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Interlaboratory Comparison of Amino Acid Enantiomeric Ratios in Fossil Pleistocene Mollusks

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Enantiomeric (D/L) ratios of eight amino acids in three homogeneous powdered fossil Pleistocene mollusk samples have been compared. Eleven laboratories have contributed results to this effort. Three gas chromatographic methods and one ion-exchange chromatographic method were used. In general, the coefficients of variation (c.v.) are best for alanine, glutamic acid, and aspartic acid (3–8%). For leucine and phenylalanine, the c.v.'s are between 5 and 10%. For isoleucine, proline, and valine the c.v.'s range from 10 to 18%. Individual laboratories usually report analytical precision of between 2 and 5%, but it is clear that significant *differences* between laboratories' results are often encountered, and that these analytical differences could, in some cases, lead to very significant differences (25% or more) in age estimates based upon enantiomeric ratios. Multiple analyses of desalted hydrolyzates of these powder samples suggest that interlaboratory differences are often caused by instrumental and/or derivatization procedures rather than the wet-chemical sample preparative steps. It is proposed that the powder samples described here be used in all future publications of fossil amino acid enantiomeric ratio data.

INTRODUCTION

In 1980 the first systematic effort at interlaboratory comparison of amino acid enantiomeric (D/L) ratios in fossil mollusks was published (Kvenvolden, 1980). This study involved the distribution and analysis of a homogeneous powdered sample of a fossil *Saxidomus*, a common mollusk in Pleistocene marine terrace deposits of the Pacific coast of the United States. Eleven laboratories participated in this interlaboratory exercise, each reporting enantiomeric ratios as determined by either gas chromatography (GC) or liquid (ion-exchange) chromatography (LC). Few laboratories, either then or now, routinely employ both GC and LC for enantiomeric ratio determinations, and because these methods do not easily produce enantiomeric ratios for the same amino acids, there is an inherent lack of comparability in the results currently available from these laboratories.

This report presents results for a second phase of the interlaboratory comparison work. Several new laboratories have become involved in amino acid geochronology and stratigraphy since the first interlaboratory comparison, and the need for abundant reference samples (with different D/L values) has become clear as attempts have been made to compare results produced from different laboratories. The occasional sharing of samples among a few laboratories has permitted some such comparisons (e.g., Bada *et al.*, 1979), but such samples are usually not available to many investigators and, unless rather large, do not remain as long-term working reference samples. In order to eliminate this problem of the lack of abundant, natural fossil reference material, three such reference samples, all powdered fossil mollusks, were prepared in large quantities (roughly 3 kg each) and distributed to 20 laboratories between December 1981 and October 1982. Each laboratory has enough of each sample so that routine analyses can be made or so that new methods (or analysts) can be evaluated for their reliability. Three samples were used in the present interlaboratory study in order to compare results over a

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wide range of enantiomeric ratios (for most amino acids, these ranges were between D/L values of 0.20 and 0.90).

SAMPLES AND PREPARATIVE METHODS

The samples distributed to all laboratories have been labeled as ILC-A, ILC-B, and ILC-C. The samples consist of either *Saxidomus* (ILC-A) or *Mercenaria* (ILC-B and ILC-C). All were collected by the author between May 1980 and July 1981. The localities and samples used for this interlaboratory study are summarized in Table 1. The particular localities were chosen because of their known abundant supplies of well-preserved samples. The aminostratigraphy of all three localities has been discussed previously [Cape Blanco (Kennedy, 1978; Kennedy *et al.*, 1982); Calabash and Mark Clark Pits (Wehmiller and Belknap, 1982; McCartan *et al.*, 1982)].

The samples used in this study were distributed to all amino acid geochemical laboratories that specifically requested participation. Notification of the availability of the samples was made via letters to the 11 laboratories that participated in the original study (Kvenvolden, 1980) and to about 15 other individuals whom it was thought would be interested. All recipients of the first announcement of this project were requested to inform others of the availability of the samples. The laboratories that received the samples are listed in Table 1. For a variety of reasons, several of these laboratories were not able to report data within the time frame of the project, but they are listed here so that the current sample distribution is known.

Mollusks used for preparation of the powdered reference samples were scrubbed with a brush in mild detergent to remove all associated sediment material. They were then subjected to extensive washing in dilute HCl solutions, followed by rinsing with tap water and then distilled water. The HCl washes removed between 10 and 20% of each mollusk valve that was used in preparation of the powders. All the mollusk valves used were nearly complete but none was articulated. Valves with any

TABLE 1. LOCALITY AND SAMPLE INFORMATION AND PARTICIPATING LABORATORIES

Sample	Location	Lat., Long.	Mean annu. temp.
ILC-A	Cape Blanco, Oreg.	42.8°N, 124.53°W	10°C
Genus: *Saxidomus*, approx age: 50,000 yr, Collected: 7/81, Reference: Kennedy *et al.* (1982).			
ILC-B	Mark Clark Pit, Charleston, S.C.	32.83°N, 80.03°W	19°C
Genus: *Mercenaria*, approx age, 100,000 to 250,000 yr, Collected: 5/80, Reference: Wehmiller and Belknap (1982).			
ILC-C	Calabash Pit, Calabash, N.C.	33.89°N, 78.56°W	18°C
Genus: *Mercenaria*, approx age, early Pleistocene, ca. 1 million yr, Collected: 5/80. Reference: Wehmiller and Belknap (1982).			

