Structure–Activity Relationships of Neuromedin U. V. Study on the Stability of Porcine Neuromedin U-8 at the C-Terminal Asparagine Amide under Mild Alkaline and Acidic Conditions

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Porcine neuromedin U-8 (X-Asn-NH_2, X=H-Tyr-Phe-Leu-Phe-Arg-Pro-Arg) is occasionally unstable in the biological fluids used for bioassay as well as in the acidic solutions used for purification of synthetic peptides. In this study, HPLC examination of an incubate solution of X-Asn-NH_2 revealed that the main decomposition products in Tyrode's solution (pH 7.4) were either α- or β-monocarboxylic acid analogs (X-Asn-OH or X-Asp-NH_2), and that no dicarboxylic acid analog (X-Asp-OH) was produced. Further investigation, employing a model peptide (Y-Asn-NH_2, Y=Benzoyl-Pro-Arg) incubated in a 0.1 M sodium bicarbonate solution at 60 °C, revealed that the decomposition of C-terminal Asn-NH_2 occurred through the formation of an aminosuccinimide intermediate (Y-Asu), at a rate faster than that of Y-Asn-Ser peptide but slower than that of Y-Asn-Gly peptide. Mild acid hydrolysis of X-Asn-NH_2 examined in a 1 M HCl solution at 60 °C yielded X-Asn-OH and X-Asp-NH_2, which further decomposed to yield X-Asp-OH. The C-terminal degradation of X-Asn-NH_2 resulted in reduced biological and immunochemical binding activities.

Key words neuromedin U; C-terminal asparagine amide; succinimide; hydrolysis; decomposition

Neuromedin U peptides, isolated originally from porcine spinal cord, were determined from various animals.[1] The C-terminal heptapeptide amide structure was highly conserved among this family of peptides.[2—4] The amide structure at the C-terminal of porcine neuromedin U-8 (X-Asn-NH_2, X=H-Tyr-Phe-Leu-Phe-Arg-Pro-Arg) was reported to have an important role on its biological activity.[1,5,6] The C-terminal asparagine amide also occurs in vasoactive intestinal peptide (VIP),[7,8] but it is not thought to be a prerequisite for the biological activity.[9] A commonly used purification procedure of synthetic peptides by RP-HPLC and gel-filtration involves their treatment with either dilute hydrochloric acid (HCl) or trifluoroacetic acid (TFA) solutions, which leads to cleavage of peptide and/or amide bonds to some extent. Bioassay procedures involve the storage of peptides in a biological fluid, where partial chemical decomposition may occur. Previously, we have reported that the pGlu-peptide bond was highly susceptible to dilute hydrochloric acid as seen in a dog neuromedin U-8 fragment.[10] Both porcine neuromedin U-8 and rat neuromedin U-23 were found to be very stable in a saline solution,[5] however, by-products appeared occasionally on the RP-HPLC chromatograms after they were suspended at room temperature in Tyrode's solution. The aim of this study is to examine the stability of neuromedin U peptides compared to various synthetic peptides under mild alkaline and acidic conditions by means of HPLC analysis. The sequences of these peptides are shown in Fig. 1. Herein, a pathway for the decomposition of the Asn-NH_2 portion of porcine neuromedin U-8 in a weak alkaline solution *via* succinimide formation is described. Further, the biological and immunochemical activities of the C-terminally deamidated neuromedin U-8 analogs are discussed.

Peptides related to porcine neuromedin U-8:

X-Asn-NH_2 (1), X-Asn-OH (2), X-Asp-NH_2 (3), X-Asp-OH (4)

X = H-Tyr-Phe-Leu-Phe-Arg-Pro-Arg

Model tripeptides and pentapeptides related to porcine neuromedin U-8:

Y-Asn-NH_2 (5), Y-Asn-OH (6), Y-Asp-NH_2 (7), Y-Asp-OH (8),
Y-Gly-NH_2 (9), Y-Gln-NH_2 (10), Y-Asn-Gly-Ala-OH (11),
Y-Asn-Ser-Ala-OH (12), Y-Asn-Ala-Ala-OH (13),
Y-Asp-Gly-Ala-OH (14), Y-Asp(β-Gly-Ala-OH)-OH (15)

Y = Benzoyl-Pro-Arg

Model tripeptides related to the C-terminal of VIP:

H-Ile-Leu-Asn-NH_2 (16), H-Ile-Leu-Asn-OH (17), H-Ile-Leu-Asp-NH_2 (18),
H-Ile-Leu-Asp-OH (19), H-Ile-Leu-D-Asn-OH (20), H-Ile-Leu-D-Asp-NH_2 (21)

Fig. 1. Porcine Neuromedin U-8 (**1**) and Synthetic Peptides (**2**—**21**)

Experimental

General HPLC analysis was performed on an apparatus equipped with a U6K injector (Waters Corp., Milford, MA, U.S.A.), a 590 and a 510 pump (Waters), an S310 Model II UV detector (Soma Optics Ltd., Tokyo, Japan), an automated gradient controller 680 (Waters), and a chromatocorder 21 (System Instruments Co., Ltd., Tokyo, Japan). Amino acid analysis was conducted on a 7300 Model amino acid analyzer system (Beckman Instruments Ltd., Fullerton, CA, U.S.A.). Fast-atom bombardment mass spectra (FAB-MS) were obtained on a JMS-DX300 mass spectrometer (JEOL Ltd., Tokyo, Japan). The optical rotations of peptides were measured with a DIP-370 digital polarimeter (Nippon Bunko Co., Ltd., Tokyo, Japan). HP-TLC analysis was carried out on precoated silica gel plates (Kieselgel 60; Merck, Darmstadt, Germany).

