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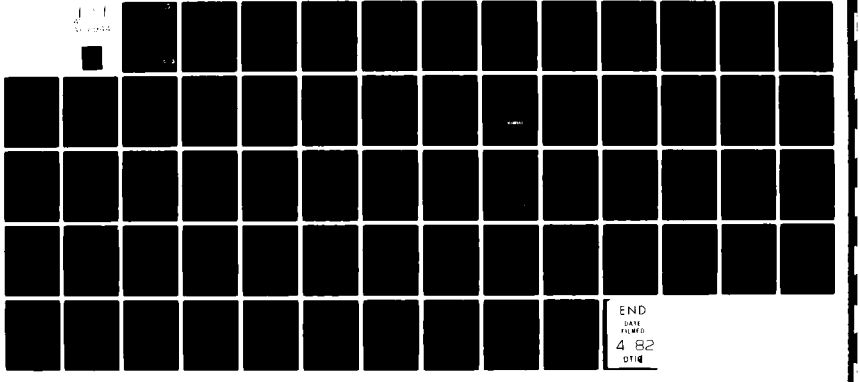

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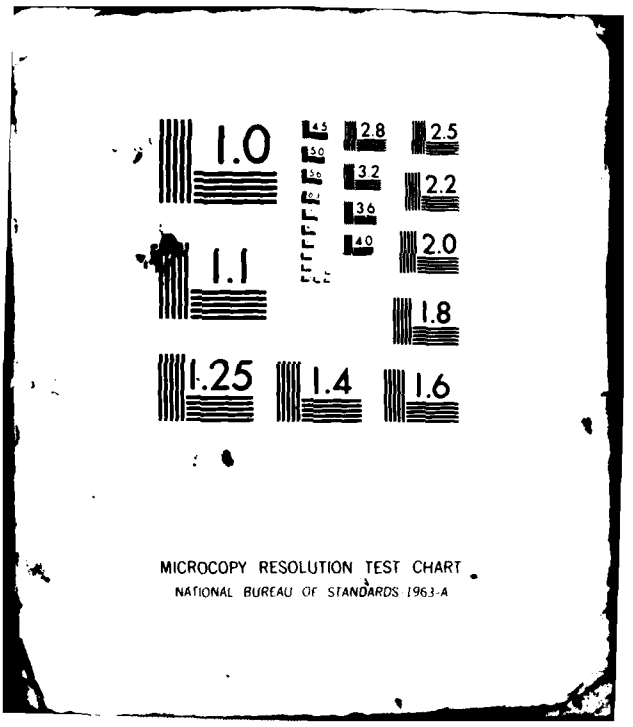
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NARCOTIC TOLERANCE AND DEPENDENCE MECHANISM: A NEUROLOGICAL CORRELATE

FINAL PROGRESS REPORT
(1972 - 1976)

May 1977

by

Horace H. Loh, Ph. D.

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San Francisco, California 94143

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Introduction

During the period of support from U.S. Army Contract DADA17-73-C-3006, the research proposal detailed in our original application was successfully carried out and subsequently resulted in a substantial number of important publications. Previous quarterly and annual reports have contained detailed summaries of our work. Additional experimental data may be found in the enclosed reprints of publications. This Final Report contains summaries of several pieces of work supported by the above named contract as well as the significance and possible practical application of the resulting information.

1. The Role of Cerebral Protein Synthesis in Opiate Tolerance Mechanism

Based on indirect evidence obtained in our own laboratories as well as by others, 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 is unknown. Our studies showed that morphine binds to brain free polysomes both in vitro and in vivo. Furthermore, morphine stabilizes 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 effects polysomes integrally via the drug's action on Mg++ concentration was investigated. However, our findings indicated

that morphine did not alter the cellular Mg++ concentration.

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 was also studied in discrete regions of the rat brain. Our results showed that chronic morphine treatment increased the ^{3}H-protein turnover in the microsomal fraction. However, acute morphine treatment increased ^{14}C-phosphatidylcholine synthesis in the hypothalamus (95%) and diencephalon (285%) and ^{3}H-protein synthesis in the hypothalamus (55%) and caudate nucleus (285%).

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. A detailed description and discussion of these studies has been published.

Another aim of our original proposal was to test any possible qualitative changes in protein synthesis due to "miscoding" of m-RNA after morphine-polysome interaction. This means that narcotic tolerance may be related to a change in the quality of cerebral proteins. In this case, narcotics need not alter the rate of turnover of brain proteins, but the tolerance development still could be blocked by inhibitors of protein synthesis. The fact that morphine binds to polysome presents the possibility that morphine could interact with the m-RNA which may, in turn, alter the coding process. The result of this "miscoding" would be to produce a "different" protein(s).

These studies were initiated in collaboration with Dr. James Meyerhoff and his associates at WRAIR. In the first phase of this work, the possibility of "miscoding" was explored, i.e., miscoding by a cell free protein synthetic system utilizing a synthetic m-RNA with known coding properties (e.g., poly U codes for phenylalanine).

Radiolabeled proteins were prepared in vitro in the presence and absence of morphine. A typical experiment would determine the relative ratio of ^{14}C-Phe to ^{3}H-Leu as well as other ^{3}H amino acids in the newly formed polypeptides utilizing poly U as the synthetic messenger. This study's significance lies in its ability to indicate whether or not morphine may induce a misreading of the genetic code causing qualitative differences in the normal brain protein.

Double labeling experiments for both in vivo and in vitro studies were used to test the possibility of "miscoding." ^{14}C-Phenylalanine (or another amino acid) was injected into control mice and ^{3}H-phenylalanine into morphinized mice (or vice versa). The mice were sacrificed, the brains removed and dissected into various regions. The brains (or discrete areas) from control and morphinized mice were combined and subjected to column chromatography. The various eluted fractions were examined for ^{3}H and ^{14}C radioactivity. The elution patterns of ^{3}H and ^{14}C-proteins will furnish information to determine whether different proteins are formed in the brain of morphine tolerant mice. A similar type of experiment was carried out in vitro by using a cell-free protein synthesis system. ^{14}C-Labeled proteins were prepared in vitro by a polysome-pH 5 enzyme system in the absence of morphine while ^{3}H-labeled protein was made in the presence of morphine. The soluble proteins

(^{3}H and ^{14}C) produced will be combined and fractionated with column chromatography. The fractions were assayed for ^{3}H and ^{14}C radioactivity.

Similar types of approaches have been employed in our laboratory using a cell-free protein synthesis system. In these studies, ^{14}C-labeled proteins were prepared in vitro by a polysome-pH 5 enzyme system in the absence of morphine while ^{3}H-labeled proteins were made in the presence of morphine. The newly formed soluble proteins (^{3}H and ^{14}C) were combined and fractionated with various chromatographic techniques. The fractions were assayed for ^{3}H and ^{14}C-radioactivity. Similarly, polysome and pH 5 enzymes isolated from morphine treated (both acute and tolerant) mice were also studied. The preliminary results of these studies follow.

2. Mechanisms of Morphine-increased Protein Synthesis (Quantitative)

In an attempt to localize the site of action of morphine in regard to protein synthesis, we have intensively studied the interaction of morphine with polysomes (as described in previous progress reports). The fact that morphine inhibits endonuclease which is responsible for the breakdown of m-RNA may, in turn, be related to the action of morphine on polysome stability. Preliminary results do not support a drug-induced ionic deficient environment as the mechanism responsible for morphine induced polysome stability. RNA synthesis in tolerant mice was studied further since preliminary results using ^{14}C-orotic acid indicated that morphine tolerant mice possess an increased rate of nuclear RNA synthesis, especially in the heavy subunit. Moreover, we have examined the effect of acute and chronic morphinization on the

following processes in a cell-free system.

a. The activation of amino acids (activating enzymes)

b. The transfer of activated amino acids to t-RNA (synthesis of aminoacyl-t-RNA and the transferase)

c. The transfer reaction (the transfer of ^{14}C-amino acid from carrier RNA to ribosomal protein), and

d. The chain initiation and release of peptides from the ribosome

To study the effect of morphine on the fidelity of translation, we found that when morphine was added to the CFPS (10^{-3} mol to 10^{-6} mol, it was ineffective at any of the concentrations studied. Poly U produced a maximal increase of phenylalanine incorporation into protein at 250 ug poly U per mg of PL protein. This is demonstrated in Fig. A-a. There was no significant difference in the incorporation of leucine (A-b) or lysine (A-c) when poly U was added to the incubation medium. Morphine alone at 10^{-4} mol had no effect on the incorporation of these amino acids into protein. In addition, morphine has no effect on the poly U directed incorporation of phenylalanine into protein. Nor does morphine plus poly U cause any significant increase or decrease in the incorporation of leucine, lysine or an amino acid mixture (A-d) into protein. This is also true of several other amino acids studied in a similar paradigm.

The lack of morphine effect is apparent irrespective of the source of PL and pH 5 enzyme. In Fig. A, the source was untreated control mice. Fig. B depicts virtually identical results in a CFPS isolated from morphine tolerant mice. The addition of exogenous morphine at 10^{-4} mol has no effect on the incorporation of phenylalanine or leucine relative to control. 250 ug of poly U produced the expected increase in phenylalanine incorpora-

tion and neither increased nor decreased leucine incorporation. Similar studies with PL and pH 5 enzyme from placebo treated mice were identical with these.

The Capacity of the CFPS for Protein Synthesis

A comparison of PL and pH 5 enzyme from morphine tolerant and placebo mice in a cross-over paradigm (Fig. C) established significant differences between the different fractions to incorporate amino acids into protein. In this series of experiments, all parameters of the CFPS were constant with the exception of the source of PL and pH 5 enzyme. The specific activity of the homologous fraction of PL and pH 5 from morphine tolerant mice is more than twice that of the homologous PL and pH 5 from placebo based upon equivalent protein content. In the case where heterologous PL and pH 5 were cross-incubated, the protein synthetic capacity of the system remained significantly elevated relative to placebo control. The purpose of the cross-over study was to ascertain if the increase in protein synthesis was due to a factor in the PL fraction or pH 5 enzyme fraction. It is apparent both fractions contribute a part to the total increase in labeled protein. The PL fraction is responsible for the major portion of the difference, but the results are more than additive at 60 minutes.

Gel Electrophoresis

An obvious question arising from these data is whether the increase in radiolabelled protein is due to a general quantitative change in the specific activity of newly formed protein, or to a qualitative change in one or a few species of protein. The total TCA precipitable fraction was solubilized and electrophoresed on acrylamide disc gels from a 60 min

incubation. No attempt was made to isolate the newly synthesized protein. The Coomassie Blue stain of such gels reflected no visual differences in the protein pattern between morphine tolerant and placebo treated CFPS. The diagram on the abscissa of Fig. D is representative. The majority of the proteins stained by the dye are due to PL and pH 5 protein, not newly synthesized protein. The gels were cut in 3 mm sections and dpm per section plotted over the diagram of the gel. It can be seen from Fig. D that there is a general quantitative increase in dpm per section along the entire length of the gel which contained protein from a morphine tolerant group.

This experiment would not accurately demonstrate small qualitative changes in specific proteins due to artifacts introduced in cutting the different gels, electrophoresis, etc. Thus, we utilized a dual label system to increase the accuracy. In these studies, the marker amino acid was either L-(^{3}H)-lysine or L-(^{14}C)-lysine. We were then able to co-electrophorese TCA precipitates from two different incubations on a single gel. Fig. E is representative of these studies. In E-a and E-b, the homologous PL-pH 5 enzyme from morphine tolerant and placebo mice were incubated with ^{3}H and ^{14}C lysine as indicated. Fractions were co-electrophoresed and the activity per section as a percent of the total in the gel was plotted. The TCA precipitates from four separate incubations were combined and co-electrophoresed in triplicate to minimize technical error. Fig. E-a serves as the morphine tolerant control and is the gel with the poorest pattern of reproducibility in the triplicate sample. If all proteins were identical and there was no technical error, the curves would superimpose, since the only difference in the samples in the label of the marker amino acid. Fig. E-b is the placebo control.

In Fig. E-c and E-d we coelectrophoresed TCA precipitates from incubations with morphine tolerant and placebo PL and pH 5 enzymes. A comparison of these graphs with control shows no evidence of a specific qualitative change in the newly synthesized protein which could account for the major differences in Fig. C.

