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Physalaemin- and Bombesin-like Peptides in the Skin of the Australian Leptodactylid Frog *Uperoleia rugosa*

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Biologically active peptides of the tachykinin and bombesin families occurring in the skin of the Australian leptodectylid frog *Uperoleia rugosa* were isolated in pure forms and their structures were determined. Uperolein-like peptide 1 was identified, as expected, as authentic uperolein; uperolein-like peptide 2 was found to have the sequence Pyr–Ala–Asp–Pro–Lys–Thr–Phe–Tyr–Gly–Leu–Met–NH_2 and may be considered a Lys^5, Thr^6-physalaemin. Bombesin-like peptide 1, in its turn, was identified as Glu(OEt)^2-litorin, and bombesin-like peptide 2 as authentic litorin. Uperolein and litorin had previously been identified in *Uperoleia rugosa* and *Litoria aurea*, respectively; the other two peptides are new. Glu(OEt)^2-litorin and Lys^5, Thr^6-physalaemin appear to have biological activities very similar to those of Glu(OMe)^2-litorin and physalaemin (or uperolein), respectively.

Keywords——*Uperoleia rugosa*; Australian leptodactylid frog; frog skin; tachykinin; uperolein; litorin; physalaemin; Lys^5,Thr^6-physalaemin; Glu(OEt)^2-litorin; active peptide

It was shown in preceding papers[2,3] that methanol extracts of the skin of the Australian leptodactylid frog *Uperoleia rugosa* contained several highly active polypeptides belonging to the tachykinin and bombesin families.

The structure of one of the two tachykinins occurring in the skin was elucidated in 1975. Uperolein was shown to be an endecapeptide with the following sequence.

Pyr–Pro–Asp–Pro–Asn–Ala–Phe–Tyr–Gly–Leu–Met–NH_2

(uperolein)

It differed from physalaemin only in having Pro^2 and Ala^6 residues in place of the Ala^2 and Lys^6 residues.

1) Locations: *a) Kasumi, Hiroshima, 734, Japan; b) Citta Universitaria, 00100, Rome, Italy; c) St. Lucia, Brisbane, 4067, Australia.*

2) A. Anastasi, V. Erspamer, and R. Endean, *Experientia*, 31, 394 (1975).

3) V. Erspamer, L. Negri, G. Falconieri Erspamer, and R. Endean, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 289, 41 (1975).

In this paper, the purification and structure elucidation of the second tachykinin (provisionally called Rogosauperolein II) will be discussed, as well as those of two bombesin-like peptides. One of them was litorin, and the other Glu(OEt)^2-litorin.

It is of interest that another leptodactylid Australian frog, *Litoria aurea*, contained litorin[4] and Glu(OMe)^2-litorin[5] in the skin.

Experimental

Amphibian Material——In all 1775 specimens of *Uperoleia rugosa* were captured in Queensland (Brisbane region) and in New South Wales during the period 1972—1975. The skin, removed from the animals immediately after sacrifice, were spread out and dried in the shade. Soon after their arrival in Italy, the dried skins (113.25 g; 0.063 g/skin) were cut into small pieces with scissors and immersed in 20 times their weight of 80% methanol. The liquid was decanted after a week and the skins were treated for another week with a similar amount of the solvent. The combined extracts were stored in a refrigerator.

Reagents and General Procedures——Alkaline alumina, activity grade I, was obtained from Merck, Darmstadt; silica gel H plates were hand made, and were activated at 110° for 1 hr before use; all reagents employed were of analytical grade.

Enzymes used were as follows: γ-chymotrypsin, Worthington Biochemicals, 83 units/mg protein; TPCK-trypsin, Worthington Biochemicals, 185 units/mg protein; carboxypeptidase A, Worthington Biochemicals, DFP-treated, 666 units/mg protein, dissolved in a 10% lithium chloride solution; amino peptidase M, Protein Research Foundation, Osaka, 10 units/vial; L-pyrrolidonecarboxylatepeptidase, a gift from Prof. D. Tsuru, Nagasaki University, prepared from *Bacillus amyloliquefaciens*, (the enzyme solution split pyrrolidonecarboxyl β-naphthylamide at a rate of 0.23 μmol/ml/min).[6]

Synthetic litorin, Glu(OMe)^2-litorin and uperolein were kindly provided by the Farmitalia S.P.A. Research Laboratories, Milan.

