CHIROPTICAL PROPERTIES OF CARBA-ANALOGUES OF OXYTOCIN: CONFORMATIONAL CONSIDERATIONS* **

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Dedicated to Professor O. Hoffmann-Ostenhof on the occasion of his sixtieth birthday.

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The circular dichroic spectra of analogues of oxytocin with structural changes in the vicinity of amino-acid residue in position 1 (cysteine) have been measured. The structural alterations included elimination of the N^{α}-amino group and modifications of the disulphide bridge (instead of —S—S— the bridging was completed with —CH_2—S—, —S—CH_2—, —CH_2—CH_2—, —S—, —CH_2— and —CH_2—S—CH_2—). Comparison of the various curves enabled us to assign dichroic bands to separate electronic transitions in the amide group, in the aromatic ring of amino-acid residue in position 2 and in the disulphide bridge, partly in discrepancy with previously published data. We demonstrated in detail the effect of the amino group of the cysteine residue on chiroptical parameters. These results were used in discussion of some conformational aspects of molecules related to oxytocin, particularly with regard to localisation of the aromatic side chain of the amino-acid residue in position 2.

Measurement of spectral properties, in particular proton and ^{13}C nuclear magnetic resonance, have enabled to construct basic concepts on the spatial arrangement of the molecules of the neurohypophysial hormones oxytocin and vasopressin, and some of their analogues (see[2–6]), with a characteristic disulphide bridge closing a cyclohexapeptide ring. Synthesis, and demonstration of a high level of biological activities, of analogues of oxytocin in which sulphur atoms of the disulphide bridge were replaced by less reactive methylene groups[7] have shown that the disulphide bridge in the oxytocin molecule does not have a high degree of functional significance[8], rather its main role is in maintaining the cyclic arrangement of the molecule. Replacement of the disulphide group by a thioether or ethylene group should, however, produce at least some small local change of conformation although the conformation of the molecule as a whole need not be changed to any considerable degree. Local conformational changes obviously in some cases permit a more effective interaction with target tissues, which along with the greater degree of metabolic stability of

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so-called carba-analogues results in the observed increase in biological activity as compared to the parent hormones. For these reasons it was considered of interest to study spatial differences in these analogues in comparison with oxytocin and deamino-oxytocin.

In the present work we have measured circular dichroism of analogues of oxytocin with structural changes primarily in the amino-acid residue in position 1 (cysteine in the parent hormones) involving either elimination of the primary amino group or changes in the disulphide group —S—S— by substitution of thioether groups (—S—CH_2—, —CH_2—S—, —S— and —CH_2—S—CH_2—) or alkylene groups (—CH_2— and —CH_2—CH_2—), *i.e.* in some cases with enlargement or contraction of the ring. Comparison of chiroptical parameters in this series of analogues (formulae *Ic*–*Ij*) and with some further analogues and the parent hormone *Ia* provided results allowing interpretation at a stereochemical level and could possibly contribute to a more detailed knowledge of the structure–biological activity relationship for neurohypophysial hormones.

EXPERIMENTAL

Substances used: Peptides* used in this work had analytical and biological properties in full agreement with published reports: oxytocin[10] (*Ia*), deamino-oxytocin[11] (*Ib*), 1-carba-oxytocin ([6,1-cystathionine]-oxytocin[12], *Ic*), deamino-1-carba-oxytocin ([6,1-α-deaminocystathionine]-oxytocin[13], *Id*), deamino-6-carba-oxytocin ([1,6-β-deaminocystathionine]-oxytocin[14], *Ie*), deamino-dicarba-oxytocin ([1,6-α-aminosuberic acid]-oxytocin[14], *If*), [1,6-deaminolanthionine]-oxytocin[15] (*Ig*), [1,6-α-aminopimelic acid]-oxytocin[15] (*Ih*), [1,6-deaminohomolanthionine]-oxytocin[16] (*Ij*), [1-alanine,6-alanine]-oxytocin[17] (*II*), N^{α}-glycyl-[4-leucine, 8-isoleucine]-oxytocin[18] (*III*).

Spectroscopic measurements: Circular dichroism spectra were measured on the Roussel - Jouan Dichrograph CD 185, model II, in cells with an optical path length of 0·01 to 1·0 cm, at a temperature (unless otherwise stated) of 22 to 25°C. Solutions were prepared by weighing of dry samples, concentrations were about 0·5 mg/ml. Spectral data are presented in values of molar ellipticity $[\theta]$ (deg. cm^2 . $dmol^{-1}$) and are not corrected for the refractive index of the solvent. As solvents we used 0·05M-HCl–KCl buffer for pH 3, 0·01M phosphate buffer for pH 7·5 and 0·05M phosphate buffer (Na_2HPO_4–NaOH) for pH 12. Hexafluoroacetone trihydrate was the commercial product of Hynes Chemical Research Co.

For measurement of temperature dependence of the spectra we used a mixture of solvents: phospate buffer pH 7·5–ethanol–glycerol[19] (1 : 1 : 1) which forms transparent glass at low temperatures. Measurements in the range 10 to 80°C were carried out in thermostated cells (Wobser 10). The temperature in the cell was measured by means of a thermoelement. For measurements at temperatures down to −100°C we used the Roussel-Jouan cryostat cooled with liquid nitrogen which maintained temperature with a precision of ±0·5°C. Analysis of the dependence of molar ellipticity at 225 nm on temperature was carried out according to reference[20].

