Specific Tritium Labeling of a Potent Gastrin Analogue

Synthesis and Pharmacological Activities of C-Terminal Gastrin Tetrapeptide Analogues

C. S. PANDE, J. RUDICK,[1] L. ORNSTEIN, I. L. SCHWARTZ, AND RODERICH WALTER

Departments of Physiology and Surgery, Mount Sinai Medical and Graduate Schools of The City University of New York, New York, New York 10029 and Medical Department, Brookhaven National Laboratory, Upton, New York 11973

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SUMMARY

The synthesis of a specifically labeled C-terminal gastrin tetrapeptide analogue, *tert*-butoxycarbonyl-L-tryptophyl-L-norleucyl-4,5-^{3}H-L-aspartyl-L-phenylalanine amide(compound IV), is described. This labeled peptide was obtained by catalytic tritiation of *tert*-butoxycarbonyl-L-tryptophyl-L-4-dehydronorleucyl-L-aspartyl-L-phenylalanine amide (II). Upon catalytic hydrogenation, the latter compound gave *tert*-butoxycarbonyl-L-tryptophyl-L-norleucyl-L-aspartyl-L-phenylalanine amide (III), which possessed physical properties identical with those of the authentic material. The biological properties of compounds II and III are compared with those of L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide (I), the natural C-terminal tetrapeptide of gastrin, with respect to gastric secretion in the frog, rat, and dog, and pancreatic secretion in the dog.

INTRODUCTION

The heptadecapeptide amides gastrins I and II, which have been isolated from the antrum of the stomach, are highly potent stimulants of gastric secretion and in addition exhibit a wide range of biological activities in the alimentary tract. The amino acid sequences of these peptides, isolated by Gregory and Tracy (1) from hog antral mucosa, have been elucidated (2), and their synthesis reported (3). One of the highlights of the biological investigations with gastrins and their synthetic intermediates was the discovery that only the C-terminal tetrapeptide [L-*tryptophyl*-L-methionyl-L-aspartyl-L-phenylalanine amide (compound I)] was required for evoking the full range of physiological activities of the natural hormones (4).

This discovery—in the light of current pharmacological concepts (5), which postulate that an essential step in the mechanism by which hormones exert their biological effects is the attachment of the agonist to specific, chemically defined portions of target tissue—offered the possibility of utilizing a tritium-labeled tetrapeptide as a model for the purpose of localizing the site of action of gastrin. In designing such a labeled analogue, a number of requirements had to be considered: (a) it was desirable to introduce the tritium label, preferably by means of catalytic reduction, in a final synthetic step into a precursor molecule, which in this way would serve as stable

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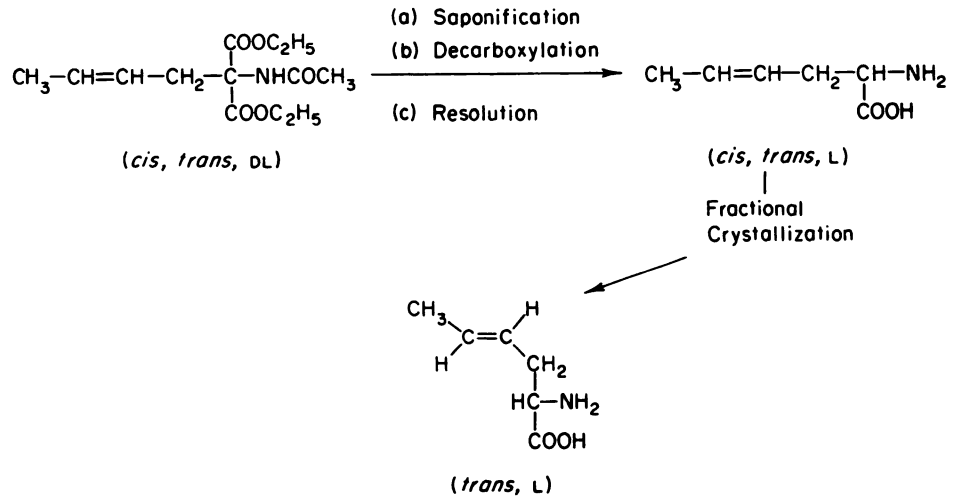
stock material; (b) the resulting labeled product should not need any further purification; (c) to avoid the danger of desulfurization during the introduction of the label by catalytic reduction, the methionyl residue present in the natural C-terminal tetrapeptide (I) should be replaced by a non-sulfur-containing amino acid residue. However, the modified tetrapeptide should exhibit both a spectrum of biological effects and an intrinsic activity identical with those of tetrapeptide I. All these criteria were apparently met by *tert*-butoxycarbonyl-L-tryptophyl-L-norleucyl-L-aspartyl-L-phenylalanine amide properties of II and III with those of I with respect to gastric secretion in the frog, rat, and dog, and pancreatic secretion in the dog.

