# THE INFLUENCE OF RESTRAINT-STRESS ON DEVELOPMENT OF TOLERANCE TO THE HEART-RATE EFFECTS OF MORPHINE

by

Karen S. Schwarz

A THESIS

Presented to the Department of Medical Psychology and the Graduate Council of the Oregon Health Sciences University in partial fulfillment of the requirements for the degree of

Master of Science

February, 1986

APPROVED:

[redacted]

.....

(Professor in Charge of Thesis)

[redacted]

.....

(Chairman, Graduate Council)

ACKNOWLEDGEMENTS

I would like to express many thanks to Chris Cunningham, my thesis advisor, for his patience and help. He has helped me develop many skills of science, including experimental design, writing and statistics. I appreciate the time and effort he has given to drill those things into my head. I would like to thank the other members of my thesis committee: Drs. John Crabbe, Dan Hatton, Hall Downes and the chairman, George Olsen for their interest and advice.

I owe a lot of my sanity, and the fact that I still have any, to all of my friends. Special thanks to Tony Parker, Adam Kramer and Kathy Whaley for helping me forget about school when necessary. Of course without the support of my fellow students, school wouldn't have been any fun at all! Thank you Ann, Glenda, Denni, Karie and Martin. Also, thanks to all of the members of Chris' lab. It has been my observation over the past few years that Ginger Ashworth is a wonderful source of necessary information about how to deal with red tape. Just as important, she has been a great counselor--THANK YOU GINGER!!

This thesis is dedicated to three very special people. First to my mother, who is my very favorite experimental psychologist. She had a great influence in my choice of careers. Second, to my father, whose belief in education and pride in his daughter have kept me in pursuit of knowledge. Third, to Seth Stevens, who has given me a lot of respect and encouragement. Seth has managed to be my best friend, partner, teacher and therapist throughout this whole project.

Finally, thanks to my siblings, Janice, David and Todd for sharing strength and love. I am lucky to be part of such a fantastic, close-knit family.

This research was funded by a National Heart, Lung and Blood Institute traineeship and the N.L. Tartar Research Fund.

ABSTRACT

Morphine, administered intravenously to rats, causes an initial bradycardia followed by tachycardia. Restraint-stress augments the bradycardia and attenuates the tachycardia. The present study assessed tolerance development to the heart-rate effects of various doses of morphine in restraint-stressed and freely-moving rats.

Male albino rats were assigned to one of four dose groups: 0 (saline), 2, 4 or 8 mg morphine/kg body weight. Within each dose group there were both restrained (R) and unrestrained (U) rats matched in pairs by weight. Following an habituation phase, during which all rats were exposed to the experimental chamber but received no injection, the tolerance acquisition phase began. During acquisition, all rats received one infusion daily for 12 days according to dose group assignment. After acquisition, the rats were exposed to each dose in a counterbalanced order to obtain within-group dose-response data. Magnitude of tolerance was assessed by comparing the responses of the saline groups with those of the drug groups. Heart rate was recorded for 1 hr before (baseline) and 2 hrs after infusion and analyzed in 5-min sample periods.

In general, heart rate was elevated initially due to handling and decreased over the 1-hr pre-infusion baseline period. Over the 2-day blocks, mean baseline heart-rate declined slightly in Dose Groups 0 and 2. In Dose Groups 4 and 8, mean baseline heart-rate increased and was significantly higher relative to Dose Groups 0 and 2. Upon morphine infusion, heart rate decreased, then increased in a dose-dependent manner. In the saline groups (Groups U0 and R0), no significant change in heart rate occurred after infusion. Over 2-day blocks during the

acquisition phase, a significant attenuation in the magnitude of bradycardia occurred in all morphine dose groups except Group R8. Group U8 also showed a significant attenuation in the magnitude of tachycardia; however, no significant change in tachycardia occurred in any other groups.

During the tolerance test phase, separate comparisons among the R and U groups were made. Tolerance to the bradycardic effect was revealed in Groups R4, R8, U2 and U4, but not in Groups R2 and U8. Sensitization to the tachycardic effect was revealed in Groups U2, U4 and U8 but not in any of the R groups.

These results are consistent with Solomon and Corbit's (1974) opponent-process theory which states that for a given response (e.g., bradycardia) to a stimulus (morphine), there is an opposing response (tachycardia) generated which serves to decrease the magnitude of the first response. According to this theory, chronic morphine exposure leads to strengthening of the tachycardia and is reflected as a decrease in bradycardia (tolerance) and an increase in tachycardia (sensitization). This opponent-process theory also suggests that the increase in baseline heart rate over blocks seen in Dose Groups 4 and 8 may be the result of conditioning. For example, the opponent process was conditioned to environmental cues which elicited tachycardia before morphine injection.

TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

Opiates are used extensively in medical treatment for patients in pain, and are also abused extensively for recreational purposes. With prolonged use, many of the effects of opiates diminish, i.e., drug tolerance develops. The factors affecting development of tolerance to opiates need to be defined in order to gain a better understanding of how best to treat patients in chronic pain, how to avoid dependence and how to treat an addicted person effectively. This thesis is especially concerned with the role that stress might play in the development of tolerance to opiate drug effects.

In terms of the study of drug abuse, many popular theories posit that although initial drug-taking is often due to peer pressure or curiosity about a drug's so-called euphoric effects, chronic drug use often occurs to alleviate stress or anxiety (Dohner, 1972). However, stress might also act to accelerate the development of tolerance (Peris, 1984), requiring an individual to take more drug in order to experience the same level of euphoria or relaxation obtained initially.

In terms of chemotherapy, hospitalized or chronically-ill people often receive repeated doses of analgesic medications such as opiates. These people are often under stress because of the pain caused by their physical condition and the corresponding emotional response. Knowing how stress affects development of tolerance to opiates may aid in establishing drug treatment and dosing regimens.

Tolerance is formally defined as a reduced drug effect to a given dose following repeated administrations of the same dose of drug (Dews, 1978). Tolerance can also be defined by showing that a drug dosage,

administered repeatedly, must be increased in order to produce effects of equal intensity or duration as those originally observed.

According to both Dews (1978) and Jaffe (1980) there are two kinds of tolerance distinguished on the basis of the processes they involve: dispositional and functional. Dispositional tolerance refers to the modification of the physicochemical processes which handle the drug in the body so that reduced concentrations are present at the sites of drug action; for example, an alteration in absorption, distribution, or inactivation of drug. Functional tolerance refers to adaptive changes in the receptive cells such that the response is reduced in the presence of the same concentration of drug. At any one time dispositional and functional tolerance may occur alone or together.

The predominant type of tolerance to opiates is functional; that is, there is an adaptation of receptive cells in the nervous system to the drug's action (Jaffe, 1980). Tolerance to morphine develops rapidly and is characterized by a shortened duration and decreased intensity of the physiological effects (Jaffe, 1980).

The basis of development of tolerance to morphine appears to include changes in the second messenger system of the receptive cells. When an opioid binds to a receptor, there is a decrease in adenylate cyclase activity with a resultant decrease in amount of cyclic adenosine monophosphate (cAMP) which serves as a second messenger to activate various cellular functions (Sharma, Klee & Nirenberg, 1975). After two or three days of incubation of cells in the presence of morphine, Sharma et al. found that the levels of adenylate cyclase activity were equal to those prior to drug exposure. Therefore, it appears that cells "adapt" to or compensate for the initial inhibitory effect of morphine.

Other researchers have investigated the effect of opioids on levels of calcium ion which may also act as a second messenger. Ross (1977) found that acute morphine treatment in mice reduced calcium levels in various fractions of nervous tissue while chronic morphine treatment increased calcium levels above normal in those fractions. The calcium ion influences many cellular processes, one of which is regulation of adenylate cyclase; therefore, the reduced levels of adenylate cyclase activity may be related to the reduction in calcium levels.

Morphine's effect on calcium-regulated cellular activity may be the underlying cause for the depressant effects of the drug. Such activity increases concurrently with development of tolerance to the physiological effects of opiates, and therefore cellular activity is not as depressed; however, an increase in dose results in renewed depression of cellular activity as well as the reappearance of the physiological effects. The changes in calcium levels occur primarily in areas of the brain or body occupied by opiate receptors.

The opiate receptors are widely distributed throughout the CNS. Specifically, there are receptors located in and around components of the vagal system, including the nucleus tractus solitarius, vagus nerve, and nucleus ambiguus (Atweh & Kuhar, 1977a; Chang & Cuatrecasas, 1980). These nuclei in the brain stem are major CNS centers for parasympathetic control of the heart. There are also receptors in the adrenal medulla, sympathetic ganglia, the arcuate nucleus of the hypothalamus and the medial nucleus of the thalamus (Atweh & Kuhar, 1977b); these areas are part of sympathetic nervous system and also affect the cardiovascular system.

Morphine's Effect on the Cardiovascular System

In general, it has been suggested that morphine increases parasympathetic and decreases sympathetic tone (Holaday, 1983). Fennessy and Rattray (1971) studied the effects of morphine on the cardiovascular system in urethane-anesthetized rats. A transient, sharp decrease in blood pressure and bradycardia occurred with doses (from 0.1 mg/kg and up) administered intravenously (i.v.). Following pre-treatment with atropine, the depressor effect of 10 mg/kg and 100 mg/kg morphine was greatly reduced and the bradycardic effect was abolished. After a bilateral vagotomy, the depressor effect was replaced by an increase in blood pressure, and this pressor reponse was several times greater in the 100 mg/kg dose group than the 10 mg/kg group. These results suggest that the depressor response to morphine as well as the bradycardic effects are vagally mediated. The pressor response to morphine following vagotomy appears to be mediated by catecholamines because the pressor response is greatly reduced following treatment with phentolamine, an $alpha_1$ adrenergic antagonist (Fennessy & Rattray, 1971). This pressor effect may be related to morphine's effect on the release of epinephrine from the adrenal medulla, but is masked in an intact animal due to predominant control by the parasympathetic system on the heart. In other words, when both the parasympathetic system and the sympathetic system are stimulated, a net decrease in heart rate results due to dominance of parasympathetic influence over sympathetic influence (Berne & Levy, 1981).

Morphine and other opioids are known to evoke release of histamine from rat mast cells (Sydbom & Terenius, 1985), and it is thought that the depressor response is at least partially due to histamine (Fennessey

& Rattray, 1971; Moss & Roscow, 1983). However, a study in which anesthetized rats were pretreated with antihistamine, either pheniramine or mepyramine, showed that neither the depressor effect nor the bradycardia produced by morphine (10 mg/kg) were reduced (Fennessey & Rattray, 1971).

Morphine's effect on the cardiovascular system is dependent on several variables: species, dose, route and site of administration, whether the animal is conscious or anesthetized, and the rate of infusion (Fennessy, 1969; Fennessy & Ortiz, 1968; Presman & Schotz, 1943). Rapid i.v. injection of morphine results in marked tachycardia (Fennessy & Ortiz, 1968), whereas, slow infusion causes no change or a decrease in heart rate (Presman & Schotz, 1943; Stein, 1976). In anesthetized cats, 0.4 mg/kg morphine administered intracisternally produced a decrease in heart rate, but the same dose administered into the lateral ventricle produced an increase in heart rate (Feldberg & Wei, 1977, 1981). Anesthetized dogs administered 7.5 mg/kg morphine i.v. responded with a transient decrease in heart rate, whereas, conscious dogs showed an increase in heart rate (Fennessy, 1969). Type of anesthesia may also be an important factor affecting direction of the heart-rate response to morphine (Sitsen, Van Ree & De Jong, 1982). A biphasic heart rate response, bradycardia followed by tachycardia, after i.v. morphine infusion has been reported in conscious squirrel monkeys (Byrd, 1983).

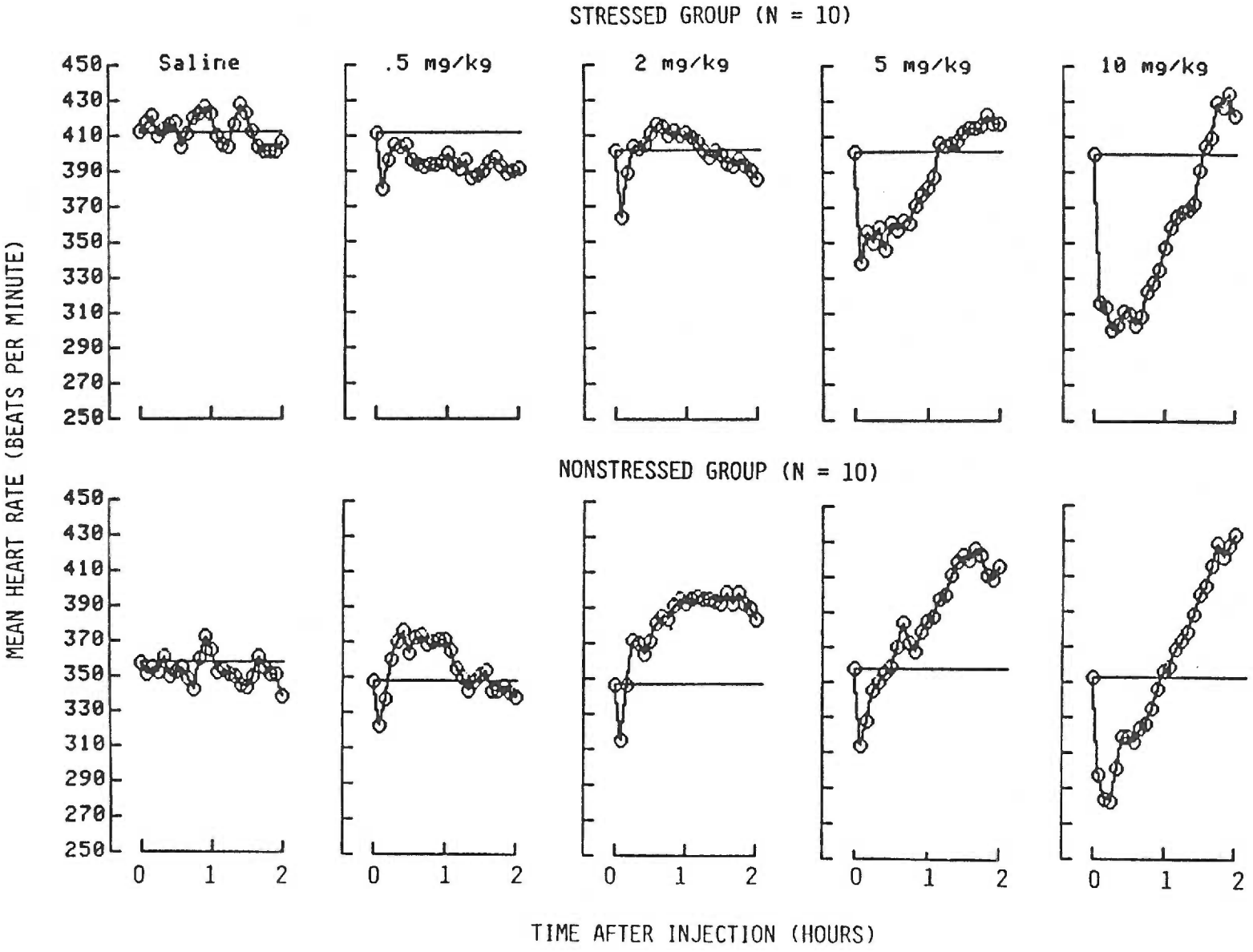
In general, studies performed on rats, anesthetized or conscious, restrained or unrestrained, have reported only a bradycardic response to i.v. infusions of morphine. Stein (1976) reported that in freely-moving rats after a 10-min habituation period, morphine administered i.v.

produced a dose-dependent decrease in heart rate. Stein's dependent measure was the lowest heart rate recorded during a 5-sec period within 30 sec after infusion. However, in a different study, in which heart rate was measured for 2 h following i.v. administration, the response to morphine was characterized by an initial decrease followed by an increase in heart rate. Both of these effects were more pronounced as dose increased, and at a 10 mg/kg dose, the initial bradycardia lasted for 1 h following injection, while the tachycardia lasted for at least 1 h following the bradycardia (Schwarz, Peris & Cunningham, 1985). The bottom row of panels in Figure 1 illustrates this biphasic heart-rate response to several doses of morphine in freely-moving rats. It is possible that Stein did not observe any tachycardia because he did not measure heart rate long enough after administering the drug.

Development of tolerance to the heart-rate effects of morphine has been studied in both the dog and rat (Fennessey & Ortiz, 1968; Stein, 1976). Fennessey and Ortiz administered rapid i.v. infusions of several doses of morphine producing tachycardia in dogs. Eight infusions were administered at 15-min intervals, and tolerance to the tachycardic effect of morphine was evident by the fifth infusion. After 4 days another infusion was given and no tachycardia occurred. Stein administered slow i.v. infusions of morphine producing bradycardia in rats. The rats were given two infusions per day at 10-30 min intervals. Stein reported that tolerance developed to the bradycardic effects in the third week of the study.

Overall, development of tolerance to the heart-rate effects of morphine has been observed to occur to tachycardic effects in dogs and to the bradycardic effects in rats; however, no data on the development

Figure 1. Mean heart rate (BPM) during the 5-min period before infusion and the 2-h period after infusion. The panels in the top row depict the cardiac responses of rats in the restraint-stressed group; those in the bottom row show the responses of rats in the Nonstressed group. Each individual panel plots the reaction to one dose of the drug as a function of time (Schwarz et al., 1985).


STRESSED GROUP (N = 10)
Saline
.5 mg/kg
2 mg/kg
5 mg/kg
10 mg/kg
NONSTRESSED GROUP (N = 10)
MEAN HEART RATE (BEATS PER MINUTE)
450
430
410
390
370
350
330
310
290
270
250
0
1
2
TIME AFTER INJECTION (HOURS)

of tolerance to both effects concurrently, as in the biphasic heart-rate response to morphine, have been reported in rats.

Stress and Drug Effects

Stress may interact with morphine, by altering the initial heart-rate response and/or by altering development of tolerance to the heart-rate effects of morphine. Stress normally causes activation of the sympathetic nervous system and, therefore, tachycardia. However, rather than attenuating morphine's vagal bradycardic response, recent findings suggest that stress augments the decelerative response to morphine.

Data showing that stress alters the heart-rate reactions to morphine in rats were obtained by Schwarz et al. (1985) who examined the effects of restraint-stress on the heart-rate response to five doses of morphine (0, 0.5, 2.0, 5.0, 10.0 mg/kg). Baseline heart-rate differed between restraint-stressed and non-stressed groups by about 50 beats per minute (BPM), the restrained group having the higher heart rate. As described above, the response to morphine was an initial bradycardia followed by tachycardia. The initial bradycardia was larger in magnitude and longer in duration in the restraint-stressed rats, and the tachycardia occurred sooner and was of greater magnitude in the freely-moving rats. These effects were more pronounced as dose increased (see Figure 1).

Effects of morphine on temperature are also augmented by stress (Stewart & Eikelboom, 1981). Stewart and Eikelboom used degree of novelty of the experimental apparatus as their stressor. They found that the hyperthermia caused by the low dose of morphine and the hypothermia caused by the highest doses were more pronounced in the

non-habituated rats relative to the habituated animals. Stewart and Eikelboom proposed that there may be an additive effect due to the stress-induced release of endorphin. That is, if the thermoregulatory action of stress-induced endorphin is similar to that of morphine, then the effect of a certain dose of morphine is greater in a stressed than a non-stressed animal. Another study by Schwen and Jones (1984) showed that 5 and 30 mg/kg of morphine administered i.p. produced no change in core temperature. However, when rats were restrained and rectally probed, morphine produced a sharp fall in body temperature.

Stress has also been shown to alter the thermic response to ethanol by augmenting the hypothermic effect of ethanol (Peris, 1984), even though stress itself causes an increase in body temperature. In both cases stress augmented drug responses even when the effects of stress itself were opposite in direction to those produced by the drug.

Stress and Tolerance

Because stress affects drug responses so profoundly, it is possible that stress also affects development of tolerance to drug effects. Adams, Yeh, Woods and Mitchell (1969) conducted experiments which they claimed indicated a drug-stress interaction as a factor in the development of tolerance to morphine analgesia. In their study, all rats were exposed to either a hot plate or an ambient temperature plate before injection of either saline or morphine. After injection, some rats were placed back in their home cage (Group NT), while other rats were again tested on the plate (Group T). For the tolerance test, all rats were exposed to a hot plate both before and after morphine administration. Analgesic tolerance was evident in both T groups which received both morphine and experience on the plate, hot or ambient.

Tolerance did not develop in Group NT which was placed back in the home cage after morphine, nor in the groups previously administered saline and given experience on the plate. The requirement of experience on the plate while intoxicated by morphine for the development of tolerance is an example of a drug-test interaction because tolerance only occurred in those animals who experienced the plate while intoxicated by morphine. Since the drug-test interaction occurred both in rats receiving experience on the hot plate and those experienced on the ambient temperature plate, Adams et al. suggested a drug-stress interaction because both these groups showed signs of stress when initially placed on the plate.

In response to the experiments by Adams et al. (1969), Gebhart, Sherman and Mitchell (1972) conducted two experiments to determine the influence of stress on the development of tolerance to the analgesic effect of morphine. The stress variables were swim stress (SS) in Experiment 1 and auditory stress (AS) and restraint stress (RS) in Experiment 2. Rats were assigned to groups based on the kind of stress they would receive or to Groups T and NT, which were identical to those in the Adams et al. study except that an ambient temperature plate was not used. During the acquisition phase in both experiments, rats in each group were assigned to receive either morphine (m) or saline (s). None of the rats in the stressed groups received experience on the hot plate prior to the test session. During the test all rats received morphine and were tested on the hot plate both before and after injection. The results of Experiment 1 indicated tolerance only in Group Tm, replicating the result of Adams et al. There were no differences among the other groups. The test results of Experiment 2

showed tolerance in all groups treated previously with morphine, Groups Tm, NTm, ASm and RSm. Groups Tm showed a significantly lower response latency than Groups NTm, ASm or RSm. Pathologic gastric alterations were evident in the rats in Groups ASm, ASs, RSm and RSs but not in Groups Tm or Ts when examined at autopsy. Therefore, Gebhart et al. concluded that stress plays no role in the tolerance produced in experienced animals tested on the hot plate. These results support those of Adams et al. but do not support the suggestion of a drug-stress interpretation.

The effects of stress on the development of tolerance to tetrahydrocannabinol (THC) have also been investigated (Carder, 1978). During pretreatment, Carder exposed rats to either THC, propylene glycol paired with shock or propylene glycol alone (i.e., the vehicle solution). Body temperature was measured during a shock-free tolerance test in which rats were administered either THC or propylene glycol. The only group not to show tolerance was the group pretreated with propylene glycol alone. The group exposed to shock without THC demonstrated an attenuated response similar to the group pretreated with THC, even though the test situation provided their first exposure to THC. The design of Carder's study did not permit evaluation of a drug-stress interaction because no rats were pretreated with both THC and shock. Carder's study, however, did provide evidence of an additive relationship between stress and tolerance to a physiological effect (temperature) of a drug.

Stress also affects tolerance development to ethanol. A recent study by Peris (1984) examining the effects of stress on tolerance development to the hypothermic effect of ethanol resulted in the

stressed rats becoming tolerant faster than the non-stressed rats. There were no differences between stressed and nonstressed rats in plasma or brain levels of ethanol (Peris & Cunningham, 1985).

In general, although Gebhart et al. (1972) ruled out a drug-stress interpretation in their experiments on tolerance to morphine analgesia, both Carder's (1978) and Peris' (1984) studies measuring the thermic response to drugs suggest an effect of stress on tolerance.

Opioids and Stress

Forced immobilization, or restraint, as a stressor has been studied in rats by several researchers. Kvetnansky et al. (1978) examined the effects of immobilization stress on plasma levels of epinephrine, norepinephrine and corticosterone. Blood samples were collected via an intra-arterial cannula. Initial, control samples were obtained from undisturbed animals. The plasma levels of catecholamines in control rats were very low. Immobilization produced a 40-fold increase in epinephrine and a 6-fold increase in norepinephrine. The maximal levels occurred at about 20 min, after which time levels of both norepinephrine and epinephrine began to decrease. After about two hrs, both norepinephrine and epinephrine remained relatively constant at approximately equal levels which were significantly greater than those in nonstressed control animals. The catecholamine levels remained elevated throughout the duration of restraint which lasted four hrs in this study. Immobilization also caused a significant increase in plasma corticosterone suggesting that restraint activates the adrenal glands. These results are supported by others (Keim & Sigg, 1976) who have demonstrated an increase in adrenal reactivity to adrenocorticotrophic hormone (ACTH) as well as an increase in plasma corticosterone after

restraint. Stress also results in the release of both ACTH and β-endorphin from the pituitary (Guillemin, 1978). The physiological effects of stress are primarily due to stimulation of the sympathetic nervous system and include tachycardia, hyperthermia, bronchial dilation, mydriasis as well as many other effects (Mayer, 1980).

The fact that β-endorphin and ACTH are derived from the same prohormone and are present in the same secretory granules in the corticotrophic cells of the pituitary suggests a role for endorphins in stress. Stressors such as restraint, limb fracture, heat stress and foot shock cause a decrease in adenohypophyseal endorphin content and an increase in plasma levels of endorphin and ACTH (Amir, Brown & Amit, 1979). The presence of enkephalin in the autonomic ganglia and adrenal medulla suggests that stress may be a releasing stimulus for this ligand also (Holaday, 1983). Morphine has been reported to influence epinephrine synthesis and storage and to stimulate directly and indirectly, via the splanchnic nerve, the release of epinephrine from the adrenal medulla (Anderson & Slotkin, 1976). Anderson and Slotkin used subjects whose left adrenal glands were denervated prior to exposure to morphine and found that in both adrenal glands there was increased catecholamine secretion and increased storage vesicle synthesis (dopamine β-hydroxylase activity). The fact that these effects occurred in the denervated adrenal implies a direct effect of morphine. Therefore, it is possible that circulating endorphins could also affect release of epinephrine via opiate receptors on the adrenal medulla.

