I. GENERAL INTRODUCTION

General Introduction

Endocrine neurones are the effector cells of neurohemal organ. Their main secretory product is a hormone which is released at the terminal membrane in response to the depolarization caused by the cation potentials arriving from the perikaryon. In mammal brain endocrine neurones are largely restricted to the hypothalamus, where they occur as two distinct populations. One includes the magnocellular neurones of the supraoptic (SON) and paraventricular (PVN) nuclei. Cells of both these nuclei elaborate and release the hormones oxytocin and vasopressin. The other comprises the parvicellular neurones that synthesize and secrete the releasing hormones responsible for regulation of the adenohypophysis. These are diffusely scattered throughout the hypothalamus, but are most densely concentrated in the region of the arcuate nucleus and in the periventricular zone. Whereas the hormones are produced in the above-mentioned region, the neurohypophysis is an integral part of this system. However, its function is not that of hormone production but of storage. Because of the anatomic proximity the hypothalamo-neurohypophysial system functions as a single integral unit.

Antidiuretic hormone (ADH) is synsthesized and packed in neurosecretory granules with an intragranular protein, neurophysin. The demonstration of axon flow of neurosecretory granules from the

perikarya in the hypothalamus to the posterior lobe of the pituitary and subsequent release into the blood stream has been an important historical chapter in our understanding of neurosecretion. Immunohistochemical studies using antibodies to vasopressin and neurophysin demonstrated that the magnocellular system is more diffusely distributed throughout the hypothalamus than was previously appreciated. In addition, vasopressin and oxytocin are formed in both the supraoptic and paraventricular neurones.

Swaab et al. (1975) using immunofluorescent labeling showed that oxytocin cells comprised 31% of the supraoptic and 40% of the paraventricular nuclei, whereas vasopressin cells comprised 53% of the supraoptic and 50% of the paraventricular nuclei with 10-20% of cells showing no staining reactions. Since the supraoptic nucleus of the rat contains about three times more neurosecretory cells than the paraventricular nucleus (Bandaranayake, 1971) it would appear that the former may be quantitatively more important for release of both vasopressin and oxytocin.

For many years it has been known that some neurosecretory cells lie outside of the main body of the supraoptic and paraventricular nuclei in what has been termed the accessory neurosecretory nuclei (Bandaranayake, 1971). Recent immunofluorescent work has shown that a number of neurophysin-containing cells are also to be

found in the suprachiasmatic nucleus (De Mey et al., 1974; Zimmerman et al., 1975). Moreover these nuclei contain a significant amount of radioimmunoassayable vasopressin (George and Jacobowitz, 1975). No one knows where these suprachiasmatic cells project to but there is evidence that median eminence contains neurophysin (Zimmerman et al., 1975). There is a significant amount of both neurophysin and vasopressin in hypophysial portal blood (Zimmerman et al., 1973).

The major pathway of secretion of the antidiuretic hormone (ADH) is the supraoptico-hypophysial tract to the posterior lobe. But additional secretory pathways have been demonstrated. One of the most unexpected findings of the immunocytochemical work on neurophysin and vasopressin was the presence of fibre terminals around portal capillaries (Parry and Livett, 1973; Zimmerman et al., 1973, 1975). Vandesande et al., (1974) suggested that they may come from the suprachiasmatic nucleus in the rat. There may be another pathway for vasopressin which involves the cerebrospinal fluid (CSF). The presence of vasopressin in CSF has been known for some time and experiments by Vorheer et al. (1968) suggested that the hormone was secreted into CSF in the dog. The source of secretion of vasopressin into CSF is not fully established. However, more recent transmission and scanning electron microscopic studies in mammals have demonstrated a variety of free nerve endings

in the floor of the third ventricle, particularly at the level of the median eminence and anteriorly near the organum vasculosum of the lamina terminalis (Scott et al., 1974). The origin of these fibres is thought to be the SON or the PVN. If, in fact, these fibres do originate in the SON or PVN, they may be related to the presence of ADH in the CSF.

The function of the secretory pathway of vasopressin and neurophysin found in tanycytes in some studies of rats (Zimmerman et al., 1975) and monkeys (Robinson and Zimmerman, 1973) suggested that these specialized ependymal cells in the infundibular recess of the third ventricle may take up neurophysin and possibly vasopressin from CSF and transport it to portal blood. This interpretation is based on the current concept that the major function of tanycytes is to transport rather than synthesize neuropeptides (Knowles, 1972; Knigge and Scott, 1970).

The pathways(s) involved in the activation of the endocrine cells are largely unknown. In all probability, these cells receive a continuous excitory input from outside the hypothalamus because the activity of endocrine neurones in the paraventricular nuclei is reduced by hypothalamic deafferenation (Dyball and Dyer, 1971). In addition, since interneurones behave somewhat differently from endocrine cells, the final integration of the input probably

occurs in the effector cells itself. However, interneurones may be responsible for some coordination of the activity of the secretory cells, as for example in the synchronous discharge of oxytocin cells in suckled rat. Interneurones close to the endocrine neurones may also be involved in the process of recurrent inhibition.

Synaptic excitation of neurosecretory cells in SON and PVN of the hypothalamus appears to be requisite for the release of vasopressin because afferent nerve impulses from peripheral receptors must be transmitted to the neurosecretory cells in the SON and PVN. The identity of chemotransmitters is still elusive. Shute and Lewis (1966) have shown that in SON, PVN, and mammillary nuclei some neurons contain small or moderate amounts of acetylcholinesterase (AchE) in their cell bodies and both the nuclei receive a rich adrenernic innervation. The early work of Pickford (1939, 1947), O'Connor (1945), and Verney (1947) and Cross and Harris (1952) suggested that acetylcholine (Ach) acted as an excitory and adrenaline as an inhibitory chemotransmitter for the release of both hormones and that the action of Ach involved nicotinic receptors. The effect of microiontophoretic application of putative transmitters on unit activity in antidromically identified neurosecretory cells in the SON and PVN has now been investigated (Barker et al., 1971; Cross et al., 1971; Dreifuss and Kelly, 1972). These studies

support the presence of an excitory nicotinic and an inhibitory muscarinic action of Ach and an inhibitory action of noradrenaline (NA) mediated by β-adrenoreceptors. On the other hand, Milton and Patterson (1971) showed that microinjection of NA in the SON in the cat releases vasopressin. Other experiments in which drugs were injected into the cerebroventricles in the dog suggest that the release of vasopressin is mediated by both muscarinic and α-adrenergic receptors and inhibition of release by β-adrenoreceptors (Bhargava et al., 1972).

Thus, in spite of the controversy, there is a general agreement that the neurones receive both cholinergic as well as adrenergic innervation. However, the results must be interpreted with caution since it has been shown by electrophysiological technique that complicated interconnections exist between these two nuclei: the electrical stimulation of SON activates or inhibits cells of PVN and vice versa (Yamashita et al., 1970).

RESUME OF LITERATURE

The Antidiuretic Hormone (ADH)

Historical Background

Extracts of the posterior pituitary were first identified to have biological activity in the last half of the nineteenth century when vasodepressor as well as vasopressor activity was described (Heller, 1974). By 1913 the antidiuretic effect of vasopressin

was established, and its therapeutic benefit in patients with central diabetes insipidus was demonstrated (Farini, 1913; von Den Veldin, 1913). In spite of early studies which suggested that the posterior pituitary did not have the appearance of a gland which was actively synthesizing hormones and in spite of the known neural connections with the hypothalamus (Cajal and Raymon, 1911) it was not appreciated that the neurohypophysis was part of the hypothalamic unit until the classic studies of Bargmann and Scharrer (1951) and Scharrer and Scharrer (1954). These investigators applied the chromealums-hematoxylin stain to the hypothalamus, and they were able to trace the posterior pituitary neurosecretory material along axons in the pituitary stalk to cell bodies in the SON and PVN of the hypothalamus. Furthermore, after transection of the pituitary stalk, Gomori-positive material accumulated above the transection and was lost from the posterior pituitary (Bargmann and Scharrer, 1951). This clearly established that the posterior pituitary hormones were synthesized in the hypothalamic nuclei and transported by axonal flow to the posterior pituitary. The posterior pituitary, which was previously considered the site of synthesis of hormone was shown to be a locus of storage and release.

At about the time that the Gomori-positive pathways to the neurohypophysis were being characterized by anatomists, biochemists,

pharmacologists and physiologists were identifying the active principle of the gland. Bioassay data from posterior pituitary extracts had shown a variety of actions: vasopressor, antidiuretic, galactogenic and uterotonic. The studies of Acher, et al. (1956) demonstrated that mild treatment (changes in pH and electrodialysis) could reversibly separate two biologically active hormones, vasopressin and oxytocin, from a carrier protein termed neurophysin. The loosely combined neurophysin and the peptide hormones formed the Van Dyke protein. Amino acid studies of vasopressin by du Vigneaud (1956) proved that vasopressor and antidiuretic actions of the posterior pituitary existed in a peptide which was separate from the peptide with oxytoxic activity. As bioassays were developed it was shown that oxytocin and vasopressin were concentrated in extracts of the SON and the PVN confirming the presence of hormone peptides throughout the system which had been described using Gomori stains (Heller, 1966). The identification of two individual hormones led to studies of the sites of synthesis and the concept emerged that the SON mainly formed vasopressin and the PVN, oxytocin. More recent data obtained by immunological methods demonstrate that this interpretation was an oversimplification. Both hormones are found in both nuclei, but axons of the PVN travel through the SON enroute to the posterior pituitary and therefore lesions in the SON can destroy most of the supraoptic and paraventricular system.

As mentioned earlier both the neurons contain both hormones. But since SON of the rat contains three times more neurosecretory cells than PVN (Bandaranayake, 1971) it would appear that the former may be quantitatively more important for release of the hormone. It is generally considered that SON is largely concerned with the synthesis of vasopressin and the PVN with oxytocin.

