ION-INDUCED CHANGES IN ELECTRON SPIN RESONANCE SPECTRA OF SPIN-LABELED OPIATE RECEPTORS

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ABSTRACT

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Chandramallika Das

This study has shown a conformational change in the morphine receptor site in the presence of Na^+ ions by means of ESR techniques. Morphine receptor preparation can be specifically labeled at sulfhydryl sites by maleimide spin label. A conformational change in the opiate receptor structure will be indicated by a change in the spectral parameter that represents the degree of mobility, e.g. the rotational correlation time. The study provides a biophysical proof for the hypothesis that the receptor is present in two interconvertible conformations; one is the "agonist conformation" or "no sodium state" and the other is the "antagonist conformation" or "sodium state". Spin labels attached to the receptor will show different rotational correlation times depending on the state of the receptor. It is confirmed also that a decrease in the mobility of the spin label is not an effect of ionic strength or of cations in general, but a unique property of sodium to stimulate the antagonist conformational state. K^+ ion has no effect on the spin label on the morphine receptor.

Addition of Mn^{+2} to the spin labeled morphine receptor results in an interfering spectrum while addition of Mg^{+2} causes no significant change in the spectrum.

The effects of morphine on receptor conformation cannot be studied by means of ESR techniques using isothiocyanate spin label since it does not attach to the specific sites of the receptor preparation.

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CHAPTER I

INTRODUCTION

1.1 Definition of Problem

The present study has employed the spin label techniques to provide direct physico-chemical evidence for the hypothesis of sodium-induced conformational changes in the opiate receptor site proposed by Snyder et al [1] based on drug binding studies only.

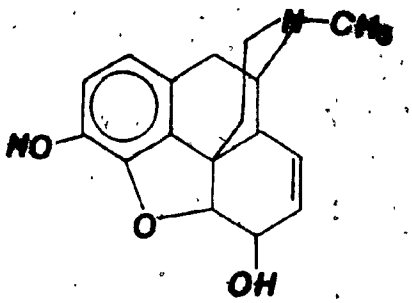
1.2 Introduction to the Opiate Receptor Concept

Biochemical identification of opiate receptor binding [2,3,4] has permitted the characterization of many properties of the pharmacologically relevant opiate receptor, including regional variations in the brain [5,6], occurrence in specific clones of neuroblastoma cultures [7], phylogenetic variations [8], localization to synaptic membranes [9] and the influence of enzymatic treatment [10,11] sensitivity to monovalent cations [12,13], divalent cations [14], and protein modifying agents [15,16].

Identification of receptor site for pharmacological action of opiates is extremely significant in elucidating how these drugs relieve pain, elicit euphoria, and addiction.

Narcotic analgesics or opiate agonists are defined as central nervous system (CNS) depressants that releive pain without, at the same time inducing loss of consciousness. They are addictive and act at the specific sites in the CNS and are thereby differentiated from other CNS depressants such as barbiturates and anaesthetics that act more generally.

Tolerance and physical dependence are mainly associated with these opiate drugs, that can occur to a variety of drugs also, such as alcohol and barbiturates. The alkaloid morphine, which is the best known narcotic analgesic, has the following structure:



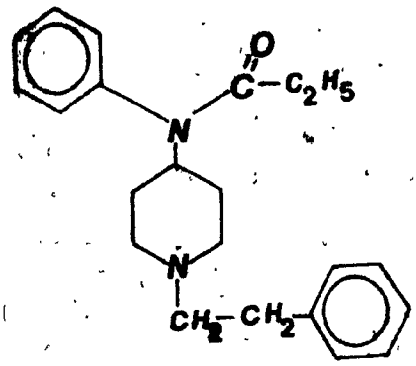
MORPHINE

Morphine and the related drugs that exert analgesic effects are generally referred to as opiate agonist molecules.

The main points which support the view that opiate actions must involve highly selective sites or "receptors" are as follows:

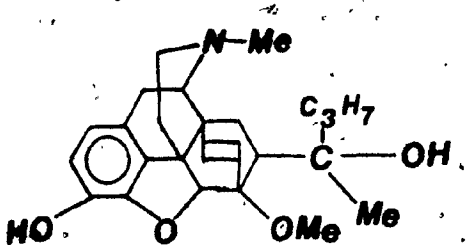
1) The Overall Similar Structure of Opiate Agonist Molecules. In spite of different novel structures characterized as having morphine-like actions, no significant analgesic has yet been identified which lacks a basic centre (at physiological pH, the nitrogen, atom is protonated, forming a cation) or aromatic features. The -OH group attached to the phenyl group is not an absolute necessity for the narcotic analgesic activity but contributes some activity.

There are also many potent analgesics which do not follow the simple structure activity relationship, e.g. fentanyl.



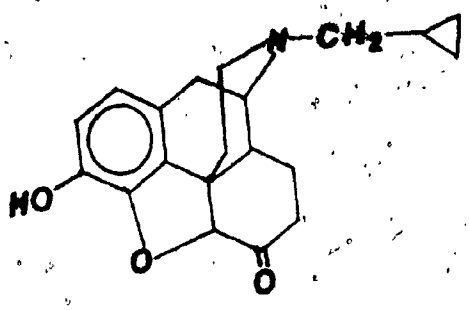
FENTANYL

Etorphine, the most potent narcotic analgesic known, has the following structure. Etorphine has a high degree of lipid solubility so it gets into the brain very quickly resulting in an extremely high parenteral activity.



ETORPHINE

2) The existence of opiate antagonist also favors the receptor concept. Opiate antagonists are drugs closely related in structure to the corresponding opiate analgesics, which have no analgesic or euphoric effect themselves but which can completely reverse actions of opiate analgesics. The example of a potent antagonist is naltrexone, which has the following structure,



NALTREXONE

3) The fact that small structural changes which are likely to alter the physical properties of the molecules to only minor degrees may bring about dramatic changes in potency, is also a proof for the existence of an opiate receptor.

4) The governing role of stereochemical features of the potency of narcotic agonists and antagonists also suggests that opiate actions must involve highly selective sites or "receptors".

Receptors are specifically located on the synaptic membrane of brain tissue. Occurrence of receptors in ileum has also been reported [17]. Opiate receptors are confined to nerve tissues [2]. A regional study of opiate receptor binding of tritiated opiates in rat brain has revealed the most binding in the corpus striatum. Receptor binding is detected in the cerebral cortex, the mid-brain and the brain stem but the binding in the corpus striatum exceeds that of the cerebral cortex more than fourfold. Receptor binding is very low, almost negligible, in the cerebellum.

Subcellular fractionation[2] of rat brain has shown the greatest enrichment of opiate receptor binding in the "microsomal" fraction which is rich in membrane fragments. About half the total opiate receptor activity can be recovered in the mitochondreal-synaptosomal fraction br the P_2 fraction. The nuclear fraction contains the least opiate receptor binding. No opiate receptor binding can be demonstrated in soluble supernatant fraction. Hence the P_2 fraction prepared from rat brain by the Simon[4] and Snyder[15] groups has been regarded as the opiate receptor since it binds opiates stereospecifically and the binding of opiates closely parallels the pharmacological potencies of opiates. In addition to its stereospecific binding properties in pharmacological concentrations, it fulfils many other criteria for drug receptors, such as saturability, reversibility and cell specificity. A sharp pH optimum at 7.4 - 7.5 for specific binding of opiates is also found, with no binding below pH 5 and above pH 10.

The isolation and molecular characterization of most membrane bound opiate receptors will be no simple task. A (^{3}H)-etorphine receptor complex has been solubilized by Simon et al.[18] from rat brain synaptosomal fraction by extraction with the non ionic detergent Brij 36T. But the receptor loses its binding capacity as soon as the bound etorphine has been taken out. The reduced affinity for etorphine binding may point to some distortion of the binding site. Because of the isolation procedure difficulties, the structural features of the receptor molecules are not very conclusive. It has been suggested that receptor is proteolipid in nature. Protein constituents of the receptor moiety

are mainly responsible for its activity because receptor molecules are deactivated by proteolytic enzymes, protein modifying agents, and heat, as shown by Pasternak and Snyder [10,15]. Low concentrations of trypsin, chymotrypsin and phospholipase A reduce opiate receptor binding markedly, while phospholipases C and D and neuraminidase have negligible influences on the binding of agonists or antagonists. Small amounts of RNA-ase and DNA-ase are without effect. Trypsin appears to decrease the number of receptor binding sites without altering its affinity for opiates, while chymotrypsin primarily lowers their affinity for opiates with little effect upon the total number of receptors. Trypsin and chymotrypsin decrease receptor binding in a biphasic fashion. As the trypsin concentration is increased it seems to destroy receptor binding in two phases, i.e. a very sensitive phase up to /μg/ml, and a less sensitive phase between 1 and 30 μg/ml. With chymotrypsin the degradation is also biphasic, with a very sensitive phase up to 0.5 μg/ml, and a less sensitive phase between 0.5 and 250 μg/ml. The biphasic decrease in binding shown by both trypsin and chymotrypsin suggests the presence of more than one population of sites with varying degrees of sensitivity to proteolysis. By contrast, the phospholipases degrade binding in a monophasic fashion. Thus the opiate receptor appears to be a membrane bound complex requiring the integrity of both proteins and phospholipids for opiate binding. Differential effects of sodium [12,13] and protein modifying agents [15,16], and proteolytic enzymes [11] on opiate agonist and antagonist binding to receptors suggest that distinct binding sites

exist for agonists and antagonists, although they may be both on the same receptor as explained by Snyder, et al.[2,4,13,19].

Low concentrations of proteolytic enzymes and reagents which affect sulfhydryl groups, e.g. N-ethylmaleimide, iodoacetamide and phenylmercuric acetate reduce opiate agonist binding with negligible effects on antagonist binding as shown by Snyder, et al.[15]. This strongly suggests that one or more -SH groups is associated with the opiate receptor which is much more important for agonist than antagonist binding. Prior treatment with opiates protects the receptor from those sulfhydryl reagents so it is likely that this group may be at or near the binding site. On the other hand, protection may be due to conformational change induced by opiate binding or by allosteric interactions. These reagents decrease the apparent number of agonist binding sites without altering their affinity and also increase the sensitivity of agonist binding to the inhibitory effects of sodium, although it is not possible to establish a causal relationship between the increase in sodium sensitivity and reagent actions.

Low concentration of sodium ions ($\geq$ 5 mM) enhances the stereospecific binding of antagonists while reducing the agonist binding to brain homogenate. This differential effect of Na^+ is highly specific since it is elicited by Na^+ and Li^+ but not by K^+ ion and other monovalent cations. It is shown that this property is not a general characteristic of cations or of high ionic strength but a property of sodium[12,13]. As per literature[12] the increase in antagonist binding

results from an enhancement of binding affinity, i.e. due to an acceleration in the rate of association but not from unmasking of new binding sites. The reduction in agonist binding in the presence of sodium is due to a decrease in binding affinity, i.e., due to an acceleration in the dissociation rate of agonist from binding sites, but no effect can be seen on the dissociation rate of antagonists.

Low physiological concentrations of Mn^{+2} and Mg^{+2} ions to some extent selectively increases the binding of agonists and reduces the binding of antagonists [14]. The differential effects produced by Mn^{+2} or Mg^{+2} ions are not as pronounced as the effects produced by Na^{+} ions. Significant effects of Mn^{+2} are produced only in the presence of the Na^{+} ion. Ca^{++} ions fail to produce such an effect.

From the studies of the differential actions of protein modifying agents and ions, a model for the opiate receptor conformation has been proposed by Snyder et al. [1] to explain the pharmacological actions of agonists and antagonists. The opiate receptor is postulated to exist in an equilibrium between two conformations which preferentially bind agonists or antagonists as shown in Figure 1.1. It is hypothesized that sodium induces a conformational change in opiate binding sites so that the transformed site exhibits greater affinity for antagonists and reduced affinity for agonists. The hypothesis is supported by the competition of agonists and antagonists for receptor sites. The opiate binding sites can vary as the receptor is transformed between two conformations: one is "Na state" or "antagonist conformation"; and the

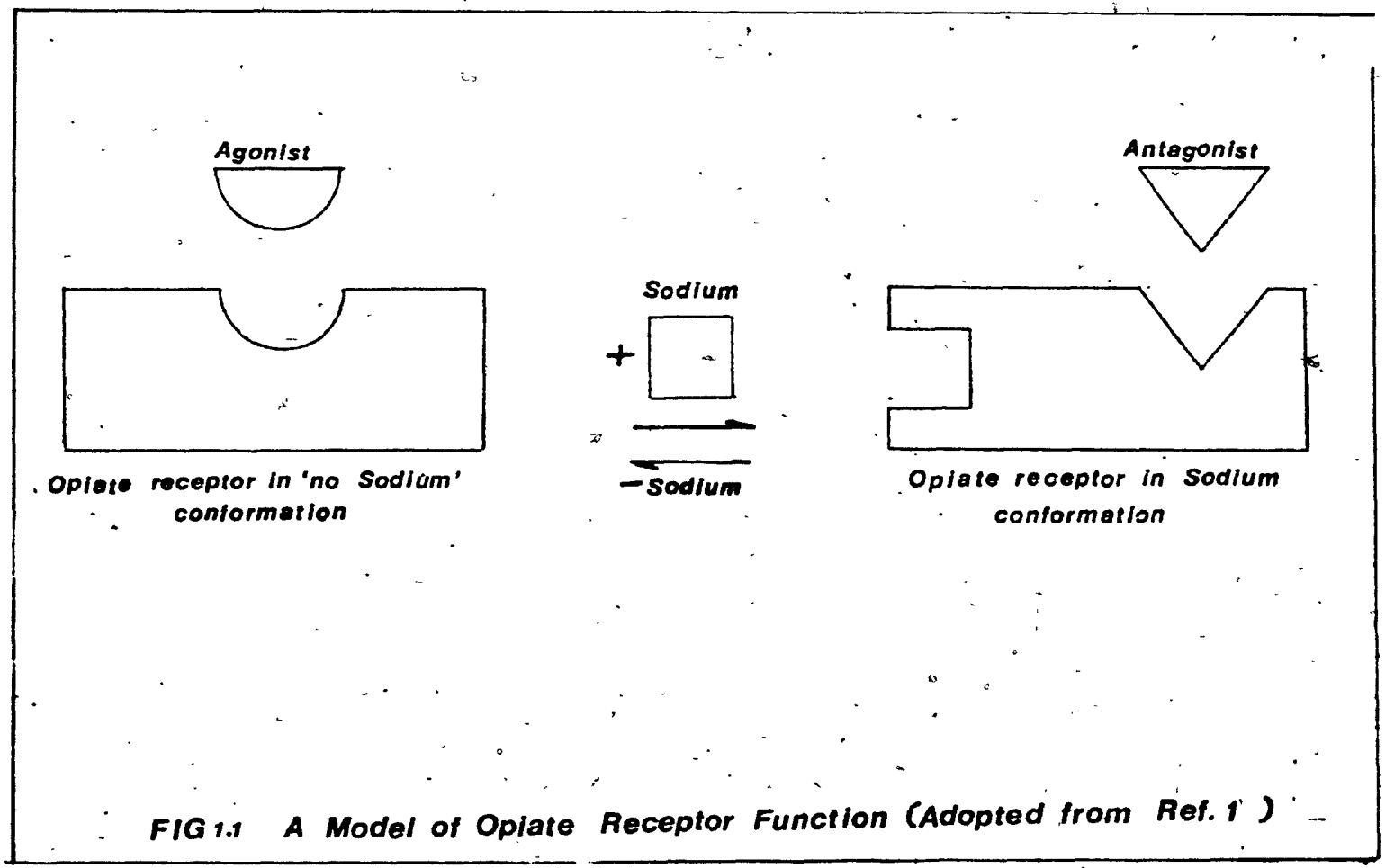


FIG 1.1 A Model of Opiate Receptor Function (Adopted from Ref. 1)

other is "no Na state" or "agonist conformation". Antagonists have greater affinity for the antagonist-sodium form of the receptor while agonists bind preferentially to the agonist-no sodium receptor state. Pharmacological actions such as analgesia occur only when a drug binds to the agonist state of the receptor so that antagonists block the effects of opiate analgesics by binding to the antagonist conformation of the receptor.

According to this model the selective decrease in agonist binding produced by protein modifying agents could be attributed to effects on sites which are involved in the interconversion of receptor conformations so that the sites freeze in the antagonist conformation. This explanation is consistent with evidence that the reagents act by enhancing sensitivity of the receptor to sodium. It also accords with the ability of reagents that act on different chemical groups to differently alter agonist and antagonist binding, since one would expect numerous chemical groups to be involved in the transition between conformational states of the receptor. Alternatively it is conceivable that the receptor contains distinct binding sites for agonists and antagonists.

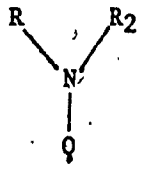
Isolation of opiate receptors has yet to be accomplished. No definitive information that relates alternations in opiate binding to tolerance dependence state has been obtained [20].

The model suggested for opiate receptor conformation to explain opiate actions is simply hypothetical, supported by strong evidence obtained through radioactive tracer techniques. This model has not yet

been confirmed by physical techniques. The present study therefore utilizes a nitroxide spin label probe and ESR spectroscopy as an effective biophysical technique complementing radioactive tracer techniques for the study of the narcotic analgesic receptor site. Eventually it might provide further new insight into the mechanism of actions of opiates.

1.3 The Spin-Label Method

Spin labeling is a versatile spectroscopic technique that employs stable organic free radicals as probes of structure of biologically important macromolecules. This method was originated by Christie & McConnell [21]. The first use of a class of stable organic free radicals known as nitroxides was reported by Stone, et al. [22]. The most successful compounds used as spin labels to date are the nitroxides of general structural formula (I)



(I)

Detailed accounts of the chemistry of nitroxides have been published [23,24].

Spin labels offer some important advantages over other structural probes including,

a) stability at pH 2-10, high and low salt concentration and tempreature from 20 - 70°C,

b) high sensitivity,

c) freedom from spectral interference (most biological systems are not paramagnetic), and

d) the absence of any adverse effects of sample opacity. In addition, labels can be prepared that are hydrophobic, hydrophilic, or amphiphilic.

Labels have been attached to macromolecular systems by covalent as well as by non-covalent bonds [25]. Most spin label studies of biological systems have used ESR of the labels [23,26,27,28,29,30].

Pure nitroxide spin labels are available commercially in combination with different attaching groups, e.g. maleimide, iodoacetamide, bromoacetamide, isothiocyanate. Each of these attaching groups has been found useful in binding to different chemical groups on protein molecules or other biological macromolecules.

Electron Spin Resonance (ESR) Spectroscopy of the Nitroxide Radical

An approximate spin Hamiltonian, $\mathcal{H}$, describing a collection of nitroxide spin labels is [31].

$$\mathcal{H} = |\beta| \underline{S}\, g\, \underline{H} + \underline{S}\, A\, \underline{I} + \text{electron - electron dipole and exchange terms}$$

where β is the electron Bohr magneton

g is the spectroscopic splitting tensor

$\underline{A}$ is the electron-nuclear hyperfine coupling tensor

$\underline{S}$ is the electron spin operator

$\underline{I}$ is the nuclear spin operator

$\underline{H}$ is the instrumental magnetic field, respectively

The electron - electron dipole and exchange terms are significant only when large local concentrations of spin labels or dinitroxide spin labels are used [32]. The Hamiltonian can be expressed as

$$\mathcal{H} = |\beta| \underline{S}\, g_o\, \underline{H} + \underline{S}\, A_o\, \underline{I}$$

for nitroxide spin labels tumbling rapidly in solution, where g_o and A_o are the isotropic components of g and $\underline{A}$.

