A CRITICAL EVALUATION OF THE APPLICATION OF AMINO ACID RACEMIZATION TO GEOCHRONOLOGY AND GEOTHERMOMETRY

K. M. WILLIAMS and G. G. SMITH
Dept. of Chemistry and Biochemistry, Utah State University, Logan, UT 84322

(Received 21 March, 1977)

Abstract. In this review we have critically evaluated the application of the diagenetic racemization of amino acids to geochronology and geothermometry. Although there has been enthusiastic support given to this new method, it is our opinion that recent developments suggest a more cautious approach. We have discussed the pitfalls and inherent complications, while outlining the advances which have been accomplished. We conclude that this is an innovative approach which will add valuable information to the scientific literature. However, since our fundamental understanding of diagenetic racemization is still limited, many of the age and paleotemperature estimates which have been assigned to fossil specimens may be unreliable.

1. Introduction

In 1860, Pasteur observed that

> All artificial bodies and all minerals have super-imposable images. Opposed to these are many organic substances (I might say nearly all, if I were to specify only those which play an important role in plant and animal life) all of which are important substances to life, are asymmetric, and indeed have the kind of asymmetry in which the image is not superposable with the object.

There is certainly a unique relationship between optical activity and life. Virtually all proteins contain exclusively the L-enantiomers of the amino acids while natural sugars are of the D-configuration. Many chemists would agree with the statement made at the *First International Symposium on the Origin of Life on Earth* by Terent'ev and Klabunovskii (1957), "we will start from the assumption that life cannot and never could exist without molecular dissymmetry".

By simulating the primitive reducing atmosphere and using appropriate energy sources the abiotic synthesis of biomonomers, such as the amino acids, has been carried out (for a review of this literature see Stephen-Sherwood and Oró, 1973). However, in all cases where optical activity has been investigated these origin of life syntheses have produced racemic mixtures of amino acids, neither enantiomer being favored. There is no generally accepted theory for the origin of optical asymmetry. As early as 1908, Van't Hoff suggested that the action of circularly polarized light might produce an asymmetric synthesis. This theory was subsequently confirmed by Karagunis and Drikos (1934) who were able to synthesize optically active tartaric acid employing polarized light. It has also been reported that the antipodes of α-bromopropionic acid decay at different rates when subjected to circularly polarized ultraviolet light (Kuhn and Braun, 1929). Unfortunately, no natural source of circularly polarized light has been demonstrated, although the 'bremsstrahlung' γ-radiation emitted during β-decay is left circularly polarized (Goldhaber *et al.*, 1957). Garay (1968) has reported the preferential decomposition of D-tyrosine over L-tyrosine when alkaline solutions of the amino acid were bombarded with

Origins of Life 8 (1977) 91–144.

β-particles. More recently, Bonner (1974) reported that D-leucine is destroyed more rapidly than L-leucine by 'left-handed' longitudinally polarized electrons. Conversely, L-leucine was found to be preferentially decomposed when irradiated with 'right-handed' electrons. However, it should be noted that no enrichment of either enantiomer was observed when DL-leucine was irradiated.

In 1968, Ponnamperuma and Gabel suggested that

> geological sediments and samples from extraterrestrial sources should be clearly examined for optical activity . . . if samples containing optically active organic molecules are discovered, they would be the most meaningful indication for the existence of life when the sediment was laid down.

(This of course would be true only if the original mixture had not racemized.)

A type II carbonaceous chondrite fell in Murchison, Australia in 1969. Eighteen amino acids were identified in the extracts of this meteorite (Kvenvolden *et al.*, 1970a, 1971a,b). Since only racemic mixtures were obtained from the hydrolytic products, the amino acids were assumed to be of abiotic extraterrestrial origin. Predominating concentrations of L-amino acids found in the Orgueil, Mokoia and Murray meteorites are believed to be the result of terrestrial contamination by microorganisms (Oró *et al.*, 1971).

Amino acids have also been identified in extracts from lunar fines at levels of 10–70 ppb (Harada *et al.*, 1971; Fox and Hare, 1973). These amino acids are probably of extraterrestrial abiotic origin since the gas chromatographic profiles appear to be mainly or entirely atypical of terrestrial contamination. There is the possibility that at least some contribution to the total amino acid content observed in lunar fines, meteorites and in simulated prebiotic experiments is the result of the hydrolysis of acid labile amino acid precursors during extraction (Matthews, 1975; Cronin, 1975).

The search for extraterrestrial optically active amino acids has thus proved unsuccessful. However, the study of optical activity in geological sediments and fossils is also of great interest and in recent years there has been a renewed study of the diagenesis of proteins and in particular in the diagenetic racemization of protein amino acids. The first report of amino acids in fossil shells, bones and teeth was by Abelson (1954). Following dissolution of the fossils in acid and hydrolysis of any proteins present, he identified the amino acids by paper chromatography. The oldest fossil examined was a fish bone of Devonian age (360 million years) which contained glycine, alanine, glutamic acid, leucine, valine and aspartic acid. In further laboratory tests, Abelson (1954) reported that these same amino acids were the most stable to heat. A more detailed investigation of alanine indicated that under mild thermal conditions, it is sufficiently stable to survive more than a billion years. After determining the differences in amino acid composition in progressively older fossil shells, Abelson (1955) suggested that "the time rate of degradation of protein in fossils could, in principle, provide a dating method". Furthermore, by "correlating isotopic measurements on shells with such dating . . . a measure of the temperature of marine environment at the time of formation of the shells" could be determined (Abelson, 1955). Jones and Vallentyne (1960) compared amino acid compositions of fossil and recent samples. On the basis of differences they

observed and using Abelson's data for the stability of alanine (Abelson, 1954), they estimated the temperature to which a sample of Green River shale could have been continuously subjected. A closer look at the thermal reaction kinetics of amino compounds by Vallentyne (1964) led him to propose a new geothermometric method based on selective destruction of certain amino acids.

However, these suggested applications of amino acid diagenesis have not been further developed. Jones and Vallentyne (1960) had originally hoped that amino acids could be used as indicators of life in the Precambrian. Since their findings indicated that amino acids are insufficiently stable to survive such a length of time, they predicted that "the best approach to understanding Precambrian life would probably come from a search for optically active substances in Precambrian sediments". The unique relationship between optical activity, life and protein amino acids turned out to be the key to the present applications of protein diagenesis to geochronology and geothermometry.

Interest in diagenetic racemization was aroused by the report that increasing proportions of D-amino acids, presumably resulting from the racemization of the protein L-amino acids, were to be found in a series of fossil shells of increasing age (Hare and Abelson, 1967). The oldest fossils studied were of Miocene age in which the amino acids were virtually racemic. Hare and Mitterer (1968) then demonstrated that extrapolation of rate data for L-isoleucine in heated Mercenaria shells (90–165° C) agreed well with a series of radiocarbon dated samples from low temperature environments. By estimating a past temperature history (9–10° C) for a fossil Mercenaria shell from Wailes Bluff, Maryland which had a D-alloisoleucine to L-isoleucine ratio of 0.32, they estimated the age of the fossil to be 70 000 y. This was the first application of amino acid racemization rate data to geochronology.

This approach has now been applied to such areas as: the dating of sediments (Bada *et al.*, 1970; Wehmiller and Hare, 1971), shells (Hare and Mitterer, 1968), bones (Bada, 1972a, Dungworth *et al.*, 1974), teeth (Helfman and Bada, 1975, 1976) and corals (Wehmiller *et al.*, 1976), to the determination of sedimentation rates on the ocean floor (Bada and Schroeder, 1972), to estimations of the average temperature which a fossil has experienced since deposition (Bada *et al.*, 1973a; Schroeder and Bada, 1973) and to stratigraphic correlations (Mitterer, 1974).

This paper gives a more critical evaluation of the applications of amino acid racemization than has appeared in previous review articles (Hare, 1974; Bada and Helfman, 1975; Bada, 1975; Dungworth, 1976; Schroeder and Bada, 1976). We particularly wish to highlight the inherent pitfalls of these applications while giving a general coverage of this new area of research interest. Calculations have been included to help the reader evaluate whether the variables discussed contribute significant errors to age and temperature estimations.

We will deal with the following topics. There is a general review of the analytical procedures used to isolate amino acids and to measure the D/L amino acid ratios. This will introduce the reader to the need to distinguish between 'bound' and 'free' amino acids and to recognize that original D/L ratios may be modified during isolation of the amino acids from the fossil speciments. As further background, a section has been

included on the derivation of the rate expression for amino acid racemization together with the suggested mechanism of racemization and factors which have been shown to modify the rates of racemization of free amino acids in aqueous solution. This is followed by a critical review of the geochronological and geothermometric applications of amino acid racemization data. Other applications as listed earlier are also examined. The concluding section deals with several potential problems which are inherent to the technique. Of particular concern are whether high temperature heating experiments reproduce diagenetic changes in fossil proteins, whether diagenetic formation of amino acids is of concern and in particular, whether contamination is a serious problem.

In our opinion the evidence suggests a cautious approach in applying amino acid racemization kinetics to these fields of interest. Undoubtedly many problems have not yet been recognized and our understanding of diagenetic racemization must be considered to be still in its infancy.

2. Methodology

The data obtained for the D/L ratios of amino acids from geological samples are dependent on the methods of isolation and analysis. Entirely different results will be obtained if one looks at the total amino acid extract as compared, for example, to the free amino acids. Some variation in data will be expected between D/L ratios determined using enzymatic, as opposed to gas chromatographic, techniques. The following discussion has been included to give the reader a better appreciation of the arguments involved in later sections.

2.1. ISOLATION OF AMINO ACIDS FROM GEOLOGICAL SAMPLES

A routine procedure has gradually emerged for the isolation of amino acids from geological samples. The sequence includes the following steps. A visual inspection of the sample and perhaps manual scraping away of the outer contaminated layers (Dungworth *et al.*, 1973). Ultrasonication has often been employed to aid in the removal of adhering dirt (Bada and Protsch, 1973; Wehmiller and Hare, 1971). In studies of foraminifera, a wet sieving procedure has been used to separate clay and other non-calcareous sedimentary contaminants (Wehmiller and Hare, 1971). For samples retrieved from special environments, such as fossil bones from the Rancho La Brea tar pits, repeated washings with cold light petroleum ether have been included in the initial cleaning phase (Wyckoff *et al.*, 1964). Fossil samples are often dried at this stage and pulverized (Dungworth *et al.*, 1975).

The cleaned fragmented fossils are then ready for extraction of the amino acids. Repeated washings with dilute hydrochloric acid give the fraction containing the 'free' amino acids leaving the protein 'bound' amino acids in the insoluble residue. (The significance of the 'free' and 'bound' fractions will be discussed later.) The combined washings may be desalted using a cation exchange column such as Dowex 50W X8 (H^+) (Bada *et al.*, 1970). The amino acids are then ready for analysis by one of the methods described below (Section 2.2).

The insoluble residue is hydrolyzed to release the protein bound amino acids. Normal hydrolysis conditions employ 6N HCl for 22–24 hr at 100–110° C under a nitrogen atmosphere. Following hydrolysis the HCl is removed by evaporation, the residue redissolved in distilled water and desalted on a cation exchange column.

Some variations on this sequence have been precipitation of calcium ions with HF following hydrolysis (Wehmiller and Hare, 1971) and passage of sediment hydrolysates through both cation (Dowex 50) and anion (Dowex 1) resins (Kvenvolden *et al.*, 1970b). To avoid variations in amino acid composition which have been reported for individual layers of Mercenaria shells (Hare, 1969), Mitterer (1975) separated the middle layer with a dental tool. Finally, collagen has been isolated from bones by dialysis against EDTA (Bada, 1972a).

Recently, Pollock *et al.* (1977) have described in detail a procedure to be used for amino acid analyses where there is interference from low concentrations of nonprotein amino acids. They have suggested that the method is particularly suitable for the analysis of soils and sediments.

In some instances, 24 hr extractions with dilute NaOH at room temperature have been employed to isolate amino acids from geological specimens (Hare, 1972; Petit, 1974b). In view of the evidence (to be presented later) which indicates the extreme rapidity of base-catalyzed racemization of protein bound amino acids, this alternative should be avoided where D/L ratios of amino acids are to be determined.

2.2. RESOLUTION OF AMINO ACID ENANTIOMERS

There are several methods available for determining the D/L ratios of amino acids. Polarimetry requires pure amino acids in relatively high concentrations and these limitations have precluded its use for geochemical analyses, although it has found some application in studies of amino acid racemization in aqueous solution (Bada, 1971, 1972b) and for the investigation of the effects of side chain substituents on base-catalyzed racemization (Sato *et al.*, 1970). Enzymatic techniques have been used to determine the proportions of D to L-amino acids in soils (Aldag *et al.*, 1971) and in some fossil materials (Hare and Abelson, 1968; Hare, 1968; Petit, 1974a,b). This method involves measuring amino acid concentrations of the hydrolyzed protein before and after treatment with L- (or D-) amino acid oxidase. The limitations of this procedure are its inapplicability for determining trace amounts of D-amino acids (Larson *et al.*, 1971) and the possibility of contamination by L-amino acids from the enzyme. Manning and Moore (1968) have described a procedure for the determination of the D and L-isomers in a given sample of an amino acid based upon an ion-exchange chromatographic separation of the diastereomeric dipeptides which result upon derivatization with an L-amino acid N-carboxyanhydride. Bada and Protsch (1973) have routinely analyzed aspartic acid isolation from bones as the L-Leu-D-Asp and L-Leu-L-Asp dipeptide derivatives.

The most sensitive and versatile methods for the resolution of amino acid enantiomers are those employing gas chromatography. There are two approaches using this technique. The enantiomeric mixture can be reacted with an asymmetric reagent to give the

corresponding diasteriomers or resolution can be achieved by chromatography of a volatile derivative of the amino acid on an optically active stationary phase. The first approach is that formulated by Charles *et al.* (1963) who resolved amino acid enantiomers as the N-trifluoroacetyl-(±) 2-n-alkanols. This method has been developed for widespread application using (+) 2-n-butanol (Pollock *et al.*, 1965) to organic geochemistry (Kvenvolden *et al.*, 1971). Amino acids which carry the hydroxyl moiety are generally converted to their O-acetyl derivatives (Pollock and Kawauchi, 1968) since the O-TFA derivatives which were initially considered (Pollock and Oyami, 1966) were found to be unstable during analysis on some polar stationary phases. The second asymmetric center may also be introduced by condensation of the amino function with a reagent such as N-trifluoroacetyl-L-prolyl chloride (Westley and Halpern, 1969) to give the diastereomeric dipeptides paralleling the approach of Manning and Moore (1968) discussed earlier. The limitation of diastereomeric preparation is that often highly optically pure reagents are not easily obtained. Thus the reagent's antipodal impurity may introduce large errors when only small relative amounts of amino acid enantiomer are present.

The second gas chromatographic approach is an elegant, accurate and sensitive technique which has been receiving increasing attention. The first optically active stationary phase, N-trifluoro-acetyl-L-isoleucine lauryl ester, was synthesized by Gil-Av *et al.* (1966) and its resolving capabilities were demonstrated by coating on glass capillary columns. Improved separations have been obtained using the N-trifluoroacetyl-dipeptide cyclohexyl esters (Gil-Av and Feibush, 1967; Koenig *et al.*, 1970; Parr and Howard, 1972). We have found N-lauryl-L-valyl-tert-butylamide (Charles *et al.*, 1975) to be a very efficient stationary phase. The latter phase has the extra benefit of higher temperature stability.

The techniques of analysis are sufficiently refined that D/L ratios should be reproducible and accurate to $\approx \pm 5\%$. Bada and Helfman (1975) have compared the racemization data obtained by Bada's group at Scripps and Kvenvolden's group at the Ames Research Center for the Del Mar Man and Sunnyvale skeletons. They indicate that there was general agreement between the Scripps and NASA results and noted that this was important because it showed the inter-laboratory reproducibility of racemization

TABLE I

Inter-laboratory reproducibility of age estimates in fossil bones[a]

Fossil bone location	Average D/L asp	Laboratory	Average age[b]
Del Mar, California	0.507	Scripps	45 200
	0.385	NASA	31 100
Sunnyvale, California	0.510	Scripps	70 400
	0.420	NASA	54 000

[a]Data given by Bada and Helfman, 1975.
[b]Calculated from Equation (17), $c = 0.14$, $k_{asp} = 1.08 \times 10^{-5}$ yr^{-1} (Del Mar); $k_{asp} = 7.0 \times 10^{-6}$ yr^{-1} (Sunnyvale).

measurements. However, the agreement of the data is, in reality, quite poor and results in large differences in estimated ages for the same samples (Table I). Part of the divergence in results may be attributed to the two different analytical techniques used. The Scripps data was for amino acids analyzed as their dipeptide derivatives on an amino acid analyzer, whereas the Ames group used gas chromatography of the N-trifluoroacetyl amino acid 2-butyl esters. Clearly there is still room for improvement here.

2.3. THE EFFECTS OF ACID HYDROLYSIS

Acid hydrolysis is the standard procedure for liberating the amino acids bound in the fossil proteins. Consequently, it should be recognized that this step may modify the original D/L amino acid ratios. There is some acid-catalyzed racemization induced by the hydrolysis and there is the possible complication of deamidation of asparagine and glutamine to aspartic and glutamic acids, respectively.

2.3.1. Racemization

As discussed earlier, a typical protein hydrolysis employs 6N HCl at 105–110° C for 22–24 hr. It has long been recognized that some racemization of the amino acids takes place when proteins are treated in this manner. Some of the earliest quantitative data was the result of the controversy over the claim that tumors characteristically contained D-glutamic acid (Kogl and Erxleben, 1939). In a rebuttal of this theory it was shown that on refluxing L-glutamic acid for 24 hr in 6N HCl, between 3 and 5% D-glutamic acid was formed (Wiltshire, 1953). Hydrolysis of horse myoglobin and beef insulin under these same conditions gave, respectively, 6.6% and 4.6% D-glutamic acid (Wiltshire, 1953).

