–15). No really major quantitative shifts in peak areas were discernible, though peak 12a was present only in the ox-bone, whereas peak 14a was present only in the fossil

Group C (peaks 16–20). Here, in the region of the middle of the run, one peak (16) was reduced in relative area by a quarter in the woolly rhinoceros digest, whereas peak 17 had more than trebled in relative area. Two small new peaks, 18a and 18b, appeared in the fossil digest and the ox-bone digests contained one peak, 20a, not found in the fossil incubate.

Group D (peaks 21–26). The major quantitative differences were the near halving in the relative area of peak 23 in the fossil collagen incubate and the absence of peak 21a.

Group E (peaks 26a–32). In this last group of peptides to be eluted (containing the smallest peptides) several major changes were observed. In the fossil digest there was a relative reduction

TABLE 8. COLLAGENASE PEPTIDE DIGESTS

| 1 | 2a | 2b | 3a | 3b | 4a | 4b |
|---|----|----|----|----|----|----|
| | | | peak area difference (WR − OB) | | relative shift (100 WR/OB − 100) | |
| | peak area (‰) | | | | | |
| peptide peak | OB | WR† | (+) | (−) | (+) | (−) |
| *Group* A (retention time 0–190 min) | | | | | | |
| 1 | 6 | 8 | 2 | — | 33 | — |
| 2 | 9 | 15 | 6 | — | 66 | — |
| 3 | 18 | 10 | — | 8 | — | 44 |
| 3a | — | 11 | — | — | — | — |
| 4 | 30 | 53 | 23 | — | 77 | — |
| 5 | 17 | 21 | 4 | — | 24 | — |
| 6 | 19 | 49 | 30 | — | 158 | — |
| 7 | 39 | 53 | 14 | — | 35 | — |
| 8 | 31 | 39 | 8 | — | 26 | — |
| 9 | 73 | 56 | — | 17 | — | 23 |
| 10 | 8 | 10 | 2 | — | 25 | — |
| 11 | 16 | 13 | — | 3 | — | 18 |
| *Group* B (retention time 190–330 min) | | | | | | |
| 12 | 195 | 181 | — | 14 | — | 7 |
| 12a | 40 | — | — | — | — | — |
| 13 | 39 | 33 | — | 6 | — | 15 |
| 14 | 28 | 26 | — | 2 | — | 8 |
| 14a | — | 6 | — | — | — | 100 |
| 15 | 3 | 6 | 3 | — | — | — |
| *Group* C (retention time 330–470 min) | | | | | | |
| 16 | 114 | 89 | — | 25 | — | 22 |
| 17 | 13 | 45 | 32 | — | 246 | — |
| 18 | 15 | 18 | 3 | — | 20 | — |
| 18a | — | 13 | — | — | — | — |
| 18b | — | 6 | — | — | — | — |
| 18c | — | 2 | — | — | — | — |
| 19 | 9 | 12 | 3 | — | 33 | — |
| 20 | 4 | 10 | 6 | — | 150 | — |
| 20a | 11 | — | — | — | — | — |
| *Group* D (retention time 470–570 min) | | | | | | |
| 21 | 11 | 7 | — | 4 | — | 36 |
| 21a | 8 | — | — | — | — | — |
| 22 | 12 | 7 | — | 5 | — | 42 |
| 23 | 23 | 13 | — | 10 | — | 44 |
| 24 | 3 | 4 | 1 | — | 33 | — |
| 25 | 6 | 5 | — | 1 | — | 18 |
| 26 | 11 | 9 | — | 2 | — | 18 |
| *Group* E (retention time 570–720 min) | | | | | | |
| 26a | — | 2 | — | — | — | — |
| 27 | 38 | 29 | — | 9 | — | 24 |
| 27a | — | 5 | — | — | — | — |
| 28 | 15 | 8 | — | 7 | — | 47 |
| 29 | 21 | 11 | — | 10 | — | 48 |
| 30 | 56 | 31 | — | 25 | — | 44 |
| 30a | — | 15 | — | — | — | — |
| 30b | — | 4 | — | — | — | — |
| 31 | 7 | 38 | 29 | — | 440 | — |
| 32 | 51 | 29 | — | 22 | — | 43 |

† In tables 8–10, OB stands for ox-bone and WR for woolly rhinoceros.
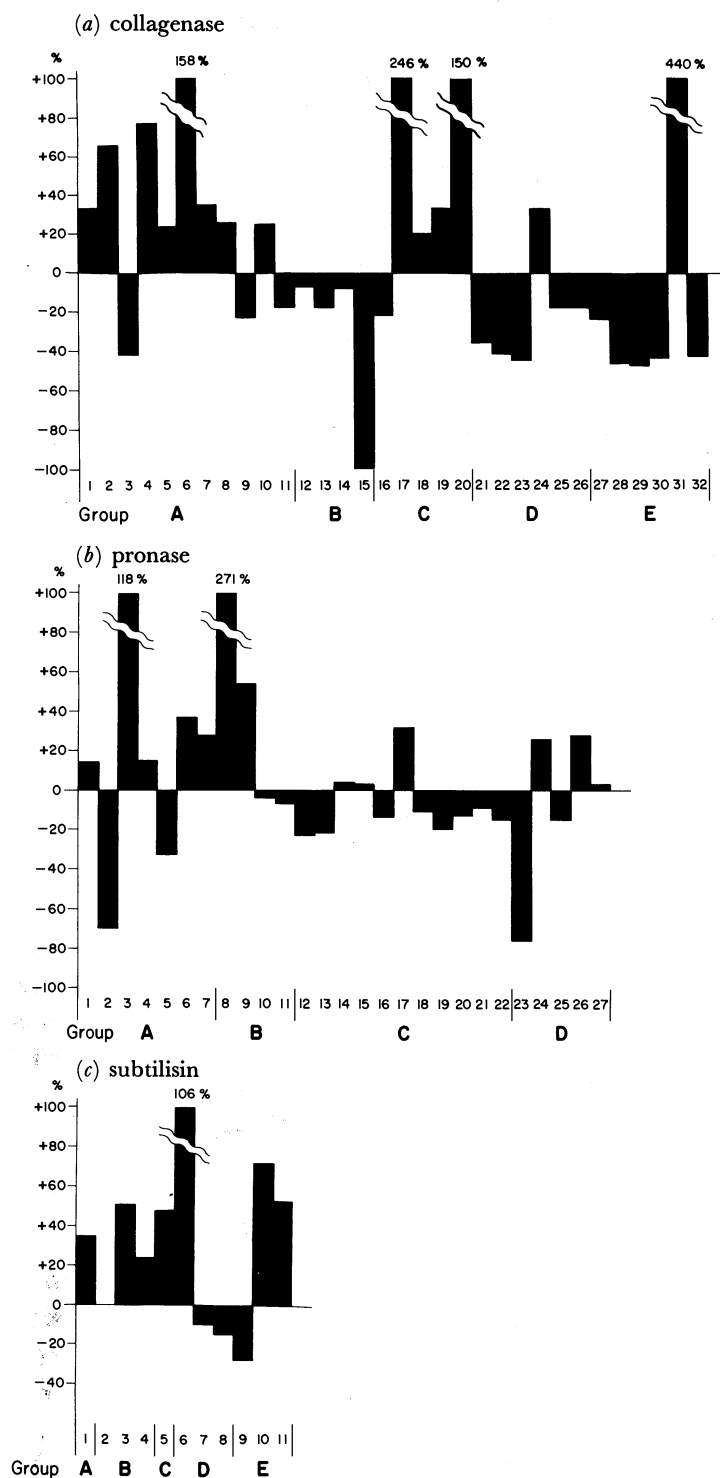
FIGURE 10. The relative abundance of the different peptides formed by enzymic digestion of fossil woolly rhinoceros and modern ox-bone collagen by collagenase, pronase and subtilisin. Peptides are grouped A–E on an approximate size basis as discussed in the text. The horizontal axis represents the modern collagen reference baseline, to which the fossil peptide digest refers: thus the fossil collagen:collagenase digest contains nearly 80 % more of peptide peak 9 (group A), and some 40 % less of peptide peak 32 (group E) than the corresponding modern collagen:collagenase digest. See tables 8–10.

