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Immunological Methods in Molecular Palaeontology [and Discussion]

Jerold M. Lowenstein, Gary Scheuenstuhl, G. Eglinton, P. Westbroek and G. Muyzer

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Immunological methods in molecular palaeontology

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SUMMARY

Variable amounts of proteins or protein fragments may survive in fossils for thousands and occasionally millions of years. The quantities are generally too small to determine accurate amino acid sequences, but even these small amounts may be immunologically detectable and may retain useful genetic information. A very sensitive solid phase radioimmunoassay has been used to analyse fossil proteins, particularly albumin and collagen, the most abundant animal proteins and those most likely to be found in fossil skin, muscle, bones and teeth. Species-specific proteins have been identified in the skin and muscle of mammoth, quagga and Tasmanian wolf, and in the bones of the extinct mastodon and Steller's sea cow. Specific albumin has also been detected in bloodstains on ancient stone weapons and in fossilized urine from the middens of cave-dwelling pack rats, porcupines and hyraxes.

1. INTRODUCTION

During the past three decades, data from the growing field of molecular evolution have been added to comparative anatomy and the fossil record as a third basis for reconstructing the evolution of life on Earth. Because all living creatures, from microorganisms to elephants, use the same genetic language and have comparable molecules of DNA, RNA and proteins, it is feasible to make quantitative comparisons either of very similar or widely divergent organisms. Rates of mutation in particular molecules seem to be roughly proportional to elapsed times, thereby providing 'molecular clocks' for timing evolutionary divergences (Wilson *et al.* 1977, Ochman & Wilson 1987).

Whereas molecular comparisons of living taxa have profoundly influenced our understanding of the evolution and relationships among extant microorganisms, fungi, plants, animals and even viruses, only relatively recently have molecular techniques been applied to clarifying the phylogenies of extinct species, which of course far outnumber their living relatives. Abelson (1954), Hare & Abelson (1965), Hare *et al.* (1980) and Wyckoff (1972) showed long ago that amino acids survive in many calcified fossils, but amino acid compositions generally provide little useful genetic information. Within the past decade, sensitive immunological techniques and mitochondrial DNA sequences have provided direct genetic comparisons of fossil and living species of animals and plants.

2. IMMUNOLOGICAL TECHNIQUES

Historically, the immunological approach was the first to show affinities between species on the molecular level. In the early years of this century, Nuttall (1904) injected the blood from hundreds of different animals into rabbits and estimated their phylogenetic distances from the amount of precipitation formed when various blood specimens were mixed with different antisera. Although the molecular structure of the serum proteins being compared was not understood, Nuttall observed that cross-reactions were stronger in more closely related animals such as humans and apes, sheep and goats, horses and donkeys, and that no cross-reactions were observed between distant groups such as mammals and reptiles.

The immune systems of rabbits and other animals are capable of generating an astronomical number of different antibodies against 'foreign' or 'non-self' proteins. The variable tips of Y-shaped antibody molecules constitute 'mirror images' of portions of the injected protein antigen. Antigenic determinants generally span six to ten adjacent amino acids that may be 'sequential' (a linear sequence) or 'conformational' (juxtaposed by the three-dimensional folding of the protein). Antibodies seem to 'recognize' abut 80% of the amino acid sequences in a protein, and the antigen–antibody reaction of a particular protein molecule such as albumin is linearly related to the proportion of amino acid that has changed as two species evolved from a common ancestor (Benjamin *et al.* 1984).

A variety of immunological techniques have been used to investigate evolutionary relationships. Nuttall's (1904) work with precipitation was earliest. Morris Goodman (1963) employed an immunodiffusion method, in which holes are bored into an agar plate, and sera and antisera poured into the holes. These diffuse through the agar, and where sera and antisera meet, lines of precipitation are formed. The density of the precipitation lines indicates semi-quantitatively the strength of the cross-reactions and hence the genetic similarity. Sarich & Wilson (1967) used the more sensitive and quantitative microcomplement fixation method for investigating evolutionary relationships among primates, and subsequently this technique was applied to phylogenetic studies of other mammals, reptiles, amphibians, insects and plants.

