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N-dansyl-(D-ala$^2$)-met$^5$-enkephalin as a Fluorescent Probe of Opiate Binding Sites in Mouse Spinal Cell Cultures

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Sanford Harvey Feldman

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# N-DANSYL-(D-ALA$^2$)-MET$^5$-ENKEPHALIN AS A FLUORESCENT PROBE OF THE OPIATE BINDING SITES IN MOUSE SPINAL CELL CULTURES

By

Sanford Harvey Feldman

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ABSTRACT

N-DANSYL-(D-ALA$^2$)-MET$^5$-ENKEPHALIN AS A FLUORESCENT PROBE OF THE OPIATE BINDING SITES IN MOUSE SPINAL CELL CULTURES

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These studies were carried out to utilize a fluorescent enkephalin derivative as a probe of the opiate receptor microenvironment. (D-ala$^2$)-met$^5$-enkephalin was successfully labeled with 1-(5-dimethylaminonaphthalene)-sulfonyl (dansyl) at the amino terminal of the pentapeptide. The fluorescent enkephalin derivative exhibited an emission maximum at 515nm in ethanol, attributed to the formation of a sulfonamide bond. Energy transfer was observed from the tyrosine residue to the dansyl group. The dansyl enkephalin emission was sensitive to the dielectric constant of several solvents.

N-dansyl-met$^5$-enkephalin was required at 60 times the concentration of the unlabelled parent peptide to elicit the same inhibition of contraction of the electrically stimulated guinea pig ileum, in the presence of 286mM ethanol. While 396.5nM met$^5$-enkephalin decreased the contraction of the ileum by 81%, adding 9nM naloxone could reduce this to only 30% inhibition, in the presence of the same concentration of opiate agonist. In the presence of 286mM ethanol, 396.5nM met$^5$-enkephalin caused an 81% decrease in the ileum contraction, which could not be reversed at all by 9nM naloxone. Ethanol was therefore felt to decrease the opiate

receptor's affinity for the opiate antagonist.

N-dansyl-(D-ala$^2$)-met$^5$-enkephalin was administered to living cultured mouse spinal cord and dorsal root ganglion cells. The emission spectrum of the fluorescent enkephalin bound to the cells in culture has a maximum at either 481nm or 504nm depending upon the type of opiate binding site. In Puck's balanced salt solution injections of unlabelled (D-ala$^2$)-met$^5$-enkephalin onto any single spinal cell exhibiting 504nm emission caused the fluorescence emission to shift to 481nm. Only about 3% of the spinal cord cells which bound the fluorescent enkephalin exhibited a 504nm emission in several of the cultures used, other spinal cell cultures had no opiate binding sites from which 504nm N-dansyl-enkephalin emission was detected. All cultures tested had 481nm emission from opiate binding sites, but not all cells were stained with the N-dansyl-enkephalin. None of the 491nm emission from N-dansyl-enkephalin bound to receptor sites could be shifted from its 481nm maximum, by injections of unlabelled enkephalin. The data was interpreted to indicate a stereospecific opiate binding site of dielectric constant of about 10 (504nm emitting), and a nonspecific binding site of dielectric constant 7. The higher dielectric constant of the stereospecific binding site indicates it may be either in closer proximity to the aqueous environment than the nonspecific binding site, or it is of a higher polarity than the nonspecific opiate binding site.

This thesis is dedicated to
Doris P. Lance, in return
for her dedication to its author

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## LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| ACTH | adrenocortiocotrophic hormone |
| ala | alanine |
| asn | asparagine |
| β-EPH | β-endorphin |
| β-LPH | β-Lipotorpin |
| β-MSH | β-melanocyte stimulating hormone |
| $Ca^{2+}$ | Calcium ion (++) |
| $CH_3$- | methyl |
| $CO_2$- | carboxyl |
| CNS | central nervous system |
| CS | cerebroside sulfate |
| $Cu^{2+}$ | Cupric ion (++) |
| DDT | N,O-didansyl-L-tyrosine |
| DME | (D-$ala^2$)-$met^5$-enkephalin |
| DMSO | dimethyl sulfoxide |
| dansyl | 1-(5-dimethylaminonapthalene)--sulfonyl |
| E | enkephalin |
| FdU | 5'-Fluoro-2'-deoxyuridine |
| γ-LPH | γ-Lipotropin |
| gly | L-glycine |
| H- | proton |
| HRP | horse radish peroxidase |

LIST OF SYMBOLS AND ABBREVIATIONS (cont.)

| | |
|---|---|
| $La^{3+}$ | Lanthanum ion (+++) |
| LE | leucine$^{5}$-E |
| $Li^{+}$ | lithium ion (+) |
| ME | methionine$^{5}$-E |
| $Mu^{2+}$ | Manganese ion (++) |
| $Na^{+}$ | Sodium ion (+) |
| NDME | N-dansyl-(D-ala$^{2}$)-ME |
| $NH_2$- | amino |
| NME | N-dansyl-ME |
| NMR | nuclear magnetic resonance |
| NT | N-dansyl-L-tyrosine |
| ODT | O-dansyl-L-tyrosine |
| -OH | hydroxyl moiety (-1) |
| PBS | Puck's balanced salt solution |
| phe | L-phenylalanine |
| PM | photomultiplier sensitivity setting |
| PS | phosphatidyl serine |
| trp | L-tryptophan |
| tyr | L-tyrosine |

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## PREFACE TO THE INTRODUCTORY SECTION

The following introductory section was written with a dual purpose in mind. It is hoped that the reader will become acquainted with the opioid literature, as well as with the rationale behind this thesis research. The first two sections ("Background" and "Characterization of the Enkephalins") were written to acquaint the reader with the historical perspective of opiate research leading to the discovery of the endogenous opioid pentapeptides. Some aspects of the proposed biosynthesis and degradation of the enkephalins are also discussed.

The following section termed the "Structural Pharmacology of the Enkephalins" contains several points extremely pertinent to the rationale behind labeling the amino terminal of enkephalins. The reader is asked to pay special attention to the subheading numbered (4), and the summary immediately following. The section on "Heterogeneous Populations of Opiate Receptors", as does the previous section, lends several clues as to why dansyl-enkephalins may retain high pharmacological activity in the guinea pig ileum assays.

The section on the "Relationship Between Opiate Receptor Binding and Ionic Composition" is for the reader's

further information about the fluctuating conformation of opiate binding sites. This section is not pertinent to the thesis research. The section on fluorescent enkephalin analogues lends credence to this author's feeling that such opiate derivatives may prove to be valuable as probes of the opiate binding site environment. The sections on distribution of opiate receptors and enkephalins in the CNS is purely for the neuro-anatomical information of the reader. Mouse spinal cell cultures were used in the study because the procedure for plating these cultures is well documented in the literature (Ransom, et.al., 1977), and they have been demonstrated to contain opiate receptors (Macdonald, et.al., 1978).

# INTRODUCTION

## Background

The historical perspective of opiate influence on man is one of both alleviation and causation of suffering. Individuals throughout history have utilized opium derivatives for their pharmacological and therapeutic properties, or else to savor their euphoric effects. Those who misuse the opiates eventually suffer the effects of tolerance-addiction and physical withdrawal. As analgesics, the opiates are the most effective drugs available. Opiates are also employed in the treatment of diarrhea and coughing, but exhibit the side effects of respiratory depression, mood modification, and altering the levels of some hormones (Miller, et. al., 1978).

In the last decade major advances have been made at the molecular level in understanding the mechanism of action of opiate derivatives.

The entity which recognizes the opiates, and initiates the biochemical and physiological changes when bound to the opiate, is termed the opiate receptor. The concept of the opiate receptor was initiated by the development of various pharmacological assay systems, that were sensitive to the effects of opiate derivatives. For example, the electrical

stimulation of the guinea pig ileum (Schaumann, 1957), or the mouse vas deferens (Henderson, et. al., 1972) would cause the tissue to contract. This was the result of acetylcholine release in the case of the ileum and norepinephrine release in the case of the vas deferens, from autonomic neurons in response to the electrical stimulation. Morphine was demonstrated to inhibit the release of these neurotransmitters in the tissues preventing their contraction. This action of opioid compounds was shown to have high specificity in the pharmacological sense. Stringent stereospecific structural requirements had to be present before an opioid would exhibit this agonistic activity. Modification of this structure produced molecules that acted as partial agonists, or antagonized this activity (Kutter, et. al., 1970; Agarwal, et. al., 1977; Shaw, et. al., 1978; Chorev, et. al., 1979). There appeared to be good correlation between the potency in these bioassays and the analgesic potency in vivo. This suggested the analgetic effects were mediated by a receptor similar to that mediating the effects in the bioassays (i.e. inhibiting neurotransmitter release). Morphine was known to inhibit acetylcholine release in the cerebral cortex (Jhamandas, et. al., 1971) and caudate nucleus (Yaksh, et. al., 1975) of the cat, as well as norephinephrine release in the cerebral cortex of the rat (Arbilla, et. al., 1978).

In 1973 neuropharmacologists utilized radioactive opiate agonists and antagonists to demonstrate a

stereospecific interaction of the tritiated ligand with some entity in brain synaptic membranes (Terenius, 1973; Pert, et. al., 1973; Simon, et. al., 1973) and guinea pig ileal homogenates (Creese, et. al., 1975a). It was demonstrated that only opioid drugs interacted selectively with the opiate receptor (Snyder, 1977), and not cholinergic, adrenergic, serotonergic, or histaminergic compounds. The electrical stimulation of the periaqueductal gray in the midbrain produces analgesia which is reversible with opiate antagonists (Mayer, et. al., 1976). This signified the CNS was capable of releasing a morphomimetic substance. In light of these results, researchers set out to isolate this opioid from brain extracts, and identify it by means of the available bioassays. Extracts that would inhibit the contraction of the guinea pig ileum in an antagonist reversible fashion were found in brain (Hughes, et. al., 1975; Terenius, et. al., 1975; Pasternak, et. al., 1975), pituitary fluids (Cox, et. al., 1975; Teschemacher, et. al., 1975) and gut (Puig, et. al., 1977; Schultz, et. al., 1977). These opiate extracts produced analgesia after intraventricular injection into rats (A. Pert, et. al., 1977). The opioid activity in brain extracts was localized in the synaptosomal fraction after subcellular fractionation (Simantov, et. al., 1976a). The results of one phylogenetic study demonstrated the presence of opioid-like material in a variety of species (Simantov, et. al., 1976b). Vertebrates ranging from man to the hagfish all possess this morphomimetic factor, with

the toad possessing the highest concentration (per kg body weight). No insects thus far has been found to contain the endogenous opioid.

Characterization of the Enkephalins

In 1975 Hughes and coworkers elucidated the structure of two endogenous opioid pentapeptides isolated from porcine brain, which they named "enkephalin". These peptides had the sequences $H_2N$-tyr-gly-gly-phe-met-$CO_2$ (ME) and $H_2N$-tyr-gly-gly-phe-len-$CO_2$ (LE). In porcine brain ME was the predominant peptide (Hughes, et. al., 1975) while LE was predominant in bovine brain (Simantov, et. al., 1976a). The sequence of ME was known to be contained within the hormone β-Liptropin (β-LPH), a 91 amino acid peptide of mass 11.7 kdaltons isolated from ovine pituitaries, which also had opioid activity and contains the amino acid sequences of β-melanocyte stimulating hormone (β-MSH), (Graf, et. al., 1973).

It has been suggested that ME has a biosynthetic origin similar to that displayed by a mouse pituitary tumor cell line (Mains, 1977). A large glycoprotein termed "Big-ACTH", M.W. = 35 kdaltons, is synthesized in a pituitary cell which contains one copy of each of the amino acid sequences of adrenocorticotrophic hormone (ACTH), M.W. = 23 kdaltons, and β-LPH (Nakanishi, et. al., 1979; Pederson, et. al., 1980). There is a sequence homology common to both ACTH and β-LPH refered to as γ-LPH, which contains the amino acid sequence of β-MSH. When β-LPH is cleaved to γ-LPH, it

yields a 31 amino acid fragment from the carboxyl terminus called β-endorphin (β-EPH), M.W. = 3.5 kdaltons. The initial 5 amino acids on the carboxyl terminus of β-EPH are ME.

It would seem a needless expense of energy for a neuron to synthesize "Big-ACTH" in order to obtain the ME sequence by a series of enzymatic cleavages, and would leave unexplained the biosynthesis of LE. It is hypothesized that elsewhere in the CNS enkephalins are produced by other means, since such small peptides are not known to be manufactured on ribosomes in eucaryotes (Miller, et. al., 1978). Both enkephalins are probably derived from larger precursors, but no precursor as yet has been found for LE.