in five of the peptide areas (peaks 27, 28, 29, 30 and 32), most notably peaks 29, 30 and 32. The fossil incubate also showed the appearance of four new peaks (26a, 27a, 30a and 30b) though only 30a can be said to be large enough to be of any significance. Only one peak, 31, was larger in the fossil incubate than in the ox-bone digest.

### TABLE 9. PRONASE PEPTIDE DIGESTS

| 1 | 2a | 2b | 3a | 3b | 4a | 4b |
|---|---|---|---|---|---|---|
| | peak area (‰) | | peak area difference (WR − OB) | | relative shift (100 WR/OB − 100) | |
| peptide peak | OB | WR | (+) | (−) | (+) | (−) |
| | | | *Group* A | | | |
| 1 | 14 | 16 | 2 | — | 14 | — |
| 2 | 10 | 3 | — | 7 | — | 70 |
| 3 | 17 | 37 | 20 | — | 118 | — |
| 4 | 38 | 26 | — | 12 | — | 32 |
| 4a | — | 31 | — | — | — | — |
| 5 | 37 | 25 | — | 12 | — | 33 |
| 6 | 19 | 26 | 7 | — | 37 | — |
| 6a | — | 18 | — | — | — | — |
| 7 | 25 | 32 | 7 | — | 28 | — |
| | | | *Group* B | | | |
| 8 | 7 | 26 | 19 | — | 271 | — |
| 9 | 26 | 40 | 14 | — | 54 | — |
| 10 | 21 | 20 | — | 1 | — | 4 |
| 10a | — | 15 | — | — | — | — |
| 11 | 52 | 48 | — | 4 | — | 7 |

(*Group* C was contaminated with several enzyme 'blank' peaks which made meaningful interpretation of the peptide peaks in this group impossible.)

| | | | *Group* D | | | |
|---|---|---|---|---|---|---|
| 12 | 26 | 20 | — | 6 | — | 23 |
| 13 | 59 | 46 | — | 13 | — | 22 |
| 14 | 72 | 75 | 3 | — | 4 | — |
| 15 | 65 | 67 | 2 | — | 3 | — |
| 16 | 50 | 43 | — | 7 | — | 14 |
| 17 | 31 | 41 | 10 | — | 32 | — |
| 18 | 120 | 107 | — | 13 | — | 11 |
| 19 | 15 | 12 | — | 3 | — | 20 |
| 20 | 15 | 13 | — | 2 | — | 13 |
| 21 | 32 | 29 | — | 3 | — | 9 |
| 22 | 27 | 23 | — | 4 | — | 15 |
| | | | *Group* E | | | |
| 23 | 50 | 12 | — | 38 | — | 76 |
| 24 | 31 | 39 | 8 | — | 26 | — |
| 25 | 61 | 52 | — | 9 | — | 15 |
| 26 | 18 | 23 | 5 | — | 28 | — |
| 27 | 33 | 34 | 1 | — | 3 | — |

*Note.* Retention times for groups A–E as in table 8.

### (ii) *Pronase–collagen incubations*

Chromatograms from enzyme blank runs for the pronase incubations contained approximately 12 ninhydrin-positive peaks. Many of these peaks were relatively small but were in positions where their presence could prevent accurate quantitation of collagen-derived peptides; consequently these areas were not evaluated in peptide chromatograms from the enzyme–substrate incubates.

Both fossil and modern bone collagen preparations proved much less susceptible to attack by pronase than by collagenase and required an overnight (20 h) incubation period to effect release of sufficient amounts of peptide material for analysis (table 7). Figure 9*b* shows the peptide chromatograms from pronase digests of ox-bone and fossil collagen. Comparisons with the preceding collagenase incubate chromatogram maps (figure 9*a*) show a quite different qualitative and quantitative distribution of peptide peak areas. However, there is a similar range of large–medium–small peptides formed by the enzyme's action on both substrates, although there appear to be rather reduced numbers of middle-range peptide groups when compared to the collagenase incubates.

After eliminating enzyme blank peaks and other doubtful areas, the individual *absolute* peak areas were expressed as fractions per 1000 residues and quantitative comparisons were made between peaks with like retention times. The results are shown in table 9.

Table 7 shows that, as with the collagenase incubations, the fossil collagen was the more susceptible to enzyme attack: some 63 % more of the fossil collagen was broken down than of the modern bone over the same 20 h incubation period employed. As indicated, direct comparisons between some peak areas were complicated by the presence of enzyme blank contaminants (presumably peptides). The data in table 9 only refer to the 30 peptide areas unequivocally derived from pronase attack on collagen substrates. Nevertheless, both the fossil and ox-bone pronase digests show several peaks larger than those given by comparable enzyme incubate contaminants, indicating the probable presence of an additional 12 or more peptide regions formed in the collagen–pronase digests.

The absolute peak area values in table 9 do show differences between the pronase peptide chromatograms for the two substrates; the same is true for the collagenase–peptide chromatograms. These differences are in general more limited in variety and extent for the pronase incubates than for the collagenase incubates. There is very little evidence for any significant 'new' peptide products appearing in either incubate and the peak area and percentage shifts are generally of a lower order than found in the collagenase studies. Nevertheless a similar trend in terms of relative abundances of peptides of a given size range is apparent: overall there are relative increases in peak areas for large- to middle-sized peptides in the fossil digests, and in contrast the smaller peptides are generally lower in concentration than for modern bone.

