AN ELECTRON SPIN RESONANCE STUDY OF SYNAPTOSOME OPIATE RECEPTORS

THE PREPARATION AND USE OF A SPIN LABELED MORPHINE

EDMUND S. COPELAND, MICHAEL E. BOYKIN, JAMES A. KELLEY, *and* M. PETER KULLBERG

From the Division of Biochemistry, Walter Reed Army Institute of Research, Washington, D. C. 20012. Dr. Boykin's present address is Department of Neurology, Loyola University Medical Center, Maywood, Illinois 60153. Dr. Kullberg's present address is NIDA Addiction Research Center, Lexington, Kentucky 40511.

ABSTRACT Morphine spin labeled on the phenolic hydroxy group has been prepared using commercially available reagents and characterized by thin layer chromatography, mass spectroscopy, and electron spin resonance spectroscopy. It has been shown that morphine modified in this way retains some opiate activity, does not pass through the blood-brain barrier, and specifically binds to isolated rat brain synaptosomes. Spin labeled morphine has been shown to be an effective biophysical probe complementing radioactive tracer techniques in the study of the narcotic receptor site.

INTRODUCTION

Numerous investigations have recently demonstrated the existence of receptors in the central nervous system which stereospecifically bind opiates (for a recent review, see ref. 1). In all such binding studies, radiolabeled narcotic agonists or antagonists have been used. Thus, unbound labeled material has to removed by procedures which may destroy evidence of weak but potentially important opiate-receptor interaction (1). On the other hand, because of the nature of spin label probes, electron spin resonance (ESR) spectra of both bound and unbound probe can be obtained simultaneously within certain concentration limits so that separation of bound and free opiate is not necessary. The spin label technique is thus complementary to radiolabel investigations of the opiate receptor site.

Early studies (2) on the pharmacologic role played by various regions of the morphine molecule suggested that the cationic form of the tertiary amine and the π molecular orbital of the aromatic ring (see Fig. 1) associate with an appropriately shaped receptor surface by ionic and van der Waals forces. The edge of the molecule composed of carbons 8 through 5 and including the hydroxyls on the 3 and 6 positions, would then be on the opposite side of the molecule from the proposed interacting regions. Knowing that this early model might be incomplete (1), it was felt, nonetheless, that the most reasonable molecular site for the incorporation of a noninterfering

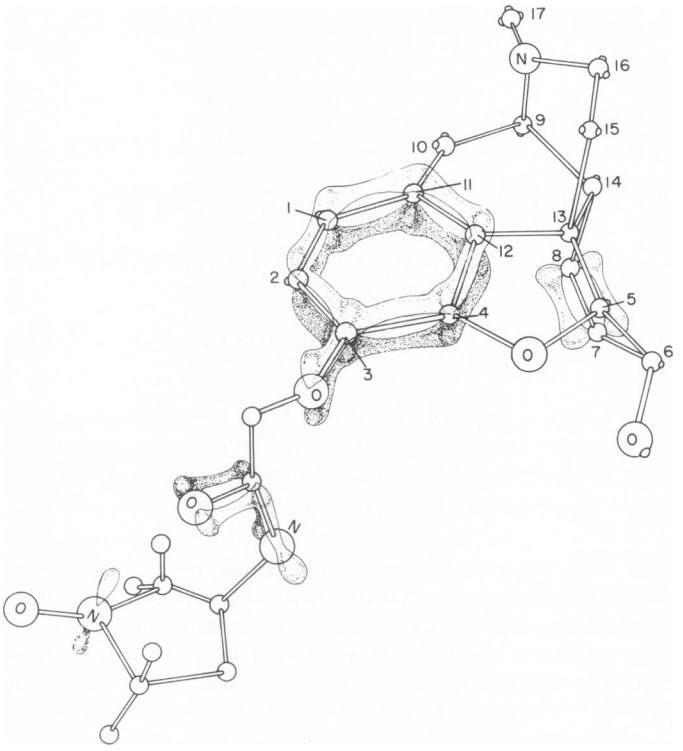


FIGURE 1 Perspective drawing of spin labeled morphine. The interacting surface of the morphine molecule according to Beckett and Casy's model (2) is on the top side in this drawing. The numbering system for the morphine molecule is indicated here. See text for further discussion.

spin label group would lie somewhere on this back-side edge. Thus, the 7,8-double bond, the 6-hydroxy, and the 3-hydroxy were potential labeling sites.

