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Intestinal Absorption of Amino Acid Derivatives: Importance of the Free α-Amino Group

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Abstract ■ The intestinal absorption of L-lysine-*p*-nitroanilide, L-alanine-*p*-nitroanilide, and glycine-*p*-nitroanilide was studied in the presence of competitive inhibitors in a perfused rat intestine. It was observed that L-lysine-*p*-nitroanilide absorption was inhibited by L-lysine methyl ester and L-arginine-β-naphthylamide but not by N_α-acetyl-L-lysine methyl esters. L-Alanine-*p*-nitroanilide absorption was inhibited by L-alanine methyl ester but not by β-alanine methyl ester. It was further observed that N_α-benzoyl-L-arginine-*p*-nitroanilide and N_α-succinyl-L-phenylalanine-*p*-nitroanilide were poorly absorbed. It was concluded that the peptidase in the brush border region that serves as the hydrolysis site requires a free α-amino group (an aminopeptidase), and that passive absorption of these compounds occurs only to a small extent.

Keyphrases □ Absorption, intestinal—amino acid derivatives, importance of the free α-amino group □ Amino acid derivatives—intestinal absorption importance of the free α-amino group □ α-Amino groups—importance of the free α-amino group in the intestinal absorption of amino acid derivatives

Recent investigations have demonstrated that brush border hydrolysis of soluble derivatives of insoluble drugs may significantly increase the absorption rate of the drug (1). Since the approach is based on enzymatic hydrolysis, the specificity of the hydrolytic enzymes in the brush border region must be investigated. While many enzymes have been reported to be present in this region (1), the aminopeptidases represent a likely site for reconversion of compounds previously studied (2–4). Furthermore, a free amino group on the substrate is required for aminopeptidase activity. The objective of this study was to determine the importance, for absorption, of a free α-amino group in amino acid derivatives of drugs.

EXPERIMENTAL

Materials—α-*N*-Benzoyl-DL-arginine-*p*-nitroanilide, *n*-succinyl-L-phenylalanine-*p*-nitroanilide, L-lysine-*p*-nitroanilide, L-lysine methyl ester, N_α-acetyl-L-lysine methyl ester, L-arginine-β-naphthylamide, L-alanine-*p*-nitroanilide, L-alanine methyl ester, β-alanine-*p*-nitroanilide, L-lysine methyl ester, β-alanine methyl ester, glycine-*p*-nitroanilide[1], and glycine methyl ester were used as received.

[1] U.S. Biochemical Corp., Cleveland, Ohio.

Perfusion Experiments—Drug absorption was measured in a perfused rat intestine segment as previously reported (1, 5). The inlet (C_o) and exit (C_m) concentrations were measured spectrophotometrically for the *p*-nitroanilide derivatives. The free *p*-nitroanilide was released by overnight (12 hr) hydrolysis of the syringe and perfusate samples after addition of sodium hydroxide (pH 11–12). A three-point analysis of the spectrum (350, 375, and 400 nm) was used in order to subtract the usually small protein background at these wavelengths.

THEORETICAL

The method of data analysis is the same as previously reported (1, 6). The method is appropriate as long as the boundary condition is linear:

$$J_w = -D\left.\frac{dc}{dr}\right|_{r=R} = P_w C_w \qquad \text{(Eq. 1)}$$

where J_w is the flux at the wall, P_w the wall permeability, and C_w the wall concentration. The dimensionless wall permeability (P_w*) is:

$$P_w^* = \frac{P_w R}{D} \qquad \text{(Eq. 2)}$$

where R is the radius of the intestine and D the solute diffusivity. The wall permeability ($^\circ P_w$*) is calculated using the measured ratio of exit to inlet concentrations (C_m/C_o) and the Graetz number (Gz) where $Gz = \pi DL/2Q$ and where L is the length of the intestinal segment and Q the fluid flow rate. The calculations follow the method previously reported (6). Diffusion coefficients are given in Table I. Analysis is based on the uncorrected $^\circ P_w$* values since the correction is small (6).