The opiate receptor affinities of β-EPH and ME are the same, however β-EPH has a more potent analgesic action than ME. ME is degraded by enzymatic cleavage of the amino terminal tyrosine residue, though other degradation modes exist (Lane, et. al., 1977). The tyr of ME is not exposed in β-EPH, imparting stability to the ME sequence from enzymatic degradation.

## Structural Pharmacology of the Enkephalins

Since their discovery, the enkephalins have been synthesized from their parent amino acids in vitro, with many structural modifications. The following conclusions have been drawn based upon which amino acid was substituted for, modified, or deleted (Miller, et. al., 1978):

1) As the length of the R-group of the amino

acid in position 5 decreases, lipophilicity decreases concommitant with pharmacological potency, thus ME is more potent an agonist than LE, The oxidation of the ME sulfur moiety to a sulfoxide reduces agonist activity. The position 5 amino acid may be cleaved, leaving reduced agonistic activity of the quadrapeptide. By reducing the position 5 carboxylic acid to an alcohol, activity is greatly enhanced.

(2) The low potency of $tyr^4$-ME and $leu^4$-LE suggests position 4 may only be a nonphenolic aromatic moiety. Analogues with $trp^4$-ME and p-$CH_3$-$phe^4$-ME have normal and enhanced activity, respectively, while p-$NH_2$-$phe^4$-ME has reduced activity.

(3) All substitutions to replace either or both of the glycines in positions 2 and 3 reduces activity. This is thought to represent a position of molecular flexibility by virtue of the absence of sterically hindering sidegroups. Thus the glycines allow the pentapeptide to achieve the proper receptor binding conformation. Asparagine (asn) substituted into position 3 reduces analgesic activity, but enhances antidiarrheal activity. This may represent a difference in the complementarity of the two

(or more) types of opiate receptors (i.e. heterogeneous receptor populations). Placing a D-conformer amino acid in positions 2 and/or 5 decreases receptor affinity, but increases the stability of the pentapeptide to enzymatic degradation.

(4) Modification of the tyr phenolic group decreases activity dramatically. The position 5 carboxyl and the position 1 amino groups may be modified without significant reduction in activity. (Terenius, et. al., 1976; Canada, 1979; Canada, et. al., 1980). There is a progressive decrease in opiate receptor affinity in going from primary to secondary to tertiary amino derivatives which is not observed in the guinea pig ileum assay (Lord, et. al., 1977). The amino terminal in opium alkaloids is a tertiary one (Kutter, et. al., 1970), not a primary α-amino group as in the enkephalins.

Thus, the only stringent structural requirement of agonist-activity is that the phenolic group of tyr and the phenyl group of phe must be held in some rigid spacial relationship, all other modifications enhance or diminish the opioid agonist activity. Energy transfer measurements from $tyr^1$ to $trp^4$ in $trp^4$-ME elucidated the distance between these aromatic moieties to be $10 \pm \mathring{A}$ (Schiller, 1977). This close distance suggests a $gly^2$-$gly^3$ β-pleated sheet bend, probably

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stabilized by a 4-1 or 5-2 hydrogen bond. NMR spectra of ME in DMSO revealed that the $gly^2$ carbonyl forms a hydrogen bond with the $met^5$ amide group holding the amino and carboxyl terminals in close proximity (Jones, et. al., 1977).

Heterogeneous Populations of Opiate Receptors

Evidence of structural differences distinguishing multiple types of opiate receptors exists. It was previously mentioned that $asn^2$ - ME is a weak analgesic but a potent antidiarrheal drug (Miller, et. al., 1978). Addition of a hydrazine moiety to the opiate antagonist naloxone forms the more selective antagonist naloxazone. Naloxazone administration to mice blocks opiate agonist analgesia but not respiratory depression (Pasternak, et. al., 1980). Rat brain homogenates exhibit two opiate binding sites. One site has high enkephalin affinity, and low naloxone and morphine affinity (enkephalin receptor). The other binding site has low enkephalin affinity, and high morphine and naloxone affinity (morphine receptor) (Chang, et. al., 1979). It was found that the mouse vas deferens bioassay gives analogous results to brain homogenate binding assays and in vivo analgesia studies, while guinea pig ileum behaves differently (Lord, et. al., 1977).

Sulfatides and phospholipids exhibit stereospecific binding to opioids. Cerebroside sulfate (CS) is a high affinity receptor for dihydromorphine (Loh, et. al., 1974), while phosphatidyl serine (PS) has a lesser affinity for

morphine (Abood, et. al., 1975). PS enhances the inhibition of the guinea pig ileal contractions elicited by morphine, while phosphatidyl choline diminishes this inhibition (Brown, et. al., 1976). PS enhances opiate agonist binding to brain membrane fragments (Abood, et. al., 1976a). Phospholipases-$A_2$ or -C inhibit opiate agonist binding to brain membrane fragments, while binding can be restored by the addition of PS (Abood, et. al., 1978). The higher the degree of saturation of the phospholipid acyl chains, the greater the opiate agonist binding (Abood, et. al., 1976). This decrease in membrane fluidity enhancing opiate agonist binding due to acylchain unsaturation appears to be in conflict with increases in temperature enhancing opiate agonist binding (Creese, et. al., 1975b). Differential scanning calorimetry demonstrated opiate agonist binding caused increased fluidity of rat brain mitochondrial membrane preparations, and was reversible both in vitro or after in vivo administration of naloxone (Hosein, et. al., 1977).

The controversy as to whether CS is one of the opiate receptors is still unresolved. A genetic leukodystrophic mutant mouse with a deficiency in brain sulfatides exhibited a decreased sensitivity to morphine compared to control mice (Law, et. al., 1978). Mouse neuroblastoma cell line (N4TG1) with a high opiate receptor density did not exhibit significant quantities of CS (Dawson, et. al., 1978). Antibodies which were made to CS and injected into peri-aqueductal gray in the intact rat inhibited the ability of

morphine and β-endorphin to produce analgesia (Craves, et. al., 1980).

## The Relationship Between Opiate Receptor Binding and Ionic Composition

Calcium ($Ca^{2+}$) Harris et al., (1976) and coworkers demonstrated that intraventricular injections of lanthanum ($La^{3+}$) into rat and mouse had antinociceptive effects. The analgesic effect produced by $La^{3+}$ could be abolished by intravenous injections of either naloxone or $Ca^{2+}$. Cross-tolerance between morphine and $La^{3+}$ was also demonstrated in morphine-tolerant mice. These researchers concluded that $La^{3+}$ physical properties allowed it to bind strongly to $Ca^{2+}$ binding sites, inhibiting $Ca^{2+}$ movement. At the presynaptic axon terminal, $Ca^{2+}$ influx in response to terminal depolarization leads to synaptic vesicle fusion to the axon terminal's membrane and subsequently neurotransmitter release. $La^{3+}$ blocks the $Ca^{2+}$ influx into the persynaptic terminal blocking neurotransmitter release. It was later shown that morphine injections blocked $^{45}Ca^{2+}$ uptake into synaptic vesicular fractions, when $^{45}Ca^{2+}$ and morphine were simultaneously administered to rats (Harris, et. al., 1977). The blockage of $Ca^{2+}$ influx into presynaptic terminals was hypothesized to be the mechanism by which opioids are able to block neurotransmitter release in the CNS as well as in bioassays (Cardenas, et. al., 1976). Cardenas, et. al., (1976) studied $^{45}Ca^{2+}$ uptake into synaptosomal fractions and found a 7-fold

decrease in $^{45}Ca^{2+}$ uptake during morphine treatment. This was reconfirmed by Guerrero-Munoz, et. al., (1979a). Conversely, calcium injections antagonize morphine inhibition of acetylcholine release in rat cerebral cortex (Jhamandas, et. al., 1978). Recently, β-EPH was found to also inhibit $Ca^{2+}$ uptake in the intact mouse brain (Guerrero-Munoz, et. al., 1979b).

Copper ($Cu^{2+}$) Marzullo, et. al., (1977) demonstrated a $Cu^{2+}$-Glutathione complex normally found in rabbit erythrocytes was capable of inhibiting binding of opiate agonists to rat brain membrane homogenates. The following transition metal ions are ranked by potency for their ability to inhibit opiate agonist binding: mercury, silver, copper, zinc, and lead. These workers later found that copper in the +2 oxidation state when complexed with glutathione exhibited a greater inhibitory effect on opiate agonist binding than either $Cu^{+2}$ or glutathione alone (Marzullo, et. al., 1980). These workers hypothesized a redox coupling between copper and the opiate receptor. Furthermore, the researchers felt that the oxidation state of the opiate receptor determines its agonist and antagonist affinities by mediating receptor conformation changes.

Sodium ($Na^{+}$) Pert, et. al., (1975) found a 60% enhancement of opiate antagonist affinity for the opiate receptor in the presence of 50mM $Na^{+}$, in the rat brain homogenate binding assay. This same enhancement could be mimicked by 150 mM lithium ($Li^{+}$), but not by potassium,

rubidium, or cesium. Similar work by Simon, et. al., (1975) on the inactivation of opiate receptor binding in brain homogenates by sulfhydril reagents, showed slow inactivation in the presence of $Na^+$ compared to controls. This confirmed the cited results, that $Na^+$ causes a conformational change of the opiate receptor making it less susceptible to sulfhydril reagents. Utilizing this $Na^+$ effect, Akera, et. al., (1975) demonstrated evidence of two types of naloxone binding sites in rat brain homogenates.

Manganese ($Mn^{2+}$) Pasternak, et. al., (1975) showed that 1mM concentrations of this divalent cation enhanced opiate agonist binding to rat brain homogenates by 43%.

## Fluorescent Analogues of Opiate Agonists

Several fluorescenct probes have been linked to the endogenous opiate agonist ME in attempts to develop a fluorescent probe of the opiate receptor (Fournie-Zaluski, et. al., 1978; Hazum, et. al., 1979a; Canada, et. al., 1980). The probe dansyl, 1-(5-dimethylaminonaphyhalene)-sulfonyl has been positioned either directly on the pentapeptide's amino terminal, or by use of N-amino alkyl dansylamides on the carboxyl terminal. The amino dansylated ME was shown to have 4% of the potency of LE, while the carboxyl dansylated ME had 86% the potency of LE (Fournei-Zaluski, et. al., 1978). The fluorescent probe rhodamine has been positioned on the ε-amino group of lysine, which was then added to the carboxyl terminal of LE in position 6. The

rhodamine LE was able to inhibit the binding of unlabeled $^{125}$I-LE to rat brain homogenate, and has been used to visualize opiate receptor clustering on neuroblastoma x glioma hybrid cell line (Hazum, et. al., 1979b).

Distribution of Opiate Receptors in Monkey Brain

Kuhar, et. al., (1973) assayed the binding of tritiated dihydromorphine to tissue homogenates made from dissected portions of monkey brain. By far the largest density of opiate receptors were located in the amygdala; less dense were the periaqueductal gray, hypothalamus, and medial thalamus; less dense still were the caudate, putamen, frontal cortex, temporal gyrus, hippocampus, interpeduncular nucleus, and the spinal cord dorsal gray matter.

Distribution of Enkephalins in Rat Brain by Immunohisto-Fluorescence

The intensity of fluorescent antibodies staining for enkephalin in the CNS is not quantitative being classified as heavy, moderate, or light. The distributions of LE and ME positively stained fibers appears to be the same, but cross-reactivity of LE antibodies with ME occurs, as does that of ME antibodies with LE. Therefore the immunofluorescence technique may not be able to distinguish the difference in regional distributions between the two enkephalin pentapeptides (Miller, et. al., 1978). The following immunofluorescent staining were reported to appear in axonal

fibers and terminals unless otherwise stated:

Telencephalon - olfactory tubercle (light), globus pallidus (heavy), candate-putamen (medium), nucleus accumbens (light), lateral septal nucleus (light), interstitial nucleus of the stria terminalis (heavy), central amygdalar nucleus (heavy), medial amygdalar nucleus (light). Stained cell bodies were found in the nucleus accumbens, caudate-putamen, central amygdalar nucleus, and the interstitial nucleus of the stria terminalis.

Diencephalon-ventromedial hypothalamic nucleus (heavy), paraventricular nucleus (heavy), medial preoptic nucleus (light), dorsomedial hypothalamic nucleus (light), subthalamic nucleus (light). Cell bodies in the medial preoptic nucleus were immunopositive.