The first term in the Hamiltonian is the electron Zeeman term representing the interaction of the magnetic moment of the unpaired electron's spin with the applied magnetic field. The electron may exist in one of the two energy states in which its magnetic moment is aligned either parallel or antiparallel to the direction of the field. Transitions between these two energy states can be induced by the application of electromagnetic radiation of the appropriate energy. At resonance the relationship is:

$$h\nu = g\beta \underline{H}$$

where h is Planck's constant

ν is the microwave frequency of the ESR spectrometer.

Electron spin resonance can be observed when either $\underline{H}$ or ν is varied. For experiment convenience it is useful to keep ν constant while $\underline{H}$ is charged. Most commercial ESR spectrometers operate in the microwave frequency range of 9×10^9 Hz or (9 GHz) so that $\underline{H}$ is approximately 3300 gauss (G).

The second term in the Hamiltonian represents the interaction between the unpaired electron and the nuclear spin, I, of the nitrogen in the nitroxide, N-O group. Since I is equal to 1 for ^{14}N, the Zeeman line will be split into 2I + 1 or three lines of equal intensity separated by A_o, the isotropic hyperfine coupling constant, and centered around g_o, the isotropic g value. In qualitative terms, the magnetic moment of the nitrogen nucleus can be aligned parallel, antiparallel, or perpendicular to the applied field. Since the magnetic field experienced by the electron is the sum of the external field and the local contribution provided by the ^{14}N nucleus, it follows that the electron experiences three different magnetic field values, each of which gives rise to an absorption line in the spectrum. For technical reasons, the commercially available ESR instruments display the spectrum at its first derivative instead of the simple absorption spectrum familiar to optical spectroscopists. The electron spin resonance (ESR) spectrum of the nitroxide radical when present at low concentration in a non-viscous solvent consists of equally spaced lines of about the same height, as shown in Figure 1.2.

The spectrum is characterized by three parameters: (1) the hyperfine splitting (A_o), i.e., the distance (G) between adjacent lines; (2) the so-called g factor (g_o), i.e., the position of the center line in the magnetic field; and (3) the peak to peak line width (G). From the combinations of these three useful spectral parameters, information

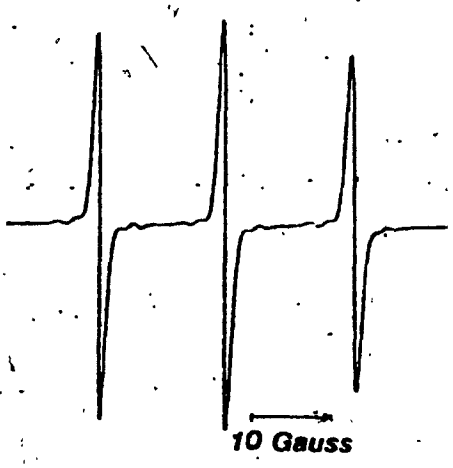
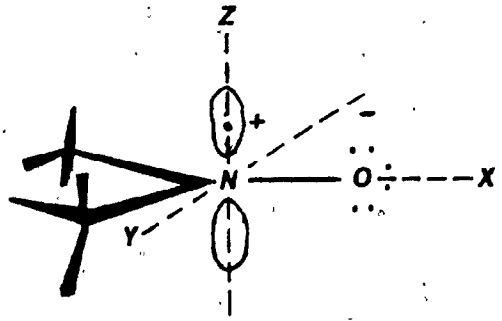


FIG 1.2 The ESR spectrum of a nitroxide radical in aqueous solution (Adopted from Ref. 43)

can be obtained about the orientation and mobility of the nitroxides radical. The ESR spectrum of the nitroxide radical is susceptible to: (a) the orientation of the radical with respect to the applied magnetic field; (b) the mobility of the radical; and (c) the polarity of the environment of the radical.

Effects of Molecular Orientation of the Radical on Spectra from the Spin Label

When a nitroxide radical is incorporated into a host crystal and the crystal axes are rotated with respect to the magnetic field, spectra similar to those of Figure 1.3 are observed. The conventional axis system for nitorxides is defined below. The unpaired electron is composed of the p_π orbitals of nitrogen and oxygen. The observed values for the components of the hyperfine tensor indicate that a substantial fraction of the unpaired electron is localized on the nitrogen atom and therefore suggest that the NO three-electron π-bond has polar character, as represented here:



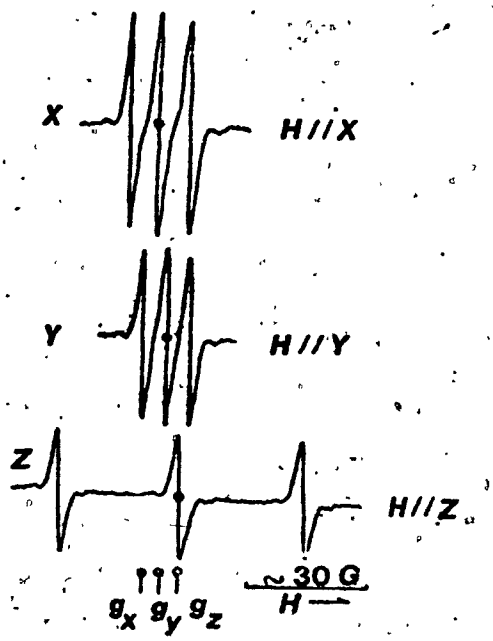


FIG 1.3 Crystal orientation effects on 1st derivative ESR spectra from single crystals of tetramethyl-1, 3-cyclobutanedione doped with di-tert-butyl nitroxide. The g-and A-tensors depend on the crystal orientation with respect to the ESR instrumental magnetic field.

(Adopted from Ref. 32)

Because the molecular orbital containing the unpaired electron is made up in part of a degenerate oxygen P_{π} orbital, the g factor for the nitroxide moiety is expected to be anisotropic [31] ($g_{xx} \neq g_{yy} \neq g_{zz}$). The hyperfine coupling tensor is also anisotropic and is most often nearly axially symmetric ($A_{zz} \neq A_{xx} \simeq A_{yy}$) because of the occupation by the unpaired electron of molecular orbital of a π symmetry [33]. Thus, the center of the magnetic field position (g factor), where ESR absorption occurs, and the separations between the hyperfine lines ($\underline{A}$, hyperfine coupling constant) will depend upon the orientation of the nitroxide with respect to the instrumental magnetic field ($\underline{H}$). As shown in Fig. 1.3, the g tensor decreases while the $\underline{A}$ tensor remains approximately constant and then increases as the magnetic field is aligned parallel to the x, y and z axis of the nitroxide crystal, respectively. The largest hyperfine splitting (A_{zz}) is observed when the magnetic field is parallel to the nitrogen π orbital (z axis). Griffith, et al. [28,34] found that di-tert-butyl nitroxide in a single crystal of tetramethyl-1-3-cyclobutanedione had the principal values of A_{xx} = 7.6 G, A_{yy} = 6.0 G, A_{zz} = 31.5 G, g_{xx} = 2.0056, g_{yy} = 2.0061, and g_{zz} = 2.0027. The assignment of the principal values was made possible by knowing the crystal structure of the host and initially assuming that nitroxide was oriented in the host crystal as a substitutional impurity. The A and g values of many nitroxides are very nearly the same. Comparison of the g and A data as obtained for different nitroxides, supports the following useful generalization;[25]

$$32G \approx A_{zz} \gg A_{xx} \simeq A_{yy} \simeq 5G;\ g_{xx} > g_{yy} > g_{zz}$$

If the polycrystalline nitroxide is dissolved in a solution of low viscosity, such as water, the triplet is observed as in Fig. 1.2. The A_o and g_o values of the three-lined spectrum are related to the single crystal data by:

$$A_o = \frac{1}{3}(A_{xx} + A_{yy} + A_{zz})$$

$$g_o = \frac{1}{3}(g_{xx} + g_{yy} + g_{zz})$$

or A_o and g_o values are each of the average of the values seen in Figure 1.3.

Effects of Molecular Motion on ESR Spectra

When the molecular motion of a nitroxide radical in dilute solution is decreased by increasing the viscosity of the solvent, the ESR lines broaden and the spectrum becomes assymmetric (Figure 1.4) with a maximum separation of $2A_{zz}$. The common terminology for these spectra are partially immobilized, strongly immobilized, and "powder spectra" (i.e., spin labels are rigidly bound to large proteins that mean the molecular motion of the spin label is completely restricted and a powder spectrum is obtained).

A parameter τ, rotational correlation time [22,32,35,36,37,38,39], which is inversely related to the tumbling rate of the nitroxide can be

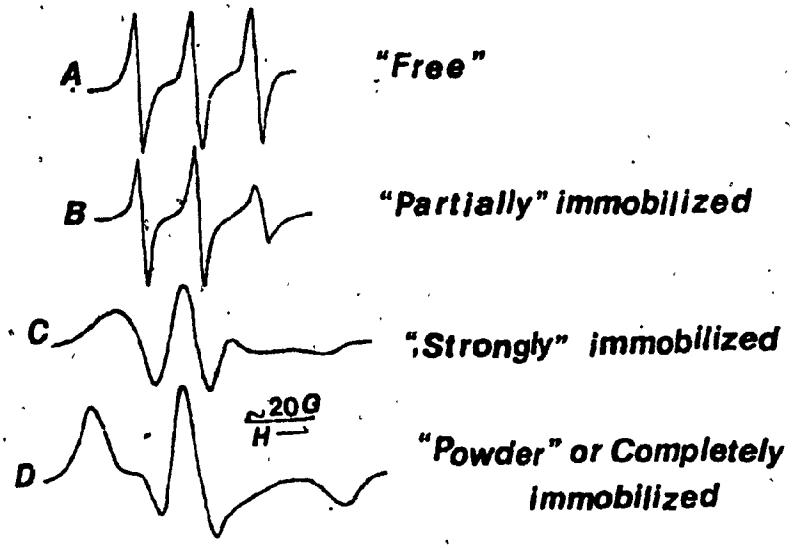


FIG 1.4 Spectral effects of increasing glycerol-solution viscosities, A to D, causing a decrease in the rotational motion of dissolved N-(1-Oxyl-2,2,6,6-tetramethylpiperidinol) spin level.

(Adopted from Ref. 32)

calculated from the spectra. The nitroxide ESR spectra are very sensitive to the rate of molecular rotation covering a range of correlation times from 10^{-11} sec (triplet) to 10^{-7} sec (a strongly immobilized spin label spectrum). This sensitivity may be utilized to describe the restricted motion of a spin label when attached to a macromolecule. The changes in the structure of the spin-labeled macromolecule, resulting in a change in the mobility of spin, will be indicated by a corresponding change in the spectral parameter of mobility, e.g., τ_c or other related parameters. Spin labels have been used, therefore, to study the conformational changes in biological macromolecules and the structure and function of membranes [25,32,40,41,42,43].

Effects of Environmental Polarity on ESR Spectra

Due to the polar character of the N-O bond, the hyperfine coupling constant A and the g factor are influenced by the polarity of environment of the radical. In general, A_o decreases while g_o increases as the solvent polarity is decreased. The nitroxide spin label, therefore, can be used as a probe for binding site polarity.

This introduction has shown that a spin label can report changes in the environment at or near the label's attachment site on a macromolecule whether the change is due to conformation and/or polarity. A detailed description of the application of ESR spectroscopy, and ESR instrumental techniques as applied to spin labeling, will be found in Ref. 44 and 45.

Following is a brief outline of the kind of information we might hope to obtain from an application of this technique:

i) Conformational changes in the binding sites or any kind of perturbation brought about by some biochemical processes can be detected.

ii) The g factor and hyperfine splitting of the spin label do vary slightly with the polarity of the solvent. Thus we have a probe into the hydrophobic or hydrophilic nature of the environment around the label.

iii) Biradical spin labels allow the study of certain intermolecular interactions, since the interactions of the two ends of the biradical is a strong function of the conformation of the biradical [21].

iv) Spin labels can be employed to study the topography of binding sites [43].

v) Spin labels provide a rapid method for the detection and quantitation of drugs and other small molecules present in biological fluids [43,46].

1.4 Purpose of Study

The main purpose of this study was to investigate the ionic influences on the opiate receptor conformation by means of spin labeling techniques. The questions we posed can be divided into three major categories:

i) To show by ESR spectroscopy that the receptor undergoes a conformational change induced by Na^+ ion but not by K^+ ion.

ii) Does the receptor undergo a conformational change opposite in nature to the Na^+ ion effect in the presence of Mn^{+2} or Mg^{+2} ions as binding studies seem to suggest?

iii) The ESR label might reflect changes due to binding of morphine. Are these changes, if any, sensitive to the Na^+ ion effect but not to the K^+ ion effect?

1.5 Experimental Limitations

The present study has the following limitations:

i) The maximum concentration of receptors obtained in the P_2 fraction was in the range of approximately 10^{-10}M but the ESR instrument available was sensitive only up to a minimum limit of spin concentration around 10^{-9}M. ESR signals will therefore be a composite of receptor-specific and non-specific labels. All possible precautions to avoid non-specific labelling have been taken.

ii) It has been assumed that spin label binding on the receptor has no effect on the conformation of the allosteric site.

iii) Isolation and purification of opiate receptors has not yet been accomplished. Therefore a crude preparation of morphine receptor was used for the study of receptor structure by means of ESR Spectroscopy. The same preparation was used by all previous authors as well, in elucidating the properties of the opiate receptor.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

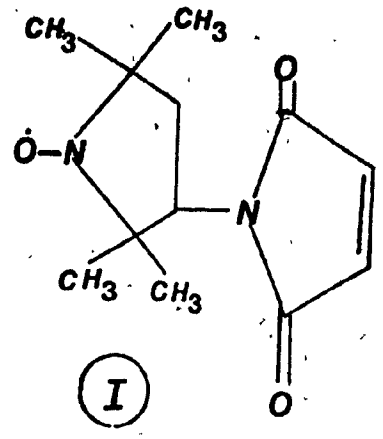
The maleimide nitroxide, 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (I), 3-(maleimidomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (II) and 3-[[2-(2-maleimidoethoxy)ethyl]carbomoyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (III) and the isothiocyanate nitroxide, e.g., 3-(isothiocyanato-methyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (IV) were purchased from Syva Corporation, Palo Alto, California and were used without further purufication. The structures of these spin labels are shown in Figure 2.1.

Morphine sulfate, N-ethylmaleimide, phenylmercuric acetate, ethyl isothiocyanate, dextrorphan and ^{3}H-dihydromorphine were supplied from different sources in our laboratory.

2.2 Preparation of Morphine Receptor

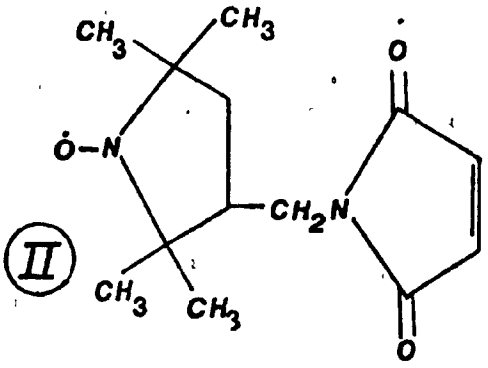
Procedures for preparing opiate receptor were mainly based on the methods used by the Snyder[15] and Simon[4] groups.

Male albino rate (180-200g) were decapitated without anesthesia and their brains were rapidly removed. The cerebellum, which contains negligible opiate binding capacity[2], was discarded and the remainder of the brain, i.e., the cerebrum (about 1.5 gms) was immediately



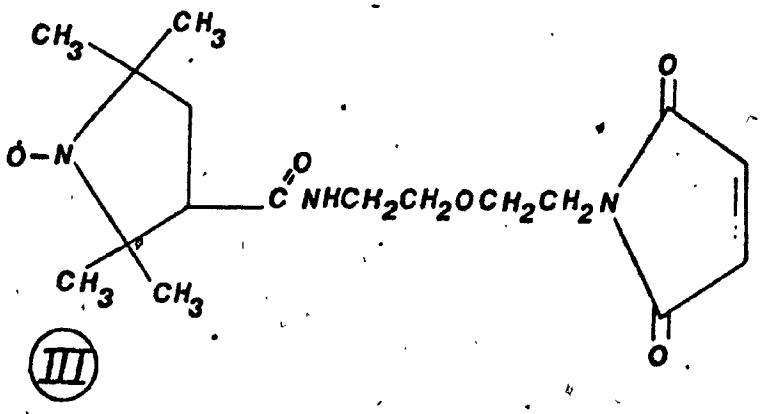
I

3-Maleimide- 2,2,5,5 tetramethyl-1-pyrrolidinyloxyl



II

3-(Maleimidomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl



III

3-[[2-(2 Maleimidoethoxy)ethyl]carbamoyl]- 2,2,5,5-tetramethyl-1-pyrrolidinyloxyl

FIG 2.1 Maleimide Spin Labels

placed in 30 volumes ice cold 50 mm tris HCl buffer (pH 7.4 - 7.5) and homogenized with 10 - 15 strokes of a motor driven homogenizer with a teflon pestle. The homogenate was centrifuged in a refrigerated centrifuge at 1000xg for 10 minutes to remove nuclei and debris. The supernatant (cold) was centrifuged at 20,000xg for 15 minutes. The pellet obtained is the fraction P_2 containing mostly synaptosomes and mitochondria. This fraction contains the most opiate binding capacity [2]. The pellet was resuspended in the original volume of fresh buffer with homogenizer. The resuspension was then incubated at 37°C for 30 minutes and recentrifuged at 20,000xg for 12 minutes and the pellet was resuspended in fresh standard buffer. This preincubation procedure increases the number of available receptors as it removes from the recptor an endogenous morphine-like factor. This substance, as characterized by Pasternak and Snyder [15], appears to be a naturally occurring ligand of the opiate receptor resembling that reported by Hughes and Kosterlitz [47,48] and is known as enkephalin. Enkephalin is composed of two pentapeptides, H-Tyr-Gly-Gly-Phe-Met-oH and H-Try-Gly-Gly-Phe-Leu-oH, as identified by Hughes, et al. [47].

Protein content in mg/ml of the P_2 fraction prepared was determined by Lowry's method [49] using crystalline bovine serum albumin as standard protein. The solution was adjusted with buffer so that homogenate protein was always between 0.5 - 2 mg/ml, a region in which binding is found to be proportional to protein concentration[4]. In practice, the

protein concentration of the P_2 fraction was kept constant throughout the study.

2.3 Spin Label Solution - Preparation, ESR Spectrum and Stability

Stock solutions of three different kinds of spin label, e.g., (I), (II), and (III) of concentration 10^{-2}M were prepared in ethanol using an analytical balance for weighing accurate amounts. Each of the three ethanolic solutions of spin labels was diluted 1:100 by tris buffer (pH 7.5).