* The nomenclature and symbols used in this paper are as recommended by IUPAC-IUB, see[9].

TABLE I

Circular Dichroic Data of Oxytocin, 1-Carba-oxytocin and Their Deamino Analogues

Compound	Solvent[a]	λ,[b] nm ($[\theta] \cdot 10^{-3}$, deg cm^2 dmol^{-1})				
		tyrosine B_{2u} band	disulphide band	complex positive band	amide π-π^* band	short wavelength tyrosine band
Oxytocin (*Ia*)	buffer, pH 7·5	281 (−0·30)	250 (+0·9)	228·5 (+ 6·6)	s 203 (−54)	195 (− 78)
	buffer, pH 3·0	282 (−0·45)	250 (+1·9)	226 (+22·3)	[c]	196 (− 70)
	HFA	280 (−1·28)	250 (+3·1)	225 (+32·0)	195 (−46)	
Deamino-oxytocin (*Ib*)	buffer, pH 7·5	275 (−0·43)	251 (+0·3)	230 (+ 1·0)	s 203 (−65)	194 (−117)
	HFA	0	259 (+0·3)	225[d] (− 5·7)	199·5 (−69)	
1-Carba-oxytocin (*Ic*)	buffer, pH 7·5	279 (−0·37)	—	228 (+ 9·2)	s 203·5 (−34)	194 (− 48)
	buffer, pH 3·0	274 (−0·29)	—	228 (+10·8)	[c]	197 (− 41)
	HFA	278 (−0·68)	—	228 (+12·8)	200 (−46)	
Deamino-1-carba--oxytocin (*Id*)	buffer, pH 7·5	280 (−0·41)	—	227 (+ 3·5)	s 200 (−69)	194·5 (− 76)
	buffer, pH 3·0	281 (−0·37)	—	228 (+ 3·0)	s 202 (−66)	[e]
	HFA	0	—	223[d] (−15·5)	200 (−80)	

[a] HFA denotes hexafluoroacetone trihydrate; [b] s denotes shoulder; [c] a poorly defined shoulder; [d] a positive band appearing as a negative minimum; [e] not measured.

RESULTS

Parameters of curves of circular dichroism of compounds $I-III$, measured under comparable conditions, are presented in Tables I and II. Assignment of bands in the circular dichroic spectra of oxytocin and its analogues to individual transitions of the chromophores present has already been published[21-23]. From our measurements it would appear that the previous interpretation must be altered or complemented in some points.

Coleman and Blout[21] assigned the intense negative band in the spectrum of vasotocin and ornithine-vasopressin at 200 nm to a short-wavelength transition in the disulphide group. However, as obvious in Table II, neither partial nor complete replacement of the disulphide bridge with methylene groups leads to a significant change in the spectra in this region, neither in aqueous nor in hexafluoroacetone media. The presence of a short-wavelength transition of the disulphide group cannot therefore be demonstrated in oxytocin substances; in any case the rotational strength is much less than that assumed by the above investigators. The positive band in the region

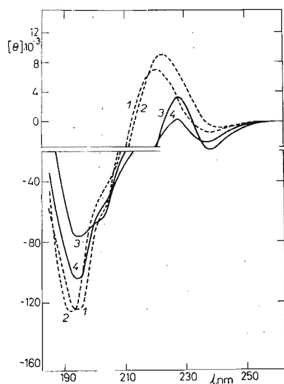


FIG. 1

Circular Dichroism of Deamino-6-carba-oxytocin (*Ie*) (*1*), Deamino-dicarba-oxytocin (*If*) (*2*), Deamino-1-carba-oxytocin (*Id*) (*3*) and of [1,6-Deaminolanthionine]-oxytocin (*Ig*) (*4*) in a Buffer of pH 7·5

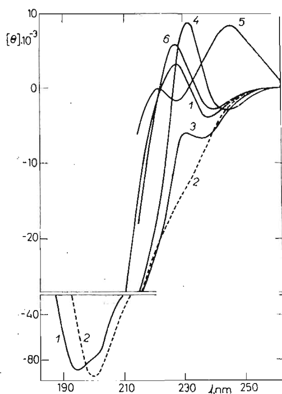


FIG. 2

Circular Dichroism of Deamino-1-carba-oxytocin (*Id*) in a Buffer of pH 7·5 (*1*), in Hexafluoroacetone Trihydrate (*2*), in 2,2,2-Trifluoroethanol (*3*), in Dioxane–Water (9 : 1) (*4*), in a Buffer of pH 12 (*5*) and in 8M Urea (*6*)

TABLE II

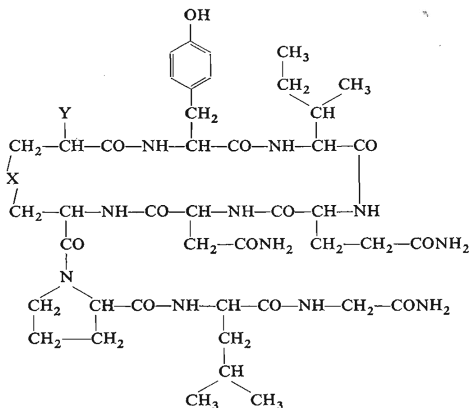
Circular Dichroic Data of Deamino-Oxytocin Analogues Modified at the Disulfide Bridge