Morphine spin labeled on the 3 position (3-SLM) is commercially available as part of a spin immunoassay kit for urinary morphine detection (3–5). We describe a simple and inexpensive method for the preparation of 3-SLM and show some of its characteristics in vitro and in vivo.

MATERIALS AND METHODS

Chemicals

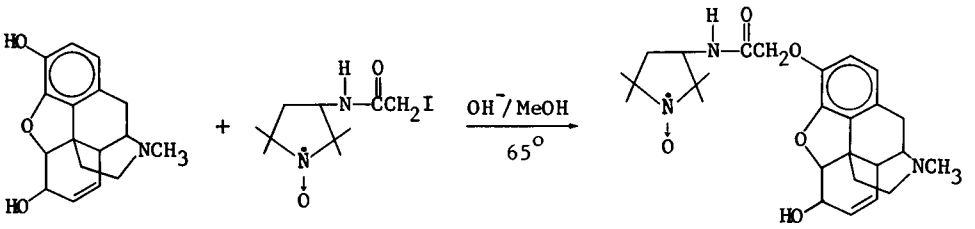
Morphine free base (I) was obtained from Mallinckrodt Chemical Works, St. Louis, Mo. and used as such. The iodoacetamide nitroxides, 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (IIa) and 4-(2-iodoacetamido)-2,2,6,6-tetramethyl-1-piperidinooxyl (IIb), and the FRAT[1] reagents were purchased from SYVA Corporation, Palo Alto, Calif. and used without further purification. Nalorphine HCl (Nalline HCl) was obtained from Merck, Sharp & Dohme,

[1] Trade names are given for the convenience of the reader and do not constitute an endorsement of the product by the authors or the U.S. Government.

West Point, Pa., and levorphanol tartrate was kindly supplied by Hoffman-LaRoche, Inc., Nutley, N.J.

Synthesis

The synthetic scheme followed for the preparation of 3-SLM is as follows:



I IIa IIIa

Morphine-free base (I) (22 mg, 0.077 mmol) was dissolved in methanol (1 ml) and freshly prepared KOH (4.3 mg, 0.077 mmol) in methanol (1 ml) was added. Then IIa (25 mg, 0.077 mmol) in methanol (1 ml) was added dropwise to the stirring solution and upon completion the reaction mixture was gently refluxed for 4 h.[2] The reaction mixture was evaporated to dryness under reduced pressure, redissolved in methanol, and applied to preparative TLC plates (Brinkman Silica Gel HF_{254}) which were developed in EtAc/MeOH/NH_4OH (17:2:1). In this system the morphine had an R_f = 0.26 and the SLM had an R_f = 0.43. The band corresponding to the SLM was scraped off the plate and the SLM was eluted from the silica gel with EtAc/MeOH (80:20). The eluant was evaporated to a yellow oil under reduced pressure. Either IIa or IIb could be attached to morphine in this way.

A perspective drawing of the reaction product (IIIa) is presented in Fig. 1. The configuration of morphine is based on X-ray crystallographic data obtained on morphine hydroiodide dihydrate (6). The sketch of the nitroxide derivative of iodoacetamide is adapted from theoretical and experimental studies (7, 8). We have no knowledge of the orientation of the spin label in relation to the remainder of the SLM molecule but presume freedom of rotation about the ether linkage at the 3 position. The spin label moiety can thus occupy a back-side position on morphine.

Mass Spectroscopic Analysis

Mass spectra of the TLC-purified reaction product (IIIa) were obtained via direct insertion probe on a Hewlett-Packard 5930A dodecapole mass spectrometer (Hewlett-Packard Co., Palo Alto, Calif.). The ion source was maintained at 180° while the probe temperature was slowly varied from ambient to 300°; spectra of 3-SLM were obtained at 250°. An ionizing current of 300 μA was employed in conjunction with an electron energy of either 70 eV or 12.5 eV.

