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DOES THE NON-SATURABLE CELL ENTRY APPLY TO THE CHARGE-FREE FORM OF AMINO ACIDS?

HALVOR N. CHRISTENSEN and MARY E. HANDLOGTEN

Department of Biological Chemistry, The University of Michigan, Ann Arbor, Mich. 48109 (U.S.A.)

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Summary

The slow cellular entry shown by neutral α-amino acids at very high concentrations appears not to arise from diffusion of the totally uncharged species through the plasma membrane of the Ehrlich cell, judging from a similarity of the rates observed for the two conformational isomers of 1-amino-2-hydroxycyclohexane-carboxylic acid. One of these isomers provides in neutral solution 4 times as large a proportion of the charge-free species as the other, and 5 times the proportion calculated for alanine.

At high levels various amino acids, whether in their D, L or DL forms, enter the Ehrlich ascites tumor cell at rates which on extrapolation [1] correspond to first-order rate constants of about 0.04 min^{-1} [2—4]. Similarly, the approach to the steady state of uptake by α,α-dicyclopropylglycine, an amino acid selected for minimal mediation of passage, corresponds to entry and exodus at rates of 0.05 min^{-1} [5]. We consider in this report the possibility that these rates for apparently non-saturable passage might represent the migration of the most lipophilic species of the amino acid, namely $RCH(NH_2)COOH$, by an unmediated penetration of the lipid matrix of the plasma membrane, admittedly at an improbably high rate. Although this structural formula is still used in some standard textbooks, it applies of course to a rare species. For example, in neutral aqueous solution alanine is calculated to be present in that form to the extent of about 4 ppm, whereas the zwitterionic form $RCH(NH_3^+)COO^-$ is, of course, predominant.

The very slow penetration of amino acids into artificial liposomes increases with the hydrophobicity of the amino acid as measured by their distribution between organic solvents and water [6, 7]. In the plasma membrane, however, the contributions of various transport-mediating systems, even those for which affinity is low, are likely to overwhelm the simple factor of

lipophilicity. It would, for example, be quite wrong to suppose that leucine enters the human red blood cell much faster than alanine, and alanine faster than glycine, because of the decreasing lipophilicity of these amino acids in the order named. Instead, the uptake of alanine and glycine is comparatively slow because the mature red cell has largely lost the Na^+-dependent transport systems [8], and because these two amino acids have successively lower affinities for the predominantly surviving Na^+-independent system of the mature mammalian red blood cell [9].

A preliminary test of the proposition that the non-saturable passage occurs in the form $RCH(NH_2)COOH$ was possible with β-alanine. Because its pK_1 values (3.6 and 10.2) are closer together than those of α-alanine (2.35 and 9.7) substantially more of the β-isomer will be present in neutral solution in the totally uncharged form than for α-alanine. Hence, β-alanine should have the higher first-order rate constant for passage at high levels. On the contrary, we found that it enters the Ehrlich cell only about one-third as fast as α-alanine and other α-amino acids do [4]. Such structural specificity leads us to suppose the non-saturable transport is mediated.

An opportunity to test the matter further by comparing molecules even more similar than α- and β-alanine was provided by our synthesis of the two conformational isomers of the serine analog, 1-amino-2-hydroxycyclohexane-1-carboxylic acid, because their two pK' values are separated by 1.2 units more in one isomer than in the other (Fig. 1). This difference should be sufficient to increase substantially the proportion of the species in question.

Bucherer-Libe synthesis. 50 mmol of 2-hydroxycyclohexanone were added to a solution of 12.5 g $(NH_4)_2CO_3$ in 50 ml water. 50 ml ethanol was added, and then 50 mmol of KCN in 2 ml ethanol/water (1 : 1, v/v). The latter

