INTERACTIONS OF NEUROHYPOPHYSEAL HORMONES WITH PROTEINS: BINDING PROTEIN (NEUROPHYSIN), RECEPTOR, INACTIVATING ENZYME

by

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Abstract

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Paula L. Hoffman

Advisor: Professor Roderich Walter

The mammalian neurohypophyseal hormones, oxytocin and vasopressin, are peptides which in the course of their "lives" in each individual animal are involved in several peptide-protein interactions. The purpose of this study is to investigate three of these interactions: hormone interaction with receptors in toad urinary bladder and mammalian kidney, with neurophysin (the "carrier protein"), and with chymotrypsin.

Bromoacetyl oxytocin was synthesized as a possible receptor affinity label. Synthetic oxytocin was acylated with bromoacetyl bromide and the product purified by gel filtration and partition chromatography. The analog fails

to enhance adenylate cyclase activity in particulate membrane preparations of rabbit kidney and toad bladder, and preincubation with bromoacetyl oxytocin nonsurmountably inhibits the stimulatory effects of neurohypophyseal hormones. This inhibition is dependent on duration and temperature of the preincubation, as well as the concentration of bromoactyl oxytocin. Fluoride-stimulated adenylate cyclase activity is not inhibited by bromoacetyl oxytocin, nor is parathyroid hormone-sensitive adenylate cyclase in rabbit renal cortex. Preincubation of the toad bladder preparation with "non-specific" peptides has no inhibitory effect. These and other results indicate that bromoacetyl oxytocin acts as a specific irreversible inhibitor of neurohypophyseal hormone action, and suggest that this analog, once radioactively labeled, may be useful as an affinity label for the isolation of neurohypophyseal hormone receptor molecules.

Localization of the binding site of arginine vasopressin and lysine vasopressin to bovine neurophysin II (NPII) by affinity labeling became feasible with the elucidation of the primary structure of the protein in this laboratory. The ionic bond between the α-amino group of arginine vasopressin, ^{14}C-labeled in the glycine residue, and the side-chain carboxyl of a residue in NPII in the specific reversible complex, was converted to a covalent bond by treatment with water-soluble carbodiimide in 4M urea. Following disulfide reduction with

dithiothreitol and alkylation with iodoacetamide, the covalent complex was partially digested with chymotrypsin (enzyme-to-substrate ratio 1:300). The resulting ^{14}C-labeled NPII fragment has a composition which allows it to be uniquely placed in positions 23 through 35 in native NPII. This identifies either the Glu in position 30 or the Asp in position 31 or both as the critical residues for binding of arginine vasopressin. Affinity labeling was repeated with lysine vasopressin labeled with ^{3}H in the tyrosine residue (to avoid loss of label due to chymotryptic hydrolysis of the alkylated hormone derivative). Chymotryptic digestion of the alkylated [^{3}H]lysine vasopressin covalent complex (enzyme-to-substrate ratio 1:3) released several labeled peptides, none of which was sufficiently purified by the techniques used to be accurately placed in the native protein. The tentative binding site identified for arginine vasopressin is discussed in terms of models proposed on the basis of spectroscopic investigations. The observed interaction between the hormone and protein tyrosines is best explained by postulating a conformation of NPII which places the protein sequence containing Tyr-49 in proximity to the proposed binding site.

Intact oxytocin, lysine vasopressin, and arginine vasopressin are resistant to the action of α-chymotrypsin when incubated at an enzyme-to-substrate ratio of 1:300.

Conversely, the bonds at the carboxyl side of aromatic residues in the acyclic, S-alkylated nonapeptides are efficiently hydrolyzed by α-chymotrypsin. These results show that the 20-membered ring of the neurohypophyseal hormones, with its compact anti-parallel β-pleated sheet structure, presents an image to the enzyme which differs significantly from that of the constituent amino acids in a linear peptide. This prevents the enzyme from recognizing a potential substrate. Neurohypophyseal hormone analogs thought to possess a less constrained 20-membered ring are susceptible to cleavage of peptide bonds in the cyclic moiety.

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Publications resulting from this experimental work:

1. Initial Studies on an Active-Site-Directed, Nonsurmountable Inhibitor of Toad Bladder Adenyl Cyclase. P. L. Hoffman, I. L. Schwartz, T. P. Dousa, O. Hechter, and R. Walter, Endocrinology 88, Suppl. A-139, 1971.

2. Bromoacetyl Oxytocin, an Irreversible Inhibitor of Adenylate Cyclase and a Possible Affinity Label for Hormone Receptors. R. Walter, I. L. Schwartz, O. Hechter, T. P. Dousa, and P. L. Hoffman, Endocrinology 91, 39-48, 1972.

3. Modulation of Enzymatic Activity by Conformational Constraints. P. L. Hoffman and R. Walter, Biophysical J. 13, 202a, 1973.

4. Tentative Identification of a Binding Site of Arginine Vasopressin to Neurophysin. R. Walter and P. L. Hoffman, Fed. Proc. 32, 567A, 1973.

5. Action of Chymotrypsin on Intact Oxytocin and a Linear Derivative. P. L. Hoffman and R. Walter, Symposium: Intracellular Protein Catabolism at Schloss Reinhardsbrunn, Friedrichroda, Germany, May, 1973.

6. Conformational Constraints within Neurohypophyseal Hormones Influencing the Action of Chymotrypsin. R. Walter and P. L. Hoffman, Biochim. Biophys. Acta, in press.

Abbreviations follow the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature, J. Biol. Chem. 242, 555 (1967) and Biochem. J. 126, 773 (1972). All optically active amino acids are of the L-configuration unless otherwise noted. Additional abbreviations used: Asu, aminosuberic acid; AcOH, acetic acid; BrOXY, bromoacetyl oxytocin; Cam, carbamoylmethyl; CM, carboxymethyl; CD, circular dichroism; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; HCOOH, formic acid; NMR, nuclear magnetic resonance; WSC, water-soluble carbodiimide (1-ethyl-3(3-dimethylamino-propyl)carbodiimide HCl); NP (I) II, bovine neurophysin (I) II; AVP, [8-arginine] vasopressin; LVP, [8-lysine] vasopressin; AVT, [8-arginine] oxytocin (arginine vasotocin).

Table of Contents

List of Tables

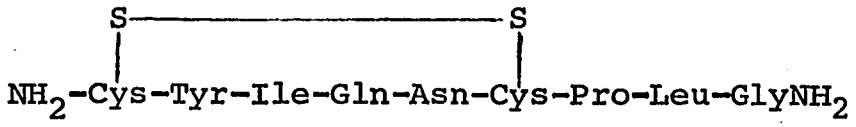
List of Figures

General Introduction

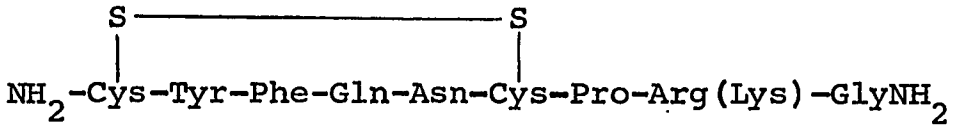
The mammalian neurohypophyseal hormones, oxytocin and vasopressin, are small peptides which in the course of their "lives" in each individual animal are involved in several physiologically important peptide-protein interactions, such as those with the carrier protein, the receptor, and inactivating enzymes. The present study is an attempt to advance our insight into some aspects of these interactions at the molecular level.

Neurohypophyseal extracts were demonstrated in the early part of this century to have five distinct biological activities: mammalian pressor (1), oxytocic (uterotonic) (2), milk-ejecting (3), avian vasodepressor (4), and mammalian antidiuretic (5). By 1949, the oxytocic principle, oxytocin, was isolated in purified form from beef posterior pituitary and was shown to consist of eight amino acids (6). DuVigneaud and co-workers determined the amino acid sequence of oxytocin (7), and later of both arginine vasopressin (8) and lysine vasopressin (9), which occurs in certain species of the Suinal group. In independent studies, Tuppy (10) proposed an identical structure for oxytocin, and Acher and Chauvet (11) for arginine vasopressin. All structures were confirmed by synthesis of the hormones by duVigneaud and associates (12-14).

The primary structures of the oxytocic and antidiuretic principles (i.e., oxytocin and vasopressin) are similar, differing only in positions 3 and 8, and are shown below:



OXYTOCIN



VASOPRESSIN

Recently, the three-dimensional conformation of oxytocin in solution has been proposed (15), primarily on the basis of circular dichroism (CD) and proton nuclear magnetic resonance (NMR) studies. The peptide is suggested to contain two β-turns, one involving the ring sequence -Tyr-Ile-Gln-Asn- and the other the acyclic portion (Fig 1). A similar configuration was proposed for lysine vasopressin (16-18). The original studies were carried out in the solvent dimethylsulfoxide, but the gross structure is presumed to be conserved in aqueous medium (19,20). The proposed conformations are supported by ^{13}C NMR and energy calculation studies (20,21), and have served, in combination with previous structure-activity studies, as a basis for considerations of hormonal activity, evolution, immunogenicity, and pathways of enzymatic degradation (16).

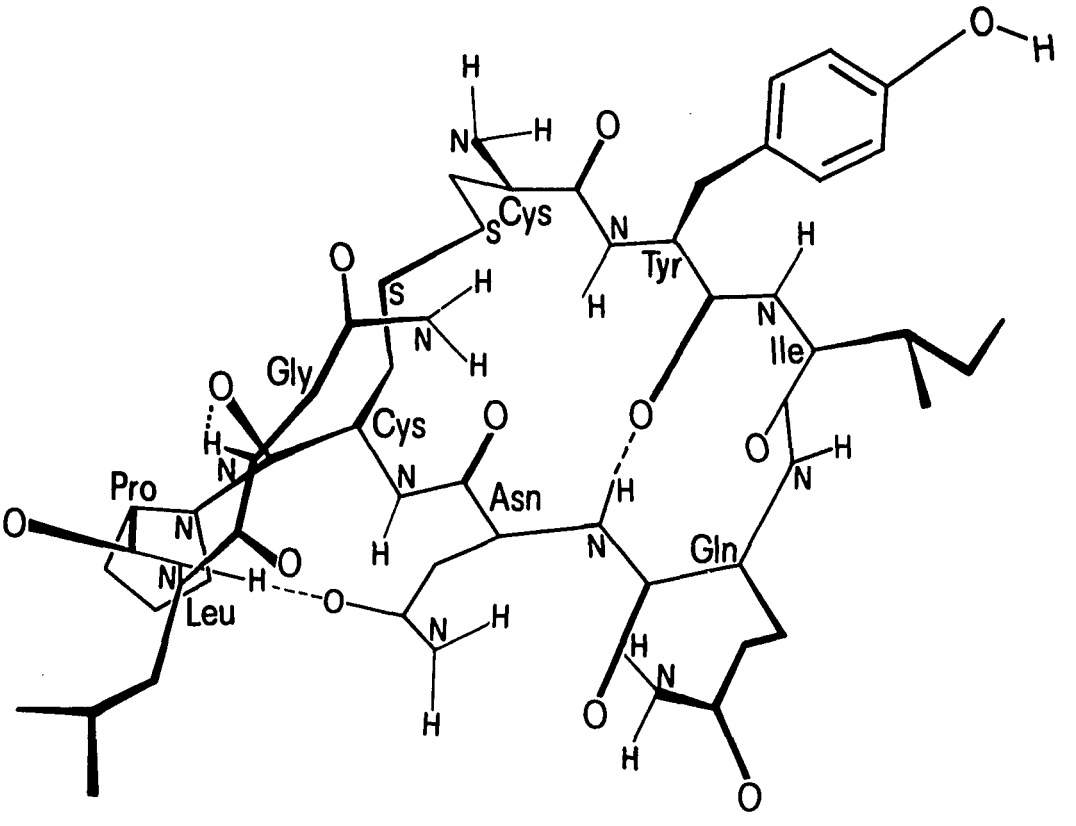


Fig. 1. Proposed three-dimensional structure of oxytocin in solution (15).

The hormones *in vivo* are synthesized in the supraoptic and paraventricular nuclei of the hypothalamus and move down hypothalamic neurons to the posterior pituitary where they are stored in neurosecretory granules and finally released (22). In the pituitary the hormones are found in association with higher molecular weight proteins, the neurophysins, which are considered to be carrier proteins for the peptides. The neurophysins have also been located immunologically in the hypothalamus and pituitary stalk (see below). There is some evidence that neurophysin and vasopressin (and presumably oxytocin with its neurophysin) share a common precursor during biosynthesis or that a common genetic unit controls their mutual synthesis (23). Release of hormones and neurophysin occurs simultaneously, apparently by exocytosis (24). The neurohypophyseal hormone-neurophysin interaction is of interest both physiologically and as an example of the more general phenomenon of interaction of carrier proteins with their ligands. The second section of the present work describes experiments aimed at location of the binding site on bovine neurophysin II for neurohypophyseal hormones.

Once the peptide hormones are released into the circulation, they must be recognized by a particular target organ in order to induce their physiological response. Recognition has for years been attributed to the presence in particular organs

of specific receptors, first defined to account for drug action (25). Receptors for peptide hormones are generally believed to be protein in nature, to account for specificity, and to be located in the plasma membrane of the cell. The interaction of oxytocin and vasopressin with their receptors in smooth muscle or kidney initiates a series of intermediate reactions leading to a final response (see below). A primary goal of hormone research has been -- and continues to be -- to elucidate hormone interaction with receptors at the molecular level, keeping in mind that a hormone receptor not only recognizes the hormonal principle, but also couples the interaction to a final effect. Isolation and characterization of such a molecule has been an elusive goal in the case of peptide hormones. The first section of this study describes synthesis and characterization of a neurohypophyseal hormone affinity label, which could covalently attach to a moiety within the receptor in order to label it during purification procedures.

The peptide hormones are finally degraded <u>in vivo</u> by a variety of specific and non-specific enzymes. A "cystine aminopeptidase" (oxytocinase) in plasma of pregnant primates is reported to degrade oxytocin (26) although its physiological importance is questionable. The most important mode of inactivation seems to occur by cleavage in the acyclic portion of the molecules to release C-terminal dipeptide or

glycinamide (27). Enzymes with this specificity have been studied in target organs such as kidney (28-30) and uterus (31) as well as in brain tissue (32) from various species. As mentioned above, the three-dimensional conformation of the hormones is valuable in predicting pathways of inactivation; the structure of the substrate can affect enzymic activity. The third section in this work discusses the interaction of neurohypophyseal peptides with an inactivating enzyme in an effort to understand how substrate conformational constraints can influence enzyme action and perhaps protect the hormone from inappropriate inactivation.

In summary, the purpose of the thesis is to investigate certain aspects of some of the in vivo peptide-protein interactions of the neurohyophyseal hormones by in vitro experiments. The three sections correspond to the three types of interaction discussed, i.e., with carrier protein, receptor, and inactivating enzyme, and contain brief reviews of the individual subject areas as well as experimental results.

SECTION 1: Affinity Label for Neurohypophyseal Hormone Receptors

Introduction

A premise of molecular endocrinology is that the selective binding of a specific hormone to a specific "receptor" is the initial event in the chain of reactions leading to the response of the target tissue. The concept of hormone receptors, although now considerably modified (as will be discussed below), originates from early ideas of drug action. In 1905 Langley introduced the idea of a specific "receptive substance" for certain drugs active at the neuromuscular junction (33). Ehrlich (34) discussed the concept of specific receptors in the action of toxins, and later mentioned specific active sites on the surfaces of antigens and antibodies as essential to interaction between them (35). The original lock-and-key concept has given way to more dynamic interpretations of drug-receptor interaction. As one example, the sigmoidal dose-response curves for the natriferic action of oxytocin on frog skin suggested to Jard et al. that the hormone receptor interaction might involve molecular mechanisms similar to those proposed for allosteric enzymes (36). In theoretical terms, a receptor may be defined as a pattern of forces which forms part of some biological system and which is complementary to a pattern of forces presented by a drug or hormone molecule, such that interaction may occur between the patterns (37). A more practical definition

of a hormone receptor combines two equally important properties: the receptor must recognize the hormone in a selective binding reaction, and the hormone-receptor complex must initiate the specific hormonal response (38). In informational terms (39) the receptor system can be regarded as a discriminator which selects a specific hormone (input signal) and an amplifier to generate an output signal: these are coupled by a transducer -- perhaps a chemical signal or a conformational change. In isolation attempts, the molecule most likely to be identified is, then, the "discriminator," but in order to qualify as a hormone receptor, it must be coupled to the rest of the system in its native form.

While the exact physical and even theoretical definition of a receptor is open to question, the theory of drug-receptor interaction has been extensively studied. The consequence of this interaction is a stimulus which leads to a measurable response of the target tissue (40). Clark (41) attempted to quantify the mechanism of drug action by applying to drug-receptor interaction the Langmuir isotherm, which deals with adsorption of gases to surfaces. He assumed that drug molecules combine with receptors at a rate proportional to the concentration of free drug and free receptor; and that the response is directly proportional to the degree

of saturation of the receptors. Implicit in the theory is the assumption of an all-or-none response at each receptor. These relationships were quite generally accepted for many years (25).

Experimental results gradually caused modification of the theory. Furchgott (42,43) and Nickerson (44) used pre-treatment with irreversible blocking agents (alkylating agents) to show that even if 99% of the receptors were blocked, evidenced for example by a shift of a histamine dose-response curve, a maximal drug effect could still be achieved. The experimental fact that response is not always linearly proportional to the number of receptors occupied leads to the hypothesis that different drugs may have varying capacities to initiate a response; i.e., occupation of different proportions of the receptors by different drugs can produce equal responses. The capacity of a drug to produce a response is called efficacy (45) or intrinsic activity (46). In the simplest case, intrinsic activity is the proportionality constant relating the number of receptors occupied to the response. It may vary between 0 and 1, giving rise to compounds called "partial agonists," which, for example, can occupy all receptors but not produce the maximal response obtained with a standard. In discussing drug action then, both affinity (inversely related to the concentration of a drug needed for a given response) and intrinsic activity

must be considered. This theory of drug action, as reviewed by Ariens (40) is the Occupation theory, stating that drug (or hormonal) response is proportional to the fraction of receptors occupied. Another theory is the Rate theory, which says the response is proportional to the number of drug-(hormone-) receptor associations per unit time (47). Both these theories predict the same dose-response relation at equilibrium. Two phenomena seen in dose-response curves, and pertinent to attempts at receptor characterization, are threshold and receptor reserve. In the first, a response is not seen until the stimulus reaches a certain value; in the second, the maximal response occurs before the receptors are saturated, i.e., addition of an inhibitor does not immediately reduce the maximal response (40). These phenomena have been demonstrated with the hydroosmotic effect of neurohypophyseal hormones in intact toad bladder (48). In these studies, saturating concentrations of an analog of oxytocin, which is itself inactive, potentiated theophylline, presumably by raising the level of cAMP (see below) above threshold. Prostaglandin E_1, a non-competitive inhibitor of the hormones, depressed the response to partial agonists, but only slightly reduced that of oxytocin, indicating receptor reserve for oxytocin. The phenomenon of receptor reserve has been confirmed in studies of adenylate cyclase activity (see below) in broken-cell preparations of toad bladder.

Thus, the maximal hormonal response in the intact tissue may be achieved with utilization of only a fraction of the cAMP generated by the hormone-receptor interaction (49; Kirchberger, M. A., Schwartz, I. L., Walter, R., Bär, H. P., and Hechter, O., unpublished). In other words, while a series of neurohypophyseal hormones and analogs all give a maximal hydroosmotic response, their ability to generate cAMP in adenylate cyclase preparations of toad bladder varies widely. Bockaert et al. (50) found that the function relating LVP-stimulated adenylate cyclase activity in pig kidney plasma membrane preparations to binding (receptor occupancy) was not linear; this may indicate the presence of binding sites not associated with adenylate cyclase activity, as was also found (51) for neurohypophyseal hormone binding to frog bladder epithelial cells. This phenomenon is somewhat different from that described above; however, the difference in hormone concentration needed for half-maximal response in intact frog bladder as compared to the broken cell system (51) suggests that receptor reserve also exists in this system. The threshold phenomenon has also been demonstrated in the toad bladder by correlation of intracellular cAMP levels with hormone-induced sodium transport measured by short-circuit current. A measurable increase in net sodium flux was observed only when tissue cAMP had increased to double the basal level (52).

Characterization of receptors, drug or hormone, has an inherent difficulty in that isolation necessarily means

removal of the receptor from its biological system, and therefore loss of response. Isolation and identification of a peptide hormone receptor in the free and bound states would represent a major advance in elucidation of the fundamental mechanism of hormone action. Structure-activity studies (53-55) and, more recently, conformation-activity studies (16,56) have attempted to map a negative outline of the receptor; often this attempt is obstructed by the many steps between hormone-receptor interaction and final effect. A large number of hormones, including many of the peptide hormones, produce their effects through stimulation of adenylate cyclase (57-59). This enzyme converts ATP to cyclic 3'5'-adenosine monophosphate (cAMP) which then acts intracellularly to affect various systems leading to the hormonal response. In many cases it appears that the cAMP generated activates a cAMP-dependent protein kinase (60). In toad bladder epithelial cells, for example, such an enzyme was demonstrated, implicating phosphorylation of an enzyme or a membrane protein in neurohypophyseal hormone-induced permeability changes (61). However, exposure of intact toad bladder to AVP or cAMP, or of toad bladder homogenate to cAMP, was reported to cause a <u>decrease</u> in phosphorylation of a membrane protein (62). It is clear that the intermediate steps between adenylate cyclase stimulation

and final effect deserve further study. Most evidence indicates that mammalian adenylate cyclase is located in the cell plasma membrane (63).

There are several criteria required to demonstrate that a peptide hormone acts via adenylate cyclase. In the case of vasopressin, for example, it was shown that: 1) vasopressin increases cAMP levels in toad bladder (64); 2) exogenous cAMP mimics the hydroosmotic and natriferic actions of vasopressin in toad bladder (65); 3) vasopressin stimulates adenylate cyclase activity in kidney and toad bladder membrane fractions, while hormones active in other systems have no effect (66-68,49). These same experiments, demonstrating a correlation between stimulation of membrane-bound adenylate cyclase activity and final hormonal effect have been performed for other peptide hormones, e.g., glucagon in liver (69,70); ACTH in the adrenals (71,72). In addition, the structural features of the molecule required for alteration of adenylate cyclase activity have been studied in a variety of systems, e.g., neurohypophyseal hormone effects on toad bladder and kidney (49,68,73,74); glucagon in liver (75) and ACTH in adrenal tumor tissue (76,77), and in rat and rabbit fat cell "ghosts" (78;79).

It was originally postulated that adenylate cyclase <u>was</u> the hormone receptor. Later studies have indicated that the

receptor (discriminator) is located in close association to the enzyme, but is a distinct entity. Several lines of evidence support this view. In rat adipose tissue and in "ghosts" obtained from the fat cells, there is an adenylate cyclase system which is activated by at least 6 hormones (ACTH, GH, catecholamines, glucagon, secretin, and LH). Combination of two or more of these hormones does not result in cAMP levels higher than those produced by the most effective hormone alone (80,81). Further, since fluoride ion stimulates adenylate cyclase in all cells tested (82) with no selectivity, it appears that fluoride acts at a point beyond the "discriminator" stage. In many studies it has been found that hormonal and fluoride stimulation can be separated (e.g.,76,83,84). Moreover, Tomasi et al. (85) could distinguish glucagon-binding activity from adenylate cyclase activity in sonicated rat liver plasma membranes.

Regardless of the relationship between the "receptor" and the adenylate cyclase system, the prevailing view is that peptide hormones in general act at the external cell surface (86). Schimmer et al. (87) showed that ACTH bound covalently to cellulose retained biological activity in cultures of adrenal cells. Similarly, Cuatrecases found covalent insulin-Sepharose to be active in fat cells (88). Agarose-ACTH was found to stimulate steroidogenesis in rat adrenal cells (89) and agarose-glucagon, lipolysis in rat fat cells (90).

However, questions have been raised regarding the validity of these results, i.e., some hormone may be free, and the response may not be physiological (91). More indirect studies involve treatment of fat cells with various proteolytic enzymes, which reduces the effects of insulin, for example, but does not affect intermediate steps leading to those effects (92,93). These cells will no longer bind insulin (94). Cuatrecasas showed that mild digestion of fat cells by trypsin causes a selective fall in the affinity for insulin and a corresponding fall in insulin-stimulated glucose transport (95). There is almost total loss of insulin-binding activity after treatment of fat cells with trypsin-agarose (96). Further, digestion of adipose cells with a combination of neuraminidase and β-galactosidase abolishes insulin effects and insulin binding (97). The action of glucagon on fat cells is abolished by trypsin, while basal and fluoride-stimulated adenylate cyclase are unaffected (98). In addition, specific antibodies to a particular hormone can rapidly reverse its effects (99). All of these experiments support the idea that the peptide hormone discriminator is located on the plasma membrane, readily accessible to the environment.

The discovery that binding of polypeptide hormones to membrane fractions of cell-free preparations can selectively affect adenylate cyclase activity has altered experimental

approaches to hormone-receptor interaction. A direct method can now be used: study of the specific binding of highly radioactive peptide hormones to cell-free preparations of their target tissue. Requirements are that the labeled hormone be biologically active, uncontaminated by unlabeled material, and of high specific activity. Rodbell et al. have examined the binding of glucagon to purified liver cell membranes which retain adenylate cyclase activity (70,100). Glucagon specifically stimulates liver adenylate cyclase at concentrations of 10^{-10} to 10^{-7} M, and radioactive glucagon binds to liver plasma membranes over the same concentration range (98). This binding is not inhibited by ACTH, secretin, or insulin, hormones which do not stimulate liver adenylate cyclase. Treatment of the membrane fractions with detergent decreases binding, and addition of dispersed membrane lipids to the system restores binding (79). In the presence of the nucleotides GTP or ATP (at high concentrations) glucagon binding was shown to be rapidly reversible, as is the activation of the adenylate cyclase system (101). By using the glucagon analog, des-histidine glucagon (DH-glucagon), as well as N- and C-terminal fragments of glucagon, these workers gained some insight into the forces responsible for the binding and action of glucagon (102). Des-histidine-glucagon inhibited glucagon binding and stimulation of

adenylate cyclase activity, while the fragments did not. It was concluded that the N-terminal histidine is essential for biological activity and contributed to binding, and that a hydrophobic near-carboxyl-terminal region was necessary for binding. On the whole, these studies indicate that glucagon-sensitive adenylate cyclase activity is identical in the whole cell and in cell-free preparations, and that binding properties of [I^{125}] glucagon to liver cell membranes closely parallel its biological properties.

The binding of ACTH to its specific "receptors" has also been studied in some detail. Roth and co-workers (72,76), using mono-iodo [^{125}I] ACTH, showed that extracts of a tumor of adrenal cortex which contained ACTH-sensitive adenylate cyclase bound the labeled hormone, while those without activity did not. Extracts of kidney, liver, skeletal, or cardiac muscle did not bind hormone and tumor extracts did not bind [^{125}I] AVP, insulin or FSH. Derivatives of ACTH inhibited the binding of [^{125}I] ACTH in proportion to their biological activity, while inactive peptides -- albumin and insulin -- did not. Unlabeled ACTH could completely abolish the binding of labeled hormone. Quantitative studies of the [^{125}I] ACTH-receptor interaction revealed the presence of two hormone-binding sites, one with high and the other with low affinity (77,103). It

was suggested that both sites are physiologically operative, giving a fine control to the hormone response. Hofmann et al. (104) used a particulate beef adrenal cortical extract and measured binding of [^{14}C] ACTH fragments. There was a significant correlation between affinity and steroidogenic potency. Study of the ability of various unlabeled fragments to displace [^{14}C] ACTH allowed the determination of the "active" and binding sites within the ACTH molecule; the specificity of the extract for binding ACTH suggested that it contained "receptors" for that hormone.

The binding of oxytocin to tissues in which this hormone either stimulates contractility or enhances membrane permeability has also been studied. Soloff et al. (105) showed that rat mammary gland (a target tissue) in vitro took up a larger amount of radioactivity from [^{3}H] oxytocin than rat abdominal muscle. This uptake was decreased by oxytocin, [4-threonine] oxytocin, LVP and [4-proline] oxytocin in order of their milk-ejecting activities. These displacement studies suggest that [^{3}H] oxytocin was bound to receptor sites in the mammary gland. More recently, oxytocin was shown to bind specifically to a particulate preparation of rat mammary tissue (binding activity paralleled biological activity as discussed) and with high affinity (physiological concentrations of hormone were used). Trypsin treatment,

p-hydroxymercuribenzoate, and 1mM ATP decreased binding, and it was suggested that the particulate preparation contains oxytocin receptors which are in part protein in nature (106). In another study (107), [^{3}H]oxytocin was shown to be taken up by rat uterine tissue *in vitro*; the uptake was again decreased by oxytocin analogs and LVP in proportion to their known oxytocic activities. A relatively inactive analog, [4-proline]oxytocin, was not competitive in the dose range tested. These results suggested that [^{3}H]oxytocin could also bind to receptor moieties in rat uterus.

Bockaert *et al.* (108) demonstrated that the time course of uptake of [^{3}H]oxytocin by frog skin epithelial cells was similar to that of the biological response. Saturation of the binding process took place over the physiological concentration range, and radioactivity was displaced by unlabeled hormones in proportion to their biological activity. Using the frog skin as the target organ these workers found it difficult to relate binding to biological response, since it had been shown that oxytocin induces two responses, the hydroosmotic and the natriferic, and that these responses are spatially or qualitatively different (109).

Further studies demonstrated thepresence in frog skin epithelium of two categories of [^{3}H]oxytocin binding sites with different affinities and maximal capacities (110). On a broken-cell preparation, both high and low-affinity

sites were again found but only the high-affinity sites were involved in adenylate cyclase activation, which is necessary for both hormonal responses (53). This does not imply that the concentration of hormone required to elicit a half-maximal response in the intact system ($3x10^{-9}$M) is identical to that needed for a half-maximal response in the broken-cell preparation ($1.5x10^{-7}$M); as indicated, there is a difference of two orders of magnitude between the high-affinity sites in the two systems. The binding of [^{3}H] oxytocin in the frog skin epithelium system was linear with protein concentration, was saturable and partially reversible.

[^{125}I] oxytocin has been shown to bind specifically to isolated fat cells (111). Binding was inhibited by unlabeled oxytocin, LVP, and AVP, and weakly by insulin. Binding was unaffected by glucagon and angiotensin.

All of these studies have demonstrated the parallel nature of the binding of peptide hormones to responsive cell membranes and the activation of adenylate cyclase. Thus, there are entities in the cell surface which both recognize the hormone, and initiate a response; in a sense, the receptor can be "seen" by the specific binding of the hormones. In addition, some light has been cast on the nature of the receptor. The abolishment of binding and stimulatory activity of glucagon by treatment of liver cell

membranes with detergent or phospholipase A, as well as decrease of binding by urea, indicates that the binding is hydrophobic, with lipids a necessary component. The earlier results with proteolytic enzymes indicated the presence of protein (and perhaps carbohydrate) in the receptor; it appears that the receptor may be proteolipid in nature, not surprising in view of its placement in the cell membrane. Further evidence for the structure of the receptor as discriminator-transducer-amplifier (or as regulatory component-transducer-catalytic component, e.g., 70,100) is also provided in these studies: des-histidine-glucagon inhibition is surmountable by glucagon, indicating that the amplifier or catalytic unit is susceptible to activation even if the regulatory unit or discriminator is blocked. In the studies with ACTH, it was noted that freezing and thawing destroyed both hormone binding and hormone-sensitive adenylate cyclase activity, while fluoride-stimulated activity remained intact.

These studies may be called characterization of the peptide hormone receptor; however, in one case isolation of a receptor has been attempted. The binding of [^{125}I] insulin to fat cell and liver membranes has to be correlated with the final hormonal effect since insulin does not stimulate adenylate cyclase. Freychet et al. discuss porcine

$[^{125}I]$ insulin binding to purified liver membranes (112). This binding was nearly completely inhibited by unlabeled insulin at physiological concentrations, while insulins from different species or insulin derivatives inhibited in proportion to their biological activities (glucose oxidation). Other peptide hormones had no effect. Binding was time- and temperature-dependent, and was rapidly reversible by excess unlabeled hormone. Cuatrecasas performed a series of studies on the binding of $[^{125}I]$ insulin to fat cells and their membrane fractions (113). He found time- and temperature-dependence of binding, and saturability. The binding was inhibited by native insulin, but not by chemically modified hormone or by ACTH, GH, prolactin, vasopressin, oxytocin, or glucagon. These results, found with metabolically active cells, indicated a "close parallel between binding and biological activation," and led to the isolation of a macromolecule involved in insulin binding (114). Liver and fat cell membranes were extracted with Triton X-100, which eliminates binding of insulin by the particulate fraction, and binding activity was found in the supernatant. Specific and reversible binding of $[^{125}I]$ insulin to soluble proteins was shown by gel filtration. As in the membrane preparations, insulin binding was inhibited by analogs in proportion to their biological activity, while inactive peptides had no effect. The liver cell insulin binding

activity was purified by affinity chromatography using insulin-Sepharose derivatives and elution with urea and detergent-containing buffers of low pH (115). If the insulin-binding studies in intact cells and broken cell preparations do represent insulin-receptor interaction (an assumption which would be more convincing using hormones known to stimulate adenylate cyclase), the close similarities in binding properties suggest that the extracted soluble protein is the receptor which was characterized. The problems with this technique are the necessity for the constant presence of detergent (116,117), and the fact that this protein is necessarily a binding protein, which does not have the property of initiating a response. This will of course be the case with any isolated receptor, but is a particularly compelling point in view of the phenomenon of "spare receptors" which are found even in adenylate cyclase preparations. Birnbaumer and Pohl (118) found that 80-90% of glucagon-specific sites in the liver plasma membranes do not participate in glucagon activation of adenylate cyclase. Gavin et al. (119) reported the preparation of "specific insulin receptors" from cultured human lymphocytes. These binding molecules are extracted in aqueous medium, and exhibit properties of the insulin receptor in the intact cell: they specifically bind $[^{125}I]$ insulin; the binding is inhibited by insulin analogs in proportion to their biological

activity, and binding is rapid and reversible. Binding is abolished by trypsin treatment. The entity isolated in these studies is, again, a binding molecule, not necessarily a receptor, and in addition the method depends on "the unique phenomenon of release of surface proteins from intact cultured lymphocytes."

A promising technique for receptor isolation is affinity labeling. In general, this approach involves the design of a labeling reagent which combines specifically and reversibly with the active site in question, and then by virtue of a suitably small and chemically reactive group forms a covalent bond with a moiety in the site (120). Affinity labeling has been extensively applied for identification of enzyme active sites and antibody combining sites. The theory of the method was proposed by Wofsy et al. (121,122) for labeling of antibody sites. Antibodies directed against the hapten p-azobenzenearsenate were treated with p-(arsonic acid)-benzenediazonium fluoborate in the presence and absence of a specific active site protector. Formation of covalent azo linkages by the diazonium group was followed spectrophotometrically, and it was shown that unprotected antibody reacted at a much higher rate than protected antibody or IgG. Labeling of unprotected antibody occurred primarily at tyrosine residues, while controls were labeled non-specifically.

