Total Solid-Phase Synthesis and Prolactin-Inhibiting Activity of the Gonadotropin-Releasing Hormone Precursor Protein and the Gonadotropin-Releasing Hormone Associated Peptide†

Saskia C. F. Milton,‖,‡ Wolf F. Brandt,§ Martina Schnölzer,‡ and R. C. deLisle Milton*,‡,‖

Regulatory Peptides Research Unit of the Medical Research Council, Department of Chemical Pathology, University of Cape Town Medical School, Observatory 7925, South Africa, and The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, California 92037

Received March 13, 1992; Revised Manuscript Received June 16, 1992

ABSTRACT: The human gonadotropin-releasing hormone precursor protein, pHGnRH (Met^{-23}–Ile^{69}) (preproGnRH), and three of its fragment peptides, pHGnRH (Asp^{14}–Ile^{69}) (gonadotropin-releasing hormone associated peptide—GAP), pHGnRH (Phe^{38}–Ile^{69}), and pHGnRH (Ser^{47}–Ile^{69}), were assembled in a stepwise solid-phase cosynthesis employing Boc/Bzl tactics and an optimized acylation schedule which included recoupling steps with hexafluoro-2-propanol to help overcome the aggregation of the pendant peptide chains of the peptidoresin during difficult couplings. Reversed-phase high-performance liquid chromatography (HPLC) purification yielded products which were characterized by analytical reversed-phase HPLC, ion-exchange chromatography, capillary zone electrophoresis, SDS–polyacrylamide gel electrophoresis, and ion-spray mass spectrometry to reveal a high degree of homogeneity. Biological characterization demonstrated that only GAP stimulated luteinizing hormone and follicle-stimulating hormone release from primary cultures of rat anterior pituitary cells, while GAP, pHGnRH (Phe^{38}–Ile^{69}), and preproGnRH all inhibited prolactin release, with the latter being the most potent at concentrations comparable to bromocryptine. However, only GAP and pHGnRH (Phe^{38}–Ile^{69}) were able to displace a labeled gonadotropin-releasing hormone agonist from binding to rat pituitary membrane preparations. This first demonstration of significant biological activity with a precursor protein also suggests that the gonadotropin-releasing and prolactin release-inhibiting functions of GAP are not mediated through the same pituitary receptors.

The structure of the human gonadotropin-releasing hormone precursor peptide (pHGnRH)[1] was derived from cDNA obtained from human placental (Seeburg & Adelman, 1984) and hypothalamic mRNA (Adelman et al., 1986) and shown to consist of a 23-residue signal sequence, the gonadotropin-releasing hormone (GnRH) decapeptide segment, a Gly-Lys-Arg processing and amidation site, and a 56-residue carboxyl-terminal extention peptide, the so-called gonadotropin releasing hormone associated peptide—GAP (Nikolics et al., 1985), which ends in a Lys-Lys-Ile sequence (Seeburg & Adelman, 1984; Adelman et al., 1986) (Figure 1). The postulated role of the former dibasic processing site has been supported by the isolation from hypothalamic neurosecretory granules of a membrane-bound enzyme, the GAP releasing enzyme, which exhibited substrate specificity for the GnRH precursor and produced the GnRH sequence extended at its C-terminus by Gly-Lys-Arg as a cleavage product (Palen et al., 1987; Harris, 1989; Rangaraju et al., 1991). Bacterially synthesized recombinant GAP was originally shown to stimulate both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release as well as inhibiting prolactin release with cultured rat pituitary cells (Nikolics et al., 1985; Seeburg et al., 1987). Several attempts were made to repeat this initial work with chemically synthesized GAP: Peninsula Laboratories has produced two lots of the synthetic peptide, the first of which proved to be inactive while the second revealed only

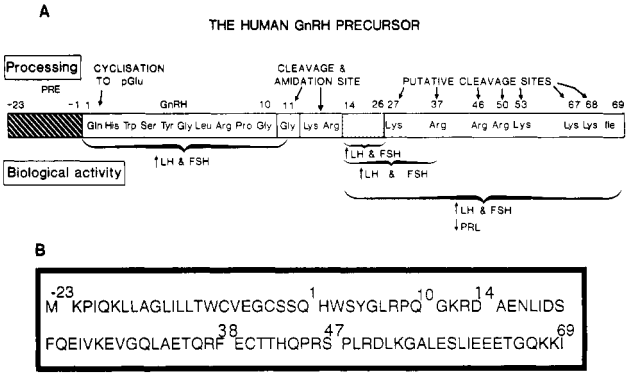

FIGURE 1: (A) Diagrammatic representation of human preproGnRH depicting the signal peptide, the GnRH sequence, and GAP. All amino acid residues involved or potentially involved in processing are indicated. The biological activity of peptide fragments that have previously been reported is also indicated (Wormald et al., 1985; Millar et al., 1986). (B) Complete amino acid sequence of the human GnRH precursor. The signal sequence, GnRH, GAP, and the fragment peptides pHGnRH 38–69 and pHGnRH 47–69 are indicated by numbering their starting residues.

† This work, which was initiated in the Department of Chemical Pathology, University of Cape Town Medical School, was supported by grants from the University of Cape Town and the Medical Research Council, South Africa, and the Markey Foundation at the Scripps Research Institute.

* Author to whom correspondence should be addressed.

‖ Department of Chemical Pathology, University of Cape Town Medical School.

‡ Present address: The Scripps Research Institute.

§ Department of Biochemistry, University of Cape Town.

[1] Abbreviations: DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; DTT, dithiothreitol; Etdt, ethanedithiol; FSH, follicle-stimulating hormone; GAP, gonadotropin-releasing hormone associated peptide; GnRH, gonadotropin-releasing hormone; HFA, hexafluoroacetone; HFIP, hexafluoro-2-propanol; LH, luteinizing hormone; Pam, (phenylacetamido)methyl; pHGnRH, human gonadotropin-releasing hormone precursor; PIF, prolactin releasing inhibiting factor; RP-HPLC, reversed-phase high-performance liquid chromatography; RT, retention time; SDS, sodium dodecyl sulfate; TEAP, triethylammonium phosphate; TFA, trifluoroacetic acid.

0006-2960/92/0431-8799$03.00/0

minimal activity (Seeburg et al., 1987). Schally and co-workers, on the other hand, have reported a synthetic product which revealed the same biological activity as the bacterially derived material (Schally et al., 1986), while Guillemin and co-workers obtained only marginal activity with their chemically synthesized peptide (Seeburg et al., 1987).

In view of these observations, further processing of the GnRH prohormone was assumed by Millar and co-workers and a 13-residue peptide, designated pHGnRH 14–26 [according to references in Seeburg and Adelman (1984) and Millar et al. (1986)] was chemically synthesized and shown to stimulate LH and FSH release with human and baboon pituitary cells in culture (Millar et al., 1986; Wormald et al., 1985). This gonadotropin release was shown to be dose and calcium dependent and was concluded to be independent of the GnRH receptor in the human pituitary cell cultures (Millar et al., 1986). The chemical synthesis of a further six overlapping peptide fragments demonstrated that the decapeptide sequence pHGnRH 17–26 contained the minimal structural features necessary to ellicit this activity (Milton et al., 1986). With rat pituitary cell cultures, however, the minimal active sequence (pHGnRH 17–26), and even the longer pHGnRH 14–26 sequence, was found to be inactive, while an analogue, [D-Trp^{22}]pHGnRH 14–26, revealed the expected gonadotropin-releasing activity (Milton et al., 1987). Similarly, in vivo studies in rats have also showed the pHGnRH 14–26 peptide to be inactive and the D-Trp analogue to be active (Yu et al., 1990). No inhibition of prolactin release was found with any of the N-terminal peptide fragments of GAP, and this encouraged the speculation that either the full GAP sequence was required or, alternatively, a fragment from the C-terminal region of GAP was responsible for this activity (Milton et al., 1986). This speculation was encouraged by the observations that while the bacterially derived GAP inhibited prolactin release from human and rat pituitary cell cultures, the disulfide-bridged dimers of this material were inactive (Abrahamson et al., 1987).

In this paper we report the solid-phase cosynthesis and characterization of a 56-residue GAP product, which both stimulates LH and FSH release and inhibits prolactin release, as well as a complete 92-residue preprohormone sequence (pHGnRH -23 to 69) which inhibits prolactin release. This is the first report of a precursor protein which is associated with a significant biological activity. Of the two other GAP fragment peptides, pHGnRH 47–69 and 38–69, which were generated during the synthesis, only pHGnRH 38–69 revealed a marginal prolactin-release inhibiting effect and receptor binding. Biological activities were evaluated by the effect of the specific peptides on gonadotropin and prolactin release from primary cultures of rat pituitary cells as well as their ability to displace a radiolabeled GnRH agonist analogue from rat pituitary cell membrane preparations.

EXPERIMENTAL PROCEDURES

Materials. Derivatized amino acids were obtained from Peninsula Laboratories Europe, St. Helens, Merseyside, UK, and Protein Research Foundation, Osaka, Japan. HFIP (99%) and HFA (98%) were obtained from Aldrich Chemical Co., Gillingham, Dorset, UK. Acetic anhydride (Aldrich Chemical Co., Gillingham, Dorset, UK), TFA (Halocarbon, Hackensack, NJ), DIEA (over CaH_2), and DCC (under reduced pressure) were distilled, and DMF (Burdick & Jackson, Muskegon, MI) was stored over a 4-Å sieve. All other solvents were HPLC grade and all chemicals met ACS standards. Bulk C4 and C18 RP-HPLC 15–20-μm silicas were obtained from the Separations Group, Hesperia, CA. The recombinant GAP, which was used for reversed-phase cochromatography, was a kind gift from K. Nikolics of Genentech (Abrahamson et al., 1987).

