PRESERVATION OF AVIAN COLLAGEN IN AUSTRALIAN QUATERNARY CAVE DEPOSITS

by R. F. BAIRD *and* M. J. ROWLEY

ABSTRACT. The small well-preserved bones of ten avian fossils, species of the quail *Turnix*, from five Australian caves, ranging in age from 9000 to 38,000 years, were tested in a radioimmunoassay for collagen. Collagen was well preserved in all cave environments studied, whether 'wet' or 'dry', and nine of ten samples tested contained collagen, from 10% to 92% of the collagen content of fresh bone. The age of the bone was not clearly related to its collagen content, although the amount of collagen detected was significantly less in the older of the two samples for three of the five caves. The moisture content of the cave was not shown to affect the preservation of collagen. This study suggests that caves may provide a favourable environment for the preservation of collagen where fossils are physically well preserved.

PHYLOGENETIC relationships have traditionally been established using morphological criteria. Recently biochemical and immunological comparisons of proteins or of DNA have also been used in determining relationships (Wilson *et al.* 1977; O'Brien *et al.* 1985). Differences between morphologically and biochemically derived phylogenies therefore have caused debate on the applicability of each method (Hillis 1987). For example, re-evaluation of the morphological evidence for primate evolution in the light of data from comparisons of proteins or of DNA, suggests that gorillas, chimpanzees and man may have shared a common ancestor as recently as 5 million years ago, as predicted by molecular evidence, and not 15–20 million years ago as first suggested from morphological evidence (Lowenstein and Zihlman 1984). Similarly, phylogenies derived by Sibley and Ahlquist (1983) using DNA hybridization are stimulating a reassessment of the origins of many Australian birds. These techniques have been extended to examine relationships of recently extinct animals, such as the quagga *Equus quagga* (Higuchi *et al.* 1984) and the thylacine *Thylacinus cynocephalus* (Lowenstein *et al.* 1981), whose preserved skins have been used as a source of organic material, but for genuine fossils, phylogenies continue to be based on morphology.

Proteins may survive for considerable periods in fossils, however: bone collagen is one of the sources of ^{14}C used to date fossils, and the characteristic amino acids of collagen have been detected in dinosaur bones 200 million years old (Wyckoff 1980). Furthermore, material which reacts with specific antibodies to collagen has been detected in mammalian fossils millions of years old (Lowenstein 1980, 1981; Rowley *et al.* 1986).

During a study of the survival of collagen in Australian fossil sites (Rowley *et al.* 1986; Rowley unpubl. data) we gained the impression that caves provided a poor environment for the preservation of collagen. By contrast, bone morphology may be excellently preserved in most caves (Baird in press). We noted that most of the material examined for collagen had been from caves with abundant moisture. This study was therefore commenced to see whether, when well-preserved bones were selected, the moisture level within the cave could be shown to influence the survival of collagen. Bone samples from two 'dry' caves were compared with those from three caves which were considered to be 'wet'. The ability to predict which samples are most likely to contain collagen would reduce sample destruction and preparation time when collagen is used for phylogenetic studies.

[Palaeontology, Vol. 33, Part 2, 1990, pp. 447–451.]

MATERIALS AND METHODS

Environment

The caves from which material was studied include Clogg's Cave (EB-2: cave numbers from Matthews 1985: 148° 11′ S, 37° 30′ E) and McEachern's Cave (G-5: 141° 00′ S, 37° 59′ 30″ E), Victoria; Koonalda Cave (N-4: 129° 50′ S, 31° 24′ E), South Australia; and Madura Cave (N-62: 127° 02′ S, 32° 00′ 30″ E) and Devil's Lair (WI-61E: 115° 03′ S, 34° 07′ E), Western Australia. Both Clogg's Cave and Devil's Lair represent caves with moist environments ('wet' caves), and Madura Cave and Koonalda Cave represent caves with dry environments ('dry' caves). These are relative terms and imply no absolute definition, but generally wet caves have damp sediments, and may have water dripping or periodic inundations, while dry caves have dust. In many subaerial caves, if *high* humidity occurs for a long enough period, there is no preservation of bone whatsoever (RFB, pers. obs.), so that the determination of 'wet' and 'dry' applies only to those caves where bone survives.

The main taphonomic accumulator was the Barn Owl (*Tyto alba*, Tytonidae, Strigiformes) for all caves except G-5, which appears to be of fluvial/pitfall origin. The material from Koonalda Cave has been secondarily sorted by fluvial action (Baird 1986).

