RACEMIZATION OF AMINO ACIDS IN WOOD
EXPERIMENTAL RESULTS, PROBLEMS, AND PERSPECTIVES

by

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ABSTRACT

When an organism (or part of an organism) dies, the amino acid substituents of its proteins and polypeptides begin to racemize from their "L" configurations to their "D" enantiomeric counterparts. The amino acid racemization reaction follows first order reversible kinetics in the heartwood of a sequoia tree (Sequoiadendron giganteum). Bound aspartic acid extracted from sequoia heartwood that had been dated dendrochronologically at 2185 B.P., had a rate constant for racemization equal to $2.11 \times 10^{-5}$ yr$^{-1}$. High temperature kinetic experiments yielded an activation energy of 22.6 kcal and a frequency factor of $1.3 \times 10^{13}$ yr$^{-1}$ for bound aspartic acid in sequoia heartwood. Insertion of the rate constant, activation energy, and frequency factor for bound aspartic acid into the Arrhenius equation yielded an average temperature of $5.0^\circ$ C, which is representative of the average temperature experienced by bound aspartic acid since the cessation of biological activity in the wood sample 2185 years ago. This average temperature value is in close agreement with weather station reports on the average temperature for the region (Windy Gulch Grove, California) in which the tree existed. Application of this method of paleotemperature detection to bristlecone pine may allow for the eventual study of paleotemperature changes over the past 8000 years.
CHAPTER 1

INTRODUCTION

Proteins of all living organisms consist of condensed chains of amino acids that exist in the L configuration (Meister, 1965; Bentley, 1969; etc.). Evidence for D amino acids in living organisms is restricted to low molecular weight polypeptides and free amino acids which are occasionally found in bacterial cell walls (Bentley, 1969). There is general agreement that fossil organic material recovered from a variety of sedimentary environments may contain proteins, peptides, and even free amino acids that at one time were constituents of once living organisms, and are not entirely the result of Recent contaminations (Abelson, 1963; Vallentyne, 1964; Hare, 1969). This is dependent on the absence of replacement, silicification, and high temperatures during diagenesis (Abelson, 1957).

Initially, the possibility of recovering biochemical information concerning the protein composition of earlier life forms led investigators to undertake amino acid analyses of marine invertebrate faunas and their associated sediments, along with the analyses of marine and terrestrial vertebrate fossil remnants. Amino acid profiles were determined for a variety of fossil and Recent
specimens found within the Phylum Mollusca and the Phylum Protozoa (Abelson, 1954a, 1954b, 1955; Hare, 1963; Degens, Spencer and Parker, 1967; Grégoire and Voss-Foucart, 1970). The feasibility of employing amino acid profiles from fossil shell material (e.g., Foraminifera) as a means of following phylogenetic changes through time has been successfully attained (King and Hare, 1972).

A detailed investigation of the extent of preservation and amino acid composition of collagen extracted from fossil and Recent bone samples has been conducted by a variety of researchers over the past twenty-two years. Amino acids have been extracted from vertebrate remnants ranging in age from the Devonian to Recent. The initial work on fossil collagen was conducted by Abelson (1954a, 1954b). He detected amino acids present in a fossil specimen of Dinichthys from the Devonian age Ohio Black Shale. More recent studies provided additional supporting evidence regarding the apparent survival of fossil collagen during long periods of time, and the facility by which protein components of collagen can be extracted from fossil bones and analyzed for their amino acid abundances (Wyckoff, Wagner, Matter and Doberenz, 1963; Ho, 1965, 1966; Armstrong and Tarlo, 1966; Doberenz and Lund, 1966; Miller and Wyckoff, 1968; Wyckoff, 1972; Ferdinand, Bartley, Cole and Bailey, 1973; Dungworth, Vincken and Schwartz, 1974; Dungworth, Vrenken and Schwartz, 1975.
Such studies of amino acids have not been limited to fossils. In a number of investigations of Tertiary and Quaternary sediments amino acids extracted from the sedimentary organic matter have been examined (Erdman, Everett and Hanson, 1956; Degens, Reuter and Shaw, 1964; Clarke, 1966; Stevenson and Cheng, 1970, 1972). Amino acids have been extracted from sediments as old as Precambrian (Harington, 1962; Schopf, Kvenvolden and Barghoorn, 1968) although the indigenous nature of these amino acids has been questioned (Abelson and Hare, 1969; Kvenvolden, Peterson and Pollock, 1969). Detailed studies of the organic constituents of carbonaceous meteorites revealed the presence of amino acids (Kvenvolden, Lawless, Pering, Peterson, Flores, Ponnampuruma, Kaplan, and Moore, 1970; Oró, Nakaparksin, Lichtenstein, and Gil-Av, 1971; Pollock, 1972; Lawless and Romiez, 1974; Nagy, 1975). Investigations of proteins, peptides and amino acids present in fossil organisms, sediments, and meteorites established a foundation and guidelines upon which future studies could be based. However, several of these studies, particularly those conducted prior to 1965, were not concerned with the optical activity of the amino acids.

In the past ten years the qualitative and quantitative capabilities of paleobiochemical investigations have advanced with the introduction of new analytical techniques and improvements in instrumentation. The utilization of gas chromatography for the
detection and separation of D and L enantiomers of amino acids provides an accurate method for the detection of racemization of amino acids extracted from marine sediments, fossil organic material, meteorites, etc. (Gil-Av, Charles and Fischer, 1965; Halpern and Westley, 1965; Pollock, Oyama and Johnson, 1965; Oyama and Pollock, 1966). Such derivatization and separation processes, using polar, non-optically active gas chromatographic columns, subsequent to the extraction and purification of amino acids from their matrix consist of three principal steps:

1. Esterification of the carboxylic acid ends of the amino acids with a pure optically active alcohol (Gil-Av, Charles and Fischer, 1965; Pollock, Oyama and Johnson, 1965). (+)-2-Butanol-2N-HCl was found to be a highly effective esterifying agent for this purpose (Oyama and Pollock, 1966).

2. Acylation of the amine ends of the amino acids with trifluoroacetic anhydride, after the reaction with an optically active alcohol, yielded derivatives which facilitate the separation of amino acid enantiomers on the gas chromatographic column (Gil-Av, Charles and Fischer, 1965). N-pentafluoropropionic anhydride has also been found to be a very useful acylating reagent. It affords far superior separation of aspartic acid diastereomers than the trifluoroacetic anhydride derivatives (Pollock, 1967, 1975).
3. Separation of the L-D and D-D diastereomers of the amino acids by gas chromatography. The columns which were found to afford maximum separation are capillary columns coated with polar polymeric materials, such as Carbowax and Ucon (Pollock, Oyama and Johnson, 1965). Carbowax-20M, 150' x 0.02" i.d. capillary columns have been found to be particularly successful when used for the separation of N-pentafluoropropionyl- (+)-2-butyl esters (Pollock, 1967, 1975).

