terminal tripeptide C_2, then a bond was split between the C end of the N-terminal pentapeptide and the amino group of lysine. Upon further action of the enzyme, tyrosine was slowly released from C_4 and finally a very slow and incomplete splitting of methionamide from C_2 was observed.

Tryptic digestion was anomalous, since two bonds were split, one of which was a lysil and the other a tyrosil bond, and the rapid and simultaneous formation of the following three peptides was observed:

T_1 [Glu, Ala, 2 Asp, Pro, Lys], T_2 [Phe, Tyr] and T_3 [Gly, Leu, Met]

From the above results the sequence of the C-terminal hexapeptide was deduced, the position of the single residues being established by the specificity of the enzymes and by the composition of the fragments. The sequence Gly-Leu was determined by the fluorodinitrobenzene procedure on the tripeptide Gly-Leu-Met-NH_2.

Under the action of carboxypeptidase lysine was slowly split from the tryptic hexapeptide T_1, thus confirming the position of lysine already deduced by the specificity of trypsine cleavage. The sequence of the five N-terminal amino acids was found by submitting T_1 to partial acid hydrolysis with 0.5 *N* acetic acid. The presence of the following fragments was consecutively observed in the acid hydrolysates:

Ac_1 [Glu, Ala], Ac_2 [H-Asp-$(OH)_2$], Ac_3 [Pro, Asp(NH_2)]-Lys-OH, Ac_4 Pyr-OH, Ac_5 H-Ala-OH, Ac_6 [Pro, Asp$(NH)_2$] and H-Lys-OH

The sequence -Pro-Asp-NH_2 was deduced by the positive reaction to isatine of the spots Ac_3 and Ac_6. The status of the glutamyl and aspartyl residues was established by the relative electrophoretic mobilities of the fragments in which they occurred.

Pharmacological actions. Physalaemin is a powerful vasodilator and hypotensive agent in dog and rabbit. It is 3 to 4 times as active as eledoisin. In the dog the i.v. threshold hypotensive dose is approximately 1 ng/kg. The pressure fall is proportional, both in intensity and even more in duration, to the dose of the polypeptide, and tachyphylaxis is completely lacking. A persistent, controllable lowering of blood pressure may be obtained with i.v. infusion of physalaemin, the threshold being 2–5 ng/kg/min. An appreciable lowering of blood pressure is also produced by subcutaneous doses of 1 µg/kg physalaemin. In the majority of experiments, hypotension produced by medium i.v. doses of physalaemin is of shorter duration than that produced by equiactive doses of eledoisin.

1 µg physalaemin is capable of counteracting completely the hypertensive action of 50–100 µg L-adrenaline or L-noradrenaline, 10–15 µg angiotensin, 30–50 µg Lys-8-vasopressin, or 3 mg nicotine. On a weight basis physalaemin is 100–700 times more potent than bradykinin, and 200–400 times more than histamine. The effect on the blood pressure of the dog and rabbit of 1 µg physalaemin is equivalent to 100–200 units of substance P.

Both in the intact and in the decapitated chicken, physalaemin is predominantly hypertensive. Pressure rise seems to be mainly due to liberation of catecholamines.

In increasing the permeability of the skin vessels of the human forearm, physalaemin is slightly more active than eledoisin[8].

Generally, physalaemin stimulates the same extravascular smooth muscles which are stimulated by eledoisin but, with the exception of the rabbit large intestine and the guinea-pig ileum, the effect is weaker than with eledoisin and tachyphylaxis is not infrequent. The rat uterus is poorly sensitive to physalaemin, and even the rabbit uterus is several times less responsive to physalaemin than to eledoisin.

Large doses of physalaemin stimulate salivary secretion in the dog, but this effect seems less intense compared with eledoisin.

Whole blood slowly inactivates physalaemin, homogenates of liver and kidney far more quickly. Generally, physalaemin is inactivated more rapidly than eledoisin[9].

In parallel assays physalaemin may clearly be distinguished from known natural polypeptides active on plain muscle, including substance P and eledoisin, which in most of their biological properties are very similar to physalaemin.

Physalaemin or strictly related polypeptides are also contained in the skin extracts of *Physalaemus centralis*, *Physalaemus bresslaui* and *Physalaemus cuvieri*.

Full reports of the experiments and results described in this paper will be published elsewhere.

