International Journal of Pharmaceutics, 62 (1990) 259–267
Elsevier

IJP 02110

Comparison of enzymic activities of tissues lining portals of drug absorption, using the rat as a model

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(Received 21 September 1989)
(Modified version received 31 January 1990)
(Accepted 8 February 1990)

Key words: Enzymic activity; Drug delivery; Aminopeptidase; Tissue esterase; Peptide delivery; Prodrug delivery

Summary

Leucine aminopeptidase and cholesteryl esterase activities of five different tissues from the rat are investigated. The tissues from the intestines, the buccal cavity, the nose, the rectum and the skin were chosen, as they represent barriers to absorption for most commonly used dosage forms. It is shown that the intestinal tissue is the most active in both enzymes. Dermal tissues are the second richest in cholesteryl esterase activity, whereas rectal tissue is the least active. Aminopeptidase activity is weakest in dermal tissues. When adjusted for protein content, dermal tissues show the highest specific esterase activity. Rectal tissue remains the least active. Based on the known relatively high permeability of buccal and nasal tissues, the low aminopeptidase and cholesterol esterase activities would suggest that those routes offer better possibilities for delivering peptides and esters. However, it should be noted that enzymic degradation is only one of the barriers to peptide absorption, and for most peptide drugs an absorption enhancer or a specific delivery system is still likely to be required for successful trans-nasal or buccal absorption.

Introduction

For a drug to be active, it must clearly reach its site of action. Except for drugs intended to have a purely external action, such as parasiticides and emollients, passage through an absorption barrier is a pre-requisite step. The efficacy of that step will depend on the physicochemical qualities of that barrier as well as its metabolic activity. Polar drugs, for example, are often poorly absorbed, whereas peptide drugs are usually mostly degraded before the systemic circulation is reached. These difficulties have led to increased research effort being directed towards enhancing the absorption of drugs, a task rendered even more urgent with the increasing availability of peptide and/or poorly absorbable drugs. If the effort is to succeed, there is a need to understand better the absorption barriers, and the present study is an attempt to make a contribution in that direction.

It is known that different possible routes of drug entry into the systemic circulation present different challenges to the drug moiety. For example, vasopressin can be effectively absorbed intranasally, but oral absorption is poor (Lundin and Vilhardt, 1986). Similarly, oral absorption of many drugs is, by common experience, highly

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efficient but transdermal absorption is poor. The present work was directed towards comparing the relative enzymic activities of tissues from different portals of entry using the rat as a model. The focus was placed on peptidase and esterase activities, the latter because of the importance of ester prodrugs (Roche, 1977; Higuchi et al., 1983; Chan and Li Wan Po, 1989) and the former because of the urgent need to obtain improved methods for the delivery of peptide drugs.

Materials and Methods

L-Leucine-β-naphthylamide dihydrochoride, *p*-dimethylaminobenzaldehyde (PDAB), (β-naphthylamide dihydrochloride), *p*-nitrophenyl acetate and *p*-nitrophenol were obtained from Sigma (Poole, U.K.), and HPLC methanol used in this work was purchased from FSA Laboratory Supplies (Loughborough, U.K.). Folin & Ciocalteu's phenol reagent was obtained from BDH (Poole, U.K.). Bovine albumin (fraction V powder, 96–99% albumin) was purchased from Sigma. The other reagents and chemicals were analytically pure grades.

Instruments

Absorbance measurements were made with a recording spectrophotometer (PU 8720 UV/VIS scanning spectrophotometer with Video-RGB Monitor CM 8533) equipped with a printer/plotter (C Philips, Model).

Preparation of tissue homogenates

The rats weighing 350–415 g were allowed free access to water prior to the experiments, but were deprived of food for 24 h prior to the study. Buccal, nasal, intestinal, dermal and rectal tissues were excised from the freshly killed animals, and the underlying muscles and fat were carefully removed where appropriate.