ESR spectra of each of the three solutions were run on a Varian E-3 spectrometer operating at 9405 MHz or x band under identical instrumental parameters. A triplet representing a freely mobile spin rapidly tumbling in solutions was obtained for each of the solutions as shown in Figure 1.2. The rotational correlation time (τ) for a very mobile free nitroxide label in solution was calculated as 0.01 ns [22].

Spin label solutions were kept at +2°C. The ESR spectra of the solutions were checked against a freshly prepared pure spin label solution after every couple of days. No significant change in the ESR spectrum was found, indicating that nitroxide spin label solutions were stable. Spin label solutions were prepared and stored in refrigerator for at least 4 weeks.

2.4 Spin-Labeling P_2 Fraction with Maleimide Spin Label

5 ml aliquotes of a freshly prepared P_2 fraction of protein concentration $\leq$ 2 mg/ml were incubated for 1 hour at room temperature with 10^{-4}M (final concentration) spin label solution in 50 mM tris buffer (pH 7.5). Incubations were terminated by centrifugation at 20,000 x g for 15 minutes and the samples were freed completely of free spin label by washing twice by centrifugation at the same speed for 15 minutes. Pellets were then resuspended in 0.5 ml standard buffer at pH 7.5. Two washings were needed to remove the unbound spin labels that might remain. The washing liquid was also checked after each wash for detecting the presence of any free spin label; none was found after two washes.

Maleimide spin labels of different chain lengths, e.g., spin label I, spin label II, and spin label III (Figure 2.1) were used for this study. The use of varied chain lengths permits a label to be selected which reflects optimum mobility to the radical. Variation in chain length may also give information about the molecular environment in which the label is attached.

In order to detect changes in structure of the labeled P_2 fraction the spin label attached to the P_2 fraction should be neither completely free nor completely immobilized since difficulty is encountered in determining any change in τ value for a completely freely mobile spectrum or a completely immobilized spectrum.

With spin label I, the spectrum was the most immobile, with spin label II the spectrum was an intermediate one; and with spin label III having the longest chain length between nitroxide radical and maleimide attaching group, the spectrum was the most mobile (see Table 3.1). So spin label II had been selected to carry out the present investigation.

Electron spin resonance (ESR) measurements were made on a Varian E-3 spectrometer operating approximately at 9405 MHz. The solutions were contained in a flat so-called "Aqueous cell" of total volume about 0.4 ml, made of fused silica. All spectra were run at room temperature and the same cell was used throughout our investigation.

The rotational correlation time (τ) which represents the degree of mobility of the spin label was calculated from the spectrum of spin-labeled P_2 fraction according to the methods used by Stone, et al.[22] and Campbell, et al.[39]. A brief discussion on the calculation of τ values is given below.

2.4.1 Calculation of the Rotational Correlation Time τ of the Spin Label

The electron spin resonance spectrum of the nitroxide label consists of three lines (assigned to the M = 1, M = o, and M = -1, ^{14}N nuclear spin quantum states, the M = 1 state corresponding to the line at lowest field).

The lines are of unequal width, the inequality being a measure of the anisotropies of the hyperfine interaction and of the g value. For the case of rapidly tumbling nitroxide radicals, the transverse relaxation time of each line $T_2(M)$ can be related to the anisotropy of the hyperfine interaction (b), the anisotropy of the g factor ($\nabla\gamma$), the nitrogen nuclear spin quantum number M, and the τ correlation time for isotropic molecular tumbling, by the equation [22,37]

$$(T_2(M))^{-1} = \tau\left\{[3I(I+1) + 5M^2]\frac{b^2}{40} + \frac{4}{45}(\nabla\gamma H_0)^2 - \frac{4}{15} b \nabla\gamma H_0 M\right\} + X \qquad (1)$$

where H_o is the applied magnetic field strength in gauss, I = 1 is the nuclear spin quantum number for ^{14}N. The quantity X represents contributions from other broadening mechanisms that do not depend on M.

Putting in M = o in equation (1), it turns out that

$$\frac{1}{T_2(0)} = \tau\left\{[3I(I+1)]\frac{b^2}{40} + \frac{4}{45}(\nabla\gamma H_0)^2\right\} + X \qquad (2)$$

Equation (1) may be rearranged in the following form,

$$\frac{1}{T_2(M)} = \tau\left\{3I(I+1)\frac{b^2}{40} + \frac{4}{45}(\nabla\gamma H_0)^2\right\} + X + \tau(5M^2)\frac{b^2}{40} - \frac{4}{15} b \nabla\gamma H_0 M \tau \qquad (3)$$

Substituting the value of $\frac{1}{T_2(o)}$ in equation (3), the above equation may be rearranged to give

$$\frac{T_2(0)}{T_2(M)} = 1 - \frac{4}{15}\tau b \nabla\gamma H_0 M T_2(0) + \frac{1}{8}\tau b^2 M^2 T_2(0) \qquad (4)$$

Putting in $M = \pm 1$; it turns out that

$$\frac{T_2(0)}{T_2(-1)} - \frac{T_2(0)}{T_2(1)} = \frac{8}{15}\tau b \nabla\gamma H_0 T_2(0) \qquad (5)$$

which is an expression which involves only the linear terms in M.

On the other hand

$$\frac{T_2(0)}{T_2(-1)} + \frac{T_2(0)}{T_2(1)} = 2 + \frac{2}{8}\tau b^2 T_2(0) \qquad (6)$$

involves the addition of the quadratic terms in M. The experimental values for $T_2(o)/T_2(\pm 1)$ are obtained from the square roots of the ratios of experimental derivative curve peak heights. The terms in equations (5 and 6) are defined below.

Definition of Terms and Units

$\nabla\gamma$ is a measure of the anisotropy of the g value,

$$\nabla\gamma = -(\beta/h)\, g_{zz} - \frac{1}{2}(g_{xx} + g_{yy})\, s^{-1} G^{-1}$$

where g_{zz} is the value of g along the z axis.

A measure of the anisotropy of the hyperfine interaction is $b = 4/3\ (A - B)s^{-1}$. A is the hyperfine coupling constant in Hz along the z axis and B is that along the x or y axis since axial symmetry is assumed for the nitroxide label (see introduction to the "ESR spectroscopy of the nitroxide radical" in section 1.3). The hyperfine coupling tensors values and g values of many nitroxides are very nearly the

same [25]. Therefore, the A and B values used to calculate the τ values of the spin-labeled opiate receptors, were taken from the literature [28]. The approximate values for A and B are

$$|A| = 87\ MHz \qquad |B| = 14\ MHz$$

These values for A and B can change slightly (15 %) in going from one nitroxide radical to another nitroxide radical or by changing the polarity of the solvent.

T_2 is a measure of the line width. $T_2^{-1}(o)$, the paramagnetic relaxation rate, is related to the peak-to-peak separation $\nabla\mu$ in gauss for the central hyperfine component of the derivative curve by the equation

$$T_2(0)^{-1} = \pi \sqrt{3}\ \nabla\mu\ 2.8\ 10^6\ rad\ Sec^{-1}$$

The $T_2^{-1}(o)$ value was measured from the experimental derivative curve.

Independent values for τ can be obtained from the linear term (equation 5) and quadratic term (equation 6). In most cases these values are not identical [22,39] and it has been found that the linear term gives values of τ that are quite sensitive to the applied microwave power [50]. It is therefore advisable to use the quadratic term (equation 6) for determination of τ unless a careful study of the microwave power dependence is done.

The calculation of the correlation time τ may be illustrated by reference to the spectrum in Figure 2.2. Experimental values for the ratios $T_2(o)/T_2(\pm 1)$ in equation (6) are obtained from the square roots of the ratios of experimental derivative curve peak heights. A_1, A_o, and A_{-1} are the measured peak-to-peak amplitudes of the low field line, center line, and high field line, respectively. "n" is the amplitude of the peak-to-peak noise as measured from the base line. The error introduced in the measurements of the ratios A_o/A_1 and A_o/A_{-1} due to the presence of noise factors in the spectrum, is

$$\pm \frac{A_1 + A_o}{A_1^2} \times 2n \text{ \& } \pm \frac{A_{-1} + A_o}{(A_{-1})^2} \times 2n, \text{ respectively.}$$

Therefore noise corrections were done on the ratios of the amplitudes of the center line to the low field line and the center line to the high field line for obtaining the correlation time within the limits of percent error from experimental spectrum.

The value of the correlation time τ of the spin-labeled (II) homogenate calculated using the parameters given below is 1.54 ns ($\pm$2.5% of error) from the quadratic term (equation 6). The value of τ for the free spin label II obtained similarly from the electron spin resonance spectrum, is 0.03 ns.

The following values of the parameters were taken from reference 39.

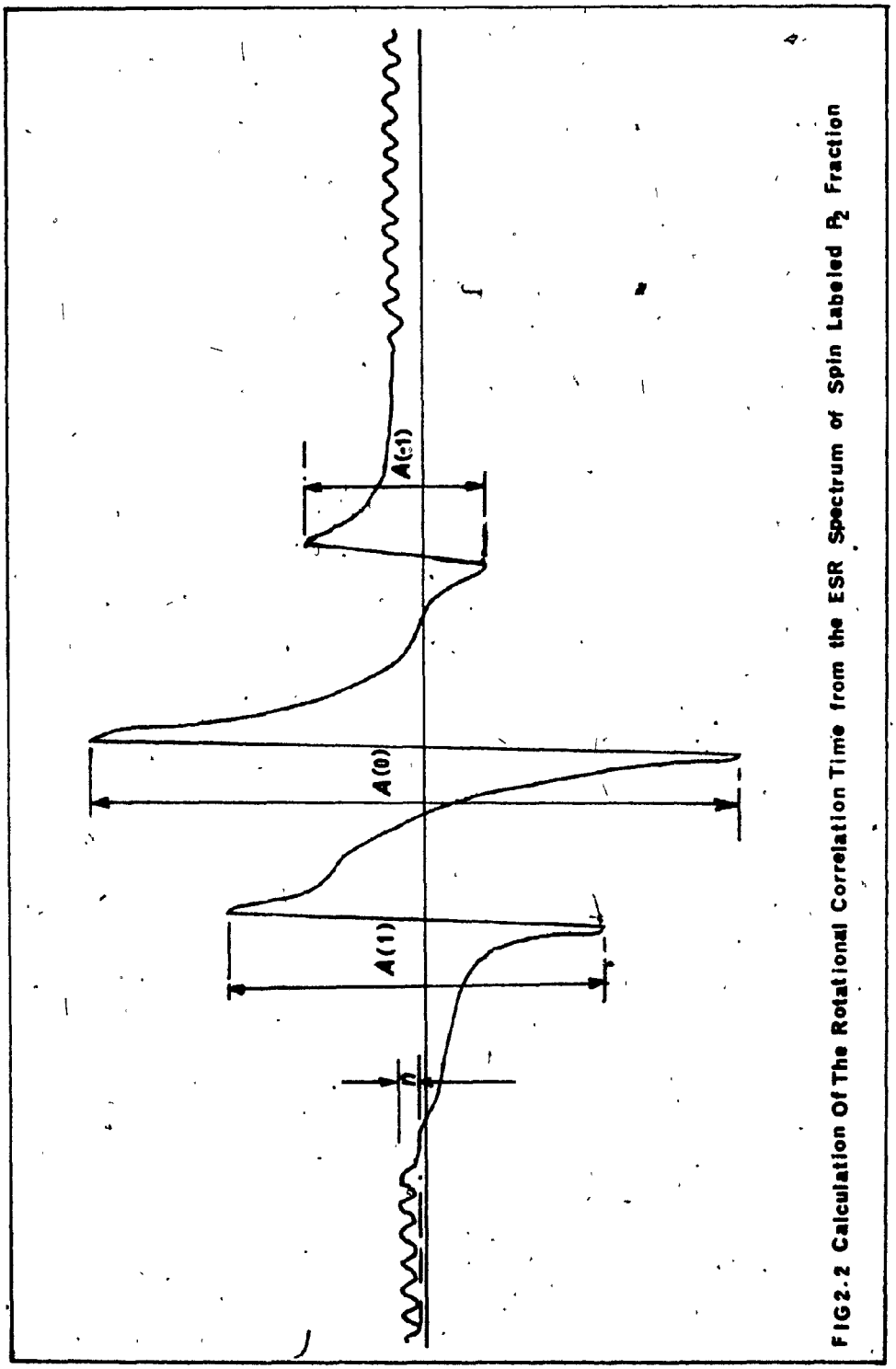


FIG 2.2 Calculation Of The Rotational Correlation Time from the ESR Spectrum of Spin Labeled P_2 Fraction

$h = 1.054 \times 10^{-27}$ erg.s, $\beta = 0.927 \times 10^{-20}$ erg.G^{-1}, g_{zz} = 2.0027, g_{xx} = 2.0089, g_{yy} = 2.0061, A = 87 MHz, B = 14 MHz, b = 3.06 $\times 10^8$ rad S^{-1}, $\gamma = 4.22 \times 10^4 S^{-1} G^{-1}$.

1 gauss is calculated to be equivalent of a line width of 2.8 MHz.

Ao/A_1 = 1.69 ($\pm$ .02) and Ao/A_{-1} = 3.59 ($\pm$ .07) as obtained from the ESR spectrum of the P_2 fraction labeled with spin label II (Figure 2.2). The values in the bracket indicate the error calculated in the respective ratio due to the noise factors.

T_2^{-1} (o) = 30.45×10^6 rad/sec as measured from the spectrum of the spin labeled (II) P_2 fraction.

Calculated rotational correlation times for three different spin labels, e.g., spin label I, spin label II, and spin label (III), attached to the P_2 fractions, are shown in Table 3.1.

2.5 Specific Spin Labeling

To label the P_2 fraction specifically, morphine sites were first protected with an opiate. Non-specific -SH groups were then blocked with unlabeled sulfhydryl reagent, e.g., N-ethylmaleimide or phenyl mercuric acetate. Morphine from specific sites was next washed out by repeated centrifugations and incubations. The P_2 fraction was then labeled with spin label (II) under the usual procedures of labeling as described in section 2.4. Spectrum of the specific spin label was then run.

The concentration, time, and reagents for blocking and protection experiments were based on the literature [15].

i) 5 ml of homogenate of protein concentration $\leq$2 mg/ml was incubated with morphine sulfate solution in tris buffer at a final concentration of 10^{-6}M for 15 minutes.

ii) The homogenates were then allowed to react with a sulfhydryl reagent, e.g., phenyl mercuric acetate (PMA) at a final concentration of 10^{-4}M for 2 minutes. Since PMA was found to be more efficient in blocking the non-specific -SH groups than N-ethylmaleimide as explained in section 3.4.2, PMA was used for this reaction.

iii) Reaction was stopped by centrifugation.

iv) Pellets were resuspended in the original volume of standard buffer, incubated at room temperature for 15 minutes followed by recentrifugation. The incubation and centrifugation were repeated twice. Since the binding of morphine was noncovalent, it could be removed this way, by simple washing.

v) Pellets with the now unprotected receptors were suspended in the same volume of fresh standard buffer followed by incubation with spin label (final concentration 10^{-4}M) for 1 hour. Reaction was terminated by centrifugation.

vi) Unbound spin labels were washed out by two centrifugations. Washing liquids were checked for free spin labels.

vii) Specifically labeled pellets or P_2 fractions were suspended in 0.5 ml standard tris buffer and the ESR spectrum of the specifically labeled P_2 fraction was run under standard instrumental parameters. This means a ten-fold increase in final receptor concentration, allowing greater accuracy in spectral determination. The rotational correlation times of the attached spin labels were calculated from the experimental derivative curves by the methods as described in section 2.4.1.

Stereo specific binding of opiates to the P_2 fraction was determined by radioactive tracer techniques, the methods commonly used in the study of opiate receptor site by the Simon and Snyder groups. These techniques were also used to determine the specificity of labeling as shown in section 3.4.2.

2.5.1 Determination of Specific Opiate Binding

Brain homogenate was prepared as described in section 2.2. Protein concentration of the homogenate was determined. Assays were performed at room temperature for 15 minutes with appropriate amount (^{3}H)-dihydromorphine, using the following procedure.

a) An aliquot of 2 ml of homogenate was incubated with 0.1 ml of 100 nanomolar of morphine for 5 minutes.

b) Another aliquot of 2 ml of homogenate was incubated with 0.1 ml 100 nanomolar of dextrorphan, the (+) isomer of Levorphanol.

Both aliquots (a) and (b) were then reacted with 0.1 ml of 1nM (^{3}H)-dihydromorphine for 15 minutes in the dark (since dihydromorphine is photosensitive). The incubation was terminated by filtration using a millipore filter under gentle suction (using a water aspirator) and the filters were subjected to two washes with 5 ml of buffer at room temperature. The filters were removed with tweezers, placed in vials and kept uncovered overnight to dry. The next day, 2 ml of Triton X-100 was added to each vial, which was then shaken for 30 minutes in a mechanical shaker. 10 ml of scintillation cocktail (17.3 gm of 2,5-diphenyloxazole (PPO) and 167 mg of p-bis [2-(5-phenyloxazolyl benzene (POPOP) in 1 litre of toluene) was then added to each vial, followed by shaking for another 15 minutes. Each vial was counted in a liquid scintillation counter with 50% efficiency.

Since dextrorphan binds to all non-specific sites but cannot bind to stereospecific opiate receptor sites, due to its wrong configuration, whereas morphine binds to both the specific and non-specific sites, the term "specific receptor binding" is used to mean the binding of (^{3}H)-dihydormorphine in the presence of 10^{-7}M dextrorphan (b) minus its binding in the presence of 10^{-7}M morphine. Hence

the specific binding is (b-a) in cpm, (results are shown in Table 3.2).

Specific binding of opiates[2] occurs rapidly at 25 - 37°C and can reach equilibrium by 15 minutes. The ratio of specific to non-specific binding at this time in brain homogenate is between 2.5 and 3.0 to 1. The ratio of specific to non-specific binding should be augmented if an opiate of high specific activity is available, permitting assays with extremely low opiate concentration. The availability of (^{3}H)-dihydromorphine[51] with high specific radioactivity has provided an important tool for studying the interaction of the opiate drug and the binding sites in the receptor. The failure of past attempts[52,53,54] was mainly due to the use of so high a concentration of opiate narcotic that the amount of non-specific binding overshadowed that of specific binding. When an appropriate concentration of an opiate drug is used, only specific binding is detected. The binding constant value for (^{3}H)-dihydromorphine[51] was calculated to be 1×10^{-9}M. As a group dihydromorphine, morphine, levorphanol, naloxone, nalorphine and levallorphan are about equally effective in competition for the binding site[2].

2.5.2 Specific Binding and Protein Concentration

Homogenate protein was always between 0.5 and 2 mg/ml, a region in which binding is proportional to protein concentration [4]. In section 2.5.1, specific binding was determined for a receptor preparation of protein concentration 1 mg/ml.

In this section, protein concentration was increased to see if the specific binding could be increased.

Two homogenate protein concentrations were taken, for example: 7.75 mg/ml of protein and 5 mg/ml of protein, and the specific bindings were determined for them by the methods as described in section 2.5.1 (Table 3.4).

