Iodinated Neurohypophyseal Hormones as Potential Ligands for Receptor Binding and Intermediates in Synthesis of Tritiated Hormones†

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ABSTRACT: [3-Iodo-Tyr^2]oxytocin (MIOT), [3,5-diiodo-Tyr^2]oxytocin (DIOT), [3-iodo-Tyr^2,Lys^8]vasopressin (MILVP), [3,5-diiodo-Tyr^2,Lys^8]vasopressin (DILVP), [3-iodo-Tyr^2,Arg^8]vasopressin (MIAVP), and [3,5-diiodo-Tyr^2,Arg^8]vasopressin (DIAVP) were synthesized by iodination of the respective hormones, purified, and characterized. All the monoiodo hormones had to be freshly prepared prior to bioassays, since on storage they gave rise to hormonal-like biological activity. The biological activities of these iodo analogues were measured in an adenylate cyclase assay employing neurohypophyseal hormone (NHH) sensitive bovine renal medullary membranes, and/or the rat oxytocic assay. In the cyclase assay, DIOT, DILVP, and DIAVP were inactive as agonists or antagonists. MIOT shows no agonistic activity in the renal cyclase system and uterus, but is a weak reversible inhibitor of oxytocin (OT) in both systems. When MIOT (10^{-4} M) was preincubated with renal membranes for 10 min at 37 °C before addition of OT, it behaved as a noncompetitive inhibitor of NHH-stimulated adenylate cyclase. MILVP and MIAVP appear to be partial agonists with K_m (half maximal response) 3×10^{-6} and 3×10^{-7} M, respectively, as determined in the cyclase assay. Upon preincubation with renal medullary membranes, MILVP (10^{-6} M) behaves as a more potent noncompetitive inhibitor of OT than MIOT. Accordingly, iodo derivatives of NHH do not exhibit sufficient affinity to serve as specific ligands to measure OT, LVP, or AVP receptors in the uterus and kidney. Study of the specificity of inhibition produced by MIOT revealed that this analogue does not act *selectively* upon NHH receptors. Thus, MIOT modified adenylate cyclase systems which do not have NHH receptors, e.g., the PTH-sensitive adenylate cyclase in bovine renal cortex and the glucagon-sensitive adenylate cyclase in rat liver. DIOT, DILVP, and DIAVP were subjected to catalytic tritiation (employing carrier free tritium) and were converted to [^{3}H]OT (25, 31, and 25 Ci/mmol), [^{3}H]LVP (26 and 23 Ci/mmol), and [^{3}H]AVP (17 Ci/mmol), respectively. These tritiated ligands have been successfully used to measure NHH receptor sites both in kidney and uterine membranes as described in other studies.

The need of radioactive neurohypophyseal hormones (NHH)[1] as ligands to measure specific receptor sites on target tissues (e.g., uterus and renal medulla) prompted this investigation of iodinated analogues of oxytocin (OT), [Lys^8]vasopressin (LVP), and [Arg^8]vasopressin (AVP). Iodinated NHH, without purification, have been used as intermediates for the preparation of [^{3}H]NHH (Agishi and Dingman, 1965; Morgat et al., 1970; Pradelles et al., 1972). Additionally, if a monoiodinated derivative of OT, LVP, or AVP labeled with ^{125}I exhibits biological activity (whether as specific agonist or antagonist), such compounds might be suitable as ligands for NHH receptor systems (Roth, 1973).

Reports on the iodination of OT have appeared; however, in most studies, the products were not fully characterized chemically and the biological results have varied.[2] In a preliminary report, Marbach and Rudinger (1974) stated that chemically characterized MIOT is an inhibitor of OT contractile activity in the rat uterus (a finding corroborated by Flouret et al., 1975) and suggested that labeled MIOT should be suitable for studying binding to uterine receptors.

In the present report, we describe the unequivocal synthesis, purification, and characterization of the [3-iodo-Tyr^2] (MI) and [3,5-diiodo-Tyr^2] (DI) analogues of OT, LVP, and AVP. The biological activity of these analogues was evaluated by assay using the NHH-sensitive adenylate cyclase system in bovine renal medullary membranes; MIOT and DIOT were also studied in the rat uterotonic assay. When the monoiodo derivatives were found to be unsuitable as high-affinity ligands to measure NHH receptors, the diiodo derivatives were tritiated to [^{3}H-Tyr^2]NHH derivatives, which retained the af-

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[1] The abbreviations employed follow the recommendations of IUPAC-IUB Commission of Biochemical Nomenclature (1972) *Biochem. J. 126,* 773–780. Additionally, the following abbreviations were used: OT, oxytocin; LVP, [Lys^8]vasopressin; AVP, [Arg^8]vasopressin; MIOT, MILVP, and AVP, [3-iodo-Tyr^2] analogues of OT, LVP, and AVP, respectively; NP, neurophysin; PTH, parathyroid hormone; NHH, neurohypophyseal hormones; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; TLC, thin-layer chromatography; TLE, thin-layer electrophoresis; TLS, thin-layer sheets.

