Proc. Natl. Acad. Sci. USA
Vol. 75, No. 9, pp. 4115–4119, September 1978
Biochemistry

Chemical determination of polypeptide hormones

(peptide fragmentation analysis/COOH-terminal α-amide groups/gastrointestinal hormone determination)

KAZUHIKO TATEMOTO AND VIKTOR MUTT

Department of Biochemistry II, Nobel Medical Institute, Karolinska Institute, S-104 01 Stockholm, Sweden

Communicated by Rolf Luft, May 25, 1978

ABSTRACT **The presence or absence of peptide hormones in tissue extracts may in certain cases be demonstrated by exposing the extracts to conditions under which characteristic fragments of the polypeptide molecule in question are formed and then analyzing for such fragments. An approximate quantitation of the hormones may also be achieved thereby. In the present work the COOH-terminal fragments of polypeptides containing characteristic α-amide groups were released enzymically and then converted into the fluorescent dansyl derivatives, which were identified by thin-layer chromatography. In this way the presence of secretin, cholecystokinin, and the vasoactive intestinal peptide in concentrates of porcine intestinal extracts were demonstrated by their COOH-terminal amide fragments: valine (or leucylvaline) amide, phenylalanine amide, and asparagine (or leucylasparagine) amide, respectively. The analytical methodology used in the present study may also be useful in devising simple and reliable chemical assay methods for the isolation of already known polypeptides and in the isolation of previously uncharacterized polypeptides from natural sources.**

Polypeptide hormones in tissue extracts are usually determined by either bioassay or radioimmunoassay. In view of the interactions between hormones *in vivo*, it is increasingly evident that bioassays of tissue extracts, which often contain several different hormones, may give ambiguous results about the presence and the concentrations of a specific hormone. Radioimmunoassay too may be complicated by the problem of crossreactivity.

The determination of polypeptide hormones by chemical means has been, in most cases, unsuccessful in spite of the importance of chemical assays in other hormone determinations. In a few cases it has been possible to use chromatographic methods of high resolution for direct physicochemical determination of the intact polypeptide hormones (1, 2), but often the peptide patterns of the extracts are so complex that this approach may not be generally applicable. As pointed out previously (3, 4), it may, however, in certain cases be possible to submit hormone-containing peptide mixtures to selective fragmentation procedures which produce characteristic fragments of known hormones and to analyze the characteristic fragments instead of whole polypeptide molecules. For many polypeptide hormones, such characteristic fragments could be (or include) the COOH-terminal amino acid α-amides which occur in such diverse hormonal polypeptides as α-melanotropin, calcitonin, cholecystokinin, gastrin, gonadoliberin, oxytocin, the pancreatic hexatriacontapeptide, secretin, substance P, thyroliberin, the vasoactive intestinal peptide (VIP), vasopressin, and others (5, 6).

In this paper we show that the presence of secretin, cholecystokinin, and VIP in peptide concentrates from porcine intestinal extracts may be demonstrated by exposing the concentrates to conditions of enzymatic degradation that release the characteristic COOH-terminal amides of these hormones and then dansylating the degradation mixtures and isolating the dansyl derivatives of the amides from the other products.

We demonstrate how this analytical methodology may be used to follow the purification of a polypeptide hormone and to reveal the occurrence in tissue extracts of peptides with COOH-terminal α-amide structures.

MATERIALS AND METHODS

Peptide and Enzyme Preparations. The pure gastrointestinal polypeptide hormones, secretin, cholecystokinin, and VIP, were prepared from porcine intestinal extracts according to Jorpes and Mutt (7), Mutt and Jorpes (8), and Said and Mutt (9), respectively. The concentrates of thermostable intestinal peptides, the methanol-soluble fraction, and the methanol-insoluble fraction were prepared as described by Mutt (10).

The following enzyme preparations were used: subtilisins (EC 3.4.21.14; subtilisin BPN′ and subtilisin Carlsberg, Sigma Chemical Co.), thermolysin (EC 3.4.24.4; Daiwa Kasei K.K., Japan), chymotrypsin (EC 3.4.21.1; TLCK-treated, α-chymotrypsin, Merk A.G.), trypsin (EC 3.4.21.4; TPCK-treated, Worthington Biochem. Co.), and elastase (EC 3.4.21.11; Worthington Biochem. Co.).

