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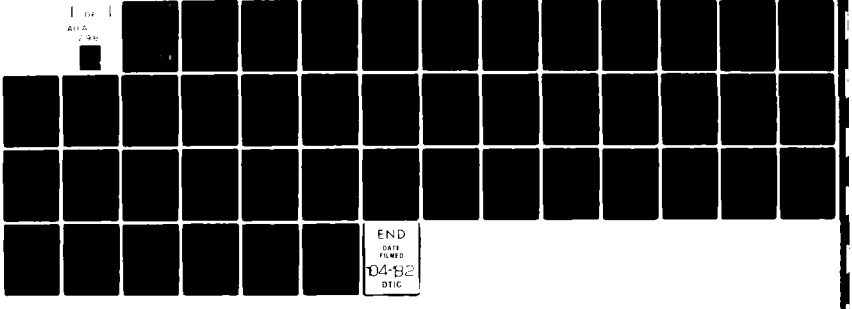

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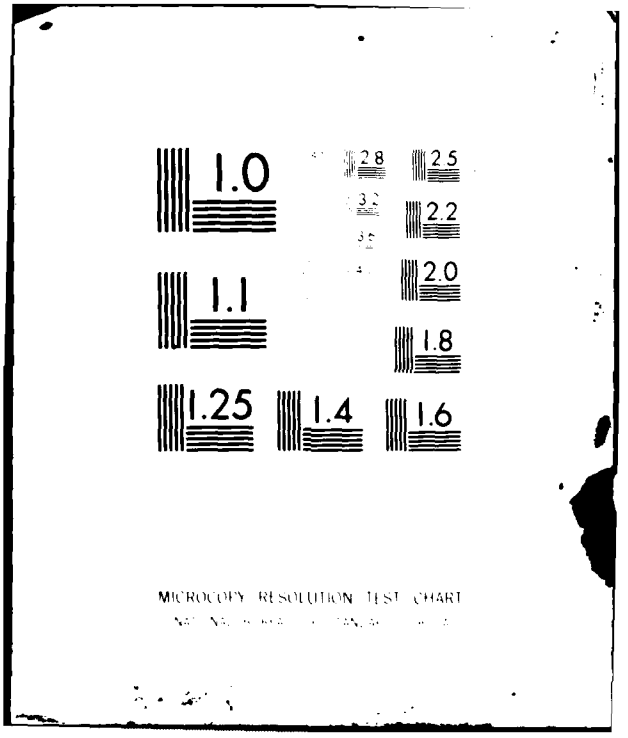
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NARCOTIC TOLERANCE AND DEPENDENCE MECHANISM: A NEUROLOGICAL CORRELATE

ANNUAL PROGRESS REPORT
(FOR THE PERIOD 1 SEPTEMBER 1974 - 31 AUGUST 1975)

BY

HORACE H. LOH, Ph.D.

SEPTEMBER 1975

SUPPORTED BY

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK, FREDERICK, MARYLAND 21701

CONTRACT NO. DADA 17-73-C-3006

UNIVERSITY OF CALIFORNIA SCHOOL OF MEDICINE

SAN FRANCISCO, CALIFORNIA 94143

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
We have examined a number of factors which may be involved in both the acute and chronic actions of morphine and related narcotics. Special emphasis was placed on the role(s) of membrane lipids and proteins. The results clearly demonstrate that both acute and chronic morphine treatments have marked effects (both inhibitory and stimulatory) on brain protein and phospholipid turnover depending on the brain region examined. Unfortunately, the effects of morphine on protein and phospholipid synthesis do not seem in any reasonable way to be associated with the known localization of

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narcotic receptors. The possibility was tested that chronic morphine treatment may lead to miscoding in the formation of m-RNA and in this way produce a qualitative change in protein synthesis. Such changes were not observed. Chronic morphine treatment was, however, found to significantly increase brain chromatin template activity.

Extensive investigations of the opiate receptor material were also performed. We were unable to find any change in the affinity or capacity of specific (^{3}H) naloxone binding to brain membranes that could be specifically associated with the development of dependence and the increased sensitivity to naloxone-precipitated withdrawal. This dilemna led us to both question the validity of the opiate tissue binding assay and to search for membrane components that might serve as suitable receptors or receptor "prosthetic groups." Our attention was drawn to cerebroside sulfate since this acidic lipid was found to be the opiate "receptor" isolated by Goldstein et al (1971). We found that in terms of relative potency and stereospecificity, cerebroside sulfate (CS) binds to narcotics in a manner that would be expected of receptor material. However, at this time, we cannot conclude that CS is the receptor. We have attempted to identify and purify the membrane bound opiate receptor. Our results suggest that the receptor is localized on the external surface of the membrane and that the receptor complex is at least partially protein, probably glycoprotein. Overall, we have examined several distinct but interrelated parameters of the mechanisms involved in the acute and chronic effects of narcotics. Potentially significant results were obtained in the areas of how morphine affects membrane synthesis and turnover and the nature of the opiate receptor.

FOREWORD

Citation of commercial organizations or trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

TABLE OF CONTENTS

A. Title of Contract: "Narcotic Tolerance and Dependence Mechanism: A Neurochemical Correlate"
DADA-17-73-C-3006

B. Period: Third Annual Progress Report (APR-3)
Covers work completed during the period
September 1, 1974 -- August 31, 1975

C. Forward:

This is the third annual progress report to be submitted under the provisions of Army supported Contract No. DADA-17-73-C-3006, "Narcotic Tolerance and Dependence Mechanism: A Neurochemical Correlate."

As described in previous quarterly reports in the past year (i.e., eighth, ninth and tenth quarterly reports), we have obtained evidence showing that a membrane sphingolipid, cerebroside sulfate (CS), is related to narcotic stereospecific binding site(s) in the central nervous system. To further support these findings, we have focused our main effort on the following areas:

1) Obtaining further data to support the theory that cerebroside sulfate may, indeed, be related to the opiate receptor on the central nervous system.

2) Studying in vivo the biological role of cerebroside sulfate in opiate action.

3) Determining that membrane acidic lipids other than cerebroside sulfate may be involved in opiate binding.

4) Using cerebroside sulfate-opiate binding as an in vitro model, to understand the mechanism of opiate receptor interaction at the molecular level.

Even though it was not reported in the past three quarterly reports, other experiments which are related to the proposed work were also performed. For

example, we have studied 1) the role of cerebral protein synthesis in morphine tolerance development; 2) the effect of acute and chronic morphine treatment on nerve membrane phospholipid turnover; and 3) the localization of opiate receptors on the nerve membrane.

