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Report

Buccal Absorption. III. Simultaneous Diffusion and Metabolism of an Aminopeptidase Substrate in the Hamster Cheek Pouch

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The simultaneous diffusion and metabolism of the D- and L-isomers of the aminopeptidase substrate, leucine-*p*-nitroanilide (LPNA), were examined *in vitro* in the hamster cheek pouch. L-LPNA was completely hydrolyzed during diffusion across the cheek pouch, whereas D-LPNA crossed the cheek pouch intact. The metabolic barrier appeared to be localized in the epithelium of the cheek pouch. Addition of an aminopeptidase inhibitor, bestatin, to both diffusion cell reservoirs resulted in decreased hydrolysis of L-LPNA. The experimental results were analyzed with a mathematical model which was developed to describe the simultaneous diffusion and metabolism processes. Using this model it was estimated that the rate of diffusion of L-LPNA across the cheek pouch was less than the capacity of the tissue to hydrolyze L-LPNA.

KEY WORDS: simultaneous diffusion and metabolism; mathematical modelling; hamster cheek pouch; leucine-*p*-nitroanilide; bestatin; aminopeptidase.

INTRODUCTION

The buccal mucosa is a potential site of absorption for some compounds that are not well suited for *per os* administration. Factors such as gastrointestinal (GI) irritation, enzymatic or chemical degradation in the gut lumen, the first-pass effect, and low solubility in GI fluids may limit the bioavailability or efficacy of conventional oral formulations. In addition, it may be desirable to administer some drugs to the buccal or other oral mucosae for local action.

The rate and extent of absorption of a compound through the buccal epithelium will be controlled by the rate of diffusion of the compound through the mucosa, and the capacity of the mucosa to metabolize the compound. There have been few reported studies dealing with the drug-metabolizing capacity of the buccal mucosa (1–3). These reports showed that there are significant levels of enzymatic activity in homogenates of oral mucosa, which may present a significant barrier to the delivery of drugs susceptible to enzymatic degradation. While useful as screening tools, results from homogenate studies may have limited applicability to intact tissues. Free access to all cell surfaces and contents in tissue homogenates may lead to overestimation of the enzymatic activity encountered by a compound diffusing through the mucosa. Most importantly, bioavailability by the buccal route is not determined by enzymatic activity alone, but by the relative rates of diffusion and enzymatic reaction.

This report presents our studies of the *in vitro* diffusion and simultaneous enzymatic degradation of the D- and L-isomers of an aminopeptidase substrate, leucine-*p*-nitroanilide (LPNA), in isolated hamster cheek pouch. The results were analyzed using a mathematical model based on Fickian diffusion with first-order chemical reaction. The model is similar to that proposed (4) for diffusion with chemical reaction in the skin, which is mathematically complex. By choosing appropriate experimental conditions, a simpler model was developed for the present work. With the simplified model estimates of the relative importance of the diffusion and metabolism processes in the viable tissue layer were made.

MATERIALS AND METHODS

L-Leucine-*p*-nitroanilide (L-LPNA), bestatin, collagenase (Type I), and *p*-nitroaniline (PNA) were obtained from Sigma Chemical Co. (St. Louis, MO), and D-leucine-*p*-nitroanilide (D-LPNA) was obtained from Serva (Westbury. NY). Sørenson's phosphate buffer (5), pH 7.40, was used in both reservoirs in the diffusion experiments. Solvents used for HPLC were of commercial HPLC grade. Water was deionized and distilled from glass.

A Side-Bi-Side diffusion apparatus was obtained from Crown Glass Co. (Somerville, NJ). The diffusional area was 0.636 cm^2 and the stirring rate was 600 rpm. The temperature and pH were maintained at 37°C and 7.40, respectively. Cheek pouches from male golden hamsters were used in

