INTRACEREBROVENTRICULAR INFUSION OF BACTRACIN INDUCES ANALGESIA AND INCREASES BRAIN β-ENDORPHIN: RELATIONSHIP TO ACUTE STRESS

BY

GURLEEN SINGH SIKAND

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DEDICATED TO

DR. VIKTOR HAVLICEK

(1929 - 1981)

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* * * *

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ABSTRACT

Protease inhibitors are widely used to protect against degradation of peptides and hormones in *in vitro* studies. Previous studies have shown that protease inhibitor bacitracin potentiated the effect of enkephalins and decreased biodegradation of β-endorphin in *in vitro* studies by inhibiting the activity of brain carboxypeptidase(s) and aminopeptidase(s). In this study, the *in vivo* effect of bacitracin on brain peptides (i.e. β-endorphin and somatostatin) was examined. Male Sprague-Dawley rats were injected with 1.5 units of bacitracin intracerebroventricularly and sacrificed by microwave irradiation at 6.5, 9.5, 15, 22, and 30 minutes postinfusion. β-endorphin content of whole brain in bacitracin injected rats was generally higher than controls, but only at 15 minutes postinfusion was the brain β-endorphin content significantly higher (by 27%) in bacitracin injected rats ($p < .05$). There was a tendency for β-endorphin levels to be higher in most brain areas examined but only in the striatum and amygdala were β-endorphin levels significantly higher. In the striatum there was a four-fold increase ($p < .05$) and in the amygdala there was a three-fold increase. These higher levels returned to control values at 30 minutes postinfusion. There were no significant differences in whole brain somatostatin content of bacitracin injected rats from controls. Bacitracin injected rats exhibited a strong analgesic response which was absent in controls. This analgesic response reached a plateau at 15 minutes postinfusion. At 30 minutes post-infusion the analgesic effect subsided. Injection of naloxone (1 mg/kg) significantly abolished bacitracin-induced analgesia. Exposure to

acute stressful stimuli (i.e. hot plate) led to a 46% decrease in brain β-endorphin content compared to control unstressed rats ($p < .01$). Similarly bacitracin injected stressed rats had lower brain β-endorphin levels (by 22%) than bacitracin injected nonstressed rats ($p < .05$). Thus bacitracin pretreatment considerably reduced the decrease (by 26%) in β-endorphin levels following stress ($p < .02$). In another group of experiments, the pre-bacitracin injection EEG record was compared with the EEG record during 5, 6 minute epochs after completion of bacitracin infusion during 30 minute postinfusion period. During the wakeful state bacitracin injected animals displayed a depressed desynchronized EEG until 18 minutes postinfusion. This depression in EEG recovered by 30 minutes postinfusion. Naloxone (1 mg/kg) abolished the occurrence of depressed and desynchronized EEG in bacitracin injected rats.

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INTRODUCTION AND LITERATURE REVIEW

The word pain is derived from the Latin word poena, meaning a penalty or punishment. In antiquity pain was viewed as something inflicted by the gods upon anyone who displeased them. However, scientific interest in understanding pain also has ancient roots. Since the initial concepts of a pain pathway by Descarte (1644) (Fig. 1) and the subsequent theories of von Frey and Goldscheider (1894), extensive research has gone into understanding mechanisms of pain. However, the basic question "what is pain and how does the body deal with pain?" remains difficult to answer. To a biologist pain is a protective mechanism which informs the individual of the presence of harmful stimuli. To a sociologist, pain or the threat of pain is an important tool of learning. The psychologist finds pain to be a subjective sensation which is modified by emotional states and conditioning influences of past experiences. Thus the difficulty in understanding pain and pain mechanisms in the body lies in the fact that the perception of pain involves a subtle blend of physiological and psychological factors. Many psychophysical studies which find a relationship between noxious stimuli strength and pain intensity (Sweet, 1959; Beecher, 1959; Hardy, 1952; Morgan, 1961) thus assume that pain is a primary sensation subserved by a direct communication system from the skin receptor to the pain center. However, a simple psychophysical relationship does not necessarily reflect equally simple neural mechanisms. This is especially true of mechanism(s) of pain. For most American soldiers wounded at the Anzio beachhead "entirely

Figure 1:

Descartes' concept of the pain pathway. He writes "If for example fire (A) comes near the foot (B), the minute particles of this fire, which as you know move with great velocity, have the power to set in motion the spot of the skin of the foot which they touch, and by this means pulling upon the delicate thread CC, which is attached to the spot of the skin, they open up at the same instant the pore, d.e., against which the delicate thread ends, just as by pulling at one end of a rope one makes to strike at the same instant a bell which hangs at the other end." (Melzack, et. al., 1968)

denied pain from their extensive wounds or had so little that they did not want any medication to relieve it, presumably because they were overjoyed at having escaped alive from the battlefield". Another, interesting yet common, example is cited by Livingston (1953). "A fisherman is sitting in one of a line of boats stretching from one sand spit to another at the mouth of a river. He suddenly feels a smashing strike, and as he lunges back to set the hook, a large salmon breaks out of the water, shaking the hook in its mouth. He realizes that his best chance for landing the salmon lies in getting ashore before his line runs out or becomes entangled with the lines of other fishermen in the neighboring boats. Fighting the salmon as he goes, he starts crossing from boat to boat to reach the spit. Once there, he runs far out on the beach and after a hard struggle lands his salmon. As he winds up his line, he looks down and sees the wet sand under his right shoe is reddening. Then he notices a long rent in his trousers and is surprised to discover a deep cut in his leg. By the time he has improvised a dressing for this wound he has found other injuries: skin scraped off three knucles, a friction burn on his right thumb and two massive bruises on his left thigh. He realizes that these injuries must have been sustained while he was crossing the line of boats. Yet he cannot recall having felt the slightest pain at the time."

A recent study by Levine et. al., (1978) showed that in a variety of painful conditions about one-third of the patients obtained significant relief from placebos.

It had been previously shown by Murray et. al., (1960) that a substance present in the posterior pituitary gland potentiated the analgesic activity of morphine-type agents. They could not determine

the mechanism of action however it was shown that the presence of a pituitary gland was required for potentiation to occur.

A. EVIDENCE FOR ENDOGENOUS ANALGESIC SYSTEM

The earliest evidence for the existence of an endogenous analgesic system came from the work of Reynolds (1969) who demonstrated that stimulation of certain brain-stem sites, in rat, produced profound analgesia. A site where electrical stimulation consistently produced analgesia was the ventrolateral periacqueductal gray. Since the initial reports, electrical stimulation produced analgesia (SPA) has been demonstrated in cat (Liebeskind, et. al., 1973), monkey (Goodman, et. al., 1975) and man (Adams, 1976; Akil, et. al., 1977; Hosobuchi, et. al., 1977; Richardson, et. al., 1973). The duration of analgesia produced by stimulation exceeds the period of stimulation in man (Adams, et. al., 1976; Hosobuchi, et. al., 1977), primates and rats (Mayer, et. al., 1974). In cat, however, SPA ceases with cessation of the electrical stimulation (Oliveras, et. al., 1974). The SPA shows tolerance with repeated exposures and this tolerance dissipates after a period of nonstimulation (Mayer, et. al., 1975). In addition there is also some evidence for crosstolerance with opiates. That is, analgesic brain stimulation loses its effectiveness in blocking pain if the animal is made tolerant to narcotics by daily systemic injections of morphine.

Analgesia, in animal studies, is usually inferred from the absence of reflexes or behaviours which are ususally elicited by exposure to noxious stimuli. The effect of focal stimulation of the brain is thus selective on those reflexes or behaviours; the animals manifesting analgesia become totally unresponsive to pain while responding normally to all other environmental stimuli. This selective action of electrical

stimulation was thought to demonstrate the presence of a separate physiological system for modulation of pain.

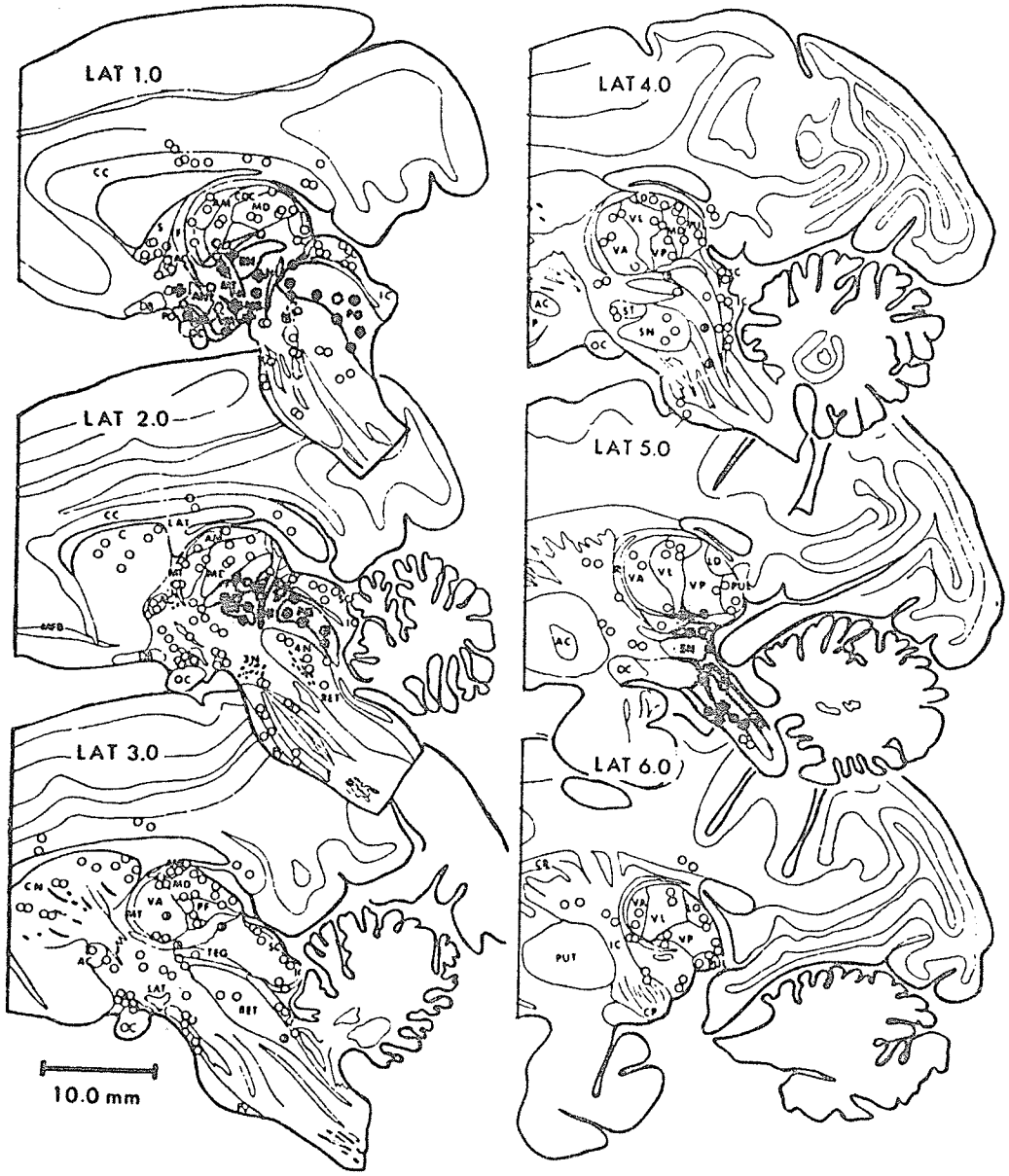
Parallel to studies demonstrating SPA were neuropharmacological studies which demonstrated microinjections of morphine in the periacqueductal gray also caused profound analgesia (Jacquet, et. al., 1976; Mayer, et. al., 1975). The primary site of morphine injection in inducing analgesia was shown to be the area located within the periventricular and periacqueductal structures in the brain-stem and diencephalon (Buxbaum, et. al., 1970; Jacquet, et. al., 1976). The action of both morphine and electrical stimulation in producing analgesia appeared to be concentrated in sites surrounding the third ventricle, cerebral acqueduct and the fourth ventricle.

Pert, et. al., (1974a,d) examined the site of morphine action with distribution of opiate receptors in the rhesus monkey. They found a positive correlation between effective analgesic sites and the presence of opiate receptors. The medial thalamus, hypothalamus, periventricular and periacqueductal gray matter were found to be most effective in producing analgesic action when injected with morphine (Fig. 2). These are also the sites which had been demonstrated to be rich in opiate receptors (Hiller, et. al., 1973; Kuhar, et. al., 1973). The amygdala, however, does not conform to this relationship between site of analgesia and presence of opiate receptors. The amygdala is an ineffective site for producing analgesia by morphine injection despite the fact that it is rich in opiate receptors. It is possible that opiate receptors in the amygdala mediate subtle psychological factors which are important in perception of pain.

Besides having similar sites of action, both electrically stimulated analgesia and analgesia produced by narcotic alkaloids have many

Figure 2:

Anatomical mapping of the analgesic sites of action of morphine in the primate brain at 6 sagittal levels from the midline in 1 mm increments (LAT 1.0 to LAT 6.0). ○ = no response following 40 μg injection; ◎ = sites that produced a threshold increase greater than 0.64 mA but less than 1.6; + = maximally active sites in which injections of the mapping dose resulted in a threshold shift of 1.6 mA or more. The active regions have been shaded in gray. AC = anterior commissure; AM = nucleus anterior medialis, ANT = anterior hypothalamic area; CC = corpus callosum; CDC = nucleus centralis densocellularis; CM = nucleus centrum medianum; CN = caudate nucleus; CP = cerebral peduncle; DB = diagonal band of Broca; F = fornix; GP = globus pallidus; HI = hippocampus; IC = internal capsule; LAT = lateral ventricle; LD = nucleus lateralis dorsalis; MB = mammillary body; MD = nucleus medialis dorsalis; MFB = medial forebrain bundle; MT = mammillo-thalamic tract; OC = optic chiasm; PF = nucleus parafascicularis; PG = periaqueductal gray matter; PO = preoptic area; PUL = pulvinar thalami; PUT = putamen; R = nucleus reticularis; RET = reticular formation; RN = nucleus reuniens; S = septum pellucidium; SC = superior colliculus; SN = substantia nigra; ST = subthalamic nucleus; TEG = tegmentum; VA = nucleus ventralis anterior; VL = nucleus ventralis lateralis; VM = ventromedial nucleus; VP = nucleus ventralis posterior.


LAT 1.0
LAT 2.0
LAT 3.0
LAT 4.0
LAT 5.0
LAT 6.0
10.0 mm

striking parallels. Both analgesia causing mechanisms are dependent on the integrity of the brain neurotransmitter system. SPA has been shown to be facilitated by serotonin and dopamine but antagonized by norepinephrine (Akil, et. al., 1972). Similarly morphine produced analgesia is affected by the brain neurotransmitters (Way, et. al., 1971). Further SPA, like morphine produced analgesia showed tolerance with repeated exposures, the tolerance dissipating after a period of nonstimulation. Addiction to morphine also reduced SPA considerably (Mayet, et. al., 1975). This suggested that morphine and electrical stimulation produced their analgesic effects by common mechanism(s).

An important observation was made by Rogers and coworkers (1978) who noted that pain threshold, in humans, is found to be lower in the afternoon than morning. Glynn and coworkers (1976) had noted earlier that patients with chronic pain reported a rise in pain sensation between 0800 and 2200 hrs. The role of opiate in this spontaneous diurnal rhythm was demonstrated by Frederickson, et. al., (1977) who showed that naloxone suppressed this circadian variation.

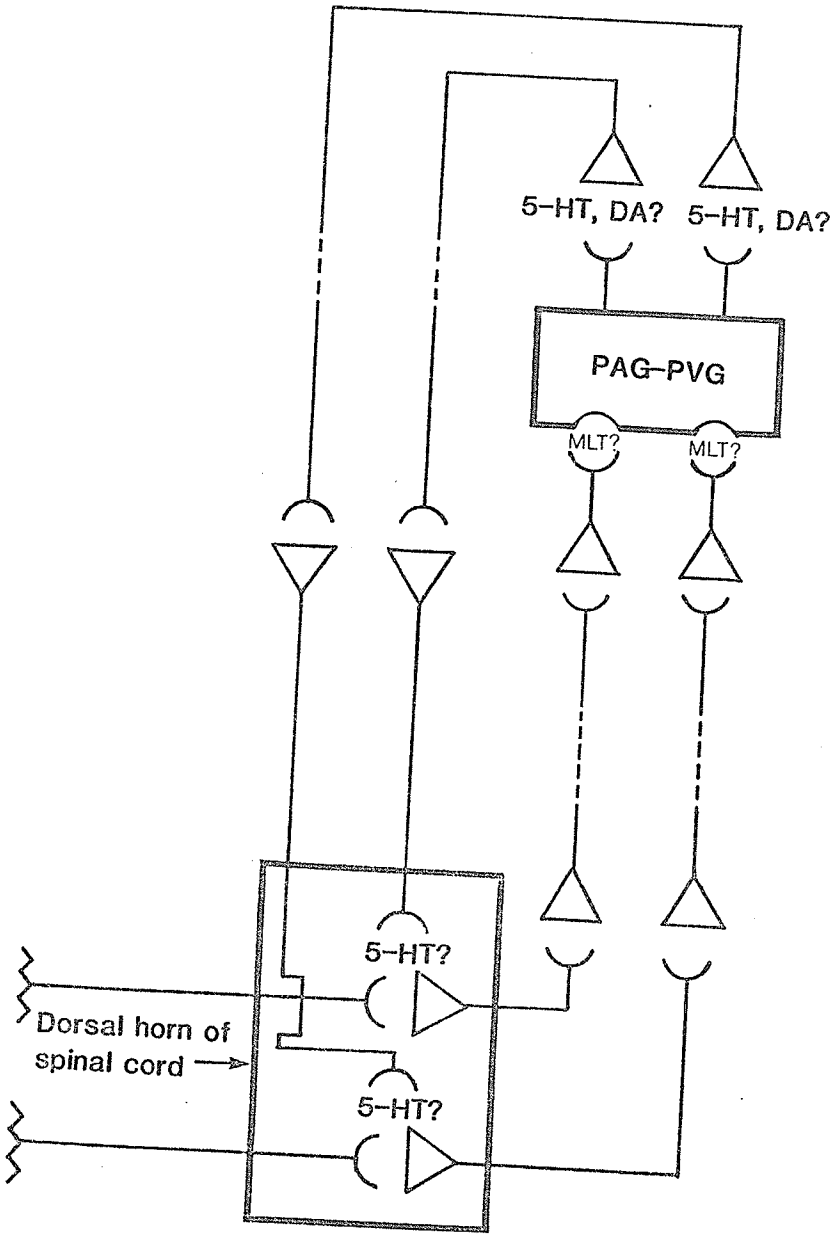
B. SCHEMATIC MODEL OF ELECTRICAL STIMULATION AND MORPHINE PRODUCED ANALGESIA

Based on the above observation, Mayer, et. al., (1974) suggested a schematic model of analgesia production by electrical stimulation and narcotic alkaloids (Fig. 3). His model was based on the assumption that the periventricular and periacqueductal gray areas contain opiate containing neurons. Electrical stimulation caused release of a "morphine-like" substance which would then bind to opiate receptors in the area thereby causing analgesia. Similarly morphine injected into this area would

Figure 3: Schematic Model of Pain Inhibition

Theoretical model of neural circuitry and neurohumoral substances involved in stimulation-produced analgesia.

Figure 3. Schematic Model of Pain Inhibition



5-HT -serotonin
DA -dopamine
MLT -morphine-like transmitter
PAG -periacqueductal gray matter
PVG -periventricular gray matter

(Mayer,et. al.,1974)

bind with opiate receptors and cause analgesia. This model, in light of recent findings, has proved strikingly accurate. It is now becoming increasingly evident that opiods are the substance released by neurons in the periventricular and periacqueductal gray areas.

C. ROLE OF ENDORPHINS IN ENDOGENOUS ANALGESIC MECHANISM

The identification and isolation of endogenous opiod peptides and the subsequent demonstration by Akil, et. al., (1976) that naloxone partially blocked analgesia caused by electrical stimulation of periacqueductal grey area, gave new insight into analgesic mechanisms that exist in the mammalian brain. Since then many studies have demonstrated the ability of opiods to produce analgesia (Table 1). Both in vitro and in vivo studies have shown that of the known opiod lipotropic fragements, β-endorphin possesses the highest opiate agonist activity (Graf, et. al., 1976a). It is about 20 times more potent than morphine when injected intracerebroventricularly (ICV) in rat (Szekely, et. al., 1977) and about 90 times more potent than morphine when injected ICV in cat (Meglio, et. al., 1977). Not only does β-endorphin possess the greatest opiate activity, the duration of its opiate action is also the longest (Graf, et. al., 1976b). It is generally believed that in vivo the analgesic potency of the opiod peptides is a function of the length of the peptide due to the higher stability of the larger peptides to degradation (Graf, et. al., 1976b). δ-endorphin, injected ICV produces a much more pronounced and prolonged analgesic effect than met-enkephalin. However, in vitro on ileum, it is less potent than met-enkephalin (Graf, et. al., 1976b). This suggests that the longer chain of the peptide perhaps played a role in protecting the active site (the N-terminal) against proteolytic

Table 1. Analgesic Effect of Opiates After Administration in Various Species

Species	Injection Method	Test Used	β-Endorphin	δ-Endorphin	γ-Endorphin	α-Endorphin	M-Endorphin	
Rat	ICV	Tail Flick	+		+	+	+	Graf, et. al., 1976
Rat	ICV	Tail Flick	+					Szekely, et. al., 1977
Rat	ICV	Tail Flick	+					Bradbury, et. al., 1977
Rat	ICV	Tail Flick	+					Bloom, et. al., 1976
Rat	ICV	Tail Flick					+	Belluzzi, et. al., 1976
Rat	ICV	Hot-Plate	+					Bradbury, et. al., 1977
Rat	ICV	Hot-Plate					+	Leybin, et. al., 1976
Mouse	ICV	Tail Flick	+		+		+	Loh, et. al., 1976
Mouse	IV	Tail Flick	+					Tseng, et. al., 1976
Mouse	ICV	Hot-Plate	+				+	Loh, et. al., 1976
Cat	ICV	Tail Pinch	+					Feldberg, et. al., 1976
Cat	ICV	Tail Pinch	+					Meglio, et. al., 1977
Cat	ICV	Tail Pinch	+				+	Smith, et. al., 1976
Man	ICV	Thermal Dolorimeter	+					Hosobuchi, et. al., 1978

+ Analgesia Observed

degradation by enzyme in the brain. The C-terminal of the ACTH molecule has been previously suggested to function similarly in protection of the molecule from enzymatic degradation (Bennet, et. al., 1974). It is also possible that the higher analgesic potency of longer opioid peptides is due to the presence of additional receptor binding site(s).

β-Endorphin administered ICV, in humans, also causes a pronounced analgesic state which is naloxone reversible (Hosobuchi, et. al., 1978). Rossier and coworkers (1979) in an important study investigated β-endorphin levels following stimulation of the periacqueductal gray for pain relief in humans. They found a significant increase in immunoreactive β-endorphin in ventricular fluid following electrical stimulation of periacqueductal gray. An innovative approach to study the role of endorphins in pain relief was taken by Tamset and coworkers (1980). They investigated the relationship between the amount of the analgesic drug Pethidine that patients would require for pain relief and the levels of endorphins in CSF. They found an inverse relationship between the mean level of Pethidine in plasma and endorphins in cerebrospinal fluid: patients with lower endorphin levels in the CSF required more Pethidine to obtain pain relief. Almay, et. al., (1978) investigated endorphin levels in CSF of patients with chronic pain. Endorphin levels in cerebrospinal fluid of patients with chronic pain were significantly lower than healthy volunteers. Thus if one was to assume that endorphin levels in cerebrospinal fluid reflect the activity of the endorphinergic system, it would seem that patients with chronic pain tend to have inadequately functioning endorphmergic systems or that the "non-physiological" stimulation exhausted the endorphinergic system of endorphin content.

A positive correlation has been demonstrated between depressive symptoms and cerebrospinal endorphin levels (Almay, et. al., 1978). People with psychiatric disorders had higher cerebrospinal endorphin levels than healthy controls. This finding taken together with the report of Knorring, et. al., (1974) that patients with depressive disorders are relatively insensitive to pain again suggests the active role of endorphin in pain relief.

It has been previously shown that D-amino acids can cause naloxone reversible analgesia in man and mice (Ehrenpreis, et. al., 1978). Cheng, et. al., (1979) studied three strains of mice; CXBX, a strain which is low in opiate receptors and thus exhibits poor morphine and electro-acupuncture analgesia, Ob/Ob, a strain which has abnormally high levels of pituitary endorphins and $B6AF_1/J$, a strain which exhibit "normal" naloxone reversible analgesia. They obtained the following rank order of analgesia: Ob/Ob>$B6AF_1$/J>CXBX which demonstrates a correlation between genetic differences in endorphinergic systems and analgesia.

D. ENDORPHINS AND ACUPUNCTURE ANALGESIA

The Chinese for some 3000 years have used acupuncture, the implantation of needles into subcutaneous connective tissue and muscles at numerous points on the surface of the body, for a variety of illnesses and malfunctions. However, its key purpose was to relieve pain. During the past twenty years, acupuncture has been successfully used to achieve analgesia during surgery. However, despite its wide-spread use, little was known about the mechanism of action of acupuncture action. It was difficult to understand how needles inserted into the body and rotated or electrically stimulated could cause analgesia at a distant portion of the body.

The discovery of endogenous opioids gave some insight into mechanism of acupuncture analgesia. Pomeranz, (1968) demonstrated that acupuncture in cats, after twenty-to-thirty minutes, raised the threshold for pain while affecting no other modalities. Similar effects were seen in mice (Pomeranz, et. al., 1976). The effects of acupuncture in both cat and mice were found to be completely naloxone reversible. Hypophysectomy led to abolition of acupuncture analgesia. Acupuncture similarly was demonstrated to cause analgesia in humans (Mayer, et. al., 1977). Sjolund, et. al., (1977) reported that following acupuncture of the lower limb, there is an increase in endorphin levels in the cerebro-spinal fluid. A recent study by Gang, et. al., (1980) gives evidence for the involvement of enkephalins in acupuncture analgesia. They found a significant increase in met-enkephalin and leu-enkephalin in the hypo-thalamus and striatum following acupuncture induced analgesia in rabbit and rat. Intraventricular injection of Bacitracin, attentuated acupuncture induced analgesia with parallel increases in enkephalin content in the brain. Naloxone at a dose of 2 mg/kg caused significant reversal of acupuncture analgesia.

Thus, these studies taken together emphasize the importance of opioids in acupuncture induced analgesia. Beside reversal of acupuncture analgesia by naloxone, the slow onset of this analgesia and the sub-sequent recovery from it are consistent with a mechanism dependent on release of a chemical substance.

E. POSSIBLE MECHANISM OF ENDORPHIN MEDIATED ANALGESIA

In the rat brain, immunoreactive β-endorphin cell bodies are present in the basal tuberal hypothalamus. These cell bodies project fibers to the anterior periacqueductal gray matter (Bloom, et. al., 1978). Thus

it seems reasonable to suggest that electrical stimulation of the periacqueductal gray matter might induce antidromic stimulation of the β-endorphin fibers thereby leading to release of β-endorphin into the third ventricle. Despite the presence of enkephalinergic fibers in the periacqueductal gray, enkephalins only produce a transient analgesia when injected ICV. Thus, it seems less likely to be the source of the profound long-lasting analgesia observed after electrical stimulation. The relationship between endorphins and opiate receptor sites effective in producing analgesia is shown in Table 2.

In animals, analgesia is usually inferred from absence of reflexes or behaviour usually elicited by noxious stimulation. The two most common parameters used to measure analgesia are tail flick and hind paw withdrawal and/or jumping, reflexes that are spinally mediated. When applied locally to analgesia producing sites in the brain, the endorphin induced analgesia was revealed to be mediated by descending pathways from the ventromedial medulla to the spinal cord via the dorsolateral funiculus (DLF) (Basbaum, et. al., 1978). These fibers terminate on the dorsal horn of the spinal cord, an area which contains neurons which are maximally sensitive to noxious stimuli. This same area contains large amounts of enkephalinergic neurons (Elde, et. al., 1976; Glazier, et. al., 1979; Hokfelt, et. al., 1977; Sar, et. al., 1978). The presence of opioids and opiate receptors in both the periacqueductal gray and superficial layers of the spinal cord raised the question of where exogenous opiates, such as morphine, act to cause analgesia. It was shown that naloxone injected into the midbrain reversed the analgesia coupled by systemically injected morphine (Tsou, et. al., 1964; Vigouret, et. al., 1973). It was further demonstrated by Basbaum,

Table 2. Relationship between endorphins, opiate receptor and analgesia producing sites

Anatomical Site	β-endorphin fibers[a]	enkephalin cells	enkephalin fibers	SPA	Opiate receptor	Opiate microinjection Analgesia
Periventricular diencephlon	+	+	+	+	+	+
Periaqueductal gray	+	+	++	+	+	+
N. Raphe Magnus	o	+	+r	+	o	+
N. Paragiganto-cellularis	o	+	+r	+	o	+
Dorsal horn	o	+	++	+	++	+

\+ present
++ large amounts
o not demonstrated
? unknown
+r present in rat, not demonstrated in cat

[a] β-endorphin perikarya have only been found in the anterobasal hypothalamus

SPA stimulation produced analgesia

(Fields, et. al., 1981)

et. al., (1977) that lesions in the DLF abolished the analgesia caused by systemic injection of exogenous opiates. This demonstrated the importance of DLF as an important pathway in mediating stimulation produced analgesia and opiate induced analgesia.

Based on these studies, Basbaum, et. al., (1978) have suggested the following model of endorphin mediated analgesia system (Fig. 4). The pain suppression system was suggested to be organized at three levels: midbrain, medulla and spinal cord. Activation of the periacqueductal gray matter by electrical stimulation, opiates or psychological factors excite the serotoninergic neurons of the raphe nucleus and neurons of the magnocellular reticular nucleus of the medulla. These nuclei then transmit the signal through fibers which pass through the dorsolateral funinculus and terminate on enkephalin containing cells in lamina $\underline{\overline{I}}$ and $\underline{\overline{V}}$, in the dorsal horn of the spinal cord. These enkephalinergic interneurons then inhibit the primary afferent neurons which predominantly contain substance P (Henry, 1976; Hokfelt, et. al., 1976) and transmit information regarding pain. These primary afferent pain transmitting fibers enter the spinal cord at the dorsal horn and ascend to the medulla. There they split into two; one group travelling medially and the second group travelling laterally. The afferent fibers travelling medially in the medulla synapse with neurons of the magnocellular reticular nuclei, which in turn project to midbrain and synapse with cells in the periacqueductal, thus establishing a negative-feedback loop. Details of the endorphinergic mediated analgesic system is shown in Figure 5. Peripheral noxious stimuli excite the primary afferents which enter the spinal cord and synapse with fibers from the dorsolateral column (a). Here, pain transmission can be inhibited by certain seg-

Figure 4: The Endogenous Pain Control System

(A) Midbrain level. The periacqueductal gray (PAG) is rich in enkephalins (E) and opiate receptor and is the important locus for stimulation-produced analgesia.

(B) Medullary level. Nucleus raphe magnus (NRM) and nucleus reticularis magnocellularis (Rmc) contain serotonergic (5HT) cells.

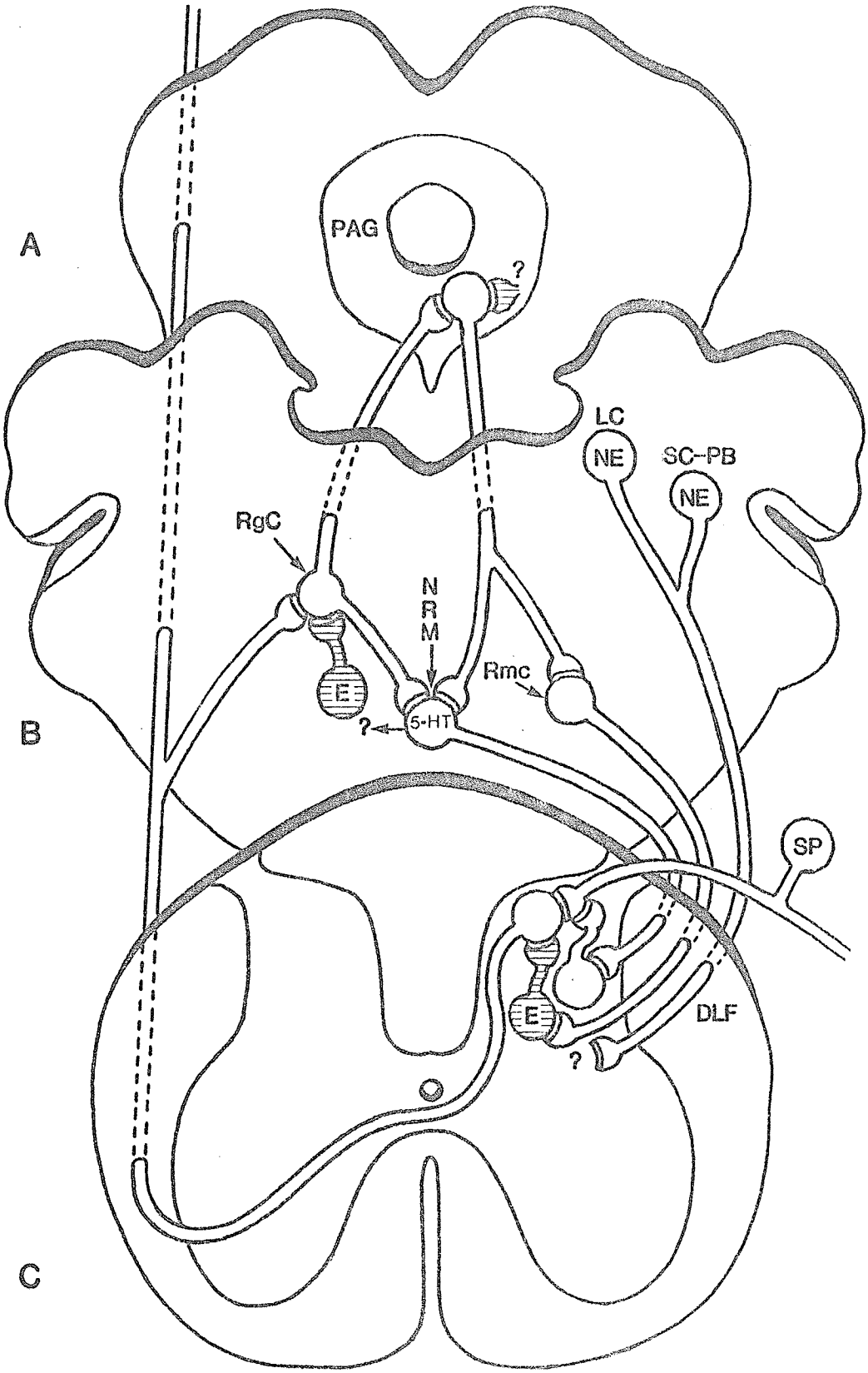
(C) Spinal level. Fibers travelling from the NRM and Rmc in the dorsolateral funinculus (DLF) terminate on cells in the dorsal horn of the spinal cord. Pain information is transmitted in substance P (SP) containing efferents project via the nucleus reticularis gigantocellularis (Rge) to PAG.

LC - locus ceruleus

SC-PB - subceruleus-parabrachialis

NE - norepinephrine

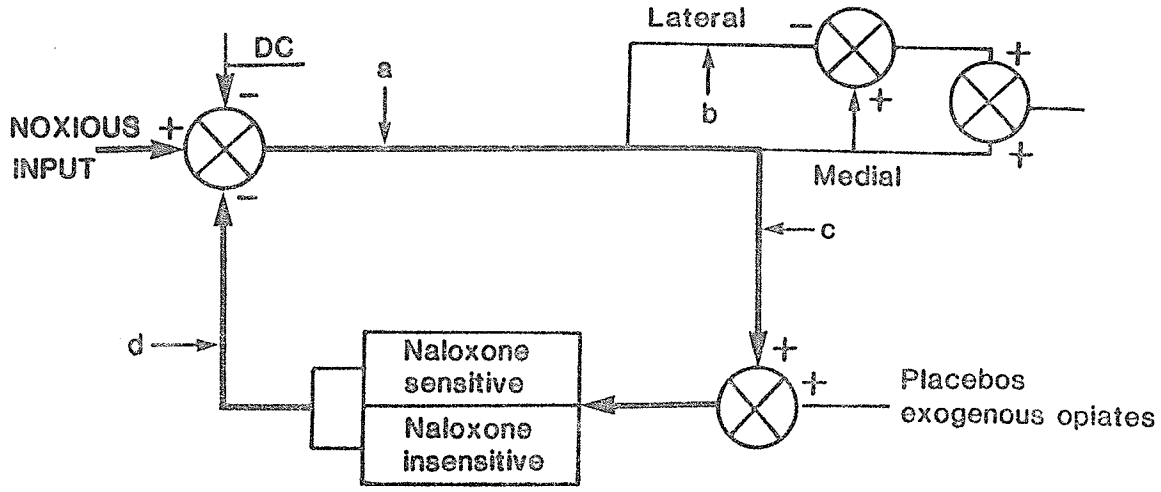
(Basbaum, et. al., 1978)


A
PAG
?
LC
NE
SC-PB
NE
RgC
NRM
Rmc
E
?
5-HT
B
SP
E
DLF
?
C

mental mechanisms which involve the enkephalinergic neurons. These mechanisms are independent of the β-endorphinergic periacqueductal gray mediated pain inhibition. Upon reaching the medulla, the pain information in the medial tract interacts with cells for the periacqueductal and periventricular gray areas, and the subsequent response is formulated. This response-information then descends down to the spinal cord via raphe nucleus through naloxone-sensitive and naloxone-insensitive pathways. This information is thus relayed on to enkephalin containing interneurons. These interneurons then inhibit the transmission of pain (Fig. 5b).

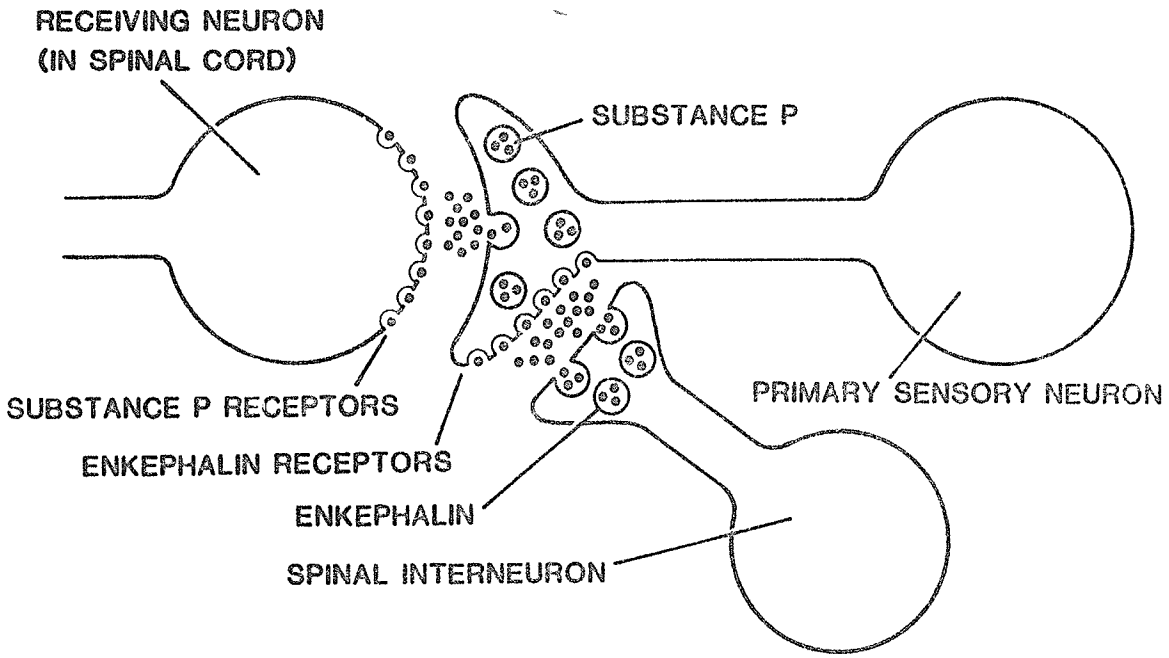
The specificity of this mechanism of pain inhibition has been corroborated by various studies. Since this endogenous analgesic system operates to limit pain, its disruption should exacerbate pain. This has been shown to occur. Naloxone has been reported to increase visceral pain in hot plate and tail-flick tests in cat and rat (Goldfarb, et. al., 1976; Jacobs, et. al., 1974). Naloxone was also shown to enhance postoperative dental pain, relative to placebos (Levine, et. al., 1978b). The most important fact about endorphin mediated analgesic system is that it is designed not to transmit afferent information but to modulate it. Thus only noxious stimuli can activate such a system besides exogenous opiates and psychological factors (Levine, et. al., 1978a). Thus if there is no afferent input to be modulated, there should be no behavioural or subjective consequences of activation of such an endorphin-mediated analgesic system.

Figure 5



a PAIN-TRANSMISSION SYSTEM

(Bausbaum et al., 1978)



b HYPOTHETICAL GATING MECHANISM

In the dorsal horn of the spinal cord, interneurons containing the peptide transmitter enkephalin make synapses onto the axon terminals of the pain neurons, which utilize substance P as their transmitter. Enkephalin released from the interneurons inhibits the release of substance P, so that the receiving neuron in the spinal cord receives less excitatory stimulation and hence sends fewer pain-related impulses to bhe brain. Opiate drugs such as morphine appear to bind to unoccupied enkephalin receptors, mimicking the pain-suppressing effects of enkephalin system.

(Iverson, 1979)

OPIATES

Morphine has been the most widely used substance for medicinal and social reasons for centuries. Its primary use in medicine has been for relief of pain. However, morphine, as morphine, has been known for less than 200 years. Before that the effects of morphine were attributed to opium which is the dried powder extracted from the exudate of the unripe seed capsules of the poppy plant. Morphine comprises 10% (by weight) of this dried powder. Sertürner, in 1805, described the isolation of a pure alkaloidal base from opium which he called morphine after Morpheus, the god of sleep.

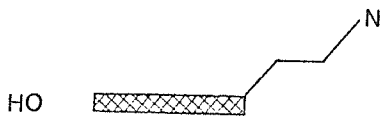
The discovery of morphine led to extensive research into development of more potent and less addictive opiates. This resulted in discovery of various structurally similar opiate agonists and antagonists. Despite this, however, the complexity in processing of pain in the central nervous system (CNS) has led to difficulty in direct measurement of pain in the laboratory. Instead we can only measure responses to the noxious stimuli. Hence a change in the response to the noxious stimuli is taken as a change in pain sensation. Still, however, opiates may alter pain responsivity across a wide range of responses, situations and species. The mechanism of action of opiates was not well understood but two lines of thought originated, opiates interrupt the transmission of painful information from the periphery to the brain, or opiates activated an endogenous pain inhibitory system. Opiates were also thought to alter the interpretation of emotional components of pain. The recent breakthrough in biochemical identification of the pharmacologically relevant opiate receptors has provided new insight into understanding of opiate analgesia and pain mechanisms in general.

Even before the original measurements of opiate receptors considerable evidence for the presence of specific site(s) for opiate alkaloids existed. Firstly, it was known that most opiates conform to a particular chemical structure (Fig. 6). They usually consist of a phenanthrene ring and a piperidine ring perpendicular to each other with a methyl substituent and a phenolic hydroxyl. The opiate molecule is usually T-shaped with two broad hydrophobic surfaces at right angles. It was also noted that the pharmacological activity was a product exclusively of levorotary opiates. The tremendous heterogeneity in pharmacological potency was also noted. For example, etorphine is 5000 to 10,000 times more potent than morphine in relieving pain in man and mammals. However, the affinity of etorphine for opiate receptors was only 20 times that of morphine (Snyder, et. al., 1975). This led to the suggestion that unlike general anesthetics, opiates do not act pharmacologically by altering membrane function in a diffuse nonspecific fashion. The presence of opiate antagonists which were structurally similar to opiates also argued strongly for the presence of specific receptors with which opiates interact.

A. DEMONSTRATION OF OPIATE RECEPTORS

Ingoglia and Dole (1970) were the first to use the observation of stereospecificity of the opiates in an attempt to identify opiate receptors. Goldstein, et. al., (1971) were the first group, who using the technique of Ingoglia and Dole, were able to demonstrate stereospecific binding in mouse brain. They incubated mouse brain extracts with [^{3}H]-levorphanol in the presence of large excess of unlabelled levorphanol or its inactive enantiomer dextrophan. Thus, stereospecific binding was that portion of the total binding of [^{3}H]-

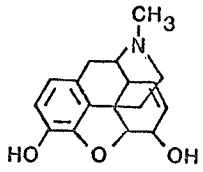
Figure 6. Structural comparison of opiate agonists and antagonists



General Conformation of Opiates

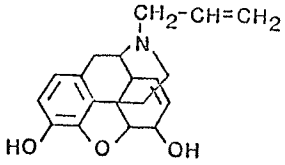
(benzene ring)

Agonists

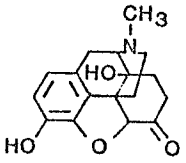


Morphine

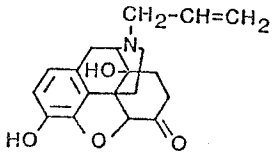
Antagonists



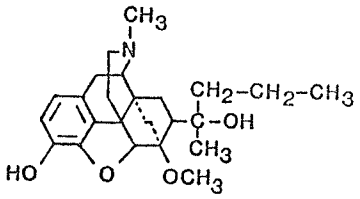
Nalorphine



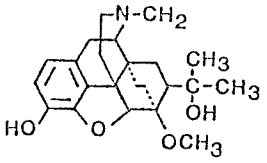
Oxymorphone



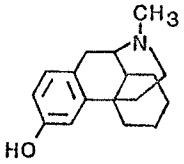
Naloxone



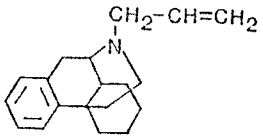
Etorphine



Diprenorphine



Levorphanol



Levallorphan

(Pert,et.al.,1974d)

Levorphanol which was prevented by levorphanol but not dextrophan. Goldstein and coworkers found that only 2% of the total binding was stereospecific. However, Pert, et. al., (1973a), Simon, et. al., (1973) and Terenius (1973a,b) all using a modified Goldstein technique independently demonostrated stereospecific saturable binding to the brain tissue. Since then many other laboratories (Hiller, et. al., 1973; Hitzeman, et. al., 1973; Klee, et. al., 1974; Lee, et. al, 1973; Wong, et. al., 1973) having suggested that the stereospecific binding of an active narcotic alkaloid indeed represents specific binding sites to which opiates bind in order to produce their pharmacological responses.