Participating Laboratories[a]

UNIVERSITY OF SOUTH FLORIDA	U.S. GEOL. SURVEY, MENLO PARK
CARNEGIE INST. WASHINGTON	UTAH STATE UNIV.
UNIV. DELAWARE	UNIV. COLORADO
SCRIPPS INST. OCEANOGRAPHY	UNIV. ALBERTA
UNIV. MASSACHUSETTS	UNIV. TEXAS, RICHARDSON
UNIV. BERGEN	
NASA–Ames Res. Center	Univ. Arizona
Lamont–Doherty Geol. Obs.	Univ. E. Anglia
California State Univ./Hayward	Univ. College of Wales
Univ. Quebec, Montreal	Univ. Calif./Riverside

[a] Laboratories listed in lowercase have received the ILC samples but were not able to report data within the time frame of this project.

chalky or poorly preserved portions were specifically avoided. X-Ray analysis of random samples from the final powdered preparations revealed less than 2% calcite. For the ILC-A and ILC-B samples, about 20 valves were used in preparing the final powder for distribution. Three large valves were used for the preparation of ILC-C.

The cleaned mollusks were broken into ca. 5-g fragments and mechanically ground to a fine mesh using a stainless-steel disk grinder. Though some heating of the powders undoubtedly occurred during this grinding process, it is thought that this heating could not have been very significant because the enantiomeric ratios observed in this study are generally quite close to those observed in shells (from these localities) that have not been ground and pulverized (Wehmiller *et al.*, 1977; Wehmiller and Belknap, 1982). The powders were sieved through a 1.0-mm (16-mesh) screen in order to remove any coarse fragments. Qualitative analysis of the powders indicated that more than 90% of the material would pass through a 63-μm (250-mesh) screen.

Powder samples for distribution were randomly scooped from plastic bags containing all of the homogenous sieved powder. Approximately 15-g portions were transferred to precleaned screw-cap glass vials, and these vials then were selected at random for shipment to participating laboratories.

In January 1982 desalted hydrolyzates were distributed to 11 laboratories in order to evaluate precision of analytical methods for sample extracts that did not have to be carried through an entire wet-chemical laboratory procedure. These hydrolyzates were prepared by dissolution of approximately 6 g of each ILC powder with concentrated HCl in a clean flask, followed by transfer of each dissolved sample to five or six separate test tubes for hydrolysis (22 hr, 110°C) after addition of an appropriate amount of HCl to bring the final concentration to 6 N. After hydrolysis, the liquids were desalted using standard cation-exchange chromatographic methods. The desalted column eluates were dried using rotary evaporators and then combined into one solution (in 1 *N* HCl) that represented the entire amino acid population of each original ILC powder. These liquid samples were desalted in individual fractions and recombined so that there would be essentially no difference between the desalting methods used for these samples and those normally enjoyed in the Delaware laboratory. Differences in the dissolution and prehydrolysis handling procedure *did* affect the actual enantiomeric ratio values observed in some of these ILC liquid samples. These procedural effects are discussed in a following section.

Four analytical methods were employed by the participating laboratories. Most of the laboratories reported results derived from ion-exchange chromatographic methods (Hare, 1975). The only enantiomeric ratio determined by this procedure is that for D-alloisoleucine/L-isoleucine. These two amino acids are diastereoisomers and have different chemical properties which permit them to be resolved using ion-exhange systems. Because of the speed of most ion-exchange analyses, and their relatively small cost and ease of sample preparation, this method appears to be the most popular among amino acid geochronology laboratories. In addition to the ion-exhange data reported by 8 laboratories, several laboratories reported results obtained by one or more gas chromatographic methods. These methods permit the determination of enantiomeric ratios of several amino acids (up to 8 in some cases) but usually do not clearly resolve the diastereoisomeric pair D-alloisoleucine/L-isoleucine. Hence there have been few opportunities to directly compare, using the same samples, results obtained by the gas- and ion-exchange chromatographic methods. Though the gas-chromatographic methods have as their major advantage the analysis of multiple enantiomeric pairs, their prime disadvan-

tage is their relative cost and longer time required for sample preparation and analysis.

The gas chromatographic methods employed in this study are as follows: (a) the (+)-2-butyl method of Kvenvolden *et al.* (1972), in which NTFA or NPFPA derivatives were chromatographed on capillary columns coated with either Carbowax 20-M, OV-225, or UCON 75-H-90,000; (b) the isopropyl method of Frank *et al.* (1977), in which NTFA or NPFPA derivatives were chromatographed on capillary columns coated with a commercial phase (or its equivalent) known as Chirasil-val (Applied Science Laboratories, State College, PA.); (c) the TPC method of Hoopes *et al.* (1978) in which NTFA-L-prolyl-peptide methyl esters were chromatographed on packed columns of SP-2250. Chromatograms representative of these methods are found in the above publications and in papers published by the different participating laboratories.