Dansylation: procedure A, described in a previous paper,[7] was employed for the peptides containing tyrosyl or histidyl residues; in other cases procedure B was used.

The dansyl-Edman procedure was performed according to the method of Gray;[8] all the reagents were redistilled, sealed under nitrogen and stored in a refrigerator.

Glass equipment, including tubes for chromatography, was siliconized with dimethyldichlorosilane and methanol.

Results and Discussion

Separation of Bombesin-like and Uperolein-like Peptides by Alumina Column Chromatography

An aliquot of the crude methanol extract corresponding to 100 g of skin was evaporated to dryness and the syrupy residue was washed with petroleum ether to remove fats, then dissolved, by stirring in a water bath (40°), in 400 ml of 95% ethanol. The liquid was loaded on 4 columns of alumina (each of 170 g) which were then eluted with ethanol-water mixtures (200 ml each) containing decreasing concentrations of ethanol.

Bombesin-like peptides emerged in the 95% and 90% ethanol eluates. Parallel bioassay showed that two different peptides were present in the eluates. The first, more abundant one, emerged in the 95_2 and 90_1 eluates and the second in the 90_2 eluate. The 95_2+90_1 and 90_2 eluates were separately rechromatographed on alumina columns, which were then each eluted with 600 ml of 95% ethanol (95_1, 95_2, 95_3) and 600 ml of 90% ethanol (90_1, 90_2, 90_3). Litorin-like peptide 1 emerged in 95_1 and 95_2 eluates, and litorin-like peptide 2 in the 90_1 and 90_2 eluates. Litorin-like activity expressed as litorin on a rat uterus preparation, was 4—5 mg in the 95_1+95_2 eluates, and 1.5—2 mg in the 90_1+90_2 eluates.

The uperolein-like peptides emerged in the 60% and 50% ethanol eluates. Authentic uperolein (peptide U-1) was present mainly in the 60% eluate, which was not used in this study.

4) A. Anastasi, V. Erspamer, and R. Endean, *Experientia*, **31**, 510 (1975).
5) A. Anastasi, P. Montecucchi, F. Angelucci, V. Erspamer, and R. Endean, *Experientia*, **33**, 1289 (1977).
6) D. Tsuru, K. Fujikawa, and K. Kado, *J. Biochem.*, **83**, 467 (1978).
7) Z. Tamura, T. Nakajima, T. Nakayama, J.J. Pisano, and S. Udenfriend, *Anal. Biochem.*, **52**, 595 (1973).
8) W.R. Gray, "Methods in Enzymology," Vol. XI, ed. by C.H.W. Hirs, Academic Press, New York, London, 1967, p. 469.

The second uperolein-like peptide (peptide U-2), which was, however, contaminated by a considerable amount of uperolein, emerged in the 50% eluate. Uperolein could be distinguished from the second tachykinin by paper electrophoresis; at pH 1.2, the electrophoretic mobility of uperolein was 0.13 Glu, while that of U-2 was 0.44 Glu. The total content of U-2 expressed as uperolein, as estimated from its action on a guinea pig ileum preparation, was approximately 5 mg.

Litorin-like Peptide (L-1). Isolation and Sequence Analysis

One-eighth of the ethanol eluates 95_1+95_2 from the alumina columns was employed for the purification of L-1. The procedures are summarized in Chart 1.