ESR Spectroscopy

ESR analyses were performed on an X-band spectrometer (E-9, Varian Associates, Palo Alto, Calif.) using either a TE_{104} dual cavity or a TE_{011} cylindrical cavity when indicated. Unless otherwise specified, 100 kHz modulation frequency at 2 G modulation amplitude was em-

[2] Morphine plus one equivalent of base generates a phenoxide anion at the three position and insures nucleophilic displacement by this site.

ployed. Using the dual cavity, it was found that power saturation of the nitroxide triplet began at about 40 mW incident power when the sample was held in a capillary tube suspended by a quartz ESR tube within a quartz variable temperature insertion Dewar. Thus 40 mW was the usual power employed. When the cylindrical cavity was used, 10 mW incident power was employed. Sample holders included the standard flat cell, the low temperature flat cell (James F. Scanlon Co., Solvang, Calif.) or the FRAT capillary tube. In controlled temperature studies (*vide infra*) a variable temperature Dewar (Scanlon) was used with a controlled temperature apparatus (Varian) and the temperature was checked using a Cu-Constantan thermocouple and found to be constant within $\pm 1.5°$. Free radical concentrations were determined using an on-line digital computer (Varian S-122) to perform double integrations of both unknown and standard spin label samples. The S-122 was also used to measure the line widths necessary for rotational correlation time determinations.

Preliminary Biological Studies

In Vivo Injections of 3-SLM. Male weanling mice were intraperitoneally (i.p.) injected with 3-SLM in 0.9 ml normal saline, pH 7.5 at a dosage of 24 mg/kg morphine equivalents. Controls were injected with normal saline. Animals were observed for 1 hr for evidence of grossly apparent opiate induced behavioral or physiological effects (lethargy, staggering, respiratory depression, altered threshold to tail clamping).

In another set of experiments, male weanling mice were injected intracerebrally (right central hemisphere, frontal lobe) with 0.2 ml 3-SLM in normal saline, pH 7.5, at a dosage of 0.30 mg/kg morphine equivalents. Controls were injected with 0.2 ml normal saline. The animals were observed for grossly apparent behavioral and physiological effects as before.

Intraventricular injections of 3-SLM in adult male Sprague-Dawley rats, 250–300 g (Simonsen Labs., Gilroy, Calif.) were also performed. Using reported coordinates (9), 30 μl of 3-SLM in normal saline, pH 7.5, at a dosage of 0.05 mg/kg morphine equivalents was injected. 30 min postinjection the animals were sacrificed by decapitation and the brains were rapidly removed and placed in cold (0–4°) 0.32 M sucrose, 0.05 M Tris-HCl, pH 7.5. Brains from six animals were pooled and homogenized (six strokes at 800 rpm), then centrifuged at 12,000 *g* for 30 min in a Sorvall Model RC-2 centrifuge (DuPont Instruments, Sorvall Operations, Newtown, Conn.). The supernate and pellet were separated for subsequent ESR analyses. Animals were also injected with 3-SLM intraperitoneally rather than intraventricularly to determine general organ distribution patterns in liver, heart, and brain. Adult male rats, as before, were injected i.p. with 0.9 ml 3-SLM in normal saline at 50 mg/kg morphine equivalents. Animals were sacrificed 15 min postinjection and the brain, liver, and heart rapidly removed and placed in 0.32 M sucrose as in previous experiments. The organs were homogenized at 800 rpm as before and aliquots of the whole organ homogenates were taken for ESR analyses.

In Vitro Synaptosomal Studies. Male Sprague-Dawley rats, 250–300 g, were sacrificed by decapitation and the brains rapidly removed and placed in 0.32 M sucrose, 0.05 M Tris-HCl, pH 7.5 at 0–4°. Synaptosomes were isolated by differential and density gradient ultracentrifugation methods (10, 11). In four successive experiments, synaptosomes containing 8.4, 9.0, 10.0, and 10.5 mg/ml protein (biuret [12]), in 0.32 M sucrose, pH 7.5 (Tris-HCl, 0.05 M), were incubated with 3-SLM. 1 ml aliquots of synaptosome suspensions were incubated in a Dubnoff metabolic shaker under the following conditions: (A) 3-SLM, 1 μM, at 0°; (B) 3-SLM, 1 μM, + nalorphine, 1 mM, at 0°; (C) 23° for 5 min, + 3-SLM, 1 μM, at 23° for 10 min; (D) nalorphine, 1 mM, for 5 min at 23°, + 3-SLM, 1 μM, 10 min at 23°. Incubations were terminated by placing the samples in ice-water slurries at 0°. Samples were kept at 0° until ESR observations (4°) were made using the small flat cell and variable temperature apparatus described previously.

