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Synthesis of the Vasoactive Intestinal Peptide (VIP). III.[1] The Sequence 7–13

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The protected heptapeptide derivative *t*-butyloxycarbonyl-L-threonyl-β-benzyl-L-aspartyl-L-asparaginyl-*O*-benzyl-L-tyrosyl-L-threonyl-nitro-L-arginyl-L-leucine methyl ester was prepared by stepwise chain lengthening. The protecting groups on the side chains of arginine, tyrosine, and aspartic acid residues were removed by hydrogenolysis and the partially deprotected heptapeptide ester converted to the hydrazide, an intermediate in the synthesis of the (porcine) vasoactive intestinal peptide (VIP).

After the removal of the *tert*-butyloxycarbonyl group, the heptapeptide ester was exposed to the action of trypsin which split off its C-terminal residue, L-leucine methyl ester. The hexapeptide was then exposed to chymotrypsin, which cleaved it into an acidic, and a basic fragment. The former was, under the conditions used, indistinguishable on paper chromatography and paper electrophoresis from the tetrapeptide threonyl-aspartyl-asparaginyl-tyrosine which had previously been isolated from natural VIP, of which it comprises the sequence 7–10. Similarly, the basic fragment was indistinguishable from threonyl-arginine, the sequence 11–12 of VIP. This intestinal peptide increases visceral blood flow and reduces blood pressure in the dog, and also causes relaxation of different smooth muscle preparations, e.g., the trachea of guinea pigs. The principal aim of the present synthesis is to provide independent evidence for the sequence of (porcine) VIP.

Isolation of a vasoactive intestinal peptide (VIP) was described by Said and Mutt (*1*), who also established the amino acid composition (*2*) and the sequence (*3*) of the single chain octacosapeptide. In previous publications (*4*, *5*) from these laboratories, the syntheses of the C-terminal hendecapeptide (VIP_{18-28}) and of the N-terminal hexapeptide (VIP_{1-6}) were reported. The present paper deals with the preparation of the partially protected heptapeptide derivative *t*-butyloxycarbonyl-L-threonyl-L-aspartyl-L-asparaginyl-L-tyrosyl-L-threonyl-L-arginyl-L-leucine hydrazide (XII), a compound that, after conversion to the corresponding azide, serves as an intermediate in the synthesis of the complete sequence of the hormone.

[1] For paper no. II in this series, cf. *Bioorg. Chem.* 2, 87 (1972).

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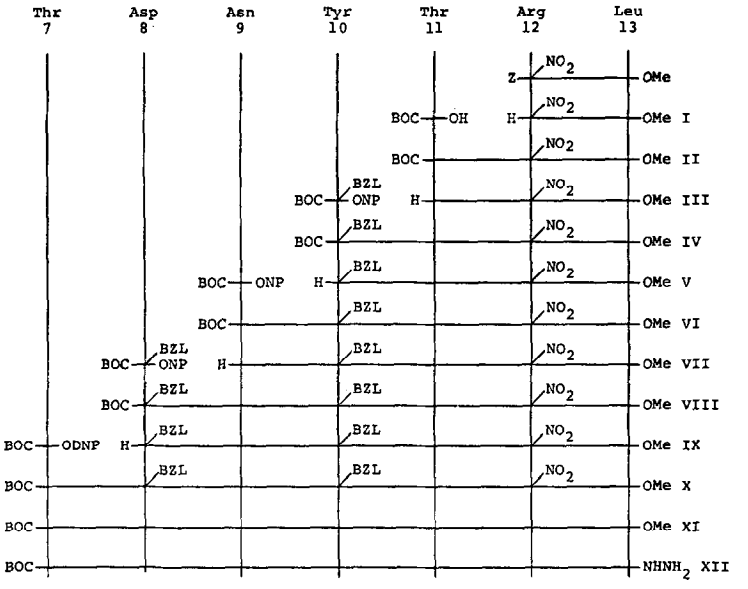
The heptapeptide derivative XII was built by stepwise chain lengthening (*6*), starting with the known protected dipeptide benzyloxycarbonyl-nitro-L-arginyl-L-leucine methyl ester (*7–9*). After removal of the benzyloxycarbonyl group with HBr in acetic acid, the resulting amine (I) was acylated with *t*-butyloxycarbonyl-L-threonine with the aid of 1-ethoxy-2-ethyl-1,2-dihydroquinoline (EEDQ) (*10*). After deblocking the resulting tripeptide derivative (II) with trifluoroacetic acid, the chain was lengthened by the incorporation of *t*-butyloxycarbonyl-*O*-benzyl-L-tyrosine in the form of its *p*-nitrophenyl ester (*11*). The next two residues, L-asparagine and *β*-benzyl-L-aspartic acid, were introduced in the same manner. The fully protected heptapeptide (X) was obtained by the application of *t*-butyloxycarbonyl-L-threonine-2,4-dinitrophenyl ester (*12*). The benzyl groups from the side chains of the aspartyl and tyrosine residues and the nitro group from the guanidino function of the arginine moiety were simultaneously removed by catalytic hydrogenation and the partially protected heptapeptide methyl ester (XI) was converted to the corresponding hydrazide (XII). The synthesis is summarized in Chart I.