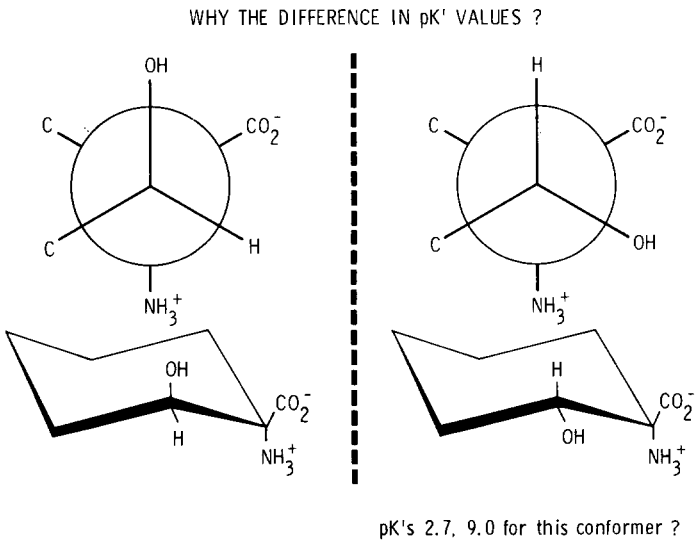

Fig. 1. Plausible conformations for the two isomeric analogs of serine. An equatorial hydroxyl group falling between the carboxyl and amino groups in the principal product of the Strecker synthesis (right) could account for the unusually low separation of pK_1' and pK_2'. We have been assisted by conversations on this question with Dr. J.A. Shafer.

addition redissolved the crystals separating on the first alcohol addition. The bottle was closed tightly and the well-mixed contents held at 50°C for 15 h. The hydantoin was crystallized by evaporating in vacuo to about 80 ml. 3.2 g of the product was hydrolyzed in a sealed tube at 214°C for 18 h in 25 ml of glacial acetic acid + 25 ml 6 M aqueous HCl. The solvent was evaporated off in vacuo and the product taken up in water. Automated column chromatography with ninhydrin color development showed 92% of a faster migrating, more soluble isomer (b), and 8% of a slower-eluting peak (a).

The solution was adjusted to pH 4.5 with IR4B resin in its basic form. The aqueous solution was taken to dryness and extracted to remove alcohol-soluble contaminants. The alcohol-washed product was crystallized twice from hot water to yield 1.5 g of crystals, now 97% isomer b and 3% isomer a. The (*carboxyl*-^{14}C)-labeled isomers were separated in chromatographic purity, using the resin column of the amino acid analyzer from the product of a small-scale Bucherer-Libe reaction with K^{14}CN.

Strecker synthesis. A solution of 50 mmol each of the same ketone, NH_4Cl and KCN in 13 ml water + 26 ml EtOH, was held at 50°C overnight in a sealed tube. The contents were evaporated to remove alcohol, and then hydrolyzed 6 h in 6 M HCl. The solution was taken to dryness, and the amino acid hydrochloride extracted from the residue with ether/ethanol (1 : 1, v/v) in repeated portions. The extracted material in aqueous solution was adjusted to pH 4.5 with IR4B resin. Two crystalline crops from hot water totalled 1.45 g (18%), which analyzed by automated column chromatography as 97% of the a isomer, and 3% of the b isomer with the same elution times as obtained for the corresponding Bucherer-Libe products.

Methyl esters. Solutions of 200 mg portions of these products in 20 ml MeOH were saturated with dry gaseous HCl, allowing the temperature to rise to boiling. After standing 48 h, the solutions were taken to dryness and the hydrochlorides of the esters crystallized by adding ethyl acetate to concentrated solutions in methanol. The products showed correct equivalent weights for neutralization and saponification by usual procedures.

Titrations with 1.00 M NaOH were made at 25°C for 0.1 M solutions brought to $\Gamma/2 = 0.15$ with NaCl. The pK' values yielded by symmetrical titration curves were as follows:

	pK_1'	pK_2'	pK_E' (methyl ester)
Isomer a (Strecker)	2.7	9.0	7.4
Isomer b (Bucherer)	2.1	9.6	7.4

The value for the methyl ester was used as the intrinsic value (pK_E') for the dissociation of the amino group of the species $RCH(NH_3^+)COOH$ on alkali titration. We calculate according to the procedure described by Edsall and Wyman [10] with the equation

$$\frac{[NH_3^+CHRCOO^-]}{[NH_2CHRCOOH]} = \frac{[H^+]\cdot[NH_3^+CHRCOO^-]}{[NH_3^+CHRCOOH]} \div \frac{[H^+][NH_2CHRCOOH]}{[NH_3^+CHRCOOH]}$$

The right-hand fraction is approximated then from the pK' of the methyl ester, and the middle fraction by observed pK_1' of the amino acid.

