High Resolution Nuclear Magnetic Resonance Studies of the Conformation of Luteinizing Hormone Releasing Hormone (LH-RH) and its Component Peptides

By **Phillipus L. Wessels** and **James Feeney,*** National Institute for Medical Research, Mill Hill, London NW7 1AA
Harry Gregory and **James J. Gormley**, I.C.I. Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire

The ^{1}H and ^{13}C n.m.r. spectra of luteinizing hormone releasing hormone (LH-RH) decapeptide. <Glu-His-OMe. <Glu-His-Trp-Ser-Tyr-Gly. Trp-Ser-Tyr-Gly. and Leu-Arg-Pro-GlyNH_2 have been studied. In aqueous solution the observed J_{NC} coupling constants are consistent with the molecules being in random coil configurations. There was no evidence for strong intramolecular hydrogen bonding involving CO · · · HN The similarities in ^{1}H and ^{13}C chemical shifts between model peptides, the component peptides, and the LH-RH decapeptide suggest no specific side-chain interactions. In particular there is no stacking of the aromatic rings. The ^{13}C chemical shifts of the β- and γ-^{13}C carbons in Pro in LH-RH indicate Pro to be in a *trans*-configuration.

THE suggestion that secretion of anterior pituitary hormones is controlled *via* the hypothalamus was first made by Harris in 1937.[1] Since that time evidence has gradually been accumulated showing that chemical transmitters are released at nerve endings in the hypothalamus and that these agents are transported by a system of portal blood vessels to the anterior pituitary gland. The so-called releasing factors then cause secretion of the appropriate pituitary hormones into the general circulation.[1]

Intensive efforts have been made to isolate and characterise the active entities and two have now been clearly identified.[2,3] The thyrotrophin releasing factor (TRF) has been shown to have the tripeptide structure <Glu-His-ProNH_2.[2,*] Many structural variants of this molecule have been prepared and examined biologically and there is considerable interest in its mode of action. The second releasing factor, that for luteinizing hormone (LH-RH), has been shown to be a basic polypeptide composed of ten amino-acids <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2.[3] The structure of the ovine releasing factor is the same as the porcine.[4] It has been demonstrated that this molecule will also cause secretion of follicle-stimulating hormone in addition to luteinizing hormone in a variety of species. Again many analogues have been synthesised and examined for biological activity and broadly these studies have shown that amino-acid substitution at most positions in the chain causes a reduction in potency but many of the analogues on record do retain detectable levels of activity. In particular shortening of the peptide chain has a marked effect. Omitting the *N*-terminal pyroglutamic acid causes a major loss of activity [3] whereas omission of the *C*-terminal glycine gave a nonapeptide with over 10% of the activity of the parent molecule.[5] Further deletion of proline gives an octapeptide of barely detectable activity but substitution of the proline by glycine in the nonapeptide leaves significant activity.[6]

Further efforts to provide understanding of the mechanisms whereby gonadotrophins are released are being carried out using a variety of pituitary tissue and isolated cell systems.

Previous speculations on the mode of action of TRF and LH-RH have drawn attention to the similarity of the *N*-terminal dipeptide and *C*-terminal primary amide and gave rise to the suggestion that there may be similar pituitary receptors for the two agents.[7] This led to the hypothesis that possibly the decapeptide adopts a conformation such that the ends of the molecule come together in a similar manner to TRF with hydrogen bonding across the chain as in a β-pleated sheet. It has also been suggested [8] that there could be stacking of the indole and phenolic rings parallel to each other as a result of π–π bond interactions.

To comment on such proposals and to attempt to establish structure–activity relationships for LH-RH we have investigated the conformational structure of LH-RH and its component peptides in solution using high resolution n.m.r. techniques.

Gibbons and his co-workers [9] and other workers [10–13] have demonstrated how one can use ^{1}H n m.r. data to

* <Glu is the symbol used for pyroglutamic acid.

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comment on the conformational situation involving the α-CH–NH (ϕ) dihedral angles and the α-CH–β-CH_2 (χ) dihedral angles and to detect the presence of intramolecular hydrogen bonds in the peptides. For small non-cyclic peptides in aqueous solution such as gastrin pentapeptide one observes a distribution of allowed conformations because of the flexibility about the bonds related to the dihedral angles ϕ, ψ and, χ.[10,†] The basis of the n.m.r. method relies on relating the observed three bond H–H spin coupling constants to the appropriate dihedral angles ϕ [12] and χ.[11] For a situation where the observed vicinal H–H coupling constant is a weighted averaged value of the coupling constants in the different conformations it is sometimes possible to calculate the fractional populations of the different conformers. Thus for α-CH–β-CH_2 side-chain fragments where we have most of the molecules in the three staggered conformations and where there is a large difference between the values of J_{gauche} (2·56 Hz) and J_{trans} (13·6 Hz) it is possible to obtain reasonable estimates of the conformational populations.[11] However, for the α-CH–NH backbone fragment where we only have a single vicinal H–H spin coupling constant J_{NC} and where the energetically favoured dihedral angles ($\phi = -100$ to $-60°$) do not have such widely differing J_{NC} values (8—3 Hz) it is much more difficult to obtain unambiguous conformational information from the measured vicinal coupling constants. The J_{NC} coupling constants can be related to the dihedral angle ϕ by the Bystrov–Karplus [12] equation $J_{NC} = 8{\cdot}9\cos^2(300 + \phi) - 0{\cdot}9\cos(300 + \phi) + 0{\cdot}9\sin^2(300 + \phi)$. From consideration of the ϕ–J_{NC} Bystrov–Karplus [12] relationship and potential energy diagrams given in ref. 9 it is seen that a measured vicinal J_{NC} coupling constant of 7·8 Hz could arise from a fixed conformation with one of four possible ϕ dihedral angles (−150, −90, +50, +70°) or from a distribution of conformations such that the averaged J_{NC} value is 7·8 Hz. In fact, the J_{NC} value expected from the distribution of conformations in a random coil situation can be shown to average to 7 ± 1 Hz.[9] Thus for a non-cyclic small peptide which shows no evidence for strong, intramolecular, hydrogen-bonding interactions and which gives J_{NC} values from 7 to 8 Hz one must assume it to have a random coil configuration.

