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Prodrugs of peptides. 8. In vitro study of intestinal metabolism and penetration of thyrotropin-releasing hormone (TRH) and its prodrugs

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Summary

The feasibility of improving the poor oral bioavailability of the tripeptide TRH (pGlu-His-Pro-NH_2) was examined using the prodrug approach. The prodrugs studied were various *N*-alkoxycarbonyl derivatives formed by reacting TRH with the appropriate chloroformates at its imidazole moiety. In vitro metabolism studies with rabbit and rat intestinal homogenates showed that TRH and the prodrugs were rapidly degraded in the rabbit homogenate by virtue of prolyl endopeptidase which cleaves the C-terminal proline amide moiety. Whereas TRH showed a high stability in rat gut homogenates, the prodrug derivatives were readily degraded, partly due to prolyl endopeptidase and partly due to non-specific esterases. In vitro penetration studies using the modified Ussing chamber showed that the prodrugs did not improve the penetration of TRH across the jejunal, ileal and colonic segments of the rat. It is concluded that although the prodrugs are much more lipophilic than TRH in terms of octanol-buffer partition coefficients, the greater susceptibility of the derivatives to undergo enzymatic degradation more than offsets the improved lipophilicity characteristics. Prodrugs suitable for improving the oral absorption of TRH should not only possess a certain lipophilicity, but should also be resistant towards the prolyl endopeptidase enzyme.

Introduction

In recent years, thyrotropin-releasing hormone (TRH) (pGlu-L-His-L-Pro-NH_2) has attracted much attention as a potential drug for the management of various neurologic and neuropsychiatric disorders including depression, brain injury, acute spinal trauma, schizophrenia and Alzheimers disease (for reviews see Jackson, 1982; Metcalf, 1982; Griffiths, 1985, 1986, 1987; Horita et al., 1986; Loosen, 1988; Metcalf and Jackson, 1989). However, the clinical utility of TRH is hampered by its rapid enzymatic inactivation in the blood and clearance as well as by its poor access to the brain (Metcalf, 1982; Hichens, 1983; Griffiths, 1987; Loosen, 1988). The lipophilicity of TRH is very low (Bundgaard and Møss, 1990) and this may be a primary reason for the limited ability of the peptide to penetrate the blood-brain barrier (Nagai et al., 1980; Banks and Kastin, 1985; Zlokovic et al., 1988).

We have recently reported that these problems of rapid enzymatic inactivation and poor lipophilicity of TRH may be overcome by bioreversible derivatization of the peptide (Bundgaard and Møss, 1990). The derivatives developed are *N*-alkoxy-

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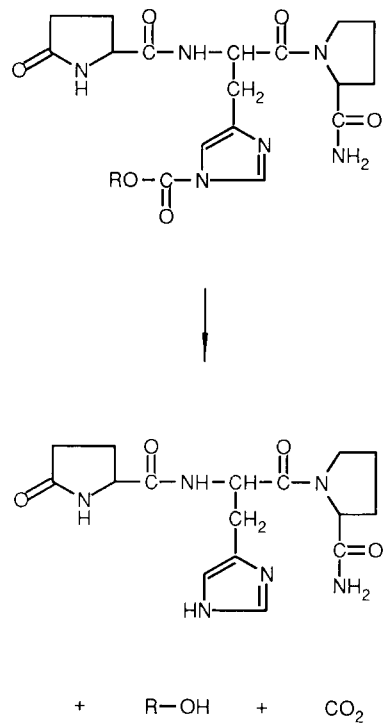
carbonyl derivatives of TRH formed by *N*-acylating the imidazole group of the histidine residue with various chloroformates. These derivatives are totally resistant to cleavage by the TRH-inactivating pyroglutamyl aminopeptidase serum enzyme, but are readily bioreversible as the parent TRH is formed quantitatively from the derivatives by spontaneous hydrolysis or by plasma esterase-catalyzed hydrolysis (Scheme 1). Besides by protecting the parent TRH against inactivation in plasma and hence being potentially useful to prolong the duration of action of TRH in vivo, the *N*-alkoxycarbonyl prodrug derivatives possess greatly increased lipophilicity relative to TRH as assessed by octanol-buffer partition experiments (Bundgaard and Møss, 1990). This property may render the prodrug forms more capable of penetrating the blood-brain barrier or various other biomembranes than the parent peptide. Thus, we have recently reported that while TRH does not penetrate human skin in vitro to any measurable extent, the lipophilic *N*-octyloxycarbonyl derivative (**VI**) of TRH shows a high penetrating capacity (Møss and Bundgaard, 1990b). The favourable skin penetration properties of this derivative along with its ready conversion to the parent TRH during or following skin penetration suggested the utility of the prodrug for transdermal delivery of TRH.

