studied by electron microscopy (*7, 12, 13, 31*), RB12A44 is the only one in which tochilinite and cronstedtite have been found. A specific search for these phases might reveal a higher abundance, but it is unlikely to exceed 10% of these IDPs. Therefore, if layer-silicate IDPs are a representative sampling of the asteroid belt, then CM chondrites cannot dominate the main belt, despite their similarity to the C reflectance class of asteroids (*28*). Both layer-silicate IDPs and C class asteroids could be samples of a broad spectrum of CM-like materials sharing common physical properties (for example, reflectance), but having significant mineralogical differences. For example, most layer silicate IDPs contain smectite rather than serpentine, and although the degree of aqueous alteration is variable from one IDP to another, it is often less than the matrices of CM chondrites (*13, 31*).

The identification of a CM-type IDP confirms predictions, based on calculations of particle orbital dynamics, that asteroidal material should be present among stratospheric IDPs (*6, 11*); most likely, materials from a variety of asteroidal parent bodies are present. However, only a subset of the IDPs have been studied. Most research has been focused on fine-grained IDPs because study of the variety of minerals they contain provides the best means for understanding their origins; coarser-grained IDPs have not yet been studied in detail. (Because of their coarse grain size, most meteorites could not be linked to a specific class of parent body on the basis of analysis of a 10-μm fragment.) In this study, we were able to make a strong link between RB12A44 and CM chondrites only because CMs are very fine grained and can be mineralogically distinct on a micrometer scale. If all layer-silicate IDPs are indeed of asteroidal origin, future sample studies combined with studies of dust dynamics should provide new information about geochemical conditions among the asteroids.