In this way, we obtain the proportions for the totally uncharged species of 20 ppm for isomer a and 5 ppm for isomer b. Thus, the abundance of the totally uncharged species is 4 times as great for isomer a as it is for isomer b, even though their isoionic points are the same. The predicted difference in lipophilicity of the two isomers was supported by the relation for their partition coefficients between water and *n*-octanol [6]. Isomer a showed a partition coefficient, water/octanol, of 75; isomer b, of 165. The values for leucine and alanine, for comparison, are 33 and 520 [6].

Non-saturable migration. Measures of uptake by the Ehrlich ascites tumor cell during 1 min in the concentration range 10—100 mM (10—81, for the less-soluble isomer) gave on extrapolation according to the method of Akedo and Christensen [1] first-order constants of 0.016 and 0.017 min^{-1} for the two isomers. The rates could not differ by a factor of 2 (Fig. 2). Furthermore, isomer a at high levels appears to enter the cell only about half as fast as alanine, even though its totally uncharged species is relatively five times as abundant, and the molecule undoubtedly more lipophilic.

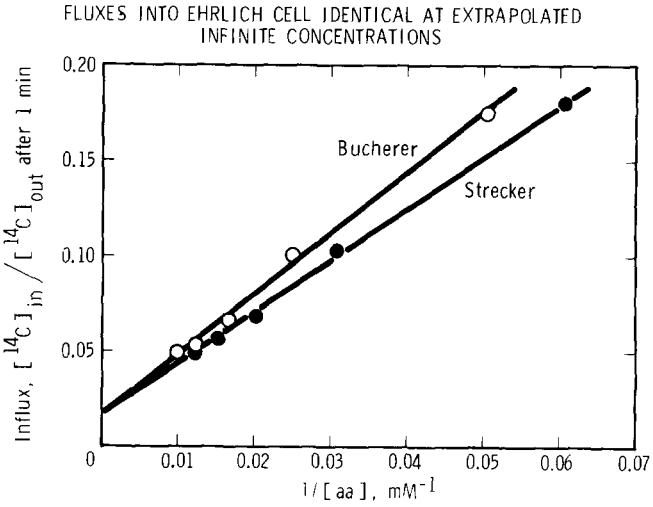

Fig. 2. Extrapolation of 1-min influx into the Ehrlich cell for the two isomeric serine analogs to infinite concentrations. The lines have been drawn by calculation of least squares.

Discussion

We have mentioned elsewhere that cell entry mediated by System L is 2.6 times as fast for isomer a as for isomer b, as measured by V/K_m [11]. A similar difference was seen, however, for the analogous isomers in which a 2-methyl group replaces the 2-hydroxy group. The pK' values of the latter two isomers show little difference. Hence the advantage for mediated uptake of isomer a of the hydroxy amino acid appears not to arise from the unusual nearness of its two pK' values, but more likely from steric factors. These 2 isomers, which may be considered valine analogs, show partition coeffi-

cients of 18 (Strecker product) and 32 (Bucherer product) between water and *n*-octanol.

Interest has occasionally fallen on the isoelectric point of neutral amino acids as perhaps an important determinant of transport. When the two pK' values are separated by 6 or more pH units, however, the proportion of the two species without net charge changes only very slowly with pH. If one of the charged species were the preferred transport substrate, the rate parameters should depend more on the value of pK_1' or pK_2' than on the isoionic point.

The present results show that the passage of the two test isomers at high concentrations is not a simple function of the concentration of the species totally without charge. Accordingly, the process allowing such passage of an amino acid into the cell is more complex than its non-specific diffusion across the lipid barrier.

Acknowledgement

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