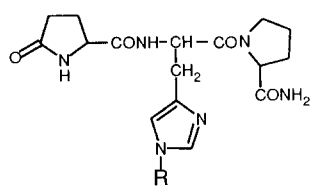
The objective of the present study was to determine whether these prodrugs (**I–IX**) could be utilized to improve the oral bioavailability of TRH. Although orally administered TRH has been reported to enhance thyroid stimulating hormone release in man (Haigler et al., 1972; Rabello et al., 1974; Schurr et al., 1985), the bioavailability is only about 1–2% (Yokohama et al., 1984a; Duntas et al., 1988). In rats a similarly low oral bioavailability (0.2–1.5%) has been found whereas a somewhat higher extent of absorption (4–13%, depending on the dose) is seen in dogs (Yokohama et al., 1984a). Evidence has been provided for the implication of a carrier-mediated transport system in the absorption of TRH in rats and dogs (Yokohama et al., 1984b).

The very low lipophilicity of TRH may be a main reason for its poor passive transport across the intestinal membrane. The logarithmic value of its partition coefficient (P) between octanol and pH 7.4 buffer solution is only −2.46 (Bundgaard and Møss, 1990) and this value is far from being favourable for gastrointestinal absorption (Yalkowski and Morozowich, 1980; Dressman et al., 1985). Enzymatic degradation of the peptide at the absorption site may also contribute to the poor bioavailability although Yokohama et al. (1984b) have reported that TRH is stable in the presence of gastrointestinal proteolytic enzymes and in rat intestinal homogenates.



Scheme 1.

Since the *N*-alkoxycarbonyl prodrug derivatives of TRH are much more lipophilic than the parent peptide in terms of octanol-buffer partition coefficients (Fig. 1), it was thought that such prodrugs may be capable of improving the gastrointestinal absorption of TRH. To assess this possibility we have examined the in vitro penetration characteristics of TRH and some of the prodrugs across isolated intestinal segments of the rabbit and rat using the Ussing chamber technique. Furthermore, the stability of the compounds in intestinal homogenates has been determined.



		log P
TRH	R = H	-2.46
I	R = $C(=O)-OCH(CH_3)_2$	-0.80
II	R = $C(=O)-OC_4H_9$	-0.47
III	R = $C(=O)-OCH_2CH(CH_3)_2$	-0.44
IV	R = $C(=O)-OC_6H_{13}$	0.71
V	R = $C(=O)-O$-cyclohexyl	0.60
VI	R = $C(=O)-OC_8H_{17}$	1.88
VII	R = $C(=O)-OCH_2CH(C_2H_5)C_4H_9$	1.82
VIII	R = $C(=O)-OCH(C_2H_5)_2$	0.20
IX	R = $C(=O)-OCH(C_3H_7)_2$	1.20

Fig. 1. Structures and logarithmic partition coefficients (P) of TRH and its prodrug derivatives. P is the partition coefficient between octanol and aqueous buffer of pH 7.4. The log P values for TRH and compounds **I–VII** are experimental values (Bundgaard and Møss, 1990) whereas the values for the compounds **VIII** and **IX** are calculated on basis of the π substituent values (Hansch and Leo, 1979).

Materials and Methods

Chemicals

TRH was obtained from Carlbiotech A/S, Copenhagen. TRH-OH (*p*Glu-His-Pro) and bacitracin were purchased from Sigma, St. Louis, U.S.A. The *N*-alkoxycarbonyl-TRH derivatives **I–X** were prepared by reacting TRH with the appropriate chloroformate as previously described (Bundgaard and Møss, 1990). *N*-Benzyloxycarbonyl-glycyl-L-prolyl-L-alanine (Z-Gly-Pro-Ala) and *N*-benzyloxycarbonyl-glycyl-L-proline (Z-Gly-Pro) were purchased from Bachem, Switzerland.