### (iii) *Subtilisin–collagen incubations*

Compared with the slower-acting pronase, subtilisin showed a much higher rate of attack, producing sufficient peptide material for chromatograms from each substrate during an hour of incubation. Unfortunately the incubated control subtilisin preparation used was found to be contaminated with numerous peptide components which severely limited interpretation and evaluation of the substrate chromatograms. Only a small fraction of the peptides appearing on the chromatograms could be directly compared unequivocally and these are listed in table 10. The data are considered too limited for any other than the following few general conclusions to be drawn. First, the fossil collagen is, as with the other two enzymes, broken down at a greater rate than the modern ox-bone substrate (nearly $2\frac{1}{2}$ times the rate, as table 7 shows). The relatively low α-amino N rates for the subtilisin incubates given in table 7 reflect the limitations imposed by the enzyme preparation contaminants. Only the peptides listed in table 10 were used to determine the relative rates of breakdown. The chromatograms (figure 10*c*) and data in table 10 show no really major differences between the two protein digests other than the

overall greater susceptibility to attack in the fossil already commented upon. However there is again the possible inference (table 10, column 4) of raised levels of the larger peptide components with concomitant reduction in the smaller peptides in the fossil digests. Apart from the observation that this conforms to the general pattern observed with the collagenase and pronase digests it would be unwise to read more into the results from the subtilisin incubates.

### TABLE 10. SUBTILISIN PEPTIDE DIGESTS

| 1 | 2a | 2b | 3a | 3b | 4a | 4b |
|---|---|---|---|---|---|---|
| | peak area ‰ | | peak area differences (WR − OB) | | relative shifts (100 WR/OB − 100) | |
| peptide peak | OB | WR | (+) | (−) | (+) | (−) |
| | | | *Group* A | | | |
| 1 | 54 | 73 | 19 | — | 35 | — |
| | | | *Group* B | | | |
| 2 | 19 | 19 | 0 | — | — | — |
| 3 | 63 | 95 | 32 | — | 51 | — |
| 4 | 63 | 78 | 15 | — | 24 | — |
| | | | *Group* C | | | |
| 5 | 168 | 243 | 75 | — | 48 | — |
| 5a | 260 | — | — | — | — | — |
| | | | *Group* D | | | |
| 6 | 45 | 93 | 48 | — | 106 | — |
| 7 | 55 | 50 | — | 5 | — | 10 |
| 8 | 59 | 50 | — | 9 | — | 15 |
| 8a | — | 36 | — | — | — | — |
| 8b | — | 51 | — | — | — | — |
| | | | *Group* E | | | |
| 9 | 150 | 108 | — | 48 | — | 28 |
| 10 | 25 | 43 | 17 | — | 72 | — |
| 11 | 40 | 61 | 21 | — | 53 | — |

*Note.* Retention times for groups A–E as in table 8.

## (d) Discussion

The first observation arising from these comparative studies on fossil and modern collagen enzymic dissolution by the three different collagenolytic enzymes is that in each instance the demineralized collagen from the woolly rhinoceros fossil is broken down at a markedly greater rate than the corresponding modern ox-bone preparation. In terms of $\alpha$-amino N released in unit time, collagenase attacks the fossil collagen at about twice the rate, pronase at $1\frac{1}{2}$ times, and subtilisin at $2\frac{1}{2}$ times.

From these results, it appears that the demineralized fossil collagen is in a form more prone to enzymic proteolysis and it is reasonable to conclude from this that involved in the various mechanisms and processes attending fossilization and subsequent events there were changes to the overall organic matrix that caused a greater net susceptibility to enzyme attack in the systems employed experimentally *in vitro*. This might reflect some degree of a general denaturation which altered the structure of the original protein, so rendering it more available to attack by the enzymes. Alternatively the increased susceptibility might reflect removal, during fossilization, of connective tissue ground substance which, by exposing the collagen fibrils more to the environment, provides greater access and vulnerability to enzymic attack.

Comparisons of the peptide fingerprints from the pronase and subtilisin digests of the fossil

and modern bone show few major specific differences other than overall quantitative changes. Thus, with the pronase incubates, virtually no new peptide products appear in either incubate, though the quantitative data on absolute peak area sizes do indicate an increase of the larger-to middle-sized peptides over the smaller ones (table 9). The observation that the fossil bone is broken down faster probably reflects, as argued above for collagenase, some change in overall accessibility, or alternatively a denaturation effect favouring the initial stages of the attack, with a consequent larger number of the more susceptible peptide bonds broken down in unit time. In the hydrolysis stages subsequent to this, however, it would appear that the enzymic hydrolysis of these larger peptide fragments first formed is apparently more limited in the fossil tissue. This interpretation would explain the observation that in the fossil digests generally there is a shift towards higher ratio of large to small peptide units in the fossil incubates than in the modern bone preparations.

As examined in detail in the Results, nine peptide peaks are found in the fossil collagen–collagenase incubates that are not present in the modern bone digests. These are indicated in table 8 by the lower case suffix (3a; 18a, b, c; 30a; etc.). In contrast there are three peaks (12a, 20a, 21a) present in the modern bone digests that are absent from the fossil. Comparisons of each peak or peak retention time area on a peak area unit per 1000 basis show significant increases or decreases in the relative amounts of the various peptide components present in the modern and fossil bone collagenase digests. These are shown in columns 3a and 3b of table 8: column 3a shows the larger quantity ($+$) in peak area units per 1000 in the fossil bone digest relative to the level found in the same peak retention time areas in the comparable ox-bone digest; column 3b shows the peak areas where lesser amounts ($-$) of peptide peak components are found in fossil collagen digests. Column 4 records these shifts on a numerical basis relative to the ox-bone levels.

From column 3 it can be seen that the group A larger peptides show the greatest number of peptide peak area increases relative to the modern bone pattern. Peptide groups B and C, which contain the medium-sized peptide components, show a scattering of both gains and losses compared with the levels found in the modern bone digests; but in small peptide elution zones on the chromatogram (groups D and E) it can be seen that *reductions* in peptide levels in fossil digests relative to modern bone predominate (see figure 10 also).

Overall then the fossil–collagenase digests are characterized by:

(i) greater amounts of the larger molecular mass peptides;

(ii) lower levels for the smaller peptide components; and

(iii) the presence of nine peptide components not found in the bone digests and the absence of three peptides that are found in the bone digests.