This work will be briefly summarized in the following sections and the detail described in the enclosed **Appendices.**

D. Summary of Experimental Work:

1. Cerebral protein synthesis: Based on indirect evidence obtained in our laboratories (*1-4*) as well as by others (*5-8*), it has been concluded that development of tolerance to and physical dependence on narcotics is related to cerebral protein synthesis. In our studies of morphine effects on protein synthesis in mouse brain, we have found that mice tolerant to morphine have a higher rate of protein synthesis. The mechanism of this phenomenon in unknown. Our studies, in an attempt to understand this mechanism, have shown that morphine binds to brain free polysomes both in vitro and in vivo. Furthermore, the stabilizing effect of morphine prevents them from breaking down. These results suggested that these effects of morphine on brain free polysomes may be related to the mechanism of morphine-increased protein synthesis in tolerant mice. Furthermore, the mechanism of morphine stabilization of brain polysomes is, at least partially, related to its inhibitory action on RNase, the enzyme responsible for the breakdown of m-RNA. The possibility that morphine affects polysome's integrity via the drug's action on Mg++ concentration was also tested, since brain polysomes are very sensitve to low Mg++ concentrations. These studies demonstrated that both acute and chronic morphine treatment did not alter brain concentration of Mg++ (for details, see Res. Comm. Chem. Path. Pharmacol., 1975, **Appendix D).**

The effect of acute and chronic morphine treatment on the synthesis and turnover of ^{3}H-leucine labeled protein and ^{14}C-choline labeled phosphatidylcholine also was studied in discrete regions of the rat brain. Our results showed that chronic morphine treatment increased the ^{3}H-protein turnover in the microsomal fractions. However, acute morphine treatment increased ^{14}C-phosphatidylcholine synthesis in the hypothalamus (95%) and diencephalin (285%) and ^{3}H-protein synthesis in the hypothalamus (55%) and caudate nucleus (285%) (Biochem. Pharmacol. 23:1753-1765, 1974; Appendix E).

As stated in the "Specific Aims" of our original proposal, the effect of morphine on "individual proteins which play an important role in regulation of brain functions" will be studied. In this regard, we have examined in detail the effect of acute and chronic treatment of morphine on adenyl cyclase, tyrosine hydroxylase, and tryptophan hydroxylase. High doses of morphine stimulated the activity of adenyl cyclase but did not show any direct action on the other two enzymes. However, the action of morphine on the enzyme may not be causally related to narcotic addiction.

Another objective of our original proposal was to test any possible "qualitative" changes in protein synthesis due to "miscoding" of m-RNA after morphine-polysome interaction. Experiments have been carried out to test this intriguing and logical hypothesis. These studies were done in collaboration with Dr. James Meyerhoff and his associates in the Department of Neurochemistry, Division of Neuropsychiatry, WRAIR (Director: Col. Harry Holloway). During the past 18 months, a tremendous effort was devoted to this problem from both laboratories. Experimental results, though negative, did show conclusively that narcotic drugs (at

least morphine) did not cause "qualitative changes" in brain protein synthesis. The results of these collaborating efforts are summarized in Appendix F which is the draft of a paper which will be submitted to the Journal of Neurochemistry for publication.

2. Regulatory mechanism of protein synthesis: Some aspects of the regulatory mechanism on protein synthesis which is altered by chronic morphine treatment (i.e., tolerance-addiction) have been considered and investigated. In this regard, we have studied one aspect, that is, the possible implications of some expression in relation to narcotic tolerance development. Other investigators have reported that synthesis of RNA in E. coli is inhibited in the presence of levorphanol, a morphine analog. The synthesis of RNA in HeLa cells is also inhibited in the presence of levorphanol and levallorphan. Becke et al have shown that the most intense and consistent inhibitory effect of levorphanol is upon ribosomal RNA synthesis, although the non-ribosomal RNA is also inhibited. On the other hand, recent reports have shown that the inhibition of ribonucleic acid synthesis resulted in antagonizing the development of morphine-induced analgesic tolerance. The authors concluded that the development of tolerance to the analgesic effect of morphine in animals can be reduced or prevented by several drugs which, in different ways, inhibit the synthesis of RNA and/or protein in the brain.

In attempts to study RNA metabolism in chronic morphine treated animals, Datta and Antopol reported that the chronic administration of morphine produced dose-dependent decreases in uridine and thymidine incorporating abilities of liver and brain homogenates and of their subcellular fractions, such as nuclei, mitochondria and microsomes.

In our studies of DNA dependent RNA biosynthesis with purified chromatins, we found that when mice are rendered tolerant to morphine by pellet implantation, the rate of chromatin-dependent UTP incorporation is increased. The increase is not due to the presence of more DNA, since the specific activity is calculated per mg DNA. Omission of chromatin from RNA polymerase resulted in negligible incorporation. Removal of histone from chromatin did not change the fact that the tolerant group still showed higher activity than the placebo group. Chromatography of the histones proteins isolated from both control and tolerant mice showed no different in either quantity or quality. However, after the acidic proteins were extracted, the chromatin isolated from tolerant animals showed less template activity than that from the placebo group. This indicates that the acidic protein may play an important role in regulating chromatin-template activity and that they may have been altered during tolerance development. In in vivo studies, this morphine effect may be blocked with naloxone pellet. Naloxone itself produces no significant increase in chromatin activity. This indicates that the morphine-induced change in chromatin activity is a "narcotic specific" action. The first paper in this series will appear in Biochemical Pharmacolog-, October issue, 1975 (Appendix G).

Further studies indicate that chronic morphine treatment resulted in an increase of phosphorylation of acidic protein in nuclei and this is not due to a decrease in phosphoprotein phosphotase activity but may be related to an increase in nuclei protein kinase activity (see Appendix H).

3. Endogenous membrane binding sites (receptor ?) and model system for the mechanism of opiate-receptor interactions: Major advances and contributions in fundamental narcotic research have been the identification and isolation of opiate receptor from CNS by Snyder, Simon, Terenius, Goldstein and their associates. Using methods developed by these investigators to study opiate-receptor binding, we have studied the following: (1) The possible changes in quality and/or in quantity of opiate receptors after animal addiction to opiates; (2) Location of the opiate receptor - Is it located inside or outside of the synaptic plasma membrane? (3) The role of membrane constituents in opiate receptor interactions.

Some progress has been made with regard to questions 1 and 2 and the data has been published in two separate papers: (a) Life Sciences 14:2393 1974; (b) Life Sciences 16:1809, 1975.

However, our main effort has been centered on the third problem. We have attempted to search for endogenous binding site(s) of opiate receptor ?). Our results demonstrated that several acidic lipids which are normal membrane constituents, bind to opiates stereospecifically. Among the acidic lipids examined, we found that cerebroside sulfate (CS) binds etorphine and naloxone stereospecifically with the highest affinity. The relative potency of 25 narcotics studied in preventing the binding of etorphine and naloxone to cerebroside sulfate correlated well with their reported analgetic activity. The data indicate similarities between cerebroside sulfate and a purified opiate receptor from mouse brain which has been reported to be a proteolipid by Dr. A. Goldstein's group from Stanford University. Explanations for the apparent proteo-like behavior of the opiate receptor are provided. Together with other chemical

analysis, we have conclusively shown that the opiate receptor isolated by Goldstein et al (1971) is indeed cerebroside sulfate. The work, which was solely supported by this contract (DADA 17-73-C-3006) has been published and confirmed by other groups (Life Sciences 14:2231, 1974). Much progress has been made and a mechanism of opiate action has also been proposed based on this work. This is described in detail in Appendices I, J and K.