SON and PVN

Ultrastructure

The structure of SON and PVN reflect their secretory function. The perikarya of the cells are larger (30-40 μm) than the surrounding nonsecretory neurons of the hypothalamus. They have fewer dendrites (Leontovich, 1969). The cells contain an extensive endoplasmic reticulum (with varying degrees of dilatation) a distinctive perinuclear Golgi region, a large nucleus dense lysosomal body and most significantly, membrane-bound neurosecretory granules (0.1-0.3 μm in diamter) (Sloper and Bateson, 1965). Under usual conditions of fixation, the latter have a dense cored appearance (Morris and Cannata, 1973) and contain neurosecretory materials (La Bella et al., 1962; Pickup et al., 1973; Pelletier et al., 1974). The unmyelinated axons of the neurosecretory cells which usually emerges from the dorsal pole of the perikarya has the appearance of a thick dendrite, rather than a typical axon-hillock and is

unbranched (Leontovich, 1969). At intervals there are dilatations in which neurosecretory granules, mitochondria and microtubules are common (Rechardt, 1969). On entering the neurohypophysis, the axons make numerous expanded contacts with the base membrane of fenestrated capillaries. These terminal dilatations, which number 2000-6000 per cell (Cross et al., 1975) contain many neurosecretory granules, mitochondria and microvesicles. There are also non-terminal dilatations (600-4000 per cell) containing large numbers of granules, but no microvesicles (Cross et al., 1975).

The subcellular organelle in neurosecretory cells undergo pronounced morphological changes in response to an increased demand for the hormone secretion. Stimulating secretion of vasopressin in rats by osmotic stress results in a 25% increase in cytoplasmic area (Morris and Dyball, 1974; Ellman and Gan, 1971) with widening of the endoplasmic reticulum and extension of the perinuclear Golgi apparatus (Hatward and Jennings, 1973; Morris and Dyball, 1974). Ultrastructural changes induced by dehydration may commence very early. Halton and Walters (1973) observed an increase in the number of rat SO cells containing multiple nucleoli after only 2 hours of water deprivation. Regression of ultrastructural response which is associated with an increased lysosomal activity occurs within about two days (Morris and Dyball, 1974). The regulatory mechanisms governing the

subcellular changes during hypersecretion are unknown, although it has been suggested (Halton and Walters, 1973) that SO cells may release a tropic substance from their endings which acts as a feedback signal to the cell body.

Because of their obvious relation to neurosecretion, much attention has been paid to changes in number, size, and appearance of neurophypophysial system. The most consistent feature is a loss of granules and this has been shown to correlate with a fall in the hormone content (Morris and Cannata, 1973). Conversely, an experimentally induced increase of granule numbers is associated with an elevated hormone content (Dyer et al., 1973).

Action Potential

Classically, nerve cells observing various functions have been identified by ablation, stimulation or recording of intracellular potentials. All three techniques have been used to identify the magnocellular cells of the SON. Hayward and his colleagues (1974) have performed a number of studies on the electrical activity of magnocellular cells in the conscious monkey in various conditions. He recently reviewed the results of these investigations (Hayward, 1974). Three types of cells identified by antidromic stimulation were classified on the basis of spontaneous firing pattern as 'silent' (3%), continuously active (63%), and bursters (21%). Hayward

postulated that the silent group represented cells in which synthesis and transport were the predominant activities; that the continuously active cells were in a state of tonic secretion; and that the buster group were those from which the hormone was released in a pulsatile fashion. Under normal conditions, some 20 to 30% of the neurosecretory cells projecting to the neurohypophysis show a 'phasic' (Dyball, 1971; Koizumi and Yamashita, 1972; Wakerly and Lincoln, 1971) or low frequency 'burster pattern' (Hayward and Jennings, 1973).

Dyball (1971) studied the action potentials of SON cells by injecting hypertonic NaCl into the carotid artery of urethane anesthetized rats. He found a significant increase in the action potential activity in the SON, identified by antidromic stimulation. The electrical activity was completed in one minute, though the peak concentration of plasma ADH occured at 3 minutes. In conscious monkeys, Vincent et al. (1972) reported that cells in SON fell into two classes: one responding monophasically to intracarotid injections (1 ml of 2.7% NaCl in 5 seconds) and the other responding biphasically to the same stimuli, usually be excitation followed by inhibition. Cross et al. (1975) reported that the predominant effect of intravenous or intracarotid injections of hypertonic saline is to cause an increase in the firing of SON and PVN.

However, not all cells are excited. Dyball (1971) describes a portion of cells in the rat which displayed several seconds of inhibition after intracarotid saline injection.

Action potentials in the SON and PVN are dependent on the usual neuronal Na^+ entry mechanism, for in in vitro preparations removal of external Na^+ (Ishida, 1970) or introduction of tetradotoxin into the bathing fluid (Dreifuss et al., 1971) blocks spike conduction induced electrical stimulation. However, Na^+ entry during action potential does not appear to contribute to the process of hormone release as the secretory response to an excess in external K^+ is potentiated, not reduced, in Na^+-free media (Douglas and Poisner, 1964). The secretory response to electrical stimulation is also potentiated when external Na^+ is reduced (Dreifuss et al., 1971).

Mechanism of Hormone Synthesis

It is now well established that oxytocin and vasopressin are synthesized in the perikarya of the SON and PVN and transported down their axon to the neurohypophysis for storage and release. The hormones are stored in granules in association with carrier protein-neurophysin (Pickering et al., 1975; Pickup et al., 1973; Cross et al., 1975).

Initial hormone synthesis probably involves a ribosomal step because vasopressin production can be inhibited by puromycin, both

in vivo (Sachs et al., 1969) and in vitro (Pearson et al., 1975). Also in organ cultured hypothalamus neurohypophyseal system, the ability to synthesize vasopressin develops in parallel with rise in RNA levels (Pearson et al., 1975) and may be inhibited by bromotubercidin, an inhibitor of RNA synthesis (Pearson et al., 1975). It has been suggested that although hormone synthesis starts on ribosome attached to the rough endoplasmic reticulum, active hormone is not formed until a later stage, perhaps at the site of the Golgi region where packaging into granules occur (Sachs et al., 1969; Pearson et al., 1975). The final synthetic step may involve cleavage of a large precursor molecule (Cross et al., 1975). Final completion of hormone synthesis probably occurs as the neurosecretory granule is transported toward the neurophysis.

Transport

Transport of neurosecretory granules down the hypothalamo-neurohypophysial stalk is extraordinarily fast (1-4 mm/hr) compared to the rate of axoplasmic flow (1 mm/day) (Pickering et al., 1975). There may also be a slow (0.5 mm/day) transport mechanism (Norstrom, 1975). Transport rates go up with a greater demand for hormone secretion (Norstrom and Sjostrand, 1972). Microtubules are involved in the transport of granules as evidenced by the fact that colchicine treatment (which causes disruption of microtubules) leads to a

damming up of neurosecretory granules (Pearson et al., 1975). Also there is a correlation between microtubular number and transport activity (Grainger and Sloper, 1974).

Storage

In common with most peptide-secreting endocrine cells, the neurosecretory cells of SON and PVN store vast quantities of hormone in relation to the daily requirements for release. The neurophypophysis of the rat may contain some 500-600 mU of oxytocin and vasopressin whereas less than 50 mU are released daily (Pickering et al., 1975). Recent autoradiographic work (Heap et al., 1975) has emphasized the dynamic nature of the storage mechanism. Granules first appear at the terminal dilatation. A maximal demand for hormone may then cause the old granules to be transported to the terminal. The newest granules with these terminal dilatations might constitute the so-called 'readily releasable pool' of hormone (Sachs et al., 1969). However, the whole concept of a readily-releasable pool of hormone has been questioned recently by Nordmann (1975) who suggests that the fall-off in hormone output during severe stimulation may have little to do with the nonavailability of hormones, but rather with an inactivation of its calcium channel within the terminal membrane. The old granules which are not released, are broken down by lysosomal activity (Cross et al., 1975)

which may be transported in a retrograde direction to the perikarya for reincorporation into new hormone.

There is good evidence to suggest that hormone is distributed between at least two pools within the nerve terminals of the neural lobe. Acute stimulation of the gland in vivo and in vitro (Thorn, 1966; Sachs et al., 1967; Weinstein et al., 1960) only elicits release of a small portion of the gland hormone from a readily releasable pool. Most of the neural lobe hormone appears to be in a larger and less accessible store. Sachs and Haller (1968) showed that the readily releasable pool represents about 2% of the total hormone stored in the neural lobe. Thorn (1965, 1966), from in vitro experiments, calculated 5-10% for this pool.

Release of the Hormone

In order to reach the blood the hormone within the neurosecretory granules has to overcome several barriers: 1) the membrane of the granule; 2) the membrane of the nerve terminal; and 3) the basement membrane of the fenestrated endothelium of the neurohypophysial capillaries. The latter is thought to be by simple diffusion. Only the first two barriers are traversed by an active process.

