Vol. 91, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 28, 1979 Pages 614-622

SYNTHESIS AND BIOLOGICAL ACTIVITY OF GLYCOSYLATED ANALOGS OF SOMATOSTATIN

S. Lavielle[1], N. Ling, P. Brazeau, R. Benoit, T. Wasada*,
D. Harris, R. Unger* and R. Guillemin

Laboratories for Neuroendocrinology
Salk Institute for Biological Studies
La Jolla, California 92037
and
*Southwestern Medical School
University of Texas
Dallas, Texas 75235

Received October 10, 1979

Summary

The synthesis by solid-phase methodology of two glycosylated analogs of somatostatin [Glc-Asn^5]-SS and [NAcGlc-Asn^5]-SS is described. These two analogs have been biologically tested on the secretion of pituitary growth hormone, pancreatic glucagon and insulin. The results show that glycosylation of somatostatin on the Asn^5 residue decreases by a hundred fold the inhibition activity on GH release when tested in vitro. In vivo, since the activity is similar to somatostatin the carbohydrates are probably removed by some enzymatic reaction and thus liberate the full activity of somatostatin.

Introduction

Somatostatin (SS), Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH, first isolated from ovine and porcine hypothalamus as an inhibitor of growth hormone release (2), has subsequently been shown to have a broad spectrum of inhibiting properties, namely on the secretion of pancreatic insulin (3) and glucagon (4). It also has a a wide distribution in the body tissues, particularly in discrete cells of the gut and pancreas (5) and in the central nervous system (6). However, the peptidergic nature of this hypothalamic messenger dictates a very short biological half-life, 2-4 min for this molecule (7), which limits its use as therapeutic agent. Consequently, after its characterization in 1973, a search (8) for more potent and longer-acting analogs of somatostatin was carried out in many laboratories which led to an appreciation of the structural requirements for somatostatin biological activities, in particular that Asn^5 could be substituted or even deleted with retention of biological activity of the analog (8,9).

The carbohydrate substituents of the glycoprotein hormones, FSH, LH, TSH, HCG have many functions, one of which is to impart the hormones with an ex-

0006-291X/79/220614-09$01.00/0

tended biological half-life (10). As a result, we reasoned that the introduction of a carbohydrate moiety in somatostatin might decrease the metabolic clearance rate of somatostatin. In the L-asparaginyl-glycosylamine linkages, so far only N-acetylglucosamine has been detected and the amino sugar linked O-glycosidically to peptide-bonded serine or threonine residues in glycoproteins seems to be invariably N-acetylgalactosamine (11). Since the Asn^5 residue of somatostatin could be substituted it was logical to introduce a sugar moiety linked to this amino acid. We have synthesized two [CHO-Asn^5]-somatostatin analogs: [NAcGlc-Asn^5]-SS,I, and [Glc-Asn^5]-SS, II by a combination of classical organic chemistry and solid-phase peptide synthesis methodology and have tested their immunoreactivity as well as bioactivity. This note is the first report on some of the biological activities of these glycosylated analogs of somatostatin.

Materials and Methods

Derivatized amino acids* used in the synthesis were of the L-configuration and purchased from Bachem Inc. The N^{α}-amino function was protected exclusively with the Boc group. Side-chain functional group protection consists of 2-chlorobenzyloxycarbonyl for Lys, p-methoxybenzyl for Cys, benzyl for Thr and Ser. TFA and DMF were distilled before use. HOBt was recrystallized from MeOH. All other solvents were reagent grade and not further purified.

Amino acid analyses were determined on peptide hydrolysates using a Beckman/Spinco Model 119 amino acid analyzer. Optical rotations were measured in a Perkin-Elmer Model 141 polarimeter. Ascending thin layer chromatography (TLC) on silica gel was performed on Eastman Chromatogram sheet no. 13191, the spots were detected by ninhydrin reagent. High pressure liquid chromatography (HPLC) was performed with a Waters Associates Model 204 liquid chromatography system equipped with two M-6000 A pumps, a 660 solvent programmer, a Schoeffel variable UV detector, a Spectra-Physics "Minigrator" and a linear instrument 456 recorder. Separation was accomplished on a 25 cm X 0.45 cm Dupont Zorbax ODS column in isocratic mode with the indicated percent composition of acetonitrile in buffer A at a flow rate of 1.5 ml/min and 0.1 absorbance full scale at 210 nm. Buffer A was 0.25 N triethylammonium at pH 3.0 (12) and the amount of peptides injected was 10 to 20 µg.

