Vol. 96, No. 2, 1980
September 30, 1980

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 725-734

ISOLATION AND AMINO ACID COMPOSITION OF TWO SOMATOSTATIN-LIKE PEPTIDES FROM OVINE HYPOTHALAMUS: SOMATOSTATIN-28 AND SOMATOSTATIN-25

Peter Böhlen, Paul Brazeau, Robert Benoit, Nicholas Ling, Frederick Esch and Roger Guillemin

Laboratories for Neuroendocrinology
The Salk Institute for Biological Studies
La Jolla, California 92037

Received August 4,1980

SUMMARY

Two peptides having somatostatin-like immunoactivity and *in vitro* bioactivity were purified to homogeneity from ovine hypothalamic extracts by means of affinity chromatography, gel filtration, cation exchange chromatography and reverse-phase high-performance liquid chromatography. Both peptides contain all the amino acids of somatostatin; thus it is likely that they are structurally related to the tetradecapeptide somatostatin. One of the peptides contains 28 amino acids with a composition identical to that of somatostatin-28, the N-terminally extended somatostatin recently isolated from porcine intestine and characterized by Pradayrol et al. (11). The second peptide, somatostatin-25, has three amino acid residues less but otherwise the same composition as somatostatin-28. Based on an *in vitro* bioassay and quantitation by amino acid analysis both peptides appear to be more potent than somatostatin.

INTRODUCTION

During the isolation of the ovine hypothalamic tetradecapeptide somatostatin in our laboratory (1) we had observed the presence of multiple forms of bioactive somatostatin. The size heterogeneity of hypothalamic (2-4) as well as pancreatic (4-7) and intestinal (7,8) bioactive and immunoactive somatostatin-like substances has since been confirmed by other laboratories. Since the somatostatin-like substances aside from the tetradecapeptide have higher molecular weights, the existence of precursor or prohormone forms of somatostatin-14 was postulated (9). Recently Pradayrol et al. have isolated from porcine intestinal tissue (10) and characterized (11) somatostatin-28 which contains the sequence of somatostatin at the C-terminal end and is

Abbreviations: CLIP, Corticotropin-like Intermediate Lobe Peptide; CM, Carboxymethyl cellulose; HPLC, High Performance Liquid Chromatography; TFA, Trifluoroacetic Acid; TRH, Thyrotropin-Releasing-Hormone.

0006-291X/80/180725-10$01.00/0

extended at its N-terminal by 14 amino acids. The primary structure was found to be H-Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH, suggesting that somatostatin-28 may indeed be a precursor of somatostatin-14.

While screening side fractions generated during the original thyrotropin-releasing-hormone (TRH) isolation program for the presence of a growth-hormone-releasing factor we found bioactive and immunoactive somatostatin-like material with a molecular weight greater than that of the tetradecapeptide. We report here the isolation from this material and the amino acid composition of two closely related N-terminally extended somatostatins one of which is identical to somatostatin-28 reported by Pradayrol (11) and the other corresponding to (Des-Ser^1,Ala^2,Asn^3)-somatostatin-28, here termed somatostatin-25. Complete sequencing of these two peptides has been achieved and will be reported elsewhere (12).

MATERIALS AND METHODS

The starting material was a side fraction obtained during the isolation of TRH (13) from 350,000 ovine hypothalami. It consisted of those substances present in an acetic acid tissue extract which eluted in the void volume during Sephadex G-25 gel filtration; 131 g of this material was used for the isolation program.

