Conformation of Gonadotropin Releasing Hormone†

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ABSTRACT: The conformation of the gonadotropin releasing hormone (Gn-RH), whose primary sequence is pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2, and of several of its structural analogues has been studied by circular dichroism, optical rotatory dispersion, and fluorescence spectroscopy. The effects of pH, guanidine, and temperature on fluorescence emission have also been examined. Titration data demonstrate that the histidine and tyrosine residues are free of any mutual interactions. The similarity of emission spectra in water and in guanidine hydrochloride solutions precludes significant interactions between the fluorescent groups and other residues. Neither the temperature nor the pH profiles of the emission intensities of either tyrosine or tryptophan reveal any fixed secondary structure in Gn-RH. Both the extent of alkaline quenching and the distance of 10–11 Å calculated from Förster energy transfer theory are in accord with a randomly coiled structure with only one residue between tyrosine and tryptophan. Furthermore, the circular dichroism spectrum and optical rotatory dispersion do not exhibit any contributions from peptide bonds in an ordered structure, although there is a perturbation of the peptide absorption region due to overlapping bands from side-chain chromophores. Gn-RH, therefore, appears to behave as a random coil polypeptide in water devoid of any intrachain residue interactions. This nonordered structure in Gn-RH and the lack of any significant differences in the physical-chemical properties of the hormone analogues indicate that a predetermined solution conformation is not required for biological activity. In contrast to its behavior in water, Gn-RH in trifluoroethanol exhibits a conformational transition, with the formation of a β structure. Differences in conformational changes exhibited by several analogues in trifluoroethanol may be relevant to their relative biological activities at the receptor site.

The gonadotropin hormone releasing hormone (Gn-RH)[1] is a decapeptide isolated from porcine and bovine hypothalamic glands (Schally et al., 1971; Burgus et al., 1972). Its amino acid sequence is pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Baba et al., 1971; Matsuo et al., 1971). This releasing factor and a variety of its analogues have now been synthesized in substantial quantities (Monahan et al., 1973).

The availability of Gn-RH and related peptides has made a comparative examination of physicochemical properties feasible. Studies have been made of optical rotatory dispersion and circular dichroism, and of fluorescence and titration behavior. The central focus of this investigation has been on the relation between these properties and conformations of the decapeptide in solution. A comparison of conformations of Gn-RH with structural analogues of varying biological activity could give some insight into the biologically active structure.

Experimental Section

Materials

Samples of the releasing factor Gn-RH and its analogues were kindly supplied by W. F. White of Abbott Laboratories. The peptides[1] examined are listed in Table I.

Thyrotropin releasing hormone (TRH) was also a gift of W. F. White. Asp[1],Ile[5]-angiotensin I (mol wt 1295), Asp[1],Ile[5]-angiotensin II (mol wt 1045), and bradykinin (mol wt 1059) were commercial samples (Spectrum Medical Industries, Los Angeles), chromatographically pure.

N-Acetyl-L-tyrosinamide and *N*-acetyl-L-tryptophanamide were purchased from Sigma Chemical Co. and were recrystallized from acetone and ethanol. *N*-Acetyl-L-tryptophan, *N*-acetyl-L-tyrosine, *N*-acetyl-L-tyrosine ethyl ester, L-tyrosine methyl ester, L-tryptophan methyl ester, glycyl-L-tyrosine, and L-tryptophanyl-L-tyrosine were purchased from Cyclo Chemical Co. and used without further purification.

N-Acetyl-L-tryptophan methyl ester and *N*-acetyl-L-tryptophan ethyl ester were prepared according to Huang and Niemann (1951), *N*-acetyl-L-tyrosine methyl ester was prepared by the procedure of Thomas et al. (1951), and *N*-acetyl-L-tryptophanylglycyl-L-tyrosine was prepared by following the method of Hoffman et al. (1957).

Guanidine hydrochloride was obtained from Sigma Chemical Co. Solvents for optical studies were of spectral grade. All other chemicals were the best quality available commercially.

The concentrations of Gn-RH solutions were determined from the optical density of neutral solutions at 280 nm. The molar extinction coefficient, ϵ_{280}, established from the average of six determinations on solutions of varying concentrations, was 6084 M^{-1} cm^{-1}. This value corresponds to $E_{1\,cm}(1\%)$ 44.3 which is in excellent agreement with $E_{1\,cm}(1\%)$ 44.4 reported to us by W. F. White. The concentrations of peptides lacking tyrosine were determined from ϵ_{280}^{Trp} = 5440 and of those lacking tryptophan from ϵ_{275}^{Tyr} = 1380. The concentrations of angiotensin solutions were

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[1] Abbreviations used are: Gn-RH, follicle stimulating hormone, luteinizing hormone releasing hormone; in hormone analogues the superscript following the amino acid designates the substituted position; the deletion of an amino acid is represented by the prefix des-; CBz, carbobenzoxy, *i*-Boc, isobutyloxycarbonyl; TRH, thyrotropin releasing hormone; ϵ_λ, molar extinction coefficient at the wavelength λ; F_3EtOH, trifluoroethanol.

Table I: Some Gn-RH Analogues Examined.

Compound	Hormonal Activity (%)	Ref
Gn-RH	100	Burgus et al., 1972
Phe^2-Gn-RH	4–7	Fujino et al., 1972a
Des-His^2-Gn-RH	Negligible	Vale et al., 1972
Phe^3-Gn-RH	0.1	*a*
OCl-Tyr^5-Gn-RH	5–8	Fujino et al., 1972a
OMe-Tyr^5-Gn-RH	10	*a*
CBz-Tyr^5-Gn-RH	0.1	*a*
Ser^5-Gn-RH	0.2–1	*a*
Orn^8-Gn-RH	6–12	Fujino et al., 1972a
Lys^8-Gn-RH	11–28	Fujino et al., 1972a
i-Boc-Lys^8-Gn-RH	Not available	
Des-Gly^{10}-propylamide-Gn-RH	200–300	Fujino et al., 1972b
Des-Gly^{10}-pyrrolidinamide-Gn-RH	70–80	Fujino et al., 1972b
Des-Gly^{10}-piperidinamide-Gn-RH	Not available	
Des-Gly^{10}-morpholinamide-Gn-RH	20–30	Fujino et al., 1972b
Des-Gly^{10}-*n*-butylamide-Gn-RH	Not available	
Des-Gly^{10}-dimethylamide-Gn-RH	Not available	

[a] W. F. White, personal communication.

determined from ϵ_{275}^{Tyr} = 1379 (Paiva et al., 1963) and of bradykinin solutions from ϵ_{258}^{Phe} = 378.

pH values were obtained with a Corning Model 12 pH meter equipped with a Corning semimicro combination electrode.

