PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Immunological Reactions from Fossil Material

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Immunological reactions from fossil material

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Genetic relations among living species can be deduced from biochemical as well as morphological similarities, but our understanding of fossil species has depended entirely on their morphology. Residual proteins in fossils might provide genetic information, but their small quantity and chemical alterations due to time and environmental agents have prevented the obtaining of species-specific analysis. This report describes a radioimmunoassay capable of detecting extremely small amounts of fossil proteins, such as collagen and albumin. Species-specific proteins have been identified in a frozen Siberian mammoth, a Pleistocene bison, and a series of human fossils that includes Neanderthal, *Homo erectus* and *Australopithecus robustus*. This technique promises to provide molecular data on the genetic affinities of fossil and living species.

Introduction

Study of organic evolution by means of the comparative anatomy of fossil and living species is limited in its precision by at least two factors: the unavoidably subjective element in anatomical interpretation, and the varying rates of morphological change in different lineages. Biochemical comparison of different species minimizes the subjective factor, for base or amino acid sequences, or immunological reactions, can be replicated in many laboratories. Furthermore, there is increasing evidence that, unlike morphological change, the rate of DNA base substitutions, and hence of amino acid substitutions in proteins, is a fairly constant function of time, so that the number of differences in the comparable DNA or proteins of two species is a measure of their time of divergence from a common ancestor (Wilson *et al.* 1977). Frogs, for example, have changed morphologically relatively little in the past 100 Ma in comparison to all the placental mammals, in particular the primates. Yet frog proteins have undergone as much change during this interval as those of mammals (Wilson *et al.* 1977).

Phylogenetic trees constructed from the biochemical similarities of homologous DNA and proteins have helped to clarify the evolutionary relations of living species (Ayala 1976), but our understanding of fossil species has continued to depend almost exclusively on their anatomical characteristics. In some cases, the conclusions drawn from biochemical and anatomical data are irreconcilable, as in the question of the phyletic status of the Miocene hominoid *Ramapithecus*, considered by many anthropologists to be a hominid (Simons 1977). Analysis of DNA and 40 different proteins shows 99 % identity between humans and chimpanzees (King & Wilson 1975). Sarich & Cronin (1976) find equal closeness between human, chimpanzee and gorilla proteins, based on the immunological cross reactions of the albumins and transferrins, and conclude that the three species diverged from a common ancestor about 5 Ma ago. If this is correct, then *Ramapithecus*, which lived some 8–20 Ma ago, could not have been 'human' (Zihlman & Lowenstein 1979). The issue continues to be disputed whether DNA and proteins, or fossil jaws and teeth, are the best criteria for phyletic status.

This controversy, like many others in evolution, might be clarified if one could make biochemical as well as morphological comparisons between the fossil and the living species. Biochemical analyses of fossils, consisting mostly of amino acid abundances, have not yielded species-specific information due to the small quantity of residual proteins and the chemical changes that they have undergone (Wyckoff 1972). When I began this project about three years ago, I hoped to overcome both the quantitative and the qualitative problem by developing a radioimmunoassay for fossil collagen. With radioimmunoassay it is possible to measure even tiny amounts (nanograms or picograms) of proteins, and I reasoned that even broken down proteins might retain some of their original species-specific sequences. Collagen is the principal protein of bone (about 20% of fresh bone) and is so tough and insoluble that collagen fibrils have been seen by electron microscopy in dinosaurs 200 Ma old (Wyckoff 1972). While testing fossils for collagen, I used antisera to albumin as a control and discovered, to my surprise, that serum factors as well as collagen may survive in fossils for millions of years.