Intestinal penetration studies

Male Sprague-Dawley rats, weighing 300–350 g, were killed and segments of the jejunum, ileum and colon were excised and freed of the underlying serosal tissues. The isolated segments were mounted in a modified Ussing chamber exposing a surface area of 0.50 cm^2. Then 5.0 ml of a glutathione bicarbonate Ringer's (GBR) solution (Hull et al., 1974), pre-adjusted to pH 7.4 and having an osmolality of 300 ± 15 mosM, was added to the reservoir bathing the serosal side. An equal volume of GBR solution (pH 7.4) containing TRH or prodrug in a concentration of 3 mM was thereafter added to the mucosal side. Mixing in each chamber was achieved by bubbling a mixture of 95% O_2/5% CO_2 at a rate of two to four bubbles per s. The temperature was maintained at 37°C. At predetermined times, 200 µl samples were taken from the serosal side and immediately replaced by an equal volume of GBR solution. In order to stabilize the prodrugs against degradation, 200 µl of a 0.15 M zinc sulphate solution (pH 5.0) was added and the samples were analyzed by HPLC as described below.

Penetration studies using intestinal segments of the albino rabbit were performed in a similar way.

Stability of TRH and its prodrugs in gut homogenates

The intestinal segments isolated from rats or rabbits as described above and comprising jejunum, ileum and colon were homogenized in a 0.05 M phosphate buffer solution (pH 7.4) containing 0.15 M sodium chloride to provide a 1:5 dilution (w/v). An Ultra-torrax TP-18/10 homogenisator was used while maintaining the temperature at 2–5°C. The homogenates were centrifuged at 1000 rpm at 5°C for 10 min and the supernatants obtained used for the hydrolysis studies.

TRH and its prodrugs were incubated at 37°C in the gut homogenates at an initial concentration of 10^{-4} M. At various intervals samples of 250 µl were withdrawn and added to 250 µl of a 0.1 M solution of zinc sulphate in order to deproteinize the samples. After immediate mixing and centrifugation at 13000 rpm for 3 min, 20 µl of the clear supernatant was analyzed by HPLC as described below.

HPLC assays

TRH and its derivatives were determined by reversed-phase HPLC procedures. In the stability studies the HPLC system used consisted of a Kontron 420 HPLC pump, a Kontron 432 LC detector operated at 215 nm and a Rheodyne 7125 injection valve with a 20-μl loop. A reversed-phase Supelcosil LC-8-DB column (33 × 4.6 mm) containing 3 μm particles and protected with a Supelguard LC-8-DB precolumn (both from Supelco Inc., U.S.A.) was used for the assay of the TRH prodrugs. The column was eluted at 1.0 ml min^{-1} with mixtures of acetonitrile and 0.1% v/v phosphoric acid, the concentration of acetonitrile (10–40% v/v) being adjusted for each compound to give an appropriate retention time (3–8 min). For the analysis of TRH as well as TRH-OH a ChromSep column (100 × 4.6 mm) packed with Microspher C-18 (3-μm particles) and supplied with a Chrompack guard column (both from Chrompack) was eluted at ambient temperature with a mobile phase consisting of methanol-0.1% phosphoric acid (2:98 v/v), the flow rate being 1 ml min^{-1}. It was ensured that the various degradation products of TRH (Møss and Bundgaard, 1990a) did not interfere with the determination of TRH using this HPLC procedure. Quantitation of the compounds was done by measuring the peak heights in relation to those of standards chromatographed under the same conditions.

In the intestinal penetration studies the HPLC system used consisted of a Merck Hitachi apparatus comprising a pump model L-6200, a variable UV-detector L-4200 operated at 220 nm and an autosampler model 655A-40 thermostated at 5°C. Data acquisition and processing was performed with a Merck Hitachi HPLC-Manager model D-6000. The column used was a ChromSep column as specified above and it was eluted using a gradient procedure with a mixture of acetonitrile and 0.1% phosphoric acid containing 5×10^{-3} M triethylamine. From 0 to 9 min the concentration of acetonitrile was increased linearly from 2 to 50%; from 9 to 13 min the concentration was decreased from 50 to 2%, the latter concentration being maintained for additional 10 min. Quantitation of the compounds was done by measuring peak areas in relation to those of standards chromatographed under the same conditions. The sensitivity of the assay was about 0.5 μg ml^{-1} of TRH and its prodrugs.