Pert, et. al., (1973b) further found that there was a direct relationship between pharmacological potency of various opiates in producing or antagonizing analgesia and their affinity for binding sites. That is, the more potent the narcotic opiate is in producing analgesia, the greater is the affinity for opiate receptors (Table 3). To ensure specificity of binding to the opiate receptors, Pert and coworkers also studied binding affinities of nonopiate drugs to opiate receptors. As can be seen in Table 3, the nonopiate drugs had no affinity for the opiate receptors. A critical demonstration of the specificity of opiate receptor binding came from Wilson and coworkers (1975). They showed that slight alterations in the side-chain length of a homologous series of opiates caused a great variation in analgesic potencies and corresponding opiate receptor binding affinity (Table 4). It was noted that for a wide variety of opiates, the affinity for the opiate receptor sites is the same in brain and guinea pig intestine (Creese, et. al., 1975). It was further demonstrated that there existed a close relationship between stereospecific binding in the brain and the potency of opiates in inhibiting intestinal muscle contraction. These studies

Table 3. Relative potencies of drugs in reducing stereospecif
^{3}H-Naloxone binding to Rat Brain homogenate

Drug	ED_{50} (nM)	No effect at 0.1 mM
(−)-Etorphine	0.3	Phenobarbital
(−)-Etonitazene	0.5	Norepinephrine
Levallorphan	1	Atropine
Levorphanol	2	Pilocarpine
(−)-Nalorphine	3	Arecholine
(−)-Morphine	7	Colchicine
(−)-Cyclazocine	10	γ-Aminobutyric acid
(−)-Naloxone	10	Bicuculline
(−)-Hydromorphone	20	Serotonin
(−)-Methadone	30	Carbamylcholine
(±)-Pentazocine	50	Neostigmine
(+)-Methadone	300	Hemicholinium
Meperidine	1,000	Histamine
(±)-Propoxyphene	1,000	Glycine
(+)-3-Hydroxy-N-allyl-morphinan	7,000	Glutamic acid
Dextrorphan	8,000	Δ9-Tetrahydrocannabinol
(−)-Codeine	20,000	Acetylsalicylic acid
(−)-Oxycodone	30,000	Caffeine

Values represent means from 3 log-probit determinations, each using 5 concentrations of drug.
ED_{50} = concentration of drug required to inhibit stereospecific ^{3}H-naloxone binding by 50%.

(Pert,et.al.,1973b)

Table 4. Relationship between analgesic potency of ketobemidones and opiate receptor binding affinity

Compound	Hot-plate analgesic (mM) ED_{50}	Inhibition of ^{3}H-naloxone binding (1 nM) ED_{50}		Ratio $\frac{+NaCl}{-NaCl}$
		No sodium	100 mM Sodium	
Methyl	2.1 (1.4-2.8)	7-10	70	7-10
Ethyl	67.2 (52.0-87.0)	400	1500-2000	3.8-5
Propyl	16.0 (13.2-19.1)	200	800-1000	4-5
Butyl	4.6 (3.8-5.9)	50	600-700	12-14
Pentyl	0.78 (0.62-1.0)	8	30	3.8
Hexyl	7.5 (5.5-10.3)	20	40	2
Heptyl	9.0 (7.0-11.6)	20	40-50	2-2.5
Octyl	26.5 (20.2-34.9)	200	200	1
Nonyl	inactive	700	700	1
Decyl	inactive	500	600-700	1.2-1.5

For hot-plate analgesic 95% confidence limits are shown in parentheses; ED_{50} = nM concentration of drug required to inhibit stereospecific ^{3}H-naloxone binding by 50%. Binding was performed using mouse brain homogenates. Eight mice were used at each dose and a minimum of 5 doses for each compound was used.

(Wilson,et.al.,1975)

thereby suggested uniformity of opiate receptor receptor type in the brain and the intestinal muscle.

B. REGIONAL DISTRIBUTION OF OPIATE RECEPTORS IN THE BRAIN

The regional distribution of opiate receptor is of extreme importance since it can reveal a relationship between opiate receptor localization and regional brain function. Kuhar, et. al., (1973) and Hiller, et. al., (1973) did a detailed in vitro binding study of regional distribution of opiate receptor in the brain of monkey (Table 5) and man (Table 6). In general, the amygdala had the greatest amount of opiate receptors, with the anterior amygdala having twice as many as the posterior amygdala. The periacqueductal gray had the same amount of opiate receptors as the anterior amygdala. There were no regional variations in the amount of receptors in the hypothalamus; however, the thalamus and cerebral cortex has marked regional differences. There is considerable similarity in regional distribution of opiate receptor in monkey and man.

Autoradiographic studies have also been used to study regional distribution of the opiate receptor. Pert, et. al., (1975) using tritiated dihydromorphine, which is a potent opiate antagonist, autoradiographically revealed an uneven distribution of opiate receptors which at a gross level paralleled regional distribution of opiate receptor measured by in vitro binding studies. Several of the areas which have been demonstrated to be rich in opiate receptors thus are the leading candidates for site of action of opiates in causing analgesia. These sites include the substantia gelatinosa of the dorsal horn of the spinal cord, the substantia gelatinosa of the spinal trigeminal nucleus, the intralaminar nuclei, dorsomedial thalamus, periacqueductal gray

Table 5. Opiate receptor binding in Monkey Brain

Region	Stereospecific dihydromorphine binding (fmol/mg protein)	
Cerebral hemispheres		
Frontal pole	11.9 ± 1.4	(4)
Superior temporal gyrus	10.8 ± 2.5	(3)
Middle temporal gyrus	7.1	(1)
Inferior temporal gyrus	6.0	(1)
Precentral gyrus	3.4	(2)
Postcentral gyrus	2.8	(2)
Occipital pole	2.3 ± 0.5	(4)
White matter areas		
Corpus callosum	<2	(2)
Corona radiata	<2	(2)
Anterior commissure	5.4	(2)
Fornix	<2	(2)
Optic chiasm	<2	(2)
Limbic cortex		
Anterior amygdala	65.1 ± 21	(4)
Posterior amygdala	34.1 ± 4.2	(4)
Hippocampus	12.5 ± 2.2	(4)
Hypothalamus		
Medial hypothalamus	24.2 ± 3.2	(4)
Anterior hypothalamus	24.3 ± 3.7	(3)
Posterior hypothalamus	24.7 ± 1.4	(3)
Hypothalamus	23.2	(1)
Mammillary bodies	5.0	(1)
Thalamus		
Medial thalamus	24.6 ± 1.6	(3)
Lateral thalamus	7.8	(2)

Region	Stereospecific dihydromorphine binding (fmol/mg protein)	
Extrapyramidal areas		
Caudate nucleus (head)	19.4 ± 2.3	(4)
Caudate nucleus (body)	9.0 ± 1.2	(3)
Caudate nucleus (tail)	8.9 ± 3.0	(3)
Putamen	11.7 ± 1.9	(6)
Globus pallidus	7.7	(2)
Internal capsule	5.4	(2)
Midbrain		
Superior colliculi	10.6 ± 2.0	(3)
Inferior colliculi	6.7 ± 0.7	(3)
Interpeduncular nucleus area	13.7 ± 1.5	(4)
Raphe area	8.2	(2)
Periaqueductal gray	31.1 ± 4.6	(4)
Cerebellum-lower brainstem		
Pons (ventral)	1.4	(2)
Cerebellar cortex	<2	(2)
Dentate nucleus	1.9	(2)
Floor of fourth ventricle	6.3 ± 1.3	(3)
Pyramidal tract	3.0	(2)
Lower medulla	5.8	(2)
Spinal cord (thoracic)		
Dorsal column (white)	3.1	(2)
Lateral cord (white)	3.3	(1)
Gray matter	8.8	(2)

Values represent the mean ± SEM followed in parentheses by the number of determinations. Each determination was calculated from triplicate incubations with 1 nM ^{3}H-dihydromorphine in the presence of 100 nM levorphanol or dextrorphan. Five rhesus monkeys (*Macaca mulatta*) were decapitated 2 h after anesthesia with 30 to 45 mg/kg of sodium pentobarbital injected intraperitoneally. The brain was removed within 5 min and dissected rapidly on ice.

(Snyder,et.al.,1975)

Table 6. Opiate receptor binding in Human Brain

High binding (0.44-0.23 pmol/mg protein)	
Olfactory trigone*	Centromedian nucleus of thalamus*
Amygdala*	Preoptic area and supra optic nucleus*
Septal nuclei*	Cingulate gyrus*
Supra orbital gyrus of frontal lobe*	Dorsomedian nucleus of thalamus*
Parahippocampal gyrus*	Frontal lobe cortex*
Periventricular gray matter*	Pulvinar of thalamus
Temporal lobe*	
Moderate binding (0.21-0.15 pmol/mg protein)	
Caudate nucleus	Olfactory bulb
Parietal lobe cortex	Periaqueductal gray
Hypothalamus*	Putamen
Ventral anterior nucleus of thalamus	Ventral posterolateral nucleus of thalamus
Low binding (0.12-0.07 pmol/mg protein)	
Occipital lobe cortex	Cerebellar cortex
Corpora quadrigemina	Pretectum
Hippocampus*	Substantia nigra
Globus pallidus	Area postrema
Very low binding (0.05-0 pmol/mg protein)	
Mammillary bodies	Olives
Medullary sensory nuclei	Dentate nucleus of cerebellum
Cerebral white matter	Tegmentum of mid pons
Posterolateral nucleus of thalamus	Pineal gland
Red nucleus	Pituitary gland

*Indicates components of the limbic system or regions associated with the limbic system.

(Hiller,et.al.,1973)

(Atweh, et. al., 1977; Pert, et. al., 1977) and the floor of the fourth ventricle (Vigouret, et. al., 1973).

C. PROPERTIES OF OPIATE BINDING SITES

1. Opiate binding is stereospecific. Levorotary opiates are the pharmacologically functional opiates. This is best exemplified by Levorphanol (which is levorotary) and dextrophanol (which is dextrorotary). Levorphanol binding affinity is four times that of dextrophanol (Pert, et. al., 1973a; Simon, et. al., 1973).

2. The stereospecific opiate binding is saturable and both agonist and antagonist bind with high affinity (Stahl, et. al., 1977).

3. Binding of all agonists is inhibited by Na^+ while the binding of antagonist is enhanced (Pert, et. al., 1974a). Other alkali metals such as K^+, Rb^+ and Cs^+ have no regulatory effect while Li^+ has some effect. Anions such as F^-, Cl^-, Br^-, I^-, SO_4^{-2} and SCN^{-2} all enhance binding of antagonist. However, Manganese and Magnesium salts depress antagonist binding while enhancing agonist binding (Pasternak, et. al., 1975a).

4. Binding is inhibited by proteolytic enzymes (Pasternak, et. al., 1973; Simon, et. al., 1973) and a wide variety of protein reagents including sulfhydryl reagents (Terenius, 1973b). Binding is also inhibited by treatment with phospholipase A (Pasternak, et. al., 1973) however phospholipase C had no effect (Simon, et. al., 1973) (Table 7).

5. Binding is temperature sensitive. Maximum binding occurs at 35^oC while only 10% occurs at 4^oC and 0% at 55^oC (Pert, et. al., 1973a).

6. The optimum pH for opiate binding ranges from 6.5 to 8.0 (Pert, et. al., 1973a).

Table 7. Effect of protein-modifying reagents and enzymes on receptor binding of opiate agonists and antagonist

Agents	Percent of control	
	Antagonist binding	Agonist binding
Protein-modifying reagents		
N-Ethylmaleimide (0.1 mM)	85	7
Iodoacetamide (5 mM)	90	5
Mersalyl acid (10 μM)	76	23
N-Bromosuccinimide (10 μM)	110	18
Iodine (20 μM)	73	52
2-Methoxy-5-nitrobenzyl bromide (1 mM)	65	50
2-Hydroxy-5-nitrobenzyl bromide (10 mM)	72	47
Enzymes		
Trypsin (1 μg/ml)	65	28
Chymotrypsin (4 μg/ml)	70	15
Phospholipase A (50 ng/ml)	55	15

Antagonist binding utilized 1 nM ^{3}H-naloxone; agonist binding employed 0.6 nM ^{3}H-dihydromorphine, both assayed in 100 mM NaCl.

(Snyder, 1975)

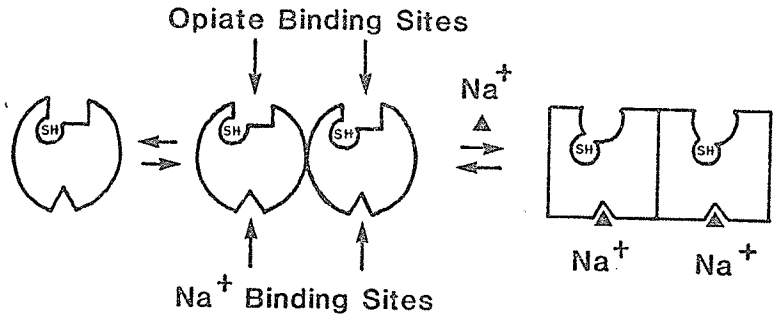
D. CONFORMATION OF OPIATE RECEPTOR

Two allosteric models of the opiate receptor have been proposed. Both are based on the selective action of the sodium ion. Several studies have reported a positive cooperativity for the binding of both agonist and antagonist in brain slices but not in broken cell (homogenized) preparations (Davis, et. al., 1977; Simon, et. al., 1975c). In line with this Simon, et. al., (1975a) proposed a model of opiate receptor where the receptor is represented as a dimer (Fig. 7a). Sodium ions bind to the allosteric site on the receptor which causes the receptor to undergo a conformational change. This conformational change leads to an alteration in the binding site which now binds antagonists with greater affinity than agonist. And in the absence of sodium ions agonist bind to the receptor with greater affinity. The dissociation of the dimeric receptor into its subunits is presented as one possible explanation for the observation that some "Na^+-free" conformer are still present in the presence of 100 mM NaCl (Simon, et. al., 1975d). A second model is proposed by Pert and coworkers (1974b) (Fig. 7b). They take into account the finding that the maximal number of binding sites remain the same irregardless of whether they are measured by methods using labelled agonist or antagonist (Creese, et. al., 1975; Pert, et. al., 1974b). This led them to propose that there were actually two reversible conformations of the receptor which existed at equilibrium. One conformation is preferred in the presence of sodium ions and this has a greater affinity for the antagonist. The other conformation is preferred in the absence of sodium ions and this has greater affinity for the agonist. Thus, in the high sodium in vivo environment, most of the receptors would prefer the antagonist, thus relatively high

Figure 7. Models of Opiate Receptor Function

a

Simon's model

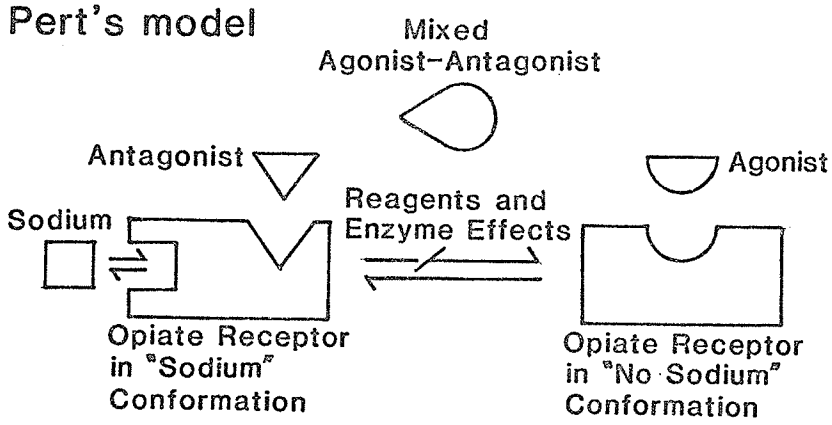


Monomer Dimer

(Simon, et. al., 1975c)

b

Pert's model



(Pert, et. al., 1974b)

amounts of agonist would be required to have agonist binding. Thus physiological opiate agonist binding results from conversion of the opiate receptor into a conformation which can no longer bind sodium. The interconversion of the two opiate receptors presumably involves folding, unfolding, aggregation, disaggregation or other modifications in the protenious structure.

E. EVIDENCE FOR MULTIPLE OPIATE RECEPTORS

Lord, et. al., (1977) presented the first evidence that multiple opiate receptors exist. This work was later confirmed by Simon, et. al., (1980). These studies demonstrated that naloxone is six times more effective in competing with labelled naloxone than leu-enkephalin, and opiate agonist. However, leu-enkephalin is eight times more effective in binding competition with labelled leu-enkephalin than labelled naloxone. Lord and coworkers (1973) proposed that this was evidence for two types of opiate receptors; μ, which has high affinity for opioid alkaloids, and δ, which has high affinity for opioid antagonist. Evidence for the existence of these two receptors is also supported by other studies (Cheng, et. al., 1979). However, there are also many similarities between μ and δ receptors. They both contain a single essential SH which is equally accessible to alkylation (Simon, et. al., 1975b). They also exhibit similar sensitivity to phospholipase A2 from the venom of Russell's viper and similar restoration of activity is followed by treatment of membrane with 1% bovine serum albumin (Lin, et. al., 1978). The two receptors are found in widely different ratios in different regions of the brain. The caudate nucleus and the periventricular gray region primarily have δ receptors. In thalamus, primarily μ receptors are found. The cortex and amygdala

have relatively high amounts of δ receptors. Martin (1967) had also provided evidence for two classes of receptors, namely σ and K, in the dog brain. There is now evidence that there exists heterogeneity even among μ type receptors (Bonnet, et. al., unpublished work, from Simon, et. al., 1981).

Pert and coworkers (1981) using a different classification system have also found heterogeneity among opiate receptors. It has been previously shown that "GTP regulatory subunits" couple receptors to their effectors (e.g., adenylate cyclase, Dodd, et. al., 1978) in the fluid mosaic model of the cell membrane (Singer, et. al., 1972). Pert and coworkers divided the opiate receptors, in the brain, into two classes: GTP-sensitive and GTP-insensitive. Their distribution is shown in Figure 8 and their properties are indexed in Table 8. The GTP-sensitivity is a reflection of the ability of the opiate receptors to bind to other membrane components. Thus it is quite plausible to suggest that there is actually only one receptor; however, the active site of this receptor can undergo numerous deformations depending on the proximity of its association with other membrane components, as in the case with muscarinic receptors (Birdstall, et. al., 1976) and dopamine receptors (Kebabian, et. al., 1979).

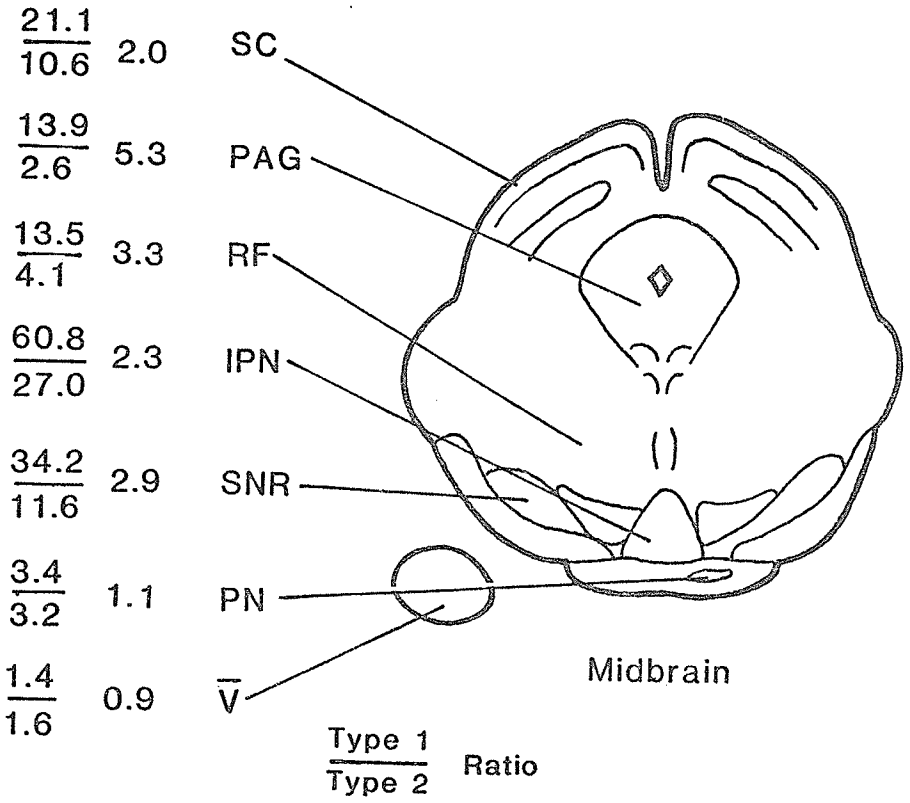
F. DISCOVERY OF ENDOGENOUS OPIATES

The discovery of opiate receptors in the central nervous system of all vertebrates from hagfish to man led to the search for endogenous ligands of the opiate receptors.

Murray, et. al., (1960) had first demonstrated the presence of an opiate like substance in the pituitary. However, it was Hughes (1975a) who first demonstrated that a peptide present in the brain extract had

Figure 8.

Quantitation of Type 1 and Type 2 receptor distribution determined by counts of auto-radiographic grain density of rat brain



SC -superior colliculus

PAG -periacqueductal gray

RF -reticular formation

IPN -interpenduncular nucleus

SNR -substantia nigra pars reticulata

PN -pontine nuclei

V -trigeminal nerve

(Pert, 1981)

Table 8. Characteristics of Type 1 and Type 2 opiate receptors

Type 1	Type 2
GTP (2 μM) sensitive	GTP (2 μM) resistant
μ-oid ligand selectivity	δ-oid ligand selectivity
Morphine more potent than leu-enkephalin	Leu-enkephalin more potent than morphine
Sodium discriminates between agonist/antagonist	No sodium effect
Warm (25-37°C) incubation temperature required to visualize [^{3}H]agonist binding	Temperature insensitive
Sensitive to opiate antagonists	Insensitive to opiate antagonists
Postsynaptic to opiatergic neurons	Extrasynaptic, presynaptic?
Capacity for coupling to effectors through GTP-sensitive "N" (19) subunit	Lacks "N" subunit coupler
Barely detectable in invertebrates	Predominate in invertebrates
Heterogeneous, discrete distribution	Diffuse distribution
Increases in cortex with increasing functional complexity	No progression, marks ancient limbic areas
Flexible modulation capacity?	Inflexible, hard-wired?

(Pert, 1981)

morphine-like activity on smooth muscle, and this peptide was shown to compete for opiate binding sites in in vitro studies (Pasternak, et. al., 1975b, 1976; Terenius, et. al., 1975). Subsequently Hughes and coworkers (1975b) isolated, characterized and synthesized two naturally occurring pentapeptides which he collectively named "enkephalins", which in Greek means "in the head". The two enkephalins were named met-enkephalin and leu-enkephalin and occurred in the ratio of approximately 3 to 1. The pharmacological actions of these two peptides are similar to those of morphine on guinea pig ileum and mouse vas deferens, two tissues which are known to possess receptor sites. The action of both peptides was reversed by a wide variety of opiate antagonists (Hughes, et. al., 1975b). Sequencing studies subsequently revealed that met-enkephalin was a N-terminal pentapeptide of β-Lipotropin (β-LPH), a peptide of pituitary origin discovered by Li, (1964) in sheep pituitary glands. During the course of isolation of melanotropins from camel pituitary, Li, et. al., (1975) obtained an untriakontapeptide whose amino acid sequence was identical to the C-terminal 31 amino acid residue of ovine β-LPH (Li, et. al., 1976a). β-Endorphin was subsequently obtained from porcine pituitary (Bradbury, et. al., 1976a; Graf, et. al., 1976a), sheep pituitary (Chretien, et. al., 1976), and from human pituitary gland (Chretien, et. al., 1976; Li, et. al., 1976b). Shortly thereafter various other residues of β-LPH were found in extracts of brain and pituitary all of which had some opiate activity (Fig. 9). Of all the β-LPH residues, β-endorphin by far is the most potent opioid (Bloom, et. al., 1976; Bradbury, et. al., 1976b,c; Cox, et. al., 1976; Graf, et. al., 1976b; Loh, et. al., 1976). The regional distribution of met-enkephalin, leu-enkephalin and

Figure 9.

Fragments of B-LPH (1-91) with Opiate Activity

β-endorphin[a] (C-fragment)	61 — 91
δ-endorphin[b] (C′-fragment)	61 — 87
γ-endorphin[c]	61 — 77
α-endorphin[d]	61 — 76
Met-enkephalin[e]	61 65
Leu-enkephalin[f]	Tyr-Gly-Gly-Phe-Leu

a. Bradbury et. al, 1976: Chretien, et. al, 1976b
Graf, et. al, 1976a : Li, et. al, 1976a,b

b. Feldberg, et. al, 1976: Guillemin, et. al, 1977

c. Ling, 1977: Ling, et. al , 1976 a,b

d. Guillemin, et. al, 1976: Ling, 1977: Ling, et. al., et.1976a

e. Hughes, et. al., 1975

f. Hughes, et. al., 1975

β-endorphin, as measured by radioimmunoassay, is shown in Tables 9, 10, and 11.

G. SYNTHESIS OF OPIOID PEPTIDES RELATED TO β-LPH

The synthesis of opioid peptides related to β-LPH is intertwined with synthesis of peptides related to ACTH. Eipper and coworkers (1977) using a double antibody immunoprecipiation procedure demonstrated that both ACTH and β-endorphin were contained within a single 31K precursor molecule. This observation, along with several others which demonstrated a close relationship between ACTH and β-LPH families of peptide (Abe, et. al., 1969; Gilkes, et. al., 1975; Hirata, et. al., 1976; Lowry, et. al., 1976; Pelletier, et. al., 1977; Phifer, et. al., 1974), has led to the suggestion that perhaps there is a common biosynthetic precursor for peptides of ACTH and β-LPH families. This common precursor is now referred to as Pro-opiomelanocortin. Nakanishi and coworkers (1979) were the first to delineate the amino acid sequence of pro-opiomelanocortin in bovine intermediate pituitary. The structure of pro-opiomelanocortin is shown in Figure 10. The post-translational processing of pro-opiomelanocortin is shown in Figure 11. The clevage sites separating the 16K fragment, ACTH, γ-LPH and β-endorphin are separated by a pair of amino acids. The formation of β-endorphin from the precursor molecule could theoretically occur after a single proteolytic cleavage. However, this does not occur. Instead β-LPH is formed first and it is then cleaved to form γ-endorphin and β-LPH. Since β-LPH contains the sequence of met-enkephalin, it had been widely believed that it is a cleavaged product of β-LPH. However, a growing amount of evidence now suggests that met-enkephalin is not formed from either β-LPH or β-endorphin but instead has its own precursor (Lewis, et. al., 1978;

Table 9. Regional Distrubution of Immunoreactive Met-enkephalin in the Brain

Brain Regions	Rat Rossier, et. al., 1977 ng/mg tissue	Rat Yang, et. al., 1977 ng/mg protein	Rat Miller, et. al., 1978 pmol/mg protein	Rat Llorens-Cortes, et. al., 1977 nmole/g	Bovine Simantov, et. al., 1977 pm/g wet wt.	Bovine Lindberg, et. al., 1979 ng/mg protein	Human Edsen, et. al., 1980 pm/g wet wt.
Cerebellum	280± 30	.36±0.02	< 0.01	0.05±0.01		0.75±0.19	
Midbrain	960± 30	1.1 ±0.17	1.90±0.1	0.20±0.05		1.66±0.31	
Hypothalamus	3600±210	4.8 ±1.3	0.4 ±0.7	0.47±0.04	110		141±22
Thalamus			1.04±0.08		60	2.28±0.14	
Striatum	3360±330	9.2 ±1.7	5.97±0.7	1.23±0.07			
Hippocampus	390± 30	0.71±0.14	0.91±0.1	0.05±0.01		1.18±0.14	56±16
Septum	2550±210						64±17
Cortex					100		
Frontal Cortex				0.27±0.04		1.45±0.4	46±17
Amygdala					300	1.65±0.14	26±10
Pons						2.77±0.71	
Medulla		2.6 ± 0.34				2.37±0.19	
Caudate N.					90		116±40
Globus Pallidus			6.7 ±0.8				1163±216
Whole Brain	750± 60	3.0 ±0.76	0.88±0.1				

Table 10. Regional Distribution of Immunoreactive Leu-enkephalin in the Brain

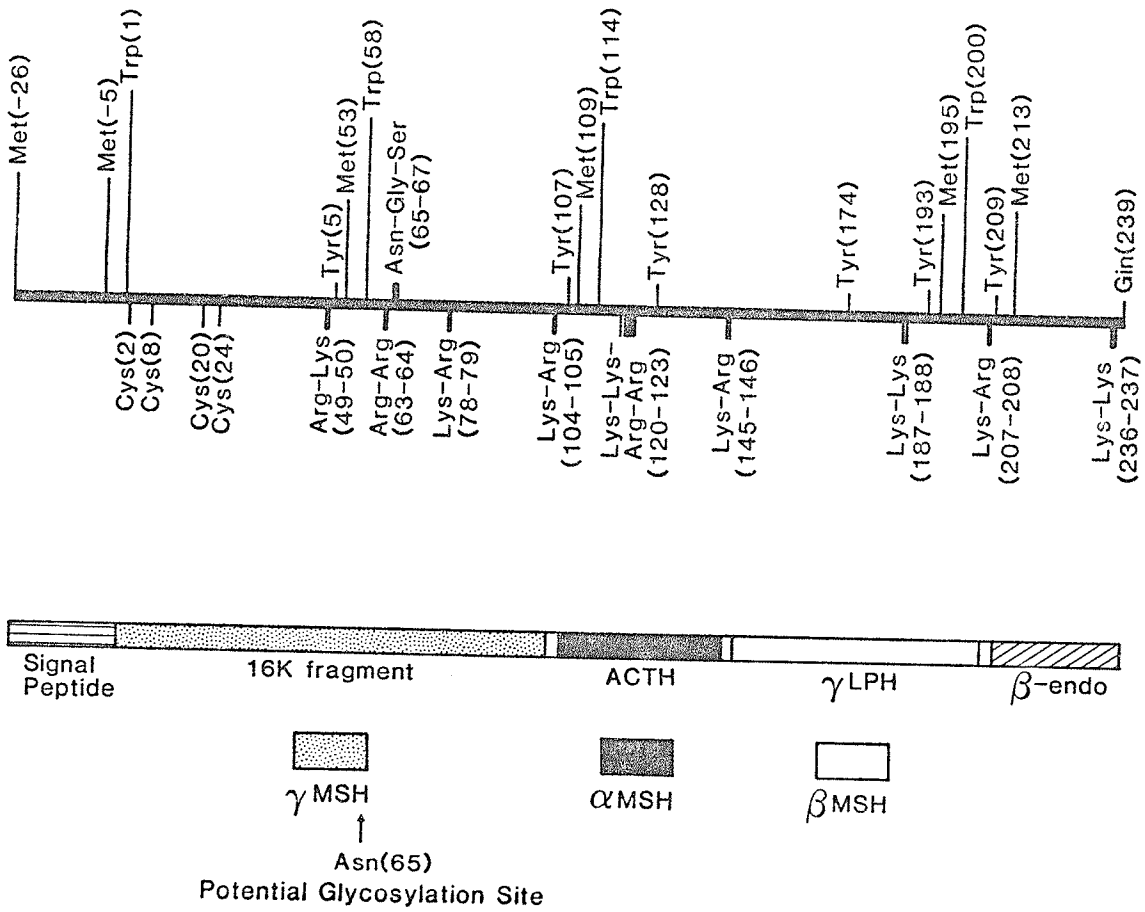
Brain Regions	Rat Rossier, et. al., 1977 ng/mg tissue	Rat Yang, et. al., 1977 ng/mg protein	Rat Miller, et. al., 1978 protein	Rat Llorens-Cortes, et. al., 1976 nmole/g	Bovine Simantov, et. al., 1977 pm/g wet wt.	Bovine Lindberg, et. al., 1979 ng/mg protein	Human Edsen, et. al., 1977 pm/g wet wt.
Cerebellum	5± 1	0.13±0.03	< 0.01	0.006±0.001	0.3	0.05±0.02	100
Midbrain	32± 1	0.13±0.04	0.21±0.01	0.011±0.002	1.1±0.3	0.27±0.03	
Hypothalamus	120± 7	0.69±0.2	0.40±0.07	0.114±0.05	1.5±0.3		270
Thalamus			0.34±0.01			0.29±0.04	
Striatum	112±11	1.3 ±0.26	0.62±0.03	0.174±0.005	2.5±0.4		
Hippocampus	13± 1	0.06±0.01	0.06±0.01	0.005±0.001	0.3	0.12±0.12	
Septum							
Cortex							
Frontal Cortex				0.043±0.016		0.11±0.02	
Amygdala						0.14±0.03	100
Pons						0.17±0.03	
Medulla		0.26±0.08				0.12±0.01	
Caudate N.							450
Globus Pallidus	85± 7		0.77±0.09				
Whole Brain	25± 2	0.22±0.05	0.21±0.06		0.4±0.2		

Table 11. Regional Distribution of Immunoreactive β-Endorphin in the Brain

Brain Regions	Rat Rossier, et. al., 1977 ng/g tissue	Rat Hollt., et. al., 1978 ng/g tissue	Rat Ogawa, et. al., 1979 ng/g tissue	Rat This Study ng/g tissue	Bovine Krieger, et. al., 1977 ng/g tissue	Monkey Matsukura et. al., 1978 pg/mg wet wt.	Human Wilkes, et. al., 1980 pg/100 µg protein	Human Lightman, et. al., 1979 pg/mg wet tissue
Cerebellum	none		77± 20	60± 10		124± 22	none	
Hindbrain	179± 5		628± 73	181± 19				65
Midbrain	207±14		573±243	263± 43				
Hypothalamus	490±30	113±30	4941±796	4370±681	19.9± 5			396±136
Thalamus	329±19		260± 52	196± 20		309± 92		837±456
Striatum	none			34± 9				
Hippocampus	none			36± 11				168± 83
Septal Nuclei			87± 12	365± 26				
Septum	234±34				15.0			
Cortex	none			10± 2	7.0		none	
Amygdala				300± 30			5.5±1.5	
Pons					9.3	387±152	3.3±0.9	
Whole Brain	108± 8			280± 60				

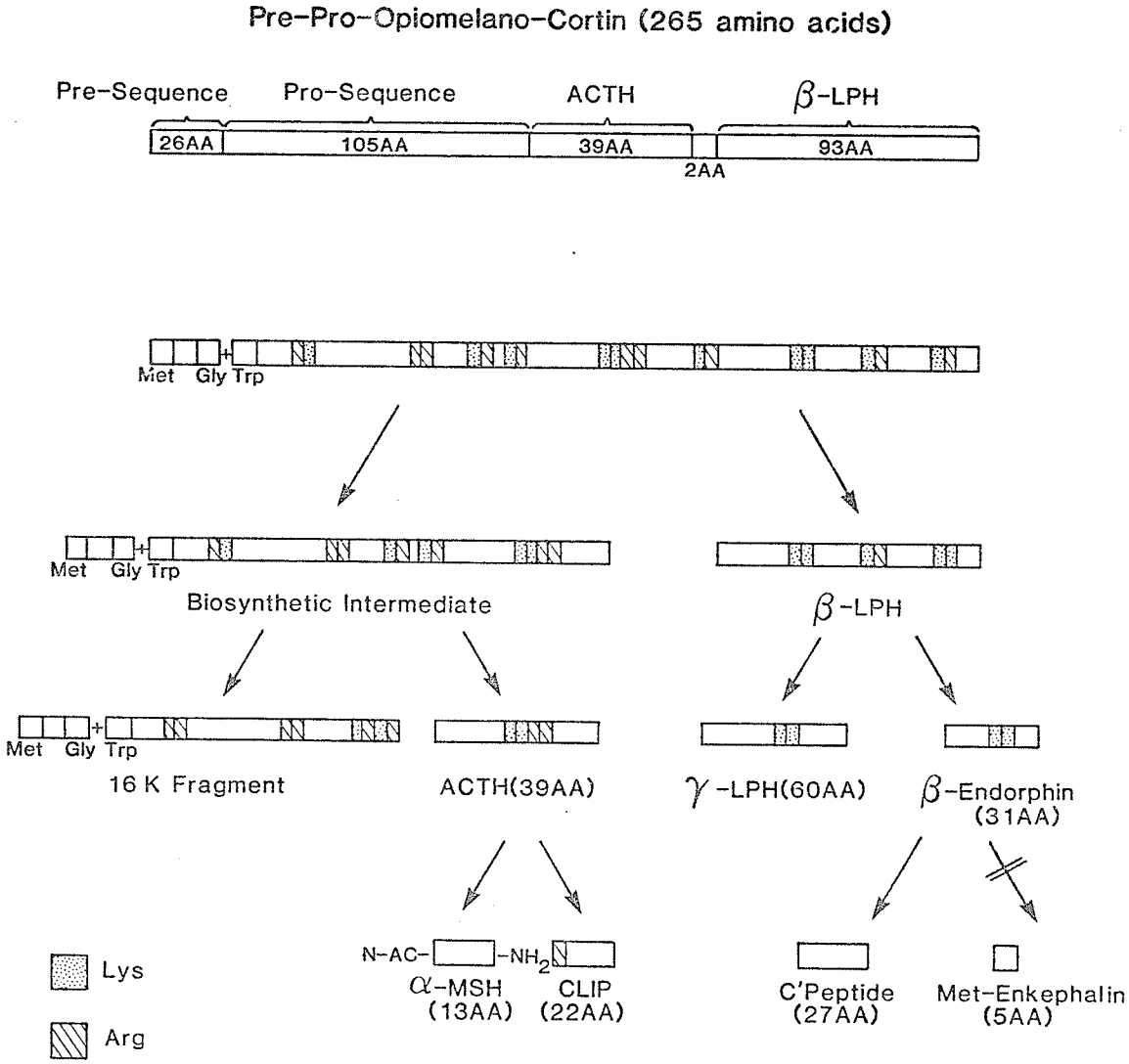
Figure 10.

Structure of the common precursor to peptides related to the ACTH and β-LPH family : Pro-Opiomelanocortin



(Eipper,et.al.,1980)

Figure 11.



(Modified from Chretien,et.al.,1980:Eipper,et.al.,1980)

Mains, et. al., 1977). The function of the 16K fragment during post-translational processing is unknown. However, it has been shown that the amino terminal region of the 16K fragment is highly conserved among species (Hakanson, et. al., 1980; Kuelmann, et. al., 1979; Nakanishi, et. al., 1979), thereby suggesting that it might have an important function. The CLIP and γ-LPH fragments of the precursor, on the other hand, exhibit most wide species-specific variation (Eipper, et. al., 1979; Li, et. al., 1976c; Lowry, et. al., 1979).

Since ACTH, endorphins and lipotropins are also present in the brain, it is likely that these, as in the case of the pituitary originate from pro-opiomelanocortin. However, it is likely that the post-translational processing of the precursor might be different in the brain.

H. ELECTROPHYSIOLOGICAL STUDIES WITH ENDORPHINS

It has been previously demonstrated that low doses of narcotic alkaloids induce slow wave high voltage synchronous EEG and higher doses lead to slow wave high voltage hypersynchronous EEG in rat (Aliosi, et. al., 1980; Cahen, et. al., 1944), rabbit (Aliosi, et. al., 1980; Gangloff, et. al., 1957; Silvestri, et. al., 1956) and dog (Chin, et. al., 1961; Winkler, et. al., 1952).

In the rat, cortical and subcortical EEG recordings have been found to be a highly sensitive index of central endorphin action. Ionotophoretic application of endorphins reveal that most of the responsive cells in the cerebral cortex, brain stem and thalamus are inhibited whereas the pyramidal cells in the hippocampus are exclusively excited (Elde, et. al., 1976).

Aloisi and coworkers (1980) investigated the effects of met-enkephalin, leu-enkephalin and enkephalin derivatives on EEG from cortical and subcortical areas in rat and rabbit. In both animals they detected a biphasic EEG response. Immediately after ICV infusion the EEG pattern was desynchronized. Twenty to thirty minutes later, there was return of synchronized EEG with some scattered spikes accompanied by a loss of response to noxious stimuli, exopthalamus, stupourous state, catatonia and rigidity. Morphine, administered ICV, causes similar modifications of EEG activity and behaviour. Havlicek, et. al., (1978) studying the EEG responses to ICV injected β-endorphin in rats have noted that 0.1 μg of β-endorphin led to non-significant depression of EEG power spectra and 50 μg of β-endorphin led to EEG hypersynchrony with significant increase in power in all frequencies (Fig. 12).

Several studies have indicated that dopamine interacts with various functions of morphine (Cowan, et. al., 1975; Ungerstedt, et. al., 1969). Since morphine is also known to increase brain dopamine levels, it has been suggested by Keane (1975) that dopaminergic mechanisms in the brain were perhaps mediating the slow wave high voltage EEG activity changes following morphine infusion. To test this hypothesis Neale, et. al., (1978) studied the EEG activity following local intracerebral injection of opiates and enkephalins into the forebrain, a region which envelops the dopamine rich striatum. The changes in EEG activity following injection of met-enkephalin are shown in Figure 13. There is a significant depression of EEG activity at 15 minutes after met-enkephalin injection. The EEG activity subsequently returned to normal by 30 minutes post-injection. Thus, they noted that narcotic alkaloids generally induce slow wave high

Figure 12: Dose-response Changes in the EEG Power Spectrum After ICV Administration of β-endorphin

A - control

B - 2 μg of β-endorphin

C - 50 μg of β-endorphin

Vertical lines indicate S.E.