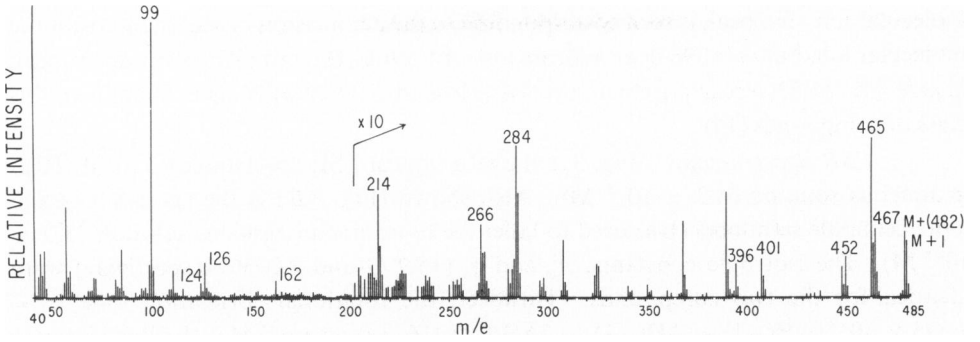


FIGURE 2 Mass spectrum of 3-spin labeled morphine. The mass spectrum was obtained at 70 eV by controlled fractional vaporization of TLC-purified 3-SLM from a probe directly inserted into the ion source of a Hewlett-Packard 5930A dodecapole mass spectrometer.

RESULTS

Characterization of Spin Labeled Morphine

Mass Spectrometry. The fragmentation pattern shown in Fig. 2 resulted at 70 eV and is in all respects characteristic of the proposed product. The peak due to the molecular ion (*m/e* 482) is readily apparent. The M+1 peak is much more intense than would be expected from the contribution of normally encountered isotopes (85% instead of the expected 31% of the molecular ion); this may be partly due to hydrogen radical abstraction from water adsorbed in the ion source or glass capillary sample holder by the even electron molecular ion species[3] (13, 14). The mass spectrum also offers strong evidence that the spin label has been attached to the 3 position rather than the 6 position. The peak at *m/e* 465 indicates loss of a hydroxyl radical from C-6 (see Fig. 1 for numbering), a cleavage that is common to the mass spectra of those morphine alkaloids that possess a 6-hydroxyl allylic to the 7,8 double bond (15). Also present are relatively intense peaks at *m/e* 124 and 162 which denote an unsubstituted 6-hydroxyl in ring C. Ions indicative of an unsubstituted phenol at the 3 position are not present or are found one mass unit lower than expected, e.g., *m/e* 284 and 214. This suggests cleavage of the spin label followed by fragmentation of the alkaloid nucleus. In addition, a ferric chloride test gave no indication of the presence of the free phenol.

Although the fragmentation of the alkaloid part of the molecule is readily apparent, the mass spectrum is dominated by cleavage occurring in the spin label moiety. The presence of a tetramethylpyrrolidine nitroxide is clearly visible from the high mass end of the spectrum[4] (14). The peak at *m/e* 467 indicates loss of a methyl radical, while *m/e* 452 denotes loss of an additional methyl radical or extrusion of NO from the

[3] A greatly simplified spectrum resulted at 12.5 eV with a relative decrease in the intensity of the *M*+1 peak compared to the molecular ion peak. This indicates that there is not an analogous impurity present, e.g., the corresponding hydroxylamine (which would also possess a much more intense *M*-15 peak at *m/e* 468) and that the ion in question requires considerable energy for its formation.

[4] J. A. Kelley and J. A. Cella, unpublished results.

molecular ion. No peak is seen corresponding to the elimination of isobutene from the molecular ion, but m/e 396 does indicate loss of both C_4H_8 and NO. An intense peak at m/e 409 (M-73, a rearrangement involving loss of C_3H_7NO) is also common to this class of compounds (14).

