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# The Partial Characterization of the Opiate Binding Capacity of Thirteen Day Old Embryonic Chick Brain

by

Robert J. Hickey

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirement for the degree of Doctor of Philosophy, The City University of New York.

1979

This manuscript has been read and accepted by the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

1/25/79
date

Maxwell Eidinoff
Chairman of the Examining Committee Prof. Maxwell Eidinoff

1/26/79
date

Executive Officer

The City University of New York

ABSTRACT:

While much previous work has characterized the opiate receptor from the brain of adult animals, we set out to characterize opiate binding in the embryonic chick. This approach offers two advantages; first, the immature nervous system of the embryonic chick might exhibit novel characteristics; second, one can easily follow the developmental course of the opiate receptor in the emerging nervous system. Preparations from thirteen day old embryonic chick brain were found to bind stereospecifically substantial amounts of synthetic narcotics such as naloxone and etorphine, while less developed brain tissue stereospecifically bound only limited amounts of these same opiates.

Specific inorganic ions, enzymatic degradations, subcellular fractionations, gross anatomical dissections, incubation studies, kinetic studies, and chromatographic techniques were used to define and characterize the embryonic opiate receptors.

The sensitivity of thirteen day old embryonic chick brain preparations to the actions of lipases, proteases, thermal denaturation, pH, and incubation studies suggests the presence of a single protein or a mixture of proteins that have the capacility of binding opiates stereospecifically. The sensitivity of the stereospecific binding capacity to phospholipase activity implicates phospho-

lipids as a component of the binding processes. The increased level of binding of naloxone at lower temperatures and the unexpectedly prolonged half-life of the naloxone-receptor complex at 9 $^{o}$C may be the result of a lipid participating either as an opiate receptor, or interacting with a protein.

Scatchard analyses of specific inorganic cation effects indicates the presence of multiple binding sites. Various inorganic cations induce conformational changes that differentially modify the receptor's ability to bind agonist and antagonist narcotics. Sodium ions are more effective than magnesium and manganous ions in inducing conformational changes that result in high and low affinity narcotic binding sites. Conformational changes are also induced by altering the temperature. Although synthetic congeners of morphine and opioid peptides compete with naloxone and etorphine for common receptors, the possibility of there being several classes of receptor sites cannot be discarded.

As the fetal nervous system matures, there is a dramatic increase in the total stereospecific narcotic binding capacity that develops, mainly in the frontal lobes. As synaptogenesis proceeds, the binding capacity shifts from the crude microsomal to the crude synaptosomal fraction. The stereospecific binding capacity becomes more resistant to autodegradation and to the

effects of increased ionic strength as the fetal nervous system matures.

Embryonic brain tissue contains at least one endogenous opioid substance that occupies a significant percentage of the receptor sites. Preparations of adult chicken brain contain amounts of an endogenous opioid substance that exceed those levels found in the embryonic chick brain.

DEDICATION:

This work is dedicated to my mother, Stella, my family, and my friends: Linda Malkas, Zenowij Majuk, David Klein, and Daniel Hryb for providing the understanding and moral support needed to complete this project.

ACKNOWLEDGEMENTS:

I wish to express my gratitude to my understanding family and friends, and especially my mother, Stella, for providing both encouragement and sustenance during my many long experiments.

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I especially wish to thank Dr. James Hogg for his reviews and in-depth discussions of both my second level proposal and this manuscript. I am especially grateful to him for the unselfish donation of so many hours of his time which were spent reading, reviewing, discussing, re-reading, re-reviewing, and re-discussing every aspect of this dissertation. (Without his effort, this work might still be in the preparative stages.) I also deeply appreciate his advice and concern, whichwere shared with me throughout my stay at the college.

I also wish to thank Drs. Raymond Disch and Daniel Sverdlich for their advice, instruction, and assistance

in using the Queens College computer facility, and David Klein for leavening the burden of research with absurdity and advice. I especially wish to thank him for his advice during the initial phases of this project.

I also extend my appreciation to the following graduate and undergraduate students who, (in spite of their assiatance), helped in the completion of this work: Patty Boiko, Mary Lovell, Jeffery Garret, David Goldstein, Michael Huchital, Richard Lewis, Suzanne Myrick, Kaya Panikian, Alan Schechter, David Shanker, Gary Silverstein, Ruth Solomon, Ewa Wajnberg, and Victoria Zeitoni.

My deepest appreciation is extended to the following individuals for devoting their own time to help me with this project: Irene Cheteyane, Mark Ladenheim, Linda Malkas, and Dr. Pradhabudas Palan. I also wish to thank the following individuals for their avid, (if not always helpful), technical assistance: Samuel Anteby, Nicholas Detsis, Larry Feldman, Thomas Hayden, Robert Pfeffer, Kenneth Pospisil, Randy Smith, and Robert Wurman. Finally, I wish to thank the departmental secretaries for their clerical assistance, which was of considerable value in the day to day operations of the lavoratory and the classroom.

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| | |
|---|---|
| SSB | stereospecific binding |
| BSA | bovine serum albumin |
| SDS | sodium lauryl(dodecyl) sulfate |
| EDTA | ethylenediamine tetraacetic acid |
| EGTA | ethyleneglycobis(beta-aminoethyl ether)-N,N' tetraacetic acid |
| PPO | 2,5 diphenyloxazole |
| POPOP | (1,4-bis(2-(5-phenyloxazolyl))-benzene |
| Tris | tris-(hydroxymethyl)aminomethane |
| HEPES | N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid |
| NEM | N-ethyl maleimide |
| Methionine Enkephalin | $NH_2$-TYR-GLY-GLY-PHE-MET-OH |
| Leucine Enkephalin | $NH_2$-TYR-GLY-GLY-PHE-LEU-OH |
| $Ala_3$-Met-Enkephalin | $NH_2$-ALA-ALA-ALA-TYR-GLY-GLY-PHE-MET-OH |
| alpha endorphin | $NH_2$-TYR-GLY-GLY-PHE-MET-THR-SER-GLU-LYS-SER-GLN-THR-PRO-LEU-VAL-THR-OH |
| gamma endorphin | $NH_2$-TYR-GLY-GLY-PHE-MET-THR-SER-GLU-LYS-SER-GLN-THR-PRO-LEU-VAL-THR-LEU-OH |
| Beta-Lipotropin | $NH_2$-TYR-GLY-GLY-PHE-MET-THR-SER-GLU-LYS-SER-GLN-THR-PRO-LEU-VAL-THR-LEU-PHE-LYS-ASN-ALA-ILE-ILE-LYS-ASN-ALA-HIS-LYS-LYS-GLY-GLN-OH |
| ficomole | is defined as $1.0 \times 10^{-14}$ moles. |

INTRODUCTION:

Opium is obtained from the milky extract of the unripened seed pod of the poppy plant, Papaver somniferum. From an extract of this plant Friedrich Serturner isolated morphine (named after the Greek god of dreams, Morpheus) in 1803, and in 1832 Pierre-Jean Robiquet isolated codeine. These two opiates are the only two naturally occurring opiates found in opium powder prepared from poppy seeds. Morphine comprises 10 percent of the dry weight of opium powder, while another 0.005 percent is composed of codeine.

Opium has been used medically throughout Europe and the Orient since the Middle Ages. The toxicity and addictivness of morphine were not realized until the drug had become an essential feature of nineteenth century medicine. The significant social problem posed by returning wounded soldiers of the U.S. Civil War who were addicted to morphine prompted the search for a non-addicting opiate analgesic.

By the turn of the century medicinal chemists had produced thousands of derivatives of naturally occuring opiates and synthetic opiate-like drugs, but with little success. The Bayer company introduced heroin, (acetylated morphine), as a non-addicting opiate analgesic. Various modifications of morphine and its synthetic congeners ultimately produced the presumably

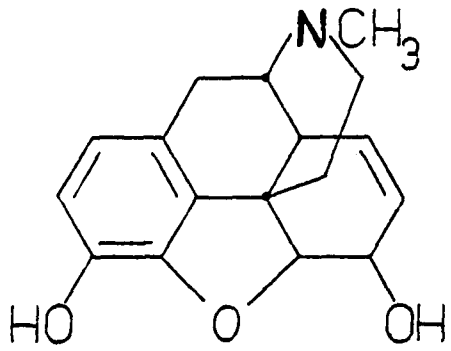
non-addicting meperidine, (demerol), around 1940, with disastrous consequences. Continued research resulted in compounds which ranged analgesically from inactive to several thousand times more potent than morphine.

Two distinct classes of opiate drugs emerged from all of this work. One class comprised the agonists; compounds eliciting morphine-like effects. The other class comprised the antagonists; drugs which specifically reversed the effects of agonists, figures 1 and 2. Most of the antagonists however, were found to have some agonistic properties. A specific subclass of antagonists were found to have some of the beneficial effects of the agonists with little of their deleterous effects. This specific subclass of opiates with mixed agonist-antagonist properties when studied clinically revealed the potent analgesic activity and limited addicting potential of these opiates. Pentazocine, which was probably the most useful of mixed agonist-antagonist opiate produced, was used clinically as an opiate analgesic with a limited addicting potential.

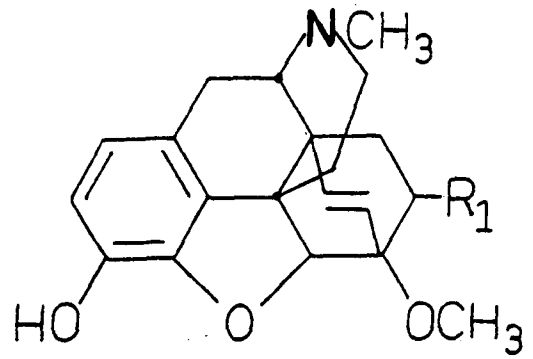
Several questions concerning opiates should be considered before proceeding. How do the opiates function? Do they bind to specific molecules, (ie., receptors)? What is the chemical nature of these molecules, (ie., protein, lipid, carbohydrate)? Why should there be receptors for

Figure 1. Structural similarities between some pharmacologically active agonists and antagonists.

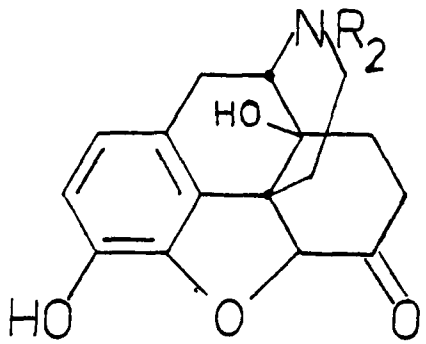
Figure 2. Structural similarities between some pharmacologicallly active agonists and antagonists.



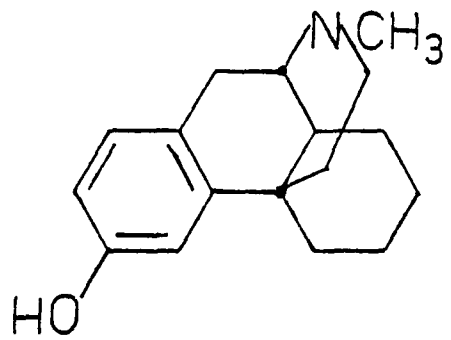

MORPHINE



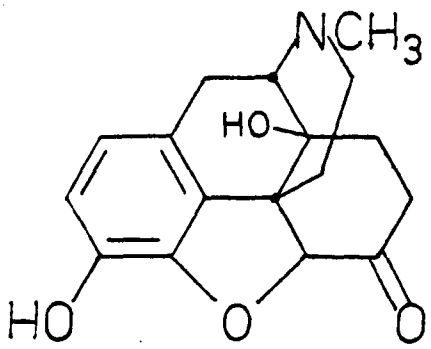

ETORPHINE



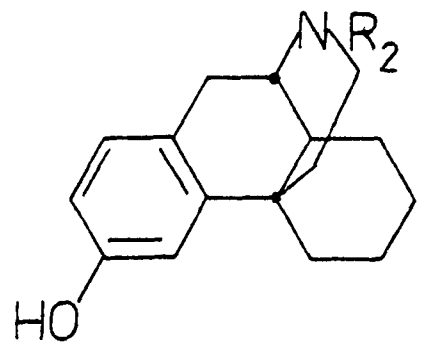

NALOXONE



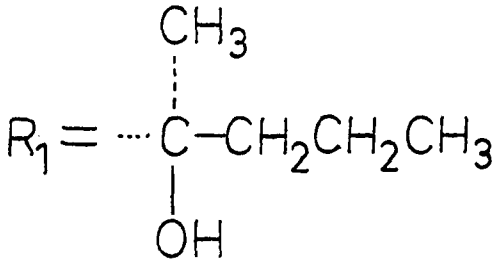

LEVORPHANOL

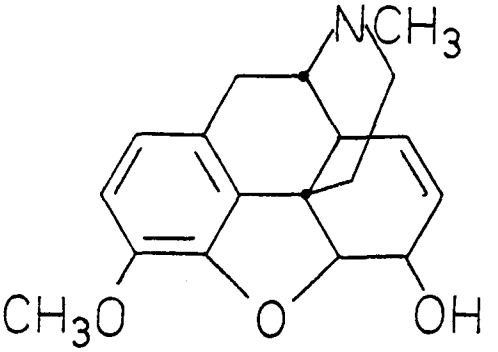



OXYMORPHONE

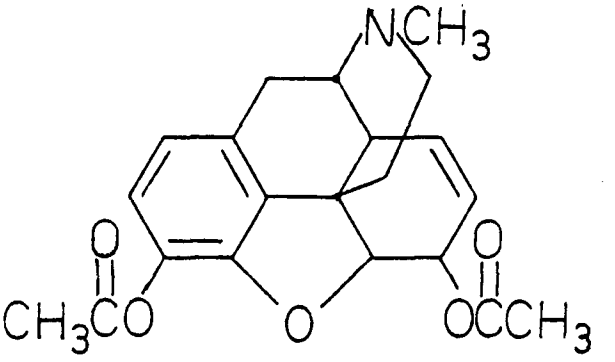



LEVALLORPHAN

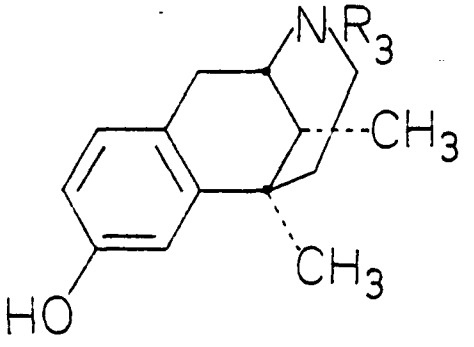
| | |
|---|---|
| $R_1 =$  | ANTAGONIST FORMING STRUCTURE<br>$R_2 = -CH_2CH{=}CH_2$ |



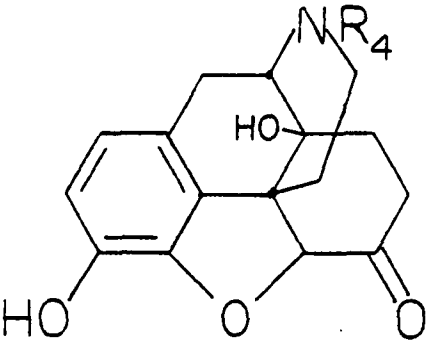

CODEINE



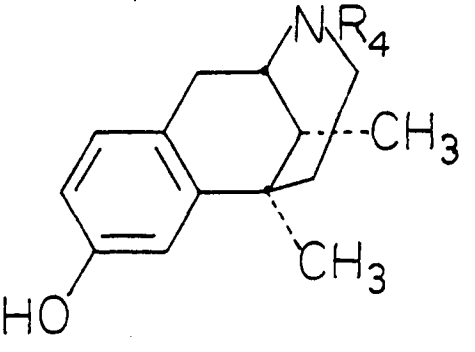

HEROIN



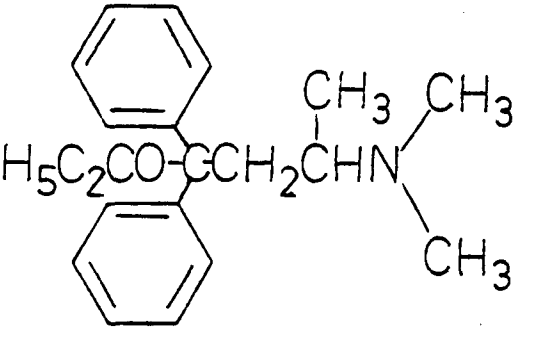

PENTAZOCINE




NALTREXONE




CYCLAZOCINE




METHADONE

| | |
|---|---|
| $R_3 = -CH_2CH=C-(CH_3)_2$ | ANTAGONIST FORMING STRUCTURE<br>$R_4 = -CH_2-CH$ (CH2, CH, CH2) |

opiate analgesics? Where might such receptors be localized on the neurons? Could the specific actions of opiate drugs be the result of opiates interacting with receptors in specific anatomical brain regions referred to as nuclei? Do opiates bind to any other organ? Is there some endogenous brain substance that interacts with the receptor(s)?

Some of these questions could not have been imagined by medicinal chemists who were trying to separate the analgesic and addicting properties of morphine and its congeners.

The systematic synthesis of various compounds based on the morphine structure was time consuming, and often yielded compounds with unpredictable properties. Even though some limited success was achieved, the nature of the interaction between an opiate and the brain was still a mystery. Several models of the opiate-brain interaction were proposed, (Becket and Casey 1954; Bentley and Lewis 1973; Portoghese, 1966; Feinberg, et al., 1976), and all of them employed some form of a receptor molecule that would bind an opiate stereospecifically.

Goldstein et al., (1971), used the idea of stereospecific binding to describe an assay system for opiates that was modified by Pert and Snyder, (1973a), and later used by all of the workers in this field as the standard assay procedure. Goldstein et al., (1971), figure 3, described how

opiates might be able to interact with a brain homogenate. They reasoned that opiates could bind in three distinct ways: 1) by becoming trapped or dissolved within the membrane or within membrane fragments in a non-saturable way; 2) by interaction of the positively charged nitrogen atom of the opiate with the available anionic groups present on the membrane; 3) by opiates with a D(-) configuration interacting stereospecifically with the receptor.

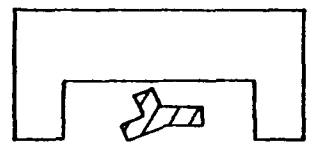
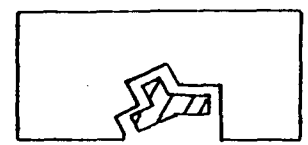
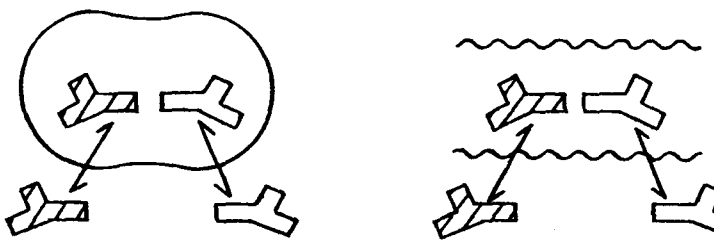
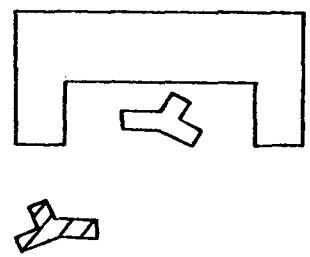
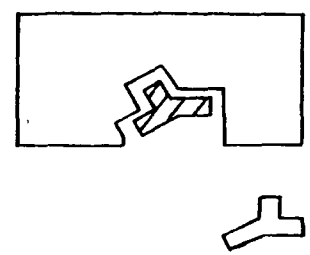
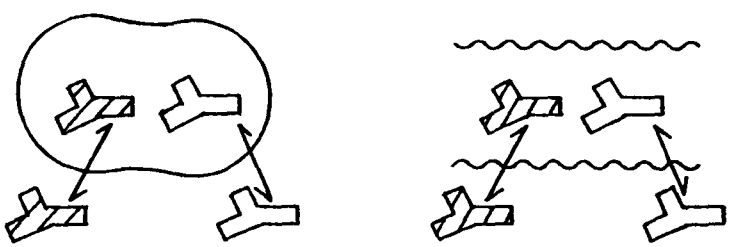
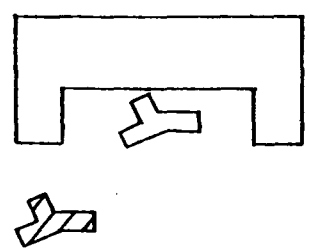
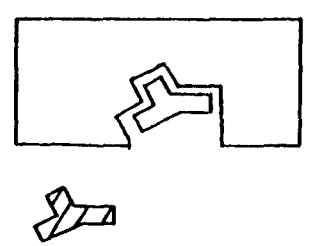
In order to evaluate stereospecific binding on the basis of this proposal, the enantiomers dextrorphan and levorphanol were chosen (Goldstein, et al., 1971). (Dextrorphan is an analgesically inactive agonist, while levorphanol is a very potent agonist, that is pharmacologically active at nanomolar concentrations.) The assay procedure, illustrated diagrammatically in figure 3, sections 2 and 3, was the primary step in helping to elucidate many of the properties of the opiate receptor. (The cross hatched symbol represents radioactive levorphanol.) Section 2 indicates that homogenates are preincubated for five minutes with a one hundred fold swamping excess of dextrorphan, which participates in the non-saturable and the saturable non-specific forms of binding. Radioactive levorphanol is added to this homogenate, where it interacts with the non-saturable and stereospecific components of the

homogenate and to a very limited extent with the non-specific component. Section 3 indicates that a one hundred fold swamping excess of non-radioactive levorphanol is incubated with homogenate for five minutes prior to incubating the homogenate with radioactive levorphanol.

Because non-radioactive levorphanol participated in all three types of binding the non-saturable binding component is the most readily available to radioactive levorphanol. After subjecting both sets of homogenates to a suitable washing procedure (Pert and Snyder, 1973a; Pert and Snyder, 1973b), the amount of radioactivity bound to homogenates in section 3 is subtracted from the amount remaining bound in section 2, and the difference is the measure of stereospecific binding.

The ability of opiates to bind stereospecifically with a pharmacologically relevant receptor was demonstrated (Pert et al., 1973a; Pert and Snyder, 1973b, Creese and Snyder, 1975a; Goldstein et al., 1971; Simon et al., 1973; Lee et al., 1975), and a preliminary subcellular fractionation study (Goldstein et al., 1971), suggested that the receptor was a lipid or a lipoprotein because of the ability of some lipid component found to bind opiates stereospecifically (Goldstein et al., 1971; Loh et al., 1974) in the crude nuclear fraction. Acidic lipids were found to bind opiates stereospecifically (Loh et al.,

Figure 3. The three types of drug binding associations described by Goldstein et al., (1971). Enantiomers were drawn as mirror images, and radioactive compounds are denoted by the cross-hatched symbols.

| | TRAPPED AND ABSORBED | NONSPECIFIC BINDING | STEREOSPECIFIC BINDING |
|---|---|---|---|
| 1 |  |  |  |
| 2 |  |  |  |
| 3 |  |  |  |

1974; Abood and Hoss, 1975), but with much less selectivity than brain homogenates, and with some degree of saturation (Loh et al., 1974). The synaptosomal fraction was later shown (Pert et al., 1974b), to contain the highly selective opiate receptor.

The "opiate-receptor" interacts selectively only with opiate-like drugs (Pert and Snyder, 1973b; Simon et al., 1973), and opiate binding does not require any metabolic energy (Simon et al., 1973).

The kinetics of naloxone binding indicated that the interactions of naloxone were too rapid to be measured by the filtration assay at the standard assay temperature (Pert and Snyder, 1973b). Naloxone binding was shown to be a reversible process (Pert and Snyder, 1973b), and the dissociation of bound naloxone followed first order kinetics with respect to naloxone (Pert and Snyder, 1973b).

The binding characteristics of agonists and antagonists were differentiated by means of alkylating reagents (Pasternak et al., 1 975b, Wilson et al., 1975), enzymatic degradation (Pasternak and Snyder, 1975d; Simon et al., 1973) and specific ion effects (Pert and Snyder, 1973b; Pert and Snyder, 1974a; Simon et al., 1975a; Simon and Groth 1975c; Pasternak et al., 1975c). Inorganic ions such as sodium (Pert and Snyder, 1974a; Simon et al., 1975a), manganese, and

magnesium (Pasternak et al., 1975c) were particularly useful in modifying the receptors.

Sodium ions were found to increase the amount of naloxone bound to washed brain particulates (Pert and Snyder, 1974a), far more than for crude homogenates (Pert and Snyder, 1974a; Pert and Snyder 1973b). Washed particulates assayed with sodium ions exhibited enhanced association of the antagonist (Simon et al., 1975a) and an accelerated dissociation of the agonist from the receptor (Pert and Snyder, 1974a). In contrast, divalent cations such as manganese and magnesium stabilize agonist-receptor interactions, while accelerating the dissociation of antagonists (Pasternak et al., 1975c).

Manganous and magnesium ions, which enhance agonist binding slightly in the absence of sodium (Pasternak et al., 1975c), dramatically increase agonist binding in the presence of sodium (Pasternak et al., 1975c). The selective enhancement of the binding of agonists by manganous ions appears to result from a countering of the sodium effect (Pasternak et al., 1975c). There is no apparent role for calcium in the opiate binding process (Pasternak et al., 1975c).

The opiate receptor appears to resemble the dopamine, serotonin, alpha-adrenergic, and muscarinic-cholinergic receptors (Guillemin, et al., 1976; Creese et al., 1975; Snyder and Bennett, 1976). All five seem to utilize a two

state receptor capable of differentiating between the agonist and antagonist forms of their respective neurotransmitters.

The opiate receptor appears to exist in either of two forms. The binding affinities of agonists and antagonists are comparable for receptors in the sodium-free form (Pert and Snyder 1973b), while the sodium-form binds antagonists with a much greater affinity than agonists (Pert and Snyder, 1974a). Brain receptors are believed to be in the sodium form due to endogenous sodium levels. This concept readily explains the greater in vivo binding potency of antagonists as compared to agonists. Newly synthesized opiates are now graded according to a sodium index in order to determine their relative capacities to act as agonists or antagonists (Creese and Snyder, 1975a).

The brains of all vertebrates appear to contain opiate receptors with essentially the same specificity for opiates. Since opiates such as morphine are not endogenous metabolites produced by vertebrates the remarkable specificity of the opiate receptor suggests the presence of some molecule in vertebrate brain that was conserved throughout evolution. The existence of such a conserved molecule is consistent with the observation that analgesia could be produced by the stimulation of the periaqueductal gray matter of the brain stem of rats (Snyder, 1977b), and

that naloxone could antagonize this effect. The presence of such a molecule in brain tissue was confirmed by Hughes, (1975a), and found to inhibit electrically induced muscle contractions of the vas deferens in mouse, and the small intestine of the guinea pig. Naloxone antagonized the inhibitory effect of the endogenous substance which was identified as a mixture of two peptides, each containing five amino acids. The sequences were determined to be TRY-GLY-GLY-PHE-MET or TRY-GLY-GLY-PHE-LEU, and the compounds were named methionine enkephalin and leucine enkephalin, respecitively. Simantov and Snyder, (1976c; 1976d), isolated the same two peptides from bovine brain tissue, but in a different ratio from that obtained by Hughes et al., 1975. The ratio of methionine to leucine enkephalin not only varied from species to species, but was shown to vary in different brain regions as well (Simantov and Snyder, 1976e; Smith et al., 1976; Gentlemen et al., 1976).

Variations in the amount of enkephalin contained in different brain regions parallels the regional distribution of the opiate receptor sites (Hughes, 1975; Pasternak et al., 1975a; Simantov and Snyder, 1976e). The anatomical distribution of the enkephalins was mapped by immunohistochemical mapping techniques (Elde et al., 1976), and found to parallel the distribution of receptors observed by autoradiography (Atweh

and Kuhar, 1977a). These peptides may serve as neurotransmitters because they are contained in the nerve terminals (Simantov and Snyder, 1976c) of specific neurons (Elde et al., 1976). Potassium depolarizes synaptosomes in a calcium dependent fashion (Smith et al., 1976), releasing enkephalin (Bayon et al., 1978). All vertebrates seem to contain similar amounts of this peptide (Simantov and Snyder, 1976d) in the brain and in the gastro-intestinal tract.

Enkephalins act as inhibitory neurotransmitters (Satoh, et al., 1974; Bird and Kuhar, 1976; Bradley et al., 1976; Korf et al., 1974; Lewis et al., 1978; Bayon et al., 1978). Whether the inhibitory effect of enkephalin is pre-synaptic and/or post-synaptic is unclear (Lamotte et al., 1976; Zieglgansberger and Fry, 1976).

Enkephalin can inhibit the transmission of a nerve impulse in two ways. First, it might alter the ability of sodium ions to cross the post-snyaptic membrane. Second, it might reduce the amount of neurotransmitter released by the pre-synaptic membrane.

Hyperpolarization occurs when the permeability of the post-synaptic membrane for chloride ions exceeds the permeability of this membrane for sodium ions (Young and Snyder, (1973). Zeiglgansberger et al., 1976), conclude that enkephalin must act post-synaptically because

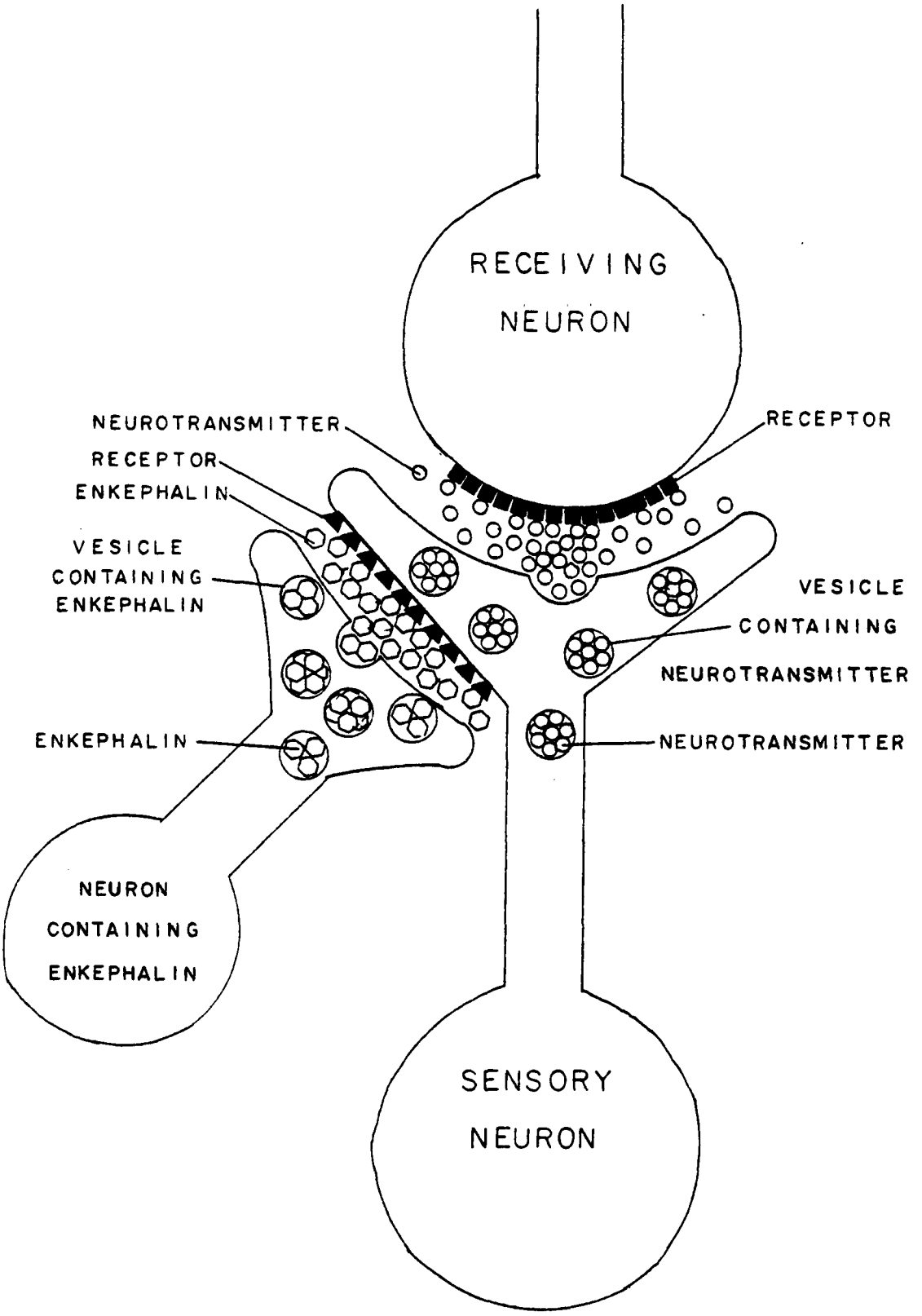
a pre-synaptic site of action cannot adequately explain the blockade of glutamate-induced excitations measured in their experiments. They postulate that enkephalin alters the sodium channels, resulting in the hyperpolarization of the post-synaptic membrane, and decreasing the sodium conductance normally elicited by glutamate.

If enkephalin containing neurons can form axo-axonic synapses, figure 4, then the enkephalins may also act pre-synaptically to inhibit neurotransmission by increasing the permeability of the pre-synaptic membrane to sodium ion; partially depolarizing it. An impulse traveling along such a "modified nerve fiber" would release less neurotransmitter; resulting in a decreased firing rate of the receiving neurons.

There should be no alteration of the pre-synaptic membrane potential if enkephalin acts pre-synaptically, at the nerve terminal. Lamotte, et al., (1976), conclude that enkephalin acts pre-synaptically because the opiate binding capacity of dorsal root horn ganglia can be halved when excitatory neurons, which interact with enkephalin neurons, are degenerated surgically (Lamotte et al., 1976). This hypothesis was supported by Atweh and Kuhar, (1977a), who observed a decrease in the number of pre-synaptically localized autoradiographic granules when the vagus nerve was cut in the neck. Zeiglgansberger et al., (1976), have shown that

Figure 4. A diagram of an axo-axonic synapse.

# AXO-AXONIC SYNAPSE



enkephalin and morphine decrease both the spontaneous firing and the glutamate induced firing of neurons in the cerebral cortex and the corpus striatum, but neither substance seems to affect the membrane potential. The seemingly conflicting set of interpretations of the data of Zeiglgansberger et al., (1976), is consistent with the existence of axo-axonic synapses in some areas of the brain, while axo-dendritic or even axo-somatic synapses may function post-synaptically in other areas of the brain (Snyder and Simantov, 1977). Gamma-amino butyric acid is an example of a neurotransmitter that exhibits both types of inhibitory mechanisms (Snyder and Simantov, 1977).

Enkephalin competes with naloxone for the opiate receptor five times more effectively in the absence of sodium ions than in the presence of sodium ions, suggesting that enkephalin has the properties of an agonist (Simantov and Snyder, 1976e; Simantov and Snyder, 1976f).

On a relative scale, morphine is less potent in competing with enkephalin for the receptor than it is when it competes with other opiates for the receptor. While the reason for the reduced activity of morphine in the presence of enkephalin is unclear, Snyder and Simantov, (1977), speculate that the difference may arise from either some sort of differential alteration in the induced fit of the opiate and enkephalin with the receptor, or

because morphine and enkephalin may interact with different receptors.

Enkephalin is very susceptible to proteolytic digestion (Simantov and Snyder, 1976f; Bayon et al., 1978; Knight and Klee, 1978). An endopeptidase that presumably degrades enkephalin in vivo was recently isolated (Knight and Klee, 1978), and found to be inhibited by antibiotic compounds such as bacitracin and puromycin (Knight and Klee, 1978). Chemically modified enkephalins are more resistant to proteolysis than synthetic or natural enkephalin (Pert et al., 1976b), and some of these modified peptides have significantly improved analgesic properties (Snyder, 1977b). One of these derivatives is 30,000 times more potent than methionine enkephalin when injected directly into the brain. However, it is only 1/5 as active as morphine if taken orally; demonstrating its limited ability to withstand proteolytic digestion (Snyder, 1977b). Analgesically, synthetic enkephalins are reported to be at least one hundred times weaker than partially purified enkephalins (Pert et al., 1976b). This may be due to substances associated with the partially purified enkephalins, and which protect it from proteolytic digestion.

The cross tolerance to enkephalin and morphine (Waterfield et al., 1976), exhibited by smooth muscle cells implies that opioid peptides are also addictive. Loh et al., (1976), have also

shown that rats can be made physically dependent on a large peptide named beta-endorphin. The amino terminus of this peptide begins with the amino acid sequence of methionine enkephalin.

The enkephalins are the major opioid peptides found in the brain (Snyder and Simantov, 1977). Almost no enkephalin can be detected in the pituitary by enkephalin radioimmunoassay, even though high levels of radioactively labeled receptor are observed in this gland. Lewis et al., (1978), hypothesize that any enkephalin found in the pituitary may result from the degradation of beta-endorphin, which can be detected in large amounts in the pituitary (Bradbury et al., 1976; Snyder and Simantov 1977b), but which is present at low levels in the brain. Bloom et al., (1978), using immunocytochemical staining techniques, discovered the presence of longer neurons, which contain beta-endorphin. These neurons are distinct from the shorter enkephalin containing neurons and from those pituitary cells that contain beta-endorphin. The neurons and nerve fibers that contain beta-endorphin are found throughout the diencephalon (Bloom et al., 1978).

Regional variations in the brain levels of methionine enkephalin and leucine enkephalin (Snyder and Simantov, 1977b), seems to reflect a differential metabolism for these two peptides because methionine enkephalin is turned over at a much faster rate than leucine enkephalin (Bayon et

al., 1978). Gillin et al., (1978), have shown that chronic treatment of rats with lithium ions increases the methionine enkephalin content of the nucleus caudatus and the globus pallidus of the striatum by almost 250 percent. They propose that lithium slows the release of methionine enkephalin from enkephalin containing neurons found in the striatum.

Bloom et al., (1978), suggested that the functions of the opioid peptides may be more diverse than that which is simply implied by the term "opioid peptide". They urged that a functional characterization of the beta-endorphin and enkephalin neuronal systems be done. In keeping with this type of characterization studies have revealed that both beta-endorphin and the enkephalins (Loh et al., 1976; Meglior et al., 1977; Bloom et al., 1976; Belluzzi et al., 1976; Buscher et al., 1976; Pert A., 1976; Pert C. B. et al., 1976) have analgesic properties. Enkephalin acts primarily on dopaminergic neurons (Plotnikoff et al., 1976). Endorphins are thought to reduce the response to thermal and other noxious stimuli (Holaday et al., 1978), triggered in common neuronanatomical pathways by heat.

Enkephalin acts primarily on dopaminergic neurons (Plotnikoff et al., 1976), Schizophrenia, which seems to be related to excessive levels of a leucine-endorphorin (Arehart-Treichel, 1977), and the presence of almost double the normal amount of

brain dopamine receptors (Arehart-Treichel, 1977), may be the result of a metabolic imbalance that primarily affects the metabolism of some of the opioid peptides; implying a behavioral role for at least some of these peptides.

Beta-endorphin and corticotropin are reported to be released simultaneously from the pituitary (Lewis et al., 1978; Liotta et al., 1978; Mains and Eipper 1978; Rubinstein et al., 1977), thereby suggesting that they may have some unknown co-functional role. Beta-endorphin, which presumably interacts strongly with the opiate receptor (Guillemin et al., 1976), in the pituitary (Simantov and Snyder 1976c; Simantov and Snyder 1976d; Simantov and Snyder 1976f; Elde et al., 1976; Smith et al., 1976), may regulate the release of anti-diuretic hormone (Snyder and Simantov, 1977b). Opiates may mimic the effect of beta-endorphin because opiates also stimulate the release of the anti-diuretic hormone from the pituitary (Snyder and Simantov, 1977b). Lewis et al., (1978), suggest that the enkephalins may function primarily as neurotransmitters while beta-endorphin may function as a hormone. The presence of the neurotransmitters, dopamine and norepinephrine, in the brain and the production of the hormone epinephrine by the adrenal gland may serve as an example of such a neurotransmitter/hormonal metabolic pathway.

Many biologically active peptides are

synthesized from larger precursor peptides. The opioid peptides may likewise be processed from larger precursor peptides (Lewis et al., 1978). The first such precursor peptide found in the pituitary was the 31K-ACTH (adrenocorticotropic hormone) molecule (Mains et al., 1977; Roberts and Herbert, 1977). This molecule can be split into ACTH and beta-lipotropin. The human pituitary and the rat pars distalis appears to contain much greater amounts of beta-lipotropin than of beta-endorphin (Liotta et al., 1978), and enkephalin appears to be almost completely absent from the pituitary (Snyder and Simantov, 1977b). However, beta-lipotropin can yield melanocyte stimulating hormone, alpha-endorphin, beta-endorphin, gamma-endorphin, and methionine enkephalin. Lewis et al., (1978), have found two large precursor-like peptides in rat and guinea pig brain, which are different from the 31K-ACTH molecule and its processed peptides, and which yield peptides with opioid activity when trypsinized. These other precursor-like peptides (Lewis et al., 1978), may be the precursors of leucine enkephalin and its analogous peptides since the 31K-ACTH molecule cannot be the source of leucine enkephalin in the brain. Furthermore, beta-lipotropin has never been isolated from the brain, thus suggesting that the biosynthesis of brain enkephalin may follow a different route from the pathway described for the pituitary.

