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Synthesis and Characterization of 2-Nitro-5-azidobenzoylglycyloxytocin, an Oxytocin Photoaffinity Label†

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ABSTRACT: The oxytocin analogue, 2-nitro-5-azidobenzoylglycyloxytocin (NAB-Gly-oxytocin), has been synthesized and purified. The analogue is a full agonist for the stimulation of osmotic water flow in the toad urinary bladder (one-half maximal activity at 3.2×10^{-6} M). It also enhances [^{14}C]urea permeability in this tissue. Repetitive photolysis in the presence of NAB-Gly-oxytocin (8×10^{-6} M) results in a progressive permanent inhibition of oxytocin stimulated urea permeability but does not alter hormone induced ^3H_2O movement. The inhibition is dependent on the photogeneration of the aryl nitrene intermediate and is relieved by protecting the hormone receptor with excess oxytocin (10^{-6} M) during the photolysis. These results suggest that the photodependent permanent inhibition of the response to oxytocin in the toad bladder is due to covalent incorporation of the photoaffinity label, NAB-Gly-oxytocin, into the hormone receptor.

The initial event in the action of peptide hormones is the binding of these ligands to specific receptors in the plasma membrane of target tissue cells (Cuatrecasas, 1974; Kahn, 1975). Recently there has been increasing interest in the identification, characterization, and isolation of these receptors. Several peptide hormone receptors have been identified by observing the specific, reversible binding of radioactively labeled hormones in isolated cells and membrane preparations from target tissues (Kahn, 1975; Catt & Dufau, 1977). However, even during mild purification procedures an alteration and/or loss of the specific binding properties for the ligand is observed as the receptor is solubilized away from the membrane lipid bilayer. Additionally, receptor solubilization often uncouples the receptor from any functional marker, e.g., adenylate cyclase, that could be followed through successive purification steps. It is necessary, therefore, to devise means of labeling hormone receptors covalently, in situ, and follow the incorporated label as a means of monitoring the isolation procedures.

The application of photoaffinity labeling to the problem of biological receptor identification has been discussed (Knowles, 1972; Cooperman, 1976). Photoaffinity labeling has a distinct advantage over chemical affinity labeling since the interaction of the ligand analogue with the receptor of interest can be characterized prior to covalent incorporation (dark experiments). The highly reactive intermediates, usually carbenes or nitrenes, generated in the binding site during photolysis increase the potential for specific labeling of the receptor, even in complex biological systems. Photoaffinity labeling has found wide application in the investigation of enzymes (Singh et al., 1962; Shafer et al., 1966), antibody binding sites (Fleet et al., 1972; Yoshioka et al., 1973; Choy et al., 1975; Converse & Richards, 1969), and nucleotide receptors (Guthrow et al., 1973; Haley, 1975; Pomerantz et al., 1975; Brunswick & Cooperman, 1971; Schafer et al., 1976).

In the present study we report the synthesis and purification of the photoactivated hormone analogue 2-nitro-5-azidobenzoylglycyloxytocin (NAB-Gly-oxytocin). The hormone oxytocin was chosen for these studies because its amino acid sequence (Figure 1) allows controlled derivatization and large quantities of the synthetic peptide were available. Additionally, the design of the photolabile hormone analogue was greatly facilitated by the availability of extensive structure-activity data (Rasmussen et al., 1963; Sawyer & Manning, 1973; Rudinger et al., 1972; Walter et al., 1971, 1967; Walter, 1971). These structure-activity studies made it clear that coupling of the photoactive reagent to the N-terminal amine of oxytocin would bring the aryl azide in close contact with the receptor, but not completely interfere with the binding specificity of the receptor for the hormone. We found that NAB-Gly-oxytocin retained a high affinity for the antidiuretic hormone receptor in the isolated toad urinary bladder. The derivatized hormone was a potent agonist for the stimulation of osmotic water flow (one-half maximal activity at 3.2×10^{-6} M) as well as stimulating [^{14}C]urea diffusion in this tissue. Photolysis of the toad bladder in the presence of NAB-Gly-oxytocin led to a permanent inhibition of the oxytocin enhanced change in [^{14}C]-urea permeability. Evidence that this permanent inhibition is due to covalent labeling of the antidiuretic hormone receptor by the photoactivated oxytocin analogue is provided by the fact that this inhibition is dependent on the presence of the azido