** $p \leq .01$

(Havlicek, et. al., 1978)

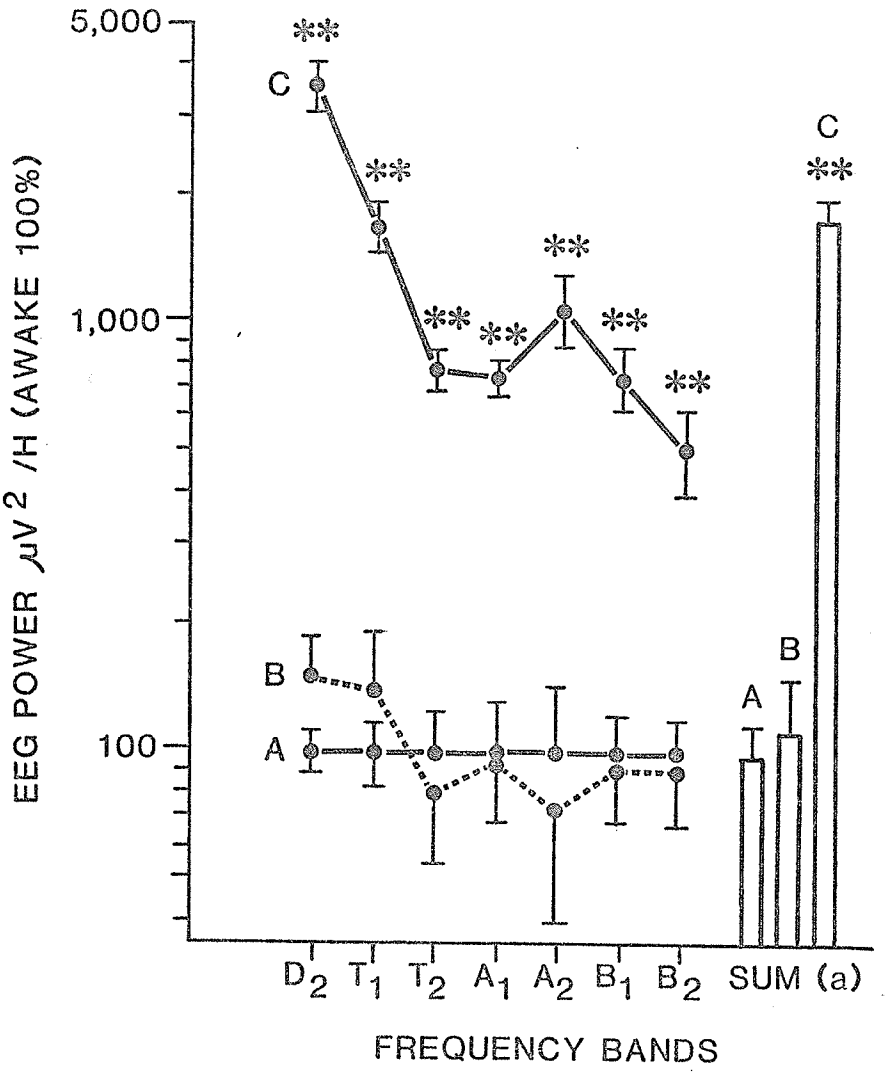

5,000
1,000
100
EEG POWER μV² /H (AWAKE 100%)
C
B
A
**
D2 T1 T2 A1 A2 B1 B2 SUM (a)
FREQUENCY BANDS

Figure 13: EEG Changes Following Injection of 100 μg Met-enkephalin into the Left Caudate-putamen

A. at the time of injection

B. at 4 min. after injection

C. at 15 min. after injection

Electrode placements:

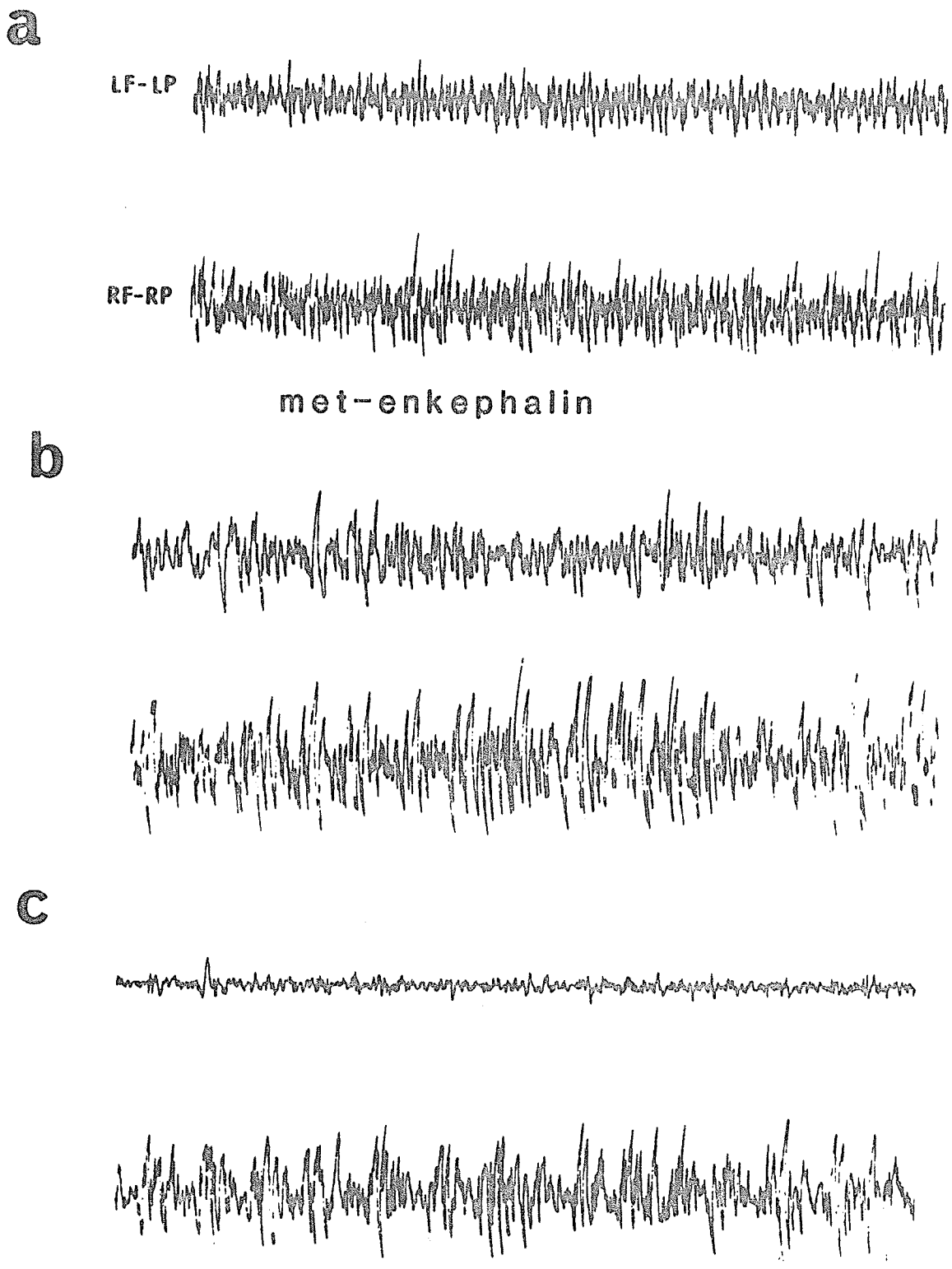
LF - left frontal cortex

RF - right frontal cortex

LP - left parietal cortex

RP - right parietal cortex

(Neal, et. al., 1978)


a
LF-LP
RF-RP
met-enkephalin
b
c

voltage EEG whereas the enkephalins produce an initial slow wave state, which then develops into a very flat, almost isoelectric EEG pattern. In light of this study, it is interesting to note that Havlicek et. al., (1978) had noted a similar depression following ICV infusion of a small dose of β-endorphin in the brain.

I. THE ROLE OF ENDORPHINS IN ACUTE STRESS

It is well known that specific psychological conditions, like stress and anxiety can modify spinal excitability (Bathien, 1971; Bathien, et. al., 1972, 1978) and the perception of pain (Hall, et. al., 1954; Hill, et. al., 1952).

To establish that endogenous opiates have a significant role in pain inhibition, the natural stressors that activate them have to be identified. The most promising evidence comes from recent studies that have demonstrated that various stressors can cause profound analgesia (Akil, et. al., 1978; Hayes, et. al., 1976). It is theorized that stressful stimuli recruits pain inhibitory mechanism(s), in the central nervous system, which are mediated by opiates. The opiates are thought to mediate the concurrent or subsequent changes in response to stress. However, direct evidence for the involvement of endorphins, is minimal and controversial. Various researchers (Akil, et. al., 1978; Madden, et. al., 1977; Wesche, et. al., 1979) have reported that following foot-shock or heat-stress there is an increase in regional or whole brain enkephalin content. However, Rossier, et. al., (1978) reports a decrease in enkephalin content in the hypothalamus. Fratta, et. al., (1977) were not able to detect any changes in met-enkephalin content in the brain after stress. Chance, et. al., (1977, 1978) and Bonnet, et.

al., (1976) have reported that following psychological stress (i.e., conditioned fear; social isolation) there is an increase in brain opiate receptor occupancy.

It has previously been shown that stress causes release of β-endorphin from the pituitary gland (Guillemin, et. al., 1977). Amir, et. al., (1979) further implicated the importance of pituitary by demonstrating that hypophysectomy blocked stress induced analgesia. This led Frata, et. al., (1977) and Bergland, et. al., (1979) to suggest that β-endorphin released from the pituitary following stress could travel via the hypophyseal portal system back into the areas of the brain that mediate analgesia. If this was the case, β-endorphin levels should be higher in the brain following stress. Rossier and coworkers (1977b) have reported that following foot-shock induced stress, there was a six fold increase in plasma β-endorphin levels but brain β-endorphin levels were not altered (i.e., pituitary β-endorphin did not accumulate in the brain) and the levels of plasma β-endorphin are well below those needed to produce analgesia with systemic β-endorphin administration.

Millan, et. al., (1981) reported that, in rat, stress induced release of both brain and pituitary β-endorphin. They found a 20% decrease in hypothalamus and a 55% decrease in periventricular tissue, an area which represents the primary projection target of the central network of β-endorphinergic neurons. They did not detect any alterations in brain enkephalin content. Bodnar, et. al., (1979) have shown that some forms of stress analgesia are disrupted by lesions of the arcuate nucleus, because the lesion leads to depletion of brain β-endorphin content. Lewis, et. al., (1980) have reported that rats which have developed tolerance to morphine exhibit considerably less stress

induced analgesia. This development of cross tolerance between morphine and stress analgesia provides further evidence that stress induced analgesia is opiate mediated.

The above findings taken together provide considerable evidence that activation of the brain endorphinergic system might be an integral part of the pain relief mechanism during stress. The endorphins released from the pituitary gland concomitantly with adrenocorticotropin hormone in response to stress may play an important role in the global response of the organism to stress.

J. OTHER PHYSIOLOGICAL EFFECTS OF ENDOGENOUS OPIATES

Besides having a prominent role in analgesia and pain relief during stress, the endogenous opiods have various other effects in the central nervous system. Direct injection of β-endorphin into ventromedial hypothalamus leads to food intake in satiated rats (Grandison, et. al., 1977). It also led to increased consumption of liquid diet in midly satiated rats (Kenney, et. al., 1978). β-endorphin, in rats, induces a profound epileptic state (Henriksen, et. al., 1978; Havlicek, et. al., 1978). This epileptic state is the result of action of β-endorphin on limbic structures (French, et. al., 1977). Endorphins also affect the "central thermostat" of the body which is located in the hypothalamus. Body temperature is lowered by α and β-endorphins whereas γ-endorphin elevates it. Naloxone blocks these effects (Bloom, et. al., 1976). However, Stewart and coworkers (1979) have found that direct injection of β-endorphin into the hypothalamus produces hyperthermia. Emotional hyperthermia in rats is also thought to be mediated by endorphins (Blasig, et. al., 1978). Naloxone itself however has no effect on thermoregulatory response

to cold or pyrogen induced hyperthermia (Blasig, et. al., 1979).

Various studies have suggested that endorphins may play an important role in mental diseases (Bloom, et. al., 1976; Terenius, et. al., 1976a). Endorphins have been associated with mania, depression and schizophrenia in humans. It is proposed that endorphins mediate neuronal systems which regulate satisfaction and reward (Stein, et. al., 1978). They have been demonstrated to mediate learning in various learning paradigms, e.g., the conditioned emotional response, preference for signaled shock, conditioned taste aversions and learned helplessness (Riley, et. al., 1980). The central neurotransmitter is also affected by endorphins. Met-enkephalin inhibits release of acetycholine (Jhamandas, et. al., 1977), adrenalin (Taube, et. al., 1976) and substance P (Jessel, et. al., 1977). β-Endorphin causes release of dopamine, increases midbrain serotonin levels (Izumi, et. al., 1977) and decrease turnover of acetycholine in cerebral cortex, hippocampus globus pallidus and nucleus accumbens (Moroni, et. al., 1977). β-Endorphin also has an effect on hypothalamic and anterior pituitary hormones. Injected ICV, β-endorphin causes release of growth hormone, prolactin (Kato, et. al., 1978) and ACTH (Holaday, et. al., 1979). However, contradictory results have been obtained concerning the effects of naloxone on opioid mediated release of growth hormone and prolactin (Gold, et. al., 1975; Martin, et. al., 1979). Thyrotropin-stimulating hormone, luteinizing hormone and follick-stimulating hormone secretion are depressed by endorphins (Bruni, et. al., 1976; Domino, et. al, 1973; Drouva, et. al., 1976).

K. SOMATOSTATIN

Somatostatin was first isolated by Brazeau, Krulick and coworkers (1972) from sheep hypothalamus. It has also been isolated from porcine hypothalamus (Schally, et. al., 1976) and its tetradecapeptide structure determined (Burgus, et. al., 1973; Ling, et. al., 1973). Subsequently, the presence of immunoreactive somatostatin was demonstrated in extra hypothalamic brain, pancreas and the gastrointestinal tract (Vale, et. al., 1976; Arimura, et. al., 1975; Kronheim, et. al., 1976; Patel, et. al., 1975). The distribution of immunoreactive somatostatin in the rat brain from various studies is indexed in Table 12.

a) Role of Somatostatin in Stress

Physiological studies have shown that somatostatin, both in vivo and in vitro, actively inhibit the release of growth hormone (Brazeau, et. al., 1973; Martin, et. al., 1974). In addition it is known to inhibit release of thyrotropin, prolactin (Vale, et. al., 1974) and ACTH from the pituitary (Tyrrell, et. al., 1975), insulin from the pancreas, gastrin from the gut (Bloom, et. al., 1974) and a variety of other exocrine secretions of the pancreas (Boden, et. al., 1975; Creutzfeldt, et. al., 1975) and the stomach (Bloom, et. al., 1974).

Various studies have shown that acute or chronic exposure to stressful stimuli leads to inhibition of growth hormone secretion (Tache', et. al., 1978; Terry, et. al., 1976). This inhibition of pituitary growth hormone secretion was thought to be due to alterations in release of hypothalamic hormones (Tache', et. al., 1976, 1977). Terry, et. al., (1976) thus proposed that growth hormone secretion, from the pituitary, was inhibited during stress due to the release of growth hormone release-inhibiting hormone somatostatin. They demon-

Table 12. Regional Distribution of Immunoreactive Somatostatin in Rat Brain

Brain Regions	Arimura, et. al., 1975 ng/mg wet wt.	Kronheim, et. al., 1976 ng/mg wet wt.	Epelbaum, et. al., 1977 ng/mg wet wt.	Patel, et. al., 1978 ng/mg wet wt.
Hypothalamus	2.12	1.105	1.397	2.3
Median-Emmence			15.47	
Ventromedial Nucleus				0.44
Olfactory Lobe		0.152		0.52
Cerebellum	0.02	0.047		
Cerebral Cortex	0.03	0.098	0.093	0.03
Pineal				
Brain Stem	0.05	0.135		0.67
Spinal Cord		0.452	0.93	0.08
Basolateral Amygdala			0.351	
Corticomedial Amygdala			0.272	
Preoptic Area			0.338	

strated that antiserum to somatostatin prevented the stress-induced inhibition of growth hormone secretion in rats. Terry, et. al, (1980) further investigated the effect of acute stress on regional brain somatostatin content. They found that following acute stress, somatostatin levels in various brain regions (Table 13) significantly decreased.

The exact function of somatostatin mediated inhibition of growth hormone secretion is not known, however, it seems possible that this might play a role in the overall defensive response of the organism to stress.

b) Somatostatin and Analgesia

Havlicek, et. al., (1976) have previously shown that somatostatin and morphine induced central effects that had certain similar behavioural and electrophysiological manisfestations. Subsequently Rezek, et. al., (1977) demonstrated that somatostatin injected ICV produced naloxone reversible analgesia. However, the potency of somatostatin in in vitro opiate binding assays is very minimal. This striking disparity between in vivo analgesic potency and in receptor assay has raised doubts about the interaction of somatostatin with opiate receptors in vivo. However, similar dissociation between in vivo and in vitro activity is also observed for androsterone sulfate (Labella, et. al., 1977) and ACTH (Terenius, 1976b). The mechanism(s) that underlie somatostatin-induced naloxone reversible analgesia are yet unclear. It is possible that somatostatin interacts with a class of receptors which can in turn interact with opiate receptors. It might be that somatostatin evokes release of endogenous opiates which subsequently cause analgesia.

Table 13.

Effects of stress on somatostatin-like immunoreactivity (SLI) in selected brain nuclei in male rats

Brain region	*SLI (pg/μg protein ± S.E.M.)*	
	Control (n = 13)	*Stress (n = 12)*
N. caudatus (medial part)	11.6 ± 0.9	9.6 ± 0.5*
N. accumbens	24.8 ± 1.9	22.4 ± 2.9
N. interstitialis striae terminalis	31.8 ± 2.4	31.9 ± 3.1
N. preopticus medialis	11.9 ± 1.7	10.1 ± 1.6
N. periventricularis	56.8 ± 2.8	53.5 ± 5.5
N. suprachiasmaticus	24.2 ± 2.6	20.5 ± 2.2
Median eminence	679.1 ±96.2	362 4 ±32.6**
N. arcuatus	67.5 ± 7.3	56.9 ± 4.8
N. ventromedialis	17.2 ± 1.5	14.4 ± 1.6
N. amygdaloideus centralis	20.1 ± 3.2	14.6 ± 1.6
N. interpeduncularis	4.2 ± 0.5	4.1 ± 0.5
Periaqueductal gray	13.6 ± 1.3	13.7 ± 1.5

* $P < 0.05$; ** $P < 0.001$ vs sham, based on unpaired Student's *t*-tests.

(Terry,et.al.,1980)

OBJECTIVES

The objectives of this study were to examine:

1. In Vivo alteration in brain β-endorphin and somatostatin levels using protease inhibitor bacitracin.

2. Electroencephalographic changes following alterations in the brain β-endorphin level.

3. The effect of acute stress on brain β-endorphin and somatostatin levels.

4. The occurence of analgesia following exposure to a noxious stimuli.

RATIONALE FOR EXPERIMENTAL APPROACH

Various studies have previously reported the induction of naloxone reversible analgesia by either electrical stimulation of various brain sites, ICV infusion of endogenous opioids or by exposure to stress. In animal studies analgesia is usually inferred from the absence of reflexes or behaviours which are usually elicited by exposure to noxious stimuli (Table 14). Since exposure to noxious stimuli itself cause analgesia, a major problem in previous studies lies in dissociating the analgesia caused by infusion of opioids and that due to release of endogenous opioids as a consequence of the analgesia testing method. In our study of analgesia we chose an analgesia testing paradigm which by itself did not lead to analgesia.

Protease inhibitors are widely used to protect against degradation of peptides and hormones in in vitro studies (Nars, et. al., 1972; Trautschold, et. al., 1967). Trasylol (Trautschold, et. al., 1967), L-1-tosylamide-2-phenylethyl chloromethyl ketone, P-tosyl-L-arginine methyl ester-HCl (Richert, et. al., 1977), ε-amino caproic acid (Parson, et. al., 1977), thiorphan (Roques, et. al., 1980), puromycin (Schwartz, et. al., 1980), catopril (Cushman, et. al., 1977), Benzyloxycarbonyl derivaties of amino acid hydroxamates (Blumberg, et. al., 1981), Bacitracin (Patthy, et. al., 1977) and various other (Partington, et. al., 1979; Patey, et. al., 1981) protease inhibitors have been in wide use. Parson and coworkers (1977, 1979) have demonstrated that protease inhibitors protect subcutaneously injected peptide hormones against local degradation. Miller, et. al., (1977) have shown that bacitracin potentiated the effect of enkephalins in vitro. Bacitracin has also been shown to decrease biodegradation

Table 14

NOCICEPTIVE STIMULATION IN ANALGESIC TESTS

Method of stimulation	*Site of stimulation*	*Species*	*Response*	*References*
Mechanical				
Hair or bristle	Skin	Man	Verbal report	von Frey (1895, 1922), Head (1920)
Hair or bristle	Muco-epithelial junctions	Man	Verbal report	Seevers and Pfeiffer (1936)
Hair or bristle	Cornea	Rabbit	Extension of local anesthesia with nupercaine following IV admin. of analgesic	Hotovy (1956)
Pinch	Tail	Rat	Vocalization, biting, struggling	Barbour and Maurer (1920)
Pressure, gross	Tail	Cat	Vocalization, biting, struggling	Eddy (1928)
Pressure, clip	Tail, toe	Mouse, rat	Vocalization, biting, struggling	Haffner (1929)
Pressure, clip	Multiple toes	Guinea pig	Vocalization, biting, struggling	Collier *et al.* (1961)
Pressure, forceps	Tail	Mouse, rat	Vocalization, biting, struggling	Molitor and Latven (1937), Friend and Harris (1948), Chen (1956)
Pressure, rod	Tail	Rat	Vocalization, biting, struggling	Eagle and Carlson (1950), Brodie *et al.* (1952)
Electrical				
Electrodes	Skin	Man	Verbal report	von Helmholtz (1851)
Electrodes	Skin	Rabbit	Skin twitch	DeSalva and Monteleone (1963)
Electrodes	Scrotum	Rat	Vocalization, biting, struggling	Macht and Macht (1940)
Electrodes	Tail	Rat	Vocalization, biting, struggling	Burn *et al.* (1950)
Electrodes	Tail	Rat	Vocalization, biting, struggling	Charpentier (1961a,b, 1963)
Electrodes	Tooth pulp	Dog	Head jerk, vocalization	Koll and Keffert (1938), Koll (1952)
Electrodes	Tooth pulp	Dog, (man)	Head jerk, vocalization (verbal report)	Goetzl *et al.* (1943)
Electrodes	Tooth pulp	Dog	Head jerk, chewing	Soehring and Becher (1949)
Electrodes	Tooth pulp	Rabbit	Head jerk	Boreus and Sandberg (1955)
Electrodes	Tooth pulp	Guinea pig	Head jerk, vocalization	Yim *et al.* (1955)
Floor Grid	Feet	Rat	Vocalization, biting, struggling	Radouco-Thomas *et al.* (1956)
Floor Grid	Feet	Rat	Flinch-jump, vocalization	Sivadjian (1936)
Electrodes	Rectum	Rat	Vocalization, biting, struggling	Evans (1961)
Thermal				Gibson *et al.* (1955), Collins *et al.* (1964)
Radiant heat	Skin	Man	Verbal report	Goldscheider (1884), Alrutz (1897)
Radiant heat	Forehead; skin blackened	Man	Verbal report	Oppel and Hardy (1937), Hardy *et al.* (1940, 1952)
Radiant heat	Tail	Rat	Tail flick	D'Amour and Smith (1941), Davies *et al.* (1946)
Radiant heat	Dorsum	Rat	Dorsal skin flinch	Ercoli and Lewis (1945), Cahen *et al.* (1948)
Radiant heat	Dorsum, natural dark skin	Guinea pig	Dorsal skin flinch	Winder *et al.* (1946)
Radiant heat	Dorsum	Dog	Dorsal skin flinch	Andrews and Workman (1941)
Hot plate	Feet	Mouse	Dancing, licking or blowing on forepaws, escape	Woolfe and Macdonald (1944), Eddy *et al.* (1950), Eddy and Leimbach (1953), Chen and Beckman (1951)

(Lim, et.al., 1967)

of β-endorphin in vitro studies (Patthy, et. al., 1977) by inhibiting the activity of brain carboxypeptidase(s) and aminopeptidase(s). In view of the studies with bacitracin, it was decided to use bacitracin, in vivo, to alter endogenous β-endorphin levels in the brain. Our contention was that if bacitracin could decrease brain β-endorphin biodegradation, it should lead to an increase in β-endorphin content in the brain. We could subsequently examine if higher levels of brain β-endorphin could cause or facilitate the occurence of naloxone reversible analgesia.

To substantiate the biochemical effects of bacitracin on brain β-endorphin content, it was deemed necessary to do an electrophysiological study since, at least in the rat, cortical and subcortical EEG recordings have been found to be a highly sensitive index of central endorphin action.

Since, we had decided to use a stress-inducing paradigm which did not itself cause analgesia (i.e., control rats subjected to this stressful exposure pattern did not exhibit analgesia), the elevated levels of brain β-endorphin in bacitracin injected rats would provide an ideal model for examining the effect of β-endorphin in inducing naloxone reversible opiate analgesia.

MATERIALS AND METHODS

A. PREPARATION OF CM-23 CELLULOSE COLUMN

a) Activation of CM-23 Cellulose Ion-exchange Material

A weighed amount of CM-23 cellulose (Sigma Chemical Co.) ion-exchange material was stirred in 15 vols. (i.e., volume of liquid/dry weight of CM-23) of 0.5 N NaOH and was allowed to stand for 30 minutes. Then the supernatant was decanted and the CM-23 cellulose ion-exchange material washed with distilled water, in a funnel, until the effluent was at intermediate pH (i.e., pH = 8). Then the CM-23 cellulose was stirred in 15 vols. of 0.5 N HCl and allowed to stand for 30 minutes. The supernatant was then decanted and the CM-23 cellulose washed with distilled water until the effluent was at pH 5.3. The incubation of CM-23 cellulose with 0.5 N HCl and the subsequent washing was repeated. Then a 0.02% (w/v) concentration solution of NaN_3 was added to the CM-23 cellulose ion-exchange material which was then stored at $4^{o}C$ until use.

b) Packing of CM-23 Cellulose Column

A chromatographic column (0.7 cm i.d. x 20 cm length - Bio-Rad, Mississauga) was packed with activated CM-23 cellulose. This involved pouring the slurry of CM-23 cellulose ion-exchange material into the column which had been previously equilibrated at $4^{o}C$. The column was then eluted at a rate of 1 drop/5 seconds with 0.002 M ammonium acetate buffer at pH 4.6 until the pH of the effluent was within 0.02 pH units of the pH of the buffer.

B. IODINATION OF β-ENDORPHIN

Camel β-endorphin was radio-iodinated using a modified lactoperoxidase method of Thorel and Johannson (1971). To a test tube containing 5 µg of camel β-endorphin (Peninsula Labs, San Carlos, California) dissolved in 5 µl of 0.1 M acetic acid, the following were added:

1. 25 µl of 0.4 M sodium acetate (pH = 5.6)
2. 25 µl of (1 mCi) of $Na^{125}I$ (New England Nuclear)
3. 25 µl of (25 µg) of lactoperoxidase (Calbiochem, La Jolla, Calif.) is dissolved in 0.1 M acetic acid of pH 5.6)
4. 10 µl of 30% H_2O_2 at 1:15,000 dilution
   wait for 1.5 minutes
5. 10 µl of 30% H_2O_2 at 1:15,000 dilution
   wait for 1.5 minutes
6. 1.0 ml of 0.002 M ammonium acetate (pH = 4.6)
7. 50 µl of 5% (w/v) bovine serum albumin (BSA) (Sigma Chemical Co.)

This reaction mixture was then poured into the CM-23 cellulose ion-exchange column. As soon as the reaction mixture was added to the column, collection of 75-drop fractions was begun. Before collection of fractions, 3 drops of Trasylol (10,000 K.I.U./ml - Boehringer Ingelhein, Canada) and 3 drops of 5% BSA were added to the collecting tubes. Fractions 1-7 were obtained by elution of the CM-23 cellulose packed column with 0.002 M ammonium acetate (pH = 4.6). Fractions 8-26 were obtained by elution of the column with 0.2 M ammonium acetate (pH = 4.6) and fractions 21-60 were obtained by elution of the column with 2 M ammonium acetate (pH = 4.6). The elution profile

of camel β-endorphin iodination reaction mixture is shown in Figure 14. The immunoactivity of each fraction from the third peak of radioactivity was determined by a double antibody radioimmunoassay (RIA).

C. RADIOIMMUNOASSAY FOR β-ENDORPHIN

The RIA was carried out in glass test tubes (10x75 mm - Fisher). The diluent for all reagents was 0.14 M sodium phosphate buffer containing 25 mM EDTA and 0.5 per cent BSA, pH 7.4. The following was added to the test tubes:

1. 100 µl of 0.14 M sodium phosphate buffer (pH = 7.4)
2. 100 µl of 1:2 Trasylol solution
3. 100 µl of Camel β-endorphin standards or sample. Camel β-endorphin standards were of the following concentrations: 0, 0.1, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 7.5, 10, 25, 50, 75, 100, 250, and 500 ng/ml
4. 100 µl of 1:10,000 rabbit anti-β-endorphin serum (1^{st} antibody)

To determine nonspecific binding, neither standards nor rabbit anti-β-endorphin serum were added to the above reaction ingredients.

The above mixture was then incubated for 24 hours at $4^{o}C$ after which the following was added:

5. 100 µl of 20,000-30,000 cpm of tracer

After another incubation for 24 hours at $4^{o}C$ the following ingredients were added:

6. 100 µl of 1:15 sheep anti-rabbit serum (2^{nd} antibody)
7. 100 µl of 1:350 normal rabbit serum

After incubation for 24 hours at $4^{o}C$ the final additive was:

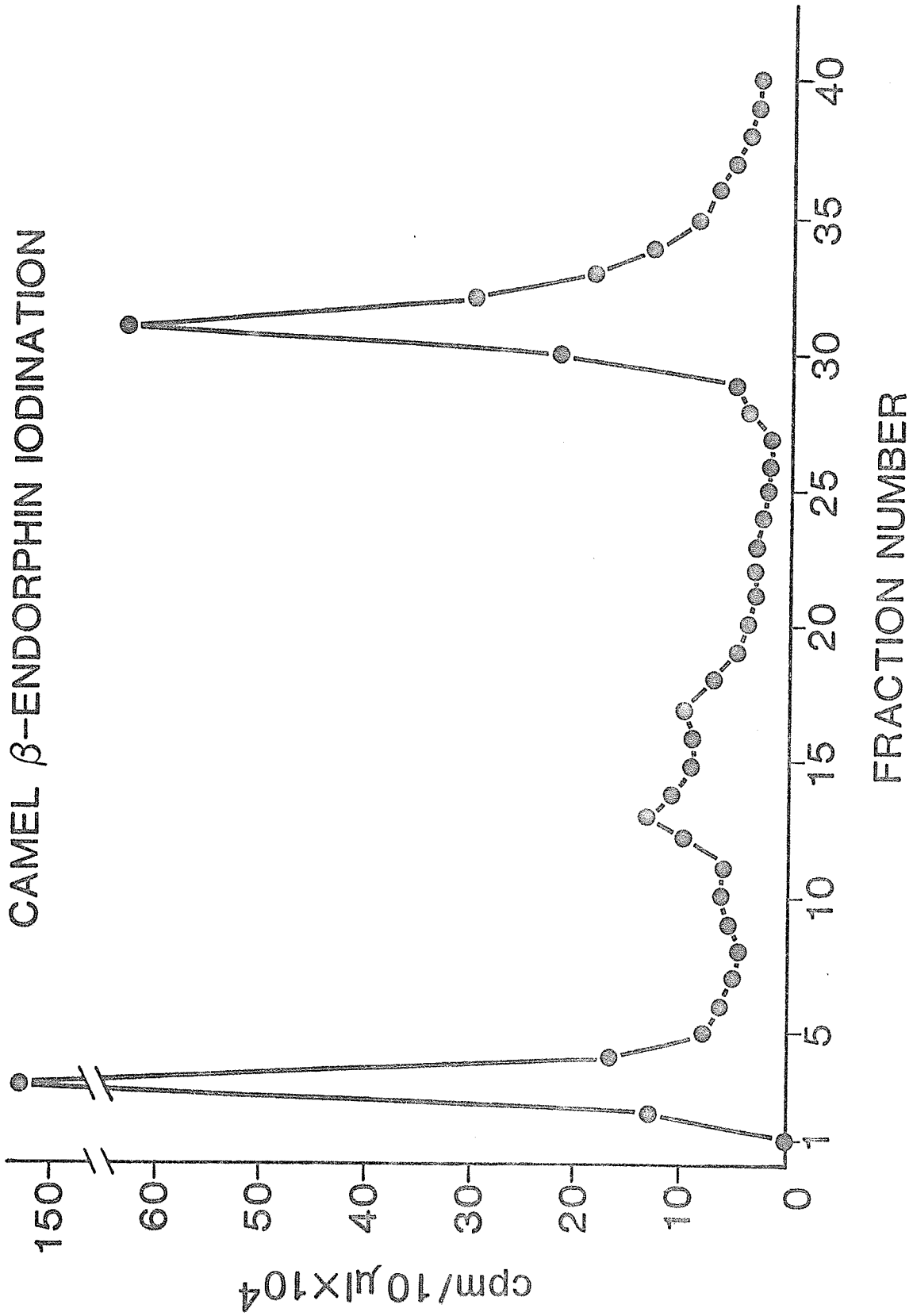
8. 1.0 ml of 0.14 M sodium phosphate buffer (pH = 7.4)

Figure 14: Elution profile of Camel β-endorphin Iodination Reaction Mixture from CM23 Cellulose Chromatographic Column

The first peak represnts free iodine.

The second peak represents damaged tracer.

The third peak represents immunoreactive β-endorphin tracer.


CAMEL β-ENDORPHIN IODINATION
cpm/10 μl x 10⁴
150
60
50
40
30
20
10
0
1
5
10
15
20
25
30
35
40
FRACTION NUMBER

This above reaction mixture was then centrifuged at 3,200 xg for 30 minutes. The supernatant was then decanted and the pellet counted in an automatic Beckman gamma counter for one minute. Specific binding of greater than 40 per cent and nonspecific binding of less than 10 per cent is considered essential for this assay. The sensitivity of this assay ranges from 0.25-0.5 ng/ml as shown in Figure 15 and the standard curve is linear between 0.5 to 40 ng/ml. Anti camel-β-endorphin-specific antibody was generated in rabbits by using conventional immunological procedures (Yoshimi, et. al., 1978). The cβ-endorphin antiserum generated does not cross-react with α- and γ-endorphin, both enkephalins, α-MSH, ACTH, vassopressin, insulin, glucagon, TRH, LH-RH, bombesin, myelin basic protein, growth hormone, prolactin, morphine and naloxone. However, it has a 50 per cent cross-reactivity with ovine β-lipotropin (Fig. 16 - Lee, 1979; Ogawa, et. al., 1979). The intrassay variation ranges from 3 to 5.5% and the interassay variation ranges from 15.3 to 18.1% (Table 15).

D. IODINATION OF SOMATOSTATIN

Tyr^1-somatostatin was radio-iodinated using a modified lactoperoxidase method of Epelbaum, et. al., (1977). To a test tube containing 5 µg of Tyr^1-somatostatin (SRIF - Peninsula Labs, San Carlos, California) dissolved in 5 µl of 0.1 M acetic acid the following were added:

1. 25 µl of 0.4 M sodium acetate (pH = 5.6)
2. 25 µl (1 mCi) of $Na^{125}I$ (New England Nuclear Co.)
3. 25 µl (25 µg) of lactoperoxidase (dissolved in 0.1 M acetic acid)
4. 10 µl of 30% H_2O_2 at 1:15,000 dilution

Figure 15: Standard curve for β-endorphin RIA. Serial dilutions of brain extracts yield curves parallel to the standard curve

Circles: Hypothalamic extracts (diluted 1:10)

Triangles: Midbrain extracts

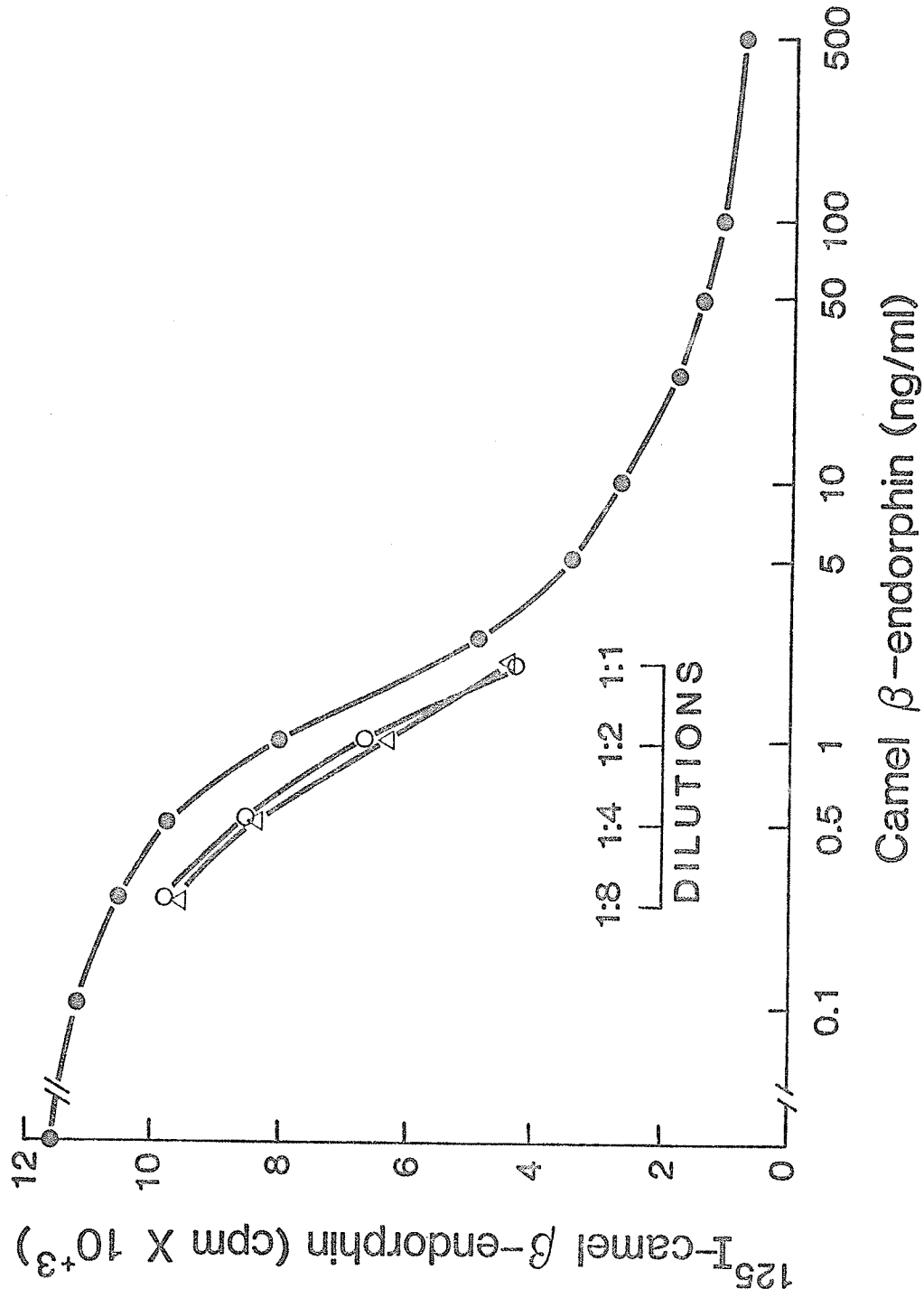

^{125}I–camel β–endorphin (cpm X 10^{+3})
12
10
8
6
4
2
0
0.1
0.5
1
5
10
50
100
500
Camel β–endorphin (ng/ml)
DILUTIONS
1:8
1:4
1:2
1:1

Table 15. Intraassay and Interassay Variation of Radioimmunoassay for β-Endorphin

β-Endorphin concentration in ng/ml of samples a, b, and c

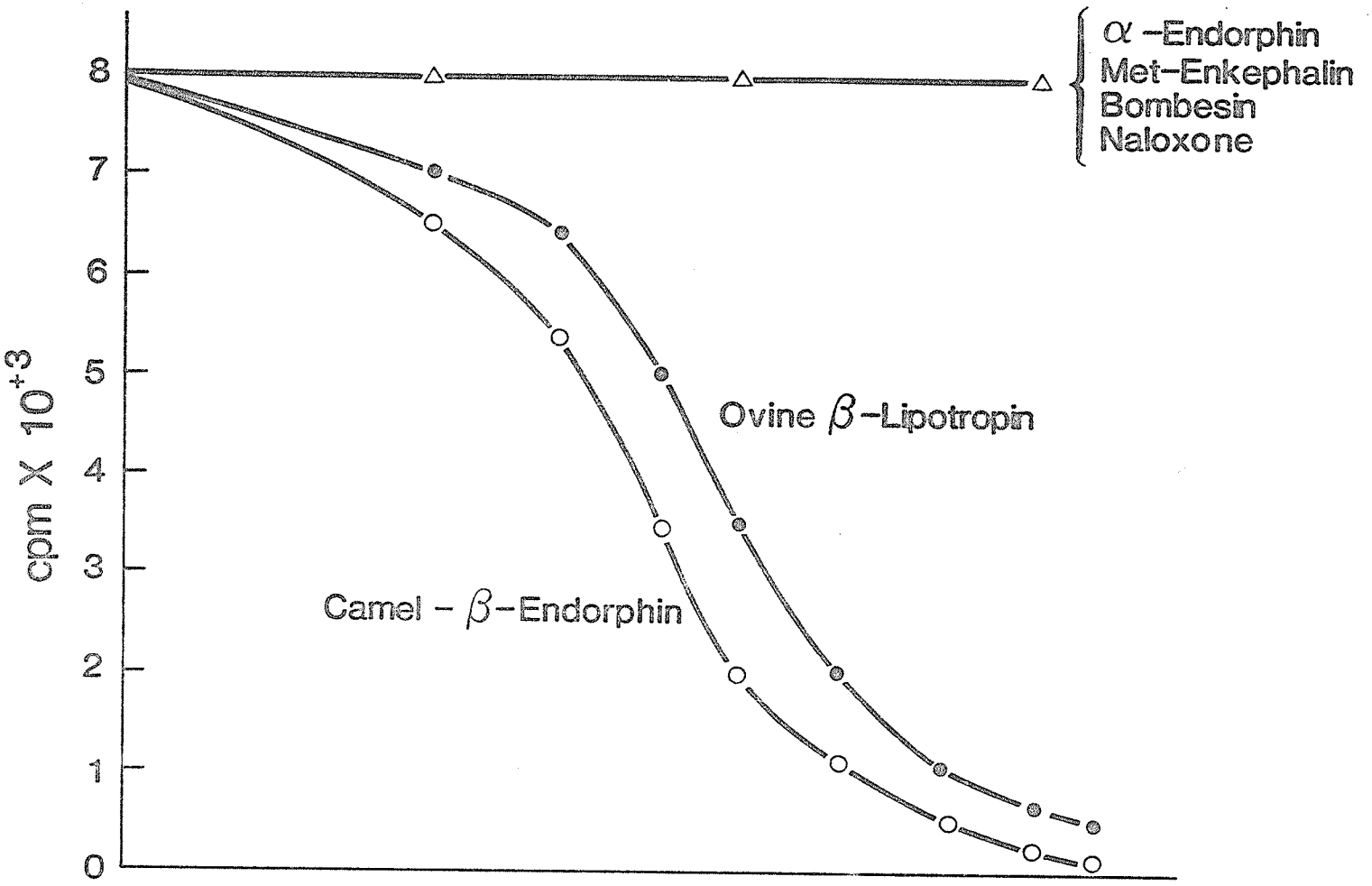
	Intraassay			Interassay		
	a	b	c	a	b	c
	10.6	4.4	.79	10.4	5.5	.71
	11.2	5.0	.80	9.1	3.9	.80
	10.6	4.7	.83	11.6	6.1	.69
	9.8	5.1	.76	8.6	5.0	.95
	9.5	5.3	.79	12.4	5.4	1.12
	10.7	4.6	.82	8.6	6.2	.75
	11.0	4.7	.83	10.14	5.85	.96
	9.6	4.9	.79	14.6	6.1	.68
	9.9	5.2	.76	9.4	4.1	.65
	10.5	4.8	.79	8.69	4.9	.81
$\overline{X}_S$	10.34	4.87	0.769	10.35	5.46	0.812
SD_S	0.566	0.269	0.024	1.88	0.831	0.144
CV	5.4%	5.5%	3.0%	18.1%	15.3%	17.7%

$\overline{X}_S$ = Mean concentration (ng/ml) of sample

SD_S = Standard deviation of sample mean

CV = Co-efficient of variation ($SD_S/\overline{X}_S$)

Figure 16. Characteristics of Camel ß-Endorphin Antiserum



(Lee, 1979)

Wait for 1.5 minutes

5. 10 µl of 30% H_2O_2 at 1:15,000 dilution

   Wait for 1.5 minutes

6. 100 µl of 200 µg/ml sodium azide solution made in 0.002 M ammonium acetate solution of pH 4.6

7. 1.0 ml of 0.002 M ammonium acetate (pH 4.6)

8. 50 µl of 5% (w/v) bovine serum albumin

The reaction mixture was then poured into the chromatographic column which had previously been packed with CM-23 cellulose ion-exchange material. As soon as the reaction mixture was added to the column, collection of 75-drop fractions was begun. Before collection of fractions, 3 drops of Trasylol (10,000 K.I.U./ml - Boehringer Ingelheim, Canada) and 3 drops of 5 per cent BSA were added to the collecting tubes. Fractions 1-8 were obtained by elution of the CM-23 cellulose ion-exchange material packed column with 0.002 M ammonium acetate of pH 4.6. Fractions 9-40 were obtained by elution of the column with 0.2 M ammonium acetate of pH 4.6. The elution profile of Tyr^1-somatostatin iodination reaction mixture chromatographed on CM-23 cellulose is shown in Fig. 17. The specific activity of each fraction from the second peak of radioactivity was determined by a double antibody radioimmunoassay.

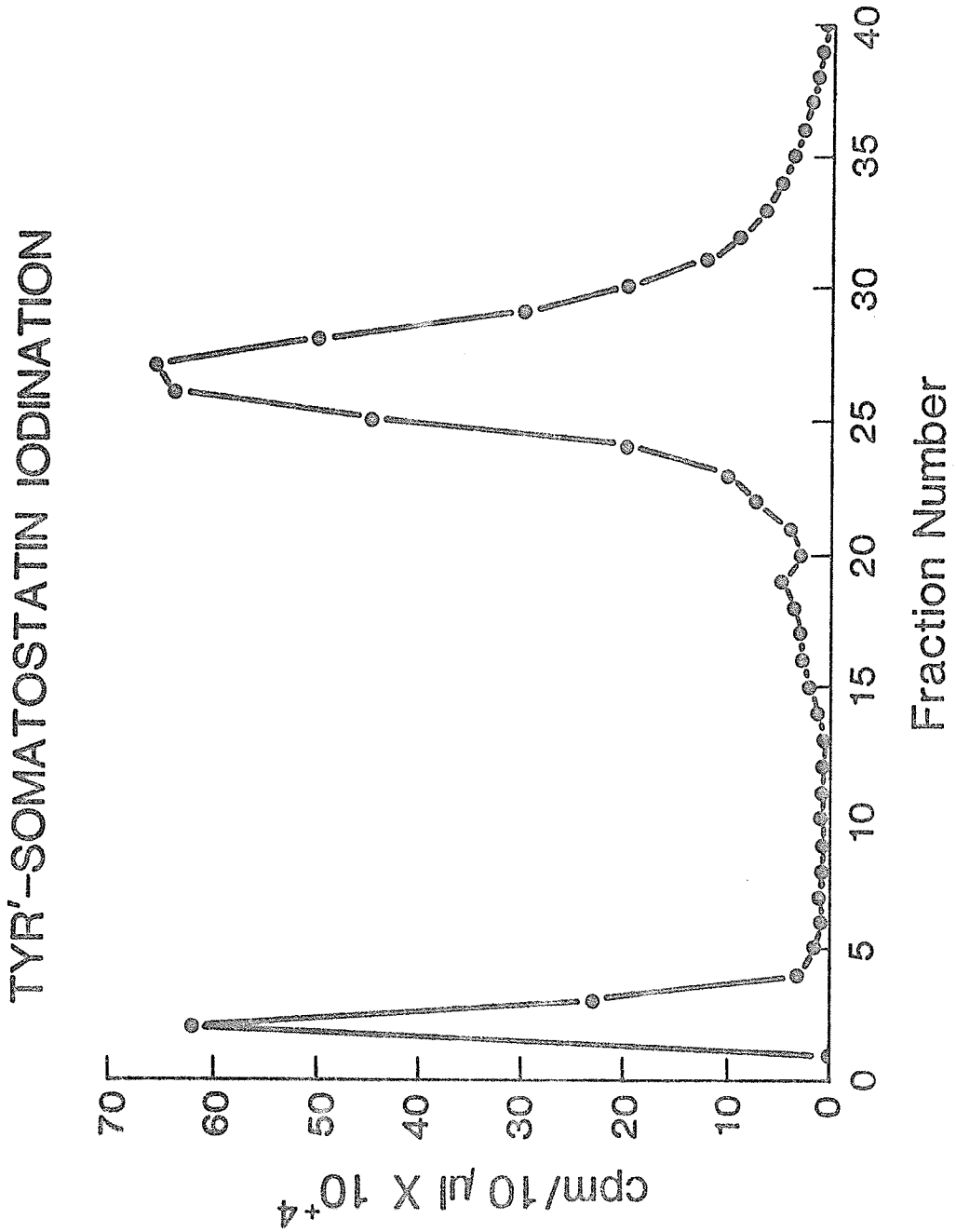
E. RADIOIMMUNOASSAY FOR SOMATOSTATIN

The diluent for all reagents was 0.14 M sodium phosphate buffer containing 25 mM EDTA and 0.5 per cent BSA, pH 7.4. The double antibody RIA was carried out in glass test tubes (10x75 mm - Fisher). The following were added to the test tubes:

Figure 17: Elution Profile of Tyr-somatostatin Iodination Reaction Mixture from CM23 Cellulose Chromatographic Column

The first peak represents free iodine.