Evidence from the studies cited on alternation of opiate tolerance development by inhibitors of protein synthesis provided the impetus to undertake the present study. The initial stages were designed to examine the teleologically attractive hypothesis that a tolerance-dependence protein might be responsible for the observed pharmacologic action of prolonged morphine administration. Other data indicating that morphine binds to and stabilized PL (Stolman and Loh, 1975) suggested the possibility that opiates could induce errors in translation of m-RNA resulting in aberrant proteins, a situation analogous to streptomycin dependence in certain bacteria. The present data do not substantiate any morphine induced ambiguity in translation of m-RNA.

When the drug was added in vitro to a CFPS isolated from untreated, placebo implanted or morphine implanted mice (i.e., tolerant-dependent) it had no effects on the incorporation of several labeled amino acids into protein. The effect of adding exogenous synthetic m-RNA, polyll, was also as expected in all cases. It produced an increased incorporation of Phe and no other amino acid. When morphine was added in conjunction with polyll there was no detectable increase in the incorporation of any of the amino acids examined other than Phe. Thus, within the limits of detection in this particular system, we are unable to demonstrate any morphine induced ambiguity of translation in the proteins synthesized by

free PL of whole brain.

A direct comparison of the protein synthetic activity of PL and pH 5 enzyme from chronically morphinized mice revealed that both fractions were capable of promoting incorporation of amino acids into protein at a rate greater than the respective controls. This increase is not apparent after the acute administration of morphine (Clouet and Ratner, 1967, 1968) and is in addition to the effect of morphine on transcription (Lee et al., 1975). Electrophoretic analysis of the proteins synthesized indicates a general increase in the specific activity of all proteins rather than an alteration in one or two species. This is in agreement with recently published data from an in vivo study in tolerant-dependent rats (Lang et al., 1975). It also mitigates against the concept of a specific tolerant-dependent protein(s) and suggests a more general drug induced homostatic change which occurs during the development of tolerance and dependence.

Although we found no significant difference in brain or body weight in drug treated mice, tolerance development usually produces some weight loss. This sort of nutritional deficit should cause a decrease in the capacity of a CFPS to synthesize protein (Migliorini and Manchester, 1971), the opposite of what we actually found. Amino acid supply also has a great effect on control of protein synthesis (Munro et al., 1975). In most experiments the endogenous amino acid pools were the only source of amino acids; the labeled amino acid did not contribute significantly. However, this seems to be an unlikely explanation for the observations herein since chronic morphine administration does not alter the endogenous amino acid pools (Clouet and Neidle, 1970; Clouet and Ratner, 1971). In some experiments, additional exogenous amino acids were added to the incubations

without qualitative effects. Moreover, this could not be the sole explanation since in the crossover studies (Fig. C), the PL fraction from tolerant dependent mice, which does not contain the amino acids pool, was more active than the pH 5 fraction.

This suggests that something occurs during development of tolerance to morphine which affects a compound present in both the PL and pH 5 fraction or that multiple events are occurring. Both the amino acyl ligases and initiation factors are present in the PL and pH 5 fractions and either may be a primary control factor in protein synthesis (Pain and Clemens, 1973). We are in the process of analyzing these macromolecules in tolerant-dependent mouse. Morphine may also exert an effect through a more complex mechanism. There is ample evidence that ethanol, another central depressant to which tolerance and physical dependence develops, produces a decrease in brain protein synthesis and that this is due to an effect on several of the different aspects of the synthetic process (Noble and Tewari, 1975).

Finally, it may not be necessary to postulate an effect on one or several of the macromolecules directly involved in protein synthesis to explain the increase seen after tolerance development. Morphine interacts with cerebroside sulfate *in vitro* in such a way that many of the criteria for a morphine receptor complex are fulfilled (Loh *et al.*, 1974). Morphine also may interact with several other lipids (Cho *et al.*, 1976). Lipids are known to be an integral component of the protein synthetic matrix *in vivo* and extraction and reconstitution of lipid *in vitro* decreases and increases respectively cell free protein synthesis (Hradec, 1975). It

has been shown that the amino acyl ligases which are usually isolated as soluble enzymes may, in fact, exist as a super molecule complex in close association with the PL *in vivo*. Under very mold conditions, these complexes have been obtained and characterized (Bandyopadhyay and Deutscher, 1971; Vennegoor and Bloemendal, 1972). The lipids have been extracted and characterized (Bandyopadhyay and Deutscher, 1973). It is postulated that the lipid serves as a medium to orient the various components of the protein synthetic matrix. Morphine may act during tolerance development to increase the effectiveness of this matrix to synthesize protein.

FIGURE LEGENDS A - E:

Fig. A - Effect of morphine and poly U on the ability of the CFPS to incorporate amino acids into protein. Incubation medium consists of: ATP generating system; buffered salts pH 7.6, PL:pH 5 enzyme (1:4) isolated from naive mice; radiolabeled amino acid; in addition - contains 10^{-4} mol morphine; - 250 ug poly U; - 10^{-4} mol morphine and 250 ug poly U. Each point is the mean of 4 determinations. Fig. A-a - L-(^{3}H) phenylalanine, Fig. A-b - L-(^{14}C)-leucine, Fig. A-c - L-(^{3}H) phenylalanine, Fig. A-b - L-(^{14}C)-leucine, Fig. A-c - L-(^{3}H)-lysine, Fig. A-d - L-(^{3}H) amino acid mixture. Results are expressed as dpm of amino acid incorporated into protein per mg of PL protein.

Fig. B - Effect of morphine and poly U on the ability of a CFSP isolated from chronically morphine tolerant morphinized mice to incorporate amino acids into protein. Incubation medium consisted of: ATP generating system; buffered salts pH 7.6, PL:pH 5 (1:4) isolated from morphine tolerant mice; radiolabeled amino acid. In addition, contains 10^{-4} mol morphine, - 250 ug poly U, - 10^{-4} mol morphine plus 250 ug poly U. Each point is the mean of 4 determinations. Fig. B-a - L-(^{3}H) phenylalanine, Fig. B-b - L-(^{14}C) leucine. Results are expressed as dpm of amino acid incorporated into protein per mg PL protein.

Fig. C - A comparison of the ability of the PL and pH 5 enzyme from morphine tolerant and placebo mice to incorporate L-(^{3}H)-phenylalanine and L-(^{14}C) leucine. Δ - PL and pH 5 enzyme from morphine tolerant mice; ■ - PL from morphine tolerant pH 5 from placebo; ● - PL from placebo

pH 5 from morphine tolerant; ○ - PL and pH 5 from placebo. Each point is the mean of 4 determinations. Results are expressed as dpm of amino acid incorporated into protein per mg of PL protein. The ratio of PL: pH 5 enzyme was 1:4. All points are significantly different from control, at 30 and 60 min, $p > .01$.

Fig. D - Protein pattern seen after acrylamide gel electrophoresis of the TCA precipitated incubation medium. The Coomassie Blue staining pattern is depicted along the abscissa and dpm per 3 mm gel section superimposed. ● is PL and pH 5 enzyme (1:4) from morphine tolerant mice; ■ is PL and pH 5 enzyme (1:4) from placebo mice. All other conditions were identical. The protein fractions electrophoresed were combined aliquots from 4 different incubations. There were no visually detectable differences in the Coomassie Blue stain between tolerant and placebo. The amount of labeled amino acid incorporated into TCA precipitable fraction was consistently greater in the morphine tolerant group.

Fig. E - Representative patterns of radioactivity from dual labeled gels. Two gractions were electrophoresed on each gel. In the fraction designated by the closed circle, ● , the marker amino acid was L-(^{3}H) lysine, by the open circle, ○ , L-(^{14}C) lysine. In E-b, the PL and pH 5 enzyme were from morphine tolerant mice in both cases, thus the only difference is the itotopic label of the lysine. Similarly, in E-a, both PL and pH 5 are from placebo. Therefore, E-a and E-b are controls for comparison with E-c and E-d in which the PL and pH 5 are from different sources as indicated. A qualitative change in protein synthesis would be indicated by a difference between ● and ○ within a single gel section in E-c and E-d, which is greater than any difference seen within a single section in E-a or E-b.

We conclude there are no major qualitative differences between the proteins synthesized *in vitro* by PL - pH 5 from morphine tolerant and placebo mice.

Figure A

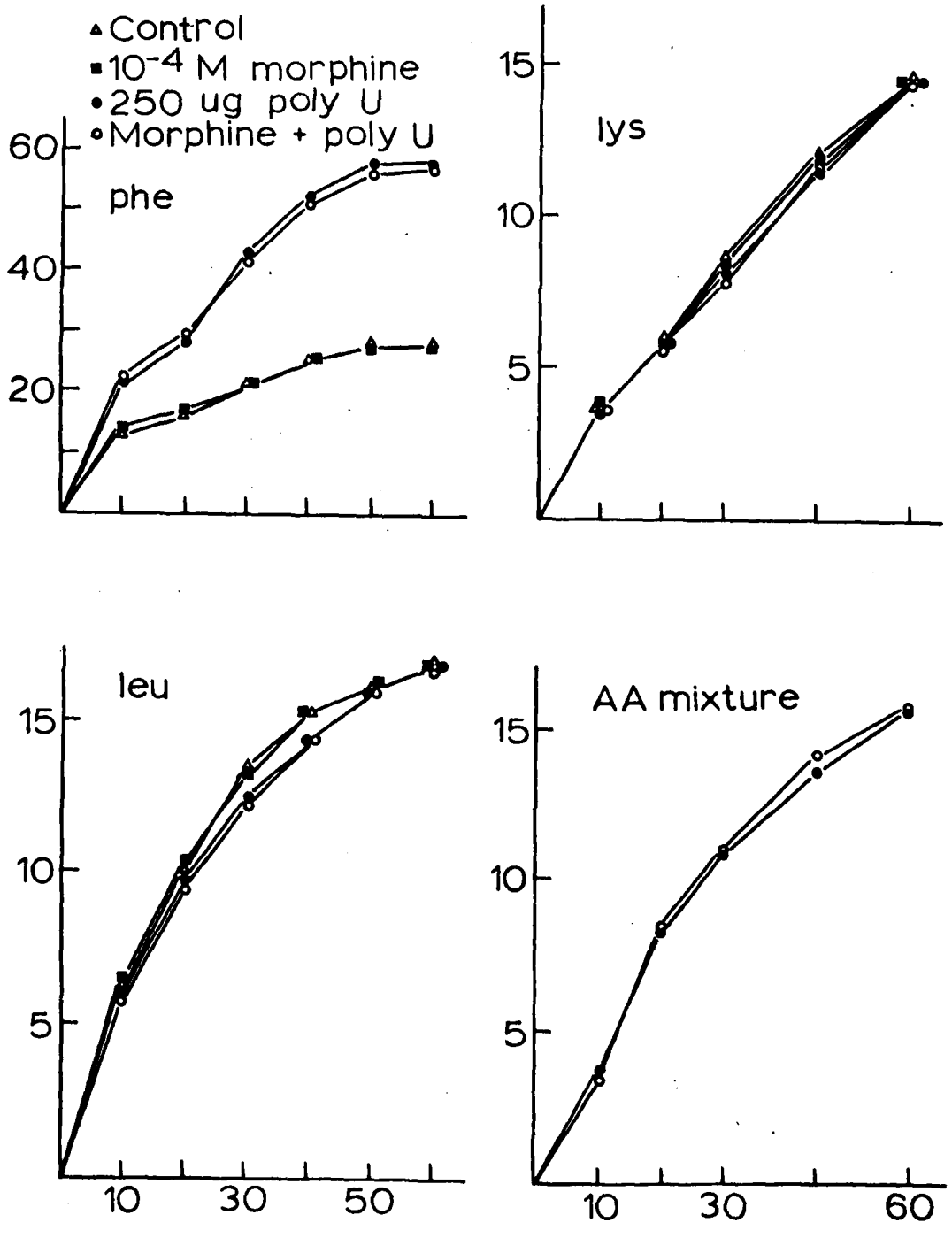
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Figure B