As the author was also one of the analysts to contribute results to the study, it was necessary that he not be aware of any results from other laboratories until all results from the Delaware laboratory were reported. Therefore all results were communicated directly to Dr. B. P. Glass of the Geology Department at the University of Delaware, who retained them until the author's results were also reported. Compilation of data began in April 1982 and a preliminary report on this study was made at the 1982 Meeting of the Geological Society of America (Wehmiller, 1982).

RESULTS

Results from this study are reported in Table 2. All results are presented with the assumption that they would have been of "publication quality" and that no further modification of the data would be required. Results are given for powder hydrolyzates (samples carried through the entire wet-chemical procedure for a laboratory and for the liquid samples prepared at Delaware. Analyses of free amino acids (those present in a sample due to diagenetic hydrolysis and extractable by simple dissolution of the shell material) were supplied by many of the ion-exchange laboratories but by only one of the gas chromatography labs. Analytical precision values were reported by most laboratories and these are also presented in Table 2.

In November 1982 all the currently available results were compiled and communicated to every laboratory that had received the reference samples. A few laboratories reported minor modification of their results or requested that data reported earlier be considered as "preliminary" and not included in any statistical compilation of the data. All the current and "best" data are included in Table 2, and preliminary results are so indicated.

In several cases multiple data sets were reported from a single laboratory. Often more than one gas chromatographic method was used by the same laboratory and in a few cases an ion-exchange laboratory ratios as determined by both peak height and peak area calculations. Multiple data sets from a single laboratory were not pooled to calculate an average for that laboratory. Although this procedure might introduce some bias into the overall mean value for any enantiomeric ratio, it was felt that it was more important to report all results from a given laboratory so that the true range of results would be better documented.

In the original design of this project, it was intended that no laboratories would be specifically identified in the final report. This was also the policy of Kvenvolden (1980). Nevertheless, in a group discussion at the 1982 Meeting of the Geological Society of America it was agreed that all future publications by participating amino acid laboratories should either report new results for these Interlab Samples or identify their results as reported here. Only some sort of identification procedure such as this will permit free and open comparison of results produced by different labo-

TABLE 2. ENANTIOMERIC RATIO DATA FOR EACH SAMPLE, IDENTIFIED BY INDIVIDUAL LABORATORY AND ANALYTICAL METHODS

Amino acid analysis

ILC-A powder

LAB	ALA	VAL	LEU	ILEU	PRO	ASP	PHE	GLU	METHOD
A	0.357 0.003	0.146 0.008	0.192 0.007	0.239 0.009	0.262 0.013	0.387 0.007	0.224 0.009	0.195 0.005	P-TFA
A	0.373 0.008	0.208 0.009	0.218 0.008	0.254 0.021	0.317 0.006	(0.377) 0.011	0.248 0.011	0.215 0.010	B-TFA
B				0.16 0.02					I EX
B	0.35	0.12	0.19	0.17	(0.27)	0.43	0.23	0.20	P-TFA
C				0.25 0.018					I EX
C	0.35 0.017	0.14 0.015	0.21 0.010		0.29 0.006	0.37 0.015	0.28 0.03	0.20 0.006	B-PFP
D	0.339 0.007		0.141 0.014			0.401 0.011			P-TFA
E*				0.182 0.004					I EX
E*	0.365 0.011	0.136 0.009	0.202 0.001			0.379 0.005	0.238 0.027	0.187 0.008	P-TFA
E*	0.367 0.017	0.169 0.014	0.205 0.006	0.183 0.005	0.303 0.016	0.33 0.023	0.255 0.005	0.224 0.013	B-TFA
F				0.151 0.01					I EX (H)
F				0.142 0.01					I EX (A)
G				0.193					I EX
G	0.395 0.035		0.21 0.014		0.22 0.028		0.205 0.035		TPC
H	0.388 0.033	0.196	0.211 0.045			0.355 0.010	0.244 0.032	0.204 0.011	P-PFP
H	0.357 0.026		0.182 0.039			0.373 0.013	0.226 0.024	0.195 0.018	P-PFP
L				0.176 0.008					I EX
M				0.14					I EX