Precipitation, immunodiffusion, and microcomple-

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Table 1. *Sensitivity of immunological methods in phylogeny*

method	antigen detectable (μg)	antibody required (μg)	reference
precipitation	1000	1000	Nuttall (1904)
immunodiffusion	100	100	Goodman (1963)
complement fixation	10	10	Sarich & Wilson (1967)
radioimmunoassay	0.001	0.001	Lowenstein (1980)

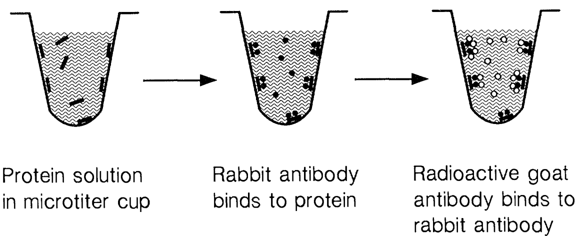

Figure 1. Solid-phase double-antibody radioimmunoassay proceeds in three steps. First, protein from solution binds to plastic microtitre well. Second, rabbit antibody binds to protein. Third, ^{125}I-labelled goat anti-rabbit γ-globulin binds to rabbit antibody. Radioactivity increases both with the amount and the immunological specificity of the original protein antigen.

ment fixation require approximately 1000, 100 and 10 μg of protein antigen, respectively, and comparable amounts of antibody (table 1) – although antigen is generally the limiting factor. Concentrations of protein in fossil materials are usually much too low for detection by any of these methods. Therefore, when we began to look for species-specific proteins in fossils about 15 years ago, we turned to radioimmunoassay (RIA), which is capable of detecting nanogram (10^{-3} μg) or even picogram (10^{-6} μg) quantities. After trying several different methods, we settled on a solid-phase double-antibody technique (Lowenstein 1980, 1981). This method not only detects proteins in many fossils, but it makes possible quantitative cross-reactions between taxa such as mammals, reptiles and amphibians (Lowenstein 1981), or different species of algae (Olsen-Stojkovich *et al.* 1986) that have been evolving separately for hundreds of millions of years.

Antisera to purified proteins, to protein mixtures such as whole serum, or to proteins extracted from fossil material are raised in rabbits stimulated by injections at two-week intervals. The assay is done in the wells of plastic microtitre plates, in three steps (figure 1). First, the protein antigen, in solution, is placed in the wells, where some of the protein binds to the plastic. Second, rabbit antisera raised against homologous and heterologous proteins (for example, to the albumins of various species) are placed in the wells. The closer the relationship, the more antibody binds to the plastic-bound protein. Third, radioactive (^{125}I-labelled) goat antibody raised against rabbit γ globulin (GARGG) is placed in the wells and binds to the rabbit antibody. The radioactivity in each well, measured in a scintillation counter, indicates both the amount of protein antigen present and its species-specificity. Some investigators now use enzyme-linked immunosorbent assay (ELISA) methods, which are very similar to RIA but use second antibodies linked to colour-forming enzymes rather than to a radioactive isotope.

3. RADIOIMMUNOASSAY OF FOSSIL MATERIAL

(*a*) *Frozen mammoth tissue*

Obviously the ideal specimen for fossil protein analysis is one frozen in the Siberian permafrost for 40000 years. A baby mammoth (*Mammuthus primigenius*), 'Dima', exhumed near Magadan in 1977, provided such an opportunity. A small piece of thigh muscle was extracted in buffer solution. RIA demonstrated the presence of serum albumin in a concentration of about 1 μg ml^{-1}, about 1% of the amount found in the muscle of a modern elephant (Prager *et al.* 1980; Lowenstein *et al.* 1981). Although most of this albumin was shown by Sephadex filtration to be in an aggregated state, indicating *post mortem* change, immunologically it was more than 99% identical to albumins of the living African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephants. Antiserum made in a rabbit by injection of mammoth extract reacted much more strongly with elephant albumin than with the albumins of other species.

Experts on proboscidean evolution have differed in their interpretations of the relationships between the mammoth, African, and Asian elephants. Based on dental similarities, most have thought the mammoth more closely related to the Asian species. With RIA it is possible to focus on the small differences between pairs of very similar proteins by the method of competitive inhibition. Soluble protein of one species 'competes' in a microtitre well with solid-phase protein adhering to the plastic. The more similar are the proteins of the two species, the more antibody will bind to the liquid phase, which has a 100-fold excess of protein, and the less will bind to the solid phase (Lowenstein *et al.* 1981). Mammoth albumin was found in this way to be equally different from the albumins of the two extant elephant species, suggesting an evolutionary trichotomy too close to call.

Collagen similar to that found in elephants was also detected by RIA in extracts of mammoth tissue (Lowenstein 1985; Shoshani *et al.* 1985), but collagen is such a conservative protein with such a slow rate of evolution that it is not possible to distinguish among the collagens of the three elephant species.