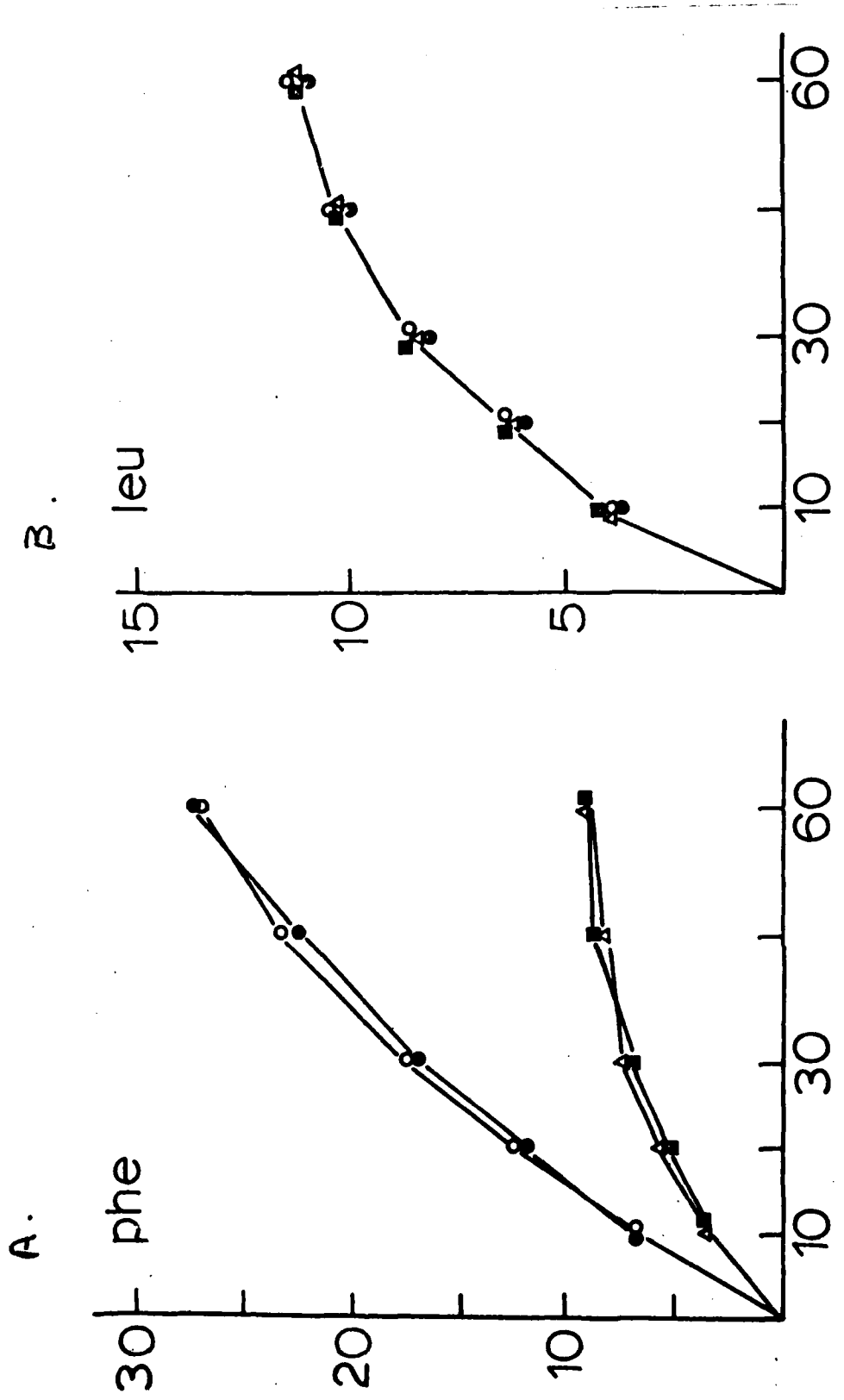
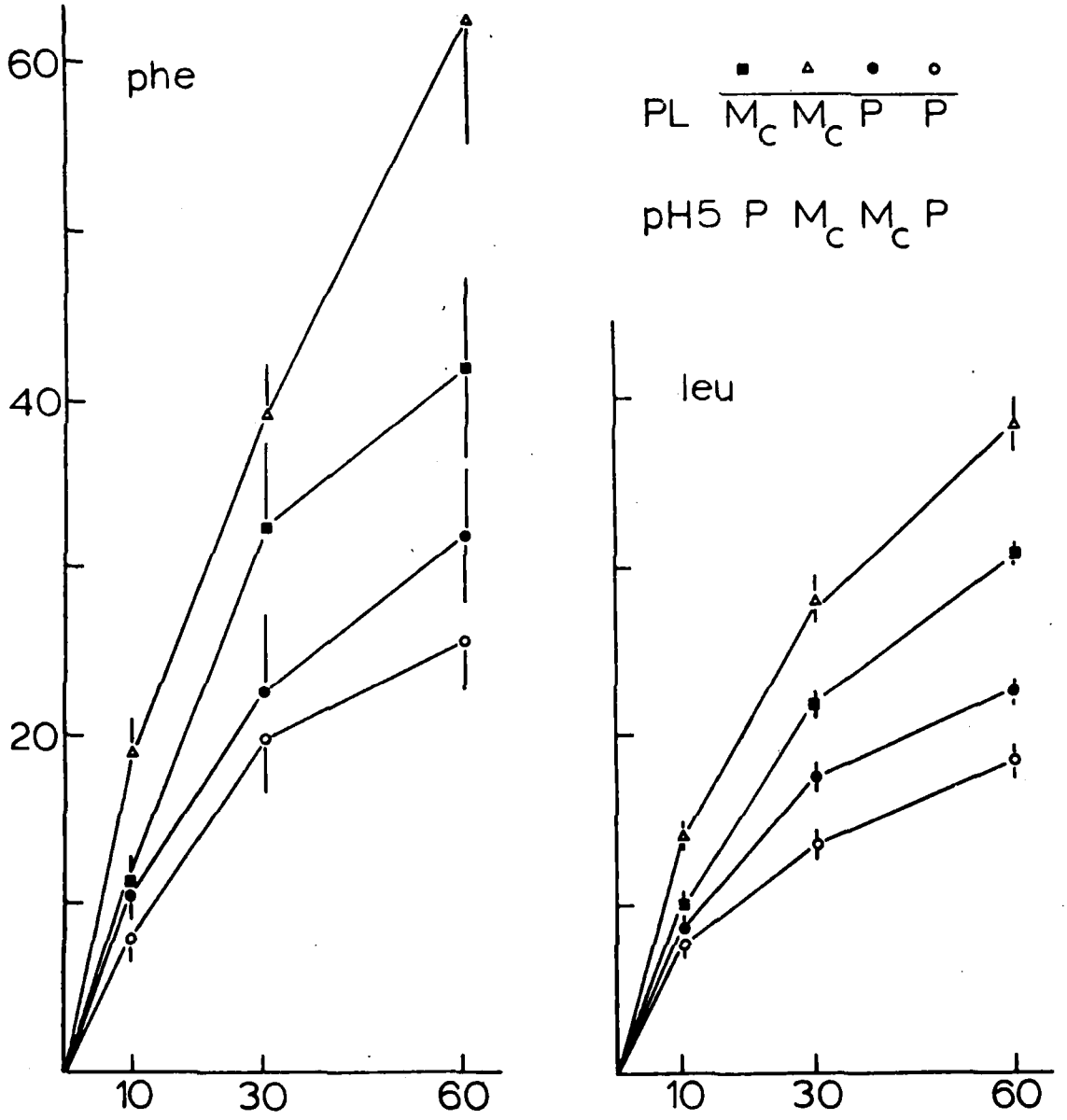


Figure C



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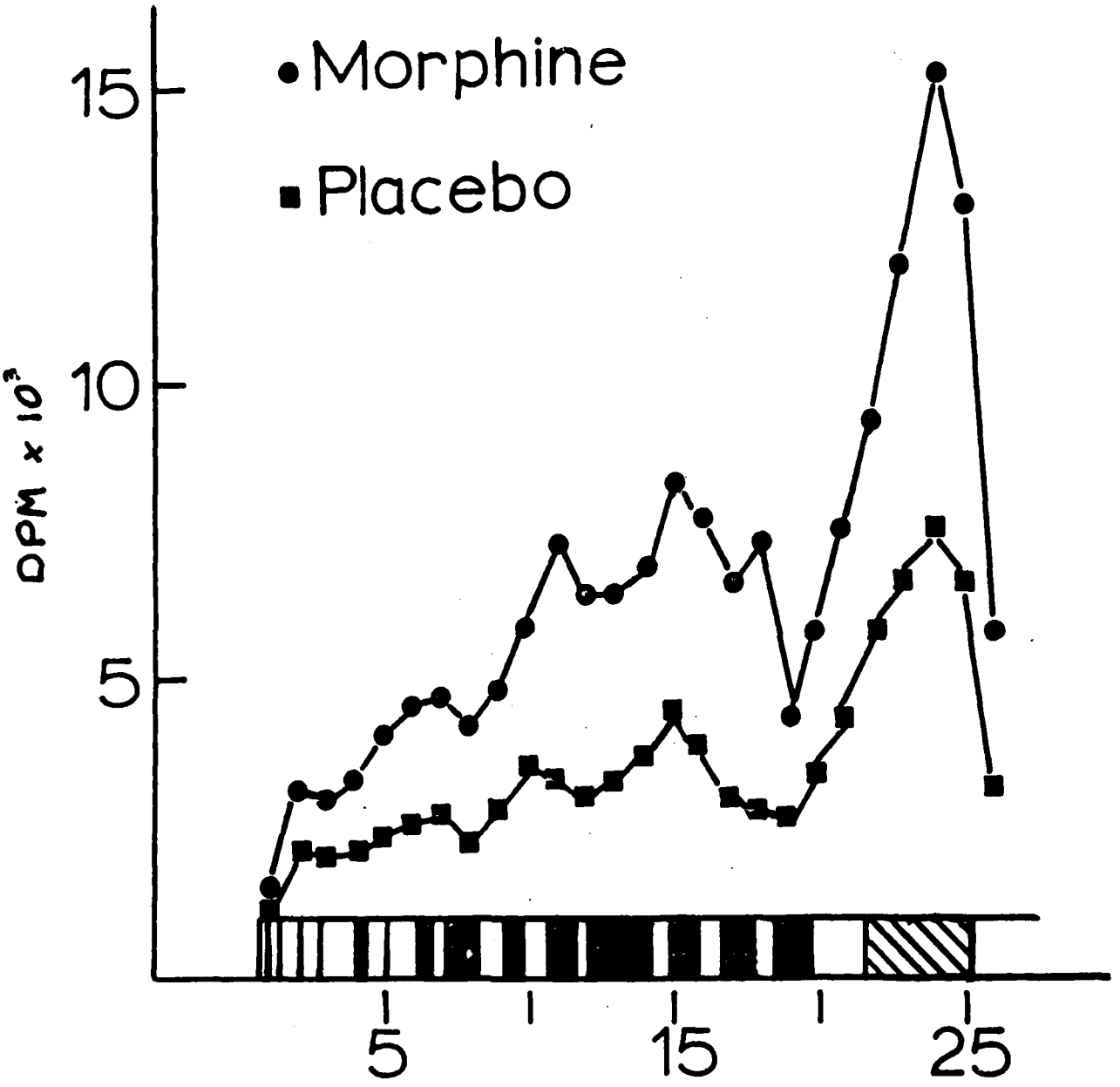
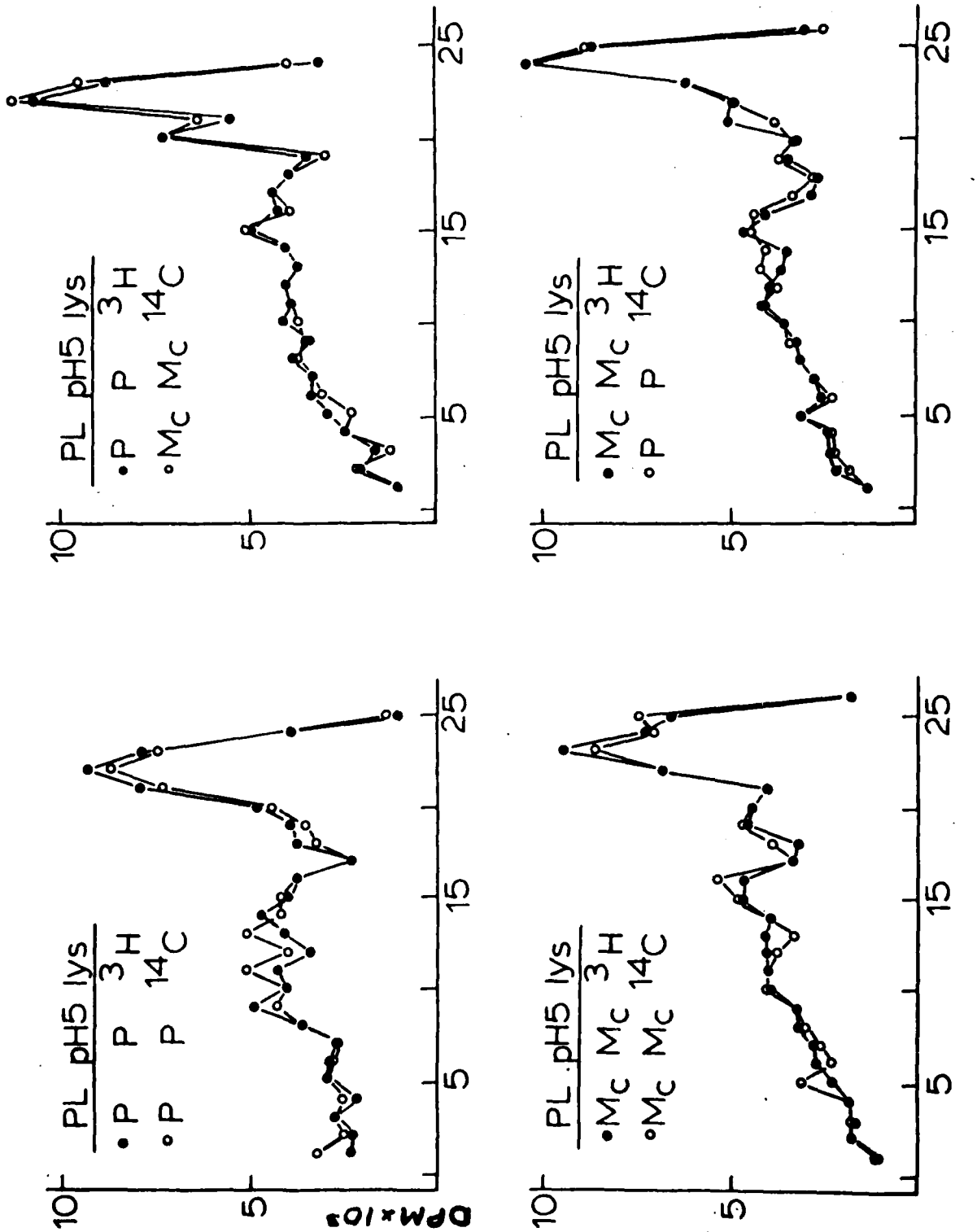