Early studies on enzyme active sites were carried out by Schoellmann and Shaw (123,124) with chymotrypsin. The reactive group used was a chloromethylketone attached to the substrate-like N-tosylphenylalanyl residue. Specific, stoichiometric inactivation was observed, and the chloromethyl ketone was shown to alkylate a histidyl residue. In a later study, the chloromethylketone of N-tosyl-L-lysine was successfully used as an affinity label for trypsin (125).

These are examples of what Baker (126) calls an "endo" mechanism, in which alkylation occurs within the active site. Baker suggested the term "active-site-directed irreversible inhibition" as a more general description of covalent bond formation within an enzyme-inhibitor complex. The studies of Baker and co-workers on various enzymes usually involve the "exo" mechanism, in which the reactive group is placed on the inhibitor so that it can bridge to a nucleophilic group on the enzyme surface, outside the active site. Baker's interest (127) lies in inactivation of enzymes for therapeutic purposes, rather than identification of residues present in the active site, making the exo method a valuable one; for receptor isolation covalent bond formation should occur within, not close to, the receptor itself.

The design of active-site directed irreversible inhibitors for use with enzymes and antibodies is facilitated by the relatively small size of the substrate or hapten, as well

as by use of purified or partially purified proteins. The main problem is specificity, which is enhanced in affinity labeling as compared to other methods, by the original formation of the non-covalent complex. This two-step reaction cannot be overemphasized. A reversible, specific complex is first formed between enzyme and inhibitor or antibody and modified hapten; then a rapid and selective neighboring group reaction occurs between the leaving group and a nucleophilic moiety on the enzyme or antibody. The objective of such studies using, for example, radioactive inhibitors, is the isolation of a specifically labeled peptide fragment, recognized by the large quantity of label on it as compared to any other fragment (128). There have been extensive studies on enzyme active sites using this technique, and they have often been valuable in recognizing critical residues which are not uniquely reactive (129-132).

The application of affinity labeling to receptors again presents the problem of specificity, particularly since the receptors cannot be purified before covalent reaction takes place. The choice of a proper reactive group, and its location on the hormone are both important factors. The method lends itself to the isolation of peptide hormone receptors since it allows specificity and coupling to physiological processes to be demonstrated before isolation,

and eliminates the problem of receptor denaturation during purification procedures. A potential peptide hormone affinity label must be tested for covalent bond formation and for selectivity of receptor interaction.

This study describes the synthesis of bromoacetyl oxytocin (Fig. 2) and a series of experiments which indicate that this analog may serve as an active-site-directed irreversible inhibitor of neurohypophyseal hormone interactions with receptors in toad bladder and kidney tissue.

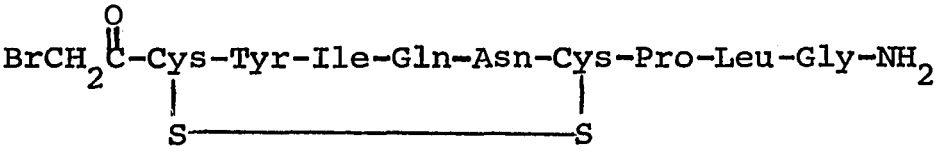


Fig. 2. Structure of bromoacetyl oxytocin.

Materials

Protected amino acids were purchased from Fluka; L-alanine was purchased from Mann Research Labs. O-Nitrophenylsulfenyl-glutamine and asparagine N-hydroxysuccinimide esters were prepared according to the method of Meyers et al. (133). Synthetic oxytocin samples were prepared by the solid phase method (134,135) and by classical methods of peptide synthesis (136). Arginine vasotocin (AVT) and arginine vasopressin (AVP) were prepared by the solid phase method (137,138) and possessed rat pressor (139) activities of 250 U./mg and 395 U./mg respectively. The [5-valine] oxytocin used was the same as that described earlier (140). Parathyroid hormone was supplied by Dr. J. Cort, Czechoslovak Academy of Science, Prague, and [4-leucine] oxytocin by Dr. V. Hruby, University of Arizona, Tucson. Crystalline glucagon was purchased from Eli Lilly and Co., and ACTH from Schwarz-Mann.

TLC plates (silica gel G) were purchased from Quantum Industries, PEI-cellulose TLC plates from Brinkmann, and Sephadex from Pharmacia. Bromoacetyl bromide was purchased from Aldrich Chemical Co. and redistilled under reduced pressure prior to use. ATP-α-^{32}P came from International Chemical and Nuclear Corp. and unlabeled nucleotides from Schwarz-Mann. Toads (Bufo marinus) came from National Reagents and male New Zealand White rabbits from Marland Breeding Farms.

Methods

Melting points were determined with a Thomas-Hoover capillary melting point apparatus, and are not corrected. Nuclear magnetic resonance (NMR) spectra were recorded with a Varian T-60 spectrometer. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn., and by Atlantic Microlabs, Atlanta, Ga. Thin-layer chromatograms were developed with the chlorine-tolidine reagent (141).

Amino Acid Analysis

Amino acid analysis was performed according to the general method of Spackman, Stein and Moore (142). Samples were hydrolyzed *in vacuo* in 6N HCl for 22 hr at 110C. The residue after drying over NaOH was redissolved in 0.2N citrate buffer, pH 2.2, and analyzed on a Beckman 121C amino acid analyzer.

Purification of Oxytocin

In the solid phase method, the protected nonapeptide of oxytocin was cleaved from the resin by suspending the peptide-resin in ethanol which was then saturated with NH_3 at 4C and stirred at room temperature under pressure for 60 hr (143). Protected nonapeptide of oxytocin was extracted by stirring in dimethylformamide (DMF) and evaporating the extract to dryness; nonapeptide resulting from this treatment or from classical methods of synthesis was partially purified

by precipitation from a DMF-ethyl ether mixture. The nonapeptide, N-Cbz-(SBz)Cys-(OBz)Tyr-Ile-Gln-Asn-(Sbz)Cys-Pro-Leu-GlyNH_2 was deprotected by treatment with sodium in liquid ammonia (144), oxidized to hormone in dilute solution with ferricyanide (145), concentrated to a small volume and subjected to partition chromatography on a Sephadex G25 column (97x2.5cm). The column was equilibrated with both phases of the solvent system n-BuOH/benzene/pyridine/acetic acid/water (600:200:100:1:900) and eluted with the upper phase (146). Fractions containing peptide were located by the Folin-Lowry method (147), pooled with the addition of water, concentrated and lyophilized. Material from the peak with the R_f of oxytocin (0.24) was assayed in conscious chickens (148) using the method of Coon (149).

Synthesis of L-Alanine Ethyl Ester HCl (L-AlaOEt)

Ten g L-alanine (0.11 mole) was suspended in 150 ml absolute ethanol. A stream of dry HCl gas was passed through the solution without cooling until the amino acid was dissolved. The reaction mixture was cooled in an ice bath and kept at 4C for 90 min, then at room temperature for 4 hr. After concentrating, the oily residue was triturated with ethyl ether and left overnight in the cold. The crystals formed were isolated by filtration: Yield: 91%, m.p. 73-76°.

Synthesis of N-Bromoacetyl-L-Alanine Ethyl Ester

L-alanine ethyl ester (0.17g, 1.0mmole) was dissolved

in 2 ml N $NaHCO_3$ at 4C. The pH was adjusted to 8.5 with a saturated solution of Na_2CO_3. Bromoacetyl bromide (0.4g, 2mmole) dissolved in 1.0 ml dry tetrahydrofuran was added dropwise over 15 min, keeping the pH at 8.5 with Na_2CO_3. The mixture was stirred at 4C for 15 min and then extracted 5 times with 5.0 ml ethyl acetate. The organic layer was washed with water, N HCl and N $NaHCO_3$, dried over sodium sulfate, and concentrated to dryness. The resulting oily residue was triturated with ethyl ether to give a white powder which was characterized by thin layer chromatography, proton NMR spectroscopy, and elemental analysis; m.p. 61-62°.

Treatment of Bromoacetyl Alanine Ethyl Ester with Ammonia

Bromoacetyl alanine ethyl ester (0.01g, 0.04mmole) was dissolved in 2 ml cold concentrated NH_4OH. The mixture stood at 4C for 1 hr, then was taken to dryness, dissolved in water, and the pH adjusted to 10. The aqueous layer was extracted 5 times with 5 ml ethyl acetate, then saturated with NaCl and again extracted with ethyl acetate. The combined organic layers were concentrated to dryness and gave a white powder.

Synthesis of Bromoacetyl Oxytocin (BrOXY)

Synthetic oxytocin (10mg, 10μmole) was dissolved in 1.0 ml of N $NaHCO_3$ and allowed to react at 0C for 1.5 hr with 0.1g (0.5mmole) of bromoacetyl bromide dissolved in 1.0 ml 1,4-dioxane, keeping the pH between 8 and 9 by

appropriate titration with 1.5N Na_2CO_3. The progress of the reaction was monitored by thin layer chromatography on silica gel, using the solvent system n-BuOH/acetic acid/H_2O (4:1:5). The reaction mixture was neutralized by addition of glacial acetic acid, applied to a Sephadex G15 column (100x0.9cm) equilibrated with 50% acetic acid, and eluted with the same solvent at 4C (150). The peptide peak, located according to the method of Lowry et al. (147), was pooled and lyophilized. The stability of bromoacetyl oxytocin was assessed by exposing it to various solvents and monitoring changes by thin layer chromatography, in order to choose a suitable solvent for partition chromatography. The product was then further purified by partition chromatography on Sephadex G25 at 4C. The column (70x0.9cm) was equilibrated with both phases of the solvent system 0.1% acetic acid/sec. butanol (1:1, v/v), and eluted with the upper phase of this solvent system. The product was again located by Folin color, pooled, diluted with 0.1% acetic acid and lyophilized.

Treatment of BrOXY with Ammonia

BrOXY (2mg, ~2μmole) was dissolved in 3.0 ml dimethylsulfoxide at room temperature. Ammonia, freshly distilled from Na, was bubbled through this solution for 30 min, and the reaction mixture was allowed to stand at room temperature for another 30 min. Ammonia and other volatile components were removed by evaporation in vacuo. The resulting residue

was diluted with glacial acetic acid and placed on a Sephadex G15 column (110x0.9cm), equilibrated with 50% acetic acid, and eluted with the same solvent. By the Lowry method a single, symmetrical peak was detected. The content of tubes associated with this peak was pooled, diluted with a large excess of water, and lyophilized. The product, glycyl-oxytocin, was subjected to avian vasodepressor assay and amino acid analysis.

Preparation of Adenylate Cyclase

Adenylate cyclase from toad urinary bladder was prepared as described by Bär et al. (49). Bladders of pithed toads exsanguinated by ventricular perfusion were removed and rinsed in amphibian Ringer's solution (containing 1.0mM $CaCl_2$, 2.0mM KCl, 2.4mM $NaHCO_3$, and 111.0 mM NaCl, equilibrated with air). Epithelial cells were scraped from the mucosal surface and collected in ice-cold 0.225M sucrose (containing 0.1mM ethylene glycol bis-(2-aminoethyl)tetraacetate (EGTA) and 0.01mM tris-(hydroxymethyl)amino methane (Tris), pH 7.5 (sucrose buffer). Cells were washed twice and homogenized in sucrose buffer with a "tight-fitting" glass homogenizer. The homogenate was washed and centrifuged twice at 600g (10 min) in a Sorvall RC-2B refrigerated centrifuge and quick-frozen in dry ice-acetone. The enzyme was stored at -70C and thawed just before use. Adenylate cyclase from rabbit renal medullary tissue was prepared by the method

of Dousa et al. (69). Medullary tissue was dissected and homogenized in 0.25M sucrose containing 5.0mM Tris, pH 7.5, 3.0mM $MgCl_2$ and 1.0mM ethylene-diamine tetraacetate (EDTA). The homogenate was centrifuged at 600g (10 min) and the pellet was resuspended in the same solution but without sucrose and washed twice. Adenylate cyclase from rabbit renal cortical tissue was prepared in the manner described for renal medullary tissue. The renal preparations were frozen and stored at -70C and thawed just before use. Protein concentration was determined by the method of Lowry et al. (147).

Adenylate Cyclase Assay

The assay for toad bladder adenylate cyclase activity was carried out according to the method of Bär and Hechter (151). In a total of 0.05 ml, final concentrations of reaction components were: 45mM Tris, pH 8.0; 5mM $MgCl_2$; 0.1% bovine serum albumin (BSA); 7mM phosphoenol pyruvate (PEP); 0.1mg/ml pyruvate kinase; 0.01mg/ml myokinase; 0.1mM ATP-α-^{32}P ($\sim 10^5$-10^6cpm), 0.5mM cyclic 3',5'-AMP (cAMP), 20µl (0.5-2.5mg/ml) adenylate cyclase enzyme preparation (in sucrose buffer, pH 8.0). After a 20-min incubation at 37C, 0.05 ml of a solution containing 4mM ATP, cAMP and 5'-AMP was added, and samples were heated for 3 min at 100C. After centrifugation supernatants were transferred to new tubes and freeze-dried. The residues were dissolved in about

5µl water and chromatographed on PEI-impregnated cellulose TLC plates. The cAMP and the ATP, ADP, AMP plus P_i spots were located under UV, cut out and counted in toluene scintillation fluid (8.0g PPO (2,5-diphenyl-oxazole) and 0.4g POPOP (1,4-bis [2-(5-phenyl-oxazolyl)] -benzene)/2L) on a Packard model 3003 liquid scintillation spectrometer. The ratio of cAMP to substrate (percent conversion) was calculated. Adenylate cyclase in renal medullary and cortical tissue was assayed as above with the following modifications in the incubation medium: a final volume of 0.05 ml contained 40mM Tris, pH 7.5, 0.1mg/ml BSA, 0.1mg/ml creatine kinase, 4mM $MgCl_2$, 0.1mM ATP-α-^{32}P, 0.1mM EDTA, 25mM creatine phosphate, 0.5mM cAMP and 20µl (0.5-1.0mg/ml) enzyme protein. Assays were carried out for 20 min at 37C, and percent conversion of ATP to cAMP determined as described above. In most cases at least three experiments (in duplicate) were performed using different enzyme preparations.

Treatment of Adenylate Cyclase Preparation with Neurohypophyseal Hormones and Analogs

The toad bladder enzyme preparation was preincubated at various temperatures for various periods of time, and with different concentrations of hormonal peptides (e.g., BrOXY or oxytocin) dissolved in sucrose buffer pH 8.0. Controls were preincubated under identical conditions except that no hormonal peptides were included. Generally,

no attempt was made to "wash out" the BrOXY before an aliquot of the preincubation mixture was challenged with hormone (e.g., AVT, AVP, etc.) in the adenylate cyclase assay system. The cAMP production was measured for a period of 20 min thereafter. In some experiments attempts were made to wash out the effects of preincubating the cyclase preparation with BrOXY or oxytocin: in these experiments the cyclase preparation was diluted with sucrose buffer alone or with buffer containing urea, and incubated for 5 min at room temperature and for 5 min at 0C. After centrifugation (600g, 10 min) the enzyme preparation was suspended in the same medium and kept for 5 min at 0C, then diluted tenfold with sucrose buffer and centrifuged. The enzyme preparation was again washed in a large volume of sucrose buffer, then resuspended in the same buffer and challenged with hormone in the adenylate cyclase medium. The kidney preparations were less stable than the toad preparation, exhibiting a loss of sensitivity to hormone when treated as described above. Therefore, the kidney enzyme preparation and peptide (BrOXY) were added to the final adenylate cyclase assay medium containing hormone (AVP) but not containing ATP-α-^{32}P and preincubated for 10 min at 37C; the labeled nucleotide was then added, and the incubation was continued for an additional 20 min.

Results

Purification of Oxytocin

Partition chromatography of the oxidized nonapeptide of oxytocin gave three Folin-positive peaks (Fig 3). Peak I is polymerized material, peak II (R_f 0.37) is hemi-1-D-cysteine oxytocin and peak III (R_f 0.24) is oxytocin (146). Material from peak III had an avian vasodepressor activity of 453 U/mg. In general, the yield from 100 mg protected nonapeptide was about 15mg oxytocin. Oxytocin used in this study was prepared from a single solid phase synthesis, and from a single stepwise synthesis.

Characterization of Bromoacetyl L-Alanine Ethyl Ester

This compound was used as a model to optimize reaction conditions for the synthesis of bromoacetyl oxytocin. The yield of bromoacetyl alanine OEt was only 10%, as only material needed for characterization was isolated. The product gave a single spot on thin layer chromatography in the solvent system benzene/ethyl acetate/acetic acid (1:1:0.1). The assigned proton NMR spectrum is shown in Fig.4. Elemental analysis gave the following results: Calc. for $C_5H_8NO_3Br$: C 35.31, H 5.08, N 5.88, Br 33.56; Fd.: C 35.57, H 5.30, N 5.93, Br 33.32.

The time course of the reaction of bromoacetyl alanine ethyl ester with ammonia was followed by thin layer

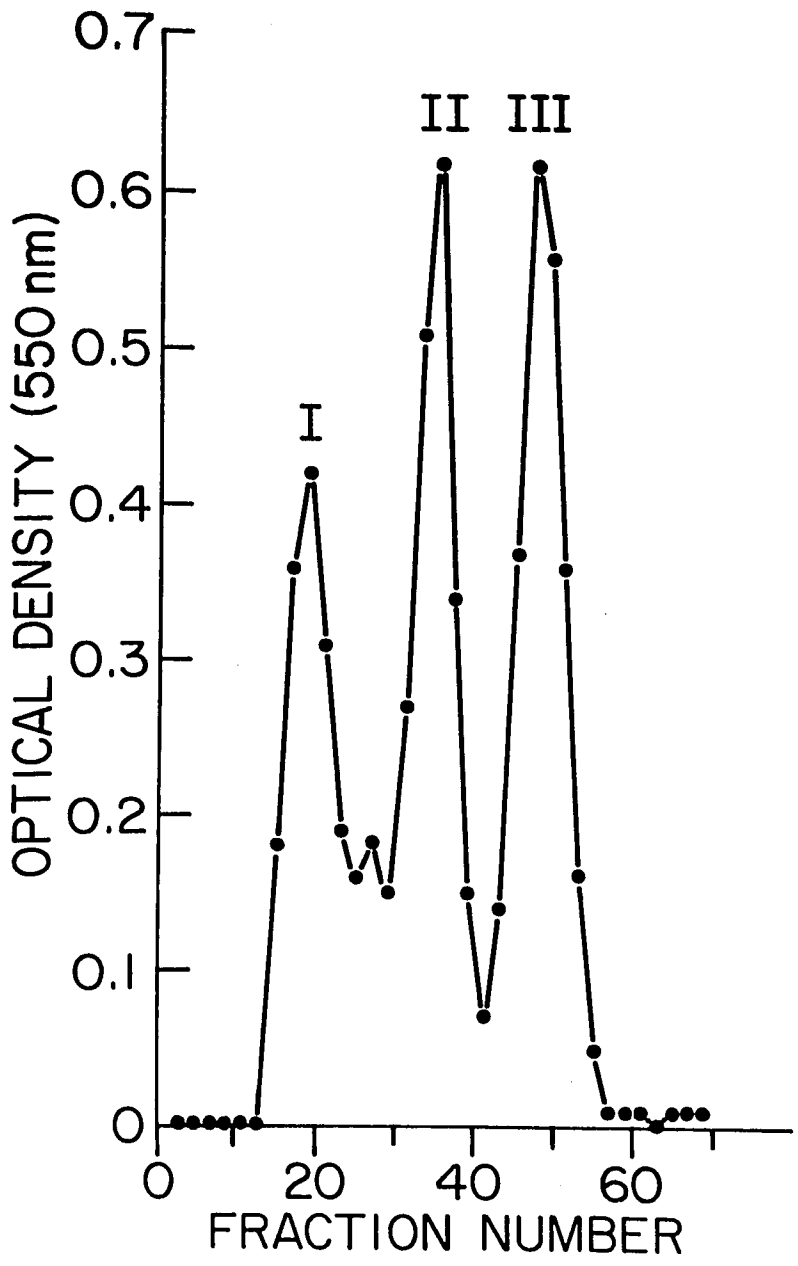


Fig. 3. Partitition chromatography of deprotected and oxidized nonapeptide of oxytocin derived from solid phase synthesis. Chromatography is on Sephadex G25 in the solvent system n-BuOH/benzene/pyridine/acetic acid/H_2O (600:200:100:1:900, upper phase). Peak I=polymeric material, Peak II= Hemi-l-D-cysteine oxytocin, Peak III=oxytocin.

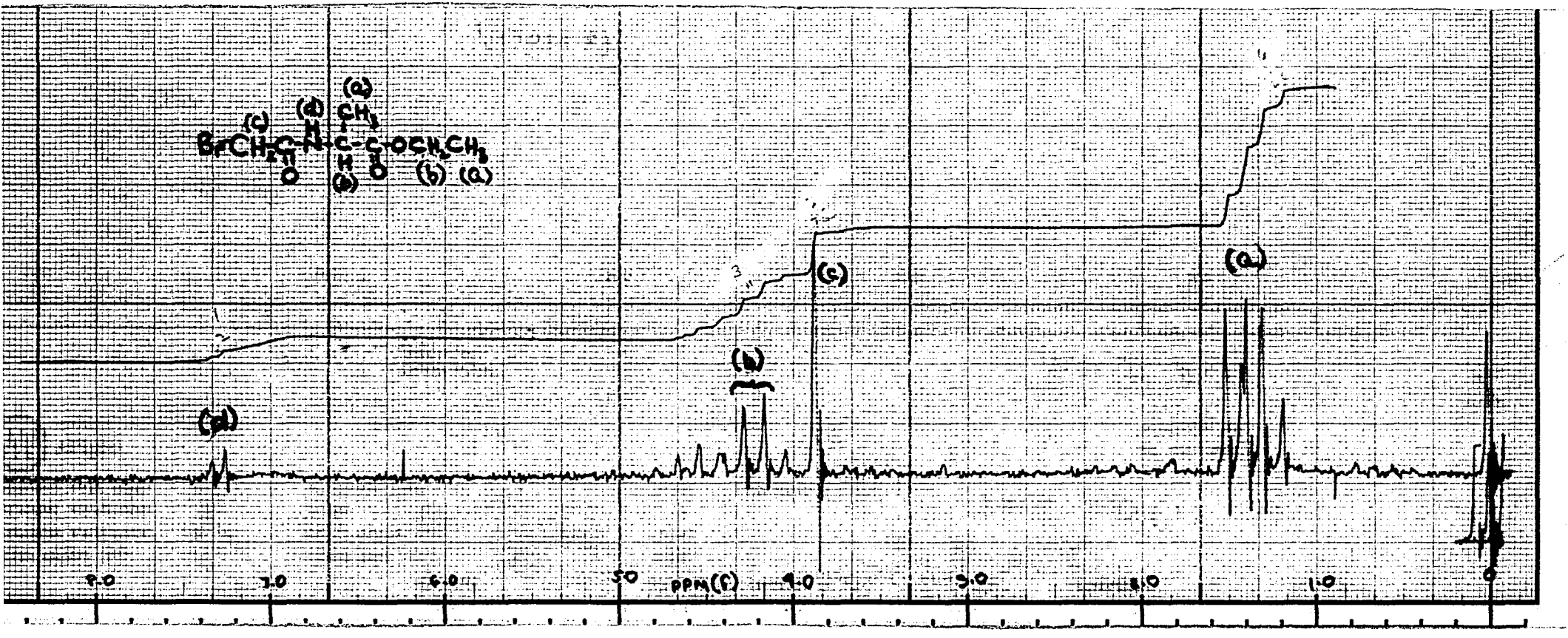


Fig. 4. Assigned NMR spectrum of bromoacetyl alanine ethyl ester. The spectrum was run on 30mg of sample in $CDCl_3$, using a Varian T-60 NMR spectrometer, at 60 MHz. Tetramethylsilane is the internal standard.

chromatography (Fig. 5). The starting material is converte to product within 45 min; the product after acid hydrolysi had amino acid ratios of Gly 1.0, Ala 0.97.

Characterization of BrOXY

After gel filtration of the reaction mixture, BrOXY w contaminated with unreacted oxytocin (Fig. 6). Exposure of BrOXY to some partition chromatography solvent systems for 24 hr caused decomposition (Fig. 7). The system sec.BuOH/0 acetic acid (1:1) was chosen for purification since BrOXY appeared to be stable in this system. BrOXY gave a single symmetrical peak (R_f 0.59) on partition chromatography (Fig. 8). A hydrolyzed sample of material from this peak gave the following ratios of ninhydrin-active components with isoleucine taken as 1.0: Asp 1.0, Glu 0.99, Pro 1.0, Gly 0.98, Cys 0.9, Ile 1.0, Leu 1.0, Tyr 0.9, and NH_3 2.99 Elemental analysis: Calculated for $C_{45}H_{68}N_{12}O_{13}S_2Br$: Br 7 Found, 6.74. The average yield of various preparations wa approximately 5.5mg (50%).

When the purified material was chromatographed on silica gel, it was noticed that decomposition occurred in some cases. This was demonstrated by running BrOXY twice on a single plate, the second run at a right angle to the first. On each run, BrOXY separated into 2 compone This result was not consistently reproducible from one batch of thin layer plates to another.

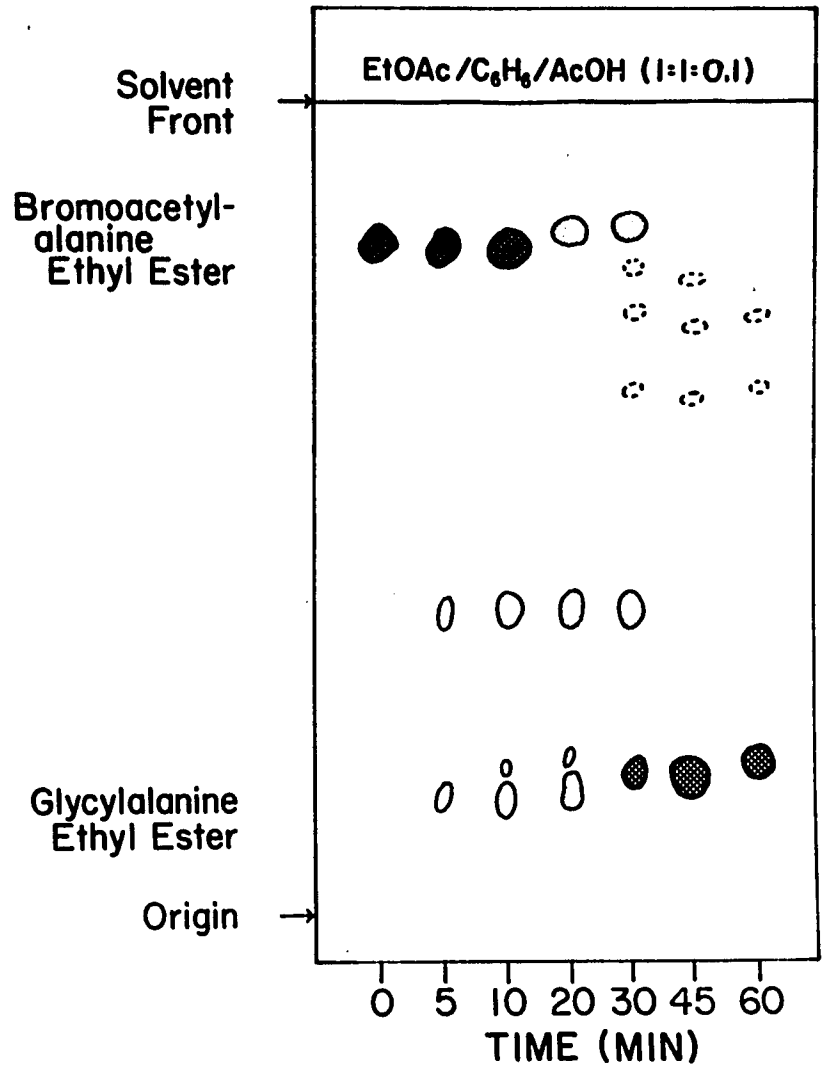


Fig. 5. Time course of the reaction of bromoacetyl alanine ethyl ester with ammonia. The bromoacetylated amino acid was dissolved in concentrated NH_4OH and aliquots were spotted on the silica gel G thin layer plates after the reaction had proceeded for the indicated times. The plate was run with the indicated solvent, and developed with chlorine-tolidine reagent. The spots between the starting material and final product were not identified; glycylalanine ethyl ester was characterized by amino acid analysis of the reaction mixture after 60 min.

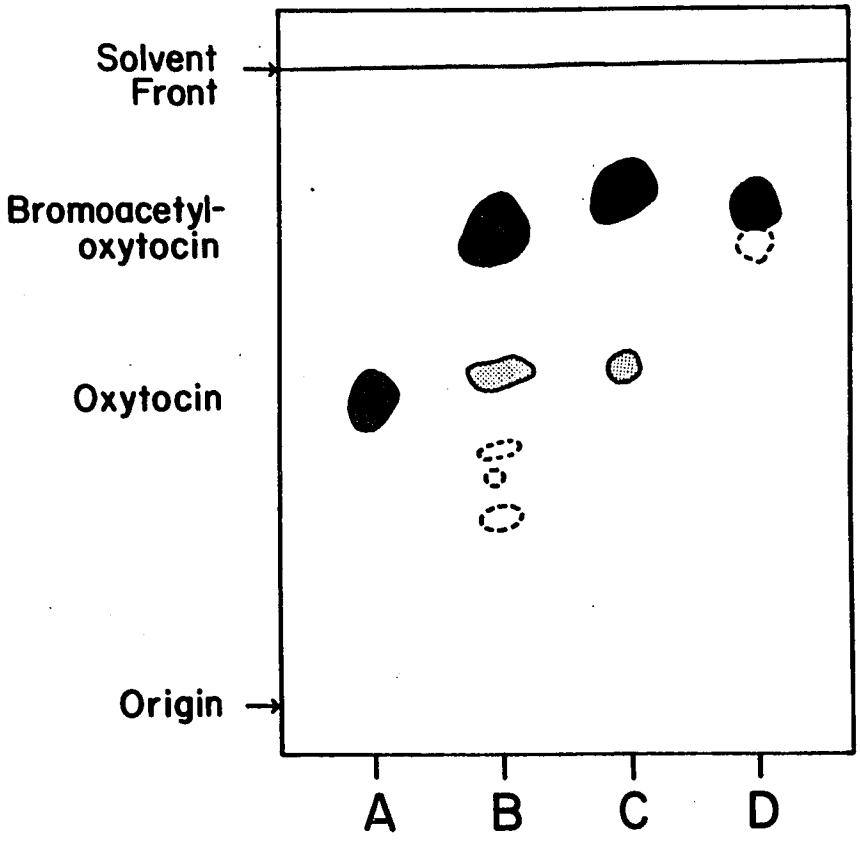


Fig. 6. Thin layer chromatography on silica gel G, using the upper phase of the solvent system n-BuOH/AcOH/H_2O (4:1:5), of A: oxytocin, B: reaction mixture of oxytocin and bromoacetyl bromide, C: bromoacetyloxytocin after gel filtration on Sephadex G15, D: bromoacetyl oxytocin after partition chromatography on Sephadex G25.

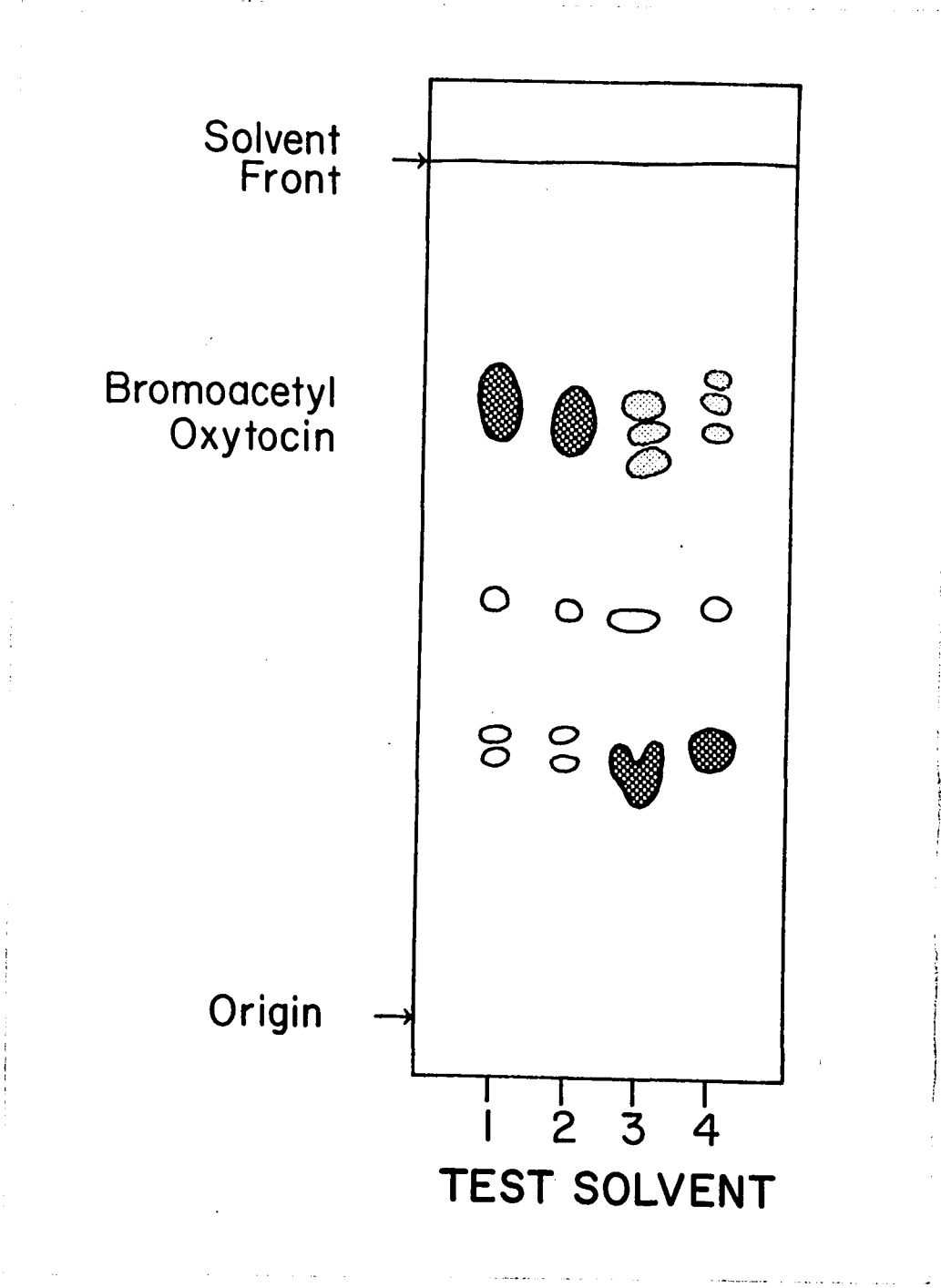


Fig. 7. Thin layer chromatography on silica gel G, in the solvent system used in Fig. 6, of bromoacetyl oxytocin after exposure for 24 hr to four solvent systems for partition chromatography. Solvent 1: sec.BuOH/0.05% AcOH (1:1), upper phase. Solvent 2: sec.BuOH/0.1% AcOH (1:1), upper phase. Solvent 3: n-BuOH/benzene/pyridine/ AcOH/H_2O (600:300:13.5:31.5:855), upper phase. Solvent 4: n-BuOH/benzene/pyridine/AcOH/H_2O (6:2:1:0.01:9), upper phase.