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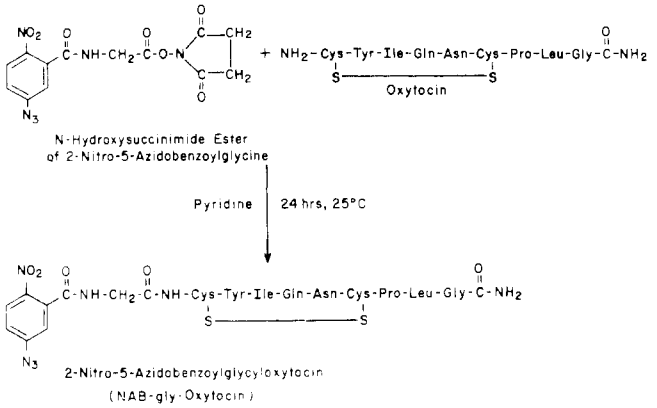

FIGURE 1: Structure of oxytocin and synthesis of NAB-Gly-oxytocin. Synthetic reaction carried out as described in Materials and Methods.

functional group and that the inhibition is prevented by photolysis in the presence of saturating concentrations of native oxytocin.

Materials and Methods

Preparation of Oxytocin. The protected nanopeptide Z-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-GlyNH_2 was the generous gift of Dr. E. D. Nicolaides, Park Davis and Co., Detroit, Mich. The protecting groups were removed by reduction with sodium in liquid ammonia and the intramolecular disulfide bond formed by oxidation with a solution of potassium ferricyanide (Walti & Hope, 1973). Oxytocin was purified by gel filtration on Bio-Gel P-2 (Bio-Rad, Richmond, Calif.) equilibrated with 50% acetic acid (Manning et al., 1968). The biological activity of the isolated peptide was assayed by measuring the hydroosmotic response in the isolated toad urinary bladder (Bentley, 1958). Half-maximal activity was induced by 7.1×10^{-9} M hormone, in good agreement with previous reports of oxytocin activity in this system (Rasmussen et al., 1963).

Synthesis of NAB-Gly-Oxytocin. Four milligrams of the *N*-hydroxysuccinimide ester of 2-nitro-5-azidobenzoylglycine (Galardy et al., 1974; 11 μmol) and 5 mg of oxytocin (5 μmol) were dissolved in 50 μL of pyridine in a 2.0-mL glass centrifuge tube. After 24 h at 25 °C in the dark, the reaction mixture was diluted with 1 mL of water and the solution lyophilized.

Partition Chromatography. Sephadex G-25 fine (Pharmacia, Piscataway, N.J.) was used as the support and the solvent system employed was butanol:hexane:water (3:1:4; Yamashiro, 1964). All procedures were carried out at 4 °C. For the purification of NAB-Gly-oxytocin, the Sephadex G-25 column (1 × 65 cm) was equilibrated first with the aqueous and then the organic phase of the solvent system. The lyophilized reaction mixture was dissolved in 1 mL of the organic phase, applied to the top of the column, and eluted with the organic phase (3–5 mL/h); 2-mL fractions were collected. The peptide material was detected by the Lowry method (Lowry et al., 1951) and recovered by lyophilization.

Hydroosmotic Assay. Paired hemibladders were removed from doubly pithed toads (*Bufo marinus,* National Reagents, Bridgeport, Conn.) and incubated in Ling-Ringer solution (composition in mM: NaCl, 92.7; KCl, 2.5; $CaCl_2$, 1.0; $MgSO_4$, 1.2; $NaHCO_3$, 7.8; NaH_2PO_4, 2.0; Na_2HPO_4, 1.2; pH 7.4; osmolarity $200–220 \times 10^{-3}$ osM/kg of H_2O; Ling, 1962). Hemibladders were tied to glass cannulae and the mucosal surface was bathed with dilute (1:5) buffer. The rod and attached hemibladder were bathed in a full strength buffer. Osmotic water flow was determined gravimetrically (Bentley, 1958). After a 30–60-min equilibration period, baseline water movement was determined (<1 mg/min) for two 10-min periods. The serosal medium was then changed to Ling-Ringer buffer containing the desired final concentration of agonist. Weight loss, i.e., waterflow, was determined over the next 20–30 min with weighings every 10 min. The first two weighing periods after agonist addition were combined for the determination of the dose–response curve. Bladders were then washed by successive changes of the serosal Ling-Ringer buffer until water flow returned to baseline (30 min). The mucosal bathing solution was changed before addition of another agonist dose.