Compound	Solvent[a]	λ,[b] nm ([θ] . 10^{-3}, deg cm^2 $dmol^{-1}$)			
		complex positive band[c]			
		tyrosine B_{1u} band	amide n-π^* band	amide π-π^* band	short wavelength tyrosine band
Deamino-6-carba-oxytocin (*Ie*)	buffer, pH 7·5	220·5 (+ 7·2)		s 203 (−57)	194 (−124)
	buffer, pH 12	243·5 (+ 2·5)	218 (+15·6)	—	—
	HFA	—	219[d] (− 4·0)	195·5 (−94)	—
Deamino-dicarba-oxytocin (*If*)	buffer, pH 7·5	222·5 (+ 9·1)		s 203 (−54)	192·5 (−129)
	buffer, pH 12	242·5 (+ 3·0)	218·5 (+11·3)	—	—
	HFA	227[d] (−10·0)	216[d] (−18·3)	200·5 (−64)	—
[1,6-α-Aminopimelic acid]- -oxytocin (*Ih*)	buffer, pH 7·5	223 (+ 9·6)		s 202·5 (−55)	194 (−100)
	buffer, pH 12	242 (+ 7·3)	219·5 (+11·0)	—	—
	HFA	223·5[d] (−12·8)		200·5 (−75)	—
Deamino-1-carba-oxytocin (*Id*)	buffer, pH 7·5	227 (+ 3·5)		s 200 (−69)	194·5 (−76)
	buffer, pH 12	245·5 (+ 8·4)	221·5[d] (0)	—	—
	HFA	223[d] (−15·5)		200 (−80)	—
[1,6-Deaminolanthionine]- -oxytocin (*Ig*)	buffer, pH 7·5	227 (+ 0·9)		s 201·5 (−61)	194 (−104)
	buffer, pH 12	246·5 (+ 3·3)	221[d] (− 4·0)	—	—
	HFA	226·5[d] (−12·8)	216[d] (−17·0)	200 (−62)	—
[1,6-Deaminohomolanthionine]- -oxytocin (*Ij*)	buffer, pH 7·5	225·5 (+ 1·8)		s 203 (−69)	193 (−130)
	buffer, pH 12	245 (+ 5·4)	220[d] (0)	—	—
	HFA	—	218[d] (−20·7)	198·5 (−81)	—
Deamino-oxytocin (*Ib*)[e]	buffer, pH 12	245 (+ 6·8)	221[d] (− 7·0)	—	—

[a] HFA denotes hexafluoroacetone trihydrate; [b] s denotes shoulder; [c] values of two bands are given if they can be distinctly seen; [d] positive bands appearing as negative minima or shoulders; [e] see also Table. I.

of 250 nm can be assigned without doubt to a transition of the disulphide group[21-23], modification of which results in its disappearance (Figs. 1, 2). It is, however, doubtful whether a transition of the same chromophore explains the negative extremum[23] observed between 230 and 240 nm in oxytocin at raised temperature or in dioxane solution. The same extreme which deepens with increased temperature or in dioxane solution (Figs 1–3) has also been observed in analogues without the disulphide group. The explanation of this extremum can probably be found in n-π^* transitions of the amide group (their long-wavelength tale, see[24]).

Detailed measurements in the region of the shortest wavelength have shown that the short-wavelength negative extremum is by no means a simple one (Figs 1, 4, Tables I, II). In all molecules containing tyrosine in position 2 we found in a neutral medium



Ia; X = S—S, Y = NH_2
Ib; X = S—S, Y = H
Ic; X = CH_2—S, Y = NH_2
Id; X = CH_2—S, Y = H
Ie; X = S—CH_2, Y = H
If; X = CH_2—CH_2, Y = H
Ig; X = S, Y = H
Ih; X = CH_2, Y = H
Ij; X = CH_2—S—CH_2, Y = H

Ala-Tyr-Ile-Gln-Asn-Ala-Pro-Leu-Gly-NH_2

II

Gly-Cys-Tyr-Ile-Leu-Asn-Cys-Pro-Ile-Gly-NH_2

III

a shoulder, more or less observable, at 200 to 203 nm, and a maximum at 193 to 195 nm, which is not present in compounds modified in position 2 (ref.[24]). In hexafluoroacetone we have observed in these parts of spectra only a negative maximum which usually, for instance in all deamino derivatives, lies at the site of the original shoulder at 200 nm (Figs 4, 5, Table II), *i.e.* the short-wavelength negative band disappears and at the same time the intensities of the aromatic bands B_{1u} and B_{2u} of the tyrosine residue (Table I) markedly decrease. These facts suggest that we can assign the negative maximum at 193 to 195 nm in all probability to electronic transition E_{1u} of the aromatic chromophore of tyrosine. The second, longer wavelength band appearing as a shoulder in a neutral medium and as a peak in hexafluoroacetone would then be assigned to π-π^* transition of the amide groups.