For the test compounds used in the present report (*e.g.*, L-lysine-*p*-nitroanilide) it is assumed that hydrolysis at the wall provides the driving force for transport to the wall, with the released *p*-nitroanilide being taken up by the wall (1). The wall permeability is, consequently, a heterogeneous reaction rate constant. The general case has been discussed (1) and gives:

$$P_w = \alpha D \operatorname{Tanh}(\alpha\delta_E) \qquad \text{(Eq. 3)}$$

or

$$P_w^* = \alpha R \operatorname{Tanh}(\alpha\delta_e) \qquad \text{(Eq. 4)}$$

where

$$\alpha^2 = k/D \qquad \text{(Eq. 5)}$$

where k is the first-order reaction rate constant, and δ_E is the (unknown) thickness of the enzyme layer. Two special cases of Eq. 4 are:

High reactivity ($\alpha\delta_e > 1$):

$$P_w^* = \alpha R \qquad \text{(Eq. 6)}$$

Table I—Diffusion Coefficients

Compound	D, × 10^6, cm^2/sec
Lysine-*p*-nitroanilide	6.7
Alanine-*p*-nitroanilide	7.0
Glycine-*p*-nitroanilide	7.4

Low reactivity ($\alpha\delta_E < 1$):

$$P_w^* = \alpha^2\delta_E \qquad \text{(Eq. 7)}$$

Assuming Michaelis-Menten kinetics at the wall and limiting substrate concentration to the first-order region, the first-order reaction rate constant (k) (Eq. 5) becomes:

$$k = V_{max}/K_m \qquad \text{(Eq. 8)}$$

where V_{max} (moles/liter/second) is the maximal velocity and K_m (moles/liter) is the Michaelis constant. In the presence of a competitive inhibitor[2] (*e.g.*, lysine-*p*-nitroanilide in the presence of lysine methyl ester) Eq. 8 becomes:

$$k = V_{max}/\left(1 + \frac{I}{K_I}\right)K_m \qquad \text{(Eq. 9)}$$

where I is the inhibitor concentration (in the enzyme layer) and K_I the inhibition constant. Combining Eqs. 5, 6, 7, and 9 gives:

High reactivity case:

$$P_w^{*2} = (V_m R^2/DK_m)[1/(1 + I/K_I)] \qquad \text{(Eq. 10)}$$

Low reactivity case:

$$P_w^* = (V_m \delta_E/DK_m)[1/(1 + I/K_I)] \qquad \text{(Eq. 11)}$$

Both Eqs. 10 and 11 can be linearized, but this aspect is not explored in this report. The main point of Eqs. 10 and 11 is that P_w^* is reduced in the presence of a competitive inhibitor. This investigation uses as substrates the *p*-nitroanilide derivatives and the remaining compounds as competitive inhibitors.

Rough estimates of the inhibition constants (K_I) can be made on the basis of this model. From Eqs. 6–11 the following relationships can be obtained:

High reactivity:

$$K_I = I/\{[P_w^*(S)/P_w^*(I)]^2 - 1\} \qquad \text{(Eq. 12)}$$

Low reactivity:

$$K_I = I/\{[P_w^*(S)/P_w^*(I)] - 1\} \qquad \text{(Eq. 13)}$$

where $P_w^*(S)$ is the (dimensionless) substrate permeability and $P_w^*(I)$ is the substrate permeability in the presence of the inhibitor. The inhibitor concentration, $[I]$, would be the concentration in the enzyme layer which is unknown. However, using as $[I]$ its value in the profusing solution allows the calculation of an apparent K_I.

RESULTS AND DISCUSSION

The experimental C_m/C_o ratios and Graetz numbers (Gz), along with the calculated dimensionless wall permeabilities ($^\circ P_w^*$), are presented in Tables II–XIV. Figure 1 presents a summary of the results.