A number of investigators succeeded in separating D-L enantioomers of amino acids by using optically inactive alcohols (i.e., methanol, isopropyl alcohol, etc.) in the esterification step of their derivatization procedure, and subsequently injecting the derivatives onto gas chromatographic capillary columns coated with an optically active stationary phase (Gil-Av, Feibush and Charles-Sigler, 1966; Koenig, Parr, Lichtenstein, Bayer and Oró, 1970; Nakaparksin, Birrell, Gil-Av and Oró, 1970; Nakaparksin, Gil-Av and Oró, 1970; Lockmueller and Souter, 1973; Charles, Beitler, Feibush and Gil-Av, 1975). It has been shown that N-trifluoroacetyl-isopropyl esters of amino acids can be separated on a gas chromatograph equipped with a 500' x 0.02" capillary column coated with N-trifluoroacetyl-L-valyl-L-valine cyclohexyl ester (Nakaparksin, Birrell, Gil-Av and Oró, 1970).
The initial success of Pollock, Gil-Av and their co-workers has created a widespread interest in the application of gas chromatography to the separation of various Suites of biological and abiological amino acids (Gehrke and Stalling, 1967; McBride and Klingman, 1967; Pollock and Kawauchi, 1968; Casagrande, 1970; Zumwalt, Kuo and Gehrke, 1971; Hardy, 1972; Gehrke, 1973; Darbre, 1974; Iwase, 1974a; Moodie, 1974). A number of experiments (some quite successful) have also been made to modify the esterifying and acylating reagents used in the synthesis of the amino acid diastereomers (Huseky, 1974; Iwase, 1974b; MacKenzie and Tenaschuk, 1974; Nambara, Goto, Taguchi and Iwata, 1974; Sakamoto, Kajiyama and Tonami, 1974).

While gas chromatography is one of the more successful methods to separate low concentrations of amino acids into their respective D and L stereoisomeric forms, investigations have not been limited to this particular analytical technique. Ion exchange chromatography which separates dipeptide derivatives (L-D and L-L) of D and L amino acids are in widespread use (Manning and Moore, 1968). This amino acid analytical technique has been found to be particularly useful for isoleucine and alloisoleucine. These amino acids are epimers, and do not require dipeptide synthesis prior to injection on amino acid analyzers using ion exchange
chromatography (Bada, Luyendyk and Maynard, 1970; Kvenvolden, Peterson and Brown, 1970; King and Hare, 1971; Wehmliller and Hare, 1971).

Enzymatic techniques employing L-amino acid oxidase have also been used to separate D and L enantiomers, but this technique is not widely applied because of experimental problems (Hare and Abelson, 1968; Larson, Snetsinger and Waibel, 1971; Petit, 1974). For additional reviews of the analytical techniques presently employed in the study of amino acid enantiomers, the reader is referred to review articles by Kvenvolden, Peterson and Pollock (1971), Bada and Schroeder (1975), Kvenvolden (1975) and Dungworth (1976).

Various methods have been pursued for analyzing unknown amino acids that are not readily identified by coinjection. Combined gas chromatography-mass spectrometry and mass spectrometry are used for the identification of unknown amino acids as well as organic compounds that interfere (overlap or partially overlap) with amino acid peaks during gas chromatographic analysis (Merritt and Robertson, 1967; Simmonds, Shulman and Stembridge, 1969; Lawless and Chada, 1971).

Gas chromatography and ion exchange chromatography enabled investigators to detect and analyze amino acids at nanogram levels. Consequently, the utilization of such sensitive techniques has required extreme caution to exclude the inadvertent introduction of
potential contaminants into the sample. Amino acids of varying concentrations have been detected in distilled water, HCl, NH₄OH, dichloromethane, butanol, etc. (Hare, 1965; Sidle, 1967; Rash, Gehrke, Zumwalt, Kuo, Kvenvolden and Stalling, 1972; Hamilton and Myoda, 1974). With respect to the distillation of hydrochloric acid, Wolman and Miller (1971) suggested using only the first half of a redistilled portion, as the second half (especially the last quarter) has been found to be highly contaminated with amino acids. It is probably safest to simply generate aqueous HCl with purified gaseous HCl in distilled water, previously established to be free of amino acid contaminations. The handling of glassware during the analysis of amino acids must be done with no or minimum direct contact with human skin. There are a number of reports of amino acid contaminations arising from one or two fingerprints (Hamilton, 1965; Oró and Skewes, 1965; Rash, Gehrke, Zumwalt, Kuo, Kvenvolden and Stalling, 1972). Caution must be observed with regard to saliva, hair, and threads from fibers of fabric (Hamilton, 1965).

The development of the aforementioned analytical techniques for the separation of D and L amino acid enantiomers has broadened the spectrum of potentially retrievable information from fossil organic material and ancient sediments. It is common knowledge that upon death of an organism, the amino acid constituents of its various proteins and peptides begin to racemize at different rates
from their "L" configuration to their "D" counterparts (Hare and Mitterer, 1967; Hare and Abelson, 1968; Bada, Luyendyk and Maynard, 1970). More importantly, it seems that in organic systems not subject to abnormal temperature effects after death, the kinetics of racemization appear to initially follow a first order reversible rate law (Hare and Mitterer, 1969; Bada, Luyendyk and Maynard, 1970; Wehmiiller and Hare, 1971). The chemical reaction mechanism of the racemization of amino acids has been suggested by Bada and Schroeder (1975) to proceed via the formation of a carbanion intermediate. Thus, if it is possible to calculate the first order rate constants for the racemization reactions of amino acids, the age of samples may be determined from the degree of racemization that has taken place. The determination of the rate constants for amino acids released by proteins and peptides through hydrolysis of fossil materials and sediments has proven to be a rather formidable task. First, to calculate a rate constant, the absolute age of a corresponding sample has to be known. Second, the rate constant is highly dependent on past temperatures, water content, pH, etc. The first investigations of racemization and their application to dating samples involved the study of marine sediments and fossil shells extracted from sediments (Hare and Mitterer, 1969; Bada, Luyendyk and Maynard, 1970; Kvenvolden, Peterson and Brown, 1970; Wehmiiller and Hare, 1971; Bada and Schroeder, 1972; Mitterer,
1972; Kvenvolden, Peterson, Wehmliller, and Hare, 1973; Akiyama, 1975; Mitterer, 1975). Minimal temperature fluctuations at the bottom of sufficiently deep bodies of water, of course, facilitated calculation of ages (Wehmliller and Hare, 1971). A number of experiments have also been conducted to determine the rate constant for the racemization of isoleucine to alloisoleucine in fossil shell and calcareous sediments by laboratory (high temperature) kinetic experiments (Hare and Mitterer, 1967, 1969; Bada, Luyendyk and Maynard, 1970; Wehmliller and Hare, 1971; Bada and Schroeder, 1972; King and Hare, 1972; Mitterer, 1972). Extensive research by Mitterer (1975) lends evidence that temperature relationships can be deduced from such an approach. A variety of problems have been considered by Hare and his co-workers that relate to the applicability of racemization to marine sediments and incorporated fossils. In a classic paper, Hare and Mitterer (1969) presented experimental evidence which showed that it is possible that amino acid contaminants may be introduced into fossil shells from the surrounding aqueous environment. Wehmliller and Hare (1971) have also shown that amino acids in old fossil material do not necessarily follow first order kinetics mainly due to diagenetic processes. They have attributed this to the increasingly complex relationship of the constituents of fossils with their sedimentary environments during diagenesis.
In addition to utilizing amino acid racemization as a dating technique, Mitterer (1974) has also employed racemization as a stratigraphic correlation technique. Bada used racemization for dating Pleistocene bone (Bada, 1972a; Turekian and Bada, 1972; Bada and Protsch, 1973; Bada, Kvenvolden and Peterson, 1973; Bada, 1974; Bada, Schroeder and Carter, 1974; Bada, Schroeder, Protsch, and Berger, 1974; Bada and Schroeder, 1975). As was noted before, dating by amino acid racemization of samples, such as Pleistocene bone, is complicated by the fact that terrestrial temperature fluctuations may make it difficult to establish the racemization rate constants. Bada and his co-workers were able to obtain rate constants for the racemization of aspartic acid in Pleistocene bone by analyzing samples that have been dated by the carbon-14 method (Bada and Protsch, 1973; Bada, Schroeder, Protsch and Berger (1974). Bada (1972a) and Dungworth, Schwartz and Van De Leemput (1976) have also investigated the kinetics of racemization of amino acids in bone (fossil and modern) by conducting laboratory (high temperature) kinetic experiments. The racemization technique may be used to establish trends in paleotemperature fluctuations (Bada, Protsch and Schroeder, 1973; Schroeder and Bada, 1973). Amino acid racemization has also been used for paleotemperature estimates on Pleistocene fossil shell material (Mitterer, 1975). In addition to the effects of temperature on the degree of racemization of amino acids in fossil
bone, Hare and his co-workers demonstrated the importance of water concentration and leaching on the amino acid composition and racemization in ancient bone (Hare, 1973; Hare, Ortner and Von Endt, 1974). It is also important that the protein bound amino acid fraction be studied independently of the free amino acid fraction since contaminations from both L-amino acids and D-amino acids (e.g., bacteria) are possible.