Bone samples

The taxa studied were *Turnix varia* (Painted Button-quail) for Clogg's Cave, McEachern's Cave and Devil's Lair, and *Turnix* sp. cf. *T. velox* (Little Button-quail) for Koonalda and Madura Caves (see Appendix). These species have been chosen because of the abundance of their elements in the deposits and the presence of this genus in a number of deposits.

A number of criteria were used in selecting material for study, including: the elements were all from the distal end of the humerus, the elements were complete before processing, the elements lacked damage or alteration to their surfaces, and the elements had a uniform light coloration. In some cases one or two of the criteria were not upheld because of the scarcity of material of appropriate geological age (i.e. different elements [youngest WI-61e = incomplete femur, and oldest = incomplete tarsometatarsus], incomplete elements [oldest N-4 = distal end humerus, youngest N-62 = proximal end humerus, and oldest N-64 = distal end humerus] and discoloured elements [oldest WI-61e = dark brown]). Table I gives the chronological distribution of the elements.

Preparation of the fossils

Fossils were ground to a fine powder, decalcified with 10 volumes of 0·5 M-EDTA, pH 7·5, then re-extracted with 10 volumes of 0·5 M acetic acid. The remaining bone powder was resuspended in 10 volumes of phosphate-buffered saline (PBS), pH 7·3, for testing. Extractions were carried out in siliconized glassware throughout, to minimize loss of protein on the sides of the tubes. Bones from modern *Turnix varia* and *Dromaius novaehollandiae* (Emu) were defatted by sequential extraction using acetone and ethyl ether, then air-dried and treated similarly to the fossils. All extractions were carried out at 4 °C.

Radioimmunoassay

A solid-phase radioimmunoassay for collagen was carried out on flexible polyvinyl microtitre plates. Wells were coated with 50 μl of EDTA extract, acetic acid extract, or bone powder suspended in PBS and held overnight at 4 °C in a moist chamber. After coating with antigen, the plates were washed 3 times with PBS containing 1% skimmed milk powder and 0·05% Tween 20 (Blotto), and then washed 6 times with distilled water. The plates were then exposed to 200 μl of Blotto for 2 hours at room temperature, to coat residual sites on the plastic, and again washed as above. The assay system was completed by adding 50 μl of antiserum dilution to each well; the plates were kept overnight at 4 °C, and then washed as before. Antibody binding to the plates was detected using protein A from *Staphylococcus aureus*, labelled with 50 μl ^{125}I, 50,000 cpm, with a specific activity of 40 μCi/μg, which was added to each well. The plates were kept overnight at 4 °C, washed as before, cut, and the activity bound to the wells was counted on a gamma counter. Under the conditions of the assay, the amount of radioactivity was proportional to the amount of collagen bound to the plate.

Each sample was tested in quadruplicate, using rabbit anti-collagen antiserum to measure specific binding to collagen, and normal rabbit serum to measure non-specific binding. In addition, each serum was tested on

uncoated wells of the plates, to determine the 'background' binding observed in the absence of fossils. Each sample was counted for 10 minutes, to increase the sensitivity of the assay. Modern *Turnix varia* and *Dromaius novaehollandiae* were included for comparison, and a control of soluble collagen from *Dromaius novaehollandiae* in PBS, in doubling dilutions from 5 μm/ml, was included on each plate.

Antibodies to collagen

Type I collagens were extracted from bird skins by pepsin digestion and purified by differential salt precipitation (Chung and Miller 1974). Rabbits were immunized subcutaneously with 5 mg of collagen in complete Freund's adjuvant initially, and in PBS 4 weeks later; antibodies reacted predominantly with native collagen and minimally with denatured collagen, as reported previously (Timpl 1982). The antiserum chosen for this study was a rabbit antibody to chicken collagen, selected because it contained the greatest reactivity to denatured collagen of any antiserum tested. Although it reacted most strongly with chicken collagen, it gave 85% of that reactivity with purified *Turnix* collagen.

RESULTS

The results of this study are summarized in Table 1. Samples of bone from modern *Turnix varia* and *Dromaius novaehollandiae* were included for comparison. However, the amount of collagen extracted from the sample of *Turnix varia* was approximately 60% of that extracted from the *Dromaius novaehollandiae* sample, and was lower than the amount obtained from some of the fossils. This bone was from a specimen that had been cleaned of flesh by exposure to dermestid beetles (*Dermestes maculatus*), in a warm moist atmosphere over several weeks. By contrast, the taphonomic accumulator for most fossils studied was the Barn Owl (*Tyto alba*), which implies that the bones would have been cleaned of flesh within hours, and excreted in a dry pellet. After preliminary drying, collagen is much more resistant to subsequent hydrolytic decomposition than is collagen, which remains moist (Wyckhoff 1972). The bone from the *Dromaius novaehollandiae* was from a freshly killed bird, which had been stored at −20 °C since death. This difference in preparation may have affected the preservation of collagen. Therefore in Table 1 the amount of collagen found in the bones is expressed as a percentage of that extracted from the *Dromaius novaehollandiae* bone.