Figure 2 is a graph of the data in Table II where the lysine-*p*-nitroanilide permeability was studied as a function of concentration. The results do not provide any evidence of saturation. While saturation would be expected at some point, it apparently would require concentrations above the 0.8 mM used in these studies. All subsequent studies were done using a lysine-*p*-nitroanilide concentration of 4×10^{-5} M. At this concentration the enzymatic reaction at the wall is in the apparent first-order region, and the analysis in the theoretical section would apply.

The results for the L-lysine-*p*-nitroanilide permeability in the presence of the competitive inhibitors L-lysine, L-lysine methyl ester, N_α-acetyl-L-lysine methyl ester, and arginine-β-naphthylamide are given in Tables IV–VII and Fig. 1. L-Lysine and N_α-acetyl-L-lysine methyl ester did not reduce the L-lysine-*p*-nitroanilide permeability at concentrations of 4×10^{-4} M. L-Lysine methyl ester and arginine-β-naphthylamide did significantly (t test) reduce the L-lysine permeability. These results are consistent with the hydrolysis site being an enzyme of the aminopeptidase type, *i.e.*, requiring a free α-amino group. The fact that L-lysine itself is not a good competitive inhibitor is probably related to the fact that it is the product of the enzymatic reaction. The surface enzyme responsible for hydrolysis apparently does not show significant product inhibition[3].

[2] Compounds used are actually competitive substrates, but since the assay is specific for only one of the substrates, the other acts as a competitive inhibitor (7).

Table II—L-Lysine-*p*-nitroanilide Dimensionless Wall Permeability *versus* Concentration

Concentration, × 10^5 M	$[C_m/C_o]$	Gz, × 10^2		$^\circ P_w^*$[a]	log C_o
4	0.84	1.97		5.4	
4	0.91	1.64		2.2	
4	0.75	1.64		∞	
4	0.78	2.63		8.4	−4.4
4	0.93	1.31		1.9	
4	0.90	1.23		3.9	
			$\overline{^\circ P_w^*}$	4.4 (1.2)	
8	0.87	1.074		12.2	
8	0.88	1.40		4.6	−4.1
			$\overline{^\circ P_w^*}$	6.5 (3.7)	
40	0.81	2.0		8.2	
40	0.82	2.0		7.4	−3.4
40	0.85	2.0		4.5	
			$\overline{^\circ P_w^*}$	6.7 (1.1)	
80	0.85	2.0		4.3	−3.1

[a] Values in parentheses are the standard error of the mean.

Table III—L-Lysine-*p*-nitroanilide [a] Dimensionless Wall Permeabilities

C_m/C_o	Gz, × 10^2		$^\circ P_w^*$
0.84	1.97		5.5
0.91	1.64		2.2
0.78	2.63		8.4
0.91	1.23		3.9
0.87	1.07		12.2
0.89	1.40		4.7
0.86	2.0		4.3
0.82	2.0		8.3
0.82	2.0		7.5
0.85	2.0		4.6
		$\overline{^\circ P_w^*}$	6.2
		SEM	0.9

[a] 4×10^{-5} M.

Table IV—L-Lysine-*p*-nitroanilide Dimensionless Wall Permeability *versus* L-Lysine Concentration

[L-Lysine-*p*-nitroanilide] × 10^5 M	[Lysine] × 10^3 M	$[C_m/C_o]$	Gz, × 10^2		$^\circ P_w^*$
8	5	0.83	1.074		131.0
40	5	0.61	2.0		∞
40	5	0.84	2.0		5.1
80	5	0.83	2.0		5.8
80	5	0.83	2.06		6.0
80	5	0.78	2.60		7.3
				$\overline{^\circ P_w^*}$	6.0
				SEM	0.5

Table V—L-Lysine-*p*-nitroanilide [a] Dimensionless Wall Permeability in the Presence of L-Arginine-β-naphthylamide [a]

C_m/C_o	Gz, × 10^2		$^\circ P_w^*$
0.93	1.81		1.34
0.92	1.32		2.45
0.90	1.23		4.41
0.95	1.32		1.4
		$\overline{^\circ P_w^*}$	2.4
		SEM	0.72

[a] 4×10^{-5} M.