Results and Discussion

Stability of TRH and its prodrugs in gut homogenates

The stability of TRH and the *N*-alkoxycarbonyl derivatives **I–IX** was determined in 20% rabbit and rat intestinal homogenates. Under the experimental conditions used the disappearance of the compounds followed first-order kinetics as illustrated by the plots in Fig. 2. The observed half-lives for the degradation are listed in Table 1 along with half-lives in pure buffer solution of pH 7.4.

Considerable differences are seen between the stability of the compounds in rabbit and rat gut homogenates, the rabbit tissue being most degradative. The species variation is in particular apparent for TRH in that the compound showed less than 5% degradation after incubation for 6 h in the rat gut homogenate whereas a half-life of only 10 min was observed for the gut homogenate of the rabbit. The high stability of TRH in the rat gut homogenate is in agreement with similar findings by Yokohama et al. (1984b) and Safran et al. (1982), but in disagreement with those by Brewster et al. (1981). The latter authors reported a half-

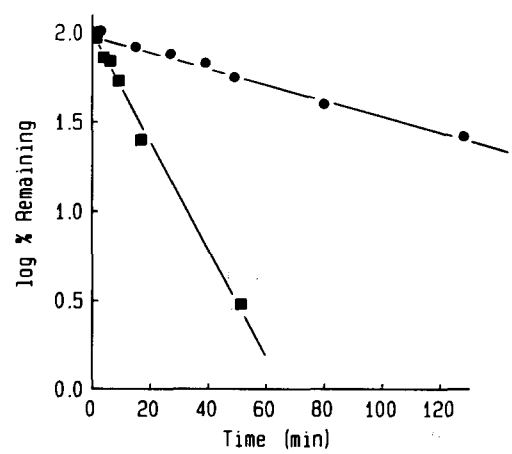


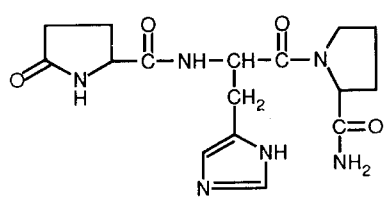
Fig. 2. Plots showing the first-order kinetics of degradation of TRH in 20% rabbit gut homogenate with (●) or without (■) addition of 1 mM bacitracin (37°C).

TABLE 1

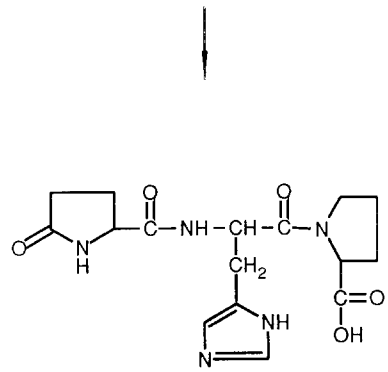
Half-lives ($t_{1/2}$) for the degradation of TRH and its prodrugs in 0.02 M phosphate buffer solution (pH 7.4) and in gut homogenates at 37°C[a]

Compound	$t_{1/2}$			
	Buffer pH 7.4[b]	20% rabbit gut homogenate	20% rabbit gut homogenate containing bacitracin (1 mM)	20% rat gut homogenate
TRH	Stable	10 min	63 min	>10 h
I	35.8 h	25 min		109 min
II	19.0 h	14 min	81 min	65 min
III	19.0 h	6 min	79 min	
IV	17.9 h	8 min		18 min
V	36.8 h	5 min	38 min	38 min
VI	17.5 h	4 min		102 min
VII	20.4 h			64 min
VIII				78 min
IX	133 h			45 min

[a]The homogenates were derived from a mixture of the jejunum, ileum and colon.
[b]The stability data for TRH and the derivatives **I–VII** are from a previous study (Bundgaard and Møss, 1990).



TRH



TRH-OH

Scheme 2.

life of degradation of TRH of 4 h in a 10% rat gut homogenate at 37°C.