2.5.3 Synaptosome Preparation and Specific Binding of (^{3}H)-Dihydromorphine

The P_2 fraction contains both synaptosome and mitochondria. Synaptosomes were separated from mitochondria by the methods described as follows:

i) The P_2 fraction after preincubation at 37°C for 30 minutes were suspended in 1.3 ml of 0.32M sucrose in tris HCl (pH 7.5).

ii) Homogenates were layered on gradient of 2 ml 1.2M sucrose and 2 ml 0.8M sucrose followed by centrifugation at 100,000 g for 90 minutes in an ultracentrifuge.

iii) The band obtained between the two layers was called the synaptosome fraction. Mitochondria were collected at the bottom.

iv) Layers at the interface (i.e., the synaptosomes) were collected and diluted with equal volumes of 0.32M sucrose in tris buffer.

v) Synaptosomes suspensions were centrifuged at 28,000 rpm for 40 minutes.

vi) Synaptosomal pellets were suspended in tris HCl buffer (pH 7.5) and the protein content was determined.

vii) Stereospecific binding of (^{3}H)-dihydromorphone to snyaptosome fractions was determined by the methods as described in section 2.5.1.

2.6 Determination of Concentration of Bound Spin Labels from Labeled P_2 Fractions

The concentration of spin labels bound to the specific site of the P_2 fraction was determined. The bound spin concentration was also determined when the P_2 fraction was labeled totally, i.e., when neither the specific nor the non-specific sites were blocked by unlabeled sulfyhydryl reagents.

A 5 ml aliquot of freshly prepared P_2 fraction of known protein concentration was labeled with spin label (II) as described in section 2.4.

Another aliquot of 5 ml of known protein concentration was labeled specifically with spin label (II) as described in section 2.5.

Labeled pellets were then suspended in two 0.5 ml volumes of standard buffer. Each suspension was then adjusted to 10% NaOH. Samples were heated to 40 - 50°C in water bath for 20 - 30 minutes and kept overnight. This method allowed the protein structure to be denatured, thereby making the bound spin free. ESR spectra were run after neutralizing the solutions with citric acid since NaOH might have damaged the quartz of the thin-walled aqueous cell.

Triplets characteristic of unbound spin labels were obtained for each of the solutions and the amplitudes of the center lines (now of normal amplitudes, i. e. not broadened) were compared with the height of the center line of the triplet from a pure spin label solution of known concentration under identical instrumental settings.

This method of bound spin concentration determination is an approximate one only, since the amplitudes of the lines of an ESR spectra can vary slightly according to the exact position of the sample cell in the cavity for given instrumental settings. While comparing the ESR spectra cells have to be withdrawn each time from the cavity. This experiment was replicated thrice.

2.7 Ionic Influences on ESR Spectrum of Specifically Labeled P_2 Fraction

Sodium chloride, potassium chloride, manganese chloride, and magnesium sulfate solutions were used for the studies of the sodium effect, potassium effect, manganese effect, and magnesium effect, respectively.

2.7.1 Effects of Na^+ Ion

Freshly prepared P_2 fractions of protein concentration 0.58 mg/ml were labeled specifically with maleimide spin label as described in section 2.5. Labeled pellets were incubated for 30 minutes at 37°C in 0.5 ml of standard buffer containing 0, 5, 25, 50, 100, and 200 mM concentration of Na^+ ions. The ESR spectrum was run for each of the incubations under identical conditions. The rotational correlation time (τ) was calculated from each of the spectra and the values were compared with the value obtained from sample containing no Na^+ ions. Na^+ ions at different concentrations (25 - 200 mM) were also added to the non-specifically labeled P_2 fraction and the rotational correlation values were determined from the spectra after each addition.

For studies of the reversibility of the sodium effect, specifically labeled P_2 fractions were incubated for 30 minutes at 37°C

in 100 mM NaCl in 0.05M tris buffer (pH 7.5). Samples were freed of salt containing buffer by centrifugation at 20,000 g for 15 minutes. Pellets were washed and resuspended in 0.5 ml tris buffer. An ESR spectrum was run and τ values were calculated.

2.7.2 Effects of K^+ Ion

Specifically labeled P_2 fractions were incubated at 37°C for 30 minutes in 0.5 ml of standard tris buffer containing 0, 50, 100, and 200 mM K^+ ions: The ESR spectrum was run for each of the samples under identical conditions. A τ value was calculated from each of the spectra. The experiment was replicated five times.

2.7.3 Effects of Divalent Cations

The ions used were Mn^{+2} ions and Mg^{+2} ions. Similar experiments were carried out with samples containing different concentrations of Mn^{+2} ions, or Mg^{+2} ions. However, each sample contained 100 mM of Na^+ ion in addition to divalent cations. The concentrations used for both Mn^{+2} and Mg^{+2} ions were 1 mM, 0.1 mM and 0.05 mM in presence of 100 mM of Na^+ ions. One blank sample was prepared containing only 100 mM of Na^+ ion but no Mn^{+2} ions or Mg^{+2} ions. For each of the sample ESR spectra were run and rotational correlation times were calculated.

2.8 ESR Studies Using Isothiocyanate Spin Label

A nitroxide spin label containing the isothiocyanate attaching group was used. A solution of 10^{-4}M concentration was prepared

with that spin label and the ESR spectrum was run under standard instrumental settings.

2.8.1 Specific Labeling

Freshly prepared P_2 fraction (protein concentration 0.58 mg/ml) were incubated with morphine sulfate solution of a final concentration of 10^{-6}M in tris buffer for 15 minutes.

To block the non-specific $-NH_2$ or -OH groups, the reaction mixtures were incubated with a freshly prepared 10^{-4}M ethyl isothiocyanate solution for 2 minutes at room tempreature. The reactions were terminated by centrifugation at 20,000 g for 15 minutes. The pellets were resuspended in the original volume of buffer, incubated at room temperature for 15 minutes, and recentrifuged.

The incubation and centrifugation were repeated twice to wash out morphine completely. The pellets were resuspended in the original volume of buffer, incubated with isothiocyanate spin label of final concentration 10^{-4}M for 1 hour at room temperature, and centrifuged. Free spin labels were washed out by two centrifugations. Labeled pellets were then suspended in 0.5 ml of standard buffer. The ESR spectrum was run for each labeled homogenate under standard instrumental settings.

Radioactive tracer techniques as described for maleimide spin in section 2.5.1 were used to verify the specificity of labeling with isothiocyanate spin label.

2.8.2 Determinations of Concentrations of Bound Isothiocyanate Spin Labels

Similar experiments as described for the maleimide spin label in section 2.6 were carried out to determine the bound isothiocyanate spin concentrations in the P_2 fraction labeled with isothiocyanate spin label by the methods as described in the previous section 2.8.1. The experiment was replicated twice.

2.8.3 Effects of Morphine, Sodium and Potassium on Spectra from Isothiocyanate Spin Labeled P_2 Fractions

Effects of 10^{-6}M morphine, 100 mM sodium and 50 mM potassium ions were investigated separately in three different aliquots of P_2 fractions labeled with isothiocyanate spin label under identical conditions. An ESR spectrum was run for each of the samples and τ values were calculated at different time intervals to detect any change in mobility due to additions of morphine or Na^+ ions or K^+ ions when the isothiocyanate label binds to $-NH_2$ groups of the P_2 fraction. Na^+ ions or K^+ ions were added to labeled samples containing morphine to detect sensitivity of the morphine binding to a sodium or potassium effect.

CHAPTER III

RESULTS OF STUDY

3.1 General

This study has concerned mainly the use of spin labeling techniques in the study of the opiate receptor site. Numerous investigations have demonstrated the existence of receptors in the central nervous system which stereospecifically bind opiates [55]. In all such binding studies radiolabeled narcotic agonists or antagonists have been used. In order to study the opiate receptor site, we isolated the P_2 fractions which contain a high concentration of specific receptors, from rat brains. The stereospecific binding of (^{3}H)-dihydromorphine, a potent opiate agonist, to this P_2 fraction preparation was determined by the methods, commonly used by the Simon and Snyder groups, as described in section 2.5.1. Radioactive tracer techniques were used to determine the number of receptors present in the freshly prepared P_2 fraction. Radioactive drug binding studies were also performed to verify the specificity of labeling of the P_2 fraction with spin labels (see section 3.4.2). Since the number of receptor sites is in the picomolar range and the ESR instrument is sensitive up to a

maximum limit of 10^{-9}M, we have tried to increase the numbers of receptors in the P_2 fraction by increasing the concentration of protein in the P_2 fraction (section 2.5.2) and by separating synaptosomes from the mitochondria-synaptosomal fraction, the P_2 fraction (section 2.5.3).

In labeling the receptors with spin labels, the concern of specificity is most important. The P_2 fraction used is a crude preparation, so many non-specific -SH groups may be present besides the specific -SH groups, and therefore there may be interference that might overshadow the changes introduced in the specific sites. To minimize this effect the procedures followed in specific labeling are outlined in section 2.5. A quantitative approach was also made to verify the specificity of labeling by determining the amount of spin labels attached to the specific sites (see section 2.6). The effects of ions on the opiate receptor conformation were studied by ESR techniques using maleimide spin labels. On the other hand, isothiocyanate spin labels were used to study the effects of morphine on the opiate receptor structure since maleimide spin labels eliminate opiate binding.

Results obtained from all those studies are shown in this chapter. In general, studies of the effects of sodium and potassium ions on the ESR spectra of the opiate receptors with maleimide spin probes attached to specific sites, yielded interesting results.

3.2 Spin Labeling of the P_2 Fraction

The ESR spectra of the brain homogenates labeled with maleimide spin label I, spin label II, and spin label III were almost similar in shape.

The ESR spectrum of the spin labeled P_2 fraction as shown in Figure 3.2 is markedly different from that of the free label (Figure 3.1) under similar conditions. The changes in the ESR spectra arise from an increase in the tumbling (or correlation time) time of the label when attached to the proteins of the homogenate [22]. The values of the correlation time τ of the labeled homogenates and of the free labels are given in Table 3.1. Analysis of the ESR spectrum shows that the hyperfine coupling constant (A_o) of the spin labeled homogenate (13.9G) is the same as that of the free label (14G), suggesting that the polarity of the environment of the spin label is almost unchanged on binding to the brain homogenate. This, together with the value of the rotational correlation time obtained for the spin labeled (II) homogenate (1.5 ns) which is much shorter than the tumbling time of the molecule (100 ns) suggests that the spin label is on the surface of the P_2 fractions and has considerable segmental mobility [39].

The value of the correlation time τ, of the spin labelled (II) P_2 fraction calculated from the quadratic term (equation (6), section 2.4.1) is 1.54 ns ($\pm$ 2.5% error). The correlation time for the free nitroxide radical (II) obtained similarly from the ESR spectra at the same magnetic field, is 0.035 ns ($\pm$ 12.5% error). The basis of the calculation is outlined in section 2.4.1.

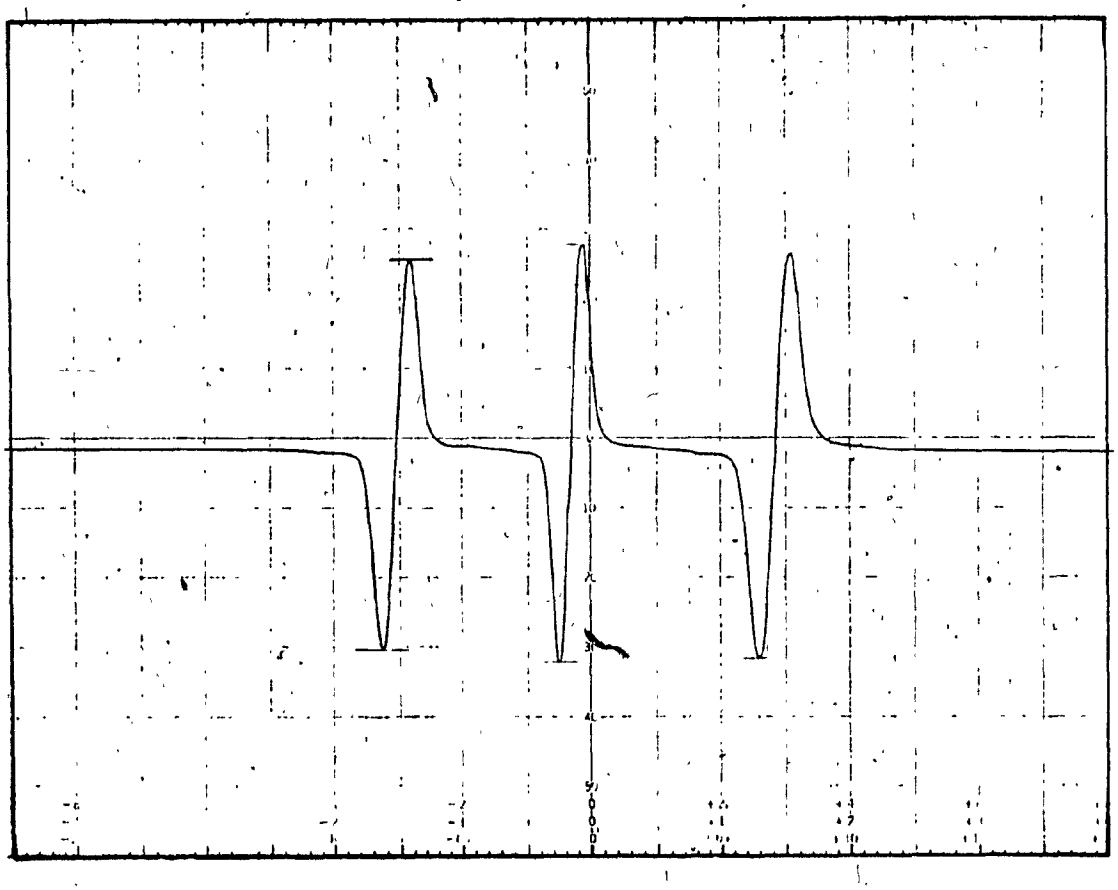


FIG 3.1 The ESR Spectrum Of Free Spin Label II (100 μM) in Tris Buffer (pH 7.5)

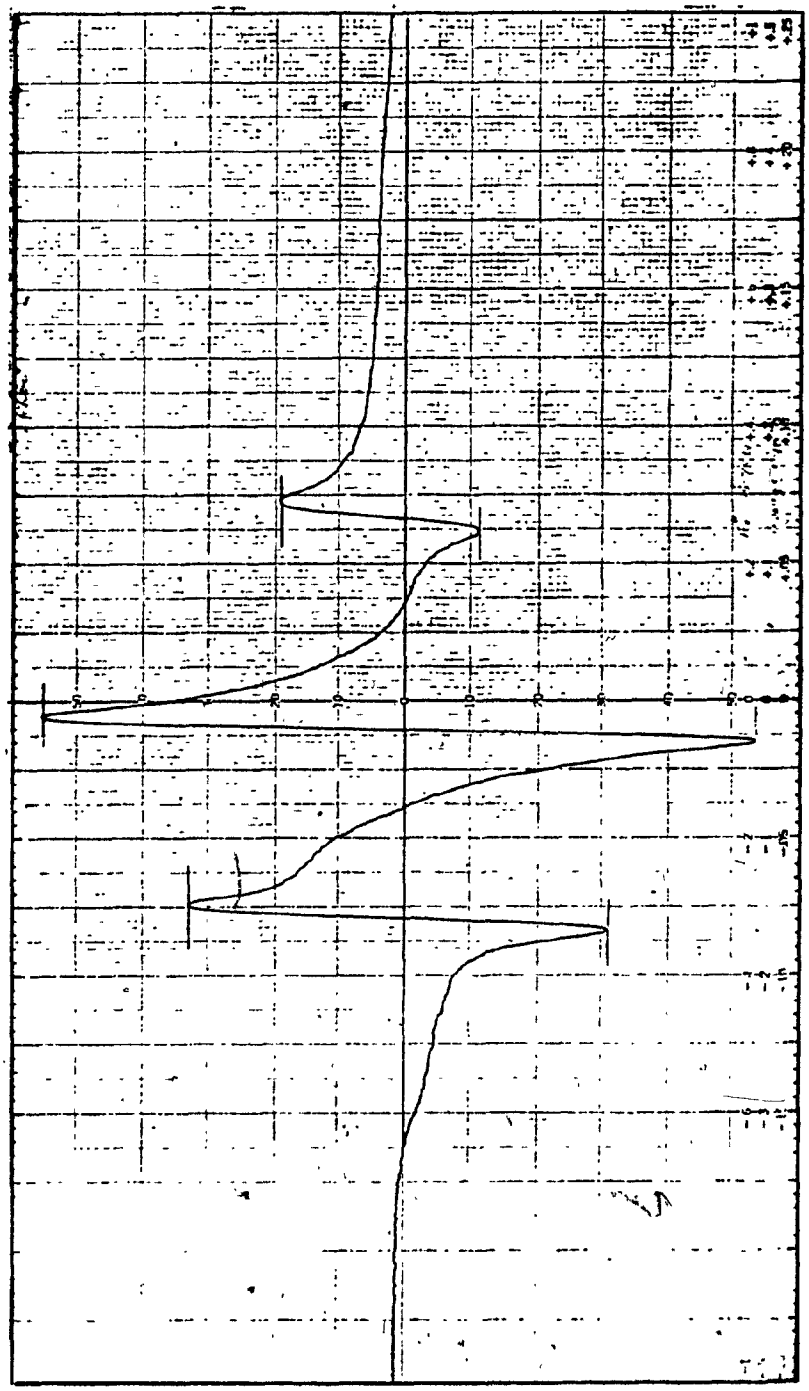


FIG 3.2 The ESR Spectrum Of Spin Labelled (II) P_2 Fraction

Table 3.1

Correlation times for spin-labeled P_2 fraction

The correlation times (τ) were calculated from equation (6) as described in section 2.4.1. τ values are expressed as the mean of all the determinations.

Sample	τ Values	Percent error
Free label I	0.04 ns	± 6.0%
Free label II	0.03 ns	±12.5%
Free label III	0.03 ns	±16.0%
Spin-labeled (I) P_2 fraction	2.51 ns	± 3.0%
Spin-labeled (II) P_2 fraction	1.54 ns	± 2.5%
Spin-labeled (III) P_2 fraction	0.62 ns	±11.0%

P_2 fraction with maleimide spin labels (10^{-4}M) in 50 mM tris buffer (pH 7.5) reacted for 1 hour at room temperature, washed, and centrifuged two times and finally concentrated into 0.5 ml tris buffer.

The spectrum of freshly prepared P_2 fraction without the label was run under the same instrumental settings. No peaks, but only a base line was obtained even after accumulating the ESR spectra for 25 scans indicating that the P_2 fraction did not contain any paramagnetic center of its own. Hence the ESR peaks obtained in the spectrum of the labeled P_2 fraction were only due to the attached spin labels used for labeling.

3.3 Specific Labeling

The spectrum obtained from the specifically labeled P_2 fraction (Figure 3.3 [a]) looks like the spectrum obtained for a labeled P_2 fraction when specificity is not concerned. The spectrum for the specifically labeled P_2 fraction shows a much lower spin concentration (obtained at a higher receiver gain) since a large proportion of the non-specific-SH groups are being blocked by PMA; and the previously protected specific sites are occupied by the spin labels. Although the spin labels now bind to the opiate sites specifically, there are certainly some residual non-specific labels which cannot be abolished completely. The calculated mean rotational correlation time of the spin label attached to the specific sites of the P_2 fraction, is 0.48 ns (S.D. $\pm$ .1) indicating that the specifically attached spin label II has considerable mobility.