Riassunto. Viene descritto il procedimento che ha permesso l'isolamento e il chiarimento della struttura chimica della *fisalemina*, il principale dei polipeptidi attivi della pelle di *Physalaemus fuscumaculatus*, anfibio dell'Argentina. La fisalemina, strettamente vicina all'eledoisina anche da un punto di vista chimico è, come questa, dotata di potente azione sulla muscolatura liscia vasale ed extravasale. Essa è nettamente distinguibile, mediante saggi paralleli, da tutti gli altri polipeptidi biogeni finora noti.

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[8] G. DE CARO and L. FARRUGGIA, to be published.

[9] M. B. NOBILI, to be published.

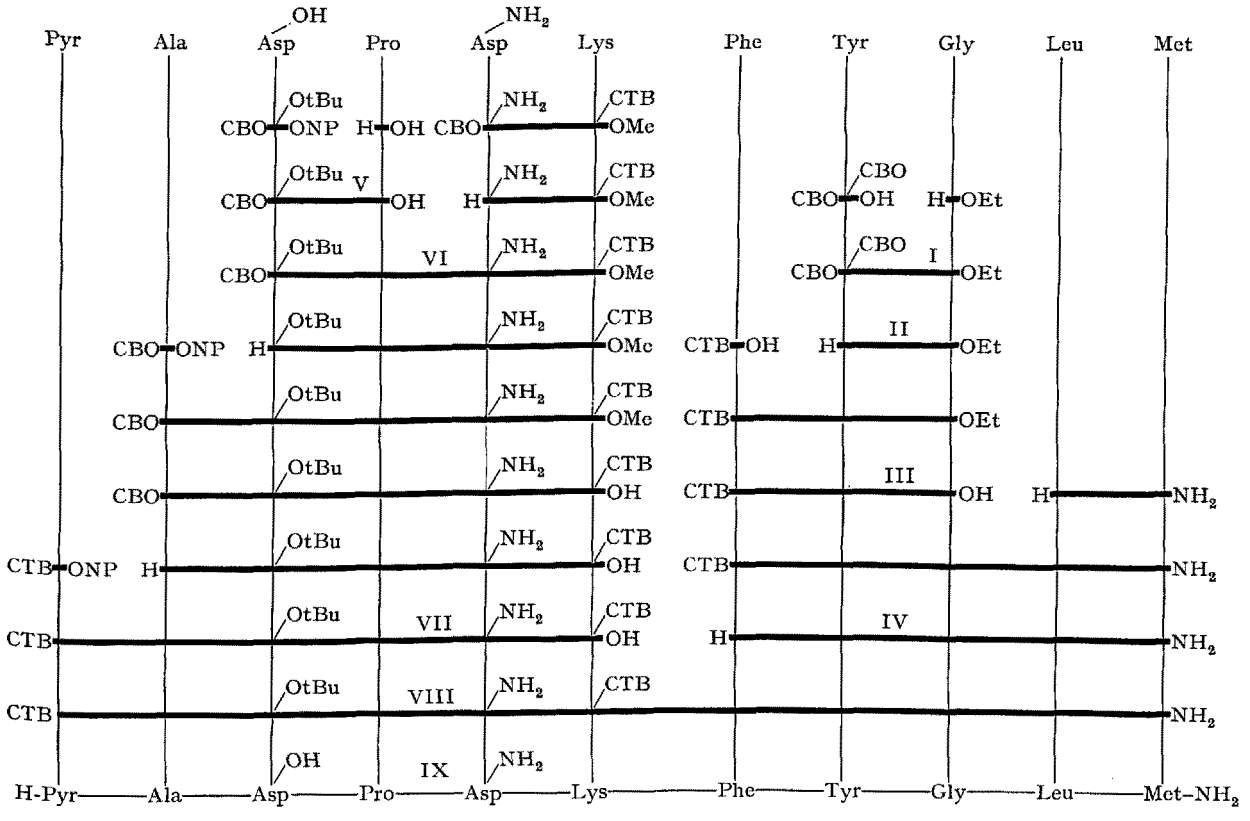
Synthesis of Physalaemin

We report the synthesis of a peptide of a formula H-Pyr-Ala-Asp-(OH)-Pro-Asp-NH_2-Lys-Phe-Tyr-Gly-Leu-Met-NH_2 according to scheme I[1]. The product was identical with natural physalaemin[2].