Table I: Schedule for Optimized Extended Manual Cosynthesis of PreproGnRH and Fragment Peptides

step	reagents and operations	mix time (min)
1a	CF_3COOH/CH_2Cl_2 (3:2 v/v)—5% 1,2-ethanedithiol	2
1b	CF_3COOH/CH_2Cl_2 (3:2 v/v)—5% 1,2-ethanedithiol	2
1c	CF_3COOH/CH_2Cl_2 (3:2 v/v)—5% 1,2-ethanedithiol	20
2	2-propanol—0.1% indole wash 2×	1 each
3	DIEA/DMF (1:19 v/v), MeOH, DIEA/DMF (1:19 v/v)	1 each
4	MeOH wash 3×, CH_2Cl_2 wash 3×	1 each
5a(i)	3 equiv of preformed -OBt ester in DMF	<300
5a(ii)	DMF wash 3×, MeOH wash 3×, CH_2Cl_2 wash 3×	1 each
5b(i)	hexafluoro-2-propanol/CH_2Cl_2 (≥1:9 v/v) wash	2
5b(ii)	3 equiv of preformed symmetric anhydride in CH_2Cl_2 + hexafluoro-2-propanol (≥1:9 v/v)	<300
5b(iii)	MeOH wash 3×, CH_2Cl_2 wash 3×	1 each
6a(i)	Gln(Xan), Asn(Xan), and Asn: 3 equiv of preformed -OBt ester in DMF	<300
6a(ii)	DMF wash 3×, MeOH wash 3×, CH_2Cl_2 wash 3×	1 each
6b(i)	hexafluoro-2-propanol/CH_2Cl_2 (≥1:9 v/v)	2
6b(ii)	3 equiv of preformed -OBt ester in DMF	<300
6b(iii)	DMF wash 3×, MeOH wash 3×, CH_2Cl_2 wash 3×	1 each
7	acetic anhydride/hexafluoro-2-propanol/CH_2Cl_2 (2:1:7 v/v/v) (when indicated)	30
8	MeOH wash 3×, CH_2Cl_2 wash 3×	1 each

Reversed-Phase High-Performance Liquid Chromatography. Preparative RP-HPLC was accomplished with a Beckman Prep 350 system connected to a Waters Associates PrepPAK 1000 radial compression chamber fitting cartridges (5.5 cm × 30.0 cm) packed with either Vydac 15–20-μm C18 or C4 silicas and operated at flow rates of 50–75 mL/min. Analytical RP-HPLC was performed using Beckman 110A solvent metering pumps, a Waters Associates Model 450 variable-wavelength detector, and a Model 730 data module. Vydac C18 and C4 columns (4.6 mm i.d. × 25 cm) provided the stationary phase and elution was accomplished with a binary buffer system at a flow rate of 2.0 mL/min. Buffer A consisted of either 0.25 M H_3PO_4 adjusted to pH 6.5 with triethylamine (TEAP), 0.1 M ammonium acetate adjusted to pH 6.5 with concentrated acetic acid, or 0.1% HFA/0.1% TFA. Buffer B consisted of 60% CH_3CN in TEAP buffer, 0.1 M ammonium acetate, pH 6.5, containing 60% CH_3CN, or a 90:10 mixture of CH_3CN and H_2O containing 0.1% HFA and 0.1% TFA (v/v).

Peptide Synthesis. *t*-Boc-Ile-OCH_2-Pam-resin, 0.40 meq of -NH_2/g of resin (by picrate titration; Gisin, 1972), was prepared following the standard methodology (Mitchell et al., 1976; Tam et al., 1979) as previously described (Milton & Milton, 1990). Cosynthesis was accomplished manually starting with 15.0 g of resin in a filter-frit reaction vessel and removing portions of protected peptidoresin at the correct stages of the synthesis to obtain the target peptides. A double-coupling protocol was used with modifications to accommodate the use of HFIP (Table I) and a maximal protection scheme with the following Boc amino acid derivatives was used: cyclohexylaspartic acid and -glutamic acid, formyltryptophan,

(bromocarbobenzoxy)tyrosine, and (benzyloxymethyl)histidine. Glutamine and asparagine carboxamides were protected with the xanthenyl group except for Asn[17], which was coupled as the unprotected Boc derivative. All amino acid derivatives were acylated using 3 equiv of the preformed oxybenzotriazole-activated ester in DMF (preactivated with 1 equiv of DCC for longer than 2 h in ice and filtered) for up to 5 h. All aminoacylations were monitored by quantitative ninhydrin assay (Sarin et al., 1981) and were repeated, if this was necessary, until an acylation extent greater than 99.5% was achieved. This was accomplished either by returning the preformed oxybenzotriazole-activated ester to the reaction vessel for a longer time or by recoupling with a 3-fold molar excess of preformed symmetric anhydride (preactivated for 30 min in CH_2Cl_2 or CH_2Cl_2/DMF, 9:1, at room temperature and filtered) in 10–20% (v/v) HFIP in CH_2Cl_2 for up to 5 h after an initial HFIP/CH_2Cl_2 prewash (Table I; Milton & Milton, 1990). HFIP is a corrosive reagent and should be used with caution. Postcoupling acetylation with acetic anhydride/HFIP/CH_2Cl_2, 1:1:3, was performed for 1 h (Milton & Milton, 1990), as required. Repetitive removal of the N^{α}-amino-protecting *t*-Boc groups was accomplished with three washes (2, 2, and 20 min) of TFA/Etdt/CH_2Cl_2, 12:1:7, and neutralization with two washes of DIEA/DMF, 1:19 (v/v). The last *t*-Boc group of each peptide was removed with TFA prior to hydrogen fluoride deprotection and cleavage from the resin (Noble et al., 1976).

On completion of chain assembly of the pHGnRH 47–69 sequence and vacuum drying, 32.87 g of peptidoresin was obtained, which was split in the ratio 1:4 (w/w), and synthesis was continued with the larger portion. Similarly, 31.44 g of the completed pHGnRH 38–69 sequence peptidoresin was split in the ratio of 1:3 and synthesis was continued with the larger portion, and subsequently, 27.30 g of the pHGnRH 14–69 sequence peptidoresin was divided into two equal portions. The synthesis was continued with one of these portions, and on completion of the pHGnRH -23 to 69 sequence, 15.57 g of peptidoresin was obtained after drying. The *t*-Boc removal from the fully protected pHGnRH -23 to 69 Pam resin was followed by treatment with piperidine in DMF to remove the formyl group from tryptophan residues (Plaue & Briand, 1988). The partially deprotected peptidoresins were all treated with hydrogen fluoride (S_N2/S_N1 protocol; Plaue & Briand, 1988; Tam et al., 1983) to remove the remaining side-chain protecting groups and to cleave the peptides from their resin. After trituration and extraction [with aqueous NH_3 in the presence of 2% (v/v) β-mercaptoethanol, pH 10.0], the crude peptides were lyophilized. The crude peptide lyophilysates were dissolved and applied onto a cartridge packed with C4 (pHGnRH -23 to 69) or C18 (pHGnRH 14–69, 38–69, and 47–69) silica and fitting a Beckman Prep 350. The peptides were eluted with a gradient of acetonitrile in either 0.1% HFA/0.1% TFA (pHGnRH -23 to 69) or 0.1 M ammonium acetate, pH 6.5 (pHGnRH 14–69, 38–69, and 47–69), and the eluant was monitored by analytical RP-HPLC so that cuts could be made and often rerun until pure product was obtained and lyophilized (Rivier et al., 1984; Hoeger et al., 1987). HFA is highly toxic and an irritant and should be used with caution.

Amino Acid Analysis. Gas-phase hydrolyses of lyophilized products were performed with 5.7 N HCl at 150 °C (Bidlingmeyer et al., 1984) and amino acid determinations were accomplished using a RP-HPLC system with precolumn (dimethylamino)azobenzenesulfonyl chloride detection (Knecht & Chang, 1986). No corrections were made for serine and threonine destruction.

Sequence Analysis. Gas-phase sequence analysis (Hewick et al., 1981; Brandt et al., 1984) was accomplished with the quantitation of the phenylthiohydantoin amino acid derivatives by RP-HPLC (Lottspeich, 1985).

Capillary Zone Electrophoresis. Capillary zone electrophoresis was performed on purified peptide products using a Beckman Model P/ACE System 2000 and a Supelco CElect-P175 (hydrophilic/polar) 50-cm × 75-mm capillary. Samples (1.0 mg/mL) were dissolved in 0.1% TFA, injected with pressure, and electrophoresed at 30 °C in 0.1 M sodium phosphate buffer, pH 2.5, at a constant voltage of 15 kV and 94 mA. Reduction of the pHGnRH 14–69 (GAP) peptide sample in 0.01 M sodium phosphate/0.06 M sodium borate buffer, pH 8.0 (1.0 mg/mL), was accomplished with 5.0% (v/v) β-mercaptoethanol for 1.5 h.

Ion-Exchange Chromatography. Ion-exchange chromatography was performed on a Pharmacia Precision column (Mono Q) 1.6/5 with detection at 254 nm. Buffer A consisted of 20 mM bis-Tris (in a 10:90 mixture of CH_3CN and H_2O), pH 6.2, and buffer B was 0.5 M NaCl in buffer A, pH 6.2. The flow rate was 0.1 mL/min and the gradient used was 0–100% buffer B in 2 mL. Purified pHGnRH 14–69 was dissolved (2 mg/mL in buffer A, pH 8.0) and reduced in the presence of 5.0% (v/v) β-mercaptoethanol for 1.0 h.

SDS–Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis of crude and purified peptide products was performed with slab gels containing acrylamide in a concentration gradient from 7 to 15% (w/v) according to the method of Laemmli (1970). Samples consisted of 0.125–0.250 mg of peptide with DTT, boiled for 6 min. The SDS–polyacrylamide gel was electrophoresed with a Hoefer Scientific Instruments (SE 600) vertical slab electrophoresis unit and power pack at a constant current of 6.0 mA for 18 h at 4 °C, after which the gel was fixed in 30% (v/v) methanol containing 10% (v/v) acetic acid in water and then stained with 0.1% (w/v) Coomassie Blue, 30% (v/v) methanol, and 10% (v/v) acetic acid in water for 3 h prior to destaining for 48 h in 30% (v/v) methanol and 10% (v/v) acetic acid in water.