3.4 Radioactive Tracer Techniques in the Study of Receptor Site

1 nanomolar (10^{-9}M) (^{3}H)-dihydromorphine was used to carry out the following experiments. The methods followed are described in the section 2.5.1.

3.4.1 Determination of the Number of Available Receptors

A stereospecific binding experiment with (^{3}H) dihydromorphine was done in five sets. Incubation of the P_2 fraction of protein of concentration 1 mg/ml, with (^{3}H)-dihydormorphine of high specific activity at concentrations around 10^{-9}M results in significant binding (Table 3.2), much of which is stereospecific as defined by Goldstein, et al.[22], i.e., it is prevented by morphine but not by dextrorphan, the inactive (+) isomer of levorphanol.

As shown in Table (3.2), 540 Cpm is the specific binding of (^{3}H)-dihydormorphine per mg of protein in the P_2 fraction. Specific binding represents 0.084 picomoles/mg protein for the P_2 fraction. Hence these P_2 fraction preparations specifically bound about 0.84×10^{-13} moles/mg protein. If it is assumed that one molecule of (^{3}H)-dihydromorphine occupies one binding site, multiplication with Avogadro's number gives a value of 5×10^{10} binding sites per mg of protein in the P_2 fraction.

Table 3.2

Determination of stereospecific binding of (^{3}H)-dihydromorphine (DHM) and the calculation of number of available receptors

2 ml of the P_2 fraction (1 mg/ml of protein) were allowed to react with (^{3}H)-dihydromorphine (1 nanomolar), 14000 cpm radioactivity, assayed as described in section 2.5.1. The concentration of the unlabeled drugs (morphine and dextrorphan) was 10^{-7}M. Results of a representative experiment are given as the mean of triplicate determinations which varied less than 10%. Specific binding is expressed as the mean ± S.D. of five separate determinations.

Experiment No.	(^{3}H)-DHM binding in presence of Dextrorphan (b) in cpm	(^{3}H)-DHM binding in presence Morphine (a) in cpm	Specific (^{3}H)-DHM binding (b-a) in cpm	Mean specific binding ± S.D. in cpm	Specific binding (cpm)/ mg protein	No. of available receptors/ mg protein
1	1710	748	961	1080±103	540±51	≈0.084 picomoles
2	2022	811	1211			
3	1868	751	1117			
4	1815	826	989			
5	1870	750	1120			

The concentration of specific opiate receptors reported here (.09 picomoles/mg protein) is quite consistent with the values determined previously [2,4,51] using radioactive labels (0.1 picomoles/mg protein). The present findings and those reported earlier lend strong support to the existence of specific receptors for opiate narcotic in nervous tissues. The receptor sites are mainly located in the synaptosomal membrane of the P_2 fraction and have a density of approximately 5×10^{10} sites per mg protein. P_2 fractions were prepared fresh throughout the investigation though the specific bindings were not significantly reduced when homogenates were maintained at -20°C for 3 weeks.[2]

3.4.2 Verification of Specificity of Labeling

Radioactive tracer techniques were used to see whether specific labeling is achieved in our experiments. The whole method can be divided into five sets, as shown in Table 3.3. The samples from the first set or control experiment were treated exactly the same way as described in section 2.5.1 for determining the number of stereospecific binding sites. The number of receptor sites were calculated as 0.72×10^{-13} moles/mg protein, the value is in good agreement with the value obtained in section 3.4.1.

In the second set around 0.087×10^{-13} moles/mg protein concentration has been found to be the number of morphine sites still available after treatment with phenyl-mercuric acetate for 2 minutes since phenyl-mercuric acetate reduces the specific binding by about 88%,

it can be said that one or more -SH groups are located on the surface of the opiate receptors and are responsible for binding of opiates. When the P_2 fraction is treated with ethylmaleimide, another sulfhydryl reagent, at the same concentration and for 2 minutes, the number of morphine receptors available for binding has been found to be around 33% of control, meaning that it is a less efficient blocking agent. Hence treatment with ethylmaleimide reduces specific binding by about 67% of the control so it was decided to use PMA as the blocking agent.

In the fourth set of experiments, the phenyl mercuric acetate inhibition of a specific binding was considerably reduced by preincubation with morphine, suggesting that specific sites are protected by opiates. Hence the protection experiment will leave all the morphine sites available for labeling with the spin label used.

In the fifth set when the homogenate was reacted with spin label II for 1 hour after protection experiment, the specific binding of (^{3}H)-DHM was found to be only 2% of the control. Hence the specific binding has been reduced by 98% of the control; that means the specific binding is almost negligible. This proves that spin labels can occupy about 98% of the total morphine sites present, or more precisely mostly all the specific sites have been labeled, though the possibility that the spin label II may bind to various non-specific sites in addition to the specific sites cannot be ruled out.

Table 3.3

Verification of specificity of labeling

2 ml P_2 fraction (protein concentration 1 mg/ml) aliquots were taken for each set of experiment. Samples from set 2 and set 3 were reacted with PMA (10^{-4}M) or ethylmaleimide (10^{-4}M) for 2 minutes, respectively. Reactions were stopped by centrifugation. Samples from set 4 and set 5 were incubated with morphine (10^{-6}M) for 15 minutes after which they were allowed to react with PMA (10^{-4}M) for 2 minutes. The homogenates were centrifuged at 20,000 x g for 15 minutes. The pellets were resuspended in the original volume of standard buffer incubated at 37°C for 15 minutes and recentrifuged. The incubation and centrifugation were repeated twice. The pellets were resuspended in original volume and assayed as described in section 2.5.1. Assays were performed with 1 nM (^{3}H)-DHM. Values are the means of duplicate determinations. The experiment was replicated twice. Percentage values in the last column were determined by comparing specific binding of the treated samples with the specific binding of unreacted tissue, i.e., control experiment.

Set No.	Experiment	Specific Binding cpm/mg (± S.D.) Protein	Morphine Receptor Concentration	Binding %
1	Control Experiment	473 ± 40	0.072 picomoles /mg protein	100%
2	Blocking with PMA for 2 min.	55 ± 10	0.0087 picomoles /mg protein	12%
3	Blocking with EM for 2 minutes	154 ± 12	0.0242 picomoles /mg protein	33%
4	Protection Experiment	508 ± 20	0.0799 picomoles /mg protein	107%
5	Labeling with Spin Label II for 1 hour	9 ± 5	0.0014 picomoles /mg protein	2%

3.4.3 Specific Binding and Protein Concentration

The purpose for this experiment was to find out whether the concentration of specific sites can be increased when the protein concentration of the homogenate is increased. Results are shown in Table 3.4, from which it appears that binding is not increased at all, in fact the binding has been reduced when the protein concentration is high. The reasons causing that reduction in a number of receptors are not really known. The one explanation may be that an increase in protein masses may slow down the filtration time by clogging the filter pores during the assays with (^{3}H)-dihydromorphine. The longer the filtration time the more chances of washing out bound (^{3}H)-dihydromorphine from the specific sites of the receptors, thereby resulting in reduction in the number of receptor sites. The result of this experiment is not consistent with the binding process, suggesting some limitations in our assays with (^{3}H)-dihydromorphine.

Table 3.4

Specific binding and protein concentration

2 ml aliquots of P_2 fraction were taken and assayed as described in section 2.5.1 with 1 mM (^{3}H)-DHM. Values are given as the mean ± S.D. of triplicate determinations.

Protein Concentration mg/ml	Specific Binding cpm	Specific Binding /mg Protein	Concentration of Morphine Receptors
7.75	1734 ± 120	111.8 cpm/mg Protein	0.17×10^{-13} moles/mg Protein
5.0	1277 ± 90	127.7 cpm/mg Protein	0.20×10^{-13} moles/mg Protein
1.0	1080 ± 103	540.0 cpm/mg Protein	0.84×10^{-13} moles/mg Protein

3.4.4 Synaptosomes and Stereospecific Binding of (^{3}H)-dihydromorphine

The P_2 fractions prepared contain both mitochondria and synaptosomes. Mitochondria are relatively inactive in binding of (^{3}H)-dihydromorphine whereas the specific binding has occurred mainly on the synaptosomal membranes. Therefore, one might think that the presence of mitochondria in the P_2 fraction may result in the reduction in the number of specific binding sites. Synaptosomes, freed from mitochondria, were prepared, and the specific binding was determined to see if the presence of mitochondria in the P_2 fraction could interfere with the specific binding of (^{3}H)-dihydromorphine.

Our results for specific binding in synaptosomes were too low and not very reliable since a portion of the synaptosomes passed through the GF/F filters, the smallest pore size available, during filtration resulting in a considerable decrease in the number of receptors. On studying thoroughly the various results obtained by various workers for specific binding it is concluded that contamination of mitochondria with synaptosomes in the P_2 fraction does not really cause any reduction in specific binding. It might have been perhaps profitable to use isolated synaptosome membranes as a means of concentrating receptor sites. The very low yield of such membranes, however, made this approach entirely impractical as it is expensive and time-consuming.

We could, however, increase the concentration of labeled receptors, once the label was covalently bound to the P_2 fraction. A ten-fold increase used in our standard procedure (see section 2.5) therefore makes our data much more credible, as otherwise the concentrations we had to measure would have been at the borderline of detectability on the instrument we had to use.

3.5 Determination of Bound Spin Concentration

The methods used to determine the bound spin concentration after specific labeling as well as after labeling for specific plus non-specific sites, are outlined in section 2.6. When the homogenate is treated simply with spin label as described in section 2.4, the label will be able to bind on all sites both specific and non-specific; therefore, the concentration determined will be the total bound spin concentration. But if the homogenate is spin labeled specifically as described in section 2.5, the method of concentration determination will indicate the amount of spin that goes on specific sites only. Results obtained for spin concentration determination are shown on Table 3.5. In order to obtain measurable spectra, the label was freed by treatment of the sample with NaOH. The spectrum becomes a triplet characteristic of freely mobile spin rapidly tumbling in solution as in

Figure 1.2. The heights of the two middle peaks of two spectra were determined and were compared with those obtained from a series of pure spin label solutions of known concentrations under the same instrumental settings.

The concentrations of pure spin label II solutions measured were 1×10^{-4}M, 4×10^{-6}M, 2×10^{-6}M and 2×10^{-7}M. From the amplitudes of the lines of those spectra it has been shown that the signal height (SH) was directly proportional to the concentration at a particular receiver gain (Rg). It has been shown that at a particular concentration signal height is directly proportional to receiver gain. If both receiver gain and concentration vary then signal height is jointly proportional to concentration and receiver gain. The proportionality constant (K) (see table 3.5) had been determined from the mid-signal heights of different spin solutions of various concentrations. When determining the signal heights standard deviations due to noise factors introduced at different times and due to different positions of the cell in the cavity are taken into consideration. Standard deviation (SD) is calculated as 1.72 for the mean peak height of 21.26. Since the SD is high for low signal heights, it is more useful to consider the greater heights obtained at higher receiver gain when the proportionality constants are determined.

The mean value of proportionality constant (K) is calculated as 17.11 ± 1.5 (SD) cm lit/mole. Mid-peak heights are determined from labeled homogenate and specifically labeled homogenate after treatment with NaOH are are expressed as 19.2 cm and 11.02 cm, respectively. The data (Table 3.5) are the means of triplicate determinations. Instrumental parameters except receiver gain are kept constant throughout the experiment. Concentrations determined are (1.47 ± 0.26) x 10^{-5}M/mg of protein and (3.75 ± 0.49) x 10^{-7}M/mg of protein for total amount of spin attached and specific amount of spin attached, respectively. This experiment was replicated three times.

The number of receptors calculated from the binding experiment with (^{3}H)-dihydromorphine is in the range of 10^{-10}M/mg of protein. But the value determined for the amount of specific spin attached contradicts that value. The attached spin concentration is much higher than the expected specific value so it can be said that the value obtained for specifically bound spin contains the specific binding as well as some non-specific binding too, that means there are still some non-specific sites which are not blocked by phenyl-mercuric acetate (PMA). But in that case non-specific binding is 98% less than that obtained in the case of labeling homogenate without pretreatment with morphine and PMA, i.e., when the homogenate is labeled non-selectively. Non-specific binding cannot be cut down completely since the spectrum would not possibly

Table 3.5

Spin concentration determination

Middle peak heights were determined from the triplet obtained after treatment with NaOH to labeled homogenate as described in section 2.6. Data are the means of triplicate determination. Experiment was replicated twice. Standard deviation calculated include the combined effects of noise introduced at different times and for different positions of the cell in the cavity (± 1.73 cm for the mean peak height of 21.26 cm). Proportionality constant K was determined from middle peak heights of different solutions of pure spin label of known concentration since signal height ∝ receiver gain x concentration when both concentration and receiver gain vary. K = signal height/ receiver gain x concentration.

	Number of Experiment	Protein concentration mg/ml	Middle peak height cm	K cm. lit /mole	Receiver gain	Bound spin conc. = signal height/receiver gain xK/ mg protein
Total spin concentration	1	4.77	18.90 ± 1.5	17.11 ± 1.5	3.2×10^3	$(1.47 \pm 0.26) \times 10^{-5}$M
	2	4.77	19.50 ± 1.6	17.11 ± 1.5	3.2×10^3	
Specific spin concentration	1	1.70	10.70 ± 0.9	17.11 ± 1.5	2.0×10^5	$(3.76 \pm 0.49) \times 10^{-7}$M
	2	1.70	11.30 ± 0.9	17.11 ± 1.5	2.0×10^5	

be detectable by the instrument. The spectrum for only the specific sites, i.e. in the range of 10^{-10} will be theoretically impossible to be detectable by the ESR instrument since it is sensitive up to 10^{-7}M (10^{-9}M on signal averaging) of spin concentration to the maximum limit.

Major discrepancy in comparing the number of opiate receptors by (^{3}H)-dihydromorphine binding as described by Synder, et al. and spin label binding is quantitative. Specific binding of (^{3}H)-dihydromorphine amounted to about 10^{-9} - 10^{-10} M/mg of protein, while spin labels were shown to be present in concentrations of 10^{-7} M/mg of protein. There are two possible explanations for this discrepancy.

(1) The determination of dihydromorphine binding is not accurate, even though in agreement with literature data. The fact that the number of binding sites is not proportional to protein concentration (see Table 3.4) points to this possibility. Normally one would expect to be able to determine number of binding sites regardless of their concentrations. The failure of this in the morphine receptor site determination casts doubt on the accuracy of the determination between 0 - 2 mg of protein as well, and it could, in fact, be much higher.

(2) We know from our own experiments as well as those of Snyder, et al., that it is not possible to protect all receptor sites with morphine or occupy all other sites with N-ethylmaleimide or phenyl-mercuric acetate and therefore there is considerable overlap between

"specific" and "non-specific" spin labels. It is conceivable therefore, that our "specific" labels are in fact a mixed population, some on, some near the receptor site that undergoes conformational change in the presence of NaCl. In addition, there is no way of deciding how many spin labels attach on each opiate receptor; the number is not necessarily one label per site. It is therefore quite likely that only a certain population of the "specific" labels are in fact specific. Therefore, while the total spin concentration is 100 times higher than expected on the basis of (^{3}H)-dihydormorphine binding, only a fraction of these reflect the conformational transition taking place, and show immobilization. In fact, the spectral changes observed are relative small (see Table 3.6). Our change between 0.5 and 1.2n sec of τ value (rotational correlation time) is equivalent to a viscosity change between 6.6 and 11.5 centipoise, which is equivalent to an increase from 50% glycerol to 60% glycerol. A "powder spectrum" corresponds to about 110n sec or 900 centipoise or 98% glycerol, keeping in mind, that the absolute viscosity scale and τ show a linear double logarithmic correlation. This just serves to illustrate that our observed changes move on a very narrow position of the rotational correlation scale observable in our experimental arrangement. This also shows that an immobilization of a large proportion of spin labels would result in larger observable change; or, that a small fraction of the attached spin labels undergoes a restriction in motion.

Since the spectra we observe are the average of all labels present, it is obvious that our observed τ values are the composite of all populations, those that react strongly and are immobilized and those that perhaps show no change in mobility at all. The truly specific labels may therefore undergo a much stronger immobilization than indicated by the τ values, but by the same token may be numerically the same order of magnitude as the number of receptor sites indicated by the (^{3}H)-dihydromorphine binding data. The separation of these populations of spin labels would be possible only by line shape analysis, a technique that was not possible with the instrumentation at our disposal.

The foregoing speculation should therefore be taken as a caution in the interpretation of our data, which ought to be considered approximate only, until further refinement.

3.6 Conformational Change Introduced by Ions

The change in conformation, if introduced by ions, can be studied in the presence of spin label by means of ESR spectroscopy. Since the ESR spectrum of a spin label is sensitive to the mobility of the label it can provide direct evidence of a conformational change in a macromolecule. The main part of this research was to see if there is any conformational change introduced in the opiate receptor in the presence of Na^+, K^+, Mn^{+2} and Mg^{+2} ions.

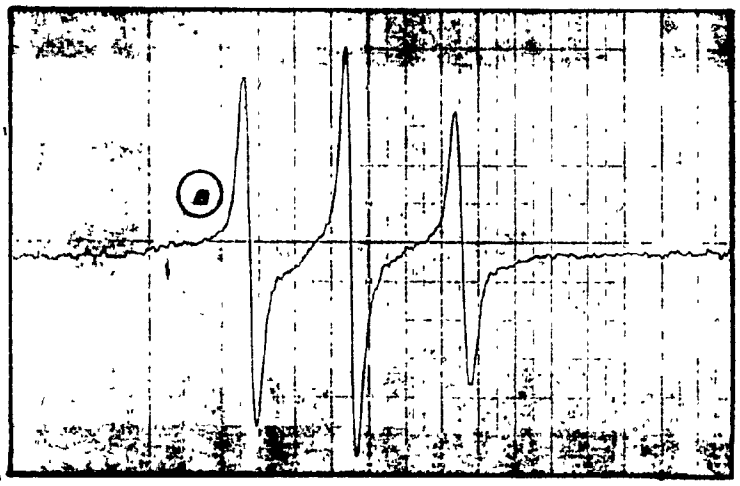
It has been assumed that Na^+ ions might affect the conformation of opiate receptors so that they will have different affinities for antagonists and agonists. In two different conformations, one "no-Na state" and one "Na state", the spin label is supposed to have different rotational correlation times or mobilities in the absence and in the presence of Na^+ ions respectively.