The enzymatic degradation of TRH in the rabbit intestinal homogenate was found to be due mainly to prolyl endopeptidase cleaving the C-terminal proline amide residue to yield the acid TRH-OH (pGlu-His-Pro) (Scheme 2). As seen from Fig. 3 the degradation of TRH was accompanied by an almost quantitative formation of deamidated TRH, i.e. TRH-OH. Further evidence for the involvement of this enzyme, also called post-proline cleaving enzyme, was provided by performing the stability study in the presence of bacitracin which is a fairly specific inhibitor of pro-

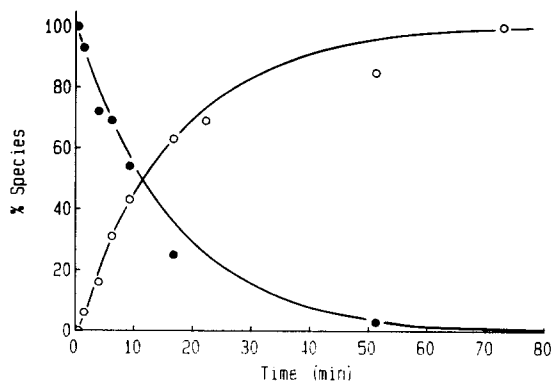


Fig. 3. Time courses for TRH (•) and TRH-OH (○) during degradation of TRH in a 20% rabbit intestinal homogenate at 37°C.

lyl endopeptidase (Tate, 1981; Browne and O'Cuinn, 1983). As seen from the data in Table 1, the presence of bacitracin in a concentration of 1 mM considerably slowed the rate of TRH degradation. The cleavage of TRH by prolyl endopeptidase has previously been reported (Knisatschek and Bauer, 1979; Safran et al., 1982; Wilk, 1983). In the study by Safran and co-workers on the degradation of TRH in various rat organ homogenates the greatest TRH deamidase activity (i.e. prolyl endopeptidase) was seen in liver, lung and kidney whereas homogenates of ileum, pancreas and skeletal muscle only showed little activity. In contrast, the prolyl endopeptidase activity in the rabbit small intestine has been reported to be high compared with that of other organs of the rabbit (Orlowski et al., 1979).

Prolyl endopeptidase is a serine protease containing a thiol group at the active site (Wilk, 1983). It is activated and stabilized in the presence of sodium edetate (EDTA) and dithiothreitol (Knisatschek and Bauer, 1979; Walter et al., 1980). When EDTA (5 mM) and dithiothreitol (2 mM) were added to the gut homogenates, the half-lives shown in Table 2 were not changed, but the rate of degradation adhered often more strictly to first-order kinetics over longer reaction times.

That the greatly varying stability of TRH in the rabbit and rat gut homogenates can be ascribed to difference in prolyl endopeptidase activity was further supported from stability experiments with Z-Gly-Pro-Ala. When this known substrate for prolyl endopeptidase (Walter and Yoshimoto, 1978; Walter et al., 1980) was incubated in the rabbit gut homogenate, it degraded with a first-order half-life of 7 min to yield Z-Gly-Pro as revealed by HPLC. In the rat gut homogenate, on the other hand, Z-Gly-Pro-Ala was only slowly hydrolyzed, the half-life observed being 65 h.

Considering the stability of the TRH derivatives **I–IX** (cf. Table 2), it was rather unexpectedly found that these compounds were more unstable than the parent TRH, especially in the gut homogenate of the rat. Only compounds **I** and **II** were more stable than TRH (in rabbit gut homogenate).

There are two possible degradation pathways for these *N*-alkoxycarbonyl derivatives: cleavage of the terminal proline amide moiety by prolyl endopeptidase to give the corresponding *N*-alkoxycarbonyl derivative of TRH-OH and cleavage of the carbamate bond by unspecific esterases as in plasma (Bundgaard and Møss, 1990) to yield TRH (Scheme 3). HPLC analysis of the reaction solutions showed that both of these routes are involved.

In the rabbit gut homogenate the major route of degradation was found to be cleavage by prolyl endopeptidase. A peak emerged on the chromatograms and subsequently disappeared, the disappearance being accompanied by the formation of a peak with the same retention time as TRH-OH. The intermediate was most likely the corresponding *N*-alkoxycarbonyl derivative of TRH-OH, since it showed the same retention time as the compound formed by treating TRH-OH with the appropriate chloroformate in acetonitrile (cf. Bundgaard and Møss (1990)).

