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Absorption kinetics of β-alanine as model compound in rat small intestine

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Contradictory results have been reported on intestinal β-alanine absorption, although a generalized view is that it could be a passive, nonmediated process. Since previous data from our laboratory suggested that some competition arises between intestinal absorption of the γ-amino acidic drug baclofen and β-alanine, a rat jejunum in situ study was undertaken in order to gain insight into the mechanism of β-alanine absorption. Perfusion solutions with initial β-alanine concentrations ranging from 0.3 to 56 mM were used. The β-alanine absorption was clearly identified as a saturable process which obeys Michaelis-Menten equation kinetics, as assessed through two computer-assisted procedures based on differential and integrated forms of this equation. Parameter values found were: $V_m = 3.88–4.72$ mg · ml^{-1} · h^{-1} (43.6–52.9 mM · h^{-1}), and $K_m = 0.97–1.13$ mg · ml^{-1} (10.9–12.7 mM). Statistical analysis does not account for the existence of significant parallel passive diffusion pathways (less than 0.2 h^{-1}).

Introduction

Intestinal absorption mechanisms of α-amino acids have been extensively studied, both in animals (in vitro and in situ methods) and in humans. Absorption has been shown to be, in the great majority of the cases, an active transport process. Several carrier-mediated mechanisms for neutral, basic and acidic α-amino acids have been demonstrated in the small intestine and their Michaelis-Menten kinetics characterized [1–4].

On the other hand, absorption mechanisms of β, γ and δ-amino acids have received much less attention. Although an independent carrier system has been ascribed to the absorption of γ- and δ-amino acids in man [4], and the existence of an active component in the absorption of carnitines together with passive non-saturable pathway has been postulated [5], reports on other particular substances are contradictory. Thus for β-alanine (which can be considered as a model compound) both mediated [6] and nonmediated [7–9] uptake from the intestinal tract has been reported. These contradictory results could be related to the main technique used for absorption measurements, i.e., the everted segments of rat or hamster small intestine (jejunum and ileum) in vitro. This procedure lacks blood supply and could lead to inconclusive results when absorption rates are slow, since K_m determinations become unfeasible due to methodological limitations. On the other hand, in vitro results may not coincide with those observed in vivo [10].

In a recent paper [11], we have shown strong evidence of carrier-mediated absorption in the rat small intestine for baclofen, a spasmolytic drug structurally related to γ-amino butyric acid (GABA). It was thought that some carrier system or systems mediating absorption of γ- or β-amino acids might be responsible for this behaviour, since preliminary experiments demonstrated that the intestinal absorption of baclofen was clearly inhibited by β-alanine even at moderate concentrations of the amino acid in perfusion fluids, as will be pointed out later. The aim of this paper is to gain insight into the intestinal absorption mechanisms of β-alanine as a β-amino acidic model compound, by the use of an in situ perfusion method, the advantages of which have been described elsewhere [11,12]. It is a preliminary step toward explaining the possible mechanisms involved in absorption of other β- or γ-amino acids and related drugs.

Abbreviation: AIC, Akaike's information criterion.

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Materials and Methods

Experimental absorption technique

Experiments were performed on male Wistar rats weighing 200–290 g, fasted for 20 h but with free access to water. The rats were anaesthetized 1 h before surgery by an intraperitoneal injection of ethylurethane.

The in situ rat gut technique [13] modified as previously described [11,12,14], was employed. In short, a midline incision was made and a fraction of the middle intestine of about 35 cm in length (measured 30 cm from pylorus) was cannulated. After rinsing with physiological saline in order to eliminate fecal residues and debris (25 ml), 5 ml of an isotonized solution of β-alanine (0.025, 0.20, 1.0, 2.5 or 5.0 mg/ml, corresponding to 0.28, 2.25, 11.2, 28.1 or 56.2 mM of the amino acid, respectively), buffered to pH 7.6 [15], was perfused at 37°C. The amino acid concentration remaining in the perfused solution was measured every 5 min for a total of 30 min, through samples of 0.1 ml. Five animals for each lot were employed and the average concentration values were used for kinetic calculations.