Other Materials. Dansyl chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride) was purchased from Pierce Chemical Co. Dansyl amino acids were obtained from Calbiochem. A.G. Dansyl amino acid α-amides and dansyl peptide α-amides were prepared from the corresponding amides by the reaction with dansyl chloride (11). Polyamide thin-layer sheets were purchased from Cheng Chin Trading Co., LTD., Taipei, Taiwan. The anion exchange resin Dowex 1 × 2 analytical grade was obtained from Bio-Rad Laboratories, U.S.A. All glassware used for the quantitation of the dansyl derivatives was pretreated with dichlorodimethylsilan in toluene (10%, vol/vol) and rinsed with water and acetone before use.

Enzymatic Fragmentation and Dansylation of Peptides. A crude or purified peptide sample, containing 0.1–10 nmol of polypeptide α-amide (sample size usually not exceeding 1 mg), was introduced into a siliconized glass test tube (4 mm inner diameter × 50 mm length). The sample was dissolved in 20 μl of a suitable buffer containing 40–200 μg of the proteolytic enzyme. The buffer used in the present study was either 0.2 M sodium bicarbonate/carbonate pH 9–10 or 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7–8/10 mM Ca^{2+}. The reaction tubes were sealed with Parafilm and incubated at 37° for 4–20 hr or at room temperature for 24–72 hr. The degradation products were lyophilized and then redissolved in 20 μl of 0.2 M sodium bicarbonate/carbonate, and 20 μl of dansyl chloride in acetone (5–20 mg/ml) was

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: VIP, vasoactive intestinal peptide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

added. The concentration of the dansyl chloride solution added was chosen according to the approximately estimated content of groups reactive to dansyl chloride in the sample. Initial pH of the buffer was selected to maintain pH of the solution at 9.5–10 during the dansyl reaction.

The reaction was allowed to proceed for 1 hr at 37° in the dark. Then 5 μl of pyridine (spectroscopic grade) was added to remove traces of free dansyl chloride that had remained in the solution. After a further 15 min of incubation at 37°, the reaction mixture was dried under reduced pressure. To the dried residue was added 100 μl of the aqueous phase of an ethyl acetate/water mixture, followed by 50 μl of the organic phase. The two phases were then mixed vigorously on a Vortex mixer and the mixed phases were separated by centrifugation in a clinical centrifuge. The organic phase was carefully transferred to another small test tube and the aqueous phase was re-extracted with two further 50-μl portions of the organic phase. The extracts were combined and evaporated to dryness.

For application to the thin-layer sheet, the residue was dissolved in 10–20 μl of a mixture of 0.2 M sodium bicarbonate/carbonate, pH 9.5 and ethanol (1:1, vol/vol). One to 5 μl of the solution was applied to the sheet.

Thin-Layer Chromatography on Polyamide Layer. The sample containing dansyl derivative(s) of the α-amide(s) was applied on a 7.5 × 7.5-cm polyamide layer sheet (washed overnight in chloroform) in a spot size of 2–7 mm in diameter. The first dimension was developed in pyridine/heptane (30:70, vol/vol) and was repeated three times in the same dimension to achieve a better separation. Between developments the sheet was dried briefly in a stream of cold air. Development in the second dimension in chloroform/heptane (70:30, vol/vol) was also repeated three times. The spots were localized with the aid of a UV lamp.

Semiquantitation of Dansylated Amides. The dansyl derivatives were eluted from the polyamide layer by cutting the appropriate area from the sheet and eluting it with 3 ml of chloroform (spectroscopic grade) in a siliconized reaction vial at room temperature for 1 hr, in a manner similar to the elution of the dansyl amino acids described by Airhart *et al.* (12). The eluate was then filtered through a siliconized glass filter (a small chromatographic glass tube, 6 mm inner diameter × 150 mm long, with no. 2 glass filter was used without applying pressure or vacuum). The filtrate was directly transferred into a siliconized fluorescence cuvette and the fluorescence intensity was determined at excitation 355 nm and emission 485 nm by a spectrophotofluorimeter against a blank prepared from the same sheet.