Figure E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The Mechanism of Morphine on Translation

Since the last report on this project, experiments were begun to determine at which level of the translation process opiates exert an effect. There is very little information available in the literature on the isolation, purification and study of the various enzymes and nucleic acids necessary for translation in brain tissue. A good deal of time was spent making modifications of techniques suitable for preparations of these factors from other tissues or prokaryotic cells. There has been considerable success in these efforts and we now have stockpiled several hundred milligrams of pure, highly active amino acyl ligase from mouse and rat brain. We also have purified brain soluble RNA from these same animals. Smaller amounts of pure stripped ribosomes are made on an "as needed" basis. The initiation factors I, II, II and peptidyl transferase have been prepared in a crude form and need further purification before studies can be undertaken. It would be premature to comment on the results of these enzymatic studies at this time. The optimal conditions of enzyme, substrate, isoacceptor tRNA, Mg++ and ATP are different for each amino acid. Current efforts are dedicated to determining the optimal parameters for each amino acid before comparisons are made between control and drug treated groups. Other research support is being sought to enable completion of the final stage of this research.

3. The Role of Gene Expression on Morphine Tolerance Development

The synthesis of ribonucleic acid (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. Recent reports have shown that the inhibition of ribonucleic acid synthesis antagonizes the development of morphine-induced analgesic tolerance. The authors concluded that tolerance development to the analgesic effect of morphine in animals can be reduced or prevented by several drugs which, in different ways, inhibit RNA synthesis and/or protein in the brain. However, the difficulty of interpreting experiments in which drug treatment results in severe disturbance of essential aspects of cell metabolism should also be emphasized.

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. Castles et al indicated in their study that newly formed RNA from brains of tolerant rats was not lost as rapidly as RNA from brains of non-tolerant rats.

In our studies, attempts were made to determine the chromatin template activity in the brains of tolerant and non-tolerant mice in order to establish if the changes in RNA metabolism during tolerance development were due to the alleviation of chromatin template activity.

Our preliminary experiments indicated that the rates of DNA dependent RNA polymerase from E. coli K-12 were linear up to 2 hours when mouse brain chromatin was used as DNA template. The UTP-H^3 incorporation into RNA was proportional to the amount of DNA added up to 30 ug. In routine assay, less than 20 ug of DNA was used. Tolerant chromatin showed higher template activity than non-tolerant one. The rate from tolerant chromatin was linear up to at least 50 ug DNA, whereas the non-tolerant chromatin began to level off above 30 ug DNA. Table 1 shows that the chronic treatment of morphine resulted in increased specific activities of brain chromatin which served as DNA template. Omission of chromatin or exogenous RNA polymerase from the reaction mixture showed negligible incorporation, thus the difference cannot be accounted for by the RNA polymerase contamination in isolated chromatin. Additions of morphine yp to 0.1 mM into the reaction medium showed no significant change in the rates of UTP-H^3 incorporation.

However, when the chromatins were washed excessively, the chromatin template activity from non-tolerant animals increased almost 2-fold. The tolerant chromatin activity remained essentially the same (Table 2). Therefore, the template activity in tolerant animals after excessive washing was about the same or slightly lower than placebo activity.

If there is an inhibitor(s) present in the nuclei which has been removed during the wash, one would expect the inhibitor to inhibit the chromatin dependent UTP incorporation if added back to the reaction

medium. The supernatant collected from the seven steps of excessive wash was lyophilized thoroughly to remove all the salts. The dialysis cellulose bag has a 3000 molecular weight cutoff. This means that molecules bigger than 3,000 would be retained in the bag; otherwise they would go into the dialysate. The concentrated and dialyzed solution was then added back to the reaction mixture. The results show that if the wash contains an inhibitor, the inhibitor must have a molecular weight of less than 3,000 because there is no inhibition of UTP incorporation in the presence of the concentrate.

Table 3 shows the result when the histones were removed from chromatins with H_2SO_4 extraction. The histone-free chromatins showed approximately a 6 to 10-fold increase in their template activities. However, excessively washed chromatin from placebo treated animals still shows higher activity than the regularly washed placebo chromatin. Apparently, the difference in their template activity cannot be due to the difference in the histones.

The data described above show that when the mice are rendered tolerant to morphine with morphine 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 activities are calculated based on per mg DNA. Omission of chromatin or RNA polymerase showed negligible incorporation. Removal of the histone increases the rate of incorporation by 6 to 10-fold. However, the DNA protein complex (free of histone) obtained from tolerant animals still shows higher template activity than the placebo control. Hodgson et al reported that DNA devoid of all proteins from either placebo or tolerant rats gave identical rate of UTP incorporation. Therefore, the data suggested that the non-histone protein which complexed

with DNA may have been altered during the morphine tolerance development. This was supported by the results shown in Table 4.

During the preparation of chromatin, if one washed the nuclei and chromatin excessively, i.e., 10 times more volume for each step of wash than the regular preparation, one observes a 2-fold increase in placebo chromatin-directed UTP incorporation, whereas the tolerant chromatin remains the same. Some components which control the activity of DNA dependent UTP incorporation must have been removed during the excessive wash. The chromatin activity from tolerant animals remains the same regardless of the washing procedure indicating that the controlling factor is either not present or is very labile in tolerant animals. However, the concentrated and dialyzed wash showed no inhibitory activity when added back to the reaction medium. Therefore, if there is a regulator which was removed, it must be smaller than 3000 m.w. Further investigation is being carried out to identify the possible regulator.

TABLE 1

EFFECT OF CHRONIC MORPHINE TREATMENT ON CHROMATIN TEMPLATE ACTIVITY ISOLATED FROM MOUSE BRAIN

Treatment	Specific Activity ± S.E. UTP-H^3 incorporation nmoles/mg DNA
Non-Tolerant	41.96 ± 3.38 (9)*
Tolerant	65.55 ± 7.87 (9)*

* Number of preparations performed. $P < 0.01$.

TABLE 2

THE EFFECT OF EXCESSIVE WASHING ON CHROMATIN ACTIVITY IN DIRECTING UTP-H^3 INCORPORATION INTO RNA

Treatment	Specific Activity ± S.E. UTP-H^3 Incorporation nmoles/mg DNA
Regular Wash	
Non-Tolerant	47.75 ± 2.60 (4)[a,b]
Tolerant	80.75 ± 15.70 (4)[a]
Excessive Wash	
Non-Tolerant	84.50 ± 7.93 (4)[b]
Tolerant	72.12 ± 8.73 (4)

a. $p < 0.05$

b. $p < 0.01$

TABLE 3

COMPARISON OF CHROMATIN TEMPLATE ACTIVITIES AFTER THE REMOVAL OF HISTONES

Chromatin (Histone Bound)	Specific Activity UTP-H^3 Incorporation nmoles/mg DNA
Non-Tolerant	
Regular Wash	47.6
Excessive Wash	66.6
Tolerant	
Regular Wash	73.3
Excessive Wash	53.6
Chromatin (Histone Free)	
Non-Tolerant	
Regular Wash	282
Excessive Wash	458
Tolerant	
Regular Wash	380
Excessive Wash	360

Table 4

COMPARISON OF CHROMATIN* TEMPLATE ACTIVITIES
AFTER THE REMOVAL OF ACIDIC PROTEINS

Treatment	Specific Activity ± S.E. UTP-H^3 Incorporation nmoles/mg DNA
Non-Tolerant	62.25 ± 2.75
Tolerant	82.90 ± 0.7
Non-Tolerant (-acidic proteins)	101.50 ± 7.6
Tolerant (-acidic proteins)	55.50 ± 6.6

* The chromatins were prepared according to the regular washing procedure described in the Methods.

In order to ascertain whether or not the phenomenon is narcotic specific, a 10 mg pellet of naloxone, a morphine antagonist, was implanted with the morphine pellet. It can be seen from the data in Table 5 that in the regular wash chromatins, naloxone clearly abolished the effect of morphine. We also observed that the acute morphine effect on chromatin template activity was similar to that of saline control. It should be noted here that similar phenomena were observed in the rat. Table 6 indicates that chromatin isolated from morphine tolerant rats showed activity approximately 50% higher than chromatin isolated from placebo controls.

Table 5

EFFECT OF NALOXONE ON CHRONIC MORPHINE-INDUCED CHROMATIN ACTIVITY

Treatment	Specific Activity UTP-H^3 Incorporation nmoles/mg DNA
Placebo-Placebo	87.5
Naloxone-Placebo	95
Placebo-Morphine	135
Naloxone-Morphine	95

The chromatins were prepared according to the regular washing procedure.

Table 6

EFFECT OF CHRONIC MORPHINE TREATMENT ON CHROMATIN* ACTIVITIES IN RAT BRAINS

Treatment	Specific Activity ± S.E. UTP-H^3 Incorporation nmoles/ΔOD_{260} mμ DNA
Non-Tolerant	8.25 ± 0.25
Tolerant	12.70 ± 0.70
$p < 0.01$	

* The chromatins were prepared according to the regular washing procedure described in the Methods.

These studies showed that regulation of brain protein synthesis was altered following chronic morphine treatment. Moreover, one possible site of action is the gene. Also, our work indicated that the non-histone proteins are responsible for the mechanism of the morphine-induced changes in gene expression. The non-histone proteins in cell nuclei have been implicated in the regulation of DNA template activity in chromatin and the heterogeneity of these proteins makes it difficult to study each protein with respect to its function and regulation. We chose to study the phosphorylation of nuclear proteins, since the phosphorylation of the nuclear proteins, especially non-histone proteins, has been suggested as a means of positive gene regulation. The enzyme responsible for protein phosphorylation, i.e., protein kinase, was also measured.