Hydrogen ion concentrations are also known to play an important role in determining racemization rates in bones (Hare and Mitterer, 1969; Hare, 1971; Bada, 1972b). A number of studies have shown that alkaline conditions in the sedimentary environment can drastically affect the racemization rates of amino acids (Pollock and Frommhagen, 1968; Hare, 1971; Provansal, Cuq and Cheftel, 1975). It is also important to note that pH and other environmental factors appear to have different effects on protein bound amino acids and free amino acids (Hare, 1971). It is well known that to some extent hydrolysis during sample preparation must also be viewed as a potential source of distortion of racemization data. Problems encountered in amino acid dating have been reviewed by Hare (1974).

For several years biochemists have been investigating the protein and amino acid contents of seeds, plants, and woody tissues of trees (Fowden, 1959; King and Bayley, 1965; Durzan and Chalupa, 1968; Scurfield and Nicholls, 1970; Ramaiah, Durzan and Mia, 1971;
Osinovskil and Skrigan, 1975; etc.). The changes of the amino acid content of living cells of plants and trees affected by climatic fluctuations have been described (Benko, 1969; Durzan, 1971; Reuther, 1975; etc.).

Amino acid racemization has been detected in samples of fossil wood (Lee, Bada and Peterson, 1976). Radiocarbon dated samples of wood were used to calculate a rate constant for the racemization of proline. These investigators also conducted high temperature kinetic experiments on bristlecone pine, and constructed a high temperature Arrhenius plot for the epimerization of isoleucine to alloisoleucine. The dependence on radiocarbon dates is a limitation in the calculation of rate constants for amino acid racemization in wood. The standard deviation of radiocarbon dates limits the reliability of the rate constants determined for any specific sample. The radiocarbon method also proves inapplicable when samples of limited quantity (i.e., less than 250 mg) are being investigated. The use of dendrochronologically dated wood samples provides an absolute control on the age of the samples and thus insures the accuracy of rate constants determined for bound amino acids in wood.

As stated previously, racemization at a constant temperature has been shown to follow first order reversible kinetics. The following is an integrated form of a first order reversible rate expression derived by Wehmliller and Hare (1971):
\[ \ln \left( \frac{Xe - X}{Xe} \right) = -2kt \quad \ldots \ldots \ldots \ldots (1) \]

\( X_e \) is the \( D/(D+L) \) ratio for a specific amino acid at equilibrium, \( X \) is the \( D/(D+L) \) ratio for a specific amino acid at time \( t \), and \( k \) is the rate constant for the racemization reaction. The relationship of the rate constant for the racemization reaction to temperature can be seen in the Arrhenius equation:

\[ k = Ae^{\frac{-E^*}{RT}} \quad \ldots \ldots \ldots \ldots (2) \]

where \( k \) is the rate constant, \( A \) is a constant known as the pre-exponential or frequency factor, \( E^* \) is the energy of activation, \( R \) is the gas constant, and \( T \) is the temperature in degrees Kelvin. The Arrhenius equation can be rewritten in the following form:

\[ \ln k = \ln A - \frac{E^*}{RT} \quad \ldots \ldots \ldots \ldots (3) \]

A plot of \( \ln k \) versus \( 1/T \) gives a straight line, the slope of which is \( E^*/R \), and the \( y \)-axis intercept is equal to \( \ln A \).

A dendrochronologically dated wood sample can be analyzed for bound \( D \) and \( L \) amino acids. A rate constant can be assigned to any specific bound amino acid by the utilization of equation (1). If the activation energy and frequency factor can be determined for a bound amino acid in the wood sample, it would be possible to use the
Arrhenius equation (3) to estimate the average temperature experienced by the bound amino acid in question since the time of its formation.

Both aspartic acid and glutamic acid are known to racemize relatively quickly compared with other amino acid substituents common to proteins. The calculation of rate constants, activation energies, and frequency factors for bound amino acids located in the heartwood of a sequoia tree (*Sequoia* _den* dendron giganteum*) has concentrated on bound aspartic acid and glutamic acid, because of the young ages of the samples being extracted from the tree (samples ranged in age from 75 B.P. to 2185 B.P.). Proline has also been studied in an attempt to compare the most recent evidence concerning proline's rapid rate of racemization in wood samples (Lee, Bada, and Peterson, 1976) with its rate of racemization in the sequoia tree.
CHAPTER 2

EXPERIMENTAL

Derivatization of Amino Acid Standards

Proteins, peptides, and amino acids exist in micromole/gram concentrations in the heartwood and sapwood of most trees (Scurfield and Nicholls, 1970). These low concentrations can be detected in the laboratory by the utilization of a gas chromatographic method. A Perkin-Elmer F-11 capillary column gas chromatograph coupled to an Infotronics CRS 208 electronic integrator (which is used to calculate the areas under amino acid peaks on the gas chromatograms) was employed to detect D and L amino acids extracted from the heartwood and sapwood of Sequoiadendron giganteum.

Initially, a standard mix of amino acids was prepared and subsequently employed to adjust the various parameters of the gas chromatograph for maximum separation of D and L amino acid derivatives. The standard mix of L amino acids was derivatized in the following manner:

1. Esterification with 0.3 ml acidified (±)-2-butanol for 3 hours at 100°C.

2. Excess butanol is evaporated under a stream of purified electronic grade nitrogen.
3. Acylation with 1.8 ml dichloromethane and 0.2 ml pentafluoropropionic anhydride (Peninsular Chemresearch Inc., Gainesville, Florida) for 2 hours at room temperature.

4. Evaporation of excess dichloromethane (CH₂Cl₂) and pentafluoropropionic anhydride (PFP) under a stream of purified electronic grade nitrogen.

5. Addition of dichloromethane as solvent for the injection of the derivatives into the gas chromatograph.

The above preparation and all additional laboratory work were conducted using acid clean glassware (hot, conc. H₂SO₄:HNO₃, 85:15 v/v) and freshly distilled solvents that were all reagent or pesticide grade.