Additional evidence concerning release of endogenous opioids during stress is stress-induced analgesia. Bodnar, Kelly, Spiaggia and Glusman

(1978) showed that following a cold-water swim (2°C for 3.5 min), rats displayed a transient, significant increase in pain threshold, as measured using a grid shock, which lasted up to 2 h. With chronic stress (one swim daily for 14 days), the pain threshold returned to pre-stress levels. Another study by Bodnar, Kelly, Spiaggia, Pavlides and Glusman (1978) showed that naloxone (10 mg/kg), an opioid receptor antagonist, partially attenuated the increased threshold to pain. Pilcher and Browne (1983) used restraint as a stressor and tested for analgesia using paw pinch pressure or tail immersion in hot water (50 °C). Restraint significantly altered the nociceptive thresholds for both heat and pressure: following restraint-stress, the threshold for heat increased while the threshold for pressure decreased. Naloxone partially reduced the analgesia to heat but had no effect on pressure. Mr1452 ((-)-N-(3-Furylmethyl)-α-normetazocine methansulphonate), another opioid receptor antagonist, caused a greater attenuation of analgesia to heat than naloxone, and potentiated the hyperalgesia to pressure (Pilcher & Browne, 1983). Naloxone is primarily a mu receptor antagonist, and Mr1452 has greatest affinity for kappa receptors (Pilcher & Browne, 1983). It is possible that stress-induced analgesia is mediated by receptors other than mu receptors, such as kappa receptors (Pilcher & Browne, 1983) or delta receptors (Bodnar et al., 1978) since naloxone only partially attenuates the analgesia. However, it is also possible that a non-opiate system is at least partly involved in stress-induced analgesia.

Additional support for stress-induced analgesia being mediated at least in part by endogenous opioids is lent by studies performed by Akil, Madden, Patrick and Barchas (1976) and Madden, Akil, Patrick and

Barchas (1977). Both studies examined the effects of acute and chronic inescapable footshock on tail flick latency as well as whole brain levels of endogenous opioid activity. A high correlation ($r = 0.92$) was found between tail flick latency and endogenous opioid activity level, i.e., acute stress caused a significant rise in endogenous opioid peptide activity in the brain and a concurrent increase in tail flick latency. The analgesic response was partially reversed by naloxone. Brain levels of opioid activity were not measured in naloxone-treated subjects.

## Rationale

Because there is evidence that stress affects the physiological reactions to morphine and possibly the development of tolerance to morphine, this study was designed to compare the rate and pattern of development of tolerance to the heart-rate effects of morphine in stressed and non-stressed rats. Heart rate was chosen as the physiological measure because there is evidence that the endogenous opiate system plays a role in autonomic cardiovascular regulation (Holaday, 1983). This is supported by the close proximity of receptors to cardiovascular centers in the brainstem and hypothalamus, and by studies showing that injection of morphine into these areas of the brain results in profound cardiovascular effects (Feldberg & Wei, 1977, 1978). The heart-rate effects of systemically-administered morphine have been studied with particular attention to the bradycardic effects (Fennessey & Ortiz, 1968; Stein, 1976). Tolerance development to the bradycardic effects of morphine has also been reported (Stein, 1976). The present study differed from Stein's in that Stein administered morphine i.v.

after a 10 min habituation period and recorded for only 30 sec after drug administration. This study included a 1-hr habituation period before injection and heart rate was recorded for 2 hr after injection.

The length of habituation was important because, in general, the level of baseline heart rate appears to affect the predominant direction of change from baseline heart rate after morphine administration (Schwarz et al., 1985). Specifically, unrestrained rats given acute exposure to morphine responded with a short duration bradycardia followed by a longer duration tachycardia, whereas, restrained rats, whose baseline heart rates were higher than those of unrestrained rats, showed a longer initial bradycardia followed by a lesser degree of tachycardia relative to unrestrained rats (Figure 1). After 1 hr in the experimental chamber, the subjects' heart-rates have usually stabilized. The purpose for recording 2 hr after injection was to find out if tolerance developed only to the bradycardic effects, only to the tachycardic effect or to both effects. This permitted evaluation of the suggestion by Seevers and Deneau (1963) that tolerance develops only to depressant effects and not to stimulant effects of morphine.

There were various ways in which stress might affect tolerance development to opioids. Because stress has been reported to increase the rate of tolerance development to certain drug effects (e.g., Peris, 1984), it was possible that restrained rats would show a greater degree of tolerance than the nonrestrained rats. However, stress could act to decrease the rate or magnitude of development of tolerance, so that rats repeatedly exposed to both restraint and morphine would not develop tolerance at all during the experiment or to a lesser degree than the nonstressed rats (e.g., Sherman, Strub & Lewis, 1984).

This experiment was designed to compare tolerance to the heart-rate effects of morphine in restrained and freely-moving rats that have received one of four doses of morphine chronically. The doses of morphine were 0.0 (saline), 2.0, 4.0 and 8.0 mg/kg/infusion. Rats were randomly assigned to groups based on dose, and within each dose group there were both restrained and unrestrained rats. After an habituation phase, during which all rats were exposed to the experimental chamber but received no injection, the acquisition phase began. During acquisition, all rats received one infusion of morphine or saline daily for twelve days. After acquisition, the rats were exposed to each dose of morphine and saline in a counterbalanced order to obtain within-group dose-response curves. Magnitude of tolerance was assessed by comparing the dose-response curves of the saline groups with those of the drug groups. A greater degree of tolerance is represented by a greater shift to the right of the curve. In this way, the curves of stressed rats can be compared to those of nonstressed rats.

It was hypothesized that tolerance would develop to the bradycardic effect but not to the tachycardic effect as suggested by Seevers and Deneau (1963). A greater magnitude of tolerance was expected to develop in the restraint-stressed groups since stressed-induced activation of the endogenous opioid system would result in a higher dose of opioid in the stressed rats. Likewise, a greater magnitude of tolerance was expected to develop in the high dose group (8 mg/kg) relative to the lower dose groups (2 and 4 mg/kg).

## Method

### Subjects

The subjects were 64 adult male albino rats, weighing an average of

402 g at the start of testing, and were purchased from the Holtzman Company of Madison, Wisconsin. These rats were housed individually in a colony room (ambient temperature = 25 °C) with a 12-hr light-dark cycle. Food and water were available ad lib except during the 180-min experimental sessions. The experimental sessions occurred during the light portion of the 24 hr cycle.

Surgical Procedure

Three days before the start of the experiment, a venous catheter and two cardiac electrodes were implanted in each rat. All subjects were allowed two days of recovery before the experiment began.

Catheter construction. The design of the catheter was modeled after Weeks (1972). In general, the catheter consisted of various sizes of polyethylene tubing melted together and melted to silastic tubing. The silastic tubing made up the intravascular portion of the catheter. For a more detailed description of the catheter construction, see Appendix A.

Cardiac Electrodes. The cardiac electrodes consisted of eight to ten strands of 32 ga stainless-steel wire wound together and covered to the tip with PE 100 (1.2 mm i.d. x 1.7 mm o.d.) tubing for insulation. A Molex pin was attached to the ends of each electrode and inserted into a nylon housing.

Anesthesia. Halothane gas in oxygen was used as the anesthetic. The flow of Halothane gas was controlled by a Calibrated Vaporizer (Ohio Medical Products). The flow of oxygen, which was constant throughout the surgery, was monitored by an Airco oxygen flowmeter. The oxygen flowmeter was set at 0.5 l/min.

The rat was placed in a Nalgene bottle and administered a loading

dose of Halothane gas (4-5% concentration in oxygen) until the animal was unconscious. The concentration of Halothane was then reduced to 1-2%, and delivered to the rat via a nose cone.

Surgical Procedure. As soon as the rat was fully anesthetized, 0.1 ml Crysticillin, an antibiotic of penicillin in procaine suspension, was injected i.m. into the left hind leg. The rat was shaved in a 2 $cm^2$ area just above the clavicle on both sides of the chest. An antiseptic solution (Betadine) was rubbed on the shaved areas to cleanse the skin and wipe away loose hair.

A 0.5 cm incision was made in the shaved area to the left of the midline using a scalpel (#15 blade), and the superficial muscle was exposed. A 156 cm length of 32 ga wire were looped 5 times through the superficial muscle and covered with PE 100. A dorsal incision (1.5 cm) was made 3 cm below the skull, and a second electrode was loosely looped through the dorsal superficial muscle five times and covered with PE 100. The ventral electrode was tunneled subcutaneously to the dorsal incision where it was looped once with the dorsal electrode. The loop was sutured to the superficial muscle, and the incision was closed with 000 suture silk. Molex connector pins were crimped onto the ends of each electrode and protected with nylon housing plugs.

A pulse was then located under the shaved area to the right of the ventral midline, and a 1 cm incision was made. The incision was at approximately a $20^{\circ}$ angle to the midline.

Using two fine-tipped curved tissue forceps, the fascia was carefully pulled away to expose 5-7 mm of the right jugular vein. The tissue surrounding the vein was removed so that the points of the forceps could be slid under the vein in order to pull two strands of 000

silk suture, 15 cm long, under the vein to use as ligatures. The ligatures were then situated at each end of the exposed vein and a single knot loop in each ligature was tied loosely around the vein. A pair of straight hemostatic forceps were attached to the ligatures so that the thread was weighted down, thus causing the vein to be raised slightly and the circulation to be cut off.

The beveled end of a 20 ga hypodermic needle was bent to where the tip was at a 90° angle to the stem of the needle. The tip was inserted into the vein so that the tip was parallel to the vein. A saline-filled cannula (as described previously) was inserted into the vein under the needle tip in a direction towards the heart. The needle tip was then removed and the pressure was released from the proximally-placed thread so that the silastic tip of the cannula slid completely into the vein.

The loose single knot loop in the proximal ligature was then tightened around the vein and catheter just enough to hold the catheter in place but not tight enough to cut off flow in the catheter. Blood flow was checked by drawing blood into the catheter and flushing saline back into the vein. The distally-placed ligature was tightened around the vein in order to cut off circulation, and then looped around the PE 10 portion of the catheter just distal to the 5-mm piece of heat shrink tubing to anchor the catheter to the vein. Blood flow was checked again to make sure the knots around the catheter were not too tight. The fascia covering the midline muscle was then cleared and a pocket was formed just rostral to the midline muscle. Silk suture (000) was tied in a knot around the catheter between the two knobs closest to the looped PE 10 portion of the catheter, and using a curved suture needle on the other end of this thread, a portion of midline muscle was

traversed by the needle to which the catheter was anchored. The knots were tied loosely around the muscle tissue in order to prevent necrosis. The PE 10 loop was then tucked into the pocket, and blood flow was checked again.

The rat was then turned over and a 2 $cm^2$ area was shaved on the back of its neck and head. Betadine solution was rubbed on this shaved area, and a .5 cm long incision was made just below the edge of the skull. A 15.5 cm long piece of 13 ga stainless steel hypodermic tubing was used to tunnel subcutaneously about four centimeters down the back to the left of the midline and then caudally around the right foreleg to the area where the catheter was anchored. A 20 cm piece of #12 steel music wire was inserted through the tube and attached to the open end of the catheter, and the catheter was pulled through the tunnel to exit through the dorsal incision. The catheter was then anchored to the subcutaneous tissue on the back of the neck in the same way as to the midline muscle.

The rat was turned over on its back, the ventral incision was filled with Bactofura wound powder and sutured with 000 silk. The dorsal incision was sutured and then treated with Bactofura powder. The flow of the catheter was checked again. The halothane gas was turned off, and the rat was allowed oxygen for 5 min. After surgery, each rat was fitted with a harness made of foam padding and velcro strips (Weeks, 1972) to which the catheter and electrodes were attached. This harness served to protect the catheter and electrodes as well as to provide an easy way to attach the catheter and electrodes to the drug infusion and electrical apparatus.

In the event that a catheter or electrode was damaged, repairs were

made immediately after a session with the minimal amount of stress possible to the rat. If the catheter or electrodes were damaged beyond repair, the subject was discarded from the study. To test for patency of the catheter, each rat was infused i.v. with .1 ml (10 mg/ml concentration) methohexital sodium (Brevital), a fast-acting, short-acting barbiturate, at the end of the experiment.

Apparatus

Restrained animals were placed in cylindrical restrainers (6.5-7 cm x 16.8 cm) made of 3 mm stainless steel rods formed into circles and mounted flush, approximately 1 cm apart, to the inside surface of three plastic railings (6.25 mm) (Anderson, Plant & Paden, 1967). One end of the restrainer contained a circular door through which the subject was inserted and removed. Unrestrained rats were placed inside a 21 x 21 x 23 cm container with clear plastic and aluminum walls, a door in one of the sides and ventilation holes at top and a stainless-steel grid floor. The restrainers and containers were placed inside larger (63 x 64 x 60 cm) sound- and light-attenuating chambers (mean ambient temperature = 23.1 $\pm$0.084 $^{o}$C).

Two lengths of wire with thermoplastic insulation were attached to the rat with Molex connectors housed in nylon plugs. In the unrestrained rats, these wires ran through a spring to a mini 3-channel fluid swivel with three electrical circuits (Ealing Corporation/Harvard Bioscience) mounted onto the top of the container. Wires led from the swivel to an amplifier. After amplification, the ECG signal was sent to a peak detector (Shimizu, 1978) and a one-shot trigger in order to convert the R-wave into digital signal. A PDP/8F computer calculated and recorded the interbeat interval from the digital signal. In the

restrained rats, the wires attached to the electrodes led directly to the amplifier; no swivel was included.

The cannulas were attached to polyethylene tubing. In the unrestrained rats, PE 50 tubing (0.58 mm i.d. x 0.96 mm o.d.) was attached to one of the fluid swivels and ran down the spring with the electrical wires and exited at a point 3 cm above the end of the spring. The tubing exited the spring through a 2 cm piece of 13 ga stainless steel hypodermic tubing to which was attached a 7 cm length of spring. Mounted to the end of this piece of spring, was the end of a 1-ml disposable syringe, cut off at the 2-ml mark. This syringe-end fit snugly into the modified hypodermic needle hub attached the cannula.

The drug was automatically infused by a Harvard Apparatus Compact Infusion Pump set at an infusion speed of 1.0 ml/min. The drug reservoirs were 5 ml syringes with luer-lock tips mounted on the infusion pump. Plastic stop cocks were attached to the tip of the syringes and PE 20 (0.35 mm i.d. x 1.05 mm o.d.) connected the syringe with the swivel. In the restrained rats, PE 50 connected the syringe directly with the rat.

Procedure

Rats were randomly assigned to four dose groups: 0.0 (saline), 2.0, 4.0 and 8.0 mg morphine/kg body weight. Within each dose group there were both restrained and unrestrained rats matched in pairs by weight (see Table 1). Eight rats were originally assigned to each group. However, subject attrition reduced the cell sizes (see Results section).

Each experimental session lasted 3 hr per day and the complete experiment required 19 sessions with successive sessions approximately

Table 1. The experimental design, group labels and cell sizes.

| | MORPHINE DOSES (mg/kg) | | | |
|---|---|---|---|---|
| | 0.0 | 2.0 | 4.0 | 8.0 |
| Restrained R | Group R0 n=6 | Group R2 n=6 | Group R4 n=5 | Group R8 n=5 |
| Unrestrained U | Group U0 n=7 | Group U2 n=8 | Group U4 n=7 | Group U8 n=7 |

Table 2. The procedure for all subjects.

| | DAY | TREATMENT |
|---|---|---|
| | 1 | Surgery |
| | 2 | Recovery |
| | 3 | Recovery |
| | 4 | Habituation 1 |
| PHASE 1 | 5 | Habituation 2 |
| | 6 | Habituation 3 |
| | 7 | D1 |
| | 8 | D2 |
| | 9 | D3 |
| | 10 | D4 |
| | 11 | D5 |
| | 12 | D6 |
| PHASE 2 | 13 | D7 |
| | 14 | D8 |
| | 15 | D9 |
| | 16 | D10 |
| | 17 | D11 |
| | 18 | D12 |
| | 19 | TT1 |
| | 20 | TT2 |
| PHASE 3 | 21 | TT3 |
| | 22 | TT4 |

24 hr apart (see Table 2). The subjects were run in squads of four animals each. At the beginning of each session, prior to placement in the chambers, each rat was taken from the colony room to a room in which they were weighed and then kept in the transport cart for 10 min. During this time, the previous squad was removed from the chambers, the shavings were changed, and the infusion tubing was cleared of drug from the previous squad and replaced with drug for the next squad of rats.

The experiment was divided into three phases. Phase 1 was the habituation phase and lasted three days. During this phase, all rats were weighed and placed in the chambers in order to habituate the animals to the experimental procedure and apparatus. No infusion was given.

Phase 2 was the tolerance-acquisition phase. This phase lasted 12 sessions, during which each animal received an infusion of drug 1 hr after placement in the chamber. The dose received corresponded to the dose group to which the subject belonged. Heart rate was monitored during the 1 hr before and 2 hr after infusion. The concentrations of drug were calculated using the previous day's weight measurement, which was found not to vary appreciably after the habituation phase. All rats received 0.5 ml of drug infused over a 30-sec period of time.

Following the tolerance-acquisition phase, each rat received each dose of morphine to assess development of tolerance in the drug groups and to obtain within-group dose-response curves. This phase lasted 4 days. The order of doses given to each subject during this tolerance-test phase was counterbalanced to fulfill certain criteria. First, on each tolerance-test day, all rats were assigned doses of morphine such that each dose of morphine was received by two restrained

and two unrestrained rats in each dose group. Second, across the dose orders chosen, a dose followed each other dose equally often to control for any possible effects a previous dose may have had on a certain dose following it.

Twelve animals (i.e., three squads) were scheduled to be run in each replication. One squad was run during the early morning, one during the mid-morning, and one during the afternoon. Six experimental replications were required to obtain a group size of eight animals. All dose groups were represented during each replication and were balanced across replications for time of day. Rats were purchased separately for each replication.

Data Analysis

Interbeat intervals (IBIs) were recorded by a PDP/8F computer during each minute of the 3-h session. As a means of eliminating errors, all IBIs that differed more than 20 msec from the previous IBI were ignored. Furthermore, all IBIs greater than 300 msec ($\leq$ 200 bpm) or less than 80 msec ($\geq$ 750 bpm) were ignored. The mean IBI recorded during each minute was translated into an average heart-rate (bpm) and stored on a floppy disk by an Apple II+ microcomputer.

These scores were averaged over 5-min periods. The first hour of each session before morphine infusion (Baseline) was used to analyze handling effects and any effects of the previous days' exposure to treatment. The average from the last 5-min period during the first hour of the session was used to calculate post-infusion change scores by subtracting the baseline score from each successive 5-min post-injection score. This permitted analyses of the magnitude of change as well as the pattern of change over the 2 hr post-injection period.

## Results

The data from twelve rats were discarded. Three subjects in the restraint-stressed group were sacrificed because of procedural problems, i.e., one rat had a negative Brevitol test at the end of the study, and two rats were discarded due to problems with the restrainers. Two additional restrained rats died after their first exposure to the 8 mg/kg dose of morphine, probably because of respiratory arrest. Seven other rats were sacrificed or died because of poor health associated with abnormal weight loss (average = 37 g over 2-3 days), respiratory disease or abnormal swelling in the neck area. Of these seven, four were restrained and three were unrestrained. This left 52 subjects overall: 23 restrained and 29 unrestrained rats. The number of subjects in each dose group is shown in Table 1. A Chi Square test performed on the distribution of dead and live subjects in Groups R and U revealed no relationship between restraint and attrition ($\chi^2(1) = 2.11$).

Mean heart rate was calculated for each 5-min period of habituation, tolerance acquisition and the tolerance test sessions. For the Habituation phase, all 36 sample periods were analyzed together; however, during the Acquisition phase, baseline scores and change scores were analyzed separately. During the Tolerance Test phase, baseline scores, change scores and post-infusion heart rate scores were analyzed separatly using Analysis of Variance (ANOVA). All $p$ values of .05 or less were considered significant. In the cases where higher-order interactions occurred, significant lower-order interactions or main effects are not reported.

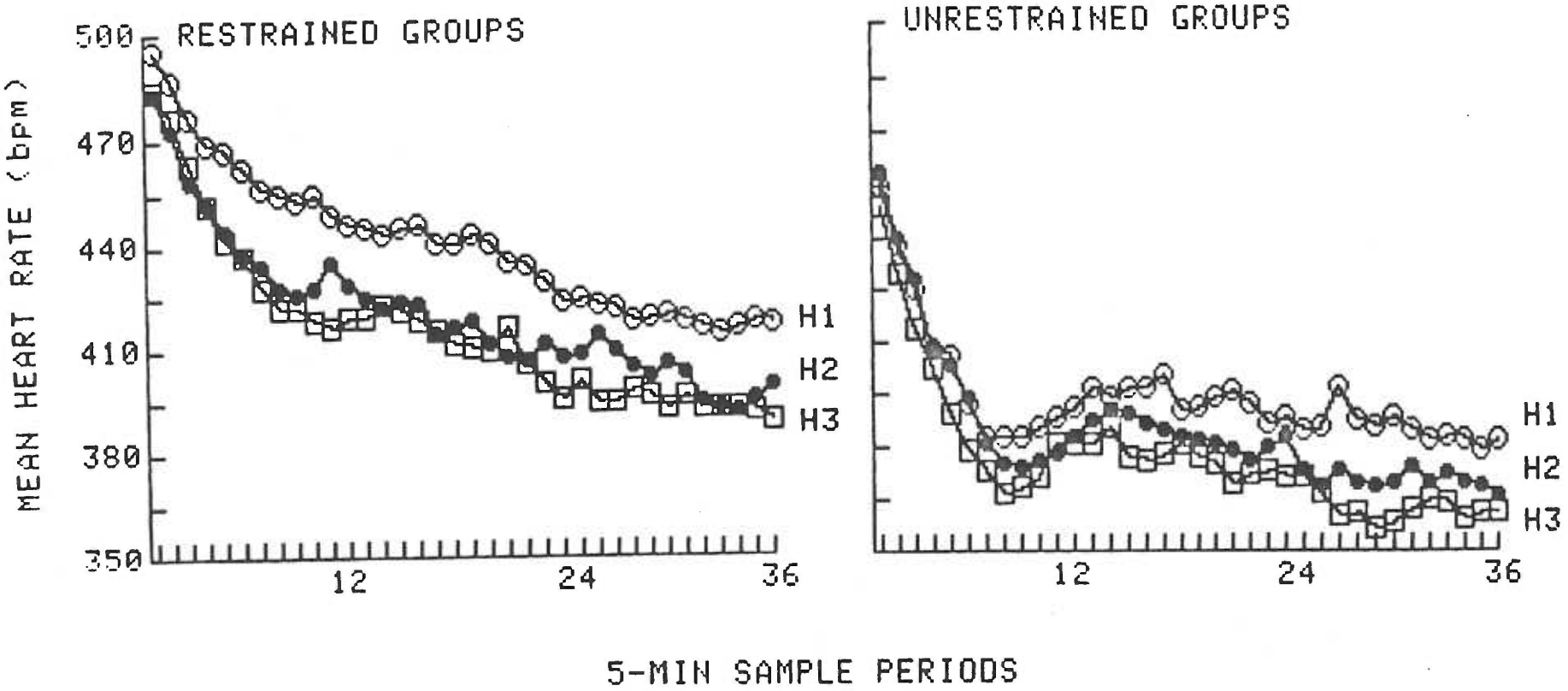
Approximately 4.6% of the total number of 5-min mean scores were missing, primarily due to equipment problems. In these cases, a mean of the surrounding scores was inserted, and the degrees of freedom were properly adjusted acording to the method of Linton and Gallo (1975).

Habituation

Heart rate during the three habituation sessions is plotted in Figure 2 over days and sample periods (collapsed across dose groups) for both the Restrained (left) and Unrestrained (right) groups. As can be seen in the graph, heart rate was initially elevated after placement in the chambers. Heart rate then decreased over the first hour; this decrease was greater in the Unrestrained than the Restrained subjects (69.5 vs. 59 bpm). In the Restrained rats, heart rate continued to decrease throughout each session. The heart rate was lowest on the third day for both Restrained and Unrestrained groups.

A four-way ANOVA in which Restraint and Acquisition Dose (a "dummy" factor) were the between-group factors and Days and Sample Periods were the within-group factors, revealed a significant Restraint x Acquisition Dose x Days x Sample Periods interaction ($F\{210,2652\} = 1.22$). None of the subjects had received morphine during this phase so the involvement of the Dose variable most likely indicates a sampling error. Separate analyses of the Restrained and Unrestrained subjects revealed an Acquisition Dose x Days x Sample Periods interaction in the R groups ($F\{210,1251\} = 1.25$) and a Days x Sample Periods interaction in the U groups ($F\{70,1401\} = 1.32$). Main effects of Days ($F\{2,41\} = 9.43$) and Sample Periods ($F\{35,875\} = 33.76$) were also revealed in the analysis of the U groups' data, but there were no significant interactions or main effects involving Dose.

