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ADVANCES IN THE ISOTOPE ANALYSIS OF BIOGENIC PHOSPHATES AND
THEIR UTILITY IN ECOPHYSIOLOGICAL STUDIES OF
AQUATIC VERTEBRATES

by

Lois Jane Roe

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A Dissertation Submitted to the Faculty of the
DEPARTMENT OF GEOSCIENCES
In Partial Fulfillment of the Requirements
For the Degree of
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In the Graduate College
THE UNIVERSITY OF ARIZONA

1998
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Lois Jane Roe entitled "Advances in the Isotopic Analysis of Biogenic Phosphates and their Utility in Ecophysiological Studies of Aquatic Vertebrates" and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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STATEMENT BY AUTHOR

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DEDICATION

This dissertation is dedicated to my parents, George and Barbara Roe, for never asking me what I was going to do with a degree in geology and for their unwavering financial, logistical and emotional support over many years of education, and to the memory of the late Thomas Hoering, who was instrumental in helping me get started in my isotopic studies of biogenic phosphates.
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ABSTRACT

Distinguishing marine and freshwater animals in the fossil record is a long-standing problem in paleontology. The physiological tolerances of extinct animals usually are inferred from environmental indicators and/or on the physiology of nearest living relatives. These types of evidence are often ambiguous and may be confounded by factors such as post-mortem transport and polymorphism in the living relatives. A solution to this problem is to combine these types of data with analyses of the oxygen isotope compositions of the phosphate ($\delta^{18}O_p$) and the carbon isotope compositions of the carbonate ($\delta^{13}C_sc$) of teeth and bones, to determine whether the ingested water and diet, respectively, were fresh or marine. The power of this approach is illustrated here in a study of the early evolution of cetaceans (whales, dolphins and porpoises). Changes in $\delta^{18}O_p$ and $\delta^{13}C_sc$ of the teeth and bones of early cetaceans documented here indicate that fully marine cetaceans existed by the middle Eocene and that some species exploited both marine and freshwater environments.

This isotopic approach requires the avoidance of isotopically altered specimens. For this reason, the second component of this work deals with criteria for recognizing isotopically altered fossils. In contrast to one recent study, I found a positive correlation between $\delta^{18}O_p$ and $\delta^{18}O_sc$ not only in mammals but also in fish and reptiles. This correlation can be used as a test of whether the original isotopic composition is preserved in fossil specimens. Another approach to this problem is to make analyses of samples taken along growth transect of a fossil tooth or bone. Growth-transect analyses could resolve whether within-species isotopic variation represents differences in preservation or ontogenetic shifts in diet or habitat. In support of this goal, a new method for the analysis of phosphate oxygen is presented. This new method differs from all previous methods in that it involves no chemical reaction, but rather high-temperature (>725°C) equilibrium oxygen isotope exchange between CO$_2$ and Ag$_3$PO$_4$. As the amount of CO$_2$ is controlled
by the analyst, small phosphate samples may be analyzed, making this method potentially useful for growth-transect analyses.
CHAPTER 1. INTRODUCTION

The Importance of Distinguishing Marine and Freshwater Animals in the Fossil Record

Fishes and aquatic vertebrates in general are closely tied to their environments and are sensitive to physical and chemical changes in their aqueous medium. Many animals encounter such changes spatially, because they regularly or at different life stages move between marine, brackish (those with salinities between approximately 1,000 and 20,000 milligrams per kilogram of total dissolved solids or 1-20 parts per thousand; Drever, 1997) and freshwater habitats. Some groups have encountered such changes on longer temporal scales, having made transitions between marine and freshwater or vice versa during the course of their evolutionary history. Biologists studying modern organisms know that brackish environments, such as estuaries, serve as nurseries for many species of both invertebrate and vertebrate animals, and individuals are recruited to both freshwater and marine ecosystems from these marginal marine settings. It is not difficult to see why. These environments are heterogeneous and nutrient-rich and probably allow organisms to more finely divide existing resources. The ecological benefits of these systems should favor evolutionary diversification as well.

The fossil record is replete with examples of localities that are difficult to classify as strictly freshwater or marine and also with examples of evolutionary transitions between marine and freshwater environments, in both directions. It is, therefore, important to be able to distinguish marine, brackish and freshwater animals not only for the purpose of understanding environments through time, but for understanding evolutionary diversification. Paleontologists and geologists have tended to focus on classifying fossil localities or depositional settings as freshwater or marine environments, with brackish environments being some sort of unfortunate, confused area in between. This view is implicit in a recent paper by Schultze (1995). There are a great many examples of fossil localities that are difficult to classify, among them the Bouse Formation (see Spencer and
Patchett, 1997 and references therein) of Arizona and California and the Joggins Formation of Nova Scotia (see Schultze, 1995). These are generally regarded as geological "problems." Here I argue that this view is backwards. Rather than viewing these localities as problematic, we need to view them as evidence that may lead to recognition of the importance of marginal marine environments in evolutionary diversification. If we do this, we begin to recognize some gaps in our knowledge and approaches in several areas. I will outline a few such gaps below.

The Environment of Vertebrate Origins

The debate over the habitat of origin of early vertebrates (Nelson's [1989] Craniata) illustrates the reason I have undertaken the research I describe here. This debate is a long-standing one which dates back at least to 1892 (Halstead, 1985). Throughout most of the history of the debate, the evidence has come primarily from physiology of modern organisms and the nature of fossil occurrences of various vertebrate lineages in marine and non-marine depositional settings (Romer, 1967; Bray, 1985; Halstead, 1985; Gans, 1989). As summarized by Pough et al. (1996), the almost complete absence of vertebrate remains from early Paleozoic marine deposits was presented by paleontologist Alfred Sherwood Romer as an argument for an origin of the earliest vertebrates in freshwater; accordingly, the occurrence of ostracoderm fragments in an apparent marine fauna from the Ordovician Harding sandstone was attributed by Romer to post-mortem transport of freshwater fluvial fossils into a marine environment because of the worn condition of the fossils. A second argument for a freshwater origin, offered by physiologist Homer Smith, is that the existence of the glomerular vertebrate kidney is critical to freshwater fish because they naturally gain water by osmosis and must get rid of it; he considered this fact as support of the hypothesis of a freshwater origin of vertebrates.
On the other side of the argument, the fact that all protochordate and deuterostome invertebrates are marine is widely regarded as evidence of a marine origin of vertebrates (see Pough et al., 1996). The fact that lampreys and hagfish, which, being jawless, are the outgroups of jawed vertebrates (gnathostomes), are largely marine, also gives some support to the notion of a marine origin. As it turns out, however, examination of the kidneys of hagfish and also sharks, contradicts Smith's idea that a glomerular kidney is essentially related to osmoregulation. As Pough et al. (1996) point out, a glomerular kidney is not strictly associated with ion balance but serves more general excretory functions, such as removing toxic nitrogenous wastes and returning glucose to the circulatory system. They argue that once a glomerular kidney had evolved, only minor modifications would have been be required in order to make it an efficient water-excreting device in freshwater organisms.

Given the nature of the fossil and physiological evidence adduced so far in the debate, it seems that several lines of work are needed. The first is a geochemical study of the fossil remains of early vertebrates, to determine in which habitat the organisms were truly living. Through isotopic analyses, it is possible to test for post-mortem transport by analyzing the bones and teeth of the fossil organisms, whose isotopic composition preserve a record of the water in which they lived. The second type of effort needed is a better study of character evolution. What does it mean to be marine? How difficult is it for organisms to move between environments in ecological or evolutionary time? Judging from the fact that there exist so many euryhaline or diadromous fishes (McKeown, 1984; McDowall, 1988), I would suggest that it is not as difficult as the vertebrate origins debate might suggest.

A final, most important point: why has no one ever suggested that vertebrates originated in brackish water or that the earliest vertebrates were euryhaline (tolerant of a wide range of salinities)? Why is the debate always presented as a strict dichotomy? As
I noted above, brackish water environments serve as ecological "nurseries", from which many types of organisms are recruited into both freshwater and marine ecosystems. A brackish water origin of vertebrates would in fact provide the most parsimonious explanation of both the fossil and physiological evidence, because it reconciles the lack of vertebrates in the earliest Paleozoic marine settings with the physiological and phylogenetic hints of a marine origin.

The Importance of Marine-Freshwater Distinctions in Biogeography

Evolutionary diversifications of primary freshwater fish (those restricted to freshwater) are generally regarded to be directly related to geologic events such as the rifting of landmasses, or the uplift of mountains, and for this reason freshwater fish distributions contributed to the development of the school of thought known as vicariance biogeography (e.g., Rosen 1975, 1978). But a particular primary freshwater fish group may not always have been restricted to freshwater. And although inferences based on the physiology of nearest living relatives in a phylogenetic framework, and the principle of parsimony are strong, specific cases may be unresolvable by this means. For example, polymorphism in physiological tolerances within a nearest living relative clade would make it difficult to resolve when the character transformation actually occurred. Even in cases, where one has the good fortune to have a well-resolved phylogenetic hypothesis are nearest living relatives more ancestral and

Osmoregulation, Environmental Change and Mass Extinctions

Growing evidence of major changes in atmospheric pCO$_2$ and resultant changes in the pH of marine and freshwater environments during times of mass extinctions (e.g., Knoll et al., 1996; Retallack, 1996; Isozaki, 1997) and also during times of evolutionary innovations, such as the evolution of air-breathing (e.g., Ultsch, 1996; Ultsch and
Jackson, 1996), has provided a new impetus for understanding the evolution of osmoregulation of aquatic vertebrates. Increasing recognition of the need to evaluate mass extinctions and character evolution in a rigorous phylogenetic framework (e.g., Patterson and Smith, 1987) places this need in its larger context.

As described above, understanding the evolution of osmoregulation in fishes and other aquatic vertebrates is critical to understanding their diversification and biogeography. It may also be critically important to understanding the patterns of extinctions of aquatic vertebrates during mass extinction events. Osmoregulation is fundamentally linked to acid-base balance (Evans, 1993). Some mass extinctions, such as the one that occurred at the end of the Permian, are thought to have been related to large changes in oceanic and atmospheric CO2 levels (Knoll et al., 1996). Such changes would have affected the pH of the oceans and fresh waters and would have most severely affected those organisms which can not easily adjust to large changes in ambient water pH. It follows that organisms whose osmoregulatory systems are critically dependent on constant pH will be less able to adjust physiologically to conditions which radically alter environmental pH. A good modern example of this is the high mortality rates of salmon in Scandinavian waters due to acid rain.

Graham et al. (1995) made predictions (or more accurately "postdictions") about the effect of excess oxygen on terrestrial organisms. No equivalent treatment of aquatic vertebrates across the Permo-Triassic (P-T) boundary has yet been published, but many of the arguments offered by Ultsch (1996) for the evolution of air-breathing in the Devonian have direct bearing on this issue. If the pH changes hypothesized (i.e., for the end-Permian mass extinction) really did occur, they ought to be truly conspicuous in the record of aquatic vertebrates. Another related point that is probably worth investigating is the possibility that the composition, thickness or degree of imbrication of scales on the bodies of fishes might be related to osmoregulation. Perhaps, for example, the peg and
socket scales of lepisosteids (gars) have been retained not because they are an effective defense (the usual evolutionary just-so story) but because they help prevent water/ion transfer across the integument.

Methods Previously Used to Make Marine-Freshwater Distinctions

Given the evident importance of making marine-brackish-freshwater distinctions in the fossil record, how do we get such information from fossils? Generally, inferences about the physiology of extinct animals are based on environmental indicators or on the physiology of nearest living relatives. Environmental indicators, such as sedimentary structures and associated fauna or flora are helpful if no transport has occurred or if it can be recognized, but these indicators give only an instantaneous view of the organism’s life. Movement by the organism between environments are difficult to detect using sedimentary or faunal indicators, unless the organism in question is preserved in multiple environments.

In contrast, inferring the physiological attributes of an organism from those of its nearest living relative(s) is independent of the organism’s occurrences in depositional environments and not confounded by transport. Nearest living relatives may be evolutionarily quite far removed from the organism of interest and may not be informative because too much evolution has occurred. In addition, if the nearest living relatives are polymorphic with respect to the character of interest, it may be impossible to reconstruct the character states of the fossil species of interest. An example of such a case is that of archeocetes (early whales). Archeocetes made the transition from terrestrial to marine life within their lineage. The living groups closest to the base of the archeocete clade are perissodactyls and artiodactyls, which are fully terrestrial. The living cetaceans closest to the most derived archeocetes are fully marine. The only inference that can be made using these constraints is that somewhere within archeocetes, the environmental
transition was completed. No further inferences based on the biology of living relatives can be made.

A New Approach to Making Marine-Freshwater Distinctions

Here I suggest that the best approach to solving problems such as the environment of origin of early vertebrates and the environmental transition of early whales is an integrative one that combines environmental (e.g. sedimentological and faunal), phylogenetic and geochemical approaches. Geochemical analyses can provide information on character states that can be used in evaluating evolutionary patterns on a phylogenetic tree (cladogram) obtained independently using morphological and/or molecular data. The patterns of character evolution, for example, the origin of seawater ingestion, can then be evaluated in this context. If it is known that a certain group started out as freshwater or terrestrial and became marine, then this progression should be reflected in some manner in the pattern of character transformation. The number of times that character arose can be determined and age constraints (based on the known ages of the fossil occurrences) can be placed on the character evolution. The ecological breadth of a clade can also be determined and hypotheses relating character evolution and diversification can be tested. For example, did a group diversify before or after it became marine? Finally, if it can be demonstrated that no post-mortem transport of fossils has occurred, environmental data, such as sedimentary indicators and biotic associations, can be compared with the phylogenetic and geochemical data to check for consistency, and add to what is known of the nature of the habitat.

Format of this Dissertation

The first part of this dissertation is devoted to exploring the utility of using isotopic data in marine-freshwater distinctions and to applying it to a case study of one specific,
well-defined clade, the Cetacea, in the course of its physiological transition from a terrestrial life to a fully marine life (Appendix A). To do this, I employed two isotopic systems: the carbon isotope ratios ($^{13}\text{C}/^{12}\text{C}$) of the structural carbonate of teeth and bones and the $^{18}\text{O}/^{16}\text{O}$ of the phosphate in teeth and bones. Carbon isotope ratios are good discriminators of marine versus freshwater (or terrestrial) diet and oxygen isotope ratios record the predominant environmental water ingested by the animal. In combination, these types of data provide information on where an animal spent most of its time, and for what purpose. By making analyses of multiple cohabitant species, the amount of ecological similarity and overlap between organisms can be determined.

An essential part of applying these geochemical approaches is obtaining isotopically unaltered specimens for analysis. Follow the isotopic study of early cetacean evolution (Appendix A) is a manuscript (Appendix B) dealing with how isotopically altered specimens are recognized. This work was designed to test the utility of a correlation between the oxygen isotope composition of the phosphate ($\delta^{18}\text{O}_p$) and that of the structural carbonate ($\delta^{18}\text{O}_{sc}$) in teeth and/or bones of aquatic vertebrates for comparison with previous work on mammals and a controversial study of lower vertebrates (fishes, amphibians and reptiles).

Also pertinent to the subject of detecting isotopic alteration was the realization from the cetacean study, that fine-scale sampling along a growth transect of a fossil tooth or bone could contribute both to studies of biology and phosphate diagenesis. Such fine-scale sampling could resolve the question of variation found between specimens of the same species (for example, Ambulocetus and Gandakasia) represents differences in preservation between specimens or real, biological phenomena, such as ontogenetic shifts in habitat and/or diet. In support of this goal, I present, in Appendix C, a new contribution to the growing body of methods for the analysis of phosphate oxygen. This new contribution differs from all previous approaches in that it involves no chemical
reaction, but only a high-temperature isotopic exchange between two phases, CO₂ and Ag₃PO₄. Demonstration of this exchange method opens the door to the manipulation of CO₂ sample size, and may make it possible to work with sample-sizes much smaller than those which currently are employed with more conventional approaches (those not involving a laser or Thermal Ionization Mass Spectrometry).

