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Synthesis of specifically tritium-labelled oxytocin and lysine-vasopressin with high specific activity

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SUMMARY

Highly tritium-labelled oxytocin and lysine-vasopressin have been synthesized. L-tyrosine-3-^3H was prepared from L-3-iodotyrosine and tritium gas in methanolic potassium hydroxide with palladium on active carbon as a catalyst. The tritiated oxytocin and lysine-vasopressin were obtained from the tritiated tyrosine and the hepta-peptides of oxytocin and lysine-vasopressin, respectively, by the p-*nitrophenyl ester method. The peptides had a high degree of purity and the specific radioactivity was 2.9 C/mmole or 5.9 μC/IU for oxytocin and 1.9 C/mmole or 6.5 μC/IU for lysine-vasopressin. The high radioactivity of the peptides permits extensive studies of their properties* in vivo. *The stability of the tritium label is discussed.*

INTRODUCTION

In many respects the posterior pituitary hormones, oxytocin and vasopressin, are convenient examples for the study of physiological and biochemical properties of peptide hormones. Their chemical characteristics are well known, and the biological methods for standardization of the oxytocic and vasopressor activity are comparatively simple and precise. The hormones have, however, high biological activity, a fact which makes it necessary to use isotopically labelled peptides for tracing them *in vivo*. The first synthesis of oxytocin and lysine-vasopressin [1,2] has been followed by only one paper describing synthesis

of the peptides with radioactive isotopes. Du Vigneaud *et al.* [3] used tritium-labelled leucine in the synthesis of oxytocin, and achieved a hormone preparation with a specific radioactivity of 130 mC/mmole or 130 μC/mg. Du Vigneaud *et al.* [3] and other authors [4-6] also labelled oxytocin, lysine-vasopressin and arginine-vasopressin with tritium, using different modifications of the Wilzbach's tritium gas exposure technique. Agishi and Dingman [7] iodinated oxytocin and treated the iodooxytocin with tritium gas under acidic conditions, under which the iodine is replaced by tritium and hydrogen. The highest specific radioactivity of the tritiated hormones obtained by the different methods was 0.23 C/mmole or 230 μC/mg oxytocin [7]. Gilliland and Prout [8] who used radioiodinated ^{131}I-oxytocin for immunological studies, calculated their iodooxytocin to have an initial specific radioactivity of 20.8 mC/mg.

The present paper describes a method for the synthesis of specifically tritium-labelled oxytocin and lysine-vasopressin giving high specific radioactivity in good yields *. Because both hormones are known to be readily inactivated in tissues and blood from pregnant women, the aim of the work was to prepare compounds not giving too many possible radioactive metabolites *in vivo*. This was achieved by labelling *one* of the common amino acid residues of oxytocin and lysine-vasopressin. The tyrosine residue (see Fig. 1) seems to be well suited for this purpose. It is situated next to the N-terminal half-cystine residue and, therefore, two to four radioactive degradation products could be expected to arise after the attack *in vivo* of aminopeptidases alone [10], or together with reducing enzymes [11]. This applies, if the label is placed in the tyrosine residue, i.e., the opened nonapeptides, obtained after cleavage of the peptide bond between cystine and tyrosine, the reduced forms of the intact peptides and nonapeptides, respectively, and free tyrosine. Moreover, the phenyl group in tyrosine offers a position where tritium could be placed with ease, yielding a product of reasonable stability and, by using the *p*-nitrophenyl ester method as introduced by Bodanszky *et al.* [12, 13], use of the radioactive substance could be avoided until the later stages of the work.

In this work L-tyrosine-3-^{3}H was prepared from L-3-iodotyrosine and tritium gas in alkaline methanol, and with palladium on active carbon as a catalyst, mainly according to Birkofer and Hempel [14]. From the tritiated tyrosine, the proper protected derivative, the O-benzyl-N-carbobenzoxy-*p*-nitrophenyl ester of tyrosine, was prepared prior to condensation with the heptapeptides of oxytocin and lysine-vasopressin, respectively, according to Bodanszky *et al.* [12, 13]. From the octapeptides obtained and the N-carbobenzoxy-S-benzyl-*p*-nitrophenyl ester of cysteine, the nonapeptides were synthesized. Their protecting groups were split off with sodium in ammonia,

* A preliminary report has already been published [9].

