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Kinetics and Mechanism of the Facile Cyclization of Histidyl-prolineamide to Cyclo (His-Pro) in Aqueous Solution and the Competitive Influence of Human Plasma

JUDI MØSS AND HANS BUNDGAARD

Royal Danish School of Pharmacy and PharmaBiotec Research Center, Department of Pharmaceutical Chemistry, 2 Universitetsparken, DK-2100 Copenhagen, Denmark

Abstract—A crucial point in the biosynthesis of cyclo (His-Pro), an endogenous and biologically active cyclic dipeptide, is the spontaneous cyclization of its precursor L-histidyl-L-prolineamide (His-ProNH$_2$). In this study the kinetics and mechanism of the cyclization process has been investigated. His-ProNH$_2$ was found to be converted quantitatively to cyclo(His-Pro) in aqueous solution at pH 2–10 and 37°C, the rate of cyclization being maximal at pH 6–7. Buffer substances such as phosphate (pH 6–7·4) were found to catalyse the cyclization. The bell-shaped pH-rate profile observed was accounted for by assuming spontaneous and specific acid- and base-catalysed reactions of the His-ProNH$_2$ species in which the imidazole group is protonated and the primary amino group unprotonated. The much more rapid rate of cyclization of His-ProNH$_2$ ($t_{\frac{1}{2}}$ of 140 min at pH 6–7 and 37°C) relative to other proline-containing di- and tripeptides studied was suggested to be due to an intramolecular general acid catalytic effect by the protonated imidazole group. In the presence of human plasma enzymatic hydrolysis of His-ProNH$_2$ competed with the cyclization and predominated greatly at 80% plasma concentration.

Cyclo(His-Pro), or histidyl-proline diketopiperazine, is a biologically active cyclic dipeptide endogenous to a variety of tissues and body fluids of animals and man (for reviews see Prasad et al 1982; Peterkofsky et al 1982; Prasad 1988). Cyclo(His-Pro) was first identified in human urine by Perry et al (1965) and later discovered by Prasad & Peterkofsky (1976) as a metabolic product of thyrotropin-releasing hormone (TRH, pGlu-His-ProNH$_2$) derived through the action of pyroglutamate aminopeptidase on TRH in the brain. This enzyme cleaves amino-terminal pyroglutamic acid from TRH and the L-histidyl-L-prolineamide (His-ProNH$_2$) subsequently cyclizes non-enzymatically to cyclo (His-Pro) (Prasad & Peterkofsky 1976; Prasad et al 1977) (Fig. 1). Since its discovery, cyclo(His-Pro) has been shown to elicit a number of behavioural, endocrine and central nervous system-related biological activities (for reviews see Peterkofsky et al 1982; Prasad et al 1982; Prasad 1984). A number of these effects associated with cyclo(His-Pro) are similar to those of TRH, whereas others are either opposite or unrelated to those of TRH (Prasad et al 1982).

To understand the mechanism(s) by which cyclo(His-Pro) brings about its different biologic activities, information on the origin of the peptide or the pathways of its biosynthesis is needed. Besides being formed from TRH through the reaction sequence shown in Fig. 1, two additional sources of cyclo(His-Pro) have recently been suggested (Prasad et al 1987): processing of the TRH prohormone, prepro TRH, which contains five copies of the TRH sequence, to yield cyclo(His-Pro); and synthesis from constituent amino acids (L-His, L-Pro and Gly) through an as-yet unknown mechanism.

Correspondence to: H. Bundgaard, Royal Danish School of Pharmacy, Dept. of Pharmaceutical Chemistry, 2 Universitetsparken, DK-2100 Copenhagen, Denmark.