Water reabsorption was evaluated separately in five animals for each data set, by measurement of the remaining volume at 30 min according to a previously reported method [14]; the significance of the differences between means of each set was assessed through a one-way ANOVA test and a subsequent Peritz *F*-test, which is, undoubtedly, a most robust statistical test for multiple comparisons [16]. A water loss of more than 10% at 30 min implies correction of experimental data for reabsorption [13]. In order to prevent adsorption to the mucosa or tissue uptake, as well as dilution effects [11–14], the initial nonperfused sample concentration was excluded from kinetic calculations.

Assessment of nonlinearities

The features of the in situ perfusion method employed here offer the possibility of investigating the existence of nonlinearities in the β-alanine absorption process. When such nonlinearities are assessed and analyzed, they can provide useful information about possible absorption kinetics of the solute [17].

In order to achieve this, preliminary fits of the classical first-order equation to data:

$$\ln A = -k_{ap} \cdot t + \ln A_0 \qquad (1)$$

should be performed. In Eqn. 1, A values represent the remaining concentrations of β-alanine found in luminal contents at the sampling times, t, and A_0 are the calculated intercept values at zero time for each set of data. Apparent absorption rate constants or 'pseudoconstants', k_{ap}, can then be determined for each concentration data set (mean of five animals) from the slope of the corresponding lines and compared statistically.

In addition, it is convenient [11,12] to perform, using the same sets of data, zero-order fits according to the expression:

$$A = -k_0 \cdot t + A_0 \qquad (2)$$

Analysis and interpretation of the results obtained will supply useful information on absorption kinetics. The first-order expression (Eqn. 1) represents the lower limit of the saturable absorption kinetics; in these conditions, the quotient V_m/K_m would equal approximately k_{ap}. Therefore, when k_{ap} pseudoconstants significantly decrease as the initial solute concentration in the perfusion fluid is increased, the fitting of the Michaelis-Menten equation to data is strongly recommended because this behaviour may indicate the existence of a carrier-mediated transport [17]. To ascertain the significance of the differences found, the k_{ap} values found for each data set were subjected to an ANOVA test and to a subsequent Peritz *F*-test, as indicated above.

On the other hand, correlation coefficients found for fitting the zero-order equation (Eqn. 2) to the experimental data are also illustrative as a supplementary criterium. Since the upper limit of any saturable process is zero-order kinetics, when such coefficients tend to increase as the initial concentration of the solute in data sets is increased, the advisability of fitting the Michaelis-Menten equation to data can also be evidenced [11,12].

Global fits of linear and nonlinear equations to data

Absorption kinetics of β-alanine was then examined using two general computer procedures based on the application of differential and integrated equations (Michaelis-Menten, first-order and combined mixed and first-order kinetics). Global fits were performed, including all sets of data for each equation.

Michaelis-Menten and combined equations were tested in order to ascertain the absorption kinetics of β-alanine, but single first-order fits were also performed. Although this latter type of kinetics can only be assayed, strictly speaking, when there is no evidence of nonlinearities, it is always useful to do so because its statistical comparison with the remaining fits (Michaelis-Menten and combined kinetics) provides a more reliable and realistic procedure for selecting the best absorption model. To achieve this, the Akaike's information criterion, namely AIC [18], was used; the smaller AIC value indicates the best fit.

As pointed out above, two forms of equations, embracing all direct-fitting procedures, have been assayed: differential and integrated expressions. Linear transforms of the differential equation have not been used

because the intrinsic difficulties involved in interpreting the results [19,20] are perhaps increased when fractional data sets are available, as occurs in our case [11]. Statistical comparison was made between models (Michaelis-Menten, first-order and combined equations) for each lot of methods (i.e., differential and integrated) because AIC figures are comparable only when the same variables have been used for fitting purposes (i.e. $-dA/dt$ and A for the differential form, and A and t for the integrated form).

Differential equations. The Michaelis-Menten differential expression in its classical form was fitted to data

$$-\frac{dA}{dt} = \frac{V_m \cdot A}{K_m + A} \tag{3}$$

by using the iterative computer procedure recommended by Yamaoka and Nakagawa [21] for differential equations, based on numerical solutions. As experimental data, the remaining β-alanine percentage values shown in Table II, expressed in mM (i.e., multiplied by the starting β-alanine concentration, in $mg \cdot ml^{-1}$, and divided by $100 \cdot 89.09$, this latter being the molecular weight of β-alanine) were introduced in the program. Calculations were performed globally for all remaining β-alanine concentrations, that is, for the five sets of data.

