

THE EFFECTS OF MORPHINE ON THE
HYPOTHALAMO-NEUROHYPOPHYSEAL SYSTEM

by

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ABSTRACT

The acute administration of an analgesic dose (1mg/kg) of morphine sulfate to conscious and hydrated rats produced a pronounced antidiuretic response. This was accompanied by an increase in urine osmolality and a decrease in free water clearance in the normal and Brattleboro rats heterozygous for the diabetes insipidus trait. The response was comparable to that of an exogenous dose of vasopressin. Antidiuresis was also observed in the homozygous D.I. rats. Since these rats are incapable of synthesizing vasopressin, the antidiuresis must be mediated by other mechanism(s). When arterial blood pressure was monitored, it was found that the mean arterial pressure decreased sharply immediately after the morphine injection in all animals. This can account for the antidiuresis in the homozygous D.I. rats. In the normal and heterozygous D.I. animals, it is quite possible that both morphine-mediated release of vasopressin and hypotension are responsible for the decrease in urinary flow. In addition, hypotension per se may act as a stimulus for vasopressin release.

To study the chronic effects of morphine, rats were rendered tolerant and physically dependent by two means: multiple injections and pellet implantation of morphine sulfate. In contrast to the antidiuretic effects of acute morphine administration, chronic treatment resulted in polyuria. Using a vasopressin radioimmunoassay (RIA), it was found that rats implanted with a morphine

pellet for 3 days had a significantly lower neurohypophyseal store of vasopressin (744.3 ± 27.9 ng, $n=6$) as compared to the placebo pellet implanted controls (1024.1 ± 66.0 ng, $n=6$). This depletion was replenished as the animals developed tolerance to the drug. Abrupt withdrawal of the drug from physically dependent animals also produced a significant depletion of the neurohypophyseal vasopressin stores - from 902.4 ± 37.0 ng ($n=6$) to 638.3 ± 36.0 ng ($n=6$). In contrast to rats implanted with morphine pellets, no significant changes in the neurohypophyseal vasopressin stores were observed in those injected daily with morphine for 2 weeks. Withdrawal from the drug in these animals also did not produce any detectable changes in the vasopressin stores. A withdrawal symptom, reduction in body weight, monitored after 24 hr of abstinence suggested that the degree of physical dependence in these animals is very light.

^3H -naloxone binding performed on whole brain homogenate of rats injected with morphine for 2 weeks revealed no significant changes in the number of binding sites (q). The affinity constant (K_d) was augmented from a control value of 5.33 nM to 22.37 nM in the morphine-injected rats. The changes in q and K_d suggested the presence of morphine in the whole brain homogenates. Moreover, the K_d was restored to the control value in animals withdrawn from the drug for 24 hr. Vasopressin or oxytocin did not have any direct effect on the ^3H -naloxone binding. Thus, it is unlikely that the facilitation of

morphine tolerance by the neurohypophyseal peptides is mediated by their direct action on the opiate receptor.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
ADH	antidiuretic hormone
AVP	arginine vasopressin
AVT	arginine vasotocin
CH ₂ O	free water clearance
Ci	curie
CNS	central nervous system
Cos	osmolar clearance
CSF	cerebrospinal fluid
D.I.	diabetes insipidus
fmol	fentomole
g	gram
GFR	glomerular filtration rate
hr	hour
i.p.	intraperitoneal
kg	kilogram
LVP	lysine vasopressin
M	molar
M.A.P.	mean arterial pressure
mCi	millicurie
mEq	milliequivalent
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mmol	millimole
mOsm	milliosmole
ng	nanogram
nM	nanomolar
pM	picomolar
Pos	plasma osmolality
RBF	renal blood flow
RIA	radioimmunoassay
μl	microliter
V	urinary flow rate

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INTRODUCTION

I. Effects of Acute Morphine Administration

The acute administration of morphine and other opiate agonists has been known to exert a variety of behavioral, cardiovascular, gastrointestinal and endocrine effects (1,2). In man, morphine produces a feeling of drowsiness, euphoria, peripheral vasodilation and a marked decrease in the motility of the gastrointestinal system (2,3). In addition, it produces a state of catatonia and waxy flexibility in experimental animals (4). Clinically, it is used extensively in the alleviation of pain. In contrast to most non-narcotic analgesics, morphine has a relatively selective effect in diminishing the subjective aspect of pain as well as raising the pain threshold (5). It has several undesirable side effects, however, most notably the development of tolerance and physical dependence, and the depression of respiration.

Several endocrine systems are known to be altered by painful or stressful stimuli. The classic example is the activation of the ACTH-cortisol axis in response to stress (6). Altered endocrine states are also known to be associated with certain changes in psychological status or mood, such as anxiety and depression. Hence, it is conceivable that psychoactive drugs which alter the mood or the perception of painful stimuli by the animal would lead to corresponding changes in endocrine status. These changes, however, are not

predictable at present. For example, morphine would be expected to have an inhibitory effect on ACTH secretion in view of its ability to block the perception of pain and stress. Yet experimental evidence has shown that morphine stimulated the release of ACTH (7-9). Several other endocrine systems were shown to be affected by morphine. Both growth hormone (7,10,11) and prolactin (10,12-14) secretion were stimulated by the acute administration of the drug. It had an inhibitory effect on the release of the pituitary gonadotrophins, FSH and LH (15). Chronic treatment with the drug resulted in the alteration of testicular function in the male (15). Irregular menstrual cycle has also been reported in human female drug addicts (16).

Morphine has also been shown to be a potent antidiuretic agent (17-20). At doses as low as 0.4 mg/kg, morphine produced a detectable antidiuretic effect on water-loaded and ethanol anesthetized rats (17). Several studies (17-20) have ascribed this antidiuretic effect to the release of AVP, as proposed initially by de Bodo (21). Antidiuresis, for example, was shown to be abolished by neurohypophysectomy (21). This implicated the involvement of the hypothalamo-neurohypophyseal system. Prior administration of an opiate antagonist, such as nalorphine, was shown to prevent the onset of antidiuresis (19). This suggested that the action of morphine is mediated through opiate receptors. Caution, however, must be exercised in the interpretation of these studies.

Factors affecting urinary output have, by far, been poorly controlled and have not been conclusively eliminated. These factors included the potentiation of the antidiuretic action of AVP, cardiovascular changes and a direct effect of the drug on renal function.

Although antidiuresis is the predominant response, the administration of morphine has been shown to cause diuresis in some cases. In patients with pulmonary edema, morphine produced a period of diuresis (22). Diuresis was also observed when morphine was given to a hydropenic rat (23). However, the same dose given to a water-loaded rat produced antidiuresis. Thus, the effects of morphine appeared to be dependent upon the physiological status and the state of hydration of the animal.

Antidiuresis may be brought on by a number of factors. For example, changes in the cardiovascular system can cause a sharp decrease in urinary flow. This is highly probable in view of the powerful cardiovascular effects of morphine (24-27). In conscious human subjects, a 15 mg dose of morphine was shown to induce peripheral venous and arteriolar vasodilation (24). This, in turn, led to a significant decrease in systemic blood pressure. Renal function can be drastically altered by changes in systemic blood pressure (28-29). Thus, hypotension may conceivably be responsible for the decrease in urinary flow. In addition, it may also serve as a stimulus for the release of AVP (30). Most studies have indicated that the

cardiovascular effects of morphine are mediated centrally (24-27). This, however, does not exclude the possibility of a peripheral action. Green et al. (31) have shown that morphine increased the hepatic venous resistance. This in turn led to an increase in the rate of ascites fluid formation and in pooling of fluids in the peritoneal cavity. The resulting decrease in plasma volume can directly alter renal function as well as stimulate the release of vasoactive peptides (30). In the rat, the cardiovascular response to morphine appeared to be dependent upon the state of consciousness of the animal. Hypertension and tachycardia were observed in the conscious, unanesthetized rat (25,26). The same dose given to a chloral hydrate anesthetized rat produced the opposite effects, namely hypotension and bradycardia.

Lastly, antidiuresis may also be explained by a direct effect of the drug on renal function. A reduction in RBF and GFR was observed by Handley and Keller (32) in normal and hypophysectomized dogs after the administration of morphine. Contrary to the results presented by de Bodo (21), they found that antidiuresis was not abolished by hypophysectomy (32). This has led them to conclude that antidiuresis was caused by the reduction of RBF and GFR. However, the validity of their experiment is questionable in view of the abnormally low RBF and GFR in their experimental animals.

II. Effects of Chronic Morphine Administration

Prolonged treatment with morphine or opiate agonists has been known to induce tolerance and physical dependence (33). Tolerance can be defined as the development of insensitivity to the drug upon repeated exposure. Thus, increasingly larger doses must be given to obtain the effects observed with the original dose. Tolerance is often assessed by comparing the analgesic potency of the drug before and after its repeated use. Physical dependence, on the other hand, refers to an altered physiological state whereby the continued administration of the drug is necessary to prevent the onset of a stereotyped withdrawal or abstinence syndrome. The withdrawal syndrome encompasses a constellation of behavioral and physiological signs. In the rat, this included hyperactivity, jumping, wet dog shaking, teeth chattering, vocalization, aggressive behavior, body weight loss and elevation of body temperature. Assessment of the degree of dependence is based mainly on the incidence of occurrence and/or intensity of each symptom.

Quantitative assessment of the degree of tolerance and physical dependence is often a complicated task. This is due to the fact that various signs of tolerance and precipitated withdrawal do not increase concomitantly with increasing tolerance or dependence. Writhing and wet dog shaking, for example, were shown to be the predominant abstinence signs in moderately dependent animals (34). These signs decreased or even

disappeared with increasing dependence, and were replaced by more vigorous motor excitation such as jumping (34). Moreover, quantification based only on one specific sign may not be adequate, especially in studies involving pre-treatment with another drug. Such pre-treatment may conceivably interfere with the neural mechanism underlying one specific sign without affecting the basic mechanism responsible for tolerance and physical dependence.

A. Induction of Tolerance and Physical Dependence

Animals can be made tolerant and physically dependent by various means. Early studies relied mainly on the daily injection of morphine (35). One major disadvantage with this procedure, other than the fact that it is laborious, is the time required to induce a reasonable degree of tolerance and physical dependence. This may be due to the fluctuation in the circulating level of the drug between injections. The dosage and the time interval between injections are crucial in these studies. The animal may go through a period of withdrawal if the dosage per injection is low or the time interval between injections too long, which in turn may affect the measured response variable. Water intake and urine excretion measurements, for example, are often influenced by the dosage and injection schedule used, because such measurements are often made over a time period much longer than that required for the clearance of the drug. Rats pre-treated with morphine were shown to consume food and water voraciously shortly after a morphine injection.

Moreover, food and water intake decreased significantly as the effect of the drug wore off (36). These changes would not have been detected were the measurements made over 24 hr. Therefore, the ideal method to study the chronic effect of a drug is to deliver the drug on a continuous and steady basis. Morphine base pellets designed by Gibson and Tingstad (37) have been used extensively in studies requiring tolerant and physically dependent animals. Maximal tolerance and dependence can be induced with this method over a very short period of time (38) as compared to that of the multiple injection of morphine.

B. Physiological Changes Associated With Chronic Morphine Administration

For most animals, the development of tolerance and physical dependence seem to occur simultaneously. Changes in several physiological parameters have been observed in the physically dependent states (39,40). Respiratory rate, for example, was depressed in chronic drug users (40). This has been shown to be primarily due to the depression of the respiratory center and the decrease in sensitivity of the center to CO₂ (41). Blood pressure, pulse rate and body temperature were also persistently elevated in physically dependent animals. Moreover, tolerant and physically dependent animals often respond differently to a single morphine injection. Whereas antidiuresis (17-21) and inhibition of drinking (42) were often seen after morphine administration to a naive animal, polyuria and polydipsia are the predominant

responses encountered in a physically dependent animal (36, 42-44). Thus, there appeared to be a complete reversal of some of the effects of the drug with repeated use.

Studies on the effects of a drug on water balance are frequently complicated by the close link between water intake and urine excretion. It is difficult to examine the two processes independently. A drug capable of inducing excessive water loss will invariably cause polydipsia, and vice versa. Hence, it is not clear whether drinking in response to morphine was due to the modification of the animal's perception of thirst or a salt and water imbalance caused by the drug. The inhibition of drinking after the administration of morphine to a naive animal may have been due to the sedative and euphoric effects of the drug; the animal may have been temporarily incapacitated or its sensation of thirst may have been masked by the euphoric effect of morphine. On the other hand, the polydipsia observed in tolerant and dependent animals may possibly be due to water imbalance caused by the excessive water loss. There is evidence to suggest that morphine has an inhibitory effect on AVP release in a tolerant animal (17). Thus, the resulting water diuresis may have caused a temporary state of dehydration and stimulated water consumption.

Salt and water metabolism may further be complicated by the apparent nephrotoxic effect of the narcotic drugs. Several reports have cited the coexistence of renal disease and narcotic addiction (45-47). The

statistical evidence available thus far is controversial, and the existence of a specific heroin-associated nephropathy is at best circumstantial. There is no unequivocal proof that heroin or any of the opiates are capable of causing renal failure. Various etiological agents have been implicated by researchers to explain the onset of renal disease in drug addicts (45-50). These range from heroin to the ubiquitous viral particle. Richter et al. (48) have suggested that adulterants or substances used to dilute the heroin can act as antigens and promote the deposition of immunoglobulins on the glomerular basement membrane. Some adulterants (49) as well as immunoglobulins (50) have been detected in kidneys of drug addicts with renal disease. Rhabdomyolysis or skeletal muscle necrosis is another well known cause of acute renal failure found in drug addicts (51). It was originally described in patients with crush syndrome (52). Since acute renal failure is not a common feature of rhabdomyolysis, it is likely that other factors are essential to bring about the renal disturbance. Imbalance in calcium and phosphate metabolism are among two of the most likely candidates (53,54). The actual mechanisms responsible for rhabdomyolysis following heroin addiction remain to be proven.

C. Tolerance to and Physical Dependence on the Endogenous Opiates

The recent discovery of endogenous peptides with opiate-like activity (55) posed an intriguing question to drug addiction researchers. These peptides have been shown to act competitively with morphine on the opiate receptor (56). There were high expectations that these opioid peptides may be used in place of morphine as an analgesic, without the impending development of tolerance and physical dependence. However, tolerance and dependence were shown to be easily evoked by the repeated administration of the endogenous opioid peptides, β -endorphin and the enkephalins (57,58). The possible advantage of tolerance and dependence on these endogenous peptides is not clear. It is possible that shifts in the level of responsiveness to these peptides may serve as a mechanism to regulate their actions on specific neural circuits. The development of tolerance and dependence may thus be viewed as an adaptive response to the excessive influence of the opioid peptides or exogenous opiates.