Synthesis. Coupling of Boc-(p-methoxybenzyl)Cys to the chloromethyl resin (0.9 meq Cl/g, Lab. Systems, Inc) was performed by the Monahan and Gilon procedure (13) which resulted in a substitution of 0.24 to 0.38 mmol Cys per gram resin. Coupling of the other Boc-amino acids to the resin was accomplished according to previously described procedure (14) using 1 mmol/g of Boc-amino acid per gram resin in CH_2Cl_2 and in 10% DMF/CH_2Cl_2 for Boc-Trp plus one equivalent 2 M DCCI in CH_2Cl_2 for two hours, except for Asn and the two glycoasparagine derivatives. Asn was coupled as its p-nitrophenyl ester in DMF for overnight and the two glycosylated asparagine derivatives were coupled with an equivalent amount of DCCI and 1.5 eqt of HOBt in 50% DMF/

* Symbols for amino acids and derivatives are according to IUPAC-IUB recommendations published in J. Biol. Chem. 247, 977-983 (1972). Other abbreviations are DCCI = dicyclohexylcarbodiimide; TFA = trifluoroacetic acid, DMF = dimethylformamide, HOBt = 1-hydroxybenzotriazole.

TABLE 1. SYNTHESIS OF SS AND GLYCOSYLATED SS

PEPTIDE	g. of starting RESIN (substitution in mmol/g)	YIELDS in mg after cyclization	after gel filtration	after de-O-acetylation	partition chromatography (over-all yield)
Somatostatin	6 (0.24)	1350	605		334 (15%)
[NAcGlc-Asn^5]-SS	2 (0.36)	882	362	305	131 (10%)
[Glc-Asn^5]-SS	3 (0.36)	1120	645	601	242 (13%)

CH_2Cl_2 for overnight. The protected peptide-resins were treated with 1.5 ml anisole and 10 ml hydrogen fluoride per gram peptide-resin at -20° for 0.5 hr and 0° for 0.3 hr. The work-up of the hydrogen fluoride cleavage reactions was as before (14) and the peptide solutions were diluted to 800 ml with degassed water and added dropwise to a ferricyanide solution to form the disulfide bond as described by Rivier et al (15). After oxidation the peptides were chromatographed on both anion- and cation-exchange resins as described (15) and lyophilized. The peptides were then submitted to gel filtration on Sephadex G-25 fine followed by de-O-acetylation of the sugar moiety for the two glycopeptides with a saturated solution of ammonia in methanol. Final purification was carried out on partition chromatography on Sephadex G-25 fine with the eluent system 1-butanol-acetic acid-water (4:1:5) (Table 1).

Trypsin Digestion. SS, [NacGlc-Asn^5]SS, [Glc-Asn^5]SS (175 µg, ∿100 nmoles) were digested with 5 µg of trypsin (Sigma) in 100 µl of 0.1 M NH_4OAc - 0.001 M $CaCl_2$ at pH 8.0 for 15 hours at 37° as described (16). The digestion was terminated with two drops of glacial HOAc and the solution was lyophilized twice.

In Vitro Growth Hormone Assay: Anterior pituitaries were obtained from young male rats (150 g). The glands were washed three times in a HEPES phosphate buffer containing 1% BSA and digested in a HEPES buffered solution pH 7.35 containing trace of calcium, 4 mg/ml of collagenase CLS-I (Worthington Co.) and 10 µg/ml of DNase (Boëhringer, Ingleheim). The tissues were digested for 45 to 55 min using a burned-tip Pasteur pipette to triturate. After digestion the cells were centrifuged and the enzyme solution discarded. After two washes in 3:1:1; HAMS F-12; DMEM, BGJb medium obtained from Gibco Lab., supplemented with 1 g/l of glucose, 10 mmoles of HEPES, 0.25% BSA from Worthington 614H Gibco; the cells were then plated in a multiwell plate and kept in an incubator under 100% humidity at 37°C, with 5% CO_2 atmosphere. Two days later, the cells were washed and the media replaced with 25% FCS supplemented media. Rat growth hormone was measured using RIA-GH kits provided by the NIH Pituitary Agency.

