2 fragments which were separated by high-voltage electrophoresis at pH 1.9.

The smaller fragment (Chym-2) was soon identified as the C-terminal tripeptide-amide, total acid hydrolysis showing that it was composed of 1 mole each of methionine, aspartic acid and phenylalanine and the sequence being established by degradation with LAP and by end group determination with the DNS-Cl and FDNB techniques on both the tripeptide itself and the C-terminal dipeptide-amide obtained by controlled removal of methionine with LAP. The state of the β-aspartyl and C-terminal carboxyl groups was deduced by the results of degradation with LAP and by qualitative determination of the net charge of the peptide according to the location of the spot on the electropherogram at pH 5.8.

The larger chymotryptic fragment (Chym-1) had anionic character on high-voltage electrophoresis at pH 1.9, was negative to ninhydrin and positive to the tryptophan and tyrosine reagents; its acid hydrolysates contained 2 moles of glutamic acid and 1 mole each of aspartic acid, tyrosine, threonine and glycine while 1 mole of tryptophan was readily liberated upon a short digestion with carboxypeptidase-A. By submitting Chym-1 to digestion with subtilisin 2 fragments were obtained: a tetrapeptide containing tyrosine, anionic at pH 1.9 (Sub-1), and a tripeptide containing tryptophan, neutral at pH 5.8 and basic at pH 1.9 (Sub-2). The sequence H-Thr-Gly-Trp-OH of Sub-2 was readily obtained by controlled degradation with CAP-A and LAP and determination of the N and C-terminal residues by dansylation and hydrazinolysis, respectively.

Tyrosine was identified as the C-terminal residue of Sub-1 upon digestion with CAP-A of the fragment previously heated for a few minutes at 100° in HCl *M* (removal of sulphate). The relative positions of the glutamyl and aspartyl residues were established by partial acid hydrolysis of Sub-1 with 0.5 *M* acetic acid which yielded free aspartic acid and tyrosine, and a dipeptide containing 2 moles of glutamic acid identified as pyroglutamyl-glutamine by its electrophoretic behaviour and by direct comparison with a synthetic sample.

Synthesis has confirmed the structure of caerulein proposed in this paper[4]. Natural caerulein was indistinguishable from the synthetic peptide in every respect, including parallel bioassay.

It may be seen that the structure of caerulein shows a close resemblance to that of the gastrins, especially to that of the gastrins-II[5]. In fact, the C-terminal pentapeptide and the N-terminal pyroglutamyl residue are the same for the 2 peptides, and similarly both peptides contain a tyrosinyl residue as sulphate ester.

Quite recently MUTT and JORPES[6] found that the C-terminal dipeptide, and most probably the C-terminal pentapeptide of cholecystokinin-pancreozymin, was the same as that of gastrin. Consequently, it is the same as that of caerulein.

A full report of the experiments and results described in this paper will be published elsewhere.

Riassunto. Vengono descritti i procedimenti che hanno permesso di giungere all'isolamento e al chiarimento della struttura della caeruleina, decapeptide attivo della pelle della *Hyla caerulea*. Vengono messi in luce gli stretti rapporti di struttura esistenti fra la caeruleina e le gastrine, soprattutto le gastrine-II, e fra la caeruleina e la colecistochinina-pancreozimina.

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[4] L. BERNARDI, G. BOSISIO, R. DE CASTIGLIONE and O. GOFFREDO, Experientia 23 (1967).

[5] H. GREGORY, P. M. HARDY, D. S. JONES, G. W. KENNER and R. C. SHEPPARD, Nature *204*, 931 (1964).

[6] V. MUTT and J. E. JORPES, Biochem. biophys. Res. Commun. *26*, 392 (1967).