Figure 2. Mean heart rate plotted over 5-min sample periods and three habituation days (H1, H2 and H3). The panel on the left depicts the heart rate in the restrained groups, while the panel on the right depicts heart rate for unrestrained rats. These data are collapsed across acquisition dose.


RESTRAINED GROUPS
UNRESTRAINED GROUPS
MEAN HEART RATE (bpm)
500
470
440
410
380
350
12
24
36
H1
H2
H3
5-MIN SAMPLE PERIODS

As can be seen in the right panel in Figure 2, the greatest difference in heart rate over the 3-hr session for Unrestrained Groups occurs between the first day (H1) and subsequent days (H2 and H3) in the later part of the session. This observation is supported by analyses of various sample periods which revealed significant effects of Days in Sample Periods 27 and 36 ($F\{2,41\}$ = 17.25 and 6.86, respectively) but not in Sample Periods 1 or 12. This accounts for the Days x Sample Periods interaction in the Unrestrained Groups.

Figure 3 shows the heart rate during each habituation day over sample periods and acquisition dose group. The panels on top are of the Restrained dose groups, while those on bottom are of the Unrestrained groups. There appears to be no difference among acquisition dose groups in the Unrestrained subjects. As can be seen in Figure 3, the greatest difference between Acquisition Dose groups in the Restrained subjects appears to be early in the session. Followup analyses of each habituation day for R groups revealed Acquisition Dose x Sample Periods interactions on all three days ($F\{105,595\}$ = 1.62 for H1, $F\{105,664\}$ = 1.86 for H2 and $F\{105,662\}$ = 1.28 for H3). Analyses of various sample periods from each day revealed significant main effects of Acquisition Dose during Sample Period 1 of each habituation day ($F\{3,19\}$ = 4.84, 4.63 and 3.53 for H1, H2 and H3, respectively) but not during Sample Periods 12 or 27 on any of the days.

Figure 4 shows heart rate in each acquisition dose condition for the Restrained groups over sample periods and habituation days. These are the same data shown in the top panels of Figure 3. The greatest difference between habituation days is between H1 and subsequent days (H2 and H3). This difference appears largest in Group R4, and somewhat

Figure 3. Mean heart rate plotted as a function of acquisition dose group and 5-min sample periods. Panels in the top row depict data for restrained subjects on successive habituation days (Hab1, Hab2, Hab3). Panels on the bottom depict heart rate in unrestrained subjects.

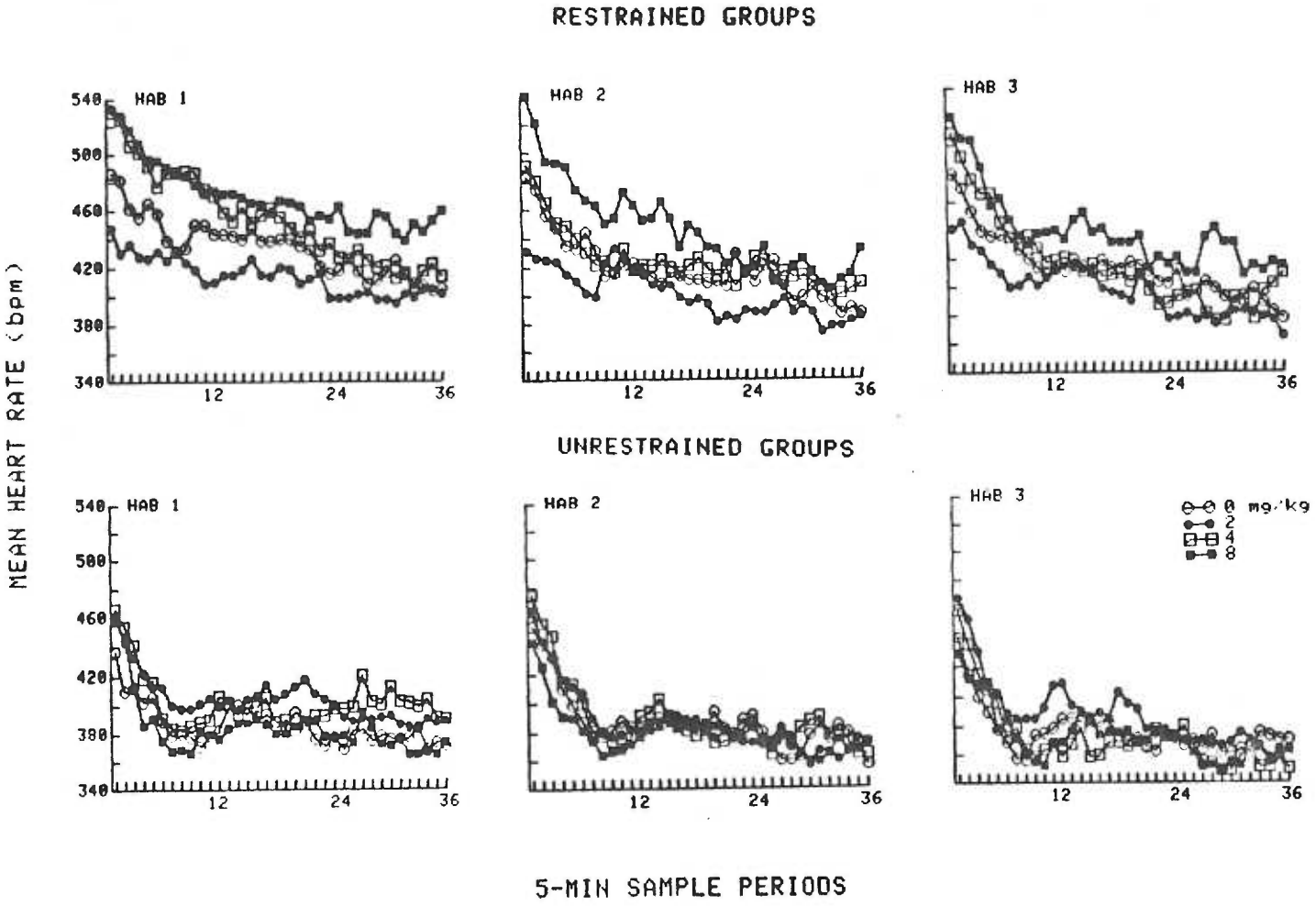
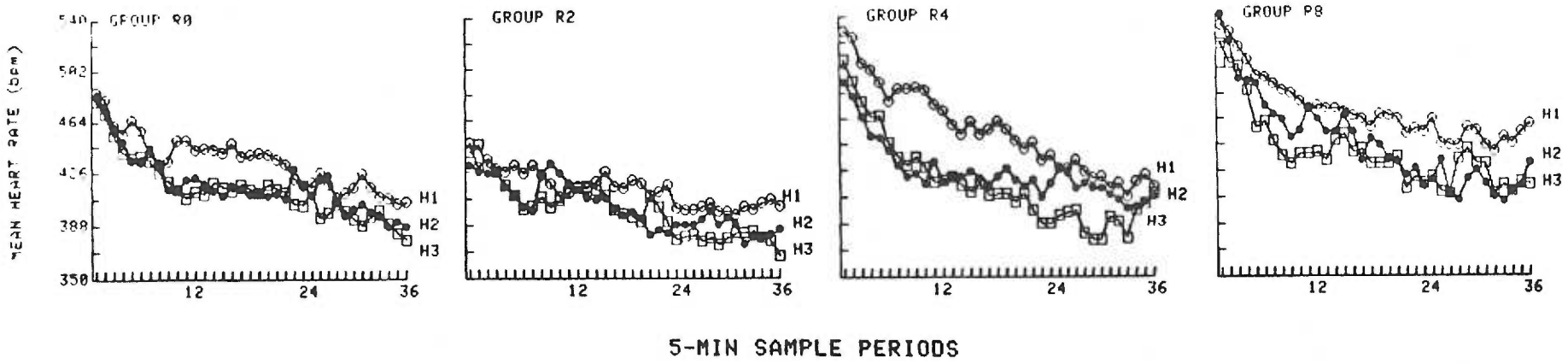

RESTRAINED GROUPS
HAB 1
HAB 2
HAB 3
UNRESTRAINED GROUPS
HAB 1
HAB 2
HAB 3
0 mg/kg
2
4
8
MEAN HEART RATE (bpm)
540
500
460
420
380
340
12
24
36
5-MIN SAMPLE PERIODS

Figure 4. Mean heart rate plotted over 36 5-min sample periods and habituation days (H1, H2 and H3). Each panel depicts the heart rate of an acquisition dose group. Data for restrained groups only are shown here.


MEAN HEART RATE (bpm)
GROUP R0
GROUP R2
GROUP R4
GROUP R8
H1
H2
H3
12
24
36
5-MIN SAMPLE PERIODS

smaller in Groups R8 and R0. In Group R2, the difference between days appears to be relatively small. These observations help explain the Acquisition Dose x Days x Sample Periods interaction seen in the R groups. Followup analyses of each dose group revealed main effects of sample periods in all dose groups ($F\{35,210\}$ = 15.88 for Group R0, $F\{35,175\}$ = 10.69 for Group R2, $F\{35,140\}$ = 20.97 and 27.76 for Group R4 and R8, respectively). There were no main effects or interactions involving days. Thus, although the three-way interaction was not explained statistically, it can be explained graphically as seen in Figure 4.

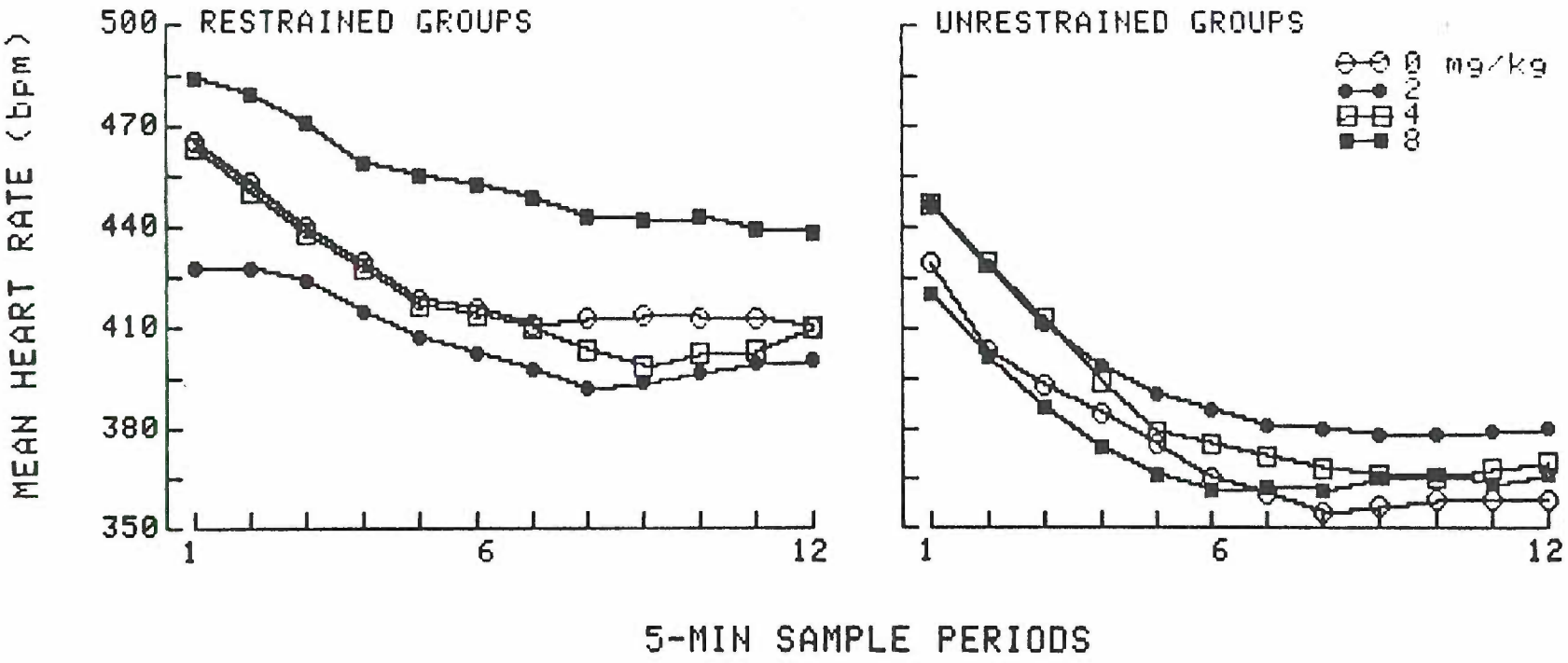
Summary: Habituation

Heart rate was initially elevated due to handling and placement in the experimental chamber. This effect was temporary, especially in the Unrestrained subjects whose heart rate had usually stabilized by the end of the first hour. In the Restrained groups, heart rate continued to decline throughout the entire 3-hr session. A sampling error appears to have contributed to a difference between dose groups in the Restrained rats. Although this difference occurred on all three habituation days, there was no difference among dose groups by the end of the first hour on any of the days which is important because during the acquisition phase morphine will be administered at the end of the first hour. In general, over the three habituation days, the greatest decline occurred between H1 and H2.

Acquisition: Baseline

Baseline (pre-infusion) heart rate of the eight groups is graphed in Figure 5 as a function of sample periods. The data are collapsed

Figure 5. Pre-infusion baseline heart rate of the four dose groups in the restrained (left) and unrestrained (right) conditions is plotted as a function of sample periods. The data are collapsed across blocks.

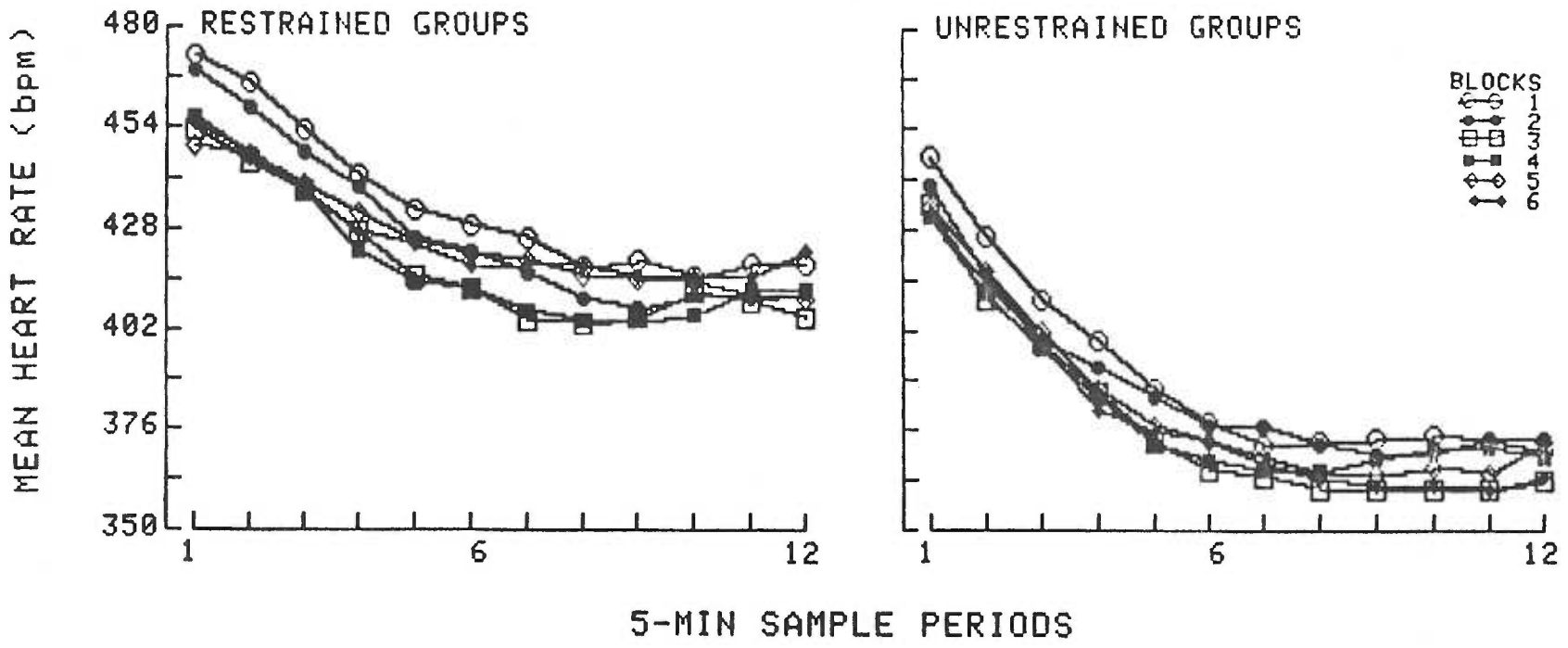

MEAN HEART RATE (bpm)
500
470
440
410
380
350
RESTRAINED GROUPS
UNRESTRAINED GROUPS
0 mg/kg
2
4
8
1
6
12
1
6
12
5-MIN SAMPLE PERIODS

across the six 2-day acquisition blocks. In general, heart rate was elevated initially in all groups and decreased by an average of 46 bpm in the Restrained Groups and 67 bpm in the Unrestrained Groups. Overall, heart rate in the Restrained Groups was higher than that in the Unrestrained Groups. Although the heart rate in Group R8 appears high relative to all other groups, analyses of the Restrained Groups' data suggested this difference was not significant (see below).

In all four-way ANOVAs performed on the data from the tolerance acquisition phase, the between-group variables were Restraint (R vs. U) and Dose (0, 2, 4 or 8 mg/kg), and the within-group variables were Blocks (2-day means) and 5-min Sample Periods. The ANOVA on the Baseline data revealed a significant Restraint x Blocks x Sample Periods interaction ($F\{55,2391\} = 1.50$). Followup analyses showed significant Blocks x Sample Periods interactions in the Restrained Groups ($F\{55,1043\} = 2.27$) but not in the Unrestrained Groups. Main effects of Blocks ($F\{5,123\} = 2.54$) and Sample Periods ($F\{11,275\} = 144.13$) were significant in the Unrestrained Groups suggesting consistent changes in heart rate over blocks in each sample period (see right-hand panel, Figure 6). Figure 6 also shows that the Blocks x Sample Periods interaction in the Restrained Groups appears to be due to the fact that there is a greater difference between blocks during the first few sample periods than during the latter sample periods. Analyses of the data for Group R during Sample Periods 1, 4 and 12 of all six Blocks supported these observations and revealed a significant effect of Blocks in Sample Periods 1 and 4 ($F\{5,95\} = 6.44$ and 5.35, respectively) but not in Sample Period 12.

The Restraint x Blocks x Sample Periods interaction is, therefore,

Figure 6. Mean heart rate during the baseline period (1-hr period before morphine infusion) is plotted as a function of 5-min sample periods and 2-day blocks. The left panel shows the data for Groups R, while the panel on the right shows the data for Groups U.


MEAN HEART RATE (bpm)
480
454
428
402
376
350
RESTRAINED GROUPS
UNRESTRAINED GROUPS
BLOCKS
1
2
3
4
5
6
1
6
12
1
6
12
5-MIN SAMPLE PERIODS

explained by the Blocks x Sample Periods interaction found in Group R but not Group U and also by significant Restraint x Sample Periods interactions found in Blocks 1, 3, 5 and 6 ($F$s{11,484} = 2.21, 3.35, 6.56 and 6.26, respectively) but not in Blocks 2 or 4. Figure 7 shows Groups R vs. U in each Block, collapsed across Dose Groups. In general, the difference between Groups R and U is less during Sample Period 1 than later sample periods because the heart rate appears to decline more in the U Groups than R Groups. Analyses in Sample Periods 1, 6 and 12 which revealed significant effects of restraint in all blocks and during all sample periods analyzed except Sample Period 1 in Block 3 (see Table 3 for the F values from these analyses).

There was no main effect of Dose in the overall analysis; however, there were various two-way interactions in which dose was a factor. Figure 8 shows the baselines for each dose group collapsed across Restraint and Blocks. During the first part of the hour, the heart rates in all of the dose groups appear to be about the same; however, after about 30 min the heart rate in Dose Group 8 remains elevated relative to the other three dose groups. These observations are supported by a significant Dose x Sample Periods interaction ($F${33,484} = 1.79); however, analyses in individual sample periods revealed no effects of Dose.

Figure 9 illustrates the mean baseline heart rate in each block for each dose group. There appears to be very little difference between dose groups during the first four blocks, but in Blocks 5 and 6, Dose groups 4 and 8 appear to have higher mean heart rates than Dose Groups 0 and 2. These observations are supported by a significant Dose x Blocks interaction ($F${15,218} = 3.18). Followup analyses of dose in each block

Table 3. Analyses of the effects of Restraint gave these F values for Sample Periods (SP) 1, 6 and 12 for each Block. Degrees of freedom were 1 and 44 for each F value. N/S denotes a non-significant effect of Restraint.

| | SP1 | SP6 | SP12 |
|---|---|---|---|
| Block 1 | 7.39 | 24.41 | 29.95 |
| 2 | 10.02 | 30.76 | 12.04 |
| 3 | N/S | 22.08 | 19.09 |
| 4 | 8.22 | 31.44 | 16.07 |
| 5 | 4.62 | 37.43 | 15.11 |
| 6 | 6.99 | 25.02 | 43.59 |

Figure 7. Mean heart rate of the restrained (R) and unrestrained (U) groups during the baseline period. Each individual panel plots heart rate as a function of sample periods during each block, collapsed across dose.

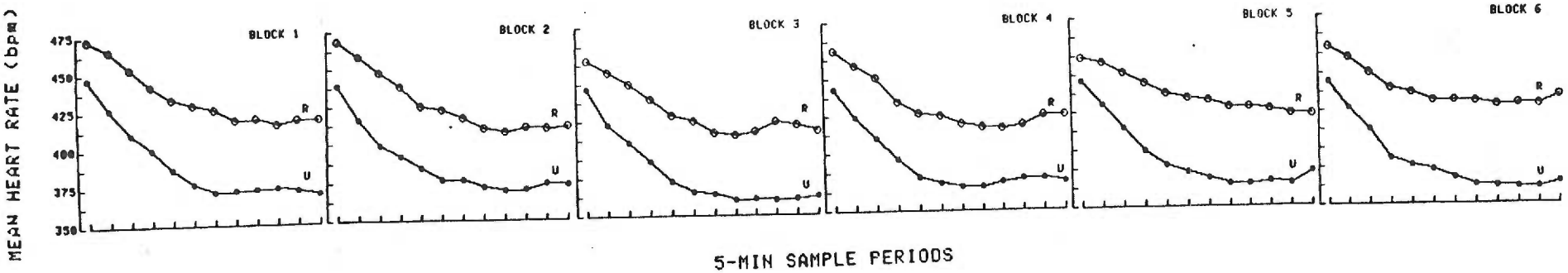

MEAN HEART RATE (bpm)
475
450
425
400
375
350
BLOCK 1
BLOCK 2
BLOCK 3
BLOCK 4
BLOCK 5
BLOCK 6
R
U
5-MIN SAMPLE PERIODS

Figure 8. Mean heart rate during the baseline period is plotted as a function of sample periods and dose. The data are collapsed across restraint and 2-day blocks.