Methods

Analytical Ultracentrifugation. The molecular weight of Gn-RH was determined by sedimentation equilibrium, using a Spinco Model E analytical ultracentrifuge equipped with an RTIC temperature unit and ultraviolet scanner optical system (Schachman, 1963). Centrifugation was carried out at 20° at an angular velocity of 60 000 rpm for 50 hr. Gn-RH was dissolved in 0.1 *M* KCl and the solution had an initial optical density of 0.5. A 12-mm double-sector cell with sapphire windows and Kel-F double-sector centerpiece was used. The partial specific volume of Gn-RH, 0.780 ml/g, was estimated from the amino acid composition and apparent specific volumes of the amino acid residues (Cohn and Edsall, 1943).

Optical Rotatory Dispersion (ORD); Circular Dichroism (CD). The optical rotatory parameters were measured at room temperature with a Cary Model 60 spectropolarimeter equipped with a Model 6002 CD attachment. Optical path lengths varied from 0.1 to 20 mm, depending on wavelength and on concentration. Reproducibility of specific rotations was ±2%. Measurements were made as far down near 190 nm as possible by appropriate reduction of the optical path. Scanning speeds were 1 nm/min or less. Except where noted, all compounds were dissolved in water and used immediately. Peptide spectra were recorded for solutions of peptide concentration 0.5–2 mg/ml. A spectral curve was obtained for each solvent before and after recording each sample. Each sample was usually scanned twice (three times at low wavelengths).

The ORD data were plotted according to the Moffitt equation (Moffitt, 1956) with λ_0 taken as 218 nm, and values of b_0 and a_0 were calculated from the slope and intercept on the linear graph.

All the CD data have been converted to molar ellipticities $[\theta]$ expressed in units of deg cm^2/dmol (Djerassi, 1960). The circular dichroism attachment was periodically standardized with a 0.1% solution of *d*-10-camphorsulfonic acid (Aldrich) which was assumed to have $[\theta] = +7146$, or $\Delta\epsilon = \epsilon_L - \epsilon_R = 2.17$ at 290 nm (Parrish and Blout, 1971; Quadrifoglio and Urry, 1968).

Electrometric Titration. The titrations were done at 25° with a Radiometer Model 4 pH meter using a G2222B type glass electrode and a saturated calomel electrode. Before and after each titration the pH meter was calibrated with phosphate buffer. Three samples of 5 m*M* Gn-RH in 0.1 *M* KCl were titrated by addition of 0.1 *M* HCl down to pH 2, then addition of 0.1 *M* KOH up to pH 12, and, finally, addition of HCl back to pH 2. The titrant was added, from a calibrated syringe driven by a micrometer graduated to 0.01 mm, so as to change the pH 0.005 to 0.1 unit after each addition. Solvent blanks were titrated in the same way. pK_a values were determined graphically from a plot of milliequivalents of acid or base added vs. pH.

Spectrophotometric Titration. These titrations were carried out in a Cary 14 spectrophotometer at 25° using a 1-cm path length square cell. Known volumes of 0.1 *M* KOH or 0.1 *M* HCl were added to 3 ml of solution in borate buffer (ionic strength 0.1). The pH was measured in the spectrophotometric cell after each addition. Mixing was accomplished by slowly bubbling nitrogen through the sample until the pH became stable. The spectrum was measured with a neutral buffer solution as the reference blank. pK_a values were obtained from graphs of absorbance (at 295 nm) vs. pH.

Fluorescence Measurements. Emission spectra were recorded with a Hitachi-Perkin-Elmer MFP-2A spectrofluorimeter using 1-cm^2 covered fluorescence cells. The temperature of the solutions was maintained at 25° by circulating water through the jacket of the cell holder. When the temperature dependence of fluorescence was measured, emission spectra were recorded 30 min after the water had been circulating at the desired temperature. Solutions had optical densities less than 0.1 at the wavelength of excitation. Hormone quantities of less than 50 μg were adequate for measurement of intensities. Excitation was at 278 nm unless otherwise indicated. Tyrosyl and tryptophyl emissions were observed at 305 and 350 nm, respectively.

The pH dependence of fluorescence was obtained by titrating the solutions in the fluorescence cells with small amounts of 0.1 *M* KOH or 0.1 *M* HCl using a Beckman-Spinco microtitrator. The final volume change was less than 0.1%. The absorbance of Gn-RH remains constant with pH since 278 nm is an isosbestic point of tyrosyl ionization. The degree of phenolic ionization, α, as a function of pH was determined from the optical density at 295 nm.

Quantum yields were determined by excitation of the hormone and of standards at the same wavelength and incident intensity. The quantum yields of the standards, *N*-acetyl-L-tryptophanamide and *N*-acetyl-L-tyrosinamide, were assigned values of 0.13 (Chen, 1967) and 0.065 (Edelhoch et al., 1968b), respectively.[2] The quantum yield of emission

[2] There has been some uncertainty as to the correct value for the quantum yield of tryptophan. Teale and Weber (1957) reported a value of 0.20. Here the value obtained by Chen (1967) was used.

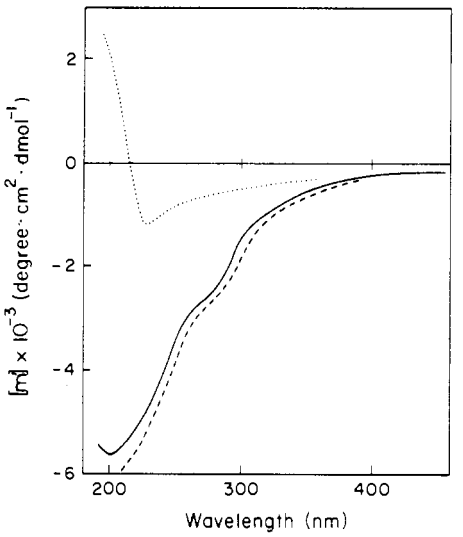

FIGURE 1: Optical rotatory dispersion of Gn-RH in water, guanidine hydrochloride, and trifluoroethanol. The results represent averages of six spectra taken in water (—), two in guanidine hydrochloride (- - -), and two in trifluoroethanol (···).