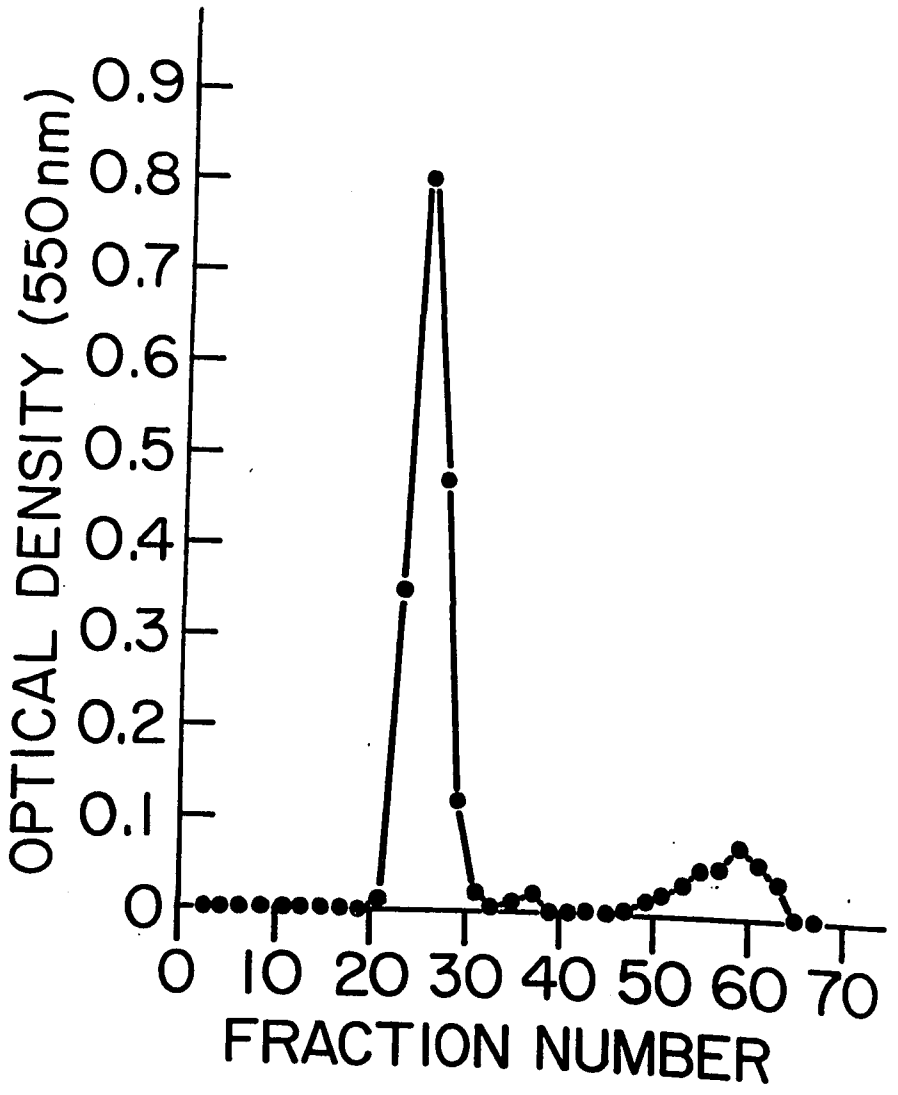


Fig. 8. Partition chromatography of bromoacetyl oxytocin on Sephadex G25 in the solvent system sec.BuOH/0.1% AcOH. For details see text. The column was equilibrated with lower and upper phases of the solvent system and eluted with upper phase. Aliquots of alternate fractions were tested by the Lowry method for protein content.

An aliquot of BrOXY was converted by treatment with ammonia to glycyl-oxytocin. After gel filtration this product had an avian vasodepressor activity of 0.75 U/mg comparable to that reported for glycyl-oxytocin prepared by a different route (152,153). Glycyl-oxytocin prepared from BrOXY also completely inhibited the response to the USP Posterior Pituitary Standard in the vasodepressor assay, and exhibited a protracted action, as reported previously. After acid hydrolysis of glycyl-oxytocin the following ratios were obtained with Glu taken as 1.0: Asp 0.9, Glu 1.0, Pro 1.0, Gly 1.8, Cys 0.9, Ile 1.0, Leu 1.0, Tyr 0.9, and NH_3 3.1.

Effect of Bromoacetyl Oxytocin on Adenylate Cyclase Activity

When BrOXY was added to adenylate cyclase preparations of either the toad bladder or rabbit renal medulla, it had no stimulatory activity at any of the concentrations tested (up to 10^{-4}M) (Fig. 9). Control experiments carried out with the same preparations established that the toad bladder cyclase responded to AVT, the natural hormone of the toad, and the renal medullary preparation was stimulated by AVP, the natural antidiuretic hormone of rabbits.

Effect of BrOXY on Neurohypophyseal Hormone-Stimulated Toad Bladder Adenylate Cyclase

In preliminary experiments, BrOXY was preincubated with toad bladder adenylate cyclase preparation under a variety

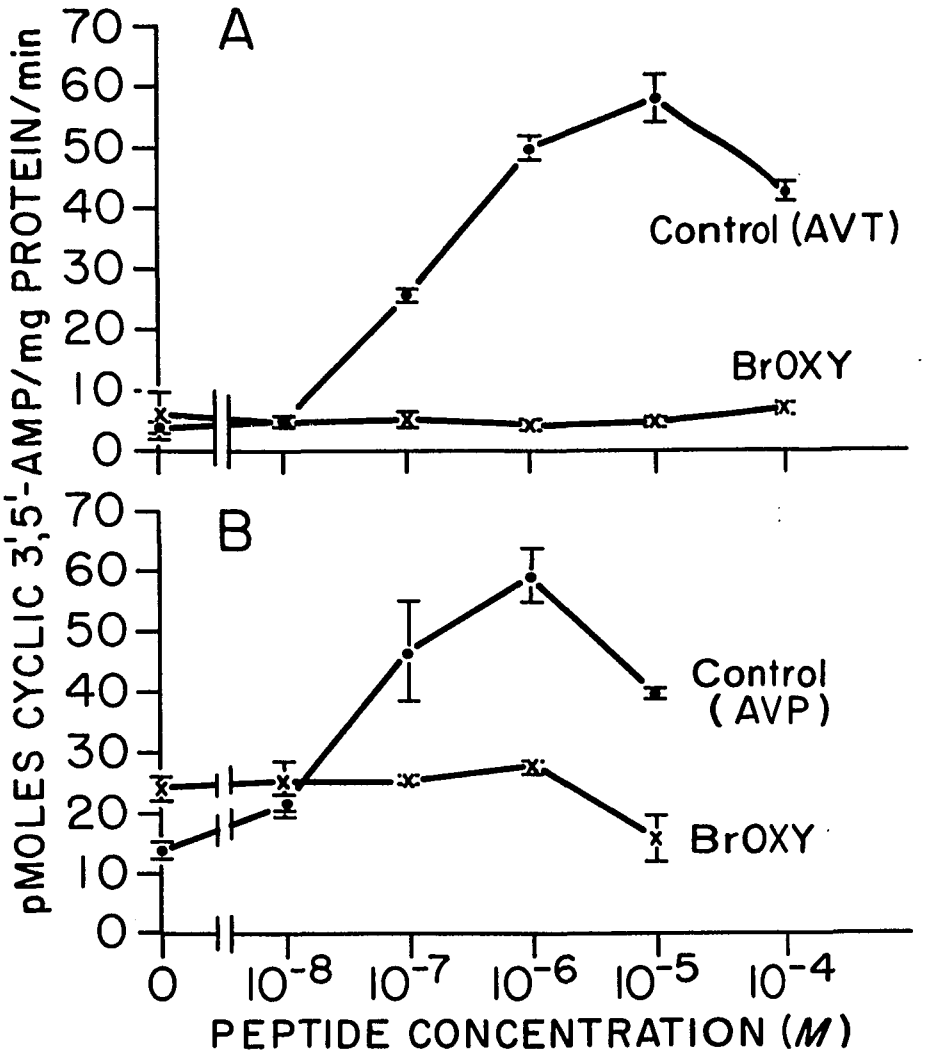


Fig. 9. A. Stimulation of toad urinary bladder adenylate cyclase (pmole cAMP/mg of adenylate cyclase protein/min) as a function of increasing concentrations (10^{-8}-10^{-4}M) of AVT and BrOXY. Each point represents a mean of 4 or more experiments; in this and subsequent figures vertical bars represent standard errors of the means. B. Stimulation of renal medullary adenylate cyclase as a function of increasing concentrations (10^{-8}-10^{-4}M) of AVP and BrOXY.

of conditions (at a final concentration of 10^{-4}M). BrOXY non-surmountably inhibited the response both to AVT and to oxytocin. Inhibition of the oxytocin response is shown in Fig 10: preincubation was carried out for 10 min at 0C. BrOXY inhibition of the response to AVT is more pronounced and is dependent on time (Fig. 11) and temperature (Fig.12) of preincubation. Fig. 11 shows that BrOXY inhibition increases linearly with preincubation time up to 10 min. The enzyme preparation begins to lose sensitivity if preincubation is carried out for 15 min or longer. The inhibition is greater when preincubation is carried out at 37C than at 0C, as shown in Fig.12. The effect of temperature is also seen when oxytocin is the stimulating hormone (Fig.13). Fig.14A shows that inhibition of the AVT-stimulated response is related to the concentration of the antagonist in the preincubation mixture. Inhibition increases sharply over a small concentration range (10^{-5} - 10^{-4}M) and is almost complete at 10^{-4}M. The response of rabbit medullary adenylate cyclase to AVP is also non-surmountably inhibited by BrOXY, and this inhibition is also concentration-dependent (Fig.14B).

Effect of BrOXY on Fluoride-Stimulated Adenylate Cyclase Activity

In contrast to the inhibition of AVT and oxytocin stimulation following preincubation of the cyclase preparation with 10^{-4}M BrOXY (10 min at 37C) this preincubation treatment

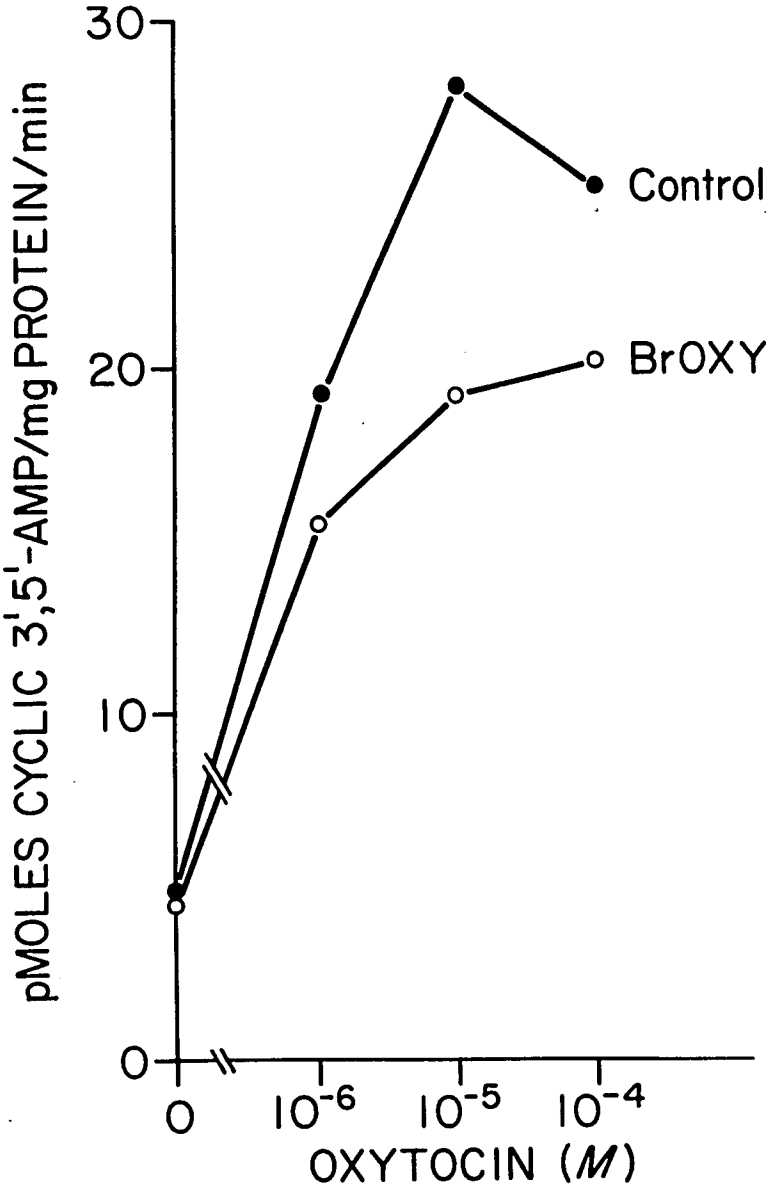


Fig. 10. Inhibition by 10^{-4}M BrOXY of the oxytocin-stimulated production of cAMP in the toad bladder adenylate cyclase preparation. Preincubation was for 10 min at 0C. Each point represents the average of two experiments.

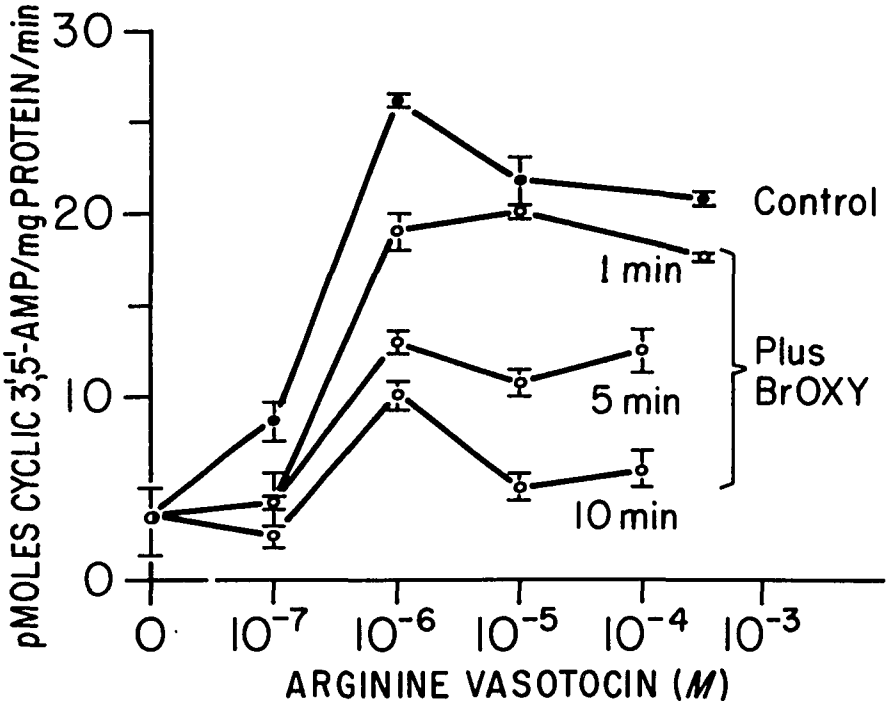


Fig. 11. Inhibition by 10^{-4}M BrOXY of the AVT-stimulated production of cAMP in the toad bladder adenylate cyclase preparation as a function of time of preincubation at 37 C.

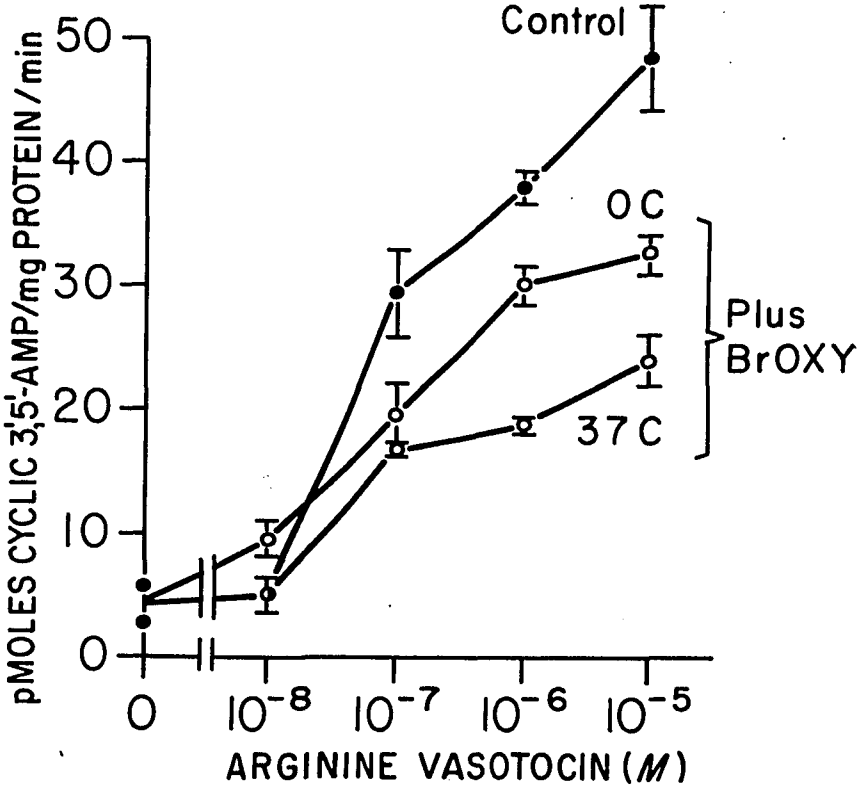


Fig. 12. Inhibition by 10^{-4}M BrOXY of the AVT-stimulated production of cAMP in the toad bladder adenylate cyclase preparation as a function of temperature of preincubation (10 min).

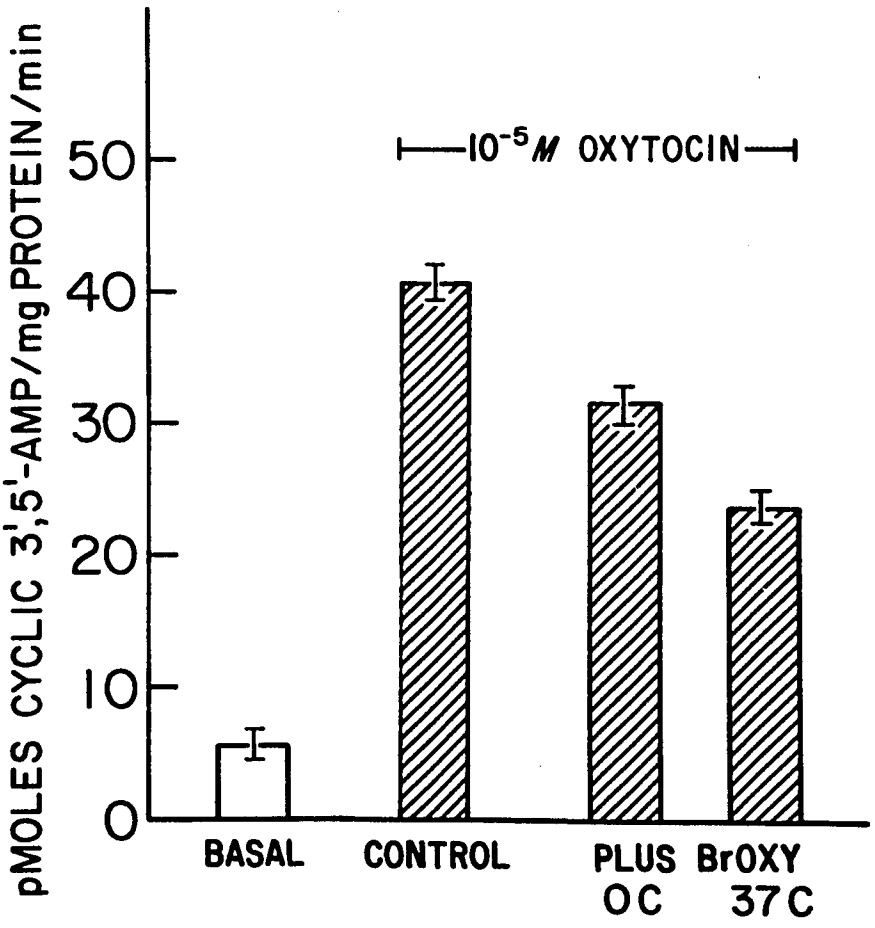


Fig. 13. Inhibition by 10^{-4}M BrOXY of the oxytocin-stimulated production of cAMP in the toad bladder adenylate cyclase preparation as a function of temperature of preincubation (10 min).

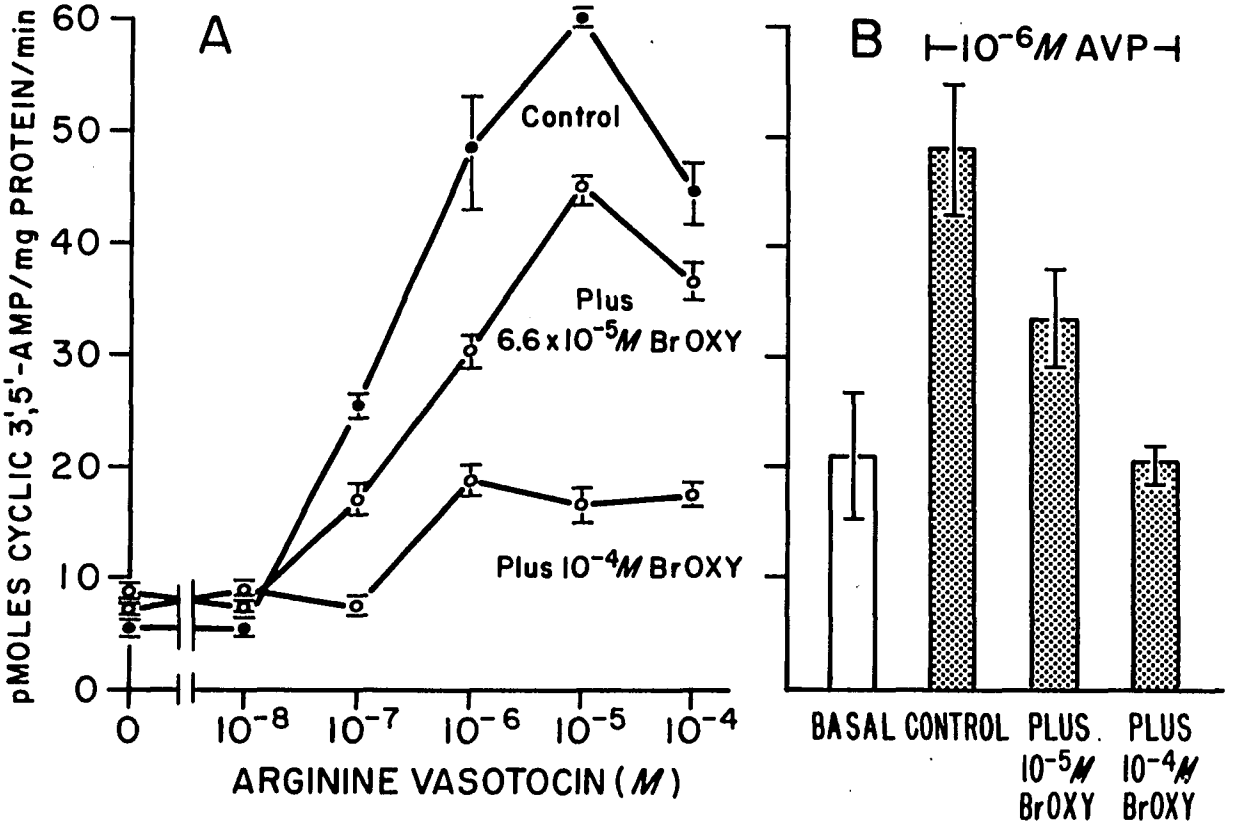


Fig. 14. Inhibition of hormone-stimulated production of cAMP in adenylate cyclase preparations as a function of BrOXY concentration. A. Inhibition of the response to AVT of toad bladder adenylate cyclase. B. Inhibition of the response to AVP of renal medullary adenylate cyclase.

did not significantly affect the response of cyclase preparations to fluoride ion (Fig. 15).

Effect of BrOXY on Mammalian Renal Adenylate Cyclases

Fig. 16 shows that BrOXY (10^{-4}M) preincubated for 10 min at 37C with rabbit renal medullary cyclase, markedly decreased the response induced by 10^{-6}M AVP; sodium fluoride stimulation was not affected by this treatment. The effect of BrOXY in mammalian renal cyclase systems is specific to the extent that BrOXY pre-treatment of rabbit renal cortical cyclase caused no inhibition of the response evoked by an optimal concentration of parathyroid hormone (PTH) ($\sim 10^{-7}$M).

Preincubation of Toad Bladder Adenylate Cyclase with "Non-Specific" Hormonal Peptides

To further test the specificity of the inhibition of the adenylate cyclase response to neurohypophyseal hormones by BrOXY, the adenylate cyclase was preincubated with a variety of peptides. Preincubation with glucagon and ACTH, at concentrations of 10^{-4}M, produced no significant inhibition of the response to AVT (10^{-7}M). Similarly, preincubation with 10^{-4}M [5-valine] oxytocin, an analog with extremely low affinity for the intact toad bladder (154) and for the adenylate cyclase preparation derived from the toad bladder (49), failed to decrease significantly the response to AVT (Fig. 17).

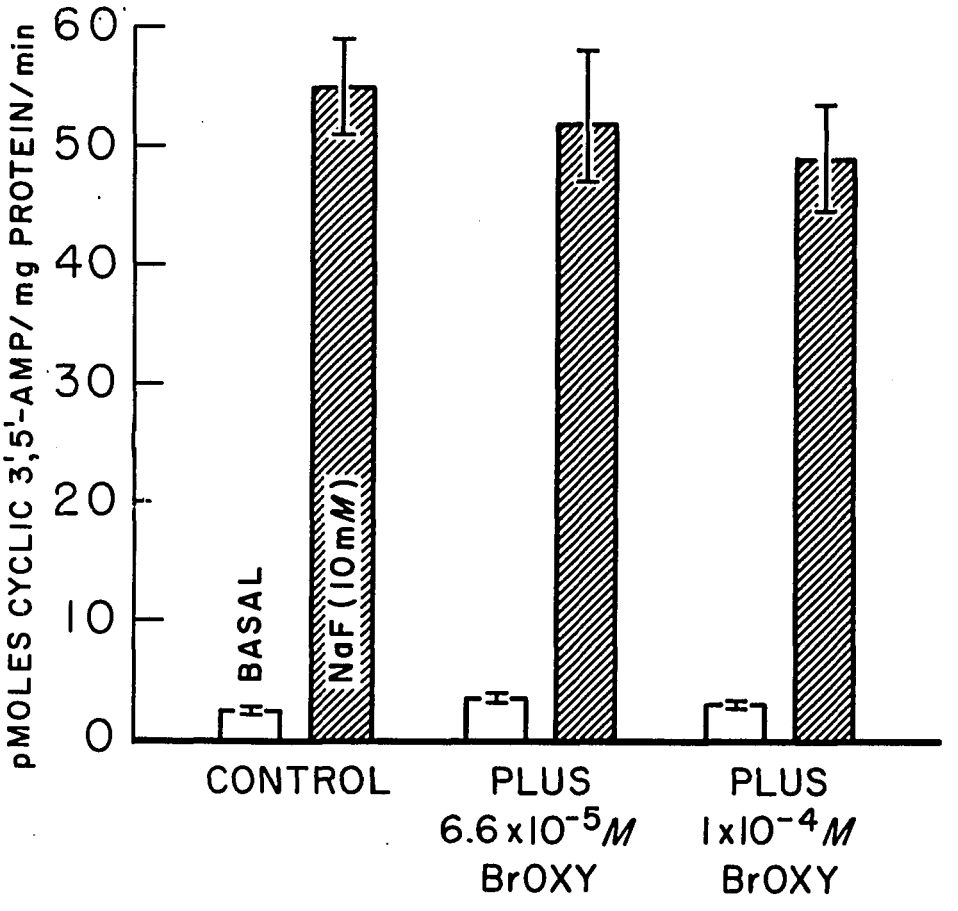


Fig. 15. Effect of preincubation with 10^{-4}M and 6.6×10^{-5}M BrOXY on stimulation of toad bladder adenylate cyclase by fluoride ion.

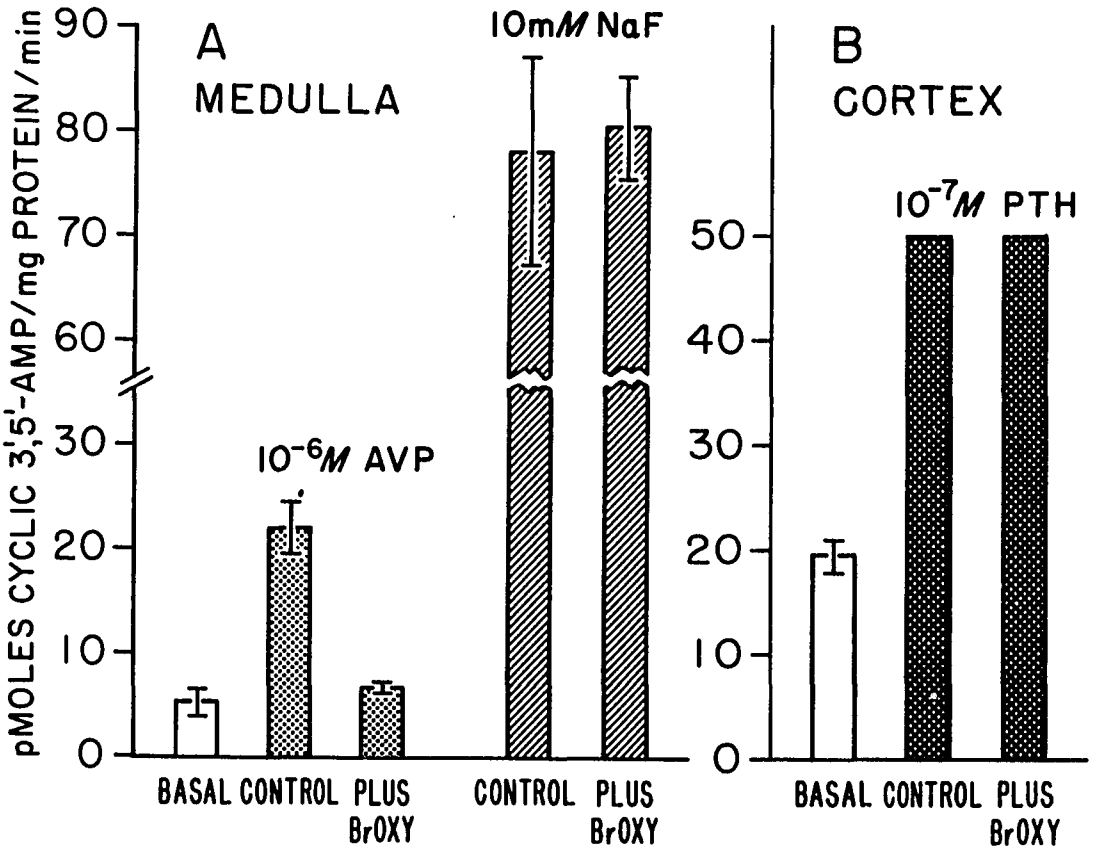


Fig. 16. Preincubation of renal adenylate cyclase preparations with 10^{-4}M BrOXY. A. Effect on stimulation by AVP and sodium fluoride of cAMP production in the renal medullary enzyme preparation. B. Effect on stimulation by parathyroid hormone of cAMP production in the renal cortical enzyme preparation. Data are mean values from a representative experiment.

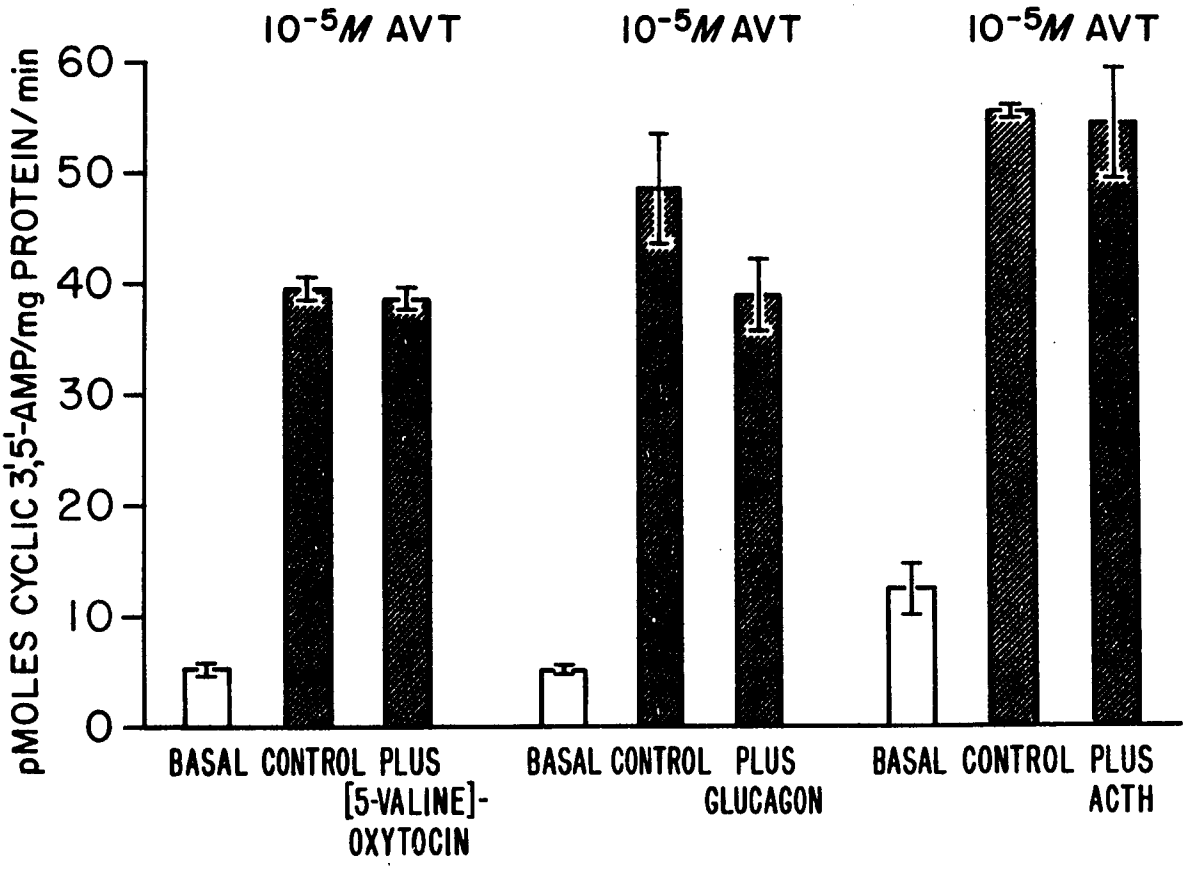


Fig. 17. Effect of preincubation with 10^{-4}M [5-valine] oxytocin, glucagon, or ACTH on AVT stimulation of toad bladder adenylate cyclase.

