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SYNTHESIS AND FLUORESCENCE PROPERTIES OF N-DANSYL-MET^{5}-ENKEPHALIN; AND, MICROSPECTROFLUOROMETRIC CHARACTERIZATION OF OPIATE RECEPTOR SITES ON CULTURED AMYGDALOID CELLS

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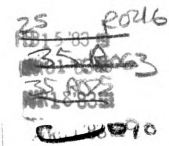

SYNTHESIS AND FLUORESCENCE PROPERTIES OF N-DANSYL-MET^5-ENKEPHALIN; AND, MICROSPECTROFLUOROMETRIC CHARACTERIZATION OF OPIATE RECEPTOR SITES ON CULTURED AMYGDALOID CELLS

By

Robert Gene Canada

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ABSTRACT

SYNTHESIS AND FLUORESCENCE PROPERTIES OF
N-DANSYL-MET^{5}-ENKEPHALIN; AND, MICROSPECTROFLUOROMETRIC
CHARACTERIZATION OF OPIATE RECEPTOR SITES ON
CULTURED AMYGDALOID CELLS

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The objective of this investigation was to develop a new technique for visually mapping the distribution of opiate receptor sites on the soma and/or processes of single cells in cultures from the amygdaloid region; to examine the conformation of opioid pentapeptides; and to examine the nature of the opiate receptor site(s). These goals were achieved by attaching a dansyl group (a fluorescent molecular probe) to the amino terminal of methionine-enkephalin, yielding N-dansyl-methionine-enkephalin. It is known that the emission of the dansyl group undergoes a large red shift when dansyl chloride is changed to dansyl amides (e.g. N-dansyl-amino acids); the emission peak for free unbound dansyl chloride in ethanol was at 480 nm; the fluorescence emission maximum of N-dansyl-met-enkephalin was found at 530 nm; in ethanol; and, the excitation spectrum had a maximum at 360 nm and a secondary peak at 270 nm. The fluorescence emission intensity of N-dansyl-met-enkephalin at 530 nm was established to linearly change as a function of concentration, having

a fluorescence coefficient equal to 1 x 10^5 M^{-1}. The fluorescence emission intensity and maximum of N-dansyl-met-enkephalin were found to be sensitive to the dielectric constant of the medium; whereby increasing the ethanol concentration in aqueous solution, decreased the dielectric constant of the system, thus causing a blue shift and an increase in the emission maximum and intensity, respectively. (D-alanine^2)-methionine-enkephalin was also labeled at the amino terminal with a dansyl group, because of its resistance to aminopeptidase degradation. The fluorescence characteristics of N-dansyl-(D-ala^2)-met-enkephalin were identical to those of N-dansyl-met-enkephalin; the CH_3 group of alanine (vs. the H of glycine) does not appear to affect the fluorescence properties of the attached dansyl group.

In order for dansyl to be a suitable probe, the dansylated enkephalin must possess the ability to bind to opiate receptor sites. N-dansyl-(D-ala^2)-met^5-enkephalin was found to inhibit the specific binding of tritiated naloxone to rat brain homogenates and slices; and, cold naloxone markedly reduced the binding of tritiated N-dansyl-(D-ala^2)-met^5-enkephalinamide to homogenates; suggesting that the N-dansyl-opioids can bind to opiate receptor sites. N-dansyl-(D-ala^2)-met^5-enkephalinamide was determined to occupy the same binding sites on brain slices as non-dansylated (D-ala^2)-met^5-enkephalinamide. However, the receptor affinity of N-dansyl-(D-ala^2)-met^5-enkephalinamide was less than that of (D-ala^2)-met^5-enkephalinamide. These results suggest that the dansyl probe in N-dansyl-enkephalin does not prevent the binding of the enkephalin moiety to the receptor site.

The amygdalar nuclei have been shown to possess a large amount of opiate receptor binding activity and to be involved in the behavioral

aspects of pain. Cells were taken from the amygdalar nuclear region of rat brain and were maintained in dissociated cell cultures; and, in the presence of N-dansyl-met-enkephalin, their fluorescence emission spectra were registered by microspectrofluorometry. The binding of N-dansyl-met-enkephalin to the cells caused a blue shift in its fluorescence peak to 440 nm and a shoulder around 480 nm, indicating that the micro-environment dielectric constant of the 1-dimethylaminonapthalene ring was substantially decreased upon cellular binding. It is argued that the hydrophobicity of the opiate receptor sites caused the blue shift in the fluorescence peak of the bound N-dansyl-met-enkephalin. The bound N-dansyl-met^5-enkephalin appeared as a continuous turquoise covering on the surface membrane of the cell, with discrete blue patches at soma-soma and process-soma contacts; the blue clusters were varied in size and shape. The binding of N-dansyl-met^5-enkephalin to cultured brain cells was inhibited by naloxone.

Studies of the fluorescence properties of free and bound N-dansyl-enkephalin give information on the microenvironmental dynamics at the receptor site, and on the specific location of enkephalin receptors on single cells.

This dissertation is dedicated to the People.

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INTRODUCTION

A. Long-term Objective

The pain-relieving action of opiates is a powerful tool in clinical medicine. However, opiate addiction (e.g. morphine, meperidine (Demerol), codeine, dextropropoxyphene (Darvon), heroin, etc.) has become a major social problem in this country, motivating the search for the mechanism by which the brain modulates pain perception. This led to the discovery that the opiate receptors were receptors for enkephalins, produced by the brain and involved in regulation of pain perception, pathogenesis of schizophrenia, physiological response to stress, long-term memory, etc. This raises the possibility of finding a nonaddictive opiate or a biophysical methodology to elicit analgesia without toxicity and addiction liability. The characterization of the opiate receptor may lead to the achievement of this goal and a deeper understanding of the molecular-neural mechanisms involved in mind-body interactions (Snyder, 1977).

B. Background

The existence of a sterospecific opiate receptor was first biochemically demonstrated in 1973 by Pert and Snyder. Using radioactivity labeled D(-)-naloxone, a potent opiate receptor antagonist, and its competition for the opiate receptor with various opiate agonists and their corresponding structurally analogous antagonist derivatives,

they established that the opiate receptor is localized in the cell membrane of nervous tissue and that the pharmacological potency of the opiates closely parallels their binding affinity for the receptor (Pert and Snyder, 1973).

It has been established that the binding of the opiate antagonist (naloxone, nalorphine, or levallorphan) to the receptor is greatly enhanced in the presence of sodium ions, while the receptor binding of the opiate agonist (oxymorphone, dihydromorphine, or levorphanol) is diminished (Snyder, 1977; Pert and Snyder, 1974; Pert et al., 1973). Therefore, the physiological concentration of sodium in the extracellular fluid bathing the membrane of the neuron should greatly favor the receptor binding of the opiate antagonist over that of the opiate agonist. The lithium ion is the only other ion which can mimic somewhat the action of Na^+, probably because its atomic radius and biological activity are similar to those of Na^+. The other monovalent cations, rubidium, cesium and potassium, and the divalent cations, calcium, manganese and magnesium, cannot discriminate between the opiates, depressing the receptor binding of both agonists and antagonists. This strongly suggests that the differential influence of Na^+ on opiate receptor binding is highly specific (Pert and Snyder, 1974).

Pert and Snyder (1974; Snyder, 1977) hypothesized that the opiate receptor has a specific site for binding the sodium ion, and that the binding of sodium allosterically transforms the opiate receptor from a conformation which readily binds the agonist to a conformation that more readily binds the antagonist; the microenvironment of the opiate binding site may change as the opiate receptor shifts back and forth between the agonist and antagonist conformations. They suggested that

opiate binding site may exist in an equilibrium between two distinct conformations, and that the binding of Na^+ induces a structural transformation in the binding site which promotes the dissociation of the opiate agonist (Pert and Snyder, 1974).

Since the stereospecific receptor binding of naloxone is temperature and pH-dependent, binding occurs most rapidly at 37°C and pH 7.4 (Pert and Snyder, 1973). The binding of naloxone may be totally abolished by heating homogenates at 55°C, or by lowering the pH below 5 or raising it above 10. This indicates that the opiate receptor may have proteins and lipids as major components, undergoing denaturation at extreme temperatures and pH; in fact, low concentrations of the proteolytic enzymes trypsin or chymotrypsin, or the fatty acid hydrolyzing enzyme phospholipase A, or the detergents Triton-X 100, deoxycholate, or sodium dodecyl sulfate have been shown to degrade the receptor (Pert et al., 1973).

A number of protein-modifying reagents have been shown to differentially influence the binding of the opiates agonists and antagonists (Pasternak et al., 1975, a). The most effective discrimination of agonist and antagonist binding occurs with protein-modifying reagents that preferentially react with sulfhydryl groups. At low concentrations, these reagents (p-aminophenyl-mercuric acetate and p-chloromercuribenzoate) can strongly inhibit the binding of the opiate agonist (dihydromorphine) without appreciably affecting the binding of the opiate antagonist (naloxone). It is suggested by Pasternak et al. (1975, a) that these reagents may interact with a reactive sulfhydryl group at or near the opiate binding site on the opiate receptor, and that this sulfhydryl group is much more important for the

binding of the opiate agonist to the opiate receptor than for the receptor binding of the opiate antagonist. Also, protein-modifying reagents like N-bromosuccinimide, which readily reacts with tryptophan and tyrosine residues, and carbodiimide, which primarily reacts with carboxyl groups, can effectively discriminate between the receptor binding of the opiate agonist and antagonist. This indicates that a reactive carboxyl group and aromatic amino acids may be at the opiate binding site, playing a very important role in the binding of the agonist to the opiate receptor. There are other protein-modifying reagents that have no effect on the receptor binding of the opiates. Pasternak et al. (1975, a) suggested that the selective destruction of the agonist receptor sites by the above reagents may be attributed to their effect on the chemical groups that are responsible for the interconversion of the receptor conformations; or, it is possible that the opiate receptor has two different sites for binding the opiate agonist and antagonist (Pasternak et al., 1975, a). In any case, the microenvironmental conditions at the binding site of the agonist-receptor complex are not exactly identical to that of the antagonist-receptor complex.

The regional distribution of the opiate receptor in the brain of monkey and man has been evaluated in detail by Kuhar et al. (1973). Using the binding of ^{3}H-dihydromorphine to homogenates of dissected monkey brain regions, they found that the amygdala possessed the greatest concentration of ^{3}H-dihydromorphine binding; the posterior amygdala displayed roughly 50% of the binding of the anterior amygdala. The periaqueductal gray was found to have the next highest opiate receptor binding, displaying slightly less binding than the posterior

amygdala. The amygdala nuclei are portions of the limbic system, largely concerned with emotional behavior; this brain region may mediate the euphoric effects of the opiates on emotional pain. Also, the regional distribution of the opiate receptor in the brain and spinal cord remarkably resembles that of the paleospinothalamic pain system; the periaqueductal gray is an important way station in the intergration of sensory information concerned with physical pain (Snyder, 1977). The cerebellum and white mater such as the corpus callosum, corona radiata, fornix and optic chiasm possessed a very low or a non-detectable amount of opiate receptor binding (Kuhar et al., 1973; Snyder, 1977). Kuhar et al. (1973) found that the map of the regional distribution of the opiate receptor in the human brain largely looks like that of the monkey brain, with the amygdala and thalamus displaying the greatest concentration of binding sites. Opiate receptor binding has been detected in the CNS of all vertebrates examined, but opiate receptor binding is not present in the invertebrate animal (Simantov et al., 1976).

Enkephalin is the proposed neurotransmitter which combines with the opiate agonist receptor conformation, and subsequently leads to the inhibition of the neuron (Hughes, 1975; Hughes et al., 1975, a, b; Pasternak et al., 1975, b; Terenius and Wahlström, 1975, a, b). Enkephalin has been purified from extracts of pig brain, and has been shown to inhibit neurally evoked contractions of the mouse vas deferens and guinea pig myenteric plexus; therefore, passing the standard test for opiate agonist receptor binding. A dose-response curve for enkephalin in the mouse vas deferens strikingly parallels the dose-response curve of the stereospecific opiate agonist normorphine, indicating that enkephalin and normorphine have a common site of action on

the opiate receptor (Hughes et al., 1975, a). Using a different approach, Terenius and Wahlström (1975, a) demonstrated that rat brain extracts possessed the endogenous enkephalin that can inhibit the opiate receptor binding of ^{3}H-dihydromorphine to the synaptic plasma membranes of rat brain preparations and to the guinea pig ileum. This receptor blocking activity of enkephalin was found to be reversible and competitive. Also, they established that enkephalin extracted and purified from human cerebrospinal fluid behaves very similar to the enkephalin purified from the rat brain extracts; both extracts displayed a five-fold reduction in their competitive affinity upon the addition of sodium (the competitive affinity of an opiate agonist is reduced, in the presence of sodium ions, but the competitive affinity of the opiate antagonist is not affected). The above findings suggest that enkephalin may be an endogenous substance which stereospecifically binds to the opiate receptor. Terenius and Wahlström (1975, b) found that the level of enkephalin in the human cerebrospinal fluid varied among different individuals and that the level of enkephalin was lower in patients afflicted with trigeminal neuralgia than in other individuals. Childers and Snyder (1977) reported that they were not able to detect enkephalin activity in human cerebrospinal fluid (obtained post-mortem) the non-detectable activity of enkephalin may be due to its prior degradation by brain enzymes before radioimmunoassay determination; Terenius and Wahlström (1975, b) employed frozen-fresh cerebrospinal fluid from a living patient, thus greatly diminishing the enzymatic destruction of enkephalin. Using radioimmunoassay, Sullivan et al. (1977) reported that the levels of met-enkephalin in normal human cerebrospinal fluid were 3.1 1 pmoles/ml.

Hughes et al. (1975, b) have isolated enkephalin from pig brain extracts and determined its amino acid sequence as two-related pentapeptides NH_3^+ - Tyr - Gly - Gly - Phe - Met - COO^- and NH_3^+ - Tyr - Gly - Gly - Phe - Leu - COO^-. These two enkephalins differ structurally by the last amino acid in their sequence and are designated as much as methionine-enkephalin and lencine-enkephalin (Simantov and Snyder, 1976, a, b). As alluded to above, the naturally-occurring enkephalins are rapidly destroyed by aminopeptidases and carboxypepticases, and the primary mode of degradation is the cleavage of the Try - Gly amide bond (Lane et al., 1977; Craviso and Musacchio, 1977). Synthetic analogues of enkephalin have been developed, which are not susceptible to destruction by brain enzymes and have biological properties similar to the endogenous enkephalin, via the substitution of glycine at the 2-position with D-alanine and/or the modification of one or both terminal with N-methyl and C-amide groups (Roemer et al., 1977; Marks and Grynbaum, 1977; Bradbury et al., 1977; Pert et al., 1976).

It has been indicated that enkephalin is a neurotransmitter for specific neuronal systems which mediate the integration of sensory information involved in the behavioral aspects of physical and emotional pain (Snyder, 1977). Immunohistochemical and autoradiographic mapping of the CNS have revealed that enkephalin nerve terminals parallel the regional distribution of opiate receptors (Snyder, 1977; Sar et al., 1977); in rat brain, a dense population of enkephalin terminals have been demonstrated in the head of the caudate, globus pallidus, hypothalamus, amgydala, periaqueductal central grey, and paraventricular nucleus of the thalamus (Watson et al., 1977; Bloom et al., 1977). Hughes (1975) suggested that enkephalin forms part of a central pain

suppressive system and subserves some other unidentified neurochemical role in the brain. The enkephalins have been shown to suppress the stimulus-evoked release of substance P from superfused slices of the spinal trigeminal nucleus from rat brain; but, the enkephalins do not suppress its release from all substance P-containing regions of CNS; substance P is a proposed excitatory neurotransmitter, released from the nerve terminals of primary sensory fibers involved in the transmission of pain (Jessell and Iversen, 1977). Buscher et al. (1976) have shown that intracerebroventricular administration of met-enkephalin can induce analgesia in mice, and that the analygesic effects can be inhibited by subcutaneous injections of naloxone. The enkephalins have been found to modulate the release of neurotransmitters from specific catecholaminergic and cholinergic neurons by presynaptic inhibition (Jhamandas et al., 1977; Pollard et al., 1977). Similar to morphine, the endogenous opioid peptides and their analogues have been reported to regulate neuroendocrine functions in stimulating the pituitary release of prolactin and growth hormone (Cusan et al., 1977). In addition, substantial amounts of enkephalin has been detected in the gastrointestinal tract of many animals, suggesting a hormonelike function for enkephalin outside of the CNS (Snyder, 1977). A number of investigators have reported that the enkephalins may be involved in the pathogenesis of schizophrenia and in the physiological response to stress (Akil et al., 1978; Barchas et al., 1978). When administered immediately following training, enkephalin has been shown to facilitate long-term memory of a learned response in rats (Stein and Belluzzi, 1978).

C. Rationale

The rationale behind using biophysical cytochemistry to investigate the structural-functional dynamics of the neuronal opiate receptor, is that: 1. By labeling the synthetic met-enkephalin and (D-ala^2)-met-enkephalin with a fluorescent molecular probe and by studying the fluorescence properties of the labeled-enkephalins and the labeled-enkephalin-opiate receptor complex under various conditions (Na^+, pH, temp., etc.), we can make inferences about the nature of the agonist receptor site and of the endogenous enkephalin pentapeptide. The emission properties of the fluorescent molecular probe should respond to environmental alterations. Since the utilization of this technique will be dependent upon the similarity in the binding affinity and the activation of the enkephalin-opiate receptor by free enkephalin and dansyl labeled enkephalin, we will experimentally determine the binding characteristics of the dansylated enkephalin. 2. Fluorescence microspectrophotometry would allow for the application of qualitative as well as quantitative cytochemistry to opiate receptors on single neurons. Fluorescent molecular probes have been used in conjunction with cell cultures to examine various cellular components (Canada, 1976, a, b; Anderson and Cohen, 1977, 1974; Anderson et al., 1977; Chignell, 1973; West and Lorinez, 1973).