ILC-B powder

LAB	ALA	VAL	LEU	ILEU	PRO	ASP	PHE	GLU	METHOD
A	0.749 0.014	0.444 0.014	0.542 0.022	(0.906) 0.18	0.644 0.013	0.747 0.009	0.605 0.025	0.435 0.010	P-TFA
A	0.754 0.023	0.597 0.022	0.582 0.028	(0.931) 0.061	0.686 0.013	(0.71) 0.01	0.632 0.018	0.446 0.016	B-TFA
B				0.45 0.02					I EX
B	0.71	0.38	0.46	0.47	(0.58)	0.73	0.54	0.40	P-TFA
C				0.61 0.006					I EX
C	0.69 0.006	0.38 0.021	0.48 0.026		0.59 0.012	0.67 0.032	0.60 0.015	0.43 0.01	B-PFP
D	0.682 0.011		0.423 0.006	0.519 0.030		0.721 0.009			P-TFA
E*				0.578					I EX
E*	0.770 0.023	0.429 0.037	0.535 0.028			0.712 0.014	0.611 0.029	0.415 0.023	P-TFA
E*	0.734 0.024	0.467 0.006	0.543 0.019	0.630 0.031	0.662 0.039	0.673 0.026	0.645 0.017	0.458 0.019	B-TFA
F				0.54 0.02					I EX (H)
F				0.50 0.02					I EX (A)
G				0.485					I EX
G	0.725 0.021		0.47 0.042		0.395 0.007		0.435 0.021		TPC
H	0.766 0.008		0.488 0.007			0.689 0.006	0.605 0.038	0.441 0.012	P-PFP
H	0.668 0.086	0.447 0.071	0.444 0.015			0.694 0.008	0.574 0.014	0.433 0.008	P-PFP
L				0.559 0.027					I EX
M				0.48					I EX

ILC-C powder

LAB	ALA	VAL	LEU	ILEU	PRO	ASP	PHE	GLU	METHOD
A	0.96 0.005	0.83 0.039	0.860 0.009	(1.85) 0.036	0.860 0.016	0.981 0.025	0.929 0.022	0.853 0.016	P-TFA
A	0.981 0.017	1.01 0.12	0.89 0.011	(1.74) 0.016	0.906 0.002	(0.846)	0.922 0.006	0.872 0.018	B-TFA
B				1.1 0.05					I EX
B	0.97	0.80	0.85	1.2	(1.0)	0.87	0.86	0.83	P-TFA
C				1.27 0.02					I EX
C	0.92 0.04	0.81 0.02	0.83 0.02		0.83 0.015	0.83 0.044	0.92 0.037	0.87 0.029	B-PFP
D	0.935 0.005		0.749 0.001			1.033 0.021			P-TFA
E*				1.14					I EX
E*	0.981 0.027	0.83 0.028	0.847 0.007			0.90 0.006	0.942 0.09	0.783 0.028	P-TFA
E*	0.94	0.93	0.857 0.015	1.23	0.905 0.007	0.882 0.011	0.957 0.011	0.912 0.017	B-TFA
F				1.08					I EX (H)
F				1.00					I EX (A)
G				1.12					I EX
G	0.945 0.021		0.78 0.028		0.555 0.007		0.67 0.028		TPC
H	0.945 0.061		(0.496) 0.092			0.808 0.039	0.822 0.049	0.836 0.020	P-PFP
H	0.878 0.129		(0.439) 0.047			0.846 0.044	0.841 0.025	0.842 0.024	P-PFP
L				0.986 0.02					I EX
M				0.87					I EX

ILC-A liquid

LAB	ALA	VAL	LEU	ILEU	PRO	ASP	PHE	GLU	METHOD
A	0.402 0.001	0.156	0.270 0.008	0.240 0.003	0.33 0.018	0.442 0.004	0.258 0.003	0.223 0.001	P-TFA
C				0.25					I EX
C	0.40	0.15	0.27		0.39	0.46	0.34	0.28	B-PFP
D	0.386 0.001					0.42			P-TFA
E*	0.390 0.019	0.214 0.015	0.245 0.003	0.2055 0.002	0.363 0.011	0.404 0.004	0.314 0.013	0.234 0.002	B-TFA
F				0.19 0.01					I EX (H)
F				0.176 0.01					I EX (A)
G				0.134					I EX
G	0.50		0.29		0.26				TPC
L				0.176 0.012					I EX

ILC-B liquid

LAB	ALA	VAL	LEU	ILEU	PRO	ASP	PHE	GLU	METHOD
A	0.745 0.002	0.387 0.015	0.546 0.001	(0.744) 0.014	0.699 0.061	(0.75) 0.03	0.605 0.01	0.433 0.006	P-TFA
C				0.63					I EX
C	0.71	0.38	0.59		0.66	0.71	0.64	0.45	B-PFP
D	0.713 0.003		0.396 0.001						P-TFA
E*	0.683 0.017	0.437 0.005	0.527 0.009	0.657 0.031	0.646 0.016	0.688 0.028	0.659 0.003	0.446 0.008	B-TFA
F				0.585 0.02					I EX (H)
F				0.53 0.02					I EX (A)
G				0.518					I EX
G	0.7				0.43				TPC
L				0.527 0.014					I EX

ILC-C liquid

LAB	ALA	VAL	LEU	ILEU	PRO	ASP	PHE	GLU	METHOD
A	0.93 0.001	0.828 0.002	0.856 0.013	(1.49) 0.03	0.85 0.009	1.01 0.01	0.882 0.01	0.81 0.007	P-TFA
C				1.24					I EX
C	0.88	0.83	0.88		0.89	0.83	0.95	0.87	B-TFA
D	1.01					1.00			P-TFA
E*	0.909 0.001	0.869 0.051	0.869 0.016	1.21 0.04	0.909 0.023	0.892 0.013	0.907 0.013	0.920 0.028	B-TFA
F				1.16					I EX (H)
G				1.125					I EX
G	1.0				0.63				TPC
F				1.05					I EX (A)
L				1.07 0.08					I EX