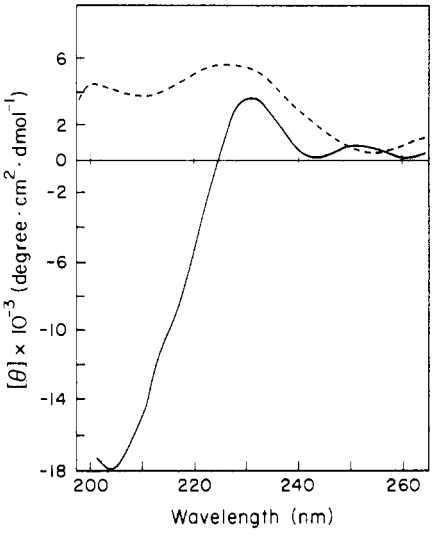

FIGURE 2: Circular dichroism spectra of Gn-RH (—) and a mixture of constituent amino acids (- - -); 27°, pH 7.

for any compound was calculated by comparing the absorbance at the exciting wavelength and the area of the emission spectrum with the standard according to the equation

$$\frac{\phi_{samp}}{\phi_{ref}} = \frac{A_{ref}}{A_{samp}} \frac{OD_{samp}}{OD_{ref}}$$

where A is the area under the emission curve, ϕ the quantum yield, and OD the absorbance. When tryptophan quantum yields were to be determined, excitation was at 295 nm. By exciting at both 278 and 295 nm one can evaluate the contribution from tyrosine residues to the hormone emission spectrum.

To enhance the possibility of observing the transfer of excitation energy, solutions of Gn-RH and its analogues were excited at 270 nm where the ratio of the molar extinction coefficients of *N*-acetyl-L-tyrosinamide to *N*-acetyl-L-tryptophanamide is maximal. The emission spectra were normalized at a wavelength above 350 nm where only tryptophan fluorescence occurs.

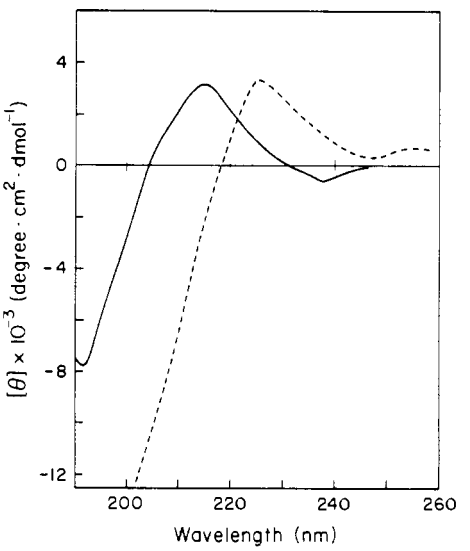

FIGURE 3: Circular dichroism spectra of the thyrotropin releasing hormone (—) and *N*-acetyl-L-tryptophanylglycyl-L-tyrosine (- - -) in aqueous solution.

Results

Ultracentrifugation. In a 1×10^{-4} *M* aqueous solution (with 0.1 *M* KCl, pH 6.8), the molecular weight of Gn-RH was found to be 1380. This compares favorably with 1374, the value calculated from the primary structure. There was no sign of aggregation at this concentration, which is approximately that used for the other physicochemical experiments.

Optical Rotatory Dispersion. The ORD of Gn-RH in the wavelength region of 200–400 nm is illustrated in Figure 1. The major trough occurs between 205 and 210 nm with $[m']_{205} = -5600$. The ORD was not dependent on pH between 2 and 11. The dispersion in 6 *M* guanidine hydrochloride resembled that of the aqueous solution of Gn-RH although the rotation at 205 nm was somewhat stronger ($[m']_{205} = -6200$). These magnitudes are small compared to those found in synthetic polypeptides (Blout et al., 1962; Iizuka and Yang, 1965) but the spectra are simple and resemble those of random chain polypeptides.

A linear relationship is obtained when the data are fitted to the Moffitt equation. When λ_0 is set at 218 nm, $b_0 = 40$ and $a_0 = -270$.

The ORD spectrum of Gn-RH in trifluoroethanol, a helix-promoting solvent, is also included in Figure 1. It shows a distinct minimum at 226 nm ($[m']_{226} = -1200$) and a crossover at 215 nm. The lower wavelength band around 205 nm disappears with the emergence of a peak below 200 nm. This behavior is characteristic of the dispersion found for a peptide containing some β structure (Beychok, 1967).

Circular Dichroism. The CD spectrum of Gn-RH is shown in Figure 2. The major dichroic activity is centered around 205 nm ($[\theta]_{205} = -18\,000$), the region of the ellipticity band in a random coiled polypeptide. In addition there is a maximum at 230 nm. This weaker band probably arises from features other than secondary structure. There is no negative band at 222 nm, the location of the $n-\pi^*$ transition of the peptide bond in an α helix (Holzwarth and Doty, 1965). When the CD spectrum is compared to those of poly(L-glutamic acid) or poly(L-lysine) in their helical forms, it is apparent that there is no helical structure in synthetic Gn-RH.