Thalamus weak to medium staining occurred in the following groups: anterior medial nucleus, anterior ventral nucleus, ventromedial nucleus, ventral thalamic nucleus, lateral habenular nucleus, nucleus periventricularis rotundis cellularis, thalamic reticular nucleus and the medial thalamic nucleus.

Mesencephalon - periaqueductal gray (heavy), reticular formation (heavy), substantia nigra zona compacta (moderate), interpeduncular nucleus (heavy), locus coeruleus (moderate), lateral and medial parabrachial nuclei (light), pontine raphe nuclei (light); cranial nerve motor nuclei IV, VII, X, and XII (all light); nucleus ambiguus (light), magnocellular reticular nuclei (light), nucleus of the tractus

solitarius (light), and the spinal trigeminal nucleus (light).

Spinal cord - laminae I, II, VI, VII, and X are all heavily immunopositive with the substantia gelatinosa of lamina II staining most heavily.

Simantov, et. al., (1977) injected the tritiated agonist, diprenorphine and fluorescent antibodies to enkephalins simultaneously into the rat. The two stains overlapped in all areas except the caudate-putamen and cerebral cortex, which have many receptors but low concentrations of enkephalins. In the spinal cord gray matter few opiate receptors were found but enkephalins were abundant.

Recently antibody-HRP conjugates to LE were used to demonstrate the presence of enkephalin in the intermediolateral autonomic nucleus of the sacral spinal cord of the cat (Glazer, et. al., 1980).

## Specific Aims of this Thesis Research

It can be concluded from parts of the literature review, that a definitive knowledge of the opiate receptor structure and microenvironment is still largely lacking. The development of fluorescently labelled opioids with biological activity, and which are suitable probes of this microenvironment, may yield information about the locale of the membrane bound receptor and the density of opiate receptors on the membrane surface.

The objectives of this investigation are to fluorescently label the neurotransmitter ME: to characterize the

fluorescent enkephalin spectroscopically; and to utilize the fluorescent enkephalin as a probe of the dielectric constant of opiate binding sites on living dissociated spinal cell cultures using microspectrofluorometry.

In order to accomplish these objectives the following methods were used: (1) the labeling of ME, DME, and tyr on the amino terminal with a dansyl group; (2) the study of the absorption and fluorescent properties of NDME, NME, and NDT; using NDT as a model molecule; (3) the assay of the pharmacological potency of N-dansyl enkephalins and the parent peptides using the guinea pig ileum bioassay; (4) the study of the fluorescence emission of NDME bound to opiate binding sites in living dissociated mouse spinal cell cultures.

# MATERIALS AND METHODS

## Materials

Methionine$^5$-enkephalin (ME) was purchased from Pierce Chemical Company (Rockford, Illinois) and the (D-alanine$^2$)-methionine$^5$-enkephalin (DME) was purchased from Peninsula Laboratories, Inc. (San Carlos, California). The naloxone was a donation from Endo Laboratories, Inc. (Garden City, New York).

Purified 1-(5-dimethylaminonaphthalene)-sulfonyl chloride (dansyl-Cl), dansyl-amide (dansyl-$NH_2$), dansyl-glycine (dansyl-gly), dansyl-γ-amino-butyric acid (dansyl-GABA) O-dansyl-L-tyrosine (ODT), Didansyl-L-tyrosine (DDT), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), potassium chloride (KCl), magnesium sulfate septahydrate ($MgSO_4 \cdot 7H_2O$), potassium phosphate ($KH_2PO_4$), calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), glucose, sucrose, N-ethylmorpholine (NEM), glutamine (gln), HEPES buffer, penicillin, steptomycin, and phenol red were all purchased from Sigma Chemical Co. (St. Louis, Missouri). Spectral grade chloroform, acetone, and methanol were purchased from Matheson, Coleman, and Bell; Manufacturing Chemists (Norwood, Ohio). Chromerge, 1-nitroso-2-naphthol, and nitric acid were purchased from Mallinckrodt, Inc. (St. Louis, Missouri). Absolute ethanol

(200 proof) was secured from INC Chemical Group, Inc. (Terre Haute, Indiana). Toluene, distilled in glass, was obtained from Burdick and Jackson Lab, Inc. (Muskegon, Michigan). Nin-sol ninhydrin aerosol, 0.25% ninhydrin in N-butanol, was obtained from Pierce Chemical Co. (Rockford, Illinois). Silica Gel-G pre-coated glass plates (layer thickness of 0.25 mm) for thin layer chromatography (without fluorescent indicator) were purchased from E. Merck (Darmstadt, Germany).

ICR-Swiss timed pregnant mice were purchased from Harlan Sprague-Dawley, Harlan, Indiana (Indianapolis, Indiana). Eagle's modified minimal essential media, fetal calf serum, and horse serum were obtained from Grand Island Biological Co. (Grand Island, New York). The incubator was a Napco 322 from National Appliance Company, with a temperature variation of $\pm$ 0.5°C.

All fluorescence and absorption spectra were taken at the National Institute of Health (Bethesda, Maryland) using a Cary-14 Spectrophotometer from Applied Physics Corp. (Morovia, California) and an Aminco-Bowman Spectrophotofluorometer; both instruments were calibrated to $\pm$ 2 nm using a mercury light source. A Leitz Ortholux-microspectrofluorometer with epi-illumination according to Ploem (1967) was constructed with grating excitation monochromator, two continuous overlapping interference filters functioning as emission monochromator, and an Aminco-Bowman microphotometer used to drive the photomultiplier and chart recorder were used to take fluorescence spectra of the NDME in cultures.

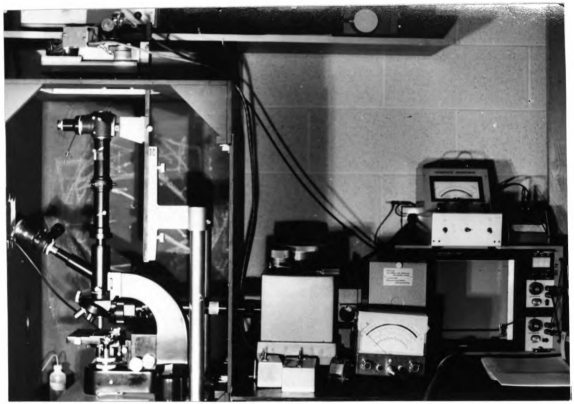


FIGURE 1: Epi-illumination microspectrofluorometer-constructed for taking fluorescence spectra of N-dansyl-enkephalins administered to living mouse spinal cell cultures. Instruments from right to left are: Aminco microphotometer and chart recorder, voltmeter-emission-wavelength readout and osram lamp, excitation grating monchrometer, Leitz ortholux microscope and the emission monochrometer with interference filters.

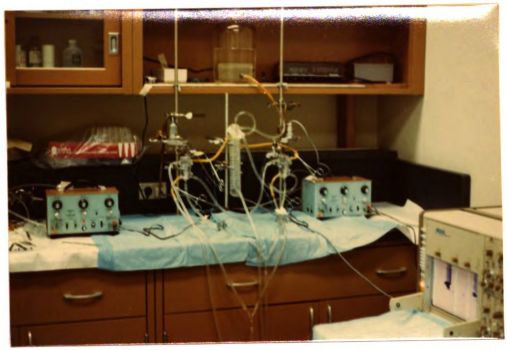


FIGURE 2: The guinea pig ileum bioassay equipment to assay two ileum preparations simultaneously. Instruments from right to left are Gould chart recorder, Grass stimulator, 37°C ileum bath, 37°C coil bath for incoming Kreb's media.

This instrument was calibrated to ± 5nm using a mercury light source (seen in Figure 1).

The guinea pig ileum assay utilized a Grass Force Displacement Transducer (FT-03-8), a Grass S-9 Stimulator, and a Gould Brush chart recorder (model 2400), see Figure 2.

## Methods

### 1. The labeling of ME, DME, and tyr with the fluorescent probe dansyl, and partial characterization of the dansyl derivatives.

The standard reaction conditions for the dansylation of an amino acid or peptide were according to the procedure of Felgner, et al. (1977; Gray, 1972). All glassware was precleaned in chromerge for three hours, rinsed overnight in distilled water, and finally rinsed in deionized water. Dansyl-Cl in anhydrous acetone, was reacted with ME, DME, or tyr in NEM:$H_2O$ (1:1,v/v); the reactants were at a 5:1 molar ratio of dansyl/peptide at millimolar concentrations. The reaction was allowed to proceed one hour while stirred in the dark at room temperature. The reaction was stopped by the addition of 4 molar equivalents of sodium hydroxide, and stirred in the dark for an additional ten minutes. The reaction mixture was dried under a stream of nitrogen in a 46°C water bath. The dried reaction mixture was then resuspended in anhydrous methanol for further purification.

Purification of N-dansyl derivatives was accomplished by separation of the reaction products utilizing thin-layer chromatography (TLC). A 20 x 20cm silica gel-G plate was

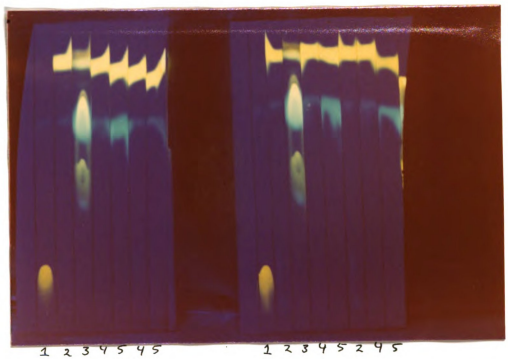
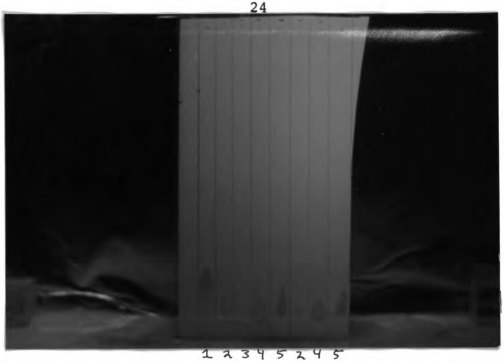



FIGURE 3: Thin layer chromatographic separation of dansylated L-tyrosine and enkephalin derivatives is shown. The O-dansyl-L-tyrosine and N,O-didansyl-L-tyrosine are standards obtained from Sigma Chemical Co. The N-dansyl derivatives were prepared using an N—ethylmorpholine buffer as described in the "Materials and Methods" section.

1. O-dansyl-L-tyrosine (Sigma)
2. N-dansyl-L-tyrosine
3. N,O-didansyl-L-tyrosine (Sigma)
4. N-dansyl-met$^5$-enkephalin
5. N-dansyl-(D-ala$^2$)-met$^5$-enkephalin

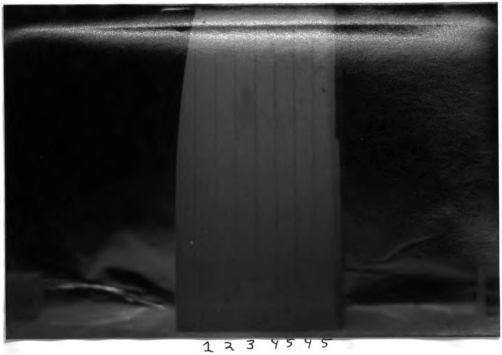
cleaned by elution in two dimensions with the eluent which was also used during purification, 6:10 (v/v) toluene/ ethanol. The cleaned TLC plate was then dried for one hour at 70°C to remove any adhering moisture. Aliquots of the resuspended reaction mixture were transferred to the clean, dry TLC plate 2cm from its base. The plate was redried under a stream of nitrogen, and more resuspended reaction solution was layered onto the plate 2cm from the base. In my experience, up to 10mg of N-dansyl derivatives could easily be purified on a single 20 x 20cm plate in this manner. The TLC plate containing dried reaction solution was placed in a developing tank, with the eluent 1.0cm in depth. The solvent front migrated 19cm in 200 minutes. Long-wage ultraviolet irradiation was used to help visualize the separated dansyl derivatives (Mineralight UVSL-25; San Gabriel, California), see Figure 3.

