Whether addition or substitution predominates depends on the extraction conditions and on the neutral organophosphorus compound used. High concentrations and basic character for the neutral organophosphorus compound B seem to promote substitution, whereas a not so basic neutral organophosphorus compound like TBP, in low concentrations seems to favor addition.

A full account will be published in this journal in the near future.

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Formulation of Active Peptide Structure by Quantitative End Group Determination and Bioassay

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The determination of the structure of bradykinin has mostly relied upon an end group determination with fluoro-2,4-dinitrobenzene using chymotryptic fragments of the peptide and subsequent degradation by the Edman procedure.[1,2] In general the C-terminal amino acid has been determined after incubation with carboxypeptidase. Continuing beyond phenylalanine in position five was not advisable due to the accumulation of disturbing artefacts with the Edman technique. The enzymatic procedures involve a number of time consuming operations, which might be simplified by applying the straightforeward degradation as described by Edman.[3-6] Combined with a quantitative end group determination at the N-terminal and bioassay of the biologically active peptide, the formulation of the structure of the kinin peptides would be simplified, particularly in view of the fact that several bradykinin-like peptides have been isolated from blood plasma, which have in common the nonapeptide sequence in bradykinin at the same time as quantitative differences occur in respect to their biological activities. Since however also species differences may occur,[7] the sequence of amino acids also becomes essential for the complete formulation of the active peptide structure.

In earlier work [8,9] we have shown that the molar proportions of the 2,4-dinitrophenyl-amino acids in bradykinin may be estimated with small amounts of pure isolated peptide from bovine and human plasma. The amino acid sequence in bovine bradykinin obtained with snake venom from the total plasma has not been as yet completely determined [10] by chemical methods. In the present study we have attempted to formulate the active peptide structure by determining the unity ratio between the peptide amount deduced

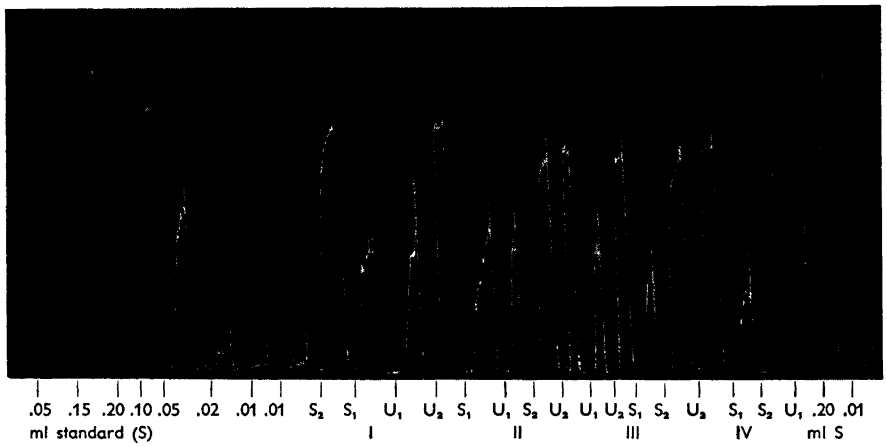

Fig. 1. Estimation by 4-point assay on the isolated guinea pig ileum of the amount of desalted synthetic bradykinin used for the quantitative N-terminal end group determination. The ileum was suspended in a 6.5 ml bath of aerated Tyrode solution at 37°C. The standard error of the assay $s = \sqrt{14} = 3.74$ represents about 7.3 % of the mean $\overline{Y} = 51$ mm for all determinations (12). The following doses were given in four groups (I—IV):
S_1 = 0.05 ml standard bradykinin (BRS-640) solution 5 μg/ml
S_2 = 0.10 ml ,, ,, ,, ,,
U_1 = 0.04 ml peptide solution (diluted in 0.15 M sodium chloride)
U_2 = 0.08 ml ,, ,, ,, ,, ,, ,,

through a quantitative determination of end group arginine and the amount estimated in bioassay. For this purpose pure synthetic smooth muscle contracting peptide (bradykinin BRS-640, Sandoz Ltd.) was used, with the following structure:

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH
1 2 3 4 5 6 7 8 9

The amino acid sequence of pure natural bradykinin [10] released from bovine plasma with *Bothrops jararaca* venom was also performed in a nine cycles degradation with the Edman method in its three stage form [4,5] (*cf.* Blombäck *et al.* 1965) [11] in comparison with the straight-foreward degradation with the synthetic nonapeptide. The synthetic bradykinin used in these experiments was obtained by desalting ampoule material containing synthetic bradykinin (BRS-640) by a similar paper chromatography as used for the final purification of the natural peptide.[10] By this procedure a smaller arginine reactive component with the electrophoretic mobility $E_{6.2} = 0.28 \times$ the synthetic bradykinin on paper, was also removed. The eluated salt free peptide was standardized by a 4-point assay on the isolated guinea pig ileum [12] as shown in Fig. 1. A peptide amount corresponding to 1.16 μmole of active bradykinin was coupled with phenylisothiocyanate. PTH-arginine was identified by the Sakaguchi reaction after paper electrophoresis (Beckman Spinco Model R, 2 hours, 10—15 V/cm, 8 mA/cm, 0.02 M phosphate buffer pH 6.2).

The quantitative determinations of the phenylthiohydantoins (PTH-amino acids) were performed using the Beckman DK-1 spectrophotometer and microcuvettes. The ultraviolet absorption spectra were run with dilutions in absolute ethanol from the ethyl acetate and water extracts, respectively, containing the PTH-amino acids obtained in the different cycles.[3,11] The following molar extinction coefficients obtained with pure PTH-amino acids (Mann Research Laboratories) were used: (in brackets the $\varepsilon_{245}/\varepsilon_{269}$) [13]PTH-arginine 10 035 (0.42), PTH-proline 14 500 (0.65), PTH-phenylalanine 16 400 (0.47), PTH-serine 15 300 (0.54) and PTH-glycine 14 650 (0.47). The recovery of PTH-

arginine obtained from the synthetic peptide was 1.175 μmole, giving a ratio of 1.01 between the N-terminal arginine and the μmole of peptide as estimated by the smooth muscle contracting activity (Fig. 1). Together with the close resemblance obtained when comparing the absorbancy ratios obtained with the pure and the N-terminal PTH-arginine (0.45) this indicates that the reaction between peptide arginine and phenylisothiocyanate was quantitative.

The synthetic peptide was further degraded through nine cycles and the identification of the PTH-amino acids at each cycle was performed by thinlayer chromatography using four different solvent systems. The details of these procedures will be described in a later publication. Identification was performed according to Edman and Sjöquist [14] by a fluorescent technique and subsequent spray with iodine azide. Care was taken to correlate these two reactions in localizing the spots. Reference mixtures of pure PTH-amino acids were used dissolved in ethyl acetate. The amounts used varied between 1 to 1.6 μg (PTH-proline 2.4 μg) per spot to obtain an easily identified reaction in ultraviolet light (254 mμ).

Approximately 0.5 μmole (0.47) of natural bradykinin was used for a nine cycles degradation, as calculated by the determination of N-terminal PTH-arginine. As also observed with the synthetic peptide, serine in the sixth cycle apparently due to destruction [5] revealed itself by the appearance of more artefacts as obtained in any of the other degradation cycles, although small amounts of PTH-serine could also be identified by chromatography. The following PTH-amino acids were recovered (μmoles through the cycles two to eight): PTH-proline 0.70, PTH-proline 0.46, PTH-glycine 0.47, PTH-phenylalanine 0.42, (PTH-serine 0.31), PTH-proline 0.44, and PTH-phenylalanine 0.26.

The C-terminal arginine was detected in the eighth cycle as the free amino acid, as it could be identified by the Sakaguchi reaction after paper electrophoresis ($E_{6.2}$ = 1.24 PTH-arginine). A quantitative determination of the free arginine [9] in the water extract corresponding to the phenylalanine cycle yielded 0.16 μmole (as arginine hydrochloride). Due to the presence of an unidentified second weaker arginine reactive spot by paper electrophoresis, this estimation must be considered approximate. Performance of a subsequent ninth cycle however yielded only a small amount of PTH-arginine (0.042 μmole by ultraviolet absorption measurement) sustaining that most of the C-terminal arginine of the peptide is found simultaneously with PTH-phenylalanine in the eighth degradation cycle. This may be caused by an increased solubility of arginine salts with organic acids into nonpolar solvents.

Clearly the nonapeptide sequence of natural bradykinin could be shown in a straightforeward degradation through eight cycles with the Edman method. The combination as outlined above of N-terminal end group determination and a suitable biological assay should considerably facilitate the formulation of structure with this type of active peptides.

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