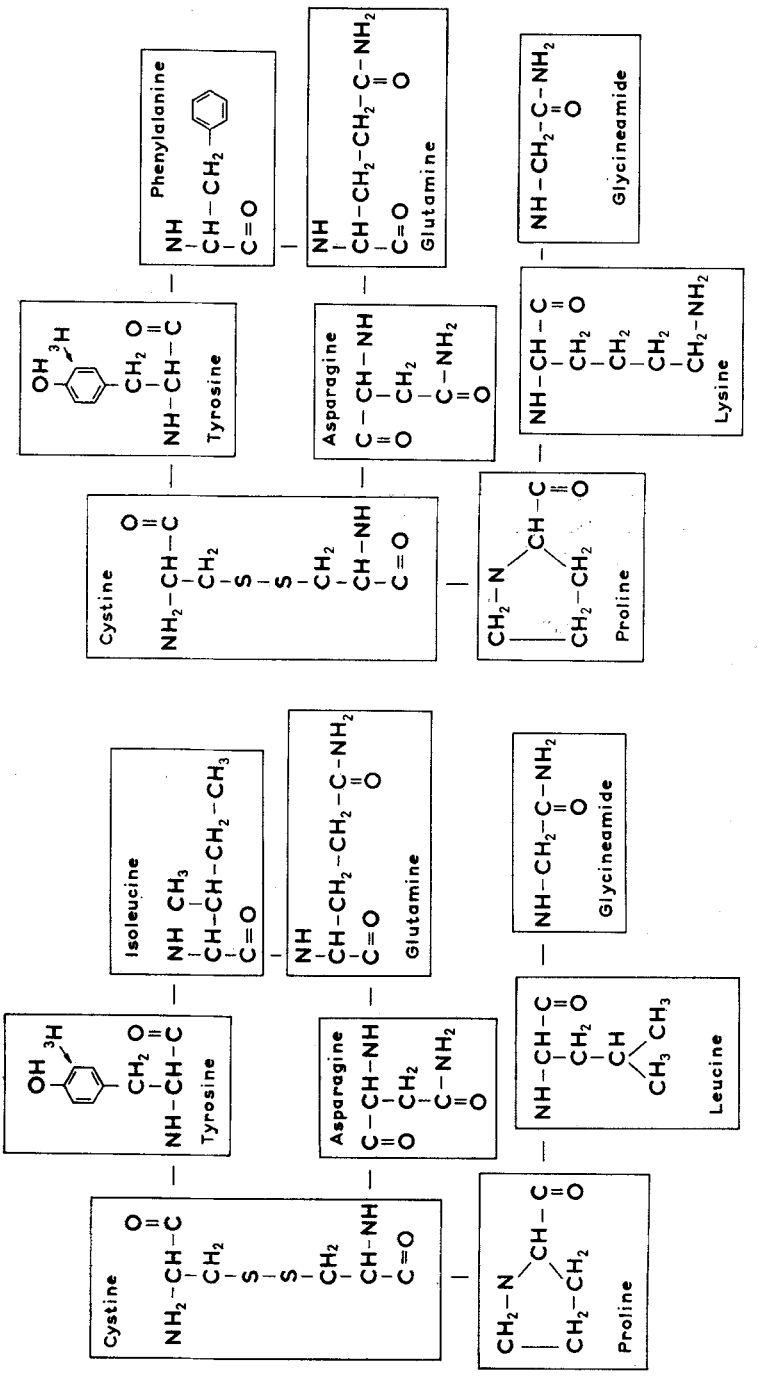

FIG. 1*a*. Tritiated oxytocin (Tyr^2-3-^{3}H-oxytocin).

FIG. 1*b*. Tritiated lysine-vasopressin (Tyr^2-3-^{3}H-Lys^8-vasopressin).

The arrows show the place of the tritium atoms.

after which the biologically active peptides were obtained after oxidation with air. The procedure is summarized in Fig. 2 *.

Methods

Tritium activity was measured by liquid scintillation counting in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 314 AS. The sample (up to 50 μl) was dissolved in 2 ml of abs. ethanol and 15 ml of a toluene solution (toluene 1,000 ml, dimethyl POPOP 0.3 g, PPO 5 g; Packard Instrument Co.). The counting efficiency was calculated by use of an internal standard of tritiated toluene. The tritium distribution on paper or thin-layer chromatograms was investigated in a window-free Nuclear Chicago 4 π Actigraph II, Model 1032, chromatogram scanner and by autoradiography with Ilford G-4.

Oxytocic activity was determined with the rat uterus *in vitro*, according to [15].

Vasopressor activity was determined on the rat according to Dekanski [16].

Chromatography was performed by the descending technique on paper (Whatman No. 1) or by thin-layer chromatography on silica gel, using Eastman Chromatogram Sheet, Type K 301 R, with fluorescence indicator.

Gel filtration was carried out on Sephadex G-15 (Pharmacia, Uppsala, Sweden) with water regain 1.5 ± 0.2 g/g and particle size 40-120 μ.