The general structure of the prepared amino acid derivatives is shown in Figure 1. These amino acid standards are chemically indistinguishable from diastereomeric derivatives produced by esterifying racemic amino acid mixtures with optically pure (+)-2-butanol. For maximum separation of D and L amino acid derivatives 1 μl aliquots of the prepared derivatives were injected onto a Carbowax-20M 150' x 0.02" i.d. capillary column. The column was held isothermally at 90° C for 20 minutes and then programmed to 150° C at 1°/minute. The injection port temperature was held at 140° C, and the helium flow was set at 8.5 ml/minute. Figure 2
Figure 1. General Amino Acid Derivative (N-pentafluoropropionyl-(\(\pm\))-2-butyl esters).
Figure 2. Gas Chromatogram of Standard D and L Amino Acid Derivatives.

A Carbowax-20M 150' x 0.02" i.d. capillary column was used. The He flow was 8.5 ml/minute. The oven program was 90° C isothermal for 20 minutes; then 90° C to 150° C at 1° C/minute. The program was finished isothermal at 150° C.
shows the gas chromatogram for the separation of D and L diastero-meric derivatives of the standard mix of amino acids.

Amino Acid Racemization in *Sequoiadendron giganteum*

D/(D+L) ratios of amino acids present in fossil and modern wood samples can be used to calculate rate constants for the racemi-zation of these specific amino acids found in wood. The following procedure was established to detect D and L enantiomers of amino acids present in small quantities (i.e., less than 300 mg) of wood (Zumberge, Engel, Nagy, and Bannister, 1976). Wood samples of various ages from a sequoia tree (*Sequoiadendron giganteum*), accurately dated by dendrochronology, were investigated. The dated wood samples were provided by the Laboratory of Tree-Ring Research, The University of Arizona. The sequoia tree grew in the Windy Gulch Grove of California at latitude 36° 47'N, longitude 118° 51'W, and at an elevation of 6800 feet. Shallow 1/4" wide holes were first drilled in the surface of the section at the position of rings formed during the following dates: 2185 B.P., 1600 B.P., 975 B.P., 600 B.P., and 75 B.P. This step removes possible sur-face contamination. Next, 3/16" diameter holes, approximately 3/4" deep were then made into the previously drilled shallow holes, and the wood fragments were collected for each precise time interval. All of these samples came from the heartwood of the tree except for
the youngest sample (75 B.P.), which is from the sapwood. Very slow revolutions of a variable speed drill were used in order to minimize heating the wood during sample extraction. The drill bits were rinsed in distilled hexane and triple distilled water prior to drilling each sample. The wood fragments from each time interval were slowly and carefully ground to less than 20 mesh in acid cleansed mullite mortars with acid cleaned pestles. Subsequent to grinding, 100 to 200 mg samples of wood powder were washed with 1.5 N HCl (distilled 6 N HCl diluted with triple distilled water) and then with distilled water in acid cleaned fritted glass filters. This process removes non-bound or free amino acids. The residues were placed in separate 125 ml round bottom flasks. Twelve ml of distilled 6 N HCl were added to each flask. The bound amino acids were subsequently hydrolyzed at 100° C for 24 hours. The hydrolyzates were filtered in fritted glass filters and the filtrates (containing the amino acids) were evaporated to dryness under a stream of purified electronic grade nitrogen. The residues were dissolved in 2 ml of 0.06 N HCl and placed on Bio Rad AG 50W-X8, 50-100 mesh (Bio Rad Laboratories, Richmond, California) cation exchange columns. The ion exchange columns were prewashed repeatedly before each sample application with 2 N NaOH, distilled water, distilled 6 N HCl, and again with distilled water. After the samples were placed on top of the columns, 30 ml of distilled water containing one drop of
phenolphthalein (a basic color indicator) were passed through the column. The amino acids were then eluted with 40 ml of .93 N \( \text{NH}_4\text{OH} \). Thirteen ml of \( \text{NH}_4\text{OH} \) were collected at the solvent front. The \( \text{NH}_4\text{OH} \) solutions were subsequently evaporated to dryness under nitrogen. The (+)-2-butanol (99% optically pure from Norse Laboratories, Santa Barbara, California) was acidified by passing dry HCl gas through the alcohol to obtain 2-4 N solutions. Three-tenths ml of the acidified (+)-2-butanol were added to the purified amino acid hydrolyzates and esterified for 3 hours at 100° C. The excess butanol was evaporated under nitrogen and the residues were dissolved in 1.8 ml of dichloromethane. Two-tenths ml of PFP were added to the solutions, and the acylation reactions were allowed to proceed at room temperature for 2 hours. The mixtures were then carefully transferred to reaction vials, evaporated to dryness under nitrogen, and redissolved in dichloromethane. One \( \mu \)l aliquots were injected into a Perkin-Elmer F-11 gas chromatograph. Gas chromatography of these derivatives followed the same procedure described above for the analysis of the standard mix of amino acids.

Contamination Control

Experiments were conducted to determine whether or not the free amino acids removed from sequoia heartwood samples by the 1.5 N HCl wash prior to hydrolysis were 1) more highly racemized
than the bound amino acids and 2) potentially the result of contaminations by bacteria. A sequoia heartwood sample age 2185 B.P. that had previously been powdered, was placed in a fritted glass filter and washed three times with 1.5 N HCl and three times with triple distilled water. The filtrate was evaporated to dryness under nitrogen. The remaining residue from the filtrate and the washed wood were hydrolyzed separately in 12 ml of distilled 6 N HCl for 24 hours at 100° C and derivatized for injection into the gas chromatograph in a manner identical to the aforementioned procedure for the sequoia heartwood and sapwood samples of varying ages.

D amino acids are commonly found in the cell walls of bacteria. Ikawa (1964) has stated that 90% of the aspartic acid found in the cell walls of lactic acid bacteria (*Leuconostoc citrovorum*) exists in the D form. A unique collection of samples provided by the Laboratory of Tree-Ring Research at The University of Arizona allowed for the investigation of the affect of bacteria on the analysis of free and bound amino acids extracted from stored and rotten wood. In 1952, a douglas fir on Mount Lemmon (near Tucson, Arizona) fell down, at which time intact portions of the tree were collected and stored at the Laboratory of Tree-Ring Research at The University of Arizona. The stump of the tree was left in place on Mount Lemmon. In the fall of 1975 samples from this same stump were collected. Much of the wood appeared severely rotten, decayed, and/or
weavered. Some of the tree rings, however, were still identifiable in the decayed samples. Samples were extracted from the ring formed in 1699 A.D. from both the decayed and stored samples. Both samples were washed in fritted glass filters with 1.5 N HCl and triple distilled water. The washed wood and filtrates of the stored and rotten wood samples were analyzed for their D and L amino acid abundances by the same procedure described above for the analysis of the filtrate and washed wood of the sequoia heartwood sample age 2185 B.P.

**High Temperature Kinetic Experiments**

The following procedure was employed in an attempt to calculate the activation energies and frequency factors for protein bound aspartic acid, glutamic acid, and proline in the heartwood of the same sequoia tree that was analyzed in the preceding experiments. Shallow 1/4" wide holes were first drilled into the surface of a section of the sequoia tree that consisted of a composite of young heartwood samples ranging in age from 1683 A.D. -1781 A.D. This step removed possible surface contaminations. Five grams of heartwood were extracted for the kinetic experiments in a manner identical to the extraction procedure previously described for the heartwood and sapwood sequoia samples of varying ages. The heartwood was subsequently ground to less than 20 mesh in an acid cleaned mullite
mortar with an acid cleaned pestle. A portion of this powdered heartwood was divided into six pairs of samples of slightly varying weights (Table I).