CHART I



A sample of compound XI was treated with trifluoroacetic acid and the heptapeptide methyl ester (XIa) digested with aminopeptidase M (*13*). Amino acid analysis of the digest showed the components in the expected ratios.

A second sample of the heptapeptide methyl ester was cleaved with trypsin into leucine methyl ester and a neutral peptide. The peptide was separated from the ester, and from the free leucine formed by hydrolysis of part of the latter, on a column of Sephadex-LH20, and then cleaved with chymotrypsin into an acidic and a basic fragment. These fragments were separated on carboxymethyl cellulose and then

subjected to paper chromatography and paper electrophoresis in parallel with two of the chymotryptic cleavage products, threonyl-aspartyl-asparaginyl-tyrosine, and threonyl-arginine, of the N-terminal tryptic fragment of natural VIP (*3*). Under the

FIG. 1. Paper electrophoresis of synthetic and natural peptides obtained by chymotryptic degradation of VIP_{7-12} (cf. Experimental Section): (1) VIP_{7-10} syn., (2) VIP_{7-10} syn. + VIP_{7-10} nat., (3) VIP_{7-10} nat., (4) VIP_{11-12} syn., (5) VIP_{11-12} syn. + VIP_{11-12} nat., (6) VIP_{11-12} nat.

conditions used, the acidic and the basic substances, respectively, from the two sources, migrated indistinguishably from each other (Fig. 1). These comparisons support the correctness of sequences 7–13 (Fig. 2) of VIP.

Thr-Asp-Asn-Tyr-Thr-Arg-Leu
7 8 9 10 11 12 13

FIG. 2. Sequence 7–13 of VIP.

EXPERIMENTAL

A. Synthesis[4]

Capillary melting points are reported uncorrected. Thin-layer chromatograms (silica gel, Merck) were developed with the solvent systems: A: *n*-butanol–acetic acid–water (4:1:1); B: *n*-butanol–pyridine–acetic acid–water (30:20:6:24); C: chloroform–methanol (9:1); D: ethyl acetate–pyridine–acetic acid–water (60:20:6:11). Spots were revealed by uv, charring with ammonium hydrogen sulfate (*14*), modified Rydon–Zahn reagent (*15*), and Sakaguchi reagents. For amino acid analysis, samples were hydrolyzed with constant boiling hydrochloric acid in evacuated, sealed ampoules at 110°C for 16 hr, and analyzed by the method of Spackman, Stein, and Moore (*16*) on a Beckman-Spinco 120C instrument.