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diffusion studies. Immediately after sacrifice by CO_2 suffocation, cheek pouches were excised and rinsed with Sørenson's phosphate buffer (pH = 7.40). For epithelial preparations, the epithelial cell layer was isolated using the procedure described by McCoy (7), which involves incubation of the tissue with collagenase. Tissue samples were mounted in the diffusion cell with the mucosal surface toward the donor reservoir. Buffer (3.0 ml) was added to both donor and receiver reservoirs simultaneously. A 10-min preincubation period was allowed before an aliquot of a stock solution of compound was added to the donor reservoir. D-LPNA and PNA stock solutions were prepared in acetonitrile. The volume of the stock solutions added was such that the concentration of acetonitrile did not exceed 1% (v/v). Samples (100 μl) were taken and replaced with the same volume of fresh buffer. An aliquot (50 μl) was injected for HPLC analysis. In experiments with bestatin, the tissues were soaked in solutions of bestatin for 15 min prior to mounting in the diffusion cells. Bestatin was then present in both reservoir solutions at the same concentrations as in the soaking solutions.

Samples were analyzed by HPLC. The HPLC system consisted of a Water Associates (Milford, MA) Model 6000A pump, Model U6K injector, and Model 440 UV absorbance detector. The column was packed by a literature procedure (6) with a stationary phase of Hypersil-OD (4.6 × 150 mm, 5-μm particles) obtained from Shandon (Sewickley, PA). The mobile phase composition was 40:60 acetonitrile:0.05 *M* NaH_2PO_4, pH 3.0, and the flow rate was 1.5 ml/min. Under these conditions, the retention times of D- and L-LPNA and PNA were 3.5 and 2.8 min, respectively, and the sensitivity of the assay for each was about 1 μ*M*.

RESULTS AND DISCUSSION

When solutions of L-LPNA were placed in the donor reservoir of the diffusion cell, no L-LPNA could be detected in the receiver reservoir. However, PNA, a product of hydrolysis of L-LPNA, was observed in both the donor and the receiver reservoirs. The donor and receiver reservoir concentrations of PNA increased linearly with time (Fig. 1). In all cases, the total amount of PNA produced during the experiments was <10% of the initial amount of L-LPNA. The slopes of the PNA reservoir concentration vs time profiles were obtained through linear regression analysis and are referred to in the following discussion as rates of PNA production.

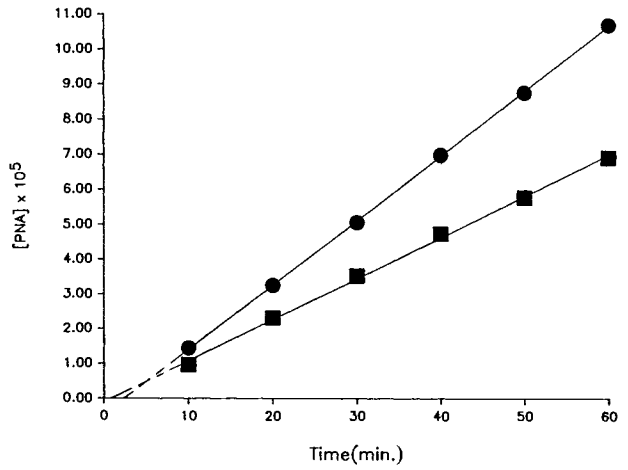

Fig. 1. Typical plot of PNA concentration in donor (●) and receiver (■) reservoirs vs time. Lines represent regression lines.

Table I. Effect of Donor Reservoir Concentration (C_d) of L-LPNA on Rate of PNA Production in the Receiver Reservoir

C_d[a]	Rate of PNA production[b]
5.00	8.61 ± 2.55 (4)
2.50	4.00 ± 1.10 (4)
1.25	2.13 ± 0.96 (3)
0.25	0.267 ± 0.059 (4)

[a] $M \times 10^3$.
[b] Mol/min; mean ± SD × 10^9 for (n) determinations.

The rate of PNA production in the receiver reservoir was found to increase linearly with the initial donor reservoir concentration (C_d) of L-LPNA as summarized in Table I and Fig. 2. The donor reservoir concentrations of PNA were not quantitated in these experiments. The linear increase in PNA production with increasing donor reservoir concentrations of L-LPNA suggests that the metabolic reaction rate is linear with concentration of substrate in this concentration range.