[3] The proteolytic enzyme, trypsin, for example, does not show significant product inhibition. Apparently the carboxyl group generated by either amide or ester hydrolysis of the substrate and its subsequent ionization significantly reduces the binding constant for the product to the enzyme.

Table VI—L-Lysine-*p*-nitroanilide [a] Dimensionless Wall Permeabilities in the Presence of *N*-Acetyl-L-lysine-Methyl Ester [b]

C_m/C_o	$Gz, \times 10^2$		^oP_w*
0.86	1.55		6.1
0.92	1.23		8.3
0.86	1.32		9.9
0.88	1.32		6.7
0.82	1.73		11.9
0.82	1.89		10.5
		$\overline{^oP_w}$*	8.8
		SEM	0.9

[a] 4×10^{-5} *M*. [b] 4×10^{-4} *M*.

Table VII—L-Lysine-*p*-nitroanilide [a] Dimensionless Wall Permeability in the Presence of L-Lysine Methyl Ester [b]

C_m/C_o	$Gz, \times 10^2$		^oP_w*
0.91	1.32		3.1
0.91	1.32		2.9
0.96	1.40		1.0
0.87	1.73		4.2
0.94	1.32		1.8
0.94	1.20		1.9
0.88	1.81		3.5
0.87	1.89		4.0
		$\overline{^oP_w}$*	2.8
		SEM	0.4

[a] 4×10^{-5} *M*. [b] 4×10^{-4} *M*.

Table VIII—L-Alanine-*p*-nitroanilide Dimensionless Wall Permeabilities

C_m/C_o	$Gz, \times 10^{-2}$		^oP_w*
0.82	1.56		13.6
0.78	1.82		32.9
0.85	1.82		5.1
0.84	1.56		8.9
0.86	1.29		7.9
0.88	1.29		5.4
0.72	1.82		∞
0.88	0.963		11.5
0.87	0.963		14.3
0.87	1.34		7.2
		$\overline{^oP_w}$*	11.9
		SEM	2.8

[a] 4×10^{-5} *M*.

Table IX—L-Alanine-*p*-nitroanilide [a] Dimensionless Wall Permeabilities in the Presence of L-Alanine Methyl Ester [b]

C_m/C_o	$Gz, \times 10^{-2}$		^oP_w*
0.82	1.91		7.5
0.87	1.91		3.2
0.86	1.91		3.8
0.81	1.91		9.5
0.91	1.27		3.2
0.95	1.27		1.3
0.96	1.27		0.97
0.94	1.37		1.2
		$\overline{^oP_w}$*	3.9
		SEM	1.1

[a] 4×10^{-5} *M*. [b] 4×10^{-3} *M*.

The results for the L-alanine-*p*-nitroanilide permeability in the presence of L-alanine methyl ester and β-alanine methyl ester (Tables VIII–X) are also consistent with the hydrolysis site being an enzyme of the aminopeptidase class. The β-alanine methyl ester, with the primary amino group on the β-carbon rather than the α-carbon, is not a good competitive inhibitor. This is consistent with structural specificity of an aminopeptidase enzyme.

The results for N_α-benzoyl-arginine-*p*-nitroanilide and N_α-succinyl-phenylalanine-*p*-nitroanilide, (Tables XI and XII) provide direct evidence for the importance of a free α-amino group. Both compounds are poorly absorbed as evidenced by their very low permeabilities. The

Table X—L-Alanine-*p*-nitroanilide [a] Dimensionless Wall Permeabilities in the Presence of β-Alanine Methyl Ester [b]

C_m/C_o	$Gz, \times 10^{-2}$		^oP_w*
0.843	1.24		19.6
0.843	1.55		9.4
0.848	1.82		5.8
0.810	1.56		27.1
0.853	1.56		7.5
0.789	1.38		∞
0.881	1.34		5.6
0.827	1.38		23.1
0.843	1.55		9.4
		$\overline{^oP_w}$*	13.5
		SEM	3.0

[a] 4×10^{-5} *M*. [b] 4×10^{-3} *M*.