Morphine Sulfate Challenge Test. In Vivo Growth Hormone Assay. Young male rats 100-125 grams, fed ad lib for a week before the assay, were used. Two days prior to the bioassay date the animals were fasted. This procedure had been shown to annihilate any pulsative secretion of GH in male rats (17). The animals were injected subcutaneously in 0.5 ml of saline -90, -60 and -30 min respectively before decapitation, with saline as control, or 100, 20 and 4 µg of analog or somatostatin. Twenty minutes before decapitation the animals were injected i.p. with morphine sulfate in 0.5 ml of saline at a dose of 1 mg/kg of body weight. Ninety minutes following the first somatostatin and/or analog injection the rats were swiftly decapitated and blood collected from the trunk in a heparinized plastic tube. From the plasma

obtained by centrifugation at 2,000 g, a dilution of 1:10 of each sample was made for evaluation of GH secretion in plasma by RIA.

Glucagon and Insulin Assays (18). Two normal mongrel dogs (23 kg, 26 kg) and two alloxan treated dogs (30 kg, 32.5 kg of 2.5 yrs duration) were included in the present studies. The diabetic dogs were maintained on their usual insulin treatment consisting of 8-13 U NPH insulin administered twice daily with their meals. About 36 hours before the test, their usual doses of insulin were discontinued. All animals were fasted overnight. Two types of protocol were used: (1) The intravenous infusion study in which a stepwise increase in the dose of SS or its analogs (9.14 - 60.95 X 10^{-3} µmol) was employed; (2) A subcutaneous injection of 1 mg of the analogs in diabetic dogs was studied. Each test material was dissolved in normal saline solution containing 1% BSA just before use. Blood samples were drawn at the times indicated and were collected in tubes containing 1.2 mg EDTA and 1000 KIU Trasylol/ml of blood.

Radioimmunoassays. The first antibody to somatostatin was raised in a sheep (Barbar) and used at a final dilution of 1/50,000 (19). The second one was raised in a rabbit (SS-7) and used at a final dilution of 1/25,000. Both antisera did not cross-react with 28 endogenous peptides and amines up to a concentration of 1 µg/ml. Iodinated Tyr^1—SS was used as a tracer. The antigenic determinant for the sheep antibody is the segment 4 to 10 of somatostatin.

Results and Discussion

We have previously reported (20) the synthesis of two glycopeptides [CHO-Asn^1] Phe-Phe-Trp-Lys-OH containing a 2-acetamido-2-deoxy-β-D-glucopyranosyl or a β-D-glucopyranosyl moiety coupled to the Asn. The two glycosylated asparagines were synthesized by classical organic chemistry and were introduced by solid-phase methodology into the sequence of the peptides; using the same two glycosylated asparagines and similar solid-phase techniques we have now prepared the two glycosomatostatins I and II. After purification these two glycopeptides were found to have the correct amino acid

TABLE 2. PHYSICAL CONSTANTS OF SS PEPTIDES

PEPTIDES	$[\alpha]_D^{23}$ (a)	HPLC (b)	TLC (c) BAW	TLC (c) BPyA
Somatostatin	-31.9	25.7 min (99%)	0.36	0.39
[NAcGlc-Asn^5]-SS (I)	-22.4	11.8 min (≥99%)	0.32	0.38
[Glc-Asn^5]-SS (II)	-29.3	13.9 min (96%)	0.31	0.39

(a) C = 1 in 1% AcOH

(b) See Materials and Methods; isocratic 23.2% acetonitrile in Buffer A

(c) BAW: 1-butanol - acetic acid - water (4:1:5), BPyA: 1-butanol - pyridine - 0.1% acetic acid (20:12:45). Loads varied from 20 to 40 µg per shot.