Mass Spectrometry. A SCIEX API III triple-quadrupole mass spectrometer (Sciex, Inc., Thornhill, Ontario, Canada) equipped with an atmospheric pressure ionization source was used in these investigations. Samples were dissolved in 15% CH_3CN/0.1% TFA/H_2O (+0.1% HFA in the case of pHGnRH -23 to 69) at 1 mg/mL concentrations and directly infused into the ion spray interface of the mass spectrometer with a syringe pump (Harvard Apparatus, South Natick, MA) at a flow rate of 5 μL/min. The ion spray voltage was 5 kV. Positively charged ions, generated by the ion spray interface, were sampled into the analyzer region of the mass spectrometer through a 100-μm i.d. orifice with 60 V applied. The vacuum in the analyzer region was maintained by the use of a helium cryogenic pump, and a curtain of ultrapure dry nitrogen gas (1–2 L/min) was applied to the atmospheric side of the sampling orifice to prevent contaminants and solvent vapor from entering the vacuum and to help declustering and desolvation of the highly charged sample droplets.

Mass calibration was accomplished with a mixture of polypropylene glycols in the positive-ion mode and single MS data were obtained by scanning quadrupole 1 from m/z 400 to 2400 in 5 s/scan with a step size of 0.5 dalton (Covey et al., 1991). Data were acquired onto an Apple Macintosh IIfx computer and were processed using the data analysis program MacSpec 3.11b25 (Sciex). Molecular masses were calculated

Table II: pHGnRH -23 to 69 (Including 14–69, 38–69, and 47–69) ⟨P_c*⟩ Values and Acylation Yields (%)[a]

	-23			-20					-15			
	Met	**Lys**	**Pro**	**Ile**	**Gln**	**Lys**	**Leu**	**Leu**	**Ala**	**Gly**	**Leu**	**Ile**
⟨P_c*⟩	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.97	0.97	0.96	0.97	0.97
yield	99.86	±	99.81	99.35	99.55	99.50	99.75	99.67	99.74	99.83	99.21	99.57
		-		99.69	99.80	99.81	99.81	99.89	99.78		99.88	99.73
				99.77								99.87
Ac_2O	*99.91*	*99.87**	*99.89*	*99.88*	*99.95*	*99.89*	*99.89*		*99.83*	*99.89*	*99.62*	*99.93*

	-10					-5					1	
Leu	**Leu**	**Thr**	**Trp**	**Cys**	**Val**	**Glu**	**Gly**	**Cys**	**Ser**	**Ser**	**Gln**	**His**
0.97	0.97	0.97	0.98	0.98	0.98	0.98	0.97	0.97	0.97	0.97	0.97	0.97
99.13	99.59	99.12	99.62	99.27	99.51	99.83	99.83	99.89	99.90	99.88	99.87	96.81
99.82	99.80	99.81	99.89	99.74	99.79							99.80
99.93	*99.93*	*99.88*		*99.89*	*99.89*	*99.88*	*99.91*				*99.91*	*99.93*

		5					10					15
Trp	**Ser**	**Tyr**	**Gly**	**Leu**	**Arg**	**Pro**	**Gly**	**Gly**	**Lys**	**Arg**	**Asp**	**Ala**
0.97	0.97	0.97	0.96	0.96	0.96	0.95	0.94	0.93	0.93	0.93	0.93	0.93
99.87	99.89	99.79	99.86	99.89	±	99.83	99.90	99.76	99.62	99.61	99.38	99.67
					-			99.89	99.99	99.79	99.92	99.91
99.97		*99.89*			*99.81**	*99.85*				*99.92*		

				20					25			
Glu	**Asn**	**Leu**	**Ile**	**Asp**	**Ser**	**Phe**	**Gln**	**Glu**	**Ile**	**Val**	**Lys**	**Glu**
0.94	0.93	0.93	0.94	0.94	0.93	0.93	0.94	0.94	0.94	0.95	0.95	0.95
99.34	97.12	99.72	99.68	99.78	99.86	99.51	98.76	99.45	99.09	99.53	99.14	99.48
99.95	99.33	99.99	99.78	99.97	99.95	99.80	99.92	99.83	99.87	99.80	99.80	99.34
	99.70											99.90
	99.88		*99.99*			*99.90*		*99.93*	*99.96*	*99.98*	*99.95*	*99.86*

	30					35					40	
Val	**Gly**	**Gln**	**Leu**	**Ala**	**Glu**	**Thr**	**Gln**	**Arg**	**Phe**	**Glu**	**Cys**	**Thr**
0.96	0.95	0.95	0.96	0.96	0.96	0.96	0.97	0.97	0.97	0.98	0.98	0.98
99.68	99.86	98.00	99.61	99.78	99.50	99.34	99.86	99.90	99.83	99.95	99.96	99.90
99.70		99.01	99.83	99.97	99.79	99.70						
		99.69										
99.91	*99.96*	*99.92*	*99.88*		*99.93*	*100*	*99.95*		*99.96*			*99.97*

			45					50				
Thr	**His**	**Gln**	**Pro**	**Arg**	**Ser**	**Pro**	**Leu**	**Arg**	**Asp**	**Leu**	**Lys**	**Gly**
0.98	0.98	0.98	0.96	0.96	0.94	0.91	0.92	0.92	0.91	0.92	0.92	0.88
99.90	91.16	-	99.89	99.80	-	99.52	98.74	96.60	99.23	99.64	99.80	99.92
	92.10	-		99.85	-	99.86	99.30	98.18	99.52	99.80		
	99.92			99.93			99.61	98.66				
								99.29				
								99.52				
99.93		*99.97**	*99.94*	*99.95*	*100**	*99.91*	*99.98*	*99.91*	*99.95*	*99.99*	*99.98*	*100*

55					60					65		
Ala	**Leu**	**Glu**	**Ser**	**Leu**	**Ile**	**Glu**	**Glu**	**Glu**	**Thr**	**Gly**	**Gln**	**Lys**
0.89	0.90	0.91	0.88	0.90	0.92	0.93	0.95	0.97	0.97	0.84	0.85	0.82
99.76	99.67	99.69	99.52	99.83	99.82	99.87	84.91	98.98	96.89	99.69	99.97	99.98
99.76	99.85	99.74	99.74				97.25	99.59	99.02	99.94		
							99.04		99.41			
							99.52					
99.98	*99.99*	*100*	*99.97*	*99.90*	*99.91*		*99.94*	*99.94*	*99.91*			

69		
Lys	**Ile-OCH$_2$-Pam-Resin**	Average coupling yields: First = 99.21%
0.74		Last = 99.83%
99.99		After capping = 99.93%

[a] Asterisks indicate backgrounds obtained after Pro. ± and –: isatin test (Kaiser et al., 1980), result not included in calculation of average coupling yields.

from the multiply-charged ions by using the HyperMass program and molecular weight mass spectra were constructed from the corresponding m/z spectra containing multiple charge states by the use of the ReconstructHyperMass program (both in MacSpec 3.11b25) (Schnölzer, Jones, Alewood, and Kent, unpublished experiments).

Biological Activities. The peptides were tested for their ability to stimulate or inhibit the release of LH, FSH, and prolactin from primary cultures of rat anterior pituitary cells obtained from adult male Long-Evans rats using previously described methodology (Vale et al., 1972). After 4 days the cells were washed five times with medium and triplicate cultures were incubated for 3 h in the presence of stock solutions of the test peptides, which were prepared by dissolving lyophilysates in medium and adjusting the pH to 7.4. pHGnRH -23 to 69, however, was first treated with HFA and then diluted with medium to a final concentration of 0.1% (v/v) HFA, and the pH was adjusted to 7.4. The harvest fluids obtained after

Table III: Yield and Homogeneity of the Synthetic pHGnRH Fragment Peptides

peptide	yield[a] mmol	yield[a] %	homogeneity[b] (%)	C18 RP-HPLC buffer system[c]	gradient (20 min)	RT[d] (min)	peptide content[e] (%)
pHGnRH 47–69	0.12	10.13	96.67	S_1	39–54% buffer B	9.52	93.50
pHGnRH 38–69	0.04	3.46	97.90	S_1	42–57% buffer B	9.10	65.00
pHGnRH 14–69	0.05	2.78	98.20	S_1	49–64% buffer B	10.76	100.00
pHGnRH –23 to 69	0.02	1.58	99.77	S_2	49–64% buffer B	9.76	83.00
				S_2	49–64% buffer B	18.74 (C4)	

[a] Overall yield based on the starting substitution of the aminoacyl resin. [b] Ratio of the integrated area under the major peak vs the total integrated areas at 210 nm. [c] S_1: buffer A = TEAP, pH 6.5; buffer B = a 60:40 mixture of CH_3CN and buffer A. S_2: buffer A = 0.1% TFA/0.1% HFA; buffer B = a 90:10 mixture of CH_3CN and buffer A. [d] RT, retention time. [e] MW/mass.

Table IV: Amino Acid Analysis of PreproGnRH and Fragment Peptides

amino acid	pHGnRH 47–69 found	pHGnRH 47–69 expected	pHGnRH 38–69 found	pHGnRH 38–69 expected	pHGnRH 14–69 found	pHGnRH 14–69 expected	pHGnRH –23 to 69 found	pHGnRH –23 to 69 expected
Asx	0.73	1	1.16	1	4.05	4	4.30	4
Thr	0.9	1	2.55	3	3.93	4	5.00	5
Ser	1.80	2	2.16	2	2.92	3	5.40	6
Glx	4.34	5	7.21	7	14.82	14	17.1	17
Pro	0.91	1	1.99	2	1.73	2	nd[a]	2
Gly	1.85	2	2.00	2	2.81	3	8.30	8
Ala	1.18	1	1.28	1	2.75	3	4.50	4
Cys			nd	1	nd	1	nd	3
Val					2.04	2	2.80	3
Ile	2.32	2	1.88	2	4.31	4	5.80	6
Leu	4.00	4	4.09	4	6.42	6	12.10	12
Phe			0.63	1	2.00	2	2.00	2
Lys	3.25	3	2.91	3	4.34	4	6.10	7
His			0.67	1	1.01	1	nd	2
Arg	1.00	1	1.34	2	3.30	3	5.00	5
Met							0.90	1
Tyr							1.00	1

[a] nd, not determined.

