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Differential distribution of D and L amino acids between the 2′ and 3′ positions of the AMP residue at the 3′ terminus of transfer ribonucleic acid

(aminoacyl-tRNA/adenylate esters/chiral selectivity in 2′–3′ distribution)

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ABSTRACT **Amino acids esterified to the ribose group of 5′-adenylic acid (AMP) constantly migrate between the 2′ and 3′ positions of the ribose at a rate of several times per second, which is slower than the rate of peptide-bond synthesis (15–20 per sec). Because the contemporary protein-synthesizing system only incorporates amino acids into protein when they are at the 3′ position of the AMP at the terminus of tRNA, the value of the equilibrium constant relative to the 2′ and 3′ positions is of considerable interest. Differences between D and L isomers in this regard might be especially revealing. We have used *N*-acetylaminoacyl esters of AMP as models for the 3′ terminus of tRNA and find that glycine and the L amino acids consistently distribute predominantly to the 3′ position (≈67% 3′, ≈33% 2′), but D amino acids distribute to that position generally to a lesser extent and in a manner inversely related to the hydrophobicity of the amino acid side chain. This consistency of the L amino acid preference for the 3′ position, combined with the inconsistency of the D amino acid preference, may be one reason for the origin of our contemporary protein-synthesizing system, which forms the peptide bond preferentially with L amino acids and only when they are in the 3′ position of the ribose moiety of the AMP residue at the 3′ terminus of every tRNA.**

For all practical purposes, *in vivo* template-directed protein synthesis proceeds with the incorporation of only L amino acids. This is true in spite of the fact that there are a number of reports that D amino acids can participate in essentially all of the reactions involved, including peptide-bond synthesis (1–3). The preference for L appears to be due to preferences at each of several steps, resulting in a cumulative preference for the L isomer of 4 orders of magnitude. A basic question remains, though, as to why these enzymatic preferences for the L amino acids have evolved. In fact, it has been difficult to show how abiological reactions could result in preferential synthesis or degradation of one isomer. Stereoselective reactions, which could have served as bases for the origin and evolution of the protein-synthesizing system, would seem more fruitful subjects of study. In that vein Profy and Usher (4, 5) have shown some stereoselectivity for the L isomer in esterification reactions with amino acids and oligo- or polynucleotides. They also have discussed in some detail the fact that amino acids esterified to ribonucleotides constantly migrate between the 2′ and 3′ positions of the ribose and have emphasized the possible significance of studies on the differential distribution of D and L amino acids between the 2′ and 3′ positions. The interest is in the fact that amino acids esterified to the AMP residue at the 3′ terminus of the tRNA are constantly migrating back and forth several times a second (6), and the amino acid is only incorporated into protein when it is in the 3′ position (7–10). Furthermore, it was established by Taiji *et al.* (6) that the rate of amino acid transacylation between the 2′ and 3′ positions (1–11 per sec) of adenosine or 5′-adenylic acid (AMP) is slower than the rate of peptide-bond formation (15–20 times per sec) (11). Taiji *et al.* (10) also showed that peptidyl (and N-blocked amino acids) have even slower 2′–3′ transacylation rates. It then becomes an interesting question as to whether D and L amino acids distribute to the 3′ position of AMP (as a model of the 3′ terminus of tRNA) differently. Profy and Usher (4) did find a preference of the D isomer of *t*-butoxycarbonylalanine for the 3′ position when esterified to diinosine monophosphate (IpI). They found excess D isomer in the 3′ position, which might be due in part to the bulky *t*-butoxycarbonyl group. However, we have now shown that, of the five L amino acids tested, all distributed 65–68% to the 3′ position and 32–35% to the 2′ position regardless of the character of the side chain. On the other hand, a variable (40–67%) distribution was generally expressed by the more hydrophobic D amino acids to the 3′ position, with the percentage decreasing as the hydrophobicity of the side chain increased. Glycine equilibrated at 67–69% 3′ ester. Other data here suggest a molecular basis for the tendency of hydrophobic D amino acids to be more attracted to the 2′ position.