Up to the present time the n.m.r. method has not been used to obtain conformational information for the ψ dihedral angle (α-CH–CO bond) although this is possible in principle by ^{15}N substitution of the peptide and by using a Karplus type relationship for the $J_{^{15}N\text{–}CCH}$ coupling constants as measured on the α-CH absorption bands.[14]

It should be mentioned that there is no reason to suppose that the most populated conformation in solution will be the conformation which is bound most strongly by the receptor or that the hormone conformation when

† For definition of ϕ and ψ see I.U.P.A.C.–I.U.B. Commission of Biochemical Nomenclature Report (*Biochemistry*, 1970, **9**, 3471).

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TABLE 1

(a) ^1H Chemical shifts of some component peptides of LH-RH in H_2O and DMSO solution

Compd.	<Glu-His OMe		Trp-Ser-Tyr-Gly		Leu-Arg-Pro-Gly-NH_2	
Frequency (MHz)	100	220	100	220	100	100
Temp. (°C)	30	20	30	20	30	30
Solvent	H_2O	$(CD_3)_2SO$	H_2O	$(CD_3)_2SO$	H_2O	$(CD_3)_2SO$
pH	2·2		3·0		2·7	
Internal ref.	DSS	TMS	DSS	TMS	DSS	TMS
<Glu						
$C_\alpha H$		4·01				
$C_\beta H$	2·50; 1·96	2·25; 1·83				
$C_\gamma H$	2·40	2·08				
His						
$C_\alpha H$	($J_{A\alpha} + J_{B\beta}$ 14 Hz)	4·50				
$C_\beta H$	3·40; 3·23	2·92				
C(2)H	8·63	7·57				
C(4)H	7·33	6·83				
OCH_3	3·79	3·61				
Trp						
$C_\alpha H$			—	3·99		
$C_\beta H$			3·26 (J_{AV} 7·3 Hz)	3·21; 2·99		
C(2)H			7·10	7·21		
C(4)H			7·50	7·70		
C(5)H			7·00	6·93		
C(6)H			7·16	7·09		
C(7)H			7·47	7·35		
N(1)H				10·98		
Ser						
$C_\alpha H$			(J_{AV} 5·6 Hz)	4·38 ($J_{A\alpha}$ 5·0, $J_{B\alpha}$ 6·5 Hz)		
$C_\beta H$			3·70	3·64; 3·52 (J_{AB} 11·5 Hz)		
Tyr						
$C_\alpha H$				4·44		
$C_\beta H$			2·87	2·9;		
C(2)H, C(6)H			7·10	7·06		
C(3)H, C(5)H			6·79	6·63		
Gly						
$C_\alpha H$			3·85	3·72		
Leu						
$C_\alpha H$						3·78
$C_\beta H$					1·7	1·5
$C_\gamma H$					1·7	1·5
$C_\delta H_3$					0·95 (J_{AV} 5·6 Hz)	0·83 (J_{AV} 5·5 Hz)
Arg						
$C_\alpha H$						4·46
$C_\beta H$					1·7	1·5
$C_\gamma H$					1·7	1·5
$C_\delta H$					3·23	3·09
$N_\epsilon H$					7·14	7·88
N(1)H, N(2)H					6·65	7·04
Pro						
$C_\alpha H$						4·20
$C_\beta H$					2·30; 2·1	2·0; 1·9
$C_\gamma H$					2·1	1·9
$C_\delta H$					3·7	3·7—3·3
Gly						
$C_\alpha H$					3·91	3·54
$CONH_2$					7·09; 6·55	7·32; 6·82

TABLE 1 (*Continued*)

(b) ^{1}H Chemical shifts of <Glu-His-Trp-Ser-Tyr-Gly and LH-RH in H_2O and DMSO solutions

