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Primary Structure of Gonadotropin-releasing Hormone from Lamprey Brain*

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The primary structure of gonadotropin-releasing hormone (GnRH) isolated from whole brains of lamprey is pGlu-His-Tyr-Ser-Leu-Glu-Trp-Lys-Pro-Gly-NH_2. This unique decapeptide was isolated and purified from brain extracts by reverse-phase high performance liquid chromatography. The structure of the peptide was established from chymotryptic fragments that were identified by protein sequence analysis and fast atom bombardment mass spectrometry. The peptide reacts with an antiserum raised against mammalian GnRH and is structurally identified as a member of the GnRH family by the amino and carboxyl termini of $pGlu^1$-His^2 and Pro^9-$Gly^{10}NH_2$, the conservation of Ser^4 in the internal segment of the molecule and its length of 10 amino acids. For the first time, amino acid substitutions are found in positions 3 and 6, critical for biological potency and conformation, respectively. Additionally, a second form of GnRH (lamprey II GnRH), representing about 10% of the total GnRH immunoreactive material in the brain, was isolated; its amino acid composition differs by 3 residues from lamprey I GnRH. Synthetic lamprey I GnRH elevates plasma estradiol in adult female lampreys.

The pituitary gonadotropins are released in most vertebrates by a factor, gonadotropin-releasing hormone (GnRH[1]), synthesized in the brain. This factor, isolated in mammals from hypothalami of pigs (Matsuo *et al.*, 1971) and sheep (Burgus *et al.*, 1972) and from placentas of humans (Tan and Rousseau, 1982), is a decapeptide (Fig. 12). Mammalian GnRH injected into submammalian species induces various reproductive events including gonadotropin release (Crim *et al.*, 1978; Peter, 1983; Sherwood, 1986a). However, distinct forms of GnRH are clearly present in submammalian vertebrates as shown by recent immunological, chromatographic (King and Millar, 1980; Sherwood, 1986b; Sherwood *et al.*, 1986), and structural studies. Two forms of GnRH exist in chicken brains (King and Millar 1982a 1982b; Miyamoto *et al.*, 1982, 1983, 1984) and a distinct form in salmon brain (Sherwood *et al.*, 1983). The major form of GnRH in amphibian brain has the same amino acid composition as mammalian GnRH (Rivier *et al.*, 1981a), but two minor GnRH forms also exist, which are chromatographically and immunologically similar to salmon GnRH (Sherwood *et al.*, 1986).

It is of considerable interest to know if members of the most ancient class of vertebrates, Agnatha, also contain GnRH material. The only living members of this class are the hagfish and lamprey. Injections of a synthetic mammalian GnRH analogue advance ovulation and induce steroidogenesis in lampreys (Sower *et al.*, 1983, 1985). And yet these agnathans lack the hypothalmo-hypophysial pathways present in other vertebrates for GnRH. They have neither a portal blood system as in tetrapods and certain primitive fish nor GnRH nerve fibers terminating in the pituitary as in most teleosts (Ball, 1981). The GnRH-staining fibers appear to terminate on a layer of connective tissue above the pars distalis or in the neurohypophysis (Crim *et al.*, 1979a, 1979b; Nozaki and Kobayashi, 1979; Nozaki *et al.*, 1984). Lamprey GnRH apparently diffuses toward the pituitary gonadotropes or reaches them by an unknown path (Gorbman, 1980, Nozaki *et al.*, 1984). Earlier we confirmed that lamprey, but not hagfish, brains contain a GnRH-like molecule that can be detected by some, but not all, antisera raised against mammalian GnRH. This immunoreactive material eluted on HPLC in the same position as mammalian GnRH (Sherwood and Sower, 1985). However, it was clear that structural analysis was needed to determine if the GnRH-like peptide in this primitive vertebrate was similar to the amphibian-mammalian GnRH molecule or, if not, could offer clues about the nature of an ancestral GnRH molecule. Our current structural studies show that lamprey brain contains two distinct forms of GnRH (Table 2). The structure of the major form shows the molecule is a unique peptide (Fig. 5) with only 50% homology to mammalian GnRH. However, it clearly contains a framework which is common to all vertebrate GnRH family members.

MATERIALS AND METHODS AND RESULTS[2]

DISCUSSION

The decapeptide reported here is the first peptide from the lamprey brain to be structurally analyzed. The structure of

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[1] The abbreviations used are: GnRH, gonadotropin-releasing hormone; HPLC, high performance liquid chromatography.

[2] Portions of this paper ("Materials and Methods," "Results," Figs. 1–4 and 6–11, Footnote 3, and Tables 1 and 3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-3082, cite the authors, and include a check or money order for $10.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

this lamprey peptide has a unique internal amino acid sequence; only the amino and carboxyl termini of pGlu[1]-His[2] and Pro[9]-Gly[10]NH_2 identify this molecule as a possible member of the GnRH family (Fig. 12). The molecule has 50% homology with mammalian and chicken I GnRH and 60% homology with salmon and chicken II GnRH. The amino acid sequence of lamprey GnRH shows that the length, termini, and probably amino acids 1–3, thought to be required for biological effect, are the important parts of the GnRH framework. The length of the molecule has remained stable for at least 500 million years, the approximate time in evolution when the ancestors to jawed vertebrates separated from the jawless agnathans. Considering the essential role GnRH plays in reproduction, it is surprising that the molecule has not been more highly conserved.

The blocked amino terminus of the molecule prevented microsequencing of the intact peptide. The amino terminus was determined from the tripeptide pGlu[1]-His[2]-Tyr[3] after chymotryptic digestion (Fig. 5). The presence of pGlu[1] was then deduced by finding Glu in the amino acid analysis; the mass spectra of the tripeptide and intact peptide were consistent with the proposed structure.

The amino acid substitutions which have occurred between lamprey GnRH and other vertebrate GnRH peptides may offer clues about function. Studies of GnRH analogs have shown that changes in single residues may lead to considerable potency and conformational changes. Folding is thought to occur at a 6–7 β turn in mammalian GnRH (Kopple, 1981; Struthers *et al.*, 1985) and presumably also in salmon and chicken I and II GnRH. In mammalian GnRH, certain substitutions in the 6-position produce enhanced biological potency (Rivier *et al.*, 1981b). These substitutions may stabilize a preferred backbone conformation. It remains to be determine if the negatively charged Glu[6] in lamprey GnRH would alter the biological potency and folding of mammalian GnRH. This could be tested with synthetic Gly[6]-lamprey GnRH in lamprey and with Glu[6]-mammalian GnRH in mammals.

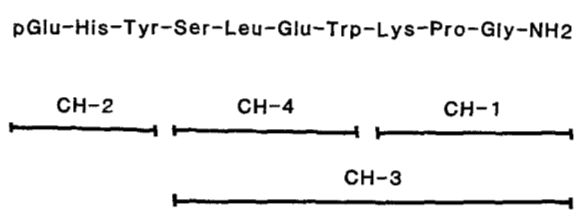

FIG. 5. **Proposed sequence of lamprey GnRH.** The fragments isolated from chymotryptic digest are labeled as CH-1, CH-2, CH-3, and CH-4 based on the order in which they eluted from HPLC as shown in Fig. 4. The identity of each fragment is based on amino acid composition (Table 2), mass spectrometry (Figs. 7–10), and amino acid sequencing (Table 3).