Diffusion Assay. Hemibladders were mounted between halves of a double compartment lucite chamber (Goodman et al., 1969). Each chamber was filled with Ling-Ringer buffer which was kept circulating by a stream of moist air. After a 30-min equilibration period, ^3H_2O or [^{14}C]urea (3.5×10^5 cpm/mL) was added to the mucosal chambers. Diffusion was determined by sampling the radioactivity appearing in respective serosal chambers every 20 min. After each 20-min period, the serosal bathing solutions were replaced with fresh buffer. At the indicated times, agonists were added to the appropriate serosal bathing solutions.

Photolysis Experiments. Hemibladders were stretched as a planar sheet on a metal rack (4 × 4 cm). The bladder was washed in buffer for 30–60 min by gentle shaking in a Petri dish. The hemibladder was placed mucosal surface down on the surface of the buffer in a Petri dish; Ling-Ringer buffer with or without hormone analogue was applied to the serosal surface and equilibrated for 15 min. Half the hemibladder was protected with black plastic and the mucosal surface of the tissue was then exposed to a Hanovia 450-W high pressure mercury immersion lamp in a water-jacketed borosilicate immersion well fitted with a Corning No. 3220 immersion filter to eliminate light at wavelengths ≤ 320 nm. The bladder was then washed by shaking in a Petri dish for 30 min with five changes of buffer before the serosal surface was reequilibrated in preparation for the next photolysis. Biological activity was determined by measuring ^3H_2O or [^{14}C]urea diffusion.

Absorbance spectra were recorded using a Cary 15 spectrophotometer. Thin-layer chromatography (TLC) was carried out on glass plates coated with Silica Gel G (Brinkmann) and zinc silicate as a fluorescent indicator. The solvent system employed was butanol–acetic acid–water (4:1:5). Paper electrophoresis was carried out as described previously (Barnes et al., 1972). Two buffer systems were employed: 20.0 mM sodium carbonate (pH 10.5) and 20 mM sodium acetate (pH 5.0). Whatman no. 1 filter paper was used as the support medium for electrophoresis. A potential difference of 230 V was applied across the supporting medium for 1 h at room temperature. Oxytocin was detected by fluorescamine spray (Fluram, Hoffmann-La Roche, Nutley, N.J.; Udenfriend et al., 1972). The derivatized peptide was detected by fluorescent quenching of the paper when excited at 365 nM by a hand held lamp.

All solvents were redistilled prior to use and all chemicals were reagent grade.

Results

Synthesis and Purification of NAB-Gly-Oxytocin. We have utilized the *N*-hydroxysuccinimide ester of 2-nitro-5-azidobenzoylglycine (Anderson et al., 1964; Galardy et al., 1974) to couple a photolabile aryl azide to the N-terminal amino group of the peptide hormone oxytocin (Figure 1). NAB-Gly-oxytocin was purified from the reaction mixture by Se-

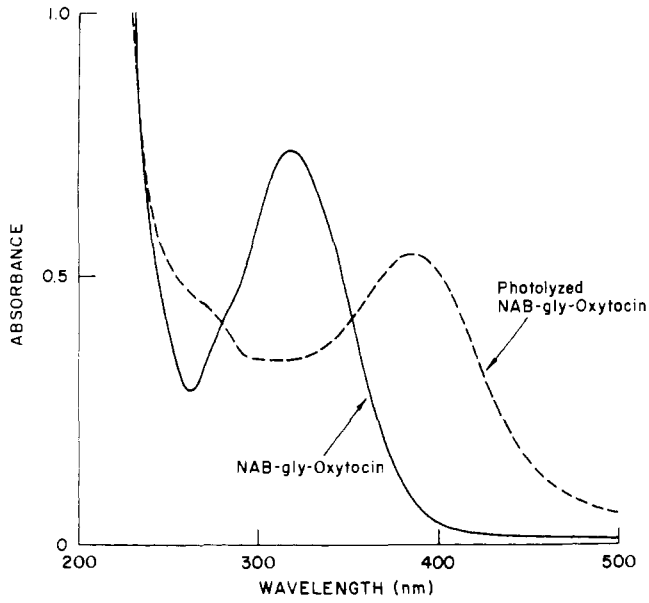

FIGURE 2: The effect of photolysis at ≥320 nm on the ultraviolet-visible absorbance spectrum of NAB-Gly-oxytocin. NAB-Gly-oxytocin was dissolved in Ling-Ringer buffer (pH 7.4) and irradiated at ≥320 nm for 1 min.