Free D-Alloisoleucine/L-Isoleucine Values in ILC Powder Samples

LAB	ILC-A	ILC-B	ILC-C	METHOD
B	0.45 0.02	0.95 0.05	1.35 0.05	I EX
E*	0.44	0.88	1.21	I EX
E*	0.46	1.02	1.35	B-TFA
F	0.53 0.03	1.08	1.40	I EX (H)
F	0.48 0.02	0.98	1.32	I EX (A)
L	0.416 0.016	0.944 0.029	1.34 0.02	I EX
P	(0.495) 0.021	(1.123) 0.015	(1.55) 0.049	I EX

Note. * denotes Delaware laboratory. Laboratory H reported results by the same GC method produced with different columns over a 3-month interval. I EX (A), ion-exchange peak area measurements; I EX (H), ion-exchange peak height measurements; B-TFA, NTFA-(+)-2-butyl derivatives; B-PFP, NPFPA-(+)-2-butyl derivatives; P-TFA, NTFA-isopropyl derivatives; P-PFP, NPFPA-isopropyl derivatives; TPC, L-prolyl derivatives. Numbers in parentheses are preliminary and were not included in statistical data reductions; numbers beneath each D/L value are individual lab precision values.

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ratories. Because enantiomeric ratios for mollusks from all of the localities used in this interlab study have been published by the Delaware laboratory [ILC-A, Cape Blanco, Oreg. (Wehmiller *et al.*, 1977; Kennedy *et al.*, 1982); ILC-B, Charleston, S.C., and ILC-C, Calabash, N.C. (Wehmiller and Belknap, 1982; McCartan *et al.*, 1982)], the results for the ILC samples obtained by the Delaware laboratory are identified in Table 2.

DISCUSSION

Table 3 and Figures 1 and 2 present statistical condensations of the results presented in Table 2. Mean and median values for all determinations are given in Table 3, along with standard deviations (SD) and the coefficient of variation (C.V.), expressed in percent. Given below are statements that relate to relative precision and/or level of agreement among different methods for each amino acid. The results for the powder samples are considered first, followed by a discussion of the results for the liquid samples.

A general statement can be made about the level of precision reported by most laboratories in comparison to the overall precision of results summarized in Table 3. Figure 1 shows that where the typical standard deviation reported by most laboratories for their own analyses is between 2 and 5%, the coefficient of variation for all the reported results is much greater. For the powder samples, the median of all the coefficients of variation is about 9.6%; for the liquid samples, the median C.V. is about 6.5% (Fig. 1). These values are not specific for any single amino acid but rather represent the entire range of standard deviations observed for all amino acids studied here.

In considering the statistical interpretation of these results, the possibility of nonrandom errors must also be evaluated. If a particular laboratory reported enantiomeric ratios that were always consistently higher or lower than the grand mean value for each of the three samples, then a systematic difference in that laboratory's analytical method for that particular amino acid would be implied. In these cases the median must be considered to be the best value for a particular enantiomeric ratio, and the standard deviation must be interpreted to represent more than merely random analytical error. Examples of this type of systematic deviation are seen for almost all the amino acids, and several significant ones are mentioned below.

TABLE 3. SUMMARY OF INTERLABORATORY ANALYTICAL DATA

AMINO ACID		ILC A POWDER	ILC A LIQUID	ILC B POWDER	ILC B LIQUID	ILC C POWDER	ILC C LIQUID
ALA	mean	0.364	0.415	0.725	0.710	0.946	0.946
	median	0.361	0.40	0.726	0.71	0.945	0.93
	s.d.	0.017	0.047	0.034	0.023	0.029	0.057
	c.v.	4.7	11.3	4.7	3.2	3.1	6.0
VAL		0.159	0.173	0.449	0.401	0.868	0.842
		0.146	0.156	0.444	0.536	0.852	0.869
		0.030	0.035	0.068	0.031	0.084	0.023
		18.9	20.2	15.2	7.7	9.7	2.7
LEU		0.196	0.268	0.497	0.515	0.833	0.868
		0.203	0.27	0.484	0.536	0.852	0.869
		0.021	0.018	0.049	0.083	0.043	0.012
		10.7	6.7	9.8	16.1	5.2	1.4
ILEU (IEX)		0.174	0.185	0.525	0.558	1.071	1.129
		0.168	0.176	0.50	0.53	1.10	1.125
		0.033	0.041	0.055	0.048	0.112	0.075
		18.9	22.2	10.5	8.6	10.5	6.6
ILEU (GC)		0.212	0.223	0.54	0.657	1.215	1.21
		0.21	0.223	0.565	0.657	1.215	1.21
		0.036	0.024	0.081	-	0.015	-
		16.8	10.8	15.0	-	1.2	-
FREE ILEU (I EX)		0.463		0.967		1.324	
		0.45		0.95		1.34	
		0.044		0.073		0.070	
		9.5		7.5		5.3	
PRO		0.278	0.336	0.595	0.61	0.81	0.82
		0.29	0.346	0.644	0.653	0.86	0.87
		0.034	0.056	0.105	0.121	0.13	0.13
		12.2	16.7	17.6	19.8	16.0	15.8
ASP		0.378	0.432	0.705	0.699	0.894	0.933
		0.374	0.431	0.703	0.70	0.876	0.946
		0.028	0.025	0.028	0.015	0.079	0.087
		7.4	5.8	4.0	2.1	8.8	9.3
PHE		0.239	0.304	0.583	0.634	0.873	0.914
		0.238	0.314	0.60	0.64	0.92	0.91
		0.020	0.041	0.059	0.027	0.089	0.034
		8.4	13.5	10.1	4.3	10.1	3.7
GLU		0.203	0.246	0.432	0.443	0.849	0.866
		0.202	0.234	0.433	0.446	0.848	0.87
		0.011	0.030	0.017	0.009	0.035	0.055
		5.4	12.2	3.9	2.0	4.1	6.4