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MATERIALS AND METHODS

All of the *N*-acetylamino acids, carbonyldiimidazole, AMP, carboxylic acids, and 99.8% ^2H_2O were obtained from Sigma and used without further purification.

We synthesized a series of *N*-acetylaminoacyl esters and a series of carboxylic acid esters of AMP using essentially the carbonyldiimidazole method of Gottikh *et al.* (12) in predominantly aqueous solvent (≈25% dimethylformamide) at pH 7.0 and purified them using HPLC. *N*-Acetylamino acids were used because they prevent peptide-bond formation and because they are more stable against hydrolysis than are the free amino acid esters, as shown by Gilbert (13) and us (unpublished data). The major part of the research was a determination of the equilibrium constants for the distribution of these various *N*-acetylamino acids and carboxylic acids between the 2′ and 3′ positions of the ribose moiety of AMP at pH 7 and 25°C. Two methods, HPLC and NMR, were used to determine these equilibrium constants, and substantially the same answers were obtained with the two methods.

The HPLC method, which is similar to the procedure used by Taiji *et al.* (10), was done with a C_{18} reverse-phase column. By operating the column at pH 2, where the 2′–3′ migration is virtually stopped, the 2′ and 3′ esters and usually any contaminating D or L isomer can be separated easily from one another. The following HPLC procedure was used. A sample of crude ester at pH 2.0 was injected into the Laboratory Data Control apparatus (the Waters Associates column was 3.9 mm in diameter and 30 cm long with C_{18}

μBondapak packing of 5-μm particles) with a pumping rate of 0.8–1.0 ml/min. The products were eluted with 0.05 M phosphoric acid (pH 2; pH not adjusted) containing different amounts of HPLC-grade methanol for different esters (8% for Ac-Ala, 15% for Ac-Val, and 20% for Ac-Leu and Ac-Phe esters). For Ac-Trp-AMP esters, a slower elution was required. At 0.6 ml/min and with 8% methanol/0.05 M phosphate, pH 2, all four products, the 2′ D, 2′ L, 3′ D, and 3′ L esters, could be separated in that order. A portion of the larger product peak (3′ ester) was collected and lyophilized to remove methanol, reconstituted to 0.05 M phosphate, and incubated at 25°C and pH 7.0 for 20 min to allow 2′–3′ equilibration. The pH was again lowered to 2.0 with 4 M HCl, and the sample was again run through the HPLC, integrating the 2′ and 3′ peaks. By dropping the pH to 2, the 2′/3′ distribution is frozen because transacylation is very near zero at this pH. From the relative integrations, the percentage of each isomer and the equilibrium constant (K_{eq} = % 3′/% 2′) were calculated. At least three HPLC samples were run for each ester. Alternately, a μBondapak phenyl column (Waters Associates) was also used, in which case somewhat lower methanol concentrations were required. Fig. 1 shows a typical separation of Ac-Phe-AMP ester.

The acetyl blocking group tends to promote racemization during the synthesis reactions. Even so, the racemization was slow enough (≈4 hr for completion) so that the identification of 2′ D and 3′ D ester peaks in the HPLC separations was relatively easy starting with pure *N*-acetylamino acids. In all cases, when working with the amino acids, we were cognizant of the necessity of separating the contaminating enantiomer. This was generally quite easy; however, in using the preparative HPLC column for the NMR study of the Ac-L-Val-AMP ester, we were never quite able to separate out the Ac-D-Val-AMP ester and, thus, were not able to make a confirming NMR run for that one only. The NMR experiments allowed precise identification of the various HPLC peaks.