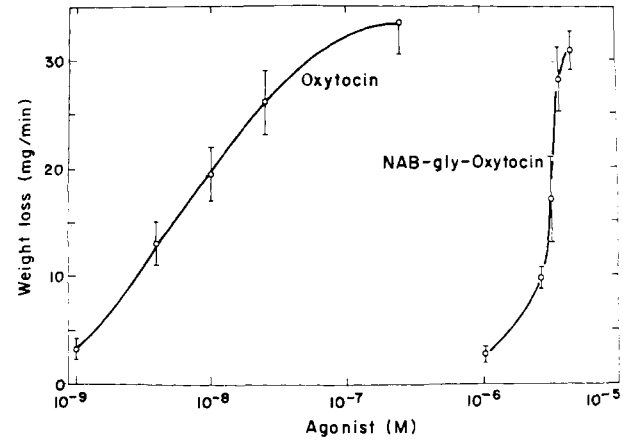

FIGURE 3: Comparison of the hydroosmotic response to oxytocin and NAB-Gly-oxytocin. Paired hemibladders were tied to glass cannulae and incubated in Ling-Ringer buffer as described in Materials and Methods. Baseline water movement was determined for two 10-min periods. Agonist was added by changing the serosal bathing medium and water flow monitored over the next 20–30 min with weighings every 10 min. The first two weighings were combined for the determination of the dose-response curve (oxytocin, $n = 8$; NAB-Gly-oxytocin, $n = 6$; bars = SEM).

phadex G-25 partition chromatography as described in Materials and Methods. The peptide eluted from the column as a single peak, $R_f = 0.68$. The NAB-Gly-oxytocin recovered from the partition column ran as a single spot on TLC ($R_f = 0.31$). Conversion of oxytocin to NAB-Gly-oxytocin was essentially quantitative under the reaction conditions described as monitored by thin-layer chromatography.

Chemical Characterization. NAB-Gly-oxytocin was determined to be free of contamination by native oxytocin after a single pass through the partition column by spraying the TLC plates with fluorescamine, a very sensitive reagent for primary amines (Udenfriend et al., 1972). Under our experimental conditions as little as 10 ng of native oxytocin can be detected. Routinely 50 μg of NAB-Gly-oxytocin was chromatographed. Under these conditions no native oxytocin was detected. Thus the preparation of NAB-Gly-oxytocin contained less than 0.02% native oxytocin. The failure of NAB-Gly-oxytocin to form a fluorescent adduct with fluorescamine is evidence that the photolytic aryl azide is covalently coupled to the amino terminus of oxytocin. Further evidence for the position of derivatization was obtained by paper electrophoresis at pH 10.5 and pH 5.0. Both NAB-Gly-oxytocin and native oxytocin migrate toward the anode relative to the neutral marker sucrose at pH 10.5 which is close to the pK of the phenolic side chain of tyrosine at position 2 in the amino acid sequence of oxytocin (see Figure 1). At pH 5.0, which is well below the pK of the N-terminal amine, oxytocin moves toward the cathode and migrates well beyond NAB-Gly-oxytocin which coelectrophoreses with sucrose. The electrophoretic mobility of NAB-Gly-oxytocin compared with oxytocin at the two different pHs demonstrates a single derivatization of oxytocin at the N terminus.

The absorbance spectrum of NAB-Gly-oxytocin (Figure 2) is characterized by a single peak ($\lambda_{max} = 317$ nm) due to the nitro aryl azide and a shoulder near 280 nm contributed by the tyrosine at position 2 of oxytocin ($\lambda_{max} = 275$ nm). Upon photolysis (1 min) with a high pressure mercury lamp (≥320 nm), a stable spectrum was obtained that retained the shoulder near 280 nm, but the major absorbance peak was shifted ($\lambda_{max} = 383$ nm) toward higher wavelengths. This red spectral shift is attributed to a solvolysis reaction of the nitrene intermediate generated by photolysis of the aryl azide.