It is suggested that these results may indicate modifications that have occurred at specific sites in the woolly rhinoceros collagen polypeptide chains, either during fossilization or as a consequence of post-fossilization environmental changes in the peptide bone regions susceptible to collagenase attack. The hypothesis proposed to explain the nature of these changes induced in the fossil collagen matrix, as indicated by the three enzyme hydrolysis studies, is best understood by reference to figure 11. At the top of figure 11 is represented modern collagen with the hypothetical amino acids (1, 2, 3, etc.) linked in chains by peptide bonds. The broad arrows indicate those bond sites that are attacked first (stage I) owing to their greater accessibility and/or susceptibility. The thin arrows show peptide bonds that are broken subsequently (stage II) in the larger peptide fragments first formed. Clearly this is no more than a broad generalization
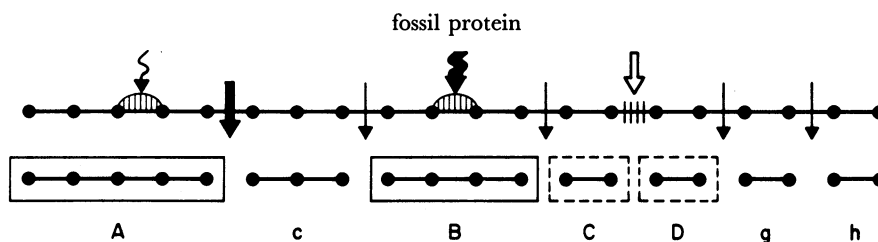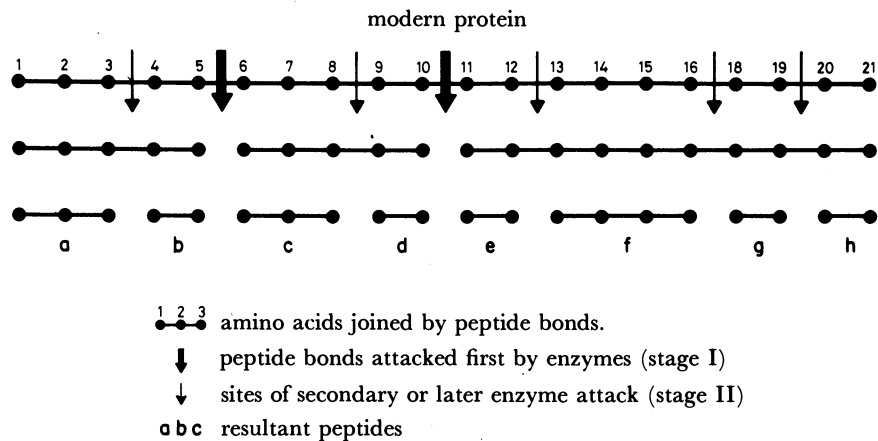
FIGURE 11. Diagrammatic representation of the hypothesis explaining observed differences between fossil and modern collagen enzyme digests.

of the sequential pattern of enzymic attack, but is represented in this simplified form to clarify the argument.

The final result of the enzyme–modern collagen substrate incubation is to produce the series of peptides a, b, c, ..., g, h, etc. that form the consequent fingerprint map, characteristic for the particular enzyme concerned. For fossil proteins we postulate three possible modifications to collagen that can account for the changes in the fossil fingerprint maps observed in our experimental results.

*Type* 1. The stage I susceptible bond sites are modified in one of two ways.

(A) Some side chain modifications and/or conformational changes render these stage I bonds more resistant to breakdown than the normal modern collagen rate. This is represented by the symbol

(B) Matrix modifications such as removal of ground substance, side chain alterations or conformational changes make the substrate overall more susceptible to enzymic hydrolysis.

*Type* 2. (A) A similar modification to 1A but operating at stage II peptide bond breakage; this makes the bond more resistant and is represented in the diagram by

(B) A peptide bond previously not attackable or available to the enzyme becomes vulnerable or exposed to attack. This is represented by the symbol

The effects of these various types of modification will be reflected in the chromatogram maps as follows.

*Type* 1A. The appearance of *new* larger peptide units concurrent with a reduction in the number of smaller peptides. For example, the bond 10–11 is rendered more resistant, with the ultimate diminution or loss of peptides d and e at stage II, and a concomitant appearance of the new peptide 'B' in the incubate.

*Type* 1B. These will have the overall effect of producing a much more rapid, stage I, general dissolution of the fossil matrix.

*Type* 2A. These are essentially similar in effect to type 1A modifications, resulting in the formation of a larger peptide at the expense of smaller peptide unit components: for example a reduction in the tripeptide a and dipeptide b in, or their elimination from, the fingerprint, with the associated appearance of the pentapeptide A. Here, however, A will have been formed in stage I of the ox-bone collagen breakdown to be subsequently broken down in stage II to form the peptides a and b. Fossil matrix modifications of this type would result in either

(*a*) appearance of greater amounts of larger peptides along with allied losses of the smaller units, or

(*b*) appearance of a peptide (A) associated with reductions in levels of the smaller peptides. Alternative (*b*) will operate when stage II breakdown of, say, bond 3–4 is complete in the modern bone incubation.

*Type* 2B. Here the development of new enzyme-vulnerable peptide bonds (e.g. 14–15) will result in a modified (fossil) matrix producing on enzymic incubation the two dipeptides C and D at the expense of the tetrapeptide f, which forms in the modern bone digest. In effect, these will be recognized as 'new' peptides in the modern bone collagen digest, compared with the fossil one, i.e. there will be peptides present in bone digests (e.g. f) that are not found in fossil incubates.

Consideration of these hypothetical models in the context of the actual experimental results obtained leads to the following conclusions.

(i) Since with all the enzyme incubation studies the fossil matrix is found to be attacked much faster than the modern matrix equivalent, then type 1B modifications (affecting stage I enzymic attack) must predominate in all instances. This observation suggests that some removal

or modification of matrix ground substance, or protein conformational changes (for which the electron microscopy studies (§4) provide some circumstantial support), has occurred in the fossilization and/or post-fossilization environment. Type 2B modifications will have a similar effect and there is some evidence from the collagenase studies that they may have occurred. However, their presence must be of a more limited nature, because of the relatively few 'new' peptides arising in the bone digests. They cannot, therefore, be major causal factors of the greater collagenolytic susceptibility observed in the fossil matrix preparations.

(ii) The observation in all the fingerprint maps of increased levels of larger peptide units, with concomitant reduction in the smaller units, points to the presence of type 1A and type 2A modifications. These will operate predominantly at stage II enzyme attacks and be most evident with the more specific mode of a collagenase attack. These types of modification can account for the appearance of the 'new' peptides found in the fossil collagen–collagenase fingerprint maps.

(iii) The occurrence of type 2B modifications at stage II enzymolysis level is again indicated by the collagenase studies alone and accounts for the detection of 'new' peptides found in the bone digests, new in the sense that they are *not* found in comparable fossil digests.

With the pronase and subtilisin incubations the type 1B and 2B modifications are considered to predominantly determine the observed increased rate of dissolution of the fossil collagen matrix. These two enzymes are probably insufficiently substrate-specific to pick out the more sophisticated levels of matrix changes revealed by the collagenase enzyme studies.

Evidence for or against one or more of the above hypotheses should result from future studies on the individual peptide levels in fossil and modern bone collagenase incubates over an extended period. Comparisons of formation rates of peptides present in the fossil digests, yet absent in the ox-bone digest, and *vice versa*, could indicate the relative contributions of the different type I and type 2 modifications. With the increasing sensitivity of amino acid analysers and sequencing methodology it may even be possible eventually to investigate such peptides, isolated from fossil digests, and identify the location and nature of any modification involved.