The tissues were blotted dry on tissue paper, weighed and placed in a ground-glass homogenizing tube with an appropriate volume of ice-cold physiological saline to produce a 10% (w/v) tissue homogenate suspension. Homogenization was carried out at 10000 rpm at 4–8 °C and centrifuged at $7500 \times g$ at 5 °C for 15 min. The supernatant was stored at −18 °C until required for analysis which was always within 1 week. During this period of storage no significant decrease in enzymic activity could be detected.

Determination of leucine aminopeptidase activity

The method used was that developed by Goldberg and Rutenberg (1958) as modified by Takenaka and Takahashi (1962). L-Leucine-β-naphthylamide hydrochloride was used as the substrate. Hydrolysis leads to the formation of β-naphthylamine which is subsequently coupled with *p*-dimethylaminobenzaldehyde to produce a highly coloured compound which can be quantified by absorbance measurement at 465 nm. Absorbance was assumed to be directly related to concentration but linearity was checked by using a volume of tissue homogenates ranging from 20 to 150 μl. For intestinal homogenates, smaller volumes in the range of 10 to 50 μl were used for constructing the calibration curves owing to higher enzymic activity in the intestines.

Procedurally, 0.5 ml of a 1.37 mM solution of leucine-β-naphthylamide hydrochloride was preincubated in a test tube at 37 °C. A suitable predefined volume of tissue homogenate was then added to be followed by pH 7.0, 0.1 M phosphate buffer to a volume of 1.2 ml. The mixture was incubated at 37 °C for 30 min and the reaction quenched with 2 ml of 0.2 M hydrochloric acid. Subsequently, 2 ml of a 4% (w/v) dimethylaminobenzaldehyde solution were added, and the mixture vortexed prior to a further 10 min incubation at 37 °C. The absorbance of the solution was then read. For the control, physiological saline was used instead of the tissue homogenates.

Determination of cholesterol esterase activity

Cholesterol esterase activity was measured by the method developed by Sih et al. (1963), with some minor modifications as described below. A suitable volume of supernatant from the tissue homogenate was added to a spectrophotometric cell containing 100 μg of *p*-nitrophenyl acetate in 0.1 ml of methanol and 0.1 M, pH 7.0 phosphate

buffer to a volume of 3 ml, including the volume of enzymic solution. The reference cell contained 2.9 ml of 0.1 M, pH 7.0 phosphate buffer and 0.1 ml of methanol containing 100 μg of *p*-nitrophenyl acetate. The absorbance of the test solution relative to the control was monitored at 400 nm at room temperature to measure the concentration of *p*-nitrophenol formed at 90 s post-addition of enzyme. Calibration curves were constructed using different volumes of enzyme solution. For the tissue homogenates, volumes of supernatant ranging from 100 to 500 μl were used, except for intestinal tissue homogenate (12.5–125 μl) and rectal tissue homogenate (25–200 μl) to adjust for differential activities.

Determination of protein content

Protein concentration in the supernatant solutions was measured by the method proposed by Lowry et al. (1951), using bovine serum albumin as reference compound. 0.2 ml of enzyme-containing supernatant solution was added to 5 ml of 0.1 M sodium hydroxide solution containing 0.1% sodium bicarbonate, 0.0005% copper sulphate pentahydrate and 0.001% potassium tartrate. The resulting solution was thoroughly stirred and allowed to stand for 10 min at 25 °C. 0.5 ml of Folin-Ciocalteau reagent was then added, and the solution mixed and incubated at 25 °C for 30 min. The absorbance was read at 500 nm, and the concentration of the protein calculated by interpolation onto the calibration curve.

Statistical analysis

One-way analysis of variance (ANOVA) was used for testing the null hypothesis of no difference in cholesterol esterase activity, aminopeptidase activity or protein concentration in the tissue homogenates. Two-way analysis of variance, using an additive model with animal number and tissue as factors, indicated no significant inter-animal difference in enzyme or protein concentrations at a significance probability of 0.05.

When ANOVA led to rejection of the null hypothesis, multiple-range testing was carried out using the Neuman-Keul method (Elliott, 1986).