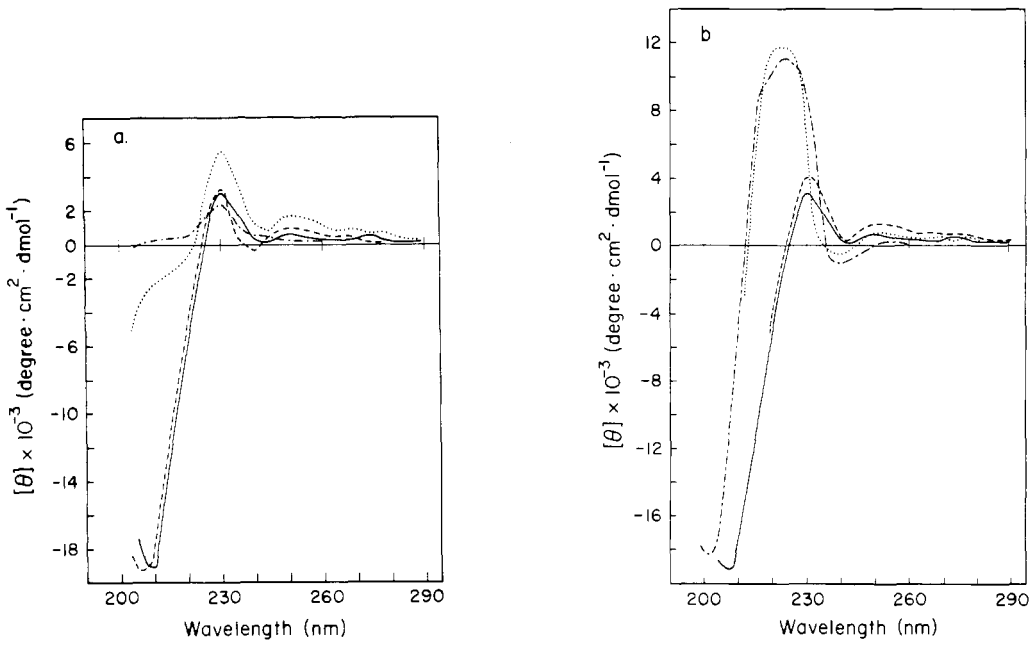

FIGURE 4: Circular dichroism of Gn-RH and some related peptides in aqueous solution; 27°, pH 7. (a) Gn-RH, OCl-Tyr^5-Gn-RH (—); Ser^5-Gn-RH (- - -); OMe-Tyr^5-Gn-RH (···); CBz-Tyr^5-Gn-RH, (- · -); (b) Gn-RH, (—); des-Gly^{10}-propropylamide-Gn-RH (- - -); Phe^3-Gn-RH (- · -); Phe^2-Gn-RH (···).

Dichroic structural investigations of Gn-RH are complicated by the presence of tyrosine and tryptophan in the molecule. The contributions of the n–π^* and π–π^* transitions of the peptide bond are obscured by the optical activity of the side-chain chromophores as indicated by the CD spectrum of a mixture of the constituent amino acids (Figure 2). To assess the contributions arising from the side chains, the CD spectra of *N*-acetyl-L-tyrosinamide, *N*-acetyl-L-tryptophanamide, histidine, and phenylalanine in water were taken. Tyrosine and tryptophan have strong ellipticity bands between 220 and 230 nm. Phenylalanine and histidine exhibit positive ellipticity bands around 215 nm. The CD spectra of these compounds were essentially unchanged in trifluoroethanol. The wavelengths of the maximum ellipticity of phenylalanine, histidine, tyrosine and tryptophan all fall within the region of the CD bands of an α helix (Legrand and Viennet, 1965). The CD of a β structure also has considerable negative ellipticity in this region (Beychok, 1967).

CD spectra were also taken of the thyrotropin releasing hormone (TRH) pGlu-His-Pro-NH_2, which contains three of the amino acids present in Gn-RH and is not likely to exist in either a helical or β structure; and of another model tripeptide, *N*-acetyl-L-tryptophanylglycyl-L-tyrosine. The spectrum of TRH (Figure 3) closely resembles that of histidine, with a maximum at 215 nm and a strong negative trough at 192 nm. There is a small but distinct minimum at 238 nm which would be expected if TRH exists essentially as a random structure. The CD of *N*-AcTrpGlyTyr (Figure 3) shows a strong positive peak at 225 nm, arising from the contributions from tyrosine and tryptophan, and an intense negative trough at 200 nm, which may be due to the peptide chromophore in a random conformation.

The CD spectra of several peptide analogues of Gn-RH are shown in Figure 4. The spectra are similar, exhibiting maxima between 220 and 230 nm, minima near 240 nm, and strong negative trends below 215 nm.

Phe^2-Gn-RH has low but distinct biological activity. The strong CD contribution from phenylalanine (217 nm) is readily apparent in Figure 4b and obscures the tyrosine and tryptophan ellipticities. In fact, if the contribution from free phenylalanine is subtracted and that of histidine is added, the resulting spectrum is similar to that observed for Gn-RH. Substitution of Trp in position 3 of Gn-RH by phenylalanine leads to a loss of biological activity but the far-ultraviolet CD spectrum is similar to that of Phe^2-Gn-RH. Thus, changes in these spectra can be accounted for by changes in the side-chain chromophores; the insertion of the phenylalanine ring in place of tryptophan or the addition of a third aromatic residue does not appear to alter the secondary conformation of the molecules as determined by CD.

The presence of tyrosine OH in position 5 is not essential for activity. Substitution at this position with OMe-Tyr results in a lower but not a complete loss of activity. The CD spectra of several Gn-RH peptides substituted at position 5 are illustrated in Figure 4a. Again, tyrosine and tryptophan contribute maxima at 230 nm. CBz-Tyr^5-Gn-RH has negligible hormonal activity but its spectrum is similar to those of the other tyrosine analogues, although the intensity below 210 nm is considerably less negative.

Comparison of the biological activities of *i*-Boc-Lys^8-Gn-RH, Lys^8-Gn-RH, and Orn^8-GnRH demonstrated the necessity of a positively charged group at position 8 (Chang et al., 1972). The CD spectra of Orn^8-, Lys^8-, and *i*-Boc-Lys^8-Gn-RH are virtually identical with the spectrum of natural Gn-RH, containing arginine in position 8. The spectra of Ser^5-Gn-RH and des-His^2-Gn-RH, which exhibit negligible Gn-RH activity, show the same maximum at 230 nm and the intense negative band at 205 nm as does natural Gn-RH. Thus, CD reveals no particular overall conformational features in these analogues associated with activity or inactivity.

Gn-RH analogues possessing unusually high Gn-RH activity (>100%) comprised the last group studied. All of these compounds exhibited similar spectra so only the spectrum of des-Gly^{10}-Pro-propylamide is reproduced in Figure 4b. Once again the spectrum had a maximum near 230 nm and a minimum around 205 nm.

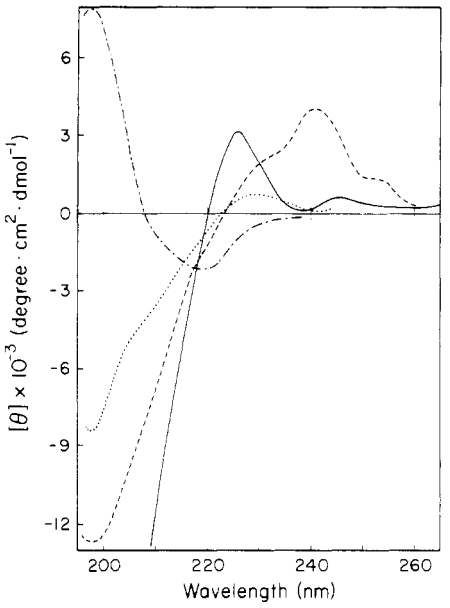

FIGURE 5: Solvent effects on the circular dichroism spectra of several Gn-RH peptides. Circular dichroism of Gn-RH in water, trifluoroethanol (F_3EtOH), and trifluoroacetic acid. Gn-RH (—); 20% F_3EtOH, and 80% H_2O (···); 100% F_3EtOH (- · -); 20% CF_3COOH and 80% F_3EtOH (- - -).