Each of the twelve samples listed in Table I was placed separately in an acid cleaned fritted glass filter and washed three times with 1.5 N HCl (distilled 6 N HCl diluted with triple distilled water) followed by three washings with triple distilled water. As mentioned previously, this preliminary acid wash was performed to remove free amino acids that may or may not have been indigenous to the wood samples. The samples were dried for 3 hours under a stream of purified electronic grade nitrogen. Samples 2 through 6 (A and B) were placed in ten separate pyrex tubes. Sufficient quantities of triple distilled water were added to each sample to reconstitute the 86% moisture content at the dry weight (i.e., the natural water content of sequoia heartwood). These moistened samples were sealed in their respective pyrex tubes under a nitrogen atmosphere. Tubes 2(A and B), 3(A and B), 4(A and B), 5(A and B), and 6(A and B) were heated at 125°C for 336 hours, 137°C for 168 hours, 145°C for 96 hours, 151°C for 24 hours, and 166°C for 48 hours, respectively. Two tubes (e.g., 2A and 2B, etc.) were heated at each temperature so that duplicate samples could be analyzed to verify the results. The heating times were varied proportional to the temperature to which the tubes were exposed. Subsequent to heating
TABLE I. Weights of Heartwood Samples, Heating Times, and Heating Temperatures.

<table>
<thead>
<tr>
<th>Sample (mg)</th>
<th>Sample (mg)</th>
<th>Heating Time (hours)</th>
<th>Heating Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a. 145.0</td>
<td>1b. 174.5</td>
<td>0</td>
<td>---</td>
</tr>
<tr>
<td>2a. 131.8</td>
<td>2b. 153.6</td>
<td>336</td>
<td>125</td>
</tr>
<tr>
<td>3a. 125.0</td>
<td>3b. 162.3</td>
<td>168</td>
<td>137</td>
</tr>
<tr>
<td>4a. 144.1</td>
<td>4b. 137.0</td>
<td>96</td>
<td>145</td>
</tr>
<tr>
<td>5a. 177.3</td>
<td>5b. 139.5</td>
<td>24</td>
<td>151</td>
</tr>
<tr>
<td>6a. 132.8</td>
<td>6b. 135.5</td>
<td>48</td>
<td>166</td>
</tr>
</tbody>
</table>
which was done in ovens that were found to maintain a constant temperature, $\pm 0.5^\circ C$), the sealed tubes were allowed to cool to room temperature. Each sample was removed from its sealed tube and placed in a round bottom flask (125 ml). Twelve ml of distilled 6 N HCl were added to each flask. The samples were hydrolyzed for 24 hours at 100$^\circ$ C. After hydrolysis the hydrolyzates were run through acid cleaned fritted glass filters. The filtrates (which contained the amino acids) were evaporated to dryness under a stream of purified electronic grade nitrogen. The residues were redissolved in 2 ml of 0.06 N HCl and placed on Bio Rad AG 50W-X8, 50-100 mesh cation exchange columns to separate the amino acids from organic impurities in the residues. The amino acid residues were eluted from the cation exchange columns, derivatized, and detected by gas chromatography in a manner identical to the procedure described above for the detection of D and L amino acids in sequoia heartwood and sapwood samples.

To determine the amount of racemization that had occurred in the heated samples, it was necessary to calculate the amounts of D and L amino acids present in the sequoia heartwood prior to heating. For this purpose samples 1(A and B) were not heated (Table I). They were, however, washed, hydrolyzed, and derivatized in a manner identical to the procedure outlined for the heated samples.
Although a percentage of the racemization detected for amino acid enantiomeric pairs is actually real, 1.5% of the racemization detected for any particular D-L amino acid pair is caused by impurities in the optically pure (+)-2-butanol. Another 0.5% error can be attributed to racemization caused during the 24 hour hydrolysis step of the above procedures. Impurities in the (+)-2-butanol were detected by derivatizing pure L-leucine with the pure butanol and, subsequent to acylation with PFP, detecting the amount of D-leucine present in the sample by gas chromatography. The %D amino acid error caused by racemization during hydrolysis was determined by hydrolyzing pure L-aspartic acid for 24 hours, derivatizing it with (+)-2-butanol and PFP, and subsequently analyzing the %D-aspartic acid present by gas chromatography. Throughout all of the experiments conducted above sample blanks were continuously run through the procedural steps to insure that the amino acids detected were derived entirely from the samples under investigation.
CHAPTER 3

RESULTS

Calculation of Rate Constants for the Racemization of Aspartic Acid in Sequoia Heartwood and Sapwood Samples

Heartwood and sapwood samples dated 2185 B.P., 1600 B.P., 975 B.P., 600 B.P., and 75 B.P. were extracted from the sequoia tree and subsequently analyzed for their D/(D+L) bound amino acid ratios by the procedure described in Chapter 2. Aspartic acid was found to have one of the faster racemization rates in the sequoia samples. Application of equation (1) yielded rate constants for aspartic acid racemization in the five samples investigated. The rate constants for aspartic acid racemization are listed in Table II. A minimum of 4 gas chromatographic injections was done for each wood sample. The range of values for the rate constants in Table II is attributed to slight discrepancies in the numerous gas chromatographic injections done for each sample.

D/(D+L) ratios for aspartic acid that were used to calculate the rate constants in Table II were initially adjusted for 2%D-aspartic acid that had been caused by (-)-2-butanol impurity and racemization during hydrolysis. Equation (4), shown below, was used to correct the initial D/(D+L) ratios of aspartic acid that were obtained from
TABLE II. Calculated Rate Constants (k) for the Racemization of Bound Aspartic Acid in Sequoia Tree-Rings.

<table>
<thead>
<tr>
<th>Age (Years B.P.)</th>
<th>Arithmetic Mean (k) (x 10^{-5} yr^{-1})</th>
<th>Range (x 10^{-5} yr^{-1})</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>2185</td>
<td>2.11</td>
<td>1.99</td>
<td>2.31</td>
</tr>
<tr>
<td>1600</td>
<td>2.16</td>
<td>1.94</td>
<td>2.59</td>
</tr>
<tr>
<td>975</td>
<td>2.05</td>
<td>1.74</td>
<td>2.51</td>
</tr>
<tr>
<td>600</td>
<td>3.75</td>
<td>3.17</td>
<td>4.08</td>
</tr>
<tr>
<td>75</td>
<td>12.67</td>
<td>6.67</td>
<td>22.67</td>
</tr>
</tbody>
</table>
the gas chromatographic peak areas of the amino acids (Pollock, 1975).

\[
\%D = \frac{50(A-x)}{50-x} \quad \ldots \quad (4)
\]

where \(\%D\) is equal to the actual percentage of D isomer, \(A\) is the apparent percentage of D isomer from the gas chromatographic peak areas of the samples, and \(x\) is equal to the correction factor (the percentage of (-)-2-butanol and the extent of D isomer formed during hydrolysis).