Owing to the multidisciplinary nature of the project and the international scope of the field work, the cetacean study (Appendix A) was necessarily a collaborative one. The idea for this project has its roots in my master's degree study at the University of Michigan. My original idea was to apply this approach to some key problems in fish evolution, but in fact, my first opportunity to apply this approach came when my former colleague Hans Thewissen when he began his own field project on early whales in 1991. Together we realized that what I had in mind for fish would work equally well for cetaceans and so, when he began finding new whale specimens in Pakistan and India, we began a pilot project in the laboratory of Jim O'Neil, my geochemistry professor from the University of Michigan. When I began Ph.D. work at the University of Arizona, we decided to build on the pilot project and I continued my isotopic work in the laboratory of my dissertation advisor, Jay Quade. Jim O'Neil prepared several of the samples for the pilot project, but I have done the majority of the laboratory work myself.

The diagenesis study (Appendix B) was entirely my own effort, and one which I completed entirely in the lab of Jay Quade. The development of the CO₂-Ag₃PO₄ equilibration method (Appendix C), however, was also a collaborative effort with Jim O'Neil. In fact, it is a direct outgrowth of an earlier method I helped develop with Jim and two of his students (O'Neil et al., 1994). This first method involves the liberation of oxygen from Ag₃PO₄ at high temperatures (≥1200°C) and the subsequent conversion of that O₂ to CO₂. The method I describe here in Appendix C is based on the observation that the residue of the Ag₃PO₄ reaction continued to exchange its oxygen with CO₂. At
that time I wondered whether the \( \text{Ag}_3\text{PO}_4 \) itself might behave the same way the residue did. I reasoned that if it did, we should be able to develop a high-temperature analogue of the \( \text{CO}_2-\text{H}_2\text{O} \) equilibration method employed in the determination of the oxygen isotope composition of water samples.
PRESENT STUDY

The methods, results, and conclusions of this work are presented in the papers appended to this thesis. The following is a summary of the most important findings presented in these papers.

Summary of Appendix A: Early Evolution of Cetacean Osmoregulation

In the first part of my dissertation work (Appendix A), I employed the standard method for carbonate analysis (McCrea, 1950) and the method I helped develop for phosphate oxygen isotope analysis (O'Neil et al., 1994). I made isotopic analyses of the carbonate and phosphate of 10 species of modern cetaceans and 11 species of Eocene cetaceans. The modern specimens were all obtained from existing collections of the institutions listed Appendix A. The Eocene specimens from India were found and collected by Sunil Bajpai, Ashok Sahni, and Hans Thewissen. Taseer Hussain and Hans Thewissen led the teams which collected Eocene cetaceans in Pakistan; I participated in their 1996 expedition.

The purpose of the modern specimens was to provide an empirical test of the hypothesis that marine-freshwater differences in the oxygen and carbon isotope compositions of cetacean teeth and bones would be apparent and to determine the magnitude of these differences. Our analyses confirmed that these differences were indeed apparent in the teeth and bones of modern cetaceans. With the modern specimens as a frame of reference, we were then able to interpret the results we obtained for the Eocene specimens. Our comparison focused on relative difference rather than actual values because of potential climatic and environmental differences between the modern and Eocene habitats of the animals. We found that the difference between marine and freshwater $\delta^{18}O_p$ values was virtually identical. In contrast, the marine-freshwater
difference in $\delta^{13}C_{sc}$ values was actually more pronounced in the Eocene species than in the modern species, probably due to migration of the modern species.

The most important results of this study are as follows. First, we documented the water and dietary requirements of each of the species in our study and found that not only were there fully freshwater and fully marine species, but that some species and made use of more than one environment—something not evident from environmental or phylogenetic data. Second, we determined that fully marine cetaceans existed by the middle Eocene. And finally, we found that changes in water use and diet were decoupled at least in some species, an observation which helps to explain how cetaceans were able to differentiate so rapidly early in their history.

Summary of Appendix B: Recognizing Phosphate Diagenesis

As in the first paper, the methods I employed in the second component of my dissertation research were the standard method for carbonate analysis (McCrea, 1950) and the method I helped develop for phosphate analysis (O'Neil et al., 1994). All specimens in this study were modern; no fossils were included. The impetus for this study was the existence of conflicting bodies of work on the existence and significance of a correlation between the oxygen isotope composition of the phosphate ($\delta^{18}O_p$) and the oxygen isotope composition of structural carbonate ($\delta^{18}O_{sc}$) in a given animal's teeth and bones. Kolodny and Luz (1991), in a study of modern and fossil fishes, demonstrated that the $\delta^{18}O$ values of the structural carbonate and the phosphate are not well correlated in modern fishes, but are correlated in a number of fossils they analyzed. They inferred from this pattern that a correlation between these two variables indicated an isotopic resetting of both the phosphate and carbonate and proposed the existence of this correlation in fossils as a test of diagenesis. In contrast, both Bryant et al. (1996) and Iacumin et al. (1996) found a strong correlation between these two variables in their studies of
terrestrial mammalian taxa from around world. This led me to wonder whether there was a fundamental difference either between ectotherms and endotherms or between terrestrial and aquatic (freshwater or marine) animals that could explain the discrepancies between the results of Kolodny and Luz (1991) and the those of the other two groups, Bryant et al. (1996) and Lacumin et al. (1996).

To test this idea, I obtained a suite of samples representing 21 species of marine fish and aquatic mammals. This set of samples would allow me to test both possible explanations. I made oxygen isotope analyses of the phosphate ($\delta^{18}O_p$) and structural carbonate ($\delta^{18}O_{sc}$) and found a strong positive correlation ($r^2 = 0.76$) between $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ in both fishes and aquatic mammals. Moreover, the equation relating these two variables, $\delta^{18}O_p = 1.008 \delta^{18}O_{sc} - 9.34$, is in good agreement with the results obtained for terrestrial mammals by Bryant et al. (1996) and Lacumin et al. (1996). When these data are separated by taxonomic groups, the correlations remain generally high, but the slopes and intercepts of the equations vary. These differences in slope and intercept are probably due to physiological factors that affect the way in which oxygen is incorporated into teeth and bones. Regardless of these differences, the existence of correlations in both fishes and mammals refutes the claim of Kolodny and Luz (1991) that there is no correlation between these two variables in modern fishes. These data also illustrate that these differences are due not to physiological differences between ectotherms and endotherms, but to some other cause. Most importantly, the existence of such a correlation in fossil specimens therefore should be considered an indication not of isotopic alteration, but of preservation of the original isotopic composition of a given bone or tooth. Use of the $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ as a test of diagenetic alteration will nevertheless require better characterization of the numerical relations between $\delta^{18}O_p$ and $\delta^{18}O_{sc}$. Such characterization is likely to result in a better understanding of water and ion balance in modern aquatic mammals.
Summary of Appendix C: A new method for determining $\delta^{18}O_p$

Unlike the first two components of this dissertation, this effort did not involve the use of an existing method, but rather was designed to develop a new method for the oxygen isotope analysis of phosphates. The method I developed involves no chemical reaction, but instead consists of a high-temperature equilibrium exchange of oxygen isotopes between two phases, carbon dioxide ($CO_2$) gas and trisilver orthophosphate ($Ag_3PO_4$) in either the solid or liquid state. This method is a low-temperature analog of the conventional $CO_2$ -H$_2$O method used in the determination of the oxygen isotope composition of water. In the $CO_2$-H$_2$O equilibration method, $CO_2$ of a known quantity and known isotopic composition is equilibrated at approximately $25^\circ$C with H$_2$O of a known quantity but an unknown isotopic composition. In this method, the unknown is the isotopic composition of the water, which is determined by measuring the final oxygen isotope composition of the $CO_2$ and by applying the empirically determined equilibrium fractionation factor between $CO_2$ and H$_2$O at $25^\circ$C.

To test the viability of using a high-temperature version of this method with $CO_2$ and $Ag_3PO_4$, I prepared a batch of $Ag_3PO_4$ samples of approximately the same mass ($51 +/- 2$ mg) from the same source and loaded them into 6 millimeter Vycor tubes. I then placed the sample tubes on a vacuum line and transferred 20 to 35 micromole aliquots of $CO_2$ with one of two isotopic composition. Once I had sealed off the tubes, I heated each at a temperature between $725^\circ$C and $825^\circ$C for a different amount of time to determine the optimum conditions for the reaction.

The most important result of this work was the demonstration that equilibrium oxygen isotope exchange occurs between $CO_2$ and $Ag_3PO_4$—both when $Ag_3PO_4$ is in the solid phase and when it is in the liquid phase. I found that equilibrium can be reached in approximately 1.5 hours at temperatures as low as $725^\circ$C, making this method feasible for
routine analysis. Equilibration time may be decreased by keeping one end of the furnace open. Exchange too near the phase transition (the melting point of Ag$_3$PO$_4$ is 849°C) results in unstable, crossing-over behavior. The fractionation factor between CO$_2$ and Ag$_3$PO$_4$ at 725°C is approximately $5.65\%$. The advantages of this method are: (1) it is the simplest, fastest, and least expensive method available for the analysis of phosphate oxygen; (2) it requires no dangerous fluorinating agents; and (3) it can be at temperatures low enough to allow the analyses to made in Vycor (glass tubes) without complications due to exchange between the oxygen in the tube. As a result, equilibrated sample vessels can be scored and cracked directly into the inlet of a mass spectrometer.

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Isotopic approaches to understanding the terrestrial to marine transition of the earliest cetaceans

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1. ABSTRACT

The transition of the earliest cetaceans from terrestrial to marine animals involved not only the evolution of novel locomotory and acoustic systems, but required physiological innovations as well. The ability to dispose of excess ions acquired during the incidental or deliberate ingestion or uptake of seawater may have been prerequisite to the transoceanic dispersal of whales in the middle Eocene. Locomotory and acoustic adaptations are studied through the morphology of fossils, but physiological transitions often have no morphological correlates. Here we present an isotopic study of the physiological transition of the earliest whales, designed to determine when whales began to ingest seawater (either incidentally or deliberately) and to eat marine prey. Our approach involves the determination of oxygen isotope compositions of the phosphate (PO$_4^{3-}$) and the carbon isotope compositions of the carbonate (CO$_3^{2-}$) of the teeth and bones of modern and Eocene cetaceans. This comparative approach provides a otherwise unobtainable insights into a freshwater to marine physiological transition in the fossil record.

Changes in the carbon isotope compositions of the carbonate ($\delta^{13}C_{\text{car}}$) and the oxygen isotope compositions of the phosphate ($\delta^{18}O_{\text{ph}}$) from lower to higher values through time record the transition of early cetaceans to a fully marine life by the middle Eocene. Although this transition was rapid, it did not take the form of a simple progression. Rather, the fine structure of the transition was mosaic. The earliest cetaceans, *Ichthyolestes, Nalacetus*, and *Pakicetus*, from the early to middle Eocene lower Kuldana Formation of Pakistan, relied on either terrestrial food or low-$^{13}C$ marine food from the remnant Tethyan epicontinental sea. *Ambulocetus* and *Gandakasia*, from the geologically slightly younger upper Kuldana Formation, also relied on terrestrial or low-$^{13}C$ marine food, but appear to have been euryhaline, so that they fall into two categories simultaneously. *Attockicetus*, from the overlying middle Eocene Kohat Formation, has
much higher $\delta^{13}C_{sc}$ values than any of the cetaceans and almost certainly ate marine food, but continued to ingest primarily freshwater. All later cetaceans, with the possible exception of *Andrewsiphius* from the middle Eocene Harudi Formation of Kachchh, India, appear to have been fully marine. *Gaviacetus*, *Indocetus*, and *Remingtonocetus* from the Harudi Formation, and *Georgiacetus* from the middle Eocene McBean Formation of Georgia, all had $\delta^{13}C_{sc}$ and $\delta^{18}O_p$ values consistent with a marine diet and seawater ingestion.

A cetacean osmoregulatory system capable of handling the high concentrations of ions associated with life in the ocean had evolved by the middle Eocene. The evolution of this new osmoregulatory system was largely decoupled from the consumption of marine food. This decoupling allowed the apparent niche differentiation among early cetaceans, and helps to explain how the evolutionary diversification of cetaceans was able to occur so rapidly. In addition, although there is some uncertainty concerning the mechanisms by which modern cetaceans maintain osmotic balance, this decoupling implies that water ingested incidentally during feeding is not an important factor in determining the osmotic balance in modern cetaceans. Clarification of the mechanisms of modern cetacean osmoregulation is needed in order to interpret whether the evolutionary change we document isotopically represents (a) the evolution of seawater drinking behavior, (b) an increased metabolism of food and/or body fat to produce freshwater, (c) the evolution of more efficient kidneys, or (d) the evolution of more permeable skin.

2. INTRODUCTION

The fossil record is replete with examples of evolutionary transitions between marine and freshwater environments, in both directions. Perhaps the most striking and best documented example of such a transition is the evolution of cetaceans (whales, dolphins,
and porpoises) from the extinct group of terrestrial mammals called mesonychians. This transition, first hypothesized by Van Valen (1966), occurred in the temporally and geographically restricted setting of the Paleogene remnant Tethyan epicontinental sea (Gingerich et al., 1983) and adjacent terrestrial ecosystems. These environments lay in the zone of convergence between the Indian Plate and southern Eurasia during the early stages of the continent-continent collision that ultimately produced the Himalaya Mountains.

The evolution of cetaceans from terrestrial land-mammals included profound changes in many aspects of their anatomy and biology. Many of those changes, such as those that occurred in the locomotory and hearing systems, have been documented through studies of the morphology of fossils (e.g., Thewissen and Hussain, 1993; Gingerich et al., 1994; Thewissen et al., 1994). But the morphological transitions are only part of the story. In order to become fully marine and independent of freshwater, cetaceans had to evolve physiologically as well. They had to develop the ability to cope with the excess salt load associated with seawater ingested either voluntarily (e.g., as drinking water) or incidentally (e.g., in the course of eating), or with excess ions present in food, such as marine invertebrates, which generally do not regulate their osmotic pressures and may have ionic concentrations in excess of those in seawater (Schmidt-Nielsen, 1997).

Most modern cetaceans are fully marine and never enter freshwater, but there are some species which are known to enter brackish waters of low salinities (e.g., Phocoena phocoena; Andersen and Nielsen, 1983), and the four species of highly endangered freshwater river dolphins known as platanistoids are exclusively freshwater. Although the platanistoids are secondarily derived from marine species (Messenger, 1994; Muizon, 1994) and were not part of the original transition of cetaceans, they are important in the context of this study because they provide us with an appropriate modern freshwater group to compare with modern marine cetaceans.
When did the cetacean osmoregulatory system become able to handle the excess salt load associated with ingesting seawater and feeding in the oceans? The answer to this question is key to understanding the evolution of cetaceans, as this adaptation was probably prerequisite to the worldwide dispersal of cetaceans in the middle Eocene (e.g., Hulbert and Petkewich, 1991; Albright, 1996).

The stable isotope ratios of carbon ($^{13}$C/$^{12}$C) and oxygen ($^{18}$O/$^{16}$O) can provide such information, and changes in these ratios have the potential to elucidate the timing and nature of the transition of early cetaceans from terrestrial to marine mammals. Our goal is to answer several fundamental questions: (1) When in geologic time did cetaceans become fully marine? (2) Was the transition to marine life a gradual or abrupt one? (3) Was the transition to seawater ingestion synchronous with the transition to a marine diet or did one precede the other? (4) To what extent did early whales differentiate ecologically?

Our approach involves five components, phosphate oxygen isotope analyses of the teeth and bones of (1) modern cetaceans and (2) Eocene cetaceans; carbon isotope analyses of structural carbonate from the teeth and bones of (3) modern and (4) Eocene cetaceans; and (5) an assessment of the degree to which diagenesis may have affected our isotopic results.

3. BACKGROUND

3.1 Ion and Water Balance in Modern Cetaceans

3.1.1 Overview

To understand the terrestrial to marine transition of the earliest cetaceans, one must first appreciate how osmoregulation works in modern cetaceans and how it relates to the oxygen isotope composition of cetacean teeth and bones. Mammals, including those that are aquatic, lose water through respiration, skin evaporation, urination, defecation,
lacrimation, and lactation (Bentley 1971; Ridgway, 1972), and most drink freshwater to replace this loss. Some mammalian species have kidneys which can rid the body of salt and urea by producing urine more concentrated than their body fluids, but most mammals are not able to concentrate their urine very strongly. Many, including humans, die if they drink only sea water because their kidneys are unable to concentrate the ions sufficiently and too much water is lost (Schmidt-Nielsen, 1997). As a result, most mammals avoid drinking seawater and can not survive without freshwater (Schmidt-Nielsen, 1964).