Experimental

L-Tyrosine-3-^3H (II) **

L-3-Iodotyrosine (Koch-Light Lab. Ltd., Colnbrook, England) was hydrogenolyzed in an all-glass apparatus constructed by Gostonyi and Walde [17]. Tritium gas (1.30 mmole, containing 66 Curie) was transferred to activated uranium chips *in vacuo* and bound as uranium hydride (U^3H_3) at about 25° C. 338 mg (1.10 mmole) of L-3-iodotyrosine (I) and 100 mg of palladium-active carbon (10%) were suspended in 2.2 ml of 1.5 M methanolic potassium hydroxide and cooled in liquid nitrogen. The apparatus was evacuated to a pressure less than 0.001 mm Hg. By warming on the uranium hydride to 300-400° C, the tritium was released and transferred to the reaction vessel with a Toepler pump. It was allowed to react with the iodotyrosine at about 25° C during stirring for 2 h, after which time all the tritium was consumed, as indicated by the decrease in pressure.

The tritiated tyrosine was isolated from the reaction mixture after filtration with Hyflo Super Cel by precipitation at pH 6. The precipitate was

* Abbreviations used : Bz, benzyl; Cbz, carbobenzoxy; Tos, *p*-toluenesulphonyl. All optically active amino acids have the L configuration.

** The Roman figures refer to the numbering of the reactants in Fig. 2.

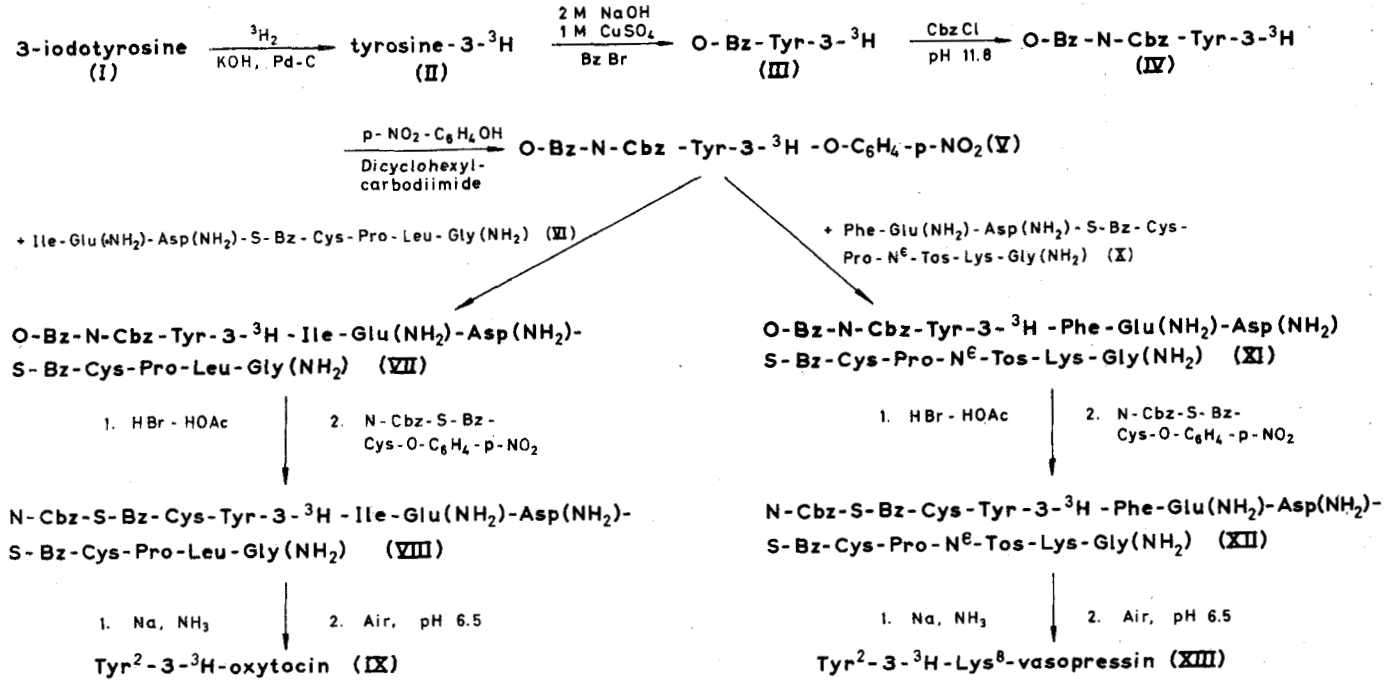

FIG. 2. Summary of the synthesis of Tyr^2-3-^{3}H-oxytocin and Tyr^2-3-^{3}H-Lys^8-vasopressin.

washed with water, ethanol and ether and dried *in vacuo*. Yield 160 mg, 0.87 mmole (80%). 160 mg of non-radioactive L-tyrosine were mixed with the active material, the mixture dissolved in 1 N HCl and reprecipitated at pH 6, washed and dried as before. Yield 300 mg, containing 7.2 C/mmole.