D. Biochemical Mechanisms of Tolerance

Several hypotheses have been proposed to explain the development of morphine tolerance and physical dependence (59). Lampert et al. (57) and Collier et al. (60) have suggested that tolerance and dependence may be due to the alteration of adenylyl cyclase activity of the target cell. Morphine and endogenous opioid peptides have

been shown to inhibit adenylyl cyclase activity in cells possessing opiate receptors (57,61). Prolonged exposure to the drug resulted in an enhanced synthesis of adenylyl cyclase (62). Thus, the cells adapt to the inhibitory effect of morphine by increasing the synthesis of the enzyme. This allows them to maintain a normal level of cellular cyclic AMP and to appear tolerant to the inhibitory effect of morphine on adenylyl cyclase. At this stage, the cells are dependent upon the continued presence of morphine to maintain a normal level of cyclic AMP. The abrupt withdrawal of the drug would result in the disinhibition of adenylyl cyclase and synthesis of an abnormally high level of cyclic AMP. This has been associated with neuronal hyperexcitability (62) and may account for the high level of activity in the CNS during withdrawal. Tolerance and dependence can, therefore, be regarded as normal processes which help to regulate the adenylyl cyclase coupled synaptic communication.

Several other biochemical changes were noted in dependent animals. Turnover rates of several neurotransmitters were altered in physically dependent animals (63,64). This has been thought to be responsible for the behavioral changes observed during periods of intoxication and withdrawal. Elevated levels of brain enkephalin has also been observed in morphine dependent rats (65). Kosterlitz and Hughes (66) proposed that tolerance and dependence might be explained by the reduction of neuronal enkephalin release as the normal

functions of these peptides were replaced by the exogenous opiate, morphine. Malfroy et al. (67) on the other hand, suggested that the increased levels of a high-affinity enkephalin-degrading peptidase serves as a mechanism of tolerance and dependence. Both these hypotheses imply that symptoms of withdrawal are due to the lack of action of the enkephalin-containing neurons on their respective target cells.

Changes in receptor affinity or number of binding sites can conceivably alter the responsiveness to a drug (68). In most biochemical pathways, feedback control is exerted on the initial step of a cycle. The binding of a drug molecule to the receptor is often considered the initial step of drug action. Hence, alterations in the ligand-receptor binding or the concentration of the receptor can not be excluded as a principal site of control. Enhancement of the number of opiate binding sites has been reported by Pert and Snyder (69) in morphine-treated mice.

E. Studies Linking AVP and Memory to Morphine Tolerance and Dependence

Aside from the biochemical changes, morphine tolerance and dependence have been linked to learning and memory processes of the animal (70,71). Memory storage, according to the current dogma (72), consists of at least two stages. The first stage involves alterations in electrical events and/or metabolic processes shortly after the learning experience. Storage at this stage is

temporary and is often referred to as short-term memory. Long-term memory is considered more permanent in nature and involves a process of information consolidation. This is accompanied by metabolic changes, and growth of axon collaterals and neural connectivity (73). A number of studies have shown that AVP and its analogues have a facilitating effect on memory consolidation (74-76). In view of this, it is probable that the peptide also plays a role in the development of morphine tolerance and dependence. Krivoy et al. (77) have shown that chronic treatment of mice with a biologically inert AVP analogue, desglycinamide⁹-lysine⁸ vasopressin (DG-LVP), facilitated the development of tolerance. In addition, hereditary D.I. (Brattleboro strain) rats not only exhibited memory deficiency but also impairment in the development of morphine tolerance as compared to their normal or heterozygous littermates (78). The biochemical mechanism by which AVP and its analogues facilitate memory consolidation is as yet unknown. The ability of DG-LVP to protect the animal against puromycin-induced amnesia (79) suggested that AVP affects memory processes through the alteration of protein metabolism in the CNS.

Facilitation of morphine tolerance may also be mediated by a possible action of AVP on the opiate receptors. Interactions between endocrine systems, and between endocrine systems and the CNS have been well documented (80-82). It is of interest to speculate that a hormone - AVP in this case - may modulate or exert some

influence on the opiate receptors. Alternatively, AVP may exert its effect on the opiate receptors through the release of ACTH. The secretion of ACTH is known to be potentiated by AVP (83,84). Furthermore, ACTH and its analogues have been shown by Terenius et al. (85) to interfere with the binding of the opiates to the receptor.

As with AVP, the other neurohypophyseal hormone, oxytocin, also appeared to facilitate the development of morphine tolerance and physical dependence (86). Moreover, this activity was shown to reside predominantly on the C-terminal tripeptide, pro-leu-gly-(NH₂). Tocinamide, the N-terminal ring structure of oxytocin, was found to be inactive (86). It is questionable whether oxytocin and its by-products play a significant role in the Brattleboro rats. Although these rats exhibited an impairment in the development of morphine tolerance (77), they are capable of synthesizing oxytocin.

Most studies on the physiological role of the neurohypophyseal peptide thus far have been directed at their extraneuronal actions. In order to understand their actions on the CNS, knowledge of their presence and the route by which these peptides reach the CNS are important. AVP and oxytocin have been found in various hypothalamic nuclei (87,88). It is not clear whether these peptides are synthesized in all the nuclei in which they were detected or are selectively taken up by the hypothalamic neurons after being synthesized elsewhere. Nevertheless, their presence in these nuclei suggests a possible neuronal

connection and/or function. Neural pathways containing AVP and/or oxytocin have been shown by Buijs (89) to spread out from the paraventricular nucleus towards various areas of the brain. These areas included the hippocampus, amygdala and various medullary nuclei. Some of these pathways have been thought to be the anatomical basis for the behavioral effects of the peptides (86,90). The neurohypophyseal peptides have been shown to be present in both axons and dendrites of the hypothalamic and extrahypothalamic fibers (89). There is evidence that these two peptides may function as neurotransmitters in these neurons. Iontophoresis of AVP has been shown to exert an inhibitory effect on a large percentage of neurons in the supraoptic nucleus (91). The other neurohypophyseal hormone, oxytocin, has an inhibitory effect on paraventricular nucleus neurons (92). The general ideas emerging from these studies are that AVP and oxytocin act as neuromodulators and general regulators of neuronal membrane properties. In contrast to the classical neurotransmitters, their actions appear to be of longer duration.

Aside from direct neuronal contact, the neurohypophyseal hormones may be transported throughout the CNS via the cerebrospinal fluid (CSF). There is morphological evidence of connections between the neurosecretory cells and the infundibular recess of the third ventricle (93). This suggested that AVP and oxytocin may be taken up and/or secreted into the CSF from neuron

terminals. Immunoreactive AVP has been detected by Dogterom et al. (94) in dog, human and rat CSF. Its presence in the CSF has been implicated in the conditioned avoidance behavior of the rat (95). Removal of AVP in the CSF by the intracerebroventricular administration of AVP-specific antibodies (96) resulted in a marked reduction of avoidance behavior.

There is no doubt that the endogenous opioid peptides play a significant role in modulating various neuroendocrine functions (97). As in most endocrine systems, proper functioning requires some form of feedback control. With regards to the hypothalamo-neurohypophyseal system, the elaboration of AVP in CNS structures may act as a mechanism to prevent the excessive influence of the endogenous opioid peptides. Stimulation of AVP release into the systemic circulation by the presence of excess endogenous opioid peptides or exogenous opiates may cause an unnecessary imbalance in the animal's salt and water metabolism. One method of diminishing such stimulative influence is to promote the development of target cell tolerance. Thus, the release of AVP either as a neurotransmitter or into the CSF may serve to facilitate the development of tolerance and prevent the hypersecretion of AVP into the systemic circulation.

III. Objectives of the Current Study

The present experiments were designed to examine the effects of both acute and chronic morphine treatment on the hypothalamo-neurohypophyseal system. It was apparent from the literature that the morphine-induced antidiuretic response was governed by at least two factors: the state of hydration and level of consciousness of the animal. In this study, the acute effects of morphine were examined using unanesthetized and hydrated rats. The release of AVP is known to be inhibited in hydrated animals or by a decrease in plasma osmolality (98,99). If the morphine-induced antidiuresis is mediated by AVP secretion from the neurohypophysis, then the drug must overcome the inhibitory effect of plasma hypoosmolality. To further elucidate the role of AVP in the induction of antidiuresis, the responses of Brattleboro rats (100) with familial hypothalamic D.I. were compared to that of their normal and heterozygous littermates. The homozygous D.I. rats have served as excellent controls in many studies involving AVP release and actions. Trauma and technical difficulties often encountered in hypophysectomy can thus be avoided with the use of these animals. The presence of an antidiuretic response in the normal rats coupled to the failure of the homozygous D.I. rats to respond to morphine would conclusively prove that antidiuresis is AVP mediated.

The effects of chronic morphine treatment on the

hypothalamic and neurohypophyseal AVP stores were also studied. Changes in AVP storage were determined by radioimmunoassay (RIA). Attempts were made to correlate these changes with the animal's daily water intake and urine output. Polyuria often encountered in physically dependent animals may be due to the depletion of the neurohypophyseal AVP stores. Changes in neurohypophyseal AVP stores may also provide some insight to the mechanism of morphine tolerance. Studies on the facilitation of morphine tolerance by the neurohypophyseal peptides (77,78) have been based exclusively on the exogenous application of these peptides. There is no study thus far on the possible effects of chronic morphine administration on the hypothalamo-neurohypophyseal system. The measurement of the hypothalamic and neurohypophyseal stores of AVP might shed some light on the possible interactions between the neurohypophyseal peptides and morphine.

METHODS

I. Acute Morphine Experiments

Male Brattleboro rats, homozygous and heterozygous for the hypothalamic diabetes insipidus (D.I.) trait, were studied. Normal male rats from the Long-Evans strain, the strain from which the Brattleboro rats were derived, were used as control. The rats, weighing 200-300 g, were anesthetized with ether. The right carotid artery was exposed by a midline incision and cannulated with a heparin-filled PE-50 tubing (Clay-Adams). The cannula was passed subcutaneously to the back of the neck and brought out through a small incision. The carotid blood pressure was monitored with a pressure transducer (Statham) and recorded on a u.v. recorder (S.E. Lab. Ltd.). For intravenous infusion, the lateral tail vein was cannulated using a PE-10 tubing.

Urine samples were collected by catheterizing the urinary bladder. The bladder was exposed by a small midline incision. A small opening was made in the apex and a flare-tipped catheter was inserted. The catheter was held in place with a suture and care was taken to minimize the amount of dead space in the bladder. The bladder was then gently guided back into the abdominal space with the tip of the catheter protruding out the abdominal incision.

After surgery, 2.5 mg of Mepivacaine (Winthrop) was injected subcutaneously around the incision sites. This local anesthetic was used in order to lessen the

surgical pain which may stimulate the release of AVP. The animal was then allowed to recover for 2 hours before commencing the experiment. After this recovery period, the rat was hydrated with a solution of 0.3% NaCl and 1.6% glucose, infused intravenously at a rate of 51 μ l/min. The infusion solution was similar to that used by Inturrisi and Fujimoto (17) with the exception that ethanol was omitted and a slower infusion rate was chosen. Urine samples were collected at 10 min intervals. Morphine sulfate (BDH Chemicals) was dissolved in 0.9% NaCl and injected into the tail vein at volumes of 20-30 μ l to give a dose of 1 mg/kg body weight. Morphine injections were given only after the establishment of a constant urine flow rate for at least two control periods. The results were expressed as the percentage reduction in the urine excretion rate after morphine injection.

The urine osmolality (Uos) was measured by freezing point depression (Osmette, Precision System). Osmolar clearance (Cos) and free water clearance (CH_2O) were calculated using the following formulae:

$$Cos = Uos \times V / Pos$$

$$CH_2O = V - Cos$$

Where V is the urine excretion rate and Pos the plasma osmolality.

II. Chronic Morphine Experiments

Male Wistar rats, weighing 200-250 g, were housed individually in metabolic cages. Food and water were given ad lib. The room was maintained at 22° C with a 12 hr light-dark cycle (light on from 0700-1900 hr).

The rats were rendered physically dependent on morphine by two means: multiple injection and pellet implantation. For the multiple injection method, 24 rats were divided into 3 equal groups. Two groups were injected intraperitoneally (i.p.) with increasing doses of morphine sulfate twice daily for 14 days (101). The control rats were injected with an equal volume of 0.9% NaCl. On the fourteenth day, the control group and one morphine-treated group were sacrificed one hour after their daily injection. Daily morphine injections were withheld from the second morphine-treated group for 24 hr before sacrifice. This group will be referred to as the withdrawal group.

For the pellet implantation method, morphine pellets containing 75 mg morphine sulfate (Allen & Hanbury's), were prepared according to the method of Gibson and Tingstad (37). Thirty-six rats were divided into 6 equal groups and placed individually in metabolic cages. After two days of housing, four groups were implanted with morphine pellets in the dorsal subcutaneous space under light ether anesthesia. The remaining two groups served as controls and were implanted with placebo pellets composed of the inert ingredients. Studies (33,34)

have shown that the absorption of morphine from each pellet rapidly decreased after a few days and tended to plateau after a week. Hence, a second pellet was implanted 3 days after the first pellet in order to maintain a constant delivery of the drug. To examine the changes in the neurohypophyseal stores of AVP, a morphine-implanted group was sacrificed after 1, 3 and 5 days of treatment. One of the control groups was also sacrificed on day 5. Withdrawal was precipitated on one morphine-implanted group on day 5 by surgically removing the implanted pellets. The implanted placebo pellets were also removed from the remaining control group. Both groups were sacrificed 3 days after the excision of the pellets. Alteration in body weight during the withdrawal period was used as an index of physical dependence (102).

III. Extraction of the Neurohypophyseal Peptides

On previously designated days, the treated rats were sacrificed by decapitation. Trunk blood was collected into a test tube and allowed to clot for 1 hr. The clot was removed by low speed centrifugation (1400 x g for 10 min) and the serum stored at -20° C. Serum osmolality was measured by freezing point depression (Osmette), and serum sodium by flame photometry (Instrumentation Lab., model 143). After the decapitation, the neurohypophysis was quickly separated from the adenohypophysis and frozen at -20° C. The hypothalamus, according to the boundaries set by Bie and Thorn (103), was removed and frozen at -20° C. The tissues (neurohypophysis and hypothalamus) were then

suspended in 6-10 volumes of ice cold 0.2 M acetic acid (pH 2.85) and homogenized in a Potter-Ehlvejehm assembly at 300 RPM for 30 sec. The homogenate was centrifuged at 6000 x g for 30 min. The pellet was discarded and the supernatant lyophilized. The lyophilized powder was stored at -20° C.

IV. Opiate Receptor Binding

Brains from rats injected chronically with saline or morphine were quickly removed after decapitation. Since the cerebellum has been shown to be devoid of receptor activity (104), it was removed and discarded. Each brain was homogenized in 10 ml of ice cold 50 mM Tris-HCl buffer (pH 7.4) for 30 sec. The homogenate was centrifuged at 12,000 x g for 10 min. The pellet was re-suspended in 10 ml of Tris-HCl buffer and centrifuged a second time. The final pellet was suspended in 5 ml of Tris-HCl buffer. This suspension had an average protein concentration of 16.33 ± 0.16 mg/ml (n=22) as estimated by the Folin-Lowry method (105).