Figure 3 is a plot of the natural logarithm of the ratio 
\((X_e - X)/X_e \quad [X_e = D/(D+L)\) at equilibrium, which is equal to 0.5, and \(X = D/(D+L)\) at time \(t\)\] for bound aspartic acid in the sequoia samples versus time. From the straight line shown in Figure 3 it appears that the racemization of bound aspartic acid in the three older heartwood samples follows first order reversible kinetics. Bound aspartic acid in the sapwood sample (75 B.P.) and youngest heartwood sample (600 B.P.) apparently racemizes at a faster rate than in the older heartwood samples. Environmental differences may account for this discrepancy in racemization rates. Old heartwood of a sequoia tree contains approximately 86% water \([(\text{weight } H_2O/\text{weight dry wood}) \times 100]\) while the sapwood and younger heartwood samples contain almost three times more water (U.S. Department of Agriculture, 1974).
Figure 3. The Natural Logarithm of the Ratio $(X_e - X)/X_e$ Versus Time for Bound Aspartic Acid in Sequoia Wood Samples.

$X_e = D/(D+L)$ at equilibrium = 0.50, and $X = D/(D+L)$ at time $t$. The 2% D-aspartic acid correction was applied first (equation 4). The dashed straight line through the three oldest heartwood samples and the origin indicates that bound aspartic acid racemization follows reversible first order kinetics in the older heartwood.
Contamination Study

A comparison of the amount of racemization in the free amino acid fraction extracted from the 2185 B.P. sequoia heartwood sample with the 1.5 N HCl wash with the amount of racemization detected in the bound amino acid fraction of this sample is shown in Figure 4. The free amino acids in the wash contained 26.1% D-aspartic acid compared with the 6.2% D-aspartic acid detected in the bound amino acid fraction. Free amino acids in the wood sample may be racemizing faster than the bound amino acids. The possibility of contamination of the heartwood by bacteria containing D amino acids in their cell walls cannot be eliminated, although sequoia heartwood is known to be resistant to microbial attack and subsequent decay. It is also resistant to attack by natural, dilute mineral and organic acids (U.S.D.A., 1974).

The results of the douglas fir contamination study provided information concerning the possibilities of sample contamination by microbial attack. The bound amino acid fractions extracted from the stored and decayed 1699 A.D. samples contained 3.4% and 3.5% D-aspartic acid, respectively. The free amino acid fractions (extracted with 1.5 N HCl washes) of the stored and decayed 1699 A.D. wood samples contained 4.1% and 8.9% D-aspartic acid, respectively. Bound aspartic acid in the stored and weathered wood samples does not appear to have been contaminated with D-aspartic acid. The free
Figure 4. Free Versus Bound Amino Acids in 2185 Year Old Sequoia Heartwood.

Column type and column program are given in Figure 2.
amino acid fraction of the decayed wood, however, has been highly
contaminated with D-aspartic acid. Caution must be taken in the
amino acid analysis of wood samples. The bound amino acid fractions
are more likely to be indigenous to the tree rings investigated than
are the free amino acid fractions.

**Kinetic Study**

The heated and unheated samples that were used in the high
temperature kinetic study were analyzed for their D and L amino acid
content (as described in the procedure) by gas chromatography.
Between 4 and 6 gas chromatographic injections were done for each of
the duplicate samples in an effort to obtain the most reliable D/(D+L)
amino acid ratios possible. Figure 5 is a gas chromatogram of the
unheated sequoia heartwood, age 1683-1781 A.D. Figures 6, 7, 8, 9,
and 10 are representative gas chromatograms of the 125°C, 137°C,
145°C, 151°C, and 166°C heated sequoia heartwood samples,
respectively. Table III lists the extent of racemization of aspartic
acid, glutamic acid and proline in the 125°C, 137°C, 145°C, and
151°C heated samples along with their corresponding rate constants
(in natural logarithmic form) calculated from equation (1). The 166°C
samples experienced a high degree of amino acid degeneration. The
D/(D+L) ratios for these samples could not be determined with any
degree of certainty. The D/(D+L) ratios in Table III were corrected
Figure 5. Gas Chromatogram of D and L Amino Acid Derivatives in Unheated Sequoia Heartwood.

Figure 5 is a gas chromatogram of N-pentafluoropropionyl- (+)-2-butyl ester diastereomeric derivatives of D and L enantiomers of amino acids detected in unheated sequoia heartwood, age 1683-1781 A.D. Column conditions are the same as in Figure 2.
Figure 6. Gas Chromatogram of D and L Amino Acid Derivatives in Sequoia Heartwood Heated at 125°C for 336 hours.

Column conditions are the same as in Figure 2.
Figure 7. Gas Chromatogram of D and L Amino Acid Derivatives in Sequoia Heartwood Heated at 137° C for 168 Hours.

Column conditions are the same as in Figure 2.
Figure 8. Gas Chromatogram of D and L Amino Acid Derivatives in Sequoia Heartwood Heated at 145° C for 96 Hours.

Column conditions are the same as in Figure 2.
Figure 9. Gas Chromatogram of D and L Amino Acid Derivatives in Sequoia Heartwood Heated at 151°C for 24 Hours.

Column conditions are the same as in Figure 2.
Figure 10. Gas Chromatogram of D and L Amino Acid Derivatives in Sequoia Heartwood Heated at 166°C for 48 Hours. Column conditions are the same as in Figure 2.
TABLE III. High Temperature Rate Constants of Aspartic Acid, Glutamic Acid, and Proline.

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>1/T (x 10^{-3} °K^{-1})</th>
<th>Time (Hrs.)</th>
<th>% (\frac{D}{D+L}) \pm 1\sigma</th>
<th>ln (k yr^{-1})*</th>
</tr>
</thead>
<tbody>
<tr>
<td>125</td>
<td>2.51</td>
<td>336</td>
<td>20.1 ± 4.3 25.2 ± 3.6 12.2 ± 2.1</td>
<td>1.73 2.12 1.16</td>
</tr>
<tr>
<td>137</td>
<td>2.44</td>
<td>168</td>
<td>19.2 ± 3.2 27.1 ± 2.9 13.7 ± 1.6</td>
<td>2.35 2.93 2.01</td>
</tr>
<tr>
<td>145</td>
<td>2.39</td>
<td>96</td>
<td>22.0 ± 2.3 24.4 ± 1.8 7.5 ± 1.4</td>
<td>3.12 3.32 1.76</td>
</tr>
<tr>
<td>151</td>
<td>2.36</td>
<td>24</td>
<td>25.0 ± 3.1 30.9 ± 2.4 8.2 ± 1.8</td>
<td>4.71 5.10 3.27</td>
</tr>
</tbody>
</table>

*Calculated from equation (1) after correcting [equation (4)] for initial D concentrations of unheated wood: D-asp = 4.0%; D-glu = 3.0%; and D-pro = 1.7%.
for initial D concentrations of amino acids in the wood samples by the use of equation (4), where the correction factor (x) was equal to the percentage of (-)-2-butanol, the extent of D isomer formed during hydrolysis, plus the amount of D isomer naturally present in the unheated heartwood sample. The total D amino acid abundances in the unheated heartwood samples were used as the correction factors for the D/(D+L) ratios detected in the heated heartwood samples (Table III).

Changes in amino acid abundances invariably occurred in the heated heartwood samples compared with the unheated samples. In the unheated heartwood samples, aspartic acid was the most abundant amino acid detected on the carbowax gas chromatographic column. Aspartic acid was found to decrease in abundance relative to the other amino acids detected in the heated heartwood samples (125° C, 137° C, 145° C, and 151° C samples). Aspartic acid was virtually absent in the 166° C wood samples (Figure 10).