## 6. CONCLUSIONS

The study of fossil proteins has proven fraught with difficulties, not least the equivocal interpretation of the results of the numerous analyses that have accumulated over the past two decades. Following upon the discovery that amino acids were preserved in fossils over periods of many million years, the field of amino acid geochemistry has developed significantly. Initially there was the accumulation of raw data, but the stage has now been reached when the methods used for acquisition of such data must be subjected to more rigorous analytical controls and evaluation. It is now evident that the amino acid analyses of a fossil sample cannot be used as a basis for interpretative evaluations until additional key tests and checks have been made. Armstrong & Halstead Tarlo in 1966 first emphasized the necessity of analysing the enclosing sediments as well as the fossil itself in order to determine the likely provenance of the organic matter that was being analysed, and the importance of including, routinely, such environmental controls is clearly vindicated by the results reported in this study. The evidence obtained indicates that collagen as a biochemical entity does not remain unchanged or intact for long in geological terms, although under certain highly specialized conditions it may possibly survive from as far back as the Mesozoic. Biochemical analysis of Palaeozoic material is almost

invariably merely detecting the general background of organic debris common to both fossil and sediment. The source of this organic matter is not only the surrounding sediment but also organisms concerned with the processes of decomposition of animal matter. Apart from such contamination which occurred during the natural processes of burial and decay, by far the most intractable problem is that of present day contamination acquired during collection and storage. Here at least the degree of racemization observed for certain amino acids can provide an accurate assessment of modern contamination in the older fossils. It is considered essential that monitoring the degree of racemization should become a routine aspect of all fossil amino acid analyses. Only with such data is one able to decide whether or not a particular analysis is trustworthy.

With the data from such analyses, it is therefore possible to be guided in the selection of the more worthwhile specimens for further study, for example by electron microscopy.

Even though amino acid analysis of a fossil sample may give a close approximation to a modern collagen compositional profile, it is now evident that there may have been diagenetic changes to the peptides, not detected in the amino acid analyses. Although there may be remains of collagen molecules in fossils ostensibly structurally intact, there is now evidence of subtle degradative changes, not found in comparable modern material. It is here that the digestion of such fossil collagens by proteolytic enzymes can provide more specific information on important modifications at particular residue sites within the intact molecules that are not evident from routine amino acid analysis. In summary, when dealing with fossil material suspected of containing proteinaceous matter, the routine analysis of both the fossil and surrounding sediment, and the monitoring of the degree of racemization is a prerequisite to any further study and interpretation. The next stage in the development of the subject should be in the area of peptide fingerprint studies. Here it seems clear that the more meaningful results are likely to come in the first instance from studies of the collagen remnants present in Pleistocene fossils, which should form a reference point for comparison with older fossil calcified tissues.

On the basis of their results in this investigation, it is the authors' opinion that, for the present at least, there seems little hope that direct study of the evolution of proteins based on fossil material can be achieved from analysis of the amino acid patterns alone. Nevertheless, there does seem to be every likelihood of obtaining new and significant information on the process of protein diagenesis through time, by using and developing the peptide fingerprinting approach described here. In conjunction with the fossil collagen immunospecifity technique of Lowenstein (1980, 1981), this gives promise of future developments in the field of fossil collagen studies and evolutionary relationships.

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## Appendix 1. Amino acid analysis

### Buffer preparations

In the single column system the following were required: pH 3.05, 0.2 M; pH 4.25, 0.2 M; pH 5.25, 0.8 M. In the dual column system the following were required: pH 3.05, 0.2 M; pH 4.40, 0.2 M; pH 5.00, 0.35 M (for basic column loading).

For the 0.2 M buffers, a 9 l stock solution was prepared as follows: sodium citrate.$2H_2O$ (Analar, 147.1 g) and sodium hydroxide (Analar, 20 g) were dissolved in distilled, deionized water, and made up to 9 l and stored in a stock buffer bottle. Of this stock solution 2.25 l portions were boiled for 5 min to remove $NH_2$, covered with foil, allowed to cool to room temperature, then titrated to the required pH with HCl (Aristar, 6 M), with use of a pH meter. For the 0.8 M buffer the procedure was as above, but NaCl was added to the stock solution before $NH_3$ was boiled off.

Finally, for the 0.35 M buffers, citric acid (Analar, 61.25 g) and sodium hydroxide (Analar, 36.0 g) were dissolved in 2.25 l of distilled deionized water, then boiled for 5 min, cooled and titrated as previously.

All of the buffers were made up to 2.5 l with distilled deionized water, after the following additions had been made.

| buffer pH ... | 3.00 | 4.25 | 4.65 | 5.00, 5.25 |
| --- | --- | --- | --- | --- |
| thiodiglycol (25%) | 40 ml | 40 ml | 40 ml | — |
| octoic acid | 2.5 ml | 2.5 ml | 2.5 ml | 2.5 ml |
| Brij (30%) | 5.0 ml | 5.0 ml | 5.0 ml | 5.0 ml |

The ninhydrin solution was made up 1 day in advance, to allow time for stabilization, as follows. Methyl cellosolve (3125 ml), sodium acetate buffer (4 M, pH 5.5, 1250 ml) and distilled deionized water (625 ml) were mixed together and then nitrogen was bubbled through the mixture for 20 min. Ninhydrin (B.D.H., 60 g) was added and nitrogen was bubbled through the mixture again. When the ninhydrin had dissolved, stannous chloride (1.67 g) was added, and nitrogen was bubbled through until colour of the solution lightened. Ninhydrin solution was stored, covered with Parafilm, at 4 °C till the following day. The sodium acetate buffer (4 M, pH 5.5) was prepared as follows: sodium acetate $3H_2O$ (Analar, 2720 g) was dissolved in 2 l of hot distilled deionized water, then cooled to room temperature; glacial acetic acid (Aristar, 500 ml) was added and then the solution was made up to 5 l with distilled deionized water at room temperature. The flask was kept covered to avoid absorption of $NH_3$.