Some interesting data have been published on the racemization that occurs when the nonapeptide bradykinin (L-Arg-L-Pro-L-Pro-Gly-L-Phe-L-Ser-L-Pro-L-Phe-L-Arg) is hydrolyzed (Manning and Moore, 1968). Bradykinin and a control containing a proportional mixture of the pure L-amino acids were similarly treated (6N HCl, 22 hr, 110° C). The amounts of racemization observed for the free and bradykinin bound serine, proline and arginine agreed very well. But for phenylalanine, the free and bradykinin forms racemized 1.4 and 3.9%, respectively. Furthermore, hydrolysis of L-Glu-L-Phe gave 1.9% D-phenylalanine. This they took to indicate that the Phe-5 of bradykinin is racemized to a greater degree than Phe-8. Two extreme examples of the effect of structure on the amount of racemization induced by hydrolysis are the reports of complete inversion of L-proline to D-proline in an ergot alkaloid (Jacobs and Craig, 1935; Stoll *et al.*, 1951) and complete racemization of isoleucine in the sequence L-Ile-L-Cys of bacitracin (Craig *et al.*, 1954).

The purpose of the above discussion is to illustrate that

(a) the same amino acid may racemize at different rates during acid hydrolysis depending upon its environment in the protein chain, and

(b) that heating a free L-amino acid as a control may not always be a reliable indicator for the degree of racemization induced during protein hydrolysis (Manning, 1971).

TABLE II

The percent racemization of free amino acids induced by hydrolysis conditions (6N HCl)

Conditions	*Amino acid:*	Ser	Ala	Arg	Val	Leu	Ile	Glu	Phe	Pro	Asp	Lys	Met	*Reference*
Temperature	Time													
Reflux	6hr	–	22	–	19	5	–	–	–	–	–	–	–	(Aldag *et al.*, 1971)
105°C	24hr	–	1.1	–	0.3	1.3	0.5	–	–	–	–	–	–	(Nakaparksin *et al.*, 1970)
120°C	24hr	–	3.7	–	0.6	2.1	1.4	–	–	–	–	–	–	
110°C	24hr	–	0.5	–	0.2	0.8	0.3	1.9	0.1	1.7	1.7	–	–	(Hare and Hoering, 1973)
110°C	22hr	0.4	–	1.6	–	–	–	–	1.4	2.2	–	–	–	(Manning and Moore, 1968)
110°C	18hr	0.5[a]	–	–	–	–	–	3.3	–	–	3.7	–	–	
110°C	22hr	0.4	1.0	1.6	0.7	1.3	1.0	–	–	2.2	–	3.0	2.2	(Manning, 1970)

[a] Ser + Thr.

TABLE III

The percent racemization induced during acid hydrolysis (6N HCl) of protein and peptides

Protein	*Conditions*		*Amino Acids*										Reference
	Temp.	Time	Ala	Glu	Val	Ile	Leu	Pro	Arg	Phe	Asp	Ser	
Pneumococcal C-Polysaccharide	110°C	22hr											(Manning, 1971)
Bradykinin	110°C	22hr	–	–	–	–	–	2.4	1.7	3.9	–	–	(Manning and Moore, 1968)
Ribonuclease	110°C	18hr	–	4.2	–	–	–	–	–	–	4.4	0.2[a]	(Manning and Moore, 1968)
Mammoth collogen[b]	105°C	(24hr)	1.2	2.7	0.7	–	1.6	–	–	2.6[c]	3.0	–	(Dungworth *et al.*, 1976)
Horse myoglobin	Reflux	24hr	–	6.6	–	–	–	–	–	–	–	–	(Wiltshire, 1953)
Beef insulin			–	4.6	–	–	–	–	–	–	–	–	(Wiltshire, 1953)

[a] Ser + Thr.
[b] Obtained by difference of 4.75hr hydrolysis from the 24hr hydrolysis as suggested by Dungworth *et al.*
[c] 48hr.

TABLE IV

The effect of hydrolytic racemization on age estimates of a fossil bone

% D-Asp from hydrolysis	0	1	2	3	4	5	6.5
Constant, $t = 0$ [a]	0	0.020	0.041	0.062	0.083	0.105	0.140
Calculated date [a] (yr)	22 400	21 700	21 000	20 300	19 600	18 800	17 700

[a] Calculations based on Equation (17): $\ln\left(\frac{1 + D/L}{1 - D/L}\right) - C = 2\,k_{asp}\,t$.

$k_{asp} = 1.48 \times 10^{-5}$ yr^{-1}, D/L asp = 0.32 (Bada and Protsch, 1973).

The heating of free amino acids has often been a suggested control for protein hydrolysis in geochronological applications of amino acid racemization data (Hare and Hoering, 1973; Nakaparksin *et al.*, 1970). Some typical figures obtained for treatment of free amino acids under hydrolysis conditions are shown in Table II. Apart from the unusually high results reported by Aldag *et al.* (1971), racemization is between 0.1 and 3.7% with the variability for any one amino acid less than 2%. Some scattered data for the racemization of amino acids during hydrolysis of proteins is given in Table III. The variability in both tables may be due partly to the different methods of analysis.

Bada and Protsch (1973) determined a D/L aspartic acid ratio of 0.07 for a modern bone following acid hydrolysis. This gave a $t = 0$ value of 0.14 in Equation 1 which was then used for all subsequent determinations of fossil bone ages. Since fossil collagen and modern bone collagen show some definite compositional differences (Dungworth *et al.*, 1975; Wyckoff *et al.*, 1963) a variability of several percent might be expected for the amount of hydrolysis induced racemization between the two collagens on the basis of the data presented above. Acid hydrolysis may produce the variability in estimated ages shown in Table IV. Table IV demonstrates that a 2% variation in the amount of D-aspartic acid produced during acid hydrolysis causes a corresponding 6% variation in the estimated age. This source of variation should be recognized, whether or not it is significant in terms of geological ages.

2.3.2. Deamidation of asparagine and glutamine

Protein and peptide bound amide groups are quantitatively split off during acid hydrolysis (Wilcox, 1967). Thus asparagine and glutamine are deamidated to give aspartic and glutamic acids. Considering that aspartic acid has been so strongly promoted as the amino acid for dating bones (Bada and Helfman, 1975), it is surprising that the possible conversion of asparagine to aspartic acid has been almost completely ignored. The only references to this potential problem have been by Petit (1974a,b) and Schroeder (1974). Petit believed that an advantage of his enzymatic technique was that it avoided an acid

hydrolysis which would convert any L-asparagine to L-aspartic acid. Schroeder (1974) estimated that the deamidation half-life of asparagine at 2° is between 1 and 100 years, while the half-life for the racemization of aspartic acid in the insoluble fraction from foraminifera is probably several hundred thousand years. He concluded "that the amide is unlikely to be present at all in Recent shells". These estimations were based on data for the deamidation half-times of a series of 34 asparagine containing pentapeptides in phosphate buffer at pH 7.4–7.5, ionic strength 0.15–0.20 and 37° (Robinson, 1974). Whether this data is directly applicable to protein in a geological matrix is not known. There is some evidence that at least under appropriate conditions, asparagine is present in Pleistocene fossil bones. In his amino acid analyses of fossil bones from the Rancho La Brea tar pits and Recent bones, Ho (1965) reported ammonia concentrations. If the ammonia values do represent the amides in the samples (asparagine + glutamine), then the proportion of amides to aspartic or glutamic acids is much the same in both Recent and fossil bones. Given that asparagine may not deamidate too rapidly, at least in the sterile, anhydrous environment of the Rancho La Brea tar pits, a complex set of conditions arise. The L-asparagine will be deamidating with time to L-aspartic acid as well as racemizing to D-asparagine. The D-asparagine may also deamidate to yield D-aspartic acid. Any asparagine left during sample preparation will be quantitatively converted to the corresponding enantiomer of aspartic acid on acid hydrolysis.

If the deamidation rate constant is indeed orders of magnitude greater than the racemization rate constant for aspartic acid, associated errors are negligible. This potential problem does warrant further study.

It should be apparent then that the method of isolation and analysis of amino acids from fossils may be expected to result in some variability in the determined D/L ratios. Acid hydrolysis may modify the original ratios by an acid-catalyzed racemization or by deamidation. Furthermore, it is important that there is no doubt as to which amino acid fraction is being analyzed whether protein bound, soluble peptide or free.

3. Theory and Kinetics of Amino Acid Racemization

3.1. DERIVATION OF THE RATE EXPRESSION FOR AMINO ACID RACEMIZATION

The general racemization reaction for amino acids may be expressed by Equation (1)

$$\text{L-Amino Acid} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{D-Amino Acid} \,, \tag{1}$$

where k_1 and k_2 are the forward and reverse rate constants, respectively, and the equilibrium constant $K = k_1/k_2$. The rate expression for the reaction is

$$-\frac{\mathrm{d}[\mathrm{L}]}{\mathrm{d}t} = k_1[\mathrm{L}] - k_2[\mathrm{D}] \,, \tag{2}$$

where [L] and [D] are the respective concentrations of the L and D enantiomers. Integration of Equation (2) gives

$$\ln\left(\frac{1+[\mathrm{D}]/[\mathrm{L}]}{1-K'[\mathrm{D}]/[\mathrm{L}]}\right) = (1+K')k_1 t + C\,, \tag{3}$$

where $K' = 1/K = k_2/k_1$. The constant of integration C, will be zero if at time zero there is no D enantiomer present, otherwise

$$C = \ln\left(\frac{1+[\mathrm{D_0}]/[\mathrm{L_0}]}{1-K'[\mathrm{D_0}]/[\mathrm{L_0}]}\right). \tag{4}$$

Bada and Protsch (1973) found that for a modern bovine bone carried through the same analytical steps as for the fossil bones the ratio of D/L aspartic acid was 0.07. The calculated value for the $t = 0$ term was thus $C = 0.14$.

In general, for free amino acids in aqueous solution, $k_1 = k_2$ and Equation (3) reduces to

$$\ln\left(\frac{1+[\mathrm{D}]/[\mathrm{L}]}{1-[\mathrm{D}]/[\mathrm{L}]}\right) = 2\,kt + C\,. \tag{5}$$

Another useful expression is that for the half-life, τ, for a given racemization reaction. This occurs for the time at which there is 25% of the D-enantiomer and 75% of the L-enantiomer. The general expression derived for τ from Equation (3) is

$$\tau = \frac{\ln 2}{k_1 + k_2}\,, \tag{6}$$

or,

$$\tau = \frac{\ln 2}{(1+K')k_1}\,. \tag{7}$$

It can be shown that the D/L ratio of an amino acid at the half-life time is given by

$$(\mathrm{D/L})_\tau = \frac{K}{K+2}\,. \tag{8}$$

The foregoing equations will be referred to in subsequent discussions and calculations. They are necessary for a complete understanding of this review.

3.2. ISOLEUCINE AND THE EQUILIBRIUM CONSTANT *K*

An important exception to the generalization that $k_1 = k_2$ is the case of isoleucine. L-Isoleucine has asymmetric centers at both its α- and β-carbon. Heating causes

TABLE V

Values reported for the Isoleucine epimerization equilibrium constant, K_{Ile}

K_{Ile}	Temperature (°C)	Environment	Reference
1.30	≈2	Early Miocene foraminifera (*t*) [b]	(King and Hare, 1972a)
1.4	≈2	Miocene sediments (*f*)	(Wehmiller and Hare, 1971)
1.29	130	6N HCl (*f*)	(Nakaparksin *et al.*, 1970)
1.3	140	Mercenaria shells (*t*)	(Hare, 1969)
1.25	[a]	Aqueous solution (*f*)	(Hare and Mitterer, 1968)
1.38	148	Modern bones (*b*)	(Bada, 1972a)
1.28	150	Fossil bone (*b*)	(Dungworth *et al.*, 1973)
1.4	[a]	Alkaline solution (*f*)	(Hare and Mitterer, 1966)

[a] Temperature not specified.
[b] *f* = free isoleucine, *b* = protein bound isoleucine, *t* = total isoleucine.

TABLE VI

The effect of the equilibrium constant K, on half-life determinations of Isoleucine

K	k_1 [a] x 10^7 (sec^{-1})	τ (hr)
1.00	143.4	6.7
1.25	50.4	21.2
1.30	48.2	22.6
1.35	46.4	23.8
1.40	44.9	25.0

[a] Rate constants determined from data for the racemization of isoleucine at 161.9° C, pH 7.6, ionic strength 0.50 (Smith *et al.*, 1976).

racemization (or more strictly, epimerization in the case of diastereomers) at only the α-carbon to produce the diastereomeric derivative D-alloisoleucine. (Inversion of the β-carbon would give the enantiomer D-isoleucine but this is not observed during simulated or natural diagenesis.) In this epimerization equilibrium, the forward rate constant k_1 is greater than the reverse constant k_2 and thus K becomes greater than unity. Reported values for K vary between 1.25 and 1.40 (Table V).

At times the value of K_{Ile} has been arbitrarily assigned (Wehmiller *et al.*, 1976). The effect of K_{Ile} on the half-life of the isoleucine epimerization at an elevated temperature (161.9° C) is demonstrated in Table VI (Smith *et al.*, 1976). Extrapolation of our data to 25° C gives a half-life of 9000 yr or 8200 yr depending on whether K_{Ile} is assigned a value of 1.30 or 1.40, respectively. To avoid unnecessary errors an accurate value of K_{Ile} should be determined for each fossil under study. Bada (1971) assumed a value of $K_{\text{Ile}} = 1.0$ when he calculated the half-life for isoleucine epimerization at 25° C (pH 7.6, ionic strength 0.5) as 34 700 yr. Our data (Table VI) indicates that the error from this assumption is much greater than the 10% claimed. In fact, in a more recent publication in which $K_{\text{Ile}} \approx 1.4$, Bada (1975) has recalculated the isoleucine epimerization half-life at 25° C to be 48 000 yr. (This age may also be in error, since Bada stated that for isoleucine, the half-life is the time required for the alloisoleucine/isoleucine ratio to reach 0.345. For $K = 1.40$, the ratio is 0.412 (see Equation (8).)

Although no temperature dependence has been established for K_{Ile}, it has been suggested that "by analogy with isotopic equilibrium, a simple $1/T$ relation might be expected

$$(\text{Alloisoleucine/Isoleucine})_{\text{equ.}} = 1 + \approx 109/\text{T},$$

with the constant 109 being fitted to the observed data" (Wehmiller and Hare, 1971). This was based on the observation that ratios no lower than 1.25 had been recorded at 165° C, and ratios had not exceeded 1.4 at ambient temperatures. The data presented in Table V, however, does not support this view and there is no obvious trend evident.

Values of $K_{\text{IIe}} = 1.4$ have been reported for example, at both elevated and ambient temperatures. Temperature is more likely to affect K_{IIe} for protein bound isoleucine than for the free amino acid. It is clear that the effects of unknown factors are operative in the different values recorded for the equilibrium constant for modern bone and fossil bone (which were determined at almost the same temperature).

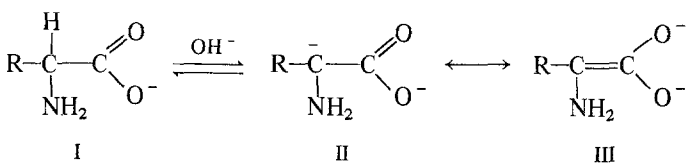
Isoleucine has been selected in the past for dating because it is stable, has a useful half-life and because it epimerizes to give its diastereomer D-alloisoleucine, which is readily separable for analysis by conventional ion-exchange chromatography. Until the temperature and environmental dependence of K_{IIe} are more fully understood, the possible source of error that the uncertainty of K_{IIe} introduces to age estimates based on isoleucine racemization, should be recognized.

It has been inferred that for amino acids with one asymmetric center $k_1 = k_2$, i.e., $K = 1.0$. Petit (1974b) has discussed this assumption and has presented evidence that although it may be valid that $k_1 = k_2$ for the free amino acid, the forward and reverse rate constants "may be quite different in the protein environment". He has estimated that the "differences in the steric interactions between the D and L forms in a protein environment . . . may be as high as 1 kcal mole^{-1}, which is several percent of the activation energy in the free amino acid". Assuming that a 1 kcal mole^{-1} difference could arise, what effect would this have on the half-life of isoleucine in a fossil bone? The following activation parameters have been published for isoleucine in a fossil bone (Dungworth *et al.*, 1973): $E_a = 30.5$ kcal mole^{-1}, $A = 8.28 \times 10^{16}$ yr^{-1}. Substituting this data into the Arrhenius equation for a temperature of 7° C gives a rate constant $k_1 = 1.326 \times 10^{-7}$ yr^{-1}. If $k_1 = k_2$, then the half-life is $\approx 2.61 \times 10^6$ yr. On the other hand, if we assume that the activation energy for the forward reaction is $E_{a1} =$ 30.5 kcal mole^{-1}, but in the reverse reaction $E_{a2} = 29.5$ kcal mole^{-1} (and with the A values the same), then $k_1 = 1.326 \times 10^{-7}$ yr^{-1} and $k_2 = 7.992 \times 10^{-7}$ yr^{-1} and the half-life is a vastly different $\approx 0.744 \times 10^6$ yr. This then is a further unexplored complication in the application of amino acid racemization rate data to geochronology.

Before proceeding with factors that affect the racemization rates of free amino acids in aqueous solution the mechanism of racemization will be presented.