ESR Spectroscopy. Fig. 3*A* shows a typical ESR spectrum of 3-SLM (IIIa) in aqueous solution (4.2×10^{-5} M). Also shown (Fig. 3*B*) is the spectrum of the iodoacetamide spin label (IIa) used to label the morphine in aqueous solution (3.0×10^{-5} M). The isotropic constants, A_o and g_o (15.9 G and 2.0056, respectively) were identical for the two compounds. However, the rotational correlation time, $\tau = 1.395 \times 10^{-8}[\Delta H(+1) + \Delta H(-1) - 2\Delta H(0)]$ s (16, 17) where ΔH is the line width at half height in gauss at $M = +1, -1$, and 0, respectively, of the actual absorption spectrum, did increase when the spin label was attached to morphine ($\tau_{IIa} = 5.2 \times 10^{-10}$ s;

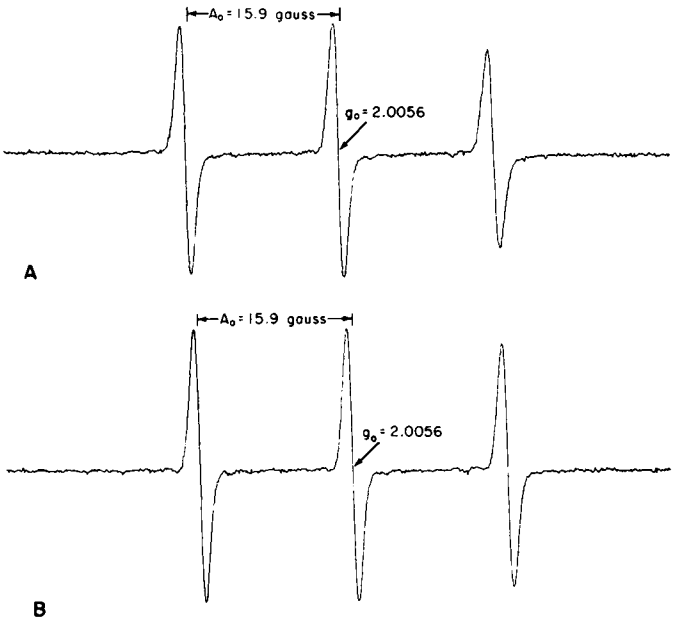


FIGURE 3 (*A*) First derivative ESR spectrum of a 4.2×10^{-5} M aqueous solution of 3-SLM. The spectrum was obtained using a TE_{011} cylindrical cavity with the sample in a capillary tube. The incident power was 10 mW and the 100 kHz modulation frequency had an amplitude of 0.1 G. The field was swept at 3 G/min using a 0.3 s filter time constant. The spectral data were processed directly by an on-line digital computer to obtain line widths at half height of the absorption peaks which were 0.720, 0.707, and 0.782 G for the $M = +1$, 0, and -1 hyperfine lines, respectively. These values were used as described in the text to calculate the rotational correlation time. The isotropic constants, A_o and g_o, are indicated. (*B*) First derivative ESR spectrum of a 3.0×10^{-5} M aqueous solution of the pyrrolidine nitroxide iodoacetamide derivative used to label 3-SLM. The observation conditions were the same as in *A*. The corresponding line widths at half height were 0.682, 0.682, and 0.720 G, respectively. The isotropic constants, A_o and g_o, were identical to those found when this spin label moiety was attached to morphine (*A*) as would be expected.

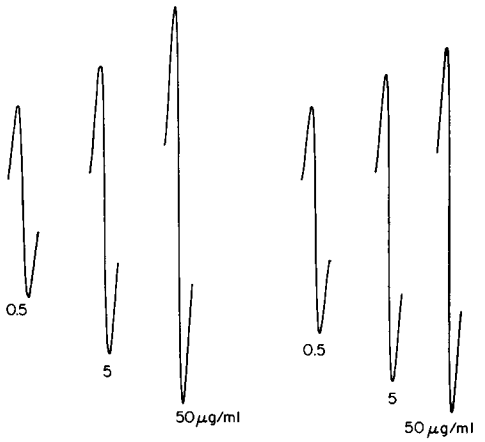


FIGURE 4 Comparison of the interaction of commercial and synthesized 3-SLM with morphine antibody. Equimolar quantities of commercial (FRAT reagent B) (left) and synthesized (right) 3-SLM were added to morphine antibody (FRAT reagent A) such that all morphine binding sites were occupied. (Titrations used to determine this equivalence point are not illustrated.) Aliquots of each antibody solution were then combined with morphine standard solutions having concentrations of 0.5, 5.0, and 50 μg/ml, respectively. The partial ESR first derivative spectra shown illustrate the increase in amplitude of the $M = +1$ line as progressively more 3-SLM is displaced from the antibody. The ESR spectra depicted were obtained using a TE_{104} dual cavity with the samples in capillary tubes. The temperature was held at 21°. The incident power was 40 mW and the modulation amplitude was 2 G.

