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Studies on single-dose tolerance and dependence on narcotic drugs in-vivo and in-vitro

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STUDIES ON SINGLE-DOSE TOLERANCE AND DEPENDENCE ON NARCOTIC DRUGS IN-VIVO AND IN-VITRO.

by

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DISSERTATION

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TO:

Professors
Fernando Huidobro T.
E. Leong Way
Two Colossus
of the Western Opiate Pharmacology.

Father and Godfather of my
awakening in Science.
Masters and morning Star in my passion for life...
Your love and tenderness dwell deep
in my latin heart.

Padre y padrino
en mi inquieto despertar por la Ciencia.
Guias y lucero de mi pasion por la vida...
Vuestro carino y desvelo
anidan hondo en mi corazon latino.

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Cuando miro hacia atras y hacia arriba y me pregunto
a quien voy a agradecer los inmensos beneficios
de estos anos de alegre y fecunda vida de estudiante bohemio
se me arranca una sonrisa jubilosa al recordar tantas y tan generosas personas.
Me arrastra una Nebulosa mental, y floto en una nube de ensuenos.
Gracias Amigos,
gracias Estados Unidos, la tierra generosa y de la abundancia.

No quisiera nombrar nombres, porque mi efimera memoria me va a traicionar.
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Dejame Musa poeta, recordar a todos ellos en Mi querido Cuate Adron,
Caballero de alma noble y Ciencia exacta.
Mi Cuate envuelves en tu persona generosa,
en tu bigote reconciliador, comunitario y sabio
todos estos anos,
todas estas emociones,
carinos
progresos humanos y cientificos
y la alegria de esplendidas celebraciones con limpia alma latina.
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generadas por experimentos nascientes, juveniles.
De la sonrisa espontanea de la dama de Blancos Cabellos
conciencia viva de mi mision de farmacologo latinoamericano.
De las horas celosas con mi jefe, amigo y maestro,
del carino de mis companeros....

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se me arranca del corazon.
Lleva tantas emociones, amistades alegrias,
que hincha las velas de mi rustico corazon andino.
Me lleva a pleamar, guiado por las estrellas del Norte,
por el tacto del joven manana.

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seria nula,
sin el amoroso carino Mariano
por el desarrollo de mi persona como un "hombrecillo de Ciencias".
Como olvidar la Comunidad de Saint John of God
representada por B-EN Sanchez, hombre y angel, musica y bondad?
Los amaneceres de Tosca?
Los suenos con la batuta de Ozawa y de de Waart?
Y mi arraigo por la naturaleza y la belleza del pacifico Oceano?
sus noches cuajadas de estrellas retractiles, y de lunas invertidas?
Y la inspiracion filosofal, revestida de entusiasta sonrisa juvenil
en cabellos de seda purisimos, simbolo de etrnidad?

En estos anos de plenitud bohemia tambien he conocido la ternura de los mios:
de la familia y de sus tesoros amorosos, derrochados en mi:
la guia en la conquista de las cumbres Andinas.
He saboreado la dulzura Pecosita,
nacida en un sencillo pero tierno Corazon de Oro
Y los ninos:
Los kissitos de la inquieta Pulguita y su hermana Marissa,
Sarita, mi ahijada Principesa.
Queeny, la nina del cabello dorado, electromagnetico,
que me derrite con su sonrisita; su hermano
Noah, ciencia ficcion, ojos sonadores y astutos...
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Julie, la ninita Virgen anticipacion al encuentro Mariano absoluto y eterno.

Miro hacia arriba,
y en la compania de Cassiopea, Andromeda y las Osas
delineo mi compromiso Universal, vestido de blanco y de azul,
bordado en Ciencias y Filosofia.
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que con su tintinear me invita a nuevos planetas;
a comprometer mi paternidad por vocacion: educar.

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engranan en la organicidad de mi desarrollo personal.
Han crecido vigorosos para hacerme JUAN y tambien PABLO,
para con humildad recordar quien soy: Juan Pablo
si, Juan y Pablo.

Una sonrisa me eleva y me hace uno con el Principito.
Con su planeta, con su rosa, con la cuncuna que el queria para mariposa.
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al saberme feliz,
llamado,
filial.

Gracias, SI, MUCHAS GRACIAS,
Gracias amigos, padres, maestros y hermanos.

A orillas de Lake Tahoe,
Incline Village, Enero 1978.

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ABSTRACT

In mice, single-dose tolerance to the antinociceptive effect or to the thermic responses of opiates, alkaloids or endogenous peptides, was demonstrated using an adequate initial priming dose of morphine or B-endorphin and allowing an interval of time for its development. Single-dose tolerance to the in-vitro effects of opiates in the isolated myenteric plexus longitudinal muscle preparation of the guinea pig ileum was also demonstrated. Evidence of tolerance was indicated by the fact that when the antinociceptive response to opiates was assessed by the hot plate and the tail flick procedures, a shift in the dose response curve of morphine to the right occurred after an adequate single priming dose of opiates. The doses of opiates to produce single-dose tolerance were proportional to the sensitivity of the animals to the different effector responses. The threshold dose necessary to produce tolerance to the antinociceptive effect of morphine was found to be 3-4 times greater than the median analgesic dose of morphine. Higher doses of morphine did not enhance further the development of tolerance, suggesting a saturation level in the degree (amount) of tolerance produced. In the case of the antinociceptive effect of B-endorphin in mice, a dose of about twice the median antinociceptive dose was required to evidence significant tolerance, and higher doses did not increase proportionally the amount of tolerance, confirming a saturation level in single-dose tolerance production to opiate's antinociception.

B-endorphin also produced single-dose tolerance to its hyperthermic response. Tolerance, however, was evidenced with doses lower than the median effective dose50. In the longitudinal muscle preparation of

the guinea pig ileum, single-dose tolerance to endorphins was maximal with doses of opiates around the effective dose50. The time courses of single-dose tolerance revealed that a variable interval of time was necessary to develop maximal tolerance after the priming dose of an opiate. The time course of tolerance development to the antinociceptive effect of mrophine was more prolonged in the case of morphine (three days) than B-endorphin (12-24 hours), while the time course for the hyperthermic reponse of B-endorphin evidenced maximal tolerance at about 48 hours after the priming dose. In-vitro, tolerance was demonstrable about half an hour after the application of a priming dose of endorphins. The development of single-dose tolerance, both in-vivo and in-vitro, was accompanied by a parallel development of physical dependence as measured by naloxone precipitated withdrawal. A mild abstinence behavior was observed compatible to the degree of tolerance produced by either opiate alkaloids or peptides.

Single-dose tolerance to the effect of the opiates was blocked in a dose dependent fashion by the simultaneous administration of naloxone with the priming dose of the opiates, suggesting that this kind of tolerance is a true opiate action. Furthermore, to reinforce this conclusion, studies were designed to examine the development of acute cross-tolerance among opiate drugs. Cross-tolerance was evidenced by a decrease in analgetic response to methadone three days after a single priming dose of morphine and a decrease in morphine response after a single dose of methadone. Likewise, cross-tolerance to the antinociceptive effect of B-endorphin was crossed to morphine and vice versa. In the case of the hyperthermic response of B-endorphin, single-dose tolerance was crossed from morphine or the endorphins to B-endorphin. The pentapeptides, leucine and

methionine enkephalin and D-ala2-Met5 enkephalinamide induced cross-tolerance to B-endorphin. These results suggest that acute or single-dose tolerance is an opiate specific effect and that probably opiate alkaloids and peptides share a common mechanism of action. The development of single-dose tolerance to the antinociceptive effect of morphine or B-endorphin was inhibited by pretreatment of mice with cycloheximide or actinomycin-D prior to the priming dose of an opiate.

Single-dose tolerance to the antinociceptive effect of morphine was also blocked by 5,6-dihydroxytryptamine and perhaps enhanced by L-tryptophan. Cyclic 3',5'-adenosine monophosphate did not affect single-dose tolerance. The results suggest that single-dose tolerance to morphine or B-endorphin involves the synthesis of some macromolecule (s) and support previous findings in this laboratory involving an association with serotonin.

INTRODUCTION.

HISTORY OF B-ENDORPHIN AND DRUG DEPENDENCE.

ON THE SEARCH FOR THE ENDOGENOUS MORPHINE.

One of the most challenging concepts in biology is the notion that cells communicate with each other to maintain a perfect and dynamic balance of the "internal milieu" of the organism as proposed by C. Bernard. For this purpose, evolution provided creatures with an elaborate system of selected chemical messengers (neurotransmitter substances, hormones, etc.) that efficiently transduce an external or internal stimuli into a chemical signal as a response to the environmental stimulation. The chemical messengers interact stereoselectively with their respective target tissues to produce "a change" that will ultimately be transduced into a biological response. Drugs have been extremely powerful tools in understanding this physiological scheme so that the development of pharmacology has been intimately associated to physiology. Drugs are known to interact mainly at two different sites in this simplified scheme: either modifying the synthesis or release of the messenger, let's call this site a "pre synaptic event". Drugs are also known to modify the interaction of the hormone or neurotransmitter with its target organ, or to alter the metabolism and distribution of the messenger. This case could be in general referred to as a "post synaptic event". The fundamental underlying principle of the chemical messenger concept is the existence of a selective "receptor-substance". In simple words, the receptor is a biomolecule that selectively recognizes the chemical messenger. The first step in the "recognition reaction" is the binding of the messenger to its specialized receptor site to produce a cellular modification (an ion permeability change? the activation of a specific enzyme?) that will ultimately cause the physiological

response of the tissue to the presence of the transmitter or the hormone.

Opium has been known to humankind for a very long time. Egyptians, Babylonians, and Chinese cultures profited from its beneficial therapeutic uses as well suffered from its misuse for recreational effects. However, the story of morphine in therapeutics and biological research is relatively novel. Morphine, the chief active ingredient of opium was isolated from opium latex more than 150 years ago by Serturner, a German apothecary. Its chemical structure was established and fully confirmed by chemical synthesis and physicochemical methods only during the past 20-22 years. It soon became apparent from structure activity studies that morphine and morphine-like compounds as well as other synthetic derivatives exhibit a remarkable stereo selectivity. Only one enantiomer of the opiate exhibits pharmacological properties, while the other isomer was found virtually inactive. Such is the case of a morphinan derivative of morphine. l(-) levorphanol was found to be 3-4 times more potent than morphine in producing analgesia in man (Grewe et al., 1949; Schnider and Grussner, 1949). However, its stereo isomer, d(+) dextromorphan is virtually inactive. A similar, though not as dramatic, case was found with the diphenylpropylamine derivatives. It is well documented that l methadone is more active as an analgesic than d methadone. Very recently, the enantiomers of morphine have been made available by chemical semisynthesis. The d(+) enantiomer of morphine was proved to be inactive as an analgesic in rodents (Jacquet et al., 1977). Furthermore, opiates exhibit most extraordinary structure activity relationships. A slight chemical modification in the opiate structure can profoundly alter the pharmacological profile of the opiate, converting it from an agonist

to an antagonist. For example, the substitution of an allyl group (-CH2-CH=CH2) for the methyl group (-CH3) on the nitrogen of morphine or oxymorphone, a potent morphine-like agonist, converts the opiate agonist into a very selective opiate antagonist (Hart and McCawley, 1944; Kosterlitz and Watt, 1968; Harris et al., 1969). The allyl derivate of oxymorphone, naloxone, is a "pure opiate antagonist", used currently in human clinics to treat opiate overdosage.

Such a dramatic specificity and stereo selectivity of drug action among narcotic drugs induced many investigators to believe that there are specific endogenous morphine receptor(s) in neural tissue. To explore this hypothesis, Goldstein and co-workers (1971) pioneered binding studies of narcotic drugs to brain tissue. Two basic pharmacological principles gave theorical support to the working hypothesis. First of all, the initial step in drug action has to be the stereoselective interaction of an agonist with its receptor substance. Secondly, the binding of the agonist with the putative brain receptor should correlate with the in-vivo pharmacological potency of the opiates. In other words, dextrorphan, the inactive opiate stereo isomer should bind mainly, if not exclusively to non-opiate specific sites of the tissue, while levorphanol, the active isomer, should bind to the receptor plus the opiate nonspecific sites. The strategy for discovering the putative opiate receptor through binding studies, was to preincubate brain membranes with dextrorphan to saturate the tissue nonspecific binding sites, and then add radio labelled levorphanol. Under conditions of a ten-fold excess dextrorphan over levorphanol the nonspecific sites would be saturated, allowing for levorphanol to bind to the "opiate receptor". After the binding reaction reached equilibrium, excess radioactive opiate ligand was removed by filtration

of the membranes under vaccum. Non-specific binding could be reduced by repeated washing of the membranes with cold buffer. Following these principles, Goldstein et al., 1971 reported evidence of stereo-specific opiate receptor binding to mouse brain membranes . However, the amount of stereo-specific radioactive levorphanol bound was dissapointingly low and not convincing. It accounted for less than 2% of all the levorphanol bound, and a selective distribution of the receptor could not be established. Subsequently in 1973, Pert and Synder (1973), Simon et al.(1973) and Terenius (1973) using a radio labelled opiate agonist or antagonist with high specific activity to perform the binding, showed a dramatic increase in the percentage of the radio labelled opiate bound to purified brain membranes. This improvement allowed for studies of the binding constants and selectivity of binding among opiate and non-opiate drugs. It was soon established by these groups of researchers that opiate drugs displaced the radio labelled narcotic bound with affinities proportional to the in-vivo pharmacological potency, and that the binding was selective to opiate drugs. Moreover, the agonist binding was displaced in a competitive fashion by naloxone. These data were interporeted to mean that the binding was probably occurring at the endogenous opiate receptor.

Further investigations have demonstrated that both opiate agonists as well as opiate antagonists bind to synaptosomal brain membranes. The binding is characterized by its saturability, its stereoselectivity, and high affinity of binding (dissociation constants for agonist-and opiate binding in the range of 100 pM to 100 nM). Binding is displaced in a

competitive fashion by opiate agonists and antagonists. The binding affinity constants of the compounds correlated well with their pharmacological antinociceptive potencies and these bindings provided strong circumstantial evidence for the presence of endogenous opiate receptors in brain tissue. Subsequent to these basic pharmacological studies, the existence of the endogenous opiate receptors in brain tissue has been further documented by autoradiographic techniques applied to brain tissue following an injection of a radioactive narcotic drug. This procedure allowed the mapping of opiate receptor density in mammalian brain (Kuhar et al., 1973; Hiller et al., 1973; Pert et al., 1975; Atweh and Kuhar, 1976; Pert et al., 1976) and the correlation of opiate binding with sensitive brain areas of morphine action. Thus, it is of extreme interest that opiate receptors were found to be abundant in selective areas of the spinal cord, brain stem (nucleus of the tractus solitarius and nucleus commisuraus), thalamus-hypothalamus and cortex.

However, considering the well documented experimental evidence for endogenous opiate receptors; from an evolutionary point of view it seemed capricious for nature to evolve an "opiate receptor system" if the body did not have an endogenous ligand(s) for these sites. Obviously, morphine has not been found in mammalian brain extracts. It is likely improbable, and a most fortuitious coincidence, that such a sophisticated receptor system exists for an exogenous compound like morphine, to which it never has been exposed. Furthermore, how could such a system evolve among vertebrates if these animals were hardly ever exposed to poppies or to opium? These philosophical considerations raised the need to look for endogenous morphine-like ligands which could shed light on the functional role of the brain opiate receptor. The endogenous

ligand clearly had to fulfill the pharmacological criteria of mimicking the effects of morphine both in-vivo as well as in-vitro systems. Furthermore, the effects of morphine and the endogenous ligands should be prevented or blocked by naloxone. In addition, the brain distribution of ligands should correlate with brain areas with high density in opiate receptors. Two in-vitro assay systems were available to the Herculean task of identification and characterization of the putative opiate-like ligands. One was the binding assay of opiates to purified brain membranes, the other in-vitro assay system used for these studies is the myenteric plexus of the longitutional muscle of the guinea pig ileum. In this last preparation, narcotic drugs are well documented to produce a depression of the electrically stimulated muscle contraction. The inhibition of the electrically induced twitch produced by opiates is blocked and reversed by naloxone. (For a review on this preparation, see Kosterlitz and Waterfield, 1975.)

Using these two in-vitro assay methods, J. Hughes and co-workers (1975; 1975a; 1976b) at Aberdeen succeeded in identifying, purifying, and characterizing both chemical and pharmacologically two pentapeptides with potent morphine-like activity. The pentapeptides were named enkephalins (word derived from the Greek meaning "from the brain") and were initially extracted from porcine brains. Both peptides had a common chemical primary structure, except for the fifth amino acid. The common segment was shown to be NH2-tyr-gly-gly-phe. The fifth amino acid was either methionine-OH (methionine enkephalin, MW 574) or leucine-OH (leucine enkephalin, MW 556) (Hughes, 1975; Hughes et al., 1975b). These two peptides were found on porcine brain on a 3:1 ratio.

Early during isolation steps it became apparent that these peptides were liable to proteolytic enzyme degradation. Incubation of the peptides with brain extracts or with carboxypeptidase A or leucine amino peptidase rapidly caused a complete loss of the opiate activity of these compounds (Hughes, 1975; Hughes et al., 1975a). The enkephalins exhibited potent morphine agonist behavior in-vitro, but evidenced a transient, short lived, antinociception in rodents (Belluzzi, et al., 1976; Graf et al., 1976). This extremely short duration of action is most likely due largely to rapid in-vivo enzymatic degradation.

Following the discoveries of Hughes and co-workers, Simantov et al. (1976) and Pasternak et al. (1976) confirmed the presence of two enkephalins in bovine brain. but the concentration of leucine enkephalin was higher than met-enkephalin. To date, enkephalins have been isolated from the mouse, rat, guinea pig, monkey, rabbit, and human brain, as well as the ileum of the guinea pig (Hughes, 1975; for a review see Synder and Simantov, 1977). Furthermore, endogenous ligands have been described not only in mammalian brain, but also in pituitary glands by Cox et al. (1975) and Goldstein (1976), human cerebro spinal fluid by Terenius and Wahlstrom (1975), the blood of rodents and humans (Pert et al., 1976), and the myenteric plexus of the guinea pig ileum (Hughes et al., 1975a). Other endogenous opiate ligands from the pituitary have been chemically and pharmacologically characterized. The isolation of alpha, beta, and gamma endorphin, from pituitary-hypothalamus homogenates has been reported by C. H. Li and co-workers, and R. Guillemin and co-workers (Li and Chung, 1976a; 1976b; Lazarus et al., 1976; Cox et al., 1976; Guillemin et al., 1976).

The distribution of the endogenous opiate ligands is not homogenous in the mammalian brain but it correlates remarkably well with the sites of higher density of opiate receptors (Simantov, 1976; Simantov, 1977). The distribution of brain endorphin has been studied either by isolation of the peptides from different brain regions, or by using immunohistochemical techniques (Elde et al., 1976). With this fine subcellular localization technique, both the endogenous opiate receptors as well as the endogenous opiate ligands have definitely been established as constituents of the mammalian brain. Eric Simon in 1975 (as cited by A. Goldstein, 1976) proposed to name all morphine-like endogenous material with the generic name of endorphins. This name was coined meaning to denote "endogenous morphine-like" substances. Thus, although enkephalins mean specifically the pentapeptides of structure tyr-gly-gly-phe-met or leu, they are chemically endorphins.

Hughes et al. (1975b) in the original report of the chemical structure of enkephalins noted that the sequence of methionine enkephalin was contained in the primary sequence of B-lipotropin (B-LPH). This molecule was characterized by Li and co-workers in 1965 from mammalian pituitaries. Further examination of B-LPH and the pituitary gland, culminated in the isolation and chemical identification of a 31 amino acid peptide from camel hypophysis and human pituitaries which corresponded to a fragment of B-LPH, the amino acid sequence 61-91. This polypeptide isolated by Li and Chung (1976a; 1976b) was named B-endorphin; it was soon found to be a potent analgesic compound in rodents. In analogy to enkephalins, B-endorphin (B-EN) proved to be as active as morphine when tested in in-vitro assay systems (Bradburry et al., 1976; Cox et al., 1976; Lazarus et al., 1976). However, in contrast

to enkephalins, B-EN was shown to be a potent antinociceptive agent when injected in rodents either directly into the brain or by intravenous route. The antinociception produced by B-EN in rodents was dose dependent and long lasting (Loh et al., 1976a; Tseng et al., 1976b). In comparison to morphine, B-EN is about 20-30 times more active than morphine on a molar basis. Furthermore, no basic difference in antinociceptive potency was found between B-EN extracted from camels and B-EN from human brains (Li et al., 1977). B-LPH segments 61-76 (alpha-endorphin) or B-LPH 61-77 (gamma-endorphin) proved to be much less active both in-vivo or in-vitro as compared to B-EN, which apparently is the pharmacologically most active endorphin known (Law and Loh, 1977).

Apparently, all the endorphins identified up to date are derived from a common macromolecule that is B-LPH. This protein would act like a "pro endorphin" molecule which on degradation would yield the different endorphins. Indeed, it has been recently shown that incubation of B-LPH with brain homogenates results in the formation of alpha, beta, and gamma endorphins (Austen et al., 1977). Recently, evidence has accumulated indicating that the incubation of endorphins with brain extracts will yield all kinds of smaller peptides (Kromer et al., 1976; Hambrook et al., 1976; Austen and Smith, 1977). Furthermore, Lane et al. (1977) suggested a specific brain subcellular localization for leucine-enkephalin hydrolyzing enzymes. However, a brain selective endorphin-metabolizing system has not been identified.

At present little is known about the genesis and distribution of the endorphins in the brain and pituitaries. It has been reported that endorphins are present both in the adeno and intermediate hypophysis and in certain

brain nuclei. In addition, 3-4 weeks after hypophysectomy, brain levels of B-endorphin were not affected (Cheung and Goldstein, 1976; Rossier et al., 1977) nor apparently were those of B-lipotropin (Watson et al., 1977). This suggests that the central and pituitary origin of endorphins are independent and different one from another. Furthermore, recent immunofluorescent experiments conducted by Guillemin and co-workers with antiserum specific for enkephalins and B-endorphin, demonstrated the existence of different fiber-like structures for brain endorphins and enkephalins (Rossier et al., 1977). These results could be tentatively interpreted to indicate the existence of different brain pathways containing each class of endorphin.

Thus, until more information is available, the origin, metabolism and anatamical distribution of the endorphins in both pituitaries and brain remain a fascinating and yet highly speculative subject. Likewise, a specific function for the endorphins both in the central nervous system and periphery, remain an open question, and a challenge to the understanding of peptide brain physiology and the pharmacology of opiates.

PHARMACOLOGY OF ENDORPHINS.

In-Vitro Preparations.

As already discussed, enkephalins and B-endorphin resemble morphine-like compounds in the opiate binding assay to purified synaptic membranes from mammalian brain. In addition, the radio labelled opiate ligand is displaced from its binding site in a competitive fashion by other opiate analogs (both peptides or alkaloids) and by naloxone,

the selective opiate antagonist. The dissociation constant obtained from the binding studies is within the range of 1nM, indicating a high binding affinity to the receptor site. Endorphins also show potent opiate-like activity in preparations of the longitudinal muscle of the guinea pig ileum or the mouse vas deferens. The IC50's (the dose of opiate that will cause 50% depression of the electrical twitch) are in the range of 100nM, B-endorphin being the most active, about twice more active than morphine on a molar basis, followed by methionine and leucine enkephalin. Alpha and gamma endorphin evidence little if any opiate activity at all.

Structure activity studies in the longitudinal muscle of the guinea pig ileum with synthetic analogs of the native peptides show interesting results: D-ala2-B-EN, is as active as B-EN itself. However, D-leu5-B-EN or D-met5-B-EN are 100 to 25 times less active respectively than B-EN. Interestingly, L-ala2-B-EN is about 20 times less active than B-EN, and about 10 times less active than D-ala2-B-EN. All these in-vitro opiate-like effects are blocked and reversed by naloxone (Yamashiro et al., 1978b).

In-Vivo Effects in Rodents.

Enkephalins when injected intracerebroventricularly in mice or rats produce a transient antinociception of extremely short duration of action (Belluzzi et al., 1976; Buscher et al., 1976; Graf et al., 1976). The main reason for the fugacity of effect has been attributed to the rapid metabolism of the peptides in the brain. A proof that this might be the case was the demonstration by Pert (1976) that D-ala2 enkephalin amide, a synthetic enkephalin resistant to enzymatic hydrolisis, produced a dose and time

dependent antinociception when injected into the cerebral ventricules of rodents. Recently, Yamashiro et al. (1978a) have reported that two thiazolidine derivatives of enkephalin: D-met2, thz5-enkephalinamide and D-thr2, thz5-enkephalinamide are equipotent and 4 to 5 times more potent than morphine injected i.v. In contrast to enkephalins, B-EN produces a dose dependent, long lasting antinociception in rodents. In mice, B-EN was shown to be about 20-30 times more active than morphine (Loh et al., 1976). B-EN injected intracerebroventricularly in rats also induced a dose dependent catalepsy that was almost instantly reversed by naloxone. B-EN also produced a sialpgogic seizure effect in rats (Holaday, 1977) and circling and stereotypy after intranigral application of B-EN (Iwamoto and Way, 1977).

Structure activity studies devoted to examine the antinociceptive effect of B-EN analogs in mice have demonstrated that D-ala2-B-EN is equipotent with B-EN, while L-ala2-B-EN is about eight times less active. D-met5 or D-leu5-B-EN were 100 and 500 times less active than B-EN respectively. Interestingly, D-ala2-D-leu5-B-EN was only 10 times less active than B-EN (Yamashiro et al., 1978b). The interpretation of these results suggest that both metabolism and a preferred molecular conformation of the peptide chain can partially account for the biological activity of the compounds. No antagonist opiate-like peptide has yet been found. The original suggestion of Ungar et al. (1976) that arg(met enkephalin, B-LPH 60-65) had antagonist effects has not been confirmed. Indeed, Law et al. (1977) and Chang et al. (1976) have demonstrated that this hexapeptide behaves like an agonist rather than an antagonist opioid. Recently, Pert et al. (1977) reported that the N-allyl derivative met5-enkephalin showed evidence

of being a partial opiate agonist.

Other in-vivo opiate-like effects of B-EN and enkephalins is the effect of these peptides in the pituitary-hypothalamus axis. B-EN or leucine enkephalin increase the release growth hormone and prolactin hormone (Dupont et al., 1977a; 1977b; Cocchi et al., 1977). Guillemin et al. (1977) have recently demonstrated that ACTH is released in conjunction with B-EN from adenohypophysis in response to stress. B-EN microiontophoretically applied to single neurones was shown by Frederickson et al. (1976) and Nicoll et al. (1977) to inhibit the spontaneous firing or glutamate evoked firing of cells from cerebral cortex, caudate nucleus, thalamus and lateral reticular nucleus but to increase the rate of firing of hippocampal cells.

A challenging question about endorphins is whether or not these compounds produce tolerance and dependence after continued administration. Experimental work both in mice and rats has shown that the enkephalins and B-endorphin can produce tolerance. The development of tolerance is accompanied by a parallel development of dependence. It was elegantly shown by Wei and Loh (1976) that after a continual brain infusion of met-enkephalin or B-EN for 72 hours into the periacqueductal gray area of rats, evident signs of dependence were clearly demonstrated by a challenge dose of naloxone. These results have been fully confirmed by Tseng et al. (1977). They presented evidence indicating that after 3-4 injections of human B-EN in rats, tolerance develops to the analgesic, hypothermic and cataleptic effect of B-EN. Furthermore, Tseng et al. (1976a) and Szekely et al. (1977) showed cross-tolerance to the antinociceptive effect of morphine and B-endorphin

in mice, suggesting a common underlying mechanism of action for the opiate alkaloids and peptides. Tolerance has also been demonstrated to the effect of enkephalins and B-EN in the longitudinal muscle of the guinea pig ileum (Waterfield et al., 1976). In this case, tolerance was also crossed between the opiate alkaloids and the peptides, further supporting the suggestion of a common mechanism of tolerance production. Tolerance was also observed at the single cell level after microiontophoresis of the peptide to cells of different brain areas (Zieglgansberger et al., 1976a; Zieglagansberger and Fry, 1976b). Nicoll et al. (1977) further observed that B-EN failed to inhibit the firing of single neurones after application of endorphins in rats implanted with morphine pellets.

Of great interest has been the report that a hybrid cell line derived of glioma x neuroblastoma cells has opiate receptors (Klee and Nirenberg, 1974). Using this cell as a neuronal model, it has been possible to establish that opiate-alkaloids and peptides develop tolerance and dependence on repeated exposure to opiate drugs. Acutely, morphine or enkephalins inhibit the basal level and the PGE1 stimulated activity of adenyl cyclase in these cells. Upon prolonged exposure to opiate-like drugs, higher activity of adenylate cyclase is evidenced, probably to overcome the depressed level of cAMP in the cells with opiates. Higher concentration of opiates are then required to inhibit adenylate cyclase to the same degree as it was inhibited by the first dose of an opiate (Sharma et al., 1975). Furthermore, in cell cultures exposed to morphine or enkephalins for a long time, the base activity of adenylate cyclase is higher than that of cells naive to narcotic drugs, evidencing signs of a cellular adaptation. When the opiates were removed from the cell cultures, in an attempt to reproduce a withdrawal situation, the level of cAMP rose dramatically.

An immediately apparent explanation was that on opiate withdrawal, the inhibitor substance was also removed causing an increase of cAMP due to the increase synthesis of adenyl cyclase during tolerance development.

THEORIES OF NARCOTIC DRUG DEPENDENCE.

Tolerance and physical dependence appear to be intimately associated. In humans, as well as in animals, especially rodents, it is very well documented that considerable tolerance to the antinociceptive effect of morphine and related alkaloids develops after repeated administration of opiates. Tolerance can be determined by demonstrating an increase in the median dose of morphine to produce antinociception in a population of mice after repeated exposure to morphine. Using the modified Maggiolo-Huidobro morphine pellet, an 8-15 fold increase in the morphine effective dose50 is reached after three days of morphine pellet implantation (Way et al., 1969). Tolerance to morphine is associated with a concomitant development of dependence. In rodents, dependence is quantified by the determination of the dose of naloxone needed to produce a stereotyped jumping behavior, characteristic of the antagonist precipitated morphine withdrawal syndrome. Parallel to the development of tolerance, Way et al. (1969) documented a dramatic at least 70-fold increase in sensitivity to the opiate antagonist. Thus, experimental laboratory animals in agreement with clinical data have confirmed the dual effect of morphine addiction: the development of tolerance coupled with dependence. Based on this data, Way et al. (1969) hypothesized that a common underlying mechanism might be responsible for both effects.

Substantial experimental evidence supports this contention. Drugs known to accelerate the production of tolerance in mice, also increase the production of dependence. Such is the case with cAMP (Ho et al., 1973b) or L-tryptophan (Ho et al., 1975). Likewise, drug manipulations that decrease the development of tolerance will also decrease the development of dependence. Such is the case with drugs like parachlorophenylalanine (Ho et al., 1972) or 5,6 dihydroxytryptamine (Ho et al., 1973a), opiate antagonists, or inhibitors of protein synthesis (see reviews of Smith et al., 1971 and of Way (1972).

Tolerance to narcotic drugs is thought to be a central phenomenon. It is visualized as an adaptational response of the organism (brain) to the repeated exposure of opiates. Tolerance does not develop to all the actions of morphine at the same rate nor to the same extent. For example, tolerance to the antinociceptive effects of morphine and related opiates develops quite rapidly, in a matter of hours in mice. The same is true with tolerance to the euphoric effect of opiates or to the respiratory depressant effect of morphine in humans or rodents. However, little tolerance is observed in humans to the constipating, antidiarrheal effect of opiates, or to pupillary contriction in humans.
This information suggests that the phenomenon of tolerance must be selectively compartmentalized in the central nervous system. Only the cells sensitive to opiates will develop tolerance and the rate of tolerance development is apparently different for each morphine effect or brain region. From a mechanistic point of view, this fact might indicate that assuming an equal brain distribution of an opiate after systemic administration, not all the areas, and so not all the morphine sensitive sites

will develop tolerance at equal rates. Thus tolerance must occur at specific cells and with slightly different characteristics in each case.

Several hypothesis have been advanced to explain tolerance and physical dependence to narcotic drugs. Even though the mechanism of action of opioid drugs is still obscure, which unfortunately masks a real understanding of opiate pharmacology. Any hypothesis to explain tolerance and dependence to narcotic drugs should consider answering the following well documented facts:

1. Morphine tolerance-dependence is crossed among narcotic opiates, but not to other CNS depressant drugs.
2. Tolerance-dependence is blocked or diminished by drugs that block protein synthesis in the brain.
3. The acceleration in the development of tolerance and dependence, caused by drugs such as cAMP, or the precursors of 5-hydroxytryptamine metabolism.
4. The existence of different types of tolerance, some of which persist for a prolonged time.

It should be kept in mind that any biochemical opiate effect observed in vitro with naive or tolerant-dependent tissue has to be paralleled by similar observations in-vivo using naive or tolerant-dependent animals. Also the hypothesis should explain the biochemical opiate effects under opiate withdrawal, both in-vivo as in-vitro.

Carefully controlled animal studies have indicated that tolerance is not related to opiate absorption, since tolerance to morphine is achieved by all routes of opiate's administration. Tolerance is unlikely to be due to

metabolism or to opiate distribution. Indeed, Cochin and Axelrod (1959) and Takemori (1960) demonstrated that morphine depressed the N-demethylating activity of rat livers and the glucuronide conjugation of morphine respectively, and that both enzymatic activities are inhibited during the development of tolerance. Thus, metabolism of opiates is inconsequential with theories of tolerance and not of magnitude to explain a 10 fold tolerance to opiates. Goldstein et al. (1974) have summarized the hypothesis to explain tolerance to opiates in two kinds of theories. One kind postulates that tolerance is related to the the number or quality of opiate receptors themselves. The other theory states that the basic problem is not at the receptor level but that tolerance is due to the fact that the effects of opiates are antagonized or compensated by changes elsewhere in other neuronal pathways.

In the first hypothesis, an exhaustive research was conducted to ascertain whether the number of opiate receptors is reduced or altered, rendering animals less sensitive to opiates. For this purpose, the opiate receptor binding technique to purified brain membranes was used. Results from independent groups of researchers concluded that there was no significant difference in the number or the affinity of the opiate receptor to narcotic drugs in-vivo (Synder et al., 1973; Hitzemann et al., 1974; Klee and Streaty, 1974) or in-vitro (Cox and Padhya, 1977). Another site of action for narcotic drugs is a direct interaction of opiates on enzyme systems. The depression theory was advanced by Shuster (1961). In the original proposal of this hypothesis receptors were visualized in a broad sense of the word, and included enzymes or other functional macromolecules. The theory postulates that opiates, or other drugs producing dependence, by inhibiting an enzyme that synthesizes a chemical crucial

for cell activity, like a neurotransmitter or a neuromodulator. The enzyme is itself regulated by its product so that if the chemical is decreased, the enzyme level will be increased to balance the appropriate level of the chemical affected. The initial drug effect is the result of the decrease concentration of the chemical, but since the decrease leads to an increase in the synthesis of the enzyme, a new steady state of the chemical is produced, resulting in tolerance. When the drug is removed, the excess synthesis of the chemical, is physiologically expressed as the abstinence or withdrawal syndrome.

The enzyme depression theory has recently gained support from two different lines of research. One is centered on cAMP and its cellular regulation, while the other line focused on the effect of Ca++ on brain metabolism during morphine administration. Klee and Niremberg (1974), Sharma et al. (1975), Klee and Niremberg (1976) discovered that a hybrid cell, a neuroblastoma x glioma (NG108-15), maintained in cell culture conditions, has opiate stereo-selective receptors. Morphine-like alkaloids, as well as opiate peptides inhibit in these cells the enzyme adenylate cyclase, decreasing in a dose dependent fashion the cellular basal cAMP levels. The blockade was reversed by (-) naloxone, but not by (+) naloxone. Tolerance to the agonist effect occurred on repeated exposure to opiates. Thus, cAMP levels initially depressed by narcotics, returned to base line levels without a change in the number of the cells opiate receptors (Sharma et al., 1975; Lampert et al., 1976). On opiate removal or administration of naloxone, full expression of the newly formed adenylate cyclase was evidenced in tolerant cells that lead to high cAMP levels in the cells. Unfortunately at the present time, this model has very little in-vivo experimental

support so as to validate its role in animals. Francis and co-workers (1976) have recently described in rats the quasi morphine withdrawal syndrome which is induced in naive rats by the administration of drugs that elevate cAMP in the brain. This is the first pharmacological evidence in support of this cAMP model of tolerance and dependence.

The other line of research has focused on the effects of opiates and Ca++ metabolism in the brain. Ca++ antagonizes the antinociception produced by opiates as first shown by Kakunaga et al. (1968) and later confirmed and expanded by Harris et al. (1975b, 1976). In mice pretreatment with a dose of 15-30 mg/kg morphine produced a significant reduction of the Ca++ brain level, associated to the synaptosomal (SP2) fraction (Ross and Cardenas, 1974; Harris et al., 1976). Tolerance developed to this effect since in animals implanted with a morphine pellet, the SP2 fraction evidenced about a two-fold increase in Ca++ levels. This effect was blocked by naloxone administered in conjunction to the morphine tablet (Harris et al., 1976). Recently, Cerreta et al. (1977) and Guerrero-Munoz et al. (1977) have demonstrated that morphine blocks in-vitro and in-vivo the Ca++ uptake to mice brain synaptosomes. Interestingly, synaptosomal preparations from tolerant-dependent animals show an increase uptake of Ca++. Thus, there seems to be correlation between the in-vivo and in-vitro effects of opiates in Ca++ brain metabolism.

These two pieces of research altogether tend to indicate that an enzyme controlling a neurohormone, or a Ca++ transport mechanism in the brain could be related to the mechanism of tolerance and dependence to opiates in rodents. At present it is not clear the exact locus of

action or the physiological implication of these two sites of opiate action. Further research is obviously needed to expand and validate in depth these hypothesis.

The second kind of theory of narcotic tolerance and dependence also assumes that opiates disturb a homeostatic balance in the organism, but describes the phenomenon in physiological terms. The primary drug effect is compensated by the activation of pathways to counterbalance the the initial effect of the narcotic. Martin (1968) based on the effect of narcotic agonists antagonists proposed a homeostatic and redundancy model . Tolerance is due to the opening of redundant pathways within the CNS when the primary pathway is blocked by the action of the narcotic drug. With drug withdrawal, activity in the primary pathway is restored, which in combination with continuing activity in the redundant pathway results in a rebound hyperexcitability of the pathways initially depressed by the narcotic drug. Little or no experimental evidence is yet available to support this particular hypothesis. Another theory often discussed in this second kind of hypothesis is that of "disuse supersensitivity" by Jaffe and Sharpless (1968). This theory is based on the well documented peripheral neurohumoral supersensitivity following organ denervation as proposed originally by Cannon and Rosenblueth (1949). In essence, the theory is founded on the fact that opiates will initially produce the inhibition of a given system, but that the tissue will react to the inhibition developing an increased sensitivity to counteract the initial effect. The supersensitivity is thought to begin as soon as input is reduced. Abrupt withdrawal of the drug or its displacement by an antagonist restores input to supersensitive elements, producing a "rebound" hyperactivity in the very systems that were depressed by the drug.

(Jaffe and Sharpless, 1968). This model does not require that the drug be present on the ultimate neuronal receptor itself. A related theory postulates that alternations in neural input cause an increase in the number of receptors, which in turn may be "active" or "silent". An increase in receptors for neurotransmitters would account for tolerance, and the rebound effects occur when drug withdrawal restores normal neurotransmitter activity to a system with excess receptors (Collier, 1966).

Cochin (1970) proposed an immunological model of tolerance to explain the lag time required in the development of tolerance, and especially to account for the prolonged persistence of certain forms of opiate tolerance. Little evidence is available to sustain this hypothesis, especially when confronted with the fact that tolerance does not develop uniformly to all the actions of opiates, as would be expected from such a model system.

The discovery of the endogenous morphine-like peptides opens a new avenue of research in relation to the mechanism of tolerance-dependence to opiates. It is possible that the endorphins are related to the mechanism of tolerance, but at present this contention is speculative. The only relationship between tolerance and brain endorphins is the recent report of Simantov and Synder (1976) who reported that brain endorphins were increased in tolerant-dependent rats. However, the same laboratory has confirmed that the chronic administration of morphine failed to alter brain inmmunoreactive enkephalin levels in morphine tolerant rats (Childers et al.,1977). Similarly, Fratta et al.(1977) could not detect differences in the enkephalin content of the hypothalamus or striatum of tolerant-dependent rats. Further research is needed to understand the relation between the endogenous opiate peptides and the mechanism of action of morphine and the development

of tolerance.