Acid Hydrolysis of Dansylated Amides to Dansylated Amino Acids. After chromatography, the dansylated amide was eluted from the polyamide sheet by cutting off the area containing the dansyl derivative and incubating it with 100 μl of chloroform in a small hydrolysis tube at room temperature for 1 hr. The piece of the polyamide sheet was then removed from the tube and the eluate was evaporated to dryness under reduced pressure. The residue was redissolved in 50 μl of 6 M HCl, and the tube was sealed under high vacuum. The hydrolysis was carried out at 105° for 18 hr. The solvent was removed from the hydrolysate under reduced pressure; the residue was redissolved in 10 μl of 50% pyridine (vol/vol) and subjected to thin-layer chromatography on a polyamide sheet (5 × 5 cm) in the solvent systems described by Woods and Wang (13).

RESULTS

Separation and Identification of Amides as Dansyl Derivatives. Amino acid α-amides and peptide α-amides were separated from free amino acids and other peptides by dansylation of the mixtures followed by extraction with ethyl acetate and thin-layer chromatography on polyamide sheets of the materials extracted into the organic phase. Provided the extraction is carried out under alkaline conditions, the dansyl derivatives that contain no free carboxyl groups are preferentially extracted into the organic phase while those with free carboxyl groups largely remain in aqueous solution. Consequently, the dansyl derivatives of amino acids and peptides are retained and those of amino acid and peptide α-amides extracted. Exceptions are the dansyl derivatives of arginine amide and of arginine-containing peptide α-amides. The didansyl derivatives of histidine, lysine, and tyrosine amides are extracted, whereas their monodansyl derivatives are largely retained. The dansyl derivatives in the organic phase were purified further by thin-layer chromatography on polyamide sheets. The chromatography was carried out in the hydrophobic solvent systems, pyridine/heptane (30:70, vol/vol) in the first dimension and chloroform/heptane (70:30, vol/vol) in the second dimension. This chromatography also served to identify the α-amides (Fig. 1).

For application to the chromatographic sheet the dansyl derivatives were dissolved in an ethanolic alkaline buffer. The dansyl derivatives with free carboxyl groups form salts with the alkali that are insoluble in the hydrophobic organic solvent systems used for the chromatography and thus remain at the origin together with dansyl derivatives of arginine amide and arginine-containing peptide α-amides. The dansyl derivatives of other amino acid amides and of many peptide α-amides migrate during the chromatography.

Initially we found that the strong anion exchanger Dowex 1 × 2 in its hydroxyl form could be used to remove most of the free amino acids and peptides by eluting the amides with water