(*b*) *Recently extinct quagga and Tasmanian wolf*

Both the quagga (*Equus quagga*) and the Tasmanian wolf (*Thylacinus cynocephalus*) have been exterminated during the past century by human activities, and each has had a disputed pedigree clarified by analysis of fossil proteins and DNA.

The quagga was striped on its face and forequarters like a zebra and chestnut-toned on its body and hindquarters like a horse. Huge herds of quaggas

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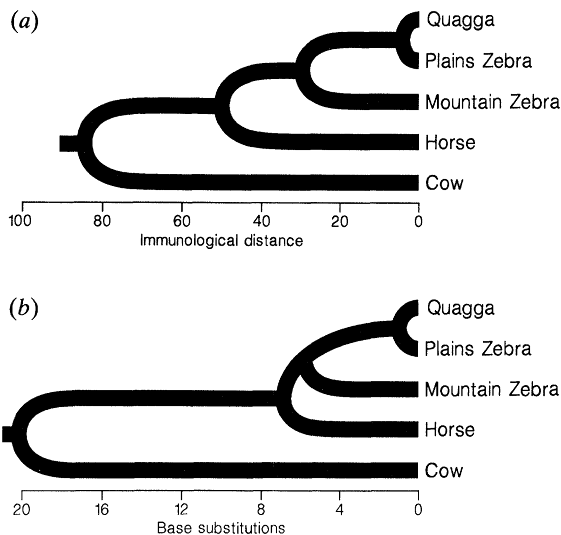

Figure 2. Comparison of immunological and DNA phylogenies derived from quagga skin. Both (*a*) RIA analysis of quagga skin proteins and (*b*) mitochondrial DNA show the quagga to be much closer to the plains zebra than to any other living equid. In contrast, morphologists have suggested three different phylogenies: (i) the one supported here, (ii) a quagga–horse pairing, and (iii) the quagga equidistant from the three zebras.

roamed the plains of Cape Province in South Africa during the Nineteenth Century until human hunting and destruction of their habitat wiped them out. Systematists have formed three different opinions about quagga relations within the genus *Equus*: (i) its closest relative was the domestic horse; (ii) it was a subspecies of the plains zebra; (iii) it was equally distant from the three surviving species of African zebras, namely, plains, mountain and Grevy's zebras.

RIA of extracts of quagga skin from museums showed quagga proteins to be six times more similar to those of the plains zebra than to those of other zebras, horses and asses (Lowenstein & Ryder 1985). In a striking confirmation of this conclusion, Higuchi *et al.* (1987) sequenced mitochondrial DNA from quagga skin and found that it differed by only two base substitutions from a plains zebra sequence, by 12 bases from the mountain zebra, and by 14 bases from the horse (figure 2).

The Tasmanian wolf or thylacine, a marsupial carnivore, was doomed when cattlemen and sheepherders settled in Tasmania and paid a bounty for its pelts. Though reported 'sightings' of the thylacine in Tasmania and elsewhere in Australia continue to the present time, the last known individual died in a zoo in 1933. Most systematists have joined the Tasmanian wolf with the other Australian marsupial carnivores, the dasyurids. However, its remarkable dental and skeletal resemblance to the extinct South American *Borhyaena* has led a vocal minority to conclude that it was a relict borhyaenid, only distantly related to the dasyurids (Archer 1976).

RIA of thylacine albumin extracted from skin and muscle specimens from museums showed a close relationship with the dasyurids that implied a divergence time of only 6–10 Ma, and a much more distant relationship with South American marsupials (Lowenstein *et al.* 1981). Again this finding was confirmed by sequencing of mitochondrial DNA from skins, which showed a consensus of four substitutions between *Thylacinus* and *Dasyurus* (implying a divergence time of 10–20 Ma) and 11 substitutions between *Thylacinus* and *Philander*, a South American opossum (Thomas *et al.* 1989). These findings contrast markedly with a dentally derived cladistic phylogeny showing the thylacine more closely related to the opossum than to the dasyure (figure 3).

(*c*) *Albumin and collagen in fossil bone*

Whereas fossilized soft tissues are rare, fossil bones and teeth are common and abundant, and many of these hard tissues preserve immunologically detectable protein or protein fragments. An American mastodon (*Mammut americanum*), 'Elmer', was unearthed in Michigan and dated by ^{14}C at 10000 years. Bone powder was decalcified with EDTA and tested with antisera against elephant albumin and elephant collagen (Shoshani *et al.* 1985). These reactions were disappointingly weak and non-specific. When the mastodon material was injected into rabbits, however, the resultant antisera reacted specifically with elephant serum and elephant collagen, showing that immunological determinants were present in the bone and could be amplified by the immunization process.