After chromatography the spots not corresponding to N-dansyl derivatives were scraped from the plate, and the plate was eluted in the second dimension to collect all the N-dansyl derivative on a small area of silica gel. The N-dansyl derivative on the silica gel was scraped into a clean testtube and eluted from the silica gel by successive resuspension in anhydrous methanol and centrifugation. Each centrifugation lasted three minutes on a 'Waco Separator' (Wilkins and Anderson Co., Chicago, Illinois). The supernatants were pooled for a final ten minute centrifugation. The pooled centrifuged supernatant was placed in




FIGURES 4a,b: The Ninhydrin reaction (above) and 1-nitroso-2-naphthol reaction (below) were performed on the plates shown in Figure 3. The faint red reaction products indicate free $\alpha$-amino groups (above) and free phenolic hydroxyl groups of tyrosine (below). The numbers below these figures identify the substance which was chromatographed in Figure 3.

1. O-dansyl-L-tyrosine (Sigma)
2. N-dansyl-L-tyrosine
3. N,O-didansyl-L-tyrosine (Sigma)
4. N-dansyl-met$^5$-enkephalin
5. N-dansyl-(D-ala$^2$)-met$^5$-enkephalin



a clean, dry preweighed beaker, and the methanol was evaporated under a stream of nitrogen at 46°C in a water bath. In this manner the reaction yield could be calculated, and the N-dansyl derivative could be resuspended in any solvent desired. The stock solutions of N-dansyl derivatives were stored at -4°C, in the dark.

The ninhydrin reaction was used to test for the presence of free amino terminals on unlabeled ME, DME, or tyr or on non-amino dansyl derivatives (Pataki, 1968). After chromatography and evaporation of the solvent, some plates were treated with ninhydrin spray and heated for 30 minutes at 60°C. The ninhydrin positive spots were identified by their purple color (Figure 4a).

The 1-nitroso-2-napthol reaction was used to detect the unreacted phenolic group of N-dansyl derivatives (Baily, 1967). Some of the TLC plates with eluted reaction mixture were sprayed evenly with a 0.1% solution of 1-nitroso-2-naphthol in 70% ethanol until they appeared a faint yellow color. The TLC plate was then dried, sprayed evenly with concentrated nitric acid, and placed in a 70°C oven for one hour. The 1-nitroso-2-naphthol positive spots were identified by their red color on the yellow-green background (Figure 4b).

2. The assessment of pharmacological potency of the N-dansyl-enkephalins.

The pharmacological potency of NME and NDME compared to the parent peptides was assayed according to the procedure

of Schaumann (1957). The segment of intestine extending from a point 10cm from the stomach to a point 10cm from the large intestine was removed from a guinea pig. The lumen was perfused with 200ml of Kreb's ringers solution (118mM NaCl, 27.2mM $NaHCO_3$, 4.8mM KCl, 1.2mM $MgSO_4 \cdot 7H_2O$, 1.0mM $KH_2PO_4$, 11.1mM glucose, and 1.8mM $CaCl_2$) and the rinsed ileum was then placed in a filled 500ml beaker of Kreb's ringer's solution with oxygen bubbling through it. A 1.0cm strip of ileum was then cut from the larger segment, lacerated longitudinally through the lumen, and tied at one end with a 3.0cm length of 000 suture. The ileum was then held stationary at one end between two insulated electrodes with the other sutured end tied to a mechanotransducer The secured ileum was placed in a bath containing 14.5ml of Kreb's ringers at the 37°C with oxygen gently bubbling through it. This entire procedure was done without placing any tension on the ileum segment, but merely suspending it, extended vertically, in the bath. The ileum was electrically stimulated to contract using the following stimulus parameters: 0.2Hz, 10 msec duration, at 100 volts. Contractions of the ileum were transformed to a voltage via a piezo-electric mechanotransducer, and were displayed by a chart recorder with the following settings: 25mm/sec, transducer at 25mv full scale, 5 Hz filter, and a DC-amplifier setting of 1.0 volts.

All stock solutions of labeled and unlabeled enkephalin were added in 0.5ml aliquots to the ileal bath (a 1:30

dilution). Seven types of controls were run: 1) unlabeled enkephalin in Kreb's ringers; 2) unlabeled enkephalin in Kreb's ringers with 286mM ethanol in the bath; 3) 286mM ethanol in the bath; 4) 9nM naloxone in Kreb's ringers; 5) 9nM naloxone in Kreb's ringers with 286mM ethanol in the bath; 6) 9nM naloxone and unlabeled enkephalin in Kreb's ringers; 7) 9nM naloxone and unlabeled enkephalin with 286mM ethanol in the bath. Concentrations of labeled and unlabeled enkephalins added to the bath ranged from nanomolar (nM) to tens of micromolar (μM) in order to obtain dose-response curves. The dose of drug was plotted logarithmically against the decrease in ileum contraction (expressed as a percentage by the equation [1-(inhibited contraction height/normal contraction height)] x 100. The N-dansyl enkephalins were placed in stock solutions of 8.58m ethanol Kreb's ringers (which became 286mM in the bath after the 1:30 dilution).

3. The culturing procedure for mouse spinal cord cells.

The culture procedures for mouse spinal cord cells were performed according to the technique of Ransom et al. (1977), with only slight modifications. Spinal cords with dorsal root ganglia were taken from 12-day-old mouse embryos obtained from timed-pregnant mice which were anesthetized with $CO_2$ and killed by cervical dislocation. The embryos in their endometrial sacs were removed and placed in a modified D1SGH saline media. Each spinal cord took about one minute

to remove.

DlSGH is comprised of (in grams per liter): 8g NaCl, 0.4g KCl, 0.045g $NaHPO_4 \cdot 7H_20$, 0.03g $KHPO_4$, 0.0015g Phenol Red, 2 x $10^5$ units of penicillin, 0.2g streptomycin-HCl, 1.1g glucose, and diluted to one liter with water. To one liter of this DlSGH were added 6.0g of glucose, 15.0g of sucrose, and 2.38g of HEPES buffer. The modified DlSGH is adjusted to a pH of 7.3 and an osmolarity of 330mOSM.

Following dissection, up to four or five spinal cords were placed in an empty sterile 60mm petri dish and minced with iridectomy scissors until the mass of tissue appeared almost gelatinous. This minced tissue was taken up in 1.5ml of nutrient medium with a sterile Pasteur pipette, and transferred to a sterile 15ml culture tube. The nutrient medium (MEM 10/10) consisted of 80% Eagle's minimal essential media with added glucose (6g/liter), 10% fetal calf serum, 10% inactivated (56°C for 30 minutes) horse serum, and $NaHCO_3$ (1.5g/liter). The tissue fragments in the culture tube were mechanically dissociated by trituration, using the Pasteur pipette to take up and expel the suspension 5-10 times. The resulting suspension was allowed to settle for two minutes, and the supernatant was removed and saved. One milliliter of MEM 10/10 was added to the remaining pellet and the trituration procedure was repeated using a Pasteur pipette with a slightly narrowed orifice (flamed tip). After again allowing the suspension to settle, the supernatant was again removed and added to the suspension previously collected.

This cycle of resuspension and trituration was repeated until the supernatant volume reached 1.0ml per spinal cord (i.e., 4-5ml). This final suspension was plated on 60mm diameter tissue culture plates (Corning) by adding 1.5ml of the suspension (about $4.5 \times 10^6$ cells) to plates containing 3.0ml of MEM 10/10 and a collagen coated 20 x 20mm coverslip (see below) which had been preincubated for at least one day so that the pH and temperature of the medium were equilibrated to the incubation conditions (10% $CO_2$ - 90% air atmosphere and 34°C).

Collagen coated coverslips were prepared in the following manner: 50mg of acid soluble calf skin collagen (Sigma) was treated as if it were sterile, and was placed in 100ml of 1:100 (v/v) glacial acetic acid/$H_2$). The mixture was stirred at room temperature for one hour. Several 20 x 20mm (No. 1 thickness) coverslips were placed in a large crystallizing dish and the collagen solution was poured over the coverslips. The crystallizing dish with coverslips and collagen solution was placed in a 60°C oven overnight to evaporate the solution to dryness. These collagen coated coverslips were individually wrapped in tissue, autoclaved, and then placed in 60mm sterile petri dishes for use in culturing.

The freshly plated cultures were incubated for 3-5 days before their first media change with 4.0ml of MEM 10/10, after 4.0ml of the initial plating media was removed. The second medium change was done after two more days and if, as

was usually the case, the nonneuronal background cells were confluent at this time, the change was made using medium which did not include fetal calf serum (MEM 10), and to which was added 5'-fluoro-2'-deoxyuridine (FdU) plus uridine (final concentrations of 15 and 35 µg /ml, respectively). Occasionally the FdU treatment was postponed until the third media change if the background cells were not confluent at the time of the second change. The MEM 10 with FdU and uridine were changed after two days and all subsequent changes were made three time a week using MEM 10 alone. Cultures prepared in the above manner were allowed to mature three weeks prior to study. Several cultures were stained by the method of Sevier and Munger (1965), and the cells thus observed are illustrated in Figures 5-10.

All solutions which were to come in contact with the cultures were sterilized by filtration through a Gelman 0.2µm millipore filter both prior to storage in a refrigerator, and prior to use on the cultures. Because a laminated air-flow culture hood was not available for use, scrupulous sterile techniques had to be practiced. The disinfectant detergent Micro-Quat was sprayed heavily in the air in a room which was sealed off at all entrances. All surfaces near where culture work was to be performed were scrubbed and sprayed with the disinfectant. Full sterile garb was worn during culture plating and medium changes. All ventilation was stopped in the sterile room during these procedures. A small culture box was constructed of plexiglass, which

FIGURE 5: A three week-old mouse spinal cell culture stained by Sevier-Munger (1965) reduced silver technique. Lower left is a dorsal root ganglion cell.

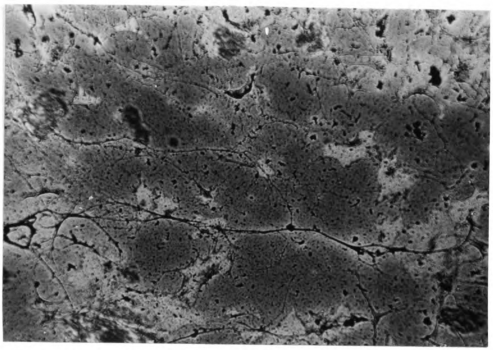


FIGURE 6: Sevier-Munger stained three week-old spinal cell culture, centered is a tripolar cell with a fine diameter axon descending in the picture.

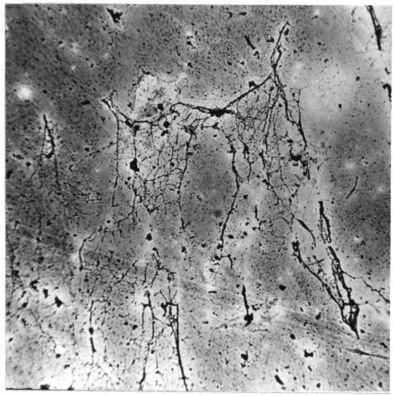


FIGURE 7: A highly branched neuron in a three-week-old spinal culture stained by Sevier-Munger method.

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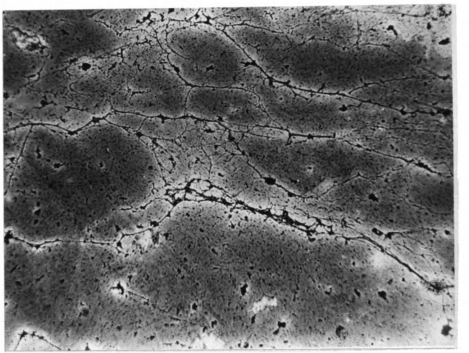


FIGURE 8: Centered is a small Sevier-Munger stained bipolar neuron from a three-week-old mouse spinal cell culture.

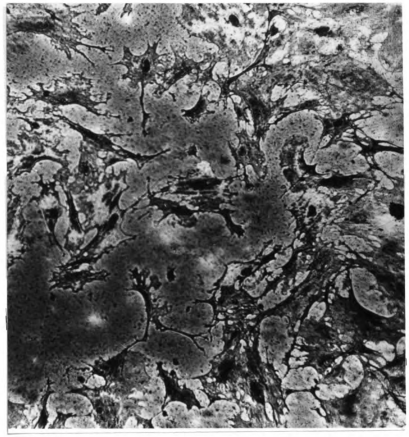


FIGURE 9: A cluster of fibroblast-like cells stained by Sevier-Munger reduced silver technique in a three-week-old spinal cell culture.