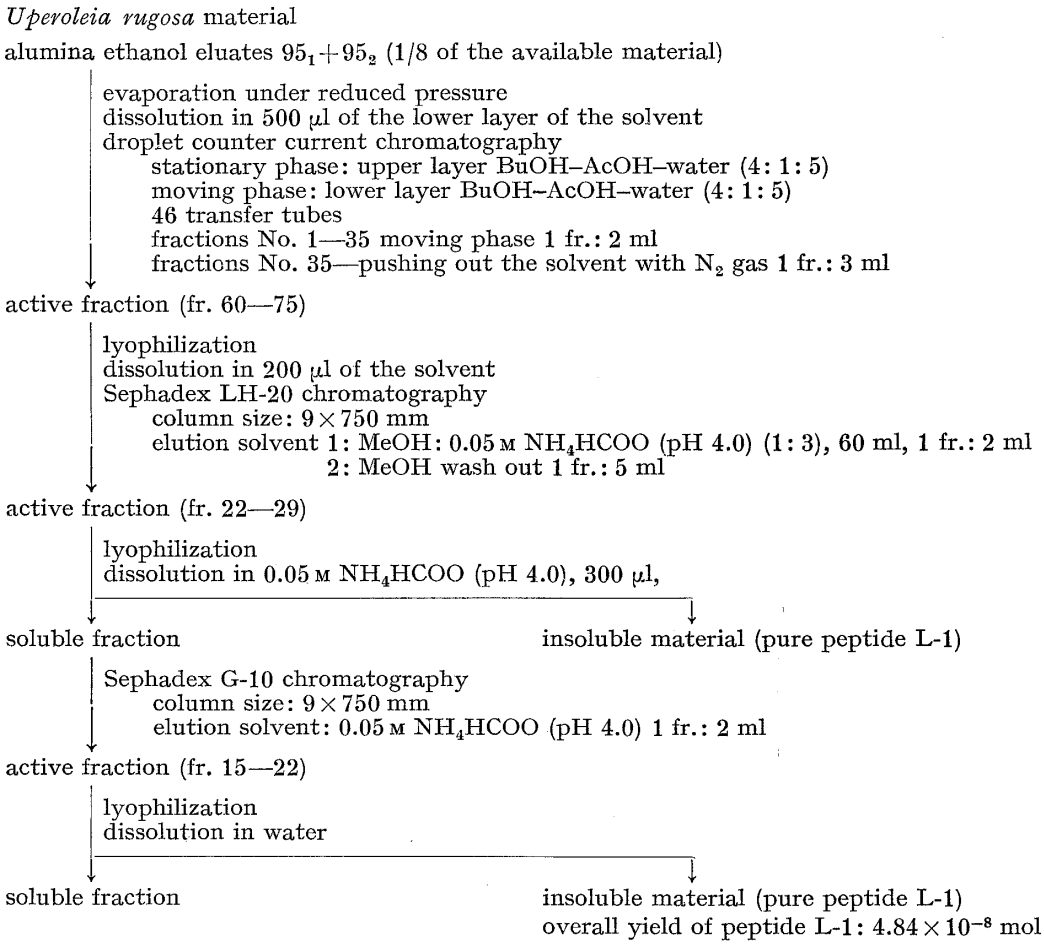

Chart 1. Purification of Litorin-like Peptide 1(L-1)

Peptide L-1 became insoluble in distilled water or dilute salt solution as it was purified. The insoluble material in the lyophilizate of the active fractions from Sephadex LH-20 consisted essentially of pure peptide L-1 according to the results of amino acid analysis after hydrolysis with 6 N HCl at 110° for 24 hr. The amino acid composition was as follows: His_1, Glu_2, Gly_1, Ala_1, Val_1, Met_1, Phe_1, Trp (UV absorption)$_1$. The N-terminal amino acid could not be detected by the dansyl method.

Peptide L-1(2.5 μg) was dissolved in 50 μl of 0.1 N triethylamine bicarbonate buffer (pH 8.0). After the addition of 10 μl of γ-chymotrypsin (100 μg/ml) the mixture was incubated at 37° for 10 hr. The material was dansylated and subjected to thin layer chromatography on silica gel H. One of the dansyl fragments was identified as dansyl methionine amide both on silica gel H using an ether: acetone (9: 1) solvent system, and by polyamide layer chromatography, benzene: acetic aicd (9: 1).