The second peak represents immunoreactive somatostatin tracer.


TYR'-SOMATOSTATIN IODINATION
cpm/10 µl X 10⁺⁴
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
Fraction Number

1. 100 µl of 0.14 M sodium phosphate buffer (pH 7.4)
2. 100 µl of 1:2 Trasylol solution
3. 100 µl of somatostatin standards or sample. Somatostatin standards were of the following concentrations: 0, 0.1, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 7.5, 10, 25, 50, 75, 100, 250, and 500 ng/ml
4. 100 µl of 1:10,000 rabbit anti-somatostatin serum (1^{st} antibody)

To determine nonspecific binding, no standards nor rabbit anti-somatostatin serum were added to the above ingredients.

The above mixture was then incubated for 24 hours at room temperature after which the following was added.

5. 100 µl of 20,000-30,000 cpm tracer

After another incubation for 24 hours at room temperature the following ingredients were added:

6. 100 µl of 1:15 sheep anti-rabbit serum
7. 100 µl of 1:350 normal rabbit serum

After incubation for 24 hours at room temperature the final additive was:

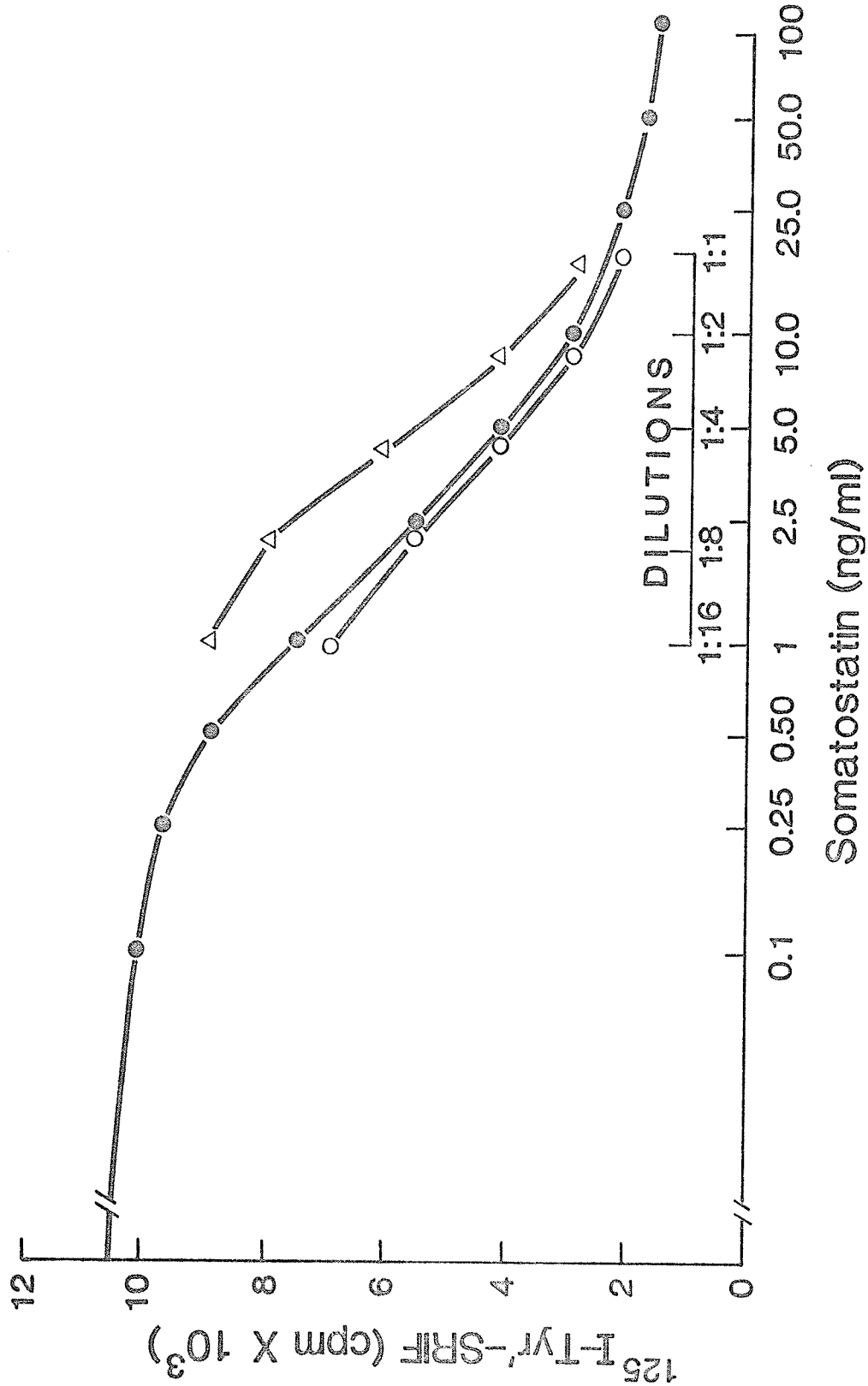
8. 1.0 ml of 0.14 M sodium phosphate buffer (pH = 7.4)

This above reaction mixture was then centrifuged at 3,200 xg for 40 minutes. The supernatant was then decanted and the pellet counted in an automatic Beckman gamma counter for one minute. Specific binding of greater than 40 per cent and nonspecific binding of less than 5 per cent is considered to be essential for this assay. The sensitivity of this assay ranges from 0.1 - 0.5 ng/ml as shown in Figure 18, and the standard curve is linear between 1 - 40 ng/ml. The antisomatostatin-specific antibody was generated in rabbits by immunizing them with somatostatin conjucated with bovine albumin.

Figure 18: Standard Curve for Tyr-somatostatin RIA. Serial Dilutions of Brain Extracts Yield Curves Parallel to the Standard Curve

Circles: Hypothalamic extracts

Triangles: Cortical extracts


^{125}I-Tyr'-SRIF (cpm X 10^3)
12
10
8
6
4
2
0
0.1
0.25
0.50
1
2.5
5.0
10.0
25.0
50.0
100
Somatostatin (ng/ml)
DILUTIONS
1:16
1:8
1:4
1:2
1:1

This antisomatostatin antibody showed no crossreactivity with TRH, LHRH, enkephalins, α-endorphin, β-endorphin, β-LPH, ACTH, porcine glucagon, vassopressin, oxytocin, secretin, prolactin, growth hormone, luteinizing hormone and FSH (Havlicek ant Friesen, 1979). The intra-assay variation ranges from 3.0 to 5.2 per cent while the interassay variation ranges from 9.0 - 13.7 per cent (Table 16).

F. PREPARATION OF BRAIN SAMPLES

Animals were placed in a transparent plastic box (5" x 11" x 6½") which was then placed in a microwave oven (Philips model HN1124). The animals were sacrificed by microwave irradiation at the power of 2.2 kw for 15 seconds followed by 1.1 kw for 10 seconds. The brains were quickly removed and when necessary dissected on ice according to modified guidelines of Glowinski and Iversen (1966). The method of brain dissection employed is illustrated in Figure 19. The weight of the whole brain or dissected parts of the brain were determined on a Mettler H51 balance. Individual brain areas were homogenized in 2 ml glass homogenizers (Fisher Instruments) and individual whole brains were homogenized in 7 ml glass homogenizers (Fisher Instruments). The homogenizing solution used was 0.1 N acetic acid (Table 17). The homogenate was then centrifuged at 3,200 xg for 30 minutes. The supernatant was collected and the pellet resuspended in original homogenizing volume of 0.1 N acetic acid, and centrifuged again at 3,200 xg for 30 minutes. The second supernatant was combined with the first for peptide analysis. The brain extracts were then frozen at -70°C and thawed one week later for radioimmunoassay.

Table 16. Intraassay and Interassay Variation of Radioimmunoassay for Somatostatin

Somatostatin concentration in ng/ml of samples d, e, f, g, and h

	Intrassay			Interassay		
	d	e	f	g	h	f
	9.8	4.6	.84	13.25	3.1	.84
	9.6	4.9	.80	11.64	2.4	.69
	10.1	5.1	.81	15.36	2.6	.71
	9.9	5.3	.79	16.79	2.45	.73
	10.4	5.1	.85	17.46	2.57	.89
	9.3	5.6	.76	15.73	2.8	.80
	9.7	4.8	.81	14.25	2.7	.85
	10.6	5.0	.80	18.10	2.96	.71
	11.3	5.2	.81	17.45	2.1	.79
	9.1	5.0	.79	12.8	2.63	.69
$\overline{X}_S$	9.98	5.08	0.806	15.28	2.611	0.77
SD_S	0.486	0.266	0.024	2.10	0.294	0.697
CV	4.8%	5.2%	2.99%	13.7%	11.2%	9.05%

$\overline{X}_S$ = Mean concentration (ng/ml) of sample

SD_S = Standard deviation

CV = Co-efficient of variation ($SD_S/\overline{X}_S$)

Figure 19: Rat Brain Dissection Procedure

a. Cross-sections c, d, and e were cut downwards from the basal surface of the brain, thereby separating cerebellum, hindbrain, mid-section and frontal brain respectively. The olfactory bulbs, located rostrally to section e, were removed. Cuts were made at a + f and g to separate sections from hypothalamus. Brain regions are coded numerically as follows:

1 - cerebellum
2 - hindbrain
3 - striatum
4 - septal nuclei
5 - amygdala
6 - hypothalamus
7 - thalamus
8 - midbrain
9F - frontal cortex
9P - parietal cortex
10 - hippocampus
11 - pituitary
12 - nucleus accumbens
13 - pyriform cortex
14 - entorhinal cortex

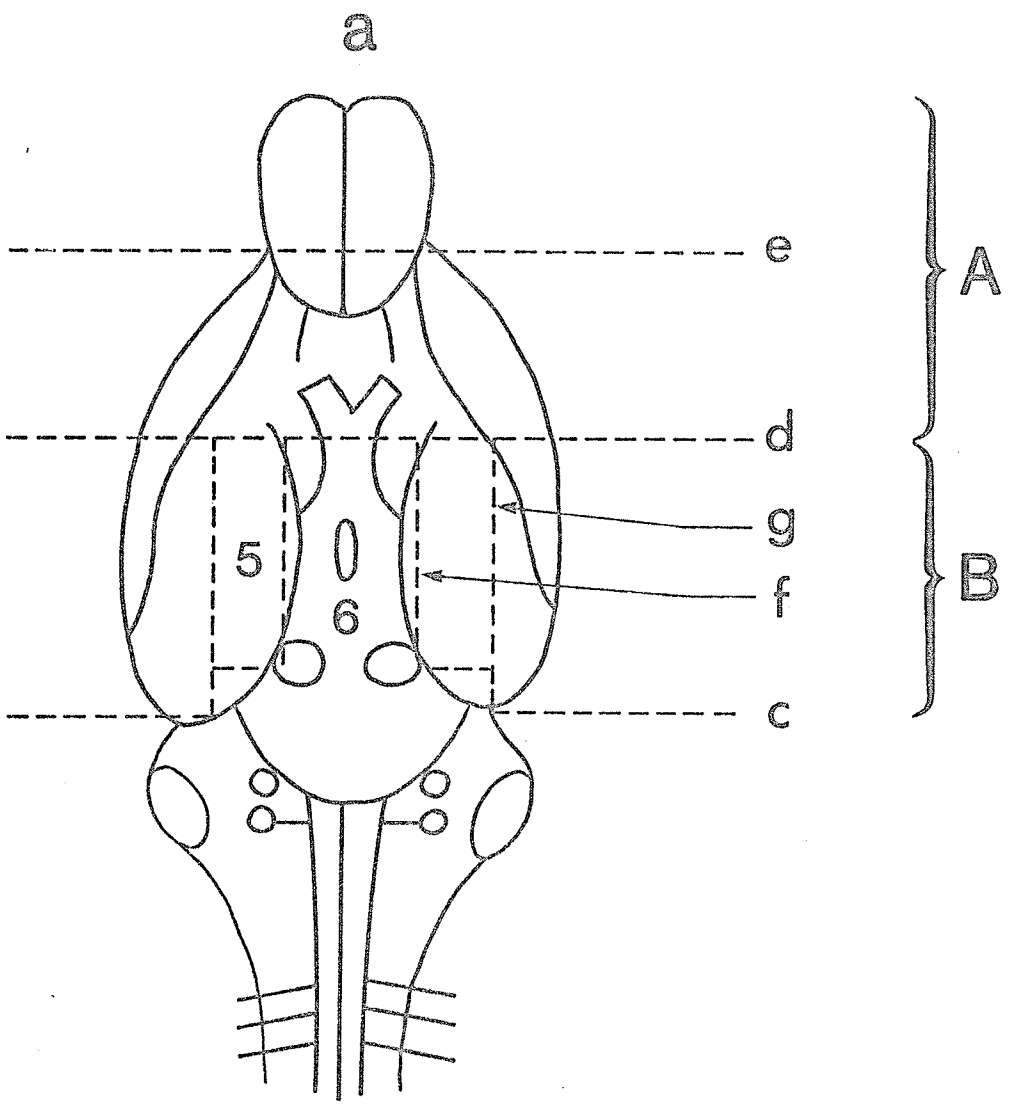
b. Lateral view of the rat brain indicating location of each brain region. The frontal section of the brain is designated section A and the midsection is designated section B.

c. Cross-sectional view of section A as viewed from dissection cut d.

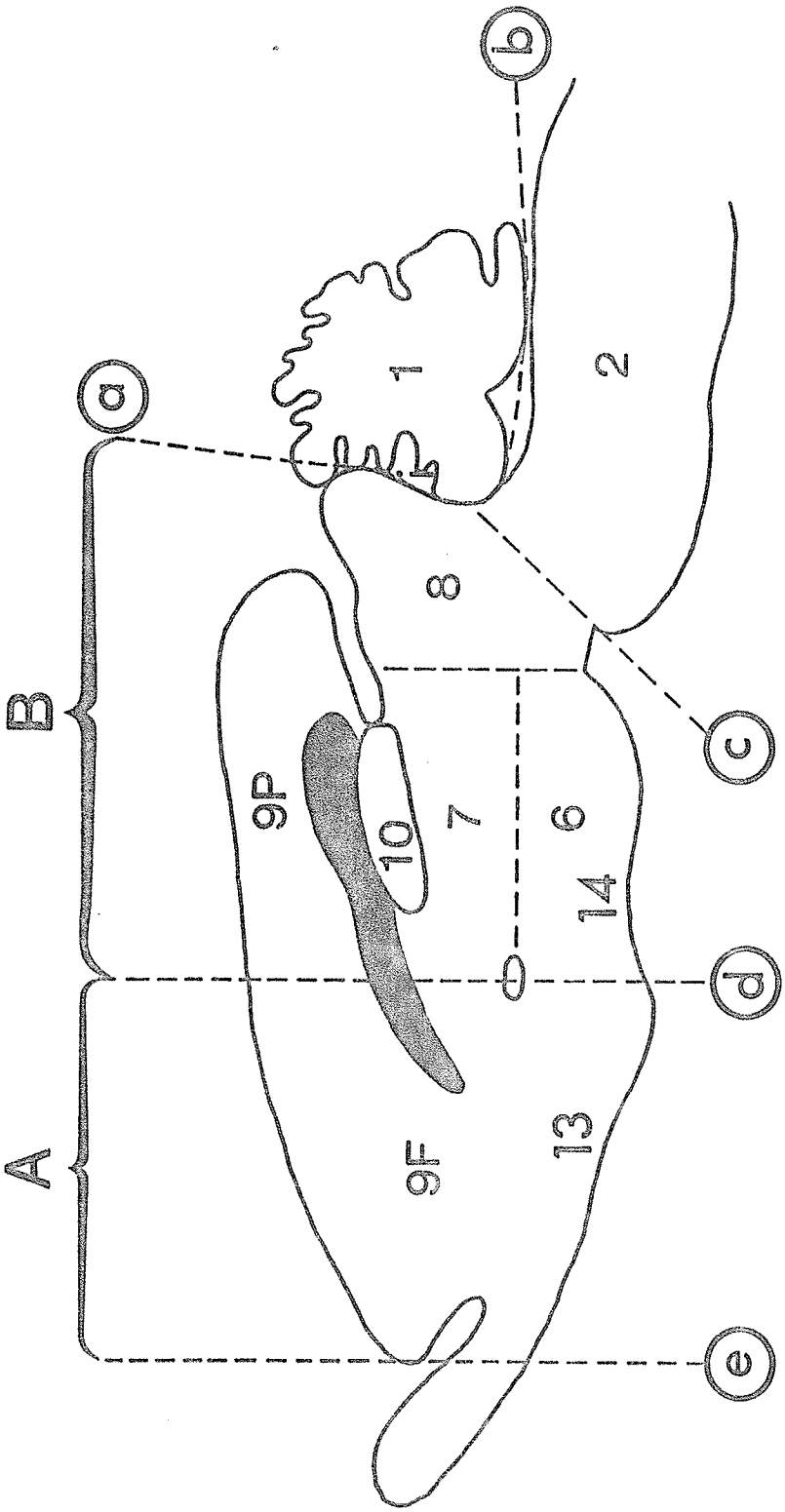
d. Cross-sectional view of section B as viewed from dissection cut d.

(Glowinski and Iverson, 1966)

Figure 19.



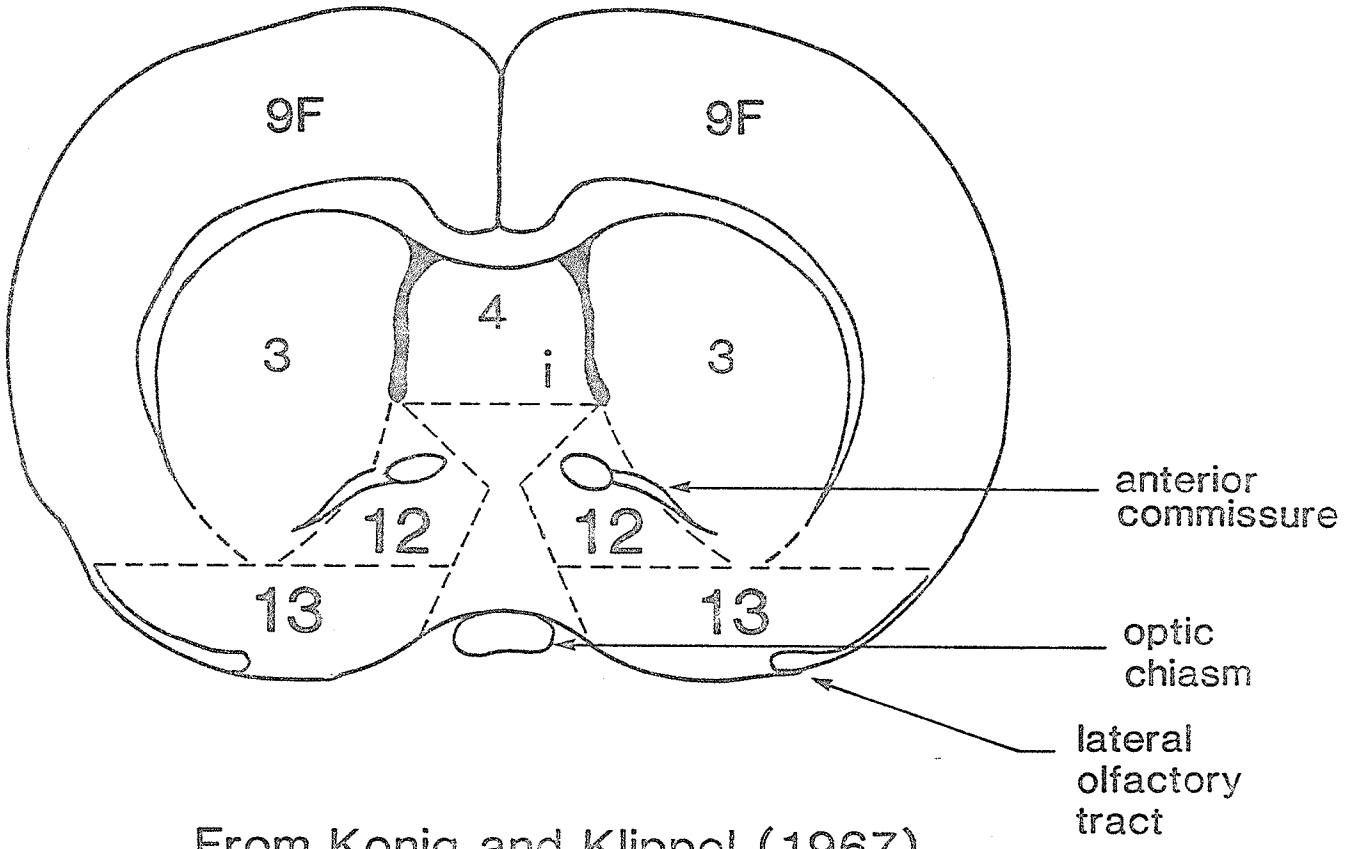
From Glowinski and Iversen (1966)



From Glowinski and Iversen (1966)

b

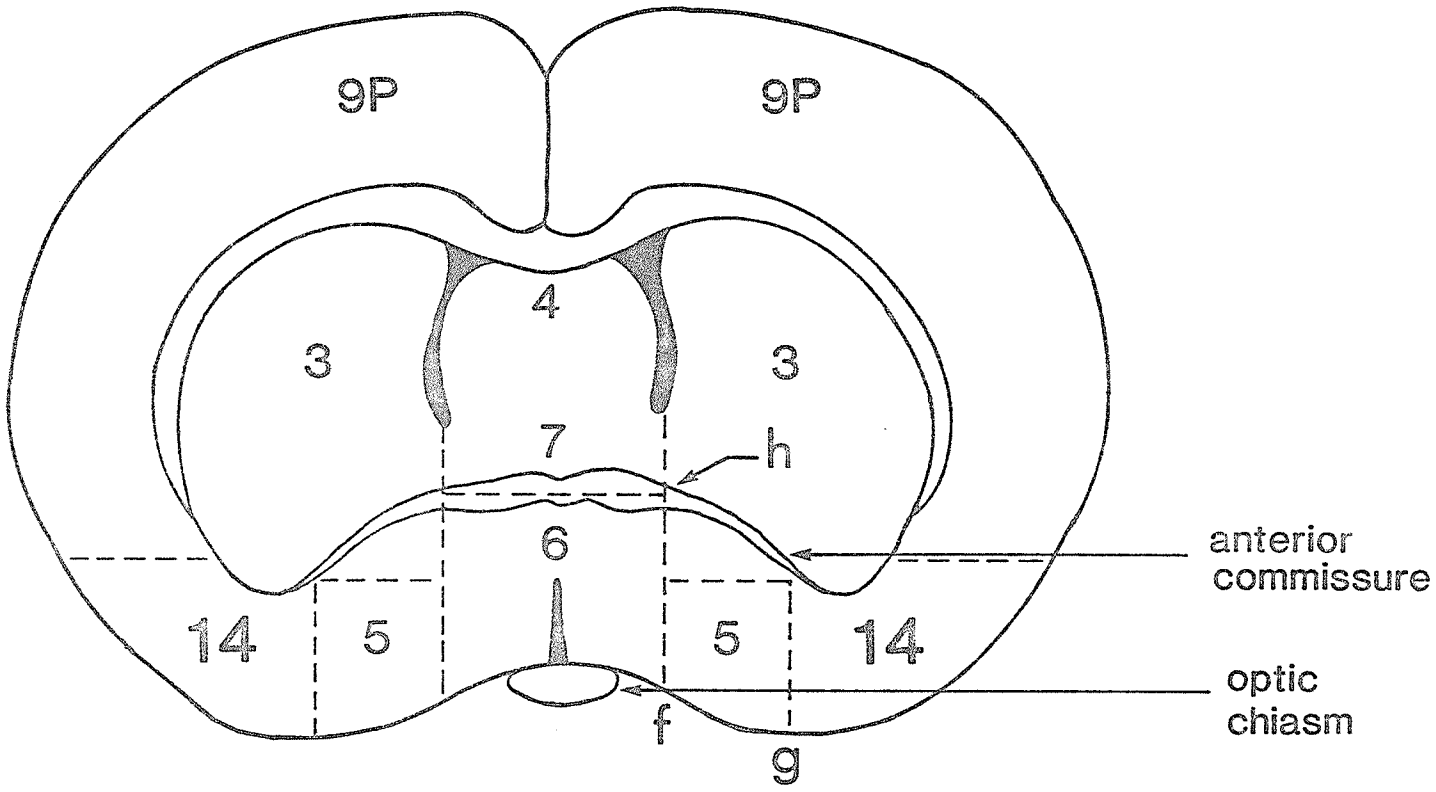
SECTION A



From Konig and Klippel (1967)

C

SECTION B



From Konig and Klippel (1967)

d

Table 17

	Brain Region	Volume of 0.1 N Acetic Acid Used per Extraction
1.	N. accumbens	1.0 ml
2.	N. septalis	1.0
3.	Thalamus	1.0
4.	Amygdala	1.0
5.	Cerebellum	1.5
6.	Hindbrain	1.5
7.	Hippocampus	1.5
8.	Striatum	1.5
9.	Midbrain	1.5
10.	Cortex	3.0
	Whole brain	7.0

G. CANNULATION

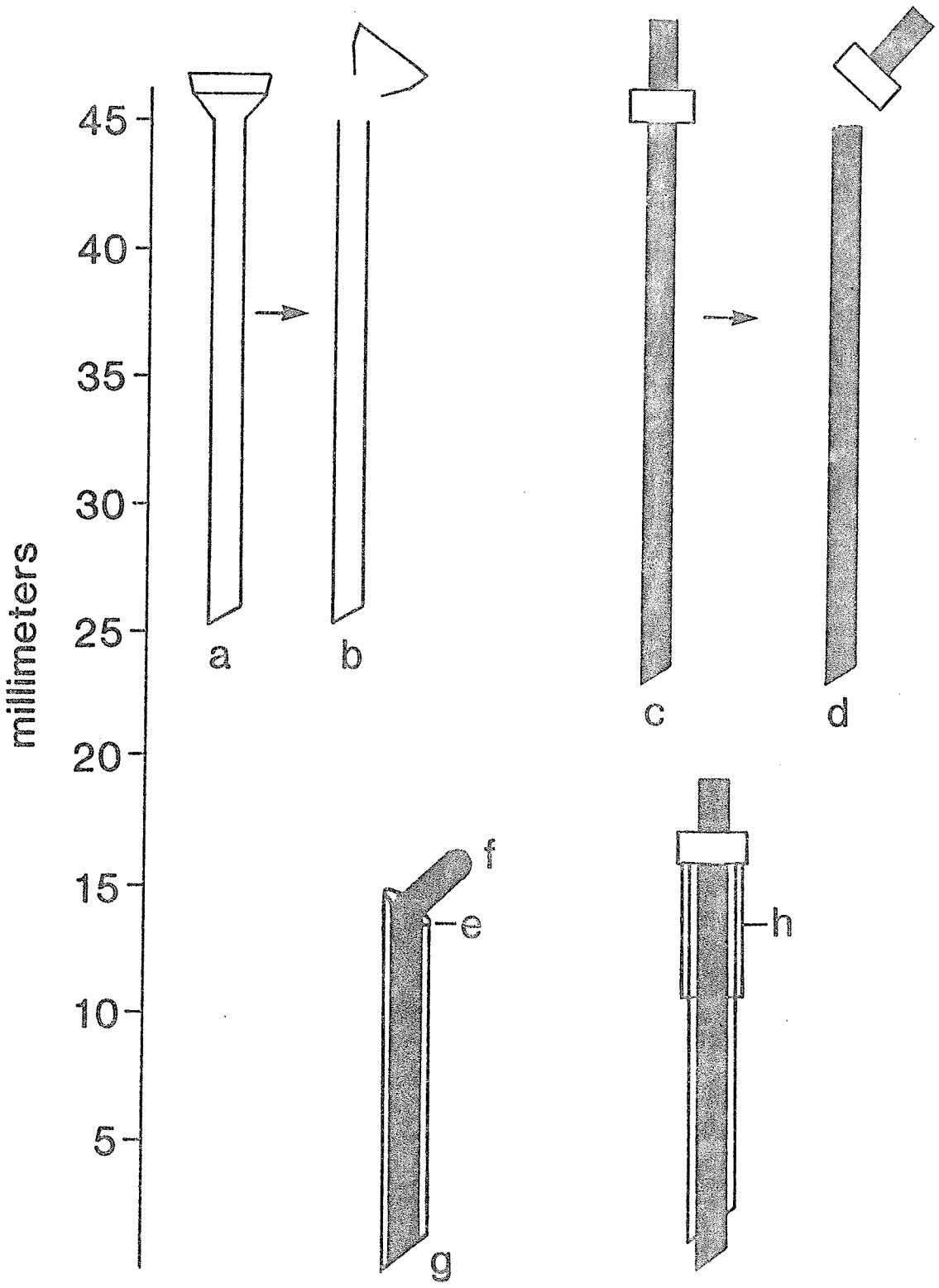
1. Preparation of Cannula

The cannulation system consisted of the guide cannula, stylet and injection cannula, all constructed of stainless steel. A 23 g, 1 inch Yale disposable needle (Fig. 20a) was cut on the Dremel emery wheel to obtain a 18 mm stainless steel tube (Fig. 20b). Next a 30 g, 3/4 inch dental needle (Fig. 20c) was cut to a length of 22 mm (Fig. 20d) and fitted inside the guide cannula with 4 mm protruding from the top end of the guide cannula. Then, by holding the top of the guide cannula against the emery wheel, a notch (Fig. 20e) was cut into the side of the tubing. The 4 mm of tubing protruding from the top of the guide cannula was then bent at a 45^{o} angle away from the guide cannula (Fig. 20f). The opposite end of the guide cannula was then bevelled sharply to the length of 15 mm. The injection cannula was made from a 30 g, 3/4 inch dental needle which was cut to a length of 16 mm. A 5 mm cuff (Fig. 20h) of Tygon microtubing (.020 x .060 cm - Norton Plastics and synthetics Division) was inserted over the tube and glued in place by RTV translucent adhesive sealent (General Electric Co.). The top end of the injecting cannula was attached to a .010 x .040 cm tygon microtubing which was then connected to a 50 µl Hamilton syringe (Sage Instruments, Mass., U.S.A.). The syringe was placed on a syringe pump.

2. Implantation of Cannula

Konig and Klippel (1967) rat brain stereotaxic atlas was used to locate the co-ordinates for the right lateral ventricle. Male Sprague-Dawley rats weighing between 180 - 225 grams were

Figure 20



anesthetized with sodium pentabarbitol (35 mg/kg - Abbot Laboratories, Canada) and chloral hydrate (460 mg/kg - Merck Co. Ltd.) according to doses suggested by Valenstein (1961). The rat head was firmly placed into the stereotaxic apparatus with ear bars. An incision was made on the skin directly above the site of implantation and the skull was exposed by reflecting the connective tissue. Using a dental drill, two partial holes through the skull were made and two optical screws (5½ barrel thread) were fixed on the skull. Then, after locating the site of cranial penetration for the guide cannula with its inset stylus, a hole was made through the skull and the cannula lowered to desired position. Then the entire wound was covered by dental cement to keep the cannula firmly in place.

H. ANALGESIA TESTING PROTOCOL

Rats were placed in a 20x21x25 cm cage which was equipped with a grid floor to permit detection of changes in fine motor co-ordination. After an habituation period of 30 minutes two units of Bacitracin (UpJohn Co.) were dissolved in 10 µl of artificial CSF and infused over 150 seconds. Controls were injected with artificial CSF. After completion of intracerebroventricular (ICV) infusion, rats were placed on a Mod 35-D Analgesia meter (IITC Inc., New Jersey) set at 55°C ± 0.1°C for periods of 30 seconds/trial. A group of 23 rats was placed on the Analgesia meter 10 times during 30 minutes post-infusion period. In this type of analgesia testing procedures rats typically exhibited two distinct behavioural responses; withdrawal of the hindpaw accompanied by vigorous licking, and jumping which ultimately leads to escape from the noxious stimuli. Hence analgesia was measured by

recording the time elapsed before the onset of hindpaw licking and/or jumping and the number of hindpaw licks and jumps. Activity, in terms of diagonal movement on the surface of the Analgesia meter was also recorded during the exposure period. Various doses (1 mg/kg, 5 mg/kg and 10 mg/kg) of opiate antagonist Naloxone (Endo Labs Inc., New York) were injected to determine whether the observed analgesic response was mediated by opiates. Naloxone was injected intraperitoneally one minute after completion of bacitracin infusion.

I. STRESS INDUCING PROTOCOL

Thirty-four rats were infused with two units of bacitracin ICV and subjected to prolonged, intermittent exposure to the hot plate. Thirty-four control rats were infused with artificial CSF. Animals were exposed for periods of 30 seconds/trial for various intervals during a 30 minute post-infusion period. Rats were sacrificed at 0, 9.5, 15, 22, and 30 minutes after completion of infusion.

J. ELECTROENCEPHALOGRAPHIC STUDY

Six male Sprague-Dawley rats weighing 180 - 225 grams were anesthetized with sodium pentobarbital (35 mg/kg) and chloral hydrate (160 mg/kg) according to doses suggested by Valenstein (1961). Stainless steel electrodes were implanted in the epidural space over the sensorimotor cortex and fixed in place with dental acrylic. Electroencephalographic (EEG) recordings were taken for at least 60 minutes before infusion of bacitracin or control and subsequently for 120 minutes thereafter. Rats were placed in an electrically shielded chamber 20x21x25 cm which was equipped with a grid floor. Bipolar

recordings were obtained using a Grass Model 78 polygraph with 7P511 EEG amplifiers (time consultant 250 msec) and recorded on a Hewlett Packard 3960 Instrumental FM tape recorder. The record was divided into 11 second intervals. Rothman's version of the Fast Fourier Transform (FFT) alogrithm (Rothman, 1968) was used to perform FFT off-line on a PDP8/E computer. Samples of 10.24 sec duration for each channel of EEG signal were digitized at a frequency of 100 Hz and a 256 point spectrum was generated between 0.10 and 25 Hz with resolution of 0.098 Hz per point. All frequency data generated was reduced to nine integrated values, eight of which represent one frequency band each, and the ninth represents the total sum (Table 18).

K. STATISTICAL ANALYSIS

Statistical calculations were done using:

a) unpaired two sample student's t test

b) two way analysis of variance and Duncan's multiple range test for multiple comparisons.

Table 18

Delta 1	(D1)	= 0.10 - 1.48 Hz
Delta 2	(D2)	= 1.56 - 3.51
Theta 1	(T1)	= 3.61 - 5.57
Theta 2	(T2)	= 5.66 - 7.52
Alpha 1	(A1)	= 7.62 - 9.47
Alpha 2	(A2)	= 9.57 - 12.50
Beta 1	(B1)	= 12.60 - 17.48
Beta 2	(B2)	= 17.58 - 25.0
SUM		= 1.56 - 25.0

RESULTS

β-Endorphin content of whole brain in bacitracin injected rats was generally higher than controls at 0, 6.5, 9.5, 15 and 22 minutes after completion of bacitracin infusion (Fig. 21a-d). However, only at 15 minutes post-infusion was the brain β-endorphin content significantly higher (by 27%) in bacitracin injected rats ($p < .05$; Student's t test). At 30 minutes post-infusion brain β-endorphin level in bacitracin injected rats returned to control values (Fig. 21c). In bacitracin injected rats sacrificed at 6.5 minutes post-infusion, β-endorphin content was similar to control rats in striatum and cortex, lower in cerebellum and hippocampus and there was a tendency for levels to be higher in thalamus, hindbrain, septal nuclei, midbrain and hypothalamus (Fig. 22a-d). However, only in the Amygdala was the β-endorphin content higher in bacitracin injected groups ($p < .05$; Student's t test). In bacitracin injected rats sacrificed 15 minutes after completion of infusion, there was a tendency for β-endorphin levels to be higher in most brain areas (except cerebellum, where there was a slight decrease) (Fig. 23a-d). However, only in the striatum and Amygdala were β-endorphin levels significantly higher. In the striatum there was a four fold increase ($p < .05$; Student's t test) (Fig. 23a) and a three fold increase in the Amygdala ($p < .01$; Student's t test) (Fig. 23c).

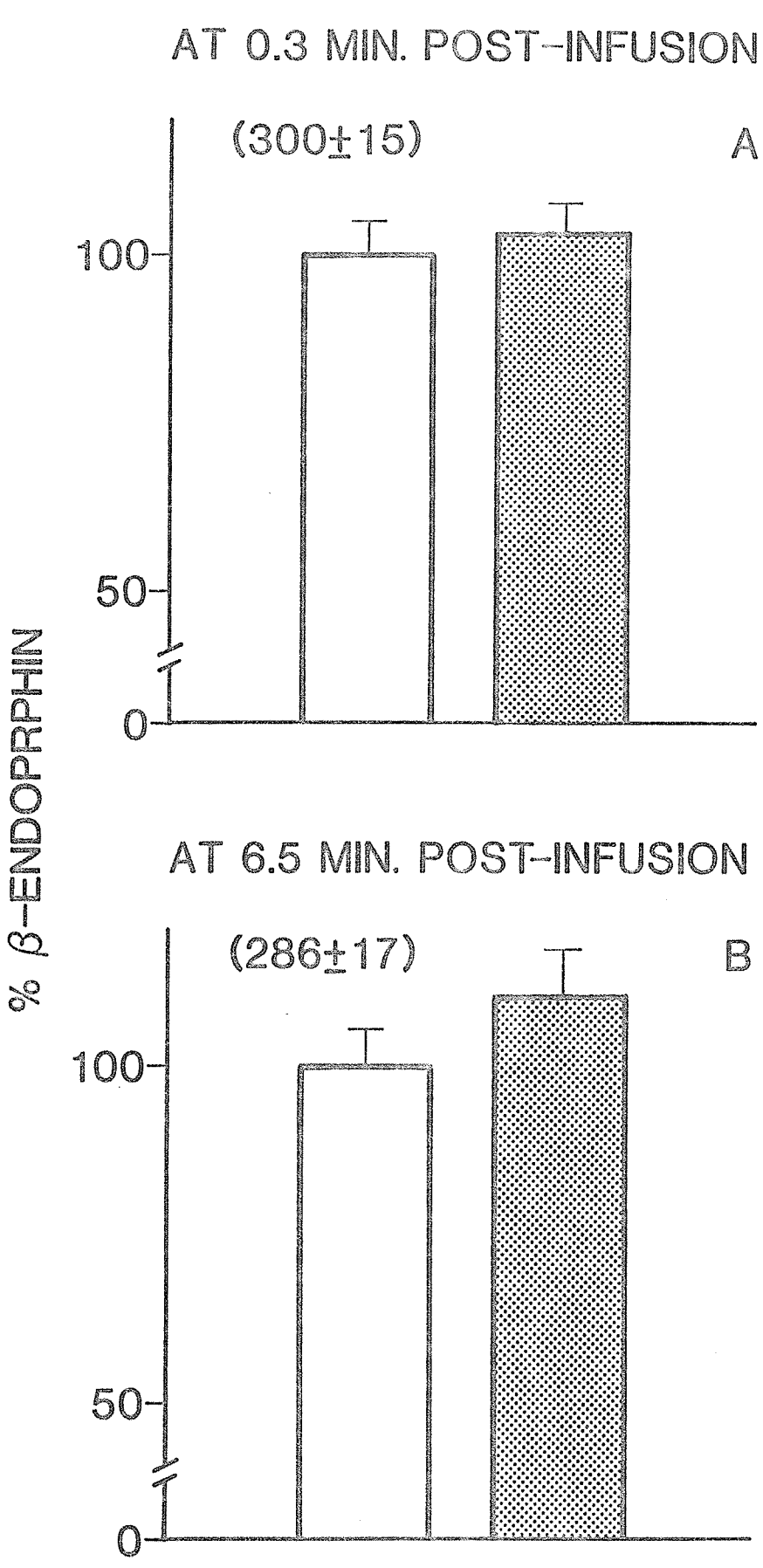
When bacitracin injected rats were tested for analgesia, the following pattern of pain threshold response was obtained (Fig. 24). Bacitracin injected rats exhibited a strong analgesic response which was absent in controls. This analgesic response reached a plateau 15

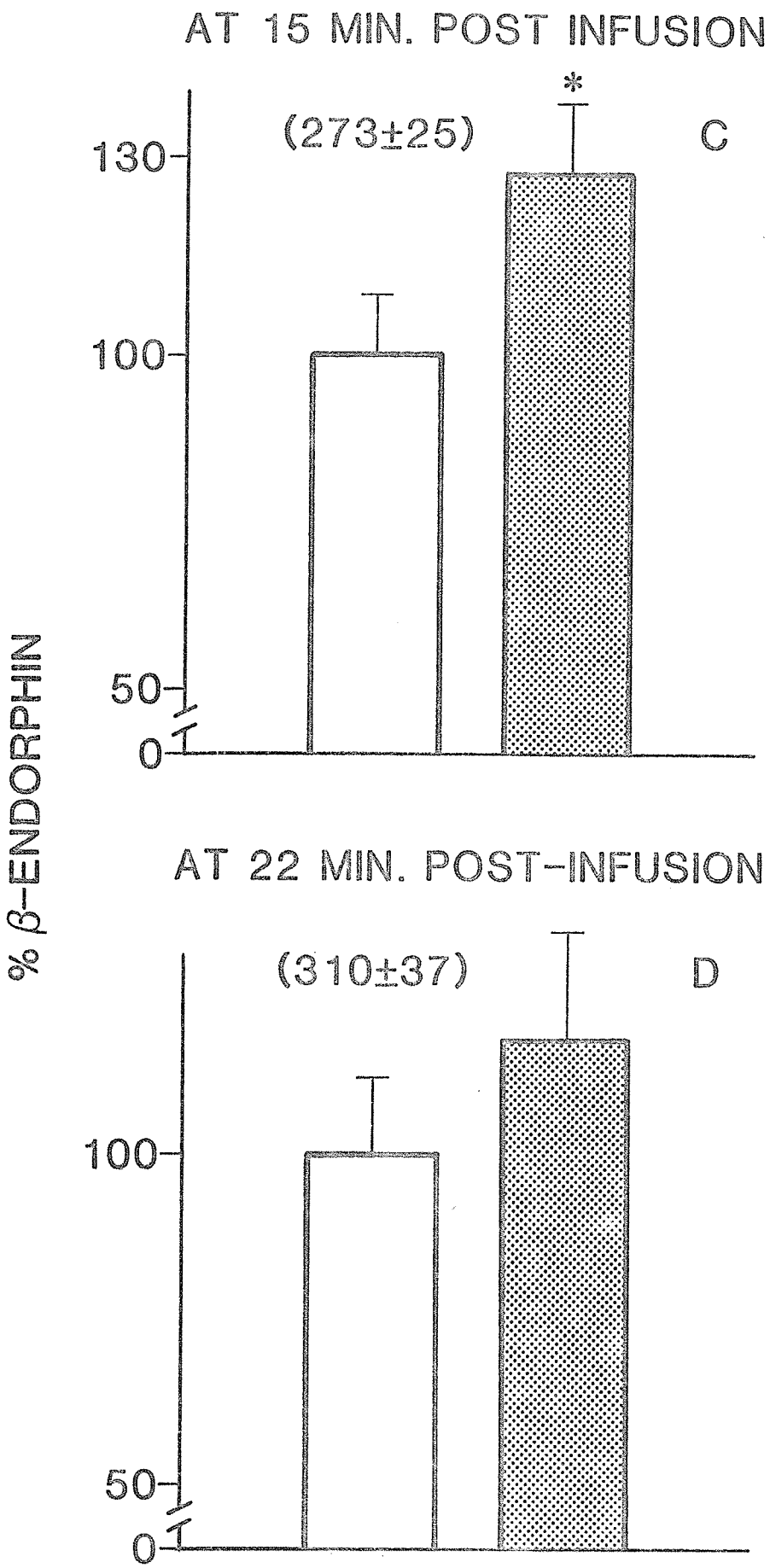
Figure 21: β-Endorphin Content of Whole Brains of Rats Sacrificed at Various Time Intervals After Completion of Bacitracin Infusion

Open Bar: Control (n = 15)

Solid Bar: Bacitracin (n = 15)

* Difference between control and bacitracin treated significant at $p \leq .05$ (Student's t test)