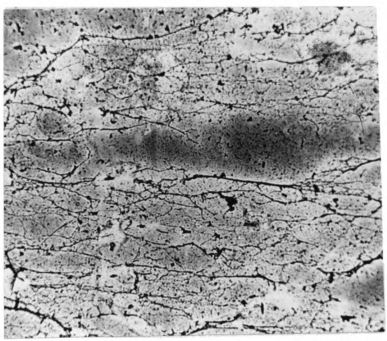


FIGURE 10: An area of finely branched neuropil demonstrating network interconnection of neurons, from a Sevier-Munger stained three-week-old spinal cell culture.

contained two fluorescent germicidal lamps and one visible fluorescent light. The cultured box was also scrubbed and sprayed with Micro-Quat on both the inner and outer surfaces.

When contamination occurred, a media change with FdU often retarded both bacterial and fungal growth. This treatment allowed contaminating colonies to be removed prior to growing over the spinal cells by the use of a sterile Pasteur pipette, and did not appear to harm the spinal cell culture growth.

4. The administration of NDME to living mouse spinal cell cultures: microspectrofluorometric characterization.

A mature mouse spinal cell culture was positioned in a plexiglass holder which also held two pipettes near the base of the inside of the 60mm culture dish. A peristaltic pump was connected via 1/8 inch diameter tygon tubing to the two pipettes so that one pipette acted as an inlet and the other as an outlet. A 34°C water bath held several 100ml bottles of Puck's balanced salt solution (PBS; 0.11mM, $CaCl_2 \cdot 2H_2O$, 3.8mM KCl, 0.61mM $KH_2PO_4$, 0.625mM $MgSO_4 \cdot 7H_2O$, 1.27mM NaCl, 14.3mM $NaHCO_3$, 1.08mM $Na_2HPO_4 \cdot 7H_2O$, 6.1mM Glucose, pH = 7.3 sucrose used to adjust osmolarity to 330m OSM). The peristaltic pump was used to fill the culture dish with PBS, while simultaneously removing the MEM 10.

A culture was perfused with the PBS using the above apparatus, on the stage of the microspectrofluorometer at room temperature, at 6ml/min for three minutes followed by a

10 minute temperature equilibration period. One of the following two methods was used in the interaction of NDME with the spinal cell culture: 1) 5.8ml of PBS was in the culture dish initially; 0.2ml of 1.732 mM DNME in ethanol was added followed by a ten minute wait (572 mM ethanol and 5.77μM NDME in the culture dish); the peristaltic pump was started at 6ml/min for two minutes; the pump was stopped and several emission spectra were taken, with the use of a 20x quartz glass objective, of various NDME stained cells ($\lambda_{ex}$ = 340 nm); a single NDME stained cell was visualized and its emission spectrum was taken, 1.0ml of 1.13 mM ME in PBS is added to the culture followed by a one minute wait (161μM ME in the dish). The single cell previously visualized had its emission spectrum retaken; 2) 4.8ml of PBS in the culture dish initially; 1.0ml of 1.13 mM ME in PBS was added to the culture followed by a one minute wait; 0.2ml of 1.732 mM NDME in ethanol was added followed by a ten minute wait (245mM ethanol, 151μM ME, and 49.5μM NDME in the culture dish); the peristaltic pump was started at 6ml/min for two minutes; the pump was stopped and several emission spectra are taken of various stained cells ($\lambda_{ex}$ = 340nm).

Several types of controls were run to distinguish artifacts from actual NDME interaction with cells in culture including: 1) emission spectrum of a 60mm culture dish containing a collagen coated coverslip and PBS only; 2) emission spectrum of spinal cell cultures in PBS only; 3) emission spectrum of spinal cell cultures in PBS with 1.0ml

of 1.13mM ME in PBS added to 6.0ml of PBS.

## RESULTS WITH DISCUSSION

### Synthesis and Spectroscopic Properties of N-dansyl-enkephalins and N-dansyl-L-tyrosine

#### 1. Synthesis

ME, DME, and tyr were successfully labeled with a dansyl group at their amino terminals. The reaction involved the formation of a sulfonamide bond between the amino group of tyrosine, and the sulfonyl group of dansyl. The tertiary amino group in N-ethylmorpholine (NEM) in its protonated quarternary form I feel probably forms a strong ionic bond to the phenolic hydroxyl of tyrosine (in its basic form) preventing its dansylation. When the NEM is mixed with water a vigorous endothermic reaction occurs, most probably due to the protonation of NEM. The fluorescent enkephalin and tyr derivatives gave negative results to the ninhydrin test, indicating that the amino terminals of the peptides were complexed with the dansyl probe (see Figure 4a). As would be expected, unreacted ME, DME, and tyr were found to remain at the TLC origin by the ninhydrin reaction. ODT standards were ninhydrin positive, while DDT standards were negative. The 1-nitroso-2-naphthol reagent indicated the presence of a free phenolic group on the fluorescent N-dansyl derivatives, while ODT and DDT were negative, and dansyl

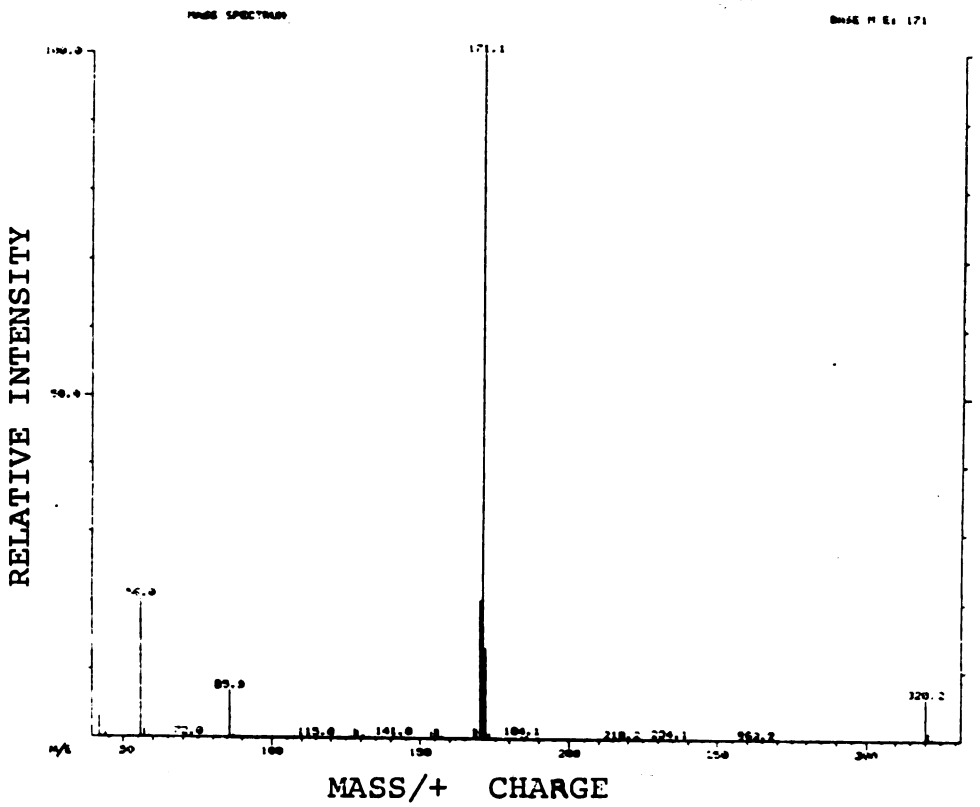



FIGURE 11a: Mass spectrum by electron impact of N-dansyl-L-tyrosine at 70eV. The prominent m/e = 171 peak is 1-dimethylaminonaphthalene. The peak at m/e = 235 is 1-dimethylaminonaphthalene-5-sulfonyl.

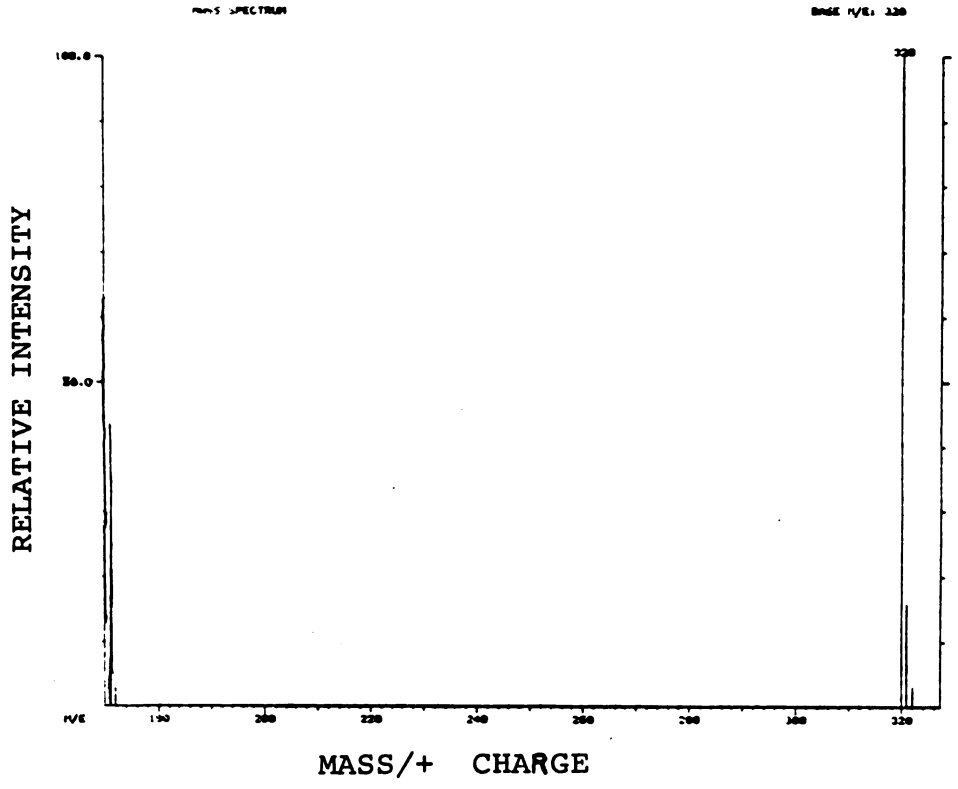



FIGURE 11b: Mass spectrum by electron impact of N-dansyl-met$^5$-enkephalin at 20eV. The prominent m/e = 171 peak is 1-dimethylaminonaphthalene.

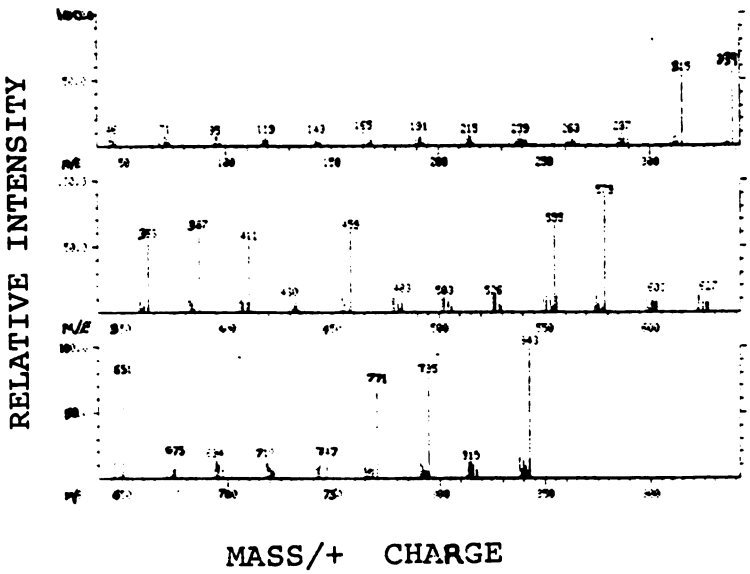



FIGURE 12a: Mass spectrum by methane chemical ionization of N-dansyl-met$^5$-enkephalin. Peaks at m/e = 818, m/e = 828, and m/e = 843 correspond to n + 15 ($-CH_3$), n + 29 ($-CH_2CH_3$) and n + 43 ($-CH_2CH_2CH_3$) peaks respectively. The calculated molecular weight of N-dansyl-met$^5$-enkephalin is 806.9 g/mole.