The tritiated tyrosine was found to be radiochemically homogeneous in thin-layer and paper chromatography.

O-Benzyl-N-carbobenzoxy-L-tyrosine-3-^3H (IV)

Tritiated tyrosine (296 mg, 1.62 mmole) was suspended in 0.83 ml of 2 N NaOH at 40-50° C and 0.83 ml of 1 N $CuSO_4$ was added (Wünsch *et al.* [18]). After 10 min, the copper complex obtained was dissolved in 6.5 ml of methanol and 0.83 ml of 2 N NaOH. After centrifugation and washing of the undissolved residue with 0.3 ml of a mixture of methanol, 2 N NaOH and water (8.6 : 2.3 : 1.1), the centrifugate and washing were pooled and transferred to a wide 15-ml reaction vessel. Benzyl bromide (205 µl, 1.72 mmole) was added and the mixture was vigorously stirred under nitrogen for 75 min. The O-benzyl-tyrosine-copper complex formed precipitated, and the mother liquor was sucked off, after which the precipitate was washed with 2 × 1.6 ml of methanol-water (1 : 3.5), 2 × 1.6 ml of ethanol and 2 × 1.6 ml of ether. The copper complex was decomposed with 2 ml of 1 N HCl and the O-benzyl-tyrosine precipitate (III) obtained was washed with 3 × 2 ml of 1 N HCl, 2 ml of water, 2 ml of 1 N NH_3, 2 ml of water, 2 ml of ethanol and 2 × 2 ml of ether. Compound III was dried *in vacuo* and weighed 218 mg (0.80 mmole, 49%). Compound III was suspended in 7.5 ml of water. During vigorous stirring, 320 µl of carbobenzoxy chloride (5.1 mmole/ml) were added successively in the course of 1 h. The reaction mixture was kept at pH 11.8 in a pH-stat with totally 530 µl of 5 N NaOH. The clear solution was washed by stirring with 4 × 5 ml of ether, and the pH was adjusted to about 1 with 1.5 ml of 5% HCl. Compound IV then precipitated; it was washed with 3 × 4 ml of water and dried *in vacuo*. Yield 316 mg (0.75 mmole, 97%).

O-Benzyl-N-carbobenzoxy-L-tyrosine-3-^3H-p-*nitrophenyl ester* (V)

Compound IV was dissolved in 3 ml of ethyl acetate, transferred to a glass-stoppered test tube, and centrifuged. The undissolved residue was washed with 1 ml of ethyl acetate, and dried (weight 11 mg). The centrifugates were transferred to another test tube with a glass stopper and cooled in an ice-bath. 111 mg (0.80 mmole) of *p*-nitrophenol were added and dissolved in the ethyl acetate, and 156 mg (0.76 mmole) of N,N'-dicyclohexylcarbodiimide in 1 ml of ethyl acetate were brought to the solution. After 30 min in the ice-bath, the tube was kept at room temperature for a further 6.30 h. The tube was centrifuged, and the supernatant transferred to a pear-shaped 50-ml glass flask. The precipitate (N,N'-dicyclohexylurea) was washed with 3 + 2 × 2 ml of ethyl acetate, dried and weighed (154 mg, 0.69 mmole). The ethyl acetate

was distilled off *in vacuo* and the residue washed with 4 × 2 ml of ether. The remaining ester (compound V) was dried and weighed. Yield 360 mg, 0.68 mmole (87%). It was dissolved in 1.2 ml of dimethylformamide, giving a total volume of 1.40 ml.

Compound V had a specific radioactivity of 7.7 C/mmole and was chromatographically homogeneous in neutral thin-layer systems as well as in acidic systems. No non-radioactive by-products could be detected with ninhydrin or chlorine-o-tolidine. In basic systems, the ester is partly hydrolyzed, giving rise to two radioactive compounds, one of which is O-Bz-N-Cbz-tyrosine-3-^{3}H and the other the active ester as compared with reference substances.

Tyr^2-3-^{3}H-oxytocin (IX)