The idea of using fluorescently labeled enkephalin to study the activation-sites of opiate receptors (i.e. conformational changes, number, binding rate, etc.) on single intact neurons is unprecedented, although the notion of using fluorescently labeled drugs to visually map the location of specific neurotransmitter receptor sites on cells is not new. In 1974, Anderson and Cohen (1977; Anderson et al., 1977)

visualized the distribution of acetylcholine receptors in vertebrate skeletal muscle fibers by using α-bungarotoxin labeled with either fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate. They found that the fluorescent staining on the muscle fiber was limited to the neuromuscular junction; the fluorescence pattern of the junctional folds was displayed as intense transverse bands occurring at about 0.5-1 micron intervals and the adjacent subsynaptic membrane had a lower fluorescence intensity than the junctional folds; the fluorescence intensity was found to decline abruptly at the border between the synaptic and extrasynaptic muscle membrane and was not detected on the nearby extrasynaptic muscle membrane. The fluorescent conjugates of α-bungarotoxin have been used to study the effects of innervation on the distribution of acetylcholine receptors on muscle cells, in culture (Anderson et al., 1977; Anderson and Cohen, 1977); small fluorescent patches on innervated muscle cells were found restricted to the path of nerve contact, indicating that innervation induces the aggregation of acetylcholine receptors at sites of nerve-muscle contact; one or more discrete fluorescent patches were found on muscle cells not contacted by nerve. The translocation of discrete dye-toxin-receptor patches, within the sarcolemma of muscle cells, have been shown to occur spontaneously or in response to innervation, in culture (Anderson et al., 1977; Anderson and Cohen, 1977). The pharmacological potencies of dye-toxin conjugates were found to be lower than that of the unlabeled toxin; the loss of potency tends to vary as a function of the number of dye molecules bound per α-bungarotoxin macromolecule (Anderson and Cohen, 1974). The nicotinic acetylcholine receptor binding specificity of the dye-toxin conjugates was tested by the ability of reversible nicotinic

agents such as curare and carbachol to decrease or virtually abolish the fluorescent staining of the muscle membrane; and, the exposure of the muscle fibers to muscarinic blocking agents, like atropine and neostigmine, had no significant effect on the fluorescence intensity of the dye-toxin-receptor complexes; and, pre-treatment with unlabeled α-bungarotoxin was found to prevent fluorescent staining of the muscle membranes (Anderson and Cohen, 1977, 1974; Anderson et al., 1977).

Why should we use the dye-enkephalin conjugates to probe the activation-site of the opiate receptor? The structural mode for the enkephalin pentapeptide is suggested to be a β-conformation (Khaled et al., 1977; Snyder, 1977). The molecular structure of tyrosine is found in a number of opiate agonists. The tyrosine terminal of the enkephalin pentapeptide readily binds to the stereospecific activation-site of the opiate receptor; the benzene ring of tyrosine is in precisely the same orientation as the benzene ring A of morphine and its OH moiety may participate in a hydrogen bond with the receptor site (Snyder, 1977). The non-polar phenylalanine and methionine residues of enkephalin must be inserted into a hydrophobic environment of the opiate receptor, thus securing the wobbly pentapeptide at the activation-site; the benzene ring of the phenylalanine residue has a specific binding location at the receptor site, which stablizes the agonist receptor conformation, similar to the benzene ring F of potent opiate agonists (Snyder, 1977). Like other opiates (Nachtmann and Spitzy, 1975), the molecular structure of the opioid peptides have several positions suitable for binding a dye molecule, the NH_2 and OH groups of tyrosine and the carboxyl terminal. A variety of molecular groups have been attached to the amino and/or carboxyl termini of enkephalin without inhibiting its opiate receptor

binding stereospecificity (Pert et al., 1977, 1977; Bradbury et al., 1977; Roemer et al., 1977). The pharmacological potency of met-enkephalin has been found to be greater than that of leu-enkephalin and at least three times more potent than morphine (Hughes et al., 1975, b). The reason for using the (D-ala^2)-met-enkephalin is that, it is very resistant to enzymic degradation, dissimilar to met-enkephalin which is easily destroyed (Pert et al., 1976; Roemer et al., 1977; Marks and Grynbaum, 1977). The opiate receptor site must have the capacity to recognize a specific ligand and to initiate action in response to ligand binding. Opiate receptor binding refers only to binding to the receptor site and not the initiation of action. Since the enkephalin is the endogenous ligand for the opiate receptor, the "opiate receptor" is actually the "enkephalin receptor."

Why should we use the dansyl group as the fluorescent probe for the molecular structure of enkephalin and the enkephalin-opiate receptor complex? The dansyl group has been employed as a probe in fluorometric analysis of amino acids/peptides and in protein binding and structure studies (Chignell, 1973; Gross and Labouesse, 1969; Stryer, 1968; Chen, 1967). Dansyl chloride has been demonstrated to react with the unprotonated amino group of tyrosine and N-terminal residues of peptides, proteins and met-enkephalin (Fournie-Zaluski et al., 1978, a; Felgner and Wilson, 1977; Gros and Labouesse, 1969). This is taken to suggest that the amino terminal of enkephalin may be reacted with dansyl chloride. The fluorescence of dansylated amino acids is yellow and very weak in water, their emission peaks and quantum yields are at about 580 nm and less than 0.1, respectively. But, when placed in an environment of

low dielectric constant, the emission of the dansyl amino acid is shifted towards the blue and its quantum yield increased significantly (e.g. 500 nm and 0.70, respectively, for dansyl DL-tryptophan in dioxane). This strong green fluorescence has permitted the fluorometric determination of some dansyl amino acids down to 1×10^{-9} M (Chen, 1967). The hydrophobicity of the environment should affect the fluorescence characteristics of dansyl enkephalin in the same manner as other dansyl derivatives. A number of dansylated amino acids (methionine, valine, tryptophan, glutamate, proline and glycine) have been shown to bind to proteins having hydrophobic binding sites such as bovine serum albumin and sperm whale apomyoglobin. Concomitantly, the dansyl emission peak is shifted towards the blue (to 480 - 500 nm) and there is a substantial increase in fluorescence quantum yield (to 0.4 - 0.7) (Chen, 1967; Chignell, 1973). The dansyl enkephalin may be used as a probe for the agonist binding-site on the enkephalin-opiate receptor if the environment of dansyl is altered as a result of binding to the opiate receptor. In this case, the fluorescence characteristics of dansyl enkephalin bound to the opiate receptor should be different from that of the free dansyl enkephalin; since the agonist receptor site has a hydrophobic nature.

Has the opiate receptor been demonstrated on neurons maintained in cell cultures? The first demonstration of opiate receptor binding on cells grown in cultures was done by Klee and Nirenberg (1974). They found that a neuroblastoma x glioma hybrid cell line (NG 108-15) has opiate agonist receptors, located in their plasma membranes, capable of binding morphine with high affinity. The displacement of the stereospecific binding of ^{3}H-dihydromorphine by non-radioactive morphine and other opiates was used to assay for the opiate receptor binding of the

cells. They found that the relative binding affinities of the test opiates to NG 108-15 cells closely approximated those found for the rat brain homogenates and match well to their pharmacological potencies as reported for the guinea pig ileum. In addition to opiate receptors, these cells were found to have neuronal characteristics such as choline acetyl-transferase, intracellular acetylcholine, long neurities, electrically excitable membranes and nicotinic acetylcholine receptors. Klee and Nirenberg (1974) calculated that the average NG 108-15 cell contains approximately 3×10^5 opiate receptors. This large concentration of opiate receptors per cell is similar to the number of nicotinic acetylcholine receptors estimated for cultured chick sympathetic ganglion neurons (Klee and Nirenberg, 1974); and, is more than enough for accurate analyzation by fluorescence microspectrophotometry, since at least one dansyl molecule may be bound per opiate receptor. Recently, Crain et al. (1977) demonstrated that morphine sulfate, etorphine and levorphanol can stereospecifically bind to opiate receptor sites in the dorsal horn regions (substantia gelatinosa) of spinal cord cross-sectional explants cultures, with attached dorsal root ganglia. They reported that, in the dorsal horn, the negative slow-wave responses evoked by focal dorsal root ganglia stimulli can be selectively depressed by exposure to opiate agonists. Naxolone was found to restore the opiate-blocked cord responses and, in cultures not exposed to opiates, naloxone was found to increase the amplitude and duration of the dorsal root ganglia-evoked dorsal cord responses. Simon et al. (1977) established that the neurities of dorsal root ganglia nerve cells were capable of stereospecific opiate receptor binding, in culture, providing evidence for presynaptic binding of opiates.

The rationale behind growing neurons from the amgydala nuclear complex in dissociated cell culture, is that a large population of neurons possessing enkephalin receptors have been demonstrated in this brain region and because different neuronal types as characterized by their cultural morphology can be readily identified in culture. Neurons maintained in dissociated cell culture have a number of advantages for use with fluorescent probes. The dissociated cell cultures can provide individual neurons. The neurons are grown in an environment that can be controlled and easily manipulated. In addition, they are readily assessible to diffusible materials and any unbound excess probe can be washed off freely. Furthermore, neurons and neuronal contacts in a monolayer cell culture are simple to observe and their fluorescence easily analyzed with microspectrofluorometry in the same petri dish, without mutilating the cells and without insulting their cell membrane integrity. It is evidenced that cell cultures in combination with fluorescent probes furnish a valuable model system to study the molecular interactions of the enkephalin-opiate receptor system in single neurons.

D. Specific Aims

The objective of this investigation is to fluorescently-label a neurotransmitter (enkephalin) and to employ it as a probe for the opiate agonist receptor site, in order to gain information about the local environment of the receptor site and its location on the single fixed neuron using microspectrofluorometry. In order to achieve these goals the following approach was used: 1. The labeling of methionine-enkephalin and (D-alanine^2)-methionine-enkephalin by a dansyl group.

2. The study of the fluorescent properties of enkephalins and N-dansyl-enkephalins. 3. The interaction of N-dansyl-enkephalins with receptor sites in rat brain homogenates and slices to assess their specific binding via the inhibition of ^{3}H-naloxone or ^{3}H-(D-ala^{2})-met^{5}-enkephalinamide binding. 4. The fluorescent labeling of receptor sites on cultured cells by N-dansyl-enkephalin. 5. The study of the emission properties of the enkephalin-dansyl-receptor complex on single cells in culture. A chief purpose of this inquiry is to introduce a novel technique in studying the opiate receptor interactions of intact neurons. This affords an advantage over existing techniques by allowing us to visually map and quantitatively follow controlled environmental modifications of the opiate receptor sites on intact neurons.

EXPERIMENTAL PROCEDURES

A. Materials

Methionine^5-enkephalin (met-enkephalin) was obtained from Pierce Chemical Company (Rockford, IL), and the (D-alanine^2)-methionine^5-enkephalin ((D-ala^2)-met-enkephalin) was obtained from Peninsula Laboratories, Inc. (San Carlos, CA). The naloxone was a donation from Endo Laboratory, Inc. (Garden City, NY). Tritiated naloxone, met-enkephalin and (D-ala^2)-met-enkephalin were purchased from New England Nuclear (Boston, MA).

Purified dansyl chloride (1-dimethylaminonaphthalene-5-sulfochloride), dansylamide and dansyl glycine were acquired from Sigma Chemical Company (St. Louis, MO) as well as Trizma base, Tris(hydroxymethyl)aminomethane. Stock solutions of dansyl chloride were made up in spectral grade acetone from Matheson, Coleman and Bell, Manufacturing Chemists (Norwood, OH) and stored at 4°C in the dark. Benzene, butyl alcohol, chloroform, ethyl acetate, ethyl ether, formaldehyde solution, formic acid, iodine, nitric acid, 1-nitroso-2-naphthol and sodium bicarbonate were purchased from Mallinckrodt, Inc. of Paris, Kentucky and St. Louis, MO. Absolute ethyl alcohol (200 proof) was secured from INC Chemical Group, Inc. (Terre Haute, IN). Toluene, distilled in glass, was obtained from Burdick and Jackson Laboratories, Inc. (Muskegon, MI). Nin-sol ninhydrin aerosol spray, 0.25% ninhydrin reagent in n-Butanol, was obtained from Pierce Chemical Company (Rockford, IL). All reagents were of

analytical quanity and were used without further purification. The thin-layer chromatography was performed on glass plates pre-coated with silica gel 60 (without fluorescent indicator) from E. Merck, Darmstadt, Germany; having a layer thickness of 0.25 mm.

Timed pregnant Sprague-Dawley rats were purchased from Spartan Research Animals Company and permitted to give birth in our animal care room. The dissociated neurons were cultivated with 17% fetal calf serum in minimum essential medium, at 37°C (Canada, 1976, a). The minimum essential media, l-glutamine and fetal calf serum were obtained from Grand Island Biological Company (Grand Island, NY). The incubator was a Napco 322 from the National Appliance Company, with a temperature variation of $\pm$ 0.5°C.

B. Methods of Procedure

1. The labeling of met-enkephalin and (D-ala^2)-met-enkephalin with the fluorescent molecular probe, dansyl chloride.

The standard conditions for the dansylation of the enkephalin were as follows: 3.0 ml of 5 x 10^{-3} M dansyl chloride in acetone was added to 3.0 ml of 2 x 10^{-3} M enkephalin in 0.05 M sodium bicarbonate buffer pH 8.3. The acetone solution was added to the buffer solution to prevent the acetone from rising on the glass walls and limiting the labeling reaction (Nachtmann and Spitzy, 1975). The reaction solution was immediately mixed for 30 minutes at 46°C in a constant temperature shaker water-bath, set at 140 osc/min (Precision Scientific Company, Chicago, IL). After mixing, the reaction solution was allowed to cool to room temperature. The reaction solution was kept in the dark during mixing and cooling. It is important that the volume of buffer solution equal that of the dansyl chloride solution (Nachtmann and Spitzy, 1975).

Purification of the N-dansyl-enkephalins and separation of the reaction by-products were accomplished by thin-layer chromatography (TLC). 0.01 ml aliquots of the reaction solution were placed as spots on silica gel 60 plates, approximately 2 cm from the bottom of the plate; each plate was 5 x 20 cm. The spots were allowed to dry in a desiccator. One-dimensional chromatographic development was achieved by ascending solvent flow in a covered tank. In our studies, the best solvent system for the separation of N-dansyl-enkephalins was the toluene-ethanol (6:10, v/v) system. The solvent front migrated approximately 19 cm in 200 min. The solvent depth was at 1.0 cm in the developing tank, and the atmosphere was permitted to equilibrate for 24 hours prior to TLC. Long wave ultraviolet irradiation was employed to visualize the separated dansyl derivatives (Mineralight UVSL-25, San Gabriel, CA; caution, wear protective glasses). The entire TLC procedure was performed in the dark and ultraviolet irradiation was kept at a minimum to minimize the photolytic degradation of the dansyl derivatives (Pouchan and Passeron, 1975).

After chromatography and evaporation of the solvent, the spots corresponding to N-dansyl-enkephalin were scraped off the plates into a test tube. The dansyl enkephalin was eluted from the silica gel via refrigeration with 10.0 ml of absolute ethanol for 24 hours. The silica was removed from solution by centrifugation, for 5 minutes, with a 'Waco Separator' (Wilkens and Anderson Company, Chicago, IL). The supernatant was extracted from the test tube, and employed in the fluorescence and absorption measurements, as well as in the opiate receptor binding radioassays. The stock N-dansyl-enkephalin solution was stored at -4°C, in the dark.

The ninhydrin reaction was used to test for any non-labeled enkephalin (Pataki, 1968; McCaldin, 1960). After chromatography and evaporation of the solvent, some plates were treated with ninhydrin spray and heated to 60°C for 30 min. The ninhydrin positive spots were identifiable by their purple color.

The 1-nitroso-2-naphthol reaction was used to detect the free phenolic group in N-dansyl-enkephalin (Greenstein and Winitz, 1961; Bailey, 1967). In a test tube, 1.0 ml of the elution was mixed with 1.0 ml of doubled distilled water. Five drops of 0.1% (wgt) 1-nitroso-2-naphthol in 75% ethanol was added to the tube along with 5 drops of concentrated nitric acid. After mixing, the test tube was heated in a water-bath at 32°C for 3 min. The reaction positive mixture turned red in color.

2. The assessment of the opiate receptor binding of the N-dansyl-enkephalins.

The specific binding of the dansylated enkephalins were assayed according to the procedures of Davis et al. (1977). Brain slices or homogenates from male Sprague-Dawley rats were used, and prepared in a cold room (5°C). The basal ganglia and diencephalon were homogenized with a motor-driven Teflon-pestle homogenizer (Potter-Elvehjem), and/or 0.5 mm thick slices from the same brain areas were prepared with a McIlwain tissue chopper. The brain slices and homogenates were incubated, for 20 min at 37°C in a gyrotory water bath, with 3 nM of either tritiated naloxone or (D-ala^2)-met^5-enkephalinamide in the presence of varying concentrations of dansylated enkephalins or cold (D-ala^2)-met^5-enkephalinamide. Solutions were made with 0.05 M tris-HCl buffer pH 7.4. The brain slices were homogenized immediately after the incubation

period. Duplicate aliquots of both tissue preparations were filtered on nitrocellulose membranes (0.8 μm pore size, Millipore Corporation, Bedford, MA), with the assistance of a vacuum pump. The millipore filters were dissolved in 1.0 ml of ethylene glycol monomethyl ether. Liquid scintillation spectrometry was used to measure the radioactivity of the samples (Davis et al., 1977).