TABLE 1. Results of the analysis on collagen in avian fossils (*Turnix* spp.) of late Quaternary age from caves distributed across southern Australia. Results are expressed as mean counts per minute of radioactivity bound ± 1 standard deviation

Site	Cave environment	Age (years)	Bone sample		Collagen % (% of fresh collagen)
			Anti-collagen	NRS	
Fresh bone (*Dromaius novaehollandiae*)		—	18,100 ± 2,300	500 ± 110	—
No antigen		—	39 ± 4	42 ± 26	—
Clogg's Cave	Wet	8,720 ± 230	11,160 ± 3,400	210 ± 120	64
		17,720 ± 840	5,300 ± 940	170 ± 60	29
Devil's Lair	Wet	11,960 ± 140	2,400 ± 350	85 ± 18	13
		32,480 ± 1,250	4,500 ± 450	160 ± 4	25
Madura Cave	Dry	18,990 ± 220	16,650 ± 5,600	360 ± 110	92
		37,880 ± 3,520	67 ± 2	45 ± 6	—
Koonalda Cave	Dry	13,700 ± 270	6,300 ± 1,700	100 ± 10	35
		> 20,600–< 21,550	1,800 ± 370	68 ± 9	10
McEachern's Cave	Wet	9,920 ± 270	3,200 ± 760	520 ± 180	18
		14,880 ± 240	3,700 ± 1,400	450 ± 160	21

Although the amounts of soluble collagen in the EDTA and acetic acid extracts were normally at or below 1 % of the total, and therefore are not considered significant in the current study, those measurements from the McEachern's Cave elements yielded anomalously high counts (i.e. about 10 % and about 5 % of the total, respectively).

DISCUSSION

In previous studies on the preservation of collagen in fossils, samples of fossil bone obtained from cave deposits have contained very little collagen, irrespective of the age of the sample (Rowley *et al.* 1986; Rowley unpubl. data). This study was commenced to evaluate the effect of a damp cave environment on the preservation of collagen in bones. However, contrary to expectations, samples tested from both 'wet' and 'dry' caves contained significant residual collagen: one sample aged 18,990 ± 220 years contained 92 % of the collagen detected in a modern bone sample. Therefore, the humidity of the cave does not appear to be a critical factor in the preservation of collagen. Other factors which we believed might have influenced collagen preservation included the geological age of the sample and its preservation history and size. The age of the bone was not clearly related to its collagen content, although the amount of collagen detected was significantly less in the older of the two samples for three of the five caves (Clogg's Cave, Madura Cave, and Koonalda Cave), and not significantly different for McEachern's Cave, the cave for which the age difference between levels was least. The effects of preservation remain untested since we purposely chose samples with similar taphonomic histories.

In contrast to our previous studies, however, these avian bones have been extremely well preserved, with an undamaged surface and minimal breakage. Such good preservation of small bones, each weighing less than 1 g, suggests that the microenvironments experienced by these bones may have been unusually favourable. Thus, Murray and Goede (1977) have shown that the preservation of elements in a cave environment is directly related to the weight and volume of the specimen. In our previous studies, the bones tested were much larger and less well preserved. Although good physical preservation is not an absolute indication of good preservation of collagen, and the only sample tested which did not contain measurable collagen was also rated as very well preserved, good structural preservation has previously been noted to be associated with preservation of amino acids of collagen (Wyckoff 1972).

Acknowledgements. We extend our thanks to the following people and their respective institutions for the use of material in their care in destructive analysis: Dr T. H. Rich (Museum of Victoria), Mr N. Pledge (South Australian Museum) and Dr K. McNamara (Western Australian Museum). M.J.R. was supported by a National Research Fellowship.

REFERENCES

BAIRD, R. F. 1986. The avian portions of the Quaternary cave deposits of southern Australia and their biogeographical and palaeoenvironmental interpretations. Unpublished Ph.D. thesis, Monash University.

——., in press. The taphonomy of late Quaternary cave localities yielding vertebrate remains in Australia. *In* RICH, P. V., BAIRD, R. F., MONAGHAN, J. M. and RICH, T. H. (eds.). *Vertebrate palaeontology of Australasia.* Chapman and Hall, London.