N-Cbz-Ile-Glu(NH_2)-Asp(NH_2)-S-Bz-Cys-Pro-Leu-Gly(NH_2) (200 mg, 0.207 mmole), m.p. 231-233° C and $[\alpha]_D^{20}$-47° (*c* 1.0, dimethylformamide), obtained according to Bodanszky and Du Vigneaud [12] was suspended in 0.80 ml of water-free acetic acid in a test tube, and allowed to swell for 20 min. 1.2 ml of hydrogen bromide-acetic acid (3.2 g HBr/10 ml HOAc) was added, and the peptide dissolved by careful swirling of the tube. After 1 h, 8 ml of ether were added to precipitate the free heptapeptide hydrobromide (VI), which was centrifuged, washed with 4 × 3 ml of ether and dried *in vacuo*. Compound VI was dissolved in 0.6 ml of dimethylformamide and neutralized with 0.18 ml of triethylamine. 0.50 ml of dimethylformamide containing 130 mg (0.246 mmole) of compound V was then added. This means that the radioactive tyrosine ester derivative (V) is added in 20% excess, which is used to force the reaction towards completion. The excess of the ester is more readily removed in the subsequent washing procedure than the heptapeptide derivative would be, if the peptide is used in excess. The tube was left at room temperature for 8 h, when the protected octapeptide (compound VII) was formed. Compound VII was precipitated with 8 ml of ethyl acetate, centrifuged, and washed with 3 × 3 ml of ethyl acetate, 3 × 3 ml of ethanol and 2 × 3 ml of ethyl acetate. Compound VII was dried *in vacuo*. Yield 248 mg, 0.203 mmole (98% as estimated from the heptapeptide).

The free octapeptide was obtained from compound VII after it had remained for 75 min in HBr-HOAc, followed by precipitation, washing and drying in the same way as in preparation of the free heptapeptide (VI). It was dissolved in 0.7 ml of dimethylformamide and 0.20 ml of triethylamine. N-Cbz-S-Bz-Cys-*p*-nitrophenyl ester (115 mg, 0.246 mmole, 120% of the theoretical amount) in 0.5 ml of dimethylformamide were added to the solution. After 9 h, the protected nonapeptide formed (VIII) was precipitated with 8 ml of ethyl acetate, centrifuged, washed, and dried in the same way as the octapeptide. Yield 244 mg, 0.184 mmole (91%).

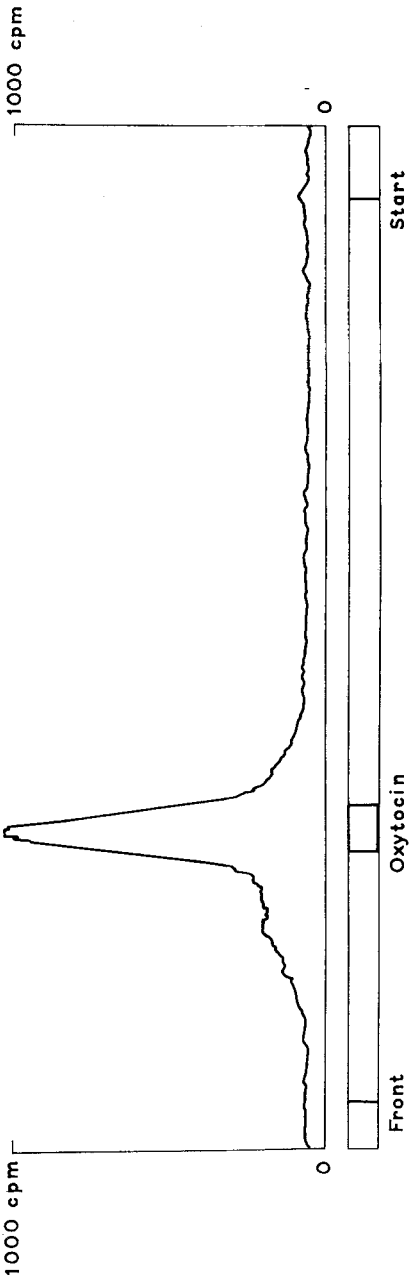

FIG. 3. Descending paper chromatography of 0.2 μC of tritiated oxytocin and 10 μg of non-radioactive oxytocin in *n*-butanol-acetic acid-water (2 : 1 : 2). The upper part shows the distribution of radioactivity and the lower part that of ninhydrin-reactive substances.

Compound VIII (237 mg, 0.179 mmole) was transferred to a 250-ml wide-neck flask with flat flange. In the flask — which was protected against humidity with soda lime — about 80 ml of NH_3 were condensed in a nitrogen atmosphere at —50° C - —60° C. Sodium in 2-4 mg pieces (totally 45 mg) was added during stirring, until the dark blue colour persisted for 20 min, whereafter 113 mg of NH_4Cl were added. The ammonia was allowed to evaporate, and the residual reduced oxytocin was dissolved in 600 ml of oxygen-free water and oxidized with air at pH 6.5 for 3 h. The solution of oxytocin formed was made up to 1,000 ml with ethanol, and the pH adjusted to 3 with HOAc. Yield 80,000 IU of oxytocic activity. The specific radioactivity was 5.9 μC/IU or, when 500 IU constitutes 1 mg of oxytocin, 2.9 C/mmole. The amount of oxytocin obtained was thus 160 mg or 0.16 mmole (88%).