Note. Unless otherwise indicated, results from all methods were combined for calculations of means and standard deviations (s.d.). c.v. = coefficient of variation.

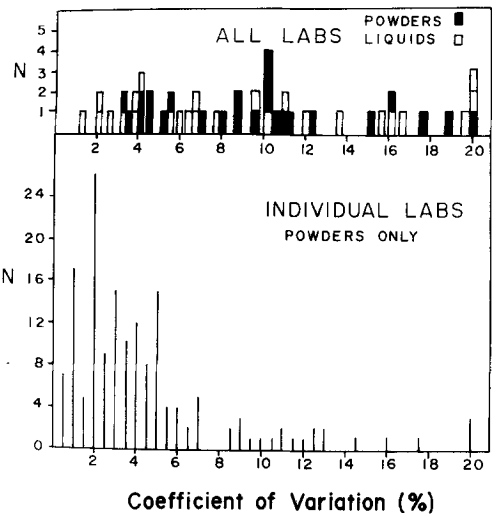

FIG. 1. Coefficients of variation (C.V.) for data from all laboratories compared with analytical precision reported by individual laboratories. Upper figure shows C.V.'s (not specifically related to individual amino acids) derived from all reported results, for powder and liquid samples. The median C.V. for the powders is about 9.5%; that for the liquid samples is about 6.5%. The lower figure shows a histogram of the analytical precision values (also calculated as coefficients of variation) reported for all the amino acids measured by an individual laboratory. These figures show that while the precision of results for any one laboratory will usually be between 2 and 5%, the precision of all results combined from several laboratories will often be significantly larger.

Alanine. The C.V. values for all three powder samples are less than 5%, and there does not appear to be any significant difference in D/L-alanine values determined by any of the three GC methods used here. Four sets of alanine results are consistently either above or below the grand mean values for all three samples, but in only two cases are these deviations greater than 6%. The C.V.'s for the alanine results seen here are much better than those reported by Bada *et al.* (1979) for bones (ca. 14–35%).

Valine. The C.V.'s for ILC-A and ILC-B are quite large, and there appears to be a distinct trend for those valine D/L values determined by the butanol method to be higher than those values determined by the isopropanol method. Interferences with D-valine are known to occur with at least some of the column/program combinations used with the butanol method. Slight interferences with L-valine are also observed with the isopropanol method, depending upon programming conditions, so both methods probably suffer from interferences which have contributed to the relatively poor precision. Six sets of valine results were either above or below the grand mean values for all three samples, in some cases by more than 10% (laboratories A, B, and C).

Leucine. The C.V. for leucine steadily decreases with increasing D/L values. There is a significant trend for the D/L-leucine values determined by the butanol method to be higher than those determined by the isopropyl method. Experience in this laboratory has shown that serine will interfere with L-leucine (causing D/L leucine values to be lower than those determined by the butanol method) in typical temperature programs using the Chirasil-val column for NTFA-isopropyl derivatives. The chromatogram shown by Frank *et al.* (1977) shows several potentially interfering peaks

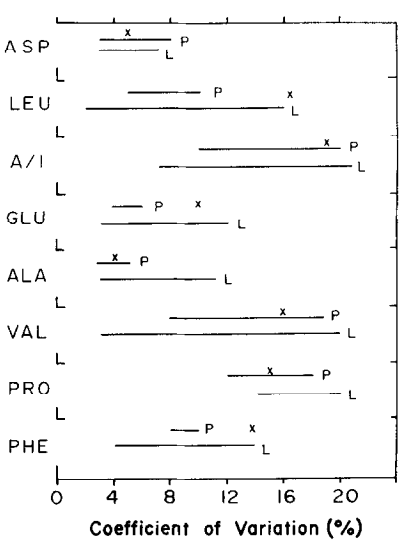

FIG. 2. Ranges of the coefficients of variation (C.V.) for each amino acid, for data from all laboratories. Results for the powder (P) and liquid (L) samples are compared with the data reported by Kvenvolden (1980), shown by X's.