Results and Discussion

Figs 1 and 2 illustrate the linear calibration curves observed, using different volumes of supernatant solution for the tissue homogenates for both leucine aminopeptidase and cholesteryl esterase activities thus validating the use of absorbance as an indirect measure of enzymic activities. Figs 3–5 illustrate the different enzymic activities and protein concentrations observed in the five different tissue homogenates. It is clear from Fig. 4 that cholesteryl esterase activity is highest in the intestinal tissue, a result which is in agreement with the data reported by Garren and Repta (1988) who compared buccal and intestinal tissues only. Table 1 gives the ANOVA table for the comparison of esterase activity while Table 2 gives the results of the multiple range test. The data would indicate that the nasal, buccal and dermal tissues have the same cholesterol esterase activities but

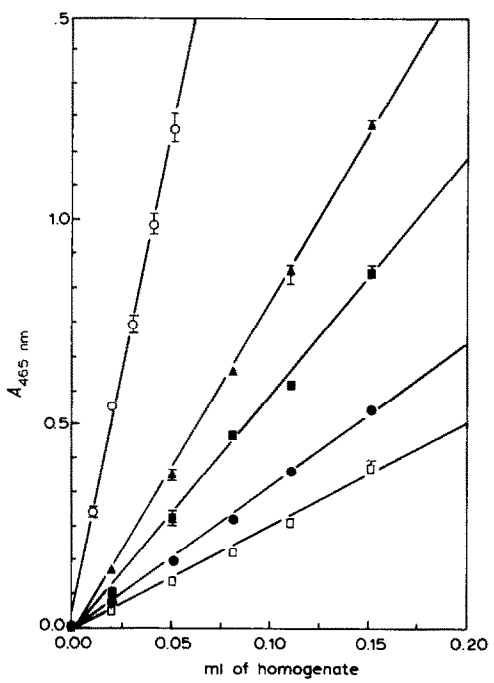

Fig. 1. Typical plots for the determination of aminopeptidase activity in the tissue homogenates. □, dermal ($y = -0.0077 + 2.5949x$; $R = 0.9967$); ●, nasal, ($y = -0.0103 + 3.5799x$; $R = 0.9992$); ■, buccal ($y = -0.0152 + 5.8572x$; $R = 0.9982$); ▲, rectal ($y = -0.0203 + 8.2225x$; $R = 0.9993$); and ○, intestinal ($y = 0.0629 + 23.1870x$; $R = 0.9994$).

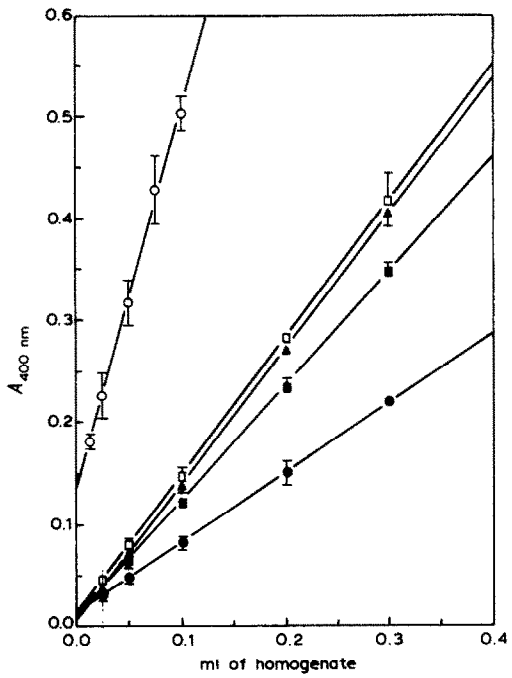

Fig. 2. Typical plots for the determination of cholesterol esterase activity in the tissue homogenates. □, dermal ($y = 0.013 + 1.3539x$; $R = 0.9973$); ▲, nasal ($y = 5.4712e-3 + 1.3357x$; $R = 0.9997$); ■, buccal ($y = 8.6010e-3 + 1.1348x$; $R = 0.9991$); ■, rectal ($y = 1.2743e-2 + 0.6923x$; $R = 0.9966$); ○, intestinal ($y = 7.3549e-2 + 4.5343x$; $R = 0.9699$).

higher than that of rectal tissues and lower than that of intestinal tissues.