[2] Thus, it has been reported that [3-^{125}I-Tyr^2]oxytocin, [^{125}I]MIOT, retained stimulatory activity in a toad bladder cyclase assay, stimulated glucose oxidation in fat cells, and showed specific binding to fat cells; however, binding of this labeled analogue could not be detected in other OT target tissues, e.g., uterus or renal medulla (Thompson et al., 1972). MIOT has been reported to be inactive in the avian depressor assay (Morgat et al., 1970), but an active agonist in a rat pressor assay (Gilliland and Prout, 1965).

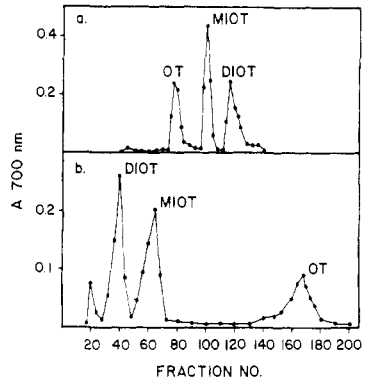

FIGURE 1: Separation of the products of iodination of OT. (a) The products of OT (90 mg) iodination were separated by gel permeation chromatography on a Sephadex G-25 column (2.4 × 115 cm); solvent 1% AcOH; fraction size 5 mL/tube; flow rate 0.5 mL/min. Aliquots of 0.1 mL were used for Folin–Lowry determination. (b) Iodinated products of OT (90 mg) were partitioned on a Sephadex G-25 column (2.4 × 115 cm) with solvent system A; fraction size 5 mL/tube; flow rate 25–30 mL/h; 0.1-mL aliquots for Folin–Lowry determination.

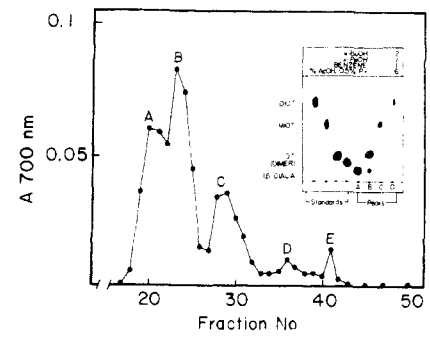

FIGURE 2: Separation of products following hydrogenation of DIOT. DIOT (1.6 mg) was hydrogenated as described in the text. The products were subjected to gel filtration using a Sephadex G-25 column (0.9 × 50 cm); solvent 1% AcOH; fraction size 1 mL; 0.2-mL aliquots were used for Folin–Lowry determination. The products eluted (designated A to E) were compared to standards on silica gel TLC (inset) using the upper phase of solvent system A as developing solvent.

finity and specificity characteristic of the parent hormone for uterus and renal membranes.

Experimental Section

Optical rotations were measured with a Rudolph polarimeter (precision ±0.1°). UV spectra were recorded in a Cary 16 recording spectrophotometer. Enzymatic digestion of peptides was accomplished with papain (General Biochemicals) and leucine aminopeptidase (Miles). For column chromatography, silica gel 60 (Brinkman), particle size 0.063–0.2 mm, was used. TLC was performed on Eastman Chromagram TLS with fluorescent indicator. When partition solvent systems were used, the lower phase was sprayed on the TLS and development was accomplished with the upper phase. Partition solvent systems used for column chromatography or TLC were: (A) *n*-BuOH–*n*-PrOH–benzene–1% AcOH containing 0.5% Py (12:1:1:16); (B) *n*-BuOH–EtOH–Py–0.5% AcOH (5:1:1:8); (C) *n*-BuOH–AcOH–H_2O (4:1:5); (D) *n*-BuOH–EtOH–Py–1% AcOH (15:2:5:24); (E) *n*-BuOH–EtOH–benzene–Py–1% AcOH (4:1:0.1:1:7); (F) *n*-BuOH–AcOH–Py–H_2O (15:3:10:12); (G) *n*-BuOH–EtOH–Py–1% AcOH (14:2:5:24). Gel permeation chromatography was conducted on Sephadex G-15 or G-25 (Pharmacia). TLE was performed on Eastman Chromagram TLS with fluorescent indicator, in a Brinkmann-Desaga apparatus at 400 V for 2–4 h. Solvents used were reagent grade. Tritiations were performed by New England Nuclear Corp. Iodinations were performed on synthetic NHH prepared by the solid-phase method and purified by gel filtration and partition chromatography on Sephadex G-25 as reported elsewhere (Flouret et al., submitted). The biological activity of the synthetic NHH in the bovine renal medullary adenylate cyclase assay was identical to synthetic standards obtained from Drs. R. Walter and I. Schwartz; AVP was also tested in the rat antidiuretic assay and found to be highly potent (465 units/mg) and OT was tested in the oxytocic and in the avian depressor assays and found to have high potency, 500 and 512 units/mg, respectively.