The existence of peptides with opioid activity in the pituitary gland was first demonstrated by Cox et al., (1975), and it was postulated that at least one other pituitary peptide that differed in sequence from beta-lipotropin (Gentlemen et al., 1976), was also present. This other peptide has not been found, but opioid peptides, from the pituitary and the brain, such as enkephalin, were found to inhibit the electrically induced contraction of guinea pig ileum (Hughes, 1975; Terenius and Wahlstrom, 1974). All of the endorphins produced in the pituitary begin with the sequence of methionine enkephalin. Alpha-endorphin can be processed from residues 61-76 of beta-lipotropin, and was found to be a relatively poor analgesic in comparison to beta-endorphin (Snyder 1977a; Snyder, 1977c). Beta-endorphin, which contains residues 61-91 of beta-lipotropin, was at least 4-5 times more potent an analgesic than methionine enkephalin (Simantov and Snyder, 1976f; Bradbury et al., 1976; Cox et al., 1976). Gamma-endorphin, (residues 61-77), contains only one more residue than alpha-endorphin, but it induces rage in rats (Snyder, 1977a; Snyder, 1977c).

Anodynin, a substance apparently produced in the pituitary gland and isolated from the blood, is a potent analgesic when injected into the brains of rats (Pert et al., 1976c). It also competes with opiates for the opiate receptor.

Enkephalin, gastrin, somatostatin, vasoactive intestinal peptide and substance-P have a dual localization in the gastro-intestinal tract and the brain. Pearse, (1976), suggested that they may function both as neurotransmitters in the brain and as part of the Amine Precursor Uptake and Decarboxylation cell systems. The existence of enkephalin in the gastro-intestinal tract suggests that enkephalin may function to regulate the peristaltic activity of the intestine. Only the brain and the gastro-intestinal tract contain enkephalin and opiate binding capacity, a fact which may be a consequence of the development of both regions from the same anatomical layer in the embryo (Snyder, 1977a).

The amount of enkephalin localized in various brain regions (Hughes, 1975; Pasternak et al., 1975; Simantov and Snyder, 1976e), closely parallels the opiate binding capacity of these same regions (Pert and Snyder 1973; Miller et al., 1973; Kuhar et al., 1973). Specific autoradiographic labeling techniques were developed (Pert and Snyder, 1975; Pert et al., 1975), and used by Atweh and Kuhar, (1977a; 1977b), to visualize the receptors in the various brain regions. Opiate receptors were localized in the caudal spinal trigeminal nucleus and the laminae II and III of the rexed, representing the substantia gelatinosa (Atweh and Kuhar, 1977a). Vagus nerve fibers, which pass through the medulla

oblongata on the way to the tractus solitarius, and which traverse the inferior cerebellar peduncle and spinal trigeminal tract, contain opiate receptors, as does the tractus solitarius. The tractus solitarius processes visceral sensory input from the glossopharyngeal cranial nerves and the vagus nerve. Receptors were also found in the nucleus commissuralis, formed in the mid-line by the merging of the nuclei of the solitary nucleus (Atweh and Kuhar, 1977a).

The localization of opiate receptors in the solitary nucleus which processes visceral sensory stimuli may explain how opiates depress gastric secretion, inhibit the cough reflex, alter sinus node reflexes, and elicit orthostatic hypotension. The area postrema also contains opiate receptors, and is the site at which opiates induce nausea and vomiting.

The effects of morphine on the hormonal secretions of rats may be related to the presence of opiate receptors in the nuclei associated with the fibers of the accessory optic pathway. This pathway is reported (Haykow et al., 1960; Marg, 1964; Moore et al., 1968), to be associated with the hypothalamus and the pineal glands.

The thalamus, which contains a high concentration of opiate receptors, is primarily concerned with analgesia. Opiate receptors were localized in the medial thalamic nucleus, the perventricular nucleus, the habenular complex, and

the intra-laminar nucleus.

The infundibulum of the hypothalamus contains the greatest receptor concentration in the hypothalamus. These receptors may be associated with the receptors of the posterior pituitary (Simantov and Snyder 1976a), which contains beta-lipotropin and other opioid peptides.

The hypothalamus, the amygdala, and the corpus striatum are part of the limbic system, which processes the emotional component of pain. These areas are probably associated with the euphoric effects produced by opiates rather than with the analgesic effects, and they contain large numbers of opiate receptors.

Synthetic opiates and opioid peptides may achieve their physiological actions by modulating the levels of adenylate cyclase (Collier and Roy, 1974; Sharma et al., 1975a; Sharma et al., 1975b; Minneman and Iversen, 1976; Racagni et al., 1976; Gullis et al., 1975; Klee and Streaty, 1974; Simantov and Snyder, 1976g; Klee et al., 1975). Cyclic adenosine monophosphate (c-AMP), acts as a second messenger; mediating the actions of several peptide hormones and neurotransmitters (Vaughan, 1960; Birnbaumer and Rodbell, 1969; Vaughan and Murad, 1969; Bar and Hechter, 1969; Butcher, 1970). Adenylate cyclase appears to be the primary enzyme affected by insulin, adrenocorticotropic hormone (ACTH), thyroid stimulating hormone, glucagon, (Vaughan, 1960),

and the biogenic amines which act at the beta-adrenergic and muscarinic-cholinergic synapses (Snyder and Simantov, 1977b). Other neurotransmitter systems such as the gamma-amino butryic acid, nicotinic cholinergic and glycine systems alter the ionic permeability of the synapses.

Drugs which alter the levels of c-AMP can alter the actions of opiates, and even mimic withdrawal (Collier et al., 1974b). Morphine alters the amount of prostaglandin E sensitive adenylate cyclase in rat brain and neuroblastoma-glioma cell hybrids (Collier and Roy, 1974a; Sharma et al., 1975a). The effectiveness of opiates on adenylate cyclase parallels their affinity for the receptor (Sharma et al., 1975a). Cyclic-AMP levels are depressed by opiates (Sharma et al., 1975b; Klee et al., 1975), while cyclic-GMP levels are increased in stritial tissue slices (Minneman and Iversen, 1976), neuroblastoma-glioma cell lines (Gullis et al., 1975), and in the corpus striatum after the in vivo administration of opiates (Racagni et al., 1976). The L-dopamine sensitive adenylate cyclase of mouse caudate nuclei is inhibited by morphine at low levels, but high levels of morphine activate the enzyme (Tang and Cortzias, 1978).

Tolerance and withdrawal symptoms produced by substances with opioid activity can be explained in terms of the basal level of adenylate cyclase.

The inhibition of adenylate cyclase activity resulting from the chronic exposure of the enzyme to opioid substances results in an increase in the basal level of adenylate cyclase (Sharma et al., 1975b). Tolerance develops as more enzyme is produced, presumably to maintain basal levels of c-AMP. Studies performed on live rats (Klee and Streaty, 1974); and in cell culture (Sharma et al., 1975b), show that as tolerance develops the inhibition of the rate of neuronal firing, induced by morphine, decreases, despite the fact that the binding of the opiates is unaffected by chronic exposure. Tolerance in rats correlates with a doubling of their enkephalin levels (Simantov and Snyder, 1976g), which implies that enkephalin acts presynaptically. Withdrawal can be explained in the following manner. Neurons exposed to both enkephalin and opiates would have depressed levels of c-AMP, which could inhibit the release of additional enkephalin. So long as the opiates are present, enkephalin would not be released because the opiates are inhibiting the excitatory neurons; which mimic the actions of the endogenous enkephalin. As basal levels of c-AMP are regained, tolerance develops. Depriving the neurons of opiates leaves them momentarily devoid of both enkephalin and exogenous opiate, and restores the uninhibited activity of adenylate cyclase. Cyclic-AMP levels soon exceed basal levels because of the uninhibited activity of the

original and recently synthesized adenylate cyclase molecules. Withdrawal symptoms, which seem to abate after an hour, correlates with the apparent release of newly synthesized enkephalin (Simantov and Snyder, 1976g).

The actions of opiates on neurotransmitters have been considered by many individuals (Winter, 1965; Way, 1973; Vogt, 1973; Way and Shen, 1971; Weinstock, 1971). Considerable confusion, however, has resulted because of poorly controlled experiments, anatomical and physiological complications, and variations in the selectivity and the sensitivity of the assays used to study these effects.

Neither norepinephrine nor acetylcholine appear to be directly involved in analgesia (Way, 1973; Vogt, 1973; Way and Shen, 1971; Weinstock, 1971). Dopamine and serotonin metabolism is affected by morphine (Yarbrough et al., 1973; Gunne et al., 1969; Gauchy et al., 1973; Clouet and Ratner, 1970; Puri et al., 1973; Pleuvry and Tobias, 1971; Buxbaum et al., 1973; Tenen, 1967; Harvey et al., 1968), though the amount of dopamine appears to be equal in both naive and tolerant rats. The higher turn-over rate of dopamine in tolerant rats as compared to naive rats (Gunne et al., 1969; Gauchy et al., 1973; Clouet and Ratner, 1970; Puri et al., 1973), may be in response to the effect of opiates on norepinephrine and serotonin (Pleuvry and Tobias,

1971; Buxbaum et al., 1973).

Serotonin, which is associated with the paleospinothalamic pain system, is also turned over more rapidly in tolerant animals (Yarbrough et al., 1973; Dole, 1970; Shen et al., 1970; Azmitia et al., 1970; Way et al., 1968). Neither tolerance nor dependence develops rapidly if sertonin biosynthesis is inhibited (Weinstock, 1971; Shen et al., 1970; Way et al., 1968; Feinberg, M. P., 1972; Sparkes and Spencer, 1971; Samanin and Bernasconi, 1972), or if tryptaminergic tracts are surgically lesioned (Samanin and Bernasconi, 1972; Samanin et al., 1970; Samanin et al., 1973; Samanin and Valzelli, 1972; Gebhart and Mitchell, 1973). Analgesia is also antagonized by these modifications. Morphine increases the transport of tryptophan into the brain (Cheney et al., 1971), and serotonin administered intraventricularly has a slight analgesic effect, that is antagonized by naloxone (Ho et al., 1973c). Cyclic-AMP, which is involved in the development of tolerance, accelerates the biosynthesis of serotonin (Ho et al., 1973a; Ho et al., 1973b).

The properties of morphine and some of its synthetic congeners are determined by their configuration and the types of substituents placed on the molecules. These molecules are rigid, T-shaped, and contain a flat hydrophobic surface, a positively charged nitrogen atom, and a

non-essential phenolic type of hydroxyl group. Because opiates with a D(-) configuration are the only analgesically active opiates, Beckett and Casey, (1954), hypothesized that some molecule interacted stereospecifically with these drugs.

Antagonists could be produced by substituting N-allyl, N-cyclopropyl, or N-cyclobutyl groups for the N-methyl group of the corresponding agonists. To account for the effects of agonists and antagonists, Portoghese, (1966), suggested that separate hydrophobic domains within the receptor interacted with these two classes of drugs. Bentley and Lewis, (1973), proposed a model that included a hydrophobic site that was apparently required to accommodate certain aromatic portions of the tetrahydrothebaine and the oripavine classes of opiates.

Feinberg et al., (1976), have recently proposed a model which explains the binding characteristics observed during in vitro opiate binding studies. The receptor is postulated to exist in two inter-convertible forms that are modulated by sodium ion. Both forms contain a lipophilic site comparable to the one proposed by Beckett and Casey, (1954).

Agonists bind to the sodium-free form of the receptor, which is stabilized by divalent cations. In addition, certain bulky hydrophobic groups presented by certain classes of agonists interact with a special agonist-stabilized domain that may

be responsible for the enhanced potency of these agonists. Morphine, which cannot approach this special site, cannot stabilize the agonist form of the receptor. Because the sodium-free form of the receptor is not stabilized by morphine, antagonists can eject morphine from the receptor.

Sodium ions, which induce the sodium-form of the receptor, enhance the association of antagonists with an antagonist-stabilized domain, but alter the availability of the agonist stabilized domain by making it unavailable to the antagonists. The antagonist-stabilized domain locks the receptor into a form that favors the binding of antagonists. Presumably, this conversion occurs when the antagonist domain interacts with the pi-electrons of the N-allyl group or the molecular configuration of the N-cyclopropyl methyl and N-cyclobutyl methyl groups of the antagonists.

Agonist site interactions preclude the antagonistic properties expected for N-allyl derivatives of the opiates of the ketobemidone, meperidines, and the N-phenylmethylmorphan classes. As a consequence of the association of these drugs with the special agonist stabilizing domain, these antagonists behave as though they were agonists. The benzene ring in these drugs presumably interacts with the agonist binding site of the receptor, while the N-allyl group is unable to associate with the antagonist-stabilized

domain.

The specific chemical nature of the opiate receptor has eluded investigators because of the difficulty in isolating an active opiate-receptor (Simon et al., 1975b; Lowney et al., 1973). Several lines of evidence (Pert and Snyder, 1973b; Simon et al., 1973; Simon and Groth, 1975; Creese et al., 1975c; Pasternak et al., 1975b; Pasternak and Snyder, 1975; Wilson et al., 1975), suggest that the receptor is made of protein and possibly lipid (Loh et al., 1974; Abood and Hoss, 1975). Lipids may also interact with the receptor to stabilize it (Simon et al., 1975b). Acidic lipids, which are capable of binding opiates stereospecifically (Loh et al., 1974; Abood and Hoss, 1975), bind these drugs with very little of the specificity that is characteristic of the opiate-receptor (Simon et al., 1973; Pert and Snyder, 1973b; Creese and Snyder, 1975). Both Pasternak et al., (1975b), and Wilson et al., (1975), were able to distinguish the activity of opiate agonists and antagonists by using specific protein reagents that presumably modified amino acids on the receptor. Simon and Groth, (1975), showed that the receptor apparently contains a reactive sulfhydryl group that is buried within the "active-site" of the receptor.

Despite all of this work many questions remain unanswered. Do multiple receptors exist? What are the specific physiological functions of

the opioid peptides? What are the metabolic pathways for the synthesis and degradation of these opioid substances? If any of these peptides function as regulators or hormones, what are their targets? Finally, the question this dissertation will attempt to answer is: How does the opiate receptor of embryonic chick brain compare with the opiate-receptor studied in adult mammals?

Comparing the similarities of the opiate binding phenomena in embryonic chick to that of the adult rat will improve the understanding of prenatal opiate addiction, and provide the first conclusive evidence for the remarkable similarities of the opiate binding phenomena across Class lines. Evidence will be presented which is consistent with the hypothesis of there being more than one class of opiate receptor. Some developmental aspects of the opiate binding phenomena will also be considered in order to confirm the existence of an endogenous opioid substance, which is presumably involved in regulating some components of embryonic neurotransmission.

MATERIALS AND METHODS:

I) Reagents:

A) Common Organic and Inorganic Chemicals:

The following reagents were obtained from J. T. Baker: potassium hydroxide, potassium chloride, potassium bicarbonate, potassium citrate, potassium carbonate, potassium dihydrogen phosphate, sodium acetate, sodium chloride, sodium monohydrogen phosphate, sodium dihydrogen phosphate, citric acid, manganese chloride, magnesium sulfate, and dimethyl sulfoxide. Rubidium fluoride was obtained from Alfa Products, and Allied Chemical supplied sodium carbonate, sodium bicarbonate, and sodium hydroxide. Glycine, Trizma, and HEPES buffer, as well as 2,5 diphenyloxazole, (PPO), and (1,4bis(2-(5-phenyloxazolyl))- benzene), (POPOP), were obtained from the Sigma Chemical Company. Cesium chloride was purchased from the City Chemical Corporation, and EDTA and SDS were supplied by Fisher Scientific. Disodium EGTA, disodium calcium EDTA, disodium manganese EDTA, and disodium magnesium EDTA were purchased from K and K Laboratories. Toluene was obtained from both Aldrich and J. T. Baker, and it was redistilled before using it in the scintillation "cocktail". Premixed scintillation cocktails were obtained from Amersham/Searle (Phase Combining

System), New England Nuclear (Aquasol), and beckman (Ready Solv-EP).

B) Non-radioactive Opiates and Synthetic Opioid Peptides:

The Hoffman-LaRoche company donated beta-hydroxyl n-allyl morphan, dextrorphan, levorphanol, and levallorphan. Cyclazocine and pentazocine were donated by the Sterling-Winthrop Research Institute, and naloxone was donated by Endo Laboratories. Peninsula Laboratory donated both the methionine and leucine enkephalins, alpha-endorphin, and ALA3-methionine enkephalin. Etorphine, naloxone, and naltrexone were sent to us by the National Institute of Drug Abuse.

C) Radioactive Opiates and Opioid Peptides:

The National Institute of Drug Abuse supplied tritiated etorphine (20.6-23.0 Curies/millimole). Tritiated naloxone (19.6-23.0 Curies/millimole), and methionine enkephalin (23 Curies/millimole) were purchased from New England Nuclear.

D) Proteins:

All of the following enzymes and bovine serum albumin were purchased from the Sigma Chemical Company: Alpha-chymotrypsin, (bovine pancrease, type I, recrystallized three times, salt free, containing 60 Sigma units/mg); Trypsin, (bovine, type XI, dicyclohexylcarbodiimide treated, recrystallized once, salt free, containing 7500 Sigma units/mg); Protease, (Streptomyces griseus, type VI, 30 percent calcium acetate, containing 3-4 Sigma units/mg); Phospholipase C, (Cl. Welchii, type I, 3.6 Sigma unit/mg); Phospholipase D, (cabbage, type I, 19 Sigma units/mg); and Bovine Serum Albumin, (fraction V, 96-99 percent pure, remainder is mostly globulins).

E) Miscellaneous:

Embryonic chicks were purchased from SPAFAS Incorporated, and glass fiber filters (GF/B) were made by Whatman. LSV-50 plastic scintillation vials were supplied by Yorktown Research. Adult chichen heads were donated by Alfredo of Station Live Poultry.

## II) METHODS:

### A) Preparation of Embryonic Chick Brain Homogenate:

After placing a thirteen day old embryonic chick brain on an ice cold petri dish, the cerebellum and adhering meninges were removed, and the brain was stored in an ice cold beaker until a sufficient weight of brain tissue had been collected. After weighing the brains they were placed in an A. H. Thomas type B Brendler homogenizer fitted with a teflon pestle to which was added 10 ml of a 5 mM tris-HCl buffered 0.32 M sucrose (referred to as the buffered sucrose solution). The brains were homogenized, at 900 rpm using a Fisher Dynamix stirred, with six strokes of the teflon pestle. This concentrated homogenate was cooled on ice for 30 seconds, before rehomogenizing it by the same procedure. A 1 percent (w/v) dilute homogenate was prepared by adding enough ice-cold buffered sucrose to an aliquot of the concentrated homogenate.

B) The Standard Assay Procedure:

Except for the assay temperature, which was 39 degrees, the assay procedure was essentially that of Pert and Snyder, (1973b), figure 3. The assay was conducted in 0.32 M sucrose buffered at pH 7.4 at 39 degrees using 5 mM tris-HCl. (The optimum assay temperature was determined by running the standard assay at all of the temperatures indicated in figure 6, and cooling the homogenate on ice prior to filtering.) Either radioactive naloxone or etorphine was used as the labeled drug unless specified otherwise, and dextrorphan and levorphanol were used as the non-radioactive enantiomeric drugs.

Two milliliter aliquots of the dilute homogenate were placed in six 10 X 75 mm test tubes, and incubated at 39 degrees for five minutes. Either 0.1 uM dextrorphan or levorphanol was added into one of two sets of three tubes, and incubated for five minutes at 39 degrees before incubating both sets of tubes for 10 minutes with either 2 nM tritiated naloxone or 1 nM tritiated etorphine. Samples were filtered after cooling them in an ice bath for fifteen minutes, unless noted otherwise.

The incubated homogenates were filtered by pouring the contents of each tube into a 15 ml stainless steel screened Millipore filter

apparatus containing a Whatman (GF/B) glass fiber filter. Each tube was rinsed with three 2 ml aliquots of ice-cold 10 ml tris-buffer (pH 7.4), and placed into the respective funnel, before washing each filter with three 5 ml portions of the same buffer. After removing the filters from the apparatus, they were placed on paper towels to dry. When the filters dried they were placed into 20 ml plastic scintillation vials, which contained 1 ml of 5 percent SDS. Then each vial was filled with 10 ml of scintillation fluid. The vials were vortexed, allowed to stand at room temperature overnight, and vortexed again. They were counted for five to 10 minutes/cycle at a fluid dependent efficiency of from 28-38 percent, using three counting cycles in a Beckman LSC-200 counter.

Stereospecific binding was determined from the difference in the bound radioactivity measured in the presence of dextrorphan and levorphanol, respectively. Unless otherwise indicated all of the stereospecific binding measurements were reported as binding /milligram of protein.

C) Preparation of Washed Particles from the Concentrated Homogenate:

A washed particulate fraction, was prepared at 4 degrees from concentrated homogenate by centrifuging the homogenate for 10 minutes at 49,000 g. After discarding the supernatant the pellet was resuspended with 10 ml of buffered sucrose, and recentrifuged for 10 minutes at 49,000 g. After again discarding the supernatant, the pellet was resuspended and recentrifuged as before. The supernatant was discarded and the pellet vortexed with 10 ml of the buffered sucrose prior to rehomogenizing it with four strokes of a chilled hand driven glass pestle homogenizer. A 0.8 percent (w/v) suspension, that was used to study the endogenous opioid compound and the salt effects, was prepared by adding enough buffered sucrose to an aliquot of the washed homogenate.

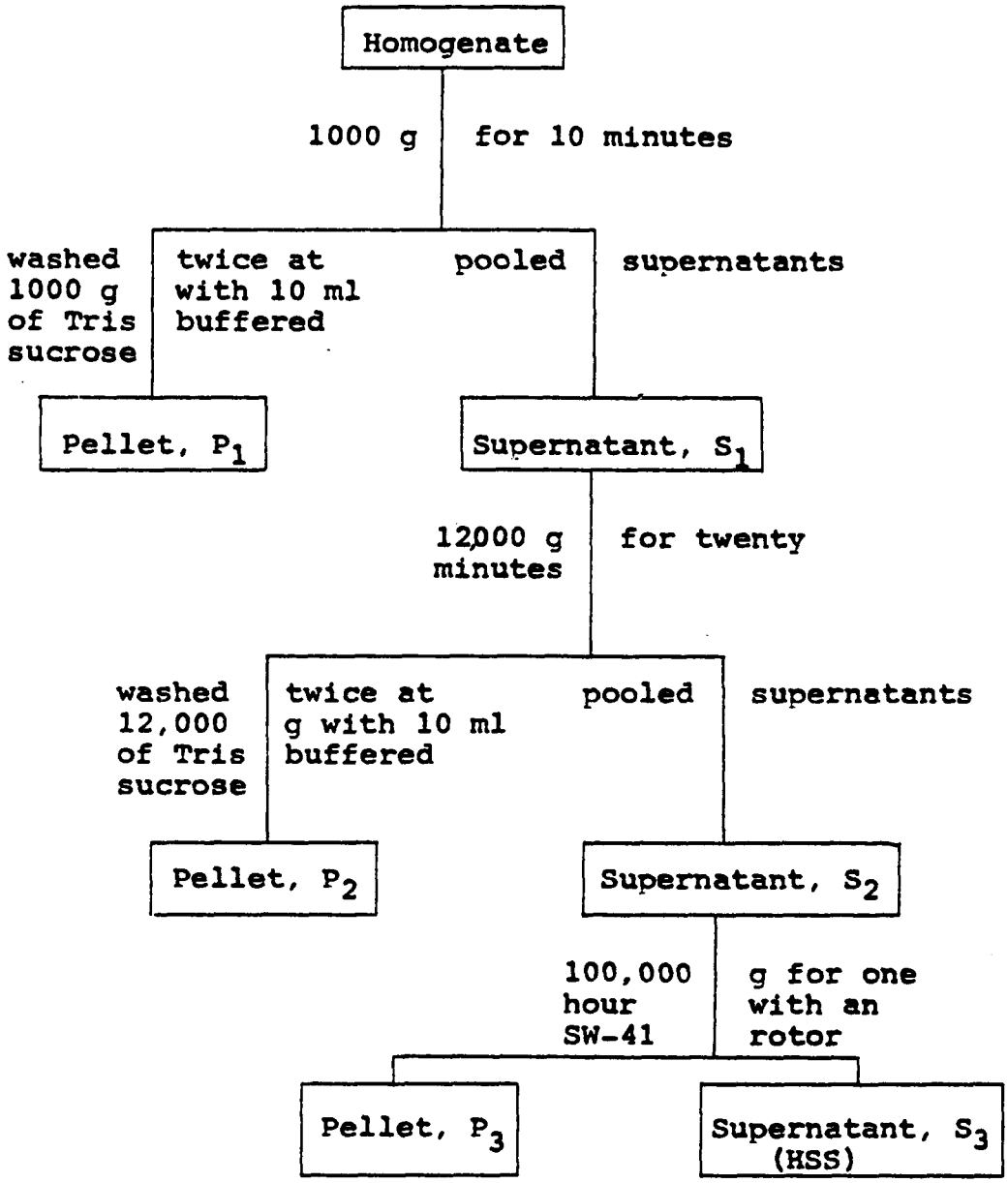
D) Subcellular Fractionation Studies:

Subcellular fractions were prepared at 4 degrees using the procedures of Whittaker and Barker, (1971), and Goldstein et al., (1971), figure 5. A 10 percent (w/v) buffered sucrose homogenate (methods II, A), was centrifuged at 1000 g for 10 minutes. After saving the supernatant (S1), the pellet (P1), was resuspended by gently vortexing it with 10 ml of buffered sucrose before repeating the centrifugation. The supernatant was pooled with S1, and P1 was washed again. The pellet was resuspended with 3 ml of buffered sucrose and stored on ice, while the supernatant was combined with S1 and centrifuged for 20 minutes at 12,000 g. The second pellet (P2), obtained from this centrifugation was resuspended by gently vortexing it with 10 ml of buffered sucrose, and recentrifuged for 20 minutes at 12,000 g. The wash supernatant was pooled with S2, and the washed P2 pellet was washed again. P2 was resuspended with 3 ml of buffered sucrose, and this wash supernatant was also pooled with S2. The entire S2 fraction was centrifuged at 100,000 g using an SW-41 swinging bucket rotor for an hour in a Beckman L2-65B ultracentrifuge. The high speed supernatant (HSS) was used directly in the assay, and the pellet (P3), was resuspended with 1 ml of buffered sucrose.

Untreated dilute homogenate and the HSS were

used in the standard assay without subsequent dilution. P1 and P2 were diluted 1:10, and P3 was diluted up to 13 ml prior to conducting the standard assay (methods II, B).

Figure 5. The flow chart describing the subcellular fractionation of the embryonic chick brain homogenate by the procedure of Whittaker and Barker, (1971).


Homogenate
1000 g
for 10 minutes
washed 1000 g of Tris sucrose
twice at with 10 ml buffered
pooled
supernatants
Pellet, $P_1$
Supernatant, $S_1$
12,000 g minutes
for twenty
washed 12,000 of Tris sucrose
twice at g with 10 ml buffered
pooled
supernatants
Pellet, $P_2$
Supernatant, $S_2$
100,000 hour SW-41
g for one with an rotor
Pellet, $P_3$
Supernatant, $S_3$ (HSS)

E) Protein Determination:

Protein content in assayed homogenates was determined by utilizing two modifications of the Lowry procedure (Dawson et al., 1968). Prior to the addition of the copper sulfate-tartrate solution, homogenates were incubated for fifteen minutes with 0.2 ml of a 5 percent SDS - 0.5 N sodium hydroxide solution (Lees and Paxman, 1972). The second modification was the use of only 0.1 ml of the 1.0 N Folin-phenol solution.

Aliquots of homogenates produced absorbances which were compared with the absorbances of fraction V of the standard bovine serum albumin (0-50 ug total protein) in order to determine the total protein content of the homogenates, (Appendix 1, figure 1).

F) Thin Layer Chromatography:

Eastman chromagram series 6061 plastic backed silica gel sheets without the fluorescent indicator were used in the following solvent systems described by the National Institute of Drug Abuse and the New England Nuclear Company. Naloxone was chromatographed in chloroform methanol acetic acid (100:60:2), ethanol acetic acid water (6:3:1), or chloroform ethanol ammonia (90:10:4 drops). Etorphine was examined in either ethyl acetate acetic acid water (6:3:1), or chloroform methanol ammonia (90:10:4 drops).

G) Stability of the Opiates Under Assay Conditions:

The ability of a 49,000 g washed particulate fraction to bind opiates was studied by thin layer chromatography using embryonic chick brain tissue homogenized in 5 ml of water. The homogenate was washed twice at 49,000 g for fifteen minutes at 4 degrees by resuspending the pellet in 5 ml aliquots of water before resuspending the final pellet in 3 ml of water. Fifty microliters of tritiated etorphine or naloxone were added to each of two tubes containing 1 ml aliquots of the washed particulates. After incubating the mixture for 10 minutes at 39 degrees, it was precipitated with 3 ml of absolute ethanol, stored for six hours at 4 degrees, and centrifuged in the cold for 15 minutes at 12,000 g. The supernatant was decanted, concentrated to dryness, and resuspended in 0.1 ml of water. After storing this suspension overnight at 4 degrees, 0.9 ml of absolute ethanol was added to each tube and incubated at 65 degrees for two minutes, cooled on ice, and centrifuged at 12,000 g for fifteen minutes. The supernatants were concentrated, brought to 100 ml with absolute ethanol, and 20 ul from each tube was plated and developed in the appropriate solvent system.

The chromatograms were removed and dried in air once the appropriate solvent system ran 10 cm beyond the origin. Chromatograms were then

sectioned into 1 cm strips and scraped into scintillation vials, before adding 10 ml of scintillation fluid. The vials were counted for 10 minutes using two counter cycles.

H) Procedures Used for the Enzymatic Digestion of the Opiate Receptors of Embryonic Chick Brain Particulates:

1) Protease: Several concentrations of Protease VI from Cl. Welchii (Sigma; 3.4 units/mg) were incubated with washed embryonic chick brain particulates for 30 minutes at 37 degrees and pH 7.4. The digestion was terminated by centrifuging the particulates at 12,000 g and 4 degrees for 10 minutes, and recentrifuging the pellets after resuspending them with 5 ml of tris-buffered sucrose. The washed pellets were resuspended to their original volume with the buffered sucrose, prior to testing the binding capacity of the washed particulates with tritiated naloxone.

2) Trypsin: Several concentrations of bovine trypsin pretreated with dicyclohexylcarbodiimide (Sigma; 7500 units/mg) were incubated with washed particulates for 15 minutes at 25 degrees and pH 7.4 prior to repeating the washing procedure described for the particulates treated with protease.

3) Alpha-chymotrypsin; (Sigma; 60units/mg). The particulates of embryonic chick brain were incubated with alpha chymotrypsin (Sigma; 60 units/mg), for several periods of time at 39

degrees and pH 7.4 prior to conducting the standard assay in the presence of the enzyme.

4) Phospholipase C: Following the procedure of Yang, (1969), several concentrations of phospholipase from Cl. Welchii (Sigma; 3.6 units/mg) were incubated with washed embryonic chick brain particulates for 30 minutes at pH 7.3 in 5 mM tris-malate buffer at 38 degrees in the presence of 2 mM calcium chloride. The enzymatic digestion was stopped by using the washing procedure described for protease prior to conducting the standard assay with tritiated naloxone.

5) Phospholipase D: Using the procedure of Tyhach et al., (1976), several concentrations of phosphlipase D type I, from cabbage (Sigma; 19 units/mg) were shaken for 15 minutes with washed embryonic chick brain particulates at 28 degrees and pH 5.6 acetate buffer containing 40 mM calcium chloride and 10 percent (v/v) freshly washed diethyl ether. The enzymatic degradation was stopped by the washing procedure described for particulates treated with protease, and the standard assay was conducted on the washed particulates using tritiated naloxone as the labeled drug. Assayed particulates were filtered without prior cooling.

RESULTS:

A) Standard Assay Conditions:

The assay conditions described by Pert and Snyder, (1973b), served as a guide while determining the optimum assay conditions used in this study, tables 1, 3, 7 and figures 6, 8-11, 13, 17-18, 23, 24.

Stereospecific binding capacity as a function of embryonic age was studied in subcellular fractions derived from embryonic chick brain, table 1. Marker enzyme distributions were not used to determine the relative purity of each fraction because the experiment was only conducted to compare the binding capacity as a function of age. Subcellular fractions were obtained using a well defined procedure (Whittaker and Barker, 1971), described in the methods, section D.

The binding capacity of homogenates increased with embryonic age, as did the binding capacity of the cell fractions. The drop in the binding capacity of the crude nuclear fraction (P1), derived from thirteen day old embryonic chick brain is not significant.

Table 1: The stereospecific binding of tritiated naloxone to cellular fractions as a function of the age of chick embryos.

| Sample | Binding (ficomoles/mg of protein) 7 day | 10 day | 13 day | 16 day |
|---|---|---|---|---|
| Homogenate | 1.15 | 3.33 | 3.29 | 4.82 |
| $P_1$ | 0.61 | 1.16 | 0.97 | 2.41 |
| $P_2$ | 1.89 | 5.06 | 13.7 | 17.2 |
| $P_3$ | 12.2 | 21.0 | 14.0 | 15.1 |
| HSS | 0.29 | 0.34 | 1.19 | 0.58 |

All of the subcellular fractions were obtained by the procedure described in section C of the methods. The values are reproducible to ± 7.0 %. $P_1$ refers to the crude nuclear fraction; $P_2$ refers to the crude mitochondrial-synaptosomal fraction; $P_3$ refers to the crude microsomal fraction; HSS refers to the high speed supernatant obtained at 100, 000 X g.

B) The Magnitude and Distribution of Avian Opiate Receptors:

As the embryo progresses from its 10th to its 13th day the crude mitochondrial-synaptosomal fraction (P2) shows a dramatic increase in its binding capacity, and a corresponding decrease in the binding capacity of the crude microsomal fraction (P3). The total binding capacity of both fractions (P2 and P3) increases; though P2 demonstrates the most rapid increase in binding capacity.

Table 2 shows that adult and embryonic chickens and several other mammals bind naloxone stereospecifically, and at comparable levels. The adult chicken, the mouse, and the rat bind naloxone to almost the same extent when compared on the basis of protein content. The magnitude of naloxone bound to mouse and rat brain homogenates is about two or three times higher than the amount bound to adult and embryonic chick.

Gross anatomical dissections of thirteen day old embryonic chick brains and adult chicken brains, tables 3-4, reveal a similar distribution in the percentage of naloxone bound by each brain region. Whole adult brain binds almost eight times as much naloxone as does the whole embryonic brain. The percentage of naloxone bound to the frontal lobes increases as the embryo matures, while all of the other lobes show corresponding

decreases in the binding capacity.

Table 2: The stereospecific binding of tritiated naloxone to brain homogenates obtained from several species of animals.

| Animal | Binding (ficomoles/mg of protein) |
|---|---|
| thirteen day embryonic chick | 3.49 ± 0.03 |
| adult chicken | 4.18 ± 0.03 |
| mouse | 4.50 ± 0.02 |
| rat | 4.71 ± 0.30 |
| rabbit | 2.12 ± 0.10 |

Unwashed homogenates were prepared and assayed according to the procedures outlined in sections A and B of the methods.

Table 3: The gross anatomical distribution of the stereospecific binding of tritiated naloxone to embryonic chick brain homogenates.

| Brain Region | Binding fmole/mg of protein | Binding fmole/brain | Percent Bound to Whole Brain |
|---|---|---|---|
| Whole | 2.02 ± 0.1 | 152 ± 6.8 | 100 ± 4.5 |
| Frontal | 2.58 ± 0.0 | 80.8 ± 0.9 | 53.3 ± 0.6 |
| Optic | 1.63 ± 0.1 | 30.6 ± 0.9 | 20.2 ± 0.6 |
| Cerebellum | 0.98 ± 0.0 | 14.7 ± 0.2 | 9.70 ± 0.1 |
| Brain Stem | 1.62 ± 0.0 | 24.3 ± 0.6 | 16.0 ± 0.4 |

The total binding recovered from the different regions of the dissected embryonic chick brain represents 99.2 ± 1.7 percent of the binding observed in whole brain.

Dissections were performed using the discussion of Murry, D. S. and Jeffree, G. M. as a guide.

Table 4: The gross anatomical distribution of the stereospecific binding of naloxone to adult chicken brain homogenates.

| Brain Region | Binding fmole/mg of protein | Binding fmole/brain | Percent Bound to Whole Brain |
|---|---|---|---|
| Whole | 2.28 ± 0.0 | 1200. ± 16.8 | 100 ± 1.4 |
| Frontal | 3.54 ± 0.1 | 758.6 ± 11.4 | 63.2 ± 1.0 |
| Optic | 2.05 ± 0.0 | 199.7 ± 3.2 | 16.6 ± 0.3 |
| Cerebellum | 0.44 ± 0.0 | 66.0 ± 3.0 | 5.5 ± 0.3 |
| Brain Stem | 1.78 ± 0.0 | 122.6 ± 3.0 | 10.2 ± 0.3 |

The total binding recovered from the different regions of the dissected embryonic chick brain represents 95.5 ± 1.7 percent of the binding observed in whole brain.

Dissections were performed using the discussion of Murry, D. S. and Jeffery, G. M. as a guide.

C) Temperature Effects:

Homogenates bind more naloxone at zero degrees than at 39 degrees, figure 6. The peak at 20 degrees is not as well formed as the peak at 39 degrees. Thirty nine degrees was used as the assay temperature whenever chick brain homogenates were used because this temperature is believed to be physiological for chicks. The gradual decrease in the amount of stereospecifically bound naloxone retained on the filters may be the result of the temperature dependent dissociation of bound naloxone. Such a dissociation of naloxone might occur during the filtering process; leaving the filters with less tritiated naloxone bound to the retained homogenate. The decrease in stereospecific binding at temperatures exceeding 40 degrees is not due to the thermal decomposition of the naloxone, figure 7, but instead results from the apparent denaturation of the receptor sites, figures 9-10.

A Scatchard analysis, table 5, of the peaks at zero and 39 degrees, figure 6, revealed that the cause of the increased naloxone binding at zero degrees was a 20 percent rise in the number of binding sites, and a 74 percent rise in the overall affinity constant of the binding process. The Scatchard analysis appeared to follow multiple binding site theory, and showed that the affinity constant of at least one set of sites increased,

Figure 6. The stereospecific binding of naloxone to embryonic chick brain homogenate as a function of the assay temperature. The standard assay (methods, section B) was conducted at the temperatures indicated and the homogenates were cooled on ice prior to filtering.