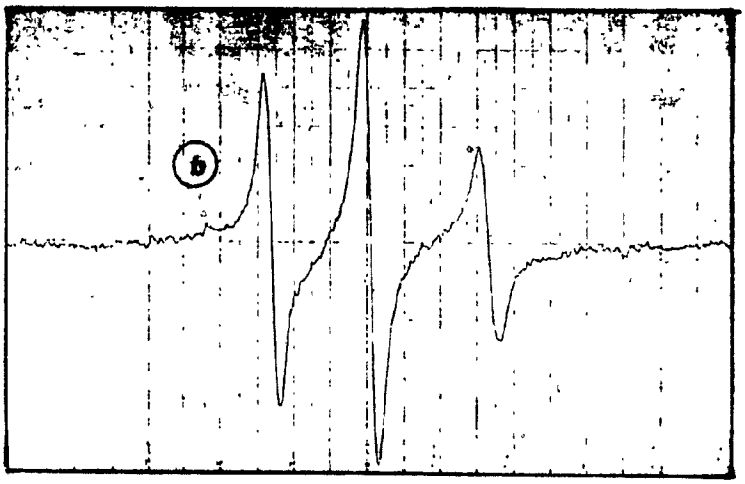
Since K^+ ion has no effect on the binding of agonists and antagonists, it is expected that the addition of K^+ ion in various concentrations will not cause any change in the τ values.

According to the literature[14] the effects of divalent cations are the reverse. So one might expect that the change in τ value in the presence of Mn^{+2} or Mg^{+2} ions would be in a direction opposite to that obtained in the presence of Na^+ ion.

3.6.1 Effects of Na^+ Ions on the ESR Spectrum

Addition of Na^+ ions causes a marked change in the ESR spectrum of specifically labeled P_2 fraction (Figure 3.3). The effect is most noticeable on the high field line while the center component remains almost unchanged. In the presence of 100 mM NaCl, the correlation time is increased to 1.12 ns (SD $\pm$ .18) from 0.48 ns (SD $\pm$ 0.1) as indicated in Table 3.6. This provides direct evidence of Na^+ ion induced conformational change in the opiate receptor. Although the amplitude of the lines of an ESR spectrum can vary according to the exact position of





(a) Specifically Labelled (II) P_2 Fraction Alone, Protein (0.6 mg/ml)

(b) With 100 mM Nacl in 50 mM Tris Buffer (pH 7.5) at Room Temperature

FIG 3.3 The Effect Of Na^+ ion On The *ESR* Spectrum Of Specifically Labelled P_2 Fraction

the sample cell in the cavity for given instrumental settings, the relative amplitudes of the three lines in the spectrum will remain unchanged. For this reason the ratios of two lines (the center to the low field, and the center to the high field) have been used to characterize the spectrum of a given sample. The ratio, the center to the high field is increased from a value of 1.48 (S.D. $\pm$ .11) to 2.31 (S.D. $\pm$ .3) by the addition of 100 mM Na^+ to the specifically labeled homogenate. The change in ratio can be used as a measure of the Na^+ ion induced conformational change in the opiate receptor (i.e., a measure of the function of state). So 1.48 represents one state which is called "no sodium state" or "agonist-conformation" and 2.31 value represents "sodium state" or "antagonist conformation".

Hence the two states may be described as follows:

$$\underset{1.48\ (\pm\ .11)}{\text{"No Na State"}} \underset{-Na^+}{\overset{+Na^+}{\rightleftharpoons}} \underset{2.31\ (\pm\ .3)}{\text{"Na State"}}$$

The change in high field to center line is more significant than the change in the ratio of low field to the center, hence it is more useful to consider the former ratio as a measure of the function of state.

Effects of Na^+ ions were studied at five different concentrations 0 mM, 5 mM, 25 mM, 50 mM, 100 mM and 200 mM. This experiment was

repeated ten times. The details of the results are shown on Table 3.6. Figure 3.4 shows a plot of the mean values of rotational correlation time values (τ) against the concentration of Na^+ ions, and Figure 3.5(a) shows a plot of the extent of the conformational change, i.e., the percentage of maximal change in the τ value as a function of the concentration of Na^+ ions, and compares this with the percentage of maximal change obtained in (^3H)-naltrexone binding (Fig. 3.5(b)) in the presence of Na^+ ions at the same concentration range as studied by Simon, et al. [12]. The data for the plot of Figure 3.5 (b) were taken from the literature [12].

Addition of Na^+ ion causes an increase in the rotational correlation time value. The increase is a maximum for 100 - 200 mM of Na^+ ions. A 200 mM concentration value is considered as the maximal concentration of Na^+ ions to produce maximal amount of antagonist conformation, i.e., about 100% of the states are in the antagonist state or the maximum immobility of the label is introduced. Na^+ ion can exert its effect from concentration as low as 5 mM, and the effect increases as the concentration of Na^+ ion is increased. In the range between (25 - 200) mM the rotational correlation time is almost constant, i.e., the mobility is more or less the same within the concentration range from 25 - 200 mM of Na^+ ions.

The results are in agreement with the literature data (as shown in Figure 3.5) obtained from the (^3H)-naltrexone binding studies

Table 3.6

Effects of Na^+ Ions on the ESR Spectra of Specifically Labeled P_2 Fraction

Spectral parameters were calculated as described in section 2.4.1. $T_2^{-1}(0) = 38.07 \times 10^6$ rad sec^{-1} as measured for each spectrum. Protein concentration = 0.58 mg/ml. Total protein = 2.9 mg. Spin label II on specific sites. Final volume = 0.5 ml. Additives = NaCl solution at different concentrations. Data are the means of five determinations. Values in the bracket indicate standard deviations. Noise corrections were included in the standard deviations for each line ratio measured.

Concentrations of NaCl solutions added mM	Spectral Parameters			Percentage of maximal change in the τ value
	Line ratios A_0/A_1	Line ratios A_0/A_{-1}	Rotational correlation time (τ) ns	
0	1.15 (± .09)	1.48 (± .11)	0.48 (± .1)	0%
5	1.21 (± .12)	1.76 (± .2)	0.69 (± .16)	35%
25	1.35 (± .1)	2.14 (± .26)	1.08 (± .15)	85%
50	1.38 (± .09)	2.33 (± .23)	1.13 (± .14)	90%
100	1.36 (± .12)	2.31 (± .3)	1.12 (± .18)	94%
200	1.37 (± .12)	2.41 (± .3)	1.16 (± .18)	100%

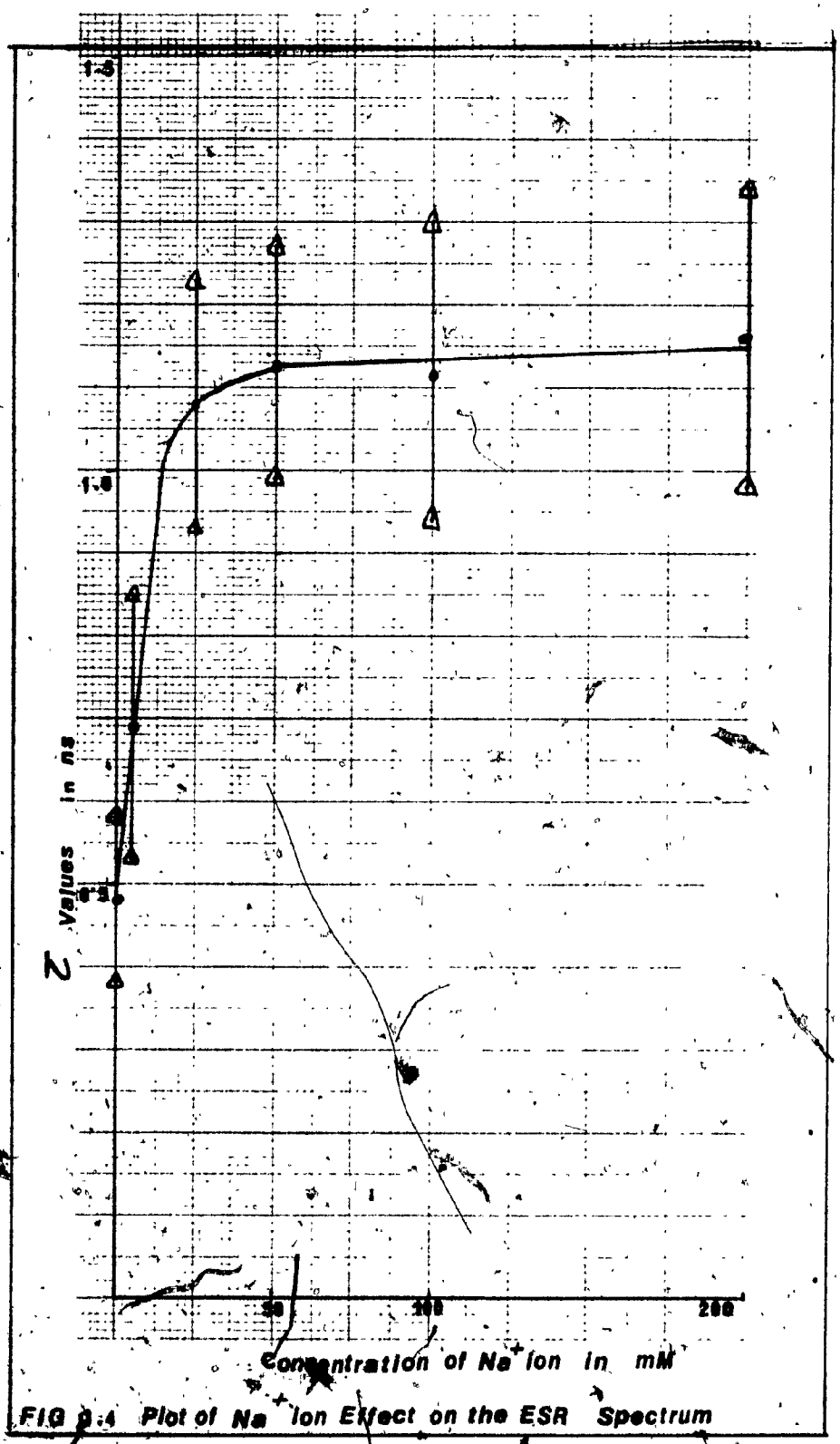


FIG 3.4 Plot of Na^+ ion Effect on the ESR Spectrum

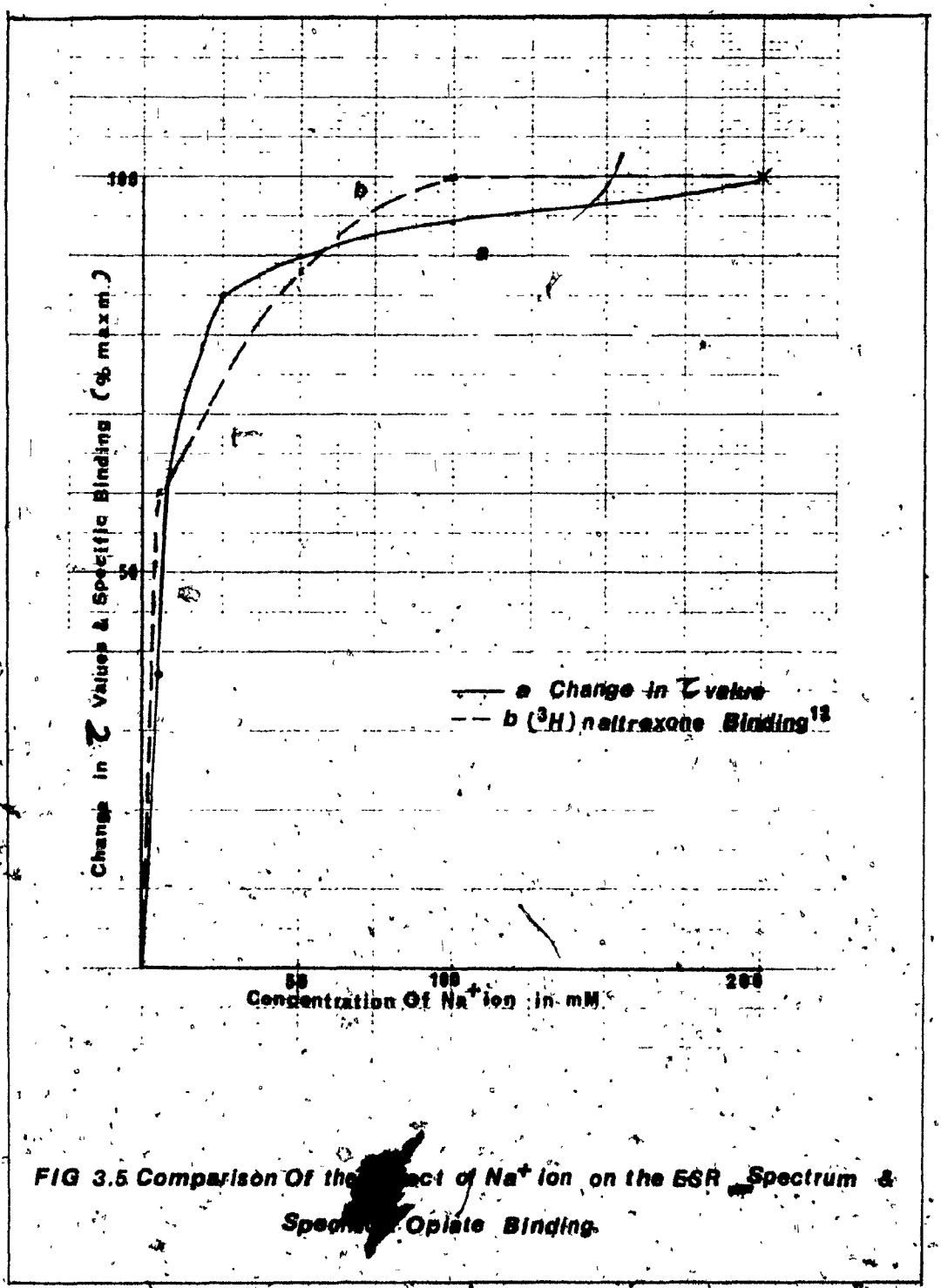


FIG 3.5 Comparison Of the [illegible]ect of Na^+ ion on the ESR Spectrum & Specific Opiate Binding.

on the nature of the sodium effect, confirming that an increase in antagonist binding in the presence of sodium is due to a conformational change in the opiate binding sites so that the transformed sites exhibit greater affinity for antagonists.

P_2 fractions were labeled with spin label II and the electron spin resonance spectra were run. τ values were calculated from the spectra. Na^+ ions at a concentration range (25 - 200 mM) were added to the P_2 fraction labeled non-selectively. τ values were determined from the spectra after each addition. But no change in τ value has been found, even at concentrations as high as 200 mM of Na^+ ion, that means the mobility of the spin label remains almost the same (1.54 ns) in contrast to the finding that Na^+ ion causes a change in mobility of the spin label when P_2 fractions are labeled specifically.

Ion effects are generally reversible. It was shown that the effects produced by the maximum concentration of Na^+ ions (100 mM) could be reversed when the Na^+ ions were removed from the incubation medium. If the Na^+ ions are removed by centrifugation and washing the rotational correlation value becomes 0.55 ns. Hence the effects produced by Na^+ ions are reversed since the τ value decreases from the value 1.12 ns obtained by adding 100 mM Na^+ ions to the initial value of about 0.55 ns.

3.6.2 Effects of K^+ Ion on the ESR Spectrum

The effects of K^+ ions were investigated on four specifically labeled samples with different concentrations of K^+ ions added: i) 0 mM; ii) 50 mM; iii) 100 mM; and iv) 200 mM of K^+ ion. The experiment was repeated five times. Results are shown on Table 3.7.

Addition of K^+ ion does not cause any marked changes in the ESR spectrum of spin labeled homogenate. Relative amplitudes of the three lines in the spectrum remain almost unchanged in the presence of different concentration of K^+ ion and also in the absence of K^+ ions. Ratios of the low field to center or the high field to center, that represent the function of state, remain more or less constant in the presence of K^+ ions. The changes obtained are very small and within the limits of standard deviation.

The rotational correlation value obtained for the spin labeled homogenate without K^+ ion is 0.53 ($\pm$ .1) ns, almost the same value (0.48 $\pm$ .1) ns as obtained for τ without ions, while studying the Na^+ ion effect. No change in the value of the rotational correlation time is obtained for specifically labeled homogenate in the presence of K^+ at any concentration. A plot of τ values as a function of concentration of K^+ ions is shown in Figure 3.6.

Table 3.7

Effects of K^+ ion on the ESR spectrum of specifically labeled P_2 fraction (protein 0.6 mg/ml)

Experimental conditions: Total protein = 3 mg, spin label II on specific sites, final volume = 0.5 ml. Data are the means of five determinations. The experiment was repeated five times. Values in the bracket indicate standard deviations. Spectral parameters and noise factors were calculated as described in section 2.4.1 $T_2^{-1}(0) = 38.07 \times 10^6$ rad/sec as measured for each spectrum.

Concentrations of KCl solutions added	Spectral Parameters			Remarks
	Line ratios A_o/A_1	Line ratios A_o/A_{-1}	Rotational correlation time (τ) ns	
0	1.21 (± .13)	1.52 (± .17)	0.53 (± .1)	No change in the value of τ in the presence of K^+ ion up to concentration 200 mM. Changes obtained are within the standard deviation.
50	1.18 (± .1)	1.55 (± .16)	0.54 (± .1)	
100	1.16 (± .14)	1.54 (± .21)	0.51 (± .16)	
200	1.19 (± .15)	1.57 (± .25)	0.55 (± .14)	

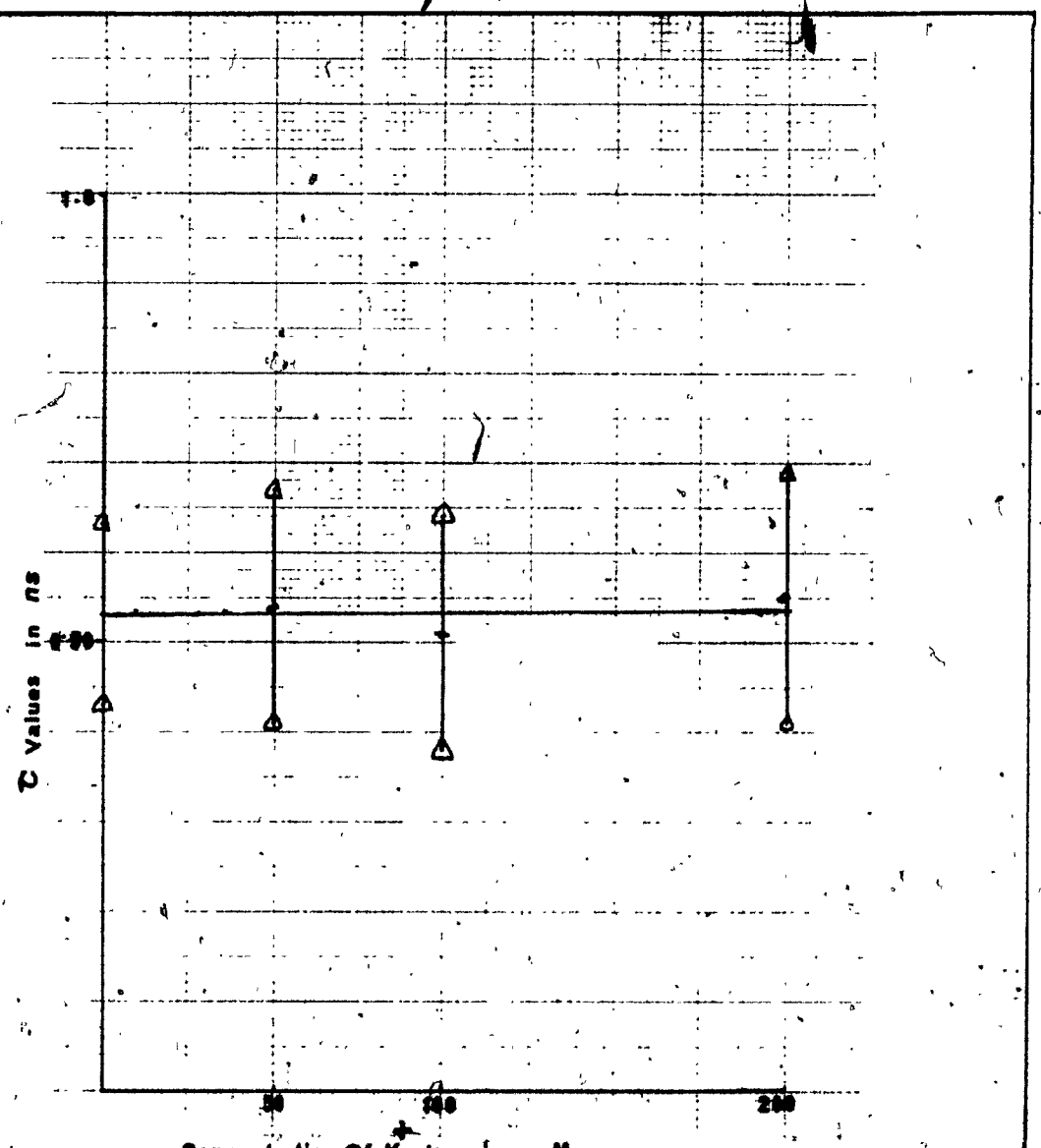


FIG 3.6 Plot of K^+ Ion Effect on the ESR Spectrum

Hence the Na^+ ion up to concentration limit of 100 mM decreases the mobility of spin attached to the specific sites of the opiate receptor due to the conformational change introduced in the specific sites; whereas K^+ ion has no effect on the conformation since the mobility of the attached spin remains more or less the same.