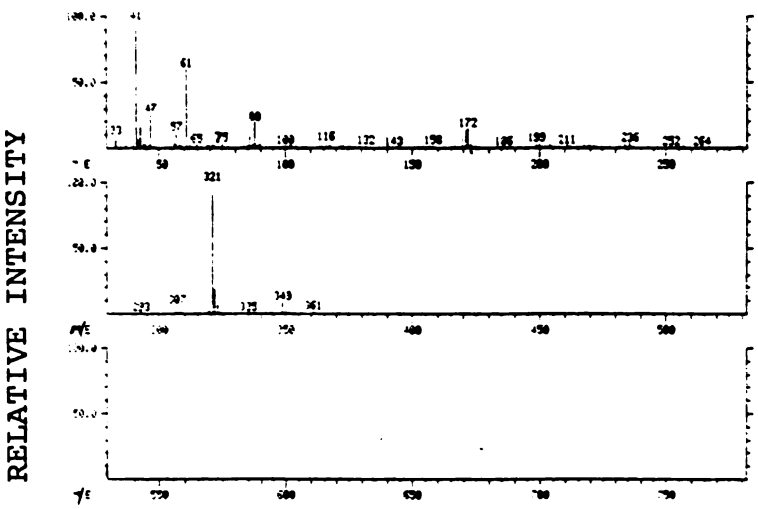



FIGURE 12b: Mass spectrum by methane chemical ionization of N-dansyl-L-tyrosine is very similar to the spectrum of this compound obtained by 20eV electron impact. The m/e = 172 peak, and the m/3 = 236 peaks correspond to the n + 1 (H-) 1-dimethylaminonaphathalene and n + 1 (H-) 1-dimethylaminonaphthalene-5-sulfonyl moieties, respectively.

sulfonic acid was positive (see Figure 4b). All N-dansyl derivatives had an Rf = 0.91, while the DDT standard had an Rf = 0.61, and the ODT had an Rf = 0.15 in the solvent system used. Therefore the position of the dansyl group on the tyrosyl residue determined the Rf value of the TLC purification system, molecular weight had no influence (i.e. NDME, NME, and NDT all had the same Rf value). The reaction yield for the N-dansyl derivatives was 85%.

Mass spectroscopy was used to characterize NT and NME. Electron impact mass spectroscopy of either compound yielded only two recognizable peaks: m/e = 171 which is 1-dimethylaminonaphthalene (Marino et al., 1968) and m/e = 235 which is 1-dimethylaminonaphthalene-5-sulfonyl (Seiler et al., 1971) (see Figures 11a,b). Chemical ionization by methane bombardment of NME yielded peaks at N + 15 (parent compound + $CH_3$-) N + 29 (parent compound + $CH_3CH_2$-) and N + 43 (parent compound + $CH_3CH_2CH2$-) positions corresponding to a parent monodansylated-ME compound (mass spectrometer was calibrated to $\pm$ 4 mass units in this mass range). The molecular weight of NME was calculated to be 806.9g/mole (Figure 12a). Chemical ionization of NDT gave a spectrum similar to the spectrum obtained by electron impact (Figure 12b).

2. Spectroscopic properties

The fluorescence spectra (uncorrected) of NDT and NDME were determined to be change identically in all solvents tested (Table 1). The fluorescence emission maxima in

TABLE 1

Fluorescence emission maxima of N-dansyl-(D-ala$^2$)-met$^5$-enkephalin and N-dansyl-L-tyrosine in solvents of different dielectric constants

| | Solvent | Dielectric | NDT λ max | NDME λ max |
|---|---|---|---|---|
| 1. | Water | 78.5 | *531 | *531 |
| 2. | Methanol | 32.6 | 520 | 520 |
| 3. | Ethanol | 24.3 | 515 | 515 |
| 4. | Acetone | 20.7 | 513 | 513 |
| 5. | Chloroform | 4.8 | 492 | 492 |
| 6. | Cyclohexane | 2.023 | 451 | 5 |

* The actual concentration of water was 99.8% with 0.2% ethanol

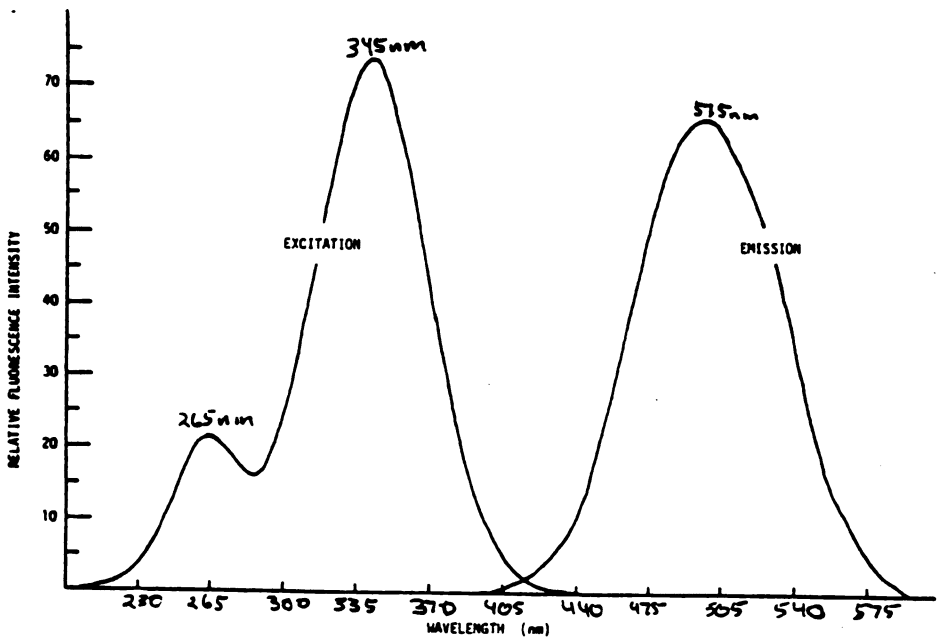



FIGURE 13: Fluorescent spectra of N-dansyl-L-tyrosine, N-dansyl-(D-ala$^2$)-met$^5$-enkephalin, and N-dansyl-met$^5$-enkephalin are all identical to that picture in ethanol.

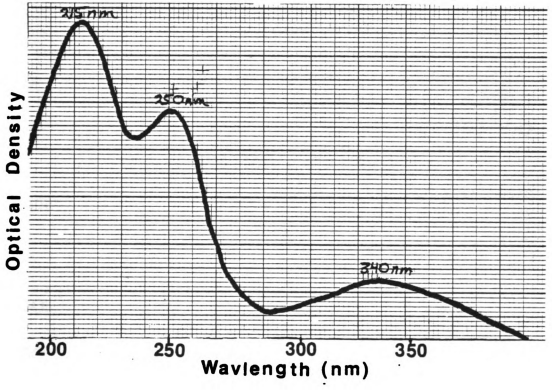
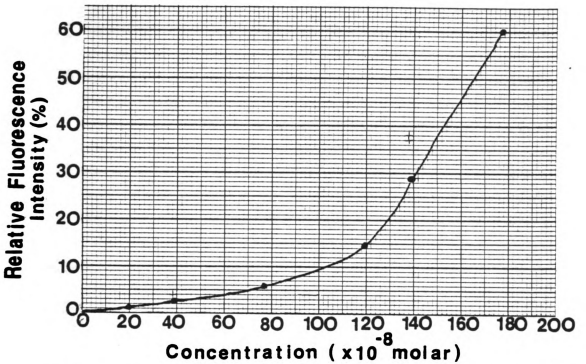
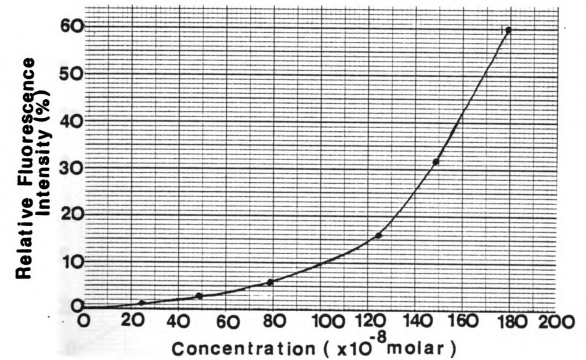
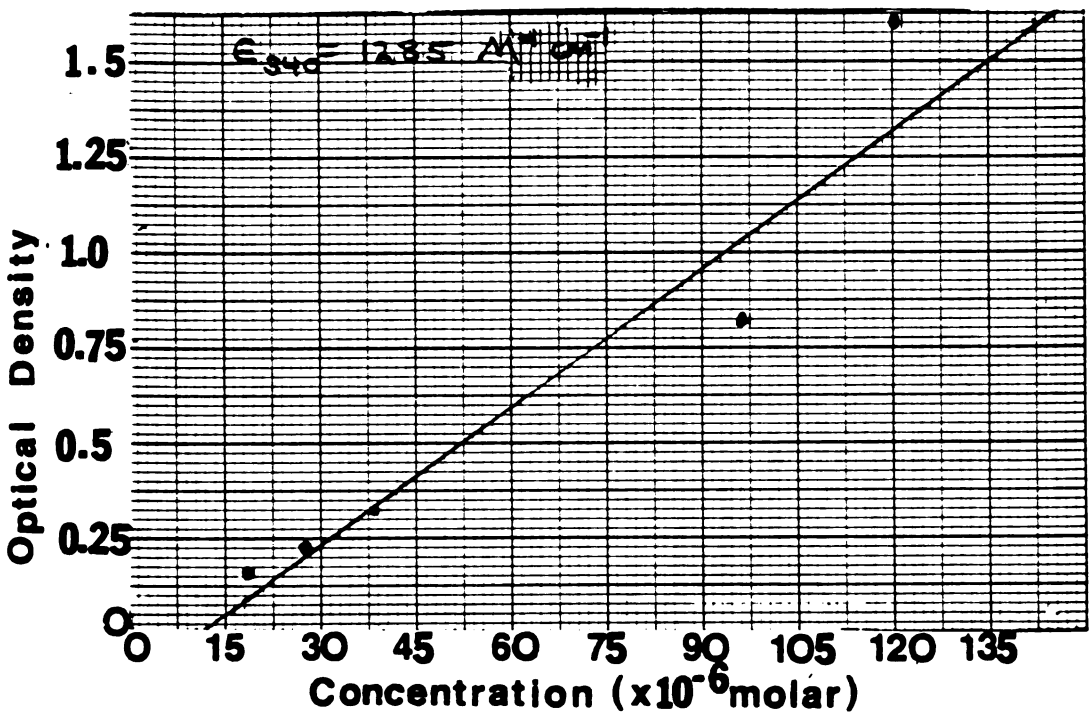



FIGURE 14: Absorption spectra of N-dansyl-L-tyrosine, N-dansyl-(D-ala$^2$), met$^5$-enkephalin, and N-dansyl-met$^5$-enkephalin are all identical to that pictured, but differ in their molar extinction coefficients, in ethanol.

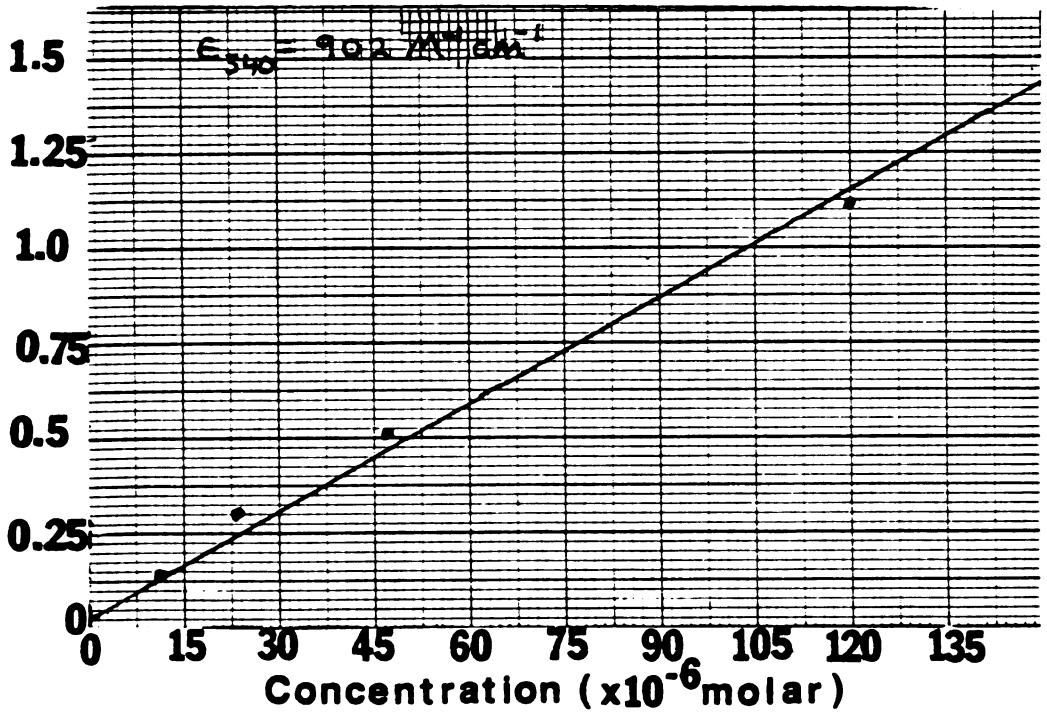



FIGURES 15a,b: Fluorescence emission intensity at 515nm as a function of concentration in ethanol of N-dansyl-(D-ala$^2$)-met$^5$-enkephalin (above, a) and N-dansyl-L-tyrosine (wavelength of excitation is 340nm).