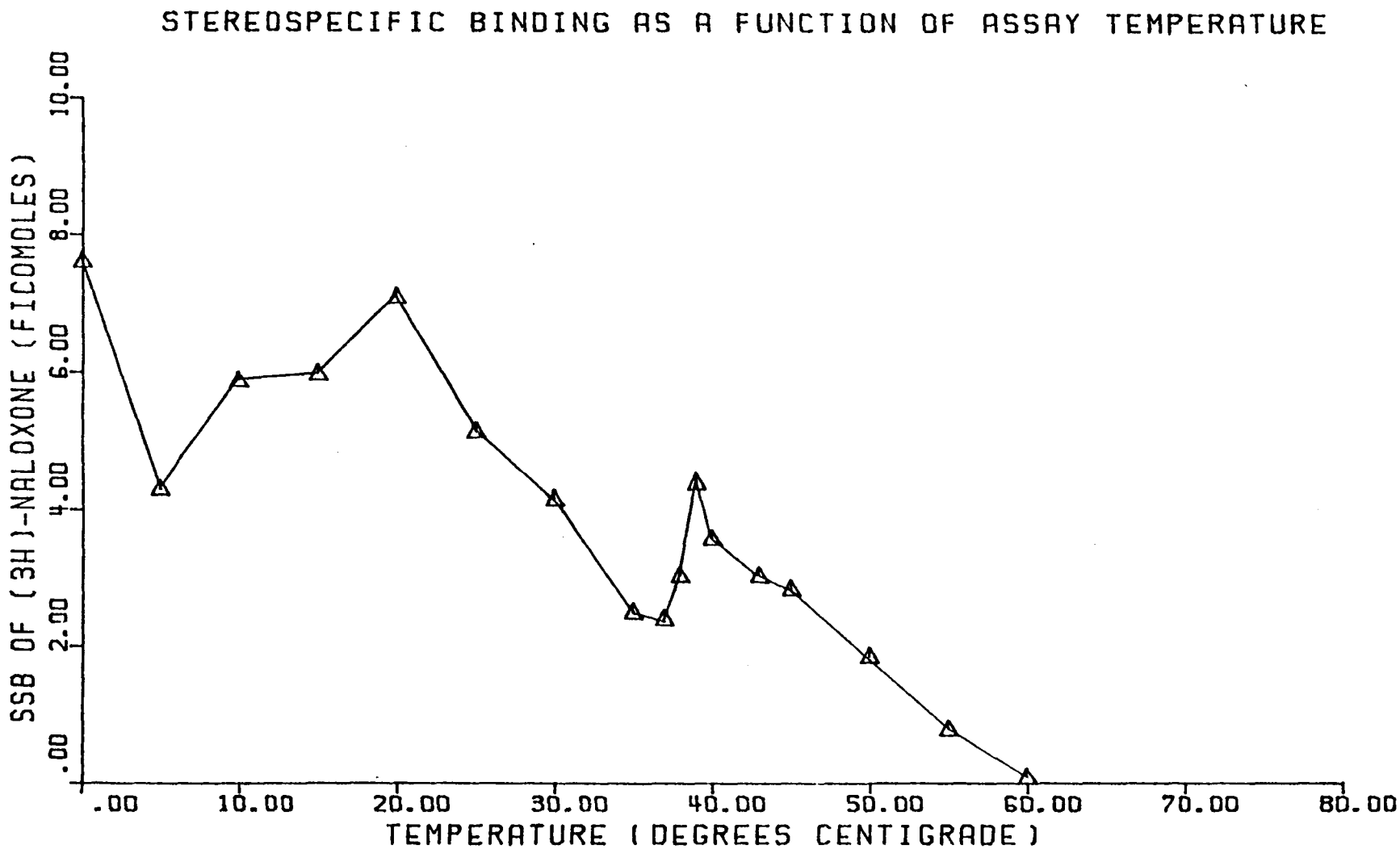
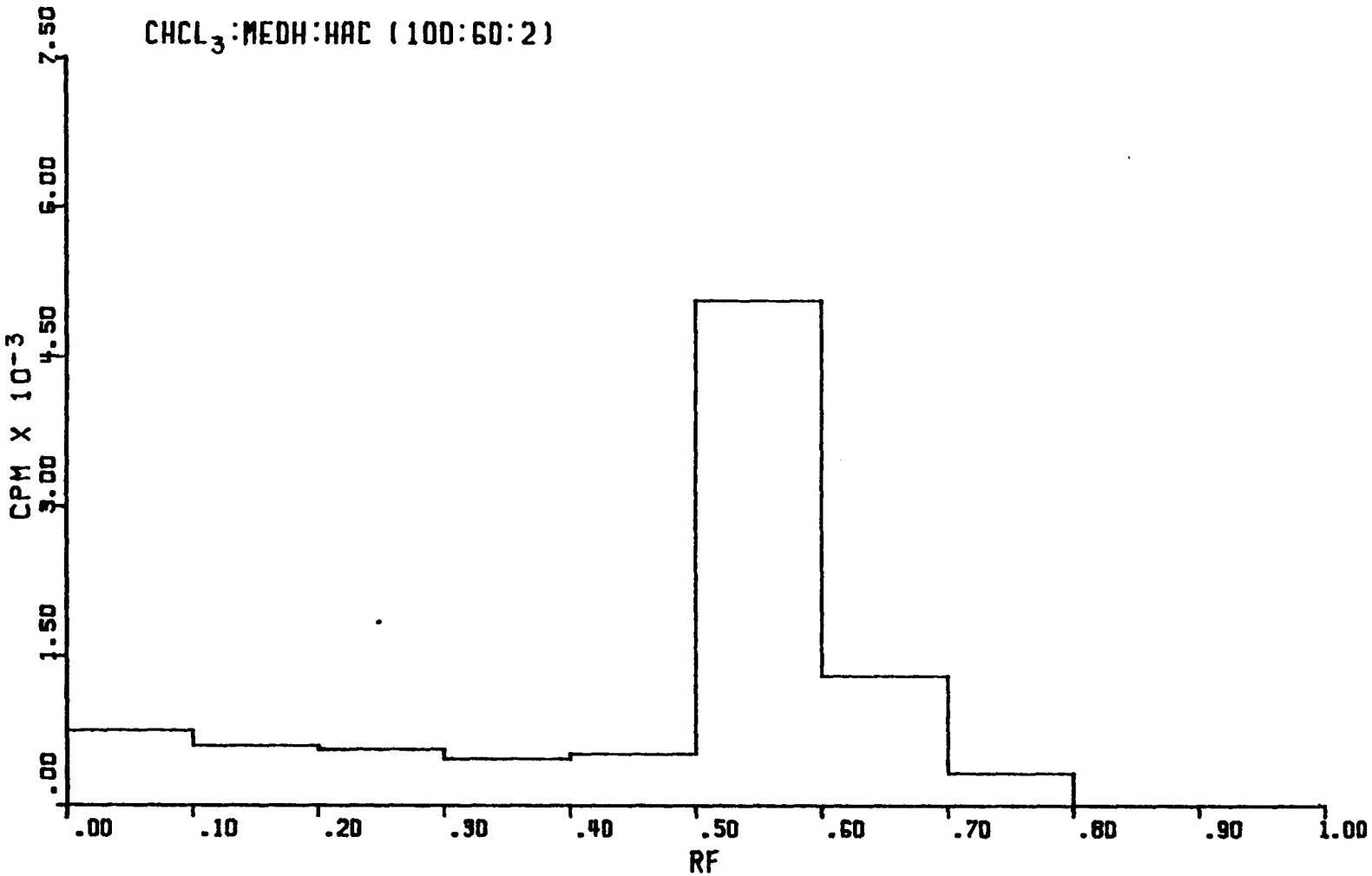

STEREOSPECIFIC BINDING AS A FUNCTION OF ASSAY TEMPERATURE
SSB OF (3H)-NALOXONE (FICOMOLES)
.00
2.00
4.00
6.00
8.00
10.00
.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
TEMPERATURE (DEGREES CENTIGRADE)

Figure 7. The stability of heated naloxone. Naloxone was heated at 65 $^{o}$C for ten minutes, cooled and 20 ul were spotted on a plastic backed thin layer chromatography plate along side 20 ul of naloxone that had been kept at 4 $^{o}$C. The chromatogram was developed in chloroform-methanol-acetic acid (100:60:2), and sectioned into 1 cm. strips. The strips were scraped into vials, and after adding 10 ml of scintillation fluid, they were counted for 10 minutes using three counter cycles.

NALOXONE HEATED TO 65°C FOR 10 MINUTES

$CHCL_3$:MEOH:HAC (100:60:2)



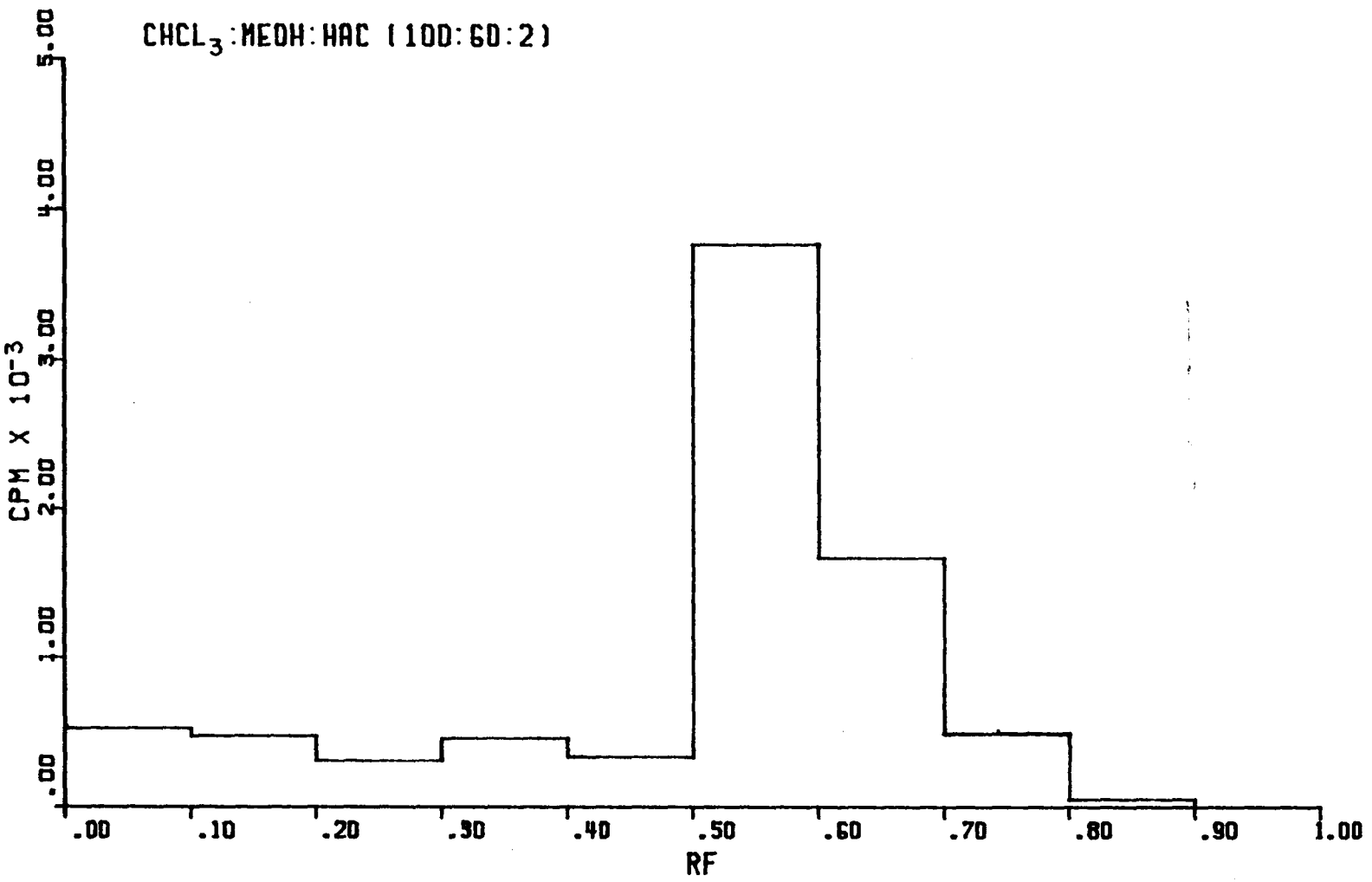
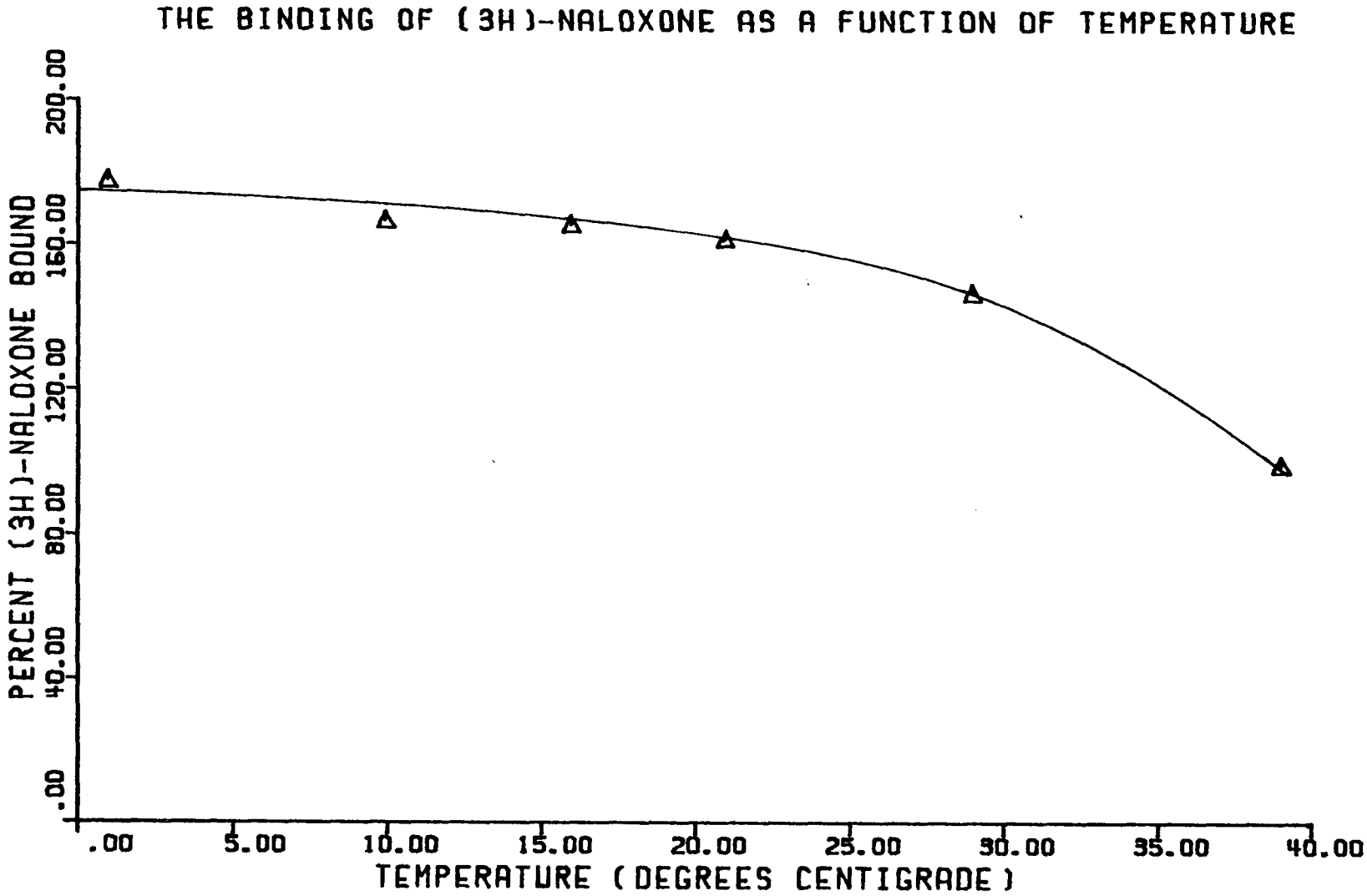

UNHEATED NALOXONE
CHCL3:MEOH:HAC (100:60:2)
CPM X 10-3
5.00
4.00
3.00
2.00
1.00
.00
.00
.10
.20
.30
.40
.50
.60
.70
.80
.90
1.00
RF

Table 5: Binding site parameters derived from the Scatchard analysis of tritiated naloxone binding at 0 °C and 39 °C to embryonic chick brain particulates.

| Constants | Temperature ( °C ) 0 | 39 | Percentage of change from 0 °C |
|---|---|---|---|
| $N_o \times 10^{+14}$ | 11.9 | 10.3 | 15 |
| $K_o \times 10^{-9}$ | 0.57 | 0.33 | 74 |
| $K_1 \times 10^{-9}$ | 1.08 | 0.81 | 33 |
| $K_2 \times 10^{-9}$ | 0.79 | 1.64 | 108 |

$N_o$ represents the number of moles of binding sites per milligram of protein; $K_o$ represents the affinity constant ($M^{-1}$) for the overall binding process; $K_1$ refers to the binding constant for the low affinity sites; $K_2$ represents the binding constant for the high affinity binding sites.

Figure 8. The effect of placing assayed washed particulates on ice prior to filtering. Washed particulates, derived from embryonic chick brain homogenates, were incubated with either 0.1 uM dextrorphan or 0.1 uM levorphanol for five minutes in different flasks before incubating both flasks with 4 nM tritiated naloxone for ten minutes. Immediately after the incubation with naloxone the flasks were placed in an ice bath, and three (2 ml) aliquots were filtered and washed three times with ice-cold 10 mM tris-HCl buffer. The temperature of the assayed homogenates were recorded just prior to withdrawing an aliquot.

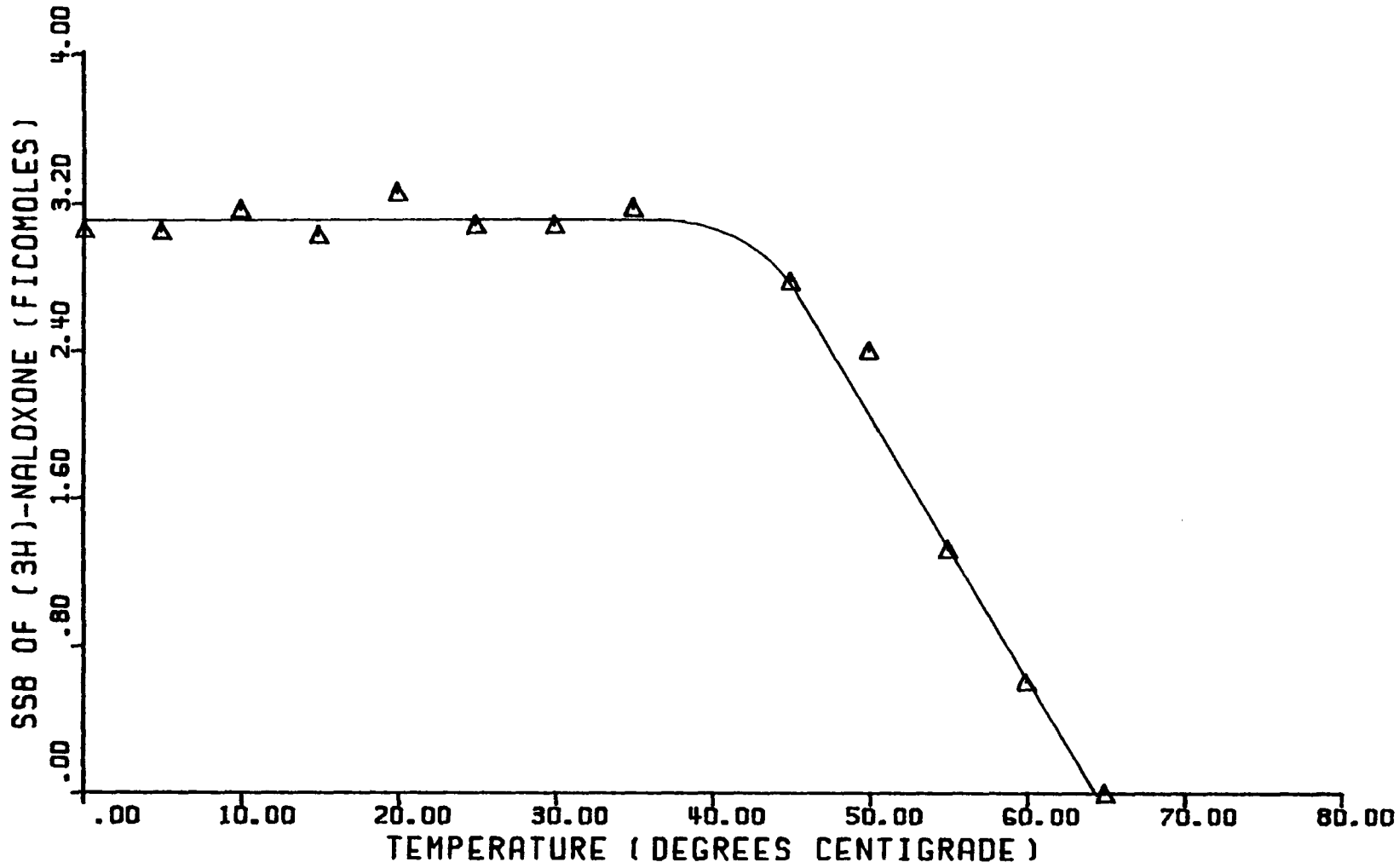

THE BINDING OF (3H)-NALOXONE AS A FUNCTION OF TEMPERATURE
PERCENT (3H)-NALOXONE BOUND
.00
40.00
80.00
120.00
160.00
200.00
.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
TEMPERATURE (DEGREES CENTIGRADE)

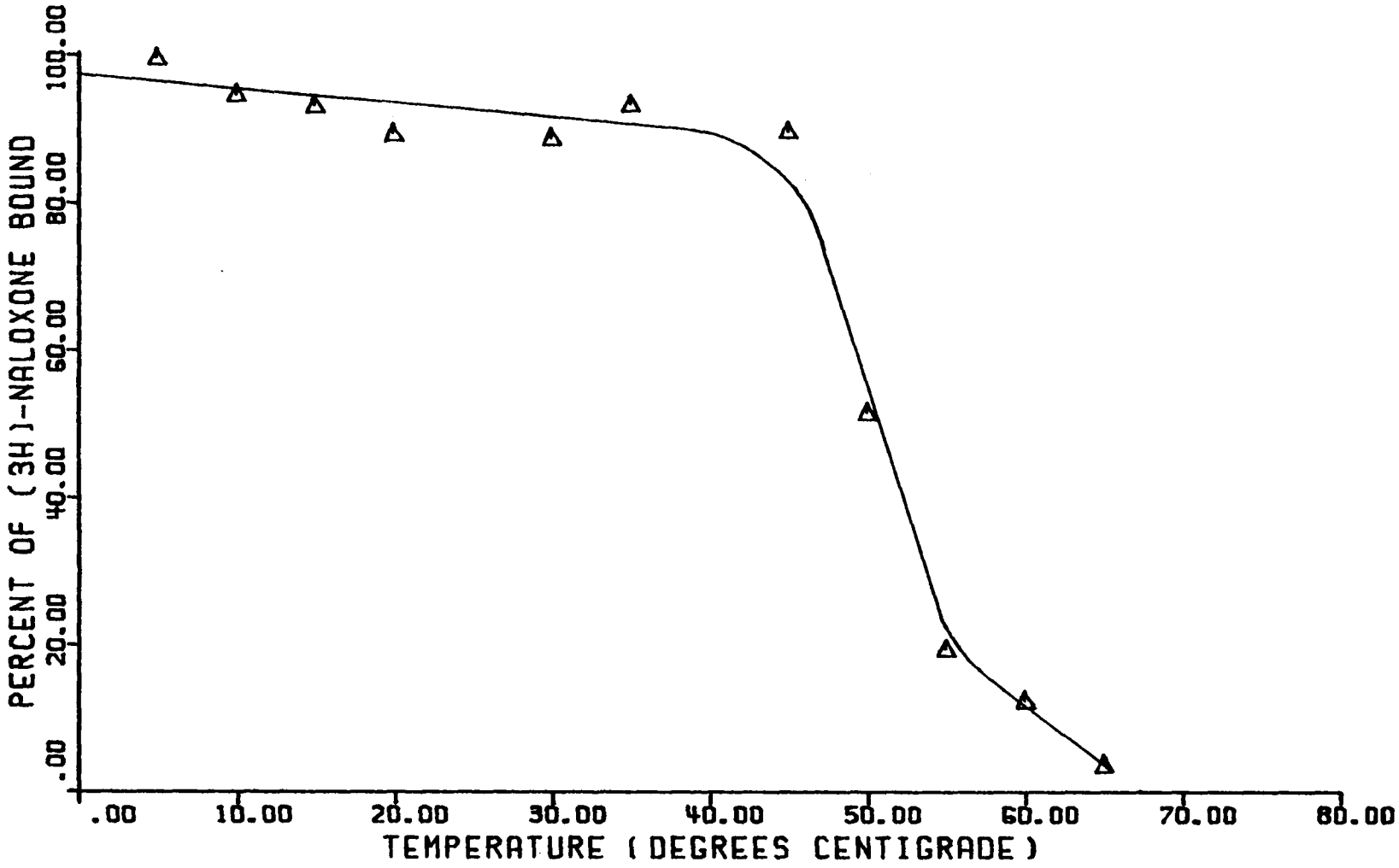
while that of the other set of sites decreased by over fifty percent. These results are sufficient to explain the 74 percent rise in the stereospecific binding of naloxone observed in figure 8.

The apparent thermal denaturation of the receptor sites, figure 9-10, is observed at temperatures exceeding 45 degrees. The binding capacity is unaffected at temperatures at or below 39 degrees. HEPES was also used to investigate this point because its pH changes only slightly as the temperature is raised, ($\Delta$pH=0.003/degree), and because HEPES is a zwitterionic buffer. The binding capacity of embryonic chick brain homogenate was obliterated after a 10 minute incubation at 65 degrees, despite the insignificant change from pH 7.4 calculated to have occurred at this temperature. This suggested that the denaturation of the homogenate is due to thermal effects, and not to alterations in the pH of the buffered sucrose during the incubation.

Figure 9. The thermal stability of the stereospecific opiate binding capacity of embryonic chick brain homogenate preincubated for ten minutes at the indicated temperatures in 5 mM tris-HCl buffered (pH 7.4) 0.32 M sucrose. The standard assay was conducted after equilibrating the homogenates at 39 $^{o}$C.

Figure 10. The thermal stability of the stereospecific opiate binding capacity of embryonic chick brain homogenate preincubated for ten minutes at the indicated temperatures in 10 mM HEPES buffered (pH 7.4) 0.32 M sucrose. The standard assay was begun after equilibrating the homogenates at the assay temperature, and HEPES buffer was used because it is a zwitterionic buffer that undergoes negligible thermally induced shifts in its pH.


THERMAL STABILITY OF THE RECEPTOR
SSB OF (3H)-NALOXONE (FICOMOLES)
4.00
3.20
2.40
1.60
.80
.00
.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
TEMPERATURE (DEGREES CENTIGRADE)


THERMAL STABILITY OF THE RECEPTOR (USING HEPES)
PERCENT OF (3H)-NALOXONE BOUND
100.00
80.00
60.00
40.00
20.00
.00
.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
TEMPERATURE (DEGREES CENTIGRADE)

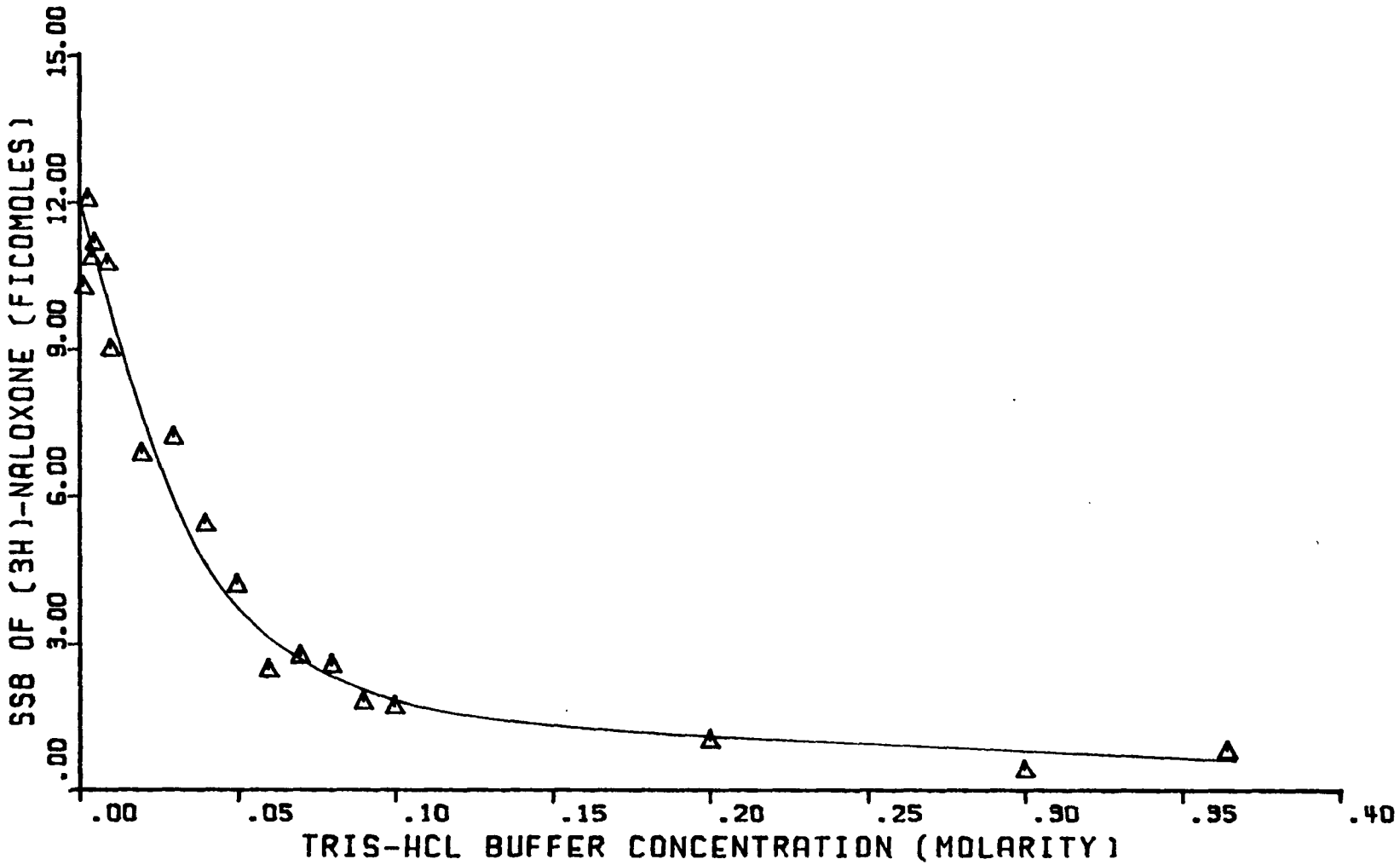
D) Buffer Concentration Effects:

The stereospecific binding of naloxone to embryonic chick brain homogenate is reversibly inhibited, table 6, by high concentrations of buffer used to buffer the sucrose, figures 11-12. Tris-HCl (5 mM) was used to buffer the sucrose solutions because the level of stereospecific binding was relatively uninhibited at this buffer concentration, and because a buffer concentration as low a 3 mM was able to maintain the pH at 7.4. Fifty millimolar tris-buffered sucrose inhibited the binding capacity of embryonic homogenates by over sixty percent. This same concentration had no apparent effect on the stereospecific binding capacity of rat brain homogenates (Pert and Snyder, 1973b). HEPES buffer, figure 12, had similar effects on the binding capacity of embryonic chick brain homogenates.

Table 6, demonstrates the reversibility of this buffer effect, for both agonists and antagonists, and it suggested that repeated washing of the homogenate could enhance the binding of these drugs by almost 50 percent. This enhancement was examined, figures 29-30, and table 11, and found to arise presumably from the removal of some endogenous inhibitor by incubating a particulate fraction under suitable conditions, and repeating the washing procedure described in the methods, section C.

Figure 11. The effect of the tris-HCl buffer concentration on the stereospecific binding of naloxone to embryonic chick brain homogenate. Tris-HCl buffered sucrose (0.32 M) solutions were added to concentrated homogenate prepared in unbuffered 0.32 M sucrose. The buffered sucrose was prepared by adding aliquots of concentrated tris-HCl buffer, pH 7.4, to 0.32 M sucrose solutions before diluting the concentrated homogenate with the buffered sucrose. The pH was maintained by buffer concentrations of 3 mM or greater.

Figure 12. The effect of HEPES buffered sucrose on the stereospeicific binding of naloxone to embryonic chick brain homogenate. HEPES buffered sucrose was prepared according th the procedure described in figure 11. The pH of the homogenate was also maintained at a buffer concentration of 3 mM or greater.


EFFECT OF [TRIS-HCL] ON THE SSB OF (3H)-NALOXONE TO EMBRYONIC BRAIN
SSB OF (3H)-NALOXONE (FICOMOLES)
15.00
12.00
9.00
6.00
3.00
.00
.00
.05
.10
.15
.20
.25
.90
.95
.40
TRIS-HCL BUFFER CONCENTRATION (MOLARITY)

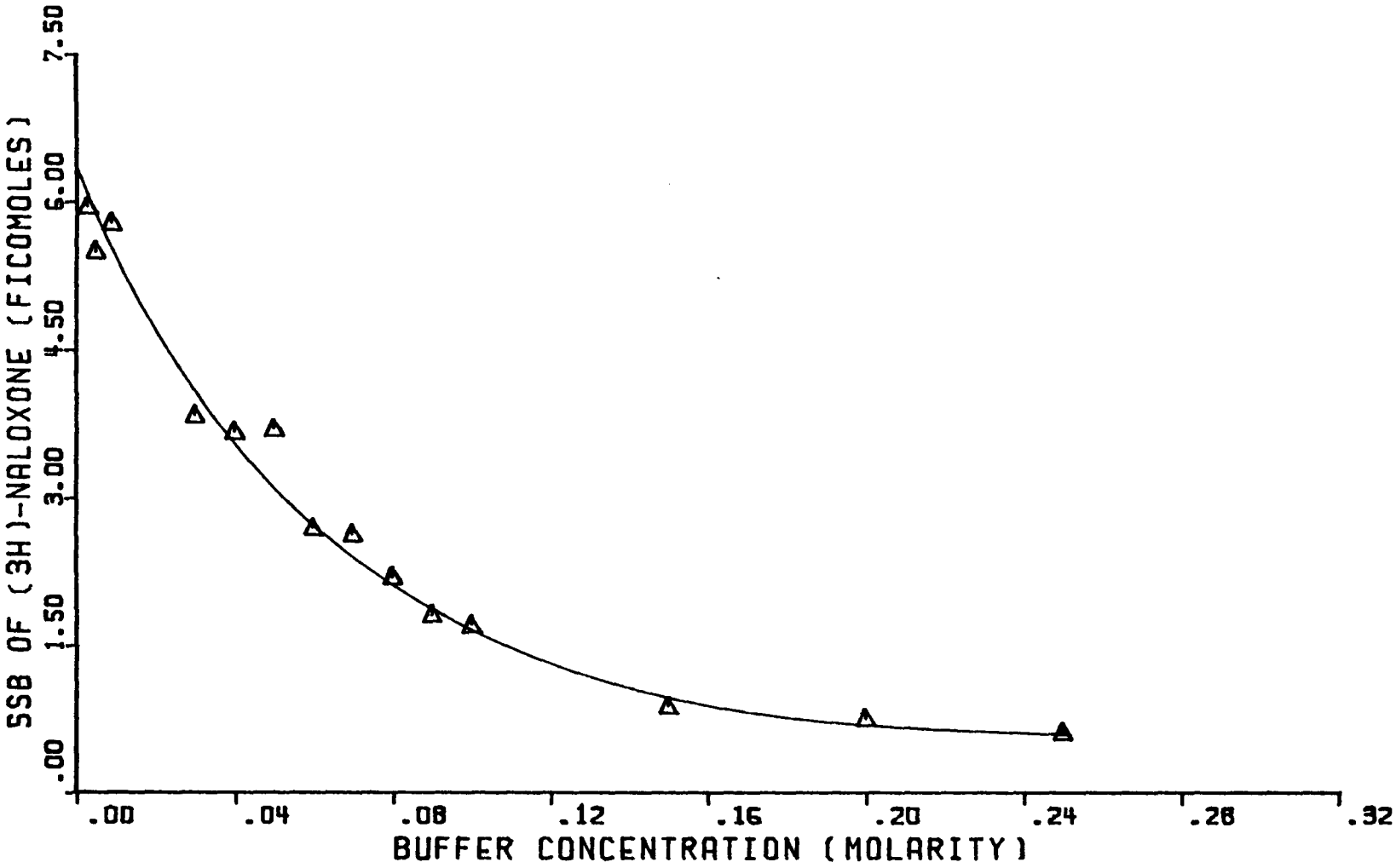

EFFECT OF [HEPES BUFFER] ON THE SSB OF (3H)-NALOXONE
SSB OF (3H)-NALOXONE (FICOMOLES)
7.50
6.00
4.50
3.00
1.50
.00
.00
.04
.08
.12
.16
.20
.24
.28
.32
BUFFER CONCENTRATION (MOLARITY)

Table 6: The reversal of the buffer induced inhibition of stereospecific binding by consecutive washings with buffered sucrose.

| Buffer Concentration ( mM ) | Naloxone (Percent bound with | Etorphine 5 mM buffer) |
|---|---|---|
| 5.0 | 100 ± 0.6 | 100 ± 1.9 |
| 50.0 | 62.1 ± 4.4 | 57.6 ± 0.6 |
| 500.0 | 13.8 ± 1.9 | 15.6 ± 0.2 |
| 50.0 | 89.2 ± 2.7 | 83.2 ± 4.1 |
| 5.0 | 148 ± 2.7 | 132 ± 1.9 |

All of the particulates were examined using the standard assay procedure, and all of the centrifugations were done at 4 $^{o}$C.

Washed particulates were suspended in 5 mM tris-buffered sucrose and aliquots were placed in four centrifuge tubes. After centrifuging the particulates at 49,000 g for ten minutes, the supernatant was discarded, and all of the pellets were resuspended with the original volume of 50 mM tris-buffered sucrose. One tube was assayed, while the others were centrifuged again at 49,000 g for ten minutes. The supernatants were discarded, and all of the pellets resuspended with the original volume of tris-buffered sucrose. One tube was assayed, and the others recentrifuged at 49,000 g for 10 minutes. The pellets were washed once with 15 ml of 50 mM tris-buffered sucrose, and after recentrifuging at 49,000 g for ten minutes, the pellets were resuspended with the original volume of 50 mM tris-buffered sucrose. One tube was assayed according to the standard procedure, and the other was recentrifuged at 49,000 g for ten minutes. The pellet was resuspended in 15 ml of 5 mM tris-sucrose, recentrifuged, and the pellet was resuspended with 5 mM tris-sucrose, prior to being assayed.

All of the particulates were derived from embryonic chick brain.

E) Washing Procedure Effects:

Figure 13 indicates that washing embryonic chick brain homogenate by the procedure described in the methods, section C, leads to a forty percent increase in the binding of naloxone to homogenates assayed with various concentrations of sodium chloride. Sodium chloride, which increases the stereospecific binding of naloxone to embryonic chick brain homogenates by almost 20 percent at low concentrations, figures 13 and 36, produces a sixty percent binding enhancement in washed particulates derived from this homogenate, figure 14. Washed particulates derived from adult chicken brain homogenate, figure 15, and rat brain homogenate, figure 16, show a more stable and sustained sodium effect than particulates derived from embryonic chick brain, figure 14. The washing procedure described in the methods, section C, resulted in binding enhancements that seemed to be due to the removal of some endogenous substances, that depress the opiate binding capacity of the homogenates. Table 7, indicates the concentrations of sodium and potassium found at each stage of the washing procedure, and clearly demonstrates that endogenous sodium and potassium are almost completely removed by the washing procedure.

Figure 13. The stereospecific binding capacity of embryonic chick brain homogenate (⊙) and washed particulates (△), as a function of the sodium ion concentration. An aliquot of the concentrated homogenate was washed using the procedure described in the methods (section C), and both the dilute homogenate (methods, section A) and the washed particulates (methods, section C) were assayed with 2.0 nM tritiated naloxone using the standard assay procedure. Both preparations were placed into tubes containing various aliquots of sodium chloride and a volume of tris buffered sucrose sufficient to make a final assay volume of 2.2 ml.

THE INCREASE OF THE SODIUM EFFECT BY WASHING THE HOMOGENATE

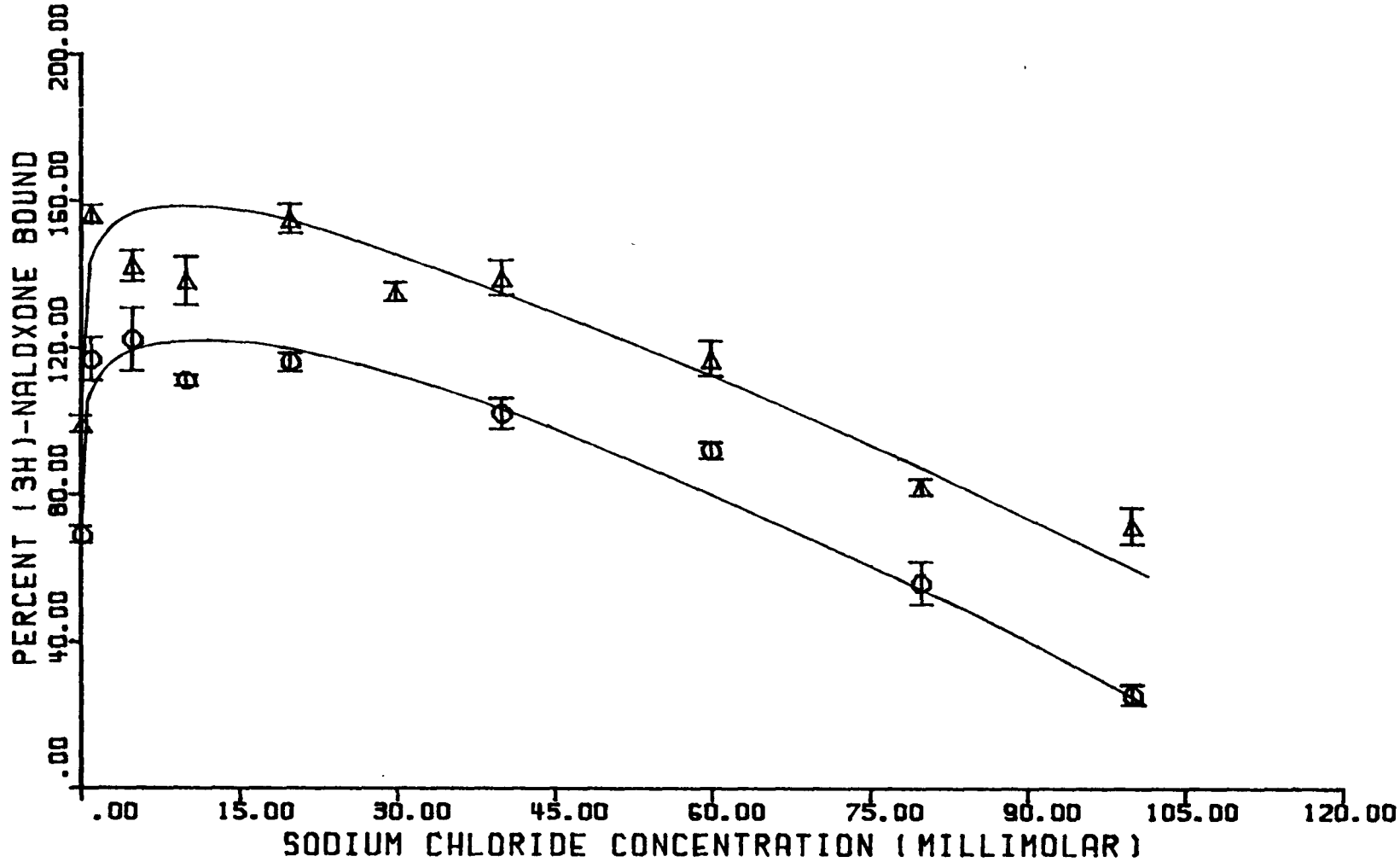


Figure 14. The effect of sodium chloride on the stereospecific binding of tritiated naloxone to washed embryonic chick brain particulates. Six 2 ml aliquots of washed particulates were placed into tubes containing the appropriate aliquots of sodium chloride and sufficient tris buffered sucrose to bring the assay volume to 2.2 ml. Particulates were examined by the standard assay procedure, and filtered without prior cooling. Tritiated naloxone (2 nM) was used in the standard assay.