Table V: Sequence Analysis of PreproGnRH and Fragment Peptides

degradation cycle	pHGnRH 38–69 PTH[a] amino acid	pHGnRH 38–69 yield (nmol)	pHGnRH 14–69 PTH amino acid	pHGnRH 14–69 yield (nmol)	pHGnRH –23 to 69 PTH amino acid	pHGnRH –23 to 69 yield (nmol)
1	Phe	2.773	Asp	0.866	Met	0.844
2	Glu	2.694	Ala	1.649	Lys	0.405
3	Cys	1.376	Glu	1.668	Pro	0.271
4	Thr	1.399	Asn	1.567	Ile	0.200
5	Thr	1.063	Leu	1.271	Gln	0.200
6	His	1.228	Ile	0.381	Lys	0.154
7	Gln	1.206	Asp	0.855	Leu	0.160
8	Pro	0.805	Ser	0.562	Leu	0.264
9	Arg	0.934	Phe	0.743	Ala	0.156
10	Ser	0.531	Gln	0.646	Gly	0.170
11	Pro	0.341	Glu	0.510	Leu	0.158
12	Leu	0.379	Ile	0.184	Ile	0.100
13	Arg	0.333	Val	0.173	Leu	0.168
14	Asp	0.310	Lys	0.425	Leu	0.202
15	Leu	0.435	Glu	0.320	Thr	0.019
16	Lys	0.404	Val	0.166	Trp	0.017
17	Gly	0.182	Gly	0.184	Cys	*b*
18	Ala	0.241	Gln	0.175	Val	0.051
19	Leu	0.288	Leu	0.194	Glu	0.036
20	Glu	0.127	Ala	0.197		
21	Ser	0.166	Glu	0.126		
22	Leu	0.252	Thr	0.194		
23	Ile	0.026	Gln	0.101		
24	Glu	0.114	Arg	0.099		
yield at	Phe^{38}	78%	Ala^{15}	100%	Met^{-23}	74%

[a] PTH, phenylthiohydantoin derivative. [b] Not found.

incubation were assayed by radioimmunoassay for LH, FSH, and prolactin content (rLH, rFSH, and rPRL RIA, NIADDK, intraassay c.v. ranging from <3.7 to <6.2%).

GnRH receptor binding studies were performed as previously described (Milton et al., 1987) using the 10000*g* crude membrane pellet from adult male Long-Evans rat anterior pituitary homogenate incubated with labeled [D-Ala^6,$N^{\alpha Me}$-Leu^7,Pro^9-NHEt]GnRH (obtained from J. E. Rivier, Salk Institute, La Jolla, CA, labeled using the chloramine-T method) in the presence of varying concentrations of GnRH or the test peptides.

RESULTS AND DISCUSSION

The synthesis of the 92-residue precursor peptide was approached with a stepwise solid-phase strategy (Merrifield, 1963) so that the assembly of the peptide chain could be interrupted after residues Ser^{47}, Phe^{38}, and Asp^{14} and the peptidoresin split to generate pHGnRH 47–69, pHGnRH 38–69, and GAP. Chemical tactics (Table I) were chosen with a view to a predictive method for difficult sequences (Milton et al., 1990) which ascribes a Chou and Fasman (1978) type coil conformational parameter, P_c* (Milton et al., 1990), value to each residue (Table II). This value is suggested to directly reflect the likely ease of acylation for the particular residue (Milton et al., 1990). The synthesis was therefore accomplished manually with real-time monitoring of aminoacylations (Kaiser et al., 1970; Sarin et al., 1981) to ensure the best possible product at the first attempt (Table II). Predicted difficult couplings ($\langle P_c^* \rangle < 1$) were typically continued for up to 5 h (with interruptions to determine the extent of acylation) or until no further improvement was seen. Recouplings were accomplished with preformed symmetric anhydrides in $HFIP/CH_2Cl_2$ (Milton & Milton, 1990) and continued until the desired acylation level was achieved. It was, however, noticed during the course of the synthesis that the concentration of HFIP needed to be increased to achieve full resin swelling with increasing peptide length. Acetylation with acetic anhydride in $HFIP/CH_2Cl_2$ (Milton & Milton, 1990) was performed when the extent of aminoacylation could not be made to exceed 99.85% (Sarin et al., 1981) by repeated recoupling. Table II includes the yields obtained for each of the acylation steps as well as the cumulative $\langle P_c^* \rangle$ value for each residue. The number of recouplings that was required for the successful incorporation of an amino acid residue generally correlates with the difficulty predicted by the P_c* value, except in the case of the first four aminoacylations (Milton et al., 1990) and random-type difficult couplings (Meister & Kent, 1983), such as the β-branched amino acids and residues with bulky side-chain protecting groups (such as tosylarginine, (benzyloxymethyl)histidine, and xanthenylglutamine; Milton et al., 1990), which also sometimes required extensive recoupling.

As the phenomenon of sequence-related incomplete aminoacylations is attributed to β-sheet hydrogen bonding of the protected peptides chains on the resin (Live & Kent, 1982,

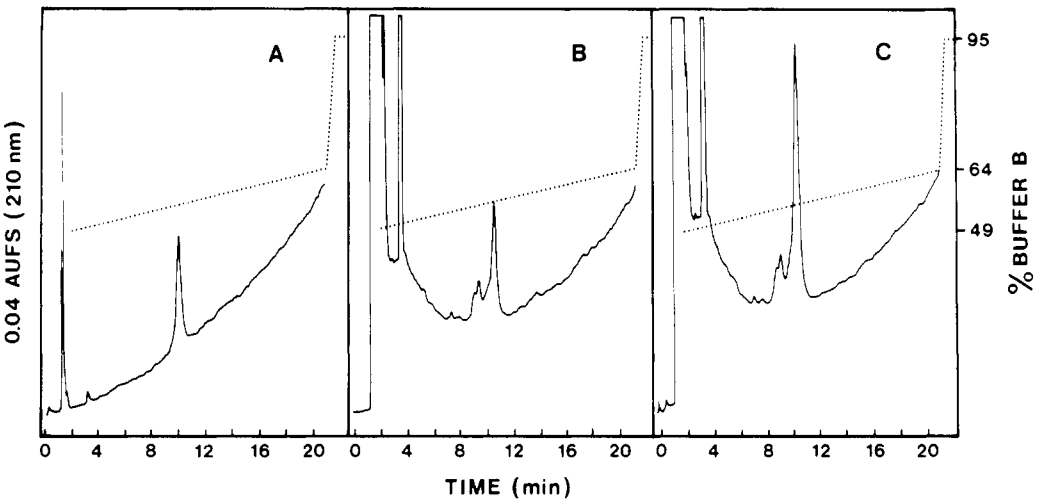

FIGURE 2: RP-HPLC cochromatography of synthetic and bacterially derived pHGnRH 14–69 (GAP). (A) Approximately 0.25 mg of synthetic pHGnRH 14–69 with 0.20 mg of dithiothreitol. (B) Approximately 0.30 mg of bacterially derived pHGnRH 14–69 with 0.20 mg of dithiothreitol (dissolved to a concentration of 10^{-5} M in minimal essential medium containing antibiotics). (C) Approximately 0.25 mg each of synthetic and bacterially derived pHGnRH 14–69. Column: Vydac C18 (218TP54). Flow: 2 mL/min. Elution: 49–64% buffer B, 20 min. Buffer A, 0.25 M triethylammonium phosphate, pH 6.5 (TEAP); buffer B, 60% CH_3CN in buffer A. Detector: 0.04^{210nm} absorbance unit full scale.

1984; Meister & Kent, 1983; Kent et al., 1984; Kent, 1985, 1988; Kent & Clark-Lewis, 1985; Milton et al., 1990), a Boc/Bzl protection scheme was adopted with TFA/CH_2Cl_2 (6:4) N^{α} deprotection to ensure complete removal of the temporary protecting group at each step (Kent, 1988). Although the mild conditions associated with orthogonal chemistries, such as *Fmoc/O-tBu*, provided an attractive alternative to Boc/Bzl tactics, the occurrence of the sequence-related incomplete aminoacylations with DMF or CH_2Cl_2 as the coupling solvents would be likely to be compounded by incomplete removal of the N^{α}-protecting group as has previously been observed (Dryland & Sheppard, 1986; Fox, 1990; Fox et al., 1990, 1991). An Ile-OCH_2-Pam-resin link was used to ensure resistance to repetitive acidolysis (Mitchell et al., 1976; Kent & Clark-Lewis, 1985; Kent, 1988) and neutralizations, with DIEA (2%), and aminoacylations, with preformed oxybenzotriazole-activated esters (Stewart & Young, 1984), were accomplished in DMF, an aprotic polar solvent which inhibits the aggregation of protected peptide chains (Kent & Clark-Lewis, 1985; Kent, 1988). Asparagine carboxamides were protected with the xanthenyl group, except for Asn^{17} (where a terminating side reaction had previously occurred; S.C.F.M., unpublished experiments).