REFERENCES AND NOTES

1. S. A. Sandford, *Fundam. Cosmic Phys.* **12**, 1 (1987).
2. E. Anders, *Icarus* **24**, 363 (1975); G. W. Wetherill and C. R. Chapman, in *Meteorites and the Early Solar System*, J. F. Kerridge and M. S. Mathews, Eds. (Univ. of Arizona Press, Tucson, AZ, 1988), pp. 35–67.
3. E. Anders, in *Asteroids: An Exploration Assessment*, D. Morrison and W. C. Wells, Eds. (*NASA Publ. CP2053*, National Aeronautics and Space Administration, Houston, TX, 1978), pp. 316–335.
4. M. E. Lipschutz, M. J. Gaffey, P. Pellas, in *Asteroids II*, R. P. Binzel, T. Gehrels, M. S. Mathews, Eds. (Univ. of Arizona Press, Tucson, AZ, 1989), pp. 740–777.
5. Z. Sekanina, *Astron. J.* **90**, 827 (1985).
6. S. A. Sandford, *Icarus* **68**, 377 (1986); G. J. Flynn, *ibid.* **77**, 287 (1989); S. A. Sandford and J. P. Bradley, *ibid.* **82**, 146 (1989).
7. L. S. Schramm *et al.*, *Meteoritics* **24**, 99 (1989).
8. J. P. Bradley and D. E. Brownlee, *Science* **231**, 1542 (1986).
9. F. Verniani, *Space Sci. Rev.* **10**, 230 (1960).
10. J. S. Dohnanyi, in *Cosmic Dust*, J. A. M. McDonnell, Ed. (Wiley-Interscience, New York, 1978), pp. 527–605.
11. G. J. Flynn, *Proc. Lunar Planet. Sci. Conf.* **20**, 363 (1990).
12. K. Tomeoka and P. R. Buseck, *Earth Planet. Sci. Lett.* **69**, 243 (1984); I. D. R. MacKinnon and F. J. M. Rietmeijer, *Rev. Geophys.* **25**, 1527 (1987); K. L. Thomas, M. E. Zolensky, W. Klock, D. S. McKay, *Lunar Planet. Sci.* **XXI**, 1250 (1990); K. Tomeoka and P. R. Buseck, *Nature* **314**, 338 (1985).
13. J. P. Bradley, *Geochim. Cosmochim. Acta* **52**, 889 (1989).
14. R. E. Grimm and H. Y. McSween, Jr., *Icarus* **82**, 244 (1989).
15. Instruments used for this study were JEOL JSM-35C, 840, and 6400F SEMs, a 200-keV 2000FX AEM, and a 400-keV 4000FX AEM. All instruments were equipped with solid-state x-ray energy-dispersive spectrometers.
16. J. P. Bradley, D. E. Brownlee, P. Fraundorf, *Science* **226**, 1432 (1984).
17. K. Thiel, J. P. Bradley, R. Spohr, *Nucl. Tracks Rad. Meas.* **15**, 685 (1988); *ibid.*, in press.
18. J. T. Armstrong and P. R. Buseck, *Anal. Chem.* **47**, 2178 (1975).
19. G. Cliff and G. W. Lorimer, *J. Microsc.* **103**, 302 (1975); P. Sheridan, *J. Electron Microsc. Tech.* **11**, 41 (1989).
20. H. Y. McSween, Jr., *Geochim. Cosmochim. Acta* **51**, 2469 (1987).
21. K. Tomeoka and P. R. Buseck, *Nature* **306**, 354 (1983).
22. ———, *Geochim. Cosmochim. Acta* **49**, 2149 (1985).
23. K. Tomeoka, H. Y. McSween, Jr., P. R. Buseck, in *Proceedings of the Second National Institute for Polar Research Symposium on Antarctic Meteorites* (1989), p. 221.
24. M. E. Zolensky, *Meteoritics* **19**, 346 (1984).
25. E. Zolensky and H. Y. McSween, Jr., in *Meteorites and the Early Solar System*, J. Kerridge and M. S. Mathews, Eds. (Univ. of Arizona Press, Tucson, AZ, 1988), pp. 114–143.
26. L. Fuchs, E. Olsen, K. H. Jensen, *Smithson. Contrib. Earth Sci.* **10** (1973).
27. N. I. Organova, V. A. Drits, A. L. Dmitrik, *Am. Mineral.* **59**, 190 (1974); D. J. Barber, A. Bourdillon, L. A. Freeman, *Nature* **305**, 295 (1980); I. D. R. MacKinnon and M. E. Zolensky, *ibid.* **309**, 240 (1984).
28. J. Gradie, C. Chapman, E. Tedesco, in *Asteroids II*, R. P. Binzel, T. Gehrels, M. S. Mathews, Eds. (Univ. of Arizona Press, Tucson, AZ, 1989), pp. 316–335.
29. J. Wasson, W. V. Boynton, C. Chou, *Moon* **13**, 121 (1975).
30. R. N. Clayton, T. K. Mayeda, D. E. Brownlee, *Earth Planet. Sci. Lett.* **79**, 235 (1986).
31. M. S. Germani, J. P. Bradley, D. E. Brownlee, *ibid.* **101**, 162 (1990).

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A New Method for Carbon Isotopic Analysis of Protein

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The reaction of ninhydrin with amino acids can be used in carbon isotopic studies of protein. The reaction can be applied to extract as carbon dioxide only peptide-bonded carbon in proteinaceous material, thus avoiding most, if not all, contaminants. Test radiocarbon dates on ancient bone indicate that the method provides reliable ages, and stable carbon isotopic data suggest that our understanding of isotopic dietary reconstruction needs detailed examination. The technique should also be useful in biochemical tracing experiments and in global carbon budget studies, and the underlying principle may be applicable to other isotopes and molecules.

AGE AND DIETARY INFORMATION obtained from carbon isotopic determinations on ancient bone is often contentious (*1, 2*) because of difficulties in isolating material uncontaminated by either diagenetic processes or laboratory procedures. Purity requirements are stringent, especially for radiocarbon dating of old samples, where admixtures of a few parts per thousand of contaminant carbon can cause serious errors. In most of the methods developed to solve this problem, an attempt is made to eliminate all possible contaminants from the sample (*3, 4*), whereas a few are designed for the extraction of only reliable material (*5–7*). In all cases, the material isolated is burned to yield the CO_2 used in isotopic analyses. Burning does not discriminate against contaminants and is known to be a source of carbon contamination (*8*). These methods do not provide internal measures of quality; one is never certain of sample purity and hence of the integrity of the result.