For affinity chromatography a γ-globulin fraction was prepared from 650 ml sheep anti-somatostatin serum (14) by precipitation in 40% (wt/vol) ammonium sulfate followed by dialysis. The γ-globulin fraction was reacted with 340 g CNBr-activated Sepharose 4B according to standard procedures in this laboratory (14). The gel was equilibrated with 10 mM sodium phosphate pH 7.3 and packed into a column (75 x 5.2 cm). The starting material (131 g) was dissolved in 6.0 liter of 10 mM sodium phosphate pH 7.3 and passed through the affinity column at a flow rate of 400 ml/h. The column was then washed with 3.0 liter of 10 mM sodium phosphate pH 7.3 and the retained material eluted with 3.0 liter of 25 mM hydrochloric acid. The acid eluate was lyophilized and subjected to gel filtration on a column of Sephadex G-50 fine (97 x 10 cm) which was eluted with 30% (vol/vol) acetic acid. The column was calibrated with [Leu^5]-β_h-endorphin (Mr 3443), corticotropin-like intermediate lobe peptide (CLIP, Mr 2463) and somatostatin (Mr 1636). Active fractions from the Sephadex column were further purified by ion-exchange chromatography on a CM-32 carboxymethyl cellulose column (18 x 1.8 cm, Whatman) eluted with a linear gradient generated by adding 1 liter of 1 M ammonium acetate pH 6.5 to 1 liter of 0.01 M ammonium acetate pH 4.5 in a mixing flask. Final isolation was achieved by high performance liquid chromatography (HPLC) on two different reverse phase systems. One HPLC system consisted of an Altex Model 332 gradient liquid chromatograph equipped with microprocessor (Altex, Berkeley CA), RP 18 reverse phase column (25 x 0.4 cm, 10 μm particle size; Brownlee, Santa Clara, CA) and a fluores-

camine stream-sampling detection system (15) for fluorescence monitoring of preparative HPLC. The mobile phase was a gradient of n-propanol in pyridine formate, pH 3.0 (0.36 M pyridine titrated to pH 3.0 with formic acid) as described (16). The second HPLC system consisted of a Waters Model 204 gradient liquid chromatograph (Waters Assoc., Milford MA), RP 18 column (same as above) and a variable wavelength UV detector (Schoeffel). The mobile phase was 0.1% (vol/vol) trifluoroacetic acid (TFA) in an acetonitrile gradient (17). All chromatographic steps were performed at room temperature. Further experimental details are contained in the legends to the figures.

For assay of somatostatin-like activity an *in vitro* bioassay and a radioimmunoassay were used. For bioassay *in vitro* (18) monolayer tissue cultures of rat adenohypophyses were incubated with aliquots of column fractions and the inhibition of growth hormone release measured. For radioimmunoassay antibodies to somatostatin were used as previously described (14). The antiserum showed no cross-reactivity with 31 peptide hormones and biogenic amines. It predominantly reads the somatostatin sequence between residues 4 and 12 (Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr).

A Liquimat III amino acid analyzer (Kontron, Zürich, Switzerland) with an o-phthalaldehyde fluorescence detection system and a proline conversion accessory (19) was used for amino acid analysis. 50-100 pmol peptide was hydrolyzed for 24 h in 100 µl constant boiling hydrochloric acid containing 2% (vol/vol) thioglycolic acid in sealed evacuated tubes. Under these hydrolysis conditions all amino acids including tryptophan were quantitated. Proline and cysteine, which coelute, were determined by hydrolyzing a second peptide aliquot after conversion of cysteine to cysteic acid with performic acid (details of the amino acid analysis procedure will be published elsewhere). N-terminal amino acid analysis was performed by the dansyl method (20).

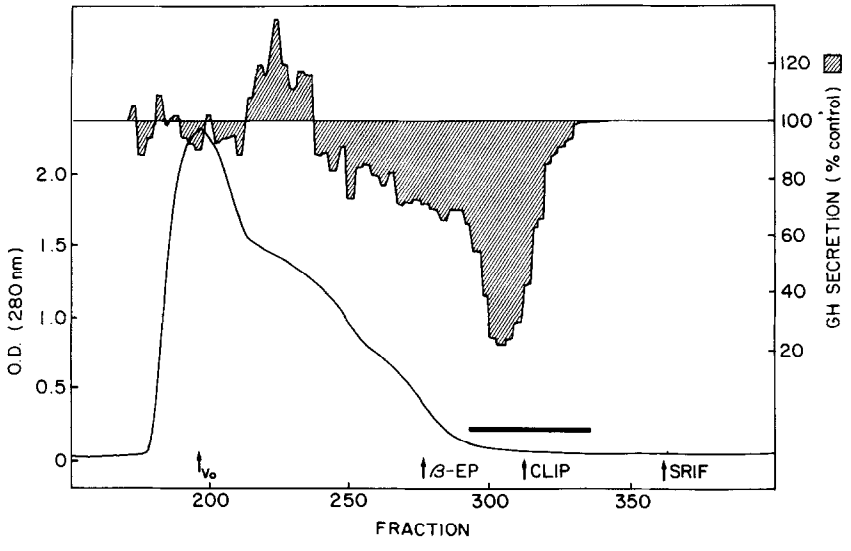

Fig. 1. Gel filtration of somatostatin-like material on a Sephadex G-50 fine column. 3.52 g of the hydrochloric acid eluate from affinity chromatography was dissolved in 40 ml of 30% (vol/vol) acetic acid. The flow rate was 112 ml/h. Fractions of 19 ml were collected and monitored manually at 280 nm for UV absorbing material. Bioassay was performed on an aliquot of each fraction (0.15 µl). Fractions 295-335 (as marked) were pooled and lyophilized for further purification. Arrows indicate the location of marker peaks for molecular weight calibration.