AT 0.3 MIN. POST-INFUSION
(300±15)
A
100
50
0
% β-ENDOPRPHIN
AT 6.5 MIN. POST-INFUSION
(286±17)
B
100
50
0


AT 15 MIN. POST INFUSION
(273±25)
C
*
130
100
50
0
% β-ENDORPHIN
AT 22 MIN. POST-INFUSION
(310±37)
D
100
50
0

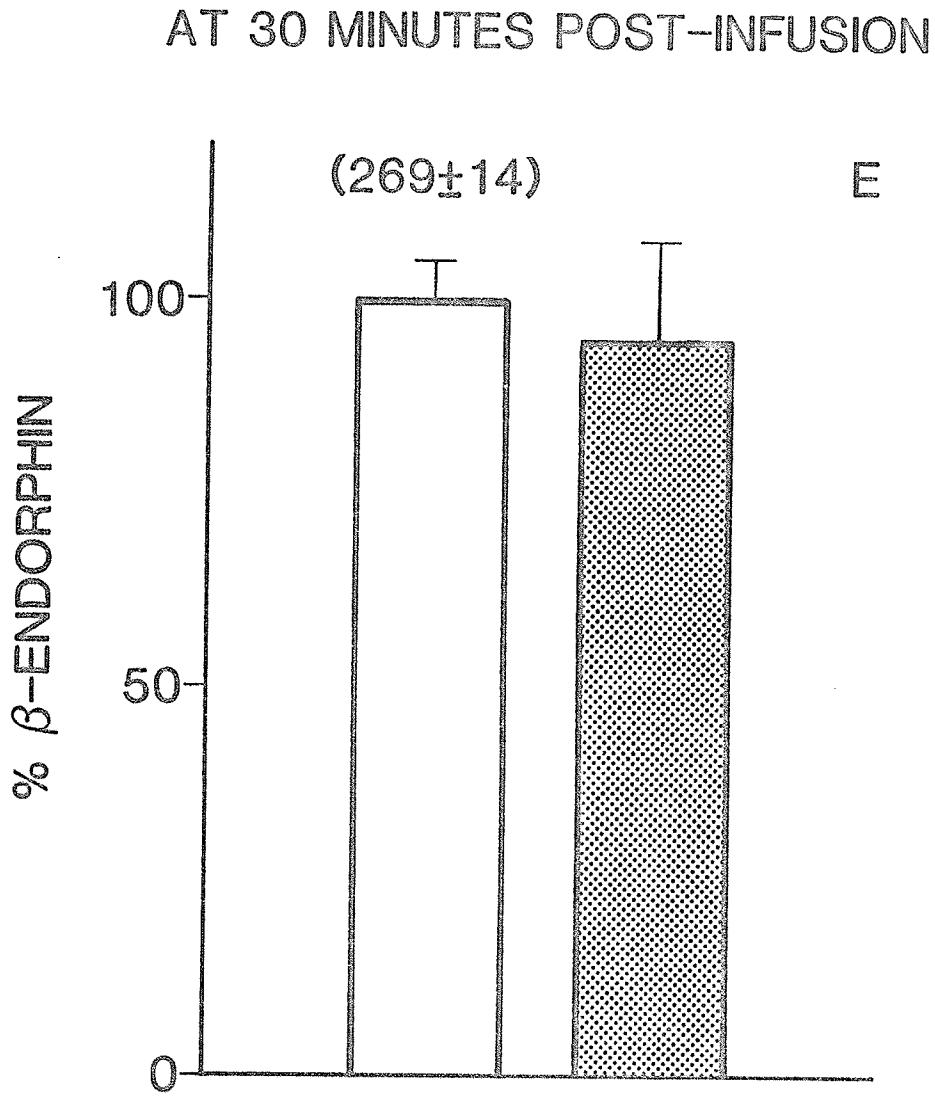

AT 30 MINUTES POST-INFUSION
(269±14)
E
100
50
0
% β-ENDORPHIN

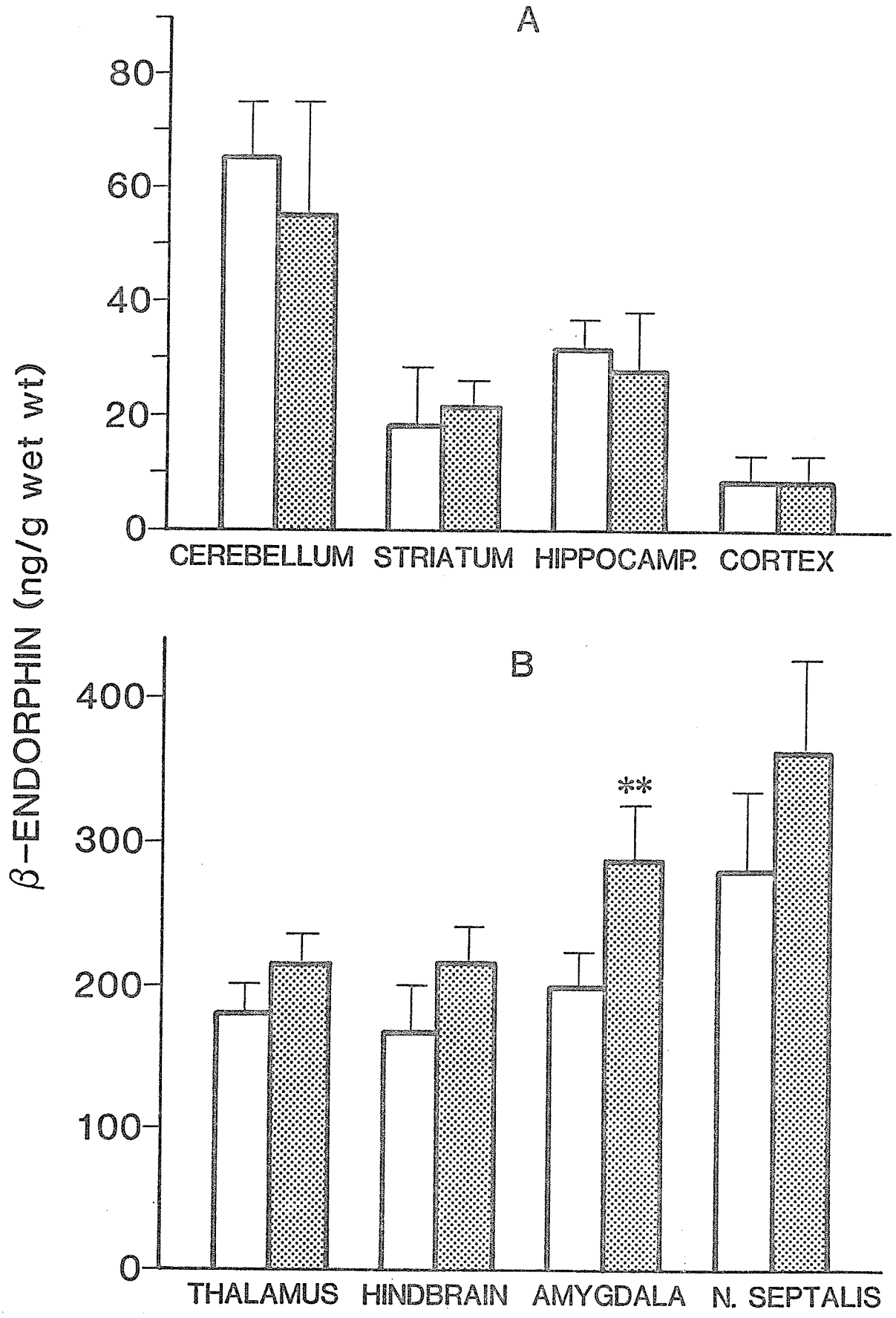
Figure 22: β-Endorphin Content of Brain Regions of Rats Sacrificed 6.5 Minutes After Infusion of Bacitracin

Open Bar: Control (n = 8)

Solid Bar: Bacitracin (n = 8)

Means and S.E.M. are shown

** Difference between control and bacitracin significant at $p \leq .05$ (Student's t test)


A
80
60
40
20
0
CEREBELLUM
STRIATUM
HIPPOCAMP.
CORTEX
β-ENDORPHIN (ng/g wet wt)
B
400
300
200
100
0
**
THALAMUS
HINDBRAIN
AMYGDALA
N. SEPTALIS

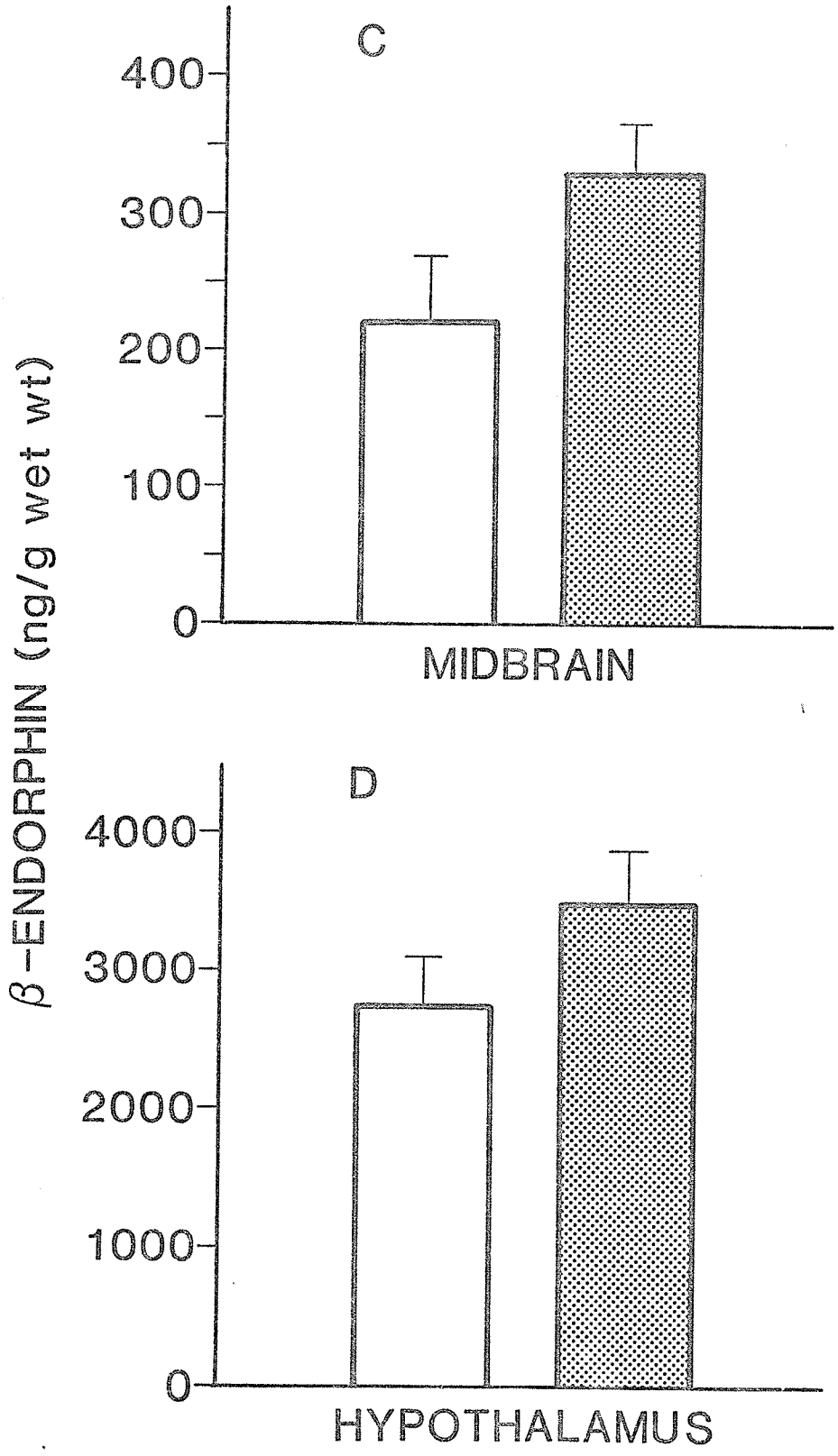

C
400
300
200
100
0
MIDBRAIN
D
4000
3000
2000
1000
0
HYPOTHALAMUS
β-ENDORPHIN (ng/g wet wt)

Figure 23: β-Endorphin Content of Brain Regions of Rats Sacrificed 15 Minutes After Infusion of Bacitracin

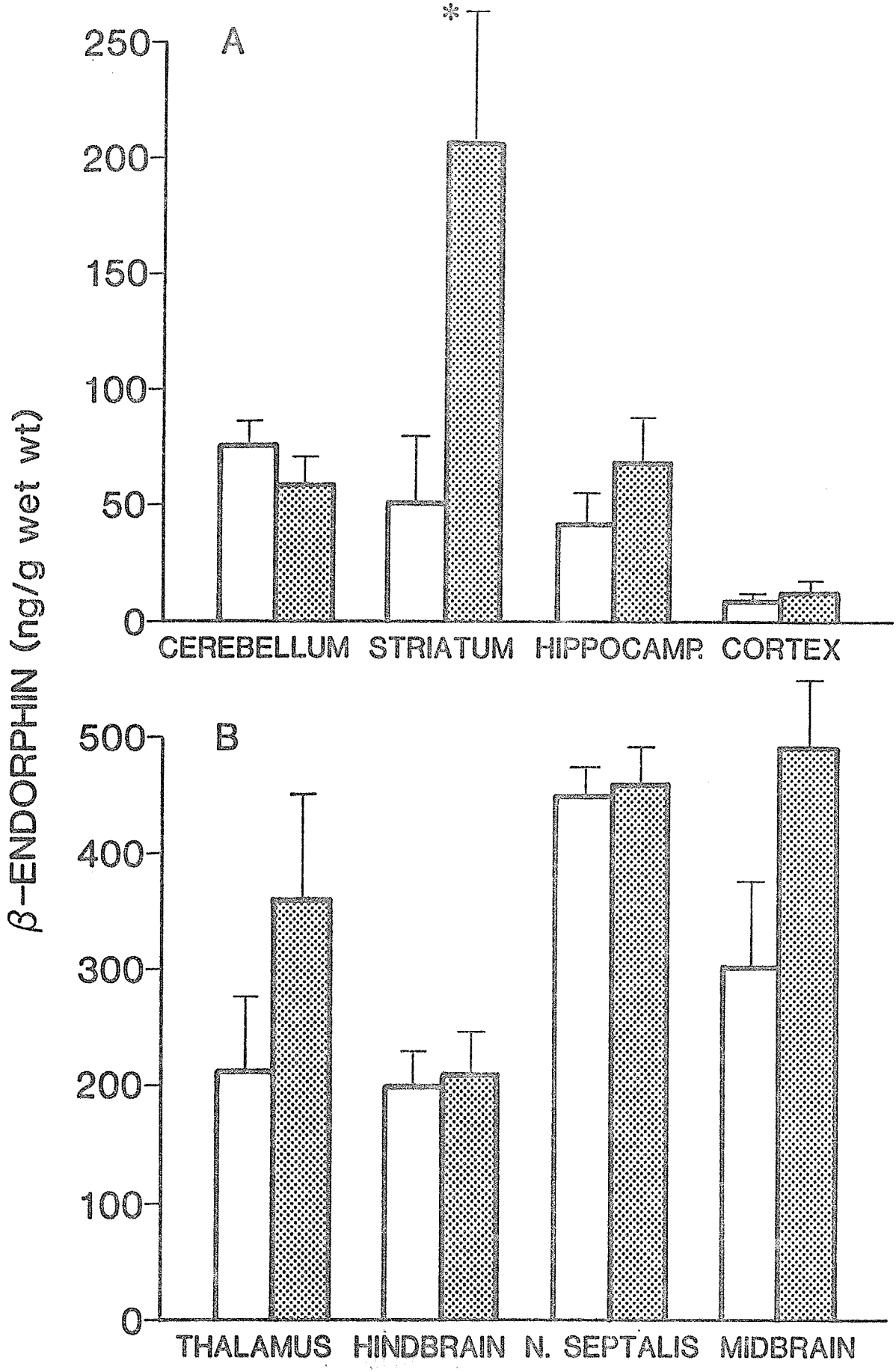
Open Bar: Control (n = 8)

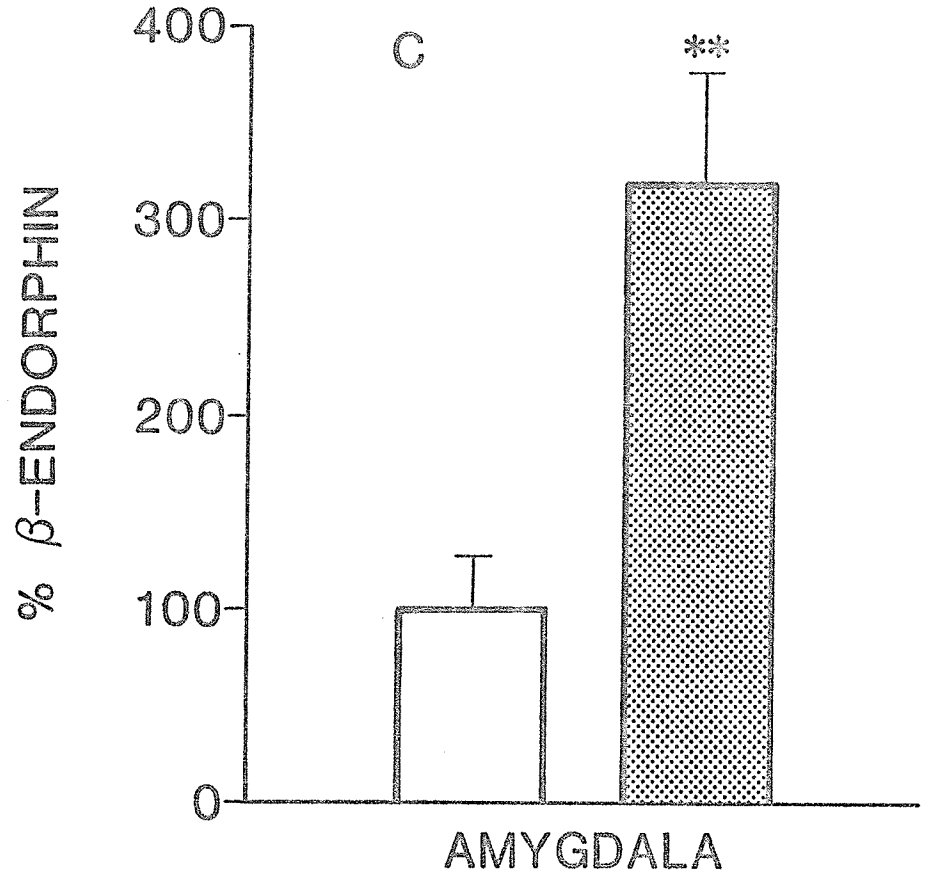
Solid Bar: Bactracin (n = 8)

Means and S.E.M. are shown

* Difference between control and bacitracin significant at $p \leq .05$ (Student's t test)

** Difference between control and bacitracin treated significant at $p \leq .01$ (Student's t test)


A
B
β-ENDORPHIN (ng/g wet wt)
250
200
150
100
50
0
500
400
300
200
100
0
*
CEREBELLUM
STRIATUM
HIPPOCAMP.
CORTEX
THALAMUS
HINDBRAIN
N. SEPTALIS
MIDBRAIN


C
**
400
300
200
100
0
% β-ENDORPHIN
AMYGDALA

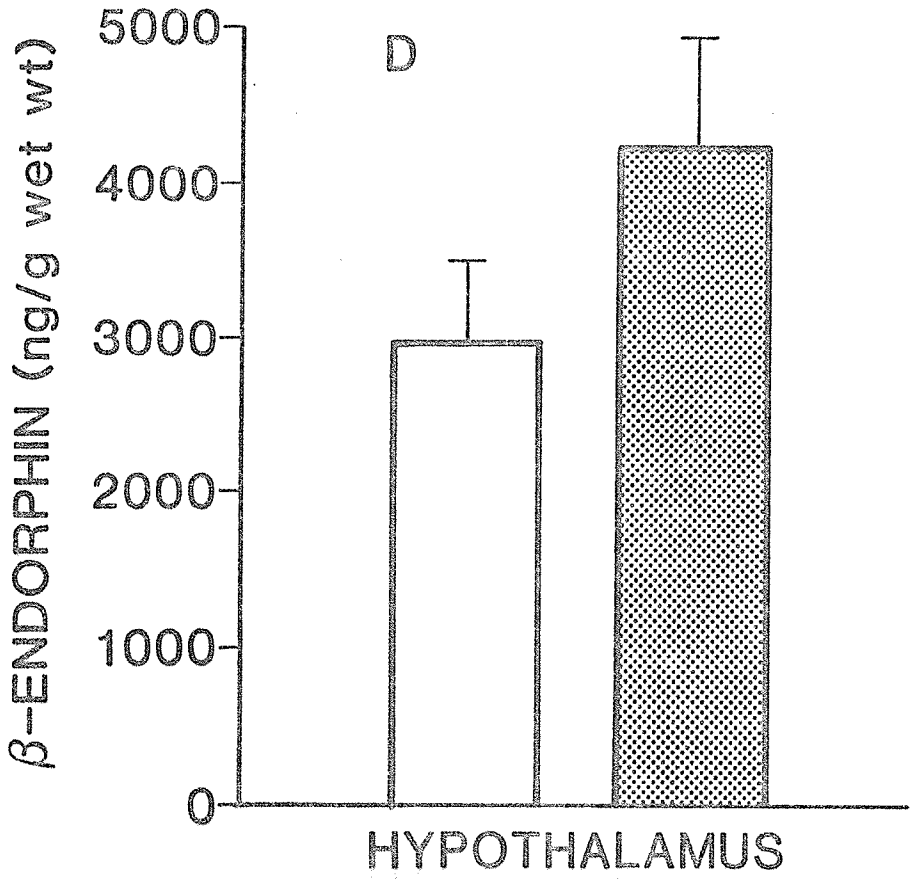
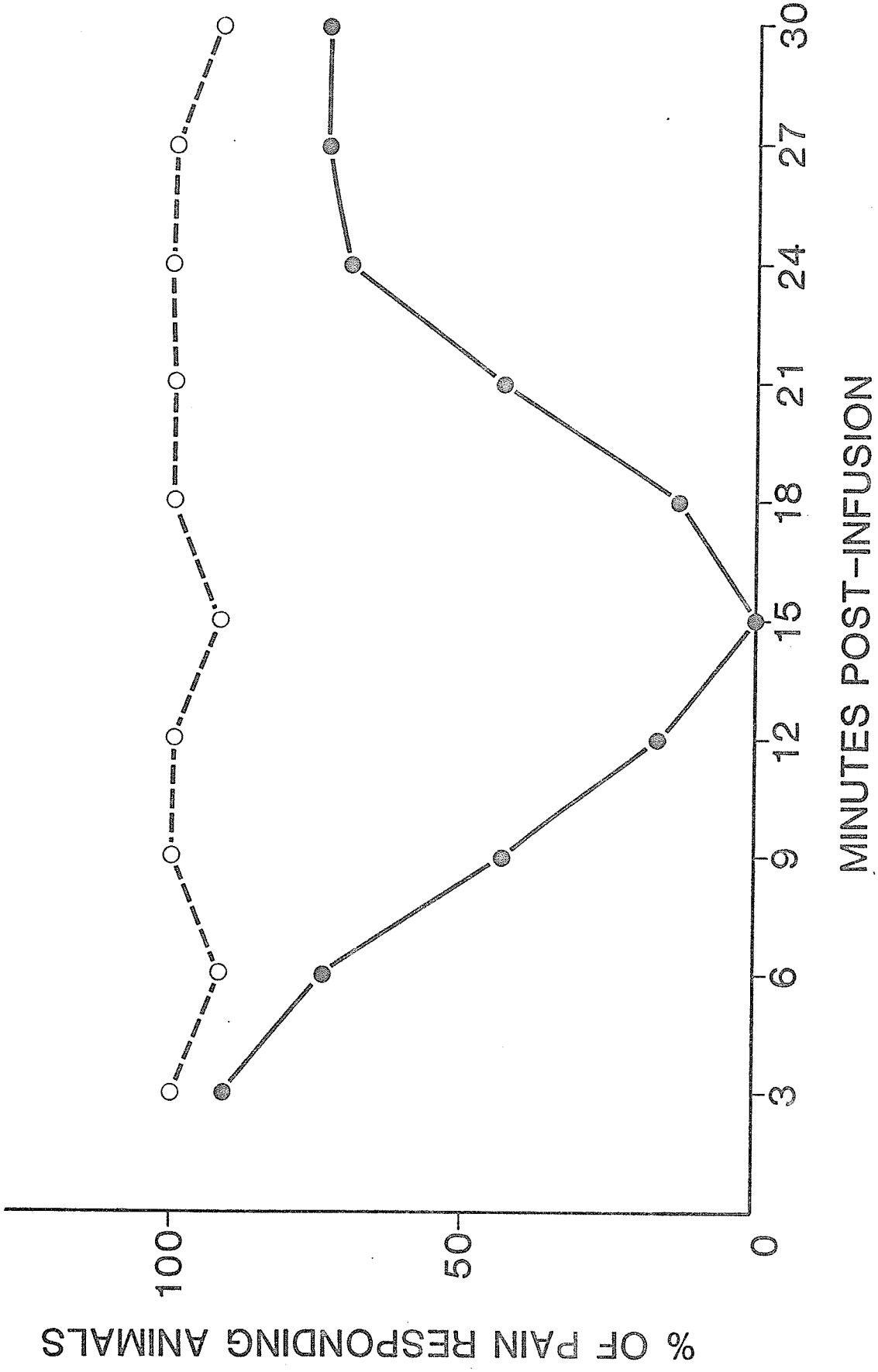

D
5000
4000
3000
2000
1000
0
β-ENDORPHIN (ng/g wet wt)
HYPOTHALAMUS

Figure 24: Effect of Bacitracin on Pain Threshold of Rats Exposed to Hot-Plate

Open Circle: Control (n = 15)

Solid Circle: Bacitracin (n = 23)


% OF PAIN RESPONDING ANIMALS
100
50
0
3
6
9
12
15
18
21
24
27
30
MINUTES POST-INFUSION

minutes after completion of infusion of bacitracin. Hence at 15 minutes post-infusion bacitracin injected rats exhibited total analgesia. There was no difference in the activity of bacitracin injected and control rats during monitoring of the analgesic response (Fig. 25). Injection of naloxone (1 mg/kg) significantly abolished bacitracin-induced analgesia (Fig. 26). A higher dose of naloxone (10 mg/kg) was less effective in antagonizing this analgesia (Fig. 27). An intermediate dose of naloxone (5 mg/kg) was still not as effective in antagonizing the analgesic response as 1 mg/kg of naloxone (Fig. 28). There was no difference in the activity of bacitracin, naloxone (1 mg/kg), bacitracin + naloxone (1 mg/kg) injected rats during the monitoring of the analgesic response. However, bacitracin + naloxone (10 mg/kg) injected rats exhibited significantly more activity than control, bacitracin, naloxone (1 mg/kg), bacitracin + naloxone (1 mg/kg) and bacitracin + naloxone (5 mg/kg) injected rats (Fig. 29). Although this higher degree of activity in bacitracin + naloxone (10 mg/kg) injected rats decreased to a lower level by 30 minutes post-infusion, it was still significantly higher than other groups.

In rats sacrificed at 9.5 minutes post-infusion and 4 intervals of exposure to stressful stimuli there was an indication that stress leads to a decrease in β-endorphin content. However, the decrease following stress at this time interval was not significant (Fig. 30). Rats sacrificed 15 minutes after infusion and 6 intervals of exposure to acutely stressful stimuli had significantly lower brain β-endorphin content following acute stress ($p < .01$; Student's t test) (Fig. 31a). Similarly bacitracin injected stressed rats had lower β-endorphin

Figure 25: Activity of Rats Exposed to Hot-Plate During 30 Minutes Post-Infusion Period

Open Circle: Control (n = 15)

Solid Circle: Bacitracin (n = 15)

Means and S.E.M. are shown.

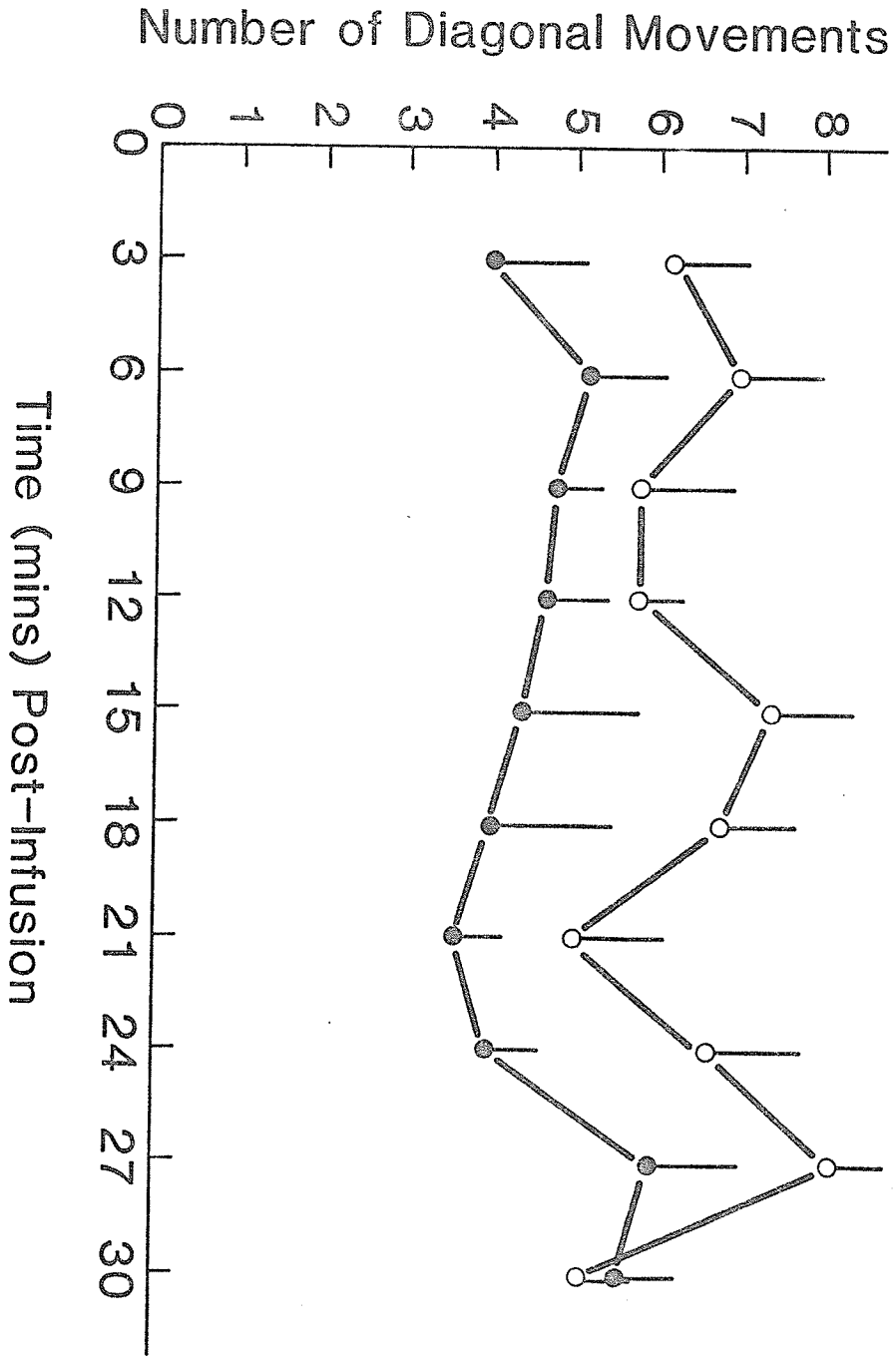

Number of Diagonal Movements
0
1
2
3
4
5
6
7
8
Time (mins) Post-Infusion
0
3
6
9
12
15
18
21
24
27
30

Figure 26: Effect of Naloxone (1 mg/kg) on Bacitracin Induced Analgesia During 30 Minute Post-Infusion Period

Solid Circle: Bacitracin (n = 15)

Solid Triangles: Bacitracin + Naloxone (1 mg/kg) (n = 15)

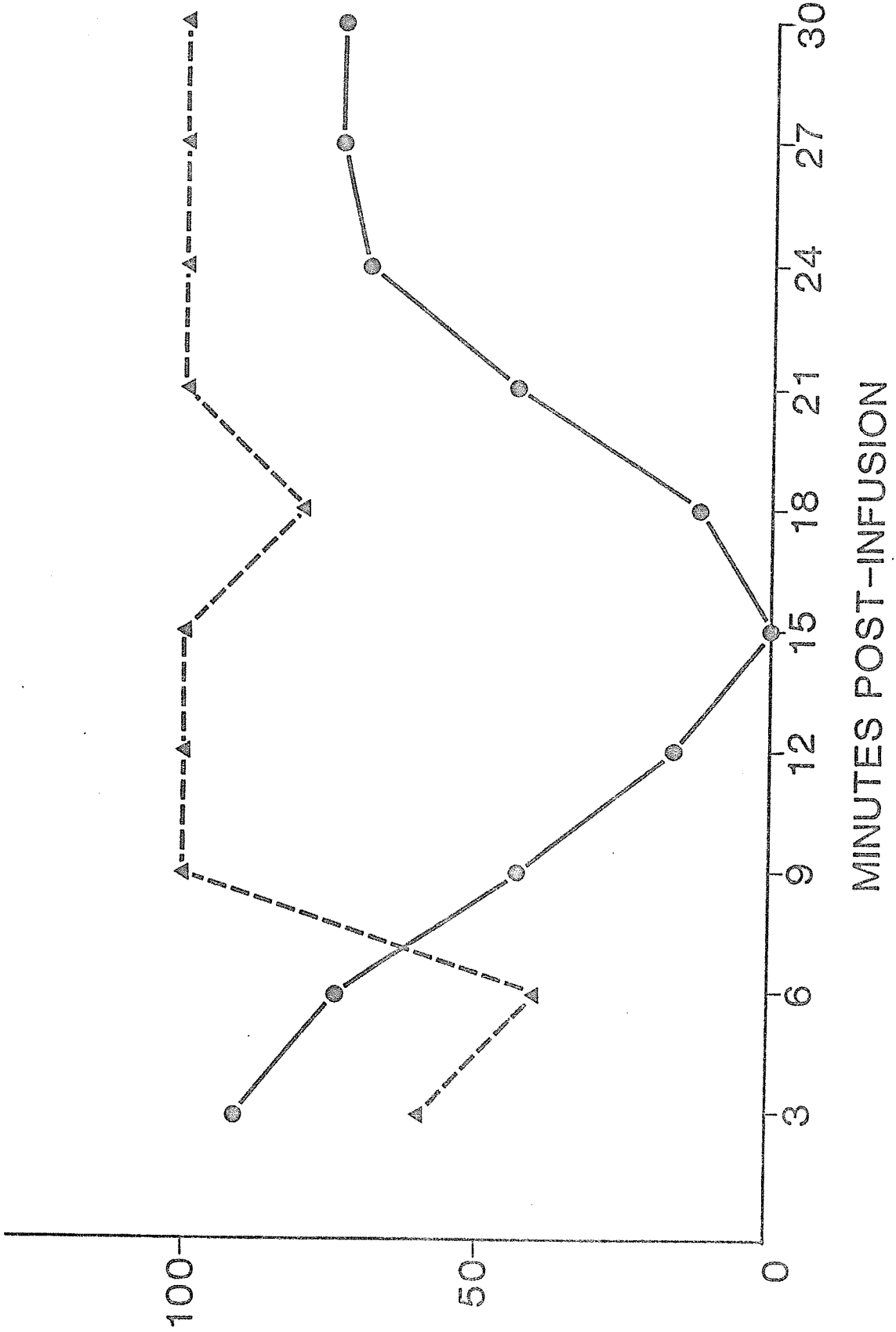

% OF PAIN RESPONDING ANIMALS
100
50
0
3
6
9
12
15
18
21
24
27
30
MINUTES POST-INFUSION

Figure 27: Comparison of Effects of Naloxone (10 mg/kg) and Naloxone (1 mg/kg) on Bacitracin-induced Analgesia During 30 Minutes Post-Infusion Period

Solid Circle: Bacitracin (n = 15)

Solid Triangle: Bacitracin + Naloxone (1 mg/kg) (n = 15)

Solid Diamond: Bacitracin + Naloxone (10 mg/kg) (n = 15)

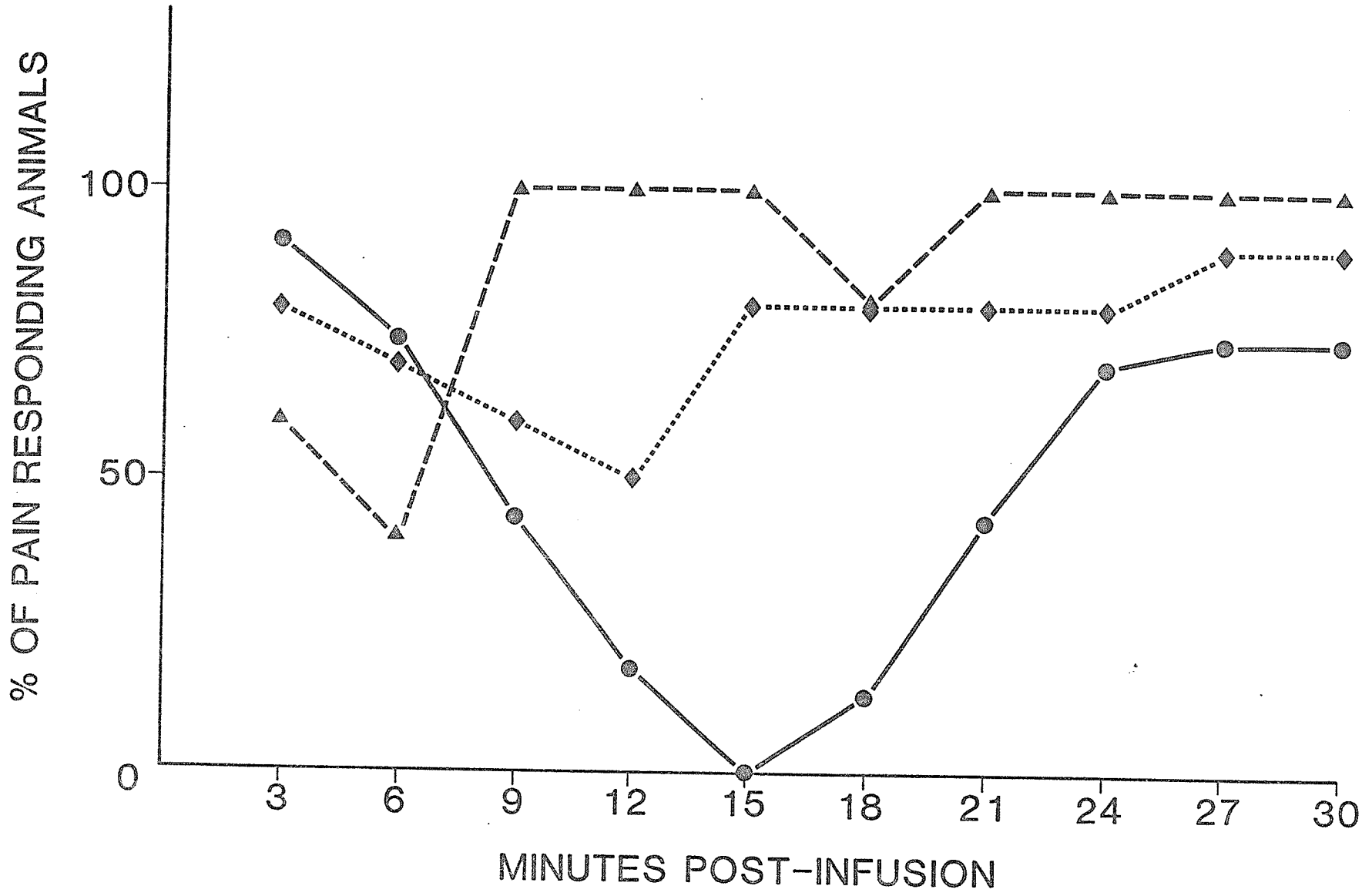

% OF PAIN RESPONDING ANIMALS
100
50
0
3
6
9
12
15
18
21
24
27
30
MINUTES POST-INFUSION

Figure 28: Comparison of Effects of Naloxone (5 mg/kg) and Naloxone (10 mg/kg) on Bacitracin-Induced Analgesia During 30 Minutes Post-Infusion Period

Solid Circle:	Bacitracin	(n = 23)
Solid Diamond:	Bacitracin + Naloxone (10 mg/kg)	(n = 15)
Open Diamond:	Bacitracin + Naloxone (5 mg/kg)	(n = 8)

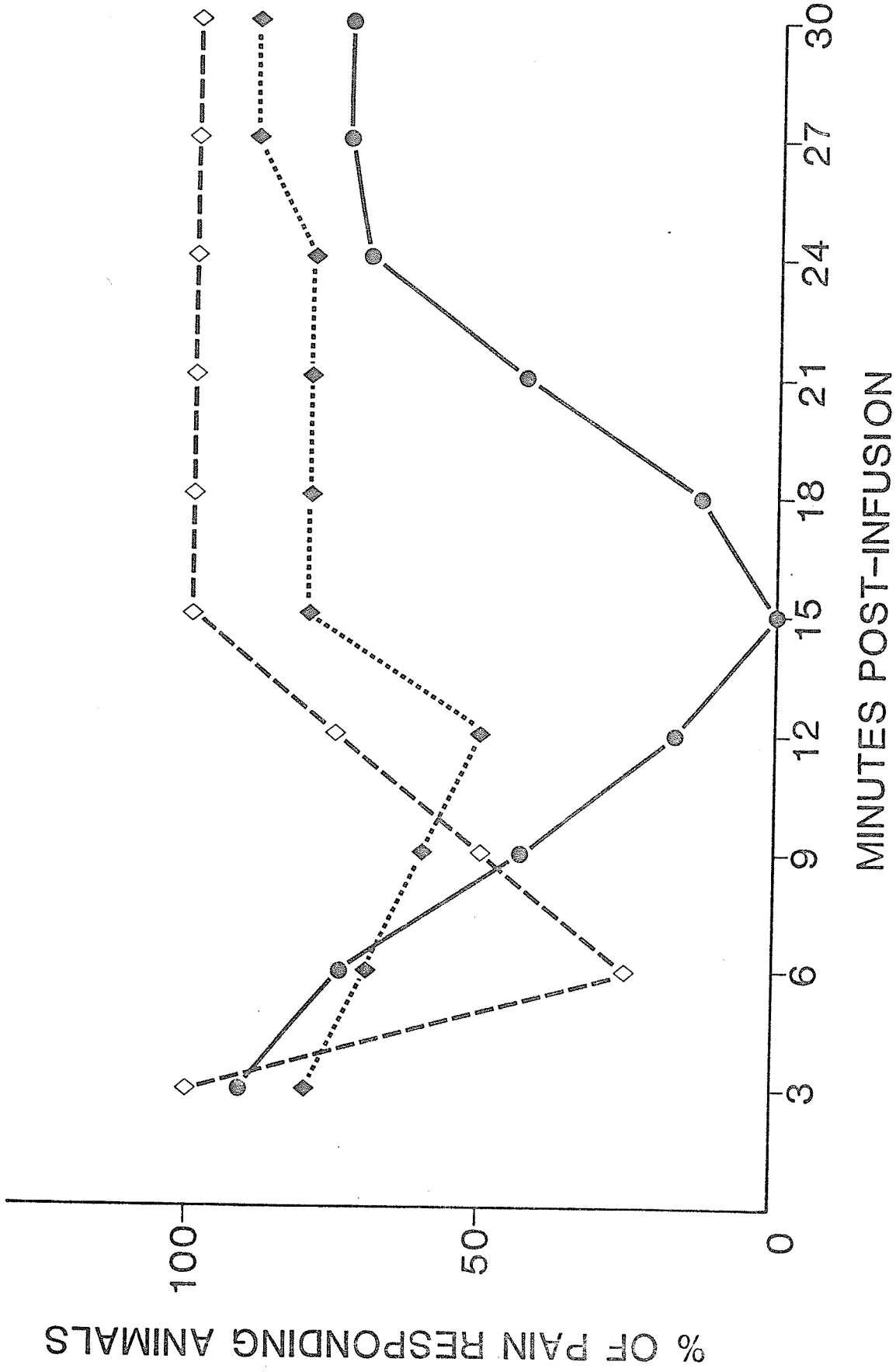

% OF PAIN RESPONDING ANIMALS
100
50
0
3
6
9
12
15
18
21
24
27
30
MINUTES POST-INFUSION

Figure 29: Activity of Rats During 30 Minutes Post-Infusion Analgesia Monitoring Period

Solid Circle: Bacitracin

Open Triangle: Naloxone (1 mg/kg)

Solid Triangle: Bacitracin + Naloxone (1 mg/kg)

Solid Triangle: Bacitracin + Naloxone (10 mg/kg)

Means and S.E.M. are shown.