A receptor-binding assay similar to that described by Pert and Snyder (106) was used. A 100 μ l aliquot of the brain homogenate, containing approximately 1.5 mg protein, was incubated with 2.33 nM of 3 H-naloxone (New England Nuclear, at 17.15 Ci/mmol) at 4° C for 60 min. Non-specific binding was determined by adding 115 nM of levallorphan (Hoffman-LaRoche) to tubes containing the brain homogenate and 3 H-naloxone. All assays were performed in triplicate. After the 60 min incubation

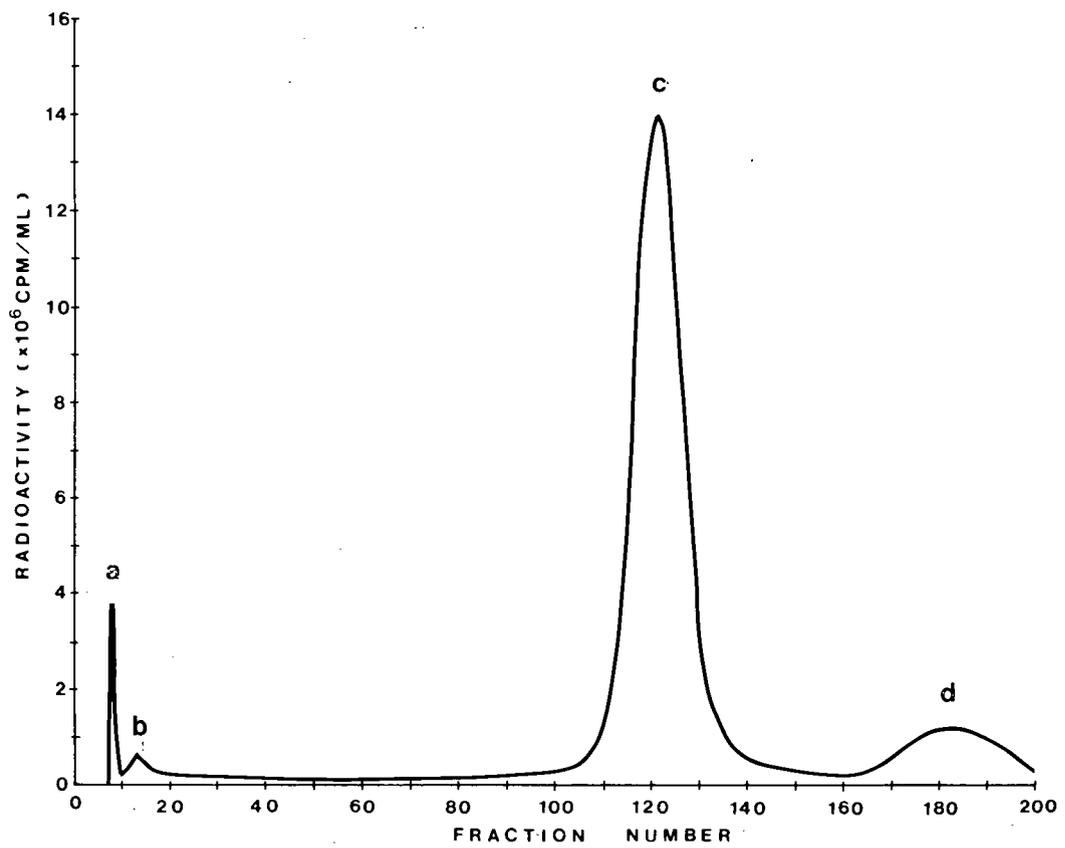
period, the samples were filtered through Whatman GF/B glass-fiber filters (24 mm diameter) and washed twice with 5 ml volumes of ice cold Tris-HCl buffer. The filters were partially dried under infra-red lamp and the protein digested overnight with 1 ml Protosol (New England Nuclear). 10 ml of Omnifluor (New England Nuclear) was added to each scintillation vial and the radioactivity was counted in a Beckman (LS-233) scintillation counter. Stereospecific binding was determined by subtracting the binding which occurred in the presence of the opiate antagonist, levallorphan, from that which occurred in its absence. The non-specific binding in the presence of excess levallorphan was not altered by adding 100 mM NaCl, which is known to enhance the binding of opiate antagonists (107). This showed that the amount of levallorphan used was adequate to bind to all opiate receptors in the tube.

V. Radioimmunoassay (RIA) of Vasopressin

The assay was performed according to the method described by Burget and Wilson (108). AVP (Spectrum Med. Ind.) was iodinated by a modification (109) of the chloramine-T method described by Greenwood et al. (110). 10 µg of synthetic AVP was dissolved in 10 µl of 0.05 M acetic acid (pH 3.0) and 15 µl of 0.5 M phosphate buffer (pH 7.4). 10 µl of 1 mg/ml chloramine-T (Eastman) and 1.5 mCi of ^{125}I (Amersham) were added to the solution. The reagents were allowed to react for 50 sec. The reaction was stopped by the addition of 100 µl of 25% BSA (Pentex).

In addition, 200 μ l of Bio-Rad anion exchanger, AG1-X10 at 250 mg/ml, was added to help bind the unreacted ^{125}I as well as the complex polyiodides. The ion exchange beads were then removed by low-speed centrifugation. The resulting supernatant, containing the iodinated AVP, was separated and purified on a CM Sephadex C-25 column (9 x 800 mm) equilibrated with 0.6 M sodium acetate buffer (pH 4.85). The elution profile of the iodination mixture on CM Sephadex C-25 is shown in fig. 1. Iodinated BSA was the first derivative to be eluted from the column and it showed no binding to the antiserum (GP-13). This was followed by several small radioactive peaks corresponding to unreacted ^{125}I and complex polyiodides. The next radioactive derivative to be eluted was mono-iodinated AVP, followed by di-iodinated AVP.

FIGURE 1. Chromatography of ^{125}I -AVP on CM Sephadex C-25 column (9 x 800 mm). The column was equilibrated with 0.6 M acetate buffer (pH 4.85) at a flow rate of 18 ml/hr. Each fraction consisted of 70 drops (or 5 ml). The order of elution was iodinated BSA (a), unreacted ^{125}I and the complex polyiodides (b), mono-iodinated AVP (c) and di-iodinated AVP (d).



The amount of mono- and di-iodinated AVP obtained was dependent on the reaction time. A longer reaction time (>45 sec) favored the production of more di-iodinated AVP.

Standardized posterior pituitary extract, supplied by Dr. R. E. Weitzman (Harbor Gen. Hospital, Torrance, Calif.) at 2.1 IU/ml, was used in the preparation of the standards. The RIA buffer was 0.15 M sodium phosphate (pH 7.2) with 0.25% normal rabbit serum. The antiserum used (GP-13) was obtained from guinea pigs immunized against synthetic LVP coupled to BSA by the carbodiimide reaction (111). In the assay, the antiserum (GP-13) was used at a final dilution of 1:90,000. This antiserum showed no cross-reactivity with oxytocin (112) and AVT (fig. 2). Although the antiserum was raised against LVP, it exhibited a greater preference for AVP. It cross-reacted with AVP at a concentration of 13 pM whereas LVP cross-reacted at 100 pM. An AVP analogue, desamino-dicarba AVP, was also tested and it cross-reacted at 45 pM (fig. 3). Hence, the GP-13 antiserum appeared to consist of at least two populations of antibodies; one specific against the N-terminus and the other against the region bounded by the disulfide bridge.

FIGURE 2. Inhibition of binding of ^{125}I -AVP to antiserum GP-13 by the neurohypophyseal peptides. Oxytocin (o) and arginine vasotocin (\square) showed no inhibition of binding up to a concentration of 500 pM/L. The antiserum (GP-13) showed greater preference for AVP (\bullet) than for LVP (\blacksquare).

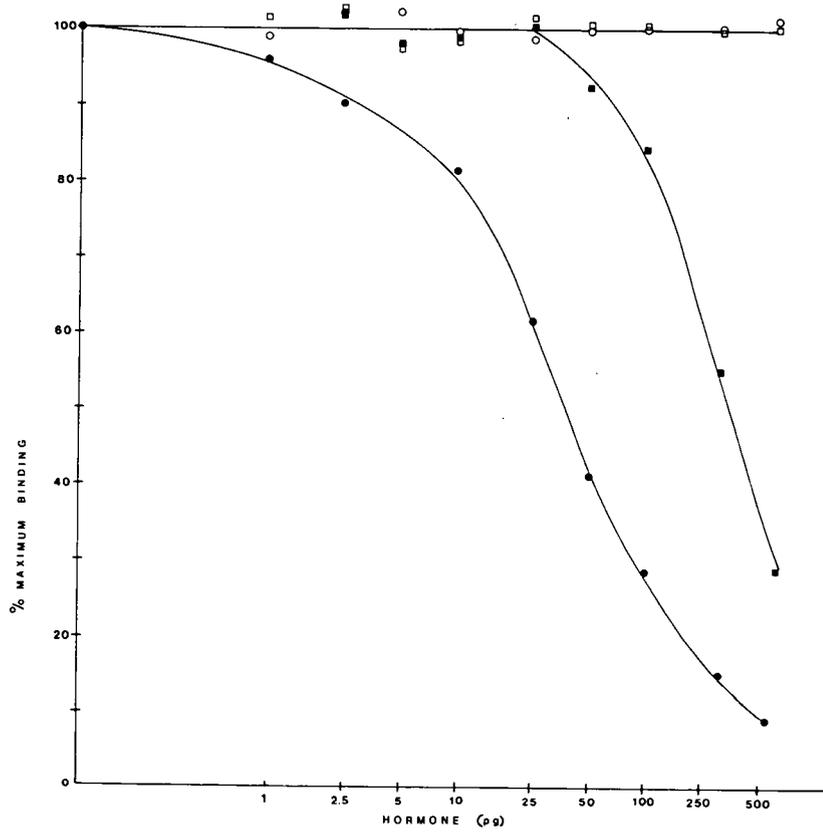
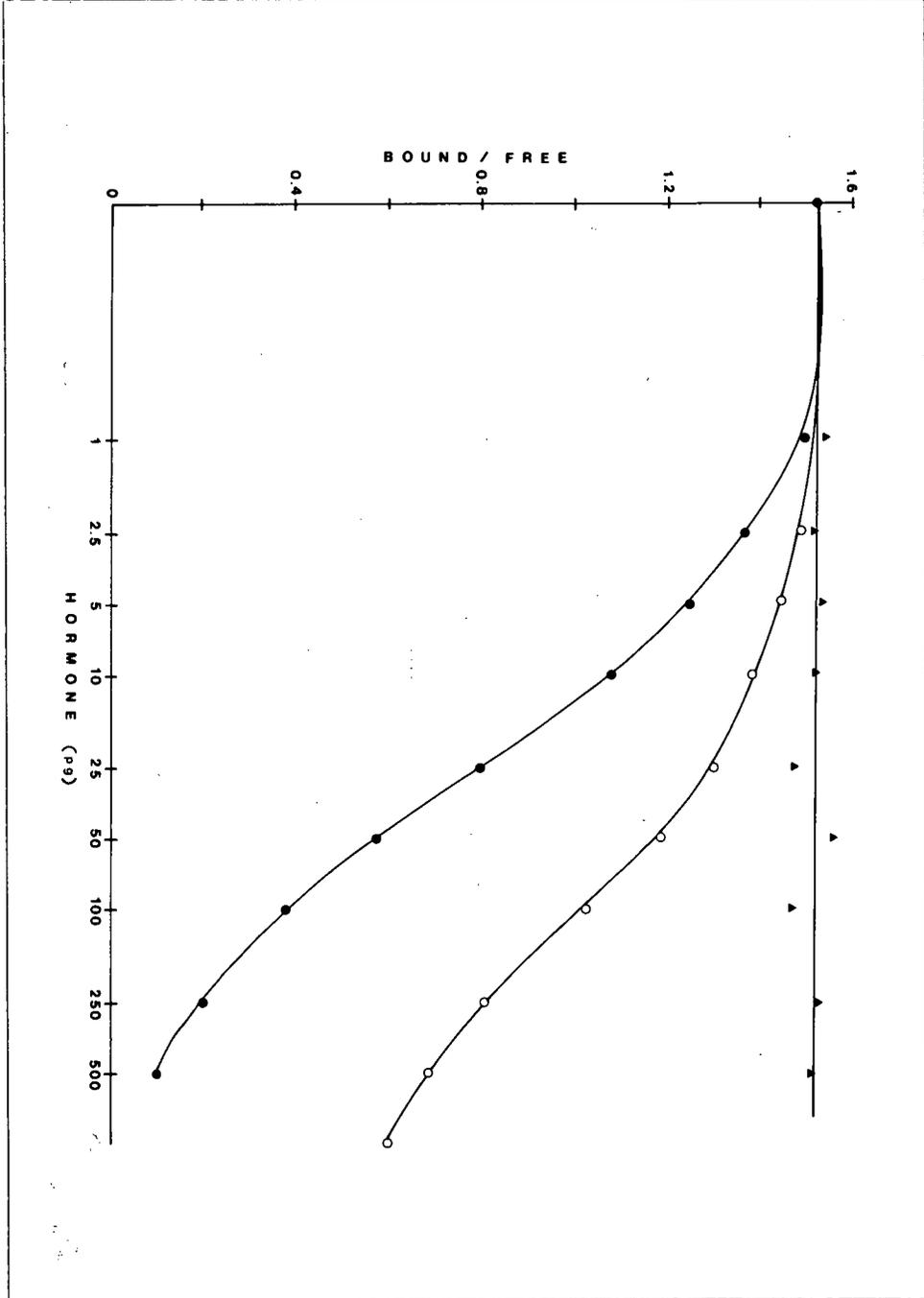


FIGURE 3. Comparison of effectiveness of an AVP analogue and leu-enkephalin in the displacement of ^{125}I -AVP from the antiserum, GP-13. The AVP analogue, desamino-dicarba AVP (○) showed lesser cross-reactivity as compared to AVP (●). The endogenous opioid peptide, leu-enkephalin (▲) did not depress the binding of ^{125}I -AVP.



The limit of detection of the standard curve was arbitrarily defined as 80% of the initial or maximal binding. This is more than the three standard deviations of initial binding often used by other authors. Using this condition, the limit of detection was about 13 fmol of AVP (13.6 ± 1.9 fmol, $n=15$), and 50% displacement of ^{125}I -AVP occurred at 52 fmol (52.1 ± 3.6 , $n=15$). The intra-assay variation at 50 fmol level was 3.2% ($n=18$). The inter-assay variation using pooled dog plasma was 13.9% ($n=15$).

The lyophilized tissues to be assayed were dissolved in the RIA buffer. A 1:500 dilution was made for the posterior pituitary extract and a 1:10 dilution for the hypothalamic extract. The final volume of each RIA tube was 1.0 ml. The assay was incubated at 4°C for 3 days. Separation of bound and free ^{125}I -AVP was accomplished by using dextran-coated charcoal (2.5 mg/ml dextran T-70, 25 mg/ml Norit A charcoal in 0.15 M phosphate buffer, pH 7.2). The results were expressed as the amount of ^{125}I -AVP bound divided by the total amount added versus the logarithm of unlabelled AVP present. Linearization was done by expressing the response variable in logit (113).