Based on the same principle, global fits of the classical first-order equation in its differential form, to the data

$$-\frac{dA}{dt} = k_{ap} \cdot A \tag{4}$$

were performed and compared with those found for Eqn. 3 with the aid of the above mentioned AIC method. In Eqn. 4, k_{ap} represents the apparent first-order rate constant (or pseudoconstant) which globally satisfies the data found for all β-alanine remaining concentrations.

Using the same procedure, fits of Michaelis-Menten and first-order combined equations to the data were developed:

$$-\frac{dA}{dt} = \frac{V_m \cdot A}{K_m + A} + k_{ap} \cdot A \tag{5}$$

and the results compared with those obtained for Eqns. 3 and 4 through the Akaike's method.

Integrated equations. Fits of the integrated equations that represent both nonlinear and linear kinetics were also performed in order to obtain a greater safety compass in the interpretation of the results. First, the integrated form of the Michaelis-Menten equation [17]:

$$t = \frac{1}{V_m}\left(A_0 - A + K_m \cdot \ln\frac{A_0}{A}\right) \tag{6}$$

was fitted to experimental data. This was achieved through a nonlinear least-squares procedure described by us in former papers [11,12]. In short, the MULTI program was utilized [22], with a subroutine written and incorporated into the program in order to treat the remaining concentration, A, as a dependent variable. This leads, for given parameters (i.e., V_m, K_m and A_0) and experimental time (t) values, to the calculation of any theoretical concentration, A. At the end of fitting, V_m and K_m values that globally satisfy the data, as well as corrected A_0 intercepts for each perfusion set were found, so that complete and continuous plots of A against t could be obtained, as represented in Fig. 1. Calculations were performed globally, as in the differential method, for all remaining concentrations, expressed in mM of β-alanine, as formerly described, by using the average values (means of five animals per set).

Based on the same principle, global fits of first-order kinetics to the data

$$A = A_0 \cdot e^{-k_{ap} \cdot t} \tag{7}$$

as well as fits of combined Michaelis-Menten and first-order kinetics to the data, according to the mixed equation [17,23]

$$t = \frac{1}{k_{ap} \cdot K_m + V_m}\left[K_m \cdot \ln\frac{A_0}{A} + \frac{V_m}{k_{ap}} \ln\frac{(A_0 + K_m)k_{ap} + V_m}{(A + K_m)k_{ap} + V_m}\right] \tag{8}$$

were performed and compared with those found from Eqn. 6 through the same statistical tests as above. The use of the subroutine for Eqn. 7 is, of course, unnecessary, since A is in explicit form.

Analytical procedures

Samples were tested for β-alanine content by reversed-phase HPLC, using a Waters joint (Waters Ass., Milford), constituted by a Model 590 pump, a Model 481 Lambda-Max detector (at 495 nm), and a Data Module. A 150×4.6 mm Spherisorb S-5 ODS-2 analytical column (Phase Separations, Ltd., Queensferry) in conjunction with a 5 mm Guard-Pak cartridge (Microbondapak C-18) was used. A mixture of acetonitrile and 0.05 M phosphoric acid, adjusted to pH 1.9 with 10 M sodium hydroxide in proportions of 20:80 was employed as mobile phase.

Intestinal samples were centrifuged at 3000 rpm for 10 min and diluted with a calculated volume of water in order to attain the linearity range necessary for their further quantification. Derivatization with 4-chloro,7-nitrobenzofurane was achieved according to the procedure described by Ahnoff et al. [24], although the operation was carried out at room temperature for 24 h instead of by heating. After derivatization, 5 μl of the solution were injected in the chromatograph. Thanks to the simplicity of the procedure no internal standard was

TABLE I

Water reabsorption in perfusion tests

Percent of remaining volume at 30 min. No significant differences between the five sets of data were found using an ANOVA test ($F = 0.474$; $P = 0.754$). A subsequent Peritz F-test did not allow for significant differences between any particular set (means of five animals ± S.D.). Mean percent water reabsorption at 30 min was less than 10% (8.2 ± 0.12). n.s., not significant.