3.3. THE MECHANISM OF AMINO ACID RACEMIZATION

The accepted mechanism for the racemization of free amino acids in aqueous solution is still that proposed by Neuberger (1948). He suggested that for base-catalyzed racemization the first step is abstraction of the α-proton by the base to produce the carbanion II. This carbanion is then stabilized by resonance with III.



I II III

Neuberger predicted that

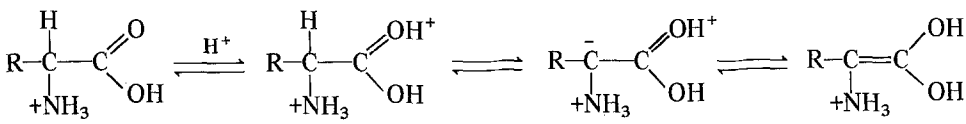
(a) any substitution on the carboxyl group would enhance racemization by abolishing its charge, thus allowing greater ease of ionization of the α-proton;

(b) introduction of an electronegative substituent on the β-carbon or on the amino group would promote racemization.

That abstraction of the α-proton is involved in racemization has been demonstrated by the finding that the percentage racemization and deuteration are identical (within experimental error) for the racemization of amino acids and their derivatives in deuterated acetic acid (Matsuo *et al.*, 1970) and in sodium deuteroxide-deuterium oxide and sodium methoxide-deuterated methanol (Matsuo *et al.*, 1967). Furthermore, in the racemization of amino acids in tritiated HCl, it was reported that the incorporation of tritium into the α-position is proportional to the amount of racemization within 1% for 10 protein amino acids studied (Manning, 1970).

Studies substantiating prediction (a) have been published (Matsuo *et al.*, 1967) and results in accordance with (b) have also been reported (Sato *et al.*, 1970; Bada, 1972b). Sato *et al.*, (1970) concluded from their base catalysis studies that the relative susceptibility of a series of N-benzoyl amino acid anilides to racemization "is proportional to the electronegativity of the α-substituent (σ^*) for derivatives of phenylglycine". It has been concluded that this same factor also determines the relative rates of racemization of amino acids in bone collagen (Bada *et al.*, 1973b). On the basis of recent studies (Smith *et al.*, 1976) it is suggested that this is an oversimplification and that relative racemization rates are determined by a complex combination of inductive, resonance, steric, proximity and solvent effects.

For acid-catalyzed racemizations, Neuberger (1948) proposed that the carboxylic acid (IV) first acts as a proton acceptor giving (V). The positive charges then promote ionization of the α-carbon and the resulting polar form (VI) will be stabilized by resonance with the nonpolar form (VII).



IV V VI VII

On the basis of these models Neuberger (1948) predicted that the removal of the positive and negative charges by peptide formation would facilitate racemization by both acid and base catalysts. Furthermore, the amide linkage can be considered a resonance hybrid of (VIII) and (IX), and the dipolar form (IX) was expected to facilitate ionization of the asymmetric carbon.

VIII IX

The conclusion was that "dipeptides should be more readily racemizable by bases than the free amino acids themselves, and this effect should become more marked as the length of the peptide chain increases". The effect of the form of the amino acid upon racemization will now be taken up.

3.4. THE RACEMIZATION OF 'BOUND' VERSUS 'FREE' AMINO ACIDS

Amino acids are known to racemize at different rates depending on whether they are in solution as the free amino acid or are incorporated into a protein chain (protein 'bound' amino acids). Levene and Bass (1929) found that nine amino acids kept in a solution of 1N NaOH for 25 days were stable and there was virtually no measurable racemization. Serine has been found to be unracemized after 22 hr at room temperature in excess 1N NaOH (Crawhall and Elliott, 1951). On the other hand, when polyserine was incubated at 30° in 0.6N or 1.0N NaOH solution, there was measurable racemization after only 20 min (Table VII). Significant racemization has also been observed when albumin, casein, edestin, fibrin and gelatin are treated with dilute alkali (Dakin and Dudley, 1913; Levene and Bass, 1929). The increased racemization in proteins was ascribed by Dakin (1912) to the internal keto-enol tautomerism, possible only for protein bound amino acids. More recently significant racemization was observed in 0.5N NaOH extracts of humic and fulvic acid soil fractions (Pollack and Frommhagen, 1968). The observation that protein bound amino acids are much more susceptible to base-catalyzed racemization than their free amino acid counterparts is consistent with Neuberger's theory discussed earlier. An interesting application of this susceptibility has been the prolongation of the activity of α-melanocyte-stimulating hormones on treatment with dilute alkali (Pickering and Li, 1964; Geschwind and Li, 1964). Racemization alone was found to be sufficient to explain the prolongation phenomenon. In a similar study with α-melanotropin, 0.1 M alkali at 60° C induced serine, methionine, histidine, phenylalanine and arginine to be racemized to an extent of 50–70%, glutamic acid, tyrosine and tryptophan 30–40%, and lysine, proline and valine to an extent of 10% or less (Lande and Lerner, 1971). The racemization was shown to occur without concomittent peptide degradation or other chemical changes. However, for amino acids isolated from fossils and sediments, the free

TABLE VII

Racemization of poly-L-serine in aqueous sodium hydroxide[a]

	% D-serine in total serine recovered Time of incubation (min)			
Medium	20	40	60	120
0.6N NaOH	8.0	7.4	17.7	22.3
1.0N NaOH	14.6	24.0	28.5	36.0

[a] Bohak and Katchalski, 1963.

amino acids are invariably more racemized than the protein bound amino acids (Dungworth *et al.*, 1973; Bada, 1975). Hare (1971) concluded that "at the time an amino acid is liberated from a peptide bond in an alkaline environment it is in an activated state that enhances the racemization reaction". However, we feel there is sufficient evidence to make this theory very unlikely. Levene and Pfaltz (1925; 1926a,b) found that ketopiperazines racemized on standing in 1 equivalent of dilute alkali (0.1 n NaOH) but when treated with stronger base (1.0 N, 5 to 15 equivalents) there was no racemization. Similarly on treating gelatin with 0.1 N and 1.0 N alkali there was racemization, but not with 3.0 N alkali (Levene and Bass, 1927). In both instances the reasonable conclusion was that in stronger base the rate of hydrolysis was greater than the rate of racemization. Once the amino acids were in the free state (or as the dipeptides from the ketopiperazines) they were not as susceptible to racemization nor did the hydrolysis itself induce racemization. In determining the D-alloisoleucine to L-isoleucine ratios in the free and total fractions of heated proteins, Schroeder (1974) concluded that there was no epimerization caused by peptide-bond hydrolysis. Bada and Schroeder (1972) also felt that the concerted mechanism was untenable on the grounds that hydrolysis and racemization involve different carbon centers and therefore should be independent.

It is more likely that the reason free amino acids are more racemized than those which are protein bound in geological samples is due to catalysis by trace concentrations of metal ions as proposed by Bada (1975) (see Section 3.5).

Clearly the diagenetic racemization of a protein will not be a simple process, but a complex interaction of hydrolysis rates and catalytic effects complicated by the fact that for each state that the amino acid passes through – protein bound, peptide and free amino acid – there is some characteristic racemization rate. It might be predicted that the most likely geochronological application of amino acid racemization data to succeed would be that which isolated only one particular state of the amino acid for study. Analysis of the protein fraction is the best of the three since it is less susceptible to contamination and probably not significantly affected by metal catalysts. The only problem might be the decreasing amounts of intact protein remaining as the age of the fossils increase. In fossil oyster shells for example, there is a rapid decrease in the protein content at first, although this loss becomes minimal for the Oligocene and more ancient fossils. Pleistocene fossils contain twenty times as much protein as Pliocene specimens which in turn have about three times the protein content as older specimens (Matter *et al.*, 1969).

3.5. FACTORS AFFECTING THE RACEMIZATION RATES OF FREE AMINO ACIDS IN AQUEOUS SOLUTION

One important variable known to greatly influence the racemization of free amino acids is pH. In the pH range of 5 to 8, however, Bada (1972b) reports that racemization is relatively pH independent. Consequently, it has been suggested that by choosing fossil material with a calcareous or an hydroxylapatite matrix, the effects of pH might be

TABLE VIII

Reported Arrhenius parameters for the racemization of free amino acids in aqueous solution

Amino Acid	Dungworth *et al.* (1973) (pH 7.0)		Bada (1971) (pH 7.6)		Smith *et al.* (1976) (pH 7.6)		Nakaparksin *et al.* (1970) [b] (6N HCl)
	E_a(kcal mole^{-1})	A(s^{-1}) x 10^{-9}	E_a(kcal mole^{-1})	A(s^{-1}) x 10^{-10}	E_a(kcal mole^{-1})	A(s^{-1}) x 10^{-8}	E_a(kcal mole^{-1})
Asp			31.0	17.03	–	–	–
Ala	29.4	3.15	30.9	4.09	28.5	20.72	25
Val	29.1	0.69	–		28.6	8.36	31
Leu	29.3	2.03	–		27.7	5.85	25
Ile	28.9	1.19	31.4	3.03	27.9 [c]	4.57	–
Phe	–		28.6	0.356	24.0	0.173	–
α-Ab [a]	–	–	–	–	–	–	25

[a] α-amino-n-butyric acid.

[b] A values not reported.

[c] K_{Ile} was assigned a value of 1.40. We are presently investigating the alloisoleucine/isoleucine equilibrium at elevated temperatures.

minimized since the matrix would act to buffer the sample against pH fluctuations (Dungworth *et al.*, 1973). This appears to be a reasonable precaution.

The racemization of free amino acids is very significantly catalyzed by metal ions, particularly Cu^{2+} and Mg^{2+} (Bada, 1971; Bada, 1975). For amino acids which are bound up in a protein chain, metal catalysis should be negligible. We have recently found (Smith *et al.*, 1976) that ionic strength is a further variable. Preliminary studies indicate that at a pH of 7.2, ionic strength does not affect the rate of racemization of free amino acids, but at pH 10.0 increasing the ionic strength causes a corresponding increase in the racemization rate. It is not yet known whether this is also true for the amino acids when in the protein form.

From the results of high temperature aqueous solution kinetic studies, activation energies have been determined for the racemization of several amino acids (Dungworth *et al.*, 1973; Bada, 1971; Smith *et al.*, 1976). As evidenced by the scatter in data (Table VIII), accurate activation parameters are not readily obtained. It should be emphasized that a 1% error in the determination of the activation energy will result in a corresponding 20% error in any calculated data.

A study of the kinetics of racemization of free amino acids has shown that metal ions, pH and ionic strength contribute significantly to the complexity of diagenetic racemization. These factors help explain the nonlinearity of racemization kinetics which have been observed in some total fossil hydrolysates. Protein bound amino acids racemize much more readily than free amino acids in basic solution as predicted by Neuberger's theory. In diagenetic racemization the reverse is observed due to the catalytic effects of metal ions on free amino acids. The susceptibility of protein bound amino acids to racemization by base serves as a precautionary warning against base extraction of protein from fossil specimens. Finally, significant variability in the Arrhenius parameters for isoleucine may be expected as the result of the uncertainty of the value of the isoleucine equilibrium constant.

4. Applications of Amino Acid Racemization Kinetics to Geochronology

The three major categories of geological samples to which amino acid racemization data has been applied for geochronological determinations are fossil shells, fossil bones and deep sea sediments. The technique has been more successful with shells and bones than with sediments. Each category has its own characteristic problems and consequently, each will be dealt with separately. Finally, some miscellaneous applications – corals, woodrat middens and teeth – will be briefly reviewed.

4.1. SHELLS

Shells consist of a layered calcium carbonate matrix with the organic material enclosed within the crystalline structure (Meenakshi *et al.*, 1971). The normal crystal type is aragonite or calcite. It has been shown that each calcified layer has a different amino acid

composition and that calcite matrices are consistently higher in the ratio of acidic to basic residues than aragonite matrices (Hare, 1963). Following an injury, new shell is regenerated which differs in composition from the normal shell (Saleuddin and Hare, 1970). The dense matrix provides protection of the protein from bacterial attack and helps to minimize leaching away of the organics by ground waters (Abelson, 1963). Historically, it was a study of the D-alloisoleucine content of progressively older shells which first indicated a correlation between amino acid racemization and age. D-alloisoleucine is a non-protein amino acid and was not found in the shell protein of living animals. As the age of the shell increased, however, so too did the proportion of D-alloisoleucine to L-isoleucine (Hare and Mitterer, 1966). This observation prompted an investigation of the enantiomeric distribution of other amino acids. Thus it was found that Recent shell fragments contained only the L-amino acids while in the oldest fossils the amino acids were almost racemic (Hare and Abelson, 1967). Hare and Mitterer (1968) heated fragments of modern shell in water in tubes sealed under nitrogen to study further the kinetics of the epimerization of isoleucine with time. Extrapolation of the Arrhenius plot so obtained (based on the total isoleucine, both free and bound) was reported to agree with data from a series of radiocarbon dated fossil shells from areas with average temperatures of 23° to 24° C. The equation of the line fitted to these results was later reported (Hare, 1974) to be

$$\log k_1 (\mathrm{yr}^{-1}) = 16.70 - 6150/T \tag{9}$$

Data from foraminiferal tests from a deep sea core also fitted the extrapolated line quite well. The good correspondence was taken to indicate that laboratory heating simulated the natural diagenetic changes in the fossils. Using these results, an Upper Pleistocene Mercenaria shell with a D-alloisoleucine to L-isoleucine ratio of 0.32 and a probable average temperature history of 11°–12° was assigned an age of 60 000 yr. This was the first application of amino acid racemization data to age estimations.

The epimerization rate of isoleucine in foraminiferal tests would be expected to be at least similar to that in shells since both are calcareous matrices. But it is surprising that the above data agrees so well, particularly in the light of a later report that even within the class of fossil planktonic foraminifera there is a species effect upon the L-isoleucine epimerization (King and Hare, 1972a; see Section 4.2).

On the basis of their findings Hare and Mitterer (1968) suggested 3 potential uses of amino acid enantiomeric ratios in fossils:

(a) if an accurate temperature is known, an approximate age can be calculated;

(b) effective incubation temperature can be estimated for well-dated material;

(c) stratigraphic correlations can be made particularly for deep sea cores where temperatures are uniform and constant.

Although these applications have been explored, the geochronological significance of the racemization of amino acids from fossil shells has not been widely investigated. Reports have appeared by Mitterer who has estimated ages and diagenetic temperatures of Pleistocene deposits from Florida (Mitterer, 1975), correlated Pleistocene deposits of

southern Florida (Mitterer, 1974), and estimated paleotemperatures for the southeastern United States based on isoleucine epimerization in Mercenaria (Mitterer, 1972).

Mitterer (1975) established that the kinetics of epimerization were first order in Mercenaria up to an alloisoleucine/isoleucine ratio of ≈ 0.90. Extrapolation of an Arrhenius plot for elevated temperature heating experiments was found to pass through the data points obtained from a series of seven radiocarbon dated Mercenaria fossils of Late Holocene age and known diagenetic temperature. A least squares line fitted to all this data had the equation

$$\log k_1 (\mathrm{yr}^{-1}) = 17.29 - 6417/T \tag{10}$$

The integration constant in Equation (3) determined by Mitterer from the analysis of several modern samples which were not heated was 0.018. It is interesting to compare an age estimated using Mitterer's Equation (9) and Hare's Equation (10). With a D/L ratio of 0.18, $K = 1.30$, $T = 294^\circ$ K, $C = 0.018$ and substituting the data into Equation (3) and Equations (7) or (8) gives an age of $\approx$5760 yr or $\approx$2770 yr (for respectively, Mitterer's data and Hare's data).

It must be concluded that there is quite a large uncertainty in age estimations of shells based on extrapolation of the Arrhenius parameters for the epimerization of isoleucine. A calibration approach (see Section 4.3) may be more successful. Mitterer (1975) has used this technique to assign ages to Pleistocene marine deposits of Florida which were in accord with radiometric evidence. Shells should not be very susceptible to microbial contamination but are susceptible to species effects (Section 6.4).

4.2 MARINE SEDIMENTS

Sedimentary deposits are a complex mixture of detrital particles, clay minerals, iron and manganese oxides together with the calcareous matter of foraminifera and shells. In one study, a Recent sediment (few thousand year old) was found to contain $3\ \mu\ \mathrm{mol\ g}^{-1}$ (Erdman *et al.*, 1956). The amino acid concentrations at the sediment-sea water interface are ≈ 0.1–$2\ \mathrm{mg\ g}^{-1}$ of dry sediment (Bada *et al.*, 1970). With progression down the sedimentary column there is a steady decline in total amino acid concentration. The acidic amino acids have been reported to decrease most rapidly followed by the hydroxy-amino acids with the neutral amino acids the most stable (Aizenshtat *et al.*, 1973).

The first report on the racemization of amino acids in marine sediments was by Kvenvolden *et al.* (1970). They found a trend of increasing differences in the % D-bound amino acids with depth for total sediments from the Saanich Inlet, British Columbia. There were some irregularities in this progression but the overall difference of $\approx$ 1–7% D-amino acids between the top and bottom of the 35 m core was believed to reflect the diagenetic racemization which had taken place over the $\approx$ 9000 yr since deposition of the core began. Based on the extreme values of 7 of the amino acids studied and assuming

first order kinetics, expected half-lives of racemization lay between 28 000 and 220 000 yr and complete racemization was estimated to take between $\approx 3 \times 10^5$ and 2×10^6 yr.