[3-Iodo-Tyr²]oxytocin (MIOT). To a solution of OT (90 mg, 90 μmol) in H_2O (4.5 mL) was added MeOH (4.5 mL), 12 M NH_4OH (0.46 mL), and a 3.9% solution of I_2 in $CHCl_3$ or MeOH (0.60 mL, 90 μmol). After 10 min at room temperature, the colorless solution was treated with glacial acetic acid (0.66 mL) to pH 6. The iodinated peptides were applied to a Sephadex G-15 column (1.5 × 45 cm) and eluted with 50% AcOH. Fractions corresponding to the main peak (Folin–Lowry color) were pooled and lyophilized yielding 100 mg of product. The latter product was purified further by partition chromatography on a Sephadex G-25 column (2.0 × 72 cm), with solvent system A, by the procedure described by Yamashiro (1964) for the purification of OT. Peptide eluted (Figure 1b) had the following R_f's: DIOT, 0.46; MIOT, 0.30; OT, 0.11. The yield of products was MIOT, 37 mg; DIOT, 32 mg; OT, 12 mg. Further purification of MIOT and DIOT by gel filtration on Sephadex G-25 with 1% AcOH yielded 29 mg of DIOT and 26 mg of MIOT. MIOT thus obtained was homogeneous on TLE and on TLC, $R_f^A = 0.56$; $[\alpha]^{24}_D$ −17° (*c* 0.5, 1 N AcOH); $\lambda_{max}^{H_2O}$ = 282 nm (ϵ = 2360), $\lambda_{max}^{OH^-}$ = 305 nm (ϵ = 4110), shoulder at 289 nm (Figure 2). Anal calcd for $C_{45}H_{77}N_{12}O_{18}S_2I{\cdot}C_2H_4O_2{\cdot}4\ H_2O$: C, 42.5; H, 6.16; N, 13.3. Found: C, 42.7; H, 6.13; N, 13.3. Amino acid analysis (Spackman et al., 1958) of a sample of MIOT hydrolyzed with 6 N HCl at 110 °C for 20 h, showed the following amino acid molar ratios with Gly taken as 1.00: Asp 0.99, Glu 1.01, Pro 1.03, Gly 1.00, $(Cys)_2$ 0.62, Ile 0.95, Leu 0.96 and Tyr 0.83. Enzymatic hydrolysis of OT and MIOT (papain and leucine aminopeptidase) showed Tyr (R_f^A = 0.12) and monoiodotyrosine (R_f^A = 0.41), respectively, on TLC. Recovered OT ($[\alpha]^{24}_D$ −22.0° (*c* 0.5, 1 N AcOH); expected (Ressler and du Vigneaud, 1957) $[\alpha]^{24}_D$ −23.1° (*c* 0.51, 1 N AcOH)) was fully active in the adenylate cyclase assay. Thus, oxytocin appears to be recovered essentially unchanged.

[3,5-Diiodo-Tyr²]oxytocin (DIOT). A sample of DIOT (35 mg) prepared as shown above was homogeneous on silica gel TLC, R_f^A OT = 0.28, MIOT = 0.56, and DIOT = 0.76, and on TLE, with electrophoretic mobilities of OT = 3.9 cm, MIOT = 3.5 cm, DIOT = 2.5 cm; $[\alpha]^{25}_D$ −7.9° (*c* 0.5, 1 N AcOH); $\lambda_{max}^{H_2O}$ = 286 nm, (ϵ = 2200), shoulder at 295 nm; $\lambda_{max}^{OH^-}$ = 311 nm (ϵ = 5380). Anal calcd for $C_{45}H_{74}N_{12}O_{17}S_2I_2{\cdot}C_2H_4O_2{\cdot}3\ H_2O$: C, 39.2; H, 5.67; N, 12.3. Found: C, 39.4; H, 5.43; N, 12.3. A sample of DIOT digested with papain and leucine aminopeptidase and analyzed by TLC, as described for MIOT, showed diiodotyrosine (R_f^A = 0.59).

[3-Iodo-Tyr²,Lys⁸]vasopressin (MILVP). LVP (90 mg) was iodinated as described above for the preparation of MIOT. The reaction mixture was subjected to three successive gel permeation chromatography steps which gave elution profiles similar to the ones obtained in the iodination of OT: (a) Sephadex G-15 (50% AcOH), (b) Sephadex G-25 (1% AcOH),

and (c) repeat of step b, yielding 22 mg of MILVP and 22 mg of DILVP. MILVP has $\lambda_{max}^{H_2O}$ = 282 nm (ϵ = 2660), shoulder at 289 nm; $\lambda_{max}^{OH^-}$ = 306 nm (ϵ = 4040); $[\alpha]^{24}_D$ −22.4° (*c* 0.5, 1 N AcOH). MILVP was homogeneous on TLC, R_f^B = 0.23, and on TLE. Anal calcd for $C_{46}H_{64}N_{13}O_{12}S_2I \cdot 2C_2H_4O_2$: C, 46.4; H, 6.00; N, 14.0. Found: C, 46.1; H, 5.57; N, 14.0.

[*3,5-Diiodo-Tyr*^{2},*Lys*^{8}]*vasopressin* (*DILVP*). DILVP was obtained either as a byproduct of the preparation of MILVP or, alternatively, by iodination of LVP using an excess of iodine. Thus, using 90 mg of LVP and 1.2 mL of 3.9% I_2 in MeOH the yield is 49 mg (50%). DILVP is homogeneous on TLC, R_f^B = 0.47, and on TLE; $[\alpha]^{24}_D$ −15.8° (*c* 0.5, 1 N AcOH); $\lambda_{max}^{H_2O}$ = 286 nm, shoulder 294 nm (ϵ = 2530), $\lambda_{max}^{OH^-}$ = 311 nm (ϵ = 5810). Anal calcd for $C_{46}H_{63}N_{13}O_{12}S_2I_2 \cdot 2C_2H_4O_2 \cdot 2H_2O$: C, 41.1; H, 5.16; N, 12.9. Found: C, 41.0; H, 5.16; N, 12.4.