The conservation of Ser[4] in all 5 GnRH peptides implies an important role because Ser has the largest mutation probability of the amino acid residues (Schulz and Schirmer, 1979). Hydrophilic residues such as Ser are usually on the protein surface and changed more frequently. The stability of Ser in the evolution of GnRH may be due to the importance of the hydrogen bonds which Ser[4] is believed to make with Pro[9] for maintaining the folded molecule (Struthers *et al.*, 1985).

The first report of a substitution in the functional region of the GnRH molecule is Tyr[3] in lamprey GnRH; the other four GnRH peptides have Trp[3]. The functional region, which is considered to be residues 2–3 or 1–3, is necessary for release of luteinizing hormone and follicle-stimulating hormone from the pituitary in mammals (Schally and Coy, 1977; Rivier *et al.*, 1981b). The Tyr[3] to Trp[3] change may reflect the high mutation probability (Schulz and Schirmer, 1979). A dramatic increase in potency is produced by the Tyr to Trp interchange in mammalian (m) GnRH; Tyr[3]-m GnRH has only 0.1–0.4% potency compared to native Trp[3]-m GnRH in a mammalian assay (Schally and Coy, 1977). This implies that lamprey and mammalian GnRH receptors have different requirements because native Tyr[3]-lamprey GnRH binds and has intrinsic activity in its own species as shown by the release of steroids after treatment with synthetic lamprey GnRH. Whether the evolutionary change to Trp[3] was due to potency enhancement or receptor requirements might be partially answered by comparing Trp[3]- and Tyr[3]-lamprey GnRH in lampreys.

The amino acid substitutions in position 7 give some indication of the order in which the GnRH peptides may have evolved. Trp[7] appears in lamprey, salmon, and chicken II GnRH; Leu[7] is in chicken I and mammalian GnRH. It seems likely that lamprey, salmon, and chicken II evolved first or from an ancestral molecule with Trp[7]. The substitution to Leu[7] must have been coincident with or prior to the appearance of chicken I and mammalian GnRH. This substitution

TABLE 2

Amino acid compositions of lamprey GnRH and its chymotryptic peptides

The values given are molar ratios. The numbers in parentheses are the nearest integral ratio. Leucine was used as the basis of the ratios for lamprey I GnRH, lamprey II GnRH, and lamprey I fragments CH-3 and CH-4; lysine was used for CH-1 and tyrosine for CH-2.

	Lamprey I GnRH	Peptides				Lamprey II GnRH
		CH-1	CH-2	CH-3	CH-4	
Glutamic acid	1.7 (2)		1.1 (1)	0.6 (1)	1.0 (1)	0.4 (1)
Serine	0.7 (1)			0.3 (1)	0.7 (1)	0.3 (1)
Glycine	0.7 (1)	1.0 (1)		0.9 (1)		1.0 (1)
Histidine	1.2 (1)		1.0 (1)			1.5 (2)
Proline	1.1 (1)	1.0 (1)		1.0 (1)		1.3 (1)
Tyrosine	1.0 (1)		1.0 (1)			
Leucine	1.0 (1)			1.0 (1)	1.0 (1)	1.1 (1)
Lysine	1.2 (1)	1.0 (1)		0.3 (1)		
Tryptophan	1[a] (1)	ND[b]	ND	ND (1)	ND (1)	
Isoleucine						0.6 (1)
Phenylalanine						1.0 (1)
Yield						
nmol	25.65	9.86	18.27	10.73	7.15	
%		44	82	48	32	

[a] Tryptophan was estimated from absorbance measurements at 280 nm.
[b] Not determined.

FIG. 12. **Comparison of primary structures of the five known vertebrate GnRH molecules.** The *boxes* show the residues which lamprey shares with all other family members. Lamprey GnRH shows 50% homology with mammal and chicken I GnRH; it shows 60% homology with salmon and chicken II GnRH because of the shared Trp residue in position 7.

conserves the bulky nonpolar side chain.

In position 5 it is difficult to tell if either His^5 (chicken II) or Tyr^5 (salmon, chicken I, and mammal) evolved first from Leu^5 in lamprey or if each evolved separately from Leu^5. Since multiple forms of GnRH exist in many submammalian species, both the Tyr^5 and His^5 form can exist simultaneously as in chicken. More primary structures are needed to determine the speciation of the 5-position in GnRH.

The most variable position is 8; each of the GnRH peptides has a different amino acid. The difference in neutral or basic amino acids in position 8 is the basis of a functional differentiation in the oxytocin (neutral)/vasopressin (basic; Arg/Lys) family. There is little evidence for such a division in GnRH. It is known that the salmon (Leu^8) GnRH-like molecule appears to act as a neurotransmitter in frog sympathetic ganglia (Eiden and Eskay, 1980; Jan *et al.*, 1983; Jones *et al.*, 1984) or in fish retina (Stell *et al.*, 1984). However, mammalian (Arg^8) GnRH may act as a local hormone in placenta and both salmon and mammalian GnRH have gonadotropin-releasing properties (Peter *et al.*, 1985). Rather, the modifications in position 8 may reflect changes in receptor molecules and binding sites in the respective species. For example, lamprey has Lys^8, but the substitution of Lys into mammalian GnRH produces a molecule with only 7.6% luteinizing hormone releasing hormone activity in a mammalian bioassay (Schally and Coy, 1977).

The amphiphilic secondary structure of lamprey, chicken I, and mammalian GnRH illustrates another common feature of the family. The β-pleated sheet conformation of mammalian GnRH shows that the amino acids have alternating hydrophobic and hydrophilic side chains (Kaiser and Kézdy, 1984; Struthers *et al.*, 1985) as does chicken II GnRH. Likewise, lamprey GnRH has hydrophobic residues $pGlu^1$-Tyr^3-Leu^5-Trp^7-Pro^9 alternating with His^2-Ser^4-Glu^6-Lys^8-$Gly^{10}NH_2$. The other 2 GnRH molecules do not fit the alternating pattern as well. In solution the folded molecule is thought to have a hydrophobic core with H bonding between Trp^3 and Gly^{10}, His^2 and Gly^{10}, and Ser^4 and Pro^9; this bonds amino and carboxyl termini together. However, when GnRH is exposed to a water/lipid inteface such as a membrane, it may become extended with the hydrophobic side chains on one side and the hydrophilic on the other.