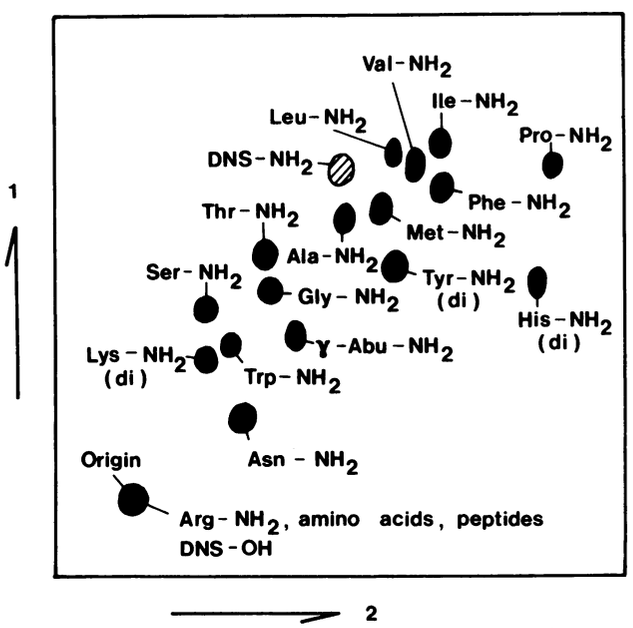

FIG. 1. Two-dimensional separation of dansyl (DNS) amino acid amides on a polyamide layer sheet. A mixture of dansyl amino acid amides (0.1 nmol each) in the presence of 22 dansyl amino acids and 30 dansyl di- and tripeptides was applied to a polyamide layer sheet (7.5 × 7.5 cm). First dimension: pyridine/heptane (30:70, vol/vol); second dimension: chloroform/heptane (70:30). The solvent development was repeated three times in each dimension.

from the column. This ion-exchange method for separating the amides prior to dansylation did, however, not seem to offer distinct advantages over the selective extraction method described above.

Enzymatic Degradation of Gastrointestinal Peptides. Enzymatic hydrolysis of the polypeptide α-amides, porcine secretin, cholecystokinin, or VIP, by endopeptidases produced a number of fragments, including the COOH-terminal peptide α-amide or amino acid α-amide. The α-amide was isolated and identified as described in *Materials and Methods*. Subtilisins and chymotrypsin cleaved off valine amide from the COOH terminus of the heptacosapeptide amide secretin (Fig. 2*A*).

Treatment of secretin with thermolysin at a neutral pH produced mainly valine amide from the COOH terminus; however, only leucylvaline amide was released by the same enzyme at pH above 10. The presence of phenylalanine amide was shown in the degradation products of cholecystokinin by treatment with thermolysin (Fig. 2*B*). Asparagine amide was obtained by treatment of VIP with subtilisins or chymotrypsin, while leucylasparagine amide was released by treatment with thermolysin.

Areas of the thin-layer sheets containing spots of the dansyl amides were cut off and eluted with chloroform. After evaporation of the chloroform, the eluted materials were hydrolyzed in 6 M HCl at 105°. The acid hydrolysis produced the corresponding dansyl amino acids from the dansyl amino acid amides and the NH_2-terminal dansyl amino acids from the dansyl peptide α-amides. These dansyl amino acids were then identified by thin-layer chromatography on polyamide sheets in the solvent systems described by Woods and Wang (13). The presence of valine, phenylalanine, and aspartic acid as the COOH-terminal amino acid residues in secretin, cholecystokinin, and VIP, respectively, were confirmed after acid hydrolysis of the dansyl derivatives eluted from the spots of the thin-layer sheets.

Presence of Polypeptide α-Amides in Porcine Intestinal Extracts. The presence or absence of intestinal polypeptides with COOH-terminal α-amide structures was investigated in crude concentrates from porcine intestinal extracts by degrading these concentrates with endopeptidases, dansylating, and isolating the dansylated α-amides as described in the previous sections. The concentrates were obtained as described by Mutt (10): Boiled hog upper intestines were extracted with 0.5 M acetic acid and the peptides adsorbed from the extract to alginic acid were eluted with 0.2 M HCl and precipitated with NaCl at saturation. Enzymatic hydrolysis with thermolysin revealed that this concentrate of the thermostable intestinal peptides contained peptides with COOH-terminal amides of valine, phenylalanine, isoleucine, leucine, leucylasparagine, and others. The concentrate was purified further by extraction in 66% ethanol and by chromatography on Sephadex G-25 in 0.2 M acetic acid. The preparation thus obtained was extracted with methanol (10). The methanol-soluble fraction contained secretin and VIP, and the methanol-insoluble fraction contained cholecystokinin (10). It was found that the thermolytic degradation products of the methanol-soluble preparation contained large amounts of valine amide and leucylasparagine α-amide in accordance with the presence of secretin and VIP in this fraction, while those of the methanol-insoluble material contained a large amount of phenylalanine amide, in accordance with the presence of cholecystokinin. In addition to those three amides from the known gastrointestinal hormones which occur in these fractions, it was found that the methanol-soluble fraction contained polypeptides with amides of isoleucine, alanine, leucine, methionine, phenylalanine, and tyrosine and that the methanol-insoluble fraction contained the amides of isoleucine, alanine, leucine, and valine (Fig. 3). A number of other amides, which were not identified, presumably short peptide α-amides, were also present in the chromatograms in both fractions. The enzymatic degradation products of these fractions by subtilisins or chymotrypsin produced different amide patterns in the chromatogram, indicating the presence of polypeptide α-amides with valine, isoleucine, asparagine, glycine, and others. It could therefore be expected that the treatment with some other enzymes would reveal the presence of still more peptides with COOH-terminal amide structures in the extracts. Further fractionation of the methanol-soluble material by CMC ion-exchange chromatography and counter-current distribution produced peptide preparations containing a number of peptides with COOH-terminal α-amides that had not been detected in the earlier preparations, probably due to the corresponding peptide amides being present in very low concentrations in the earlier preparations.