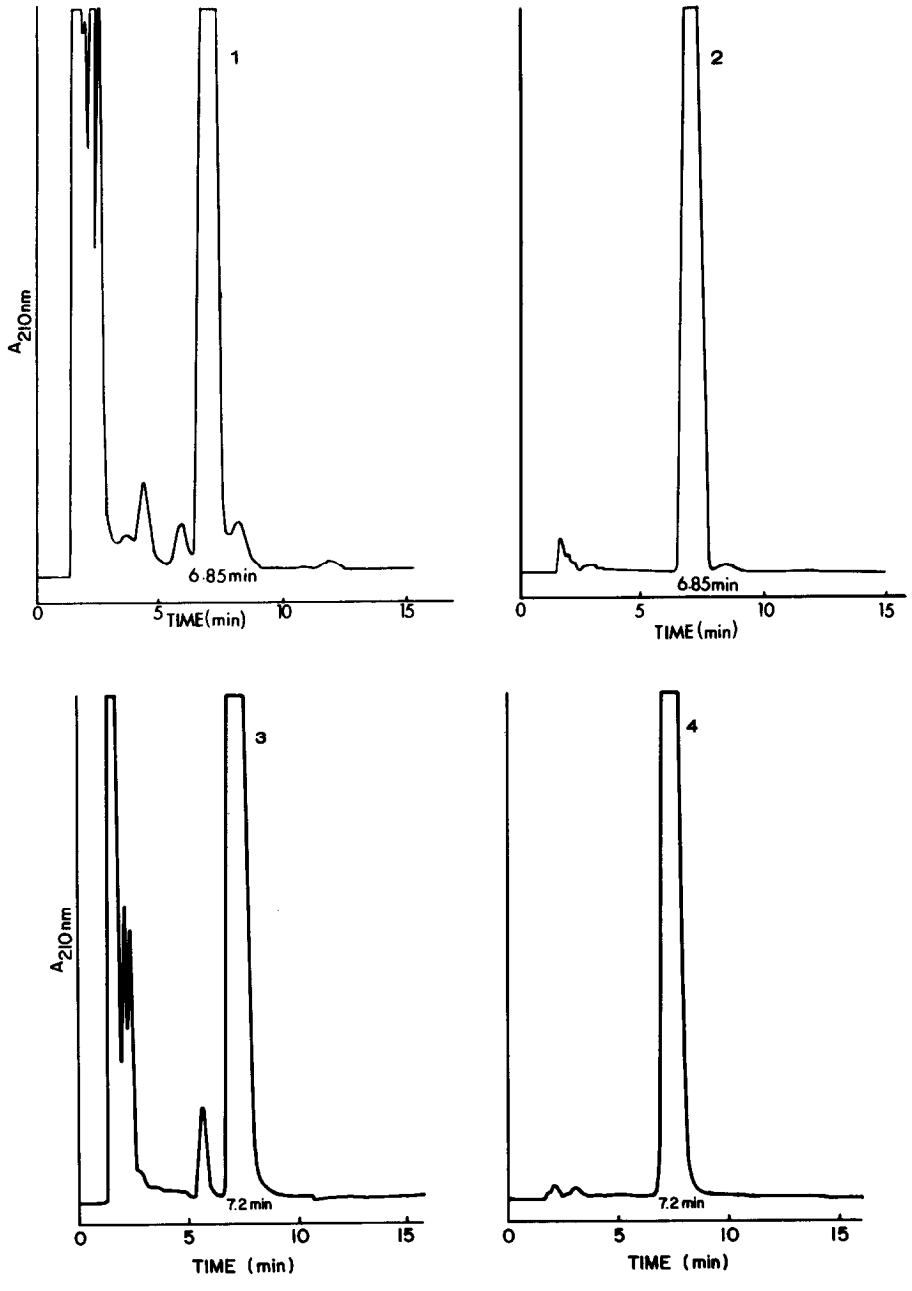

FIGURE 1: HPLC of the trypsin digests of [NAcGlc-Asn^5]-SS, (1), and [Glc-Asn^5]-SS, (3), and of the corresponding references glycopeptides [NAcGlc-Asn^1]-Phe-Phe-Trp-Lys (2) and [Glc-Asn^1]-Phe-Phe-Trp-Lys (4).

composition (data not shown) and further characterized by optical rotations, TLC in two solvent systems and HPLC (Table 2). The trypsin digestion of these two glycosomatostatins yielded the pentapeptide fragment comprising

residues 5 to 9 which on TLC (in two solvent systems) and HPLC showed the same R_f values and retention times (Figure 1) as the two corresponding model compounds [NAcGlc-Asn^1]-Phe-Phe-Trp-Lys-OH and [Glc-Asn^1]-Phe-Phe-Trp-Lys-Oh, that we have synthesized and characterized by mass spectrometry (20).