It is unclear how the osmoregulatory system of marine cetaceans handles ingested seawater.

Several mechanisms involved in maintaining osmotic balance have been identified: (1) osmotically-driven transfer of water but not ions across the integument (= percutaneous or transcutaneous flux; Hui, 1981; Andersen and Nielsen, 1983); (2) active drinking (Telfer et al. 1970); (3) metabolism of food and/or body fat (Bentley, 1963) to produce fresh water; and (4) concentration of ions in urine to conserve water (Ridgway, 1972). In addition, water conservation may also be aided by concentration of lipids in milk during lactation (reviewed in Schmidt-Nielsen, 1997), lower respiratory rates, and undersaturation of water in expired air (Kirschner, 1991).

Kohn (1996) modeled the oxygen isotope systematics of cetaceans as being dominated by the transcutaneous flux of water. Using the results of Hui (1981), Andersen and Nielsen (1983) and Sokolov et al. (1994), he calculated that the transcutaneous flux constituted 98% of the total water flux. On this basis, he suggested that the phosphate oxygen isotope compositions of cetacean teeth and bones should record the isotopic composition of the water in which the animals live (Kohn, 1996). If the transcutaneous flux dominates the water fluxes in all cetaceans, measurements of the oxygen isotope compositions of cetacean teeth and bone phosphate would not reflect drinking water, diet and species-specific physiological variables as they do in terrestrial
animals (Bryant and Froelich, 1995; Kohn, 1996; Kohn et al. 1996), but the environment in which the animals live (Kohn, 1996). But is this true of all cetaceans?

Until recently, it was thought that cetacean skin was impermeable to both solutes and water (Ridgway, 1972; see review in Kirschner, 1991), in part because sweating does not work underwater (Ridgway, 1972). Recent general physiology texts (e.g., Schmidt-Nielsen, 1997) refer to cetaceans and other higher marine vertebrates as more similar to terrestrial mammals than to fish, because, with respect to salt and water balance, they are physiologically isolated from the surrounding sea water. The results of some recent studies (Hui, 1981; Andersen and Nielsen, 1983; Sokolov et al., 1994), however, suggest that this assumption of physiological isolation may be at least partially incorrect. Hui (1981) demonstrated experimentally that cetacean skin is impermeable to sodium, but permeable to water, which will flow into the animal when the osmotic pressure inside the animal is high enough to create the necessary gradient. Andersen and Nielsen (1983) demonstrated that the flow of water could occur in both directions, depending on the ionic concentrations of the external aqueous environment. The results of these experiments stand in contrast to the results of other experiments which seemed to indicate more important roles for drinking (Telfer et al., 1970), urine concentration (Ridgway, 1972) and metabolism of food and body fat (Bentley, 1963; Telfer et al., 1970). In most of these earlier experiments, however, skin impermeability was either implicitly (e.g., Bentley, 1963) or explicitly (Fetcher and Fetcher, 1942; Ridgway, 1972) assumed and was therefore not included in calculations of the other fluxes. Only Telfer et al. (1970) attempted a direct test of permeability, but their test involved the monitoring of a solute--radioactive $^{24}$Na--rather than the water itself. Given Hui's (1980) demonstration that cetacean skin is permeable to water but not to sodium, Telfer et al.'s (1970) test was not adequate to understand what is happening to the water, only to the solutes, and in this regard, is quite consistent with Hui's (1980) results. The importance of drinking,
metabolic water, and the concentration of ions in the urine, may therefore need to re-evaluated.

3.1.2 Implications for understanding cetacean $\delta^{18}O_p$

Understanding which processes exert control over the osmotic balance in modern cetaceans is important for studies such as this one, because if the transcutaneous flux really dominates in modern cetaceans, it would be reasonable to infer that the first appearance of marine isotope signatures in cetacean teeth and bones would reflect evolution of a permeable integument. If, on the other hand, drinking dominates the maintenance of water and ion balances in modern cetaceans, then the same isotopic evidence would be interpretable as the initiation of seawater drinking by cetaceans. Similar arguments could be made for kidney evolution, and other possible mechanisms.

In spite of the uncertainty regarding the mechanisms of osmoregulation in modern cetaceans, we can still establish when cetaceans became independent of freshwater and able to eat marine food. We accomplish this by determining empirically the nature and magnitude of oxygen and carbon isotope differences between modern freshwater and marine cetaceans. These measurements provide a framework for interpreting isotopic analyses of fossil species. The basis of these measurements in modern systems follows next.

3.2 Oxygen Isotope Variations in Marine and Fresh Waters

Oxygen isotope ratios ($^{18}O/^{16}O$) of marine and fresh waters are different as a result of the kinetic and equilibrium fractionations that result from the physical processes of evaporation and condensation operative in the hydrologic cycle (e.g., Epstein and Mayeda, 1953; Craig, 1961a). For example, $H_2^{16}O$ molecules preferentially evaporate (move from the liquid into the vapor phase) and $H_2^{18}O$ molecules preferentially condense
out as precipitation. Evaporation is a kinetic process, and the water vapor produced by evaporation from the ocean has been found to be more depleted in $^{18}$O than would be the case if the vapor formed in isotopic equilibrium with ocean water (Craig and Gordon, 1965). In spite of the kinetic nature of this fractionation, the oxygen isotope compositions of water vapors formed over ocean waters have been found to be consistently 11 to 14‰ lower than those of the oceans (Craig and Gordon, 1965), with the exact fractionation dependent on the humidity and isotopic composition of water vapor already in the air at the time (Gat, 1981). As this vapor in the air mass moves inland, it progressively condenses out, thereby causing the clouds to become further depleted in $^{18}$O (Epstein and Mayeda, 1953; Fig. 1). As the clouds become progressively more depleted in $^{18}$O, the precipitation becomes more depleted in $^{18}$O as well. In this way, the physical processes of the hydrologic cycle produce an isotopic difference between the oceans and fresh waters, such that fresh waters have lower ratios of $^{18}$O/$^{16}$O than do the oceans. This difference is expressed in $\delta^{18}$O values, where delta (δ) represents the deviation, in parts per mil (‰), of the $^{18}$O/$^{16}$O of the sample from that of Standard Mean Ocean Water (SMOW). Samples with ratios of $^{18}$O/$^{16}$O that are lower than those of seawater have lower (more negative) $\delta^{18}$O values (see Methods section). The magnitude of this fractionation is partly a function of temperature, but the isotopic composition of any given body of freshwater also depends on the proportion of the clouds that have condensed out as precipitation by that point, how much evaporation has occurred, and what mixture of different waters and transport between phases has occurred (Whelan, 1987). In addition, the same progressive fractionation that causes $\delta^{18}$O values to decrease as one moves farther inland, also produce results in decreasing $\delta^{18}$O values as one moves to higher elevations and higher latitudes (Craig, 1961a; Dansgaard, 1964).
In this study, latitudinal variation is not a problem because the species which are most
critical to distinguish as marine or freshwater lived in relatively close proximity to each
other in, or around the margin of, the remnant Tethys. These early and middle Eocene
deposits of the remnant Tethys all occur today at latitudes of 33°40' +/-10', and although
their latitude may have been slightly different during the Eocene, their proximity to each
other changed only slightly (on the order of a few kilometers) as a result of folding and
faulting in the region. Our other samples--*Andrewsiphius, Gaviacetus, Indocetus,* and
*Remingtonocetus* from Kachchh, India (23°30' +/- 10' N latitude) and *Georgiacetus,* from
Burke County, Georgia; approximately 31° N latitude)--occurred in pelagic, open marine
settings which are more readily distinguishable from terrestrial environments.

Also, although we do not know the Eocene elevations of the land surrounding the
remnant Tethys, significant elevation would only have increased the isotopic difference
between the local fresh waters and the Tethys, by adding water depleted in $^{18}$O from
precipitation at higher elevations. As a result, our lack of knowledge of paleoelevations
is not a problem.

How do we determine what magnitude of difference is indicative of a difference
between marine and fresh waters? Although this difference depends in part on how far
inland the meteoric water condensed out of the vapor phase, we can determine a
minimum magnitude of the marine-freshwater difference by considering known
fractionation factors. The mean $\delta^{18}$O value of vapor forming over the ocean at 25°C is
-13%, or 13% more negative than Standard Mean Ocean Water (SMOW) (Fig. 1); the
$\delta^{18}$O value of the rain forming from that vapor would be 10% more positive or -3%
(SMOW). The net result is that fresh waters consisting of pure, unevaporated rainwater
would be about 3% more negative than the ocean (Fig. 1). Precipitation forming farther
inland (or groundwater) would have lower $\delta^{18}$O values, and could lower the values of
freshwater near the coast by mixing. A river near the coast would thus have a $\delta^{18}$O value
of ≤ -3‰ if no evaporation takes place. Evaporation of water would increase the \( \delta^{18}O \) value. At lower temperatures, the magnitudes of the differences would be slightly greater; at higher temperatures, they would be slightly less (for equilibrium fractionation factors between liquid water and water vapor at these temperatures, see Friedman and O'Neil, 1977).

An empirical test of this three per mil difference is a comparison with data on the isotopic composition of meteoric waters from small islands or stations very near to the ocean, where only one cycle of evaporation and precipitation is possible. One such site is the station of Malan, South Africa, which is located at 33°97′ S latitude, and receives rain with a mean \( \delta^{18}O \) value of approximately -3.5‰ (SMOW), at a mean annual temperature of 15.9°C (International Atomic Energy Agency, 1986). The average elevation of Malan is 44 meters above sea level, which is too low to produce any detectable fractionation in the precipitation. Similarly, meteoric water falling on Midway Island, which is located at 28°22′ N latitude and has an average elevation of 13 meters above sea level, has an average \( \delta^{18}O \) value of -1.81 (International Atomic Energy Agency, 1986).

Our strategy, therefore, is to exploit the persistence through geologic time of the hydrologic cycle (Kolodny and Luz, 1991), in order to document the habitat use (marine versus freshwater) of cetaceans in the fossil record. To do this, we need a proxy record of the water of the animals’ habitats. This record exists in teeth and bones, whose oxygen isotope compositions are linearly related to the oxygen isotope composition of the water ingested, which is in turn related to the oxygen isotope composition of the environmental water in which an animal lives or which an animal primarily consumes (Longinelli and Nuti, 1973a, b; Kolodny et al., 1983; Bryant and Froelich, 1995).

The precise nature of that linear relation varies with diet and physiology (Kohn, 1996; Kohn et al., 1996), and possibly body size (Bryant and Froelich, 1995). The body water
of most terrestrial mammals is derived primarily from the water ingested directly as drinking water (Luz et al., 1984; Luz and Kolodny, 1985; Luz and Kolodny, 1989; Bryant and Froelich, 1995). Contributions from other sources of oxygen, such as food and atmospheric oxygen, are generally small compared to that from drinking water (Luz et al., 1984; Bryant and Froelich, 1995). Some notable exceptions occur in arid environments, however, where animals must get much of their water from their food (Schmidt-Nielsen, 1964). Where an animal's food consists largely of leaves, from which substantial evaporation takes place, the oxygen isotope composition of an animal's teeth and bones reflects relative humidity (Ayliffe and Chivas, 1990; Luz et al., 1990). There may also be species-specific physiological effects which influence the isotopic composition of the body water and therefore of teeth and bones (Kohn et al., 1996).

How is the oxygen isotope composition of environmental water related to the oxygen isotope composition of the phosphate (PO$_4^{3-}$) in the teeth and bones of marine and freshwater cetaceans? Kohn (1996) inferred that the rapid, transcutaneous (percutaneous) exchange of water documented in several cetacean species by Hui (1981), Andersen and Nielsen (1983) and Sokolov et al. (1994) dominates all other oxygen fluxes, and concluded that oxygen isotope compositions of body water should track the isotopic composition of ambient water perfectly. An implicit assumption of this model is that the gradient(s) needed to drive that flux, which is osmotic in nature, exist continuously throughout the life of the animals, which may not be the case.

In spite of this and other possible complications, there is abundant empirical evidence of an isotopic difference between marine and freshwater cetaceans. Yoshida and Miyazaki (1991) and Barrick et al. (1992) presented evidence of a linear relation between the oxygen isotope composition of environmental water and the oxygen isotope composition of the teeth and bones of modern cetaceans. Yoshida and Miyazaki (1991) determined the oxygen isotope compositions of both marine and freshwater cetaceans and
found a 3-7‰ difference between the δ18O values of freshwater and marine species. The presence of a 3‰ minimum difference is in good agreement with our minimum estimate of the difference between marine and fresh waters based on hydrologic data and is further borne out by our recent analyses (Thewissen et al., 1996b), which yielded a 2‰ minimum difference between both modern and fossil cetaceans. We therefore employ analyses of the oxygen isotope compositions of the phosphate in the teeth and bones of the earliest cetaceans to determine which of these animals ingested freshwater and which ingested seawater.

3.3. Carbon Isotope Ratios (13C/12C) of Marine and Fresh Waters

Like the oxygen isotope compositions of natural waters, the carbon isotope compositions of both the organic and inorganic constituents of natural fresh waters are depleted in the heavier isotope—in this case 13C—relative to the corresponding substances in the oceans (Clayton and Degens, 1959; Fig. 2). This difference is recorded in the carbon isotope compositions of both biogenic and abiogenic carbonates (Clayton and Degens, 1959; Keith et al., 1964; Allen and Keith, 1965; Keith and Parker, 1965). Consequently, δ13C values of carbonate phases (δ13Csc) are useful in distinguishing marine and freshwater environments and organisms (Keith et al., 1964; Allen and Keith, 1965; see review in Fry and Sherr, 1984).

The teeth and bones of vertebrates contain structural carbonate and the carbon isotope composition of this carbonate has been demonstrated to reflect the carbon isotope composition of an animal's diet in terrestrial animals, with a 12‰ offset (e.g., Lee-Thorp and van der Merwe, 1987; Quade et al., 1992). A relation between the carbon isotope composition of tooth and bone carbonate and diet has not been demonstrated in aquatic vertebrates, but that between diet and bone collagen of aquatic vertebrates has (DeNiro and Epstein, 1978; Schoeninger and DeNiro, 1984; Ames et al., 1996; Hobson et al.,
In modern ecosystems, carbon isotope differences between marine and freshwater animals are evident in analyses of the soft tissues (Schoeninger and DeNiro, 1984; Keegan and DeNiro, 1988; Little and Schoeninger, 1995; Smith et al., 1996), although overlap of the carbon isotope compositions of groups such as sea grasses and terrestrial C₄ plants must be taken into account (Keegan and DeNiro, 1988; Little and Schoeninger, 1995).

Here we employ analyses of the carbon isotope compositions of the structural carbonate in the teeth and bones of the earliest cetaceans to determine which of these animals ate marine and which ate freshwater or terrestrial food. We begin by making analyses of the structural carbonate of the teeth and bones of modern cetaceans in order to test empirically the existence of a difference between the carbon isotope composition of the tooth and bone carbonate of marine and freshwater animals. These analyses of modern cetacean tooth carbonate provide the framework for evaluating the results of our analyses of the carbon isotope compositions of fossil cetaceans. In addition, although the first fossil and isotopic evidence of C₄ plants does not appear until the Miocene, we analyzed pedogenic carbonates and the teeth and bones of herbivores in order to check for the presence of C₄ plants in the Eocene Tethyan systems.

4. MATERIALS AND METHODS

4.1 Samples

4.1.1 Modern Cetaceans

Our aim was to include as broad a sampling as possible of modern freshwater and marine cetaceans, with a range of body sizes, locations and diet. The modern cetaceans represented in our sample varied in typical body weight from 35 kg (the Tucuxi dolphin, Sotalia) to 40,000 kg (the sperm whale, Physeter), thus spanning nearly the full range of cetacean body sizes and most of the range of mammalian body sizes considered by
Bryant and Froelich (1995). Latitudinally, our specimens spanned temperate (*Phocoena*) to tropical (*Inia*) latitudes. Their diets varied from primarily invertebrates (*Physeter*) to predominantly fish (*Tursiops*), and a combination of fish and amniote prey (*Orcinus*).

