Peptidic Hormone Interactions at the Molecular Level -Preparation of Highly Labelled ^{3}H Oxytocin

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SUMMARY

The tritium labelling of oxytocin has been attained by a two step procedure. First, di-iodo oxytocin is prepared. After a survey of various iodination reagents, ICl has been selected as it reacts much more rapidly with the tyrosyl ring than with the disulfide bridge, which under mild conditions remains unaffected. The di-iodo derivative, nevertheless, has lost most or all of its biological activity. Catalytic substitution of the peptide bound iodine by tritium results in labelling the hormone and in restoration of the biological activity. The ^{3}H oxytocin obtained retains 90 % of its hydrosmotic activity and about 65 % of its avian depressor activity. Its specific radioactivity reaches 36 Ci/mMole, a value allowing further studies of the hormone at a molecular level.

Experimental endocrinology has first defined the physiological significance of the posterior pituitary hormones. Their mode of action is now under investigation. This prospect would be greatly facilitated if labelled hormones were available, provided their specific radioactivity is high enough to enable their detection at physiological concentrations, 10^{-9} to 10^{-10} M. Labelled posterior pituitary hormones have already been obtained by different technique. The Wilzbach method, used by Schwartz *et al.* [1] and by du Vigneaud *et al.* [2] leads to extensive degradation and to specific radioactivities of about 0.1-15 mCi/mMole. The incorporation by synthesis of labelled aminoacid into the peptide chain constituting the hormones as achieved by du Vigneaud *et al.* [2]

and by Sjöholm *et al.* [3] has given interesting results, 2 to 3 Ci/mMole, at the cost however of a difficult set of operations. Finally catalytic dehalogenation, as used by Agishi and Dingman [4] has made possible the preparation of oxytocin with specific activity of 230 mCi/mMole. As the latter method is much simpler in principle than the previous techniques, it has been subjected to extensive study. The present report shows that it is possible to halogenate oxytocin, a tyrosine containing peptide, by a reagent leaving the disulfide bond unharmed, and to substitute the halogen by a tritium atom under conditions leading to specific radioactivities up to 35 Ci/mMole which are in the range required for physiological experiments. The technique described is applicable to a number of other compounds provided they contain aromatic rings. For instance labelled actinomycin, puromycin, as well as angiotensin II [5], have been prepared.

MATERIAL AND GENERAL METHODS.

Synthetic oxytocin was kindly provided by Dr Boissonas (Sandoz, Bâle). It was dissolved in a buffer, pH = 4.6, and admixed with methyl-parahydroxybenzoate as preservative.

The halogenation reagents were a iodide-iodate mixture in preliminary experiments, and iodine monochloride (from Rhône Poulenc) in later experiments. ^{131}ICl was obtained from New England Nuclear Corporation. The reduction catalyst was palladium dispersed on $CaCO_3$ prepared by Engelhardt. Sephadex G 15 from Pharmacia, Biogel P2 as well as the ion exchanger AG 11 A8 have been provided by BioRad Laboratories. Peptide estimations were made by absorbance measurements at 275 nμ (pH 4.5) or by the Folin method [6].

Biological tests — the biological activity of oxytocin was controlled either *in vitro* on the frog bladder by the hydroosmotic test as described by Bourguet and Jard [7] or *in vivo* on the hen, by the avian blood depressor test [8]. Synthetic oxytocin (Sandoz) was used as standard in these determinations.

Radioactivity measurements — tritium was measured by liquid scintillation with a Nuclear Chicago counter. ^{131}I was determined by crystal scintillation in a Packard 410 A Auto Gamma Counter.

(*a*) *Preparation of iodo-oxytocin.*

Oxytocin contains one tyrosin residue and one cystine closing a six membered ring. Preliminary experiments using iodide-iodate mixture resulted in compounds containing more than two I atoms per molecule, indicating that halogenation was not limited to the aromatic ring of tyrosin and attacked the disulfide bridge, leading to alteration of the whole structure. Therefore different halogenation reagents have been thoroughly investigated on model systems until the present favourable reagent and conditions have been determined : the halogenation compound chosen is ICl, used at neutral pH. As shown