t-*Butyloxycarbonyl*-L-*threonyl-nitro*-L-*arginyl*-L-*leucine methyl ester* (*II*). A sample of benzyloxycarbonyl-nitro-L-arginyl-L-leucine methyl ester[5] (*7–9*) (3.85 g, 8 mmole) was treated with 4 *N* HBr in AcOH (32 ml). After 1 hr at room temperature, the dipeptide hydrobromide (I) was precipitated with ether (100 ml), washed with more ether (2 × 50 ml), filtered, and dried over NaOH. The hydrobromide (I) was dissolved in a mixture of DMF (5 ml) and CH_2Cl_2 (25 ml), and the amine liberated by the addition of triethylamine (2 ml). *t*-Butyloxycarbonyl-L-threonine[6] (2 g, 9 mmole) and EEDQ (*10*) (2.5 g, 10 mmole) were added and the mixture stirred overnight. Next morning, 50 ml more CH_2Cl_2 was added, the solution washed with a 20% solution of citric acid, H_2O, 0.5 *N* $KHCO_3$ and H_2O (each 50 ml), dried and evaporated. The residue solidified on trituration with ether; it was filtered and washed with ether: yield, 3.7 g. On tlc, II moves with an R_fC of 0.30. An impurity with R_f 0.39 is also present.[7] The protected tripeptide ester was purified by silica gel chromatography on a 2.5 × 78 cm column, prepared with $CHCl_3$, 3% MeOH in $CHCl_3$ eluted the impurity, 5% MeOH in $CHCl_3$ eluted 2 g (45.6%) of the desired tripeptide, which has no well defined mp: $[\alpha]_D^{25}$ −13° (*c* 1, DMF); tlc R_fA 0.62, R_fB 0.66, R_fC 0.30. Amino acid analysis: thr, 0.9; Arg + Orn + NO_2 − Arg, 1.0; Leu, 1.0.

Anal. Calcd for $C_{22}H_{41}N_7O_9$ (547.6): C, 48.3; H, 7.5; N, 17.9. Found: C, 48.5; H, 7.7; N, 17.6.

t-*Butyloxycarbonyl*-O-*benzyl*-L-*tyrosyl*-L-*threonyl-nitro*-L-*arginyl*-L-*leucine methyl ester*(*IV*). The protected tripeptide II (3.52 g, 6.4 mmole) was dissolved in TFA (15 ml). After 10 min, the TFA was evaporated *in vacuo*, dry ether (200 ml) was added, and the product was collected by filtration. It was washed with dry ether (100 ml) and dried *in vacuo* over NaOH and P_2O_5 to give III, the tripeptide ester trifluoroacetate: 3.42 g (95%); mp 95–97°C; tlc R_fA 0.45, R_fB 0.67.

[4] The following abbreviations are used: DMF (dimethylformamide); TFA (trifluoroacetic acid); EEDQ (1-ethoxy-2-ethyl-1,2-dihydroquinoline).

[5] This protected dipeptide methyl ester was obtained in homogeneous form (tlc), mp 161–162°C. The mp was unchanged on recrystallization from ethanol. Lit.: mp 162–163.4°C (7), 170–171°C (8), and 160–161°C (9).

[6] Purchased from Bachem, California.

[7] From its nmr spectrum ($CDCl_3$), the material seems to be ethoxycarbonyl-nitroarginyl-leucine methyl ester. Since the proposed mechanism (10) for EEDQ couplings involves an ethoxycarbonyl mixed anhydride intermediate, the formation of this by-product could have been anticipated.