RESULTS AND DISCUSSION

By means of affinity chromatography, 3.52 g of material containing the somatostatin-like substances was obtained from the bulk of 131 g of starting extract. Upon further purification of the active material by Sephadex G-50 chromatography (Fig. 1) a major immunoactive (data not shown) and bioactive somatostatin-like fraction with an apparent molecular weight of ca 3000 was collected. This fraction (62 mg) was subjected to ion-exchange chromatography on CM-32 carboxymethyl cellulose (Fig. 2.). Two zones of immuno- and bioactive somatostatin-like material eluted from the column. They were pooled separately and designated CM-53-54 and CM-57-60. After lyophilization, they yielded 4.4 and 5.0 mg, respectively. For final purification of the two zones containing the active materials reverse phase HPLC was used.

CM-53-54 was first chromatographed (Fig. 3a) using the pyridine formate/n-propanol system with a propanol gradient. The fractions containing the bio- and immunoactivity were pooled, lyophilized and rechromatographed

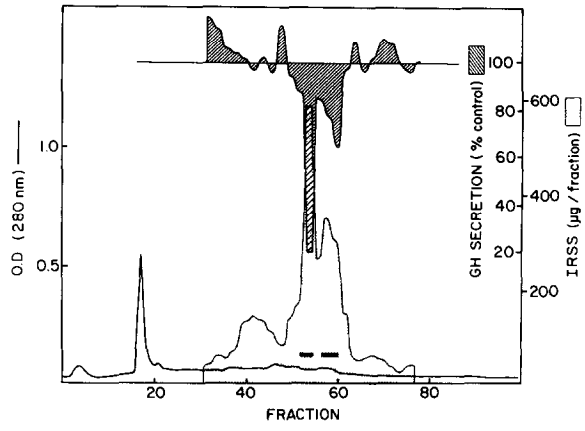

Fig. 2. Ion-exchange chromatography of the gel filtration fractions 295-335 on a CM-32 carboxymethyl cellulose column. The dry material (62 mg) was dissolved in 10 ml 0.01 M ammonium acetate pH 4.0 and loaded onto the column which was eluted at a flow rate of 71 ml/h. Fractions of 9.5 ml were collected and monitored manually at 280 nm for UV absorbing substances. Aliquots of each fraction (2 µl) were subjected to bio- and immunoassay. Two zones of activity, fractions CM-53-54 and CM-57-60, were pooled separately (as marked) and lyophilized.