VI. Radioimmunoassay (RIA) of Oxytocin

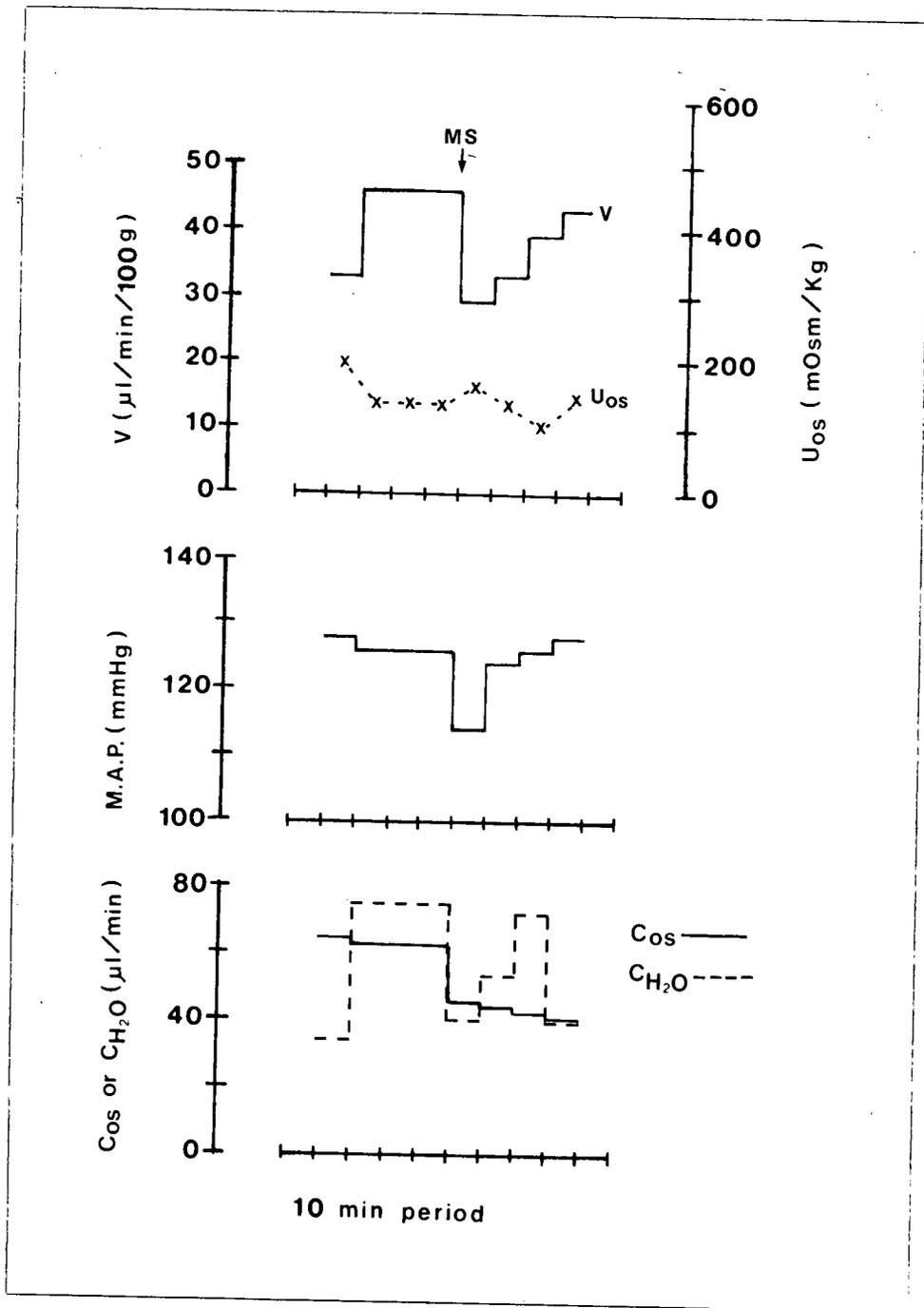
Acetic acid extracts of the neurohypophysis from the pellet implantation series were also assayed for oxytocin. The RIA procedure has been described previously (114). The antiserum used, GP-4, has been shown not to cross-react with arginine and lysine vasopressins, vasotocin, angiotensin I, calcitonin or epinephrine (114). The limit of detection of this assay was about 25 fmol of oxytocin. The intra- and inter-assay variations were similar to that of the AVP radioimmunoassay.

RESULTS

I. Acute Effects Of Morphine

The animals were hydrated by infusing intravenously a hypotonic glucose-saline solution. This was infused slowly to prevent any abrupt changes in plasma osmolality. Urine excretion rate was observed to increase gradually. Once a steady urine flow rate was obtained for at least two 10 min collection periods, morphine sulfate was injected into the tail vein cannula. A moderately low dose, 1 mg/kg, was chosen. This dose resulted in a marked antidiuresis in the hydrated normal and D.I. (Brattleboro) rats. An example of the response of a normal Long-Evans rat is shown in fig. 4. The antidiuretic response occurred almost immediately after the morphine injection. It generally lasted for 10-20 min and there was a gradual recovery to the pre-injection excretion rate. A rebound phenomenon was observed in several rats during the recovery phase. The rate of urine excretion was seen to increase beyond the pre-injection level before returning back to baseline.

FIGURE 4. An example of the renal and cardiovascular response of a normal Long-Evans rat to a single dose (1mg/Kg) of morphine sulfate. The drug was injected (MS) after establishing a steady urinary flow for at least two 10 min collection period. Urinary flow was corrected to 100 g body weight. The dashed line indicates changes in urine osmolality (Uos). Changes in mean arterial pressure (M.A.P.) were monitored through an indwelling carotid cannula. Corresponding changes in osmolar clearance (Cos) and free water clearance (CH₂O) are shown in the bottom graph.



The responses of heterozygous and homozygous D.I. rats, shown respectively in fig. 5 and fig. 6, were comparable to that of the normal Long-Evans rats. Morphine also produced a marked reduction in the rate of urine excretion in these animals. The period of antidiuresis was brief (10-20 min) with subsequent return to pre-injection level. As with the normal rats, a rebound phenomenon was observed in several rats.

In all three groups of rats, 1 mg/kg of morphine sulfate produced a mild but noticeable sedative effect. The animals remained immobile or catatonic for 5-15 min after the injection. Most animals, however, appeared to have recovered from the sedative effect of the drug and were fully conscious within 20 min.

Table I summarizes the effects of morphine on urine excretion, urine osmolality and arterial blood pressure. At 1 mg/kg, morphine caused over 50% (n=6) reduction in urine flow in the normal rat. The same dose caused a 46% (n=6) reduction in the heterozygous D.I. group. Although this reduction was not as great as that observed in the normal group, the difference was not significant. Homozygous D.I. rats also showed an antidiuretic response. A 25% (n=6) reduction in urine excretion was observed after the morphine injection. This was significantly less than that of the normal and heterozygous D.I. groups.

FIGURE 5. An example of the renal and cardiovascular effects of morphine sulfate (1mg/Kg) on a heterozygous D.I. (Brattleboro) rat. Legend is the same as that in fig. 4. The response to a test dose of AVP (at 50 μ g) was also included for comparison.

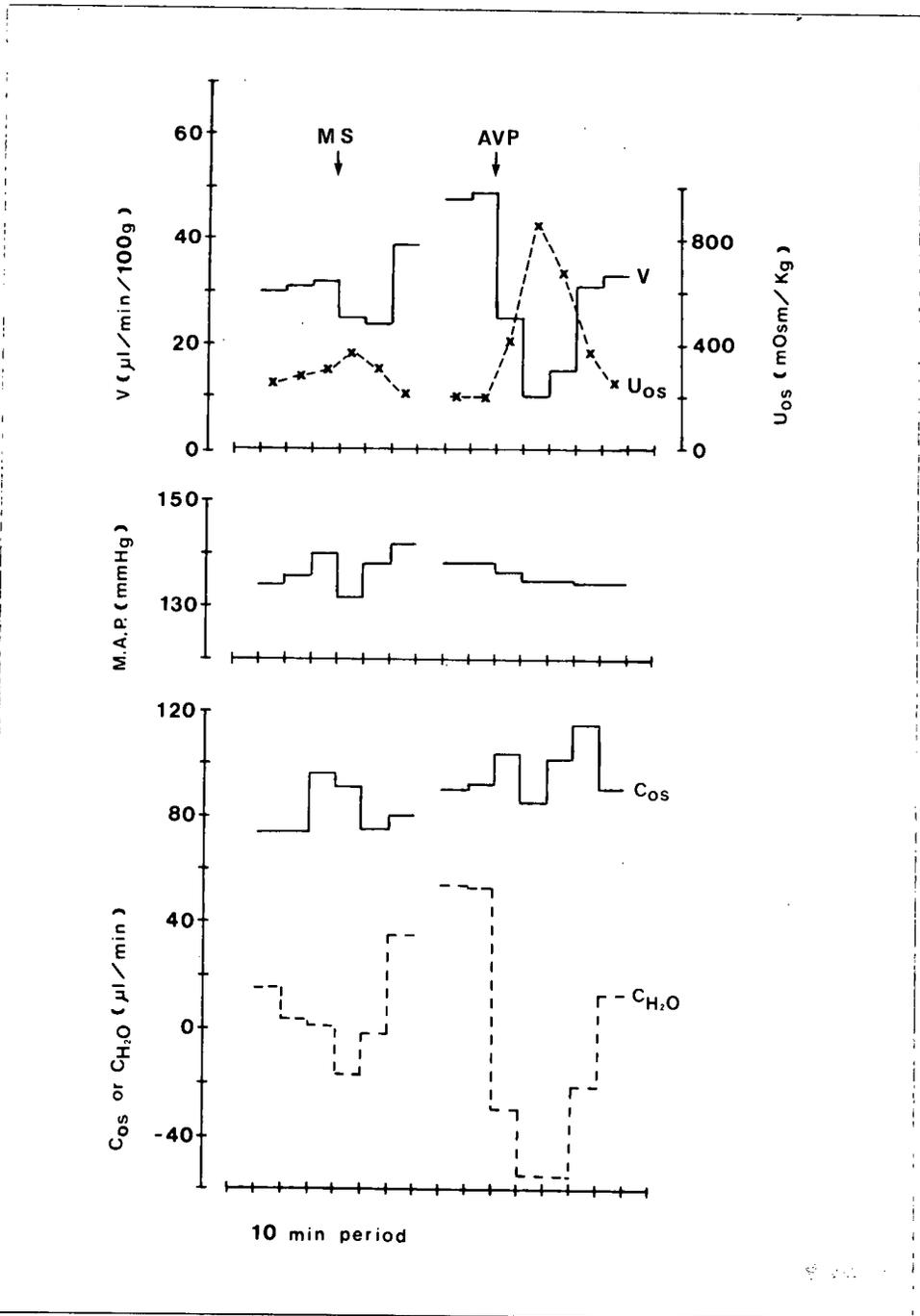


FIGURE 6. An example of the renal and cardiovascular effects of morphine sulfate (1mg/Kg) and AVP (10 μ g) on a homozygous D.I. (Brattleboro) rat. Legend is the same as that in fig. 4.

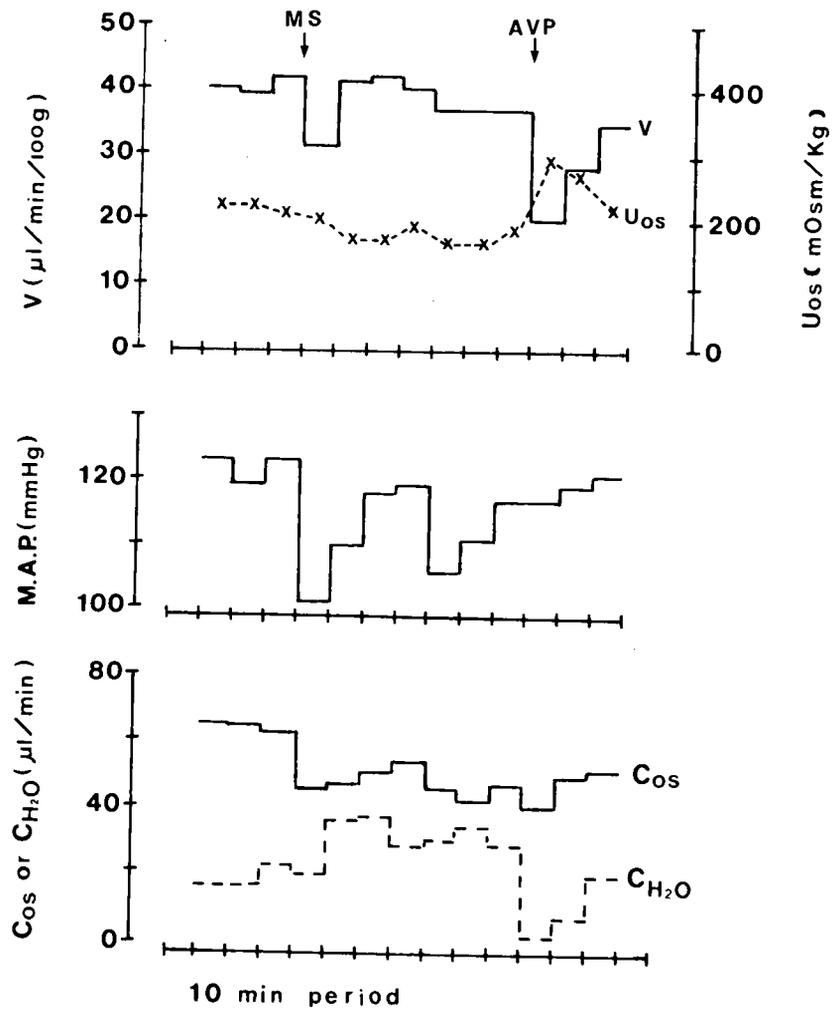


TABLE I. Changes in mean arterial pressure, urine flow rate and urine osmolality in Long-Evans (LE), heterozygous D.I. (HE-DI) and homozygous D.I. (HO-DI) rats after a 1 mg/Kg dose of morphine sulfate. The values represent the mean \pm SEM and each group comprises of 6 animals. * Values in the HO-DI are significantly different ($p < 0.05$) as compared to the LE group.

GROUP	MEAN ARTERIAL PRESSURE (mmHg)	URINE FLOW RATE (μ l/min)	URINE OSMOLALITY (mOsm/Kg)
	%Reduction	%Reduction	%Increase
LE	8.4 \pm 1.2	51.4 \pm 9.1	54.1 \pm 20.7
HE-DI	11.3 \pm 1.5	45.9 \pm 9.9	64.8 \pm 18.5
HO-DI	16.7 \pm 1.0	25.1 \pm 7.7	9.0 \pm 8.3

When blood pressure was measured simultaneously, an interesting phenomenon was observed. Contrary to the reports given by Gomes et al. (25,26), a decrease in arterial blood pressure was observed. In the normal Long-Evans group, this amounted to an average of about 8% (n=6) reduction (Table I). Both Brattleboro groups gave greater reductions, with the arterial pressure reduced by as much as 16% (n=6) in the homozygous D.I. group. The decrease observed in the heterozygous D.I. rats ($11.3 \pm 1.5\%$, n=6), however, was not significantly different from that of the normal rats.