Effect on In Vitro Growth Hormone. Peptides I and II were studied in 5-point bioassay with reference to somatostatin for their ability to inhibit the spontaneous secretion of GH by the monolayer cultures of dispersed rat anterior pituitary cells. The activity found for these two glycopeptides is low, of the order of 0.1% for peptide I and 1% for peptide II compared to somatostatin (data not shown).

Effect on In Vivo Growth Hormone. When tested in rats in which secretion of growth hormone is acutely stimulated by morphine injection, the two analogs are at least as potent as somatostatin (Fig. 2) for inhibiting the secretion of growth hormone.

Effect on In Vivo Insulin and Glucagon. Both peptides I and II showed the apparent suppression of glucagon and insulin at the minimal dose used in the intravenous infusion study on either fasted normal dogs or alloxan treated dogs. After discontinuing the SS or analog infusion, glucagon and insulin levels rapidly rebounded to above the preinfusion levels in almost the same fashion - but slightly delayed in the analog studies. In the experiments using somatostatin in diabetic dogs a decrease in plasma glucose was observed, however, no change in glucose was observed with the analogs (data not shown).

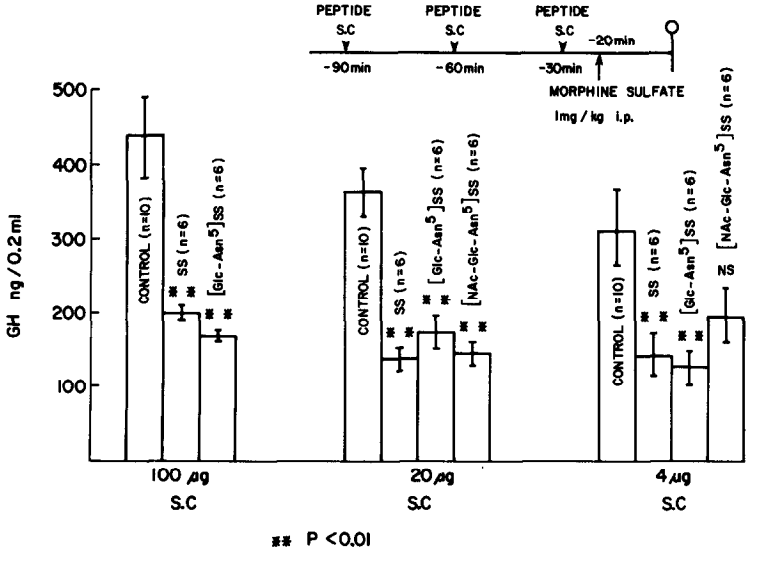

FIGURE 2: Morphine sulfate challenge test - In vivo growth hormone radioimmunoassay.