Sea cows are among the closest living relatives of the elephants. The Pacific dugong and three Atlantic manatee species are extant. Steller's sea cow (*Hydrodamalis gigas*), a resident of the Commander Islands off the Kamchatka Peninsula, U.S.S.R., was slaughtered to extinction by sailors and seal hunters in 1768. A skull in the University of California Berkeley Museum of Vertebrate Zoology provided an opportunity to test the affinity of Steller's sea cow with the four living species (Rainey *et al.* 1984). Unlike the much older mastodon bone, extracts of *Hydrodamalis* reacted strongly with anti-sea-cow albumin and showed the closest relationship, as expected, with the dugong.

By cross-reacting the tissue extracts and antisera of the mammoth, mastodon and Steller's sea cow with each other and with comparable sera and antisera of the living species of elephants and sea cows, it was possible to generate an immunological family tree that includes these three extinct species (Figure 4).

(*d*) *Bloodstains on ancient weapons*

Stone tools and weapons have provided archaeologists with vital information about the culture, lifestyle and tool-making and tool-using skills of ancient humans. Surprisingly, bloodstains on such weapons may survive sufficiently well over millennia to indicate the species of animals that were hunted or butchered.

Loy (1983) has reported identification of species from bloodstains on ancient stone weapons, by using the technique of haemoglobin recrystallization. To be reliable, this method would require the preservation of three-dimensional protein structure over thousands of

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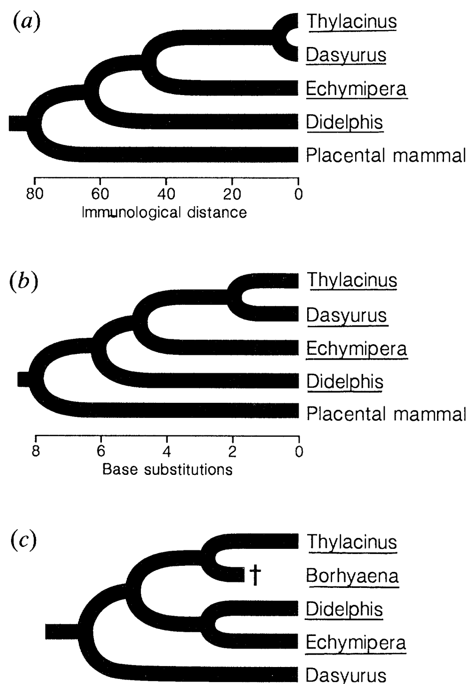

Figure 3. Comparison of immunological, DNA and dentally derived phylogenies of the Tasmanian wolf. Both (*a*) RIA and (*b*) mitochondrial DNA analysis of thylacine skin confirm its affinity with the Australian marsupial carnivores, the dasyurids. In contrast, (*c*) a phenogram based on analysis of tooth morphology places the thylacine with the extinct South American borhyaenid and the living opossum (*Didelphis*). *Echymipera*, the Australian bandicoot, is also placed differently in the dental scheme to the two molecular ones.

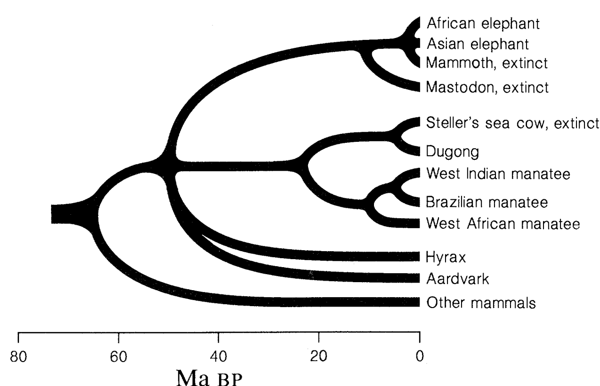

Figure 4. An immunological albumin tree of the 'paenungulates' – elephants, sea cows, hyrax and aardvark – that includes three extinct species.

years: an extremely unlikely eventuality. Immunological methods like RIA, however, can readily detect protein fragments that have undergone the inevitable diagenetic changes, as long as they retain some species-specific sequences. Loy provided stone spear points approximately 3000 years old from British Columbia. Each point was examined visually for bloodstains or discolorations. A drop of distilled water placed on the stain was scraped and stirred with the plastic tip of a micropipette, and the liquid was then transferred for RIA analysis. Of some ten implements tested, four gave specific reactions (Lowenstein 1988): two deer, one bison and one human (table 2).