TABLE I: Hydroosmotic Activity of NAB-Gly-oxytocin in the Toad Urinary Bladder.[a]

Agonist	M	n	Weight loss, mg/min
NAB-Gly-oxytocin	3.8×10^{-6}	5	27.1 ± 3.2
Prephotolyzed NAB-Gly-oxytocin	3.7×10^{-6}	3	21.2 ± 0.6
Rechromatographed NAB-Gly-oxytocin	3.8×10^{-6}	6	32.0 ± 1.5

[a] Hydroosmotic assay was carried out as described in Materials and Methods. Data are presented as the mean ± SEM.

Biological Activity. NAB-Gly-oxytocin stimulates osmotic water flow in the isolated toad urinary bladder (Figure 3). Although the derivatized hormone is a weaker agonist than oxytocin, it still retains considerable affinity for the antidiuretic hormone receptor (one-half maximal activity at 3.2×10^{-6} M) and has full agonist activity. When NAB-Gly-oxytocin was rechromatographed on the Sephadex G-25 partition column, the product maintained constant specific activity (Table I) indicating that the observed agonist activity was intrinsic to the hormone analogue. NAB-Gly-oxytocin (6.5×10^{-6} M) was also a full agonist in stimulating [^{14}C]urea permeability in the toad bladder (Figure 4).

Photolysis Experiments. Because of the difficulty in photolyzing tissue mounted as sacs and the requirement for large quantities of hormone derivative, photolysis experiments were conducted on tissue mounted on a rack as a planar sheet. When the serosal surface of the toad bladder was equilibrated with buffer alone, repeated photolysis had no effect on oxytocin stimulated ^3H_2O or [^{14}C]urea permeability. Also photolysis (5×1 min) in the presence of 9.6×10^{-6} M 2-nitro-5-azidobenzoylglycine had no effect on the subsequent response to native oxytocin (data not shown). Incubation of the bladder with NAB-Gly-oxytocin (8×10^{-6} M) prior to photolysis resulted in a permanent inhibition of the oxytocin initiated increase in [^{14}C]urea diffusion but had no effect on ^3H_2O diffusion (Figures 5A and 5B and Table II). The extent of the inhibition of the hormone induced increase in urea permeability

TABLE II: Effects of Photolysis in the Presence and Absence of NAB-Gly-oxytocin and Oxytocin on Toad Bladder Response to Oxytocin.[a]

[NAB-Gly-oxytocin] (M)	[Oxytocin] (M)	Time of photolysis	n	% inhibition of [^{14}C]urea[b] permeability
0	0	5 × 1 min	3	0
8×10^{-6}	0	3 × 1 min	2	36.1, 23.7
8×10^{-6}	0	5 × 1 min	6	49.9 ± 3.2
8×10^{-6}	10^{-6}	5 × 1 min	3	13.9 ± 0.7

[a] Photolysis experiments and [^{14}C]urea diffusion assays were carried out as described in Materials and Methods. [b] The percentage of inhibition due to photolysis was calculated by comparing the urea permeability 40 min after oxytocin with baseline urea permeability in photolyzed and control tissue.

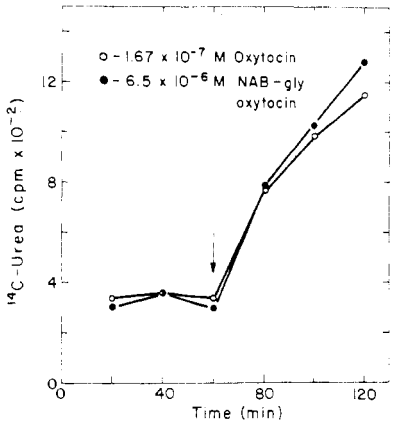

FIGURE 4: Comparison of the effect of NAB-Gly-oxytocin and oxytocin on [^{14}C]urea permeability. Hemibladders were mounted between halves of a lucite diffusion chamber and incubated with Ling-Ringer buffer as described in Materials and Methods. [^{14}C]Urea diffusion was determined by changing the serosal bathing medium every 20 min and counting an aliquot for radioactivity. At 60 min, NAB-Gly-oxytocin (6.5×10^{-6} M) and oxytocin (1.67×10^{-7} M) were added to the appropriate serosal bathing solutions.