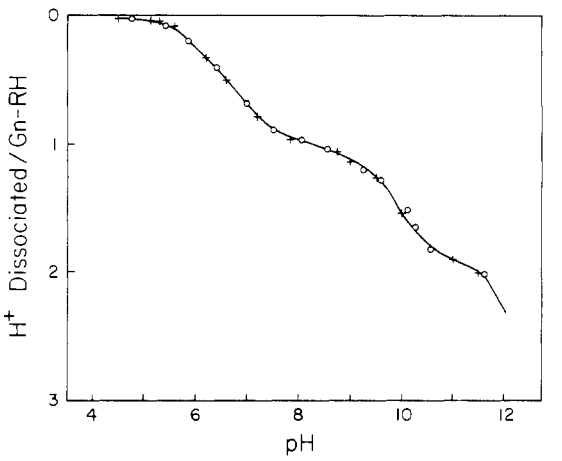

FIGURE 6: Titration curve of Gn-RH in 0.1 *M* KCl at 25°: (+) titration with HCl; (O) titration with KOH. The curve is a theoretical one calculated with pK_a values 6.6, 10.0, and 12.5.

To investigate the possible perturbation of Gn-RH structure by solvent and pH, the spectra of Gn-RH in acidic and basic solutions, guanidine hydrochloride, and trifluoroethanol were analyzed. Similar CD spectra were obtained at pH 2.5 and pH 11.0 as at pH 7 although at high basicity there is a shift of the maximum to higher wavelengths and the ellipticity decreases slightly. A shoulder appears at 223 nm but the strong negative band at 205 nm is not affected. These same spectral shifts are observed in *N*-acetyl-L-tyrosine ethyl ester (Legrand and Viennet, 1965) at pH 13. The shoulder at 223 nm can be attributed to tryptophan. Ser^5-Gn-RH, where tyrosine is replaced, was found to have the same CD spectrum at pH 11 as the native hormone below pH 6.

The CD spectrum of Gn-RH was also measured in 6 *M* guanidine hydrochloride, a solvent in which little or no organized structure remains in proteins (Tanford et al., 1967). The spectrum in 6 *M* guanidine was essentially the same as that in water although there is a slight decrease in the intensity of the ellipticity band at 230 nm.

To see if Gn-RH is intrinsically capable of forming a helical or β structure, CD measurements were recorded in trifluoroethanol (F_3EtOH), a helix-inducing solvent (Figure 5). In 20% F_3EtOH/water (v/v) the ellipticities at 230 and 205 nm were diminished. In 100% F_3EtOH an entirely different spectrum was observed. There is a major negative trough at 218 nm ($[\theta]_{218} = -2000$), a crossover at 207 nm, and a peak at 198 nm. The features correspond to the electronic transitions associated with the peptide bond in a β structure. The lower magnitude of the ellipticity may be a result of the positive contribution in this region from the side-chain chromophores. As mentioned earlier, spectra of model compounds of tyrosine and tryptophan changed significantly when the solvent was changed from water to F_3EtOH.

To evaluate the effect of a disrupting solvent on this organized structure, trifluoroacetic acid, a helix-breaking solvent, was added to the F_3EtOH solution of Gn-RH to give a 20% CF_3COOH/F_3EtOH (v/v) solution. Although the CD spectrum (Figure 5) did not return completely to that of the original water solution of Gn-RH, there were positive bands around 240 nm and a negative band near 198 nm.

Phe^3-Gn-RH and OMe-Tyr^5-Gn-RH exhibited somewhat different behavior in trifluoroethanol. The spectrum of the latter resembled that of Gn-RH in F_3EtOH with a negative trough at 218 nm. However, there was also a less pronounced minimum at 205 nm. The spectrum of Phe^3-Gn-RH exhibited the same features as that of a water solution of this compound although the intense positive ellipticity may be obscuring a negative trend. These results suggest that these two analogues may fold somewhat differently in F_3EtOH than does Gn-RH. The near-ultraviolet CD spectra of all analogues containing tyrosine and tryptophan were normal compared to that of the free amino acids. There were several poorly resolved positive bands between 260 and 290 nm which correspond to the indole and benzene moieties of tryptophan and tyrosine, respectively. The substitution of tyrosine derivatives blocked at the phenolic oxygen did not alter the nature of these bands. When tryptophan was replaced by phenylalanine the intensity of the band diminished. There was little change in the spectrum when serine was substituted for tyrosine.

Acid–Base Titrations. The curve obtained for the electrometric titration of Gn-RH at 25° is shown in Figure 6. The dissociations of the imidazolium, phenolic, and guanidinium groups are well-separated and there are no apparent long-range electrostatic interactions between them. The pK_a's of the histidine and tyrosine residues can be ascertained directly from the titration curve and are 6.60 and 10.0, respectively. Titration errors in the high pH region are large but the entire curve can be fitted if the arginine residue is assigned a pK_a of 12.5.

The pK_a values of the phenolic group calculated from spectrophotometric titrations performed at 25 and 35° were 10.00 and 9.86, respectively. This titration was reversible as was the electrometric one. The enthalpy of ionization, calculated from the equation $\Delta H = 2.303R[\Delta(\text{pH})/\Delta(1/T)]$ at each of several stages in the ionization, was found to be 5.8 kcal/mol. Spectrophotometric titrations of an equimolar mixture of *N*-acetyl-L-Tyr-NH_2 and *N*-acetyl-L-Trp-NH_2, and of *N*-acetyl-L-Trp-Gly-Tyr yielded the same pK_a value for tyrosine. These results and the presence of isosbestic points at 267.5 and 278 nm throughout the pH range of

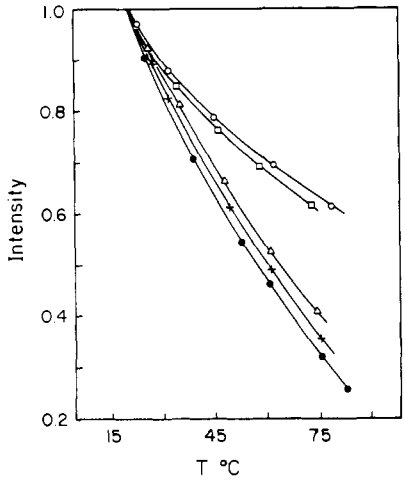

FIGURE 7: The temperature dependence of emission intensity of Gn-RH and several analogues. All solutions are 1×10^{-4} *M*. Initial emission intensities have been normalized to the same value at 20°. Emission of Gn-RH, OMe-Tyr^5-Gn-RH(+), Phe^3-Gn-RH (□), *N*-Ac-L-Trp-Gly-L-Tyr (●), Ser^5-Gn-RH (△), *N*-Ac-L-Tyr-NH_2 (○).