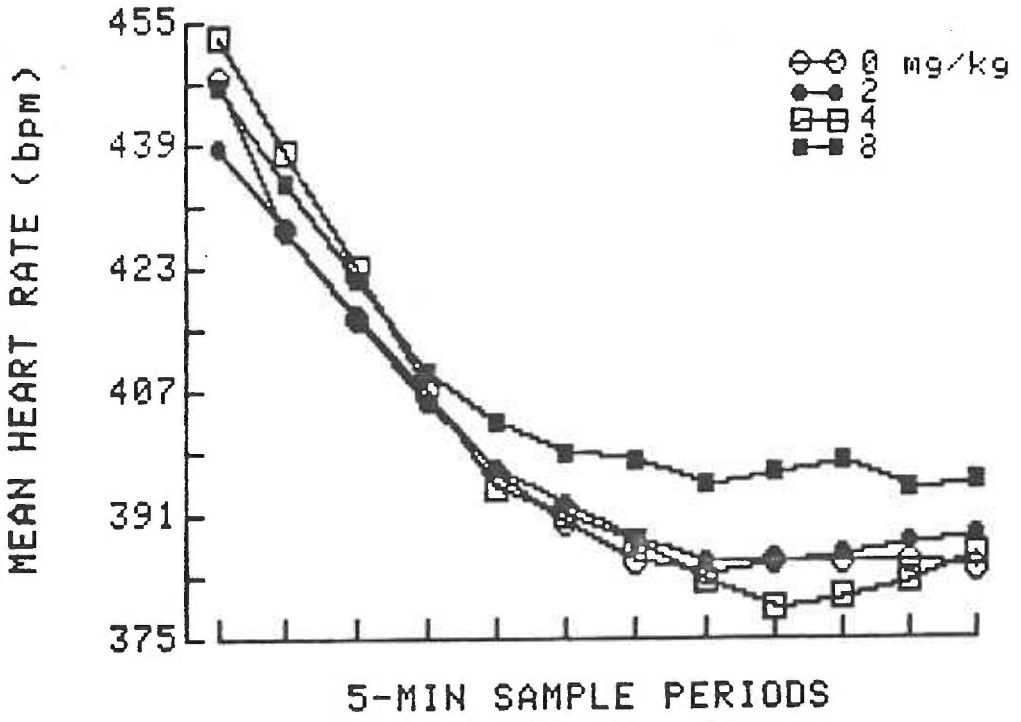
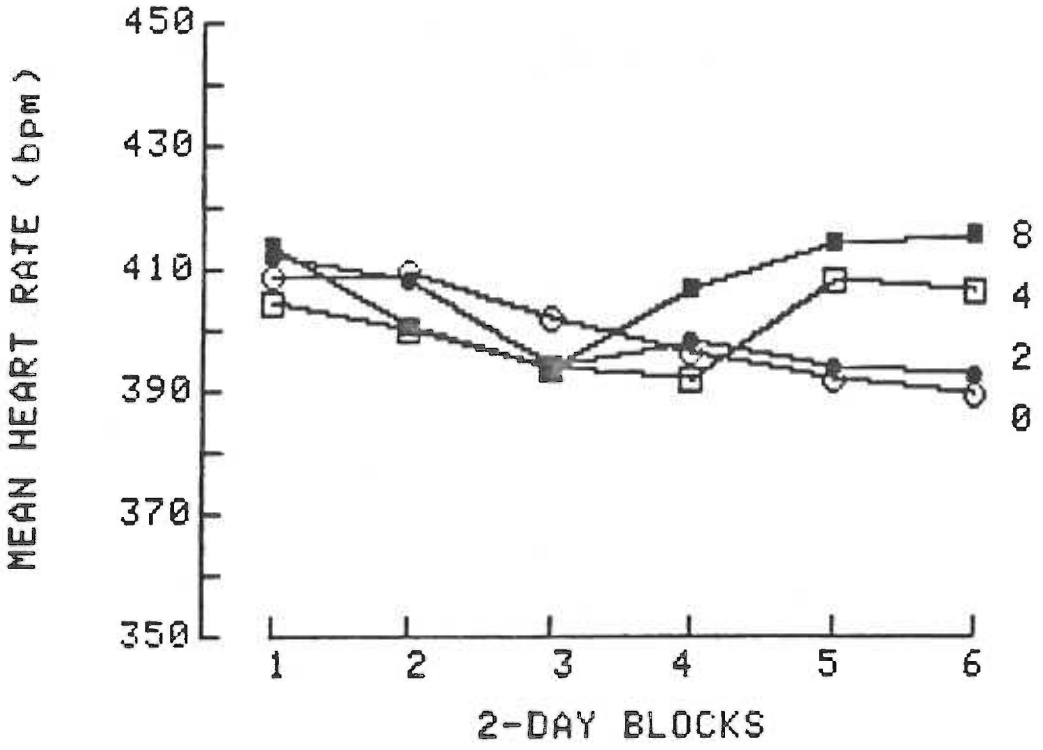

455
439
423
407
391
375
MEAN HEART RATE (bpm)
0 mg/kg
2
4
8
5-MIN SAMPLE PERIODS

Figure 9. Mean heart rate collapsed across sample periods during the baseline period. The data depicted here, collapsed across restraint, show how the baselines changed over blocks for each dose group. The numbers to the right of the graph correspond to dose (mg/kg).


MEAN HEART RATE (bpm)
450
430
410
390
370
350
1
2
3
4
5
6
2-DAY BLOCKS
8
4
2
0

revealed significant effects of Dose on Blocks 5 and 6 ($F$s{3,44} = 3.08 and 3.52, respectively) but not on Blocks 1 through 4.

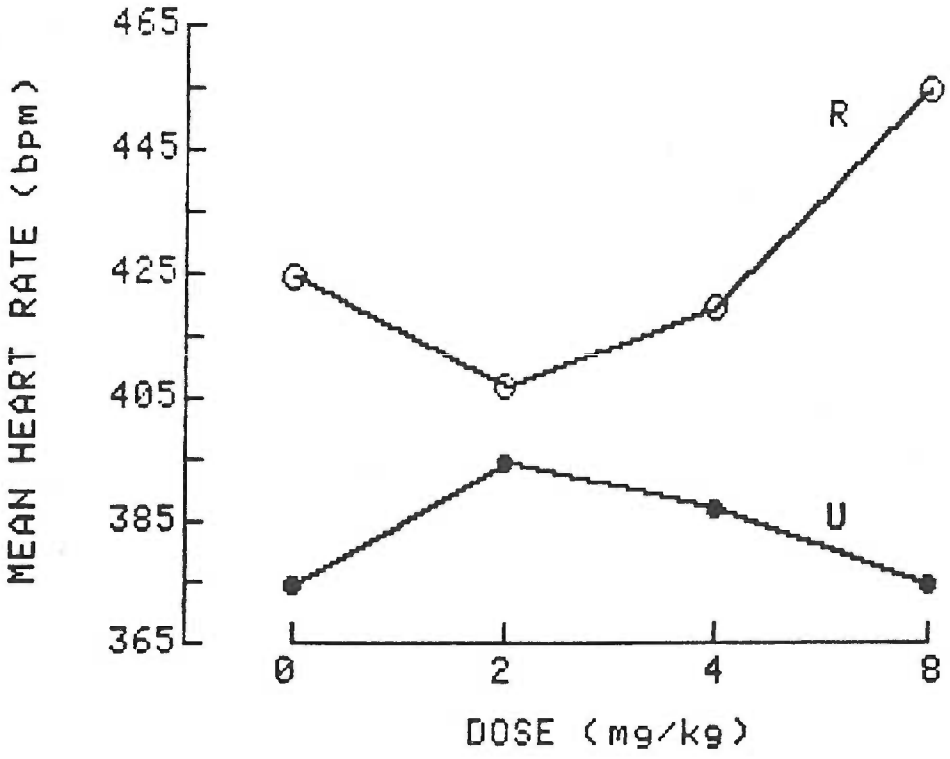
An initial expectation in this study was that if baseline heart rate was plotted as a function of dose and restraint, as in Figure 10, the two lines would be parallel as well as straight because dose was not expected to affect heart rate during the pre-injection period, especially 24 hrs after injection. However, the differences between Groups R and U vary as a function of dose. A significant Restraint x Dose interaction ($F$\{3,44\} = 3.37) supports this observation. Followup analyses at each dose yielded significant effects of Restraint only in Dose Groups 0 and 8 ($F$\{1,12\} = 16.02 and $F$\{1,10\} = 30.42, respectively).

Summary: Baseline

In general, the pattern of change in heart rate over the 1-hr baseline period was an initial elevation, presumably due to handling, followed by a decrease over the hour. The heart rate in Restrained rats was higher than that in Unrestrained rats. However, the difference between Groups R and U at each dose was only significant in Dose Groups 0 and 8. During the baseline period, the decline in heart rate was greater in the Unrestrained rats relative to Restrained rats.

Over 2-day Blocks, there was generally a decline in baseline probably because of habituation to the handling procedure and apparatus. There was a greater decline in initial elevation in Restrained rats as compared with Unrestrained rats. There was also a greater decline in baseline heart rate overall in the low Dose Groups (0 and 2 mg/kg) relative to high Dose Groups (4 and 8 mg/kg) indicating there is some effect of repeated administration of a relatively high dose of morphine (e.g., 4 and 8 mg/kg) on an animal's heart rate, even 24 hrs after

Figure 10. Mean heart rate of the restrained (R) and unrestrained (U) groups during the baseline periods is plotted as a function of dose. The data are collapsed across sample periods and blocks.


MEAN HEART RATE (bpm)
465
445
425
405
385
365
R
U
0
2
4
8
DOSE (mg/kg)

receiving the dose.

Acquisition: Post-infusion

The mean heart rate change scores of the eight groups are shown, collapsed across blocks, in Figure 11. In general, as shown in previous studies, morphine produced a biphasic change in heart rate: bradycardia followed by tachycardia. The bradycardia was greater in magnitude and longer in duration in the restrained rats, while the tachycardia was of greater magnitude and duration in the unrestrained rats.

Figure 11 also shows that the form of the response differs as a function of dose. Specifically, there was a greater decrease in heart rate in Dose Group 8 with longer time to reach peak tachycardia relative to the lower doses. In the lower doses, the initial bradycardia was rapidly replaced by tachycardia which was sustained in Dose Group 4 relative to Dose Group 2.

Change scores rather than post-infusion heart rates were analyzed because of the differences between the R and U groups in baseline heart rate. A four-way ANOVA on these data revealed a significant Restraint x Dose x Blocks x Sample Periods interaction ($F\{345,5028\} = 1.22$). Followup analyses comparing Groups R and U at each dose revealed a significant Restraint x Blocks x Sample Periods interaction in Dose Group 8 ($F\{115,1149\} = 1.43$) but not in any other Dose Groups.

The data for the three other dose groups can be seen in Figure 12 which plots change in heart rate as a function of sample periods and restraint, collapsed across blocks. The responses of the R and U rats diverge over the post-infusion sample periods. In Group R0, heart rate continues to decline throughout the session, while heart rate in Group U0 remains stable. In Groups U2 and U4, there is a very large increase

Figure 11. Heart rate is plotted as a function of dose and time after infusion during acquisition. This figure shows how the form of the response varies as a function of dose as well as restraint. The heart rate for restrained groups is plotted in the left panel, while that of the unrestrained groups is plotted on the right.

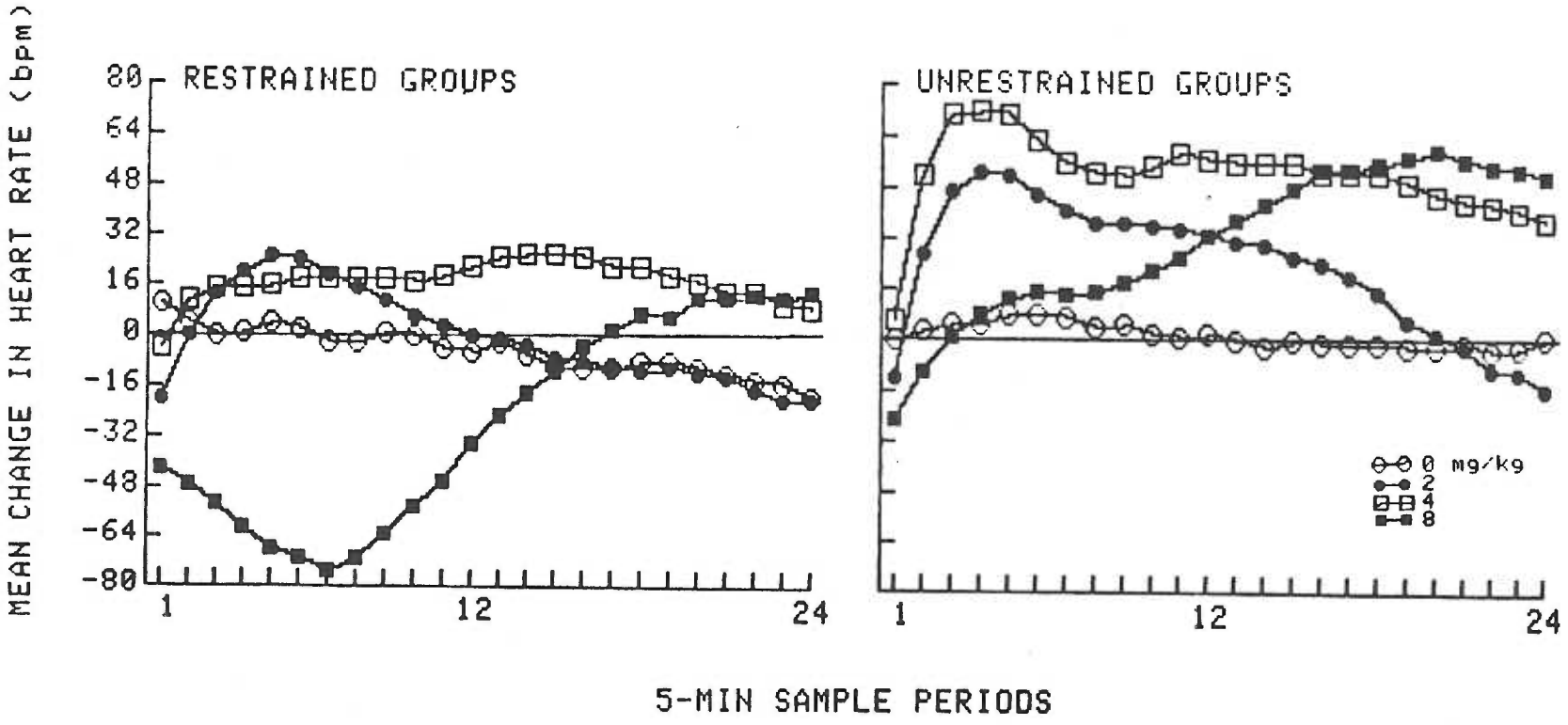
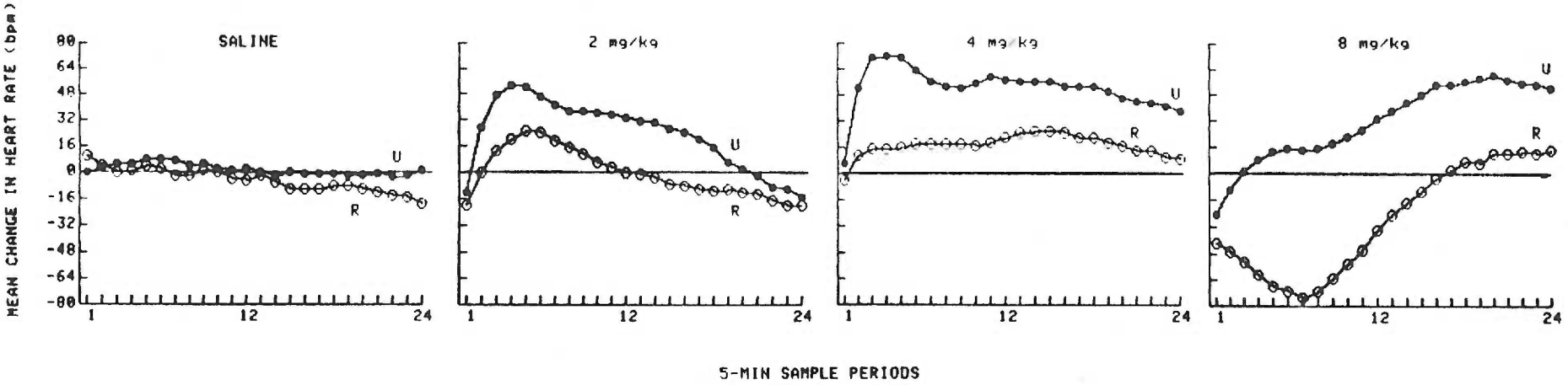

MEAN CHANGE IN HEART RATE (bpm)
RESTRAINED GROUPS
UNRESTRAINED GROUPS
80
64
48
32
16
0
-16
-32
-48
-64
-80
1
12
24
0 mg/kg
2
4
8
5-MIN SAMPLE PERIODS

Figure 12. Mean change in heart rate from baseline during the 2-hr period after infusion. Each panel plots the reaction of restrained (R) and unrestrained (U) groups to their dose of drug as a function of time after infusion. These data are collapsed across all 12 days of acquisition.


MEAN CHANGE IN HEART RATE (bpm)
80
64
48
32
16
0
-16
-32
-48
-64
-80
SALINE
2 mg/kg
4 mg/kg
8 mg/kg
U
R
1
12
24
5-MIN SAMPLE PERIODS

in heart rate relative to baseline, but in Groups R2 and R4, there is a slight decrease followed by a slight elevation relative to baseline. The heart rate in Groups R2 and U2 converge at the end of the session. These observations are supported by Restraint x Sample Periods interactions in Dose Groups 0 and 4 ($F\{23,276\} = 1.72$ for Dose 0 and $F\{23,230\} = 1.95$ for Dose 4) but not in Dose Group 2. Note that a Restraint x Sample Periods interaction in Dose Group 8 is qualified by the Restraint x Blocks x Sample Periods interaction which is described below.

Figure 13 plots heart rate as a function of sample periods and blocks collapsed across restraint condition. The general form of the response in Dose Groups 2 and 4 was one of initial bradycardia replaced immediately by tachycardia relative to baseline. Dose Group 0 showed a slight decline over the 2-hr post-injection period. Significant Blocks x Sample Periods interactions were revealed in Dose Groups 2 and 4 ($F\{115,1353\} = 2.11$ and $F\{115, 1146\} = 2.42$, respectively) but not in the Saline Group. Figure 13 suggests that the magnitude of bradycardia decreased over blocks, but the tachycardia remained relatively unchanged. The greatest difference between blocks is during Sample Period 1 for all Dose Groups except Saline. This observation is supported by separate analyses of Sample Periods 1, 6 and 24 in Dose Groups 2 and 4 which revealed significant effects of Blocks in Sample Period 1 ($F\{5,59\} = 6.62$ for Dose Group 2 and $F\{5,50\} = 6.92$ for Dose Group 4). No significant Blocks effect was found in Sample Periods 6 and 24. A main effect of Sample Periods was revealed in Dose Group 0 ($F\{23,276\} = 4.96$).

Figure 14 shows the heart-rate response of Group R8 and Group U8 as

Figure 13. Mean change in heart rate from baseline plotted as a function of sample periods and blocks. Each individual panel plots the reaction of one group to its dose of drug. These data are collapsed across restraint.

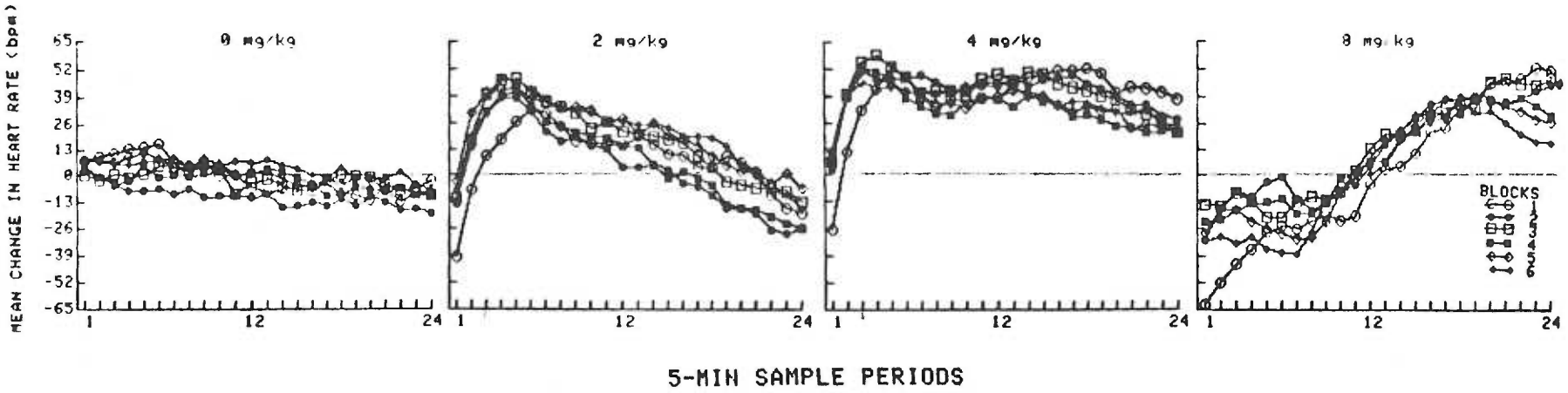
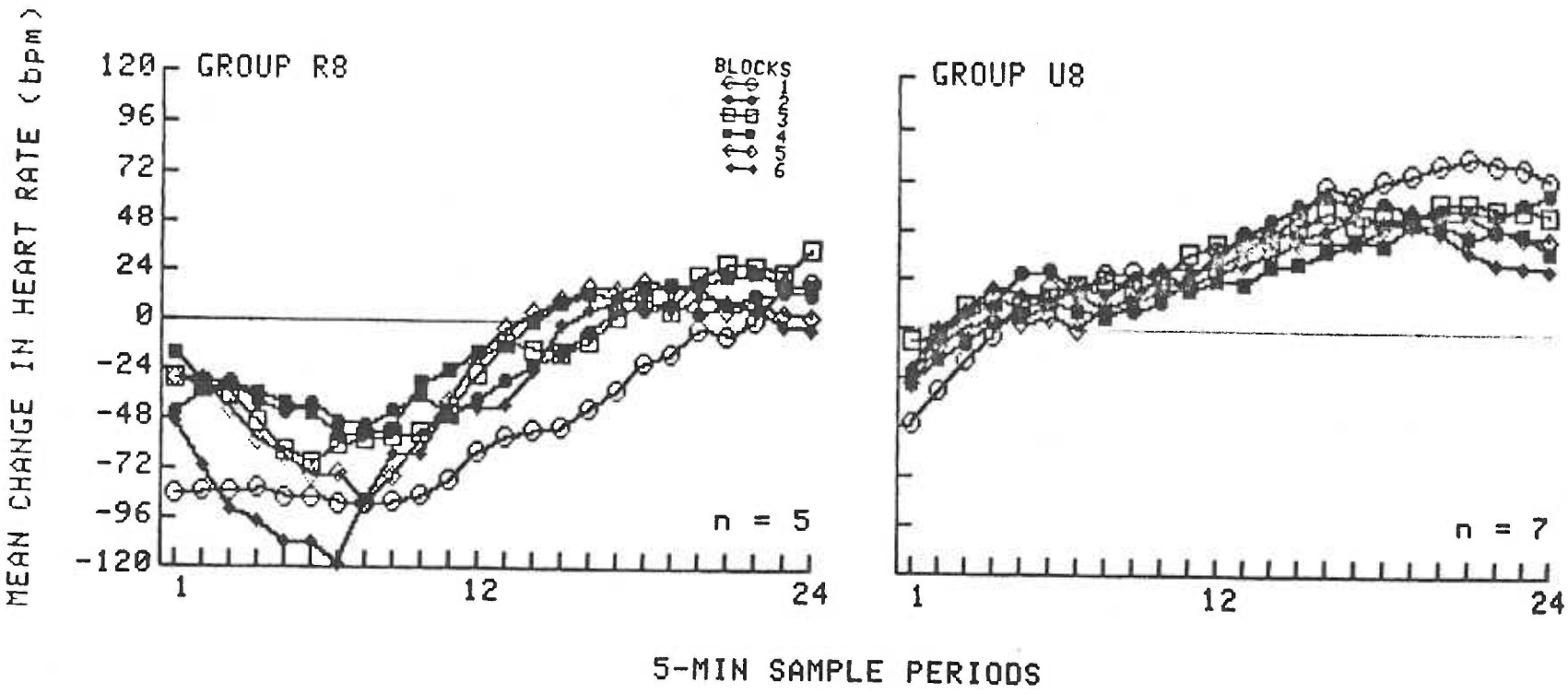

MEAN CHANGE IN HEART RATE (bpm)
65
52
39
26
13
0
-13
-26
-39
-52
-65
0 mg/kg
2 mg/kg
4 mg/kg
8 mg/kg
1
12
24
BLOCKS
1
2
3
4
5
6
5-MIN SAMPLE PERIODS

Figure 14. Mean change in heart rate from baseline during the 2-hr period after infusion of an 8 mg/kg dose of morphine. Each panel shows the responses of rats in Group R8 (left panel) and Group U8 (right panel) as a function of blocks and time after infusion.


MEAN CHANGE IN HEART RATE (bpm)
120
96
72
48
24
0
-24
-48
-72
-96
-120
GROUP R8
BLOCKS
1
2
3
4
5
6
n = 5
1
12
24
GROUP U8
n = 7
1
12
24
5-MIN SAMPLE PERIODS

a function of sample periods and blocks. The form of the response is different in each Group: the bradycardia in Group R8 is longer in duration relative to Group U8, and in Group U8, the bradycardia was quickly replaced by tachycardia. In general, the initial bradycardia appeared to decrease in magnitude over blocks for Groups U8 but not for Group R8. Figure 14 shows that the bradycardia in Group R8 remained long in duration and even increased in magnitude in Block 6, while the degree of bradycardia in Group U8 remained stable after Block 3. In contrast, the magnitude of tachycardia did not change uniformly over blocks in either Group R8 or U8. A significant Blocks x Sample Periods interaction was revealed in Group U8 ($F\{115,690\} = 1.95$) but not in Group R8. Main effects of Sample Periods were revealed in both Groups R8 and U8 ($F\{23,92\} = 20.85$ for Group R8 and $F\{23,138\} = 17.57$ for Group U8). Subsequent analyses of Sample Periods 1, 12 and 24 in Group U8 revealed no effect of blocks during any sample periods; however, a contrast analysis of the difference between Block 1 and all other Blocks in Sample Period 1 versus that in Sample Period 24 revealed a significant decrease over blocks in both the bradycardia and tachycardia ($F\{1,690\} = 27.96$). This is the source of the Blocks x Sample Periods interaction.

Additional analyses of Restrained and Unrestrained Groups revealed a significant Dose x Blocks x Sample Periods interaction in the Groups U ($F\{345,2849\} = 1.29$) but not in Groups R. The three-way interaction in Group U is due to a significant Blocks x Sample Periods interaction in Dose Groups 2 and 4 ($F\{115,780\} = 1.56$ for Dose Group 2 and $F\{115,689\} = 1.70$ for Dose Group 4), but not Dose Group 0. A significant Dose x Sample Periods interaction revealed in both the Restrained ($F\{69,437\} =$

10.98) and Unrestrained groups ($F\{69,575\}= 15.84$) lend further support to the observations made in Figure 11.

Summary: Post-infusion

The change in heart rate after morphine infusion varied in form as a function of restraint and dose. In general, there was a more positive change in the Unrestrained Groups as compared to the Restrained Groups, and the biggest differences between R and U were during the later sample periods relative to the early sample periods (see Figure 12). However, at the highest dose, there was a more prolonged initial decrease in heart rate in Group R8 relative to U8. Both the bradycardic and tachycardic portions of the response increased as a function of dose.