Table XI—*N*-α-Benzoyl-arginine-*p*-nitroanilide [a] Dimensionless Wall Permeabilities

C_m/C_o	$Gz, \times 10^{-2}$		^oP_w*
0.963	1.48		0.8
1.06	1.32		0.0
1.03	2.64		0.0
0.913	1.92		1.8
0.982	3.85		0.1
0.955	1.58		0.9
1.035	1.23		0.0
1.082	1.23		0.0
0.978	1.15		0.6
0.969	1.15		0.8
		$\overline{^oP_w}$*	0.5
		SEM	0.2

[a] 4×10^{-5} *M*.

Table XII—Succinyl-L-phenylalanine-*p*-nitroanilide [a] Dimensionless Wall Permeabilities

C_m/C_o	$Gz, \times 10^{-2}$		^oP_w*
1.000	1.24		0.0
1.11	1.27		0.0
1.04	2.55		0.0
0.969	1.41		0.7
0.954	2.82		0.5
0.892	7.07		0.5
1.003	1.65		0.0
0.993	1.65		0.1
1.103	1.68		0.0
1.131	1.29		0.0
1.021	1.20		0.0
1.004	1.20		0.0
		$\overline{^oP_w}$*	0.1
		SEM	0.04

[a] 4×10^{-5} *M*.

Table XIII—Glycine-*p*-nitroanilide [a] Dimensionless Wall Permeability

C_m/C_o	$Gz, \times 10^2$		^oP_w*
0.78	2.19		13.7
0.87	1.82		3.7
0.76	2.92		8.6
0.90	2.10		1.9
0.85	1.91		4.8
0.87	1.64		4.3
0.89	1.55		3.61
0.77	1.55		∞
0.83	1.55		12.0
0.95	1.27		1.32
0.88	1.27		5.4
0.88	1.64		3.54
0.86	1.8		4.01
		$\overline{^oP_w}$*	5.6
		SEM	1.1

[a] 4×10^{-5} *M*.

Table XIV—Glycine-*p*-nitroanilide [a] Dimensionless Wall Permeability in the Presence of Glycine Methyl Ester [b]

C_m/C_o	$Gz, \times 10^2$		$°P_w*$
0.84	1.92		6.4
0.88	1.55		4.6
0.91	1.55		2.5
0.88	1.55		4.5
0.88	1.28		6.6
0.91	1.28		3.1
0.87	1.74		4.1
0.87	1.74		4.5
0.86	1.83		5.1
0.90	1.65		2.9
		$\overline{°P_w*}$	4.4
		SEM	0.4

[a] 4×10^{-5} *M*. [b] 4×10^{-3} *M*.

fact that L-arginine-β-naphthylamide is a good competitive inhibitor for L-lysine-*p*-nitroanilide, while the N_α-benzoyl-arginine-*p*-nitroanilide is not absorbed well at all, further illustrates the importance of a free α-amino group. In addition, the low permeabilities for N_α-benzoyl-arginine-*p*-nitroanilide and N_α-succinyl-phenylalanine-*p*-nitroanilide suggest that passive absorption for both compounds is very small. This is expected since both compounds are ionized at pH 7.4. It further suggests that passive permeation of the other compounds studied is not significant, providing further evidence (in addition to the observed competition) that the absorption mechanism is through membrane hydrolysis (1).