A solution of III (3.42 g, 61 mmole) in DMF (13 ml) was neutralized with triethylamine (0.85 ml). *t*-Butyloxycarbonyl-*O*-benzyl-L-tyrosine *p*-nitrophenyl ester (*11*) (3 g, 6.1 mmole) was added and the reaction mixture kept slightly basic by the addition of small amounts of the same base. After 3 days, the mixture was diluted with ethyl acetate (300 ml), washed with 5% citric acid (60 ml), with 0.5 *N* ammonium hydroxide until free of *p*-nitrophenol, 5% citric acid (20 ml), water (3 × 20 ml) and saturated sodium chloride solution (20 ml). It was dried over sodium sulfate, filtered and evaporated *in vacuo*. The residue was dissolved in methanol (15 ml) and precipitated by the addition of cold water (200 ml). The product was collected by filtration, washed with water (30 ml), and dried in air. Yield: 4.8 g (98%); mp 99–101°C (softens at 89–90°C). The mp remains unchanged after reprecipitation from methanol–water and then from ethyl acetate–light petroleum ether. $[\alpha]_D^{25}$ −7° (*c* 1, DMF); tlc R_fA 0.80, R_fB 0.80, R_fC 0.36.

Anal. Calcd for $C_{38}H_{56}N_8O_{11}$ (800.9): C, 57.0; H, 7.0; N, 14.0. Found: C, 56.7; H, 7.1; N, 14.0.

t - *Butyloxycarbonyl* - L - *asparaginyl* - O - *benzyl* - L - *tyrosyl* - L - *threonyl* - *nitro* L - *arginyl*-L-*leucine methyl ester* (*VI*). The protected tetrapeptide IV (4.8 g, 6 mmole) was dissolved in TFA (20 ml). After 8 min, the TFA was evaporated *in vacuo*. Dry ether (200 ml) was added; the product was collected by filtration, washed with ether (100 ml) and dried *in vacuo* over NaOH and P_2O_5 to give 4.78 g (98%); mp 73–76°C; tlc R_fA 0.68, R_fB 0.69.

The tetrapeptide ester trifluoroacetate V (4.78 g, 5.9 mmole), triethylamine (0.82 ml) and *t*-butyloxycarbonyl-L-asparagine *p*-nitrophenyl ester[8] (2.14 g) were dissolved in DMF (18 ml). After 2 days, more active ester (706 mg) was added. Next day, the reaction mixture was poured slowly into ethyl acetate (200 ml), the precipitate filtered, washed with ethyl acetate (4 × 15 ml) and dried in air to give 3.72 g; mp 169–170°C. The filtrate was concentrated to a small volume and a second crop (1.27 g, mp 169–170°C) was isolated by the addition of ethyl acetate (200 ml). The total yield is 92%. Reprecipitation of a sample from methanol–water raised the mp to 171–172°C; $[\alpha]_D^{25}$ −16.5° (*c* 1, DMF); tlc R_fA 0.74, R_fB 0.80, R_fC 0.27.

Anal. Calcd for $C_{42}H_{62}N_{10}O_{13}$ (915.0): C, 55.1; H, 6.8; N, 15.3. Found: C, 55.1; H, 6.8; N, 15.5.

t - *Butyloxycarbonyl* - *β* - *benzyl* - L - *aspartyl* - L - *asparaginyl* - O - *benzyl* - L - *tyrosyl* - L-*threonyl-nitro*-L-*arginyl*-L-*leucine methyl ester* (*VIII*). The protected pentapeptide VI (4.58 g, 5 mmole) was dissolved in TFA (20 ml). After 8 min, the TFA was removed *in vacuo* and the product isolated as described for compound III. Yield: 4.31 g (95%); mp 82–84°C; tlc R_fA 0.53; R_fB 0.66.

The pentapeptide ester trifluoroacetate VII (4.27 g, 4.6 mmole), triethylamine (0.64 ml) and *t*-butyloxycarbonyl-*β*-benzyl-L-aspartic acid *p*-nitrophenyl ester (*17*)