Material and methods

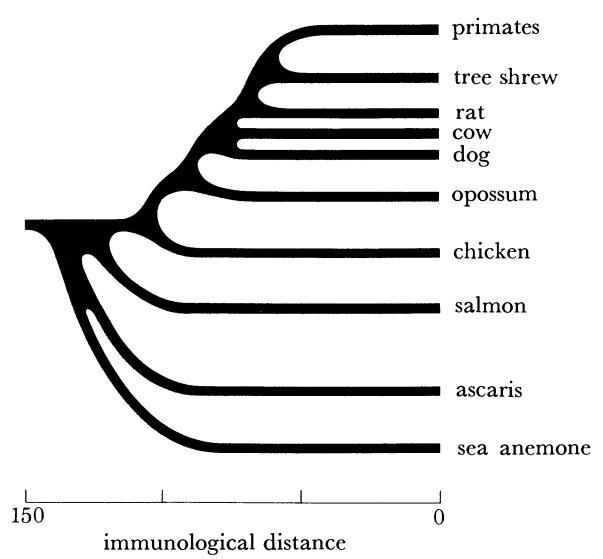
A solid phase radioimmunoassay was developed for collagen, albumin and other proteins (Lowenstein 1980). First, the fossil extract or protein solution is pipetted into the cups of a polyvinyl microtitre plate, allowed to remain for 1 h at room temperature, then washed out. Secondly, rabbit antibody to the specific protein is pipetted into the cup, allowed to remain for 24 h, then washed out. Finally, ^{125}I-labelled goat anti-rabbit gamma globulin (GARGG) is placed in the cups, allowed to remain for 24 h, then washed out. The cups are cut from the plate and the radioactivity measured in a scintillation counter. In this double antibody assay, radioactivity increases with the amount of the target protein in the cup. First, some of the protein binds irreversibly to the plastic. Secondly, some of the rabbit antibody binds to the protein. Finally, the radioactive goat antibody binds to the rabbit antibody.

Collagen was extracted from the skin of various species by acetic acid 0.5 M and purified by repeated salt precipitation. Antibodies were raised in rabbits by intramuscular injection of 5 mg of type I collagen dissolved in 1 ml of acetic acid (0.05 M), emulsified with Freund's complete adjuvant. After 3 weeks, a second intraperitoneal injection of 5 mg of collagen without adjuvant was given, and the rabbits were bled 2–3 weeks later. Antibodies to mummy and *Homo erectus* fossil bone were made by injecting rabbits with a mixture of fossil extracts and insoluble residue. The albumins and anti-albumins, as well as the mammoth muscle extract and anti-mammoth serum were obtained from Dr V. M. Sarich, Dr E. M. Prager and Dr A. C. Wilson, Biochemistry Department, University of California, Berkeley. Fossil bone fragments were ground to a fine powder and extracted for 1 week at room temperature with EDTA (0.2 M, pH 7.4), to decalcify. The residue was further extracted for 1 week with acetic acid, 0.5 M. The EDTA and acetic acid extracts were tested by radioimmunoassay for collagen and albumin.

Results

First it was necessary to establish that collagen evolution follows a pattern similar to that of other proteins. Collagen, the main structural protein of metazoans, from sponges to man, is a triple helix with approximately 1000 amino acids in each helix and with extensive homology between species (Balazs 1970). Every third amino acid is glycine and 10–15% are hydroxy-

proline. Mammalian collagens differ among themselves in amino acid composition by only about 5% (Fietzek & Kuhn 1976). In looking for species differences, the immunological approach has advantages over direct sequencing, in that the rabbit makes antibodies mostly against those determinants that differ from its own collagen and so amplifies the differences while minimizing the homology (Furthmayr & Timpl 1976).



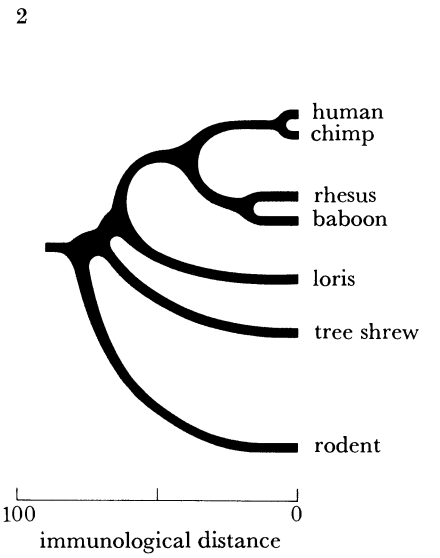


FIGURE 1. Collagen phylogeny constructed from immunological cross reactions (l.c.). Immunological distance, $D_1 = -100 \lg C_1$, where C_1 is a measure of cross reaction between two species, by radioimmunoassay; cross reactions between mammals and invertebrates are detectable.