The fluorescence spectra of N-dansyl-enkephalins bound to opiate receptor sites in purified membrane fragments was measured in solution with a spectrophotofluorometer. The procedures for the isolation of membrane fragments were adapted from Fleischer and Packer (1974). Sucrose gradients were formed in ultracentrifuge tubes with a gradient fractionator in a cold chest (5°C). The solutions were made with 0.05 M tris buffer pH 7. The formation of a gradient was with the following solutions: 4 ml of 60% sucrose, 8 ml of 50% sucrose, 8 ml of 40% sucrose, 6 ml of 60% sucrose, 4 ml of 20% sucrose and 3 ml of 15% sucrose. The sucrose gradients were stored in the cold chest until use. The rhinencephalon, amygdala and hypothalamus from four Sprague-Dawley rats were homogenized with 5 ml of 7% sucrose in a glass homogenizer; the rats were anesthetized with ether before brain removal. The homogenates were poured into clinical centrifuge tubes, and centrifuged for 5 min at a setting of 30 in a clinical centrifuge. The supernatants were decanted off into small test tubes and labeled "homogenate"; the remaining pellet contains unbroken cells and nuclei (e.g. Fleischer and Packer, 1974, p. 70). The homogenates were applied to the top of the sucrose gradients; 7% sucrose was added to the gradients to match the weight of each bucket. The gradients were ultracentrifuged for 2 1/2 hr at

25,000 rpm (Beckman model L3-40 Ultracentrifuge). After centrifugation, the gradient fractionator was employed to fractionate the gradients; the optical density of each fraction was measured at 280 nm (Beckman model DB spectrophotometer). The microsomal fractions of the gradients were pooled and placed in centrifuge tubes, with 0.05 M tris buffer pH 7. The tubes were inserted in the Beckman #30 rotor and centrifuged for 30 min at 27,000 rpm. The sucrose was washed from the pellet; where the pellet was resuspended in 0.05 M tris buffer pH 7 and centrifuged for 30 min at 27,000 rpm in the #30 rotor, twice. After washing, the pellet was suspended in 3 ml of 0.05 M tris buffer pH 7, and labeled "purified membrane fragments." Fifty µl aliquots of the membrane preparation were mixed with 3 ml of varying concentrations of N-dansyl-enkephalin; immediately thereafter, their fluorescence spectra were recorded with a spectrophotofluorometer.

The protein concentrations of the purified membrane preparations were determined by the Lowry method (Lowry et al., 1951). 1.0 ml of 0.1 N NaOH was added to sample tubes containing 25 µl or 50 µl of "purified membrane fragments" and to standard curve tubes which contains 1.0 ml of bovine serum albumin at 0 to 190 µg. Five µl of Lowry D were added to each tube and mixed; Lowry reagent D is prepared by mixing together Lowry B, Lowry C and Lowry A, (1:1:98 v/v/v). The tubes were incubated for 30 min at 45°C and then permitted to cool to room temperature. 0.5 ml of Folin reagent E were added to each tube and immediately mixed vigorously and allowed to stand at room temperature for 30 min. Folin reagent E was made by diluting 5 ml of Folin Phenol with 5 ml of double distilled H_2O (1:1 v/v). The optical density at 560, 600 and 660 nm for each standard curve tube was obtained (Beckman

model DB spectrophotometer) and plotted against the protein concentration within the tube; making three standard curves. The protein concentrations of the sample tubes were calculated from their optical densities at 560, 600 and 660 nm, using the standard curves; three wavelengths were used to improve accuracy.

3. The culture procedures for single amygdaloid cells and the staining of culture cells with N-dansyl-enkephalins.

The culture procedures for single amygdaloid cells are a modification of the process outlined by Canada (1976, a) and will be described here in detail. The location and condition of the area to be utilized in the preparation and growing of the cells are critical. The entire working area and room were sterilized to prevent contaminates invading the cell culture and disrupting the neuronal development. Mikro-Quat detergent-germicide deodorizer (Economics Laboratory, Inc., St. Paul, MN) served this purpose; the work area was scrubbed first with diluted and second with concentrated Mikro-Quat. The room was sealed off, and sprayed with Staphene disinfectant spray and air sanitizer (Vestal Laboratories, St. Louis, MO), to eliminate air-born germs and bacteria. All glassware (i.e. culture tubes, serological pipets and extra media bottles) were sterilized in the autoclave for 30 min. The necks and caps of the media bottles and of the culture tubes were heated with a blue flame, before and after use. Prior to use, the shaft of each pipet was heated by blue flame (and permitted to cool). The surgical instruments were rinsed in ethanol from water, before flaming with a blue flame. The instruments were sterilized before each surgical step; care was taken to make sure that the surgical instruments were at room temperature before touching animal tissue. Betadine surgical scrub (The Purdue Frederick Company, Norwalk, CT) was used to wash hands before

animal surgery. The entire culture procedures employed aseptic techniques.

The basic neuron medium consists of 17% fetal calf serum in minimum essential medium with Earle's salts. The minimum essential medium was purchased without glutamine to prevent prior degradation of the glutamine. Therefore, 0.0292 g of l-glutamine was added to 100 ml of Eagle's minimum essential media with Earle's salts but without glutamine. Twenty ml of deactivated fetal calf serum was mixed with this solution. (The fetal calf serum was inactivated in a constant temperature water bath at 56°C for 40 minutes.) This mixture was labeled Neuron Medium. Prior to use, half of the medium was stored at 4°C to prevent contamination of the entire lot. The rest was kept sealed at room temperature until use.

Before brain removal, the animals were anesthetized with ether, and their skins were sterilized by submersion in a solution of concentrated iodine in 70% ethanol, and then rinsed in 70% ethanol. The first cut, with scissors, was across the rat's shoulders and back of its neck. The second cut was made perpendicular to the first cut, from the back of the neck and forward across the head to the tip of the nose. The first and second cuts formed an upside-down "T." The skull was skinned and exposed. The whole brain was removed via a calvarium dislocation, where a horseshoe-shaped cut was made along the perimeter of the skull. This cut was made to penetrate the soft bone but not the brain. Hemostatic forceps were employed to gently raise the skull cap and expose the brain. The spinal cord, cranial nerves and remaining ligaments were shipped with micro-scissors; after flaming, the micro-scissors were rapidly cooled by dipping the blades in media. The micro-scissors were

then inserted under the brain, and used to lift the brain out of the skull into a culture dish containing minimum essential media without glutamine and without fetal calf serum. The brain removal procedures were performed under the hood; the amygdalar dissection and mechanical dissociation were performed in a work area away from the hood and airflow.

For amygdalar cultures, the amygdalar nuclei were dissected from the brains of 15 days old postnatal rats, using the olfactory tubercle, rhinal fissure, optic chiasma and pyriform cortex as external limiting boundaries. The cerebellum was omitted from "whole-brain" cultures. The dissected material was placed inside a culture dish top and dissociated via mincing into a fine mash with two scalpels (No. 11 blades); the scalpels were rapidly cooled after flaming by dipping the blades into medium. The mash was scaped off the surface of the dish and placed into a culture tube (20 x 150 mm) utilizing a bone cleaner cooled in medium, then 2.0 ml (10 ml for whole-brain cultures) of Neuron Medium were added to the tube. The dissociated material and media were briskly agitated for 5 minutes with a vortex. This mixture was allowed to stand, undisturbed, for 30 minutes at room temperature, in order that the non-dissociated material or debris could settle (by gravity) to the bottom of the tube. The nervous tissue was mechanically dissociated without the utilization of trypsin to reduce unwanted chemical interferences and to increase cell attachment to the surface of the culture dish. 1.0 ml (2.0 ml for whole-brain cultures) of the supernatant was pipetted from the culture tube and placed in the center of a 60 mm diameter sterilized plastic culture dish (Corning Company). The culture was placed in the incubator for 24 hours; this permitted the dissociated cells to attach

to the bottom surface of the dish, in a restricted area. The internal environment of the incubator was 5% CO_2 in air at 37°C and humidified with bidistilled water. Next, 6 ml of Neuron Medium at 37°C were introduced to the culture. After three weeks of incubation, 5 ml of Neuron Medium at 37°C were added to the culture. Three weeks later, the old media was siphoned off and 7 ml of fresh Neuron Medium at 37°C were added to the culture. This neuron feeding procedure was continued to allow the cultures to develop satisfactorily. The age of the cells in culture was equated to the age of the animal at the time of sacrifice plus the number of days spent in culture. The cells in culture first met a morphological criterion (extension of processes, contacts) before staining with N-dansyl-enkephalins.

The entire N-dansyl-enkephalin binding procedure was performed on fixed cells in culture, at room temperature, involving:

1. Removal of medium and cell fixation. After rinsing away the medium with physiological saline (0.87% NaCl), the cells were fixed and transferred from fixative into an aqueous environment:

(a)	Physiological saline	30 sec.
(b)	Physiological saline	30 sec.
(c)	Physiological saline	30 sec.
(d)	3.7% formaldehyde in 0.05 M tris buffer pH 7.3	1 hr.
(e)	0.05 M tris buffer pH 7.3	60 sec.
(f)	0.05 M tris buffer pH 7.3	60 sec.
(g)	0.05 M tris buffer pH 7.3	60 sec.

2. Staining process. N-dansyl-enkephalin was introduced to the cultured cells.

   (a) 1×10^{-6} M N-dansyl-enkephalin in 0.05 M tris buffer pH 7 and 1% ethanol — 30 min.

3. Rediffusion process. The washing away of the unbound excess N-dansyl-enkephalin with buffer.

   (a) 0.05 M tris buffer pH 7.3 — 60 sec.

   (b) 0.05 M tris buffer pH 7.3 — 60 sec.

   (c) 0.05 M tris buffer pH 7.3 — 60 sec.

The solutions were slowly added to the cultures in 5.0 ml allotments; solutions were removed from the cultures by siphon. Immediately following the rediffusion process, the fluorescence emission spectra of the cells were registered by the microspectrofluorometer; the cells were sealed in culture with tris buffer, under cover-slip. The areas of the cells bodies were determined by projecting and tracing the cell outlines on paper. A planimeter was used to measure the areas of the cells' profiles.

C. Instrumentation

The absorbance of solutions were measured with a Cary model 15 spectrophotometer or Beckman model DB spectrophotometer. The fluorescence excitation and emission spectra of solutions were obtained with an Aminco-Bowman spectrophotofluorometer (American Instrument Company), using 1 cm path length cuvettes. The cell cultures were observed with a Unitron Mic-1234 inverted microscope.

Photomicrographs and fluorescence emission spectra of the cultured cells were registered with a microspectrophotometer constructed from commercially available components. The microscope was a Leitz Ortholux,

with fluorescence attachments. The light source was a Xenon arc lamp, type XBO 150. The Xenon lamp was powered by a power supply which produces a constant current that maintains the light output within $\pm$ 1% (E. Leitz, New York). A tungsten lamp, with a transformer, was used to visually locate the cells under low intensity white light.

For fluorescence excitation of cells, in incident light, the energy source was focused into the entrance slit of a Leitz Fluorescence Vertical Illuminator (according to Ploem). The excitation wavelength was produced by exciting filters, 4 mm BG 38 and 2 mm UG 1, approximately 365 nm. Dichroic beam-splitting mirror, TK 400, was used also. Fluorescence from the cells were filtered through built-in suppression filter, K 400, and through a Sharp-Cut filter, CS 3-73 #3389 (Corning Company) contained in the suppression filter slide. The emitted radiation was collected and projected into a 4 in. diameter, 180°, wedge interference filter (400 - 700 nm).

The monochromatic radiation was focused onto a R 446S potted photomultiplier tube (American Instrument Company). The high-voltage for the phototube was produced by two Heathkit IP32 regulated power supplies (800 volts). The photoelectric responses were amplified by an instrument modelled after the Aminco Solid-State Blank-Substract Photomultiplier Microphotometer 10-180. Fluorescence emission spectra were recorded by a Aminco X-Y Recorder. The Farrand Microscope Spectrum Analyzer was used to measure the fluorescence intensity at 480 nm, consisting of beam-splitting eyepiece, photomultiplier photometer and microammeter.

RESULTS AND DISCUSSION

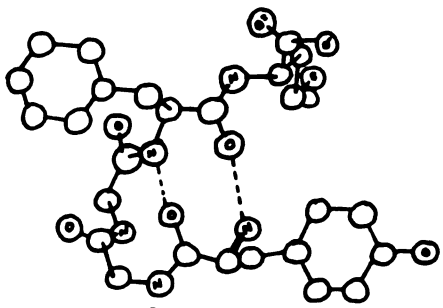
A. Synthesis and Fluorescence Properties of N-dansyl-enkephalins

1. Synthesis

Met^5-enkephalin and (D-ala^2)-met^5-enkephalin were successfully labeled with a dansyl group at their amino terminals, see Figure 1. The synthesis of the N-dansyl-enkephalins was through the formation of a sulfonamide bond between the amino group of tyrosine and the sulfonyl nucleophilic substitution. In these reactions the unprotonated amino group served as a nucleophilic reagent attacking the susceptible sulfonyl sulfur and displacing the chloride ion. It is known that acid chlorides can readily react with primary amines to form substituted amides (Morrison and Boyd, 1966). This sulfonamide linkage is a very stable covalent bond with a tetrahedral bond angle. The major side reaction is the hydrolysis of dansyl chloride to dansyl sulfonic acid.

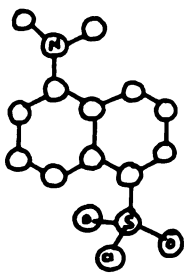
Dansyl chloride has been shown to react easily with unprotonated amines and phenols (Felgner and Wilson, 1977; Nachtmann and Spitzy, 1975; Weiner et al., 1972; Hartley, 1970; Gros and Labouesse, 1969). The relative reactivities of the amino and phenolic groups of tyrosine are determined by the pH of the reaction mixture (Felgner and Wilson, 1977; Hartley, 1970). As the pH of the reaction mixture increases from acid to base, the unreactive protonated amino group (NH_3^+) of tyrosine is shifted into its reactive basic form (NH_2); and, at pH values below 9.5, the rate of hydrolysis of dansyl chloride to dansyl sulfonic acid

Figure 1. This is an intuitive structure of N-dansyl-met^5-enkephalin. Met^5-enkephalin is in a -conformation stabilized by antiparallel hydrogen bonding between the tyrosine and phenylalanine amino acids. The structure for met^5-enkephalin is an X-ray diffraction interpretation by Smith and Griffin (Science, vol. 199, p. 1214, 1978).

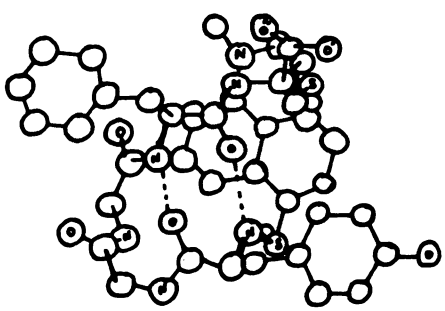


met^5-enkephalin

+



dansyl chloride



N-dansyl-met^5-enkephalin

chloride

is low and essentially constant. However, above pH 9.5, the hydrolytic reaction becomes significantly greater (Gros and Labouesse, 1969). Since the pH of the amino group of tyrosine is 9.1 and the Ph of tyrosine's phenolic hydroxyl is 10.1 (Hartley, 1970; Felgner and Wilson, 1977), the reaction rate of the phenolic group is greatly diminished, compared with the reaction rate of the amino group at pH values below 9.1 and, the maximum reactivity of the phenolic hydroxyl is expected to be at pH 11 or more (Felgner and Wilson, 1977; Gros and Labouesse, 1969).

A reaction with the phenolic hydroxyl of tyrosine was avoided because there are implications that this group is crucial for the opiate receptor binding of the enkephalins (Snyder, 1977; Cusan et al., 1977). The dansylation of the phenolic hydroxyl was minimized by choosing appropriate labeling conditions such as pH and temperature. By having the reaction mixture at pH 8.3, substantial amounts of N-dansyl-enkephalins were formed. This pH was high enough to have the amino terminals of enkephalin in the NH_2 form, since the pH of the amino group of tyrosine was lowered further by the gly-gly-phe-met side chain of enkephalin (Gros and Labouesse, 1969). The enkephalins were also dansylated at pH 9.2, but this pH is not optimal because of the increased hydrolytic activity and reactivity of the phenolic hydroxyl.

The reaction mixture must be buffered in order to neutralize the hydrochloric acid, which is continuously formed during the hydrolytic and labeling reactions. Felgner and Wilson (1977) have demonstrated that the amounts of N-dansyl-tyrosine produced is dependent on the buffer in solution. They found that a reaction mixture containing a bicarbonate buffer can generate significantly greater amounts of N-dansyl-tyrosine (30 times) than a solution containing a borate buffer at a similar pH.

It is recommended a 50% acetone with bicarbonate buffer appears satisfactory as the reaction solution (Felgner and Wilson, 1977; Hartley, 1970; Gros and Labouesse, 1969). The acetone was employed to solubilize the dansyl chloride and to suppress the ionization of the amino group (Hartley, 1970).