[8] Prepared according to the procedure described for the corresponding benzyloxycarbonyl derivative in *Biochem. Prep.* **10**, 122 (1963); mp 163–164.5°C, $[\alpha]_D^{25}$ −36°C (*c* 1, DMF). Lit.: mp 157–158°C, $[\alpha]_D^{28}$ −36.9°C (*c* 1.001, DMF), E. Bayer, G. Jung and H. Hagenmaier, *Tetrahedron* **24**, 4853 (1968); mp 161–162.5°C, $[\alpha]_D^{25}$ −44°C (*c* 1.3, DMF), ref. 11; mp 163°C, G. R. Marshall and R. B. Merrifield, *Biochem.* **4**, 2394 (1965); mp 157–158°C, $[\alpha]_D$ −45.3 (*c* 1, DMF), E. Schröder and E. Klieger, *Ann.* **673**, 208 (1964); mp 178–180°C, $[\alpha]_D^{22}$ −36°C (*c* 2, DMF), E. Sandrin and R. A. Boissonnas, *Helv. Chim. Acta* **46**, 1637 (1963).

(2.65 g) were dissolved in DMF (10 ml). After about 16 hr, the reaction mixture was poured slowly into ethyl acetate (200 ml), the product filtered, washed with ethyl acetate (4 × 15 ml), ether (2 × 10 ml), and air dried to give 4.36 g (86%); mp 187–188.5°C. Reprecipitation of a sample from DMF–water did not change the mp. $[\alpha]_D^{25}$ −16° (*c* 1, DMF); tlc R_fA 0.77, R_fB 0.74, R_fC 0.34.

Anal. Calcd for $C_{53}H_{73}N_{11}O_{16}$ (1120.2): C, 56.8; H, 6.6; N, 13.8. Found: C, 56.5; H, 6.7; N, 13.6.

t - *Butyloxycarbonyl* - L - *threonyl* - *β* - *benzyl* - L - *aspartyl* - L - *asparaginyl* - O - *benzyl*-L-*tyrosyl*-L-*threonyl-nitro*-L-*arginyl*-L-*leucine methyl ester* (*X*). The protected hexapeptide VIII (3.92 g, 3.5 mmole) was dissolved in TFA (15 ml). After 10 min, the TFA was removed *in vacuo* and the product isolated as described for compound III. Yield: 3.89 g (98%); mp 193–194°C; tlc R_fA 0.59, R_fB 0.69.

The hexapeptide ester trifluoroacetate IX (3.89 g), triethylamine (0.48 ml) and *t*-butyloxycarbonyl-L-threonine-2,4-dinitrophenyl ester (*12*) (5.25 mmole) were dissolved in DMF (12 ml). The reaction mixture was kept slightly basic by the addition of small amounts of the same base. Next day, it was poured slowly into ethyl acetate (200 ml). The product was filtered, washed with ethyl acetate (4 × 10 ml) and ether (4 × 10 ml), and air dried to give 2.9 g (68%); mp 194–196°C. A second crop was isolated from the combined filtrate, yield 500 mg (12%), mp 194–196°C. Reprecipitation of a sample from DMF–ethyl acetate and from DMF–water did not raise the mp. $[\alpha]_D^{25}$ −18° (*c* 1, DMF); tlc R_fA 0.8, R_fB 0.8, R_fC 0.19.

Anal. Calcd for $C_{57}H_{80}N_{12}O_{18}$ (1221.3): C, 56.0; H, 6.6; N, 13.8. Found: C, 56.0; H, 6.4; N, 13.8.

t - *Butyloxycarbonyl* - L - *threonyl* - L - *aspartyl* - L - *asparaginyl* - L - *tyrosyl* - L - *threonyl*-L-*arginyl*-L-*leucine methyl ester* (*XI*). The fully protected heptapeptide X (2.69 g, 2.2 mmole) was suspended in 90% aqueous methanol (500 ml), containing acetic acid (0.6 ml), and hydrogenated in the presence of 10% Pd/C catalyst (820 mg) for 48 hr. The reaction mixture was filtered from the catalyst and evaporated to a small volume. Ethanol (10 ml) was added and the suspension reevaporated. The residue was then suspended in ethanol (10 ml) and precipitated with ether (200 ml). The product was filtered, washed with ether (30 ml) and dried in air. Yield: 2.03 g (92%); mp 171–173°C (dec.). The mp remained unchanged on reprecipitation from methanol with ether. $[\alpha]_D^{25}$ −21° (*c* 1, DMF); tlc R_fA 0.51, R_fB 0.67. Amino acid analysis: Thr, 2.0; Asp, 2.0; Tyr, 0.9; Arg, 1.0; Leu, 1.0; NH_3, 2.0.