(n = 15 for each group)

* Difference between bacitracin + naloxone and control groups (bacitracin and naloxone (1 mg/kg) significant at $p < .05$ (Duncans Multiple Range Test)

** Difference between bacitracin + naloxone group and control groups (bacitracin and naloxone (1 mg/kg)) significant at $p < .01$ (Duncans Multiple Range Test)

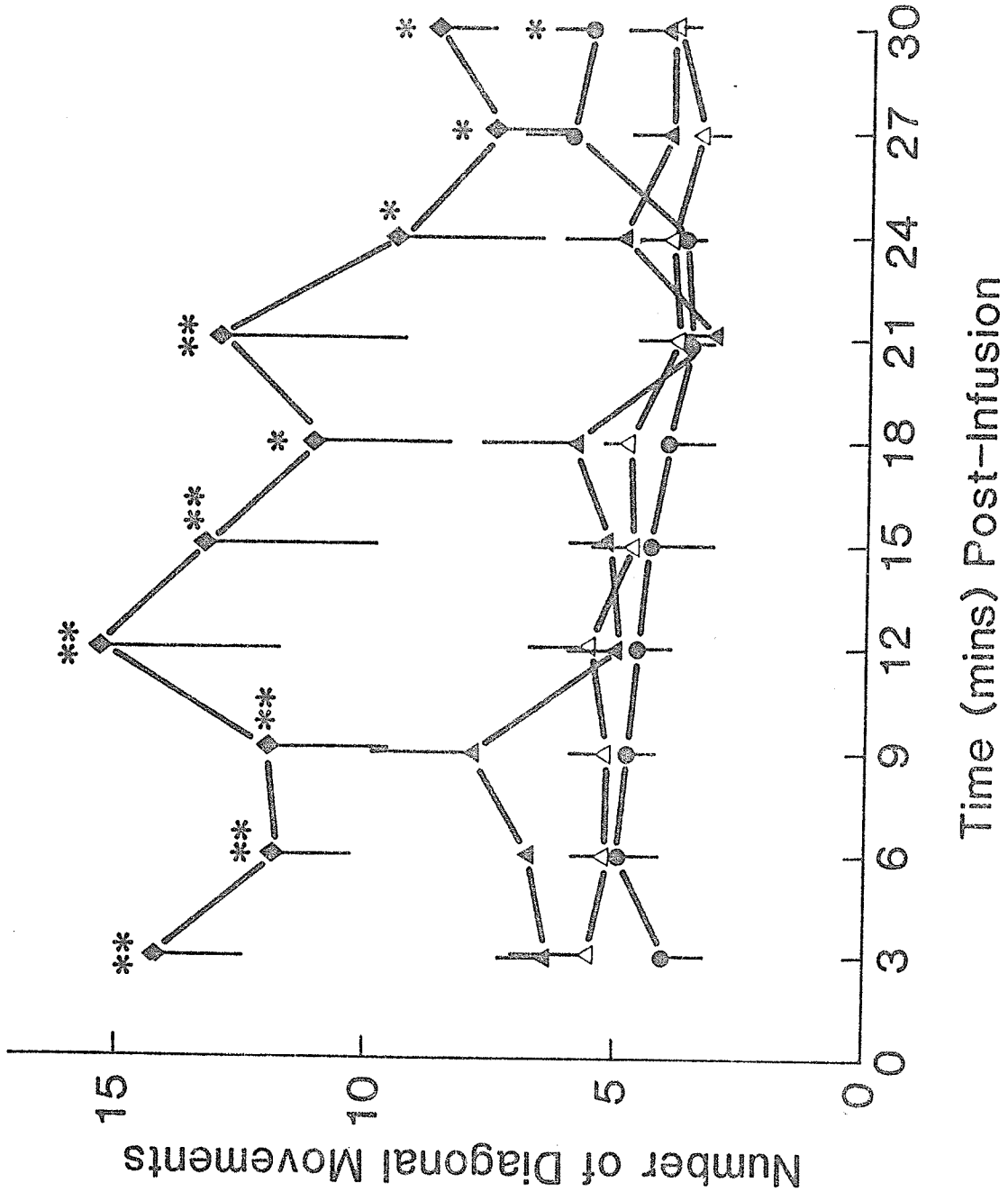

Number of Diagonal Movements
0
5
10
15
0
3
6
9
12
15
18
21
24
27
30
Time (mins) Post-Infusion

Figure 30: β-Endorphin Content of Whole Brains of Rats Sacrificed at 9.5 Minutes Post-Infusion After 4 Intervals of Exposure to Hot-Plate

Open Bar: Control

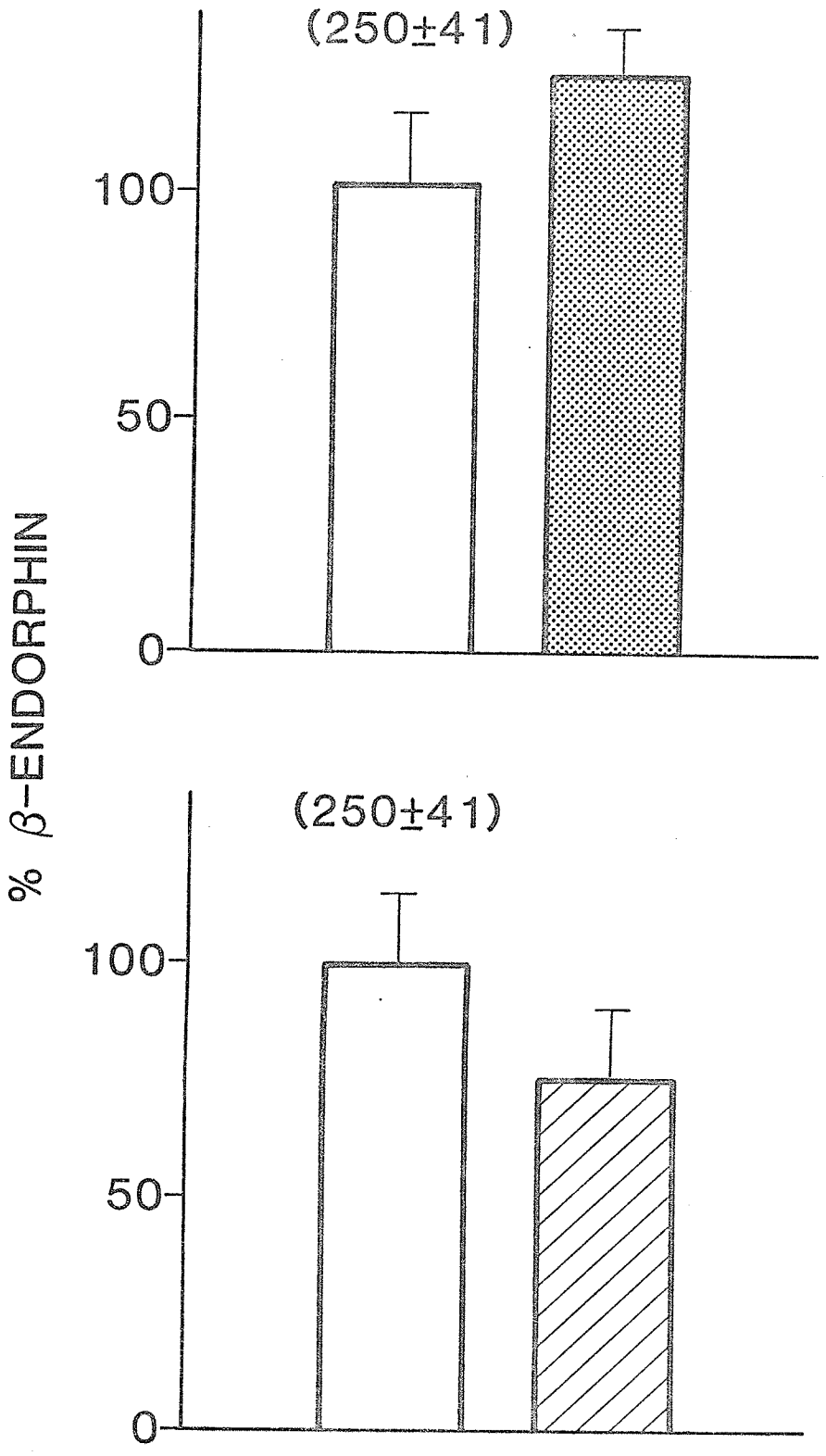
Thin Cross-Hatched Bar: Stressed

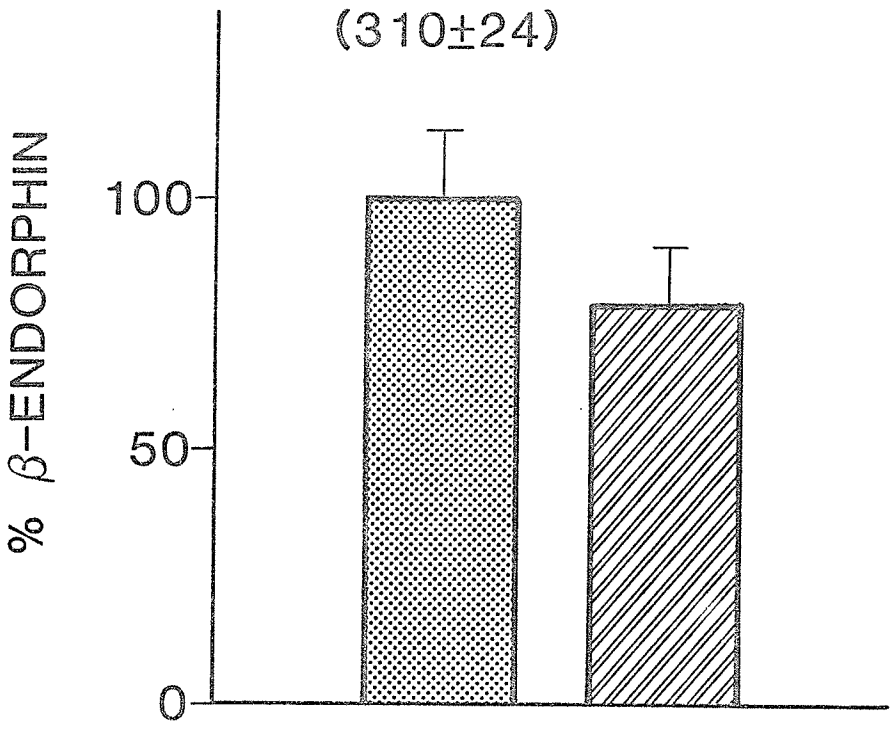
Solid Bar: Bacitracin

Thick Cross-Hatched Bar: Bacitracin-stressed

Means and S.E.M. are shown

(n = 4 for each group)


(250±41)
100
50
0
% β-ENDORPHIN
(250±41)
100
50
0


(310±24)
% β-ENDORPHIN
100
50
0

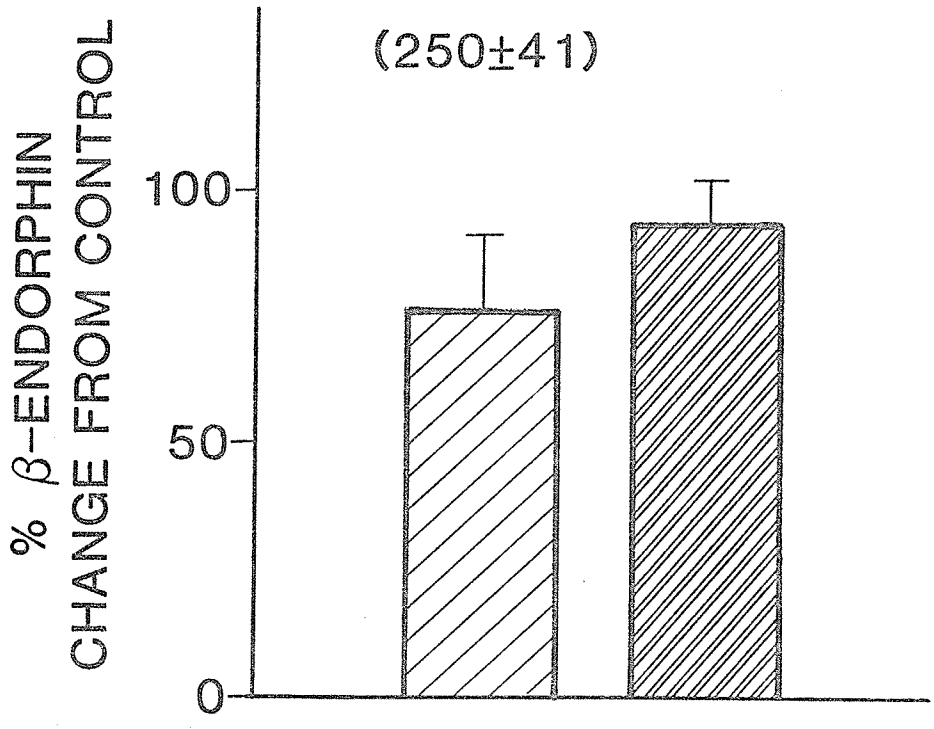

(250±41)
% β-ENDORPHIN
CHANGE FROM CONTROL
100
50
0

Figure 31: β-Endorphin Content of Whole Brains of Rats Sacrificed at 15 Minutes Post-Infusion After Six Intervals of Exposure to Hot-Plate

Open Bar: Control

Thin Cross-Hatched Bar: Stressed

Solid Bar: Bacitracin

Thick Cross-Hatched Bar: Bacitracin-stressed

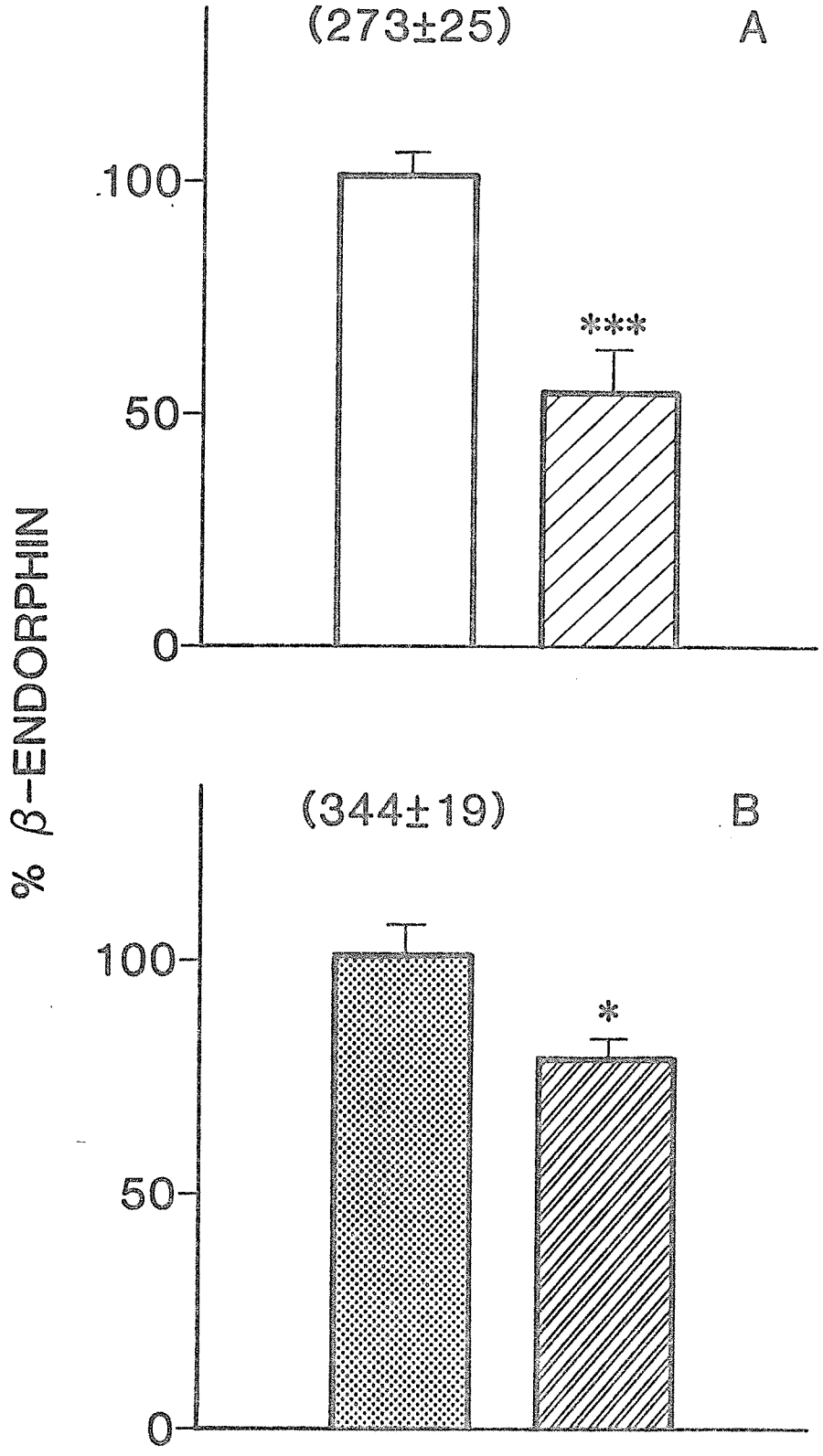
Means and S.E.M. are shown.

(n = 12 for each group)

* Difference between bacitracin and bacitracin-stressed significant at $p < .05$ (Student's t test)

** Difference between stressed and bacitracin-stressed significant at $p < .02$ (Student's t test)

*** Difference between control and stressed significant at $p < .01$ (Student's t test)


(273±25)
A
100
50
0
***
% β-ENDORPHIN
(344±19)
B
100
50
0
*

levels (by 22%) than bacitracin injected nonstressed rats ($p < .05$; Student's t test) (Fig. 31b). Thus bacitracin pretreatment considerably reduced the decrease (by 26%) in β-endorphin levels observed in the stressed rats ($p < .02$; Student's t test) (Fig. 31c). In rats sacrificed 22 minutes after infusion and 9 intervals of exposure to stress, β-endorphin levels, in the brain, were lower but not significantly in stressed rats (Fig. 32a-b). Bacitracin pretreatment again reduced the decrease in β-endorphin levels caused by stress, however, it was not significant (Fig. 32c). By 30 minutes post-infusion and 11 intervals of exposure to stressed stimuli, brain β-endorphin levels returned to control values (Fig. 33a-c).

In rats sacrificed 15 minutes after completion of bacitracin infusion there was a significant increase in somatostatin content in the septal nuclei ($p < .02$; Student's t test) (Fig. 34b) and hypothalamus ($p < .05$; Student's t test) (Fig. 34c). Other brain areas demonstrated no significant changes (Fig. 34a-c). Overall in the whole brain there were no significant changes in somatostatin content of bacitracin injected rats (Fig. 35a). Stress caused a decrease in brain somatostatin content in the control group of rats ($p < .001$; Student's t test) (Fig. 35b). However, in bacitracin injected rats stress did not significantly lower brain somatostatin levels (Fig. 35c). Thus bacitracin pretreatment significantly reduced the decrease in brain somatostatin content caused by stress ($p < .05$; Student's t test) (Fig. 35d). The stress induced decrease in brain somatostatin content was evident even at 22 minutes post-infusion ($p < .02$; Student's t test) (Fig. 36). Again at 22 minutes post-infusion bacitracin prevented stress induced decrease of brain somatostatin content (Fig. 37). By 30 minutes post-infusion and 11 intervals of exposure to stressful stimuli brain somatostatin levels returned to

Figure 32: β-Endorphin Content of Whole Brains of Rats Sacrificed at 22 Minutes Post-Infusion After 9 Intervals of Exposure to Hot-Plate

Open Bar: Control

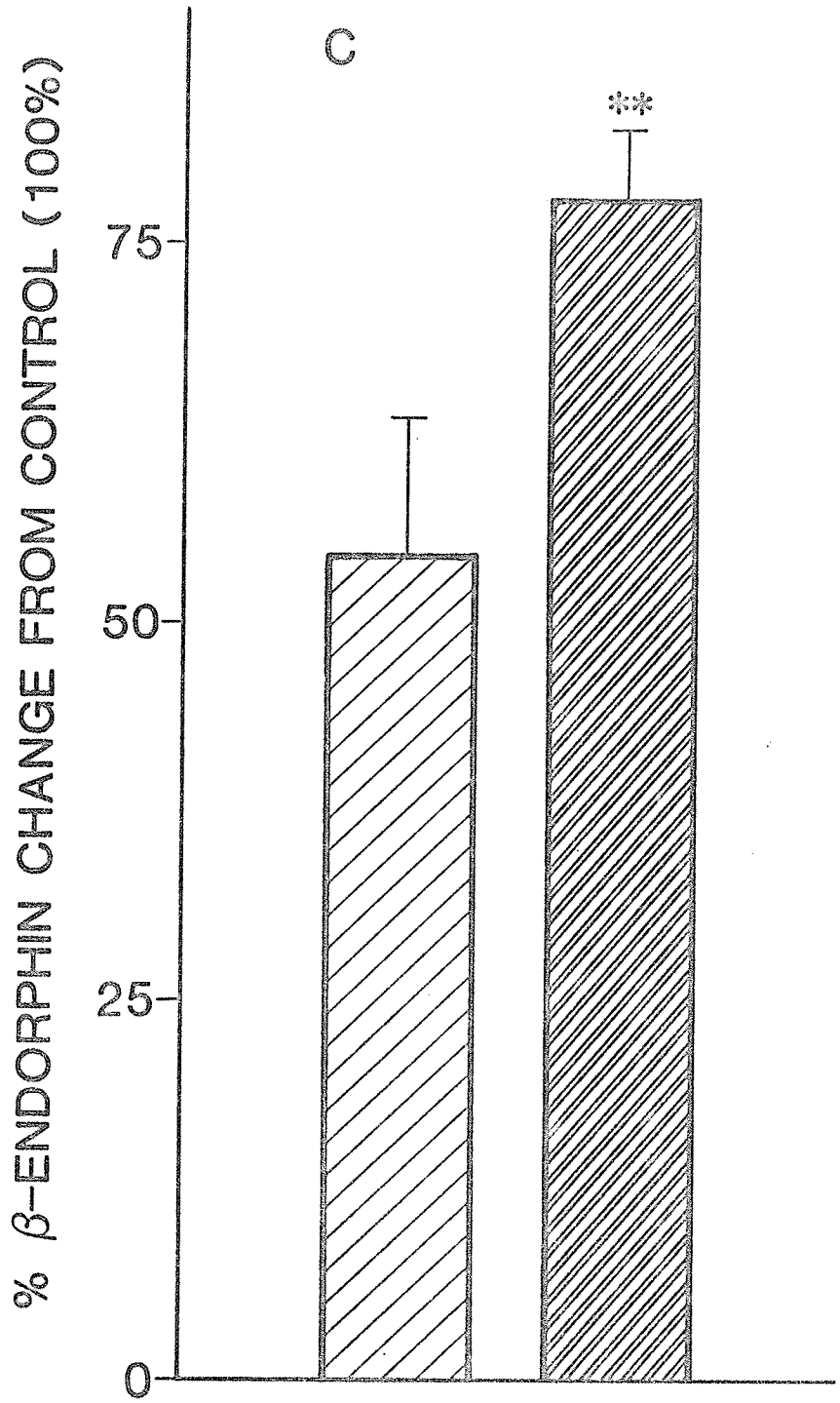
Thin Cross-Hatched Bar: Stressed

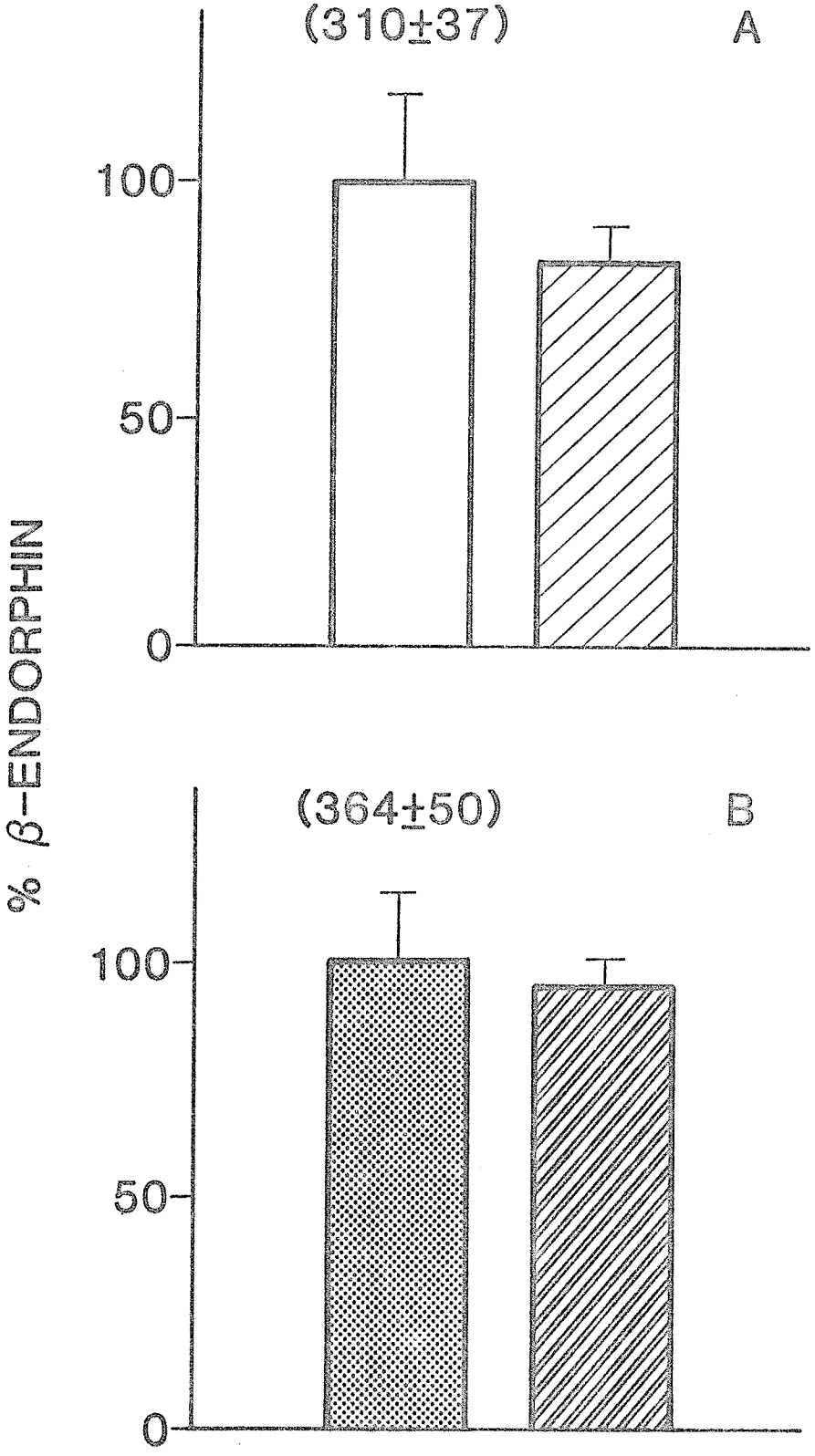
Solid Bar: Bacitracin

Thick Cross-Hatched Bar: Bacitracin-stressed

Means and S.E.M. are shown.

(n = 8 for each group)


C
**
75
50
25
0
% β-ENDORPHIN CHANGE FROM CONTROL (100%)


(310±37)
A
100
50
0
% β-ENDORPHIN
(364±50)
B
100
50
0

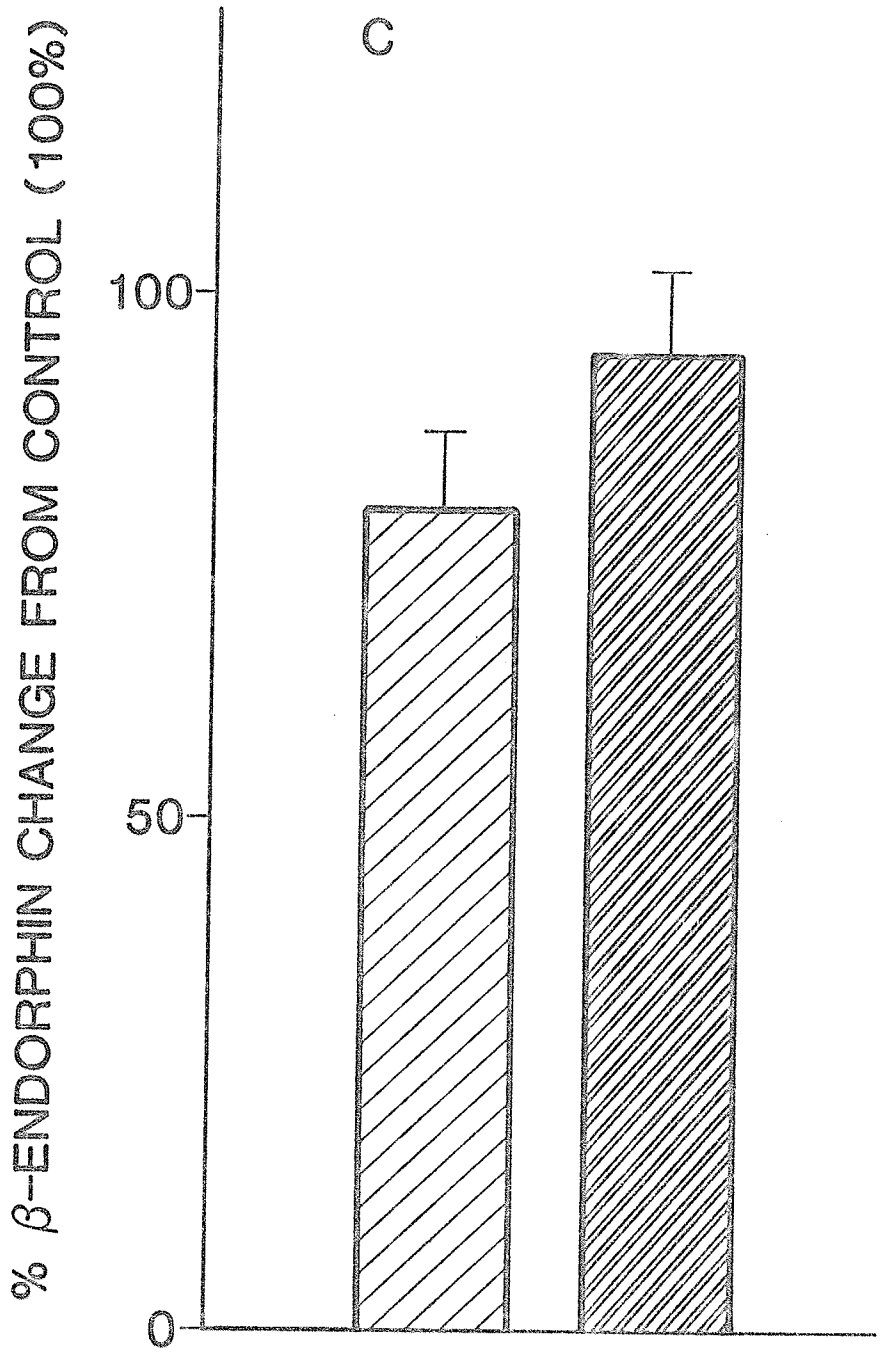

C
% β-ENDORPHIN CHANGE FROM CONTROL (100%)
100
50
0

Figure 33: β-Endorphin Content of Whole Brains of Rats Sacrificed at 30 Minutes Post-Infusion After 11 Intervals of Exposure to Hot-Plate

Open Bar: Control

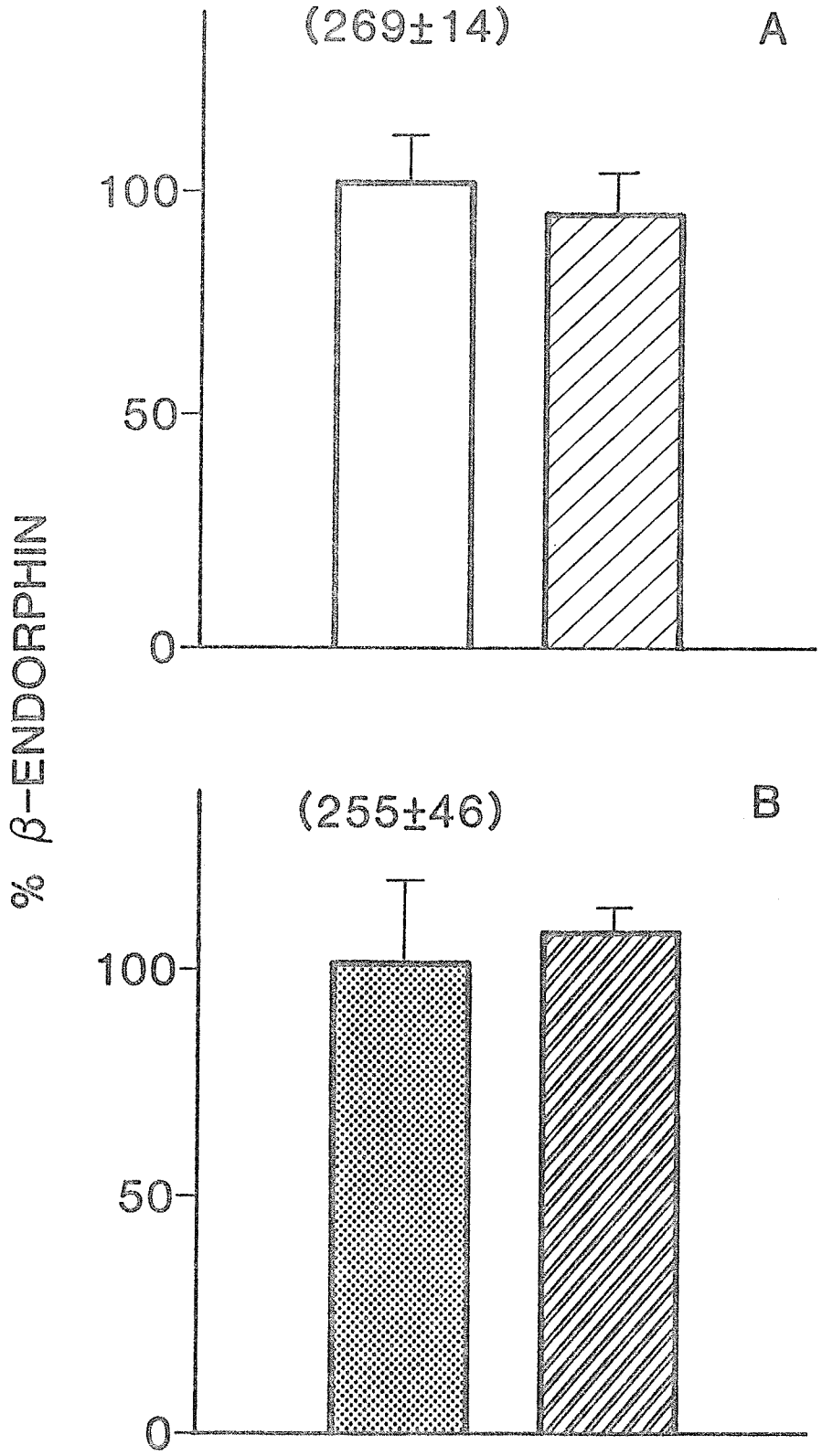
Thin Cross-Hatched Bar: Stressed

Solid Bar: Bacitracin

Thick Cross-Hatched Bar: Bacitracin-Stressed

Means and S.E.M. are shown.

(n = 10 for each group)


(269±14)
A
100
50
0
% β-ENDORPHIN
(255±46)
B
100
50
0

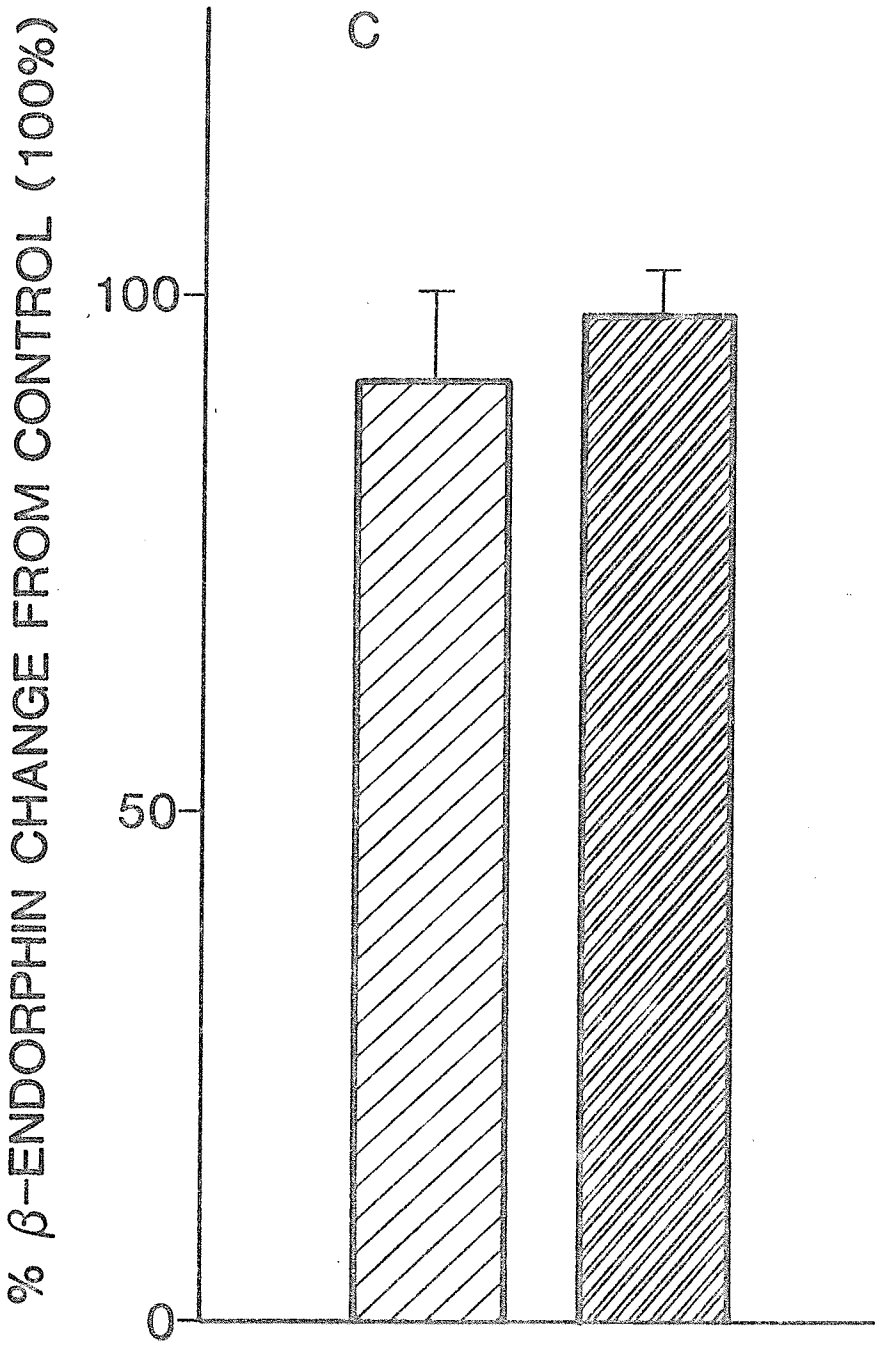

C
% β-ENDORPHIN CHANGE FROM CONTROL (100%)
100
50
0

Figure 34: Somatostatin Content of Brain Regions of Rats Sacrificed 15 Minutes After Infusion of Bacitracin

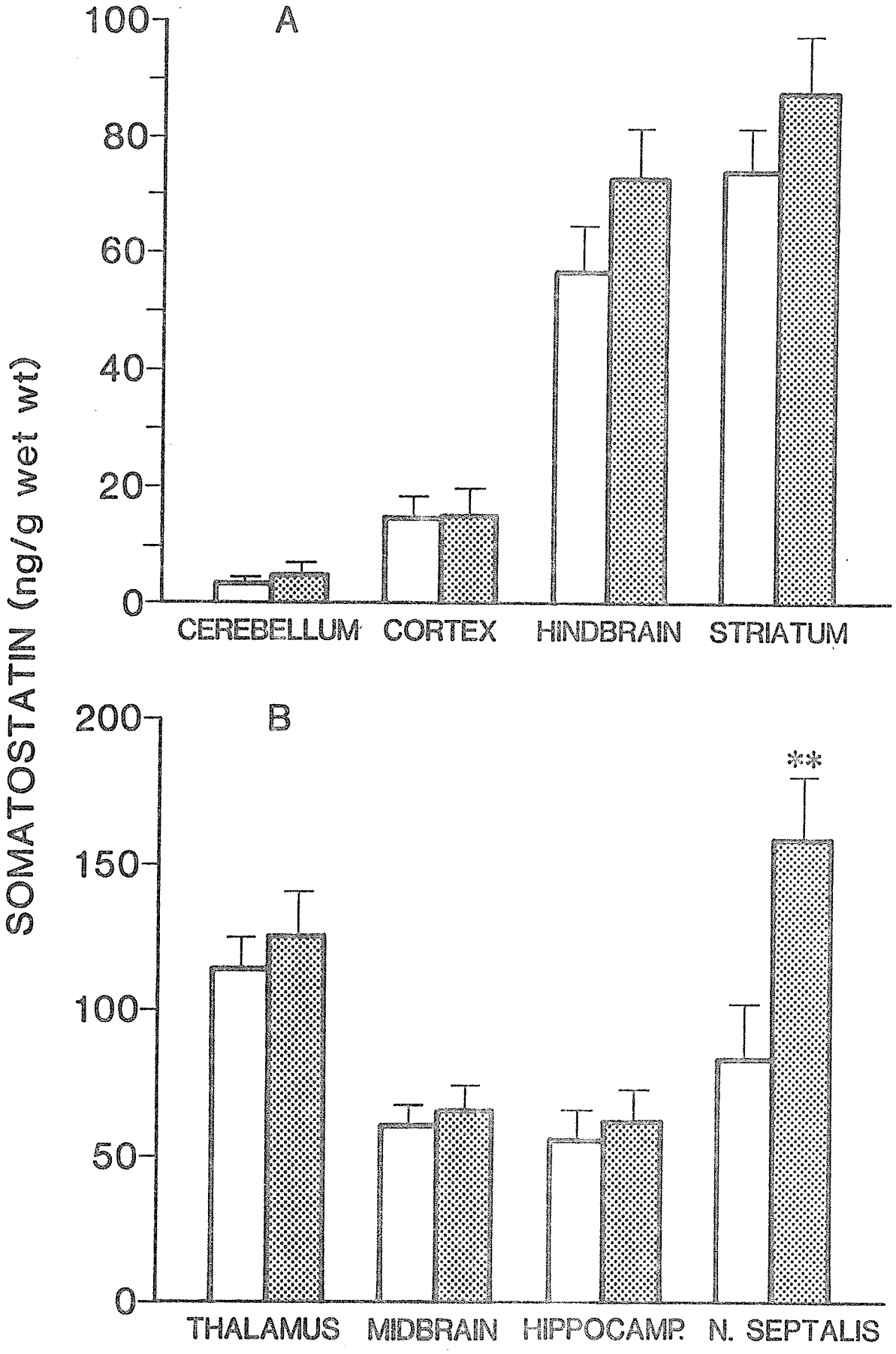
Open Bar: Control (n = 8)

Solid Bar: Bacitracin (n = 8)

Means and S.E.M. are shown.