Semiquantitation of Polypeptide Hormones by Fragmentation Analysis. The semiquantitative analysis of the polypeptide hormones secretin and cholecystokinin was carried out by the quantitation of the characteristic COOH-terminal amide fragment released by the enzymatic hydrolysis. After isolation of the dansylated COOH-terminal fragment of the polypeptide on polyamide thin-layer sheet, the spot containing the amide was cut off and eluted with chloroform. The fluorescence intensity of the dansyl α-amide was then measured with a fluorimeter. The fluorescent material was quantitatively recovered from the thin-layer sheet in this manner within the

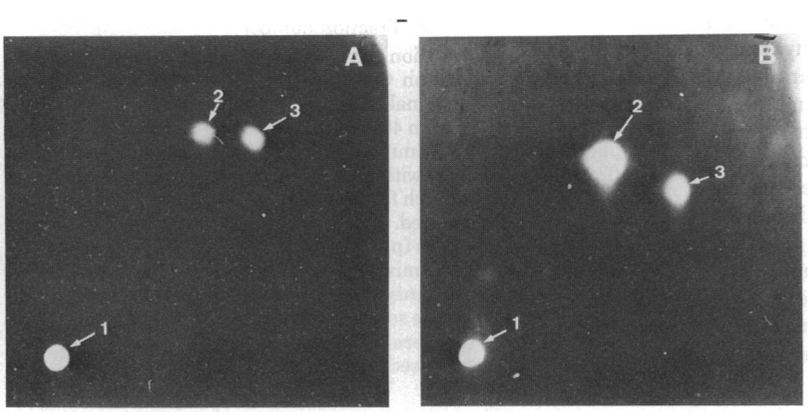

FIG. 2. Separation and identification of COOH-terminal amino acid amides from the gastrointestinal polypeptide hormones. (*A*) Porcine secretin (1 nmol) was hydrolyzed with subtilisin BPN′ at pH 10 and 37° for 4 hr. (*B*) Porcine cholecystokinin was degraded with thermolysin at pH 7.5 and 37° for 4 hr. The hydrolysates were dansylated and the resulting dansyl derivatives of the amides were isolated and identified. Two-dimensional thin-layer chromatography on polyamide sheet indicated the presence of: (*A*) *1*, origin; *2*, dansyl-NH_2; and *3*, dansyl-valine amide; (*B*) *1*, origin; *2*, dansyl-NH_2; and *3*, dansyl-phenylalanine amide. The presence of large amounts of dansyl-NH_2 in these chromatograms may be due to the presence of ammonium salts in these preparations. It may, however, also arise from the side reactions of amino acids with dansyl chloride (14). Because of its distinct blue fluorescence, the presence of dansyl-NH_2 is a convenient standard marker in the chromatograms.