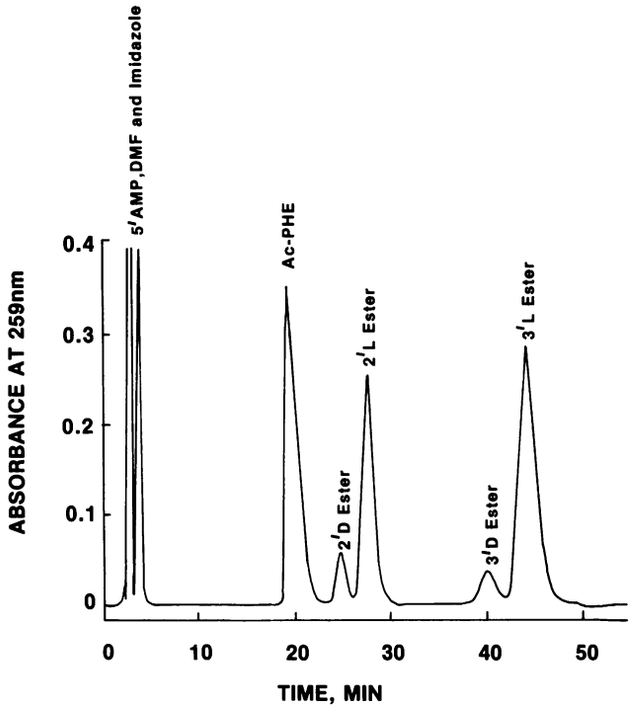

FIG. 1. HPLC separation of Ac-Phe-AMP ester prepared from Ac-L-Phe. Note some D isomer is present. When preparation started with Ac-D-Phe, the peaks labeled D ester predominate. The column was Waters Associates μBondapak phenyl (5-μm particles), 3.9 mm in diameter and 30 cm long. The pumping rate was 1.0 ml/min with 11% (vol/vol) methanol/0.05 M phosphoric acid, pH 2. DMF, dimethylformamide.

In essentially every case, the 3′ ester (which was in most cases the larger of the two product peaks) was eluted after the 2′ ester. The NMR method below allowed us to determine more explicitly which peaks were due to the 2′ ester and which were due to the 3′ ester.

The NMR method of Taiji *et al.* (6) depended on the fact that when the 2′ or 3′ position of AMP is esterified, the signal for the proton (H-2′ or H-3′) that is attached to the same carbon moves downfield 1.0 ppm (6). Interestingly, the signals for the H-2′ and H-3′ protons do not overlap except in two cases (Ac-D-Phe-AMP and Ac-D-Trp-AMP esters) in which we integrated the corresponding H-1′ peaks, which are well separated. Decoupling experiments made it possible to identify which H-1′ peaks were due to the 2′ ester and the 3′ ester. For the proton NMR procedure, crude samples were run through a preparative C_{18} reverse-phase column (two columns in series, each being 7.8 mm × 61 cm with 50-μm packing) with 0.001 phosphate buffer (pH 2.0) containing various amounts of methanol for the different esters (30–35% for Ac-Trp-AMP, Ac-Phe-AMP, and Ac-Leu-AMP; 20% for Ac-Val-AMP; and 15% for Ac-Ala-AMP esters) with a pumping rate of 2.0 ml/min. After free AMP, amino acid, imidazole, and reaction solvent (dimethylformamide) were eluted, the combined 2′ and 3′ esters were collected. The samples were adjusted to p^{2}H 6.5 (p^{2}H = pH + 0.4), lyophilized to dryness, dissolved in 3–4 ml of ^2H_2O, lyophilized again, reconstituted in ^2H_2O to give a concentration of 0.004 M at p^{2}H 6.5, and maintained at −70°C until the NMR assay. The ^{1}H NMR was done at 300.131 MHz on a Nicolet 300/WB Fourier transform NMR spectrometer in the pulse mode. Sufficient scans were accumulated to give clear signals of the H-1′, H-2′, and H-3′ protons generally in the range of 5.4–6.2 ppm. The H-1′, H-2′, and H-3′ protons were all integrated, allowing two independent estimates of the percentage of 3′ ester (there is a separate H-1′ signal for the H-2′ and the H-3′ esters). Sodium 3-trimethylsilyl [2,2,3,3-$^2H_4$]propionate was used as an external reference.

Because the NMR results actually obtained at the increased pH agreed with the HPLC results (which involved equilibration at pH 7 and then decrease of the pH to 2 to freeze the distribution), we know that the distributions were not altered by the HPLC procedure at low pH. Furthermore, the agreement between the two methods assures us that the 20-min equilibration time used in the HPLC experiments was sufficient.