Complete assembly of each peptide was followed by *t*-Boc removal (Noble et at., 1976), and the formyl group of tryptophan, in the case of pHGnRH -23 to 69, was removed with piperidine in DMF (Plaue & Briand, 1988) prior to concomitant side-chain deprotection and cleavage from the resin with hydrogen fluoride in a S_N2/S_N1 procedure (Tam et al., 1983) which allowed the trituration and isolation of the intermediate partially deprotected peptidoresins (Plaue & Briand, 1988). Extraction of the cleaved peptide products from the resin was accomplished with aqueous NH_3 in the presence of β-mercaptoethanol, followed by lyophilization. Purification of the crude peptide products by preparative C18 RP-HPLC (Rivier et al., 1984; Hoeger et al., 1987) (in the presence of DTT for the Cys-containing peptides) utilized a 0.1 M ammonium acetate, pH 6.5/CH_3CN buffer system for pHGnRH 47–69, 38–69, and 14–69. The crude pHGnRH -23 to 69 lyophilysate, however, was insoluble in the usual RP-HPLC buffer systems, even with facilitation, and required a mobile phase consisting of aqueous 0.1% hexafluoroacetone trihydrate (HFA)/0.1% TFA and CH_3CN (90:10 in the B buffer). HFA was chosen to mimic the β-sheet-disrupting properties of the HFIP used with success for difficult couplings during synthesis (Milton & Milton, 1990; Narita et al., 1988). After their respective lyophilizations, 400.0 mg (0.12 mmol) of product, representing a 10.13% overall molar yield (based on the starting substitution of the resin), was obtained for the pHGnRH 47–69 sequence, and 299.5 mg of peptide product (0.04 mmol, representing a 3.46% overall molar yield) was obtained for the pHGnRH 38–69 sequence (Table III). The pHGnRH 14–69 sequence yielded 330.9 mg of product (0.05 mmol, representing a 2.78% overall molar yield), and the pHGnRH -23 to 69 sequence yielded 345.0 mg, representing a 1.58% overall molar yield (0.02 mmol) (Table III). The loss of yield during the synthesis of these peptides, in spite of the optimized acylation tactics, is attributable to attrition by side reactions at each step of chain assembly (Kent, 1985), while losses during purification are dependent on the level of purity of the final product. The excision of relatively small amounts of peptide side products which have physical and chemical properties similar to the target compound often results in disproportionally large losses of product (Sikakana et al., 1991).

RP-HPLC analysis of each peptide revealed 96.67% homogeneity (based on the ratio of the integrated area under the main peak vs the total integrated areas recorded at 210 nm on analytical C18 RP-HPLC) for pHGnRH 47–69, 97.90% homogeneity for pHGnRH 38–69, and 98.20% homogeneity for pHGnRH 14–69 and revealed 97.31% and 99.77% homogeneity on C4 and C18 silica columns, respectively, for pHGnRH -23 to 69 (Figure 4) (Table III). The amino acid compositions of the four peptides were consistent with their anticipated sequences (Table IV), and Edman degradation of three of the peptides confirmed the N-terminal sequence for 24 cycles (pHGnRH 38–69 and 14–69) and 19 cycles (pHGnRH -23 to 69) (Table V).

On C18 RP-HPLC the synthetic GAP (pHGnRH 14–69) coeluted with a bacterially derived GAP (Figure 2) and capillary zone electrophoresis (Jorgenson & DeArman Lukacs, 1983) revealed two peaks of approximately the same height in 0.1% TFA (Figure 3A). After reduction in the presence of 5% (v/v) β-mercaptoethanol for 1.5 h, however, they merged into one high-purity peak (Figure 3B). Ion-exchange chromatography (Clezardin et al., 1985) of this peptide also

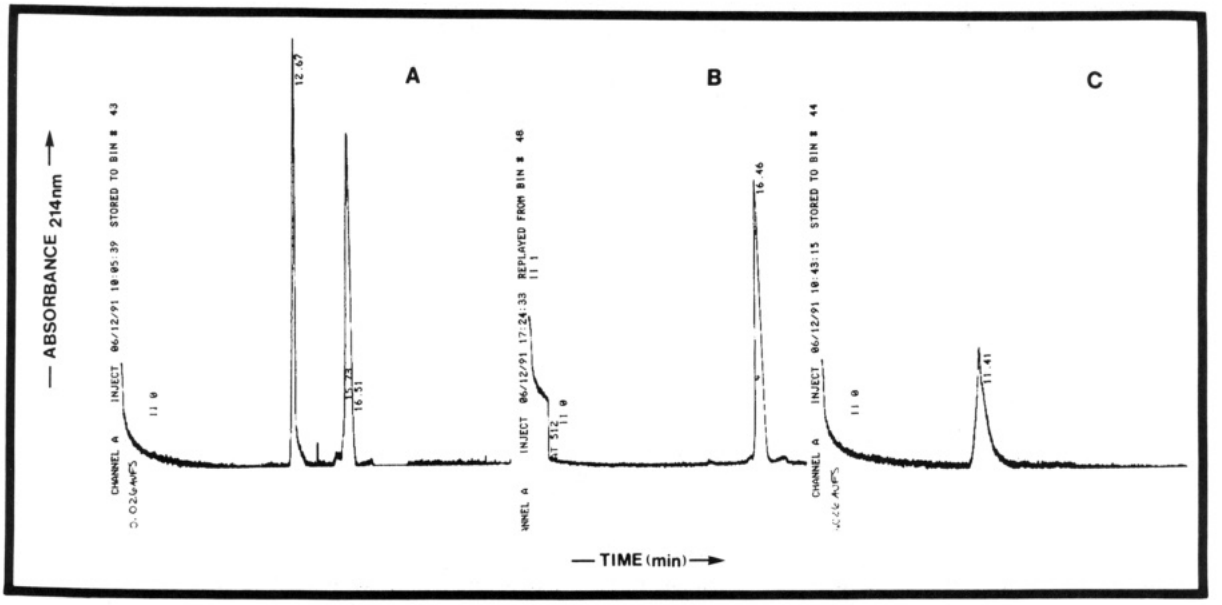

FIGURE 3: Capillary zone electrophoresis of pHGnRH 14–69 and pHGnRH -23 to 69. (A) pHGnRH 14–69 prior to reduction. (B) pHGnRH 14–69 in 0.01 M sodium phosphate/0.06 M sodium borate buffer, pH 8.0 (1 mg/mL) with 5.0% (v/v) β-mercaptoethanol for 1.5 h. (C) pHGnRH -23 to 69. Samples A and C were dissolved in 0.1% (v/v) trifluoroacetic acid. Samples were injected with pressure and electrophoresed in 0.1 M sodium phosphate buffer, pH 2.50.

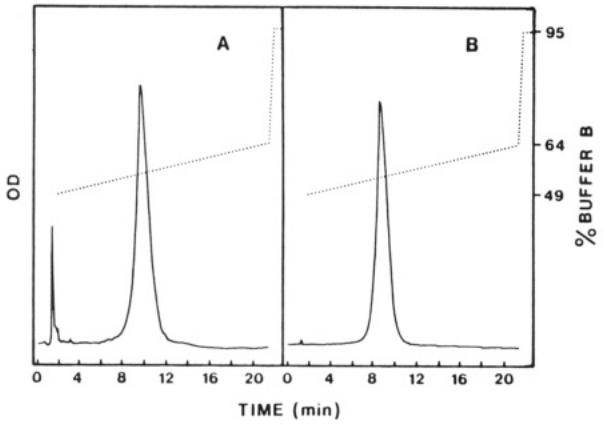

FIGURE 4: Analytical RP-HPLC characterization of synthetic pHGnRH -23 to 69. (A) Approximately 0.40 mg of pHGnRH -23 to 69 with ~0.20 mg of dithiothreitol, 1.0^{210nm} AUFS. (B) Approximately ~0.125 mg of pHGnRH -23 to 69 with ~0.1 mg of dithiothreitol, 0.04^{280nm} AUFS. Column: Vydac C18 (218TP54). Flow: 2 mL/min. Elution: 49–64% buffer, 20 min. Buffer A, 0.1% trifluoroacetic acid/0.1% hexafluoroacetone; buffer B, CH_3CN/H_2O (90:10) containing 0.1% trifluoroacetic acid and 0.1% hexafluoroacetone (v/v).

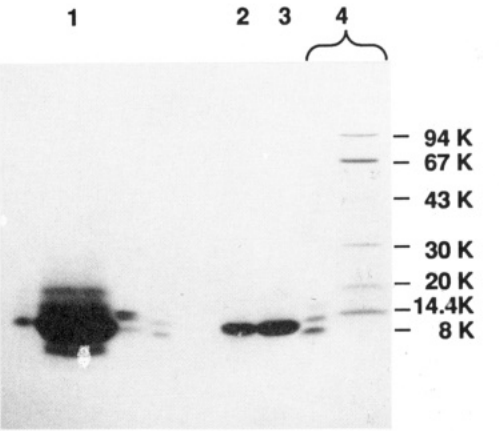

FIGURE 5: SDS–polyacrylamide gel electrophoresis of pHGnRH -23 to 69. Samples (125–250 μg) were dissolved and boiled for 6 min in the presence of dithiothreitol and electrophoresed on a 7–15% (w/v) slab gel for 18 h at 4 °C at a constant current of 6 mA. The gel was fixed and stained with Coomassie blue prior to photography. Lane 1: Crude post-HF pHGnRH -23 to 69 (250 μg). Lane 2: Purified pHGnRH -23 to 69 (125 μg). Lane 3: Purified pHGnRH -23 to 69 (250 μg). Lane 4: Molecular weight markers.

revealed one major peak (with a small contaminant peak which was shown to be due to dimer formation by analysis with capillary zone electrophoresis; results not shown). The differing extents of dimerization in the two samples derived from the same lyophilysate suggest that oxidation is occurring during sample preparation and that the purified peptide product is not substantially contaminated by the disulfide-bridged dimer.