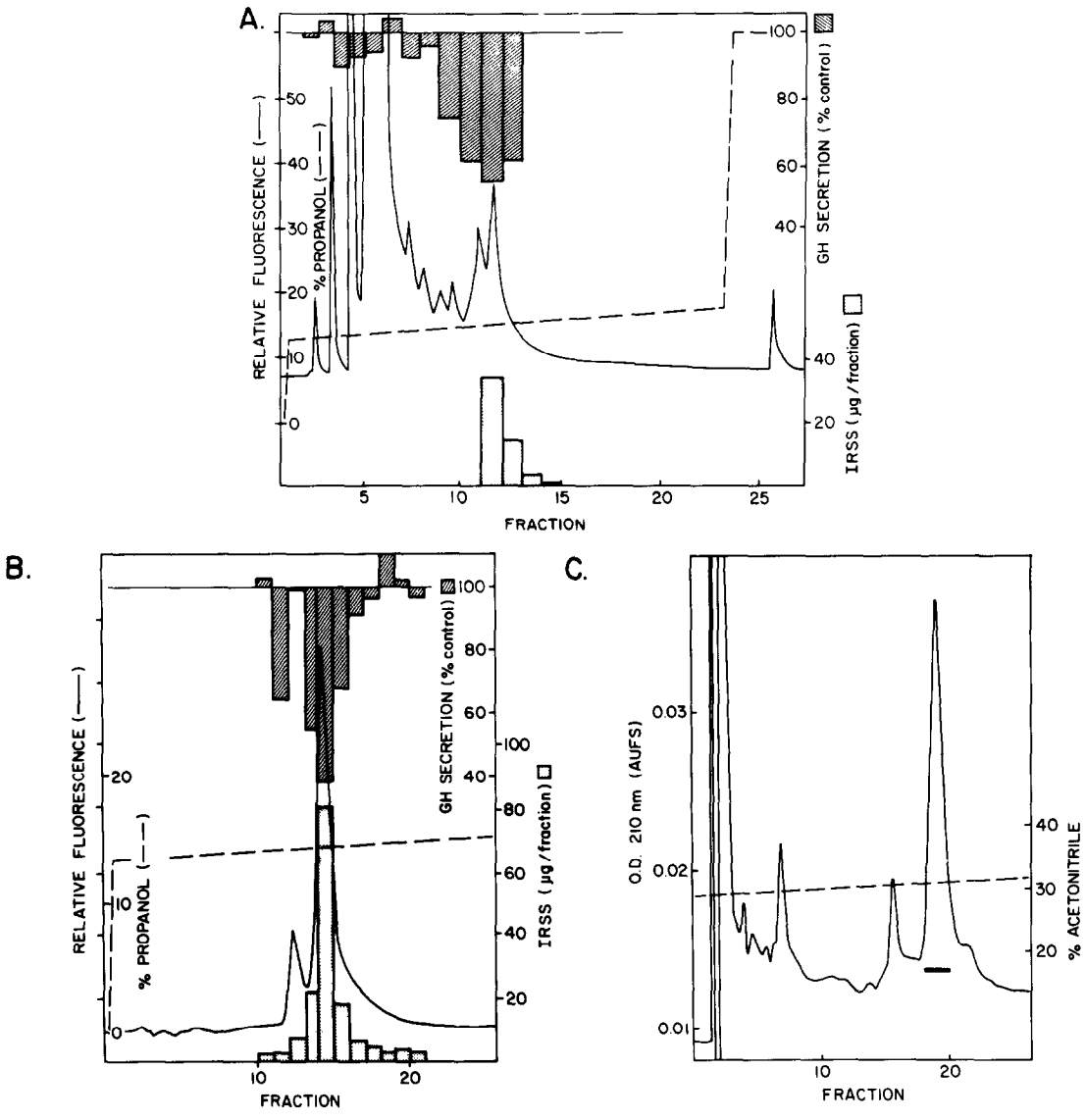

Fig. 3. Reverse phase HPLC of CM-53-54 (somatostatin-28).

a) 4.4 mg was dissolved in 0.5 ml 0.2 M acetic acid and applied to the RP 18 column of the Altex liquid chromatograph. The column was eluted at a flow rate of 0.6 ml/min with the pyridine formate/ n-propanol mobile phase. 5% of the eluate was diverted to the fluorescamine detection system. Fractions of 1.8 ml were collected and aliquots of 1 µl used for bioassay and radioimmunoassay. Fractions 12-14 were pooled as marked and lyophilized.

b) Rechromatography of fractions 12-14 from the first HPLC using the same system (See Fig. 3a) with a shallow n-propanol gradient. Fractions 14-16 were individually lyophilized. Aliquots were used for bio- and immunoassay (0.25 µl) and structural studies. All other conditions were as in Fig. 3a.

c) Rechromatography of fraction 14 from the second HPLC (See Fig. 3b) on the Waters liquid chromatograph using an RP 18 column which was eluted at a flow rate of 42 ml/min with a shallow acetonitrile gradient. The peak zone was collected as marked and subjected to amino acid analysis.

in the same buffer system with a shallow gradient of n-propanol (Fig. 3b). One major fluorescent peak was obtained which corresponded to the peak of bio- and immunoactivity. Chromatography of a portion of this material in a second reverse phase HPLC system using the same type of column but another mobile phase with different solute selectivity (Fig. 3c) indicated that the isolated peptide was essentially pure. Dansylation of an aliquot of fraction 15 (Fig. 3b) did not reveal any N-terminal amino acid. The experiment was not repeated due to scarcity of material.