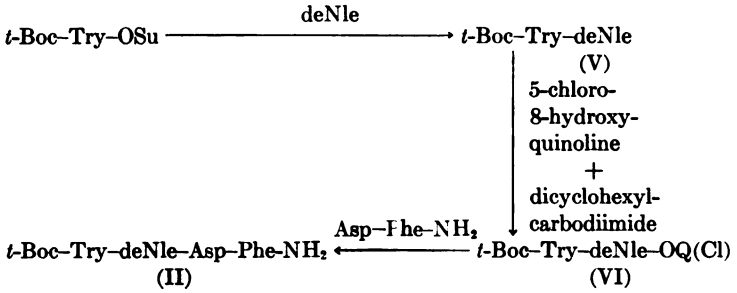
A key amino acid in the synthesis of IV was L-2-amino-*trans*-4-hexenoic acid. For the preparation of this amino acid, ethyl acetamidomalonate was alkylated with crotyl bromide; the resulting *C*-alkylated ethyl acetamidomalonate was saponified according to the general procedure described by Albertson and Archer (7), and the DL-2-acetamido-4-hexenoic acid was then resolved with acylase as described by Greenstein *et al.* (8) (see Scheme 1). Amino



SCHEME 1

(6), an analogue of the gastrin C-terminal tetrapeptide in which the L-methionyl residue has been replaced by an L-norleucyl residue.

In this communication we report (a) the synthesis of the precursor *tert*-butoxycarbonyl-L-tryptophyl-L-4-dehydronorleucyl-L-aspartyl-L-phenylalanine amide (II); (b) the conversion of II to *tert*-butoxycarbonyl-L-tryptophyl-L-norleucyl-L-aspartyl-L-phenylalanine amide (III) by catalytic hydrogenation; (c) the conversion of II to *tert*-butoxycarbonyl-L-tryptophyl-L-norleucyl-4,5-^{3}H-L-aspartyl-L-phenylalanine amide (IV) by catalytic tritiation; and (d) a comparison of the pharmacological acid analysis of a sample of this resolved material revealed, aside from the major peak, the presence of a second, ninhydrin-active component (approximately 20%). The possibility that the minor component was a structural isomer of the desired product, such as L-2-amino-3-methyl-4-pentenoic acid, had to be explored. Such an allylic rearrangement had probably been encountered in the case of the alkylation of the carbanion of ethyl acetamidocyanoacetate with crotyl bromide (9) and had in fact been established for related alkylating reactions (10–12). During our condensation reaction, however, such a rearrangement did not occur, because catalytic hy-



SCHEME 2

drogenation of another sample of the resolved material gave optically pure norleucine as the sole product.