Peptide Synthesis The peptides used in this study were synthesized through a solid-phase methodology employing Boc-amino acids, as previously reported for neuromedin U analogs.[11] Protected peptides were constructed either on a benzhydrylamine resin or a chloromethylated polystyrene resin using a peptide synthesizer (ABI 433A; Applied Biosystems, Foster City, CA, U.S.A.). All reagents and protected amino acid derivatives were obtained from Watanabe Chemical Industries Ltd. (Hiroshima, Japan). The protected peptide resin was treated with an anhydrous hydrogen fluoride (HF) solution containing 10% anisole under ice cooling for 45 min in a Teflon HF apparatus (Peptide Institute Inc., Osaka, Japan). After evaporation of HF *in vacuo*, the residual peptides were lyophilized and purified by RP-HPLC on a column of YMC-pack ODS-AM S-5 120 Å (20×150 mm) using a 0.1% TFA–acetonitrile (CH_3CN) solvent system, followed by gel-filtration on a column of Toyopearl HW-40 (super fine) (1.5×47 cm, Tosoh Co., Tokyo, Japan) with 25% CH_3CN in 5 mM hydrochloric acid as an eluent. Hy-

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drolysis of the synthetic peptides for amino acid analysis was performed by reacting with 6 M HCl vapor at 130 °C for 3 h in the same manner as reported previously.[5] The amino acid analysis data of various synthetic peptides is shown in Table 1, while their FAB-MS analysis data and characteristics are shown in Table 2.

Stability Test The peptide solutions related to porcine neuromedin U-8 were prepared under ice cooling at a concentrations of 4×10^{-4} M in a) Tyrode's solution (pH 7.4) of the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.1, NaH_2PO_4 0.4, $NaHCO_3$ 12, D-glucose 5.6, b) 0.1 M $NaHCO_3$, c) 2% NH_4OH (ammonia solution), or d) 1 M HCl. The peptide solutions related to VIP were prepared at a concentration of 4×10^{-3} M. To examine the decomposition reaction, these peptide solutions were divided into ten aliquots (100 μl each) in polypropylene tubes (2 ml), which were tightly capped and kept at 60 °C (or exceptionally at 4 °C, for peptide solutions in 2% NH_4OH) in a thermostatically regulated apparatus. Samples were removed from the apparatus at an appropriate time (shown in Figs. 2—

Table 1. Amino Acid Analysis of the Synthetic Peptides

Peptides	Asp	Pro	Leu	Tyr	Phe	Arg	NH_3	Other amino acid(s)	
1	1.06 (1)	0.97 (1)	1.02 (1)	0.97 (1)	1.97 (2)	2.00 (2)	1.87 (2)		
2	1.03 (1)	0.99 (1)	1.00 (1)	0.88 (1)	2.09 (2)	2.02 (2)	0.93 (1)		
3	0.98 (1)	0.97 (1)	0.14 (1)	0.95 (1)	1.96 (2)	2.01 (2)	1.09 (1)		
4	1.05 (1)	0.92 (1)	1.10 (1)	0.99 (1)	1.97 (2)	1.97 (2)	0.71 (0)		
5	1.01 (1)	0.96 (1)	—	—	—	1.03 (1)	2.10 (2)		
6	0.98 (1)	0.90 (1)	—	—	—	0.95 (1)	1.08 (1)		
7	1.00 (1)	0.96 (1)	—	—	—	1.04 (1)	0.96 (1)		
8	0.99 (1)	1.00 (1)	—	—	—	1.00 (1)	0.20 (0)		
9	—	0.93 (1)	—	—	—	1.00 (1)	1.12 (1)	Gly 1.07 (1)	
10	—	0.95 (1)	—	—	—	1.01 (1)	2.03 (2)	Glu 1.04 (1)	
11	1.02 (1)	0.93 (1)	—	—	—	1.01 (1)	1.24 (1)	Gly 1.04 (1)	Ala 1.00 (1)
12	1.05 (1)	1.05 (1)	—	—	—	1.04 (1)	1.26 (1)	Ser 0.85 (1)	Ala 1.00 (1)
13	1.04 (1)	1.04 (1)	—	—	—	1.03 (1)	1.07 (1)	Ala 1.89 (2)	
14	1.00 (1)	1.02 (1)	—	—	—	0.99 (1)	0.47 (0)	Gly 1.01 (1)	Ala 0.99 (1)
15	1.01 (1)	0.96 (1)	—	—	—	1.02 (1)	0.29 (0)	Gly 1.03 (1)	Ala 0.98 (1)
16	1.13 (1)	—	0.95 (1)	—	—	—	2.15 (2)	Ile 0.92 (1)	
17	1.08 (1)	—	0.98 (1)	—	—	—	1.40 (1)	Ile 0.94 (1)	
18	1.10 (1)	—	0.98 (1)	—	—	—	1.33 (1)	Ile 0.93 (1)	
19	1.09 (1)	—	0.98 (1)	—	—	—	1.42 (1)	Ile 0.93 (1)	
20	1.05 (1)	—	1.02 (1)	—	—	—	1.34 (1)	Ile 0.94 (1)	
21	1.06 (1)	—	1.00 (1)	—	—	—	0.27 (0)	Ile 0.95 (1)	

Hydrolysis at 130 °C for 3.0 h by 6 M HCl vapor containing phenol (3%). Number in parentheses are theoretical values.