Starting β-alanine concentration in perfusion fluids (A_i, μg/ml)	Percent remaining volume at 30 min (ml)	Peritz F-test significance	
		A_i sets compared	P values
25	4.59 (±0.28)	25– 200	0.734 (n.s.)
		25–1000	0.793 (n.s.)
		25–2500	0.832 (n.s.)
200	4.64 (±0.24)	25–5000	0.843 (n.s.)
		200–1000	0.814 (n.s.)
1000	4.55 (±0.12)	200–2500	0.339 (n.s.)
		200–5000	0.846 (n.s.)
2500	4.49 (±0.18)	1000–2500	0.646 (n.s.)
		1000–5000	0.347 (n.s.)
5000	4.67 (±0.16)	2500–5000	0.754 (n.s.)

TABLE III

Nonlinearities in β-alanine absorption

Statistical comparison between first-order rate pseudoconstants, k_{ap}, found at different starting β-alanine concentrations in the perfusion fluids, through a Peritz F-test consecutive to an ANOVA test ($F = 154.6$; $P < 0.0001$). Nonlinearities in absorption are clearly evidenced, strongly suggesting the fitting to Michaelis-Menten kinetics. n.s., not significant

Compared β-alanine initial perfusion concentrations (μg/ml)	Peritz F-test significance (P values)
25– 200	0.267 (n.s.)
25–1000	0.0007
25–2500	< 0.0001
25–5000	< 0.0001
200–1000	0.002
200–2500	< 0.0001
200–5000	< 0.0001
1000–2500	< 0.0001
1000–5000	< 0.0001
2500–5000	< 0.0001

necessary. Calibration curves covering the entire range of β-alanine concentrations in diluted luminal samples (2.5–25.0 μg/ml, equivalent to 0.14–1.4 nM injected volume) were prepared in triplicate. Excellent linear plots relating peak area and β-alanine concentration were obtained, with practically no intercept. Variation coefficients ranging from 1.6 to 4.4 were found for standard samples.

Results

Water reabsorption at 30 min for the five sets of data is shown in Table I. Since a mean loss of less than 10% was observed and no significant differences were found between data set mean values, correction of experimental data for water reabsorption was thought to be unnecessary.

The time course of disappearance of remaining concentrations (A, means of five animals per set), as well as the apparent first-order rate constants, k_{ap}, and zero-order rate constants, k_0, calculated for each set of data according to Eqns. 1 and 2, respectively, are shown in Table II. In Table III the statistical comparison between the apparent first-order pseudoconstants is shown; it clearly indicates the existence of nonlinearities in β-alanine absorption.

In Table IV, parameter values and statistical AIC figures found after fitting differential and integrated forms of the Michaelis-Menten, first-order and com-

TABLE II

Luminal disappearance of β-alanine and limiting pseudoconstants

Percent average β-alanine concentration, relative to initial (A_i) remaining in the jejunal fluid at each sampling time (± S.D., means of five animals). Pseudo first- and zero-order absorption rate constants fitting each data set and correlation coefficients are also shown.

Sampling time (min)	Percent remaining β-alanine in jejunal fluid for each starting concentration (A_i, μg/ml)				
	$A_i = 25$	$A_i = 200$	$A_i = 1000$	$A_i = 2500$	$A_i = 5000$
5	69.27 (±2.72)	73.82 (±4.25)	67.86 (±2.17)	83.60 (±3.63)	78.51 (±3.18)
10	49.16 (±3.04)	51.73 (±2.70)	52.59 (±1.72)	70.82 (±0.66)	72.15 (±2.74)
15	34.48 (±3.59)	37.46 (±2.50)	40.41 (±1.92)	60.63 (±1.87)	65.93 (±2.92)
20	24.04 (±2.48)	27.34 (±1.30)	30.57 (±2.02)	52.59 (±1.57)	60.06 (±2.60)
25	17.19 (±2.70)	19.87 (±1.62)	23.35 (±1.81)	46.91 (±1.84)	54.20 (±2.68)
30	12.67 (±2.38)	14.37 (±1.12)	17.92 (±2.12)	38.67 (±2.04)	49.81 (±3.13)
k_{ap} (h^{-1})	4.117 (±0.36)	3.897 (±0.25)	3.224 (±0.23)	1.793 (±0.17)	1.109 (±0.11)
r	0.999	0.999	0.999	0.998	0.999
k_0 (%/min)	2.225 (±0.06)	2.302 (±0.17)	1.984 (±0.07)	1.739 (±0.16)	1.161 (±0.10)
r	0.968	0.969	0.984	0.991	0.999

TABLE IV

Kinetic parameters in β-alanine absorption

Parameter values found after fitting the selected equations (Michaelis-Menten, first-order and combined kinetics, both in differential and integrated form) to the data. Statistical figures found for each fit are also shown, indicating a specialized transport mechanism for β-alanine.

