Dating Pleistocene Archeological Sites by Protein Diagenesis in Ostrich Eggshell

A. S. BROOKS, P. E. HARE, J. E. KOKIS, G. H. MILLER, R. D. ERNST, F. WENDORF

Eggshells of the African ostrich (*Struthio camelus*), ubiquitous in archeological sites in Africa, have been shown by laboratory simulation experiments to retain their indigenous organic matrix residues during diagenesis far better than any other calcified tissue yet studied. The rate of L-isoleucine epimerization to D-alloisoleucine follows reversible first-order kinetics and has been calibrated for local temperature effects and used to estimate the age range of stratified archeological sites. Age estimates are consistent with radiocarbon dates from several stratified archeological sites. With adequate calibration, this technique can provide accurate ages to within 10 to 15 percent for strata deposited within the last 200,000 years in the tropics and the last 1,000,000 years in colder regions such as China.

THE ORIGIN OF ANATOMICALLY modern humans is controversial (*1*). Much of the debate centers around the dating and stratigraphic provenience of key hominid fossils and associated archeological industries from Africa and the Near East (*2–9*). Most of these finds fall in a "gap" between the currently effective ranges of radiocarbon and potassium-argon dating procedures (*10*). In the age range from 40 to 200 ka (thousand years ago), many paleoanthropological finds have been provisionally dated by correlation with the oxygen isotope record or recognized glacial stages on the basis of stratigraphy, biostratigraphy, or paleoenvironment. Such dating is uncertain because of possible microenvironmental variability and problems with low-latitude climatic models of the last 200,000 years.

In this report, we describe an alternate approach to establishing chronologies in this time range that is based on protein diagenesis in ostrich eggshell. Protein diagenesis in mineralized tissues is the basis of several potential dating systems for archeological applications. These include methods based on the rates of protein hydrolysis, amino acid epimerization and racemization, and amino acid decomposition reactions. Because these are chemical reactions, reaction rates are temperature-dependent so that both time and temperature are variables. In principle, two or more reactions with different activation energies can be solved simultaneously to determine both time and temperature; however, the various activation energies are not greatly different and the approximate values presently available are insufficient to independently calculate both time and temperature.

Racemization of the L-amino acid isomers (epimerization for diastereomers) to a mixture of D and L isomers is perhaps the most useful diagenetic reaction for dating calcified tissues. In a closed system it follows reversible first-order kinetics and can be applied to a longer time range than radiocarbon dating.

Racemization ages of mollusk shells have generally been in agreement with ages determined by other techniques (*11–19*), but racemization ages on bone (*20*) have not (*21*). Bone, being relatively porous and inhomogeneous, is particularly susceptible to ground-water leaching, which preferentially removes denatured collagen and other soluble fractions (*22, 23*). Mollusk shell, on the other hand, is less permeable than bone to ground-water leaching, and its calcium carbonate ($CaCO_3$) composition provides a buffer against low *p*H conditions.

A reliable dating technique based on protein diagenesis requires a common archeological material that is resistant to humidity and *p*H effects. We believe ostrich eggshell (OES) is such a material (*24–28*). Fragmentary eggshells of the large ratite bird *Struthio* (modern species: *camelus*) are ubiquitous in African prehistoric (and historic) sites. From as early as 1.8 Ma (million years ago) at Olduvai Gorge (*29*), humans used the eggs for food, water containers, and, after 30 ka, for beads. In many northeast African sites, OES is the only identifiable faunal indicator, because its dense carbonate matrix (primarily calcite) is extremely resistant to decay processes. OES is also common in Middle Eastern sites (*30*), and eggshells of now extinct, cold-adapted ostriches are known from Pleistocene sites in China and Mongolia (*31*). The earliest members of the genus *Struthio* date to the early Miocene (*32*).

Earlier studies (*33*) of OES have focused on its utility for reconstructing prehistoric climates; shell thickness (*34*) or stable isotopes (^{13}C, ^{18}O) have been used as indicators of ostrich diets and habitats (*35*). The $^{13}C/^{12}C$ ratios of the shell are indicative of the ostriches' dietary intake. Radiocarbon assays on OES fragments have generally yielded consistent age estimates, another indication that the carbonate matrix typically is not contaminated (*36*). The organic component, bound in a calcite matrix, is about 3% of the eggshell mass, 50 to 100

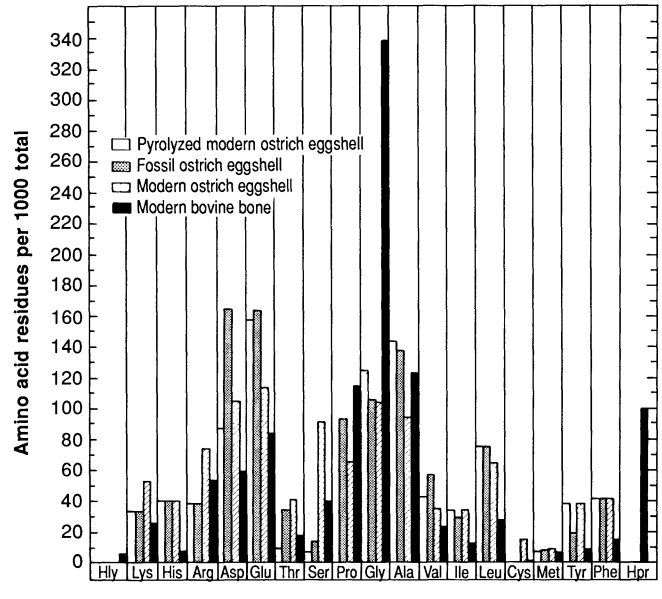

Fig. 1. Relative amino acid composition of ostrich eggshell and modern bovine bone material (*22*); Hly, hydroxylysine; Hpr, hydroxyproline.