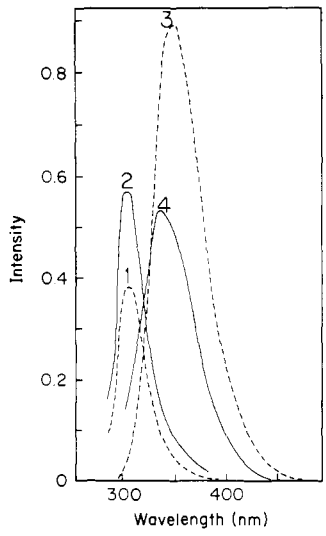

FIGURE 8: The fluorescence of Gn-RH and Phe^3-Gn-RH in water and trifluoroethanol. Emission spectra of Phe^3-Gn-RH in water (curve 1) and F_3EtOH (curve 2); Gn-RH in water (curve 3) and F_3EtOH (curve 4).

the Gn-RH titration indicated that the tyrosine hydroxyl was free of detectable interactions and that there was no irreversible structural change as the pH increased.

Fluorescence. Emission Characteristics. As an alternative approach to the detection of tertiary interactions between aromatic residues in Gn-RH, the fluorescence parameters of Gn-RH, Phe^3-Gn-RH, Ser^5-Gn-RH, and OMe-Tyr^5-Gn-RH were investigated. All spectra were taken at the same excitation wavelength (289 nm) with solutions of identical concentrations (1×10^{-4} *M*). Phe^3-Gn-RH (lacking a Trp) has an emission spectrum with a maximum at 305 nm, characteristic of tyrosine alone. The quantum yield of phenylalanine fluorescence is negligible when excitation is at wavelengths greater than 270 nm so the emission of Phe^3-Gn-RH arises from tyrosine fluorescence only. The emission maxima of Gn-RH, Ser^5-Gn-RH, and OMe-Tyr^5-Gn-RH occur at 350 nm, the wavelength characteristic of the indole maximum of simple tryptophan peptides in aqueous solution.

There is no significant fluorescence at 305 nm, the region of tyrosyl emission, for Gn-RH, Ser^5-Gn-RH, or OMe-Tyr^5-Gn-RH. This is not unexpected since the tyrosyl and tryptophanyl chromophores are separated by only one residue. Energy transfer from tyrosine to tryptophan has been found to occur with high efficiency in the tripeptide Trp-Gly-Try-(Edelhoch et al., 1967). The relative quantum yields of tryptophanyl emission in the peptides, determined from the emission spectra using a reference value of 0.13 for tryptophan, were 0.09 in both Gn-RH and OMe-Tyr^5-Gn-RH. The quantum yield of tyrosine in Phe^3-Gn-RH was considerably lower, 0.04.

The effect of guanidine hydrochloride on the emission intensity of the hormone analogues was also studied. The shapes of the spectra were the same, the emission maxima remaining at 350 and 305 nm for tryptophanyl and tyrosyl residues, respectively. A comparison with the effect of guanidine on the fluorescence intensity of *N*-Ac-Try-NH_2, *N*-Ac-Tyr-NH_2, and *N*-Ac-Trp-Gly-Tyr demonstrated that this denaturant up to 6 *M* concentrations has little or no effect on the tryptophan fluorescence, but there is some effect on tyrosyl fluorescence. The fluorescence intensity of the tryptophan peak at 350 nm decreased by 5% in *N*-Ac-Trp-Gly-Tyr and by about 15% in the tyrosyl compounds. The changes in emission intensities of the Gn-RH peptides were in the same direction but smaller.

Temperature Effects. The thermal dependence of fluorescence emission has been studied in trytophan and tyrosine, synthetic polypeptides of tryptophan and tyrosine, and hormones containing these residues (Edelhoch and Lippoldt, 1969; Steiner and Edelhoch, 1963b; Gally and Edelman, 1964). There is a gradual decline in the quantum yields of tryptophan and tyrosine with increasing temperature, the former changing more rapidly than the latter. The temperature dependence of tyrosyl and tryptophanyl emission in Gn-RH and several analogues is illustrated in Figure 7 along with data for *N*-Ac-Trp-NH_2, *N*-Ac-Tyr-NH_2, and *N*-Ac-Trp-Gly-Tyr. A monotonic decline was observed in all compounds over the temperature range 20–80°. The intensity returned to its original value on cooling the solution to 20°. The peak locations and overall shapes of the emission spectra remained constant throughout this temperature range.

Effect of Organic Solvent. Since CD observations indicate that Gn-RH forms an ordered structure in trifluoroethanol, a study of the fluorescence parameters in this solvent was undertaken. The fluorescence spectra of Gn-RH and Phe^3-Gn-RH in water and in 100% F_3EtOH are compared in Figure 8. The emission maximum for tyrosine remains at 305 nm whereas the tryptophan emission shifts toward the blue, the maximum appearing at 340 nm. This shift to a lower wavelength is expected since tryptophanyl fluorescence maxima located at wavelengths greater than 340 nm reflect an exposure to aqueous solvent (Teale, 1960). Solvents of decreasing polarity increase the quantum yield and shift the emission spectrum of indole toward lower wavelengths (Teale, 1960) but only increase the yield of tyrosine without altering its spectrum. As expected, the quantum yield of Phe^3-Gn-RH is larger in F_3EtOH than in water. However, the opposite was found for native Gn-RH, which indicates that the fluorescent residues may be involved in some interactions not found in water. The fluorescence spectrum of OMe-Tyr^5-Gn-RH in F_3EtOH was similar to that of Gn-RH in F_3EtOH but the quantum yield was somewhat increased rather than decreased compared to

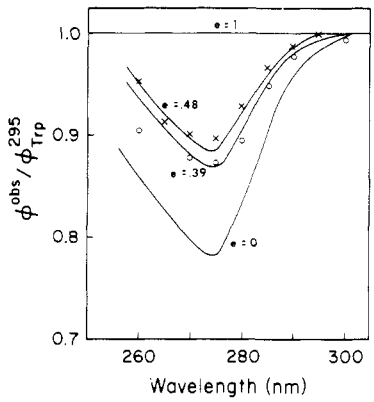

FIGURE 9: Wavelength dependency of tryptophanyl quantum yield. The x's represent the experimental data for N-Ac-L-Trp-Gly-L-Tyr and the circles, the data for Gn-RH. The curves are theoretical ones obtained from an analysis of the absorption spectra of the amino acids and correspond to different efficiencies of transfer, e.