Gas chromatograms of the heated wood samples contained a variety of unidentified peaks which are tentatively classified as breakdown products of amino acids and other unknown organic compounds that survived sample preparation prior to derivatization. A few of the breakdown products had retention times similar to amino acids in the samples, therefore causing difficulty in obtaining accurate D/(D+L) ratios. Breakdown product interference was particularly
noticeable with L-leucine. A comparison of L-leucine in the unheated wood sample (Figure 5), with the peak labeled "?" (that had the same retention time as L-leucine) in the 166° C heated sample (Figure 10) reflects the high degree of interference experienced by a number of the amino acid derivatives in the heated samples.

Proline, aspartic acid, glutamic acid, phenylalanine, and glycine did not appear to be interfered with in the gas chromatograms of the heated samples. Table IV shows the fluctuations in relative abundances of these five amino acids with respect to each other that occurred in the various high temperature experiments of the kinetic study. The following changes in relative amino acid abundances were detected in the heated and unheated sequoia heartwood samples: in the unheated sample, asp > gly > glu > pro > phe in abundance; in the 125° C sample, pro > glu > phe > gly > asp in abundance; in the 137° C sample, pro > gly > glu > phe > asp in abundance; in the 145° C sample, pro > glu > gly > phe > asp in abundance; in the 151° C sample, pro > glu > gly > phe > asp in abundance; in the 166° C sample, pro > gly > phe > glu > asp in abundance.

Aspartic acid is unstable in the temperature range of 125° C to 166° C. Glutamic acid appears to be relatively stable in the range of 125° C to 151° C, but decomposes at a faster rate when heated at a temperature of 166° C. Proline and phenylalanine remain stable within the temperature range that has been investigated. The
### TABLE IV. Changes in Relative Amino Acid Abundances in Heated and Unheated Sequoia Heartwood Samples (%).

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Unheated</th>
<th>125°C</th>
<th>137°C</th>
<th>145°C</th>
<th>151°C</th>
<th>166°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>glycine</td>
<td>18.69</td>
<td>10.99</td>
<td>18.58</td>
<td>20.81</td>
<td>20.73</td>
<td>27.14</td>
</tr>
<tr>
<td>proline</td>
<td>16.99</td>
<td>38.62</td>
<td>42.23</td>
<td>30.85</td>
<td>33.88</td>
<td>39.26</td>
</tr>
<tr>
<td>aspartic acid</td>
<td>35.44</td>
<td>7.98</td>
<td>7.35</td>
<td>4.78</td>
<td>5.65</td>
<td>4.01</td>
</tr>
<tr>
<td>phenylalanine</td>
<td>10.52</td>
<td>20.33</td>
<td>15.85</td>
<td>16.66</td>
<td>18.56</td>
<td>22.62</td>
</tr>
<tr>
<td>glutamic acid</td>
<td>18.37</td>
<td>22.08</td>
<td>16.00</td>
<td>26.91</td>
<td>21.19</td>
<td>6.95</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>
decomposition of glycine appears to be a time dependent reaction. Glycine remains stable when heated between 137°C and 166°C for the times indicated in Table I. However, glycine begins to decompose when heated at 125°C for a longer duration of time (336 hours). Table V lists comparative ratios for the five amino acids that are discussed in Table IV.

The accuracy of these ratios may be limited by a number of factors. The abundances of the amino acids were determined by measuring peak intensities produced by a flame ionization detector. Glutamic acid and aspartic acid both contain two carboxylic acid functionalities. Upon esterification with butanol, these acidic amino acids increase their molecular weight by 57 grams/mole relative to the neutral and basic amino acids. These higher molecular weights may enhance the peak intensities of aspartic acid and glutamic acid. Basic amino acids in a similar manner may react with twice as much PFP when two or more amine positions are available for attack during acylation. Drastic differences in the size of 'R' groups of different amino acids may also slightly alter the ratios shown in Table V. The ratios listed in Table V should be considered as rough estimates, particularly in cases such as gly/pro, gly/phe, gly/glu, where the ratios approach 1.

Figures 11, 12, and 13 show the plots of \( \ln k \, yr^{-1} \) versus the inverse of the temperature (°K) for aspartic acid, glutamic acid, and
TABLE V. Comparative Ratios for Amino Acids that Were Consistently Detected in Heated and Unheated Sequoia Heartwood Samples.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Unheated</th>
<th>125°C</th>
<th>137°C</th>
<th>145°C</th>
<th>151°C</th>
<th>166°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>gly/pro</td>
<td>1.10</td>
<td>0.28</td>
<td>0.44</td>
<td>0.67</td>
<td>0.61</td>
<td>0.69</td>
</tr>
<tr>
<td>gly/asp</td>
<td>0.53</td>
<td>1.38</td>
<td>2.53</td>
<td>4.35</td>
<td>3.67</td>
<td>6.72</td>
</tr>
<tr>
<td>gly/phe</td>
<td>1.30</td>
<td>0.54</td>
<td>1.17</td>
<td>1.25</td>
<td>1.12</td>
<td>1.20</td>
</tr>
<tr>
<td>gly/glu</td>
<td>1.02</td>
<td>0.50</td>
<td>1.16</td>
<td>0.72</td>
<td>0.98</td>
<td>3.91</td>
</tr>
<tr>
<td>pro/asp</td>
<td>0.48</td>
<td>4.84</td>
<td>5.74</td>
<td>6.45</td>
<td>6.00</td>
<td>9.72</td>
</tr>
<tr>
<td>pro/phe</td>
<td>1.62</td>
<td>1.90</td>
<td>2.67</td>
<td>1.85</td>
<td>1.83</td>
<td>1.74</td>
</tr>
<tr>
<td>pro/glu</td>
<td>0.93</td>
<td>1.75</td>
<td>2.64</td>
<td>1.15</td>
<td>1.60</td>
<td>5.65</td>
</tr>
<tr>
<td>asp/phe</td>
<td>3.37</td>
<td>0.39</td>
<td>0.46</td>
<td>0.29</td>
<td>0.30</td>
<td>0.18</td>
</tr>
<tr>
<td>asp/glu</td>
<td>1.93</td>
<td>0.36</td>
<td>0.46</td>
<td>0.18</td>
<td>0.27</td>
<td>0.58</td>
</tr>
<tr>
<td>phe/glu</td>
<td>0.57</td>
<td>0.92</td>
<td>0.99</td>
<td>0.62</td>
<td>0.88</td>
<td>3.26</td>
</tr>
</tbody>
</table>
Figure 11. An Arrhenius Plot of Aspartic Acid Determined from High Temperature Kinetic Experiments.

The vertical error bars represent the range of values for the natural logarithms of the rate constants that were a consequence of the values obtained from integrated peak area measurements of the gas chromatographic runs done for each set of samples.
Figure 12. An Arrhenius Plot of Glutamic Acid Determined from High Temperature Kinetic Experiments.

The vertical error bars represent the range of values for the natural logarithms of the rate constants that were a consequence of the values obtained from integrated peak area measurements of the gas chromatographic runs done for each set of samples.
Figure 13. An Arrhenius Plot of Proline Determined from High Temperature Kinetic Experiments.

The vertical error bars represent the range of values for the natural logarithms of the rate constants that were a consequence of the values obtained from integrated peak area measurements of the gas chromatographic runs done for each set of samples.
proline, respectively. It appears that for aspartic acid and glutamic acid the temperature dependence of the rate constant does not follow the Arrhenius relationship at 151°C. It should also be noticed that the lower temperature values for proline do not fall on a straight line. The Arrhenius plots indicate that the activation energies for the racemization of bound aspartic acid, glutamic acid, and proline are not constant values over the entire range of temperatures that were investigated (i.e., the slopes of these lines are fluctuating).