A. S. Brooks and J. E. Kokis, Geobiology Program, Department of Anthropology, George Washington University, Washington, DC 20052, and Geophysical Laboratory, Carnegie Institution of Washington, Washington DC 20008.
P. E. Hare, Geophysical Laboratory, Carnegie Institute of Washington, Washington, DC 20008.
G. H. Miller, Center for Geochronological Research, Institute of Arctic and Alpine Research and Department of Geological Sciences, University of Colorado, Boulder, CO 80309.
R. D. Ernst, Center for Geochronological Research, Institute of Arctic and Alpine Research, University of Colorado, Boulder, CO 80309, and Department of Geosciences, University of Arizona, Tucson, AZ 85721.
F. Wendorf, Department of Anthropology, Southern Methodist University, Dallas, TX 75275.

times that in most mollusk shells.

We used pyrolysis experiments to simulate and accelerate diagenetic processes. Eggshell fragments were sealed in hydrolysis tubes and heated at controlled temperatures (*37–39*) for up to 16 weeks to determine the rate of diagenetic change as a function of temperature; various leaching environments were also simulated. Amino acid concentrations were determined as in (*39*).

Figure 1 compares the relative amino acid composition of various eggshell preparations to that of bone (*22*). In contrast to bone, OES contains no hydroxyproline or hydroxylysine but does contain a moderate concentration of cystine. Comparison of the composition of pyrolyzed and fossil OES with that of the untreated sample also showed significant differences. The less stable amino acids such as serine and threonine were depleted, whereas no significant change in the relative concentrations of the more stable amino acids (glutamate, glycine, valine, isoleucine, and leucine) was detected. The concentration of glutamate per gram of shell apparently changes least over time, and the results of the experiments suggest that it can be used as a reference to evaluate the decomposition rates of other amino acids (*40*).

After 70 hours of continuous leaching at 105°C, OES retained 99% of its original stable amino acid content, in contrast to bone, which lost 80% of its organic material. Molluscan fossils show a slower rate of loss than bone but were still depleted by 60% when the epimerization ratio of D-alloisoleucine to L-isoleucine (A/I) reached approximately 0.6 (*12*). In contrast to the results of earlier experiments with bone, OES samples heated under different conditions of leaching exhibited little difference in protein diagenesis, either in terms of protein hydrolysis, decomposition of particular amino acids, or A/I ratio. Because serine, a common contaminant in laboratory reagents as well as in natural ground waters, is nearly absent in the oldest archeological samples we examined, contamination from the uptake of amino acids from the environment was not significant. In the simulations of leaching, amino acid concentrations in the water in which eggshell fragments were immersed during pyrolysis did not increase. Thus leaching did not occur even though low molecular weight free amino acids were abundant in the eggshell matrix in the later stages of the experiments.

All of these results confirm the integrity of OES. This material more nearly approximates a closed system than any other organic material yet recovered from archeological sites.

L-Isoleucine has two chiral carbon atoms and epimerizes during protein diagenesis to its diastereoisomer, D-alloisoleucine. Most studies on mollusk shells and foraminifera tests have demonstrated, however, that the reaction does not follow reversible first-order kinetics over the entire range of the reaction. From our experiments, isoleucine epimerization in OES closely followed reversible first-order kinetics when the A/I ratio was below 1.0 (Fig. 2). The reaction rate may be expressed as $\ln (k_1) = C - H/RT$ where k_1 is the forward rate constant, H is the activation energy, T is temperature in kelvins, and R and C are constants. Combining the rate constants calculated from high-temperature pyrolysis experiments with those from series of early Holocene ^{14}C-dated samples from the eastern Sahara (Egypt and Sudan) and from South Africa yields an activation energy, E_a, of 30.01 kcal/mol and a value for C of 40.37 (Fig. 3); the standard error for E_a is 0.16.

Where temperature history is known or derived from other data (for example, obsidian hydration), particularly for deeply buried samples, A/I ratios in eggshell can be used to estimate age or, where age is known, to estimate temperature. At normal sediment temperatures in the tropics and subtropics (18° to 26°C), this should allow accurate dating of samples from 1 to 200 ka, depending on temperature history. The technique could be accurate to 1 Ma or older for samples from cold climates (northern China, Mongolia). The accuracy of such amino acid dates is dependent on uncertainties in the derived Arrhenius parameters and temperature reconstructions. If the reaction rate is calibrated at independently dated levels, the Arrhenius parameters are eliminated from the age equation and uncertainties are due primarily to possible differences in temperature between levels to be dated and that of the calibration horizon. Calibrated epimerization-based dates to twice the age of the calibrated sample can be made with a precision of 15%; increasing temperature uncertainties raise the error to 30% at three times the age of the calibration level. Extrapolation to greater ages is more uncertain. Interpolation can provide dates with a precision of 10% or greater (*40*).