Capillary zone electrophoresis of the pHGnRH -23 to 69 peptide revealed a single broadened peak which was not totally symmetrical (Figure 3C). It may be that there was not sufficient discrimination to separate closely related impurities in this case, or on the other hand, the peptide may be quite pure and the shape of the peak may be related to other technical factors. Also, no evidence of dimerization was seen with this sequence during capillary zone electrophoresis (compared to the synthetic GAP) and this may be due to a tendency to intrachain disulfide formation. A single band was obtained with pHGnRH -23 to 69 on SDS-polyacrylamide gel electrophoresis (Figure 5), and treatment of the peptide with hydrogen peroxide (Milton et al., 1988) afforded a product which, consistent with a [Met-$(O)^{-23}$] derivative, eluted earlier on C18 RP-HPLC than its unoxidized counterpart (results not shown). Ion spray mass spectrometry of all four of the peptides revealed the presence of the anticipated molecular ion in each case (Figure 6), and on the basis of these data a high degree of homogeneity was assumed for each of the synthetic peptide products of the cosynthesis.

The biological characterization of the synthetic GnRH precursor protein, GAP, and their fragment peptides followed that of the original recombinant GAP product, with LH and FSH release as well as the inhibition of prolactin release providing the pertinent criteria (Nikolics et al., 1985). In addition, the synthetic products were tested for their ability to displace a radiolabeled GnRH agonist analogue from rat pituitary cell membrane preparations (Millar et al., 1986).

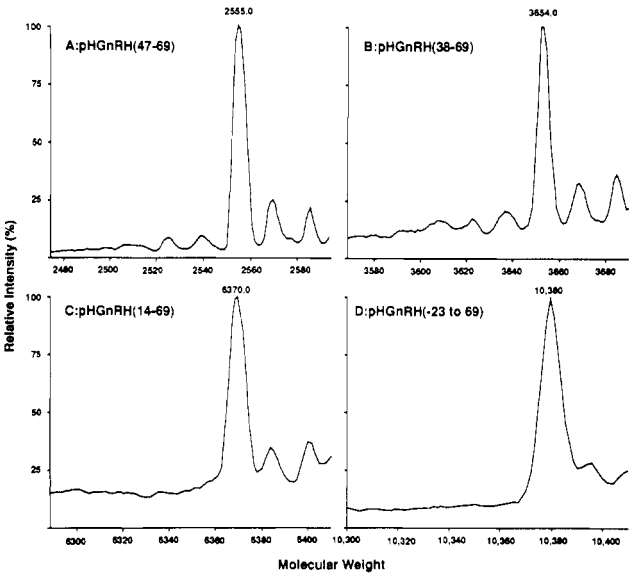

FIGURE 6: Ion spray mass spectrometric characterization of the synthetic pHGnRH peptides. In the deconvoluted mass spectra shown, the raw m/z data have been sorted to yield all molecular species within a range of 200 Da. This deconvolution procedure mathematically reduces the multiple charge states observed for a given molecular species to a single molecular mass. (Upper left) Deconvoluted spectrum of pHGnRH 47–69, observed molecular mass 2555.0 ± 3 Da. Calculated mass: (monoisotopic) 2554.41 Da; (average) 2555.94 Da. (Upper right) Deconvoluted spectrum of pHGnRH 38–69, observed molecular mass 3654.0 ± 3 Da. Calculated mass: (monoisotopic) 3653.90 Da; (average) 3656.16 Da. (Lower left) Deconvoluted spectrum of pHGnRH 14–69, observed molecular mass 6370.0 ± 3 Da. Calculated mass: (monoisotopic) 6367.25 Da; (average) 6371.13 Da. (Lower right) Deconvoluted spectrum of pHGnRH -23 to 69, observed molecular mass 10380 ± 3 Da. Calculated mass: (monoisotopic) 10374.37 Da; (average) 10380.97 Da.

pHGnRH 47–69 failed to display any significant pituitary membrane binding (GnRH receptor), gonadotropin-releasing activity, or inhibition of prolactin release with rat pituitary cells (Figures 7–10). Although pHGnRH 38–69 also failed to stimulate the release of gonadotropins from cultured rat pituitary cells (Figures 7 and 8), it was found to inhibit the release of prolactin from rat pituitary cells in culture (3 experiments, $p < 0.01$) to a marginal extent (Figure 9) as well as displacing labeled agonist from rat pituitary membrane preparations at concentrations of 1×10^{-5} and 1×10^{-6} M (Figure 10). pHGnRH 14–69 (GAP) caused a significant calcium-dependent (data not shown) and dose-dependent release of LH (3 experiments, $p < 0.001$) and FSH (3 experiments, $p < 0.05$) (Figures 7 and 8) and inhibited prolactin release (3 experiments, $p < 0.001$) (Figure 9) from cultured rat pituitary cells, although less than reported for the bacterially derived biosynthetic material (Nikolics et al., 1985; Wormald et al., 1985). pHGnRH 14–69 also displaced labeled agonist from membrane preparations (1×10^{-5} M). As this peptide was observed to oxidize readily to form disulfide-bridged dimers during HPLC and capillary zone electrophoresis (Figures 2 and 3), it was treated with the usual amount of DTT used in binding studies (1 mM DTT), and with 2 and 3 times that amount (2 and 3 mM DTT). However, no increase in membrane binding by the peptide was observed (Figure 10B). Similar titrations were also performed with DTT on its own (and with HFA as control for pHGnRH -23 to 69) in the absence of test peptides without observing any effect on basal values of LH, FSH, and prolactin release or on receptor binding (data not shown).

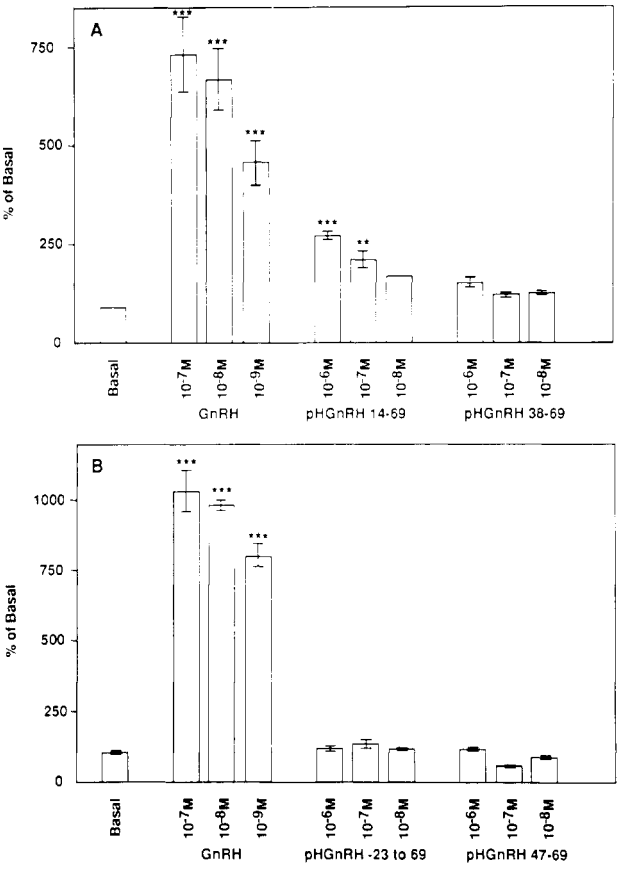

FIGURE 7: LH release from cultured rat pituitary cells after a 3-h incubation in the presence of the test peptides. (A) LH release increased in a dose-dependent fashion with GnRH and pHGnRH 14–69, while pHGnRH 38–69 was inactive at a concentration of 1×10^{-6} M. (B) LH release increased in a dose-dependent fashion with GnRH, while pHGnRH -23 to 69 and pHGnRH 47–69 were inactive at a concentration of 1×10^{-6} M. Values are means ± SEM. Astrisks indicate significant differences from basal values: (**) $p < 0.01$; (***) $p < 0.001$.

pHGnRH -23 to 69 was ineffective in releasing both LH and FSH from cultured rat pituitary cells at a concentration of 1×10^{-6} M (Figures 7 and 8) and failed to displace labeled agonist from membrane preparations (Figure 10). The polypeptide did, however, inhibit the release of prolactin (3 experiments, $p < 0.001$) (Figure 9) from cultured rat pituitary cells to an extent greater than even that observed for the synthetic GAP (pHGnRH 14–69) and calculated for the recombinant GAP of Nikolics et al. (1985). This inhibition of prolactin release can be seen to be approximately equivalent to the inhibition obtained with bromocryptine, a potent dopamine agonist (Fink, 1985), at the same concentrations (Figure 9B). The likelihood exists that pHGnRH -23 to 69 is processed in the cell culture system and that proGnRH (pHGnRH 1–69) or shorter fragments are responsible for the observed prolactin release inhibition, and it can be argued that the presence of inactive dimers (Abrahamson et al., 1987) was responsible for the reduced activity of pHGnRH 14–69 and pHGnRH 38–69. Completely reduced, these peptides would then inhibit prolactin release to the same extent as pHGnRH -23 to 69 (which does not seem to dimerize as readily, although it may form an intrachain disulfide bond). Both pHGnRH 14–69 and 38–69 were therefore reduced (treatment with DTT at room temperature and pH 8.0 for 3 h prior to desalting by RP-HPLC), but no increase in prolactin release inhibition was observed for these two peptides (data not shown). It is more likely that the hydrophobic leader sequence of the GnRH precursor, pHGnRH -23 to 69, enhances the receptor binding