FIGURES 16a,b: Optical density as a function of concentration in ethanol of N-dansyl-(D-ala$^2$)-met$^5$-enkephalin (above) and N-dansyl-L-tyrosine (below). Molar extinction coefficients are at 340nm are $\varepsilon_{340}^{NDME}$ = 1285 $M^{-1}cm^{-1}$ and $\varepsilon_{340}^{NDT}$ = 902 $M^{-1}cm^{-1}$, respectively. The N-dansyl-L-tyrosine graph appears more linear than that of N-dansyl-(D-ala$^2$)-met$^5$-enkephalin.



ethanol, and the excitation spectra had a primary maximum at 340nm and a secondary peak at 260nm, in ethanol (Figure 13). The substitution of the peptide chain in NME and NDME for the carboxyl OH group of NDT had no effect on the fluorescence spectra of the respective attached dansyl groups. The absorption spectra of NDME, NME, and NDT were identical, in ethanol, having maxima at approximately 215nm, 250nm and 340nm (Figure 14). The change in fluorescence intensity as a function of change in concentration of NDME and NDT remains nearly identical for the two compounds (Figure 15a,b). However, the extinction coefficients at 340nm of these two compounds differ ($\varepsilon_{340}^{NDME} = 1285$, $\varepsilon_{340}^{NDT} = 902$), (Figure 16a,b).

NDME, NME, and NDT each have only two moieties that absorb in the near UV and which exhibit fluorescence. These are the tyrosyl and dansyl moieties. There is considerable spectral overlap between the tyrosyl emission and the dansyl absorption spectra leading to efficient singlet-singlet energy transfer (Stryer, 1968) from the tyrosine residue to dansyl group in NDME, NME, and NDT. Thus, the tyrosyl fluorescence emission was quenched, and only emission from the dansyl group occurred upon excitation of the tyrosyl residue. Energy transfer from the tyrosyl to the dansyl group was evidenced by the fluorescence excitation of NDME, NME, and NDT which show an excitation peak at 260nm, in the

TABLE 2

Effect of Substituent Basicity on the Emission of N-dansyl-derivatives in Ethanol

| | Compound | $\lambda_{ex}$ = 340nm | λ max |
|---|---|---|---|
| 1. | Dansyl hydroxide | | 455nm |
| 2. | Dansyl chloride | | *460nm |
| 3. | Dansylamide | | 500nm |
| 4. | Dansyl glycine | | 505nm |
| 5. | Dansyl-γ-amino-n-butyric acid | | 505nm |
| 6. | N-dansyl-l-tyrosine | | 515nm |
| 7. | N-dansyl-met$^5$-enkephalin | | 515nm |
| 8. | N-dansyl-(D-ala$^2$)-met$^5$-enkephalin | | 515nm |

* The measurement was made on a freshly prepared solution since dansyl-Cl reacts with ethanol to form a sulfonic ester with an emission peak at about 520nm.

wavelength range of tyrosyl fluorescence excitation (Figure 13).

The emission spectra of NDME and NDT were observed to shift toward longer wavelengths in solvents of high dielectric constant (Table 1). The excited state of the dansyl group has a much higher dipole moment than the ground state. Thus, in water the emission maximum of the probe shifted toward the red by 1500cm$^{-1}$ compared to its emission maximum in chloroform.

The fluorescence emission maxima of dansyl derivatives in ethanol were found to depend upon the nucleophilic strength of the substituent attached to the 1-dimethylamino-naphthalene-5-sulfonyl group. The emission maximum of the dansyl fluorophor shifted toward longer wavelengths as the basicity of the attached substituent increased, according to OH < Cl < $NH_2 \leq$ NHR (Table 2). The emission of dansyl hydroxide (1-dimethylaminonaphthalene-5-sulfonic acid) was 45nm less than that of dansylamide.

## The Pharmacological Potency of N-dansyl-enkephalins in Relation to the Parent Peptides

The first time an attempt was made to dissolve NDME or NME in an aqueous solution, their low solubilities in water become readily apparent. It was possible to get the NDME and NME in 286mM ethanol in water, a 50% (by volume) glycerol-water solution and a 25% propylene (by volume) glycol-water solution. A solution of 10% (by weight)

FIGURE 17: Dose response curves of met$^5$-enkephalin assayed using the guinea pig ileum, is displayed (below) using the presence and absence of 286mM ethanol. The activity of 9nM naloxone with 396.5nM met$^5$-enkephalin or 11.8μm N-dansyl-met$^5$-enkephalin in the presence or absence of 286mM ethanol (EtOH) are displayed as points on this graph. The brush recorder readout is also shown (above).

1. met$^5$-enkephalin in Kreb's ringers'
   1a - 19.9nM
   1b - 39.6nM
   1c - 199.3nM
   1d - 396.5nM

2. 286mM ethanol in Kreb's ringers
   2a - initial 2 minutes
   2b - 10 minutes
   2c - 20 minutes

3. met$^5$-enkephalin and 286mM ethanol in Kreb's ringers
   3a - 19.9nM
   3b - 39.6nM
   3c - 199.2nM
   3d - 396.5nM

4. 5.9μM N-dansyl-met$^5$-enkephalin and 286mM ethanol in Kreb's ringers

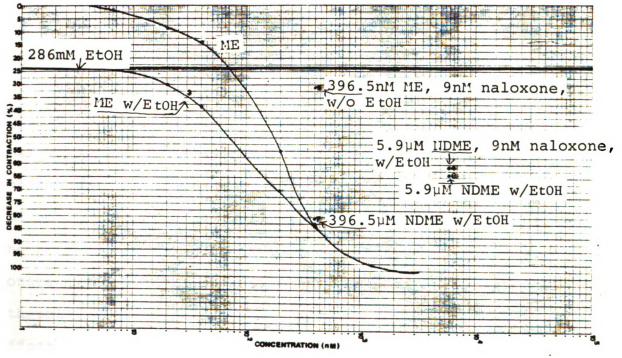
5. 9nm naloxone and 396.4nM met$^5$-enkephalin in Kreb's ringers

6. 9nM naloxone in Kreb's ringers

7. 9nM naloxone, 396.5nM met$^5$-enkephalin, and 286mM ethanol in Kreb's ringers

8. 9nM naloxone, 5.9μM N-dansyl-met$^5$-enkephalin and 286mM ethanol in Kreb's ringers


FIGURE 17




Of Opioid Agonist

Pluronic polyol (polyethylene-oxy-polypropylene) F-108 complexed with NDME and NME, to form precipitates. Of the three NDME (and NME) solutions described above, the 286mM ethanol control had the least inhibitory effect (40 ± 10%) on the electrically-induced contraction of the guinea pig ileum.

Because ethanol was going to be present in the assay, it was necessary to determine what effect it might have on the inhibition of guinea pig ileum contractions by opiates. A dose response curve of ME both in the presence and absence of 286mM ethanol is displayed in Figure 17. A line is drawn in this figure indicating the percentage of inhibition due to ethanol. The ME assay in ethanol begins to show further inhibition from this 286mM ethanolic inhibition line. Initially the inhibitory effects of 286mM ethanol and the ME appeared to be additive (see Figure 17). At the point of maximum inhibition of contraction by ME (396.5nM, 81% inhibition) the presence of 286mM ethanol did not appear to add an inhibitory effect of its own on the ileal contractions. The presence of a 9nM naloxone had no effect on the ileum contractions, or on the inhibitory effect induced by 286mM ethanol. The 9nM naloxone was able to reduce the inhibitory effect of 396.5nM ME by 70% (a 50.1% reversal of the ME effect shown as a point in Figure 17). In the presence of 286mM ethanol and 9nM naloxone, the 396.5nM ME was again able to inhibit the ileum contraction by 81% (shown in Figure 17). The presence of 286mM ethanol appeared to nullify the opioid

FIGURE 18: Dose response curves of met$^5$-enkephalin, N-dansyl-(D-ala$^2$)-met$^5$-enkephalin, and N-dansyl-met$^5$-enkephalin assayed using the guinea pig ileum, is displayed (below) in the presence of 286mM ethanol. Amino terminal dansylation decreased the potency of met$^5$-enkephalin 60-fold. Dansylation of the amino terminal of (D-ala$^2$)-met$^5$-enkephalin caused an 11-fold potency decrease compared to unlabelled met$^5$-enkephalin.

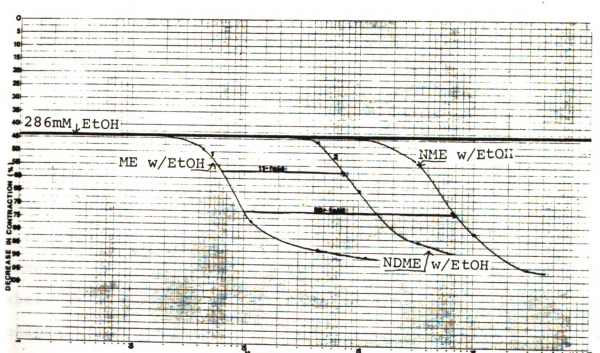
1. met$^5$-enkephalin and 286mM ethanol in Kreb's ringers
   - 1a - 0.4nM
   - 1b - 1.3nM
   - 1c - 4.3nM
   - 1d - 13nM
   - 1e - 43.4nM
   - 1f - 130nM
   - 1g - 434nM
   - 1h - 1.3μM
   - 1i - 26nM

2. N-dansyl-(D-ala$^2$)-met$^5$-enkephalin and 286mM ethanol in Kreb's ringers
   - 2a - 10nM
   - 2b - 33nM
   - 2c - 100nM
   - 2d - 333nM
   - 2e - 1.0μM
   - 2f - 3.3μM
   - 2g - 5.0μM

3. N-dansyl-met$^5$-enkephalin and 286mM ethanol in Kreb's ringer
   - 3a - 333nM
   - 3b - 1.0μM
   - 3c - 3.3μM
   - 3d - 10μM
   - 3e - 29μM

4. 286mM ethanol (EtOH) in Kreb's Ringers


FIGURE 18




Of Opioid Agonist

agonist effect reversal capacity of the 9nM naloxone.

The results presented above meant that reversal of NDME with 9nM naloxone would probably not be demonstratable in the presence of 286mM ethanol. Figure 18 shows dose response curves for ME, NME, and NDME in the presence of 286mM ethanol. From Figure 18 it can be seen that a concentration of NME at 60-fold greater than that of ME was needed to elicit the same inhibition of the ileum contraction, while 11-fold greater concentrations of NDME produced this same inhibition. As expected, 9nM naloxone was shown to be unable to reverse the inhibition of NME or NDME in the presence of 286mM ethanol (See the chart recorder response of Figure 18).

Another interesting result was elucidated in the ileum bioassay. When ME or DME were introduced to the assay solution, their full inhibitory effects were apparent within 10 seconds. The NME and NDME took 35 seconds to reach their maximum inhibitory effects on the ileal contractions. When ME had been in the ileum bath for 30 seconds, a decrease in the inhibition of ileum contraction was visible, which was not observed with NME even after one minute has passed. This result may be explained by postulating that the enzyme which degrades ME is less efficient on NME due to the dansyl label. This 'enkephalinase', so named since it degrades enkephalins, appears to be released from the intestine into the bathing medium. The bathing medium could then decrease the potency of any stock solution which had become contaminated with it.