Fig. 5 and Table 3, however, show that the protein contents of the different tissue extracts were different with the intestinal tissue being particularly rich in protein. The nasal and dermal tissues had the same amounts but less than buccal, rectal and intestinal tissues in that respective order (Table 4). When the cholesteryl esterase activity is standardised in relation to protein content (absorbance per mg of protein), the tissues behave as different populations (Tables 5 and 6 and Fig. 6) with the rectal tissues having the lowest specific activity, followed by intestinal and buccal tissue as a second population of higher activity, and by nasal tissues and dermal tissues, respectively. Standardisation with respect to protein content helps to isolate the contribution made by the esterase to the total protein content. The high specific cholesteryl esterase activity of the skin is interesting and may well reflect an important biological function.

It is known that sebum and sebaceous secretions have high levels of cholesteryl esters (Yardley, 1983; Williams and Elias, 1987). Steroidal esters are major components of lanolin and wool fat. This observation has potentially important implications, since if the differences in enzymic activities of the different tissues are mirrored in human subjects then the use of ester produgs for transdermal delivery can be rationalised further. Indeed, some earlier work on corticosteroid esters for topical application have indicated that by stereochemical manipulation, it is possible to signifi-

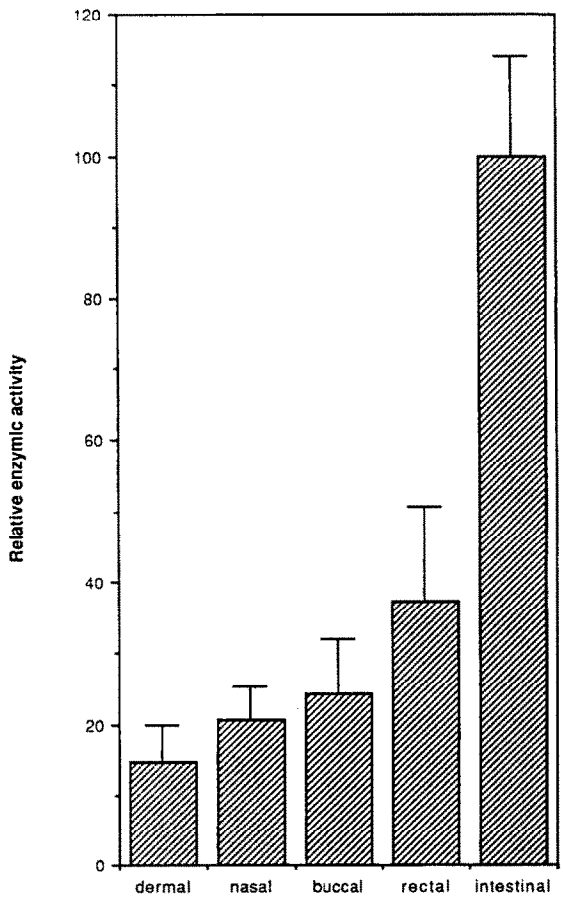

Fig. 3. A comparison of the aminopeptidase activity (mean ± SD, $n = 5$) of different tissues from the rat. The enzymic activity of intestinal tissue homogenate is taken as 100%.