Several hypotheses have been put forward regarding the release of the hormone. Douglas (1974) has given an excellent review on these different hypotheses. One hypothesis on the mechanism of

hormone discharge is that molecular disperson occurs within the granule, followed by release of the hormone into the cytoplasma and diffusion out of the nerve terminal. According to another hypothesis, the microvesicles of the neurosecretory terminals are supposed to contain acetylcholine which is somehow involved in the release. However, Lederis and Livingstone (1970) showed that microvesicular fraction of the neurohypophysial homogenate contained little acetylcholine activity. The most convincing hypothesis is that hormone release occurs by simple fusion of the neurosecretory granule membrane with the axon membrane - a process of exocytosis (Douglas, 1974; Dreifuss, 1975). The sequence of events is as follows. The first event is depolarization of the axonal terminal membrane. Hormone release from neurophysis in vitro may be triggered by electrical stimulation (Dreifuss, 1971; Ishida, 1970) even after action potential activity is blocked by tetrodotoxin (Tox) or a reduced extracellular sodium concentration (Nordman and Dreifuss, 1972). Depolarization of the new terminal, by increasing extracellular potassium, is a powerful method for releasing hormone (Douglas and Poisner, 1964). The next important event is the movement of calcium into the neurosecretory terminal. Neurohypophysis in a calcium-free medium show diminished hormone release (Douglas and Poisner, 1964; Dreifuss and Nordmann, 1974). In addition, the

application of ionophores (which form membrane channels for calcium movement) greatly increase the hormone output (Nakazato and Douglas, 1974; Russel, et al., 1974). The action of calcium once inside the neurosecretory terminal is unknown. Calcium might form ionic bridges between the negatively charged granule membrane and the negatively charged intracellular side of the axon membrane (Dean, 1974). Fusion of the granule and cell membranes is facilitated by their similarity of structure (Dreifuss, 1975). Fusion results in discharge of all the granular contents, which explains the release of the neurophysin as well as the hormone (Cheng and Freisen, 1973; Legros and Franchimont, 1972). All the above events are accomplished extremely rapidly and thus it is not surprising that good ultrastructural evidence for exocytosis has only recently been obtained (Nagasawa, et al., 1970; Dreifuss et al., 1973; Dreifuss et al., 1974).

A problem which had to be overcome before the exocytosis theory could be finally accepted was the fate of the neurosecretory granule membrane, for without some means of recapture there would be an unacceptable expansion of the neurosecretory cells. Elegant studies with horseradish peroxidase (Nagasawa et al., 1971) or tritiated H_2O (Nordman et al., 1974) have demonstrated the occurance of pinocytosis, whereby membrane is recaptured as small vesicles.

Neurophysin

McArthur (1931) and Rosenfield (1940) reported evidence for the presence of a large polypeptide having both oxytoxic and vasopressor activities. In 1942, van Dyke and his colleagues showed that the addition of NaCl to an acid extract of bovine neurophyses resulted in precipitation of a protein having all the biological action of posterior pituitary extract in the proportion found in the gland. This 'van Dyke protein' had a molecular weight of about 30,000 and was rich in cystine (Black and van Dyke, 1952). In 1956 Archer et al. isolated the protein part of the complex and coined the name 'Neurophysin'.

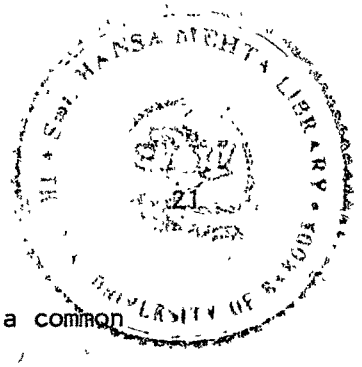
Neurophysin is elaborated by perikarya of the hypothalamo neurohypophysial neurones. Each vertebrate species elaborates a family of neurophysins, generally two major and one minor component (Pickering et al., 1974). The neurophysins are found within the same secretory granules as the hormones (Ginsburg and Ireland, 1963, 1966; Dean and Hope, 1966, 1967) and are thus packaged along with them in the perikarya.

Because of the readiness with which hormone and protein associate, it has been suggested that neurophysin functions as an intracellular carrier molecule to keep the hormone within the granule as they pass along the axons and become stored in nerve terminals

(Ginsberg, 1968). Others suggest (Sachs et al., 1969; Pickering et al., 1974) that the same biosynthetic process is concerned in the formation of the neurophysin and their associated hormones; e.g., the protein precursor of vasopressin found by Sachs and Takabataka (1964) is also the precursor of a specific vasopressin - neurophysin.

The hormone neurophysin complexes are formed primarily by electrostatic bonds between carboxyl groups in neurophysin and the free terminal NH_2 group in the peptide (Stouffer et al., 1963; Ginsburg and Ireland, 1964). However the fact that synthetic analogues of oxytocin with substituents for the tyrosine and leucine residues do not form complexes with neurophysin suggests that secondary hydrophobic bonds also normally contribute to the forces of association (Breslow and Abrash, 1966).

It now appears that the carrier proteins in the ox (Hollenberg and Hope, 1968; 1969), the pig (Uttenthal and Hope, 1970; Buford et al., 1971) and the rat (Burford et al., 1971; Burford and Pickering, 1972) contain three components or separate neurophysins, two major and one minor component. These do not show differential binding of the hormones <u>in vitro</u>, but there is good evidence that one of the major neurophysins is stored specifically with oxytocin and the other with vasopressin, possibly in separate neurosecretory granules (Dean, et al., 1968). It has also been suggested that hormone and



neurophysins may be linked biosynthetically as part of a common 'prohormone precursor' (Sachs et al., 1969; Buford and Pickering, 1972).

Porcine neurophysin II (associated with lysine-vasopressin, LVP) has been localized by immunofluorescent histochemistry mainly in the SON of the pig (Livett, et al., 1971). Correspondingly, in neurohyphophysial extracts from the rat, the neurophysin associated with oxytocin is preferentially labeled when 35S cystine injected into the PVN (Burford et al., 1971). These results are consistent with the known distribution of the two hormones in the SON and PVN.

In many of the species investigated, there is a third neurophysin component whose significance is obscure. From a study of the pattern of labeling of the three neurophysin in normal and heterosygous Brattleboro rats, Cross et al. (1975) concluded that the minor neurophysin component is a metabolic product of oxytocin-neurophysin and arises largely after the neurosecretory granules have arrived in the neural lobe. They further proposed that the neurosecretory granules must contain an enzyme which can affect the conversion of oxytocin-neurophysin to the minor component and this enzyme 'maturase' exists for the maturation of pro-vasopressin and pro-oxytocin, and further that oxytocin-neurophysin has a sensitive bond that can be slowly cleaved by the enzyme to give the minor neurophysin.

Mechanism of Hormone Synthesis

It is now well established that oxytocin and vasopressin are synthesized in the perikarya of the PVN and SON cells and transported down their axons to the neurohypophysis for storage and release. The hormones are stored in granules in association with carrier proteins, neurophysin-I for oxytocin and neurophysin-II for vasopressin (Pickering et al., 1975; Pickup et al., 1973; Cross et al., 1975).

Cross et al. (1975) assume that the initial synthesis of the prohormone (precursor) is a ribosomal step since it is blocked with puromycin (Takabataka and Sachs, 1964) and almost certainly occurs on the endoplasmic reticulum. After packaging the prohormone into the secretory granule, maturation continues during the axonal journey to the neural lobe.

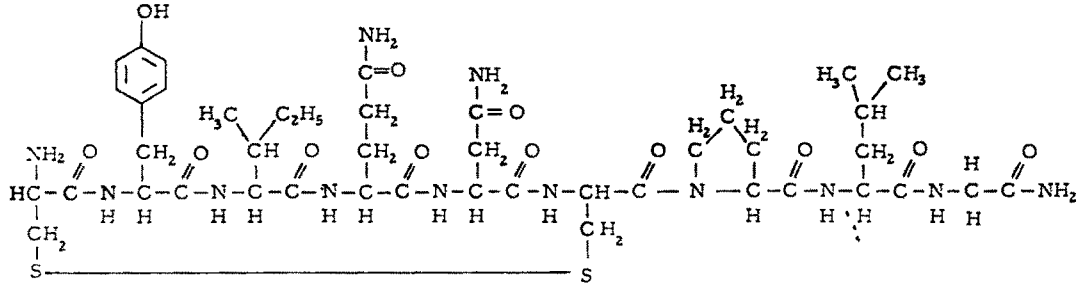
Structure-Activity Relations of ADH (SAR)

The isolation of arginine vasopressin (AVP) from beef glands and lysine-vasopressin (LVP) from hog gland and the structure elucidation and synthesis of these hormones were accomplished by du Vigneaud and associates (Turner et al., 1951; Popenoe et al., 1952; du Vigneaud et al., 1957; du Vigneaud, 1960; du Vigneaud, 1955; du Vigneaud et al., 1958). The amino acid sequence of AVP was elucidated independently by Acher and Chauvet (1953). The structure of AVP differs from the structure of oxytocin only in that a phenylalanine residue replaces the isoleucine residue in position 3 and an arginine residue replaces the leucine residue in position 8 (Fig. 1). In turn the structure of LVP differs from that of AVP only in that a lysine residue replaces the arginine residue. The neurohypophysis of most of mammals contain oxytocin and AVP. In the pig arginine is replaced by LVP. The occurance of both forms in wart-hogs and peccaries may represent a transitional stage in evolution (Lederis, 1969).

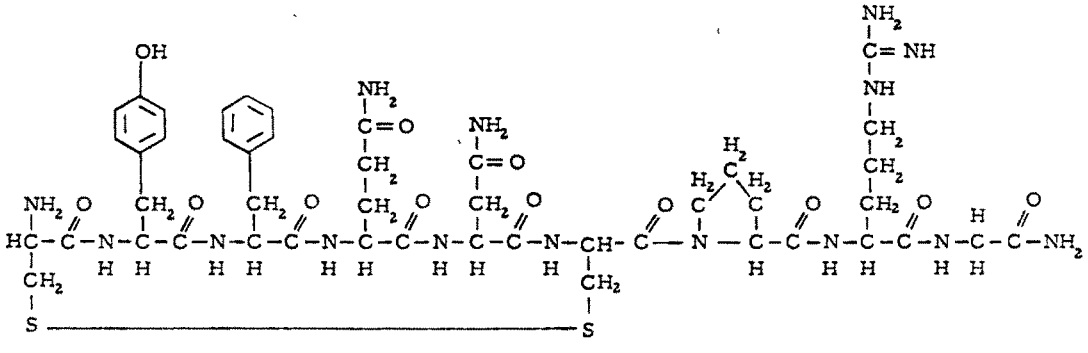
In lower vertebrates, four other neurohypophysial hormones have been identified which may be considered chemically as variants of oxytocin with different amino acid residues in position 4 or 8 or both (Boissonnas and Guttmann, 1968). Arginine-vasotocin (Arg^8-oxytocin) is a hybrid analogue consisting of the tripeptide tail of AVP attached to the ring of oxytocin. It is a naturally occuring

Figure 1

Structure of arginine vasopressin and oxytocin.