CHUNG, E. and MILLER, E. J. 1974. Collagen polymorphism: characterization of molecules with the chain composition [I(III)] in human tissues. *Science*, **183**, 1200–1201.

HIGUCHI, R., BOWMAN, B., FREIBERGER, M., RYDER, O. A. and WILSON, A. C. 1984. DNA sequences from the quagga, an extinct member of the horse family. *Nature*, **312**, 282–284.

HILLIS, D. M. 1987. Molecular versus morphological approaches to systematics. *Annual Review of Ecology and Systematics*, **18**, 23–42.

LOWENSTEIN, J. M. 1980. Species-specific proteins in fossils. *Naturwissenschaften*, **67**, 343–346.

—— 1981. Immunological reactions from fossil material. *Philosophical Transactions of the Royal Society of London*, Series B, **292**, 143–149.

——, SARICH, V. M. and RICHARDSON, B. J. 1981. Albumin systematics of the extinct mammoth and Tasmanian wolf. *Nature*, **291**, 409–411.

—— and ZIHLMAN, A. L. 1984. Human evolution and molecular biology. *Perspectives in Biology and Medicine*, **27**, 611–622.

MATTHEWS, P. G. 1985. *Australian karst index*. Australian Speleological Federation, Melbourne, 322 pp.

MURRAY, P. and GOEDE, A. 1977. Pleisocene vertebrate remains from a cave near Montague, N.W. Tasmania. *Records of the Queen Victoria Museum*, **60**, 1–30.

O'BRIEN, S. J., NASH, W. G., WILDT, D. E., BUSH, M. E. and BENVENISTE, D. E. 1985. A molecular solution to the riddle of the giant panda's phylogeny. *Nature*, **317**, 140–144.

ROWLEY, M. J., RICH, P. V., RICH, T. H. and MACKAY, I. R. 1986. Immunoreactive collagen in avian and mammalian fossils. *Naturwissenschaften*, **73**, 620–623.

SIBLEY, C. G. and AHLQUIST, J. E. 1983. Phylogeny and classification of birds based on the data of DNA–DNA hybridization. *Current Ornithology*, **1**, 245–292.

TIMPL, R. 1982. Antibodies to collagens and procollagens. *Methods in Enzymology*, **82**, 472–498.

WILSON, A. C., CARLSON, S. S. and WHITE, T. J. 1977. Biochemical evolution. *Annual Review of Biochemistry*, **46**, 573–639.

WYCKOFF, R. W. G. 1972. *The biochemistry of animal fossils*. Scientechnica, Bristol, 152 pp.

—— 1980. Collagen in fossil bones. *In* HARE, P. E. (ed.). *Biogeochemistry of amino acids*. 17–22. John Wiley & Sons, New York. 558 pp.

R. F. BAIRD
Department of Earth Sciences
Monash University, Clayton
Victoria 3168, Australia
and
Department of Ornithology
Museum of Victoria, Abbotsford
Victoria 3067, Australia

M. J. ROWLEY
Department of Earth Sciences
and
Centre for Molecular Biology and Medicine
Monash University, Clayton
Victoria 3168, Australia

Typescript received 16 August 1988
Revised typescript received 1 August 1989

APPENDIX

Avian fossil material used during the course of this study, arranged with the youngest material from each locality above the older material. Cave numbers are from Matthews (1985). Abbreviations: com. = complete, dist. = distal, fem. = femur, hum. = humerus, incom. = incomplete, MV = Museum of Victoria, prox. = proximal, SAM = South Australian Museum, WAM = Western Australian Museum.

Identification	Element	Museum number	Cave	Cave no.
Turnix sp. cf. *T. velox*	Com. right hum.	SAM P.26117	Koonalda	N-4
Turnix sp. cf. *T. velox*	Dist. end right hum.	SAM P.261132	Koonalda	N-4
Turnix varia	Incom. right fem.	WAM 73.10.1451	Devil's Lair	WI-61e
Turnix varia	Incom. right tmt.	WAM 86.7.47	Devil's Lair	WI-61e
Turnix varia	Com. right hum.	MV P.183347	Clogg's	EB-2
Turnix varia	Incom. left hum.	MV P.1834377	Clogg's	EB-2
Turnix sp. cf. *T. velox*	Prox. end right hum.	MV P.184897	Madura	N-62
Turnix sp. cf. *T. velox*	Dist. end right hum.	MV P.184902	Madura	N-62
Turnix varia	Incom. right hum.	MV P.161181	McEachern's	G-5
Turnix varia	Com. right hum.	MV P.161131	McEachern's	G-5