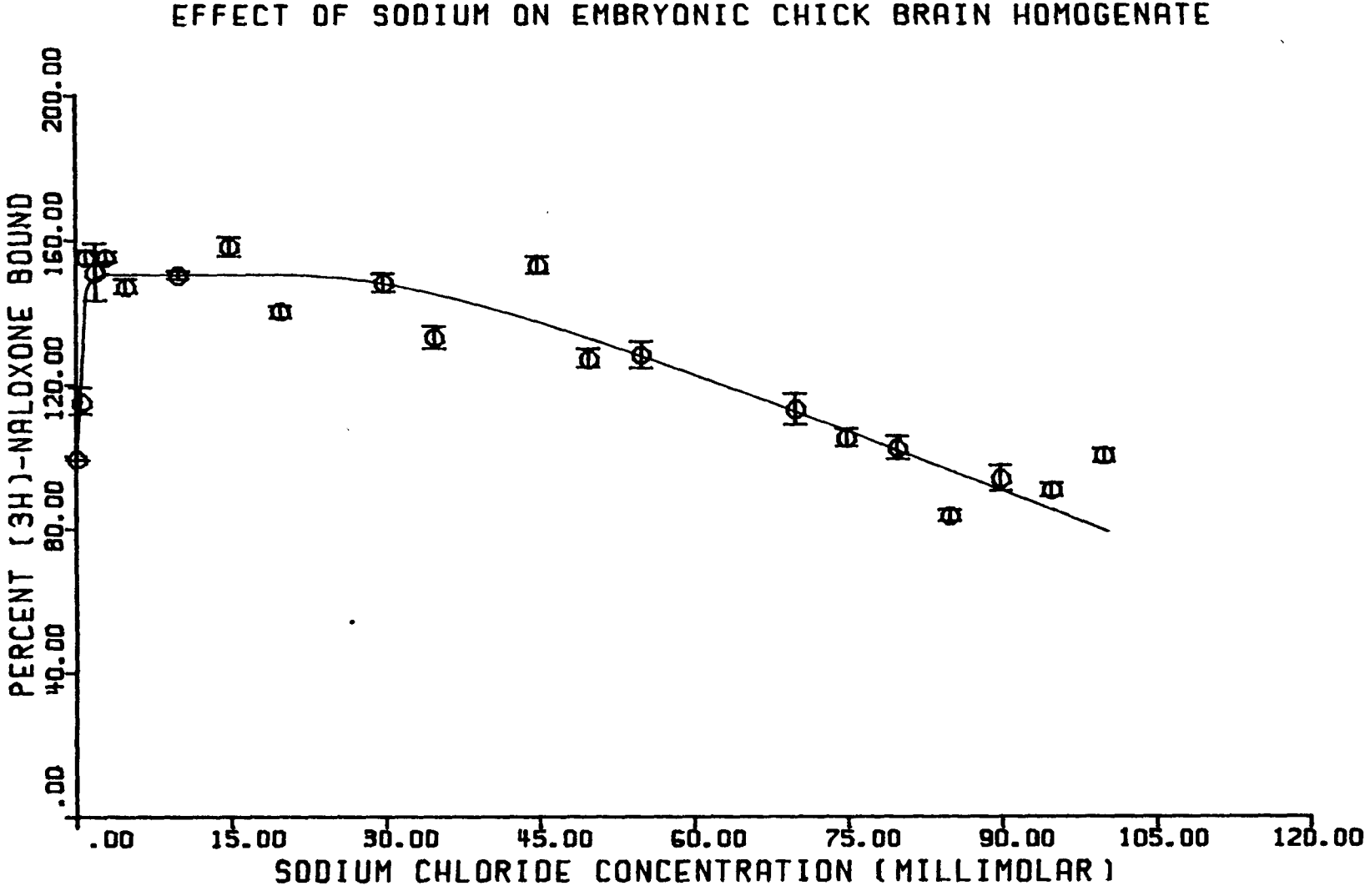

EFFECT OF SODIUM ON EMBRYONIC CHICK BRAIN HOMOGENATE
PERCENT (3H)-NALOXONE BOUND
.00
40.00
80.00
120.00
160.00
200.00
.00
15.00
30.00
45.00
60.00
75.00
90.00
105.00
120.00
SODIUM CHLORIDE CONCENTRATION (MILLIMOLAR)

Figure 15. The effect of sodium chloride on the stereospecific binding of tritiated naloxone to washed adult chicken brain particulates. Six 2 ml aliquots of washed particulates were placed into tubes containing an appropriate aliquot of sodium chloride and sufficient tris buffered sucrose to maintain the assay volume at 2.2 ml. Particulates were examined by the standard assay procedure, and filtered without prior cooling.

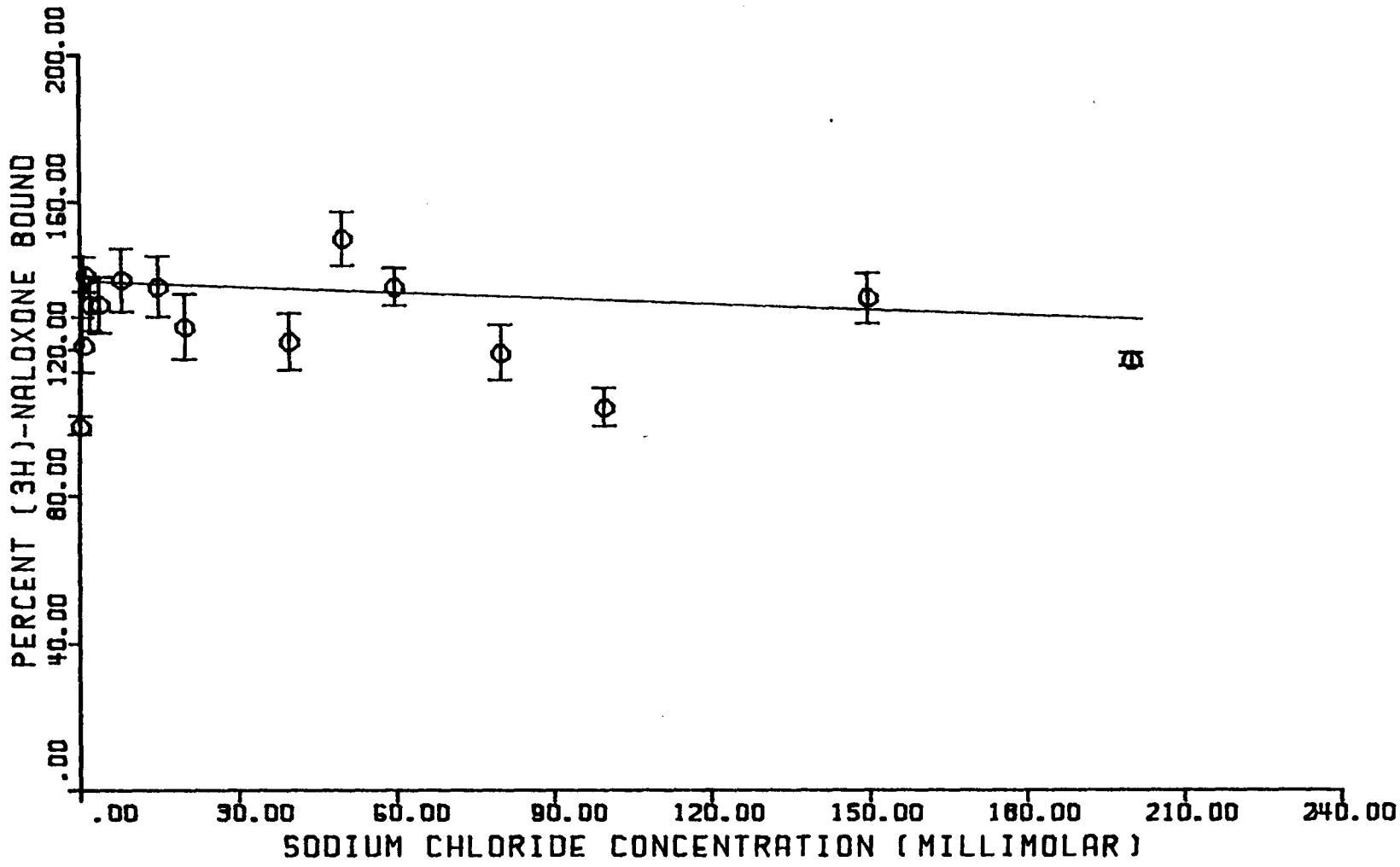

EFFECT OF SODIUM ON ADULT CHICK BRAIN HOMOGENATE
PERCENT [3H]-NALOXONE BOUND
.00
40.00
80.00
120.00
160.00
200.00
.00
30.00
60.00
90.00
120.00
150.00
180.00
210.00
240.00
SODIUM CHLORIDE CONCENTRATION [MILLIMOLAR]

Figure 16. The effect of sodium chloride on the stereospecific binding of tritiated naloxone to washed adult rat brain particulates. Six 2 ml aliquots of washed particulates, prepared with 50 mM tris buffered sucrose pH 7.4, were placed into tubes containing aliquots of sodium chloride and enough tris buffered sucrose sufficient to bring the assay volume to 2.2 ml. Particulates were examined by the standard assay procedure, and filtered without prior cooling.

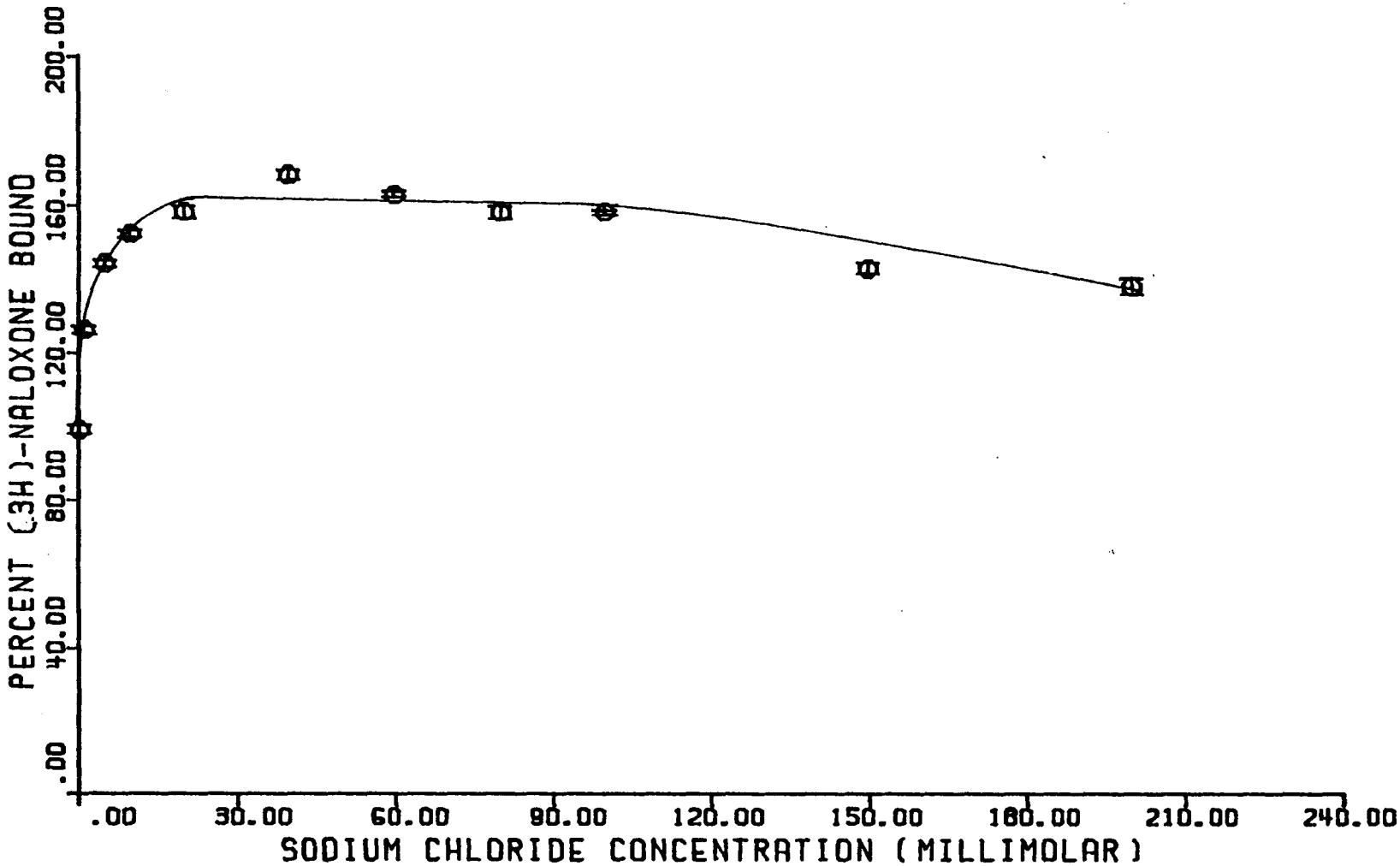

SODIUM EFFECT IN RAT BRAIN HOMOGENATE
PERCENT (3H)-NALOXONE BOUND
.00
40.00
80.00
120.00
160.00
200.00
.00
30.00
60.00
90.00
120.00
150.00
180.00
210.00
240.00
SODIUM CHLORIDE CONCENTRATION (MILLIMOLAR)

Table 7: The retention of sodium and potassium ions at the various stages in the washing procedure of embryonic chick brain homogenate.

| Sample | Sodium Content (milliequivalents/liter) | Potassium Content (milliequivalents/liter) |
|---|---|---|
| Homogenate | 15.2 | 14.8 |
| Supernatant | 13.4 | 11.7 |
| First Wash | 4.1 | 3.4 |
| Second Wash | 1.6 | 1.4 |
| Washed Pellet | 0.1 | 0.2 |
| Tris-Sucrose | 0.1 | 0.05 |

Homogenates were washed in tris-sucrose ( 0.005-0.32 M ) by centrifuging them at 49,000 g for ten minutes at 4 $^{o}$C. The supernatant was decanted and the pellet resuspended in the tris buffered sucrose. This entire procedure was repeated again before assaying the washed pellet.

F) Protein and PH Effects:

For the binding of naloxone to embryonic chick brain homogenate a linear dependence on the protein content was found between the range 0.2-0.5 mg/ml, figure 17. Above 1.8 mg/ml of protein the filtration flow rate decreases rapidly, thus leading to an artifactual decrease in the binding capacity of the homogenate (that actually results from warming).

The stereospecific binding of naloxone to embryonic homogenate exhibits two peaks at pH 6.7 and 7.4, figure 18. An assay pH of 7.4 was chosen because it was considered to be physiological, and because it was the major binding peak. Presumably, the sodium ion content of the phosphate buffer was responsible for the enhanced pH profile obtained while using this buffer. Below pH 5.7 and above pH 9.0 the opiate binding capacity of embryonic chick brain homogenates is negligible.

Figure 17. The effect of homogenate concentration on the stereospecific binding capacity of embryonic chick brain homogenate. Successively larger aliquots of concentrated homogenate were added to enough buffered 0.32 M sucrose to make 15 ml of homogenate prior to assaying it by the standard procedure.

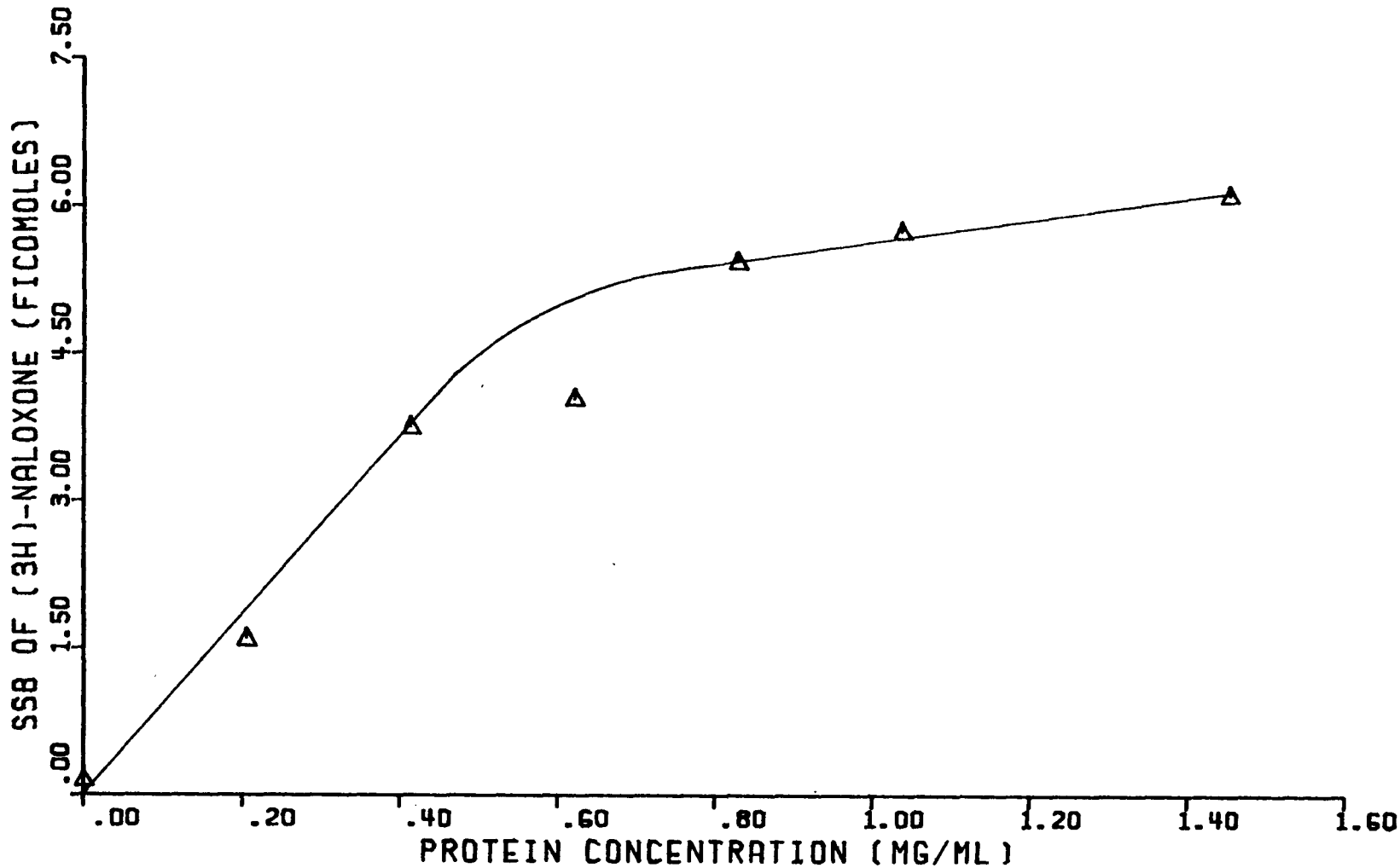
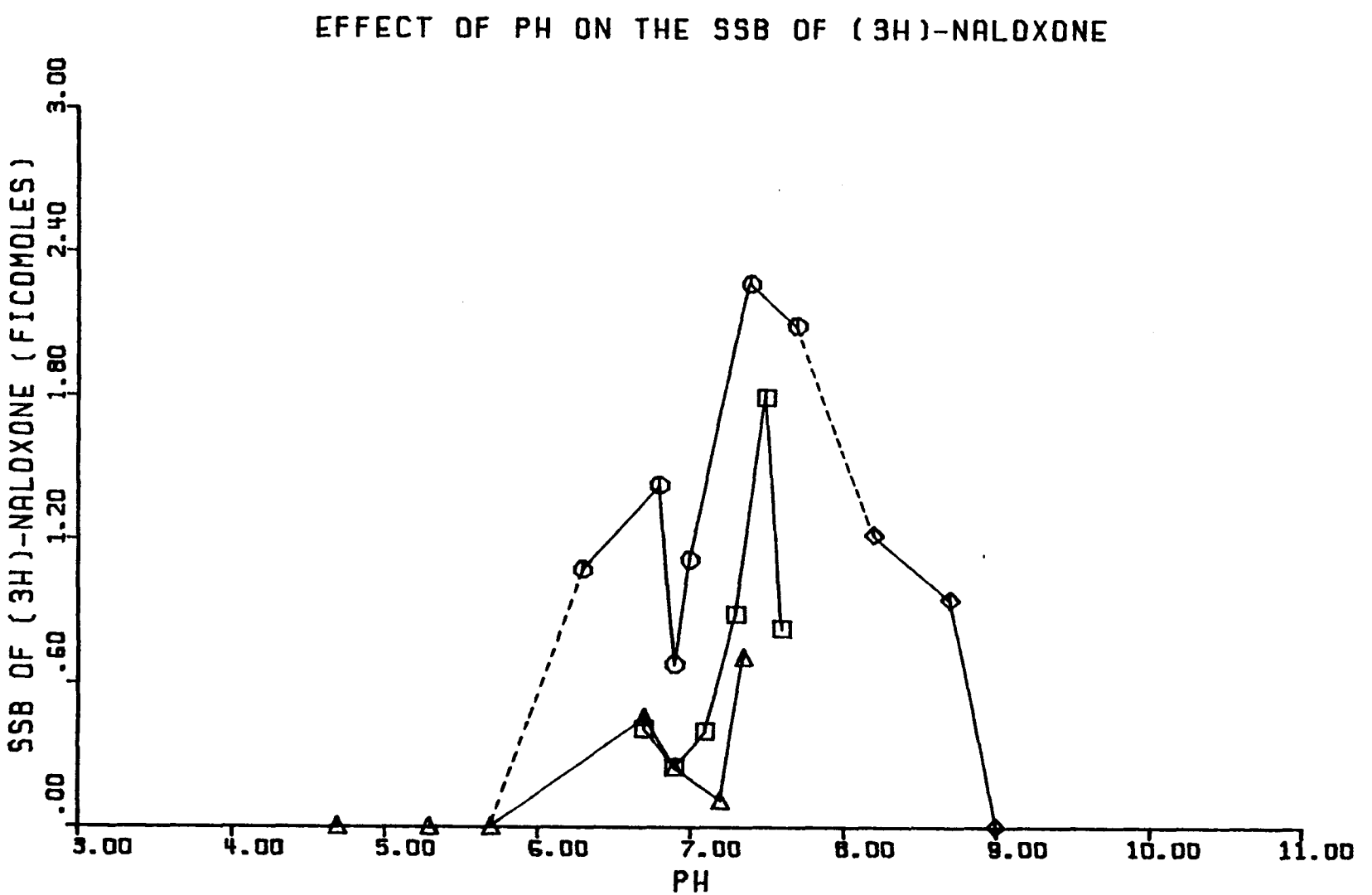

EFFECT OF PROTEIN CONCENTRATION ON SSB OF (3H)-NALOXONE
SSB OF (3H)-NALOXONE (FICOMOLES)
.00
1.50
3.00
4.50
6.00
7.50
.00
.20
.40
.60
.80
1.00
1.20
1.40
1.60
PROTEIN CONCENTRATION (MG/ML)

Figure 18. The effect of pH on the stereospecific binding capacity of embryonic chick brain homogenate. The following buffer systems were prepared by mixing aliquots of the components of each system (Data for Biochemical Research, 1969a), and measuring the resulting pH on a Corning model 7 pH meter with a Ag/AgCl//KCl microelectrode: citric acid-potassium phosphate (pH 4.6-7.3), (△); tris-HCl (pH6.7-7.5), (⊡); sodium hydrogen phosphate-potassium dihydrogen phosphate (pH 6.2-7.8), (⊙); and glycine-potassium hydroxide (pH 8.2-9.0), (◈). Aliquots of concentrated homogenate were added to tubes containing chilled 5 mM buffered 0.32 M sucrose solutions, and the pH was measured again.


EFFECT OF PH ON THE SSB OF (3H)-NALOXONE
SSB OF (3H)-NALOXONE (FICOMOLES)
.00
.60
1.20
1.80
2.40
3.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00
11.00
PH

G) Thin Layer Chromatographic Analyses:

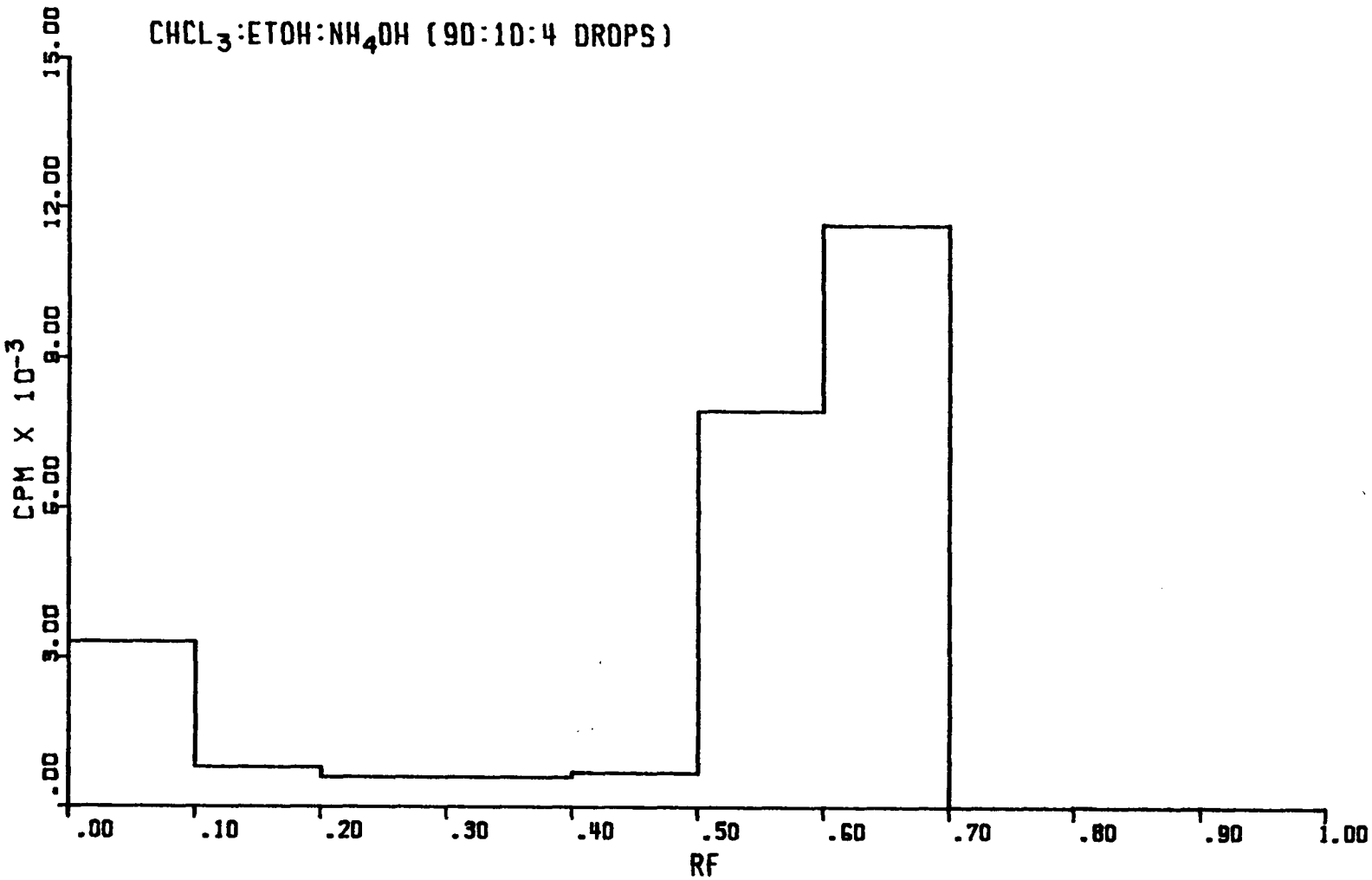
Thin layer chromatographic analysis, figures 19-22, of tritiated etorphine and naloxone incubated in the presence or absence of washed particulates shows almost identical Rf values, table 8. Minor discrepencies in the Rf values presumably resulted from a slight curvature of the solvent front across two of the chromatograms. Any discrepancy resulting from this solvent front curvature was probably compounded by the sectioning technique used to analyze the thin layer chromatogram.

Figure 19-20. The stability of naloxone incubated with embryonic chick brain particles during the course of a standard assay. The solvent system used is indicated on the figure.

Figure 21-22. The stability of etorphine incubated with embryonic chick brain particles during the course of a standard assay. The solvent system used is indicated on the figure.

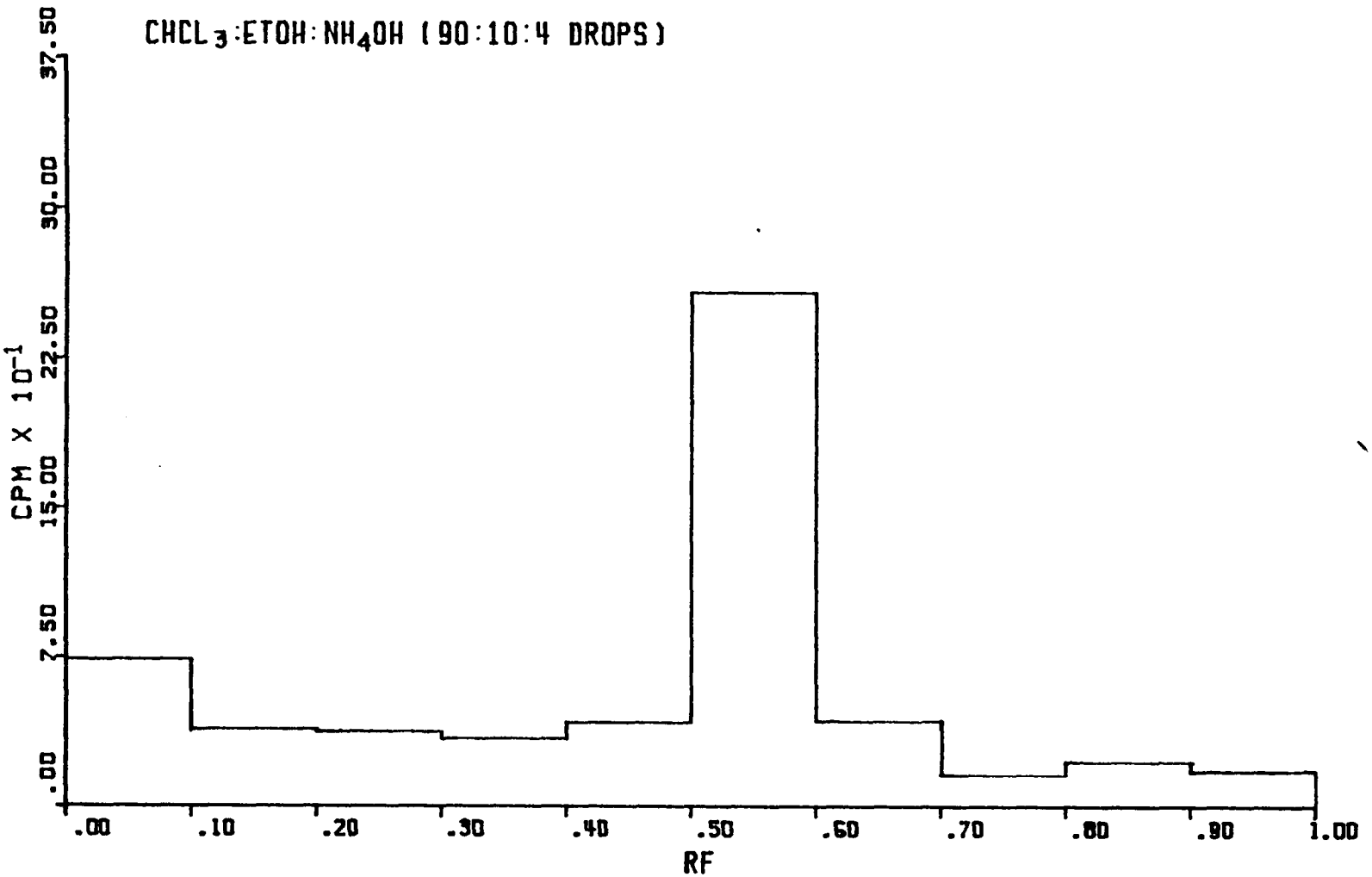
UNTREATED NALOXONE

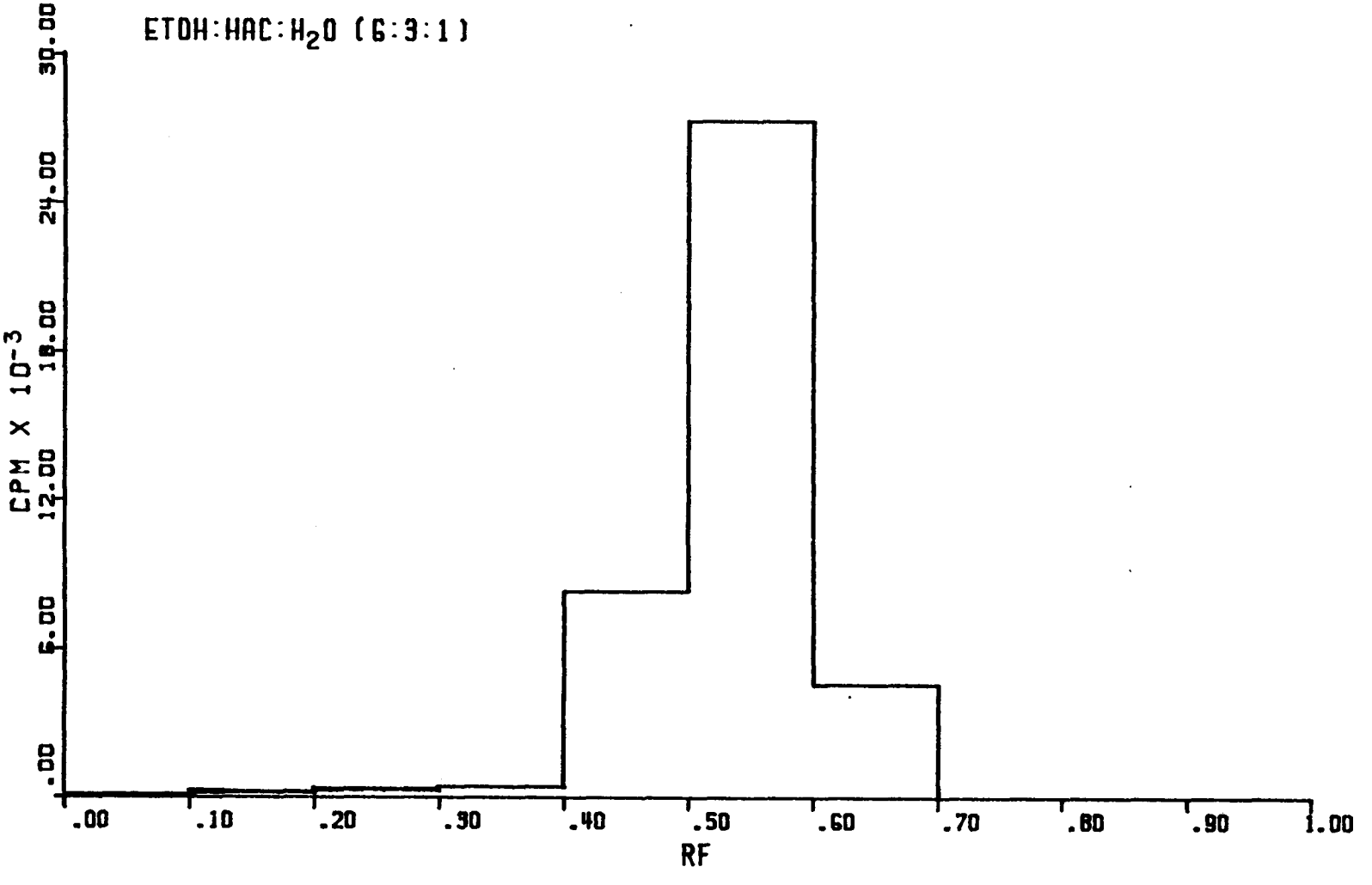
$CHCL_3$:ETOH:$NH_4OH$ (90:10:4 DROPS)



NALOXONE EXPOSED TO HOMOGENATE:

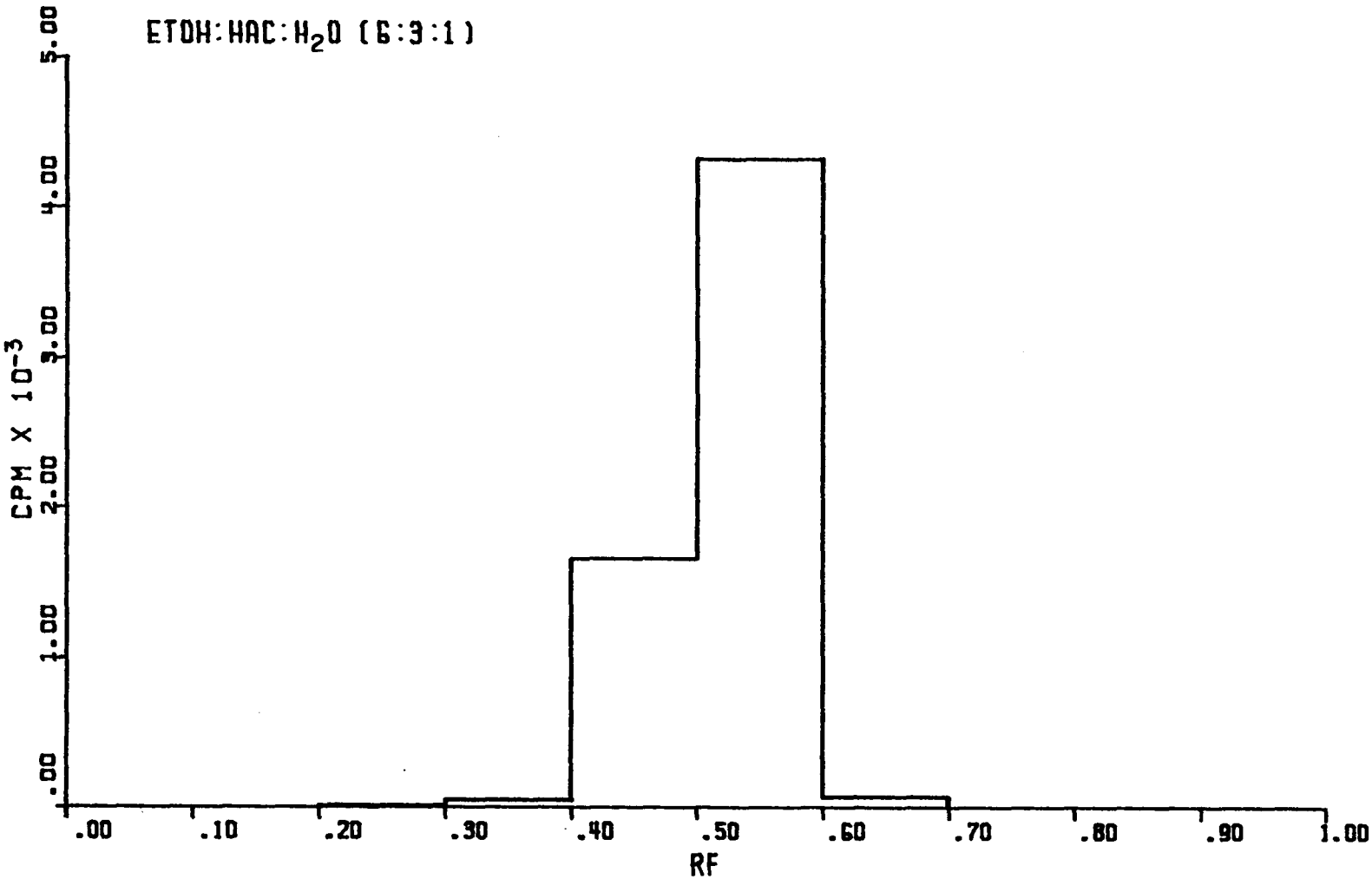
$CHCL_3$:ETOH:$NH_4OH$ (90:10:4 DROPS)