Even though cerebroside sulfate binding to opiates fulfill most of the criteria used to identify opiate receptors in the CNS, indirect evidence (as described in Appendices I, J and K) also strongly suggest the involvement of cerebroside sulfate in opiate analgetic action, in vivo. However, it is not possible to prove nor is it our intention to claim that CS is "the opiate receptor" --- even though other investigators have suggested that CS may be a part of the opiate receptor which may function as a "prosthetic group" in the receptor (Snyder, S.H., ed., Opiate Receptor Mechanism - 10).

4. Localization of opiate binding site on nerve membrane:

In the past two years, several laboratories have demonstrated the existence of a high affinity, stereospecific and apparently saturable narcotic binding material in brain tissue (Pert and Snyder, 1973; Simon et al, 1973; Terenius, 1973). A number of the binding materials' characteristics have been determined. The material is principally proteinaceous in nature; binding is sensitive to sulfhydral reagents but is insensitive to DNAase, RNAase and neuraminidase (Pasternak and Snyder, 1974; Simon et al, 1975). Binding is markedly inhibited by phospholipilase A treatment but it is not clear if this effect is specifically

related to the loss of the fatty acid or to membrane disorganization (Heilbronn and Cedergren, 1971; Pasternak and Snyder, 1974). The binding of agonists but not antagonists is inhibited by Na+ (Pert et al, 1974; Simon et al, 1975). The synaptic plasma membrane has the highest subcellular density of narcotic binding material (Terenius, 1973; Hitzemann et al, 1974; Pert et al, 1974). These data are consistent with the idea that morphine acts by altering synaptic function (Paton, 1957; Domino and Wilson, 1972; Kosterlitz et al, 1973; Kosterlitz and Waterfield, 1975).

Despite the extensive characterization of the binding material, there have been no studies designed to determine if the binding material is located primarily on the external or internal (cytoplasmic) surface of the nerve membrane.

In a previous study (Hitzemann et al, 1975), it was determined that intact nerve ending particles (NEP) could be subjected to mild tryptic digestion without changing NEP morphology, presumably the trypsin attacks only those proteins which are solely or partially located on the external surface of the NEP. Since narcotic binding is markedly sensitive to proteolytic enzymes, it should be possible to determine if narcotic binding material is located on the external or internal surface of the synaptic plasma membrane (SPM) by incubating intact and lysed NEP with trypsin and then determining binding capability. Briefly, the data obtained from such experiments indicate the following. (1) The narcotic binding material is located on the external surface of the membrane. (2) The accessibility of trypsin to the binding material is blocked by Na+ (>50 mM), suggesting that Na+ alters the membrane confirmation. (3) On the basis of the disc gel electrophoresis studies, approximately 40

percent of the membrane protein is lost before a decrease in binding activity is observed. (4) Some evidence suggests the binding material is a glycoprotein and perhaps a sulfated glycoprotein. (5) The receptor material has a high molecular weight (<100,000). This is in agreement with the recent report of Simon et al (1975) who have solubilized receptor material with a molecular weight of approximately 300,000. (6) Interestingly, the binding material has approximately the same molecular weight as the glycoprotein whose synthesis is increased during tolerance development.

5. Morphine and nerve membrane phospholipid turnover: In our previous work (Hitzemann and Loh, 1974, 1975), we have suggested that narcotic tolerance and dependence development are associated with the enhanced synthesis of some synaptic plasma membrane (SPM) proteins. Interestingly, the increased protein synthesis was observed in SPM derived from a GABA enriched population of subcortical nerve ending particles (NEP) and not from a population of NEP enriched in NE. This observation on specificity of effect complimented the work of Ho et al (1974) who demonstrated that the GABA system plays an important role in tolerance and dependence development. We decided to examine the possibility that narcotic tolerance-dependence development is also associated with increased phospholipid synthesis in the SPM-GABA but not the SPM-NE. Rats were implanted with either 2 morphine or 2 placebo pellets 24 hr prior to the administration of 100 uCi of ^{32}Pi and 20 uCi of ^{3}H-choline. The animals were sacrificed 1, 3 and 24 hr after isotope injection. SPM were prepared from the subcortex and the specific activities of the SPM phospholipids were determined. The data indicated that tolerance development either inhibited

or had no effect on the incorporation of ^{32}P or ^{3}H-choline into both SPM-GABA and SPM-NE phospholipids. There are several possible explanations for these data. (A) Changes in SPM phospholipid synthesis are unrelated to tolerance and dependence development. (B) Changes in SPM phospholipid synthesis that are related to tolerance development may be occurring in SPM associated with small regions of the brain and thus were not measured in the present study. (C) Rather than tolerance development being associated with enhanced phospholipid synthesis, perhaps tolerance develops to an acute narcotic effect on phospholipid synthesis. Based on the work of Mule (1966, 1967, 1970), this explanation already has a considerable amount of experimental verification.

As an extension of the previous work of Mule (1966, 1967, 1970), we examined the effect of acute and chronic morphine treatment on the incorporation of ^{32}Pi and ^{3}H-glycerol into microsomal and NEP phospholipids from 4 subcortical brain regions; the neustriatum, brainstem, midbrain and diencephalon. Briefly, the data indicate that acute morphine treatment markedly stimulates microsomal and NEP phospholipid synthesis in the midbrain and to a lesser degree in the brainstem. The neostriatum and the diencephalon were relatively unaffected. In the midbrain the synthesis of all phospholipids was stimulated but the acidic phospholipids (phosphatidylserine and phosphatidylinositol) showed the greatest effect (>300% increase). As in the SPM studies, phospholipid synthesis in the midbrain was either decreased or showed no change in the chronically morphinized group. This data indicates that habituation to the acute drug effect has developed. Presently, we are conducting experiments to determine whether or not the administration of large doses of morphine to the tolerant animals will

reintroduce the acute drug effect. At this time, no results from these experiments are available.

Since morphine stimulates the incorporation of both ^{3}H-glycerol and ^{32}Pi into all phospholipids, the narcotic effect is not similar to the non-specific stimulation of phosphatidic acid and phosphatidylinositol synthesis observed after the introduction of many weak bases, e.g., amphetamine, chlorpromazine, acetylcholine. The narcotic effect is somewhat similar to but much larger than the effect of NE or dopamine on phospholipid synthesis in vitro (Abdel-Latif et al, 1974).