The extent and rate of dansylation of enkephalin is dependent on the absolute concentrations of dansyl chloride and of the reactive species rather than the mere excess of reagent over peptide (Hartley, 1970; Gros and Labouesse, 1969). To guarantee a large concentration of reactants, the reaction mixture was made with 10^{-3} M enkephalin and a 2.5-fold stoichiometric excess of dansyl chloride. Higher concentrations of enkephalin and/or dansyl chloride were found to be difficult to solubilize and interferred with the purification of N-dansyl-enkephalin.

The dansylation reaction was dependent upon the temperature of the reaction mixture. The synthesis of N-dansyl-enkephalin was improved by raising the reaction temperature from 21°C to 46°C because the rate of labeling and the rate of dissociation of NH_3^+ into NH_2 and H^+ increases as a function of temperature and is greater at 46°C than at 21°C (Gros and Labouesse, 1969; Nachtmann and Spitzy, 1975). The 46°C reaction temperature was high enough to loosen the conformation of enkephalin, thereby decreasing steric hinderances, and thus making the reactive NH_2 group more susceptible to dansylation and increasing the solubility of dansyl chloride in acetone. High reaction temperatures (66°C and above) resulted in an increased rate of hydrolysis rather than of dansylation due to the greater rate of dissociation of H_2O into OH^- and H^+ (Gros and Labouesse, 1969; Nachtmann and Spitzy, 1975).

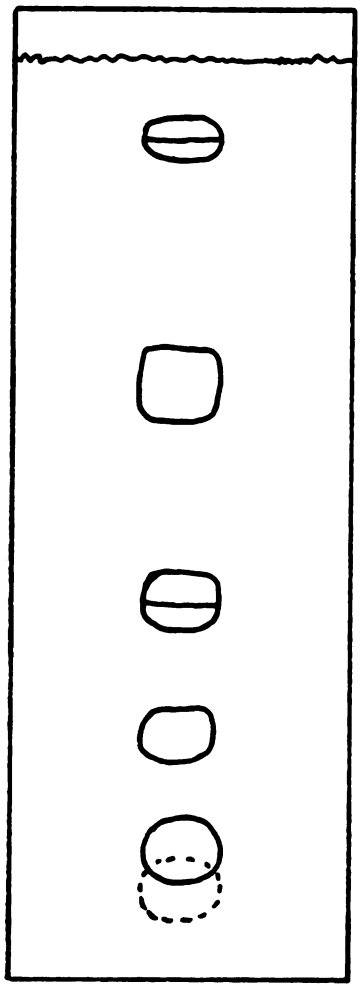
Gros and Labouesse (1969) have reported that the α-amino groups of peptides can be 95% dansylated in 5 minutes at pH 9.5 and 20°C. They found that under the same conditions it takes five times longer for the phenolic hydroxyl of tyrosine residues in proteins to 95% dansylated. Nachtmann and Spitzy (1975) have shown that the dansylation of ephedrine, an analogue of adrenaline, increases with respect to the reaction time, and that ephedrine can be completely derivatized in 20 minutes. A reaction time of 30 minutes was sufficiently long enough to permit the optimum dansylation of enkephalin.

In some reaction mixtures, 0.1 N NaOH was employed to stop the labeling reaction. Any remaining dansyl chloride was rapidly hydrolyzed to dansyl sulfonic acid via the overpowering OH^- catalyzed reaction (Gros and Labouesse, 1969). Unfortunately, this served to increase the dansylation by-products which interferred with the purification process. For this reason it was preferred to omit the NaOH and to not use a tremendous amount of dansyl chloride. A reaction temperature of 46°C over a prolonged period of time (30 minutes) facilitated the hydrolysis of the excess dansyl chloride.

2. Identification of dansyl derivatives

The dansyl derivatives were purified by silica gel 60 thin-layer chromatography. Identification of the derivatives were by their fluorescent color, Rf value was calculated from spots visualized under long-wave ultraviolet light on chromatograms, and by their fluorescence emission maximum in ethanol (excitation at 340 nm), see Figure 2. The Rf values were calculated from the following equation:

$$\text{Rf value} = \frac{\text{migration distance of the substance}}{\text{distance of the front from start}}$$



Chromatogram

der. #	deriv. name	color	Rf	Fl
sol. frt.	--	--	1.00	--
1.	dansylamide	blue green yellow	0.91	516 nm
2.	dansyl sulfonic acid	blue	0.65	474 nm
3.	N-dansyl-met^5-enkephalin	yellow-green	0.37	530 nm
4.	?	orange	0.22	548 nm
5.	?	orange	0.07	--
6.	initial spot	--	0.00	--

Figure 2. Identification of dansyl derivatives by silica gel 60 thin-layer chromatography.

The reaction by-products, dansylamide and dansylsulfonic acid, were easily separated and identifiable. The migration of dansylamide which traveled closely with the solvent front and possessed a Rf value of approximately 0.9, was faster than that of dansylsulfonic acid. Dansylamide migrated as two distinct but inseparable bands, a greenish-blue and greenish-yellow band, which may depict a molecular charge difference between the NH_2 and NH_3^+. The fluorescence forms of dansylamide emission maximum of the designated dansylamide was at 516 nm in ethanol, which was identical to the emission maximum of a commercially obtained sample of dansylamide. The number two dansyl derivative, having a Rf value of 0.65, was identified as dansylsulfonic acid because of its bright blue color. The reaction by-product dansylsulfonic acid displayed a fluorescence emission peak at 474 nm in ethanol. The number three dansyl derivative migrated as two closely opposed, yellow-green, bands traveling at a much slower rate than the #2 derivative and having a Rf value of approximately 0.4. The fluorescence of the first band was more intense than the second, which may suggest a concentration gradient. The identification of N-dansyl-met^5-enkephalin and N-dansyl-(D-ala^2)-met^5-enkephalin was based on the bright yellow-green fluorescence of N-dansyl amino acids/peptides in thin-layer chromatography (Felgner and Wilson, 1977; Weiner et al., 1972; Hartley, 1970; Chen, 1967). The fluorescence emission maxima of the N-dansyl-enkephalins were obtained at 530 nm in ethanol. The fluorescence and chromatographic properties of the above dansyl derivatives were in agreement with those reported for dansylamide, dansylsulfonic acid and N-dansyl-peptides (Weiner et al., 1972; Hartley, 1970; Gros and Labouesse, 1969; Woods and Wang, 1967).

Since the ninhydrin reagent is known to react with α-amino acids and amines to yield a purple colored product, in thin-layer chromatography (Pataki, 1968; McCaldin, 1960), the ninhydrin reaction was used for the detection of non-labeled met^5-enkephalin, and for the verification that N-dansyl-met^5-enkephalin fraction did not have a free N-terminal amino group. The number three dansyl derivative was negative to the ninhydrin reaction; N-dansyl-met^5-enkephalin cannot interact with the ninhydrin reagent because its N-terminal amino group is not available for reaction. Only the position on the chromatogram corresponding to the number five dansyl derivative was positive to the ninhydrin reaction. The chromatographic properties of pure met^5-enkephalin was found to be similar to the number 5 derivative, and the pure met^5-enkephalin was ninhydrin positive. This suggests that the number 5 spot may represent the migration of the initial spotting material and may contain the remaining non-labeled met^5-enkephalin.

The phenolic hydroxyl of enkephalin is believed to be necessary for its' opiate receptor binding behavior. 1-Nitroso-2-naphthol has been shown to react specifically with the phenolic group of tyrosine residues (Felgner and Wilson, 1977; Greenstein and Winitz, 1967; Bailey, 1967), and was used to detect for a free phenolic group in N-dansyl-met^5-enkephalin and N-dansyl-(D-ala^2)-met^5-enkephalin. The number 3 dansyl derivative (yellow-green, 530 nm, Rf = 0.4) was treated with 1-nitroso-2-naphthol, in the presence of nitric acid, and was found to yield the characteristic red color of a positive reaction. Also, pure met^5-enkephalin reacted to give a red color. The existence of the phenolic hydroxyl in N-dansyl-enkephalin might permit the receptor binding of the opioid analogue to be similar to the non-dansylated enkephalin.

There is not enough experimental data to accurately identify the number four dansyl derivative on the chromatogram (Figure 2). However, available information suggests that it may represent a stable O-dansyl derivative of enkephalin. The chromatographic properties of the number 4 derivative were very similar to those of a commercially obtained sample of O-dansyl tyrosine. The fluorescence of the unknown derivative was determined to be identical to the fluorescence of O-dansyl tyrosine, both having a burnt orange color on silica gel plates and an emission maximum at 548 nm in ethanol. The number 4 dansyl derivative and O-dansyl-tyrosine have low Rf values, and both displayed a negative reaction to the 1-nitroso-2-naphthol reagent. In contrast, the photolytic degradation of the unknown dansyl derivative was negligible during ultraviolet irradiation, unlike the high photolability displayed by O-dansyl-tyrosine (Felgner and Wilson, 1977).

The fluorescent spots corresponding to dansylamide and dansylsulfonic acid were found to fade as a function of time exposed to ultraviolet irradiation. The fluorescence of dansylamide completely disappeared after a few minutes of irradiation, fading much more rapidly than the bright blue fluorescence of dansylsulfonic acid. No significant loss of fluorescence was observed for N-dansyl-met^5-enkephalin and N-dansyl-(D-ala^2)-met^5-enkephalin during UV irradiation. The negligible photodegradation of N-dansyl-enkephalin on silica gel plates is in agreement with the results reported by Felgner and Wilson (1977) on the photolability of N-dansyl-tyrosine and dansyl glycine on polyamide plates. In contrast, Pouchan and Passeron (1975) have demonstrated that the fluorescence of dansyl glycine on silica gel G plates decreases as a function of UV irradiation time, following first order kinetics.

Nachtmann and Spitzy (1975) have observed that dansyl derivatives of ephedrine, emetine and morphine linearly decrease as a function of excitation. It is evident that the various dansyl derivatives may undergo photochemical degradation in thin-layer chromatography, which must be considered when computing concentrations. (Note photolytic degradation was not detected in solutions devoid of silica gel, and the dansyl derivatives were very stable against solvent degradation when stored in absolute ethanol.)

Chromatographic separation of the reaction by-products and N-dansyl-enkephalin was attempted with a number of different solvent systems. The best separation was achieved with a toluene-ethanol (6:10, v/v) system (Figure 2). The addition of a more polar solvent to the system interferred with the separation of the dansyl derivatives. Good separation was not achieved with toluene-ethanol-water or 25% NH_3OH (6:10:4, v/v/v) systems, but the Rf values of the dansyl derivatives were in the same immediate range. The toluene-water (3:1, v/v) system gave the worst results. Thus, increasing the polarity of the solvent system decreased the extent of separation. The highly toxic benzene-methanol systems were able to adequately separate the dansyl derivatives. Other effective solvent systems were found that had various ratios of toluene and ethanol.

Without proper equipment (e.g. densitometer), it was not possible to determine the absolute amount of N-dansyl-enkephalin produced in the process. The reaction and purification conditions were found to affect the yield, and were designed to give the optimum production of N-dansyl-enkephalin. The pH of the reaction mixture should permit only the amino group of enkephalin to be in the unprotonated form to produce a

monodansylated derivative of the N-terminal tyrosine. The chromatographic solvent system must give a wide separation between the dansyl derivatives for complete purification. The photolability of the dansyl derivatives may cause considerable error unless the UV exposure time is held at a minimum and the same for each derivative including standards (Pouchan and Passeron, 1975). Since this investigation was concerned with the synthesis, fluorescence and opiate receptor binding of N-dansyl-enkephalin, the absolute yield was not determined. It was estimated that the amount of N-dansyl-enkephalin produced in the process was at least 30% of the initial concentration of enkephalin, based on the reported production of N-dansyl-tyrosine (Felgner and Wilson, 1977; Gros and Labouesse, 1969).

3. Fluorescence and absorption spectra of N-dansyl-enkephalins

The fluorescence characteristics of N-dansyl-(D-ala^2)-met^5-enkephalin were determined to be identical to those of N-dansyl-met^5-enkephalin in ethanol, see Figures 3 and 4; the fluorescence emission maximum was obtained at 530 nm, and the excitation spectrum had a primary maximum at 356 nm and a secondary peak at 272 nm. The substitution of the CH_3 group of D-alanine for the H of glycine in N-dansyl-(D-ala^2)-met^5-enkephalin had no effect on the fluorescence properties of the attached dansyl group. The fluorescence emission peak of the free unreacted dansyl chloride was at 480 nm in ethanol. It is known that the emission of the dansyl group undergoes a large red shift when dansyl chloride is changed to a dansylamide derivative, e.g. N-dansyl-amino acids, see Figure 4 (Hartley, 1970). The formation of the sulfonamide bond between the amino group of tyrosine and the sulfonyl group of dansyl was the major factor for the (50 nm) shift to higher wave-lengths in

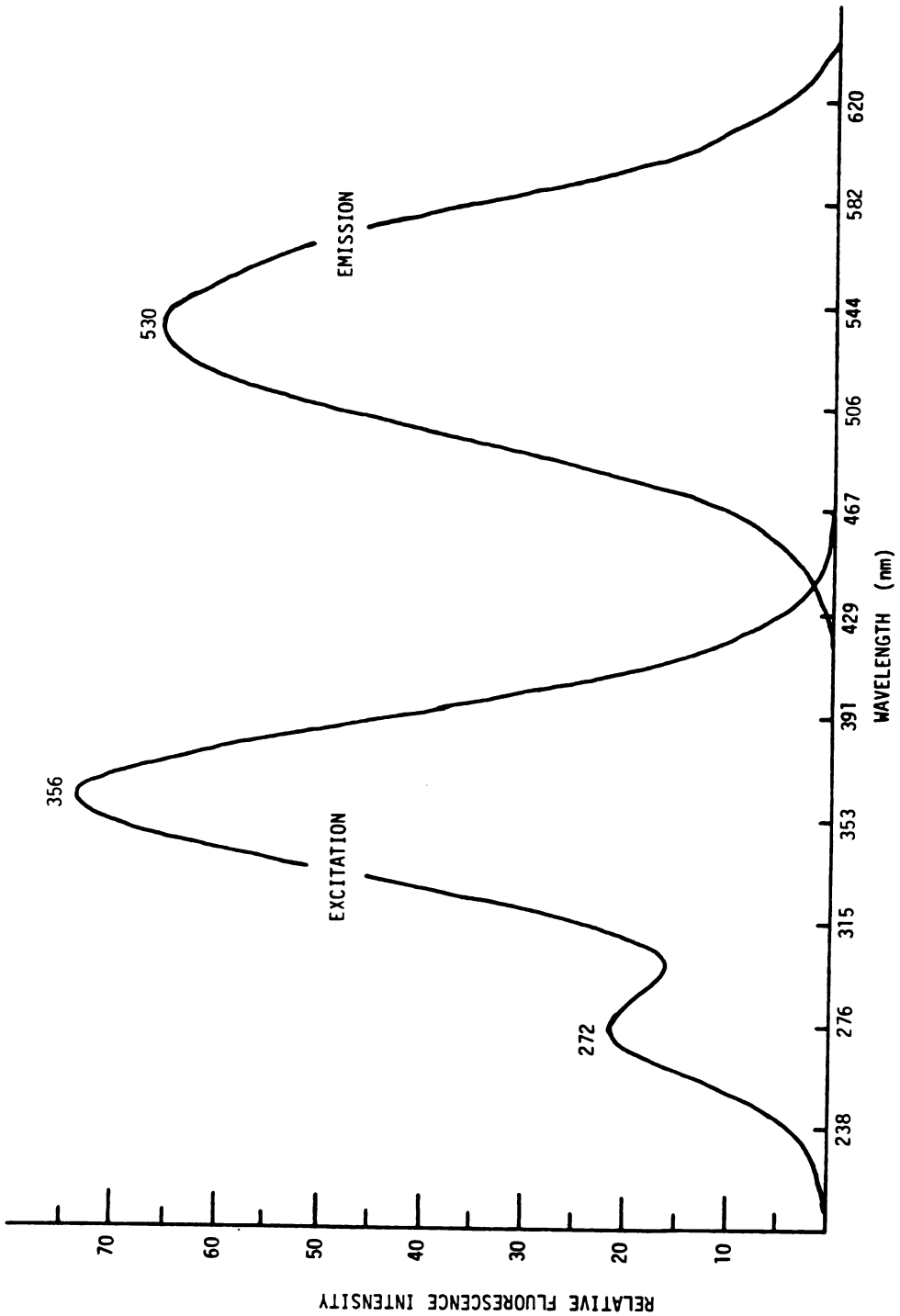


Figure 3. Fluorescence excitation and emission spectra of N-dansyl-(D-ala^2)-met^5-enkephalin in C_2H_5OH.