The effects of repeated exposure to the lower doses of morphine (0, 2 and 4 mg/kg) generally varied as a function of dose. There was no significant change in response to Saline over the 12-day acquisition period. In Dose Groups 2 and 4 the greatest change over blocks occurred as a decrease in initial bradycardia between Block 1 and Block 2 which was very similar to the changes over blocks in Group U8.

Over the 12-day acquisition phase, there was no consistent change in response in Group R8, whereas there was a decrease in magnitude of the initial bradycardia and the tachycardia in Group U8 with the greatest change being between Blocks 1 and 2 indicating tolerance to both effects. There was no significant change in the magnitude of tachycardia over the acquisition period in any other morphine groups; however, as the magnitude and duration of bradycardia decreased, the latency of occurrence of tachycardia also decreased.

Tolerance Test: Baseline

Figure 15 shows the pre-infusion baseline heart rate for all eight groups collapsed across test dose. As during the Acquisition Phase (see Figure 5), the heart rate in Group R8 appeared much higher than that of other groups. Also, as seen in previous phases, there was a greater decline in heart rate over the one-hour period in the U groups relative to the R groups (55 vs. 25 bpm).

In the four-way ANOVA performed on the baseline tolerance test data, the between-group factors were Restraint and Acquisition Dose, and the within-group factors were Test Dose and Sample Periods. The overall analysis revealed a Restraint x Sample Periods interaction ($F\{11,484\} = 9.11$) which supports the observations made about Figure 15, i.e., that there is a greater decline in heart rate in the U groups than the R groups.

Figure 16 shows the mean heart rate of the pre-infusion baseline hour for the R and U groups as a function of acquisition dose, collapsed across test dose and sample periods. This figure resembles Figure 10 from the Acquisition Phase in that the difference between R and U is greatest in Acquisition Dose Groups 0 and 8. There is a smaller difference in Dose Group 4 and very little difference in Dose Group 2. These observations are supported by a Restraint x Acquisition Dose interaction ($F\{3,44\} = 4.67$). Follow-up analyses at each acquisition dose revealed effects of Restraint at Doses 0 and 8 ($F\{1,12\} = 20.8$ for Dose 0 and $F\{1,10\} = 30.39$ for Dose 8) but not at Doses 2 or 4. Separate analyses of R and U groups revealed a significant Acquisition Dose x Sample Periods interaction in Group R ($F\{33,209\} = 1.84$) as well as a main effect of Acquisition Dose ($F\{3,19\} = 4.13$). A significant effect of Sample Periods was revealed in Group U ($F\{11,275\} = 1.38$); no

Figure 15. Mean heart rate during the 1-hr pre-infusion baseline period during the Tolerance Test Phase is plotted for each acquisition dose group in both the restrained (left panel) and unrestrained (right panel) conditions. The data are collapsed across test dose.

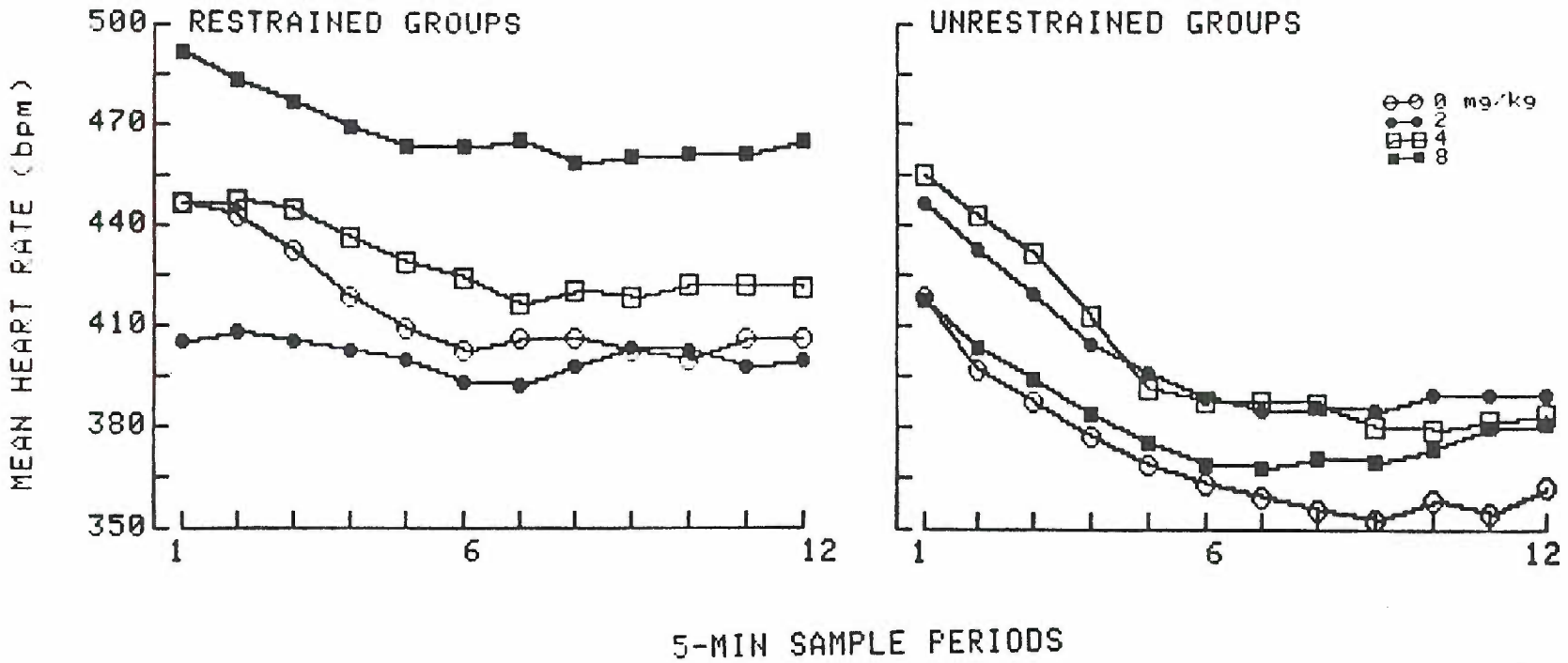
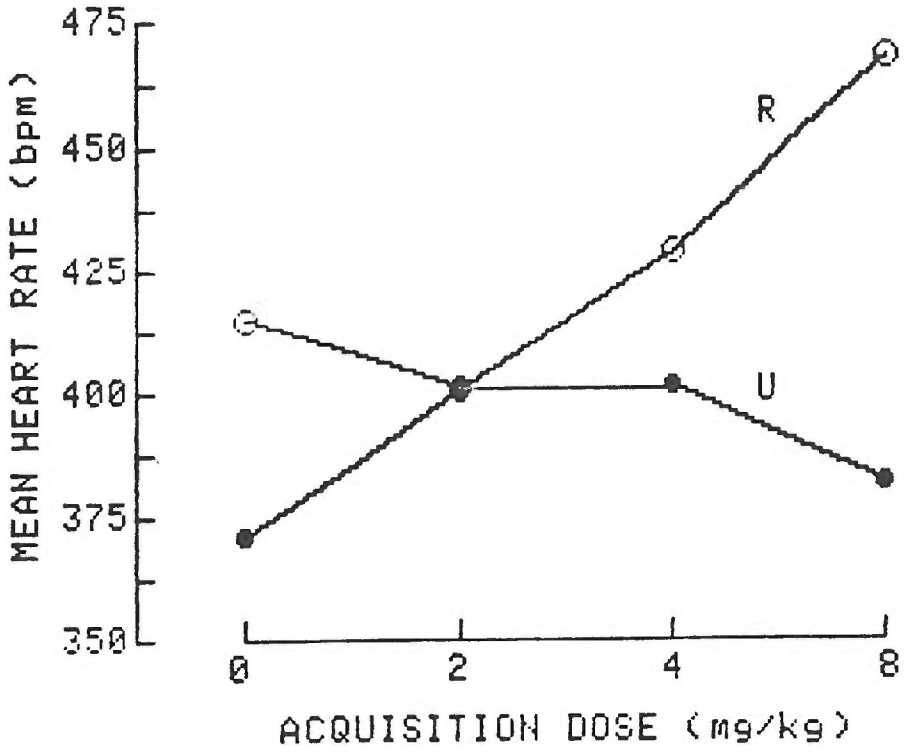

RESTRAINED GROUPS
UNRESTRAINED GROUPS
MEAN HEART RATE (bpm)
500
470
440
410
380
350
1
6
12
0 mg/kg
2
4
8
5-MIN SAMPLE PERIODS

Figure 16. Mean heart rate during the baseline hour is graphed as a function of restraint and acquisition dose. These data are collapsed across test dose.


475
450
425
400
375
350
MEAN HEART RATE (bpm)
R
U
0
2
4
8
ACQUISITION DOSE (mg/kg)

main effect of or interactions involving acquisition dose were found in Group U.

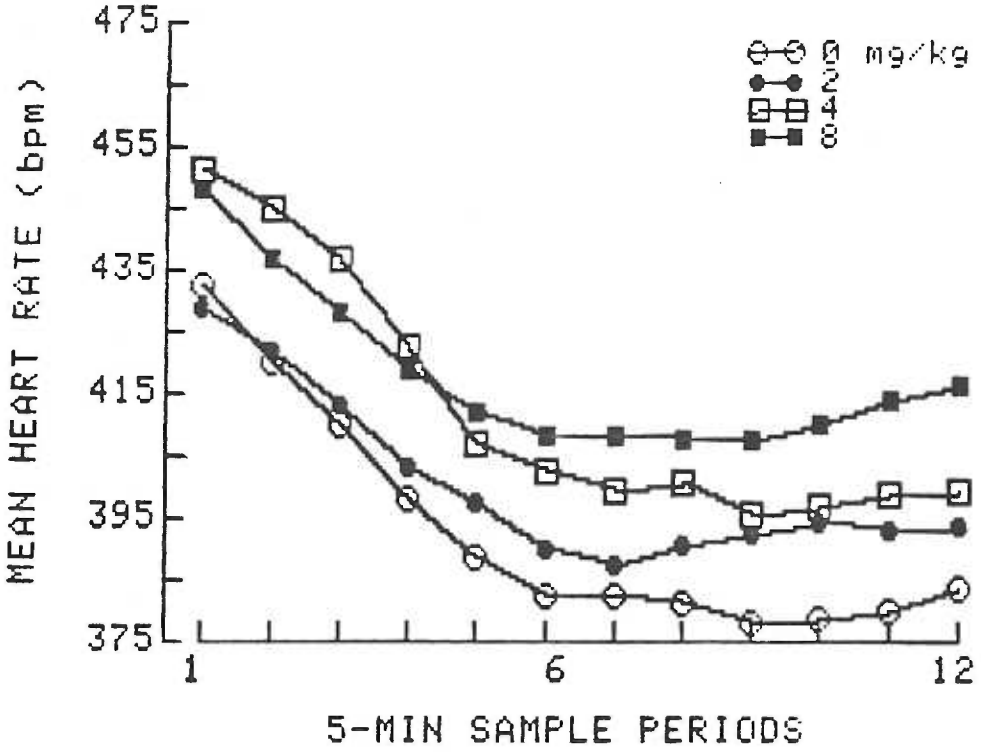
Figure 17 shows the heart-rate response of each acquisition dose group collapsed across restraint. Overall, the decline in heart rate was greater in Acquisition Dose Groups 0 and 4 (48 and 52 bpm, respectively) relative to Groups 2 and 8 (35 and 32 bpm, respectively). This observation is supported by an Acquisition Dose x Sample Periods interaction ($F\{33,484\} = 1.56$). Separate analyses of Sample Periods 1, 6 and 12 revealed effects of Acquisition Dose during Sample Periods 1 and 12 ($F\{3,44\} = 3.1$ and 3.2, respectively) but not during Sample Period 6 suggesting greater differences in heart rate among acquisition dose groups early and late in the hour but not throughout the whole hour. As in the Acquisition Phase, the baseline heart rate was higher in Dose Groups 4 and 8.

Generally, tolerance test dose did not appear to affect mean heart rate during baseline. However, the four-way ANOVA revealed a Restraint x Acquisition Dose x Test Dose interaction ($F\{9,126\} = 2.96$). Follow-up tests indicated that the effect of Test Dose was only significant in Group R2 ($F\{3,15\} = 7.66$) and not in any other acquisition group. This three-way interaction qualifies the Restraint x Acquisition Dose interaction already discussed above; however, since the effect of test dose was only found in Group R2, and the order of test doses was counterbalanced, it is possible that this effect was due to sampling error.

Summary: Baseline

In general, baseline heart rate was elevated initially, presumably due to handling, and declined over the pre-infusion hour. The decline

Figure 17. Mean heart rate during baseline is graphed as a function of 5-min sample periods and acquisition dose. The data are collapsed across restraint and test dose.


475
455
435
415
395
375
MEAN HEART RATE (bpm)
1
6
12
5-MIN SAMPLE PERIODS
0
2
4
8
mg/kg

was greater in the U groups relative to R groups. Differences between R and U groups in mean baseline heart rate were significant in Acquisition Dose Groups 0 and 8 but not in Groups 2 and 4.

Differences due to acquisition dose were revealed early and late in the hour, just before infusion. These results suggest the presence of a learned compensatory increase in heart rate due to expectancy of morphine, especially in the Acquisition Dose Group 8. The pattern of results suggest that the R groups contributed more to the acquisition dose differences than the U groups, but since there was no Restraint x Acquisition Dose x Sample Periods interaction in the overall analysis, some of the dose differences were due to differences within the U groups.

Tolerance Test: Post-infusion

Figure 18 shows the heart rate after infusion of each test dose for all eight groups. Generally, as shown previously, morphine produced a biphasic heart rate response: bradycardia followed by tachycardia. In Figure 18, bradycardia and tachycardia are defined by comparing the heart-rate response of each group after morphine (2, 4 or 8 mg/kg) with the response after saline (open circles). Figure 19 shows the same data, but in terms of change from pre-infusion baseline. Both figures illustrate that the bradycardic portion of the response was greater in the R groups (top row of panels), while the tachycardia was generally greater in the U groups (bottom row) relative to the R groups.

Both figures also show that the form of the response differed as a function of test dose. Specifically, the magnitude of bradycardia in each group was greatest following Test Dose 8. After Test Doses 2 and 4, the initial bradycardia was quickly replaced by tachycardia which was

Figure 18. The post-infusion heart rate is graphed as a function of tolerance test dose and time after infusion. Panels A and E show athe responses of Dose Group 0; panels B and F graph the responses of Dose Group 2; panels C and G show Dose Group 4; panels D and H show Dose Group 8. The top row of panels shows the responses of the R groups, and the bottom row, the U groups.

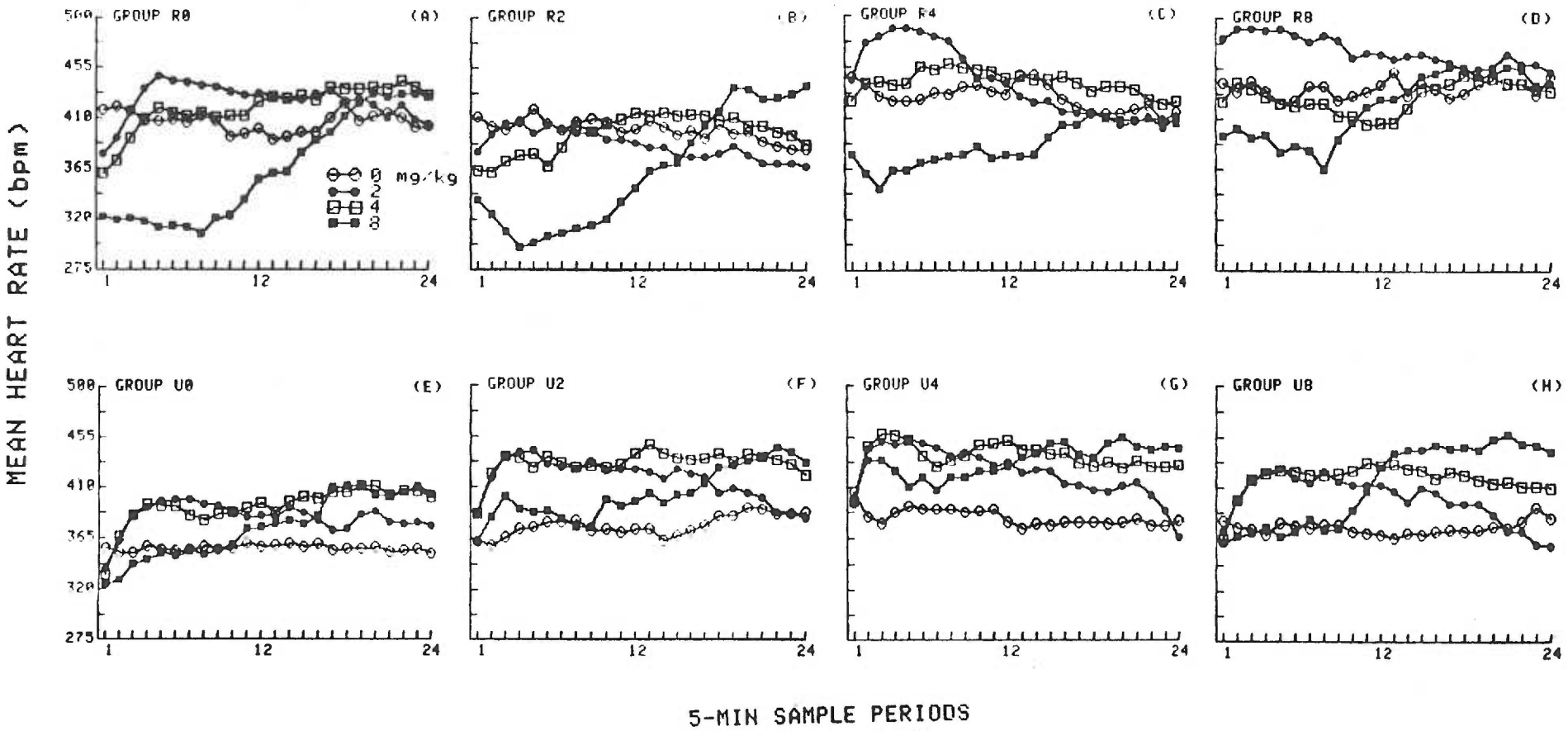
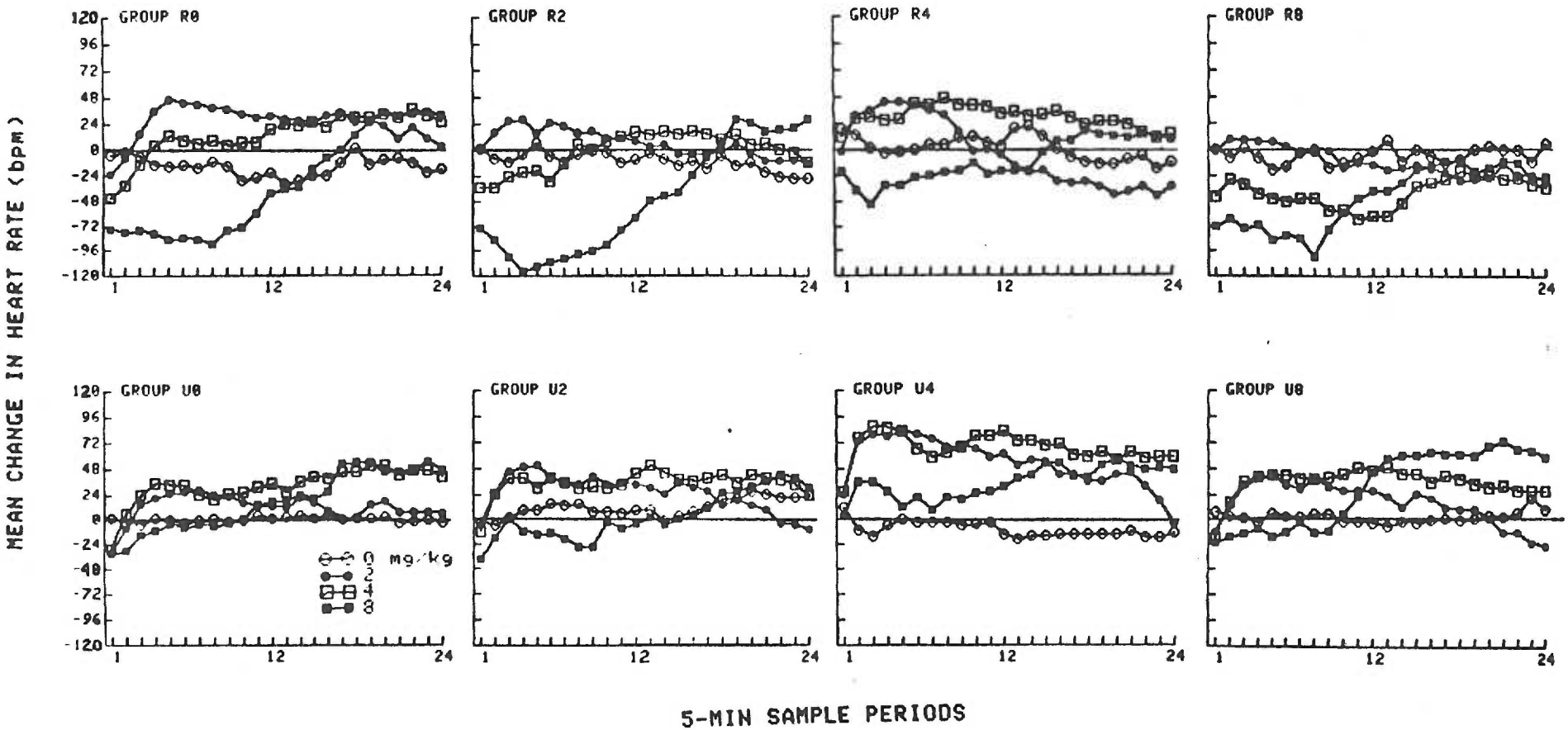

MEAN HEART RATE (bpm)
GROUP R0
(A)
GROUP R2
(B)
GROUP R4
(C)
GROUP R8
(D)
GROUP U0
(E)
GROUP U2
(F)
GROUP U4
(G)
GROUP U8
(H)
0 mg/kg
2
4
8
500
455
410
365
320
275
1
12
24
5-MIN SAMPLE PERIODS

Figure 19. Post-infusion change scores are shown for all eight acquisition groups. Each panel shows the response of a given acquisition group to each test dose of morphine. The top row of panels shows the responses of the R groups, and the bottom row of panels shows the response of the U groups. The line drawn through zero is baseline.


GROUP R0
GROUP R2
GROUP R4
GROUP R8
GROUP U0
GROUP U2
GROUP U4
GROUP U8
120
96
72
48
24
0
-24
-48
-72
-96
-120
1
12
24
0 mg/kg
2
4
8
MEAN CHANGE IN HEART RATE (bpm)
5-MIN SAMPLE PERIODS

longer lasting in Test Dose 4 relative to Test Dose 2. In Group U4 (see panel G, Figure 19), no bradycardia relative to baseline occurred. The differences due to test dose are more clearly seen in the R groups.

Because treatment-related differences in baseline developed during acquisition and were still present during tolerance testing, two different analyses were performed on the post-injection tolerance test data: one analysis was applied to change scores and the other to post-infusion heart rate. The change-score analysis was done to compare drug effects on magnitude of change from baseline in various groups regardless of the level of baseline heart rate. Post-infusion heart rate was analyzed to compare differences due both to drug effects and residual effects of baseline. The two analyses revealed almost parallel results; however, the differences were felt to be important enough to pursue explanation of both.

In all four-way ANOVAs performed on the data from the Tolerance Test Phase, the between-group variables were Restraint and Acquisition Dose, and the within-group variables were Test Dose and Sample Periods. Both the change-score ANOVA and heart-rate ANOVA revealed a significant Restraint x Acquisition Dose x Test Dose x Sample Periods interaction ($F\{207,2853\} = 1.41$ for both ANOVAs).

Follow-up analyses were carried out separately for R and U groups, and involved a series of two-group comparisons between the saline group (drug-naive subjects) and each acquisition dose group (drug-experienced subjects). The intent of these comparisons was to determine the degree of tolerance to the heart rate effects of morphine by comparing drug-experienced subjects with drug-naive subjects that had received the same amount of handling. In each of the two-group comparisons, a Test

Dose x Sample Periods interaction was revealed. The degrees of freedom and F values are given in Table 4. The source of this interaction has already been described as a difference in the form of the heart-rate response as a function of test dose. The interactions of importance for the determination of tolerance are those involving acquisition dose group.

Figure 20 shows the heart-rate responses of Groups R0 and R2 collapsed across test dose. The panel on left depicts the change from baseline while the panel on the right shows the heart rate. Both figures show that the bradycardia and tachycardia are slightly greater in Group R0 relative to Group R2. However, these observations were not supported statistically. Only a main effect of Sample Periods was revealed in both analyses ($F\{23,253\} = 11.84$ for both the change-score analysis and the heart-rate analysis). The lack of an effect of acquisition dose indicates the lack of tolerance to the heart-rate effects of morphine in Group R2.