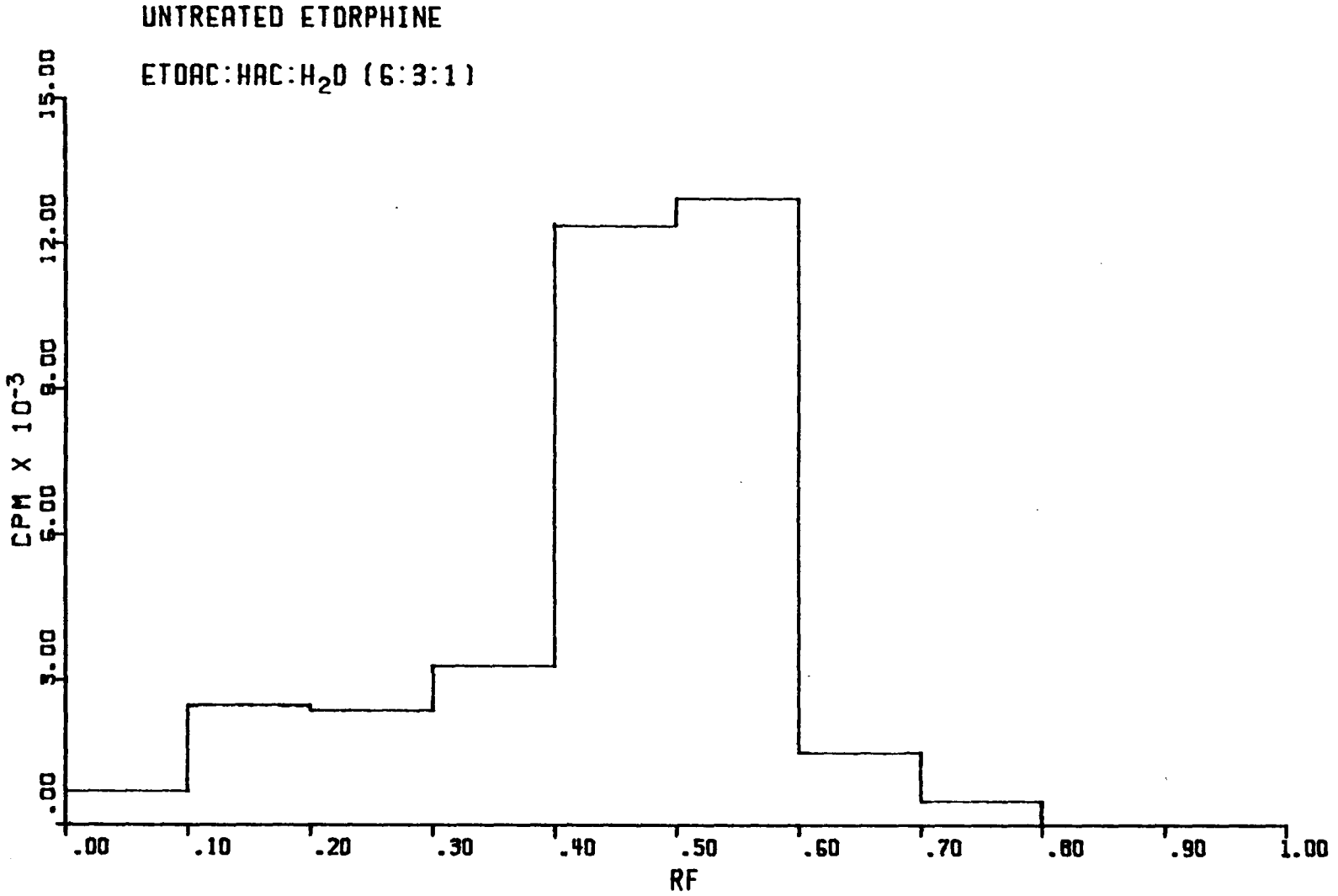



UNTREATED NALOXONE
ETOH:HAC:$H_2O$ (6:3:1)
CPM X $10^{-3}$
.00
6.00
12.00
18.00
24.00
30.00
.00
.10
.20
.30
.40
.50
.60
.70
.80
.90
1.00
RF

NALOXONE EXPOSED TO HOMOGENATE

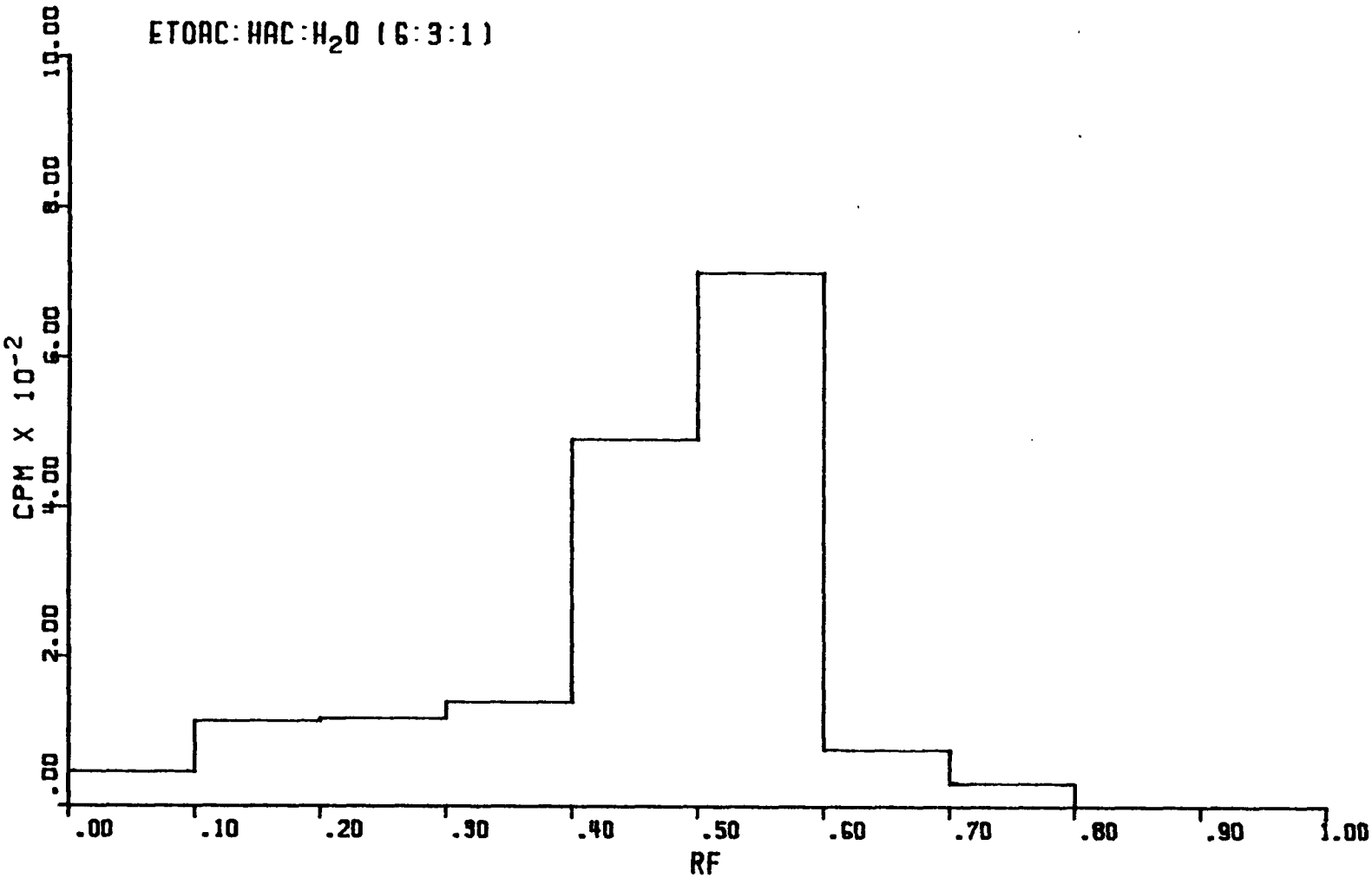
ETOH:HAC:$H_2O$ (6:3:1)

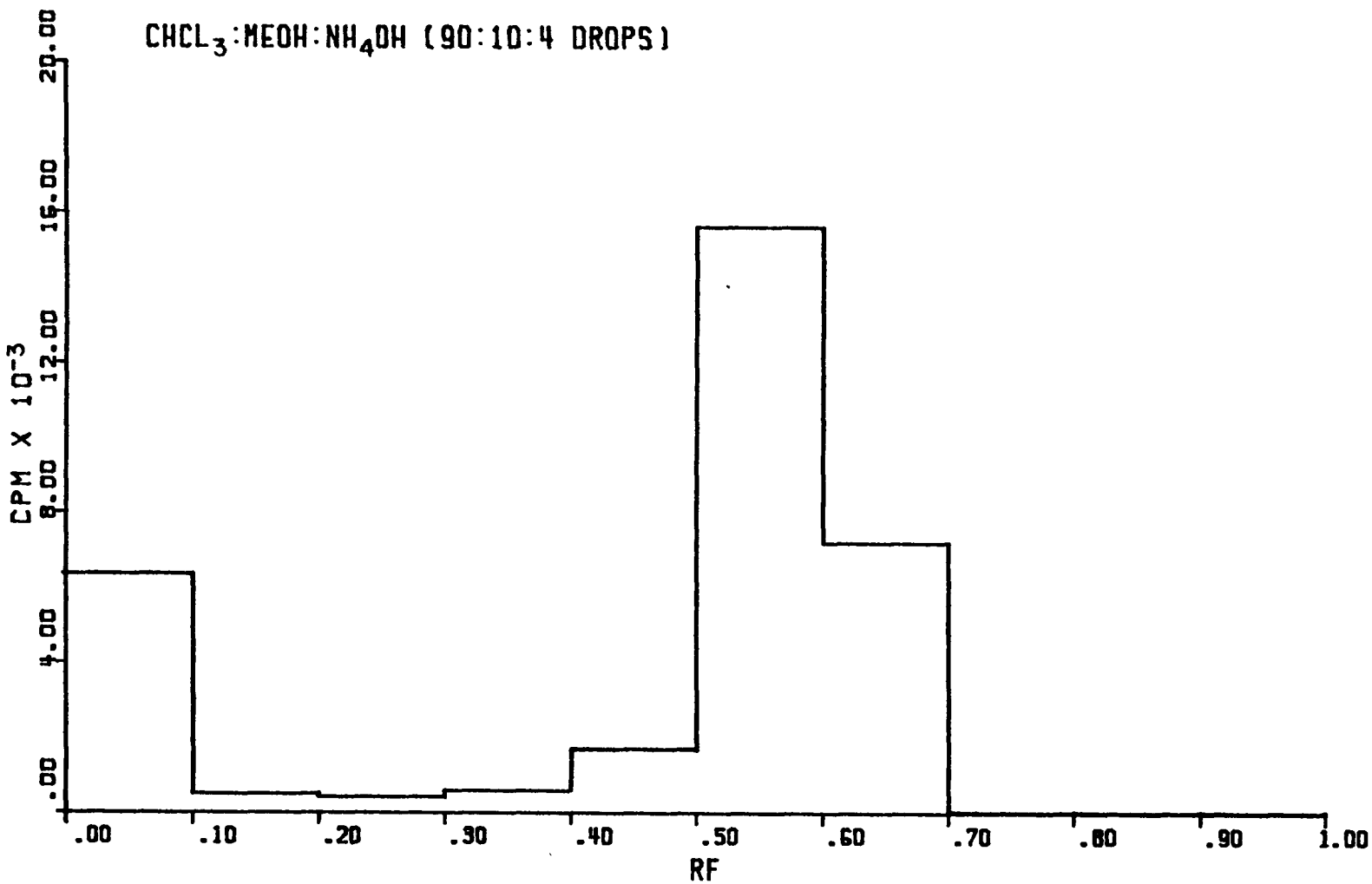



UNTREATED ETORPHINE
ETOAC:HAC:H2O (6:3:1)
CPM X 10-3
15.00
12.00
9.00
6.00
3.00
.00
.00
.10
.20
.30
.40
.50
.60
.70
.80
.90
1.00
RF

ETORPHINE EXPOSED TO HOMOGENATE:

ETOAC:HAC:$H_2O$ (6:3:1)




UNTREATED ETORPHINE
CHCL3:MEOH:NH4OH (90:10:4 DROPS)
CPM X 10-3
20.00
16.00
12.00
8.00
4.00
.00
.00
.10
.20
.30
.40
.50
.60
.70
.80
.90
1.00
RF

ETORPHINE EXPOSED TO HOMOGENATE

$CHCL_3$:MEOH:$NH_4OH$ (90:10:4 DROPS)

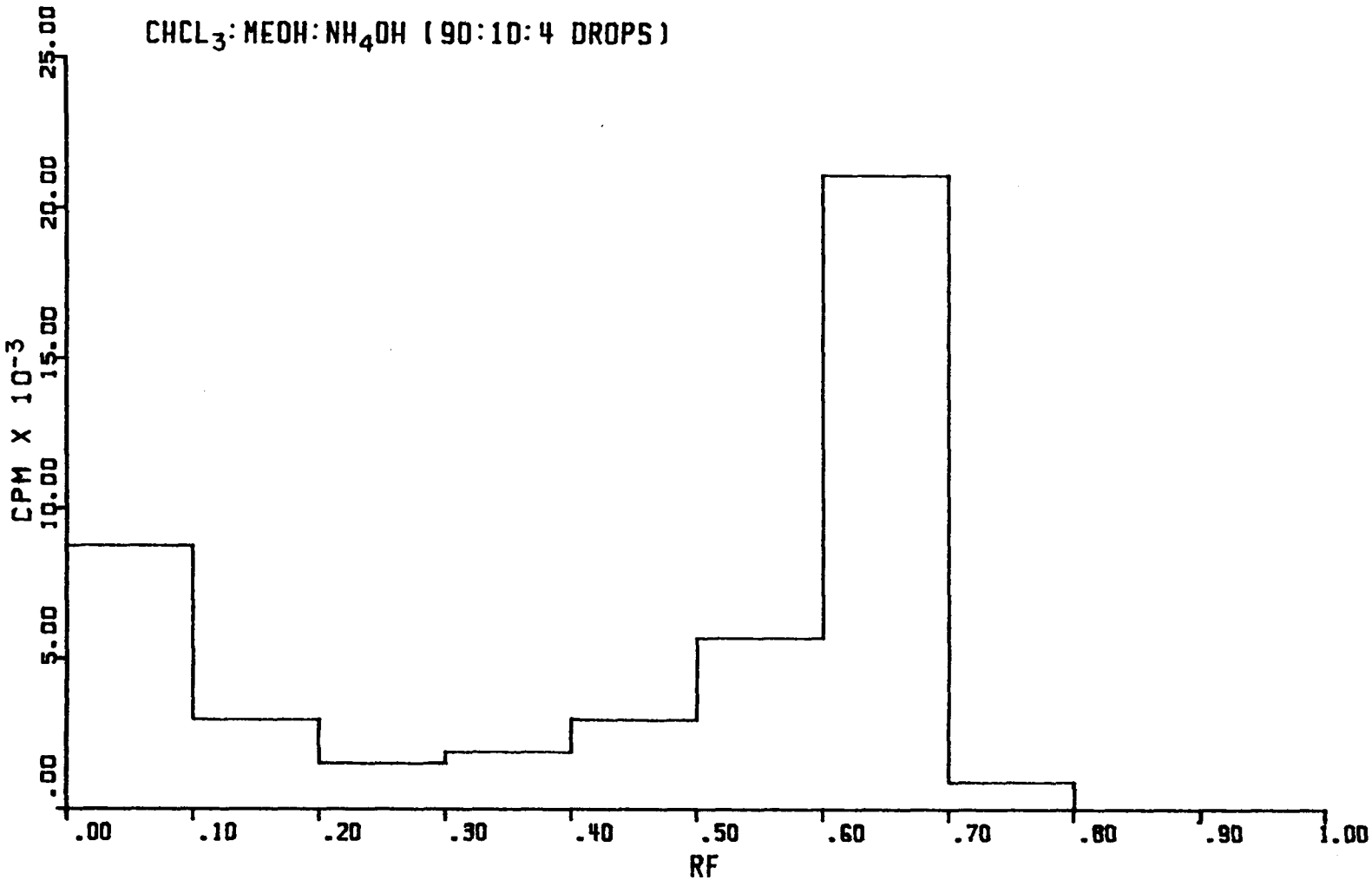


Table 8: Thin layer analysis of etorphine and naloxone incubated with washed particles from embryonic chick brain.

| Opiate | Solvent | $R_f$ authentic opiate | $R_f$ preincubated opiate | $\Delta R_f$ |
|---|---|---|---|---|
| Naloxone | A | 0.62 | 0.56 | -0.06 |
| Naloxone | B | 0.53 | 0.53 | 0.00 |
| Etorphine | C | 0.51 | 0.52 | 0.01 |
| Etorphine | D | 0.58 | 0.63 | 0.05 |

Solvent A - chloroform:ethanol:ammonia (90:10:4 drops).

Solvent B - ethanol:acetic acid: water (6:3:1).

Solvent C - ethylacetate:acetic acid:water (6:3:1).

Solvent D - chloroform:methanol:ammonia (90:10:4 drops).

Five milliliters of washed particles from embryonic chick brain were prepared according to the procedures described in sections A and C of the methods using freshly distilled water in place of the buffered sucrose. The pellets were resuspended in 5 ml of water during the washing procedure, and the twice washed pellet was resuspended to a volume of 3 ml with water. One milliliter was added to each of two Corex centrifuge tubes, and incubated for 15 minutes with 50 ul of tritiated etorphine or naloxone. The suspension was precipitated with 3 ml of absolute ethanol, and stored at 4 $^{o}$C for six hours. The tubes were then centrifuged at 12,000 g for 15 minutes. The supernatant was removed and concentrated to dryness, and resuspended in 0.1 ml of water. The tubes were stored overnight at 4 $^{o}$C, and in the morning they were brought to 1.0 ml with absolute ethanol. After heating each supernatant fraction for two minutes at 65$^{o}$C, they were cooled to 4 $^{o}$C and centrifuged at 12,000 g for fifteen minutes. The supernatants were concentrated and brought to 100 ul with absolute ethanol. Twenty microliters from each tube was spotted on silica gel plates and developed in the appropriate solvent system.

B) Naloxone Distribution and Saturation Binding:

Tritiated naloxone binding to embryonic chick brain homogenate was studied as a function of time, figure 23. The association of naloxone with the homogenate was rapid, and plateaued within 2-4 minutes of adding the drug. The amount of naloxone bound to homogenates previously incubated with levorphanol was constant throughout the period studied. A 10 minute incubation with tritiated naloxone was chosen as a standard assay condition because the level of naloxone bound to homogenates incubated with dextrorphan was constant for almost fifteen minutes. Within twenty minutes of having added naloxone, and after an initial rise in naloxone binding, the homogenates incubated with dextrorphan, or just tris-buffered sucrose, showed a decrease in naloxone binding.

The stereospecific binding of naloxone to embryonic chick brain homogenate is half saturated at a tritiated naloxone concentration of 1.5 nM, and saturates at a tritiated naloxone concentration exceeding 10.5 nM, figure 24.

Figure 23. The time course for naloxone binding to embryonic chick brain homogenate. Individual flasks containing 60 ml of homogenate were incubated with either 0.1 uM dextrorphan (△), 0.1 uM levorphanol (□), or a comparable aliquot of tris-sucrose (○) for five minutes prior to assaying all three flasks with tritiated naloxone for up to one hour. At specified times three 2 ml aliquots were withdrawn from each flask, filtered, and washed with three 5 ml aliquots of ice-cold 10mM tris-HCl buffer.

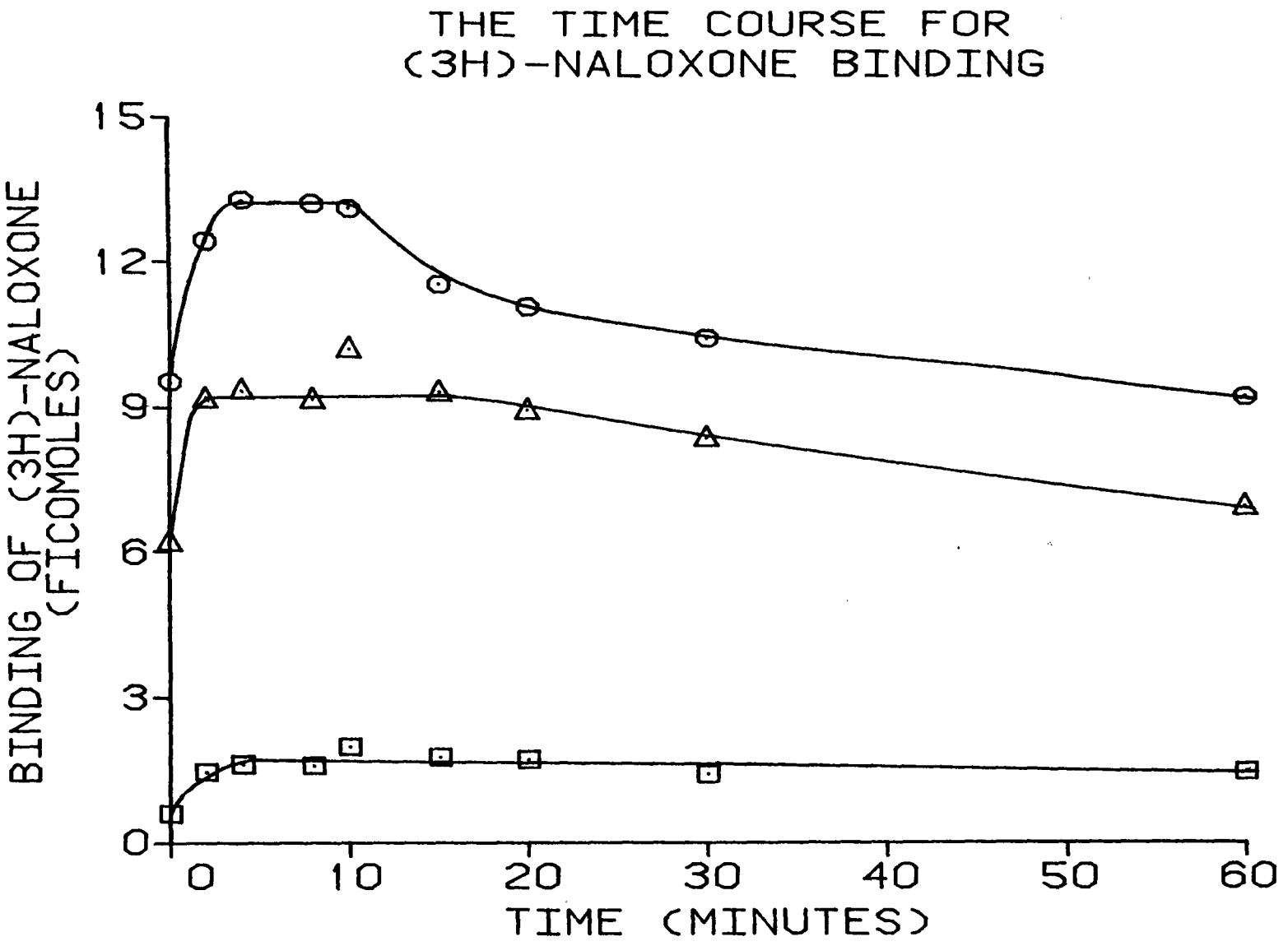
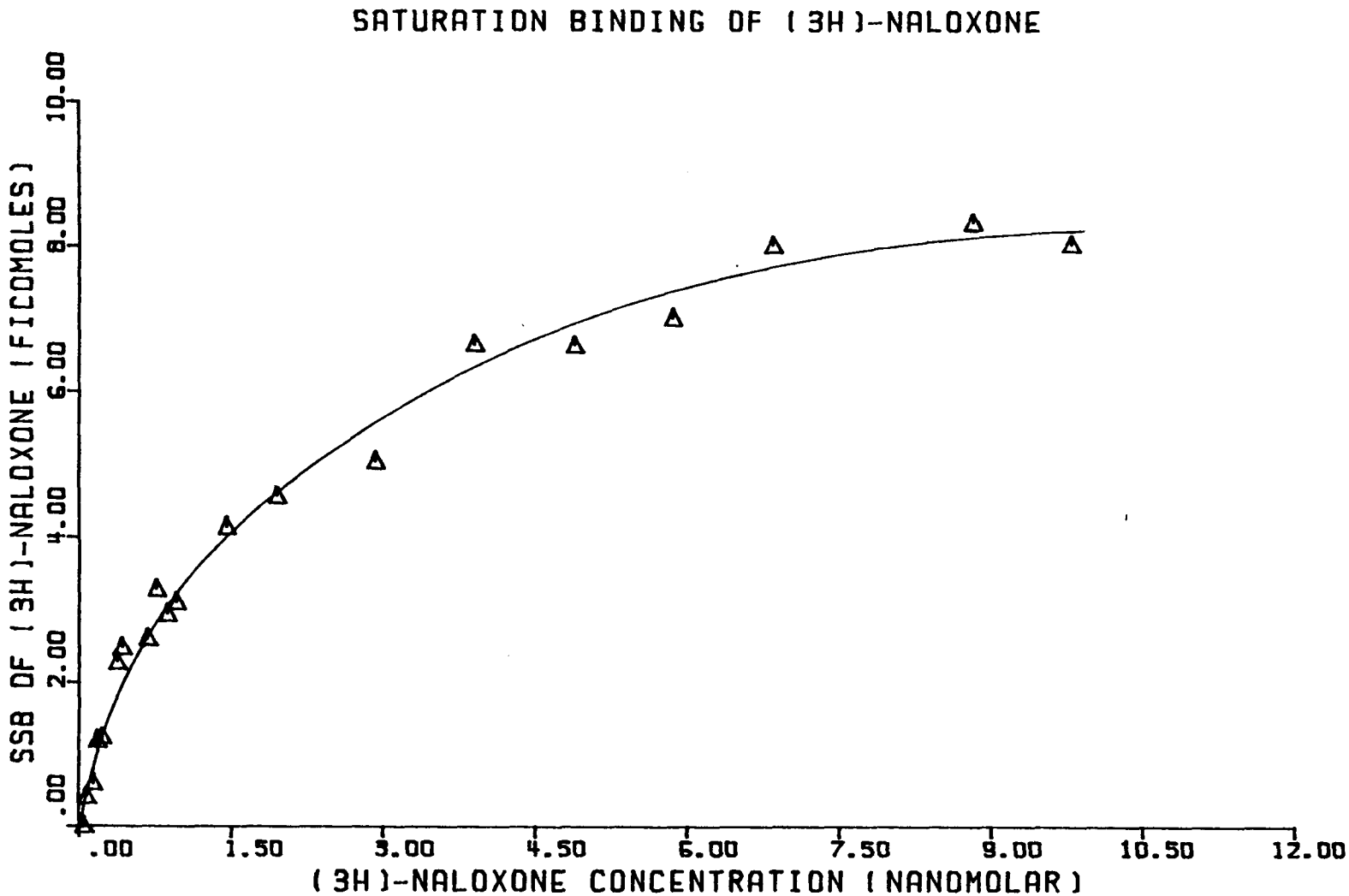

THE TIME COURSE FOR
(3H)-NALOXONE BINDING
BINDING OF (3H)-NALOXONE
(FICOMOLES)
15
12
9
6
3
0
0
10
20
30
40
50
60
TIME (MINUTES)

Figure 24. The saturability of the stereospecific binding capacity of embryonic chick brain particulates. Washed particles were incubated for five minutes with standard assay concentrations of dextrorphan or levorphanol prior to incubating these particulates for ten minutes with successively larger aliquots of tritiated naloxone. Particulates were filtered without any prior cooling. Two milliliter aliquots of washed particulates were added to tubes containing complementary aliquots of tris-buffered sucrose necessary to maintain a constant assay volume.


SATURATION BINDING OF (3H)-NALOXONE
SSB OF (3H)-NALOXONE (FICOMOLES)
.00
2.00
4.00
6.00
8.00
10.00
.00
1.50
3.00
4.50
6.00
7.50
9.00
10.50
12.00
(3H)-NALOXONE CONCENTRATION (NANOMOLAR)

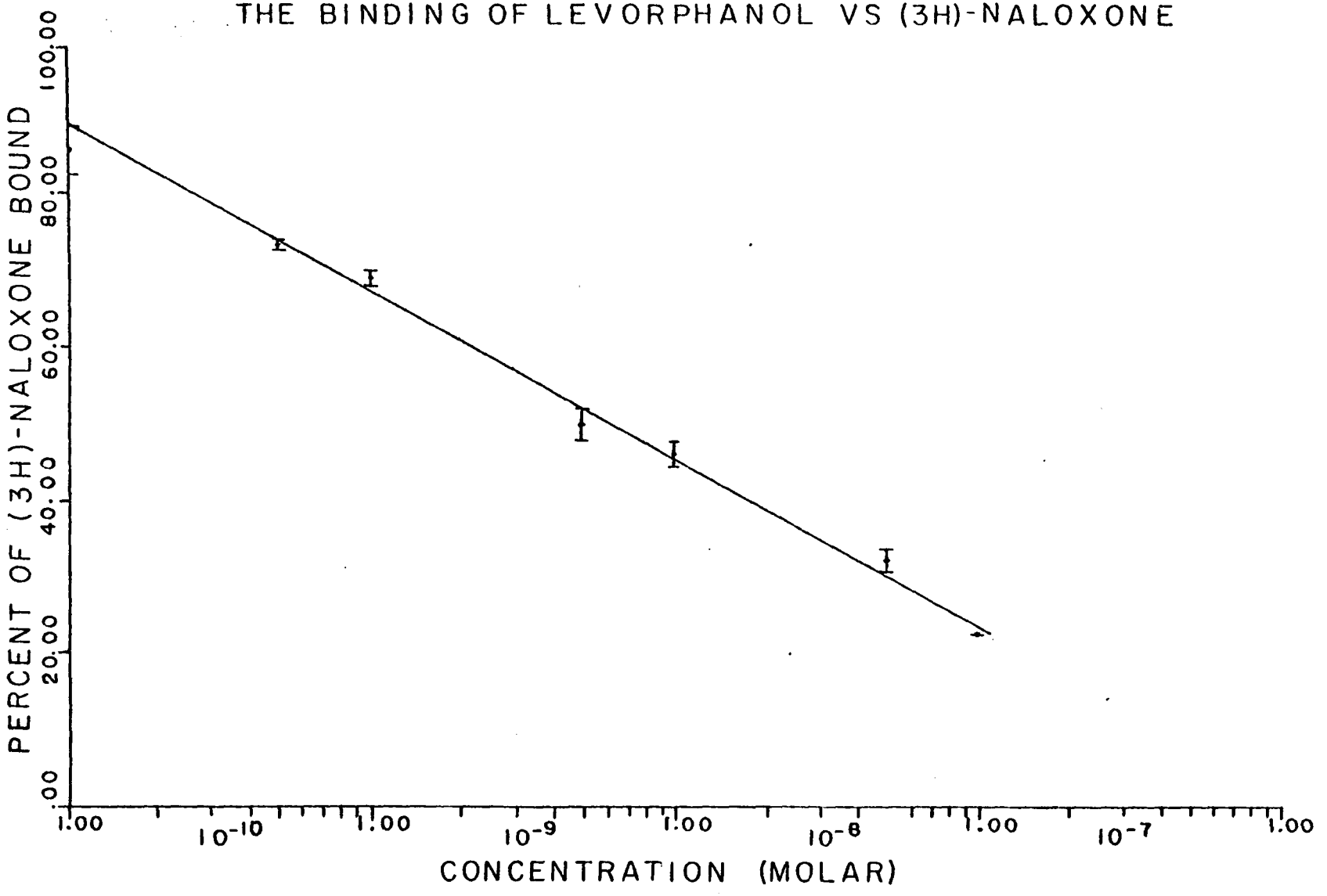
I) Competition Studies:

A semi-log plot of the concentration of levorphanol required to reduce the binding of tritiated naloxone by fifty percent (IC-50), figure 25, reveals the IC-50 of levorphanol to be approximately 6.65 nM. Naloxone binding is unaffected by 0.1 nM levorphanol, though at higher concentrations levorphanol competes with naloxone in a concentration dependent fashion.

The IC-50 value for various opioid compounds versus tritiated naloxone and etorphine, table 9, suggests as does figure 25, that all of these compounds compete with one another for the same receptor sites.

The endogenous opioid peptides leucine enkephalin and methionine enkephalin have similar IC-50 values in competing with naloxone. Leucine enkephalin is only about half as effective as methionine enkephalin in reducing the binding of etorphine. The derivative ALA3-methionine enkephalin is almost 300 times more potent in reducing the binding of naloxone than it is in inhibiting etorphine binding. Alpha-endorphin, a peptide which contains the methionine enkephalin sequence as its first five amino acids residues, was the least effective opioid peptide studied. Concentrations of alpha-endorphin 100,000 to 2 million times those of unlabeled naloxone and etorphine, respectively, appear to inhibit the

Figure 25. The binding of levorphanol versus tritiated naloxone. The ability of levorphanol to reduce the binding of tritiated naloxone to washed embryonic chick brain particulates was studied by incubating the washed particulates for five minutes with several different concentrations of levorphanol. Tritiated naloxone, (4 nM), was incubated with the particulates for 10 minutes and the particulates were filtered without cooling. The concentration of levorphanol producing a fifty percent reduction in the stereospecific binding of tritiated naloxone was reported as the $IC_{50}$ value for levorphanol. This same procedure was used to report all of the $IC_{50}$ values reported in table 9.


THE BINDING OF LEVORPHANOL VS (3H)-NALOXONE
PERCENT OF (3H)-NALOXONE BOUND
100.00
80.00
60.00
40.00
20.00
.00
1.00
10-10
1.00
10-9
1.00
10-8
1.00
10-7
1.00
CONCENTRATION (MOLAR)

binding of both tritiated naloxone and tritiated etorphine. Etorphine is the most potent opiate studied, and has an IC-50 that makes it almost 25 times more potent than naloxone in reducing the binding of the competing radioactive opiate. The enantiomers dextrorphan and levorphanol reduce the binding of tritiated etorphine and naloxone to washed embryonic chick brain. Levorphanol is approximately 15,000 times more potent than dextrorphan, and only three times less effective than naloxone in reducing the binding of etorphine. Levorphanol is about 30 times weaker than etorphine in reducing the binding of tritiated naloxone. Levallorphan, which is the antagonistic form of levorphanol, is almost twice as potent as levorphanol in reducing the binding of tritiated naloxone, and almost four times more potent than levorphanol in reducing the binding of tritiated etorphine. The mixed agonist-antagonist cyclazocine is equally potent in its ability to reduce tritiated naloxone and etorphine binding. The IC-50 of cyclazocine is intermediate between those of levorphanol and levallorphan. Pentazocine, which is also a mixed agonist-antagonist, is almost fifty times weaker than cyclazocine, and inhibits the binding of naloxone and etorphine with IC-50 values that are comparable to those of cyclazocine. The very potent antagonist naltrexone is only slightly more potent than naloxone in inhibiting the binding of

etorphine. Levallorphan is slightly more potent than naltrexone, and naltrexone is slightly more potent than cyclazocine. All of this data confirms the well-known facts that an opiate antagonist is generally more effective than an opiate agonist in inhibiting the binding of some other opiate, and that mixed agonist-antagonists have potencies that are intermediate to those of comparable agonists and antagonists.

Table 9: The $IC_{50}$ values for the binding of various opioid compounds versus the binding of tritiated naloxone and tritiated etorphine.

| Compound | $IC_{50}$ ($^3H$)-Naloxone ( nM ) | $IC_{50}$ ($^3H$)-Etorphine ( nM ) |
|---|---|---|
| Leucine Enkephalin | 122 | 368 |
| Methionine Enkephalin | 110 | 200 |
| $Ala_3$-Met-Enkephalin | 6,400 | 215,000 |
| Alpha-Endorphin | 34,000 | 400,000 |
| Etorphine | 0.18 | ------- |
| Naloxone | ------ | 4.5 |
| Levorphanol | 6.7 | 12.0 |
| Dextrorphan | 5,850 | 183,000 |
| Levallorphan | 3.8 | 3.5 |
| Cyclazocine | 4.8 | 4.3 |
| Pentazocine | 108 | 132 |
| Naltrexone | 4.1 | 3.5 |

The $IC_{50}$ values were determined by incubating washed particles from embryonic chick brain with several concentrations of opioid compound for five minutes at 39 $^oC$, and then incubating them with either 4 nM tritiated naloxone or 1 nM tritiated etorphine for ten minutes. The washed particles were filtered without any prior cooling on ice, and counted as described in the methods. The concentration of opioid compound reducing the binding of the tritiated compounds by fifty percent was the $IC_{50}$ concentration of the non-radioactive compound.

J) Association and Dissociation Studies:

Stereospecifically bound naloxone can be almost totally displaced by 10 nM unlabeled naloxone in a concentration dependent fashion, figure 26. Little additional displacement of bound radioactive naloxone occurs at non-radioactive concentrations exceeding 50 nM. The rapid loss in binding at low concentrations of naloxone, figure 26, was studied at three different temperatures, figure 27, in order to determine the rate of dissociation of stereospecifically bound naloxone. A semi-log plot of the data indicated that the dissociation process followed first order kinetics, but it was too rapid to follow accurately at 39 degrees. At nine, nineteen, and 29 degrees respectively, the dissociation rates for the naloxone-receptor complex were $8.70X10^{-17}$ mole/sec, $5.88X10^{-16}$, mole/sec, and $1.64X10^{-15}$, and the complex had a half-life of 324 ± 15 seconds, 30 ± 9 seconds, and 13 ± 1 seconds. At 9 and 19 degrees a rapid loss in the amount of bound tritiated naloxone was followed by a rapid rebinding of the labeled naloxone. Dissociation progressed normally, shortly after the rebinding phenomena was completed. The dissociation rate at 19 degrees is almost half the rate at 29 degrees, and six times the rate observed at 9 degrees.

The rate of association of stereospecifically

Figure 26. The exchange of tritiated naloxone bound stereospecifically to embryonic chick brain homogenate with non-radioactive naloxone. Homogenates of thirteen day old embryonic chick brain were incubated for five minutes with either 0.1 uM dextrorphan or levorphanol and for eight minutes with 2 nM tritiated naloxone. The homogenates were incubated with several concentrations of unlabeled naloxone for another five minutes, and cooled on ice prior to filtering.