Bada *et al.* (1970) observed a similar trend of increasing epimerization of L-isoleucine with increasing depth below the sediment-sea water interface for a 5 m sediment core taken from the Atlantic Fracture zone. Total sediment hydrolysate was used in this study. The data fitted the simplified first order rate expression (which may be derived for the case where racemization has not progressed more than $\approx$ 15%).

$$\ln\left(1 + \frac{\text{D-Alloisoleucine}}{\text{L-Isoleucine}}\right) = k'(\text{depth of burial}) + C\,. \tag{11}$$

A combination of Equation (11) and the simplified form of Equation (3) gives

$$\text{Sedimentation Rate} = k/k'\,. \tag{12}$$

The sediment was assumed to have initially contained only L-isoleucine, i.e., $C = 0$, and the value of k' was determined to be $k' = 2.85 \times 10^{-4}$ cm^{-1}. Assuming that the equilibrium ratio of D-alloisoleucine to L-isoleucine was 1.0 they estimated that the

$$\text{Depth of 50\% Racemization} = \ln 2/2k' \approx 12 \text{ m}\,. \tag{13}$$

A more realistic value for the equilibrium ratio is $K = 1.40$ (Wehmiller and Hare, 1971) for which the ratio of D-alloisoleucine/L-isoleucine for 50% racemization is 0.412 and thus the

$$\text{Depth of 50\% Racemization} = \frac{\ln 2}{(1 + K')k'} \approx 14 \text{ m}\,. \tag{14}$$

Furthermore, they assumed that the rate of interconversion in the sediment was the same as that determined for the epimerization of free isoleucine in aqueous solution (pH 7.6, 2° C, $k = 1.20 \times 10^{-7}$ yr^{-1}). On this basis a sedimentation rate (4.2 mm/1000 yr) and consequently an age of the bottom of the core of 1.23 my, was estimated. These data were supposedly "in close agreement with values determined in the general vicinity by both paleomagnetic and radioactive nuclide decay techniques" (Bada, 1971).

In a study of the foraminiferal fraction from deep sea cores (Wehmiller and Hare, 1971) it was found that the constituent total amino acids showed an initial rate of racemization almost an order of magnitude faster than the rate observed for free amino acids at a comparable pH and temperature. Also the kinetics of epimerization of the foraminiferal isoleucine were first order only up to a D-alloisoleucine/L-isoleucine ratio of $\approx$ 0.25, corresponding to an age of $\approx$ 400 000 yr at these temperatures. One further important observation made by Wehmiller and Hare (1971) was that when only the free amino acid fraction of the foraminifera was analyzed, the D-alloisoleucine/L-isoleucine ratio was significantly higher than that for the total hydrolyzate. These results indicated that the sedimentation rate and age of the core determined by Bada *et al.* (1970) were in serious error. In fact, Wehmiller and Hare estimated a sedimentation rate of 3.1 cm/1000

yr based on the method of excess ^{230}Th (Ku, 1965) for which the bottom of the 5 m core would have an age of $\approx$ 161 000 yr.

Bada and Schroeder (1972) were thus prompted to reexamine their findings for core CH96-G12. Elevated temperature kinetic studies with the total sediment indicated that first order kinetics were obeyed only up to D-alloisoleucine/L-isoleucine ratios of 0.3–0.4.

The data fitted to an Arrhenius plot gave

$$\log k(\mathrm{yr}^{-1}) = 15.77 - 5939/T\,. \tag{15}$$

At 2° K, $k = 1.6 \times 10^{-6}$ yr^{-1}. With this data a revised estimate was given for the sedimentation rate of 5.4 cm/1000 yr and an age of the bottom of the core of 96 000 yr which at least agrees better with the age calculated by Wehmiller and Hare given above.

Aizenshtat *et al.* (1973) also applied the approach of Bada *et al.* (1970 to date a 478 m core from the Vema Fracture Zone at 1.2 my. They did not specify, however, whether the rate constant (k) used in the calculation was that for the racemization of free isoleucine in solution or isoleucine in heated sediment.

An extension of Wehmiller and Hare's study (1971) to amino acids other than isoleucine from the foraminifera of two deep sea cores confirmed earlier findings. D/L ratios of amino acids did increase with depth and consequently with age but the kinetics of racemization of the total foraminiferal amino acids did not fit a first order kinetic model. The free amino acids were more racemized than the total amino acids (Kvenvolden *et al.*, 1973).

A further complication, even for studies using the much cleaner foraminiferal fractions of sediments, is the species effect mentioned in Section 4.1. King and Hare (1972a) studied D-alloisoleucine/L-isoleucine ratios in 29 samples of seven species of foraminifera isolated from six dated horizons in an Atlantic deep sea core. Each species was found to exhibit a different degree of epimerization at a given time horizon.

One surprising result is that the kinetics of epimerization of isoleucine in the total heated sediment were first order up to a D-alloisoleucine/L-isoleucine ratio of $\approx$ 0.35 (Bada and Schroeder, 1972) while the foraminiferal fraction apparently is first order only up to a ratio of $\approx$ 0.25 (Wehmiller and Hare, 1971). Considering the much more complex nature of a total sediment as compared to the foraminiferal fraction, there is perhaps an anomaly here. (The explanation may lie in the values of the equilibrium constant of 1.0 and 1.25 used respectively in the two studies).

It must be concluded that at the present state of our knowledge amino acid racemization dating of deep sea cores can at the best only be expected to give a rough approximation of the age even when accurate temperatures are known.

4.3. BONES

A dry modern bone has the following approximate composition (Berger *et al.*, 1964); 50% calcium phosphate, 10% calcium carbonate, 25% collagen and 5 to 10% bone fat.

The remaining 5 to 10% includes mucopolysaccharides, calcium fluoride, magnesium phosphate, sodium salts and heavy elements such as iron and manganese. The bone mineral which is principally calcium phosphate has the hydroxyapatite structure. Berger *et al.* (1964) have likened the structure of bone to that of a brick wall. "The bricks are apatite; the mortar consists of citrate, carbonate and other ions; and the collagen fibers act as reinforcing strands in the loosely assembled inter-crystalline matrix."

The open, porous structure of bone makes it particularly susceptible to the leaching action of ground waters (Hare, 1974). The free amino acids generated by hydrolysis of the collagen tend to be leached from the bone (Dungworth *et al.*, 1975; Hare *et al.*, 1975). As a result, although bones have a higher protein content than shells at first, after $\approx 10\,000$ yr or more shells and bones may have comparable amounts of protein left (Berger *et al.*, 1964).

The interesting feature of the protein of bone is that 90–95% of it is in the form of the unique substance, collagen (Bada, 1972a). The peculiar feature of collagen is the regular ordering of the amino acid residues such that every third position is glycine. Collagen is further characterized by having high concentrations of glycine, proline and hydroxyproline. Collagen becomes soluble in hot water as it dissociates to the protein gelatin. The amino acid compositions of collagen and gelatin are very similar (Eastoe, 1955, 1957). Although the amount of collagen in a fossil bone decreases with time, appreciable amounts of collagen have been found in bones 100 000 yr old (Heizer and Cook, 1952) while evidence has been presented for the preservation of collagen in a bone from the Miocene (25 my old; Isaacs *et al.*, 1963).

Although it has been reported that the proteinaceous material in fossil bones is collagen which has not undergone serious alteration (Wyckoff *et al.*, 1963; Wyckoff and Davidson, 1976a), a comparison of typical analyses for fossil bones and modern collagens shows some dissimilarities in composition. The fossil collagens do contain relatively large amounts of glycine, alanine and glutamic acid but lack the characteristic collagen amino acids hydroxylysine and hydroxyproline (Armstrong and Tarlo, 1966; Ho, 1965). There is evidence to suggest that the initial collagen undergoes diagenetic alteration to produce a 'fossil collagen' which then is relatively stable to further changes in composition with time (Dungworth *et al.*, 1974).

Fossil bone is the source material which has received the greatest attention in the application of amino acid racemization data to geochronology. This work dates from 1972, when Bada (1972a) first reported the age estimation of fossil bone using the epimerization of isoleucine. Elevated temperature studies with modern bone showed that the epimerization reaction follows reversible first order kinetics up to an alloisoleucine/isoleucine ratio of ≈ 1.1 with the Arrhenius plot for this data giving

$$\log k(\mathrm{yr}^{-1}) = 19.41 - 7304.0/T\,. \tag{16}$$

[The calculations of half-life for the isoleucine racemization reaction based on this data as given by Bada (1972a) are incorrect. The half-life ratio of alloisoleucine/isoleucine is given by Equation (8) and for $K = 1.38$, this will equal 0.408 (cf Bada's 0.345). At 20° C,

and from Equation (16), $k = 3.1220 \times 10^{-6}$ yr^{-1}. Substituting this data into Equation (7), gives $\tau = 129\,000$ yr (cf Bada's 110 000 yr).]

The data given by Bada (1972a) indicated that "in the temperature range of most natural environments, the epimerization reaction of isoleucine could provide an important tool for dating Pleistocene bones in the range beyond that applicable to radiocarbon." Two bones which he analyzed by this technique gave an estimated age and an estimated accumulation rate of manganese on a shark vertebra in good agreement with other evidence.

One of the limitations of the isoleucine dating procedure recognized by Bada was the need for an accurate evaluation of the average temperature experienced by the bone (an uncertainty of ±2° C would yield an age with error of ≈±50%). In order to overcome this problem Bada and Protsch (1973) introduced the 'calibration' technique using the racemization of aspartic acid rather than isoleucine. The basis of this innovative idea is as follows. A bone from a particular site is assigned an age (t) on the basis of a radiocarbon measurement and the D/L ratio of aspartic acid is also determined for this same 'calibrant' bone. For aspartic acid, $K = 1.0$ and Equation (3) reduces to

$$\ln\left(\frac{1 + \mathrm{D/L}}{1 - \mathrm{D/L}}\right) - C = 2\,k_{\mathrm{asp}}\,t\,. \tag{17}$$

Substituting the data into Equation (17) (for which Bada and Protsch determined, $C = 0.14$) gives an *in situ* k_{asp} for this particular deposit. This site has now been calibrated. By measuring the D/L aspartic acid ratio of other bones from the same general area and using the *in situ* k_{asp}, Equation (17) can be used to estimate their ages without any knowledge of the average temperature to which these bones have been subjected. It is claimed that the only condition which has to be fulfilled in using this dating procedure is that the average temperature experienced by the calibration sample must be representative of the temperature experienced by other samples from the deposit which are to be dated (Bada and Protsch, 1973). Bada *et al.*, (1974a,b) believed that bones with ages of ≈ 15 000 to ≈ 20 000 yr are suitable calibrants for the dating of bones which are much older (Bada and Deems, 1975). Supporting this claim is the oxygen isotope evidence of Shackleton and Opdyke (1973).

The reason aspartic acid is more suited to the calibration procedure than isoleucine is that aspartic acid racemizes much more rapidly than isoleucine epimerizes. Consequently, in the time datable by radiocarbon (<40 000 yr) a sufficient degree of racemization of aspartic acid takes place to allow an accurate determination of the D/L ratio. (Incidentally, using aspartic acid removes the effects of the uncertainty of the equilibrium ratio of alloisoleucine/isoleucine, which has been discussed earlier).

Advantages of the aspartic acid dating technique over radiocarbon methodology are (Bada and Protosch, 1973):

(a) it exceeds the viable dating range of radiocarbon dating. For average temperatures between 15 and 20° C, the effective range of dating is 5000 to 100 000 yr;

(b) only gram quantities of bone are required, compared to the hundreds of grams of material needed for radiocarbon. By using expendable faunal material for the calibration, very little hominid material need be used for the amino acid dating.

In order to show the reliability of the calibration procedure, Bada *et al.* (1974a) demonstrated the concordance of collagen-based radiocarbon and aspartic acid racemization ages for 11 fossil bone samples from 10 different geographical sites. In this paper 2 bones which were to old for radiocarbon dating were assigned aspartic acid ages. A bone from the upper Ndutu Beds in Olduvai Gorge was dated at 56 000 yr and a bone fragment from Rhodesian Man from Broken Hill, Zambia, was assigned an age of 110 000 yr. Several bones from Nelson Bay and Klasies River Mouth in Southern Cape Province, South Africa, have also been dated using aspartic acid racemization giving ages in close agreement with ages deduced from oxygen isotope and faunal evidence (Bada and Deems, 1975). The extent of aspartic acid racemization in 5 Californian Paleo-Indian skeletons has also been used to suggest that man was present in North America at least 50 000 yr ago (Bada *et al.*, 1974b).

Despite the preceding evidence demonstrating the reliability of the calibration procedure, it has not been free of criticism (Bender, 1974) and some of the data is in error. Hare (1974) has pointed out some major discrepancies between the 'calibrated rate' of racemization and the present climate of the areas reported by Bada *et al.* (1974a). Thus, amino acids in fossils from Murray Springs, Arizona, had the fastest racemization rate of amino acids from the 10 sites listed with a D/L aspartic acid ratio of 0.33 for a bone dated at 5640 yr. In contrast, amino acids in samples from Olduvai Gorge had a much slower racemization rate with a D/L ratio of 0.32 in a sample dated at 17 550 yr. The conclusion from this data is that Murray Springs has a 5–6° C higher temperature than Olduvai Gorge (see Section 5.1) when, in fact, the reverse is true. It was Hare's conclusion that probably few of the published amino acid dates are reliable, but despite problems with the calibration procedure, a useful tool would be developed. He suggested that data derived from a combination of different amino acid racemization rates would be of value (Hare, 1974).

Since 1974, a research group under Graham Dungworth at the University Nijmegen, The Netherlands, has been studying amino acid racemization in bones along the lines

TABLE IX

Abundances of D-Enantiomers in fossil collagen from a Pleistocene deer antler [a]

Amino acid	Internal	External	Anterior
D-Alanine	8.5	9.0	5.6
D-Valine	13.1	13.6	6.0
D-Alloisoleucine	22.6	22.6	8.1
D-Leucine	13.2	12.8	10.5

[a] Dungworth *et al.*, 1975.

suggested by Hare, and their data has illuminated several interesting facets of amino acid racemization in fossil bones. It is our opinion that Dungworth *et al.*'s research has contributed significantly to a better understanding of the problems associated with amino acid dating. We agree with their contention that a cautious approach should be taken in applying the technique to absolute geochronological dating.

A study of the compositions of fossil collagens resulted in the publication of some interesting data on the racemization of amino acids in different sections of a deer antler (posterior and anterior to the bony burr), of Pleistocene age (Dungworth *et al.*, 1974). Internal and external abundances of the D-enantiomers in the posterior section of the bone (as determined by gas chromatography on an optically active phase) were in good agreement within experimental error. But data recorded for the anterior section were significantly different from that determined for the posterior fragment (Table IX).

Based on the Arrhenius Equation (16) determined by Bada (1972a) for the epimerization of isoleucine in modern bone, where $K = 1.38$, $C = 0.028$ and the temperature is 7° C, the age estimates for the Pleistocene deer antler would be $\approx 1\,069\,000$ yr or $\approx 281\,000$ yr, depending on whether the data for the posterior or anterior sections of the bone are respectively used. Such large differences in D/L ratios are inexplicable on the basis of either temperature differences or contamination in such a local environment. The suggestion by Dungworth *et al.* (1974) that homogeneity of D/L ratios should be evaluated before data are applied to age estimations is a good one.

In the light of this evidence, the data from Bada's group must be considered to be suspect since reasonable homogeneity has been verified in very few samples (Bada and Helfman, 1975). In a more detailed report on the amino acid composition of the Pleistocene deer antler, it was concluded that there were two distinct fossil collagens with widely different amino acid compositions, anterior and posterior to the burr (Dungworth *et al.*, 1975). The conclusion was that the kinetics of racemization of protein are related to protein structure and composition (Dungworth *et al.*, 1973). This conclusion is supported by evidence that a species specific amino acid composition of calcified tissue can be identified with each of 16 species of planktonic foraminifera isolated from a sedimentary core (King and Hare, 1972b) and that alloisoleucine/isoleucine ratios at

TABLE X

Rate constants for the racemization of the Alanyl residue in collagen at 150.0 ± 0.1°C (Dungworth *et al.*, 1976)

Sample	Age	$k_{ala}^{150^\circ}$ (hr^{-1})	Reference
Modern Bone	0 yr	3.16×10^{-2}	Bada and Protsch (1973)
Human Bone (Aztec)	700 yr	3.21×10^{-2}	Dungworth *et al.* (1976)
Mammoth Bone	–	2.30×10^{-2}	Dungworth *et al.* (1976)
Deer Bone	Pleistocene	4.92×10^{-3}	Dungworth *et al.* (1976)
Walrus Bone	Pleistocene	2.50×10^{-3}	Dungworth *et al.* (1976)

equivalent time horizons are also species dependent (King and Hare, 1972a). Consequently, it is clear "that kinetic data derived from a study of an individual specimen cannot be applied to fossil collagen from other sources" (Dungworth *et al.*, 1973).

Dungworth *et al.* (1973) carried out incubation studies with fossil bones and confirmed that the racemization process for the bound amino acids follows first order reaction kinetics. Using the Arrhenius parameters so determined, they extrapolated to a temperature of 7° C to estimate the burial period of a fossil Walrus femur of Pleistocene age. The significant finding here was that the age estimate was dependent on which amino acid was used for the dating. The ages assigned using the abundance of D-alanine, D-valine, D-alloisoleucine and D-leucine were, respectively, 24×10^4, 36×10^4, 63×10^4 and 11×10^4 yr. This scatter in ages throws more doubt on the reliability of published fossil bone ages based on the racemization data from a single amino acid.