WHY SINGLE DOSE TOLERANCE AND DEPENDENCE?

The problem of drug addiction and dependence is a challenging human problem. Its socio-economical as well as its ethical and moral implications are of major humanitarian consideration. This together with the altruism of conquering physical and spiritual well-being are the main motivations to discover animal models in order to investigate in detail the fundamental basis of drug addiction. The development of models to study the biochemistry and pharmacological basis of addiction to narcotic drugs has been of primordial importance. Once animal models were made available, it became possible to initiate studies on the neurochemical mechanisms of tolerance and dependence to narcotic drugs. Rodents and specifically mice have been demonstrated to be appropriate laboratory animals. Rodents develop high degree of tolerance to morphine and other opiates. Furthermore, mice develop dependence as evidenced by the abstinence syndrome elicited by the administration of opiate antagonist or abrupt opiate withdrawal (Maggiolo and Huidobro, 1961 and Way et al 1969).

Mice can be rendered highly tolerant-dependent to morphine by a simple procedure as described originally by Maggiolo and Huidobro (1961). Way et al. (1969) modified the thecnique to obtain quantitative data of tolerance and dependence. The procedure consists in the implantation of a specially formulated tablet containing 75 mg of morphine base (Gibson, 1971) on the subcutaneous tissue of the mouse. After 72 hours of pellet implantation, a high degree of tolerance and dependence can be demonstrated to morphine antinociception. About a 10-fold elevation of the morphine AD50 (refers to

the dose of opiate needed to produce analgesia in 50% of the population) is generally obtained with this procedure. This indicates about 10-fold tolerance to the antinociceptive effect of morphine. This decrease in sensitivity is coupled to at least a 70-fold increase in sensitivity to the effects of naloxone. The supersensitivity to the antagonist in causing the precipitated withdrawal response has been interpreted to indicate narcotic dependence. One of the reasons for the success of the morphine pellet implantation method in contrast to the daily repeated administration of morphine or related opiates is the continuous and approximately constant release of morphine from the tablet, at least during the first day of pellet implantation. With this convenient technique, numerous studies have been conducted to study the neurochemical basis of narcotic drug tolerance and to assay for drugs to "cure" human addiction.

However, in spite of the considerable interest and attention focused in a model system to produce tolerance and dependence, little is known about the first initial stages of addiction. From human experience it is known that an important variable in the process of tolerance is the frequency of opiate administration. If it is erratic, minimal tolerance develops. However, the persistent and continual use of narcotics leads very soon to the need of escalating doses to obtain the euphoric and rewarding effects of the drug.

It seemed of considerable interest to study in detail the kinetics of tolerance development to the antinociceptive effect of narcotics and to determine the dose needed to produce tolerance and dependence. For this purpose, I chose the mouse as the laboratory animal-model because of its practical and economical convenience and also because tolerance

to opiates is particularly easy to induce in mice. Furthermore, tolerance has been best studied in the mouse and particularly in this laboratory. In addition, abundant literature is available on the neurochemical mechanisms of tolerance development to morphine in mice. For reviews, see Way, 1972; Takemori, 1975; Clouet and Iwatsubu, 1975.

Hypothetically, tolerance (as any other pharmacological effect) should be a dose-dependent phenomenon. Furthermore, tolerance should be initiated after a priming dose. In the case of a morphine pellet implant, the high degree of tolerance-dependence attained is likely due to the continual absorption of morphine. This will produce high morphine levels so that a suficient priming dose is always maintained. Under these conditions, the "tolerance producing system" is being continually stimulated, accounting for the level of tolerance induced. Obviously, tolerance to opiates is a dynamic process that depends on the rate of induction and the rate of loss of tolerance. A proper balance between these two rates determines the actual degree of tolerance achieved by the animals. There is no reason to believe that both rates are equal, on the contrary, Way et al.(1969) have commented that the rate of morphine tolerance loss apparently is faster than that of tolerance induction.

With these thoughts in mind, I was interested to study specifically 2 variables in the early induction of tolerance to opiates. These were the dose and the lag time between the first administration of an opiate and the maximal expression of tolerance. For these purposes, I used a single dose of morphine and opiate peptides. This procedure had previously demonstrated the effective production of single dose tolerance (Huidobro-Toro et al., 1975). For this purpose, two doses of opiates were used.

The first dose, called the priming dose, was used to initiate tolerance. The second dose was used to reveal the degree of tolerance induced by the priming dose. The priming doses ranged within wide limits: lower than the median dose to produce an opiate-like effect to 20-40 times this dosage. The lag time between the the priming dose and the second dose of the opiate also varied from hours to weeks. Results show that tolerance occurs to several narcotic effects after a single-dose of an opiate. Tolerance induction is strickly dose-dependent as was predicted. Priming doses to initiate the development of tolerance need to be larger than the dose needed to produce an opiate effect in 50% of the population. The lag time period is crucial and is variable for different opiate effects.

EXPERIMENTAL PART I:

SINGLE-DOSE TOLERANCE AND DEPENDENCE TO THE ANTINOCICEPTIVE EFFECT OF MORPHINE AND B-ENDORPHIN IN MICE.

1.1 INTRODUCTION.

The first part of the project describes studies on the antinociceptive effect of B-endorphin (B-EN) in mice in comparison with that of morphine. Single-dose tolerance to and dependence on morphine and B-EN, as well as single-dose cross-tolerance between the two compounds are examined. Attempts to characterize the mechanism of action of opiates were made by studying the influence of some neurochemicals on the development of tolerance and dependence to opiate drugs.

1.2 MATERIALS AND METHODS.

1.2.1 Generalities: animals and chemicals used.

All experiments were performed on Swiss-Webster mice, raised at the vavarium of the Catholic University of Chile, or on ICR mice from Simonsen Laboratories, Gilroy, California.

The animals were housed six per cage and allowed free access to food and tap water. Food consisted of Purina mouse chow pellets. Animals were always maintained in the animal room provided by the animals care facilities at U. C. S. F. The mouse room was maintained at 22$\pm$ 0.5 oC, with a 12 hour artificial light cycle (6:00 am - 6:00 pm). Mice were allowed to adjust for 3-4 days to the animal room conditions prior to any experimentation. All analgesic determinations were done in the same room.

Chemicals used and their purchasers:

Camel B-endorphin (B-EN) and human B-endorphin (hB-EN) were prepared by synthesis and provided by Li et al. (1976b); Li et al.

(1977). B-EN or hB-EN were dissolved in sterile saline (Travenol) at a concentration of 1 ug/ul, and stored in plastic vials at -20 °C in aliquots of 100 ug each. Since a gradual loss in activity was noted with thawing between each experiment, a fresh aliquot was used for each experiment. Each sample was kept on ice, and sonicated for 15 seconds immediately before use; the potency was determined by assay for analgetic activity. Part of the variation in the effect of the peptide, apart from biological variability might be due to absorption to container surfaces (Bloom et al. 1976). Morphine sulfate and methadone hydrochloride were purchased from Mallinckrodt Chemical Works (St. Louis, Missouri). Naloxone hydrochloride was a gift from Endo Laboratories (Garden City, New York). Actinomycin D and cycloheximide were obtained from Sigma Chemical Co. (St. Louis, Missouri). 6-hydroxydopamine and 5,6-dihydroxytryptamine were obtained from Regis Chemical Co., Chicago, Illinois); L-tryptophan and cyclic adenosine-3'-5'-monophosphate from Calbiochem (San Diego, California). All dosages of the alkaloids were expressed as the free base, and the components were dissolved in isotonic saline unless otherwise stated in the methods, and injected in volumes of 0.1 ml/10 g of mouse.

1.2.2 Measurement and quantification of antinociception.

1.2.2a Hot plate method:

Swiss-Webster mice of both sexes were tested exclusively by the hot plate at 56 oC as described by Woolfe and MacDonald (1944). For any determination, in all the experiments reported, at least mice were tested twice on the hot plate prior to the experiments

to obtain the base-line latency of response; the mean ± S. E. being 6 ± 2 seconds. The reaction time was redetermined 1/2, 1, 2, 4 and 5 hours after the i.p. injection of morphine or methadone using a 30-second cut-off time. Antinociception was expressed quantitatively according to Cox et al. (1968) as the index of analgesia, defined as T1-T0/Tmax-T0. T0 and T1 represent the mean reaction time before and after a narcotic, respectively; Tmax represents maximal or cut-off time of exposure of the animals to the hot plate. Thus, with maximal analgesia the index would be 1.0. The index of analgesia was then plotted vs. time in hours and the area under the time-effect curve, area of analgesia (AA), was determined. The area in square centimeters was used as an indication of the degree of analgesia.

Dose effect curves of morphine and methadone were obtained using i.p. doses in the range of 5 - 50 mg/kg for morphine, and 5-25 mg/kg methadone respectively. The time course of the analgesic effect for each dose in each mouse was determined, and the area under the effect vs. time-course curve. The AA was calculated individually for each mouse, allowed statistical evaluation of the effect of each opiate dose studied. Dose effect curves were obtained by plotting the dose of narcotic drugs vs. the AA produced by the respective opiate.

1.2.2b Effect of morphine on tail-flick antinociception.

The tail-flick method by D'Amour and Smith (1941) was used on male ICR mice weighing 20 - 25 g each. After the base line response (1.1-1.5 seconds) was established, the reaction time was

redetermined 30 minutes after an i.p. injection of morphine. A 3.5-second delay in the tail-flick reaction time to thermal stimulus was used as a quantal response to at least three doses of morphine using at least eight animals per dose. The median analgetic dose (AD50) of morphine and its 95% confidence limits were estimated by the method of Litchfield and Wilcoxon (1949). Inasmuch as the absolute AD50 varied even with animals from the same supplier, comparisons between test and control groups were always made on the same day, with the same batch of animals. In these studies, the morphine AD50 ranged between 4 and 12 mg/kg. The effect of various drugs on the AD50 were compared by determining the AD50 potency ratio after and before treatments.

1.2.2c Quantification of B-endorphin and morphine antinociception after intracerebroventricular administration.

Intracerebroventricular (icv) injections of B-EN or morphine were done according to Haley and McCormick (1957), and Harris et al., (1975a). Under slight ether anesthesia a midline incision was made through the scalp to expose the skull. Injections were made into the lateral ventricles 1 - 2 mm lateral to bregma through the coronal suture, using a 27 gauge needle, sleeved with polyethylene tubing so that 2.5 mm of the needle penetrated the skull. The volume injected was 10 ul/mouse. Prior experimentation established that the procedure did not affect the tail-flick response. Each experimental group consisted of 12 mice. Protocols were designed so that mice were not exposed to the noxious stimuli more than 4-5 times since repeated exposures resulted in an increase in

the control latency time. To reduce the variability, each experimental series was carried out on the same day and approximately at the same time, employing the same batch of mice. Paired experiments were always performed using a vehicle injected group as the control. Antinociception was expressed as "% of analgesia", calculated from the expression (T1-To)/(T2-To) x100, where T0 refers to the control latency time before the drug, T1 to the experimental values obtained at the different intervals (1/2, 1, 2 or 4 hours) after drug injection, and T2 to maximal analgesia set at the cut-off time. After plotting on graph paper % analgesia against time, the area under the curve was cut out and its weight determined using an analytical balance. The area under the curve (AA) is an expression of the total analgesic response: which considers the intensity and the duration of action of the opiate. The AA was used to compare the effect of the opiates. The technique of weighing the AA obtained from each mouse allowed the calculation of the mean AA $\pm$ SEM for the different doses of B-EN. The weighing method to determine the AA introduced an error of less than 1%. The AAs were expressed in arbitrary units (U2), and were analyzed statistically using the students "t" test. Significance was set at the P 0.05 level. The median analgesic dose of B-EN (AD50) and its 95% confidence limits were estimated according to Litchfield and Wilcoxon (1949).

1.2.2d Estimation of B-endorphin AD50 and its blockade by naloxone. Three groups of 12 mice each were used. Mice were injected with doses of 0.2, 0.6 and 2.0 ug B-EN icv/mouse. Antinociception was measured 15, 30 or 60 minutes after the administration of B-EN. The % of analgesia of each mouse was calculated and analyzed statistically. To determine the AD50, the % of analgesia was plotted in log probit graph paper, and the 95% confidence limits of the AD50 were estimated by the method of Litchfield and Wilcoson (1949).

In another set of experiments, the complete time course of the antinociception produced by doses of B-EN was followed. The mean AA $\pm$ SEM produced by each dose of B-EN was calculated, and plotted vs. the log dose of B-EN.

To examine whether naloxone, the opiate antagonist, blocked the antinociceptive response of B-EN, three groups of mice were used. The first group was injected simultaneously with 600 ng hB-EN icv/mouse and injected with 10 ml/kg saline s.c., and the AA of this control was determined. The second and third groups of mice were treated with 1 or 4 mg/kg naloxone s.c. in conjunction to 600 ng hB-EN. The AA of the two groups was determined and compared to that obtained in the hB-EN-saline control group.

1.2.3 Assessment and quantification of single-dose tolerance. The following general experimental procedure was followed. Tolerance induction was initiated by the injection of a priming dose (PD) of an opiate. To assess the development of tolerance,

a challenge dose of a narcotic drug was administered at different time intervals following the PD. The second dose of the opiate was injected at least 5-6 hours after the priming dose, when the tail-flick latency had returned to control, pre-drug levels. Tolerance was evaluated by comparing the AA produced by the challenge dose of an opiate in a naive or saline pretreated group of mice to that produced by the challenge dose of the opiate in a narcotic primed group. A significant decrease in the AA of the test was taken as indication of tolerance development. On other occasions, at varying intervals following the PD, morphine dose effect curves were obtained using 3 groups of eight mice for each determination. The morphine AD50 after opiate pretreatment was established and compared to that obtained in saline primed, naive mice. Results were expressed as a potency ratio (the morphine AD50 in morphine-primed mice divided by the morphine AD50 in saline treated animals). The ratio was analyzed statistically according to Litchfield and Wilcoxon (1949). In general, a ratio of two or more was found to be statistically different at a P less than 0.05.

Single-dose tolerance could then be defined as a statistically significant decrease of the AA produced by the second dose of an opiate drug (alkaloid-type or peptide) as compared to the effect obtained with the priming dose. Alternatively, tolerance was defined as a significant shift of an opiate dose effect curve to the right, caused by morphine or pretreatment with other opioids. At least a two fold increase in the opiate potency ratio between saline and opioid treatment mice to show statistical significance.

1.2.4 Determination of single-dose tolerance to morphine and B-endorphin in mice and rats.

1.2.4a Single-dose tolerance to morphine in mice.

Separate groups of mice were injected i.p. with various amounts of a priming dose (PD) of morphine. At fixed time intervals thereafter the degree of tolerance developed was established by the decrease in response to a challenge dose of morphine. Two strains of mice and different procedures were used for assessing tolerance. In the first method, a total of 18 groups of Swiss-Webster mice (n = 10-12) were given a PD of morphine varying from 5-50 mg/kg i.p. The AA for each dose was determined immediately (day 0) and subsequently in other groups 1, 2 and 3 days later after challenge with a second dose of morphine equal to the original PD. If the AA of the experimental group was less than that of the control at day 0, tolerance to the antinociceptive effect of morphine was considered to have developed. In general, a difference of at least 20% was necessary to attain this level of significance.

To establish single-dose tolerance in ICR mice, separate groups of animals were simultaneously primed i.p. with saline or with 15, 60 or 200 mg/kg of morphine, but were not tested on the tail-flick machine. The four groups were divided into four subgroups and the responsivity of each group (n = 24-30) to morphine was assessed either on the 1st, 3rd or 7th day after priming. The development of tolerance was assessed comparing the morphine AD50 obtained in the morphine vs. saline-tested groups.

1.2.4b Single-dose tolerance to B-endorphin in mice.

Groups of mice were injected icv with a priming dose of the peptide to induce the development of tolerance. At different time intervals following the priming dose, a second dose of B-EN was administered to study if tolerance had developed to the priming dose. As an illustration of the protocol used to establish single-dose tolerance, 2 groups of mice were administered 0.6 ug of B-EN icv (7.2 nMol/Kg) at time zero. The time course of the antinociception was determined in one group, and its AA was obtained. The second group of animals was reinjected with 0.6 ug B-EN after the tail-flick reaction time of the first group had returned to baseline value (5 hours after the injection). In this way, the second group of mice was not tested on the tail-flick machine until the second exposure to B-EN. The AA of the second dose of B-EN was determined and compared to the group that received only one dose of B-EN.

To study the relation between the initial dose of B-EN and the degree of single-dose tolerance , increasing doses of B-EN were used as the priming doses. To perform this particular experiment, four groups of mice were employed; each group was injected with 0.1, 0.2, 0.6 or 2 ug B-EN icv respectively. Twelve hours after the priming dose, each group was administered a second dose of B-EN. The time course of the analgesia produced by the second dose of B-EN was established, and the AA was calculated. The AA produced by the different doses of B-EN was compared to that obtained in naive, non-primed mice.

1.2.4c Single-dose tolerance to B-endorphin in rats.

In an additional set of experiments, Sprague-Dawley rats (160-200g from Simonsen Laboratories, Gilroy, CA) were used to establish the development of single-dose tolerance. Rats were implanted three days before the administration of peptides with a guide cannulae (L shaped steel cannulas, made from 21-gauge disposable needles) in the lateral ventricles as previously described by Wei et al. (1973). Three groups of rats were used. Two groups (six animals each) were injected with a priming dose of 10 ug B-EN icv/rat at time zero. Anti-nociception was measured on one of the primed groups, and the AA was determined. The other, pretreated group, received a second dose of 10 ug B-EN six hours after the priming dose, The AA obtained in the naive group of rats was compared to that produced by the second dose in the primed group. The third group (three rats) received twice saline icv and served as the control group.

1 - 2. 5 Time course of single-dose tolerance development.

1 - 2. 5a Morphine.

The time course of tolerance development to a PD of 20 mg/kg of morphine i.p. was followed in ten groups of mice. In one of the groups, the AA for this dose was determined and this value was taken to be the control response. On each subsequent day for nine consecutive days, a group selected at random was challenged with the same dose of morphine and the AA was determined; the last group was tested 12 days after the PD. The decrease in AA noted for a given day relative to the control response obtained on the first day was plotted against the number of days.

1.2.5b B-Endorphin.

To establish the time course of single-dose tolerance development to B-EN, 7 groups of mice were used. All groups were administered with a priming dose of 0.6 ug B-EN icv at time zero. In one of the groups, the time course of the antinociception produced by the priming dose of 0.6 ug B-EN icv was followed, and the AA for this dose was determined. This value was taken as the control response. At 6, 12, 24, 36, 48 or 96 hours after the priming dose of B-EN, a group selected at random was challenged with the same dose of B-EN. The time course of each B-EN administration was established, and the AA was determined. The mean AA $\pm$ SEM noted for each administration of B-EN in the time interval studied was plotted against the time in hours following the priming dose.

An identical protocol as outlined for a priming dose of 0.6 ug B-EN was followed to study the time-course of single-dose tolerance development to a priming dose of 2.0 ug of B-EN icv/mouse.

1 - 2.6 Relation between dose and time in the development of tolerance to morphine.

In order to further explore the relation between the dose of morphine and the lag time required to express tolerance, the following protocol was designed. Groups of mice were implanted s.c. with a 75 mg morphine pellet in the subcutaneous tissue of the back according to the technique of Way et al. (1969). The pellets were wrapped in a fine nylon mesh to facilitate their removal The pellets were removed 6, 12 or 24 hours after implantation,

and the sensitivity of the mice to morphine was assayed 66, 60 or 48 hours following pellet removal. As a control, groups of mice were implanted with morphine pellets for 72 hours, and the morphine AD50 was determined in this case six hours after the pellet removal. At this time, morphine brain levels were barely detectable (Way et al., 1969). As a control group, animals were implanted for 72 hours with placebo pellets and the morphine AD50 was established. Results were expressed as the morphine AD50 ratio of each experimental group divided by the morphine AD50 of the placebo implanted group. Tolerance at any interval was established by a significant increase in the morphine AD50 after the pellet implantation.

1 - 2.7 Assessment of single-dose physical dependence.

1 - 2.7a Morphine.

The degree of physical dependence developed after a PD of morphine was assessed in ICR mice by estimating the amount of naloxone needed to precipitate withdrawal jumping from a platform, as described by Way et al. (1969). Groups of eight animals each were injected i.p. with a PD of 15, 60 or 200 mg/kg of morphine. On days 1, 3, 4 and 7 after the PD, mice were injected with doses of 1, 10, 30 or 100 mg/kg of naloxone s.c. and the proportion of animals jumping within 15 minutes was determined. Control animals were given saline instead of morphine on day 0, but thereafter were treated in the same way as those primed with morphine.

The effect of 5,6-DHT, L-tryptophan, or 6-OHDA was also

studied on physical dependence. The pretreatment with these drugs was the same as outlined in the tolerance studies. Control animals received saline instead of the morphine PD.

1.2.7b B-endorphin.

To establish whether single-dose tolerance was associated to the development of a short term physical dependence, the same animals that were used to study the time course of single-dose tolerance were used. Three to four hours after the second dose of 0.6 or 2.0 ug B-EN icv, mice were administered 10 mg/kg naloxone i.p. Precipitated withdrawal signs were observed for a period of 30 minutes following the injection of naloxone on a circular platform two feet high. Precipitated abstinence behavior was quantified according to the ranking score method of Huidobro and Maggiolo (1961). This method scores 1 point for mild abstinence syndrome, escalating to 5 points for complete withdrawal.

1-2.8 Single-dose cross-tolerance studies.

1-2.8a Morphine and methadone.

Two separate experiments were performed on Swiss-Webster mice using the hot plate procedure. In the first experiment, a PD of 15 mg/kg of morphine or saline was administered i.p. and 72 hours later each group was divided into 5-6 subgroups (n = 10-12) for determining their sensitivity to either morphine or methadone. The AA after varying doses of morphine or methadone was determined and plotted as a dose effect function as previously described. In the second experiment, a PD of 15 mg/kg of methadone or saline was injected i.p.; 48 hours

later the animals in each group were divided into two subgroups and the AA's were determined after challenge with varying doses of either morphine or methadone.

1.2.8b B-endorphin and morphine and vice versa.

Two cross experiments were performed. In the first, 600 ng of B-EN was injected icv at time zero, and five hours later, the animals were challenged with 1 ug of morphine icv. The second group received 1 ug of morphine icv at time zero and five hours later mice were given 600 ng of B-EN. After the second administation, the AA for each compound was determined. The control groups for these series, were injected at time zero with saline and five hours later with either 600 ng B-EN or 1 ug of morphine. The AA of the saline treated and opiate pretreated animals were compared. To complete this set of experiments, four groups of mice from the same batch of animals as those employed for the cross-tolerance studies above, were used to control the development of acute tolerance by B-EN or morphine. One group was administered with 600 ng of B-EN, and one with 1 ug of morphine icv and two groups were not primed. The AA of each group was determined. Five hours later, each primed group received a repeat dose of the same compound and the AA to this challenge dose was determined. The non-primed controls received the same challenge dose of B-EN or morphine for the AA estimation.

In another set of experiments, six groups of eight mice each were used. Three groups were injected with 600 ng B-EN icv and the

other three groups with saline icv. Six hours later, three different doses of morphine were given i.p. and the AA of each morphine dose (5, 10 and 20 mg/kg) was calculated, and compared between the peptide and saline primed group.

1.2.9 Effect of opiate antagonists on the development of single-dose tolerance.

1.2.9a Effect of nalorphine on morphine.

To study the interaction of nalorphine, a partial opiate agonist, on the development of single-dose tolerance to morphine, a group of Swiss-Webster mice was primed with a PD of 10 mg/kg morphine i.p. and simultaneously injected with 10 mg/kg nalorphine. The time course of antinociception was determined and analgesia was quantified calculating the AA. Twenty-four hours later, a second dose of nalorphine was administered; and 48 hours following the PD, animals were challenged with 10 mg/kg of morphine. The analgesic effect of the challenge dose was established and the AA of morphine was calculated. In the control group, mice were primed with morphine but nalorphine was substituted for saline. The AA of the PD and the challenge dose of morphine was compared in both groups.

1.2.9b Effect of naloxone on B-endorphin.

Naloxone was injected in conjunction with of B-EN, in 2 sets of experiments. In the first one, 1 mg/kg naloxone s.c. was administered simultaneously with a priming dose of 600 ng hB-EN. As a control, a paired group of mice was injected with saline and hB-EN. Twelve hours later, 600 ng hB-EN was administered

and the AA was determined to establish the degree of tolerance developed. In the second series of experiments, 2 mg/kg naloxone was administered s.c. simultaneously with a priming dose of 600 ng hB-EN icv. One hour later, a second injection of 2 mg/kg naloxone was given to the mice. Controls were mice injected with saline instead of naloxone. A challenge dose of 600 ng hB-EN was administered 12 hours after the priming dose of the peptide. As a control for each series of experiments, a group of mice was primed with saline icv and saline s.c., and 12 hours later administered with a challenge dose of 600 ng hB-EN .

1.2.10 Effect of protein synthesis inhibitors on the development of single-dose tolerance.

1.2.10a Effect of cycloheximide on single-dose tolerance to morphine. In experiments using Swiss-Webster mice and the hot plate method, cycloheximide (30 mg/kg i.p.) was given 30 minutes prior to a 15 mg/kg PD or morphine. Three days later, a challenge dose of 15 mg/kg of morphine was administered and the AA was determined. Comparisons were made with animals receiving saline instead of cycloheximide before a PD of morphine and with non-primed animals challenged with morphine only.

With ICR mice, cycloheximide (30 mg/kg i.p.) was administered 30 minutes before a PD of morphine (60 mg/kg i.p.). Twenty-four or 72 hours later, the AD50 for morphine was determined using the tail-flick method. Control animals were morphine-

primed animals pretreated with saline and non-primed animals.

1.2.10b Effect of protein synthesis inhibitors on the development of single-dose tolerance to B-endorphin.

To study whether the synthesis of de novo protein is involved in the development of single-dose tolerance, a DNA and a protein synthesis inhibitor were used in conjunction to the administration of a priming dose of B-EN. Three groups of mice were used in this experiment. The first group was pretreated with 0.35 mg/kg actinomycin D i.p., and the second group with 30 mg/kg cycloheximide i.p. As control, a third group was injected with 10 ml/kg saline i.p. Half an hour after the administration of these drugs, 0.6 ug B-EN was injected icv per mouse. Twelve hours after the priming dose of B-EN, all the animals were injected with a second dose of 0.6 ug B-EN. The AA produced by the second dose was calculated. In this protocol, any significant increase in the AA over the AA obtained in the saline pretreated group was indicative of blockade of tolerance development.

1.2.10c Effect of cycloheximide on single-dose morphine-methadone cross-tolerance.

Swiss-Webster mice were pretreated with 30 mg/kg i.p. of cycloheximide or saline. Thirty minutes later, a PD of 15 mg/kg morphine or saline was injected and 72 hours after the mice were challenged with methadone (15 mg/kg) and the AA was determined. In a second set of experiments 15 mg/kg methadone was used as the priming drug and the AA of 15 mg/kg morphine was estimated 48 hours later.

1.2.11 Effect of various agents on single-dose tolerance development to morphine.

The studies were performed on ICR mice using the tail-flick procedure. All experiments were paired using the vehicle for dissolving the test drug. The PD of morphine was 60 mg/kg i.p.

1.2.11a 6-hydroxydopamine (6-OHDA).

The compound was dissolved immediately before use in saline containing 0.1% ascorbic acid. Intracerebral injections were given under light ether anesthesia 40 hours prior to the PD of morphine and 72 and 96 hours after the PD; the morphine AD50 was determined. The dose (28 ug of 6-OHDA base 10 ul) and conditions of pretreatment with 6-OHDA were those reported by Friedler et al. (1972) to produce a 66% and 30% depletion of norepinephrine and dopamine, respectively.

1.2.11b Cyclic adenosine-3'5'-monophosphate (cAMP).

A dose of 10 mg/kg of cAMP or saline was injected into the tail vein of ICR mice two hours before the PD of morphine. After 72 hours, the AD50 for morphine for each group was estimated. The cAMP treatment was identical to that described by Ho et al. (1973b) to enhance tolerance development after morphine pellet implantation.

1.2.11c L-tryptophan.

A dose of 75 mg/kg of tryptophan or saline was administered i.p. every 24 hours for a total of three times. Two hours after the initial dose of tryptophan a PD of 60 mg/kg of morphine was

given. Seventy-two hours later, the morphine AD50 was determined in these groups and in a group of naive mice. This pretreatment schedule for L-tryptophan was similar to that previously used in our laboratory (Ho et al., 1975) to enhance the development of tolerance after morphine pellet implantation.

1.2.11d 5,6-dihydroxytryptamine (5,6-DHT).

The creatinine sulfate dihydrate (base equivalent 0.436) was dissolved immediately before use in saline containing 0.1% ascorbic acid. A dose of 2.7 mg/kg of 5,6-DHT was injected icv (60 ug/10 ul/mouse) under light ether anesthesia. After 24 hours, the PD of morphine was given and the morphine AD50 was determined 72 hours later (96 hours after the 5,6-DHT). It was established previously (Ho et al., 1973a) that this pretreatment with 5,6-DHT produced a 40% fall in brain 5-hydroxytryptamine, without affecting catecholamine levels, and inhibited the development of chronic morphine tolerance.

1.2.12 Effect of tolerance on brain morphine uptake.

Brain levels of morphine were estimated by the procedure of Kupferberg et al. (1964). Animals were injected with a PD of 60 mg/kg of morphine i.p. and 72 hours later a challenge dose of 40 mg/kg morphine was injected i.p. Determinations of morphine levels in brain were made 30 minutes after the last dose of morphine. The control group was primed with saline and challenged 72 hours later with the same dose of morphine.

1.3 RESULTS.

1.3.1 Opiate antinociception, dose effect responses.

1.3.1a Morphine and methadone in mice.

The median antinociceptive doses (AD50) of the opiates and strain of mice used in the two assay methods of this study, are shown in Table I-1. Swiss-Webster mice, assayed on the hot plate test for antinociception, were slightly more sensitive to morphine than methadone. ICR Simonsen mice assayed on the tail-flick test, were more sensitive to the effect of morphine than the Swiss-Webster mice studied on the hot plate.

1.3.1b B-endorphin in mice.

B-EN produced a prompt dose-dependent and long-lasting antinociception in mice. The AD50 (and its 95% confidence limits) ranged between 380.0 (149.6 - 965.2) ng/mouse 15 mins. after icv administration, to 265.0 (101.5 - 685.5) ng/mouse 30 minutes after injection. At doses of B-EN producing significant antinociception comparable to morphine, none of the overt behavioral effects of morphine (Straub tail and increased locomotor activity) were evident. No significant differences were observed in the antinociceptive effect of the peptide obtained from human pituitaries (hB-EN) as compared to that from the camel (B-EN). The hB-EN AD50 value, and its 95% confidence limits, obtained 30 minutes after injection was 210 (105-420) ng/mouse. (Table I-1). B-EN exhibited a parallel dose effect curve to hB-EN (Figure 1.1), and proved to be on a molar bases, about 25 fold more potent than morphine injected icv.

Table I-1

AD50 values of different opiates in mice.

	AD50 mg/kg (95% C.L.)[a]
a) Hot plate test	
Morphine i.p. Swiss-Webster	8.0 (5.63 - 11.36)
Methadone i.p. Swiss-Webster	10.0 (7.87 - 12.7)
b) Tail flick method	
Morphine i.p. ICR, Simonsen	6.5 (5.0 - 8.5)
Morphine icv ICR, Simonsen	0.030 (0.01 - 0.07)
B-EN icv ICR, Simonsen	0.012 (0.004 - 0.033)
hB-EN icv ICR, Simonsen	0.008 (0.004 - 0.016)

[a] Antinociception was determined 30 minutes after the opiate administration. Values in parenthesis denote the 95% confidence limits.

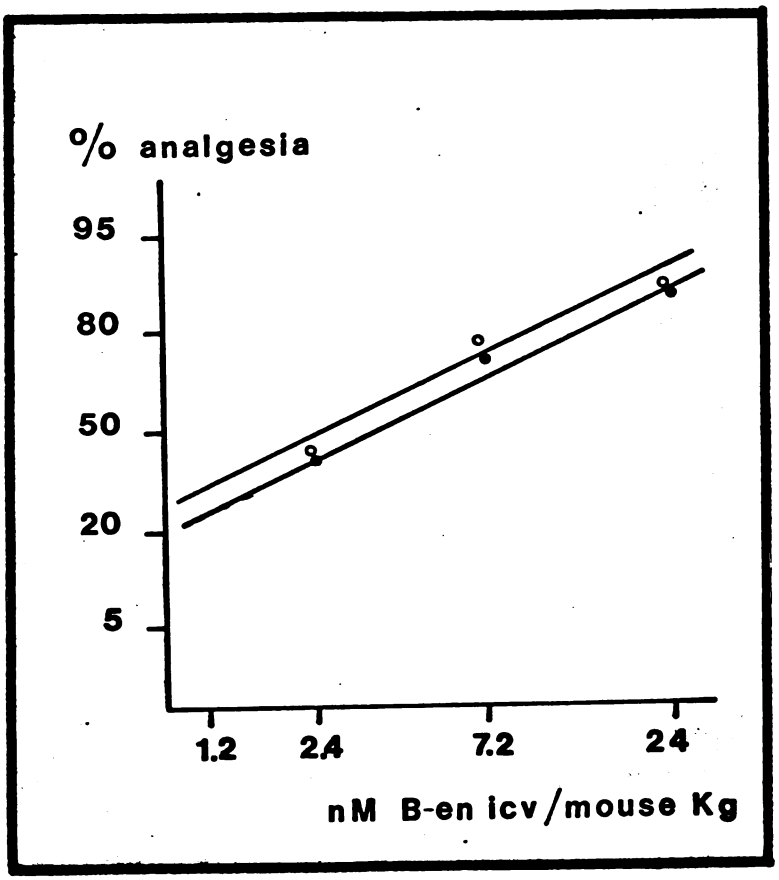

Figure 1.1

Comparison of the analgetic response of human and camel B-Endorphin in mice.

Each point represents the % of analgesia obtained with 12 mice. Solid circles represent antinociceptive responses 30 minutes after icv administration to camel B-Endorphin; open circles to human B-Endorphin. The median antinociceptive dose was 265 ng/mouse for human B-Endorphin and 300 ng/mouse of camel B-Endorphin.

In all the subsequent experiments a dose of 600 ng B-EN or hB-EN (7.2 nMol/Kg) was used. This dosage consistently produced 60-70% analgesia 30 minutes after icv administration, and the effect lasted for about 4 hours. Figure 1.2 shows the time course of the antinociception of 600 ng B-EN icv/mouse. It can be observed that the response reached peak effects as early as 30 minutes following administration; and that intense antinociception was maintained for the first 2 hours after which the response decayed linearly, and disappeared by 4-5 hours.

1.3.1c Effect of naloxone on B-endorphin antinociception in mice. Naloxone blocked the antinociceptive response produced by B-EN in mice. The administration of 1 or 4 mg/kg naloxone in conjunction with the injection of 600 ng B-EN/mouse produced a dose dependent blockade of B-EN antinociception, as evidenced by significant decreases in the AA (Table I-2). This data is in agreement with previous data reported by Loh et al., (1976).

1.3.2 Single-dose tolerance development to opiates.

1.3.2a Morphine in mice.

Development of single-dose tolerance to the analgetic effect of morphine was demonstrated in 2 strains of mice using different procedures for assessing antinociception. With the hot plate procedure, development of tolerance was demonstrated in Swiss-Webster mice by a decrease in the AA 1, 2 and 3 days after priming with various single doses of morphine greater than 10 mg/kg. Doses of 5 and 7.5 mg/kg of morphine, although producing effective analgesia, failed to induce tolerance develop-

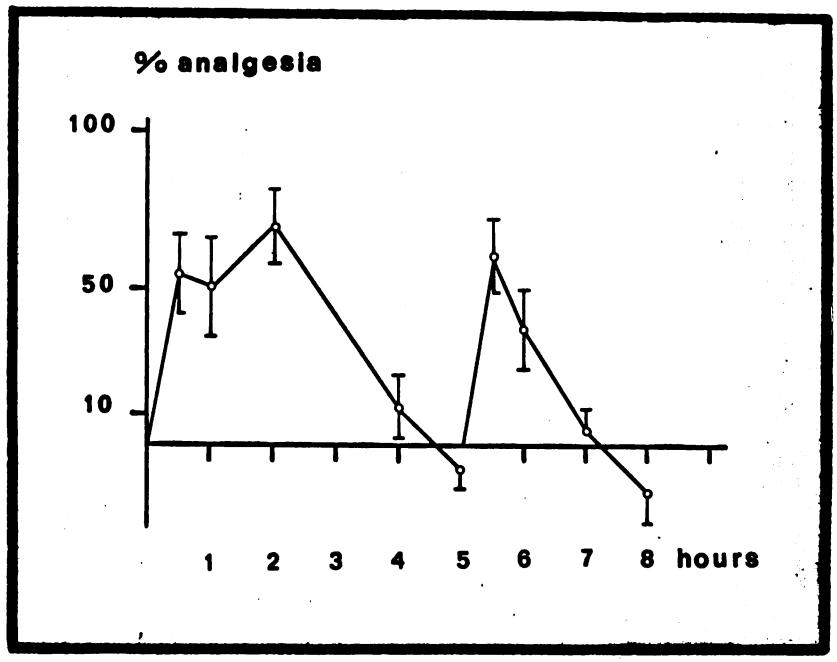

Figure 1.2

Development of single-dose tolerance after a single and second administration of 600 ng B-Endorphin.

Two groups of mice were injected with 7.2 nMol B-EN icv/kg (600 ng/mouse) at time zero. In one group antinociception was determined 0, ½, 1, 2, 4 and 5 hours after injection. The other group was injected with a repeat dose of B-EN 5 hours after the priming dose (when the tail-flick latency in the first group had returned to control), and the time course of antinociception was followed. Circles represent the mean % of analgesia of 12 mice, bars denote the S.E.M. To establish tolerance, the areas under the time course effect of each injection were compared. In this particular experiment, the second dose of B-EN produced an area of anlagesia of only 43.7% the response in nonprimed, control animals.

Table I-2

Effect of naloxone in the antinociceptive response of 7.2 nMol B-Endorphin/Kg

Pretreatment[a]	Area of Analgesia $\bar{x} \pm$ SEM (U^2)
Saline sc.	125.94 ± 34.15 (12)
1 mg/Kg naloxone sc.	55.84 ± 28.10 (12)
4 mg/Kg naloxone sc.	20.80 ± 6.66 (11)

[a] Pretreatment was done concomitantly to the icv. administration of 600 ng B-EN/mouse (7.2 nMol B-EN/Kg). Number in parenthesis indicates animals in each group.

ment. As shown in Figure 1.3, after a PD of 10-50 mg/kg of morphine, tolerance was evidenced by a shift in the dose-response curve to morphine downward and to the right. The decrease in the AA was significant on day 3 and greater than on day 1. The effects on day 2 were intermediate and not shown. Although there was increasing analgesia with increasing doses of morphine for initiating tolerance development, the doses between 10-50 mg/kg all produced about the same relative decrease (40-60%) in the AA.

The results obtained by the tail-flick on ICR mice were consistent with previous findings, although higher PD doses of morphine were necessary to demonstrate significant tolerance. Tolerance to a single dose of morphine was evidenced by a parallel shift to the right of the dose response curve of the animals receiving morphine as compared with saline controls. The increase in the morphine AD50 after 15, 60 and 200 mg/kg of morphine was about 2-fold although the level of significance for the lowest dose was equivocal. As shown in Figure 1.4, the effect was maximal 3-4 days after a PD of morphine, and the effect was lost after one week.

1.3.2b B-endorphin in mice and rats.