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## APPENDIX 2. AMINO ACID ASSAYS

TABLE 11. CAENOZOIC FOSSILS AND SEDIMENTS: AMINO ACID ANALYSIS

| specimen no. … | 7 | 7 | 7 | 7 | 7 | 6 | 6 | 6 | 5 | 5 | 4 | 3 | 2 | 1 | 1 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | sediment residue | sediment supernatant | Emys residue | Emys suspension | Emys supernatant | sediment residue | sediment supernatant | Australopithecus | Coelodonta (B) residue | Coelodonta (B) supernatant | Coelodonta (A) | Mammuthus | Homo sapiens | ungulate residue | ungulate supernatant | oxbone collagen |
| **Amino acid content (‰)** | | | | | | | | | | | | | | | | |
| Hyp | — | — | — | — | — | — | — | — | — | — | 132 | 120 | 123 | 11 | 119 | 101 |
| Asp | — | 27 | 83 | 72 | 47 | 34 | 4 | 275 | 95 | 103 | 54 | 72 | 42 | 4 | 53 | 51 |
| Thr | — | 11 | 18 | 42 | 26 | 21 | 5 | 100 | 54 | 43 | 16 | 18 | 18 | 2 | 25 | 18 |
| Ser | — | 32 | 55 | 276 | 151 | 75 | 23 | 139 | 53 | 37 | 44 | 21 | 26 | 3 | 28 | 35 |
| Glu | — | 35 | 92 | 15 | 25 | 71 | 86 | 126 | 140 | 120 | 92 | 110 | 78 | 15 | 77 | 75 |
| Pro | — | 53 | 103 | 36 | 54 | 26 | — | 196 | 41 | 40 | 146 | 154 | 134 | 37 | 137 | 116 |
| Gly | 15 | 191 | 93 | 167 | 109 | 167 | 125 | 85 | 140 | 133 | 270 | 246 | 309 | 405 | 302 | 317 |
| Ala | 14 | 67 | 51 | 96 | 51 | 208 | 63 | 20 | 120 | 129 | 120 | 123 | 114 | 118 | 111 | 113 |
| Val | — | 21 | 46 | 32 | — | 31 | — | — | 63 | 64 | 21 | 19 | 28 | 32 | 25 | 19 |
| Cys | — | 37 | — | — | — | — | — | — | — | 24 | — | 12 | — | — | — | — |
| Met | — | — | — | — | — | — | — | 6 | — | 18 | — | 4 | 11 | 13 | 11 | 5 |
| Ile | 11 | 12 | 38 | 30 | 69 | 26 | 96 | 9 | 44 | 39 | 8 | 7 | 26 | 25 | 22 | 13 |
| Leu | 220 | 262 | 36 | 21 | 26 | 41 | 49 | 14 | 68 | 71 | 19 | 16 | — | 70 | — | 28 |
| Tyr | 6 | 2 | 10 | 17 | — | — | — | 16 | 17 | 14 | — | 8 | — | — | — | 5 |
| Phe | 162 | 37 | — | 17 | 14 | 145 | 41 | 15 | 27 | 32 | 10 | 12 | 14 | 39 | 11 | 18 |
| Hyl | — | — | — | — | — | — | — | — | 9 | — | — | — | 3 | 14 | 6 | 5 |
| Orn | 64 | 110 | — | 7 | 42 | 27 | — | — | 14 | 6 | — | 20 | 2 | 4 | 3 | 2 |
| Lys | 93 | 42 | 26 | 18 | 17 | 79 | 74 | — | 7 | 51 | 14 | — | 24 | 77 | 27 | 24 |
| His | 106 | 21 | 124 | 9 | 32 | — | — | — | 18 | 31 | — | 2 | 1 | 2 | 1 | 5 |
| Arg | 309 | 40 | — | 13 | — | 49 | 13 | — | 49 | 45 | 53 | 35 | 48 | 108 | 34 | 49 |
| Gln | — | — | — | 7 | — | — | 85 | — | — | — | — | — | — | — | — | — |
| Asn | — | — | — | 1 | — | — | 61 | — | — | — | — | — | — | — | — | — |
| unknown | — | — | — | 121 | 319 | — | 276 | — | 27 | — | — | — | — | — | — | — |
| **Racemization** | | | | | | | | | | | | | | | | |
| percentage D-allo-Ile in total Ile | n.d. | n.d. | 47 | 28 | n.d. | n.d. | n.d. | 5 | 8 | 2.5 | 5 | 3 | n.d. | n.d. | n.d. | |
| **Fossil protein content** | | | | | | | | | | | | | | | | |
| total mass of amino acids as a percentage of sample mass | 0.002 | 0.00025 | 0.01 | 0.01 | 0.003 | 0.001 | 0.001 | 0.02 | 0.08 | 0.08 | 1 | 0.25 | 1 | 2 | 0.5 | |

Abbreviation n.d. stands for not detected; a dash (—) indicates absence in analysis.

TABLE 12. MESOZOIC FOSSILS AND SEDIMENTS: AMINO ACID ANALYSES

| | 8 dinosaur supernatant | 8 dinosaur residue | 9 siluroid supernatant | 9 siluroid residue | 10 Pachythrissops supernatant | 10 Pachythrissops residue | 10 sediment supernatant | 10 sediment residue | 11 Cetiosaurus supernatant | 11 Cetiosaurus residue | 11 sediment supernatant | 11 sediment residue | 12 ichthyosaur supernatant | 12 ichthyosaur residue | 13 Triassic fish supernatant | 13 Triassic fish suspension | 13 Triassic fish residue |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Amino acid content* (‰) | | | | | | | | | | | | | | | | | |
| Hyp | 192 | — | — | — | — | — | 110 | — | — | — | 110 | — | | — | — | — | — |
| Asp | 296 | 124 | 128 | 162 | 393 | 189 | 53 | 187 | 86 | 85 | 35 | 99 | | 174 | 101 | 5 | 65 |
| Thr | 167 | 76 | 9 | 56 | 14 | 11 | 42 | 105 | 15 | 33 | 19 | 45 | | 66 | 34 | 92 | 18 |
| Ser | — | 64 | 154 | 155 | 19 | 82 | 86 | 146 | 53 | 86 | 72 | 148 | | 5 | 97 | 62 | 63 |
| Glu | — | 86 | 20 | 113 | 29 | 71 | 9 | 42 | 104 | 92 | 64 | 53 | | 96 | 59 | — | 75 |
| Pro | — | 29 | — | 29 | — | — | — | — | 54 | 90 | 117 | — | | 51 | — | — | 53 |
| Gly | 34 | 141 | 21 | 78 | 90 | 135 | 99 | 192 | 209 | 210 | 250 | 185 | | 57 | 309 | 394 | 195 |
| Ala | 16 | 103 | 17 | 165 | 46 | 55 | 103 | — | 100 | 94 | 99 | 96 | | 68 | 173 | 208 | 272 |
| Val | — | 63 | — | 25 | 11 | 20 | — | — | 33 | 40 | 29 | 46 | | 17 | — | 35 | 43 |
| Cys | | | | | | | | | | | | | | | | | |
| Met | | | | | | | | | | | | | | | | | |
| Ile | 29 | 37 | 38 | 36 | 8 | 18 | 11 | 40 | 24 | 34 | 12 | 38 | | 80 | 25 | 109 | 43 |
| Leu | 29 | 46 | 49 | 51 | 15 | 27 | 353 | 35 | 28 | 59 | 43 | 36 | 110· | 128 | 23 | 50 | 31 |
| Tyr | — | 9 | 10 | 9 | — | 5 | 7 | 73 | 6 | 25 | 2 | 6 | 46 | 39 | 12 | 6 | — |
| Phe | 14 | 27 | 23 | 21 | 33 | 31 | 84 | 84 | 55 | 54 | 30 | 50 | 800 | 74 | 20 | 40 | 26 |
| Hyl | — | 19 | — | — | 13 | 42 | — | — | — | — | — | — | — | — | — | — | — |
| Orn | — | — | — | — | — | — | 126 | — | 34 | 11 | 48 | 40 | — | — | — | — | — |
| Lys | 45 | 7 | — | 7 | 86 | 103 | 15 | — | 15 | 26 | 19 | 46 | — | — | — | — | — |
| His | 15 | 13 | 9 | 11 | 12 | 12 | 12 | — | 6 | 4 | — | 14 | 17 | 3 | 9 | — | 10 |
| Arg | 10 | 52 | 4 | 14 | 2 | 8 | — | — | 13 | 15 | 20 | 99 | 27 | 42 | 6 | — | 26 |
| Gln | — | 52 | — | 57 | — | — | — | 97 | — | — | 30 | — | — | 60 | — | — | — |
| Asn | — | 16 | — | 8 | — | — | — | — | — | — | — | — | — | 42 | — | — | — |
| unknown | 154 | 6 | 519 | — | 228 | 191 | — | — | 163 | 39 | — | — | — | — | 133 | — | 78 |
| *Racemization* | | | | | | | | | | | | | | | | | |
| percentage D-*allo*-Ile in total Ile | n.d. | 27 | 40 | 15.5 | n.d. | n.d. | n.d. | n.d. | 29 | 15 | n.d. | n.d. | n.d. | 12 | 25 | 43 | 33 |
| *Fossil protein content* | | | | | | | | | | | | | | | | | |
| total mass of amino acids as a percentage of sample mass | 0.0004 | 0.004 | 0.0005 | 0.002 | 0.0011 | 0.0012 | 0.0008 | 0.0004 | 0.0007 | 0.001 | 0.0024 | 0.0009 | 0.00007 | 0.0004 | 0.00017 | 0.00004 | 0.00016 |