[*3-Iodo-Tyr*^{2},*Arg*^{8}]*vasopressin* (*MIAVP*). AVP (60 mg) was iodinated as described for OT. For purification of MIAVP, the crude reaction mixture was chromatographed on: (a) Sephadex G-15 (50% AcOH), by gel filtration; (b) Sephadex G-25 (solvent E, yielding R_f^E = 0.45), by partition chromatography; and (c) Sephadex G-25 (1% AcOH), by gel filtration, yielding 17.4 mg of analogue; $[\alpha]^{24}_D$ −9.3° (*c* 0.43, 1 M AcOH); $\lambda_{max}^{H_2O}$ = 282 nm (ϵ 2565), $\lambda_{max}^{OH^-}$ = 306 nm (ϵ = 4030). Anal calcd for $C_{50}H_{78}N_{15}O_{19}S_2I \cdot 2C_2H_4O_2 \cdot 3H_2O$; C, 43.4; H, 5.68; N, 15.2. Found: C, 43.5; H, 5.63; N, 15.0. MIAVP was homogeneous on TLC, R_f^F = 0.68, R_f^C = 0.31, and on TLE.

[*3,5-Diiodo-Tyr*^{2},*Arg*^{8}]*vasopressin* (*DIAVP*). DIAVP was obtained as a by-product of the above experiment. On Sephadex G-25 partition chromatography, DIAVP has R_f^E = 0.58. The yield of DIAVP was 14.6 mg; $[\alpha]^{24}_D$ −5.9° (*c* 0.34, 1 M AcOH); $\lambda_{max}^{H_2O}$ = 286 nm (ϵ = 2420), $\lambda_{max}^{OH^-}$ = 311 nm (ϵ = 5300). Anal calcd for $C_{50}H_{77}N_{15}O_{19}S_2I_2 \cdot 2C_2H_4O_2 \cdot 3H_2O$: C, 39.8; H, 5.14; N, 13.9. Found: C, 39.7; H, 5.14; N, 13.9. DIAVP was homogeneous on TLC, R_f^F = 0.68 and R_f^C = 0.39, and on TLE.

Model Hydrogenation of DIOT. In a Parr shaker, DIOT (1.63 mg, 1.185 μmol) was dissolved in MeOH (1 mL) and the solution was diluted further by the addition of EtOAc (2 mL) and AcOH (2 drops), 5% Pd on $CaCO_3$ (81.4 mg) was added, and the reaction mixture was hydrogenated at 55 psi with shaking for 40 min. The reaction mixture was filtered through Hiflo-Super Cel and the latter was washed with four 1-mL portions of MeOH. AcOH (2 drops) was added to the combined filtrate, and the solvents were removed under vacuum. The reaction products were routinely monitored at this point by TLC with solvent A. The product was analyzed by gel filtration on Sephadex G-25 column (0.9 × 44 cm) with 1% AcOH. The three major peaks detected (Figure 2) were A (probably [diAla^{1,6}]OT, see below), B (OT), and C (MIOT), and also minor peaks D (DIOT) and E (unknown). The product of peak B (0.3 mg estimated at 275 nm by UV spectrometry) was subjected to affinity chromatography (described later) on Neurophysin–Sepharose (preparation described below). The purity of OT was evaluated by TLC (system A) and the yield (0.21 mg, 19%) was determined by UV spectrometry. An adenylate cyclase assay showed the product to be as active as a standard OT preparation.

On the Identity of Peak A. In one experiment, the hydrogenation of DIOT (1.83 mg) was performed as above, with 183 mg of catalyst and 4-h reaction time. The product isolated showed only peaks A and E. Peak A yielded 0.92 mg; UV $\lambda_{max}^{1\%AcOH}$ 275 nm. A TLC analysis (Figure 2, inset) showed an identical R_f value with peak A obtained as a by-product in the preparation of OT as described above. An amino acid analysis showed the presence of 2 mol of Ala per mol of the other amino acids and absence of Cys, suggesting that this product is [diAla^{1,6}]OT. An adenylate cyclase assay showed negligible but detectable biological activity, probably due to contamination with a trace of OT. On affinity chromatography, this product showed no interaction with the Neurophysin–Sepharose and was eluted with the buffer front.

Neurophysin Isolation. Acetone-dried bovine posterior pituitary powder (5 g) was extracted following the procedure of Hollenberg and Hope (1968), yielding fractions A (493 mg of protein) and B (21 mg of NHH). From fraction A, 44 mg of Neurophysin (NP) was obtained by gel filtration chromatography on Sephadex G-50 (1% AcOH). Alternatively, we accomplished the purification of NP by affinity chromatography. Thus, fraction A (5 mg) was dissolved in 0.1 M NH_4OAc buffer (pH 5.7) and applied to a column of LVP–Sepharose (1 mL). Elution was performed with the same buffer. After elution of proteins, NP was eluted with 0.1 M HCOOH. The NP prepared by either gel filtration or affinity chromatography was subjected to polyacrylamide gel electrophoresis on thin plates employing a Tris-sulfate buffer at pH 9.0, at 325 V for 55 min. The electrophoretic patterns for the NP prepared by either method were essentially equal, and similar to the pattern previously published by Breslow et al. (1971).