Cysteine	Tyrosine	Isoleucine	Glutamine	Aspar-agine	Cysteine	Proline	Leucine	Glycine amide
1	2	3	4	5	6	7	8	9



Cysteine	Tyrosine	Phenyl-alanine	Glutamine	Aspar-agine	Cysteine	Proline	Arginine	Glycine amide
1	2	3	4	5	6	7	8	9

H-Cys-Tyr-Phe-Glu (NH_2)-Asp-(NH_2)-Cys-Pro-Arg-Gly-NH_2

hormone in the pituitary of the chicken (Munsick et al., 1960). It is known to occur in all non-mammalian vertebrates and may be considered as the probable ancestor of all other active neurohypophysial principles (Sawyer, 1961).

Variation in Position 1

Substitution of the amino group in the 1-hemicystine residue - Sar-Cyst-Ille^{3}Leu^{8} - vasopressin proved to have low antidiuretic activity. When amino acid residues or short peptide chains attached to the terminal amino group of oxytocin and Lys^{8}-vasopressin, they have been characterized as 'hormonogene' (Zaoral and Sorm, 1965; Kasafirek et al., 1966). A hormonogene is defined as a derivative whose biological effects, particularly in vivo are wholly or largely due to its conversion to the active hormone by enzyme action.

The replacement of the amino group by a hydrogen in the 1-hemicystine residue (β-mercapto-propionic acid in place of cystein) and Lys^{8}-vasopressin increased the antidiuretic and lowered the pressor activity (Kimbroung et al., 1963; du Vigneaud et al., 1960).

Variation in Position 2

Methylation of tyrosine hydroxyl group of vasopressin, Tyr (Me)^{2}-Lys^{8}-vasopressin has decreased antidiuretic activity and a slight pressor activity, attended by inhibition of the response to vasopressin in the rat; but it has pressor activity unattended by inhibition in rabbits and cats (Siedel et al., 1963; Vogel Hergott, 1963).

In the $Tyr(Et)^2$ analogue the activites are further reduced and the inhibitor properties accentuated (Zaoral et al., 1965). The replacement of phenolic hydroxyl function by hydrogen yields vasopressin analogues with a high ratio of pressor to antidiuretic activity, an effect which is exactly opposite to that when the phenolic hydroxyl group is blocked (Huguenin, 1966).

Variation in Position 3

This is one of the three loci in the native hormones in which structural alterations have occured in the course of evolution. Therefore a considerably greater tolerance to replacements in this position might be expected than appears to be the case. Attempts to replace the phenylalanine residue by other aromatic residues; e.g., tyrosine (Boissonnas and Guttman, 1960) and trypotophan (Guttmann and Boissonnas, 1960) lead to analogue which are almost completely devoid of biological activity. Aliphatic side chain in position 3 participates actively in lipophilic binding (Nesvadba, et al., 1963; Rudinger and Krefci, 1962). Support for this was produced by the finding that $Thr(Me)^3$-Leu^8-vasopressin, an analogue sterically approximating oxytocin, exhibits an activity spectrum which resembles that of the parent hormone. The presence of the methylene group as such appears to be required to restore full biological activity (Chimiak and Rudinger, 1965).

Variation in Position 4

The deletion of one methylene group from the side chain in position 4 of Lys^8-vasopressin led to $Asp(NH_2)^4$-Lys^8-vasopressin (Boissonnas et al., 1963; Zaoral, 1965). The antidiuretic activity of this analogue evaluated conventionally by the total antidiuretic response shows a different dose-dependence from that of the standard: the analogue appears to be about 8 times more potent than Lys^8-vasopressin at dose levels of 10^{-9} mg and about three times as potent at dose levels of 10^{-8} mg. At still higher doses, the analogue seems to be less active than the parent hormone.

Variation in Position 5

This locus is most important for the manifestation of neurohypophyseal hormone activities, a conclusion derived from a comparison of the biological potencies of analogs with comparable replacements in position 4 and 5. In vasopressin series, Ser^4-Lys^8-vasopressin is inactive (Boissonnas et al., 1963). Furthermore, the syntheses and pharmacological evaluations of Ile^3-Abu^4-Leu^8 and Ile^3-Ala^5-Leu^8-vasopressin (Guttmann and Boissonnas, 1963; du Vigneaud et al., 1963), two analogs in which the carboxamide groups of the glutamine and the asparagine residues at position 4 and 5 respectively, were replaced by hydrogen, also showed a disparity in sensitivity toward structural changes on position 4 relative to position 5.

Variation in Position 6

The disulfide bridge between the tow hemicystine residues in position 1 and 6 is functional importance. It has been suggested that an essential step in hormone-receptor interaction is the formation of a mixed disulfide (SH) group in tissue receptors (Fong et al., 1960; Rasmussen et al., 1960). This view seems no longer to be tenable since synthetic analogues of oxytocin with one or both of the sulphur atoms replaced by CH_2 group retain biological activity (Pliska et al., 1968). However, reduction of the S-S bond with tiols, such as sodium thioglycolate disrupts the ring and causes almost total loss of activity of both hormones (Bisset, 1961).

Variation in Position 7

This position has received little attention. The synthesis and pharmacological evaluation of Ile^3-D Pro^7-Leu^8-vasopressin was reported (Ferraro and du Vigneaud, 1966). Here the structural change at position 7 affected rat antidiuretic activity to a lesser degree than rat pressor activity.

Variation in Position 8

To date, no less than five natural structural variations have been detected in this locus; viz; arginine in arginine vasopressin, and arginine vasotocin, lysine in lysine-vasopressin, leucine in osytocin, isoleucine in mesotocin and isotocin and finally glutamine in glumitocin. One of the first structure-activity relationships

to be observed was the shift from a predominantly oxytocin (galactobolic, uterotonic) spectrum when a neutral amino residue was replaced with a basic amino acid residue in position 8 (Boissonnas et al., 1961; Katsoyannis and du Vigneaud, 1958). A striking dissociation of the antidiurectic and pressor activities is observed upon stereoisomeric replacements; viz; the replacement of basic L-amino acid residues by D-amino acid residues has an unequivocally detremental effect on the pressor activity, but a variable effect on the antidiuretic activity.

Variation in Position 9

Apparently the glycinamide moiety in position 9 constitutes a biologically functional locus in the mammalian neurophypophyseal hormone molecule which is second in importance only to the asparagine moiety in position 5 (Walter et al., 1967). The replacement of the glycinamide function by a glycine methylamine or sarcosinamide function yields analogs with drastically reduced activities. It appears that the tripeptide amide moiety serves a strikingly different function in determining activity in nonmammalian vertebrates as compared with mammalian vertebrates.

Physiological Regulation of ADH Release

There are two physiological stimuli for the synthesis and release of ADH. First, the plasma osmolality, which acts via the osmoreceptors located in or near the hypothalamus. Second, the blood

volume which through induced changes in systemic arterial (P_A) and venous (Pvs) pressure, acts via the stretch receptors or volume receptors located in the carotid sinus, aortic arch and left atrium.

Osmoreceptors

As early as 1947, Verney, in a series of now classical experiments, demonstrated that a sustained five minute infusion of a hypertonic solution of saline or sucrose (but not urea which enters the cell) into the arterial blood supply of hypothalamus of dogs reduced a pre-established water diuresis. The changes in renal response were brought about by as little as 1 to 2% change in the osmolality of the perfusing arterial blood. On the basis of these findings, Verney proposed that there must be receptor cells in or near hypothalamus sensitive to changes in the effective osmotic pressure of the extracellular fluid bathing them. He called these cells 'osmoreceptors'. Subsequent studies (Jewel and Verney, 1957; Jewel, 1963) located these osmoreceptors in the vicinity of the supraoptic nucleus (SON of the hypothalamus.

According to the osmoreceptor concept developed by Verney and his associates, an increase in the osmolality of the blood supplying the osmoreceptor cells leads to an increase in the cellular uptake of osmotically active solutes accompanied by osmotic entry of water. This would result in the swelling of the osmoreceptor cells which would in turn stimulate the receptor endings to increase their neural

discharge. The increase in the frequency of impulses arising from the osmoreceptor cells will eventually lead to an increase in the synthesis of ADH by the hypothalamus-pituitary system and its release into the blood. The resulting increase in $(ADH)_p$ will then cause an increase in osmotic reabsorption of water from the distal and collecting tubules, thereby reducing CH_2O. Although the details of how the increase in the osmolality of the extracellular fluid to which the osmoreceptors are exposed, leads to an increase in neural discharge and eventual increase in synthesis and release of ADH remains entirely speculative, considerable evidence supports the osmoreceptor concept.

Location of the Osmoreceptors

Durham and Novin (1970) recorded slow potential changes from the region of SON in response to infusion of hypertonic solution of NaCl into rabbit carotid artery. Their conclusion was that SON contains osmoreceptors which respond to rapid changes in osmolality of the circulating body fluid.

Holland et al. (1959) and Clemente et al. (1957) have suggested on the basis of the fact that the pattern of EEG was affected by the infusion of hypertonic saline, the location of the osmoreceptors other than those in the hypothalamus (i.e., in olfactory tubercle and in the medial reticular formation respectively). The result of Saito et al. (1969) of hypertonic saline infusion in intact dogs and

dogs with diencephalic islands revealed that the isolated diencephalon was able to release ADH in response to osmotic stimuli-supporting the concept that the osmoreceptor is located in the hypothalamus.