In summary, the data indicate that associated with the acute administration of morphine is a marked increase in the turnover of membrane phospholipids which occurs primarily in the midbrain, the presumed locus of morphines nociceptive effect. Habituation (and perhaps tolerance) develops to the acute morphine effect. In our opinion, these data provide the most dramatic example of how narcotics can alter the synthesis of membrane constituents.

E. Personnel Involved in these Studies:

1. Dr. Horace H. Loh, Principal Investigator. No salary obtained from this contract.

2. Dr. Robert J. Hizemann, 1/3 time. No salary obtained from this contract.

3. The following persons are supported full time or part time by this contract:

   a) T. M. Cho, M.S.
   b) J. S. Cho, M.S.
   c) Nancy Lee, Ph.D.
   d) David Brase, Ph.D.

F. Publications Supported by this Contract;

1. Hitzemann, R.J. and Loh, H.H.: Characteristics of the binding of ^{3}H-naloxone in the mouse brain. Soc. for Neuroscience, Nov. 7-10, San Diego, Calif., p. 350, 1973.

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G. References:

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H. APPENDICES

Appendix A -- Ninth Quarterly Report, 10 December 1974 (Contract No. DADA17-73-C-3006)

Appendix B -- Tenth Quarterly Report, 15 March 1975 (Contract No. DADA17-73-C-3006)

Appendix C -- Eleventh Quarterly Report, 15 June 1975 (Contract No. DADA17-73-C-3006)

Appendix D -- Stabilization of Brain Free Polysomes by Morphine

Appendix E -- Effect of Morphine on the Turnover and Synthesis of (Leu-^{3}H)-Protein and (Ch-^{14}C)-Phosphatidylcholine in Discrete Regions of the Rat Brain

Appendix F--The Effect of Morphine Tolerance and Dependence on Cell Free Protein Synthesis

Appendix G -- The Effect of Chronic Morphine on Brain Chromatin Template Activities in Mice

Appendix H -- Apparent Protein Kinase Activity in Oligodendroglial Chromatin after Chronic Morphine Treatment

Appendix I -- Stereospecific Binding of Narcotics to Brain Cerebrosides

Appendix J -- Opiate Binding to Cerebroside Sulfate: A Model System for Opiate-Receptor Interaction

Appendix K — A Model System for Opiate Receptor Interaction

APPENDIX A

NINTH QUARTERLY REPORT ON U.S. ARMY RESEARCH CONTRACT

"Narcotic Tolerance and Dependence Mechanism: A Neurochemical Correlate"

Contract Number:	DADA-17-73-C-3006
Name of Contractor:	University of California, San Francisco
Principal Investigator:	Horace H. Loh, Ph.D.
Telephone Number:	415-666-1951; 415-666-1826
Date of Report:	December 10, 1974

During the past contractual year of support by DADA-17-73-C-3006, we have spent considerable effort in the search for endogenous narcotic binding sites in the brain (receptor ?). As a result of these studies, we have obtained conclusive evidence showing that a membrane sphingolipid, cerebroside sulfate, may be related to narcotic binding in the brain.

Cerebrosides were shown to bind etorphine and naloxone stereospecifically with high affinity. The relative potency of several narcotic analgesics in preventing the binding of etorphine and naloxone to cerebrosides correlated well with their reported intraventricular analgetic activity. The data indicate similarities between cerebroside sulfate and a purified opiate receptor from mouse brain which has been reported to be a proteolipid. Explanations for the apparent proteo-like behavior of the opiate receptor are provided, too.

Besides cerebroside sulfate (CS), other membrane acidic lipids have also been considered and their binding to narcotic agonists as well as antagonists have been tested (Figures 1 and 2). In these studies, we not only observed the binding of agonists or antagonists to acidic lipids differently, but also observed a difference in physical properties

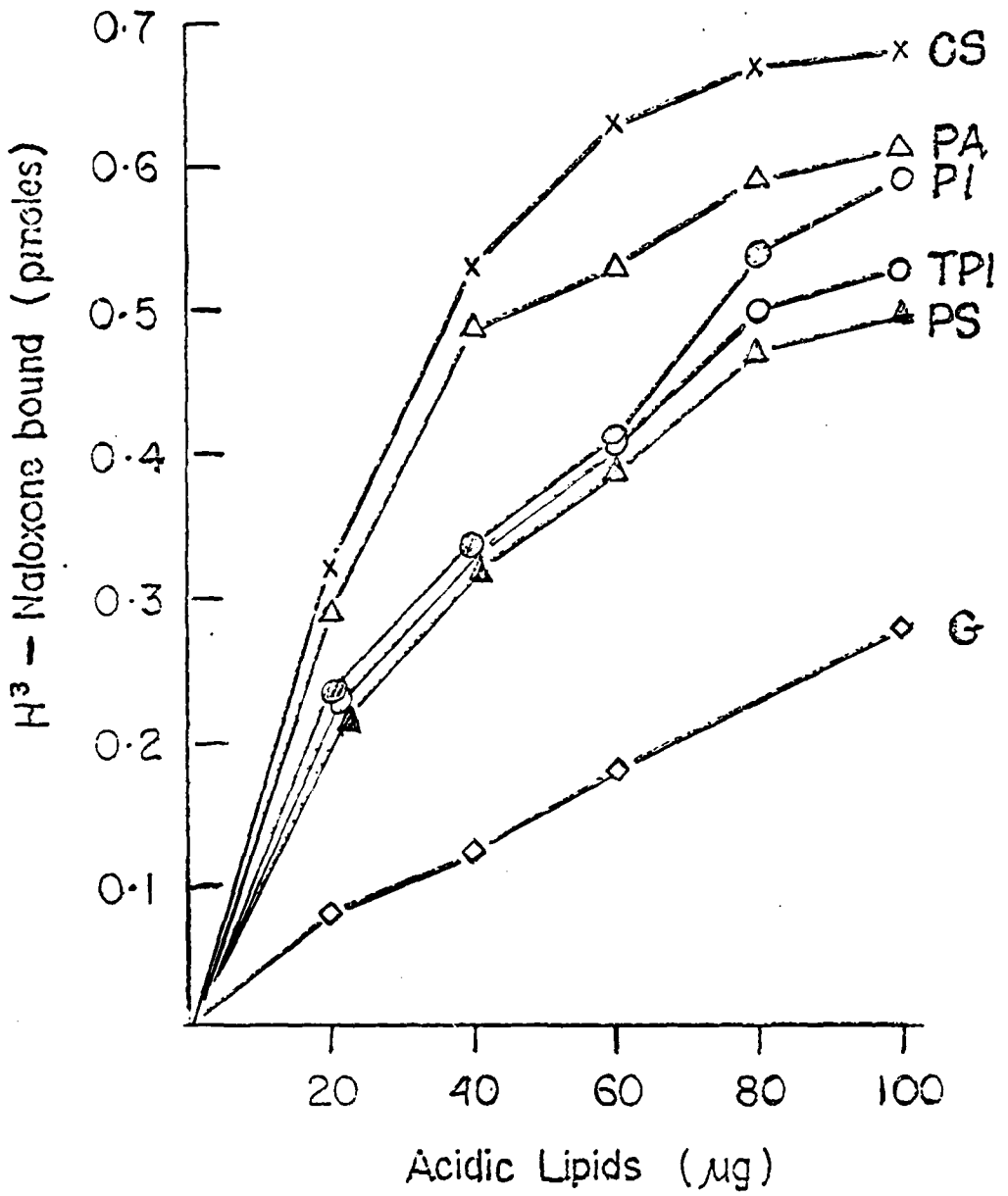
between acidic lipids -- agonist complex and acidic lipid-antagonist complex (Figures 3 and 4).

