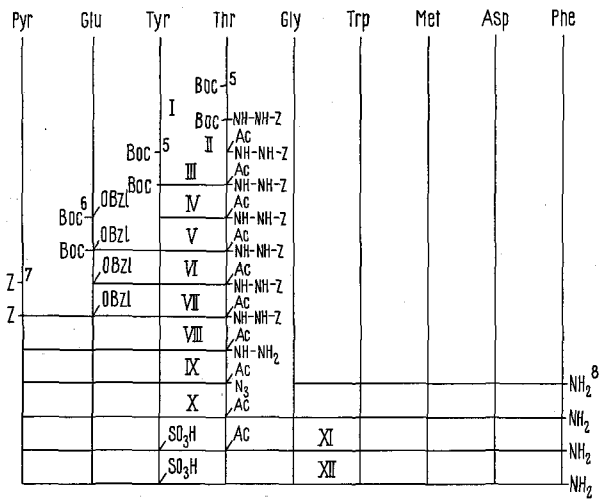
$[\alpha]_D^{22}$ −9.2°, $c = 1$, DMF. *Anal.* Calcd. for $C_{48}H_{52}N_6O_{14}$: C 61.5; H 5.6; N 9.0. Found C 61.8; H 5.8; N 8.9).

Hydrogenation of VII in DMF in the presence of Pd/C 10% gave the key intermediate Pyr-Glu-Tyr-Thr(Ac)-$NH-NH_2$ (VIII) (99% yield; m.p. 193–194°; $[\alpha]_D^{22}$ +2.8°, $c = 0.6$, DMF; $E_{1.2} = 0.48$ Glu) which was converted into the azide IX by treatment at −25° with HCl/THF 2N and *t*-butyl nitrite, and condensed with the pentapeptide $^+H_2$-Gly-Trp-Met-Asp-Phe-$NH_2 \cdot Cl^-$ [8] in DMF at −12° for 4 days to give the nonapeptide Pyr-Glu-Tyr-Thr(Ac)-Gly-Trp-Met-Asp-Phe-NH_2 (X) (83% yield; m.p. 208 to 210°; $[\alpha]_D^{22}$ −15°, $c = 1$, DMF; $E_{5.8} = 0.28$ Glu. *Anal.* Calcd. for $C_{56}H_{69}N_{11}O_{17}S \cdot {}^1/_2 H_2O$: C 55.6; H 5.8; N 12.7. Found C 55.7; H 5.9; N 12.3).

The product was next treated in pyridine-DMF with 7 equivalents of SO_3/pyridine complex for 5 h. After evaporation of the solvent in vacuo and dissolution of the residue in 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 (XI) which 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 which was purified by elution from DEAE – Sephadex (OH^-) with 1 *M* $(NH_4)_2CO_3$ buffer and deionized on Amberlite CG-50 (H^+). The nonapeptide Pyr-Glu-Tyr(SO_3H)-Thr-Gly-Trp-Met-Asp-Phe-NH_2 (XII) so obtained (48% yield; $E_{1.9} = 0.57$ Cys(SO_3H); $E_{5.8} = 0.50$ Glu; 0.39 Cys(SO_3H)) was found to be homogeneous and showed the same electrophoretic and chromatographic properties, the same behaviour towards chymotrypsin and subtilisin, and the same degradation pattern and biological properties as natural phyllocaerulein, thus confirming the formula previously deduced from degradation experiments.



Synthesis of phyllocaerulein.

Riassunto. Viene riportata la sintesi della piroglutamil-glutamil-tirosil(0-solfato)-treonil-glicil-triptofanil-metionil-aspartil-fenilalanilamide, un nonapeptide identico per proprietà chimiche, fisiche e biologiche alla Phyllocaeruleina.

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Milano (Italy), 30 September 1968.

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The Enzymatic Degradation of Phyllocaerulein and Analogs

Phyllocaerulein, a nonapeptide with a structure very similar to, and activity spectrum identical with that of caerulein[1,2], has been isolated from the skin of a South American amphibian *Phyllomedusa sauvagi*[3]. The structure of phyllocaerulein has been proved by synthesis[4]. As shown in Figure 1, it differs from that of caerulein only in the lack of glutamine and in the substitution of 1 aspartyl with a glutamyl residue.

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

Phyllocaerulein Pyr-Glu-Tyr(S)-Thr-Gly-Trp-Met-Asp-Phe-NH_2

The sequential analysis of phyllocaerulein has been based, like that of caerulein, on the enzymatic degradation with chymotrypsin and subtilisin. The purpose of this communication is to describe briefly a discrepancy that has been observed in the behaviour of the 2 structures, otherwise so similar, towards the enzymatic attack.

Chymotrypsin behaved in the same way with both peptides. The hydrolysis occurred at the carboxyl side of tryptophan and 2 fragments were produced. In contrast with caerulein, the N-terminal fragment of phyllocaerulein was found to be free of aspartic acid and this immediately confirmed that phyllocaerulein contained only 1 aspartyl residue.