in the leucine region for the NPFPA derivatives as well. Only by sacrificing resolution of D-leucine from proline can this interference (with the NTFA derivatives) be avoided. The alternative that we have employed is to destroy the TFA serine derivative by addition of methanol to the completed derivative followed by refrigerated overnight storage (Pollock and Kawauchi, 1968). By following this procedure, good agreement between isopropyl and butanol has been observed (Table 2, data for laboratory E). It is not clear how many of the leucine results reported here might have been affected by such interferences, especially because so many different columns and derivatives were used, but this is an important analytical issue to resolve because leucine enantiomeric ratios have often been used in comparisons of GC results with LC data for D-alloisoleuine/L-isoleucine values. Calculations based on the relative amount of serine (from ion-exchange data) in the three ILC samples indicate that the leucine results for the ILC-A and ILC-B samples could have been reduced by 10–15% if there was moderate serine interference with L-leucine, but that the ILC-C sample would not have been affected by this problem because there is very little serine remaining in this sample. Five data sets for leucine were consistently above or below the grand means for all three samples; in two of these cases (laboratories A and D) the deviations from the mean were 10% or more.

Isoleucine. The C.V.'s for the three samples show a decreasing trend with increasing D/L value just as in the case of leucine. There is a trend for D-alloisoleucine/L-isoleucine values determined by GC to be somewhat greater than those determined by ion exchange, but GC data reported by certain labs are quite close to the mean values for all of the ion-exchange data (see also free amino acid D-allo/L-iso results). Therefore it appears that some GC methods might serve to link the growing body of GC and LC data. It should be noted that a few isoleucine results were reported as "preliminary" because they were obtained on ion-exchange analyzers that were not devoted to maximum resolution in the isoleucine region. This was the case for at least two laboratories (E and P). Four data sets for isoleucine as determined by LC were either consistently above or below the grand mean for this amino acid; in two cases (laboratories C and M) these deviations were often greater than 15%.

Proline. The overall precision of the proline results is greatly affected by the analytical methods represented. The TPC method (lab G), in spite of the fact that it shows good precision (Table 2), is in major disagreement with other methods for D/L-proline determination. Many labs using the isopropyl method did not report D/L-proline values because this amino acid is rarely well-resolved by this derivatization scheme. The proline results obtained the the butanol method show C.V.'s on the order of 5%.

Aspartic acid. The C.V. for aspartic acid is generally quite good (between 3 and 8%). Comparable standard deviations were usually observed for aspartic acid in bone samples shared by three laboratories, as reported by Bada *et al.* (1979). There is a tendency for the D/L-aspartic values determined by the butanol method to be lower than those determined by the isopropyl method. The latter method generally resolves the aspartic acid enantiomeric derivatives better than does the former method. Five data sets for aspartic acid showed results either consistently above or below the grand mean, but most of these deviations were less than 5% of the mean value.

Phenylalanine. The precision of the phenylalanine results is, as in the case of proline, greatly affected by the data obtained by the TPC method (laboratory G). There is a clear trend for the TPC data to be much lower than those obtained by the other two GC methods, and there is also an indication that the D/L-phenylalanine values obtained

by the butanol method are slightly higher than those obtained by the isopropyl method. This trend has been noted previously (e.g., Wehmiller and Emerson, 1980), but it is not clear with which method the problem might lie. Phenylalanine closely elutes with glutamic acid in the isopropyl scheme, so it seems likely that there might be some interference in this region of the chromatogram. However, D/L-phenylalanine values of up to 1.15 are often observed in "old" samples analyzed by the butanol method (Wehmiller, unpublished) so there may be some interference with phenylalanine for at least some analytical methods employing the butanol derivatives. Only two laboratories (E—butanol and G) reported phenylalanine results consistently above or below the grand means for all three samples, but only those for laboratory G deviated by more than 10%.

Glutamic acid. This amino acid shows excellent precision (2–5%) among all the laboratory results. These results represent a significant improvement over the C.V.'s (10% or greater) reported by Kvenvolden *et al.* (1972) and Bada *et al.* (1979). There is a slight tendency for the D/L-glutamic acid values obtained by the butanol method to be greater than those obtained by the propanol method. This trend becomes magnified in the older, most extensively racemized samples. As mentioned above, interferences in the phenylalanine–glutamic region of the chromatograms of the isopropyl derivatives could be the cause of this discrepancy between the two methods. Three data sets for glutamic acid were consistently either above or below the grand means for individual samples, but all but one of the deviations were less than 8%.