Table 2. FAB-MS Analysis and Characteristics of Synthetic Peptides

Peptide	Formula	FAB-MS[a] Found (m/z) (Calcd for $[M+H]^+$)	$[\alpha]_D^{21}$ (c=0.5) (50% AcOH)	HPLC[b] t_R (min)	HP-TLC[c]	
					Rf^1	Rf^2
1	$C_{54}H_{78}N_{16}O_{10}$	1111.6172 (1111.6165)	−99.9°	52	0.88	0.88
2	$C_{54}H_{77}N_{15}O_{11}$	1112.6002 (1112.6005)	−30.4°	58	0.41	0.38
3	$C_{54}H_{77}N_{15}O_{11}$	1112.6002 (1112.6005)	−38.0°	60	0.42	0.43
4	$C_{54}H_{76}N_{14}O_{12}$	1113.5848 (1113.5845)	−36.8°	75	0.41	0.36
5	$C_{22}H_{32}N_8O_5$	489	−57.7°	25.0	0.50	0.36
6	$C_{22}H_{31}N_7O_6$	490	−55.2°	26.8	0.36	0.35
7	$C_{22}H_{31}N_7O_6$	490	−62.3°	28.6	0.43	0.39
8	$C_{22}H_{30}N_6O_7$	491	−60.2°	32.8	0.33	0.37
9	$C_{20}H_{29}N_7O_4$	432	−63.6°	27.1	0.52	0.39
10	$C_{23}H_{34}N_8O_5$	503	−63.1°	27.4	0.51	0.38
11	$C_{27}H_{39}N_9O_8$	618	−64.9°	22.8	0.37	0.34
12	$C_{28}H_{41}N_9O_9$	648	−70.4°	22.3	0.36	0.33
13	$C_{28}H_{41}N_9O_8$	632	−75.9°	23.9	0.40	0.35
14	$C_{27}H_{38}N_8O_9$	619	−70.7°	24.3	0.31	0.35
15	$C_{27}H_{38}N_8O_9$	619	−56.0°	23.9	0.26	0.30
16	$C_{16}H_{31}N_5O_4$	358	+3.9°	17.0	0.61	0.45
17	$C_{16}H_{30}N_4O_5$	359	+0.1°	17.8	0.47	0.42
18	$C_{16}H_{30}N_4O_5$	359	−11.8°	20.3	0.56	0.47
19	$C_{16}H_{29}N_3O_6$	360	+6.9°	24.5	0.45	0.45
20	$C_{16}H_{30}N_4O_5$	359	−0.3°	16.1	0.46	0.40
21	$C_{16}H_{30}N_4O_5$	359	+23.4°	21.9	0.57	0.46

a) High-resolution FAB-MS for peptides **1**—**4**. *b*) HPLC conditions: column, J'sphere ODS-H80 (4.6×250 mm); UV detection, 210 nm, flow rate, 0.6 ml/min for peptides **1**—**4** and 0.8 ml/min for peptides **5**—**21**; elution, 19% MeCN in 0.1% TFA (isocratic manner) for peptides **1**—**4**, and linear gradient from 8 to 16% MeCN (30 min) in 0.1% TFA for peptides **5**—**10** and **16**—**21**, or linear gradient from 8 to 28% MeCN (30 min) in 0.1% TFA for peptides **11**—**15**. *c*) HP–TLC solvent: Rf^1: *n*-BuOH : pyridine : AcOH : H_2O (30 : 20 : 6 : 24), Rf^2: *n*-BuOH : AcOEt : AcOH : H_2O (1 : 1 : 1 : 1).

6), neutralized under ice cooling and kept in a freezer at −40 °C until analysis. RP-HPLC employing a J'sphere ODS H-80 (4.6×250 mm, YMC Co., Japan) column was used for quantitative analysis, where the peak area at a wavelength of 210 nm was integrated. The decomposition products isolated from HPLC peaks were assigned by amino acid analysis and FAB-MS, and the structure was confirmed by co-elution on HPLC with authentic synthetic peptides.

Biological Activity of Neuromedin U-8 Analogs The biological activity of synthetic peptides was evaluated by a contraction assay on a chicken crop smooth muscle preparation as described in a previous paper.[6] Briefly, a strip of smooth muscle from the chicken crop was mounted longitudinally at a resting tension of 0.5 g in an organ bath (10 ml) containing Tyrode's solution at 30 °C. The tissue preparation was equilibrated for 60 min, and was then challenged twice with carbachol (2×10^{-6} M), and once with X-Asn-NH_2 (**1**) added cumulatively up to 3×10^{-6} M. After re-equilibration for 30—40 min, increasing concentrations of **1** were added up to 3×10^{-6} M, and after a 30 min resting period by those of an analog (**2**, **3**, or **4**) up to 3×10^{-6} M. The pharmacological parameters, RA value (relative activity; EC_{50} of **1**/EC_{50} of an analog), maximum contraction (efficacy; %) [(maximum effect induced by 3×10^{-6} M of an analog/maximum effect induced by 3×10^{-6} M of **1**)×100] were calculated from the cumulative concentration–response curves. These experiments were repeated 5 to 7 times.