The skeletal remains most often attributed to the earliest modern humans in Africa are associated with stone industries grouped under the term "Middle Stone Age" (MSA), which refers to industries characterized by tools made on flakes by a variety of prepared core techniques. With the exception of a few sites in Zaire and Zimbabwe, all with possible stratigraphic admixtures, the MSA appears to have ended in most of Africa by the time of the earliest reliable radiocarbon

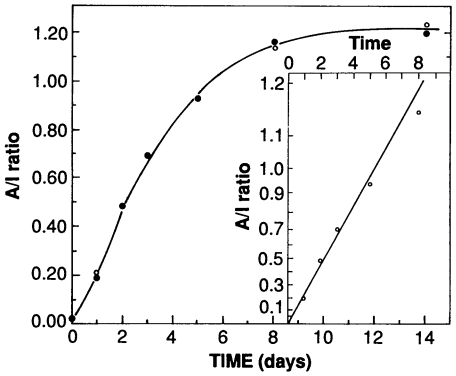

Fig. 2. Plot of the A/I ratio as a function of time based on high-temperature pyrolysis experiments of modern material at 144°C in both water vapor (●) and excess water (○) environments. The correspondence for the two data sets is strong evidence that there is little difference in the apparent rate of racemization for the two environments. The data in the inset are plotted as log[(1 + D/L)/(1 − 0.8 D/L)] versus time.

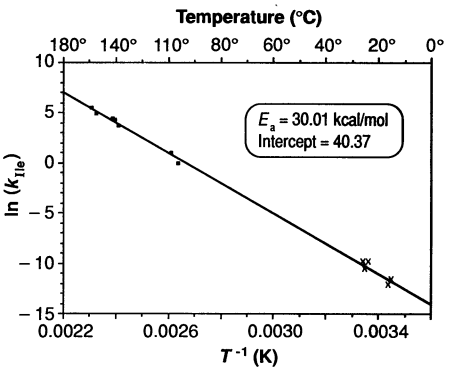

Fig. 3. Arrhenius diagram for isoleucine epimerization in eggshell of *Struthio camelus* based on high-temperature pyrolysis experiments of modern material at 160°, 157°, 146°, 144°, 142°, 110°, and 106°C, and radiocarbon-dated Holocene control (x) at ambient temperatures (*44*).

dates at about 30 ka (*41*). The age of most MSA sites remains problematic; all the sites that have yielded human remains have stratigraphic problems or disturbance that could have produced an admixture of older and younger materials. Protein diagenesis in OES not only can provide accurate age estimates for MSA sites where temperature is known or inferred from radiocarbon calibration of younger levels but also can indicate the degree of disturbance through comparison of data from different fragments within the same level.

We discuss the dating of two MSA sites to illustrate the consistency of the data generated and the potential uses of this technique in archeology. Each of the sites includes Holocene material (in an adjacent locality in the case of Bir Tarfawi) and extends to MSA levels with an estimated age range of >75 ka. Each site represents a "worst case" sce-

nario for protein decomposition dating, as both are open-air pan or lake margin localities in desert regions on or near the temperate-tropical border, and thus they were exposed to extreme diurnal and seasonal temperature variations as well as to seasonal or occasional flooding.

The first site to be analyzed, ≠Gi, is an open-air pan-margin site in the northwest Kalahari desert, on the Botswana-Namibia border (*42, 43*). Three main cultural units are distinguished: MSA with finely made bifacial and unifacial points and scrapers (level 4), an intermediate industry with blades but few formal tools (level 2C), and at least two horizons of Later Stone Age (LSA) materials (levels 2A and 1A), dating at the top to within the last 200 years. Separating level 2C from the underlying MSA and overlying LSA are two horizons of lacustrine limestone, reflecting intervals of relatively humid climate.

The MSA materials at ≠Gi document several behaviors normally associated with anatomically modern humans in Howieson's Poort levels of disputed age or in LSA or Upper Paleolithic contexts younger than 30 ka. These include (i) over 400 projectile points of standardized shape and size apparently modified for hafting; (ii) association with the remains of large, dangerous, or elusive prey species (*Phacochoerus*, *Pelorovis*, *Equus* cf. *capensis*, *Megalotragus*); and (iii) use of grindstones. The antiquity of the LSA materials is also of interest, because these do not appear to change throughout the sequence and can be linked at the top to the modern San or Bushman inhabitants of the area.

Epimerization results on OES from ≠Gi have been obtained, together with conventional and tandem accelerator mass spectrometry (TAMS) ^{14}C ages (Fig. 4). The level 4 coefficient of variation for A/I values is low, 4.9%, compared to the usual figure of 10 to 15% for mollusks and other systems. The lack of vertical stratification apparent in the epimerization results within level 4 corresponds to the results of stratigraphic and taphonomic analyses of the site and probably reflects a combination of trampling disturbance and relatively short accumulation time. A piece of eggshell at the base of unit 2C with an A/I ratio of 0.534 provided a radiocarbon age of 34.0 ± 1.1 ka (sample AA-3302), which is consistent with conventional radiocarbon age estimates by Stuckenrath on the under- and overlying limestone as well as with other dates for early post-MSA industries in south central Africa (*41*). The effective temperature is calculated to be 20.5°C, and the age of level 4, by extrapolation, is 65 to 85 ka (Figs. 4 and 5). This age conforms to thermoluminescence (TL) estimates on sediments from the top of level 4 (77 ± 11 ka).