A crucial point in the formation of cyclo(His-Pro) through either of these biosynthetic pathways is the final step involving spontaneous cyclization of His-ProNH$_2$, a precursor common to all three pathways, to cyclo(His-Pro). Prasad et al (1977) reported a half-life of approximately 40 min for the cyclization in 0·1 M phosphate buffer of pH 7·2 at 37°C whereas a half-life of about 160 min has been observed by Bauer & Nowak (1979) for the cyclization in 0·05 M Tris buffer of pH 7·4 at 37°C. Bauer & Kleinkauf (1980) also reported an initial rate of cyclization in 0·1 M phosphate buffer of pH 7·4 at 37°C of 1·5% min^{-1} which corresponds to a half-life of 44 min if first-order kinetics is assumed. There is no evidence for an enzymatically catalysed cyclization of His-ProNH$_2$ (Bauer et al 1978; Matsui et al 1979; Bauer & Kleinkauf 1980; Prasad et al 1987) nor is there any detailed information on the kinetics of the cyclization of His-ProNH$_2$ in aqueous solution. In view of the importance of this reaction with regard to both the biosynthesis of cyclo(His-Pro) and the metabolic fate of TRH (Browne & O'Cuinn 1983; Griffiths et al 1983; Bauer 1988; Wilk et al 1988) we have studied the rate of cyclization of His-ProNH$_2$ in aqueous solution at 37°C as a function of pH, buffer species and concentration. In addition, the influence of human plasma on the stability of His-ProNH$_2$ has been investigated.

Materials and Methods

HPLC was carried out with a system consisting of a Kontron 420 LC 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 (150 × 4·6 mm) packed with 3 μm particles and equipped with a Supelguard LC-8-DB guard column (Supelco, Inc., USA) was used and eluted at ambient temperature. For analysis of plasma, a Nova-Pak CN Radial Pak (Waters) column

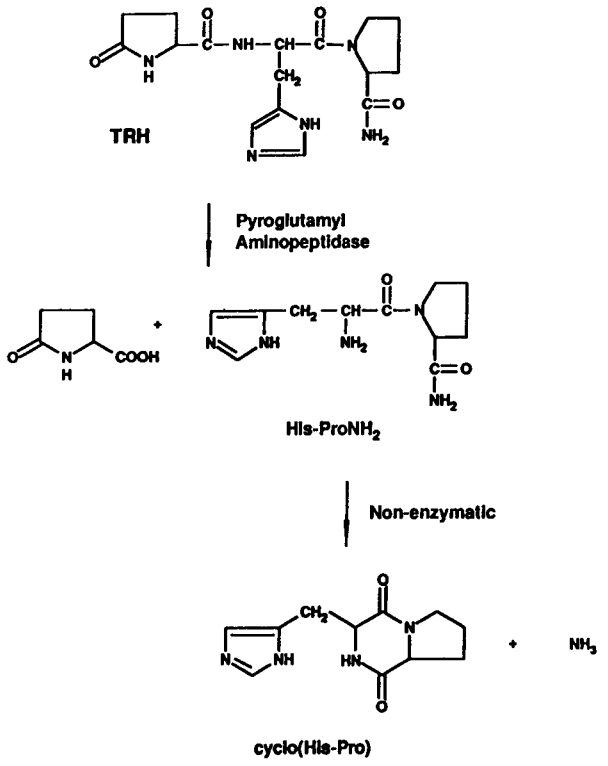


FIG. 1. Metabolic degradation of TRH by enzymatic hydrolysis by pyroglutamyl aminopeptidases to yield pyroglutamic acid and L-histidine-L-prolineamide followed by spontaneous or non-enzymatic cyclization of the latter to yield histidyl-proline diketopiperazine (cyclo(His-Pro)) and ammonia.

(100 × 8 mm) equipped with a Resolve CN Guard Pak precolumn was used. pH was measured with a Radiometer Type PHM 26 meter.

His-ProNH$_2$ (as the dihydrochloride salt) was kindly supplied by Calbiotec A/S, Copenhagen. Cyclo (His-Pro) and TRH were purchased from Sigma Chemical Company, St. Louis, USA; the di- and tripeptides His-Pro, Tyr-ProNH$_2$, Ala-Pro-Gly and Gly-Pro-Ala and cyclo (Gly-Pro) were obtained from Bachem AG, Bubendorf, Switzerland.