Synthesis of Caerulein

We report the synthesis of a peptide of the formula H-Pyr-Gln-Asp-Tyr(SO_3H)-Thr-Gly-Trp-Met-Asp-Phe-NH_2 according to the following scheme. The product was found to be identical with natural caerulein[1,2]. Condensation of Boc-Tyr with Z-$NHNH_2$ via the mixed anhydride in THF afforded the protected hydrazide (I) (85% yield; m.p. 118–120°; $[\alpha]_D^{20} - 1.7°$, c 1, DMF. *Anal.* Calcd. for $C_{22}H_{27}N_3O_6$: C 61.5; H 6.3; N 9.8. Found C 61.3; H 6.3; N 9.9) which was treated with HCl/AcOH 1.3 N to give $^+H_2$-Tyr-NHNH-Z.Cl^- (II) (90% yield; m.p. 100°; $[\alpha]_D^{23} + 38°$, c 1, AcOH 95%; $E_{1.2} = 0.80$ Glu. *Anal.* Calcd. for $C_{17}H_{19}N_3O_4$. HCl: C 55.8; H 5.5; N 11.5. Found C 55.7; H 5.7; N 11.2). Boc-Asp(OBzl) was condensed, via the mixed anhydride, with II in THF/DMF in the presence of one equivalent of TEA to give Boc-Asp(OBzl)-Tyr-NHNH-Z (III) (70% yield; m.p. 138–140°; $[\alpha]_D^{21} - 21°$, c 1, DMF. *Anal.* Calcd. for $C_{33}H_{38}N_4O_9$: C 62.4; H 6.0; N 8.8. Found C 62.3; H 6.1; N 8.8).

Compound III, after treatment with HCl/AcOH to give IV ($E_{1.2} = 0.65$ Glu), was condensed with Boc-Gln, via the mixed anhydride, to afford the protected peptide Boc-Gln-Asp(OBzl)-Tyr-NHNH-Z(V) (85% yield; m.p. 173–175°; $[\alpha]_D^{23} - 22°$, c 1, DMF. *Anal.* Calcd. for $C_{38}H_{46}N_6O_{11}$. $^1/_2$ H_2O: C 59.1; H 6.1; N 10.9; O 23.8. Found C 59.0; H 6.0; N 10.8; O 23.8).

Z-Pyr was condensed, via the mixed anhydride, with $^+H_2$-Gln-Asp (OBzl)-Tyr-NHNH-Z.Cl^- (VI) ($E_{1.2} = 0.59$

[1] A. ANASTASI, V. ERSPAMER and R. ENDEAN, Experientia *23*, 699 (1967).

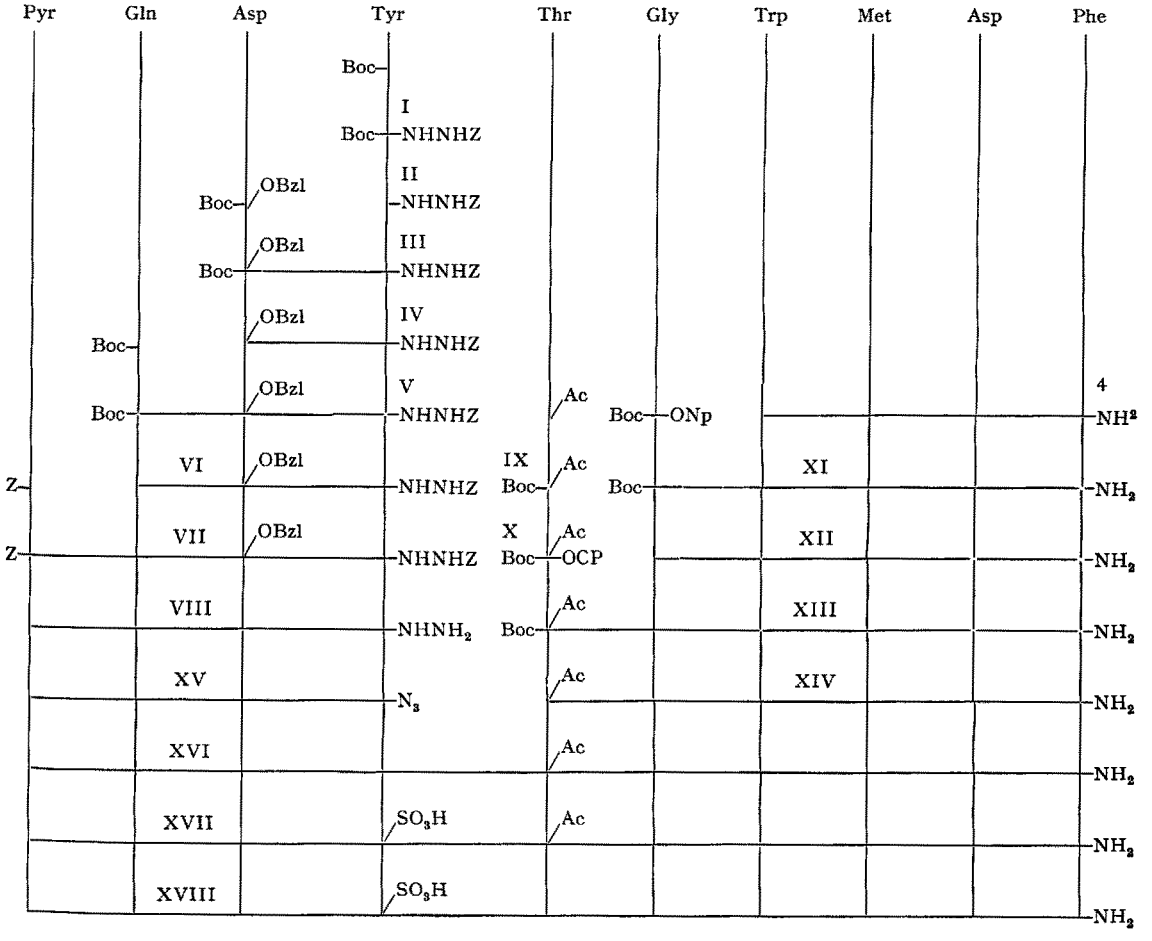
[2] All the amino acids have the L-configuration. The following abbreviations are used throughout this paper[3]: Z, carbobenzyloxy; Boc, carbo-*ter*-butyloxy; TEA, triethylamine; THF, tetrahydrofuran; DMF, dimethylformamide; DMSO, dimethylsulfoxyde; DCEA, dicyclohexylamine; DCCI, dicyclohexylcarbodiimide; ONp, *p*.nitrophenyl ester; OCP, 2,4,5-trichlorophenyl ester; Cys (SO_3H, cysteic acid.