Marked tolerance to a single injection of B-EN developed rapidly in mice. The effect of a second dose of 0.6 ug B-EN icv (administered when the animals appeared to have recovered from the antinociception induced by the initial dose of B-EN, as evidenced by a return of the tail-flick latency to baseline)

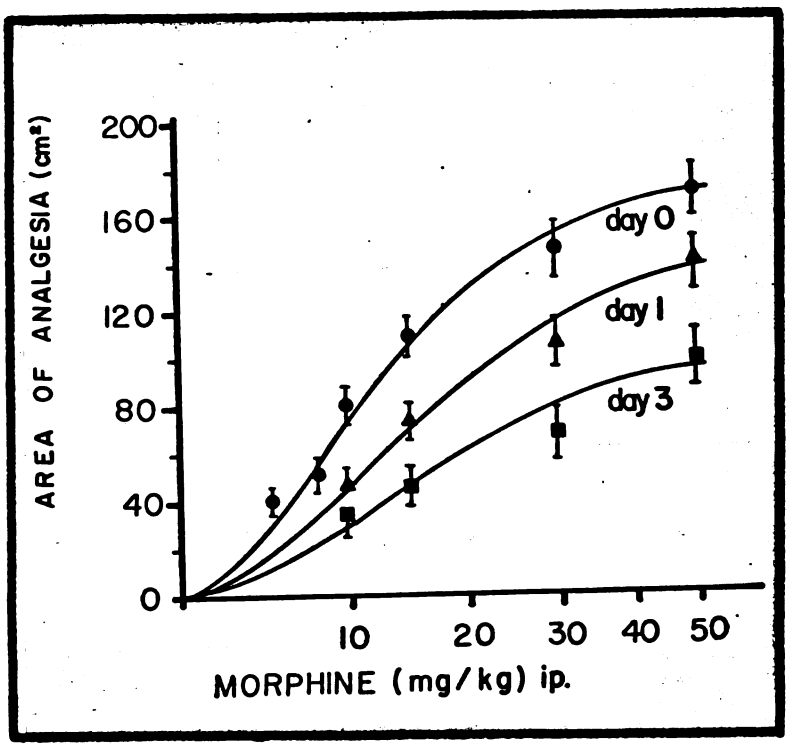

Figure 1.3

Development of single-dose tolerance to morphine in Swiss-Webster mice.

Varying single doses of morphine were administered and the analgetic response to morphine was determined (day 0). The test was repeated with each dose in separate groups of animals after 1, 2 and 3 days. The Area of Analgesia was plotted against dose. The curve for day 2 fell between those for days 1 and 3 and has been omitted. Each point represents the mean response on at least 10 mice $\pm$ S.E.M. By the third day, the difference in the area of analgesia was statistically different, with a P value of less than 0.05.

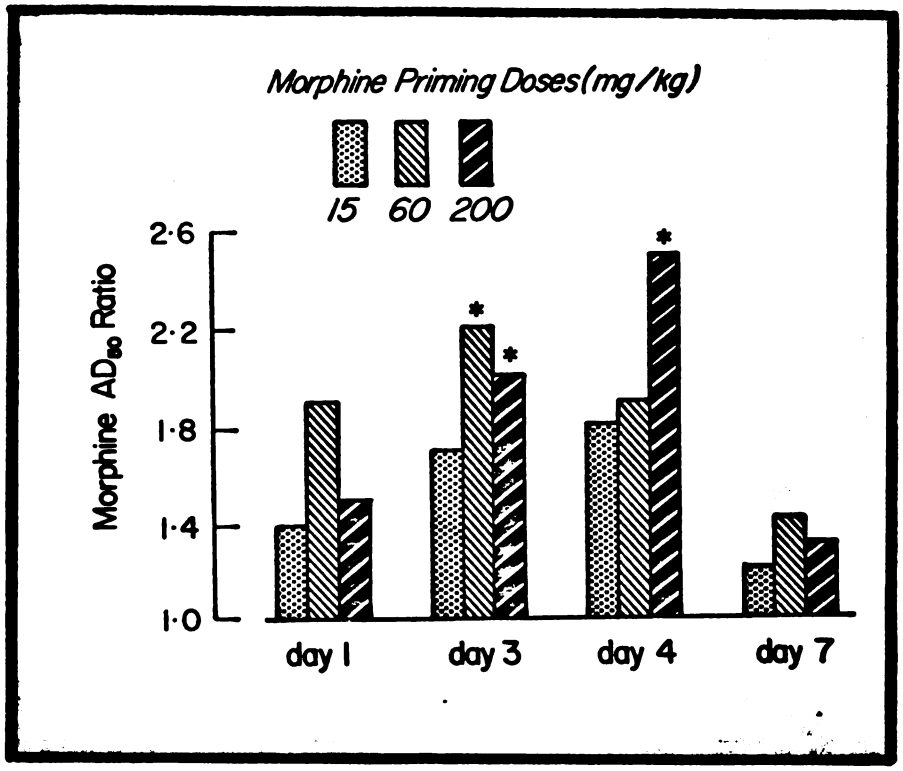

Figure 1.4

Single-dose tolerance development in ICR mice.

Separate groups of animals were given various priming doses of morphine, 0 (saline), 15, 60, and 200 mg/kg, and on the 1st, 3rd, 4th, and 7th day after the priming dose, the tail-flick morphine AD50 of each group was determined. The results of a given day are expressed as a ratio of the AD50 of the group primed with morphine over that of the group primed with saline (day 0). In these experiments ratios greater than 2 (*) were establish to be statistically significant, with a P value less than 0.05.

was more than 50% decreased as compared to the effect obtained with the first dose of B-EN. The maximal response obtained 30 minutes after the second injection of B-EN was not significantly reduced, but the time course of the analgesic response was considerably diminished, accounting for the significant decrease in the AA. In the particular experiment shown in Figure 1.2, the AA produced by the first dose of B-EN was 145.0±21.3 U2 which was diminished to 62.7±13.1 U2. The difference in the AA between the first and the second dose of B-EN was of 56.7%, with a P value less than 0.01.

The development of single-dose tolerance to the antinociceptive effect of B-EN was dose-dependent. B-EN in the range of 1.2-2.4 nMol/Kg although analgesic, did not develop tolerance, which was demonstrable using a larger priming dose. Figure 1.5a illustrates a dose response curve of the antinociception produced by B-EN in naive mice, and in mice pretreated with different doses of B-EN. The dose response curve of B-EN, in opiate primed animals, illustrates that the antinociception of low doses of B-EN was not altered, while that of higher doses of B-EN was decreased and did not recover maximal effect. These results are best analyzed in Figure 5.1b, where results are expressed as a percentage of the AA reduced. A linear relation can be appreciated, demonstrating that the development of single-dose tolerance is dependent on the priming dose, up to a plateau level.

At the only dose of B-EN used in rats to produce single-dose

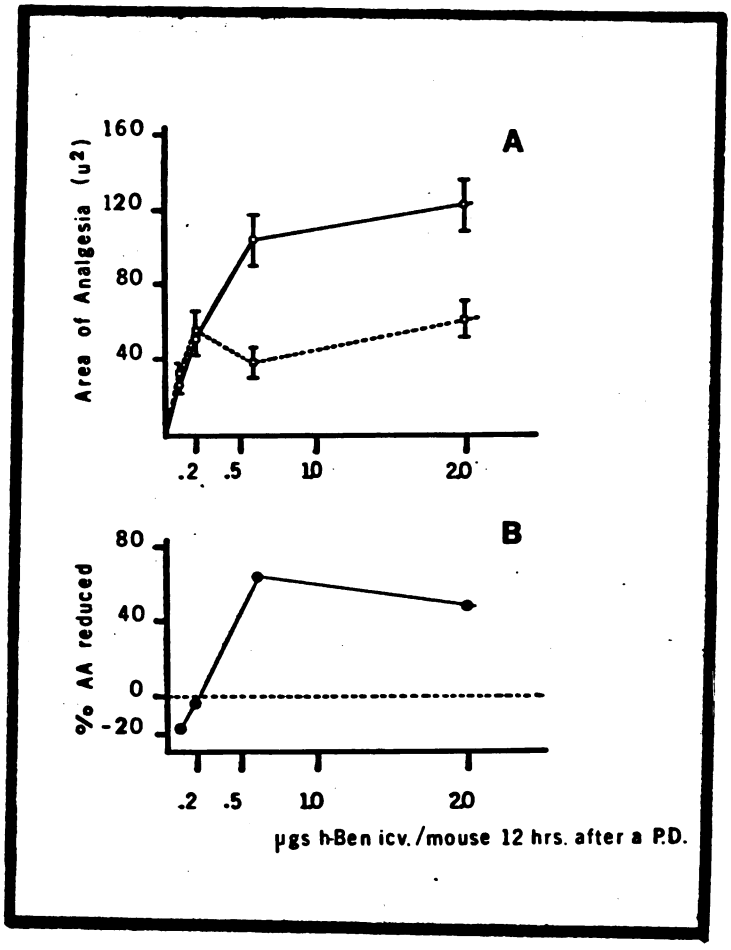

Figure 1.5

Effect of various priming doses of B-Endorphin on the development of single-dose tolerance.

Two groups of mice were administered with a priming dose of 100 ng hB-EN icv at time zero, and the area of analgesia of one group was determined. Twelve hours later, the second group of mice was challenged with 100 ng hB-EN to determine the difference in the AA between control, nonprimed and hB-EN primed groups. A similar procedure was followed with priming doses of 200, 600 and 2000 ng hB-EN.

A. Dose effect curve of antinociception. Abscissa represents the AA, ordinate log-dose of hB-EN icv. Solid line:effect of hB-EN in naive mice; dash line in animals primed 12 hours before. Bars denote the S.E.M.

B. Represents the AA as reduced by the priming dose, plotted versus the log-dose of the priming dose.

tolerance, there was a 42.1% reduction in the AA produced by the second dose of 10 ug B-EN icv/rat, as compared to the effect of the priming dose of B-EN. The mean AA ± SEM of the priming dose was 141.5 ± 45.4 U2 (n = 6) which was diminished to 81.9 ± 19.1 U2 (n = 6) six hours after the priming dose.

1.3.3 Temporal course of single-dose tolerance development to morphine and B-endorphin in mice.

1.3.3a Morphine.

The peak (maximal) tolerance to a priming dose of morphine, in the range of 10-50 mg/kg in Swiss-Webster mice, occurred at 72 hours. The mean time course of tolerance development for 10 separate groups to a PD of 20 mg/kg of morphine is shown in Figure 1.6. Measurement of AA after a challenge dose of 20 mg/kg of morphine indicated that development of tolerance to morphine as measured by the percentage of decrease in AA, was significant 3 to 4 days after the PD and that nearly complete recovery occurred after one week.

1.3.3b Time course of B-endorphin tolerance.

Measurement of the AA after the challenge dose of 0.6 or 2.0 ug of B-EN /mouse at various intervals after the priming dose of B-EN at time zero, indicated the development of tolerance to the antinociceptive effect of B-EN. Single-dose tolerance reached its maximal effect 12 hours after a priming dose of 0.6 ug B-EN. In this case, the AA of 0.6 ug B-EN was reduced to more than 60% of the initial effect of B-EN

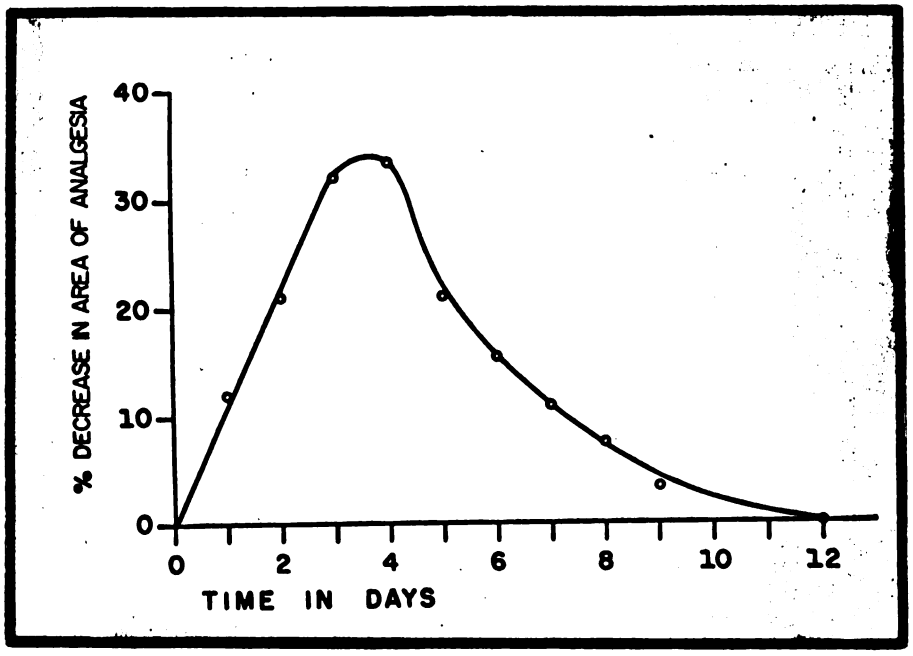

Figure 1.6

Time course of single-dose tolerance to morphine in Swiss-Webster mice.

A priming dose of 20 mg/kg i.p. was injected in 10 groups of mice. In one of the groups, the AA was determined for this dose. On each subsequent day, the analgetic response to the same dose of morphine was determined in a group selected at random. The decrease in the AA was estimated as described in Material and Methods. A decrease of 20% or more in the AA was significant with a P value less than 0.05.

(Figure 1.7a). Tolerance was completely lost 48-96 hours after the priming dose, when the AA of the priming dose of B-EN was nearly completely recovered. Its is evident that tolerance was maximal at about 12 hours after the priming dose, and was lost within 2-3 days. An almost identical time course as described for a priming dose of 0.6 ug B-EN was found with a priming dose of 2.0 ug B-EN (data not shown). Single-dose tolerance followed a linear increase up to 12 hours, where it reached maximal expression, and decayed completely by 48-72 hours. Maximal tolerance showed a decrease in the AA of approximately 50%, with a P value less than 0.01.

1.3.4 Effect of dose and time on the development of chronic tolerance. The rate of tolerance development seemed to be faster during the earlier hours of the exposure to a morphine pellet. During the first 6, 12 and 24 hours of pellet implantation, the morphine AD50 increased 1.76, 2.61 and 3.8 times respectively as compared to placebo controls (Figure 1.8). Morphine dose effect curves were displaced to the right in a parallel fashion. After 72 hours of pellet implantation, a 7.8-fold increase in tolerance was evidenced by the increase in the morphine AD50 (Fig. 1.9). Interestingly, after only 24 hours of morphine exposure but allowing a period of 48 hours before the AD50 determination, 50% of the tolerance was built up. Similarly, the data shows that after 12 hours of pellet implantation, 33.76% of the total tolerance had developed. It is evident that the rate of tolerance development does not remain linear, rather it tends to plateau. This effect is illustrated in Figure 1.8. Data indicates that the

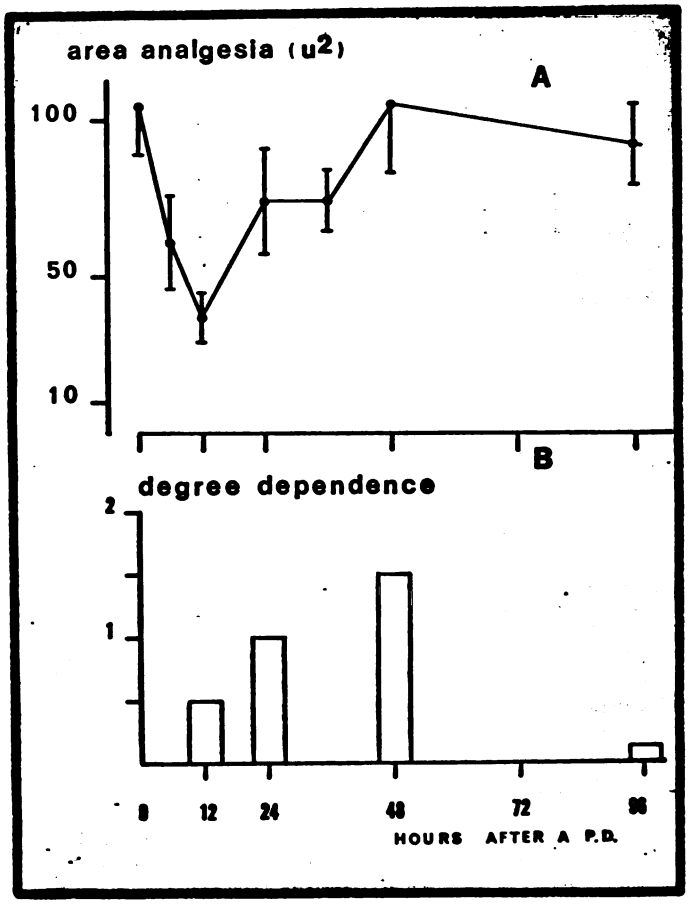

Figure 1.7

Time course of single-dose tolerance to and physical dependence on B-Endorphin in mice.

A priming dose of 0.6 ug/mouse (7.2 nMol/kg) hB-EN was injected icv in 7 groups at time zero. In one of the groups, the area of analgesia (AA) immediately determined for this dose. At different time intervals following the priming dose, the AA to a challenge injection of hB-EN was determined in a group of primed mice selected at random.

A. The AA obtained for each injection was plotted versus the time after the priming dose. Circles represent the mean AA (12 mice each), bars denote the S.E.M.

B. Development of dependence. Four hours after the challenge dose of hB-EN, Mice were injected with 10 mg/kg naloxone s.c., and observed for morphine-like withdrawal signs. Abscissa: intensity score of dependence vs. time interval between the priming and challenge dose of hB-EN.

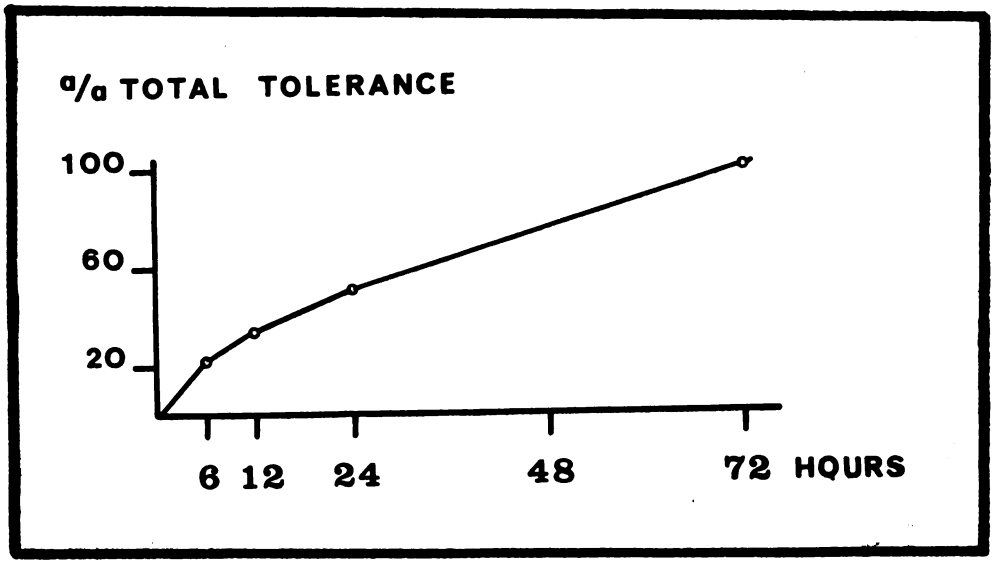

Figure 1.8

Rate of tolerance development to morphine after morphine pellet implantation for varying durations.

A 75 mg morphine pellet was implanted in different groups of mice for 6, 12, 24 and 72 hours. After removal of the pellets, the morphine AD50 was determined respectively in each group after 66, 60, 48 or 6 hours. A uniform period of 72 hours was allowed between pellet implantation and the morphine AD50 determination in all groups. The potency ratio relative to the group of mice implanted for 72 hours was calculated. The results are expressed as % of tolerance developed compared to the group implanted with morphine for 72 hours.

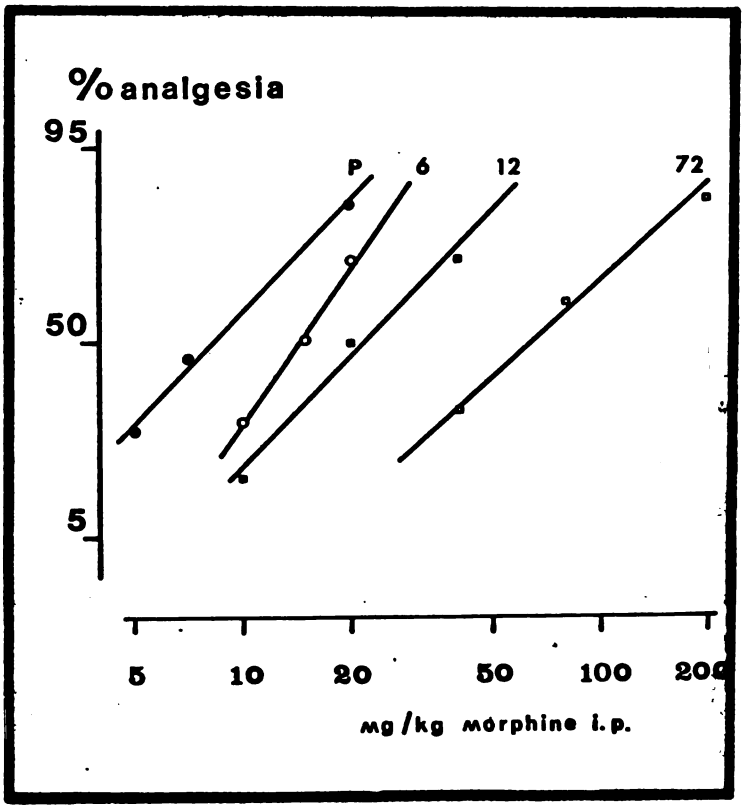

Figure 1.9

Dose effect curves of morphine obtained after implantation of a morphine pellet for varying durations.

Conditions of this experiment are described in the legend of figure 1.8. Mice were implanted with a morphine pellet for 6, 12 and 72 hours. P represents placebo treated mice.

degree of tolerance development depends on its rate of induction and degradation, and that these two variables are dynamically related. When the data of this experiment is compared to that of Way et al. (1969), in which the morphine pellet was implanted for 12-24 hours and the morphine AD50 was determined immediately, results are very different. Previous data of Way et al. (1969) indicated that after 24 hours of implantation the morphine AD50 was nearly doubled, while present results evidence an almost 4-fold increase in the AD50. These results can be interpreted to imply the importance of a lag time in the full development of tolerance, as was shown after a single-dose of morphine.

1.3.5 Development of dependence after single doses of morphine or B-endorphin.

1.3.5a Morphine.

Single doses of morphine produced only minor physical dependence in ICR mice as measured by antagonist precipitated withdrawal. On the first, third or fourth day after a PD of morphine either 15 or 60 mg/kg, naloxone did not elicit the jumping response. The doses of naloxone were as high as 100 mg/kg s.c. and produced mild convulsive seizures in mice. However, with a PD of 200 mg/kg of morphine, weak withdrawal similar to that described by Huidobro and Maggiolo (1961), was evoked 5-20 minutes after 30 or 100 mg/kg naloxone. This behavior consisted of sniffing and exploration, accompanied by hyper excitability and jumping in some cases. No jumps were observed in control saline-pretreated mice. With PD's of 15-

50 mg/kg of morphine in Swiss-Webster mice and a dose of 10 of 10 mg/kg of nalorphine i.p. a weak abstinence syndrome was preciopitated after 10-40 minutes. An additional series of experiments were carried out in which a second dose of morphine, equal to the PD, was injected 30 minutes before the dose of naloxone on the first, third or fourth day after the morphine PD. Recently, Brase et al., 1976 have shown that in Simonsen Swiss-Webster mice rendered dependent by morphine pellet implantation, such a dose of morphine could sensitize the animals to naloxone and result in precipitated withdrawal jumping even after the animals had been withdrawn from morphine for several weeks. However, in the present series of experiments no enhancement in the jumping response could be elicited. Likewise, no modification in jumping behavior was demonstrable in animals pretreated with 5,6-DHT, L-tryptophan or 6-OHDA for 1, 3 or 4 days and primed subsequently with 60 mg/kg of morphine; negative results were observed also after a second dose of morphine.

1.3.5b Effect of a single dose of B-endorphin on development of dependence in mice and rats.

Single doses of B-EN produced minor physical dependence as measured by antagonist precipitated withdrawal. The administration of 10 mg/kg naloxone i.p. to mice primed with either 1 or 2 icv injections of B-EN, produced a behavioral syndrome characterized mainly by a stereotyped rubbing of the nose with the front paw, wet shakes, teeth chattering, and a curious walking posture; jumping behavior was rarely observed. Almost

none of these signs were observed in the saline-control group. Single-dose dependence although minor was consistently reproduced. It was observed to be maximal at about 48 hours after priming doses of 0.6 or 2.0 ug B-EN icv, and largely over after 96 hours. The time course of dependence was almost identical at both priming doses of B-EN studied. Figure 1.7b shows the temporal course of dependence after a priming dose of 0.6 μg B-EN. It is of interest that the peak of precipitated dependence was observed at a time when tolerance was minimal.

In rats, the same animals used to study development of tolerance were injected with 10 mg/kg naloxone 4 hours after the second dose of B-EN. The animals that received a single dose of B-EN exhibited a 1.5 score, while those receiving two doses of B-EN had a 1.0 score. The saline treated group had a 0 score. These results confirm those obtained in mice, evidencing that some degree of dependence to B-EN developed concurrently with tolerance.

In summary, the results indicate that concurrent to the development of single-dose tolerance to the antinociceptive effects of morphine or B-EN, some degree of physical dependence also develops. The degree of dependence, as evidenced by the behavioral signs precipitated by naloxone challenge are mild, but distinguishable. Thus, the degree of tolerance was comparable to the degree of dependence.

1.3.6 Single-dose cross-tolerance.

1.3.6a Morphine and methadone.

Swiss-Webster mice injected with a PD of 15 mg/kg of morphine 72 hours previously, became tolerant to both morphine and methadone. Figure 1.10a shows that the dose-response curve for the analgesic effect of morphine was shifted to the right by morphine pretreatment when compared to saline controls. Figure 1.10b indicates that the dose-response curve to metha done was also shifted to the right, indicating cross-tolerance between morphine and methadone.

Cross-tolerance between methadone and morphine was also demonstrated by methadone pretreatment. These experiments were performed 48 hours rather than 72 hours after a priming dose of methadone since it was established in preliminary tests that tolerance was maximal 48 hours after a PD of 15 mg/kg of methadone. Pretreatment with a single dose of methadone decreased the antinociceptive response to methadone. The AA of 15 mg/kg of methadone that was 121.2 cm2 at day 0, decreased to 89.5 cm2 on day 1, 56.7 cm2 on day 2 and was 64.4 cm2 on the 3rd day after the PD 15 mg/kg of methadone. In addition, a PD of 15 mg/kg of methadone shifted the dose-response curve of morphine to the right (Figure 1.10c). Thus, the degree of shift of the dose-response curve produced by a single dose of either morphine or methadone for itself or its surrogate was approximately the same, suggesting that the degree of cross-tolerance induced by each agent was similar in magnitude.

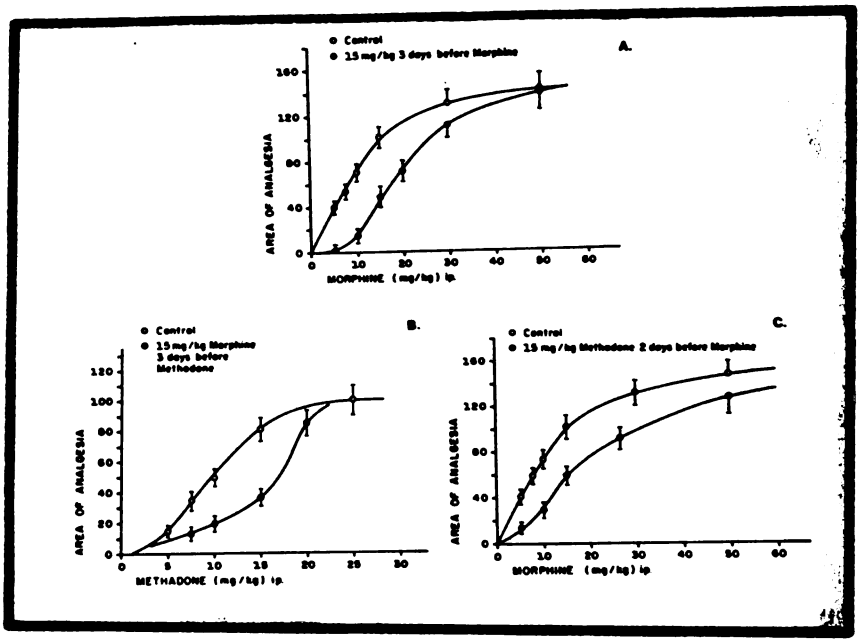

Figure 1.10

Demonstration of cross-tolerance between morphine and methadone.

Swiss-Webster mice were given a priming dose of 15 mg/kg i.p. of either morphine or methadone. After three days, the area of analgesia (AA) was determined after challenge with various doses of morphine and methadone (●). The control groups were pretreated with saline and are represented by the open circles (o).

A. Single-dose tolerance to morphine antinociception.

B. Single-dose cross-tolerance between morphine and methadone.

C. Single-dose cross-tolerance between methadone and morphine.

The AA obtained 48 hours after after the priming dose in the saline treated group had a P value of less than 0.05 as compared to the effect of the priming dose.

1.3.6b B-endorphin and morphine.

A priming dose of B-EN, that reduced the effect of a second dose of B-EN, produced an equivocal decrease in the effect of morphine. Interestingly, priming with an equianalgetic dose of morphine also reduced the effect of B-EN but not that of a second dose of morphine (Tables I-3 and I-4). As indicated by group D in Table I-3, 1 ug of morphine icv decreased the antinociception of 600 ng of B-EN by 48%. In the reverse experiment, pretreatment with B-EN only reduced the effect of morphine by 27% and this was not at a level of statistical significance. However, the results in the second set of experiments suggested that this difference may be quantitative rather than qualitative and was likely a matter of selection of the dose of morphine. When mice were primed with 0.6 ug of B-EN and challenged six hours later with 10 and 20 mg/kg of morphine intraperitoneally significant tolerance to the lower but not to higher dose of morphine could be demonstrated (Figure 1.11). The third group of mice given 5 mg/kg of morphine did not produce enough increase in the AA for making comparisons.

1.3.7 Effect of opiate antagonists on the development of single-dose tolerance.

1.3.7a Effect of nalorphine on morphine tolerance.

Simultaneous pretreatment of Swiss-Webster mice with morphine and nalorphine reduced the development of single-dose tolerance to morphine. In the saline pretreated control group a challenge dose of 10 mg/kg morphine 48 hours after the PD evidenced a significant 35% reduction of the AA, indicating the

Table I-3

Development of acute cross-tolerance between morphine and B-Endorphin.

Group	Pretreatment[a]	Challenge	n	Area of Analgesia $\bar{x} \pm$ SEM (U^2)
A	Saline	Morphine	24	106.85 ± 11.30
B	Saline	B-EN	24	99.98 ± 13.56
C	B-EN	Morphine	12	88.84 ± 11.20
D	Morphine	B-EN	12	56.88 ± 9.09*

The dose of morphine was 1 ug/mouse; B-EN 0.6 ug/mouse.

[a] Pretreatment time was 5 hours prior to challenge.

*P less than 0.01 as compared to the saline pretreated control.

Table I-4

Antinociceptive response to morphine and B-Endorphin after single and two successive injections within a 5-hour interval.

Pretreatment[a]		Challenge	n	Area of Analgesia $\bar{x} \pm$ SEM (U^2)
-		Morphine	12	70.70 ± 13.75
Morphine		Morphine	12	71.83 ± 13.31
-	B-EN	Sample #1	12	145.0 ± 21.26
B-EN	B-EN	Sample #1	12	62.73 ± 13.15*
-	B-EN	Sample #2	12	39.27 ± 10.13
B-EN	B-EN	Sample #2	12	6.81 ± 4.47*

The dose of morphine was 1 ug/mouse; B-EN 0.6 ug/mouse icv.

[a] Pretreatment 5 hours before challenge.

*P less than 0.01 as compared to its corresponding control.

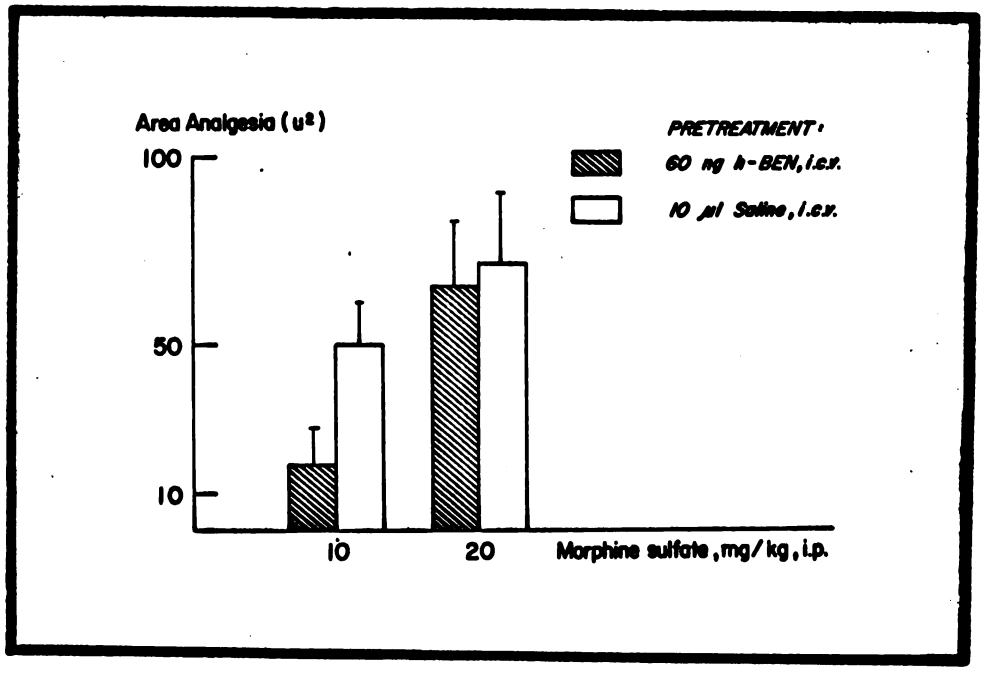

Figure 1.11

Single-dose tolerance between B-Endorphin and morphine.

To ascertain the development of cross-tolerance between these two opiates, 3 groups of 8 mice each were pretreated with 7.2 nMol B-EN/kg, and 3 groups with 10 ul saline icv. Six hours later, animals were challenged with morphine sulfate (5-10-20 mg/kg i.p.), and the area of analgesia was determined. Results after 5 mg/kg of morphine are not shown because no significant antinociception was obtained.

development of tolerance. In contrast, the group treated with 10 mg/kg nalorphine in conjunction to the PD of morphine, evidenced only a 10.8% reduction in the AA produced by the challenge dose of morphine. The AA value obtained was not significantly different from the AA produced by the PD of morphine in the saline pretreated group (Table I-5). Nalorphine also blocked the antinociceptive effect of the PD of morphine evidencing its partial agonist effects (Table I-5).

1.3.7b Effect of naloxone on B-endorphin tolerance.

Naloxone blocked the development of single-dose tolerance to the antinociceptive effect of B-EN in a dose dependent fashion. Naloxone at a dose of 1 mg/kg blocked significantly the antinociception produced by B-EN, and reduced the development of tolerance to a priming dose of B-EN. The AA produced by the challenge dose of 600 ng hB-EN in the B-EN-saline pretreated group was significantly lower than the AA produced in the saline-saline pretreated group, establishing the development of single-dose tolerance (the AA's were 63.6 $\pm$ 9.0 U2 vs. 113.1 $\pm$ 9.4 U2 respectively). However, when animals were primed with B-EN + naloxone, and challenged later with B-EN the AA was 100.8$\pm$20.2 U2. This was significant (P less 0.05) when compared to the B-EN + saline pretreated control group. Increasing the dosage of naloxone to 4 mg/kg administered as 2 doses of 2 mg/kg, spaced an hour apart, showed unequivocal results in that tolerance was almost completely antagonized by the naloxone pretreatment. As can be observed in Figure 1.12, the B-EN-naloxone primed group

Table I-5

Effect of nalorphine on single-dose tolerance to morphine in Swiss-Webster mice.

	$\bar{X}$ AA ± SEM (U^2) 10 mg/kg morphine i.p.		
Pretreatment	Time 0	48 Hours	% Reduction
Saline	137.0 ± 7.0	89.00 ± 5.0	35.0[a]
Nalorphine, 10 mg/kg	86.1 ± 5.3[b]	122.25 ± 6.0	10.7

[a] P less than 0.01 as compared to the AA at time 0.

[b] P less than 0.01 as compared to the control, saline pretreated group.

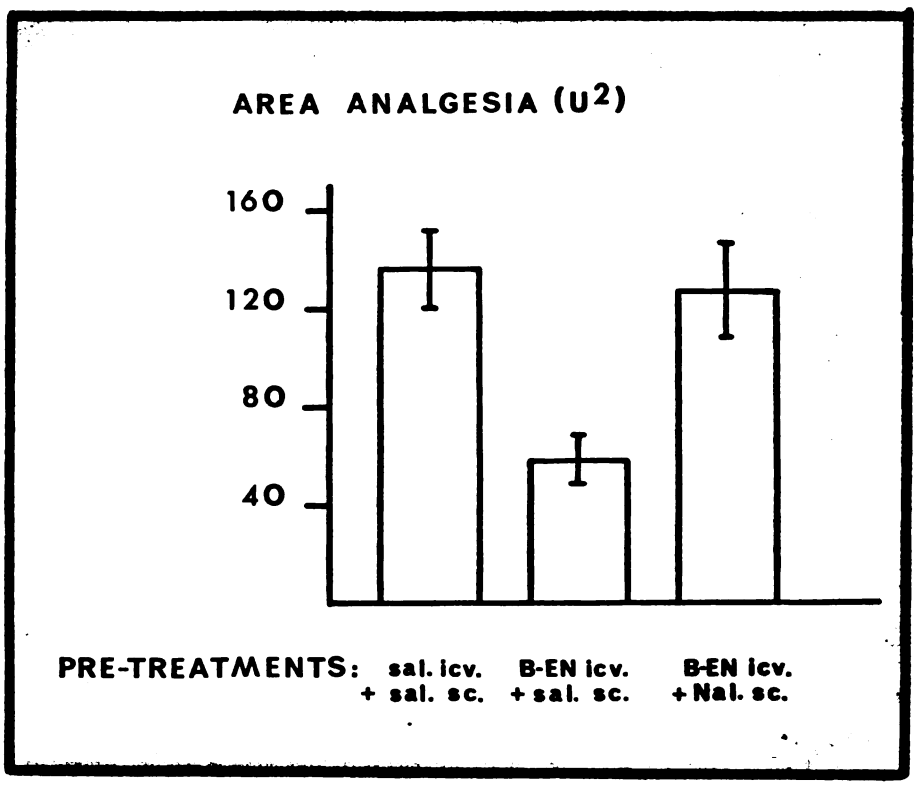

Figure 1.12

Effect of naloxone on the development of single-dose tolerance to B-Endorphin.

Three groups of mice were employed. The first group was pretreated with 10 ul saline icv, and 10 ml/kg saline s.c. The second group was primed with 0.6ug hB-ENplus saline s.c.; the third group received 2 mg/kg naloxone instead of saline in conjunction with hB-EN. An hour after, the second group was re-injected with saline s.c., and the third group with a second dose of naloxone. Twelve hours later, all three groups were administered with a challenge dose of 0.6ug hB-EN and the area of analgesia was determined. Each group was composed of 12 mice except for the first which had 11 animals. Bars represent the S.E.M.

reacted to the challenge dose of hB-EN with an AA almost identical to that obtained in the saline-saline pretreated group. A control standard AA of 600 ng hB-EN icv in this particular shipment of mice was 105.0 ± 13.8 U2. Thus, these results illustrate that naloxone can block the development of single-dose tolerance by hB-EN.

1.3.8 Effect of drugs that inhibit DNA replication or protein synthesis on single-dose tolerance.

1.3.8a Effect of cycloheximide on morphine tolerance.

Cycloheximide completely blocked the development of single-dose tolerance to morphine in both Swiss-Webster and ICR mice. As can be seen in Table I-6, 72 hours after a PD of 15 mg/kg of morphine in Swiss-Webster mice, the AA after morphine decreased about 50% relative to the AA of animals primed with saline. However, in animals given cycloheximide 30 minutes before the PD of morphine, the AA was nearly identical to that of untreated primed animals challenged with morphine. Similarly, in ICR mice, cycloheximide prevented the develop ment of single-dose tolerance. In mice primed with 60 mg/kg of morphine for 3 days, evidence of tolerance indicated by a two-fold increase in the morphine AD50, whereas in the group pretreated with cycloheximide, the AD50 was nearly identical with the group which did not receive a PD of morphine (Table I-6).

1.3.8b Effect of actinomyin D and cycloheximide on B-endorphin tolerance.

Table I-6

Effect of cycloheximide on tolerance development to a single priming dose of morphine.

Pretreatment	Morphine Dose(s) mg/kg i.p. [1] Priming	[2] Challenge	Analgesic Effect [3] AA cm^2
Swiss-Webster Mice			
None	0	15	87.2 + 11
Saline	15	15	37.9 + 6.0
Cycloheximide	15	15	93.2 + 12.4*
ICR Mice			[4] AD50 mg/kg
None	0	5, 10, 20	12.0 (7.6 - 19.0)
Saline	60	10, 20, 40	24.5 (19.1 - 31.4)
Cycloheximide	60	5, 10, 20	12.5 (9.1 - 17.2)*

1 - 30 minutes before PD.