Specimens of modern cetaceans were supplied by several institutions, including the American Museum of Natural History (AMNH; specimen abbreviation AMNH-M), the National Museum of Natural History (Smithsonian; specimen abbreviation USNM), and the National Oceanic and Atmospheric Administration's National Marine Fisheries Service (NMFS; specimen abbreviations WEE and FKB) laboratory in La Jolla, California. Because the methods we used to determine oxygen and carbon isotope ratios are destructive, we analyzed specimens of more common, less endangered species whenever possible. Specimens of some of the more common species we analyzed were not catalogued in regular museum collections and lacked provenance data. In sampling species that are found in both freshwater and seawater, however, we analyzed only specimens with good provenance data. Our specimen of *Sotalia* (USNM 571461), a genus that sometimes enters fresh waters, was collected in the sea off the coast of Trinidad. Similarly, we analyzed samples of freshwater dolphins of known provenance. These included one specimen of *Platanista gangetica* (USNM uncatalogued specimen from the Ganges River); three of *Inia geoffrensis* (WEE 069 from the Orinoco River; USNM 396166 from the San Fernando River, Venezuela; and USNM 406801 from near San Juan, Venezuela); and one of *Lipotes vexilfer* (AMNH-M 57333) from Tung Ting Lake in Hunan Province in the People's Republic of China. All of our modern specimens were teeth, except for our sample of the rare Yangtze River dolphin *Lipotes*, of which only a rib fragment was available.
4.1.2 Eocene Cetaceans

We analyzed fossil cetacean samples from the early Eocene Kuldana and middle Eocene Kohat Formations of Punjab, northern Pakistan, the middle Eocene Harudi Formation of Kachchh in western India, and the late middle Eocene McBean Formation of Georgia, U.S.A. Details of the stratigraphy and locality maps are provided by Williams (this volume). Cetaceans from the lower Kuldana Formation included *Ichthyolestes*, *Pakicetus*, and *Nalacetus*, all from a single locality in the Kala Chitta Hills of Punjab, Pakistan (Thewissen and Hussain, in press). This locality (H-GSP Locality 62, West and Lukacs, 1979), has recently been interpreted by Aslan and Thewissen (1996) as a freshwater channel deposit that may be similar depositionally to the Barbora Banda locality described by Wells (1983). The associated fauna consists exclusively of terrestrial and freshwater vertebrates and planorbid gastropods.

Our cetacean specimens from the upper Kuldana Formation included the holotype of *Ambulocetus natans* (HGSP-18507; H-GSP Locality 9209), and several specimens referred to that species (H-GSP Locality 9207). H-GSP localities 9207 and 9209 are stratigraphically continuous along strike and are approximately 90 meters higher in the section than H-GSP Locality 62 and approximately three kilometers away (Thewissen et al., 1996a) from the latter. The depositional environment represented by localities 9207 and 9209 was near-shore marine (Wells, 1984; Thewissen et al., 1996a). The non-cetacean taxa (pycnodontid fish, oysters and crabs) found at these localities are consistent with this environmental interpretation.

Our samples from the overlying Kohat Formation included a single specimen of *Attockicetus* (Thewissen and Hussain, in press), the first whale recovered from that formation. This specimen includes the dorsal part of the skull with a few intact teeth.

Our specimens of middle Eocene (Lutetian) fossils from northern India come from the Rato Nala locality of the Harudi Formation of Kachchh District, Gujarat State, northern
India. We have analyzed Andrewsius, Gaviacetus, Indocetus, and Remingtonocetus (Sahni and Mishra, 1975; Kumar and Sahni, 1986; Bajpai and Thewissen, this volume).

From the U.S.A., we analyzed a tooth of the recently discovered protocetid whale Georgiacetus Hulbert 1995 from the middle Eocene McBean Formation of Burke County, Georgia (Hulbert and Petkewich, 1991; Hulbert, this volume).

4.2 Analytical Methods

Wherever possible, the enamel and dentine of fossil teeth were separated using a dental drill. Specimens that were too fragmentary to allow such separation are listed in the table as enamel and dentine (e,d or d,e depending on the relative amounts of each). Most modern cetacean teeth have very thin (~1mm) tooth enamel and/or are relatively small (e.g., the teeth of Phocoena). Because these teeth yield such small amounts of enamel, and diagenesis is not a concern when analyzing modern specimens, we analyzed most of the modern teeth whole.

Following separation, where applicable, samples were ground in a mortar and pestle, treated with approximately 3% sodium hypochlorite (NaOCl) to oxidize organic matter, and rinsed five times with de-ionized distilled water. Samples being prepared for phosphate analyses were then placed in a drying oven at approximately 80°C and, once dry, reground to a fine powder. Fossil samples that were used in carbonate analyses were subjected to a 1M acetic acid (CH₃COOH (aq)) treatment to remove secondary, non-structural carbonate, following the suggestion of Lee-Thorp and van der Merwe (1991) that this step solves the problem of diagenetic overprinting documented, for example, by Nelson et al. (1986). After the acetic acid leaching step, samples were again rinsed five times with de-ionized distilled water to ensure the removal of any acetate residue. Omitting this step results in the liberation of isotopically light CO₂ during the phosphoric reaction step (J. Quade, unpubl. data).
Diagenesis is a concern when analyzing fossils, especially in settings where the depositional environments have repeatedly alternated between marine and freshwater through time, as happened during the Paleogene in the remnant Tethyan region of South Asia. It is generally agreed that tooth enamel is the best hard tissue to analyze because it is generally more densely crystalline and more resistant to alteration than are dentine, cementum and bone (Lee-Thorp and van der Merwe, 1991; Ayliffe et al., 1992, 1994; Koch et al., 1994). For this reason, we analyzed tooth enamel wherever possible, but as some specimens of interest did not have tooth enamel, we chose not to limit ourselves to analyses of enamel in order to sample as many taxa as possible. To ascertain that our analyses of dentine and bone were meaningful, we made comparative analyses of bone, dentine and enamel from several specimens to determine how our results would change if we used dentine and/or bone instead of enamel. Because we did not have adequate amounts of bone, dentine and enamel from the cetaceans from the lower Kuldana Formation, we analyzed instead two anthracobunid specimens (H-GSP 83-31p and H-GSP 96214) and two brontothere specimens (H-GSP 18478 and H-GSP 96034). In addition, as a test of our pretreatment steps, we made analyses of treated and untreated aliquots of several samples and compared their isotopic compositions.

In order to determine whether the phosphate oxygen had been altered, we made analyses of the crystallinity of our specimens using Fourier Transform Infrared Spectroscopy (FTIR) according to the method described by Shemesh (1990). We discovered, however, that this method is highly sensitive to the presence of adsorbed water, which causes crystals to clump together and makes the sample appear less crystalline (= have a lower crystallinity index, or C.I.). Progressive removal of adsorbed water under vacuum increased the crystallinity of our samples, from below Shemesh's (1990) crystallinity index threshold of 4.0 to above it. Unfortunately, without a way of
standardizing for the amount of adsorbed water, we are unable to relate our results to Shemesh's (1990) crystallinity index.

Extractions of the carbon and oxygen in the carbonate (CO$_3^{2-}$) component of the teeth and bones of our samples were performed using the standard phosphoric acid (H$_3$PO$_4$ (aq)) reaction method of McCrea (1950) at 50°C, until the reactions ceased, and no further CO$_2$ bubbles were produced. Carbonate standards (IAEA-C1, Carrara Marble and NBS-18, carbonatite) reacted completely within 30 minutes. Tooth enamel samples generally required a longer reaction time of three hours or more, but extensive regrinding of the tooth samples after pretreatments and prior to loading lessened their reaction times considerably. Oxygen isotope analyses of the phosphate (PO$_4^{3-}$) component were made using the trisilver phosphate (Ag$_3$PO$_4$) thermal decomposition method of O'Neil et al. (1994) at 1200°C. The end product of both the carbonate and phosphate extraction methods is CO$_2$, which we analyzed on Finnigan MAT Delta-S series 251 gas source mass spectrometers in the Stable Isotope Laboratories at the University of Arizona and the University of Michigan. Analytical precision of the mass spectrometric analyses of our samples was +/-0.06‰ or better for large (>50 µmol) samples; smaller samples had analytical uncertainties better than or equal to +/-0.10‰.

We report our isotopic results in the standard delta (δ) notation as the deviation, in parts per mil (‰), of the isotopic ratios of the sample from those of the PDB and SMOW standards for carbon and oxygen respectively, where δ = [(R$_{sample}$-R$_{standard}$)/R$_{standard}$] * 1000 and R = $^{18}$O/$^{16}$O or $^{13}$C/$^{12}$C. Wherever possible, we made duplicate analyses of specimens. Intra-specimen variation observed in duplicate analyses was generally less than 2‰. Where intra-specimen variation was greater than 2‰, the range of values closely matched the inter-specimen (=inter-individual) range of values, suggesting that this variation is a primary feature. For this reason, we have chosen not to treat the intra-sample and intra-taxon variation of the samples statistically, in recognition of the
possibility that this variation may represent the movement of the organisms between environments. In such a case, the ranges of delta values, rather than any measure of central tendency, will be the key to understanding the biology of these extinct species. We therefore present our data as ranges of values for each taxon, rather than as means with associated standard errors or standard deviations.

5. RESULTS

5.1 Oxygen Isotope Compositions (δ¹⁸O<sub>p</sub>) of Tooth and Bone Phosphate

5.1.1. Modern cetaceans

Phosphate δ¹⁸O values (δ¹⁸O<sub>p</sub>) obtained for the modern cetaceans (Table 1; Fig. 3a) range from +10.8‰ to +19.6‰ (SMOW), with a 2.4‰ gap between the ranges of the freshwater and marine species. The freshwater cetaceans have values in the range +10.8 to +15.7‰ and the marine cetaceans have values between +18.1 and +19.6‰. These results are very similar to those obtained by Yoshida and Miyazaki (1991), whose measurements of the δ¹⁸O<sub>p</sub> values of freshwater cetaceans ranged from +11.1 to +13.3‰, while those of the marine cetaceans they analyzed ranged from +16.7 to +18.6‰, with a 3.4‰ gap between the freshwater and marine ranges. Similarly, Barrick et al. (1992) analyzed seven specimens (representing four species) of modern marine cetaceans and obtained a range of δ¹⁸O values of 18.3 to 19.9‰ (SMOW), in excellent agreement with our measurements.

5.1.2. Eocene cetaceans

The Eocene fossil cetaceans we analyzed had δ¹⁸O<sub>p</sub> values spanning a range almost identical to that of the modern species (Table 2; Fig. 3b). Two of the three cetaceans we analyzed from the lower Kuldana Formation, *Nalacetus* (n=3) and *Pakicetus* (n=7) had
\( \delta^{18}O_{p} \) values between +15.0 to +16.6\%o; the third cetacean from the lower Kuldana, *Ichthyolestes* (n=3), had a slightly larger range of values, from +13.8 to +16.3\%o. Some of these values (4 out of 10) fall in the range of values of modern freshwater cetaceans, but slightly more (6 out of 10) were higher. In contrast, *Ambulocetus* and *Gandakasia* from the upper Kuldana Formation had a wide range of \( \delta^{18}O_{p} \) values of the modern cetaceans, overlapping modern freshwater and marine values. *Ambulocetus* (n=13) \( \delta^{18}O_{p} \) values ranged from +13.0 to +20.1\%o; *Gandakasia* (n=5), which is also an ambulocetid (Thewissen et al., 1996a) had a narrower range of \( \delta^{18}O_{p} \) values, from +14.0 to +17.9, but that may reflect a smaller sample size.

The Kohat Formation is the only pelagic marine unit in Pakistan from which we have cetaceans. One cetacean, *Attockicetus* (a remingtonocetid), has been collected from this unit. This specimen had a \( \delta^{18}O_{p} \) value of +16.6\%o, at the high end of the range of values of *Pakicetus* from the lower Kuldana Formation.

*Gaviacetus, Indocetus,* and *Remingtonocetus* from the pelagic marine limestone of the Harudi Formation in western India had \( \delta^{18}O_{p} \) values between +18.2 and +21.8\%o. These values either overlap or are more positive than the \( \delta^{18}O_{p} \) values of the modern marine cetaceans. They are also 1.5 to 5.2\%o higher than the \( \delta^{18}O_{p} \) values of the cetaceans from the lower Kuldana Formation.

### 5.2 Carbon isotope Compositions (\( \delta^{13}C_{sc} \)) of Tooth and Bone Carbonate

#### 5.2.1. Modern cetaceans

Our carbon isotope analyses of the structural carbonate (\( \delta^{13}C_{sc} \) values) of modern cetacean teeth and bone (Table 1; Fig. 4a) demonstrate that there is a difference in the carbon isotope composition of freshwater and marine cetaceans. The modern freshwater cetaceans have \( \delta^{13}C_{sc} \) values between -17.1 and -11.6\%o (PDB), whereas, with one
exception, all the modern marine cetaceans primarily had $\delta^{13}C_{sc}$ values between -10.9 and -7.8‰. The exception is the $\delta^{13}C_{sc}$ value of -13‰ (PDB) we obtained for a specimen of the sperm whale, *Physeter*. A second specimen of *Physeter* had a much lower $\delta^{13}C_{sc}$ value of -8‰, which is within the range of $\delta^{13}C_{sc}$ values of all the other marine cetacean species we analyzed. One possible explanation for this difference in values between the two individuals of *Physeter* is migration. It is possible that the specimen of *Physeter* with the more negative value migrated into and fed on giant squid (the primary component of sperm whale diets) that were feeding in food webs based on plankton with more negative $\delta^{13}C_{sc}$ values. It is known that the carbon isotope composition of plankton in the oceans varies on the order of 5‰ (e.g., Rau et al., 1982), and the magnitude of this difference is essentially identical to the magnitude of the range of values of our specimens. The plankton with the most negative $\delta^{13}C$ values is found at the highest latitudes, although there is no single correlation between latitude and plankton $\delta^{13}C$ that applies to all oceans (Rau et al., 1982). We therefore conclude that, although there is some overlap in the ranges of $\delta^{13}C_{sc}$ values between freshwater and marine cetacean species, the general pattern, for cetaceans that do not migrate long distances, is that marine species have higher $\delta^{13}C_{sc}$ values than freshwater species.

5.2.2. Eocene Cetaceans

A difference between the earliest cetaceans from the Kuldana Formation of Pakistan and those from the Kohat, Harudi and McBean Formations and the slightly younger cetaceans from (Table 2; Fig. 4b) is apparent in the carbon isotope composition of the structural carbonate of these animals. The earliest whales, *Ichthyolestes*, *Nalacetus*, and *Pakicetus*, from the lower Kuldana Formation, had $\delta^{13}C_{sc}$ values between -14 and -12‰ (PDB), in the range of modern freshwater cetaceans. The mesonychian had a higher
δ¹³C_sc value of -10.1‰, which is similar to the values obtained for Eocene herbivores from the Kuldana lower Formation (to be published elsewhere). That value also overlaps with the range of values obtained for marine species, but the available environmental and phylogenetic evidence suggests that it is unlikely that mesonychians consumed a marine diet. The upper Kuldana cetaceans, *Ambulocetus* and *Gandakasia* had very similar values. *Gandakasia* specimens (n=4) had values between -14.0 and -11.9‰; *Ambulocetus* specimens (n=9) had values between -14.2 and -10.6‰, with most (7 out of 9) of these falling in the -14.2 to between -12.0‰ range, essentially identical to the cetaceans from the lower Kuldana.

In striking contrast to the Kuldana cetaceans, *Attockicetus* from the Kohat Formation, three of the four cetaceans from the Harudi Formation—*Gaviacetus*, *Indocetus*, and *Remingtonocetus*—and *Georgiacetus* from the McBean Formation, all have δ¹³C_sc values between -10.7 and -5.2‰, very similar to the range of values we obtained for modern marine cetaceans. The fourth cetacean from the Harudi Formation, *Andrewsiphius*, had a δ¹³C_sc value of -13.4‰ (n=2)—a value very similar to those of the Kuldana cetaceans.