FIGURE 2. Primate collagen phylogeny. As is true for many other proteins, human and chimpanzee collagens are nearly identical.

As has been done with many other proteins, I have constructed a collagen phylogeny (figures 1, 2) based on cross reactions between species, ranging from sea anemone to primates. For comparison, the albumin phylogeny, determined by the same radioimmunoassay technique, is shown in figure 3 and 4. Both agree as to the near identity of the proteins of humans and African apes. These results differ from previous immunological phylogenies only in that the standard methods, immunodiffusion and complement fixation, fail to give cross reactions when the divergence times of two species are greater than *ca.* 100 Ma. The sensitivity of the radioimmunoassay makes it possible to detect cross reactions between protein species that have been evolving separately for hundreds of millions of years. In figure 5, the immunological distances (defined in the legend to figure 1) are calibrated against time for those species having reasonably good geological dating. The primates, the most disputed group, are interpolated. Values for the last 100 Ma, for collagen and albumin, are nearly identical. The collagen curve tends toward its asymptotic limit sooner than the albumin curve does, because of the relative weakness of anti-collagen antibodies (Furthmayr & Timpl 1976). Nevertheless, cross reactions between human and invertebrate collagens can be detected. In principle, a fossil species might be placed on one of the phylogenetic trees by its relative distance from the living species. Before this can be done in practice, however, more needs to be known about the effects on apparent immunological distances due to degenerative changes in fossil proteins.

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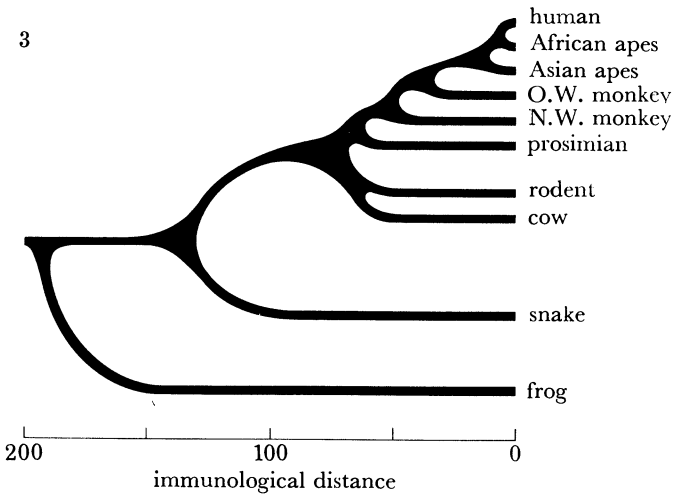

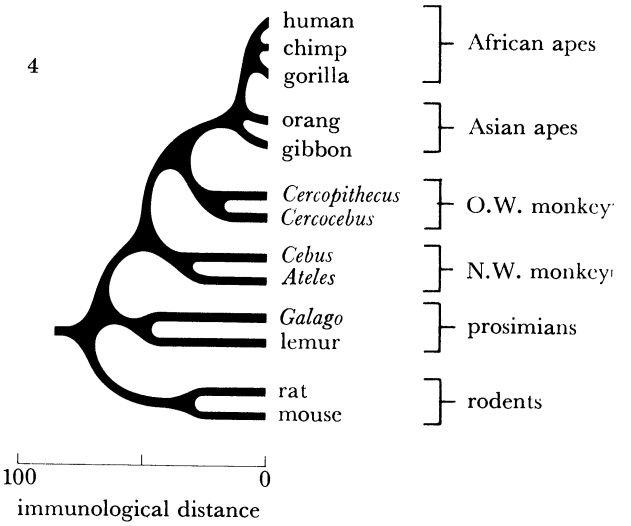

FIGURE 3. Albumin phylogeny constructed from radioimmunoassay data. This is similar to albumin trees generated from other immunological techniques, with the difference that radioimmunoassay detects cross reactions between species that diverged more than 100 Ma ago.