The epimerization results also suggest two distinct periods of LSA occupation, an earlier one (level 2A) during the terminal Pleistocene and a later Holocene occupation (level 1A). The earlier age of level 2A is further supported by sedimentological evidence that the level 2A sediments represent a more arid climate than at present (*42*). Stratigraphic disturbance, in the form of pits excavated by the LSA inhabitants into the MSA horizons, is reflected in a distinct grouping of OES pieces between levels 1A and 2A with anomalously high A/I ratios; one of these (A/I ratio = 1.23) yielded a TAMS ^{14}C age of >37.2 ka (sample AA-3303). The localization of disturbance at this horizon and its absence elsewhere are important in any site interpretation and could not be demonstrated by other dating techniques.

A similar open-air site, BT-14, is located at the opposite end of Africa in the northern portion of the Bir Tarfawi lake basin in southern Egypt (*44–46*). OES fragments from four successive lake horizons (Grey Lakes 1 to 3 below, Olive-Green Lake above), all associated with Middle Paleolithic artifacts, were analyzed. As at ≠Gi, the coefficients of variation in each lake deposit are very low (2.0% on 23 separate OES preparations from Grey Lake 1, for example), and A/I ratios increase with stratigraphic depth (*45*).

Holocene age samples of OES were recovered from surface scatters a few kilometers to the east of Bir Tarfawi as well as from the nearby basin of Bir Sahara East. Effective sediment temperature in this region today, as measured directly with thermal sensors, averages 26.4°C. Several samples with known A/I ratios were directly dated by radiocarbon dating, and the results used to calculate the rate constant reflected in Fig. 3.

Using OES A/I ratios and radiocarbon or uranium-series dates from each site to calibrate the epimerization rate constant, the ages of older MSA levels have been estimated. These estimates also conform to estimates based on stratigraphic, faunal, typological, and geochronological analyses (*47*). In addition, the technique has demonstrated stratigraphic integrity or admixture within particular levels.

Ostrich eggshells are found outside of Africa in the dry regions of Asia and as far north as Mongolia. Eggshells of other ratite

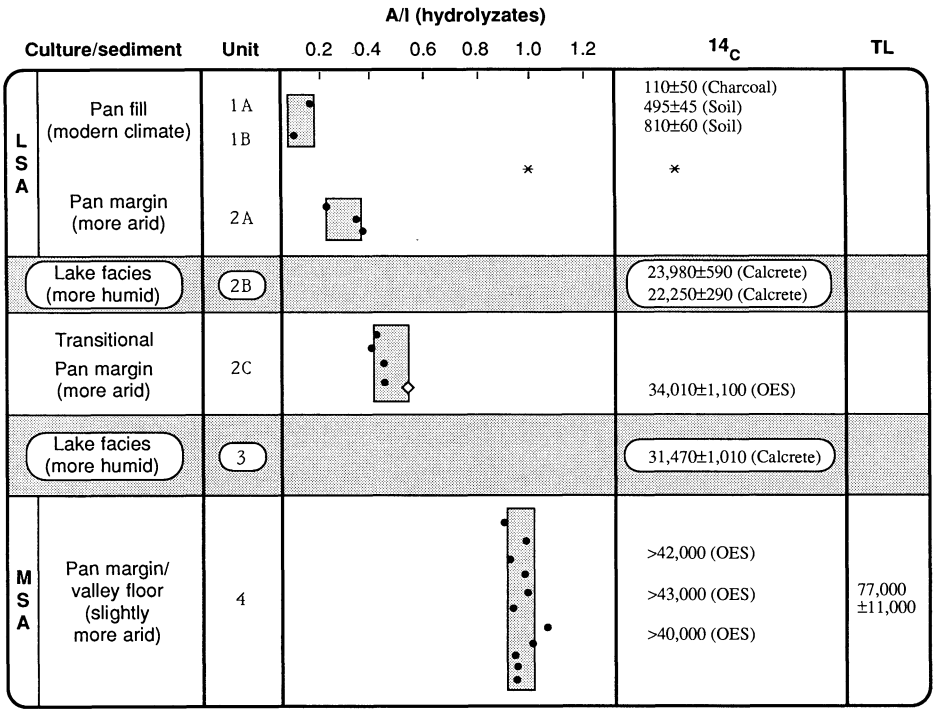

Fig. 4. Stratigraphy of ≠Gi (*41*) showing four archeological units (early and late LSA, transitional MSA/LSA, and MSA). The lower units are separated by lacustrine deposits. Amino acid data from the separate archeological units are plotted. The enclosing envelope represents the mean ± 1 SD of the measurements. A sample from level 2C (◇) was directly calibrated with radiocarbon (sample AA-3302). Three samples from the top of level 2A with high A/I ratios (*) almost certainly derive from the lower MSA horizons because of extensive pit digging by LSA inhabitants. One of these yielded a ^{14}C date of >37.2 ka [AA-3303]. In addition to the level 2C TAMS ^{14}C date, the other dates shown here were published as (from top to bottom): S1-4098, 4091A, 4091C, 4090, 4647, 4648, 4097, 4095, and 4094.