Haberick (1968) has suggested that part of the osmoreceptor mechanism is located in the liver and is affected by the osmotic pressure of the portal blood. However, there is little change in the afferent impulses arising from perfused liver when ionic concentration is varied by ±5%; so that the argument for hepatic osmoreceptors of the classic type is unconvincing (Andrews and Orbach, 1975).

Osmoregulation

The increase in ADH secretion associated with dehydration may be reproduced over a shorter time scale by administering hypertonic saline solution by either an intravenous, intracarotid, intraperitonial or intraventricular route. The relation between the intensity of osmotic stimulation and the rate of ADH secretion has been quantitatively determined in the conscious rat by Dunn and co-workers (Dunn et al., 1973). The osmotic threshold for ADH secretion (290-293 mOsm/kg) was just below the normal plasma osmotic pressure (mean 294±1.4 mOsm/kg). Thus during normal conditions of hydration, vasopressin cells appear to be minimally activated. Dunn and co-workers also found that, as plasma osmotic pressure was increased above the osmotic threshold by intraperitoneal injection of saline solution,

an almost exactly proportional increase in ADH level occured; the correlation between these two variables was always above 0.9. Furthermore the slope of the regres ion line was such that even a 1% increase above the normal osmotic pressure regularly produced a significant change in ADH levels. These findings fully validate the earlier claims, concerning the extraordinary sensitivity and precision of the osmoreceptor mechanism for regulating ADH secretion (Verney, 1947).

In conformity with what is known about stimulus-secretion coupling within the hypothalamo-neurohypophysial system (Cross et al., 1975) the predominant effect of intravenous or intracarotid injection of hypertonic saline solutions is to cause an increase in the firing of SON and PVN (Koizumi and Yamashita, 1972; Haywood and Jennings, 1973; Dreifuss and Kelly, 1972). However, not all cells are excited. Dyball (1971) described a proportion (30-40%) of cells in the rat which displayed several seconds of inhibition after intracarotid saline solution injection. In the unanesthetized monkey, the same procedure evokes in many SON cells a biphasic (excitory-inhibitory) pattern of firing (Hayward and Vincent, 1970) which appear to be exclusively present in neurones projecting to the neuro-hypophysis (Hayward and Jennings, 1973).

Volume Receptors

Share has investigated during the last several years the relationship between ADH release and the cardiovascular receptors and

reported that a decrease in blood volume (Share, 1962) as sensed by receptors located in the left atrium (Share, 1965) and/or aortic arch (Share, 1962) and carotic sinuses (Share, 1962, 1965, 1966, 1967) causes ADH release. Although thereis little doubt that the arterial baroreceptors play an important role in the control of ADH release, the bulk of the currently available experimental evidence is consistent with the view, originally expressed by Gauer and Henry (1963) that a reduction in the activity of the arterial receptors is primarily responsible for the increase in the plasma ADH concentration which follows small to moderate reductions in blood volume. On the other hand, Rocha e Silva and Rosenber (1969) have suggested that the arterial baroreceptors play a dominant role in the hemorrage-induced release of ADH.

A large hemorrhage is required to release detectable release of ADH. A better explanation is the exponential (rather than linear) relationship between percentage of blood depletion and hormonal response (Dunn et al., 1973; Shade and Share, 1975). This exponential stimulus-response relationship for hemorrhage contasts with the linear relationship for osmotic change (Dunn et al., 1973). Thus under normal condition of hydration, the secretion of ADH probably represents a homeostatic response initiated by an osmotic rather than a volumetric stimulus.

The importance of the spinal cord for ADH release in response to hemorrhage has been reported by Saito (Saito et al., 1969). On the other hand, direct nervous connections between the diencephalon and the midbrain were proved to be indispensable by the results of the experiments on the decerebrated dogs and the dogs with diencephalic islands (Saito et al., 1969). On the basis of hemorrhage experiments, it seems clear that ADH release in response to hemorrhage is caused by a 'receptor' located in the nervous system below the level of the midbrain and other than the spinal cord (Saito et al., 1969).

The importance of vagal afferents in ADH release after hemorrhage was pointed out by Share (1967). Clark and Rocha e Silva (1967) reported that in cats, section of vagus nerves or denervation of the carotid sinus caused suppression of ADH response by hemorrhage and the degree of suppression was more striking in vagotomized cats. Saito et al. (1969) support this finding.

Left artrial receptors have recently been given considerable attention. Of particular importance is the report by Johnson et al. (1970) that small increases in left atrial transmural pressure, which are within the physiological range can inhibit vasopressin release. The plasma ADH concentration decreased linearly with increases in atrial transmural pressure of up to 7 cm H_2O. Although there is no question that distention of the left atrium will inhibit vasopressin release, there has been no conclusive demonstration that a decrease

in left atrial volume per se will result in an increased release of this hormone. Goetz et al. (1970) have attempted to provide such a demonstration in dogs prepared with a pericardial pouch around the atria. In the conscious animal, an increase in pressure within this pouch, decreasing atrial transmural pressure, failed to result in the expected increase in the plasma ADH concentration, although urine flow and urinary sodium excretion were decreased. The reabsorption of solute free water was unchanged. As the authors point out, one explanation for this apparently anamolous finding is that the receptors which function in the control of ADH release may be located along the edge of the peripheral pouch and would thus be little affected by the atrial tamponade. An alternative explanation that the atrial receptors are not tonically active in the control of vasopressin release is most unlikely in view of the overwhelming evidence that distention of the left atrium can inhibit the release of this hormone (Gauer and Henry, 1963; Gauer et al., 1970; Share, 1969).

Evidence continues to accumulate that changes in intrathoracic blood volume, presumably acting via atrial receptors, affect the plasma ADH concentration. Six hours after comatose patients with severe lesions of the central nervous system were changed from 15° to 20° head-up position to a 5° to 10° head-down position, the blood ADH concentration fell an average of 0.8 microunits/ml (Auger et al.,

1970). When the patients were returned to the head-up position, the ADH concentration rose again. Similar findings were obtained in a normal volunteer. Bengele et al. (1969) found that when rats were subjected to prolonged centrifugation, the blood ADH concentration fell to a minimum level in five days and then returned to control values over the next four days. One explanation for the fall in ADH concentration is a pooling of blood in the chest, the result of the position of the rats in the centrifuge.

Interrelationship between Volume and Osmotic Factors

Since it is widely held that extracellular fluid volume is conserved at the expense of concentration under extreme circumstances, it is of interest to examine the interrelationship between the volume and osmotic elements of ADH control system. Moore and his associates (1967) have attempted this in the conscious sheep. In water deprivation, both elements of this control system act together to stimulare vasopressin release. There is an increase in the plasma osmolality and a reduction in blood volume which is reflected by a reduction in left atrial pressure (Zehr et al., 1969). Hypotonic or isotonic expansion of extracellular fluid volume in either the normally hydrated or dehydrated sheep resulted in a reduction in the plasma ADH concentration, regardless of the direction of the changes in the plasma osmolality (Zehr et al., 1969). In a subsequent paper (Johnson et al., 1970) they showed that with small changes in plasma

osmolality and blood volume, neither receptor system appears to dominate the other in the control of ADH release. In these experiments comparisons were made of the effects of an isovolemic reduction in plasma osmolality, isotonic changes in blood volume, and combinations of osmotic and volume stimuli. The stimuli applied individually resulted in the expected changes in the plasma ADH concentration. When volume and osmotic stimuli were combined, the effects on the plasma ADH concentration was roughly additive. Thus the plasma ADH concentration was unchanged when 1-2% reduction in plasma osmolality was combined with a hemorrhage of 10% of the estimated blood volume. Similarly, in hydrated human subjects, the plasma osmolality at which the water diuresis was inhibited was higher when the increase in the osmotic pressure of the plasma was achieved by infusing hypertonic saline, increasing extracellular volume (Moses and Miller, 1971).

Olsson and McDonald (1970) reported that the injection of hypertonic solutions of sodium chloride or sucrose into the arterial supply to the head in the ewe did not inhibit a water diuresis until approximately 30% of the administered water load was excreted. This was not due to a lack of responsiveness of the kidney to ADH. The authors suggested that it may have been due to a volume effect overriding an osmotic stimulus.

Thirst and ADH

Since the thirst mechanism and ADH both act to maintain plasma volume, it is not surprising that attempts have been made to find an interrelationship between the two systems. The possibility of an interrelationship has been investigated by several workers including Szczepanska-Sadowska et al. (1974). They describe experiments in which they observed the drinking response in intact and left side vagotomized animals. The threshold of drinking defined in terms of the drinking response to an osmotic stimulus (i.v. infusion of 0.75 M NaCl) was reduced by subpressor amounts of ADH. This reduction was proportional to the dose infused over the range 22-47 μU/ml plasma. ADH on its own never caused drinking. The concentrations of ADH reached would not be found in normal circumstances, although they might occur in some pathophysiological conditions. The response was unaffected by left cervical sympathectomy. In contrast, infusions of pressor dose of vasopressin (406 μU/ml) elevated the drinking threshold to i.v. hypertonic saline infusions and this response was affected by cervical sympathectomy. However, how ADH lowers the drinking threshold is not clear, but it could be due to increased excitability of the system controlling water intake in response to osmotic stimulation and/or modification of the permeability of the cell membrane of osmoreceptors.

Kozlowski and Szezepanska-Sadowska (1975) subsequently showed that volume expansion exerts an effect antagonistic to that of ADH on the thirst mechanism. The osmotic thirst threshold was increased by a 15% expansion of the blood volume produced by i.v. infusion of dextran solution. No significant change was seen when blood volume expansion was performed during infusion of ADH sufficient to produce ADH concentration of about 30 μU/ml. It would then look as though there is a complex relationship between plasma ADH, osmolality, blood volume and the thirst mechanism. These various parameters are integrated to maintain the body fluid volume within narrow limit.