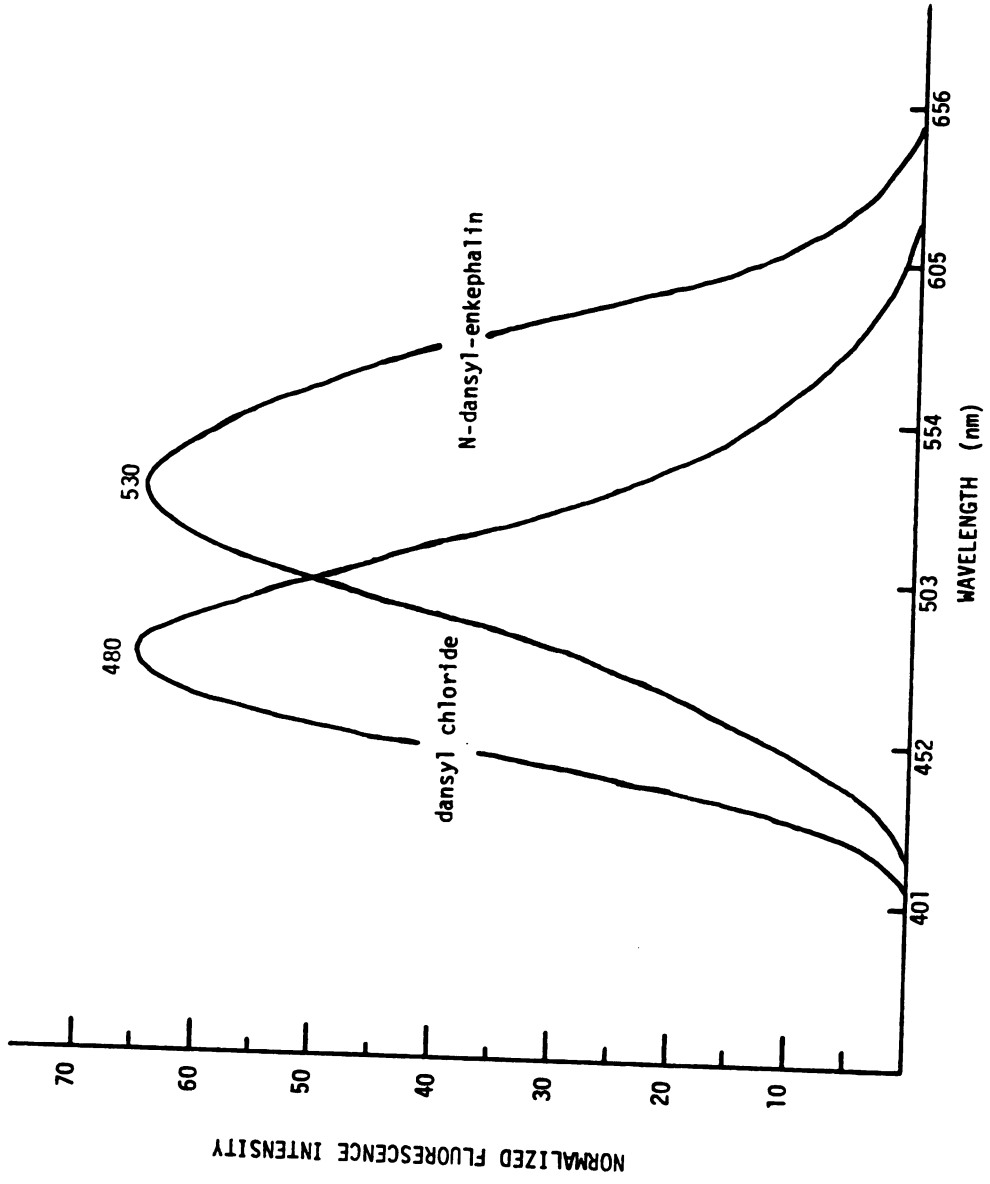


Figure 4. Fluorescence emission spectra of dansyl chloride and N-dansyl-met^5-enkephalin in C_2H_5OH.

N-dansyl-enkephalin, since dansylamide and N-dansyl-tyrosine both displayed an emission peak at 516 nm in ethanol (exc 340 nm); and also, the presence of the gly-gly-phe-met side groups caused the emission maximum of dansyl to red shift further.

The fluorescence emission spectrum of met-enkephalin was found to closely resemble that of the free tyrosine residue with a maximum at 330 nm in 0.05 M $NaHCO_3$ buffer pH 8.3 (exc 270 nm) due to the greater fluorescence quantum yield of the tyrosine amino acid (over that of phenylalanine) and due to Förster type singlet-singlet energy transfer from the phenylalanine residue to the tyrosine residue. The emission peak of tyrosine has been shown to shift to longer wavelengths with increasing pH (Bishai et al., 1967); likewise, the fluorescence emission maximum of met^5-enkephalin was found to increase to 334 nm at pH 9.5 in the tris buffer which at pH 9.5 can permit a small percentage of the phenolic groups of met-enkephalin to become partially unprotonated, causing the very small (4 nm) shift in wavelength. The 330 nm value for the fluorescence emission maximum of met-enkephalin at pH 8.3 corresponds to a tyrosine with a protonated phenolic group.

The attachment of the dansyl group to the N-terminal of met^5-enkephalin was confirmed by its distinct ultraviolet absorption spectrum, as compared to that of the unbound dansyl group. The absorption spectrum of N-dansyl-met^5-enkephalin was found to have maxima at 260 and 342 nm, and a pronounced peak at 268 nm in ethanol. In comparison, the absorption spectrum of dansyl chloride in ethanol was established to have two maxima at 260 and 360 nm. When dansyl chloride reacts with amino acids or peptides, its absorption maximum at 360 nm is known to blue shift to shorter wavelengths (Gros and Labouesse, 1969; Hartley,

1970; Chen, 1967). The absorption maximum at 360 nm was determined to shift to 335 nm for dansyl glycine in ethanol. The ultraviolet absorption spectrum of met^5-enkephalin was found to be very similar to tyrosine, having a maximum at 275 nm and a molar absorptivity of approximately 1600, in 0.05 M $NaHCO_3$ pH 8.3. It is suggested that the pronounced peak at 268 nm in the absorption spectrum of N-dansyl-met^5-enkephalin corresponds to the tyrosine residue and the two maxima at 260 nm and 342 nm corresponds to the dansyl chromophore.

The fluorescence and absorption changes of the dansyl group were taken as verification of the synthesis of N-dansyl-met^5-enkephalin, and are consistent with the information in the literature on N-dansyl peptides (Hartley, 1970; Gros and Labouesse, 1969; Chen, 1967). Fournie-Zaluski et al. (1978) have synthesized N-dansyl-met^5-enkephalin, and employed NMR to confirm the position of the dansyl group, the presence of the phenolic proton at 9.3 ppm and the sulfamidic NH at 8.8 ppm.

4. Related compounds

The fluorescence emission maximum of a dansyl derivative in ethanol was found to depend upon the nucleophilic strength of the substituent attached to the dimethylaminonaphthalene-sulfonyl group, in that, the emission maximum of the dansyl chromophore shifted towards longer wavelengths as the basicity of the substituent increased, according to $OH < Cl < NH_2 \leq NHR$, see Table 1. The emission maximum of dansyl hydroxide, i.e. dansyl sulfonic acid, was 42 nm less than that of dansylamide, which contains the strong base NH_2 as dansyl substituent. Note: the OH and Cl ions are very weak bases. The nature of the R group in dansyl derivatives with NHR substituents was determined to affect the extent of red shift in the dansyl fluorescence, but the size of the R group

Table 1. Fluorescence emission maximum of various dansyl derivatives in ethanol (exc λ 340 nm).

Compound	λ max
1. dansyl sulfonic acid	474 nm
2. dansyl chloride	480 nm
3. dansylamide	516 nm
4. dansyl glycine	519 nm
5. N-dansyl-l-tyrosine	516 nm
6. O-dansyl-l-tyrosine	548 nm
7. N,O-didansyl-l-tyrosine	522 nm
8. N-dansyl-met^{5}-enkephalin	530 nm
9. N-dansyl-(D-ala^{2})-met^{5}-enkephalin	530 nm

was determined to have no effect on fluorescence, e.g. compare the emission maximum of N-dansyl-l-tyrosine at 516 nm to that of dansyl glycine at 519 nm and of N-dansyl-met^5-enkephalin at 530 nm in ethanol. The emission maximum of met^5-enkephalinamide-$(CH_2)_2$-dansyl has been reported at 520 nm in ethanol (Fournie-Zaluski et al., 1978, a) compared to 519 nm for dansyl glycine.

The dansylation procedures (in Section II, Experimental Details) were employed on free l-tyrosine, in order to obtain the fluorescence emission maximum of N-dansyl-l-tyrosine in ethanol and to confirm the synthesis of N-dansyl-met^5-enkephalin. Under similar TLC conditions, the fluorescence and chromatographic properties of N-dansyl-l-tyrosine were identical to those reported by Felgner and Wilson (1977) for a commercially obtained sample of dansyl tyrosine, thus, verifying the formation of N-dansyl-l-tyrosine. N-dansyl-l-tyrosine was determined to be positive to the l-nitroso-2-naphthol reaction, indicating that the phenolic hydroxyl of tyrosine was not dansylated. The fluorescence emission maximum of N-dansyl-l-tyrosine was found at 516 nm in ethanol. Since the unprotonated phenolic hydroxyl of tyrosine is considerably more reactive (basic) than the unprotonated amino group of tyrosine (Hartley, 1970), the fluorescence emission maximum of O-dansyl-l-tyrosine was found at 548 nm, red shifted by 32 nm, (see Table 1). Additionally, the electroegativity of oxygen is greater than that for nitrogen; one expects a greater solvent shift in a polar media. The fluorescence emission maximum of N,O-didansyl-l-tyrosine was found at 522 nm, close to the emission maximum of N-dansyl-l-tyrosine.

5. Energy transfer

N-dansyl-met^5-enkephalin has only two structures capable of fluorescence, the phenol and dansyl moieties. Since there is considerable spectral overlap between the tyrosine emission spectrum and the dansyl groups absorption spectrum, efficient energy transfer from the tyrosine residue to the nearby ligand was established to occur in N-dansyl-met^5-enkephalin via the Förster's theory of singlet-singlet energy transfer (Fournie-Zaluski, 1978, a, b; Stryer, 1978, 1968), in that, the fluorescence emission of the tyrosine residue was completely quenched, and only the emission of the dansyl group had occurred upon excitation of the tyrosine residue at 270 nm. Energy transfer from tyrosine to dansyl was evidenced by the fluorescence excitation spectrum of N-dansyl-(D-ala^2)-met^5-enkephalin in ethanol (ems 530), which showed an additional excitation peak at 272 nm in the wavelength range of the tyrosine's absorption band, see Figure 3. The excitation spectrum of the free dansyl chloride in ethanol, displayed only one peak at 328 nm; which shifted to 356 nm when reacting with the enkephalins. The above results are in complete agreement with those reported by Fournie-Zaluski et al. (1978, a, b) on the energy transfer in met^5-enkephalin dansylated at either the N- or C-terminals. The energy transfer from the tyrosine to the dansyl group has been demonstrated by a dramatic 94% decrease in the fluorescence emission of tyrosine at 305 nm as compared to non-labeled met^5-enkephalin alone, at the same concentration in 0.05 M tris HCl buffer pH 7.4 (exc 275 nm) (Fournie-Zaluski et al., 1978, a).

The Förster theory of singlet-singlet energy transfer has been employed to elucidate the conformational behavior of the enkephalins via evaluation of intramolecular distance between donor and acceptor

chromophores (Fournie-Zaluski et al., 1978, a, b; Schiller, 1977; Stryer, 1978, 1968). Fournie-Zaluski et al. (1978, b) have determined that the intramolecular distance between tyrosine and dansyl in met^5-enkephalin-$(CH_2)_2$-dansyl is 13.7 Å, which favors a folded conformation (β) for met^5-enkephalin. Schiller (1977) has used the energy transfer from tyrosine to tryptophan in the biologically active (trp^4)-met^5-enkephalin to study the conformational behavior of met^5-enkephalin. In this analogue, the phenylalanine residue of met^5-enkephalin was replaced by tryptophan. He calculated that the Tyr-Trp separation is 9.3 Å in aqueous solutions at pH 1.5 and 5.5, suggesting a folded conformation (β) for both the cationic and zwitterionic forms of the analogue (Schiller, 1977). The attachment of a dansyl group to the N- or C-terminal of (trp^4)-met^5-enkephalin may possibly be useful for conformational analysis of met^5-enkephalin, since the spectral overlap of the tryptophan emission spectrum and the dansyl absorption spectrum is considerably better than that of tyrosine and dansyl (Chen, 1967).

6. Fluorescence intensity changes of N-dansyl-met^5-enkephalin as a function of concentration

The amount of N-dansyl-met^5-enkephalin in solution should be reflected in the fluorescence intensity at 530 nm. In this experiment, the emission spectra of N-dansyl-met^5-enkephalin were recorded as a function of its concentration in ethanol. The fluorescence emission intensities were found to increase linearly as the N-dansyl-met^5-enkephalin concentration increased from 6×10^{-7} M to 3×10^{-5} M, indicating that the emission of N-dansyl-met^5-enkephalin was proportional to its concentration in ethanol. The relative intensity for each concentration is graphically displayed in a semilogarithmic plot in Figure 5. The slope of the line was

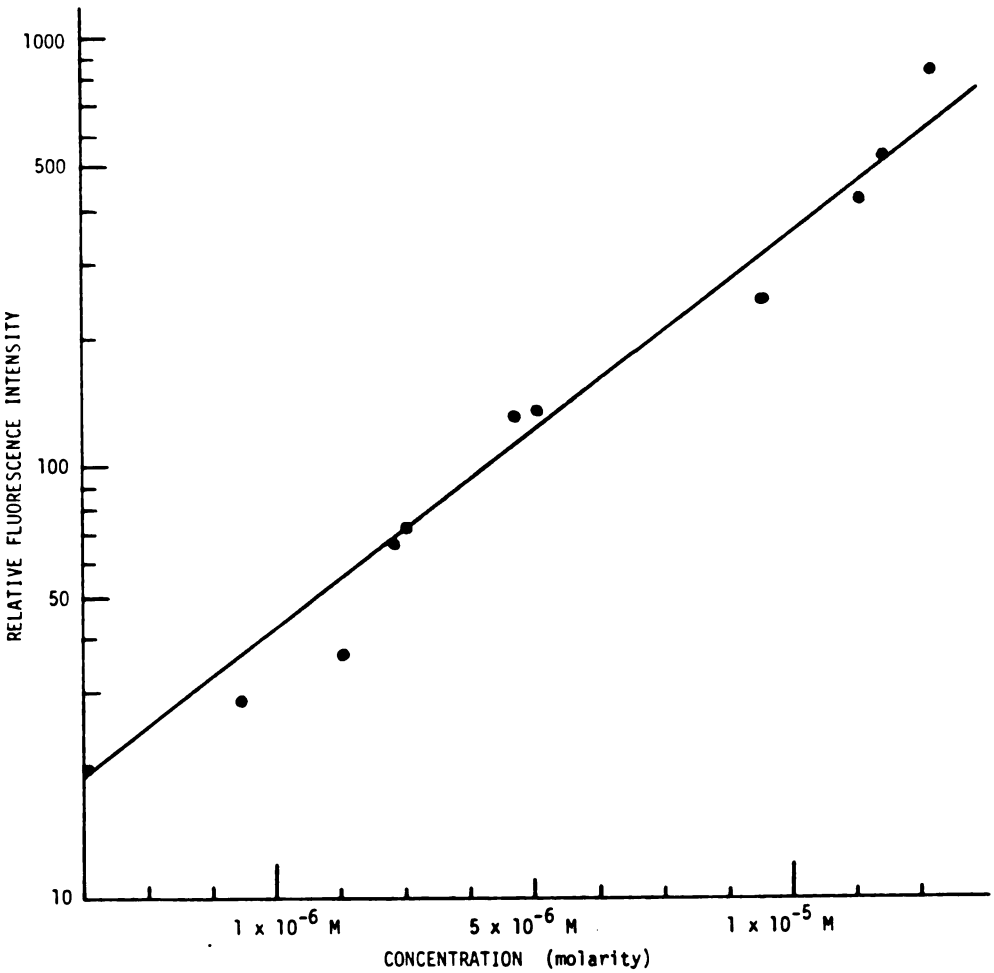


Figure 5. Fluorescence intensity of N-dansyl-met^5-enkephalin as a function of concentration.

calculated to be equal to 1 x 10^5 per molar. This calibration curve may be used to determine the amount of N-dansyl-met^5-enkephalin in a solution of unknown concentration. Also, the fluorescence emission maximum of N-dansyl-met^5-enkephalin was established to decrease slightly at lower concentrations (data not shown), reflecting an aggregation effect caused by the hydrophobicity of the molecule at higher concentrations.

7. Effects of solvent dielectric constant on N-dansyl-met^5-enkephalin fluorescence.

The dansyl group has been identified as a fluorescent molecular probe of polarity (Stryer, 1968), and the fluorescence properties of N-dansyl derivatives of amino acids, peptides and met^5-enkephalins have demonstrated a sensitivity to the dielectric constant of the environment (Fournie-Zaluski et al., 1978, a; Hartley, 1970; Chen, 1967). The emission maximum of 3.6 x 10^{-6} M N-dansyl-met^5-enkephalin was established to shift 15 nm towards shorter wavelengths from water (dielectric constant, 78.5 D) to ethanol (dielectric constant, 25.8 D) (Figure 6), accompanied by approximately a four-fold increase in fluorescence intensity. Inversely, the excitation maxima of N-dansyl-met^5-enkephalin were shifted towards the longer wavelengths as a function of the ethanol concentration in water (Figure 7); similar to the shifts in wavelength for the dansyl absorption maxima of N-dansyl-met^5-enkephalin, which was due to H-bonding effects.