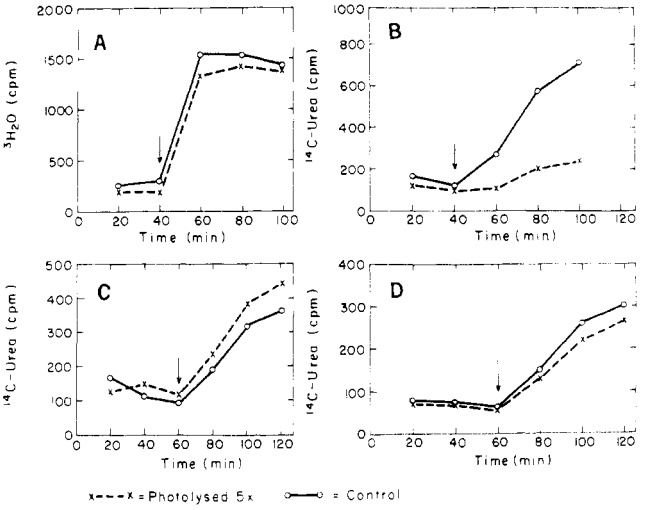

FIGURE 5: The effect of photolysis (5×) on the presence of NAB-Gly-oxytocin on the response of the toad bladder to native oxytocin. The tissue was mounted on a rack, exposed to NAB-Gly-oxytocin (8×10^{-6} M) for 15 min, and photolyzed at ≥320 nm for 1 min as described in Materials and Methods. Control tissue was also exposed to NAB-Gly-oxytocin, but was covered with black plastic during photolysis. The tissue was then washed in Ling-Ringer buffer with gentle shaking for 30 min. After repetition of this procedure for a total of five times, the tissue was mounted in a lucite chamber and ^3H_2O (A) or [^{14}C]urea permeability (B) determined. After establishing baseline permeability, both halves of the hemibladder were challenged with oxytocin (8.4×10^{-9} M). (C) The effect of photolysis (5×) in the presence of NAB-Gly-oxytocin plus native oxytocin on the response of the toad bladder to oxytocin. Experimental conditions were identical with those employed in A and B except that the incubation medium before photolysis contained native oxytocin (10^{-6} M) in addition to NAB-Gly-oxytocin (8×10^{-6}). Photolysis was repeated five times before determination of [^{14}C]urea permeability. (D) The effect of photolysis (5×) in the presence of prephotolyzed NAB-Gly-oxytocin on the response of the toad bladder to native oxytocin. Experimental conditions were identical with those employed in A and B except that the NAB-Gly-oxytocin was photolyzed 2 min prior to incubation with the tissue. After exposing the tissue to the prephotolyzed material the repetitive photolysis procedure was carried out. After each washing fresh prephotolyzed NAB-Gly-oxytocin was added to the tissue. After the fifth photolysis [^{14}C]urea permeability was determined.

was dependent on the time of photolysis. The response in tissue photolyzed 3 × 1 min was inhibited approximately 30% compared with control, while tissue photolyzed 5 × 1 min in the presence of NAB-Gly-oxytocin was inhibited 50%. When, however, tissue was incubated in the presence of saturating concentrations of native oxytocin (10^{-6} M) prior to repetitive photolysis, the irreversible inhibition of oxytocin enhanced [^{14}C]urea permeability was prevented (Figure 5C and Table II). Under these conditions only 14% inhibition was observed as compared with 50% inhibition under incubation conditions where the receptor was not protected by native oxytocin during the photolysis.

To investigate the possibility that the photolysis dependent inhibition was due to chromophore catalyzed photooxidation at the receptor site we examined the properties of prephotolyzed NAB-Gly-oxytocin. The azide of NAB-Gly-oxytocin was prephotolyzed (2 min) in dilute solution and the resulting compound examined for biological activity and ability to irreversibly inhibit hormone stimulation of [^{14}C]urea permeability following photolysis. Prephotolyzed NAB-Gly-oxytocin retained its ability to stimulate osmotic water flow (Table I), but had no effect on oxytocin stimulated [^{14}C]urea transport after 5 × 1 min photolysis (Figure 5D).