Immunochemical Reactivity of Neuromedin U Analogs Neuromedin U radioimmunoassay (RIA) system was established in the same manner as reported previously.[12] Briefly, an anti-neuromedin U-8 antiserum (ASH-15) was obtained from a rabbit after ninth subcutaneous injection of bovine serum albumin (BSA)-conjugate of **1** at the N-terminal Tyr, and used for RIA at a final dilution of 30000. Iodination of **1** was carried out with ^{125}I Na by 1,3,4,6-tetrachloro-3α,6α-diphenyl-glycouril (Pierce Co., U.S.A.) and the labeled antigen was purified by RP-HPLC. Separation of free and bound tracers was performed by the dextran-charcoal method.

Results and Discussion

Stability of Porcine Neuromedin U-8 (1) in Tyrode's Solution at 60 °C and in 2% Ammonia Solution at 4 °C The RP-HPLC chromatogram shown in Fig. 2A represents the decomposition mixture of porcine neuromedin U-8 (X-Asn-NH_2, **1**) in Tyrode's solution (pH 7.4) at an elevated temperature of 60 °C. The separation of the decomposition products was attained by RP-HPLC with an isocratic elution of 19% acetonitrile in 0.1% trifluoroacetic acid (TFA) at a low flow rate (0.6 ml/min). The peaks 2 and 3 were analyzed as the partially hydrolyzed peptides of X-Asn-NH_2 either at the α-amide or at the β-amide, since these peaks co-eluted with authentic synthetic peptides, X-Asn-OH (**2**) and X-Asp-NH_2 (**3**), respectively. The size of a small-unknown product peak 4 increased in the first 2—3 h, then decreased, and almost completely disappeared in 10 h.

The degradation and accumulation curves of X-Asn-NH_2 (**1**, peak 1), X-Asn-OH (**2**, peak 2) and X-Asp-NH_2 (**3**, peak 3) are shown in Fig. 2B. The half-life period ($t_{1/2}$) of X-Asn-NH_2 (**1**) was 3.6 h, and the molar ratio of the products X-Asn-OH (**2**) and X-Asp-NH_2 (**3**) was about 4.5 to 1.

The degradation products of peaks 2, 3, and 4 were isolated by RP-HPLC. Peak 4 was assumed to be the aminosuccinimide form (X-Asu, MW $C_{54}H_{75}N_{15}O_{10}$: 1094.282), which was supported by FAB-MS analysis data values of 1094 ($[M+H]^+$). FAB-MS analysis data of materials isolated from peaks 2 and 3 showed the same value of 1112 ($[M+H]^+$), and both peaks co-eluted with synthetic X-Asn-OH (**2**, $C_{54}H_{77}N_{15}O_{11}$; 1112.297) and X-Asp-NH_2 (**3**, $C_{54}H_{77}N_{15}O_{11}$; 1112.297), respectively.

It was observed that the incubation of X-Asn-NH_2 in a 2% ammonia solution at 4 °C led to a greatly increased amount of the unknown product peak 4 (Figs. 2C, D). The time courses for X-Asn-NH_2 (**1**) and its decomposition products in

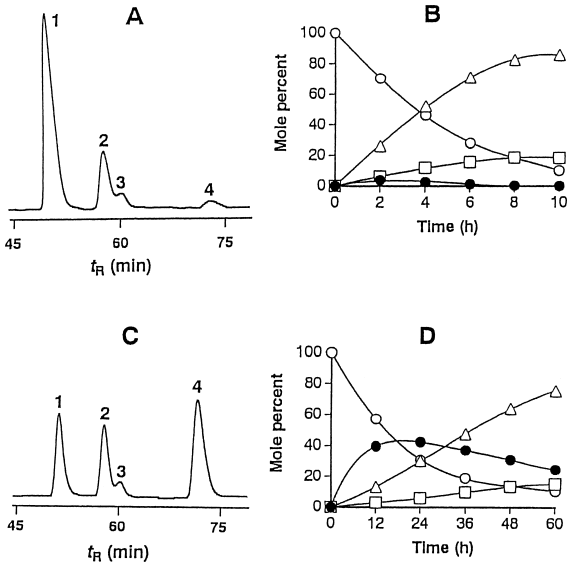

Fig. 2. HPLC Profile and Time Courses for Porcine Neuromedin U-8 and Its Hydrolysates during Incubation in Tyrode's Solution at 60 °C (A, B), and in 2% Ammonia Solution at 4 °C (C, D)

HPLC conditions: column, J'sphere ODS H-80 (4.6×250 mm); elution, 19% MeCN in 0.1% TFA; flow rate, 0.6 ml/min; detection, 210 nm. Peak 1 and ○; X-Asn-NH_2 (**1**), peak 2 and △; X-Asn-OH (**2**), peak 3 and □; X-Asp-NH_2 (**3**), peak 4 and ●; presumably X-Asu.