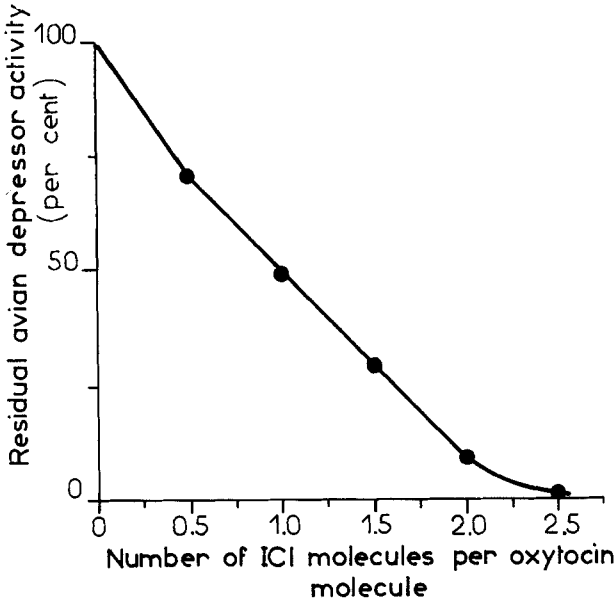

FIG. 1. *Iodination of oxytocin* : correlation between its avian depressor activity and the level of iodination.

on Figure 1, the reaction was nearly stoichiometric, as judged by loss of biological activity as a function of the amount of ICl added to oxytocin (iodo-oxytocin is virtually devoid of biological activity [9]).

1. *Removal of the aromatic preservative* — the commercial oxytocin sample used, 10 μmoles, dissolved in 25 ml 10^{-3} M acetate buffer pH 4.6 was filtered on a Biogel P2 column (40 × 2 cm) equilibrated with 10^{-3} M phosphate buffer pH 6.4, rate 0.2 ml/min. The oxytocin eluate was concentrated to 2.2 ml. About 20 % of the biological activity was lost at this stage.

2. *Iodination* — 25 μMoles of ICl in O.25 ml of methanol were very slowly added, together with ^{131} ICl as tracer to the 2.2 ml of the concentrated oxytocin solution placed at 0°. The addition of ICl required 10 minutes under magnetic stirring. The mixture was kept for 20 minutes or more at 0° and the iodine was then reduced by addition of an excess of 0.1 M thiosulfate. It should be noticed that a more rapid addition of ICl produced a peptidic precipitate. The iodination of tyrosin shifts the oxytocin spectra to longer wave lengths (Fig. 2). The extent of tyrosine halogenation was deduced first from the spectrum shift, and second, by direct measurement of the peptide bound ^{131}I. For the later purpose, an aliquot of the reaction mixture was placed on Whatman paper n° 1 impregnated with 7.10^{-2} M pyridine-acetic

acid buffer pH 6.5, and submitted to electrophoresis for 1 hr in a potential gradient of 12 volts/cm (Fig. 3). Iodination was calculated from the radioactivity recovered, the amount of peptide in the aliquot, and the specific activity of ^{131}I in the reaction mixture. Usually, nearly two I were bound per oxytocin molecule.

3. *Removal of inorganic salts* — the mixture of iodo-oxytocin and inorganic salts was filtered on an ion exchange resin AG 11 A8 50-100 mesh, column 5 × 1.2 cm, at the rate of 0.2 ml/min. Iodooxytocin is recovered in the filtrate. This fraction had virtually no biological activity.

In some experiments, it happened that iodination was not complete. To attain a high specific radioactivity in tritium, it was then necessary to separate oxytocin from iodo-oxytocin. This was achieved by adsorption on Sephadex G 15 (column 40 × 2 cm), equilibrated and eluted with a 10^{-3} M phosphate

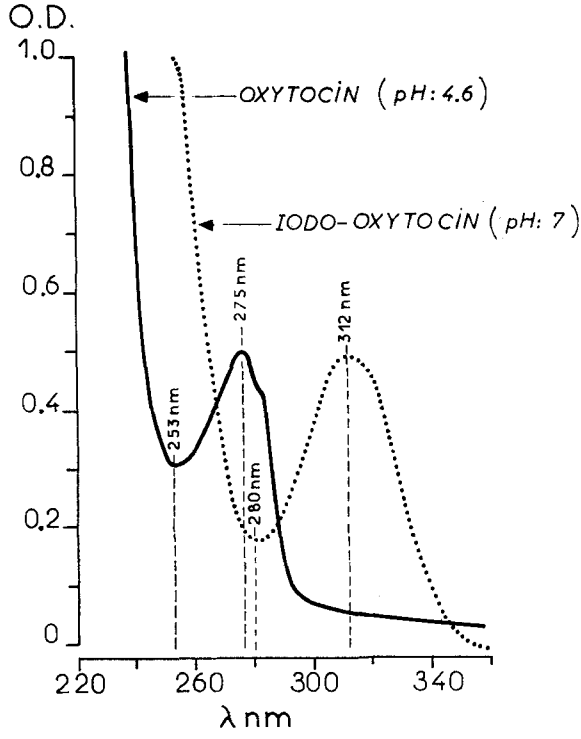

FIG. 2.