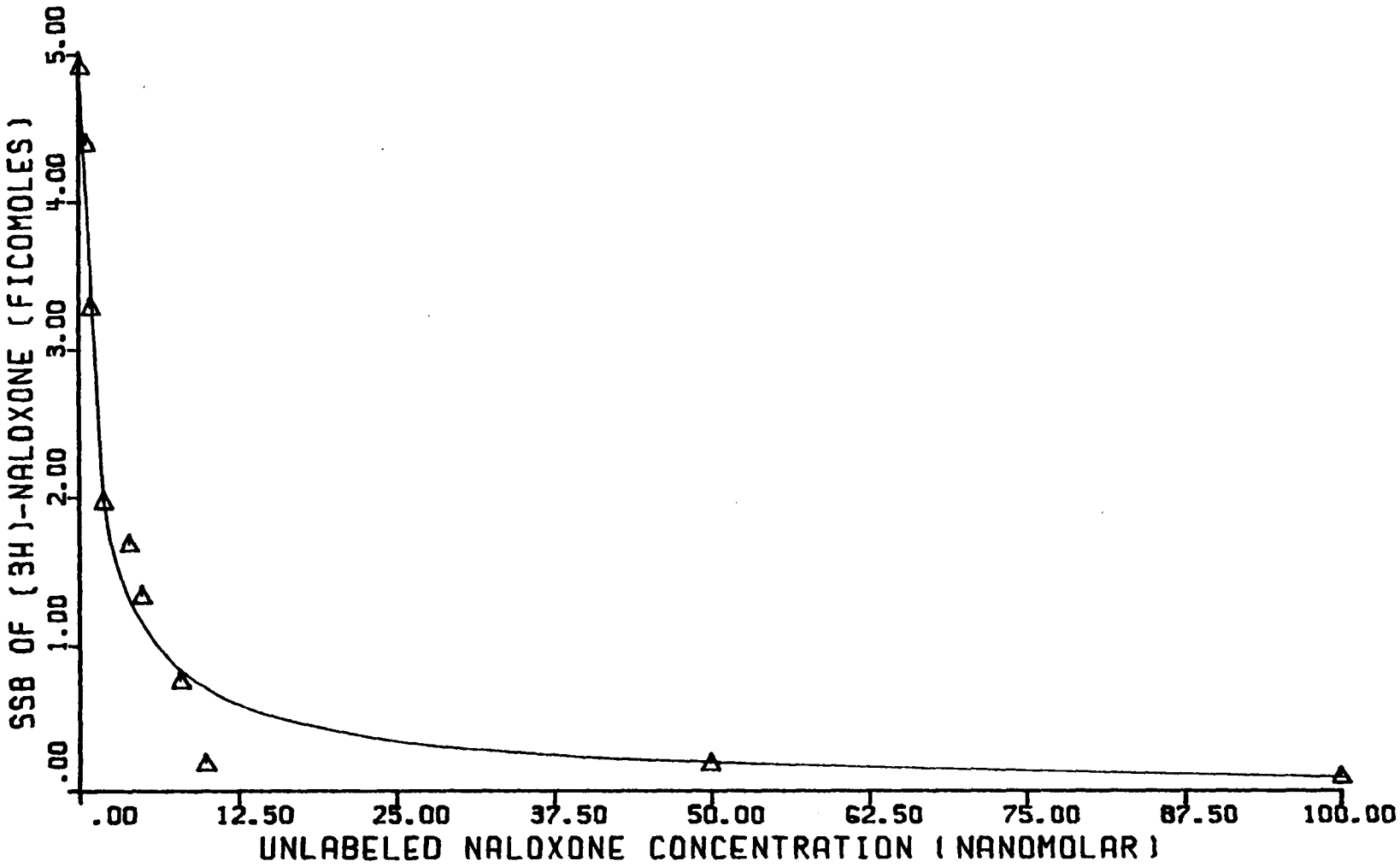
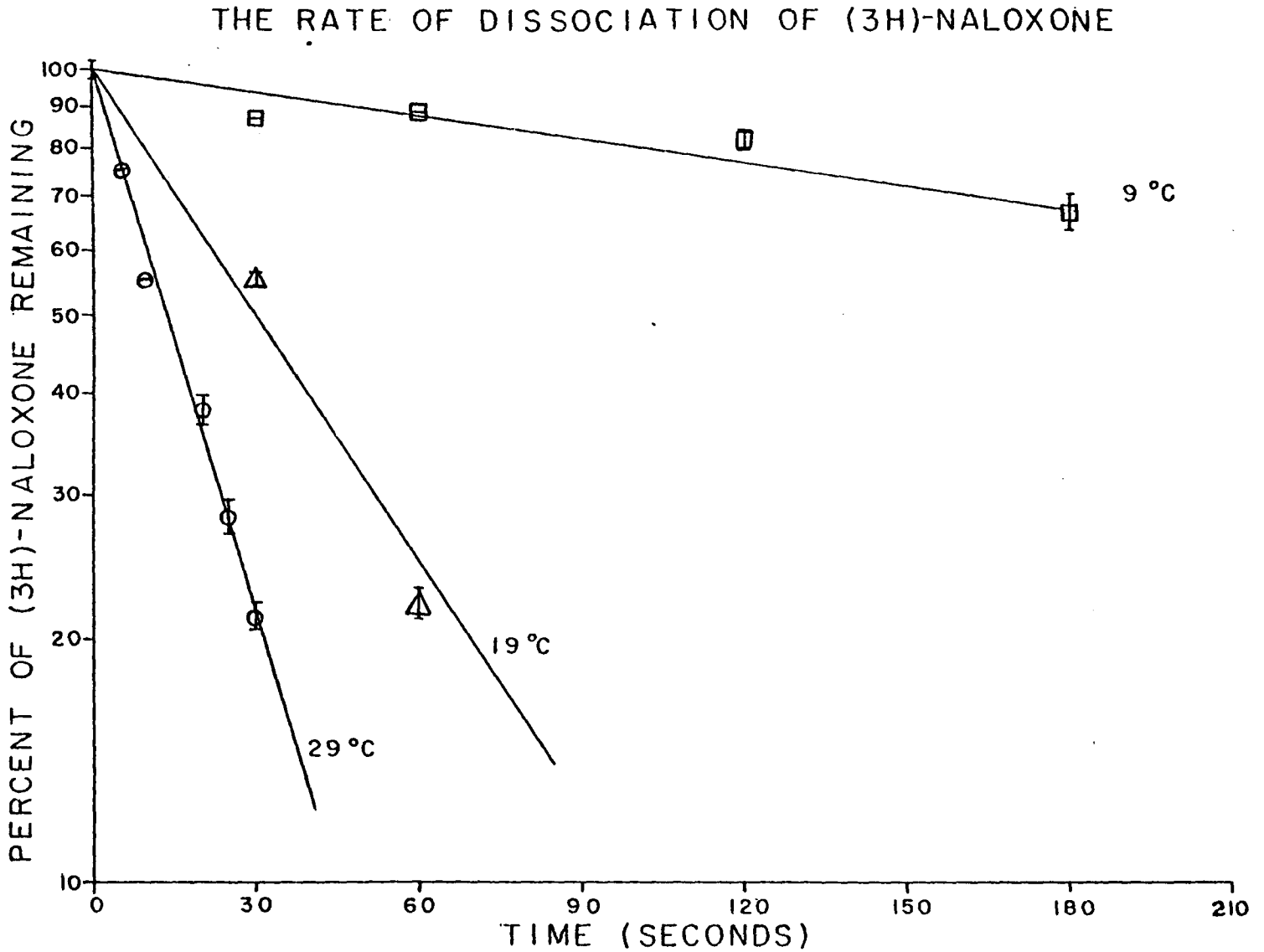
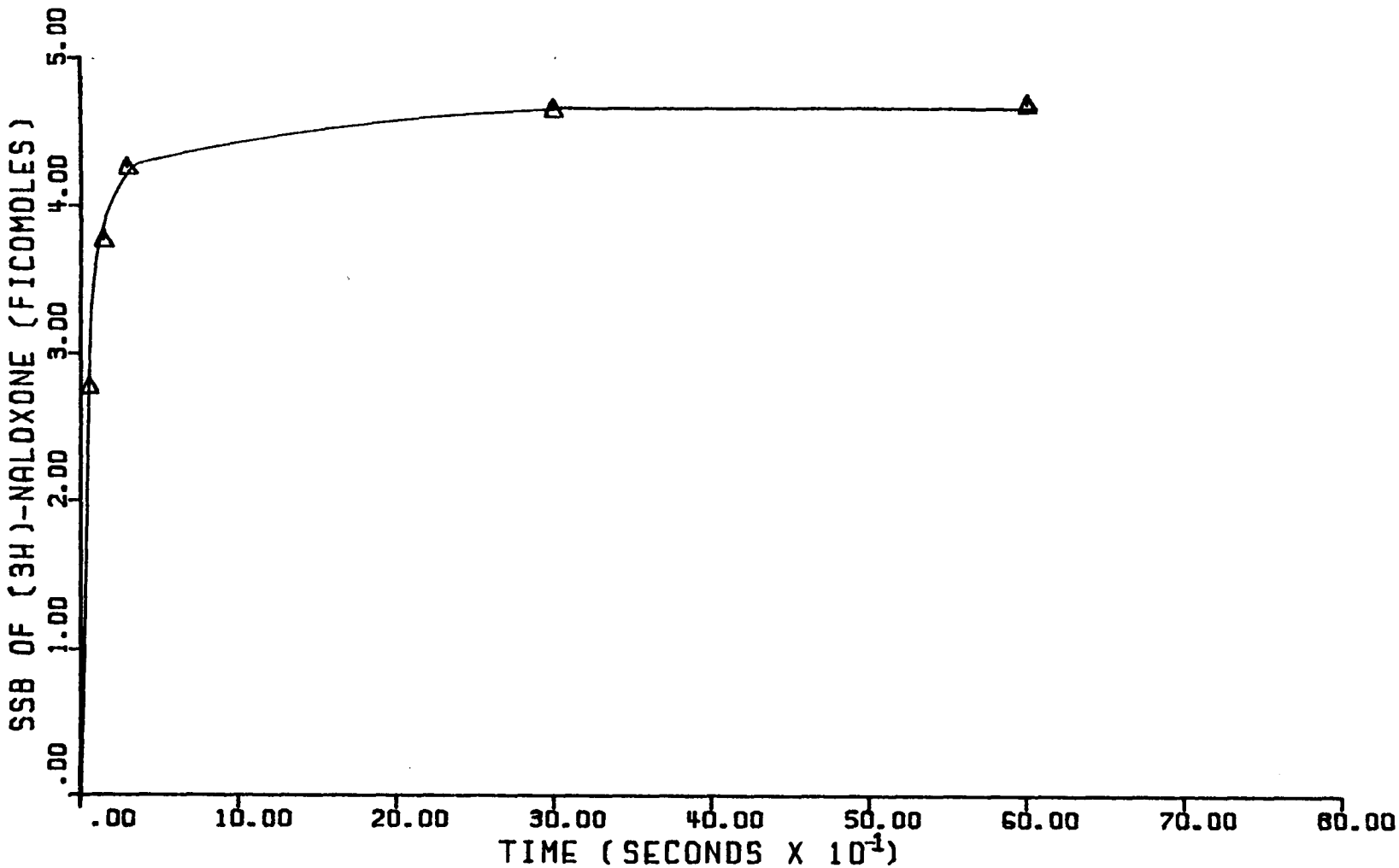

DISPLACEMENT OF (3H)-NALOXONE WITH UNLABELED NALOXONE
SSB OF (3H)-NALOXONE (FICOMOLES)
5.00
4.00
3.00
2.00
1.00
.00
.00
12.50
25.00
37.50
50.00
62.50
75.00
87.50
100.00
UNLABELED NALOXONE CONCENTRATION (NANOMOLAR)

Figure 27. The rate of dissociation of stereospecifically bound tritiated naloxone from embryonic chick brain homogenate. A five hundred fold excess of non-radioactive naloxone was added to each of two flasks incubated according to the standard assay procedure with 0.1 uM dextrorphan or levorphanol, and 2 nM tritiated naloxone. Three 2 ml aliquots were withdrawn from each flask at the specified times, and filtered without cooling. The dissociation rate was studied at nine, nineteen, and twenty nine degrees because of the immeasurably rapid dissociation rate observed at 39 degrees.


THE RATE OF DISSOCIATION OF (3H)-NALOXONE
PERCENT OF (3H)-NALOXONE REMAINING
100
90
80
70
60
50
40
30
20
10
0
30
60
90
120
150
180
210
TIME (SECONDS)
9 °C
19 °C
29 °C

bound naloxone at 29 degrees has an initial rate of $5.6X10^{-14}$ mole/sec, figure 28. Within fifty seconds the stereospecific association of naloxone with embryonic chick brain homogenate is essentially complete. Assaying the homogenate at 39 degrees, figure 23, results in the completed association of stereospecifically bound naloxone within two minutes, and a constant amount of stereospecifically bound naloxone for over fifteen minutes.

Figure 28. The rate of the stereospecific association of tritiated naloxone with embryonic chick brain homogenate. Tritiated naloxone (2 nM) was added to flasks containing homogenate preincubated at 29 $^{o}$C for five minutes with either 0.1 uM dextrorphan or levorphanol. Three 2 ml aliquots of homogenate were withdrawn at the times specified, and filtered without cooling. The 29 $^{o}$C assay temperature was choosen because of the immeasurably rapid association rate observed at 39 $^{o}$C.


RATE OF ASSOCIATION OF (3H)-NALOXONE AT 29 DEGREES
SSB OF (3H)-NALOXONE (FICOMOLES)
.00
1.00
2.00
3.00
4.00
5.00
.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
TIME (SECONDS X 10-1)

K) Enzymatic Degradation:

Homogenates and washed particulates derived from embryonic chick brain homogenate are sensitive to proteolytic digestion, table 10. Clostridial protease and chymotrypsin decrease the binding capacity of washed particulates and homogenates very rapidly at 39 degrees. Trypsin causes almost a sixty percent loss in the binding capacity of washed particulates at the highest concentration of enzyme used in the study. Prebinding etorphine to washed embryonic particulates does not protect its binding capacity from tryptic digestion.

Phospholipase C and phospholipase D also significantly reduce the binding capacity of washed embryonic chick brain particulates, but because of the harsh conditions of incubation, which produced significant denaturation of the opiate receptor, the data are not tabulated. The degree to which the loss in the opiate binding capacity can be attributed to phospholipase activity was estimated by comparison to particulates incubated with boiled enzyme.

Table 10: Proteolytic digestion of the embryonic chick brain opiate receptor.

Percentage of SSB without Enzyme

| Protease conc. units/ml | binding | Trypsin conc. units/ml | binding | Chymotrypsin time minutes | binding |
|---|---|---|---|---|---|
| 0.0 | 100 ± 0.0 | 0.0 | 100 ± 2.3 | 0.0 | 100 |
| 0.5 | 19.5 ± 4.5 | 1.0 | 100 ± 0.3 | 15.0 | 27.4 |
| 1.0 | 9.0 ± 0.4 | 2.0 | 67.4 ± 1.8 | 30.0 | 13.3 |
| 5.0 | 4.4 ± 0.8 | 5.0 | 55.3 ± 1.8 | 45.0 | 11.0 |
| 10.0 | 0.8 ± 0.8 | 10.0 | 42.3 ± 0.1 | ---------- | |
| 10.0[1] | 96.5 ± 3.7 | 10.0[1] | 100 ± 0.4 | 45.0[2] | 100. |

1.) Enzymes used at these concentrations or for this length of time were boiled for ten minutes before being added to the washed embryonic chick brain particulates.

L) Endogenous Opioid Compound Studies:

The presence of an endogenous inhibitor that could be washed out of the homogenate, table 6, was examined using washed embryonic chick brain particulates, figures 29-30, tables 11, and adult chickens, table 12. Preincubating embryonic chick brain particulates, figure 29, as a function of temperature, and in the absence of 20 mM sodium chloride, led to an 18 percent rise in the binding capacity when the particulates were incubated above 10 degrees. A similar effect was noted for particulates incubated with 20 mM sodium chloride, but above 20 degrees the binding capacity decreased by over 30 percent. Preincubating embryonic chick brain particulates for 10 minutes at 39 degrees, figure 30, in the absence of sodium chloride, resulted in a sharp peak in the binding capacity, that rapidly disappeared upon continuing the incubation. Incubating the preparation of particulates for 10 minutes with 20 mM sodium chloride resulted in an initial increase in the binding capacity that equaled the binding enhancement observed after the same period of incubation in the absence of sodium chloride. The rate of loss in the binding capacity of both particulates was similar, although the preparation incubated with sodium chloride had a higher binding capacity than the suspension incubated without sodium chloride.

Figure 29. The effect on the stereospecific binding capacity of embryonic chick brain particulates preincubated as a function of temperature. Aliquots of washed particulates(15 ml) were incubated at the specified temperatures for twenty minutes either in the absence (⊙), or the presence (△) of 20 mM sodium chloride, and then washed twice at 49,000 g for ten minutes with ice-cold 5 mM tris-buffered 0.32 M sucrose. The pellets were resuspended with the original volume of tris-buffered sucrose, and assayed according to the standard assay procedure using 2 nM tritiated naloxone. The assayed particulates were filtered without cooling.

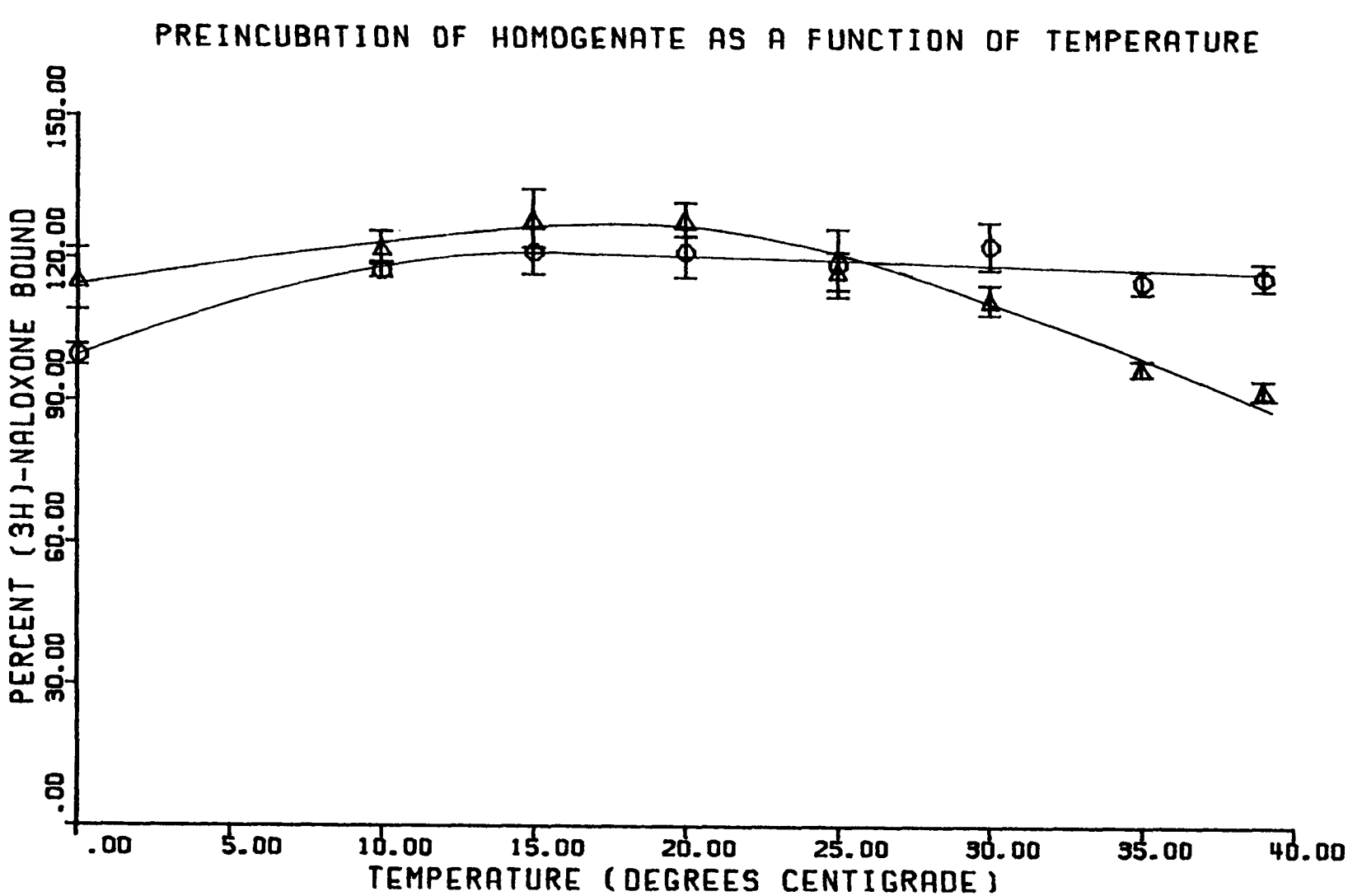

PREINCUBATION OF HOMOGENATE AS A FUNCTION OF TEMPERATURE
PERCENT (3H)-NALOXONE BOUND
150.00
120.00
90.00
60.00
30.00
.00
.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
TEMPERATURE (DEGREES CENTIGRADE)

Figure 30. The effect on the stereospecific binding capacity of washed embryonic chick brain particulates preincubated as a function of time at 39 °C. Fifteen milliliters of washed particulates were incubated at thirty nine degrees for specified intervals of time, in the absence (⊙) or the presence (△) of 20 mM sodium chloride, and washed twice at 49,000 g for 10 minutes with ice-cold 5 mM tris-buffered 0.32 M sucrose. The pellets were resuspended with 15 ml of tris-buffered sucrose, and assayed with 2 nM tritiated naloxone according to the standard assay procedure. The assayed particulates were filtered without cooling.

PREINCUBATION OF HOMOGENATE AS A FUNCTION OF TIME

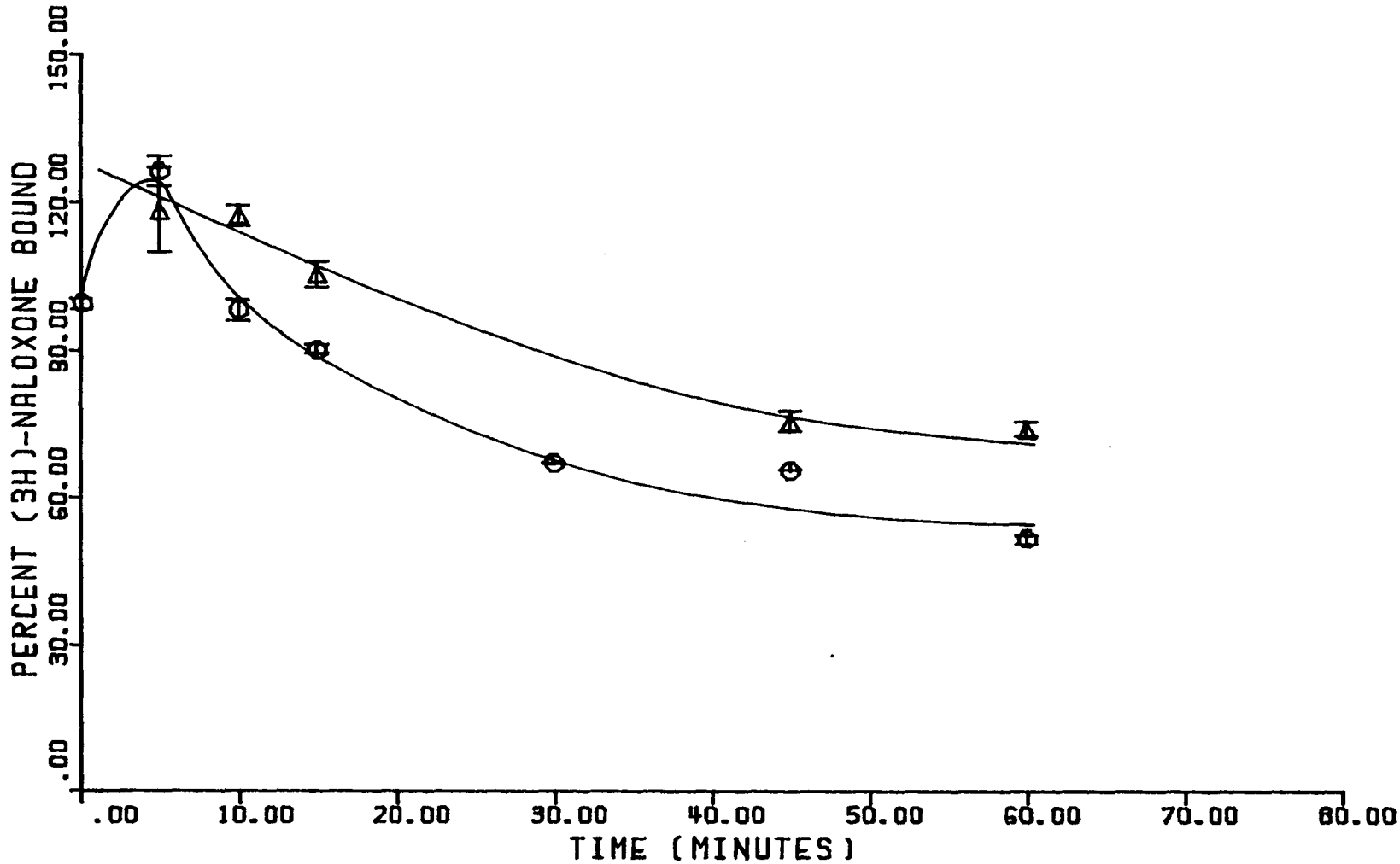


Table 11: The ability of an endogenous opioid compound to inhibit the stereospecific binding of etorphine to particles from embryonic chick brain.

| Concentration Opioid Substance (mg/ml) | Fraction | SSB (fmoles/mg of protein) | Percentage of Fraction B |
|---|---|---|---|
| 0.0 | A | 4.51 ± 0.1 | 54.5 ± 1.2 |
| 0.0 | B | 8.27 ± 0.1 | 100 ± 0.7 |
| 4.0 | C | 7.69 ± 0.1 | 93.0 ± 1.7 |
| 8.1 | D | 7.90 ± 0.1 | 95.5 ± 0.6 |
| 20.2 | E | 7.38 ± 0.2 | 89.2 ± 1.8 |
| 80.8 | F | 1.84 ± 0.1 | 22.2 ± 0.2 |

Fraction A refers to unincubated washed particulates; Fraction B refers to particulates that were prepared as described in the legend and assayed without adding any endogenous opioid subatance; Fractions C-E refer to washed particles that were assayed with successively larger amounts of endogenous opioid substance; Fraction F refers to washed particles that were assayed with endogenous opioid substance that had been boiled for ten minutes.

Washed particles from embryonic chick brain were prepared as described in the methods except that they were finally resuspended in water to avoid concentrating the buffered sucrose. The particulates were incubated at 25 $^{o}$C for fifteen minutes and centrifuged at 17,000 g for ten minutes at 4 $^{o}$C. The pellet was washed once with tris-sucrose (0.005-0.32 M), and the supernatant was saved and concentrated to approximately 2 ml. The pellet was resuspended in buffered sucrose and 10 ul of either dextrorphan or levorphanol (0.1 uM) were incubated for five minutes with the resuspended pellet. Successively larger aliquots of the concentrated supernatant were incubated with the suspension for five minutes prior to incubating each of the six tubes with the standard assay concentration of tritiated etorphine. The suspension was filtered without any prior cooling.

Using the data from figures 29-30, a 15 minute incubation of embryonic chick brain particulates in the absence of sodium chloride at 25 degrees was performed to try to produce a substantial increase in the binding capacity of this suspension of particulates, table 12. Incubating the particulates under these conditions almost doubled the initial binding capacity. The addition of successively larger aliquots of concentrated supernatant to a suspension of particulates rewashed after the incubation resulted in a minor decrease in the binding capacity of only 11 percent. This seemingly insignificant decrease in the binding capacity was presumably due to a degradation of the endogenous inhibitor by catabolic enzymes present in the preparation. The 78 percent decrease in the binding capacity observed when a very large aliquot of boiled concentrated supernatant was added to the particulates was probably an artifact resulting from the very slow filtration rate obtained because of the hugh amount of protein added to the suspension of particulates.

The supernatants of washed particulates derived from adult chicken brain homogenates which were incubated at 39 degrees for various time intervals contained substantial levels of an endogenous inhibitor that was able to reduce the binding of naloxone by almost 35 percent, table 12. Preincubating particulates derived from adult

Table 12: The binding of tritiated naloxone to adult chicken brain particulates as a function of preincubation time at 39 °C.

| Preincubation Time (minutes) | % remaining | | Effect of Preincubation Supernatants on Binding | |
|---|---|---|---|---|
| | No Salt | NaCl (4 mM) | No Salt | NaCl (4 mM) |
| | | | (Percent remaining) | |
| 0.0 | 100 ± 1.9 | 100 ± 0.5 | 100 | 100 |
| 5.0 | 109 ± 1.1 | 103 ± 4.6 | --- | --- |
| 10.0 | 116 ± 0.3 | 104 ± 0.9 | --- | --- |
| 15.0 | 108 ± 0.8 | 129 ± 5.8 | 98 | 98 |
| 30.0 | 106 ± 1.1 | 136 ± 1.4 | 77 | 77 |
| 45.0 | 91.4 ± 1.1 | 91.8 ± 0.9 | --- | --- |
| 60.0 | 87.4 ± 0.5 | 72.5 ± 0.9 | 67 | 63 |

Washed particles from adult chicken brain were prepared according to the procedure described in section C of the methods and placed in centrifuge tubes containing either 4 mM sodium chloride or tris buffered sucrose and incubated for several periods of time at 39 °C. The incubatedparticulates were centrifuged at 49,000 g for ten minutes at 4 °C, and the supernatants from the first centrifugation were saved, in order to resuspend a series of washed pellets at the end of the centrifugation procedure. All of the pellets were resuspended in tris-sucrose (0.005-0.32 M) after being washed once with the buffered sucrose. A separate batch of this suspension of particulates was incubated for one hour and washed by the procedure just described. The final pellet was resuspended with the supernatant that was saved from the first batch of particles examined. All of the particles were assayed according to the standard procedure using 4 nM tritiated naloxone. Filtering was done without any prior cooling on ice.

chicken as a function of temperature or time resulted in binding enhancements that were similar to those obtained with embryonic chick brain particulates, figure 30, regardless of whether the assay temperature was 25 degrees or 39 degrees.

M) Stability Studies:

The loss in the binding capacity of particulates derived from both embryonic and adult chicken brain incubated as a function of either temperature or time, figures 29-30, and table 12, was studied by assaying suspensions of both types of particulates incubated for specific periods of time at the temperatures indicated, figures 31-32. Both figures illustrate that the loss in binding capacity of particulates is temperature dependent. Particulates derived from embryonic chick brain lost their binding capacity for naloxone more slowly when stored at 25 degrees than at 39 degrees. Suspensions of particulates stored at zero degrees appeared to be more stable than those stored at either 25 or 39 degrees. However, less than 25 percent of the binding capacity was retained when embryonic chick particulates were stored for more than 9 hours, regardless of the storage temperature.

Preparations derived from adult chicken brain, figure 32, were more stable than those derived from embryonic chick brain, figure 31. The loss in the binding capacity was almost complete after 9 hours at 39 degrees. Particulates retained almost 45 percent of their original binding capacity after 9 hours at 25 degrees, and less than 10 percent of the binding capacity was lost after 8 hours at zero degrees.

Figure 31. The autodegradation of washed embryonic chick brain particulates incubated at zero (◈), twenty five (△), or thirty nine (⊙) degrees. Six 2 ml aliquots of washed particulates incubated at the appropriate temperatures were withdrawn at the specified times and assayed by the standard procedure using tritiated naloxone. The assayed particulates were filtered without any prior cooling.

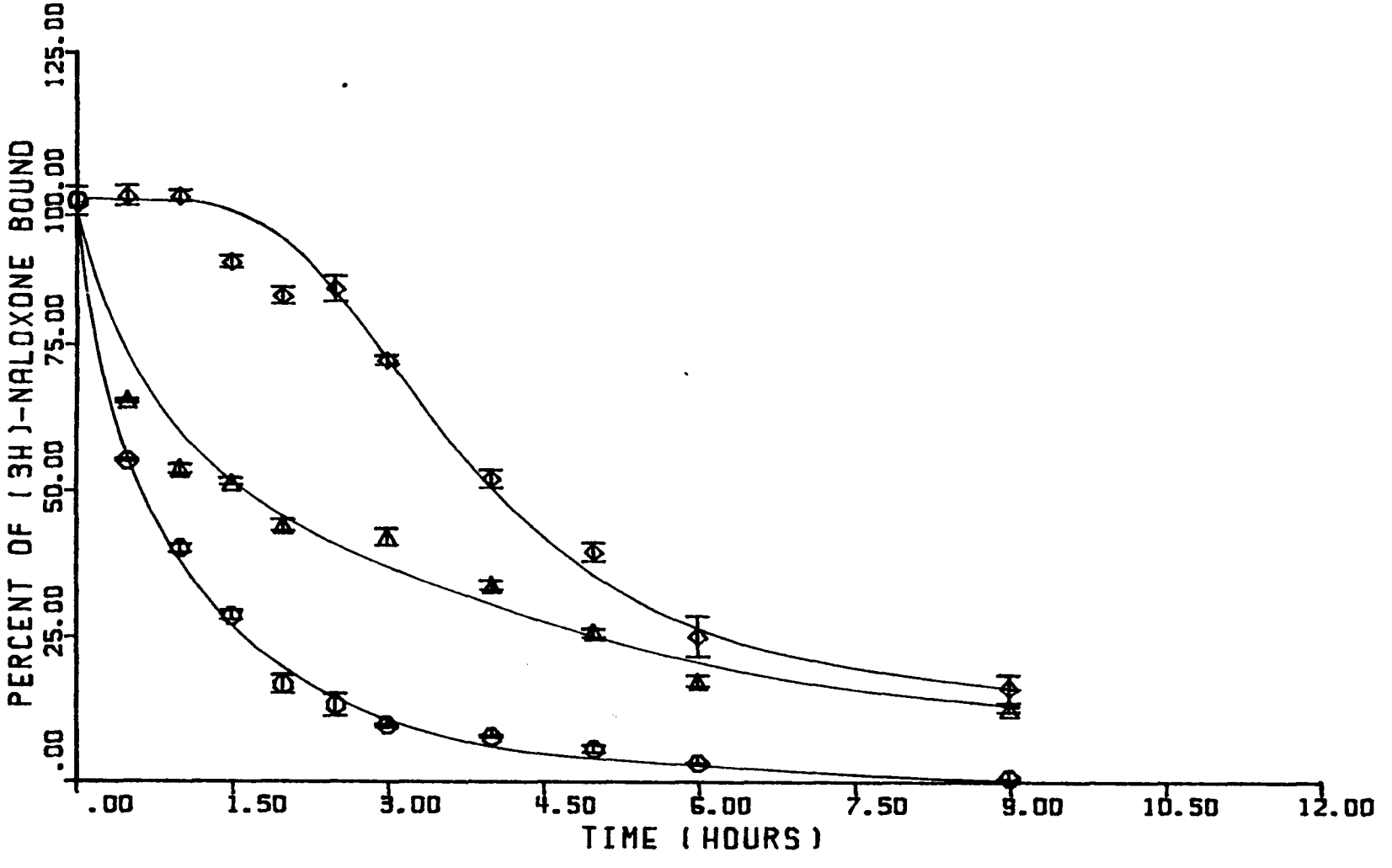
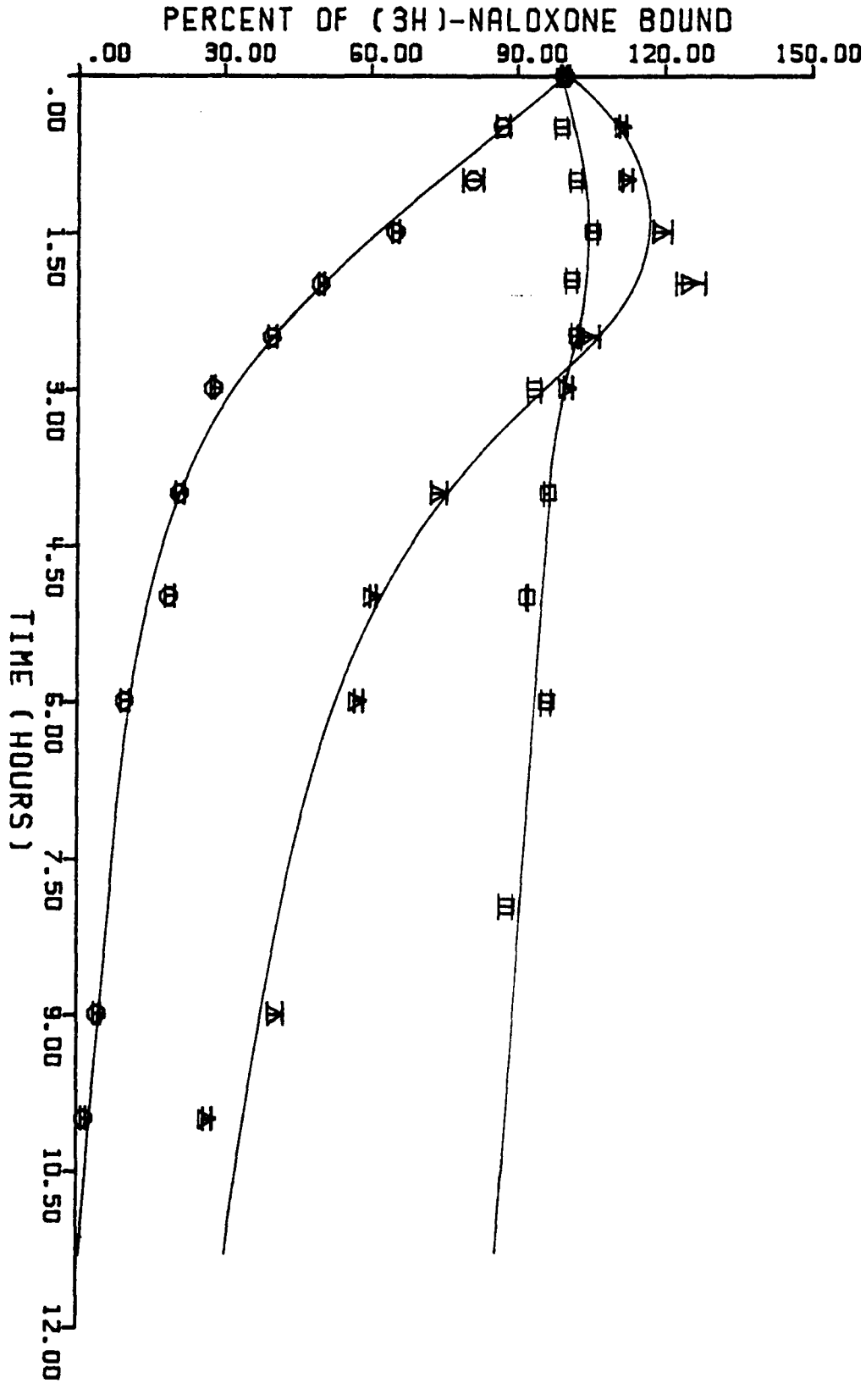

AUTODEGRADATION OF EMBRYONIC BRAIN HOMOGENATE
PERCENT OF (3H)-NALOXONE BOUND
125.00
100.00
75.00
50.00
25.00
.00
.00
1.50
3.00
4.50
6.00
7.50
9.00
10.50
12.00
TIME (HOURS)

Figure 32. The autodegradation of washed adult chicken brain particulates incubated at zero (⊡), twenty five (△), or thirty nine (⊙) degrees. Six 2 ml aliquots of washed particulates were incubated at the appropriate temperatures, withdrawn at the specified times, and assayed by the standard procedure using 2 nM tritiated naloxone. The assayed particulates were filtered without any prior cooling.




AUTODEGRADATION OF THE ADULT OPIATE RECEPTOR.

The incubation of particulates derived from adult chicken brain at 25 degrees resulted in an increase in the binding capacity that was sustained for almost 3 hours. A 7 percent rise in the binding capacity of particulates stored at zero degrees, may not be significant, although such an increase would be in agreement with the results obtained at 25 degrees. Except for adult particulates stored at zero degrees all of the particulates derived from adult and embryonic chick brain retained little or no binding capacity after being stored for 24 hours.

The autodegradation studies, figures 31-32, performed on particulates derived from adult and embryonic chicken brain suggested the action of some endogenous catabolic enzyme. Curiously, a very mild trypsin digestion, table 13, resulted in an initial rise and later a decrease, in the binding capacity of particulates derived from adult chicken brain. Boiled trypsin had no effect on the binding capacity.

The enhancement caused by trypsin, table 13, is reminiscent of the binding capacity enhancements observed when adult chicken brain particulates are incubated at 39 degrees as a function of time, table 12. Embryonic chick brain particulates incubated for short periods of time at 39 degrees, figure 30, or at several temperatures for 20 minutes, figure 29, revealed small binding capacity enhancements after being

Table 13: The effect of mild tryptic digestion on the stereospecific binding of tritiated naloxone by adult chicken brain particulates.

| Time (minutes) | Percentage SSB at Zero Time | |
|---|---|---|
| | Trypsin Concentration | |
| | 1.73 units/ml | 3.45 units/ml |
| 0.0 | 100 ± 6.9 | 100 ± 0.8 |
| 5.0 | 100 ± 6.9 | 100 ± 1.1[2] |
| 10.0 | 140 ± 4.8 | 102 ± 0.4[3] |
| 20.0 | 152 ± 2.7 | ---------- |
| 40.0 | 180 ± 3.0 | ---------- |
| 60.0 | 144 ± 1.9 | 100 ± 0.8 |

1.) Trypsin was boiled for ten minutes.

2.) Incubated with boiled trypsin for fifteen minutes.

3.) Incubated with boiled trypsin for thirty minutes.

Washed particles were incubated for various periods of time with either active or boiled trypsin, and centrifuged at 49,000 g for ten minutes. The supernatant was decanted, and the pellet was washed in tris-sucrose (0.005-0.32 M) buffer according to the washing procedure described in section C of the methods. The pellets were finally resuspended in tris buffered sucrose, and assayed according to the standard procedure. After the assay each suspension was filtered without any prior cooling in an ice bath. Adult chicken brain was used instead of embryonic chick brain because the mature brain appeared to contain more of the endogenous opioid inhibitor. The specific activity of trypsin was 7,500 Sigma units/ mg.

centrifuged and washed with tris-sucrose. Unlike adult chicken brain particulates stored at several temperatures as a function of time and assayed directly without any prior washing, figure 32, embryonic chick brain particulates treated in this manner never showed any binding capacity enhancement; regardless of the storage temperature.