5.3 Diagenetic Assessment of Bone, Dentine and Enamel δ¹³C_sc

Our comparison of bone, dentine and enamel (Fig. 5) reveals differences in the isotopic composition of the three phases for most specimens. Several important patterns are evident in this comparison. (1) First, the differences among the phases are not all in the same direction, but where the values of dentine are more than 1‰ different from bone or enamel, the dentine δ¹³C_sc values are higher (*Gandakasia*, both *Ambulocetus* specimens, *Attockicetus*, and *Georgiacetus*) and bone δ¹³C_sc values are closer to those obtained from enamel samples, a result similar to that of Koch et al. (1994). (2) Second, the greatest variation among bone, dentine and enamel δ¹³C_sc values occurs in *Ambulocetus* and *Gandakasia* specimens from the upper Kuldana Formation. This
variation may be due to diagenetic alteration in fluctuating environments or to real biological variation, and requires further investigation. (3) Finally, regardless of whether one compares bone, dentine or enamel, the relative differences among the lower Kuldana specimens, upper Kuldana, Kohat, Harudi and McBean specimens are apparent.

6. DISCUSSION

Potential differences in temperature and isotopic composition between the Eocene and modern oceans and rivers led us to expect that the actual $\delta^{18}O_p$ and $\delta^{13}C_{sc}$ values of the Eocene cetaceans might be quite different from the values of the modern species, and for this reason, we focus on relative differences within and between faunas rather than actual values. Nevertheless, not only does the 2-3$\%$ difference in $\delta^{18}O_p$ between putatively marine and freshwater Eocene cetaceans match the difference between modern marine and freshwater cetaceans, but most of the actual values of the fossils overlap the ranges of $\delta^{18}O_p$ values of the modern specimens (Figs. 3a and 3b). The notable exception to this pattern is Georciacetus, which had a $\delta^{18}O_p$ value of 23.3$\%$. As Georciacetus is the geologically youngest of the cetaceans we analyzed, it is tempting to explain this high value in terms of the ocean cooling recorded by foraminifera in the middle and late Eocene (Zachos et al., 1994). This observation runs counter to the expectation that mammalian teeth should not record changes in temperature because they are thought to form in equilibrium with body water at a nearly constant temperature. Nevertheless, Barrick et al., (1992) also found a temporal trend in their cetacean fossils that paralleled the foraminiferal record. One possible explanation for this correlation in both studies is that because teeth are more exposed to the external medium than are bones and because cetaceans are nearly continuously submerged in that medium, teeth may record more environmental changes than has previously been expected. One way to test this
hypothesis would be to measure the $\delta^{18}O$ values of teeth and bones throughout the bodies of modern cetaceans, over their full range of body sizes.

Similarly, the $\delta^{13}C_{sc}$ values of our Eocene cetaceans overlap those of our modern freshwater and marine cetaceans. In addition, the differences in $\delta^{13}C_{sc}$ values between freshwater and marine modern cetaceans correspond well to differences in $\delta^{13}C$ values obtained in analyses of collagen (Schoeninger and DeNiro, 1984; Keegan and DeNiro, 1988; Little and Schoeninger, 1995). These values can also be used as a prediction of the carbonate $\delta^{13}C$ values ($\delta^{13}C_{sc}$) by adding 7% to the collagen $\delta^{13}C$ values (Lee-Thorp and van der Merwe, 1987). Making this adjustment to the collagen values of marine cetaceans presented by Schoeninger and DeNiro (1984) yields expected $\delta^{13}C_{sc}$ values ranging from -9.4 to -5.6‰, in good agreement with our results. Unfortunately, the data of Schoeninger and DeNiro (1984) did not include any freshwater cetaceans or other aquatic mammals but analyses of non-collagen soft tissues of freshwater and saltwater harbour seals (Smith et al., 1996) yielded a 4 to 7‰ difference, consistent with the freshwater-marine $\delta^{13}C_{sc}$ values we obtained for both modern and Eocene cetaceans.

An examination of the isotopic compositions of the Eocene cetacean teeth and bones reveals a shift from more negative to more positive $\delta^{18}O$ and $\delta^{13}C_{sc}$ values (Figs. 4a and 4b), suggesting that the transition of cetaceans from terrestrial/freshwater to marine life is preserved in our samples. In detail, however, this pattern is both more complex and more informative. Hypothetically, there are four broad ecological categories into which a cetacean species may fall (Fig. 6a). These hypothetical categories are delimited according to whether an animal: (1) eats terrestrial/freshwater food and ingests freshwater; (2) eats terrestrial/freshwater food and ingests seawater; (3) eats marine food and ingests freshwater; or (4) eats marine food and ingests seawater. In general, this categorization works well because the ranges of $\delta^{18}O_p$ and $\delta^{13}C_{sc}$ values are largely non-overlapping and because most of the species analyzed are full marine or fully freshwater
(Fig. 6a). Only one specimen of the sperm whale *Physeter* has a $\delta^{13}C_{\text{sc}}$ value that overlaps those of the freshwater/terrestrial animals.

Using boundary values ($\delta^{18}O_p = +17\%o$ and $\delta^{13}C_{\text{sc}} = -11\%o$) defined for these categories using the modern data, we find that the $\delta^{18}O_p$ and $\delta^{13}C_{\text{sc}}$ values of the Eocene cetaceans place at least one taxon in each of the four hypothetical categories (Fig. 6b). The presence of taxa in categories other than fully terrestrial and fully marine suggests that the transition from a terrestrial to marine diet was not strictly coupled to the transition in ingested water. It is also clear that some of the early cetacean species, such as *Attockicetus*, had ecological and physiological requirements that could not be inferred from morphology and depositional environments.

For example, although it has been suggested that the remnant Tethys was rich in nutrients and an important source of food for *Pakicetus* (Gingerich et al., 1983), our carbon isotope analyses of all three lower Kuldana cetaceans, *Ichthyolestes, Nalacetus*, and *Pakicetus*, suggest that these animals had a terrestrial diet and were ingesting primarily, if not exclusively, freshwater. An alternative explanation is that these animals were consuming marine foods which were relatively low in $^{13}C$ in the remnant Tethys and that the increase in $\delta^{13}C_{\text{sc}}$ values evident in our data (Fig. 3b) represents a change in the isotopic composition of marine waters available through time. This seems unlikely because even cetaceans feeding on marine plankton with the lowest documented $\delta^{13}C_{\text{sc}}$ values of -23.5\%o (Rau et al., 1982) would have tooth $\delta^{13}C_{\text{sc}}$ values of approximately -11.5, or 2-3\% higher than the lowest $\delta^{13}C_{\text{sc}}$ values of the cetaceans from the Kuldana Formation. In addition, the low $\delta^{18}O_p$ values of these animals suggest that they were restricted to freshwater. Another test of these dietary interpretations would be strontium isotope ($^{87}Sr/^{86}Sr$) analyses of these fossils (currently in progress), because the strontium content--and therefore $^{87}Sr/^{86}Sr$--of vertebrate teeth and bones is controlled largely by diet (Toots and Voorhies, 1965).
In contrast to the cetaceans from the lower Kuldana, the $\delta^{13}C_{sc}$ value of the mesonychian is on the border between marine and freshwater values. It falls within the range of values we have obtained from enamel of co-occurring herbivores, but a better understanding of the ecology of the Tethyan mesonychian must await the discovery and analysis of additional fossils.

Like *Ichthyolestes*, *Nalacetus*, and *Pakicetus*, both cetaceans from the upper Kuldana, *Ambulocetus* and *Gandakasia*, appear to have had a primarily terrestrial diet, but their wide range of $\delta^{18}O_p$ values suggests that they ingested waters of a wide variety of isotopic compositions. These patterns are suggestive of a euryhaline physiology, and are consistent with the preservation of *Ambulocetus* and *Gandakasia* in nearshore marine depositional environments. *Ambulocetus* has yet to be found in any terrestrial deposits, but if *Ambulocetus* and *Gandakasia* obtained their food very near the mouths of rivers, their absence from terrestrial deposits is consistent with the available evidence.

Of all the fossils analyzed here, those from the upper Kuldana Formation appear to have been most affected by post-mortem alteration. Nevertheless, it is unlikely that diagenesis is responsible for the low $\delta^{18}O_p$ (apparent freshwater) values of the bone and dentine samples of *Ambulocetus*, because our analyses of diagenesis of the upper Kuldana samples indicate that the secondary (diagenetic) carbonate of our specimens from the upper Kuldana Formation had considerably higher $\delta^{13}C_{sc}$ and $\delta^{18}O_p$ values than did the structural carbonate of the specimens themselves. For example, an untreated aliquot of H-GSP 18473 bone had a $\delta^{13}C_{sc}$ value of -5.6‰ (PDB) and a $\delta^{18}O_p$ value of 33.4‰ (SMOW), whereas, after treatment, H-GSP 18473 bone had values of -13.7‰ and 28.8‰, respectively. These results are not conclusive, but strongly suggest that the lower values are primary. The reality of the higher values is indicated by their occurrence in enamel samples. A further test of the nature of this intra-specimen isotopic variation
would be to make fine-scale analyses along transects of the teeth of \textit{Ambulocetus} and \textit{Gandakasia} specimens in order to determine whether the variation in the isotopic compositions of our samples represents real biological variation, such as an ontogenetic habitat shift.

One early cetacean species, \textit{Attockicetus}, appears to have eaten marine food and ingested freshwater as many seals apparently do. An interesting aspect of these results is that they indicate that \textit{Attockicetus}, a remingtonocetid, was ecologically quite different from its close relative \textit{Remingtononocetus} from the Harudi Formation.

By the middle Eocene, the first fully marine cetaceans had appeared. \textit{Gaviacetus}, \textit{Indocetus}, and \textit{Remingtononocetus}, from the Harudi Formation and \textit{Georgiacetus} from the McBean Formation have $\delta^{18}\text{O}_p$ and $\delta^{13}\text{C}_\text{sc}$ values consistent with a fully marine existence. \textit{Andrewsipsihius}, which had a very negative $\delta^{13}\text{C}_\text{sc}$ value of -13.4\textperthousand, is the exception. Too little is known of the postcranial morphology of \textit{Andrewsipsihius} to allow inferences concerning its locomotory abilities, but it is unlikely that this animal was able to migrate the distances modern cetaceans do. It is therefore unlikely that it fed in food webs based on phytoplankton low in $^{13}\text{C}$, as suggested for the sperm whale, \textit{Physeter}. A more likely explanation for the low $\delta^{13}\text{C}_\text{sc}$ value of \textit{Andrewsipsihius} is that fed on terrestrial or freshwater prey, as \textit{Ambulocetus} appears to have done. The low $\delta^{18}\text{O}_p$ value of \textit{Andrewsipsihius} relative to the $\delta^{18}\text{O}_p$ values of the other Harudi cetaceans, is consistent with this latter interpretation.

A temporal pattern is thus apparent in the cetacean data when they are viewed stratigraphically. Stratigraphic ranges do not necessarily reflect the exact sequence of evolutionary divergence, however. In order to answer questions such as how many times did the physiological transition occur?, or did the evolution of this ability precede or follow evolutionary diversification?, an hypothesis of evolutionary phylogenetic relationships is needed. This phylogenetic hypothesis must be based on criteria
independent of those to be evaluated, in this case, on morphological criteria. For present purposes, I have mapped ingested water (Fig. 10) and diet (Fig. 11) onto a phylogenetic hypothesis (cladogram) taken from Thewissen (this volume).

The result of this exercise is a set of diagrams which depict sequences of character transformation very similar to those suggested by the stratigraphic mapping of data. A notable difference between the stratigraphic and phylogenetic patterns is that in the latter, the wide range of ecological strategies employed by the Remingtonocetidae is much more apparent. Whereas remingtonocetid taxa are spread out temporally in the stratigraphic diagram, they are together in the cladogram because of their status as a monophyletic group, and this highlights the ecological diversity within the clade.

Now, with these diagrams, we can answer some of the above questions. The distribution of character states on the two cladograms is consistent with a single transition to a marine diet and a seawater ingestion at the base of the clade containing remingtonocetidae plus all later cetaceans (Indocetus, etc.), with a reversal of each in different species within the Remingtonocetidae. An alternative explanation is that the transition was made independently within the Remingtonocetidae and within later cetaceans. In terms of the number of steps in the tree, these two explanations are equally parsimonious, but given that the Ambulocetidae appear to have been euryhaline, it seems more likely that the transition occurred gradually (and once) along the branch leading to the Remingtonocetidae, Protocetidae and later cetaceans.

Although diagenesis appears to have affected the carbon isotope composition of carbonate in our samples, using an acetic-acid pretreatment reveals that the broad pattern of relative isotopic differences is apparent regardless of whether the sample analyzed was bone, dentine or enamel. If these isotopic differences are a good guide, then bone is actually a better proxy of enamel composition than is dentine, a result in good agreement with those of Koch et al. (1994). On the other hand, intratooth variation on the order of
3% has been recorded in modern terrestrial mammals (Fricke and O'Neil, 1996) and could explain some of the variation observed among bone, dentine, enamel of our Eocene fossils. Moreover, some of the isotopic variation in the fossils may also be due to differences in the timing and duration of mineralization. For example, the enamel of most mammal teeth forms over a short interval of 1-2 years (generally in utero) and is not remodeled during an animal's life as bone is, so depending on the age of the animal and the specifics of its physiology, bone may provide a longer-term average or a record from later in the animal's life than that preserved in its teeth.

7. CONCLUSIONS

The earliest cetaceans, *Ichthyolestes*, *Nalacetus* and *Pakicetus*, from the lower Kuldana Formation of Pakistan, were closely tied to terrestrial sources of fresh water and food and apparently shared very similar ecologies in these two respects. *Ambulocetus* and *Gandakasia*, from the upper Kuldana Formation, also appear to have relied on terrestrial food sources, but may have been euryhaline and able to ingest seawater at least occasionally. The mesonychian from the lower Kuldana Formation probably fed on terrestrial animals, but could have been feeding in part on marine prey.

*Attockicetus* from the Kohat Formation of Pakistan appears to have eaten primarily marine food, but required freshwater, in contrast to its close relative, *Remingtonocetus*. *Gaviacetus*, *Indocetus*, and *Remingtonocetus* from the Harudi Formation, and *Georgiacetus* from the McBean Formation, appear to have been fully marine, both living and feeding at sea. *Andrewsiphius*, also from the Harudi Formation, appears to have had different ecological requirements from the other cetaceans from the Harudi Formation. It either fed on terrestrial prey or marine plankton low in $^{13}$C.

The first fully marine cetaceans had thus appeared by the middle Eocene. The evolutionary transition of cetaceans from terrestrial to marine life was thus geologically
rapid. The transition to life in seawater involved changes both in osmoregulatory physiology and diet, but these changes were not strictly coupled. This apparent decoupling of food and water requirements may have facilitated niche differentiation, and as a result, the diversification of the earliest cetaceans.

8. ACKNOWLEDGMENTS

We thank the Geological Survey of Pakistan, its Director General, Dr. M. Talib Hassan, Assistant Director Muhammad Arif, and Dr. S Mahmood Raza of the Oil and Gas Development Corporation, for their support of our fieldwork in Pakistan. Financial support was provided by an NSF-funded Fellowship from the University of Arizona Research Training Group for the Analysis of Biological Diversification (to Roe), the National Geographic Society (to Thewissen), a Pioneer Award from the Northeastern Ohio Universities College of Medicine (to Thewissen), and by National Science Foundation Awards EAR-9005717 to O'Neil; EAR-9526686 to Thewissen, and EAR-9418207 to Quade. We thank R. D. E. MacPhee, of the American Museum of Natural History, W. F. Perrin of the NOAA/National Marine Fisheries Service branch in LaJolla, and C. W. Potter of the United States National Museum (Smithsonian) for comparative recent material, H. Achyuthan for assistance with wet chemistry, D. Surge and T. Moore for assistance with illustrations, C. Beuchat, J. N. Stallone, and S. I. Madar for helpful discussions and M. J. Schoeninger for a very helpful review.
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Table 1. Isotopic compositions ($\delta^{18}O_p$, $\delta^{13}C_{sc}$) of the teeth and bones of modern cetaceans.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Specimen</th>
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<th>$\delta^{18}O_p$, $%$ (SMOW)</th>
<th>$\delta^{13}C_{sc}$, $%$ (PDB)</th>
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Platanistidae

| Platanista        | NMFS uncat. | Ganges River, India | 14.9 | --    |

Pontoporiidae

| Lipotes           | AMNH-M 57333 | Tung Ting Lake, Hunan Prov., China | 11.2 | -12.8 |
Table 2. Isotopic compositions ($\delta^{18}\text{O}_p$, $\delta^{13}\text{C}_{sc}$) of the bone (b), dentine (d), and enamel (e) of Eocene cetaceans from Pakistan, India and the U.S.A., listed in alphabetical order by family.