The active material from the second peak of ion-exchange chromatography, CM-57-60 (5.0 mg), was isolated in a similar way in two HPLC steps using the pyridine formate/n-propanol mobile phase. Again, one major fluorescent peak corresponding to the peak of bio- and immunoactivity was obtained (Figs. 4a,b). No significant further purification was achieved of this peak in the TFA/acetonitrile system (Fig. 4c). Further evidence of purity was obtained by N-terminal amino acid analysis. Only one dansylated N-terminal amino acid was found (Ser) with fraction 11 of Fig. 4b.

The amino acid composition of the two active peptides (Figs. 3c,4c) is shown in Table 1. The two peptides consist of 28 and 25 amino acids, respectively, and both contain all the amino acids present in the tetradecapeptide somatostatin. Since they are somatostatin-like with respect to their bio- and immunoactivity it may reasonable be inferred that they contain the sequence of somatostatin within their primary structure. The larger of the two peptides has an amino acid composition identical to that of somatostatin-28 isolated by Pradayrol et al. (11) from porcine intestinal tissue. The smaller peptide, somatostatin-25, has the same amino acid composition as somatostatin-28, except that one residue each of Ser, Asp and Ala is missing. It may therefore be closely related in structure to the larger somatostatin-28.

Approximately 10 and 30 nmol of pure somatostatin-28 and somatostatin-25, respectively, were isolated as determined by quantitative amino acid analysis.