The results for glycine-*p*-nitroanilide and glycine methyl ester (Tables XIII and XIV) do not fit with the above results. That is, it would be expected that glycine methyl ester would be a competitive inhibitor for glycine-*p*-nitroanilide. This suggests that glycine derivatives may be absorbed by a different mechanism (*e.g.*, passive diffusion) or that the enzyme binding constants for the glycine derivative are small. Peptide absorption studies on glycine peptides (*e.g.*, Gly-Gly) indicate that the peptide is taken up intact and is not a good substrate for the brush border peptidases (3, 8). Studies with peptide transport inhibitors need to be

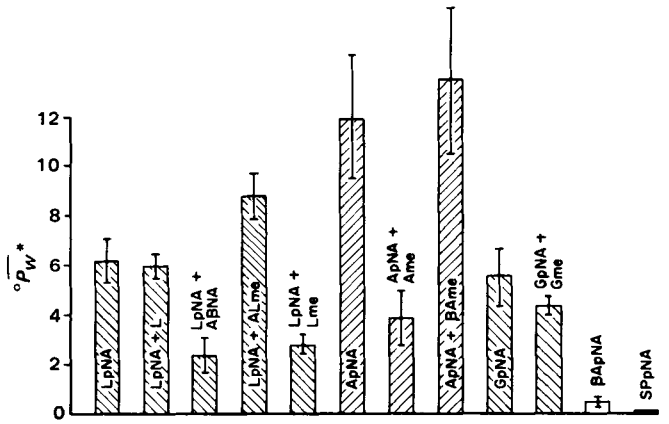

Figure 1—*Summary of intestinal wall permeability ($°P_w*$) results. Key: (LpNA) L-lysine-p-nitroanilide; (L) L-lysine; (Lme) L-lysine methyl ester; (AβNA) L-arginine-β-naphthylamide; (ALme) N-acetyl-L-lysine methyl ester; (ApNA) L-alanine-p-nitroanilide; (Ame) L-alanine methyl ester; (βAme) β-alanine methyl ester; (GpNA) glycine-p-nitroanilide; (Gme) glycine methyl ester; (βApNA) N_α-benzoyl-arginine-p-nitroanilide; (SPpNA) N_α-succinyl-phenylalanine-p-nitroanilide.*

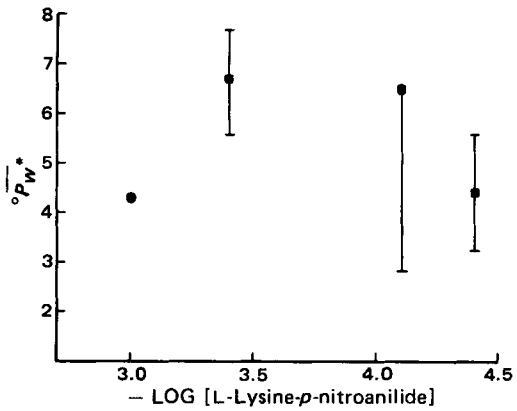

Figure 2—*Wall permeability of L-lysine-p-nitroanilide as a function of concentration in the perfusing solution.*

done to further establish the absorption mechanism for glycine-*p*-nitroanilide.

Using Eqs. XII and XIII, rough estimates of a k_I value can be made where significant inhibition is observed. For L-arginine-β-naphthylamide and L-lysine methyl ester respective values of 0.7×10^{-4} and 1.0×10^{-4} *M* are obtained for the high reactivity case, while respective values of 2.5×10^{-4} and 3.3×10^{-4} *M* are obtained for the low reactivity case. The values for L-alanine methyl ester are 0.6×10^{-4} and 2.2×10^{-4} *M* for the high and low reactivity cases, respectively. Since the inhibitor concentration in the enzyme layer is certainly lower than that in the perfusing solutions, these values would represent upper limits on the estimate.

Studies on the intestinal absorption of *p*-nitroanilide derivatives of the amino acids, L-lysine and L-alanine, in the presence of various competitive inhibitors have shown that only those compounds with a free α-amino group are good competitive inhibitors, *i.e.*, reduce the wall permeability, $°P_w*$. This suggests a free α-amino group is essential for good brush border peptidase activity. The fact that N_α-benzoyl-arginine-*p*-nitroanilide and N_α-succinyl-phenylalanine-*p*-nitroanilide are poorly absorbed is also consistent with this inference.

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