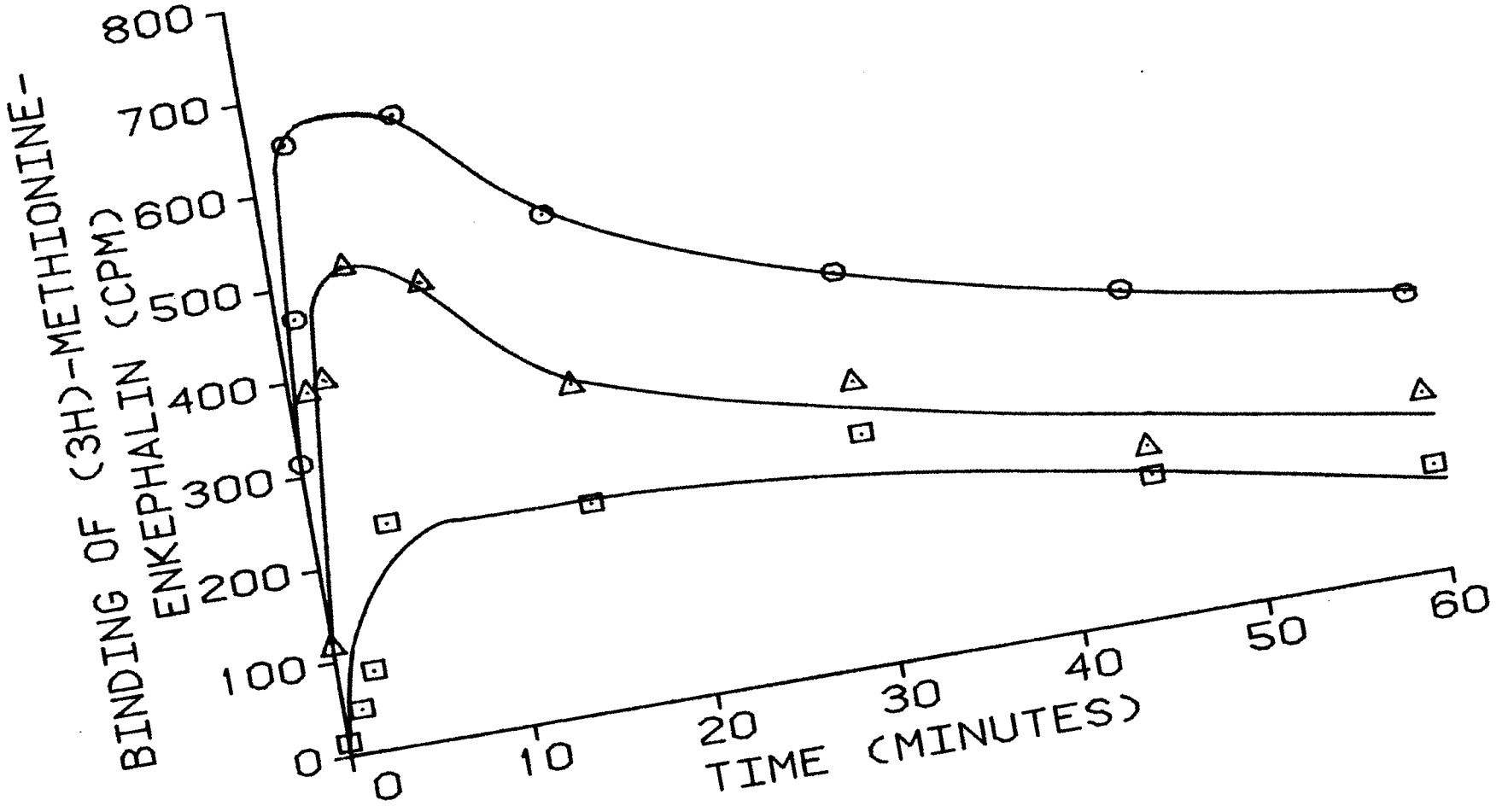
N) Tritiated Methionine Enkephalin Studies:

Since particulates from both embryonic and adult chicken brain appeared to contain some endogenous substance that interacted with the opiate receptor, the ability of washed particulates to interact with tritiated methionine enkephalin was studied, figures 33-34, because methionine enkephalin is known to be a natural opioid peptide that presumably interacts with the opiate receptor, table 9. Particulates derived from adult chicken brain were used for these experiments, because these preparations bound substantially more tritiated methionine enkephalin than did embryonic chick brain.

The time course for the binding of tritiated methionine enkephalin to particulates derived from adult chick brain, figure 33, exhibits a profile that is similar to that of the binding of naloxone to embryonic chick brain homogenate, figure 23. The binding of the enkephalin was constant for almost 25 minutes in the presence of levorphanol. Tritiated enkephalin binding peaked within 4 minutes of having been added to the particulates previously incubated in the presence of dextrorphan or tris-buffered sucrose. After 8 minutes both of these preparations showed parallel decreases in the amount of bound enkephalin. A decrease parallel to that observed with the dextrorphan incubated particulates was also seen

Figure 33. The time course for the binding of tritiated methionine enkephalin to washed particulates derived from adult chicken brain homogenate. Each of three 60 ml aliquots of washed particulates was incubated for five minutes with 0.1 uM dextrorphan (△), 0.1 uM levorphanol (◇), or a comparable aliquot of tris buffered sucrose (⊙) before incubating all three aliquots with 5.2 nM methionine-enkephalin. At the specified times three 2 ml aliquots were filtered from each flask without any prior cooling.

TIME COURSE FOR (3H)-METHIONINE-
ENKEPHALIN BINDING



after incubating particulates containing levorphanol for more than 30 minutes. The loss in the amount of methionine enkephalin bound apparently results from both the autodegradation of the adult binding capacity, figure 32, and the proteolytic action of an endopeptidase on the enkephalin (Knight and Klee 1978).

The binding of tritiated methionine enkephalin to adult chick brain particulates figure 34, is half saturated at a concentration of almost 30 nM, and is saturated at a concentration exceeding 200 nM.

A semi-log plot of the reduction of tritiated naloxone binding by methionine enkephalin, figure 35, results in an IC-50 of $1.1X10^{-7}$.

Figure 34. The saturability of the stereospecific binding of methionine enkephalin to washed adult chicken brain particulates. Successively larger aliquots of tritiated methionine-enkephalin were added to tubes containing 2 ml of washed particulates and complementary volumes of tris-buffered sucrose sufficient to provide a constant assay volume.

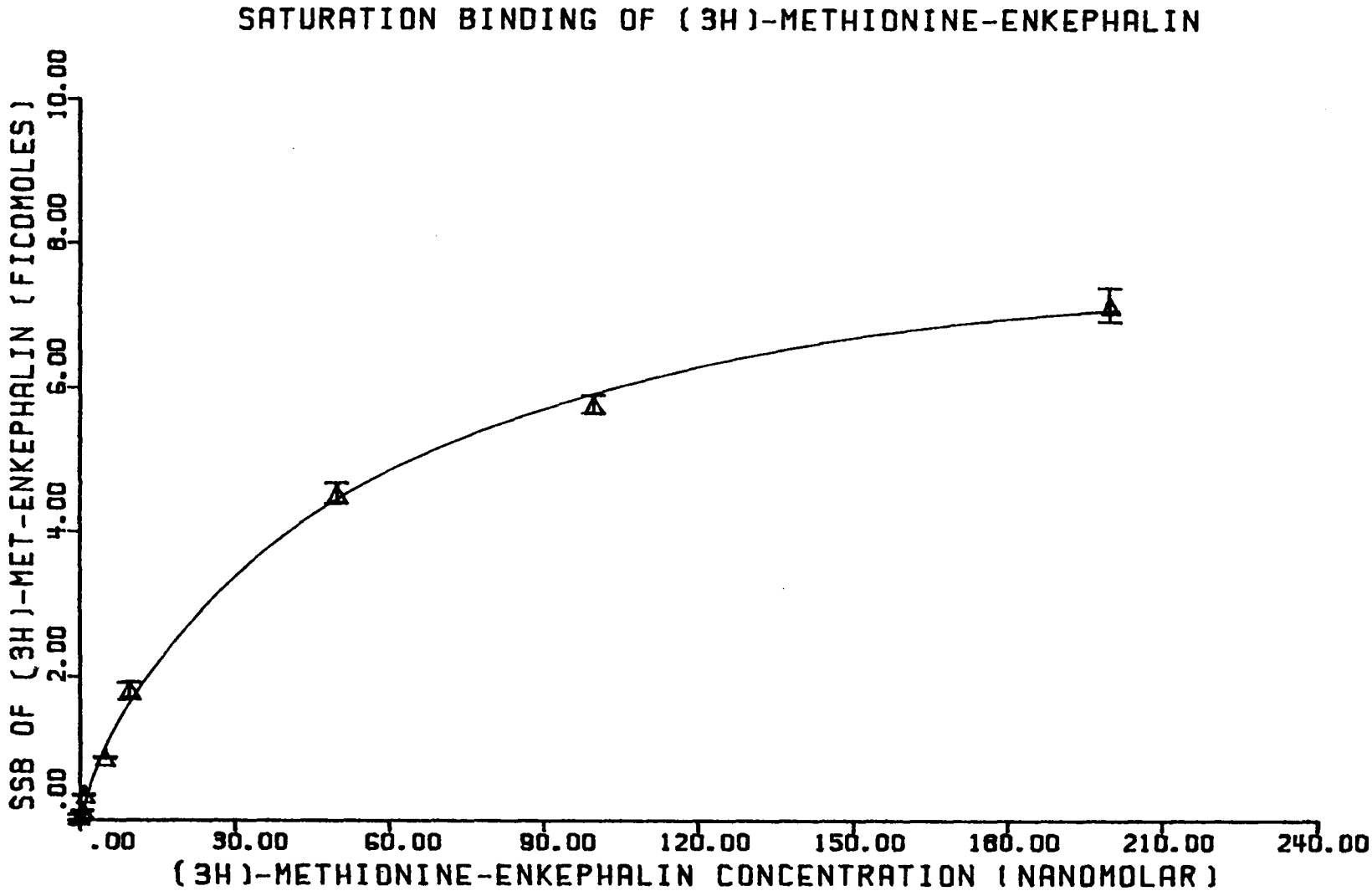
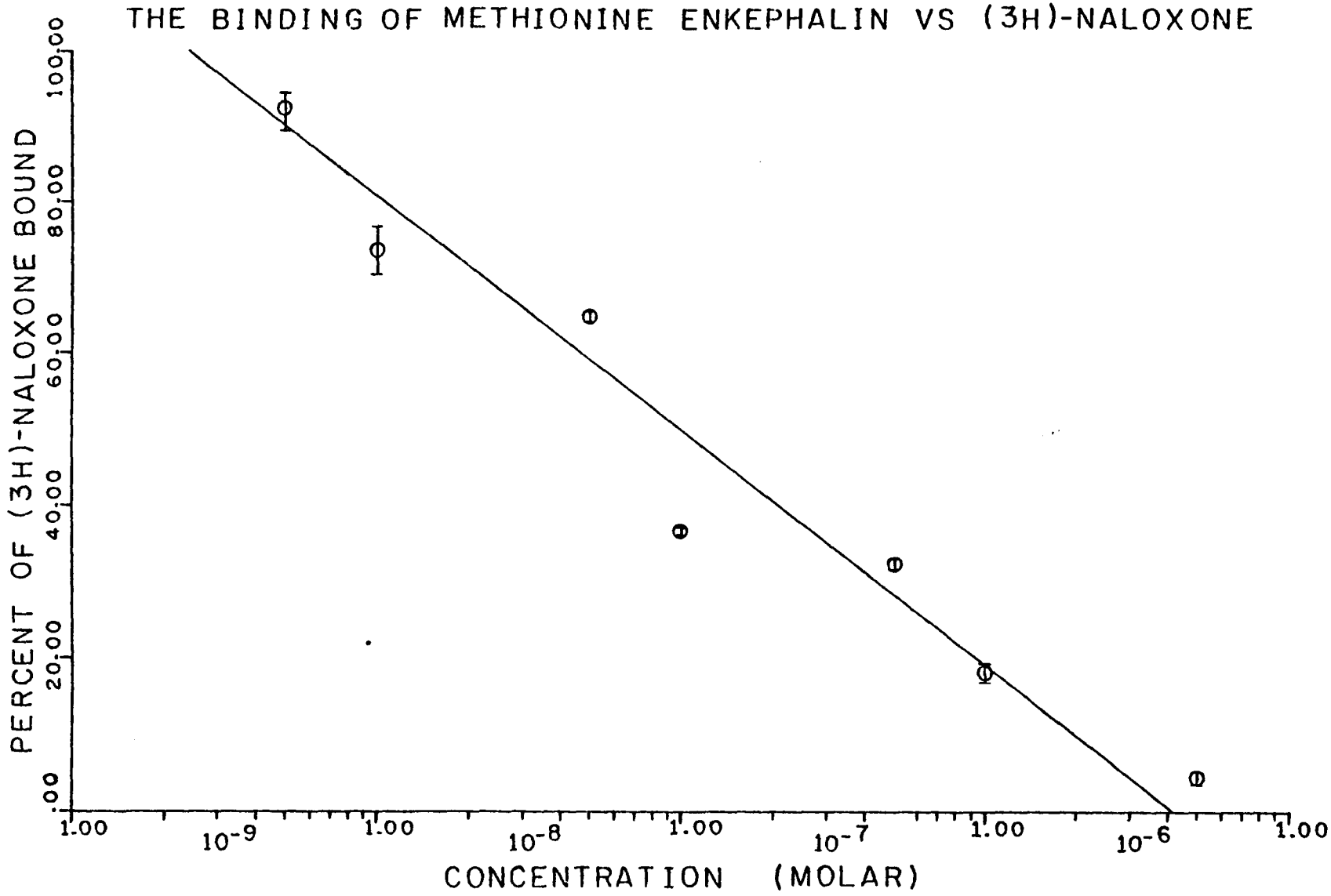

SATURATION BINDING OF (3H)-METHIONINE-ENKEPHALIN
SSB OF (3H)-MET-ENKEPHALIN (FICOMOLES)
.00
2.00
4.00
6.00
8.00
10.00
.00
30.00
60.00
90.00
120.00
150.00
180.00
210.00
240.00
(3H)-METHIONINE-ENKEPHALIN CONCENTRATION (NANOMOLAR)

Figure 35. The binding of methionine-enkephalin versus tritiated naloxone. The ability of methionine-enkephalin to reduce the binding of tritiated naloxone to washed adult chicken brain particulates was studied by incubating the washed particulates for five minutes with several different concentrations of methionine-enkephalin. Tritiated naloxone (4 nM), was then incubated with the particulates for ten minutes, and the particulates were filtered without cooling. The concentration of methionine-enkephalin producing a fifty percent reduction in the stereospecific binding of tritiated naloxone was reported as the $IC_{50}$ value for methionine-enkephalin.


THE BINDING OF METHIONINE ENKEPHALIN VS (3H)-NALOXONE
PERCENT OF (3H)-NALOXONE BOUND
100.00
80.00
60.00
40.00
20.00
.00
1.00
10-9
1.00
10-8
1.00
10-7
1.00
10-6
1.00
CONCENTRATION (MOLAR)

O) Alkali Metal Ion Effects on Naloxone Binding:

Embryonic chick brain homogenate exhibited enhanced naloxone binding in the presence of low concentrations of lithium and sodium ions, figure 36. Potassium ions led to a concentration dependent decrease in the binding capacity as did, calcium, magnesium, and manganese ions. Washed brain particulates derived from the embryonic chick, the adult chicken, and the rat, figures 13-16, showed an enhanced binding capacity over unwashed homogenates in the presence of sodium chloride. Suspensions of particulates derived from adult chicken brain and rat brain exhibit a pronounced and sustained sodium ion concentrations exceeding 100 mM. This result is in contrast to that demonstrated by preparations from embryonic chick, figure 14. The sodium ion induced enhancement of the stereospecific binding of naloxone is unaffected by the concentration of tris-buffer used in the assay figure 37; though less naloxone is bound in the presence of 50 mM tris-HCl. Sodium ions, and to a lesser extent lithium ions, enhance the binding of naloxone to embryonic chick brain particulates by almost 60 percent, figure 38. Potassium ions produce a minor enhancement at very low concentrations, but this enhancement rapidly gives way to a dramatic decrease, in the naloxone binding capacity of embryonic brain particulates. Even lower

concentrations of rubidium and cesium ions inhibit the stereospecific binding of tritiated naloxone to embryonic chick brain particulates.

Figure 36. The effect of several monovalent cations on the stereospecific binding capacity of embryonic chick brain homogenate. Two milliliter aliquots of homogenate were placed into test tubes containing aliquots of either lithium chloride (△), sodium chloride (⊙), or potassium chloride (⊡), and sufficient buffer to make a final volume of 2.2 ml. Tritiated naloxone was used in the standard assay procedure.

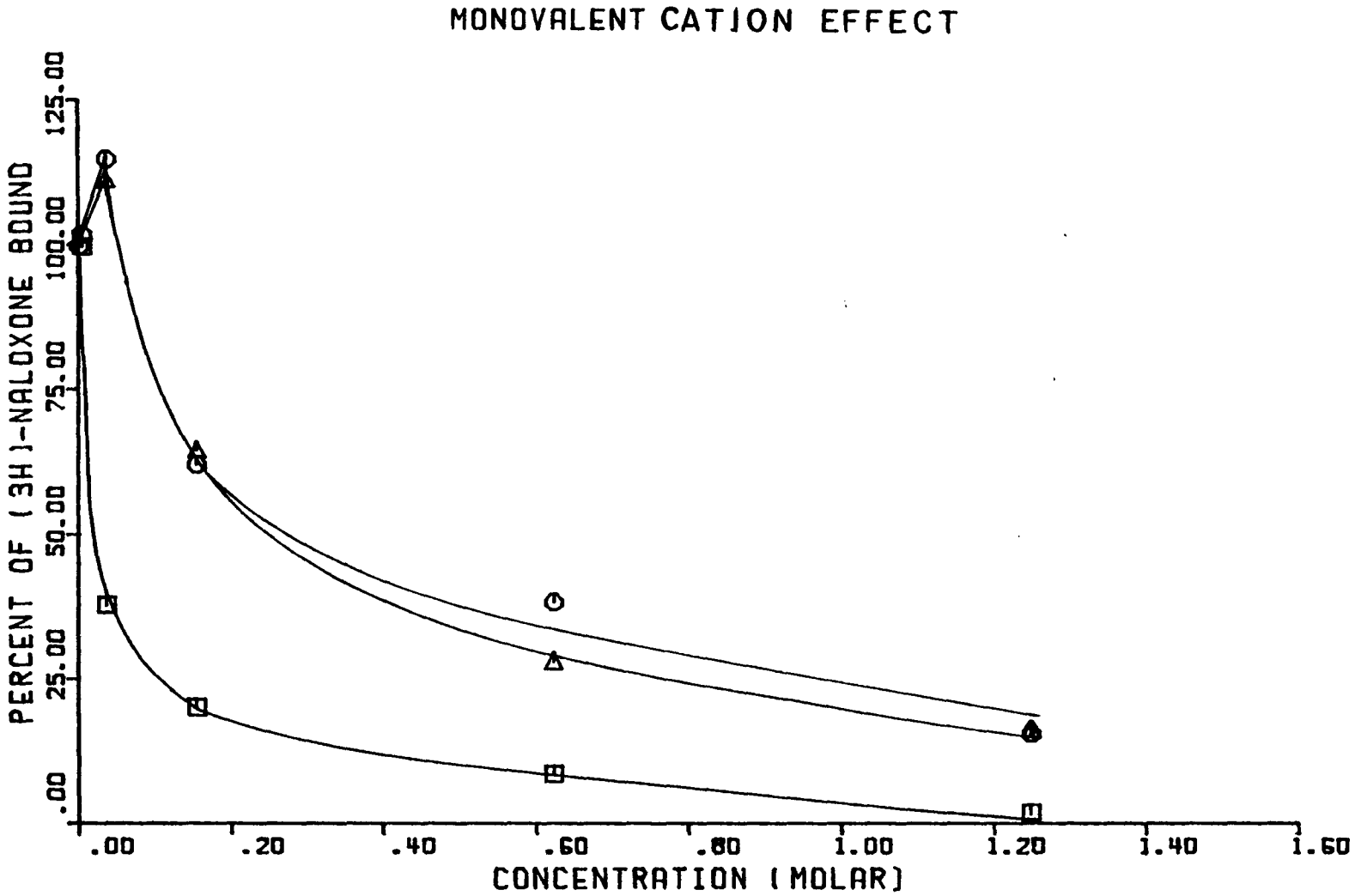

MONOVALENT CATION EFFECT
PERCENT OF (3H)-NALOXONE BOUND
.00
25.00
50.00
75.00
100.00
125.00
.00
.20
.40
.60
.80
1.00
1.20
1.40
1.60
CONCENTRATION (MOLAR)

Figure 37. The effect of tris-buffer concentration on the sodium enhanced binding of tritiated naloxone to embryonic chick brain. Embryonic chick brain particulates were prepared by washing homogenates, which were suspended in 5 mM tris-buffered 0.32 M sucrose, twice at 17,000 g for ten minutes, and resuspending the pellets at each step with either ice-cold 5 mM (⊙) or 50 mM (△) tris-buffered sucrose. The final pellets were resuspended with the appropriately buffered sucrose. Six 2 ml aliquots were placed into tubes containing the appropriate aliquots of sodium chloride and sufficient tris-buffered sucrose to make a final volume of 2.2 ml. Tritiated naloxone (2 nM) was used in the standard assay.

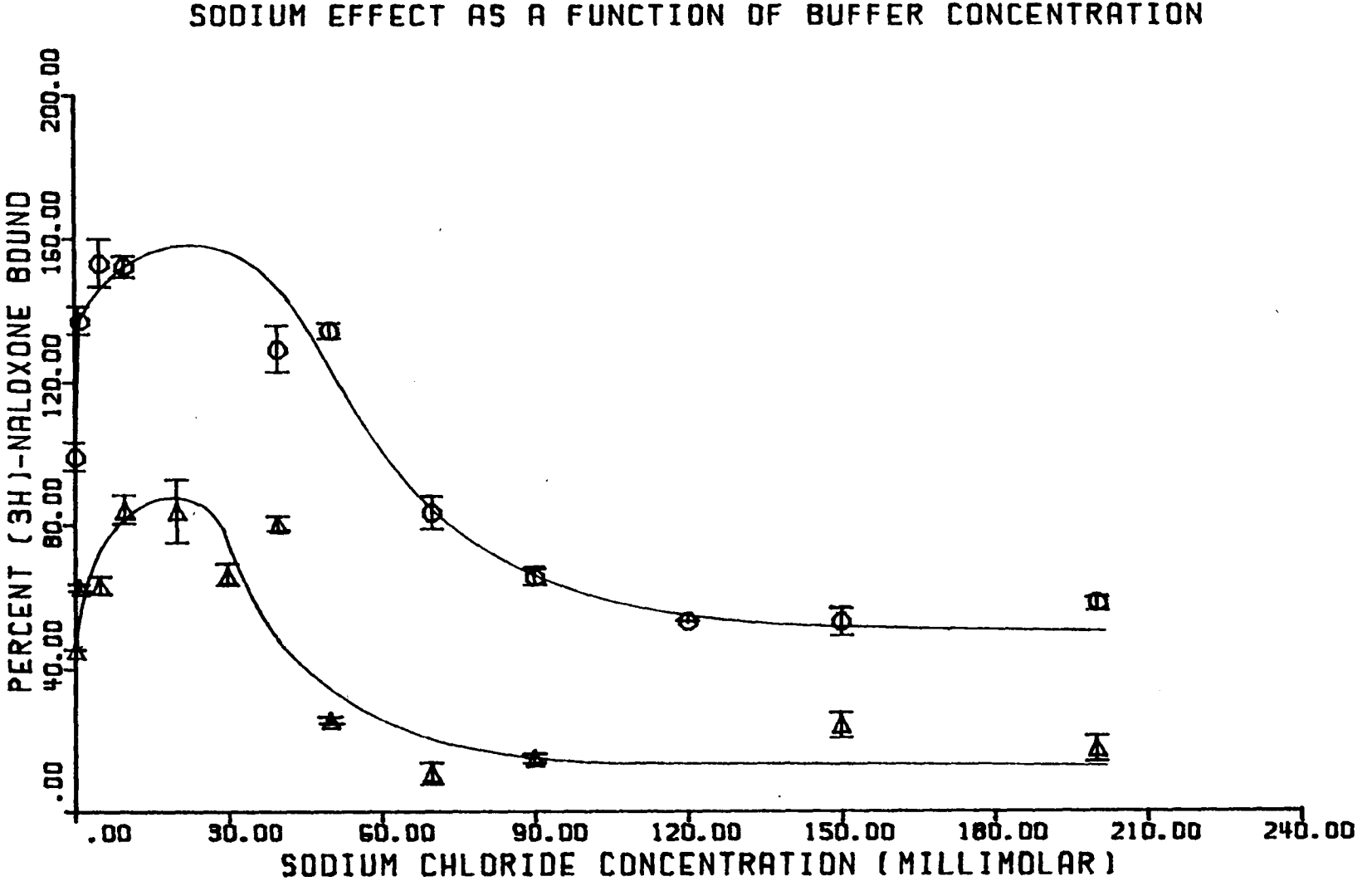
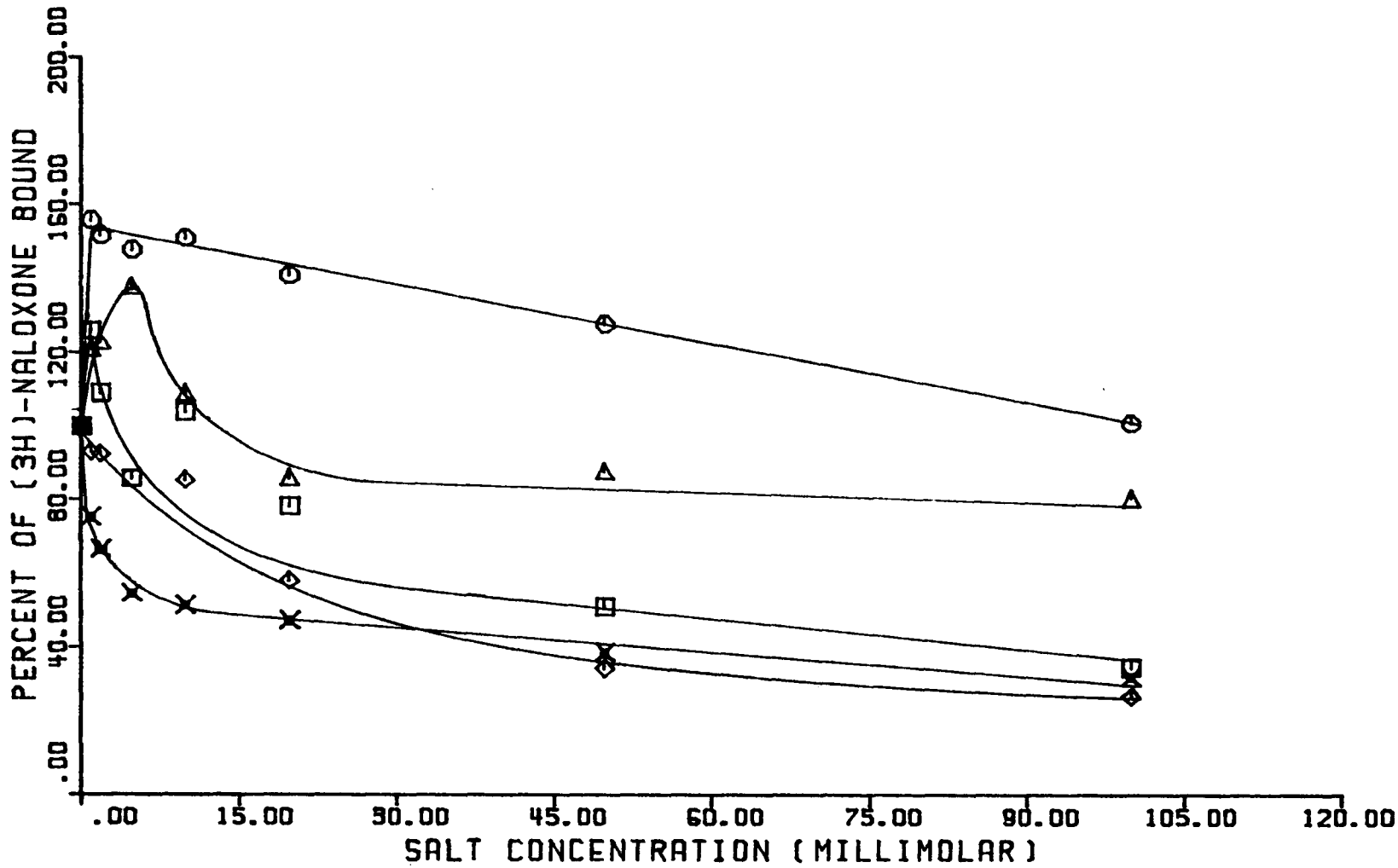

SODIUM EFFECT AS A FUNCTION OF BUFFER CONCENTRATION
PERCENT (3H)-NALOXONE BOUND
200.00
160.00
120.00
80.00
40.00
.00
.00
30.00
60.00
90.00
120.00
150.00
180.00
210.00
240.00
SODIUM CHLORIDE CONCENTRATION (MILLIMOLAR)

Figure 38. The effect of several monovalent cations on the stereospecific binding of tritiated naloxone to washed particulates derived from embryonic chick brain. Six 2 ml aliquots of washed particulates were placed into tubes containing appropriate aliquots of salt and enough tris-buffered sucrose to make a final assay volume of 2.2 ml. Lithium chloride (△), sodium chloride (⊙), potassium chloride (⊡), rubidium fluoride (◈), and cesium chloride (⌘) were incubated with the particulates for five minutes at 39 °C before beginning the standard assay. Tritiated naloxone (4 nM) was the labeled drug, and the particulates were filtered without any prior cooling.


THE EFFECT OF MONOVALENT CATIONS ON THE SSB OF (3H)-NALOXONE
PERCENT OF (3H)-NALOXONE BOUND
200.00
160.00
120.00
80.00
40.00
.00
.00
15.00
30.00
45.00
60.00
75.00
90.00
105.00
120.00
SALT CONCENTRATION (MILLIMOLAR)

P) Divalent Cation Effects:

Calcium ions have no affect on the binding of naloxone or etorphine at the concentrations of magnesium and manganese ions (0.001-0.05mM), tables 14-15, which enhance the binding of both opiates. High concentrations (1.0-100mM) of the binding of all three ions inhibit the binding of both opiates.

Table 14: The effect of divalent cations on the stereospecific binding of tritiated naloxone by washed particulates from embryonic chick brain.

| Concentration (mM) | Percentage SSB without Salt | | |
|---|---|---|---|
| | $MgSO_4$ | $MnCl_2$ | $CaCl_2$ |
| 0.00 | 100 ± 2.7 | 100 ± 1.4 | 100 ± 3.2 |
| 0.01 | 117 ± 1.9 | 118 ± 1.7 | 99.8 ± 2.0 |
| 0.02 | 124 ± 1.9 | 136 ± 1.7 | 104 ± 2.0 |
| 0.05 | 127 ± 2.2 | 124 ± 3.3 | 94.0 ± 2.9 |
| 0.10 | 121 ± 2.3 | 103 ± 2.3 | 87.0 ± 1.4 |
| 0.50 | 91.0 ± 1.2 | 83.6 ± 1.6 | 60.6 ± 1.6 |
| 1.00 | 85.2 ± 1.9 | 80.6 ± 2.7 | 43.6 ± 1.9 |
| 5.00 | 32.9 ± 2.7 | 58.2 ± 2.3 | 19.9 ± 1.1 |
| 10.0 | 26.6 ± 0.7 | 35.7 ± 1.3 | 17.7 ± 5.1 |
| 50.0 | 8.1 ± 1.6 | 14.5 ± 1.4 | 6.9 ± 0.5 |
| 100 | 0.9 ± 3.9 | 7.2 ± 1.6 | 7.0 ± 0.8 |

Particles from embryonic chick brain were incubated for five minutes at 39 $^{o}$C after being added to tubes containing the appropriate aliquot of salt solution. These washed particulates were examined using the standard assay procedure with 4 nM tritiated naloxone. The particles were filtered without any prior cooling in an ice bath.

Table 15: The effect of divalent cations on the stereospecific binding of tritiated etorphine by washed particulates from embryonic chick brain.

| Concentration (mM) | Percentage SSB without Salt | | |
|---|---|---|---|
| | $MgSO_4$ | $MnCl_2$ | $CaCl_2$ |
| 0.00 | 100 ± 1.2 | 100 ± 1.3 | 100 ± 0.5 |
| 0.01 | 117 ± 0.8 | 115 ± 0.9 | 110 ± 0.5 |
| 0.02 | 121 ± 0.7 | 126 ± 1.2 | 107 ± 0.5 |
| 0.05 | 125 ± 1.0 | 115 ± 1.1 | 98.1 ± 0.5 |
| 0.10 | 114 ± 1.3 | 107 ± 1.5 | 93.1 ± 0.6 |
| 0.50 | 86.0 ± 0.9 | --------- | 83.2 ± 0.9 |
| 1.00 | 70.7 ± 0.8 | 81.1 ± 0.6 | 70.1 ± 0.6 |
| 5.00 | 48.9 ± 0.5 | 55.9 ± 1.0 | 43.9 ± 0.4 |
| 10.0 | 36.5 ± 0.2 | 38.2 ± 0.8[1] | 31.7 ± 0.7 |
| 50.0 | 21.4 ± 0.4 | 27.3 ± 0.8[2] | 20.0 ± 0.5 |
| 100. | 16.8 ± 1.0 | 11.4 ± 0.5 | 15.2 ± 0.4 |

1.) 20.0 mM $MnCl_2$ in place of 10.0 mM $MnCl_2$.

2.) 40.0 mM $MnCl_2$ in place of 50.0 mM $MnCl_2$.

Washed embryonic chick brain particles were incubated for five minutes at 39 °C after being added to tubes containing the appropriate aliquot of salt solution. These particles were studied with the standard assay using 1 nM tritiated etorphine, and filtered without any prior cooling in an ice bath.

Q) Alkali Metal Ion Effects on Etorphine Binding:

An insignificant increase in the binding of etorphine results from the incubation of embryonic chick brain particulates with low concentrations (less than 10 mM) of sodium and potassium ions, figure 39. At higher concentrations these ions inhibit the binding of etorphine. Lithium, rubidium, and cesium ions inhibit the etorphine binding capacity of embryonic brain particulates as a function of the salt concentration. Except for lithium and sodium ions, these results are remarkably similar to those obtained with naloxone, and the corresponding decrease in the binding of etorphine by 20 mM sodium ions is independent of the type of anion associated with the sodium ion, table 16. The slightly lower binding enhancement observed for particulates incubated with sodium acetate presumably resulted from a slight shift in pH.

Figure 39. The effect of several monovalent cations on the stereospecific binding of tritiated etorphine to washed particulates derived from embryonic chick brain. Six 2 ml aliquots of washed particulates were placed into tubes containing appropriate aliquots of salt and enough tris-buffered sucrose to make a final assay volume of 2.2 ml. Lithium chloride (△), sodium chloride (⊙), potassium chloride (⊡), rubidium fluoride (◈), and cesium chloride (⌻) were incubated with the particulates for five minutes at 39 $^{o}$C prior to beginning the standard assay. Tritiated etorphine (1 nM) was used as the labeled drug, and the particulates were filtered without any prior cooling.

THE EFFECT OF MONOVALENT CATIONS ON THE SSB OF (3H)-ETORPHINE

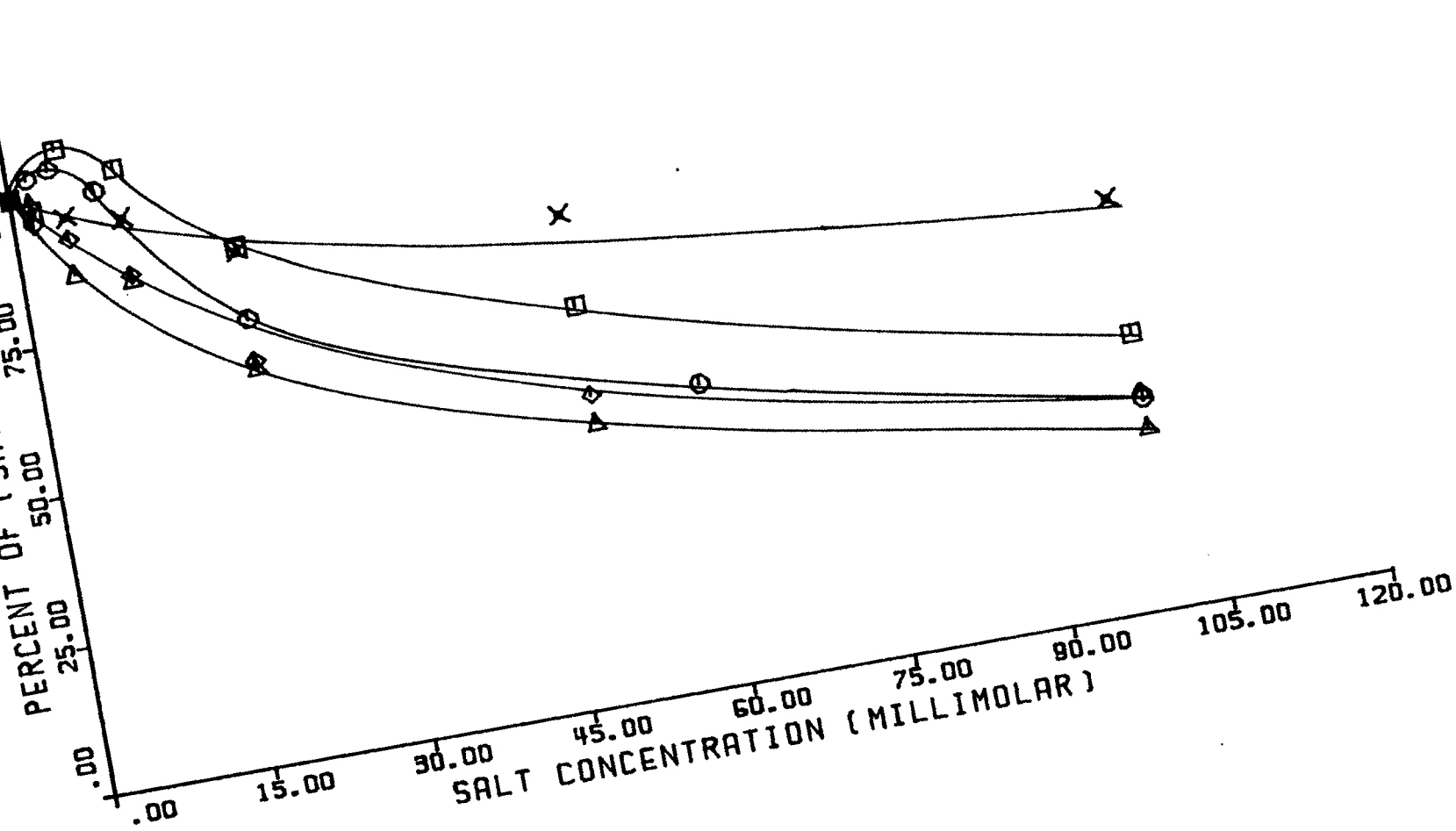


Table 16: The effect of several anions on the stereospecific binding of tritiated naloxone and etorphine by washed embryonic chick brain particulates.

| Sodium salt (20mM) | Percentage SSB without Salt | |
|---|---|---|
| | ($^3$H)-Naloxone | ($^3$H)-Etorphine |
| Control | 100 ± 1.2 | 100 ± 1.6 |
| $NaN_3$ | 153 ± 1.3 | 66.5 ± 0.6 |
| $NaNO_3$ | 168 ± 2.0 | 68.9 ± 1.0 |
| NaF | 145 ± 1.8 | 78.9 ± 2.2 |
| NaCl | 149 ± 2.5 | 62.0 ± 0.3 |
| NaBr | 150 ± 2.6 | 71.2 ± 1.0 |
| NaI | 142 ± 5.2 | 64.5 ± 1.4 |
| $NaHCO_3$ | 147 ± 3.6 | 78.2 ± 1.3 |
| $NaClO_4$ | 144 ± 0.8 | 67.4 ± 1.2 |
| NaOAc | 128 ± 2.0 | 64.4 ± 0.2 |
| NaSCN | 120 ± 1.3 | 63.7 ± 1.3 |

Either tritiated naloxone (4 nM) or tritiated etorphine(1 nM) were used in the standard assay, and washed particles from embryonic chick brain were filtered immediately after the assay without any prior cooling on ice.

R) Scatchard Analyses:

Scatchard analyses, figure 40-42, and table 17, of the ion induced binding enhancements of naloxone and etorphine revealed that these enhancements arise from alterations in both the affinity constants of the opiate receptors and the total number of receptors, present in particulates, table 17. Naloxone binds to at least two classes of binding sites, figure 40-41, The addition of sodium, magnesium, or manganese ions increases the number of naloxone binding sites, table 17. Magnesium and manganous ions increase the number of etorphine binding sites, but sodium ions decrease the number of sites available to etorphine. Sodium ions increase the dissociation of etorphine from particulates while increasing the association of naloxone with the embryonic binding sites. Both manganous and magnesium ions reduce the overall affinity constants, (Ko), of the binding sites associated with naloxone and etorphine. When the affinity constant, (Ko) of the particulates studied in the absence of any ions are compared to those incubated with either magnesium or manganous ions the affinity constants, (Ko), for etorphine are reduced by only half as much as are those for naloxone. Etorphine appears to bind to only one class of sites except when incubated in the presence of sodium ions, which seems to produce at

Table 17: Binding site parameters determined by the Scatchard analysis of the salt effects observed with washed particles from embryonic chick brain.

| Opiate | Salt | Binding Sites (moles X $10^{+14}$) | $K_o$ | $K_1$ ($M^{-1}$ x $10^{-9}$) | $K_2$ |
|---|---|---|---|---|---|
| Naloxone | ---- | 10.3 | 0.33 | 0.81 | 1.64 |
| Naloxone | NaCl | 13.7 | 0.68 | 1.09 | 1.75 |
| Naloxone | $MnCl_2$ | 12.2 | 0.12 | 0.22 | 0.39 |
| Naloxone | $MgSO_4$ | 12.4 | 0.11 | 0.31 | 0.19 |
| Etorphine | ---- | 11.4 | 4.76 | ---- | ---- |
| Etorphine | NaCl | 9.2 | 0.77 | 1.79 | 2.27 |
| Etorphine | $MnCl_2$ | 13.8 | 2.86 | ---- | ---- |
| Etorphine | $MgSO_4$ | 14.2 | 2.70 | ---- | ---- |

Sodium chloride (20 mM), manganese chloride (0.02 mM), and magnesium sulfate (0.02 mM) were preincubated for five minutes with washed embryonic chick brain particles prior to conducting the standard assay. The washed particles were filtered without any prior cooling in an ice bath.