$\tau_{III_a} = 1.2 \times 10^{-9}$ s) indicating inhibition of the nitroxide's tumbling rate by the 285 dalton morphine molecule.

Fig. 4 illustrates that added free morphine displaces commercial SLM and synthesized 3-SLM from morphine antibody[5] in a nearly identical manner. The slight differences in line amplitude may be related to the variability encountered in titrating the antibody. The $M = +1$ line of the nitroxide triplet is illustrated. Because the line amplitude is temperature dependent (19), a constant temperature of 21° was maintained for these determinations.

Preliminary Biological Studies

In Vivo Injections. In the first set of experiments, animals were injected i.p. with 3-SLM at a dose of 24 mg/kg morphine equivalents. None of the animals demonstrated any grossly apparent signs (behavioral or physiological) of central nervous system effects.

In the six mice receiving 3-SLM intracerebrally at 0.30 mg/kg morphine equivalents and observed for grossly apparent drug effects, all demonstrated lethargy, staggering gait, and moderate respiratory depression. None of the six control animals demon-

[5] Morphine antibody can recognize substituents on C-6 and the ring nitrogen as well as the degree of saturation of the C-7 to C-8 bond (18).

strated similar effects. The duration of the observed effects varied from 15 min to 1 h. Thus, even though the bulky spin label group can orient itself on the postulated backside of morphine, it has a marked effect on morphine's pharmacology, possibly on its transport into the central nervous system or on the rate at which it is metabolized after i.p. injection.

The next set of experiments was carried out to determine whether 3-SLM could be detected in brain tissue after injection directly into a brain ventricle and whether 3-SLM injected i.p. could be detected in whole brain tissue homogenate. In those rats receiving 0.05 mg/kg morphine equivalents of 3-SLM intraventricularly, 3-SLM could be detected by ESR in the whole brain homogenate. However, when 3-SLM was administered i.p. at 50 mg/kg, no 3-SLM was detectable in brain tissues isolated under the same experimental conditions. The latter animals did have readily detectable levels of 3-SLM in homogenates of nonperfused heart and liver. No attempt was made to determine whether the spin label detected in these tissues was attached to morphine.

The apparent absence of 3-SLM in brain tissue after i.p. administration could have been due to an enhanced metabolism, an inability of 3-SLM to cross the blood-brain barrier, a selective reduction of nitroxide by brain tissues or an insufficient concentration of 3-SLM in brain tissues for ESR detection ($\sim 10^{-8}$ M). If, like codeine, 3-SLM were O-dealkylated to morphine (20), i.p. administration should have produced a pharmacological effect. Enhanced metabolism to the glucuronide would have negated this effect. Reduction of the majority of the nitroxide radicals appears unlikely in view of the fact that the spin label was detectable in brain tissue after intraventricular administration. In regard to the possibility that 3-SLM may not cross the blood-brain barrier, it is interesting to note the structural similarity between 3-SLM and morphine glucuronide (Fig. 5). Hepatic morphine detoxification involves addition of the polar glucuronide moiety at the 3-position which inhibits passage through the blood-brain barrier. However, because the nitroxide moiety is relatively nonpolar compared to the sugar moiety, the two forms of morphine although sterically similar probably differ markedly in lipid solubility. It is, however, reasonable that 3-SLM would penetrate the barrier less readily than free morphine.

In Vitro Synaptosomal Binding Studies

In order to study 3-SLM binding by an opiate receptor, we isolated whole brain synaptosomes which contain a high concentration of specific opiate receptors and bind both opiates (21–23) and opiate antagonists (24). This in vitro system was also selected so that the spin labeled opiate concentration could be in a pharmacologically meaningful range and yet be detectable with ESR. Goldstein et al. (25, 26) have found that the levorphanol concentration in brain water of mice exhibiting maximal opiate analgesia is approximately 10^{-6} M. Thus, a total 3-SLM concentration of 10^{-6} M in the synaptosome preparation was employed.