RESULTS AND DISCUSSION

***N*-Acetylamino Acid D and L Isomers.** We first investigated a series of amino acids having side chains of decreasing hydrophobicity, Ac-Trp, Ac-Phe, Ac-Leu, Ac-Val, and Ac-Ala, using both D and L isomers. Ac-Gly was also included for comparison. The data from both the HPLC and NMR experiments are given in Table 1. The data for Ac-D-Phe-AMP and Ac-L-Phe-AMP esters have been presented in abstract form (14).

The reproducibility of the HPLC experiments was excellent, agreement between the two sets of data (HPLC and NMR) is remarkably good, and the conclusion to be drawn seems straightforward. The standard deviations are presented in the Table with the data. The *N*-acetylamino acid L isomers uniformly distribute to the 3′ position to about 65–68%. Glycine behaves like an L amino acid (67–69% 3′ ester). These findings are not really new, as most workers have found this approximate result with L amino acids esterified to adenosine or AMP (6, 15–17). The *N*-acetylamino acid D isomers, on the other hand, distribute to the 3′ position of AMP to a variable degree inversely related to the hydropho-

Table 1. Distribution of various *N*-acetylamino acids between the 2′ and 3′ positions when esterified to AMP

N-Acetylamino acid	Optical isomer	HPLC % 3′*	HPLC K_{eq} (3′/2′)	NMR % 3′	NMR K_{eq} (3′/2′)
Ac-Trp	L	67.6 (0.25)	2.06	65.1	1.87
	D	39.7 (0.73)	0.66	40.0	0.67
Ac-Phe	L	66.6 (1.10)	2.00	66.0	1.98
	D	49.8 (1.30)	0.99	45.8	0.85
Ac-Leu	L	67.1 (0.07)	2.04	67.1	2.04
	D	57.1 (0.49)	1.33	56.0	1.27
Ac-Val	L	65.6 (0.14)	1.90	—	—
	D	58.3 (0.07)	1.40	59.6	1.48
Ac-Ala	L	67.1 (0.00)	2.04	65.9	1.93
	D	66.3 (0.21)	1.97	67.9	2.10
Ac-Gly	—	69.1 (0.67)	2.24	67.2	2.05

*Standard deviation of the percentage of 3′ isomer is given in parentheses.

bicity of the amino acid side chain. From our preliminary ^1H NMR data, this discrimination appears to be due to the greater ability of the hydrophobic D amino acid side chain when it is in the 2′ position to interact with the adenine ring, an interaction that may be sterically more difficult when the aminoacyl group is in the 3′ position. The variation in the percentage of 3′ ester as a function of the binding constant of the amino acid for AMP (18) is shown in Fig. 2. Because the distribution of the L amino acids is independent of the hydrophobicity of the side chain, there is then some inherent property of the L configuration itself that results in its favoring the 3′ position. Two factors appear to be operating: one causes the L amino acids and glycine to consistently favor the 3′ position and the other discriminates against the D amino acids being in the 3′ position as the side chain becomes more hydrophobic. The data in Fig. 2 would suggest that the greater affinity of the amino acid (when it is in the 2′ position) for adenine is responsible for the increased amounts of the more hydrophobic D amino acids in the 2′ position. It may well be that with the L isomer, other factors more hydrophilic in nature—e.g., hydrogen bonding, dipole–dipole interactions etc.—may take place and outweigh any hydrophobic interactions that take place.

Carboxylic Acid AMP Esters. In an attempt to elucidate the role of the hydrophobic side chains in the discrimination against the more hydrophobic D amino acids being in the 3′ position, we repeated the experiment using straight-chain carboxylic acid esters of AMP. Using the HPLC and NMR methods as described, we obtained the results shown graphically in Fig. 3.

These data suggest that the D amino acids and carboxylic acids are behaving in the same manner—i.e., as the hydrophobicity increases, the distribution to the 3′ position decreases. However, because there are no amino acids with linear aliphatic side chains, we repeated the experiment with four additional carboxylic acids, isovaleric acid, 4-methylvaleric acid, 3-phenylpropionic acid, and indole-3-propionic acid which are explicit analogs of valine, leucine, phenylalanine, and tryptophan. Combined with the data for Ac-Ala, Ac-Val, Ac-Leu, Ac-Phe, and Ac-Trp from Table 1, we present the data graphically in Fig. 4.