Discussion

To be useful a photoaffinity label must embody certain basic properties (Knowles, 1972; Cooperman, 1976). (1) The derivatization of the ligand must not distort the structural features necessary for binding to the receptor of interest. (2) The specificity of the ligand–receptor interaction must be maintained and it should be possible to demonstrate that interaction before photoactivation. (3) The ligand analogue should be stable in aqueous solution prior to photolysis, but the photogenerated intermediate should react rapidly and nonselectively to ensure specific covalent incorporation. (4) It must be demonstrated that covalent incorporation is into the receptor. By saturating the binding site of the receptors with the native ligand it should be possible to protect against light-dependent incorporation of the photolabel. Finally, photolysis conditions should not damage or alter the receptor.

NAB-Gly-oxytocin possesses the requisite characteristics

of a photoactivated affinity label. NAB-Gly-oxytocin retains high affinity for the antidiuretic hormone receptor in the toad urinary bladder, and is a full agonist for stimulated osmotic water flow (one-half maximal activity at 3.2×10^{-6} M), and [^{14}C]urea diffusion.

The 300-fold shift in affinity of the hormone analogue as compared with native oxytocin (one-half maximal activity at 10^{-8} M) in the hydroosmotic assay was anticipated from previous structure–activity studies which utilized other derivatives of the N-terminal amine of oxytocin (Rasmussen et al., 1953; Walter et al., 1967; Walter, 1971). These same structure–activity studies indicated that derivatization at the N terminus of oxytocin would orient the photolabile group of the hormone analogue into the receptor and increase the potential for specific covalent incorporation. The shift in biological activity of the hormone analogue made it necessary to purify the derivative completely of all contaminating native oxytocin. The purification was achieved in a single step by the method of partition column chromatography (Yamashiro, 1964). Repeated chromatography did not alter the specific activity of NAB-Gly-oxytocin in the hydroosmotic assay (Table I).

The steepness of the log dose–response curve for NAB-Gly-oxytocin, as compared with native oxytocin, cannot be fully explained (Figure 3). A similar result, however, has been obtained with another N-terminal oxytocin derivative in a simpler biological system, closely related to the toad bladder (Roy et al., 1975). In the activation of a kidney plasma membrane adenylate cyclase preparation *N*-methanesulfonyl-(2-*O*-methyltyrosine)oxytocin displayed both weaker agonist activity and a steeper dose–response curve than either native oxytocin or 2-*O*-methyloxytocin. While the precise mechanism for these alterations in the properties of these two N-terminal oxytocin derivatives is not known, derivatization of this site in either oxytocin or 2-*O*-methyloxytocin results in a similar alteration in biological activity in both the toad bladder and the kidney plasma membrane.

The photochemistry of aryl azides is not well understood, but activation by light leads to the production of a nitrene intermediate (Knowles, 1972). Nitrenes are highly reactive toward nucleophiles and can even insert into carbon–hydrogen bonds (Rees & Gilchrist, 1969). The ability of nitrenes to react nonselectively is an important advantage in labeling hormone receptors since reactive nucleophiles have not been identified at the binding sites. A nitro group is often introduced into the ring structure of aryl azides due to the electron-withdrawing nature of this group which increase the reactivity of the photogenerated nitrene, $t_{1/2} \sim 10^{-3}$ s (Reiser & Leyshon, 1970) and it also shifts the peak absorbances of the aryl azide toward the visible spectrum. It is then possible to photoactivate aryl azides efficiently at wavelengths ≥ 320 nm, thus minimizing the possibility of destructive effects of photolysis on the receptor (Knowles, 1972). The photolability of NAB-Gly-oxytocin is demonstrated by the changes in the absorbance spectrum after brief exposure to light ($\geq$320 nm) (Figure 2). The photolysis conditions employed in these studies had no effect on the ability of the toad bladder to respond to oxytocin.

The photolysis of the toad bladder in the presence of 8×10^{-6} M NAB-Gly-oxytocin leads to a permanent inhibition of the bladder's ability to respond to oxytocin as measured in the [^{14}C]urea diffusion assay. There was, however, no inhibition of the response of tissue to native oxytocin when ^3H_2O diffusion was measured (Figures 5A and 5B). Repetitive photolysis was necessary to demonstrate the observed inhibition, and the degree of inhibition increased with the number of photolytic exposures (Table II). Although the amount of inhibition was subject to biological variability, a consistent inhibitory effect was observed. It appears that approximately 10% of the receptor sites are blocked with each photolysis. This is comparable to the amount of incorporation that has been observed in previous photoaffinity labeling studies (Cooperman & Brunswick, 1973; Haley, 1975; Hixon & Hixon, 1973). The low yield of light-dependent incorporation is attributed to the fast reaction of photogenerated intermediates with solvent during the time course of a photolysis experiment (Cooperman & Brunswick, 1973). It is also possible that the photoactivated ligand analogue dissociates from the binding site faster than it reacts. This is probably not the case in the present study since the dissociation rate constant of the hormone analogue can be estimated from previous kinetic studies of neurohypophyseal hormones (Nakahara & Birnbaumer, 1974) and is slower than the reactivity of the nitrene.