UV Spectra of oxytocin and derivatives

—— oxytocin, pH 4.6 λ_{max} 275 nm;

... di-iodo oxytocin, pH 7 λ_{max} 312 nm.

buffer, pH 6.4, rate of filtration 0.2 ml/min. It was interesting to observe that iodo-oxytocin is more retained on the column than oxytocin. It thus appears that Sephadex G 15 operates in this case as an adsorbent and not as a molecular sieve.

(b) *Tritiation of iodo-oxytocin.*

The iodo-oxytocin solution was concentrated to 1 ml in a rotatory evaporator, and placed into the tritiation vessel described on Figure 4. The catalyst (10 mg) was introduced into the cup of the tritiation vessel, which was connected to the vacuum line, as shown on Figure 5. The aqueous iodo-oxytocin solution was frozen in liquid nitrogen, and vacuum was applied to the tritiation flask, until the residual pressure was 10^{-4} torr. Pure tritium gas was then allowed to enter the flask and to flush the catalyst. The tritium gas was removed and discarded. Then 10 curies of ^{3}H gas were introduced into the flask with a Toeppler pump and the cup was inverted to drop the catalyst onto the still frozen aqueous solution. Temperature was allowed to rise until the iodo-oxytocin solution melted and the mixture was stirred with a magnet. After 20 minutes the solution was frozen again and the ^{3}H gas removed.

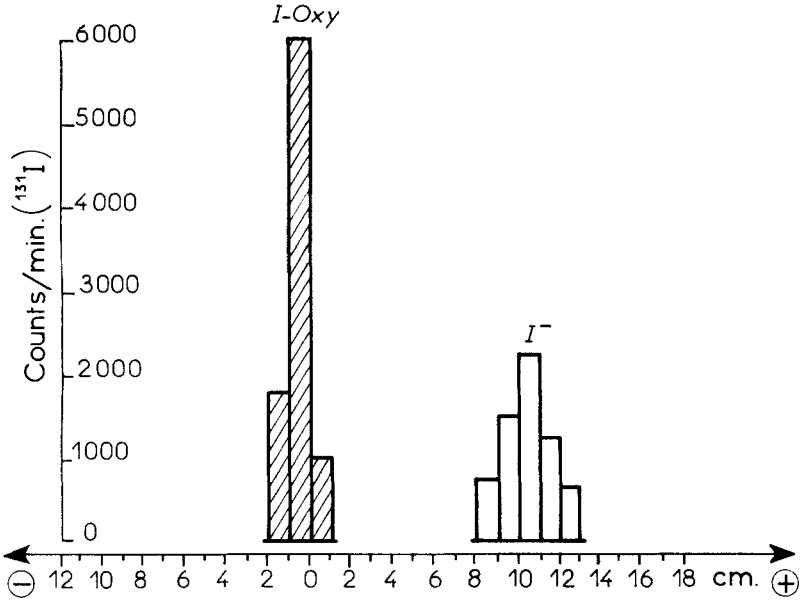

FIG. 3. *Electrophoretic separation of iodo oxytocin from iodide.* Whatman paper No. 1, pyridine-acetic acid buffer, pH 6.5, 10^{-3}M in acetic acid, potential gradient 12 volts/cm for 1 hour.

(c) *Removal of exchangeable tritium atoms.*

The catalyst was removed from the reaction mixture by filtration on Millipores (Mitex). The ionizable ^{3}H atoms were exchanged against water protons by successive dilutions with water and concentrations in a rotatory evaporator, until the amount of radioactivity in the distillate became negligible.

(d) *Final purification of ^{3}H oxytocin.*

The ^{3}H oxytocin solution was filtered on a Biogel P2 column similar to the one used in step *a*) 1. The column was developed with a 10^{-3} M acetic acid acetate buffer pH 4.6. All fractions were submitted to the following tests : spectra, measure of the biological activity and of the ^{3}H and ^{131}I content. The 60 to 160 ml elution fractions contained most of the biological activity recovered and were highly tritiated, corresponding to labelled and biologically active ^{3}H hormone. ^{131}I was absent from these fractions and appeared later in the eluate as inorganic iodide.