Condensation of glycine ethylester hydrochloride with N,O-bis-CBO-tyrosine in methylene chloride, with one equiv. of TEA, by DCCI gave ethyl N,O-bis-CBO-tyrosylglycinate (I) (82% yield; m.p. 165–166°; $[\alpha]_D^{20}$ −25°, c 0.5,

[1] All the amino acids have the L-configuration. The following abbreviations are used throughout this paper: CBO = carbobenzyloxy; CTB = carbo-ter-butyloxy; OtBu = ter-butylester; Pyr = pyroglutamyl; TEA = triethylamine; DCCI = dicyclohexylcarbodiimide; DCEA = dicyclohexylamine; E° = electrophoretic mobility of a sample pre-treated with HBr/AcOH or TFA according to the protecting group (CBO or CTB); DMF = dimethylformamide; TFA = trifluoroacetic acid.

[2] V. ERSPAMER, A. ANASTASI, G. BERTACCINI, and J. M. CEI, Exper. *20*, 489 (1964).



DMF. *Anal.* Calcd. for $C_{29}H_{30}O_8N_6$: C 65.16; H 5.66. Found: C 65.23; H 5.87) that was decarbobenzoxylated by treatment with HBr/AcOH 32% to give ethyl tyrosyl-glycinate hydrobromide (II) (100% yield; $E_{1,9}=0.77$ Leu; *Anal.* Calcd. for $C_{13}H_{18}O_4N_2\cdot HBr$: C 44.96; H 5.53; N 8.07; O 18.43; Br 23.01. Found: C 45.21; H 5.49; N 7.57; O 18.78; Br 23.31). N-CTB-phenylalanine was condensed, *via* the mixed anhydride, with II to give ethyl N-CTB-phenylalanyl-tyrosyl-glycinate (80% yield; m.p. 191–192°; $[\alpha]_D^{20}$ −16°, c 0.7, DMF; $E_{1,9}=0.5$ Leu; λ_{max} 278 mμ, $\varepsilon=1600$. *Anal.* Calcd. for $C_{27}H_{35}O_7N_3$: C 63.14; H 6.87; N 8.18. Found: C 62.85; H 7.12; N 8.18) that was next hydrolysed with NaOH to N-CTB-phenylalanyl-tyrosyl-glycine (III). (75% yield; m.p. 144–146°; $[\alpha]_D^{20}$ −14°, c 0.5, DMF. *Anal.* Calcd. for $C_{25}H_{31}O_7N_3\cdot{}^1/_2H_2O$: C 60.72; H 6.52. Found C 60.75; H 6.79.)

Condensation of III with leucyl-methioninamide hydrochloride[3] in DMF, with 1 equiv. of TEA, by DCCI afforded the protected pentapeptide N-CTB-phenyl-alanyl-tyrosyl-glycyl-leucyl-methioninamide (65% yield; m.p. 229–230°; $[\alpha]_D^{20}$ −22°, c 0.5, DMF; λ_{max} 278 mμ, $\varepsilon=1700$. *Anal.* Calcd. for $C_{38}H_{52}O_8N_6S$: C 59.32; H 7.19; N 11.53. Found: C 59.22; H 7.34; N 11.24) that was treated with HCl/AcOH 7% to give phenylalanyl-tyrosyl-glycyl-leucyl-methioninamide hydrochloride (IV) (100% yield; m.p. 120–122° dec.; $[\alpha]_D^{20}$ −66°, c 0.5, DMF; $E_{1,9}=0.44$ Leu. *Anal.* Calcd. for $C_{31}H_{44}O_6N_6S\cdot HCl\cdot{}^1/_2H_2O$: C 55.22; H 6.88. Found: C 55.04; H 7.20).

The *p*·nitrophenylester of N-CBO-O$^\beta$-t·Bu-aspartate, prepared from N-CBO-O$^\beta$-t·Bu-aspartate[3] and nitrophenol, *via* DCCI (70% yield; m.p. 84–86°; $[\alpha]_D^{20}$ +3°, c 3, $CHCl_3$. *Anal.* for $C_{22}H_{24}O_8N_2$: C 59.44; H 5.45; N 6.30. Found: C 59.55; H 5.46; N 6.40) was condensed with proline in $CHCl_3$ containing one equiv. of TEA to give N-CBO-O$^\beta$-t-Bu-aspartyl-proline (V) (95% yield; oil; DCEA salt: m.p. 132–133°; $[\alpha]_D^{20}$ −30°, c 0.5, DMF. *Anal.* Calcd. for $C_{33}H_{51}O_7N_3$: C 65.85; H 8.56; N 6.98. Found: C 65.83; H 8.53; N 7.04).