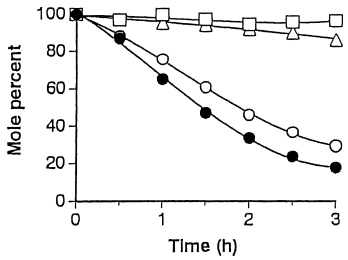

Fig. 3. Degradation Curves of Porcine Neuromedin U-8 and Model Tripeptide Amides in 0.1 M $NaHCO_3$ at 60 °C

●; X-Asn-NH_2 (**1**), ○; Y-Asn-NH_2 (**5**), □; Y-Gly-NH_2 (**9**), △; Y-Gln-NH_2 (**10**).

cold ammonia solution show that the $t_{1/2}$ of X-Asn-NH_2 (**1**) is 15 h, and that X-Asu was considerably stable under these conditions. The molar ratio of X-Asn-OH (**2**) and X-Asp-NH_2 (**3**) was about 4.5 to1.

Stability of Porcine Neuromedin U-8 in 0.1 M $NaHCO_3$ at 60 °C The decomposition reaction of porcine neuromedin U-8 (X-Asn-NH_2, **1**) was examined in a 0.1 M $NaHCO_3$ solution at 60 °C. As seen from the degradation curves in Fig. 3, the reaction proceeded faster than in Tyrode's solution at the same temperature. The half-life period ($t_{1/2}$) of X-Asn-NH_2 (**1**) was 1.5 h. The ratio of the degradation products, X-Asn-OH (**2**) and X-Asp-NH_2 (**3**) was again at about 4.5 to 1 (data not shown). When X-Asu, isolated from peak 4 in the reaction mixture of X-Asn-NH_2 (**1**) in 2% NH_4OH as described above, was kept in 0.1 M $NaHCO_3$ at 60 °C, the degradation reaction proceeded very fast. The $t_{1/2}$ of X-Asu was around 0.3 h, producing X-Asn-OH (**2**) and X-Asp-NH_2 (**3**) in a ratio of about 4.5 to 1. These results confirm that the peak 4 observed in Fig. 2C was the succinimide form. Due to the difficulty associated with separation of octapeptides **1**—**4** by HPLC (Figs. 2A, C) in time course data experiments, smaller model peptides were used for further

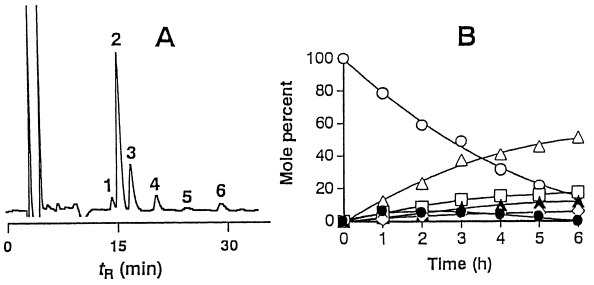

Fig. 4. HPLC Profile (A) and Time Courses (B) for H-Ile-Leu-Asn-NH_2 and Its Hydrolysates during Incubation in 0.1 M $NaHCO_3$ at 60 °C

HPLC conditions: column, J'sphere ODS H-80 (4.6×250 mm); elution, 8.8% MeCN in 0.1% TFA; flow rate, 0.8 ml/min; detection, 210 nm. Peak 1 and ▲; H-Ile-Leu-D-Asn-OH (**20**), peak 2 and ○; H-Ile-Leu-Asn-NH_2 (**16**), peak 3 and △; H-Ile-Leu-Asn-OH (**17**), peak 4 and □; H-Ile-Leu-Asp-NH_2 (**18**), peak 5 and ◇; H-Ile-Leu-D-Asp-NH_2 (**21**), peak 6 and ●; presumably H-Ile-Leu-Asu.

study.

Stability of Model Tripeptide Amides in 0.1 M $NaHCO_3$ at 60 °C In order to simplify the HPLC analysis, a model tripeptide (Y-Asn-NH_2, **5**, Y=Benzoyl-Pro-Arg), related to porcine neuromedin U-8, and its analogs were synthesized and used for the stability test. Y-Asn-NH_2 (**5**) decomposed almost as fast as X-Asn-NH_2 (**1**) in 0.1 M $NaHCO_3$ at 60 °C with the $t_{1/2}$ of 1.9 h (Fig. 3 and Table 3). On the other hand, Y-Gly-NH_2 (**9**) and Y-Gln-NH_2 (**10**) survived to a level of more than 90% after 2.5 h (Fig. 3).

In the case of H-Ile-Leu-Asn-NH_2 (**16**), a model tripeptide amide related to the C-terminal of VIP, minor peaks of the decomposition products were examined in detail. The peaks 1—6 that appeared in the incubation solution of H-Ile-Leu-Asn-NH_2 (**16**) in 0.1 M $NaHCO_3$ at 60 °C (Fig. 4A) were isolated and analyzed by FAB-MS to deduce their structures, which were confirmed by co-elution with authentic synthetic peptides. In independent experiments, the synthetic peptides, H-Ile-Leu-D-Asn-OH (**20**), H-Ile-Leu-Asn-NH_2 (**16**), H-Ile-Leu-Asn-OH (**17**), H-Ile-Leu-Asp-NH_2 (**18**), and H-Ile-Leu-D-Asp-NH_2 (**21**), co-eluted with peaks 1, 2, 3, 4, and 5, respectively. FAB-MS analysis of the isolated material from peak **6** showed the molecular ion of 341, which corresponded to H-Ile-Leu-Asu. Thus, the partial epimerization of H-Ile-Leu-Asu occurred in mild alkaline solution to produce the C-terminal D-Asn and D-Asp peptides (**20**, **21**).