3.6.3 Effects of Mn^{+2} Ion on the ESR Spectrum

According to the literature [12] the effects of divalent cations are not as significant as the Na^+ ion effect in producing the increase in agonist binding of opiate receptor. The most effective concentration of Mn^{+2} ions as studied by other workers is 1 mM in the presence of 100 mM of Na^+ ion.

Specifically labeled samples having the following concentrations of Mn^{+2} ions were used:

i) 0.05 mM of Mn^{+2} in the presence of 100 mM of Na^+ ion;

ii) 0.1 mM of Mn^{+2} in the presence of 100 mM Na^+ ion;

iii) 1 mM of Mn^{+2} in the presence of 100 mM Na^+ ion.

Since Mn^{+2} has a paramagnetic center, it interacts with the lone electron of the spin attached to the specific site of the homogenate. Therefore, the spectra obtained from the above samples are interfering, containing six peaks for Mn^{+2} ions and the three-lined spectrum for the spin in middle of the six peaks (Figure 3.7). The line intensities of the spectrum for the spin label has been decreased due to the

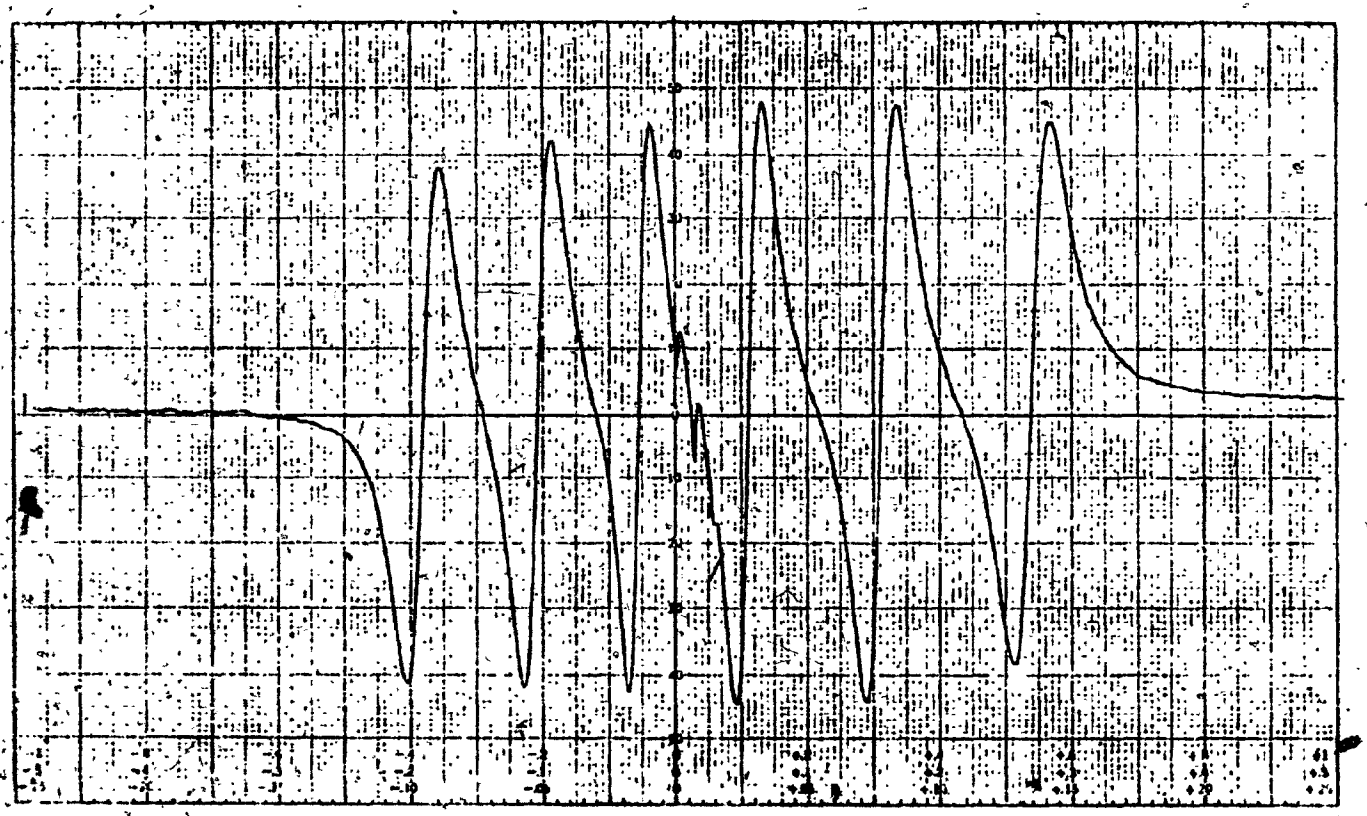


FIG 3.6 Effect of Mn^{+2} ion on The ESR Spectrum of Specifically Labeled P_2 Fraction
(Protein Concentration 0.6 mg/ml)

effects of the paramagnetic center of the Mn^{+2} ion. Line ratios can still be calculated from those interfering spectra. But the τ value determined will have no significance in determining conformational change since the line ratios have been influenced by the combined interactions of paramagnetic center on Mn^{+2} and the attached spin label. The line ratios can no longer be considered as a measure of the function of state of the opiate receptor. The τ values determined in the presence of different concentration of Mn^{+2} are shown in Table 3.8.

Since Mn^{+2} ion increases agonist binding, it is expected that rotational correlation values should decrease in the presence of Mn^{+2} (since the effect is opposite to that of Na^{+} ion). Since it is not possible to determine the τ value correctly under the influence of paramagnetic center of Mn^{+2} ion, the results obtained have no significance at all. In the presence of 0.1 mM and 0.05 mM of Mn^{+2} ion, the influence of paramagnetic center becomes less but it is not removed completely and therefore the line ratios cannot be considered as a measure of the conformational change. The most effective concentration [14] of Mn^{+2} in increasing the agonist is 1 mM and the effects are not as significant below 1 mM of concentration. This indicates that the concentrations below 1 mM will not help to show any effect of Mn^{+2} on the conformation of the opiate receptor.

Table 3.8

Effects of Mn^{+2} ions on the ESR spectrum of spin-labeled P_2 fraction.

Experimental conditions; protein concentration = 0.58 mg/ml, total protein = 2.9 mg; spin label II on specific sites, final volume 0.5 ml. Additives - Mn^{+2} ions of following concentrations indicated in the Table 3.8 in presence of 100 mM of NaCl. τ values were calculated as described in section 2.4.1.

Concentration of $MnCl_2$ solution added mM	Totational correlation time (τ) ns	Remarks
0 mM	1.15 ($\pm$ .13)	values determined have no significance on receptor's conformational change due to the interaction of paramagnetic center on Mn^{+2} ion with lone electron on the spin label.
0.05 mM	1.63 ($\pm$ .14)	
0.1 mM	1.19 ($\pm$ .13)	
1 mM	1.33 ($\pm$ .15)	

In short, the effects of Mn^{+2} ions on the opiate receptor cannot be worked out by ESR study due to the presence of a paramagnetic center on the Mn^{+2} ion.

3.6.4 Effects of Mg^{+2} Ion on the ESR Spectrum

According to the literature [14] Mg^{+2} ion in the presence of Na^{+} ion also increases the binding of agonists and decreases the binding of antagonists, but the effects produced by Mg^{+2} ion are less significant than the effects produced by Mn^{+2} in increasing agonist binding and Na^{+} ion in increasing antagonist binding. Therefore the question arises whether conformational change introduced by Mg^{+2} ion can be detected by means of ESR studies, since this method is not that sensitive up to the limit of 10^{-10}M, the concentration of opiate receptors, as mentioned preivously.

The most effective concentration of Mg^{+2} ions as studied by other workers is 0.05 mM. The results are shown in Table 3.9. The change obtained in the presence of Mg^{+2} ions, is so small that it cannot be considered as significant. The changes are almost within the standard deviations. It may be concluded that additions of Mg^{+2} ions do not cause any significant change in the conformation of the opiate receptor or the change is too small to be detected by ESR studies. Hence the addition of 0.05 mM of Mg^{+2} causes a very slight decrease in the $\mathcal{Z}$

Table 3.9

Effects of Mg^{+2} ions on the ESR spectrum of specifically labeled P_2 fraction.

Experimental conditions: protein concentration = 0.58 mg/ml total protein = 2.9 mg; spin label II on specific sites; final volume = 0.5 ml. Results of a representative experiment are given as the mean ± S.D. of triplicate determinations. The experiment was replicated twice. Spectral parameters were calculated as described in section 2.4.1. $T_2^{-1}(0) = 38.07 \times 10^6$ rad/sec.

Concentration of Mg^{+2} ions in the presence of 100 mM NaCl	Spectral Parameters			Remarks
	A_o/A_1	A_o/A_{-1}	τ ns	
0 mM	1.36 (± .12)	2.38 (± .23)	1.13 (± .11)	No significant change in τ values.
0.05 mM	1.31 (± .13)	2.0 (± .19)	0.89 (± .11)	
0.1 mM	1.35 (± .1)	2.34 (± .24)	1.11 (± .15)	
1 mM	1.42 (± .05)	2.17 (± .23)	1.06 (± .16)	

value that might have some experimental significance but is not very prominent. Additions of 0.1 mM of Mg^{+2} and 1 mM of Mg^{+2} ion cannot be worked out very well by ESR spectroscopy, as expected, but the results obtained do not contradict the literature data obtained by binding studies.

3.7 Use of Isothiocyanate Spin Label

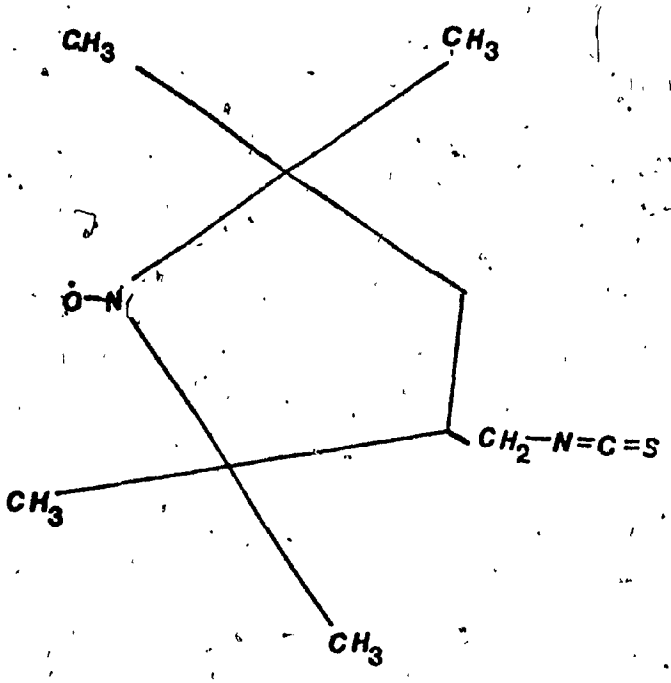
An ESR label might reflect changes due to the binding of agonist or antagonist to the receptor. For such a study, the maleimide spin labels cannot be used since those labels will attach to the -SH groups covalently and thereby eliminate the opiate binding capacity as all the agonists react with the -SH groups on the receptor since the receptor can be deactivated by pretreatment with -SH reagents.

To study the effects of drugs other labels such as isothiocyanate could perhaps be used. Isothiocyanate binds preferentially to $-NH_2$ groups and to some extent to -OH groups.

The same kind of triplet representing a freely mobile spin rapidly tumbling in solution, is obtained as shown in Figure 3.1 when the ESR spectrum for the isothiocyanate spin label used (Figure 3.8) is run under the usual conditions.

3.7.1 Labeling the Homogenate with Isothiocyanate Spin (IV)

When the brain homogenate (protein concentration 0.58 mg/ml) is labeled with isothiocyanate spin under the usual procedure of



IV

FIG 3.8 3 isothiocyanato methyl 2,2,5,5 tetramethyl-1-pyrrolidinoyloxyl

specific labeling, the ESR spectrum obtained is identical to that shown in Figure 3.2. Line ratios are calculated and τ value determined is around 0.75 ($\pm$ .09)ns.

Results from the radioactive binding experiment to verify the specificity of labeling are shown in Table 3.10. It is observed from the results that specific labeling is not obtained with the isothiocyanate spin. When the P_2 fraction is treated with ethyl isothiocyanate for 20 minutes most of the specific sites have been blocked. But a protection experiment shows that only 9% of the total morphine sites has been protected. Even though the morphine sites are not protected, the P_2 fraction was still incubated with isothiocyanate spin for 1 hour to see the extent of labeling for the sake of interest. Only 4% of the protected sites were labeled by isothiocyanate spin. The study indicates that specific labeling is not obtained with isothiocyanate label. Most of the specific sites had been blocked by unlabeled ethyl isothiocyanate. The extent of blocking depends upon the time of reaction with the blocking reagent. Specific labeling of only 4% of the total morphine sites can be neglected. It is not possible to obtain any significant results from the experiments due to failure of labeling P_2 fraction specifically.

Table 3.10

Verification of specificity of labeling by isothiocyanate label.

Values are the means of duplicate determinations. The experiment was replicated three times. Binding % control values were determined by comparing the specific binding of the reacted samples with the specific binding of unreacted tissues.

Experiment	Additions	Receptor Concentration	Binding % Control
Total morphine sites determination	None	0.08 picomoles/mg protein	100%
Blocking	E. isothiocyanate for 20 minutes	0.0007 picomoles/mg protein	0.8%
Protection experiment	Morphine for 15 minutes + E. isothiocyanate for 2 minutes	0.007 picomoles/mg protein	8.3%
Labeling with spin isothiocyanate	Morphine for 15 minutes + E. Iosthiocyanate for 2 minutes + isothiocyanate spin for 1 hour	0.003 picomoles/mg protein	4.1%

3.7.2 Determination of Bound Isothiocyanate Spin Concentration

The isothiocyanate label was freed by treatment of the sample with NaOH and a triplet characteristic of a freely mobile spin rapidly tumbling in solution was obtained. Mid-peak heights were determined. The proportionality constant (K) is determined from the signal heights of various known concentrations of pure spin label solutions. The method for determining K has been shown in section 3.5. Results obtained are shown in Table 3.11.

The mean concentration of bound isothiocyanate has been calculated as 5.82×10^{-6} ($\pm$ .22) M/mg of protein. This indicates that when the homogenate is labeled with isothiocyanate spin the amount of bound spin is about 15 times more than the amount attached when the spin label is maleimide. This can be explained on the basis that the probability of having $-NH_2$ groups is more than that of -SH groups on a protein molecule. Secondly, the percentage of non-specific binding is much more in the case of isothiocyanate spin label than that obtained in maleimide spin labeled homogenate. In any case, the concentration determined is approximate only as mentioned earlier.

Table 3.11

Determination of concentration of bound spin (isothiocyanate)

Middle peak heights are determined from the triplet obtained after treatment with NaOH of the P_2 fraction labelled with IV spin as described in section 2.6. Data are the means of triplicate determinations. The experiment was repeated twice. Standard deviations and K value were calculated as described in table 3.5. Total protein = 2.9 mg.

Experiment No.	Proportionality constant K cm lit/mole	Receiver gain	Middle peak heights cm	Mean concentration of bound spin	Concentration of bound spin/ mg protein
1	17.12 (± 1.53)	6.2×10^4	17.60 (± 1.43)	1.68×10^{-5}M (± 0.22)	5.82×10^{-6}M (± 0.22)/mg protein
2	17.12 (± 1.53)	6.2×10^4	18.10 (± 1.47)		

3.7.3 Effects of Morphine on the Spin Labeled Homogenate and their Sensitivity towards the Effects of Na^+ and K^+ Ions

τ values were determined for the attached isothiocyanate spin after the addition of various additives from the experimental spectra of spin labeled homogenate. The mean τ value has been calculated as 0.75 ns when there are no additives. After the addition of morphine (2×10^{-6}M), τ value changes from 0.75 ns to 0.71 ns. Such a small change is not significant and falls within the standard deviations calculated.

When 100 mM Na^+ ion was added to the sample, containing added morphine, no change in the spectrum was found. The τ value changed to 0.68 ns which is not very significant.

If 50 mM K^+ ion was added to the sample containing added morphine the τ value changes from 0.71 ns to 0.70 ns, i.e., within the limits of standard deviation. Results are shown in Table 3.12.

In summary, there is no detectable change of conformation in the opiate receptor due to the addition of morphine, Na^+ or K^+ ion when the spin label is isothiocyanate.

Table 3.12

Correlation times for isothiocyanate spin labeled P_2 fraction with various ligands.

τ_c values were calculated from equation 6 as described in section 2.4.1. Additions were made as described in section 2.8.3. Data are the means of triplicate determinations. Values in the bracket indicate the standard deviations.

Sample	τ_c ns
Free spin label	0.01 (± .004)
IV labeled P_2 fraction (p.c. 0.58 mg/ml)	0.75 (± .09)
IV labeled P_2 fraction + morphine (2×10^{-6}M)	0.71 (± .12)
IV labeled P_2 fraction + morphine + NaCl (100 mM)	0.68 (± .15)
IV labeled P_2 fraction + morphine + KCl (50 mM)	0.7 (± .09)

3.7.4 Individual Effects of Na^+, K^+ and Morphine on Isothiocyanate-labelled Homogenate

The results of this study are shown in Table 3.13. It has been found that 30 mimrtes after the addition of 100 mM Na^+ ion, the τ value of attached isothiocyanate changes from a value of 0.78 ns to a value of 0.77 ns. Such a small change cannot be considered significant.