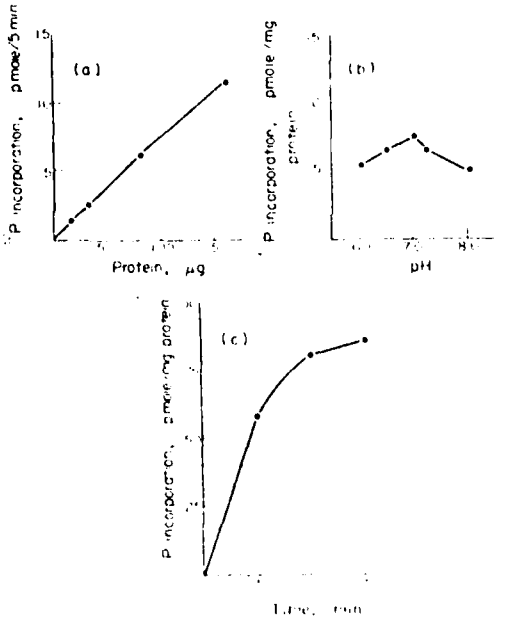
As shown in Fig. 1, we found the activity of protein kinase isolated from mouse brain chromatin to be linear up to 10 min. The zero time count was always less than 10% of the total counts and has been subtracted. The pH optimum of the enzyme was quite broad; in the range tested 9Ph 6.0 to 8.0), there was no obvious peak. Thus, in routine assay, pH 6.5 was used. The γ-(^{32}P)ATP incorporation was linear with increasing concentration of ATP up to 5 uM.

The enzyme may use its endogenous chromatin proteins as substrate. Additional histone, 40 ug/0.2 ml did not increase the amount of γ-(^{32}P)ATP incorporation. However, additional casein (40 ug/0.2 ml) resulted in 50-60% more phosphorylation. The enzyme was active when chromatin was freshly prepared; however, within a couple of days of storate at -10°,

more than 80% of the activity was lost. Thus, assay of the protein kinase activity immediately after chromatin preparation is important.

Table 7 shows that the protein kinase activity from chromatin isolated from morphine-induced tolerant mice was 65% higher than that of the control group. Addition of morphine sulfate (10^{-4} to 10^{-7} M) or cAMP (10^{-5} to 10^{-7} M) <u>in vitro</u> had no significant effect on protein phosphorylation (Table 8).

Fig. 1



Some properties of the apparent protein kinase activity in oligodendroglial-rich chromatin. The reaction mixture is as described in Methods except that in panel a various concentrations of chromatin proteins were added, in panel b, various pH values of potassium phosphate buffer were used and in panel c various time intervals if incubation were used

Table 7

Effect of chronic morphine treatment on chromatin protein kinase activities

Treatment	^{32}P incorporated (pmoles mg protein)
Non tolerant	54.9 ± 3.47 (4)*
Non tolerant + 40 μg casein reaction	81.2
Tolerant	90.9 ± 10.23 (4)*
Tolerant + 40 μg casein	112.3

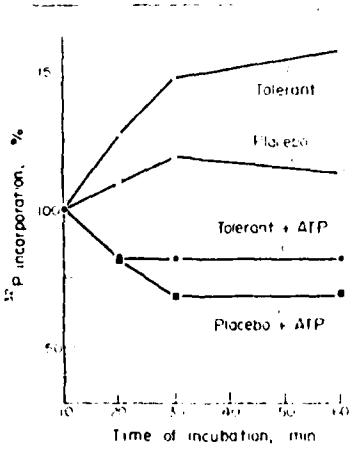
* $P < 0.01$. Number in parentheses is the number of experiments performed

Table 8

Effect of morphine and cAMP on phosphorylation of chromatin protein *in vitro*

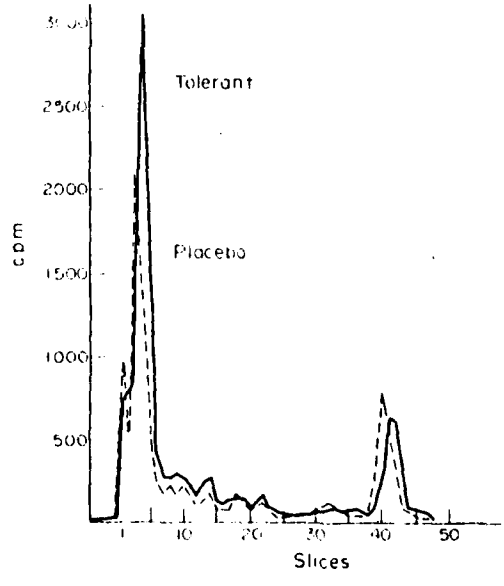
Treatment	^{32}P incorporated (pmoles/mg protein)
Control	65.8 ± 4.2
Control + morphine sulfate, 10^{-7} M	64.5 ± 1.9
Control + morphine sulfate, 10^{-6} M	56.8 ± 2.3
Control + morphine sulfate 10^{-5} M	64.8 ± 4.5
Control + morphine sulfate, 10^{-4} M	58.4 ± 4.7
Control + cAMP, 10^{-5} M	72.2 ± 5.0
Control + cAMP, 10^{-6} M	68.8 ± 2.0
Control + cAMP, 10^{-7} M	69.2 ± 5.5

Fig. 2



Turnover of previously incorporated [^{32}P]phosphate in chromatin proteins. Chromatin proteins were incubated for 10 min in the presence of [^{32}P]ATP as described in Methods. Retention of ^{32}P was measured after the addition of 5 mM of non-radioactive ATP.

Fig. 3



SDS acrylamide gel electrophoresis of mice brain chromatins. The chromatins from placebo and morphine-tolerant mice were phosphorylated and electrophoresed as described in Methods. Gels were stained by Coomassie Brilliant Blue. Gel origin is at left.

In order to determine whether or not the increase in phosphorylation of chromatin protein isolated from tolerant animals was due to a decrease in phosphoprotein phosphatase activity, the cold ATP dilution procedure was used. Fig. 2 shows that dephosphorylation was evident in both the placebo and the tolerant groups. At 20 min, dephosphorylation was identical for both preparations. At 60 min, in chromatin isolated from the placebo and the tolerant groups, 31 and 18% of the ^{32}P were lost, respectively. However, in the absence of cold ATP, chromatin isolated from tolerant animals increased phosphorylation by 56% at 60 min, whereas in chromatin isolated from placebo, phosphorylation increased less than 20%. This result indicated that protein kinase was responsible for the increase of phosphorylation in chromatin protein isolated from tolerant animals.

Electrophoresis of the Phosphorylated Chromatin Proteins

Nuclear chromatin comprises a heterogeneous mixture of proteins differing in molecular weight, amino acid composition and degree of phosphorylation. The heterogeneity of molecular sizes is indicated by the differences in electrophoretic mobility in SDS-polyacrylamide gel. The degree of phosphorylation can also be measured in the gel with ^{32}P-labeled chromatins.

Fig. 3 reveals the complicated banding pattern of chromatin protein subunits. There are at least 30 different multiple polypeptide bands ranging in molecular weight from 15,000 to 200,000 daltons calculated from proteins with known molecular weights. The most prominent bands after staining with Coomassie Brilliant Blue were the histone proteins. It has been reported that two of the histone proteins were located in the middle of the gel (slices 19-22) and two more were observed in the

lower part of the gel (slices 30-36). The specific activity of ^{32}P-labeling was low in the histone protein-rich area. In agreement with the findings of Richwood et al., this indicated that histone proteins were a rather weak substrate for this protein kinase reaction. The other bands of the gels were non-histone protein bands. The high molecular weight region (slices 1-5) represents a series of very finely separated high molecular weight protein subunits. Although the bands were rather light after staining with Coomassie Blue, it was quite clear that they are highly phosphorylated (Fig. 3). The degree of phosphorylation in this region was about 74% higher in the tolerant group than in the control group (Table 9), although the electrophoretic patterns between those two groups were similar.

Since there was no visible protein staining, the ^{32}P-labeling was quite significant in slices 38-46 (molecular weight about 7000 daltons). The intensity of phosphorylation was about the same between chromatins isolated from tolerant animals and controls. However, the band moved slightly faster in the tolerant group (Fig. 4). The shifting in mobility is a true phenomenon, since we have repeated it more than 5 times in 5 different nuclei preparations and found it to be reproducible each time. When morphine sulfate (1 mM) was added during phosphorylation in vitro of chromatin proteins isolated from control or tolerant animals, the electrophoretic pattern of this shift was unaffected.

Cyclic AMP did not stimulate the phosphorylation of chromatin protein in vitro. Electrophoretic patterns of the chromatin proteins phosphorylated with or without the presence of cAMP (5 uM) were similar, except in slices 38-46. Fig. 5 shows that this peak shifted when phosphorylated in

the presence of cAMP. This shift was observed in both types of chromatins. The data presented demonstrate the apparent protein kinase activity in oligodendroglial-rich chromatin. Since it is unclear whether or not the protein kinase(s) is a separate protein(s) or the kinase activity is inherent in the phosphoproteins themselves, we decided to refer to the activity as "apparent protein kinase activity." The addition of casein has been shown to increase ^{32}P-labeling in TCA-precipitable materials. Since casein is not a natural component of brain tissue, the significance of the stimulation of phosphorylation is unclear. There is no obvious

Degree of phosphorylation in high molecular weight region

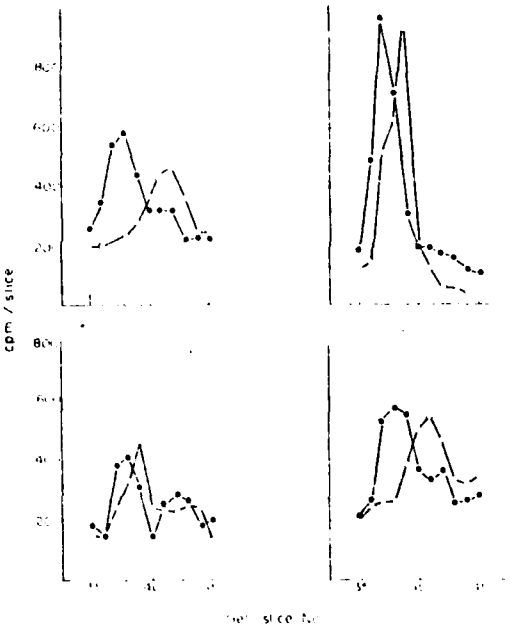
Treatment	Activity (cpm)
Placebo	1675 ± 154 (11)*
Tolerant	2935 ± 142 (11)*

* $P < 0.001$. Number in parentheses is the number of experiments performed.

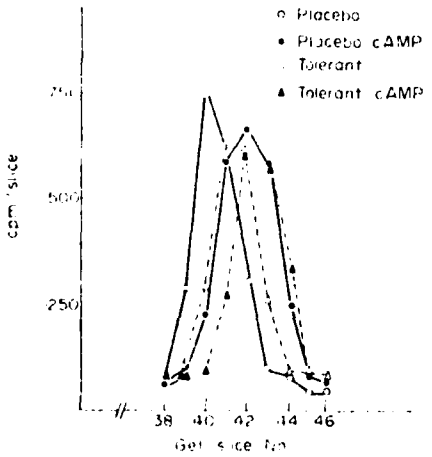
Table 9

Fig. 4 ⟶

Fig. 5



Phosphorylation in electrophoresis gel slices 35–45 from four different chromatin preparations. The chromatins from placebo and morphine-tolerant mice brain were phosphorylated and electrophoresed as described in Methods. Results from four different chromatin preparations are presented. Gel origin is at left. Key: ●, placebo; ▲, tolerant.



Effect of cAMP *in vitro* on phosphorylation in electrophoresis gel slices 38–45. The chromatins from placebo and morphine-tolerant mice brain were phosphorylated in the absence or presence of cyclic AMP (5×10^{-6} M) respectively and electrophoresed as described in Methods. Gel origin is at left.

optimum pH for this enzyme activity indicating that several enzymes may be responsible for the phosphorylation. Kish and Kleinsmith have reported the separation of protein kinase activity into 12 distinct enzyme fractions in beef liver chromatin. This possibility of multiple enzymes would also have to be carefully examined in oligodendroglial-rich chromatin preparation. Unlike the histone kinase, this protein kinase activity was very labile; 80% of the activity was lost within only a few days of storage. This presents a serious problem in further purification of the enzymes. A search for methods to stabilize the activity is in progress.