In the rat gut homogenate esterase-catalyzed cleavage of the *N*-alkoxycarbonyl bond resulting in TRH formation appeared to be a dominating degradation pathway. However, an appreciable formation of the corresponding *N*-alkoxycarbonyl derivative of TRH-OH and hence TRH-OH was

TABLE 2

*Permeability coefficients of TRH and some prodrugs (**VI**, **VIII** and **IX**) across various rat intestinal segments*

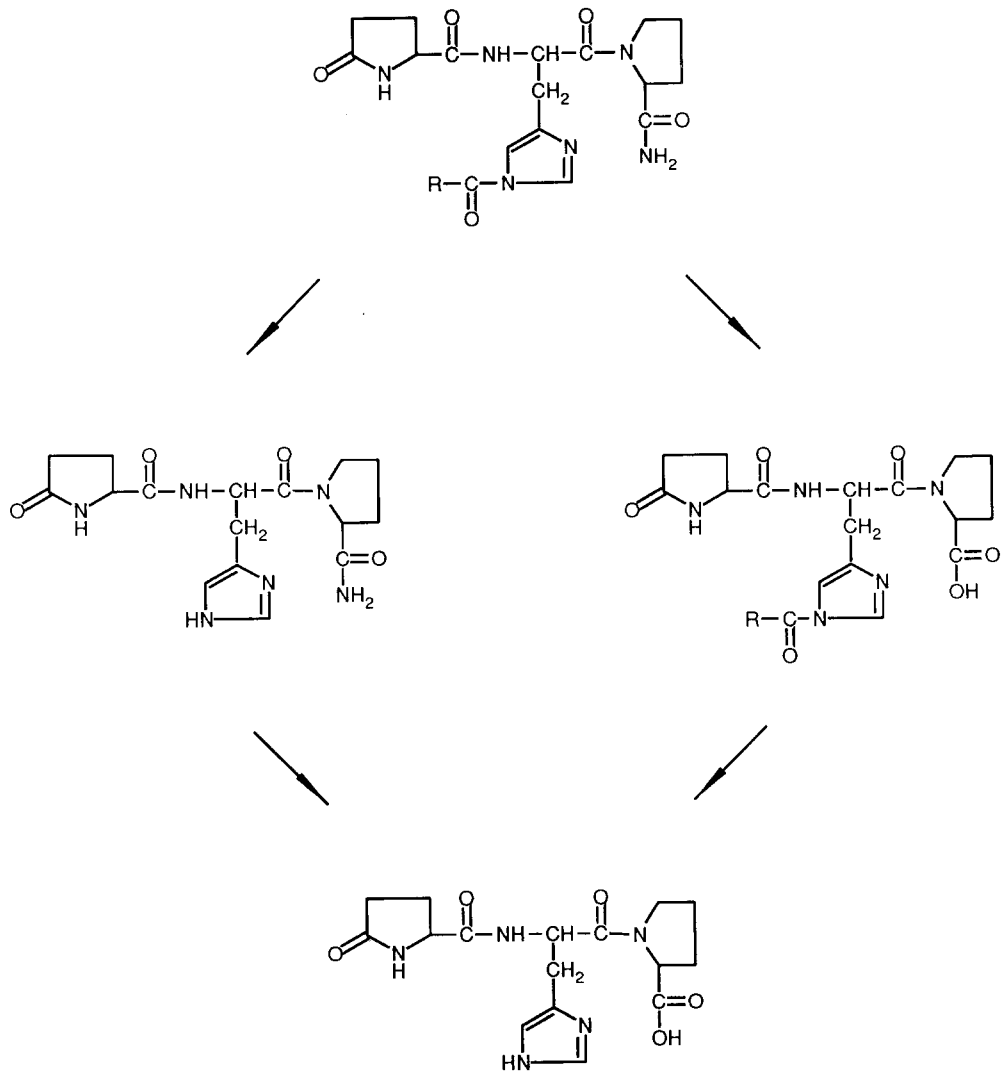
Region	Permeability coefficients ($\times 10^6$ cm s^{-1})[a]			
	TRH	VI	VIII	IX
Jejunum	8.9 ± 1.1 (4)	4.3 ± 0.8 (2)	1.5 ± 0.3 (2)	1.7 ± 0.7 (2)
Ileum	7.4 ± 0.7 (5)	5.7 ± 1.0 (2)	0.6 ± 0.1 (2)	2.7 ± 0.3 (2)
Colon	5.8 ± 0.8 (6)	2.8 ± 0.4 (2)	1.8 ± 0.1 (2)	1.4 (1)

[a]Mean ± SE; figures in parentheses represent number of determinations.