The results of the incubation experiments outlined above are presented in Table I. The ESR spectra observed were similar to that of Fig. 3 *A* and showed no significant changes in 3-SLM rotational correlation time after the various incubations. A reduc-

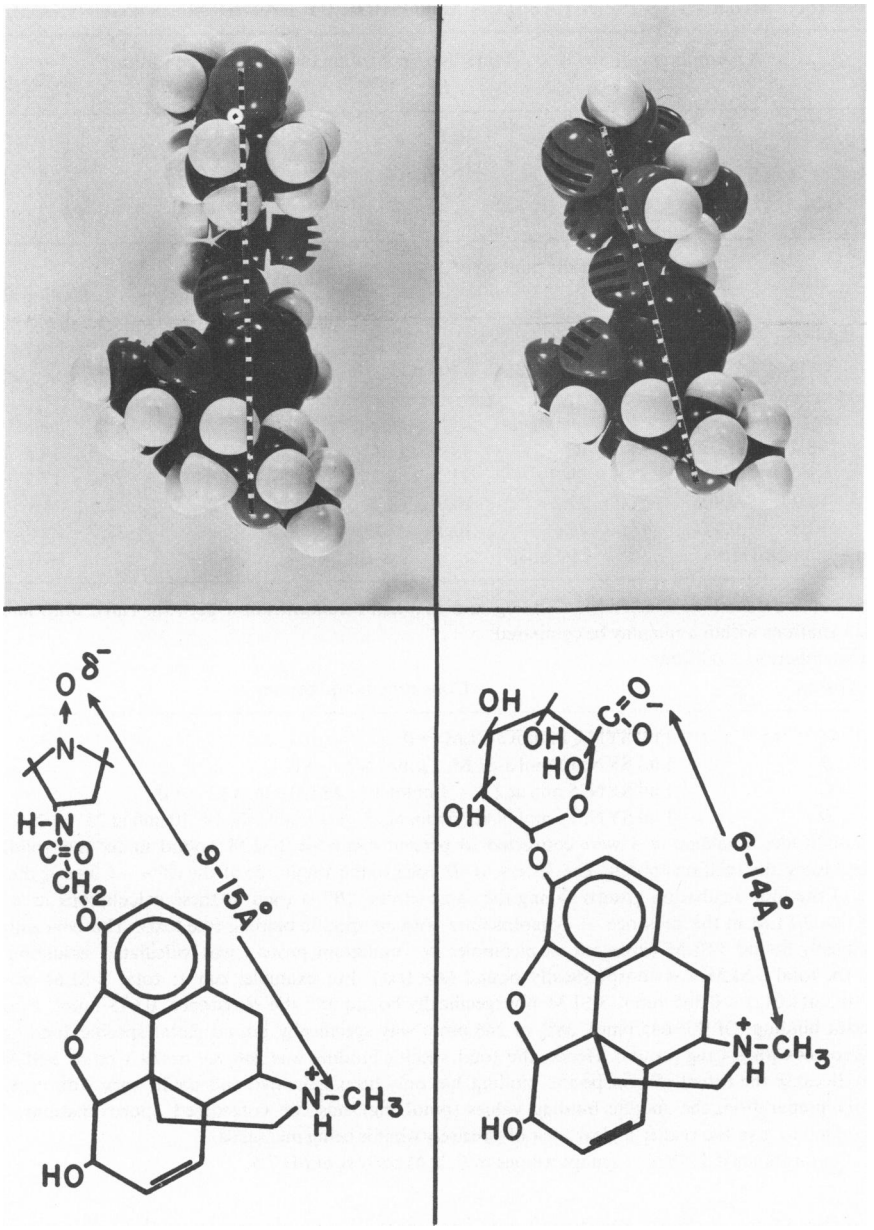


FIGURE 5 Comparison of the molecular conformations of spin labeled morphine and morphine and morphine glucuronide. Corey-Pauling-Koltun (CPK) molecular models of 3-SLM (left) and morphine glucuronide (right) illustrate that both the molecular conformation and the separations of the charged groups (dotted line) are similar. Below the CPK models, the conventional structures of the two molecules provide orientation. See text for further discussion.