Heart rate was monitored in some experiments and bradycardia was consistently observed after morphine administration. In the homozygous D.I. group, the heart rate decreased from a pre-injection level of 445 ± 7 beats/min (n=6) to 320 ± 18 beats/min (n=6). Similar changes were observed in the normal Long-Evans and heterozygous D.I. groups.

The reduction in the rate of urine excretion was accompanied by a slight increase in the urine osmolality in both the normal Long-Evans and the heterozygous D.I. groups. The homozygous D.I. group, on the other hand, produced only a small and relatively insignificant increase in urine osmolality (Table I). All three groups of rats were capable of increasing urine osmolality in response to a test dose of synthetic AVP, as shown in fig. 5 and 6. Thus, antidiuresis can not be attributed to the development of renal insensitivity to AVP.

II. Chronic Effects Of Morphine

Development of physical dependence on morphine has been known to occur with repeated use. As shown in fig. 7, rats injected daily with morphine did not gain weight as rapidly as the saline injected controls. This was significantly different, as determined by Student's t-test, by day 7. Water intake and urine output were not monitored in this series, thus the state of fluid balance of these animals was not known.

Body weight gain was also attenuated in rats implanted with morphine pellets as compared to animals implanted with placebo pellets (fig. 8). Daily water intake of the morphine-pellet implanted animals was highly variable during the treatment period. There was a noticeable decrease in water intake immediately after morphine implantation (fig. 8), which presumably is due to the non-specific behavioral disruption by morphine. Daily water intake, however, returned to levels comparable to that of placebo-pellet implanted groups on the second day of treatment. Thus, the animals appeared to have developed tolerance to the sedative effect of the drug.

FIGURE 7. Changes in body weight of rats injected daily with saline (\blacktriangle , n=8) or increasing dosage of morphine sulfate (Δ , n=14). The values represent means \pm SEM of the two groups of rats. * Values are significantly different ($p < 0.05$) as determined by Student's t-test.

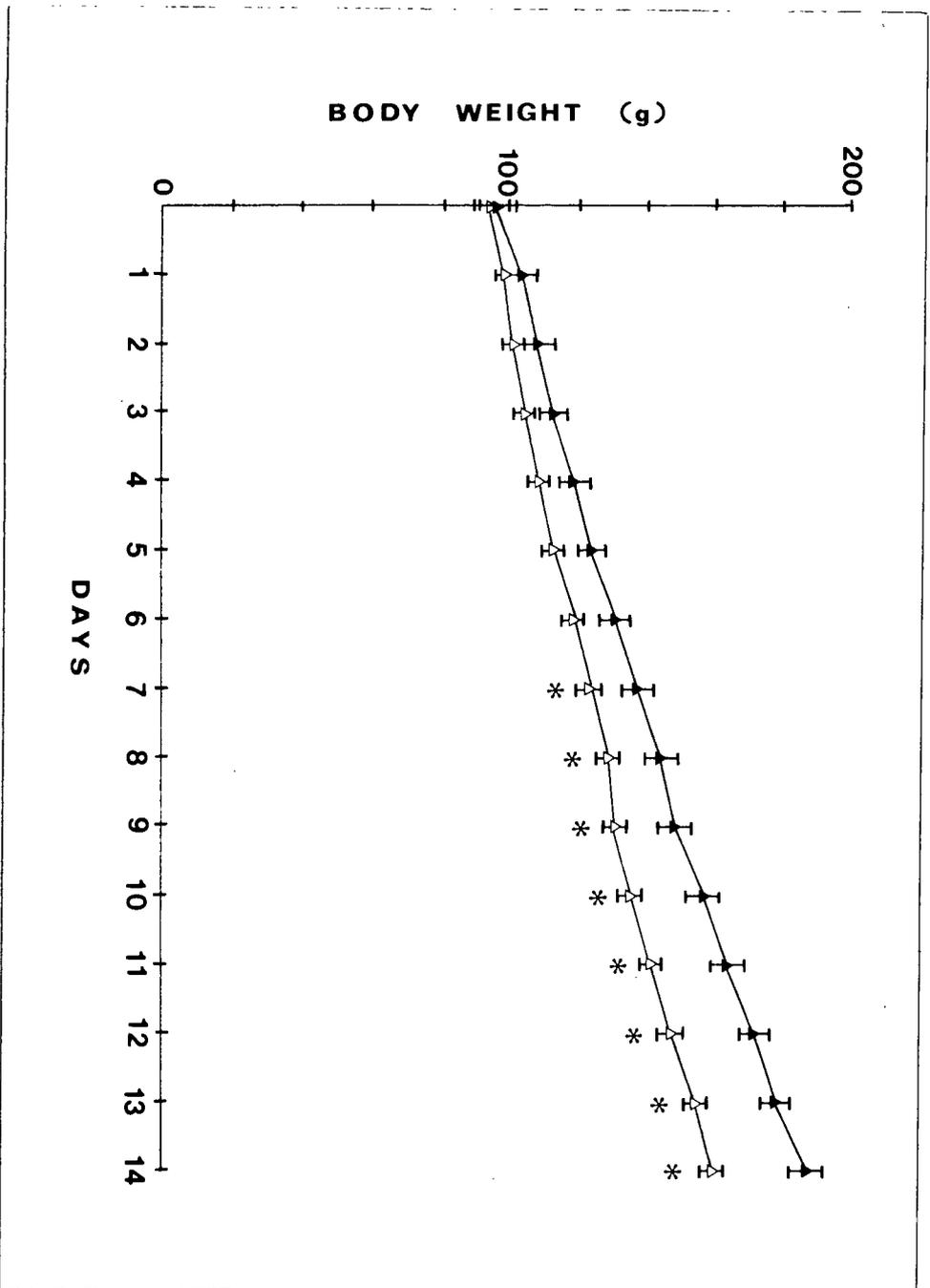
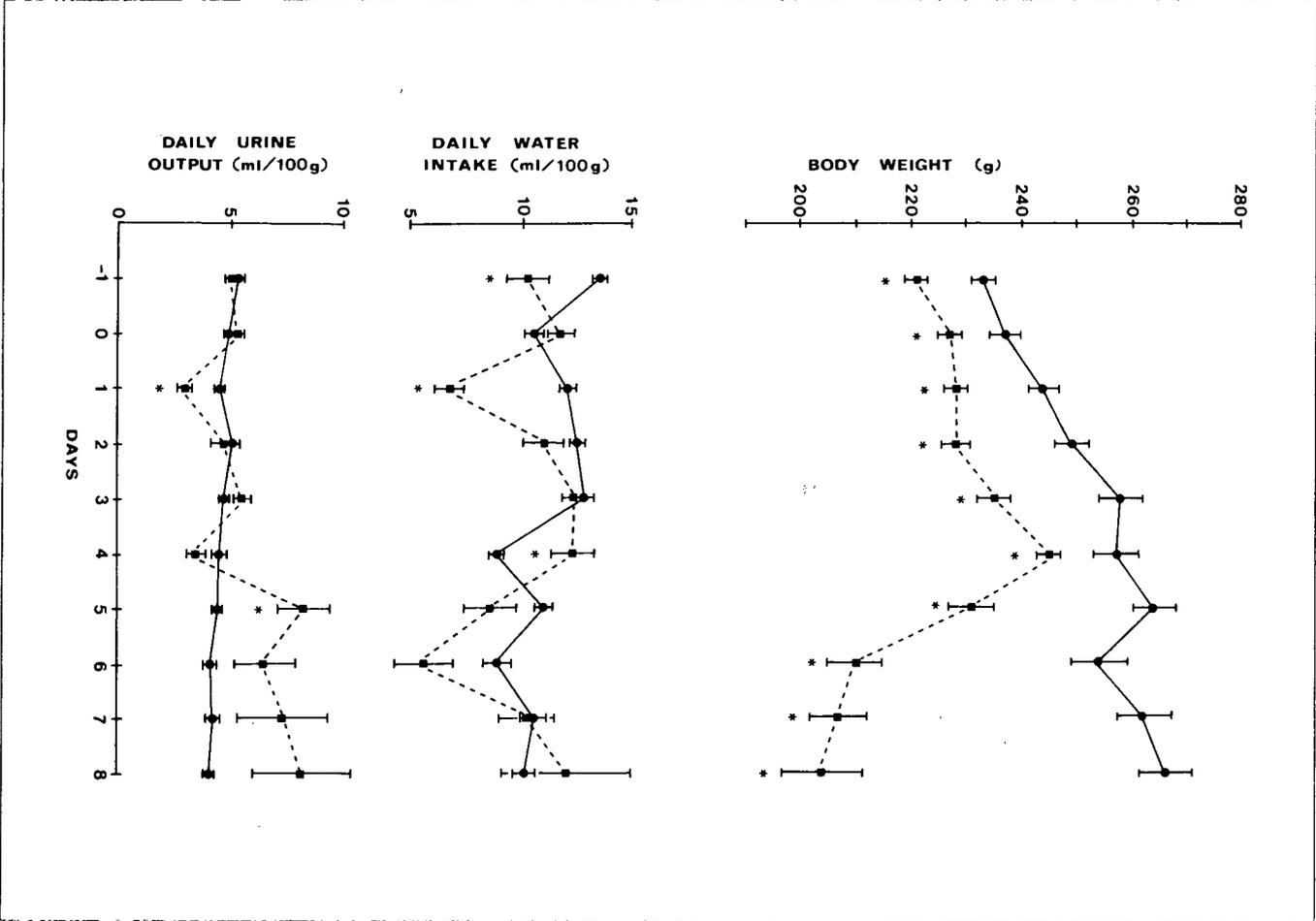


FIGURE 8. Changes in body weight, water intake and urine output of rats implanted with placebo (●-●) and morphine (■-■) pellets. Each animal was implanted with either a placebo or morphine pellet on day 0 and day 3. Both daily water intake and urine output were corrected to 100 g body weight. Withdrawal was precipitated by the excision of the implanted pellets on day 5. * Values are significantly different ($p < 0.05$) as determined by Student's t-test.



As with water intake, daily urine output was reduced on the first day of morphine-pellet implantation. Changes in water intake and urine excretion were closely related throughout the treatment period with only one exception. Urine excretion was significantly increased in the morphine-pellet implanted group on day 5, while daily water intake was significantly reduced. This change may account for the sudden decrease in body weight observed on day 5. Also, in contrast to the antidiuretic effect of a single dose of morphine, the drug appeared to promote urine formation when given chronically.

Excretion of a dark brown urine was observed in a few rats after morphine pellets implantation. Attempts to characterize the nature of the pigmentation through Sephadex G-75 were unsuccessful. There is reason, however, to believe that such pigmentation is due to a combination of hemoglobin and myoglobin, as will be discussed later.

Body weight loss is one of the physiological parameters often used in the quantitative assessment of physical dependence (103). When morphine was withheld from rats injected daily with morphine, the resulting reduction in body weight was small (fig. 9). Approximately 3% (n=8) reduction in body weight was observed 24 hr after the last injection. This implies that the degree of dependence of these animals is very light. In the morphine-pellet implanted rats, however, the surgical removal of the implanted pellets resulted in a dramatic decrease in body weight. As shown in fig. 10, the body weight decreased by

as much as 10% 24 hr after the excision of the pellets. No significant changes in body weight were observed in the placebo-pellet implanted animals.

Daily water intake was depressed slightly on the first withdrawal day but returned to control levels by the second day. Polyuria was quite marked throughout the withdrawal period. This temporary state of polyuria accompanied by the transient decrease in water intake can lead to a negative fluid balance and may account for the substantial decrease in body weight.

A. The Neurohypophyseal Stores Of AVP

Table II shows the the neurohypophyseal stores of AVP after 14 days of morphine sulfate injection. The average amount of AVP stored in the neurohypophysis of rats injected with morphine for 14 days was not significantly different from that of saline-injected control (423 ± 60 ng vs. 401 ± 32 ng). A 24 hr withdrawal from the drug also did not appear to influence the levels of AVP in the neurohypophysis (449 ± 55 ng). The AVP content in the hypothalamus of the morphine-injected (45.5 ± 3.7 ng) and withdrawn rats (34.4 ± 5.2 ng) were both lower than that of saline-injected control (50.4 ± 8.4 ng). This difference, however, was also not significant.

FIGURE 9. Changes in body weight during withdrawal in the multiple injection series. The rats (n=7) were injected with an increasing dose of morphine sulfate for 14 days prior to the withdrawal of the drug.

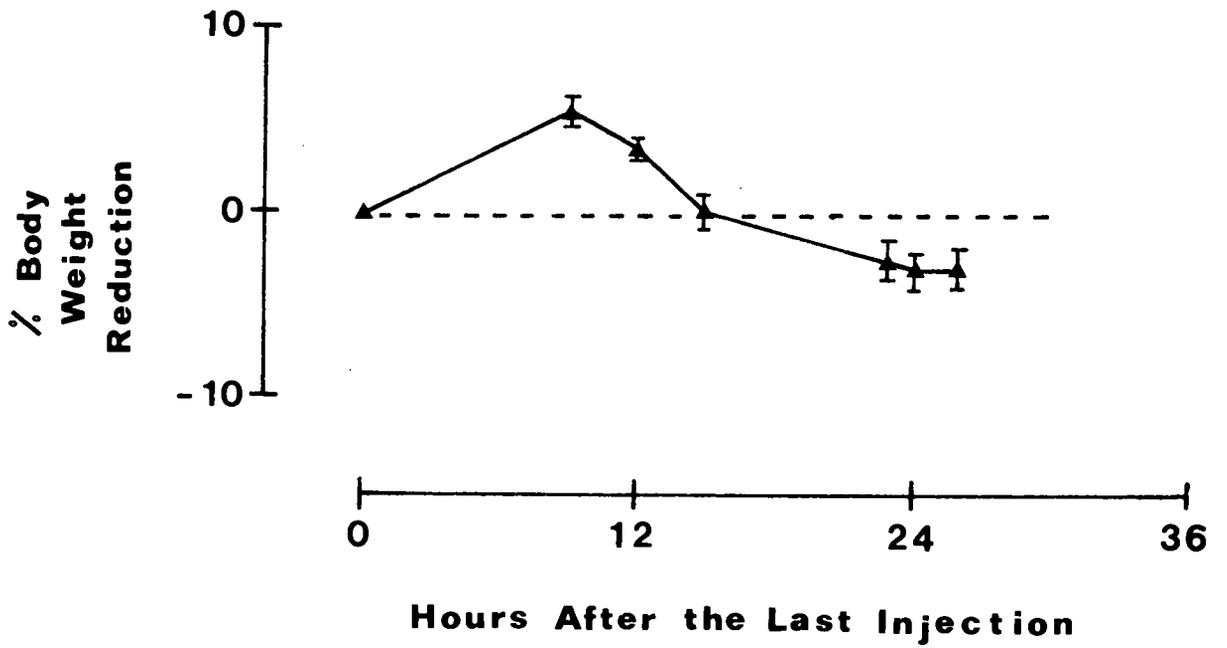


FIGURE 10. Changes in body weight during withdrawal in the pellet implantation series. The rats (n=6) were implanted with either placebo (□) or morphine pellets (○) for five days. * Values are significantly different ($p < 0.05$) as determined by Student's t-test.