Repeated crystallization of the reaction product from aqueous acetone reduced the amount of the minor component to 2.5%. The 60 Mc/sec proton magnetic resonance spectrum of the product thus secured displayed a pattern in agreement with that of 2-amino-4-hexenoic acid. The spectrum taken in deuterated trifluoracetic acid featured the multiplets of the two olefinic protons at $\tau = 4.10$ and 4.63, respectively. When the spectrum was recorded at 220 Mc/sec, these multiplets were clearly separated. Centered at $\tau = 5.66$, essentially a triplet, were the methine resonances, and at $\tau = 7.10$, those of the distorted quartet of the methylene group. The methyl resonances appeared as a doublet at $\tau = 8.25$. The presence of a *trans*-substituted ethylene grouping was supported by the infrared spectrum, which exhibited a strong band at 970 cm^{-1} characteristic of C—H out-of-plane deformation vibrations (13). It can thus be concluded that the L-2-amino-4-hexenoic acid is the *trans* isomer (hereafter referred to as L-4-dehydronorleucine) containing 2.5% of the *cis* isomer.

The L-4-dehydronorleucine was allowed to react with *tert*-butoxycarbonyl-L-tryptophan *N*-hydroxysuccinimide ester (14), and the resulting dipeptide, *tert*-butoxycarbonyl-L-tryptophyl-L-4-dehydronorleucine (V), was converted to the 5-chloro-8-hydroxyquinoline ester (VI)—an activated ester of high optical stability recently introduced into peptide chemistry (15). This dipeptide ester was coupled with L-aspartyl-L-phenylalanine amide[2] to yield the tetrapeptide, *tert*-butoxycarbonyl-L-tryptophyl-L-4-dehydronorleucyl-L-aspartyl-L-phenylalanine amide (II) (see Scheme 2). A sample of compound II was converted by catalytic hydrogenation to *tert*-butoxycarbonyl-L-tryptophyl-L-norleucyl-L-aspartyl-L-phenylalanine amide (III), previously prepared by Morley and Smith (6). Com-

[2] The synthesis of this dipeptide was reported by Davey *et al.* (16); however, in this study we evolved a more convenient preparation of this compound. L-Aspartic acid was selectively esterified in 40% yield with benzyl alcohol to give β-benzyl L-aspartate (17), then carbobenzoxylated in 75% yield (17–19), and finally converted to α-2,4,5-trichlorophenyl β-benzyl *N*-carbobenzoxy-L-aspartate in 85% yield; m.p. 89–91°, $[\alpha]_D^{22}$—26.6° (*c*, 1, in dimethylformamide) [m.p. 93–94°, $[\alpha]_D^{22}$ —22.1° (*c*, 1, in dimethylformamide) (17)]. This activated ester was allowed to react with phenylalanine amide to give β-benzyl *N*-carbobenzoxy-L-aspartyl-L-phenylalanine amide (yield, 77%), which was hydrogenated in 95% acetic acid to give the L-aspartyl-L-phenylalanine amide (17) in 71% yield. The acid hydrolysate of a sample of the dipeptide amide showed, on amino acid analysis, the following molar ratios: aspartic acid, 1.00; phenylalanine, 0.97; and ammonia, 0.97. Different preparations of this dipeptide exhibited a melting point of either 154–155° or 223–225°. Both peptides were found to be monohydrates ($C_{18}H_{17}N_3O_4 \cdot H_2O$: Calculated: C 52.5, H 6.39, N 14.1. Found: C 52.5, H 6.30, N 14.1, and C 52.4, H 6.26, N 14.1, respectively). However, all samples possessed the same optical rotation, gave similar amino acid analyses, and exhibited identical behavior on paper chromatography. This suggests that L-aspartyl-L-phenylalanine amide monohydrate is dimorphous. The literature reports a melting point of 188–189° for the unhydrated dipeptide amide (16).

parison of the physical data of III prepared in this study with those of a sample supplied by Dr. Morley revealed that both preparations possessed comparable properties.

In addition, the optical homogeneity of III was determined by digestion with leucine aminopeptidase after liberation of the terminal amino group of a sample of III by treatment with trifluoracetic acid. Quantitative amino acid analysis gave the expected ratio of the constituent amino acids, thus proving that the acylation of L-aspartyl-L-phenylalanine amide with the ester VI proceeded without racemization.