Glu), which in turn was obtained by treatment of V with HCl/AcOH 1.3 N, and the protected tetrapeptide Z-Pyr-Gln-Asp(OBzl)-Tyr-NHNH-Z (VII) (70% yield; m.p. 205–207°; $[\alpha]_D^{25}$ – 28°, c 1, DMF. *Anal.* Calcd. for $C_{46}H_{49}N_7O_{13}$: C 60.8; H 5.4; N 10.8; O 22.9. Found C 60.4; H 5.3; N 10.8; O 22.9) was in this way secured. Hydrogenation of VII in the presence of Pd/C 10% in DMF afforded the key intermediate Pyr-Gln-Asp-Tyr-$NHNH_2$ (VIII) (90% yield; m.p. 203–205°; $[\alpha]_D^{23}$ – 24°, c 1, DMSO; $E_{1.2} = 0.45$ Glu; $E_{5.8} = 0.37$ Glu. *Anal.* Calcd. for $C_{23}H_{31}N_7O_9$: C 50.3; H 5.7; N 17.8. Found C 50.2; H 5.9; N 17.4).

Boc-Gly-ONp was condensed in DMF with $^+H_2$-Trp-Met-Asp-Phe-NH_2.Cl^- [4] in the presence of 2 equivalents of TEA and 1 equivalent of 1,2,4-triazole, to give Boc-Gly-Trp-Met-Asp-Phe-NH_2 (XI) (80% yield; m.p. 196°; $[\alpha]_D^{25}$ – 27°, c 1, DMF. *Anal.* Calcd. for $C_{36}H_{47}N_7O_9S$: C 57.3; H 6.3; N 13.0; S 4.2. Found C 57.3; H 6.4; N 12.9; S 4.2) that by treatment with HCl/AcOH 1.3 N gave $^+H_2$-Gly-Trp-Met-Asp-Phe-NH_2.Cl^- (XII) (m.p. 190–192°; $[\alpha]_D^{22}$ – 17°, c 1, AcOH 95%; $E_{1.2} = 0.59$ Glu. *Anal.* Calcd. for $C_{31}H_{39}N_7O_7S$.HCl: C 54.0; H 5.8; N 14.2. Found C 53.8; H 6.0; N 13.5).