Dungworth (1976) has questioned the assumption that the Arrhenius parameters which describe the reversible first order rate of racemization of amino acids in fossil protein are invariant. He has presented data (Table X) which implies that the rate of the racemization reaction for alanine is decreasing with time, the decrease being $\approx 30\%$ over the time period datable by radiocarbon. On the assumption that the order of amino acid racemization appears to be relatively constant (see Section 6), Dungworth (1976) has suggested that the decrease in the rate of racemization may be similar in magnitude for the other amino acids. If rate constants are not invariant during the diagenetic history of the fossil, amino acid dates can be, at the best, only rough estimates of fossil ages.

Activation parameters determined in elevated temperature studies with bone collagen are clearly dependent on the fossil under study (Table XI) The activation energies reported for the racemization of amino acid residues in Mammoth collagen (Dungworth *et al.*, 1976) are not only lower than those obtained for a modern bone (Bada *et al.*, 1973b), but are also very much lower than those obtained for fossil walrus bone (Dungworth *et al.*, 1973).

With such large differences in activation energies even within the class of fossil collagens, it is by no means clear that the only conditions which need be fulfilled in using the calibration procedure of dating is that the average temperature experienced by the calibration sample must be representative of the temperature experienced by other samples from similar deposits (Bada and Protsch, 1973). Neither is it certain that a fossil of $\approx$20 000 yr is a suitable calibrant for dating older fossils. "It is apparent therefore, that Arrhenius parameters should be obtained for reliably dated fossil collagens in order to understand the limitations of using directly derived rate constants for age dating purposes" (Dungworth *et al.*, 1976).

Another interesting finding from the study of the composition and racemization of amino acids in Mammoth collagen (Dungworth *et al.*, 1976) was the identification of racemic α-amino-n-butyric acid (α-Abu) in both unheated and heated bone fragments. Prolonged heating of the Mammoth bone resulted in increasing concentrations of α-Abu but did not alter the equilibrium D/L ratio. It has been assumed that α-Abu is derived from glutamic acid by decarboxylation of the γ-carboxyl group (Hare, 1969) even though it is

TABLE XI

Arrhenius parameters for the racemization of amino acids in bone

Amino acid	Modern bone (Bada *et al.*, 1973b)		Fossil Walrus collagen (Dungworth *et al.*, 1973)		Fossil Mammoth collagen (Dungworth *et al.*, 1976)	
	E_a(kcal mole^{-1})	A(yr^{-1})	E_a(kcal mole^{-1})	A(yr^{-1})	E_a(kcal mole^{-1})	A(yr^{-1})
ala	33.4	–	29.6	4.97×10^6	25.1	6.0×10^7
val	–	–	29.7	2.42×10^6	25.4	4.0×10^7
ile	33.4	–	30.5	8.28×10^6	24.7	2.7×10^7
leu	33.4	–	27.5	1.64×10^6	23.0	2.7×10^6
glu	33.4	–	–	–	26.9	6.1×10^8
phe	–	–	–	–	23.9	1.2×10^7
asp	33.4 [a]	–	–	–	–	–

[a] Inferred to have the same E_a as the other amino acids (Schroeder and Bada, 1973).

known that aliphatic carboxylic acids do not readily decarboxylate. Furthermore, Dungworth *et al.* (1976) have pointed out that simple decarboxylation would yield L-α-Abu. The mechanism of formation of racemic α-Abu is unknown. Other nonprotein amino acids such as β-alanine and γ-amino-n-butyric acid are of interest in geochronological studies because of a suggested technique for testing for contamination (see Section 6.5.3).

In view of the many complications which have become evident through the studies by Dungworth's group, the dating of fossil bones with amino acid racemization data should be considered in its infancy.

4.4. MISCELLANEOUS APPLICATIONS

Four publications have appeared in the literature applying amino acid geochronology to woodrat middens, teeth and corals. Each of these will now be given brief mention since each contributes points of significance in the understanding of the problems associated with the amino acid dating technique.

4.4.1. Woodrat middens

In a study of racemization rate constants for aspartic acid in woodrat middens from dry caves and rock shelters in Arizona, Petit (1974b) determined what he considered to be a relatively high value for k_{asp} when compared to data published by Bada and Protsch (1973). However, because of the unknown temperature differences for Petit's and Bada's samples, such differences in rate constants may be expected. Petit has suggested, however, that the difference may be due to the methods of hydrolysis. The hydrolysis with 6N HCl used by Bada could deamidate any L-asparagine (see Section 2.3.2) thus decreasing the apparent D/L ratio, while Petit's enzymatic procedure avoided this potential problem. Although this reasoning may be sound, Petit's values for k_{asp} are no doubt high because of the extraction procedure employed. Samples were extracted by blending with 1N NaOH for 24 hr at room temperature. Evidence has already been presented that significant racemization may occur under these conditions (see Section 3.4), thus giving anomalously high D/L ratios. At present, there is insufficient data to properly assess the usefulness of studying woodrat middens by this technique.

4.4.2. Teeth

It has been reported that with increasing age there is a corresponding increase in the extent of racemization of aspartic acid isolated from human tooth enamel (Helfman and Bada, 1975). Up to $\approx$ 11% D-aspartic acid was observed in teeth from individuals of $\approx$ 70 years of age. (No net accumulation of D-aspartic acid with age was observed for haemoglobin because of its rapid turnover time of $\approx$ 120 days). Some scatter in data did occur, particularly for ages greater than 50 yr, and it was suggested that poor preservation of the enamel or small temperature differences between individuals might account for this

variability. Error from all sources was estimated at 15%. By using aspartic acid racemization in tooth dentine it was claimed that total errors could be reduced to 4% (Helfman and Bada, 1976). The standard deviation for the dentine ages was less than half that of the enamel values. Using tooth dentine from a suitable sample, they claim that an age estimate will fall within ±10% of the individual's actual age. Two suggested applications were: age at death determinations for skeletons preserved at relatively cold temperatures or within a few hundred years of burial, and examination of claims of unusual longevity among various populations.

This particular application of amino acid racemization data at least has the advantages that there is a known constancy of body temperatures of 36.9 ± 0.7° C (Spector, 1956) and particularly for the dentine which is relatively sheltered by the enamel layer, the environment should be very constant from one individual to the next.

4.4.3. Corals

The diagenetic racemization of amino acids in coralline material has been examined to determine the suitability of dating corals by this method (Wehmiller *et al.*, 1976; Wehmiller and Hare, 1970). Of 38 samples studied, 16 gave amino acid ages within 10 to 15% of their probable radiometric/stratigraphic age, while the other 22 samples gave ages which were less than 50% of the probable age. The D/L ratios often did not conform to the concept of increasing racemization being associated with increasing fossil age. Contamination and leaching were invoked as the causes for the poor correlations.

Numerous assumptions were made in this publication by Wehmiller *et al.* (1976). The equilibrium constant for the isoleucine epimerization was assigned a value of $K = 1.25$ introducing some uncertainty as previously discussed. An attempt was made to fit data from the corals to curves based on the kinetics of epimerization of isoleucine from foraminifera (Bada and Schroeder, 1972). In view of the variability of activation parameters with environment (see Table XII) it is very unlikely that they are the same for both foraminifera and corals, and there may even be a species effects for corals as has

TABLE XII

Activation parameters for the epimerization of Isoleucine in geological samples

Sample	E_a (kcal mole^{-1})	log A (yr^{-1})	Reference
Mercenaria shells	29.4	17.29	Mitterer (1975)
Mercenaria shells[a]	30.3	17.99	Mitterer (1975)
Mercenaria shells	28.1	16.70	Hare (1974)
Total sediment	27.2	15.77	Bada and Schroeder (1972)
Modern bone	33.4	19.41	Bada (1972a)
Fossil walrus collagen	30.5	16.92	Dungworth *et al.* (1973)
Fossil mammoth collagen	24.7	14.93	Dungworth *et al.* (1976)

[a] Data from radiocarbon dated fossil Mercenaria shells.

been reported to be the case for foraminifera (King and Hare, 1972a) and mollusk shells (Miller and Hare, 1975). Another assumption was that the linearity of the kinetics of isoleucine racemization held over the whole range despite the fact that in foraminifera, first order kinetics were only observed up to an alloisoleucine/isoleucine ratio of ≈ 0.35.

In our opinion there are too many assumptions in this report to properly assess the usefulness of applying amino acid racemization data to fossil corals. There is a need for a further systematic study of this particular application. Even if the assumptions made are valid, Wehmiller *et al.* (1976) have concluded that "fossil corals are of only modest value as specimens for amino acid geochronological studies".

5. Other Geological Applications of Amino Acid Racemization Kinetics

Besides the application of amino acid racemization kinetics to geochronology there have been several other areas to which this approach has been applied. These applications will now be reviewed.

5.1. AMINO ACID RACEMIZATION AND GEOTHERMOMETRY

As early as 1955, Abelson suggested that by "correlating isotopic measurements on shells" with dates based on the "time rate of degradation of proteins" in these same shells, an estimate could be made of the temperature of the marine environment at the time of their formation. Jones and Vallentyne (1960) calculated from the alanine content of a sample of Green River shale that the maximum temperature to which the shale could have been subjected during its history was 74° C. Following a study of the thermal stability of the amino acids, Vallentyne (1964) suggested that a geothermometric method "might be developed on the basis of selective destruction of amino compounds". These approaches have not been further investigated.

In reply to a letter by McKenna (1971), Bada and Luyendyk (1971) indicated that they had begun work on estimating temperature histories of sediments and fossils using known radiocarbon ages and amino acid racemization kinetics. The first application of this type was the use of the isoleucine epimerization reaction as a paleotemperature indicator (Bada *et al.*, 1973a). A hippopotamus bone from a peat layer next to a warm lithium spring at Florisbad (near Bloemfontein, Orange Free State, South Africa) had a collagen radiocarbon age of 38 680 ± 2000 yr. The alloisoleucine/isoleucine ratios were 0.46 (bone) and 0.42 (tooth). This data was substituted into the equation

$$\text{age (yr)} = \frac{\ln\left[\dfrac{1 + \text{D/L}}{1 - 0.725\,(\text{D/L})}\right] - 0.028}{(1.725)\,(10^{19.41 - 7304.0/T})} \tag{18}$$

where T is the average temperature (° K) of the bone since deposition. This equation is derived from Equation (3) with $C = 0.028$, $K = 1.38$ and using the Arrhenius data for a

modern bone (Bada, 1972a) of

$$\log k = 19.41 - 7304.0/T \tag{19}$$

i.e., an activation energy of 33.4 kcal mole^{-1}.

The average temperature was thus determined to be 26.5 ± 0.3° C which agreed well with the average temperature in the spring of ≈ 28° C. However, it should be emphasized that calculations based on Equation (18) are very susceptible to any variation in the activation parameters. As illustrated earlier in Table XII, these parameters may be variable. Those for modern bones are not necessarily the same as for fossil bones. A change in the activation energy from 33.4 to 30.0 kcal mol^{-1} gives an average temperature for the hippopotamus bone of –4° C. Thus a ±10% error in the activation energy gives a ±10% error in the temperature in °K which at ≈300 °K = ±30° C. It is clear that it is useless to evaluate the accuracy of the 26.5° C value by comparing it with the present average temperature of 28° C.

In the same way, Mitterer (1975) has estimated average diagenetic temperatues in Florida during the late Pleistocene using the epimerization of isoleucine in Mercenaria for which he found

$$\log k(\mathrm{yr}^{-1}) = 17.29 - 6417/T\,. \tag{20}$$

The same argument applies to these calculations. Mitterer's data together with the data that would have been obtained if the experimentally derived activation energy was in error by 1 kcal mole^{-1} (i.e., E_a = 30.3, cf Mitterer's E_a = 29.3) are given in Table XIII.

At the present level of understanding of diagenetic racemization, little credence can be given to absolute average temperatures determined in this manner where accuracies within a few °C are expected.

Aspartic acid racemization in fossil bones has been used to estimate glacial-postglacial temperature differences which are claimed to be reliable to 1° C (Schroeder and Bada, 1973). Three steps were involved in calculating the paleotemperatures:

(a) The D/L aspartic acid ratio and radiocarbon age were determined for each sample.

TABLE XIII

Average diagenetic temperatures in Florida during the Late Pleistocene calculated from isoleucine epimerization

Location in Florida	k(yr^{-1}) x 10^6	Present $\bar{T}$ (°C)	Pleistocene $\bar{T}$ (°C)	
			E_a = 29.3 kcal mole^{-1}	E_a = 30.3 kcal mole^{-1}
North	3.10	20.9	8.3	18.3
Central	4.12	22.0	9.8	19.9
South	5.52	23.5	11.4	21.5

Substitution into Equation (17) gave the average rate constant (same as the *in situ* k_{asp} in the calibration technique).

(b) From the known temperature dependence of the rate constant

$$k = Ae^{-E_a/RT}$$

can be derived an expression for the difference in the rate-average temperature ($\Delta\bar{T}_r$) of any two samples

$$\Delta\bar{T}_r = \bar{T}_2 - \bar{T}_1 = \frac{2.303\, R\, \bar{T}_1\bar{T}_2}{E_a} \log(k_2/k_1). \tag{21}$$

The temperature difference ($\Delta\bar{T}_r$) is not very sensitive to the values assigned to the average temperatures ($\bar{T}_1$, $\bar{T}_2$), so Schroeder and Bada (1973) substituted present mean temperatures.

(c) To determine the magnitude of the change, "the difference in average temperature for two samples spanning the termination of the last glaciation was fitted to a model for the Earth's temperature history for the past 20 000 yr". This model was based on graphs from pollen and isotopic analyses from which the equation for temperature as a function of time was

$$T(t) = \begin{matrix} T_p & t < 10\,000 \text{ yr}, \\ T_p - \Delta T & t > 10\,000 \text{ yr}. \end{matrix} \tag{22}$$

The difference in the time-average temperature ($\Delta\bar{T}_t$) for two samples can be expressed in integral form as

$$\Delta\bar{T}_t = \bar{T}_2 - \bar{T}_1 = \frac{\int T_2(t)\mathrm{d}t}{\int \mathrm{d}t} - \frac{\int T_1(t)\mathrm{d}t}{\int \mathrm{d}t}. \tag{23}$$

Combining Equations (3) and (4) gives

$$\Delta\bar{T}_t = \frac{\int T_p\, \mathrm{d}t + \int (T_p - \Delta T)\mathrm{d}t}{\int \mathrm{d}t} - \frac{\int T_p \mathrm{d}t}{\int \mathrm{d}t}. \tag{24}$$

Values obtained by Schroeder and Bada (1973) for the glacial-postglacial temperature change by equating the time-average temperature difference ($\Delta\bar{T}_t$; Equation (24)) and the rate-average temperature difference ($\Delta\bar{T}_r$; Equation (21)) are shown in Table XIV. However, it has been pointed out by McCullough and Smith (1976) that, in general, $\Delta\bar{T}_t \neq \Delta\bar{T}_r$. In fact, under the assumptions of the temperature model in Equation (22), the temperature change ($\Delta\bar{T}$) can be related to $\Delta\bar{T}_r$ exactly by the following expression (McCullough and Smith, 1976)

$$\Delta T = \frac{-RT_p^2}{E_a} \ln\left(\frac{t_2(k_2/k_1) - 10\,000}{t_2 - 10\,000}\right), \tag{25}$$

where T_p is the present temperature and t_2 is the age of the glacial-period fossil.

TABLE XIV

Average Glacial-Post Glacial temperature differences calculated from aspartic acid racemization

	ΔT (°C)	
Location	Schroeder and Bada (1973), Equation (21)	McCullough and Smith (1976), Equation (25)
Muleta Cave, Majorca, Spain	3.9	5.6
	3.7	4.8
Lukenya Hill, Kenya	4.7	7.0

Consequently, the claim by Schroeder and Bada (1973) that "the results give an estimated temperature increase which is reliable to 1°" is not valid.

Equations (24) and (25) give the corrected values, also shown in Table XIV. The corrected estimates are, in fact, in closer agreement with those deduced from isotopic and paleontological studies (Bada and Schroeder, 1976), although this is probably fortuitous.

Bada and Helfman (1975) have proposed that the k_{asp} values estimated from radiocarbon dated bones with ages less than 10 000–12 000 yr should be roughly proportional to the present-day annual air temperature of the site, assuming that the temperature of the Earth has been more or less constant during that time. To test this proposal they determined D/L aspartic acid ratios for radiocarbon dated bones from various regions of the world whose ages were less than 12 000 yr. A bone from Olduvai Gorge, Tanzania (D/L asp = 0.165; $k_{asp} = 7.0 \times 10^{-5}$ yr^{-1}) for which the present mean annual temperature is 23°C, was used as the reference. The data was substituted into Equation (15) to give the estimated average temperature at each site. These temperatures agreed within 2–3° with the present-day mean annual air temperatures. The absolute temperature values are probably not too significant. As indicated by Bada and Helfman (1975), temperature records for the sites represent only a small fraction of the time interval since bone deposition. Consequently, the 'present-mean annual air temperature' may be several degrees different to the true average temperatures experienced by the fossils. On the other hand, the trend of decreasing temperatures as represented by their data for the 11 different sites, is of some significance. There is no doubt that the Lindenmeier site, Colorado, (calculated average temperature, 12° C) has experienced a substantially lower temperature than the Olduvai Gorge site (present average temperature, 23° C).