Methyl N-CBO-asparaginyl-N$^\varepsilon$-CTB-lysinate (m.p. 170–171°; $[\alpha]_D^{20}$ −11°, c 0.7, EtOH; $E_{1,9}=1.3$ Leu. *Anal.* Calcd. for $C_{24}H_{36}O_8N_4$: C 56.68; H 7.14; N. 11.02. Found: C 57.05; H 7.28; N 11.25; lit.[4] m.p. 125–127°; $[\alpha]_D^{20}$ −3.5°, DMF) was hydrogenated to methyl asparaginyl-N$^\varepsilon$-CTB-lysinate and condensed by the mixed anhydride method (ethyl chloroformiate) with V to give methyl N-CBO-O$^\beta$-t·Bu-aspartyl-prolyl-asparaginyl-N$^\varepsilon$-CTB-lysinate (VI) (65% yield; m.p. 96–100° dec. (from AcOEt/pet. ether); $[\alpha]_D^{20}$ −40°, c 0.4, DMF; $E_{1,9}=1.1$ Leu .*Anal.* Calcd. for $C_{37}H_{56}O_{12}N_6$: C 57.19; H 7.28; N 10.81. Found: C 56.85; H 7.30; N 10.52). Next VI was hydrogenated and the resulting oil was condensed with *p*·nitrophenyl N-CBO-alaninate to give methyl N-CBO-alanyl-O$^\beta$-t·Bu-aspartyl-prolyl-asparaginyl-N$^\varepsilon$-CTB-lysinate (60% yield; m.p. 103–106° (from AcOEt/Et_2O); $[\alpha]_D^{20}$ −59°, c 1, EtOH; $E_{1,9}=1.05$ Leu. *Anal.* Calcd. for $C_{40}H_{61}O_{13}N_7$: C 56.65; H 7.26; N 11.56. Found: C 56.06; H 7.23; N 11.52). This was hydrolysed with NaOH[5] and hydrogenated to alanyl-O$^\beta$-t·Bu-aspartyl-prolyl-asparaginyl-N$^\varepsilon$-CTB-lysine (amorphous) which was condensed with *p*·nitrophenyl N-CTB-pyroglutamate[6] in DMF containing 1.2 equiv. of TEA to give N-CTB-pyroglutamyl-alanyl-O$^\beta$-t·Bu-aspartyl-prolyl-asparaginyl-N$^\varepsilon$-CTB-

[3] L. Bernardi, G. Bosisio, R. de Castiglione, and F. Chillemi, Gazz. Chim. Ital., in press.

[4] Ed. Sandrin and R. A. Boissonnas, Helv. chim. Acta *46*, 1637 (1963).

[5] Since aspartic acid is bound to a secondary amino acid (proline) there is no risk of an α-β rearrangement *via* an aspartic imide.

[6] F. Chillemi, L. Bernardi, and G. Bosisio, Gazz. Chim. Ital., in press.

lysine (VII) (75% yield; m.p. 143–148°; $[\alpha]_D^{20}$ −67°, c 0.5, EtOH; $E_{1,9}=0.53$ Leu. *Anal.* Calcd. for $C_{41}H_{66}O_{15}N_8 \cdot H_2O$: C 52.99; H 7.39; N 12.06. Found: C 52.98; H 7.28; N 12.06). Condensation of VII with IV in pyridine by DCCI[7] afforded N-CTB-pyroglutamyl-alanyl-O$^{\beta}$-t·Bu-aspartyl-prolyl-asparaginyl-N$^{\varepsilon}$-CTB-lysyl-phenylalanyl-tyrosyl-glycyl-leucyl-methioninamide (VIII) (35% yield; m.p. 155–158° dec.; $[\alpha]_D^{20}$ −59°, c 0.3, EtOH. *Anal.* Calcd. for $C_{72}H_{108}O_{20}N_{14}S \cdot H_2O$: C 56.15; H 7.21; N 12.74. Found: C 55.80; H 7.34; N 12.51) which was treated with HCl/TFA for 2 h and subjected to counter-current distribution first in *n*·butanol/EtOH/AcOH/H_2O 5:1:1:8 (40 transfers, tubes 18–30) and subsequently in H_2O/*n*·butanol/pyridine/0.1 *N* aqueous ammonia 80/34/20/1 (40 transfers, tubes 22–32) to give pyroglutamyl-alanyl-aspartyl-prolyl-asparaginyl-lysyl-phenyl-alanyl-tyrosyl-glycyl-leucyl-methioninamide, which for solubility reasons was transformed into its trifluoroacetate (IX) (40% yield; m.p. 180° dec.; $E_{1,9}=0.4$ Glu; $E_{1,9}=0.33$ Leu; $[\alpha]_D^{20}$ −56°, c 0.2 in EtOH; λ_{max} 278 mμ, $\varepsilon=1780$. *Anal.* Calcd. for $C_{58}H_{84}O_{16}N_{14}S \cdot CF_3COOH \cdot 2H_2O$: C 50.90; H 6.35; N 13.85. Found: C 51.01; H 6.25; N 13.53). IX was found to be homogeneous, and showed amino acid composition, electrophoretic mobility, behaviour towards trypsin and chimotrypsin[8] and the same biological properties as natural physalaemin, thus confirming the formula deduced from degradative experiments[2,9,10].