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$^a$ Where values obtained for a specimen span more than a 2% range, the minimum and maximum values of the range, rather than averages, are listed, separated by a comma.
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Figure 1. Schematic representation of the fractionation of oxygen isotopes in the hydrologic cycle as a result of evaporation and condensation (based on the work of Epstein and Mayeda, 1953; Craig, 1961a and b; and Craig and Gordon, 1965). Note resultant difference between the oxygen isotope compositions of the ocean and fresh waters. Delta (δ) values are in parts per mil (‰) deviation from Standard Mean Ocean Water (SMOW).
\[ \delta^{18}O = -13\% \]

\[ \delta^{18}O = 0\% \quad \text{(SMOW)} \]

\[ \delta^{18}O = -3\% \]

\[ \delta^{18}O = -5\% \]

\[ \delta^{18}O = -15\% \]

Evaporation

Ocean

Atmosphere

Continent
Figure 2. Schematic representation of the average carbon isotope composition of some major ecosystem components. Note the difference in isotopic composition of total inorganic carbon species (reported as $\Sigma$CO$_2$) and organic matter between the ocean and fresh waters. POM = particulate organic matter; DOM = dissolved organic matter. Delta ($\delta$) values are in parts per mil (‰) deviation from the Pee Dee Belemnite standard (PDB).
Figure 3. Ranges of oxygen isotope compositions of the phosphate component ($\delta^{18}O_p$) of the teeth and bones of modern cetaceans. Note the $2\%$ minimum difference between marine and freshwater taxa.
Figure 4. Ranges of oxygen isotope compositions of the phosphate component ($\delta^{18}O_p$) of the teeth and bones of Eocene cetaceans. Note the ~3%e minimum difference between the cetaceans from the lower Kuldana Formation and the Kohat Formation of Pakistan and the cetaceans from the Harudi Formation of India and the McBean Formation of Georgia. The range of $\delta^{18}O_p$ values of Ambulocetus and Gandakasia from the upper Kuldana Formation suggest that these animals may have been euryhaline.
Figure 5. Ranges of carbon isotope compositions of the structural carbonate component ($\delta^{13}C_{sc}$) of the teeth and bones of modern cetaceans. Note the difference in $\delta^{13}C_{sc}$ values between marine and freshwater taxa. The $\delta^{13}C_{sc}$ value of -13‰ obtained for one specimen of the sperm whale *Physeter* may reflect migration into and feeding in a food web based on relatively $^{13}C$-depleted plankton.
The graph compares the δ¹³C values (%o) of different cetacean species. The species include Tursiops, Stenella, Sotalia, Physeter, Delphinus, Lipotes, and Inia. The y-axis represents the species, and the x-axis represents the δ¹³C values. The species are categorized into marine and freshwater groups. The marine species include Tursiops, Stenella, Sotalia, and Physeter, while the freshwater species include Delphinus, Lipotes, and Inia.
Figure 6. Ranges of carbon isotope compositions of the structural carbonate component ($\delta^{13}C_{sc}$) of the teeth and bones of Eocene taxa. Note the difference in $\delta^{13}C_{sc}$ values between the cetaceans from the Kuldana Formation and those from the Kohat, Harudi and McBean Formations. The $\delta^{13}C$ value of -13.4% of *Andrewsiphius* may be due either to a largely terrestrial/freshwater diet or to feeding in a marine food web with relatively $^{13}C$-depleted plankton at its base.
Modern freshwater

Modern marine

\[ \delta^{13}C, \%o (PDB) \]

- Georgiacetus
- Andrewsiphius
- Gaviacetus
- Indocetus
- Remingtonocetus
- Attockicetus
- Ambulocetus
- Gandakasia
- Ichthyolestes
- mesonychian
- Nalacetus
- Pakicetus

Taxon

McBean

Harudi

Kohat

upper Kuldana

lower Kuldana

14-12 million years ago
Figure 7. Bivariate plot of the $\delta^{13}C_{sc}$ values and $\delta^{18}O_p$ values of modern cetaceans, showing the general pattern of lower values in freshwater cetaceans and higher values in marine taxa.
Modern cetaceans

\[ \delta^{13}C, \text{‰ (PDB)} \]

-4
-6
-8
-10
-12
-14
-16
-18
10 12 14 16 18 20 22 24

\[ \delta^{18}O, \text{‰ (SMOW)} \]

freshwater seawater

marine food

terrestrial food or low \(^{13}C\) marine plankton
Figure 8. Bivariate plot of the $\delta^{13}C_{se}$ values and $\delta^{18}O_p$ values of Eocene cetaceans.
Eocene cetaceans

freshwater         seawater

δ¹³C, ‰  (PDB)

δ¹⁸O, ‰  (SMOW)

marine food

terrestrial food or low ¹³C marine plankton
Figure 9. Comparison of the $\delta^{13}C_{sc}$ values of bone, dentine, and enamel of individual fossil specimens of Eocene taxa from the five geologic formations represented. The differences in $\delta^{13}C_{sc}$ values among the three phases may reflect variation in preservational integrity, original differences due to differential remodeling and time-averaging of mineralization or both. Note that the different phases all exhibit the same general pattern of isotopic values.
Figure 10. Evolution of water ingestion in early cetaceans, as determined by mapping oxygen isotope data onto a phylogenetic hypothesis of cetacean relationships based on morphological data (Thewissen, this volume). The fossil taxa shown here are only those that were analyzed in this study. Other early cetacean species not analyzed as part of this project are not included in this figure.
Ingested water (unordered)

- freshwater
- brackish water
- seawater
- polymorphic
- equivocal
Figure 11. Evolution of diet in early cetaceans, as determined by mapping oxygen isotope data onto a phylogenetic hypothesis of cetacean relationships based on morphological data (Thewissen, this volume). The fossil taxa shown here are only those that were analyzed in this study. Other early cetacean species not analyzed as part of this project are not included in this figure.
Diet (unordered)

- freshwater or terrestrial
- marine
- equivocal
The isotopic composition of carbonate and phosphate in the bones and teeth of aquatic vertebrates: significance for recognizing diagenesis and understanding marine mammal physiology (formatted for submission to the Canadian Journal of Fisheries and Aquatic Sciences)

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Abstract: Oxygen isotope analyses of the phosphate ($\delta^{18}O_p$) and structural carbonate ($\delta^{18}O_{sc}$) of 21 species of marine fish and aquatic mammals, indicate that there is a positive correlation ($r^2 = 0.76$) between $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ in both fishes and aquatic mammals. The equation relating these two variables, $\delta^{18}O_p = 1.00\delta^{18}O_{sc} - 9.34$, is in good agreement with the results obtained for terrestrial mammals by previous workers. When these data are separated by taxonomic groups, however, the correlations remain generally high, but the slopes and intercepts of the equations vary. These differences in slope and intercept are probably due to physiological factors that affect the way in which oxygen is incorporated into teeth and bones. Regardless of these differences, the existence of alternative correlations in both fishes and mammals refutes an earlier claim that there is no correlation between these two variables in modern fishes. The existence of such a correlation in fossil specimens therefore should be considered an indication not of isotopic alteration, but of preservation of the original isotopic composition of a given bone or tooth. Use of the $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ as a test of diagenetic alteration will nevertheless require better characterization of the numerical relations between $\delta^{18}O_p$ and
\[ \delta^{18}O_{sc} \]. Such characterization is likely to result in a better understanding of water and ion balance in modern aquatic mammals.

**Introduction**

The development of criteria for the recognition of diagenetic alteration of biogenic phosphates has lagged behind such studies of carbonates, in spite of the importance of biogenic phosphates in paleotemperature and paleophysiological studies. One criterion for recognition of diagenesis, proposed first by Kastner et al. (1984) and further explored by Kolodny and Luz (1991), is the existence of a correlation between the oxygen isotope composition of the structural carbonate (\( \delta^{18}O_{sc} \)) and the oxygen isotope composition of the phosphate radical (\( \delta^{18}O_p \)). Kolodny and Luz (1991) demonstrated that the \( \delta^{18}O \) values of the structural carbonate and the phosphate are not well correlated in modern fishes, but are correlated in a number of fossils they analyzed. They inferred from this pattern that a correlation between these two variables indicated an isotopic resetting of both the phosphate and carbonate.

Kolodny and Luz's (1991) suggestion that there is no correlation in modern fishes challenges the assumption that both \( \delta^{18}O_{sc} \) and \( \delta^{18}O_p \) are governed largely by temperature. The slopes of the \( \delta^{18}O_{sc} \)-water and \( \delta^{18}O_p \)-water equations are so nearly parallel (Friedman and O'Neil 1977) that they can not be used to solve simultaneously for both the temperature and isotopic composition of paleowaters. It therefore follows that if \( \delta^{18}O_{sc} \) and \( \delta^{18}O_p \) are primarily temperature-controlled, the isotopic compositions of the two would be expected to be related by a fractionation factor that is nearly constant. Certainly this should be true over the range of temperatures (approximately 4-35°C) at which modern fishes live. The lack of such a correlation would suggest that other factors, such as the physiological control of water and ion balance (=osmoregulation), may also
play a role in the determination of the isotopic composition of both $\delta^{18}O_{sc}$ and $\delta^{18}O_p$ in biogenic phosphates.

Since Kolodny and Luz (1991) published their fish data, two groups have examined the oxygen isotope composition of both carbonate and phosphate in the teeth and bones of modern terrestrial mammals. Bryant et al. (1996) found a strong correlation ($r^2 = 0.99$) between $\delta^{18}O_c$ and $\delta^{18}O_p$ in equid enamel, as did Lacumin et al. (1996) in a variety of herbivorous and carnivorous mammalian taxa from different regions of the world ($r^2 = 0.98$). At least in terrestrial mammals, there is a correlation between the $\delta^{18}O_{sc}$ and $\delta^{18}O_p$—in spite of the existence of physiological complications (Kohn et al. 1996; Kohn 1996). Lacumin et al. (1996) argued that the correlation between $\delta^{18}O_{sc}$ and $\delta^{18}O_p$ therefore could be used for recognizing diagenesis, but in a way opposite to that proposed by Kolodny and Luz (1991): they considered a strong correlation between $\delta^{18}O_{sc}$ and $\delta^{18}O_p$ indicative of preservation of original isotopic composition, rather than of isotopic resetting. Bryant et al. (1996) did not discuss the implications of their data for understanding diagenesis, but the strong correlation they observed between $\delta^{18}O_{sc}$ and $\delta^{18}O_p$ in modern equids supports the arguments presented by Lacumin et al. (1996).

Why is there a discrepancy between the observations of Kolodny and Luz (1991) and those of Bryant et al. (1996) and Lacumin et al. (1996)? At least four possibilities exist. First, the data presented by Kolodny and Luz (1991) was primarily for ectotherms ("cold-blooded" animals), whereas the data presented by Bryant et al. (1996) and Lacumin et al. (1996) was all for homeotherms ("warm-blooded" animals). Second, all of Kolodny and Luz's taxa were aquatic and therefore living in environments where they may have been exposed to more environmental $HCO_3^-$, which potentially could play a role in the determination of the isotopic composition of teeth and bone carbonate (McConnaughey and Whelan 1997; McConnaughey et al. 1997). Third, specific biological effects, such as those of coral reefs on local carbon reservoirs (e.g., Schoeninger and DeNiro 1984) may
also effect oxygen isotope compositions, if the oxygen in the CO$_2$-HCO$_3^-$-CO$_3^{2-}$ system were not in isotopic equilibrium with ambient water. In this case, the oxygen isotope composition of structural carbonate could be offset in a linear fashion. Finally, differences in pre-treatment of samples may be at least partially responsible for the observed differences. Kolodny and Luz (1991) did not employ an acetic acid leaching step in the preparation of their samples for isotopic analysis of the structural carbonate. This step is critical for removing diagenetic carbonate from fossils (Lee-Thorp and van der Merwe 1987; Quade et al. 1992), but is also used to remove labile carbonate from modern bones and teeth (Lee-Thorp and van der Merwe 1987, 1991; Bryant et al. 1996).

The purpose of the work described here was to determine whether there is a correlation between the $\delta^{18}O_{sc}$ and $\delta^{18}O_{p}$ of the bones and teeth of aquatic (both freshwater and marine) vertebrates, and if not, to determine the reasons for any the lack of correlation. This information is necessary for determining whether, and in what cases, paired $\delta^{18}O_{sc}$ and $\delta^{18}O_{p}$ values of fossils will be useful in assessing whether a given fossil has been isotopically altered.

**Materials and Methods**

**Selection and preparation of specimens**

Specimens of 33 species of modern marine and freshwater animals were supplied by several institutions, including the American Museum of Natural History (AMNH; specimen abbreviation AMNH-M), the National Museum of Natural History (Smithsonian; USNM), the National Oceanic and Atmospheric Administration (NOAA) 's National Marine Fisheries Service (NMFS) laboratory in La Jolla, California, Phil Hastings and Bob McCord of the University of Arizona (UAZ), Terry Williams of the University of California at Davis, Richard White of Tucson, Arizona (RSW), and Sentiel A. Rommel of the Florida Department of Environmental Protection.
Most of the specimens provided were already skeletonized. Specimens not already skeletonized were dissected and treated with NaOCl to remove adhering tissue. All samples were dried, ground in a mortar and pestle and placed in 50 mL centrifuge tubes. Approximately 20 mL of 3% sodium hypochlorite (NaOCl) was then added and the tubes placed in beakers in an ultrasonic bath to promote oxidation of the organic matter. Often, this treatment required several iterations of grinding and treatment to powder the specimens sufficiently. Treatments were deemed complete when the addition of fresh NaOCl to finely ground (less than 100 mesh) samples produced no further visible reaction (when bubbling ceased). Each sample was then rinsed five times with de-ionized distilled water, and finally, placed in a drying oven overnight at approximately 80°C. Once dry, all samples were reground to a fine powder to ensure efficient reaction in the subsequent steps. Extractions of the carbon and oxygen in the carbonate (CO$_3^{2-}$) component of the teeth and bones of our samples were performed using the standard phosphoric acid (H$_3$PO$_4$ (aq)) reaction method of McCrea (1950) at 50°C. Oxygen isotope analyses of the phosphate (PO$_4^{3-}$) component were made using the trisilver phosphate (Ag$_3$PO$_4$) thermal decomposition method of O'Neil et al. (1994). The end product of both reactions is CO$_2$, which was measured on Finnigan MAT delta-S series 251 gas source mass spectrometers in the Department of Geosciences at the University of Arizona and the Department of Geological Sciences Stable Isotope Laboratory at the University of Michigan. Isotopic results are reported in the standard delta ($\delta$) notation as the deviation, in parts per mil ($\%$), of the sample CO$_2$ from the PDB and SMOW standards for carbon and oxygen respectively, where $\delta = (R_{\text{sample}} - R_{\text{standard}} / R_{\text{standard}}) * 1000$ and $R = ^{18}\text{O}/^{16}\text{O}$ or $^{13}\text{C}/^{12}\text{C}$. 
Interferences produced by residual NaOCl

As will be discussed further below, the NaOCl treatments produced problems with both the carbonate and phosphate analyses. Even after five rinses, some NaOCl residue remained behind in many of the samples treated in 15 mL conical centrifuge tubes. Upon reaction with H₃PO₄, these samples produced a contaminant that was probably HCl. This contaminant was removed by running the sample gas over Ag₃PO₄ to form AgCl. Samples analyzed on the mass spectrometer with the HCl present had spuriously high δ¹⁸O values.

NaOCl also interferes with the precipitation of Ag₃PO₄ for δ¹⁸Oₚ analysis. The Cl⁻ in solution precipitates out as AgCl when the AgNO₃ ammine solution is added. This AgCl must be removed, and additional AgNO₃ added, but the low yields often result and the measured isotopic composition may be altered by the low yields. For these reasons, it is preferable to oxidize organic matter with H₂O₂ or use 50 mL centrifuge tubes which allow for more thorough rinsing.