In this report I propose an alternative method; the use of a chemical reaction that selects specific carbon atoms from proteinaceous molecules. First tests indicate that this method is accurate, practical, and easily adapted for routine use in both radio- and

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stable-isotope studies. Tests for contamination are possible in critical cases.

The reaction used is that of 2,2-dihydroxy-1,3-indandione (ninhydrin) with free amino acids. This reaction produces a purple pigment that is widely used for determining amino acid concentrations. The reaction is structurally specific, requiring the presence of carboxyl and amino groups on the amino acid. Nitrogen in the amino acid combines with ninhydrin to form the pigment, and the carboxyl carbon is released as CO_2 (*9*). Today the pigment is used for amino acid analyses, but 50 years ago, the evolved CO_2 was used for this purpose (*10*). Analyses with uncertainties ≤1% were made on samples containing as little as 40 μg of carboxyl carbon (*11*).

The CO_2 produced by this reaction seems ideally suited for carbon isotopic studies of proteins. For both stable-isotope mass spectrometry and radiocarbon dating by accelerator mass spectrometry (AMS), CO_2 is used either directly for the measurement or in the last step of sample preparation. Many natural materials will meet size requirements (≤1 mg of carbon) for both methods, and, although other molecules may react to produce interfering pigments, few react to produce CO_2. (Glutathione and sarcosine are exceptions, having active amino and carboxyl groups. Both are water-soluble and easily eliminated.) There is no reaction of ninhydrin with intact peptides or proteins, as the carboxyl and amino groups of the constituent amino acids have been combined to form peptide bonds. The chemical reactions involved are easy to perform, and, compared to the specificity of combustion, the reaction specificity gives great advantages in avoiding contaminants.

As a first test, I have applied the method to ancient bone. Bone collagen has amino acid concentrations (*12*) corresponding to about 20 mg of carboxyl carbon per gram of bone. The intact collagen trimer is insoluble, but, in old bone, diagenetic processes may have broken some of the collagen into soluble peptides and amino acids. Because ground water contains various soluble carbonaceous materials, including proteins and their degradation products, soluble material in the bone could contain contaminants. In the method presented here, only those carbon atoms forming intact peptide bonds in insoluble bone protein are isolated directly as CO_2. Thus, the only possible contaminants would be insoluble proteins that have been diagenetically formed within the bone from foreign amino acids. It is difficult to imagine such processes, but there are means to test for contaminants (as described below).

The method is as follows: First, inorganic carbon and soluble organic materials including amino acids and peptides are extracted from the bone with mild acid. (Because nonprotein contaminants do not produce CO_2, their presence is of no consequence.) Next, reacting the insoluble residue with ninhydrin without collecting gas provides additional assurance that interferences are removed. The reaction residue is then hydrolyzed to amino acids that are again reacted with ninhydrin, and the carboxyl carbon is collected as CO_2 for isotopic analyses.

For accurate isotopic analyses, CO_2 yields from the sample must be quantitative so that there is no isotopic fractionation; therefore, I investigated the utility of the old amino acid analysis recipes for the reaction (*10*). To simulate bone measurements, I made an aqueous solution of 16 reagent amino acids at concentrations similar to those for bone collagen (*12*), reacted aliquots containing carboxyl carbon in the range 0.05 to 1.02 mg, and measured the evolved CO_2. The observed yields confirm the old methods (Fig. 1) and show that they are applicable to the present problem.