The characteristic positive band of oxytocin analogues at about 225 nm has been assigned to transition B_{1u} of the tyrosine chromophore[21,23]. It appears, however, that in this region in addition to the aromatic transition there is a positive dichroic band of at least one further transition. This would appear from a number of observations. A similar positive band can be seen in substances which do not contain an

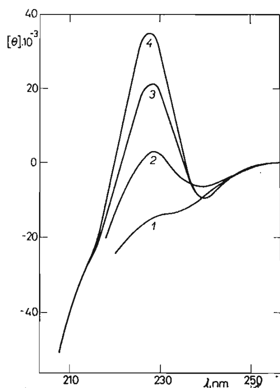


FIG. 3

Circular Dichroism of Deamino-1-carba-oxytocin (*Id*) in Water–Glycerol–Ethanol (1 : 1 : 1) at 70°C (*1*), 20°C (*2*), −40°C (*3*), −80°C (*4*)

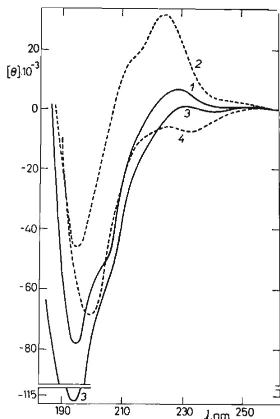


FIG. 4

Circular Dichroism of Oxytocin (*Ia*) in a Buffer of pH 7·5 (*1*), in Hexafluoroacetone Trihydrate (*2*) and of Deamino-oxytocin (*Ib*) in a Buffer of pH 7·5 (*3*), in Hexafluoroacetone Trihydrate (*4*)

aromatic amino acid or in which the aromatic amino acid in position 2 has no transition in the critical spectral region[24]. In hexafluoroacetone we can observe a short-wavelength shoulder at about 212–215 nm on the positive band of oxytocin (Fig. 4); with some deamino derivatives in hexafluoroacetone both components of the positive band can be well observed as not-very-marked maxima on a negative background (Fig. 5, curves 2 and 3). A still clearer differentiation of both components can be seen in alkaline media, where the B_{1u} band of the ionisable phenol group is bathochromically shifted (see ref.[21]). From spectra in an alkaline solution (Fig. 6, Table II) one can also estimate the relative intensities of both components of the band which depend upon the structure of heterodetic closure of the peptide ring. The positive extreme of oxytocin and its analogues which can be found in the wide range of 220 to 230 nm is therefore the result of superposition of two bands. The longer wavelength band can be assigned without doubt to the B_{1u} transition of the tyrosine side chain, the band lying at shorter wavelength, with regard to its position and hypsochromic shift in hexafluoroacetone, to a n-π^* transition of the amide group. Further justifications for this assignment become apparent from our studies of circular dichroism of linear and cyclic peptides[18,25,26] (see Discussion).

In the spectra of oxytocin analogues we therefore assign two extremes to the n-π^* transitions of peptide groups, a negative one between 230 and 240 nm and a positive one below 220 nm. A similar spectrum is not unusual and can be met with in a number of other polypeptide and oligopeptide systems (*e.g.* poly-L-lysine[27] in random-coil state, in short linear peptides[18] and in cyclic dipeptides[28]). It would appear that the presence of two energetically different n-π^* bands reveals the presence of two or more structurally different amide groups. Amide groups differentiate probably in their interaction with neighbouring structures, either with neighbouring groups in the peptide molecule or with solvent molecules. These interactions and the conformational situation of the given peptide group are in mutual relation.

Attention has already been called in the literature to the effect of the N^{α}-amino group of oxytocin and its state of ionisation on the course of CD spectra. Beychok and Breslow[22] have observed an increase in the intensity of the positive band of oxytocin (*Ia*) at 225 nm in an acid solution and suggest a direct interaction of the primary amino group with tyrosine. Urry and coworkers[23] have reported a lower amplitude of the positive band in deamino-oxytocin (*Ib*) which they explain as a decrease in thermal stability of the conformation. In the present series of oxytocin analogues, compounds of both types are present, amino and deamino analogues. The two pairs of amino and deamino analogues derived from oxytocin (*Ia*) and 1-carba-oxytocin (*Ic*) behaved in a parallel manner just as the third pair derived from oxytocin which lack the 7–9 amino acid tale. (Properties of this pair are described elsewhere[19,29]). The most marked differences in the behaviour of both types of analogues are observed in the region of the positive band at 225 nm (Table I, Fig. 4). Derivatives with a primary amino group have in neutral solution a markedly

higher intensity of the positive band. Ionisation of the primary amino group results in a further increase in band intensity, whereas in deamino derivatives a decrease in pH is without significant effect. A dramatic differentiation of both types of compounds occurs in hexafluoroacetone (Fig. 4). In amino derivatives the intensity of the positive band is still further increased, in deamino derivatives there is a marked decrease so that the positive band practically disappears. This observation therefore confirms the existence of an interaction of the primary amino group with tyrosine side chain.

In an acid medium and in hexafluoroacetone [2-O-methyltyrosine]-oxytocin and [2-pentafluorophenylalanine]-oxytocin behave in a similar manner as oxytocin[24]. The suggested interaction is therefore not conditioned by the hydroxyl group of tyrosine, either free or substituted, and is related rather to the bulkiness of the substituent in the side chain, its hydrophobic character and/or electronic properties of the aromatic nucleus (its π-electron system). The effect of distance of the primary amino group from tyrosine is illustrated by the behaviour of N^{α}-glycyl-[4-leucine-8-isoleucine]-oxytocin (*III*) (with the assumption that the changes in positions 4 and 8 do not have a significant effect on conformation at the amino terminal of the

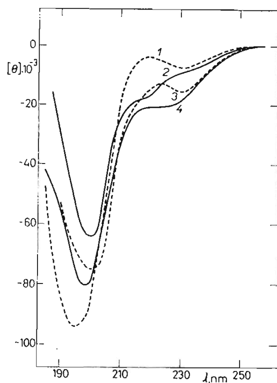


FIG. 5

Circular Dichroism of Deamino-6-carba-oxytocin (*Ie*) (*1*), Deamino-dicarba-oxytocin (*If*) (*2*), [1,6-α-Aminopimelic acid]-oxytocin (*Ih*) (*3*) and of [1,6-Deaminolanthionine]-oxytocin (*Ig*) (*4*) in Hexafluoroacetone Trihydrate