Compd.	<Glu-His-Trp-Ser-Tyr-Gly		<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH$_2$	
Frequency (MHz)	100	220	100	270
Temp. (°C)	30	20	30	18
Solvent	H_2O	$(CD_3)_2SO$	H_2O	$(CD_3)_2SO$
pH	1·8		2·3	
Internal ref.	DSS	TMS	DSS	TMS
<Glu				
$C_\alpha H$		4·00		4·02
$C_\beta H$	2·3;	2·08; 1·69	2·32;	2·02;
$C_\gamma H$	2·3	2·08	2·3	2·02
His				
$C_\alpha H$		4·61		4·60
$C_\beta H$	3·1	2·99	3·20—2·90	*ca.* 3
C(2)H	8·50	8·87	8·52	7·55
C(4)H	7·13	7·30	7·15	6·78
Trp				
$C_\alpha H$		4·61		4·60
$C_\beta H$	3·1	3·16; 2·91 (J_{AB} 15, $J_{A\alpha}$ 5, $J_{B\alpha}$ 8 Hz)	3·20—2·90	*ca.* 3
C(2)H	7·13	7·16	7·15	7·15
C(4)H	7·54	7·63	7·57	7·60
C(5)H	7·0	6·92	7·0	6·99
C(6)H	7·2	7·00	7·22	7·03
C(7)H	7·46	7·31	7·47	7·33
N(1)H	10·01	10·85	10·05	10·90
Ser				
$C_\alpha H$		4·32		4·35
$C_\beta H$		3·60; 3·52 (J_{AB} 10·6, $J_{A\alpha}$ 6, $J_{B\alpha}$ 6 Hz)	3·71 (J_{AV} 5·5 Hz)	3·60
Tyr				
$C_\alpha H$		4·49		4·4
$C_\beta H$	3·1	3·03; 2·73 (J_{AB} 13, $J_{A\alpha}$ 6, $J_{B\alpha}$ 9 Hz)	3·20—2·90	*ca.* 3
C(2)H, C(6)H	7·08	7·04	7·11	7·03
C(3)H, C(5)H	6·79	6·64	6·81	6·64
Gly				
$C_\alpha H_2$		3·78	3·89 (J_{NC} 5·5 Hz)	3·73
Leu				
$C_\alpha H$				4·4
$C_\beta H$			1·6	1·5
$C_\gamma H$			1·6	1·5
$C_\delta H_3$			0·89; 0·87	0·83; 0·80
Arg				
$C_\alpha H$				4·4
$C_\beta H$			1·6	1·5
$C_\gamma H$			1·6	1·5
$C_\delta H$			3·20—2·90	*ca.* 3
N(1)H, N(2)H			6·60	
Pro				
$C_\alpha H$				4·29
$C_\beta H$			2·3; 2·1	2·0; 1·8
$C_\gamma H$			2·1	1·8
Gly				
$C_\alpha H$			3·89	3·60
$CONH_2$			-; 6·60	

bound to its receptor will be similar to that in solution. However, any conformational changes which occur on binding will influence the energetics of the hormone binding and to this extent conformational information concerning both the free and bound hormone will be necessary for a full understanding of the interaction.

EXPERIMENTAL

The ^{1}H n.m.r. spectra were recorded at 270, 220, and 100 MHz using Bruker, Varian HR-220, Varian XL-100-15, and Varian HA-100D spectrometers. Usually the peptides were examined as solutions containing 20—30 mg in H_2O (0·5 ml) or $(CD_3)_2SO$ (DMSO) (0·5 ml). Tetramethylsilane (TMS) and sodium 4,4-dimethyl-4-silapentanesulphonate (DSS) were used respectively as internal references for the (DMSO) and aqueous solutions.

Some ^{1}H spectra were obtained in the Fourier transform mode on the Varian XL-100-15 and to overcome the computer dynamic range problem posed by the large H_2O solvent band a double resonance experiment was carried out in which the water band was irradiated throughout the experiment. Fortunately the exchange between peptide NH protons and those in the H_2O solvent is not sufficiently rapid for transfer of magnetisation to be a problem. Furthermore, because the H_2O irradiation frequency is close to the α-CH region all the peptide NH protons are decoupled in such experiments. This spectral simplification (see Figure 3) helps in identifying the locations of the different NH absorptions.

The variable temperature and H_2O–DMSO mixed solvents studies of the ^{1}H spectra of the hexapeptide were made using a hexamethyldisiloxane (HMS) external reference: these were then converted to a DSS internal reference scale by using conversion factors measured in separate experiments where both external and internal references were used.

The pH measurements were made at 22 °C using a glass electrode Radiometer model 26 pH meter.

The ^{13}C n.m.r. spectra were recorded on a Varian XL-100-15 spectrometer using Fourier Transform and proton noise decoupling facilities. D_2O Solutions were used and the solvent deuterium used as the field-frequency lock. TMS was the external reference for the ^{13}C measurements.

RESULTS AND DISCUSSION

^{1}H *and* ^{13}C *Spectral Assignments.*—The problem of spectral assignment was made somewhat easier because we had available a closely related series of peptides, namely <Glu-HisOMe, Trp-Ser-Tyr-Gly, <Glu-His-Trp-Ser-Tyr-Gly, Leu-Arg-Pro-GlyNH$_2$, <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH$_2$. The assignments were made on the basis of comparisons with the chemical shifts in model compounds and by spin-decoupling experiments and the results from the ^{1}H and ^{13}C spectra are presented in Tables 1—4 and Figures 1—5. For the ^{1}H assignments spin-decoupling experiments were carried out on the DMSO solutions where it is easy to irradiate the α-CH protons while observing NH resonances. In H_2O solutions this type of experiment is difficult because of problems associated with irradiating α-CH protons close to the water signal. However, by observing the NH resonances in H_2O–DMSO mixtures one can extrapolate the assignments in DMSO solution to those for aqueous solution.[15,16]

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[16] P. H. von Dreele, A. I. Brewster, J. Dadok, H. A. Scheraga, F. A. Bovey, M. F. Ferger, and V. du Vigneaud, *Proc. Nat. Acad. Sci. U.S.A.*, 1972, **69**, 2169.