Neurophysin–Sepharose 4B. Sepharose 4B (7 mL) was activated by cyanogen bromide (1.4 g) as described by Axen et al. (1967) and added to purified NP (67 mg) dissolved in pH 7.2 phosphate buffer (6.7 mL). After stirring for 24 h at 4 °C, the suspension was transferred to a column and the beads were washed successively with 0.1 M phosphate (100 mL, pH 7.2), 0.5 M glycine in 0.1 M phosphate (700 mL, pH 7.2), 0.1 M NH_4AcO (700 mL, pH 5.7), 0.1 M HCOOH (700 mL), and, finally, 0.1 M NH_4AcO (700 mL, pH 5.7). To determine binding capacity, OT (2.62 mg) was dissolved in 0.1 M acetate buffer pH 5.7 (0.5 mL) and was applied to a column of 1.9 mL of this preparation and the column was eluted with the same buffer. After unbound OT had been eluted, bound OT was determined (UV) by elution with 0.1 M formic acid. Bound OT amounted to 0.51 mg/mL of bed. The following peptides were not retained on the NP–Sepharose column (1 mL), eluting with 0.1 M NH_4OAc: [diAla^{1,6}]OT, [diAla^{1,6}]LVP, [diAla^{1,6}]AVP, MIOT, DIOT, MILVP, DILVP, MIAVP, and DIAVP, whereas the following peptides were retained in 0.1 M NH_4OAc and were eluted with 0.1 M HCOOH: OT dimer, LVP dimer, AVP dimer, OT, LVP, and AVP.

LVP–Sepharose 4B. To a suspension of Affi-Gel 10 (101 mg), an agarose gel (Bio-Rad) with aliphatic arms 10 Å in length with a carboxy-*N*-hydroxysuccinimide end group (Cuatrecasas and Parikh, 1972), in 0.1 M phosphate buffer (1 mL, pH 8.5) was added acetone–LVP (20 mg) (Yamashiro et al., 1967). The reaction was allowed to continue at 4 °C for 2 h. The beads were washed with the same buffer, with 1 M Gly-OMe dissolved in 0.1 M $NaHCO_3$ (6 mL) at 4 °C for 1 h (to decompose excess active ester), with phosphate buffer, and finally water. The beads were suspended in water (4 mL) and heated at 100 °C for 25 min to remove the blocking group. The beads were then washed in a column with H_2O (100 mL), 0.1 M HCOOH (100 mL), 0.1 M NH_4OAc buffer (150 mL, pH 5.7). Amino acid analysis showed 5.3 μmol of peptide/mL of gel. When NP (45 mg) in 0.1 M acetate buffer (pH 5.7) was added to 1 mL of the above affinity carrier, 6.49 mg was retained of a possible 53 mg assuming 1:1 binding of NP to LVP,

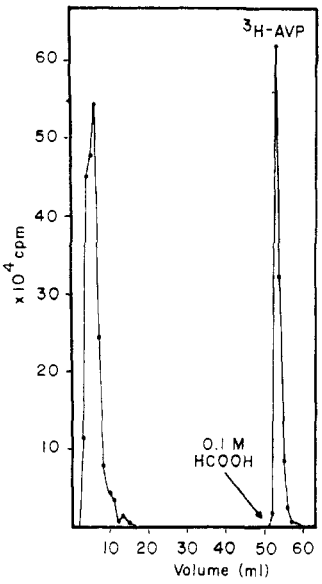

FIGURE 3: Purification of [^{3}H]AVP by affinity chromatography. The products of tritiation of DIAVP (1.9 mg) were separated on a neurophysin–Sepharose column (2 mL); eluent 0.1 M NH_4OAc buffer, pH 5.7; fraction size 1 mL; at fraction 50, the eluent was switched to 0.1 M HCOOH. Radioactivity was measured on 1-μL samples.

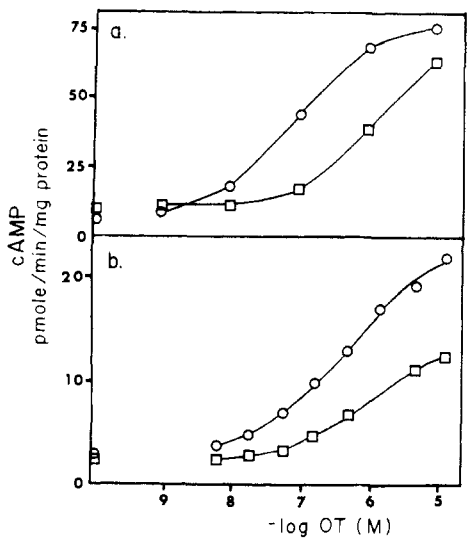

FIGURE 4: Inhibitory effect of MIOT on adenylate cyclase stimulation by OT. (a) The effect of OT upon NHH sensitive adenylate cyclase activity in a bovine renal medullary membrane preparation was measured in the absence (-O-) and in the presence (-□-) of MIOT (10^{-3} M) simultaneously added (standard assay). (b) Bovine renal medullary membranes were preincubated for 10 min with (-□-) and without (-O-) MIOT (10^{-3} M), OT was then added and adenylate cyclase response was measured (standard assay conditions).

on a molar basis.