Chromatography of the tritiated oxytocin on paper in *n*-butanol-acetic acid-water (4 : 1 : 5) showed that the main peak was preceded by small amounts of radioactivity, probably deriving from the dimer form of the oxytocin (Fig. 3). No non-radioactive substances could be detected with ninhydrin after chromatography of about 750 μC oxytocin. The monomer was isolated after gel filtration on Sephadex G-15 in 0.1 M NaOAc-HOAc at pH 5.8 (Fig. 4). When chromatographed in the butanol-acetic acid-water system, the main fraction gave only one peak in the chromatogram scanner.

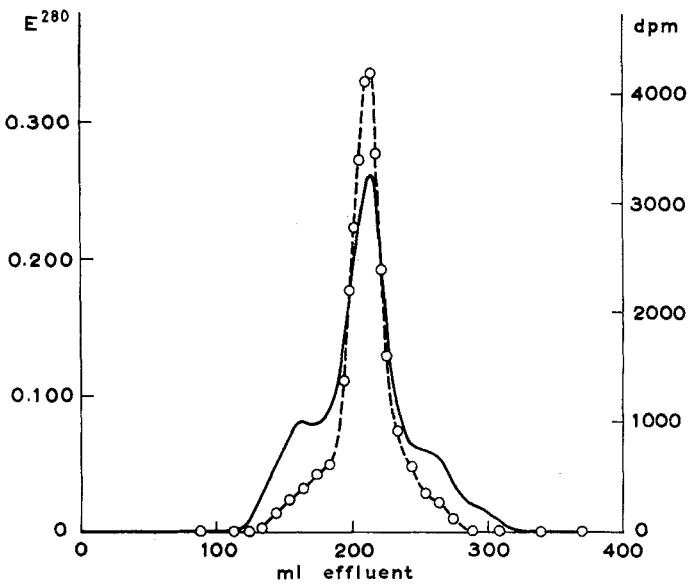

FIG. 4. Gel filtration of 2 μC of tritiated oxytocin and 5 mg of non-radioactive oxytocin on Sephadex G-15, 140 × 1.5 cm, with 0.1 M sodium acetate-acetic acid buffer, pH 5.8.

——— E^{280}; -o--o- Tritium contents.

Tyr²-3-³H-Lys⁸-vasopressin (XIII)

N-Cbz-Phe-Glu(NH_2)-Asp(NH_2)-S-Bz-Cys-Pro-N^{ε}-Tos-Lys-Gly(NH_2), (200 mg, 0.171 mmole), m.p. 200-202° C and $[\alpha]_D^{20}$-37° (*c* 1.0, dimethylformamide), was suspended in a test tube with 1.25 ml of HOAc. After swelling for 30 min, it was dissolved in 1.25 ml of HBr-HOAc (3.2 g HBr/10 ml HOAc). After 2 h at room temperature, the free heptapeptide hydrobromide (X) was precipitated with 8 ml of ether. It was centrifuged, washed with 4 × 3 ml of ether and dried *in vacuo*. Compound X was dissolved in 0.9 ml of dimethylformamide and neutralized with 0.18 ml of triethylamine. 0.45 ml of dimethylformamide containing 115 mg, 0.218 mmole (125% of the theoretical amount as earlier) of the tritiated tyrosine derivative (V) were added, and allowed to condense with compound X for 20 h at room temperature. The protected octapeptide formed (XI) was precipitated with 250 µl of HOAc and 17 ml of water, which were added during stirring and cooling. Compound XI was centrifuged, washed with 4 × 2 ml of water, 2 × 1 ml of ethanol and 4 × 1 ml of ethyl acetate, and dried *in vacuo*. Yield 209 mg, 0.147 mmole (86%).

The free octapeptide was obtained from XI and precipitated, washed and dried in the same way as that used to obtain the free heptapeptide from its protected derivative. It was dissolved in 0.9 ml of dimethylformamide and 0.15 ml of triethylamine and condensed for 18 h at room temperature with 82 mg (0.176 mmole, 120% of the theoretical amount as earlier) of N-Cbz-S-Bz-Cys-*p*-nitrophenyl ester in 0.3 ml of dimethylformamide. The reaction was stopped with 0.13 ml of HOAc, and the protected nonapeptide (XII) was precipitated with 20 ml of water. It was washed with 6 × 2 ml of water, 2 × 1.6 ml of acetone-ethyl acetate (1 : 1) and 4 × 3 ml of ethyl acetate, and dried. Yield 171 mg, 0.112 mmole (76%).

Compound XII (165 mg, 0.108 mmole) was transferred to a 250-ml wide-neck flask with flat flange, and the reduced vasopressin was prepared as described for oxytocin with about 100 ml of NH_3 and 60 mg of sodium. 220 mg of ammonium acetate were used for neutralization. The residue, after the ammonia had evaporated, was washed with 2 × 1.5 ml of ethyl acetate, after which it was dissolved in 600 ml of oxygen-free water and oxidized at pH 6.5 with air for 75 min. After oxidation, the pH was lowered to 4 with HOAc. The yield was 34,000 vasopressor units. If 1 mg of lysine-vasopressin contains 280 IU on determination in the rat, this corresponds to a total yield of 121 mg (0.115 mmole) active peptide. Estimated from compound XII, the yield thus corresponds to 106%. The specific radioactivity was 6.5 µC/IU vasopressor activity or 1.9 C/mmole.