The second form of lamprey (lamprey II) GnRH was more hydrophobic on a C_{18} HPLC column compared with the first form (lamprey I). The amino acid composition is consistent with this observation; lamprey II GnRH has Ile, Phe, and His whereas lamprey I GnRH has Glu, Lys, and Tyr, a more polar combination (Table 2). It is likely the second form is also a decapeptide with the same terminal residues as the other family members because the molecule is detected by antiserum R-42. This antiserum recognizes GnRH molecules by their conformation, which depends on their termini, but does not detect fragments or extended molecules. This second form of GnRH within the same species may be the result of gene duplication; one copy of the gene may serve the original function whereas the redundant copy may change structurally and/or functionally without harming the organism. The role of lamprey II is unknown and may remain so until the peptide is sequenced and the synthetic form tested for physiological activity.

In terms of evolution, lamprey GnRH is far removed from the other 4 peptides; the minimum nucleotide base changes required to make the substitutions are 6 to chicken II, 6 to mammal, 7 to chicken I, and 7 to salmon GnRH. This assumes a direct change from lamprey GnRH to each form; the number of base changes may be larger if intermediate forms of GnRH occurred. Only 1–4 minimum base changes are required to explain the interchanges among the other 4 GnRH peptides.

The origin of GnRH, however, is not necessarily in lamprey. Hagfish, the only other living agnathan, did not have GnRH which cross-reacted with any of several antisera in three studies (Crim *et al.*, 1979a; Nozaki and Kobayashi, 1979; Sherwood and Sower, 1985), but had small amounts in two other studies (Jackson, 1980; King and Millar, 1980). GnRH has been detected in a prochordate, the tunicates, although only by immunological means (Georges and Dubois, 1980). Invertebrates have not been reported to contain GnRH, but a considerable similarity in amino acid sequence between mammalian GnRH and yeast α_1 mating factor has been noted (Hunt and Dayhoff, 1979). Mating factor not only binds specifically to rat pituitary cells, but also releases luteinizing hormone (Loumaye *et al.*, 1982). Yeast α_1 mating factor has 6 out of 13 amino acids (Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr) which may be homologous to lamprey GnRH. Residues 5, 6, and 8 in lamprey GnRH exhibit greater homology to the yeast mating factor than do the corresponding residues in mammalian GnRH.

Our elucidation of the structure of a GnRH peptide from a primitive vertebrate may provide a basis for studying related, but phylogenetically older, molecules.

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PRIMARY STRUCTURE OF GONADOTROPIN-RELEASING HORMONES FROM LAMPREY BRAIN

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MATERIALS AND METHODS

Tissue. Whole brains from 13,000 mature male and female sea lampreys (Petromyzon marinus) were collected in June, 1984 at Hammond Bay Biological Station (Millersburg, Michigan). The fish were captured as they migrated from Lake Huron to rivers for spawning. Brains were removed and frozen.

Tissue Extraction. The extraction method was adapted from Chang and Leeman (1970). Frozen brains (928 g) were divided into 4 batches; each was processed separately until the third HPLC procedure. The brains were powdered with liquid nitrogen in a stainless steel Waring blender. Powdered tissue was added to acetone/1 M HCl(100:3, v/v), 1 g frozen brain to 5 ml. The extraction mixture was stirred for 3 h at -5° to -20°C, then filtered (Whatman No. 4). Insoluble material was reextracted in acetone/0.01 M HCl (80:20, v/v) in 40% of the volume of the original extract, stirred 5 min and refiltered. Combined filtrates were extracted 5 times with petroleum ether (b.p. 30-60 °C) at a ratio of 4:1 (filtrate:petroleum ether, v/v) to remove lipids. The final aqueous phase was reduced under vacuum to less than 1 liter, then decanted and centrifuged at 12,000 x g for 30 min. The supernatant was diluted with water to make a 1 liter volume and an aliquot was removed for radioimmunoassay (RIA)

Concentration. Each batch of extract (333 ml) was passed through 4 tandemly connected Sep-Pak C-18 cartridges (Waters Associates); the flow rate of 1 ml/min was controlled by a peristaltic pump. Bound material was eluted from the Sep Paks with acetonitrile (CH_3CN) in 0.25 M triethylamine adjusted with phosphoric acid to pH 6.5 (TEAP) as shown in Table 1. Aliquots of fractions were taken for RIA. Fractions with GnRH immunoreactivity in each batch were evaporated under vacuum to less than 2 ml, filtered (0.45 µm) and applied to HPLC-1.

High Performance Liquid Chromatography (HPLC). A Varian 5000 liquid chromatograph was set for a flow rate of 1 ml/min and 1 ml fractions were collected in all experiments listed below. Details of the 7 consecutive HPLC runs are given in Table 1. Aliquots of fractions from each HPLC run were taken for RIA. Immunoreactive fractions were combined as either early-(peak 1) or late-eluting (peak 2) fractions and reapplied for HPLC-2. Peaks 1 and 2 were run separately in all subsequent HPLC runs. The fractions from peak 1 (HPLC-7) containing the sole chromatographic peak were taken for amino acid analysis, mass spectrometry and chymotryptic digestion; the fractions from peak 2 were taken for amino acid analysis only.

Radioimmunoassay (RIA). GnRH was measured as described (Sherwood et al., 1983) except ^{125}I-GnRH was prepared using a modification of the chloramine-T (20 µg for 30 sec) method (Greenwood et al., 1963), purified on a Sephadex G-25 column (0.9 x 45 cm) and eluted with phosphate-buffered saline (1.25 g Na_2HPO_4, 0.15 g KH_2PO_4, 8.5 g NaCl, 0.1 g ethylmercurithiosalicylate sodium salt/liter) with gelatin (10 g/l), pH 7.6. Synthetic mammalian GnRH (LHRH, Peninsula Labs) was used as standard and as ^{125}I-tracer. Antiserum R-42 at a final dilution of 1:500,000 was used for assay. This resulted in 15-30% binding of the total ^{125}I-labelled GnRH (B_0) added to each reaction tube. A level of 20% inhibition of this binding was obtained at 1.2-3.3 pg of mammalian GnRH.

Amino Acid Analysis. Amino acid analysis was performed on samples hydrolyzed in vacuo for 22 h at 105°C in 6N HCl containing 1% (v/v) phenol. The samples were dried, derivatized with phenylisothiocyanate, and analyzed by HPLC as described (Bidlingmeyer et al., 1984) using a Waters liquid chromatograph.

Protein Sequence Analysis. Automated repetitive Edman degradations were performed on samples of peptides using an Applied Biosystems 470A protein sequencer. All procedures were performed as described (Marshak et al., 1984), including the identification of phenylthiohydantoin amino acid derivatives, using a Hewlett Packard 1090 liquid chromatograph and 3392 integrator.