2 - 72 hours after PD.

3 - AA = area of analgesia $\pm$ SEM.

4 - AD50 median analgetic dose and 95% confidence limits.

*P less than 0.05 in relation to saline.

Both actinomycin D and cycloheximide inhibited the development of single-dose tolerance to B-EN. In mice pretreated with these inhibitors of DNA and protein synthesis 30 minutes before the priming dose of hB-EN, the AA produced by the second injection of B-EN was significantly larger than that of the saline pretreated control group. These results (Table I-7) suggest that protein synthesis is involved in the development of single-dose tolerance to B-EN, and are consistent with those reported earlier by (Loh et al., 1969). The treatment did not affect the acute response to B-EN since cycloheximide an hour before 600 ng hB-EN did not alter the AA of the peptide.

1.3.8c Effect of cycloheximide on morphine methadone cross-tolerance.

Cycloheximide blocked the development of cross-tolerance induced by a single dose of either morphine or methadone. As illustrated in Table I-8, 72 hours after a PD of morphine, the AA of methadone was reduced about 60% when compared to control animals primed with saline. In animals pretreated with cycloheximide, however, the AA after methadone was the same as that for the group which was not primed with morphine. Similarly, 48 hours after a PD of methadone, the AA for morphine was reduced about 35% but was unchanged in mice that received cycloheximide 30 minutes before methadone.

1.3.9 Effect of various agents on the development of single-dose tolerance.

The results with 6-OHDA, cAMP, L-tryptophan and 5,6 -DHT on development of single-dose tolerance to morphine

Table I-7

Effect of protein synthesis inhibitors on single-dose tolerance to B-Endorphin.

Pretreatment (12 hours before testing)	AA of 600 ng B-EN icv/mouse $\bar{x} \pm$ SEM U^2
600 ng B-EN + Actinomycin D[a]	216.64 ± 41.55* (7)
600 ng B-EN + Cycloheximide[b]	142.85 ± 22.94* (10)
600 ng B-EN + Saline	92.76 ± 13.85 (12)

a
Actinomycin D (0.35 mg/kg i.p. 30 minutes before the priming dose of B-EN icv).

b
30 mg/kg Cycloheximide i.p. 30 minutes before the priming dose of B-EN icv.

*P less than 0.05 as compared to the B-EN primed by saline pretreated group.

Table I-8

Effect of cycloheximide on single-dose cross tolerance development to morphine and to methadone in Swiss-Webster mice.

	Agent		
1	2	3	Antinociceptive Effect
Pretest treatment	Priming	Test	AA + SEM (cm^2)
Experiment I			
a. None	Saline	Morphine	80.45 + 11.02
b. None	Morphine	Morphine	42.27 + 5.97
c. Saline i.p.	Morphine	Methadone	37.30 + 4.82
d. Cycloheximide 30 mg/kg i.p.	Morphine	Methadone	81.50 + 10.03*
Experiment II			
a. None	Saline	Methadone	81.69 + 10.83
b. None	Methadone	Methadone	35.83 + 5.03
c. Saline i.p.	Methadone	Morphine	59.07 + 5.81
d. Cycloheximide 30 mg/kg i.p.	Methadone	Morphine	82.66 + 9.23*

1 - 30 minutes before priming agent.

2 - Priming doses (PD) of morphine and methadone were 15 mg/kg i.p.

3 - Test response to 15 mg/kg i.p. 72 hours after PD of morphine and 48 hours after PD of methadone.

*Significantly different (P less than 0.05) in relation to 2 or 3.

are summarized in Table I-9.

1.3.9a 6-OHDA did not alter tolerance development to morphine although the treatment modified the antinociceptive effect of morphine. As shown in Table I-9, 40 hours after the administration of 6-OHDA, naive mice showed a decreased response to the antinociceptive effect of morphine as evidenced by a significant, almost 2-fold, increase in the AD50 when compared to vehicle-pretreated animals. After the PD of morphine, the AD50 of both 6-OHDA and vehicle-pretreated animals showed an increase in the AD50 of morphine. However, the relative change effected by 6-OHDA in the primed and non-primed group was about the same since the ratio of the morphine AD50 of vehicle and 6-OHDA-treated animals was not altered significantly. These findings are in agreement with previous results from this laboratory (Friedler et al., 1972), showing that 6-OHDA decreased morphine antinociception but did not alter the development of tolerance to morphine.

1.3.9b cAMP produced an equivocal acceleration in tolerance development after a single dose of 15 or 60 mg/kg of morphine. As noted in Table I-9 and Figure 1.13, the morphine AD50 of mice pretreated with saline and given a PD of morphine was increased slightly more than 2-fold and in the cAMP group more than 3-fold; however, the greater ratio obtained with cAMP was not at a level of significance.

1.3.9c L-tryptophan produced an equivocal enhancement of single-dose tolerance. As shown in Table I-9 saline-pretreated mice

Table I-9

Effect of pretreatment with various substances on the development of single-dose morphine tolerance.

	Morphine AD50 + 95% C.L.		
	Pre PD[1]	Post PD	After/Before ± 95% C.L.
	mg/kg		
Vehicle	4.5(3.02 - 6.70)	8.0(5.17 - 10.5)	1.77(1.02 - 3.04)
6-OHDA[2]	8.4(5.21 - 13.5)	18.0(12.3 - 26.2)*	2.14(1.17 - 3.89)
Saline	6.5(5.0 - 8.5)	14.0(8.9 - 25.2)	2.15(1.20 - 3.82)
cAMP[3]	5.0(2.8 - 9.0)	16.5(11.0 - 25.0)	3.3(1.61 - 6.73)
Saline	4.9(2.9 - 8.1)	10.5(6.40 - 17.2)	2.18(1.05 - 4.49)
Tryptophan[4]	5.2(3.4 - 8.7)	15.0(10.3 - 21.7)	2.88(1.4 - 5.60)
Vehicle	6.0(4.25 - 8.46)	21.0(16.1 - 27.3)	3.5(2.27 - 5.39)
5,6 DHT[5]	6.2(3.87 - 9.92)	9.2(6.47 - 13.0)	1.48(0.82 - 2.66) ns

1 - PD - priming dose of morphine, 60 mg/kg i.p., administered 72 hours before test.

2 - 28 µg 6-OHDA per mouse intraventricularly 40 hours before PD.

3 - 10 mg/kg cAMP i.v. 2 hours before PD.

4 - 75 mg/kg L-tryptophan i.p. daily for 3 days after PD.

5 - 60 µg 5,6 DHT per mouse intraventricularly 24 hours before PD.

*Potency ration with P less than 0.05 when compared to vehicle treated mice.

ns - no statistical significance in relation to vehicle pretreated animals.

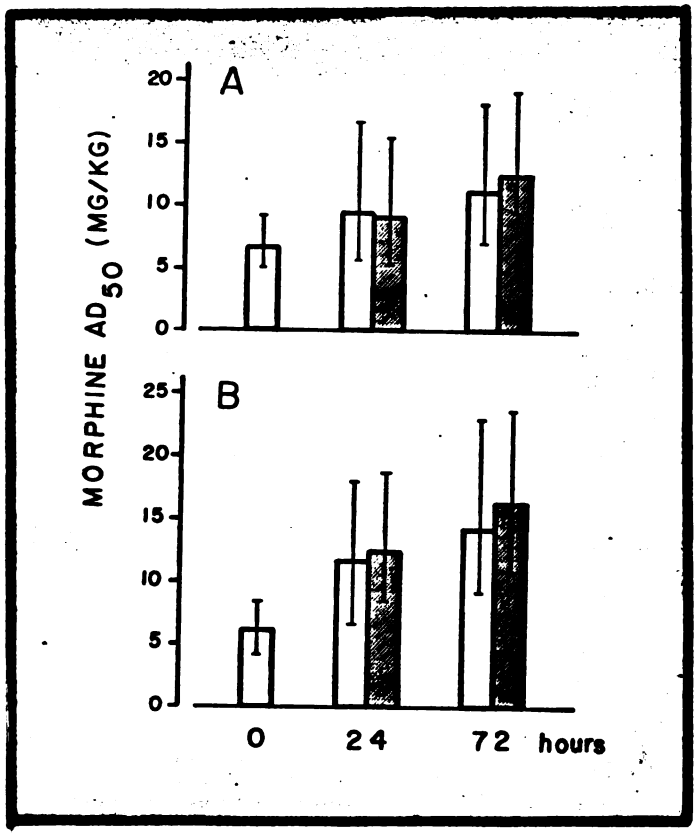

Figure 1.13

Effect of adenosine-3'5'-cylic monophosphate (cAMP) on the development of single-dose tolerance to morphine.

Groups of mice were pretreated with 10 mg/kg cAMP into the tail vein 2 hours prior to the priming dose of 15 mg/kg morphine (A), or 60 mg/kg morphine (B). Subgroups of mice from each pretreatment regimen was used at 24 or 72 hours to determine the morphine AD50. Control animals were injected with sterile saline i.v.. cAMP treated animals are represented by the shaded columns, saline by the open columns. Bars denote the 95% confidence limits.

receiving a PD of morphine showed the usual 2-fold increase in the morphine AD50. L-tryptophan treatment for three days in conjunction with the priming dose of morphine, increased the AD50 nearly 3-fold. However, this degree of increase was not significantly different from the saline groups which exhibited a 2-fold increase to the PD of morphine.

1.3.9d 5,6-DHT pretreatment inhibited the development of single-dose tolerance to morphine. Under conditions in which the morphine AD50 was not affected by 5,6-DHT, it prevented the increase in morphine AD50 after a PD of morphine. As shown in Table I-9, the increase in morphine AD50 after a PD of morphine was reduced significantly by 5,6-DHT. This finding is in agreement with previous work from this laboratory (Ho et al., 1973a) in relation to tolerance development after pellet implantation.

1.3.10 Brain uptake of morphine in acute tolerance.

Single-dose tolerance did not alter the distribution of morphine in the brain. Thirty minutes after the administration of 40 mg/kg of morphine i.p. the mean brain levels of morphine of mice previously rendered tolerant by a priming dose of 60 mg/kg of morphine three days earlier were almost the same as saline controls (0.476 ± 0.044 vs 0.467 ± 0.047 ug/g). It was established in other experiments that no morphine was detectable 72 hours after the PD of morphine.

1.4 DISCUSSION.

B-EN injected icv to mice produces a prompt and long lasting antinociception. The AD50 for the analgesic effect, as assayed by the tail-flick method, is in complete agreement to the data presented by Loh et al. (1976), Li et al. (1977), and Tseng et al. (1977). These results confirm and establish that B-EN on a molar bases is about 25-fold more potent than morphine injected icv. No difference was observed between the camel and human B-EN in terms of antinociceptive or behavioral effects, supporting previous data of Li et al. (1977). In contrast to the transient antinociception produced by enkephalins in rodents, B-EN produced a profound analgesia that lasted, depending on the dose, for 4-5 hours. The antinociception induced by B-EN in rodents was antagonized by naloxone (Loh et al., 1976). The important implication is that both substances probably interact with a same or a common pharmacological receptor, and thus provide evidence that this in-vivo effect of B-EN is a true opiate response.

Further evidence that B-EN has opiate properties, can be derived from data indicating that B-EN produces tolerance and dependence. Present results show that in mice, a single dose of B-EN induces within a few hours a state of decreased antinociceptive response to a second injection of B-EN. Moreover, tolerance is associated with some degree of physical dependence. Results with respect to acute tolerance development confirm similar observations done in cats by Meglio et al. (1977) and Hosobuchi et al. (1977). There is, however, some controversy about the of tolerance to B-EN in rats. Tseng et al.,(1976a, 1976b, 1977), and Wei and Loh (1976) reported

that tolerance occurs rapidly after daily injections or icv infusion of B-EN, while Bloom et al. (1976) did not observe tolerance to behavioral effects of B-EN, even after 7 daily injections. The present data support the affirmative findings.

The results indicate that significant tolerance to the antinociceptive effect of morphine can be produced in mice by a single optimum dose of opiates. Tolerance development was established using two different methods of evaluation and two strains of mice to demonstrate a diminution in analgetic response to a challenge dose of opiate alkaloids or peptides after the initial priming dose. In general, it can be said that the tolerance-dependence developed showed about the same characteristics in both strains of mice and that it was crossed to other narcotic agonists. The difference in dose to produce single-dose tolerance may be attributed to a difference in sensitivity of the strains to morphine, to the method employed for antinociception or a combination of both. Cross-tolerance between morphine and methadone, methadone and morphine, and between B-EN and morphine were also observed. The time course of tolerance development to a single dose of morphine was about the same in both strains, being maximal three days after the PD and disappearing completely after 8-10 days. However, in the case of B-EN (at the 2 doses used) the time course was much shorter, suggesting that B-EN can induce significant tolerance earlier than the alkaloid type of opiates. Single-dose tolerance to morphine or B-EN was paralleled by the simultaneous development of dependence. The degree of dependence developed was comparable to the degree of tolerance achieved, both being

to a moderate degree. Present results confirm previous findings of acute morphine tolerance (Eddy, 1953; Jacob and Berthelemy, 1972; Miller and Cochin, 1968; Huidobro, 1971) and define in greater detail the dose-effect relationships for producing single-dose tolerance in mice.

Single-dose tolerance to morphine in the rat has been reported but the results appear to be contradictory. Although tolerance can be manifested within several hours after a PD of morphine, as in the case of the hypothermic effect (Lotti et al., 1966) and the antinociceptive effect (Ferguson et al., 1969; Kayan et al., 1969; Huidobro and Huidobro, 1973), other reports indicate that an interval of days is necessary to develop tolerance (Cochin and Kornetsky, 1964; Kornestsky and Bain, 1968; Feinberg and Cochin, 1972). The present results show that single-dose tolerance was also observed to B-EN in rats, as early as six hours after the PD.

The type of tolerance manifested early in rats may be due to a different mechanism than that involved in the longer-lasting tolerance which has been reported to persist for weeks and even months after the first dose (Cochin and Kornetsky, 1964; Ferguson et al., 1969). Cochin (1970) suggested the possible involvement of an immunological mechanism for the protracted type of tolerance.

Several pharmacological considerations suggest that the phenomenon of single-dose tolerance to opiates is a true morphine-like effect, similar to that observed after chronic narcotic administration. First of all, tolerance development was blocked or prevented by the

simultaneous administration of an antagonist in conjunction with the agonists. Tolerance development was accompanied by some mild degree of dependence. Single-dose tolerance was dependent on the dose of the opiate administered, and required a lag time to reach full expression. Single-dose tolerance was crossed between the different opiates studied. Furthermore, single-dose tolerance is is probably associated with protein synthesis or DNA expression, since a blocker of protein synthesis or DNA replication ameliorated the development of tolerance. All these pharmacological properties are common to the development of tolerance and dependence following the chronic administration of narcotic drugs (Way, 1972). Some of these points are now discussed in detail.

Opiate antagonists, nalorphine and naloxone, not only antagonized the antinociception produced by the opioids (alkaloid-type or the peptides respectively, as used in this study) but blocked the development of tolerance. It is not surprising that opiate antagonists blocked the development of single-dose tolerance, since it is known that naloxone injections or a naloxone pellet implanted in conjunction to a morphine pellet in mice abolished the development of tolerance and dependence to morphine in mice (Shen et al., 1970; Lee et al., 1975). Smits (1976) demonstrated in mice that single-dose tolerance-dependence was reduced in a dose-dependent fashion by co-administration of naloxone. Similarly in rats, single-dose tolerance development was decreased by the simultaneous administration of morphine in conjunction to naloxone (Mushlin and Cochin, 1976; Feinberg and Cochin, 1977). We would like to interpret this information as having two fundamental consequences. First of all, it implies that

the development of tolerance is related to the activation of an opiate-naloxone sensitive receptor mechanism. It would then be expected that tolerance were blocked by naloxone in a dose-dependent fashion. Present data support this conclusion. Furthermore, the kinetic data presented for the development of single-dose tolerance to morphine or B-EN demonstrates a dose effect curve for tolerance development, and a specific time course of its effect. Second, if the development of tolerance needs the activation of an opiate-sensitive receptor, then most probably tolerance cannot be explained quantitative or qualitative by a defective "opiate receptor" system. In support of this hypothesis, Pert et al. (1974), Hitzemann et al. (1974) Klee and Streaty (1974) , have demonstrated that there is no change in the brain stereospecific opiate binding in tolerant rodents when compared to that of that of naive animals. Present results consistently show that the maximal antinociceptive response of the second-challenge-dose of B-EN is not significantly decreased, while the time course of the effect reflected quantitatively in the AA, is significantly decreased. Similar results are presented in Part 2 of this study, to the hyperthermic response of B-EN in mice. It was consistently observed that single-dose tolerance occurred to the hyperthermic response without significantly altering the maximal response of B-EN.

This argument would indicate that the development of tolerance probably occurs at a site past the receptor system, somewhere along the chain of physiological events that mediate the pharmacological response. This conclusion is of primary importance since it could help to explain the fact that opiate tolerance is a

local phenomenon that develops to almost all opiate effects, although at varying rates. This might be related to the fact that the site of tolerance need not necessarily be the same for each opiate effect, or else equal rates of tolerance development would be expected. In this connection, Part 2 of this study presents evidence indicating that single-dose tolerance to the hyperthermic effect of B-EN occurs at a lower dose level and with a different time course than that of B-EN antinociception in mice.

From a kinetic point of view, two variables are of fundamental importance in the development of tolerance: the dose of the opiate and a lag time required to express fully its development. Obviously, the net tolerance developed depends on two rates: the rate of induction, and its rate of degradation or loss. Tolerance as observed in-vivo is a dynamical equilibrium of these two rates. During the early stages of pellet implantation, the rate of induction of tolerance predominates over a minimal rate of tolerance loss. However, with the establishment of tolerance, the rate of loss becomes of importance and has to be balanced with the rate of induction. The characteristics of these two rates are at present obscure. Some experiments of the present study were aimed to gain better knowledge of this particular aspect of tolerance. The model of single-dose tolerance seemed especially appropriate to study the dose and time requirements of single-dose tolerance.

There is a threshold dose for producing single-dose tolerance and this appears to be 2-4 times greater than that for eliciting median antinociception. Doses over the threshold increase the degree of

tolerance up to a point where it reaches a plateau, suggesting saturation of the processes involved. Since tolerance develops to a considerably higher degree after repeated administration of morphine, it appears that continued maintenance of an optimal level produces cumulative effects on tolerance development. The success of the pellet implantation method (Maggiolo and Huidobro, 1961; Way et al., 1969) for producing a high degree of tolerance, therefore, is likely dependent on a constant release of morphine to active sites. In fact, after implantation of the Maggiolo-Huidobro pellet, although the level of morphine in the mouse brain is not excessively high as compared to single doses, it remains continually elevated for at least 72 hours after implantation (Patrick et al., 1975; Jeanne W. Shen and E. Leong Way, personal communication).

Single-dose tolerance needs a certain time to develop. The time course of tolerance to a single dose of morphine or B-EN are somewhat different; B-EN having a much shorter time course. The reasons for such a difference are obscure. It can possibly be related to the dose of the peptide or to its degradation by the brain. However, independent of the lag time, protein synthesis inhibitors block the development of tolerance to the opiates. This experimental fact adds support to the possibility that the lag time for tolerance development were due to the time needed to produce a specific protein (s). The development of tolerance following a single dose of opiates as well as after chronic morphine depends on the expression of DNA and protein synthesis. The evidence to support this conclusion is mainly derived from pharmacological studies on

tolerance to (Cohen et al., 1965; Cox et al., 1968; Way et al., 1968 Cox and Osman, 1970; Feinberg and Cochin, 1972; for a review see Smith, 1971) and dependence on morphine (Way et al., 1968).

In the present study, cycloheximide, a well-known inhibitor of translation, blocked the development of single-dose tolerance. In addition, cross-tolerance between morphine and methadone and between methadone and morphine were inhibited by cycloheximide. Similarly, cycloheximide or actinomycin-D blocked the development of single-dose tolerance to B-EN. The fact that cycloheximide blocked single-dose tolerance development argues in favor of the idea that narcotics can induce the synthesis of a macromolecule in the CNS which will reduce the response to morphine agonists. This molecule, is not the "opiate receptor", since cycloheximide did not alter the acute response of morphine or B-EN, in conditions under which it blocked the development of tolerance. B-EN was no exception to the finding that acute tolerance is dependent on de novo protein synthesis. This fact suggests that acute as well as chronic tolerance to opiates or endorphins could have a common biochemical mechanism. Interestingly, Lee and Loh (1976, 1977) have recently found that during chronic morphine treatment the template activity of oligodendroglial nuclei is increased. This phenomenon was shown to be an opiate selective effect. Furthermore, B-EN in-vitro enhanced the template activity of DNA. These experiments support and suggest a fundamental role of DNA expression on tolerance development to opiates and that the blockade of DNA functions during opioid administration impairs the development of tolerance and dependence.

To further examine the role of a lag time in the expression of morphine tolerance, the injection of morphine was substituted by the implantation of a morphine pellet. The tablet was implanted for 6-12 or 24 hours, at which time the pellet was removed, and a lag period of 66, 60 or 48 hours respectively was allowed before the determination of the morphine AD50 in each group. As a control, a group was implanted for 72 hours, but pellets were removed six hours before the determination of the morphine AD50. (This procedure allowed morphine brain levels to decrease to a similar extent as in the other groups employed.) After 72 hours of pellet implantation, the morphine AD50 was increased 7.8-fold. However, the group implanted with the morphine tablet for 24 hours and allowed 48 hours for induction of tolerance, had a 3.8-fold decrease in morphine sensitivity. This value shows that about 50% of the tolerance was built after only 24 hours of continual exposure to morphine. Similarly, after 12 hours of morphine implantation and allowing 60 hours for the expression of tolerance, 33.8% of the 72 hour tolerance could be accounted for. These AD50 values are significantly higher than those presented by Way et al. (1969), when the pellet was implanted for 12 or 24 hours and the morphine AD50 was determined 6-8 hours after pellet removal, without a lag period. In this particular case, little tolerance was detected after12 hours of morphine implantation, and a modest doubling of the morphine AD50 was observed after 24 hours of morphine (Way et al., 1969). These experiments considered as a whole add further proof that a lag period of time of at least 24-48 hours is needed for full development of morphine tolerance in mice. After longer periods of time, the rate of tolerance loss

can overcome that of induction, decreasing tolerance. Similar results supporting the importance of the lag time for tolerance production in rats has been recently documented by Mushlin et al. (1976).

Although the disposition of morphine is important for the initiation, maintenance and augmentation of tolerance, it is highly unlikely that single-dose tolerance can be explained in terms of drug disposition which generally involves enzyme induction at sites other than in CNS. First, there is little if any evidence to support that metabolism or excretion of morphine is enhanced materially in tolerance (Way and Adler, 1962). In the present study, an incubation period of 3 days was necessary for tolerance to become fully manifest and a single-dose of morphine is eliminated largely by 12 hours (Way and Adler, 1962). Similarly, Patrick et al. (1975) have recently shown that even 48 hours after pellet removal, when there was no detectable morphine brain levels, there was still a considerable level of tolerance. Secondly, cross-tolerance between morphine and its surrogates is well established and it would be difficult to explain tolerance in terms of the disposition of these drugs with diverse structures and potencies. Finally, brain uptake of morphine was not altered in animals which had developed single-dose tolerance. It would appear, therefore, that single-dose tolerance reflects an adaptive event occurring in the central nervous system.

The fact that single-dose tolerance needs a time to develop and that it might be linked to a saturable process, suggests that central

neuronal adaption occurs. However, the mechanism does not appear to be directly concerned with the receptor necessary for mediating the acute pharmacologic effects of morphine. The findings with 6-OHDA are certainly compatible with this contention. Pretreatment with intraventricular 6-OHDA which destroys central catecholaminergic nerve endings sites (Bloom et al., 1969) decreased the analgetic response to morphine but did not modify the development of single-dose tolerance. Based on such data, it is possible to conclude that while the catecholamines may be associated with morphine antinociception, do not appear to have a direct role in single-dose tolerance development. This view is consistent with and supported by other studies from this laboratory concerned with the assessment of the role of the catecholamines during chronic morphine treatment (Way and Shen, 1971; Friedler et al., 1972; Bhargava et al., 1973).

In contrast to the catecholamines, it has been shown that manipulations that increase the brain levels of 5-hydroxytryptamine such as L-tryptophan treatment, enhance tolerance and dependence (Ho et al., 1975), whereas drugs that decrease the levels of serotonin inhibit the development of tolerance and dependence (Way et al., 1968; Shen et al., 1970; Ho et al., 1972; 1973a). Consistent with these results, we found that destruction of serotoninergic nerve endings with 5,6-DHT (Baumgarten et al.,1972) pretreatment decreased single-dose tolerance development whereas L-tryptophan may have enhanced tolerance. Although the magnitude of the latter change was insufficient to establish statistical significance, the direction of the change was consistent with previous

findings (Ho et al., 1975). Cholinergic drugs were not studied since they seem to be mainly involved in the expression of the withdrawal effects of morphine (Way, 1972; Iwamoto et al., 1973; Bhargava and Way, 1974; Domino and Wilson, 1973).

The results obtained with cAMP on the development of single-dose tolerance to morphine were not inconsistent with earlier findings. Even though the effects obtained with cAMP were not at a level of significance, the direction of change was in agreement with the earlier findings (Ho et al., 1973b). It has been previously demonstrated that a single injection of cAMP shortly before the implantation of a morphine pellet augments (by at least 3-fold) the degree of tolerance that develops after three days. As with the results obtained with tryptophan, the low degree of tolerance produced by single-dose experiments may be inadequate for demonstrating the enhancing effect of cAMP.

Acute-cross tolerance was demonstrable between different opiates. It was evidenced by a parallel displacement of the dose effect curve of morphine or methadone to the right by the respective cross opiate. Tolerance to B-EN was crossed to morphine. It was not apparent using the dose of opiates selected probably because doses were not the adequate. However, pretreatment of mice with B-EN caused less response of morphine as evidenced by a diminished response to a median dose of morphine, but full agonist recovery occurred at a higher dose of the opiate. The inverse experiment evidenced a positive result since morphine pretreatment decreased the antinociception produced by B-EN. The finding of single-dose

cross-tolerance between B-EN and morphine and between morphine and methadone and vice versa after acute or chronic administration (Tseng et al., 1976a) can be interpreted to mean that morphine and B-EN have a similar site or mechanism of action.

Opiates might interact at a common central pathway although not necessarily need to activate the same steps in the pathway. From binding studies of morphine-like alkaloids (both agonists and antagonists) and opiate peptides to brain membranes, it seems likely that both groups of opiates interact with a common receptor site (Bradbury et al., 1976; Cox et al., 1976). The activation of the "pain opiate receptor" by both groups of narcotics could be responsible for the initiation of the antinociceptive response. By analogy, the site of tolerance activation could probably be the same for all opiates at a given effector site. Once one narcotic drug initiates (triggers) the process of tolerance induction, the other agonist also recognize this CNS adaptation and show a diminished response. Interestingly, morphine administered icv did not reveal the development of acute tolerance to its antinociceptive effect, in conditions under which it reduced the effects of B-EN. This result is in agreement to that reported by the group of Takemori et al. (1973) and Harris et al. (1976), in mice, but in contradistinction to that of Lotti et al. (1965) and Huidobro and Huidobro (1973) who reported an acute tolerance to the hypothermic or antinociceptive effect of morphine in rats after systemic administration. However, we do not understand why morphine does not produce tolerance to its own effect, under conditions which B-EN repeatedly evidenced acute tolerance in mice or in cats (Hosobuchi et al., 1977; Meglio

et al., 1977). A possibility which cannot be discarded is that both narcotics could somehow interact differently with the opiate(s) receptor(s) thus producing somewhat different effects.

Tolerance and physical dependence have been thought to have a common underlying mechanism and to develop concurrently (Way et al., 1969). In the case of single-dose tolerance to morphine, only a weak degree of abstinence could be observed to accompany the former phenomenon. A very low incidence of jumping occurred after naloxone at a time when a clear degree of single-dose tolerance was manifest in Swiss-Webster and in ICR mice three days after priming with morphine. Other studies with morphine (Way et al., 1969; Barthelemy and Jacob, 1972; Kaneto et al., 1972; Kosersky et al., 1974) and levorphanol (Cheney and Goldstein, 1971) have indicated that naloxone-induced jumping in mice develops very rapidly. Indeed, Kosersky et al. (1974) and Smits (1975) reported that after a single-dose of morphine, naloxone induced jumping is maximal 2 hours and largely disappears by 8 hours. On the other hand, single-dose tolerance was not detectable at these early periods in the ICR mice. However, with the Swiss-Webster strain, an acute tolerance to the antinociceptive effect was observed 5-6 hours after a PD of morphine. It was considerably less than that observed after 72 hours. Mice tolerant to B-EN exhibited some clear signs of precipitated withdrawal when challenged with naloxone, revealing some degree of dependence after 8-12 hours of a single dose of B-EN; or the combination of a priming + a challenge dose of B-EN. It is interesting to point that the degree of dependence developed was comparable to the degree

of tolerance: both are mild, yet detectable under the appropriate conditions of time and dosage. Early signs of dependence are known to occur in mice after about two hours of exposure to high doses of morphine, but no tolerance was found to have developed during this time (Kosersky et al., 1974; Smits, 1975). In this regard, it seems that B-EN induces an early state of acute tolerance that is paralleled by the development of dependence as is the case with chronic administration of morphine.

Even though some mild signs of dependence were discernable after a single dose, the time course of dependence did not exactly match that of tolerance. This was especially true in the case of B-EN, where maximal tolerance occurred early at around 12-18 hours after the priming dose, whereas dependence peaked at about 48. However, as will be discussed in Part 2, tolerance to the hyperthermic response of B-EN correlated much better to the time course of dependence. It is difficult to make clear differentiations with respect to the time course of single-dose tolerance and dependence development. Divergent findings with respect to the rate of tolerance and dependence development may not be meaningful if different facets of the two phenomena are being evaluated, particularly since both syndromes consist of a constellation of signs varying in intensity that appear and disappear at different rates. The sensitivity of the tests for evaluating tolerance and dependence may not be the same and may change to a different degree as each syndrome develops. To clarify these issues the assessment of both phenomena may have to be performed on a neurochemical pathway common to both processes and with equisensitive methods.

EXPERIMENTAL PART II :

PHARMACOLOGICAL CHARACTERIZATION OF THE HYPERTHERMIC RESPONSE OF B-ENDORPHIN IN MICE: MECHANISM OF ACTION AND DEVELOPMENTY OF SINGLE-DOSE TOLERANCE.

2.1 INTRODUCTION

It was of interest to characterize further the similarities in pharmacological effect between morphine and B-endorphin, specially on body temperature. From preliminary experiments it was observed that B-EN produced an hyperthermic response of quick onset, at doses lower than those used to produce antinociception. This section describes in detail the characterization of the hyperthermic response of B-ENin mice.

This study was centered on two basic questions:

1) What is the pharmacology of the hyperthermic response of B-EN? It was of special interest to study if this response was an opiate specific effect i.e., blocked and reversed by naloxone, and to study some interactions between the effects produced by B-EN and leucine or methionine enkephalin.

2) Does tolerance and dependence develop to the repeated injections of the endogenous opiate peptide?

Present results demonstrate that the hyperthermia produced by B-EN is an opiate specific, naloxone sensitive effect. The physiological-pharmacological nature of the temperature rise induced by B-EN is still unknown, but results allow to suggest that it could be due to a direct effect of B-EN on brain thermoregulatory centers. Single-dose tolerance can be developed to this central effect of B-EN using an adequate initial priming dose of B-EN and allowing at least 24-48 hours for its development.

2.2 EXPERIMENTAL PROCEDURE.

2.2.1 Measurement and quantification of the temperature effects produced by opiates.

Adult ICR mice 23-25 g (Simonsen Labs, Gilroy, California) were used throughout. Animals were housed six to each cage, fed Purina chow and allowed free access to tap water. Mice were kept in the animal room for 3-4 days before performing any experiments. The room was maintained at 22 $\pm$ 1 ^{o}C, with 40 to 41% of humidity, and a 12 hour light-dark cycle. All measurements were done on the animal room. Each experimental experimental group consisted of 12 mice.

Core temperature was measured using a digitex 581-C digital thermometer. A temperature sensitive thermocouple (15 mm diameter), immersed prior to the measurement in baby oil, was carefully introduced about an inch into the mouse rectum. To attain a stable reading, the probe was held in position for 20-30 seconds. Mice were free to move in the cages, excepting at the time of temperature measurement.

Animals were injected intracerebroventricularly (icv) according to the technique of Haley and McCormick (1957), as modified by Harris et al. (1975a). The latter workers made an incision on the scalp under slight ether anesthesia prior to making the injection as detailed in the previous section. All drug solutions were prepared freshly (or stored overnight at -20 C) disolved in sterile saline (Travenol) when injected icv. The volume of the injection was 10 ul, administered using a 50 or a 100 ul Hamilton syringe equiped with a 27-gauge needle.

Experiments were generally started around 8:00 a.m. Mice basal body temperature was recorded and then animals were injected according to the protocol to be described. B-EN and enkephalins were always administered into the brain ventricles. Core temperature was then measured 1/2, 1, 2, 4 and 8 hours after the administration of any of the peptides or other drugs. Temperature measurements allowed the determination of a time course for the effect of each substance tested. To minimize errors due to the circadian rhythms in body temperature and to diminish error contributed by individual animal variation, each animal served as its own control in each series. As a control, each animal was injected with 10 ul sterile saline icv one week after the opiate or drug injection. The time course of the body temperature change after saline was measured at the same intervals as when injected with the drug. The core temperature obtained after the administration of saline was subtracted from that of the opiate. The difference in temperature change (absolute temperature change, delta TC) was analyzed statistically (mean $\pm$ SEM for each particular determination). The total thermic response was quantified as the area under the TC-time curve for each individual mouse. The area under the curve was obtained by cutting and weighing the respective piece of surface drawn on standard graph paper. The weighing procedure had a maximal error of less than 1%. The area of temperature (AT) is an expression of time duration and intensity of drug effect. It was measured and expressed in arbitrary standardized units (time in hours x TC; square units, u2).

2.2.2 Effect of B-endorphin and enkephalins on body temperature.

Various dosages of B-EN and enkephalins were administered in groups of 12 mice each. Body temperature was measured as described above. The thermal response was quantified as the TC (oC) by subtracting the saline basal core temperature from the temperature induced by the peptides at each point following the time course. The area of temperature (AT) was determined as described above.

2.2.3 Relation between the dose of B-endorphin to produce antinociception and hyperthermic responses.

Four groups of animals were employed. Mice were injected icv with 20, 100, 600 ng B-EN or10 ul saline. Half an hour after the injection, the analgesic response was assayed by the tail-flick method as described previously (Huidobro et al., 1976). An hour after the injection, the core temperature was measured and compared to the saline treated group of animals. Antinociception was expressed as the percent of analgesia, see paragraph 1.2. The thermal response was quantified as the mean temperature change produced by B-EN.

2.2.4 Interaction of naloxone with B-endorphin.

Two different sets of experiments were performed to study the interactions between B-EN and naloxone. The first series was aimed at studying if naloxone could prevent the hyperthermic response of B-EN, and the second set of experiments to evaluate if naloxone could reverse the hyperthermic effect of B-EN.

For the first set of experiments, three groups of mice were

injected icv with 10, 100 and 200 ng B-EN /mouse and simultaneously with 2 mg/kg naloxone s.c. Core temperature was measured 30, 60 and 120 minutes after B-EN administration and compared to the temperature of the same animals injected a week later with 10 ul of saline icv and 10 ml/kg saline s.c. To study if naloxone could reverse the hyperthermic effect of 100 ng B-EN /mouse, three groups of mice were used. The first group was injected with B-EN alone. The second and third groups were injected with 2 mg/kg naloxone s.c. 30 or 120 minutes respectively after the administration of B-EN. The time course of the hyperthermia produced by B-EN was followed in the 3 groups over 8 hours. A week later, the same animals were injected with saline icv and served as the respective control groups.

2.2.5 Effect of environmental temperature on the thermal effects of B-endorphin.

To determine whether environmental temperature had an influence on the thermic effect of B-EN, experiments were conducted with mice maintained at 6 $\pm$ 1 or 32 $\pm$ 1oC. Three groups of mice were used at each environmental temperature. The first group of mice served as internal control, and received 10 ul saline icv. The second group also received saline icv while the third group was injected with 100 ng B-EN. As soon as each group was injected, the cages were placed in surroundings set at the temperatures mentioned above. Mice temperature was registered 1, 2, 4, 8 and 24 hours after injection. The second group was injected 24 hours with 100 ng

B-EN to establish the thermic response of B-EN in animals exposed to the new environment for 24 hours. As the internal control, the mice used in the first group were reinjected with saline icv.

2.2.6 Effect of calcium and manganese on B-endorphin hyperthermia. It was of considerable interest to study the influence of some divalent cations on the hyperthermia induced by B-EN, since Kakunaga et al., 1966 and Harris et al., 1975b had demonstrated that Ca++ or Mn++ antogonized in a dose dependent fashion the antinociception induced by morphine. Two types of experiments were performed. First of all, the effect of Ca++ and Mn++ was evaluated on mice temperature; then a study was conducted to examine the interaction of B-EN and these cations. Two sets of experiments were performed to analyze the possible interaction on body temperature. First, B-EN was administered in sterile saline containing the cations; and secondly, B-EN was administered in mice previously pretreated with the cations.

2.2.6a Dose effect curves of calcium and manganese injected icv on body temperature.

Groups of six mice each were administered with Ca++ or Mn++ icv. The doses administered were as follows: 0.9; 3.7; 15 or 30 µMol Ca++/kg or 3.7 and 15 µMol Mn++/kg. As a control, 10 µl saline was injected icv; temperature was registered immediately before and 30 minutes after injections. The delta temperature change before/after was calculated and compared.

2.2.6b Administration of B-endorphin in saline containing Ca or Mn.

Three groups of 12 mice each were employed. The first group was injected with 100 ng B-EN/mouse in sterile saline containing Ca++ (10 μMol Ca++/kg in a volume of 10 μl). The second group received 100 ng B-EN in saline containing 3.7 μMol Mn++/kg. As control, the third group received B-EN. Body temperature was monitored 30 and 60 minutes after injection. The body temperature change before/after drug administration was calculated for each mouse. As paired controls, two additional groups of mice received the cations alone, but otherwise treated identically.

2.2.6c Effect of calcium or manganese pretreatment on B-endorphin induced hyperthermia.

Four groups of mice were used for this experiment. Mice were pretreated with 3.7 or 15 μMol Ca++/kg, 3.7 μMol Mn++/kg or saline icv 60 minutes before the administration of 100 ng B-EN. Body temperature was recorded 30 or 60 minutes after the peptide and compared to that of the same animal immediately before the administration of B-EN.

2.2.7 Effect of indomethacin and acetylsalicylic acid (ASA) on the hyperthermia caused by B-endorphin.

It was of interest to establish whether B-EN's hyperthermia could be pharmacologically related to the rise in body temperature caused by prostaglandins, in resemblance to fever. Also we wanted to examine if the hyperthermic response of B-EN could be caused by the introduction of a foreign protein into the mouse brain. For these purposes, the following series of experiments were conducted.

2.2.7a Effect of indomethacin on B-endorphin hyperthermia.
Three groups of 8 mice each were employed. The first group was injected with vehicle i.p. (a 1% solution sodium carbonate adjusted to pH 9). The second group received 45 mg/kg indomethacin i.p., while the third group was injected with saline i.p. Four hours later, the first two groups received an injection of 100 ng B-EN and the other group had 10 µl sterile saline icv. Results were analyzed comparing the delta change in body temperature immediately before and after 1/2, 1 or 2 hours following the injection of B-EN or saline.

2.2.7b Acetylsalicylic acid (ASA) on B-endorphin hyperthermia.
Mice were implanted with a 75 mg ASA tablet subcutaneously. The tablets, St. Joseph's children's aspirin tablets, were wrapped in a fine nylon mesh to prevent pellet desintegration and to allow a more gradual salicylate absorption and to lower mortality rates. As controls, a paired group of mice was implanted with a placebo pellet. Two days after pellet implantation, both groups of mice were injected with 100 ng B-EN. Thirty minutes after injection, temperature was monitored, and mice were injected with 2 mg/kg naloxone s.c. Body temperature change before/after B-EN or naloxone was compared in the ASA vs. the placebo treated mice.