All studies were performed at 37·0 ± 0·2 or 60·0 ± 0·2°C. Hydrochloric acid, acetate, phosphate, Tris and borate buffers were used to maintain a constant pH. A constant ionic strength (μ) of 0·5 was maintained for each buffer by adding a calculated amount of potassium chloride. The reactions were initiated by adding 100 μL of a stock solution of the compounds in water to 10 mL of preheated buffer or plasma solutions, to a final concentration of about 10^{-4} M. The solutions were kept in a water-bath at 37 or 60°C and at appropriate intervals samples were taken and immediately analysed by HPLC. For analysis of plasma, samples of 250 μL were added to 250 μL of a mixture of 70% perchloric acid and 0·1 M zinc sulphate (1 : 1 v/v) to deproteinize the plasma. After immediate mixing and centrifugation for 3 min at 13 000 rev min^{-1}, 20 μL of the clear supernatant was analysed by HPLC.

For the analysis of the di- and tripeptides and their products of degradation, a mobile phase system of 0·1% phosphoric acid containing 10^{-3} M triethylamine was used. The column effluent was monitored at 215 nm, the flow rate was 1·0 mL min^{-1} and the compounds were quantified by measuring the peak heights in relation to those of standards chromatographed under the same conditions. Under these chromatographic conditions the di- and tripeptides showed retention times from 3 to 8 min.

Results and Discussion

Kinetics of cyclization of His-ProNH$_2$

The kinetics of cyclization of His-ProNH$_2$ was studied at 37°C in aqueous buffer solutions over the pH-range 2–10. At constant pH and temperature the reaction displayed strict first-order kinetics over several half-lives as illustrated in Fig. 2. Pseudo-first-order rate constants (k_{obs}) for the cyclization were calculated from the slopes of linear plots such as those shown in Fig. 2. As revealed by HPLC analysis of the reaction solutions (Fig. 3) cyclo(His-Pro) was found to be formed in quantitative amounts at all pH values studied. An example of a product analysis is shown in Fig. 4.

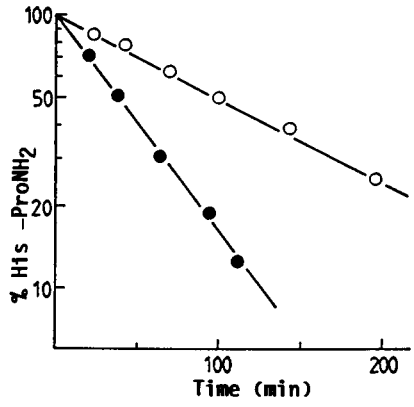


FIG. 2. Kinetics of degradation of His-ProNH$_2$ in 0·02 M (○) and 0·1 M (●) phosphate buffer solutions (pH 7·40, 37°C).

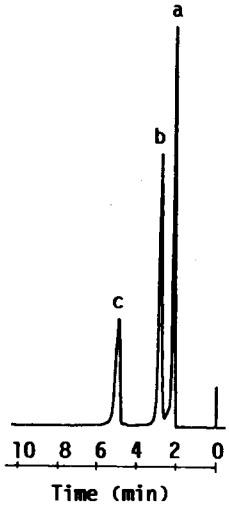


FIG. 3. Chromatogram showing the separation of cyclo(His-Pro) (c) and His-ProNH$_2$ (b) in a phosphate buffer solution; (a) is the solvent front.

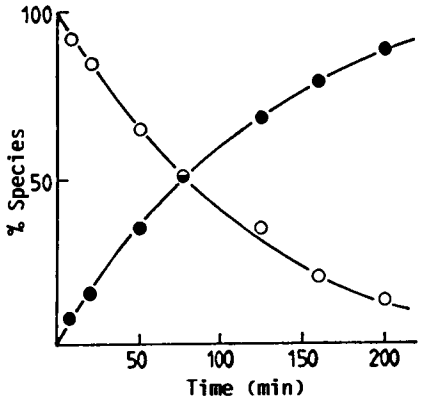


FIG. 4. Degradation of His-ProNH$_2$ (○) and formation of cyclo(His-Pro) (●) in 0·02 M phosphate buffer (pH 7·40, 37°C).