Preincubation of Toad Bladder Adenylate Cyclase with Neurohypophyseal Peptides

An interesting finding was that preincubation of the toad bladder adenylate cyclase with 10^{-4}M oxytocin (Fig.18) or AVT for 10 min at 37C gave nonsurmountable inhibition of the response to a second challenge with AVT or oxytocin in the adenylate cyclase assay similar to that observed with BrOXY. As seen in Fig.18, this inhibition is also dependent on the concentration of oxytocin in the preincubation medium, over a range of concentrations identical to that effective for BrOXY. In addition, the amount of inhibition is proportional to the length of time and the temperature of preincubation with 10^{-4}M oxytocin. When [4-leucine]oxytocin (10^{-4}M), a competitive inhibitor of the neurohypophyseal hormones, was preincubated with the enzyme for varying lengths of time prior to the addition of increasing concentrations of AVT, the response to AVT was partially inhibited. This inhibition, in contrast to that induced by BrOXY and oxytocin, did not increase with the time of preincubation (Fig.19).

The effect of oxytocin and [4-leucine]oxytocin could not be reversed by washing with sucrose buffer, or with sucrose buffer at pH 6.2. It was demonstrated that 0.5M urea had no effect on the response of toad bladder adenylate

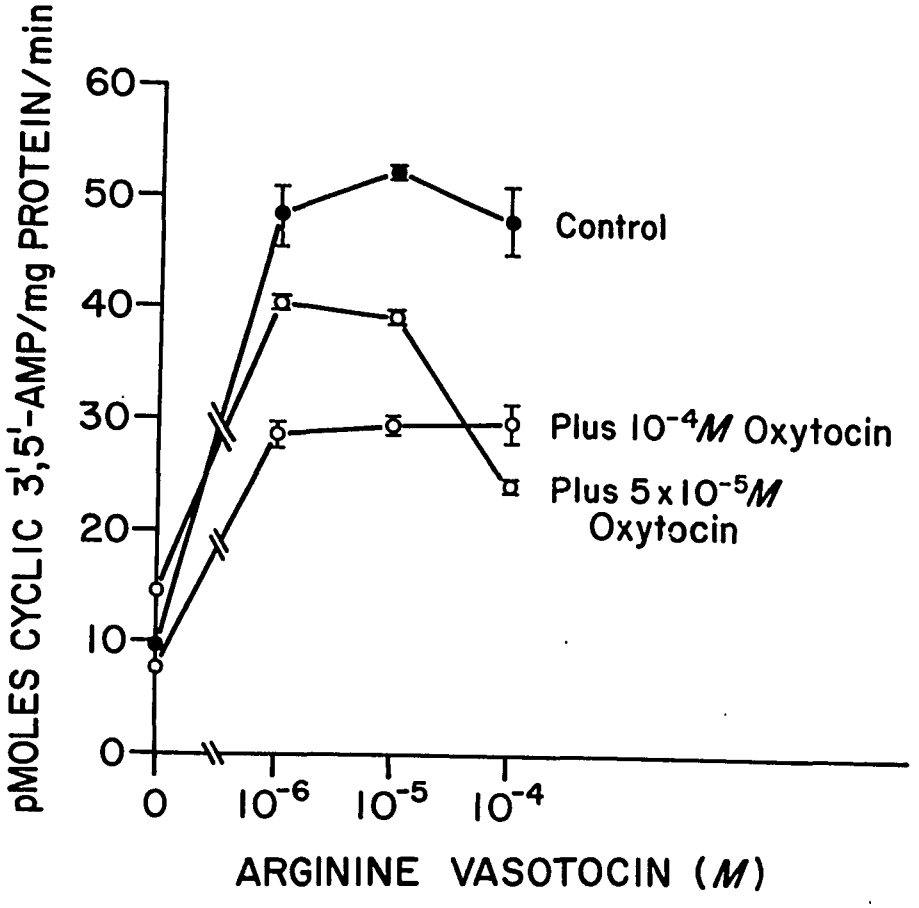


Fig. 18. Inhibition by oxytocin of the AVT-stimulated production of cAMP in the toad bladder adenylate cyclase preparation as a function of oxytocin concentration in the preincubation mixture.

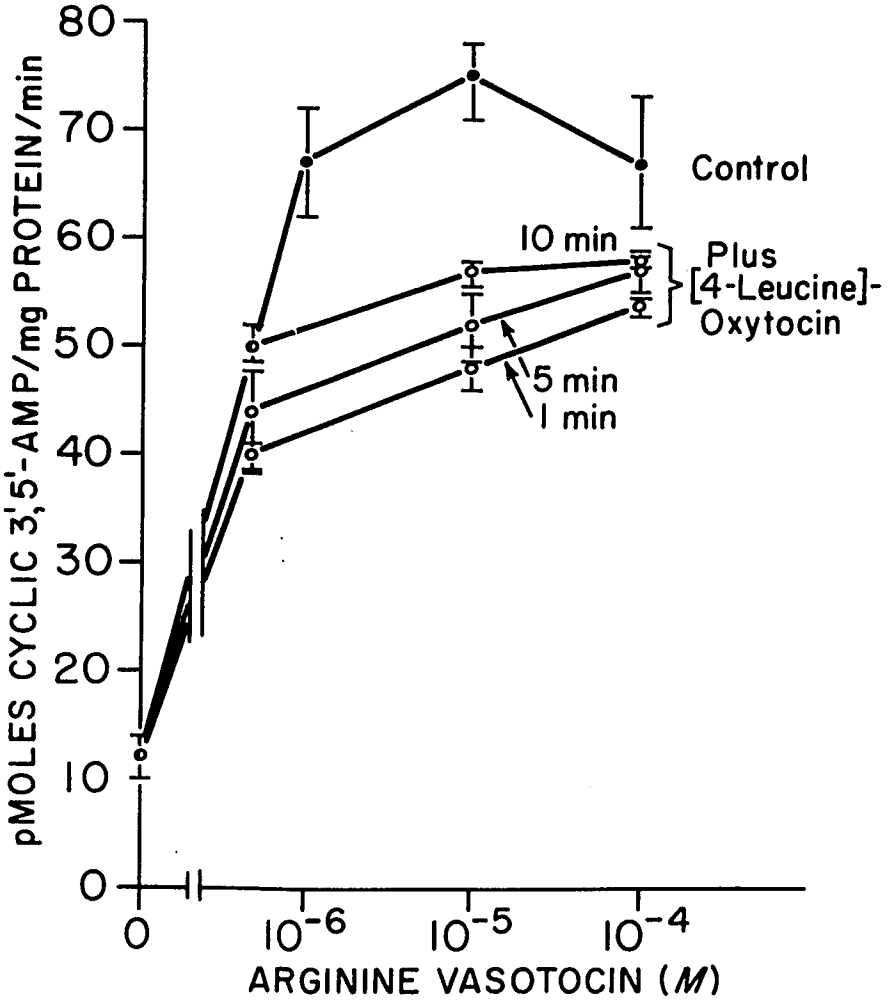


Fig. 19. Inhibition by 10^{-4} M [4-leucine]-oxytocin of the AVT-stimulated production of cAMP in the toad bladder adenylate cyclase preparation as a function of the duration of the preincubation period at 37 C.

cyclase to neurohypophyseal hormones (Fig. 20), and washing the preparation with 1.0M urea followed by several washes of sucrose buffer reversed the inhibition caused by oxytocin (or [4-leucine]oxytocin) but not that caused by BrOXY (Fig. 21

Preincubation of Toad Bladder Adenylate Cyclase with [Ileu-5] Angiotensin II

Angiotensin II was tested as an "inactive" peptide. In the original experiments, it was found that preincubation with this peptide (10^{-4}M) enhanced basal activity of the enzyme and did in fact inhibit the response to 10^{-5}M AVT. This inhibition may be more apparent than real, as a result of increased basal activity. In dose-response experiments, angiotensin II had no agonistic activity in the toad bladder adenylate cyclase system, and in later experiments was not found to be an effective inhibitor of neurohypophyseal hormone stimulation.

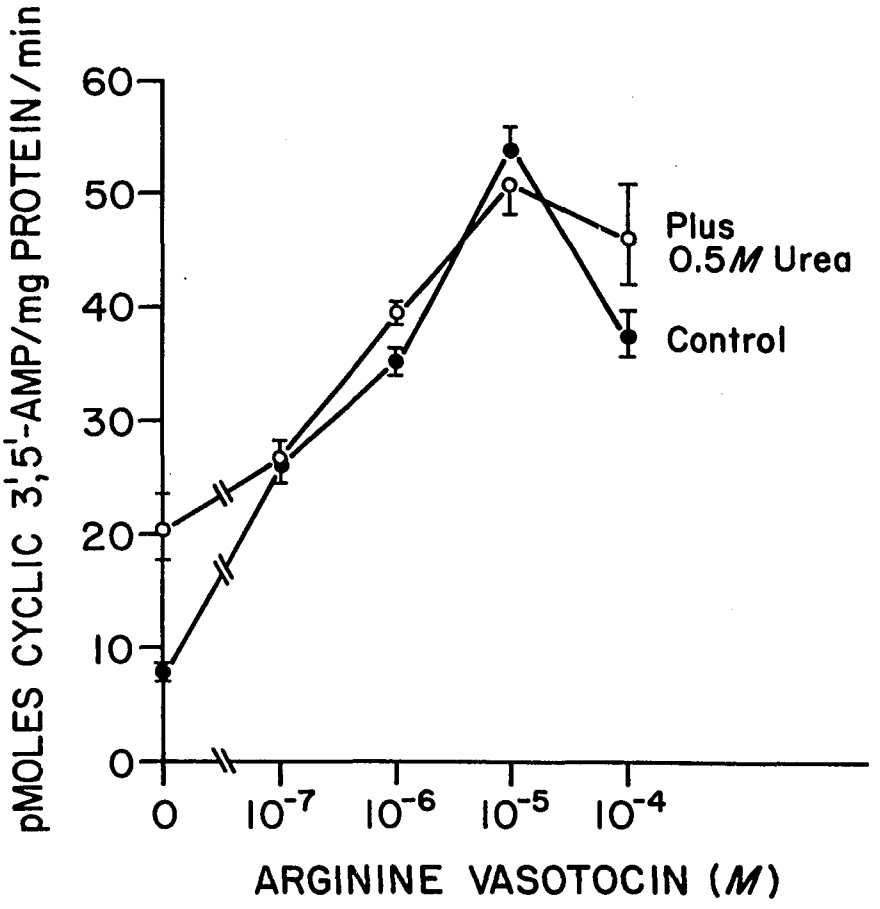


Fig. 20. Effect of 0.5M urea on AVT-stimulated production of cAMP in the toad bladder adenylate cyclase preparation. Urea was present during the adenylate cyclase assay, not as part of a preincubation.

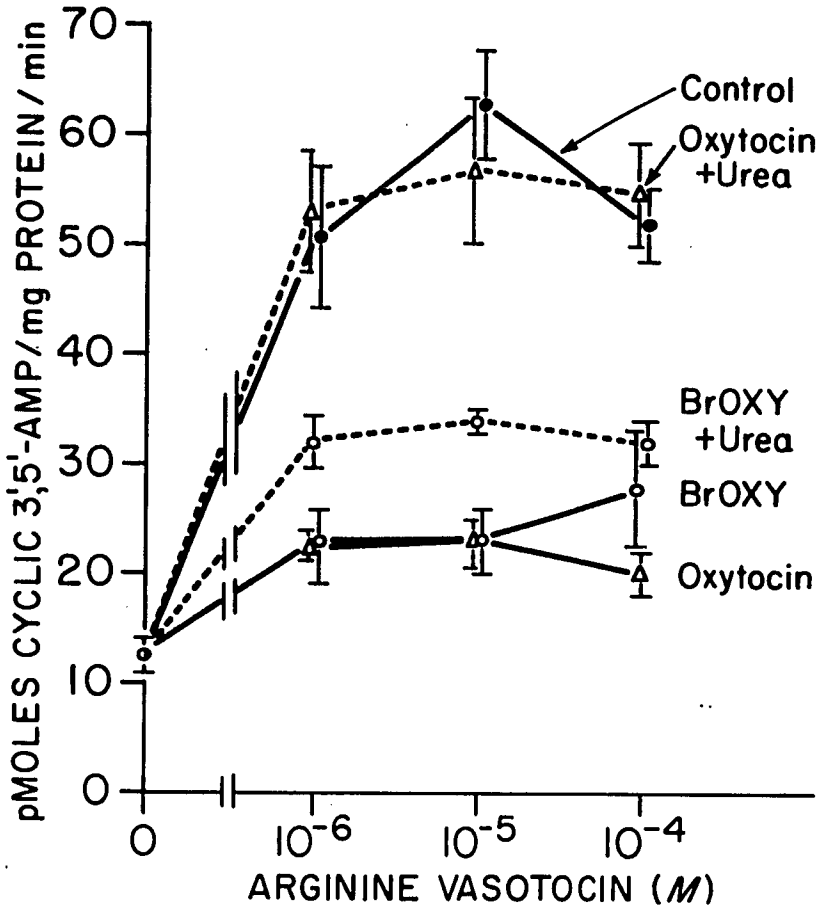


Fig. 21. Treatment of adenylate cyclase preparations with 1.0M urea after preincubation with oxytocin or BrOXY: the upper solid line and closed circles represent the adenylate cyclase response to 10^{-7}-10^{-4}M AVT after a period of preincubation in sucrose buffer in the absence of any added neurohypophyseal peptide (control). The lower solid lines with open triangles and open circles represent the adenylate cyclase responses to 10^{-7}-10^{-4}M AVT immediately after a period of preincubation in sucrose buffer containing 10^{-4}M oxytocin or BrOXY, respectively. The dotted lines with open triangles and open circles represent the adenylate cyclase responses to AVT after a similar preincubation with oxytocin and BrOXY, respectively, but followed by washout with sucrose buffer containing 1.0M urea.

Discussion

Neurohypophyseal hormones enhance the water permeability of the mammalian kidney and of the amphibian skin and bladder via a sequence of events -- the first being the interaction of the hormone with specific receptors. This interaction is coupled to stimulation of adenylate cyclase (49,68,74,75) and the hormonal information is translocated intracellularly by cyclic AMP (49,75,155). While we have found that the effects of neurohypophyseal peptides in intact target organs and broken-cell preparations derived from these organs are qualitatively similar, we have observed marked quantitative differences; i.e., greater peptide concentration is required for enzyme stimulation in the particulate system. These differences in hormonal sensitivity may be associated with the exposure of "silent receptors" or with perturbations of the receptor or the coupling (transducing) element. Roy et al. found that the homogenization of frog skin epithelial cells deteriorates the receptors, leading to a loss in their apparent affinity for the hormones (51). However, the differences may be more apparent than real, resulting from threshold and receptor reserve phenomena mentioned above, which are demonstrable both for the hydroosmotic effect of neurohypophyseal hormones and for their stimulatory effects in toad bladder adenylate cyclase preparations. Despite

these considerations, cell-free, hormone-responsive adenylate cyclase systems from neurohypophyseal hormone target organs lend themselves to the isolation of receptor molecules.

The application of affinity labeling to the problem of hormone receptor identification and characterization seems a most promising approach. The initialstep has been synthesis of a neurohypophyseal hormone analog which retains specificity for the receptors, thus forming initially a reversible hormone-receptor complex, and at the same time possesses a reactive group in order to form a covalent hormone-receptor complex in a second step.

The elucidation of the three-dimensional configuration of oxytocin in solution (15) facilitated the design of the analog. The reactive group should be located in a position which interferes minimally with hormonal affinity and specificity. Conformational studies (56,57) indicated that the side chains of residues 3, 4, 7, and 8 are not involved in maintaining the backbone conformation, and are therefore available for intermolecular interaction. This does not imply, however, that all of these side chains are necessarily constituents of the "active site" of the hormones. The oxytocin molecule is practically "featureless" on one side -- the "hydrophobic" side -- and on the other, is covered by

the hydrophilic groups of the hormone: the side chains of asparagine and glutamine, the aromatic moiety of tyrosine, and the C-terminal tripeptide. The hydrophilic area is probably involved in the expression of hormonal activity, while the hydrophobic most likely interacts with the receptor (56). Modifications within the hydrophilic area, therefore, may affect catalytic activity without drastically interfering with binding of hormone to receptor. Extensive structure-activity studies (53-55) bear out these predictions, and in combination with conformational analysis suggest the side chains of the amino acid residue in position 4, the p-position of tyrosine, and the C-terminal glycine residue as possible loci for modification to achieve affinity labels. These sites are also suggested by their relative ease of derivatization. In this study the N-terminal amino group of oxytocin was chosen as another feasible locus, since it is not important for expression of the hormonal activity of neurohypophyseal hormones (145,156).

Once the site on the hormone is decided, the type of reactive group can be chosen. Groups commonly used in affinity labeling include the sulfonyl fluoride, diazonium, and halomethyl ketone moieties (157). For this initial study, the bromoacetyl group, which has rather broad specificity, being able to react with lysine, histidine, aspartic acid,

glutamic acid, cysteine, and methionine side chains, as well as the α-amino group, was chosen. The reactivity is not so great as to preclude specificity; since the nature of the receptor is unknown it seemed wise to use a group that could react with many of the residues which might be present.

Synthesis and characterization of BrOXY were aided by use of the model compound, bromoacetyl alanine ethyl ester, which could easily be purified and identified. The reactivity of the bromoacetyl group did not seriously interfere with purification as long as nucleophiles such as pyridine were avoided. In addition it was noted that in some cases bromoacetyl oxytocin was unstable during thin layer chromatography on silica gel. Elemental analysis of BrOXY and its conversion by treatment with ammonia to glycyl-oxytocin, which was characterized biologically and chemically, demonstrated the identity of BrOXY.

A hormone affinity label is expected, <u>a priori</u>, to occupy hormone receptor sites and either stimulate or irreversibly inhibit the hormonal response. BrOXY over a wide range of concentrations has no stimulatory effect in the mammalian kidney or toad urinary bladder adenylate cyclase systems, but it significantly inhibits cyclic AMP production stimulated by the neurohypophyseal hormones (AVP, AVT, and oxytocin). The high concentrations necessary

for maximal inhibition are in line with the low affinity of N-acylated oxytocin derivatives for AVP and AVT receptors, and are not noticeably different from the hormone concentrations needed for maximal stimulation of the adenylate cyclase preparations. The formation of a covalent bond after an initial reversible complexation is in agreement with the time- and temperature-dependence of BrOXY inhibition, and the fact that inhibition of the neurohypophyseal hormone-induced response is insurmountable by increasing AVT, AVP, or oxytocin concentrations. These findings suggest that BrOXY is competing with the natural hormones for specific receptors. Since specificity of interaction is of prime importance for receptor isolation, further studies were aimed at the definition of this specificity. The responses of the toad bladder and kidney preparations to fluoride ion are neither enhanced nor inhibited by BrOXY, indicating that BrOXY affects only the hormone-sensitive component of the adenylate cyclase system. Moreover, neither glucagon nor ACTH, hormones which enhance cyclic AMP production in cyclase preparations of their target organs (75,76) has any inhibitory activity using conditions and concentrations where BrOXY causes significant inhibition of neurohypophyseal hormone-induced responses. [5-Valine]oxytocin, an analog which is incapable of producing a detectable increase in

cyclic AMP production in the toad bladder preparation (49) likewise has no inhibitory activity. Since rabbit kidney has been shown to contain two adenylate cyclases, one in the cortex and one in the medulla (68,158), analogous to rat (67), it was decided to test the effect of BrOXY on both cortical and medullary adenylate cyclases. The cortical enzyme, which is markedly stimulated by parathyroid hormone (68), is not affected by BrOXY; on the other hand, the medullary cyclase, which is stimulated by neurohypophyseal hormones, is significantly inhibited by BrOXY. All of the above results indicate that BrOXY inteferes selectively with the neurohypophyseal hormone receptor site. This interpretation is further supported by experiments in the intact toad urinary bladder, in which the hydroosmotic effect induced by 10^{-9}M AVP in one hemibladder is inhibited approximately 60% in the contralateral hemibladder preincubated for 30 min at room temperature with 10^{-7}M BrOXY. In this system, which is more sensitive than the adenylate cyclase preparation, BrOXY also has a slight stimulatory effect.

It should be noted that preincubation of the toad bladder adenylate cyclase system with 10^{-4}M oxytocin or AVT caused the enzyme to lose responsiveness to a second stimulation with these hormones in a range of 10^{-7} to 10^{-4}M. This inhibition was dependent on neurohypophyseal hormone concentration in the preincubation medium over the same range

as BrOXY, and was nonsurmountable by increasing hormone concentrations in the adenylate cyclase assay. The inhibitory activity could not be reversed by washing the enzyme preparation with sucrose buffer, but was reversible on treatment with 1.0M urea. In striking contrast, when identical experiments were carried out using BrOXY in the preincubation period, the inhibition could not be reversed by urea. (Urea is known to interfere with hydrophobic interactions between proteins.) These experiments point to a difference in the nature of the inhibition caused by oxytocin and BrOXY.

Experiments comparing the inhibitory properties of BrOXY and [4-leucine] oxytocin likewise reveal a different mechanism for diminution of AVT-stimulated cyclic AMP production. In contrast to the time-dependent inhibition by BrOXY, the inhibition by [4-leucine] oxytocin was time-independent. This latter compound, shown to be a weak competitive inhibitor of AVT in the intact toad bladder (159) and the bladder adenylate cyclase preparation (160), cannot form an irreversible link to the receptor moiety, and the weak inhibition it produces can be eliminated by treatment with urea.

A further interesting finding, not directly connected to the affinity labeling studies, was the stimulation of basal adenylate cyclase activity in the toad bladder preparation by preincubation with [Ileu-5] angiotensin II. This peptide was originally tested as a "non-specific" compound, similar to ACTH and glucagon, but the results, along with some studies indicating angiotensin activity in the intact toad bladder (161,162) prompted further investigation. Although the basal response was stimulated fairly reproducibly by preincubation with angiotensin, the peptide did not show a dose-response relationship and preincubation did not consistently inhibit the response to AVT.

The application of affinity labeling to receptor isolation has been rather limited, in spite of its advantages. The main problem is lack of specificity, which may be considerable because of spare receptors and receptors of varying affinity for the hormone. For example, attempts to isolate the adrenergic and cholinergic receptors in rat aortic strip have been unsuccessful as a result of non-specific binding of the irreversible blocking agents (163). The acetylcholinesterase of red blood cell membranes (164) and of the electroplax of electric eel (165) have been inhibited by quaternary amine derivatives: this enzyme was initially thought to be identical to the acetylcholine receptor, but

has since been demonstrated to be distinct (166). Acetylcholine sensitivity of the electroplax was abolished by p-(trimethylammonium) benzenediazonium fluoborate, and this decrease in sensitivity could be prevented by protection with D-tubocurarine (167). In later studies the acetylcholine receptor in electroplax was labeled in a two-step reaction with dithiothreitol and 4(N-maleimido)-benzyltrimethyl-^{3}H methylammonium iodide both *in situ* and in single cells (168,169). Labeling could be prevented by specific oxidation with choline thiol in both cases. In the single-cell experiments, extraction with sodium dodecyl sulfate and gel electrophoresis revealed a major peak of ^{3}H activity with a molecular weight estimated at 42,000.

The only other attempts at peptide hormone affinity labels are the p-N,N-Bis(2-chloroethyl)aminophenylbutyryl derivatives of bradykinin and angiotensin II fragments synthesized by Stewart *et al.* (170,171). Two of the latter compounds irreversibly inhibited the response of guinea pig ileum to angiotensin.

The experiments described in this study of bromoacetyl oxytocin suggest that the compound, when radioactively labeled, will be a valuable marker for isolation and characterization of the specific neurohypophyseal hormone receptors from broken cell preparations both of amphibian

bladder and mammalian kidney. In the kidney, the analog bromoacetyl AVP would be a better choice since the affinity of AVP for kidney tissue, its target organ, is higher than that of oxytocin. Specificity can be enhanced by "differential labeling" (120); i.e., treatment of the adenylate cyclase preparation or purified plasma membranes with unlabeled BrOXY in the presence of AVT or oxytocin as a protector, removal of the hormone with urea as described, and treatment with radioactive BrOXY.

SECTION 2: Localization of Neurophysin Binding Site

Introduction

An apparently homogeneous protein was isolated from ox pituitary by van Dyke et al. in 1942 (172); this preparation had oxytocic and pressor activities in a ratio of close to 1:1. Later studies (173) showed that the preparation was actually a complex mixture of peptides and proteins from which the neurohypophyseal hormones, vasopressin and oxytocin, could be removed by dialysis against dilute acetic acid, electrodialysis, protein precipitation with trichloroacetic acid, and countercurrent distribution, all methods which do not cleave covalent bonds. The protein portion of the complex was named "neurophysin" (174) and consists of a family of proteins with similar biological properties. The neurophysin exhibits no oxytocic and pressor activities, but has the ability to specifically and reversibly bind oxytocin and vasopressin.

The procedures originally devised for isolation and purification of individual neurophysins from ox pituitary (175,176) gave rise to five or six similar proteins; these findings were revised when enzymatic degradation was found to occur during the isolation step (177-179). Subsequent studies, avoiding such conditions, have identified three neurophysins in the ox pituitary (I, II, and C) (180), three in pig (I, II, and III) (181,220), three (182,183) in rat,

and 2 in human (184). Varying numbers of neurophysins have been reported in pituitaries of other species (185). The application very recently of new techniques of protein purification, such as preparative polyacrylamide disc electrophoresis, and isoelectric focusing, has led to the isolation of highly purified neurophysins (186) and has revealed that earlier preparations still were heavily contaminated despite the prevention of enzymatic breakdown.

Sawyer proposed in 1961 (187), primarily on the basis of the close association of neurophysins with neurophypophyseal hormones during extraction, that the neurophysins function as "carrier proteins" for the neurohypophyseal peptides. According to the theory of neurosecretion proposed by Bargmann and the Scharrers (22,188), neurohypophyseal hormones are synthesized in hypothalamic nerve cell bodies and carried by axoplasmic transport to nerve terminals in the posterior pituitary, where they are stored and released. There is a great deal of evidence for the close spatial and temporal (23) association of the neurophysins and the hormones during these processes. First, the high disulfide content of the neurophysins (186) indicates that they are a major component of the histochemically identified "neurosecretory material" which is present along the mammalian hypothalamo-neurohypophyseal tract (189). Recently,

neurophysins have been isolated from the hypothalamus of several species and identified by starch gel electrophoresis (190). They have also been demonstrated immunochemically throughout the porcine hypothalamo-neurohypophyseal system (191,192) as well as in monkey and ox hypothalamus, in axons running through the hypothalamus, and in nerve terminals in the posterior pituitary (193). Neurophysins have been identified in the isolated neurosecretory granules of ox pituitary, which also contain oxytocin and vasopressin (178,194,195). Finally, the release of neurophysin has been shown to accompany release of vasopressin from dog, pig, and rat pituitaries, both *in vivo* and *in vitro* (196-200).

The neurophysin proteins in the above studies are not always specifically characterized, e.g, as bovine neurophysin I or II. Oxytocin and vasopressin are thought to be synthesized in the paraventricular and supraoptic nuclei, respectively, of the hypothalamus (201,202), and transported and stored in *separate* neurons (203,204). There is growing evidence for *in vivo* compartmentalization of a specific neurophysin with a given hormone. Dean *et al.* (205) showed that in isolated bovine neurosecretory granules neurophysin I is associated with oxytocin-rich granules, and neurophysin II with vasopressin-containing granules. Robinson *et al.* (206) found an increase in circulating bovine neurophysin I (by radioimmunoassay) as a result of stimuli thought to

release oxytocin (lactation, parturition) and sometimes those which cause vasopressin release (dehydration, hemorrhage) (207-209), while neurophysin II levels rose only during vasopressin-related events. In the neurosecretory granules of pig, a close correlation was found between distributions of lysine vasopressin and porcine neurophysin I and of oxytocin and porcine neurophysin II (210). Moreover, vasopressin-releasing stimuli cause a decrease of neurophysin I in the pig pituitary (211) and a six-fold increase in the plasma concentration of neurophysin I (192).

The fact that rats with neurogenic hereditary diabetus insipidus, which are unable to synthesize vasopressin (212,213), also lack a particular neurophysin (182,214) is further support for this hypothesis. Studies on biosynthesis of vasopressin in dogs has indicated that the hormone and its associated neurophysin may actually be synthesized through a common precursor molecule, or at least be under the same genetic control (23,215). As a note of caution it should be stated that bovine NP I has been located in <u>both</u> the supraoptic and paraventricular nuclei of ox hypothalamus (193) and that both porcine NP I and porcine NP II have been located in the porcine supraoptic nucleus (192); however, there is probably some overlap in hormone

synthetic sites as well, i.e., oxytocin and vasopressin synthesis occur at both sites, with a predominance in one or the other nucleus (e.g.,216).

The structure of the isolated neurophysins has been extensively investigated. There is evidence that in vivo they exist as acidic lipoproteins (217), so that the studies described here may actually be on the apoprotein moiety. The porcine proteins have been shown to exist in different polymerizing forms varying in relative proportion with protein concentration (218), and this phenomenon of aggregation (179,219,220) complicated determination of molecular weights. Elucidation of the amino acid sequence of bovine neurophysin II (221) unequivocally demonstrated its molecular weight to be 10,041. The primary structure of porcine neurophysin I has also been determined (222), and its molecular weight is 9356. The partial sequence of bovine NP I has been reported (223) and the structures of the three proteins are shown in Fig. 22. Localization of the seven disulfide bridges of bovine neurophysin II led to definition of its complete covalent structure, shown in Fig. 35 (224).

An understanding of the nature of the interactions between the neurophysins and the neurohypophyseal hormones is important both as a guide to their relationship in vivo

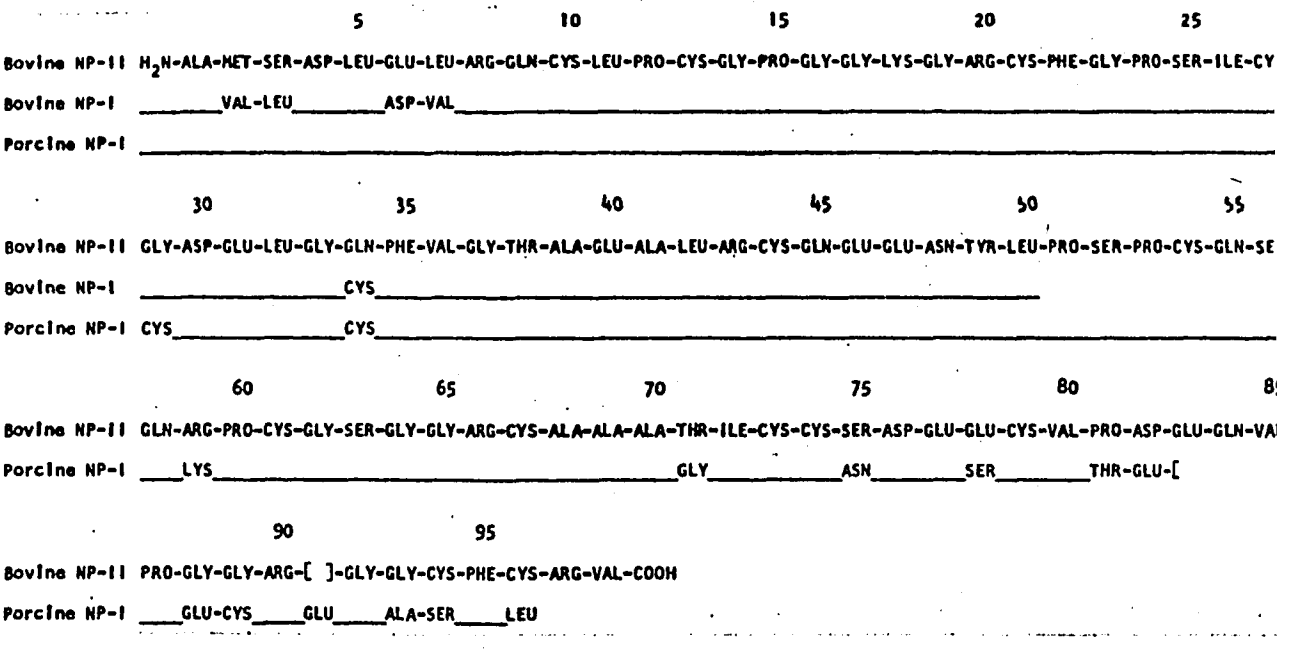


Fig. 22. Amino acid sequence of bovine neurophysin II (221), porcine NP I (222), and partial sequence of bovine NP I (223). Only those residues in bovine and porcine NP I that differ from NP II are shown.

and as a general model for protein-peptide interactions. Determination of the structure of bovine neurophysin II set the stage for localization of its hormone-binding site, with the aid of evidence gained previously concerning features of the hormones necessary for binding, the number of binding sites per neurophysin monomer, and the affinities for each hormone.

The neurophysin-neurohypophyseal hormone bond is not covalent in the isolated complex, as illustrated by the experiments of Acher et al., mentioned above. The pH-dependence of formation of the ionic complex was first demonstrated by Ginsberg and Ireland (225) for interaction between oxytocin, vasopressin, and neurohypophyseal extracts of ox and rabbit; the highest amount of binding was found to occur between pH 5.2 and 5.8 (i.e., 66% of hormone is bound at pH 5.8 as compared to 27% at pH 7.4). It was found by Stouffer et al. (226) that deamino oxytocin, in which the α-amino group is replaced by a hydrogen atom, does not bind to bovine neurophysin under conditions where oxytocin does. The combination of these results, in conjunction with titration studies (175), indicated the importance of the α-NH_2 group of oxytocin for formation of the ionic complex, and suggested that the bond is formed between the protonated amino group and an unprotonated carboxyl on neurophysin. In the above studies, hormone binding

was measured by gel filtration or equilibrium dialysis. More recent experiments, also measuring binding by gel filtration, showed that shortening of the hormonal peptide chain at the C-terminal end does not affect binding to bovine neurophysin II, while extending the N-terminus (i.e., addition of leucine, mono-, di-, or tri-glycine or phenylalanine residues) eliminates binding. This indicates once more the importance of the N-terminal amino group of vasopressin for binding, as well as the necessity for its precise location with respect to the rest of the hormone (227).

Although the α-amino group is a feature of the hormone essential to formation of the electrostatic peptide-protein complex, other factors also enter. In early studies Breslow and Abrash (175) showed that modifications of the two-position of oxytocin (substitution with D-tyrosine or isoleucine) eliminated binding, and modification of the three-position decreased binding. These results indicated hydrophobic interactions between residues in positions two and three of the hormone and groups on the protein. In this study, calculation of the energy contribution of the α-NH_2 group to the complex indicated that the electrostatic bond occurred in a non-polar environment. Later experiments (179), monitoring (by changes in proton equilibria and ellipticity) the interaction with bovine neurophysin II of a series of peptides with sequences related to the 1-3

positions of oxytocin and vasopressin, showed that the qualitative features of the hormone-neurophysin interaction are preserved. This reinforced the importance of residues two and three, and also revealed that the side-chain in position 1 plays an important role in peptide or hormone binding to neurophysin. Circular dichroism and proton titration studies of mixtures of native or nitrated (nitro-tyrosine) bovine neurophysin II confirm that peptides containing only the first three residues of the hormone contribute almost two-thirds of the binding free energy of the hormones, and that half of this energy is contributed by the cooperative binding of residues one and two (228). Moreover, binding interactions at the side chain of residue one are found to be hydrophobic, and an aromatic, hydrophobic side chain at residue two is necessary for binding. Proton NMR studies of bovine NP II interactions with hormone and model peptides again demonstrate (by line broadening of the protons of particular residues) the importance of residues one and three of the hormone (229).