Physiological Function of ADH

The most important function of ADH in the body is the regulatory role on renal handling of water in mammals. Impressive progress in elucidating the mode of action of this hormone at cellular level has recently been accomplished.

In the mammalian kidney, ADH acts on the collecting duct and on functionally similar late segments of distal convoluted tubule. The hormone binds to a specific receptor located in the basal and/or plasma (i.e. cell) membrane (also often called the basolaterla, serosal or peritubular plasma membrane). The action of ADH with its specific receptor stimulates enzymatic formation of cyclic adenosine 3',5'-monophosphate (cyclic AMP), which serves as an intracellular mediator of ADH action. Cyclic AMP, in turn, directly or indirectly elicits

an increase in the water permeability of the luminal (also called apical or mucosal) membrane and possibly of adjacent intercellular structures. Thus the cellular action of ADH can be conveniently divided into two major sequential biochemical events.

a) The interaction of the hormone with its receptor and the effect of this reaction on cyclic AMP metabolism.
b) The reactions by which cyclic AMP, in turn, may elicit changes in water permeability.

ADH-Receptor Interaction

That ADH probably acts only when presented to the peritubular surface of the cell was first suggested by the lack of antidiuretic effect when the hormone was injected retrograde into the collecting system of rat kidneys (Skadhauge, 1964). In subsequent experiments on isolated, perfused collecting tubules, an increase in water permeability was obtained only when ADH was put into the bathing medium, but not when it was added to the luminal perfusate (Grantham and Burg, 1966; Grantham and Orloff, 1968). These studies strongly suggested, although they do not prove, that the receptor for ADH is located in the basal or lateral membranes or in both.

Evidence for the binding of VP to hormone-responsive renal cells was first suggested by the autospecific binding of ^{3}H-ADH to plasma membranes of the renal medulla (Campbell et al., 1972; Bockert et al., 1973). However, these studies could not localize the binding

specifically to peritubular as opposed to luminal membranes of collecting ducts or even to plasma membranes. Nevertheless the evidence certainly seems to favour the location of ADH-specific receptors in the basal or lateral plasma membrane.

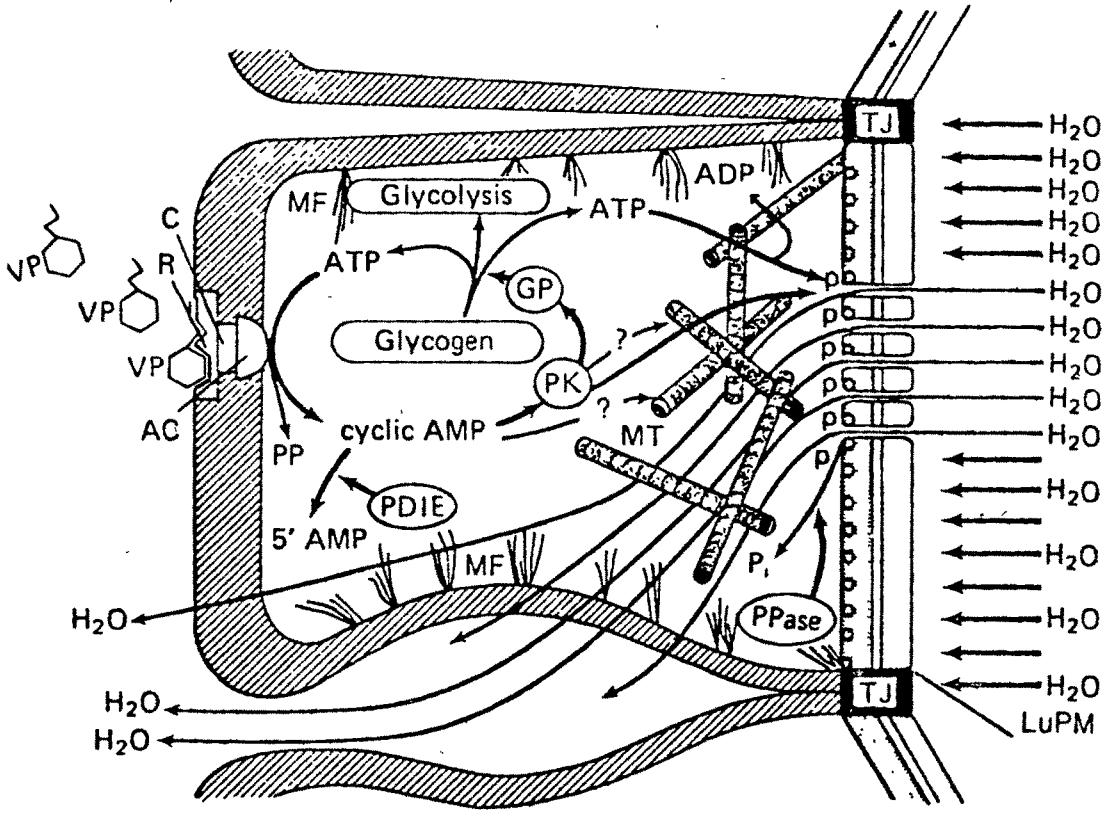
In the cell membrane, the ADH receptor is associated with adenylate cyclase, an enzyme that catalyzes the formation of cyclic AMP from adenosine triphosphate (ATP) (Perkins, 1973). The ADH receptor-enzyme system is a complex biochemical entity, which is frequently referred to in a concise way as 'ADH-sensitive adenylate cyclase'. The ADH receptor is probably the adneylate cyclase, or at least its catalylic center, faces the interior of the cell (Douse and Valtin, 1976) (Fig. 2).

Recent studies have shown that the association of the ADH receptor with adenylate cyclase, and possibly with other components of the membrane, may involve hydrophobic interactions with lipids or hydrophobic membrane proteins (Roy et al., 1975). The predominant solubilized form of the rat enzyme has an estimated mol. wt. of about 159,000 daltons, and it appears that no more than 5% of its surface is involved in the hydrophobic interactions with other components of the membrane (Neer, 1973).

Chase and Aurbach (1968) showed that most of the adenylate cyclase that is stimulated by ADH is located in the renal medulla, while a much smaller portion can be detected in the renal cortex.

Figure 2

Schema of the current view on the cellular action on vasopressin. AC, adenylate cyclase; ADP, adenosine 5'-diphosphate; 5'AMP, adenosine 5'-monophosphate; ATP, adenosine 5'-triphosphate; C, coupling component of adenylate cyclase; GP, glycogen phosphorylase; MF, microfilaments; MT, microtubules; LuPM, luminal plasma membrane; p, phosphate attached to protein; cyclic AMP, cyclic adenosine 3'5'-monophosphate; PDIE, cyclic AMP phosphodiesterase; PK, cyclic AMP-dependent protein kinase; PI, inorganic phosphorus; PP, pyrophosphate; PPase, protein phosphatase; R, receptor for vasopressin; TJ, tight junction; VP, vasopressin molecule; Loci showing water penetration through the LuPm do not necessarily mean structural pores but only areas in the membrane where water flux increases as a consequence of the action of cyclic AMP. (Reproduced with permission from Dousa and Valtin, 1976.)


TJ
H_2O
ADP
MF
Glycolysis
ATP
C
R
VP
VP
VP
ATP
GP
Glycogen
?
PK
?
MT
AC
PP
cyclic AMP
PDIE
5' AMP
MF
P_i
PPase
TJ
LuPM

The latter presumably is located in cortical collecting ducts and late parts of the distal convoluted tubule (Morel et al., 1975; Imbert et al., 1975). Imbert et al. (1975) localized the highest activity of ADH sensitive-adenylate cyclase in the collecting duct. There was also prominent ADH-stimulated activity in the outer medullary and cortical portions of the ascending limbs of Henle's loops, although the threshold and half-maximal doses of ADH required for this stimulation were higher than in collecting duct (Imbert et al., 1975). Imai and Kokko (1974) reported a small ADH-induced increase in the water permeability of thin ascending limb of Henle in rabbit.

Jard et al. (1975) have analyzed the relationship between the ability of ADH to activate adenylate cyclase and its capacity to bind onto receptors in the plasma membrane. In general they found that the similarity between the structural requirements for attachment to the receptor and resultant enzyme activation, is a non-linear function of ADH-binding unto the receptors and that it may be related to occupancy of the receptors. The onset of adenylate cyclase activation by ADH appears to be very fast, and it is rapidly though not instantaneously reversed when the concentration of ADH is decreased (Jard et al., 1975; Neer, 1973).

The functional effects of cyclic AMP may all be mediated through activation of the enzyme, cyclic AMP-dependent protein kinase

(Walsh et al., 1968; Krebs, 1972; Kuo and Greengrad, 1969) which catalyzes the transfer of phosphorus from ATP onto serine or threonine in side chains of polypeptides. A possible role of cyclic AMP-dependent phosphorylation in ADH-mediated water permeability has been suggested for renal epithelial membranes (Dousa et al., 1971). According to this hypothesis, water permeability of the luminal plasma membranes of collecting ducts and late distal tubules would be associated with a specific membrane protein, which serves as substrate for cyclic AMP-dependent protein kinase (Dousa et al., 1971, 1972). Phosphorylation of this protein would cause a change in the structure of the membrane, which in turn would increase the water permeability. The membrane can be returned to its original resting relatively water-impermeable state by enzymatic removal of phosphate from the protein through the action of protein phosphatase.

In recent years, experimental evidence has been gathered which suggests that the integrity of cytoplasmic microtubules (MT) and microfilaments (MF) is required for the cellular action of ADH (Taylor et al., 1973; Dousa and Barnes, 1974; Taylor et al., 1972). The evidence consists mainly of the fact that a variety of chemicals that disrupt MT and MF block the hydroosmotic effect of ADH both '<u>in vivo</u>' and '<u>in vitro</u>'. The mode whereby these structures are involved in ADH-controlled water permeability is not yet known.