Another sample of II was catalytically reduced in the presence of tritium gas, yielding IV with a specific activity of 212 μCi/mg. The labeled material (IV) migrated as a single spot on thin layer chromatography, and exhibited an R_F value identical with that of III. Amino acid analysis following acid hydrolysis gave the expected amino acid and ammonia ratios. It was also shown that the radioactivity was associated solely with the peak which corresponded to norleucine.

Tracy and Gregory (4) showed that the tetrapeptide I exhibited the full range of biological activity of gastrin itself. Although it was not as potent as gastrin on a molar basis, it elicited a qualitatively identical response (20). Morley *et al.* (21) have shown in several bioassays that many changes could be made at the methionine position, including replacement of the sulfur atom by a methylene moiety (tetrapeptide III), without impairing the effective response of tetrapeptide I. When we compared the full dose-response relationship of the gastric secretory action of the tetrapeptides II and III with those of I on the isolated gastric mucosa of the bullfrog (*Rana catesbiana*) (22), we found that all three agonists possess the same intrinsic activity (i.e., the ability to induce a response subsequent to receptor occupation), while the affinities (i.e., the ability to combine with a receptor) of II and III were 85–90% that of I. As in the case of I, peptides II and III proved to be potent stimulants of gastric acid secretion in the anesthetized rat and the conscious dog (Fig. 1). Moreover, in the latter assay, peptides I, II, and III were equipotent stimulants of pepsin secretion. All three peptides also provoked an equal and marked stimulatory response for enzyme elaboration from the canine pancreas.

MATERIALS AND METHODS[3]

Syntheses

L-*2-Amino-cis,trans-4-hexenoic acid.* Diethyl acetamidomalonate was alkylated with *cis,trans*-crotyl bromide, and the resulting ester was saponified and decarboxylated (7). After crystallization from hot water, DL-2-acetamido-*cis,trans*-4-hexenoic acid was isolated in 60% yield (m.p. 114–114.5°) and was subsequently resolved by selective deacetylation with hog acylase (8) to give a 59% yield of L-2-amino-*cis,trans*-4-hexenoic acid; m.p. 260° (decomposition), $[\alpha]_D^{23}$ −17.6° (*c*, 1, in 5 N hydrochloric acid).

$C_6H_{11}NO_2$

Calculated: C 55.8, H 8.53, N 10.9%

Found: C 56.0, H 8.73, N 10.8%

[3] Crotyl bromide was purchased from Aldrich Chemical Company; hog kidney acylase, from Mann Research Laboratories; and leucine aminopeptidase, from Worthington Biochemical Corporation. Thin layer chromatograms were run on silica gel G (Warner-Chilcott Laboratories); paper chromatograms were run on Whatman No. 1 paper by the descending method at 24° in the solvent system 1-butanol–acetic acid–water, 5:1:4 (v/v/v, upper phase). Tryptophan-containing compounds were stained with the Ehrlich reagent (23); other peptides were visualized according to the procedure of Zahn and Rexroth (24). Catalytic tritiation was performed by Tracerlab, Waltham, Massachusetts. Amino acids and the peptide hydrolysates were chromatographed on a Beckman 120C amino acid analyzer using Beckman "custom research" resin PA-29 packed in a 56 × 0.9 cm column maintained at 55° throughout the analysis. The buffer flow rate was 68 ml/hr, the ninhydrin flow rate was 34 ml/hr, and the recorder print speed was 1 dot/sec. The calculated developing time of the ninhydrin color was 7.7 min. Pepsin was obtained from Mann Research Laboratories, and natural porcine secretin, from the Gastrointestinal Hormone Laboratory, Karolinska Institutet, Stockholm.