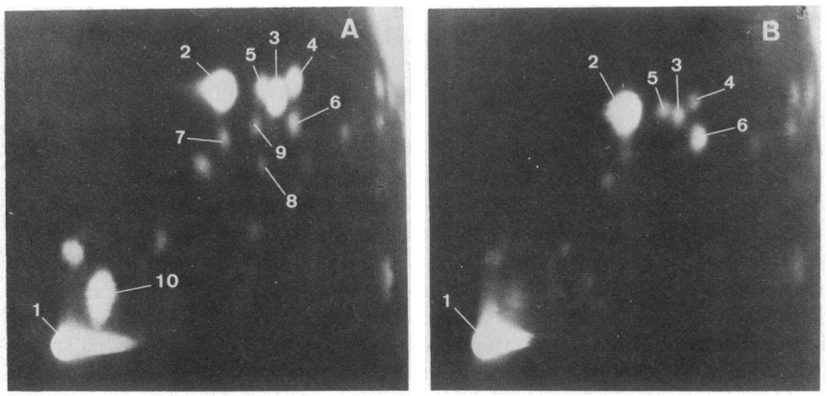

FIG. 3. The presence of polypeptide α-amides in crude peptide preparations from the intestinal extract. The methanol-soluble and -insoluble fractions prepared from the intestinal extract (10), 0.5 mg each, were dissolved in 20 μl of 50 mM Hepes (pH 7.5) containing 10 mM Ca^{2+} and 40 μg of thermolysin. The mixture was incubated at 37° for 20 hr. The reaction mixture was dansylated and the dansyl α-amides were extracted. (*A*) COOH-terminal α-amides from the methanol-soluble fraction: *1*, origin; *2*, dansyl-NH_2; *3*, dansyl-Val-NH_2; *4*, dansyl-Ile-NH_2; *5*, dansyl-Leu-NH_2; *6*, dansyl-Phe-NH_2; *7*, dansyl-Ala-NH_2; *8*, didansyl-Tyr-NH_2; *9*, dansyl-Met-NH_2; and *10*, dansyl-Leu-Asn-NH_2. (*B*) COOH-terminal α-amides from the methanol-insoluble fraction: *1*, origin; *2*, dansyl-NH_2; *3*, dansyl-Val-NH_2; *4*, dansyl-Ile-NH_2; *5*, dansyl-Leu-NH_2; and *6*, dansyl-Phe-NH_2.

limits of approximately 10 pmol–10 nmol of the dansyl derivative. Although a quantitative release of valine amide from secretin by treatment with chymotrypsin or phenylalanine amide from cholecystokinin by thermolysin was easily demonstrated with pure preparations, in the presence of other peptides and proteins in excess, the release of the amide was somewhat slower and often incomplete under the given conditions. An intensive hydrolysis or an internal standard system, such as the use of a labeled standard polypeptide, would therefore be required to determine the absolute concentration of the polypeptide in a crude preparation. In spite of this, the present simple technique was shown to be useful for the determination of relative concentrations of the polypeptide hormones during the purification procedures from the extract. In Fig. 4 the relative quantities of valine amide released from the fractions obtained after CMC column chromatography of the methanol-soluble material from the intestinal extract were compared with the bioactivity (pancreatic secretion) of secretin in the same fractions. The major peak, at fraction 20–22 contains the normal secretin with 27 amino acid residues (7). The minor peak, at fraction 3, might be due to a secretion modified during the purification procedure. The small peak at fraction 32 contains VIP (9). Since VIP exerts a pancreatic secretory activity in the bioassay method used (9), the bioactivity measured in fraction 32 may be mainly due to VIP instead of secretin. The results of the chemical assay indicated that this fraction contained little secretin.

DISCUSSION

Determination of the COOH-terminal α-amide groups has not been studied extensively in spite of the biological importance of many substances with such structures. The amide bond is relatively inert to most of chemical reactions but easily hydrolyzed, making it difficult to release selectively the COOH-terminal α-amide without deamidation. A chemical modification for stabilizing this amide bond against hydrolysis has been studied (4), but a number of difficulties limited its practical application. In the present work, commercially available endopeptidase preparations were used to analyze the COOH-terminal amide groups. The use of exopeptidases such as aminopeptidases or carboxypeptidases is, with few exceptions, inapplicable because of their deamidase activities.