As to other hormonal characteristics in relation to binding, iodination of the tyrosyl residue in LVP and oxytocin eliminated binding, in support of the above results (230). Breslow and Walter found, measuring binding by equilibrium dialysis, that substitutions of the residues in positions five and nine of oxytocin had minor effects

on binding, while substitution of the Gln in position four by ornithine slightly increased the affinity of the analog for bovine NP II (231). These results are in substantial agreement with the recent deuteron magnetic resonance studies by Glasel et al. (232) of the interaction between oxytocin and the bovine neurophysins, which indicate that the acyclic tail portion of the hormone retains a considerable degree of freedom even when the hormone is bound to neurophysin.

In addition to hormone structural features which contribute to binding, the affinity of the protein for the peptides and the number of binding sites per neurophysin monomer have been investigated. Before neurophysin molecular weights were accurately determined, one "mole" of neurophysin was reported to bind seven moles of oxytocin and four moles of arginine vasopressin (233); three moles of vasopressin; two moles of vasopressin plus one mole of oxytocin; two moles of oxytocin (234); or two moles of oxytocin per 25,000g protein (175). Binding was assessed by bioassay of the complex or equilibrium dialysis with tritiated hormones.

The discrepancies have been somewhat resolved by using more purified proteins and the correct molecular weight in the calculations. Breslow and Walter (231), with ^{14}C-labeled hormones and equilibrium dialysis, found that bovine NP II

has one binding site per monomer for LVP at physiological pH (7.38), that oxytocin is bound with the same or greater affinity, and that binding of the two is competitive. The binding constant for LVP to neurophysin II was calculated as 8.4×10^{3}/M and for oxytocin as 1.2×10^{4}/M. At the protein concentrations used in this study, bovine neurophysin II (at pH 8.1) consists of a 2:1 molar mixture of monomer and dimer (179); the finding of a single binding constant for each hormone indicates either that the protein species have equal affinities for the hormones, or that the equilibrium in the absorbence of hormone is highly in favor of the species with the higher affinity for hormone. It should also be noted that the difference in the binding constant calculated at pH 7.38 (1.2×10^{4}) compared to that at pH 5.8 (1.4×10^{5}) (175) again indicates the importance of the protonated α-amino group of the hormone for binding. Breslow et al. have recently confirmed the finding of a single binding site for LVP on bovine NP II, similar to that for oxytocin, by circular dichroism and proton titration studies (228). These latter results, however, allow for the possibility of a second, lower affinity, LVP binding site.

Camier et al. (230) also studied binding of neurophysin to tritiated neurohypophyseal hormones using equilibrium dialysis, with an NP I or II concentration of 50μM (so that the predominant molecular species is the

monomer) and a hormone concentration of 5 - 500μM. Based on their data, Scatchard plots indicate that neurophysins I and II bind oxytocin and LVP with equal affinity, and that at pH 5.7 one oxytocin or two lysine vasopressin molecules are bound per protein monomer. Each compound inhibits the binding of the other in a non-competitive manner, suggesting that the hormones interact with different binding sites on the same neurophysin molecule. The dissociation constant calculated for oxytocin and NP I at pH 5.7 is 22.9 μM; for LVP and NP I, 25.5μM. At pH 7.0, the LVP dissociation constant is 71.7μM. Values for NP II are similar.

A comparison of the binding of tritiated LVP to free or to agarose-bound bovine neurophysin II revealed that in both cases there are two populations of binding sites, one with an association constant of $10^7/M$, and one with a constant of $10^4/M$ (235). The fraction of the first population varied with varying conditions when free neurophysin was studied, but remained unchanged in the case of bound neurophysin. It was suggested that there are two types of heterogeneity in the binding sites of neurophysin II, one due to the existence of two different molecular species with different binding constants, and the other which could be accounted for by differences in the thermodynamic states of molecules within each population of binding sites.

The number of hormone-binding sites per neurophysin monomer is still questionable, and affinities, calculated using the number of sites, vary with the experimental conditions. However, the electrostatic bond between the α-amino group of oxytocin or vasopressin and an unprotonated carboxyl of neurophysin is supported by the data and serves as a starting point for localization of the hormone-binding site by affinity labeling (see Section 1). In this study we attempt to convert the ionic bond between bovine neurophysin II and radioactive hormones to a covalent one, and then to locate the labeled peptide sequence in the native protein.

Materials

[9-^{14}C-1-Glycinamide]oxytocin, ([^{14}C]oxytocin) and [9-^{14}C-1-glycinamide, 8-arginine]vasopressin ([^{14}C]AVP), each with a specific activity of 30mC/mmole, were the same as those for which we reported the syntheses earlier (236). Labeled oxytocin was kept in glass-distilled water and [^{14}C]AVP in 0.25% acetic acid containing 0.1% chlorobutanol. [2-^{3}H-tyrosine, 8-lysine]vasopressin, ([^{3}H]LVP), 12 C/mmole, was supplied by Dr. Paul Cohen. This hormone had 228U/mg pressor activity and was radiochemically pure. Aliquots were stored at -70C in 0.1% formic acid and thawed just before use. In the general method adopted for these experiments, 1.0 mg of unlabeled hormone was mixed with 0.01 mg [^{14}C]hormone to give a calculated specific activity of 0.30 mC/mmole. For the [^{3}H]LVP, 1 mg unlabeled hormone was mixed with 0.16 µg labeled hormone to give a calculated specific activity of 1.9 mC/mmole. Non-radioactive oxytocin (135), arginine vasopressin (AVP) (237), and lysine vasopressin (LVP) (238) were synthesized by the solid phase method. Labeled and unlabeled hormones were tested for avian vasodepressor activity on conscious chickens (148) according to the method of Coon (149) or for rat pressor activity (139) and the peptides exhibited the potencies recorded in the appropriate references above.

Bovine neurophysin II (NP II) was prepared from an acetone powder of bovine posterior pituitary gland (Parke-Davis, Lot 284346).

Dithiothreitol (DTT) was purchased from Calbiochem; phenylisothiocyanate from Beckman Instruments, iodoacetamide (recrystallized from water before use) from Baker Chemical Co., and α-chymotrypsin and carboxypeptidases A and B from Worthington Biochemical Corp. Dicyclohexylcarbodiimide came from Fluka, and 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide HCl from City Chemical Corp. and from Sigma. The latter chemical was stored at -20C. Sephadex was purchased from Pharmacia and IRC50 from BioRad (BioRex 70).

Methods

High voltage paper electrophoresis was carried out on strips of Whatman 3MM paper (3x60cm). Conditions were 3000V for 1 hr using either a 0.5% pyridine-5% acetic acid buffer, pH 3.5, or a 10% pyridine-0.3% acetic acid buffer, pH 6.5, on an HV 5000A Savant apparatus. Radioactivity on paper electrophoretograms was detected on a Packard model 7201 chromatogram scanner (10% efficiency for ^{14}C, ~4% efficiency for ^{3}H). Non-radioactive compounds were detected by spraying with a cadmium-ninhydrin solution (50 ml 1% ninhydrin in acetone mixed with 5 ml of a solution of 88 mM cadmium acetate in 6M acetic acid). Radioactivity in solution was detected on a Packard model 3003 liquid scintillation spectrometer (77% efficiency for ^{14}C, 65% for ^{3}H) using counting solution consisting of 125g naphthalene, 12g PPO (2,5 diphenyloxazole) and 0.3g POPOP (1,4-bis [2-(5-phenyloxazolyl)] benzene) per liter dioxane.

Amino acid analyses were carried out according to the general method of Spackman *et al.* (142): peptides were hydrolyzed *in vacuo* in 6N HCl containing 0.2% phenol and 0.1% mercaptoacetic acid (239) for 22 hr at 110C, dried over NaOH, and analyzed on a Beckman 120C amino acid analyzer equipped with a 4-5 mV range recorder for routine peptide and protein hydrolysates. Hydrolysis of phenylthiohydantoin

amino acids was carried out *in vacuo* in 1.0 ml 0.1 N NaOH for 12-14 hr at 120C. The samples were dried over H_2SO_4, and 0.25 ml 0.1 N HCl was added to the residue before analysis.

Polyacrylamide disc gel electrophoresis was run according to the method of Ornstein (240) and Davis (241) using a 7.5% gel, running pH of 9.5 in a Canalco apparatus. Gels were run at ~ 2.5 ma/gel and 600V at room temperature.

Purification of Bovine Neurophysin II

Neurophysin II was extracted from the acetone powder and purified according to the methods of Hollenberg and Hope (176) and Breslow (175). It was assayed for residual avian vasodepressor activity and its purity was assessed by polyacrylamide disc gel electrophoresis. Gels were removed from the tubes, stained by treatment for 30 min with 12.5% trichloroacetic acid (TCA) followed by Coomassie brilliant blue (242) (1 volume of a 1% aqueous solution/ 20 vol 12.5% TCA) for 1-2 hr and destained in 12.5% TCA. Gels were stored in 10% TCA in the dark; intensity of the bands increased for 48 hr.

Formation and Dissociation of the Neurophysin II-Neurohypophyseal Hormone Complex

NP II and unlabeled oxytocin or [8-arginine] vasopressin in a 1:1 molar ratio were dissolved in a small volume of 0.82M pyridine-acetate buffer, pH 5.8, applied to a Sephadex G75 column (3x37cm) equilibrated with the same buffer, and

were eluted with that buffer. Fractions of 5.5ml were collected, and protein peaks were located by the Lowry method (147). Fractions corresponding to these peaks were pooled, concentrated to a small volume, and lyophilized. An aliquot of material from the peak corresponding to the elution volume for neurophysin was assayed for hormonal activity. The rest of this material was dissolved in a small amount of 0.1N formic acid (pH 2.5) and applied to a Sephadex G25 column (100x0.9cm) equilibrated and eluted with the same buffer, in order to dissociate the complex (243). Again material from the peak corresponding to neurophysin (in the void volume) was assayed for hormonal activity.

In the case of the $[^{14}C]$- and $[^{3}H]$-labeled hormones, an aliquot of radioactive hormone was mixed with the analogous unlabeled hormone and the hormone-neurophysin II complex was formed and chromatographed as described. Peaks were located by liquid scintillation counting of 0.01ml aliquots of alternate fractions or by the Lowry method, and the extent of complexation and dissociation could be judged by the amount of radioactivity present in each fraction after the peak fractions were pooled and lyophilized.

Formation of a Covalent Neurophysin II-$[^{14}C]$ Neurohypophyseal Hormone Complex

A. Original experiments were based on the reported hydrophobic nature of the specific non-covalent neurophysin-

neurohypophyseal hormone complex. A neurophysin II-[^{14}C]oxytocin complex from the Sephadex G75 column (5 mg dry weight, ~1.5 mmole oxytocin, assuming the non-covalent complex forms on a 1:1 molar basis) was suspended in 0.5 ml freshly distilled methylene chloride. Dicyclohexylcarbodiimide (30.8 mg) was dissolved in 0.3 ml methylene chloride and added to the complex. The reaction mixture was stirred for 24 hr at room temperature.

Pyridine acetate (0.82M, pH 5.8) was added and the mixture was flash evaporated to a small volume, and chromatographed on the G75 column described above. The neurophysin peak was located by radioactivity measurements (0.02 ml from each 5.5 ml fraction), pooled, and lyophilized. This lyophilized material was subjected to gel filtration on Sephadex G25 (100x0.9cm) in 0.1 N HCOOH. Peaks were located by radioactivity and the Lowry method.

B. Further experiments used [^{14}C]oxytocin and a water-soluble carbodiimide (1-ethyl-3(3-dimethylaminopropyl)-carbodiimide HCl). Non-covalent NP II-[^{14}C]oxytocin complex (20 mg, ~2 μmole oxytocin) was dissolved in 0.5 ml of 0.82M pyridine acetate buffer, pH 5.8. Water-soluble carbodiimide (WSC) (50 mg, ~200 μmole) in 0.2 ml of the same buffer was added and the mixture stirred at room temperature for 16 hr. Then 30 mg WSC in 0.1 ml buffer was added and the reaction left for 10 hr at room

temperature. The mixture was chromatographed on the Sephadex G75 columndescribed. The radioactive peak eluting at the position of neurophysin was pooled, lyophilized, and applied in a small volume of 0.1 N HCOOH to the G25 column described. Peaks were again located by radioactivity measurement and the Lowry method.

C. The experiments described above were also carried out using [^{14}C]AVP. In the course of these experiments it was noted that the original non-covalent complexation on Sephadex G75 was superfluous, since the amount of hormone bound covalently was always much less than that bound non-covalently. NP II (2.0 mg, 0.2 μmole) and 0.01 mg (0.01 μmole) [^{14}C]AVP were dissolved in 0.2 ml 4M urea (pH adjusted to 6.1 with dilute HCl) and the mixture stirred for 30 min. 100 mg WSC was added and the reaction was carried out at room temperature for 20 hr. A small amount of 0.1 N HCOOH was added, and the mixture applied to the Sephadex G25 column (100x0.9cm) described. Peaks were located by radioactivity measurement, and fractions corresponding to various peaks were pooled and lyophilized.

Characterization of NP II- [^{14}C]AVP Covalent Complex

Several experiments were carried out to determine optimal conditions for formation of the covalent

NP II- [^{14}C]AVP complex and to test its specificity. Using the same conditions as in the preliminary experiments (i.e., 1.0 mg of NP II, 2.0 mg of WSC [10-fold molar excess], total volume 0.2 ml), the ratio of unlabeled hormone to NP II was increased while the amount of labeled hormone was kept constant, in order to determine the effect of hormone concentration on the binding of radioactivity. In addition in separate experiments angiotensin II was added (at a molar ratio of 1:100, NP II to peptide) as a "non-specific" peptide. Next, using the optimal ratio of NP II to unlabeled AVP, as determined in the first series of experiments (i.e., a 1:10 molar ratio of NP II to hormone), the amount of carbodiimide was increased over a range of 0.02 to 200 mg per mg of NP II. In the next set of experiments, the above parameters remained constant and the effect of the volume of 4M urea on binding was studied. Finally, the pH of the solution was varied over the range 5.0 to 7.0. In all cases, the percent of radioactivity bound to neurophysin II was calculated by comparison of radioactivity in the pooled and lyophilized "neurophysin" peak to the total radioactivity recovered (approximately 85-90% of that applied).

Reduction and Alkylation of the NP II- [^{14}C] AVP Covalent Complex

The covalent complex was reduced and alkylated according to the method of Liu and Meienhofer (244). In a typical reaction, 20 mg of NP II- [^{14}C]AVP complex was dissolved

in 3.0 ml 0.1M phosphate buffer, pH 8.2. N_2 was bubbled through for 15 min, and a solution of DTT in phosphate buffer (1.9 ml, 3.8 mg, 24 μmole) was added. The mixture was kept under N_2 at room temperature for 90 min, and 2.2 ml of iodoacetamide in phosphate buffer (22 mg, 12 μmole) was added. After 15 min under N_2 atmosphere, glacial acetic acid was added to adjust the pH to 4, and the mixture was concentrated to a small volume, applied to a G25 column (100x0.9cm) and eluted with 0.1 N HCOOH. Fractions of 2.3 ml were collected, and 0.01 ml aliquots were added to 5 ml counting solution for liquid scintillation counting. The fractions corresponding to the alkylated complex were pooled and lyophilized.

Chymotrypsin Digestion of NP II-[^{14}C]AVP Covalent Complex

In a typical procedure, 20 mg of S-alkylated NP II-[^{14}C] AVP complex was dissolved in 10 ml 0.2 N $NaHCO_3$, pH 8.0. α-Chymotrypsin (0.1 mg/ml in 0.2 N $NaHCO_3$) was added to give an enzyme-to-substrate ratio of 1:300 (0.67 ml). The reaction mixture was stirred at 37C for 2.5 hr. The pH was adjusted to 4.0 with 50% acetic acid, the mixture concentrated to a small volume and applied to a G25 column (100x0.9) equilibrated with 0.1 N HCOOH and eluted with the same solvent. Fractions of 2.3 ml were collected, and 0.01 ml aliquots of alternate fractions were counted. In addition,

protein was detected by the Lowry method. Fractions corresponding to all peaks were pooled and lyophilized.

Purification and Characterization of Radioactive Peaks Resulting from Chymotrypsin Digestion

Aliquots from all peaks, radioactive or unlabeled, from the gel filtration of the chymotryptic digest, were subjected to analytical high-voltage electrophoresis, 3000V for 1 hr, at pH 6.5 and 3.5. The amount used was determined on the basis of total counts in the fraction or the estimated amount of protein present. Unlabeled material was detected with cadmium-ninhydrin spray, and labeled material on the chromatogram scanner. The remaining radioactive material was then purified by partition and ion-exchange chromatography. For partition chromatography, a column of Sephadex G25 Fine (100x0.9) was equilibrated with the lower and upper phases of the solvent system n-BuOH/ethanol/pyridine/0.1 N acetic acid (4:1:1:7). The column was eluted with upper phase. Fractions of 2.2 ml were collected, and 0.01 ml aliquots of alternate tubes were counted. The column was washed with 0.82M pyridine acetate, pH 5.5.

Ion exchange chromatography was carried out on an IRC50 column (0.9x40cm). The resin was prepared in the H^+ form as described for chromatography of AVP (245). Elution buffer was 0.65M NH_4OAc, pH 6.5. Fractions of 1.0 ml were collected and 0.01 ml aliquots of alternate fractions

were counted; those containing the radioactive peptides were pooled, concentrated to a small volume, and lyophilized.

Further separation of radioactive peptides was achieved by high-voltage paper electrophoresis and elution with ~2.0 ml water or dilute acetic acid. Radioactive peptides separated by these methods were identified by amino acid and N- and C-terminal analyses.

Carboxypeptidase Digestion

Digestion with carboxypeptidases A and B was carried out by the method of Liu et al. (246). Peptide (0.048 μmole, judged on the basis of amino acid analysis)was dissolved in 0.4 ml 0.2 N $NaHCO_3$. To this solution was added 0.01 ml of carboxypeptidase B suspension, and 0.02 ml of a solution of carboxypeptidase A (0.1 ml enzyme suspension dissolved in 1.0 ml buffer containing 0.8 ml 2M NaCl and 0.2 ml 0.2M Na_2HPO_4). The reaction mixture was stirred at 37C and aliquots were taken at 30 min and 3 hr. Enzyme activity was stopped by addition of 6N acetic acid and the unhydrolyzed reaction mixture was placed on the amino acid analyzer.

Edman Degradation

Peptides were subjected to one cycle of phenylisothiocyanate degradation by a modified three-stage procedure (247). The amount of peptide was judged by amino acid analysis, and an appropriate aliquot (i.e., 0.7 μmole) was

used. The resulting thiazolinone was converted to the phenylthiohydantoin (PTH) amino acid by treatment with 0.2 ml of 1.0N HCl at 80C for 10 min. The PTH amino acids were identified by hydrolysis and amino acid analysis, and by "subtractive" amino acid analysis (248).

Formation of a Covalent NP II-[^{3}H]LVP Complex

The same general procedure used for covalent linkage of [^{14}C]AVP to neurophysin was used with [^{3}H]LVP. 10 mg NP II was mixed with 1 mg unlabeled LVP and 0.01 ml ($\sim 10^6$cpm) [^{3}H] LVP in 0.2 ml 4M urea, pH 6.0. 200 mg solid 1-ethyl-3 (3-dimethylaminopropyl)carbodiimide HCl was added after 15 min. The mixture was stirred for 24 hr at room temperature, then applied to a G25 column (100x0.9cm) and eluted with 0.1 N HCOOH. Fractions of 2.3 ml were collected and radioactivity measured in alternate fractions. The fractions corresponding to the NP II-[^{3}H]LVP complex (elution volume of NP II) were pooled and lyophilized for use in further studies. Two sets of conditions were used in these binding reactions. When the radioactive pattern of the chymotryptic digest was to be investigated, neurophysin was reacted with [^{3}H]LVP alone; so that only a very small amount of hormone but high amount of radioactivity was bound. When peptides were to be isolated and identified, the reaction was carried out with a mixture of [^{3}H]and unlabeled LVP as described, in order

to secure a higher amount of labeled peptide material. If necessary, the electrophoretogram of the chymotryptic digest in the latter case was compared with that of the former, to verify position of radioactive peaks.

Reduction and Alkylation of the NP II-[^{3}H]LVP Complex

Reduction of the NP II-[^{3}H]LVP covalent complex with DTT and alkylation of the free sulfhydryls with iodoacetamide was carried out as described for the NP II-[^{14}C]AVP complex.

Digestion of the NP II-[^{3}H]LVP Complex by α-Chymotrypsin

Digestions were carried out using conditions identical to those used for the NP II-[^{14}C]AVP complex, except for the times of incubation and the enzyme-to-substrate ratios. In preliminary experiments the ratio was 1:300 and incubation time was 2.5 hr, but later experiments used a ratio of 1:3, and incubation times 1, 2, or 10 hr.

Results

Characterization of Neurophysin II

The purity of NP II was judged by disc gel electrophoresis. Fig. 23 shows a gel with NP II after a third DEAE-Sephadex run. After staining with Coomassie blue, a method which can detect minor impurities (186), only one band is visible, even when the gel is overloaded (i.e., 0.2 mg protein). Hormonal (avian vasodepressor) activity on a sample of NP II at this stage of purification was assayed at 0.094U/mg.

Characterization of Non-Covalent Neurophysin II-Neurohypophyseal Hormone Complexes

Table 1 lists avian vasodepressor and rat pressor activities of neurophysin II before and after formation of the non-covalent oxytocin or AVP complexes, as well as activity after dissociation of the non-covalent complex by chromatography at acid pH. These results demonstrate that non-covalently bound hormone can be nearly completely dissociated from neurophysin II by this method. Fig. 24 depicts dissociation of an NP II-[^{14}C]AVP non-covalent complex by the same procedure, indicating that radioactive hormone does not bind non-specifically to the protein after chromatography in 0.1N HCOOH. [^{14}C]oxytocin and [^{3}H] LVP gave the same results. Therefore, radioactive hormone remaining associated with NP II after chromatography on Sephadex G25

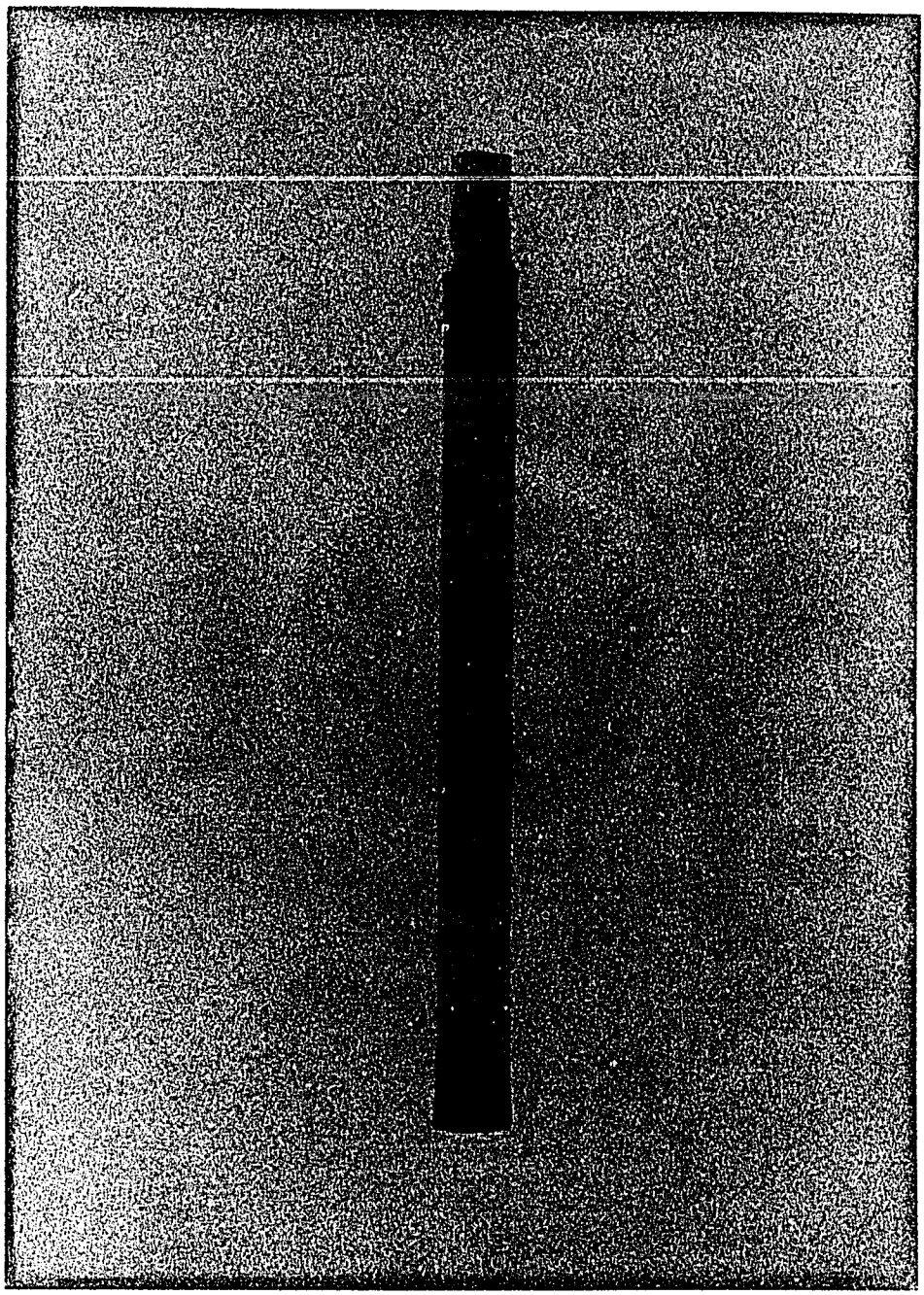


Fig. 23. Disc gel electrophoresis of bovine NP II purified by ion exchange chromatography on DEAE Sephadex (3 runs). For details of electrophoresis and staining with Coomassie Brilliant blue, see text. Direction of run is toward anode, at bottom.

Table 1. Formation and Dissociation of Neurophysin II-Neurohypophyseal Hormone Complexes[a]

	Oxytocin Complex	AVP Complex
NP II	0.094	0.96
Complex	14.2	6.33
NP II after dissociation of complex	0.040	0.88
μMoles hormone/ μMole NP II after dissociation[b]	8×10^{-4}	2.2×10^{-2}

[a]Samples were assayed for avian vasodepressor (oxytocin) or for rat pressor (AVP) activity (for details see text). All values are in units/mg. The NP II-hormone complex was purified by gel filtration on a Sephadex G75 column at pH 5.8. The complex was dissociated by gel filtration on a G25 column in 0.1N formic acid (pH 2.5).

[b]Calculated assuming oxytocin has 500U/mg avian vasodepressor activity and AVP 400U/mg rat pressor activity.

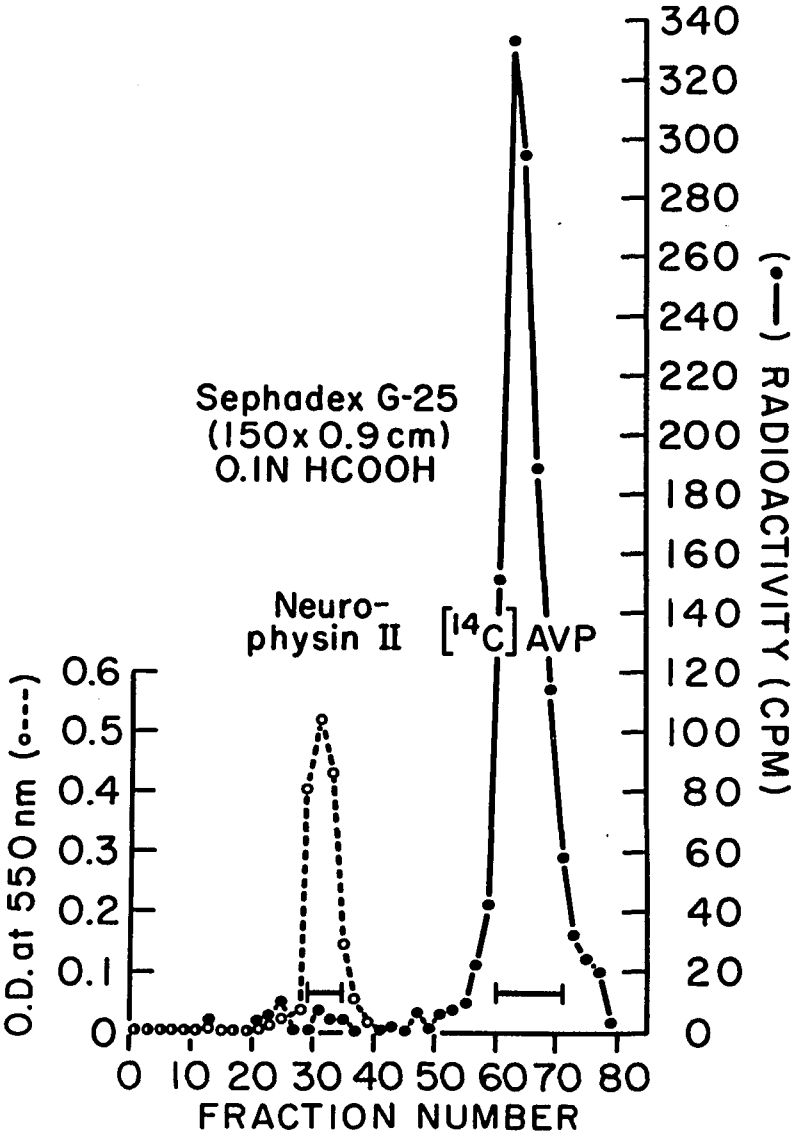


Fig. 24. Dissociation of NP II- $[^{14}C]$AVP complex by gel filtration on Sephadex G25 in 0.1N formic acid, pH 2.5.

in formic acid was considered to be covalently bound.

Formation of a Covalent Neurophysin II-[^{14}C]Neurohypophyseal Hormone Complex

Methods A and B gave either no covalent binding or binding of 1-2% of the radioactive material. The use of AVP instead of oxytocin was suggested by evidence (205,206) that AVP and neurophysin II are found in close association *in vivo*. A significant amount of AVP did not bind covalently to NP II, however, until the reaction was carried out in 4M urea, at which concentration NP II is not denatured, nor is the protein conformation significantly affected. Fig. 25 shows the gel filtration pattern of the covalent NP II-[^{14}C]AVP complex. Peak I (fractions 31-42) in the position of NP II, also contains covalently bound [^{14}C]AVP. Peak II (fractions 58-65) is a polymer of [^{14}C]AVP, formed during the carbodiimide reaction, as was demonstrated by reacting [^{14}C]AVP alone with carbodiimide. Peak III (fractions 71-79) is unreacted [^{14}C]AVP.

Conditions for Formation of Covalent NP II-[^{14}C]AVP Complex

The specificity of formation of the covalent complex of NP II and [^{14}C]AVP was tested by diluting [^{14}C]AVP with varying amounts of unlabeled AVP. Fig. 26 shows that the amount of labeled AVP bound decreases linearly as unlabeled AVP is increased in the reaction mixture. When labeled AVP was diluted with angiotensin II, which does not bind to NP II, the amount of label bound was essentially the same as if

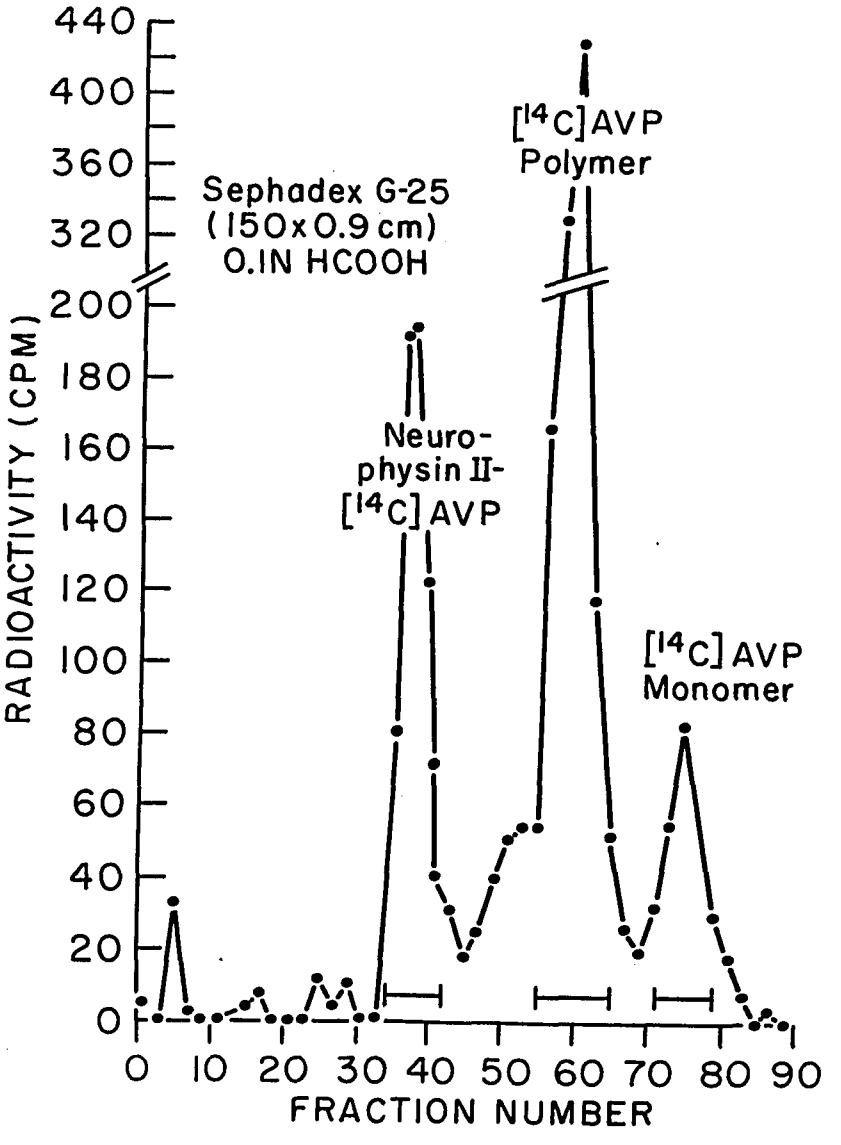


Fig. 25. Gel filtration elution pattern of reaction mixture for formation of covalent NP II- [^{14}C] AVP complex, on Sephadex G25 in 0.1N formic acid.