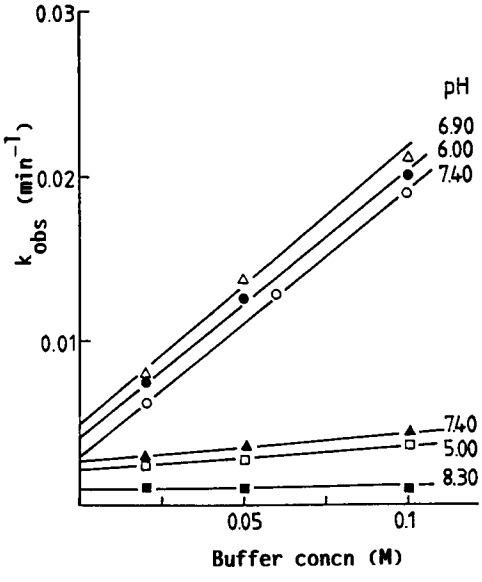


FIG. 5. The influence of buffer concentration on the rate of cyclization of His-ProNH$_2$ in aqueous solution at 37°C. The buffers shown are phosphate (pH 6·9–7·4), Tris (pH 7·4, ▲), acetate (pH 5·00) and borate (pH 8·3).

In alkaline solutions cyclo(His-Pro) was found to be unstable, presumably due to hydrolytic opening of the piperazinedione ring, although its rate of degradation in all cases was much slower than the rate of formation. Thus, in 0·02 M borate buffer of pH 9·80 (37°C) a half-life of 15·0 h was found whereas at pH 8·40 a half-life of 270 h was observed.

The cyclization of His-ProNH$_2$ was found to be subject to general acid-base catalysis by various buffer substances, to varying extents. As seen from Fig. 5 phosphate buffers exhibited a pronounced catalytic effect whereas Tris, acetate and borate were less catalytically active. The influence of pH on the rates of cyclization at 37°C of His-ProNH$_2$ is shown in Fig. 6. The values of k were obtained at each pH by extrapolation of linear plots of k_{obs} against buffer concentration (cf. Fig. 5) to zero buffer concentration. It is seen that the pH-rate profile is bell-shaped with a maximal rate of cyclization ($t_{\frac{1}{2}} = 140$ min) occurring at pH 6·5.

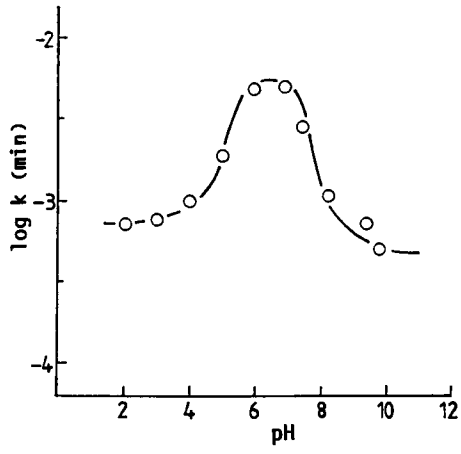
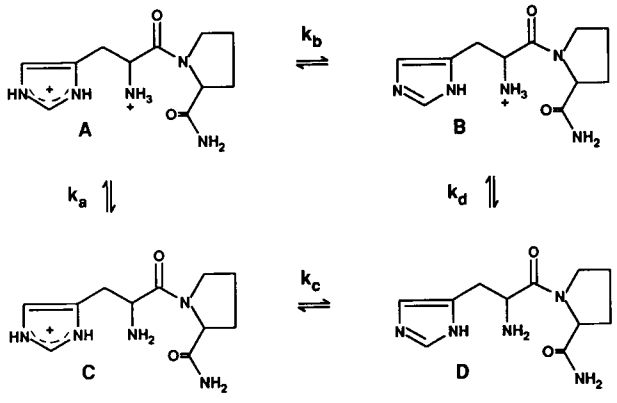


FIG. 6. The pH-rate profile for the cyclization of His-ProNH$_2$ to cyclo(His-Pro) in aqueous solution at 37°C.