Chronic morphine treatment resulted in an increase in total apparent protein kinase activity. It was evident in cold ATP dilution experiments that the increase in phosphorylation of chromatin in tolerant animals was, indeed, due to "apparent protein kinase activity." Compared to the placebo group, although the turnover rate of ^{32}P was slower in chromatin protein isolated from tolerant animals, the phosphorylation rate in the absence of cold ATP was considerably higher at all time periods. These results suggest that this may be at least partially due to the increase in protein kinase activity.

The phosphorylation of proteins occurred mainly in the acidic protein region of chromatin proteins. Electrophoresis of SDS-acrylamide gel revealed that chronic morphine treatment increased the ^{32}P incorporation in this area. It is not known if the gel slices still contain protein kinase activity. Furthermore, the ability of a one-dimensional dodecyl-sulfate-electrophoresis system to demonstrate the true complexity of chromatin non-histone proteins is limited, therefore requiring more detailed

work to elucidate this high molecular weight region. In slices 39-45, where there was no visible protein stain, significant phosphate labeling was observed. It is interesting to note that this band was similar in position to the one reported by MacGillivray and Richwood. Chronic morphine treatment shifted this band slightly; it is not known if this was the result of a change in peptide molecular weight.

It has been suggested that phosphorylation of chromatin proteins would normally increase chromatin template activity. Therefore, our observation may represent one of the positive controls of gene expression. The addition of morphine sulfate in vitro had no effect on ^{32}P-labeling of protein, indicating that morphine did not directly interfere with protein phosphorylation. Therefore, the effect of chronic morphine treatment in vivo may be via some other mechanism, indirectly affecting protein phosphorylation of chromatin proteins.

In summary, we have demonstrated apparent protein kinase activity in oligodendroglial-rich chromatin. The activity was unaffected by cAMP or morphine sulfate in vivo. Chronic morphine treatment resulted in increased phosphorylation which may be due to protein kinase activity rather than to a decrease of phosphoprotein phosphatase activity.

The increase was located primarily in high molecular weight regions of the SDS gel. As we reported previously, oligodendroglial-rich template activity increased in chronic morphine treated animals; the increase in phosphorylation of non-histone proteins observed in this study may be related. Further studies are in progress to elucidate the phosphorylation reaction and its relationship to chromatin template activities in different types of nuclei.

4. The Role of Membrane Acidic Lipids in Opiate Receptor Binding

There have been numerous attempts to elicit the mechanism of action of morphine and its surrogates in analgesia, tolerance and physical dependence. As an initial approach to studying selective narcotic-receptor interaction, the distribution and binding characteristics of the active D(-) enantiomorph in the brain have been compared either with the inactive isomer or in the presence of an antagonist. However, these attempts to demonstrate selective binding by pharmacologically active narcotics were unsuccessful because of the lack of suitable tools for the purpose. Recently, Goldstein et al, using levorphanol and dextrorphan, elaborated a procedure for demonstrating stereospecific binding and reported that mouse brain contains fraction that binds opiates stereospecifically. Subsequently, several groups of investigators using an antagonist and various narcotic agonists with high radioactivity, also have demonstrated stereospecific binding of narcotics with varying degrees of affinity for certain brain areas. The primary binding site has been identified to be in the membrane of nerve terminals at certain brain regions.

In our laboratory, we have been interested in endogenous substances in the brain which might interact with morphine agonists and antagonists in a stereospecific manner. Based on molecular models, it was found that parts of the structures of several glycolipids appear to exhibit structural complementariness to morphine and several neurotransmitters we well (Cho et al, unpublished). Indeed, the structures of cerebrosides, cerebroside sulfates and gangliosides appeared to fulfill the requisites of the

analgetic receptor postulated by Beckett and Casy and by Portoghese. Moreover, these glycolipids are optically active and capable of stereospecific interaction with narcotics, and since cerebrosides, cerebroside sulfates and gangliosides are located in the nerve membrane and nerve endings of the central nervous system, an examination of these substances for stereospecific binding to opiates was initiated.

Our preliminary studies indicated that high affinity stereospecific binding to narcotic compounds was exhibited by cerebrosides. While these studies were in progress, Lowney et al announced the isolation from mouse brain of a partially purified opiate receptor which they reported to be a proteolipid. Our further investigations revealed that the narcotic binding properties exhibited by cerebroside sulfate, separated from our commercial source of cerebroside, were similar to those of the reported opiate receptor. Inasmuch as the cerebroside sulfate which we purified contained no protein contaminants, it became incumbent upon us to compare in a more definitive fashion the characteristics of cerebroside sulfate and the mouse brain opiate receptor. Subsequently, we reported narcotic stereospecific binding to cerebrosides and provided evidence that one of the cerebrosides, cerebroside sulfate (or sulfatide), is possibly identical to the purified mouse brain narcotic receptor. We also provided explanations for the apparent proteo-like behavior of this mouse brain opiate receptor.

Narcotic Binding Studies

Stereospecific binding of H^3-etorphine at 2×10^{-8} M concentration and H^3-naloxone at 2×10^{-7} M concentration to cerebroside were studied under conditions as shown in Table 1 according to a modification of the method of Goldstein et al.

For fractionation and estimation of cerebroside sulfate and its levorphanol complex, the method of Soto et al was used. To isolate the opiate receptor, 10 brains (about 5 g) were homogenized at room temperature in 100 ml of a mixture of chloroform-methanol (C-M 2:1 v/v) and processed in the manner described by Lowney et al who essentially adapted the procedures of Folch et al. The extract was filtered, washed once with 0.2 vol of distilled water, and chilled at 5°C. 280 ml of cold diethyl ether was added to the cold $CHCl_3$ solution (70 ml). After 1 hr, the mixture was centrifuged at 5°C for 10 min at 8,000 x g. The precipitate was dissolved in 5 ml of C-M 2:1 and applied to a Sephadex LH-20 column pre-equilibrated with chroroform. The column was eluted as described previously. Aliquots (0.5 ml) of the separated fractions were dried and determined for cerebroside (C) by the phenol sulfuric acid methods or by a more specific procedure for cerebroside sulfate (CS). The absorbance of each fraction at 280 nm was also determined. Various fractions were qualitatively examined by application on precoated silica gel sheets and the chromatograms were developed by a solvent system of C:M:H_2O (35:15:2, by volume). Spots were visualized on separate plates with iodine vapor and with fluorescamine spray. Qualitative analysis for proteins and amino acids in opiate receptor fractions were carried out after dansylation and subsequent separation on a mini polyamide thin layer plate as described by Neuhoff et al.

H^3-Etorphine and H^3-naloxone binding to cerebrosides were prevented significantly by levorphanol but not by its enantiomer, dextrorphan. As shown in Table 1, the stereospecific binding of H^3-etorphine at 1000-fold excess of levorphanol plus dextrorphan was 17% of total binding.

TABLE 1

STEREOSPECIFIC BINDING OF (H^3)-NALOXONE AND (H^3)-ETORPHINE TO CEREBROSIDES

UNLABELED DRUGS	CONCENTRATION (M)	(H^3)-ETORPHINE 2×10^{-8} M (CPM ± SE)	(H^3)-NALOXONE 2×10^{-7} M (CPM ± SE)
NONE		5500 ± 70	5720 ± 110
DEXTRORPHAN (D)	2×10^{-5}	5470 ± 59	5670 ± 82
LEVORPHANOL (L)	2×10^{-5}	4561 ± 39	3988 ± 173
MORPHINE (M)	2×10^{-5}	4302 ± 23	3726 ± 51
ETORPHINE (E)	4×10^{-8}	4089 ± 20	3894 ± 108
		STEREOSPECIFIC BINDING	
D-L		909 (17%)	1682 (30%)
D-M		1168 (21%)	1944 (34%)
D-E		1390 (25%)	1776 (31%)

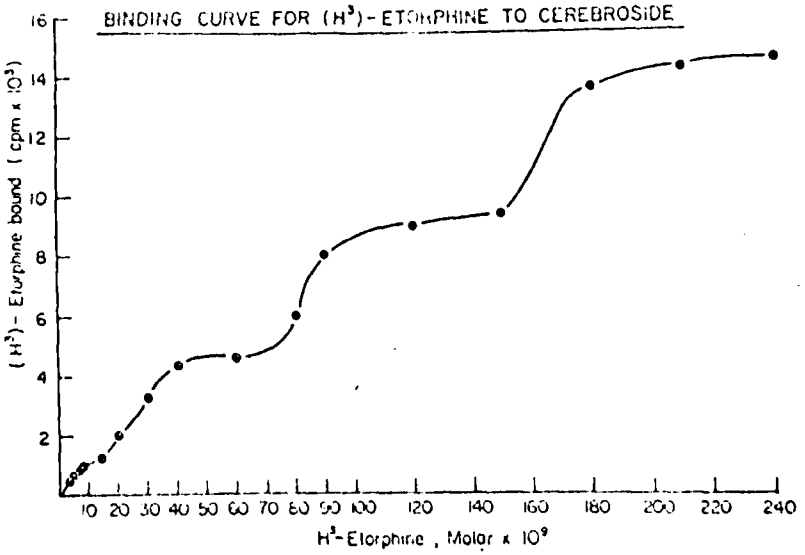
1 mg cerebroside was mixed with unlabeled drug and incubated for 2 hours. Labeled drugs were added and stereospecific binding determined as described in Methods.

For H^3-naloxone, binding at 100-fold excess of levorphanol plus dextrorphan was 30% of total binding. Similar inhibitions in H^3-etorphine and H^3-naloxone binding were observed in the presence of morphine and etorphine.

The binding of etorphine to 1.0 mgm of cerebroside was not saturable over the range 2-240 nM of H^3-etorphine. As shown in Fig. 1, four stepwise increases in binding were observed with increasing concentrations of H^3-etorphine. Additionally, the etorphine binding with increasing cerebrosides was nearly linear over the range of 0.2-0.5 mg of cerebroside, virtually constant over the range 1.0-3.0 mg of cerebroside, after which the binding slowly increased.

The concentration of dextrorphan, levorphanol, etorphine and morphine

FIG. 1



H^3-Etorphine Binding to Cerebrosides. 1 mg of cerebroside liposome was incubated at 37°C for 1 hr with increasing concentrations of H^3-etorphine (3.4 Ci/mM) in 50 mM sodium phosphate buffer.

required to inhibit the stereospecific binding of 2 x 10^{-8} M H^3-etorphine and 2 x 10^{-7} M H^3-naloxone to cerebroside was determined by log-probit analysis. As shown in Table 2, the inhibition (ID50) exhibited by these compounds closely paralleled their analgesic potencies by intraventricular administration reported by Herz et al. Levorphanol had about 10,000 times the affinity of its enantiomer, dextrorphan, in inhibiting H^3-naloxone binding and about 1,000 times that of dextrorphan in inhibiting H^3-etorphine binding. The affinity of etorphine was higher than that of morphine by 200-fold in inhibiting H^3-etorphine binding and by 13 times in inhibiting H^3-naloxone. An interesting point to note is that morphine is stronger than levorphanol in inhibiting both H^3-etorphine and H^3-naloxone binding. Consistent with this finding is the report that morphine is about 7 times more potent as an analgetic than levorphanol by intraventricular administration.