This figure shows clearly that the D amino acid distribution to the 3′ position is controlled in the same way as simple carboxylic acid analogs. Again, for the amino acids, the double discrimination seems to be working—i.e., for the L amino acids to be in the 3′ position and against the more hydrophobic D amino acids being in the 3′ position.

The present data relate most directly to the events at the 3′ terminus of every tRNA. That terminus is always an AMP residue, and amino acids are brought into protein synthesis as ribose esters of that terminal AMP. Consequently, aminoacyl esters of AMP are good models of the aminoacyl tRNA terminus. The work of Taiji *et al.* (6, 10) shows that AMP and adenosine give similar results; consequently, the double charge on AMP at pH 7 does not have dramatic effects.

The molecular mechanisms responsible for these selectivities are not clear at present, but preliminary NMR experiments suggest that the hydrophobic side chains of the D amino acids can associate more strongly with the hydrophobic adenine ring when the amino acid is in the 2′ position. The plot in Fig. 2 is consistent with this idea. Such hydrophobic interactions between both aromatic and aliphatic amino acids and the adenine ring have been documented in a previous paper (19).

The present work is especially relevant to the origin of the process of protein synthesis because the rates of 2′–3′ transacylation have been shown to be slow relative to the rate of peptide bond formation. This fact coupled with the observations in this paper have led us to the following hypothesis: The origin and evolution of these aspects of protein synthesis—i.e., the preferential use of L amino acids esterified to the 3′ position to form the peptide bond—came about because of the consistency of L amino acids and glycine to favor that position and the inconsistency of D amino acids to favor that position. Further work will be required to confirm or deny this hypothesis. Anyway, it seems somewhat incomplete in the sense that, while the AMP residue might

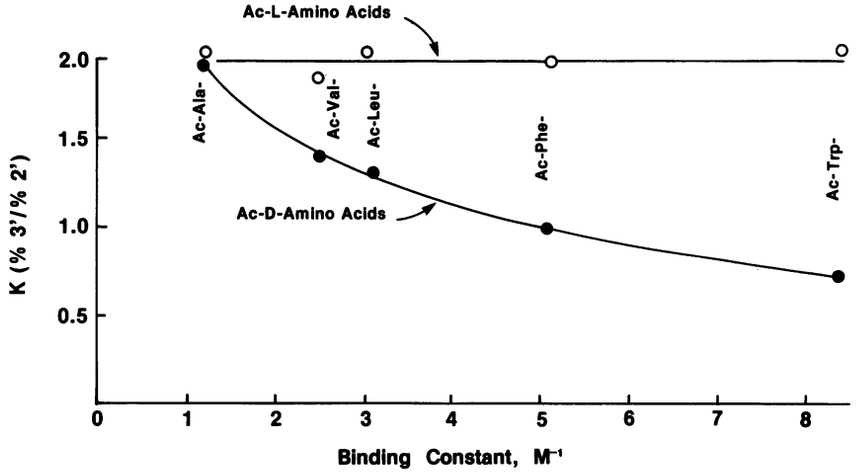

FIG. 2. The equilibrium constant at pH 7 and 25°C for distribution of *N*-acetylamino acid D and L isomers to the 3′ position when esterified to AMP, plotted as a function of the binding constants of the methyl esters of the same amino acids for AMP (18).

discriminate against hydrophobic D amino acids, it apparently would not discriminate against less hydrophobic D amino acids—e.g., D-alanine in the present study. One is encouraged to imagine the use of all four ribonucleotides in that regard and perhaps an earlier class of tRNAs that did not carry the C-C-A terminus but rather carried one of the four ribonucleotides as a terminus, each nucleotide being a discriminator for certain classes of L amino acids and against the same class of D amino acids. In fact, as Crothers *et al.* (20) pointed out, the fourth nucleotide from the 3′ terminus seems to be a discriminator nucleotide and varies in a regular way depending on the amino acid for which the tRNA serves as an adaptor. No information is available regarding discrimination between D and L amino acids by this fourth nucleotide.