Figure 21 shows the heart-rate responses of U0 and U2 as a function of sample periods. There appears to be very little difference between the change from baseline in each group (see left-hand panel), while the heart rate of Group U2 is higher than that of Group U0. Generally, the form of the response for each group is similar. These observations are supported by a main effect of Acquisition Dose in the heart-rate analysis ($F\{1,13\} = 12.35$) but not in the change-score analysis. A main effect of Sample Periods was revealed in both analyses ($F\{23,299\} = 10.2$ for both analyses). In this case, the results of the change score analysis do not indicate tolerance in Group U2, while the analysis of heart rate does indicate tolerance to the bradycardic effects and

Table 4. Results of the two-group comparisons. The degrees of freedom and F values for the Test Dose x Sample Periods interaction were the same in both the change-score and heart-rate analyses.

| | df | F |
|---|---|---|
| R0 vs. R2 | 69, 759 | 16.26 |
| R0 vs. R4 | 69, 690 | 8.75 |
| R0 vs. R8 | 69, 690 | 6.11 |
| U0 vs. U2 | 69, 897 | 5.29 |
| U0 vs. U4 | 69, 828 | 8.41 |
| U0 vs. U8 | 69, 828 | 12.66 |

Figure 20. The heart-rate response to morphine is graphed as a function of acquisition group (Group R0 and R2) and time after infusion. The panel on the left shows change from baseline, and the panel on the right shows the actual heart rate. These data are collapsed across test dose.

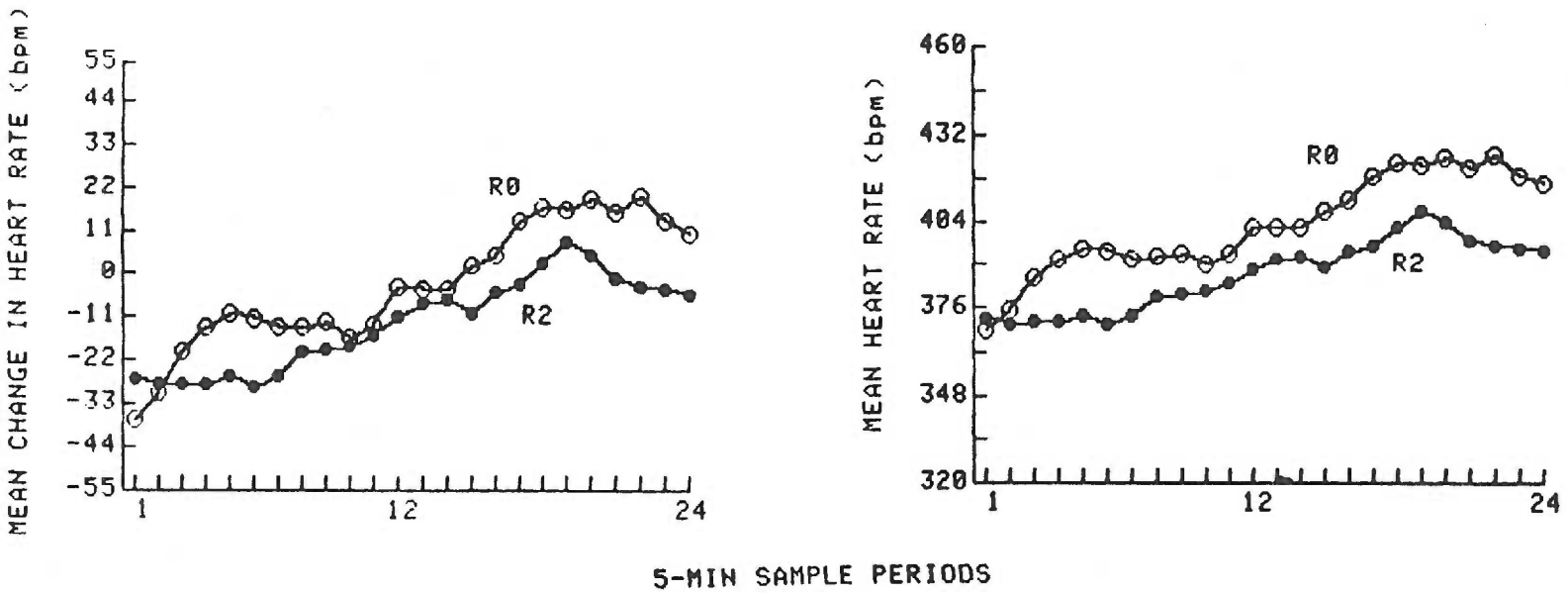
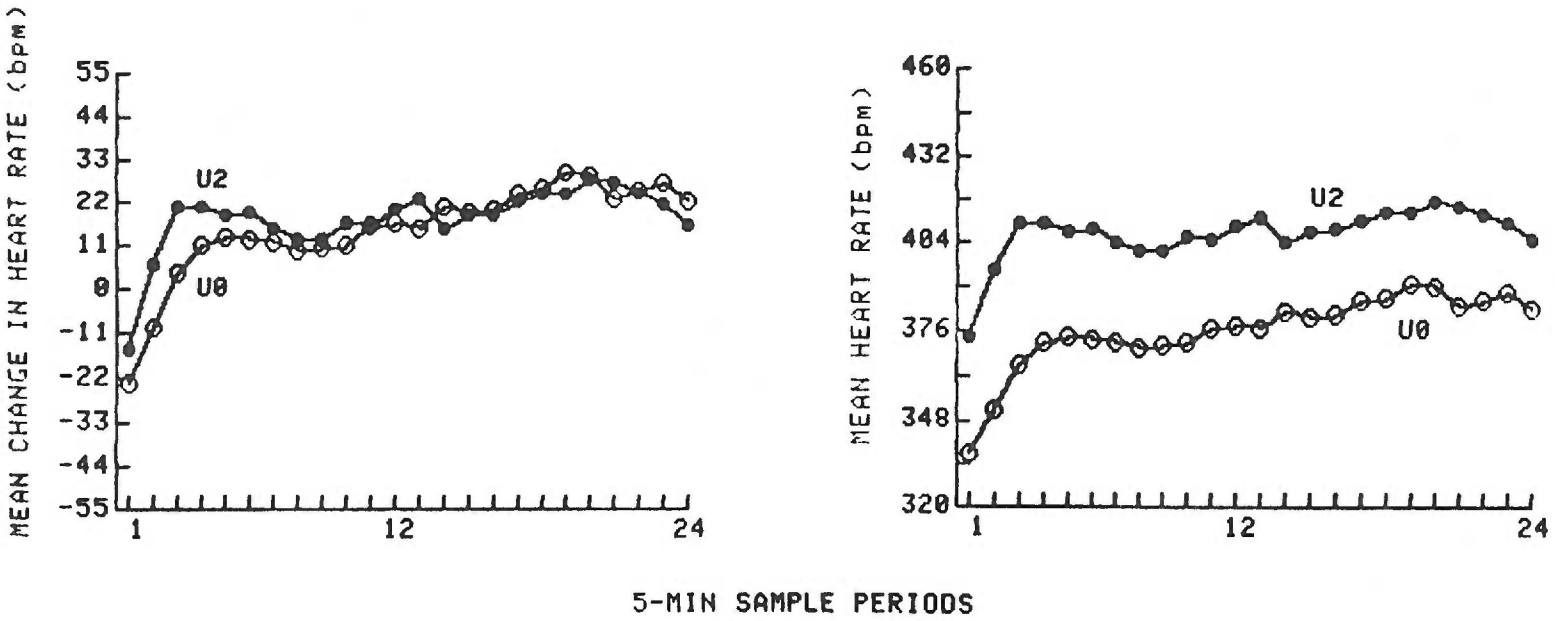

MEAN CHANGE IN HEART RATE (bpm)
55
44
33
22
11
0
-11
-22
-33
-44
-55
1
12
24
R0
R2
MEAN HEART RATE (bpm)
460
432
404
376
348
320
1
12
24
R0
R2
5-MIN SAMPLE PERIODS

Figure 21. The heart-rate response to morphine is graphed as a function of acquisition group (Group U0 and U2) and time after infusion. The panel on the left shows the change from baseline, and the panel on the right shows the actual heart rate. These data are collapsed across test dose.


MEAN CHANGE IN HEART RATE (bpm)
55
44
33
22
11
0
-11
-22
-33
-44
-55
1
12
24
U2
U0
MEAN HEART RATE (bpm)
460
432
404
376
348
320
1
12
24
U2
U0
5-MIN SAMPLE PERIODS

sensitization to the tachycardic effects of morphine (see Figure 18, panel F to compare the response of Group U2 to morphine with its response to saline).

Figure 22 shows the heart-rate response as a function of sample periods in Dose Groups R0 and R4 collapsed across test dose. The magnitude of bradycardia and tachycardia were greater in Group R0 relative to Group R4 (see left-hand panel). The heart rates of the two groups converged and were about the same by the end of the 2-hr post-infusion period (right-hand panel). An Acquisition Dose x Sample Periods interaction supports these observations ($F\{23,230\} = 7.3$ in both types of analysis). Analyses of simple effects in Sample Periods 1, 5, 12 and 24 revealed a significant effect of Acquisition Dose in Sample Period 1 ($F\{1,10\} = 8.36$ for the change-score analysis, and 11.31 for the heart-rate analysis) but not in any other sample periods analyzed. These results indicate the presence of tolerance in Group R4 to the bradycardic effect of morphine. However, these results do not indicate tolerance or sensitization to the tachycardic effect.

Figure 23 shows the heart-rate responses of Group U0 and U4 collapsed across test dose. As can be seen in the panel on the left (change scores), Group U4 responded only with tachycardia while Group U0 responded with the usual biphasic response. Both panels illustrate a greater difference between the two dose groups just after infusion with a convergence towards the end of the post-infusion period. These observations are supported by an Acquisition Dose x Sample Periods interaction ($F\{23,276\} = 7.49$). Analyses of change scores in Sample Periods 1, 5, 12 and 24 revealed effects of Acquisition Dose in Sample Periods 1 and 5 ($F\{1,12\} = 16.17$ and 5.98, respectively) but not in

Figure 22. The heart-rate response to morphine is graphed as a function of acquisition group (Group R0 and R4) and 5-min post-infusion sample periods. The panel on the left shows the change from baseline, and the panel on the right shows the actual heart rate. These data are collapsed across test dose.

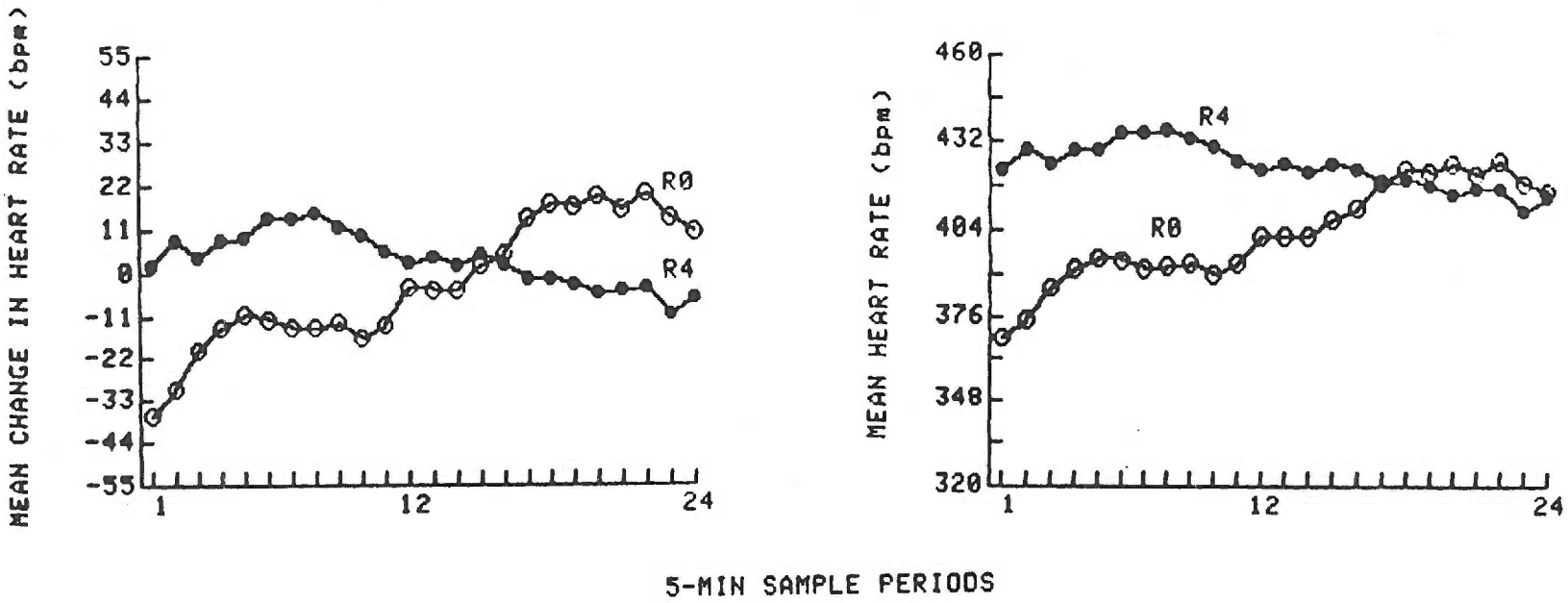
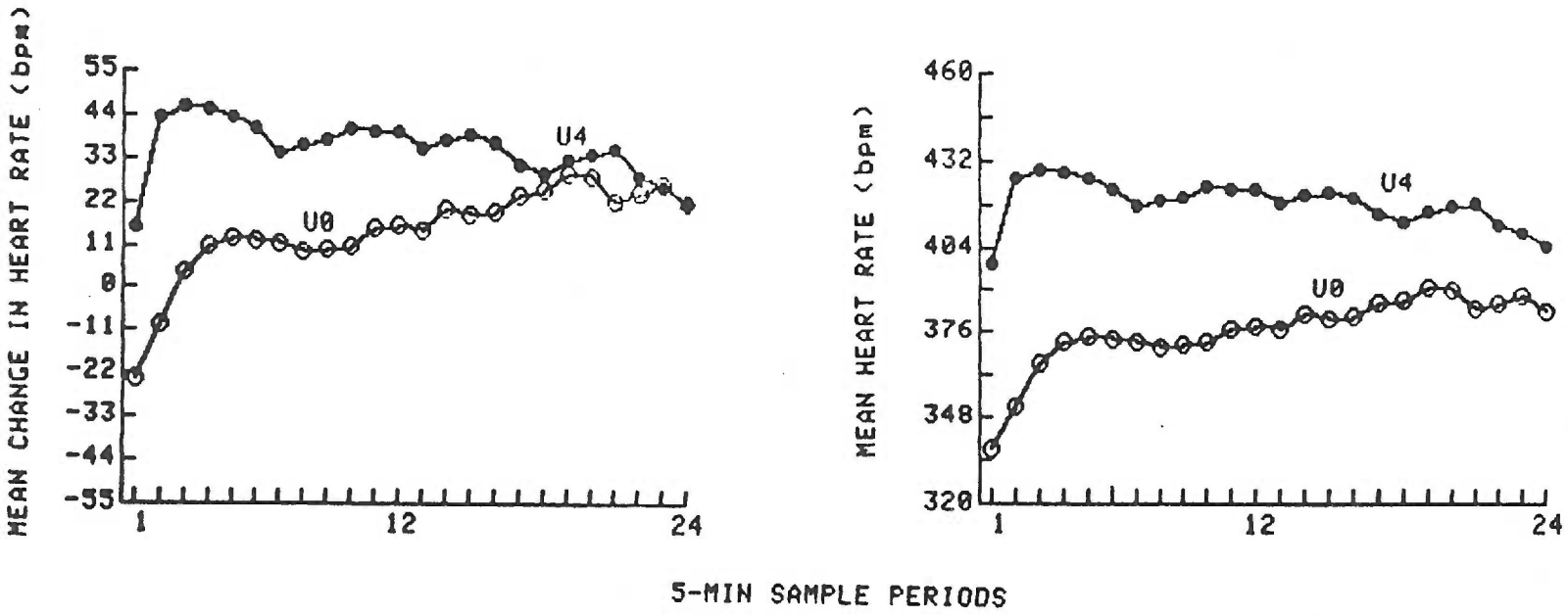

MEAN CHANGE IN HEART RATE (bpm)
55
44
33
22
11
0
-11
-22
-33
-44
-55
1
12
24
R0
R4
MEAN HEART RATE (bpm)
460
432
404
376
348
320
1
12
24
R4
R0
5-MIN SAMPLE PERIODS

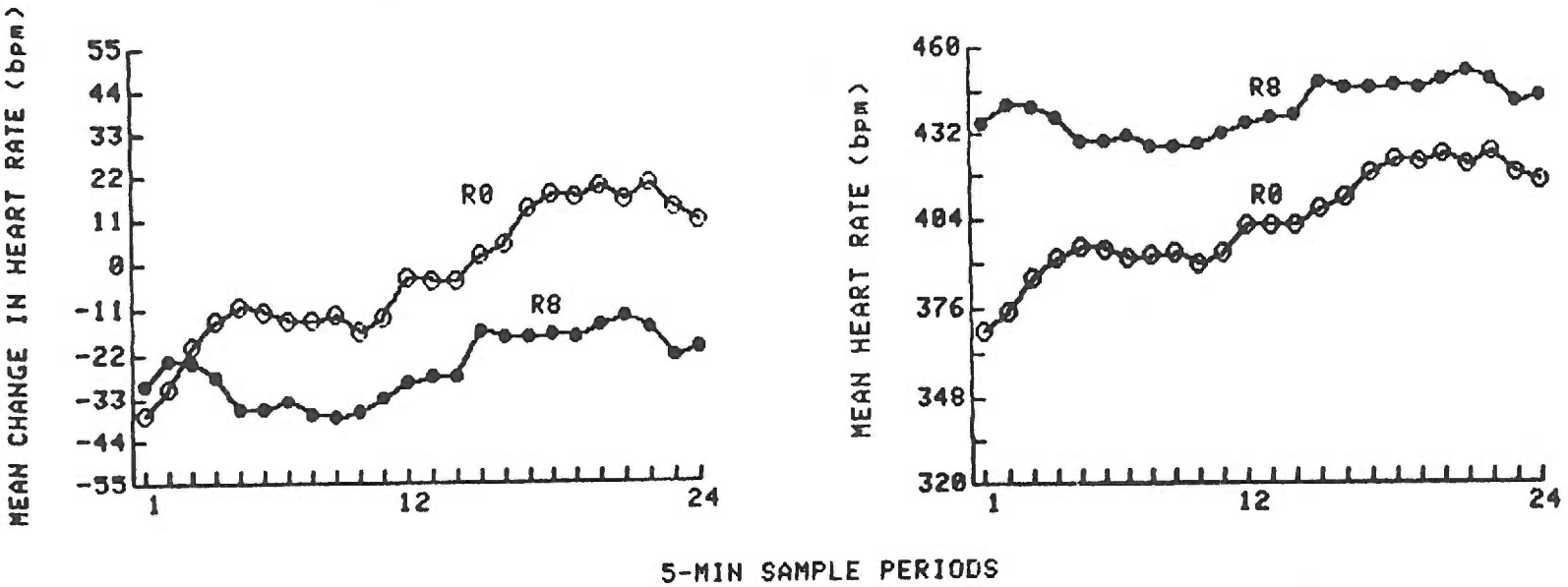
Figure 23. The heart-rate response to morphine is graphed as a function of acquisition group (Group U0 and U4) and time after infusion. The panel on the left shows the change from baseline, and the panel on the right shows the actual heart rate. These data are collapsed across test dose.


MEAN CHANGE IN HEART RATE (bpm)
55
44
33
22
11
0
-11
-22
-33
-44
-55
1
12
24
U4
U0
MEAN HEART RATE (bpm)
460
432
404
376
348
320
1
12
24
U4
U0
5-MIN SAMPLE PERIODS

Sample Periods 12 or 24. However, analyses of heart rate in the same sample periods revealed effects of Acquisition Dose on Sample Periods 1, 5 and 12 ($F\{1,12\}$ = 22.5, 34.66 and 25.55, respectively) but not in Sample Period 24. These results indicate that tolerance developed to the bradycardic effects of morphine in Group U4. Furthermore, these results imply that sensitization occurred to the tachycardic effects. The occurrence of sensitization is supported by both analyses in Sample Period 5 as well as the heart-rate analysis in Sample Period 12. Sensitization is demonstrated by the fact that the magnitude of tachycardia was greater in Group U4 (see Figure 18 panel G to compare Group U4's response to saline (open circles) with its response to the morphine test doses). Also, the peak tachycardia occurred sooner, i.e., latency for the maximum increase in heart rate to occur was less in Group U4 relative to Group U0.

Figure 24 shows the heart-rate responses of Groups R0 and R8 as a function of sample periods collapsed across test dose. As can be seen in the left-hand panel, the magnitude of initial bradycardia in each group was similar. The heart rate in Group R8 remained below baseline throughout the post-injection period, while Group R0 showed tachycardia by the end of the session. The two responses diverge over the post-infusion period. The panel on the right indicates a somewhat different story. The heart rate of Group R8 remained above that of Group R0 throughout the whole post-infusion period. The heart rates of the two groups, in contrast to the change scores, converge near the end of the post-infusion period. These observations are supported by an Acquisition Dose x Sample Periods interaction ($F\{23,230\}$ = 1.86 for both types of analyses). Analyses of individual sample periods revealed a

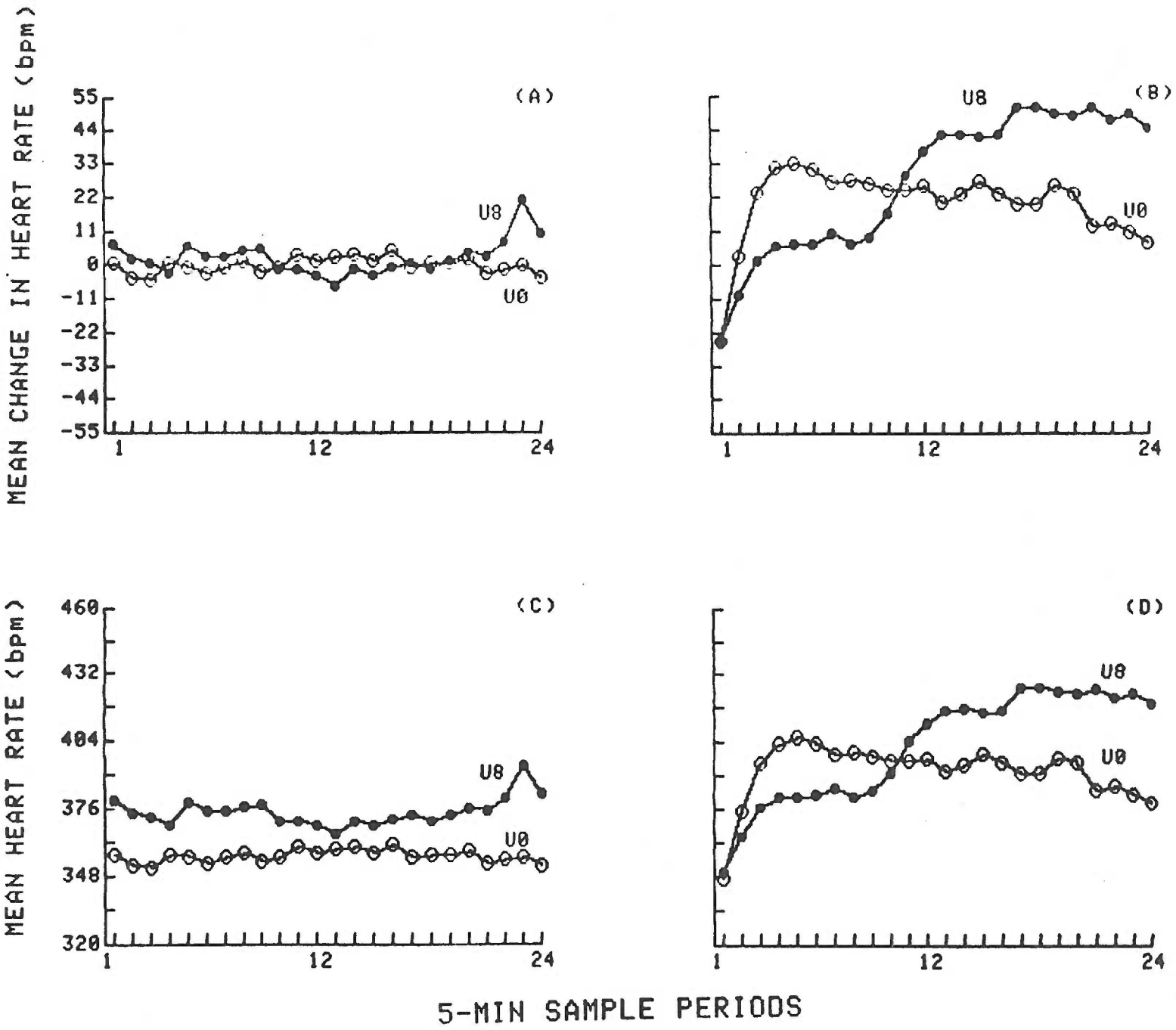
Figure 24. The heart-rate response to morphine is graphed as a function of acquisition group (Group R0 and R8) and 5-min post-infusion sample periods. The panel on the left shows the change from baseline, and the panel on the right shows the heart rate. These data are collapsed across test dose.


MEAN CHANGE IN HEART RATE (bpm)
55
44
33
22
11
0
-11
-22
-33
-44
-55
1
12
24
R0
R8
MEAN HEART RATE (bpm)
460
432
404
376
348
320
1
12
24
R8
R0
5-MIN SAMPLE PERIODS

significant effect of Acquisition Dose at Sample Period 1 ($F\{1,10\}$ = 13.85) but not at Sample Periods 5, 12 or 24 of the heart-rate response. The analyses of change scores in individual sample periods revealed no effect of acquisition dose at any sample periods analyzed. The source of the Acquisition Dose x Sample Periods interaction in the change-score analysis is illustrated in Figure 24 (left-hand panel) by the increasing divergence of the responses of Groups R0 and R8 over the post-infusion period. This figure suggests sensitization to the bradycardic effect and tolerance to tachycardic effect of morphine in Group R8. However, the Acquisition Dose x Sample Periods interaction revealed by the heart-rate analysis indicates tolerance to the bradycardic effect in Group R8, but no sensitization or tolerance to the tachycardic effect.