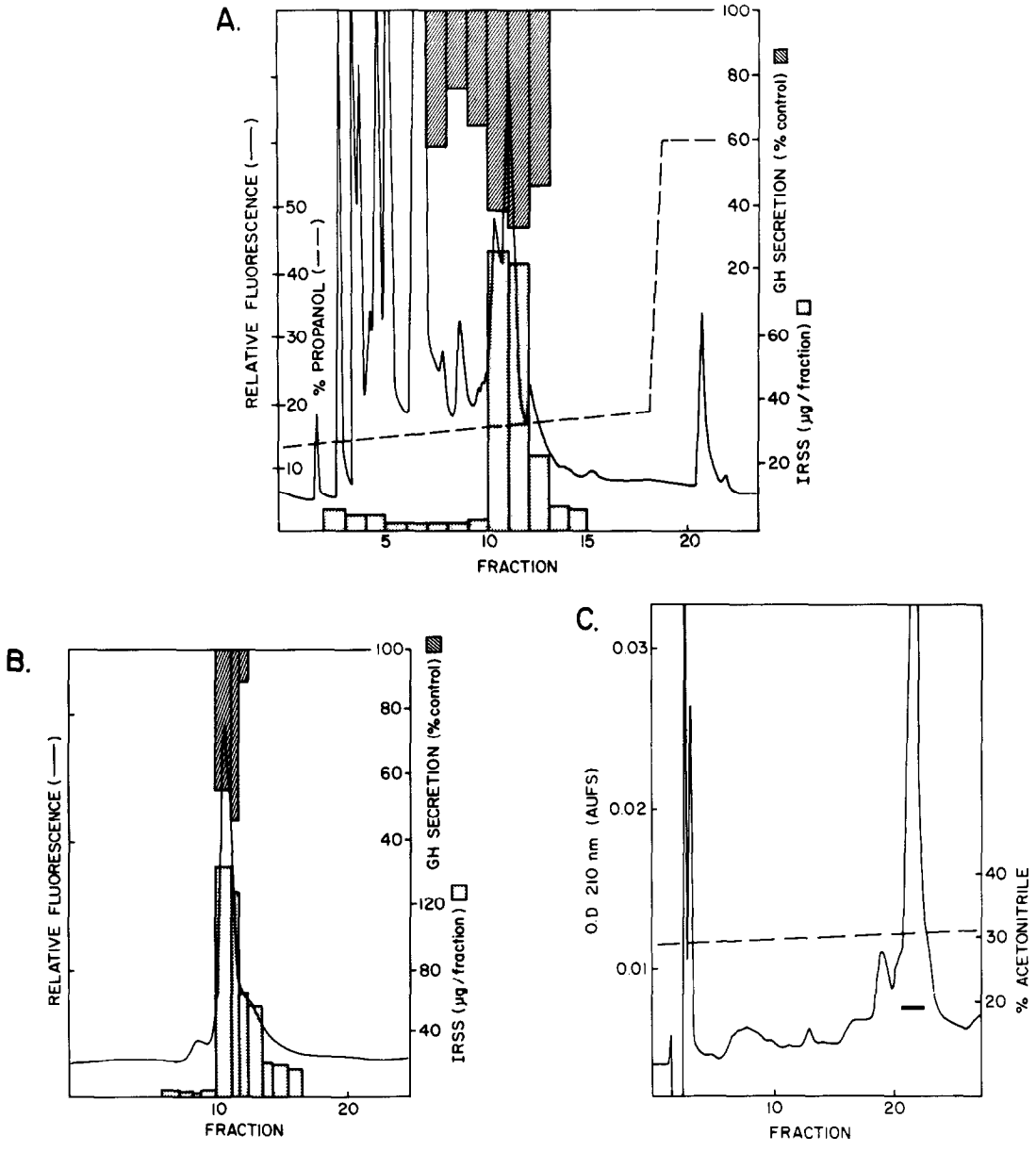

Fig. 4. Reverse-phase HPLC purification of CM-57-60 (somatostatin-25). Unless otherwise stated all conditions were as in the respective Figs. 3a-c.

a) HPLC of 5.0 mg CM-57-60. Fractions 11 and 12 were pooled as marked and lyophilized for further purification.

b) Rechromatography of fractions 11 and 12 from the first HPLC (Fig. 4a) under isocratic conditions with 13.5% (vol/vol) n-propanol in the mobile phase. The sensitivity setting of the fluorometer was 3-fold higher than in Fig. 4a. Fractions 11 and 12 were individually lyophilized and aliquots were used for bioassay, radioimmunoassay (0.25 µl) and structural studies.

c) Rechromatography of fraction 11 from the previous HPLC (Fig. 4b) in the TFA/acetonitrile system using a shallow acetonitrile gradient.

Table 1: Amino acid composition of two somatostatin-like peptides.

	Somatostatin-28 (Fig. 3)	Integer	Somatostatin-25 (Fig. 4)	Integer
Asp	2.96 ± 0.12	3	2.02 ± 0.08	2
Thr	2.01 ± 0.10	2	1.75 ± 0.11	2
Ser	2.58 ± 0.08	3	1.70 ± 0.17	2
Glu	1.46 ± 0.09	1	1.28 ± 0.19	1
Pro	1.95 ± 0.23	2	2.20 ± 0.27	2
Gly	1.41 ± 0.18	1	1.19 ± 0.36	1
Ala	3.81 ± 0.26	4	2.75 ± 0.30	3
Cys*	1.55 ± 0.32	2	2.34 ± 0.13	2
Val	0	0	0	0
Met	0.79 ± 0.16	1	1.12 ± 0.08	1
Ile	0	0	0	0
Leu	0	0	0	0
Tyr	0	0	0	0
Phe	2.75 ± 0.20	3	2.70 ± 0.03	3
His	0	0	0	0
Trp	0.42 ± 0.13	1	0.58 ± 0.09	1
Lys	2.81 ± 0.48	3	3.31 ± 0.40	3
Arg	2.40 ± 0.15	2	2.11 ± 0.02	2
		28		25

Thr and Ser values are uncorrected. Values are means ± SD (n=3).

* determined as cysteic acid.

In contrast, quantitation of the isolated materials by bioassay with synthetic somatostatin as the standard indicated the presence of about 50 and 250 nmol of the peptides, respectively. This discrepancy can only be explained by assuming that both somatostatin-28 and somatostatin-25 are more active in the *in vitro* bioassay than the tetradecapeptide. Indeed, we have recently shown that somatostatin-28 with the amino acid sequence as reported by Pradayrol et al. (11), and (Des-Ser^1,Asn^2,Ala^3)-somatostatin-28, both synthesized in our laboratory (21), are each approximately 10 times more active *in vitro* and *in vivo* than the tetradecapeptide (18,22). It is conceivable that somatostatin-14, now considered the physiologically active species, is a still active metabolic product of the larger peptide having its own biological regulatory function(s). Although it is likely that somatostatin-28 is a biosynthetic precursor of somatostatin-14, its high specific bioactivity suggests that it is a biologically significant form of somatostatin. It remains to be seen whether somatostatin-28 is released as such from cells containing immunoreactive somatostatin. Evidence in support of this

possibility has been published by Gillioz et al. (23) who found immunoreactive somatostatin with a molecular weight greater than that of the tetradecapeptide circulating in hypophyseal portal blood.