Abbreviation n.d. stands for not detected; a dash (—) indicates absence in analysis.

TABLE 13. PALAEOZOIC FOSSILS AND SEDIMENTS: AMINO ACID ANALYSES

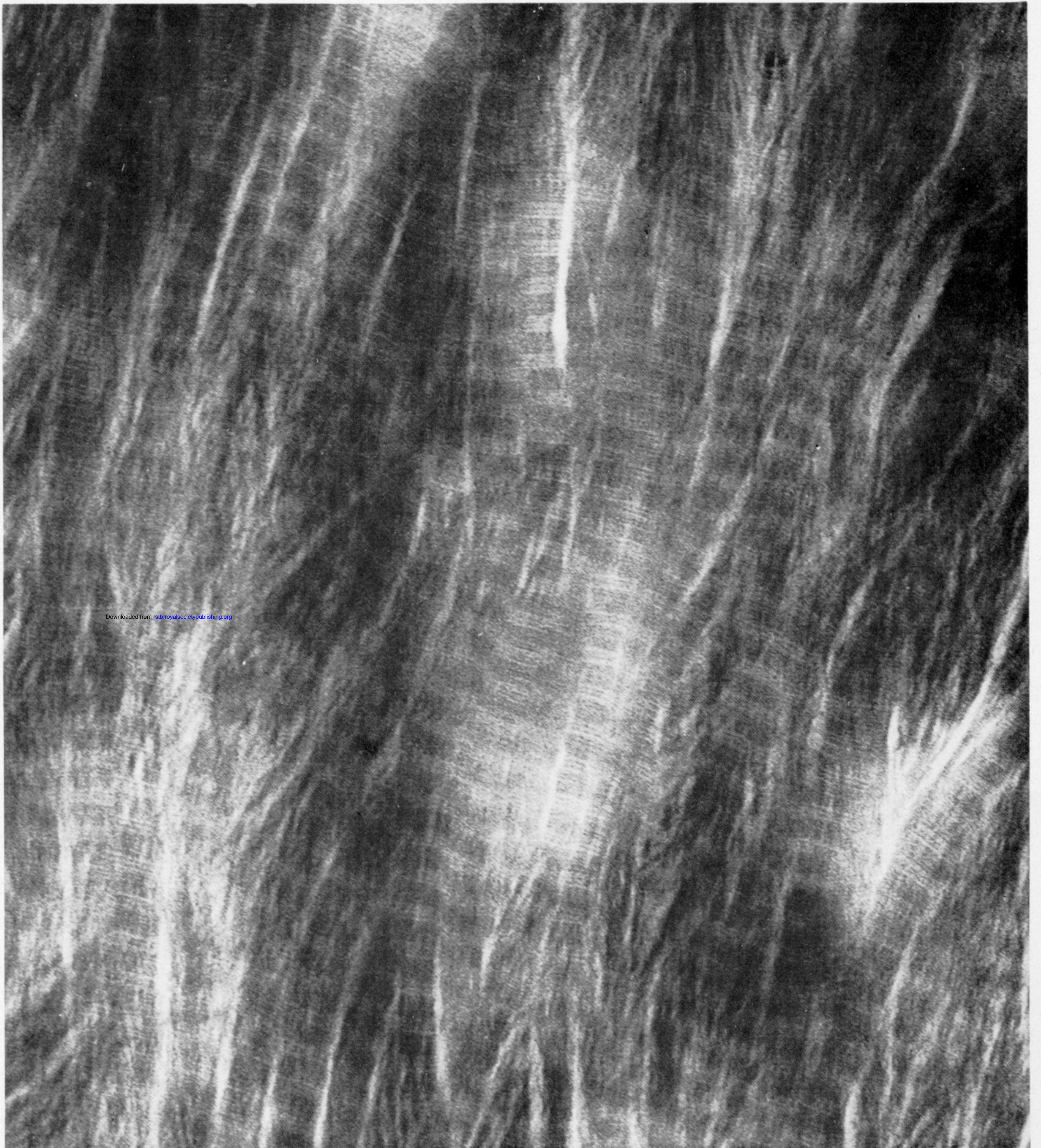| specimen no. | 14 | 14 | 14 | 14 | 15 | 15 | 15 | 15 | 16 | 16 | 16 | 16 | 17 | 17 | 17 | 18 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Plourdosteus* supernatant | *Plourdosteus* residue | sediment supernatant | sediment residue | *Plourdosteus* supernatant | *Plourdosteus* residue | sediment supernatant | sediment residue | *Plourdosteus* supernatant | *Plourdosteus* residue | sediment supernatant | sediment residue | *Wijdeaspis* | sediment supernatant | sediment residue | conodonts | hand contamination (control) |
| **Amino acid content (‰)** | | | | | | | | | | | | | | | | | |
| Hyp | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| Asp | 315 | 179 | 84 | 58 | 180 | — | 253 | 330 | — | 187 | 75 | 63 | — | 191 | — | 22 | 33 |
| Thr | 26 | 51 | 46 | 158 | 61 | 351 | 37 | 10 | 181 | 51 | 51 | 55 | — | 41 | — | — | 59 |
| Ser | 231 | 270 | 202 | 198 | 12 | — | 249 | 161 | 52 | 76 | 341 | 325 | — | 259 | — | 101 | 415 |
| Glu | 17 | 38 | 60 | 29 | 66 | 79 | 40 | 41 | 50 | 86 | 19 | 20 | — | 38 | — | 15 | 20 |
| Pro | 44 | 38 | 49 | 26 | — | — | 36 | 23 | 62 | 41 | 29 | 30 | — | 32 | — | 104 | — |
| Gly | 137 | 165 | 227 | 204 | 254 | 256 | 150 | 157 | 280 | 232 | 222 | 225 | — | 156 | — | 102 | 204 |
| Ala | 86 | 95 | 134 | 134 | 129 | 116 | 91 | 86 | 97 | 114 | 122 | 131 | — | 97 | — | 43 | 92 |
| Val | 24 | 47 | 37 | 53 | — | 33 | 34 | 24 | 24 | 47 | 36 | 37 | — | 35 | — | 28 | — |
| Cys | — | — | 29 | — | — | — | — | — | — | — | — | — | — | — | — | 107 | — |
| Met | — | — | 8 | — | — | — | — | — | — | — | — | — | — | — | — | 24 | — |
| Ile | 16 | 26 | 22 | 23 | 79 | 17 | 17 | 13 | 28 | 28 | 21 | 22 | — | 29 | 240 | 6 | — |
| Leu | 48 | 25 | 42 | 19 | 128 | 51 | 43 | 65 | 154 | 50 | 28 | 29 | — | 26 | 418 | 20 | — |
| Tyr | 16 | 18 | 9 | 20 | 51 | 9 | 19 | 12 | 6 | 4 | 7 | 8 | 1000 | 12 | 100 | — | — |
| Phe | 14 | 16 | 8 | 20 | 38 | 19 | 15 | 16 | 22 | 19 | 14 | 16 | — | 21 | 153 | 230 | — |
| Hyl | — | — | — | — | — | — | — | — | — | 23 | — | — | — | — | — | 52 | — |
| Orn | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 116 | 178 |
| Lys | 14 | 11 | 19 | 10 | — | — | 12 | 9 | 34 | — | 28 | 29 | — | 21 | 79 | — | — |
| His | 8 | 17 | 17 | 34 | 2 | 8 | 5 | 5 | 10 | 3 | 7 | 11 | — | 7 | 11 | 11 | — |
| Arg | — | 3 | 8 | 14 | — | 7 | — | — | — | 20 | — | — | — | — | — | 19 | — |
| Gln | — | — | — | — | — | — | — | — | — | — | — | — | — | 35 | — | — | — |
| Asn | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| unknown | — | — | — | — | — | 53 | — | 46 | — | 18 | — | — | — | — | — | — | — |
| **Racemization** | | | | | | | | | | | | | | | | | |
| percentage D-*allo*-Ile in total Ile | n.d. | 8.6 | 6.5 | 13 | 18 | 18.5 | n.d. | 6 | 27 | 16 | n.d. | n.d. | n.d. | n.d. | 53.8 | 36 | 40 |
| **Fossil protein content** | | | | | | | | | | | | | | | | | |
| total mass of amino acids as a percentage of sample mass | 0.0011 | 0.0018 | 0.001 | 0.002 | 0.0005 | 0.0009 | 0.0011 | 0.0012 | 0.0005 | 0.0029 | 0.0047 | 0.0026 | 0.0038 | 0.0008 | 0.0001 | 0.01 | — |