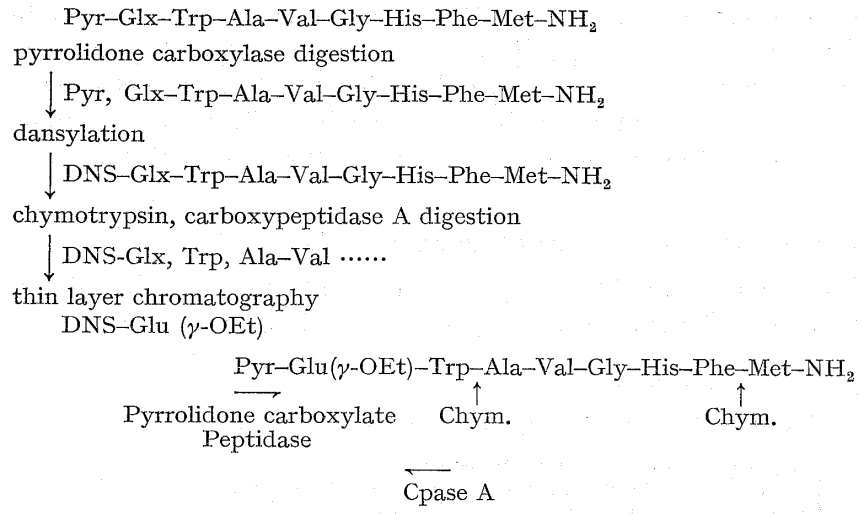

Chart 2. a Procedure for Detection of the γ-Ethyl Ester of Glutamic Acid at Position 2 of L-1

The other dansylated fragment had the same *Rf* value as DNS-Ala-Val-Gly-His-Phe on silica gel H using two different solvent systems: *n*-butanol: acetic acid: water (4:1:5) and isopropanol: methyl acetate: 28% ammonia (9: 7: 4).

Pyrrolidone carboxylate peptidase (EC 3.4.11.8) from *Bacillus amyloliquefaciens* hydrolyzes the N-terminal pyrrolidone carboxyl bond and liberates pyrrolidone carboxylic acid from the peptide.[6] A 5 μl aliquot of the enzyme solution (sp. act., 0.23 μmol/ml/min), diluted with 20 μl of 0.05 N ammonium formate (pH 6.5) containing 1 mM EDTA and 1 mM mercaptoethanol, was added to 2.5 μg of peptide L-1 and the mixture was incubated at 37° for 4.5 hr. The reaction mixture was dansylated and the fluorescent fragment was separated by thin layer chromatography on silica gel H, using *n*-butanol: acetic acid: water (4:1:5). The purified dansyl fragment was further digested at 37° for 13 hr with a mixture of γ-chymotrypsin (5 μg) and carboxypeptidase A(5 μg) in 40 μl of 0.1 N triethylamine bicarbonate buffer (pH 8.2) (Chart 2). The digested dansyl fragment showed, on polyamide layer chromatography, the same *Rf* value as γ-ethyl glutamic acid, as shown in Fig. 1.

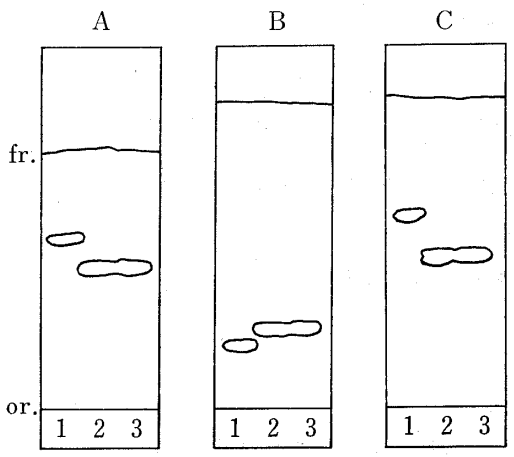

Fig. 1. Polyamide Layer Chromatogram of the Dansyl Derivatives of Glutamic Acid γ-Methyl, γ-Ethyl Ester and the DNS-Fragment from L-1

Solvent systems:
A, 1% NH_4OH; B, benzene: acetic acid (9: 1);
C, 1.5% formic acid.
1, DNS-Glu (γ-OMe); 2, DNS-fragment 3, DNS-Glu (γ-OEt).

In a similar way, DNS-Glu(γ-OMe) was obtained from synthetic Glu(OMe)^2-litorin.

Based on the above experimental results, it was concluded that peptide L-1 was Glu(OEt)^2-litorin.

Litorin-like Peptide 2(L-2). Identification with Litorin

Peptide L-2 was purified as shown in Chart 3. This peptide also became insoluble in water with the progress of purification.