Chymotryptic Digestion. Lamprey GnRH (22.35 nmol) was digested with α-chymotrypsin (Worthington) in 0.1 M ammonium bicarbonate, pH 7.8, using a final ratio of enzyme to peptide of 1:21 (w/w). The reaction was incubated for 1 h at 37°C, and then stopped by the addition of 0.01 M HCl to pH 2. The resulting peptide mixture was fractionated by HPLC on a column of octadecylsilanyl silica (Beckman-Altex; 0.46 x 25 cm; 5 µm particles) using solvent A (trifluoroacetic acid/ water, 1:1480, v/v) and solvent B (trifluoroacetic acid/water/CH_3CN, 1:148:1332, v/v/v). The programmed gradient elution is shown in Fig. 4, and was performed on a Gilson liquid chromatograph. The absorbance of the effluent was monitored at 210 nm, and fractions corresponding to peaks of absorbance were collected. Aliquots of the 4 fractions were analyzed for amino acids. After evaporation to dryness, each fraction was dissolved in 10 µL of trifluoroacetic acid/H_2O/acetonitrile, (1:740:740, v/v/v); 1 µL was removed and examined by fast atom bombardment mass spectrometry. Another aliquot was removed for protein sequence analysis.

Fast Atom Bombardment Mass Spectrometry. Underivatized lamprey GnRH (26 nmol) was dissolved in 10 µL of trifluoroacetic acid/H_2O/acetonitrile, (1:740:740, v/v/v). One µL (2.6 nmol) and 0.25 µL (650 pmol) were each separately delivered to 1 µL of liquid matrix (5:1, w/w) mixture of dithiothreitol and dithioerythritol (Finke et al., 1984)) on a gold probe for analysis from m/z 65 to m/z 2696. Thirty fast atom bombardment mass spectra were scanned from each peptide/matrix sample introduced directly into the mass

spectrometer ion source; each sample was bombarded with a beam of xenon fast atoms. Mass spectra were recorded with a Kratos MS-50 mass spectrometer fitted with a high field magnet and equipped with a Kratos DS-55 data system (Kratos Scientific Instruments, Manchester, England), previously calibrated against cesium iodide/glycerol and sodium fluoride over the mass range (Buko et al., 1983a, b).

Peptide Synthesis. Synthetic lamprey GnRH was synthesized by solid phase methodology (Barany & Merrifield, 1979) on p-methyl benzhydrylamine resin (0.61 mmol/g) using automated, repetitive coupling cycles on an Applied Biosystems 430A instrument. The amino acids were coupled as N-α-t-Boc symmetric anhydrides in various proportions of dimethylformamide and/or dichloromethane. 5-Pyrrolidone-2-carboxylic acid (pyroglutamic acid; pGlu) was coupled as the activated ester with 1-hydroxybenzotriazole. Samples of the resin were removed automatically at every cycle and analyzed for free amine content using a quantitative ninhydrin monitoring procedure (Sarin et al., 1981). Based on these assays, all coupling efficiencies were greater than 99.0%.

The peptide was cleaved from the resin and the protecting groups were removed by treatment of the resin with 1 M trifluoromethanesulfonic acid, 1 M thioanisole in anyhydrous trifluoroacetic acid (30-fold molar excess over total protecting groups) containing 10% (v/v) m-cresol for 3 h at 0°C essentially as previously described (Yajima & Fujii, 1983). The peptide was precipitated from the reaction mixture with 10 volumes of anhydrous diethyl ether, and the peptide was extracted in 1% (v/v) aqueous acetic acid containing 5 mM dithiothreitol. The peptide was purified by HPLC on a column (1 x 25 cm) of octadecylsilanyl silica (Supelco LC-18) using trifluoroacetic acid and acetonitrile in the mobile phase (as listed for HPLC-7). The structure of the peptide product was confirmed by amino acid analysis and mass spectrometry.

RESULTS

Peptide Cross-reactivity. Lamprey brain contains 8.9 ng immunoreactive (ir) GnRH/gram of brain or 0.64 ng/brain. However, the actual amount is probably higher since antiserum R-42, raised against mammalian GnRH, detects only a fraction of the lamprey GnRH. Amino acid analysis showed the final purified peptide (peak 1 from HPLC-7) contained 43.7 μg, whereas R-42 detected 4.22 μg or 1/10th of the native lamprey material. Figure 1 shows that R-42 also poorly detected synthetic lamprey GnRH.

Chromatographic Behavior. Lamprey GnRH extract eluted as 2 peaks from Sep Paks, but the 2 peaks were more clearly separated by reverse phase HPLC-1 (Fig. 2). The second peak was 7-13% of the total GnRH material in the first 3 HPLC runs, but decreased to 1% on the 4th HPLC run. In the various purification steps, the best separation of the two GnRH peaks was achieved by the conditions used for HPLC-1 and HPLC-3. In the different HPLC systems, peak 2 was more hydrophobic and eluted later compared with peak 1 except in HPLC-5. Peak 1 eluted at fractions 9-12 (HPLC-1), 27-30 (HPLC-2), 9-12 (HPLC-3), 27-28 (HPLC-4), 31 (HPLC-5), 19 (HPLC-6) and 22 (HPLC-7); peak 2 eluted at fractions 28-31 (HPLC-1), 32-33 (HPLC-2), 28-30 (HPLC-3), 31 (HPLC-5), and 26-27 (HPLC-7). Figure 3 shows peaks 1 and 2 as single chromatographic fractions achieved after 6 or 4 HPLC runs, respectively. Peak 1 is identified as lamprey I GnRH and peak 2 as lamprey II GnRH.

The total amount of ir-GnRH in the acetone/HCl extract was 8.27 μg; the total amount after the final HPLC runs was 4.24 μg by RIA for peaks 1 and 2. Thus the overall yield during the purification was approximately 50%.

The chymotryptic digest of peak 1 yielded 4 peptides, separated by HPLC. This chromatographic pattern is compared to the chymotryptic digest of synthetic mammalian GnRH (Fig. 4). None of the fragments in native lamprey I GnRH and synthetic mammalian GnRH eluted in the same position.

Amino Acid Composition. The purified lamprey I GnRH was analyzed initially by mass spectrometry (see below) and amino acid analysis. The amino acid composition of the peptide *is shown in Table 2*. The composition was similar to that of other GnRH molecules (Schally et al., 1971; Miyamoto et al., 1982; Sherwood et al., 1983), but had two unique features. First, lamprey I IGnRH contained only one residue of glycine, unlike the two residues of glycine found in mammalian (Schally et al., 1971), avian (Miyamoto et al., 1982), and teleost (Sherwood et al., 1983) GnRH molecules. Second, the lamprey I GnRH contained one residue of lysine, unlike any other known GnRH molecule. In addition, lamprey I GnRH contained two residues of glutamic acid after acid hydrolysis, similar to those found in chicken GnRH (Miyamoto et al., 1982). Thus, lamprey I GnRH appeared to be a unique chemical structure, although analysis of the degree of structural homology with other GnRH molecules required the amino acid sequence.