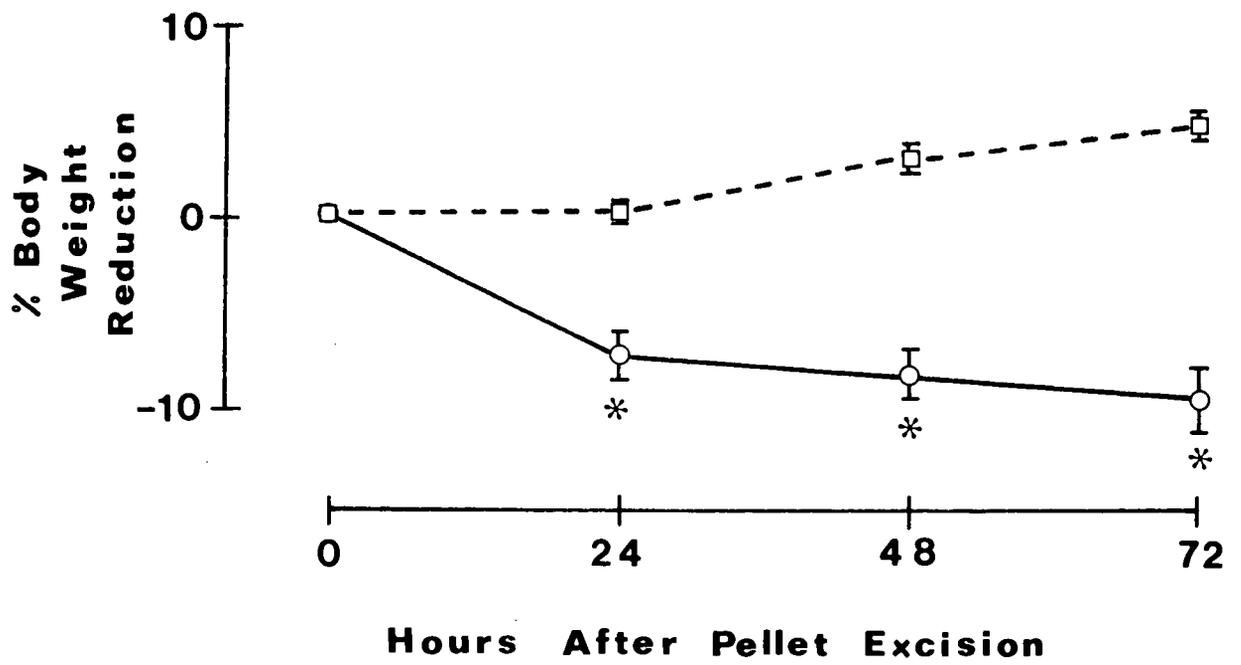
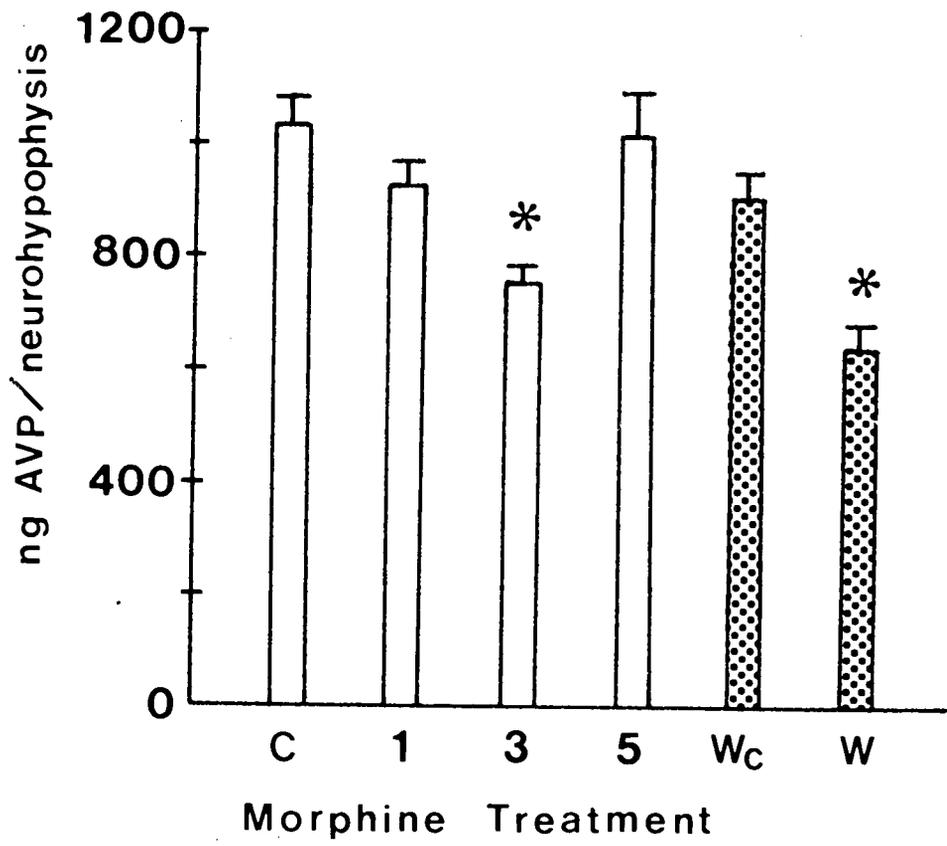


TABLE II. Hypothalamic and neurohypophyseal stores of AVP in the multiple injection series. The control group was injected with saline and the morphine-treated group with morphine sulfate for 14 days. Morphine was withheld from the morphine-withdrawn group for 24 hr after the 14 day treatment period. Each group consisted of 8 rats. No significant difference was found between the three treatments.

GROUP	HYPOTHALAMUS	NEUROHYPOPHYSIS
	(ng AVP)	(ng AVP)
CONTROL	50.44 ± 8.43	401 ± 32
MORPHINE	45.50 ± 3.67	423 ± 60
WITHDRAWN	34.43 ± 5.15	449 ± 55

Neurohypophyseal AVP stores of the morphine pellet implantation series are shown in fig. 11. Morphine treatment resulted in a significant depletion of the neurohypophyseal AVP stores. This was particularly noticeable on day 3, in which the amount of AVP stored was reduced from a control value of 1024 ± 66 ng (n=6) to 744 ± 28 ng (n=6). Continued treatment with the drug did not cause any further depletion of the neurohypophyseal AVP stores. In fact, it appeared that the neurohypophyseal stores of the hormone, which were depleted during the first three days of treatment, were being replenished by the fourth and fifth day. The amount of AVP stored on the fifth day of treatment was not significantly different from that of the placebo-pellet implanted control. If the AVP stored in the gland is maintained near the normal level with the continuous application of morphine after the fifth day, it may explain the lack of depletion of AVP in the neurohypophysis of rats given daily i.p. injection of morphine on the fourteenth day. Possible mechanisms by which the AVP stores were replenished will be discussed later.

FIGURE 11. Neurohypophyseal stores of AVP in rats implanted with placebo pellets (C) and morphine pellets for one (1), three (3) or five (5) days. Stippled bars represent neurohypophyseal AVP in rats implanted with morphine pellets for 5 days and allowed to withdraw from the drug for 3 days thereafter (W). The control group (WC) was implanted with placebo pellets and subjected to the same treatment as the withdrawal group (W). * Values are significantly different ($p < 0.05$) as determined by Student's t-test.

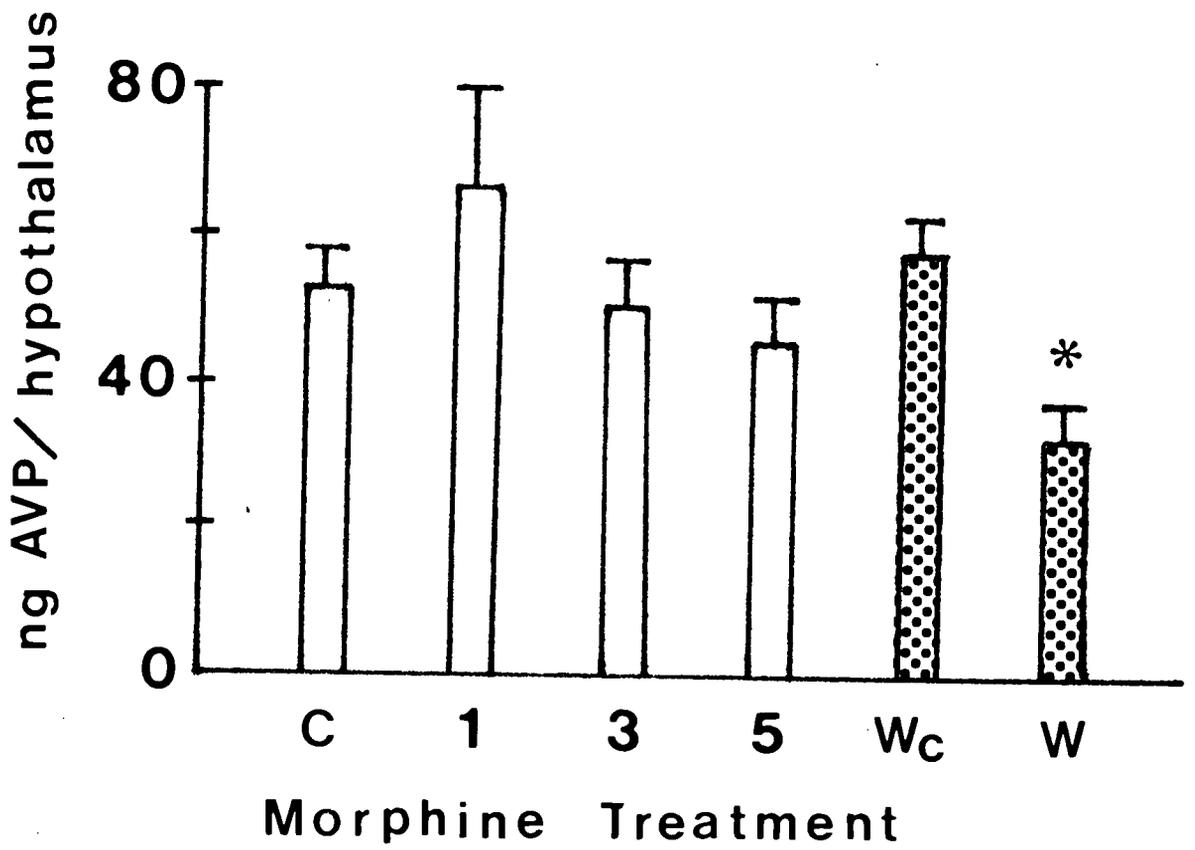


When morphine withdrawal was precipitated on the fifth day by the surgical removal of the implanted pellets, a marked reduction in the neurohypophyseal AVP stores was observed (fig 11). The levels decreased from a pre-withdrawal value of 1012 ± 70 ng (n=6) to 638 ± 36 ng (n=6) 72 hr after the excision of the pellets. Removal of the implanted placebo-pellets did not result in any significant change in the neurohypophyseal AVP stores (902 ± 37 ng, n=6) as compared to the placebo-pellet implanted controls.

Depletion of the neurohypophyseal AVP store, therefore, can only be observed during the early stages of morphine treatment. As the animal develops tolerance to and physical dependence on the drug, the neurohypophyseal stores of AVP returned to pre-treatment levels. Abrupt withdrawal of the drug from physically dependent animals again led to a depletion of the AVP stores.

During morphine treatment, no changes in the hypothalamic AVP stores were observed in the pellet implantation series (fig. 12). During withdrawal, however, a significant reduction in AVP stores was seen. The hypothalamic AVP stores were reduced from the pre-withdrawal value of 53 ± 5 ng (n=6) to 33 ± 5 ng (n=6) 72 hr after the excision of the implanted pellets. This change was significant as determined by the Student's t-test ($p < 0.03$).

FIGURE 12. Hypothalamic AVP stores in rats implanted with placebo pellets (C) or morphine pellets (1, 3 and 5). Stippled bars represent hypothalamic stores of AVP in the withdrawal group (W) and its placebo implanted control (Wc). * Values are significantly different ($p < 0.05$) as determined by Student's t-test.



The control neurohypophyseal AVP stores in the morphine-pellet implanted series (fig. 11) was much higher than that observed in the i.p. injected series (Table II). This discrepancy can neither be explained by variations in the extraction procedure nor by variations in the RIA of AVP. It may be due to the differences in the age of the animals used. Young male Wistar rats (193.5 ± 2.5 g, $n=8$) were used in the i.p. injection series, whereas adult male Wistar rats (266.0 ± 2.5 g, $n=6$) were used in the morphine-pellet implantation series. Seasonal variations may also play a role in altering the neurohypophyseal stores of AVP. The i.p. injection series was performed in the month of December, whereas the pellet implantation series was performed in May.

B. Neurohypophyseal Stores of Oxytocin

The neurohypophyseal oxytocin stores of the morphine-pellet implanted series are shown in Fig. 13. Unlike the neurohypophyseal AVP stores, oxytocin stores in the rats implanted with morphine pellets were unaltered in the first three days of treatment. However, a slight increase in the neurohypophyseal oxytocin stores from a control value of 1051 ± 52 ng ($n=6$) to 1353 ± 50 ng ($n=6$) was observed on the fifth day. This difference was significant as determined by Student's t-test ($p<0.01$). This change also coincided with the replenishment of the depleted AVP stores. The abrupt withdrawal from the drug initiated on the fifth day of treatment resulted in a significant reduction in the neurohypophyseal oxytocin

stores. The oxytocin stores fell from a pre-withdrawal value of 1328 ± 54 ng (n=6) to 901 ± 38 ng (n=6) 72 hr after the excision of the pellets.

C. Serum Osmolality and Sodium

No significant changes in serum osmolality and serum sodium were detected between the morphine and placebo pellet implanted animals (Table V). There was a slight increase in serum osmolality in the morphine withdrawn group (303.5 ± 2.6 mOsm/kg, n=6) as compared to its placebo implanted control (296.8 ± 2.4 mOsm/kg, n=6). This difference, however, was not significant as determined by Student's t-test.

FIGURE 13. Neurohypophyseal stores of oxytocin in rats implanted with placebo pellets (C) or morphine pellets (1,3 and 5). Stippled bars represent the withdrawal group (W) and its placebo implanted control (Wc). * Values are significantly different ($p < 0.05$) as determined by Student's t-test.