At present, we are employing this model system to test the properties of narcotic-receptor binding. Our hope is to understand the similarity (or difference) between agonist-receptor binding and antagonist-receptor binding.

Enclosed is an abstract of the paper which we will present at the forthcoming Federation Meetings in Atlantic City, New Jersey (April, 1975).

STEREOSPECIFIC BINDING OF NARCOTIC TO ACIDIC LIPIDS.
H.H. Loh, T.M. Cho* and Y.C. Ma*. Langley Porter Neuropsychiatric Institute and Dept. of Pharmacology, University of California, San Francisco, California 94143

In a previous paper, we have reported that cerebroside sulfate (CS) binds to etorphine and naloxone stereospecifically with high affinity. The relative potency of several narcotic analgesics in preventing the binding of etorphine to CS correlated well with their analgetic activity. Our previous data also indicated similarities between CS and a purified opiate receptor from mouse brain which has been reported to be a proteolipid (Life Sciences 14:2231, 1974). In studies with an octanol-water system, we have shown that narcotics bind to other acidic lipids stereospecifically and with high affinity. In the octanol phase, the binding of acidic lipids to morphine is higher than naloxone. In the water phase, the binding to naloxone is higher. Sodium inhibits the binding of narcotic to acidic lipids in the octanol phase. However, it enhances the binding of naloxone in the water phase. The significance of hydration of acidic lipids which may relate to narcotic binding will be discussed.
(Supported by U.S. Army Research and Development Command under Contract #DADA 17-73-C-3006).



ABBREVIATIONS:

CS:	Cerebroside sulfate
PA:	Phosphatidic acid
PI:	Phosphatidylinositol
TPI:	Triphosphoinositide
PS:	Phosphatidylserine
G:	Ganglioside

FIG. 1

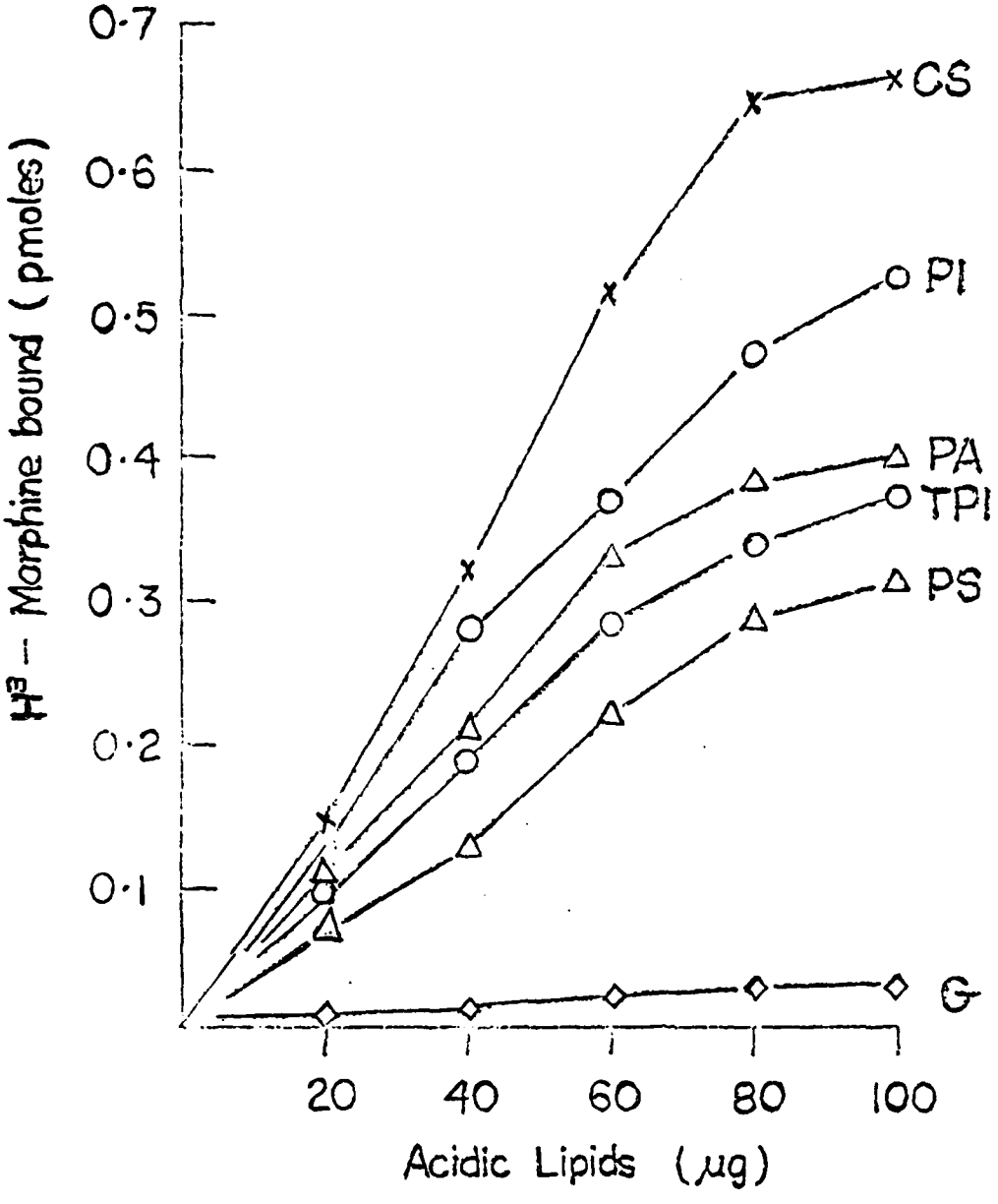


FIG. 2

ABBREVIATIONS:
CS: Cerebroside sulfate
PA: Phosphatidic acid
PI: Phosphatidylinositol
TPI: Triphosphoinositide
PS: Phosphatidylserine
G: Ganglioside