Wavelength of Emission ($\times 10^4 cm^{-1}$)

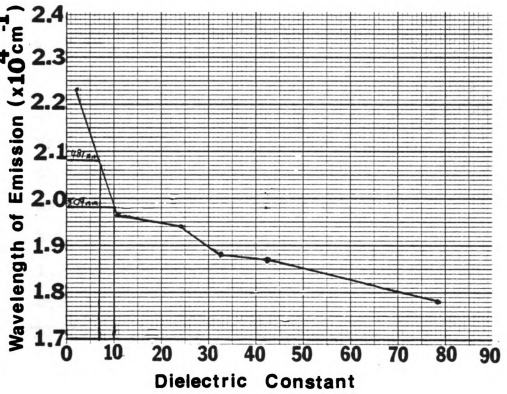



FIGURE 19: Fluorescence emission maximum as a function of solvent dielectric constant as revealed by microspectrofluorometry.

TABLE 3

Fluorescence emission maxima of N-dansyl-(D-ala$^2$)-met$^5$-enkephalin in solvents of different dielectric constants. Spectra taken by microspectrofluorometry from a 2ml sample ($3.1 \times 10^{-4}$m) in a 2mm well slide.

504nm = 19841.3cm$^{-1}$ or a dielectric constant of 10

481nm = 20790.0cm$^{-1}$ or a dielectric constant of 7

| | Solvent | Dielectric | $\lambda$ max (cm$^{-1}$) |
|---|---|---|---|
| 1. | Water | 78.5 | 17796.1 |
| 2. | Glycerol | 42.5 | 18728.0 |
| 3. | Methanol | 32.6 | 18779.3 |
| 4. | Ethanol | 24.3 | 19411.4 |
| 5. | 1,2 Dichloroethanene | 10.65 | 19666.4 |
| 6. | Cyclohexane | 2.023 | 22303.0 |

Administration of NDME to Living Dissociated Mouse Spinal Cell Cultures

Table 3 displays the results of a study of NDME fluorescence emission maxima as a function of dielectric constant which was done on the microspectroflurometer. These results are displayed as a nonlinear graph in Figure 19. The fluorescence emission of NDME administered to mouse spinal cell cultures will be compared to this graph to determine the local dielectric constant of the environment near the NDME molecular binding site(s). The emission maxima vs. dielectric constant graph was assumed to approximate linearity between data points, for ease of interpretation.

The fluorescence emission of the NDME bound to spinal cells in culture was observed as discrete blue or green patches distributed in clusters on some cells in the culture, usually on the somatic portion of the cells. The emission spectrum of the NDME clusters had a maximum at either 481nm or 504nm, the 481nm being the predominant bound NDME emission observed (98% of the NDME binding sites characterized). Sites bound with NDME exhibited the 504nm emission shifted their emission maximum to 481nm when unlabelled ME was introduced to the spinal cell culture medium. Sites bound with NDME exhibiting the 481nm emission were not shifted in their emission maximum upon introduction of unlabelled enkephalin to the culture medium. When ME was introduced to the culture medium prior to NDME, only 481nm emission could be detected from opiate binding sites. These results indicated that with a

504nm emission peak occurs from an NDME labelled opiate binding site which is stereospecific, while the 481nm emission peak of NDME is from a nonspecific binding site. From Figure 19 it was interpolated that the 504nm binding site has a dielectric constant of about 10 that was detected by the dansyl probe. The dielectric constant of the 481nm emitting site is about 7. The higher dielectric constant of the stereospecific binding site, indicates this membrane binding site may lie closer in proximity to the external aqueous media than does the nonspecific binding site, or is of a higher molecular polarity than the nonspecific binding site.

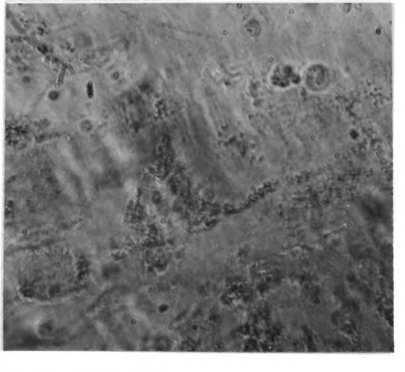
The control spectra taken of a culture which had not been administered NDME, a culture which had been administered ME, and the culture dish with no cells at all gave identical light scattering emission spectra. Some of this light scattering may also have been due to autofluorescence of nonquartz glass surfaces within the microspectrofluorometer. The scattered emission peaks were observed at 422nm, 468nm, and 527nm at the most sensitive photomultiplier setting (PM = .1). These peaks were not observed when emission spectra were taken of the NDME bound to spinal cell cultures, which were taken at a far less sensitive photomultiplier settings (PM = 3 or 10).

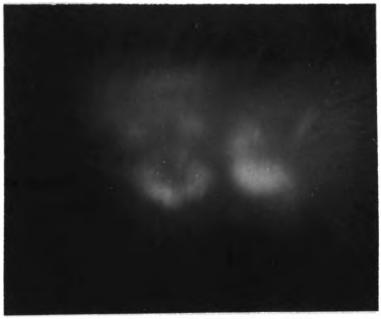
Figures 20,21, and 22 display fluorescence emission photographs (a) and phase contrast photographs (b) of several 481nm emission binding sites (taken with a 40x phase contrast objective). Unfortunately, no 504nm emission sites were

found in the two remaining cultures from which these photographs were taken. Of the 35 cultures used in this study, only 4 exhibited significant numbers of 504nm emitting binding sites. All cultures, however, exhibited the 481nm emitting binding site.

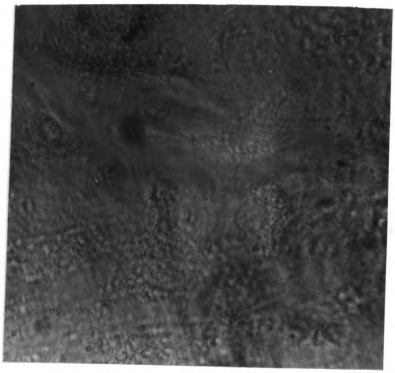
FIGURES 20a,b: A fluorescence photograph (above,a) and a phase photograph (below,b) of a group of living spinal cells in culture with binding sites for N-dansyl-(D-ala$^2$)-met$^5$-enkephalin, emission 481nm.

FIGURES 21a,b: A living pyramidal shaped cell in culture exhibiting 481nm fluorescence emission (above,a) of bound N-dansyl -(D-ala$^2$)-met$^5$-enkephalin and seen in a phase-contrast photograph (below,b).





FIGURES 22a,b: Three living mouse spinal cells exhibiting 481nm fluorescence emission of N-dansyl-(D-ala$^2$)-met$^5$-enkephalin (above,a) and in a phase-contrast photograph (below,b).



# DISCUSSION

The method of tyrosyl amino terminal dansylation was performed using a bicarbonate buffer reaction procedure (Gros et al., 1969) and compared to NEM reaction procedure. The bicarbonate and NEM reactions both yielded dansyl sulfonic acid as the major by-product. The bicarbonate buffer reaction procedure produced more dansylated reaction by-products and a lower yield of the N-dansyl-tyrosyl products, than did the NEM reaction procedure (Canada, et al., 1980).

Absorption and fluorescence spectroscopic studies demonstrated that NDT and NDME have nearly identical absorption and fluorescence emission properties in a variety of solvents. These two dansyl derivatives differ in their molar extinction coefficients. The graph of optical density vs. concentration of NDT was more linear than that of NDME (Figures 15a,b). This may be due to scattering off other moieties located in the peptide chain (i.e. the phenyl group or methionine R-group), not found in NDT. The pharmacological study of ME, DME, NME, and NDME displayed many significant results on which I would like

to speculate opiate agonist activity is likely the result of many concommitant factors. Placing the dansyl group on the amino terminal of enkephalin increased the hydrophobicity of the dansyl enkephalin compared to its parent peptide. An increase in hydrophobicity also increases lipophilicity, which is known to increase potency of opiate agonists (i.e. ME is more potent than LE; Miller et al., 1978). Primary amino groups are more potent on analgesic opiate agonists than tertiary amino groups, but this is not observed in the guinea pig ileum (Lord et al., 1977). The long latency of NME compared to ME in its inhibitory action may be due to the lesser ability of the dansylated enkephalin to cross tissue barriers in order to bind to the receptor site. The lasting inhibitory action of NME compared to ME may be due to its increased stability to enzymatic degradation, or its increased partitioning into the membrane from the aqueous media. My reported results of a 60-fold potency decrease of NME compared to ME differs from that of Fournie-Zaluskie et al., (1978). These workers may not have recognized the decrease in aqueous solubility of the dansyl enkephalins, and therefore may not have had the solution concentrations they calculated. Also, Fournie-Zaluski et al., (1978) compared NME to LE and not its parent peptide ME.

The effect observed by the presence of ethanol in the guinea pig ileum preparation has not been previously reported to my knowledge. Ethanol is a normal constituent

of the intestinal lumen, an adult human male produces about 30ml per day in his intestine by bacterial fermentation (Arnow, 1976). The ethanol affect, I feel, is likely one of a nonspecific fluidity change of a membrane's lipids, which may mediate membrane protein conformation change and thereby protein activity, (Sargent, 1980) and in this case changing the opiate receptor to an agonist binding conformation. I plan to study this ethanol effect in further detail. Increased temperature also increases opiate agonist binding (Reese et al., 1975B) as well as increasing membrane lipid fluidity.

Epi-illumination used in the microspectrofluorometry was felt to be indispensible in utilization of N-dansyl-enkephalin for probing of opiate binding sites in nerve cell cultures. The detectability of the fluorescent enkephalin depended on four parameters: 1) the membrane surface concentration of NDME; 2) the quantum yield of the NDME bound to the cell's surface; 3) the number of incident exciting photons on the bound NDME; 4) the rate of photolytic degradation of the NDME. The technique of epi-illumination focuses the excitation light upon the sample observed, so the only excitation light intensity loss occurs by reflection and scattering at glass surfaces in the microspectrofluorometer. As long as the photolytic degradation of the probe is slow, and the probe's quantum yield is reasonably high, sufficient fluorescence emission will result and be easily detected (as was observed). Emission spectra of a single NDME binding

site could be scanned several times over a period of five minutes before a detectable emission intensity decrease occurred. The amino dansylated peptides appear to be very stable to photolytic degradation (also reported for NDT by Felgner et al., 1977).

The NDME proved to be a valuable probe of the opiate binding sites in living mouse spinal cell cultures by elucidating the dielectric constants of two types of binding sites. The fact that the 504nm emitting NDME which was stereospecifically bound, shifted to 481nm on administration of unlabeled ME testifies to the high partitioning of NDME into the membrane rather than the aqueous media. The NDME was likely removed from a stereospecific binding site into a nonspecific binding site (i.e. some surrounding lipids).

The significance of this research is that it represents a novel system for the visual and quantitative analysis of opiate binding sites. The effects of drugs, ions, reagents, etc. on the structure of opiate binding sites may be determined by studying the fluorescence properties of the dansyl probe bound to enkephalin. Once the fluorescence lifetime of the NDME has been calculated at various dielectric constants, it may yield further information on the mobility of the NDME-opiate binding site complex in its lipid environment in fluorescence polarization studies (Youerabide et al., 1971).

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(1980)

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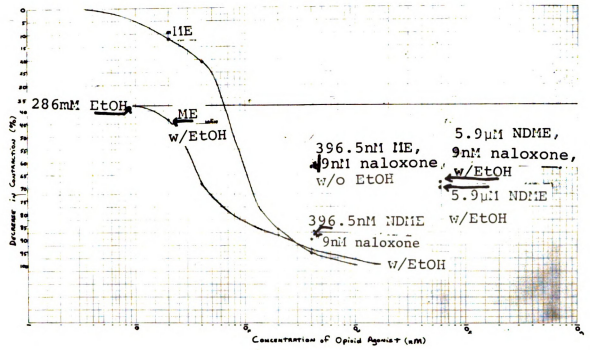
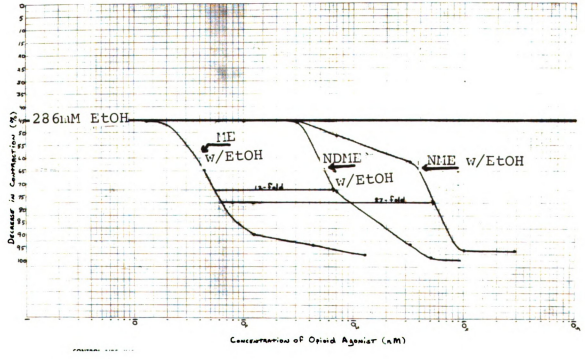



APPENDIX

Supplementary Dose Response Curves of ME in the Presence and Absence of Ethanol (EtOH) (above) Dose Response Curves of ME, NME, and NDME (below).



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