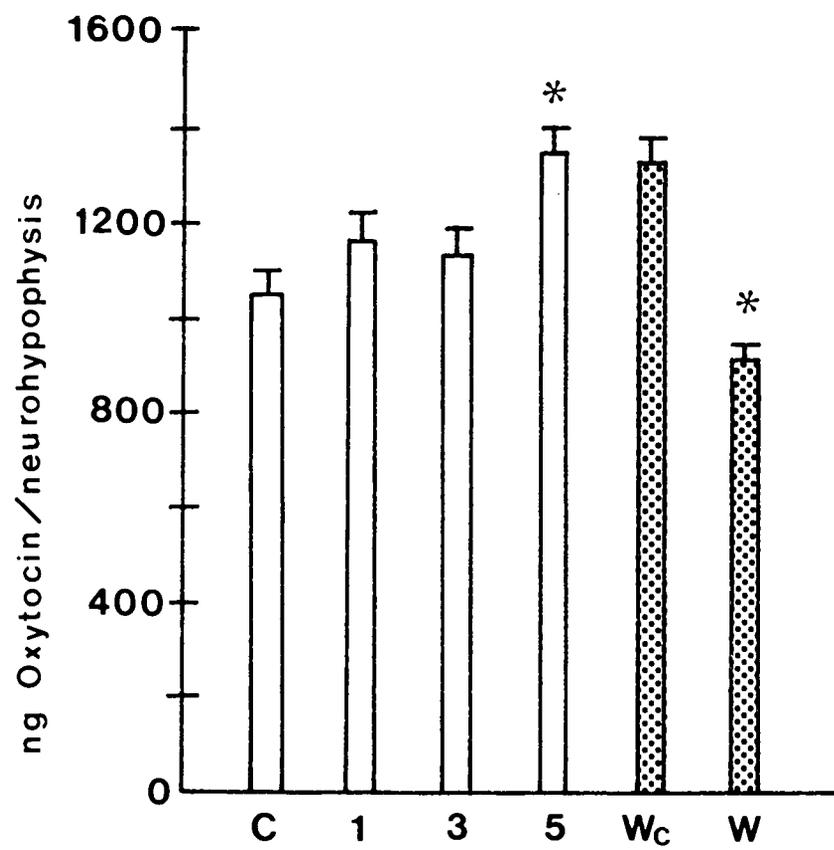


TABLE III. Serum osmolality and sodium of rats in the pellet implantation series. Each group consisted of 6 rats. The groups were classified as follows:

- C rats implanted with placebo pellets for 5 days;
- 1 rats implanted with morphine pellet for 1 day;
- 3 rats implanted with morphine pellets for 3 days;
- 5 rats implanted with morphine pellets for 5 days;
- Wc rats implanted with placebo pellets for 5 days; pellets were excised on the fifth day and the animals allowed to recover for 3 days;
- W rats implanted with morphine pellets for 5 days; pellets were excised on the fifth day and the animals allowed to recover for 3 days;

No significant difference was found amongst all groups.

GROUP	SERUM OSMOLALITY (mOsm/Kg)	SERUM SODIUM (mEq/L)
C	293.7 ± 2.0	144.8 ± 0.9
1	295.2 ± 3.4	142.7 ± 1.1
3	297.0 ± 1.8	146.0 ± 2.8
5	292.7 ± 3.2	143.8 ± 1.0
Wc	296.8 ± 2.4	145.8 ± 1.7
W	303.5 ± 2.6	147.7 ± 1.1

D. Opiate Receptor Binding

The binding of ^3H -naloxone to whole brain homogenate was depressed with chronic i.p. injection of morphine (fig. 14). Morphine abstinence for 24 hr resulted in near complete recovery of the ^3H -naloxone binding. Since the decrease in receptor binding can be explained by either a change in the number of binding sites or binding affinity, a Scatchard (115) analysis of the ^3H -naloxone binding was performed (fig. 15). The affinity constant (Kd) for ^3H -naloxone was found to be 3.2 and 5.3 nM for the control and withdrawal groups, respectively. This was increased four to seven fold (about 22.4 nM) in the morphine treated group (Table VI). A small and relatively insignificant increase in the number of receptor binding sites (q) was also observed in the morphine-treated group (Table VI). The lack of change in the number of receptor binding sites coupled to the decrease in the affinity of the receptor for ^3H -naloxone in the morphine-treated rats suggest the presence of a competitive ligand, such as morphine, in the brain homogenate. Repeated washing of the receptor preparation from the morphine-injected rats resulted in an enhancement of the ^3H -naloxone binding. Effects of the neurohypophyseal hormones, AVP and oxytocin, on the receptor binding assay were also tested. No specific or non-specific interference were detected at concentrations of 20 pM to 2 nM.

FIGURE 14. Stereospecific binding of ^3H -naloxone to whole brain homogenates from rats injected daily with saline (\bullet) or morphine sulfate (\blacktriangle) for 14 days. The third group (Δ) represents binding to whole brain homogenates from rats withdrawn from the drug for 24 hr.

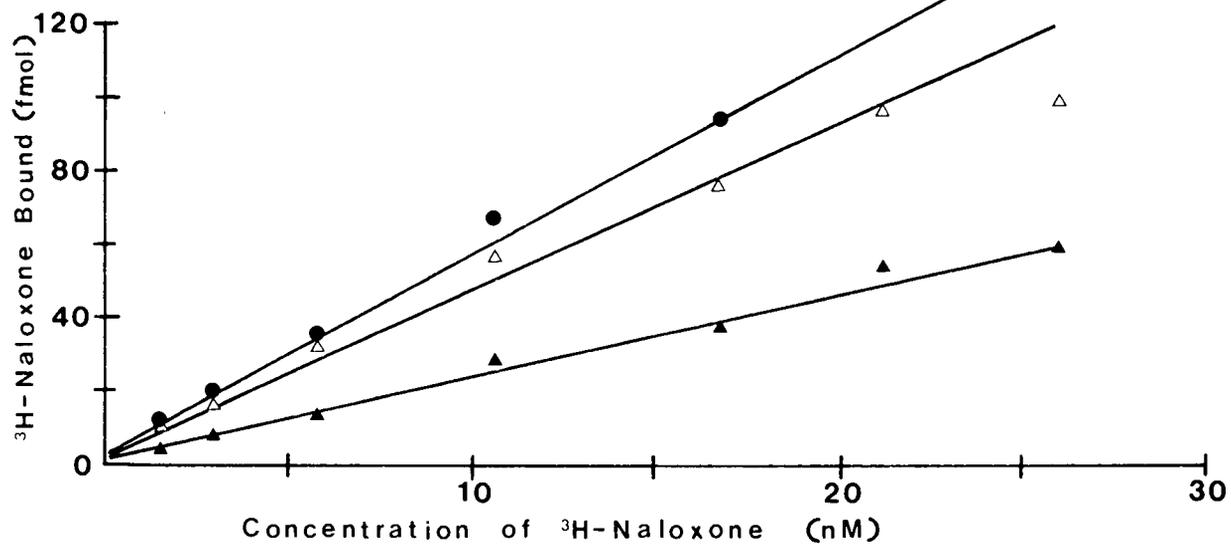


FIGURE 15. Double reciprocal (Scatchard) plot of stereospecific ^3H -naloxone binding to whole brain homogenates from rats in the multiple injection series. Legend is the same as that in fig. 14.

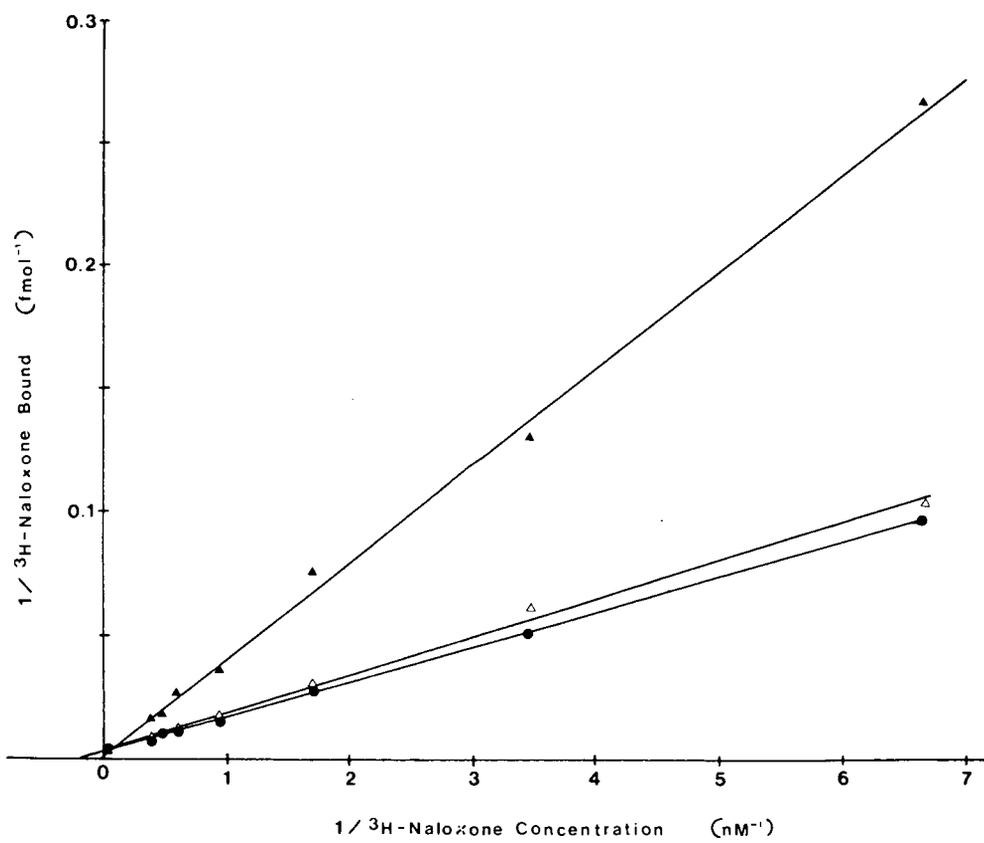


TABLE IV. Number of binding sites (q) and binding affinity (Kd) as determined from Scatchard analysis of ^3H -naloxone binding to whole brain homogenates from rats injected with morphine.

	BINDING SITES	BINDING AFFINITY
GROUP	(pmol)	(nM)
CONTROL	0.370	5.32
MORPHINE	0.588	22.37
WITHDRAWN	0.213	3.21

DISCUSSION

The present study dealt with two aspects of morphine effects on AVP function - the immediate systemic effects of a single dose administration (acute experiments) and the effects of continuous administration of the drug (chronic experiments). These two aspects will be dealt with separately in the discussion.

I. Acute Effects of Morphine Administration

One of the approaches in studying the antidiuretic action of a drug is to measure the ability of the drug to reduce the rate of urinary flow in a water hydrated animal. In this type of study, it is important to establish a constant state of hydration and water diuresis. If the action of the drug is directed at the kidneys, then factors affecting urine flow rate, such as endogenous release of AVP, must be controlled. This is the basis for using ethanol to suppress the endogenous release of AVP in the antidiuretic hormone (ADH) bioassay (116). The study reported by Inturrisi and Fujimoto (132) on the antidiuretic action of morphine utilized this feature of the ADH bioassay. One major problem in using ethanol-hydrated animal preparations is that antidiuresis mediated through the release of AVP may not be detected. The reason being that the drug in question has to counteract the suppressive effect of both ethanol and low plasma osmolality on AVP release. In view of the possibility that morphine-induced antidiuresis is mediated

by the release of AVP, ethanol was excluded from the infusion solution in these experiments. This also has an added physiological advantage in that the animals were fully conscious. The inhibition of endogenous AVP release was evident by the maintenance of a stable high urinary flow rate and low urine osmolality.

The results from the acute experiments clearly established that morphine at 1 mg/kg, induced a substantial reduction in urinary flow in the hydrated rat (Table I). This agreed well with studies by several investigators on the antidiuretic action of morphine (17-21). The same dose also produced a significant reduction in the mean arterial pressure (Table I). In terms of the renal and cardiovascular responses to morphine, both heterozygous D.I. and normal Long-Evans rats may be considered identical. However, the responses of the homozygous D.I. rat were statistically different from those of heterozygous D.I. and normal Long-Evans rats.

The reduction in urinary flow was accompanied by an increase in urine osmolality. The concentrating ability of the kidney in all three groups improved after the administration of morphine. A substantial decrease in free water clearance was also observed. In some cases, free water clearance reversed to negative values, suggesting net water reabsorption. It is difficult to accurately determine the underlying mechanism for this decrease as free water clearance is affected by a multitude of

physiological parameters. Nevertheless, these changes suggested the involvement of AVP in the heterozygous D.I. and normal rats.

The release of AVP has been known to be influenced by a number of factors. These include changes in blood osmolality, volume, and pressure (30). Its release is also affected by various hormones, water and electrolyte balance, and emotional stress (117). It has been widely accepted that the morphine-induced antidiuresis is due to the direct stimulation of the hypothalamo-neurohypophyseal system (17-21). In order to conclusively prove this, all factors affecting the release of AVP must be adequately controlled. In view of the powerful cardiovascular effect of morphine (25-27), the secretion of AVP may not be a direct result of the stimulation of the hypothalamo-neurohypophyseal system by the drug. Recent evidence indicates that blood pressure changes as small as 5% could significantly increase the plasma AVP levels (30). As much as 8 and 11% reduction in mean arterial pressure (Table I) was observed after morphine administration in the normal Long-Evans and heterozygous D.I. rats, respectively. Hence, hypotension can not be ruled out as a possible mechanism by which the drug effects the release of AVP.

If the endogenous release of AVP is the sole mechanism responsible for the antidiuretic action of morphine, one would not expect an antidiuretic response from animals incapable of synthesizing AVP. For example,

this was shown to be the case for chlorpropamide-induced antidiuresis (118). Chlorpropamide, at doses effective in causing antidiuresis in normal rats was ineffective in rats with hereditary hypothalamic diabetes insipidus (118). Morphine sulfate, on the other hand, was effective in reducing the urinary flow in the homozygous D.I. rats (Table I). Hence, mechanisms other than AVP must be involved in the induction of antidiuresis by morphine.

The antidiuresis observed in the homozygous D.I. rats was significantly less than that of the normal and heterozygous D.I. rats. This difference may be due to the lack of AVP in the homozygous D.I. rats, leading to a decreased ability of the kidneys to reabsorb water. Compared to the normal and heterozygous D.I. rats, there was hardly any change in the urine osmolality of the homozygous D.I. rat during the antidiuretic period. This again can be attributed to the lack of AVP in these animals.

Antidiuresis can be brought on by several means. For example, alterations in renal function can be induced by various vasoactive agents. The resulting changes in filtrate reabsorption, amount of filtrate formed, or both, can lead to a marked reduction in urinary flow. Antidiuresis and antinatriuresis have been shown to be induced by changes in the physical forces responsible for tubular fluid reabsorption (119) as well as by vasoactive agents such as angiotensin (120,121), epinephrine (121), and AVP (122). Changes in GFR, caused by either an

alteration in glomerular membrane permeability or renal vascular tone (123) can also cause a substantial reduction in urinary flow. Since the homozygous D.I. rats are devoid of AVP, only AVP-independent mechanisms may be used to explain the antidiuresis observed in these animals.

In view of the marked decrease in mean arterial pressure observed after morphine administration, it is highly probable that neural and hormonal compensatory mechanisms, similar to those involved in hemorrhagic hypotension (124,125) are activated. An increase in sympathetic nervous tone and circulating catecholamines has been shown to dramatically alter renal function (126,127). Reduction in renal blood flow, and reduction in salt and water excretion have been demonstrated during periods of heightened sympathetic discharge (126). An increase in circulating catecholamine levels (mainly epinephrine and norepinephrine) can cause a decrease in the total renal blood flow and an increase in filtration fraction (127). In addition, hypotension may also serve as a stimulus for the release of other vasoactive peptides (120-122). Activation of the renin-angiotensin system, for example, was shown to result in a decrease in GFR and RBF, antidiuresis and antinatriuresis (128). It is therefore likely that antidiuresis in the homozygous D.I. rat is due to changes in the cardiovascular system and renal hemodynamics brought on by neural and hormonal mechanisms other than AVP.