Petit (1974a) has used Equation (21) to calculate that the average temperature at a Tucson mountain location, Arizona (woodrat midden sample, $k_{asp} = 2.15 \times 10^{-5}$ yr^{-1}) over the past 12 400 yr has been $\approx 1.9°$ C higher than the average temperature over the past 17 500 yr at the Olduvai Gorge, Tanzania sample site (bone sample, $k_{asp} = 1.48 \times 10^{-5}$ yr^{-1}). However, application of Equation (21) requires that the temperature of each site has not fluctuated significantly from the average temperature. Presumably

this will be true for the Arizona location which has experienced only post-glacial conditions. It is not true for the Tanzania site whose history extends into the last glaciation and consequently is subject to a temperature model similar to that expressed by Equation (22). In terms of the error thus introduced in Petit's application of Equation (21), the value of 1.9° C difference in average temperatures for the two locations is not very significant. Furthermore, use of Equation (21) assumes that the rate constants from the two sites are dependent solely on temperature. For two samples of the same fossil type and from similar geographical locations (such as the two bones from Lukenya Hill, Kenya, used by Schroeder and Bada (1973)) this may be a reasonable approximation. But for a woodrat midden from Arizona and a bone from Tanzania it is unlikely this assumption can be made. Supporting our conclusion is the data already presented (see Tables XI and XII) which show the variability of activation parameters with environment.

There are many problems associated with the application of amino acid racemization kinetics to geothermometry. The method is incapable of yielding average temperatures to which samples have been subjected since deposition to an accuracy of a few ° C. The estimation of the difference in average temperature for two samples from the same location is not as susceptible to error, provided a good temperature model can be derived for the past temperature history of the Earth.

5.2. AMINO ACID RACEMIZATION AND STRATIGRAPHIC CORRELATIONS

Hare and Mitterer (1968) suggested that D/L amino acid ratios might be useful for stratigraphic correlations, "particularly in deep sea cores, because the temperatures are uniform and constant".

Mitterer (1974) has used the diagenetic epimerization of isoleucine in Mercenaria shells to study the stratigraphic units from Pleistocene sequences in Florida. The alloisoleucine/isoleucine ratio values did not form a continuous sequence but fell into discrete groups. The gaps in this data were assumed to be caused by "nondeposition or erosion of marine strata". It was concluded that deposition had taken place only during intermittent high stands of the sea. During the times of lower sea level there was deposition of nonmarine sediments together with some erosion and reworking of earlier deposited strata. The results indicate that at least seven major glacial cycles including the Holocene epoch have occurred in the past 400 000 yr. This appears to have been a useful, accurate application of racemization data.

By correlating several radiocarbon and uranium-thorium ages with alloisoleucine/isoleucine ratios, Mitterer (1974) postulated a stratigraphic column for the Pleistocene deposits of southern Florida. He also tentatively correlated the Coffee Mill Hammock Formation from southern to northern Florida. He supported the latter analysis from the temperature dependence of the alloisoleucine/isoleucine ratio which indicated that the difference in average temperature from South to North was $\approx 3°$ C which is equivalent to the current mean temperature difference. This stratigraphic correlation must be

considered much more tenuous than the other conclusions in view of the arguments presented in Section 5.1.

Miller and Hare (1975) have measured alloisoleucine/isoleucine ratios in mollusks from sections of fossiliferous units on Eastern Baffin Island, Northwest Territories, Canada. This information was used to correlate units from sections 4 km apart. Lateral correlation between these units had been hampered by a major river outlet. Since the two sections studied were in such close proximity, this application of isoleucine epimerization data to stratigraphic analysis is probably sound.

5.3. MISCELLANEOUS APPLICATIONS OF AMINO ACID RACEMIZATION

Other applications of amino acid racemization kinetics which have been suggested but not studied in detail are: the determination of thermal gradients in long sediment cores or in cores taken from areas of high heat flow (Bada and Luyendyk, 1971; Bada, 1975; Schroeder and Bada, 1976), identification of reworked fossils, and verification of radiocarbon dates (Mitterer, 1974).

6. Potential Complications

Several of the problems related to specific applications of amino acid racemization kinetics have already been emphasized. However, there are other complications which are inherent to the technique. Certain basic assumptions have to be made. Certain precautions must be taken. Before estimating ages or paleotemperatures, or in any other use of the amino acid racemization approach, the following topics should be taken into consideration.

6.1 HEATING AND DIAGENESIS

One of the basic assumptions which has to be made in determining activation parameters for application to amino acid geochronology is that high temperature heating of samples such as bones and shells, mimics those changes which occur during low temperature diagenesis of the fossil proteins. It is always possible that changes may be initiated under the action of an elevated temperature which will not be observed at lower temperatures. There is evidence both for and against the assumption.

The stabilities of free amino acids in aqueous solution (0.01 M) at elevated temperatures (216–280° C) have been studied (Vallentyne, 1964). The amino acids fell into the following five groups of increasing relative stability:

(1) aspartic acid, cystine, threonine, serine, arginine (hydrochloride);
(2) lysine (hydrochloride), histidine (hydrochloride), methionine;
(3) tyrosine, glycine, valine, leucine, isoleucine;
(4) alanine, proline, hydroxyproline;
(5) glutamic acid.

TABLE XV

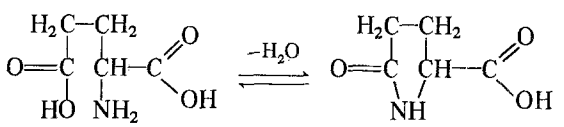
Amino acid content of various fossils [a]

Name	Approximate age (years)	Formation	Amino acid content ($\mu M\ g^{-1}$)	Principal constituents
Plesippus (prehistoric horse)	Late Pliocene 5×10^6	Hagerman Lake Beds, Idaho	0.6	Ala, gly
Plesippus (prehistoric horse, tooth)	Late Pliocene 5×10^6	Hagerman Lake Beds, Idaho	1.5	Gly, ala, leu, val, glu
Mesohippus (prehistoric horse, tooth)	Oligocene 40×10^6	White River, Nebraska	0.31	Ala, gly
Mosasaurus (dinosaur)	Cretaceous 100×10^6	Pierre Shale, South Dakota	1.8	Ala, gly, glu, leu, val
Anatosaurus (dinosaur)	Cretaceous 100×10^6	Lance, Lance Creek, Wyoming	2.8	Ala, gly, glu, leu, val, asp
Stegosaurus (dinosaur)	Jurassic 150×10^6	Morrison, Como Bluff, Wyoming	0.26	Ala, gly, glu
Dinichthys (fish)	Devonian 360×10^6	Ohio Black Shale	3.0	Gly, ala, glu, leu, val, asp

[a] Abelson, 1954.

This order compares quite favorably with those amino acids found in several fossils (Table XV; Abelson, 1954), and with the following order of increasing stability observed for pyrolyzed shells: serine, threonine, methionine, lysine, tyrosine, phenylalanine, isoleucine, leucine, proline, valine, glycine, alanine and glutamic acid (Vallentyne, 1969).

The stability of glutamic acid as compared to aspartic acid has been attributed to the ability of glutamic acid to undergo lactam formation to give pyroglutamic acid (Vallentyne, 1964; Povoledo and Vallentyne, 1964), although it is unknown whether glutamic acid can undergo this transformation while peptide bound (Dungworth *et al.*, 1976).



glutamic acid pyroglutamic acid

The observed instability of serine and threonine which is reported to increase when they are in a peptide (Vallentyne, 1964) does not agree with reports of serine and threonine being found in fossil conchiolons of lower Tertiary mollusk shells (Florkin *et al.*, 1961). Dungworth *et al.*, (1976) observed that, although there was an initial marked decrease of serine and threonine in heated mammoth collagen after only 2 days heating, this was not the case when older collagens were treated identically (Dungworth

et al., 1975). Hare and Mitterer (1968) have also observed that the amino acids still present in Miocene shells are much more stable than the same amino acids in Modern and Pleistocene shells during pyrolysis. Although less than 3% of the initial protein remained on heating oyster shells for one week, Totten *et al.* (1972) found that this protein residue was very stable. In a further eight weeks of heating only half of this residue was lost. Furthermore, the amino acid composition changed very significantly during the first week but showed little variation in succeeding weeks. Thus, changes induced by heating total amino acids in fossils may not be applicable to 'residual scleroprotein fractions' (Vallentyne, 1964) or what have also been described as 'fossil proteins' (Dungworth *et al.*, 1974).

If simulated laboratory experiments and diagenetic changes were really comparable, then heating a Pleistocene shell, for instance, should give the amino acid composition of a Miocene shell. Evidence has been presented to both support this argument (Vallentyne, 1969) and discredit it (Jones and Vallentyne, 1960). Another study on heating oyster shells indicated that "quick protein decomposition brought about by heat only roughly parallels that produced by slow aging" (Totten *et al.*, 1972). However, samples were heated dry in this work and water is believed to be an important factor in diagenesis (Hare and Mitterer, 1968; Hare, 1974).

Despite some conflicting evidence, amino acid stability studies probably support at least a reasonable parallel between diagenetic changes and those induced by elevated temperatures. Of more direct interest is whether the racemization parameters determined by heating proteins at elevated temperatures over short periods of time are the same as those which govern diagenetic racemization at low temperatures over extended periods of geological time. If this is true, then extrapolation of the Arrhenius plot from heating experiments should pass through points obtained from dated fossil material. Hare and Mitterer (1968) observed a reasonable correlation when they carried out such an extrapolation for isoleucine in Mercenaria (see Section 4.1).

Mitterer found that heated specimens of Mercenaria gave an Arrhenius plot which fitted the equation

$$\log k(\mathrm{yr}^{-1}) = 17.29 - 6415/T\,, \tag{26}$$

while data from seven natural specimens gave

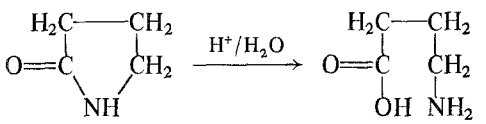
$$\log k(\mathrm{yr}^{-1}) = 17.99 - 6625/T\,. \tag{27}$$

Ages calculated using the rate constants derived from either of these equations are in quite good agreement. At least for the epimerization of isoleucine in Mercenaria there appears to be good reason to believe that high temperature laboratory racemization closely parallels low temperature diagenetic racemization. There is insufficient data at present to make a general statement concerning the equivalence of the racemization induced by heating and diagenesis for all amino acids in all fossils.

6.2. DIAGENETIC FORMATION OF AMINO ACIDS

This potential complication has been alluded to in the earlier discussion of the interconversion of asparagine to aspartic acid (Section 2.3.2). Pyrolysis experiments with 0.01 M solutions of amino acids have indicated that some of the amino acids isolated from fossil materials may be diagenetic and not those from the originally deposited protein (Table XVI; Vallentyne, 1964).

Vallentyne (1969) analyzed the total amino acid fraction after pyrolyzing Mercenaria shell fragments in water (174–252° C). Glycine and alanine concentrations were higher than for the untreated shell and γ-aminobutyric acid was identified in the products. These results parallel the findings of Table XVI. Since the pyrolyzed samples were completely lacking in serine and threonine, it is reasonable to assume that this was the source of the increased amounts of alanine and glycine. Strictly speaking, γ-aminobutyric acid is not the pyrolysis product of glutamic acid (Table XVI). Povoledo and Vallentyne (1964) found in a study of the glutamic acid-pyroglutamic acid system, that glutamic acid decarboxylates via pyroglutamic acid to give pyrrolidone. On acid hydrolysis pyrrolidone is converted to γ-aminobutyric acid.



pyrrolidone γ-aminobutyric acid

They concluded that γ-aminobutyric acid in fossil materials may be of diagenetic origin.

Dungworth *et al.* (1975) have also observed increased concentrations of certain amino acids on heating fragments of deer antler of Pleistocene age at 150° C in oxygen-free distilled water. Only the amino acid composition of the bound residues was analyzed.

TABLE XVI

Amine products from the pyrolysis of amino acids

Pyrolyzed amino acid [a]	Product(s)
glutamic acid	γ-aminobutyric acid [b]
glycine	methylamine
alanine	ethylamine
serine	glycine, alanine, ethanolamine
threonine	glycine
phenylalanine	phenylethylamine
methionine	glycine, alanine
arginine	proline

[a] 0.01 M aqueous solutions heated in sealed degassed tubes at 216–280°C. (Vallentyne, 1964).
[b] γ-aminobutyric acid is formed on acid hydrolysis of pyrrolidone; see discussion.

For the anterior section of the deer antler, heating resulted in an increase in the abundance of valine, leucine, isoleucine (and alloisoleucine) and phenylalanine. Some arginine was hydrolyzed to ornithine. A similar trend was observed for the posterior section, but in particular valine was very substantially enriched.

Of course, amino acids formed by diagenesis are indistinguishable from the amino acids originally in the deposited fossil. Little is known of the significance of the contribution of diagenetic amino acids to the overall amino acid concentrations. Consequently there is no way of predicting how much these transformations will falsify the 'true' D/L amino acid ratios.

6.3. THE EFFECTS OF CLAYS

In model experiments with the clay minerals kaolinite and montmorillonite (Hedges, 1975), no more than 10% of the initial concentration of L-valine was removed from solution, but removal increased as the amino acid concentration was decreased. Since the amount of organic matter in surface sediments is orders of magnitude greater than the associations observed with the model clays, selective removal of amino acids from sea water by clay mineral surfaces does not directly account for their concentrations in fine-grained sediments. Hedges (1975) has proposed that the organic material in sediments is largely transported to the ocean floor in particulate form where the sediment restricts the flow of oxygen and nutrients to benthic organisms, thus preserving the organic detrital matter.

Hare and Hoering (1973) isolated almost entirely L-amino acids from the clay mineral of a core for which it had been found that the foraminiferal amino acids were almost racemic. They proposed that clay-protein or clay-amino acid interactions may have resulted in stabilization of the amino acids against racemization, provided the amino acids were not recent contaminants.

We believe that contamination by recent amino acids accounts for the observed findings. This conclusion is supported by evidence that montmorillonite acts as a specific catalyst for the racemization of L-leucine and L-isoleucine (Kroepelin and Georgaras, 1968). Further studies with tyrosine have shown that the L- and D-isomers are sorbed nonpreferentially by montmorillonite. However, L-tyrosine is oxidized to polyhydric phenols and bityrosine at a greater rate than the corresponding D-isomer leading to selective enrichment of D-tyrosine (Thompson and Tsunashina, 1973).

Little is really known about the uptake and racemization of amino acids by clays. The above discussion does indicate the complexity of amino acid diagenesis in sediments and helps explain why total sediments are unsuitable for amino acid geochronological studies.

6.4. SPECIES EFFECT

Species-specific amino acid compositions have been identified with 16 species of planktonic foraminifera (King and Hare, 1972b). Furthermore, a species effect has been

observed for the epimerization of isoleucine in foraminifera, with each species exhibiting a different degree of epimerization at a given time horizon (King and Hare, 1972a). Miller and Hare (1975) have also observed a pronounced species effect upon the rates of hydrolysis and racemization in mollusk shells. Three species of mollusk from the same stratigraphic layer gave alloisoleucine/isoleucine ratios which varied between 0.09 and 0.21. (Results were consistent within a given shell species.) In principle, such an effect might exist for other fossils. These findings serve as a precautionary warning. The racemization rate of amino acids cannot be assumed to be solely dependent on temperature, nor can it be assumed that fossils with the same matrix type which have experienced the same temperature history will have the same racemization rate constants.

6.5. CONTAMINATION

Possibly one of the greatest sources of error in applying amino acid racemization data to dating is in the determination of the 'true' D/L ratio of any amino acid because of the possibility of contamination by more recent L-amino acids. The older the fossil, the lower the concentration of the original amino acids, and consequently, the greater the potential for erroneous D/L ratios. It is clear that for accurate age estimations the extracted amino acids must be both indigenous to the sample and syngenous with its deposition (Kvenvolden, 1975). It is helpful to discuss this complication from the two aspects of bacterial contamination and the contamination by ground waters percolating through the fossil. Suggested tests for contamination will then be reviewed.

6.5.1. Bacterial Contamination

Bacteria are ubiquitous and the biomass of bacteria may constitute as much as 15% of all organic material in soils, sediments and water (Messineva, 1962). There are few places where organisms cannot live and thus where organic material is not being synthesized and degraded. Hare (1969) has cryptically stated that

> when a particular organic-rich sediment was formed may not be as important to the preservation of the organic material as the question of how much alteration has been or is being accomplished by living organisms as part of the nitrogen cycle

There is some confusion as to how significant microbial activity is in deep sea sediments. ZoBell (1964) reports that large numbers of bacteria are to be found in organic-rich sediments. Bada (1971) has discussed evidence that the density of microorganisms is very low in the deep sea and others believe that microbial activity is considerably reduced in buried sub-aquatic sediments (Vallentyne, 1964). Abelson (1963) has stated that a recent shallow-water marine deposit collected from the inner continental shelf of the Gulf of Mexico at a depth of 120 cm was below the zone of major bacterial activity.

The non-protein amino acids β-alanine and γ-aminobutyric acid are believed to be

formed by the enzymatic decarboxylation of aspartic and glutamic acids, respectively, by microorganisms (Abelson, 1963; Bada, 1971; Schroeder, 1975). (Although as discussed earlier, there is evidence that γ-aminobutyric acid may also be of diagenetic origin). An analysis of the total sediment taken from a sediment depth of 13.3 m in the Caribbean Sea at a water depth of 4242 m showed that the two most abundant amino acids were β-alanine and γ-aminobutyric acid (Schroeder, 1975). Aizenshtat *et al.* (1973) observed that the percentage of β-alanine and γ-aminobutyric acid relative to the other amino acids increases dramatically down the sedimentary column for a core from the Vema Fracture Zone (13% at 100 m, 44% at 230 m, 70% at 478 m). The concentration of β-alanine in a core from the Saanich Inlet, British Columbia, increased from a trace to 10 nmole g^{-1} at 17 m and then decreased again (Kvenvolden *et al.*, 1970b). Provided there is no diagenetic origin of these amino acids, major bacterial activity is still taking place even at these depths.