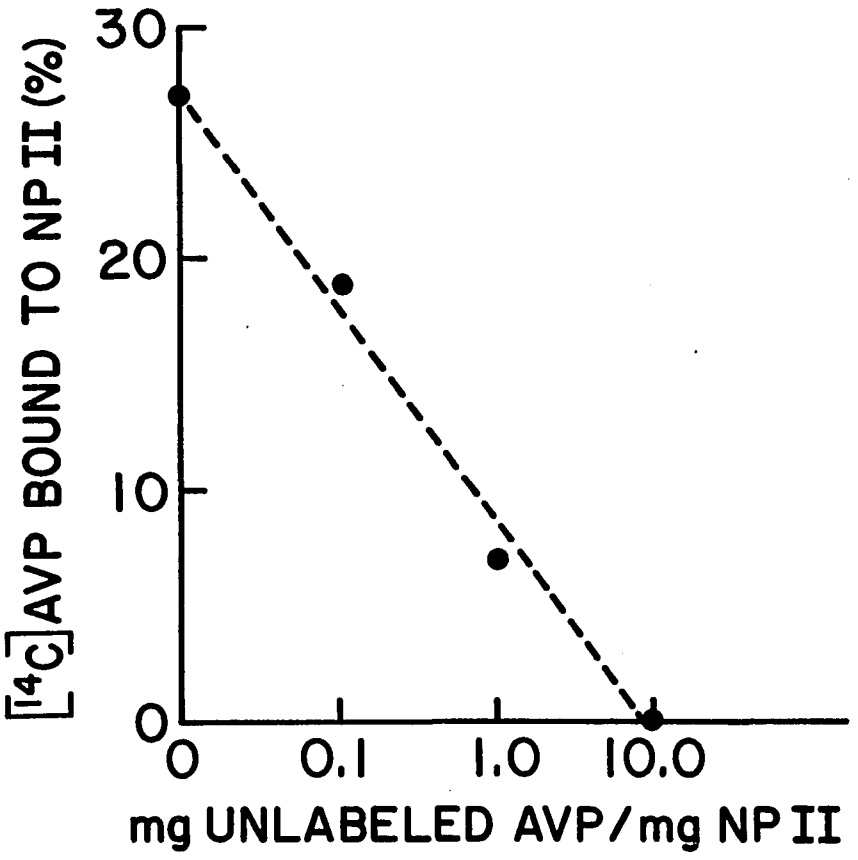


Fig. 26. Percentage of covalent binding of [^{14}C] AVP to NP II as a function of unlabeled AVP in the reaction mixture. For these experiments, 1.0mg NP II was reacted with 0.01mg [^{14}C] AVP and varying amounts of unlabeled hormone. Percent of bound radioactivity was measured by gel filtration in 0.1N formic acid. For details see text; each point represents the average of two experiments.

no unlabeled peptide was present. Another specificity experiment was the increase in amount of carbodiimide, which, up to a point, increased the amount of covalent complex formed (Fig. 27). The amount of [^{14}C]AVP covalently bound seemed to level off somewhat between 20-100 mg carbodiimide per mg NP II, and then decreased sharply as more carbodiimide was used. Volume of the reaction mixture had little effect on formation of the covalent complex, and the pH optimum for binding was 6.0-6.5 (Fig. 28). The results of these experiments indicated that optimal conditions for binding of AVP to NP II (not for binding the highest amount of radioactivity) were the following (typical example): 10 mg NP II, 10 mg unlabeled AVP and 0.2 ml (0.02 mg) [^{14}C]AVP were mixed in 2.0 ml 4M urea, adjusted to pH 6.0 before use. After 30 min, 200 mg solid 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide HCl was added. The reaction was stirred for 24 hr at room temperature, the pH adjusted to 3.0 with 0.1 N HCOOH, and gel filtration carried out as described. These experimental conditions were used for identification of the location of the covalently bound hormone.

Reduction and Alkylation of NP II-[^{14}C]AVP Covalent Complex

The NP II-[^{14}C]AVP covalent complex was reduced with DTT and gave a single radioactive peak on gel filtration, with an elution volume of 55.2 ml. Amino acid analysis showed that all half-cystine was present as S-carboxylmethyl cysteine.

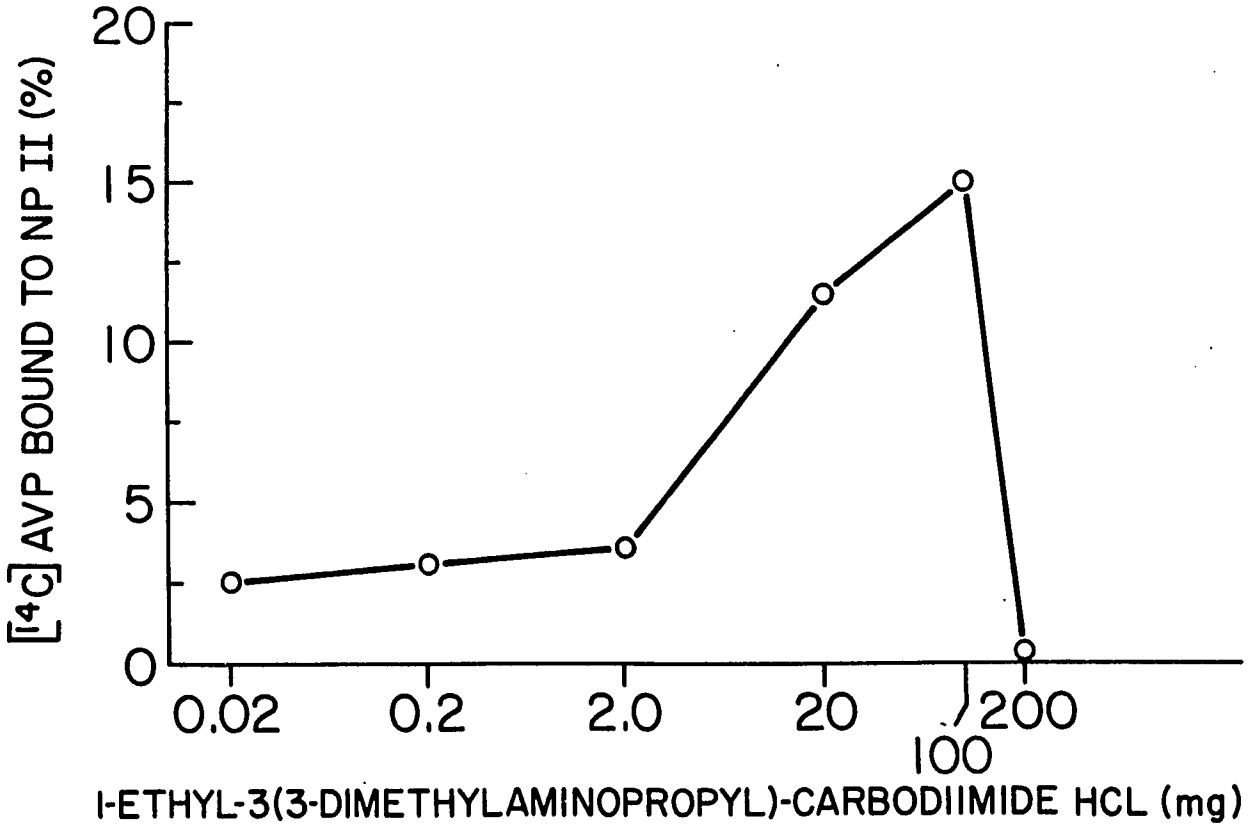


Fig. 27. Percentage of covalent binding of [^{14}C] AVP to NPII as a function of water-soluble carbodiimide concentration. One mg of NP II, 1.0mg unlabeled AVP, and 0.01mg [^{14}C] AVP were reacted with varying amounts of carbodiimide and chromatographed as described in the text; each point represents the average of two experiments.

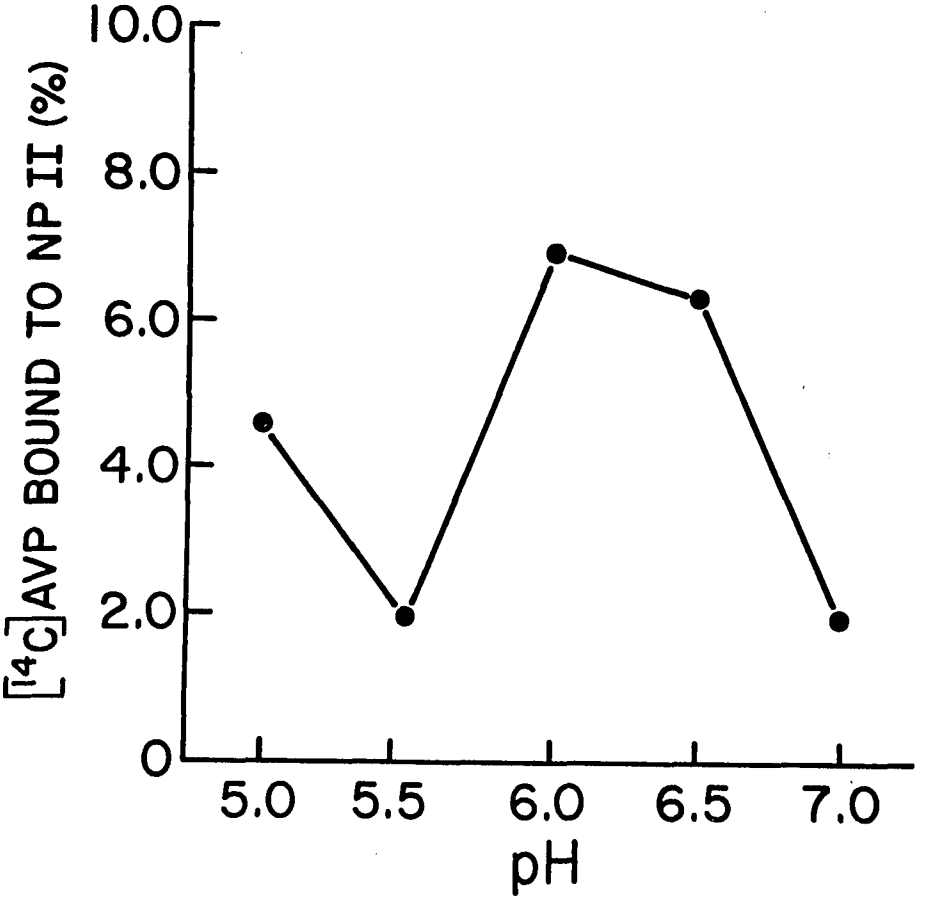


Fig. 28. Percentage of covalent binding of [^{14}C] AVP to NP II as a function of pH of the reaction mixture. For details of reaction and chromatography, see text and legends to Figs. 26 and 27.

Digestion of the NP II-[^{14}C]AVP Covalent Complex by Chymotrypsin and Identification of Radioactive Products

Gel filtration of the chymotrypsin digest of the NP II-[^{14}C]AVP covalent complex gave the pattern shown in Fig. 29. Each peak, whether located by radioactivity or Folin color, contained several peptides as demonstrated by high-voltage electrophoresis, but attention was focused on the main radioactive peak, fractions 54-69, which contained little protein. An aliquot of this material on high-voltage electrophoresis at pH 3.5 and 6.5 separated into two detectable radioactive peaks, neither of which corresponded to [S,S'-Cam,^{14}C]AVP. Cadmium-ninhydrin spray indicated several unlabeled peptides in the pooled fractions as well. It was noted that after storage at -20C an aliquot of the same material gave only one peak (the less basic) on high-voltage electrophoresis. Further purification of the main radioactive peak from the enzyme digest by partition chromatography was unsuccessful, and the material had to be washed off the column. However, ion-exchange chromatography on IRC50 using the solvent system for AVP (245) gave the pattern shown in Fig. 30. The peak eluting at fractions 10-20 corresponded to high-voltage electrophoresis peak I (the more basic peak) and that at fractions 21-38 to peak II on electrophoresis. Amino acid analysis of an aliquot of material from pooled fractions 21-38 indicated that it did not contain enough

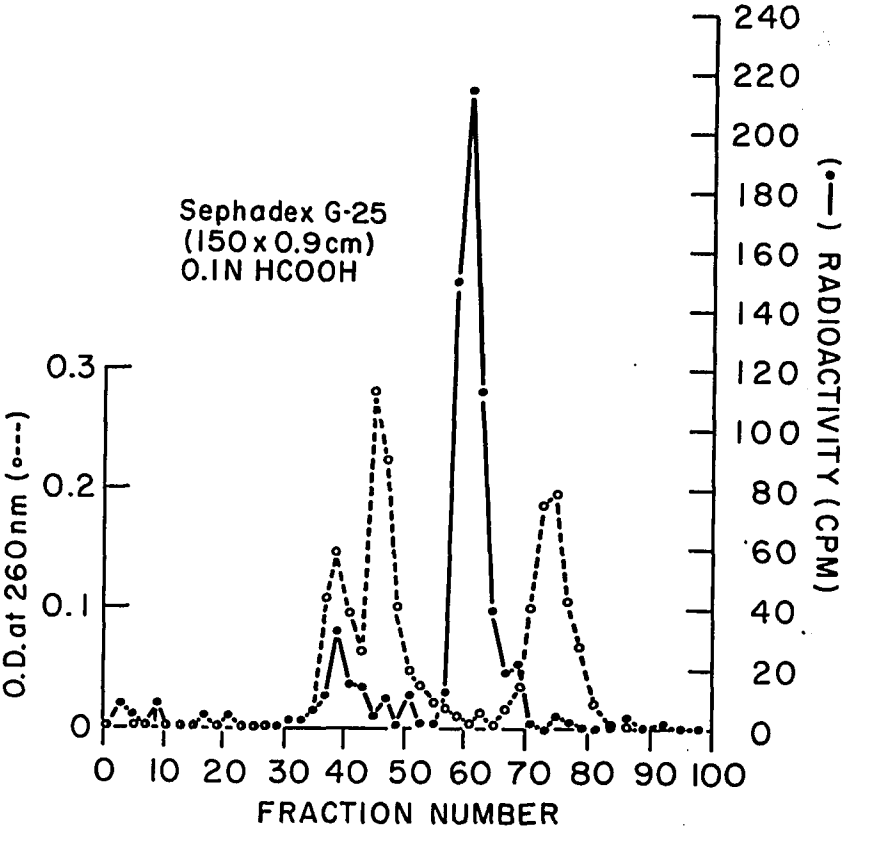


Fig. 29. Gel filtration elution pattern of the chymotryptic digest of the covalent NP II-[^{14}C] AVP complex, on Sephadex G25 in 0.1N formic acid. Digestion was carried out at an enzyme-to-substrate ratio of 1:300 for 2.5 hr at 37C. Peaks are detected by radioactivity measurements or by Folin color. The main protein peak corresponds to the elution position of alkylated NP II.

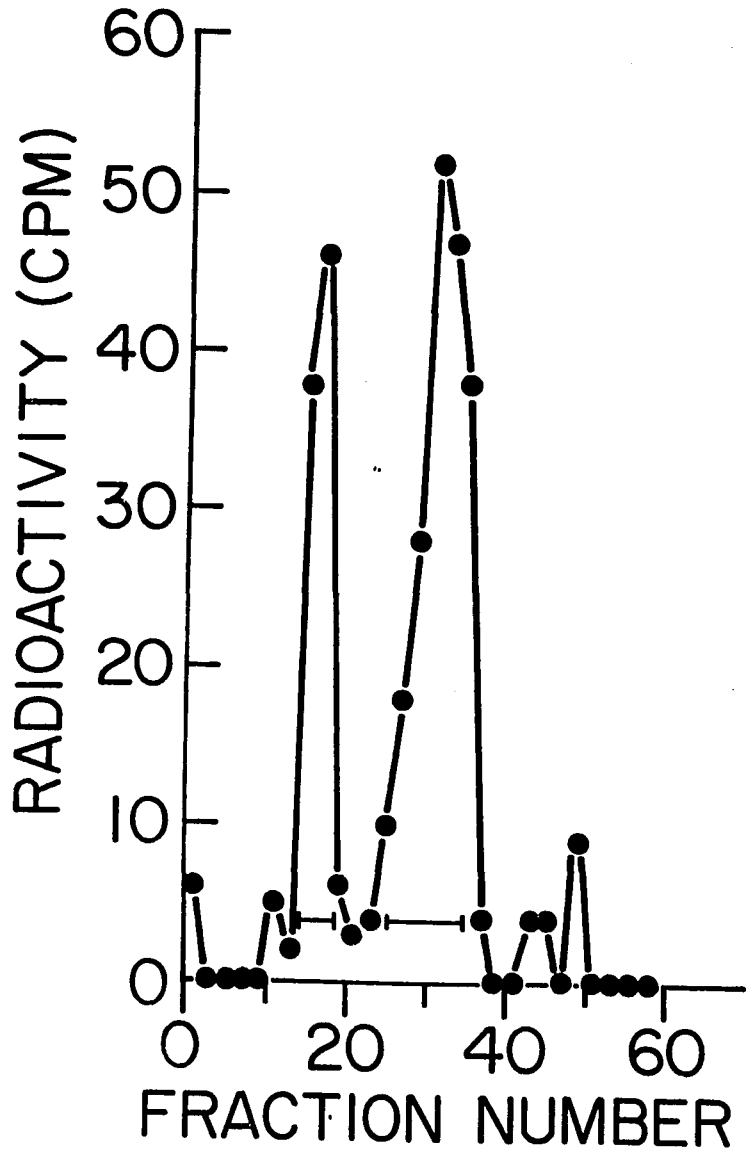


Fig. 30. Ion-exchange chromatography of the major radioactive peak from gel filtration of the chymotryptic digest of the NP II- [^{14}C] AVP covalent complex. Material from pooled fractions 56-68 in Fig. 29 was chromatographed on an IRC-50 column in 0.65N NH_4OAc, pH 6.5.

protein for an N-terminal analysis; however, by this procedure an aliquot of material from pooled fractions 10-20 was shown to consist of two peptides with N-terminal Gly and Val. Carboxypeptidase digestion of material from these same fractions released Phe and Leu, although the results were more equivocal. On high-voltage electrophoresis of the remainder of the material from pooled fractions 10-20 two radioactive peptides were detected, one of which (peak III) contained very few counts (Fig. 31). Peak III had not been detected previously (i.e., in electrophoresis of material from the main radioactive peak of gel filtration of the chymotryptic digest, or in analytical electrophoresis of the IRC50 fractions) because of its low specific radioactivity. When small aliquots of material were applied to electrophoresis, peak III was below the level of detection, since most of the radioactivity was associated with the other peaks.

Amino acid composition of the peptide material from peak III, eluted from paper, revealed that it represents a fragment which can be placed from positions 23 to 35 in native bovine neurophysin II. Edman degradation demonstrated an N-terminal Gly residue, which is in line with the positioning of the peptide fragment in the NP II sequence. Peaks I and II were shown by amino acid analysis in later experiments

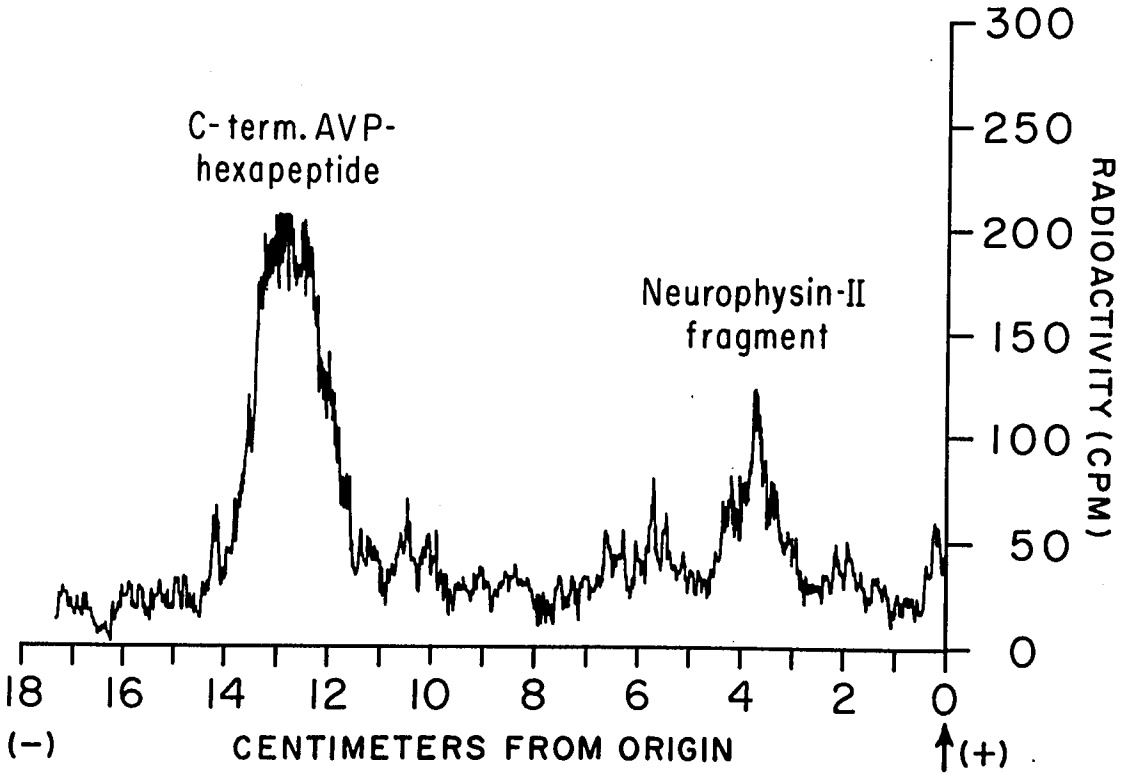


Fig. 31. Electrophoretic separation of radioactive peptides present in pooled fractions 10-20 from ion-exchange chromatography (see Fig. 30). An aliquot of the radioactive material was subjected to high-voltage electrophoresis at pH 3.5 for 1 hr at 3000V, in a buffer of 5% acetic acid-0.5% pyridine.

(and see Section 3) to have a composition corresponding to the C-terminal hexapeptide of [^{14}C]AVP. Amino acid compositions of neurophysin peptides are shown in Table 2.

Formation of a Covalent NP II-[^{3}H]LVP Covalent Complex

Fig. 32A depicts the gel filtration pattern of the reaction mixture of NP II, [^{3}H]LVP, and water-soluble carbodiimide. The three peaks are the NP II-[^{3}H]LVP complex, polymer of [^{3}H]LVP and [^{3}H]LVP monomer. The material from the peak eluting at the position of NP II was pooled and lyophilized. Because of the poor resolution of these three peaks, which always occurred when LVP was the binding hormone (see Fig. 32B which shows the gel filtration pattern of an NP II-[^{14}C]LVP covalent complex) the peak corresponding to the complex was always rechromatographed and emerged as a single radioactive peak.

Alkylation of the Covalent NP II-[^{3}H]LVP Covalent Complex

The covalent complex of NP II with [^{3}H]LVP was reduced with DTT and the resulting free sulfhydryls alkylated with iodoacetamide. Upon gel filtration of the reaction mixture, the S-alkylated complex eluted as a single, symmetrical radioactive peak with an elution volume of 75.9 ml.

Chymotrypsin Digestion of the Covalent NP II-[^{3}H]LVP Complex and Identification of Products

With an enzyme-to-substrate ratio of 1:300, and an incubation time of 2.5 hr, conditions used for chymotrypsin digestion of the NP II-[^{14}C]AVP complex, most of the radioactivity

Table 2. Amino Acid Composition of the Neurophysin II- ^{14}C AVP Covalent Complex and Its Chymotryptic Fragments

	Alkylated NP II- ^{14}C AVP Complex		Chymotryptic Products of NP II- ^{14}C AVP Complex		Labeled Neurophysin I Fragment	
Lys	2.09	(2)	0.4	(0)		
His	--					
NH_3	n.d.	(8)				
Arg	6.81	(7)	0.6	(1)		
CmCys	13.1		1.3	(1-2)	1.1	
Asp	5.6	(5)	1.2	(1)	1.0	(1)
Thr	2.0	(2)	1.0	(1)		
Ser	5.3	(6)	1.3	(1)	0.5	(1)
Glu	14.7	(14)	5.5	(6)	2.3	(2)
Pro	8.7	(8)	1.2	(1)	0.5	(1)
Gly	16.0	(15)	3.5	(3-4)	3.0	(3)
Ala	6.7	(6)	1.9	(2)		
½Cys	1.2	(14)	0.5	(1)	2.0	(2)
Val	4.0	(4)	1.0	(1)		
Met	1.0	(1)	0.3	(0)		
Ile	1.9	(2)	0.5	(1)	0.8	(1)
Leu	5.98	(6)	2.6	(2-3)	1.4	(1)
Tyr	0.88	(1)	0.3	(0)	Trace	
Phe	2.98	(3)	0.7	(1)	1.0	(1)
N-terminal	Ala		Gly, Val		Gly	

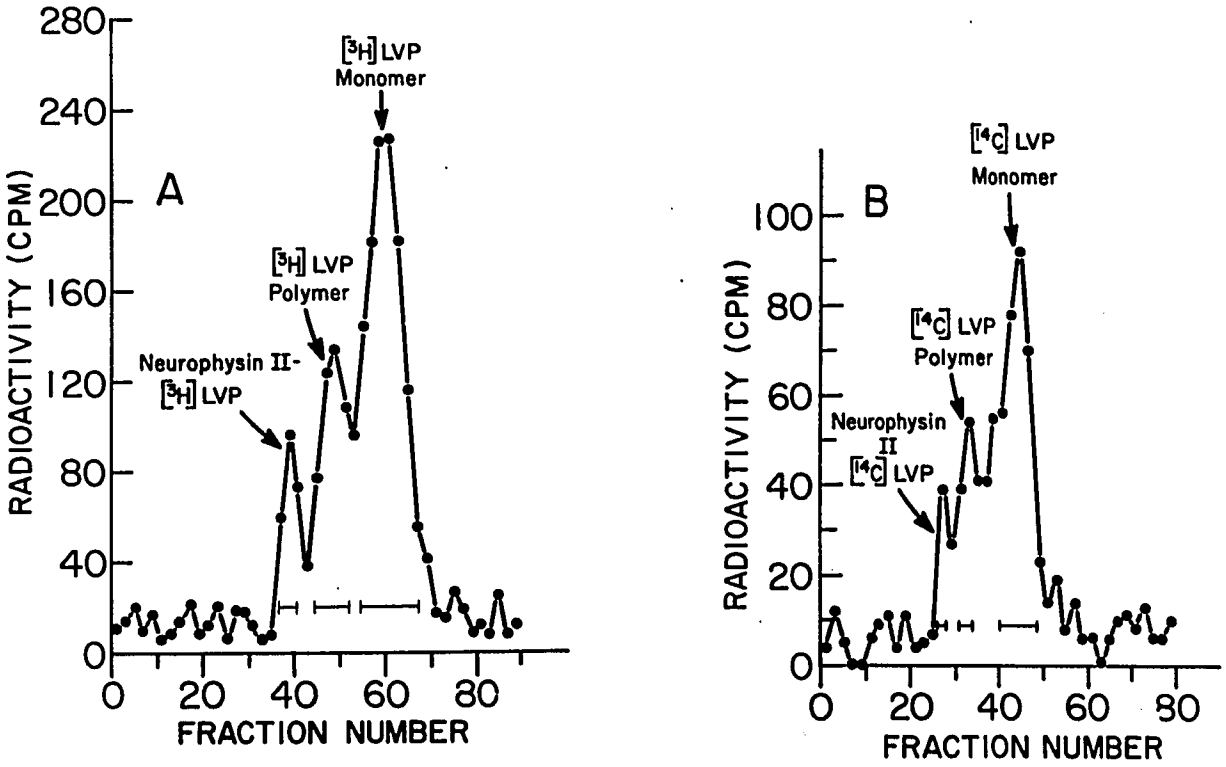


Fig. 32. Gel filtration elution patterns of NP II-LVP covalent complexes.
A: Reaction mixture of NP II with [^{3}H]LVP.
B: Reaction mixture of NP II with [^{14}C] LVP.
Both gel filtrations were carried out on Sephadex G25 in 0.1N formic acid.

eluted at the position of intact, alkylated NP II. When the amount of enzyme was increased, to give an enzyme-to-substrate ratio of 1:3, even with an incubation time of 1 hr more radioactivity was associated with later-eluting material, presumably smaller peptide fragments. As the time of incubation was increased (first to 2 hr and then to 10 hr) radioactivity eluting at the position of alkylated NP II decreased, and that associated with lower molecular weight components increased. After one hour of digestion, for example, the total amount of radioactivity in the later-eluting peaks was approximately 38% of the radioactivity recovered; at two and ten hours it was approximately 50%. Fig. 33 shows the gel filtration pattern of a 2 hr digest at an enzyme-to-substrate ratio of 1:3.

Aliquots of material from each radioactive peak were subjected to high-voltage paper electrophoresis at pH 3.5. The electrophoresis pattern of radioactive material from pooled fractions 30 to 39 (see Fig. 33) is identical to that of intact, S-alkylated NP II (with [^{3}H]LVP bound). Material from the other peaks gave the patterns shown in Figs. 34A and B. The radioactive peaks were eluted from the electrophoretogram, and aliquots of material associated with each peak were hydrolyzed and subject to amino acid analysis. On the basis of these analyses, it appeared that the peptides were impure,

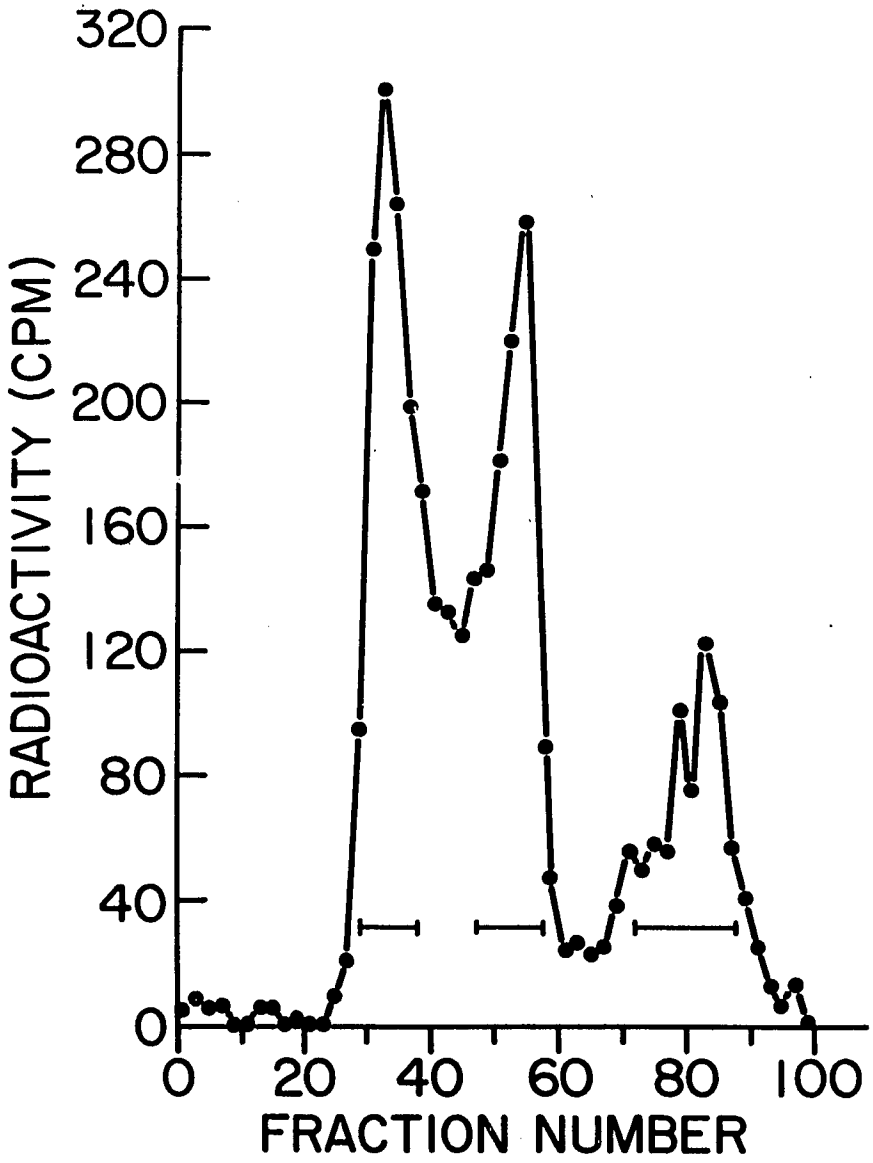


Fig. 33. Gel filtration elution pattern of the chymotryptic digest of the covalent NP II- [^{3}H] LVP complex, on Sephadex G25 in 0.1N formic acid. Digestion was carried out for 2 hr at 37C with an enzyme-to-substrate ratio of 1:3; peaks are detected by radioactivity measurement.

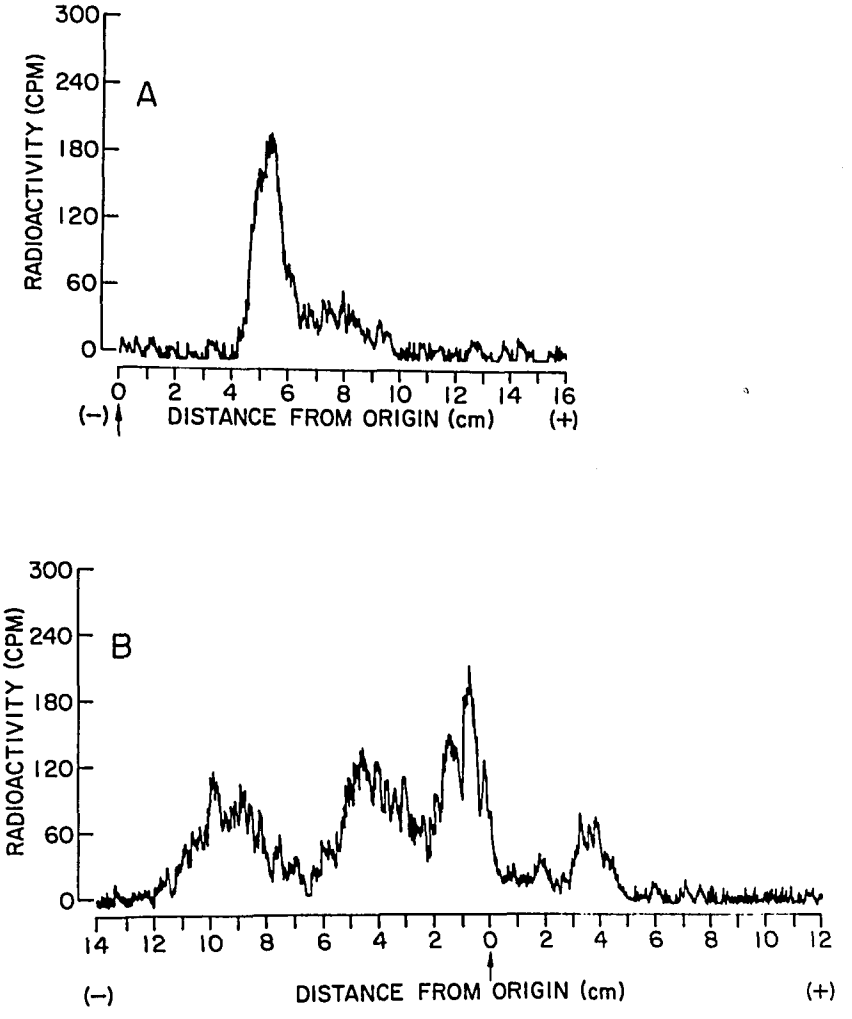


Fig. 34. Electrophoretic separation of radioactive peptides present in the chymotryptic digest of the covalent NP II-[^{3}H] LVP complex. Aliquots of material from fractions 71-89(A) and fractions 45-58(B) were subjected to high-voltage electrophoresis at pH 3.5 for 1 hr at 3000V, in a buffer of 5% acetic acid-0.5% pyridine.

and perhaps contained non-radioactive peptide fragments not separated by electrophoresis.