TABLE 2

J_{NC} Spin-coupling constants, NH proton chemical shifts, and their temperature dependencies for the component peptides of LH-RH in H_2O and DMSO solution

Compound	<Glu-His-OMe		Trp-Ser-Tyr-Gly		Leu-Arg-Pro-GlyNH$_2$		<Glu-His-Trp-Ser-Tyr-Gly	
Obs. freq. (MHz)	100	220	100	220	100	220	100	220
Temp. (°C)	30	20	30	20	30	30	30	20
Solvent	H_2O	$(CD_3)_2SO$	H_2O	$(CD_3)_2SO$	H_2O	$(CD_3)_2SO$	H_2O	$(CD_3)_2SO$
pH	2·2		3·0		2·7		1·8	
Internal ref.	DSS	TMS	DSS	TMS	DSS	TMS	DSS	TMS
<GluNH	7·85	7·83					7·62	7·71
dδ per 10 °C	6·8	4·7					6·1	5·7
HisNH	8·70	8·36					8·12	8·14
dδ per 10 °C	7·4	5·8					6·1	5·4
J_{NC}(Hz)	7·7	7·3					7·5	7·1
TrpNH							8·13	8·20
dδ per 10 °C							7·3	5·4
J_{NC}(Hz)							7·5	7·6
SerNH			8·25	8·78			8·33	8·34
dδ per 10 °C			5·5	8·5			5·8	6·0
J_{NC}(Hz)			7·9	7·0			8·0	7·8
TyrNH			8·00	8·16			7·92	8·00
dδ per 10 °C			7·6	7·0			6·2	5·7
J_{NC}(Hz)			6·8	8·2			7·2	7·1
GlyNH			8·04	8·23			8·16	8·40
dδ per 10 °C			7·0	4·4			6·7	5·4
J_{NC}(Hz)			5·8	4·7			5·5	6·0
Arg-NH					8·71	8·76		
dδ per 10 °C					7·0	4·5		
J_{NC}(Hz)					7·0	7·0		
GlyNH					8·51	8·18		
dδ per 10 °C					8·0	4·5		
J_{NC}(Hz)					5·8	5·6		

The dδ per 10 °C values were all measured in Hz at 100 MHz.

The α-CH, β-CH_2, and other side-chain proton resonances have very similar chemical shifts in the decapeptide and in its component peptides.

The ^{13}C assignments were made from comparisons with model peptides [17,18] and from considering the self-consistency of the shifts within this series of related peptides. The ^{13}C spectrum of the decapeptide LH-RH was very similar to that expected by adding together the spectra of the *C*-terminal tetrapeptide and *N*-terminal hexapeptide. Concurrent with this work Smith and his co-workers [19] have investigated the ^{13}C spectrum of the LH-RH decapeptide. Our assignments are in agreement for the aromatic region of the spectrum but we differ with their assignments for α-C of Ser, His, Trp, and Leu, β-C of Leu, and δ-C of Arg; while our assignments are more consistent with the observed ^{13}C chemical shifts in model peptides and the component peptides of LH-RH it should be realised that all the assignments are made solely on the basis of chemical shift considerations. The danger in using a model compound approach for making ^{13}C assignments is that it is possible for this to prevent the detection of chemical shift differences between the model compounds and the compound under study. However, the larger the available component fragments of the molecule the less likely is one to make assignment errors. In the case of the LH-RH decapeptide, the excellent agreement between the ^{13}C shifts of the model peptides, component peptides, and those of the LH-RH decapeptide itself allows one to make the assignments with confidence. Figure 5 shows the assignments on the high field signals of the ^{13}C spectrum of the LH-RH decapeptide. Proline is one of the residues known to be capable of causing substantial shielding effects on the ^{13}C nuclei in a neighbouring amino-acid [17] and we observe such effects in the Arg ^{13}C spectrum of LH-RH; here the α-CH signal is *ca.* 1 p.p.m. to higher field than the value normally observed for Arg when not in an -Arg-Pro- containing peptide.[17]

TABLE 3

J_{NC} Spin-coupling constants, NH proton chemical shifts, and their temperature dependencies for LH-RH in H_2O solution

Band	Chemical shifts (p.p.m.) *	dδ per 10 °C (Hz) †	J_{NC} (Hz)
A	8·52	8·1	5·5(t)
B	8·44	7·1	7·0(d)
C	8·34	6·8	7·0(d)
D	8·28	7·7	5·5(t)
E	8·24	6·8	6·7(d)
F	8·17	6·2	7·4(d)
G	8·02	7·4	6·2(d)
H	8·01	7·4	6·8(d)
<GluNH	7·65	6·8	

* Chemical shifts referred to DSS internal reference measured at 270 MHz at 18 °C. † Measured at 100 MHz.

t = Triplet (Gly); d = doublet. J_{NC} Errors: bands B, C, F, H ± 0·2 Hz; bands A, D, E, G ±0·3 Hz.