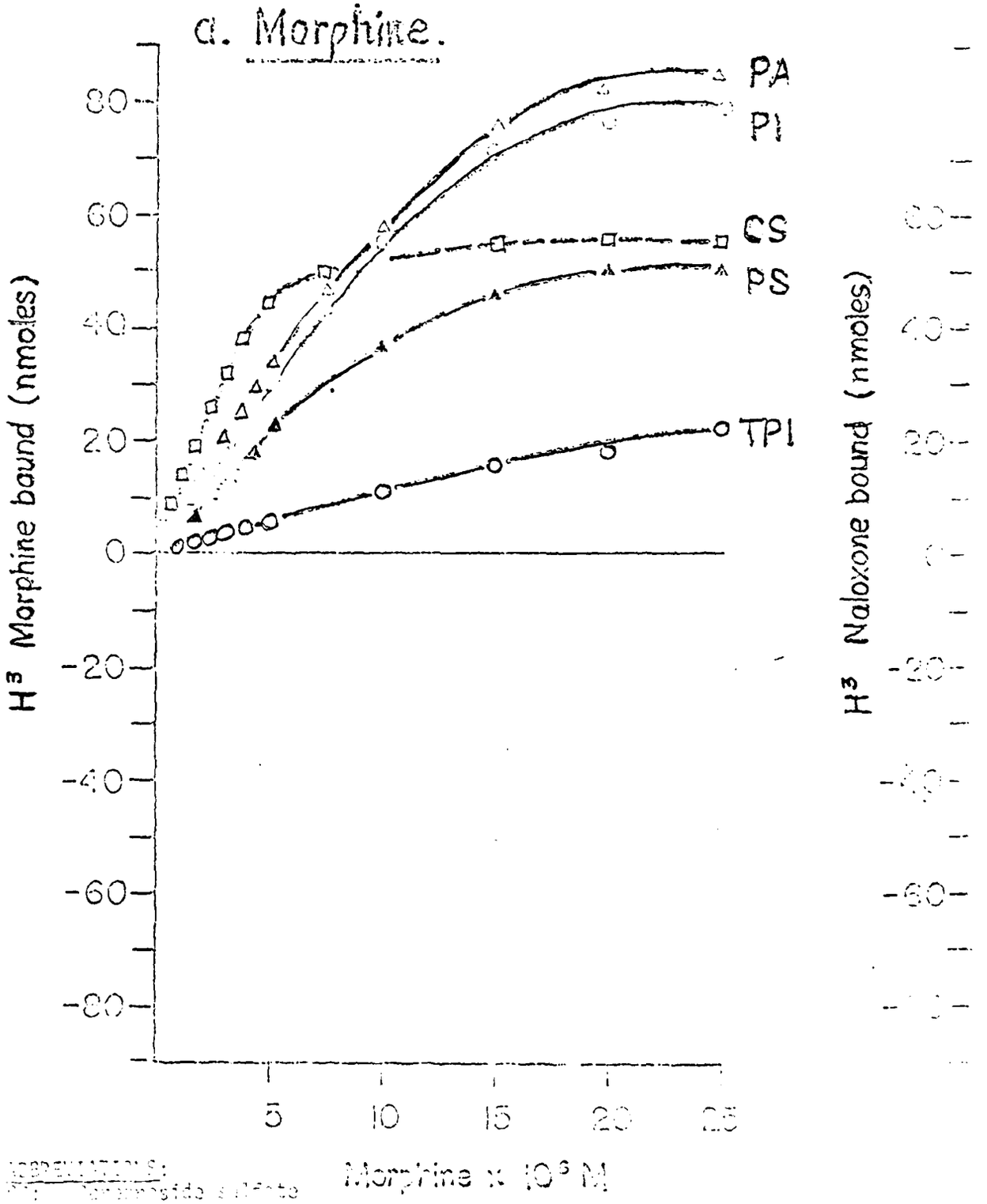

a. Morphine.
PA
PI
CS
PS
TPI
H³ Morphine bound (nmoles)
H³ Naloxone bound (nmoles)
80
60
40
20
0
-20
-40
-60
-80
5
10
15
20
25
Morphine x 10⁻⁸ M