Results

The results of paired carbonate and phosphate analyses (Table 1) for all the aquatic species analyzed here show that there is a positive correlation between δ¹⁸Oₛₑ and δ¹⁸Oₚ (Fig. 1). The correlation coefficient of this relation (0.76) is not as high as the correlation coefficients of 0.99 and 0.98 found by Bryant et al. (1996) or Lacumin et al. (1996), respectively, but the samples analyzed here include a greater variety of animals (aquatic and terrestrial mammals, and marine and freshwater fishes) and a correspondingly greater range of physiological types. Examination of the δ¹⁸Oₚ - δ¹⁸Oₛₑ relations for individual taxonomic groups generally show equivalent or higher correlations. For example, the lower vertebrates analyzed, excluding Alligator and Fistularia (Fig. 2) have a linear relation of δ¹⁸Oₚ = + .86δ¹⁸Oₛₑ - 6.2 with a least squares correlation coefficient of 1.00.
As these specific $\delta^{18}O_p$ values of these taxa looked surprisingly high, lower vertebrate $\delta^{18}O_p$ values were regressed against $\delta^{13}C_{sc}$ values, as a check of the $\delta^{18}O_p$ results (Fig. 3). When these data are plotted in this way, very good correlations are found—but only when they are divided into freshwater-terrestrial and marine categories. The equations found were: $\delta^{18}O_p = 0.996\delta^{13}C_{sc} + 30.0 (r^2 = 0.95)$ for the freshwater-terrestrial species and $\delta^{18}O_p = + 0.72\delta^{13}C_{sc} + 22.8 (r^2 = 1.00)$ for the marine species.

The mammal data were subdivided also. A regression of both river and sea otter data (Fig. 4) yields the relation $\delta^{18}O_p = + 0.72\delta^{18}O_{sc} - 1.9 (r^2 = 0.95)$, and a regression of cetacean data (Fig. 5) yields the relation: $\delta^{18}O_p = + 1.2\delta^{18}O_{sc} - 15.1(r^2 = 0.78)$, both equations differ from the overall equation but in opposite ways. The slope of the otter equation is approximately 0.28 lower than that of the overall equation; the slope of the cetacean equation is approximately 0.20 higher than that of the overall equation. Similarly, the intercept of the otter equation is approximately 7.4 more positive, whereas the intercept of the cetacean equation is 5.8 more negative.

The exception to this general pattern of fairly high correlations is the regression of the pinniped (seals, sea lion and walrus) and sirenian (Florida manatee) data (Fig. 6). Within this group, $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ are weakly correlated ($r^2 = 0.40$). If the seals are divided by taxonomic group, the otariids (eared seals, which include sea lions) and phocids (true seals) (Fig. 6), the resulting clusters are cleanly divided, but this division does not account for the scatter in $\delta^{18}O_p$. As with the lower vertebrates, the reality of this variation was checked by regression of $\delta^{18}O_p$ on $\delta^{13}C_{sc}$. Once again, the animals were divided—in this case into putatively freshwater-ingesting (phocid) versus putatively seawater-ingesting (otariid) species (Fig. 7). The resultant equation for the seawater-ingesting group is: $\delta^{18}O_p = 0.99\delta^{13}C_{sc} + 30.0 (r^2 = 0.95)$. The slope of this equation is identical to that obtained for terrestrial/freshwater lower vertebrates (and also terrestrial mammals—Bryant et al. 1996; Iacumin et al. 1996); the intercept is intermediate between
that of the terrestrial-freshwater lower vertebrates and that of the marine lower vertebrates.

**Discussion**

The positive correlation between $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ in the wide variety of taxa analyzed here is consistent with the recent findings of Bryant et al. (1996) and Iacumin et al. (1996). Direct comparison of these analyses of mammals with those of Kolodny and Luz (1991) may be unwarranted because the latter authors did not analyze mammals. Analyses of fishes presented here are directly comparable, however, and the positive correlation for at least some of the fishes and other lower vertebrates analyzed here is inconsistent with the results of Kolodny and Luz (1991), who found no correlation between $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ in modern fishes. Our results suggest that a correlation between $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ in fossil specimens should not be considered evidence of alteration, as argued by Kolodny and Luz (1991), but rather as evidence of the preservation of original isotopic composition.

The correlations between $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ are as strong as or stronger than the overall correlation when taxonomic groups are treated separately, suggesting that the differences observed may be due to physiological or ecological differences. Another possibility that may explain the results obtained for *Alligator* and *Fistularia*, is the effect of high-productivity systems on oxygen isotope fractionation. It has been established that reefs and other high-productivity systems, such as seagrass communities and marshes exert a strong influence on the carbon isotope composition of the soft and hard tissues of the animals living and feeding nearby. This effect is produced by high rates of photosynthesis, which preferentially sequesters $^{12}C$ (Swart 1983; Schoeninger and DeNiro 1984; Keegan and DeNiro 1988). Although oxygen isotope compositions are generally little affected by photosynthesis, there may be substantial effects due to
respiration (Swart 1983), which is coupled with photosynthesis. In order to produce an offset between $\delta^{18}O_p$ and $\delta^{18}O_{sc}$, however, this mechanism would have to produce isotopic disequilibrium between $\text{HCO}_3^-$ and $\text{H}_2\text{O}$ oxygen, which is probably unlikely. Further dietary analyses of lower vertebrates are needed to determine the cause(s) of offsets between $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ observed here in Alligator and Fistularia.

Much of the variation in the aquatic mammal data presented here, particularly in the case of pinnipeds, may result from differences in osmoregulatory (water and ion balance) strategies. Although much of the evidence is anecdotal, it is clear that osmoregulatory strategies vary among pinnipeds (eared seals, true seals, sea lions and walruses). In general, phocids (true seals) seem to drink only freshwater (Gentry 1981), whereas otariids (eared seals and sea lions) drink seawater at least occasionally (Gentry 1981) and odobenids (walruses) are apparently fully marine. But the field observations and experimental data are not always in agreement. For example, Gentry (1981) observed four species of otariids—Arctocephalus forsteri, Callorhinus ursinus, Eumetopias jubatus, Zalophus californianus—drinking seawater in the wild. In contrast, Costa and Gentry (1986) presented evidence that Northern fur seals (Callorhinus ursinus) do not drink seawater in the wild and in experiments performed by Pilson (1970), Zalophus californianus did not drink seawater even when excess salt was added to its diet and no freshwater was available. Field and laboratory data are in somewhat better agreement with regard to phocids: with only one exception (Gentry 1981), phocids have never been observed drinking sea water, although they may ingest a substantial amount during feeding, and the harp seal (Phoca groenlandica) is known to seek out freshwater in captivity (Renouf et al. 1990).

Modern sirenians (manatees and dugongs) live in the sea and in freshwater. The Florida manatee (Trichechus manatus) commonly swims to river mouths to drink
(Reynolds and Odell 1991) and feed. The Florida manatee (the species analyzed here) probably consumes some seawater at least incidentally (e.g., when feeding) which may explain its apparent marine $\delta^{18}O_p$ value. The West Indian manatee feeds on a wide variety of vegetation in both marine and fresh waters. The Amazon manatee, however, lives only in freshwater and consumes primarily vascular aquatic plants (Caldwell and Caldwell 1981). In contrast, the manatees' closest relatives, the dugongs (Dugong), do drink seawater (Reynolds and Odell 1991) and consume a wide variety of seagrasses.

The observational data on pinniped drinking habits is consistent with the data presented here. By comparison with the lower vertebrate results, the isotopic data presented here for pinnipeds suggest that phocids ingest relatively more freshwater than do otariids. The experimental data for these animals suggests that this is probably a simplistic concept, as some pinnipeds may drink seawater only under special circumstances (Gentry 1980), but still ingest considerable seawater when they feed. Also, some species, such as the harbour seal, have both marine and freshwater populations (Smith et al. 1996). It is important to realize, though, that it is not the behavior of drinking or not-drinking seawater that is important here, but the relative amounts of different waters ingested or obtained from the food. Water conservation mechanisms involved in lactation may also be important. Final resolution of this question will require more than analyses of teeth and bones collected after the animal dies. Measurements of in vivo dietary and water fluxes are also needed. Nevertheless, stable isotope analyses of both soft tissues (e.g. Smith et al. 1996) and hard tissues, such as those described here, are likely to be of great use in answering questions of water and ion balance not only in fossil, but also in modern species.

Regardless of the ultimate causes of the variation, these data illustrate that there are important questions that need to be resolved before the correlation between $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ can be used to assess diagenesis in fossil aquatic vertebrates. Although the
difference between $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ is generally between 7 and 9‰, the exact mathematical relation between $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ varies by group and the appropriate equation will have to be used for each case to make an accurate assessment for a given fossil. Also, even if the correlation between $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ can be established as an indicator of diagenetic alteration, it remains to be demonstrated whether a lack of correlation between $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ in fossil specimens represents a resetting of both $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ or only $\delta^{18}O_{sc}$. It is not a necessary conclusion that resetting of the $\delta^{18}O_{sc}$ is an indication of resetting of $\delta^{18}O_p$, although an inherited $\delta^{18}O_p$ may be difficult to discern from an original isotopic composition (Blake et al. 1997).

Conclusions
Contrary to the findings of Kolodny and Luz (1991), there is a correlation $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ in modern fishes and other aquatic vertebrates. The correlation is not as strong as that obtained for terrestrial mammals by Bryant et al. (1996) and Iacumin et al. (1996) ($r^2 = .76$ vs. $r^2 = .98$ and .99, respectively), probably as a result of physiological differences. The data I present here do not support the exist of a difference in the ectotherms and endotherms, but the equations relating these variables do differ between freshwater and marine species. The existence of a correlation between $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ in fossil specimens should not be considered an indication isotopic alteration, but rather of the preservation of the original isotopic composition of a given bone or tooth. Use of the correlation between $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ as a test of isotopic resetting should be made using of the specific relations between $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ for different groups of aquatic vertebrates. Further characterization of the specific relations is required and is likely to result in a better understanding of water and ion balance in modern aquatic mammals.
References


Table 1. Paired isotopic analyses of the phosphate ($\delta^{18}\text{Op}$) and structural carbonate ($\delta^{13}\text{C}_{\text{sc}}$ and $\delta^{18}\text{O}_{\text{sc}}$) of the teeth and bones of modern aquatic vertebrates.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Specimen</th>
<th>Type</th>
<th>$\delta^{18}\text{Op}$</th>
<th>$\delta^{13}\text{C}_{\text{sc}}$</th>
<th>$\delta^{18}\text{O}_{\text{sc}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pisces</td>
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<td>Osteichthyes</td>
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<tr>
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<td>DUF 1072</td>
<td>ribs</td>
<td>16.4</td>
<td>-13.3</td>
<td>26.1</td>
</tr>
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<tr>
<td>Fistulariidae</td>
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<tr>
<td><em>Fistularia sp.</em></td>
<td>UAZ uncat.</td>
<td>bone</td>
<td>22.5</td>
<td>-0.38</td>
<td>30.0</td>
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<td>Serranidae</td>
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<td>bone</td>
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<td>Scorpaenidae</td>
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<td>20.8</td>
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Carnivora

Mustelidae

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dentine  
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26.0

*Lutra canadensis*  
RW 94304  
enamel  
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-17.7  
28.2

*Lutra canadensis*  
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18.8  
-16.0  
29.3

*Lutra canadensis*  
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25.3

*Lutra canadensis*  
USNM 144  
jaw  
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-18.0  
25.0

Phocidae

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-12.9  
25.6

*Phoca hispida*  
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skull bone  
15.7  
-14.5  
25.5

Cetacea

Mysticeti

Balaenopteridae

*Balaenoptera*  
USNM 571917  
tympanic  
18.1  
-12.3  
26.9

Odontoceti

Delphinidae

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26.9

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tooth  
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Figure 1. Bivariate plot of $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ values of 21 species of modern aquatic vertebrates, showing a positive correlation between the two variables.
\[ y = 1.00x - 9.34 \]
\[ r^2 = 0.76 \]
Figure 2. Bivariate plot of $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ modern marine and freshwater lower vertebrates, showing a strong positive correlation among taxa excluding Alligator and Fistularia.
\[ y = 0.86x - 6.16 \]

\[ r^2 = 1.00 \]
Figure 3. Bivariate plot of $\delta^{18}O$ and $\delta^{13}C_{\text{sc}}$ modern marine and freshwater lower vertebrae, showing a strong correlation among all taxa (including Alligator and Fistularia). Note that the regression lines for freshwater and marine taxa have similar slopes but different intercepts.
Alligator
Fistularia
Paralabrax
Scorpaen
Trachemys

\[ \delta^{18}O_p \text{ (SMOW)} \]

\[ \delta^{13}C_{sc} \text{ (PDB)} \]

- marine
- freshwater/terrestrial

\[ y = 0.99x + 30.0 \]
\[ r^2 = 0.97 \]

\[ y = 0.83x + 22.8 \]
\[ r^2 = 1.00 \]
Figure 4. Bivariate plot of the $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ values of modern lutrines (river and sea otters), showing a positive correlation between the two variables.
$y = 0.72x - 1.91$

$r^2 = 0.95$
Figure 5. Bivariate plot of the $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ values of modern cetaceans, showing a positive correlation between the two variables.
\[
y = 1.22x - 15.1
\]
\[
r^2 = 0.78
\]
Figure 6. Bivariate plot of the $\delta^{18}O_p$ and $\delta^{18}O_{sc}$ values of modern pinnipeds and sirenian, showing a weak correlation between the two variables.
The graph shows the relationship between two variables, $\delta^{18}O_p$ (SMOW) on the y-axis and $\delta^{18}O_{sc}$ (SMOW) on the x-axis. The equation $y = 0.74x - 2.07$ with $r^2 = 0.40$ describes the linear regression line. The data points are categorized into three groups: sirenian, otariids, and phocids. Each group is represented by a different symbol, with sirenian represented by squares, otariids by a box, and phocids by small squares within the box.
Figure 7. Bivariate plot of $\delta^{18}O_p$ and $\delta^{13}C_{SC}$ modern pinnipeds and sirenian, showing a strong correlation among otariids and sirenian. Note that the slope of this regression line is identical to that of the comparable equation for marine lower vertebrates and the intercept is intermediate between marine and freshwater lower vertebrates.
The diagram shows a scatter plot with the following annotations:

- The equation of the line is $y = 0.99x + 26.7$ with $r^2 = 0.95$.
- The plot includes data points for phocids and otariids.
- There are markers for seawater ingestion and freshwater ingestion.

The axes are labeled as $\delta^{18}O$ (SMOW) and $\delta^{13}C$ (PDB).
EXCHANGE BETWEEN CO$_2$ AND Ag$_3$PO$_4$ AT HIGH TEMPERATURES: A NEW METHOD FOR DETERMINING THE OXYGEN ISOTOPE COMPOSITION OF PHOSPHATIC MATERIALS (formatted for submission to Geochimica et Cosmochimica Acta)

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Abstract—Here we report for the first time that CO$_2$ exchanges readily with Ag$_3$PO$_4$ at high temperatures (≥725°C) and propose controlled equilibrium exchange as a method of determining the oxygen isotope composition of phosphates. The advantages of this method are: (1) it is the simplest, fastest, and least expensive method available for the analysis of phosphate oxygen; (2) it requires neither fluorination nor graphite reduction and (3) it can be done in glass tubes at temperatures low enough to reduce or eliminate the problem of exchange between the oxygen in the tube and the reagents. Initial experiments done over a range of temperatures (725-1200°C) and times (10-60 minutes) indicate that exchange occurs when Ag$_3$PO$_4$ is in either the solid phase or the liquid phase (the melting point of Ag$_3$PO$_4$ is 849°C). At the lowest temperature tested, 725°C, isotopic equilibrium was reached in approximately 90 minutes with both ends of the furnace plugged and within about an hour when one end of the furnace was unplugged to increase convection. The resultant equilibrium fractionation factor, 1000 ln alpha (α) between the CO$_2$ and Ag$_3$PO$_4$ at 725°C is between 5.9 and 6.1‰. The fractionation factor decreases with increasing temperature, from about 5.2‰ at 775°C to as low as 3.6 to 3.8‰ at 875°C. At 825°C, the exchange reaction was unstable, perhaps because of
proximity to the phase transition. The exchange reaction is thus a viable method, but must be performed more than 25°C above or below the melting point of Ag₃PO₄. Use of the lowest possible temperature is recommended in order to minimize exchange with O₂ from the Vycor (SiO₂) sample tubes. Employing lower temperatures delays the achievement of equilibrium, but may be countered by unplugging one end of the furnace to increase convection.