Since the elution profile indicated the presence of two peaks for biological activity (avian depressor test), the fractions were pooled in two samples (I and II) in each of which peptide content (Folin) tritium activity, and both avian depressor activity and frog hydrosmotic activity were determined (table 1).

As compared to reference oxytocin, fraction I retained more than 90 per cent hydrosmotic activity, but only 47 per cent avian depressor activity; on the contrary, fraction II retained 84 per cent and 67 per cent activity

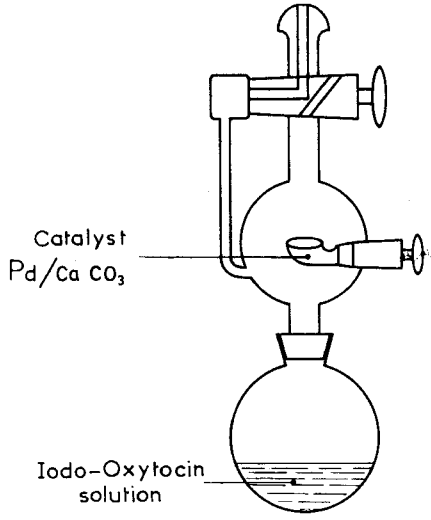

FIG. 4. *Tritiation vessel.* Its total volume is about 10 ml.

TABLE 1. Summary of the results obtained in each step of one assay.

	Peptide amount (μM)	Biological activities		Specific radio-activity (Ci/mM)	Maximum absorbance (mμ)
		Avian depressor (U/mg)	*Frog bladder* (U/mg)		
Reference oxytocin	—	450	450		
Stock oxytocin	10.0				
After Biogel P2 column	8.1	480	—		275 (pH 4.6)
Iodo-oxytocin	8.1	<1	—		312 (pH 7.0)
Tritiated hormone :					
Fraction I	0.168	210	417	35.7	275 (pH 4.6)
Fraction II	0.080	300	376	37.7	275 (pH 4.6)

respectively. The observed discrepancy between the two bioassays suggests that part of the hormone had been slightly transformed during the procedure (by deamidation for example), the resulting peptide retaining full hydrosmotic activity but decreased avian depressor activity; most of the modified hormone was eluted in the fraction I. In some other tritiation experiments, no such large difference in biological activities of ^3H oxytocin were noted.

DISCUSSION.

The substitution of a labile atom or group by ^3H is a general technique of tritiation. For aromatic compounds, the removal of a halogen under reductive conditions is often a method of choice as mentioned by Nunez *et al* [10]. In the case of oxytocin, one is dealing with a peptide containing both a tyrosine residue and a disulfide bond. Alteration of the latter results in the loss of the biological activity. The halogenation of oxytocin raises therefore the problem either of protecting the disulfide bridge or of finding conditions for specific halogenation of the tyrosine residue. Iodine, as formed by oxidation of iodide

$$5\ I^- + I\ O_3^- + 6H^+ \rightarrow 3\ I_2 + 3\ H_2O$$

is involved in secondary reactions as :

$$I\ O^-_3 + 2\ I_2 + 6H^+ \rightarrow 5\ I^+ + 3\ H_2O$$
$$I_2 + H_2O \rightleftarrows HIO + HI \rightleftarrows H_2IO^+ + I^-$$
$$I^- + I_2 \rightleftarrows I_3^-$$
$$I^- + I^+ \rightleftarrows I_2$$

The halogenation species is I^+ formed from I_2 in minute amounts. I_2 does not react readily with the tyrosyl-ring, but attacks extensively the disulfide bond.

It was therefore of interest to compare the reactivities of I^+ in the two competing reaction, halogenation of tyrosine and destruction of the S-S bond. Having observed that the former reaction is more rapid than the latter, the halogenation of oxytocin by an I^+ generating reagent has been studied. As expected the S-S bond is preserved, while the theoretical amount of iodine is fixed on the tyrosin residue. Di-iodo oxytocin is virtually devoid of biological activity. A similar finding with di-iodo angiotensin II,[5] suggests an important role for the tyrosine residue, either in the binding of these hormones onto their specific sites or in the modifications they induce.