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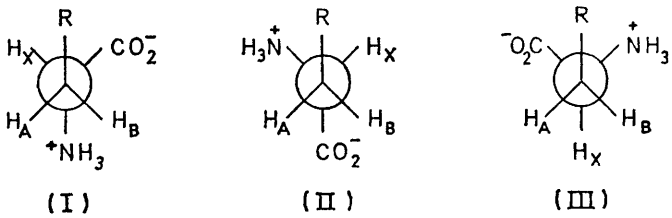
TABLE 4

^{13}C Chemical shifts and spectral assignments for LH-RH and its component peptides in D_2O solution

Compound		Trp-Ser-Tyr-Gly	Leu-Arg-Pro-Gly-NH_2	<Glu-His-Trp-Ser-Tyr-Gly	LH-RH
pD		3·10	2·65	2·55	5·45
<Glu	C_α			57·7	57·6
	C_β			26·2	26·2
	C_γ			30·4	30·3
His	C_α			55·5	55·5
	C_β			27·6	28·2 or 28·8
	C(2)			134·5	135·2
	C(4)			118·2	118·1
	C(5)			129·2	
Trp	C_α	54·6		53·3	53·4
	C_β	28·0		28·4	28·2 or 28·8
	C(2)	126·2		125·4	125·4
	C(3)	107·2		109·8	109·6
	C(4)	119·0		119·3	119·2
	C(5)	123·1		123·0	122·9
	C(6)	120·4		120·3	120·3
	C(7)	113·0		112·9	112·9
	C(8)	127·6		127·9	127·9
	C(9)	137·2		137·2	137·2
Ser	C_α	56·0 or 56·3		56·3	56·6
	C_β	62·5		62·5	62·3
Tyr	C_α	56·0 or 56·3		56·3	56·6
	C_β	37·3		37·4	37·0
	C(1)	129·1		129·1	128·9
	C(2), C(6)	131·6		131·6	131·4
	C(4)	155·5		155·5	155·5
	C(3), C(5)	116·5		116·4	116·5
Gly	C_α	43·0		42·6	43·6
Leu	C_α		52·6 or 52·9		54·0
	C_β		41·0		41·0
	C_γ		25·0 or 25·3		25·1 or 25·5
	C_δ		23·0		23·3
	$C_{\delta'}$		22·4		22·2
Arg	C_α		52·6 or 52·9		52·1
	C_β		28·6		28·2 or 28·8
	C_γ		25·0 or 25·3		25·1 or 25·5
	C_δ		41·9		41·7
	C_ϵ		157·7		157·5
Pro	C_α		61·9		61·8
	C_β		30·5		30·3
	C_γ		26·0		25·8
	C_δ		49·2		49·0
Gly	C_α		43·4		43·3
CO		173·7; 171·0; 171·3	175·5; 172·2; 171·2	182·8; 175·3; 174·1; 173·9; 171·9; 171·6	182·3; 175·4; 175·0; 174·2; 172·5; 172·3; 172·0; 171·8

Side-chain Conformations.—In some cases it was possible to carry out an ABX analysis of the α-CH–β-CH_2 multiplets and thus obtain the two vicinal H–H coupling constants. These coupling constants can be used to estimate the fractional populations p_I, p_{II}, and p_{III} of the rotamers (I)—(III) for the side chains in α-amino-acids.[20]

The measured vicinal coupling constants J_{AX} and J_{BX} are averaged values of the *gauche* (J_g) and *trans* (J_t) vicinal coupling constants in rotamers (I)—(III) and weighted according to the fractional populations. We assumed that the *gauche* and *trans* coupling constants are the same in the three rotamers and use the values J_g 2·56 and J_t 13·6 Hz obtained from model peptide studies.[11] The approximation used will lead to errors in the fractional populations but it is possible to use this approach to follow the trends in the fractional populations of the peptides studied. Often one cannot assign



the A and B protons unambiguously and in such cases it is impossible to distinguish between rotamers (I) and (II). The rotamer populations for the Ser α-CH–β-CH_2 side chains in Trp-Ser-Tyr-Gly, <Glu-His-Trp-Ser-Tyr-Gly and LH–RH decapeptide are $(p_I + p_{II}) = 0·54$; 0·62; and 0·53 and $p_{III} = 0·46$; 0·38; and 0·47: these are very similar to what is normally observed for Ser in peptides.[21] The high fractional population of rotamer (III) has been interpreted as arising from hydrogen bonding between the OH and the peptide NH groups. The Tyr and Trp α-CH–β-CH_2 side chain rotamer populations in <Glu-His-Trp-Ser-Tyr-Gly were found to be normal compared with rotamer populations measured for aromatic residues in other small peptides.[10,22] The populations of rotamer (III) for His in <Glu-HisOMe is 0·20 in contrast to the value observed (0·39) by Fermandjian and co-workers in TRF.[23] Apart from the Ser side chain it was not possible to measure any α-CH–β-CH_2 vicinal coupling constants for the LH-RH decapeptide because of extensive overlap of absorption bands even at 270 MHz.

Backbone Conformations.—To obtain backbone conformational information it is necessary to observe the multiplet splitting (J_{NC}) on the low field NH absorption bands resulting from the spin–spin interaction with the neighbouring α-CH protons. In DMSO solution and in aqueous solution at low pH value (pH < 4·0) the doublet and triplet (Gly) splittings are easily detected on the NH absorption bands (see Figures 1—4). At higher pH values in H_2O solution (pH > 6) the NH absorptions appeared as broad multiplets because of exchange with the solvent.

The assignments of the NH absorption bands were made in DMSO solution by double resonance experiments (at 270 MHz) to connect together the NH, α-CH, and β-CH_2 absorption bands; since the β-CH_2 assignments can often be made unambiguously this leads to α-CH and NH assignments. From mixed solvent studies using H_2O–DMSO mixtures we extrapolated the assignments from DMSO solution into H_2O solution for Trp-Ser-Tyr-Gly and <Glu-His-Trp-Ser-Tyr-Gly.