Paper chromatography of the tritiated lysine-vasopressin in *n*-butanol-acetic acid-water (4 : 1 : 5) and scanning for tritium (see Fig. 5) showed that the greater part of radioactivity followed lysine-vasopressin. Only small amounts of radioactivity migrated just before and after the vasopressin peak,

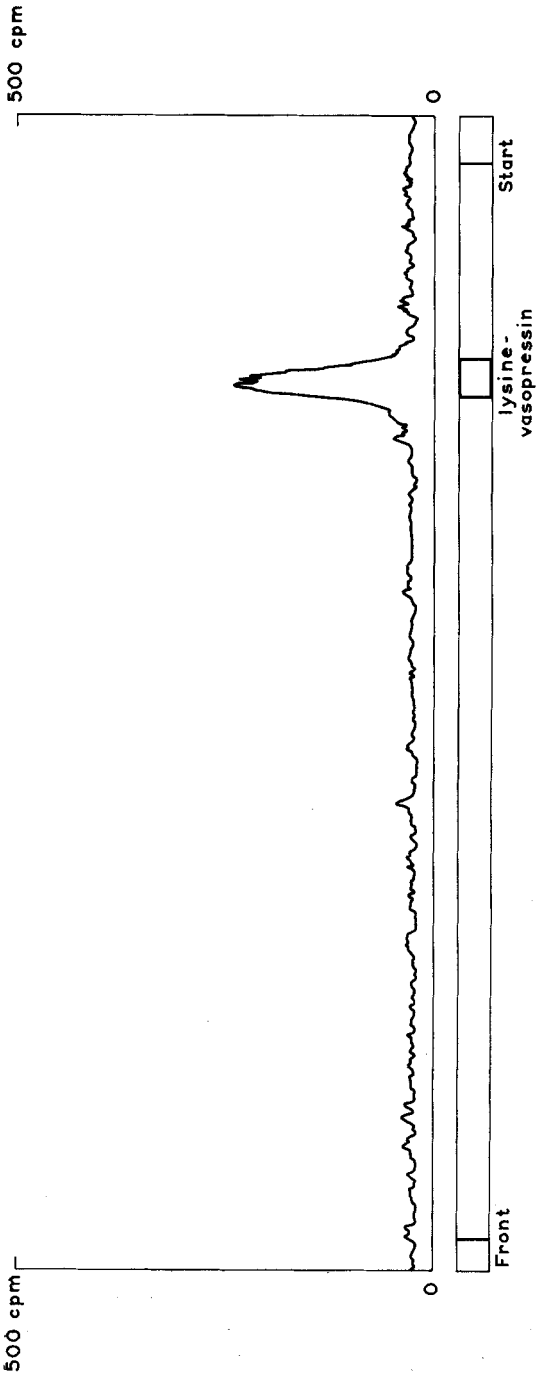

FIG. 5. Descending chromatography of 0.05 μC of tritiated lysine-vasopressin and 20 μg of non-radioactive lysine-vasopressin in *n*-butanol-acetic acid-water (4 : 1 : 5). The upper part shows the distribution of radioactivity and the lower part that of ninhydrin-reactive substances.

which could be better demonstrated by autoradiography (200 μC, exposure for 24 days). The darkening of the film from the activity, not following the lysine-vasopressin was, however, too small to be visible by reproduction. Nor could any non-radioactive substances be detected with ninhydrin after paper chromatography of 200 μC lysine-vasopressin. In the chromatographic system used no separation of the monomer and dimer forms of lysine-vasopressin could be seen. However, as shown in Fig. 6, small amounts of radioactivity,