[^{3}*H-Tyr*^{2}]*OT* ([^{3}*H*]*OT*). To DIOT (3.02 mg, 2.2 μmol) dissolved in MeOH (2 mL), EtOAc (2 mL), and AcOH (3 drops), was added 5% Pd/$CaCO_3$ (60 mg). Tritiation was carried out with 25 Ci of carrier-free $^3H_2$ for 20 min. The reaction solvents were removed as described in the model hydrogenation. The [^{3}H]OT was purified by partition chromatography on a Sephadex G-25 column (0.9 × 44 cm) with solvent A. The fractions collected showed a peak of [^{3}H]OT, R_f 0.24. The [^{3}H]OT was dissolved in 0.1 M NH_4OAc buffer (pH 5.7). The sample was applied to a column of NP–Sepharose 4B (2.0 mL) equilibrated with 0.1 M NH_4OAc buffer (pH 5.7) and the column was washed with the same buffer (30 mL) and then with 0.1 M HCOOH which elutes [^{3}H]OT. Alternatively, the [^{3}H]OT could be purified directly by affinity chromatography as above and by passing the [^{3}H]OT thus obtained through the affinity column a second time under identical conditions.

[^{3}H]OT was homogeneous, on TLC and TLE, as determined by dividing the TLS into segments and then measuring the radioactivity in each segment; UV $\lambda_{max}^{1\%AcOH}$ = 275. Based on the total radioactivity obtained and the oxytocic and/or adenylate cyclase assay of the product, the specific activity was determined. Preparations were made having specific activities of 25.0, 31.0, and 25.0 Ci/mmol. One commercial preparation having 31 Ci/mmol was purchased and analyzed by affinity chromatography, which showed 46% of the radioactivity corresponding to [^{3}H]OT. The [^{3}H]OT thus obtained had a specific activity of 20 Ci/mmol.

[^{3}*H-Tyr*^{2}]*LVP* ([^{3}*H*]*LVP*). DILVP (3 mg) dissolved in H_2O (3 mL), MeOH (3 mL) was tritiated and purified by affinity chromatography as described in the above experiment. [^{3}H]LVP was homogeneous on TLC and TLE; $\lambda_{max}^{H_2O}$ = 275 nm. The adenylate cyclase assay showed full biological activity. The specific activity of this sample was 26 Ci/mmol.

[^{3}*H-Tyr*^{2}]*AVP* ([^{3}*H*]*AVP*). DIAVP (1.9 mg) was tritiated and purified by affinity chromatography (Figure 3) as described for the preparation of [^{3}H]LVP. [^{3}H]AVP was homogeneous on TLC and TLE; $\lambda_{max}^{H_2O}$ = 275 nm. In the adenylate cyclase assay, [^{3}H]AVP was fully active showing a specific activity of 17.2 Ci/mmol.

(*a*) *Oxytocic Assay*. Peptide analogues were evaluated in an oxytocic assay (Holton, 1948). Isometric contractions of isolated uterine horns incubated at 32 °C in 10 mL of modified Van Dyke Hastings solution containing 0.5 mM Mg^{2+} (Munsick, 1960) were measured with a Grass displacement transducer and a Beckman Dynograph. The molarity of peptide which produces half-maximal response (designated as apparent K_m) was determined for each analogue relative to a synthetic preparation of oxytocin used as standard (Hechter et al., 1975). The pD_2 is the negative log of the molar concentration of OT which gives a half-maximal response. The apparent affinity of competitive antagonists was estimated in terms of pA_2, determined from the displacement of the dose-response curve along the hormone concentration axis, according to Schild (1947).

(*b*) *Adenylate Cyclase Assay*. Partially purified renal medullary membranes were prepared from perfused bovine kidneys by a poly(ethylene glycol)–dextran double-phase technique to be reported elsewhere (Nakahara T., Terada, S., Pincus, J., Flouret, G., and Hechter, O., in preparation).[3] Adenylate cyclase activity was assayed at 30 °C in a medium containing Bistris–propane (pH 8.5), 2.0 mM $MgCl_2$, 1.4 mM EDTA, 100 μM ATP, 1 mM cAMP, 20 mM creatine phosphate, and 0.2 mg/mL creatine kinase. The bovine membranes

[3] Essentially, the method involves homogenization of renal medullary tissue with 0.25 M sucrose containing 1 mM EDTA; the 1500*g* pellet obtained from the initial homogenate is then dispersed in the double-phase polymer system which contains 10 mM Tris-maleate buffer (pH 6.8), 1 mM EDTA, and 5 mM $MgCl_2$, and plasma membranes are obtained at the interface after centrifugation at 11 000*g*. In such membranes, the mean specific activity of maximally stimulated (AVP) cyclase specific activity (corrected for basal) was 700 pmol 10 min^{-1} (mg of protein)$^{-1}$; the average ratio of NHH-sensitive adenylate cyclase activity to basal was 14. When tested under identical conditions, bovine membranes prepared with the double-phase method had somewhat higher specific activities than those prepared using the methods of Fitzpatrick et al. (1969) or Birnbaumer and Yang (1974).