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[22] K. D. Bartle, D. W. Jones, and R. L'Amie, *J.C.S. Perkin II*, 1972, 650.
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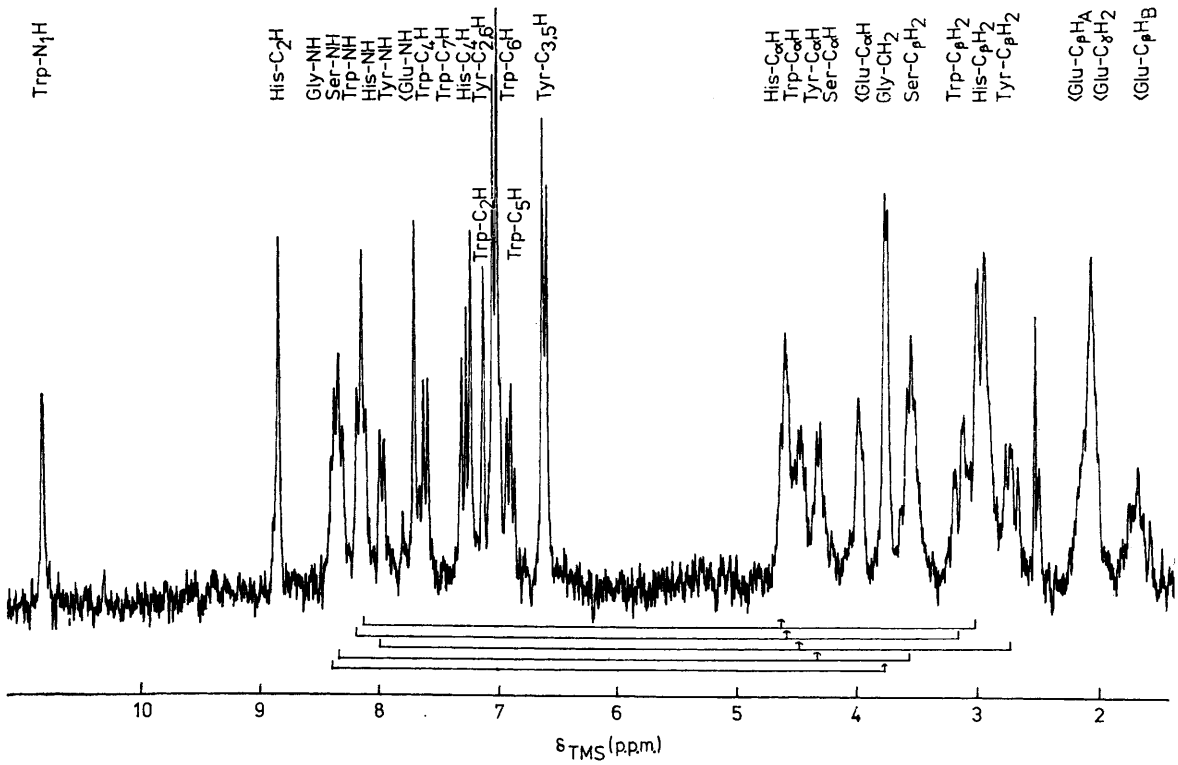

FIGURE 1 The ^1H resonance spectrum at 220 MHz of <Glu-His-Trp-Ser-Tyr-Gly in DMSO solution. The double resonance experiments are indicated on the spectrum

The NH region of the ^1H spectrum of the LH-RH decapeptide contains nine NH multiplets [the <GluNH singlet, six doublets and two triplets (Gly)] as indicated in Figure 4. Location of the positions of the over-

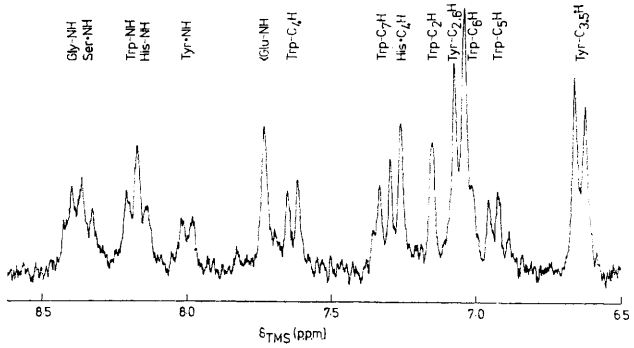

FIGURE 2 The low field region of the ^1H spectrum at 220 MHz of <Glu-His-Trp-Ser-Tyr-Gly in DMSO solution

lapping multiplets was facilitated by comparisons of the 270, 220, and 100 MHz spectra at various temperatures. Thus one can measure the J_{NC} coupling constants and the temperature dependencies of the multiplets and these are reported in Table 3. The ^1H chemical shifts are similar to those measured in <Glu-His-Trp-Ser-Tyr-Gly and the GlyNH of Leu-Arg-Pro-GlyNH_2. For example, the chemical shifts of GlyNH triplets measured in the hexa- and tetra-peptide [8·24 (Gly), 8·59 p.p.m. (GlyNH_2)] agree remarkably well with the Gly triplets in the decapeptide [8·28 (Gly) 8·52 p.p.m. (GlyNH_2)] after correction for the temperature effect. However the chemical shift agreement alone is not sufficient to provide an unambiguous assignment of the bands. At the present time we have not completed a detailed assignment for these NH absorption bands using spin decoupling as a result of the complexity of the α- and β-CH_2 region of the proton spectrum. However, be-