* Difference between control and bacitracin treated significant at $p < .05$ (Student's t test)

** Difference between control and bacitracin treated significant at $p < .01$ (Student's t test)


A
100
80
60
40
20
0
SOMATOSTATIN (ng/g wet wt)
CEREBELLUM
CORTEX
HINDBRAIN
STRIATUM
B
200
150
100
50
0
**
THALAMUS
MIDBRAIN
HIPPOCAMP.
N. SEPTALIS

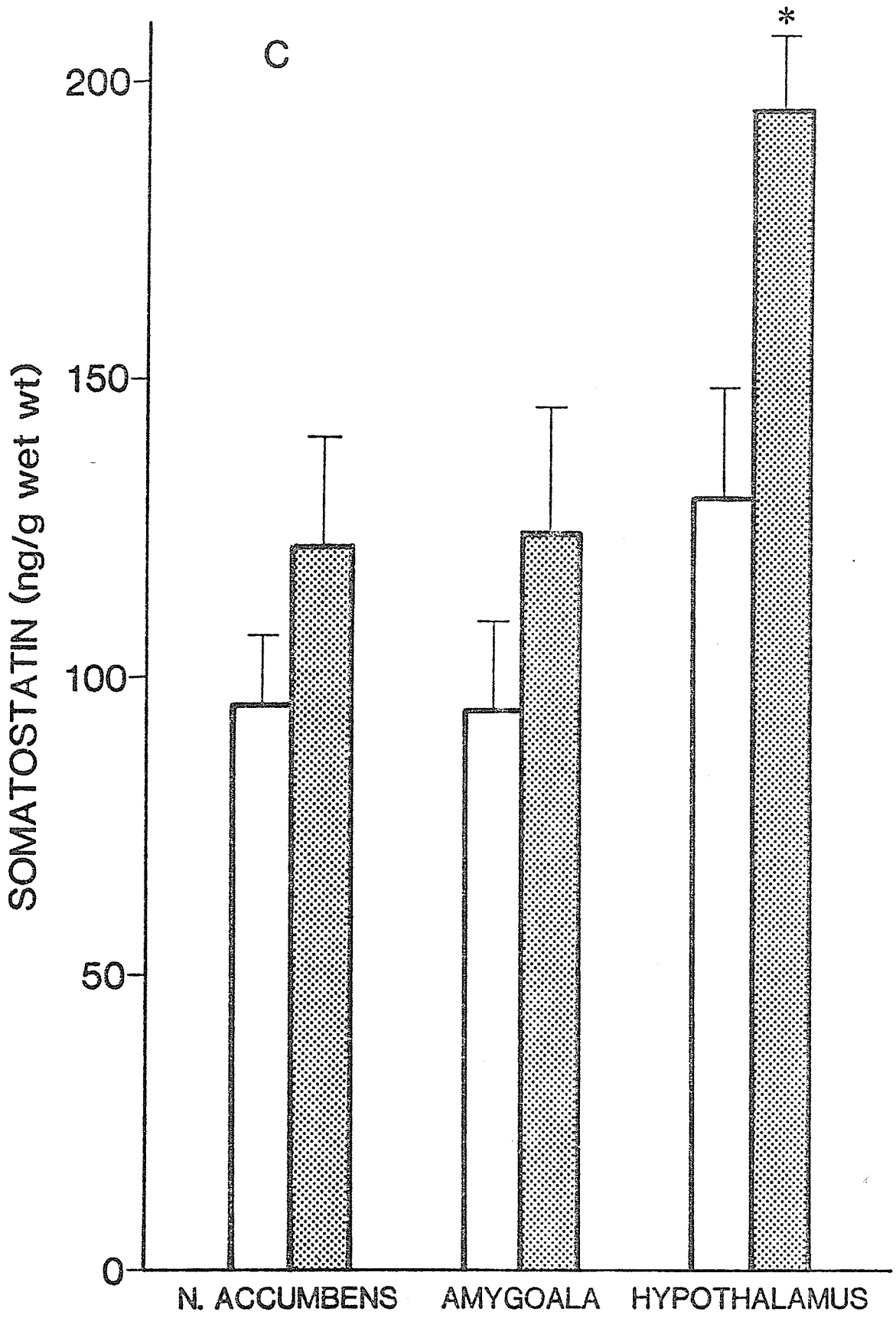

C
*
200
150
100
50
0
SOMATOSTATIN (ng/g wet wt)
N. ACCUMBENS
AMYGOALA
HYPOTHALAMUS

Figure 35: Somatostatin Content of Whole Brains of Rats Sacrificed at 15 Minutes Post-Infusion After 6 Intervals of Exposure to Hot-Plate

Open Bar: Control

Thin Cross-Hatched Bar: Stressed

Solid Bar: Bacitracin

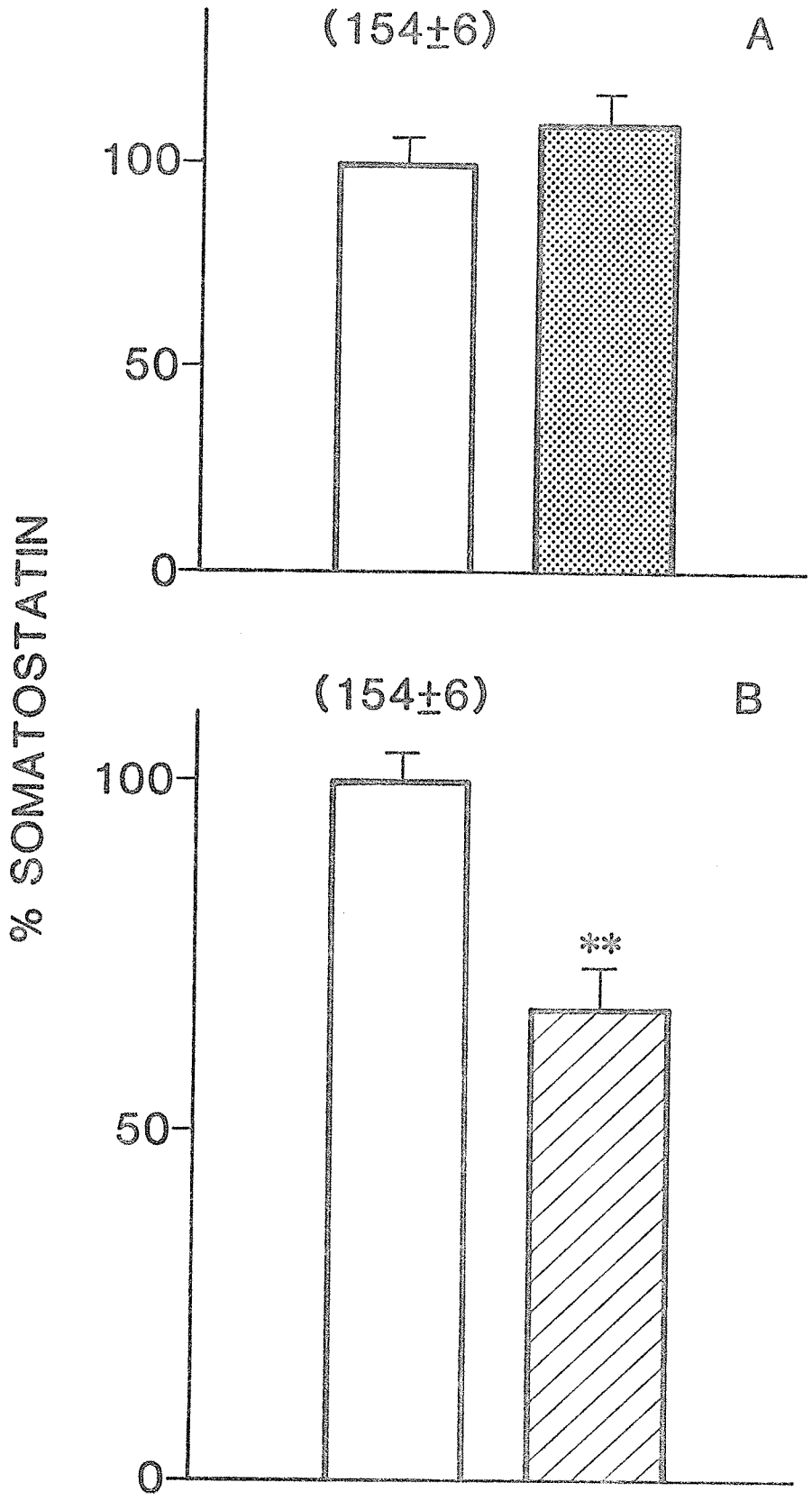
Thick Cross-Hatched Bar: Bacitracin-stressed

Means and S.E.M. are shown.

(n = 8 for each group)

* Difference between control-stressed and bacitracin-stressed significant at $p < .05$ (Student's t test)

** Difference between control and control-stressed significant at $p < .001$ (Student's t test)


(154±6)
A
100
50
0
% SOMATOSTATIN
(154±6)
B
100
50
0
**

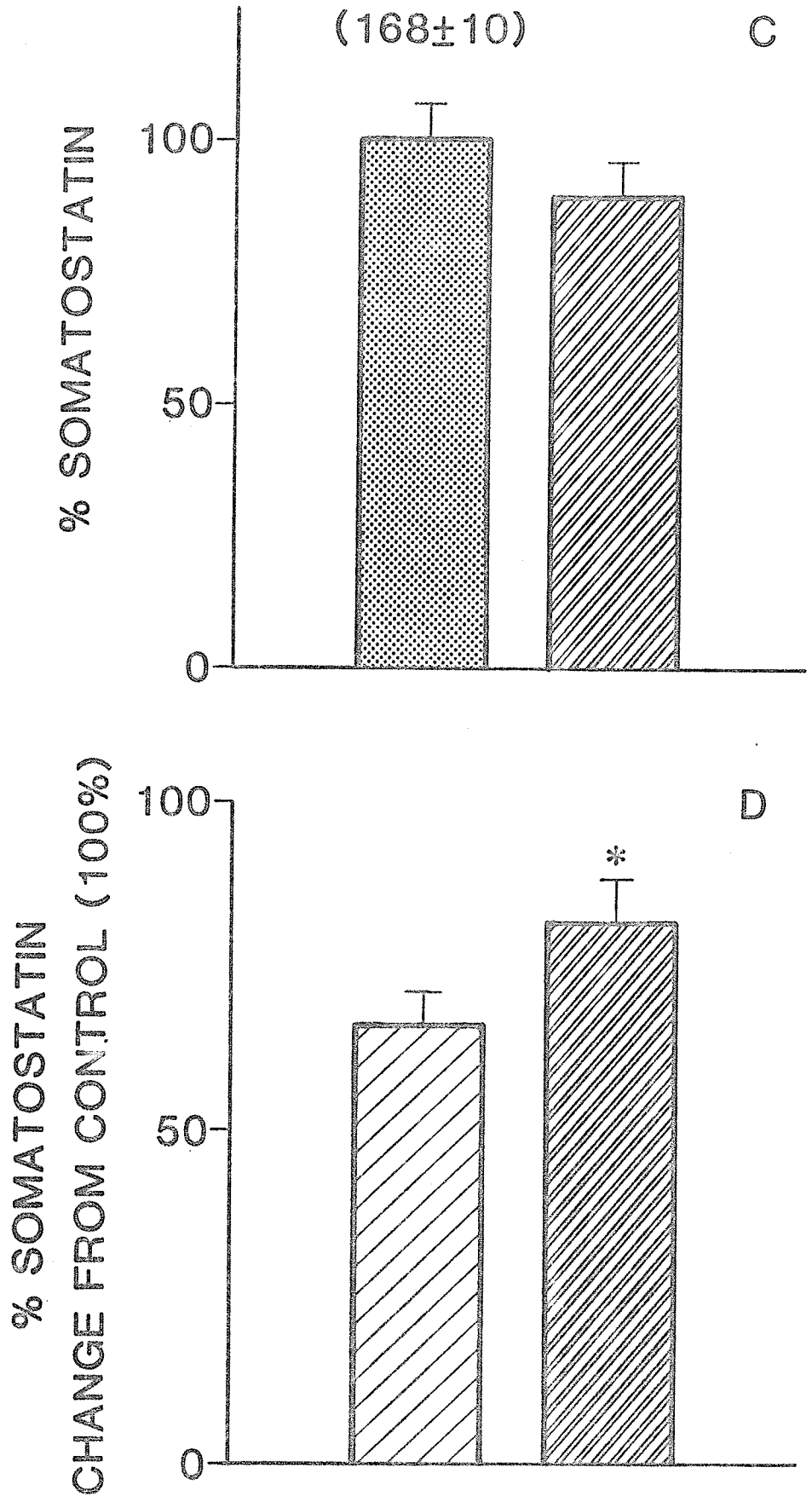

(168±10)
C
% SOMATOSTATIN
100
50
0
D
% SOMATOSTATIN
CHANGE FROM CONTROL (100%)
100
50
0
*

Figure 36: Somatostatin Levels in Rats Sacrificed at 22 Minutes Post-Infusion After 9 Intervals of Exposure to Hot-Plate

Open Bar: Control (n = 8)

Thin Cross-Hatched Bar: Stressed (n = 8)

Means and S.E.M. are shown.

** Difference between control and stressed significant at $p < .02$ (Student's t test)

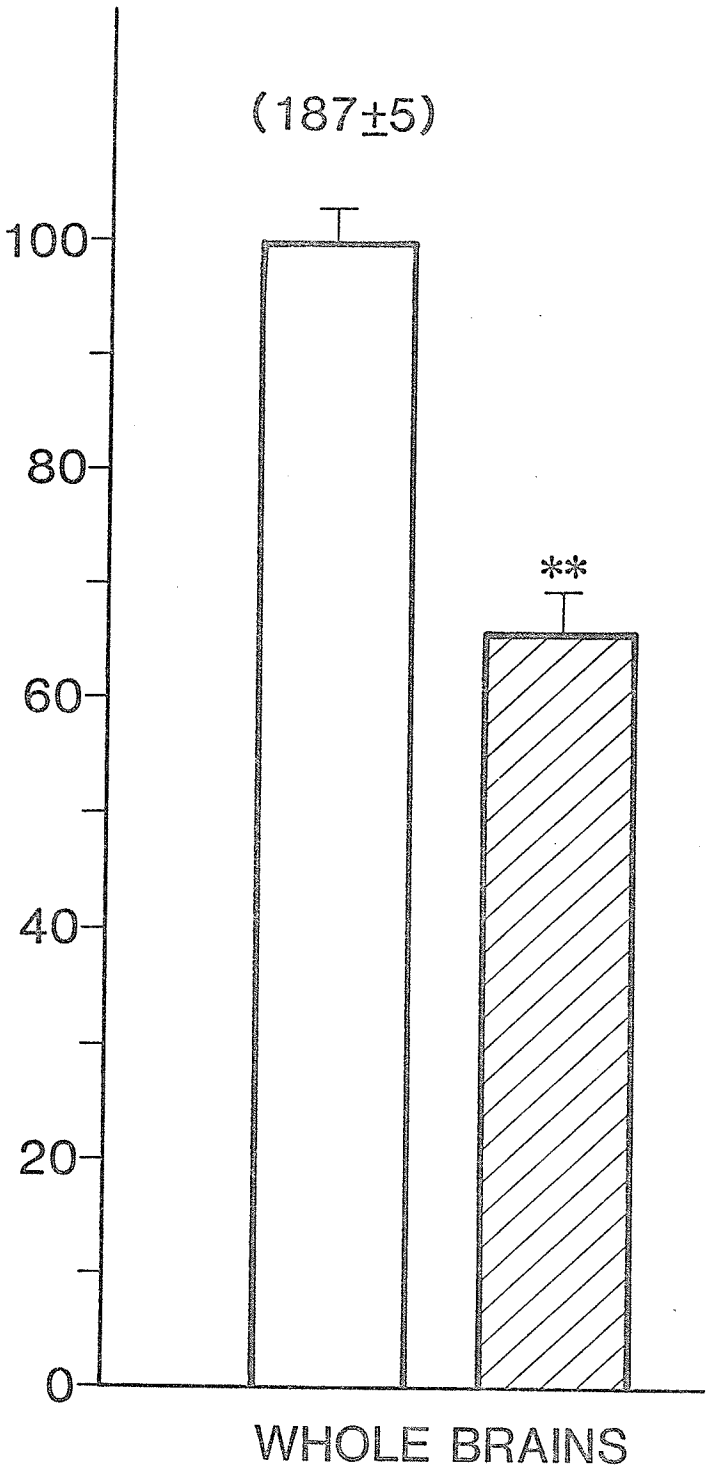
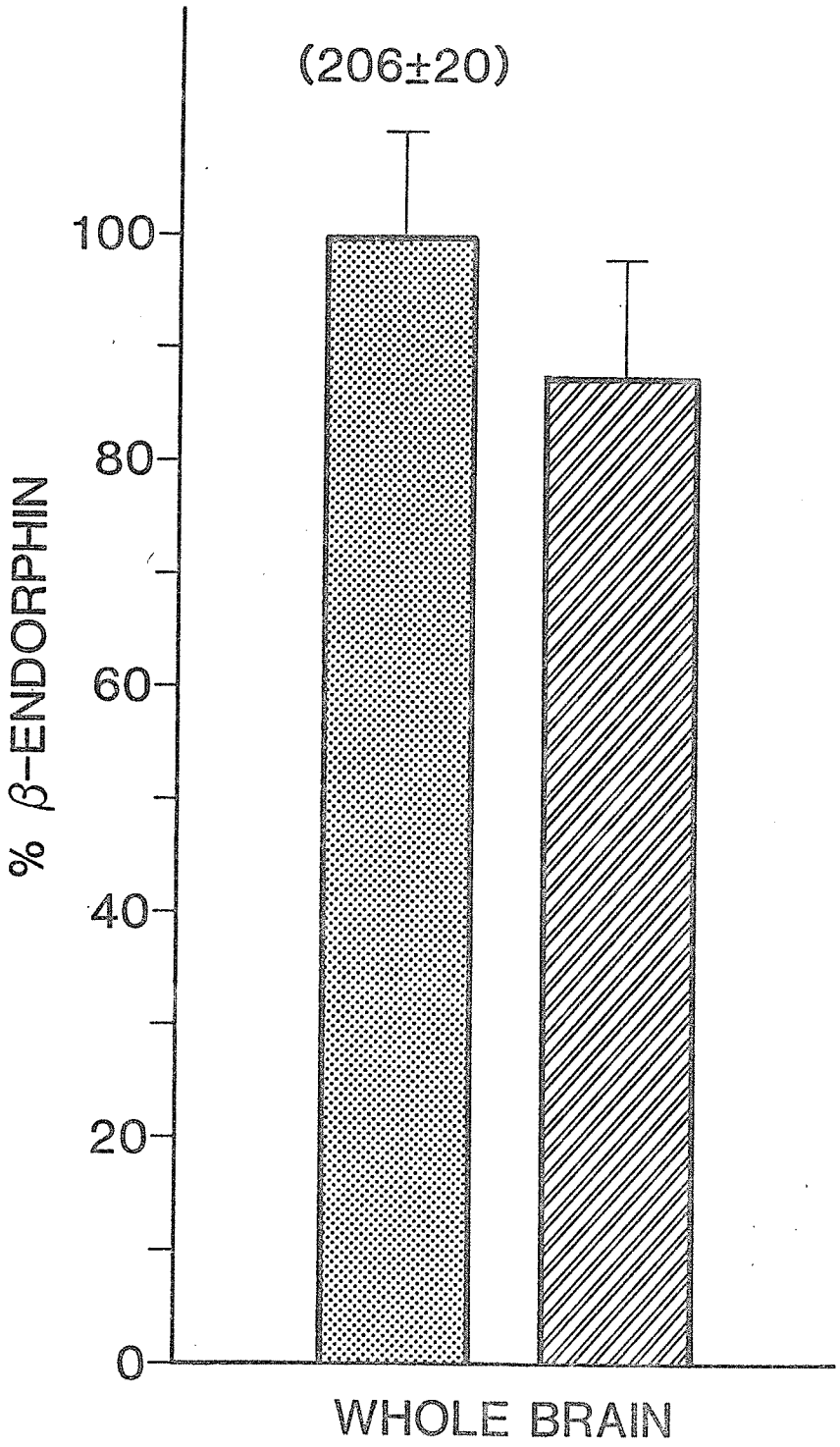

(187±5)
100
80
60
40
20
0
**
WHOLE BRAINS

Figure 37: Somatostatin Levels of Rats Sacrificed at 22 Minutes Post-Infusion After 9 Intervals of Exposure to Hot-Plate

Solid Bar: Bacitracin (n = 8)

Thick Cross-Hatched Bar: Bacitracin-stressed (n = 8)

Means and S.E.M. are shown.


(206±20)
100
80
60
40
20
0
% β-ENDORPHIN
WHOLE BRAIN

control values except for bacitracin-injected stressed rats which now had significantly higher levels ($p < .05$; Student's t test) (Fig. 38).

In another experiment, the pre-bacitracin injection EEG record was compared with the EEG record during 5, 6 minute epochs after completion of bacitracin infusion (during 30 minute post-infusion period). During the wakeful state bacitracin injected animals displayed a depressed desynchronized EEG until 18 minutes post-infusion (Fig. 39). There was a significant depression of the EEG power spectra following bacitracin infusion during this time (Fig. 40a-c). This depression of the power spectra occurred mainly in the alpha 1 and 2, and beta 1 and 2 frequency bands. This decrease in the power spectra of fast frequencies averaged about 48 per cent. There was, however, an increase, in power spectra of the slower frequencies (i.e., delta and theta). These changes in the EEG power spectra returned to control values at 30 minutes post-infusion (Fig. 40d-e). Naloxone (1 mg/kg) abolished the occurrence of depressed desynchronized EEG record in wakeful, bacitracin-injected rats (Fig. 41), i.e., there was no depression of the fast frequencies in the power spectra (Fig. 42).

Figure 38: Somatostatin Content of Whole Brains of Rats Sacrificed 30 Minutes After Infusion of Bacitracin

Open Bar:	Control	(n = 10)
Solid Bar:	Bacitracin	(n = 10)
Thin Cross-Hatched Bar:	Stressed	(n = 10)
Thick Cross-Hatched Bar:	Bacitracin-stressed	(n = 10)

Means and S.E.M. are shown.

* Difference between bacitracin-stressed and control, stressed and bacitracin significant at $p < .05$ (Student's t test)

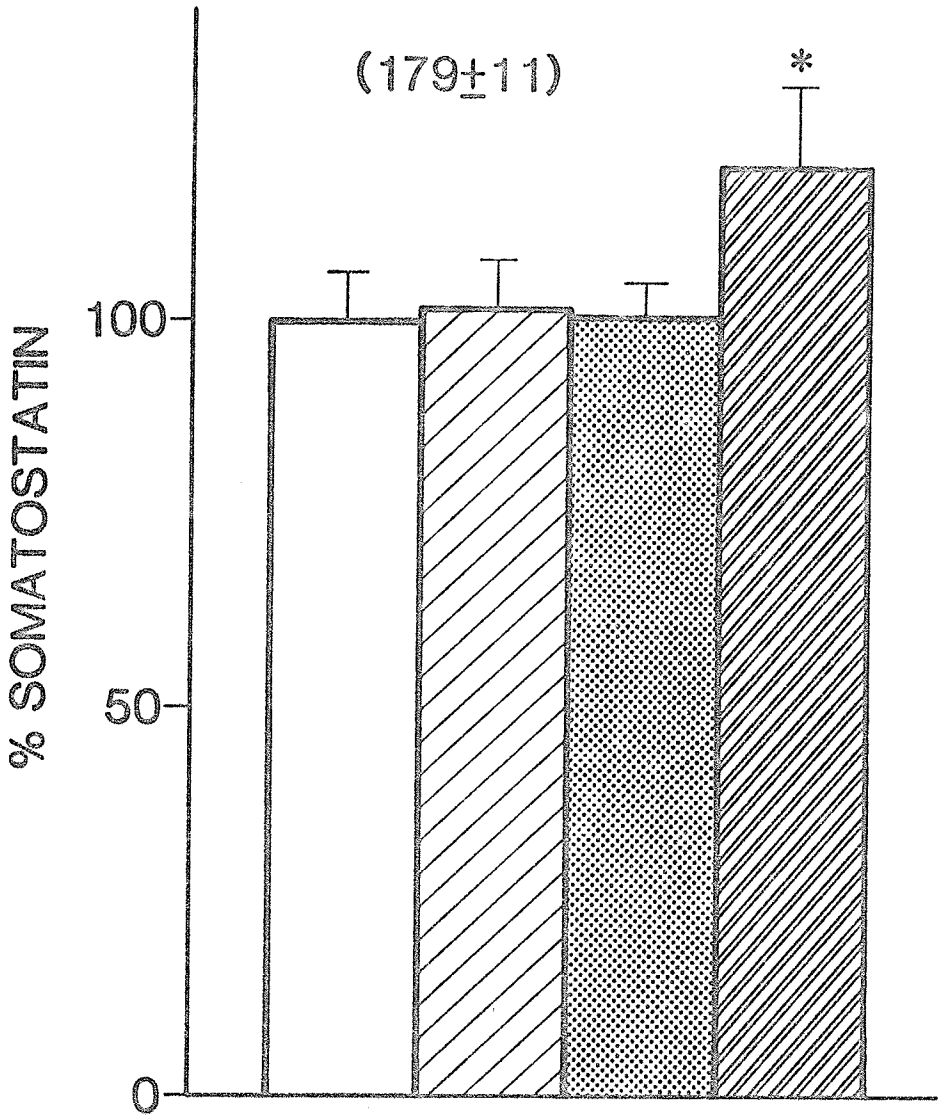

(179±11)
*
% SOMATOSTATIN
100
50
0

Figure 39: EEG Record Pre- and Post-bacitracin Infusion.

EEG record speed is 6 mm/sec.

1. Prebacitracin wakeful record

2. Post-bacitracin wakeful record is 15 minutes post-infusion

(Calibration is at lower right corner)

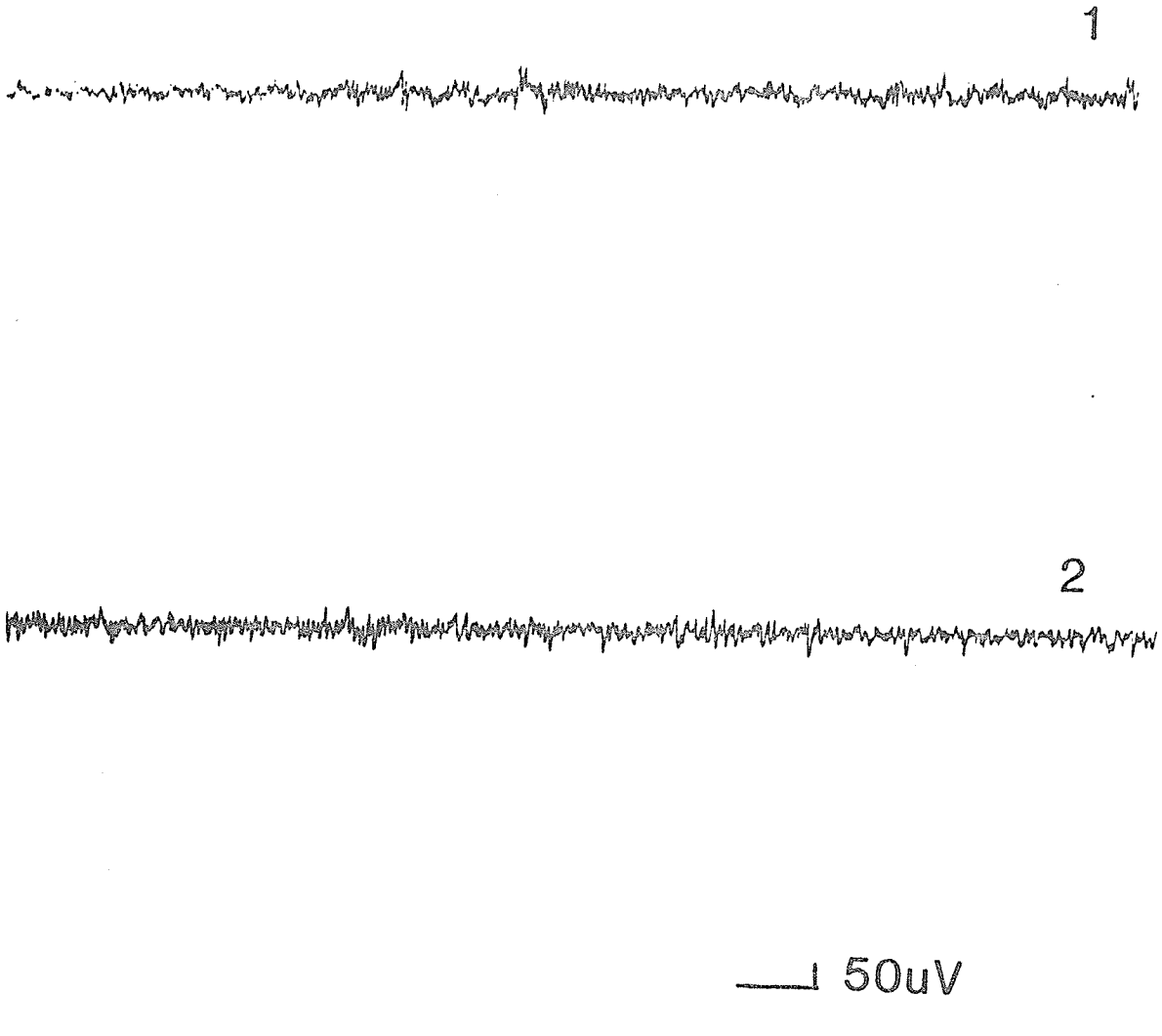

1
2
50uV

Figure 40: Power Spectrum of EEG of the Sensorimotor Cortex After Infusion with Bacitracin

Solid Line: Pre-bacitracin wakeful record

Interrupted Line: Post-bacitracin wakeful record

Vertical bars represent sum of the frequency spectrum

Open Bar: Pre-bacitracin wakeful record

Solid Bar: Post-bacitracin wakeful record

Means and S.E.M. are shown. (n = 6)

A. 0-6 minutes post-infusion of bacitracin

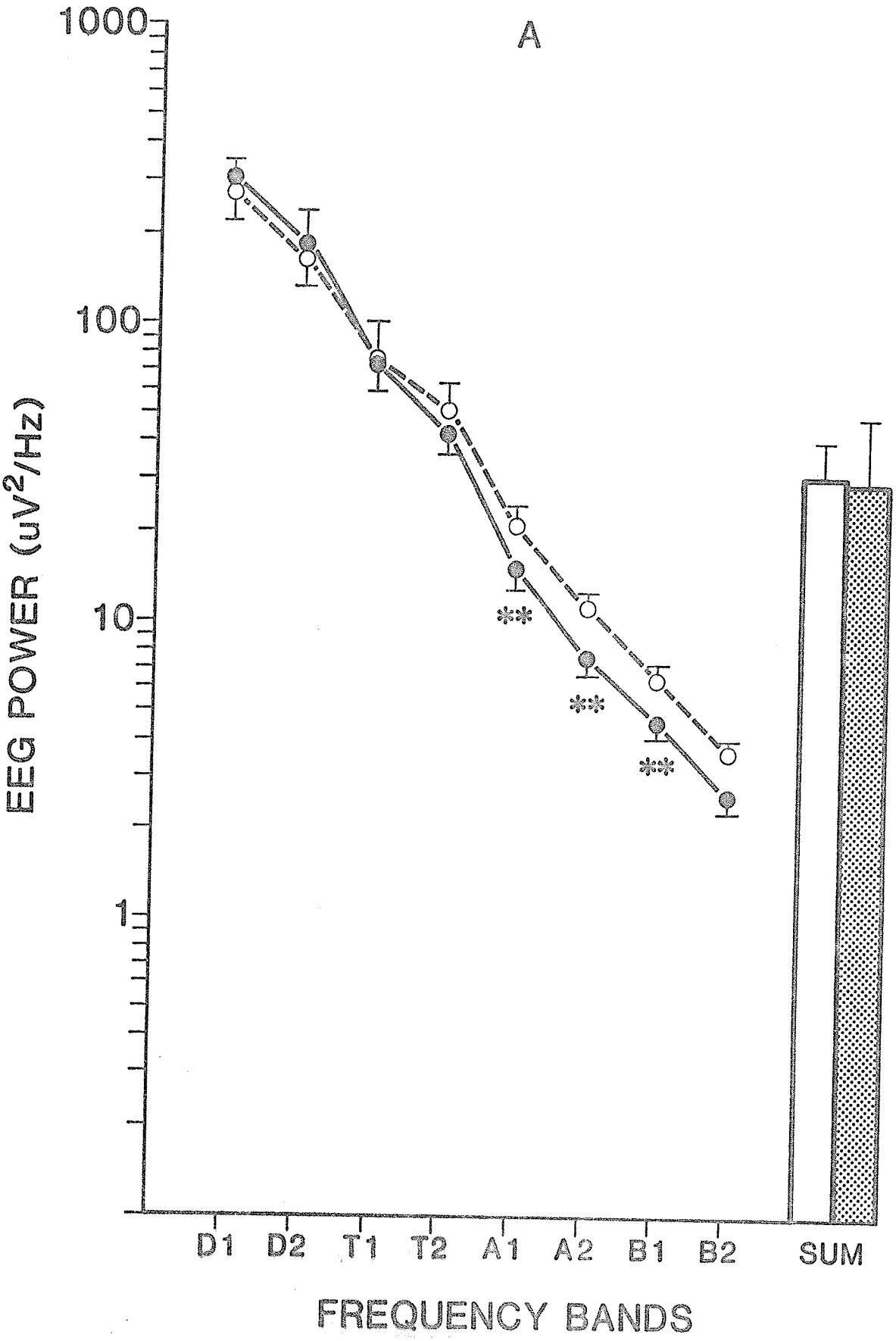
6-12 minutes post-infusion of bacitracin

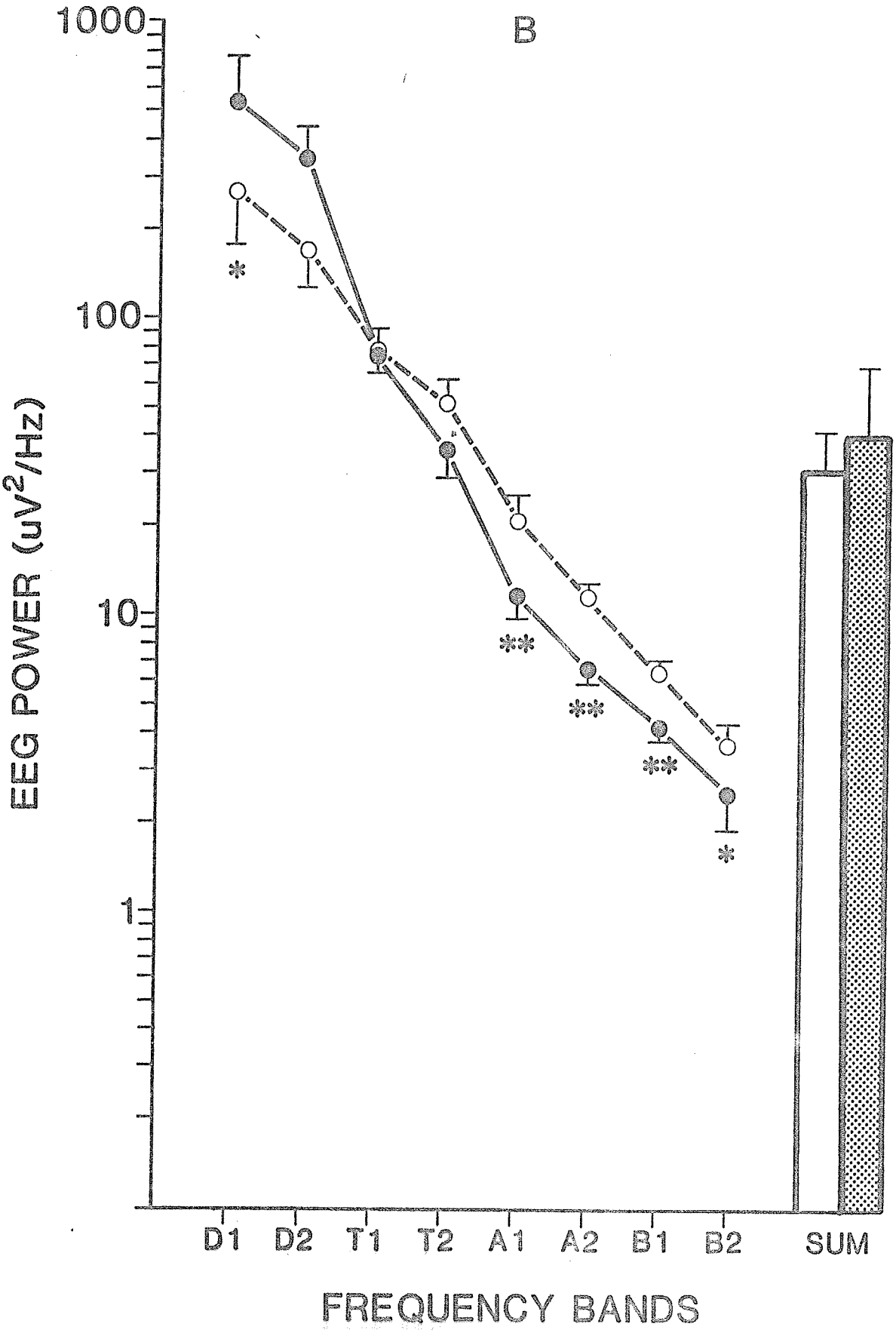
12-18 minutes post-infusion of bacitracin

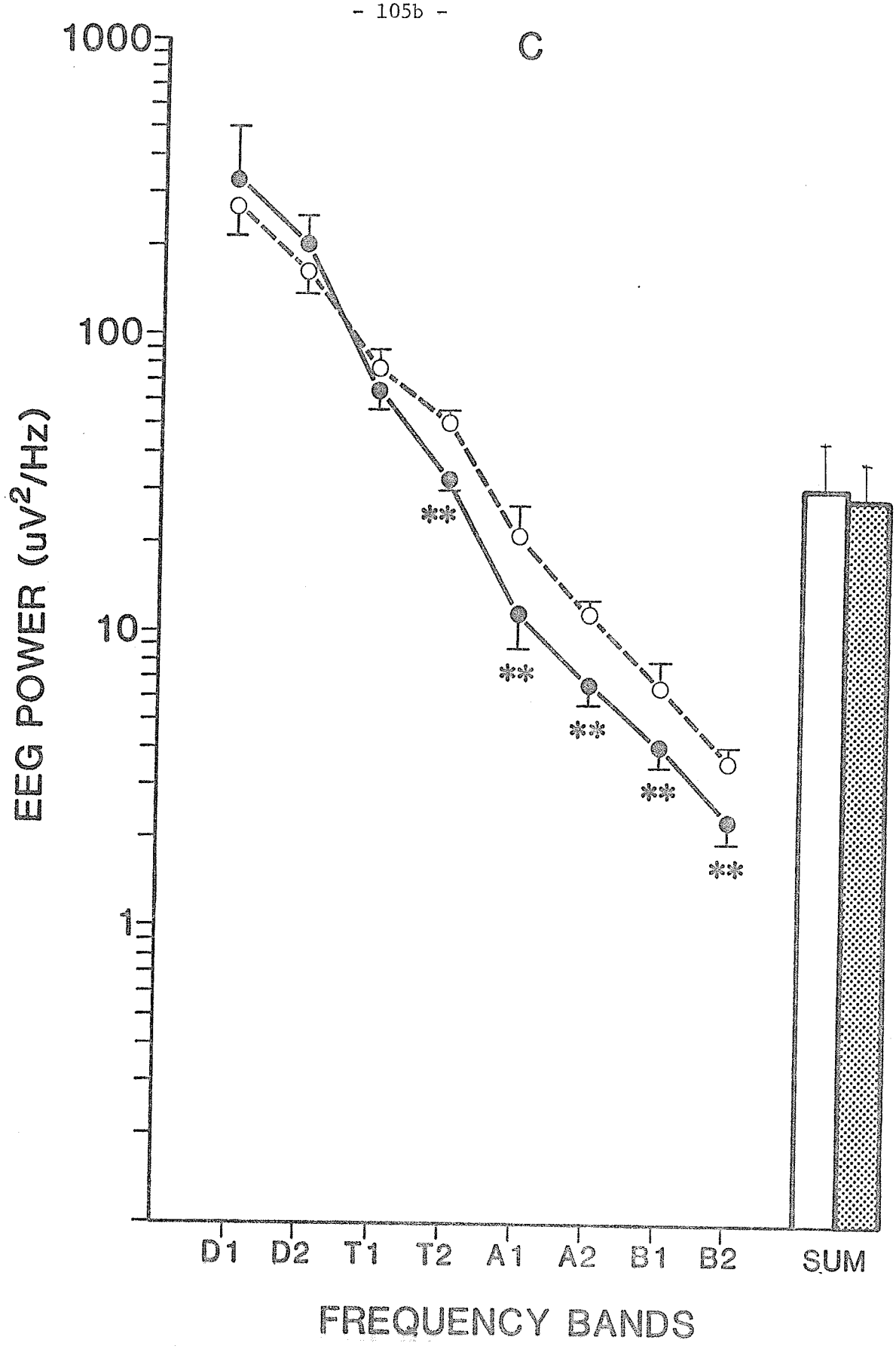
18-24 minutes post-infusion of bacitracin

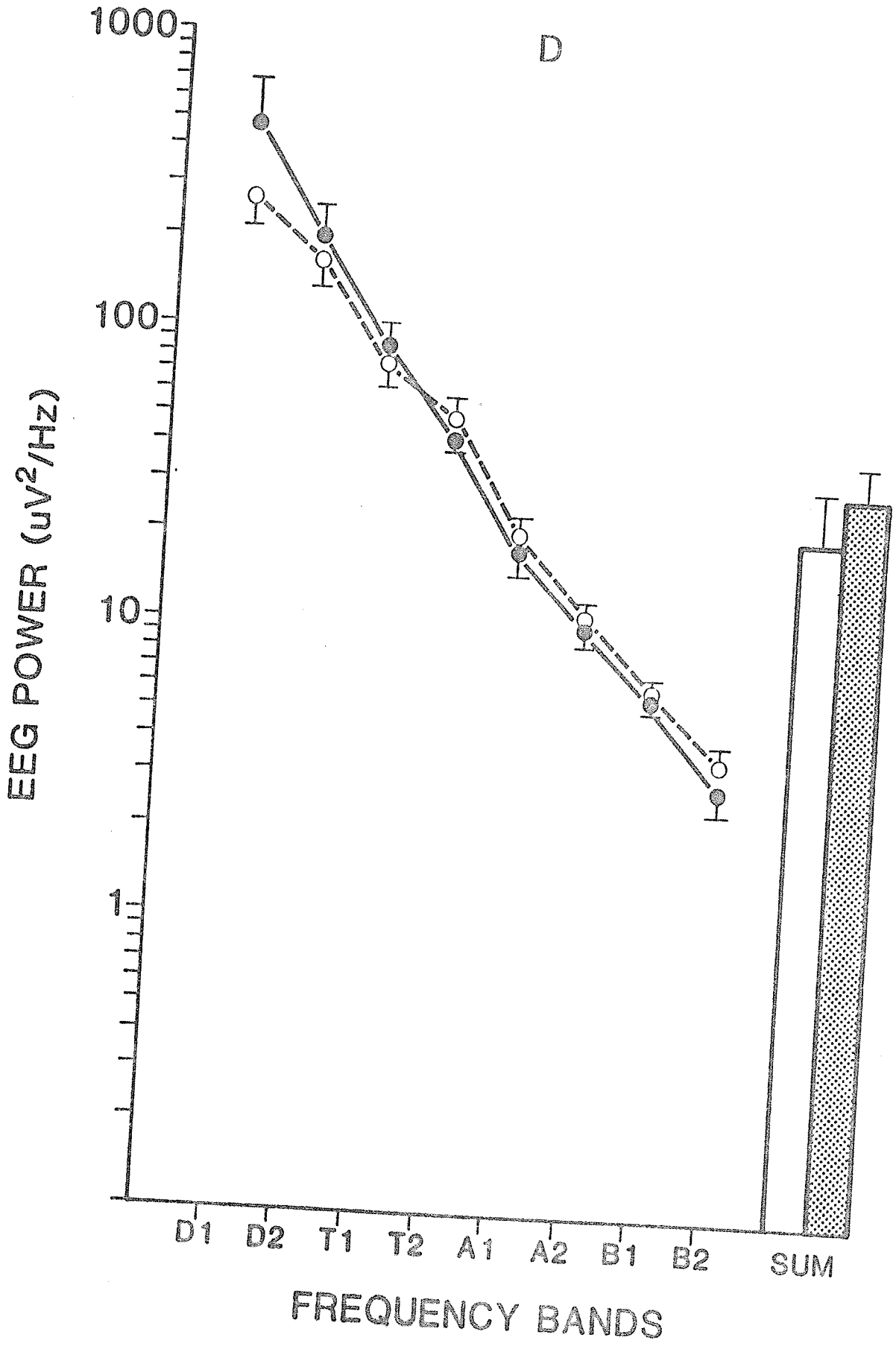
24-30 minutes post-infusion of bacitracin

** Difference between pre-bacitracin and post-bacitracin significant at $p < .01$ (Duncan's Multiple Range Test)


A
1000
100
10
1
EEG POWER (uV²/Hz)
**
**
**
D1
D2
T1
T2
A1
A2
B1
B2
SUM
FREQUENCY BANDS


B
1000
100
10
1
EEG POWER (uV²/Hz)
*
**
**
**
*
D1
D2
T1
T2
A1
A2
B1
B2
SUM
FREQUENCY BANDS


C
1000
100
10
1
EEG POWER (uV²/Hz)
**
**
**
**
**
D1
D2
T1
T2
A1
A2
B1
B2
SUM
FREQUENCY BANDS


D
1000
100
10
1
EEG POWER (uV²/Hz)
D1
D2
T1
T2
A1
A2
B1
B2
SUM
FREQUENCY BANDS

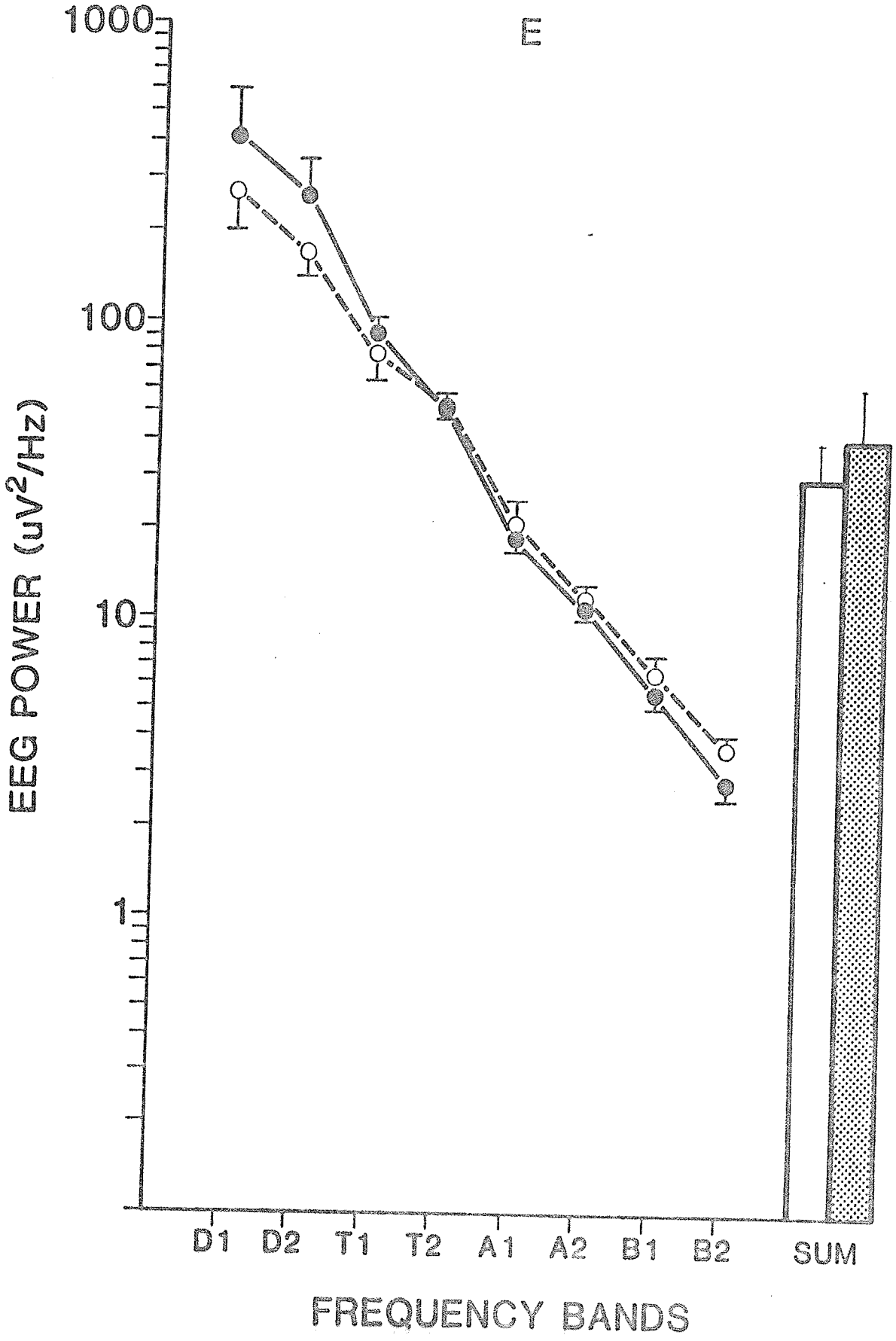

E
1000
100
10
1
EEG POWER (uV²/Hz)
D1
D2
T1
T2
A1
A2
B1
B2
SUM
FREQUENCY BANDS

Figure 41: EEG Record Pre-bacitracin and Post-bacitracin + naloxone (1 mg/kg) Infusion.