The dependence of the emission maximum of N-dansyl-met^5-enkephalin on the dielectric constant of the environment results from a reorientation of the solvent shell around the excited dansyl chromophore; since the excited state of the dansyl group has a much higher dipole moment than the ground state (Stryer, 1968; Chen, 1967). Due to the solvation

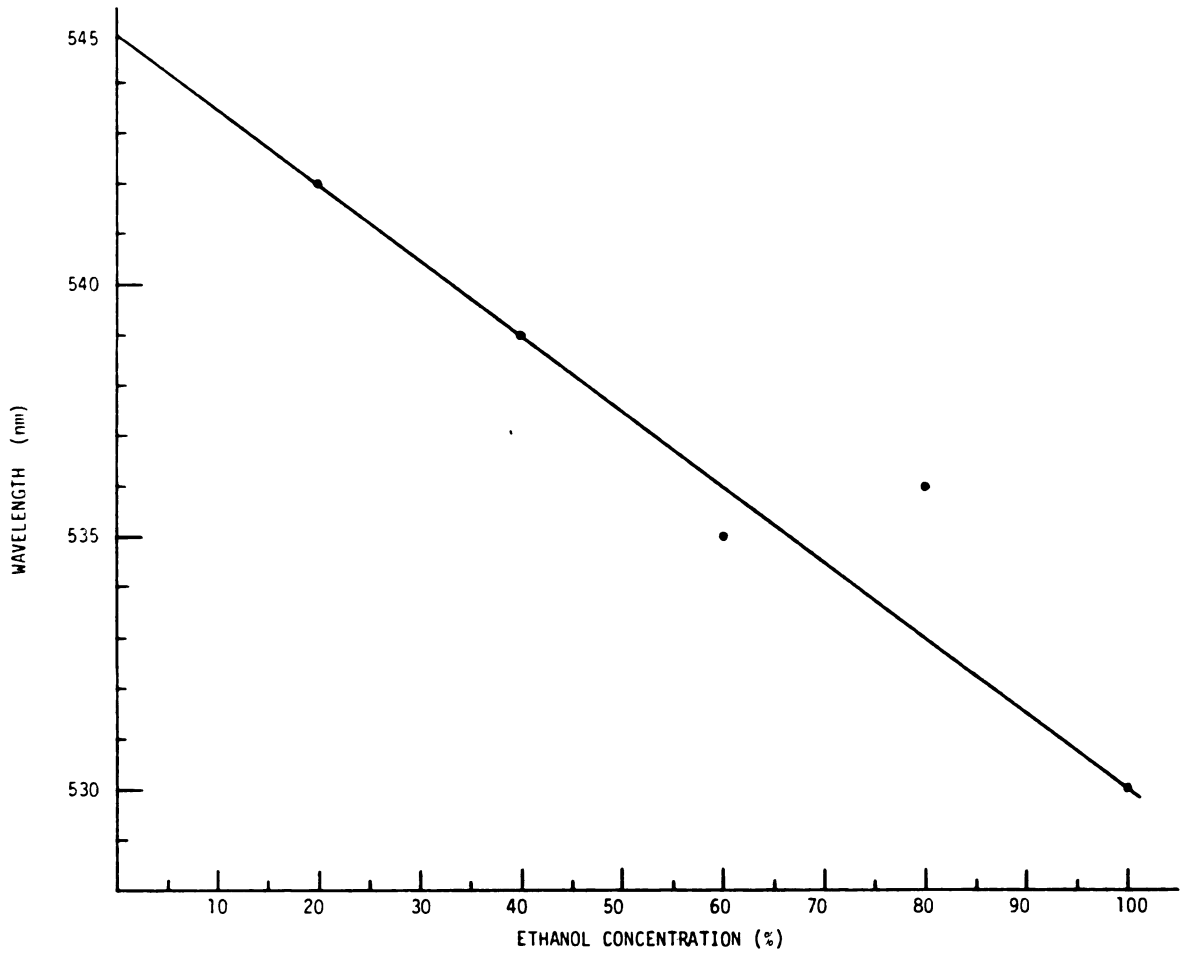


Figure 6. Fluorescence emission maximum of 3.6×10^{-6} M N-dansyl-met^5-enkephalin as a function of ethanol concentration in water (ems λ 530 nm).

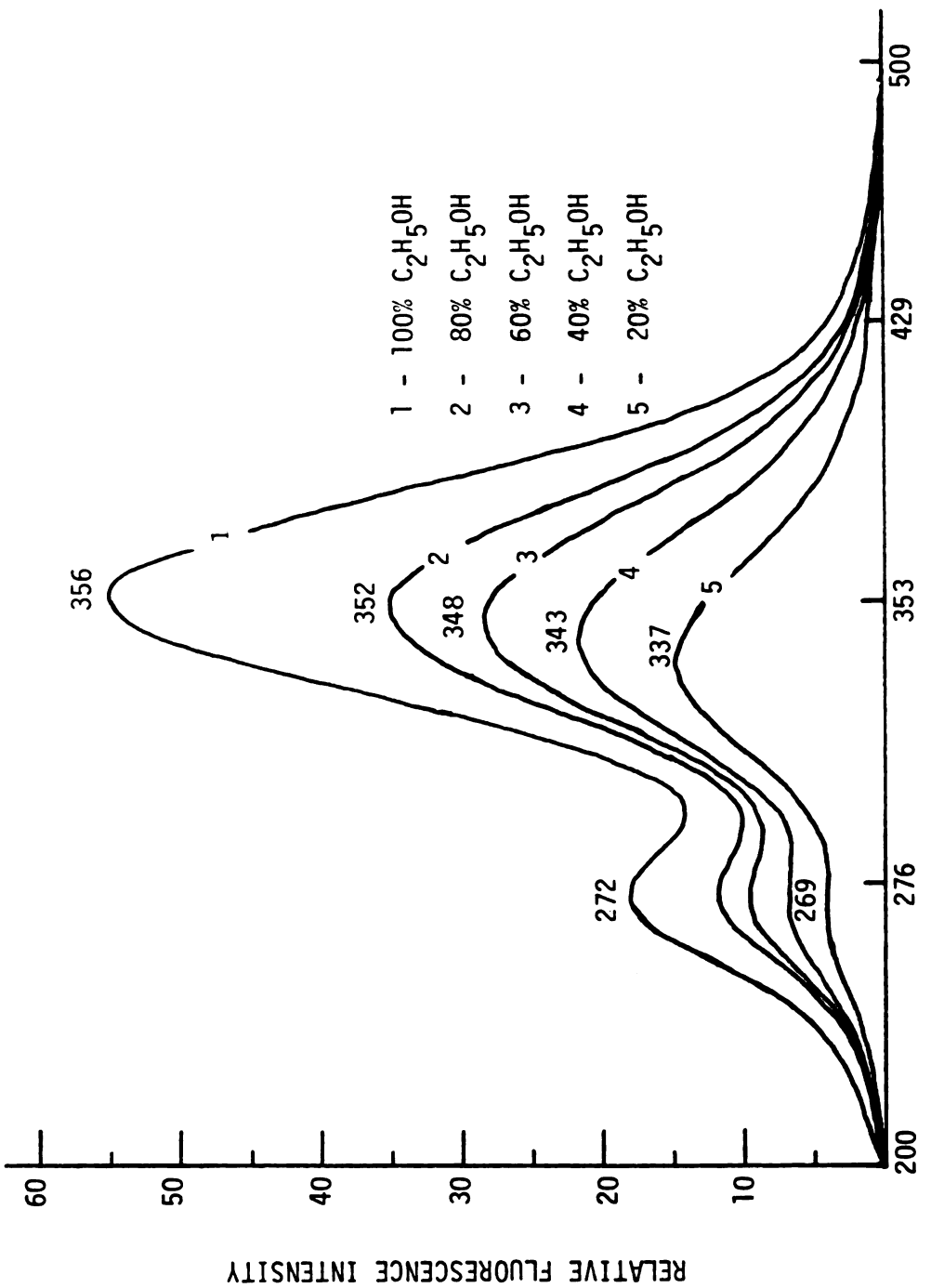


Figure 7. Fluorescence excitation spectra of 3.6×10^{-6} M N-dansyl-met^5-enkephalin as a function of ethanol concentration in water (ems λ 530 nm).

energy of the excited dansyl group via dipole-dipole interaction with the solvent molecules, a photon of lower energy is emitted by dansyl in a polar solvent. Since water is more polar than ethanol, the emission maximum of the dansyl group is shifted toward the red as the water concentration in ethanol increases (Stryer, 1968).

The dansyl group was chosen as a fluorescent molecular probe because of 1) its high quantum yield, 2) its covalent attachment to enkephalin without difficulty, and 3) its fluorescence sensitivity to environment modifications. The above experiments suggest that N-dansyl-met^5-enkephalin may be useful for the visualization of receptor sites and the investigation of ligand-receptor interactions.

B. Assessment of the Opiate Receptor Binding of N-dansyl-enkephalins

1. Inhibition of ^{3}H-naloxone binding by N-dansyl-enkephalins.

In order for dansyl to be a suitable probe, the dansylated enkephalin must possess the capacity to bind to opiate receptor sites. In accordance with the procedure outlined by Davis et al. (1975; Pasternak et al., 1975; Pert and Snyder, 1973, 1974), homogenates and slices of rat brain were used to evaluate the opiate receptor binding of N-dansyl-met^5-enkephalin and N-dansyl-(D-ala^2)-met^5-enkephalin. The opiate-like activity of N-dansyl-(D-ala^2)-met^5-enkephalin was illustrated by its ability to inhibit the binding of ^{3}H-naloxone to brain homogenates, see Table 2. The binding of ^{3}H-naloxone was markedly reduced by 40% at 100 nM of N-dansyl-(D-ala^2)-met^5-enkephalin, having a saturable binding concentration of 0.10 pmoles/mg protein. The ID 50 value for non-dansylated (D-ala^2)-met^5-enkephalin has been reported at 20 mM in the inhibition of stereospecific binding of ^{3}H-naloxone to homogenates

Table 2. Inhibition of ^{3}H-naloxone binding to brain membranes by N-dansyl-(D-ala^{2})-met^{5}-enkephalin.

Conc. (nM) N-dan-enk	^{3}H-naloxone bound in pmole/mg P	Reduction
0.00	0.16	0%
0.01	0.14	13%
1.00	0.12	25%
100.00	0.10	38%
100 nM cold naloxone	0.03	81%

(Pert et al., 1977). Present data suggests that the opiate receptor affinity of N-dansyl-(D-ala^2)-met^5-enkephalin is less than that of (D-ala^2)-met^5-enkephalin. For the brain slice, the concentration of N-dansyl-(D-ala^2)-met^5-enkephalin to produce 50% occupancy of binding sites was obtained at 10 nM and the ^{3}H-naloxone binding concentration was 0.15 pmole/mg protein. The binding of N-dansyl-(D-ala^2)-met^5-enkephalin to ^{3}H-naloxone binding sites were greater for the brain slice, because of the greater affinity and greater number of binding sites, than for the homogenate (Davis et al., 1975). N-dansyl-met^5-enkephalin failed to inhibit the binding of ^{3}H-naloxone to brain homogenates and slices, due to enzymatic degradation by aminopeptidases. However, in the presence of enzyme inhibitors, N-dansyl-met^5-enkephalin has been shown to reduce the stereospecific binding of ^{3}H-naloxone to homogenates by 50% at 74 nM, very similar to natural met^5-enkephalin which has an ID 50 value of approximately 50 to 70 nM (Pert and Bowie, personal communication). The above results suggest that the bound probe in N-dansyl-enkephalin does not appreciably disturb the opiate receptor binding of enkephalin.

2. Inhibition of radioactively labeled N-dansyl-(D-ala^2)-met^5-enkephalinamide, (tyrosyl-2-,6-^{3}H), binding by naloxone.

A successful attempt was made to synthesize a N-terminal dansyl derivative of radioactively labeled (D-ala^2)-met^5-enkephalinamide, (tyrosyl-2,6-^{3}H) according to the dansylation procedures described in Section II, Experimental Procedures, except for a few modifications due to solvent and low concentration of ^{3}H-opioid. ^{3}H-N-dansyl-(D-ala^2)-met^5-enkephalinamide (^{3}H-N-dansyl-DALA) was identified by its radioactive properties on silica gel 60 chromatograms, see Figure 8. The

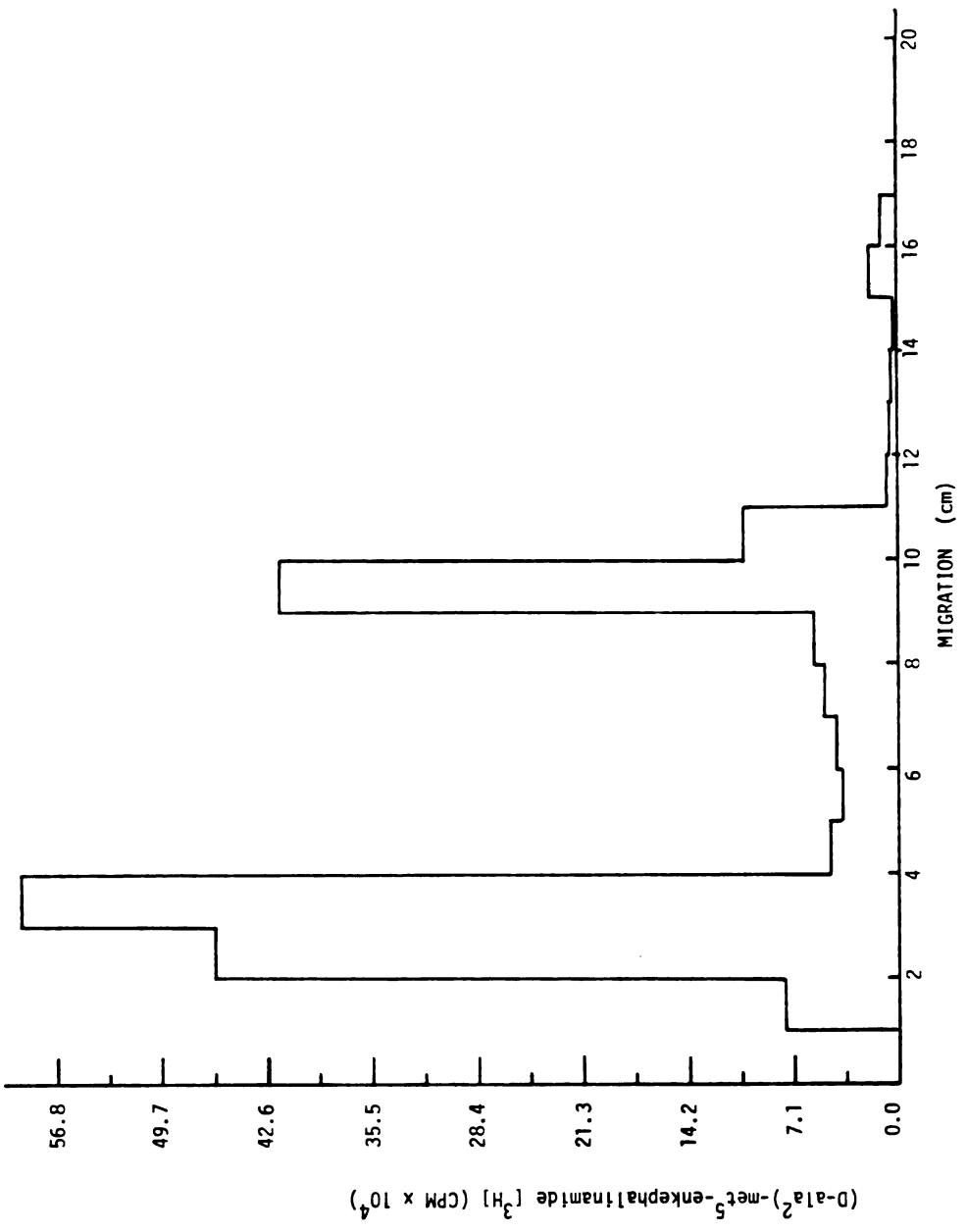


Figure 8. Identification of radioactive N-dansyl-(D-ala^2)-met^5-enkephalinamide, (tyrosyl-2,6-^{3}H), by silica gel 60 thin-layer chromatography.

high radioactivity at 10 cm was designated as ^{3}H-N-dansyl-DALA because of its migration distance from the origin since a dansyl derivative has no difficulty traveling in the toluene-ethanol system. The radioactivity maximum at 4 cm, and the radioactivity near the origin, were identified as unreacted ^{3}H-(D-ala^{2})-met^{5}-enkephalinamide which is similar to the number 5 derivative of enkephalin, see Figure 2; and, a non-dansylated peptide can not easily migrate in this solvent system.

The opiate receptor affinity of ^{3}H-N-dansyl-DALA was demonstrated by the ability of cold naloxone to inhibit its binding to brain homogenates, see Figure 9. The binding of ^{3}H-N-dansyl-DALA to brain homogenates increased as a function of opioid concentration. When 14 nM of ^{3}H-N-dansyl-DALA was incubated with the homogenates, approximately 0.20 pmoles were bound per mg protein. In the presence of 100 nM naloxone, the binding was reduced by 65% to 0.07 pmoles/mg protein. A significant amount of ^{3}H-N-dansyl-DALA was bound by the brain homogenate, at low incubation concentrations. At 3.5 nM ^{3}H-N-dansyl-DALA, the naloxone inhibition increased to 77% occupancy of binding sites, virtually abolishing the binding of ^{3}H-N-dansyl-DALA to the homogenates. Naloxone is known to have a high affinity and specificity for opiate receptor sites, and (D-ala^{2})-met^{5}-enkephalinamide has an opiate receptor affinity (K_D) of about 4×10^{-7} M to 5×10^{-7} M (Pert et al., 1976). It is concluded that, the opiate receptor binding of ^{3}H-N-dansyl-DALA is comparable to the binding of (D-ala^{2})-met^{5}-enkephalinamide.