Mechanism of cyclization

To account for this unusual pH-rate profile the ionization scheme of His-ProNH$_2$ is considered. In the pH range investigated His-ProNH$_2$ can exist in 4 different forms. These are shown in Scheme 1, in which k_a, k_b, k_c and k_d are



Scheme 1

microscopic ionization constants, describing the interconversion of the four species denoted A, B, C and D. The macroscopic ionization constants K_I (for the protonated imidazole group) and K_{II} (for the protonated primary amino group) determined by titration are related to the microscopic ionization constants as shown in equations 1–3 (Albert & Serjeant 1971):

$$K_I = k_a + k_b \quad (1)$$

$$1/K_{II} = 1/k_c + 1/k_d \quad (2)$$

$$K_I K_{II} = k_a k_c = k_b k_d \quad (3)$$

Since the formation of cyclo(His-Pro) must involve a nucleophilic attack of the unprotonated amino group of His-ProNH$_2$ on the prolineamide variety the reactive species are C and/or D. The shape of the pH-rate profile indicates that at pH 5–8 a predominant reaction is spontaneous or water-catalysed cyclization of species C while at lower and higher pH values specific acid and base-catalysed reactions of species C, respectively, make a significant contribution to the

overall rate of cyclization. Thus, the suggested reaction scheme is:

$$C \xrightarrow{k_1} \text{cyclo(His-Pro)} \quad (4)$$

$$C + H^+ \xrightarrow{k_2} \text{cyclo(His-Pro)} \quad (5)$$

$$C + OH^- \xrightarrow{k_3} \text{cyclo(His-Pro)} \quad (6)$$

where k_1 is a first-order rate constant for the spontaneous or water-catalysed cyclization of C and k_2 and k_3 are second-order specific acid (k_2) and base (k_3) catalytic rate constants for the reaction of species C. The equation for k is accordingly:

$$k = k_1 f_C + k_2 a_H f_C + k_3 a_{OH} f_C \quad (7)$$

where f_C is the fraction of His-ProNH$_2$ present as form C at a given pH and a_H and a_{OH} are the hydrogen and hydroxide ion activity, respectively. The fraction of species C can be calculated from the following equation

$$f_C = \frac{K_{II} a_H}{a_H^2 + K_I a_H + K_I K_{II}} \quad (8)$$

Equation 8 was derived from equations 1–3, the equation for mass balance and the equations defining the microscopic ionization constants, along with the assumption that $k_b \gg k_a$, i.e. $k_b = K_I$, and $k_c \gg k_d$, i.e. $k_d = K_{II}$.

Inserting equation 8 in equation 7 gives:

$$k = \frac{k_1 K_{II} a_H + k_2 K_{II} a_H^2 + k_3 K_{II} K_w}{a_H^2 + K_I a_H + K_I K_{II}} \quad (9)$$

where K_w is the ionization constant for water at 37°C, i.e. $10^{-13.62}$ (Harned & Hamer 1933). The following values of the rate and ionization constants were derived from equation 9 and the rate data at pH 2–10:

$$k_1 = 0{\cdot}21 \text{ min}^{-1}$$

$$k_2 = 1{\cdot}2 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$$

$$k_3 = 3{\cdot}2 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$$

$$pK_I = 5{\cdot}8$$

$$pK_{II} = 7{\cdot}2$$

The solid curve in Fig. 6 was constructed from these values and equation 9, and the good agreement observed between the calculated and experimental data demonstrates that the reactions proposed (eqns 4–6) adequately account for the cyclization kinetics.