The rates of PNA production were compared in diffusion experiments using either the full-thickness cheek pouch or the epithelial cell layer to determine the location of the aminopeptidase activity within the cheek pouch tissue. Table II shows the values for the rates of PNA production for the full-thickness cheek pouch and the epithelial cell layer. There was no significant difference (Student's t test, $P > 0.05$) in the values of PNA production for the two preparations. Also, when full-thickness cheek pouch or isolated epithelial tissues of equal area were incubated in solutions of L-LPNA (5.00×10^{-5} *M*), the rates of PNA production were 7.20 and 6.90×10^{-10} mol/min (means of two values) for the full-thickness cheek pouch and epithelial cell layer, respectively. These values were not significantly different (Student's t test, $P > 0.05$). PNA production by chemical degra-

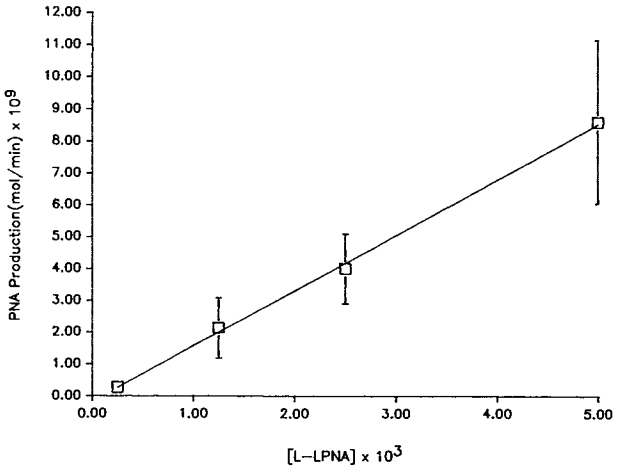

Fig. 2. PNA production in receiver reservoir as a function of C_d. The line represents the regression line ($r^2 = 0.999$) described by

$$y = (1.74 \times 10^{-6}) \cdot X - (1.71 \times 10^{-10}).$$

Table II. Rates of PNA Production from Full-Thickness Cheek Pouch and Epithelial Cell Layer

Membrane	PNA production[a]		
	Donor	Receiver	Total
Full thickness	0.95 ± 0.33 (8)	1.48 ± 0.42 (8)	2.43
Epithelial cell layer	0.620 ± 0.16 (8)	2.09 ± 0.70 (8)	2.71

[a] Mol/min; mean ± SD × 10^9 for (n) determinations.

dation of L-LPNA or hydrolysis of L-LPNA by enzymes that may have escaped from the tissues over the course of the experiment (1 hr) was shown to be negligible in preliminary studies. These results suggest that the enzymatic barrier for L-LPNA is located in the epithelial cell layer of the hamster cheek pouch. Related studies have shown that the physical barrier to diffusion is also in the epithelial cell layer (8).

It has been reported that bestatin is a competitive inhibitor of leucine aminopeptidase (9). Hence, the effect of bestatin on the diffusion and metabolism of L-LPNA was examined. At a bestatin concentration of 100 μM it was found that the rate of PNA production was significantly decreased and low levels of L-LPNA could be quantitated in the receiver reservoir (Table III). This result suggests that an enzyme inhibitor may be able to increase the amount of an enzymatically labile compound diffusing across the oral mucosa.

D-LPNA was used to determine the flux in the absence of metabolism since the physical properties of the D- and L-isomers should be the same and leucine aminopeptidase is reported (10) to be specific for substrates containing L-amino acids at the N-terminal position. The receiver reservoir concentrations of D-LPNA were found to increase linearly with time, suggesting passive diffusion. The flux value is reported as F_u in Table IV.

The results were analyzed using a mathematical model based on Fickian diffusion with first-order chemical reaction, as described in the Appendix. The model is similar to that proposed by Ando *et al.* (4) for diffusion with chemical reaction in the skin. They treated the skin as a two-ply laminate composed of the stratum corneum and the viable epidermis. Such a model is applicable in this case since the hamster cheek pouch is histologically similar to skin, with a thin keratinized layer analogous to the stratum corneum and a thicker viable tissue layer (11,12). However, that model (4) is mathematically complex. In our studies certain experimental conditions were used that made a simpler model appropriate (see Appendix).