Thirty minutes after the addition of 50 mM K^+ ion, the τ value decreases from a value of 0.78 ns to 0.65 ns. This change is also within the limits of standard deviation and therefore it may or may not be considered as significant.

Finally the addition of morphine at a concentration of 2×10^{-6}M causes a change in the τ value from 0.78 ns to 0.73 ns which also is not significant to consider as a change in mobility. The values are almost within the standard deviation that can vary up to the limit of $\pm$ 0.1 ns as seen from previous experimental determinations.

No change in the mobility or conformation has been detected after the addition of Na^+, K^+ and morphine when the homogenate is labeled with isothiocyanate spin. In conclusion, the experiment with isothiocyanate spin is a failure as predicted, since the specific sites cannot be labeled with isothiocyanate spin label.

Table 3.13

Effects of Na^+, K^+ and morphine on ESR spectrum of isothiocyanate spin labeled P_2 fraction (protein 0.55 mg/ml).

Total protein = 2.9 mg; final volume = 0.5 ml. Additives = NaCl (100 mM), KCl (50 mM) or morphine (10^{-6}M) standard buffer = 50 mM tris (pH 7.5). Temperature 25°C. ESR spectra were taken after 30 minutes of incubation with various ligands. τ values were calculated as described in section 2.4.1. Data are the means (± standard deviation) of triplicate determinations.

Addition	τ values in ns
None	0.78 (± .09)
100 mM Na^+ ion	0.77 (± .13)
50 mM K^+ ion	0.65 (± .15)
Morphine (10^{-6}M)	0.73 (± .09)

CHAPTER 4

DISCUSSION

4.1 Spin Labeling

Spin labels used for the present study are the 2,2,4,4-tetramethyl pyrrolidinyl-oxyl spin labels carrying systematically varying chain lengths in combination with two attaching groups, maleimide or isothiocyanate. Spin labels in which the attaching group is maleimide give the most valuable information regarding the conformation of opiate receptor. The maleimide group has been found useful in spin labeling proteins covalently at -SH groups in the opiate receptor. The spin label in which the attaching group is isothiocyanate mainly attaches to the protein moiety of the receptor at $-NH_2$ groups and to some extent at -OH groups. When the P_2 fractions were reacted with a maleimide spin their ESR spectra revealed that there is definite attachment of the spin labels to the P_2 fractions, since the mobility of the spin label changes from its pattern of three equally spaced lines of about the same height (triplet) representing a freely mobile spin rapidly tumbling in a solution, to a less mobile spectrum having three broadened lines of unequal heights.

4.1.1 Specific Labeling

In labeling the receptors the concern of specificity is most important. In the previous sections (2.5), a simple procedure for spin labeling opiate receptor preparation specifically at its -SH groups that are responsible for opiate activity, has been shown. Protein modifying agents which affect sulfhydryl groups differentially influence the binding of agonists and antagonists [15]. Phenyl mercuric acetate and N-ethyl maleimide both strongly inhibit (^{3}H)-dihydromorphine binding to the receptor. PMA at a concentration of 10^{-4}M is more potent than ethyl-maleimide at the same concentration in inhibiting dihydromorphine binding. Similar effects are exerted by a wide selection of sulfhydryl reagents which differ markedly in their structure and chemical mechanism of sulfhydryl inactivation, strongly suggesting the presence of a reactive sulfhydryl group associated with the opiate receptor which is much more important for agonist than antagonist binding. Prior treatment with opiates protects the receptor binding from sulfhydryl reagents. The location of the -SH group in relation to the binding site is difficult to establish. Protection experiments with high concentrations of opiates suggest that this group may be at or near the binding sites. However, it is possible that opiates protect receptor binding by conformational changes induced by binding or allosteric interaction. The mechanism by which these sulfhydryl reagents act is still not known. According to the literature[15] they appear to decrease the number of agonist molecules binding, and it

appears also that other residues, in addition to sulfhydryl groups, are important for the binding of agonists. The time allowed for the reaction with sulfhydryl reagent after treatment with the appropriate concentration of morphine, is very important. Since both ethyl maleimide and phenyl mercuric acetate bind covalently, they might displace the loosely, non covalently bound morphine from the specific sites if the period of reaction is longer. Hence the period of two minutes incubation was allowed only to decrease the number of non-specific sulfhydryl groups to a certain extent when the specific sulfhydryl groups were protected. Morphine could be washed off, followed by centrifugation. Specific sites were then labeled by maleimide spin, thereby inhibiting strongly the (^{3}H)-dihydromorphine binding, suggesting that the specific sites were labeled by maleimide spin label. Hence the spectrum obtained after labeling the homogenate specifically, will contain information on the specific sites in the opiate receptor. The spectrum reveals the mobility of the bound spins on the specific sites. Hence any change in the conformation of specific sites will be indicated by changes in the spectral parameters representing mobility of the spin. The spectrum obtained is significantly weaker than the spectrum obtained after labeling the homogenate non-selectively. The present study shows a direct physical proof that sulfhydryl groups are essential for binding of opiates.

As indicated previously in section 3.7.1, the P_2 fraction cannot be labeled specifically with isothiocyanate spin labels which preferentially affect $-NH_2$ groups and to some extent -OH groups. Like maleimide or PMA, ethylisothiocyanate at the same concentration strongly inhibits (^3H)-dihydromorphine binding to the receptor, suggesting that $-NH_2$ groups are also necessary for opiate agonist binding. The extent of inhibition depends upon the period of reaction with reagents. But prior treatment with morphine cannot protect the receptor binding from isothiocyanate reagent, unlike sulfhydryl reagent. Since protection does not occur, the location of $-NH_2$ groups responsible for agonist binding seems to remain a big question. In contrast to -SH groups, after binding of opiates to the receptor the specific $-NH_2$ groups are still exposed to binding by isothiocyanate reagents, thereby eliminating dihydromorphine binding. But the mechanism by which these isothiocyanate reagents act is still not known. Since the protection experiment fails there is no question of labeling the specific sites by isothiocyanate spin. Most of the specific sites are blocked by unlabeled ethylisothiocyanate covalently, the spin label (isothiocyanate) may label only 5% of the total morphine sites. Such a low percentage of specific labeling can be neglected and is considered as an experimental error. Since specific labeling is not achieved with isothiocyanate spin, the spectrum obtained after the usual procedure of labeling will not indicate the

conformational change if introduced in the specific sites by ligands. More precisely, the study of the opiate receptor conformation will not be possible with the use of isothiocyanate spin label.

4.1.2 Determination of Bound Spin Concentration

The amount of spin bound to the homogenate or P_2 fraction is determined by the methods described in section 2.6. Treatment with NaOH after labeling makes the bound spin free by destroying the protein structure of the opiate receptor. The spectrum obtained after the treatment of NaOH, gives a triplet characteristic of a freely mobile spin. The amplitudes of the signals of the triplet for freely mobile spin will depend upon the concentration of free spin in solution. Hence amplitude determinations of various spectra will give an idea about the concentration of spin attached. This method of spin concentration determination is an approximate one since the amplitudes of the ESR spectrum may vary according to the exact position of the cell in the cavity. The exact position cannot be maintained during comparison of the signal heights in a spectrum of NaOH treated sample with the signal heights in a spectrum of a free spin label solution of known concentration. When the homogenate is simply labeled without concern to specificity, the amount of the spin attached is much higher than that present in the homogenate when it is labeled specifically. In specifically labeled homogenate the amount of non-specific binding is reduced but it is not possible to cut down the non-specific binding totally.

The total amount of spin label bound to the receptor preparation is higher than (^{3}H)-dihydromorphine binding. This suggests that we are dealing with a high proportion of non-specific binding, or that the spin label binds to more sites on the receptor than does the opiate.

Nevertheless, opiate binding is eliminated by "specifically" bound label, and it does indeed behave in a specific way, as predicted by previous pharmacological investigations. That gives us the confidence to consider it indicative of receptor behavior, quantitative discrepancies notwithstanding.

The increase in this virtually specific binding is indeed fortunate, as spin concentration in the picomolar range could not have been detected by the instrument at our disposal.

4.2 Effects of Ligands on ESR Spectrum

Since the spin label is covalently linked to the P_2 fraction, it provides a direct probe of the conformational state of the opiate receptor. The changes in ESR spectrum of the label on addition of a ligand result from changes in the mobility of the label, i.e., from a conformational change of the lipoprotein matrix.

4.2.1 Ion-induced Changes in ESR Spectrum

The ESR spectrum from the P_2 fraction (protein concentration 0.6 mg/ml) labeled with maleimide spin label II changes when Na^+ ions at concentration (5-200)mM are added to the sample contained in a flat

aquous ESR cell. Effects are most pronounced at 50-200 mM of Na^+ ions. A spectral parameter τ_c, the rotational correlation time, inversely related to the tumbling rate of the nitroxide spin label changes significantly due to addition of Na^+ ions. Definitions of τ have been given in several publications [35,36]. The correlation time can vary between 10^{-11} to 10^{-7} sec. corresponding to very fast and very slow tumbling, respectively.

The Na^+ ion induced conformational change is detected by the maleimide spin label. Results obtained for the differential effects of Na^+ ion [12,13] and protein modifying agents [15] on the binding of agonists and antagonists are best explained by a hypothesis that Na^+ ions produce a conformational change in the opiate receptor which causes an alternation in opiate binding sites. The altered sites have increased affinity for antagonists and considerable decreased affinity for agonists. This is just a hypothesis about the structure of the opiate receptor, but so far no direct physical proof was offered for it. Hence the present study implies a biophysical proof for the hypothesis that two interconvertible conformations are indeed present in the opiate receptor; one is the agonist conformation which is represented by a state having τ of 0.48 ns that means the spin in that conformation has considerable mobility, and the other is the antagonist conformation in which the spin has decreased mobility, having a rotational correlation time of 1.12 ns. Na^+ ions

promote the antagonist conformation, reducing the proportion of agonist conformation. At 100-200 mM of Na^+ ions, the transformation to antagonist state from agonist state is almost complete.

This property of Na^+ ion in increasing the immobility of the spin or the antagonist conformation is characteristic of sodium [10] rather than a general effect of cations or of high ionic strength since no such conformational change, i.e., no change in τ value, is detected in the presence of Na^+ ion up to a concentration range of 200 mM when the P_2 fractions are labeled with the same spin label non-specifically. If this effect were due to the ionic strength but not to the conformational change on the specific site, the same kind of change in an ESR spectra would be expected in the presence of Na^+ ion of same concentration when the P_2 fraction is labeled non-specifically.

It has to be emphasized that the effect of Na^+ ion is not a general effect of cations since K^+ ion in the concentration range (100-200 mM) has no effect on the mobility of the label attached to the receptor site. This result is in accord with the results obtained previously from radioactive binding studies [12,13]. Our studies confirm the conformation of opiate receptor previously proposed [1] to explain the mechanism of action of opiate agonists and antagonists in receptor function.

The effects of Mn^{+2} ions on the conformation of opiate receptor cannot be studied by ESR techniques due to the presence of a paramagnetic center on the Mn^{+2} ion itself. Addition of Mn^{+2} ions to the

spin-labeled P_2 fraction results in a diminution of the ESR signal and gives an interfering spectrum due to the interactions of the lone electron on the spin label and the paramagnetic center on the Mn^{+2} ion. Line ratios which determine the mobility of the spin, calculated from these interfering spectra, will have no bearing on the conformational change if introduced by Mn^{+2} ions.

Addition of other divalent cations, e.g., Mg^{+2} causes no change in the spectrum of the P_2 fraction specifically labeled with maleimide spin label. This indicates that Mg^{+2} ion does not cause any conformational change in the opiate receptor site in contrast to the findings [14] that divalent cations selectively increase the agonist binding. The nature of the change introduced in the opiate receptor, that results in high affinity for agonist binding, by reducing receptor sensitivity to Na^{+} ion, is unclear. It might also be possible that the postulate in favor of the agonist conformation due to the addition of Mg^{++} ion, is correct, but the change introduced is not as significant as the change produced by Na^{+} ions, therefore, the change in conformation is not detected by the ESR techniques. This explanation seems reasonable since the technique is sensitive only up to a maximum limit of 10^{-7}M(10^{-9}M on signal averaging) concentration but the specific site concentration has been found to be around 10^{-9} to 10^{-10}M concentration, this means that a large number of non-specific sites may overshadow the effects

produced by the specific sites only. It has to be stated also that changes due to Mg^{++} are considerably less pronounced than the Na^{+} effects in the binding experiments of the Snyder groups [14], another possible reason for their undetectability by ESR.

4.2.2 Morphine Induced Changes in ESR Spectrum

The mechanism of action of agonist and antagonist with a receptor is not known. Conformational studies of receptors might give insight into their actions. Binding of opiates to the receptor may induce a conformational change in the site of action, resulting in a series of chemical and biological reactions which cause analgesia and perhaps reveal the nature of receptor coupling to adenyl cyclase. However, no morphine induced changes are observed with the P_2 fraction spin labeled by maleimide spin label. This may be due to the fact that morphine cannot bind to receptor, since the maleimide spin labels bind covalently to the sulfhydryl groups thereby eliminating opiate activity. Hence isothiocyanate spin label which reacts with amine and/or alcohol groups [32] is used for this purpose. No morphine induced and even no Na^{+} induced changes are observed with the P_2 fraction spin labeled by isothiocyanate label. The only explanation which can be given is the inability of the isothiocyanate spin to label the P_2 fraction specifically.

The ESR spectra from the P_2 fraction spin labeled with maleimide spin labels before and after the use of a blocking reagent, e.g.,

PMA or ethylmaleimide, indicates that the label probably attaches to the receptor at sulfhydryl sites. The amount of bound spin after the use of a blocking agent which affects sulfhydryl groups, has been drastically reduced. The first biophysical evidence in support of the view that sulfhydryl groups are important components for opiate actions and for differentiating the interactions of opiate agonists and antagonists with the opiate receptor, is obtained from our present study as the specific binding is inhibited completely after labeling the P_2 fraction with maleimide spin label. Specific sulfhydryl groups may be located on the surface of the receptor since the attached spin labels have considerable mobility. This work also indicates that $-NH_2$ groups may be responsible for opiate activity as ethyl-isothiocyanate (unlabeled) can eliminate specific binding. The impossibility of protecting the binding sites by preincubating with a saturating concentration of morphine before treatment with ethyl-isothiocyanate might suggest that "specific $-NH_2$" sites are different from "specific -SH" sites. The location of specific $-NH_2$ groups on the opiate receptor cannot be elucidated because of the failure of the experiment of labeling the P_2 fraction specifically with isothiocyanate spin label.

CHAPTER 5

SUMMARY AND CONCLUSIONS

5.1 General

The spin labeling technique has been introduced to investigate the structure of the opiate receptor. Spin labels used for this purpose have two types of attaching groups, maleimide and isothiocyanate. A P_2 fraction has been prepared from rat brain. Specific binding of ^{3}H-dihydromorphine has been demonstrated. Techniques for labeling the P_2 fraction specifically with maleimide spin label has been developed in a series of experiments. A study has been carried out to investigate the effects of some important ligands on the spectrum from a specifically labeled P_2 fraction. Ligands studied are Na^+ ion, K^+ ion, Mn^{+2} ion, Mg^{+2} ion and morphine. Of those Na^+ ion provides the most interesting results that are in agreement with the hypothesis of a conformational change in the specific site in the presence of Na^+ ion. The results of the study of the effects of the ligands are tabulated and plotted in graphs and their effects are discussed in detail in section 3.6.

Use of spin labeling technique in this field enabled us to study the opiate receptor conformation in understanding the mechanism of actions of opiates.

5.2 Contribution of this Study

The specific contributions of this study are:

i) Techniques are developed for labeling the P_2 fraction with spin label.

ii) The P_2 fraction can be specifically spin labeled at sulfhydryl sites. The first physical approach has been made to the postulation that sulfhydryl groups are located on the specific sites and are responsible for binding of opiates.

iii) The electron spin resonance spectrum indicates that the bound spin label has considerable mobility which is greater than that of the whole macromolecule. This together with measurements of the hyperfine coupling constant suggests that spin-labeled SH is on the surface of the receptor. Hence it indicates also that specific -SH groups on the receptor might be located on the surface of the specific sites. But this study is not enough to give such definite comments about the location of specific -SH groups. Further investigation is necessary in this area.

iv) The amount of spin bound has been determined by a method using NaOH to disrupt the protein structure of the receptor thereby making the bound spin free.

v) The conformational change induced by Na^+ ion is detected by the spin label. The previously postulated conformational change in the presence of Na^+ ion can be confirmed by a study of the spin-labeled spectrum in presence of Na^+ ion.

For the first time it has been proved by biophysical techniques that the receptor undergoes a conformational change in the presence of Na^+ ion so that the transformed site has higher affinity for binding of opiate antagonist than agonist binding.

vi) K^+ ion at the same concentration has almost no effect on the spin label. That means that K^+ ion cannot cause conformational change in opiate receptor site. This result is in agreement with the result obtained from previous binding studies in the literature [12].

vii) Addition of Mn^{+2} ion to the spin-labeled P_2 fraction produces an interfering spectrum due to the interaction of the lone electron on the spin label and the paramagnetic center of Mn^{+2} ion. Hence the effects of Mn^{+2} on opiate receptor conformation cannot be studied by means of this technique.

viii) Addition of Mg^{+2} ion causes no significant change in the spectrum. The results obtained is inconsistent with the results deduced from the binding studies. This experiment fails due to inadequate sensitivity of the techniques.

ix) No significant conformational change has been detected by the isothiocyanate spin label in the presence of Na^+, K^+ and morphine

due to inability of the spin isothiocyanate to label the P_2 fraction specifically.

5.3 Suggestion for Future Study

An understanding of the conformation of the opiate receptor site by the application of a biophysical technique should shed light on the reason for the difference in pharmacological action between drugs which differ only slightly in structure. The uniqueness of sodium ion raises the possibility that alterations in sodium binding or transport may play a role in the mode of action of opiates. Various possibilities may arise involving the design of the conformation of the narcotic receptor site for explaining the mechanism of action of opiates. This field is so big that it cannot be covered in a single study. The present study confirms some of the speculations about the conformation of the opiate receptor but a further study is necessary.

Suggestions for improving future studies are outlined as follows:

i) In the present study, the specific labeling obtained contains both specific sites as well as non-specific sites. Hence an effort should be made to cut down the amount of non-specific labeling as much as possible so that a measurable signal is still obtained. That may be

possible by using a more modern ESR spectrometer and using spin concentrations in the nanomolar range.

ii) Blocking the receptor with opiates of higher affinity (etorphine, "super-fentanyl") might improve the extent of specific labeling.

iii) Conformational changes on drug binding could be investigated by spin-labeled opiates. Some investigations along this line are in progress in our laboratory.

iv) Present study as well as previous studies by other workers uses a crude preparation of receptor to investigate the narcotic receptor site. The isolation of opiate receptor has to be accomplished for further improved studies.

v) Present study with isothiocyanate spin label is not very comprehensive. Hence further study is necessary in this field.

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