The amino acid composition determined after acid hydrolysis was the same as that of litorin. Moreover, peptide L-2 showed the same *Rf* value as litorin in thin layer chromatography on silica gel H with *n*-butanol: acetic acid: water (4: 1: 5) and *n*-butanol: pyridine: acetic acid: water (4: 1: 1: 1) (Fig. 2).

Uperoleia rugosa material
alumina ethanol eluates 90_1+90_2 (1/8 of the available material)
↓ evaporation under reduced pressure
dissolution in 1 ml of 40% MeOH
Sephadex LH-20 chromatography
column size: 9×750 mm
elution solvent 1: 40% MeOH, 1 fr.: 3 ml
2: MeOH, 1 fr.: 3 ml
active fraction (fr. 19—23)
↓ lyophilization
dissolution in 200 μl of 0.1 N acetic acid
Sephadex G-10 chromatography
column size: 9×750 mm
elution solvent: 0.1 N acetic acid, 1 fr.: 2 ml
active fraction (fr. 16—21)
↓ lyophilization
dissolution in 1 ml of water
soluble fraction
insoluble material (pure peptide L-2) 3.5×10^{-9} mol
↓ Sp-Sephadex (NH_4^+) chromatography
column size: 6×30 mm
elution solvent: H_2O
adjustment of pH to 7.4 with dil. NH_4OH 1 fr.: 1 ml
active fraction (fr. 6—8)
(pure peptide L-2) 8.0×10^{-9} mol

Chart 3. Purification of Litorin-like Peptide 2(L-2)

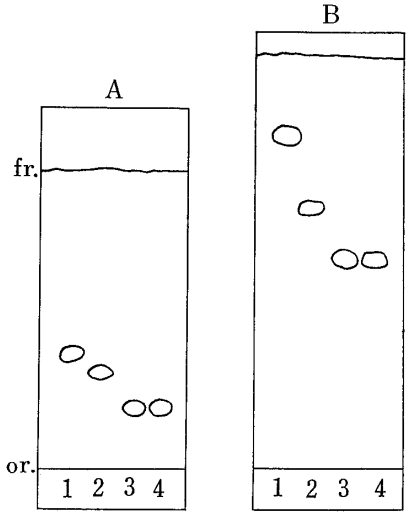

Fig. 2. Thin Layer Chromatogram of Peptide L-2, Litorin and Its Derivatives

Solvent systems:
A, *n*-BuOH: AcOH: H_2O (4: 1: 5);
B, *n*-BuOH: Pyr: AcOH: H_2O (4: 1: 1: 1).
Iodine staining
1, Glu(OEt)²litorin; 2, Glu(OMe)²litorin; 3, peptide L-2; 4, litorin.

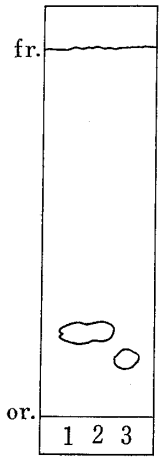

Fig. 3. Thin Layer Chromatogram of Uperolein and Peptides U-1 and U-2

Solvent system:
n-BuOH: AcOH: H_2O (4: 1: 1).
Iodine staining
1, peptide U-1; 2, uperolein; 3, peptide U-2.

Peptide L-2 was digested with γ-chymotrypsin and dansylated as described for peptide L-1. DNS-Ala-Val-Gly-His-Phe and DNS-Met-NH_2 were identified in the reaction products by thin layer chromatography on silica gel H.

Peptide L-2 was treated with pyrrolidone carboxylate peptidase, dansylated and then digested with a mixture of γ-chymotrypsin and carboxypeptidase A. Dansyl glutamine was detected by thin layer chromatography.

These results show that peptide L-2 is identical with litorin.

Purification of the Uperolein-like Peptides

The mixture of uperolein-like peptides in the 50% ethanol eluates from an alumina column could be separated into two active peptide fractions: uperolein-like peptide 1(U-1) and uperolein-like peptide 2(U-2).

U-1 was not adsorbed on an SP-Sephadex column; U-2 was eluted at approximately 0.3 N ammonium formate in a linear gradient. The two peptides were further purified as shown in Chart 4.