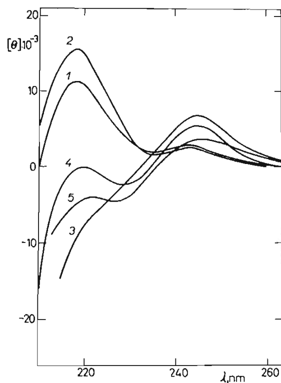


FIG. 6

Circular Dichroism of Deamino-dicarba-oxytocin (*If*) (*1*), Deamino-6-carba-oxytocin (*Ie*) (*2*), Deamino-oxytocin (*Ib*) (*3*), [1,6-Deaminolanthionine]-oxytocin (*Ig*) (*4*) and of [1,6-Deaminohomolanthionine]-oxytocin (*Ij*) (*5*) in a Buffer of pH 12

peptide chain). This compound behaves essentially as the other compounds with a primary amino group, the intensity of the positive band is, however, far less. In curves of compound *III* in neutral and acid media (Fig. 7) this band is hardly observable on the negative background of more intense bands, in hexafluoroacetone there is an increase but not to the same degree as that in the other amino derivatives. Linear nonapeptide *II*, the primary amino group of which is formally at the same distance from tyrosine as in oxytocin, shows properties of both of the discussed groups of analogues (Fig. 8). In neutral aqueous media there is a weak positive band, the spatial relation of both interacting groups is therefore in the linear peptide similar to that in cyclic analogues. Acidification of the solution to pH 2 produces an increase in the positive band as with amino derivatives, but transfer into hexafluoroacetone causes its disappearance as in deamino derivatives. The mechanism by which the intensity of the positive band in cyclic compounds is increased is therefore different in the two media. It would appear that in hexafluoroacetone this effect is in addition conditioned by a cyclic structure influencing the conformation of the critical part of the molecule. The same conclusions are suggested by circular dichroism spectra of vasopressin analogues with a primary amino group(*c.f.*[19,29]). In acid media these substances behave in the same manner as analogous molecules of oxytocin, in hexafluoroacetone, however, they behave differently. The intensity of the positive band in comparison with aqueous solutions decreases somewhat, probably because flexibility of the side chain of tyrosine is limited by interaction with the neighbouring phenylalanine residue.

All other deamino derivatives in this series have the expected properties, that is in hexafluoroacetone their positive band practically disappears (Figs 2, 5). However, in neutral media some of them have intensity of the positive band comparable with that of amino substances (Table II). In these cases the maximum of the band is at shorter wavelength which indicates that a short-wavelength n-π^* component

TABLE III

Thermodynamic Parameters of the Local Conformational Transition of Amino-and Deamino-1-carba-oxytocin in Water–Glycerol–Ethanol (1 : 1 : 1)

Compound	ΔH kcal mol^{-1}	ΔS cal grad^{-1}	$K_{0°C}$	$\Delta G_{0°C}$ kcal mol^{-1}	$K_{20°C}$	$\Delta G_{20°C}$ kcal mol^{-1}
1-Carba-oxytocin (*Ic*)	3·9 ± 0·3	13·0 ± 1·1	0·49	0·4	0·77	0·16
Deamino-1-carba-oxytocin (*Id*)	4·0 ± 0·2	13·6 ± 0·8	0·67	0·31	1·21	−0·14

is responsible for the increased intensity (compare the splitting of the positive band in alkaline medium, see p. 1278).

The effect of hexafluoroacetone, known as a helix-breaking solvent, has been described above in a number of examples. The effect of a number of other non-aqueous solvents was also briefly tested[19] (Fig. 2). 2,2,2-Trifluoroethanol, strongly polar as well, has a similar (but lesser) effect as hexafluoroacetone, although it acts as a helix-forming solvent on macromolecular peptides. Dioxane increases the intensity of the positive band even in the absence of a primary amino group. Urea and guanidine hydrochloride appear to be without any specific effect on the CD spectrum. In terms of the nature of forces stabilising the conformation of oxytocin analogues in aqueous media, it would appear that we are dealing with a nonspecific interaction of side chains (steric interaction at the Tyr-Ile bond and π-π interaction of the aromatic ring with the amide group) rather than hydrogen bonds.

The CD spectra of oxytocin analogues are markedly dependent upon temperature[23]. With increasing temperature, the intensity of the positive band decreases and there is also a hypsochromic shift of the negative extreme between 230 and 240 nm. On the other hand, a decrease in temperature results in an increased intensity of the band in certain cases to markedly high values (Fig. 3). These temperature effects are a general characteristic of the compounds studied here, without regard to the modification at position 1, the structure of the ring junction and the character of the solvent[19]. The influence of the temperature on the intensity of the positive band of the disulphide group at 250 nm is not so pronounced. Circular dichroic spectra therefore indicate a change in conformation in the region of the tyrosine residue. This change without doubt involves an increase in flexibility of the tyrosine side chain, but probably also a shift in its prevalent conformation to other values of torsion angles φ, ψ, χ.