The time courses of these peptides are shown in Fig. 4B. H-Ile-Leu-Asn-NH_2 (**16**) had decomposed by more than 40% after 2.5 h (the $t_{1/2}$ was 2.8 h, Fig. 4B). These results suggest that the C-terminal Asn-NH_2 portions of both neuromedin U-8 and VIP have higher susceptibility to alkaline solution than the other C-terminal amides, Gly-NH_2, and Gln-NH_2.

Comparative Stability Experiment with Model Asn-Gly- and Asn-Ser-Peptides in 0.1 M $NaHCO_3$ at 60 °C A model pentapeptide Y-Asn-Gly-Ala-OH (Y=Benzoyl-Pro-Arg, **11**), which is known to contain a structure involved in formation of a succinimide derivative formation, decomposed rapidly to yield Y-Asp-Gly-Ala-OH (**14**) and Y-Asp(β-Gly-Ala-OH)-OH (**15**), as confirmed by HPLC co-elution with authentic synthetic peptides. The ratio of the decomposed products **14** and **15** was about 4 to 1. A small amount of an unknown peak that appeared on HPLC chromatograms of the reaction mixture was presumed to be the succinimide intermediate (Y-Asu-Gly-Ala-OH). The time courses of peptides produced from Y-Asn-Gly-Ala-OH (**11**) are shown in Fig. 5. The $t_{1/2}$ of the other model pentapeptides (**12**, **13**), the model tripeptide amides (**5**, **9**, **10**, **16**), and porcine neuromedin U-8 (**1**) are summarized in Table 3. It is clear that the peptides bearing the C-terminal Asn-NH_2 (**1**, **5**, and **16** with $t_{1/2}$ values of 1.5, 1.9, and 2.8 h, respectively) have higher susceptibility than Y-Asn-Ser-Ala-OH (**12**, $t_{1/2}$=5.1 h) or Y-Asn-Ala-Ala-OH (**13**, $t_{1/2}$=6.8 h), and higher stability than Y-Asn-Gly-Ala-OH (**11**, $t_{1/2}$ 0.5 h) in 0.1 M $NaHCO_3$ at 60 °C.

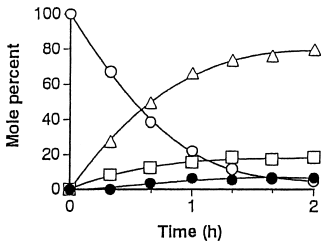

Fig. 5. Time Courses for Benzoyl-Pro-Arg-Asn-Gly-Ala-OH (Y-Asn-Gly-Ala-OH) and Its Hydrolysates during Incubation in 0.1 M $NaHCO_3$ at 60 °C

○; Y-Asn-Gly-Ala-OH (**11**), △; Y-Asp(β-Gly-Ala-OH)-OH (**15**), □; Y-Asp-Gly-Ala-OH (**14**), ●; presumably Y-Asu-Gly-Ala-OH.

Table 3. Half-Life Periods ($t_{1/2}$) of Various Peptides during Incubation in 0.1 M $NaHCO_3$ and 1 M HCl at 60 °C

Peptides	$t_{1/2}$ (h)	
	0.1 M $NaHCO_3$	1 M HCl
X-Asn-NH_2 (**1**)	1.5	1.3
Y-Asn-NH_2 (**5**)	1.9	1.6
Y-Gly-NH_2 (**9**)	—*a)*	1.1
Y-Gln-NH_2 (**10**)	—*a)*	0.8
Y-Asn-Gly-Ala-OH (**11**)	0.5	2.7
Y-Asn-Ser-Ala-OH (**12**)	5.1	3.0
Y-Asn-Ala-Ala-OH (**13**)	6.8	3.0
H-Ile-Leu-Asn-NH_2 (**16**)	2.8	1.4

X=H-Tyr-Phe-Leu-Phe-Arg-Pro-Arg, Y=Benzoyl-Pro-Arg. *a)* $t_{1/2} \gg 3$ h (see Fig. 3).

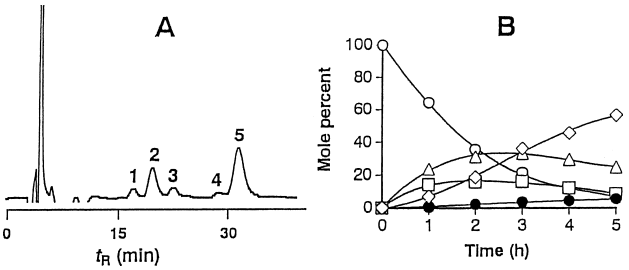

Fig. 6. HPLC Profile (A) and Time Courses (B) for Benzoyl-Arg-Pro-Asn-NH_2 (Y-Asn-NH_2) and Its Hydrolysates during Incubation in 1 M HCl at 60 °C

Peak 1 and ○; Y-Asn-NH_2 (**5**), peak 2 and △; Y-Asn-OH (**6**), peak 3 and □; Y-Asp-NH_2 (**7**), Peak 4 and ●; Y-OH, peak 5 and ◇; Y-Asp-OH (**8**).