Upon silica gel H chromatography using *n*-butanol: acetic acid: water (4: 1: 1), U-1 showed the same *Rf* value as uperolein, whereas U-2 had a lower *Rf* value (Fig. 3).

Identification of Peptide U-1 with Uperolein

Peptide U-1 had the same amino acid composition as uperolein.

Peptide U-1 and uperolein were separately treated with sodium in liquid ammonia as follows. Peptide U-1 (4 μg) was dissolved in a small stoppered glass tube, in 5 μl of absolute methanol, then 100 μl of liquid ammonia was added to the solution under cooling with dry ice

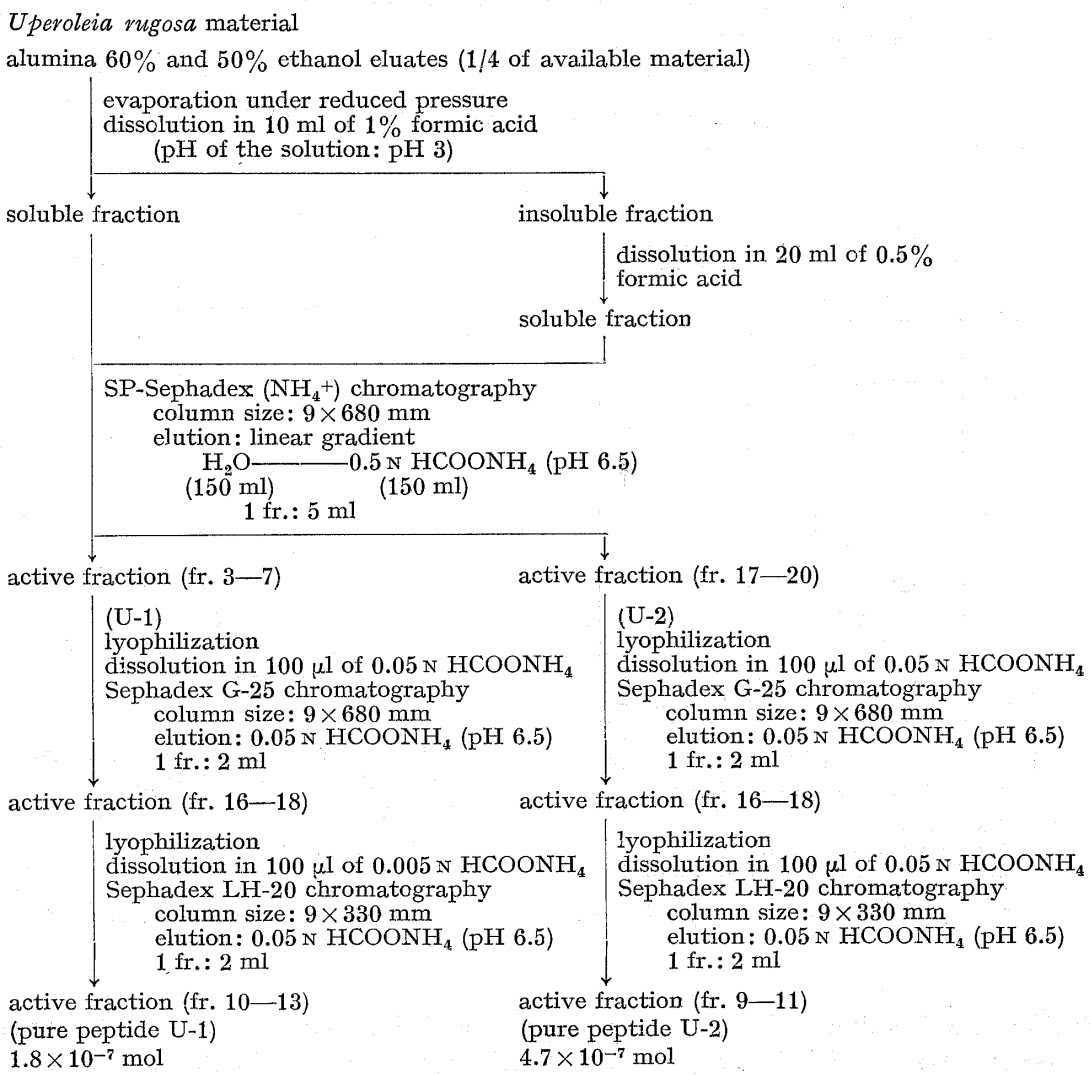

Chart 4. Purification of the Uperolein-like Peptides

acetone. Next, approximately 500 μg of sodium in a glass capillary was quickly introduced into the tube, and the metal was dissolved, with occasional agitation. The mixture was left for 10 min until the blue color diminished.