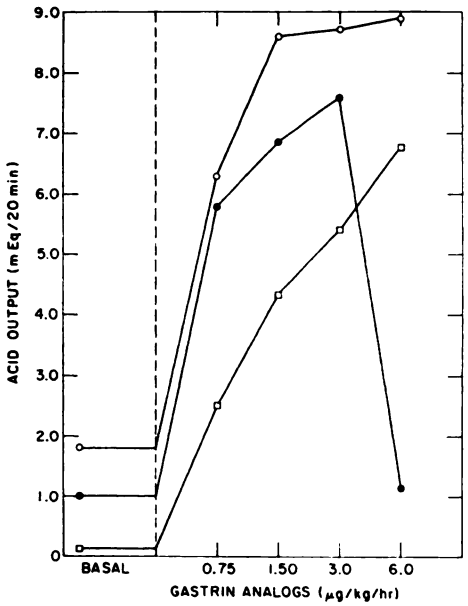


FIG. 1. *Typical dose-response curves of gastrin analogues I* (○——○), *II* (●——●), *and III* (□——□) *for acid secretion in the gastric fistula dog*

L-*2-Amino-trans-4-hexenoic acid* (L-*4-dehydronorleucine*). When a sample of L-2-amino-*cis,trans*-4-hexenoic acid (1 μmole) was applied to the acidic column of the amino acid analyzer and eluted with 0.2 N citrate buffer (pH 3.25 ± 0.002), the chromatogram showed two peaks, one emerging at 149.5 ml (peak A) and the other at 158.5 ml (peak B). These peaks corresponded to the *cis* and *trans* isomers and were present in a 1:5 ratio. Another sample of L-2-amino-*cis,trans*-4-hexenoic acid (100 mg) was hydrogenated in glacial acetic acid in the presence of 10 mg of palladium on charcoal (10%) at 30° for 5 hr. After removal of the catalyst by filtration, the solution was lyophilized and yielded quantitatively L-norleucine; m.p. 320–322° (decomposition), $[\alpha]_D^{22}$ +23.6° (*c*, 4.15, in 6 N hydrochloric acid) [$[\alpha]_D^{23}$ +23.3° (*c*, 4.15, in 6 N hydrochloric acid) (25)]. The product was homogeneous on paper chromatography and gave, on examination by amino acid analysis, only one peak corresponding to L-norleucine.

The L-2-amino-*cis,trans*-4-hexenoic acid (13 g) was subjected to repeated fractional crystallization (10 times) from 50% aqueous acetone to yield 0.8 g of the *trans* isomer, which contained 2.5% of the *cis* isomer; m.p. 261–262° (decomposition), $[\alpha]_D^{22}$ −23.7° (*c*, 1, in 5 N hydrochloric acid). The last percentages of *cis* isomer could not be removed on further fractional crystallization.

*tert-Butoxycarbonyl-*L*-tryptophyl-*L*-4-dehydronorleucine (V).* The above *trans* isomer (0.516 g), together with sodium bicarbonate (0.672 g), was dissolved in 25 ml of warm water, and the solution was cooled to room temperature. The *N*-hydroxysuccinimide ester of *tert*-butoxycarbonyl-L-tryptophan (1.614 g) was dissolved in warm ethanol (25 ml), cooled to room temperature, and added to the above aqueous solution with stirring. Ethanol was added until the solution became homogeneous, and stirring was continued for 24 hr more at room temperature. After this period the solvent was removed under reduced pressure, and the resulting syrup was dissolved in 20 ml of water and washed with ethyl acetate. The aqueous phase was separated, acidified to pH 4 with acetic acid, and then extracted with ethyl acetate. Upon evaporation of the solvent a white, amorphous powder was obtained, which was purified by precipitation from ether–petroleum ether. The purified product was homogeneous on thin layer chromatography in ethyl acetate–acetic acid (25:0.3). Yield, 1.1 g (66%); $[\alpha]_D^{23}$ + 4.0° (*c*, 5, in 95% ethanol).

$C_{22}H_{29}N_3O_5$
Calculated: N 10.1%
Found: N 9.68%

To a solution of compound V in ethanol, an equimolecular amount of dicyclohexylamine was added. Upon dilution with ether and cooling, crystals of the dicyclohexylammonium salt separated in quantitative yield; m.p. 202–203° (decomposition); they exhibited no optical rotation in ethanol and dioxane.