Could protein in old bone become so degraded that it would hydrolyze and react during the first ninhydrin treatment? If so, the subsequent reaction would not be possible. To test this possibility, I reacted weighed amounts of high molecular mass (>10 and >30 kD) peptides extracted from insoluble proteins (*5*) of several bones of ages to 50,000 years before present (B.P.). The reaction products were ultrafiltered, dried, and reweighed. Some hydrolysis did occur during the reaction (pigment was observed), but the combined hydrolysis and sample handling losses were ≤20%. The

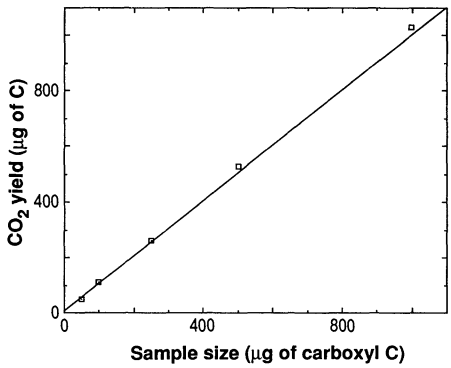

Fig. 1. Yield of evolved CO_2 as a function of the quantity of carboxyl carbon reacted. The line is a least-squares fit (slope = 1.03; SE = 0.01). The amino acid aliquots were reacted with 50 mg of ninhydrin in a 2-ml aqueous solution buffered to pH 4.7 with a mixture of citric acid and sodium citrate. The vessel containing this solution was evacuated and heated to ~110°C for ~12 min. The evolved CO_2 was separated from water and aldehyde vapors with the use of a dry-ice trap, collected with liquid nitrogen, and measured manometrically. Systematic uncertainties are estimated at ~5%, which is similar to the differences between expected and observed results.

Table 1. The four bones studied were from bison (PRB) and mammoth (PB-1 and OC-1) from northern Canada and cave bear (DB-A12) from Yugoslavia. The CAMS analyses are those from this study. All ages are given in radiocarbon years B.P. as defined in (*27*). Those CAMS ages marked with an asterisk were done by the simpler process, in which the first ninhydrin reaction was performed directly on organic material extracted from the bone with mild acid. All other CAMS data were taken on high molecular weight peptides obtained by ultrafiltration (*5*) after reaction with ninhydrin. The $\delta^{13}C$ values for whole collagen were taken on CO_2 from burned samples of high molecular weight peptides; those for carboxyl carbon on CO_2 from the ninhydrin reaction on these peptides.

Sample and lab numbers (*15*)	^{14}C age (years B.P.)	$\delta^{13}C$† (per mil)	$\delta^{13}C$‡ (per mil)
	PRB		
RIDDL-220	10,750 ± 180		
AA-1219	10,600 ± 160		
CAMS-398	10,580 ± 210	−19.4	−11.8
CAMS-399*	10,340 ± 150		
	PB-1		
RIDDL-223	20,230 ± 180		
NMC-1221	18,220 ± 310		
GSC-3053	15,500 ± 130		
CAMS-400	20,070 ± 340	−20.6	−12.8
CAMS-401*	20,080 ± 220		
	OC-1		
RIDDL-122	31,120 ± 450		
RIDDL-231	30,680 ± 600		
NMC-1235	30,490 ± 550		
CAMS-402	30,700 ± 930	−20.8	−14.0
	DB-A12		
RIDDL-744	>53,000		
CAMS-403	>54,000	−21.4	Not determined

*Simpler extraction process. † Whole collagen carbon. ‡ Carboxyl carbon.

peptides retained their structural integrity, and abundant insoluble residue was available for the second step.

As a final test, four bones of ages ranging from 10,000 to >53,000 years B.P. (Table 1) were chosen for carbon isotopic analyses. Two of these bones (PRB and OC-1) were of well-known age, as concordant results had been measured in different laboratories by different preparation methods. One bone (PB-1) was problematic, as three different laboratories had obtained three different results. The fourth bone was beyond the range of radiocarbon dating and was thus a test of sensitivity.