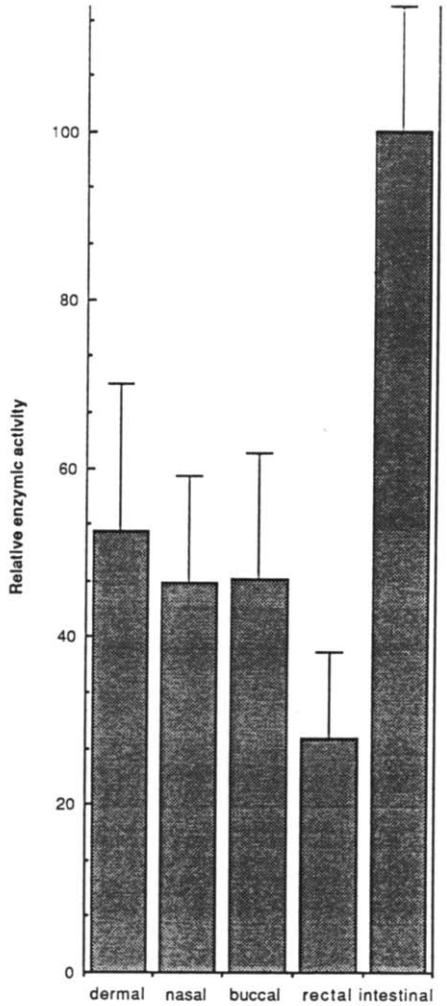

Fig. 4. Comparative cholesterol esterase activity (mean ± SD, $n = 5$) of different tissues from the rat (the enzyme activity of intestinal tissue homogenate is taken as 100%).

TABLE 1

ANOVA table for testing for differences in crude esterase activities of different tissues from the rat

Animal: rat	Analysis of variance table				Cholesterol esterase (uncorrected)
Source	d*f*	SS	MS	*F*	
Factor (tissue type)	4	0.82	0.20	38.68	< 0.001
Residual	70	0.37	0.01		
Total	74	1.19			

TABLE 2

Multiple-range test for cholesteryl esterase activity of different tissues of the rat

Population no.	Cholesterol esterase activity (uncorrected) of tissue				
	Rectal	Nasal	Buccal	Dermal	Intestinal
1		----------			
2	------				
3					--------

cantly alter the potency of the parent steroids (Cheung et al., 1985a,b). With betamethasone valerate, for example, the 17-ester is about 15 times as active as the 21-ester (McKenzie and

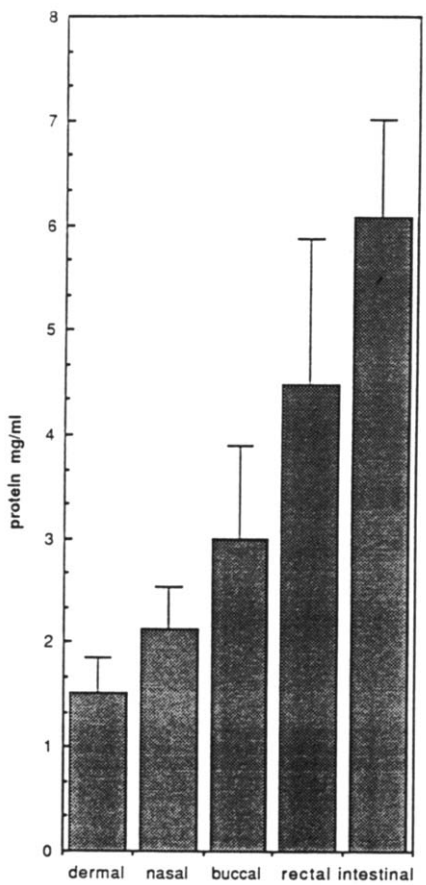

Fig. 5. Protein concentration in the different tissue homogenates. (The protein concentration was determined by using the Lowry et al. (1951) method. Each point is expressed as mean ± SD ($n = 5$).)

TABLE 3

ANOVA table for testing for differences in the mean protein enzymic contents of different tissues in the rat

Animal: rat	Analysis of variance table				Protein concentration
Source	d*f*	SS	MS	*F*	Approx. *P*
Factor (tissue type)	4	7.31	1.83	56.33	< 0.001
Residual	70	2.27	0.03		
Total	74	9.58			

TABLE 4

Multiple-range test for differences in mean protein concentration of different tissues from the rat

Animal: rat Population no.	Protein concentration in tissue				
	Dermal	Nasal	Buccal	Rectal	Intestinal
1	----------				
2			-------		
3				-------	
4					---------

TABLE 5

ANOVA table for comparing esterase activity of different tissues from the rat

Animal: rat	Analysis of variance table				Cholesterol esterase
Source	d*f*	SS	MS	*F*	Approx. *P*
Factor (tissue type)	4	4.39	1.10	36.30	< 0.001
Residual	70	2.11	0.03		
Total	74	6.50			