The Stability of Neuromedin U-8 and Model Peptides in 1 M HCl at 60 °C Incubation of Y-Asn-NH_2 (**5**) in 1 M HCl at 60 °C for 5 h yielded Y-Asn-OH (**6**), Y-Asp-NH_2 (**7**), and Y-Asp-OH (**8**), as shown in Fig. 6A. A small peak 4 was observed, corresponding to Y-OH, a product of cleavage of an internal peptide bond. No peak corresponding to Y-Asu was observed. The time courses of the peptides are shown in

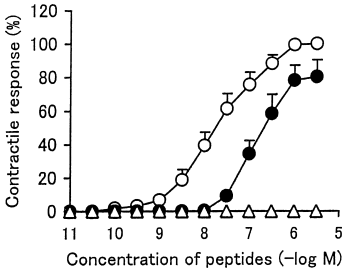

Fig. 7. Contractile Activity of Porcine Neuromedin U-8 and the C-Terminal Degradation Products on Isolated Chicken Crop Smooth Muscle Preparation

○; X-Asn-NH_2 (**1**), ●; X-Asp-NH_2 (**3**), △; X-Asn-OH (**2**) and X-Asp-OH (**4**).

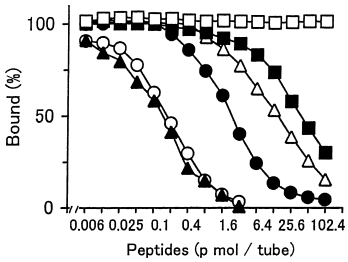

Fig. 8. Immunochemical Reactivity of Various Neuromedin U Analogs in a Neuromedin U Radioimmunoassay

○; X-Asn-NH_2 (**1**), ▲; porcine neuromedin U-25, ●; neuromedin U-8 (2—8) (H-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH_2), △; neuromedin U-8 (3—8) (H-Leu-Phe-Arg-Pro-Arg-Asn-NH_2), ■; neuromedin U-8 (4—8) (H-Phe-Arg-Pro-Arg-Asn-NH_2), □; X-Asn-OH (**2**), X-Asp-NH_2 (**3**), X-Asp-OH (**4**), neuromedin U-8 (5—8) (H-Arg-Pro-Arg-Asn-NH_2), porcine neuromedin U-8 (1—7)-OH H-Tyr-Phe-Leu-Phe-Arg-Pro-Arg-OH, neuromedin U-8 (1—7)-NH_2 (H-Tyr-Phe-Leu-Phe-Arg-Pro-Arg-NH_2) and [Gly^8]-neuromedin U-8 (H-Tyr-Phe-Leu-Phe-Arg-Pro-Arg-Gly-NH_2).

Fig. 6B; the amount of Y-Asp-OH (**8**) increased almost linearly with time. The amount of the model pentapeptide Y-Asn-Gly-Ala-OH (**11**) decreased under these conditions to yield a single product, Y-Asp-Gly-Ala-OH (**14**). As shown in Table 3, the degradation rates of the model pentapeptides Y-Asn-Gly-Ala-OH (**11**), Y-Asn-Ser-Ala-OH (**12**) and Y-Asn-Ala-Ala-OH (**13**), were similar with $t_{1/2}$ values of 2.7—3.0 h, while the $t_{1/2}$ of Y-Asn-NH_2 (**5**) was 1.9 h. These Asn-containing peptides were more stable than Y-Gly-NH_2 (**9**, $t_{1/2}$=1.1 h) and Y-Gln-NH_2 (**10**, $t_{1/2}$=0.8 h). The $t_{1/2}$ of neuromedin U-8 (**1**) was 1.3 h.

Smooth Muscle Contractile Activity and Immunochemical Cross-Reactivity of C-Terminally Deamidated Analogs of Porcine Neuromedin U-8 The biological activity of the deamidated peptides X-Asn-OH (**2**), X-Asp-NH_2 (**3**), and X-Asp-OH (**4**) was evaluated by chicken crop smooth muscle contraction assay. The deamidation products of the α-carboxamide (**2**, **4**) underwent a complete loss of the biological activity. X-Asp-NH_2 (**3**) retained a weak biological activity but the dose-response curves shifted to the right, and the maximum contractile response elicited at 3×10^{-6} M was 80% of X-Asn-NH_2 (**1**) (Fig. 7).

It was found that the neuromedin U-8 RIA system employing the antiserum ASH-15 was C-terminal specific, because the synthetic N-terminal analogs such as porcine neuromedin U-25[1,13] (Fig. 8) displaced the tracer, ^{125}I-X-Asn-NH_2, in the same manner as X-Asn-NH_2 (**1**). The same result was obtained for rat neuromedin U-23,[5,14,15] dog neuromedin U-8 bearing pGlu at the N-terminal,[6,16] and [Gly^1]-neuromedin U-8,[17] (data not shown). The displacement curves of synthetic N-terminally deleted analogs[5] such as H-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH_2, H-Leu-Phe-Arg-Pro-Arg-Asn-NH_2, and H-Phe-Arg-Pro-Arg-Asn-NH_2 showed reduced but considerable cross-reactivity. The C-terminally deleted analogs H-Tyr-Phe-Leu-Phe-Arg-Pro-Arg-OH[6] and [Gly^8]-neuromedin U-8[17] showed no activity in displacing the tracer. In this neuromedin U RIA system, the deamidation products **2**, **3**, and **4** had no cross-reactivity.