Subtilisin also broke the tryptophan bond of both peptides, and in addition hydrolyzed a second bond, giving rise to 3 fragments. However, this second point

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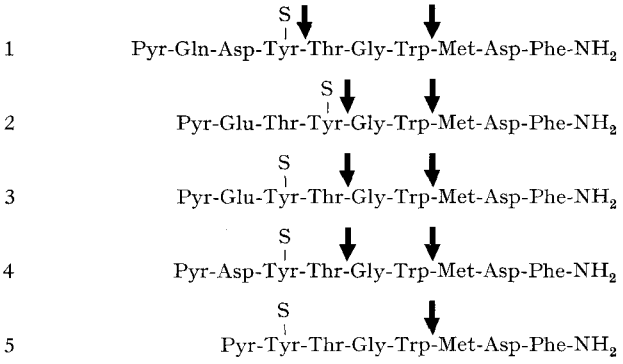
of action was not the same for the 2 peptides and this led initially to a misinterpretation of the structure of phyllocaerulein. The results of the subtilisin digestion are shown in Figure 2.

Degradation by subtilisin of caerulein and analogs



Caerulein (structure 1) was broken into 3 fragments, 2 tripeptides and an N-terminal tetrapeptide which did not contain threonine. By submitting phyllocaerulein (structure 3) to the same subtilisin digestion, an N-terminal fragment was obtained which also contained 4 residues one of which was threonine. By analogy with caerulein, it was initially thought that the bond broken was the tyrosine sulphate bond and that the threonine residue was in the next position, as in structure 2.

Further experiments showed however that this structure was not correct. Digestion with carboxypeptidase of the desulphated N-terminal tetrapeptide gave doubtful results, since the threonine and tyrosine residues were released at approximately the same rate; however, by submitting the same fragment to hydrazinolysis, free threonine was obtained and this proved that the sequence of phyllocaerulein was that of peptide 3. Furthermore, the activity spectrum of peptide 2, which was subsequently synthesized as a caerulein analog, was found to be different from that of phyllocaerulein.

Shortly afterwards it was possible to explain the discrepancy between the subtilisin degradation of caerulein and phyllocaerulein, since it was possible to examine the behaviour of 2 synthetic analogs: des-glutamine-caerulein (structure 4) and des-glutamine, des-aspartic acid-caerulein (structure 5). Peptide No. 4 was hydrolyzed at the carboxyl side of the threonine residue, giving rise to an N-terminal fragment containing threonine in the C-terminal position, while no cleavage of peptide No. 5 could be observed either at the tyrosine sulphate or at the threonine bond.

The subtilisin behaviour was also examined on the desulphated peptides and the same result was found. It was therefore evident that both the tyrosine (or tyrosine sulphate) and the threonine bonds were susceptible to the action of subtilisin but that their cleavage depended on their position in the chain. Tyrosine was preferred when it held the fourth position from the N-terminus. When tyrosine was replaced by threonine in the fourth place, the bond broken was still the fourth; but, when both threonine and tyrosine occupied a position closer than the fourth to the N-end, the enzyme could not exert any action on the bonds formed by these 2 amino acids.

Chymotrypsin has no action on tyrosine sulphate but is very specific for the tyrosine bonds. By digesting the desulphated peptides with chymotrypsin, it was found that all the tyrosine bonds were normally split, even when in the second position.

Riassunto. Vengono riportate e discusse alcune differenze osservate durante la degradazione con subtilisina dei 2 peptidi naturali ceruleina e phylloceruleina.

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Calcium as Stabilizing Factor of the Collagen Macromolecule

It is known that the stability of the collagen molecule increases with age[1], and that also the Ca content of several organs increases[2]. It has been discussed whether some relationship exists between these age changes of collagen and the increase of Ca content.

To study this we performed 2 types of experiments on rats under conditions in which an increased calcium content exists. In the first series we used artificial calcification, and in the second we studied the skin collagen (corium) of old animals. In both series we tested the stability of collagen before and after extraction of calcium. For this we estimated the total and the 'labile' hydroxyprolin (hypro) content[3].

Methods. We used Wistar-rats from this Institute's old age colony. Skin was taken immediately after death, cleaned from hair and s.c. material, defatted with ether and cut in small pieces of about 1 mm³.

Collagen was estimated as hypro after Stegemann[4], as modified by Woessner[5].

Total collagen was measured after hydrolysis with 6 *N* HCl at 110 °C for 12 h, expressed as g hypro in 100 g skin, dry weight.

'Labile' (soluble) collagen is the part which is dissolved after denaturation at 65 °C for 10 min in Ringer's solution at pH = 7.4. After hydrolysis, it is estimated as hypro and expressed as % of the total. It is known that the 'labile' (soluble) collagen decreases with aging in the skin (corium)[1] and that the decrease is larger in the dorsal than in the ventral skin[6].

Calcium was estimated after ashing about 1 g skin at 500 °C. The ash was dissolved in 2 ml 0.01 *N* HCl and calcium precipitated with 3 ml ammonium oxalate-buffer pH 5.5. The precipitate is then centrifuged and washed with H_2O. The calcium oxalate is dissolved in 0.05 *N* perchloric acid and Ca estimated in the flame photometer.

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