Thr(Ac) [5] was treated in DMF with *ter*-butyloxycarbonil azide and TEA for 5 days to give Boc-Thr(Ac) (IX) (DCEA salt, 40% yield; m.p. 180–181°; $[\alpha]_D^{23}$ + 18°, c 1, DMF. *Anal.* Calcd. for $C_{11}H_{19}NO_6.C_{12}H_{23}N$: C 62.4; H 9.6; N 6.3. Found C 62.3; H 9.4; N 6.4), that by treatment with 2,4,5-trichlorophenol and DCCI afforded the oily active ester (X) which was next condensed with XII in DMF in the presence of 1 equivalent of TEA and 1 equivalent of 1,2,4-triazole. The resulting hexapeptide Boc-Thr(Ac)-Gly-Trp-Met-Asp-Phe-NH_2 (XIII) (82% yield; m.p. 187–188°; $[\alpha]_D^{24}$ – 17°, c 1, DMF. *Anal.* Calcd. for $C_{42}H_{56}N_8O_{12}S$: C 56.2; H 6.3; N 12.5. Found C 56.1; H 6.4; N 12.1) was treated with HCl/AcOH 1.3 N to give $^+H_2$-Thr(Ac)-Gly-Trp-Met-Asp-Phe-NH_2.Cl^- (XIV) (m.p. 155°; $[\alpha]_D^{24}$ – 8°, c 1, AcOH 95%; $E_{1.2} = 0.50$ Glu. *Anal.* Calcd. for $C_{37}H_{48}N_8O_{10}S$.HCl.H_2O: C 52.2; H 6.0; N 13.2. Found C 52.3; H 5.9; N 13.2) which was condensed in DMF at – 12° for 4 days with Pyr-Gln-Asp-Tyr-N_3 (XV), obtained by treatment at – 30° of the hydrazide VIII with anhydrous HCl/THF and *n*-butyl nitrite [6]. The decapeptide Pyr-Gln-Asp-Tyr-Thr(Ac)-Gly-Trp-Met-Asp-Phe-NH_2 (XVI) (45% yield; m.p. 218–220°; $[\alpha]_D^{25}$ – 21°, c 1, DMF; $E_{5.8} = 0.32$ Glu. *Anal.* Calcd. for $C_{60}H_{75}N_{13}O_{19}S$: C 54.8; H 5.8; N 13.8. Found C 54.9; H 6.3; N 13.0) was obtained by diluting the reaction mixture with water and citric acid. The product was successively treated overnight in pyridine-DMF with a large excess of SO_3/pyridine complex. After evaporation of the solvent in vacuo and dissolution of the residue in

[3] IUPAC-IUB Commission on Biochemical Nomenclature, Biochemistry *5*, 2485 (1966).
[4] J. M. Davey, A. H. Laird and J. S. Morley, J. chem. Soc. (C) 555, (1966).
[5] M. Wilchek and A. Patchornik, J. org. Chem. *29*, 1629 (1964).
[6] J. Honzl and J. Rudinger, Colln. Czech. chem. Commun. Engl. Edn *26*, 2333 (1961).



the bottom layer (A) of the system *n*-BuOH-EtOH-H_2O (5:1:8), NaOH was added to pH 3.2 and the solution was extracted with the top layer (B) of the same system. Evaporation of the solvent left a crude residue (XVII) that was dissolved in A and made basic with NaOH to pH 11. After 3 h HCl was added to pH 3.2 and the solution extracted with B. Evaporation of the solvent left a residue of crude peptide[7] which was eluted from DEAE-Sephadex (OH^-) with 1 *M* $(NH_4)_2CO_3$ buffer[8]. A further purification was achieved by counter-current distribution in the system *n*-BuOH-EtOH-H_2O (5:1:8). After deionization on Amberlite CG 50 (H^+) the solution was evaporated in vacuo: the residue (XVIII) (m.p. 224–226° dec.; $[\alpha]_D^{20}$ − 26°, c 1, DMF; $E_{5.8}$ = 0.43 Glu; $E_{1.9}$ = 0.53 Cys (SO_3H). *Anal.* Calcd. for $C_{58}H_{73}N_{13}O_{21}S_2$: C 51.5; H 5.4; N 13.5. Found C 51.3; H 5.7; N 13.1) was found homogeneous and showed the same electrophoretic and chromatographic properties, the same behaviour towards chymotrypsin, subtilisin and the same degradative pattern and biological properties[9] of natural caerulein, thus confirming the formula deduced from degradative experiments[10,11].

Riassunto. Viene riportata la sintesi della piroglutamil-glutaminil-aspartil-tirosil(O-solfato)-treonil-glicil-triptofanil-metionil-aspartil-fenilalaninamide, un peptide identico per proprietà chimiche, fisiche e biologiche alla caeruleina.