also observed, indicating a higher susceptibility of the prodrugs to prolyl endopeptidase than underivatized TRH. The specificity of this enzyme is rather broad in terms of groups attached to the proline amide core, and hydrophobic substituents in the moiety increase the substrate reactivity (Walter et al., 1980; Wilk, 1983). Thus, the β-naphthylamide of TRH-OH is a far better substrate than the unsubstituted amide (i.e. TRH) (Andrews et al., 1980). Interestingly, the introduction of a benzyl group in the imidazole moiety of this naphthylamide further enhances the reactivity (Andrews et al., 1980). Accordingly, it appears not unlikely that the introduction of lipophilic *N*-alkoxycarbonyl groups in the imidazole moiety of TRH may make its proline amide residue more susceptible towards prolyl endopeptidase.

In vitro penetration studies

Penetration studies using the isolated jejunal, ileal and colonic segments of the albino rabbit and rat were performed with TRH and its most lipophilic prodrug (**VI**) as well as (for the rat study) with the prodrugs **VIII** and **IX** containing a branched alkyl side chain. With the rabbit intestinal



Scheme 3.

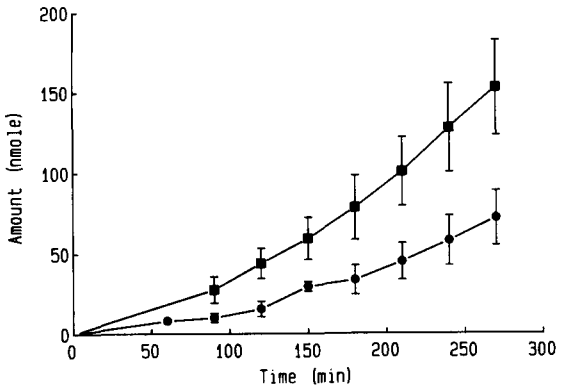


Fig. 4. Time course of penetration of TRH (■) and the prodrug **VI** (●) across the jejunum of the rat. Error bars represent standard errors in the mean values for $n = 2$–4.

segments neither TRH nor compound **VI** was found to penetrate across the intestinal segments to any measurable extent. The concentrations in the receptor cell were at all sampling times below the detection limit (0.5 μg ml^{-1}). This lack of significant penetration can most likely be ascribed to the facile enzymatic degradation of the compounds as described above.

When rat intestinal segments were used measurable amounts of the compounds were detected in the receptor phase. An example of the time course of drug penetration is shown in Fig. 4. From the slope of such plots, the permeability coefficients of TRH and its prodrugs were calculated (Table 2). No intact prodrug was seen in the receptor phase.

The results obtained show that the poor penetration of TRH across the jejunal, ileal and colonic segments is not improved by the prodrugs but rather is reduced. Although the higher lipophilicities of the prodrugs should lead to enhanced permeability, the greater susceptibility of the prodrugs to undergo enzymatic degradation than TRH (cf. Table 1) apparently more than offsets the improved lipophilicity characteristics. This degradation may predominantly occur intracellularly. Analysis of the donor cell solutions showed that about 50% of the prodrugs were degraded after 4 h.

A remark should be given on the lipophilicity. Although the *N*-octyloxycarbonyl derivative **VI** shows a log *P* value of 1.88 which normally is considered to be very favourable for oral absorption (Yalkowski and Morozowich, 1980) partition coefficients in the octanol-water system may not be a good predictor of biomembrane penetration of peptides. Ho et al. (1990) have recently shown that although the uncharged model peptides, *N*-acetyl$(Phe)_2NH_2$ and *N*-acetyl$(Phe)_3NH_2$ possess log *P* values of 1.3 and 2.8, respectively, their intestinal and buccal permeability are very, low ($\leq 5 \times 10^{-6}$ cm s^{-1}). Octanol is a hydrogen bonding solvent, and it may not be modeling the transfer into a biomembrane of peptides like TRH and the TRH prodrugs which contain several amide bonds capable of functioning as both acceptor and donor for hydrogen bonding. A better predictor of biomembrane transport of such molecules may be the partition parameter Δlog *P*, defined as log *P*(octanol/water) −log *P*(cyclohexane/water), which takes the overall hydrogen bonding ability of a compound into account (Young et al., 1988). Studies are in progress to assess this aspect for the TRH prodrugs and other peptides.

Acknowledgements

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