TABLE 6

Multiple-range test for cholesteryl esterase activity of different tissues from the rat, normalised for protein content

Population no.	Cholesterol esterase activity (corrected by protein concentration) in tissue				
	Rectal	Intestinal	Buccal	Nasal	Dermal
1		----------			
2	-------				
3				------	
4					-------

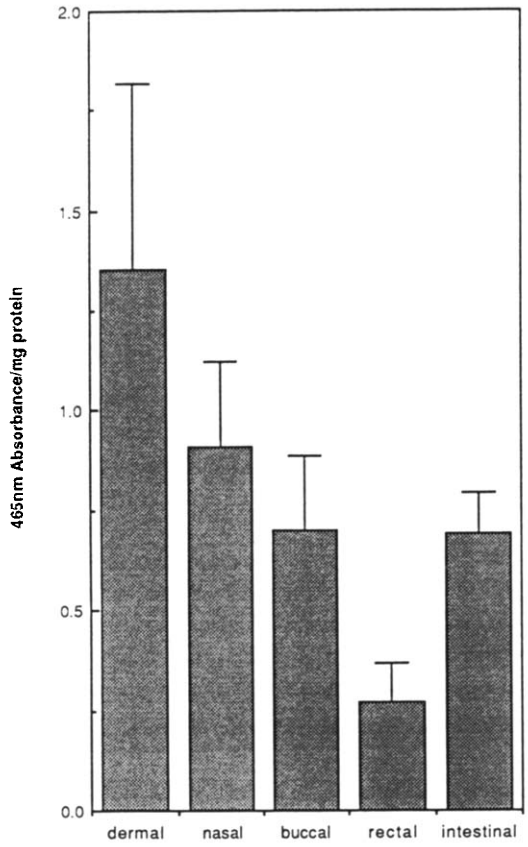

Fig. 6. Relative cholesterol esterase activity of five tissue homogenates from the rat. (Each column is expressed as mean ± SD ($n = 5$).)

Atkinson, 1964) and hydrocortisone 17-butyrate is as active as fluocinolone acetonide (McKenzie and Stoughton, 1962).

Analysis of the aminopeptidase data provides equally interesting information. ANOVA (Table 7) shows that there is clearly a difference in the leucine-aminopeptidase activities of the different tissues in the rat. The crude activity values indicate that the intestinal tissues were the most and the dermal tissues the least active. Nasal and buccal tissues had the same activity which is higher than dermal tissues but lower than rectal tissues (Table 8). Again, standardisation with respect to protein content changes the order of activity of the different tissues (Fig. 7 and Table 9). Intestinal tissue was, however, still the most active. The activities of buccal and rectal tissues formed a

TABLE 7

ANOVA table for testing for differences in crude aminopeptidase activities of different tissues from the rat

Animal: rat	Analysis of variance table				Aminopeptidase (uncorrected)
Source	d*f*	SS	MS	*F*	Approx. *P*
Factor (tissue type)	4	8.80	2.20	341.13	< 0.001
Residual	70	0.45	0.01		
Total	74	9.25			

single population. Rectal tissues could in turn not be separated from dermal and nasal tissues in their relative leucine aminopeptidase activities per unit of protein (Table 10). The results reported here contrast with those reported by Stratford and Lee (1986) who found little difference in the aminopeptidase activities of the various non-oral mucosal tissues in their work on the albino rabbit tissues, but were consistent with those of Garren and Repta (1988). The latter authors commenting on those results indicated that inter-species differences and use of different substrates may be involved. Subsequent work by Lee et al. (1987) showed that the substrate may determine whether tissue differences are observed or not. Indeed Dodda Kashi et al. (1986) showed that using enkephalins the order of tissue activity was rectal > nasal > buccal > vaginal. These aspects will clearly need further investigation.