Type of equation	Working equation	Parameter values (±S.D.)	AIC
Differential	3	V_m = 52.698 (±2.64) mM/h K_m = 12.671 (±0.71) mM	−122.31
	4	k_{ap} = 2.84 (±0.25) h^{-1}	6.05
	5	V_m = 43.578 (±4.44) mM/h K_m = 10.880 (±0.94) mM k_{ap} = 0.190 (±0.10) h^{-1}	−118.30
Integrated	6	V_m = 52.884 (±2.22) mM/h K_m = 12.705 (±0.59) mM A_0 = 47.31 (±0.53) mM	−127.05
	7	k_{ap} = 2.84 (±0.25) h^{-1} A_0 = 73.79 (±7.11) mM	5.89
	8	V_m = 44.598 (±1.08) mM/h K_m = 11.150 (±0.07) mM k_{ap} = 0.186 (±0.07) h^{-1} A_0 = 48.10 (±0.74) mM	−122.93

bined equations to the data in global form (Eqns. 3–8) are shown. In Figs. 1 and 2, graphs plotted according to Eqns. 6 and 7 have been reproduced in order to calibrate, merely by visual inspection, the characteristics of the fits; the plot for Eqn. 8 has not been reproduced because it is very similar to that found for Eqn. 6.

Discussion

Experimental absorption technique

Apart from in vitro methods, intestinal single-pass or recirculation techniques in situ [25–28] have often been used to characterize active absorption mechanisms in

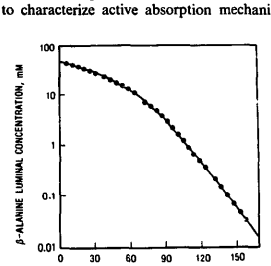

Fig. 1. Semilogarithmic continuous plot representing the β-alanine absorption from rat jejunum, according to the Michaelis-Menten kinetics (Eqn. 6). Parameter values are shown in Table IV.

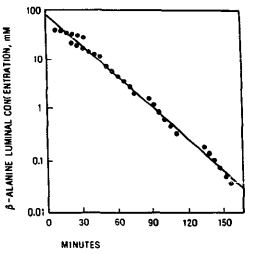

Fig. 2. Same as Fig. 1, but assuming first-order kinetics for absorption (Eqn. 7). The poorness of fit, as compared with that found for Michaelis-Menten kinetics, can be easily guessed by mere visual inspection.

small animals rather than the 'static' perfusion methods like that employed in this study. Recently, Sinko and Amidon [29] have strongly recommended steady-state single-pass perfusion methods to characterize intrinsic Michaelis-Menten kinetics and have explicitly criticized intestinal loop procedures, adducing that the diffusion rate constant of the solute in the aqueous diffusion layer adjacent to the absorbing membrane (k_d) could act as the limiting step for absorption, particularly when the compound is perfused at a very low concentration, that is, when the carrier-mediated absorption process is clearly non-saturated and has turned to first-order kinetics, being, therefore, governed by V_m/K_m, in reciprocal time. This can give rise to biased Michaelis constants when k_d is effectively less than intrinsic V_m/K_m.

In our experimental conditions, we have selected, however, the 'static' perfusion technique for several reasons.

First, it has been demonstrated that intrinsic permeability constants found by both types of methods, when normalized for perfused volumes and intestinal lengths, are virtually identical [30]. Since the procedure used in the present work will allow absorption rates that are several times greater [31], nearer to in vivo values [13] and much more suitable for kinetic calculations [11,12,31], it was selected as routine working procedure in order to characterize better the possible nonlinearities and to fit absorption models more easily to experimental data.

Second, it is true that k_d can actually limit absorption rate constants of some compounds, particularly those which can permeate the lipophilic membrane at high rates, as occurs with highly lipophilic molecules which are passively absorbed [17,31]. But this could not be the case of most actively absorbed compounds, whose

maximal absorption rate constants V_m/K_m, are not sufficiently high to be effectively limited. We have found k_d values as high as 6 h^{-1} by means of the perfusion method described here [14] for series of compounds with a molecular weight higher than that of β-alanine, a compound for which a V_m/K_m value of 4.1 h^{-1} has been found; other substances, such as baclofen or cefadroxil, show V_m/K_m values even lower, i.e., 1.5 and 3.1 h^{-1}, respectively [11,12]. For such substances the limiting effect of the aqueous diffusion layer could be reasonably minimized, thus justifying the use of the 'static' perfusion techniques like that described here.