We have also measured the temperature dependence of ellipticity at 225 nm with 1-carba-oxytocin (*Ic*) and deamino-1-carba-oxytocin (*Id*) (in a range from +70 to −100°C) and have attempted to calculate thermodynamic parameters of this conformational transition. The curves have a slightly sigmoidal character. However, we were not able to measure in the given experimental conditions at sufficiently high temperatures to reach the assumed state of complete conformational disorder. We therefore do not attach absolute importance to the thermodynamic values presented in Table III. It is, however, significant that these values are practically the same for both substances.

Marked differences in the behaviour of amino and deamino derivatives were also observed in hexafluoroacetone in the region of the aromatic transition B_{2u} (about 275 nm). In comparison with a neutral aqueous solution, this band was markedly increased in hexafluoroacetone in the case of amino substances whereas in deamino substances there was a decrease up to complete disappearance (see *e.g.* Table I).

This suggests that there is a conformational change in the tyrosine side chain in both types of substance, but presumably opposite in sign.

Interesting effects of the presence of the primary amino group can also be observed in the region of short wavelength negative bands. In neutral media amino substances show lower values of ellipticity (by 30000 to 40000 units) in the shoulder at 202 nm and the maximum at 194 nm (Table I). This effect again indicates that the primary amino group influences the conformation of the aromatic amino acid (194 nm band) but evidently also the conformation of the backbone peptide chain (π-π^* band at 200 nm). A similar situation is found in the case of the simple negative maximum observed in hexafluoroacetone (Table I). The simplified spectra in this region in hexafluoroacetone can be explained by an essential decrease of the aromatic band E_1, *i.e.* by a change in conformation in both types of analogues. Along with the fact that ionisation of the primary amino group by decrease in pH in aqueous solution does not lead to a significant change in the spectra in this region of wavelengths (not even of the B_{2u} band) this indicates that increase in the intensity of the positive band at pH 2 and in hexafluoroacetone is due to different mechanisms and in hexafluoroacetone is always associated with a change in conformation of the tyrosine residue.

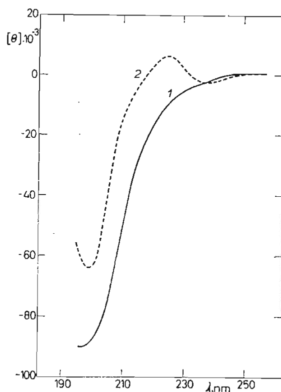


FIG. 7

Circular Dichroism of N$^{\alpha}$-Glycyl-[4-leucine, 8-isoleucine]-oxytocin (*III*) in a Buffer of pH 7·5 (*1*) and in Hexafluoroacetone Trihydrate (2)

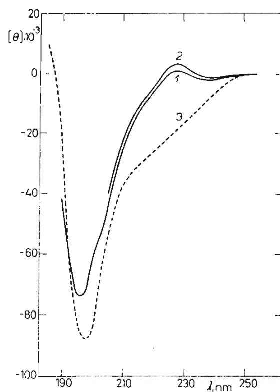


FIG. 8

Circular Dichroism of [1-Alanine, 6-alanine]-oxytocin (*II*) in a Buffer of pH 7·5 (*1*), in a Buffer of pH 2·5 (2) and in Hexafluoroacetone Trihydrate (3)

The second group of analogues in this series in which we can successfully compare dichroic spectral properties are the deamino derivatives with a modified ring closure (*Id*–*Ij*). Structural features of the ring-closing part influence the spectra of these analogues to the least degree in the region of the shortest wavelengths (Table II). In neutral aqueous media the positions of both short-wavelength bands are the same in the entire group. Values of ellipticity in the shoulder at 200 to 203 nm show only insignificant variations from mean values (with regard to the high experimental error of ± 4000). This band is associated with π-π^* transition of the amide group and its rotational strength depends primarily on the spatial relations of mutually interacting amide groups, *i.e.* on the conformation of the backbone peptide chain. It can therefore be suggested that structural changes of the disulphide bridge, or decreases or increases in ring size, do not have any basic effect on the distribution of dihedral angles of the backbone peptide chain. These experimental results are not completely compatible with the tentative suggestion of Hase and coworkers[30] that a change in the character of the heterodetic ring-closing part influences the main peptide chain as a whole. This suggestion was primarily based upon the assumption of validity of the oxytocin model proposed by Urry and Walter[31] which need not be necessarily true in all situation[32].

The negative band at 194 nm, which is associated with aromatic transition E_1 of the tyrosine chromophore, shows significantly lower values of ellipticity in 1-carba-derivative *Id*, somewhat higher values in both analogues with a constrained ring (*Ig*, *Ih*) and still higher values in the other substances. It is not surprising, however, that structural changes in the neighbourhood of the tyrosine residue (*e.g.* the position of the sulphur atom) influence the rotational strength of its electronic transitions.

In hexafluoroacetone both short-wavelength bands join in a single negative maximum as a result of a decrease in the intensity of the aromatic band and probably also a blue shift of the amide π-π^* band (Table II, Figs 4, 5). The ellipticity and wavelength of this maximum show marked fluctuation in the present series of analogues without obvious relationship to structure. This scatter would appear to be the result of changing relative intensities of bands which make up the negative maximum.