Liquid data compared with powder data (Figs. 1 and 2) perhaps one of the bigger surprises of this project was the observation that the precisions for all results obtained on the desalted liquid hydrolyzates were not much different from the precisions obtained on the powder samples. This relationship can be seen in the upper histogram of Figure 1, in which the C.V.'s for all liquid and all powder values are compared. The median C.V. for the powders is somewhat greater (9.6% vs 6.5%) than the median C.V. for the liquids, but there is significant overlap of these C.V. values. Figure 2 shows the range of C.V. values for both liquid and powder analyses for each amino acid. This figure shows that in all cases except aspartic acid, the range of C.V. values for all the data was greater for the liquid samples than for the powder samples. This observation is biased slightly by the fact that fewer laboratories reported data for the liquid samples than for the powder samples (Table 2), but when a similar comparison is made using only data from laboratories that reported both liquid and powder data, the conclusion is essentially the same. Therefore it appears that the main reason for the spread of results obtained from the powder analyses does not lie in the wet-chemical sample preparation steps but in the instrumental analyses themselves. Though this conclusion might not be surprising for the variety of GC methods used here, it is somewhat surprising for the ion-exchange methods which involve quite similar analytical schemes.

Also shown in Figure 2 (by X's) are the C.V. values reported by Kvenveldon (1980) for the previous interlaboratory comparison. The precision of the leucine, glutamic acid, and phenylalanine results observed here is significantly improved over those previously observed. Other amino acids show precisions similar to those seen in the earlier study.

Enantiomeric ratios, liquids vs powders. In the case of every amino acid, the mean D/L value observed in ILC-A liquid is greater than the mean D/L value observed in the powder. This observation is not biased by the different numbers of laboratories that reported results for the powder and liquid. There is a suggestion of the same relationship among the results for ILC-B, but no such trend is seen in the results for ILC-C. These observations are

taken as evidence of a phenomenon that has been documented by several workers at the University of Colorado Amino Acid Lab (G. H. Miller, personal communication, 1982). It has been noted that when a fossil sample is dissolved and transferred to another vessel for hydrolysis, the resultant D/L values are higher than if the hydrolysis takes place in the same vessel as the dissolution step. It appears that high-molecular-weight polypeptide material (which would typically have the least racemized amino acid population) adheres to glassware and is "lost" in each transfer step that occurs between dissolution and hydrolysis. The D/L values measured on hydrolyzates that have been depleted in this high-molecular-weight component will be artificially high. This effect was not known to J.F.W. at the time of preparation of the ILC samples, and it appears to have been a significant factor in the results for ILC-A (see discussion of methods of sample preparation). The fact that the difference between the powder and liquid D/L values decreases with increasing age (extent of racemization) of the sample is consistent with the concept that decreased amounts of high-molecular-weight material are preserved in progressively older samples.

CONCLUSIONS

It is clear from the results summarized here, as well as in Kvenvolden (1980), that some significant differences in enantiomeric ratios are encountered in interlaboratory comparisons. The best agreement among different methods is seen for aspartic acid, alanine, and glutamic acid. The most important observation to result from this work is that derivative or instrumental effects appear to be responsible for the major portion of these interlaboratory differences. It is possible that the analysis of liquid samples prepared from pure amino acids and not from fossil samples might reveal smaller differences between instrumental analyses, and the analysis of such mixtures would be an important step in the understanding of the origin of such discrepancies. In addition to the many differences that were encountered within a range about the mean of approximately 10%, there were several extreme discrepancies (usually involving proline and/or phenylalanine and their isopropyl or TPC derivatives) which suggest that these methods are not reliable for the enantiomeric ratio analysis of these particular amino acids. Smaller, but still significant, systematic differences among results for individual amino acid analyses by particular laboratories were also recognized.

Of the amino acids studied here, aspartic acid, isoleucine, and leucine have been discussed most frequently in geochronological applications. It is useful to consider what the absolute age implications would be for some of the differences in enantiomeric ratios reported here. These age implications would be dependent upon kinetic models and absolute temperatures, so they can be only qualitatively estimated. Because of the exponential nature of the time-dependence of racemization, age uncertainties are slightly greater than the analytical uncertainties. For linear kinetics, such as have been proposed for aspartic acid in bone samples, a 10% uncertainty in an enantiomeric ratio is equivalent to about 13.5% of age uncertainty. For nonlinear kinetics, which have been proposed for isoleucine and leucine in shell material, the relative age uncertainty of a given sample depends somewhat on its position in relation to overall kinetic pathways. In the early portion of the nonlinear racemization pathway (D/L < 0.25) the relationship between analytical and age uncertainities is the same as given above for aspartic acid. In the latter portion of the racemization pathway (D/L > 0.45), a 10% analytical uncertainty becomes equivalent to an age uncertainty of as much as 25%. Examples of possible age uncertainties for different ranges of D/L values can be seen in Wehmiller and Belknap (1982, Figs. 6 & 7).

Several laboratories that received these ILC samples were unable to contribute results to this study, but sufficient data were

available to form a meaningful basis for comparison with all future results. The author recommends that all future publications of fossil amino acid enantiomeric ratio data include at least one analysis of the interlaboratory samples discussed here. Only in this manner will it be possible to compare results obtained by different laboratories over extended time intervals. In addition, it is recommended that these interlaboratory samples be regularly analyzed by individual labs as an ongoing check on the consistency of analytical results. Ideally interlaboratory samples would also become available for other sample types, such as wood and bone, because the overall matrix effects of individual sample types are important components of any thorough interlaboratory comparison. In the absence of such samples, the carbonate samples described here should suffice. Additional sample material is available from the author as needed.

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