2.2.7c Effect of indomethacin and ASA on pyrogen-induced fever.
As appropriate pharmacological controls for the antipyretic-antiinflamatory drugs, the following controls were attempted. Mice were injected icv or i.v. with either E. coli or salmonella

typhosa purified lipopolyssacharides (doses in the range of 10 ng-10 μg icv/mouse or 10 mg/kg i.v.) or 10 ml/kg of a boiled milk solution (Berkeley Farms, 2% fat milk boiled for 5 minutes). As controls for the pyrogens, paired groups were injected with saline either icv or i.p., and body temperature was compared among the pyrogen-saline groups. Both indomethacin and ASA, were used to study whether they antagonized the pyrogen induced fever.

2.2.8 Assessment of single-dose tolerance development.

2.2.8a Generalities.

To test if B-EN shares with morphine the property to induce single-dose tolerance, B-EN was administered twice. The first dose was used as a priming dose; the second dose was repeated to reveal if tolerance developed as a consequence of the priming dose. The time course of the temperature change following B-EN injection was studied in each case. Single-dose tolerance was quantified by comparing the AT produced by the priming dose to that produced by the second dose, and it was defined as a significant decrease in the AT (P less than 0.05).

2.2.8b Estimation of the optimal time for single-dose tolerance development.

Six groups of mice were utilized. All groups were injected with 100 ng B-EN /mouse on day zero. In one of these groups, the body temperature was measured and the AT was calculated. This value served as a control, 100% response. Every 24 hours after the priming dose, a random animal group was injected on the opposite side of the brain with a second dose of 100 ng B-EN

per mouse. The AT was determined and compared to that produced by B-EN on the first day, (time 0) As controls, each group of mice was injected with 10 µl saline icv seven days after the second dose of B-EN.

2.2.8c Effect of dosage on single-dose tolerance development.

To study the influence of different doses of B-EN on the development of single-dose tolerance, 8 groups of mice were injected with increasing priming doses of B-EN. Twenty four hours after the initial dose, a second dose of B-EN identical to that used for priming was injected on the opposite side of the brain. In this particular set of experiments, the time interval between the first and second injection of B-EN was kept constant, but the dosage was varied. As controls for the effect of B-EN, the same animals were injected a week later with saline icv, and the colonic temperature was measured. Comparing the temperature between the saline vs. the B-EN treated mice, the AT produced by the priming and the second dose of B-EN was calculated and compared. A dose effect curve was constructed with the mean AT produced by the priming and the second dose. As a quantitative expression of tolerance, the difference in the AT produced by the first and second dose was calculated for each dose. The two tail paired student "t" test was used in this particular set of experiments.

In an additional set of experiments to evaluate the influence of brain damage produced by the icv injection per se as a cause of the diminished response of B-EN, two groups of mice were

employed. The first one was injected with 10 µl of saline icv and challenged with 100 ng of B-EN 24 hours later. The second group was injected with 100 ng B-EN on day 0 and 24 hours later with saline icv. The body temperature was measured following each injection. The saline injection served as control in each case. The AT produced by B-EN in the 24 hour saline pretreated group was compared to that produced by B-EN in the naive, nonprimed group of mice.

2.2.8d Effect of naloxone on the development of single-dose tolerance. To study if naloxone, the specific opiate antagonist, could diminish or block the development of single-dose tolerance to B-EN, the following series of experiments were performed. Three groups of mice were injected with 10, 100 or 200 ng B-EN and simultaneously administered with 2 mg/kg naloxone s.c. Twenty-four hours later each group was administered with a second dosage of B-EN equal to that of the priming dose. The AT produced by the second dose of B-EN was calculated and compared to that produced by the same dose of B-EN in animals primed with B-EN but not injected with naloxone.

2.2.8e Development of single-dose tolerance and physical dependence. It was of interest to examine if the development of single-dose tolerance was associated with the development of dependence. To resolve this question, three groups of mice were used. Mice were injected on day zero with 600 ng B-EN and 24, 48 or 96 hours after the priming dose, each group was injected the second dose of B-EN. Six hours after the second injection

of the peptide, mice were challenged with 10 mg/kg naloxone s.c. and placed on a circular platform and observed for withdrawal signs over a period of 20 minutes after naloxone. Dependence was quantified according to the ranking method of Huidobro and Maggiolo (1961). This method scores increasing points for the withdrawal behavioral signs; 1 point is for a mild increase in locomotor activity, up to 5 points for the stereotyped, withdrawal jumping behavior.

2.2.8f Effect of a priming dose of enkephalin on the development of single-dose tolerance.

It was of special interest to evaluate whether a single dose of enkephalin, acting like a priming dose, could influence the hyperthermic response of B-EN. For this purpose, doses of 1,10 or100 μg leucine or methionine enkephalin or 2,10 and 100 μg of D-ala2 enkephalinamide were administered to different groups of mice. Twenty-four hours later, all groups were injected with 100 ng B-EN and the time course of the temperature change produced by B-EN was monitored. The AT produced by both the first and second peptide injection was calculated. As controls, 2 groups of animals, from the same shipment, were used. One was injected on day 0 with saline icv and the second group was injected with 100 ng of B-EN . Twenty-four hours after the priming dose, both groups were challenged with 100 ng B-EN. Statistical analysis was performed by calculating the difference between the AT generated by 100 ng B-EN in the peptide primed animals vs. the saline treated mice.

2.2.8g Effect of different environmental temperatures on single-dose tolerance development.

To examine whether single-dose tolerance to the hyperthermic effect of B-EN develops independent of the ambient temperature, the following experiments were performed. Three groups of mice were injected with 100 ng B-EN and immediately transferred to a room at 6, 22 or 32oC, where the time course of rise in body temperature was determined. Twenty-four hours after the priming dose of B-EN, a second dose of opiate was administered to examine the degree of tolerance developed at the different environmental conditions. Comparisons between the AT produced by the first and second dose were used to establish tolerance at the environments studied. Control groups were injected twice with saline and served as the internal standard to calculate the delta temperature changes.

2.2.9 Development of cross-tolerance from morphine to B-endorphin.

To perform this experiment, mice were rendered tolerant-dependent by implantation of a 75 mg morphine pellet in the subcutaneous tissue for 72 hours as described by Way et al. (1969). After implantation for 72 hours, animals develop about a 10-fold tolerance to the antinociceptive effect of morphine (Way et al., 1969). Core temperature was compared between naive mice and tolerant-dependent mice on the third day after implantation without removal of the pellet. The tolerant-dependent mice were divided into 2 groups and injected with 100 or1000 ng B-EN. The time course of the temperature change was followed and compared to the changes in body

temperature produced by B-EN in naive control mice. As a control, one group of mice was implanted with a morphine pellet, but injected with 10 µl saline icv instead of the peptide.

2.2.10 Sources of the opiates and opiate-like peptides and other chemicals.

Human B-endorphin (hB-EN) was furnished by Li et al. (1976). Leucine and methionine enkephalin and D-ala2-methionine5 enkephalinamide (D-ala2 enkaphalinamide) were purchased from Bachem (Marina del Mar, CA). Morphine sulfate from Mallinckrodt (St. Louis, MO). Naloxone HCl was a generous gift of Endo Labs (Garden City, NY). Bovine serum albumin (fractionV) was purchased from Sigma (St. Louis, MO). Opiate peptides solutions were freshly prepared in sterile saline in plastic mini vials. Occasionally, samples were stored overnight at -20 oC. Acetylsalicylic acid tablets were purchased commercially. Indomethacin, a gift from Merck Sharpe and Dohme Research Labs (West Point, PA), was dissolved in 1% sodium carbonate, pH 9. Calcium and manganesium chloride (reagent grade) were purchased from Mallinckrodt (St. Louis, MO), and dissolved in saline for icv injections. The concentration of the peptides was expressed as ng or µg of peptide injected /mouse; as nMol B-EN injected per mouse; or as nMol B-EN/kg.

2.2.11 Statistics.

In general, the two tailed student "t" test was used to compare different AT's or the delta temperature change. Ocassionally, one tail student "t" test was used when justified. In the case

the experiments with single-dose tolerance, the paired student "t" test was used. The level of significance was set at a P value of less than 0.05.

2.3 RESULTS.

2.3.1 Effect of B-endorphin and enkephalins on body temperature and behavior.

B-EN injected icv produced a dose-dependent hyperthermic response. Hyperthermia was evident with doses as low as 100 picogram (1.2 pMol/kg). The hyperthermic response of B-EN was consistent from day to day and was found at all the doses studied. It was characterized by a fast onset and a prolonged duration of action. The time course of the hyperthermic response of 100 ng B-EN (1.2 nMol/kg) is illustrated in Figure 2.1. It can be seen that the peak effect occurred at about 30 minutes and that the effect lasted more than 8 hours. By 24 hours, the body tempeture had returned to, or was slightly below the control base line level.

The dose response curve of the hyperthermic effect of B-EN showed 3 distinct phases. Doses of B-EN in the range of 0.001-0.12 nMol/kg produced a low level non dose dependent hyperthermia. Increasing doses produced dose related augmentation in the AT up to a dose of 2.4 nMol/kg, after which increasing doses produced a considerable reduction in response. (Figure 2.2). However, even at the highest doses tested, no hypothermia was ever observed. Few or no morphine-like behavioral effects was evident up to 200 ng B-EN; however, increased locomotion and Straub tail was noted at higher doses.

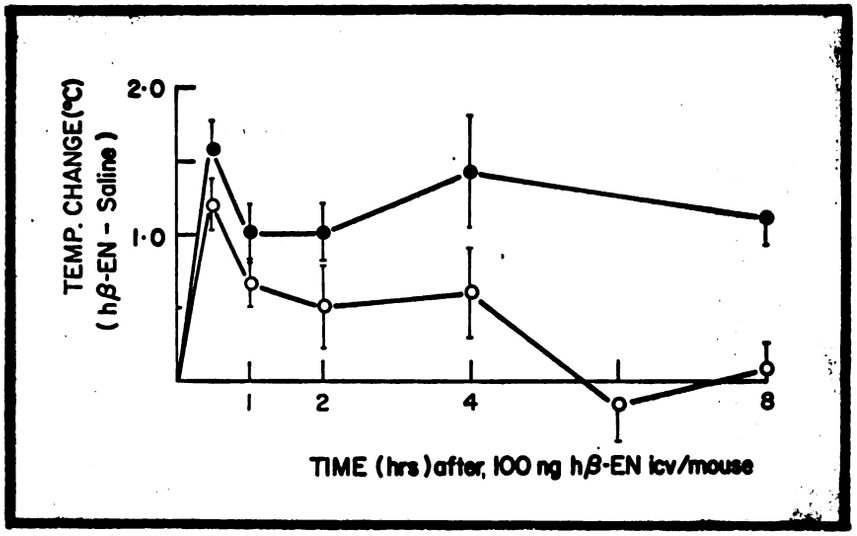

Figure 2.1

Time course of B-Endorphin hyperthermia in mice.

A group of mice was injected with a priming dose of 100 ng B-EN (1.2 nMol/kg). The temperature change was calculated by substracting the body temperature obtained after the injection of the peptide from that of 10 ul saline icv, a week after the administration of B-EN. Solid circles represent the time course of the priming dose; open circles represent the time course of a second dose of 100 ng B-EN administered 24 hours later. Bars represent the S.E.M. of a group of 12 mice.

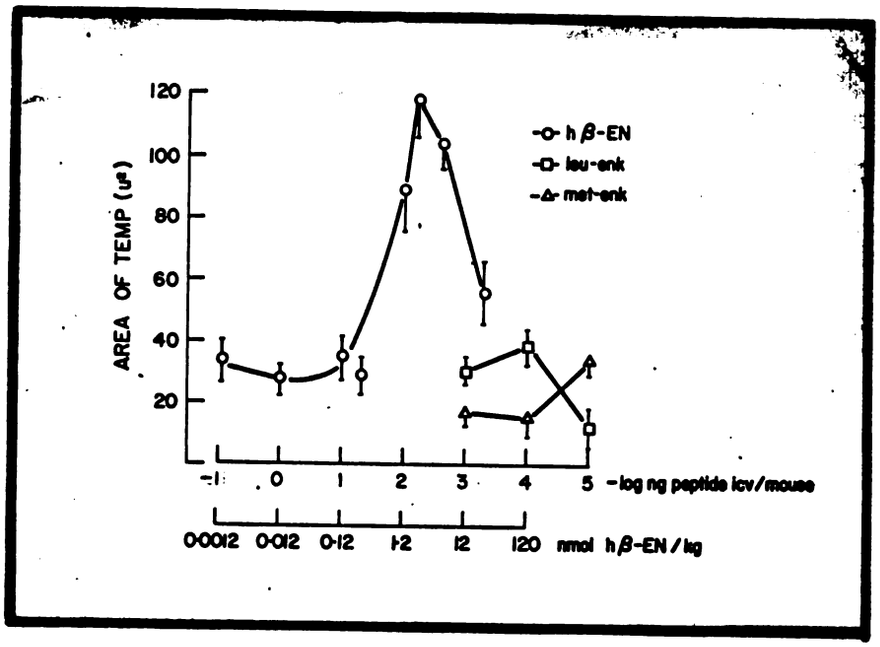

Figure 2.2

Dose effect curve of B-Endorphin and enkephalins on body temperature.

Ordinate represents the area of temperature (u^2) produced by the peptides; abscissa the dosage of the peptides. The lower scale in the abscissa expresses the concentration of B-EN in nMol/kg body weight. Each point represents the mean area of temperature of a group of 12 mice each. Bars denote the S.E.M.

The enkephalins shared with B-EN the property to produce hyperthermia, though the time course of effect was very different. However, the pentapeptides were considerably less potent, did not produce a maximal effect, and showed a shorter duration of action than B-EN. Dose effect curves for the hyperthermic response of leucine and methionine enkephalin are shown in Figure 2.2. It can be noted that the AT did not reach a comparable maximal response to that of B-EN. The curves are considerably displaced to the right, an indication of lower potency and/or shorter biological half life. Leucine enkephalin appeared to be more potent than methionine enkephalin.
The duration of the hyperthermia produced by the pentapeptides was shorter than that of B-EN, approximately 6-8 hours. Figure 2.3a illustrates the time course of the temperature changes produced by 10 μg /mouse with each of the three peptapeptides studied. The fast initial peak response characteristic of B-EN was not observed. Instead there was a modest increase in core temperature that peaked 4 hours after administration. (This time course is typical of the effect of 1-30 ug morphine, data shown in Paragraph 3.3). In contrast to the endorphins and morphine, D-ala2 enkephalinamide produced initially a dose-dependent drop in temperature (Figure 2.3b), followed by a modest but significant increase in body temperature.

At the doses of enkephalins studied, D-ala2 enkephalinamide produced marked morphine-like effects: Straub tail, increase in locomotion and exophthalmus. Leucine or methionine enkephalin produced very mild behavioral effects. No mortality

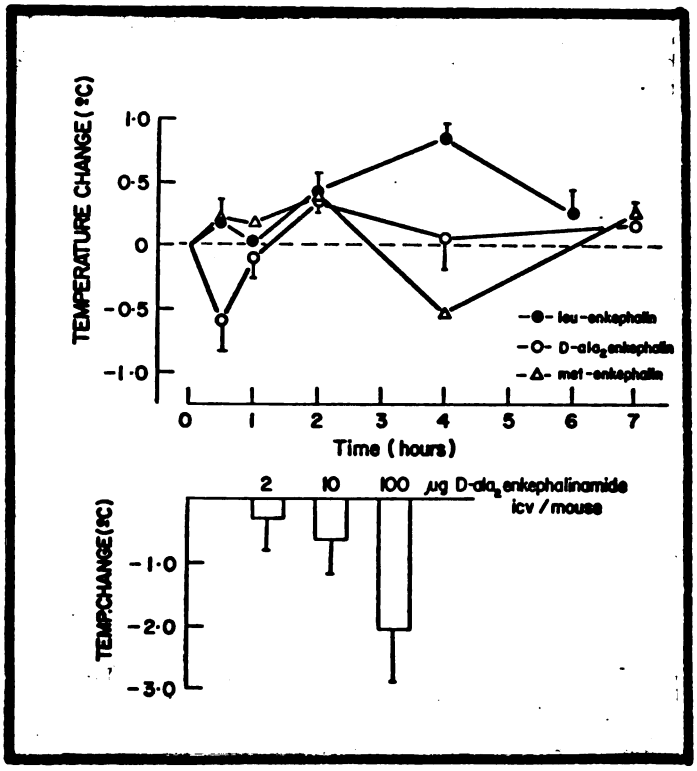

Figure 2.3

Effect of enkephalins on body temperature.

Upper panel: Time course of the temperature change produced by 10 ug enkephalin/mouse. Each group consisted of 12 mice. Bars denote the S.E.M. of the temperature change.

Lower panel: Mean decrease in body temperature 30 minutes after administration of D-ala$_2$-enkephalinamide. N equals 12 mice for the doses of 1 and 10 ug, and 10 mice for the dose of 100 ug D-ala$_2$-enkephalinamide. Bars denote the S.E.M.

was observed with 100 μg of leucine or methionine enkephalin; 2/12 animals died within 10 minutes following 100 μg of D-ala2 enkephalinamide.

To control for possible hyperthermia due to the introduction of a foreign protein to the brain, the icv injection of 100 ng albumin did not produce a significant change in core temperature 30 or 60 minutes after the injection.

2.3.2 Dose effect relation between the antinociceptive and hyperthermic responses of B-endorphin.

B-EN is more potent in producing hyperthermia than in causing antinociception. The hyperthermic response of B-EN was evident at doses lower than those causing antinociception. These results are presented in Table II-1. It can be observed that 20 ng B-EN produced hyperthermia while the same mice exhibited no appreciable antinociception. To produce analgesia, higher doses of B-EN were required. For most of these studies we have used a dose of 100 ng B-EN . This dosage falls in the linear portion of the hyperthermic dose response curve and consistently produced a marked hyperthermia, accompanied by less than 40% antinociception.

2.3.3 Blockade and reversal of the hyperthermia by naloxone.

Naloxone, the specific opiate antagonist, prevented and reversed B-EN's hyperthermia. When naloxone was injected simultaneously with B-EN, the hyperthermia was antagonized in a dose dependent fashion. The effect of naloxone lasted for at least 2 hours after injection (Table II-2). This

Table II-1

Effect of B-Endorphin on antinociception and core temperature.

B-EN ng/mouse		% Analgesia[1] ($\bar{x}$ + SEM)	Temperature Change °C[2] ($\bar{x}$ + SEM)
20	(12)	18.39 ± 8.63	1.17 ± 0.14
100	(12)	38.66 ± 7.99	1.72 ± 0.11
600	(12)	76.02 ± 9.49	1.58 ± 0.10

Numbers in parenthesis denote the number of animals employed.

1 - Determined 30 minutes after icv administration.

2 - Determined in the same animals used to study antinociception, 60 minutes after icv administration.

Table II-2

Effect of naloxone on B-Endorphin hyperthermia.

	Temperature Change ^{o}C ($\bar{x} \pm$ SEM)		
B-EN ng/mouse icv	1/2 hour[a]	1 hour	2 hours
100 + Saline s.c. (12)	1.56 ± 0.171	1.00 ± 0.187	1.01 ± 0.211
100 + Naloxone[b] (12)	0.38 ± 0.123**	0.50 ± 0.126*	0.25 ± 0.195**
200 + Saline s.c. (12)	2.02 ± 0.188	1.76 ± 0.136	1.36 ± 0.137
200 + Naloxone[b] (12)	0.77 ± 0.137**	0.54 ± 0.223**	0.53 ± 0.164**

Numbers in parenthesis are the number of mice in each group.

a - time after administration of B-EN.

b - 2 mg/kg s.c., administered simultaneously with B-EN.

*P less than 0.05.

**P less than 0.005 as compared to the saline pretreated group.

results suggest a competitive interaction between naloxone and B-EN. Furthermore, naloxone injected 30 minutes after the peptide, when the hyperthermia had fully developed, temporarily reversed the hyperthermia. Two hours after naloxone, the hyperthermia appeared and followed a time course similar to that seen without naloxone treatment (Figure 2.4). However, if naloxone is injected two hours after B-EN, it did not modify the time course of the hyperthermia (Figure 2.4). These results suggest that the hyperthermic response of B-EN is mediated by opiate specific receptors, and that B-EN might trigger a process to cause the long lasting hyperthermia. Naloxone can reverse the effect of B-EN provided it is administered soon after B-EN; if administered 2 hours apart, B-EN becomes refractory to naloxone.

2.3.4 Hyperthermic responses of B-endorphin at different environmental temperatures.

B-EN administered to mice maintained at 6 or 32 oC produced a long lasting hyperthermia independent of the environmental temperature. Figure 2.5-A shows the temperature change observed an hour after the administration of a challenge dose of 100 ng B-EN. It can be seen that at all 3 temperatures B-EN caused a prompt and significant increase in body temperature. The peak hyperthermia produced by B-EN was consistent and of similar magnitude in the acclimatized or non acclimatized mice. As can be observed in Figure 2.5-B, mice pretreated with 10 µl saline and maintained 24 hours at 6 or32 oC showed basically the same increase in body temperature as

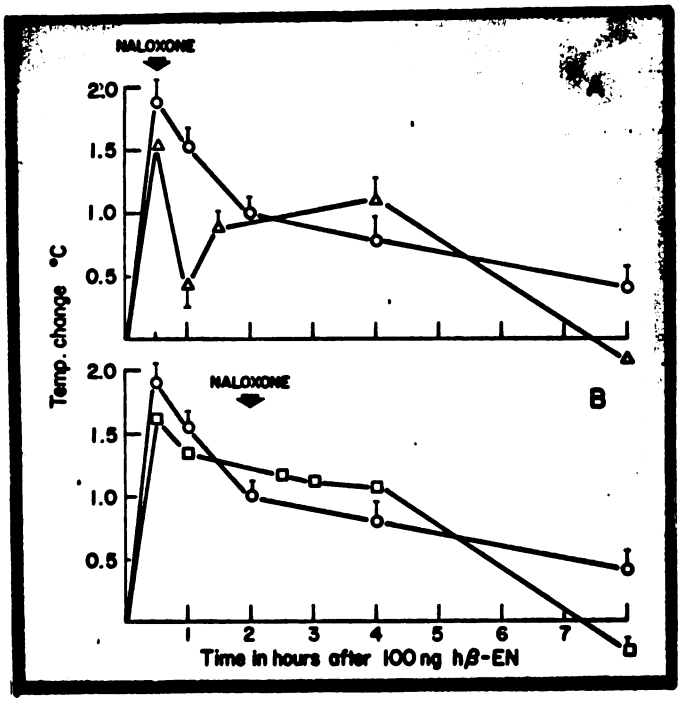

Figure 2.4

Temporal reversal of B-Endorphin's hyperthermia by naloxone.

Ordinate represents the core temperature change of 100 ng B-EN icv. Abscissa, time in hours following the administration of B-EN. Open circles denote the time course effect of 100 ng B-EN, and is valid for A and B.

Upper panel (A): 2mg/kg naloxone was injected 30 minutes after B-EN (open triangles).

Lower panel (B): 2mg/kg naloxone was injected 120 minutes after B-EN (open squares).

Points represent the mean temperature change of 12 mice, bars represent the S.E.M.

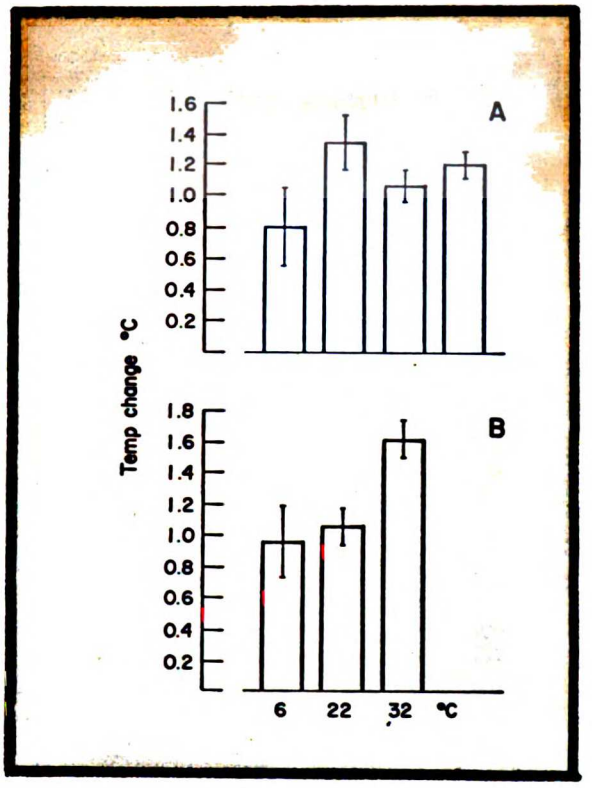

Figure 2.5

Hyperthermia of B-Endorphin at different environmental temperatures.

Columns represent the mean ± S.E.M. hyperthermia observed 60 minutes after icv injection of 100 ng B-EN.

Upper panel (A): Animals were injected at room temperature(22oC) and immediately transferred to the new environment.

Lower panel (B): Mice were acclimatized for 24 hours in the new environment.

Ordinate:room temperature (oC) matched for A and B. In A, the column to the further right represents an ambient temperature of 35 oC.

that produced by B-EN in mice not previously exposed to the new environment (Figure 2.5-B). This fact suggests that B-EN produces hyperthermia independent of ambient temperature. The AT produced by 100 ng B-EN at 6 or 32 oC was significantly reduced when compared to the AT produced by the same dose of B-EN at 22o C. (See Fig. 2.5 A and B, paragraph 2.3.7f and Table II-7.)

2.3.5 Interaction between B-endorphin and divalent cations on core temperature.

2.3.5a Effect of the administration of calcium or manganese .

Ca++ and Mn++ produced a dose-time dependent hypothermic effect in mice. As can be appreciated from Table II-3, Mn++ was considerably more potent than Ca++ in producing a fall in body temperature. The temperature change was reversible. Control base line temperature was attained within 2-4 hours following cation injections. None of the doses of the cations caused toxic or overt behavioral signs in the mice. The doses of the cations used were those reported by Harris et al. (1975b), not to produce toxic effects in mice.

2.3.5b Effect of calcium or manganese on B-endorphin hyperthermia.

The simultaneous injection of Ca++ or Mn++ with B-EN, or the pretreatment of animals with either Ca++ or Mn++ did not prevent the hyperthermic response of B-EN. As can be seen in Figure 2.6, the simultaneous injection of 3.7 μMol Mn++/kg or10 μMol Ca++/ kg did not significantly alter B-EN's hyperthermia. Ca++ or Mn++ per se caused no change in temperature after1 hour.

Table II-3

Effect of calcium and manganese on body temperature in mice.

uMol/Kg icv		Temperature Change* (^{o}C) $\bar{x} \pm$ SEM
0.93 Ca++	(6)	0.05 $\pm$ 0.17
3.75 Ca++	(6)	-0.10 $\pm$ 0.26
15.0 Ca++	(12)	-0.78 $\pm$ 0.35
30.0 Ca++	(6)	-4.40 $\pm$ 0.66
3.75 Mn++	(6)	-1.32 $\pm$ 0.62
15.0 Mn++	(6)	-4.38 $\pm$ 0.73
Saline (10 ul icv)	(6)	0.083 $\pm$ 0.28

*Determined 30 minutes after icv administration.

Number in parenthesis reflects the number of mice in each determination.

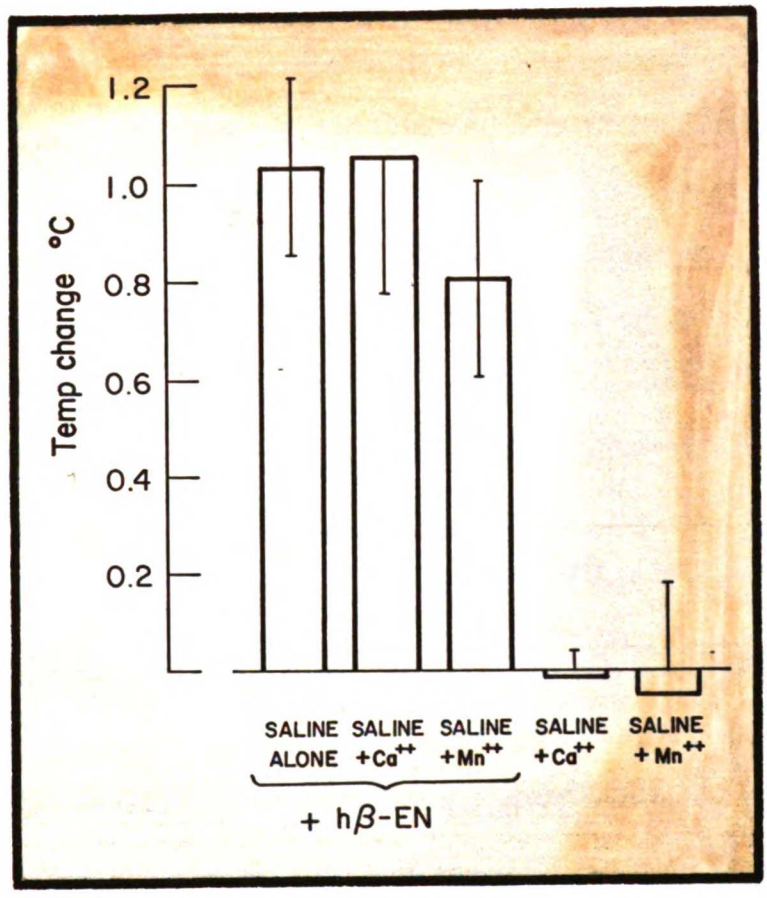

Figure 2.6

Effect of Ca^{++} and Mn^{++} on the response of B-Endorphin.

Groups of 12 mice were injected with B-EN in saline, or in saline containing Ca^{++} or Mn^{++}. Control groups received 10 uMol Ca^{++}/kg or 3.7 uMol Mn^{++}/kg. Body temperature was determined 60 minutes after icv administration. Ordinate denotes delta temperature change obtained as compared to a paired group injected with saline instead of B-EN. Bars denote the S.E.M.

On the other hand, pretreatment of mice with 3.7 or 15 μMol Ca++/kg or 3.7 μMol/kg Mn++ for an hour did not antagonize B-EN. As can be observed in Table II-4, 60 minutes after B-EN, considerable hyperthermia was demonstrable in all three cases. Harris et al. (1975b) had previously shown that Ca++ brain levels remain almost unaltered during the first 2 hours following the icv injection of 15 uMol Ca++/kg. It must be emphasized that on a molar basis the dose of Ca++ or Mn++ was about 1000-fold larger than that of B-EN; however, no antagonism to the hyperthermic effect of B-EN was evident.

2.3.6 Effect of indomethacin or acetylsalicylic acid (ASA) on B-endorphin's hyperthermia.

2.3.6a Neither indomethacin nor ASA antagonized the rise in core body temperature induced by B-EN. As shown in Figure 2.7, pretreatment of mice with indomethacin did not alter the prompt rise in body temperature that occurs 30 or 60 minutes following B-EN's administration. Similarly, in mice implanted with ASA tablets, B-EN produced a rise in temperature comparable to that obtained in mice implanted with placebo pellets. On the other hand, the B-EN induced hyperthermia was blocked by 2 mg/kg naloxone s.c. in both ASA and placebo treated mice (Figure 2.8), suggesting that salicylates even at toxic doses did not interfere with the mechanism of B-EN induced hyperthermia. (The ASA tablet implant produced about 50% mortality. The survivors did not evidence appreciable weight loss nor an alteration in core temperature as compared to the paired placebo implanted mice.)

Table II-4

Effect of calcium or manganese pretreatment on B-Endorphin induced hyperthermia.

		Temperature Change (°C) $\bar{x} \pm$ SEM B-EN 100 ng/mouse	
Ion Pretreatment*	(uMol/Kg icv)	1/2 hour	1 hour
3.7 Mn++	(6)	0.93 ± 0.33	1.56 ± 0.23
3.7 Ca++	(12)	0.56 ± 0.23	0.58 ± 0.14
10.0 Ca++	(12)	0.87 ± 0.30	1.00 ± 0.37
Saline	(12)	0.92 ± 0.45	1.10 ± 0.42

*Pretreatment was done 60 minutes before the administration of B-EN.

Number in parenthesis refers to the animals employed in each determination.

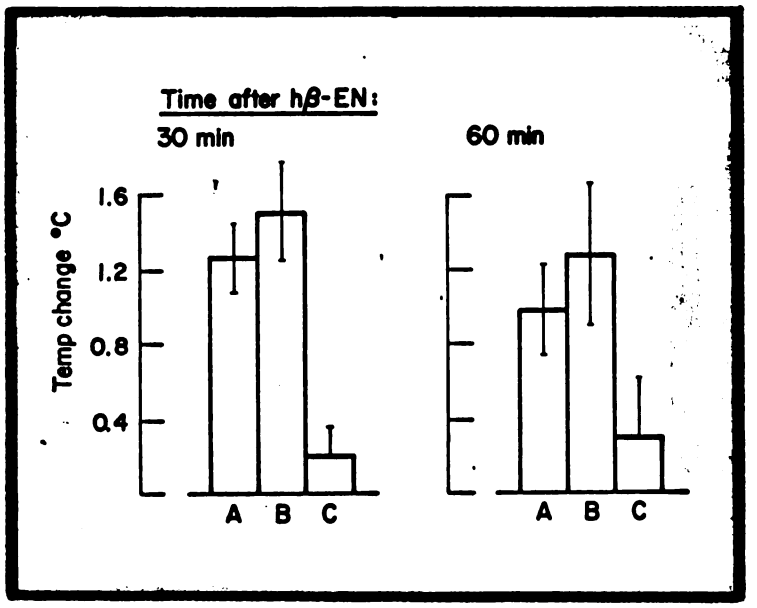

Figure 2.7

Effect of indomethacin on B-Endorphin hyperthermia.

Three groups of 12 mice were used. A was injected with the indomethacin vehicle solution i.p., B was administered with 45 mg/kg indomethacin i.p., while C was injected with saline i.p. Four hours later, groups A and B were injected with 100 ng B-EN icv, and C with 10 ul saline. Body temperature was determined 30 or 60 minutes after B-EN. Bars denote the S.E.M.

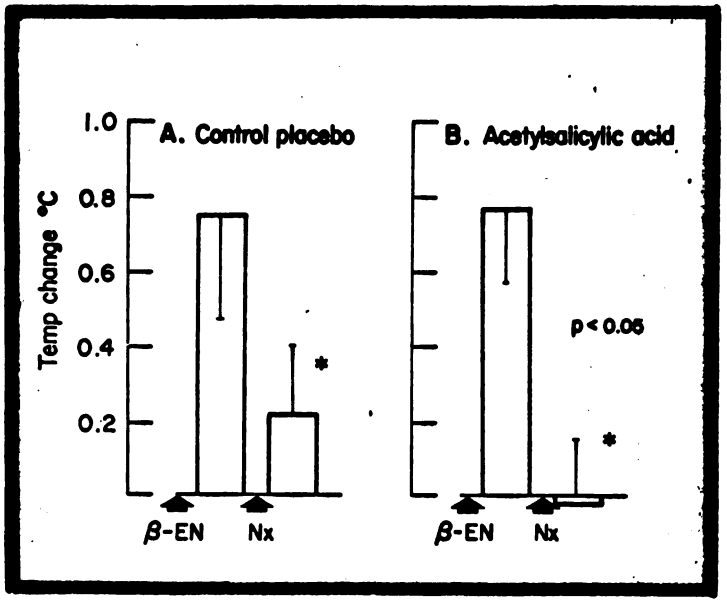

Figure 2.8

Effect of acetyl-salicylic acid on B-Endorphin hyperthermia.

Two groups of 8 mice were used. One group was implanted with a tablet containing 75 mg acetylsalicylic acid, the other group with a placebo tablet for 48 hours. At the arrows, 100 ng B-EN was injected icv, and temperature was measured 30 minutes later. At the time of the peak response, 2 mg/kg naloxone was injected s.c. Column indicate the mean temperature change, bars denote the S.E.M.

2.3.6b Effect of antipyretic drugs on pyrogen induced fever.

The administration of purified E. coli or salmonella typhosa lipopolyssacharide either icv or iv failed to produce fever in mice. However, the i.p. injection of a 5 min. boiled milk solution produced a slow onset hyperthermia that peaked three hours after injection. As can be seen from Figure 2.9, both indomethacin and acetylsalicylic acid completely counteracted the milk (pyrogen) induced fever, demonstrating that the indomethacin or salicylates were pharmacologically active under the present experimental conditions. These results suggest that the B-EN hyperthermia is not mainly mediated by endogenous prostaglandins, as it is thought to be in the case of the pyrogen induced fever.

2.3.7 Development of single-dose tolerance to B-endorphin.

The second administration of B-EN produced a significantly reduced area of temperature as compared to that of the first dose of B-EN. The peak hyperthermic response of the second administration of B-EN was not generally affected, while the duration of action was considerably diminished. This effect is represented in Figure 2.1 by the results shown in open circles. The reduction in the hyperthermic response of B-EN is not due to brain damage caused by the injection procedure or to an experimental artifact. To verify, mice were primed with saline icv and 24 hours later injected with 100 ng B-EN. There was no statistical difference in the AT produced in the saline primed group vs a control-nonprimed group. The AT produced by 100 ng of B-EN in the saline primed

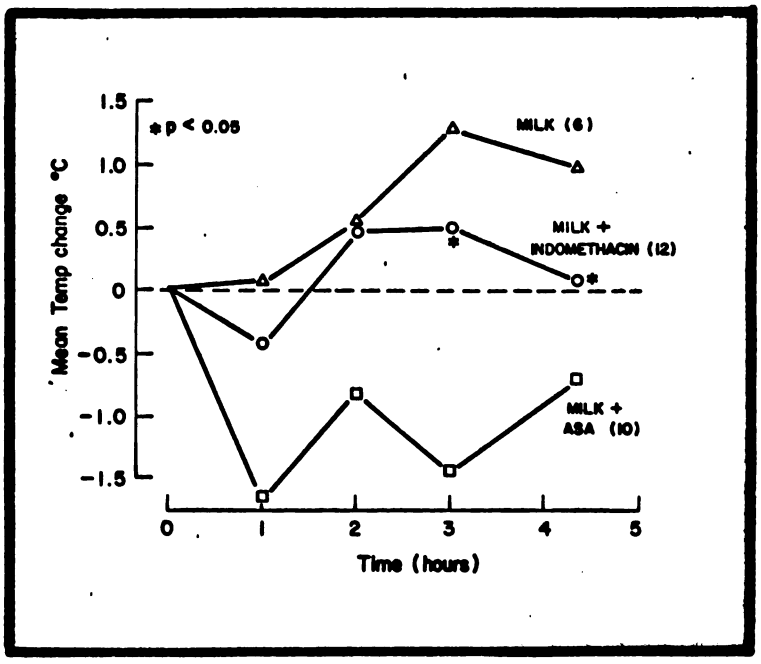

Figure 2.9

Effect of indomethacin and acetylsalicylic acid on pyrogen induced fever.

Open triangles: time course of a febrile reaction to the i.p. injection of a 5-minute boiled milk solution in six naive mice. Open circles refer to the change in body temperature of pyrogen in 12 mice pretreated with indomethacin. Open squares, refer to 10 mice pretreated with acetylsalicylic acid (ASA) for 48 hours and administered with milk pyrogen. Paired controls were treated with saline. Abscissa, time in hours after the injection of milk i.p. Ordinate: mean temperature change in oC.

group was 67.6±4.4 u2 (n=24), while in the naive control group the AT was 76.9± 5.4 u2 (n=24). As a further control, an extra group of mice was injected with 100 ng B-EN and showed a significant reduction in the AT to the second administration of B-EN: 92.0±7.4u2 vs 50.2±6.0u2. (This decrease represents a 45.4% reduction in the AT, P less than 0.01.)

2.3.7a Time course of single-dose tolerance development.

The development of single-dose tolerance to the hyperthermic effect of B-EN is dependent on both the time and dosage administered. Single-dose tolerance to a priming dose of 100 ng B-EN was maximal 24-48 hours after the initial dose, but not present seven days after the initial dose (Figure 2.10).

2.3.7b Effect of B-endorphin dosage on the degree of tolerance development.

Single-dose tolerance to B-EN's hyperthermia is dose related. Doses of 0.1-1 ng/mouse B-EN which produced hyperthermia, did not cause tolerance. At higher doses (10-200 ng), there seemed to be a direct relation between the degree of single-dose tolerance and the priming dose used to induce tolerance. A dose of 600 ng B-EN did not produce more tolerance than that attained with 200 ng (Figure 2.11). This fact suggests that there is a limit to the production of tolerance.

2.3.7c Effect of naloxone on the development of single-dose tolerance.