Morphine appeared to exert a more potent

hypotensive effect on the homozygous D.I. rats as compared to the normal and heterozygous D.I. rats (Table I). This suggested that the lack of AVP may have interfered with the cardiovascular compensatory mechanisms in these animals. Under normal conditions, AVP has been shown to be involved in the maintenance of the mesenteric vascular tone (129). Hypophysectomy was shown to cause dilatation of the mesenteric resistance vessels as well as to abolish the intestinal vasoconstrictor response to volume depletion (130). In addition, renal blood vessels appeared normally to be under the vasoconstrictor influence of AVP (131). The greater sensitivity to AVP in the diabetes insipidus animals has been shown to be partially due to the vasoconstrictor effect of the hormone (132). Hence, in the normal rat, the vasoconstrictor effect of AVP may assist in the restoration of a normal systemic blood pressure.

The rate of clearance of morphine is slow and its duration of action is generally in the order of 4-5 hr (133). Uptake of the drug by the CNS can be detected as long as 60 minutes after a single i.v. injection (134,135). Hence, the presence of the drug should provide a continuous stimulus for AVP secretion and a protracted antidiuretic period. However, the antidiuretic period observed only lasted for 10 to 20 minutes. This could either mean that the drug was cleared rapidly to below the stimulus threshold or the animal has developed rapid tolerance or tachyphylaxis to the drug. When a second dose

of morphine was administered 30 min after the initial dose, the resulting antidiuresis observed was much reduced. Therefore, the short antidiuresis period observed after the initial dose is likely due to the development of tachyphylaxis. Morphine tachyphylaxis was also observed by Inturrisi and Fujimoto (17). They hypothesized that morphine has an inhibitory effect on AVP release after one or more doses. The mechanism underlying the development of tachyphylaxis is not known at present. The chronic experiments in the current study were designed to examine the long term effects of morphine administration on the hypothalamo-neurohypophyseal system. Changes in the neurohypophyseal stores of AVP might shed some light on the effects of morphine on the release of AVP.

II. Chronic Effects of Morphine Administration

Chronic osmotic stimuli or dehydration has been known to deplete the neurohypophyseal stores of both AVP and oxytocin (136,137). Since the acute administration of morphine causes the release of AVP, it would be of interest to examine the adaptive response of the neurohypophysis upon chronic administration. Rats implanted with morphine pellets showed progressive depletion of neurohypophyseal AVP in the first three days of treatment. The neurohypophyseal stores subsequently recovered to the pre-treatment levels on the fifth day. These changes may reflect the development of tolerance to the stimulatory effects of the drug. Although tachyphylaxis can be demonstrated after a single dose

injection of morphine, the development of tolerance is reported to be maximal after 3 days (33). Hence, the depletion of AVP in the first 3 days of treatment might be due to the direct stimulation of AVP release or by morphine-induced alteration of the afferent signals to the hypothalamo-neurohypophyseal system. Presumably, the amount of hormone released during this 3 day period exceeded the biosynthetic rate. With the development of tolerance, the amount of hormone released progressively decreases, thus allowing biosynthesis to overcome the rate of release and replenish the neurohypophyseal stores of the hormone.

In rats given daily i.p. injections of morphine, the neurohypophyseal AVP stores did not show any significant difference compared to those of saline-injected controls. This may be due to the fact that the animals are tolerant to the stimulatory effects of morphine at the time of sacrifice. It is quite possible that the lack of difference be due to an adaptive change in AVP synthesis in response to chronic stimulation.

It is difficult to compare the values obtained in the multiple injection series with those of the pellet implantation series. First, the degree of dependence on morphine in these two series is different as is evident by the changes in body weight during withdrawal. A 10% body weight loss was encountered in the pellet implantation series after 24 hr of drug abstinence. The morphine injected rats, on the other hand, only exhibited a 3%

decrease in body weight over the same period of withdrawal. Secondly, the mode of delivery of the drug is different. In the multiple injection series, morphine was given twice daily at 0800 and 1800 hr. Since morphine is cleared in 4-5 hr, the animals may go through a brief period of withdrawal between injections. On the other hand, morphine was delivered at a reasonably continuous rate in the pellet implantation series. Thus, these animals are not as likely to have encountered withdrawal periods throughout the series. Thirdly, the duration of exposure to the drug varies between the two series. The rats were only exposed to morphine for 5 days in the pellet implantation series, whereas rats were given morphine for 14 days in the multiple injection series. Hence, the neurohypophyseal stores of AVP are not comparable between the two series. It is of interest, however, that the neurohypophyseal AVP of the saline injected rats (423 ± 60 ng, $n=8$) was much lower than that of the placebo-pellet implanted rats (1024 ± 66 ng, $n=6$). This is partially due to the differences in the age of the animals used. Young rats were used in the multiple injection series, whereas adult rats were used in the pellet implantation series. Adult rats have been shown to have significantly more AVP stored in the neurohypophysis than young rats (137). It is also possible that the stress caused by the daily handling and injections may have contributed to the depletion of AVP stores in the multiple injection series. Stressful and painful stimuli have long

been known to stimulate the release of AVP (117). Animals in the pellet implantation series were not subjected to stressful stimuli and, consequently, a better reflection of the chronic effects of the drug.

Depletion of neurohypophyseal AVP stores in the morphine-pellet implanted rats can be attributed to a decreased biosynthetic rate or an increased rate of release. Morphine has been known to affect the synthesis of proteins (138). Furthermore, this has been attributed to the decrease in food intake resulting from the sedative effect of the drug (138). However, starvation per se is not a likely cause for the depletion of the neurohypophyseal AVP stores. It has been shown to have no effect on AVP stores despite the presence of a slight decrease in water intake (139). This, however, does not rule out the possibility of a direct effect of morphine on the biosynthesis or transport of AVP.

Increased release, such as that occurring during dehydration (139), can also deplete the neurohypophyseal stores of AVP. This may partially explain the depletion observed after the first day of pellet implantation. Water intake in rats implanted with morphine pellets decreased significantly after the first day (Fig. 10). As water intake recovered to control levels by the second day, dehydration can be ruled out as a factor for the depletion of neurohypophyseal AVP stores observed on the third day of treatment. Since AVP was shown to be released with the acute administration of morphine (17-21), the continuous

application of the drug should deplete the stores of the hormone. A chronically elevated circulating AVP level would tend to promote water retention and development of a syndrome of inappropriate secretion of antidiuretic hormone (SIADH). The two most prominent symptoms of SIADH are reduced serum osmolality and dilutional hyponatremia (140). Serum determinations performed on rats treated with morphine revealed no changes in either serum osmolality or sodium (Table V). This raises the possibility that the hormone may not be released into the systemic circulation if the depletion of AVP is due to an increased rate of release.

In contrast to AVP, oxytocin stores in the neurohypophysis were unaltered by morphine-pellet implantation. Although the neurosecretory neurons producing these two hormones are anatomically closely associated, there is evidence that these hormones can be released independently of each other (141). Thus, stimuli causing AVP release may not necessarily affect the release of oxytocin. Also, morphine and its analogues have been shown to inhibit the suckling-induced oxytocin release (140). This may account for the lack of depletion of oxytocin stores after 3 days of morphine treatment. It may also explain the slight elevation in oxytocin stores observed on the fifth day of treatment.

Abrupt withdrawal of morphine from the physically dependent animals resulted in the depletion of both AVP and oxytocin stores. Changes in AVP storage may

partially be due to the sudden changes in water and electrolyte balance. Diarrhea (142), and a decrease in water intake (40) during withdrawal can drastically reduce the body's stores of salt and water. These changes, however, were not observed in the current study (fig. 10). Aside from the transient decrease in water intake observed on the first day of withdrawal, no significant changes were observed in the morphine or placebo pellet implanted group on the second and third day of withdrawal. There was, however, a noticeable increase in daily urine output throughout the 3 days of withdrawal. Because of the small number of animals in each group ($n=6$), this increase was not significant as determined by Student's *t*-test. Nevertheless, this change in urine excretion could have altered the animal's salt and water balance. In addition, it may account for the dramatic decrease in body weight observed during withdrawal. The generalized increase in CNS activity is equally likely to potentiate the release and subsequent depletion of the two hormones. The presence of various withdrawal signs, such as jumping, wet dog shaking, and teeth chattering, is no doubt related to the hyperactivity of the CNS.

It is clear that both acute and chronic administration of morphine affects the functioning of the hypothalmo-neurohypophyseal system. The discovery of opiate receptors in the neurohypophysis (143) raised the possibility that endorphin and other endogenous opioid peptides may regulate the neurohypophysis. Administration

of β -endorphin has been shown to stimulate the release of AVP in the rabbit (144). The presence of enkephalin innervation in the neurohypophysis and the fact that both AVP and enkephalin content were depleted during dehydration (145) further strengthen the concept of the control of AVP secretion by these peptides.

Aside from the influence of the endorphins and enkephalins on the neurohypophysis, the neurohypophyseal hormones may in turn control the actions of these endogenous opiates. Krivoy et al. (79) have reported that the neurohypophyseal hormones can facilitate the development of tolerance to and physical dependence on opiate agonists. A number of theories have been proposed to explain the mechanism of tolerance and physical dependence (59). One of these theories is the change in adenylate cyclase activity (60). Acute administration of morphine was shown to cause a transient suppression of the adenylate cyclase activity. An enhanced formation of cyclic AMP was found during the development of tolerance and physical dependence. Since AVP has been shown to exert its action on target cells by accelerating the formation of cyclic AMP (146), the possibility exists that it may be one of the compensating mechanisms in the maintenance of a stable cellular level of the cyclic nucleotide.

Alteration in opiate receptor binding has also been proposed as a mechanism of tolerance and physical dependence (68). Whole brain homogenates from rats injected daily with morphine for 14 days showed a decrease

in ^3H -naloxone binding (Table VI). In contrast to the findings by Pert and Snyder (69), no significant changes in the number of binding sites were detected. Scatchard analysis revealed that the changes are likely due to the presence of a competitive agonist. The repeated washing of the homogenate caused a progressive increase in the receptor affinity. This suggested the interference of ^3H -naloxone binding by the injected morphine. Also, changes in receptor affinity are probably caused by the presence of minute quantities of morphine in the whole brain homogenate. Direct interference of opiate receptor binding by the neurohypophyseal hormones can be ruled out as a possible mechanism of tolerance and dependence. Both oxytocin and AVP were found to have no effect on the ^3H -naloxone binding.

The temporal relationship between opiate receptor binding and tolerance to the analgesic effect of morphine appears rather weak. Pert and Snyder (69) found that enhancement of opiate receptor binding appeared 2 hr after morphine treatment, and remained at the same level for 4 days. Tolerance, on the other hand, has been shown to increase progressively and reaches a peak on the third day of treatment (35). Hence, the appearance of maximal enhancement of opiate receptor binding occurred long before the development of maximal tolerance and physical dependence. However, the lack of correlation in opiate receptor binding and tolerance development does not necessarily rule out the possible involvement of the

opiate receptors. It is highly probable that changes in receptor affinity or binding sites occur regionally in the brain. Such alterations may only occur in brain structures responsible for parameters or behavioral signs measured in the assessment of tolerance and physical dependence. Hence, they may only be detected by examining the binding in these specific structures of the brain. Crude preparations, such as whole brain homogenate, may not be able to detect such small changes.

One interesting phenomenon encountered in the pellet implantation series is the action of morphine on the kidneys. Dark brown urine was observed from a few rats 24 hr after the implantation of a morphine pellet. The excretion of dark brown urine resulting from narcotic use has been reported in human drug addicts (51). The pigmentation has been characterized as a combination of hemoglobin and myoglobin (48). This symptom is quite similar to the crush syndrome described by Schreiber (52). Regional skeletal necrosis or rhabdomyolysis were found in some patients (51) and is likely the cause of the myoglobinuria. Rhabdomyolysis may be caused by the prolonged immobilization as the animal is under the sedative effect of morphine. The possibility that rhabdomyolysis is caused by adulterants present in the heroin used by the drug addict, as proposed by Richter et al. (48), may be excluded. The morphine used in the present study is devoid of any adulterants. The likelihood that rhabdomyolysis be due to the inert binders used in

the processing of the pellets may also be excluded. Control animals were implanted with pellets made exclusively of such materials, yet myoglobinuria was never observed in these animals. It is still questionable whether the chronic use of narcotic drugs can in some unknown way predisposes the onset of renal disease. The current observation supported studies (48-51) on a possible link between renal disease and heroin addiction. It does not, however, conclusively rule out the possibility that the onset of renal disease be due to other complications associated with addiction. It would be of interest though to determine whether the renal disease improves or stabilizes with the withdrawal of the drug.

CONCLUSION

It is clear that morphine administered acutely has an antidiuretic effect. The release of AVP is at least partially responsible for the antidiuresis. The actual stimulus for AVP release, however, remains questionable. It is possible that morphine acts directly on the hypothalamic neurosecretory cells. However, the hypotensive effect of the drug can also exert a substantial influence on the release of the hormone. The application of morphine directly to an in vitro culture of the hypothalamo-neurohypophyseal complex may provide some clue to the direct effect of the drug on AVP release. It is equally likely that a portion of the antidiuresis observed is mediated by the effects of the resulting hypotension on renal function. In view of the cardiovascular effects of morphine, it would be of interest to examine its effects on the renal vasculature and the output of various urinary constituents.

When given chronically, morphine depleted the neurohypophyseal stores of AVP. This depletion, however, was transient and only lasted for 3 days. The cause of this depletion is partially due to dehydration initiated by a decrease in food and water intake. Potentiation of AVP release due to the direct action of the drug on the hypothalamo-neurohypophyseal system is an equally likely possibility. The subsequent development of tolerance and physical dependence on morphine presumably allows the animal to adapt to the effects of the drug by either

decreasing the rate of secretion or increasing the biosynthetic rate. Depletion observed when the drug was abruptly withdrawn from physically dependent animals can be attributed to the stimulatory effect of salt and water loss on AVP release as well as to the generalized hyperactivity of the CNS. The actual cause of AVP depletion during morphine treatment and withdrawal remains unsettled. Simultaneous measurements of the biosynthetic and secretion rates of the peptide may shed some light to this problem. Furthermore, if the depletion was due to the augmented release of AVP, it would be of interest to determine the eventual fate of the hormone. There is a possibility that it may be elaborated not only to the systemic circulation but also to various CNS structures and the CSF.

Enhancement of opiate receptor binding with morphine treatment as reported by Pert and Snyder (69) was not duplicated in this study. It was concluded that the minute quantities of the drug left in the whole brain homogenates severely affected the binding of ^3H -naloxone to the receptor. The possibility that AVP and oxytocin facilitate the development of morphine tolerance by interfering directly with the binding of the drug to the receptor was judged to be unlikely.

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