Dehalogenation by metal catalysts and hydrogen is in general carried out in an alkaline solution. High pHs however are not compatible with the stability of oxytocin. The use of $CaCO_3$ as carrier for palladium black partly circumvents this difficulty, which could account for the observed change in ^{3}H oxytocin biological activity profiles. Palladium black is known to catalyse an exchange between the hydrogen of water and molecular hydrogen. In the case of tritium gas and an aqueous solution of iodo-oxytocin, this exchange will take place together with the dehalogenation process. The relative rates or both reactions will be an important factor in determining the final specific radioactivity of the

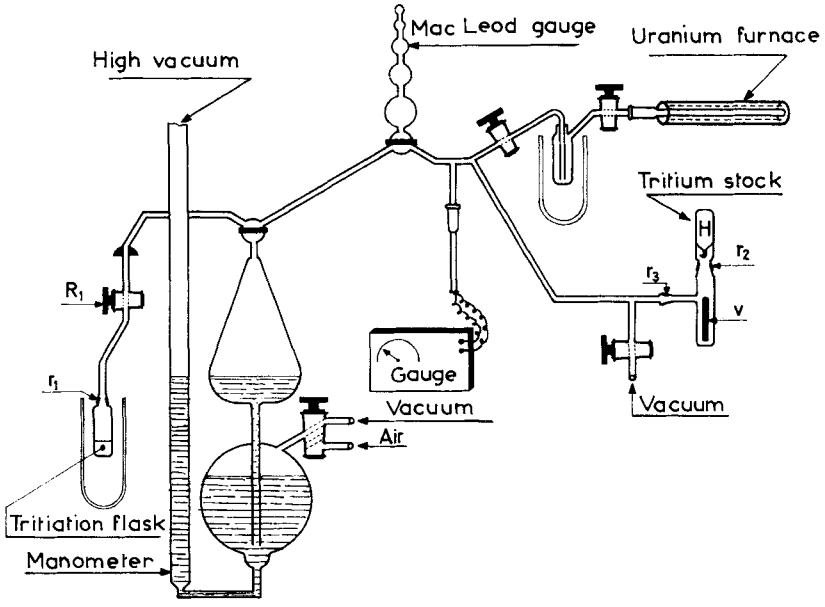

FIG. 5. Vacuum line. *Scheme of the tritium gas handling apparatus.*

labelled product. It is of considerable interest that the substitution of the two iodine atoms fixed on the tyrosin residue gives rise to a specific radioactivity of 36 C/mMole oxytocin. This fact means that the tritium gas has not been extensively diluted by the hydrogen contained in 1 g of water. Hence, the rate of water -^{1}H/gas-^{3}H exchange is very low compared to the rate of the dehalogenation process itself. The same conclusion has been reached in the dehalogenation of di-iodo angiotensin II [5].

The tritiation step is accompanied by an important loss of the peptide, due to an adsorption onto the catalyst. After removal of the latter, most of the peptide remains bound and could not be recovered even after extensive washing. Irreversible fixation of oxytocin on the walls of the glassware may also take place. These losses account for the overall yield of 2.5 % of ^{3}H oxytocin.

Stability tests are underway to determine the best conditions of storage.

REFERENCES

1. SCHWARTZ, J. L., RASMUSSEN, H., SCHOESSLER, M. A., SILVER, L. and FONG, C. T. O. — *Proc. Natl. Acad. Sci. U. S.*, **46** : 1288 (1960).
2. DU VIGNEAUD, V., SCHNEIDER, C. H., STAUFFER, J. E., MURTI, U. V. S., AROSKAR, J. P. and WINESTOCK, G. — *J. Am. Chem. Soc.*, **84** : 409 (1962).
3. SJÖHOLM, I. and CARLSON, L. — *Acta Chem. Scand.*, **20** : 259 (1966).
4. AGISHI, Y. and DINGMAN, J. F. — *Biochem. Biophys. Res. Commun.*, **18** : 92 (1965).
5. MORGAT, J. L., LAM THANH, H. and FROMAGEOT, P. — *Biochim. Biophys. Acta*, **207** : 374 (1970).
6. LOWRY, O. H., ROSEROUGH, N. J., FARR, A. L. and RANDALL, R. J. — *J. Biol. Chem.*, **193** : 265 (1951).
7. BOURGUET, J. and JARD, S. — *Biochim. Biophys. Acta*, **88** : 442 (1964).
8. COON, J. M. — *Arch. Int. Pharmaco. Syn.* LXII fasc. I. : 79 (1939).
9. FONG, C. T. O., SCHWARTZ, I. L., POPENCE, E. A., SILVER, L. and SCHOESSLER, H. A. — *J. Am. Chem. Soc.*, **81** : 2592 (1959).
10. NUNEZ, J., JACQUEMIN, C. and ROCHE, J. — *International J. of Applied Radiation and Isotopes*, **13** : 573 (1962).