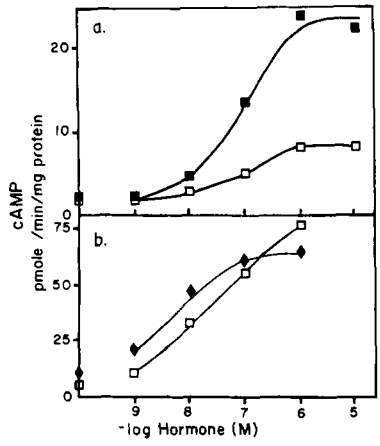

FIGURE 5: Effect of MIOT on the response of hormone-sensitive adenylate cyclase in renal cortex and rat liver membranes. (a) Bovine renal cortex membranes were assayed (using the same standard conditions used for the assay of renal medullary membranes) with PTH^{1-34} added in the absence (-■-) and in the presence (-□-) of simultaneously added MIOT (10^{-3} M). (b) Rat liver membranes were assayed (using the same standard conditions used in the assay of renal medullary membranes) with glucagon added in the absence (-□-) and in the presence (-♦-) of simultaneously added MIOT (10^{-3} M).

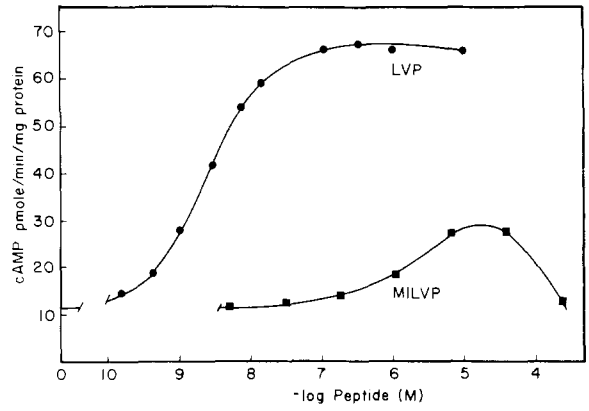

FIGURE 6: Comparison of LVP and MILVP on adenylate cyclase activity in bovine renal medullary membranes (standard assay conditions).

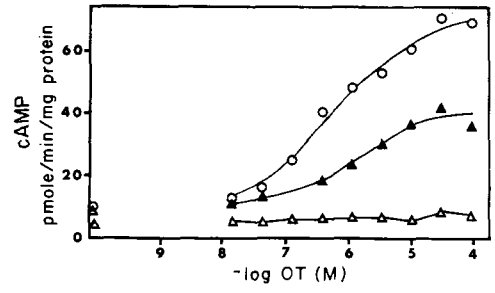

FIGURE 7: Effect of preincubation of MILVP on adenylate cyclase response to OT. Bovine renal medullary membranes were preincubated for 10 min without (-○-) and with MILVP 10^{-5} (-△-) and 10^{-6} (-▲-) M, prior to the addition of OT (standard assay).

were shown to be devoid of enzymatic activity capable of degrading [^{3}H]LVP or [^{3}H]OT.

Results and Discussion

The direct iodination of NHH, using I_2 in $CHCl_3$-MeOH-H_2O at about pH 8, is a mild and convenient method to obtain iodinated analogues in good yield without attack on either the disulfide bond or the tyrosyl-peptide bond.

MIOT was purified by successive gel filtrations on Sephadex G-25 (Figure 1a): the product purified after two gel-filtration steps had no detectable stimulatory activity in the NHH-specific renal cyclase; MIOT was also purified by preliminary partition chromatography on Sephadex G-25 (Figure 1b), or countercurrent distribution employing the same partition solvent system, supplemented with a final gel filtration on Sephadex G-25. The products obtained are identical in chromatographic systems and in the bovine cyclase system. MILVP and MIAVP were synthesized and purified by similar methods.

When tested immediately after preparation, MIOT was shown to be without agonistic activity both in the renal medullary cyclase system and in the oxytocic assay. MIOT preparations stored for several months (whether in a desiccator, in solid form, or as a frozen solution at −20 °C) show uterotonic activity and stimulatory effects on the renal medullary cyclase system. Similar results were obtained with MIAVP and MILVP. Accordingly, freshly prepared samples were tested for biological activity, since iodo-NHH appears to slowly undergo degradation to NHH with concomitant loss of I. In the adenylate cyclase assay, MIOT was a weak competitive antagonist (apparent K_i about 10^{-4}) when tested together with OT (apparent K_m 9 × 10^{-8}) (Figure 4a). Noncompetitive inhibition of OT was observed when the renal cyclase preparation was first exposed to MIOT (10 min at 37 °C) before adding OT (Figure 4b); the magnitude of the OT response was reduced about 50% without influence on either basal or fluoride-stimulated cyclase activities. MIOT was found to be a weak competitive inhibitor of OT in the rat oxytocic assay (Holton, 1948). Marbach and Rudinger (1974) and Flouret et al. (1975) reported pA_2 values of 7.0 and 7.2, respectively, whereas in the latter study OT had pD_2 = 10.5. Since [Tyr(Me)^2]OT is also an antagonist in the uterus, the inhibitory effects of MIOT appear to be due to steric effects and not to changes in acidity of the phenolic hydroxyl group, as pointed out by Marbach and Rudinger.