The question must also be raised whether somatostatin-25 has its own physiological significance or is itself a still highly active degradation product of somatostatin-28. The latter possibility cannot be excluded since the starting material was quite old and may have been partially degraded during storage at -20°C.

ACKNOWLEDGEMENTS

We wish to thank Drs. S. Udenfriend and S. Stein, Roche Institute of Molecular Biology, Nutley NJ for the valuable gift of the prototype peptide analyzer. Skillful technical assistance was provided by R. Schroeder, G. Kleeman, F. Castillo, D. Martineau and J. Addison. This work was supported by NIH grants AM-18811-05 and HD-09690-05-06, the W.R. Hearst Foundation and the National Foundation March of Dimes.

REFERENCES

1. Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier J., and Guillemin, R., (1973) Science 179, 77-79.
2. Vale, W., (1976) In Hypothalamus and Endocrine Functions (Labrie, F., Meites, J. and Pelletier, G., eds), pp 397-429, Plenum Press, New York.
3. Schally, A.V., Dupont, A., Arimura, A., Redding, T.W., Nishi, N., Linthicum, G.L.and Schlesinger, D.H., (1976) Biochemistry 15, 509-514.
4. Patel, Y. and Reichlin, S., (1978) Endocrinology 102, 523-530.
5. Conlon, J., Zyzmar, E., Vale W. and Unger, R., (1978) FEBS Lett 94, 327-330.
6. Vale, W., Ling, N., Rivier, J., Villareal, J., Rivier, C., Douglas, C., and Brown, M., (1976) Metabolism 25, 1491-1494.
7. Arimura, A., Sato, H., Dupont, A., Nishi N. and Schally, A.V., (1975) Science 189, 1007-1009.
8. Pradayrol, L., Chayvialle J.A. and Mutt, V., (1978) Metabolism 27, 1197-1200.
9. Millar, R.P., (1978) J. Endocrinol. 77, 429-430.
10. Pradayrol, L., Chayvialle, J.A., Carlquist M. and Mutt, V., (1978) Biochem. Biophys. Res. Commun. 85, 701-708.
11. Pradayrol, L., Jörnvall, H., Mutt V. and Ribet, A., (1980) FEBS Lett 109, 55-58.
12. Esch, F., Böhlen, P., Ling, N., Brazeau P. and Guillemin, R., (1980) Proc. Natl. Acad. Sci. USA, (in press).
13. Burgus, R., Dunn, T., Desiderio, D., Ward, D., Vale W. and Guillemin, R., (1970) Nature 226, 321-325.
14. Brazeau, P., Epelbaum, J., Tannenbaum, J.S., Rorstad, O. and Martin, J.B., (1978) Metabolism 27, 1133-1137.
15. Böhlen, P., Stein, S., Stone J. and Udenfriend, S., (1975) Anal. Biochem. 67, 438-445.
16. Rubinstein, M., Stein, S., Gerber L. and Udenfriend, S., (1977) Proc. Natl. Acad. Sci. USA 74, 3052-3055.
17. Bennett, H.P.J., Browne, C.A., Goltzman D. and Solomon, S., (1979) In Peptides: Structure and biological function (Gross, E. and Meienhofer, J. eds.) pp 121-124, Pierce Chemical Co., Rockford IL.

18. Brazeau, P., Ling, N., Esch, F., Böhlen, P., Benoit R., and Guillemin, R., (1980) Regulatory Peptides, (in press).
19. Böhlen, P. and Mellet, M., (1979) Anal. Biochem. 94, 313-321.
20. Hartley, B.S., (1970) Biochem. J. 119, 805-822.
21. Ling, N., Esch, F., Davis, D., Mercado, M., Regno, M., Böhlen, P., Brazeau, P. and Guillemin, R., (1980) Biochem. Biophys. Res. Commun. (in press).
22. Brazeau, P., Ling, N., Esch, F., Böhlen, P., Benoit R., and Guillemin, R., (1980) C.R. Acad. Sci. (Paris) 290, 1369-1371.
23. Gillioz, P., Giraud, P., Conte-Devolx, B., Jaquet, P., Codaccioni J., and Oliver, C., (1979) Endocrinology 104, 1407-1410.