Figure 40. The Scatchard analysis of the sodium ion induced enhancement in the stereospecific binding of tritiated naloxone to washed embryonic chick brain particulates. Six 2 ml aliquots of washed particulates were added to tubes containing either 20 mM sodium chloride (⊙) and tris-buffered sucrose, or just tris-buffered sucrose (△). Both dextrorphan and levorphanol (0.1 uM) were incubated with one set of three tubes for five minutes, and successively larger aliquots of tritiated naloxone were incubated with each set of six tubes for ten minutes. The final assay volume was adjusted to 2.2 ml with tris-buffered sucrose, and the particulates were filtered without any prior cooling.

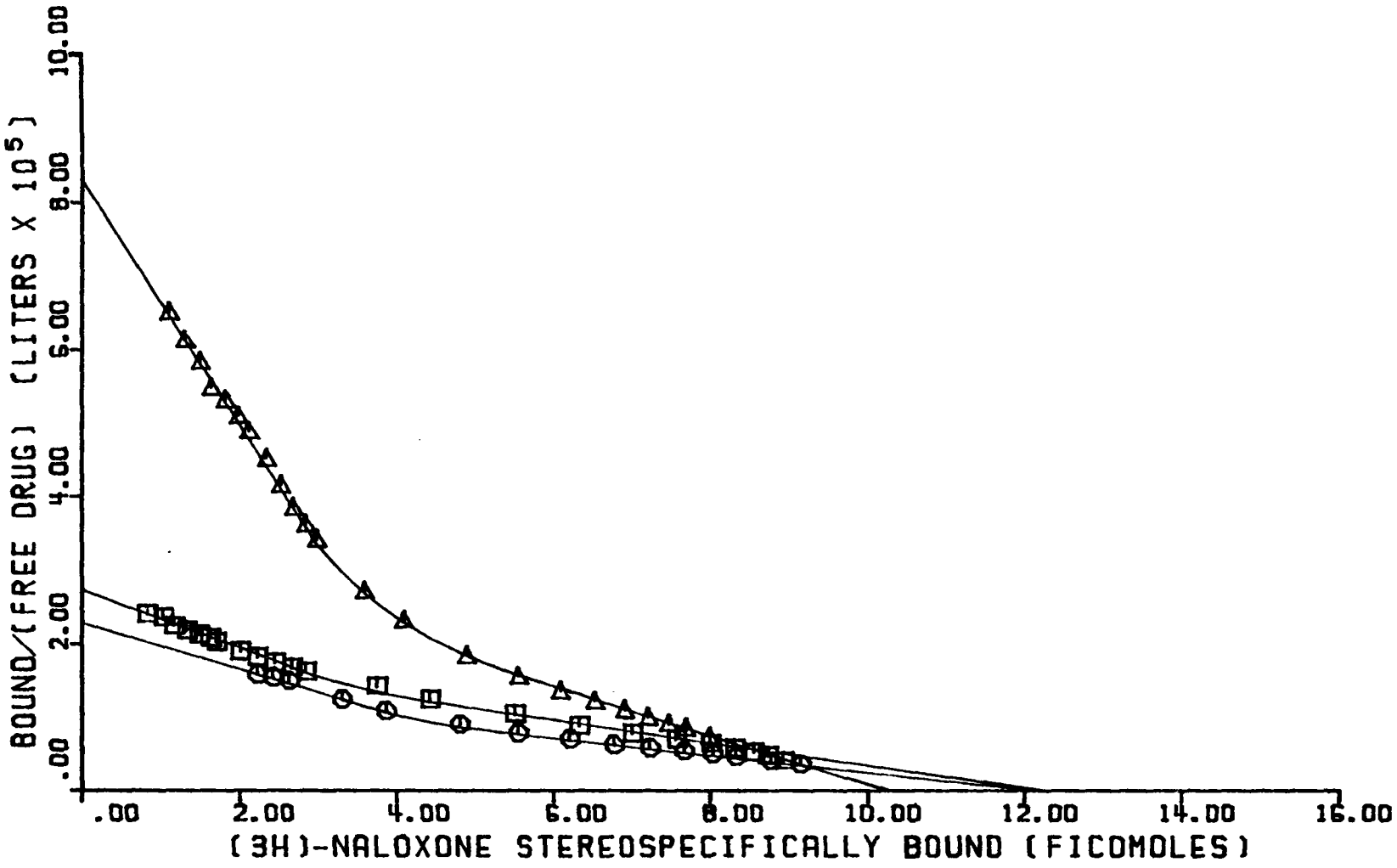
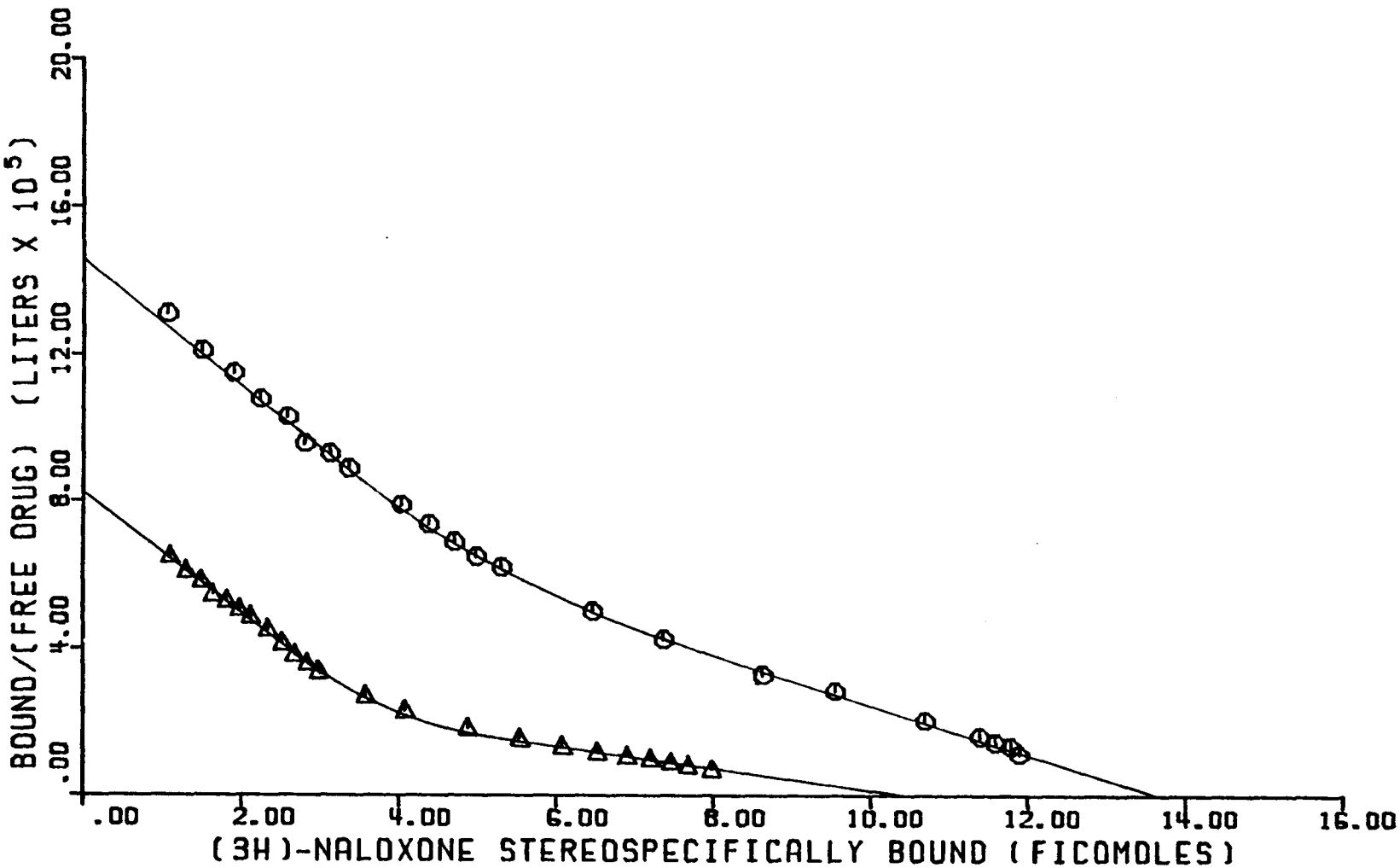
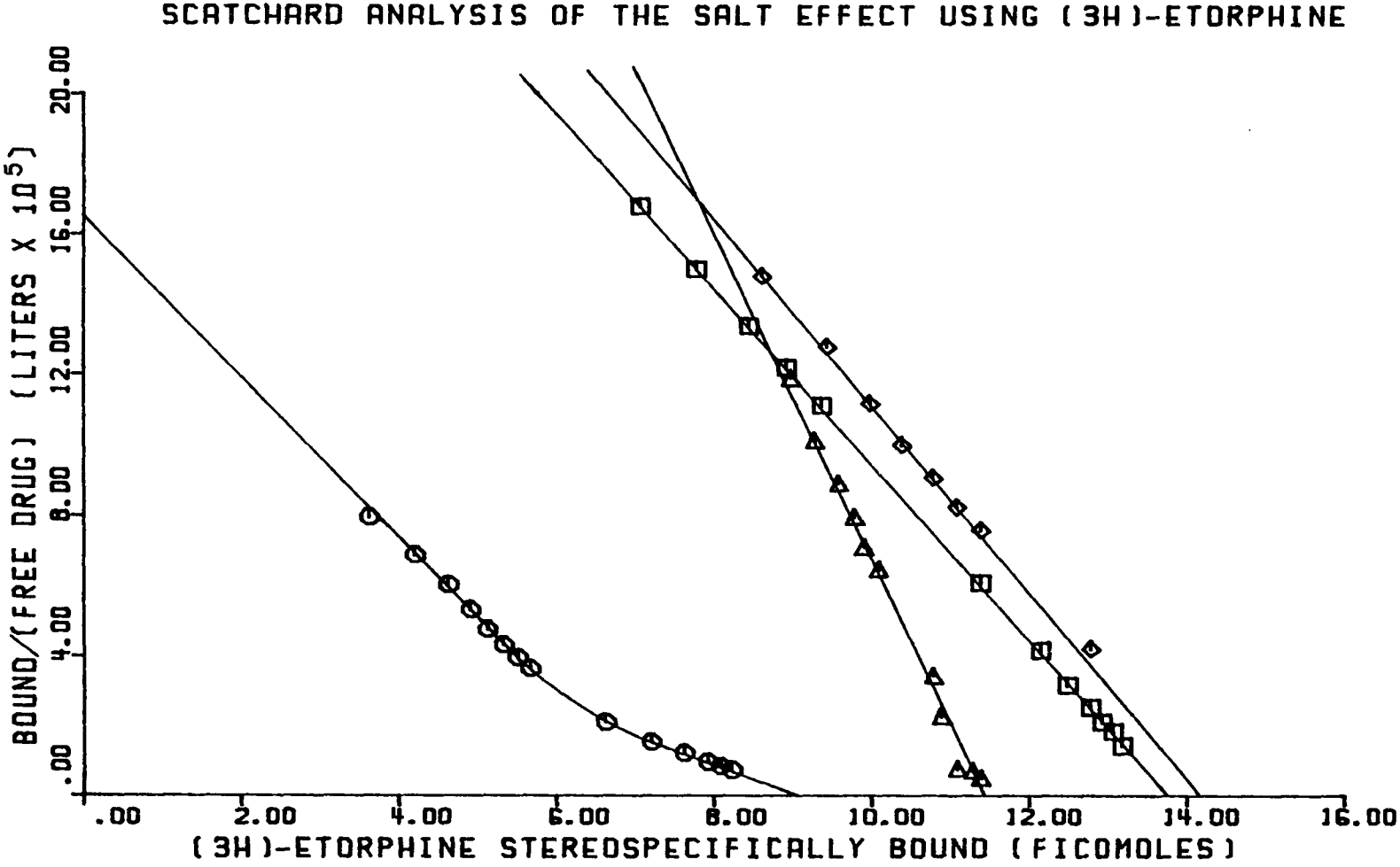

SCATCHARD ANALYSIS OF THE Mg++ Mn++ EFFECT USING (3H)-NALOXONE
BOUND/(FREE DRUG) (LITERS X 10^5)
.00
2.00
4.00
6.00
8.00
10.00
.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
(3H)-NALOXONE STEREOSPECIFICALLY BOUND (FICOMOLES)

Figure 41. The Scatchard analysis of the manganous and magnesium ion induced enhancement of the stereospecific binding of tritiated naloxone to washed particulates derived from embryonic chick brain. Particulates were incubated for five minutes in tubes containing either 0.02 mM manganese chloride (⊡), 0.02 mM magnesium sulfate (⊙), or tris-buffered sucrose (△). Standard assay concentrations of either dextrorphan and levorphanol were incubated for five minutes with each of two sets of three tubes containing the washed particulates, prior to adding successively larger aliquots of tritiated naloxone. The assay volume was adjusted to 2.2 ml by the addition of an appropriate aliquot of tris-buffered sucrose. The particulates were filtered without any prior cooling.


SCATCHARD ANALYSIS OF THE $Na^+$ EFFECT USING (3H)-NALOXONE
BOUND/(FREE DRUG) (LITERS X $10^5$)
.00
4.00
8.00
12.00
16.00
20.00
.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
(3H)-NALOXONE STEREOSPECIFICALLY BOUND (FICOMOLES)

least two types of binding sites. This sodium induced pertubation is evident from the Scatchard plot for etorphine incubated with sodium ion, figure 42. The binding constants, (K1 and K2) are almost 2 to 3 times larger than the dissociation constant reported here for particulates studied in the absence of any ion. Sodium ions increase the affinity of both the high, (K2), and low, (K1), affinity sites for naloxone in comparison to the affinity of naloxone for particulates studied in the absence of any ion. Manganous and magnesium ions reduce the affinity of the naloxone binding sites. Magnesium reduces the affinity of the high affinity sites more than it does that of the low affinity sites. This result is in contrast to those obtained for particulates incubated in the absence or presence of both sodium and manganese ions.

Figure 42. The Scatchard analysis of the cation induced alteration of the stereospecific binding of tritiated etorphine to washed particulates derived from embryonic chick brain. Particulates were incubated for five minutes in tubes containing either 0.02 mM manganese chloride (⊡), 0.02 mM magnesium chloride (◇), 20 mM sodium chloride (⊙), or tris-buffered sucrose (△). Standard assay concentrations of either dextrorphan or levorphanol were incubated for five minutes with each of two sets of three tubes containing washed particulates prior to incubating them for ten minutes with successively larger aliquots of tritiated etorphine. The assay volume was adjusted to 2.2 ml by the addition of an appropriate aliquot of tris-buffered sucrose. The particulates were filtered without any prior cooling.


SCATCHARD ANALYSIS OF THE SALT EFFECT USING (3H)-ETORPHINE
BOUND/(FREE DRUG) (LITERS X 10^5)
.00
4.00
8.00
12.00
16.00
20.00
.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
(3H)-ETORPHINE STEREOSPECIFICALLY BOUND (FICOMOLES)

S) EDTA Effects:

The ability of EDTA to almost completely reverse the calcium ion induced inhibition of etorphine binding contrasts with the partial reversal of the inhibition of the binding of naloxone with 1.0 mM EDTA, table 18. The binding capacity of particulates incubated with 1.0 mM calcium ion is increased 50 percent by the addition of 0.5 mM EDTA, and almost doubled by the addition of 1.0 mM EDTA. The binding of tritiated naloxone to particulates is inhibited approximately 30 percent by the addition of 1.0 mM calcium ion. This inhibition is only partially reversed by the addition of 0.5 mM EDTA. The inhibition of the binding of etorphine to washed embryonic chick brain particulates incubated with 1.0 mM calcium ions, prior to the addition of 2.0 mM EDTA, presumably results from the removal of some endogenous ions that are necessary for maintaining the binding capacity of the particulates. Two millimolar EDTA has no apparent inhibitory effect on naloxone binding.

Table 18: The effect of EDTA-calcium complexes on the stereospecific binding of tritiated naloxone and etorphine by washed particles from embryonic chick brain.

| EDTA Concentration (mM) | Calcium Concentration (mM) | Percentage SSB without Calcium Ion | |
|---|---|---|---|
| | | Naloxone | Etorphine |
| 0.0 | 1.0 | 70.1 ± 0.6 | 49.3 ± 1.2 |
| 2.0 | 0.0 | 100 ± 1.5 | 100 ± 5.3 |
| 0.5 | 1.0 | 84.5 ± 1.3 | 70.7 ± 2.7 |
| 1.0 | 1.0 | 84.7 ± 1.4 | 91.7 ± 1.9 |
| 2.0 | 1.0 | 84.5 ± 0.2 | 81.8 ± 2.1 |

Washed embryonic chick brain particles were incubated with calcium chloride for five minutes prior to incubating them for five minutes with EDTA. Particles were assayed according to the standard assay using either tritiated naloxone (4 nM) or tritiated etorphine (1 nM), and filtered without cooling them in an ice bath.

DISCUSSION:

Although remarkable similarities exist between the opiate binding capacity of 13 day old embryonic chick brain suspensions and preparations made from mature brain tissue, considerable differences are apparent in the magnitude and stability of the opiate-binding capacity of embryonic chick brain suspensions. Both adult and embryonic brain tissue contain substantial amounts of a bound endogenous opioid substance, yet embryonic chick brain particulates exhibit specific cation effects that are quantitatively and qualitatively different from those effects exhibited by mature rat brain particulates.

The opiate binding capacity of 13 day old embryonic chick brain has a strong resemblence to that of the adult chicken, mouse, and rat. The shift in the binding capacity from the crude microsomal to the crude synaptosomal-mitochondrial fraction (P2), table 1, as synaptogenesis proceeds in the immature brain (Szutowicz et al., 1976), correlates with the localization of the opiate binding capacity in the P2 fraction of adult rats (Pert et al., 1974b). The synaptosomes, which are biochemically and morphologically recognizable in thirteen day old embryonic chick brain (Szutowicz et al., 1976), are similar to those observed in adult chicken (Whittaker, 1969; Gray and Whittaker, 1962). The shifts in the binding

capacity from the P3 fraction to the P2 fraction, as the embryonic brain matures, suggests that opiate binding structures are formed before the synapse is completely developed (Szutowicz et al., 1976).

A crude nuclear fraction (P1) derived from the brain of mice was reported, (Goldstein et al., 1971), to contain significant amounts of opiate receptor activity, while the P1 fraction of both chick brain, table 1, and adult rats (Pert et al., 1974b), contained less than 15 percent of the total binding activity.

The amount of naloxone bound by 13 day old embryonic chick and adult chicken homogenates, which differs by approximately 20 percent, table 2, are comparable to the levels of opiate bound in rotation cultures of embryonic chick cells (Peterson et al., 1975a), and to rat homogenates (Simon et al., 1973; Pert et al., 1974c). Pert et al., (1974c), observed very little difference between the binding constants of dihydromorphine bound to animals considered to range evolutionarily from very primitive to very advanced. Anatomical studies, tables 3 and 4, confirmed the observation, (Pert et al., 1974c), that the frontal lobes of chicken brain exhibit the largest portion of opiate binding capacity, while the other lobes have correspondingly lower binding capacities. Pert et al., (1974c), found most of the binding capacity of the frontal lobes

to be localized in the olfactory bulb and archstriatum, which are the forerunners of the mammalian limbic system, (MacLean (1949); Papez 1967).

There is very little difference in the time dependent distribution of naloxone in brain homogenates prepared from rats (Pert and Snyder 1973b), and embryonic chicks, figure 23. In comparison to rat brain homogenate, which binds almost equal amounts of naloxone whether incubated in the absence or presence of dextrorphan, embryonic chick brain homogenate binds almost 30 percent less naloxone in the presence of dextrorphan. This suggests that the lipophilicity of dextrorphan may have a more significant role in the binding of naloxone to embryonic chick brain homogenates than to adult rat preparations. The discrepancy between the amount of naloxone bound may be specific to the chick, because a similar pattern of binding is seen when the time course for tritiated methionine enkephalin binding, figure 33, is studied.

The thermal stability, figure 9-10, the bimodal effect of protein concentration, figure 17, and the ability of opiates to saturate the opiate binding capacity of embryonic chick brain homogenate, figures 24, and 40-42, are remarkably similar to these same parameters exhibited by adult rat brain homogenate (Pert and Snyder, 1973b).

Both types of receptors denature above 45 degrees. The bimodal pattern for the effect of protein concentration is larger in magnitude and range for the rat, but this seems to be due to the greater binding capacity of rat brain homogenate. Chicks are reported, (Pert et al., 1974c), to have a lower binding capacity than rats, which may be the result of more structural protein and/or fewer opioid binding structures. The half saturation value for the binding of naloxone and etorphine to embryonic chick brain is similar to those values reported by Pert and Snyder, (1973b), and Simon et al., (1973), respectively. The time course, figure 23, saturation, figure 24, and IC-50 values, figure 25, and table 9 for the binding of naloxone indicate that embryonic chick brain contains pharmacologically active receptors that bind opiates stereospecifically. The IC-50 values reported for the binding of naloxone and etorphine to adult rats, (Simon et al., 1973; Pert and Snyder, 1973b; Pert et al., 1973a), are similar to those reported for embryonic chick brain, table 9, and figure 25, with agonists generally having larger IC-50 values than the antagonists.

The dual peak in the pH profile, figure 18, for the embryonic chick brain homogenate is unique to the chick system. A pH of 7.4 was chosen for the study because it correlated with the physiologically significant value reported earlier, (Pert and Snyder, 1973b). The second

peak in the pH profile may represent a second type of binding site.

Analogous displacements of tritiated naloxone by non-radioactive naloxone were observed between rat brain homogenates, (Pert and Snyder, 1973b), and embryonic chick brain homogenates. Both types of homogenate exhibit a sharp decrease in the amount of tritiated naloxone bound at low concentrations of unlabeled naloxone. The dissociation rates for naloxone bound to embryonic chick brain preparations, figure 27, and rat brain homogenates, (Pert and Snyder 1973b), are similar at temperatures above 9 degrees, but at 9 degrees a dramatic increase in the half-life of the naloxone-receptor complex is observed. Both of these assay systems exhibit first order kinetics with respect to naloxone, and immeasurably rapid dissociations of the opiate-receptor complex at 39 degrees.

Cooling assayed homogenates on ice enhances the binding of naloxone, (Creese et al., 1975b), figure 8. Like rat brain homogenate cold incresaes the number of naloxone binding sites and their affinity for naloxone. The affinity constant increases almost 5 times more than the number of receptors in embryonic preparations. Conceivably, the affinity constants are altered by a thermally induced membrane lipid transition, (McConnell et al., 1972).

The Scatchard analyses of the binding

enhancements elicited by specific ions in embryonic chick brain particulates, figures 41-43, and table 17, resembles the analyses described for rat brain preparations, (Creese et al., 1975b; Pasternak et al., 1975c; Simon et al., 1975a; Pert and Snyder, 1974a). Manganous and magnesium ions, enhance the binding of both naloxone and etorphine to levels similar to those reported by Pasternak et al., (1975c). Above 25 degrees the effects of manganous, magnesium, and sodium ions, on the binding of agonists and antagonists, are due to alterations in the number of binding sites, and not the affinity of these sites for opiates, (Pert and Snyder, 1974a; Pasternak et al., 1975c; Creese et al., 1975b; Simon et al., 1975a). This contrasts with the change in both the number of receptors and their affinity for naloxone and etorphine in embryonic chick preparations, table 17.

Alterations in the number of embryonic chick brain binding sites for antagonists elicited by sodium and for agonists elicited by sodium, magnesium, and manganeous ions, corresponds with those reported for adult rat brain, (Pert and Snyder, 1974a; Pasternak et al., 1975c; Simon et al., 1975a). Sodium ions, which reduce the binding of agonists and increase the binding of antagonists in both embryonic chick brain and adult rat brain preparations, (Pert and Snyder, 1974a), appear to modulate the number of receptor

sites for these classes of drugs, (Pert and Snyder, 1974a), figures 40-42.

Sodium ion concentrations of 100 mM were routinely used by investigators (Pert and Snyder, 1974a; Pasternak et al., 1975c; Creese et al., 1975b; Simon et al., 1975a; Lee et al., 1975), who believed that this concentration was physiologically relevant. Pert and Snyder, (1974a), observed that the differential effects for the binding of agonists and antagonists in the presence of sodium were due to the existence of high and low affinity sites for these classes of opiates. Simon et al., (1975a), indicated that while high concentrations of sodium ions (200mM), completely convert rat brain receptors to a state that only accepts antagonists, lower concentrations of sodium ions (100mM), leave a mixed population of receptors that differentially bind both classes of opiates. Sodium ions reveal the presence of high and low affinity agonist and antagonist binding sites in washed embryonic chick brain particles, figures 40-42, that are similar to those observed by Pert and Snyder, (1974a).

Adult chick brain particulates demonstrate a comparable sodium effect, figure 15, but the binding of naloxone to embryonic chick brain particulates at the lower sodium ion concentration, (100 mM), is substantially reduced, figure 14, indicating the sensitivity of the embryonic binding capacity to the ionic strength

of the medium.

The inhibition of opiate binding to embryonic chick brain homogenates as a function of buffer concentration, figures 11-12, and table 6, also attest to the sensitivity of the embryonic receptors to the ionic strength of the assay mixture. The binding capacity of adult chicken brain homogenate also decreased as the tris-buffer concentration is increased. In contrast, the sodium effect in rat brain homogenate is unaffected with 50 mM tris buffered sucrose, figure 16, while embryonic chick brain particulates bind much less naloxone with 50 mM tris-buffered sucrose than with 5 mM tris-buffered sucrose, figure 37.

Corresponding to the sodium effect observed with rat brain preparations, embryonic chick brain preparations exhibit a sodium effect that is independent of the buffer concentration, figure 37, and enhanced by the washing procedure, figure 13, used for the studies with adult rat brain, (Pert and Snyder, 1974a). Unwashed homogenates prepared from both adult rat brain, (Pert and Snyder, 1974a; Pert and Snyder, 1973b), and embryonic chick brain, figure 36, exhibit a small sodium induced enhancement of the binding of naloxone. Only sodium ions, and to a lesser extent lithium ions, increase the binding of naloxone to suspensions made from mature rats (Pert and Snyder, 1974a), chickens, and 13 day old

chick embryoe. Potassium, rubidium and cesium ions either reduce or have little effect upon the binding of agonists and antagonists, (Pert and Snyder, 1974a). This result is in agreement with the observations made using washed embryonic chick brain particulates, figures 38-39.

Calcium presumably has no role in the binding process in both embryonic chick brain preparations, tables 14-15, and adult rat brain particulates, (Pasternak et al., 1975c). EDTA is capable of inhibiting the binding of both naloxone and etorphine in embryonic chick preparations, table 18, in contrast to adult rat preparations in which EDTA only inhibits the binding of agonists, (Pasternak et al., 1975c). Anions have no obvious effect on the binding actions elicited by sodium ions in both embryonic chick brain particulates, table 16, and adult rat brain suspensions, (Pasternak et al., 1975c).

The presence of an endogenous opioid substance in adult and embryonic chick brain suspensions is comparable to the discovery of some related substance in mammals, (Hughes, 1975; Terenius and Wahlstrom, 1974; Pasternak et al., 1975a; Simantov and Snyder, 1976d; Pasternak et al., 1975b; Simantov et al., 1976b). This substance can be removed from embryonic chick brain by utilizing a procedure similar to the one used by Pasternak et al., (1975b). However, both adult and embryonic chick brain preparations lose

binding activity when incubated at the higher temperatures, figure 29, or the longer time periods, figure 30, used by Pasternak et al., (1975b), and Simantov et al., (1976b).

The apparent thermal sensitivity of the opiate binding capacity of the preparations derived from both adult and embryonic chick brain, figures 31-32, seem to be the result of an autodegradative process because both types of chicken brain particulates lose their opiate binding capacity in a temperature dependent fashion. The embryonic chick brain suspensions lose their opiate binding capacity more quickly than the adult preparations, figures 31-32. The autodegradation process can be slowed by reducing the storage temperature, but only the adult chicken brain particulates can be stored for more than 8 hours without an appreciable loss in the binding capacity. This result implies that the mature receptors are more stable than the embryonic receptors.

Adult chicken brain preparations also bind slightly larger amounts of naloxone than do those derived from embryonic chick brain. The enhanced opiate binding capacity of the adult chicken brain, in comparison to the binding capacity of embryonic chicken brain, derives from the increased size and binding capacity of the frontal lobes in the mature brain, tables 3-4.

Opiate binding capacity increases as the

embryo matures, tables 1-4. The frontal lobes of the embryonic chick differentiate at a rate that exceeds the growth of the other lobes, (Peterson et al., 1974b), the greatest density of opiate receptors is found in the frontal lobes of the chick, (Pert et al., 1974c), tables 3-4, and these lobes are analogous to the corpus striatum in mammals, (Pert et al., 1974c).

Suitable incubation conditions were chosen which represented a compromise between the autodegradative effects and the binding enhancement elicited by the incubation procedure. These conditions doubled the binding capacity of embryonic chick brain suspensions, table 11, presumably by permitting the endogenous opioid substance to dissociate from the receptor. A similar doubling effect was observed by Pasternak et al., (1975b).

The addition of supernatant to the specially preincubated washed embryonic brain particulates produced only a minor inhibition of the opiate binding capacity. This result suggests that either there are only low levels of endogenous opioid present in the embryonic preparation, or that there is a rapid degradation of the endogenous opioid substance released during the incubation procedure. The latter alternative is the more likely because adult preparations, which should contain more endogenous opioid substance, exhibit a significant inhibition of the binding of

naloxone when supernatant, that presumably contains the endogenous opioid substance, is incubated with the specially preincubated and washed adult chicken brain suspension, table 12.

An endopeptidase that specifically degrades methionine enkephalin, (which was identified as one of the endogenous opioid substances), (Simantov et al., 1976b; Hughes, 1975), was recently isolated, (Knight and Klee, 1978). This endopeptidase may degrade the endogenous opioid substance released by embryonic chick brain preparations; accounting for the inability of supernatants, that presumably contain the endogenous opioid substance, to significantly reduce the binding of etorphine, table 11.

Additional evidence, figures 32-35 and tables 6 and 13, that suggests the existence of an endogenous opioid substance in mature and embryonic chick brain is consistent with the results reported by Pasternak et al., (1975b).

The binding enhancement observed while investigating the sensitivity of the embryonic chick brain binding capacity to the assay buffer concentration, table 6, suggested that the washing procedure was "unmasking" some additional binding capacity that was not present before the embryonic suspension was washed. Allowing adult chick brain suspensions, to stand at 25 degrees, figure 32, indicates not only that they are more stable than embryonic preparations, figure 31, but that there

is an increase in the binding capacity of preparations made from mature brain tissue. This result implies that an unmasking of additional receptor sites arises from the removal of some endogenous opioid substance during the incubation.

The results in table 13 suggest that very low levels of trypsin can increase the binding capacity of chicken brain suspensions by unmasking receptor sites. Presumably this occurs when an endogenous opioid component is degraded. The data in table 13, resembles profiles described for adult and embryonic chick brain, figures 29-30, table 12, and it is consistent with the interpretation of these data.

While the endogenous opioid substance present in chicken brain preparations was not isolated and shown to be one or both of the enkephalins, as was done with the rat, (Simantov et al., 1976b), and guinea pig, (Hughes, 1975), the ability of adult chicken brain preparations to stereospecifically bind methionine enkephalin saturably, and in a pharmacologically meaningful way was confirmed, figures 33-35 and table 9.

The IC-50 values for the binding of some of the opioid peptides to adult and embryonic chick brain preparations are similar to those reported by others, (Guillemin et al., 1976; Lazarus et al., 1976; Snyder and Simantov, 1977b), and in agreement with the observation that leucine enkephalin is slightly more agonistic than

methionine enkephalin, (Snyder and Simantov, 1977b). The loss in the distribution of methionine enkephalin observed after incubating the enkephalin for several minutes, with adult chick brain particulates, figure 33, is almost three times as rapid as that seen for the binding of naloxone to embryonic chick brain, figure 23. The very short period over which the maximal stereospecific binding of methionine enkephalin can be measured is consistent with the presence of an endopeptidase that specifically degrades exogenous enkephalin, (Knight and Klee, 1978), and is similar to observations made by others, (Simantov and Snyder, 1976c; Bayon et al., 1978).

The rapid degradation of synthetic enkephalin by extensively washed rat brain membranes, (Simantov and Snyder, 1976c), contrasts with the inability of both embryonic chick brain particulates figures 13-16, and table 8, and rat brain preparations, (Pert and Snyder, 1973c), to modify synthetic opiates.

The opiate receptor seems to be either a protein or group of proteins which are associated with membrane lipids. The opiate binding capacity of adult and embryonic chick brain preparations is degraded by proteolytic enzymes, table 10, denatured at high temperatures, figures 9-10, and is linear with respect to protein concentration, figure 17. The loss of the opiate binding capacity in rat brain preparations treated

with phospholipases and proteases, (Pasternak and Snyder, 1975d), correlates with the sensitivity of embryonic chick brain suspensions to proteolytic digestion, tables 10. The decrease in the binding capacity of rat brain homogenates treated with phospholipases, (Pasternak and Snyder, 1975d), agrees with the observation made with embryonic chick brain particulates treated with phospholipase C. The ability of certain acidic lipids to bind opiates stereospecifically, (Loh et al., 1974; Abood and Ross, 1975), the isolation of a lipophilic component with opiate-binding activity, (Lowney et al., 1973), and the drop in the opiate binding capacity of rat brain suspensions treated with detergents, (Simon et al., 1975b), are consistent with the hypothesis that proteins and lipids have a role in the bindingprocess.Several investigators, (Pasternak et al., 1975b ; Wilson et al., 1975; and Simon et al., 1975c), have shown that the binding of opiates to rat brain preparations are effected by protein modifying reagents, and Simon et al., (1975c), found that at least one sulfhydryl group participates in the opiate binding process.

The existence of multiple opioid binding sites that may be responsible for the diverse actions of opiates, (Snyder and Simantov, 1977b), and opioid peptides, (Snyder and Simantov, 1977b; Holaday et al., 1978; Gillin et al., 1978), and that may interact with one or several of the

opioid peptides present in various brain regions, (Lewis et al., 1978; Bloom et al., 1978), is consistent with our data. Evidence for at least two classes of opiate binding sites in embryonic chick brain preparations, is presented in figures 6, 17-18, 27, 40-42, and tables 5 and 17.

Varying the assay temperature, figure 6, reveals three peaks in the binding capacity of embryonic chick brain homogenate; suggesting the existence of several types of receptors. The peaks at the lower temperatures may arise from thermally induced transitions in the lipid bilayer, (McConnell et al., 1972), that alter the binding activity of the receptor observed at 39 degrees. However, the dissociation of bound naloxone from embryonic chick brain preparations at 9 degrees, figure 27, implies that lower temperatures may unmask additional sites, table 5, that behave differently from those observed at higher temperatures. Scatchard analyses of the effect of salts on the binding capacity of embryonic chick brain particulates, figure 41-43 and tables 5 and 17, support the existence of at least two types of binding sites, (Klotz and Hunston 1971). Since several examples of "dual-state" neurotransmitter receptors exist, (Snyder and Simantov, 1977b), our data and the data of others, (Pert and Snyder, 1974a; Pasternak et al., 1975c), were interpreted to mean that one type of receptor exists in either a sodium form or

a sodium-free form. This interpretation does not preclude the existence of multiple "dual-state" opiate receptors. Furthermore, the bimodal distribution of the opiate binding capacity as a function of protein concentration in embryonic chick brain suspensions, figure 17, and the dual binding peaks observed at different values of assay pH, figure 18, suggest the existence of at least two types of receptors.

The existence of discrete neuronal pathways for at least two of the opioid peptides, (Bloom et al., 1978), the apparent differences in the synthetic routes leading to enkephalin in the pituitary and the brain, (Lewis et al., 1978; Liotta et al., 1978), as well as the diverse physiological effects characteristic of the opiate narcotics, (Holaday et al., 1978), implies the existence of several types of receptors that can interact with synthetic opiates and natural opioid substances to varying degrees. The difference in the agonist potency of leucine enkephalin versus methionine enkephalin, (Snyder and Simantov, 1977b), and the difference in the potency of morphine versus methionine enkephalin, (Lord et al., 1976; Simantov and Snyder, 1976e), is consistent with this hypothesis.

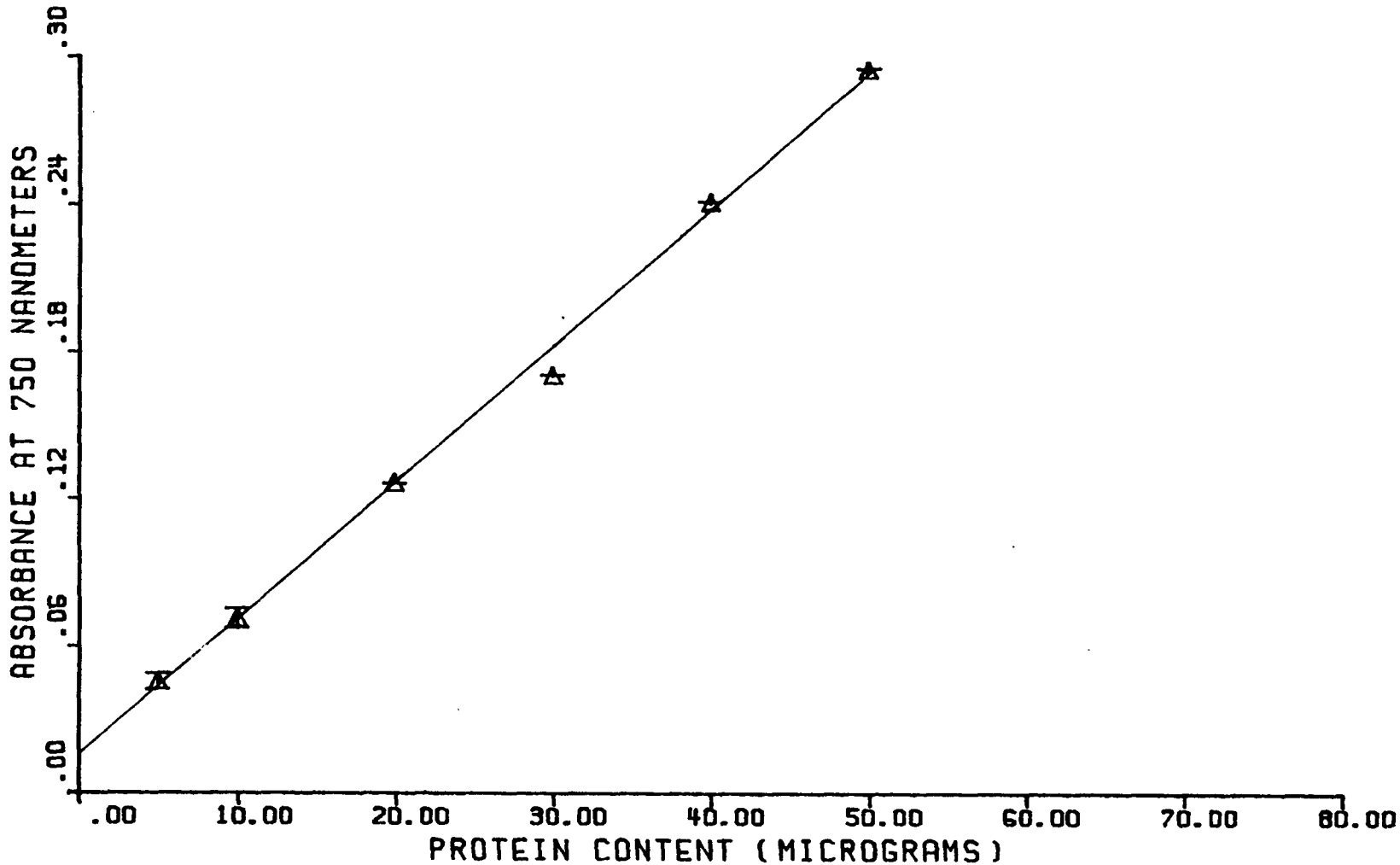
The remarkable similarity between many of the opiate binding parameters of embryonic chick brain and adult mammals confirm the view of Peterson et al., (1974a), that the chick system is a

reasonable system for evaluating opiate receptor binding. Our results and the correlation of these results with the effects of opiates on adult tissues not only confirms this opinion, but successfully answers the question, "How does the opiate receptor of embryonic chick compare with the opiate receptors studied in adult mammals?"

One need look only at the infants born to opiate addicted women to realize that while manipulating the present, we are really shaping the future.

APPENDIX I:

Figure 1: A typical standard protein content curve used to determine the protein content of the assayed brain homogenates and washed brain particulates. Bovine Serum Albumin, fraction V, (0-50 ug) was used as the standard, each time the protein content was determined by the procedure of Lowry, (Methods, section E).


A TYPICAL LOWRY STANDARD CURVE
ABSORBANCE AT 750 NANOMETERS
.00
.06
.12
.18
.24
.30
.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
PROTEIN CONTENT (MICROGRAMS)

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