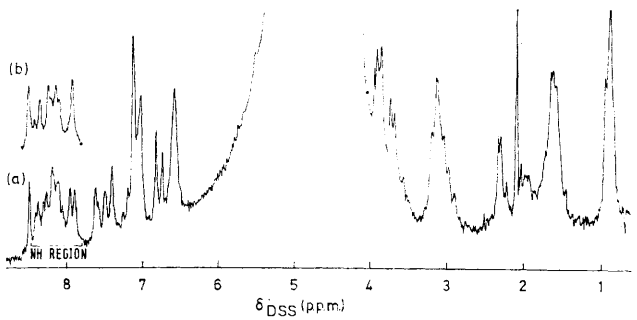

FIGURE 3 (a) The ^1H resonance spectrum at 100 MHz of LH-RH decapeptide in H_2O solution. (b) The ^1H resonance spectrum at 100 MHz of the NH absorption decoupled from the α-CH protons in H_2O solution

cause all the J_{NC} coupling constants and the NH temperature dependencies show no major difference from each other the detailed assignments will not provide significantly more conformational information.

NH *Proton Chemical Shifts.*—The observed ^1H chemical shifts for the assigned NH protons in the component

peptides of LH-RH are all *ca.* 0·2 p.p.m. to higher fields of the values estimated for NH protons from studies of Gly-X and X-Gly dipeptides.[24] It is also noted that the

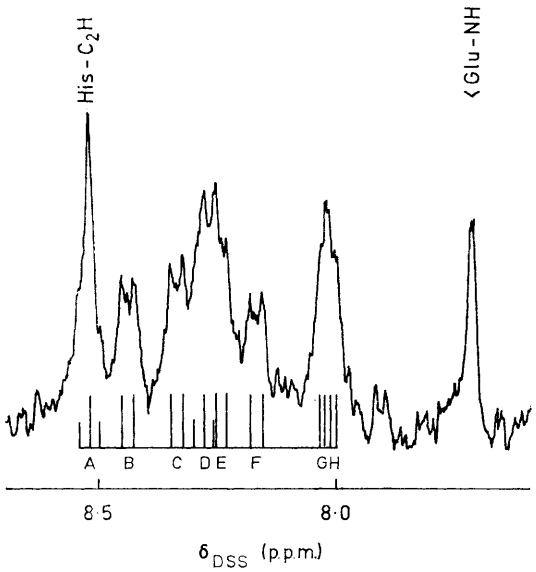

FIGURE 4 The NH region of the 270 MHz ^1H resonance spectrum of the LH-RH decapeptide in H_2O solution

NH temperature coefficients are somewhat lower (*ca.* 1 Hz per 10 °C lower) in the larger peptides than the values measured for glycyl-X dipeptides.[24] This probably reflects the slightly decreased accessibility of the involving other peptide NH protons.[15] The temperature coefficients for such peptide NH protons can be increased from the normal values by the presence of this exchange process. This could be a possible explanation for the abnormally high temperature coefficient (8·5 Hz per 10 °C) for SerNH in Trp-Ser-Tyr-Gly in DMSO which contained a trace of water. Studies on simple di- and tri-peptides[24] also indicate that *N*-terminal peptide NH protons are deshielded by 0·2—0·4 p.p.m. compared with the shielding of a similar peptide NH proton (*i.e.* between the same amino-acid residues) but located at a non-terminal position in a peptide chain.

It is clear from the observed normal temperature dependencies (*ca.* 6—8 Hz per 10 °C) of the NH protons in the LH-RH decapeptide in water and its component peptides in both water and DMSO solution that none of the NH protons is involved in strong intramolecular hydrogen bonds which could hold the molecules in a fixed conformation. The observed J_{NC} coupling constants in LH-RH are all fairly large (6·2—7·4 Hz) and are compatible with values expected for a random coil molecule.[9] However, the J_{NC} values are somewhat smaller than those observed in <Glu-His-Trp-Ser-Tyr-Gly (7·2—8·0 Hz). These higher values are consistent with a distribution of conformations as described by potential energy maps such as those calculated by Scott and Scheraga[25] for a dipeptide where the conformers exist for 70% of their time with ϕ angles in the region −80 to −105° (calculated J_{NC} 7·9 Hz).[10] The lower J_{NC} values measured in LH-RH could be accounted for by assuming

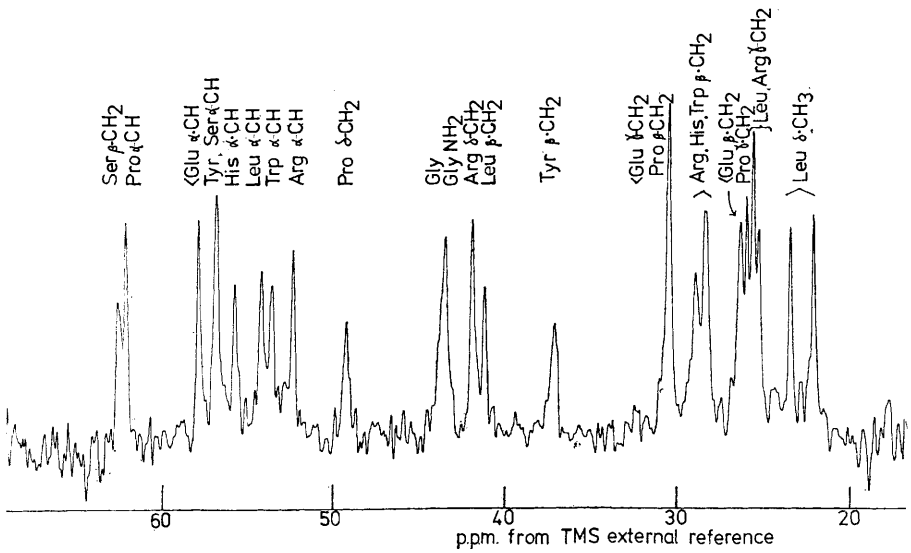

FIGURE 5 Part of the ^{13}C proton noise decoupled spectrum at 25·2 MHz of the LH-RH decapeptide in D_2O solution using the Fourier transform technique: LH-RH (40 mg) in D_2O (0·3 ml) (pH 5·45); 70,000 transients; aquisition time 0·8 s