FIGURE 4. Primate albumin phylogeny, very similar to the collagen phylogeny. Again the human protein groups are nearly identical to the protein group of the African apes.

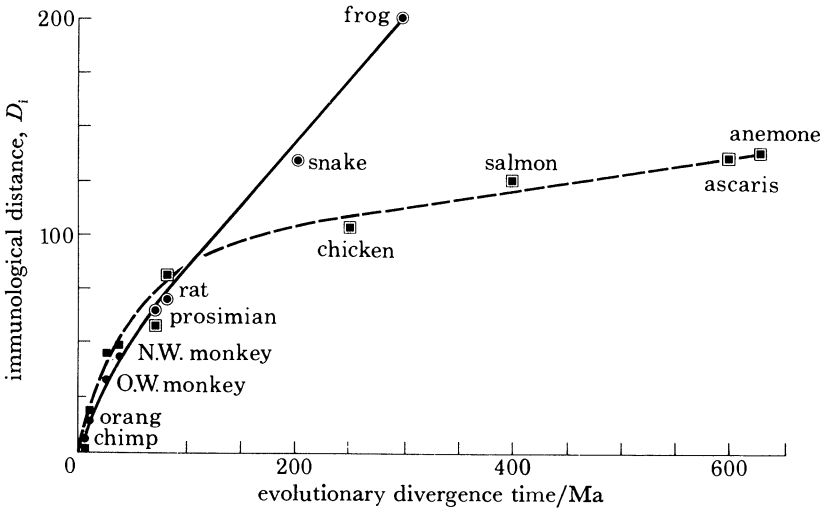

FIGURE 5. Time calibration of immunological distance (from human): ◉, ▣, geological divergence times; ●, ——, albumin; ■, – – –, collagen. Values up to about 100 Ma are nearly identical for collagen and albumin. Anti-collagen antibodies are weaker and so approach their asymptotic limit earlier.

I have applied this radioimmunoassay to the detection of residual proteins in a variety of fossils. The most likely fossil in which to find albumin would be a frozen mammoth, for the soft tissues as well as the bones are preserved at a temperature that minimizes protein breakdown. The baby mammoth known as Dima was discovered near Magadan, U.S.S.R., in 1977, and refrigerated immediately. The Evolutionary Biochemistry Group at the University of California, Berkeley, obtained a piece of muscle from this mammoth and attempted to identify albumin by micro-complement fixation and immunodiffusion. Though these efforts were initially unsuccessful, homogenized muscle injected into rabbits evoked antibodies that reacted with elephant albumin, indicating that mammoth albumin was present in small amounts. Using the

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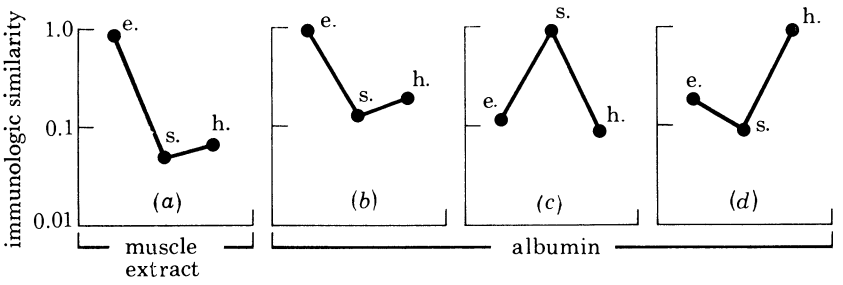

FIGURE 6. Immunological similarity of mammoth and elephant albumin: Muscle extracts from the frozen baby mammoth Dima (*a*), ^{14}C date 44 ka B.P., has anti-albumin reactions nearly identical with those of elephant albumin (*b*) and quite different from those of the sea cow (*c*) (a distant relative of the elephant) and human (*d*) albumin. A value of 1.0 indicates immunological identity, 0 indicates no cross reaction.