Figure 25 shows the heart-rate response in Group U0 versus U8 to saline (panels A and C) and to morphine, collapsed across the 2, 4 and 8 mg/kg test doses (panels B and D). The top two panels depict the response as change from baseline, while the bottom two panels graph the post-infusion heart rates. Both the change-score analysis and the heart-rate analysis comparing Group U0 with U8 revealed an Acquisition Dose x Test Dose x Sample Periods interaction ($F\{69,828\}$ = 1.84 for each analysis). As can be seen in Panels A and C, the response to Test Dose 0 is similar in both groups, and there is not much change over the two hours. A follow-up analysis of Test Dose 0 revealed no significant effects of Acquisition Dose or Sample Periods in either the change-score or heart-rate analysis. Therefore, follow-up analyses of change scores and heart rates were performed in which the three morphine test doses (2, 4 and 8 mg/kg) were combined without saline to compare the responses of Groups U0 and U8.

Figure 25. The heart-rate response of Groups U0 and U8 are graphed as a function of time after infusion. Panels A and C depict the response to the saline test dose. Panels B and D depict the response collapsed across the morphine test doses (2, 4 and 8 mg/kg). The top two panels show the response in terms of change from baseline. The bottom two panels show the post-infusion heart rate.


MEAN CHANGE IN HEART RATE (bpm)
(A)
55
44
33
22
11
0
-11
-22
-33
-44
-55
U8
U0
1
12
24
(B)
U8
U0
1
12
24
MEAN HEART RATE (bpm)
(C)
460
432
404
376
348
320
U8
U0
1
12
24
(D)
U8
U0
1
12
24
5-MIN SAMPLE PERIODS

Panels B and D in figure 25 show that there is little difference between Group U0 and U8 in initial bradycardia (see Sample Period 1), but the difference increases over the post-infusion period, with the peak tachycardia occurring in Group U0 before that in Group U8. However, the magnitude of tachycardia is greater in Group U8 relative to Group U0. Analyses of responses to the morphine test doses revealed an Acquisition Dose x Sample Periods interaction ($F\{23,276\} = 6.30$ for both analyses). Analyses of heart rate in Sample Periods 1, 5, 12 and 24 revealed significant effects of Acquisition Dose in Sample Period 24 ($F\{1,12\} = 10.79$) but not in Sample Periods 1, 5 or 12 suggesting sensitization to the tachycardic effect of morphine in Group U8. Analyses of change scores in individual sample periods revealed no effect of acquisition dose at any sample period analyzed. The source of the Acquisition Dose x Sample Periods interaction in the change-score analysis is probably due to the fact that the peak tachycardia occurred sooner in Group U0 followed by a return toward baseline, while heart rate in Group U8 continued to increase over the whole post-infusion period (see panel B, Figure 25). Therefore, the change-score analysis, like the heart-rate analysis, suggests sensitization to the tachycardic effect of morphine in Group U8 and no tolerance or sensitization to the bradycardic effect.

Summary: Post-infusion

Overall, the heart-rate response varied in all acquisition groups as a function of test dose. Specifically, there was very little variation in heart rate after receiving the saline test dose, while the morphine test doses elicited biphasic responses. The biphasic response was generally more visible in the U groups relative to the R groups

because the U groups usually demonstrated a greater degree of tachycardia.

The results of the heart-rate analyses and those of change scores introduced some discrepancies in determining whether tolerance to bradycardia and sensitization to tachycardia occurred. These results, along with the results from the Acquisition phase, are summarized in Table 5. The change-score analyses of the R groups revealed no change in morphine's effects in Group R2, and tolerance to the bradycardic effect in Group R4. In contrast, Group R8 showed sensitization to the bradycardic effect and tolerance to the tachycardic effect. The change-score analyses of the U groups revealed no change in morphine's effects in Group U2. Group U4 showed both tolerance to the bradycardic effect and sensitization to the tachycardic effect, and Group U8 showed only sensitization to morphine's tachycardic effect.

The results of the heart-rate analyses revealed that Group R2 developed no change in morphine's effects, while Groups R4 and R8 both showed tolerance to the bradycardic effect of morphine. Groups R4 and R8 did not show any change in morphine's tachycardic effect. Groups U2 and U4 demonstrated both tolerance to the bradycardic effect and sensitization to the tachycardic effect, while Group U8 showed only sensitization to the tachycardic effect.

## DISCUSSION

This project was concerned primarily with the development of tolerance to the cardiovascular effects of various doses of morphine in the rat. The effect of restraint-stress on the development of tolerance was also examined. Generally speaking, tolerance was manifest as a

Table 5. A summary of the results of the Acquisition and Tolerance Test phases are shown for each morphine acquisition group. The results of the Acquisition phase are in the two columns on the left, while the results from the two types of analyses performed on Tolerance Test data are in the four columns on the right. A line (---) indicates neither tolerance nor sensitization. Tolerance (tol) and sensitization (sens) are indicated under bradycardia (↓HR) or tachycardia (↑HR).

| | ACQUISITION | | TOLERANCE TEST | | | |
|---|---|---|---|---|---|---|
| | CHANGE SCORE | | CHANGE SCORE | | HEART RATE | |
| | ↓HR | ↑HR | ↓HR | ↑HR | ↓HR | ↑HR |
| Group R2 | tol | --- | --- | --- | --- | --- |
| R4 | tol | --- | tol | --- | tol | --- |
| R8 | --- | --- | sens | tol | tol | --- |
| U2 | tol | --- | --- | --- | tol | sens |
| U4 | tol | --- | tol | sens | tol | sens |
| U8 | tol | tol | --- | sens | --- | sens |

decrease in the magnitude of bradycardia, and sensitization was manifest as an increase in the magnitude of tachycardia and/or a decrease in the latency for the peak tachycardia to occur. Based on the results from the Tolerance Test Phase, restraint affected the change in response after repeated exposure to morphine, in that, the R groups (except R2) showed only tolerance to the bradycardic effect and no change in the tachycardic effect, whereas, the U groups showed both tolerance (except U8) and sensitization. The effect of dose on tolerance to the bradycardic effect varied as a function of restraint; i.e., in the R groups, the two high dose groups, Groups R4 and R8, showed tolerance to the bradycardic effect, while Group R2 did not. In the U groups, the two lower dose groups, Group U2 and U4, showed tolerance to the bradycardic effect and Group U8 did not.

Overall, each dose of morphine produced a biphasic heart-rate response characterized as an initial decrease in heart rate followed by an increase relative to baseline. During the Acquisition Phase, all morphine dose groups showed attenuation of the magnitude of the initial bradycardia, especially between Blocks 1 and 2 (see Figure 13). In general, the magnitude of the bradycardia did not change appreciably during the remainder of the acquisition phase. Group U8 showed tolerance over blocks to the tachycardic effect; however, no appreciable change over blocks occurred in the tachycardic portion of the response for any other group throughout acquisition. Therefore, based on the Acquisition Phase data, it appears that tolerance developed in all groups except Group R8 to the initial bradycardia, and only Group U8 showed tolerance to the tachycardia. These results contrast with those from the Tolerance Test Phase in which all groups except Groups R2 and

U8 showed tolerance to the bradycardia and Groups U2, U4 and U8 showed sensitization to the tachycardia. The Tolerance Test Phase was designed to assess tolerance/sensitization by comparing the response of drug-experienced subjects with drug-naive subjects that had received the same amount of handling. The implication of the discrepancies between the acquisition and tolerance test results is that repeated handling in the 0 mg/kg dose (saline) groups attenuated their response to morphine even though the Tolerance Test Phase provided their first drug exposure; no difference in bradycardia was apparent between Groups R0 and R2 or between Groups U0 and U8. Carder (1978) obtained similar results in his study in which rats repeatedly exposed to shock showed the same magnitude temperature response to THC as rats given repeated exposure to THC without shock. Since repeated handling appears to affect the response to drugs, conclusions about tolerance/sensitization are based on the data from the Tolerance Test Phase.

The data from the Tolerance Test Phase were separately analyzed in terms of heart rate and change scores. The results of the two analyses revealed discrepancies in concluding whether tolerance or sensitization developed to morphine's effects. For example, the results of the change-score analysis suggested sensitization to the bradycardic effect and tolerance to the tachycardic effect in Group R8, while the heart-rate analysis suggested tolerance to the bradycardic effect and no change in the tachycardic effect. In Group U2, the change-score analysis revealed no change in morphine's effects, while the heart-rate analysis revealed tolerance to the bradycardic effect and sensitization to the tachycardic effect (see four columns on the right in Table 5). The increasing baseline in Group R8 during the acquisition phase may be

related to the magnitude of bradycardia caused by morphine. For example, Galizio and Eisman (1979) have formulated a response constancy theory which asserts that a stimulus places a constant demand on a system, and thereby produces a response which reflects the level of activity in the autonomic nervous system which is necessary to compensate for the demand. Mathematically, there is a negative correlation between prestimulus levels and change scores. In Group R8, for example, the higher the baseline heart rate, the greater the negative change from baseline, and hence, the less likely it is for tolerance to be revealed.

Because differences between acquisition dose groups in baseline heart rate appeared after several exposures to morphine, it is possible that the elevated baseline heart rate in the higher dose groups was a conditioned response, opposite in direction to the initial bradycardic effect of morphine. By using change from baseline to determine the presence or absence of tolerance or sensitization, the heart rate just prior to infusion becomes zero regardless of the occurrence of a conditioned heart-rate response. In other words, when calculating the change scores, it is possible that what is defined as the baseline period is actually the end of the CS-US interval. The results of the heart-rate analysis are thought to be a more accurate description of the presence or absence of tolerance or sensitization because they include both the effects of conditioning as well as the drug-induced effects.

During the pre-infusion periods of the Acquisition Phase, the mean heart-rate of the two high dose groups (4 and 8 mg/kg) was significantly higher than that of the low dose groups (0 and 2 mg/kg) on Blocks 5 and 6 (see Figure 9). This finding suggests that there was some effect of

previous days' exposure to morphine which could be due to anticipation of the current day's treatment. Anticipation of treatment is possible because each animal was tested at the same time of day and was exposed to the same cues everyday; therefore, an animal could learn to expect drug (cf. Siegel, 1975). Siegel has shown that a hyperalgesic conditioned response can be observed in morphine-tolerant subjects when drug administration cues are followed by a placebo. In other words, Siegel's compensatory response can occur without the presence of drug itself as long as the drug administration cues are present. If this is the case, then the conditioned response in this experiment was an increase in heart rate just before drug administration. One qualification that must be made is that Siegel used a discrimination paradigm in which all subjects were given morphine in one environment and saline (placebo) in a different environment. Therefore, all subjects received the same exposure to morphine. On test days, some of the subjects were given placebo in the drug-paired environment and the others were given placebo in the placebo-paired environment. The group given placebo in the drug-paired environment exhibited the hyperalgesic conditioned response; this is an example of an associative drug effect. In the present study, the saline control group allows for conclusions to be made about the effect of repeated exposure to morphine, but does not allow for a clear-cut distinction to be made between associative and non-associative drug effects.

A residual drug effect is another possibility for the increase in mean baseline heart rate in the two high-dose groups, and could be due to changes in the autonomic nervous system following chronic treatment with morphine. For example, following daily treatment with a 10 mg/kg

dose of morphine, induction of both tyrosine hydroxylase and dopamine β-hydroxylase leading to an increase in adrenal medullary levels of catecholamines have been reported in rats (Anderson & Slotkin, 1975, 1976). Whether there was any change in uptake or release of catecholamines, however, was not clear. There is also evidence of increasing levels of plasma catecholamines with chronic exposure to a 5 mg/kg dose of morphine (Mansfield et al., 1981).

Another type of residual drug effect involves a change in the pharmacokinetics of morphine. In rats receiving either a single exposure or daily exposures for eight days to a 30 mg/kg dose of morphine, Vetulani, Melzacka, Adamus and Danek (1983) showed that eight exposures to a high dose of morphine slows the elimination of morphine such that significant concentrations of morphine are present in plasma and brain 24 hrs after the last dose. However, the high doses in the present study (4 and 8 mg/kg) are probably not high enough to elicit any changes in the pharmacokinetics of morphine.

The major finding in this study was that tolerance developed to the bradycardic portion of heart rate response, and that changes in the tachycardic portion involved sensitization. This finding is consistent with the suggestion made by Seevers and Deneau (1963) that tolerance develops only to the depressant effects and not to the stimulant effects of morphine. This finding is also consistent with those involving development of tolerance to the biphasic temperature effects and locomotor effects of morphine. Low doses of morphine (10 mg/kg and less) have been shown to induce hyperthermia, while higher doses elicit a biphasic temperature response: hypothermia followed by hyperthermia. Following repeated exposure to morphine, the hypothermia disappears and

only hyperthermia occurs (Gunne, 1960; Stewart & Eikelboom, 1981). An increase in the hyperthermic response, i.e., sensitization, to morphine after several days' exposure has also been reported (Gunne, 1960; Mansfield et al., 1981). Morphine also elicits a biphasic locomotor activity response: a decrease followed by an increase in activity (Babbini & Davis, 1972; Schnur, 1985a, 1985b; Schnur, Bravo & Trujillo, 1983; Vasko & Domino, 1978). Tolerance develops only to the hypoactive effects, while the hyperactive effects remain or even increase in magnitude (Schnur, 1985b). In all of the studies on the temperature, locomotor activity and heart-rate, the "excitatory" effects of morphine have, in some cases, increased in magnitude and in all cases have have occurred with decreasing latency, i.e., have occurred in place of the depressing effects of morphine. Each of these effects has been shown to be blocked by naloxone or naltrexone indicating that both the depressant and excitatory effects are opiate mediated.

Schnur (1985a) has developed a dual process hypothesis which states that morphine's biphasic locomotor activity response is the behavioral result of two underlying processes, one inhibitory and the other excitatory. In Schnur's studies, pretreatment with naltrexone blocked the morphine-elicited hypoactivity, and produced hyperactivity. Subsequent treatment with naltrexone, after the hypoactivity occurred, blocked the hyperactivity and even elicited hypoactivity. Schnur proposes that the two underlying processes are evoked simultaneously by morphine. Immediately following morphine administration, the inhibitory process predominates over the excitatory process and hypoactivity is the behavioral manifestation. Later, the excitatory process predominates over the inhibitory process causing hyperactivity to be the behavioral

response. Schnur's results suggest that only the predominant process is blocked by naltrexone. His explanation of the results involves an assumption that naltrexone uncovers a complementary process that is otherwise obscured, and that it is the predominant process and not the complementary process that is selectively antagonized by naltrexone.

With repeated drug exposure, Schnur (1985a) proposes that three possibilities exist about what happens to the inhibitory and excitatory processes. One possibility is that the inhibitory process decreases (i.e., tolerance) but the excitatory process remains unchanged. The second possibility is that the excitatory process increases (i.e., sensitization), but the inhibitory process remains unchanged. Both of these possibilities would lead to a decrease in hypoactivity and an increase in hyperactivity. The third possibility is that there is a change in both processes with chronic exposure to morphine.

In the present study, repeated exposures to morphine revealed a decrease in the degree of bradycardia with either no change or an increase in the degree of tachycardia. The latency of the tachycardia appeared to decrease as the bradycardia decreased (see Figure 13). The present results, while similar to Schnur's (1985a), do not lend enough information to rule out any of Schnur's possibilities about what happens to each process with repeated exposure to morphine.

Another behavioral theory to which these data apply is Solomon's opponent-process theory of motivation (Solomon & Corbit, 1974). This theory states that for a given affective, hedonic or emotional state aroused by a stimulus, there is a central nervous system mechanism which serves to decrease the magnitude of the hedonic feelings, whether they are aversive or pleasant. Initially, a primary 'a' process (e.g.,

bradycardia) is aroused by a stimulus (morphine). Next, an opponent loop generates the secondary 'b' process (tachycardia) which is opposite to the 'a' state. The loop generating the 'b' process is activated whenever any stimulus elicits a sufficient 'a' state. The 'a' and 'b' processes do not occur simultaneously as proposed by Schnur (1985a); instead, the 'a' process alone is evoked initially so that there is some time lag before the opposing 'b' process is elicited. Also, unlike Schnur's proposal, the occurrence of the 'b' process is dependent upon the occurrence of the 'a' process. Once the 'b' process begins, the two processes summate, and because 'a' is larger than 'b', the direction of 'a' predominates. The 'b' process has a long latency, recruits slowly and dies out slowly so that upon termination of the primary 'a' process, the secondary 'b' process remains activated, thus, the direction of the reponse changes, i.e., becomes opposite of the initial direction. With repeated exposure to the stimulus, 'b' will show a shorter latency of response to 'a', a quicker rise, a higher asymptote, and a longer decay time. Therefore, the peak of 'a' will be less in magnitude and the steady level will be closer to baseline.

A major difference between Schnur's (1985a) dual process hypothesis and the opponent-process theory of Solomon and Corbit (1974) is whether the opposing processes occur simultaneously and independently. Schnur says, "yes;" Solomon and Corbit say, "no." In studies measuring the heart-rate response to acute morphine exposure, pretreatment with naltrexone blocked both the bradycardia and tachycardia, while treatment after the bradycardia occurred, blocked the tachycardia but did not elicit bradycardia (Schwarz et al., 1985). The results are not consistent with Schnur's blockade study in that no complementary process

was uncovered by naltrexone, i.e., blockade of bradycardia did not result in tachycardia and blockade of tachycardia did not elicit bradycardia. These data indicate the lack of two simultaneous, independent processes underlying the bradycardia and tachycardia and lend support to Solomon and Corbit's opponent-process theory.

Each theory addresses the issue of repeated exposure to the stimulus which, in this study and Schnur's studies, is morphine. Solomon and Corbit (1974) state that the opponent process, 'b', is strengthened by use and weakened by disuse, but the primary process, 'a', is not affected by use. This idea is similar to the second of the three possibilities that Schnur (1985) gives, that is, that the excitatory process (b) increases but the inhibitory process (a) remains unchanged. Whichever theory turns out to be correct, the end result of repeated exposure to a drug is the same: tolerance to the initial effect and sensitization to the secondary effect.

The present data are consistent with Solomon's opponent process theory in that with repeated exposure to morphine, the 'b' process became stronger and the latency to occur became shorter. In Dose Groups 4 and 8 (both R and U), the 'b' process became stronger and began to occur before morphine infusion, that is, the 'b' process may have become conditioned to the environmental cues surrounding drug administration. This conditioning was seen as an increase in baseline heart rate just prior to drug administration.

Seaman (1985) has demonstrated that the higher the dose of morphine that rats receive repeatedly, the greater the amount of tolerance that develops to the analgesic effects. On the basis of this, the R groups were initially expected to show greater tolerance than the U groups

because the R groups might have had a higher dose of opioid in their system due to activation of the endogenous opioid system by stress. The results from the high dose group support this idea, in that tolerance developed in Group R8 but not in Group U8. The effect of dose on tolerance to the bradycardic effect varied as a function of restraint, i.e., Groups R8 and R4 showed tolerance, but Group R2 did not. However, in the U groups, Group U2 and U4 developed tolerance, but Group U8 did not. The results from the U groups conflict with evidence about the dose-relationship involved in tolerance. It is possible that the dose range studied was too narrow to see a clear dose-effect on tolerance in the U groups, and that activation of the endogenous opioid system by stress enlarged the dose-range enough to reveal dose-effects in the R groups. A wider range of doses (e.g., 0, 2, 4, 8 and 16 mg/kg) may reveal clearer effects of dose on the magnitude of tolerance in freely-moving rats.

There are several possibilities for why the U groups showed both tolerance (except Group U8) and sensitization while the R groups (excluding Group R2) showed only tolerance to the bradycardia. First, since the R groups do not exhibit a robust tachycardia after acute treatment with morphine, there may be a "ceiling" effect, i.e., heart rate can only increase a certain amount which is not great enough to produce a measurable change even after chronic morphine treatment. Second, the number of subjects in Groups R4 and R8 was only five, versus seven or eight in the U groups. This small 'n' led to an increase in the amount of variability and, therefore, to an increased possibility of a Type II error. The third possibility is that not enough morphine treatments were given to see sensitization to the tachycardic effects of

morphine in the R groups. At this point, the most likely is the second possibility, i.e., the effect of small group size. More data need to be collected on whether sensitization (or tolerance) can occur to the tachycardic effects of morphine in restraint-stressed rats, and if so, how many exposures to morphine are required for sensitization to occur.

Speculation about the mechanisms underlying each portion of the biphasic response to morphine involves either a cellular theory about the opioid receptors or a physiological theory about the autonomic nervous system. The cellular theory involves a two-receptor model that has been proposed to explain the thermic effects of morphine. Adler and Geller (1985) have proposed that the kappa receptor subtype mediates the hypothermic response to morphine, while the mu receptor mediates the hyperthermic response. Furthermore, Adler and Geller have proposed that the mu effects are centrally-mediated, and the kappa effects are peripherally-mediated because dynorphin$_{17}$, a kappa agonist which cannot cross the blood-brain barrier, does not affect body temperature when administered intracerebroventricularly. However, when dynorphin$_{17}$ is administered peripherally, a hypothermic response is revealed.

A two-receptor model for the cardiovascular effects of opiates has also been proposed. Laurent and Schmitt (1983) showed that intracisternal administration into anesthetized rats of either ethylketocyclazocine (EKC) or dynorphin$_{1-13}$, both kappa receptor agonists, caused a significant and long-lasting (> 45 min) decrease in heart rate. In contrast, intracisternal administration of β-endorphin or fentanyl, both mu receptor agonists, produced a significant increase in heart rate which was maximum after 30 min. These results suggest that the decrease in heart rate is caused by activation of the central

kappa receptors, and activation of mu receptors induce tachycardia. There is also evidence that activation of peripheral kappa receptors induces bradycardia. Gautret and Schmitt (1985) showed that dynorphin$_{1-13}$, administered i.v., caused a decrease in heart rate which lasted 15 min. Bilateral vagotomy attenuated the response but did not abolish it, indicating a centrally-mediated effect. In pithed rats, dynorphin$_{1-13}$, administered i.v., caused a long-lasting decrease in heart rate indicating a peripherally-mediated effect. Gautret and Schmitt concluded that the dynorphin$_{1-13}$-induced decrease in heart rate has two components: stimulation of central kappa receptors which creates an increase in parasympathic tone and a decrease in sympathetic outflow, and stimulation of peripheral kappa receptors located on the atria of the heart. If two different opioid receptor subtypes are responsible for the biphasic responses to morphine, then one implication of the present findings is that cross-tolerance between the mu- and kappa receptor subtypes does not occur. Schulz, Wuster, Rubini and Herz (1981) have reported the development of tolerance to both mu and kappa agonists. However, no cross-tolerance between the two types of receptor agonists occurred in the guinea pig ileum.

In addition to the two-receptor theory, there is also a physiological theory about the biphasic effect of morphine. For example, it has been well established that the decrease in heart rate caused by morphine is, at least in part, due to an increase in parasympathetic tone with a corresponding decrease in sympathetic outflow (Holaday, 1983). Evidence is accumulating that suggests that it is possible that the tachycardic effects are due to an increase in sympathetic outflow. For example, Feldberg and Wei (1978) showed that

morphine injected into the lateral ventricle of cats produced tachycardia presumably by stimulation of opioid receptors in and around the areas of the brain responsible for sympathetic control of the heart. Also Mansfield et al. (1981) showed that repeated administration of a 5 mg/kg dose of morphine enhanced both hyperthermia and catecholamine-releasing effects of morphine. On the basis of this, one might argue that the excitatory action of morphine on heart rate is indirect, i.e., morphine elicits an increase in plasma levels of catecholamines which cause an increase in heart rate.

Conway, Brown and Dollery (1984) conducted a study in which morphine, [D-Ala$^2$-D-Leu$^5$]enkephalinamide (DADLE), a delta receptor agonist and U50,488H, a kappa receptor agonist were used to characterize the receptor that mediates the increase in plasma catecholamines. The drugs were administered intracerebroventricularly into conscious rats. Morphine (35 nmol) produced an immediate and long-lasting increase in epinephrine and a gradual increase in norepinephrine. DADLE (35 nmol) produced a significant increase in plasma levels of epinephrine which returned to pre-injection levels within 60 min; no change in levels of norepinephrine occurred. U50,488H (39 nmol) caused an immediate, short-lasting rise in epinephrine levels which returned to pre-injection levels within 15 min. No significant change in norepinephrine levels occurred following injection of U50,488H. Dose-response studies using each agonist, revealed the rank-order of potency as DADLE $\cong$ morphine $>$ U50,488H. Because morphine and DADLE act somewhat selectively on the mu and delta receptors, respectively, Conway et al. concluded that the increase in plasma catecholamines was clearly not mediated through a kappa receptor. However, since morphine and DADLE were almost equally

potent, it was not clear if the receptor mediating the increase in catecholamines was mu or delta. The results of the study by Conway et al. begin to tie together the cellular and physiological theories about the biphasic effect of morphine. Kappa-receptor agonists known to produce a decrease in heart rate have very little effect on levels of plasma catecholamines, while mu-receptor agonists, known to cause an increase in heart rate also cause significant increases in both epinephrine and norepinephrine. It is possible that, in the present study, morphine's initial bradycardic effect was mediated by activation of peripheral and central kappa receptors causing an increase in parasympathetic tone followed a mu- (or delta-?) receptor-mediated increase in plasma catecholamines, thus causing an increase in heart rate. After repeated morphine exposure, tolerance developed in the kappa receptor leading to an attenuation of the bradycardia, while the catecholamine-releasing effects were enhanced, leading to sensitization to the tachycardic portion of the response. It is known that tolerance develops in mu receptors (cf. Schulz et al., 1981), but how tolerance would lead to an enhanced catecholamine-releasing response is not understood.