Amino Acid Sequence. All known natural GnRH peptides contain amino-terminal 5-Pyrrolidone-2-carboxylic acid residues that are resistant to Edman degradation. Therefore, lamprey I GnRH was treated with α-chymotrypsin, and four peptide fragments were purified by HPLC (Figure 4). The amino acid compositions of these fragments are shown in Table 2, and the sequencer determinations of three of these peptides are shown in Table 3. The composition and sequence of peptide CH-3 indicated that it was a fragment overlapping the subfragments, peptides CH-1 and CH-4 (Fig. 5). In addition, the sum of the yields of peptides CH-3 and CH-1 (92%) or peptides CH-3 and CH-4 (80%) was similar to the yield of peptide CH-2 (82%). Thus, the sequence analyses, amino acid compositions and yields of the peptide fragments quantitatively accounted for the entire lamprey I GnRH molecule.

Fast Atom Bombardment Mass Spectrometry. The *partial fast atom* bombardment mass spectrum from m/z 620 to m/z 1300 for lamprey I GnRH is presented in Fig. 6 but the entire mass listing for this peptide from m/z 65 to m/z 2696 was examined. The calculated $(M+H)^+$ for the peptide, based upon the amino acid analysis and assuming a 5-pyrrolidone-2-carboxylic acid, one tryptophan, and one carboxyamide, is 1226.59 amu or a nominal mass of 1227 amu (measured mass equalled 1226.54 amu). Intensities at m/z 1227, m/z 1249, and m/z 1265 are consistent with observation of the protonated molecular ion $(M+H)^+$, the natriated molecular ion $(M+Na)^+$ and the kaliated molecular ion $(M+K)^+$. It can be postulated from these molecular ion regions and Edman degradation data that lamprey GnRH contains a single carboxyamido group (otherwise the $(M+H)^+$ would appear at m/z 1228), has a molecular weight consistent with the amino acid composition including one tryptophan and probably has an amino terminal 5-pyrrolidone-2-carboxylic acid.

Information about the amino acid sequence of lamprey I GnRH can be gained from the remainder of the mass spectrum. Fast atom bombardment mass spectra of peptides display fragment ions consistent with peptide bond fission followed by various neutral losses (Buko et al., 1983a). Fission of the peptide bond with retention of the positive charge on the amino terminal fragment ion yields predominantly the aldimine ion series, (A_n-28) series, while retention of the positive charge on the carboxy-terminal fragment ion yields predominantly the ammonium ion series, NZn series. Other fragment ion series are sometimes present, but are generally not as abundant. These simple fragmentations serve as a framework for investigating the remainder of the spectrum. Two partial inferred sequences are labelled in Fig. 6.

Loss of $CONH_3$ from the protonated molecular ion $(M+H)^+$ is consistent with m/z 1182. This A_{10}-28 fragment ion, by losing Gly, would give the A_9-28 fragment ion at m/z 1125. Loss of Pro from the m/z 1125 ion would give the A_8-28 fragment ion at m/z 1027. Loss of Lys from this ion would yield the A_7-28 ion at m/z 899. The m/z 899 ion could lose Trp to give the A_6-28 ion at m/z 713. This pattern of aldimine fragment ions is consistent with a carboxy-terminal amino acid sequence of -Trp-Lys-Pro-Gly-NH_2. The ion intensity at m/z 1210 is consistent with loss of NH_3 from the $(M+H)^+$ to yield the highest mass acylium ion (Buko et al., 1983b), further suggesting that lamprey GnRH is α-carboxyamidated.

A second fragment ion series, the ammonium ion series, can be inferred from other intensities in the spectrum. Loss of an amino-terminal pGlu from the $(M+H)^+$ yields an ion at m/z 1116. This NZ_9 ion could then lose His to give the NZ_8 ion at m/z 978. Loss of Tyr from m/z 978 would yield the ion at m/z 815. This NZ_7 ion could then lose Ser to yield the NZ_6 ion at m/z 728. This ammonium ion series suggests an amino-terminal sequence of pGlu-His-Tyr-Ser-Leu-.

Examination of the entire mass listing affords assignment of complete aldimine and ammonium fragment ion series that are consistent with the amino acid composition and suggest an amino acid sequence of pGlu-His-Tyr-Ser-Leu-Glu-Trp-Lys-Pro-Gly-NH_2. These fragment ion series indicate that Glu at residue 6 is most likely a Glu and not a Gln.

After chymotryptic digestion of lamprey I GnRH, four peptides were isolated by HPLC. A portion of each peptide was analyzed by amino acid analysis and fast atom bombardment mass spectrometry. Peptide CH-1 contained Lys, Pro, and Gly; the peptide displayed a protonated molecular ion $(M+H)^+$ at m/z 300. Peptide CH-2 contained Glu, His, and Tyr and showed an $(M+H)^+$ ion at m/z 430. Peptide CH-3 contained Ser, Leu, Glu, Lys, Pro, and Gly and displayed an $(M+H)^+$ at m/z 815. Peptide CH-4 contained Ser, Leu, and Glu and showed an $(M+H)^+$ at m/z 534. Calculation of the molecular weights for peptides with these amino acid compositions did not accurately correlate with the observed protonated molecular ions. Further examination of the fast atom bombardment mass spectra and Edman degradation of peptides CH-1, CH-3, and CH-4 provided consistent complementary data.

The amino acid composition of peptide CH-1 (Lys, Pro, Gly) indicates that the peptide should display an $(M+H)^+$ at m/z 301. Instead, this peptide displayed an $(M+H)^+$ at m/z 300 (Fig. 7). Two partial fragment ion series could be inferred from the spectrum. Loss of $CONH_3$ from the $(M+H)^+$ yielded an ion at m/z 255. Loss of Gly from this A_3-28 ion would yield the A_2-28 ion at m/z 198. These two aldimine ions suggest that the carboxy terminal amino acid sequence of peptide CH-1 is -Gly-NH_2. Loss of Lys from the $(M+H)^+$ yields the NZ_2 ion at m/z 172, suggesting that the amino terminal residue of peptide CH-1 is Lys. Edman degradation identified the amino acid sequence Lys-Pro-Gly. Together, Edman degradation and mass spectrometry data suggest Lys-Pro-Gly-NH_2 as the structure for peptide CH-1.

Composed of Glu, His and Tyr, peptide CH-2 showed an $(M+H)^+$ at m/z 430 (Fig. 8). A tripeptide of this composition should display an $(M+H)^+$ of 448 amu, 18 amu higher than the observed $(M+H)^+$. Refractory to Edman degradation, this peptide probably contains pGlu at its amino terminus. Loss of CO_2H_2 and Tyr from the $(M+H)^+$ yielded A_2-28 ion at m/z 221, consistent with Tyr being the carboxy terminus of peptide CH-2. The ammonium ion series inferred from the spectrum for the intact lamprey I GnRH (Fig. 6) supports the amino acid sequence for peptide CH-2 as pGlu-His-Tyr- and places this peptide at the amino terminus of lamprey I GnRH.