TABLE 2

RELATIVE POTENCIES OF NARCOTIC ANALGETICS IN PREVENTING STEREOSPECIFIC (H^3-NALOXONE AND (H^3)-ETORPHINE BINDING TO CEREBROSIDE

	ID_{50} (M)	
COMPOUND	(H^3)-ETORPHINE	(H^3)-NALOXONE
(-)ETORPHINE	2.5 X 10^{-8}	1.5 X 10^{-8}
(-)MORPHINE	5.0 X 10^{-6}	2.0 X 10^{-7}
LEVORPHANOL	7.0 X 10^{-6}	4.0 X 10^{-7}
DEXTRORPHAN	1.0 X 10^{-3}	5.0 X 10^{-3}

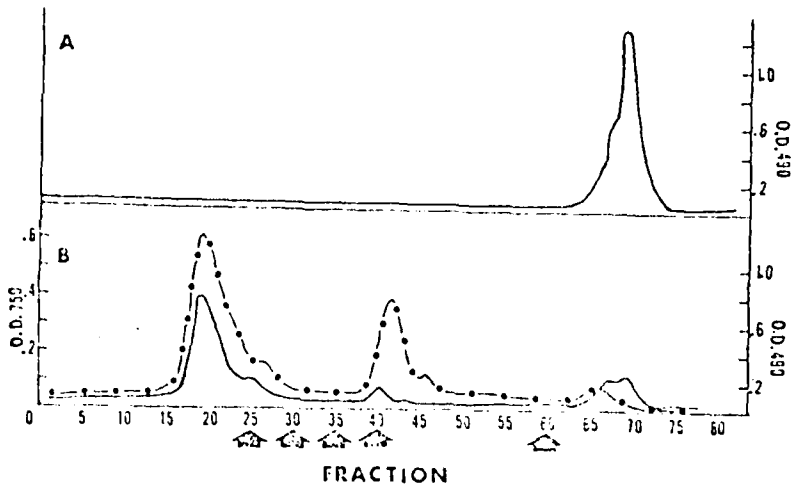
2 X 10^{-8} M H^3-etorphine and 2 X 10^{-7} M H^3-naloxone were used. The ID_{50} was defined as the concentration of drug which inhibits 50% of the stereospecific binding of H^3-etorphine or H^3-naloxone to cerebrosides. The values were determined from log-probit plots of the data.

The elution of the commercial preparation of cerebroside previously applied to the Sephadex column resulted in 3 major peaks. The first and second peaks (fractions 27 and 42) were identified as two cerebrosides by thin layer chromatography. The third peak (fraction 67) was identified as cerebroside sulfate. Determination of galactose in these fractions indicated that the commercial cerebroside preparation contained about 95% cerebroside and 10% cerebroside sulfate. It appeared to us that the cerebroside sulfate present in the chloroform:methanol (1:1) eluate was eluted in the same fraction as the proteolipid opiate receptor which Lowney et al obtained from mouse brain.

To test the possibility that CS might be the opiate receptor, levorphanol complexes of CS were prepared from commercial CS and subjected to chromatographic analysis on Sephadex LH-20 in the same manner described for the opiate receptor. Application and elution of these complexes on the column resulted in a shift of the elution pattern corresponding to that reported for the proteolipid opiate receptor. Purified cerebroside sulfate was eluted in fractions 67-72 (Fig. 2a) while the levorphanol complexes were eluted in fractions 17 - 24 (Fig. 2b). The fraction with a peak around 41 corresponded to free levorphanol. Over 90% of the total binding capacity of levorphanol was due to the CS. The pattern of the elution curve for CS and its levorphanol complex was very similar to that of the opiate receptor. The mouse brain opiate receptor was reported to be aluted around fraction 21.

On separating the opiate receptor as described by Lowney et al and subjecting it to thin layer chromatography and color development with iodine, two major spots were established to be CS (hydroxylated and non-hydroxylated) and one minor spot at the origin was not identified.

FIG. 2 (a,b)



Elution Behavior on Sephadex LH-20 of:
A - pure cerebroside sulfate (5 mgm);
B - cerebroside sulfate levorphanol complex.
The major peak around fraction 19 corresponds to the complex; the peak around 42 corresponds to free levorphanol. ——— denotes cerebroside sulfate. ●———● denotes levorphanol. The arrows indicate the fraction in which successive eluting mixtures first emerge.

When another thin layer plate was sprayed with fluorescamine, the opiate receptor fraction exhibited three very faint spots (R_f = 0.24, 0.1 and 0) while purified CS did not show any spots.

Since the purifed narcotic receptor was reported to be proteolipid in nature, we examined it and the cerebrosides as well for protein content by the fluorescamine procedure. With the opiate receptor fraction, about 20 nmoles of amine per gram tissue was obtained and, after acid hydrolysis, it was increased to 508 nmoles. The latter value is almost identical to that reported for the opiate receptor isolated by Lowney et al. When 100 nmoles hexose equivalent of the opiate receptor fraction and of commercial cerebroside were subjected to acid hydrolysis and estimated for fluorescamine fluorescene, the difference in amino nitrogen before and after acid hydrolysis for the two substances was almost identical, being 98 nmoles for the opiate receptor fraction and 110 nmoles for the pure cerebrosides (Table 3). Thin layer chromatography analysis of both acid hydrolyzates showed that only sphingosine spots were present.

A search for proteins and amino acids was conducted also in the opiate receptor fraction before and after acid hydrolysis by thin layer chromatography using the dansylation procedure on polyamide plates. With this method, which is sensitive to 5 ng of amino acids, no evidence for the presence of protein or amino acids could be detected in the separated fractions either before or after hydrolysis.

Generally speaking, stereospecific binding of morphine-like compounds is necessary but not sufficient requirement for identification of any substance as the receptor. Since many natural substances are optically active, they could interact stereospecifically with the opiate enantiomers

TABLE 3

AMINO NITROGEN CONTENT IN THE MOUSE BRAIN OPIATE RECEPTOR AND IN PURIFIED CEREBROSIDE

Preparation	Hexose Equivalent nmoles	Amino Nitrogen (nmoles) Before Hydrolysis	After Hydrolysis	Difference
Opiate Receptor	100	10	108	98
Cerebroside	100	0	100	100

An aliquot of the opiate receptor fractions and cerebrosides corresponding to 62 nmoles of hexose were dried and the residue was hydrolyzed in a sealed tube with 0.5 ml of 6N HCl at 100°C for 18 hr. The hydrolyzate was neutralized with 0.5 ml of 6N NaOH and aliquots (0.2 ml) were mixed with 1.3 ml of 0.2 M Na borate buffer pH 9.0. The solution was agitated rigorously while adding 0.5 ml fluorescamine solution (15 mg % in acetone). The fluorescence was excited at 390 mμ and the emission was read at 490 mμ in an Aminco-Bowman Spectrophotofluorometer with an external standard of 1% quinine sulfate in 0.1 N H_2SO_4 (adjusted to 100 units).

To obtain the fluorescence reading before hydrolysis, aliquots (0.5 ml) were dried and the residue was dissolved in 0.5 ml of 0.5% sodium lauryl sulfate in 0.5 N NaOH. Aliquots (0.2 ml) were mixed with 1.3 ml 0.2 M sodium borate buffer pH 9.0 and the fluorescence was developed and measured in the usual way.

with varying degrees of affinity. Indeed, compounds such as silica gel and cellulose have been reported to bind enantiomers of morphine-like compounds in different manners (Wu et al, unpublished). In addition to the demonstration of stereospecific binding of drug receptor, the correlation of its degree of affinity with drug potency should also be considered. In our experiments, narcotics not only bind to cerebroside stereospecifically (Table 1), but their binding to various drugs also parallels their reported

intraventricular analgetic potency (Table 2).

The binding of ^{3}H-etorphine to cerebroside is not staurable in the concentrations we have examined. The stepwise increase in ^{3}H-etorphine binding to cerebroside (Fig. 1) may be due to the fact that cerebrosides from commercial sources contain at least two kinds of cerebroside and cerebroside sulfate. Our preliminary data (Wu et al, unpublished) suggest that the first two apparent saturation curves at about 1.0 x 10^{-8} M and 4.0 x 10^{-8} M etorphine may be due to binding to hydroxylated and non-hydroxylated CS. Studies are in progress to obtain liposomes in yields adequate for stereospecific binding studies.

It is very interesting to note that there are striking similarities between CS and the partially purified opiate receptor recently reported by Lowney et al. CS resemble the opiate receptor with respect to its elution pattern on Sephadex column and its non-saturable nature. Moreover, the behavior of the cerebroside sulfate-levorphanol complexes on the column resembles that reported for the opiate receptor levorphanol complexes . Our preliminary data indicated that one of the glycolipids which binds narcotic stereospecifically was eluted in the same chloroform-methanol fractions as the opiate receptor. Furthermore, when this glycolipid was complexed with levorphanol, like the opiate receptor, the complexes formed could be eluted by a much less polar solvent. Thus, the elution pattern of the complexes from the two sources were virtually identical. The glycolipid was subsequently identified to be cerebroside sulfate, one of the contaminants in the commercial cerebroside.

Thin layer chromatographic studies of mouse brain extract reveal that the chloroform-methanol (1:1) fraction eluted from the Sephadex column, in which the opiate receptor is concentrated, is primarily CS. Based on the

calculated cerebroside content in this material from the galactose estimation and assuming that the fluorescence yield of sphingosine is the same as that of the leucine standard, all of the fluorescence generated from the fraction during acid hydrolysis could be accounted for by the spingosine amino group in cerebroside.

The nature of the trace fluorescamine positive material in the opiate receptor fraction detected on the thin layer silica gel plates was not identified. However, our results do not support that it could be protein, although it should be mentioned that CS is a major constituent of proteolipids. Instead, the R_f values suggest that the opiate receptor may be a phospholipid such as phosphatidylserine or a neuramine such as norepinephrine (Wu et al, unpublished). No proteins or amino acids could be identified in the acid hydrolyzates by the dansylation procedure on thin layer polyamide plates. The only compound which could be detected was sphingosine, and this substance also ixists in the hydrolyzate of pure cerebroside.

The conclusion by Lowney et al that the opiate receptor purified from mouse brain is a proteolipid was based on several premises. The procedures which were applied for the extraction of the receptor are essentially those used for the islation of proteolipids. The protein and levorphanol eluted in each fraction was monitored by both the Lowry reagent and by ultraviolet absorption. The purified fraction, which was separated, yielded a large increase in amine content by the fluorescamine method after acid hydrolysis. However, u.v. absorbance and the Lowry procedure can only be used as rough measures because of their non-specificity. Even th-

fluorescamine method which is specific for primary amines cannot be used as an absolutely reliable index for protein since γ-aminobutyric acid, β-alanine, histamine, catecholamines, amino sugars, spermine, spermidine and lipid hydrolyzate products such as sphingosine also yield intense fluorescence with fluorescamine. Based on our data, therefore, it would appear that the purified opiate receptor in mouse brain obtained by extraction and column chromatography is mostly cerebroside sulfate.

In summary, we have found a group of endogenous glycolipids which elicit stereospecific binding with high affinity to narcotics in accordance with their analgetic potency. Furthermore, we have provided evidence that one of the cerebrosides, cerebroside sulfate, behaves similarly to the purified proteolipid narcotic receptor isolated from mouse brain. Although the stereospecific binding of opiates to these glycolipids may be coincidental and unrelated to the pharmacologic effects of opiates, the possibility that cerebrosides may in some way be related to the opiate binding sites, or even the opiate "receptor," should be considered.

Since CS fulfills all the requirements as an opiate binding site, we propose to use this as a model receptor to study the molecular interactions between opiate agonist or antagonist with their receptors. One of the important problems in elucidating the mechanism of opiate-receptor interaction at the molecular level is explaining how opiate antagonist attenuates the agonist action. The displacement of agonist by antagonist does not provide a satisfactory explanation for the difference in pharmacological action between agonist and antagonist. The terms "efficacy" or "intrinsic factor" were, therefore, introduced in order to explain the difference in pharmacological action between agonists and antagonists. However, the nature of "efficacy" at the molecular level is not known. The possibility that the term "efficacy" may be related to the physicochemical properties of opiate-receptor complex has been implicated.

In opiate-receptor interaction, the importance of the electrostatic bond formation between the protonated nitrogen of opiate and the anionic group of the receptor has been emphasized repeatedly. Further studies from our laboratory, using CS as a model receptor have provided evidence that this electrostatic bond formation also distinguishes the action of agonists from antagonists. The former favor the formation of intimate ion pairs while the latter favor the formation of hydrated ion pairs. To extend these findings, we studied the partition of H^3-cerebroside sulfate (H^3-CS) between aqueous phase and non-aqueous phases (heptane and interface) in the presence of opiates, ions and interactions with other acidic lipids.