3. Opiate receptor binding of radioactively labeled (D-ala^{2})-met^{5}-enkephalinamide, (tyrosyl-2,6-^{3}H) in the presence of N-dansyl-(D-ala^{2})-met^{5}-enkephalinamide or (D-ala^{2})-met^{5}-enkephalinamide

To be an adequate probe, the binding behavior of N-dansyl-enkephalin to the opiate receptor must be similar to the binding of

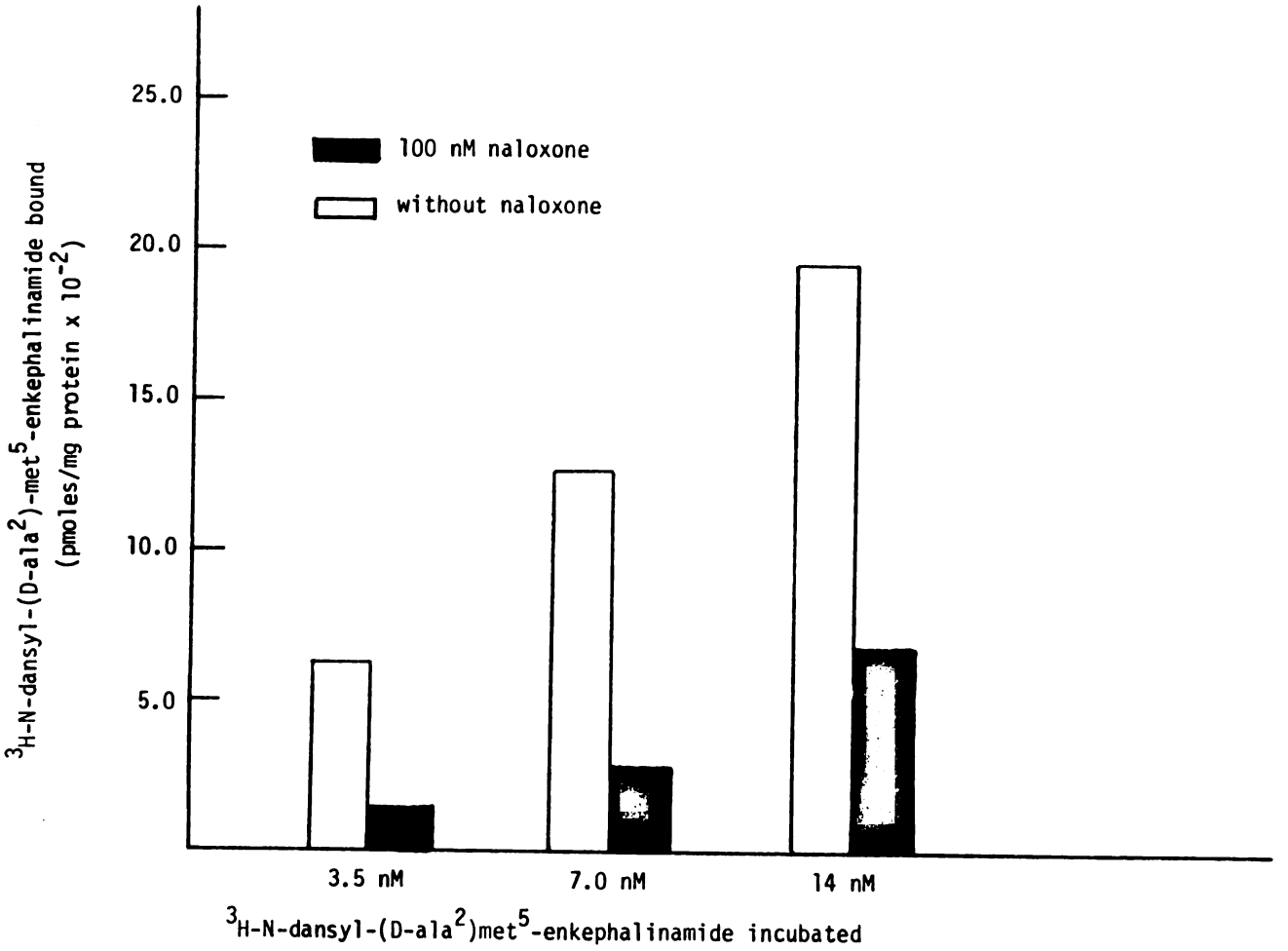
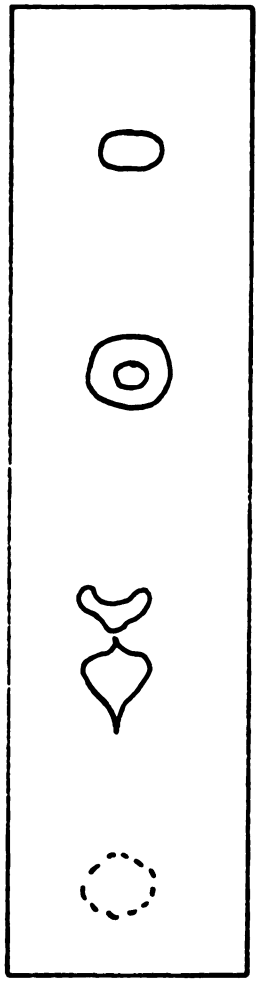


Figure 9. The binding of radioactive N-dansyl-(D-ala^{2})-met^{5}-enkephalinamide, (tyrosyl-2,6-^{3}H), to brain homogenates.

non-dansylated enkephalin. The influences of cold N-dansyl-DALA on the binding of ^{3}H-(D-ala^{2})-met^{5}-enkephalinamide (^{3}H-DALA) to brain slices were found to parallel those of cold (D-ala^{2})-met^{5}-enkephalinamide (DALA). N-dansyl-DALA was prepared by the standard procedures, as previously described. The identification of the dansyl derivatives of DALA was based on their fluorescence characteristics on silica gel 60 chromatograms and in ethanol, see Figure 10. The number one derivative displayed a bright yellow-green color on the chromatogram, and had an emission maximum at 530 nm in ethanol, like N-dansyl-enkephalin. Even though the Rf value of this derivative was different from the Rf value for N-dansyl-enkephalin, the number 1 derivative was designated as N-dansyl-DALA. The amide group at the carboxyl terminal of N-dansyl-DALA may have caused it to travel similar to dansylamide. The number 2 derivative had a yellow-green center surrounded by a bright blue band as concentric circles on the chromatogram. The emission maximum was obtained at 474 nm in ethanol and, was attributed to the high energy emission of dansylsulfonic acid, since the position of the number 2 derivative on the chromatogram was similar to that of ^{3}H-N-dansyl-DALA, see Figure 8. It is inferred that the number 2 derivative contains dansylsulfonic acid and unseparated N-dansyl-DALA. The number 3 and 4 derivatives were not identifiable but the fluorescence properties of the number 3 derivative do indicate a dansyl group without a sulfonamide bond. When exposed to UV irradiation, all of the dansyl derivatives of DALA showed some degree of photolytic degradation on the chromatograms. The number one derivative (N-dansyl-DALA) did not fade like the number 1 dansyl derivative of enkephalin (dansylamide).



der. #	deriv. name	color	Flλ
1.	N-dansyl-DALA	yellow-green	530 nm
2.	dansyl-OH and N-dansyl-DALA	yellow-green surrounded by blue	474 nm
3.	?	blue	465 nm
4.	?	orange	465 nm - 561 nm
	initial spot		

Figure 10. Identification of dansyl derivatives of (D-ala^2)-met^5-enkephalinamide by silica gel 60 thin-layer chromatography.

In the presence of N-dansyl-DALA, the binding behavior of ^{3}H-DALA to brain slices was similar to its binding in the presence of the control, DALA, see Figure 11. At 3 nM, the saturable binding of 3 nM ^{3}H-DALA was the same for both N-dansyl-DALA and DALA, at approximately 0.113 pmoles/mg protein. The opiate receptor binding of ^{3}H-DALA was markedly reduced by both N-dansyl-DALA and DALA, at high incubating concentrations (300 nM); however, the reduction by DALA was 28% greater than N-dansyl-DALA. This suggests that the opiate receptor affinity of N-dansyl-DALA is slightly less than the affinity of DALA. The number 2 derivative influenced the binding behavior of ^{3}H-DALA in a manner similar to the control (DALA) except, at low concentrations, the number 2 derivative reduced the binding site concentration of ^{3}H-DALA by 28% less than DALA, probably due to hydrophobic interactions between dansyl-sulfonic acid and the receptor site. The presence of N-dansyl-DALA or DALA were found to enhance the binding of ^{3}H-DALA to the brain slices. The saturable binding of ^{3}H-DALA alone (at 0.0 nM cold drug) was 0.062 pmoles/mg protein. However, naloxone (100 nM) inhibited the ^{3}H-DALA binding by 76%, similar to the inhibition of ^{3}H-N-dansyl-DALA binding. The opiate receptor binding of ^{3}H-DALA in the presence of the number 3 or 4 derivative was analogous to the binding of N-dansyl-DALA or the number 2 derivative, respectively, indicating opiate-like characteristics of DALA. These results conclusively demonstrate that N-dansyl-DALA and DALA occupy the same binding sites.

Fournie-Zaluski et al. (1978, a) have reported that the opiate-like activity of N-dansyl-met^{5}-enkephalin was considerably less than leu^{5}-enkephalin. They found that the K_i value for the inhibition of ^{3}H-leu^{5}-enkephalin binding to membranes by N-dansyl-met^{5}-enkephalin was 50 nM,

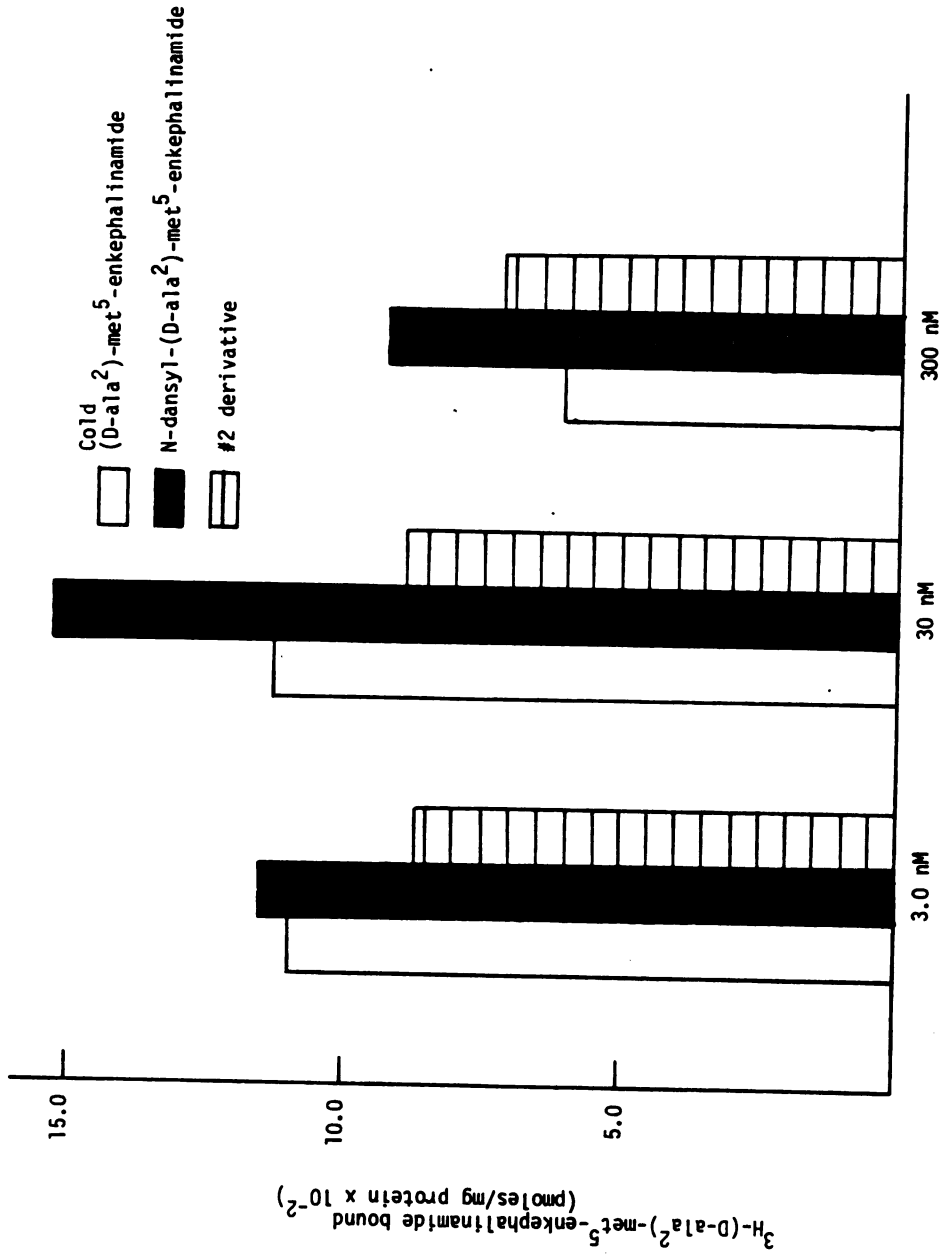


Figure 11. Opiate receptor binding of ^{3}H-(D-ala^{2})-met^{5}-enkephalinamide to brain slices in the presence of N-dansyl-(D-ala^{2})-met^{5}-enkephalinamide or (D-ala^{2})-met^{5}-enkephalinamide.

compared to 5 nM for leu^5-enkephalin; and, in the inhibition of electrically induced contractions of guinea pig ileum, N-dansyl-met^5-enkephalin had a IC 50 value of 1306 nM compared to 67 nM for leu^5-enkephalin. The low values for N-dansyl-met^5-enkephalin binding may be due to comparison to leu^5-enkephalin binding, instead of met^5-enkephalin or naloxone. However, the opiate receptor binding of (D-ala^2)-met^5-enkephalin-$(CH_2)_2$-dansyl has been shown to be equal to or better than leu^5-enkephalin. It is agreed that N-substitution of enkephalin decreases its opiate receptor affinity (Pert et al., 1976).

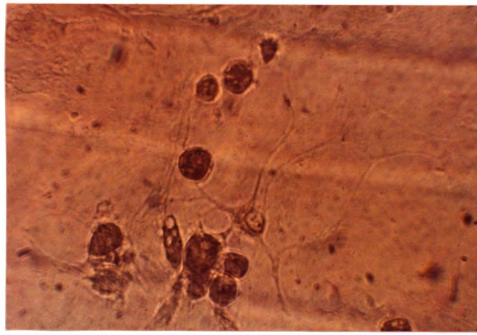
C. Interaction of Cultured Brain Cells with N-dansyl-met^5-enkephalin

1. Microspectrofluorometric characterization of amygdaloid cells by N-dansyl-met^5-enkephalin fluorescence

The mapping of opiate receptor sites on amygdaloid cells was accomplished by N-dansyl-met^5-enkephalin fluorescence; in that, enkephalin-dansyl-receptor complexes were visually located on single cells via fluorescence microscopy. The enkephalin-dansyl-receptor complexes appear as a continuous turquoise covering on the surface membrane of the cell, with discrete blue patches, see Figures 12 and 13. In photomicrograph A of Figure 12 (phase-contrast), the processes of the center cell projected towards other nearby cells and appeared to come into close opposition with their cell bodies. The left process of the center cell, having several bifurcations, extended over to a cell and appeared to wrap around approximately 30% of the cell body. A short process projected to a bottom large cell and wrapped around its cell body. In photomicrograph B of Figure 12, there was no fluorescence from the cell in the center of the picture, but the cells that were contacted by its neurites displayed a

Figure 12. Part A - This is a photomicrograph of cultured cells from the amygdalar region of a 15 days old postnatal rat brain. The cells were maintained 30 days in culture before fixation and staining for 74 hours with 2×10^{-6} M N-dansyl-met^{5}-enkephalin in tris buffer. They are seen here under low intensity white light. The cell in the center of the picture is about 20 microns long and has five processes. The nucleus of the cell is situated to one-side of the cell body and is plainly visible. (0.5 cm = 10 microns).

Part B - This is a photomicrograph of the same cells that were seen in the above picture, but using the fluorescence excitation system.

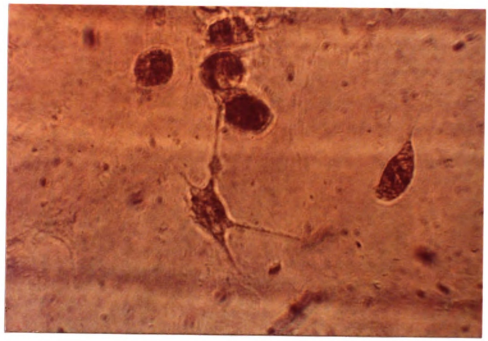


A

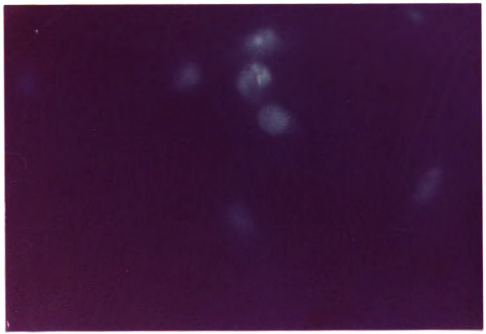
B

Figure 13. Part A - This photomicrograph is of cells that are located in a different culture region.

Part B - These are the same cells under fluorescence excitation.