Peptide CH-3 was the largest of the chymotryptic peptides with an $(M+H)^+$ at m/z 815 (Fig. 9). The amino acid composition of this peptide (Ser, Leu, Glu, Lys, Pro, Gly) would indicate an $(M+H)^+$ of 630 amu. The difference in mass between the calculated and observed $(M+H)^+$ of 185 amu could be attributed to one carboxyamide and one Trp. Edman degradation of this peptide was successful and identified an amino acid sequence of Ser-Leu-*Glu-Trp-Lys-Pro-Gly. Several ions consistent with this amino acid sequence* for peptide CH-3 appear in the mass spectrum. Loss of Ser from the $(M+H)^+$ yields the NZ_6 ion at m/z 728. Loss of Leu and Glu from this ion would yield the ion at m/z 486. An amino terminal sequence for peptide CH-3 of Ser-Leu-Glu- could be inferred from this data. Loss of NH_3 from the $(M+H)^+$ yielding the acylium ion at m/z 798 is consistent with an α-carboxyamide as the carboxy terminus of peptide CH-3.

An additional ammonium ion series could be inferred from the spectrum for peptide CH-3 beginning at m/z 831. This ion intensity is 16 amu higher than the $(M+H)^+$ at m/z 815 and could be due to oxidation of some of the Trp residues to oxyindole-alanine either during digestion of the GnRH by chymotrypsin or during separation by HPLC. Loss of Ser from m/z 831 yields the NZ_6 ion at m/z 744. Loss of Leu from this ion gives the NZ_5 ion at m/z 631. This ammonium ion could then lose Glu to yield the NZ_4 ion at m/z 502. This ammonium ion series extends to lower mass and is consistent with the Edman degradation data except that for this ion series the tryptophan is most likely an oxyindole-alanine.

With a composition of Ser, Leu, and Glu, peptide CH-4 showed an $(M+H)^+$ at m/z 534 (Fig. 10), which is 186 amu higher than the $(M+H)^+$ of 348 amu predicted by this amino acid composition. This indicates that Trp is likely to be the additional amino acid. Observation of an intensity at m/z 550, 16 amu higher than the $(M+H)^+$, indicates, in the same manner as for peptide CH-3, that some of the Trp present in peptide CH-4 has been oxidized to oxyindole-alanine. Edman degradation of peptide CH-4 identified Ser-Leu-Glu-Trp as the amino acid sequence. Two aldimine ion series could be inferred from the mass listing that would support this structure. Loss of CO_2H_2 and Trp from the $(M+H)^+$ at m/z 534 yielded *the A_3- 28 ion* at m/z 302. *Loss of* CO_2H_2 and oxyindole-alanine from the $(M+H)^+$ at m/z 550 also yielded the ion at m/z 302. The remaining intensities for this aldimine ion series were evident in the mass listing and provided data consistent with and complementary to the Edman degradation data.

Synthetic Lamprey I GnRH. The synthetic lamprey I peptide eluted on HPLC in the same position as native lamprey I material (Fig. 11). Mammalian GnRH also eluted in this position. *A chromatographic analysis of the synthetic* lamprey I peptide is shown in the lower part of Figure 11; only fraction #22, which eluted coincidently with this peak, contained ir-GnRH (middle graph, Fig. 11).

Bioassay. An injection of synthetic lamprey GnRH into 15 adult female lamprey produced a significant elevation in plasma estradiol compared with control lamprey as measured by RIA. The estradiol RIA was described previously (Sower et al., 1985). A dose of 100 or 200 μg/Kg given for 2 successive days was effective in a dose-related manner. Lamprey I GnRH was nearly as active as a mammalian superactive agonist, that is, an injection of the mammalian GnRH analog (DAla^6-des Gly^{10} ethylamide) at 50 μg/Kg stimulated an increase of estradiol comparable to a dose of 150 μg/Kg of synthetic lamprey I GnRH. Comparative effects of synthetic lamprey GnRH and an analog have been tested (Sower et al., in preparation).[3]

Second Form of Lamprey GnRH. Peak 2 from HPLC (Fig. 3) was analyzed and had the amino acid composition shown in Table 1 for lamprey II GnRH. This form also contains Trp based on absorbance at 280 nm indicating it may be similar to the other family members.

[3] Sower, S. A., Sherwood, N. M., Millar, R. P., and Marshak, D. R., manuscript in preparation.

Table 1. PROTOCOL FOR PURIFICATION OF LAMPREY GnRH BY HPLC.

HPLC no.	Material applied to column	Column	Guard column	Mobile phase[a]	Program Time (min)	Program % CH_3CN
0	Extract	C-18, Sep Paks	-	0.25 M TEAP, pH 6.5/CH_3CN	0-10 10-50	10 10-60
1	Fractions with ir-GnRH from HPLC-0	C-18, Supelco[b] 0.46 x 25 cm, 5 μm particles	C-18, Supelco 0.46 x 2 cm 5 μm particles	0.25 M TEAP, pH 6.5/CH_3CN	0-10 10-17 17-30	17 17-24 24
2	Peak 1 from HPLC-1, peak 2 from HPLC-1, applied separately	"	"	0.25 M TEAP, pH 2.5/CH_3CN	0-10 10-60	10 10-60
3	Peak 1 from HPLC-2, peak 2 from HPLC-2, applied separately	"	"	0.25 M TEAP, pH 6.5/CH_3CN	0-10 10-17 17-50	17 17-24 24
4	Peak 1 from HPLC-3	"	"	0.25 M TEAP, pH 2.5/CH_3CN	0-10 10-20 20-40	10 10-20 20
5	Peak 1 from HPLC-4, peak 2 from HPLC-3	C-18, Vydac[c] 0.46 x 25 cm 5 μm particles	None	TFA-H_2O/TFA-H_2O-CH_3CN (1:1000)/(1:3000:700) (v/v) (v/v/v)	0-50 0-50	7-57 14-65
6	Peak 1 from HPLC-5	phenyl, Vydac 0.46 x 25 cm 5 μm particles	None	0.25 M TEAP, pH 2.25/CH_3CN	0-55	5-60
7	Peak 1 from HPLC-6, peak 2 from HPLC-4	C-18 Vydac 0.46 x 25 cm 5 μm particles	None	TFA-H_2O/TFA-H_2O-CH_3CN (1:1480)/(1:148:1352) (v/v)/ (v/v/v)	0-50	10-60

[a] Mobile phase components: TEAP, triethylammonium phosphate; CH_3CN, acetonitrile; TFA, trifluoroacetic acid
[b] Supelco (Bellefonte, Pa)
[c] Vydac (the Separations Group, Hesperia, CA)

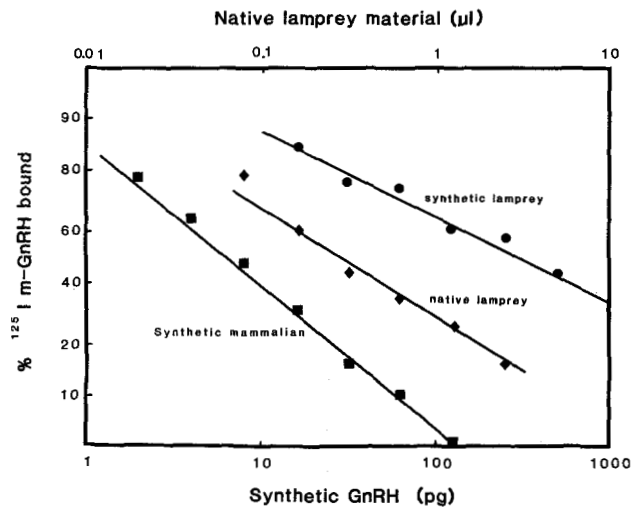

Figure 1. Displacement of mammalian ^{125}I-GnRH from antiserum R-42 by 1:2 serial dilutions of synthetic mammalian GnRH (■), synthetic lamprey GnRH (●) or native lamprey GnRH from brain extract (◆). On the ordinate, % ^{125}I m-GnRH is the amount of mammalian GnRH tracer bound by antibody R-42 in the presence of unlabelled peptide, divided by the total amount of tracer bound by the antiserum. The lamprey brain sample was an aliquot of fraction #22 from the HPLC shown at the top of Figure 11.