Based on the above information, a model to explain ADH-controlled barrier to water flow has been proposed. According to Dousa and Valtin (1976) phosphorylation can profoundly alter the physiochemical properties of a polypeptide. The increase in the number of negative charges, as well as the size of a polypeptide resulting from the covalent attachment of phosphorous to the side chains of serine and threonine, can modify the secondary and tertiary structure of the protein and influence its interaction with other components of the membrane. ADH-induced aggregation of intramembranous particles of protein might well be a cause for or consequence of increased fluidity of ADH-sensitive plasma membrane. In turn, the increased fluidity might be a result of increased diffusional permeability of these membranes for water. It is through these chain of events that MT might influence membrane permeability.

ADH would not increase the bulk flow through nonspecific increase in lumen membrane permeability to water, but it would enhance diffusion in specific areas within the membrane. Thus the primary mechanism of water permeation would be an increase in diffusional water permeability, but in specifically localized areas or 'functional pores' rather than randomly throughout the entire surface of the membrane.

MF may influence the properties of the basolateral membrane because preferential flow of water through lateral intercellular

spaces is hindered when MF are disrupted. Just how MF might affect the basolateral membrane is not known, but the fact that these filaments appear to be contractile elements suggests some sort of active role.

A role for ADH in anterior pituitary regulation of other hormones has been proposed. Interest in this area has been recently stimulated by reports that ADH is released into hypophysial portal blood (Zimmerman et al., 1973) possibly from the median eminence (Zimmerman et al., 1975; Silverman and Zimmerman, 1975). Intravenous infusion of ADH may stimulate the release of growth hormone (Meyer and Knobil, 1966) and ACTH (Clayton et al., 1963). ADH has been shown to potentiate the action of corticotropin releasing factor (Yates et al., 1971).

Pharmacological Actions of ADH

The cardiovascular actions of ADH have been reviewed by Berde and Saameli. The threshold dose for eliciting a pressor reponse is at least 20 times that required for an antidiuretic response in the same animal (Bisset and Lewis, 1962). The pressor activity of ADH is probably of physiological significance only during severe hemorrhage (Rocha e Silva and Rosenberg, 1969; McNeill et al., 1970). With angiotensin, ADH makes an important contribution to the pathology of irreversible hemorrhagic shock (Errington and Rocah e Silva, 1973). The effects of ADH on renal electrolytic excretion is still

uncertain. A natriuretic action of ADH has been claimed in the dog and rat (Brooks and Pickford, 1958; Chan and Sawyer, 1961; Chan, 1965).

Metabolism

There is little information available concerning the control of ADH metabolism. Metabolism depends to a degree on the nature of the circulating hormone. Considerable controversy has surrounded this point. Fabian (1969) reports that 30% of the ADH was bound to plasma protein. The half-life ($t_{\frac{1}{2}}$) of ADH has been reported to be 5.6 min and apparent volume of distribution of approximatly 2/3, the extracellular fluid volume. The whole body clearance for ADH is 1 litre/min. Relatively little destruction of ADH occurs in plasma. The major site of clearance is the kidney, although only a small proportion of the peptide, approximately 10% of an administered dose, appears in the urine in the biologically active form. The liver also contributes to the removal of the hormone from the circulation.

Nardacci and his associates (1975) performed partial purification of a peptidase from the kidney, and they think that a chymotrypsin-like enzyme is involved. Borth et al. (1969) have shown that both soluble and particulate fractions of porcine kidney were able to destroy the antidiuretic activity of both Lysine and Arginine vasopressin. Walter and Shlank (1975) have followed the enzymatic inactivation of ADH by rat kidney supernatent fluid and separated

degradation products and they found that the major pathway of inactivation was cleavage of the Pro-Arg, bond with the release of Arg-Gly-NH_2.

It has been shown that placental extracts can inactivate ADH and Oxytocin (Hawker, 1956). Small and Watkins (1974) using placental extracts prepared an enzyme which contained glutathione-protein transhydrogenase activity, which was specific for ADH and oxytocin.

Measurement of ADH Level in Body Fluids

Until recently the only method available to investigators to measure the circulating level of ADH was bioassay. Various methods have been developed using different animals and tissue; but every method had its own drawback. One of the main problems with the bioassay was its lack of sensitivity so that excretion and concentration of ADH from plasma and urine was required.

A satisfactory method for extraction of ADH from a sample of plasma is simply to precipitate the plasma protein with absolute alcohol and concentrate the supernatent (Bisset et al., 1967). Trichloroacetic acid may be used as a precipitant and ADH recovered from the supernatent by chromatography on Amerlite CG-50 (Share and Levy, 1966). ADH can be extracted from urine by chromatography on Amberlite, IRC-50 and dialysis (Frandsen, 1969). From body tissues, the hormone is usually extracted by hemogenising in 0.25% acetic acid (pH 3.0) and then boiling, cooling and filtering the homogenate.

A fairly satisfactory way to assay the ADH is reported by Dekanski (1952) by using the increment in blood pressure as a parameter, in anesthetized rat. Another acceptable method for assaying ADH in body fluids and tissue is by intravenous injection in rats under ethanol anesthesia, in which a constant water load is maintained (Jeffers et al., 1942; Dicker, 1953). This method is highly specific for ADH. The only likely interference is from vasodilator peptide in extracts which may cause a reflex release of an endogenous hormone by their hypotensive action (Bisset and Lewis, 1962). These can be detected by recording blood pressure simultaneously with urine flow (Bisset et al., 1967).

Vane and his co-workers (Vane, 1964; Gilmore and Vane, 1970; Vane and Williams, 1970) have applied the blood-bathed (superfused) organ technique to the measurement of the neurohypophyseal hormone in circulating blood. Relaxation of rabbit rectum is used in this assay of ADH.

Radioimmunoassay (RIA)

The disadvantages of bioassay were lack of sensitivity and specificity. This has caused, in the last two decades, a burgeoning interest to develop an assay system sufficiently sensitive to be applied to the measurement of very low hormone concentrations present in the circulation. In consequence there has been an explosive activity directed toward the adaptation of immunologic methods to the assay of hormones in blood.

The basis of hormone assays, employing immunological methods, is an antigen-antibody reaction. Although the hormone being assayed participates as the antigen, it need not be the immunizing antigen itself, provided it reacts well with the antibody. In all immunological systems, the primary reaction is the combination of one molecule of antigen (Ag) with one molecule of antibody (Ab) to form a bimolecular antigen-antibody complex (Ag-Ab), that may, depending on the valences of each of the two contributing molecular species, accumulate more molecules of antigen, antibody or both. The complex may increase sufficiently in size to form spontaneously precipitating aggregates, or the complex can be precipitated by using other chemicals; such as polyethylene glycol, or saturated ammonium sulphate.

The requirements for developing an RIA are to have an antigen, an antibody and some method of labeling the hormone. The antigen must be in the pure form to be applied in an assay system. At present all the hormones are available in a purer form for this purpose. Next one must harvest the antibody for the particular polypeptide. The usual procedure is to inject the appropriate polypeptide into a suitable animal and after a certain period of time bleed the animal for the antibody. With small polypeptides like ADH, there is another problem. Although they are antigenic,

they lack immunogenicity. Therefore in these cases the polypeptide is used as an hapten and conjugated with some immunogen like bovine serum albumin or Thyroglobulin and injected into the animal mixed with Freund's Adjuvant. In this way an antibody with sufficient sensitivity is obtained. The next step is to label the antigen with a suitable isotope. Usually ^{131}I or ^{125}I is used to label the peptide and it is purified by chromatographic procedure. The hormone must then be extracted from plasma by one of many methods available for this. Rateliff and Edwards (1971) showed that ADH could be extracted from plasma by absorption on porous glass beads (Spherosil X OA,400) followed by elution with 80% acetone in water. Other methods include the use of Fullers' Earth (Johnson, 1972), Sephadex G-25 (Robertson et al., 1970), or acetone with petroleum ether (Robertson et al., 1973). For the assay of ADH in urine, Miller and Moses (1971, 1972) used extraction by the method of Moral et al. (1974) with Amberlite CG-50.

Pathophysiology

Diabetes insipidus is the disorder of water conservation which results either from inadequacy of ADH release in response to physiological stimuli (central diabetes insipidus) or from renal unresponsiveness to the action of ADH (nephrogenic diabetes insipidus).

Defects may exist in a) the mechanism responsible for sensing plasma osmolality (the osmoreceptors), b) the neurons of the SON in

which ADH is formed or their axons in which the hormones traverses the supraoptico-neurophypophysial tract, c) the storage site of ADH in the neurohypophysis, or d) the action of ADH on the renal tubule.

The osmoreceptors may be merely 'set' too high so that the osmotic threshold for ADH release is found to be above the normal level of approximately 287.3 mOsm/kg (Mahoney and Goodman, 1968; Aubry et al., 1965; Moses and Miller, 1974). In occasional instances there is apparently complete loss of osmoreception but with retention of the capacity to release ADH in response to neurogenic stimuli simulated by intravenous administration of nicotine or acetyl-methylcholine (Mecholyl) (Bode and Crawford, 1969). In other rare instances, osmoreceptor function has been shown to be completely lost, while some control of ADH release via volume receptors is preserved (de Robertis et al., 1971). Impairment of osmoreceptor influences on ADH release is frequently associated with loss of thirst sensation which results in a profound degree of dehydration and hyperosmolality or essential hypernatremia (Mahoney and Goodman, 1968; Avioli et al., 1962; Kastin et al., 1965).

There may be pathological destruction, degeneration, or absence of the supraoptic neurons resulting in reduction or complete loss of the capacity to make ADH. This is presumed to be the mechanism whereby diabetes insipidus results from tumors or other lesions

of the pituitary and hypothalamus. There have been occasional reports of patients with idiopathic diabetes insipidus in whom the nerve cells of SON and PVN were found at autopsy to be strikingly decreased in number and depleted of Nissl granules (Blotner, 1958; Braverman et al., 1965). This abnormality has been found in some patients in whom diabetes insipidus was inherited as a Mendelian dominant (Blotner, 1958).