We describe how the presence or absence of secretin, cholecystokinin, and VIP may be demonstrated in concentrates of porcine small intestinal extracts by subjecting the concentrates to enzymic hydrolysis that releases the amides of valine (or leucylvaline), phenylalanine, and asparagine (or leucylasparagine) from the COOH termini of these three polypeptides and then analyzing for the presence or absence of the amides. The absence of the amide would mean that the corresponding hormone must have been absent from the material analyzed. The presence of the amide, on the other hand, would make the presence of the hormone in the material probable although not actually proven, since theoretically the amide in question could be derived from the COOH terminus of some other peptide. In practice, this would be unlikely for secretin or VIP, but the phenylalanine amide might to some extent be derived from intestinal gastrin in addition to cholecystokinin since the COOH-terminal structures of gastrin and cholecystokinin are identical (7). Despite such limitations we find this

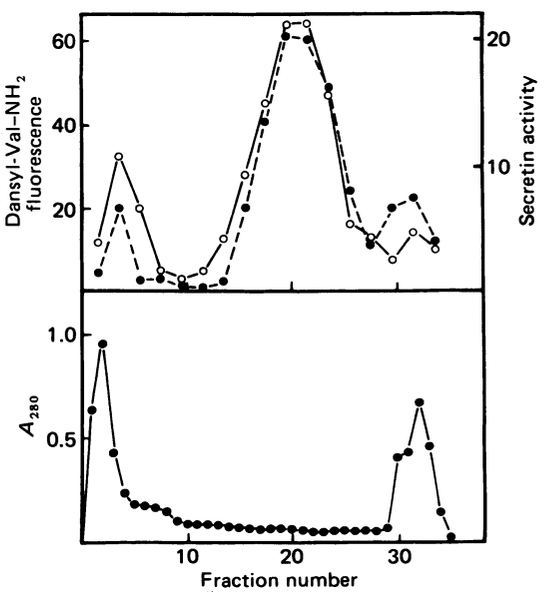

FIG. 4. Fragmentation chemical analysis of secretin during column chromatography on CMC cellulose of the methanol-soluble fraction from the intestinal extract (10). The methanol-soluble fraction (6 g) was dissolved in 480 ml of 12.5 mM phosphate (pH 8.0) and applied to the CMC column (14 × 20 cm) equilibrated with the same buffer. It was developed with 12.5 mM phosphate (pH 8.0) at flow rate of 100 ml/min. From each fraction (500 ml), 1 ml of the solution was withdrawn and lyophilized. The lyophilized samples were redissolved in 20 μl of 50 mM Hepes (pH 7.5) containing 10 mM Ca^{2+} and 40 μg of α-chymotrypsin. The mixtures were incubated at 37° for 20 hr and lyophilized. After the dansylation the resulting dansyl-valine amide released from the peptide solution was quantitated. The results of the chemical analysis (○) were compared with those of the bioassay (●) of secretin (pancreatic secretion of cat; in units/ml) described by Jorpes and Mutt (15).

chemical method of revealing the presence of a polypeptide hormone most useful, both in developing methods for the purification of hormones and for answering questions concerning the possible presence of known hormones in tissue extracts.

Selection of a specific proteolytic enzyme may improve the sensitivity and specificity of the present fragmentation technique. The use of peptidases isolated from the kidney that release the glycine amide or leucylglycine amide of oxytocin (16), for example, could be used to determine this hormone. The sensitivity of the present method, using the fluorescence of the dansyl derivatives, could also be improved by the use of isotope labeling techniques, such as the use of [^{3}H]dansyl chloride and/or [^{14}C]peptides, or the use of high-pressure liquid chromatography or gas chromatography-mass spectrometry techniques. A recent publication (17) indicates that a nonenzymatic chemical method for the removal of COOH-terminal amides from polypeptides might be of interest in connection with the present method.