Anal. Calcd for $C_{43}H_{69}N_{11}O_{16}\cdot\frac{1}{2}H_2O$ (1005.1): C, 51.4; H, 7.0; N, 15.3. Found: C, 51.2; H, 7.1; N, 15.6.

A sample (40 mg) of compound XI was treated with TFA (0.4 ml) for 10 min. After the removal of the TFA *in vacuo*, the residue was triturated with ether (10 ml), filtered, washed with ether, and dried *in vacuo* over P_2O_5 and NaOH. This heptapeptide methyl ester trifluoroacetate (XIa) was used for the comparisons with fragments of natural VIP. A portion (3.2 mg) was treated with aminopeptidase M (0.75 mg) at 37°C for 20 hr under the conditions described by Hofmann and his co-workers (*13*). Amino acid analysis of this digest gave the following ratios: Arg, 1.0; Asp, 1.0; Thr, 2.1; Leu, 1.0; Tyr, 0.9. (Asn was not calculated because it was not sufficiently separated from Thr.).

t - *Butyloxycarbonyl* - L - *threonyl* - L - *aspartyl* - L - *asparaginyl* - L - *tyrosyl* - L -

threonyl-L-arginyl-L-leucine hydrazide (*XII*). The partially protected heptapeptide XI (1.71 g, 1.7 mmole) was suspended in methanol (36 ml), cooled with ice-water, and hydrazine (97%, 4 ml) was added. A clear solution resulted. After 3 hr at room temperature, the solvent was evaporated *in vacuo* and the oily residue was dried overnight over H_2SO_4. The amorphous material was suspended in methanol (10 ml) and precipitated with ether (200 ml). The product was filtered, washed with ether (30 ml) and dried *in vacuo* over H_2SO_4. Yield: 1.64 g (97%); mp 171–172°C (dec.). After reprecipitation of a sample from methanol with ether, the mp remained the same. $[\alpha]_D^{25}$ −6° (*c* 1, DMF); tlc R_fA 0.44, R_fB 0.72, R_fD 0.13. Amino acid analysis: Thr, 2.0; Asp, 2.1; Tyr, 0.9; Arg, 1.0; Leu, 1.0; NH_3, 1.1.

For analysis, a sample was dried at 80°C and 0.1 mm for 2 hr; the determined values still indicate the presence of water.

Anal. Calcd for $C_{42}H_{69}N_{13}O_{15}$ (996.1): C, 50.6; H, 7.0; N, 18.3. Calcd for $C_{42}H_{69}N_{13}O_{15}\cdot 2H_2O$ (1032.1): C, 48.0; H, 7.1; N, 17.6. Found: C, 48.2; H, 7.1; N, 17.3.

B. Comparison of the Fragments Comprising the Amino Acid Sequences 7–10 and 11–12 from Natural VIP with the Corresponding Synthetic Peptides

Chymotrypsin (α-chymotrypsin, TLCK treated) was obtained from Merck, Darmstadt, trypsin (TPCK treated "TRTPCK") from Worthington, Sephadex LH20 from Pharmacia, Uppsala, and ammonium bicarbonate, of reagent grade, from Baker and Adamson. Carboxymethyl cellulose, CM-22, was from Whatman. The techniques used for paper electrophoresis and paper chromatography have been described elsewhere (*18*, *19*).