The facile cyclization of His-ProNH$_2$ compared with other di- or tripeptides containing a prolineamide moiety is apparent by inspection of Table 1 containing rate data for various peptides. In weakly acidic solutions (pH < 5) these peptides (except His-ProNH$_2$) were so stable that no reliable rate data could be obtained. The cyclization of Tyr-ProNH$_2$ showed a sigmoid pH-rate profile according to a rate expression containing terms for uncatalysed and hydroxide ion-catalysed reactions of the unprotonated dipeptide species. Cyclization of dipeptide esters containing a C-terminal proline residue, e.g. glycylproline ethyl ester, has previously been shown to be easier than the cyclization of peptide esters containing other C-terminal amino acids, e.g. prolylglycine ethyl ester (Rydon & Smith 1956), owing to a more

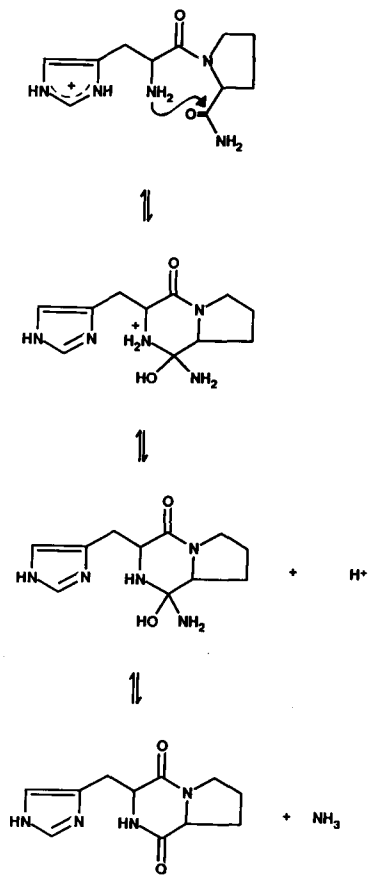
Table 1. Half-lives for the cyclization of His-ProNH$_2$ and various other proline-containing di- and tripeptides in aqueous solution at 37 and 60°C.

Compound	$t_{\frac{1}{2}}$ (ha)[a]			
pH	9·75 (60°C)	5·00 (37°C)	6·00 (37°C)	7·40 (37°C)
His-ProNH$_2$	3·5	6·1	2·4	2·8
Tyr-ProNH$_2$	8·7	480	150	130
Gly-Pro-Ala	370			
Ala-Pro-Gly	83			

[a] The values given refer to those obtained in buffer-free solutions by extrapolating k_{obs} values to zero buffer concentration.

favourable conformation of the side-chain and a weaker peptide bond. Such effects evidently also contribute to the facile cyclization of His-ProNH$_2$, but its greater reactivity relative to the other prolineamide peptides studied may be due solely to its histidine moiety.

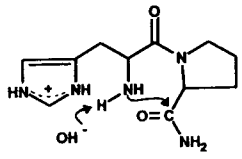
As described above, the kinetic analysis of the pH-rate profile for His-ProNH$_2$ indicated species C with a protonated imidazole moiety to be the reacting form of the compound. A plausible mechanism for its uncatalysed reaction (eqn 4) may be that shown in Scheme 2 involving a



Scheme 2

general acid catalytic effect of the protonated imidazole group. The cyclization or intramolecular aminolysis of glutamine to pyroglutamic acid has previously been found to be general acid-catalysed by various buffers (Martin et al

1964; Acree & Lee 1975) and the high reactivity of species C may be a result of *intramolecular* catalysis of the imidazolium ion. The k_3-reaction involving specific base catalytic cyclization of species C may occur as shown in Scheme 3 with



Scheme 3

hydroxide ion removing a proton from the attacking amino group in analogy with similar reactions of dipeptide esters (Martin et al 1964; Meresaar & Ågren 1968; Purdie & Benoiton 1973) or intramolecular aminolysis of amino group-containing cephalosporins (Bundgaard 1976). The apparent hydrogen ion-catalysed reaction of species C (eqn 5) accounting for the cyclization of His-ProNH$_2$ in weakly acidic solutions may be ascribed to protonation of the prolineamide moiety or to an intermolecular general acid catalytic effect of hydronium ion.