For these measurements, insoluble protein residues in 150- to 250-mg samples of bone powder were extracted as peptides of molecular mass >10 kD (*5*). These peptides were reacted with ninhydrin, ultrafiltered, hydrolyzed to amino acids, and reacted again with ninhydrin. The evolved CO_2 was collected in the second ninhydrin reaction. Because this procedure was possibly unnecessarily rigorous, two samples (PRB and PB-1) were also subjected to a simpler process. The first ninhydrin reaction was done directly on the insoluble residues of a mild HCl extraction of bone powder. These residues were then hydrolyzed, the amino acids were reacted with ninhydrin, and the CO_2 was collected. Each CO_2 sample (containing 350 to 800 μg of carbon) was converted to graphite (*8*), and the ^{14}C ages were obtained by AMS (*13*). Several determinations of system background were obtained on CO_2 made from geological doublespar that had been reacted with acid, as this most closely resembled the procedure used for the samples.

Separate CO_2 samples were prepared in the same manner for $\delta^{13}C$ (*14*) analyses. I also analyzed aliquots of unreacted high molecular weight peptides to test for isotopic differences between the peptide-bond carbon and the collagen as a whole (Table 1).

It is evident from the radiocarbon ages obtained that the method works. For the samples of well-known age (PRB and OC-1), the ages determined by this procedure were entirely consistent with the independent measurements. For sample PB-1, one measurement (RIDDL-223) (*15*) used the most selective extraction method, and the excellent correspondence with the ninhydrin data verifies both measurements. The result for the very old sample DB-A12 is remarkable for a first test, as ages of this magnitude are difficult to achieve under the best circumstances. There seems little reason to choose the more extensive reaction method over the much simpler one, but this conclusion reflects only two comparisons and needs further verification.

Bones from terrestrial northern animals like these have $\delta^{13}C$ values for whole collagen of about −19 to −21 per mil (*16–18*), and variation between individuals in a population is often <1 per mil (*17, 19, 20*). In contrast, some studies (*1, 21*) indicate that the $\delta^{13}C$ values of the constituent amino acids of collagen can differ by as much as 10 per mil. The data from the ninhydrin procedure add yet another dimension. As expected, the $\delta^{13}C$ values for the whole collagen are about −20 per mil, but the carboxyl $\delta^{13}C$ values are only about −13 per mil (Table 1). Apparently, there are large isotopic differences among different functional groups in amino acid molecules.

This approach should prove useful for carbon isotopic studies of bone. For routine radiocarbon dating, a single ninhydrin reaction may be adequate, whereas for more critical cases we can use established techniques to isolate for separate measurement either the different classes of amino acids or the individual amino acids themselves. Concordant results would provide a high degree of certainty, whereas discrepant results would give direct evidence of contamination. These first few stable isotope data on the carboxyl groups in amino acids are intriguing, and it will likely be necessary to undertake detailed isotopic studies of both the carboxyl carbon and the remaining carbon of individual amino acids to appreciate the implications. Do these data indicate the existence of an unexploited resource of information for food chain studies?

More generally, the method is applicable to any protein, and it thus opens the way to carbon isotopic measurement of a wide range of materials. Because the reaction is so specific, it should be possible to use standard biochemical methods to identify and isolate proteins without introducing further interferences. Possible applications include:

1) Isotopic studies of remnant proteins in shell, teeth, food remains, blood on stone tools (*22*), and art residues (*23*).

2) Detailed analyses of the role of proteins and their degradation products in global carbon pools. In the ocean, for example, this material is often lumped and studied together with other organic remnants as "dissolved organic carbon" (*24*).

3) Highly sensitive biochemical studies of metabolic processes using isotopically tagged amino acids, peptides, and proteins. The specificity of the ninhydrin reaction, if used with single or multiple tags and AMS detection (*25*), should allow detection of very small quantities of the tagged molecules.

Finally, the general principle should be applicable to studies of other isotopes and molecules, such as measurement of peptide-bonded nitrogen in $\delta^{15}N$ dietary studies. The ninhydrin reaction could be useful, as the pigment contains only this nitrogen, or the reaction could be modified to release the nitrogen directly as ammonia (*10*); alternatively, reactions with nitrous acid or hydriodic acid (*26*) might be useful.