Best fit lines of the three lower temperatures (125°C, 137°C, and 145°C) for bound aspartic acid and glutamic acid, which appear to give relatively straight lines, allowed for the calculation of activation energies and frequency factors for aspartic acid and glutamic acid. Bound aspartic acid was found to have an activation energy of 22.6 kcal and a frequency factor of $1.3 \times 10^{13}$ yr$^{-1}$. Bound glutamic acid yielded an activation energy of 20.0 kcal and a frequency factor of $7.9 \times 10^{11}$ yr$^{-1}$. Activation energies and frequency factors were determined from the data in Table III and Figures 11 and 12 by the application of the least squares method of analysis of the 125°C, 137°C, and 145°C points.
CHAPTER 4

DISCUSSION

Equation (1) yielded a rate constant $k$ for bound aspartic acid in the 2185 B. P. sequoia heartwood sample of $2.11 \times 10^{-5} \text{ yr}^{-1}$. The kinetic study yielded an activation energy of 22.6 kcal and a frequency factor of $1.3 \times 10^{13} \text{ yr}^{-1}$ for bound aspartic acid in sequoia heartwood. When the rate constant, activation energy, and frequency factor of bound aspartic acid are inserted into the Arrhenius equation (3), an average temperature experienced by bound aspartic acid over the past 2185 years is determined. A temperature of approximately $5^\circ \text{C}$ was calculated in this manner.

The Grant Grove and Lodgepole weather stations in California are located at $36^\circ 44' \text{N}$ latitude, $118^\circ 58' \text{W}$ longitude, 6600' elevation; and $36^\circ 36' \text{N}$ latitude, $118^\circ 44' \text{W}$ longitude, 6735' elevation, respectively. These weather stations are located in close proximity to the Windy Gulch Grove where the sequoia tree once grew. The mean annual temperatures recorded at Grant Grove for the past twenty years were approximately $8^\circ \text{C}$. The mean annual temperatures recorded at Lodgepole station for the past eight years were approximately $5.5^\circ \text{C}$ (U. S. Dept. of Commerce, 1947, 1969, 1970, 1974). The average temperature ($5^\circ \text{C}$) reflected by the sequoia heartwood
sample is remarkably similar to these actual values. An in-depth study of wood samples taken every hundred years from a sequoia tree that was in excess of 2000 years of age could potentially provide extensive information concerning past temperature fluctuations for the region in which the tree grew.

The aforementioned results, while extremely encouraging, must be interpreted with caution. The results are no more accurate than our present day knowledge of the amino acid racemization reaction in a complex organic matrix such as wood. As stressed in the introduction, a variety of factors other than time and temperature are known to influence amino acid racemization (e.g., pH, water concentration, metal ion concentrations, etc.). The effects of lignin and cellulose (both principal components of wood) on the rates of amino acid racemization have yet to be investigated in detail. Indeed, the actual structure of lignin is not completely known. Changes in glucose concentrations are known to increase the rate of degradation of alanine at high temperatures (Vallentyne, 1964). The possibility exists that the B(1-4) glycosidic linkages of cellulose were broken during the high temperature heat treatments of the wood samples. Destruction of these linkages yields glucose monomers or small polymers which may have affected the rate of degradation of certain amino acids present in the heartwood samples. It is imperative that future investigations be conducted on the effects of lignin and
cellulose on amino acid racemization and degradation at normal and high temperatures.

Important questions concerning the validity of information (i.e., activation energies and frequency factors) obtained by the use of high temperature kinetic studies on amino acid racemization must be answered prior to the continuation of this line of research. Bound amino acids experienced varying degrees of degradation during the high temperature kinetic experiments described above. It is assumed that D and L enantiomers of specific amino acids undergo degradation at identical rates during heating. However, a kinetic sample such as the young sequoia heartwood is composed primarily of L amino acids prior to heating. If degradation is an equally competitive reaction with racemization, it is possible that a greater amount of L amino acids are being destroyed than their D counterparts. This preferential degradation may distort D/(D+L) amino acid ratios.

A number of amino acids, when heated during thermal experiments have been found to degrade to, among other things, different amino acids (Vallentyne, 1964, 1968). Histidine, valine, serine, threonine, methionine, and tyrosine all produce glycine as a product during thermal degradation. Methionine and histidine have also produced traces of alanine. Arginine produces proline as a thermal degradation product (Vallentyne, 1964). Investigations of amino acid breakdown products at high temperatures must be
pursued further. It must be ascertained that degradation reactions are not occurring in heated wood samples that are producing amino acids that are principal components of the kinetic investigation (i.e., aspartic acid and glutamic acid). Amino acid "reorganization" of this nature could conceivably have distorted the information on relative amino acid abundances in the kinetic samples that was presented in Tables IV and V.

Hydrogen ion concentration is known to affect amino acid racemization. The heartwood samples reconstituted with 86% water prior to heating at 166°C, were tested for pH before and after heating. Initially these wood samples mixed with triple distilled water had a pH of approximately 5. Subsequent to heating, the pH of the water solution was found to be approximately 3. The causes of this pH drop and their affects upon racemization are currently under investigation. Glutamic acid, glycine, alanine, and phenylalanine decarboxylate when heated in the absence of oxygen during high temperature kinetic experiments (Abelson, 1954b; Vallentyne, 1964). Reactions of this nature offer a potential explanation for the drop in pH that has occurred. It is also possible that some of the bound acidic amino acids are released into the aqueous medium during heating. However, there is presently no explanation that would account for the preferential release of acidic amino acids relative to basic amino acids from the wood matrix. The large quantities of
ammonia and variety of amines produced when amino acids are heated must also be taken into consideration.

Carbon dioxide is two times as soluble as ammonia in cold water. Although carbon dioxide and ammonia are both common breakdown products of bound amino acids in the heartwood samples that were heated, a higher percentage of ammonia may have escaped as a gas when the wood samples were removed from their sealed tubes. Carbon dioxide, on the other hand, tended to remain dissolved in the water that was present in the thermal samples. The high solubility of carbon dioxide in water may have accounted for the drop in pH detected in the high temperature kinetic samples. Phenolic compounds liberated by lignin degradation may also have contributed to the pH drop.

It has been reported in the literature (Lee, Bada, and Peterson, 1976) that proline racemizes at a fast rate in fossil and recent wood samples. Initial investigations by the Laboratory of Organic Geochemistry at The University of Arizona indicated that proline was racemizing at a detectable rate in 2185 year old sequoia heartwood. However, continued improvement of column separation led to the discovery that the D-proline peak in sequoia heartwood gas chromatograms was being enhanced by an unknown organic substituent of the wood with a similar retention time. Gas chromatograms (Figures 5 through 10) clearly show this unknown compound as a
separate entity, existing with a retention time intermediate to those of glycine and D-proline. The identification of this unknown compound is currently being attempted by the application of combined gas chromatography-mass spectrometry.

The research presented in this thesis is part of a larger interdisciplinary study presently undertaken by the Laboratory of Organic Geochemistry and the Laboratory of Tree-Ring Research at The University of Arizona in an attempt to detect and interpret the occurrence of amino acid racemization in *Sequoiadendron giganteum* and *Pinus longaeva* D. K. Bailey (bristlecone pine). The eventual goal of this research project is the development of a paleotemperature indicator for the past 8000 years, and the improvement of the amino acid dating technique as applied to biogenic remains of the Pleistocene and post-Pleistocene period.
REFERENCES