A



B

considerable amount of dansyl fluorescence. The intense fluorescence from the cells contacted by the processes may indicate a large concentration of enkephalin receptors located on the postsynaptic membrane of opiate susceptible neurons. The lack of fluorescence from the center cell was understandable, because an enkephalin secreting neuron may not contain opiate receptors. In Figure 13 (photomicrograph A), the process of the center cell, leading up to the group of cells, was of particular interest. The process bifurcated just before contacting a cell, traveled across the cell body, and appeared to terminate in a specific location. In photomicrograph B of Figure 13, note the intense blue patches at the termination site of the bifurcated neurite. Also, the above cell had a discrete blue patch at a point of contact with the process. It was evidenced from these photomicrographs, that the blue clusters were varied in size and shape, and located at soma-soma or process-soma contacts between two cells. The opiate receptor binding of N-dansyl-met^5-enkephalin onto amygdaloid cells was not conclusively demonstrated by these results, because inhibition of the enkephalin-dansyl-receptor fluorescence by naloxone or enkephalin was not attempted with the amygdalar cultures here. However, the dansylated opioid was established to bind to opiate receptor sites, in brain slices and homogenates, see page

The fluorescence emission spectrum of N-dansyl-met^5-enkephalin bound to the amygdaloid cells had a maximum at 445 nm with a shoulder around 478 nm, see Figure 14. The binding of N-dansyl-met^5-enkephalin to the cell caused a blue shift in its fluorescence peak, indicating that the microenvironmental dielectric constant of the 1-dimethylamino-naphthalene ring was substantially decreased upon cellular engagement.

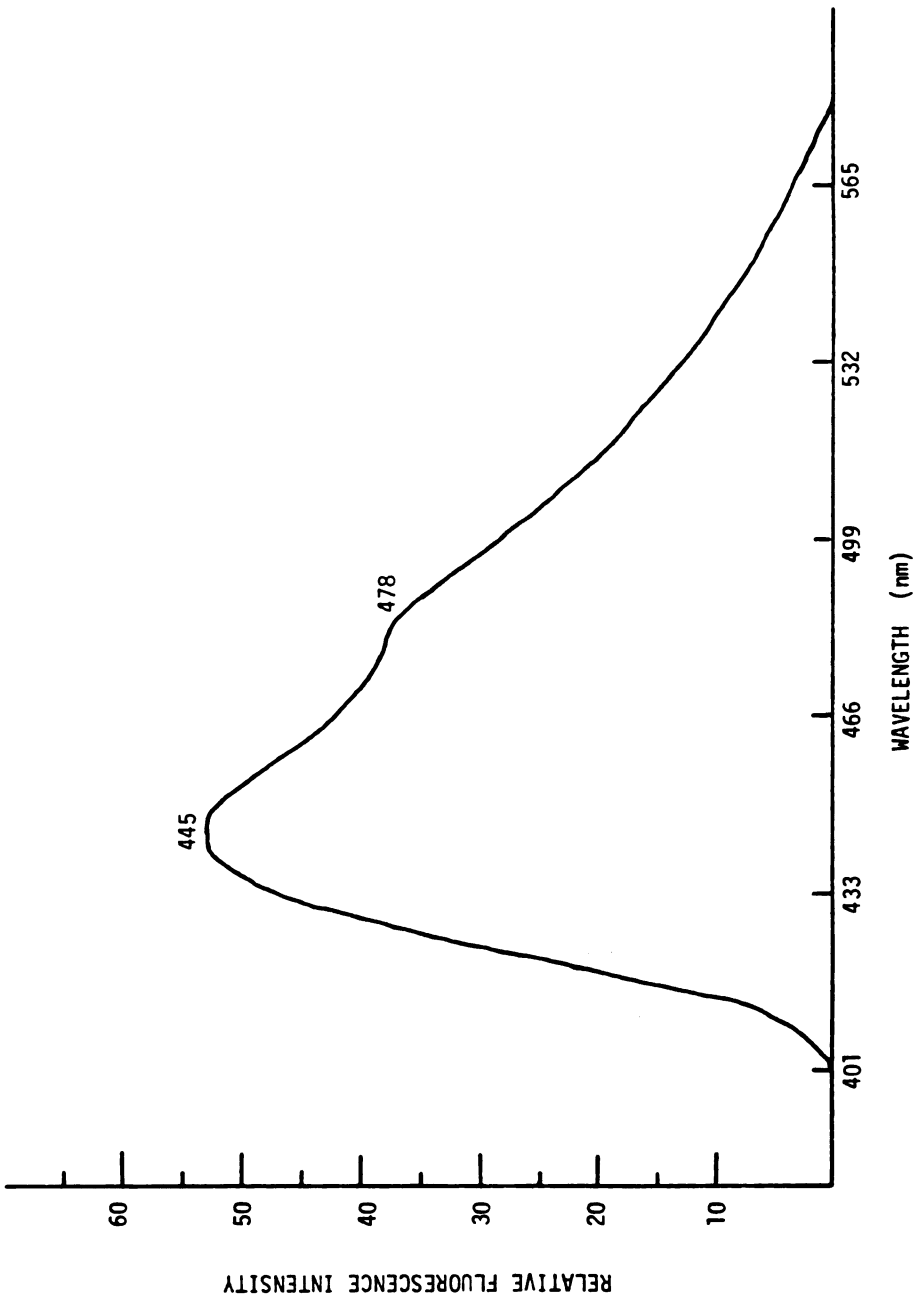


Figure 14. Fluorescence spectrum of an amygdaloid neuron stained with 2.1×10^{-7} M N-dansyl-met^5-enkephalin in 0.05 M tris buffer pH 7.01. The peak at 445 nm is attributed to light scatter from the excitation beam and the shoulder at 478 nm is due to the dansyl chromaphore.

In the solution studies with free N-dansyl-met^5-enkephalin, the dansyl fluorescence was found to be sensitive to the polarity of the environment. When the dielectric constant of the system decreased, the fluorescence maximum of N-dansyl-met^5-enkephalin shifted towards shorter wavelengths. Therefore, it is argued that the hydrophobicity of the opiate receptor site caused the blue shift in the fluorescence peak of the neuron bound N-dansyl-met^5-enkephalin.

The fluorescence intensity of enkephalin-dansyl-receptor complexes was determined to be weak, and to increase as a function of staining time. Since low fluorescence intensities could be measured by the microspectrofluorometer, the cells were stained for 30 min. However, for the photomicrographs, the staining time of the cells was prolonged to increase the dansyl fluorescence.

2. Inhibition by naloxone of N-dansyl-met^5-enkephalin binding to cells of whole-brain cultures

The binding of N-dansyl-met^5-enkephalin to cultured brain cells was evaluated by the ability of naloxone to decrease the fluorescence of the probe. The relative fluorescence intensity at 480 nm of cells of whole-brain cultures, stained with either 1.0×10^{-6} M N-dansyl-met^5-enkephalin (A), 1×10^{-4} M naloxone and 1.9×10^{-6} M N-dansyl-met^5-enkephalin (B) or 2×10^{-6} M dansyl chloride (C), was measured as a function of irradiation time, see Table 3. The emission intensity of a cell should reflect the number of dansyl molecules bound by the cell and should be proportional to the cellular concentration of enkephalin receptors. At each irradiation time, the fluorescence of cells stained under condition A was determined to be greater than the fluorescence from cells stained under the B or C condition. The brain cells stained

Table 3. Fluorescence intensity at 480 nm of cultured brain cells, stained with A, B or C (see below), as a function of excitation time; stained for 30 min. at room temp.

Continuous exc. time	Relative fluorescence intensity per cell			The percentage A is greater than	
sec.	A	B	C	B	C
5	0.470	0.393	0.333	19.6%	41.1%
15	0.350	0.302	0.265	15.9%	32.1%
30	0.290	0.252	0.227	15.1%	27.8%
60	0.235	0.210	0.191	11.9%	23.0%
90	0.209	0.187	0.174	11.8%	20.1%
120	0.194	0.170	0.161	14.1%	20.5%

A - 1.9×10^{-6} M N-dansyl-met^{5}-enkephalin only.

B - 1.9×10^{-6} M N-dansyl-met^{5}-enkephalin plus 1×10^{-4} M naloxone.

C - 2×10^{-6} M dansyl chloride only.

by dansyl chloride had the lowest fluorescence intensity. This would suggest that the affinity of N-dansyl-met^5-enkephalin for brain cells is greater than the cell affinity of dansyl chloride, since naloxone inhibited the neuron binding of N-dansyl-met^5-enkephalin. It is obvious that a percentage of the cell's fluorescence may be opiate receptor sites labeled with N-dansyl-met^5-enkephalin. The greatest difference between the fluorescence intensities of cells, stained with either A, B or C, was at 5 sec. excitation time. If the fluorescence intensity was measured in the msec excitation time range, then the intensity difference between the staining conditions would be even greater.

Like many other fluorescent molecular probes, the fluorescence emission of dansyl slowly diminishes, i.e. undergoing photolytic degradation. The concern of this experiment was the establishment of the fluorescence fading behavior for N-dansyl-met^5-enkephalin bound to brain cells. The average relative fluoresceice intensities of cells, stained under A, B or C conditions, at 480 nm, were graphically displayed as a function of irradiation interval in Figure 15. The fluorescence fading of cells, stained under A, demonstrated a somewhat faster, but similar, rate than the corresponding fading of cells, stained under B or C condition. The fluorescence fading behavior of debris stains under condition A resembles quite closely to the fading of debris stained under condition C, but differ markedly from the fading behavior of cells. (Floating material in cell culture was termed "debris.") The fluorescence of debris faded much more slowly than the fluorescence from cultured cells; scattered light does not fade. These results suggest that the fluorescence fading behavior of bound dansyl molecules is more dependent on the binding site than on the dansyl derivative.

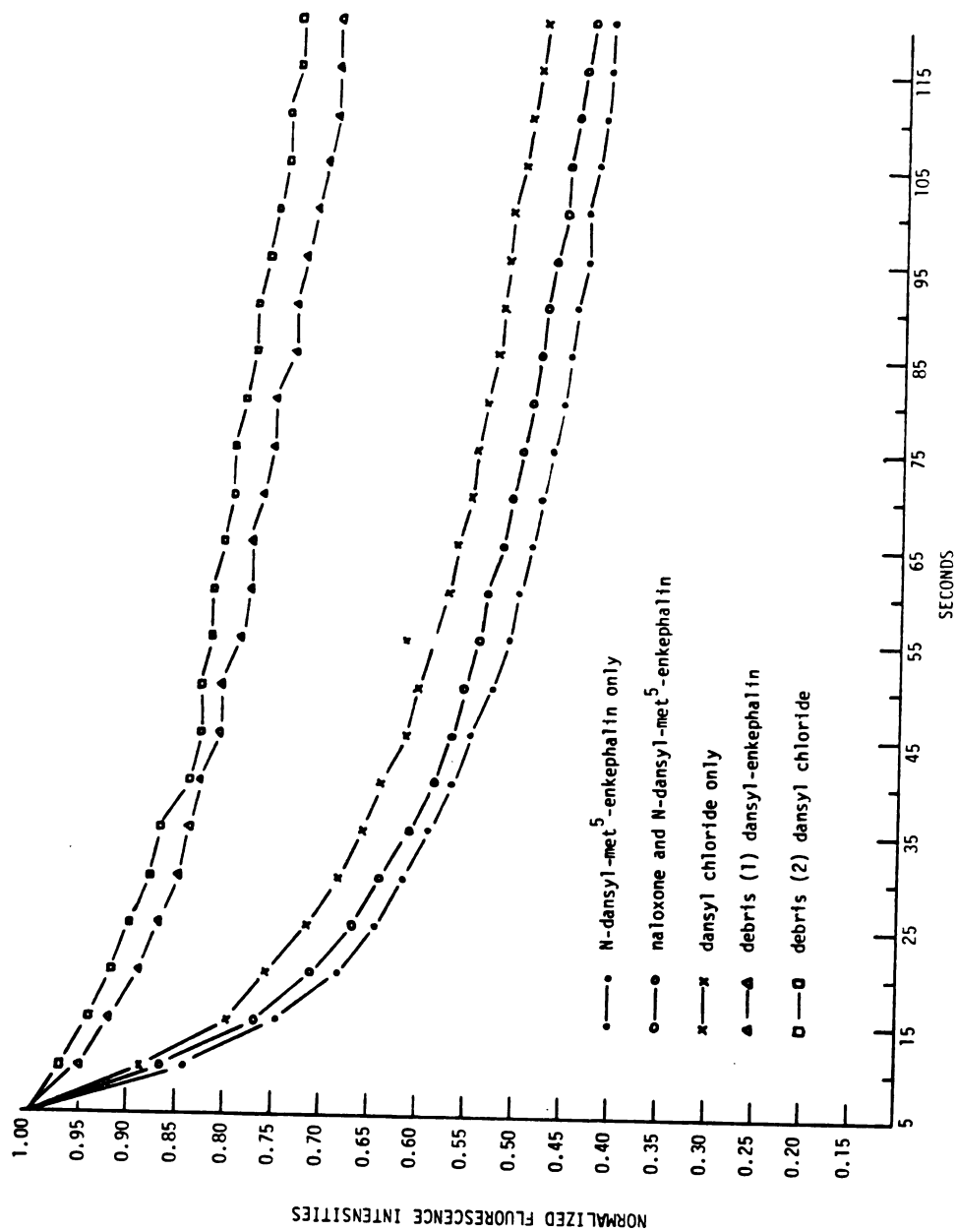


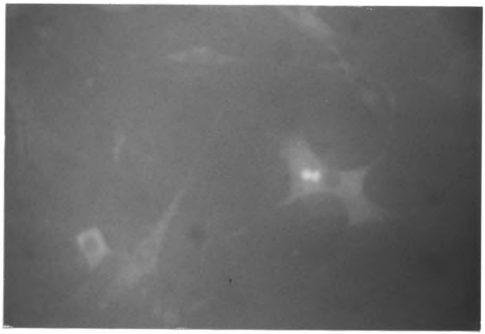
Figure 15. Fluorescence fading of cells from whole brain cultures stained under different conditions, as a function of excitation time; at 480 nm (normalized to 5 sec.).

The enkephalin-dansyl-receptor complexes on whole brain cells appeared, similar to those on amygdaloid cells, as a continuous turquoise covering on the surface membrane of the cell, see Figure 16. The two spots on the off-center cell are believed to be artifacts. It is thought that, under these conditions, a significant portion of the binding of N-dansyl-met^5-enkephalin to fixed neurons was to non-specific and specific enkephalin binding sites. However, as shown by the naloxone inhibition of binding, N-dansyl-met^5-enkephalin does bind to enkephalin receptor sites on cultured cells.

D. Interaction of Purified Membrane Fragments with N-dansyl-met^5-enkephalin

An attempt to obtain the fluorescence properties of N-dansyl-met^5-enkephalin bound to purified membrane fragments, by use of conventional spectrophotofluorometry, was unsuccessful because light scattering from the membrane fragments overshadowed any fluorescence possible from the dansyl chromophore even at very low membrane protein concentrations.

Figure 16. This is a fluorescence photomicrograph of cells from whole brain cultures of a 15 days old postnatal rat. The brain cells were maintained in culture for 15 days before fixation and staining with 1.9×10^{-6} M N-dansyl-met-enkephalin for one hour in tris buffer.



CONCLUSIONS

In this investigation, the synthesis, fluorescence characteristics, and opiate-like activity of N-dansyl-met^5-enkephalin were examined. The novel opioid was employed to visually map the distribution of opiate receptor sites on cultured neurons. These goals were achieved by the following approach: 1. The synthesis of N-dansyl-enkephalin was based on the procedures for N-terminal derivatives of peptides (Felgner and Wilson, 1977; Gros and Labouesse, 1969). The fluorescence and chromatographic properties of N-dansyl-met^5-enkephalin were in agreement with those published for N-dansyl-peptides (Weiner et al., 1972; Hartley, 1970; Gros and Labouesse, 1969; Woods and Wang, 1967). N-dansyl-enkephalin had a yellow-green color on silica gel chromatograms, and an emission maximum at 530 nm in ethanol. The ninhydrin and 1-nitroso-2-naphthol reactions verified that attachment of the dansyl group at the amino terminal of enkephalin. The fluorescence properties of N-dansyl-met^5-enkephalin were found to be sensitive to the dielectric constant of the environment, similar to the results of Fournie-Zaluski et al. (1978, a, b). 2. The opiate receptor binding of N-dansyl-enkephalin was evaluated by its ability to inhibit the binding of ^{3}H-naloxone and ^{3}H-(D-ala^2)-met^5-enkephalinamide to brain homogenates and slices. The attachment of the dansyl group to enkephalin does not greatly interfere with the opiate receptor binding of the enkephalin moiety. N-dansyl-DALA, which has a slightly less opiate receptor affinity, was found to

occupy the same binding sites as DALA. The opiate-like activity of N-dansyl-enkephalin has been confirmed by Fournie-Zaluski et al. (1978, a).

3. Opiate receptor sites on cultured brain cells were labeled by N-dansyl-enkephalin. The surface membrane of the labeled cells demonstrated a continuous turquoise color, with discrete blue clusters at soma-soma or process-soma contact points. On the basis of fluorescence wavelength shifts, it was argued that the opiate receptor site is hydrophobic in nature. Naloxone competitive inhibition established that N-dansyl-enkephalin can bind to opiate receptor sites on cultured cells.

The significance of this research is that it represents a model system that permits the visual and quantitative analyses of the opiate receptor site on the single cell. The influences of drugs, ions, reagents, etc. on the stereospecific binding of enkephalin to the opiate receptor may be determined by studying the fluorescence changes of the bound dansyl probe. The fluorescence properties of N-dansyl-enkephalin can give information on the microenvironmental dynamics at the opiate receptor site. Presently, no other methodology can directly yield this kind of information from the single cell. These analyses may enable us to interpret the functional activity of the opiate susceptible neuron by determining the specific locality of the enkephalin-opiate receptor system on the neuronal cell body or processes.

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