The radioactive CS in the absence of cation or opiate was distributed about 90% in whater phase, 9% at the interface and less than 1% in heptane phase as shown in Fig. 1. However, in the presence of increasing concen-

tration of cation or opiate, the amount of H^3-CS in water phase decreased, resulting in the increase of the radioactivity at the interface and heptane phase but the radioactivity in heptane was negligible. The degree of the partition from the aqueous micelles to the non-aqueous micelles was dependent upon the concentration of the cation, opiate or phosphatidylserine added.

FIG. 1 - A schematic representation of the distribution of H^3-CS. 1 ml of aqueous H^3-CS micelles (10 ug/ml) was mixed with 1 ml of heptane and the distribution of H^3-CS was determined.

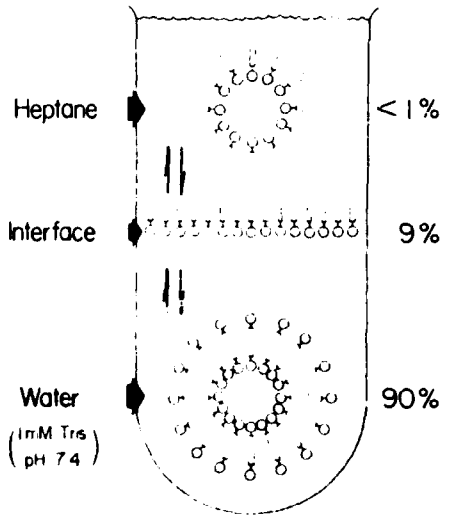


Fig. 2 shows the redistribution of H^3-CS between aqueous phase and the nonaqueous phases with increasing concentrations of Ca++ or Na+. The concentration of Ca++ and Na+ required to increase H^3-CS in the nonaqueous phases to half maximum were 5.5 mM and 600 mM, respectively. Based on these values, calcium is about 100 times more potent than sodium in inducing the transfer of H^3-CS from water to the non-aqueous phases. This is compatible with the data obtained from the turbidity studies of CS induc-d by calcium and sodium ions which indicate that the calcium salt of CS is more hydrophobic in nature than the sodium salt in CS.

The partition of H^3-CS was also studied with increasing concentration of an opiate agonist, GPA-1657, and its corresponding antagonist, GPA-2163. The latter is known to be a pure antagonist. As shown in Fig. 3, the agonist, GPA-1657, is about 30 times more potent than its corresponding antagonist, GPA-2163, in inducing the transfer of H^3-CS. The partition coefficients of GPA-1657 and 2163 between water and heptane ($P_{H/W}$) are 0.34 and 11, respectively. After correction for the drug partition, it is noted that the agonist is 100 times more potent than its corresponding antagonist. In conjunction with the result of Ca - CS and Na - CS as shown in Fig. 2, these data indicate, as we reported earlier, that agonist - CS complex is more hydrophobic than the CS complex formed with its corresponding antagonist. More importantly, this finding strongly suggests tha the electrostatic interaction between the protonated nitrogen of the drug and anionic sulfate group of the CS is crucial to distinguish the agonist from its corresponding antagonist.

The antagonism of the agonist-induced redistribution was tested in the presence of 20 uM of the antagonist. It should be noted that this amount of antagonist does not induce redistribution of CS (Fig. 3)

by itself.

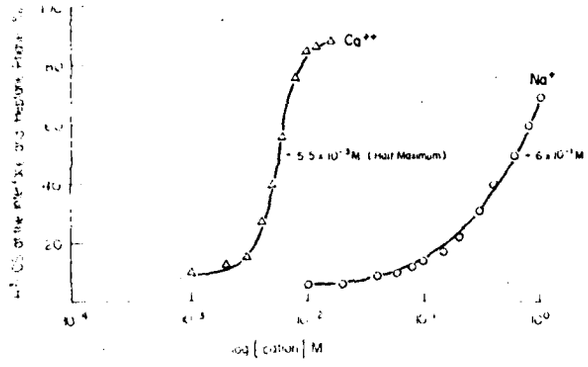


FIG. 2

Percentage of H^3-CS in the Nonaqueous Phases Induced by Ca^{++} and Na^+. Ordinate: percentage of H^3-CS at the interface and heptane phase. Abscissa: concentration of calcium or sodium in logarithm scale. The redistribution of H^3-CS (10 µg/ml) was determined with increasing concentration of Ca^{++} or Na^+ at PH 7.4 as described in Methods.

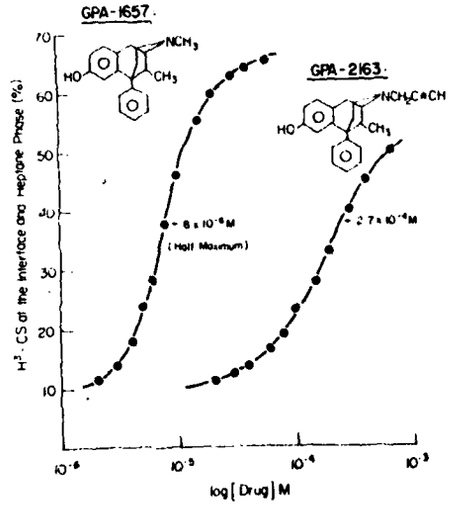


FIG. 3

Percentage H^3-CS in the Nonaqueous Phases Induced by GPA-1657 or GPA-2163. Redistribution of H^3-CS induced by GPA-1657 or GPA-2163. Ordinate: percentage of H^3CS at the interface and heptane phase. Abscissa: concentration of GPA-1657 or GPA-2163 in logarithm scale. The amount of H^3-CS in the nonaqueous phases was determined with increasing concentration of the drugs, at pH 7.4 as described in Methods. The values are derived from the means of triplicate determinations of two separate experiments.

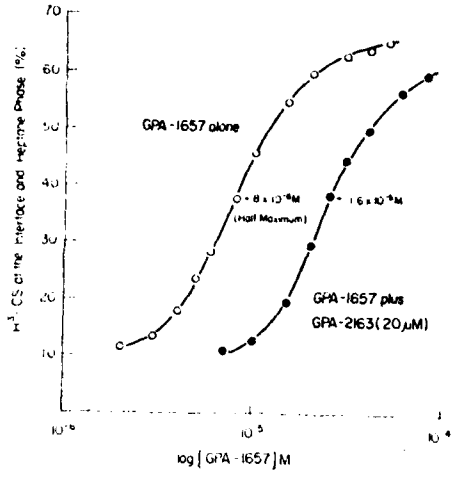


FIG. 4

Inhibition of Agonist-Induced H^3-CS in the Nonaqueous Phases by Antagonist. The percentage of H^3-CS in the nonaqueous phases induced by an opiate agonist (GPA-1657) was determined in the absence and presence of its corresponding antagonist (GPA-2163). 10 μg of H^3-CS and 20 μM of GPA-2163 were used.

The results in Fig. 4 show that the concentration of the agonist (GPA-1657) required to induce the redistribution to half maximum was 2.0 times higher than the value without the presenc ef the antagonist (GPA-2163).

Since there are acidic lipids other than CS present in membranes as native membrane constituents, the effect of phosphatidyl serine (PS), another membrane acidic lipid with a higher degree of hydration, on the agonist (GP-1657) induced redistribution of H^3-CS was also examined. The degree of distribution was determined with increasing concentrations of GP-1657. The data are shown in Fig. 5. In the presenc eof 20 ug/ml

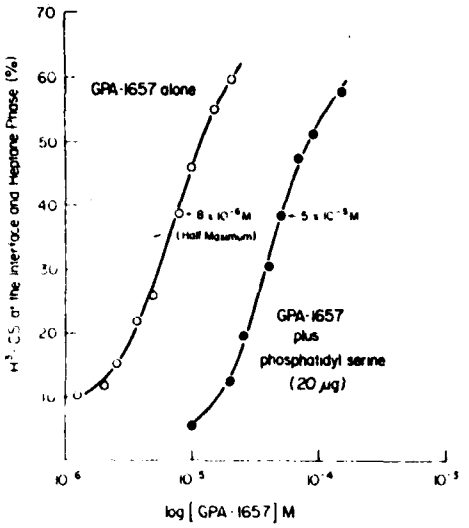


FIG. 5

Inhibition of GPA-1657-Induced H^3-CS Partition by Phosphatidyl Serine. Mixed micelles were prepared with CS (10 µg/ml) and PS (20 µg/ml), and the percentage of H^3-CS in the nonaqueous phases induced by GPA-1657 was determined as described in Methods.

of PS in the mixed micelles, the concentration of GPA-1657 needed to induce the redistribution of H^3-CS to half maximum was 6 times higher than the value obtained in the absence of PS in the micelles. These data indicate that PS is more potent than the corresponding opiate antagonist, GPA-2163, in inhibiting the redistribution. However, the antagonism of agonist induced redistribution by PS is apparently different from the antagonism by GPA-2163. The facts that PS is more polar than CS and that the redistribution of PS is not induced by the same concentration of opiate agonist (unpublished data) suggest that PS not only inhibits opiate binding to CS

but also decreases the hydrophobicity of CS by increasing the dielectric constant of the micelles. It should also be noted that the effect of PS cannot be explained by the decreases in effective drug concentration due to binding of PS to opiates. Furthermore, our preliminary data also shows that other phospholipids such as phosphatidylinositol, triphosphoinositide, phosphatidylcholine and phosphatidyl ethanol amine also inhibit the agonist induced partition of H^3-CS in the nonaqueous phases (unpublished data).

In conclusion, the data presented seem to indicate that the distribution of H^3-CS between aqueous and the nonaqueous micelles is determined by the hydrophobic-hydrophilic balance of the lipid. This balance may be dependent upon the degree of dissociation (hydration) of the electrostatic bond between cation (or opiate) and sulfate group of CS. Calcium ion as well as opiate agonist (GPA1657) tend to decrease the dissociation of the electrostatic bond, and thereby increase the hydrophobicity of CS, whereas sodium ion and opiate antagonist (GPA-2163) tend to increase the dissociation of the electrostatic bond resulting in the increase of hydrophilicity. PS, a membrane acidic lipid, with a higher degree of hydration decreased the hydrophobicity of CS by forming mixed micelles with CS.

The true significance of the change of the physicochemical properties of acidic lipids by opiate is not clear. However, if it occurs <u>in vivo</u> after the drug combines with the membrane acidic lipid, it would be tempting to speculate that this physicochemical property may be related to the conformational changes which could be coupled with a part of the receptor

mechanism leading to the pharmacologic action of opiates. In this case, the hydrophilic and hydrophobic complexes would play a role as the transduction mechanism between drug-receptor binding and the step eliciting a pharmacologic response.

PUBLICATIONS SUPPORTED IN FULL OR IN PART BY DADA17-73-C-3006

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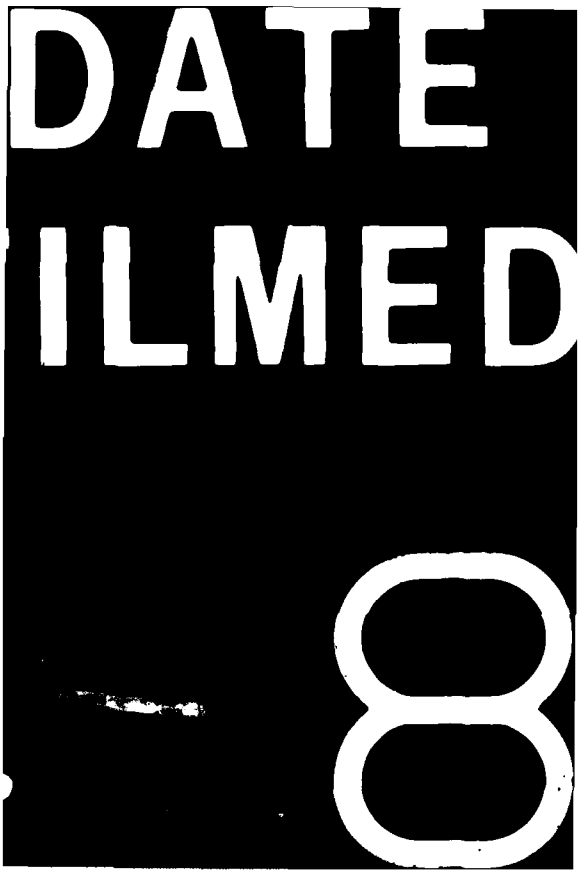
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