Two bands between 460 and 430 cm^{-1} appear consistently in strontium hydroxyapatite. These bands are not as intense as the other 2 members of ν_4. The numerical value of their frequency, however, is close in magnitude to the difference between ν_3 and ν_4, suggesting them to be difference tones[12,13].

Zusammenfassung. Es wird über eine Methode berichtet, die es erlaubt, Apatide zu unterscheiden, in denen anstelle des Calciums Strontium eingetreten ist.

V. M. BHATNAGAR[14,15]

Fisk University, Nashville (Tennessee, USA), 29th March 1967.

[12] G. HERZBERG, *Infrared and Raman Spectra of Polyatomic Molecules* (D. Van Nostrand and Co. Inc., New York 1945).

[13] J. M. STUTMAN, J. D. TERMINE and A. S. POSNER, Trans. N.Y. Acad. Sci. *27*, 669 (1965).

[14] This investigation was supported by Research Grant DE-02102-02 of the U.S. Public Health Service. I thank Prof. N. FUSON for his interest, encouragement and many helpful suggestions throughout apatite investigation. The IR-spectrum of the sample was run by Mr. K. E. STINE at Beckman Instruments Inc., Fullerton, California, and the X-ray diffraction pattern was done by Dr. R. L. COLLIN of the University of Rochester, Rochester, New York. The sample was prepared at the Infrared Spectroscopy Institute, Fisk University, Nashville, Tennessee, and the results and discussion parts were completed at Kentucky State College, Frankfort, Kentucky.

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Isolation and Structure of Caerulein, an Active Decapeptide from the Skin of *Hyla caerulea*[1]

Methanol extracts of the skin of some South-American and Australian amphibians contain a polypeptide, or closely related polypeptides, which displays a potent and relatively long-lasting hypotensive action in the dog, potently stimulates some extravascular smooth muscles and possesses a remarkable action on several external secretions of the digestive tract[2,3].

The polypeptide, called caerulein, has been obtained for the first time in a pure form from methanol extracts of the skin of *Hyla caerulea*, an Australian amphibian.

In this communication the procedures used for the isolation of caerulein and the elucidation of its structure are briefly reported.

Isolation procedure. Crude methanol extracts of the dry skin of *H. caerulea* were submitted to a short counter-current distribution between petroleum ether and aqueous 80% ethanol and then to a single distribution in a separatory funnel between 50 ml each of the aqueous and solvent phases of the system: chloroform ml 62, methanol ml 35, and acetic acid 0.5 *M* ml 40. The active material was collected in the aqueous phases freed of the fat soluble contaminants and of a large part of the pigments.

Further purification of the defatted material was obtained with a counter-current distribution of 80 transfers using the solvent system *n* butanol-acetic acid-ethanol-water (800:160:160:1280) in which caerulein showed a distribution coefficient of about 1.6. This step was repeated twice.

The partially purified material was then adsorbed on a small column of DEAE Sephadex in the OH^- form and eluted with ammonium carbonate solutions of concentrations increasing from 0.05 *M* to 1 *M*. Caerulein emerged with the higher concentration of carbonate while the bulk of contaminants emerged with the first fractions of the effluent.

The appropriate samples were finally passed through a small column of Amberlite CG-50 in the H^+ form and eluted with water. Caerulein emerged unretarded in the first fractions while the ammonium ion and the small amounts of less acidic contaminants still present were retained on the resin. The active fractions were pooled and freeze-dried.

Caerulein purified with the above procedures was homogeneous on paper electrophoresis and chromatography giving a single biologically active peptide spot which was negative to ninhydrin and positive to the Ehrlich reagent for tryptophan, to the α-nitrose-β-naphthol reagent for tyrosine and to the jodoplatinate reagent for sulphur amino acids.

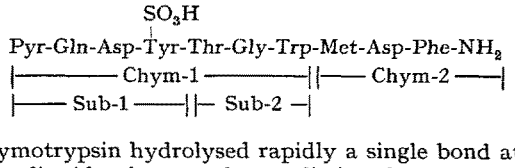
On ascending thin-layer chromatography on silica gel caerulein had an Rf of 0.2 with the solvent system *n* butanol-acetic acid-water (4:1:1) and of 0.7 with 80% aqueous ethanol. On high-voltage electrophoresis the caerulein spot migrated toward the anode at neutral and acidic pHs, its position being 0.43 relative to glutamic acid at pH 5.8 and 0.53 relative to cysteic acid at pH 1.9.

Structure. The results of the experiments summarized below were consistent with the following structure for caerulein:

Pyr-Gln-Asp-Tyr(SO_3H)-Thr-Gly-Trp-Met-Asp-Phe-NH_2

On total acid hydrolysis pure preparations gave 2 moles each of glutamic acid and aspartic acid and 1 mole each of tyrosine, threonine, glycine, methionine and phenylalanine. Tryptophan was recovered in a low yield. On alkaline hydrolysis with N $Ba(OH)_2$ tryptophan was obtained in a 1:1 ratio with respect to the other neutral amino acids and tyrosine was found in the form of a tyrosine *O*-sulphate ester.

The sequence of caerulein was deduced by the analysis of the fragments obtained upon digestion with the enzymes chymotrypsin and subtilisin which split the molecule as shown below:



Chymotrypsin hydrolysed rapidly a single bond at the carboxylic side of tryptophan, splitting the molecule into

[1] Supported in part by a grant from the Consiglio Nazionale delle Ricerche, Roma.

[2] V. ERSPAMER and A. ANASTASI, in *Hypotensive Peptides* (Eds. E. G. ERDÖS, N. BACK, F. SICUTERI and A. F. WILDE; Springer Verlag, New York 1966), p. 63.

[3] V. ERSPAMER, M. ROSEGHINI, R. ENDEAN and A. ANASTASI, Nature *212*, 204 (1966).

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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Istituto Ricerche, Farmitalia S.p.A., Milano, Istituto di Farmacologia, Università di Parma (Italy) and Zoology Department, University of Queensland, Brisbane (Australia), 11th May 1967.

[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.