The development of single-dose tolerance to B-EN was antagonized by the simultaneous administration of naloxone in conjunction with the priming dose of B-EN. The degree of

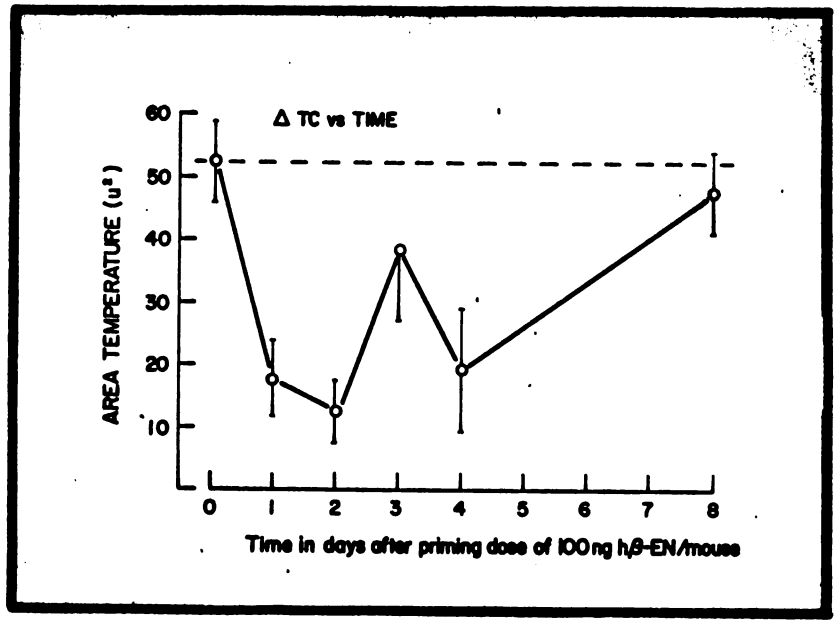

Figure 2.10

Time course of development of single-dose tolerance.

Six groups of 12 mice each were administered on day zero with a priming dose of 100 ng B-EN. In one of the groups, the area of temperature was determined for this dose of B-EN. On each subsequent day following the priming dose, the hyperthermic response was again determined in a group selected at random after challenge with the same dose of B-EN. Ordinate represent the area of temperature (u^2) obtained on consecutive days following the priming dose. Each point represents the mean area of temperature, bars the S.E.M.

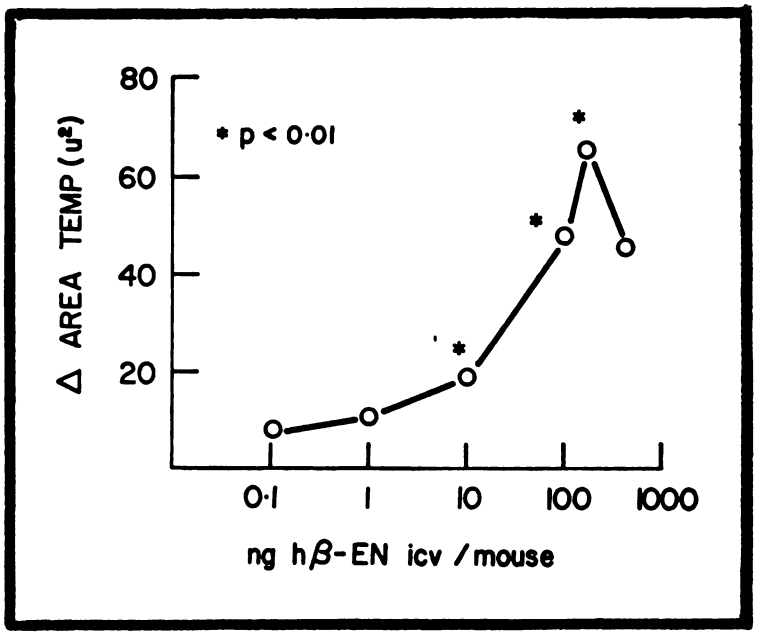

Figure 2.11

Dose effect curve for the development of single-dose tolerance.

Six groups of 12 mice each were injected icv on day zero with varying doses of B_EN. Twenty four hours after the first injection, a second dose of B-EN was injected icv on the opposite side of the brain. The area of temperature produced by both doses was determined.A decreased response to the second dose of B-EN was considered to be an indication of tolerance, and the difference in the area of temperature,an estimate of the degree of tolerance.

blockade of tolerance by naloxone was dependent on the priming dose of B-EN. With the dose of naloxone used, the blockade of tolerance was significant and almost complete to doses of 10 or 100 ng B-EN, but there was no significant antagonism of the 200 ng dose (Table II-5).

2.3.7d Demonstration of single-dose dependence.

Development of single-dose tolerance to B-EN was associated with the development of dependence as evidenced by morphine-like withdrawal signs after challenging with naloxone. Within 20 minutes of the naloxone injection, animals displayed "wet dog shakes", teeth chattering, and a peculiar form of locomotion, none of which were observed in saline pretested mice challenged with a similar dose of naloxone. Jumping behavior, characteristic of more severe dependence was observed only occasionally. The time course of the withdrawal score is shown in Table II-6. It can be seen that the naloxone precipitated withdrawal syndrome was maximal 48 hours after the priming dose, concurrent with when maximal tolerance was observed after a single dose of B-EN.

2.3.7e Single-dose cross-tolerance between enkephalin and B-endorphin.

Pretreatment of mice with leucine or methionine enkephalin and D-ala2 enkephalinamide produced cross-tolerance to B-EN. Not all the enkephalins were equally effective in producing single-dose tolerance. Leucine enkephalin was found to be the most active; it diminished by approximately 80% the effect of B-EN injected 24 hours after different priming doses (Table II-7).

Table II-5

Effect of naloxone on the development of single-dose tolerance.

B-EN icv (ng/mouse)	Area of Temperature ($\bar{x} \pm$ SEM U^2)		
	Priming Dose[a]	Second Dose	Second Dose with Naloxone Pretreatment[b]
10	34.68 ± 6.98 (12)	11.90 ± 3.47 (12)	48.47 ± 5.32* (12)
100	66.27 ± 6.89 (39)	24.49 ± 6.25 (26)	48.87 ± 4.70* (12)
200	118.32 ± 11.16 (12)	51.96 ± 9.77 (12)	67.50 ± 4.56 (12)

Numbers in parenthesis denote the number of animals.

a - Administered 24 hours before the second dose.

b - 2 mg/kg naloxone s.c. injected simultaneously with the priming dose, 24 hours before the administration of the second dose of B-EN. Area of temperature for the priming dose naloxone is not shown, see Table II-2.

*P less than 0.01 as compared to the area of temperature of the second dose of B-EN in the control group.

Table II-6

Development of single-dose dependence.

Time after the Priming Dose	Withdrawal Score (*)
24	1.0
48	1.5
96	0

Three groups of 12 mice each were primed with 0.6 ug B-EN icv/mouse. Twenty-four, 48 and 96 hours after the initial dose, a different group was reinjected with the same dose of B-EN and 6 hours later challenged with naloxone.

(*) Opiate withdrawal was precipitated with 10 mg/kg naloxone s.c. 6 hours after the second dose of B-EN. Animals primed with saline and reinjected with saline icv. 24 hours later, showed a 0 point score in withdrawal when challenged with naloxone.

Table II-7

Effect of pretreatment with the enkephalins on the hyperthermic response to B-Endorphin.

Dose of Enkephalin[2] (ug/mouse)	Area of Temperature[1] ($\bar{x} \pm$ SEM) (U^2)		
	Leucine Enkephalin	Methionine Enkephalin	D-ala$_2$ Enkephalimamide
1	16.47 ± 2.79 (10)	54.05 ± 9.27 (11)	43.62 ± 8.09* (12)
10	17.28 ± 2.85 (11)	45.34 ± 7.20 (11)	30.63 ± 6.04 (12)
100	14.38 ± 3.17 (12)	61.60 ± 5.03 (12)	25.39 ± 4.85 (9)

Number in parenthesis denotes number of animals used to obtain these results.

All the enkephalins at the doses studies show P values ranging from P less than 0.05 to P less than 0.001 in relation to the saline pretreated group, which had an AT OF 80.12 ± 4.59 (12) u^2.

1 - After 100 ng B-EN icv.

2 - Administered 24 hours before challenge with B-EN.

D-ala2 enkephalinamide was of moderate potency in reducing the effects of B-EN and exhibited a graded dose response curve in reducing the effects of B-EN. Methionine enkephalin proved to be the least active of the pentapeptides. It reduced the effect of B-EN in a non-dose dependent fashion by less than 40%. It should be noted that the lowest dose of the enkephalins used was ten times higher than the standard dose of B-EN used to produce single-dose tolerance.

The control for this set of experiments consisted of a group of mice primed 24 hours previously with 10 µl saline. Saline did not signficantly reduce the effect of B-EN (Table II-7). There seemed to be a correlation between potency of the peptides to cause changes in body temperature and the potency to induce tolerance: B-EN being the most potent followed by leucine enkephalin and methionine enkephalin being apparently the least active.

2.3.7f Effect of ambient temperature on single-dose tolerance development.

Single-dose tolerance to B-EN's hyperthermia was not evident at ambient temperatures of 6 or 32 oC. The challenge injection of B-EN, 24 hours after the priming dose, evidenced that the AT produced by the first or second administration of B-EN were equivalent. This result is in mark contrast to the data obtained with mice maintained at 22oC (Table II-8), suggesting that at these extremes of temperature single-dose tolerance does not develop so readily.

Table II-8

Effect of ambient temperature on the hyperthermic response of B-Endorphin and single-dose tolerance development.

Pretreatment[1]		Area of Temperature[2] $\bar{X} \pm$ SEM (U^2) 100 ng B-EN icv	
6° C	10 ul Saline	50.84 ± 16.02	(6)
	0.1 ug B-EN	60.36 ± 12.92	(12)
22° C	10 ul Saline	67.63 ± 4.38	(24)
	0.1 ug B-EN	29.49 ± 6.25*	(26)
32° C	10 ul Saline	40.51 ± 5.90	(12)
	0.1 ug B-EN	31.36 ± 4.94	(12)

Number in parenthesis refers to the number of animals employed.

Control paired experiments exhibited an area of temperature to a challenge dose of 100 ng B-EN of 44.54 ± 9.03 U^2 (12) at 6° C, 66.27 ± 6.89 U^2 (39) at 22° C, and 30.80 ± 5.55 U^2 (12) at 32° C.

1 - Done 24 hours prior to the challenge dose, animals acclimatized to the new ambient.

2 - Challenge dose of 100 ng B-EN icv/mouse.

*P less than 0.05 as compared to the saline pretreated group.

2.3.8 Cross tolerance from morphine to B-endorphin.

Mice implanted with a 75 mg morphine tablet for 72 hours were markedly resistant to the hyperthermic response of B-EN. The basal body temperature of the tolerant-dependent animals was significantly lower than that of control saline treated mice. The mean body temperature of the tolerant-dependent mice was 36.5 $\pm$ 0.02 (n=60) as compared to 37.3 $\pm$ 0.07 oC for the control non-implanted mice (P less than 0.01). The administration of 100 or 1000 ng B-EN to morphine-tolerant mice showed that the characteristic hyperthermia observed the first after B-EN was virtually abolished at both doses. The data also showed some indication that in a morphine tolerant-dependent animal B-EN induced a hypothermic response rather than producing hyperthermia (Table II-9). The hypothermia was maintained for 6-8 hours and gradually returned to base line within 12 hours.

2.4 DISCUSSION.

The present results show that B-EN consistently produced a dose dependent and long lasting hyperthermic response. The hyperthermia had a rapid onset in action; 30 minutes after administration the increase in body temperature reached a maximal effect, that lasted for more than eight hours. The duration of the hyperthermic response was markedly more prolonged than the duration of its antinociceptive response, attained with higher doses of B-EN. (See paragraph 1.3.1b.) The prolonged duration of the hyperthermic response of B-EN was shared to some extent by

Table II-9

Effect of B-Endorphin on the hyperthermia response in morphine tolerant-dependent mice at various times after icv administration.

B-EN icv (ng/mouse)		Temperature Change ^{o}C ($\bar{x} \pm$ SEM) Hours after B-EN Challenge 1/2	1	2
100 in control	(12)	1.60 ± 0.197	1.34 ± 0.188	0.80 ± 0.161
100 in tolerant-dependent	(12)	0.14 ± 0.499**	-0.99 ± 0.678***	-1.35 ± .932*
1000 in tolerant-dependent	(12)	0.51 ± 0.359**	0.22 ± 0.390**	-0.30 ± 0.594*

Numbers in parenthesis denote the number of mice employed.

*P less than 0.05.

**P less than 0.01.

***P less than 0.005.

the enkephalins. This fact is in contrast to the extremely brief, transient, duration of antinociception caused by the enkephalins (Belluzi et al., 1976; Graff et al., 1976) with the exception of the synthetic analog D-ala2 methionine enkephalin (Pert et al., 1976). It is important to emphasize that in mice, significant changes in body temperature were observed at doses of B-EN that exhibited little or no antinociception. This fact would imply that mice are more sensitive, in a dose related fashion, to the hyperthermia produced by B-EN than to its well known antinociceptive effect.

The precise sites and mechanisms by which the endorphins increase body temperature are presently unknown. However, presents results allow to suggest that the effect is mainly of central origin. First of all, B-EN has a fast onset of action when injected icv. Secondly, the hyperthermia occurs at doses lower than dose required to cause antinociception, suggesting that B-EN must activate specific brain cells. Thirdly, the change in body temperature observed after opiates is not mainly related to locomotor activity. In fact, few if any morphine-like behavioral effects were observed at doses causing big changes in body temperature. Recently, Wei et al., 1977 have shown that B-EN even at doses of 40 nMol/kg produced little or no effects on mice locomotion. Paragraph 3.3.1 shows evidence indicating that doses of morphine producing a marked increase in locomotion are associated with an initial decrease in core temperature. In rats, it is documented that morphine causes hyperthermia at doses producing catatonia (Cox et al., 1976). To further investigate this aspect, an experiment was carried to study whether mice crowding-huddling could be partially responsible

for B-EN's hyperthermia. For this purpose, two groups of mice were injected with 100 ng of B-EN. One group was housed as usual, 6 mice per cage, and in the other group, each of 9 mice was isolated in a smaller plastic cage. There was virtually no difference in the AT induced by B-EN in both cases. In fact, the AT of the first group was 78.1 $\pm$ 9.2 u2 vs. 66.1 $\pm$ 11.6 u2 for the mice kept in isolation. Altogether, these results suggest that at least in mice, the hyperthermic response of B-EN is probably centrally mediated.

B-EN's site of action must be anatomically close to the ventricles, the site of injection, possibly in the pre-optic anterior hypothalamus as first suggested for morphine by Lotti et al. (1965a, 1966). This brain area is now well documented to be rich in opiate receptors. (For a review, see Snyder and Simantov, 1977.) The mechanism of action might be related, among other possibilities, to a resetting of the brain thermostat or mobilization of hypothalamic factors, some of which are known to be altered by morphine or B-EN (Cicero et al., 1977; Dupont et al., 1977a; 1977b). In preliminary experiments we have observed that the icv injection of 1-10 ug of thyrotropin releasing hormone causes hyperthermia of prolonged duration. However, the connection between these two effects remains unknown. On a general physiological basis, it is unclear if the effect of B-EN is due to a reduction in heat loss or an increase in heat production or a combination of both factors. In a more strict pharmacological-physiological sense, the mechanism of action of B-EN to produce hyperthermia is unknown, but a matter of great interest. In this study, several possibilities were raised, some of which

received experimental attention. These alternatives are discussed below.

To explore the possibility that B-EN might cause a resetting of the brain thermoregulatory center, experiments were conducted with mice maintained at different ambient temperatures. The rationale for these experiments being that if B-EN causes a change in the brain thermostat, hyperthermia should be observed independent of the environmental temperature. In the case of morphine, Lotti et al. (1965a, 1966) after careful stereotaxic studies with micro-injections of narcotics at specific brain sites in rats, proposed that the site of action of morphine was directly at the thermoregulatory center. The authors suggested that the hypothermic effect of morphine on rats was the result of lowering the hypothetical brain thermoregulatory set point on the anterior hypothalamus. However, Paolino and Bernard (1968) were unable to support experimentally this hypothesis. They observed that whereas systemic or intra-cerebrally morphine produced hypothermia at 5 and 24 oC, no hypo-thermia was observed at 32 oC. Our results clearly show that at all environmental temperatures B-EN caused a hyperthermic response; no evidence was ever observed of an hypothermic effect. These results further support the suggestion that the site of action of B-EN is central in origin, probably related to the mechanism of thermoregulation in the anterior hypothalamus. The total thermic response of B-EN (AT) might be somewhat reduced at both extreme temperatures as compared to results obtained at room temperature. This apparent antagonism might be attributed among other reasons to a stress factor associated to the animal's exposure into a new

environment. It is known that ACTH or cortisterioids antagonize the effects of opiate drugs (Zimmermann and Krivoy, 1973; Gispen et al. 1976). This same explanation might be responsible for the difference in tolerance development observed at both extreme temperatures. Recently, Guillemin et al. (1977) have shown that ACTH and B-EN are concomitantly secreted by the adenohypophysis in response to stress. This information provides a tentative explanation to the reduced effect of B-EN under conditions of stress.

It was challenging to study in more detail the mechanism of B-EN's hyperthermia. The experiments designed to investigate the effect of B-EN at different environmental temperatures established that the hyperthermic response is independent of ambiental temperature but did not provide an explanation as to its mechanism of action. Few drugs are known in mice to produce hyperthermia, apart from pathophysiological conditions. It appeared of interest therefore, to explore if the temperature rise produced by B-EN was due to, or at least related in part, to the liberation of endogenous prostaglandins. Prostaglandins are the only natural neurochemicals known in mice to produce hyperthermia (Hellon 1974, and also paragraph 3.35), apart from the peptides described in this study. In mice the exogenous icv administration of prostaglandin E1 or E2 produced a rise in body temperature of fast onset but of very short duration. These effects are documented in paragraph 3.3.5. To test the possibility that prostaglandins were related to B-EN's hyperthermia two prostaglandin synthetase blockers were used. Results were negative, implying

that prostaglandins are not mainly involved in the hyperthermic response of B-EN. As an internal control, these antipyretic-antiinflamatory drugs blocked fever produced by an external pyrogen. Results were clear cut, suggesting that B-EN does not activate prostaglandin synthesis to induce fever, in clear contradistinction to the mechanism of action of bacterial pyrogens or endotoxins in mammals. (For a review, see Feldberg, 1974.) This result, in addition to other data presented in this part of the project, could be interpreted to indicate that B-EN in mice might act directly at the thermoregulatory center of the anterior hypothalamus to produce hyperthermia. If B-EN plays an important role in central thermoregulation, it would be expected that B-EN had similar effects on body temperature in other mammalians. Recently Holaday et al. (1977) have presented evidence to demonstrate that B-EN produces a hypothermic effect in rats; Tseng (1978) has expanded Holaday's studies to show that B-EN has a biphasic effect on rat core temperature. Low doses of B-EN produce a marked hyperthermia of long duration of action, while higher doses of B-EN produce a drop in temperature as was first demonstrated by Holaday et al. (1977). The hypothesis that B-EN can produce a direct effect on the brain thermoregulatory center needs further experimental support. Experiments are currently in progress to substantiate, the hypothesis.

The hyperthermic response of B-EN appears to be an opiate specific effect. Three major experimental evidences support this conclusion. First of all, B-EN hyperthermia is dose dependent and blocked by naloxone, the opiate selective antagonist. The blockade, at least within the first 30 minutes of the combined agonist-antagonist

administration, suggests a competitive interaction between naloxone and B-EN. More refined experiments are needed to study this aspect in depth, especially with varying doses of naloxone, to determine a pA2 value for the agonist-antagonist interaction. Furthermore, naloxone administered half an hour after the injection of B-EN caused a partial reversal of the hyperthermia produced by B-EN. Interestingly, when naloxone was injected two hours after B-EN, it did not reverse the effects of B-EN. An interpretation of this finding is that B-EN could trigger in the CNS a process leading to hyperthermia so that when the mechanism has already been activated, the system is refractory to the blocking effect of naloxone. This data supports the hypothesis that morphine and endorphins could be acting by resetting the brain thermoregulatory center as previously discussed. This explanation could also help to understand the prolonged duration of the hyperthermic response, which apparently is much longer than the half life of B-EN in the CNS. Secondly, tolerance develops to the hyperthermia, a characteristic common to most of the narcotic drug effects. Single-dose tolerance produced by B-EN was found to be crossed to the enkephalins, supporting that both endorphins could share a common mechanism of action. In addition, single-dose tolerance development was antagonized in a competitive fashion by naloxone. This fact further supports other experimental studies that have shown blockade of the development of tolerance to morphine by the concurrent administration of naloxone (Lee et al., 1975; Mushlin and Cochin, 1976; Feinberg and Cochin, 1977) suggesting that the development of tolerance is related to the activation of an opiate receptor. Thirdly, mice rendered tolerant-dependent to the effects of morphine proved

to be dramatically resistent to the hyperthermic effects of B-EN. This is a very interesting point which, at present, can only be documented. Part 3 of this study is dedicated to study in depth the effects of morphine on mice body temperature. It appears that the hyperthermic response to morphine is different from that of B-EN. The time courses and magnitude of effects are markedly different. However, it is of interest that cross-tolerance is demonstrable. It is possible that morphine alters some neurochemical pathway(s) that is (are) common to both morphine and B-EN, counteracting the effect of B-EN. However, independent of the mechanism of action of B-EN and alkaloid opiates to produce changes on body temperature, present results considered altogether support that the hyperthermia produced by B-EN is an opiate specific effect.

Divalent cations have been recently described to play a pivotal role in the effect of narcotic drugs (For a review, see Ross et al., 1977). Kakunaga et al. (1968) demonstrated that the administration of Ca++ altered morphine's antinociception; recently Harris et al. (1975b) extended and amplified this observation and described that Ca++ or Mn++ antagonize the antinociceptive effects of morphine. It was of interest, therefore, to investigate whether Ca++ or Mn++ could antagonize B-EN hyperthermia. Results provided a clean negative answer. Neither the simultaneous administration of the cations with B-EN, nor icv pretreatment with Ca++ or Mn++ blocked B-EN's hyperthermia. These results can be interpreted to indicate that the thermic effect of B-EN is not Ca++ sensitive. Most likely, the hyperthermia has a different biochemical mechanism of action than that of the antinociceptive response of B-EN. The

mechanism of Ca++ in morphine's effect is unclear at present to allow ample discussion. However, it must be mentioned that McGilliard et al. (1976) postulated different opiate receptors for morphine antinociception and thermic effects, based on different morphine-naloxone pA2 values. The differential effect of divalent cations on morphine's antinociception and thermic responses could be related among other possibilities to a drug receptor interaction.

As a characteristic common to opiates, it was expected that B-EN developed tolerance and dependence on repeated administration. B-EN was no exception to this opiate property. It was previously noted that single-dose tolerance developed to the antinociceptive effect of B-EN (See paragraph 1.3.5b). Present results extend the finding of single-dose tolerance to the hyperthermic response of B-EN. Single-dose tolerance was characterized by its dose effect dependency and its particular time course. As was the case with antinociception, single-dose tolerance to B-EN or morphine required an adequate priming dose to trigger the production of tolerance. The priming dose of B-EN needed to induce tolerance to analgesia or hyperthermia was proportional to the effective dose 50 of each effect of B-EN. In both cases, a plateau was found, indicative of a particular kinetics of tolerance induction. There are some diffeences in the time course of the development of tolerance. Apparently, tolerance to antinociception reached a maximal effect considerably earlier (12 hours after the priming dose) than the peak tolerance developed to the thermal response, that occured at about 48 hours after the priming dose. See paragraph 1.3.3b. This apparent discrepancy might be a reflection of the well known

differences in rate of tolerance development to the effects of morphine in animals or humans (Jaffe, 1975). Recently, McGilliard et al. (1976) have demonstrated, based on PA2 values of morphine-naloxone interactions, that the opiate receptors for antinociception and hyperthermia in mice are different. Hence, the rate of tolerance development to analgesia and hyperthermia need not be the same. The important and common fact is a lag period of time required for maximal expression of the phenomenon of tolerance after the priming dose. The meaning of this lag time and the difference in lag time to these opiate effects is not clear. A priori it could be related to the kinetics of induction of single-dose tolerance in the different cell populations, since it is shown that tolerance is not a generalized phenomenon in its genesis but of rather selective induction.

The development of single-dose tolerance to the hyperthermic effect of B-EN was found to be associated with a certain degree of dependence. There is apparently a good correlation between the time required for maximal tolerance and the development of maximal singledose dependence, as evidenced by the almost identical time courses for tolerance and dependence. This correlation agrees with the fact that the development of tolerance to morphine is paralleled by the development of dependence (Way et al., 1969). Apparently, B-EN induces tolerance-dependence earlier or or easier than morphine. In a previous section it was discussed that following a single dose of morphine, little evidence of dependence was observed whereas after a single-dose of B-EN, signs of dependence were found. It must be emphasized that both dependence and tolerance are modest, yet demonstrable. It could be

argued that the dose of naloxone used to precipitate withdrawal is high and so unspecific. As a paired control, animals injected with saline icv were always used to control the effect of 10 mg/kg naloxone s.c. No behavioral effects were noted by naloxone alone.

These results add support to the notion that a single-dose of B-EN will trigger a process in the CNS to develop tolerance. The degree of tolerance developed is related to the concentration of narcotic drug exposed during the period of opiate administration. In this respect, it is remarkable that low doses of B-EN, almost completely devoid of antinociception, can produce tolerance after a first dose. This fact should be interpreted as an indication of the extraordinary potential of endorphins to produce tolerance and dependence. Present results support the data of Wei and Loh (1976) who reported that in rats 3-19 nMol of B-EN administered by constant icv infusion to the periaqueductal gray matter for 70 hours caused marked signs of morphine-like dependence when withdrawal was precipitated by a challenge dose of naloxone.

It is of special interest that the enkephalins, especially leucine and D-ala2 methonine enkephalinamide produced single-dose cross-tolerance to B-EN. Even though the doses of the pentapeptides are much larger than the doses of B-EN, it is surprising to observe that tolerance appeared to be of considerable magnitude as compared to the moderate hyperthermia observed at these doses of enkephalins. This data is in agreement with results from Pert (1976) who showed cross-tolerance between morphine and D-ala2 methionine enkephalin and to Wei and Loh (1976) that reported development of physical

dependence after continual brain infusion of B-EN or methionine enkephalin to rats.

In general, tolerance to the body temperature changes caused by narcotic drugs is a well known effect of morphine in rats. Gunne (1960) reported that tolerance lasted for more than two weeks after cessation of drug administration; Lotti et al. (1966) showed acute tolerance to the hypothermia produced by morphine following systemic or icv injection. Acute tolerance developed in a matter of hours after a priming dose of morphine and lasted for 10 or more days. This form of tolerance was blocked or diminished by the concurrent administration of nalorphine (Lotti et al., 1965b). With this experimental background, it is neither surprising that B-EN developed single-dose tolerance to its hyperthermia, nor the time course of its development is unexpected. It seems like the brain is particularly susceptible to tolerance in relation to the thermoregulatory effects of opiates. The mechanism of tolerance remains obscure. It was repeatedly observed that the maximal hyperthermia was produced within the first 60 minutes after administration of B-EN. In the case of the second injection of B-EN, it was noted that the maximal response was not significantly reduced, whereas the total time course effect was considerably shortened. This suggests that the endorphin receptors that trigger the change in body temperature are not the sites of tolerance but that tolerance might develop at sites following the receptor interaction. It appears as if the brain could still be able to recognize the concentration of peptide available but could "turn off" faster the hyperthermic response to the second dosage of B-EN. This effect could be explained by a faster

rate of brain metabolism of B-EN or to a structural/functional alteration of the brain physiology or biochemistry after the second dose. The second alternative seems more attractive from a pharmacological point of view, although we have no data to disprove the first possibility. Among the possible alterations, specific brain neurochemicals could be implicated in the development of single-dose tolerance as was demonstrated for morphine's single-dose tolerance (See paragraph 1.3.9). The first obvious candidates are brain neurotransmitters, specifically those shown to be involved in tempe rature regulation, such as catecholamines and indolalkylamines, acetylcholine, etc. (Hellon, 1974). It is known that morphine and endor phins alter the release of various transmitters from brain or periphery (Schaumann, 1957; Montel et al., 1974; Jhamandas et al., 1975; Henderson and Hughes, 1976; Taube et al., 1976; Loh et al., 1976a). Recently it has been demonstrated that Ca++ plays a pivotal role in narcotic effects (Ross et al., 1974; Harris et al., 1976; Ross et al., 1977). The development of single-dose tolerance might involve a Ca++ effect or a combination of neurotransmitters coupled to a Ca++ brain change. Experiments are in progress to explore these alternatives.

An important question that cannot be ignored at this point is whether B-EN plays a physiological role in thermoregulation. Present results are challenging and suggesting, especially based on the doses of B-EN used, the consistency of the response and its fast onset of action. Are endorphins involved in the progress of tolerance development to opiates? At present the answer is not at all clear. Simantov and Snyder (1976) have shown an increase in brain enkephalins during tolerance development. However, further investigations from the

same laboratory have not been able to confirm this data (Childers et al., 1977). Coming back to the initial question of a of a possible function of B-EN in temperature regulation, it is of keen interest to recall the temperature misadaptations of human addicts undergoing opiate withdrawal (Jaffe, 1975). Perhaps this aspect of opiate pharmacology might give us a clue to the understanding of possible involvements of B-EN in physiology and in opiate addiction. Experiments are in progress to examine this hypothesis in laboratory animals.

EXPERIMENTAL PART III :

COMPARISON OF THE EFFECT OF MORPHINE AND B-ENDORPHIN ON BODY TEMPERATURE IN NAIVE AND MORPHINE TOLERANT-DEPENDENT MICE: A RELATION TO A NEUROCHEMICAL MECHANISM.

3.1 INTRODUCTION.

The thermoregulatory effect of morphine in mice have not been adequately explored and it seemed of special interest to study this effect and compare it to that of B-endophin (B-EN), one of the most active endorphins. B-EN injected icv in mice produced a long lasting, dose dependent hyperthermia with a rapid onset in action, which was antagonized by naloxone (paragraph 2.3.3). In contrast, morphine injected icv produced a modest hypothermia followed by a rise in temperature that peaked about four hours after drug administration.

In this part of the project, we compare the effects of intraventricular or systemic injections of morphine and B-EN on body temperature regulation in naive and tolerant dependent mice. Experiments were also designed in an attempt to analyze this information in relation to understanding the neurochemical basis of this effect.

3.2 MATERIALS AND EXPERIMENTAL PROCEDURES.

3.2.1 Animals and chemicals.

Male adult ICR Simonsen mice (23-25 g from Simonsen Labs, Gilroy, California) were housed six per cage and maintained on standard laboratory diet and tap water. Mice were kept in the animal room, maintained at 22.5 ± 0.5 °C on a 12 hour artificial light cycle, lights on 6:00 am - 6:00 pm, for 3-4 days prior to the initiation of an experiment. Each animal group consisted in general of 6-12 mice each.

Morphine sulfate was purchased from Mallinckrodt Chemical Works (St. Louis, MO) and human B-endorphin (hB-EN) was furnished by Li et al. (1977). hB-EN was freshly diluted with sterile saline (Travenol) in plastic vials immediately before usage and kept on ice. Noradrenaline hydrochloride (NA), 5-hydroxytryptamine creatine sulfate (5-HT), carbachol chloride (CCh), dopamine hydrochloride (DA), histamine dihydrochloride (Hist) were purchased from Sigma Chemical Co. (St. Louis, MO). Naloxone hydrochloride was a generous gift of Endo Laboratories (Garden City, NY). Diphenhydramine hydrochloride was commercially available from Parke-Davis. All these chemicals were dissolved freshly before use in sterile saline (Travenol). Prostaglandin E1 and E2 (PGE1 and PGE2) were a gift from Dr. J. E. Pike from Upjohn Co., Co., Kalamazoo, MI). Prostaglandins were dissolved in 95% alcohol (10 mg/ml) and diluted from this stock solution using sterile saline. Drug solutions were sonicated for 30 sec. at room temperature immediately before testing. All drug doses refer to the base, excepting for when injections of morphine sulfate or naloxone hydrochloride were made systemically.

3.2.2 Measurement and quantification of body temperature in mice. A digitex 581-C digital thermometer with a temperature sensitive probe, lubricated with Simmonds baby oil, was carefully introduced about an inch into the mouse rectum. The probe was held in position for 20-30 secondsary to attain a stable reading. Mice were free to move in their cages, excepting at the time of temperature measurement when animals were held manually

for temperature recordings.

Experiments were generally started around eight o'clock in the morning. Basal mice body temperature was recorded, and immediately thereafter animals were injected according to the corresponding experimental protocol. Core temperature was measured 1/2, 1, 2, 4 and 6 or 8 hours after opiate administration. As a control, a group of mice from the same animal shipment was always injected with saline or the vehicle used for drug administration. Furthermore, in some experiments to avoid interference with the natural temperature circadian rhythm, measurements were made daily at about the same time. To diminish error contribution by individual animal variation, each animal served as its own control. For this purpose, core temperature was monitored following drug administration and compared to that obtained in the same animals a week after when injecting with vehicle (saline) at approximately the same time of the day. The temperature values obtained at each point of the time course from the drug treated group was subtracted from that of the animals injected with vehicle or saline to obtain a mean delta change.

Opiates and other drugs were injected intracerebroventricularly (icv) or intraperitoneally (i.p.). Mice were injected icv following the technique of Haley and McCormick (1957) as described earlier, see paragraph 1.2.2c. Drug solutions were either prepared freshly or used after storage overnight at -20oC in plastic vials. Drugs were dissolved in sterile saline (Travenol) when

injected icv. The volume of injection was 10 µl, measured with a 50 µl Hamilton syringe attached to a 27-gauge needle.

3.2.3 Effect of morphine and B-endorphin on body temperature.
Opioids were injected either i.p. or icv and core temperature was measured at fixed intervals as described below.

3.2.3a Intraperitoneal administration of morphine.
Six groups of mice (n=12) were injected with 0, 1, 5, 20, 30 and 60 mg/kg and the core body temperature was monitored at different intervals. Results were expressed as the mean ± SEM delta temperature change (o C), obtained at each time interval between the morphine treated vs. the vehicle (saline) treated group.

3.2.3b Intracerebroventricular administration.
Groups of 12 mice were injected with 0.5, 5 and 30 µg morphine sulfate/mouse or 100 ng hB-EN/mouse. As a control, the same animals were injected on the opposite side of the skull with 10 µl saline seven days after the opiate injection at approximately the same time of the day. The effect of the opioids for each mouse was evaluated calculating the difference in core body temperature obtained with morphine or hB-EN vs saline. The results are expressed as the mean ± SEM of the delta temperature change (o C).

3.2.3c Co-administration of morphine and hB-endorphin.
In three groups of 12 mice each, 100 ng hB-EN/mouse was injected icv simultaneously with 5, 20 or 30 mg/kg of morphine

i.p., and the time course of the effects on body temperature was determined. In an additional set of experiments, two groups of mice were injected with 100 ng hB-EN icv but the dose of 30 mg/kg morphine was delayed 30 or120 minutes. The time course of the thermic effect of each opiate was followed and compared to that of saline-saline treated control group.

3.2.4 Effect of naloxone on morphine body temperature changes. Groups of 12 mice were injected simultaneously with 5 mg/kg morphine i.p. and 1 mg/kg naloxone s.c. or 30 mg/kg morphine and 1 mg/kg naloxone s.c. The time course of body temperature was monitored. As a control, the same mice were administered a week later with 10 ml/kg saline i.p. The mean delta temperature change (oC) was obtained by subtracting the body temperature of each mouse after the morphine-naloxone treatment from that obtained after the challlenge with saline. The results of the morphine-naloxone treatement were compared to those of mice treated with morphine alone. In a separate series of experiments, two groups of eight mice were injected with 1 or 4 mg/kg naloxone s.c. and body temperature was measured and compared to that obtained in a control group challenged with 10 mg/kg saline s.c.

3.2.5 Effect of morphine or hB-EN on body temperature in morphine tolerant-dependent mice.

Mice were rendered tolerant dependent on morphine by the subcutaneous implantation of a 75 mg morphine pellet for 72 hours. This procedure described by Way et al. (1969) produces a high

degree of tolerance to and dependence on morphine. Basal body temperature was determined before and three days after pellet implantation and compared to that in mice implanted with placebo pellets but otherwise treated similarly. As controls, each set of experiments included a group of 12 tolerant-dependent mice injected with 10 µl saline icv. This control group was used to obtain the delta temperature change produced by the opiate. The results obtained with tolerant-dependent mice were compared to those of naive mice.

3.2.5a Intraperitoneal injection of morphine sulfate.

Two groups of 12 tolerant-dependent mice were administered with 30 or 200 mg/kg morphine i.p. and the time course of body temperature was followed and compared to that of a group injected with saline. The mean delta temperature change (oC) produced by morphine in tolerant-dependent mice was compared to that obtained in naive animals. Additionally, a group of tolerant-dependent mice was injected simultaneously with 30 mg/kg morphine and 1 mg/kg naloxone s.c. Additionally, one group received saline and the other received 1 mg/kg of naloxone s.c.

3.2.5b Intracerebroventricular administration of morphine and hB-endorphin.

Five groups of 12 mice each were administered with 10, 30 or 60 µg morphine sulfate icv/mouse or 0.1 and 1.0 µg hB-EN icv/mouse. The core body temperature was monitored as usual to obtain a time course of the opiate effect. An icv dose of 200 µg morphine /mouse resulted in 100% mortality within

30 minutes of administration.

3.2.5c Combined administration of morphine and hB-endorphin.

A group of 12 tolerant-dependent mice were injected simultaneously with 100 ng hB-EN icv and 30 mg/kg morphine i.p. A second group of mice received 100 ng hB-EN icv and two hours later 30 mg/kg morphine i.p. The body temperature time courses were monitored and compared to a saline (10 µl icv)-saline (10 ml/kg s.c.) treated group to obtain the mean delta temperature change produced by the opiates.

3.2.6 Effect of neurochemical manipulations on body temperature in naive and tolerant-dependent mice.

3.2.6a Naive mice.

All drugs examined were injected icv in groups of 8-10 mice. The neurochemicals and their icv doses (ug/mouse) were the following: Noradrenaline, NA (1.64, 5.4, 16.4, 41.0); Serotonin, 5-HT (12.6, 63.0 and 126.0); Carbachol, CCh (0.8, 2.4 and 8.0); Dopamine, DA (12, 60, 120); Histamine, Hist (1.5, 7.5, 15 and 30); Prostaglandin, PGE1 and PGE2 (0.01, 0.1, 1). Core body temperature was monitored immediately before and 1/2, 1 or 2 hours after administration of drugs. The results were analyzed by comparing the mean delta temperature change (oC) before and after drug administration or by comparing the temperature change of the drug treated group to a saline treated control group. Both methods of control yielded data that were consistent.

3.2.6b Morphine tolerant-dependent mice.

Animals were rendered tolerant-dependent by the implantation of a 75 mg morphine tablet as described in a preceding paragraph (3.2.5). After 72 hours of morphine implantation, groups of 8-12 mice were challenged icv with: 41 μg NA; 126 μg 5-HT; 0.8 μg CCh; 120 μg DA; 30 μg Hist; 1 μg PGE1 or or PGE2 and the mean delta temperature (oC) change produced 1/2, 1 or 2 hours after drug administration was determined.

3.2.7 Statistics.

The mean changes on body temperature between experimental and saline (vehicle) treated groups were analyzed statistically by the two tailed unpaired student "t" test, the significance level was set at a P value of less than 0.05.

3.3 RESULTS.

3.3.1 Effect of morphine or hB-endorphin on mouse core body temperature.

Morphine independent of the route of administration produced a complex effect on body temperature. Low doses of morphine (1-5 mg/kg i.p.) produced a dose dependent hyperthermic response that was maximal an hour after injection. Increasing doses of morphine produced hypothermia followed by a modest hyperthermia that peaked 2-4 hours after injection. The time time course of these responses is illustrated in Figure 3.1 and the maximal hyper and hypothermia effect of each dose is illustrated in Figure 3.2a.