REFERENCES AND NOTES

1. T. W. Stafford, Jr., K. Brendel, R. C. Duhamel, *Geochim. Cosmochim. Acta* **52**, 2257 (1988).
2. M. J. DeNiro, *Nature* **317**, 806 (1985).
3. R. Longin, *ibid.* **320**, 241 (1971).
4. T. W. Stafford, Jr., A. J. T. Jull, K. Brendel, R. C. Duhamel, D. Donahue, *Radiocarbon* **29**, 24 (1987).
5. T. A. Brown *et al.*, *ibid.* **30**, 171 (1988).
6. R. Gillespie, R. E. M. Hedges, J. O. Wand, *J. Archaeol. Sci.* **11**, 165 (1984).
7. G. J. van Klinken and W. G. Mook, *Radiocarbon* **32**, 155 (1990).
8. J. S. Vogel *et al.*, *ibid.* **29**, 323 (1987).
9. A. L. Lehninger, *Biochemistry* (Worth, New York, 1978), p. 85.
10. D. D. Van Slyke, R. T. Dillon, D. A. McFayden, P. Hamilton, *J. Biol. Chem.* **141**, 627 (1941).
11. D. D. Van Slyke, D. A. McFayden, P. Hamilton, *ibid.*, p. 671.
12. R. E. M. Hedges and C. J. A. Wallace, in *Biogeochemistry of Amino Acids*, P. E. Hare *et al.*, Eds. (Wiley, New York, 1980), pp. 35–40.
13. The radiocarbon ages were taken on the Lawrence Livermore National Laboratory (LLNL) AMS facility, as described in J. R. Southon *et al.*, *Nucl. Instrum. Methods*, in press.
14. Stable carbon isotope ratios are measured as $\delta^{13}C$ values, defined as $\delta^{13}C$ (per mil) = $1000 \times (R_{sample} - R_{standard})/R_{standard}$, where R is the ratio $^{13}C/^{12}C$, and the standard used is the Pee Dee belemnite.
15. Previous radiocarbon measurements were obtained by the Geological Society of Canada β-counting laboratory (GSC) and by AMS laboratories at Simon Fraser University (RIDDL), the University of Arizona (AA), and Chalk River Nuclear Laboratories (NMC). Ages RIDDL-220 and AA-1219 were obtained in a cross-laboratory study of different preparation methods (D. E. Nelson and R. Gillespie, unpublished data). Results for RIDDL-122, RIDDL-231, and NMC-1235 are described in R. E. Morlan *et al.*, *Can J. Archaeol.* **14**, 75 (1990). Ages for RIDDL-223, NMC-1221, and GSC-3053 are unpublished data (D. E. Nelson; J. Cinq-Mars, personal communication) as is RIDDL-744 (D. E. Nelson and I. Turk).
16. N. J. van der Merwe and J. C. Vogel, *Nature* **276**, 815 (1978).
17. B. S. Chisholm *et al.*, *Science* **216**, 1131 (1982).
18. M. J. Schoeninger and M. J. DeNiro, *Geochim. Cosmochim. Acta* **48**, 625 (1984).
19. N. C. Lovell, D. E. Nelson, H. P. Schwarcz, *Archaeometry* **28**, 51 (1986).
20. D. E. Nelson, unpublished data.
21. P. E. Hare and M. L. F. Estep, *Carnegie Inst. Washington Yearb.* **82**, 410 (1983).
22. D. E. Nelson, T. H. Loy, J. S. Vogel, J. R. Southon, *Radiocarbon* **28**, 170 (1986).
23. T. H. Loy *et al.*, *Antiquity* **64**, 110 (1990).
24. P. M. Williams and E. R. M. Druffel, *Nature* **330**, 246 (1987).
25. J. S. Vogel *et al.*, *Nucl. Instrum. Methods*, in press.
26. E. E. Howe, in *Amino Acids and Proteins*, D. M. Greenberg, Ed. (Thomas, Springfield, IL, 1951), pp. 30–39.
27. M. Stuiver and H. Polach, *Radiocarbon* **19**, 355 (1977).

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