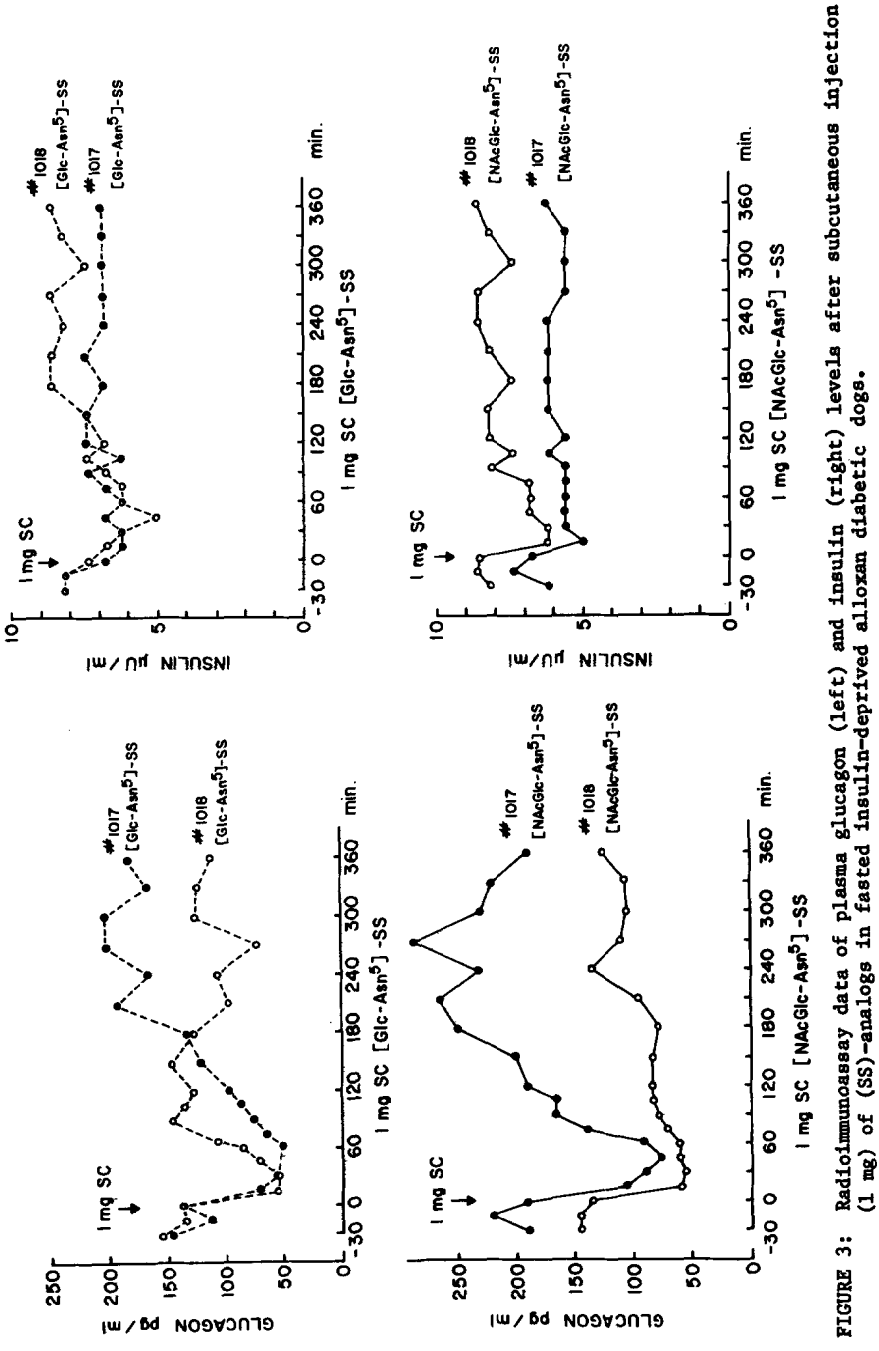

FIGURE 3: Radioimmunoassay data of plasma glucagon (left) and insulin (right) levels after subcutaneous injection (1 mg) of (SS)-analogs in fasted insulin-deprived alloxan diabetic dogs.

In diabetic dogs, the subcutaneous injection of somatostatin analogs demonstrated a significant suppression of glucagon within 15 minutes after injection and the suppression persisted for 2 hours in the case of one dog (#1018) and with a longer duration of activity than the somatostatin standard in one of the animals (Fig. 3). Despite the significant suppression of glucagon and some decrease of insulin levels, plasma glucose did not change significantly with either analog.

Immunological activities. In both antisera (sheep and rabbit) somatostatin, the two glycosylated analogs showed parallelism to the standard curve of somatostatin. While the β-glucopyranosyl moiety only slightly modified the antigenic determinant of the molecule, the 2-acetamido-β-glucopyranosyl substitution has caused a more definite alteration, increasing the ED_{50} by two and a half times (data not shown).

These results show that glycosylation of somatostatin on the Asn^5 residue - which is not a natural site for N-glycosylation (11), since a necessary but not sufficient prerequisite for N-glycosylation in vivo is the tripeptide sequence Asn-X-Ser/Thr in the peptide - somehow decreases by a hundred to one thousand fold the inhibition activity on growth hormone release when tested in vitro. In vivo, since the activity is similar to somatostatin, it is probable that the carbohydrates are removed by some enzymatic reactions in tissue or blood thus liberating the full activity of somatostatin. The experimental protocol of the in vivo experiments reported here was not designed to assess the duration of activity of the glycosylated analogs of somatostatin. The results of such studies now underway will be presented in another report.