The reaction was stopped by adding a small piece of ammonium carbonate, and the liquid ammonia was evaporated off under a nitrogen stream.

An aliquot of the reaction mixture was hydrolyzed for 24 hr at 110° with 6 N HCl. No glutamic acid or aspartic acid was found in the hydrolysate, suggesting the presence of Glu-Pro and Asp-Pro bonds.

Another aliquot of the reaction products was dansylated. A fluorescent fragment was obtained which showed the same *Rf* value, upon silica gel H chromatography, as the corresponding product from uperolein.

Although the amino acid composition of the dansylated fragment of U-1 was not studied, the above data suggest that peptide U-1 is identical with uperolein.

Sequence Analysis of Peptide U-2

The amino acid composition of peptide U-2 was as follows: Lys_1, Asp_1, Thr_1, Glu_1, Pro_1, Gly_1, Ala_1, Met_1, Leu_1, Tyr_1, and Phe_1. The composition is similar to that of physalaemin, but with one Asp residue less and one Thr residue more. The N-terminal amino acid could not be detected by the dansyl method. On treating U-2 with sodium-liquid ammonia as described above, the Asp residue was lost, suggesting the presence of an Asx-Pro bond.

Peptide U-2 (5 μg) was dissolved in 50 μl of a γ-chymotrypsin solution (1 mg/ml 0.1 N triethylamine bicarbonate, pH 8.2) and incubated at 35° overnight. The reaction product was dansylated and then chromatographed on a thin layer of silica gel H using dichloromethane: methyl acetate: methanol (20: 20: 4). One of the fluorescent peptide fragments showed the same *Rf* value as DNS-Gly-Leu-Met-NH_2, *i.e.* the chymotryptic fragment of physalaemin. This suggests that the C-terminal tripeptide of U-2 is the same as that of physalaemin.

Peptide U-2(2.5 μg) was dissolved in 50 μl of a TPCK-trypsin solution (100 μg/ml 0.1 N triethylamine bicarbonate, pH 8.2) and incubated at 37° for 5 hr. After incubation, the mixture was dansylated and chromatographed on silica gel H. Two fluorescence bands(T-1, upper band; T-2, lower band) were observed. Each fluorescent peptide was purified and hydrolyzed with 6 N HCl for 16 hr at 90°. DNS-Thr was identified in the acid hydrolysate of T-1, while ε-DNS-Lys was identified in the hydrolysate of T-2, suggesting that T-2 occupies the N-terminal portion of the peptide, and indicating the presence in the peptide of a Lys-Thr bond.

Peptide U-2(10 μg) was dissolved in 50 μl of 0.05 N ammonium formate(pH 6.5) containing 1 mM EDTA and 0.5 mM mercaptoe thanol, and 5 μl of pyrrolidone carboxylate peptidase solution was added. The mixture was incubated at 35° overnight then subjected to the dansyl Edman procedure, which permitted the following sequence to be established:

Pyr–Ala–Asx–Pro–Lys–Thr–Phe–Tyr

Asx at position 3 was considered to be the free acid in view of its electrophoretic behavior. Thus, the sequence of the Peptide U-2 appeared to be

Pyr–Ala–Asp–Pro–Lys–Thr–Phe–Tyr–Gly–Leu–Met–NH_2

i.e. that of Lys^5, Thr^6-physalaemin or Pro^2, Lys^5, Thr^6-uperolein.

Preliminary experiments indicated that $Glu(OEt)^2$-litorin has a spectrum of biological activity very similar to that of $Glu(OMe)^2$-litorin. Similarly, Lys^5, Thr^6-physalaemin is a tachykinin closely related, even from a biological point of view(including its potent sialogenic activity), to physalaemin and to uperolein.