Discussion

Elucidation of the primary structure of bovine neurophysin II (Fig. 22) set the stage for localization of the neurohypophyseal hormone-binding site by affinity labeling. Covalent binding of a radioactively-labeled neurohypophyseal hormone to neurophysin II, followed by enzymatic digestion of the peptide-protein complex, should allow isolation of a radioactively-labeled neurophysin II fragment, which, with the amino acid sequence of the protein at hand, can be readily positioned in the native neurophysin. Rather than synthesize a reactive, radioactive hormone analog, it was decided to take advantage of the nature of the ionic bond in the specific, reversible protein-peptide complex. As discussed above, the bond involves the protonated α-NH_2 group of the hormone, essential to binding, and an unprotonated carboxyl group of neurophysin. (Titration studies had suggested that this group is a side-chain carboxyl in NP II (179)). The conversion of the ionic bond to a covalent one seemed best accomplished with a dehydrating agent (carbodiimide), commonly used for formation of peptide bonds during chemical synthesis.

Since the non-covalent complex was shown to be dissociable by gel filtration chromatography at pH 2.5 (243) (Table 1 and Fig. 24), radioactive hormone associated with NP II after this treatment was assumed to be covalently bound.

Formation of a covalent bond was first attempted in a non-polar solvent, methylene chloride, with the intention of enhancing the interaction between protein and peptide (which is in part a hydrophobic one) by placing the complex in a non-polar environment. However, experiments using methylene chloride as solvent, and dicyclohexylcarbodiimide for the reaction, with the complex in suspension, failed to give covalent binding. After preliminary experiments using [^{14}C]oxytocin, [^{14}C]arginine vasopressin was used when it was decided to digest the complex with chymotrypsin in order to compare the results with those found in sequence studies. Since chymotrypsin is known to remove glycinamide from oxytocin (249, and see Section III), it was thought that the radioactive label might be lost during digestion. When these experiments were undertaken, all available evidence indicated that there was no difference in the non-covalent binding of oxytocin or vasopressin to neurophysin II (231). The percentage of [^{14}C]AVP covalently bound to NP II was no greater than [^{14}C]oxytocin either in methylene chloride or in aqueous solution using 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide HCl until the reaction was carried out in 4M urea. Urea in general decreases hydrophobic interactions between proteins; in this case it most likely interferes with interactions between different parts of the NP II

molecule, without affecting its overall conformation, and allows the carbodiimide to reach the reaction site. This hypothesis is supported by the fact that several attempts to form a covalent complex of [^{14}C]AVP and NP II in a solution which did not contain urea, i.e., 0.1M phosphate buffer, pH 5.8, 80%-, 30%-, and 10%-dioxane-phosphate buffer, and pyridine acetate buffer, pH 5.8, were unsuccessful. In all of these experiments ratios of NP II to hormone, volume of the reaction mixture, and pH were chosen on the basis of conditions which had been found to be optimal when the reaction was carried out in 4M urea. In addition, varying the ratio of NP II to hormone in the absence of urea did not enhance formation of the covalent complex. Whether the covalent coupling was achieved starting from a non-covalent complex which had been chromatographed at pH 5.8, or from a mixture of neurophysin II and [^{14}C]AVP which had equilibrated in 4M urea, pH 5.8-6.0, the results were identical.

In affinity labeling of antibodies or enzymes, certain criteria are employed to judge the specificity of the reaction (120). These include a) the stoichiometry of the reaction, and its restriction to the antibody (not IgG) or particular enzyme involved; b) rate and extent of reaction not being increased by increasing the excess of labeling reagent;

c) protection of the protein from being labeled by use of specific haptens or inhibitors.

Some of these criteria can be applied to the neurophysin II-[^{14}C]AVP experiments. For example, when radioactive hormone is diluted with increasing amounts of unlabeled hormone, the covalent binding of labeled material decreases linearly, suggesting that the reaction is limited to the specific binding site on neurophysin. Using a large excess of unlabeled hormone eliminates binding of labeled material, rather than increasing non-specific binding; this may also be considered as an example of protection. The labeling reagent in this case is actually <u>not</u> the hormone, but the carbodiimide, which creates the covalent bond. When carbodiimide concentration is increased in a certain range (Fig. 27), [^{14}C]AVP binding also increases, and then appears to plateau to some extent. When a greater amount of carbodiimide is used, binding drops almost to zero. The appearance of a plateau in the covalent binding would indicate some degree of specificity. However, it was noted that reaction of [^{14}C]AVP alone with the carbodiimide caused polymerization of the hormone, and the polymer did not bind to NP II. There is evidently competition between the two reactions, and at very high carbodiimide concentrations the hormone polymerization occurs faster than the formation of the covalent

linkage of the hormone to the neurophysin. These results do not allow interpretation of the leveling-off of binding as an indication of specificity. It should be noted that the amount of carbodiimide needed is quite high; this is probably due to the competitive reaction outlined above, to destruction of the reagent, and to steric hindrance of the "fit" of the coupling reagent into the binding site. Modification of protonated carboxyl side chains (250), which could be considered as another possible side reaction, is ruled out by the pH of the reaction mixture.

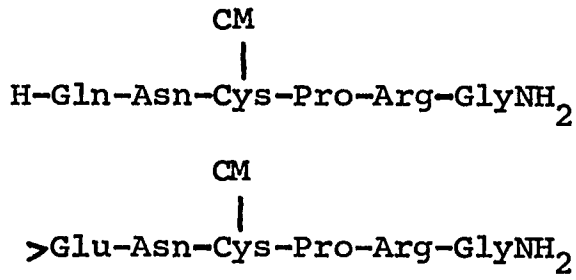
When neurophysin and [^{14}C]arginine vasopressin are reacted at a 1:10 molar ratio (1.0 mg neurophysin and 1.0 mg AVP), approximately 10% of the label is bound, the stoichiometric amount expected for a complex containing a 1:1 molar ratio of protein and peptide. The pH optimum for covalent binding occurs at 6 to 6.5, which is similar to the optimum for formation of the non-covalent complex (225).

The experiments described provide some evidence that the neurophysin-[^{14}C]AVP covalent complex is formed in a second step from the specific, reversible electrostatic interaction, and that covalent binding occurs at a minimum of non-specific sites. These are the main requirements for affinity labeling (120,166). As discussed in Section 1, the success of the method depends on isolation of a peptide

fragment with a high amount of label as compared to other fragments.

Chymotryptic digestion of the covalent complex was carried out using identical conditions to those employed in sequence studies, in order to simplify the identification of fragments obtained. Identification of radioactive peptides was complicated by the susceptibility (see Section 3) of reduced and alkylated covalently bound [^{14}C]AVP to chymotryptic cleavage at the carboxyl side of the aromatic residues (Phe and Tyr). In view of the earlier reports dealing with the treatment with chymotrypsin of oxytocin and vasopressin and their acyclic derivatives, such cleavage was unexpected (249). As a result of this hydrolysis, most of the radioactivity in the main radioactive peak detected on gel filtration of the enzyme digest was associated with the C-terminal hexapeptide of the hormone, which eluted at the same position as the neurophysin peptide fragments but was not covalently bound. Ion-exchange chromatography (Fig. 30) separated one radioactive peak from the others present in the main gel filtration fraction: both this separated peak (IRC50 fractions 21-38) and the material associated with most of the radioactivity in the other fraction (10-20) were hormone fragments, one with an N-terminal Gln and the other with an N-terminal

pyroglutamic acid (see Section 3):



After the ion-exchange step material at the elution position of the more basic peak (fractions 10-20) also consisted of two neurophysin peptides, which were identified by amino acid composition and N- and C-terminal analysis as those corresponding to positions 23 through 35 and 36 through 50 in the native protein. High-voltage electrophoresis of this material separated the hormone fragment from a labeled protein segment, the one corresponding to positions 23 through 35 of NP II, and an unlabeled protein fragment (i.e., the one corresponding to positions 36 through 50) which was not identified since it was not associated with any radioactivity. As discussed in the results section, the labeled neurophysin peptide had not been detected in original electrophoretograms because the percent of the total radioactivity bound to it was too low to permit detection until a high starting amount of radioactivity was analyzed.

The amount of radioactivity on the neurophysin peptide isolated by HVE was, as mentioned, quite low, corresponding to $\sim$0.08 nmole hormone, judged by integration of the

electrophoretogram. The amount of protein applied in the step following ion-exchange chromatography was approximately 100 nmole, and based on recovery of radioactivity after elution and amino acid analysis, the amount of labeled neurophysin peptide was ~30 nmole. The amino acid composition of the eluted labeled peptide should include both the neurophysin and AVP residues, but as could be predicted from the above results, this was not the case. The amino acid composition found corresponds to sequence 23 through 35 in the native neurophysin, and the amount of bound hormone is low enough to escape detection. At first glance, this phenomenon seems to result from cleavage of most of the hormone at the Phe-Gln bond (see Section 3), with concomitant loss of the C-terminal hexapeptide and the label; however, it could be expected that the neurophysin peptide with AVP bound would show a different electrophoretic mobility from that with the covalently attached N-terminal hormone sequence, or the free peptide. The radioactivity associated with the peptide is not at the position of the C-terminal hexapeptide fragments of AVP or the alkylated nonapeptide of AVP on the electrophoretogram, however, and the other neurophysin peptide (sequence 36 to 50) which was present in the mixture applied for electrophoresis is <u>not</u> labeled. Furthermore,

if the labeled peptide is eluted and rerun, the radioactivity is not separated from the peptide material either at pH 3.5 or 6.5.

At present these discrepancies have not been resolved. The fact that only one peptide is labeled, and that the label remains with the peptide on re-electrophoresis although alkylated AVP and its C-terminal fragments have quite different mobilities, lead us to believe that the labeled hormone is attached covalently to the NP II peptide, as opposed to being in a non-specific association. The low amount of hormone bound and resulting lack of AVP in the amino acid analysis is difficult to explain, unless the NP II peptide bound to the N-terminal AVP sequence and the NP II peptide covalently attached to the total hormone have the same electrophoretic mobility. If the isolated NP II peptide is accepted as the sequence in the native protein affinity labeled by $[^{14}C]$AVP, then the side-chain carboxyls involved in binding would belong to the aspartic acid residue in position 30 or the glutamic acid residue in position 31 in native bovine neurophysin II (Fig. 35).

Affinity labeling was repeated using LVP labeled with tritium in the tyrosine residue, in order to avoid the loss of label during chymotryptic digestion. The results of this study have failed thus far to give a unique location of the

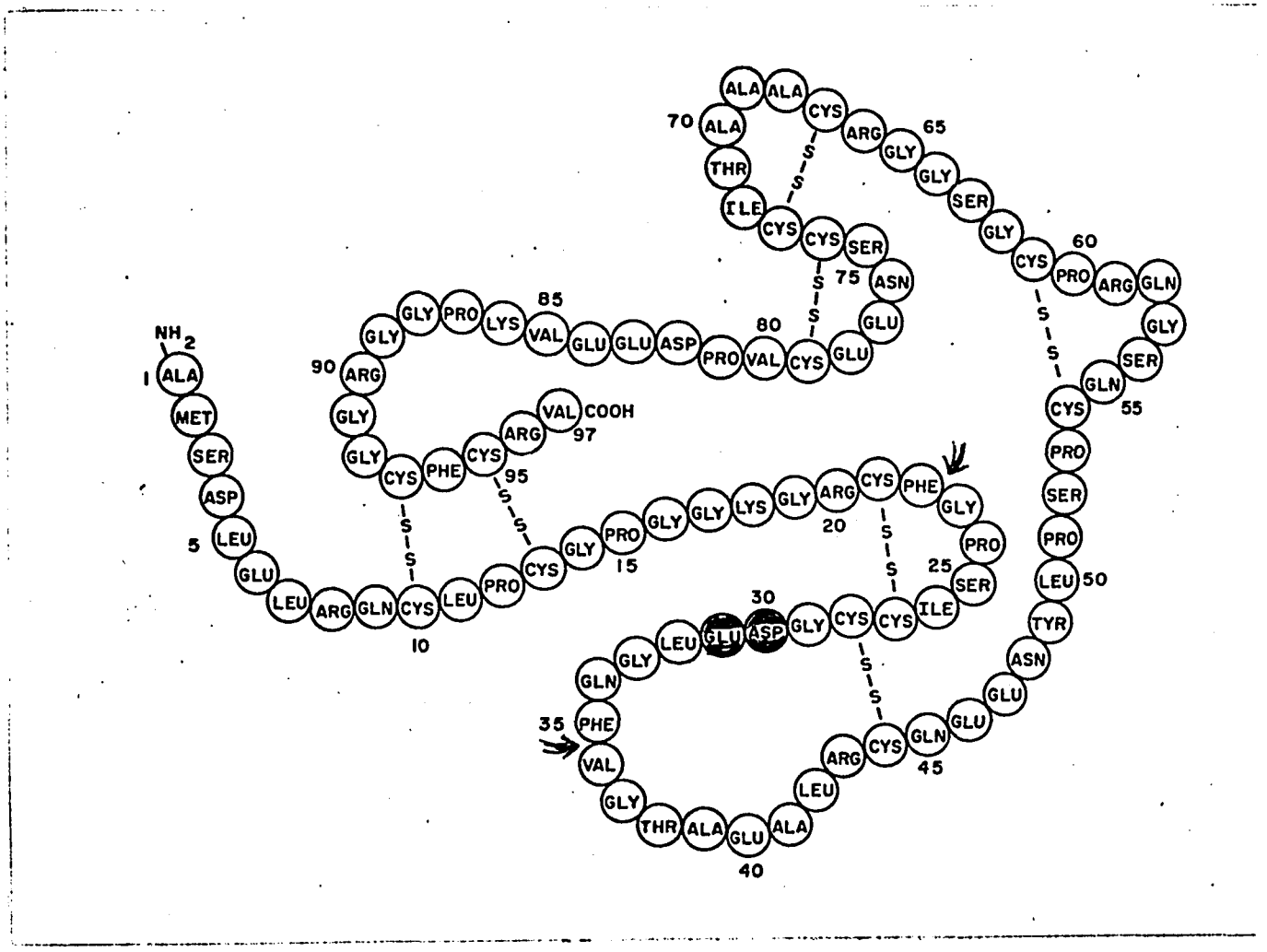


Fig. 35. Complete covalent structure of bovine NPII (224), indicating the fragment identified by affinity labeling as the binding area for [^{14}C]AVP, and the probable residues containing the carboxyl side chains involved in binding.

LVP-binding site on NP II. After chymotryptic digestion of the covalent NP II-[^{3}H]LVP complex several radioactive fragments were found by high-voltage electrophoresis (Fig. 34). After elution of material associated with these peaks, hydrolysis, and amino acid analysis it appeared that they did not correspond to known sequences in NP II. In these experiments the chymotrypsin concentration had been increased to give an enzyme-to-substrate ratio of 1:3, in order to digest the alkylated complex to a greater extent than in the studies with [^{14}C]AVP, and thus increase the amount of labeled NP II peptide released. The increased chymotrypsin concentration resulted in a large number of radioactive peptides and apparently in several unlabeled peptides, all of which could not be adequately separated by high-voltage electrophoresis. On the other hand, a lower enzyme-to-substrate ratio (1:300) cleaved so little of the alkylated complex that determination of labeled fragments was difficult, and the amount of protein associated with the radioactivity was so low that characterization was impossible. There is also some evidence that neurophysin II has two binding sites for LVP (230); this may contribute to the complexity of the results.

A comparison of the sequences of bovine neurophysin I and II shows that the N- and C-terminal portions are relatively

variable, while the central region is essentially constant (223). If oxytocin and vasopressin compete for the same binding site both on neurophysin I and II, as has been suggested (231), and considering that binding constants for the two hormones are similar, then the binding region is most likely in the constant portion of the molecule. The fragment suggested by affinity labeling is derived from this region.

The tentative conclusion reached here, that the α-amino group of AVP interacts with the carboxyl side chain at position 30 or 31 of bovine NP II, has to be correlated with results gained by spectrophotometric methods, (circular dichroism [CD], UV absorption, and especially NMR). Bovine neurophysins I and II and the hormones oxytocin, AVP, and LVP each contains a single Tyr residue. There is general agreement that the tyrosine residues of both protein and hormone are perturbed on binding. Furth and Hope (251) found that neither nitration nor O-acetylation of the tyrosine of bovine neurophysin II affected its binding capacity, but their UV absorption studies indicated both tyrosine residues were involved in binding. Changes in the CD spectrum at 280nm of bovine NP II observed by Breslow and Weis (252) upon binding to peptide hormones were attributed to changes in the environment of the

tyrosine in position two of the hormone. The protein tyrosine is also perturbed upon complexation (in agreement with the findings of Furth and Hope) as indicated by changes in ellipticity of this nitrated residue upon binding of NP II to the hormones. Involvement of the hormone tyrosine in binding is supported by the NMR studies of Balaram et al. (229) with bovine neurophysin II and model peptides; the proton resonances on the aromatic residue of the peptide hormone are differentially broadened by binding, indicating dipolar relaxation of these protons by nearby residues on the protein. NMR studies by Cohen et al. (253) of the interaction of oxytocin with bovine NP I also implicate the hormone tyrosine, and not the histidine residue of the protein, in complexation.

Furth and Hope (251) had attributed a large red-shift of the absorbence of the nitrated tyrosine of NP II upon complexation to the entrance of the protein tyrosine into a more hydrophobic environment. However, Breslow and Weis (252) found that the shift in the nitrotyrosine ellipticity to longer wavelengths could be accounted for by the increase in nitrotyrosine ionization accompanying binding, and did not necessarily indicate a more hydrophobic environment for the protein tyrosine.

The recent work of Griffin et al. (254), using both UV absorption and circular dichroism, further describes changes in the microenvironment of the aromatic residues during complex formation. During binding of oxytocin, LVP, and N-terminal tripeptide analogs of the hormones to bovine NP I, red-shifted tyrosyl absorbences indicated increasing inaccessibility of a tyrosine residue to solvent. However, when tripeptide analogs without tyrosine were bound, the tyrosyl absorption was blue-shifted, i.e., the protein tyrosine enters a more polar environment in the complex. Ellipticity changes were interpreted similarly, leading to a model for hormone-neurophysin interaction in which a conformational change of the protein allows the hormone to enter a hydrophobic environment. The results of the NMR studies by Cohen et al. suggest restriction of motion of the hormone tyrosine in the complex, while NMR studies by Balaram et al. (229) indicated that the protein tyrosine is less restricted in the complex than in the free protein, both in line with the proposed model.

Thus it appears from both spectroscopic and binding studies that the hormone-binding site lies in a hydrophobic region of the protein, and that the tyrosines of both moieties are involved in, or at least affected by, complexation.

The proximity of the protein and peptide tyrosines in the complex is suggested by Balaram et al. (229,255) on the basis of nuclear Overhauser effects, using bovine NP II and the model peptide (S-Me)Cys-Phe-Ile-NH_2. On the basis of model peptide binding and of hormone and analog binding, circular dichroism spectra and the NMR results of Balaram et al. (255), Breslow and Weis (252) had earlier proposed that the protein and peptide aromatic rings are close in the complex but that the side chains of residues 1 and 3-9, and the tyrosine OH of the hormone make no direct contact with the protein tyrosine. There is no evidence, however, for "stacking" (or π - π interaction) of the aromatic rings (228,253,255), and it was suggested by Breslow et al. (228) that the apparent requirement for aromaticity and hydrophobicity in position two of the hormone may actually be a steric requirement for a planar hydrophobic residue.

The high apparent pK of the nitrated tyrosine in bovine NP II was suggested to result from its nearness to the carboxyl side chain involved in binding; thus, the decrease in pK observed on formation of the hormone-neurophysin complex might result partly from neutralization of this carboxylate ion by the α-amino group of the peptide (252). These results, consideration of the "constant" regions of the various neurophysins (223), with the added restriction that

binding should occur only in non-duplicated regions, led Breslow and Weis (252) to speculate that the carboxyls of the glutamic acid residues in position 40, 46, or 47 (tyrosine is position 49) might be directly involved in the electrostatic bond.

The position of the binding site probably depends heavily on the 3-dimensional structure of neurophysin. These proteins have a high disulfide content which reduces the number of their possible conformers (224), implying a requirement for a specific topography in order for them to function as carrier proteins. This configuration might make one area more available for binding than another, even though their sequences might be similar, so that binding could occur in a duplicated region on the protein.

The results of the studies discussed above indicate that both hormone and protein tyrosine are perturbed on binding, that the complex is in a hydrophobic environment, and that the tyrosines may be near each other in the complex. It is not clear how this occurs, if the protein tyrosine enters a more polar environment, as suggested. It seems likely that binding induces a conformational change in the protein, such as that proposed by Griffin et al. (254). The tyrosines may be brought into proximity by this change,

even when the electrostatic (or covalent) bond between hormone and neurophysin is some distance away (i.e., at residue 30 or 31 as suggested here). Griffin et al. (254) suggested that the decrease in apparent pK of the nitrated tyrosine upon complexation might be due to its entrance into a more polar environment, so that its nearness to the carboxyl involved in binding is not essential. The binding of LVP, oxytocin, or tripeptides to bovine NP II all increase its sedimentation velocity, which indicates the possibility of conformational change (228).

The tentative conclusions reached here on the basis of affinity labeling, in combination with sequence, spectrophotometric, and binding studies may allow a definitive description of the binding site. A further important study would be X-ray diffraction of the crystalline neurophysin-vasopressin complex. The value of combining results from different methods is illustrated particularly well by the determination of active sites and mechanisms of action of enzymes such as carboxypeptidase A, ribonuclease A, lysozyme and chymotrypsin (256). In chymotrypsin, for example, His-57 was identified as part of the active site by affinity labeling, and the total mechanism could be elucidated by considering these results along with those gained from X-ray crystallography of the enzyme-substrate complex. Affinity labeling of neurophysin II with LVP

and oxytocin, labeled in the tyrosine residue, may help to determine exactly how many binding sites exist for a given hormone, and whether all hormones bind at the same site.

The exact residue interacting with an affinity label can often be identified. In general, in the case of enzymes, this is a nucleophilic moiety which contributes to the enzymatic hydrolysis of the substrate. The histidine residues at the active sites of trypsin and chymotrypsin were identified by this method, and later shown to play an important role in their catalytic mechanisms (123-125). In a study of elastase, which is also a serine protease, peptide chloromethyl ketones were used as irreversible inhibitors (257). The performic acid-oxidized, modified enzyme was subjected to acid hydrolysis, and the decrease in histidine content, along with appearance of trace amounts of carboxymethyl histidine, suggested that this enzyme also contains a His residue at the active site. The use of halomethyl ketones, followed by acid hydrolysis, leads to formation of carboxymethylated amino acids whichcan be identified by amino acid analysis. This technique has also been used for antibody combining sites (258) and for the active site of carboxypeptidase A, for example (250). The rate of active site labeling can be followed by enzyme inhibition, or, as in the present study, by uptake of

radioactivity; in the latter case the residue modified, if the label stays on during acid hydrolysis, can be confirmed by radioactivity measurements. In some cases the affinity label can be displaced by another reagent to yield a recognizable modified amino acid. Cardinaud and Baker (259), for instance, displaced a sulfonate group with mercaptoethylamine to give S-aminoethyl cysteine after hydrolysis.

The method used here for affinity labeling of neurophysin, which takes advantage of the nature of the non-covalent complex, does not create a modified amino acid residue after acid hydrolysis, since the covalent bond is a peptide bond. Whether the Asp or Glu residue is involved might be determined by using model peptides containing reactive groups; since the location of binding is already known, the position of the modified residue would be apparent. Further studies of the peptide-protein interaction might also involve synthesis of the peptide corresponding to the "constant" region of residues 10-60 in native bovine neurophysin II to see if it would bind and if its binding site were identical to that in the native protein.

SECTION 3: Chymotrypsin Inactivation of Neurohypophyseal Hormones and Analogs

Introduction

In their pioneering studies on three-dimensional protein configurations Pauling and Corey (260) described two helical structures, the γ and the α-helix, which are compatible with the various structural parameters calculated for amino acids and small peptides. A third compatible configuration, the β-pleated sheet, consists of extended peptide chains laterally hydrogen-bonded to adjacent chains. Since these predictions, an ever-increasing number of scientists have studied the relationship between amino acid sequence and protein or peptide conformation. From these studies it has emerged that in fact the particular primary structure of a protein is, within certain limits and under specific conditions, the sole determinant of its preferred conformation. Strong evidence for this hypothesis is the spontaneous refolding of polypeptide chains derived from proteins that do not contain disulfide bonds, e.g., tobacco mosaic virus coat protein and lactic dehydrogenase (261). This refolding with recovery of activity can occur even after treatment that is expected to completely disrupt tertiary structure. Further support for the hypothesis derives from the chemical synthesis of an active enzyme, ribonuclease (262,263).

In recent years, detailed models of three-dimensional protein structures have been secured, primarily by X-ray

diffraction techniques (256). Many of the proteins studied are enzymes, and probable mechanisms of their action have been outlined in light of the stable, static complexes of these enzymes with substrates or inhibitors in the crystalline form. Some techniques, particularly high-resolution proton NMR, can provide information about local environments and conformation of proteins in solution; in the instances where both methods have been used, the proposed structures, particularly in active site regions, are related (256).

Correlations are now beginning to be made between conformation and biological parameters such as enzymatic (256,264,265) and hormonal activities (16,56), biosynthesis (266), antigenic properties and immunoreactivity (267,268), and enzymatic inactivation (16,269). As an example of this type of correlation, in protein and polypeptide antigens "sequential" and conformational determinants have been distinguished (270,271), and antibodies to native proteins are directed mostly against the latter.

Conformational analyses of peptides, rather than proteins, have in the past encountered great difficulties, mainly because of the flexibility of peptides in solution, a property which also explains the lack thus far of proper crystalline preparations for X-ray studies. Recently, however, considerable progress has been made in assigning preferred

conformations to small cyclic peptides, notably model peptides and antibiotics (ionophores) (272). These peptides have structures simple enough to be determined in solution by investigation with available spectroscopic methods. An outstanding conformational feature of many of the cyclic peptides and antibiotics studied is the β-turn characteristic of anti-parallel pleated-sheet structures. Pauling and Corey originally proposed several types of β-structure (260); only two have been found to be important in protein and polypeptide conformation (273). In the first, hydrogen bonds are formed between peptide chains which are parallel in terms of the direction of propagation of the chain. In the second, bonds are formed between chains that run in opposite directions. A third form of the β-structure is the "cross-β" form, which is essentially antiparallel, but in which a single peptide chain folds back on itself to form intrachain hydrogen bonds (273). The conformation of the residues which are involved in the bending or folding portion of the chain in this conformation are said to be in a "β-turn" (273). Geddes et al. (274) reported the conformational characteristics of the β-turn in their X-ray analysis of Chyopsa silk. The β-turn is usually closed by a hydrogen bond from the peptide oxygen of residue i to the hydrogen on the peptide nitrogen of residue i+3.

Various physical techniques have been employed in experimental investigations of the β-structure, including UV spectroscopy, optical rotatory dispersion (ORD), and NMR (273). The last is most useful for determination of conformation in solution, and has been used, in conjunction with sequence studies, to identify β-turns in many peptides (272). In general, application of NMR to determination of peptide structure begins with the assignment of all resonances. This is accomplished by starting with protons located in the amino acid side chains and identifying, primarily by decoupling techniques, the coupled proton on the α-carbon, and the amide proton. Next, hydrogen-bonded protons can be identified by exchange studies and by studies on the temperature dependence of NH proton chemical shifts. Information on the αCH-NH dihedral angle is gained by determination of coupling constants between NH and αCH protons, and differences in chemical shifts of various protons can give information about the magnetic anisotropy of nearby groups.

A specific conformational feature, such as the β-turn, may be characterized by particular combinations of the above pieces of information. Based on these methods, β-turn conformations have been proposed for such cyclic antibiotics

as gramicidin S (272,275), valinomycin (272,276), the valinomycin-K^+ complex (272,277), polymyxins (272), and cyclic hexapeptides (278,279). Conformations, primarily based on CD and NMR studies have also been proposed for the cyclic neurohypophyseal peptide hormones, oxytocin (15) (Fig. 1) and its close congener lysine vasopressin (16,17,18). The β-turns found in these hormones, which are thought to be closed by hydrogen bonds, could be characterized by an amide proton resonance which is found at high field due to shielding by the end-peptide moiety (residues i+1 and i+2), and which exhibits a low temperature coefficient for its chemical shift, suggesting hydrogen bonding (280). Furthermore, there is a relatively small αCH-NH coupling constant for residue i+1 and a large one for i+2 (280). Our studies indicate that the preferred conformation of the neurohypophyseal hormones consists of two β-turns, one involving the Tyr-Ile(Phe)-Gln-Asn sequence in the 20-membered ring structure and the other the Cys-Pro-Leu(Lys,Arg)-Gly sequence in the acyclic tail portion. A prerequisite for completion of the 20-membered ring structure was the determination by CD studies of the preferred absolute configuration of the C-S-S-C group in oxytocin as a right-handed screw sense (281). The disulfide bridge of the hormones stabilizes the secondary structure by giving

rise to the unusually close packing of the cross β-structure (16). The conformations were originally assigned in the solvent dimethylsulfoxide (DMSO). Spectra obtained during the transition from DMSO to H_2O indicate some conformational changes, but the gross structure is presumed to be conserved (19,20). The extensive structure-activity studies of neurohypophyseal hormones have been reexamined from a conformational standpoint, and this three-dimensional approach also formed the basis for considerations of their evolution and immunogenicity, as well as an hypothesis concerning their most likely pathways of enzymic inactivation (16). Amide bonds within the 20-membered ring containing a β-turn should have greater resistance to enzymic attack than amide bonds present in the acyclic tail portion of the hormones. Moreover, it is expected that peptide bonds sequestered by the ring structure will be less susceptible to enzymic cleavage than bonds of identical residues exposed to the environment. Both of these hypotheses about enzymic degradation are examined and confirmed in this section. The second is borne out by two types of experiment: first, by demonstrating increased susceptibility to enzyme action of acyclic oxytocin, lysine vasopressin and arginine vasopressin derivatives as compared to the intact hormones, and second, by comparing the susceptibility of natural hormones to that of analogs which are thought to possess a looser ring structure.

Materials

[9-^{14}C-1-glycinamide, 8-arginine]vasopressin and [9-^{14}C-1-glyanamide]oxytocin, each with a specific radioactivity of 30mC/mmole, and [9-^{14}C-1-glycinamide, 8-lysine]vasopressin, specific activity 25mC/mmole, were synthesized and stored as described in Section 2 (236). For the experiments in this study, 2 mg of unlabeled hormone was mixed with 0.01 mg of [^{14}C]hormone to give a specific activity of 0.15 mC/mmole. Non-radioactive oxytocin (135), [8-arginine]vasopressin (AVP) (237) and [8-lysine] vasopressin (LVP) (238) were synthesized by the solid phase method and assayed for biological activity as described in Section 2 (139,148,149). 1,6-Aminosuberic acid (Asu) analogs of the hormones (AsuAVP, AsuLVP and Asu oxytocin) were synthesized by classical methods of peptide synthesis (282) and assayed for avian vasodepressor activity on conscious chickens (148) according to the method of Coon (149) or for rat pressor activity (139). All peptides exhibited the potencies reported in the reference above.

The reagents used are described in Section 2. Pyrollidone carboxylyl peptidase isolated from P. fluorescens was a gift of Dr. R. F. Doolittle, University of California at San Diego.

Methods

High voltage electrophoresis was carried out on strips of Whatman 3MM paper (60x3.0cm). Unless otherwise stated, conditions were 3000V for 1 hr using a 0.5% pyridine-5% acetic acid buffer, pH 3.5. Radioactivity on paper electrophoretograms was detected with a Packard Model 7201 chromatogram scanner; radioactivity in solution on a Packard Model 3003 liquid scintillation spectrometer; and non-radioactive compounds on electrophoretograms by spraying with a cadmium-ninhydrin solution, all as described in Section 2.

Amino acid analyses were carried out according to the general method of Spackman et al. (142): peptides were hydrolyzed in vacuo in 6N HCl containing 0.2% phenol and 0.1% mercaptoacetic acid (239) for 22 hr at 110C, dried over NaOH, and analyzed on a Beckman 120C amino acid analyzer.

Preparation of S-carbamoylmethylcysteine Derivatives

Oxytocin, AVP, and LVP were alkylated using the method of Liu and Meienhofer (244). In a typical procedure, 2.0 mg (2 μmole) of [^{14}C]AVP (corresponding to 300,000cpm) were dissolved in 3.0 ml 0.1M sodium phosphate buffer, pH 8.2. Nitrogen was bubbled through the solution for 10 min and

then 3.8 mg (24 μmole) of DTT in phosphate buffer was added and the mixture kept under N_2 for 90 min. A solution of 22 mg (120 μmole) of iodoacetamide freshly mixed in the same buffer was added and after an additional 20 min under N_2 atmosphere the pH of the mixture was adjusted to 3.0 with glacial acetic acid. The solution was concentrated to a small volume and subjected to gel filtration on a Sephadex G15 column (100x0.9cm) equilibrated with 0.1N formic acid, and eluted with the same solvent. Fractions of 2.3 ml were collected and 0.01-ml aliquots of alternate fractions were counted in 5.0 ml of counting solution. Fractions containing the product were pooled, concentrated to a small volume, and lyophilized. Recovery of radioactivity was about 90%, or 270,000cpm.

Incubations of Intact or Alkylated Hormones with α-Chymotrypsin

In a typical procedure 2.0 mg of intact or alkylated radioactive hormone (in the case of alkylated hormone, radioactivity now amounts to approximately 270,000cpm) were dissolved in 1.0 ml 0.2N $NaHCO_3$, pH 8.0. Chymotrypsin solution (0.067 ml of a 0.1 mg/ml solution in $NaHCO_3$, to give an enzyme-to-substrate ratio of 1:300,w/w) was added and the mixture stirred for 2.5 hr at 37C. At the end of the incubation period 50% acetic acid was added to adjust the pH to 4.0. The mixture was subjected to gel filtration

on the same Sephadex G15 column used above and eluted under the same conditions;radioactivity was measured, fractions pooled and peptides isolated as described. Again, recovery is 85-90%, or approximately 250,000 cpm.

An additional experiment was carried out in which alkylated [^{14}C] oxytocin was incubated at enzyme-to-substrate ratios of both 1:3 and 1:300, and for 2.5 and 24 hr. All of the above experiments were controlled by incubation of the appropriate peptide under identical conditions but without enzyme.