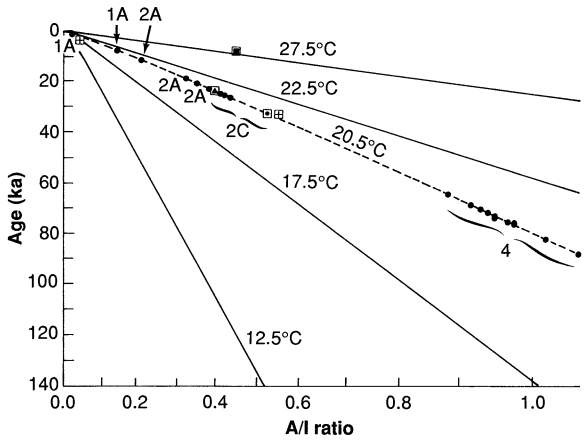

Fig. 5. Comparison of epimerization curves for ostrich eggshell from laboratory simulation data with samples from archeological sites in Africa. Temperature curves are derived from laboratory experiments at temperatures between 85° and 157°C. Five radiocarbon-calibrated archeological samples {from ≠Gi (●), Bir Tarfawi (■) [7,500 ± 80 years before present (BP) (AA-3292A)], Mumba Shelter (+) [1,980 ± 52 years BP, 33,460 ± 900 years BP (AA-3301, AA-3299)], and Ishango (▲) [25,570 ± 350 years BP (AA-3300)]} are plotted on the isotherm indicated by the combination of known age and epimerization. Samples calibrated with radiocarbon are shown enclosed in squares. The ≠Gi series (Fig. 4) is then plotted on the isotherm indicated by this calibration.

species (moas, rheas, and emus) occur in New Zealand, South America, and Australia, respectively. Pyrolysis experiments and results from natural samples indicate that at least the eggshell of the Australian ratites behaves similarly to those of *Struthio*. In Europe, some of the earliest sites associated with archeological remains of modern humans (*Homo sapiens sapiens*) have yielded fragments of owl eggs. We expect that expanded study of diagenesis in avian shells could resolve many of the chronological problems surrounding the question of modern human origins and will stimulate further research in this important time range. Initial work at Border Cave (*48*), for example, documents the stratigraphic integrity of the site as well as the age of the levels associated with anatomically modern humans.