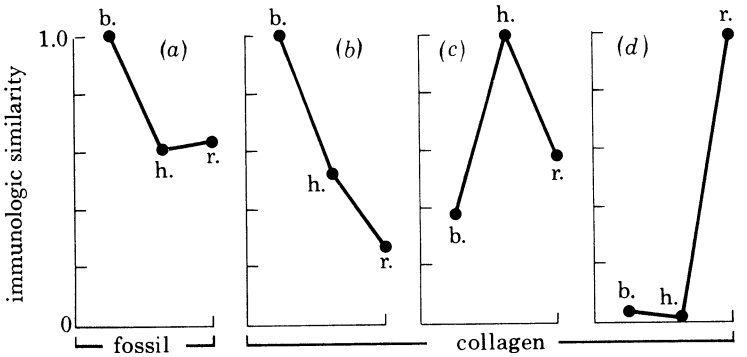

FIGURE 7. Immunological similarity of fossil bison and bovine collagen. (*a*) A North American bison bone fragment of Pleistocene age was tested as an 'unknown' and found to have strong reaction with bovine anti-collagen. Reactions of bovine (*b*), human (*c*) and rat (*d*) collagen are shown for comparison.

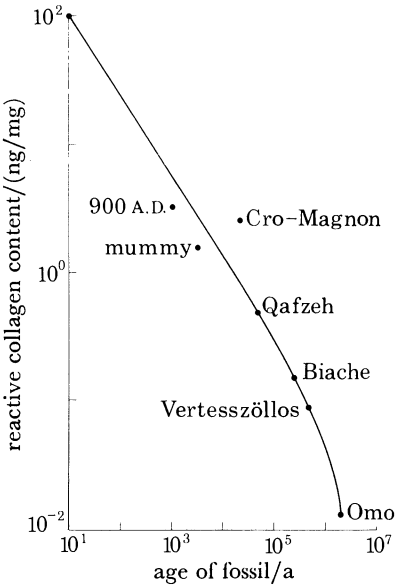

FIGURE 8. Immunologically reactive collagen in extracts of human fossil bones (nanograms of collagen per milligram of bone). The point on the ordinate is derived from a fresh femur removed at surgery; A.D. 900 (Hungarian burial); mummy (Egyptian, 800 B.C.): Cro-Magnon (from Musée de l'Homme, Paris); Qafzeh (Neanderthal, Israel); Biache (*Homo erectus*, France); Verteszöllos (*Homo erectus*, Hungary); Omo (*Australopithecus robustus*, Ethiopia).

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radioimmunoassay method, I demonstrated both by directed reaction and by inhibition that the muscle extract contained the equivalent of 3 μg of elephant albumin per millilitre (Prager *et al.* 1980). This material reacted strongly with antibody to elephant albumin and weakly with other mammalian anti-albumins (figure 6). The ^{14}C date for this mammoth is 44 ka.