Riassunto. Per condensazione fra fenilalanil-tirosil-glicil-leucil-metioninamide e N-carbo-ter-butossi-piroglutammil-alanil-β-ter-butil-aspartil-prolil-asparaginil-N$^{\varepsilon}$-carbo-ter-butossi-lisina in presenza di dicicloesilcarbodiimmide si è ottenuto l'endecapeptide VIII che per trattamento con una soluzione di HCl in acido trifluoroacetico ha fornito la piroglutammil-alanil-aspartil-prolil-asparaginil-lisil-fenilalanil-tirosil-glicil-leucil-metioninamide identica, per proprietà chimiche, fisiche e biologiche alla physalaemina naturale.

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Laboratori Ricerche Chimiche Farmitalia, Milano (Italy), May 29, 1964.

[7] More straightforward approaches to the synthesis of physalaemin having been rejected owing to the impossibility of securing crystalline intermediates, we had to take the risk of a partial racemization of the lysine residue during the condensation. We felt confident we would be able to eliminate the unwanted isomer by crystallization and we think we have succeeded, since trypsine completely splits the lysine-phenylalanine bond of our synthetic physalaemin sample.

[8] We are indebted to Dr. A. ANASTASI for these assays.

[9] We hope to be able to report in the near future the characteristic and the biological activities of a number of fragments of physalaemin and of several synthetic peptides embodying the features of both eledoisin and physalaemin.

[10] We express our appreciation to Dr. B. CAMERINO, Director of these Laboratories, for his interest in the work.

Selective Destruction in Testes Induced by Fluoroacetamide

During research carried out in order to find out substances causing hyperplasia of the epithelium of small bile-ducts, we have seen a peculiar destructive action of fluoroacetamide (FAA) on the testicular germinal epithelium.

The FAA was given orally, added to diet in the proportion of 50 mg/kg of food, to male rats weighing from 150–160 g. They were killed by exsanguination after 30, 64 and 90 days of treatment. At the end of the experimental period the body weight was increased on the average by 88% on the initial values.

In the necroscopic examination the testes revealed a notably reduced volume and such a flaccid consistence as if they were emptied of the greater part of their contents. The Table shows their weight in mg/100 g of body weight. The histological examination carried out on organs fixed in Bouin's fluid and stained by haematoxylin and eosin showed a gradual disappearance of the testicular germinal epithelium, from the most mature cells to the spermatogonia.

The tubules of the testes of the animals after 64 days of treatment were almost completely lacking in the seminal cells; only some spermatogonia and the Sertoli's cells were apparently undamaged as well as the interstitial cells. During the evolution of the regressive damage which causes the destruction of the testicular germinal epithelium, peculiar giant cells appear, perhaps by fusion

Days of treatment	Average weight ± S.E.M.
Controls (8)	0.97 ± 0.05
30 days (8)	0.55 ± 0.07
64 days (9)	0.32 ± 0.03
90 days (10)	0.30 ± 0.08

In parentheses the number of experiments performed.

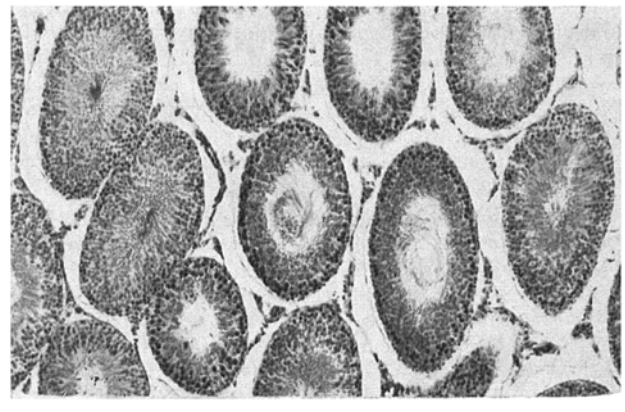

Fig. 1. Testis of normal rat (× 230).