REFERENCES AND NOTES

1. P. Mellars, *Curr. Anthropol.* **30**, 349 (1989); C. B. Stringer and P. Andrews, *Science* **239**, 1263 (1988); R. Lewin, *ibid.*, p. 1240; *ibid.* **237**, 1292 (1987).
2. H. Valladas *et al.*, *Nature* **331**, 614 (1988).
3. C. B. Stringer, *ibid.*, p. 565.
4. ———, R. Grün, H. P. Schwartz, P. Goldberg, *ibid.* **338**, 756 (1989).
5. H. P. Schwartz *et al.*, *J. Hum. Evol.* **17**, 733 (1988).
6. M. H. Wolpoff, *Am. J. Phys. Anthropol.* **78**, 326 (1988).
7. P. Mellars and C. B. Stringer, Eds., *The Human Revolution: Behavioral and Biological Perspectives on the Origins of Modern Humans* (Edinburgh Univ. Press, Edinburgh, 1989), vol. 1.
8. H. J. Deacon and V. B. Geleijnse, *S. Afr. Archaeol. Bull.* **43**, 5 (1988).
9. J. F. Thackeray, *ibid.*, p. 27.
10. The $^{40}Ar/^{39}Ar$ single-crystal laser technique may be reducing the size of this gap: R. E. Drake *et al.*, *Eos* **69**, 1502 (1988); A. L. Deino and R. Potts, *J. Geophys. Res.*, in press.
11. G. H. Miller, in *Quaternary Environments, the Eastern Canadian Arctic, Baffin Bay, and West Greenland*, J. T. Andrews, Ed. (Allen and Unwin, Winchester, MA, 1984), pp. 394–427.
12. ——— and P. E. Hare, in *Biogeochemistry of Amino Acids*, P. E. Hare, T. C. Hoering, K. King, Jr., Eds. (Wiley, New York, 1980), pp. 415–443.
13. ———, J. T. Hollin, J. T. Andrews, *Nature* **281**, 539 (1979).
14. J. F. Wehmiller, in *Biogeochemistry of Amino Acids*, P. E. Hare, T. C. Hoering, K. King, Jr., Eds. (Wiley, New York, 1980), pp. 341–355.
15. ———, *Quat. Sci. Rev.* **1**, 83 (1982).
16. ———, in *Quaternary Dating Methods*, W. C. Mahaney, Ed. (Elsevier, Amsterdam, 1984), pp. 171–193.
17. ———, *Quat. Res. (N.Y.)* **22**, 109 (1984).
18. ———, in *Dating Young Sediments*, A. J. Hurford, E. Jager, J. A. M. TenCate, Eds. (U.N. Commission for Coordination of Joint Prospecting for Mineral Resources in Asian Offshore Areas Tech. Publ. 16, Bangkok, Thailand, 1986), pp. 139–158.
19. ——— and D. F. Belknap, *Quat. Res. (N.Y.)* **18**, 311 (1982).
20. J. L. Bada and P. M. Masters, in *Peopling of the New World*, J. E. Ericson, R. E. Taylor, R. Berger, Eds. (Ballena, Menlo Park, CA, 1982), pp. 171–179; R. Protsch, *J. Hum. Evol.* **4**, 297 (1975).
21. R. E. Taylor, *Radiocarbon* **25**, 647 (1983).
22. P. E. Hare, in *Fossils in the Making: Vertebrate Taphonomy and Paleoecology*, A. K. Behrensmeyer and A. P. Hill, Eds. (Univ. of Chicago Press, Chicago, 1980), pp. 208–219.
23. J. L. Bada, *Earth Planet Sci. Lett.* **15**, 223 (1972).
24. P. E. Hare, A. S. Brooks, D. H. Helgren, J. E. Kokis, K. Kuman, *Geol. Soc. Am. Abstr. Programs* **16**, 529 (1984).
25. A. S. Brooks, P. E. Hare, J. E. Kokis, paper presented at Carnegie Institution of Washington Conference on Biomineralization and the Fossil Record (14 to 17 April 1985).
26. G. H. Miller and F. Wendorf, *Geol. Soc. Am. Abstr. Programs* **18**, 696 (1986).
27. R. D. Ernst, thesis, University of Colorado, Boulder (1987).
28. J. E. Kokis, thesis, George Washington University, Washington, DC (1988).
29. M. D. Leakey, *Olduvai Gorge*, vol. 3, *Excavations in Beds I and II, 1960–1963* (Cambridge Univ. Press, Cambridge, 1971), p. 291.
30. O. Bar-Yosef, in *Problems in Prehistory: North Africa and the Levant*, F. Wendorf and A. E. Marks, Eds. (Southern Methodist Univ. Press, Dallas, 1975), pp. 363–375; J. L. Phillips and E. Mintz, in *Prehistoric Investigations in Gebel Mughara, Northern Sinai*, O. Bar-Yosef and J. L. Phillips, Eds. (Hebrew Univ. Press, Jerusalem, 1977), pp. 149–163; G. E. Watson, B. Hesse, P. Watnish, unpublished data.
31. R. Wu and J. W. Olsen, Eds., *Palaeoanthropology and Palaeolithic Archaeology in the People's Republic of China* (Academic Press, New York, 1985).
32. S. L. Olson, in *Avian Biology*, D. S. Farner, J. R. King, K. C. Parkes, Eds. (Harcourt Brace Jovanovich, New York, 1985), vol. 8, pp. 80–238.
33. C. Carey, in *Current Ornithology*, R. F. Johnston, Ed. (Plenum, New York, 1983), vol. 1, pp. 69–103.
34. E. G. F. Sauer, *Cimbebasia* **14**, 1 (1966); *Cimbebasia Ser. A* **1**, 21 (1968).
35. Y. von Schirnding, N. J. van der Merwe, J. C. Vogel, *Archaeometry* **24**, 3 (1982).
36. H. Haas, in *Prehistoric Investigations in Gebel Mughara, Northern Sinai*, O. Bar-Yosef and J. L. Phillips, Eds. (Hebrew Univ. Press, Jerusalem, 1977), pp. 261–264; A. Long, R. B. Hendershott, P. S. Martin, *Radiocarbon* **25**, 533 (1983).
37. Pyrolysis experiments at the Carnegie Institution of Washington Geophysical Laboratory were carried out at 157°, 146°, 106°, and 85°C.
38. Pyrolysis experiments at the Center for Geochronological Research, the Institute for Arctic and Alpine Research, and the Department of Geological Sciences, University of Colorado, Boulder, were carried out at 160°, 144°, 142°, and 110°C.
39. Samples were subjected to one of two preparations. (i) Water vapor preparation: sample was placed in a small unsealed inner test tube or buried in 3 g of sterile quartzose sand; 0.5 ml of distilled water was added to the outer tube or sand before sealing. (ii) Excess water preparation: sample was immersed in 5.0 ml of distilled water in a sealed hydrolysis tube. The water was changed at regular intervals (8 hours to 1 week) to simulate ground-water leaching conditions. The shell and leach water were analyzed separately for amino acid residues. Two amino acid fractions were measured for each analysis: the free fraction and the total or acid-hydrolyzate fraction (free + peptide-bound amino acids). Analyses were carried out on automated high-performance liquid chromatographs with ion-exchange resin in narrow-bore (2 mm inside diameter) columns maintained at a constant temperature (55° or 62°C), and with postcolumn derivitization with *O*-phthalaldehyde. The fluorescent derivative was detected by a sensitive photomultiplier tube after excitation by long-wave ultraviolet light. This system allowed detection of all protein amino acids except proline and hydroxyproline and provided baseline resolution of D-alloisoleucine from L-isoleucine. Chromatograms were automatically stored on microcomputers, where the peaks were identified and edited. Most samples were analyzed two to four times. An alternate detection system with ninhydrin was used to determine the proline and hydroxyproline concentrations.
40. Samples of modern eggshell from widely separated localities (Australia, Texas, Kenya, South Africa) yielded similar concentrations of amino acids; coefficients of variation between samples for different relative amino acid concentrations ranged from 0.6 to 4.3%. The standard error in the determination of E_a is most likely due to slight inaccuracies in temperature measurement in the pyrolysis experiments as well as to uncertainties in the true average temperatures of the radiocarbon-dated samples. For a discussion of implications of a 1% uncertainty in E_a, see G. H. Miller *et al.*, *Nature* **326**, 593 (1987). In our applications, age estimates are based on a calibration of the rate constant from independently dated material at the same or nearby sites. Uncertainties in our age estimates are based on: (i) deviation from first-order kinetics (not relevant for ≠Gi or Bir Tarfawi; (ii) uncertainty in the independent age estimate; and (iii) deviation in average temperature experienced by the sample to be dated from that experienced by the calibration sample. Over ambient temperatures (−10° to +30°C), the rate of epimerization doubles for every 4°C temperature increase. In the case of an error of ~1°C, the age estimate error should be about 25%. We estimate that the likely error in both cave environments and in deeply buried open air samples is closer to 15%.
41. A. S. Brooks and P. Robertshaw, in *The World at 18,000 BP*, vol. 2, *Low Latitudes*, C. Gamble and O. Soffer, Eds. (Unwin Hyman, London, 1990), pp. 121–169.
42. D. M. Helgren and A. S. Brooks, *J. Archaeol. Sci.* **10**, 181 (1983).
43. K. A. Kuman, thesis, University of Pennsylvania, Philadelphia (1990).
44. F. Wendorf *et al.*, in *Proceedings of the XIth Congress of the Union of Pre- and Protohistoric Sciences, Lower and Middle Paleolithic of Africa*, J. D. Clark, Ed. (Mainz, Germany, 1 to 9 September 1987) (Verlag des Römisch-Germanischen Zentralmuseums, Mainz, in press).
45. G. H. Miller *et al.*, *Palaeogeogr. Palaeoclimatol. Palaeoecol.*, in press.
46. K. Kowalski *et al.*, *Quat. Res. (N.Y.)* **32**, 335 (1989).
47. P. B. Beaumont and J. C. Vogel, *Afr. Stud.* **31**, 66 (1972); *ibid.*, p. 155; J. C. Vogel and P. B. Beaumont, *Nature* **237**, 50 (1972); F. Wendorf *et al.*, *Science* **187**, 740 (1975); K. W. Butzer, P. B.