The RP-HPLC examination of the reaction mixture of X-Asn-NH_2 (**1**) after standing in Tyrode's solution verified that the main decomposed products were X-Asn-OH (**2**) and Asp-NH_2 (**3**). No peak corresponding to X-Asp-OH (**4**) appeared, however, a small peak corresponding to a succinimide intermediate derivative (X-Asu) was observed. This intermediate appeared as a major product after 24 h incubation in a cold diluted ammonia solution (Fig. 2), where the conversion of X-Asu to X-Asn-OH (**2**) and X-Asp-NH_2 (**3**) was slower, but the ratio (4.5 : 1) of the monocarboxylic derivatives after 60 h was the same as in Tyrode's or 0.1 M $NaHCO_3$ solution. The mechanism was considered to proceed through succinimide formation, with spontaneous hydrolysis promoting the degradation of neuromedin U and VIP-related peptides, specifically at their C-terminal Asn-NH_2 portions. The model peptides bearing the C-terminal Gln-NH_2 and Gly-NH_2 were considerably stable in a weak alkaline solution. Thus, glutarimide formation from peptidyl-Gln-NH_2 seemed to occur very slowly, in the same manner as previously reported for a synthetic pentapeptide.[18] A model tripeptide amide related to VIP (H-Ile-Leu-Asn-NH_2, **16**) was much more stable than a model peptide related to neuromedin U-8 (Benzoyl-Arg-Pro-Asn-NH_2, **5**). These results suggest that the stability of the C-terminal Asn-NH_2 portion was somewhat dependent on the preceding sequence of the Asn residue. Although the deamidation of Asn-Gly or Asn-Ser peptides *via* succinimide intermediates has previously been studied in detail,[19—22] the present results demonstrate that the degradation of the C-terminal Asn-NH_2 occurred faster than that of Asn-Ser and slower than that of Asn-Gly in 0.1 M $NaHCO_3$. Further examination employing H-Ile-Leu-Asn-NH_2 suggest that the degradation of Asn-NH_2 through Asu formation was accompanied by racemization, as has previously been reported for a synthetic L-Asn-Gly peptide.[23]

In a 1 M HCl solution at 60 °C, the first deamidation of Y-Asn-NH_2 (**5**) produced α- and β-monocarboxylic acids (Y-Asn-OH, **6** and Y-Asp-NH_2, **7**), which decreased after 3 h due to a second hydrolysis that brought about an increase in the dicarboxylic acid (Y-Asp-OH, **8**). Y-Asn-Gly-Ala-OH (**11**), the most susceptible peptide in a weak alkaline solution, was more stable than the three C-terminal amide peptides, Y-Asn-NH_2 (**5**), Y-Gly-NH_2 (**9**), and Y-Gln-NH_2 (**10**), in this mild acidic solution (Table 3). Mild acid hydrolysis of Y-Asn-Gly-Ala-OH (**11**) produced exclusively Y-Asp-Gly-Ala-OH (**14**), and no peak related to Y-Asp(β-Gly-Ala-OH)-OH (**15**) appeared on the HPLC chromatogram of the hydrolysate (data not shown). This result is in good agreement with that by Patel and Borchardt.[21] Thus, it can be concluded that the hydrolysis of C-terminal Asn-NH_2 in 1 M HCl proceeded independently at the α- and β-amides through an alternative pathway than alkaline hydrolysis.

The present study indicated that the C-terminal asparagine amide portions of neuromedin U and VIP were exceptionally

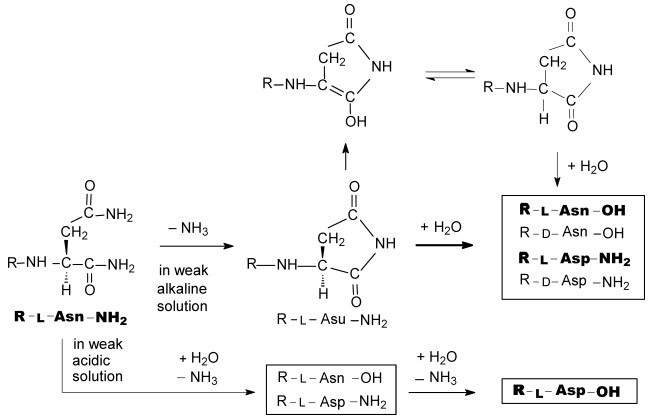

Fig. 9. Dergadation Pathway of C-Terminal Asparagine Amide Peptide (R-Asn-NH_2) under Mild Alkaline and Acid Solutions

unstable in a weak alkaline solution. The decomposition mechanism involved formation of succinimide intermediates followed by spontaneous hydrolysis (Fig. 9), in a pathway similar to the deamidation of Asn-Gly or Asn-Ser peptides. The degradation of the C-terminal Asn-NH_2 occurred faster than that of Asn-Ser but slower than that of Asn-Gly in a weak alkaline solution. Acid hydrolysis of the C-terminal Asn-NH_2 in 1 M HCl proceeded faster than that of the internal peptide bonds to a similar extent to the other primary amides to yield Asn-OH and Asp-NH_2 peptides, both of which were converted into the C-terminal Asp-OH peptides. As the deamidation at the C-terminal of neuromedin U peptides brought about a great loss in the biological and immunochemical binding activities, the chemical characteristics of neuromedin U peptides with respect to the C-terminal Asn-NH_2 structure should be kept in mind in the handling of their solutions during biological and immunological study.

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