Cleavage of leucine methyl ester from the heptapeptide ester and isolation of the residual hexapeptide. Two milligrams of the heptapeptide ester XIa was dissolved in 1 ml 1% NH_4HCO_3, and 50 μliters of a 0.2% solution of trypsin in m*M* AcOH was added. After 2 hr at 21°C, the solution was lyophilized. Paper chromatography in the *n*-butanol–acetic acid–pyridine–water (30:6:20:24) system of Waley and Watson (*20*) of a 1% aliquot of the lyophilized material in parallel with 20 μg of the original heptapeptide ester showed that the latter (R_f 0.37) had completely disappeared during the treatment with trypsin and three new substances with R_f values 0.50 (leucine), 0.76 (leucine methyl ester) and 0.08 had been formed instead. The substance with the R_f value 0.08, obviously the hexapeptide, gave, like the original heptapeptide ester, a yellow color with the cadmium-ninhydrin reagent used (*21*); the other two substances gave the ordinary reddish color.

The bulk of the lyophilized material was dissolved in 0.2 ml 0.1 *M* ammonium bicarbonate and 0.8 ml of ethanol was added to the clear solution. A slight precipitate, most probably trypsin, was collected by centrifugation and discarded. Half of the clear supernatant was chromatographed in 80% v/v ethanol which was 0.02 *M* in ammonium bicarbonate on a 0.6 × 46 cm column of LH Sephadex. Fractions of 0.5 ml each were collected at a flow rate of one fraction per 6 min. Material absorbing at 280 nm appeared in fractions 14–17. These were combined and lyophilized. The lyophilized material was found to contain the hexapeptide, migrating at R_f 0.08, in the Waley–Watson system.

Degradation of the hexapeptide with chymotrypsin and separation of the fragments formed. One milligram of the hexapeptide obtained as described in the previous section was dissolved in 0.5 ml 1% ammonium bicarbonate and 10 μliters of a 0.2% solution of chymotrypsin in m*M* AcOH was added. After 2 hr at 21°C, the degradation mixture was frozen and lyophilized. Paper electrophoresis at pH 6.4 (90 min at 50 V/cm in pyridine/acetic acid/water, 300:1.5:2700 by vol, Whatman 3 MM paper) of a 2% aliquot of the lyophilized material in parallel with 20 μg of the hexapeptide showed that the original material, which migrated 1.5 cm toward the cathode, uncorrected for electroendosmosis, had disappeared. Instead, two new fragments had been formed, both giving a yellow color with the ninhydrin reagent but one moving 9 cm toward the anode and the other 15 cm toward the cathode.

The bulk of the lyophilized material was dissolved in 0.5 ml of an ammonia/acetic acid buffer of pH 4.8 (0.02 *M* in acetic acid, 0.07 *M* in ammonia), and chromatographed in this buffer on a 0.6 × 10 cm column of carboxymethyl cellulose. Material adsorbing light at 280 nm but giving a negative phenantrenequinone reaction for arginine (*22*) appeared in fractions 3–4. They were combined and lyophilized. Fractions 6–7 did not absorb light at 280 nm, but gave a positive reaction for arginine. They too were combined and lyophilized. The material from fractions 3–4 was found to contain the substance migrating toward the anode on paper electrophoresis and that from fractions 6–7, the basic substance.

Comparison of the two fragments of the hexapeptide with the corresponding fragments from natural VIP. The fragments of natural VIP comprising the amino acid sequences 7–10, threonyl-aspartyl-asparaginyl-tyrosine, and 11–12, threonyl-arginine, respectively, were isolated from a chymotryptic degradation mixture of the N-terminal tryptic peptide of VIP as described elsewhere (*3*). On high voltage paper electrophoresis at pH 6.4, the acidic fragment from the hexapeptide and the threonyl-aspartyl-asparginyl-tyrosine from VIP were indistinguishable, when run either in parallel or in mixture (Fig. 1). They were also indistinguishable on paper chromatography in the Waley–Watson system, both as to speed of migration (R_f 0.20) and as to color tone. Similarly, the basic fragment from the hexapeptide and the threonyl-arginine from VIP were indistinguishable (R_f 0.17).

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