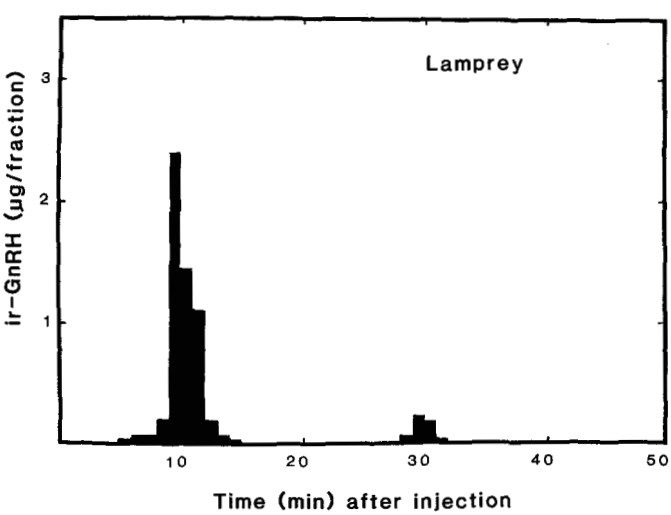

Figure 2. Reverse-phase HPLC-1 of lamprey brain extract containing immunoreactive (ir) GnRH. Active fractions eluted from Sep-Pak columns were injected onto a C-18 HPLC column. The mobile phase was acetonitrile (CH_3CN) diluted with 0.25 M TEAP, pH 6.5. Details of HPLC-1 are shown in Table 1. The early-eluting material, peak 1, was further purified and identified as lamprey I GnRH; the late-eluting material was the source of lamprey II GnRH.

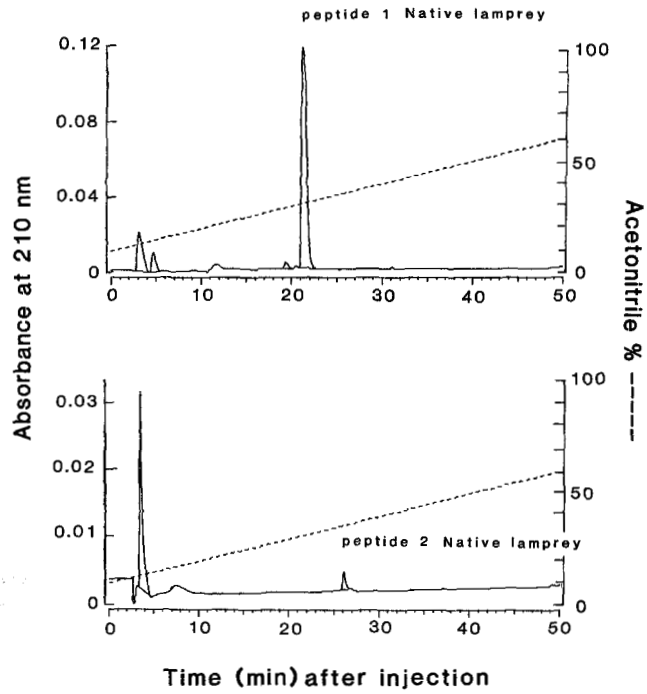

Figure 3. Reverse-phase HPLC-7 of the final purified peaks of lamprey brain peptides isolated by cross-reactivity to a mammalian GnRH antiserum. The peaks were eluted from a Vydac C-18 column. The mobile phase was composed of trifluoroacetic acid, water and CH_3CN. The gradient of % acetonitrile is indicated by the dotted line. Fractions #21-23 (peak 1) and #26-27 (peak 2) were separately taken for chymotryptic digestion (peak 1 only), amino acid analysis and mass spectrometry.

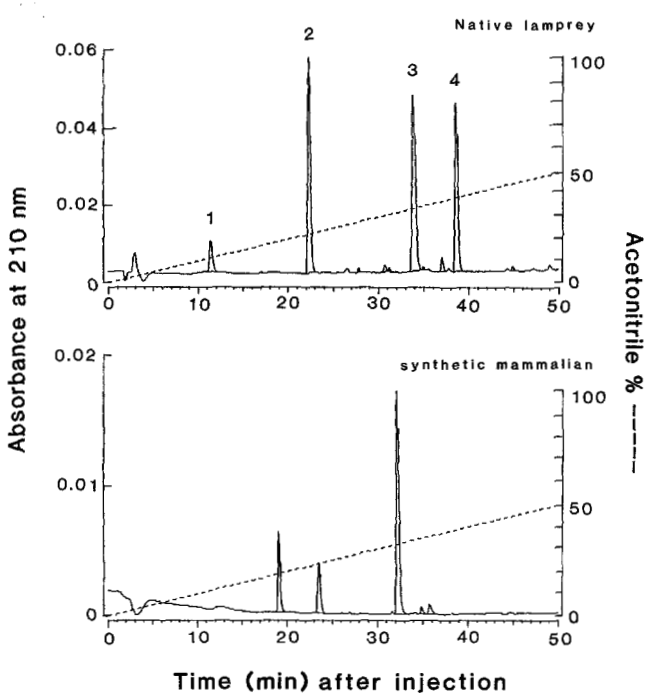

Figure 4. Reverse-phase HPLC of the chymotryptic digest of lamprey brain fractions containing ir-GnRH (top) and synthetic mammalian GnRH (bottom). The numbers 1-4 for native lamprey peaks represent the chymotryptic fragments identified as CH-1 to CH-4 in the text. The chymotryptic digest was injected onto a C-18 column. The mobile phase was the same as Figure 3.