$C_{34}H_{53}N_4O_5$
Calculated: C 68.5, H 8.72, N 9.40%
Found: C 68.3, H 8.70, N 9.33%

*tert-Butoxycarbonyl-*L*-tryptophyl-*L*-4-dehydronorleucine 5-chloro-8-hydroxyquinoline ester (VI).* To a mixture of 2.5 g of V and 1.19 g of 5-chloro-8-hydroxyquinoline dissolved in 50 ml of dry ethyl acetate at −10°, 1.1 g of dicyclohexylcarbodiimide were added. The reaction mixture was stirred for 3 hr at −10° to −15°, and for 20 hr at room temperature. A few drops of 50% acetic acid were added, and the precipitated dicyclohexylurea was filtered off. The solution was washed successively with water, 5% sodium bicarbonate solution, water, 0.5 N HCl, and water; dried over anhydrous sodium sulfate; and evaporated to dryness under reduced pressure. The residue was dissolved in ether and precipitated with petroleum ether. Because of the lability of the ester, no further purification was attempted. The yield of active ester was 1.8 g (70%); m.p. 120–125°; $[\alpha]_D^{23}$ −4.4° (*c*, 1, in chloroform).

$C_{31}H_{33}ClN_4O_5$
Calculated: N 9.71%
Found: N 9.47%

L-*Phenylalanine amide.* A methanolic solution of L-phenylalanine methyl ester hydrochloride (26) was passed through a column of anion exchange resin, Rexyn RGl (OH^-), and the resulting free ester was converted in 77% yield to L-phenylalanine amide by treatment with dry methanolic ammonia; m.p. 94.5–95.5°, $[\alpha]_D^{23}$ +21.0° (*c*, 1.75, in water) [m.p. 91–92° (16), m.p. 93–94°, $[\alpha]_D^{20}$ +20.7° (*c*, 1.75, in water) (27)].

*tert-Butoxycarbonyl-*L*-tryptophyl-*L*-4-dehydronorleucyl-*L*-aspartyl-*L*-phenylalanine amide (II).* L-Aspartyl-L-phenylalanine amide (140 mg) was dissolved in 2 ml of dimethylformamide at ice bath temperature, and 288 mg of the ester VI were added immediately thereafter to the solution. The mixture was stirred as it slowly approached room temperature, where it was allowed to remain for the next 24 hr. The solvent was removed under reduced pressure, and the residue was dissolved in ethanol and precipitated with ether. The crude product was further purified by dissolving it in 18 ml of 1-butanol and agitating the resulting clear solution with a mixture of cyclohexane (12 ml) and 0.5% aqueous acetic acid (30 ml); the lower phase was subsequently removed. This entire process was repeated twice. Finally the organic phase was evaporated to dryness under reduced pressure, and the material was crystallized in needles from acetone, giving 200 mg (59%) of II; m.p. 206–208° (decomposition), $[\alpha]_D^{23}$ −26.9° (*c*, 1, in 95% ethanol).

$C_{35}H_{44}N_6O_8$
Calculated: C 62.1, H 6.51, N 12.4%
Found: C 62.3, H 6.54, N 12.4%

On thin layer chromatography in three solvent systems [cyclohexane–ethyl acetate–methanol–acetic acid (10:10:5:1, v/v/v/v), ethyl acetate–acetic acid (20:4, v/v), and acetonitrile–water (3:1, v/v)], II was found to be a homogeneous compound.