that in water. Ser^5-Gn-RH behaved in a similar manner. The temperature dependence of fluorescence in F_3EtOH was normal. There were no sharp breaks and the change was completely reversible.

pH Effects. The fluorescence intensity of Gn-RH measured at 350 nm increased slightly when acidic solutions were neutralized to pH 7. When the solutions were back titrated to pH 2 the fluorescence intensity returned to its original value. The intensity changes are small and cannot be attributed to structural modifications but may be related to the influence of the charged state of histidine adjacent to tryptophan (Shinitsky and Goldman, 1967). The tryptophanyl fluorescence of Phe^2-Gn-RH and des-His^2-Gn-RH does not appear to be significantly affected by acid titration, and the tyrosyl fluorescence of Phe^3-Gn-RH was quenched to a lesser extent with acid.

In contrast to the small effects produced by acidification, major quenching of both tryptophan and tyrosine emission occurs in alkali. If a peptide is stable in alkali and has only one tyrosine, its ionization can be followed fluorometrically since ionized tyrosine does not fluoresce at 305 nm (Cowgill, 1963). The quenching of tyrosyl fluorescence in Phe^3-Gn-RH was similar to that of free tyrosine. The pK_a for tyrosyl ionization in this peptide determined fluorometrically was 10.1, in agreement with that obtained spectrophotometrically.

Loss of tryptophan emission by radiationless energy transfer to ionized tyrosine has been observed in the series Trp(Gly)$_n$Tyr, the loss decreasing with increasing separation of tryptophan and tyrosine residues (Edelhoch et al., 1967). This same effect would be expected in Gn-RH because of the close proximity of tyrosine and tryptophan in the primary structure. Tryptophan fluorescence is quenched in alkaline solution for several Gn-RH peptides. At high pH, the degree of quenching of tryptophan emission due to phenolic ionization can be determined by plotting the intensity of tryptophan fluorescence against α, the degree of ionization of tyrosine, and extrapolating to $\alpha = 0$ and $\alpha = 1$ to get the intensity when tyrosine is in the un-ionized and ionized forms, respectively. The tryptophanyl fluorescence of Gn-RH, Phe^2-Gn-RH, and N-Ac-Trp-Gly-Tyr is quenched by 60% upon complete ionization of phenol. The tryptophan fluorescence of Ser^5-Gn-RH and OMe-Tyr^5-Gn-RH, in which no phenolic ionization is possible, was quenched to a much lesser extent than in those peptides containing tyrosine and resembled the quenching curve of N-Ac-Trp-NH_2.

Energy Transfer. Small peptide hormones are attractive for study of radiationless energy transfer because they possess only one or two aromatic residues, the transfer efficiency is high, and the distances between residues are small. Gn-RH contains tryptophan in position 3 and tyrosine in position 5 and, therefore, lends itself to an analysis of the efficiency of singlet energy transfer between this pair of aromatic residues. With the aid of the Förster energy transfer theory (Förster, 1948, 1959), it is then possible to determine the distance between the donor and acceptor chromophores.

The absorption spectrum of Gn-RH coincides with that of a solution of the N-acetylamide derivatives of tyrosine and tryptophan in 1:1 molar proportion. One may assume, therefore, that the absorption properties of tyrosine and tryptophan incorporated in the polypeptide are the same as those of the isolated amino acids.

The fluorescence emission spectrum of tyrosine overlaps the absorption spectrum of tryptophan. In addition, there is spectral overlap between the emission of tryptophan and the absorption of ionized tyrosine at pH 11.5. Based on these overlaps energy transfer is expected from tyrosine to tryptophan and from tryptophan to ionized tyrosine. A comparison of energy transfer in Gn-RH at acid and basic pH with that of N-Ac-Trp-Gly-Tyr should give some insight into the relative positions of tyrosine and tryptophan in the hormone.

The fluorescence of Gn-RH is predominantly from tryptophan when excitation is at 278 nm but a contribution from tyrosine can be observed by comparing the emission spectrum from excitation at 278 nm with that from excitation at 295 nm where tyrosine emission is absent. By normalizing the pure tryptophan spectrum at 370 nm where there is no appreciable contribution from tyrosine, we can compute the tyrosine emission by difference. The quantum yield for tyrosine obtained in this manner was equal to 0.02 compared to the value of 0.13 for free tryptophan. The quantum yield for tryptophan at pH 11.5, obtained from the emission spectrum of tryptophan excited at 278 nm, was equal to 0.06.

The rate of transfer from an excited donor D to an acceptor A at a distance r has been analyzed by Förster (1948, 1951, 1967). We have used the Förster parameters for tyrosine and tryptophan pairs discussed by Eisinger (1969) and calculated by Eisinger et al. (1969), and taken for R_0, the distance at which the rate of energy transfer equals the loss of energy of the donor by other processes, values of 10.0 and 7.8 Å based on the quantum yields of 0.02 and 0.06 for tyrosine and tryptophan, respectively, in Gn-RH.

As in the treatment of Eisinger (1969), the efficiency of energy transfer, e, was obtained from the observed quantum yield of the peptide and the quantum yield of tryptophan determined at 295 nm, where tyrosine does not absorb. Based on the equation

$$\phi_{\text{Gn-RH}} = \phi_{\text{Trp}}^{295}(f_{\text{Trp}} + ef_{\text{Tyr}})$$

where f_{Trp} and f_{Tyr} represent the fractions of light absorbed by tryptophan and tyrosine, respectively, a plot of $\phi_{\text{Gn-RH}}/\phi_{\text{Trp}}$ at different exciting wavelengths was constructed (Figure 9). The data can be fitted best with ($f_{\text{Trp}} + ef_{\text{Tyr}}$) obtained from absorption curves of tyrosine and tryptophan and a value 0.39 for e for transfer from tyrosine to tryptophan in Gn-RH. A similar treatment for tryptophan to tyrosinate transfer at high pH gave an $e = 0.20$. The efficiency of transfer for Tyr to Trp in N-Ac-Trp-Gly-Tyr was

found to be 0.48. Once the efficiency of transfer is known the distance between the two chromophores can be obtained from the relation between efficiency of transfer, R_0, and r, investigated thoroughly by Eisinger (1969). The results for Gn-RH are listed in Table II. Since the tyrosine (donor) quantum yield is somewhat ambiguous, distances between chromophores are calculated at several donor quantum yields. These distances are between 9.5 and 14.5 Å, depending upon the quantum yield used.