The inhibitory effects of MIOT observed in renal medullary and uterine systems were not specific for NHH receptors. Thus, MIOT (10^{-4} M) modified the parathyroid hormone sensitive bovine renal cortex cyclase system (Figure 5a) and the glucagon-responsive rat liver cyclase systems (Figure 5b).

DIOT (10^{-8}–10^{-3} M) was found to be without agonistic or antagonistic activity in the uterotonic and renal cyclase assays.

In contrast to MIOT, freshly prepared MILVP had partial agonistic activity in the renal medullary cyclase system, with maximal response about 30% of LVP maximal response and K_m = 3 × 10^{-6}, whereas in the same experiment LVP had K_m = 2.5 × 10^{-9} (Figure 6). When MILVP was preincubated with renal medullary membranes and then tested with OT (Figure 7), it was a more potent noncompetitive inhibitor of OT than MIOT. MIAVP also appears to be a partial agonist in the renal cyclase system, with maximal response about 60% that of AVP maximal response and K_m = 3 × 10^{-7}, whereas AVP had K_m = 1 × 10^{-9} in the same experiment. It was difficult to definitively establish to what extent the high biological activity was due to MIAVP itself or to AVP arising through relatively rapid deiodination. MIAVP appeared to be more unstable than MILVP, which was more unstable than MIOT, suggesting that the substituent at position 8 may have a significant influence on the stability of the I–C bond in the iodo-Tyr^2 residue.

While these results establish that MIOT, MILVP, and

MIAVP interact with NHH receptors in renal membranes, they have low affinity relative to parent hormones; the potential advantage of higher specific activity of a ^{125}I label vs. a ^{3}H label (76 times higher) is cancelled by the more than 10^2-fold reduction in affinity relative to tritiated hormones. The poor stability of iodinated analogues, and evidence for their interaction with membrane sites unrelated to NHH receptors, suggest that MILVP (or MIAVP) or MIOT would not be useful ligands for NHH receptor sites in renal medulla and uterus, respectively.

Since the diiodo analogues of AVP, LVP, and OT were biologically inactive in the cyclase assay (and DIOT in the oxytocic assay as well), we studied the conversion of DIOT, DIAVP and DILVP to the tritiated hormones. It was hoped that catalytic tritiation of the latter analogues would lead to tritiated NHH of higher specific activity than those attained by Agishi and Dingman (1965), Morgat et al. (1970), or Pradelles et al. (1972) who reduced the crude iodinated NHH products without isolation (or characterization). Best conditions for hydrogenations of DIOT in model reactions were established; the products of hydrogenation (Figure 2) showed a complex mixture with 5 components: OT, MIOT, DIOT, and unknown products A and E. Product A was tentatively identified as [diAla^{1,6}]OT on the basis of amino acid analysis and was the major product when hydrogenation period was extensive. For purification of [^{3}H]NHH, a variety of systems were tried including gel filtration, partition chromatography on Sephadex G-25, and affinity chromatography on purified neurophysin (NP) bound to a solid support (Pradelles et al., 1972).[4] All three [^{3}H]NHH products purified by the techniques of gel filtration or partition chromatography on Sephadex G-25 appeared to be chemically pure, according to the criteria of UV spectrometry, TLC, and TLE, and had full biological activity in the adenylate cyclase assay and/or in the oxytocic assay. However, upon utilization of the preparations for binding studies in bovine kidney (Hechter O., Terada, S., Nakahara, T., and Flouret, G., in preparation) and rabbit uterus (Nissenson R., Flouret, G., and Hechter, O., in preparation) using techniques similar to those described by Bockaert et al. (1973), high nonspecific binding values were obtained. Only samples where NP affinity chromatography had been used in purification gave consistently low nonspecific binding, and were useful as radioactive ligands for the detection of specific sites. A commercial preparation, purchased as pure [^{3}H]OT, which had 55% labeled impurities (and high nonspecific binding)[5] could be purified to radiochemical homogeneity by affinity chromatography, with low values of nonspecific [^{3}H]OT binding. In order to obtain valid and reproducible data, it is necessary to have in-house methods for the purification of iodo or tritio derivatives of NHH, prior to chemical, physical, or biological studies.

[4] Recently, LVP-Sepharose has been prepared and used for the purification of NP by affinity chromatography. Thus, Frenoy et al. (1974) coupled LVP to cyanogen bromide activated Sepharose 4B and Flouret et al. (preliminary report, 1975) coupled acetone-LVP to Affigel-10 (an active ester derivative of Sepharose 4B), as detailed in the Experimental Section; and Edgar and Hope (1974) coupled acetone-LVP to cyanogen bromide activated Sepharose 4B.

[5] In our best preparations, nonspecific binding of [^{3}H]LVP and AVP to NHH renal receptors was about 3-7% of specific binding in the concentration range 10^{-10}-10^{-8} M; at concentrations greater than 10^{-8} M, nonspecific binding rises very sharply and exceeds specific binding. Similar results are obtained with [^{3}H]OT and uterine or myometrial membrane receptors. With the commercial preparation of [^{3}H]OT, nonspecific binding to uterine membranes was 30-40% of specific binding in the concentration range 10^{-10}-10^{-8} M.

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