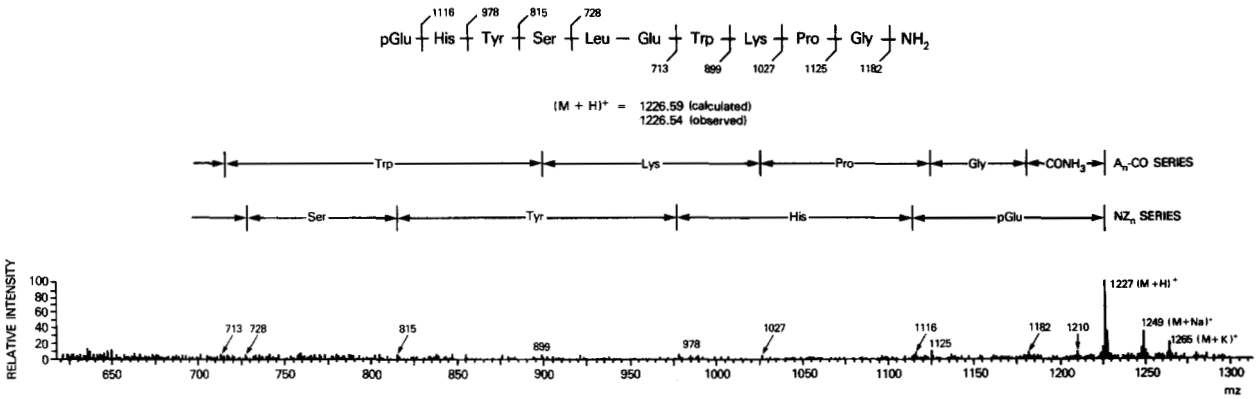

Figure 6. Fast atom bombardment mass spectrum of lamprey GnRH. The partial fast atom bombardment mass spectrum of 650 pmol of lamprey GnRH (presumptive M_r= 1226.59) is shown from m/z 620 to m/z 1310. A_n-CO and NZ_n series refer to the fragment ions originating from peptide bond fission with retention of the positive charge on the amino- or carboxy- terminal fragment ion, respectively.

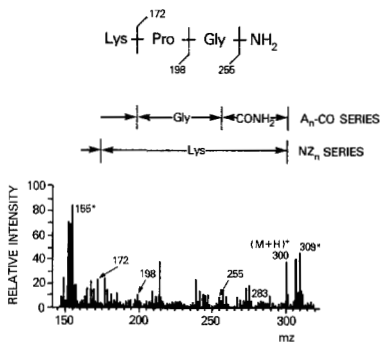

Figure 7. Fast atom bombardment mass spectrum of chymotryptic peptide CH-1 from lamprey GnRH. The partial fast atom bombardment mass spectrum of 0.99 nmol of peptide CH-1 isolated by HPLC of the chymotryptic digest of lamprey GnRH is shown from m/z 145 to m/z 320. Asterisks label those intensities attributed to the liquid matrix.

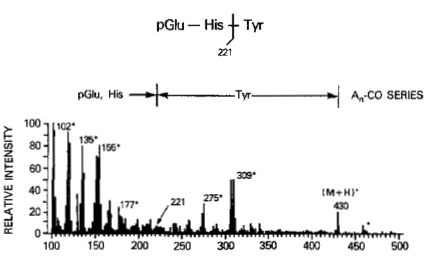

Figure 8. Fast atom bombardment mass spectrum of chymotryptic peptide CH-2 from lamprey GnRH. The partial fast atom bombardment mass spectrum of 1.83 nmol of peptide CH-2 isolated by HPLC of the chymotryptic digest of lamprey GnRH is shown from m/z 100 to m/z 300. Asterisks mark those intensities attributed to the liquid matrix.

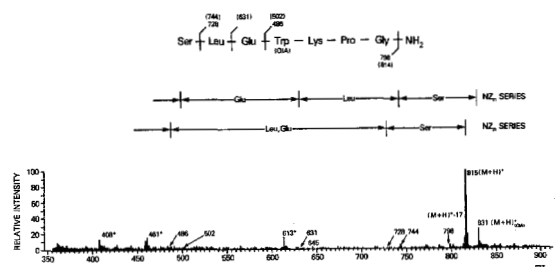

Figure 9. Fast atom bombardment mass spectrum of chymotryptic peptide CH-3 from lamprey GnRH. The partial fast atom bombardment mass spectrum of 1.07 nmol of peptide CH-3 isolated by HPLC of the chymotryptic digest of lamprey GnRH is shown from m/z 350 to m/z 910. Asterisks mark those intensities attributed to the liquid matrix.

Table 3. AUTOMATED SEQUENCER DETERMINATIONS OF PEPTIDES CH-1, CH-3, AND CH-4

CH-1 (6130 pmol)

Cycle	Residue	pmol
1	Lysine	4674
2	Proline	1027
3	Glycine	858

CH-3 (2600 pmol)

Cycle	Residue	pmol
1	Serine	250
2	Leucine	730
3	Glutamic Acid	485
4	Tryptophan	281
5	Lysine	218
6	Proline	161
7	Glycine	189

CH-4 (4440 pmol)

Cycle	Residue	pmol
1	Serine	1215
2	Leucine	5776
3	Glutamic Acid	3790
4	Tryptophan	680

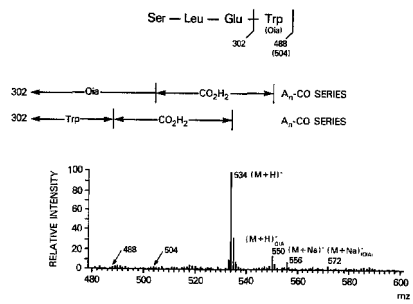

Figure 10. Fast atom bombardment mass spectrum of chymotryptic peptide CH-4 from lamprey GnRH. The partial fast atom bombardment mass spectrum of 0.71 nmol of peptide CH-3 isolated by HPLC from the chymotryptic digest of lamprey GnRH is shown from m/z 480 to m/z 600.

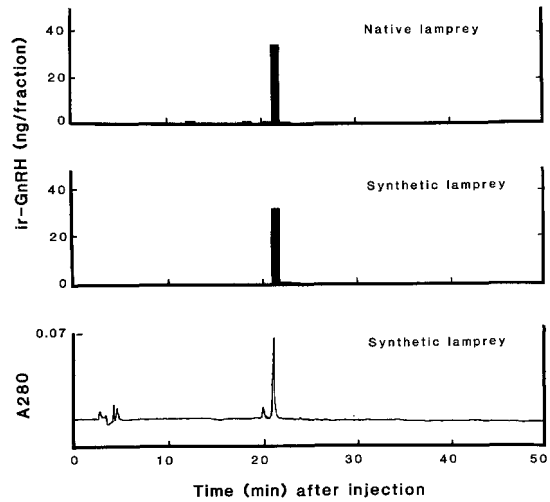

Figure 11. Reverse-phase HPLC of native lamprey brain extract containing immunoreactive (ir) gonadotropin-releasing hormone, GnRH, (top) and synthetic lamprey GnRH (middle and bottom). The native lamprey material was taken from fraction #20, HPLC-6, peak 1. The HPLC chromatograph for 5 μg of synthetic sample was measured at an absorbance (A) of 280 nm. The mobile phase and conditions were the same as HPLC-1 in Table 1. Synthetic mammalian GnRH was run on the same day with the same conditions; it also eluted in fraction #22, but is not shown.