NH protons to the H_2O solvent molecules caused by the presence of the amino-acid side chains. Such effects could also be responsible for the observed shift differences between the NH protons in the component peptides of LH-RH and the values estimated from dipeptide studies.

Exchange between H_2O protons and terminal peptide NH protons (adjacent to NH_3^+) is greater than that a shift in the potential energy minimum compared with that for small peptides such that the conformers exist for 70% of the time in the region ϕ −70 to −90°. This would not constitute a major conformational difference

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[25] R. A. Scott and H. A. Scheraga, *J. Chem. Phys.*, 1966, **45**, 2091.

in terms of determining biological activity. Evidence that no dramatic changes in conformation are taking place when the LH-RH decapeptide is formed from the *N*-terminal hexapeptide and the *C*-terminal tetrapeptide is indicated by the agreement between ^{1}H and ^{13}C chemical shifts in the side-chain resonances for the LH-RH decapeptide and its component peptides. Some small chemical shift changes are observed but they were all $<0{\cdot}1$ p.p.m. and are probably due to aromatic ring current shifts arising from non-neighbouring aromatic rings. However these shifts are small and clearly one cannot have large populations of conformations where the aromatic groups are nearer than 5 or 6 Å to other side-chain protons. The aromatic ring protons themselves show very little ring current effects on their shielding which is evidence that there is no appreciable stacking of the aromatic rings in free solution. This possibility had been suggested for the bound conformation of LH-RH in the studies of Chang and his co-workers.[8] Grant and his co-workers [7] have suggested that the LH-RH molecules might exist in a preferential conformation with the two ends of the peptide in close proximity but no n.m.r. evidence for this can be found in free solution.

The large shift differences observed for the His C(2)–H and C(4)–H resonances in DMSO and water observed in all compounds are probably due to specific interactions between the DMSO and the imidazole ring. There is also some evidence of specific solvent interactions between DMSO and tyrosine. When one makes allowance for these solvent effects (which can be seen also for dipeptides) the chemical shifts of protons and the H–H side-chain coupling constants are similar in H_2O and DMSO and thus there is no large change in conformation in these two solvents.

The His C(2)–H and C(4)–H protons in <Glu-His-Trp-Ser-Tyr-Gly in DMSO have shifts to much lower fields than normally observed (see Figure 1): however, this is clearly an ionisation effect on the imidazole ring because by adding HCl to <Glu-His-OMe similar low field shifts were observed. The His C(2)–H and C(4)–H protons in the LH-RH decapeptide, <Glu-His-OMe, and <Glu-His-Trp-Ser-Tyr-Gly in H_2O at low pH have similar shifts which indicates absence of any electrostatic intramolecular interactions involving the charged imidazole ring with amino-acid residues in the remainder of the molecule. In angiotensin II, an electrostatic interaction between terminal free CO_2^- groups and charged imidazole rings has been suggested as a structure-forming interaction.[26] Of course in the LH-RH decapeptide this type of interaction is not possible because we have a terminal $CONH_2$ group. Likewise, by examining <Glu-His-Trp-Ser-Tyr-Gly at low pH values where we have a CO_2H group one would not expect to see such electrostatic interactions with the imidazole ring.

The Ser α-CH–β-CH_2 side-chain conformation is similar in LH-RH to that in its component peptides with a high population in rotamer (III) (0·47). No other vicinal coupling constants for α-CH–β-CH_2 were obtained for LH-RH. However in the component peptides the Ser, Tyr, His, and Trp side chains give spectra from which the vicinal H_α–H_β coupling constants could be measured. The values obtained were similar to those in dipeptides showing no unusual side-chain conformational preferences.[27]

From the ^{13}C spectra it is possible to comment on the configuration of the Pro residue: [28,29] the β- and γ-^{13}C chemical shifts for Pro in Leu-Arg-Pro-GlyNH_2 and the LH-RH decapeptide are consistent with the proline being in a *trans*-configuration.

Conclusions.—Thus the n.m.r. data supports a model of LH-RH decapeptide in which we have no special structure-forming associations (such as NH · · · OC intramolecular bonds) and with a distribution of conformers typical of a random coil configuration with 70% of the conformers having ϕ in the region of -70 to $-90°$. There is no appreciable stacking of the aromatic rings and no electrostatic intramolecular interactions involving the charged imidazole ring. A similar state of affairs was found for pentagastrin in solution.[10] It might well be that for small linear hormonal peptides where the activity is concentrated over a fairly small number of directly connected residues that the active conformation of the hormone can be organised during the binding process. However, until it is possible to measure the conformation of hormonal peptides bound to their receptors one can only speculate on such matters.

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