The data reported here should be useful for predicting absorption efficiency of peptides and esters through the different portals of entry studied. The crude enzymic activities would suggest that the intestinal route presents the most

TABLE 8

Multiple-range test for differences in crude aminopeptidase activities of different tissues from the rat

Population no.	Aminopeptidase activity (uncorrected) in tissue				
	Dermal	Nasal	Buccal	Rectal	Intestinal
1		----------			
2	-------				
3				-------	
4					--------

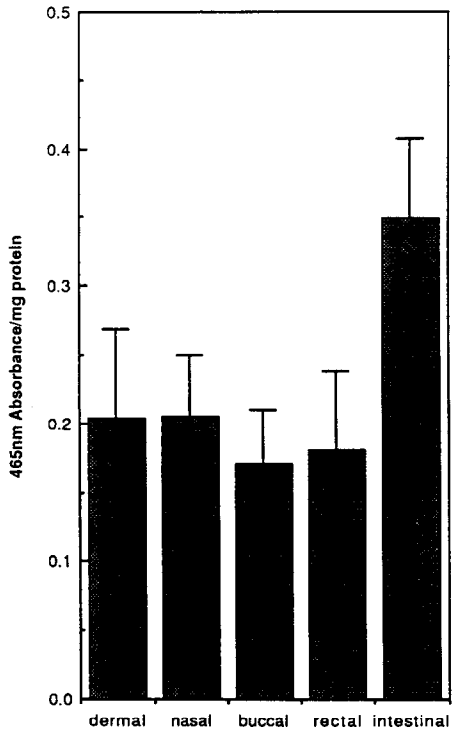

Fig. 7. Relative aminopeptidase activities of five tissue homogenates from the rat. (Each column is expressed as mean ± SD ($n = 5$).)

formidable enzymic barrier to the absorption of peptides. On the other hand the skin will present the weakest barrier in this respect. However, since the skin is relatively impermeable (Elias, 1988), the nasal route appears to be the best choice for delivering peptides, at least in the rat. The buccal and rectal routes hold intermediate positions. In-

TABLE 9

ANOVA table for testing for differences in standardised aminopeptidase activities of different tissues from the rat

Animal: rat	Analysis of variance table				Aminopeptidase (corrected for protein concentration)
Source	d*f*	SS	MS	*F*	Approx. *P*
Factor (tissue type)	4	2.90	0.72	50.15	< 0.001
Residual	70	1.01	0.01		
Total	74	3.91			

TABLE 10

Multiple-range test for difference in aminopeptidase activity of different tissues from the rat

Population no.	Aminopeptidase activity (corrected by protein concentration) in tissue				
	Buccal	Rectal	Dermal	Nasal	Intestinal
1		----------			
2	----------				
3					--------

deed, several reports have high-lighted the value of the nasal route for delivering peptides when the oral route is inefficient (Moses et al., 1983; Su et al., 1985; Mishima et al., 1987). For the esters, the rectal route appears to be the most promising (Table 2). The nasal, buccal and dermal routes represent intermediate barriers, whereas the intestinal route is again the most hostile. The dermal route has obvious disadvantages, not least is its poor permeability. However, when transdermal administration is possible, this route is ideal and, indeed, despite the limitations, the fact that administration can be stopped at will means that it will continue to attract much research interest. In our study, tissue homogenates were used for convenience and reproducibility. Such homogenates do not necessarily directly mirror the type and concentration of enzymes which a drug is exposed to during transport through the absorption barriers. Enzymes may be both cytosol-bound and enzyme-bound (Lee, 1988). Therefore allowance must be made when extrapolating our data to in vivo absorption. Nonetheless, our data should be useful first iterations in understanding peptide absorption.

Conclusion

The search for improved methods for peptide delivery is an important one. In addition to increasing permeability and transport through use of absorption enhancers (Ichikawa et al., 1980) or iontophoretic methods (Burnette et al., 1988), sequestration using surfactant molecules (Ziv et al., 1987) has also been tried. Inhibition of enzymic break-down either by introducing less labile peptide bonds or by enzyme inhibitors, also holds promise. However, for this avenue to be optimised an improved understanding of the peptidase barriers and their localisation in the body is important. The present work will hopefully be seen as a contribution in this respect within the clear constraints discussed above and by various authors.

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