Morphine injected icv produced a similar effect excepting that

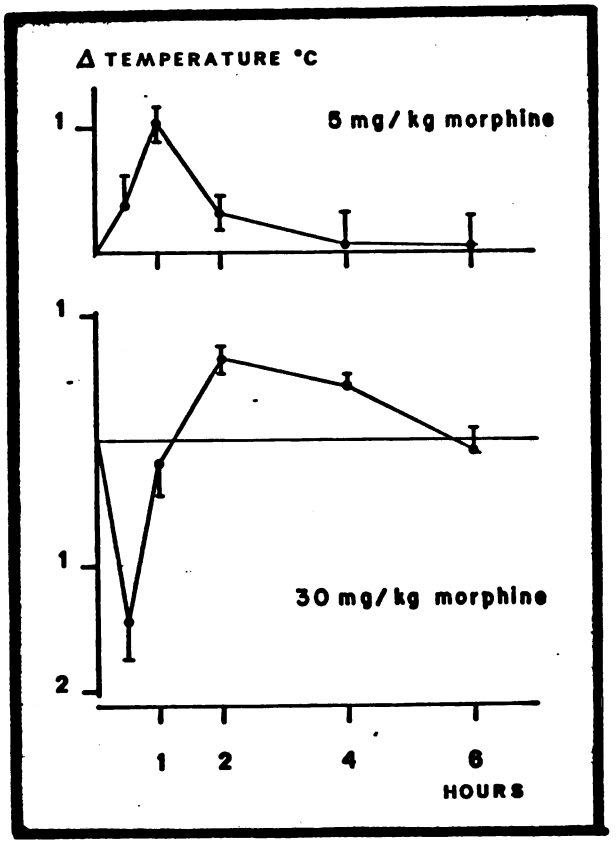

Figure 3.1

Time course of the thermic response of morphine in mice.

Mice were injected with 5 or 30 mg/kg morphine i.p., and the colonic temperature was compared to that of a control group of mice injected with saline i.p. The difference in colonic temperature between opiate and saline treated mice (delta temperature change in oC) was plotted versus time in hours.

Symbols represent the mean $\pm$ S.E.M. of the delta temperature change in each group of 12 mice.

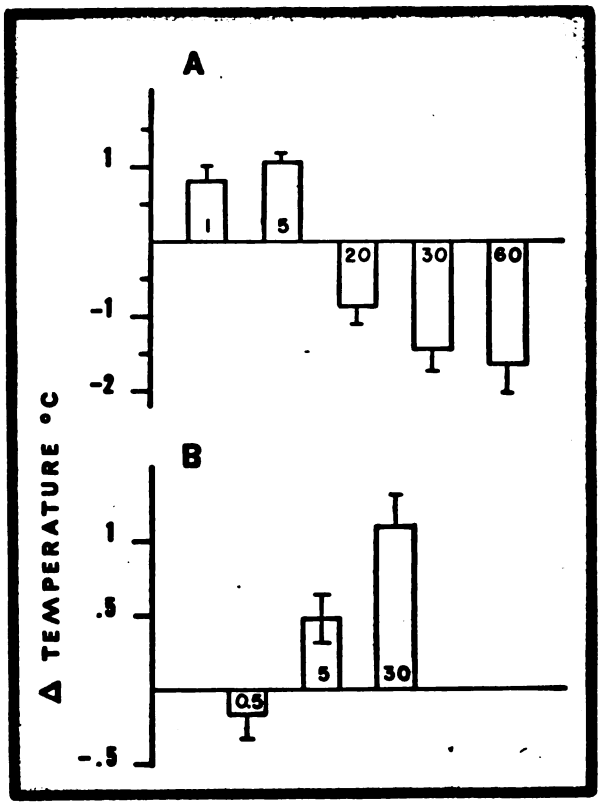

Figure 3.2

Dose effect relation of the hyper-hypothermic response of morphine in mice after i.p. or icv administration.

A. i.p. administration. Peak hyperthermic response occured at 60 minutes after administration; peak hypothermia at 30 minutes.

B. icv administration. Maximal temperature change (rise) occured 4 hours after injection.

Number refer to the dosage of morphine administered. In A, doses in mg/kg; in B, doses in ug morphine/mouse. Bars denote the S.E.M. of the temperature change, in groups of 12 mice each.

the hypothermia was markedly reduced as compared to the effect of morphine administered i.p. The hypothermia was followed by a slowly rising hyperthermic response that peaked 2-4 hours after injection. The peak hypothermia of 30 μg morphine was -0.34 ± 0.31oC as compared to -1.45 ± 0.27 oC for 30 mg/kg morphine i.p. (n=12 for each group). The maximal hyperthermic response of morphine obtained 4 hours after administration is shown in Figure 3.2b and is of about the same magnitude as that produced by morphine injected systemically.

In sharp contrast to the biphasic effects of morphine, hB-EN produced a prompt and long lasting rise of body temperature that peaked at about 30 minutes and decayed gradually in about 8 hours or more (see Figure 2.1).

The co-administration of 100 ng hB-EN and 5 mg/kg morphine i.p. did not produce additive hyperthermia (Figure 3.3a). However, the simultaneous administration of 20 or30 mg/kg morphine i.p. with 100 ng hB-EN produced hypothermia, of equal magnitude to that observed with morphine alone that was superseded a long lasting hyperthermic effect (Figure 3.3b). Apparently, morphine prevented the initial prompt rise in body temperature produced by B-EN, but once the hypothermia of morphine faded, the hyperthermia produced by hB-EN was evident. No clear additive hyperthermia of hB-EN + morphine was observed. When morphine was injected 30 or120 mins after hB-EN (at the peak or at the plateau of the hyperthermia) the rapid and pronounced morphine hypothermia

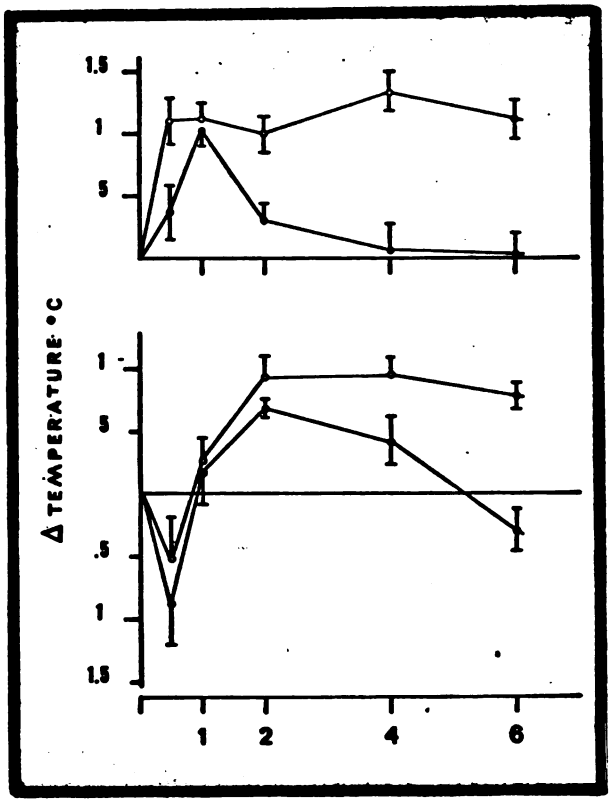

Figure 3.3

Time course of the temperature change following morphine and morphine plus hB-Endorphin.

Upper panel: Solid circles represent the the effect of 5mg/kg morphine i.p., open circles 5 mg/kg morphine plus 100 ng hB-EN/ mouse.

Lower panel: Solid circles: effect of 20 mg/kg morphine i.p., open circles idem plus 100 ng hB-EN/mouse.

Symbols represent mean of the delta temperature change. Bars represent the S.E.M. of 12 mice in each group.
For a control of 100 ng hB-EN alone, see Figure 2.1.

was still observed, but again this converted in approximately 60 minutes to a state of hyperthermia.

3.3.2 Effect of naloxone and diphenhydramine on morphine thermic responses.

Naloxone antagonized both the hyperthermic response of low doses of morphine as well as the hypothermic response of higher doses of morphine. The administration of 1 mg/kg naloxone s.c. in conjunction to 5 mg/kg morphine reduced the peak hyperthermic response of morphine (Table III-1). The same dose of naloxone also antagonized the hypothermia of 30 mg/kg morphine i.p. Under this condition, morphine produced an initial modest hyperthermia (Table III-1). Naloxone per-se at doses of 1 or 4 mg/kg s.c. did not produce a significant decrease in core body temperature.

In another experiment, the administration of diphenhydramine blocked the initial drop in body temperature produced by 30 mg/kg morphine i.p. The decrease in body temperature 30 minutes after morphine was -1.45 ± 0.27 oC (n=12), that was significantly reduced to -0.30 ± 0.33 oC (n=6) by diphenhydramine. Diphenhydramine per se cause a minor decrease in core temperature, which was of borderline significance.

3.3.3 Thermic responses of morphine and hB-endorphin in morphine tolerant-dependent mice.

3.3.3a Intraperitoneal route.

Complete tolerance was demonstrable to the body temperature and behavioral effects produced by injections of morphine in

Table III-1

Effect of 1.0 mg/kg naloxone s.c. on the thermic responses of morphine.

Dose of Morphine (mg/kg i.p.)	Temperature Change °C (x ± S.E.M.) 30 minutes saline	30 minutes naloxone	60 minutes saline	60 minutes naloxone
5	0.38 ± 0.23	0.22 ± 0.20	1.03 ± 0.13	0.31 ± 0.10*
30	-1.45 ± 0.27	-0.54 ± 0.24*	-0.19 ± 0.24	0.53 ± 0.15*

*$P < 0.01$ between naloxone and saline treated mice. Each group was composed of 12 mice each.

mice rendered tolerant by morphine pellet implantation. In tolerant-dependent mice, 30 mg/kg morphine i.p. did not produce hypothermia, but only a prompt and prolonged hyperthermia that was not only completely antagonized by 1 mg/kg naloxone, but transformed into a hypothermic response (Figure 3.4). No behavioral signs accompanied the hyperthermia of morphine. However, a dose of 200 mg/kg morphine i.p. produced the characteristic biphasic hypothermia-hyperthermia seen with higher doses of morphine, paralleled by modest behavioral signs. The injection of 1.0 mg/kg naloxone s.c. to morphine tolerant-dependent animals produced a slight but significant hypothermia of -0.55 ± 0.20 and -0.76 ± 0.27 oC at 30 or 60 minutes after administration.

3.3.3b Intracerebroventricular route.

A marked hypothermia was observed in tolerant-dependent mice by 10, 30 or 60 µg morphine /mouse. The hypothermic response of morphine in tolerant-dependent mice was significantly augmented as compared to the modest hypothermia observed in naive mice (Table III-2). The hypothermia was superseded 4 hours after the injection by hyperthermia.

The implantation of a morphine pellet produced an almost complete cross-tolerance to the hyperthermic effects of B-EN. The administration of 100 or1000 ng hB-EN in tolerant-dependent mice did not produce the characteristic prompt and long lasting hyperthermia but a brief transient rise in temperature that evolved into hypothermia within 2 hours of administration.

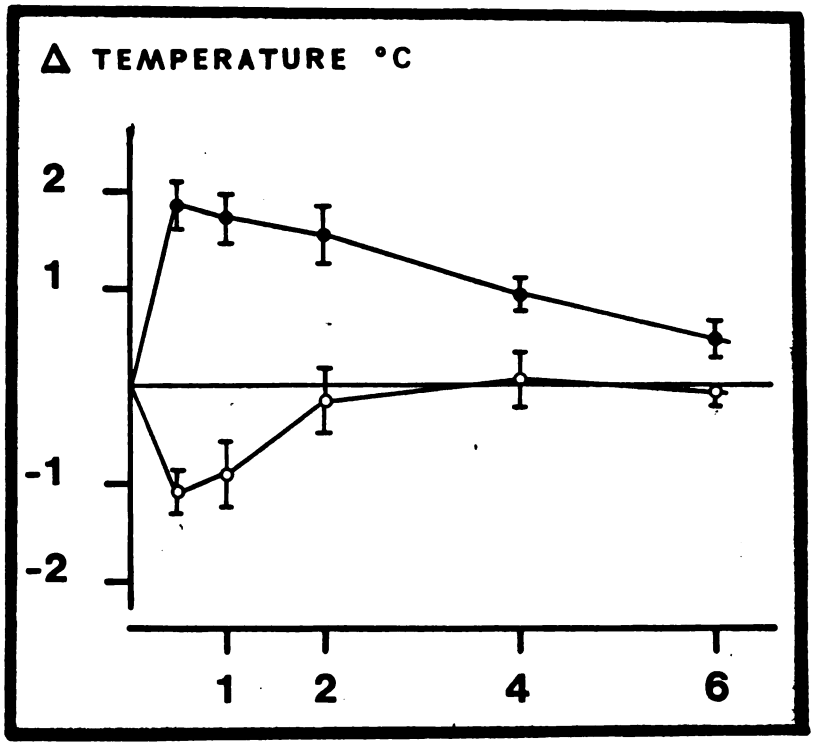

Figure 3.4

Effect of morphine and morphine plus naloxone on the colonic temperature of morphine tolerant-dependent mice.

Solid circles: 30 mg/kg morphine i.p.
Open circles: idem morphine plus 1 mg/kg naloxone s.c.
The effect of 30 mg/kg morphine i.p. alone, in naive mice is represented in Figure 3.1.

Symbols represent the mean, bars the S.E.M. of the delta temperature change in ^{o}C of 12 mice in each group.

Table III-2

Effect of intracerebroventricular morphine on colonic temperature of naive and morphine tolerant-dependent mice.

Dose of Morphine Sulfate (ug icv/mouse)	Temperature °C (x ± S.E.M.) 30 minutes	60 minutes	120 minutes
Naive mice			
0.5	-0.12 ± 0.20	0.13 ± 0.12	0.33 ± 0.07
5.0	-0.06 ± 0.19	-0.72 ± 0.19	0.17 ± 0.12
30.0	- .035 ± 0.31	0.17 ± 0.21	0.29 ± 0.11
Tolerant-dependent			
10	-2.01 ± 0.51	0.53 ± 0.33	0.56 ± 0.45
30	-3.36 ± 0.40	-1.72 ± 0.61	-0.61 ± 0.69
60	-1.88 ± 0.64	-2.08 ± 0.40	-0.12 ± 0.13

Each group was composed of 12 mice. Animals were rendered tolerant-dependent on morphine by the subcutaneous implantation of a 75 mg morphine pellet for 72 hours.

These results are presented in further detail in paragraph 2.3.8 and illustrated in Table II-8.

3.3.3c Joint administration of hB-EN plus morphine.

The combined administartion of hB-EN and morphine produced basically no alteration in core body temperature. The usual behavioral signs of morphine were not evident. Table III-3 shows the time course of the body temperature (oC) obtained in the the group treated with both opiates compared to that of mice injected with saline-saline. Furthermore, when morphine was injected 2 hours after the administration of hB-EN, no evidence of the hyperthermic effect of morphine in tolerants mice was observed within four hours.

3.3.4 Pharmacological modification of body temperature in naive and morphine tolerant-dependent animals.

Table III-4 summarizes the time course effect of the neurochemicals studied on the mouse body temperature. The doses selected were those that produced a marked and consistent temperature effect without causing apparent toxic behaviors. In naive mice, all the compounds studied produced dose related effects: 5-HT, CCh, DA, and Hist produced a marked hypothermia. PGE1 and PGE2 produced a hyperthermic response of short duration while NA produced an initial hyperthermia followed by a marked and prolonged hypothermia. In tolerant-dependent mice, the sensitivity to the chemicals studied was not dramatically affected. The only significant change observed was an intensification of the hypothermic responses of NA and Hist and a prolongation of the hyperthermic response to PGE2.

Table III-3

Effect of the coadministration of morphine and human B-Endorphin in morphine tolerant-dependent mice.

Treatment	Hours after Opiate Administration					
	0	1/2	1	2	4	6
10 uls saline icv + 20 mg/kg saline i.p. (12)	37.72 ± 0.24	37.28 ± 0.30	38.00 ± 0.27	38.03 ± 0.29	37.76 ± 0.18	37.96 ± 0.18
100 ngs hB-EN icv + 30 mg/kg morphine (12)	37.16 ± 0.48	37.02 ± 0.30	37.56 ± 0.39	37.58 ± 0.42	36.06 ± 0.30	37.05 ± 0.33

All animals were implanted with a 75 mg morphine pellet for 72 hours prior to the drug manipulations. Number in parenthesis refers to the number of mice per group.

Table III-4

Effect of neurochemicals on colonic temperature of naive and morphine tolerant-dependent mice.

Dose and Agent icv		30 minutes	60 minutes	120 minutes
41 ug NA	N (9)	1.17 ± 0.24	-1.63 ± 0.42	-1.67 ± 0.43
	T (9)	-0.61 ± 0.17*	-2.75 ± 0.27*	-3.96 ± 0.41*
126 ug 5 HT	N (8)	-3.61 ± 0.44	-3.21 ± 0.36	-1.68 ± 1.03
	T (10)	-3.01 ± 0.08	-3.16 ± 0.31	-2.08 ± 0.24
0.8 ug CCh	N (8)	-3.87 ± 0.56	-4.22 ± 0.52	-2.37 ± 0.39
	T (12)	-3.95 ± 0.23	-3.38 ± 0.33	-2.39 ± 0.48
120 ug DA	N (8)	-1.73 ± 0.62	0.26 ± 0.13	0.86 ± 0.13
	T (10)	-1.38 ± 0.63	-0.22 ± 0.50	0.90 ± 0.33
30 ug Hist	N (8)	-1.68 ± 0.27	-2.00 ± 0.34	-2.00 ± 0.41
	T (12)	-2.27 ± 0.38	-3.13 ± 0.41*	-3.95 ± 0.38*
1 ug PGE_1	N (6)	1.45 ± 0.36	-0.02 ± 0.27	-0.52 ± 0.06
	T (6)	1.50 ± 0.28	0.57 ± 0.14	-0.03 ± 0.36
1 ug PGE_2	N (6)	1.78 ± 0.15	-0.45 ± 0.18	-0.80 ± 0.19
	T (6)	1.91 ± 0.37	1.28 ± 0.27*	-0.15 ± 0.20*
10 ul saline	N (8)	-1.20 ± 0.24	-0.36 ± 0.24	0.71 ± 0.35
	T (6)	0.52 ± 0.30	0.08 ± 0.17	-0.78 ± 0.65

N = naive mice.

T = morphine tolerant-dependent mice.

Number in parenthesis refers to the number of animals used per group.

*P less than 0.05 as compared to naive, control mice.

The sensitivity to the other neurochemicals was unaltered.

3.4 DISCUSSION.

Morphine produces changes in body temperature that are dependent on the dose and animal species. (For a review, see Lomax, 1971; Lotti, 1973.) However, in most species studied morphine produces a fall in body temperature. This effect of morphine has been studied in rats and it is believed to be a stereoselective response of central origin. Lotti et al. (1965a, 1965b) elegantly showed that the direct injection of morphine and analogs into the rostral pre-optic hypothalamus caused a profound decrease in core body temperature. Two pharmacological properties characterized this drop in temperature. First the hypothermia was antagonized by the simultaneous injection of nalorphine and second, the injection of a second dose of morphine produced less or no effect, suggesting the development of tolerance after a single dose of morphine. Recent studies by Cox et al. (1976) extended this work and demonstrated that morphine in doses of less than 10 mg/kg produced hyperthermia in rats possibly by causing an elevation in the set point of central thermostats.

The present experiments show that morphine independent of the route of administration causes a biphasic response on body temperature. Moderate to high doses produce an initial drop in body temperature that is superseded gradually by a hyperthermia of slow onset. However, with lower doses of morphine (1-5 mg/kg i.p.) only a rise in core temperature is produced. These results obtained in mice are

are consistent with similar observations in rats. (For a review, see Lotti, 1973.)

The initial hypothermia produced by systemic administration of morphine can possibly be related to a peripheral rather than to a central effect of morphine. Two arguments substantiate this statement. First of all the hypothermia was counteracted by the simultaneous administration of morphine and diphenhydramine, supporting the contention that the drop in core temperature could be due at least in part to local release of histamine that causes vasodilation favoring heat loss, evidenced as a decrease in core temperature. Secondly, the intracerebral injection of morphine caused a much reduced hypothermia as compared to the i.p. route.

In contrast with the results on morphine a prompt and long lasting hyperthermia was caused by the administration of hB-EN. Part 2 of this study, describes that the hyperthermic effect of hB-EN is dose related and the effect is reversed and prevented by naloxone.

However, the time course of the peak responses of morphine and hB-EN are quite different. In rats, even when morphine is administered into the cerebral ventricles, maximal hypothermia did not occurr until 2-3 hours after injection (Lotti et al., 1965a). In mice, eventhough hyperthermia ocurred, there was nontheless a lag in the development of the peak response. On the other hand, hB-EN produced a prompt hyperthermia within less than 30 minutes of administration. These data may be interpreted to mean that morphine and B-EN

activate different receptor sites in the brain. However both the hypo-hyperthermic effects produced by morphine are blocked by naloxone indicating that all affected sites are opiate sensitive. The rise in corporal body temperature produced by hB-EN is also antagonized by naloxone. In support of this argument, Lord et al. (1976) and more recently Rossier et al. (1977) provide evidence suggesting the existence of different opiate receptor sites. Moreover, mapping for endogenous opiate ligands in the brain point to the possibility that different receptor sites may be activated separately by enkephalins and B-EN or morphine.

It has been known for a time that tolerance develops to the thermic effects of opiates. In rats the stereotaxic administration of morphine to the region of the anterior hypothalamus causes a decrease in body temperature. Tolerance develops to this morphine effect soon after the first administration of morphine (Lotti et al. 1966a). In addition, Gunne reported in 1960 the curious observation that in a rat tolerant to the effects of morphine, the further injection of morphine produced an effect opposite to that observed on the first administration of an opiate. For example, if a given dose of morphine in naive rats causes hypothermia, the same dose in a tolerant-dependent rat will cause hyperthermia. Cox et al. (1976) have observed the same phenomenon with a dose of 4 mg/kg of morphine that causes hyperthermia in naive rats but a second administration of the dose produced hypothermia. In mice, the results are analogous; 30 mg/kg morphine i.p. caused a biphasic (hypo-hyper thermia) effect on body temperature in a naive mouse, while in tolerant mice, the same dose caused a promt hyperthermia.

If morphine acts on the CNS to alter the set point of temperature control, as suggested by Lotti et al. (1965) or Lomax (1970), it is possible that as a result of tolerance development the set point (or the thermoregulatory center) is not only refractory to the effect of morphine but it also reacts to opiates in a much different way. The hypothermia produced by morphine in a naive mouse is probably mediated by a completely different neurochemical mechanism than that responsible for the hyperthermic response. Unfortunately, brain thermoregulatory physiology is a very complicated issue that depends on a constellation of variables, and multiple neurotransmitters are involved in its control. It is naive to believe that the effect of morphine could be explained by an alteration of one of these neurochemicals.

As an approach to elucidate the mechanism of the "inversion" of morphine effects in body temperature in tolerant-dependent mice, we undertook to study the effect of several putative neurochemicals postulated to be involved in thermoregulation. For this purpose, the effect of certain bioamines and prostaglandins on body temperature were compared in naive and in morphine tolerant-dependent mice. None of the chemicals elicited a change in effector sensitivity as dramatical as that produce by morphine itself. Histamine and noradrenaline induced in tolerant-dependent mice a significant enhancement of the hypothermic response. These results suggest that morphine probably interacts with more than one of these neurochemicals so that the end physiological result is a composite of the interaction with multiple sites and chemicals. On the other hand, results could be interpreted to indicate that noradrenaline and/or histamine might be

involved in the increased hypothermia observed to morphine in tolerant-dependent mice.

It is confusing to observe that in the tolerant-dependent animal the effects of morphine injected systemically are different from those obtained when morphine was injected icv. This indicates the complexity of the peripheral response of morphine which modulates the total effect produced by morphine. In the case of the systemic injection, it is difficult to evaluate the extent of peripheral versus central effects and tolerance; the final response corresponding to the net contribution between central and peripheral morphine effects.

The results obtained with neurochemicals on body temperature must be analyzed with caution, especially considering the complexity of the processes involved in the central control of body temperature. Recent studies by Jacob and Suaudeau (1977) in morphine tolerant animals have indicated that dependent rats are less responsive to the thermic effects of dopamine, and this favors the involvement of dopamine in the thermic effects of morphine. However, we did not observe significant changes in sensitivity to icv dopamine in naive as compared to morphine tolerant mice. These results emphasize that the thermoregulatory effects of neurotransmitters is markedly different from species to species (see Hellon, 1974).

In conclusion, based on the difference in the time courses between morphine and B-EN to achieve maximal changes on body temperature, it is reasonable to postulate that these opiates might have

different sites and mechanisms of action to alter body temperature in mice. The neurochemistry of these differential effects is currently under investigation.

EXPERIMENTAL PART IV :

DEVELOPMENT OF SINGLE-DOSE TOLERANCE AND DEPENDENCE TO OPIATE PEPTIDES IN THE GUINEA PIG ILEUM PREPARATION OF THE MYENTERIC PLEXUS.

4.1 INTRODUCTION.

It was of considerable interest to study if the condition of acute or single-dose tolerance to opiates observed in-vivo could also be demonstrated using in-vitro systems. If this were the case, the condition of single-dose tolerance could be generalized to different effector systems, laying a plausible foundation for a common mechanism of action.

The main purpose of this part of the project was to explore single-dose tolerance in an in-vitro system in order to investigate its kinetics of development. The myenteric plexus of the longitudinal muscle of the guinea pig ileum is a highly specific, sensitive, simple and reproducible in-vitro opiate model. This tissue shows a remarkable correlation between the potencies of opiates to inhibit the electrical twitching and to inhibit opiate binding to brain stereo-selective receptor material (Kosterlitz and Waterfield, 1975). Furthermore, this preparation has been extensively used to characterize the pharmacology of endorphins. The results indicate that under adequate conditions single-dose tolerance to B-EN and the enkephalins can be demonstrated in this preparation. Single-dose tolerance to B-EN was paralleled by the development of dependence. Dose effect curve analysis evidenced that the induction of tolerance in-vitro was dose dependent.

4.2 EXPERIMENTAL PROCEDURES.

4.2.1 Generalities.

Guinea pigs (400-450 g) from Marsh Farm, Gilroy, California

were fasted overnight before sacrifice. The morphine-like activity of the endorphins was studied in the guinea pig ileum myenteric plexus longitudinal muscle strip, that was prepared as described by Paton and Zar (1968) and Kosterlitz et al. (1970). Tissues were suspended at 37oC in a 10 ml organ bath containing Krebs-Ringer solution of the following composition in mMol/l: sodium chloride 118, potassium chloride 4.75, calcium chloride 2.54, potassium monophosphate 1.19, magnesium sulfate 1.2, sodium bicarbonate 25, glucose 11, choline chloride 0.02 and diphenhydramine 0.0001, and gassed with 95% oxygen, 5% CO2. Isometric muscular contractions were registed by means of a Grass force displacement transducer (model FT 03C) coupled to a polygraph. The tissues were maintained at 0.5 g of tension during the course of the experiment. Preparations were allowed to equilibrate for an hour before initiating electrical stimulation and during this period the tissues were washed 3-4 times every 15 minutes. At the end of each drug addition, the tissues were likewise washed before adding another concentration. Suitability of the preparations was determined by challenge with 4.5×10^{-7} M Ach to determine the sensitivity of each strip. The strips were then stimulated through 2 ring platinum electrodes with supramaximal pulses (80 volts, 5 m secs of duration) at a frequency of 0.1 Hz. Pulses were generated by a Grass S-4A stimulator. The strips were stimulated until a stable twitch was obtained. In general, the twitch tension was about 1.5 to 2g, that was maintained for the first 2 hours and decayed slowly thereafter Opiates and other drugs were added in a volume of 0.1-0.3 ml. Diffusion was complete within 2 seconds of drug application.

4.2.2 Dose response curves and determination of ID50.

Opiates were added for a period of two minutes after which the preparations were washed 3-4 times and allowed to equilibrate to recover to the previous muscular twitch tension. The opiate activity was expressed as percentage of inhibition of the electrically induced muscular twitch. The concentration of opiate to inhibit the muscular twitching by 50% (ID50) was estimated by interpolation of the log dose response curves. The 95% confidence limit of the ID50 were calculated according to Litchfield and Wilcoxon (1949).

4.2.3 Development of single-dose tolerance.

To study the development of single-dose tolerance, the following procedure was followed. Challenge doses of opiates were applied and muscular twitches were recorded continually for 30 minutes at which time the preparations had recovered to at least 95% of the initial control tension. At this time a second dose of opiate was applied and its effect was compared to that produced by the first dose of opiate. The preparations were not washed until after the application of the second opiate. Tolerance would be established if the second application of an opiate produced less of an inhibitory effect than that produced by the priming dose. Dose effect curves were performed to characterize single-dose tolerance. For this purpose, different priming doses of the opiates were used; each strip was used to study the effect of 3 priming doses spaced in a six hour interval. The preparations were washed 3-4 times at 10-15 minute intervals and allowed to recover to previous control tension.

Results were plotted as the percentage difference of the opiate effect vs. the dose, or as dose effect curves of opiate activity. For this particular series of experiments, the statistical analysis used was a two tailed paired student "t" test. Significance was set at a P value less than 0.05.

4.2.4 Effect a tetanic stimulation on opiate responses.

Increasing the frequency of electrical stimulation from 0.1 to 10 Hz produced a muscular tetanus. Then upon reverting the frequency of stimulation to 0.1 Hz, a lesser twitch response was observed, suggesting the release of an endogenous opiate-like material. This effect is reversible and blocked by naloxone or other selective narcotic antagonists (Puig et al., 1977a). This protocol was used to investigate the occurrence of cross-tolerance between the effect of the opiate-like material released by the tetanus and the effect of exogenous opiates. The responsivity with 2×10^{-7} M normorphine or 2.1×10^{-7} M B-EN was determined and the drug was removed by washing. The preparations were equilibrated until recovery to 100% control base line twitch tension; then the frequency of electrical stimulation was increased from 0.1 to 10 Hz for five minutes. Thirty-five minutes after the tetanus, the decrease twitch tension to 0.1 Hz spontaneously recovered, without washing, to 85 $\pm$ 2% of the original tension. Estimation for development of tolerance was determined by readdition of the challange dose of B-EN or normorphine. On completion of these studies, the drug was left in contact with the tissue for 20 minutes, and then washed. The electrical stimulation was turned off and the tissues were

applied with 2×10^{-7} M naloxone to determine if physical dependence had developed in parallel to single-dose tolerance. For statistical analysis, the paired student "t" test was used. Significance was set at a P value less than 0.05.

4.2.5 Opiates used and their sources.

Methionine enkephalin (M-enk), leucine enkephalin (L-enk), D-ala2-methionine enkephalinamide (D-ala2-M-enk) were purchased from Bachem (Marina del Rey, California). Human B-endorphin was a generous gift of Prof C. H. Li and normorphine was generously supplied by Dr. E. L. May from NIH. Fresh opiate peptide solutions were prepared daily. The other drugs were dissolved with distilled water in plastic mini vials. Solutions were sonicated for 15 seconds prior to use and kept on ice. The narcotic dosages are expressed in terms of the final molar concentration of the opiate base. Inorganic reagents and glucose for the Krebs-Ringer solution were purchased from Mallinckrodt (St. Louis, Missouri). Naloxone was a gift from Endo Laboratories (Garden City, New York).

4.3 RESULTS.

4.3.1 Morphine-like activity and potency of opiate peptides.

All the opiate peptides studied produced a dose dependent inhibition of the electrically induced muscular twitching of the guinea pig ileum. The depression of the muscular contraction was in all cases reversible by naloxone, confirming that this response is a morphine-like effect. B-EN and M-enk were the most active of the opiate peptides; their ID50 values were

95 nM. D-ala2-M-enk and L-enk were about equally active, but 4 times less potent than the other peptides (Table IV-1). Morphine and normorphine were as active as M-enk. These results are in complete agreement with previous data by Hughes (1975), Hughes et al. (1975a), Waterfield et al. (1977), Puig et al. (1977c) establishing that in the guinea pig ileum M-enk is as potent as normorphine or morphine and about 4 times more active than L-enk. The relative potency of these opiates expressed as the ratio of the ID50 of normorphine divided by the ID50 of the other narcotics (Table IV-1), is also in full agreement with recent data of Doneen et al. (1977).

4.3.2 Fade response of opiate peptides and the development of single-dose tolerance.

In agreement with the initial report of Hughes (1975), it was observed that the opiate response faded (Figure 4.1). The muscular twitch reached promptly pre-drug, control tension even though the preparation was not washed to eliminate the peptide. The time needed for recovery to the initial twitch tension was proportional to the dose of the opiate applied. As an example, the time required by 1.1×10^{-7} M L-enk to reach 100% recovery was 13 ± 1 min (n=5); while 6.8×10^{-7} M L-enk required 18.4 ± 1 min (n=7). Normorphine and D-ala2M-enk did not exhibit fade. These particular drugs did not evidence a significant decrease in the opiate effect for up to 30 minutes following application of the doses studied.

B-EN, M-enk, and L-enk produced single-dose tolerance that was dose dependent. The addition of a second dose of the peptide

Table IV-1

Inhibitory effects of opiate alkaloids and peptides on the electrically induced muscular twitch of the guinea pig ileum.

	$ID_{50} \times 10^{-7}M$ ($\pm$ 95% C.L.)[a]	Relative Potency[b]
Normorphine (6)	0.70 (0.21 - 2.31)	1.00
Morphine (10)	1.00 (0.35 - 2.84)	0.70
Methionine enkephalin (8)	0.95 (0.22 - 4.16)	0.73
Leucine enkephalin (8)	4.00 (1.08 - 14.72)	0.17
D-ala_2 methionine enkephalin (11)	3.25 (0.75 - 14.00)	0.21
hB-Endorphin (4)	0.95 (0.17 - 5.27)	0.73

Number in parenthesis indicates the number of experiments.

a
95% confident limits.

b
Potency relative to normorphine, ratio = 1.0.

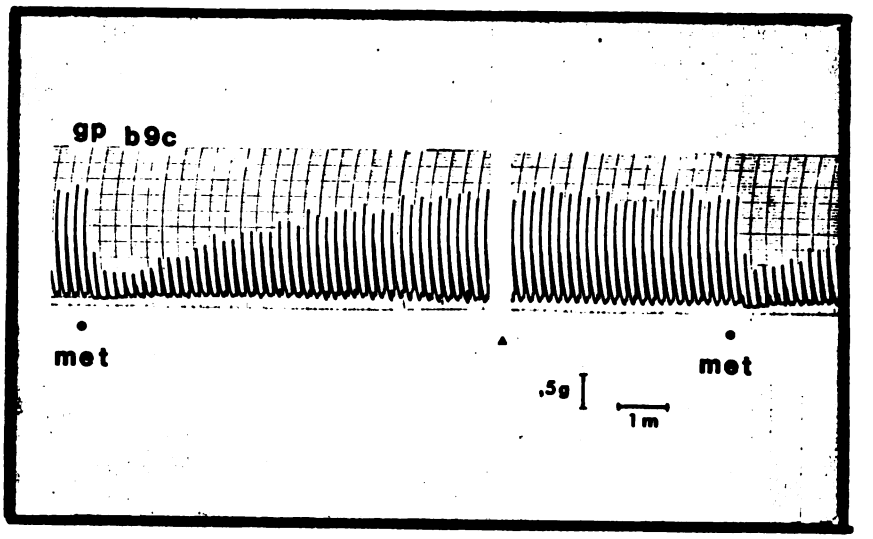

Figure 4.1

Effect of methionine enkephalin in the longitudinal muscle of the guinea pig ileum.

Dots indicate the application of 4.4×10^{-7} M M-enk. The first application of the peptide reduced the electrically induced twitching to 22%. The opiate response was not maintained, but faded rapidly to complete recovery. Twenty minutes after the application of enkephalin, a second dose was applied without washing the preparation; it reduced the twitching by only 37%. Calibrations at the right corner: tension in grams, time in minutes. At the triangle, the recording was stopped.

produced less inhibition as compared to the effect of the first opiate application. As an example of the experimental design and the opiate effects described, see Figure 4.1. A typical protocol used to study acute tolerance is illustrated in Figure 4.2. It is apparent that the second application of B-EN produced about 20% less effect than the first application. Evidence was also obtained that the induction of tolerance was associated with physical dependence. A challange dose of 200 nM naloxone produced in 3 out of 4 of the tolerant preparations a muscular contraction. Naloxone did not produce a contraction in similar naive, non-opiate treated, preparations.

Tolerance was dose dependent. Figure 4.3 shows a dose effect curve of the development of single-dose tolerance to B-EN. Even though the effect is of low magnitude, it was consistent and of statistical significance. The results for similar experiments attempting to produce single-dose tolerance in the guinea pig ileum to M-enk and L-enk are presented in Table IV-2. Plotting this data as dose effect curves demonstrated that the first application of the opiate shifted to the right and in a parallel fashion the effect produced by the second application of the peptide. In the case of L-enk and M-enk the ID50 was shifted 2.3 and 3.6 fold respectively. B-EN was the most active of the peptides in producing tolerance; it increased significantly its ID50 value 5 fold. Single-dose tolerance was not applicable to the opiate alkaloids nor to D-ala2-M-enk since they did not exhibit fade.

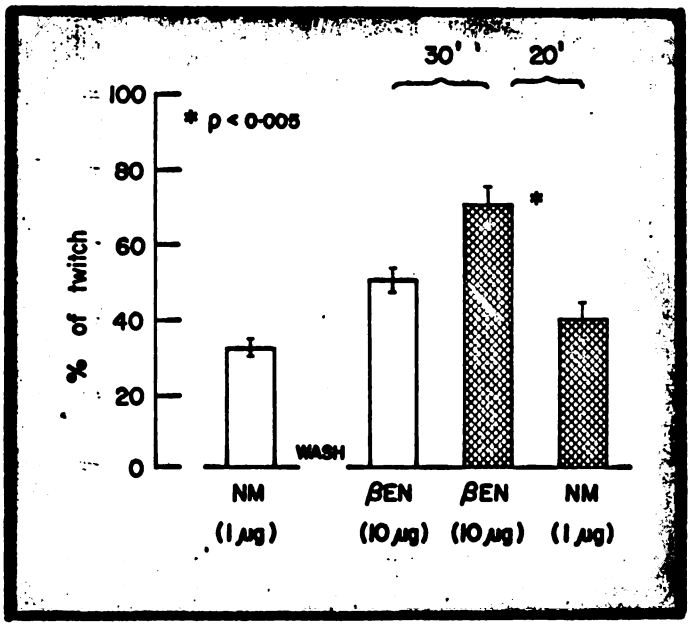

Figure 4.2

Single-dose tolerance to B-Endorphin.

First column to the left, effect of 2 x 10^{-7}M normorphine(NM). Second column, effect of 2.1 x 10^{-7}M hB-EN. The effect of B-EN faded to 100%. Thirty minutes following the peptide, a second dose of hB-EN was applied. The response was much reduced and faded in less than 20 minutes at which time the preparations were rechallenged with normorphine.

Bars represent the S.E.M. of 8 different preparations.

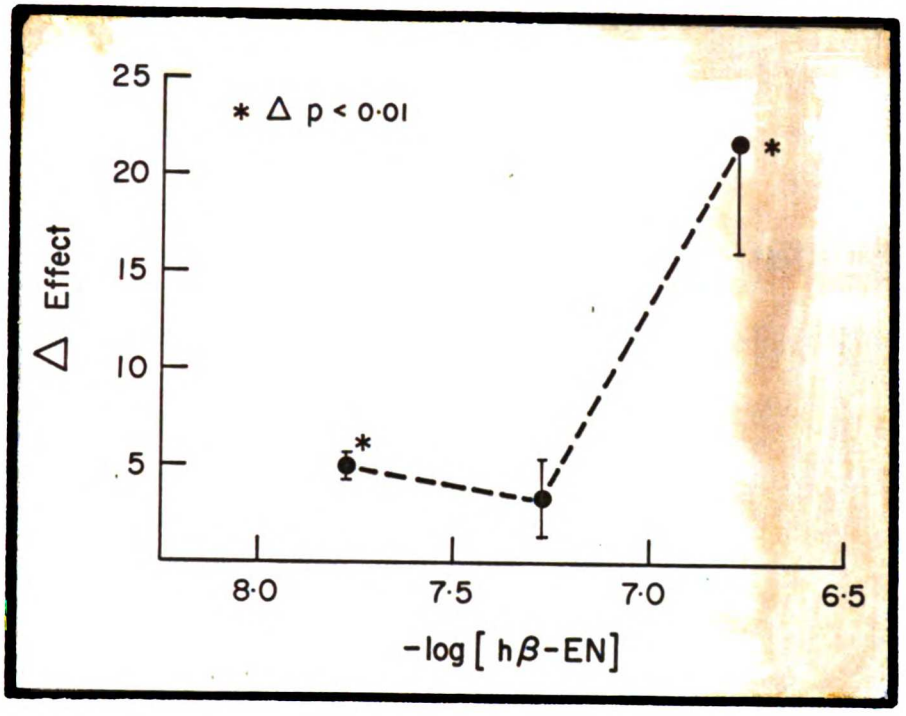

Figure 4.3

Dose effect relation of single-dose tolerance to B-Endorphin.