Table 2. *Bloodstains on 3000-yr-old spear points: identification by radioimmunoassay*

(The numbers are percentage uptake of radioactive 2nd antibody. The first two specimens react as cervids (they could be mouse or reindeer rather than elk), the third as human, the fourth as bison. Cross-reactions are usually observed between mammalian albumins but little or none between mammals and fish. As controls, weak dilutions of elk, bison, horse, rat and fish serum were tested in the same run.)

	antisera against albumins of					
specimen	elk	bison	horse	human	rat	fish
FST1–4	**6.7**	1.9	1.4	1.3	0.8	0
FST1–6	**5.0**	1.3	0.7	0.4	0.5	0
GSK7–31	0.8	0.1	0.5	**5.5**	1.1	0
GSK7–32	0.4	**6.0**	2.2	2.8	0.7	0.2

Table 3. *Albumin reactions in fossil urine by radioimmunoassay*

(The numbers are relative uptake of radioactive 2nd antibody. Species-specific reactions are shown in bold type. The deer mouse (*Peromyscus*) is related to the pack rat and wood rat (*Neotoma* spp.). Pack rat urine reacts specifically, but the older wood rat urine has a less specific pattern, although it still reacts more strongly with the murid rodents than with other species.)

		antisera against the albumins of				
urines	age BP	deer mouse	porcupine	rat	hyrax	sheep
pack rat	2440	**45**	3	6	0	0
wood rat	41300	12	7	19	6	3
porcupine	5430	3	**51**	4	4	3
stick rat	1120	6	0	**18**	8	1
hyrax	1130	12	0	1	**18**	0

We have tested a number of stone tools from museums. Most have failed to yield positive reactions except for faint traces of human albumin probably attributable to human handling. Unfortunately, most artifacts are thoroughly 'cleaned' in water or other solutions that effectively remove the traces of protein that could be diagnostic. In future, it would be desirable to collect stone artifacts in such a way that these trace bloodstains are retained and not confused by the effects of repeated human handling.

(*e*) *Fossil pack rat, porcupine and hyrax urine*

Certain cave-dwelling mammals such as North American pack rats (*Neotoma* spp.), North American porcupines (*Erethizon dorsatum*) and South African hyraxes (*Procavia* sp.) build houses known as middens which may incorporate masses of crystallized urine (Betancourt *et al.* 1990).

Crystallized urine from ^{14}C-dated middens of pack rats from Utah, porcupines from New Mexico and hyraxes from South Africa was dissolved in water and pipetted into the plastic wells of a microtitre plate. RIA analysis was done with antisera made in rabbits to the albumins of the deer mouse (*Peromyscus*, related to

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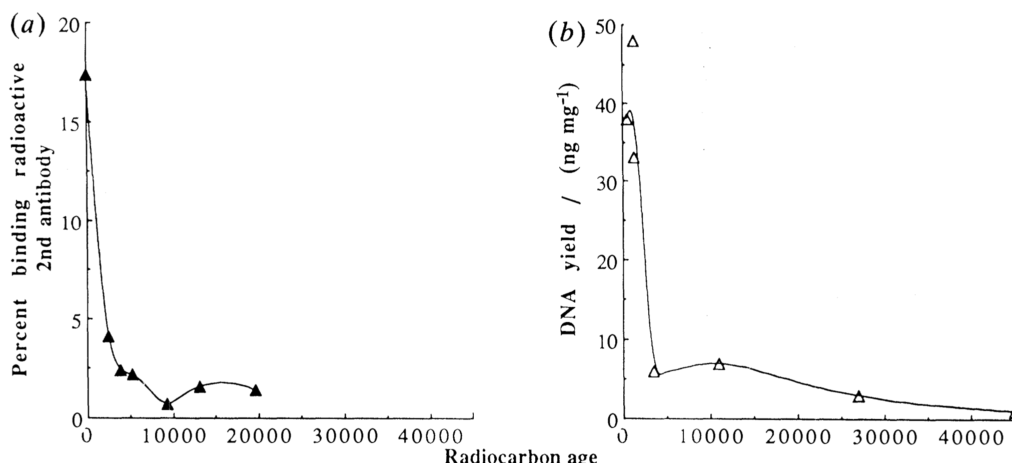

Figure 5. Similar patterns of molecular degradation through time in fossil packrat middens are shown for (*a*) albumin immunoreactivity in packrat urine by RIA from a midden series in Utah, U.S.A. and (*b*) DNA yield of plant tissue from middens in southern Nevada, U.S.A.