FIG. 3

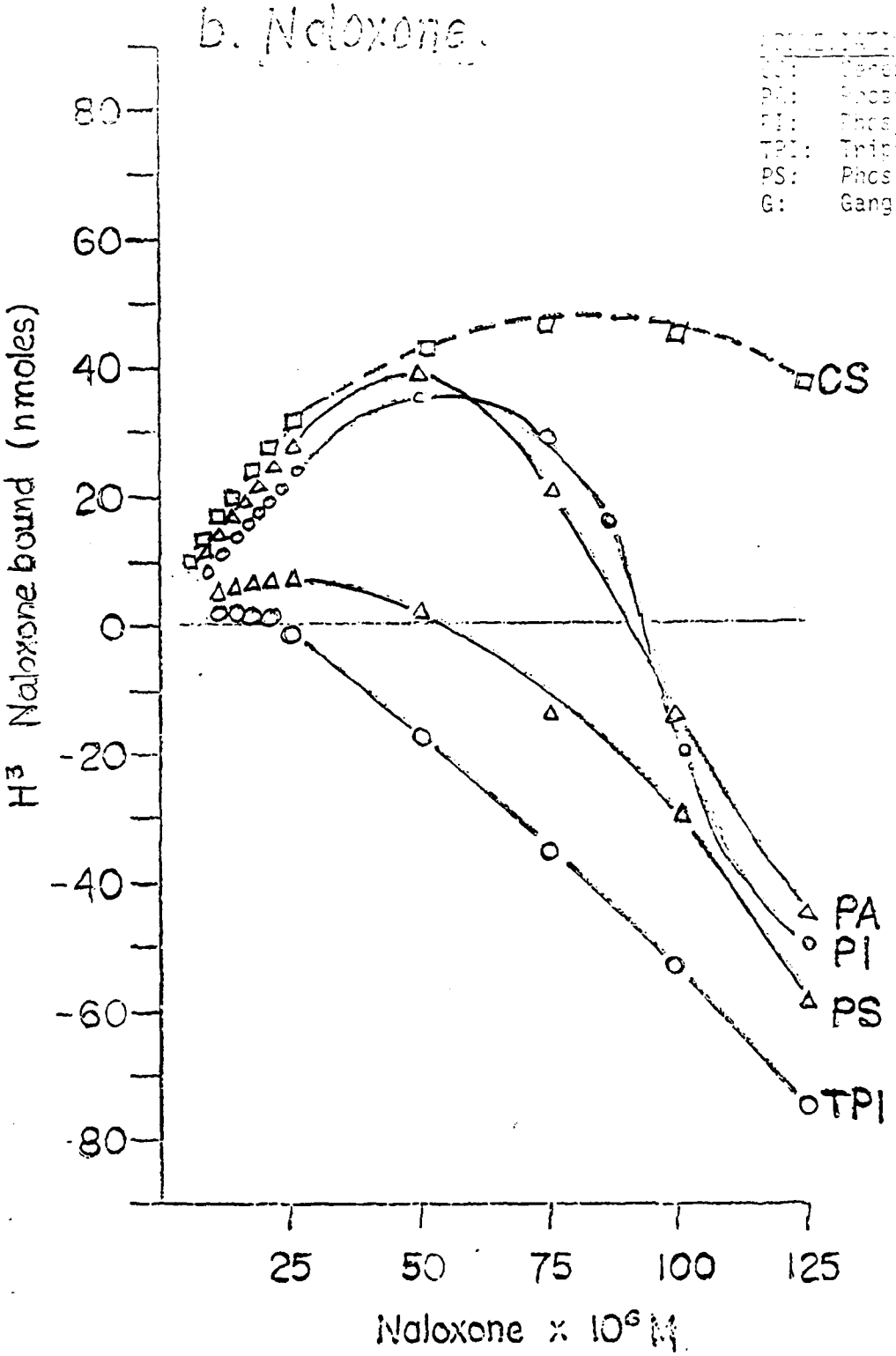

b. Naloxone.
CS: Chondroitin sulfate
PA: Phosphatidic acid
PI: Phosphatidylinositol
TPI: Triphosphoinositide
PS: Phosphatidylserine
G: Ganglioside
80
60
40
20
0
-20
-40
-60
-80
H³ Naloxone bound (nmoles)
CS
PA
PI
PS
TPI
25
50
75
100
125
Naloxone x 10⁶ M

FIG. 4

APPENDIX B

TENTH QUARTERLY REPORT ON U.S. ARMY RESEARCH CONTRACT

"Narcotic Tolerance and Dependence Mechanism: A Neurochemical Correlate"

Contract Number:	DADA-17-73-C-3006
Name of Contractor:	Univ. of Calif., San Francisco
Principal Investigator:	Horace H. Loh, Ph.D.
Telephone Number:	(415) 666-1826 or 1951
Date of Report:	March 15, 1975

Since we have obtained conclusive evidence showing that a membrane sphingolipid, cerebroside sulfate (CS) is related to narcotic binding in the brain, our task is, first, to establish an in vitro model to study the molecular interaction of narcotic to its binding sites. Using CS as the binding substance to study its binding to opiate in an organic solvent (membrane) - buffer (cellular fluid) partition system seems to be a good model. Preliminary studies indicate that the binding of CS to various opiate in this system does indeed correlate with the analgetic potential. In order to test this in vitro system further, we have examined the binding of CS to a series of opiate homologs - i.e., the ketobemidones. A complete description of the results of these studies is given below.

Bindings of N-alkylnorketobemidone to Cerebroside Sulfate

J. S. Cho, T. M. Cho, H. H. Loh[1] and E. L. Way

Langley Porter Neuropsychiatric Institute
and
Department of Pharmacology
School of Medicine
University of California
San Francisco, California 94143

Footnote: This publication#....from the Department of Pharmacology

Introduction

Among the acidic lipids examined which bind stereospecifically with narcotic analgesics, cerebroside sulfate exhibited the highest affinity (1). Moreover, the concentration of various agonists required to inhibit radioactive opiate binding to cerebroside sulfate correlated with their reported analgetic activity (2). The chromatographic behavior of the cerebroside sulfate complex formed with the agonist, levorphanol, was shown to be virtually identical to that of the complex formed with a purified opiate receptor which was isolated from mouse brain and reported to be a proteolipid (3). Subsequenlty, data were obtained consistent with the interpretation that agonists can be discriminated from antagonists by the degree of hydration of the complex formed by interaction with cerebroside sulfate, i.e., narcotic agonist cerebroside sulfate complexes tend to be hydrophilic (4). In order to test further the correlation of narcotic binding to cerebroside sulfate with analgetic activity, a study of the interaction between cerebroside sulfate and a series of N-alkylnorketobemidones was initiated. The compounds of this series are of particular interest because the lower homologs (C_1-C_4) have only agonist effects, but the higher homologs (C_5-C_{10}) have both agonistic and antagonistic character (5).

Materials and Methods

The inhibition of H^3-levorphanol tartrate (Hoffmann-La Roche) binding to cerebroside sulfate (Supelco Incorporated.) by N-alkylnorketobemidones (generously donated by Dr. E. L. May) was determined

with increasing concentrations of homologs from methyl to decyl. The degree of inhibition is expressed as a percentage of the total H^3-levorphanol binding in the absence of each homolog. The organic solvent water partition method as described previously was used (1). The amount of drug bound to cerebroside sulfate in the organic phase and at the interface was calculated by the method of Weber et al (6).

Results

The alkylnorketobemidones were found to inhibit H^3-levorphanol binding to cerebroside sulfate to varying degrees in a manner that was concentration dependent and relatable to analgetic potency. The lower homologs of norketobemidone (C_1-C_5) produced parallel shifts of the log concentration -% inhibition curve along the log concentration axis (Fig. 1). However, with the higher homologs (C_6-C_{10}), there was an increasing tendency to deviate from parallelism with increasing length of the alkyl chain due to the fact that the inhibition seemed to plateau at high concentrations; the maximum inhibition obtainable was between 60 to 80 percent. The concentration that inhibits 50% H^3-levorphanol binding to cerebroside sulfate and N-ethylnorketobemidone, the least potent analgetic, was the weakest in preventing the binding. With the higher homologs (C_6-C_{10}), the degree of the inhibition of H^3-levorphanol binding decreased with increasing length of the carbon chain and such changes are also consistent with the ranking of their analgetic potency (5).

Discussion

The parallel shifts in the log concentration- % inhibition curve for the first members of the series (C_1-C_5) suggest that the

homologs interact with cerebroside sulfate in a similar manner. However, the plateauing in response with consequential in creasing tendency to deviate from parallelism with the higher homologs (C_6-C_{10}) indicates that there may be another type of interaction with cerebroside sulfate. The decrease in the maximum inhibition and an increase in antagonistic activity seen with increasing alkyl chain length may be explained in terms of existing theories relating to dehydrated and hydrated ion pair formation. It has been suggested that the agonistic action is dependent upon agonist-receptor interaction to form a dehydrated ion pair whereas antagonistic properties are dependent upon receptor binding to form a hydrated ion pair (4).