One problem with the study by Conway et al. (1984) was that morphine is not a highly-selective mu agonist, so it is difficult to differentiate the actions of the various receptor subtypes. Further studies examining the heart-rate effects of very specific opioid receptor subtype agonists and antagonists are required to analyze the two-receptor mechanism. Studies of tolerance to specific receptor subtype agonists should also be performed. To understand the underlying physiological mechanisms, a specific $alpha_1$ adrenergic receptor

antagonist, such as phentolamine, could be used to study the input of sympathetic nervous system in the tachycardic portion of the response. Ideally, these proposed studies would use conscious subjects to eliminate the confounding effects of anesthesia.

# REFERENCES

Adams, W.J., Yeh, S.Y., Woods, L.A. & Mitchell, C.L. (1969). Drug-test interaction as a factor in the development of tolerance to the analgesic effect of morphine. Journal of Pharmacology and Experimental Therapeutics, 168, 251-257.

Adler, M.W. & Geller, E.B. (1985). Opioid receptors and body temperature in rats. Society for Neuroscience Abstracts, 11, Part 2, 1071.

Akil, H., Madden, J. IV, Patrick, R.L. & Barchas, J.D. (1976). Stress-induced increase in endogenous opiate peptides: Concurrent analgesia and its partial reversal by naloxone. In H.W. Kosterlitz (Ed.), Opiates and Endogenous Opioid Peptides (pp. 63-70). Amsterdam: Elsevier/North-Holland.

Amir, S., Brown, Z.W. & Amit, Z. (1979). The role of endorphins in stress: Evidence and speculations. Neuroscience and Biobehavioral Reviews, 4, 77-86.

Anderson, D.C., Plant, C. & Paden, P. (1967). Conditioned suppression of a running response as related to competing responses, drive, and basal skin resistance level. Journal of Comparative and Physiological Psychology, 63, 282-287.

Anderson, T.R. & Slotkin, T.A. (1975). Effects of morphine on the rat adrenal medulla. Biochemical Pharmacology, 24, 671-679.

Anderson, T.R. & Slotkin, T.A. (1976). The role of neural input in the effects of morphine on the rat adrenal medulla. Biochemical Pharmacology, 25, 1071-1074.

Atweh, S.F. & Kuhar, M.J. (1977a). Autoradiographic localization of opiate receptors in rat brain.I. Spinal cord and lower medulla. Brain Research, 124, 53-67.

Atweh, S.F. & Kuhar, M.J. (1977b). Autoradiographic localization of opiate receptors in rat brain.II. The brain stem. Brain Research, 129, 1-12.

Babbini, M. & Davis, W.M. (1972). Time-dose relationships for locomotor activity effects of morphine after acute or repeated treatment. British Journal of Pharmacology, 46, 312-224.

Bodnar, R.J., Kelly, D.D., Spiaggia, A. & Glusman, M. (1978). Stress-induced analgesia: Adaptation following chronic cold water swims. Bulletin of the Psychonomic Society, 11, 337-340.

Bodnar, R.J., Kelly, D.D., Spiaggia, A., Pavlides, C. & Glusman, M. (1978). Stress-induced analgesia: Effects of naloxone following cold water swims. Bulletin of the Psychonomic Society, 12,

125-128.

Berne, R.M. & Levy, M.N. (1981). Cardiovascular Physiology (4th ed., pp.145-181). St. Louis: C.V. Mosby Co.

Byrd, L.D. (1983). Cardiovascular effect of naloxone, naltrexone and morphine in the squirrel monkey. Life Sciences, 32, 391-398.

Carder, B. (1978). Environmental influences on marihuana tolerance. In N.A. Krasnegor (Ed.), Behavioral Tolerance: Research and Treatment Implications. NIDA Research Monograph, 18, 90-102.

Chang, K-J. & Cuatrecasas, P. (1980). Heterogeneity and properties of opiate receptors. Federation Proceedings, 40, 2729-2734.

Conway, E.L., Brown, M.J. & Dollery, C.T. (1984). Studies on the pharmacology of central opioid-induced increases in plasma catecholamines in conscious rats. Neuropharmacology, 23, 1291-1296.

Dews, P.B. (1978). Behavioral tolerance. In N.A. Krasnegor (Ed.), Behavioral Tolerance: Research and Treatment Implications. NIDA Research Monograph, 18, 18-26.

Dohner, V.A. (1972). Motives for drug use: Adult and adolescent. Psychosomatics, 13, 317-324.

Feldberg, W. & Wei, E. (1977). The central origin and mechanism of cardiovascular effects of morphine as revealed by naloxone. Journal of Physiology, 272, 99-100P.

Feldberg, W. & Wei, E. (1978). Central sites at which morphine acts when producing cardiovascular effects. Journal of Physiology, 275, 57P.

Feldberg, W. & Wei, E. (1981). Cardiovascular effects of morphine and opioid peptides in anesthetized cats. In J.P. Buckley & C.M. Ferrario (Eds.), Central Nervous System Mechanisms in Hypertension (pp. 229-233). New York: Raven Press.

Fennessey, M.R. (1969). The behavioral, cardiovascular and respiratory actions of morphine-N-oxide in the dog. European Journal of Pharmacology, 8, 261-268.

Fennessey, M.R. & Ortiz, A. (1968). The behavioral and cardiovascular actions of intravenously administered morphine in the conscious dog. European Journal of Pharmacology, 3, 177-185.

Fennessy, M.R. & Rattray, J.F. (1971). Cardiovascular effects of intravenous morphine in the anaesthetized rat. European Journal of Pharmacology, 14, 1-8.

Galizio, C.N. & Eisman, E. (1979). Cardiac conditioning in the rat: US intensity function and response constancy theory.

Psychopharmacology, 16, 537-545.

Gautret, B. & Schmitt, H. (1985). Central and peripheral sites for cardiovascular actions of dynorphin-(1-13) in rats. European Journal of Pharmacology, 111, 263-266.

Gebhart, G.F., Sherman, A.D. & Mitchell,C.L. (1972). The influence of stress on tolerance development to morphine in rats tested on the hot plate. Archives Internationales de Pharmacodynamie et de Therapie, 197, 328-337.

Guillemin, R. (1978). Peptides in the brain: The new endocrinology of the neuron. Science, 202, 390-402.

Gunne, L-M. (1960). The temperature response in rats during acute and chronic morphine administration a study of morphine tolerance. Archives Internationales de Pharmacodynamie, 79, 416-428.

Holaday, J.W. (1983). Cardiovascular effects of endogenous opiate systems. Annual Review of Pharmacology and Toxicology, 23, 541-94.

Jaffe, J.H. (1980). Drug addiction and drug abuse. In A.G. Gilman, L.S. Goodman & A. Goodman (Eds.), The Pharmacological Basis of Therapeutics (pp. 535-584). New York: Macmillan.

Keim, K.L. & Sigg, E.B. (1976). Physiological and biochemical concomitants of restraint stress in rats. Pharmacology Biochemistry and Behavior, 4, 289-297.

Kvetnansky, R., Sun, C.L., Lake, C.R., Thoa, N., Torda, T. & Kopin, I.J. (1978). Effect of handling and forced immobilization on rat plasma levels of epinephrine, norepinephrine, and dopamine-β-hydroxylase. Endocrinology, 103, 1868-1874.

Laurent, S. & Schmitt, H. (1983). Central cardiovascular effects of k agonists by dynorphin-(1-13) and ethylketocyclazocine in the anesthetized rat. European Journal of Pharmacology, 96, 165-169.

Linton, M. & Gallo, P.S. (1975). The Practical Statistician: Simplified Handbook of Statistics. Monterey, CA: Brooks/Cole Publishing Co.

Madden, J. IV, Akil, H., Patrick, R.L. & Barchas, J.D. (1977). Stress-induced parallel changes in central opioid levels and pain responsiveness in the rat. Nature, 265, 358-360.

Mansfield, J.G., Wenger, J.R., Benedict, R.S., Halter, J.B. & Woods, S.C. (1981). Sensitization to the hyperthermic and catecholamine-releasing effects of morphine. Life Sciences, 29, 1697-1704.

Moss, J. & Roscow, C.E. (1983). Histamine release by narcotics and muscle relaxants in humans. Anesthesiology, 59, 330-339.

Peris, J. (1984). Associative and nonassociative mechanisms in the development of tolerance to the thermic and cardiovascular effects of ethanol . Unpublished doctoral dissertation, Oregon Health Sciences University, Portland.

Peris, J. & Cunningham, C.L. (1985). Handling-induced enhancement of alcohol's acute physiological effects. Life Sciences, 38, 273-279.

Pilcher, C.W.T. & Browne, J.L. (1983). Effects of naloxone and Mr1452 on stress-induced changes in nociception of different stimuli in rats. Life Sciences, 33, 697-700.

Presman, D. & Schotz, S. (1943). A critical analysis of the use of intravenous morphine. Anesthesiology, 4, 53-66.

Ross, D.H. (1977). Calcium content and binding in synaptosomal subfractions during chronic morphine treatment. Neurochemical Research, 2, 581-593.

Schnur, P. (1985a). Effects of naloxone and naltrexone on morphine-elicited changes in hamster locomotor activity. Physiological Psychology, 13, 26-32.

Schnur, P. (1985b). Morphine tolerance and sensitization in the hamster. Pharmacology Biochemistry and Behavior, 22, 157-158.

Schnur, P., Bravo, F. & Trujillo, M. (1983). Tolerance and sensitization to the biphasic effects of low doses of morphine in the hamster. Pharmacology Biochemistry and Behavior, 19, 435-439.

Schulz, R., Wüster, M., Rubini, P. & Herz, A. (1981). Functional opiate receptors in the guinea-pig ileum: Their differentiation by means of selective tolerance development. The Journal of Pharmacology and Experimental Therapeutics, 219, 547-550.

Schwarz, K.S., Peris, J. & Cunningham, C.L. (1985). The effects of stress and naltrexone on the heart-rate response to morphine. Society for Neuroscience Abstracts, 11, Part 2, 1198.

Schwen, R.J. & Jones, L.C. (1984). Handling stress affects body temperature. The Pharmacologist, 26, 140.

Seaman, S.F. (1985). Growth of morphine tolerance: The effect of dose and interval between doses. In F.R. Brush & J.B. Overmier (Eds.), Affect, Conditioning and Cognition: Essays on the Determinants of Behavior (pp. 249-262). Hillsdale, NJ:Lawrence Erlbaum Associates, Inc.

Seevers, M.H. & Deneau, G.A. (1963). Physiological aspects of tolerance and physical dependence. In W.S. Root & F.G. Hofmann (Eds.), Physiological Pharmacology (pp. 565-641). New York: Academic Press.

Siegel, S. (1975). Evidence from rats that tolerance is a learned

response. Journal of Comparative and Physiological Psychology, 89, 498-506.

Sharma, S.K., Klee, W.A. & Nirenberg, M. (1975). Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. Proceedings of the National Academy of Science, 72, 3092-3096.

Sherman, J.E., Strub, H. & Lewis, J.W. (1984). Morphine analgesia: Enhancement by shock-associated cues. Behavioral Neuroscience, 98, 293-309.

Shimizu, H. (1978). Reliable and precise identification of R-waves in the EKG with a simple peak detector. Psychophysiology, 15, 499-501.

Sitsen, J.M.A., van Ree, J.M. & de Jong, W. (1982). Cardiovascular and respiratory effects of β-endorphin in anesthetized and conscious rats. Journal of Cardiovascular Pharmacology, 4, 883-888.

Solomon, R.L. & Corbit, J.D. (1974). An opponent-process theory of motivation: I. Temporal dynamics of affect. Psychological Review, 81, 119-145.

Stein, E.A. (1976). Morphine effects on the cardiovascular system of awake, freely behaving rats. Archives Internationales de Pharmacodynamie et de Therapie, 223, 54-63.

Stewart, J. & Eikelboom, R. (1981). Interaction between the effects of stress and morphine on body temperature in rats. Life Sciences, 28, 1041-1045.

Sydbom, A. & Terenius, L. (1985). The histamine-releasing effect of dynorphin and other peptides posessing the Arg-Pro sequences. Agents and Actions, 16, 269-272.

Vasko, M.R. & Domino, E.F. (1978). Tolerance development to the biphasic effects of morphine on locomotor activity and brain acetylcholine in the rat. Journal of Pharmacology and Experimental Therapeutics, 207, 848-858.

Vetulani, J. Melzacka, M., Adamus, A. & Danek, L. (1983). Changes in morphine pharmacokinetics in nervous and peripheral tissues following different schedules of administration. Archives Internationales de Pharmacodynamie, 265, 180-191.

Weeks, J.R. (1972). Long-term intravenous infusion. In R.D. Myers (Ed.), Methods in Psychobiology (Vol. 2, pp. 155-168). London: Academic Press.

# Appendix A: Cannula Construction

The jugular vein cannula was constructed using a hot air stream to first flare the end of 160-mm piece of PE 20 (polyethylene) tubing (.38 mm i.d. x 1.09 mm o.d.). A stainless steel wire attached to a 22 ga blunt needle tip was inserted into the lumen of the PE 20. The wire was slid through the PE 20 until the flared end met with the needle tip. Two pieces of heat shrink tubing, 17 mm (3/64 in. diameter) and 10 mm (1/16 in. diameter) long were placed over the flared end of the PE 20 and the 22 ga needle hub and shrunk with the hot air stream. This created an end for a blunt 22 ga needle tip to fit onto the cannula through which drug was infused. When the heat shrink tubing was cool, the needle tip and wire were removed and a longer piece of wire was slid through the lumen of the PE 20. Three knobs were then formed 25 mm, 35 mm and 45 mm from the heat shrink end of the tubing. Two more knobs were formed, one at the center of the PE 20 and one knob 10 mm from the other end. When these knobs cooled, the wire was removed and the PE 20 was set aside.

The intravascular portion of the catheter was made of a 37-mm piece of Dow Corning Medical Grade silastic tubing (.51 mm i.d. x .94 mm o.d.). The end to be inserted into the vein was cut at a $45^{\circ}$ bevel. A piece of 9 ga wire was then slid through the lumen of the silastic tubing and the silastic tubing was slid over 5 mm of PE 10, and the PE 10 was quickly melted to the silastic. A 5-mm piece of heat shrink tubing (3/64 in. diameter) was centered over the end of the silastic and shrunk tightly over the silastic-PE10 junction. When this junction had cooled, the 9 ga music wire was inserted into both the PE 20 and the PE

10 so that the plain end of the PE 20 was melted to the loose end of the PE 10 forming a knob approximately 4 mm from the knob on the PE 20.

The catheter was checked for leaks by infusing air through the catheter while it was submerged in water. When it had been established that there were no leaks, the PE 10 portion of the catheter was wrapped twice around a glass rod so that the joints of the PE 10 to the silastic and PE 20 were parallel, and then dipped into boiling water forming a loop.

The PE 20 portion was then bent in a U-shape towards the silastic end and dipped into hot water. The tip of the catheter where the three knobs are, was bent in a U-shape in the opposite direction and dipped in hot water. The loop and the bends in the PE tubing allow for greater flexibility and ease of insertion of the catheter. To the external end of the catheter, a modified 21 ga disposable hypodermic needle was attached so that the catheter could easily be attached to the drug infusion apparatus.

## Appendix B: Analysis of Body Weights

The mean body weights of each dose group are plotted in Figure 26 as a function of restraint and days during the experiment. In general, restraint plus a relatively high dose of morphine (4 or 8 mg/kg) led to a decrease in body weight over sessions. The highest dose alone led to a decrease in body weight in Group U8. An ANOVA was performed on these data using Restraint and Acquisition Dose as between-group variables and Days as a within-group variable. The ANOVA revealed a significant Restraint x Days interaction ($F\{18,792\} = 6.72$) and a Dose x Days interaction ($F\{54,792\} = 5.82$) as well as a main effect of Days ($F\{18,792\} = 5.16$).

Figure 27 graphs body weight in the R and U groups as a function of days collapsed across acquisition dose. At the beginning of the experiment, there was little or no difference between restraint conditions. However, the restraint condition led to a decrease in body weight (-22 g) while the freely-moving condition did not (+ 0.3 g). Follow-up analyses of selected days revealed a significant effect of Restraint on Days 14 and 19 ($F\{1,44\} = 5.32$ and 7.52, respectively) but not on Days 1 or 8.

Figure 28 shows the mean body weights of each acquisition dose group, collapsed across restraint condition. As can be seen in this figure, the body weight of Dose Group 0 increased over days, while that of Dose Group 2 remained relatively stable over the entire experiment. The body weights in Dose Groups 4 and 8 steadily decreased over days, with the decrease in Dose Group 8 being the largest. This observation is supported by the Dose x Days interaction described above. Follow-up

Figure 26. Mean body weight of the restrained (R) and unrestrained (U) groups are plotted as a function of days during the experiment. Each panel plots the body weight of each acquisition dose. HAB refers to the three habituation days; TT refers to the four tolerance test days.

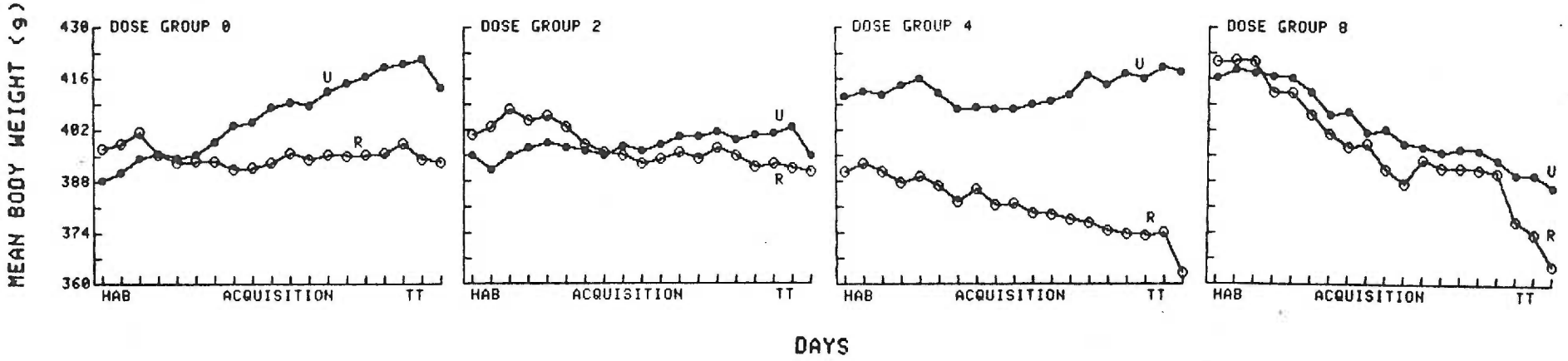

MEAN BODY WEIGHT (g)
430
416
402
388
374
360
DOSE GROUP 0
DOSE GROUP 2
DOSE GROUP 4
DOSE GROUP 8
U
R
HAB
ACQUISITION
TT
DAYS

Figure 27. Mean body weight is plotted for both restrained (R) and unrestrained (U) groups over days during the experiment, collapsed across acquisition dose. HAB refers to the three habituation days; TT refers to the four tolerance test days.

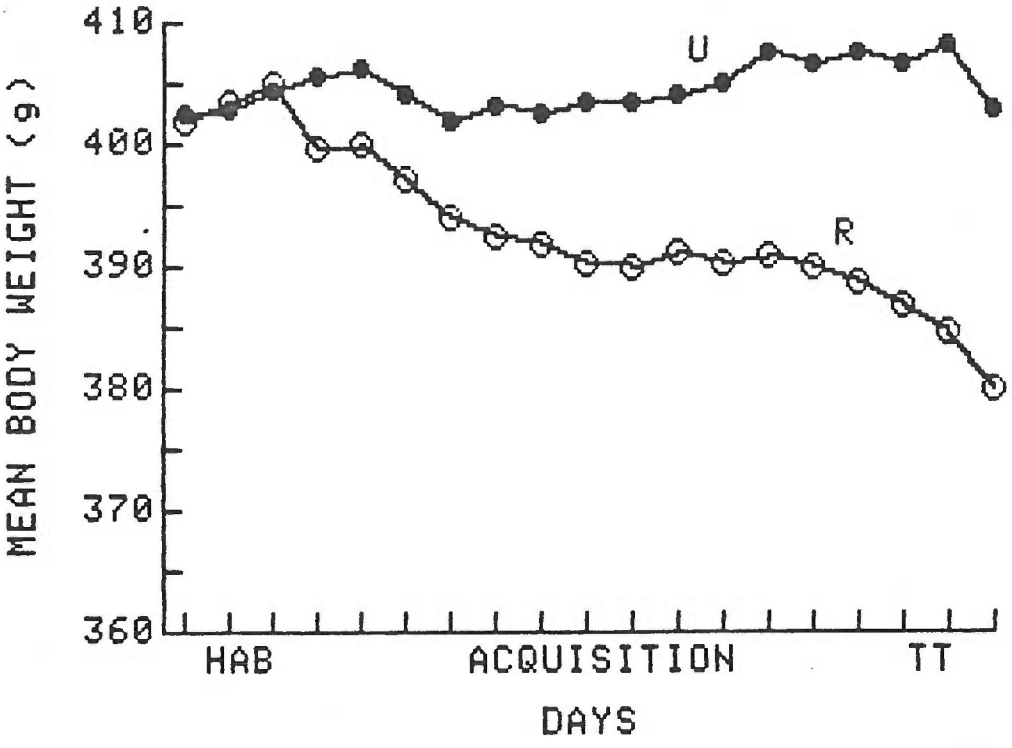
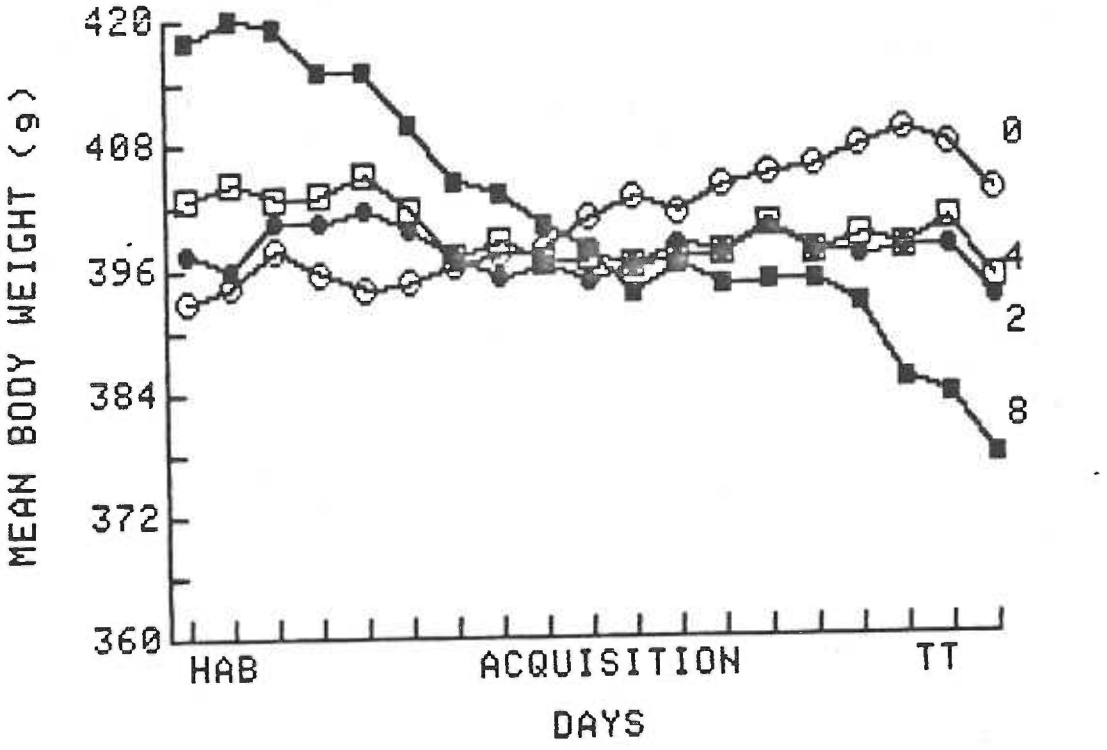

410
400
390
380
370
360
MEAN BODY WEIGHT (g)
U
R
HAB
ACQUISITION
TT
DAYS

Figure 28. Mean body weight for each acquisition dose group is plotted as a function of days during the experiment, collapsed across restraint condition. The numbers at the right of the graph refer to the acquisition dose group. HAB refers to the three habituation days; TT refers to the four tolerance test days.


MEAN BODY WEIGHT (g)
420
408
396
384
372
360
HAB
ACQUISITION
TT
DAYS
0
4
2
8

analyses of each dose group revealed a significant effect of Days in Dose Groups 0, 4 and 8 ($F\{18,216\} = 3.57$ for Dose 0, and $Fs\{18,180\} = 1.83$ and 9.24 for Doses 4 and 8, respectively) but not in Dose Group 2.