Table III. Effect of Bestatin on Rate of PNA Production from Full-Thickness Cheek Pouch

[Bestatin][a]	PNA production[b]		L-LPNA flux[b]
	Donor	Receiver	
0	5.60 ± 0.98 (4)	4.00 ± 1.10 (4)	ND[c]
100	3.09 ± 0.18 (3)	2.34 ± 0.46 (3)	1.47 ± 0.30 (3)

[a] μM.
[b] Mol/min; mean ± SD × 10^9 for (n) determinations.
[c] Not detected.

Table IV. Flux Values[a] Determined in Diffusion Experiments Using Different Cheek Pouch Preparations

Value	Full thickness	Epithelium
F_u	2.07 ± 0.64 (4)	3.00 ± 1.60 (8)
F_d	0.95 ± 0.33 (8)	0.62 ± 0.16 (8)
F_r	1.48 ± 0.42 (8)	2.09 ± 0.70 (8)

[a] Mol/min × 10^9 ± SD for (n) determinations. C_d of L-LPNA = 2.5 × 10^{-3} M.

F_u, the flux of D-LPNA into the receiver reservoir, represents the situation where no metabolism occurs. F_d and F_r, the values for the flux of PNA into the donor and receiver reservoirs, respectively, are obtained from experiments where L-LPNA is metabolized in the cheek pouch tissue. F_u, F_d, and F_r were calculated according to Eqs. (3)–(5) and are summarized in Table IV. With these values, Eqs. (6) and (7) were solved iteratively to give values of αL, which are summarized in Table V. The values of αL range from about 1 to 3, indicating that the capacity of the tissue for metabolism of the substrate is greater than the rate of diffusion of the substrate through the tissue. This result is in agreement with the experimental observation that no intact L-LPNA diffused through the cheek pouch. Although the hamster cheek pouch and human buccal mucosa exhibit some morphological and probably some biochemical differences, the results of these studies suggest that human buccal mucosa may present a significant barrier to diffusion of intact peptides with enzymatic susceptibilities.

The value of $(\alpha L)^2$ is an indication of the magnitude of the ratio of metabolic capacity to diffusion rate. The values of $(\alpha L)^2$ obtained suggest that the metabolic capacity of the tissue may be up to 10 times greater than the diffusion rate of L-LPNA. Due to differences in tissue preparation, values of αL could not be calculated for the enzyme inhibition experiments. However, such information could be obtained with this model, and comparison of values of αL would be useful in determining the utility of enzyme inhibitors to enhance the absorption of enzymatically labile compounds.

APPENDIX

The schematic representation of the experimental system used in development of the mathematical model is shown in Fig. 3. In general, the model describes the transport process as an initial partitioning of LPNA from the donor reservoir into the tissue, followed by simultaneous dif-

Table V. Values of αL Obtained According to Equations (6) and (7)

Membrane	Equation	αL
Epithelial cell layer	6	0.805
Full thickness	6	1.23
Epithelial cell layer	7	2.98
Full thickness	7	3.07

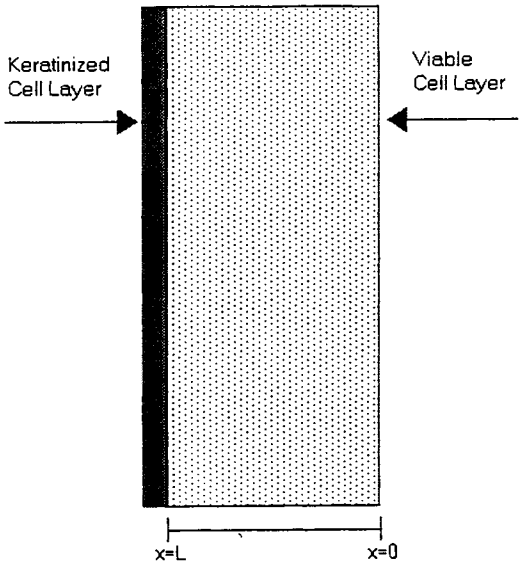

Fig. 3. Schematic representation of the diffusion model.

fusion and metabolism in the viable tissue layer. Diffusion is modelled explicitly only in the viable tissue layer. In addition, the following conditions and assumptions were maintained:

(1) Transport through the keratinized layer is treated as simple partitioning from the donor phase to the viable tissue.
(2) The enzymatic reaction is first-order and occurs only in the viable cell layer. This is supported by the linear dependence of PNA production on initial L-LPNA concentration and the experimental results.
(3) Steady-state conditions exist at all times.
(4) The donor reservoir concentration of LPNA was maintained constant, with ≤10% decrease over the course of the experiments.
(5) Sink conditions exist for LPNA in the receiver reservoir and PNA in both donor and receiver reservoirs.
(6) Metabolic enzymes are distributed homogeneously throughout the viable cell layer.
(7) The subepithelial layers provide no diffusional resistance and are devoid of enzymatic activity, as shown by previous work (1) and the experimental results.

The differential equations describing the diffusion and reaction of reactant (L-LPNA) and product (PNA) within the viable cell layer at steady state are:

$$0 = D_r \cdot d^2C_r/dx^2 - k \cdot C_r \quad (1)$$

$$0 = D_p \cdot d^2C_p/dx^2 + k \cdot C_r \quad (2)$$

where

C_r = [L-LPNA] in viable cell layer
D_r = diffusivity of L-LPNA in viable cell layer
C_p = [PNA] in viable cell layer
D_p = diffusivity of PNA in viable cell layer
k = first-order reaction rate constant
x = position within viable cell layer

The position $x = L$ corresponds to the side of the viable cell layer closest to the donor reservoir, while the position $x = 0$ corresponds to the side closest to the receiver reservoir.

With the model assumptions, the boundary conditions are

at $x = 0$, $C_r = 0$ and $C_p = 0$
at $x = L$, $C_r = P \cdot C_d$ and $C_p = 0$

where

C_d = [L-LPNA] in donor reservoir
P = partition coefficient

P is a composite partition coefficient relating the concentration in the donor reservoir to the concentration in the viable cell layer at $x = L$. It includes partitioning from the donor reservoir into the keratinized layer, transport through that layer and partitioning into the viable cell layer. The value of P is expected to be constant within and between experiments since two experimental conditions were met: (i) the concentration in the donor reservoir was constant within an experiment and was the same for all experiments; and (ii) transport (through the keratinized layer) is at steady state. The latter was assumed since steady-state flux through the whole tissue was observed.

The solutions to Eqs. (1) and (2), subject to the boundary conditions, give the concentrations of reactant and product as a function of position in the tissue. From these results, the flux of product into the donor and receiver reservoirs can be calculated.

Flux of PNA into donor reservoir:

$$F_d = -(D_r \cdot P \cdot C_d/L) \cdot \{1 - (\alpha L) \cdot \coth(\alpha L)\} \quad (3)$$

where

$$\alpha = (k/D_r)^{1/2}$$

Flux of PNA into receiver reservoir:

$$F_r = (D_r \cdot P \cdot C_d/L) \cdot \{1 - (\alpha L) \cdot \operatorname{csch}(\alpha L)\} \quad (4)$$

Flux of D-LPNA into receiver reservoir (no enzymatic reaction):

$$F_u = D_r \cdot P \cdot C_d/L \quad (5)$$

In Eqs. (3)–(5), the dimensionless term (αL) appears. The square of this term is the ratio of the reaction rate, $1/k$, to the diffusion rate, L^2/D_r (i.e., D_r/kL^2). Two different implicit expressions for (αL) can be developed using Eqs. (3)–(5). They are

$$(F_u + F_d)/F_u = (\alpha L) \cdot \coth(\alpha L) \quad (6)$$

$$(F_u - F_r)/F_u = (\alpha L) \cdot \operatorname{csch}(\alpha L) \quad (7)$$

Determination of values for F_u, F_d, and F_r under the appropriate experimental conditions allowed estimations of αL by iteratively solving Eqs. (6) and (7). Values of $(\alpha L)^2$ give an indication of the magnitude of the ratio of metabolic activity to diffusion rate. The calculation of αL by Eq. (6) involves determining the difference between two small numbers (F_u and F_d). For this reason there is more error involved in calculation of αL by Eq. (6) than by Eq. (7).

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