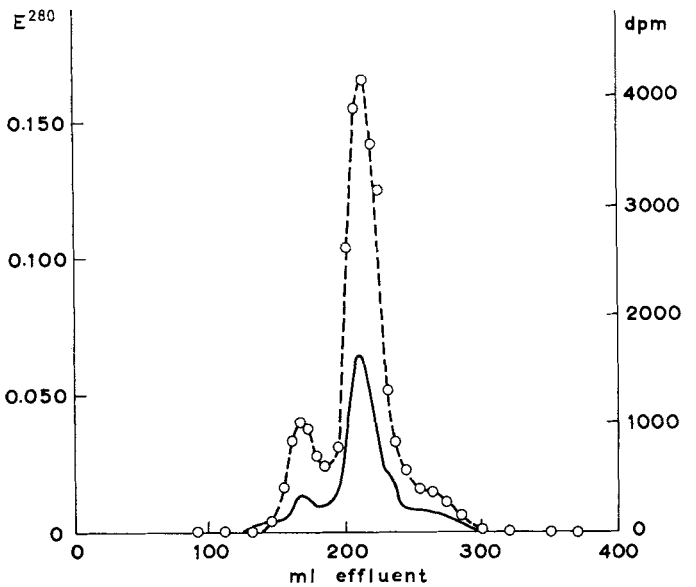

FIG. 6. Gel filtration of 1.5 μC of lysine-vasopressin and 4 mg of non-radioactive lysine-vasopressin on Sephadex G-15, 140 × 1.5 cm, with 0.1 M sodium acetate-acetic acid buffer, pH 5.8.

——— E^{280}; -o--o- Tritium contents.

probably deriving from a dimer form as concluded from the elution volume, are eluted before the main fraction by gel filtration on Sephadex G-15 in 0.1 M NaOAc-HOAc, pH 5.8.

DISCUSSION

Because of the very short range of the β-particles from tritium, most of the energy from the radiation of tritiated compounds will be absorbed by the compound itself when stored as solids or in concentrated solutions, resulting in pronounced destruction. In a preliminary experiment, no special precautions were taken in this respect, and synthesis was performed during normal working hours. In this case, most of the tritiated tyrosine was lost

after 10 days. As it is not practical to carry out synthesis in dilute solutions, the final work was performed in the quickest way, with as short reaction times as possible. Thus, all the work — from tritiating of 3-iodotyrosine to final dilution of the oxytocin and vasopressin — was finished in totally 90 h. Before benzylation of the tritiated tyrosine was complete, a weak darkening was seen, but in the subsequent work no signs of radiolysis could be observed, and the yields were consistently high. The purity of the compounds obtained was also good, and the final peptides contained very small amounts of radioactivity, which could not be attributed to oxytocin or lysine-vasopressin, respectively. The dimerization of the peptides was also minimized to about 5-10 %, by performing the oxidation of the reduced peptides in dilute solutions. During storage at +2 C at a concentration of about 0.5 mC/ml in 40 % ethanol-water, no new radioactive products could be detected chromatographically after 18 months.

When the compound is labelled with only one tritium atom, this fragment will be non-radioactive and will not cause disturbances in tracer studies, unless it reacts with an unmodified molecule. This is, however, unlikely to occur in dilute solutions. However, if the molecule is labelled with two or more tritium atoms, the residual fragment after decomposition of a tritium nucleus will be radioactive. The biological activity is then lost faster than the radioactivity so that, finally, the latter will not be a true measure of the compound in question. Consequently, it is preferable to use monolabelled compounds, provided that their specific radioactivity is high enough for the relevant purpose.

The specific radioactivity of tyrosine-3-^{3}H and O-benzyl-N-carbobenzoxy-tyrosine-3-^{3}H-*p*-nitrophenyl ester obtained was the same, about 7.5 C/mmole. The final peptide products had, however, much lower specific activity, i.e. 2.9 C/mmole and 1.9 C/mmole for oxytocin and lysine-vasopressin, respectively. This decrease is a result of an electrophilic attack of protons during treatment of the peptides with hydrogen bromide-acetic acid to cleave the protecting groups, resulting in substitution of tritium for hydrogen. The ortho and para positions in phenols are more susceptible to such substitutions, owing to their higher electronegative property. Similar substitutions were noted by Kresge and Chiang [19], who found that the exchange of tritium in 1,3,5-trimethoxy-benzene-2-^{3}H was acid-catalyzed, and by Martin and Morlino [20], who heated tyrosine in deuterium chloride solution at 100° C. They were able to demonstrate by proton magnetic-resonance spectroscopy that the hydrogens in the ortho position to the hydroxyl group were exchanged by boiling for 18 h in 2.4 N DCl. Such a substitution must, however, be negligible at physiological pH values, but during storage at pH 3-4 about 20 % of the tritium was lost as tritiated water after 18 months. The comparatively lower specific activity of lysinevasopressin is probably a result of the longer treatment with and the larger amount of hydrogen bromide-acetic acid

used for the cleavage reaction, making more protons available for the substitution.

Although the specific activity figures are higher than any previously reported for either hormone, they can be further increased by not diluting the active tyrosine with "cold" tyrosine and, in particular, by placing the tritium in the meta position to the phenyl hydroxyl group, thus diminishing substitution of the former during peptide synthesis. The specific radioactivities are, however, high enough to allow extensive studies *in vivo* of the distribution, metabolism and intracellular localization of oxytocin and vasopressin. The possibilities should thereby be increased for attaining better knowledge of the function and mode of action of these hormones especially and of peptide hormones in general.

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