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11th May 1967.

[7] Electrophoretic analysis reveals that the residue contains caerulein, de-sulphated caerulein and polisulphated caerulein (probably mixed anhydrides aspartic acid $-SO_3$).

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

[9] We are indebted to Prof. V. ERSPAMER for these assays.

[10] We wish to thank Dr. B. CAMERINO, Director of this Research Institute, for his interest in this work.

[11] The synthesis of a number of fragments and analogues of caerulein is currently under way.

Pharmacological Actions of Caerulein[1]

The dacapeptide caerulein (natural caerulein from *Hyla caerulea* skin[2] and synthetic caerulein[3]) displayed a number of pharmacological actions on vascular and extravascular smooth muscles as well as on some external secretions.

Action on systemic blood pressure. The i.v. injection of caerulein caused in the dog a pressure fall which was satisfactorily proportional to the dose, especially in its duration. It lasted considerably longer than that caused by bradykinin or physalaemin. Tachyphylaxis was either lacking or moderate. The threshold i.v. dose of caerulein ranged between 0.01 and 0.1 μg/kg, but even doses of 100–1000 μg/kg could be tolerated and recovery was complete. The threshold dose by i.v. infusion was 5–20 ng/kg/min. Considerably larger doses were required by s.c. route.

The polypeptide lowered the blood pressure also in humans and in the rabbit. In the cat its action was erratic, less intense and there was often tachyphylaxis; in the rat it generally caused a hypertensive or biphasic response.

By intradermal injection into the human forearm caerulein increased the capillary permeability and caused a reaction which was approximately half as intense as that caused by bradykinin. The polypeptide, however, was 5000 times less active than bradykinin on the permeability of the skin capillaries of the guinea-pig.

Action on extravascular smooth muscle. Caerulein generally displayed a poor stimulant action on isolated preparations of intestinal and uterine smooth muscle. The most important exception was the isolated gall bladder. That of the guinea-pig was contracted by concentrations of caerulein as low as 1 ng/ml, that of the rabbit and the sheep by concentrations as low as 0.2 ng/ml and 0.1 ng/ml, respectively.

The in situ gall bladder of the guinea-pig was tremendously sensitive to caerulein, the i.v. threshold dose being 0.2–0.6 ng/kg. There was an excellent dose/response relationship and there was no sign of tachyphylaxis, even over long periods of time. One μg caerulein was equiactive to 40–50 Ivy dog units of cholecystokinin, i.e. to 7–15 μg of pure cholecystokinin-pancreozymin[4].

Like that of the guinea-pig, the in situ gall bladder of the dog was potently contracted by caerulein, the threshold dose being apparently of the order of a few ng/kg. In normal humans, caerulein produced a contraction of the gall bladder at i.v. doses as low as 1–2 ng/kg (BRAIBANTI et al., personal communication).

Another aspect of the stimulant action of caerulein on the in situ gastrointestinal smooth muscle was represented by emesis and diarrhoea as seen in the intact conscious dog after administration of the polypeptide. The threshold emetic dose was approximately 0.5 μg and 2 μg/kg, by i.v. and s.c. route, respectively.

In an anaesthetized dog provided with a denervated fundic pouch, caerulein produced contraction of the gastric musculature at i.v. doses as low as 2–6 ng/kg.

Action on secretions associated with the digestive tract. (a) *Gastric secretion.* In conscious dogs provided with denervated fundic pouches, the s.c. injection of caerulein stimulated both the acid flow and the volume of gastric juice. The threshold dose was 0.1–0.5 μg/kg and the magnitude of the responses was proportional to the dose administered. The effects produced by a single s.c. dose

[1] Supported by grants from the Consiglio Nazionale delle Ricerche, Roma.

[2] A. ANASTASI, V. ERSPAMER and R. ENDEAN, Experientia *23*, 699 (1967).

[3] L. BERNARDI, G. BOSISIO, R. DE CASTIGLIONE and O. GOFFREDO, Experientia *23*, 700 (1967).

[4] A generous sample of synthetic human gastrin-I was kindly set at our disposal by Dr. R. C. SHEPPARD, The Robert Robinson Laboratories, University of Liverpool, and generous samples of pure secretin and of pure cholecystokinin-pancreozymin by Prof. E. JORPES, Department of Chemistry, Karolinska Institutet, Stockholm.