*tert-Butoxycarbonyl-*L*-tryptophyl-*L*-norleucyl-*L*-aspartyl-*L*-phenylalanine amide (III).* A solution of 50 mg of II in 30 ml of methanol was hydrogenated in the presence of 8 mg of palladium on charcoal for 6 hr. The catalyst was then filtered off, and the solvent was removed under reduced pressure. The resulting product melted at 196–198° (decomposition), $[\alpha]_D^{22}$ −29.2° (*c*, 1, in dimethylformamide) [m.p. 217–218° (decomposition), $[\alpha]_D^{24}$ −29.6° (*c*, 1, in dimethylformamide) (6)]. Amino acid analysis of the acidic hydrolysates of a sample of the peptide gave the following ratios of ninhydrin-positive substances: norleucine, 1.02; aspartic acid, 1.03; phenylalanine, 1.0; and ammonia, 1.14. The tryptophan was accounted for after base hydrolysis of another sample of tetrapeptide (norleucine to tryptophan = 1.00:0.94). Another sample (1.7 mg) of peptide III was treated with an excess of trifluoracetic acid for 30 min. The trifluoracetate of L-tryptophyl-L-norleucyl-L-aspartyl-L-phenylalanine amide was digested with leucine aminopeptidase (0.47 mg of enzyme in 0.1 ml of buffer). Upon amino acid analysis, the following molar ratios for the amino acids were obtained: tryptophan, 0.92; aspartic acid, 1.0; norleucine, 1.16; and phenylalanine, 0.92. The product was homogeneous on thin layer chromatography and exhibited

the same R_F as a sample of III prepared by an alternative route (6) in the solvent systems described above for II.

tert-Butoxycarbonyl-L-tryptophyl-L-norleucyl-4,5-^{3}H-L-aspartyl-L-phenylalanine amide (IV). The peptide II (30 mg) was catalytically tritiated in a manner analogous to that described for the preparation of III. After removal of catalyst, replaceable tritium, and solvent, the compound was obtained as a dry powder possessing an activity of 212 μCi/mg as measured in a Packard Tri-Carb scintillation counter using Bray's scintillation solution standardized with tritiated *n*-hexadecane. A sample of IV was applied on a thin layer plate and developed with ethyl acetate–acetic acid (25:3, v/v). The chromatogram was cut into horizontal sections, each of which was eluted with 3 ml of absolute ethanol in a liquid scintillation counting tube. For tritium counting, 12 ml of 0.3% solution of 5-phenyl-2-(4-biphenyl)oxadiazole in xylene were added. When counts per unit of time were plotted against tube numbers (tube 1 contained the section corresponding to the starting point of the chromatogram), a single, narrow, symmetrical peak with an R_F value identical with that of III was obtained. A sample of IV was hydrolyzed in 6 N HCl at 110° for 18 hr and then analyzed for ninhydrin-reactive material. The expected molar ratios of amino acids and ammonia were obtained. Tryptophan was not determined in this peptide. Radioactivity, monitored in an anthracene-packed flow cell connected directly to the bottom of the analytical column in the Tri-Carb counter, was associated only with the peak corresponding to norleucine.

Gastric Secretory Assays

Isolated frog mucosa. Assay of the three compounds was carried out on the bullfrog (*Rana catesbiana*). Bullfrogs, deprived of all food, were kept at room temperature until they were killed by decapitation. The stomachs were removed, and the gastric mucosa was separated from the muscular layers as an intact sheet. The gastric mucosa was immediately mounted between two chambers containing the following solutions: solution 1, on the submucosal (nutrient) side, 85.3 mM NaCl, 3.4 mM KCl, 1.8 mM $CaCl_2 \cdot 2H_2O$, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.8 mM KH_2PO_4, 17.8 mM $NaHCO_3$, and 11.0 mM glucose; solution 2, on the secretory side, was the same except for the omission of $NaHCO_3$, $MgSO_4 \cdot 7H_2O$, and KH_2PO_4 (21). A mixture of 95% oxygen and 5% carbon dioxide was bubbled through the nutrient solution, while 100% oxygen was passed through the bathing fluid on the secretory side. All incubations were carried out at room temperature (21–24°). The acid formed on the secretory side was titrated automatically and continuously to pH 5.5 with 0.1 N sodium hydroxide using a Radiometer TTT111C pH meter and automatic titrator.