The extent of formation of a dehydrated ion pair for a given ionic concentration is greater, the smaller the size of the cation and the lower the dielectric constant of the solvent (7). Also, the bond strength of a dehydrated ion pair is stronger than that of a hydrated ion pair since the attraction (affinity) between positive and negative charge is inversely proportional to the dielectric constant of solvent used. With respect to the noralkylketobemidones, therefore, the binding of the smaller size cations of the lower homologs (C_1-C_5) would favor the formation of a dehydrated ion pair rather than a hydrated ion pair. However, as the length of carbon chain increases, the size of the cation would be larger and this would favor a decrease in the formation of the dehydrated ion pair and an increase in the hydrated type. Thus, the binding of the higher homologs (C_6-C_{10}) would tend to be more as hydrated than as the dehydrated ion pair. This interpretation provides

an explanation for the increasing deviation of the higher homologs (C_6-C_{10}) from parallelism and their lower efficacy. The fact that the binding of naloxone, a pure antagonist, to cerebroside sulfate is through the hydrated ion pair mechanism (4) and the fact that the higher homologs (C_6-C_{10}) also have a partial antagonistic activity (5) are compatible with such an interpretation.

It has been suggested that opiates could stabilize the resting state of an excitable membrane or decrease its rate of conformational change from the resting to the excited state by interacting with acidic lipids present to form a hydrophobic complex. The stabilization and the decreased rate of conformational change may relate to the analgetic action of the opiate (1) and also serve to explain the "efficacy" or "intrinsic activity" postulated by Stephenson (8) and by Ariens (9).

Acknowledgements

This investigation was supported by the U.S. Army Medical Research and Development Command under Contract No. DADA17-73-C-3006.

[1] Recipient of a National Institute of Mental Health Research Scientist Development Award, K2-DA-70554.

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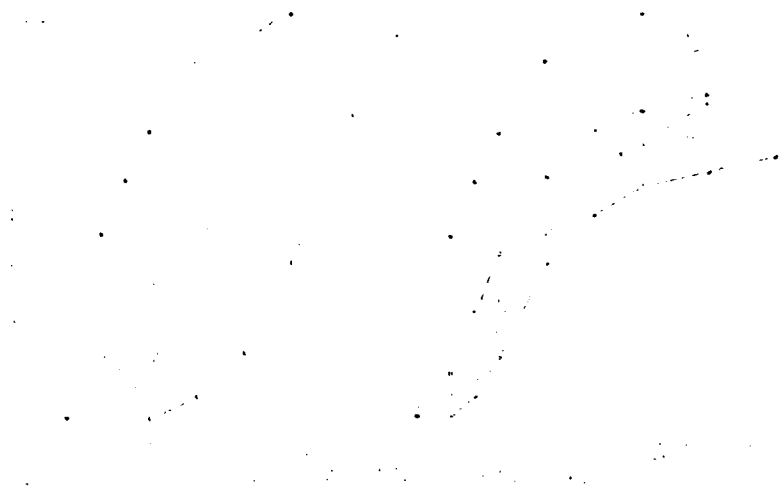
Figure 1. Log concentration-%inhibition Curves for N-alkylnorketobemidoses



The various concentration of N-alkylnorketobemidone that inhibits 1uM of H^3-levorphanol binding to 10 µg CS by 50% was determined in a manner as described in the method.

Table I. Relative potencies of N-alkylnorketobemidone derivatives in preventing H^3-levorphanol binding to cerebroside sulfate.

N-Derivatives	Hot plate ED50 (µmoles/kg)	ID50 (M)x10^{-6}
Methyl	2.82	1.1
Ethyl	67.25	12.0
Propyl	16.01	2.5
Butyl	4.59	1.6
Amyl	0.78	0.5
Hexyl	7.53	1.9
Heptyl	8.98	2.4
Octyl	26.50	2.8
Nonyl	inactive	3.8
Decyl	inactive	6.8

APPENDIX C

ELEVENTH QUARTERLY REPORT ON U.S. ARMY RESEARCH CONTRACT

"Narcotic Tolerance and Dependence Mechanism: A Neurochemical Correlate"

Contract Number:	DADA-17-73-C-3006
Name of Contractor:	University of California, San Francisco
Principal Investigator:	Horace H. Loh, Ph.D.
Telephone Number:	415-666-1826 or 415-666-1951
Date of Report:	June 15, 1975

As described previously (Eighth, Ninth and Tenth Quarterly Reports), we have obtained evidence showing that a membrane sphingolipid, cerebroside sulfate (CS), is related to narcotic stereospecific binding site(s) in the central nervous system. During the past several months of support from Contract #DADA-17-73-C-3006, we have focused our main effort on the following areas:

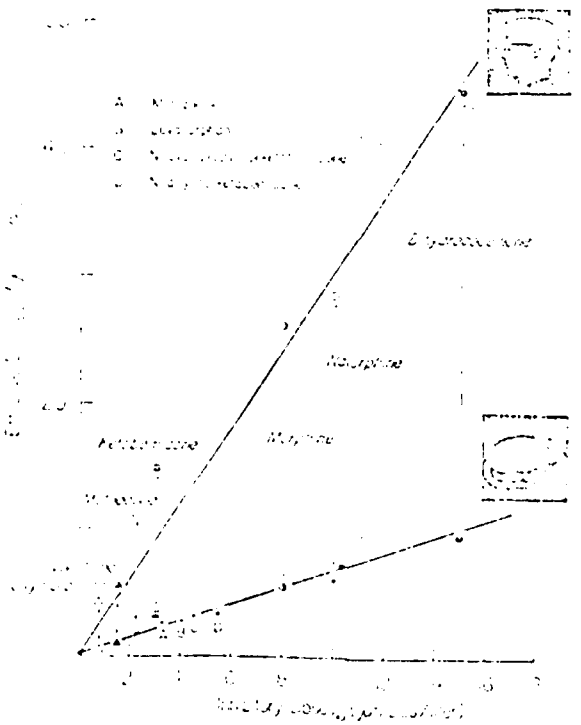
1) Obtaining further data to support the theory that cerebroside sulfate may, indeed, be related to the opiate receptor on the central nervous system.

2) Studying *in vivo* the biological role of cerebroside sulfate in opiate action.

3) Determining that membrane acidic lipids other than cerebroside sulfate may be involved in opiate receptor binding.

4) Using cerebroside sulfate-opiate binding as an *in vitro* model, to understand the mechanism of opiate receptor interaction at the molecular level.

In this report, I will describe some results with regard to the first point mentioned. In these studies, we have determined the affinities to cerebrosude sulfate of eleven narcotics with different chemical structures. We have found, as summarized in the following figure, that the affinities of these opiate bindings to cerebroside sulfate correlate very well with their pharmacologic potencies in both the mouse and man.



Correlation between the ID50 and analgetic activity (ED50). The ID50 is the concentration of the narcotic analgetics required to inhibit the binding of 5×10^{-8} M, H^3-levorphanol (3 Ci/mmole) with 4 µg of cerebroside sulfate by 50%. The analgetic activities (ED50's) of the drugs were taken from E.L. May et al and converted into umoles/kg.

The results indicate further that cerebroside sulfate may be related to the opiate receptor *in vivo*. I have incorporated this important data into one of our recent papers which will be published by *Life Sciences* in the July, 1975 issue. Enclosed is a preprint of this work.

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