Log-dose plot of tolerance development. Abscissa dose of hB-EN, ordinate: % of the difference in opiate response between the effect of the first and second dose of hB-EN.
Circles denote the mean, bars the S.E.M. of four preparations in the case of the two lower doses of B-EN, and eight samples in the case of the larger dose of B-EN.

Table IV-2

Single-dose tolerance to methionine and leucine enkephalin in the guinea pig ileum.

Agonist	Final (M)	n	Opiate Activity First Application	Second Application
M-enk				
	5.48×10^{-9}	8	62.8 ± 5.5	72.4 ± 3.5
	1.82×10^{-8}	8	39.7 ± 5.4	49.5 ± 5.9*
	4.40×10^{-7}	9	35.0 ± 2.9	49.6 ± 3.9**
L-enk				
	3.0×10^{-9}	3	86.4 ± 1.3	89.0 ± 2.0
	1.10×10^{-8}	5	59.9 ± 0.2	69.1 ± 7.4
	3.0×10^{-8}	9	52.5 ± 7.3	58.1 ± 5.4*
	6.8×10^{-8}	7	41.1 ± 6.0	45.3 ± 7.6

n - Refers to the number of GPi LM preparations studied in each case.

*P less than 0.05.

**P less than 0.01 by the double paired students "t" test.

4.3.3 Cross-tolerance between the opiate-like material released by tetanic stimulation and exogenous B-endorphin.

The post tetanic opiate-like activity faded rapidly analogously to the effect produced by the exogenous application of endorphins. Cross-tolerance was established between the opiate-like material released by tetanus and the addition of B-EN, as revealed by a significantly reduced opiate effect obtained after the tetanus (Figure 4.4 and 4.5). The reduction in the opiate activity was about 20%, for both B-EN and normorphine. The application of 0.2 uM naloxone produced a muscular contraction in four out of seven of the preparations evidencing some degree of dependence.

4.4 DISCUSSION

The present results indicate that in the guinea pig ileum M-enk is about 4 times more active than L-enk or D-ala2-M-enk and that M-enk and normorphine are equipotent. Results also indicate that B-EN is as active as normorphine in the guinea pig ileum assay; its ID50 value was about 95 nM. Since the demonstration of Waterfield et al. (1976) of cross-tolerance in the guinea pig ileum between morphine and the enkephalins, it is generally assumed that all the opiates interact in the myenteric plexus of the guinea pig ileum at a common receptor site. This hypothesis is supported by experimental evidence indicating that naloxone antagonizes the effect of both the opiate-alkaloids and the peptides in the guinea pig ileum with the same dissociation constant (Waterfield et al., 1977) or as recently shown by Vaught and Takemori (1977) enkephalins and morphine

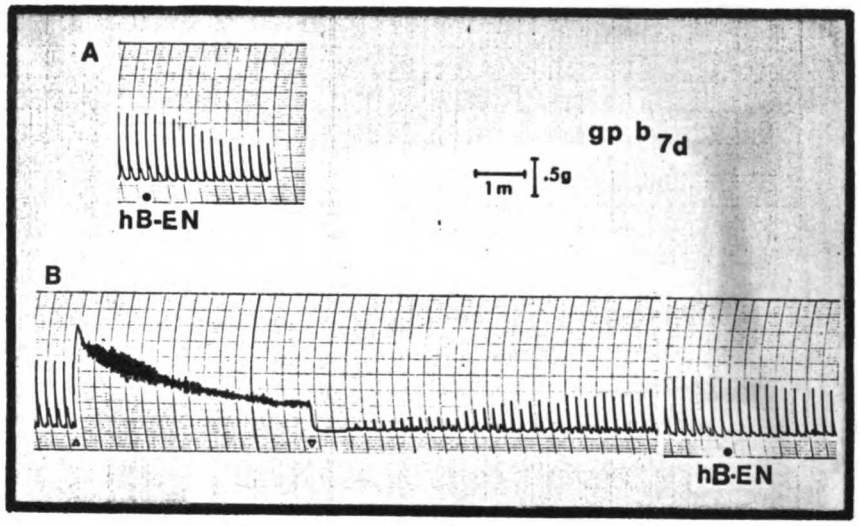

Figure 4.4

Tetanization of the guinea pig ileum longitudinal muscle, and effect of exogenous hB-EN.

A. At the dot, application of 2.1 x 10^{-7}M hB-EN and rapid wash. The opiate produced a 50% reduction of the twitch tension.

B. Tetanus applied for 5 minutes. Prior to the tetanus, 10^{-5}M choline was added to the bath. This particular preparation recovered the twitch tension in thirty-five minutes to 86% of its initial tension. The second application of hB-EN reduced the twitch by 14.3%.

Between A and B 30 minutes.
Calibrations in A, valid for both panels: tension in grams, time in minutes.

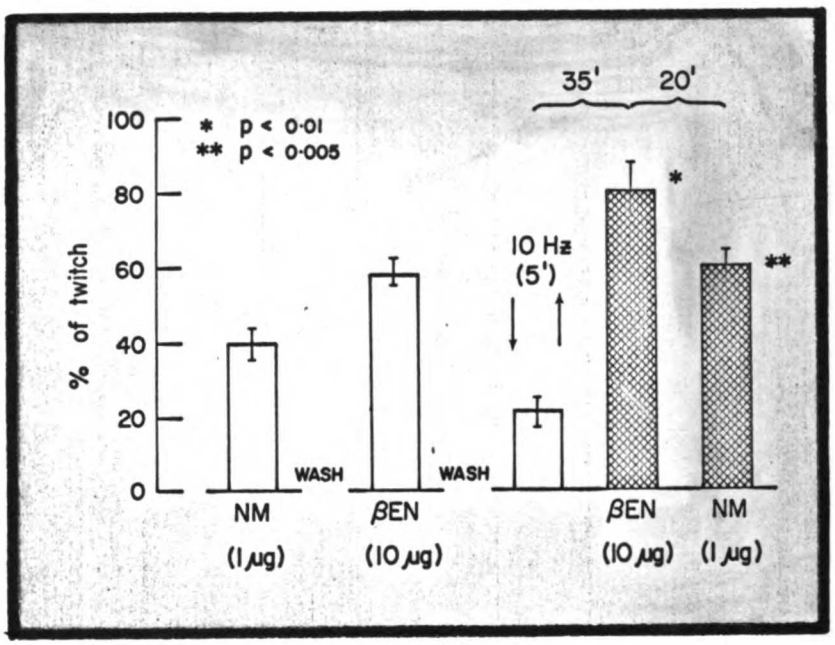

Figure 4.5

Development of cross-tolerance between the post tetanic opiate-like effect and exogenous B-Endorphin.

Tissues were challenged with 2 x 10^{-7} M normorphine (NM), and 30 minutes later challenged with 2.1 x 10^{-7} M hB-EN. Tetanus was applied for 5 minutes, see arrows. The addition of a second dose of hB-EN or normorphine produced a significantly reduced effect as compared to its control prior to the tetanus.

Columns represent the % of electrical twitching, bars the S.E.M. derived from 8 different preparations.

have a same opiate naloxone pA2 value. From a pharmacological point of view, this fact is of great interest especially considering the differences in molecular sizes of these opiates (more than a 10-fold difference in molecular weight between morphine and B-EN), and their chemical nature.

Single-dose tolerance to the opiate peptides is demonstrable in the guinea pig ileum provided that some experimental requirements were fulfilled. A fundamental condition required in the guinea pig model to study the development of single-dose tolerance was that the preparation should be allowed to recover from the effect produced by the priming dose without washing, prior to the addition of the second opiate. This condition should simulate the in-vivo situation where the second opiate dosage is administered at a time when no opiate effects are detectable after the priming dose. Fortunately, the opiate activity of the peptides faded so that generally within less than 30 minutes of administration, the twitch tension fully recovers.

It is of interest to discuss the nature of fade and its relation to acute tolerance. Theoretically, there are various alternatives to explain the fade. It could be due to desensitization, to a metabolic inactivation, to a process of drug redistribution, etc. It is highly unlikely that the fade response is due to a desensitization type phenomenon. First of all, the time to 100% recovery (fade), was proportional to the dose of the opiate peptide. If fade were due to desensitization, an inverse time-dose relationship should be expected. Furthermore, a second dose of the peptide should have been completely ineffective rather than producing a diminished response. Of the two remaining

alternatives, it is possible to postulate a metabolic inactivation or degradation of the peptidfe as a cause for fade. It is well known that opiate peptides are enzymatically degraded within minutes both in-vivo and in-vitro (Hughes, 1975; Hughes et al., 1975a; Kromer et al., 1976; Hambrook et al., 1976; Puig et al., 1977c). If this were so, a direct relationship should be found between the time to 100% recovery of the twitch and the doses of the peptides. The present results substantiate this contention. Furthermore, D-ala2-M-enk, an opiate resistant to enzyme degradation, should show no fade. This was exactly the case found experimentally. This data can be interpreted to indicate that fade is mainly due to enzymatic degradation of the peptides rather than to desensitization or redistribution of the opiate in the tissue.

However, if fade can be explained based mainly on the basis of enzymatic degradation occurring in-vitro, the development of tolerance can not be explained by a degradation metabolic process. The argument is based on the assumption that if the opiate activity faded completely due to tissue metabolism, it should be expected that the second application of the opiate should be fully active. This was not the case since the preparations consistently showed less of an opiate effect to the second administration. Obviously, something more is involved. It seems highly probable that the reduction of the response was related to the development of acute tolerance. As an evidence that this might in fact be the case, 75% of the preparations that exhibited tolerance to the application of 2.16×10^{-7} M B-EN responded to the addition of naloxone with a muscular contraction indicating a certain degree

of dependence. This particular effect of naloxone has been interpreted to be a withdrawal sign in the guinea pig ileum since the response does not occur in naive, nonopiate-treated preparations (Ehrenpreis et al.,1972; Villareal and Dummer,1973; Frederickson et al.,1976). In summary,the data shows evidence indicating that the decreased effect produced by the second application of opiate peptides in the guinea pig ileum is not due to a fade artifact, but it rather favors the possibility of true opiate tolerance development.

The 2-5 fold degree of tolerance obtained with a single dose of opiate peptide is not comparable to that obtained in guinea pigs implanted with morphine pellets for 3 days. In the latter case, a 3-10 fold tolerance was reported by Goldstein and Schultz, 1973; Schultz and Herz, 1976; Cox and Padhya, 1977. However, it is possible that since tolerance is dependendent on the priming dose, that the degree of tolerance could be increased if the tissues were continually exposed to the narcotic drugs.

In our studies we have avoided the procedure of repeated exposure to narcotics to produce tolerance, to circunvent the equivocal results reported by Fennessy et al. (1969). The present data does not offer an answer to the question whether normorphine or D-ala2-M-enk are capable of producing acute tolerance. Since this two opiates did not exhibit a fade response, it was not possible to study tolerance using the present model system. However, this does not mean that they do not produce acute tolerance. The use of a more adequate model, like the one developed by Schoham and Weinstock (1974), where the drug concentration was constantly maintained by

continous perfusion would be suitable to solve this aspect. However, based on theoretical considerations that acute tolerance is but an early manifestation of the chronic phenomenon, it is very possible that the opiate alkaloids will induce an acute state of tolerance that could be increased or accelerated during continual exposure to narcotics. Tolerance to opiates in the guinea pig ileum is well established, although its mechanism has not been extensively studied.

It is possible that following the application of a tetanus to the longitudinal muscle of the guinea pig ileum, a morphine-like substance is liberated from the myenteric plexus and appears in the incubation fluid. The chemical identity of the substance is unknown; preliminary data by Schultz et al., (1977) favors a peptide, but evidence is not conclusive. Evidence supporting this hypothesis has been described by Puig et al. (1977a, 1977b). They interpreted the post tetanic opiate activity to be due to the release of an opiate-like material from the myenteric plexus. In support of this conclusion, present results show cross-tolerance between the putative endogenously released opiate and normorphine or B-EN applied exogenously. These results are qualitatively and quantitatively similar to those obtained after two consecutive peptide applications to the ileum preparation. These findings add pharmacological support to the hypothesis for the release of an endogenous opiate from the myenteric plexic upon sustained electrical stimulation.

EXPERIMENTAL PART V : APPENDIX

5-HYDROXYTRIPTOPHAN AND THE DEVELOPMENT OF SINGLE-DOSE TOLERANCE TO THE ANTINOCICEPTION OF B-ENDORPHIN.

A. 1 INTRODUCTION.

Recently, Meglio et al. (1977) described that after a single intracerebroventricular administration of B-endorphin (B-EN) in cats, an acute state of tolerance to its antinociceptive effects developed. This type of acute tolerance was prevented by the administration of 5-HTP. It was shown that the injection of 5-HTP could restore almost completely the antinociceptive response to B-EN, once single-dose tolerance to B-EN was produced. This observation was the starting point of this study. The objective of this part of the project was to repeat the interaction of B-EN and 5-HTP in mice and to gather more information about the mechanism of 5-HTP in B-EN tolerant dependent mice.

A. 2 MATERIALS AND METHODS.

Camel B-endorphin (B-EN) prepared by synthesis, was furnished by Li et al. (1976b). B-EN was dissolved in sterile saline (1ug/ul) and stored in plastic vials at -20 ^{o}C in aliquots of 100 µg each. Naloxone hydrochloride was a generous gift from Endo Labs (Garden City, New York). 5-hydroxytryptamine creatine sulfate complex (5-HT) and 5-hydroxy-L-tryptophan (5-HTP) were purchased from Sigma Chemical Co. (St. Louis, Missouri). Naloxone and 5-HTP were dissolved in distilled water, except for 5-HT, which was injected icv dissolved in isotonic sterile saline. Doses are expressed as their respective free bases. The volume of i.p. injections was equivalent to 0.1 ml/10 g of body weight.

A. 2. 1 Measurement and quantification of antinociception.

Adult male ICR mice (23-25 grs) supplied by Simonsen Labs (Gilroy, California) were used in these studies. B-endorphin (B-EN) or morphine were injected intracerebroventricularly (icv) according to Haley and McCormick (1957) under slight ether anesthesia after incision of the skull. The volume injected was 10 µl/mouse. Each experimental group consisted of 12 mice. Protocols were designed so that mice were not exposed to the noxious stimuli more than 4-5 times, since repeated exposures resulted in an increase in the control latency time. To reduce the variability, each experimental series was carried out on the same day and approximately at the same time, employing the same batch of mice.

Antinociception was assayed by the tail-flick method as described by D'Amour and Smith (1941). Prior to drug administration, the control latency to the noxions stimuli was established for each mouse and 30, 60, 120 or 240 minutes after the drug measurements of the tail-flick reaction were repeated. A 2.1-fold increase in the latency of the reaction time was used as a cut off time in order to avoid the burning of the animal's tail. Antinociception was expressed as "% of analgesia", calculated from the expression (T1-T0)/(T2-T0) x 100, where T0 refers to the control latency time before the drug, T1 to the experimental values obtained at the intervals following injection, and T2 to maximal analgesia set at the cut off time of 5 secs. After plotting on graph paper the percent of analgesia against time, the area under the curve was cut and weight

on an analytical balance. The area of analgesia (AA) was obtained for each mouse. It was expressed in arbitrary units (AA U2). The AA values were analyzed statistically using the student "t" test. Significance was set at the P 0.05 level.

A. 2. 2 Reversibility of the acute tolerance produced by B-endorphin by 5-hydroxy-L-tryptophan (5-HTP) or 5-hydroxytryptamine (5-HT).

Four groups of mice were used. All the animals were injected icv with 600 ng B-EN at time 0. The first group was used to control the time course effect produced by the peptide and to obtain the control AA. It was established that the increase in latency after B-EN returned to base line control within 5 hours. At this time the remaining 3 groups of mice were administered a second 600 ng dose of B-EN on the opposite side of the brain. The AA in one of these groups was determined to establish the degree of acute tolerance to the first dose of B-EN. After 150 minutes the remaining two groups were challenged with either 80 mg/kg 5-HTP i.p. or saline i.p. and retested immediately for analgesia. The AA produced by 5-HTP was compared to that produced by saline.

Two additional experiments were performed to study the anti-nociceptive effect of 5-HTP in B-EN treated animals. In the first series, a group of mice received 600 ng of B-EN at time 0, but instead of receiving the second dose of B-EN at 5 hours, they were injected with saline icv 150 minutes later. Mice received 80 mg/kg 5-HTP i.p. and the AA was determined. The second group received either 80 mg/kg 5-HTP or saline i.p.

30 minutes before the administration of 600 ng B-EN. The AA of B-EN was determined in the usual manner.

An identical protocol as outlined above was used to study whether 5-HT produced the same effect as 5-HTP. The only difference in methodology was the mode of administration. 5-HT (47 ug) or saline (10 µl) was administered icv per mouse. Tests to check whether the doses of 5-HTP or 5-HT produced per se antinociception were also performed.

A. 2. 3 Effect of naloxone on the 5-HTP induced antinociception.
In order to study if the antinociception produced after the administration of 5-HTP was an opiate mediated effect, it was of interest to determine if it could be blocked by naloxone. The protocol consisted of 4 groups of mice. The first group was used to quantify the AA of 600 ng B-EN and the second to establish acute tolerance to the effect of the priming dose of B-EN. The third and fourth groups received 10 min before the administration of 80 mg/kg 5-HTP i.p. 1.0 mg/kg naloxone s.c. or 10 ml/kg saline s.c. respectively. The AA produced by 5-HTP with and without naloxone pretreatment was determined as previously. An additional control group received 1.0 mg/kg naloxone 10 minutes before the injection of 5-HTP.

A. 2. 4 Estimation of the optimal time for 5-HTP antinociception.
To study the optimal time of the 5-HTP induced antinociception in mice tolerant to B-EN, 80 mg/kg 5-HTP was injected i.p. at different time intervals before or after the second dose of B-EN.

Six groups of mice were employed. All groups were injected with 600 ng B-EN icv at time 0. The first group was used to obtain the control AA produced by B-EN. The second group received five hours after the first dose a second 600 ng dose of B-EN and the degree of tolerance developed was assessed by determining the AA. The third group received 5-HTP 30 minutes before the second dose of B-EN while the fourth, fifth, and sixth groups were injected with 5-HTP one, two and four hours after the second dose of B-EN respectively. The AA produced by 5-HTP was calculated and subtracted from the residual antinociception produced by the second dose of B-EN.

A. 3 RESULTS.

A. 3. 1 Antinociceptive effect of 5-HTP and 5-HT after development of acute tolerance to B-endorphin and the effect of naloxone.

5-HTP produced significant antinociception in mice tolerant B-EN and had just recovered from a second dose of B-EN. The administration of 5-HTP i. p. two hours after the second dose of B-EN, when the control tail-flick latency had returned to base line, produced a brief period of analgesia which was maximal at about 30 minutes (Figure A. 1). Similarly, in a more completely controlled series of experiments, after mice were given two successive injections of B-EN spaced five hours apart, an injection of 5-HTP but not saline produced an antinociceptive response. The AA produced by 5-HTP (Table A-I, group B) was 4-fold greater than its saline controls. Eighty mg/kg 5-HTP per se produced negligible analgesia (Group D); 5-HTP antinociception could be obtained only in mice tolerant to the effect of B-EN. Thus, mice receiving saline

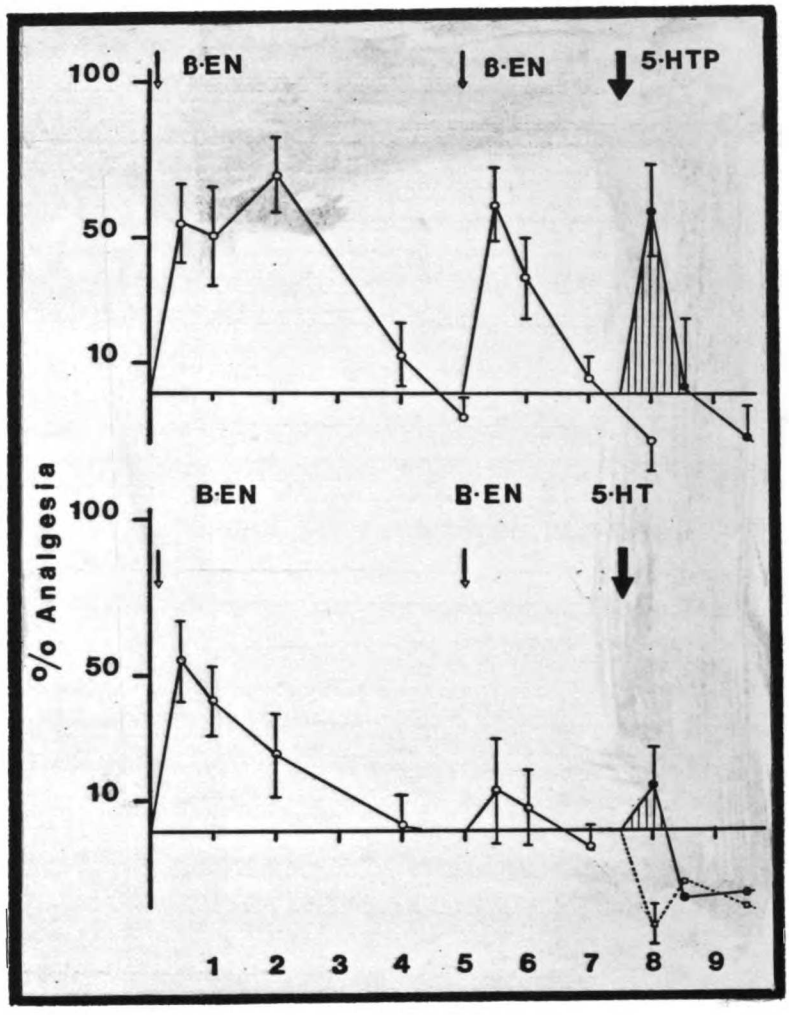

Figure A.1

Time course of acute tolerance development to the antinociceptive effect of B-Endorphin and the antinociception of 5-hydroxytryptophan (5-HTP) or serotonin (5-HT).

Upper panel: At the arrows, B-EN or 5-HTP.

Lower panel: At the arrows, B-EN or 5-HT.

Dose of B-EN was 0.6 ug B-EN/mouse, 80 mg/kg 5-HTP, or 47 ug/mouse of 5-HT. Symbols represent the mean, bars the S.E.M. of 12 mice in each group. In the lower panel, squares and dotted line represent saline icv,as a control for the injection of 5-HT.

Table A-1

Production of antinociception by 5-hydroxytryptophan (5-HTP) in mice after two doses of B-Endorphin spaced five hours apart.

Group	Pretreatment	Challenge	n[a]	AA x ± S.E.M.[b]
A	B-EN + B-EN	Saline	12	10.6 ± 5.3
B	B-EN + B-EN	5-HTP	12[c]	40.3 ± 11.0*
C	B-EN + Saline	5-HTP	12	7.4 ± 3.5
D	-	5-HTP	12	5.3 ± 2.0
E	Saline	B-EN	12	112.0 ± 29
F	5-HTP	B-EN	12	89.7 ± 24

a
Refers to the number of mice used per group.

b
Area of analgesia in U^2 ± S.E.

B-EN = 600 ng B-EN/mouse.

5-HTP = 80 mg/kg 5-HTP i.p.

c
80 mg/kg 5-HTP was injected i.p. two hours after the second dose of 600 ng B-Endorphin.

In the case of groups E and F, mice were pretreated with saline or 5-HTP i.p. respectively 30 minutes before the administration of B-EN.

*P less than 0.05 in relation to its saline control.

instead of a second dose of B-EN and subsequently challenged with 5-HTP failed to exhibit analgesia (Group C). Furthermore, 5-HTP did not enhance B-EN acutely since naive animals pretreated with 5-HTP prior to challenge with B-EN (Group F) did not yield a higher AA than the saline controls (Group E). Although not statistically significant, the change effected by 5-HTP was in the opposite direction with less analgesia noted. Thus, the effects of 5-HTP are dependent upon the tolerant state appearing after the second dose of 5-HTP. Forty mg/kg 5-HTP produced equivocal effects.

5-HT (47 ug/mouse) produced a significant though transient antinociception in B-EN tolerant animals (Figure A.1b). Again, it should be noted that this dose of 5-HT alone produced a negligible AA in animals not previously primed with B-EN.

A.3.2 Effect of naloxone on the 5-HTP induced antinociception. Naloxone at a dose of 1 mg/kg failed to block the antinociception produced by 5-HTP in animals rendered tolerant to B-EN by 2 successive injections. As shown in Table A-2, the AA produced by 5-HTP when administered 2.5 hours after the second dose of B-EN was about the same in the naloxone and saline treated groups. An additional control group showed that the combined treatment of naloxone + 5-HTP produced no significant analgesia. Naloxone 1 mg/kg s.c. significantly blocked the antinociception produced by B-EN. The AA produced by 600 ng B-EN icv/mouse was diminished from 125.0 ± 34.0 U^2 to 66.0 ± 27 U^2 AA, and had a P value less than 0.05.

Table A-2

Failure of naloxone to block 5-hydroxytryptophan (5-HTP) induced antinociception in B-Endorphin tolerant mice.

Pretreatment[1]	Challenge[2]	n	AA[3]
B-EN + B-EN	Saline + 5-HTP	23	23.9 ± 6.2**
B-EN + B-EN	Nx + 5-HTP	18	22.5 ± 7.0**
-	Nx + 5-HTP	12	5.4 ± 1.9

(1) B-EN 600 ng icv spaced five hours apart.

(2) 5-HTP 80 mg/kg i.p. two hours after the second B-EN dose; naloxone, 1 mg/kg or saline 10 minutes before 5-HTP.

(3) Means area of analgesia ± S.E.M.

**P less than 0.01 compared to non B-EN treated mice.

A. 3. 3 Time course of the antinociceptive effect of 5-HTP.

In another series of experiments, to further characterize the antinociception produced by 5-HTP in the B-EN tolerant mice a dose of 80 mg/kg was injected 30 minutes before the second dose of B-EN. The usual decrease in response to B-EN was noted after successive injections; the AA after a second dose of B-EN being only 39% of the original response. However, the injection of 5-HTP before the second dose of B-EN restored the original response to B-EN; the AA of the 5-HTP treated group was about the same (112%) as the original response to B-EN. The antinociceptive effect induced by 5-HTP in B-EN tolerant mice was dependent on its time of administration following the second dose of B-EN. As already mentioned and illustrated in Figure A. 2, the antinociceptive effect of 5-HTP was larger the closer it was administered to the second dose of B-EN. 5-HTP was significantly less effective when administerd 150 minutes or four hours after the second dose of B-EN.

A. 4 DISCUSSION.

The finding that 5-HTP produced a noticeable AA only in mice that had received 2 doses of B-EN (or B-EN and morphine or vice versa) might be related at least in part to the mechanism of action of B-EN or to the genesis of acute tolerance. 5-HTP by itself did not produce any appreciable degree of analgesia nor did it potentiate the antinociception of a single dose of B-EN. However, after 2 doses of B-EN, 80 mg/kg 5-HTP i.p. invariably potentiated the analgesia of the second injection of B-EN confirming the observations of

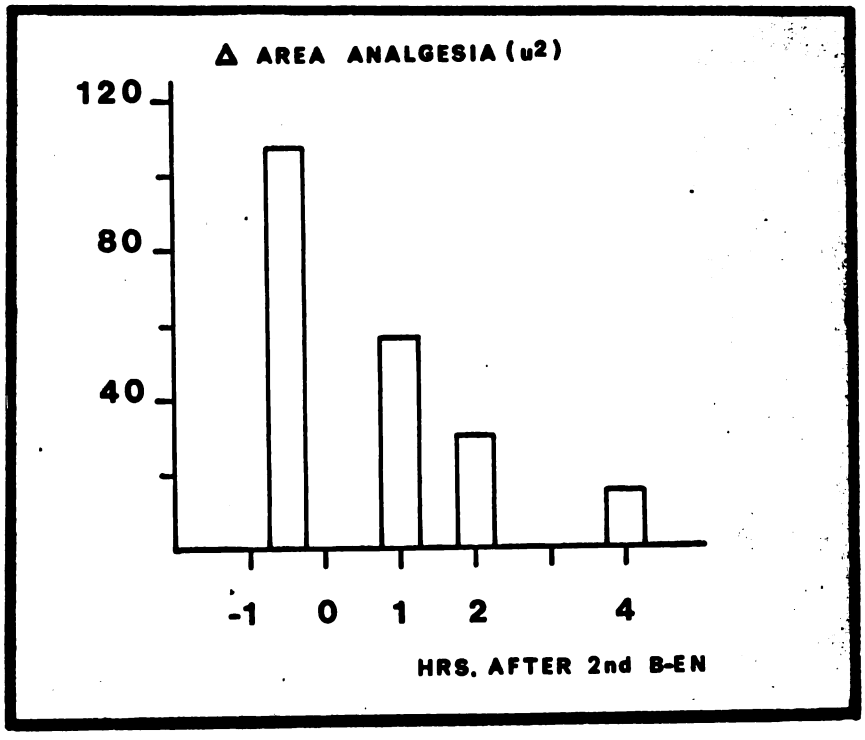

Figure A.2

Estimation of the optimal time for producing 5-hydroxytryptophan (5-HTP) antinociception in mice rendered tolerant to B-Endorphin.

Four different groups of mice were injected with 0.6 ug B-EN at time zero. Six hours later, a second icv injection was performed. Eighty mg/kg 5-HTP was injected at different intervals before or after the injection of the second dose of B-EN. Antinociception was measured and the area of analgesia was obtained. To calculate the antinociception produced by 5-HTP, the residual analgesic effect produced by the second dose of B-EN, was substracted from that of 5-HTP.

Hosobuchi et al. (1977) and Meglio et al. (1977). The effect was more evident in cats than in mice but the phenomenon appears easily reproducible in both species. There are two intriguing facts about the effect produced by 5-HTP. Firstly, the antinociception was not blocked by naloxone, suggesting that the analgesia is not a direct opiate-mediated effect despite that it is an analgesic response. Secondly, there is an apparent relation between the time course of the second dose of B-EN and the antonociception produced by 5-HTP. When 5-HTP was injected 30 minutes before the second dose of B-EN, it almost restored the AA to that produced by the first dosage of B-EN. However, the potentiation was less effective when 5-HTP was administered two and half hours after the second dosage. This suggests that the synergism was dependent on the presence of B-EN in the brain. It is possible that B-EN induced a state of increased CNS sensitivity to certain chemicals. In the present experiments, the increased sensitivity was manifested by the potentiation of the analgesia produced by 5-HTP. The specificity of this postulated sensitivity is unknown, but apparently extends to 5-HT since the administration of 5-HT, under similar conditions, produced the same effect as 5-HTP. An alternative is that 5-HTP could potentiate the decreased effect of the second administration of B-EN. However, this alternative is less likely since naloxone did not block the effect of 5-HTP.

It might be argued that the effect of 5-HTP occurred at a different sitefrom that of the opiate receptor. There is evidence that morphine's analgesia as well as the electrical stimulation produced analgesia are mediated at least in part by serotonergic neurones.

Both of these responses have been demonstrated to be reduced by pharmacological manipulations that decrease brain 5-HT levels, and are restored by administration of 5-HTP (Akil and Liebeskind, 1975; Gorlitz and Frey, 1972; Tenen, 1968; Vogt, 1973; Yaksh et al., 1976). On the other hand, manipulations to increase brain 5-HT levels potentiated morphine analgesia (Dewey et al., 1970; Redjemi et al., 1974; Samanin and Valzelli, 1971; Sparkes and Spencer, 1971). These results are in line with studies of lesions of midbrain nuclei that postulate the involvement of 5-HT pathways in the mediation of the effects of morphine and stimulation produced analgesia (Akil and Liebeskind, 1975; Proudfit and Anderson, 1975; Samanin et al., 1970). The relationships between serotonergic pathways and the effect of B-EN remain to be studied, but it should be anticipated that the findngs should bear a close relationship to those established for morphine.

In conclusion, this data suggests that acute tolerance to B-EN may involve the functional inactivation of a critical serotonergic link in the pathways responsible for pain modulation. This inactivation may be overcome and the acute tolerance reversed by administration of exogenous 5-HT or 5-HTP.

FINAL DISCUSSION.

FINAL DISCUSSION.

The fundamental question that motivated these studies was whether morphine and related opiates can produce in-vivo acute tolerance and dependence. In other words, is tolerance and dependence established with the first dose of opiate administration? The animal model used for this investigation was the laboratory mouse, since rodents are known to develop a high degree of tolerance and dependence after continued exposure to morphine (Huidobro and Maggiolo, 1961; Way et al., 1969). The approach to study this experimental question was to ascertain if the second administration of an opiate would produce less effect, as an indication of the induction of tolerance by the first exposure . For this purpose, two doses of each of the opiates studied were generally administered. The initial or priming dose served to initiate, trigger, the development of tolerance while the second injection of the opiate was used to reveal the degree of tolerance developed by the first dose.

This acute type of tolerance has been poorly characterized pharmacologically. In mice, Eddy (1953) was the first to observe that if there is a 72-96 hour interval between the first and second injection of low doses of morphine, the effect of the second dose of morphine was reduced. Similar conclusions were noted by Huidobro and Huidobro (1972) and Jacob and Berthelemy (1972) in mice. In rats, this problem is inconsistent. Cochin and Kornetsky (1964) reported that tolerance was present after weeks or months of the first injection and proposed an immunological model to account for this effect (Cochin, 1970). Other investigators like Lotti et al. (1966), Lomax and Kirkpatrick (1967), Ferguson et al. (1969), or Huidobro and Huidobro (1973) have demonstrated

an acute tolerance of much shorter duration and faster onset.

A simple reduction of the opiate's effect caused by a previous, first exposure to the narcotic is not a sufficient condition to establish the development of central adaptive tolerance. Several additional experimental criteria are required before single-dose tolerance can be related to this phenomenon. First of all, tolerance should be a dose and time process. Secondly, the development of tolerance should be accompanied by a parallel development of physical dependence. Thirdly, the development of tolerance is expected to be reduced by the simultaneous administration of an opiate antagonist in conjunction with the priming dose of the opiate. In the fourth place, single-dose tolerance should be generalized, crossed, to different types of narcotic drugs so that cross-tolerance should be demonstrable among the different narcotics. Fulfillment of these requirements allows the interpretation that the reduction of the opiate effect caused by the priming dose is a true opiate tolerance.

In these studies three different opiate effects have been examined and attempts were made to document experimentally in each case, the development of single-dose tolerance. The first part of this work was concerned with the antinociceptive effect of morphine and like alkaloids as well as peptides. The second and third part were studies on the hyperthermic response of the endogenous opiate peptides in comparison to those of morphine. Finally, the fourth part was devoted to study the development of tolerance to endorphins in-vitro, using the longitudinal muscle of the myenteric plexus of the guinea pig ileum. This preparation has been well documented as a model for the effect of opiates in the brain

(Kosterlitz and Waterfield, 1975).

The present results demonstrate that a single-dose of an opiates can induce a measurable tolerance. First of all, this was shown to be the case with the antinociceptive effect of morphine, methadone and B-EN. Different strains of mice were used to document this effect utilizing two experimental procedures for evaluation of the analgesic activity of the opiates. Single-dose tolerance was also shown to the hyperthermic effect of B-EN in mice. The effects of the endogenous peptides on body temperature were unknown at the time we initiated these studies so that a physiological investigation was undertaken to characterize this effect. Before studying the problem of single-dose tolerance, it was established that the hyperthermic effect of B-endorphin is an opiate specific effect, blocked and reversed by naloxone. Furthermore, it was found that endorphins and morphine produced cross-tolerance to the effects of B-endorphin. Finally, single-dose tolerance was also established and demonstrated in the isolated preparation of the longitudinal muscle myenteric plexus of the guinea pig ileum. In all the pharmacological preparations examined, single-dose tolerance was established to be a dose dependent effect.

In general, the degree of tolerance developed varied with the dose of of the opiate each effect was assessed. These findings agree with human clinical data where it is known that the development of tolerance to opiates is not a uniform process to all the effects of narcotic drugs (Jaffe, 1975). Tolerance to opiates develops to different degrees and at different rates depending on the effector system under study. As an illustration of this fact, it has been recently documented that in mice

(McGuillard et al., 1977). Furthermore, Holladay (1977) has observed that in rats the rate of tolerance development to the antinocicpetive effect of B-endorphin was different from the rate of tolerance to catalepsy or the sialogogic seizures produced by B-EN in rats. McGuillard et al.(1977) elaborated on the notion of different opiate receptors for each of these effects of morphine as a basis to explain their restuls. It is also possible that the rate of tolerance was different for each effect, based on the concentration of morphine attainable at each anatomical site. It cannot be excluded at present that the kinetics of tolerance induction might not be the same at all brain regions sensitive to opiates. Further experiments along these lines are required.

Apart from the dose and the sensitivity of each effector system to the development of tolerance, a lag time was found to be crucial for the maximal development of tolerance to morphine. This was shown by studying the time course of tolerance after a single dose of an opiate or after varying the time of exposure to a 75 mg morphine tablet. In the latter case, it was surprising to find that 50% of the total tolerance to the antinociceptive effect of morphine was built up with the dose of morphine absorbed from the pellet during the first 24 hours following pellet implantation, provided that a 48-hour periof of time was allowed after pellet removal to express tolerance. In conclusion, there is a consierable lag time for the manifestation of maximal tolerance or that tolerance reaches a maximal degree hours after the removal of the opiate tablet from the animal. In general, from a pharmacological point of view, it appears that single-dose tolernace shares a common mechanism of action to that observed after chronic exposure to narcotics.

Apart from all the general characteristics of tolerance already discussed, it was of interest to examine if single-dose tolerance was significantly reduced by drugs that interfere with the synthesis of proteins. Actinomycin D and cycloheximide, two drugs that block the process of protein synthesis at different sites, were used for these studies. Actinomycin D blocks the transcription of DNA, preventing the formation of a new mRNA; cycloheximide blocks the translation of the mRNA into the nascent primary sequence of proteins. Experimentally both drugs evidenced a common effect, i.e., to prevent the development of single-dose tolerance or single-dose cross-tolerance without blocking the acute effect of the opiate. This evidence has been interpreted to mean that a macromolecule can be responsible for the process of tolerance. The crucial question is related to the nature of this macromolecule and its function in opiate tolerance. Obviously the molecule is not an opiate receptor, or related to the synthesis of the opiate(s) receptor(s); since tolerance to opiate drugs was obliterated without an appreciable alteration of the acute response of opiates. This conclusion is in complete agreement to previous data from this laboratory indicating similar results (Way et al., 1968; Loh et al., 1969; Shen et al., 1970).

Speculation favors the notion that this macromolecule could be related to the synthesis and/or metabolism of 5-hydroxytryptamine in the brain. In agreement with results obtained from experiments using a 75 mg morphine pellet to induce a high degree of tolerance, the administration of L-tryptophan seemed to increase to degree of single-dose tolerance while pretreatment wth 5,6-dihydroxytrytamine effectively reduced the degree of tolerance developed. Furthermore, in the case of the administration of B-EN, it was shown that 5-HTP

could somehow counteract the development of tolerance to a second dose of B-EN. The correct interpretation of these results might seem premature but data certainly suggest that the metabolism of serotonin is somehow involved in the development of tolerance as first evidenced by Way et al. (1968) and Loh et al. (1969).

The present results tend to support that the pharmacological administration of endogenous opiate peptides or opiate alkaloids produce an adaptational change in the CNS that will influence the second or a future exposure to the opiate. The site of the change is probably not related to the opiate receptor itself but rather to a process coupled to the opiate receptors. Thus this data tend to support the enzyme expansion theory of tolerance development as proposed by Shuster (1961).

The present findings raise a challenging question . Why do the endogenous peptides produce tolerance and dependence? Why is tolerance produced by the endogenous opiates quantitatively as much if not more than that produced by the opium alkaloids? It is obvious that we do not understand the mechanism of action of the endogenous peptides nor the mode of action of the morphine-like alkaloids. However, since the endogenous peptides (granted we are using pharmacological doses injected to the brain ventricles rather than the biophase of the specific opiate receptors), are capable of producing tolerance and dependence, indicates that the potential to develop tolerance is inherent to the function of these peptides at concentrations higher than that ordinary occuring in a normal state. If narcotic tolerance is by definition a mechanism that renders an effector cell resistant to the effect of an opiate agonist, tolerance might be the physiological expression of the

refractoriness of the brain to the effect of its own endogenous substances. Morphine or the opium alkaloids and chemical derivatives might function then by mobilizing or mimicking the effect of these endogenous substances. Tolerance to morphine-like alkaloids can be a consequence of a physiological unbalance in the function of the endogenous peptides. The CNS responds to the unbalance by a temporal adaptation that renders certain brain cells partially refractory to the effect of an opiate agonist but supersensitive to an opiate antagonist. Experiments are urgently needed to solve this challenging question.

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