A very characteristic relationship to the structure of the heterodetic closure of the ring can again be observed in the complex positive band at 225 nm. The given deamino derivatives can be subdivided into two groups (Table II). The first, including deamino-6-carba-oxytocin (*Ie*), deamino-dicarba-oxytocin (*If*) and [1,6-α-amino-pimelic acid]-oxytocin (*Ih*), show in neutral aqueous solutions an intense positive band, the maximum of which lies at relatively short wavelengths (220 to 223 nm, Fig. 1). With these analogues in alkaline media, we meet with two positive bands. In addition to the tyrosine band at 243 nm there is a more intensive one (n-π^*) at 218–219 nm (Fig. 6). In the second group, including deamino-oxytocin (*Ib*), deamino-1-carba-oxytocin (*Id*) and [1,6-deaminolanthionine]-oxytocin (*Ig*) we find in neutral media a positive band of far lower intensity and at higher wavelength (226–230 nm).

In alkaline solutions, analogues of the second group show only one band belonging to tyrosine. The intensity of the n-π^* band, if observable at all, is very low. Analogues in the first group, which have a much higher n-π^* component in the complex positive band, can be formally characterised by the presence of a methylene group instead of a sulphur atom in amino-acid residue in the position 6. Analogues in the second group retain the sulphur atom at this position. An exception is [1,6-deaminohomolanthionine]-oxytocin (*Ij*) which structurally belongs to group 1, spectrally to group 2. The isolated aromatic band, as can be seen from its intensity in alkaline media (Table II, Fig. 6), is much less sensitive to modifications of the disulphide bridge and its changes do not meet with changes in the n-π^* component.

DISCUSSION

Conformational Considerations

A number of specific problems are met with in trying to use circular dichroic spectra for analysis of conformation of molecules of the size of oxytocin. Such molecules are not sufficiently large that their spectra can be interpreted in terms of regular structures such as helical conformations. On the other hand these molecules are too complex to allow all structural components to be identifiable by means of spectroscopical methods as it was possible with other types of organic molecules. From this point of view it is an advantage that in circular dichroism of oxytocin-like compounds, in addition to electronic transitions of the peptide bond, there are also manifest transitions of chromophores in the side chain of comparable intensity which in favourable cases allow us to localise the source of the observed spectral change to a given part of the molecule. Similarly, one can in certain cases take advantage of differentiated properties of amide groups.

Despite this the number of distinct bands in the circular dichroic curves of oxytocin and similar compounds is far less than the number of structural elements giving rise to the absorption in the accessible region of the spectrum. Most of the molecule contributes little of a specific nature to modelling of the curve. A favourable combination of factors allows us to interpret with some degree of accuracy changes of parameters of the positive band at 225 nm as a change in conformation in the critical region of the heterodetic ring-closing part and amino-acid residues 1 and 2. It cannot, however, be excluded that the same structural changes in the molecule do not result in significant conformational changes in other parts of the molecule where the circular dichroic spectrum is insensitive. Two or more simultaneous structural changes can compensate one for the other in their spectral effect. For these reasons meaningfull conformational conclusions can be derived only by comparing spectra of analogues with structural modifications at a single site in the molecule. With these facts in mind, we can attempt to discuss what influence is imposed on the conformation of the

critical part of the molecule by individual structural features altered in the present series: *a*) by the presence or absence of the amino group of the amino-acid residue in sequence position 1; *b*) by the character of the heterodetic ring-closing part.

Since an increase in temperature in neutral media causes qualitatively (and approximately quantitatively) the same spectral changes in amino and deamino derivatives, the nature of the stabilising interactions disturbed by temperature in both types of compounds are probably similar. The primary amino group in this case plays a role only as a steric factor. Transfer of the substance into hexafluoroacetone is always associated with marked conformational changes because this solvent disturbs the interactions stabilising conformation in an aqueous solution by its polarity and acidity. In deamino derivatives the effect of increased temperature and hexafluoroacetone are similar. The result of both are obviously similar conformational changes (essentially an increase of conformational freedom of the entire molecule). On the other hand, in amino derivatives, hexafluoroacetone has the opposite effect – an increase in the intensity of the positive band similar to the effect of decreasing pH in aqueous solution. In these cases we are obviously dealing with the acidity of hexafluoroacetone changing the ionisation of the primary amino group and thus causing a different conformational change than in deamino derivatives. The basis of this could be attractive interaction between the positive charge of the amino group and the π-electron system of the aromatic ring (with simultaneous nullification of other forces stabilising conformation) which increases the population of local conformations resulting in a positive band at 225 nm.

The intensity of the n-π^* component of the positive band is clearly related to the presence and position of the sulphur atom. Modification of the heterodetic ring-closing part obviously influences local conformation in its immediate neighbourhood, *i.e.* residue 1 and 2, particularly in the side chains. The sulphur atom connected to residue 6 or in the enlarged ring of [1,6-deaminohomolanthionine]-oxytocin (*Ij*) retains a similar spatial relation to its immediate environment as in the original molecule of deamino-oxytocin (*Ib*). The contribution of this local conformation to dichroic absorption in the region of the positive band at 225 nm is either insignificant or negative. It is possible that in analogues of this group the sulphur atom is oriented towards the same side of the ring as the aromatic side chain. There can be some degree of interaction between the free electron pairs of this atom and the electron system of the aromatic nucleus leading to the fixation of the local conformation which would be different, in the same part of the molecule, in compounds without a sulphur arom at this position. However, the phenomena of dichroic absorption are very sensitive to all structural changes. One can stress here deamino-vasopressin[29] which as opposed to deamino-oxytocin (*Ib*) has an intensive positive n-π^* component, apparently as a result of changes in conformation caused by stacking interaction of neighbouring aromatic nuclei.