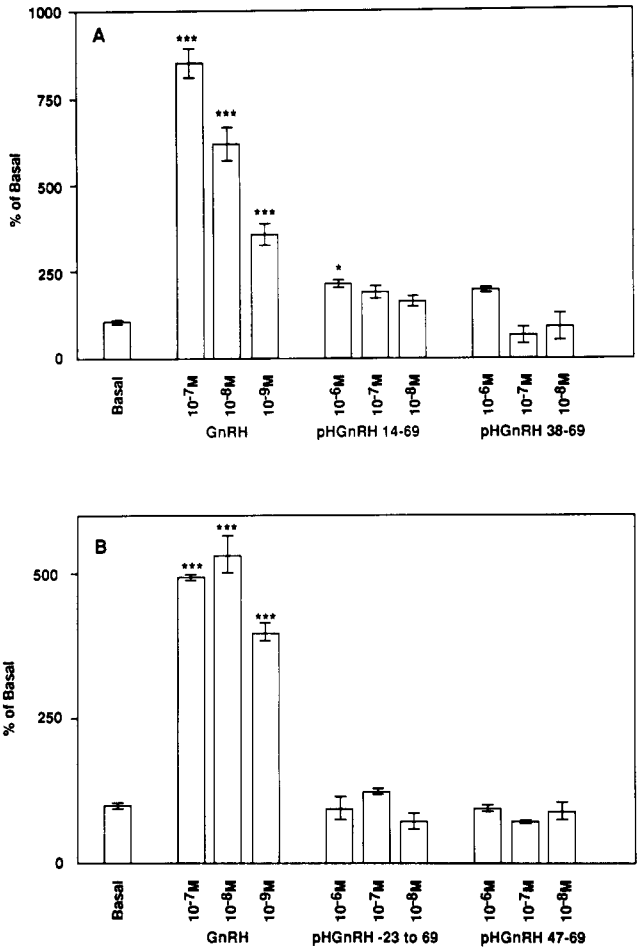

FIGURE 8: FSH release from cultured rat pituitary cells after a 3-h incubation in the presence of the test peptides. (A) FSH release increased in a dose-dependent fashion with GnRH and pHGnRH 14–69, while pHGnRH 38–69 was inactive at a concentration of 1×10^{-6} M. (B) FSH release increased in a dose-dependent fashion with GnRH, while pHGnRH -23 to 69 and pHGnRH 47–69 were inactive at a concentration of 1×10^{-6} M. Values are means ± SEM. Asterisks indicate significant differences from basal values: (*) $p < 0.05$; (***) $p < 0.001$.

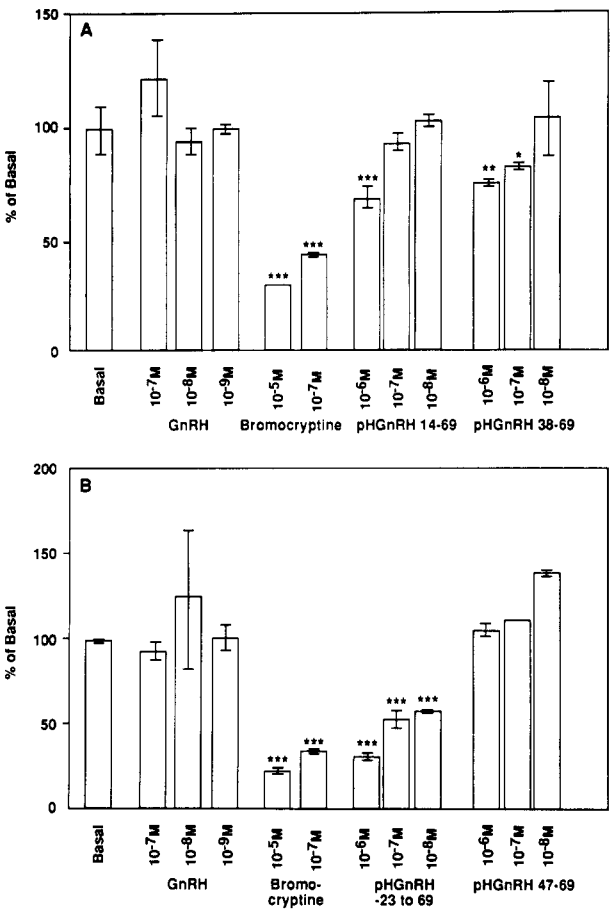

FIGURE 9: Prolactin release from cultured rat pituitary cells after a 3-h incubation in the presence of the test peptides. (A) Prolactin release was inhibited in a dose-dependent fashion by bromocryptine, pHGnRH 14–69, and pHGnRH 38–69, while GnRH failed to stimulate or inhibit the release of prolactin. (B) Prolactin release was inhibited in a dose-dependent fashion by bromocryptine and pHGnRH -23 to 69, while GnRH and pHGnRH 47–69 failed to stimulate or inhibit the release of prolactin. Values are means ± SEM. Asterisks indicate significant differences from basal values: (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$.

of this molecule or that it folds preferentially to allow higher affinity binding.

Mammalian reproduction requires that the appropriate hormones (LH, FSH, and prolactin) be released at the correct times and in an appropriate amount and fashion from the anterior pituitary (Leong et al., 1983; Fink, 1985), and this is generally accomplished via the hypophysial portal system by neuropeptides from the median eminence of the hypothalamus (Ziporyn, 1985; Hazum & Conn, 1988). Prolactin secretion, however, is predominantly under an inhibitory control which cannot be entirely accounted for by the action of dopamine (Demarest et al., 1984) and 4-aminobutyric acid (Schally et al., 1977), the only known physiological inhibitors. Seeburg and co-workers have suggested GAP as a major regulator of prolactin secretion (PIF, prolactin release inhibiting factor) and that the generation of gonadotropin-releasing and prolactin release inhibiting activities from the same precursor protein may account for the known inverse relationship of LH–FSH and prolactin levels during *certain* physiological states of reproduction and lactation (Nikolics et al., 1985). However, this model has been criticized as overly simplistic as it does not explain (and even contradicts) most of the other known physiological phenomena associated with these processes (Fink, 1985). Nevertheless, the strong prolactin release inhibiting activity of the synthetic preproGnRH and GAP, reported in this paper, is seen to emphasize a physiological role in reproduction for proGnRH and its smaller fragments after excision of the leader sequence.

The prolactin release inhibiting activities of the four peptides, here reported, are consistent with a proposed minimal active sequence for receptor binding. All three peptides (preproGnRH, GAP, and pHGnRH 38–69) as well as the proGnRH sequence share a common sequence around Cys^{40} and it has also been reported that the disulfide-bridged dimer of GAP is inactive in the inhibition of prolactin release (Abrahamson et al., 1987). The observation that preproGnRH does not displace GnRH agonist in the membrane binding assays is most easily explained by the existence of a receptor regulating prolactin release which is separate from the GnRH receptor. The gonadotropin-releasing activity of GAP involves the latter receptor while its prolactin release inhibition involves the former—i.e., GAP would contain two minimal active sequences, one in the N-terminal region for gonadotropin release (Milton et al., 1986) and the other associated with Cys^{40} for the inhibition of prolactin release.

In summary, on the basis of the analytical methods described (RP-HPLC, capillary zone electrophoresis, ion-exchange chromatography, SDS–polyacrylamide gel electrophoresis, amino acid analysis, sequence analysis, and mass spectrometry), we would conclude that the manual solid-phase synthesis strategy,

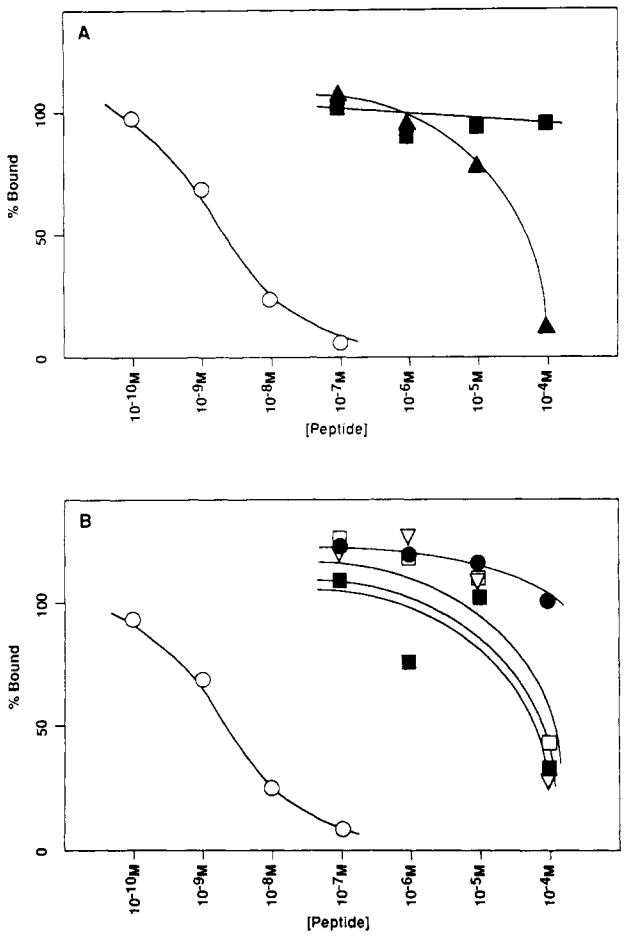

FIGURE 10: Effect of increasing amounts of synthetic peptide on the binding of ^{125}I-labeled [D-Ala^6, N$^{\alpha Me}$-Leu^7,Pro^9-NEt]GnRH agonist to crude rat pituitary cell membrane preparations is shown. (A) GnRH (○), pHGnRH 38–69 (▲), and pHGnRH 47–69 (■): GnRH and pHGnRH 38–69 displaced the labeled GnRH agonist, while pHGnRH 47–69 failed to compete for GnRH receptors. (B) GnRH (○), pHGnRH -23 to 69 (●), pHGnRH 14–69 + 1 mM dithiothreitol (□), pHGnRH 14–69 + 2 mM dithiothreitol (■), and pHGnRH 14–69 + 3 mM dithiothreitol (▽): GnRH and pHGnRH 14–69 displaced labeled GnRH agonist, while pHGnRH -23 to 69 failed to compete for GnRH receptors. The presence of increasing concentrations of dithiothreitol up to 3 mM did not affect pHGnRH 14–69 binding. Values are means ± SEM.

here employed, yielded reasonable quantities of homogeneous peptides. These peptides were evaluated in an in vitro biological system previously used to demonstrate the activity of bacterially derived recombinant GAP, and the synthetic product, equivalent to that sequence, was shown to exhibit the same activity. Although only the prolactin release inhibiting activity of preproGnRH is demonstrated here, the prohormone should exhibit the same biological activity associated with the precursor protein, and this should stimulate interest in proGnRH and other hormone precursors as well as offering a potential for the development of a new family of pharmacologically active agents for the inhibition of prolactin release.