In this discussion of the possible mechanisms accounting for the high reactivity of His-ProNH$_2$ it should be noted that although equation 9 and hence the reactions described by equations 4–6 fully account for the observed kinetics of cyclization, several kinetic equivalent reactions can be formulated. Thus, a specific acid-catalysed reaction of species D is equivalent to the spontaneous reaction of species C (eqn 4) and similarly, a spontaneous reaction of species D is equivalent to the hydroxide ion-catalysed reaction of species C (eqn 6). This can be seen from the equation describing the fraction of His-ProNH$_2$ present as species D:

$$f_D = \frac{K_I K_{II}}{a_H^2 + K_I a_H + K_I K_{II}} \qquad (10)$$

Furthermore, since species C and B are related by the tautomerization constant K_T:

$$K_T = [B]/[\overset{+}{C}] = k_b/k_a \simeq K_I/K_{II} \qquad (11)$$

reactions involving species B can also account for the kinetics observed. However, this appears less likely because of the non-nucleophilicity of the protonated primary amino group in this species.

Influence of plasma

The rate of degradation of His-ProNH$_2$ was studied at 37°C in 0·01 M phosphate buffer solution of pH 7·40 containing varying concentrations of human plasma. An accelerated rate of degradation was observed with increasing plasma concentrations as seen from Fig. 7. Thus, at 80% human plasma the half-life of degradation was 5·2 min compared with 125 min in pure 0·01 M phosphate buffer. With increasing plasma concentrations the amount of cyclo(His-Pro) formed decreased markedly and inversely proportional to the rate increase. These findings demonstrated that human plasma catalyses the degradation of His-ProNH$_2$ by a reaction competing with cyclization to cyclo(His-Pro). The plasma-catalysed reaction may either be hydrolysis of the prolineamide bond to yield His-Pro or hydrolysis of the peptide bond yielding His and ProNH$_2$. Both the former

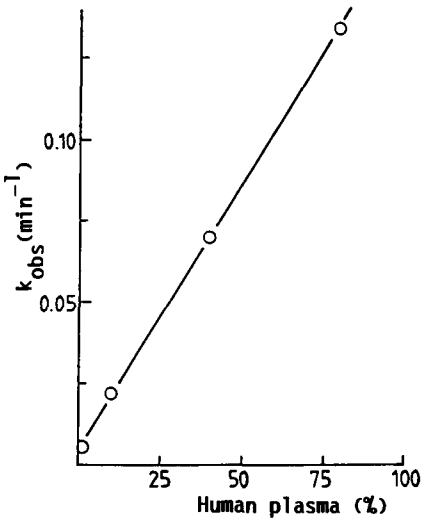


FIG. 7. The influence of human plasma on the rate of degradation of His-ProNH$_2$ in 0·01 M phosphate buffer solution (pH 7·40, 37°C).

reaction involving a post-proline dipeptidyl-aminopeptidase and the latter reaction involving an imidopeptidase have been demonstrated to occur upon incubation of His-ProNH$_2$ with various brain tissues (Matsui et al 1979; Bauer & Kleinkauf 1980; O'Connor & O'Cuinn 1986). Because of the inability of the HPLC method to separate these products from signals arising from the plasma, a distinction between these two possible routes of plasma-catalysed degradation could not be made.

Since buffer substances such as phosphate markedly catalyse the rate of cyclization of His-ProNH$_2$ but do not catalyse enzymatic reactions, attention should be paid to this fact in investigations of the fate of this compound, e.g. in studies on the biosynthesis of cyclo(His-Pro) and metabolism of TRH. Thus, the use of a high phosphate buffer concentration may disturb any enzymatic degradation competing with the non-enzymatic cyclization process. For example, as shown above, the half-life of cyclization of His-ProNH$_2$ in 0·1 M phosphate buffer of pH 7·4 at 37°C is only 36 min compared with 112 min in 0·02 M phosphate buffer and 247 min at pH 7·4 in the absence of phosphate ions.

Acknowledgement

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