Beaumont, J. C. Vogel, *J. Archaeol. Sci.* **5**, 317 (1978); T. Volman, in *Southern African Prehistory and Palaeoenvironments*, R. G. Klein, Ed. (Balkema, Rotterdam, 1984), pp. 169–220.
48. G. H. Miller and P. B. Beaumont, *Geol. Soc. Am. Abstr. Programs* **21**, 235 (1989).
49. This research was supported by grants and fellowships from the Geophysical Laboratory, Carnegie Institution of Washington (J.E.K.), the George Washington University Committee on Research (A.S.B.), and the National Science Foundation (NSF) (BNS88-05555 to G.H.M., and BNS85-18574 to F.W.). Collection of samples was supported by NSF grants SOC75-14227 and BNS76-19633 (to A.S.B. and J. Yellen) and BNS85-18574 (to F.W.), as well as by grants from the National Geographic Society (to A.S.B., 1980), the L. S. B. Leakey Foundation (to K. A. Kuman, 1980, 1981), and a Fulbright Africa Research Fellowship (to A.S.B., 1982 to 1983). Additional samples were provided by M. Mehlman (Mumba Shelter) and J. Deacon (Boomplaas). Faunal identifications at ≠Gi were provided by R. G. Klein. Radiocarbon dates were supplied by R. Stuckenrath, C. V. Haynes, A. Long, and D. J. Donahue with the assistance of L. Toolin and A. J. T. Tull; and uranium-series dates were provided by H. P. Schwarcz, supported by grants from the Natural Sciences and Engineering Research Council of Canada and the Social Sciences and Humanities Research Council of Canada. A. J. Franklin and W. Hornyak provided thermoluminescence dating estimates on sediments from ≠Gi, supported by NSF grant BNS-83-19298. K. Kuman, G. el-Gamassy, D. Goter, and L. Bracewell assisted in the collection and laboratory analysis of eggshell samples. We acknowledge valuable discussions with numerous colleagues over the last year, especially N. Tuross for comments on an earlier version of this report, and we thank the anonymous referees for their helpful comments.

25 September 1989; accepted 1 February 1990

Molecular Cloning and Expression of a Complementary DNA for Inositol 1,4,5-Trisphosphate 3-Kinase

KWAN YONG CHOI, HA KUN KIM, SANG YEOL LEE,*
KYUNG HO MOON,† SANG SOO SIM, JAE WON KIM,
HONG KEUN CHUNG, SUE GOO RHEE‡

A complementary DNA (cDNA) clone that encodes inositol 1,4,5-trisphosphate 3-kinase was isolated from a rat brain cDNA expression library with the use of monoclonal antibodies. This clone had an open reading frame that would direct the synthesis of a protein consisting of 449 amino acids and with a molecular mass of 49,853 daltons. The putative protein revealed a potential calmodulin-binding site and six regions with amino acid compositions (PEST regions) common to proteins that are susceptible to calpain. Expression of the cDNA in COS cells resulted in an approximately 150-fold increase in inositol 1,4,5-trisphosphate 3-kinase activity of these cells.