Anesthetized rats. Male Sprague-Dawley rats, ranging in weight from 180 to 220 g (average, 206 g), were used in these experiments. The animals were housed in individual cages with wide wire mesh bottoms to prevent coprophagia. They were deprived of food for 48 hr prior to each experiment but were allowed water *ad libitum.*

Assay of the secretagogues was performed by a modification of the method of Ghosh and Schild (28). Under urethane anesthesia (1.75 g/kg intramuscularly), a No. 6 French soft polyethylene infant's nasogastric catheter was passed down the esophagus so that its tip lay just distal to the cardioesophageal sphincter. The esophagus was ligated around the catheter in the neck to prevent retrograde leakage of perfusion fluid. A second catheter was introduced into the stomach through a duodenostomy; the pylorus was ligated around the catheter, with care not to impair the blood supply to the duodenum. The stomach was gently flushed with 100 ml of warm solution of 0.9% sodium chloride at 37° using a Harvard constant infusion pump to maintain the flow rate at 1.0 ml/min. The perfusate was collected at 10-min intervals, and each sample was analyzed individually for titratable acidity. Intravenous injections of each stimulant were made in volumes of 0.5 ml via the

dorsal vein of the penis. No more than four intravenous injections of each stimulant were administered to a rat. Ninety minutes were allowed to elapse between each dose.

Conscious dogs. Three mongrel dogs weighing 16–22 kg were prepared with gastric fistulas. In each animal, a Thomas cannula (29) was inserted just proximal to the pyloric gland area near the greater curvature. At least 4 weeks elapsed before starting the experiments. All animals were deprived of food for at least 18 hr before each test but were allowed free access to water. None of the animals was used more than twice a week, and never on successive days.

The animal was placed in a modified Pavlov frame and basal secretion was collected; if there was any spontaneous secretion, the experiment was discontinued for that day. An intravenous infusion of 0.15 M NaCl was continued throughout each test. Each substance was administered intravenously at an initial dose of 0.375 μg/kg/hr. Gastric secretion was collected at 10-min intervals. When a plateau of secretion had been reached (usually after 60 min), the dose of each substance was doubled, until doubling the dose did not increase the volume flow rate of gastric juice. The doses ranged from 0.375 to 6 μg/kg/hr. Only one substance was used on any one animal on any single day. At least three experiments were carried out with each substance on each animal.

Ten-minute samples of gastric juice were analyzed individually for volume, titratable acidity, and pepsin concentration. The acidity was determined by titration to pH 7.0 with 0.01 N NaOH. Pepsin was measured by a modification of Hunt's method (30) using bovine serum albumin as substrate; 1 unit was equivalent to the peptic activity of 3 μg of crystalline pepsin.[8]

Pancreatic secretion in the conscious dog. Three mongrel dogs were prepared with Thomas cannulae (29) in the duodenum, placed opposite the major pancreatic duct. The accessory pancreatic duct was ligated and divided in each animal. A gastric cannula was placed in the most dependent part of the stomach, just proximal to the pyloric gland near the greater curvature; this was kept open during subsequent experiments to prevent acid contamination of the duodenum. At least 4 weeks elapsed before commencement of the study. The animals were deprived of food for 18 hr prior to each experiment but were allowed free access to water.

A continuous infusion of 0.15 M NaCl was administered intravenously throughout each experiment. The Thomas and gastric cannulae were opened, and the pancreatic duct was cannulated under direct vision with a glass cannula. This was kept in place, and pure, uncontaminated pancreatic secretion was collected utilizing the adapter and test tube described by Rudick and Dreiling (31). Pancreatic secretion was collected at 10-min intervals. Natural porcine secretin[3] was injected intravenously at a rate of 0.6 μg/min. When a secretory plateau had been reached, either substance I, II, or III was given as a single intravenous injection of 6 μg/kg. Only one substance was given to each animal on any one day; at least three tests were carried out with each substance on each animal.

Ten-minute samples were analyzed individually for volume, bicarbonate concentration (with a Technicon AutoAnalyzer), and enzyme content. The enzyme concentration of the pancreatic juice was measured as the total protein concentration (32), which was determined by measuring absorbance at 280 mμ with a Beckman DB spectrophotometer. Bovine serum albumin was used as standard.

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