Neotoma), porcupine (*Erethizon*) and hyrax (*Procavia*). Species-specific albumin was identified in these specimens, as shown in table 3 (Lowenstein *et al.* 1991).

The rate of degradation of albumin was estimated by comparing urine of different ages from five different pack rat middens in the same cave, ranging in age from 2440 to 19700 years BP. A two-phase decline in immunoreactivity was observed: a rapid first phase, in which some 80% of the original reactivity was lost with a halftime of 800 years, and a slower second phase with a halftime of about 25000 years (figure 5). DNA yields from plant remains in the middens also decline in a manner very similar to that of albumin.

CONCLUSIONS

Sensitive immunological techniques such as RIA are providing new information about the evolutionary relationships of fossil species, the culture of ancient stone toolmakers and the preservation of biomolecules in fossil skin, muscle, bone, blood and urine.

In recent years, evolutionary biochemists, palaeontologists and the general public have become excited by the finding of mitochondrial DNA in some subfossils such as the quagga. The new polymerase chain reaction (PCR) method makes it possible to amplify tiny amounts of DNA by factors of a million or more and so to obtain partial DNA sequences from extinct species. This advance in technique raises the question of whether there is still a role for the immunological approach.

We have shown here that radioimmunoassay yields the same phylogenetic results as DNA sequencing in the cases of the recently extinct quagga and Tasmanian wolf, and the time curves are similar for survival of proteins and DNA in pack rat middens. There are probably many instances in which proteins but not DNA survive in older fossils. The two approaches are clearly complementary to each other, and there will be circumstances in which either or both will be preferable.

G. H. F. Nuttall presented his first results on immunological 'blood relationships' between animal taxa before the Royal Society in 1901. It is appropriate that now, ninety years later, in that same forum, we discuss the extension of Nuttall's pioneering work to include immunological studies on fossil species.

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Discussion

G. Eglinton (*Organic Geochemistry Unit, School of Chemistry, University of Bristol, U.K.*). How does the immunological reaction work in terms of the specific target sites (e.g. the six or seven amino acids that interact); if one amino acid changes or racemizes, how does the reaction register as a percentage of the original one?

J. M. Lowenstein. Racemization of amino acids would greatly reduce binding of antibody specific to the original group of amino acids. The use of monoclonal antibodies known to bind with specific configurations of amino acids would be useful for defining these diagenetic changes.

G. Eglinton. The fall-off in percentage reactions with geological age: does Professor Lowenstein think it is due to partial damage to each reaction zone or to diminishing content of completely unaltered zones?

J. M. Lowenstein. In a series of pack rat urine specimens from the same cave, ranging in age from 2000 to 20000 years, I observed a two-phase decline in immunological reaction, the first having a half-life of 800 years, the second 25000 years. The rapid decline may be due to loss of conformational determinants, those due to amino acids brought together by folding of the protein, the second, slower decline probably reflects processes such as racemization and fragmentation of the protein.

P. Westbroek (*Geobiochemistry Unit, Departmental of Biochemistry, University of Leiden, The Netherlands*). Sometimes 'spurious' immunological reactions are found, even among extant organisms. We had, for example, strong reactions between a systematically specific antiserum directed against macromolecules from a particular brachiopod and an extract from a coral skeleton. One should be aware of this phenomenon.

G. Muyzer (*Geobiochemistry Unit, University of Leiden, The Netherlands*). Significant positive results have been recorded by using antibodies produced against osteocalcin from recent alligator bones and tested in a dot immunobinding assay (DIBA) on EDTA-extracts of dinosaur bones. These results were confirmed by using another immunological assay, in which fossil osteocalcin has to compete with recent [^{125}I]-labelled osteocalcin. Although no systematically specific results could be obtained, supporting evidence of the presence of osteocalcin in these bones was obtained from Gla determination. Apart from its abundance in osteocalcin and its close interaction with the mineral phase, Gla is an excellent chemical marker for the presence of this molecule in fossil bones. It cannot be synthesized by bacteria, so using the necessary precautions in handling fossil samples, the presence of Gla must point to the presence of osteocalcin in the sample. The dinosaur bones that were positive in the immunological assays, also contained high concentrations of Gla. These results strongly suggest that osteocalcin or original fragments thereof are still preserved in bones of dinosaurs.

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