Acknowledgements

We wish to thank R. Schroeder and D. Martineau for their excellent technical assistance as well as B. Gayer for secretarial help. This research was supported by NIH grants, AM-18811-05, HD-09690-05, 1-RO1-AM-18179, 1-MO1-RR-00633, AM-2700-16, the Contract Number NO1-AM62219 and the William Randolph Hearst Foundation.

References

1. S. LAVIELLE is an IAMOV-post doctoral fellow, 1977-1979, D.G.R.S.T., France.
2. P. BRAZEAU, W. VALE, R. BURGUS, N. LING, M. BUTCHER, J. RIVIER, R. GUILLEMIN, Science 179, 77 (1973).
3. K.G.M.M. ALBERTI, N.J. CHRISTENSEN, S.E. CHRISTENSEN, A.P. HANSEN, J. IVERSEN, K. LUNDAEK, K. SEYER-HANSEN, and H. ORSKOV, Lancet 2, 1299 (1973).
4. J.E. GERICH, M. LORENZI, V. SCHNEIDER, J.H. KARAM, J. RIVIER, R. GUILLEMIN and P. FORSHAM, N. Engl. J. Med. 291, 544 (1974).

5. a) T. HOKFELT, S. EFENDIC, C. HELLERSTROM, O. JOHANSSON, R. LUFT and A. ARIMURA, Acta Endocrinol. 80 (Suppl. 200), 1, (1975); b) L. ORCI, D. BAETENS, and C. RUFENER, Horm. Metab. Res. 7, 400 (1975); c) M. DUBOIS, Proc. Natl. Acad. Sci. USA, 72, 1340 (1975).
6. W. VALE, C. RIVIER, M. PALKOVITIS, J.M. SAAVEDNA, M. BROWNSTEIN, Endocrinology 94, A128 (1974).
7. T.W. REDDING and E.J. COY, Endocrinology 94, A156 (1974).
8. J. RIVIER, M. BROWN, C. RIVIER, N. LING and W. VALE (1976), in Proceedings of the 14th European Peptide Symposium (ed. A. Loffet), 427-451, and references therein.
9. D. SARANTAKIS, J. TEICHMAN, E.L. LIEN, R.L. FENICHEL, Biochem. Biophys. Res. Commun. 73, 336 (1976).
10. W.H. DAUGHADAY (1974) in Textbook of Endocrinology, (ed. E. Williams) W.B. Saunders Co., 31-54.
11. R.D. MARSHALL and A.NEUBERGER (1972) in Glycoproteins, 2nd Edition, (ed. A. Gottschalk) Elsevier Publ., Amsterdam, 453.
12. J. RIVIER, R. WOLBERS and R. BURGUS in Peptides, Proc. 5th Amer. Peptide Symposium (1977), (eds. M.Goodman and J. Meienhofer) John Wiley and Sons, Inc. Publ., New York, 52-55.
13. M.W. MONAHAN and C. GILON, Biopolymers 12, 2513 (1973).
14. N. LING, Biochem. Biophys. Res. Commun. 74, 248 (1977).
15. J. RIVIER, R. KAISER and B. GALYEAN, Biopolymers 17, 1927 (1978).
16. N. LING, R. BURGUS and R. GUILLEMIN, Proc. Natl. Acad. Sci. USA, 73, 3942 (1976).
17. G. SCHAFFER TANNENBAUM, O. RORSTAD, P. BRAZEAU, Endocrinology 104, 1733 (1979).
18. V. SCHUSDZIARRA, J. RIVIER, R. DOBBS, M. BROWN, W. VALE and R. UNGER, Horm. Metab. Res. 10, 563 (1978).
19. J. EPELBAUM, P. BRAZEAU, D. TSANG, I. BRAVER and J.B. MARTIN, Brain Res. 126, 309 (1977).
20. S. LAVIELLE and N. LING, submitted for publication.