EEG record speed is 6 mm/sec

1. Pre-bacitracin wakeful record
2. Post-bactracin + naloxone (1 mg/kg) wakeful record at 15 minutes post infusion

(Calibration is at lower right corner)

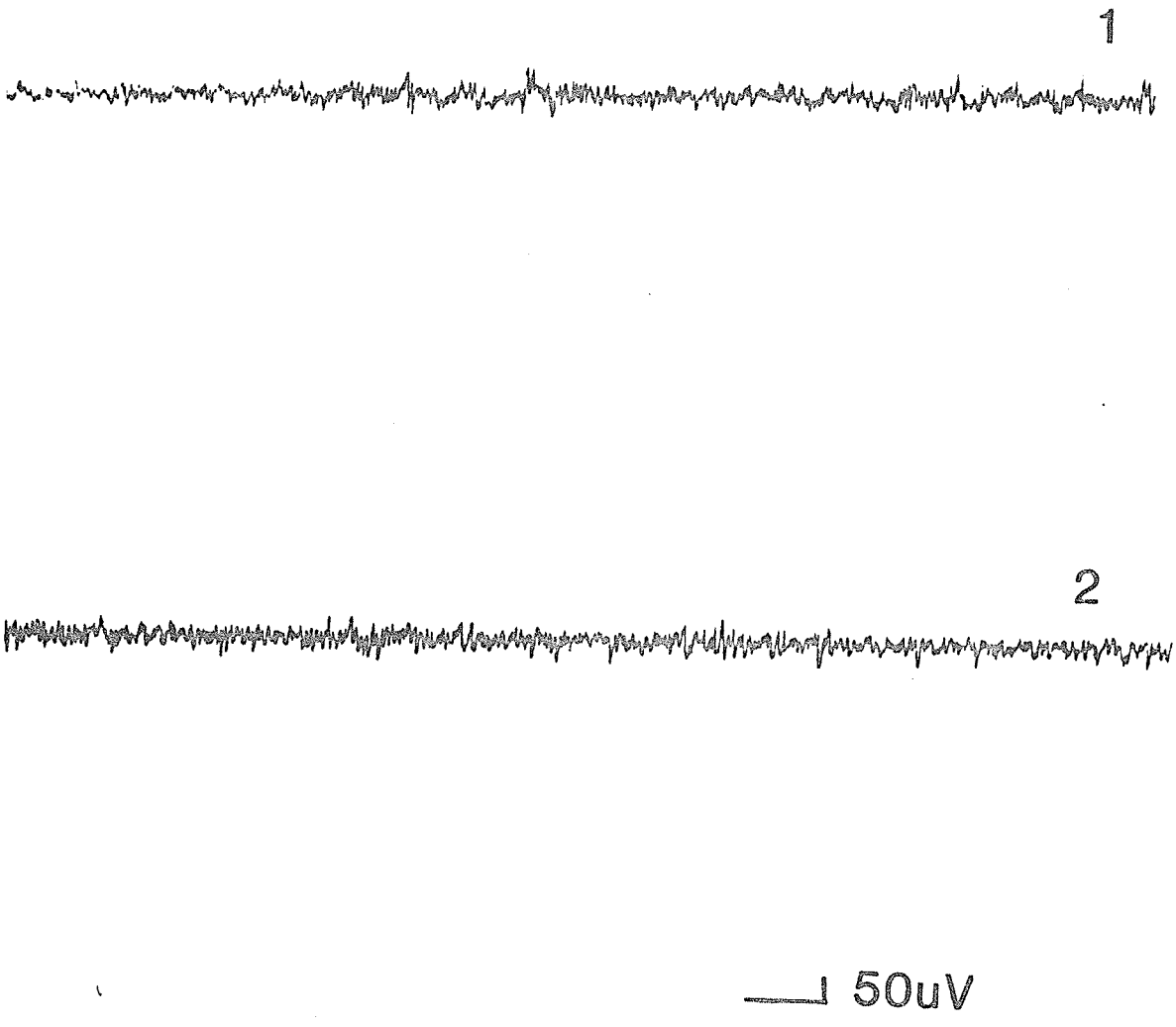

1
2
50uV

Figure 42: Power Spectrum of EEG of the Sensorimotor Cortex After Infusion with Bacitracin and Injection of Naloxone (1 mg/kg)

Solid Line Pre-bacitracin wakeful record

Interrupted Line: Post-bacitracin + naloxone wakeful record

Vertical bars represent sum of the frequency spectra.

Open Bar: Pre-bacitracin wakeful record

Interrupted Solid Bar: Post-bacitracin + naloxone wakeful record

Means and S.E.M. are shown. (n = 5)

A. 0-6 minutes post-infusion of bacitracin + naloxone

B. 6-12 minutes post-infusion of bacitracin + naloxone

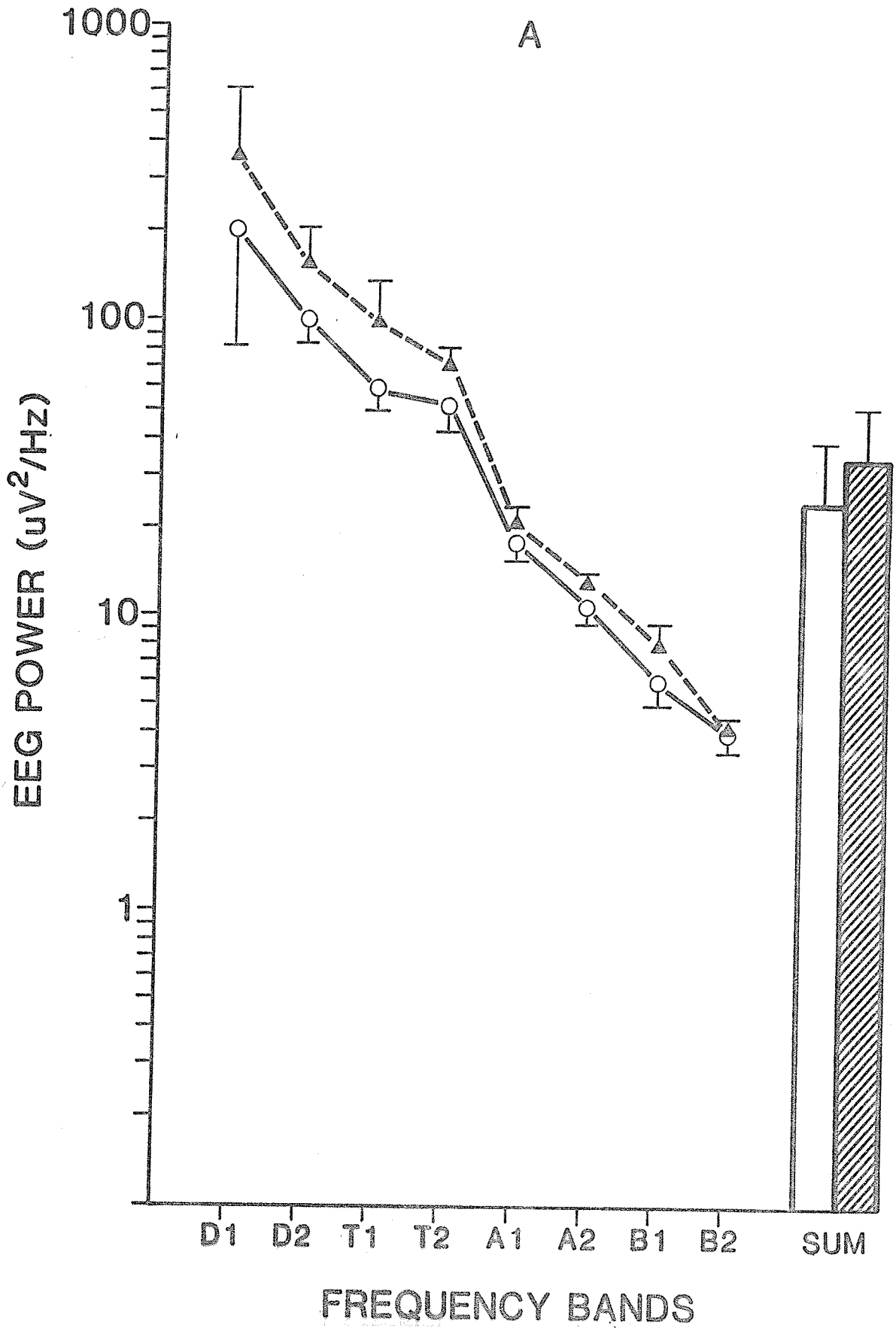
C. 12-18 minutes post-infusion of bacitracin + naloxone

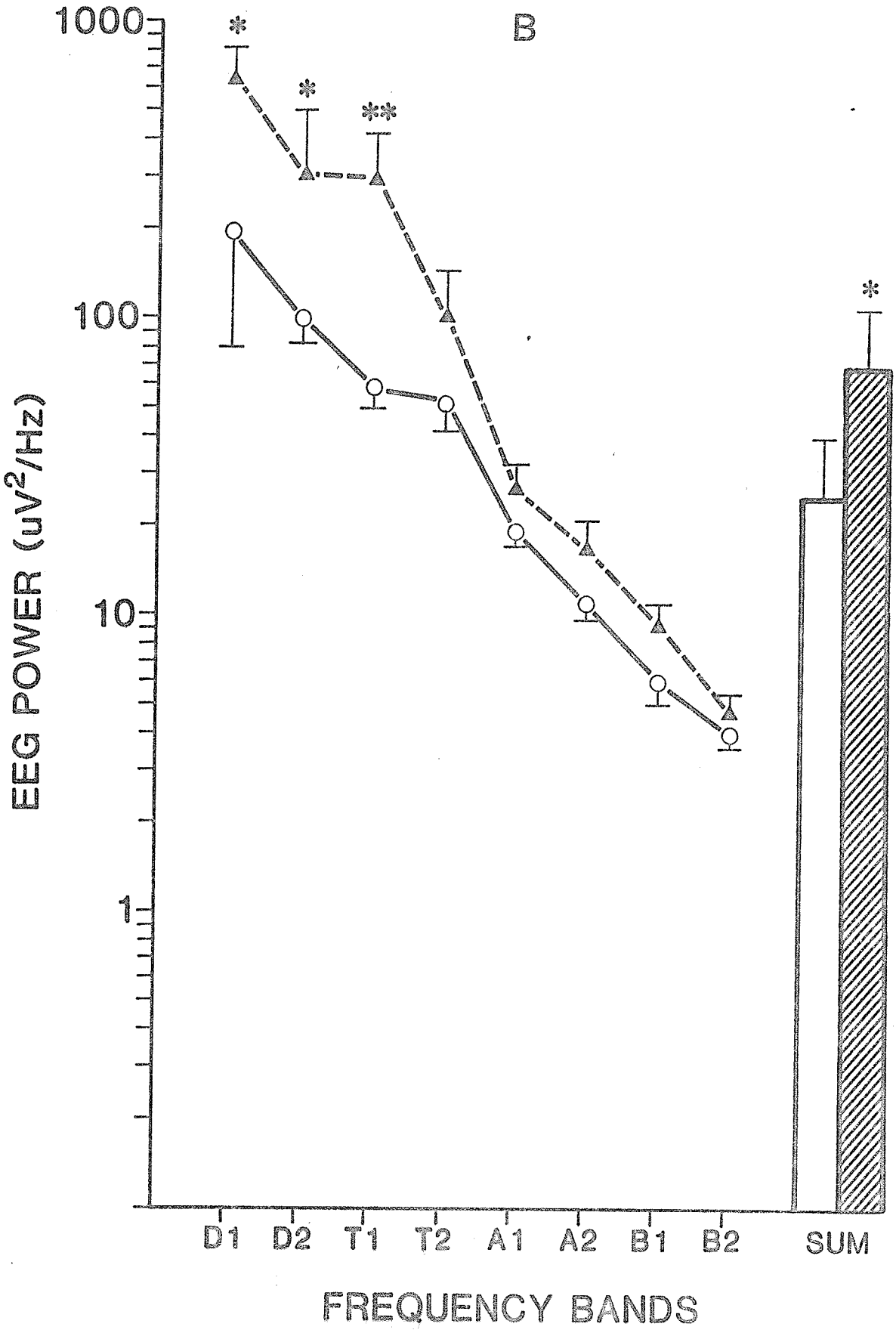
D. 18-24 minutes post-infusion of bacitracin + naloxone

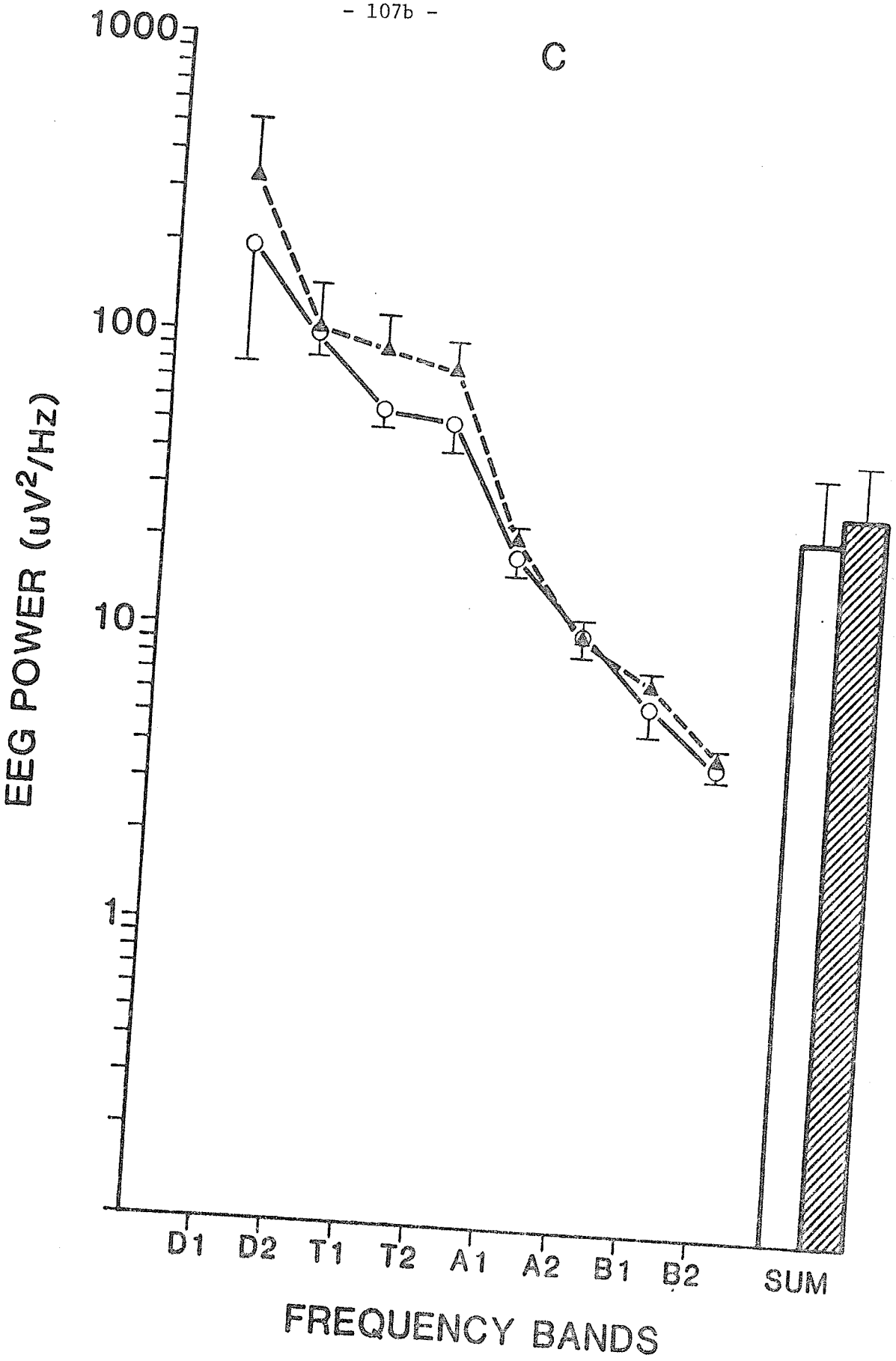
E. 24-30 minutes post-infusion of bacitracin + naloxone

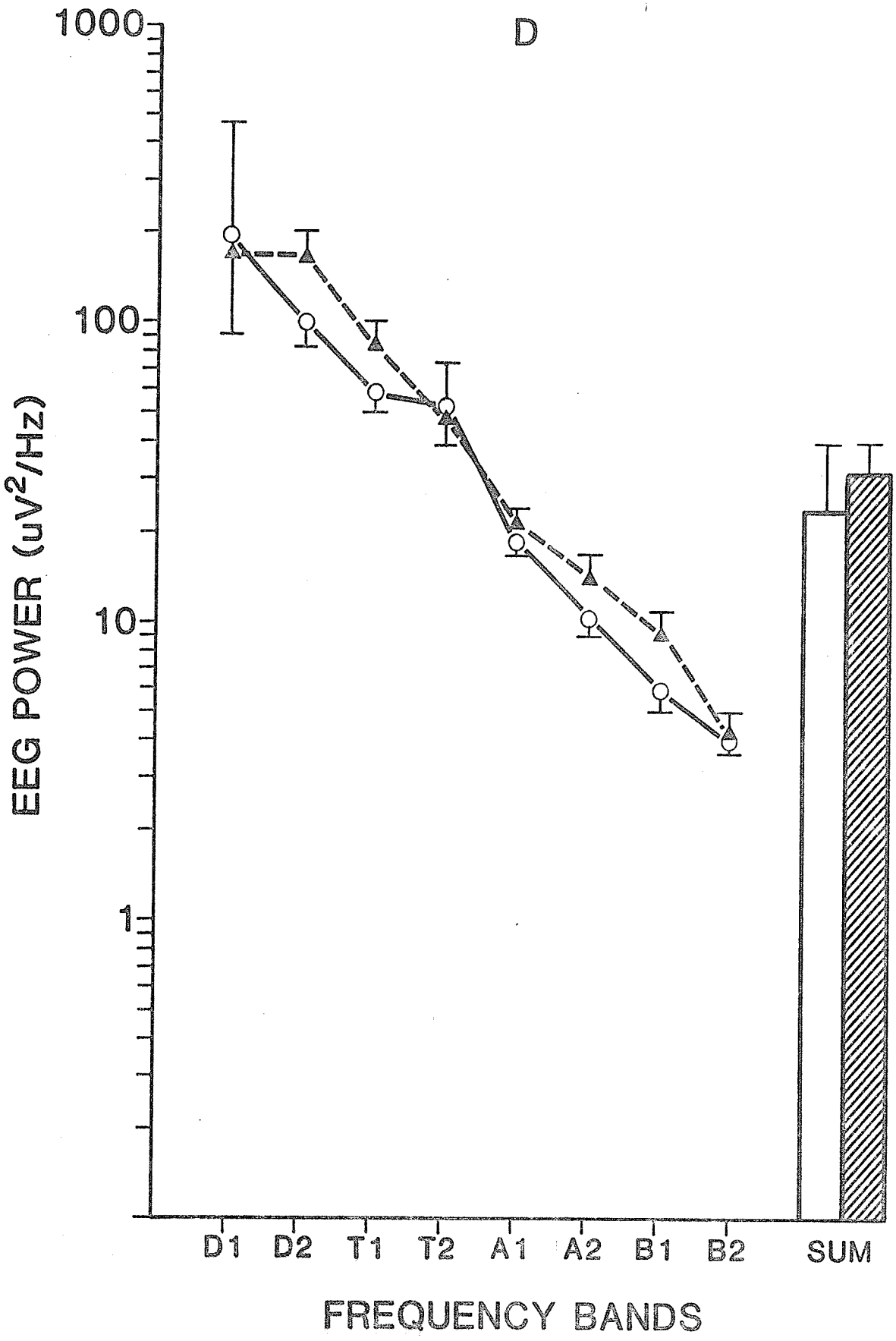
* Difference between pre-bacitracin and post-bacitracin + naloxone significant at $p < .05$ (Duncan's Multiple Range Test)

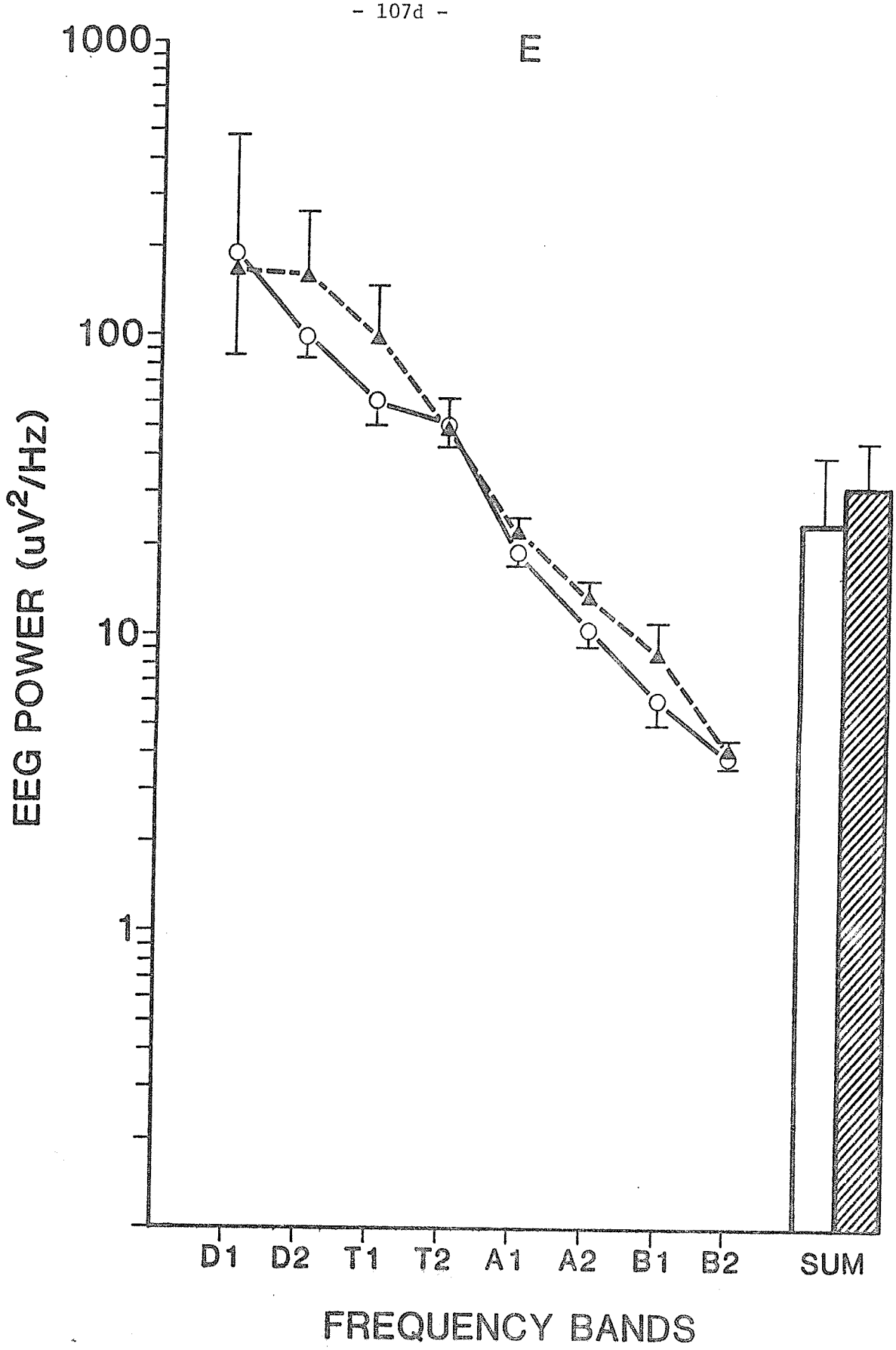
** Difference between pre-bacitracin and post-bacitracin + naloxone significant at $p < .01$ (Duncan's Multiple Range Test)


A
1000
100
10
1
EEG POWER (uV²/Hz)
D1
D2
T1
T2
A1
A2
B1
B2
SUM
FREQUENCY BANDS


B
1000
100
10
1
EEG POWER (uV²/Hz)
*
*
**
*
D1
D2
T1
T2
A1
A2
B1
B2
SUM
FREQUENCY BANDS


C
1000
100
10
1
EEG POWER (uV²/Hz)
D1
D2
T1
T2
A1
A2
B1
B2
SUM
FREQUENCY BANDS


D
1000
100
10
1
EEG POWER (uV²/Hz)
D1
D2
T1
T2
A1
A2
B1
B2
SUM
FREQUENCY BANDS


E
1000
100
10
1
EEG POWER (uV²/Hz)
D1
D2
T1
T2
A1
A2
B1
B2
SUM
FREQUENCY BANDS

DISCUSSION

A. BACITRACIN AND BRAIN β-ENDORPHIN LEVELS

It has been previously reported that bacitracin potentiates the effect of enkephalins (Miller, et. al., 1977) and decreases biodegradation of β-endorphin (Patthy, et. al., 1977), in vitro. Our hypothesis was that if bacitracin could decrease peptide biodegradation, in vivo, it should lead to an increase in brain β-endorphin content. Brain β-endorphin content was 27 per cent higher in bacitracin injected rats than control rats at 15 minutes post-infusion. The increased brain β-endorphin level is most likely due to decrease in metabolic breakdown of basally secreted β-endorphin. The gradual decrease in β-endorphin level in bacitracin injected rats to control values at 30 minutes post-infusion was understandable since bacitracin itself is susceptible to degradation by brain enzymes.

Since the action of bacitracin is not specific for β-endorphin, brain levels of another peptide, namely somatostatin, were also examined. However, we found no significant changes in brain somatostatin levels following infusion of bacitracin. It might be that the basal rate of secretion of somatostatin is lower than that of β-endorphin, hence there is an insignificant accumulation of somatostatin to be detected. The significant increases in β-endorphin levels in the striatum and Amygdala following bacitracin infusion might reflect an increased rate of β-endorphin turnover in these regions. It is interesting to note that we detected this in two regions of the brain which are ineffective sites for the production of analgesia, which is the

most potent function attributed to β-endorphin. The increased turnover of β-endorphin in striatum and Amygdala might reflect the importance of endorphins in normal everyday regulatory functions of limbic systems such as control of mood and behaviour. Various observations (Lal, 1975; Neal, et. al., 1978) suggest that opiates, in general, affect the activity of the nigrostriatal dopaminergic systems. For instance, high doses of β-endorphin cause hypokinesia, catatonia and muscular rigidity, effects which are easily inhibited by dopamine agonist. The view that action of opiates involve the dopaminergic systems in the striatum has been substantiated by evidence that the striatum is rich in opiate receptors. The Amygdala has the greatest amount of opiate receptors with the anterior Amygdala having twice as many as the posterior Amygdala. Various other observations have also suggested a close interaction between β-endorphin and Amygdala. For instance, lesions on the pyriform cortex overlying the Amygdala (Green, et. al., 1957) and peripheral injection of enkephalin or β-endorphin (Veith, et. al., 1978) both markedly increase sexual activity. Limbic structures have long been known to be involved in learning behaviour. Now recent evidence has shown that β-endorphin also plays a role in the learning process (Riley, et. al., 1980). Intravenous injection of β-endorphin causes an increase in movements such as chewing and licking (Catlin, et. al., 1978). Similarly increases in chewing and licking movements have also been seen following stimulation of limbic structures (Ganong, 1969). And, of course, the epileptogenic effect of β-endorphin on limbic structures has been well documented.

B. BACITRACIN INDUCED CHANGES IN EEG

The depression of the EEG activity during the wakeful state, in bacitracin injected animals further supports our view that bacitracin does indeed lead to accumulation of endogenous opiates in the brain. This observation of depression of EEG 15 minutes following infusion of bacitracin and the subsequent recovery at 30 minutes post-infusion is strikingly similar to the depression seen by Neale, et. al., (1978) following local intracerebral injection of met-enkephalin in the basal ganglia. It could be that bacitracin leads to an accumulation of endogenous endorphins in this region which thereby leads to depression of EEG activity, as was the case in the study done by Neale, et. al., (1978). Whether the depression in EEG activity observed in bacitracin injected rats is due to accumulation of met-enkephalin, β-endorphin or both yet remains unclear. It is well known that the amount of opiate required to cause electrophysiological changes is much less than that required to cause behavioural effects. Thus despite the fact that the profound analgesia exhibited by bacitracin injected rats is most likely due to accumulation of β-endorphin, the EEG changes could be due to the accumulation of met-enkephalin and/or β-endorphin.

Analysis of the EEG power spectrum revealed that the faster requencies were depressed in bacitracin injected rats. The appearance of fast frequency alpha waves is dependent on the degree of activity in either the brain stem or the thalamic portions of the reticular activating system. Transection of the fiber tract from the thalamus to the cortex causes the development of delta waves in the cortex. Thus the delta waves are a result of some independent mechanisms in the cortex (Guyton, 1976). The depression of the faster frequencies

and the increase in power spectra of the slower frequencies might be a reflection of the low activity in the reticular activating system. That is, the signals from the reticular activating system, which normally pass through various limbic structures, on the way to the cortex might have been depressed in bacitracin injected rats. Narcotic analgesics, such as Pethidine, are known to depress cerebral activating and waking mechanisms which are usually mediated by reticular and hippocampal system (Monnier, et. al., 1967).

The mechanism by which endogenous opioid might modify spontaneous EEG becomes apparent when one correlates (1) the close association of distribution of opiate receptors and their endogenous ligands in the limbic structures and (2) the fact that transmission of signals from the reticular activating system is usually through various limbic structures.

C. STRESS AND β-ENDORPHIN

Acute stress clearly leads to a significant decrease in brain β-endorphin content. This is in agreement with Millan's work (1981) who found a similar decrease in β-endorphin content in hypothalamus and periventricular tissue following acute stress. However, despite this release of β-endorphin in both control and bacitracin injected stressed rats, relief of pain from the noxious stimuli in the form of analgesia was observed only in the bacitracin injected rats. One possible explanation for this observation is shown in Fig. 43. Analgesia is thought to result from binding of endogenous opiate ligands with opiate receptors. Under normal physiological conditions the basal rate of secretion and degradation of opiates is kept at such

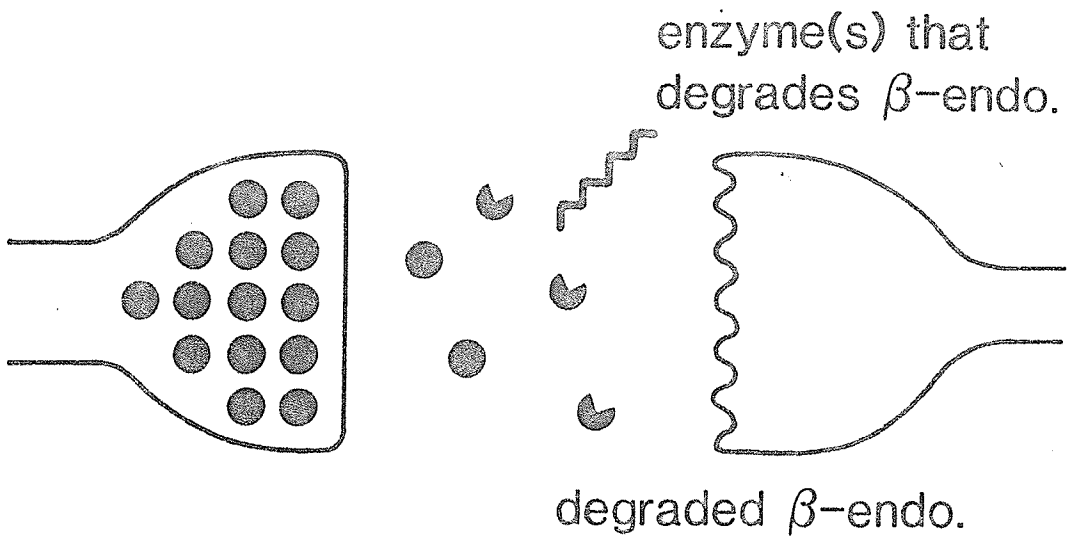
a balance that there isn't sufficient accumulation of endogenous opiate peptides to cause analgesia. However, this balance between β-endorphin synthesis and degradation is altered by bacitracin. Thus in unstressed animals β-endorphin levels are noted to be higher in bacitracin injected rats than control rats at 15 minutes post-infusion (Fig. 43a,b). However, with repeated exposures to stress, which causes release of β-endorphin, one would expect a larger accumulation of β-endorphin in bacitracin injected rats than control rats due to the action of bacitracin in decreasing biodegradation. This is consistent with our results since we find that bacitracin pretreatment reduced the stress induced decrease in brain β-endorphin levels by 26 per cent. Since it seems reasonable to expect that in both control and bacitracin injected rats stress caused a similar release of β-endorphin, the higher levels of β-endorphin in bacitracin injected rats must be of biodegradation (Fig. 43C,D). Thus as a critical minimum number of opiate receptors are bound, the onset of analgesia is observed. As more and more β-endorphin accumulates the analgesic response becomes stronger and stronger until it reaches a plateau at 15 minutes post-infusion and then begins to subside probably because bacitracin itself is being degraded. With subsequent stress exposures there is a paradoxical increase in brain β-endorphin levels of stressed rats to equal those in unstressed rats (i.e., at 30 minutes post-infusion and 12 intervals of stress). Thus it might be that previous stress exposures lead to a differential effect on β-endorphin levels. Such a differential effect on behaviour and opioid levels following stress has also been noted by Madden, et. al., (1977).

Figure 43: One Hypothetical Model of Bacitracin Action

Let's suppose it takes binding of at least seven molecules of β-endorphin to opiate receptors to cause analgesia.

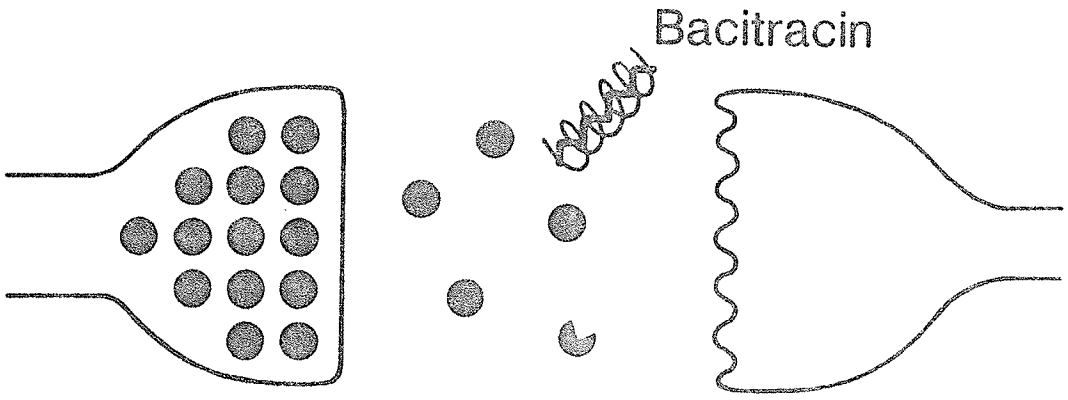
Figure 43

A. CONTROL (CSF injected rats)



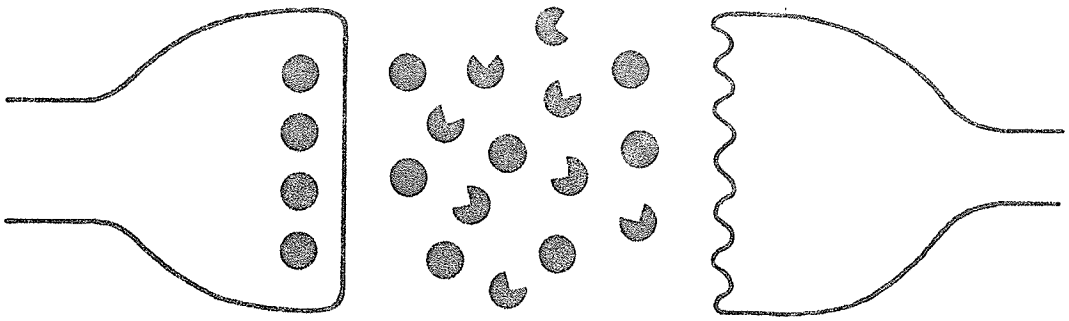
Number of molecules available to bind to opiate receptors = 2 ∴ no analgesia.

B. BACITRACIN INJECTED RATS



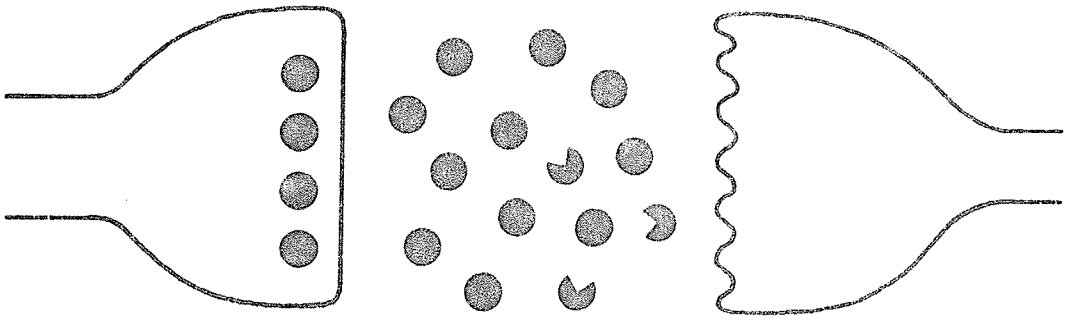
Number of molecules available to bind to opiate receptors = 4 ∴ no analgesia

C. CSF INJECTED STRESSED RATS



Number of molecules available to bind to opiate receptors = 7 ∴ no analgesia

D. BACITRACIN INJECTED STRESSED RATS



Number of molecules available to bind to opiate receptors = 11 ∴ ANALGESIA

The results following acute stress, from this study, provides clear evidence that (a) acute stress leads to release of β-endorphin in the brain and (b) the released β-endorphin may play a role in modulation of pain during acute stress.

The fragmentary or contradictory evidence on the role of endogenous opiates in mediating relief from stress that has been reported by various studies (see introduction) in our opinion is due to the fact that different studies have used different stress paradigms. Lewis, et. al., (1980) have demonstrated qualitative and quantitative differences in stress paradigms used in stress studies. They claim that there are two independent substrates which mediate stress analgesia and only one of these is acted upon by opioids. The substrate which is recruited during stress depends on the temporal properties of the stressful stimuli: it's duration and it's pattern (i.e., continuous vs intermittent). They showed that inescapable foot-shock caused profound naloxone reversible analgesia only when the shock was delivered intermittently for 30 minutes rather than continuously for three minutes. Another source of contradiction in results from different studies is due to the fact that pain responsivity follows a diurnal rhythm. Weshce, et. al., (1979) have shown that in the morning the levels of brain met-enkephalin in mice exposed to hot-plate stress increased significantly compared to unstressed animals. However, in the afternoon hot-plate stress lead to a decrease in brain met-enkephalin content.

D. β-ENDORPHIN AND ANLGESIA

The occurrence of profound analgesia at the same time (15 minutes post-infusion of bacitracin) as the endogenous build up of β-endorphin content in the brain provides striking evidence for the involvement of β-endorphin in analgesia. The slow onset of analgesia, which reaches a plateau at 15 minutes after bacitracin infusion, is understandable since even direct infusion of β-endorphin and the subsequent plateau in the analgesic response occurs at least 15 minutes after infusion (Graf, et. al., 1976b). Even though our study did not measure levels of met-enkephalin during the analgesic phase, it is unlikely that met-enkephalin played a significant role in bacitracin induced analgesia. This is because Graf, et. al., (1976b) have shown that the analgesic potency of the opiate peptides is a function of the length of the peptide due to the higher stability of the larger peptides to degradation. That is, whereas met-enkephalin, in vivo in rat, is relatively inefficient in producing analgesia, β-endorphin and δ-endorphin produce profound analgesia. Since our antiserum does not cross-react with the two enkephalins, or alpha- and gamma-endorphin, we feel that the analgesia observed in rats, after bacitracin infusion, is most likely due to a genuine increase in levels of brain β-endorphin, and to a much lesser degree by enkephalins.

Since both β-endorphin and naloxone have been reported to have higher affinity for "μ" opiate receptors (Lord, et. al., 1977), the antagonism of bacitracin induced analgesia by naloxone (1 mg/kg) further supports our view that bacitracin induced analgesia is due

to elevated levels of β-endorphin. An interesting observation with the use of naloxone was that doses of naloxone greater than 1 mg/kg (i.e., 5 mg/kg and 10 mg/kg) seem to be less effective in antagonizing the observed analgesia. This lack of effectivenss of naloxone at higher doses has been observed in many other studies. It has been suggested that naloxone at low doses acts as an opiate antagonist, while at higher doses naloxone acts as an agonist. There are various observations to substantiate this claim (Franks, 1975; Lasagna, 1965; Levine, et. al., 1979; McMillan, et. al., 1970) however the pharmacological basis for these observations is not yet well understood. We have noticed a significant increase in the activity of rats with higher doses of naloxone (i.e., at 5 mg/kg and 10 mg/kg). This increased movement resulted in ensuring that neither the forepaws or the hindpaws touched the surface of the hot plate for an extended length of time. Thus, if one was not measuring the activity in such rats but only the latency to the hindpaw lick or jump or the number of jumps, one would quite understandably record longer latencies for such responses and fewer jumps, as was the case in our study, since the animals are receiving some relief from the noxious stimuli merely because they are moving more on the surface of the hot plate. However, when the activity of these rats is examined together with their responses to noxious stimuli, it becomes evident that our observation that naloxone at higher doses is less effective in antagonizing bacitracin induced analgesia is solely not correct. We feel that the fragmentary or often contradictory evidence that exists for the role of naloxone, as an antagonist at low doses and

as an agonist at higher doses, is probably due to the fact that different studies are using different parameters to measure analgesia, without giving much consideration to the thought that naloxone itself has a physiological effect which then might modify the need for that particular behavioural response which is being monitored.

E. SOMATOSTATIN AND STRESS

This study also revealed that somatostatin is released during stress. Since growth hormone secretion is known to be inhibited during stress, and the fact that somatostatin has been shown to inhibit release of growth hormone, it seems reasonable to suggest that somatostatin is at least partly responsible for stress induced growth hormone suppression. These results are in agreement with the results of Terry, et. al., (1980) who have demonstrated similar decreases in somatostatin levels in various hypothalamic regions following acute stress. An interesting observation in this study was that continued exposure to stress paradoxically caused an increase in brain somatostatin levels of stressed animals to equal those in unstressed animals (i.e., at 30 minutes post-infusion and 12 intervals of stress). The reasons for this differential effect on brain somatostatin levels remains unknown. It might be that the somatostatin induced growth hormone suppression mechanism might be operable for only the initial duration of the stress and subsequently some other growth hormone suppression mechanism takes over, or that an increase in somatostatin synthesis occurs to correct the deficit created by acute stress.

F. DIFFERENCES IN CONTROL VALUES OF β-ENDORPHIN AND SOMATOSTATIN IN BRAIN / BRAIN AREAS OF CONTROL RATS FROM DIFFERENT EXPERIMENTS

It is quite possible that differences in age, body weight (180-250 gm), time of day at which rats were sacrificed (8-12 p.m.), as well as intra- and interassay variation could have contributed to the variability in both β-endorphin and somatostatin measurements obtained in different experiments (Table 19). In addition variation in these peptides could also be due to non-specific stress effects of handling prior to sacrifice. Since this non-specific stress effect is rat-specific, slight variations in the level of stress experienced could greatly affect brain concentrations.

Table 19. Comparison of IR- β-endorphin and IR- Somatostatin in Brains of Control Rats from Different Experiments from this Study and Comparison with Other Studies

BRAIN REGION	β-ENDORPHIN (ng/g wet wt.)				SOMATOSTATIN (ng/g wet wt.)	
	This Study I	This Study II	West., 1980	Lee., 1979	This Study	West., 1980
Cerebellum	63.4± 9.8	75.4± 6.4	36 ± 5	77± 20	3.6± 1.2	7.4± 0.72
Striatum	19.2± 5.4	49.4±23.0	31 ± 7.5		72.3± 9.1	81 ±11
Hippocampus	25.1± 7.3	41.3± 7.9	60 ± 6		52.9±10.2	74 ± 5
Cortex	4.6± 1.4	9.6± 1.9	7.5± 2.4		17.3± 2.4	18 ± 4.5
Thalamus	190 ±10	210.4±16.1	194 ±33		109.1± 6.9	116 ±14
Hindbrain	182.1±12.4	195.4±21	147 ±18	628± 73	58.6± 8.6	63 ±20
Amygdala	195 ±14.1	406 ±60	188 ±30		94.3±14.9	260 ±37
N. Septalis	291.6±21	448.3±28.6			81.6±20.4	239 ±35
Midbrain	222.6±30.4	304.6±78.6	195 ±11	326± 70	54.4± 8.1	111 ±28
Hypothalamus	2896 ±456		4401 ±425	4945±796	132 ±18.6	417 ±95
Whole Brain	n = 15	300 ±15		601±103		
	n = 15	286 ±17		540±104		
	n = 15	273 ±25				
	n = 15	310 ±37				
	n = 10	269 ±14				
	n = 10	250 ±41				

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