Loss of the neurohypophysis (by surgical excision or disease) does not cause persistent diabetes insipidus unless the pituitary stalk is sectioned or destroyed far above the posterior lobe, thus resulting in degeneration of the cell bodies in the SON (Laszlo and Wied, 1966). An intact stalk is able to release vasopressin adequately in response to physiological stimuli.

Nephrogenic diabetes insipidus results from impairment or complete loss of the antidiuretic action on the collecting tubule. This may be the consequence of potassium depletion, hypercalcemia, various renal tubular lesions (chronic pyelonephritis, obstructive uropathy, acute tubular necrosis, myeloma, amyloidosis), various drugs (methoxylflurane anesthesia, lithiums and demethylchlortetracycline therapy), sickle cell disease, Sjogren's disease and renal transplantation (Moses et al., 1976). There is also an idiopathic form of nephrogenic diabetes insipidus which is an inherited disorder. This appears to result from a defect in the intracellular

release of cAMP in response to the action of ADH on the cells of the collecting tubule. Thus, the increase in urinary cAMP which normally follows ADH infusion is considerably diminished or absent in patients with nephrogenic diabetes insipidus (Finchman and Brooker, 1972; Bell et al., 1974). Whether this is due to a defect in the ADH receptor, or the adenylate cyclase enzyme or the response of the renal tubule to adenylate cyclase is not known. The administration to these patients of cAMP or dibutyryl cAMP has generally failed to induce a convincing ADH-like action on the collecting tubule (Proesmans, 1975), although an antidiuresis has been observed in consequence of circulatory effects of the administered cAMP and a fall in glomerular filtration rate (Jones et al., 1972).

ADH deficiency in central diabetes insipidus may be treated by replacement therapy with various forms and derivatives of vasopressin or by giving drugs which enhance the action and perhaps increase the release of endogenous hormone.

Aqueous vasopressin has a very short duration of action and is used only in acute diabetes insipidus following hypophysectomy. Vasopressin tannate suspension in oil is the standard preparation, given by subcutaneous injection in doses of 2.5 or 5.0 U every 24-72 hours. Lysine vasopressin (Lypressin) nasal spray is effective for a few hours (Dashe et al., 1964). DDAVP (1-deamino-8-D-arginine vasopressin) has recently been introduced as a nasal spray (Andersson

et al., 1974) and its more prolonged antidiuretic action (13-22 hr) is the basis for its effectiveness when administered in doses of 10-20 µg once or twice daily (Ward and Fraser, 1974).

Patients who have some residual capacity to secrete vasopressin may be treated with oral agents as chlorpropamide 200-500 mg once daily (Miller and Moses, 1970), clofibrate, 500 mg four times daily (Moses et al., 1973), or carbamazepine, 400-600 mg daily. Combination of these drugs may be used in the presence of panhypopituitarism. All these drugs stimulate secretion of ADH. Chlorpropamide and clofibrate also enhance the antidiuretic action of submaximal concentrations of ADH.

Thiazides are more effective form of therapy available in nephrogenic diabetes insipidus. These drugs probably act predominantly by natriuresis which lowers the glomerular filtration rate and increases reabsorption of fluid in the proximal tubule (Moses et al., 1976).

SIADH (Syndrome of Inappropriate ADH Secretion)

SIADH is a disorder in which there is continual release of ADH unrelated to plasma osmolality. Since patients with the syndrome are unable to excrete a dilute urine, ingested fluids are retained, with consequent expansion of the extracellular volume and the development of dilutional hyponatremia. The amount of ADH released and the elevation of urinary osmolality which it produces are considered to be inappropriate only in relation to the level of plasma

osmolality or serum sodium concentration. The hallmark of SIADH thus, is hyponatremia due to water retention, in the presence of urinary osmolality which is less than the maximum dilute (Bartter and Schwartz, 1967).

SIADH may occur in association with a large number of clinical disorders (Bartter and Schwartz, 1967). By far the most common cause is malignancy making up 67% of the cases. Of the malignancies most are lung carcinoma, with 70% of these histologically proven to be small cell or oat cell carcinoma (Moses et al., 1976).

Malignant lung cells obtained from patients with SIADH have been shown to be capable of synthesizing, storing and releasing ADH (George et al., 1972; Vorherr et al., 1974).

The treatment of SIADH is by restricting fluid intake to about 800-1000 ml daily. In some cases the intravenous administration of 200-300 ml of 5% saline solution over several hours is used to raise the serum sodium level. The administration of large doses of furosemide along with hypertonic saline has also been advocated as a rapid means of correcting severely symptomatic hyponatremia (Hartman et al., 1973). At present no drugs are available which are clinically useful in suppressing ADH release from a tumore.

Introduction to the Thesis

It is well established that ADH is synthesized in the magnocellular system of the hypothalamus in clusters of cells which form the SON and PVN. The hormone is synthesized and packaged in the neurosecretory granules with an intragranular protein, neurophysin. The axonal flow of the neurosecretory granules from the perikarya in the hypothalamus to the posterior lobe of the pituitary and subsequent release into the blood has been clearly demonstrated.

There is a consistent and mutually reinforcing body of evidence about the 'osmoreceptor' concept. Equally convincing is the evidence considering the 'osmoreceptor' as a separate entity from that of SON. As mentioned earlier plasma osmolality and blood volume are two important stimuli to hormone release. There is considerable evidence that this hormonal system is also sensitive to many pharmacological agents, designated as 'nonosmotic stimulants'. However, the mechanism by which these agents elicit hormone release is controversial. Whatever the mechanism, it appears that synaptic excitation of the neurosecretory cells in the SON and PVN of the hypothalamus is required for hormone release.

Shute and Lewis (1966) have shown that in SON, PVN and mammilary nuclei some neurones contain small or moderate amounts of acetylcholinesterase (AchE) in their cell bodies. Both the SON and PVN

receive a rich adrenergic innervation. Early studies by Pickford (1939, 1947) showed that acetylcholine (Ach), injected intravenously into anesthetized, atropinized dogs causes antidiuresis, due to the release of ADH. She further demonstrated that the effective dose of Ach is smaller when injected via the carotid artery (Pickford and Watt, 1951). In addition, di-isopropylphosphorofluoridate (DFP) when injected into the SON in dogs was shown to produce a profound and long-lasting antidiuresis (Pickford, 1947). These effects of Ach and AchE-inhibitors indicate that mediation at the neuronal synapses in SON is cholinergic. They also suggest that the postsynaptic neurones bearing the cholinergic receptor might be the neurosecretory nerve whose processes or axons form the supra-optic-hypophyseal tract and whose terminals form the neural lobe.

Walker (1957) on the basis of pharmacological effects of nicotine on ADH release suggested an analogy between peripheral autonomic ganglia and neurones forming the SON and PVN. On the other hand, Bisset and Walker (1957) and Supek and Eisen (1953) studied the effects of nicotine and various ganglionic blocking drugs on the release of ADH and found that ganglion blocking drugs do not block the antidiuretic effects or prevent the rise in ADH induced by nicotine. The former suggested that any synapse which exists at the SON is dissimilar in its pharmacological properties to synapses

at autonomic ganglia. Harris et al (1969) found that a ganglionic-blocking agent, pempidine, injected intravenously into cat was ineffective in blocking the release of ADH by osmotic stimulation. De-Wied and Laszlo (1967) obtained no inhibition of osmotic stimulation with another ganglionic-blocking agent, mecamylamine. Recently Schrier et al. (1974) found that intracarotid injection of nicotine into anesthetized dogs was ineffective in eliciting ADH release while an intravenous route was effective. Their interpretations of these findings was that the release of ADH was the result of an indirect rather than direct action on the hypothalamic neurones, due to nicotine-induced sympathetic changes in hemodynamics. In separate studies the same authors showed that the diuresis produced by the α-adrenergic stimulant, noradrenaline and the antidiuresis produced by the β-adrenergic stimulant, isoproterenol, are also due to the hemodynamic changes induced by these drugs. Finally, Malvin (1971) showed that angiotensin (A-II) when injected into the carotid artery of conscious animals released ADH, and suggested a direct effect of A-II at some level of hypothalamico-hypophyseal system, while Share and Levy (1973) could not reproduce it.

Thus, there is a considerable amount of controversy regarding the pharmacological action of these drugs with respect to their

effects on the synapse(s) at the SON. One reason for this controversy may be due to the lack of a sensitive assay to quantitate the hormonal release. Many of these measurements have been made indirectly by free water clearance (TcH_2O) of the test animal. In a few studies where bioassays have been used to quantitate the hormone release, the sensitivity of the assay system may not have been sufficient to detect small changes. Another reason may be the use of anesthetic agents, which seem to alter the functional properties of the osmoregulatory system (Vatner and Braunwald, (1975).

The present study was undertaken to reinvestigate the effects of various pharmacological agents on the supraoptico-hypophysial system as given under the following: 1) to verify the presence of a synapse at or near SON and characterize the nature of the synapse in comparison with the peripheral autonomic ganglia. 2) To test whether these effects of these agents are indirect, due to changes in the systemic hemodynamics or a direct action, due to the presence of specific receptors. 3) To try to interpret the mechanisms by which these various drugs affect the hormonal release.

These were accomplished by studying two parallel experimental models: an '<u>in vivo</u>' system, using conscious dogs and an '<u>in vitro</u>' system using an hypothalamic island consisting SON, pituitary stalk,

posterior pituitary and hypothalamic region. The hormone released was measured by a very sensitive radioimmunoassay. The results are interpreted in the light of findings of two independent experimental set ups and the conclusion reached regarding the mechanisms of the action of the drug on SON was based on their known mechanism of action on peripheral autonomic ganglia.

It is expected that this study will help to resolve the discrepancies found in the literature, and increase our understanding of the physiology of ADH releasing system.