It has been suggested that the dense calcareous structures of shells and foraminifera protect the organic matter from attack by microorganisms (Abelson, 1963; Schroeder, 1975). However, fossil bones have a much more open structure and consequently are susceptible to invasion and modification by microorganisms. Dungworth *et al.* (1976) believed that the inversion of the normal leucine/valine racemization order (see Section 6.5.3) in a Pleistocene deer antler could be attributed to the occurrence of amino acid distributions that were typical of bacterial and not of collagenous origin. Some conditions are more amenable to fossil preservation and protection than others. The Rancho La Brea tar pits apparently provide a sterile, anaerobic environment in which the water concentration is very low (Abelson, 1963). Consequently, bacterial contamination (as well as contamination by ground waters) of fossil bones from this site should be minimal (Wyckoff *et al.*, 1964).

Not only is there the possibility of modification of L-amino acid concentrations by bacteria but also bacteria are known to concentrate certain D-amino acids within their cell walls. Recent evidence indicates that very significant amounts (7–18%) of D-alanine, D-aspartic acid and D-glutamic acid are present in contemporary soils and probably originated from microorganisms (Pollock *et al.*, 1977). Very low concentrations of the D-enantiomers of valine, isoleucine, leucine, proline and phenylalanine were observed. Consequently, the above authors have alerted investigators of geological sediments to this possible complication in using amino acid racemization as an age dating technique.

6.5.2. Ground Water Contamination

The other main source of possible contamination is the introduction of modern L-amino acids by percolation of ground waters through the sample. This is possible not only for the porous bones but also for the more dense shells and foraminifera. Even the low permeability Onverwacht cherts may have been host rather than source rocks for some of the organic compounds which are now in them (Sanyal *et al.*, 1971). This form of

contamination explains the presence of only the L-enantiomers of the amino acids being identified in a sample of the Fig Tree chert (Kvenvolden *et al.*, 1969).

Bada *et al.* (1973b) have likened the diffusion process of introducing amino acid contaminants to the introduction of secondary carbonate into bones and the difficulties this presents for radiocarbon dating. Some of the fossil bones studied by Bada *et al.* (1973) were clearly contaminated. Armstrong and Tarlo (1966) determined amino acid concentrations of a bone of Upper Jurassic age ($\approx$ 135 my) together with sediments immediately surrounding the bone and sediments between 0.5 cm and 2 cm away from the bone. They concluded that while some of the amino acids detected in the fossil were original, others had been introduced by diffusion. Provided only L-amino acids are introduced, D/L ratios can still be used to estimate the minimum age of a fossil, if the contamination is not too great (Bada *et al.*, 1973).

6.5.3 Tests for Contamination.

There are some tests which may be applied to help assess the possibility of contamination in a fossil sample.

It is generally considered that for samples whose ages are Early Miocene or older, amino acids should be racemic and any evidence that this is not the case can be taken as proof of contamination. For example, a fossil shell with an age of 180 my was found to contain amino acids at levels substantially above background controls. However, the amino acids were only of the L-configuration and hence were identified as contaminants (Dungworth *et al.*, 1974). At the other end of the time scale, Kvenvolden (1973) has suggested that, in general, modern sediments or fossils will not contain the non-protein amino acids alloisoleucine (from isoleucine), ornithine and citrulline (from arginine), α-aminobutyric acid and γ-aminobutyric acid (from glutamic acid) and β-alanine (from aspartic acid). However, as indicated earlier γ-aminobutyric acid and β-alanine can be expected to be found in sediments as the result of microbial activity.

Schroeder (1975) has discussed procedures which have been used to check that shells and foraminifera from deep sea sediments are properly cleaned. He lists them as

(a) direct visual inspection to determine the presence or absence of clay minerals

(b) repeated ultrasonication/decantation cleaning cycles until no further change in the amino acid spectrum and/or D/L ratios is observed

(c) observation that the amino acid enantiomeric equilibrium has been established in very old samples.

A further, more specific test proposed by Schroeder (1975) was based on the observation that although β-alanine and γ-aminobutyric acid were abundant in marine sediments, they were completely absent in thoroughly cleaned foraminifera from those sediments. He suggested that this might also be applicable to fossil bones, teeth, and carbonate shells from sedimentary strata on land. Of relevance to this test are the findings

of Wyckoff and Davidson (1976b) who have studied the composition of gelatins isolated from fossil bones. The gelatins all contained significant concentrations of β-alanine except that from a bone which had come from the Rancho La Brea tar pits. Assuming that β-alanine is of microbial origin, there are two conclusions which can be drawn from these results:

(a) the Rancho La Brea tar pits do provide a sterile environment as indicated earlier, and

(b) the isolated 'gelatin' fractions were not pure gelatin but were contaminated by microbial degradation products or by microbial antibiotics.

However, γ-aminobutyric acid may be of diagenetic origin as previously discussed. Also Wehmiller *et al.* (1976) report that γ-aminobutyric acid and β-alanine have been identified in fossil shells that otherwise appear 'clean' and in a coral sample that seemed to contain racemic amino acids.

Wyckoff *et al.* (1963) have noted that visual inspection of a fossil bone is not sufficient to check for invasion by microorganisms because the presence of actively growing molds may not be macroscopically apparent. Electron microscopic examination is required for this check. It is instructive that an examination of the Murray meteorite has shown that it contains 6000 viable bacteria per gram, a figure similar to that for soil. Another meteorite (Mokoia) had 1800 viable bacteria/g (Oró *et al.*, 1971). β-Alanine and γ-aminobutyric acid have been identified in the Murcheson and Murray meteorites (Kvenvolden, *et al.*, 1970a, 1973), which in itself may be indicative of microbial contamination.

In the study of fossil corals, Wehmiller *et al.*, (1976) made several observations relating to tests for contamination. Data giving the maximum degree of racemization was taken to be most nearly correct on the basis that contamination would presumably introduce L-amino acids. Poorer reproducibility was generally observed in triplicate analyses where contamination was suspected. Fossils which had undergone recrystallization (from aragonite to calcite structure) were significantly contaminated, although some aragonitic samples had lower alloisoleucine/isoleucine ratios than totally recrystallized samples of the same age. Serine and threonine concentrations were a qualitative index of the amount of contamination. As indicated earlier, these two amino acids are relatively unstable and their concentrations in fossils decrease rapidly with age. Schroeder and Bada (1976) have reported that in a deep sea core threonine decreased by 50% and serine by 66% over $\approx$ 700 000 yr. This is to be compared with the less than 5% decomposition observed for the more stable amino acids. For corals older than 100 000 yr, Wehmiller *et al.* (1976) found that samples which agreed the best with model kinetics had less than 1 mole% threonine plus serine. In an analysis of planktonic foraminifera, King and Hare (1972a) decided that nearly half of the samples were contaminated on the basis of their anomalously high threonine and serine concentrations. These same samples were also characterized by high glycine/alanine ratios.

In principle, contaminants which have been introduced into a fossil from ground

TABLE XVII

Amino acid content of Mercenaria[a]

	Amino acid content (μmol g^{-1})		
Age	Protein bound	Soluble protein or peptide	Free
Recent	33.0	1.5	<0.35
Pleistocene	2.1	2.25	1.0
Miocene	0	0	0.75

[a] Abelson, 1955.

waters should be readily removed by acid washing and sonication. In some, but not all cases, Wehmiller *et al.* (1976) did observe an increase in the alloisoleucine/isoleucine ratios of the residual sample following acid washing.

The collagen method of radiocarbon dating was introduced to overcome problems of contamination by secondary carbonate (Longin, 1971). For the study of racemization in fossil bones, many of the contamination problems should be reduced if in the same way, only the pure collagen component is isolated for analysis.

Since the conchioline of shells has the same physico-chemical property as bone collagen, it may also be extracted by the same method in order to overcome contamination problems (Longin, 1971). As indicated in earlier discussion, the protein bound content of fossils decreases with time. Typical data for Mercenaria shells are shown in Table XVII (Abelson, 1955).

Particularly for bones there is the possibility that they have been heated prior to deposition and thus have a high degree of racemization. Bada *et al.* (1974a) have pointed out that burnt bones have a 'distinctive amino acid pattern' (not described) which readily distinguishes them from normal unheated samples.

It has been suggested that a further test for contamination in bone is the order of the racemization rates of the amino acids. Bada *et al.* (1973b) have stated that

> since the racemization reactions of amino acids in bone have the same Arrhenius activation energies, the relative order of rates of racemization determined at elevated temperatures should also exist at the lower temperatures at which fossil bones are found in nature, provided the bone has not been contaminated with amino acids from more recent sources. It is likely that contamination would introduce only L-amino acids to the bone and would thus alter the predicted sequence.

The predicted sequence for uncontaminated bone was aspartic acid > alanine = glutamic acid > isoleucine ≈ leucine. Dungworth *et al.* (1976) have gathered together published data showing that the order of racemization determined for amino acids in aqueous solution and modern bone at elevated temperatures is generally observed by amino acids from fossil bones. Those which deviate from the order are presumed to be contaminated.

However, this reasoning is only valid if the racemization activation energies are the same for all the amino acids. Although Bada *et al.* (1973b) did report that this was the case ($E_a \approx 33.4$ kcal $mole^{-1}$) for a heated modern bone, data for Walrus collagen

TABLE XVIII

Effect of temperature on the order of amino acid racemization

Sample description	T(°K)	Order [a]	Reference
Aqueous solution	400	ala>leu>ile>val	Dungworth *et al.* (1973)
	283	ala>ile>leu>val	
Walrus collagen	400	ala>ile>leu>val	Dungworth *et al.* (1973)
	280	leu>ala>val>ile	
	280 [b]	asp>ala>ile>leu>val	
Mammoth collagen	400	asp>glu>ala>phe>ile>leu>val	Dungworth *et al.* (1976)
	283	leu>phe>ala>ile>glu>val	
	Natural [c]	asp>phe≈glu>leu>ala>val	

[a] Calculations based on Arrhenius Parameters given in the corresponding references.
[b] Order for the natural unheated specimen. Estimated temperature ≈ 7° C.
[c] Order for the natural unheated specimen. No estimated temperature reported.

(Dungworth *et al.*, 1973), Mammoth collagen (Dungworth *et al.*, 1976) and free amino acids in aqueous solution (Dungworth *et al.*, 1973; Smith *et al.*, 1976) have shown a diversity of activation parameters. If these parameters differ, one must extrapolate the data to the temperatures of the natural environment before predicting the expected uncontaminated racemization order. In many instances, the predicted low temperature order is not the same as that at elevated temperatures nor is it the same as the order found in the natural unheated fossil (Table XVIII). Consequently, at our present level of understanding, it is not clear that the order of racemization given by Bada *et al.* (1973b) is a valid criterion for contamination.

Contamination is a complication which is inherent to the amino acid dating technique and is a problem which may not be readily recognized. Certain precautionary measures which may be taken are: microscopic inspection of the fossil for microbial invasion, and careful acid washing. The presence of the non-protein amino acids β-alanine and γ-aminobutyric acid may be indicative of contamination by microorganisms with the reservation that in some cases, γ-aminobutyric acid may be of diagenetic origin. Concentrations of the unstable amino acids serine and threonine can also be used as a qualitative index of contamination. In our opinion, the order of racemization of the amino acids is not a reliable indicator. In general, the D/L amino acid ratios from a sample which is not appreciably contaminated can at least provide a minimum age.

6.6. OTHER ENVIRONMENTAL CONSIDERATIONS

Hare (1974) has shown that when a bone is heated in the complete absence of water, essentially no racemization is observed. Bada and Helfmann (1975) have noted that the aspartic acid racemization rate for bones from the Rancho La Brea tar pits (k_{asp} = 2.8×10^{-6} yr^{-1}) is about four times slower than for bones from nearby soil environments ($k_{asp} = 10.8 \times 10^{-6}$ yr^{-1}). They believe that this represents the extremes

in the possible humidity changes of the environment. Nevertheless, some precaution should be taken to choose samples from environments of similar humidity, since a four-fold error in the estimated rate constant also represents a four-fold error in the estimated age of the fossil.

Hare (1974) has also discussed the possible problems of leaching. As the collagen is hydrolyzed, the free amino acids and small soluble peptides will be leached from the bone. Since these are the amino acids which are the most racemized, there is a selective removal which leaves the less racemized protein bound amino acids. The measured D/L amino acid ratios will be subject to variation depending on the amount of leaching the sample has experienced. Hare (1974) found almost an order of magnitude difference in the isoleucine epimerization rate for bone fragments heated only in water vapor compared with fragments heated with a large excess of water. He believed that the original calibration curves by Bada (1972a) and Bada *et al.* (1973b) which were based on studies where the bone was heated with only the indigenous water present were not a realistic assessment of the naturally occurring diagenetic changes. This is a further environmental complication overlooked in early studies, although if only the collagenous protein bound amino acid fraction is analyzed, this problem may not be significant.

6.7. TEMPERATURE ESTIMATION

The biggest problem associated with the amino acid dating technique is determining an accurate average temperature history for the fossil under study. A relatively small uncertainty of $\pm 2^{\circ}$ C yields an estimated age whose uncertainty is $\approx \pm 50\%$. It was for this reason that the calibration procedure was introduced. Provided the calibrant has experienced the same average temperature as the sample to be dated no knowledge of the actual temperature history is required.

Apparently deep ocean temperatures have not fluctuated more than 1 or 2° C even for glacial to non-glacial transitions and so present day temperatures are a reasonable approximation to average temperatures over geological time periods. Bada (1972a) has argued that caves have also provided a constant temperature history, although temperature fluctuations during glaciation periods were greater than for the deep sea.

7. Conclusions

The method of isolation and analysis of amino acids from fossils may be expected to result in some variability in the determined D/L ratios and consequently estimated ages. Precautions should be taken to ensure that base extractions do not cause significant racemization, and standard hydrolysis conditions should be established. There is a need for a study of the survival of asparagine (and glutamine) in fossil protein and the contribution, if any, that asparagine makes to the measured D/L aspartic acid ratios following acid hydrolysis. It should also be clear whether total, free, or protein bound amino acids are being analyzed. Inter-laboratory reproducibility of results should no

longer be a problem, since gas chromatography on optically active phases and ion-exchange chromatography of diastereomeric dipeptides are now reliable, accurate methods of analysis.

Factors which are known to influence the racemization rates of free amino acids such as metal ions, ionic strength, clays and pH indicate the complexity of diagenetic racemization. Bound amino acids are probably less susceptible to these environmental factors and consequently are more likely to yield more reliable, reproducible data. Bound amino acids racemize faster than free amino acids as predicted by Neuberger's theory. However, in natural systems the free amino acids racemize more rapidly as the result of metal catalysis. The scatter in reported Arrhenius parameters for the racemization of the free amino acids is evidence of the difficulty of obtaining accurate values for these parameters. This in turn indicates the uncertainties of age estimates based on extrapolation from this type of data to natural diagenetic temperatures.

Fossil bones have received the most attention in the application of amino acid racemization data to geochronology and geothermometry. Although the porosity of bone makes it more susceptible to contamination, the relative success of the technique with bone may be attributed to the unique protein collagen which gives an added factor of conformity from one fossil to the next. Foraminifera perhaps represent the best approximation to a closed system and from this point of view are the fossils most likely to give accurate age estimates. Shells are probably impervious to bacterial contamination and should yield useful racemization data. Total sediments are far too complex and are of limited use for amino acid dating, as are corals. Quite good data can apparently be obtained for teeth which represent samples from well-defined environmental and temperature conditions. There is insufficient data to properly assess the usefulness of studying woodrat middens by this technique.

The determination of absolute average temperatures is a highly tenuous application, particularly in view of the uncertainty of temperature models for the Earth's history. On the other hand, the estimation of temperature differences is not subject to such large errors, and useful data may be obtained in this manner.

Several possible complications should be recognized. There is the problem of diagenetic generation of amino acids and of contamination by bacteria or by more recent amino acids. There may be no way to recognize errors contributed from these sources, although the presence of β-alanine and γ-aminobutyric acid may in some cases be a valuable indicator of bacterial contamination. The concentrations of the unstable amino acids serine and threonine may also serve as a qualitative indicator of recent contamination. Variable humidity may alter racemization rates and elevated temperature kinetics may only roughly parallel diagenetic racemization. Rates of racemization have been shown to be species dependent in mollusk shells and foraminifera. In principle this may also be true of other fossils. There is evidence to suggest that the Arrhenius parameters which describe the racemization of amino acids in fossil collagens are not invariant. A further complication is that activation parameters have been found to be different in modern bone, a fossil Walrus collagen and a fossil Mammoth collagen. This

indicates that one cannot extrapolate data from high temperature racemization studies of modern bone to estimate ages in fossils with any certainty.

The use of the calibration procedure offers the possibility of offsetting the uncertainties of temperature histories experienced by the fossils and of some environmental effects.

It is our opinion that the amino acid dating technique is still in its infancy. Much of the most recent data encourages a more cautious approach. Once dates and ages appear in the popular scientific literature, they are hard to erase. Although in specific cases the calibration procedure may give age estimates to better than ±15%, it is felt that generally errors are much greater. Fossil ages calculated from estimated average temperatures suffer from the susceptibility of this age to minor changes in temperature. Since the estimated average temperature cannot be more accurate than ±2° C in most cases, the corresponding ages can be given only to an accuracy of ±50%. Despite the limitations of the procedure, it is no doubt useful where no other method of dating is available or when used in conjunction with other dating techniques. Provided the problems are carefully evaluated for each application and taken into account when specifying the uncertainty of any estimated age, etc., amino acid racemization data will contribute useful information to the scientific literature.