Identification of Enzymic Digestion Products

Radioactive material obtained from gel filtration of enzyme incubation mixtures was subjected to high voltage electrophoresis as described. In the case of experiments with [^{14}C] oxytocin the electrophoresis was also carried out at 1000V for 1.5 hr at pH 3.5 in order to test for glycinamide. In all cases the appropriate hormone or alkylated hormone was run as marker. In general, for elution 10% of the material was applied for electrophoresis; this amounts to ~25,000cpm; recovery from elution was about 60%. For analytical purposes only half this amount was spotted. Radioactive peaks were eluted with approximately 2.0 ml water or dilute acetic acid into ignition tubes;

the material was lyophilized, hydrolyzed, and subjected to amino acid analysis.

Performic Acid Oxidation of Intact Hormone and Enzymic Digest of Intact Hormone

Intact [^{14}C] hormone and aliquots of desalted hormone-enzyme digest were oxidized with performic acid according to the method of Brown and Hartley (283). In brief, the material to be oxidized was spotted on Whatman 3MM paper and the paper exposed to the vapors of performic acid (20 ml 98% formic acid and 1.0 ml 30% H_2O_2, freshly mixed) for 3 hr. After allowing the vapors to evaporate, products were separated by high voltage electrophoresis.

Digestion of Chymotryptic Peptides with Pyrollidone Carboxylyl Peptidase

The enzyme was prepared and incubated with substrate as described (284). An A-25 pellet was thawed and dissolved in 0.5 ml of 0.1M phosphate buffer, pH 7.3, containing 1mM EDTA and 10 mM β-mercaptoethanol. The solution was dialyzed against three changes of the same buffer for three hr at 4C. The $O.D._{280}$ of the enzyme solution was adjusted to approximately 1.0 and the enzyme was incubated with the eluted chymotryptic peptides in a ratio of 100 µl enzyme solution to 0.1 µmole peptide. After 20 hr the enzyme was inactivated by addition of 1.0 ml of absolute EtOH, the mixture was dried and redissolved in water, and aliquots were taken for

electrophoresis as described, along with aliquots taken at zero time. As controls, enzyme and peptides were incubated in buffer alone, and >Glu-Leu-Gly-NH_2 was incubated with the enzyme.

Edman Degradation

Peptides were subjected to one cycle of phenylisothiocyanate degradation by a modified three-stage procedure (247). The resulting thiazolinone was converted to the phenylthiohydantoin amino acid by treatment with 0.2 ml of 1.0N HCl at 80C for 10 min. An aliquot of the "subtractive" peptide was hydrolyzed and subjected to amino acid analysis.

Treatment of Intact, Unlabeled Hormones with α-Chymotrypsin

Hormones were dissolved at a concentration of 2 mg/ml in 0.2N $NaHCO_3$ pH 8.0. Chymotrypsin was dissolved in the same buffer at a concentration of 1 mg/ml, and aliquots were added to the hormone solution to give a final enzyme-substrate ratio of 1:3. Incubations were carried out for 24 hr at 37 C with aliquots removed after 3 and 10 hr. The enzyme reaction was stopped by addition of 50% AcOH to adjust the pH to 4.0, followed by lyophilization. Controls were incubated identically, except that 0.2N $NaHCO_3$ was added in place of chymotrypsin solution. Residual hormonal activity was determined by the pressor assay, with anesthetized male rats as described in the United States Pharmacopeia

(139). Lyophilizates were taken up in 0.9% NaCl and diluted to approximately the same level of activity as the standard (USP Posterior Pituitary Reference Standard, containing 22 mU/ml pressor activity). Each determination was carried out on two rats, and the experiment was performed in duplicate.

Results

Alkylation of Hormones

[^{14}C]oxytocin, [^{14}C]LVP or [^{14}C]AVP was reduced with DTT and the resulting free sulfhydryls were alkylated with iodoacetamide. H-Cys(S-Cam)-Tyr-Ile-Gln-Asn-Cys(S-Cam)-Pro-Leu-[^{14}C]Gly-NH_2 and H-Cys(S-Cam)-Tyr-Phe-Gln-Asn-Cys(S-Cam)-Pro-Arg(Lys)-[^{14}C]Gly-NH_2 gave upon gel filtration a single radioactive peak with an elution volume of ~90 ml. Both the oxytocin and vasopressin derivatives moved upon electrophoresis towards the cathode and gave single spots with mobilities of 0-0.8 cm (oxytocin derivative), 9.5 cm (AVP derivative), and 8.5 cm (LVP derivative). Under identical conditions intact [^{14}C]oxytocin, [^{14}C]AVP, and [^{14}C]LVP showed mobility values of 9.0 cm, 13.9 cm, and 13.8 cm. Amino acid compositions of the alkylated hormones are shown in Table 3.

Digestion of [^{14}C]Vasopressins and S-alkylated [^{14}C] Vasopressins by Chymotrypsin

Gel filtration of the chymotryptic digest of intact [^{14}C]AVP gave one major radioactive peak, eluting at fractions 35 to 50 (Fig. 36A). Material from this peak traveled on electrophoresis with the same mobility as [^{14}C]AVP (Fig. 37A). Elution of the radioactive material from the electrophoretogram, hydrolysis, and amino acid

Table 3. Amino Acid Composition of Alkylated Peptides

	S,S'-dicarbamoylmethyl-cysteine, [^{14}C] AVP	S,S'-dicarbamoylmethyl-cysteine, [^{14}C] LVP	S,S'-dicarbamoylmethyl-cysteine, [^{14}C]oxytocin
Arg	1.0	---	---
Lys	---	1.0	---
CMCys	1.9	2.4	2.0
Asp	1.0	0.9	1.0
Glu	1.0	0.8	0.9
Pro	1.0	0.7	0.7
Gly	1.1	0.7	0.9
Ile	---	---	1.0
Leu	---	---	1.0
Tyr	0.9	1.0	0.7
Phe	1.0	1.0	---

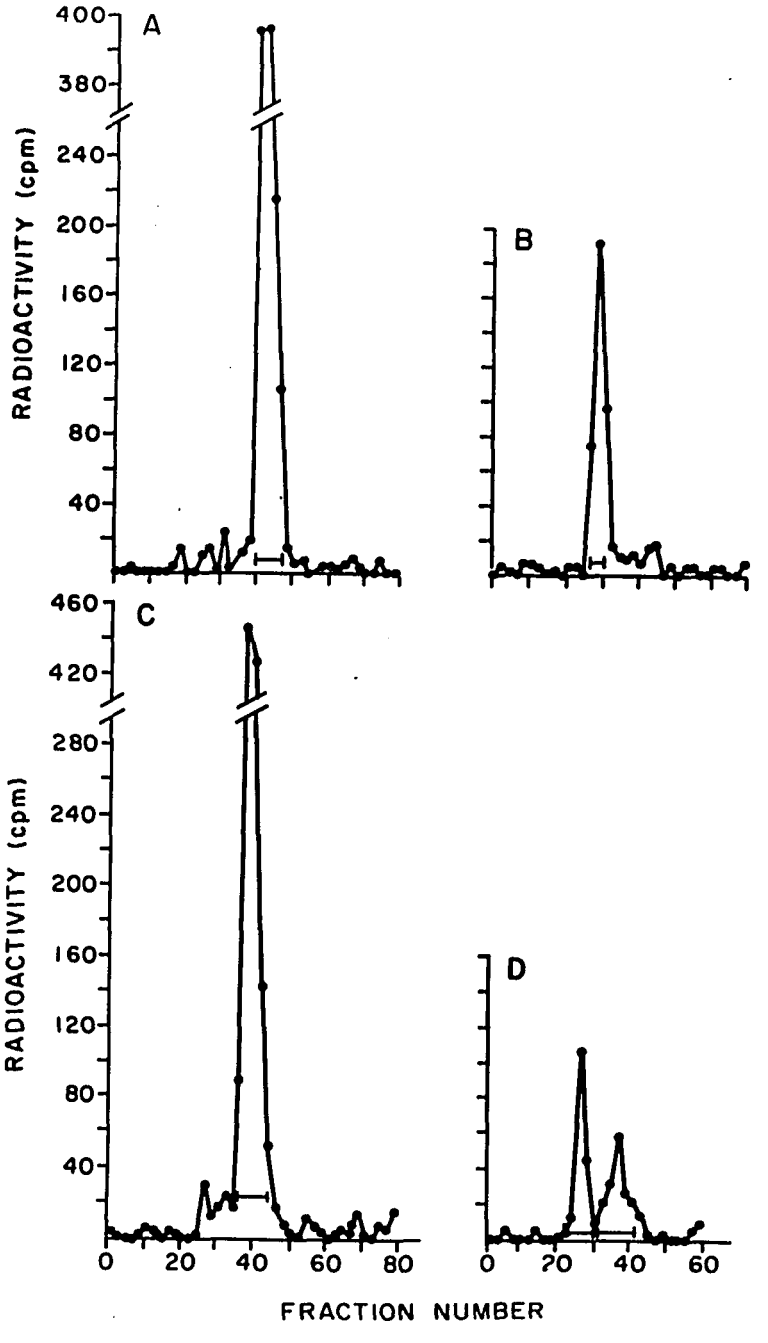


Fig. 36. Gel filtration elution pattern of chymotryptic incubation of intact [^{14}C] AVP(A) and [^{14}C] oxytocin(C), and of S,S'-dicarbamoylmethylcysteine- [^{14}C] AVP(B) and [^{14}C] oxytocin(D) on Sephadex G15 with 0.1N HCOOH. The following fractions were pooled and used for further study: A 35-50; B 24-34; C 35-45; and D 23-30 and 31-42.

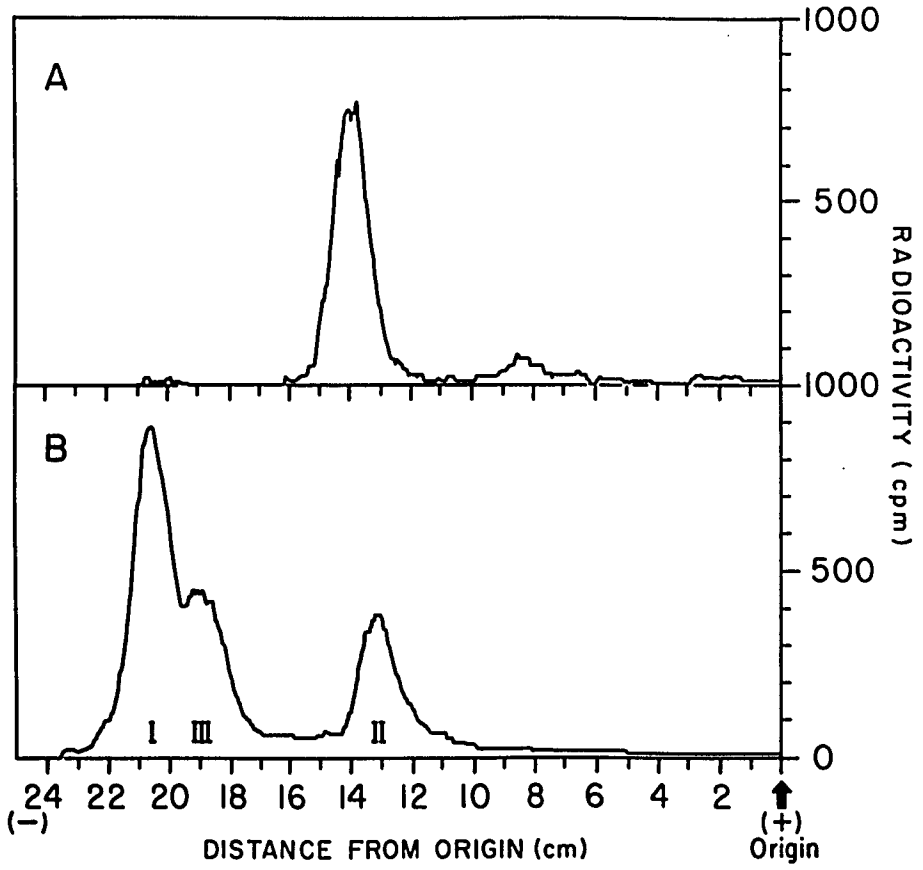


Fig. 37. Electrophoretic separation of radioactive products from the chymotryptic incubation of [^{14}C] AVP(A) and of S,S'-dicarbamoylmethylcysteine-[^{14}C] AVP(B). An aliquot of pooled radioactive material from the gel filtration was subjected to high voltage electrophoresis at pH 3.5 for 1 hr at 3000V, in a buffer of 5% acetic acid-0.5% pyridine.

analysis gave a composition identical to AVP (Table 4). When an aliquot of the digest of intact [^{14}C]AVP was treated with performic acid before electrophoresis, it gave the same pattern as performic acid-treated [^{14}C]AVP.

Gel filtration of the chymotryptic digest of S-alkylated [^{14}C]AVP gave a single peak eluting from fraction 24 to 34 (Fig. 36B). Material from this peak was resolved by electrophoresis into two components, the more basic (peak I) with a mobility of 21.0 cm towards the cathode and the other (peak II) with a mobility of 13.0 cm (Fig. 37B). Elution, hydrolysis, and amino acid analysis revealed that the composition of both was the same and corresponded to that of the C-terminal hexapeptide of AVP (Table 4). It was noted that peak II showed only a light ninhydrin color, although it contained three times as much material as judged by amino acid analysis. Furthermore, after storage at room temperature, or in the freezer (-20C), the proportion of the two peaks in the digest mixture changed, i.e., amount of radioactivity under peak I decreased and that under peak II increased; however, no attempt was made to quantify these changes. If peak I, after elution, was rerun on high-voltage electrophoresis, it separated again into the two peaks; this phenomenon was not found with peak II.

Table 4. Composition of Peptides Derived from Chymotryptic Digests of Neurohypophyseal Hormones

	Intact AVP	Alkylated AVP I	II	III (shoulder)	Intact LVP	Alkylated LVP I	II	III (shoulder)	Intact Oxytocin	Alkylated Oxytocin I	II
Arg	1.0	0.8	0.8	0.9	---	---	---	---	---	---	---
Lys	---	---	---	---	1.0	0.6	0.8	0.9	---	---	---
CMCys	---	0.5	0.6	0.6	---	0.7	0.7	0.7	---	1.1	1.4
½Cys	1.4	---	---	---	1.4	---	---	---	1.4	---	---
Asp	1.1	1.2	1.0	1.1	0.9	1.1	1.0	1.0	1.1	1.1	1.0
Glu	0.9	1.0	1.0	1.0	0.8	0.9	1.0	1.0	1.0	1.0	1.0
Pro	0.9	1.0	1.1	0.9	0.7	1.0	1.0	0.7	1.0	1.0	1.0
Gly	1.0	1.0	1.3	0.7	0.8	1.0	1.0	0.7	1.1	0.7	1.2
Ile	---	---	---	---	---	---	---	---	1.2	0.9	0.8
Leu	---	---	---	---	---	---	---	---	1.2	1.0	1.0
Tyr	0.8	---	---	---	1.0	---	---	---	0.7	---	0.7
Phe	1.0	---	---	0.6	1.0	---	---	0.9	---	---	---

In some experiments, a shoulder (peak III) was observed on peak I of the chymotryptic AVP digest; this shoulder had the amino acid composition shown in Table 4; indicating that it is the C-terminal heptapeptide of AVP. Chymotryptic digests of intact and S-alkylated [^{14}C]LVP gave identical electrophoretic patterns to the [^{14}C]AVP incubations except that peaks I and III were resolved in the digest of S-alkylated LVP (peak I, 21.9 cm, peak III, 20.0 cm, and peak II,13.7 cm from the origin). Amino acid composition of each of the peaks of the [^{14}C]LVP digest is included in Table 4.

Incubation with Pyrollidone Carboxylyl Peptidase

Peaks I and II resulting from the chymotryptic digest of [^{14}C]AVP were each incubated with pyrollidone carboxylyl peptidase. Electrophoresis of the digest of peak II revealed a new spot which migrated identically with authentic pyroglutamic acid (1.0 cm toward the anode); both compounds were identified with cadmium-ninhydrin spray. At the same time the radioactivity located at the position of peak II decreased nearly to zero and a new radioactive peak moving 15.0 cm toward the cathode appeared. Surprisingly, the same results were obtained with the digest mixture of peak I; this was explained by the control

incubation (peptide of peak I without enzyme) in which it was demonstrated that after 20 hr at 30C in the buffer used for enzyme digestion, the electrophoretic pattern of material originally associated with peak I became identical to that of peak II.

Edman Degradation of Chymotryptic Peptides

Peaks I and II resulting from the chymotryptic digests of [^{14}C]AVP and [^{14}]LVP were subjected to one cycle of the manual Edman degradation. The resulting phenylthiohydantoin amino acids were difficult to identify, but amino acid analysis of "subtractive" peptides in both cases revealed that the N-terminal Gln was removed from peak I, but not from peak II (Table 5).

Digestion of [^{14}C]oxytocin and S-alkylated [^{14}C]oxytocin by Chymotrypsin

Gel filtration of the digest of intact [^{14}C]oxytocin gave the pattern shown in Fig. 36C; with a single peak from fraction 35 to 45. The electrophoretic pattern of this material is shown in Fig. 38A; again only one peak is detected which corresponds in its electrophoretic mobility to intact hormone. Elution of this peak yielded a peptide with an amino acid composition identical to oxytocin (Table 4). In another experiment we found that performic acid oxidation

Table 5. Amino Acid Composition of "Subtractive" Peptides After One Cycle Edman Degradation of Chymotryptic Peptides from $[^{14}C]$AVP and $[^{14}C]$LVP

	$[^{14}C]$AVP		$[^{14}C]$LVP	
	Peak I	Peak II	Peak I	Peak II
Lys	---	---	1.0	1.1
Arg	1.0	1.2	---	---
CMCys	1.2	1.1	1.1	1.0
Asp	0.9	1.0	1.0	1.0
Glu	0.4	0.9	0.2	0.7
Pro	1.0	0.8	1.0	0.7
Gly	1.2	1.0	0.8	0.6

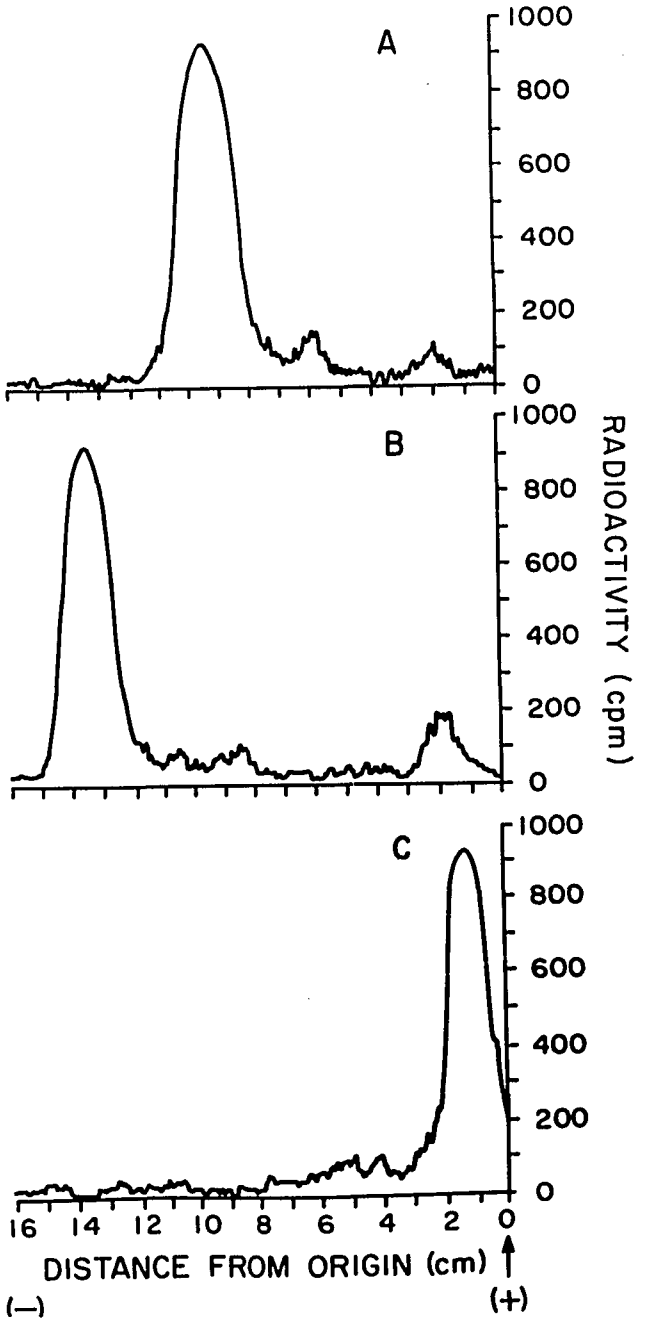


Fig. 38. Electrophoretic separation of radioactive products from the chymotryptic incubation of [^{14}C] oxytocin(A) and S,S'-dicarbamoylmethylcysteine- [^{14}C] oxytocin (aliquot from fraction 21-30(B) and 31-42 (C) from gel filtration; see Fig. 36,D). Same experimental condition as given under Fig. 37.

of this material before electrophoresis gave a pattern identical to performic-acid oxidized [^{14}C]oxytocin.

The chymotrypsin digest of S-alkylated oxytocin gave upon gel filtration the elution pattern shown in Fig. 36D. Two peaks were present, one eluting at fractions 23 to 30, and the other between 31 and 42. The amino acid composition of samples of both peptides is shown in Table 4. Aliquots (10%) taken from each of these peaks traveled as a single component on electrophoresis (Fig. 38B and C). Elution followed by amino acid analysis gave the composition of ninhydrin-active components shown in Table 4. Peak I (fraction 31-42) has a composition identical to S-alkylated oxytocin and peak II to the C-terminal heptapeptide of oxytocin, H-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2.

Under our experimental conditions for ch[illegible] digestion of [^{14}C]oxytocin, no release of [^{14}C]Gly-NH_2 was detected. However, when [^{14}C]oxytocin was incubated with chymotrypsin at an enzyme-substrate ratio of 1:3, [^{14}C]Gly-NH_2 was released. The amide was identified by comparison to authentic glycinamide (detected with Cd-ninhydrin spray). After the 2.5-hr chymotryptic digest about 10% of the radioactivity was present as H-Gly-NH_2, and after 24 hr of incubation all radioactivity was associated with H-Gly-NH_2.

Inactivation of Unlabeled Hormones and Analogs by Chymotrypsin

Several unlabeled neurohypophyseal hormones and analogs (none of which contained a Leu-Gly-NH_2 sequence) were treated with α-chymotrypsin for varying time intervals. Residual hormonal activity was determined by bioassay. The results shown in Table 6 show that LVP and AVP are in general considerably more resistant to inactivation by chymotrypsin than their corresponding 1,6-Asu- analogs. The initial decrease of activity of LVP and AVP sometimes observed may be due to non-specific binding of hormone to enzyme, similar to that seen in other studies (285).

Table 6. Hormone Inactivation by α-Chymotrypsin[a]

		Zero Time	Incubation (2.5 hours)	Inactivation (in %)	Incubation (10 hours)	Inactivation (in %)	Incubation (24 hours)	Inactivation (in %)
LVP	C	255	284	(27,29)	227	(35,25)	161	(33,23)
	E	276	208		147		108	
AVP	C	340	368	(9.6,10)	331	(0,0)	384	(58,48)
	E	356	333		350		162	
Asu LVP	C	---	5.3	(0)	4.6	(44)	5.3	(96)
	E	---	6.2		2.3		0.3	
Asu AVP	C	---	30	(59,72)	31	(90,80)	21	(98,98)
	E	---	12		3		0.35	
Asu [Arg^8] oxytocin	C	---	21	(69,72)	21.4	(78,96)	30	(97,98)
	E	---	6.6		4.8		0.98	

[a]Numbers represent U/mg rat pressor activity in control (C) or experimental (E) samples. Values are the averages from determinations on two rats from one experiment; the results of the duplicate experiment were, however, practically identical and the percent of inactivation in the second experiment is the second value in the parentheses.

Discussion

The inactivation of neurohypophyseal hormones by purified enzymes has been studied in some detail (26). In early investigations dealing with the characterization of naturally occurring and synthetic neurohypophyseal hormones (286-289), α-chymotrypsin was used to inactivate oxytocin. Vasopressins, which were found to be resistant to the action of this enzyme, were degraded by purified trypsin (8). Barth et al. (249) later identified C-terminal glycinamide as the sole product released from both intact oxytocin and some acyclic derivatives of the hormone upon incubation with α-chymotrypsin, and confirmed glycinamide release from lysine-vasopressin by trypsin. The results of the chymotrypsin experiments were unexpected, since the enzyme fails to attack the peptide bonds of the aromatic residues -- normally its preferred specificity -- and instead cleaves the usually less susceptible leucine peptide bond (290).

When we repeated the experiments of Barth et al.(249) using similar experimental conditions but oxytocin specifically [^{14}C]labeled in the glycine residue (236) we confirmed glycinamide release, with all radioactivity being associated with free glycinamide after a 24-hr incubation.

However, incubation of intact [^{14}C]oxytocin with chymotrypsin at a low enzyme-to-substrate ratio did not

produce cleavage in any part of the hormone molecule. Similarly, intact [^{14}C]AVP and [^{14}C]LVP were not cleaved by chymotrypsin. Performic acid oxidation of these enzyme-hormone digests gave only one radioactive spot, identical to performic acid-oxidized intact hormone, confirming that chymotrypsin left the peptide chain intact.

In contrast, the acyclic derivatives, [S,S'-dicarbamoylmethylcysteine, ^{14}C]hormones, were attacked by α-chymotrypsin even at the low enzyme-to-substrate ratio. Barth *et al.* (249) had reported that the enzyme did not split any peptide bond other than the Leu-Gly-NH_2 linkage in the two acyclic oxytocin derivatives, S,S'-dimethyl dihydrooxytocin and 1,6-dialanine oxytocin. In the present study, identification of a peptide with an amino acid composition corresponding to the sequence H-Ile-Gln-Asn-Cys-Pro-Leu-[^{14}C]Gly-NH_2 clearly demonstrates cleavage of the Tyr-Ile bond of the nonapeptide. Similarly, identification of peptides corresponding in their composition to H-Gln-Asn-Cys-Pro-Arg(Lys)-[^{14}C]Gly-NH_2 and H-Phe-Gln-Asn-Cys-Pro-Arg(Lys)-[^{14}C]Gly-NH_2 in the digests of the S-alkylated vasopressins indicates cleavage at the Tyr-Phe and Phe-Gln peptide bonds. The data do not exclude the possibility that α-chymotrypsin first attacks the Tyr-Phe peptide bond and then releases Phe from the heptapeptide in a fast step. The amount of S-alkylated hormone cleaved was not quantified, but the gel

filtration pattern of the chymotryptic digest of [S,S'-(Cam)cysteine-^{14}C]oxytocin reveals that about 50% cleavage occurs. In the case of S-alkylated [^{14}C]AVP and [^{14}C]LVP it appears that complete breakdown takes place even at the low enzyme-to-substrate ratio and in the short incubation time used.

The presence of two radioactive peaks with different electrophoretic mobilities but the same amino acid composition in the digests of the [^{14}C]vasopressins will be noted. Several lines of evidence suggest that the N-terminal Gln residues cyclized partially to pyroglutamic acid during proteolytic digestion and subsequent purification steps: (a) the difference in intensity of the Cd-ninhydrin color of the two spots, (b) the change in proportion upon storage of the two peaks obtained from chymotryptic digestion, and (c) the presence of only one fragment with the composition of the heptapeptide sequence in the oxytocin digest in which there is no peptide with an N-terminal Gln. Moreover, Edman degradation of the peptide from peak I of both [^{14}C]AVP and [^{14}C]LVP chymotryptic digests, removed an N-terminal Gln while peptide from peak II remained intact (Table 5). If a peptide contains an N-terminal glutamine which has been converted to pyroglutamic acid, no reaction with

phenylisothiocyanate and no degradation of the peptide occurs; so a peptide which contains glutamic acid and which is not degraded may be tenatively concluded to have an N-terminal Gln which has cyclized (291). The release of pyroglutamic acid from peptide of peak II by pyrollidone-carboxylyl peptidase definitively demonstrates that cyclization occurs. Peptide from peak I is converted to peptide from peak II under the conditions of the enzyme digest, and therefore also releases pyroglutamic acid, demonstrating the ease with which the N-terminal glutamine can cyclize.

In summary, intact [^{14}C]oxytocin and [^{14}C]vasopressins are not attacked in the ring portion by α-chymotrypsin, although at a high concentration the enzyme does release [^{14}C]glycinamide from oxytocin. In contrast, once the disulfide bond is eliminated the enzyme readily attacks at its points of preferred specificity, i.e., at the carboxyl side of aromatic residues (Fig. 39).

It is becoming apparent that the informational content of a protein resides in its amino acid sequence insofar as that sequence determines conformation. Susceptibility to proteolysis is now one of the more generally used criteria for detecting a change in a protein's conformation (269). As early as 1938 Linderstrøm-Lang (292) realized that disruption of the tertiary structure of a protein

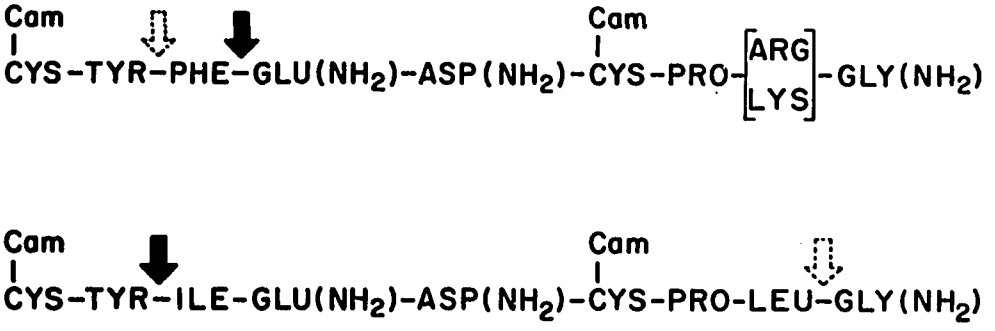


Fig. 39. Sites of cleavage of acyclic analogs of oxytocin, arginine- and lysine-vasopressin by α-chymotrypsin.

increased the rate of its hydrolysis by proteases. Studies on ribonuclease A reveal that thermal denaturation of this protein leads to increased susceptibility to the action of ficin (293), trypsin, and chymotrypsin (294,295). In addition, a strongly-bound ribonuclease inhibitor, 3'-cytidylate, prevents cleavage of the protein by trypsin, chymotrypsin, and subtilisin, even though the binding site is far removed from the bonds reported to be cleaved (296). The pH optimum of pepsin has been ascribed to the structure of its substrates: i.e., at pH 3-4, proteins such as albumin are denatured and can be hydrolyzed without difficulty (297). Furthermore it has been demonstrated that the disulfide linkages in serum albumin prevent its hydrolysis by trypsin (298,299). Studies on the action of papain have given similar results (300). In the conversion of proinsulin to insulin peptide bonds of certain Lys and Arg residues are sensitive to trypsin action, and are cleaved to release insulin and C-peptide, while the bonds of other basic residues are not attacked (266). A general explanation of both the labilization and stabilization of proteins to proteolytic action is conformational rearrangements which alter the accessibility of the critical peptide bonds to the enzyme(269). The three-dimensional organization of the

protein substrate therefore imposes limitations on enzyme activity. These are superimposed on the "specificity" profile of the enzyme deduced from small acyclic polypeptide substrates, which possess maximal conformational freedom.

With low molecular weight peptides little is known about conformational influences on enzymic degradation, due in part to the fact that only recently have preferred structures of peptides been elucidated. However, there have been some indications of constraints imposed on enzymic hydrolysis of small peptide substrates. For example, cyclic pentapeptide cyclo(Gly-Lys-Gly-Lys-Gly) is resistant to trypsin hydrolysis, whereas the corresponding linear peptide is hydrolyzed rapidly (301). Similar results have been obtained with cyclo(Gly-Gly-Lys)$_2$ and its acyclic congener (302). Furthermore, the size of certain synthetic peptides contraining glycine and lysine influences the rate of their hydrolysis by trypsin (303).

The inability of α-chymotrypsin to cleave after the aromatic residues of the neurohypophyseal hormones represents another example. The peptide bonds of the aromatic residues, as part of the 20-membered β-structure of the hormones, are apparently not accessible to α-chymotrypsin. Thus, even in a relatively small peptide, which may be expected to be flexible in solution, and which in fact has

been judged on the basis of deuteron magnetic resonance to have "apparent flexibility" (232), there is enough rigidity to prevent enzymatic attack at a normally preferred site. The acyclic tail portion, while it also forms a β-structure in solution, is less rigid and, as shown with oxytocin, susceptible to enzymatic attack. Once the disulfide bond of the hormones is eliminated, and the peptide backbone exhibits maximal conformational freedom, bonds formerly sequestered in the ring structure become available for enzymic cleavage.

Even a less drastic change in the topography of the hormonal peptides can alter susceptibility to chymotryptic cleavage. The rate of inactivation by α-chymotrypsin of neurohypophyseal hormone analogs in which the N-terminal amino group is replaced by a hydrogen atom and the disulfide bridge by an ethylene moiety (282,304,305), is significantly greater than that of the parent hormones (Table 6). In this context it is important that Sakakibara and Yamanaka (personal communication) have observed that chymotrypsin cleaves Asu-oxytocin at the carboxyl side of Tyr. From an earlier study it was tentatively concluded that the replacement of sulfur by an ethylene bridge perturbs the backbone conformation of the peptide. Now we suggest that one of the effec

of the chemical modification is to loosen the β-conformation of the 20-membered ring. As a result, peptide bonds within the ring become susceptible to enzymatic cleavage.

In a different type of study, Schally et al. showed that the anti-parallel dimer of LVP, which has a 40- rather than a 20-membered ring and consequently is expected to have a more flexible structure than LVP, is susceptible to the action of α-chymotrypsin (306).

These results have implications for the study described in Section 2. Neurophysin II was found not to be susceptible to cleavage by chymotrypsin unless the seven disulfide bonds are reduced. Since reduction must take place after formation of the covalent protein-peptide complex, the neurohypophyseal hormone will also be reduced, and be attacked at the sites identified. This is demonstrated by the finding of the C-terminal hexapeptide of $[^{14}C]$AVP as one of the labeled products released during digestion of the covalent complex, and illustrates the need for a different location of label in the hormone during such affinity labeling studies.

The results presented here also have implications for the in vivo degradation of the neurohypophyseal hormones. Enzymatic attack on these hormones will preferably occur at points in the acyclic portion, while conformational

constraints may protect the ring portion. Chymotrypsin-like enzymes present in rat uterus and rat kidney release glycinamide from oxytocin, and in general tissues seem to contain high levels of enzymes which can attack the Leu-Gly-NH_2 bond. Moreover, human uterus, kidney of some species, and rat brain tissue contain enzymes capable of releasing the C-terminal dipeptide of both oxytocin and AVP (for summary see 16 and 27). The study described here seems to lend support for the hypothesis (16) that cleavage of the disulfide bond of the hormones, followed by attack on peptide bonds formerly in the ring, is another important mode of <u>in vivo</u> hormone inactivation.

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