Third, these latter methods yield, undoubtedly, more realistic absorption rates and would reflect better the in vivo processes [13]. Since our main objective was to determine actual rather than intrinsic Michaelis constants, the use of the reported technique seems to be entirely reasonable.

Absorption kinetics of β-alanine

Relatively recent reports [8,9] claim a nonspecialized absorption mechanism for β-alanine in vitro, because of its nonconcentrative characteristics and the lack of inhibition in the presence of dinitrophenols. Preliminary observations with the in situ rat gut preparation made in our laboratory showed, however, that the intestinal absorption of baclofen – which was clearly characterized as a Michaelis-Menten process [11] – was inhibited by β-alanine, as shown in Fig. 3; while the V_m value of baclofen remained nearly constant in the absence and in the presence of 2.25 mM β-alanine, the K_m value became greater in the latter condition, a phenomenon which could be indicative of a competitive inhibition. Consequently, one can reasonably assume that a common carrier-mediated mechanism accounts for the intestinal absorption of both substances, closely related in chemical structure, and a reevaluation of the absorption kinetics of β-alanine through an in situ technique seemed to be desirable in order to explain the above observations.

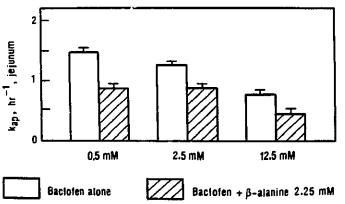

Fig. 3. Comparative absorption rates (as measured by the apparent first-order rate constant, k_{ap}, in the rat jejunum in situ) found for baclofen at variable concentrations, in the absence and in the presence of β-alanine in the perfusion fluids (2.25 mM). In all instances, the differences were significant ($P < 0.001$).

From the experimental results obtained, it becomes evident that β-alanine absorption is not merely a concentration independent process that can be described by first-order kinetics as is generally assumed. Nonlinearities (Table III) and the correlation coefficients shown in Table II for apparent zero-order kinetics emphasize the convenience of fitting the Michaelis-Menten equation to the data [17]. As can be seen from Table IV, statistical AIC figures for first-order global fits are hardly comparable with those obtained for nonlinear model equations. Therefore, passive diffusion kinetics should be discarded as the main absorption pathway for β-alanine. Fig. 2 indicates the poorness of the first-order fit as compared with the nonlinear adjustment shown in Fig. 1.

Concerning nonlinear absorption fits, both differential and integrated equations satisfy the experimental data and provide similar V_m and K_m parameter values. It is difficult, however, to discriminate, from the AIC figures shown in Table IV, the relative goodness of the two nonlinear fits (i.e., Michaelis-Menten and combined kinetics). It is possible, of course, that a passive component contributes to global absorption of β-alanine, but this contribution would probably be of minor importance if compared with the saturable transport mechanism. The limiting value of the latter absorption pathway (i.e., at very low concentration in perfusion fluids, when saturation phenomena are virtually absent, so that k_{ap} should equal V_m/K_m) is about 4.2 h^{-1}, according to Eqns. 3 or 6, and about 4.0 h^{-1} according to Eqns. 5 or 8. Consequently, the passive component (less than 0.2 h^{-1}) would allow only for about 5% of the global absorption at this concentration range; at concentrations near to saturation, probably unusual in practice (as would be 56 mM), the passive component contribution would be of about 14%. Some literature reports have shown that a significant nonsaturable component coexists with active transport in the intestinal absorption of a number of neutral amino acids, such as L-histidine [9] or L-phenylalanine [27], whereas in other instances, such as L-lysine, only the saturable mechanisms seem to be operative [27]. In light of the present results, β-alanine could rather belong, for practical purposes, to the second type of compound.

Another point of interest lies in the high absolute value found for the absorption rate of β-alanine through the rat gut preparation used here as compared with the values found in the in vitro tests [6–9], thus emphasizing the importance of having a good blood supply during the experiments when absorption kinetics have to be fully characterized.

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