1. INTRODUCTION

The last several years have seen tremendous progress in the development of methods for the analysis of the oxygen isotope compositions of phosphate minerals (Crowson et al., 1991; O'Neil et al., 1994; Stuart-Williams and Schwarcz, 1995; Cerling and Sharp, 1996; Sharp and Cerling, 1996; Holmden et al., 1997). All of these methods involve chemical reactions which liberate oxygen in varying yields from the phosphate, either in the form of O₂ or as CO₂. The yields obtainable may place constraints on the amount of original phosphate needed for analysis. Two of these methods are especially suited to the analysis of small samples. The method described by Cerling and Sharp (1996) and Sharp and Cerling (1996) makes possible the analysis of very small samples through the use of a laser, but is not well-suited to making the necessary pretreatments for removal of secondary carbonate or iron/magnesium oxyhydroxides from permineralized fossils, a much bigger problem for bone than for tooth enamel. In contrast, the thermal ionization method of Holmden et al. (1997) can be used with extremely small samples, and like several of the other methods, employs purification of the phosphate as Ag₃PO₄ and methods making it compatible with pretreatments. Their method does not really solve the problem of sample size, however, because they precipitated large amounts of Ag₃PO₄ and took small aliquots for TIMS analysis. The difficulty of obtaining good yields in the wet chemical procedures used to make the Ag₃PO₄ remain a limiting factor.
In the course of developing a thermal decomposition method for the analysis of Ag₃PO₄ (O'Neil et al., 1994), we discovered that the solid residue produced by the decomposition of Ag₃PO₄ readily exchanged its oxygen with carbon dioxide introduced after the reaction. This led us to consider whether Ag₃PO₄ itself might also exchange its oxygen with CO₂, without any prior reaction. Initial experiments (Roe et al., 1994) indicated that this exchange did occur over temperatures ranging from 750°C to 1100°C and that equilibrium was easily reached within minutes at temperatures above the melting point of Ag₃PO₄, which is 849°C (Table 1; Fig. 1). One problem with this type of exchange, however, is that the Ag₃PO₄ may briefly exchange as a solid upon quenching, so that exchange actually occurs with the Ag₃PO₄ in both the liquid and solid phases. In addition, the potential of the Vycor tubing to exchange its oxygen with the samples is greater at higher temperatures.

The purpose of the present work was to investigate the possibility of isotopic exchange between the two phases at lower temperatures. In particular, we wanted to better evaluate the time-dependent equilibration and fractionation factors of the system at temperatures below the melting point. Lower temperature exchange would decrease and possibly eliminate the exchange of oxygen between the Vycor tubing and the samples contained therein.

2. EXPERIMENTAL METHODS

2.1 Preparation of Ag₃PO₄ samples

In order to ensure isotopic homogeneity and achieve fairly uniform sample size, the Ag₃PO₄ used was prepared by the dissolution of a large amount 2.50 g of KH₂PO₄ in one liter of deionized water. This solution was thoroughly mixed and allowed to sit overnight. The solution was then placed in a 50 mL burette, and dispensed in 3.3 mL increments into 50 mL or 100 mL beakers. To each of these sample solutions, 5 mL of
AgNO₃-NH₄NO₃ ammoniacal solution was added (O'Neil et al., 1994). Sample beakers were then placed on a hotplate and evaporated at a temperature of about 50-60°C. The Ag₃PO₄ was then filtered on Supor-200 0.20 micron membrane filters, placed in a petri dish, dried overnight and weighed. Drying temperature is not critical at this stage because samples are later subjected to a 550°C heating under vacuum to remove trapped water and possible organic contaminants (though these are generally not present in samples prepared from KH₂PO₄).

2.2 Choice and preparation of CO₂ samples
Two CO₂ gases with different δ¹⁸O values were used in these experiments, in order to approach equilibrium exchange from opposite directions. The δ¹⁸O values of the two reference gases were +7.4‰, and +17.7‰, and the δ¹⁸O of the Ag₃PO₄ prepared from the KH₂PO₄ was +12.0‰. Thus, the gases were both approximately 5‰ different from the Ag₃PO₄ used. The CO₂ with the lower δ¹⁸O value was commercially produced tank gas which required only a minor amount of cryogenic purification. The second CO₂ gas was produced from Palabora (South Africa) carbonatite (122-10) using the H₃PO₄ (aq) reaction method of McCrea (1950) and required only slightly more cryogenic purification than did the tank gas. Approximately 2,000 micromoles of each gas was loaded into one of two identical glass reservoirs with double stopcocks isolating a volume of tubing for aliquotting. Each time gas was expanded into the aliquotting volume, it was equilibrated for at least two minutes.

2.3. Exchange experiments
Thirty approximately 50 mg samples of Ag₃PO₄ precipitated from pure KH₂PO₄ were placed in 6mm Vycor (pure SiO₂) tubes, evacuated and preheated at 550°C to remove any trapped water and drive off any organic contaminants. A 20-35 micromole aliquot of one
of the two different CO₂ reference gases was frozen into each tube, and the tube sealed. These amounts were chosen to both (1) approximate reasonable sample sizes of phosphatic material and (2) to ensure that the ratio of O₂ in the Ag₃PO₄ to that in the introduced CO₂ was fairly high. A 50 mg sample of Ag₃PO₄ contains the equivalent of 239 micromoles of O₂ and our Ag₃PO₄ samples ranged from 47.1 mg (225 micromoles O₂) to 51.5 mg (246 micromoles), so that our ratios varied from about 7 to 10. The samples were then placed in a handmade nichrome holder designed to place the bottom of the sample tube at the center, near the hotspot of a Thermolyne 21100 tube furnace for the specified amount of time. Two sets of 725°C trials were performed, one with both ends of the tube furnace plugged with silica wool, the other with one end of the tube furnace unplugged. During all of the 775°C and 825°C trials, the open end was plugged with SiO₂ wool. At the end of the desired time, samples were withdrawn rapidly from the furnace and quenched in a large (two liter) beaker of room-temperature water. The CO₂ samples were expanded into the inlet system of the mass spectrometer without purification. Fractionation factors were calculated as 1000 ln α, where α = (1000 + δ¹⁸O_T)/(1000 + δ¹⁸O_O), where δ¹⁸O_T is the theoretical value of the CO₂ after the heating, assuming equilibration with no fractionation. The value of δ¹⁸O_T was determined by a simple mass balance calculation. The second variable, δ¹⁸O_O, is the observed final value of the CO₂ after the heating.

3. RESULTS

The results of our trials at 725°C, 775°C and 825°C (Table 2; Figs. 2-6) confirm that exchange between CO₂ and solid Ag₃PO₄ does occur and indicate that equilibrium is reached within about 90 minutes, depending on the initial δ¹⁸O value of the CO₂ used, and whether one end of furnace was left unplugged. Pure Ag₃PO₄ is a bright canary yellow, but has a tendency to change to a light olive color during precipitation and
filtration, possibly due to photoreactivity. The 550°C preheating step does not change the olive color of the crystals of already pure Ag₃PO₄, but after the exchange reactions, the Ag₃PO₄ is always bright canary yellow once again, suggesting that pure Ag₃PO₄ remained.

In all tests reported here, the resultant isotopic composition of the CO₂ was different from its initial value, and had a δ¹⁸O value higher than that of the initial δ¹⁸O value of the Ag₃PO₄. At the lowest temperature used, 725°C, isotopic equilibrium was reached within about an hour (Fig. 1) when one end of the furnace was unplugged, with a resultant equilibrium fractionation factor (1000 ln α) between the CO₂ and Ag₃PO₄ of 5.9 to 6.1‰ (Fig. 3). When both ends of the furnace were plugged, samples heated at 725°C did not reach equilibrium within an hour (Fig.). The best-fit lines for these data were logarithmic, as expected, and were solved simultaneously to determine that the time to equilibration (point of intersection of the two lines) under these conditions was 93 minutes. Solving either equation for y then yields an expected value of 5.65‰ for 1000 ln α.

The fractionation factor decreases with increasing temperature. At 775°C (Fig. 5), it is approximately 5.2‰. At 825°C it is approximately 4.7‰, although the fractionation is unstable. The direction of the fractionation changes direction after 45 minutes (Fig. 6). In terms of magnitude, however, these results are consistent with initial results indicating a lower value of 3.6 to 3.8‰ at 875°C (Fig. 1; Roe et al., 1994).

4. DISCUSSION

Oxygen isotope exchange between CO₂ (g) and Ag₃PO₄(s) occurs well below the melting point of Ag₃PO₄. At the lowest temperature tested, 725°C, equilibrium can be reached in 1 to 1.5 hours. In order for this method to be practical for routine measurements of δ¹⁸Oᵊ, isotopic equilibrium must be reached within a reasonable amount of time. This
requirement appears to be easily met even at 725°C. Isotopic equilibration of different phases is generally faster at higher temperatures, and the results obtained here are consistent with this general pattern. Increasing the temperature of the exchange reaction could therefore increase the reaction speed and improve efficiency. The results of trials done at 775°C and 825°C, provide a test of this possibility.

The experiments described here were designed to demonstrate an asymptotic approach of isotopic equilibrium from two directions. This behavior is most obvious in the results from the 725°C experiments conducted with both ends of the furnace plugged (Fig. 4). Comparison of these results with the 725°C experiments conducted with one end of the furnace open (Fig. 3) suggests that increased convection inside the sample tube (produced by a larger temperature gradient from the furnace hotspot in the center to the furnace opening) may be used to decrease equilibration time.

In contrast to the predicted behavior of samples heated at 725°C (Figs. 3 and 4), the results obtained at 775°C and 825°C do not exhibit the expected convergence indicative of an approach to equilibrium. There is good consistency among the values obtained for the gases at 775°C, but no strong indication of equilibrium. One possible explanation of this behavior is interference of oxygen in the glass tubing. Tests at 825°C, with no Ag₃PO₄ present, showed that exchange between SiO₂ and CO₂ does occur within an hour. During the exchange reactions, Ag₃PO₄ contains the vast majority of the O₂, and should "swamp out" any such effect of the SiO₂ tubing, but it is a possible that somewhere between 725°C and 775°C, there is a transition point at which O₂ begins to diffuse out of the SiO₂ tubing.

The apparent crossing-over behavior of the samples run at 825°C, however, may be due to an entirely different factor, the proximity to the phase transition. If this crossing-over phenomenon is real, it may be due to an incorrect thermocouple in the furnace. If the temperature was actually ≥849°C, the Ag₃PO₄ may have changed from solid to liquid
state during the experiments. A second less likely possibility is that the melting point of
the Ag3PO4 was lowered by the presence of an impurity. This explanation is unlikely
because of the purity of the KH2PO4 from which the Ag3PO4 was made.

In any case, it appears that an exchange reaction between gaseous CO2 and solid
phase Ag3PO4 is viable even at 725°C, although an hour may be impractical if samples
are heated one at a time. It is possible that this inconvenience may be circumvented by
batch loading of samples, but at present, it is not known whether batch loading in a tube
furnace would allow all samples to be heated in the same way. Keeping one of the end
furnace open decreases the amount of time required to reach equilibrium but may
produce slightly less consistent results (Figure 3).

Another possible solution is to increase the temperature to 750°C. Experiments at
750°C, together with SiO2-O2 exchange experiments below 825°C, are the next steps in
this study. For the present purposes, these latter tests will be made at 725°C and 750°C,
but similar tests at temperatures between 550°C and 725°C also be useful as a way of
determining whether a slightly higher temperature would be preferable in the preheating
of samples for removal of contaminants.

5. CONCLUSIONS
Exchange between CO2 and Ag3PO4 occurs both when Ag3PO4 is in the solid phase and
the liquid phase. Equilibrium can be reached in approximately 1.5 hours, making
controlled exchange a viable method of determining the oxygen isotope composition of
phosphates, at temperatures as low as 725°C. Equilibration time may be decreased by
keeping one end of the furnace open. Exchange too near the phase transition (849°C)
results in unstable, crossing-over behavior. The fractionation factor between CO2 and
Ag3PO4 at 725°C is approximately 5.65‰.
Acknowledgments—We thank Nat Lifton for supplying the tank CO₂ and Mark Barton for the Palabora carbonatite 122-10 from South Africa from which CO₂ used in these experiments was made. Support for this work was provided by Jay Quade, by NSF EAR-9005717 to JRO, and conducted while LJR was supported by a University of Arizona Fellowship from the Research Training Group for the Analysis of Biological Diversification and by a Graduate College Dean's Fellowship.

REFERENCES


Table 1. Initial results of equilibrium oxygen isotope exchange experiments between CO$_2$ and Ag$_3$PO$_4$ as a function of temperature.

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Table 2. Oxygen isotope exchange between CO₂ and Ag₃PO₄ as a function of temperature between 725°C and 825°C.

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Figure 1. Summary diagram of results of initial experiments above and below the melting point of Ag₃PO₄. Note that Ag₃PO₄ made from two sources with different initial δ¹⁸O values, were used in these experiments. Also note the similarity in fractionation factors between CO₂ and Ag₃PO₄ at temperatures above the melting point of Ag₃PO₄.
melting $T$ of $\text{Ag}_3\text{PO}_4$

$1000 \ln \alpha$, (\%)

Temperature ($^\circ$C)

$\text{Ag}_3\text{PO}_4$ from NBS-120c
$\text{Ag}_3\text{PO}_4$ from $\text{KH}_2\text{PO}_4$
Figure 2. Summary diagram of results of present experiments done below the melting temperature of Ag₃PO₄. Note that after 10 minutes of exchange, virtually all samples had fractionation factors between 5 and 6.5%, and that the fractionation factors decrease with increasing temperature.
Figure 3. Oxygen isotope fractionation between $\text{CO}_2$ and $\text{Ag}_3\text{PO}_4$ at 725°C, with one end of the tube furnace unplugged. Note asymptotic behavior of fractionation factor of Lifton Dilution $\text{CO}_2$ samples, indicating that isotopic equilibrium is being approached.
$y = 0.54 + 3.10 \log x$

$r^2 = 0.71$
Figure 4. Oxygen isotope fractionation between CO$_2$ and Ag$_3$PO$_4$ at 725°C, with both ends of the tube furnace plugged with SiO$_2$ wool. Note convergence of fractionation factors of both Lifton Dilution CO$_2$ samples and Palabora Carbonatite CO$_2$ samples, indicating that isotopic equilibrium is being approached. Simultaneous solution of the best fit lines (logarithmic relations) provides an approximation of the time needed to reach isotopic equilibrium.
$y = -0.16 + 2.95 \log x$
$r^2 = 0.99$

$y = 7.12 - 0.74 \log x$
$r^2 = 0.96$

- Palabora carbonatite CO$_2$
- Lifton dilution CO$_2$
Figure 5. Oxygen isotope fractionation between CO$_2$ and Ag$_3$PO$_4$ at 775°C. The nearly constant values of the fractionation factor (1000 ln $\alpha$) suggests that isotopic equilibrium was reached within 20 minutes. The difference in fractionation factors between the two gases used is as yet unexplained and may indicate differential interference from the oxygen in the Vycor tubing.
In a graph, the $1000 \ln \alpha$ (in %) is plotted against time, minutes. The graph shows two sets of data points: ■ for Palabora carbonatite CO$_2$ and ▲ for Lifton dilution CO$_2$. The x-axis represents time in minutes, ranging from 0 to 70, while the y-axis represents $1000 \ln \alpha$ in increments of 0.2, ranging from 4.6 to 5.6.
Figure 6. Oxygen isotope fractionation between CO$_2$ and Ag$_3$PO$_4$ at 825°C. Note apparent crossing-over of fractionation factors of the two gases. This may be due to close proximity to the phase transition (melting point) and suggests that exchange between CO$_2$ and Ag$_3$PO$_4$ in the solid phase is probably better done at temperatures more than 25°C below the melting point.
REFERENCES