ACKNOWLEDGMENTS

We thank the National Pituitary and Hormone Program (NIADDK) for providing radioimmunoassay material, C. Miller and J. E. Rivier for capillary zone electrophoresis and ion-exchange chromatography, R. Prescott, A. Jones, J. Morta, and J. Rodrigues for technical assistance, and R. P. Millar and S. B. H. Kent for providing laboratory facilities.

REFERENCES

Abrahamson, M. J., Wormald, P. J., Seeburg, P., & Millar, R. (1987) *Endocrinology 121*, 1913.
Adelman, J. P., Mason, A. J., Hayflick, J. S., & Seeburg, P. H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 179–183.
Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984) *J. Chromatogr. 336*, 93–104.
Brandt, W. F., Alk, H., Chauhan, M., & von Holt, C. (1984) *FEBS Lett. 174*, 228–232.
Chou, P. Y., & Fasman, G. D. (1978) in *Advances in Enzymology* (Meister, A., Ed.) Vol. 47, pp 45–148, John Wiley & Sons, New York.
Clezardin, P., McGregor, J. L., Manach, M., Boukerche, H., & Dechavanne, M. (1985) *J. Chromatogr. 319*, 67–77.
Covey, T. R., Huang, E. C., & Henion, J. D. (1991) *Anal. Chem. 63*, 1193–1200.
Demarest, K. T., Riegle, G. D., & Moore, K. E. (1984) *Endocrinology 115*, 2091–2097.
Dryland, A., & Sheppard, R. C. (1986) *J. Chem. Soc., Perkin Trans. 1*, 125–137.
Fink, G. (1985) *Nature 316*, 487–488.
Fox, J. (1990) *Biochem. Soc. Trans. 18*, 1308–1310.
Fox, J., Newton, R., Heegard, P., & Schafer-Nielsen, C. (1990) in *Innovation and Perspectives in Solid Phase Synthesis* (Epton, R., Ed.) pp 141–153, SPCC (UK) Ltd., Birmingham, England.
Fox, J. E., Newton, R., & Stroud, C. H. (1991) *Int. J. Pept. Protein Res. 38*, 62–65.
Gisin, B. F. (1972) *Anal. Chim. Acta 58*, 248–249.
Harris, R. B. (1989) *Arch. Biochem. Biophys. 275*, 315–333.
Hazum, E., & Conn, P. M. (1988) *Endocr. Rev. 9*, 379–386.
Hewick, R. M., Hunkapiller, M. W., Hood, L. E., & Dreyer, W. J. (1980) *J. Biol. Chem. 256*, 7990–7997.
Hoeger, C., Galyean, R., Boublik, J., McClintock, R., & Rivier, J. (1987) *BioChromatography 2*, 134–142.
Jorgenson, J. W., & DeArman Lukacs, K. (1983) *Science 222*, 266–272.
Kaiser, E., Colescott, R. L., Bossinger, C. D., & Cook, P. I. (1970) *Anal. Biochem. 34*, 595–598.
Kaiser, E., Bossinger, C. D., Colescott, R. L., & Olsen, D. B. (1980) *Anal. Chim. Acta 118*, 149–151.
Kent, S. B. H. (1985) in *Peptides: Structure and Function, Proceedings of the 9th American Peptide Symposium* (Deber, C. M., Hruby, V. J., & Kopple, K. D., Eds.) pp 407–414, Pierce Chemical Co., Rockford, IL.
Kent, S. B. H. (1988) *Annu. Rev. Biochem. 57*, 957–989.
Kent, S. B. H., & Clark-Lewis, I. (1985) in *Synthetic Peptides in Biology and Medicine* (Alitalo, K., Partanen, P., & Vaheri, A., Eds.) pp 29–57, Elsevier Science Publishers, B. V. Amsterdam.
Kent, S. B. H., Hood, L. E., Beilan, H., Meister, S., & Geiser, T. (1984) in *Peptides 1984, Proceedings of the 18th European Peptide Symposium* (Ragnarsson, U., Ed.) pp 185–187, Almqvist and Wiksell, Stockholm, Sweden.
Knecht, R., & Chang, J.-Y. (1986) *Anal. Chem. 58*, 2375–2379.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Leong, D. A., Frawley, L. S., & Neill, J. D. (1983) *Annu. Rev. Physiol. 45*, 109–127.
Live, D. H., & Kent, S. B. H. (1982) in *Elastomers and Rubber Elasticity* (Mark, J. E., & Lal, J., eds.) ACS Symposium Series 193 (Comstock, M. J., Series Ed.) pp 501–515, American Chemical Society, Washington, DC.
Live, D. H., & Kent, S. B. H. (1984) in *Peptides: Structure and Function, Proceedings of the 8th American Peptide Symposium* (Hruby, V. J., & Rich, D. H., Eds.) pp 65–68, Pierce Chemical Co., Rockford, IL.
Lottspeich, F. (1985) *J. Chromatogr. 326*, 321–327.
Meister, S. M., & Kent, S. B. H. (1983) in *Peptides: Structure and Function, Proceedings of the 8th American Peptide Symposium* (Hruby, V. J., & Rich, D. H., Eds.) pp 103–106, Pierce Chemical Co., Rockford, IL.

Merrifield, R. B. (1963) *J. Am. Chem. Soc. 85*, 2149–2154.
Millar, R. P., Wormald, P. J., & Milton, R. C. de L. (1986) *Science 232*, 68–70.
Milton, R. C. deL., Wormald, P. J., Brandt, W., & Millar, R. P. (1986) *J. Biol. Chem. 261*, 16990–16997.
Milton, R. C. deL., Mayer, E., Walsh, J. H., Rivier, J. E., Dykert, J., Lee, T. D., Shively, J. E., & Reeve, J. R. (1988) *Int. J. Pept. Protein Res. 32*, 141–152.
Milton, R. C. deL., Milton, S. C. F., & Adams, P. A. (1990) *J. Am. Chem. Soc. 112*, 6039–6046.
Milton, S. C. F., & Milton, R. C. deL. (1990) *Int. J. Pept. Protein Res. 36*, 193–196.
Milton, S. C. F., Millar, R. P., & Milton, R. C. deL. (1987) *Biochem. Biophys. Res. Commun. 143*, 872–879.
Mitchell, A. R., Erickson, B. W., Ryabtsev, M. N., Hodges, R. S., & Merrifield, R. B. (1976) *J. Am. Chem. Soc. 98*, 7357–7362.
Narita, M., Honda, S., Umeyama, H., & Obana, S. (1988) *Bull. Chem. Soc. Jpn. 61*, 281–284.
Nikolics, K., Mason, A. J., Szonyi, E., Ramachandran, J., & Seeburg, P. H. (1985) *Nature 316*, 511–517.
Noble, R., Yamashiro, D., & Li, C. H. (1976) *J. Am. Chem. Soc. 98*, 2324–2328.
Palen, T. D., Harris, R. B., Wypij, D. M., & Wilson, I. B. (1987) *Peptides 8*, 21–24.
Plaue, S., & Briand, J. P. (1988) Chapter 2: Solid-Phase Peptide Synthesis, in *Synthetic Polypeptides as Antigens* (van Regenmortel, M. H. V., Briand, J. P., Muller, S., & Plaue, S., Eds.) Laboratory Techniques in Biochemistry and Molecular Biology *19* (Burdon, R. H., & Knippenberg, P. H., Series Eds.) Elsevier, Amsterdam.
Rangarajan, N. S., Xu, J.-F., & Harris, R. B. (1991) *Arch. Biochem. Biophys. 290*, 418–426.
Rivier, J., McClintock, R., Galyean, R., & Anderson, H. (1984) *J. Chromatogr. 288*, 303–328.
Sarin, V. K., Kent, S. B. H., Tam, J. P., & Merrifield, R. B. (1981) *Anal. Biochem. 117*, 147–157.
Schally, A. V., Redding, T. W., Arimura, A., Dupont, A., & Linthium, G. L. (1977) *Endocrinology 100*, 681–691.
Schally, A. V., Olsen, D. B., Gulyas, J., Szoke, B., Horvath, J., Karashima, T., Redding, T. W., Nikolics, K., & Seeburg, P. H. (1986) 68th Annual Meeting of the American Endocrine Society (Siitori, P., Ed.) Anaheim, CA, Abstract.
Seeburg, P. H., & Adelman, J. P. (1984) *Nature 311*, 666–668.
Seeburg, P. H., Mason, A. J., Stewart, T. A., & Nikolics, K. (1987) *Rec. Prog. Hormone Res. 43*, 69–98.
Sikakana, C. N. T., Wessels, P. L., Fourie, L., Flanagan, C. A., & Milton, R. C. deL. (1991) *S. Afr. J. Sci. 87*, 364–?75.
Tam, J. P., Kent, S. B. H., Wong, T. W., & Merrifield, R. B. (1979) *Synthesis 12*, 955–957.
Tam, J. P., Heath, W. F., & Merrifield, R. B. (1983) *J. Am. Chem. Soc. 105*, 6442–6455.
Vale, W., Grant, G., Amoss, M., Blackwell, R., & Guillemin, R. (1972) *Endocrinology 91*, 562–572.
Wormald, P., Milton, R. C. deL., Abrahamson, M., Duflou, J., Roberts, J., Brandt, W., & Millar, R. P. (1985) *S. Afr. J. Sci. 81*, 579.
Yu, W. H., Millar, R. P., Milton, S. C. F., Milton, R. C. deL., & McCann, S. M. (1990) *Brain Res. Bull. 25*, 867–873.
Ziporyn, T. (1985) *J. Am. Med. Assoc. 253*, 469–476.