In that regard, phenylalanine, leucine, isoleucine, valine, and methionine, all hydrophobic amino acids having A as their middle anticodonic letter, also have adenosine as the discriminator nucleotide in their tRNAs. The tRNAs for tryptophan have either an adenosine or guanosine in the discriminator position. Therefore, there is also an element of coding involved here in that AMP serves best to discriminate against the D isomer of those amino acids for which A is the middle anticodonic letter. In preliminary experiments exploring the 2′–3′ distribution of amino and carboxylic acids esterified to the other three ribonucleotides, it appears that results with GMP will be very similar to those with AMP. However, UMP appears to be somewhat different in that its discrimination against hydrophobic amino acids being in the 3′ position is not so pronounced.

Finally, we need to relate the information in this paper to studies on the comparative rates of incorporation of D and L amino acids into protein. Calendar and Berg (1) reported that D-tyrosine could be esterified to $tRNA^{Tyr}$ and incorporated into protein at about one-sixth the rate of the L isomer with poly(U,A) as a template. Yamane and Hopfield (2) in following this up found D-tyrosine was esterified to $tRNA^{Tyr}$ more slowly than was the L isomer. In 1981, Yamane *et al.* (3) studied in some detail the comparative rates of utilization at several stages of protein synthesis of D- and L-tyrosine. These experiments were done with Ac-L-Phe-tRNA as donor and using U-U-U-U-A-C as a template. While the selectivity was not absolute at any point, there was a definite preference for the L isomer at each step. Aminoacylation of tRNA showed a 25-fold preference for the L isomer; a 25-fold preference for the L isomer in elongation factor Tu–GTP complex formation; a 10-fold preference for the L isomer in binding that complex to the ribosome; and a 5-fold excess of the L isomer in peptide formation. They calculated a discrimination of about 10^4 for the L isomer based on these combined preferences.

Because the amino acid (tyrosine) that Yamane *et al.* (3) worked with is quite hydrophobic (21), we would expect the D isomer to distribute about 50% (K_{eq} = 1.0) to the 3′ position, whereas the L isomer should distribute 67% (K_{eq} = 2.0). We have found that, in similar experiments with unblocked D- and L-phenylalanine, the distributions are the same as for the *N*-acetyl-D- and -L-phenylalanine. The differential distribution might well be responsible for the 5-fold preference for the L isomer in peptide-bond formation. In addition to the intrinsic preference of the L isomer for the 3′ position, it is further possible, and this can be explored with NMR, that the L amino acid esterified to the 3′ position of two adjacent AMP residues allows an organization and orientation that will properly position adjacent aminoacyl-AMP esters, allowing more efficient peptide bond formation.

In summary, while the data here cannot explain the preferences for the L amino acid in all steps of protein synthesis, it may well explain the origin of the preferential

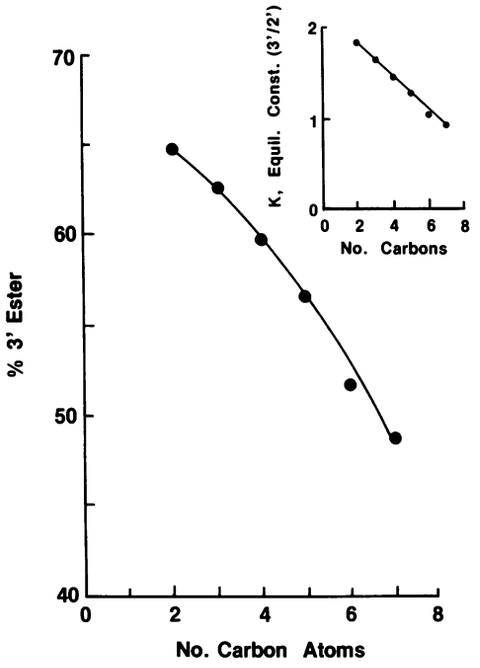

FIG. 3. The variation in equilibrium percentage of the 3′ ester of AMP at pH 7 and 25°C as a function of the number of carbons in the linear carboxylic acid. (*Inset*) The same data presented as equilibrium constants (K_{eq} = % 3′/% 2′). HPLC experiments were done essentially as described in the text; triplicate runs were made in each case. All points were also determined by ^1H NMR with good agreement. Standard deviations of the percentage of 3′ ester were: acetic (2.0), propionic (0.06), butyric (0.21), valeric (0.21), caproic (0.24), and heptanoic (0.15).