A relatively close model for the discussed part of the molecule of the oxytocin

series are peptides with the sequences Gly-L-Phe-L-Leu, or Gly-L-Phe(OMe)-L-Leu which have been studied in this laboratory in other connections[18]. Linear peptides (tripeptides and hexapeptides) with this sequence at the amino terminal show in hexafluoroacetone (comparable data in aqueous solvents are not available because of the insolubility of these substances) an intensive positive band at 218 or 225 nm (according to the character of the aromatic chromophore) and a negative band above 238 nm. Here as well the positive band has a complex character and its n-π^* component is probably associated with the carbonyl group of the aromatic amino-acid residue. On the other hand, cyclohexapeptides of the same sequence not having a primary amino group show a positive band in trifluoroethanol but not in hexafluoroacetone. Their spectra in the latter solvent are strikingly similar to that of deamino-oxytocin derivatives.

The presence of a positive band at 225 nm in the circular dichroic spectra of oxytocin analogues is therefore probably given by the sequence of the amino terminal tripeptide L-Cys-L-Tyr-L-Ile and its conformation, including the side chains of the first two amino-acid residues. The main source of rotational strength of this band is the aromatic nucleus of tyrosine, both in its own dichroic band and with its vicinal effect on the n-π^* transition of the carbonyl group in its environment. In this conformation the free primary amino group of the neighbouring cysteine residue is able to interact with the side-chain of tyrosine and limit its available conformational space. The nature and intensity of the interaction depends upon the character of the medium. We can gain some information on the type of these local conformations from the linear peptide molecules referred to. For tripeptides Gly-L-Phe-L-Leu and Gly-L-Phe(OMe)-L-Leu (blocked at the amino terminal) proton magnetic resonance has shown torsion angle φ of the aromatic amino acid practically zero, *i.e.* an extended conformation should be present[33]. The side chain of the aromatic amino acid is oriented towards the amino terminal of the molecule, so that the C_β-C_γ bond lies between the C_α-H and N atoms (torsion angle $\chi_1 = 60°$). Analogy of structure and similarity of the spectra of circular dichroism suggests the concept that in oxytocin-like molecules a significant population has an analogous local conformation at least in hexafluoroacetone. In this local conformation, which is very plausible on analysis of models, there is close contact between the aromatic component and the primary amino group and also the aromatic nucleus and the heterodetic ring-closing part. In hexafluoroacetone there is no interference with this position of the tyrosine side-chain from the acyclic tripeptide carboxyl terminal of the molecule, bound to the primary amino group by a hydrogen bond in aqueous solution according to Walter and coworkers[34]. The lower intensity of the positive band at 225 nm, observed with the amino derivative dissolved in neutral aqueous solution, could then be explained by rotation of the tyrosine side chain above the plane of the cyclohexapeptide ring.

Biochemical Aspects

The high degree of biological activities of monocarba-analogues of oxytocin show that the disulphide group is not important for producing the biological effect. There is, however, the possibility that the mechanism of action of oxytocin and its carba-analogues is different. Since the basic mechanism of interaction is not known, we can neither support nor exclude the latter possibility. Some indirect evidence exists that the effects of oxytocin and its carba-analogues are similar, such as the same time course of the actions and the same slopes of log-dose-*vs*-response curves[35].

The similar spatial arrangements of oxytocin and its carba-analogues determined on the basis of measurement of their circular dichroism curves is a further indirect support for the concept that the mechanism of action is the same. It is known, from studies of the relationship of biological activity to chemical structure using synthetic analogues, that spatial arrangement is important for a high degree of biological potency. To some extent, however, the presence of separate functional groups also plays a role both in local spatial arrangement and in biological activity. This effect is manifest quantitatively in various ways and in some cases there are even qualitative changes (inhibition). These facts can be understood only on the basis of knowledge of detailed spatial arrangements. If we interpret a change in conformation of tyrosine in deamino-oxytocin on transfer from water to hexafluoroacetone as a certain degree of conformational freedom in reaction with a receptor, then steric interaction between N^{α}-amino group of cysteine and the tyrosine residue in sequence position 2 requires the molecule to take up a functionally less favourable conformation. This could be the explanation of why practically all deamino analogues have a higher degree of biological activity than their corresponding amino derivatives. An increase in biological activity does not occur in those deamino analogues where the amino derivatives have such a structural alteration in which there can be no interaction between amino group and tyrosine side chain. For instance, [2-D-tyrosine]-deamino-oxytocin[36] has a lower activity than [2-D-tyrosine]-oxytocin[36], practically the same activities (with a few exceptions related to difficulties in testing) are shown by [2-phenylalanine]-oxytocin[37] and its deamino analogue[38]. Similar activities are also shown by [2-isoleucine]-oxytocin[39] and [2-isoleucine]-deamino-oxytocin[39].

The biological activities of both monocarba-analogues in all basic pharmacological tests are higher than the activities of oxytocin, and in most cases also higher than deamino-oxytocin. It is interesting in this regard that in no tests are these analogues equally active, usually in a given assay there is a difference by at least a factor of 2. This differentiation of activities is obviously very complex in nature and not only conformational arrangements of both thioether bonds (1-carba- and 6-carba), *i.e.* steric „fit'' with the receptor (which can be different for each separate target organ), play a role but also the rate of elimination of both analogues from the receptor

compartment[40] and to some degree the rate of biological inactivation[41] will also participate.

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