INOSITOL 1,4,5-TRISPHOSPHATE [I(1,4,5)P_3], a product of phospholipase C activity on phosphatidylinositol 4,5-bisphosphate, is metabolized through two separate pathways: sequential dephosphorylation to inositol or phosphorylation to inositol 1,3,4,5-tetrakisphosphate [I(1,3,4,5)P_4] (*1–3*). I(1,4,5)P_3 is a second messenger and releases Ca^{2+} from vesicular, nonmitochondrial intracellular stores by binding to a specific receptor (*4*). I(1,3,4,5)P_4 also appears to be a second messenger and seems to function synergistically with I(1,4,5)P_3 in the control of Ca^{2+} homeostasis by regulating the transfer of Ca^{2+} from the I(1,4,5)P_3-insensitive pool to the I(1,4,5)P_3-sensitive pool (*3, 5, 6*). Although preliminary, the I(1,4,5)P_3-I(1,3,4,5)P_4 "duet" model can explain many complex, and sometimes seemingly contradictory, experimental results (*3, 7*). Therefore, I(1,4,5)P_3 3-kinase (IP3K), which catalyzes the adenosine triphosphate (ATP)–dependent phosphorylation of I(1,4,5)P_3 to I(1,3,4,5)P_4, not only provides an important branch point in the pathway of inositol phosphate metabolism, but also occupies a central position in regulating the availability of two Ca^{2+}-mobilizing second messengers.

Consistent with such a central role, IP3K appears to be the target of multiple cellular regulatory mechanisms. IP3K activity in rat brain increases 14-fold during development from fetus to adult (*8*) as a result of an increased synthesis of enzyme (*9*). IP3K is a Ca^{2+}-calmodulin–dependent enzyme (*10–13*) and may be modulated by protein kinase C (*14*). IP3K may also be a target of protein-tyrosine kinases, because IP3K activity increased by six- to eightfold in v-*src*–transformed cells (*15*).

IP3K has been purified from rat (*11*) and bovine (*12*) brains and from pig aortic smooth muscle (*13*), and its activity has been associated with polypeptides of 93,000 (*13*), 53,000 (*11*), 52,000 (*12*), 38,000 (*12*), and 35,000 daltons (*12*). These results could be due to the presence of multiple isozymes or to limited proteolysis. Inherent problems in studies on IP3K are its low concentration in tissues and its low recovery during purification. We have now isolated a cDNA clone that encodes IP3K.

A cDNA library was prepared from rat brain total polyadenylated [poly(A)$^+$] RNA with the use of the λgt11 vector system. Recombinant phages were screened with a mixture of ten monoclonal antibodies to the 53-kD IP3K from rat brain (*16*). Nine immunologically positive clones were isolated from about 1×10^6 transformants. The two longest inserts, pIP3K-I (1.85 kb) and pIP3K-II (2.15 kb), were then completely sequenced (*17*). The 1853-bp pIP3K-I has one open reading frame and carries coding sequences for all 17 tryptic peptides that had been isolated from the 53-kD IP3K from rat brain (*18*). This reading frame contains two methionine codons (at nucleotide positions −27 and +1) upstream of the amino acid sequence determined by direct analysis of a tryptic peptide. The ATG triplet of nucleotide residues +1 to +3 is likely to be the translational start site because the sequence immediately surrounding it (CGGGCATGG) better matches the Kozak consensus sequence (*19*) CC(A/G)CCATGG than does that surrounding the ATG triplet at nucleotide residues −17 to −25 (GGGAGATGA). Attempts to identify the amino acid sequence at the NH_2-terminus of the protein directly from purified 53-kD IP3K by Edman degradation failed because of blockage by an unidentified modifying group (*20*). A translational termination codon (TGA) occurs in-frame after codon 1350, which specifies arginine. The 5′ noncoding region is 72 bp long and rich in G and C residues. The 3′ noncoding region is 378 bp long, excluding the poly(A) tail, and contains an AATAAA polyadenylation signal 13 bp upstream from the poly(A) tail. Thus, pIP3K-I would encode a mature protein of 449 amino acids with a calculated molecular mass of 49,853 daltons (Fig. 1).

The complete sequence of pIPK-II (*20*) revealed that the 3′ 1472 nucleotides were identical to the 3′-end nucleotides of pIP3K-I. However, the 683 nucleotides at the 5′ end did not contain any sequences corresponding to the amino acid sequence of IP3K tryptic peptides and contained multiple stop codons. This suggests that pIP3K-

K. Y. Choi, H. K. Kim, S. Y. Lee, K. H. Moon, S. S. Sim, J. W. Kim, S. G. Rhee, Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892.
H. K. Chung, Department of Biochemistry, College of Medicine, Seoul National University, Seoul, Korea.

*Present address: Department of Biochemistry, Kyongsang National University, Chinju, Korea.
†Present address: Department of Pharmacy, Kyongsung University, Pusan, Korea.
‡To whom correspondence should be addressed.