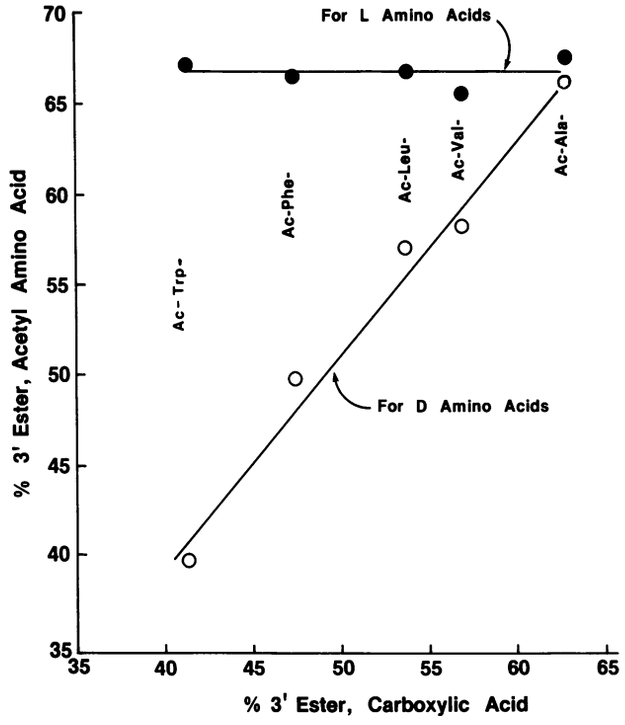

FIG. 4. The percentage of the 3′ ester at pH 7 and 25°C for *N*-acetylamino acids esterified to AMP plotted versus the data for the corresponding carboxylic acid analogs of the amino acids. All points were determined both by the HPLC method (triplicates) and by ^1H NMR except for Ac-L-Val-AMP, which has not been determined by ^1H NMR because the preparative HPLC column could not separate some contaminating D isomer. Standard deviations calculated for the carboxylic acid analogs (see text) of the amino acids were as follows: propionic (0.06), isovaleric (0.26), 4-mevaleric (0.21), phenylpropionic (0.57), and indole-3′-propionic (2.0).

combined use of the L isomer in the 3′ position for the formation of the peptide bond. Certainly a premise is that enzymatic systems tend to originate and evolve based on most probable molecular realities that yield results favorable to survival. In the present case those results would be successful synthesis of proteins required for survival.

There are several questions that arise as a result of this work. For example, the data suggest that hydrophobicity is an important factor in determining how a D amino acid will distribute between the 2′ and 3′ positions. One would then predict that a more hydrophilic amino acid such as Ac-D-Asn would distribute the same as Ac-L-Asn. We have found that to be approximately true in preliminary experiments (both NMR and HPLC). The L isomer was 68.8% 3′ and the D isomer was 62.0% 3′. Consequently, we feel the same trend will be noted with other hydrophilic amino acids.

Another question of interest is whether there would be selectivity in the esterification reaction itself when using a racemic mixture of amino acids. In fact, we have shown that esterification of AMP with racemic Ac-Phe-imidazolide proceeds with a continual enrichment in the D isomer. It appears that initial attack of both D and L amino acids is at the 2′ position. Because the L isomer, more than the D isomer, distributes away from the 2′ position to the 3′ position, subsequent attack at the 2′ position with elimination of the moiety at 3′ should gradually increase the D/L ratio. This selectivity will be the subject of a separate paper.

What effect would one see if the AMP contained L ribose instead of D ribose? One might then expect the distributions to reverse in their selectivities—i.e., all D amino acids might then prefer the 3′ position, and L amino acids might vary in their preference for the 3′ position. However, in the absence of experiment, this must be labeled as pure speculation.

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