Acknowledgements

This work has been supported by a research grant from NASA, NSG-7038 for which we express our sincere thanks and appreciation. We also wish to acknowledge the valuable input and criticism of the manuscript by Dr. T. Emery and Dr. E. A. McCullough, Jr., of the Department of Chemistry and Biochemistry, Utah State University, Logan, Utah.

References

Abelson, P. H.: 1954, *Carnegie Inst. Washington Yearb.* **53**, 97.
Abelson, P. H.: 1955, *Carnegie Inst. Washington Yearb.* **54**, 107.
Abelson, P. H.: 1963, in I. A. Breger (ed.), *Organic Geochemistry*, Pergamon Press, Oxford, N.Y., p. 431.
Aizenshtat, Z., Baedecker, M. J., and Kaplin, I. R.: 1973, *Geochim. Cosmochim. Acta* **37**, 1881.
Aldag, R. W., Young, J. L., and Yamamoto, M.: 1971. *Phytochem.* **10**, 267.
Armstrong, W. G. and Tarlo, L. B. H.: 1966, *Nature* **210**, 481.
Bada, J. L., Luyendyk, B. P., and Maynard, J. B.: 1970, *Science* **170**, 730.
Bada, J. L.: 1971, in J. D. Hem (ed.), *Non-Equilibrium Systems in Natural Water Chemistry, Adv. in Chem. Series* **106**, 309. (Amer. Chem. Soc., Washington, D.C., 1971.)
Bada, J. L. and Luyendyk, B. P.: 1971, *Science* **172**, 503.
Bada, J. L. and Schroeder, R. A.: 1972, *Earth Planet. Sci. Lett.* **15**, 1.
Bada, J. L.: 1972a, *Earth Planet. Sci. Lett.* **15**, 223.
Bada, J. L.: 1972b, *J. Amer. Chem. Soc.* **94**, 1371.
Bada, J. L., Protsch, R., and Schroeder, R. A.: 1973a, *Nature* **241**, 394.
Bada, J. L., Kvenvolden, K. A., and Peterson, E.: 1973b, *Nature* **245**, 308.

Bada, J. L. and Protsch, R.: 1973, *Proc. Nat. Acad. Sci. U.S.A.* **70**, 1331.
Bada, J. L., Schroeder, R. A., Protsch, R., and Berger, R.: 1974a, *Proc. Nat. Acad. Sci. U.S.A.* **71**, 914.
Bada, J. L., Schroeder, R. A., and Carter, G. F.: 1974b, *Science* **184**, 791.
Bada, J. L. and Helfman, P. M.: 1975, *World Archael.* **7**, 160.
Bada, J. L.: 1975, *Naturwiss.* **62**, 71.
Bada, J. L. and Deems, L.: 1975, *Nature* **255**, 218.
Bada, J. L. and Schroeder, R. A.: 1976, *Science* **191**, 103.
Bender, M. L.: 1974, *Nature* **252**, 378.
Berger, R., Horney, A. G., and Libby, W. F.: 1964, *Science* **144**, 999.
Bohak, Z. and Katchalski, E.: 1963, *Biochemistry* **2**, 228.
Bonner, W. A.: 1974, *J. Mol. Evol.* **4**, 23.
Charles, R., Fischer, G., and Gil-Av, E.: 1963, *Israel J. Chem.* **1**, 234.
Charles, R., Beitler, U., Feibush, B., and Gil-Av, E.: 1975, *J. Chromatogr.* **112**, 121.
Craig, L. C., Hausman, W., and Weisiger, J. R.: 1954, *J. Amer. Chem. Soc.* **76**, 2839.
Crawhill, J. C. and Elliott, D. F.: 1951, *Biochem. J.* **48**, 237.
Cronin, J. R.: 1975, *Carnegie Inst. Washington Yearb.* **74**, 617.
Dakin, H. D.: 1912, *J. Biol. Chem.* **13**, 357.
Dakin, H. D. and Dudley, H. W.: 1913, *J. Biol. Chem.* **15**, 263.
Dungworth, G., Vincken, N. J. and Schwartz, A. W.: 1973, in B. Tissot and F. Bienner (eds.), *Advances in Organic Geochemistry*, Editions Techniq., Paris, p. 689.
Dungworth, G., Vincken, N. J. and Schwartz, A. W.: 1974, *Comp. Biochem. Physiol.* **47B**, 391.
Dungworth, G., Vrenken, J. A. Th., and Schwartz, A. W.: 1975, *Comp. Biochem. Physiol.* **51B**, 331.
Dungworth, G.: 1976, *Chem. Geol.* **17**, 135.
Dungworth, G., Schwartz, A. W., and van de Leemput, L.: 1976, *Comp. Biochem. Physiol.* **53B**, 473.
Eastoe, J. E.: 1955, *J. Biol. Chem.* **61**, 589.
Eastoe, J. E.: 1957, *J. Biol. Chem.* **65**, 363.
Erdman, J. G., Marlett, E. M., and Hanson, W. E.: 1956, *Science* **124**, 1026.
Feibush, B.: 1971, *Chem. Commun.* 544.
Florkin, M., Grégoire, C., Briteaux-Grégoire, S., and Schoffeniels, E.: 1961, *C. R. Acad. Sci., Paris* **252**, 440.
Garay, A. S.: 1968, *Nature* **219**, 338.
Geschwind, E. I. and Li, C. H.: 1964, *Archives Biochem. Biophys.* **106**, 200.
Gil-Av, E., Feibush, B., and Charles, R.: 1966, *Tetrahedron Lett.* 1009.
Gil-Av, E. and Feibush, B.: 1967, *Tetrahedron Lett.* 3345.
Goldhaber, M., Grodzins, L., and Sunyar, A. W.: 1957, *Phys. Rev.* **106**, 826.
Harada, K., Hare, P. E., Windsor, C. R., and Fox, S. W.: 1971, *Science* **173**, 433.
Hare, P. E.: 1963, *Science* **139**, 216.
Hare, P. E. and Mitterer, R. M.: 1966, *Carnegie Inst. Washington Yearb.* **65**, 362.
Hare, P. E. and Abelson, P. H.: 1967, *Carnegie Inst. Washington Yearb.* **66**, 526.
Hare, P. E. and Mitterer, R. M.: 1968, *Carnegie Inst. Washington Yearb.* **67**, 205.
Hare, P. E.: 1969, in G. Eglington and M. T. J. Murphy (eds.), *Organic Geochemistry: Methods and Results*, Springer-Verlag, New York.
Hare, P. E.: 1971, *Carnegie Inst. Washington Yearb.* **70**, 256.
Hare, P. E.: 1972, *Carnegie Inst. Washington Yearb.* **71**, 592.
Hare, P. E. and Hoering, T. C.: 1973, *Carnegie Inst. Washington Yearb.* **72**, 690.
Hare, P. E.: 1974, *MASCA Newsletter* **10**, 1.
Hare, P. E., Miller, G. H., and Tuross, N. C.: 1975, *Carnegie Inst. Washington Yearb.* **74**, 608.
Hedges, J.: 1975, *Carnegie Inst. Washington Yearb.* **74**, 604.
Helfman, P. M. and Bada, J. L.: 1975, *Proc. Nat. Acad. Sci. U.S.A.* **72**, 2891.
Helfman, P. M. and Bada, J. L.: 1976, *Nature* **262**, 279.
Heizer, R. F. and Cook, S. F.: 1952, *Am. J. Phys. Anthropol.* **10**, 289.
Ho, T-Y.: 1965, *Proc. Nat. Acad. Sci. U.S.A.* **54**, 26.
Isaacs, W. A., Little, K., Currey, J. D., and Tarlo, L. B.: 1963, *Nature* **197**, 192.
Jacobs, W. A. and Craig, L. C.: 1935, *J. Biol. Chem.* **110**, 521.
Jones, J. D. and Vallentyne, J. R.: 1960, *Geochim. Cosmochim. Acta* **21**, 1.

Karagunis, G. and Drikos, G.: 1934, *Z. Physik. Chem.* **26**, 428.
King, K. and Hare, P. E.: 1972a, *Carnegie Inst. Washington Yearb.* **71**, 596.
King, K. and Hare, P. E.: 1972b, *Science* **175**, 1461.
Koenig, W. A., Parr, W., Lichtenstein, H. A., Bayer, E., and Oró, J.: 1970, *J. Chromatogr. Sci.* 8, 183.
Kögl, F. and Erxleben, H.: 1939, *Zeits. Physiol. Chem.* **285**, 57.
Kroepelin, H. and Georgaras, K.: 1968, *Z. Natursforch. B.* **23**, 1266.
Ku, T.-L.: 1965, *J. Geophys. Res.* **70**, 3457.
Kuhn, W. and Braun, E.: 1929, *Naturwiss.* **17**, 227.
Kvenvolden, K. A., Peterson, E., and Pollock, G. E.: 1969, *Nature* **221**, 141.
Kvenvolden, K. A., Lawless, J. G., Pering, K., Peterson, E., Flores, J., Ponnamperuma, C., Kaplan, I. R., and Moore, C.: 1970a, *Nature* **228**, 923.
Kvenvolden, K. A., Peterson, E., and Brown, F. S.: 1970b, *Science* **169**, 1079.
Kvenvolden, K. A., Peterson, E., and Pollock, G. E.: 1971a, *Adv. in Org. Geochem.*, Pergamon Press, Oxford. Braunschweig (1972), p. 387.
Kvenvolden, K. A., Lawless, J. G., and Ponnamperuma, C.: 1971b, *Proc. Nat. Acad. Sci. U.S.A.* **68**, 486.
Kvenvolden, K. A.: 1973, *Space Life Sci.* **4**, 60.
Kvenvolden, K. A., Peterson, E., Wehmiller, J., and Hare, P. E.: 1973, *Geochim. Cosmochim. Acta* **37**, 2215.
Kvenvolden, K. A.: 1975. *Ann. Rev. Earth Planet. Sci.* **3**, 183.
Lande, S. and Lerner, A. B.: 1971, *Biochim. Biophys. Acta* **251**, 246.
Larson, D. M., Snetsinger, D. C., and Waibel, P. E.: 1971, *Anal. Biochem.* **39**, 395.
Levene, P. A. and Pfaltz, M. H.: 1925, *J. Biol. Chem.* **63**, 661.
Levene, P. A. and Pfaltz, M. H.: 1926a, *J. Biol. Chem.* **68**, 277.
Levene, P. A. and Pfaltz, M. H.: 1926b, *J. Biol. Chem.* **70**, 219.
Levene, P. A. and Bass, L. W.: 1927, *J. Biol. Chem.* **74**, 715.
Levene, P. A. and Bass, L. W.: 1929, *J. Biol. Chem.* **82**, 171.
Longin, R.: 1971, *Nature* **230**, 241.
McCullough, Jr. E. A. and Smith, G. G.: 1976, *Science* **191**, 102.
McKenna, M. C.: 1971, *Science* **172**, 503.
Manning, J. M. and Moore, S.: 1968, *J. Biol. Chem.* **243**, 5591.
Manning, J. M.: 1970, *J. Amer. Chem. Soc.* **92**, 7449.
Manning, J. M.: 1971, *J. Biol. Chem.* **246**, 2926.
Matsuo, H., Kawazoe, Y., Sato, M., Ohnishi, M., and Tatsuno, T.: 1967, *Chem. Pharm. Bull.* **15**, 391.
Matsuo, H., Kawazoe, Y., Sato, M., Ohnishi, M., and Tatsuno, T.: 1970, *Chem. Pharm. Bull.* **18**, 1788.
Matter, P., Davidson, F. D., and Wyckoff, R. W. G.: 1969, *Proc. Nat. Acad. Sci. U.S.A.* **64**, 970.
Matthews, C. N.: 1975, *Origins of Life* **6**, 155.
Meenakshi, V. R., Hare, P. E., and Wilbur, K. M.: 1971, *Comp. Biochem. Physiol.* **40B**, 1037.
Messineva, M. A.: 1962, in S. I. Kuznetsov (ed.), *Geological Activity of Microorganisms*, Consultants Bureau, New York.
Miller, G. H. and Hare, P. E.: 1975, *Carnegie Inst. Washington Yearb.* **74**, 612.
Mitterer, R. M.: 1972, in H. R. von Gaertner and H. Wehner (eds.), *Advances in Organic Geochemistry*, Oxford, Pergamon Press, 1971.
Mitterer, R. M.: 1974, *Geology* **2**, 425.
Mitterer, R. M.: 1975, *Earth Planet. Sci. Lett.* **28**, 275.
Nakaparksin, S., Gil-Av, E., and Oró, J.: 1970, *Anal. Biochem.* **33**, 374.
Neuberger, A.: 1948, *Adv. in Prot. Chem.* **4**, 297.
Oró, J., Nakaparksin, S., Lichtenstein, H., and Gil-Av, E.: 1971, *Nature* **230**, 107.
Parr, W. and Howard, P. J.: 1972, *Angew. Chem.* **84**, 586.
Pasteur, L.: 1860, Second Lecture delivered before the Chemical Society of Paris, in G. M. Richardson (ed.), *The Foundations of Stereo Chemistry*, American Book Company, New York, 1901.
Petit, M. G.: 1974a, *Anal. Lett.* **7**, 215.
Petit, M. G.: 1974b, *Quat. Res.* **4**, 340.
Pickering, B. T. and Li, C. H.: 1964, *Arch. Biochem. Biophys.* **104**, 119.
Pollock, G. E., Oyama, V. I., and Johnson, R. D.: 1965, *J. Gas Chromatogr.* **3**, 174.

Pollock, G. E. and Oyama, V. I.: 1966, *J. Gas Chromatogr.* **4**, 126.
Pollock, G. E. and Kawauchi, A. H.: 1968, *Anal. Chem.* **40**, 1356.
Pollock, G. E. and Frommhagen, L. H.: 1968, *Anal. Biochem.* **24**, 18.
Pollock, G. E., Cheng, C. N., and Cronin, S. E.: 1977, *Anal. Chem.* **49**, 2.
Ponnamperuma, C. and Gabel, N. W.: 1968, *Space Life Sciences* **1**, 64.
Povoledo, D. and Vallentyne, J. R.: 1964, *Geochim. Cosmochim. Acta* **28**, 731.
Saleuddin, A. S. M. and Hare, P. E.: 1970, *Can. J. Zoology* **48**, 886.
Sanyal, S. K., Kvenvolden, K. A., and Marsden, S. S.: 1971, *Nature* **232**, 325.
Sato, M., Tatsuno, T., and Matsuo, H.: 1970, *Chem. Pharm. Bull.* **18**, 1794.
Schroeder, R. A. and Bada, J. L.: 1973, *Science* **182**, 479.
Schroeder, R. A.: 1974, *Ph. D. Thesis,* University of California, San Diego.
Schroeder, R. A.: 1975, *Earth Planet. Sci. Lett.* **25**, 274.
Schroeder, R. A. and Bada, J. L.: 1976, *Earth Science Reviews* **12**, 347.
Shackleton, N. J. and Opdyke, N. D.: 1973, *Quat. Research* **3**, 39.
Smith, G. G., Williams, K. M., Astill, D. M., Sivakua, T., and Wonnacott, D. M.: 1976, Paper 226; Presented by K. M. W. at the 172nd ACS National Meeting, San Francisco, California.
Spector, W. S.: 1956, *Handbook of Biological Data,* Saunders, Philadelphia, p. 343.
Stephen-Sherwood, E. and Oró, J.: 1973, *Space Life Sciences* **4**, 5.
Stoll, A., Hofmann, A., and Petizilka, T.: 1951, *Helv. Chim. Acta* **34**, 1544.
Terent'ev, A. P. and Klabunovskii, E. I.: 1957, in A. I. Oparin (ed.), *First International Symposium on the Origin of Life on Earth,* Pergamon Press, New York, 1959.
Thompson, T. D. and Tsunashina, A.: 1973, *Clays and Clay Minerals* **21**, 351.
Totten, D. K., Davidson, F. D., and Wyckoff, R. W. G.: 1972, *Proc. Nat. Acad. Sci. U.S.A.* **69**, 784.
Vallentyne, J. R.: 1964, *Geochim. Cosmochim. Acta* **28**, 157.
Vallentyne, J. R.: 1969, *Geochim. Cosmochim. Acta* **33**, 1453.
Van't Hoff, I.: 1908, in *Die Lagerung der Atome in Raume*, Vierweg, Braunschweig 1908.
Wehmiller, J. F. and Hare, P. E.: 1970, *Geol. Soc. Amer. Abstr.* **2**, 718.
Wehmiller, J. F. and Hare, P. E.: 1971, *Science* **173**, 907.
Wehmiller, J. F., Hare, P. E., and Kujala, G. A.: 1976, *Geochim. Cosmochim. Acta* **40**, 763.
Westley, J. W. and Halpern, B.: 1965, *Biochem. Biophys. Res. Commun.* **19**, 361.
Wilcox, P. E.: 1967, in C. H. W. Hirs (ed.), *Methods in Enzymology* **XI**, 63. Academic Press, New York and London.
Wiltshire, G. H.: 1953, *Biochem. J.* **55**, 46.
Wyckoff, R. W. G., Wagner, E., Matter, P., and Doberenz, A. R.: 1963, *Proc. Nat. Acad. Sci. U.S.A.* **50**, 215.
Wyckoff, R. W. G., McCaughey, W. F. and Doberenz, A. R.: 1964, *Biochim. Biophys. Acta* **93**, 374.
Wyckoff, R. W. G. and Davidson, F. D.: 1976a, *Comp. Biochem. Physiol.* **54B**, 201.
Wyckoff, R. W. G. and Davidson, F. D.: 1976b, *Comp. Biochem. Physiol.* **55B**, 95.
ZoBell, C. E.: 1964, *Recent Res. Fields Hydrosphere, Atmosphere Nucl. Geochem.* **83**.