Abbreviation n.d. stands for not determined; a dash (—) indicates absence in analysis.

TABLE 14. LIVING FOSSILS: AMINO ACID ANALYSES

| | Latimeria scale | Latimeria tooth | Latimeria skin | Macropoma | Neoceratodus | Protopterus | Lepidosiren | Chirodipterus | Sphenodon bone | Sphenodon skin |
|---|---|---|---|---|---|---|---|---|---|---|
| specimen no. … | 19 | 19 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 25 |
| **Amino acid content (‰)** | | | | | | | | | | |
| Hyp | 77 | 70 | 70 | — | 71 | 77 | 82 | — | 83 | 87 |
| Asp | 58 | 59 | 53 | 105 | 52 | 63 | 56 | 63 | 66 | 56 |
| Thr | 20 | 24 | 21 | 16 | 26 | 33 | 27 | 36 | 29 | 25 |
| Ser | 42 | 54 | 51 | 84 | 40 | 45 | 44 | 187 | 64 | 66 |
| Glu | 72 | 80 | 79 | 62 | 83 | 88 | 86 | 30 | 93 | 79 |
| Pro | 121 | 107 | 107 | — | 142 | 125 | 121 | 32 | 99 | 106 |
| Gly | 343 | 305 | 335 | 196 | 311 | 284 | 308 | 203 | 285 | 307 |
| Ala | 115 | 116 | 123 | 69 | 121 | 112 | 113 | 96 | 90 | 100 |
| Val | 17 | 24 | 19 | — | 24 | 29 | 28 | 29 | 28 | 25 |
| Cys | — | — | — | — | — | — | — | — | — | — |
| Met | 3 | 6 | 1 | 32 | — | — | — | — | 19 | 17 |
| Ile | 10 | 13 | 12 | 37 | 13 | 15 | 14 | 16 | 38 | 32 |
| Leu | 23 | 30 | 25 | — | 25 | 30 | 29 | 114 | — | — |
| Tyr | 3 | 3 | 2 | — | 7 | 2 | 1 | 12 | 20 | 19 |
| Phe | 14 | 17 | 16 | 26 | 19 | 18 | 14 | 90 | 7 | 4 |
| Hyl | — | — | — | — | 5 | 5 | 7 | — | 1 | 1 |
| Orn | 17 | 22 | 18 | — | 1 | 1 | 1 | 22 | — | — |
| Lys | 3 | 3 | 1 | — | 18 | 17 | 13 | 5 | 21 | 23 |
| His | 4 | 4 | 2 | — | 1 | 1 | 3 | — | 2 | 1 |
| Arg | 41 | 45 | 45 | — | 38 | 51 | 49 | 65 | 50 | 52 |
| Gln | 5 | 6 | 1 | 15 | 1 | 1 | 2 | — | 3 | 1 |
| Asn | 5 | 7 | 4 | 43 | 2 | 1 | 1 | — | 3 | 1 |
| unknown | — | — | — | 317 | — | — | — | — | — | — |
| **Racemization** percentage D-allo-Ile in total Ile | — | — | — | 30 | — | — | — | 20 | — | — |
| **Fossil protein content** total mass of amino acids as a percentage of sample mass | — | — | — | 0.005 | — | — | — | n.d. | — | — |

Abbreviation n.d. stands for not determined; a dash (—) indicates absence in analysis.

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FIGURE 8. Transmission electron micrograph of collagen preparation from demineralized woolly rhinoceros specimen (magn. × 121 000).