Table II: Distance between Tyrosine and Tryptophan for Several Donor Quantum Yields.

Compound	e^a	$r/R_0{}^b$	r (Å) for ϕ_D 0.01	0.02	0.05
Gn-RH (pH 7)	0.39	1.20	10.7	12.0	14.3
			r (Å) for ϕ_D 0.06	0.10	
Gn-RH (pH 11.5)	0.20	1.35	10.5	11.3	
			r (Å) for ϕ_D 0.01	0.02	0.05
N-Ac-TrpGlyTyr-NH_2	0.48	1.10	9.8	11.0	13.1

[a] Efficiency of energy transfer. [b] Ratio of donor–acceptor separation to Förster distance.

Discussion

In aqueous solution both ORD and CD give no indication of any ordered structure in the natural Gn-RH or in any of the peptide analogues examined. Thus optical rotatory properties point to a random arrangement of side chains of residues in the hormone, all residues being exposed to the aqueous environment. Furthermore, the similarity in behavior of the active peptide and its inactive analogues indicates that biological effectiveness is not associated with a preferred conformation of the hormone in solution. Similar conclusions have been reached recently by Marche et al. (1973). Comparable behavior has also been observed for the nonapeptide hormone bradykinin for which circular dichroism studies reveal a flexible molecule possessing several different conformations (Cann et al., 1973).

The titration behavior of Gn-RH is also consistent with a nonordered structure for the decapeptide. The pK_a values observed are normal and indicate that the imidazole, phenol, and guanidine groups of histidine, tyrosine, and arginine, respectively, are exposed to the solvent and free of any neighboring interactions. The pK_a of 7.0 for the imidazole in glycylhistidine and 6.2 for that in histidine illustrate the influence of the charged carboxyl and amino groups on the imidazole moiety. Gn-RH has neither charged group and would be expected to have an intermediate pK_a; the observed value is 6.60. Comparison of this pK_a with that of histidine in Gly-Gly-His-NH_2 (pK_a = 6.61), Gly-His-Leu (pK_a = 6.68) (Muramatsu et al., 1963), pGlu-His-NH_2 (pK_a = 6.7) (Grant et al., 1972), and Val-His-Pro-Phe (pK_a = 6.6) (Zimmer et al., 1972), respectively, also indicates that there is nothing unusual about the ionization properties of histidine in Gn-RH. For tyrosine, the observed pK_a of 10.0 obtained from spectrophotometric and direct titration of Gn-RH is close to the value found in several oligopeptides (Fermandjian et al., 1972), as well as in the free amino acid. Although the arginine group was not titrated, the full experimental titration data could be fitted to a theoretical curve (needing no corrections for electrostatic interactions) when pK_a's = 6.6, 10.0, and 12.5 were assigned to the imidazole, phenol, and guanidine side chains, respectively. Thus, the normal pK_a values of the titratable groups of Gn-RH suggest that there are no direct interactions between these groups and neighboring residues.

Fluorescence investigations of several peptide hormones have been useful in determining various types of folding, and Förster energy transfer theory has been applied to the calculation of donor–acceptor distance (Eisinger, 1969; Steiner and Edelhoch, 1963b). The emission maxima of Gn-RH and its analogues containing tryptophan appear between 348 and 350 nm, and coincide with the emission maxima of small model tryptophan peptides of low molecular weight (e.g., Tyr-Trp, *N*-Ac-Trp-Gly-Tyr) in aqueous solution. The lack of tyrosyl emission in Gn-RH can be explained by energy transfer between the phenol and indole moieties; emission from tyrosine is also not observed in the tripeptide Tyr-Gly-Trp (Edelhoch et al., 1967) where the chromophores are also separated by just one residue. The position of the 350-nm Trp maximum is stable at high temperatures, in strong acid or base, and in guanidine. These results indicate that the tryptophan moiety is surrounded by solvent molecules and does not reside in a discrete hydrophobic region of the hormone. Furthermore, the monotonic decline in intensity of tryptophan emission with increasing temperature and the indifference of emission intensity to 6 *M* guanidine are also in accord with this interpretation.

A slight decrease is observed in emission intensity as the pH of Gn-RH is decreased from 7 to 2. The fluorescence intensity of tryptophan in the dipeptide histidyltryptophan is reduced by protonation of the imidazole ring (Shinitsky and Goldman, 1967). The titration effect below pH 7 in the hormone could also be the response to the ionization properties of the single histidine adjacent to tryptophan.

A much greater degree of quenching is observed at alkaline pH. When the Tyr^5 is substituted at the phenolic oxygen with a nonionizable group the quenching observed in Gn-RH is probably due to the ionization of tyrosine rather than a structural modification. The decrease of tryptophan fluorescence by energy transfer to ionized tyrosine has been reported in numerous peptides and proteins (Edelhoch and Lippoldt, 1969; Steiner and Edelhoch, 1963a,b). Edelhoch and coworkers (1967) have also examined this quenching of tryptophan at high pH for the series Trp-$(Gly)_n$-Tyr with n varied from 0 to 4. The maximum degree of quenching falls from 85% for Trp-Tyr to 50% for Trp-Gly_4-Tyr. The 60% quenching found in Gn-RH is consistent with these values.

If an α-helical model for Gn-RH is constructed, a minimum separation between the tryptophan and tyrosine of 14 Å and a maximum of 17.6 Å is obtained, corresponding to a linear average of 15.8 Å. When similar measurements are made on a model of the hormone folded between Tyr^5 and Gly^6, the closest distance between Trp and Tyr is 8.8 Å and the farthest 11.2 Å, the average being 10.0 Å. Assuming that no secondary structure exists other than a random coil, one finds that the two chromophores could come as close as 5 Å if held in a relatively parallel position, whereas the farthest distance apart would be 14 Å. The average distance in a random coil would be about 10 Å. The average value calculated for the distance between tyrosine and tryptophan from Förster theory is 10.6 Å. This is consistent with both a folded model and a random coil, but is too short for an α helix. If Gn-RH were held in a rigid conformation, the tryptophan and tyrosine could still be free to interact with the

solvent and the fluorescence properties would be normal as observed. However, the CD and ORD data should indicate the presence of secondary structure and they do not. Furthermore, if a β structure were present one would expect disruption by guanidine which is not observed. The possibility of an aggregate of two or more Gn-RH molecules is ruled out by ultracentrifugation measurements. Thus only a nonordered conformation for the decapeptide is consistent with all of the experimental observations.

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