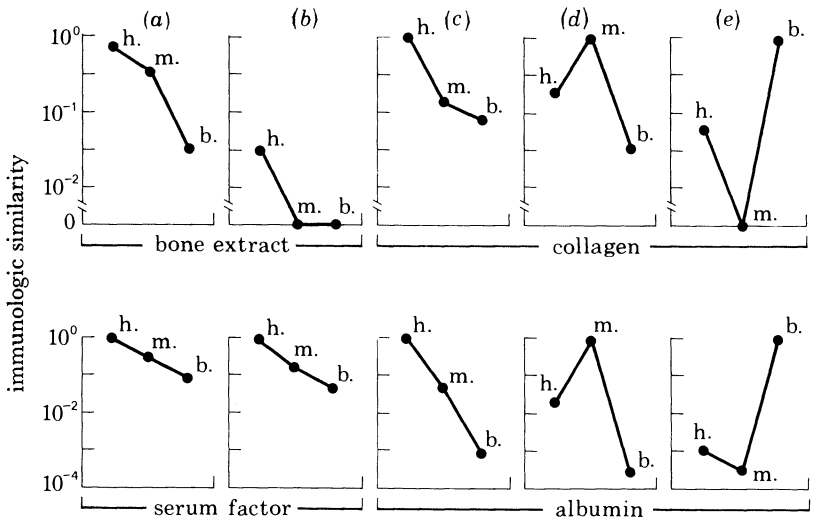

FIGURE 9. Reactive collagen and serum factor in two human fossils, (*a*) radius of an Egyptian mummy 3 ka old and (*b*) mandible of *Homo erectus* 0.5 Ma old. Top: reactions of fossil extracts with human (h.), monkey (m.) and bovine (b.) anti-collagens; the reactions of human (*c*), monkey (*d*) and bovine (*e*) collagen are shown for comparison. Bottom: reactions of antibodies made in rabbits to the two human fossils, with human (h.), monkey (m.) and bovine (b.) serum. Reactions of human, monkey and bovine anti-albumins are shown for comparison. The anti-fossil antibodies did not, however, react with pure albumin, which implies that the immunogen is another serum factor.

Professor F. Jenkins, Museum of Comparative Zoology, Harvard University, provided me with three sets of fossil bone fragments identified only as being of Pleistocene age. When tested, one gave a strong reaction with antibody to bovine collagen (figure 7), the others gave no reaction. The reacting specimen was then identified as a North American bison; the others were horse and mastodon, for which I had no related anti-collagens. None of the three gave reactions with specific anti-albumins.

I have tested a range of human fossils (figure 8) that includes Egyptian mummy, Cro-Magnon, Neanderthal, *Homo erectus* and *Australopithecus robustus*. All gave positive collagen reactions, though the amount decreased with time. Hydroxyproline determination, a measure of collagen, revealed that the amount of collagen decreased with the age of the fossil and also there was a decrease in reactivity of the remaining collagen, due no doubt to chemical changes. The collagen remaining retained species specificity, reacting more strongly with antibody to human collagen than with other anti-collagens (figure 9). Rabbits were immunized with extracts from two human fossils, the mummy and the Verteszöllos *Homo erectus* (the only two of which there was sufficient quantity for this experiment) and produced antibodies that reacted more strongly with human serum than with the serum of other species (figure 9). Though these antibodies reacted with serum in a pattern similar to that of anti-albumins, they did not react with purified albumin; therefore, it appears that some unidentified serum factor other than albumin has survived in these fossils.

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Discussion

While one might expect to find identifiable proteins in a frozen Siberian mammoth, it is perhaps surprising that collagen, albumin and other serum factors survive in fossil bones that have been subjected to ambient temperatures and to the effects of water, chemical and bacterial action for thousands or millions of years. One clue to this survival comes from my control studies on human bone ash. Bone powder was heated at a temperature of 850 °C until the nitrogen content was reduced to a negligible amount. Yet an EDTA extract of this material consistently gave radioimmunoassay evidence of small but definite amounts of surviving human collagen and albumin. From this, and from the fossil results, it seems that the calcium apatite matrix in which the proteins are embedded provides considerable protection against the destructive effects of temperature and chemical agents. Furthermore, proteins need not survive intact to account for these reactions, as most immunological determinants consist of a few adjacent amino acids, so that fragments could retain immunological reactivity. The mammoth albumin was shown by Sephadex chromatography to be mostly in an aggregated form, yet it reacted nearly identically to elephant albumin.

Obviously, a great deal more systematic work needs to be done before phylogenetic interpretations can confidently be made from fossil immunological data. Nevertheless, these early results offer the hope that we may be able to obtain useful molecular genetic information from many fossils.

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