To investigate the possibility that the permanent inhibition of oxytocin stimulated urea transport in the toad bladder is due to receptor inactivation by photooxidation of critical amino acid side chain (Means & Feeney, 1971; Scoffone et al., 1970), the photolysis experiments were repeated using prephotolyzed NAB-Gly-oxytocin. The prephotolyzed hormone analogue retained its affinity for the antidiuretic hormone receptor as measured in the hydroosmotic assay (Table I), but repetitive photolysis of the toad bladder equilibrated with prephotolyzed NAB-Gly-oxytocin (8×10^{-6} M) had no effect on oxytocin stimulated urea transport in this tissue (Figure 5D). These experiments demonstrate that the inhibition of oxytocin enhanced [^{14}C]urea diffusion observed after photolysis of the toad bladder in the presence of NAB-Gly-oxytocin is dependent on photogeneration of the aryl nitrene intermediate which is necessary for covalent bond formation.

Incubation of the toad bladder with 2-nitro-5-azidobenzoylglycine prior to repetitive photolysis (5×) had no effect on the ability of the tissue to respond to native oxytocin in the [^{14}C]urea diffusion assay. This demonstrates that photoactivation of the aryl azide does not lead to permanent inhibition of the tissue response to native hormone unless the reactive nitrene intermediate is in close contact with the antidiuretic hormone receptor. The positioning of the photolabile reagent within the receptor is achieved by means of the affinity binding properties of the hormone analogue NAB-Gly-oxytocin.

That the irreversible inhibition of the tissue response to native hormone is due to covalent receptor labeling is suggested by the protection experiment (Figure 5C). Preincubation of the serosal surface of the toad bladder with 8×10^{-6} M NAB-Gly-oxytocin plus 10^{-6} M native oxytocin prior to photolysis of the toad bladder relieved the permanent inhibition. Thus, by incubating the bladder with a concentration of oxytocin that saturates the antidiuretic hormone receptors the NAB-Gly-oxytocin is prevented from interacting with the receptor and undergoes a reaction with solvent upon photolysis.

The observation that photolysis in the presence of NAB-Gly-oxytocin inhibits the response to native hormone when urea permeability is measured but has no effect upon the oxytocin induced increase in ^3H_2O diffusion was unexpected (Figures 5A and 5B). At present this cannot be explained. It is possible that photolysis in the presence of NAB-Gly-oxytocin alters some effector system, unrelated to the membrane receptor, unique to the control of tissue urea permeability. However, the experiments discussed above, particularly the "protection experiment", indicate that this is not likely. The apparent dissociation of the urea and H_2O response under our photolysis conditions suggests that there may be more than one type or class of antidiuretic hormone receptor in the toad uri-

nary bladder: one involved in the control of urea permeability; another in the control of H_2O permeability. Previously evidence for multiple antidiuretic hormone receptors has been reported. Incubation in 10 mM calcium did not alter antidiuretic hormone dependent urea and sodium transport, but inhibited hormone stimulated water movement (Petersen & Edelman, 1964). Also, when oxytocin and arginine vasotocin were assayed successively in the same tissue at a concentration that produced equal stimulation of water permeability, the stimulation of transcellular sodium transport by oxytocin was 2.5-fold greater than the stimulation by arginine vasotocin (Bourguet & Morel, 1967). Thus, these studies as well as our present results support the notion that there is more than one class of antidiuretic hormone receptor in the toad urinary bladder.

To fully characterize the covalent incorporation into the receptor and to utilize the photoaffinity label to isolate the hormone receptor, it will be necessary to introduce a radioactive marker into the hormone analogue. It is possible to incorporate tritium into either the photoreagent or into the tyrosine of oxytocin but very high radiospecific activity would be necessary ($\geq$30 Ci/mmol) since receptor sites compose a very small percentage of the plasma membrane protein (Kahn, 1975; Bockaert et al., 1970).

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