Another area in which the fragmentation technique of peptide analysis may find important applications is in the isolation of hitherto unknown polypeptide hormones. Purification of polypeptide hormones that have not previously been isolated is usually followed by bioassay. Radioimmunoassay may be used when the hormone to be isolated crossreacts with antibodies to some known substance or as a detection device to find a chemically unknown hormone that affects the concentration of a known hormone in a test system. This latter principle was, for instance, used by Brazeau *et al.* (18) for the isolation of somatostatin. In the present paper it is shown that porcine intestinal extracts contain a variety of unknown polypeptides with COOH-terminal α-amide structures, including the amides of alanine, glycine, isoleucine, leucine, methionine, and tyrosine, in addition to the known polypeptides with valine (secretin), phenylalanine (cholecystokinin), and asparagine (VIP) amides. The methionine amide might be derived from substance P (19), but no naturally occurring polypeptides with COOH-terminal isoleucine or leucine amides seem to have been hitherto isolated. It is therefore of interest to isolate and investigate the biological properties of these substances from the intestinal tissue.

The present paper describes a fragmentation analytical technique based on characteristic COOH-terminal α-amide groups of polypeptides. The principle of fragmentation analysis may, however, also be applied to other characteristic fragments of polypeptides, such as NH_2-terminal blocked groups, unusual amino acid residues, or sequences similar to those of already known biologically active polypeptides. Such chemical assay systems may provide simple and inexpensive polypeptide determinations that are independent of bioassays or radioimmunoassays.

We acknowledge helpful discussions with Dr. David Eaker and Dr. Thomas J. McDonald. The investigation was supported by the Swedish Medical Research Council through Grant B77-13X-01010-12B.

1. Gruber, K. A., Stein, S., Brink, L., Radhakrishnan, A. & Udenfriend, S. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 1314–1318.
2. Krummen, K. & Frei, R. W. (1977) *J. Chromatogr.* **132,** 429–436.
3. Mutt, V. (1973) in *Endocrinology 1973, Proceeding of the Fourth International Symposium, London* (William Heinemann Medical Books Ltd, London), pp. 100–116.
4. Mutt, V. (1976) *Clin. Endocrinol. Suppl.* **5,** 175s–183s.
5. Dayhoff, M. O. (1972) in *Atlas of Protein Sequence and Structure,* ed. Dayhoff, M. O. (National Biomedical Research Foundation, Silver Spring, MD), Vol. 5, pp. D173–D227.
6. Hunt, L. T. & Dayhoff, M. O. (1976) in *Atlas of Protein Sequence and Structure,* ed. Dayhoff, M. O. (National Biomedical Research Foundation, Silver Spring, MD), Vol. 5, Suppl. II, pp. 113–145.
7. Jorpes, J. E. & Mutt, V. (1973) in *Secretin, Cholecystokinin, Pancreozymin and Gastrin,* eds. Jorpes, J. E. & Mutt, V. (Springer-Verlag, Berlin), pp. 1–179.
8. Mutt, V. & Jorpes, J. E. (1968) *Eur. J. Biochem.* **6,** 159–162.
9. Said, S. I. & Mutt, V. (1972) *Eur. J. Biochem.* **28,** 199–204.
10. Mutt, V. (1978) in *Gut Hormones,* ed. Bloom, S. R. (Churchill-Livingstone, Edinburgh), pp. 21–27.
11. Gray, W. R. (1972) in *Methods in Enzymology,* eds. Hirs, C. H. W. & Timasheff, S. N. (Academic Press, New York), Vol. 25, pp. 121–138.
12. Airhart, J., Sibiga, S., Sanders, H. & Khairallah, E. A. (1973) *Anal. Biochem.* **53,** 132–140.
13. Woods, K. R. & Wang, K. T. (1967) *Biochim. Biophys. Acta* **133,** 369–370.
14. Neadle, D. J. & Pollitt, R. T. (1965) *Biochem. J.* **97,** 607–608.
15. Jorpes, J. E. & Mutt, V. (1966) *Acta Physiol. Scand.* **66,** 316–325.
16. Walter, R. (1976) *Biochim. Biophys. Acta* **422,** 138–158.
17. Parham, M. E. & Loudon, A. M. (1978) *Biochem. Biophys. Res. Commun.* **80,** 1–6.
18. Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier, J. & Guillemin, R. (1973) *Science* **179,** 77–79.
19. Chang, M. M. & Leeman, S. E. (1971) *Nature (London) New Biol.* **232,** 86–87.