TABLE I

SPECIFIC BINDING OF SPIN LABELED MORPHINE BY RAT BRAIN SYNAPTOSOMES

A. Amplitude of the $M = -1$ line* (cm) according to incubation condition‡

Run	*A*	*B*	*C*	*D*
1	9.2	10.8	6.7	8.7
2	11.1	12.5	8.3	11.3
3	6.4	8.0	5.2	5.8
4	7.8	8.7	4.6	7.9

B. Specific binding of 3-SLM by synaptosomes‖

	Protein conc.	Total 3-SLM	Binding: Total [(*B* − *C*)/*B*]		Binding: 0° [(*B* − *A*)/*B*]		Binding: 23° [(*A* − *C*)/*B*]		Binding at 23° prevented by NAL§ [(*D* − *C*)/*B*]	
	mg/ml	*nmol/ml*	%	*pmol/mg*	%	*pmol/mg*	%	*pmol/mg*	%	*pmol/mg*
1	8.4	1.012	38.0	29.3	14.8	11.4	23.1	17.8	18.5	14.3
2	9.0	1.012	33.6	24.2	11.2	8.1	22.4	16.1	24.0	17.3
3	10.0	0.996	35.0	22.3	20.0	12.7	15.0	9.6	7.5	4.8
4	10.5	0.997	47.1	*28.6*	10.3	*6.3*	36.8	*22.4*	37.9	*23.0*
Mean ± standard error				26.1 ± 1.7		9.6 ± 1.5		16.5 ± 2.6		14.9 ± 3.8

*Because of differences in spectrometer tuning and in 3-SLM concentration between runs, only line amplitude variations within a run may be compared.

‡Key to incubation conditions:

Group	Constituents and treatment
A	1 ml SYN,§ 1 nmol 3-SLM → 0°
B	1 ml SYN, 1 nmol 3-SLM, 1 μmol NAL → 0°
C	1 ml SYN, 5 min at 23° + 1 nmol 3-SLM, 10 min at 23° → 0°
D	1 ml SYN, 1 μmol NAL, 5 min at 23° + 1 nmol 3-SLM, 10 min at 23° → 0°

‖Line amplitudes tabulated in *A* were converted to percent available 3-SLM bound under the conditions described using the relations noted. The letters, *A–D*, refer to the amplitude of the $M = -1$ line of the ESR spectra of the four incubation groups having the same letters. "*B*" is used in these calculations as a measure of free 3-SLM in the presence of synaptosomes with no specific binding (see text). The concentration of specifically bound 3-SLM expressed as picomoles per milligram protein was calculated assuming that 36% of the total 3-SLM was nonspecifically bound (see text). For example, run 1: total 3-SLM is 1.012 nmol, (0.36)(1.012) = 0.365 nmol 3-SLM nonspecifically bound and the remainder, 0.645 nmol, available for specific binding. Of this 645 pmol, 38% or 246 pmol was specifically bound (total specific binding) by synaptosomes with 8.4 mg protein. Hence, the total specific binding was 246/8.4 or 29.3 pmol 3-SLM/mg protein. Because the extent of nonspecific binding has only been estimated and could vary with each synaptosome preparation, the specific binding values (pmol/mg) must be considered approximations. They are presented to give the reader a clear idea of precisely what is being measured.

§NAL = nalorphine HCl, SYN = synaptosomes in 0.32 M sucrose at pH 7.5.

tion of the $M = -1$ line amplitude[6] was interpreted as a decrease in the concentration of free 3-SLM due to synaptosome binding (27, 28). The baseline for no specific[7]

[6] Either of the $M = \pm 1$ lines can be monitored since each is located in a field region little perturbed by the spectrum of strongly immobilized spin label.

[7] Since 3-SLM specific binding was not measured by using morphine analog stereoisomers, e.g., levorphanol vs. dextrorphan, the observed binding cannot strictly be termed *stereo*specific (1), but is *pharmacologically* specific (29).

3-SLM binding was provided by the synaptosome samples held at 0° with 1 mM nalorphine and 1 μM 3-SLM (group B). The high concentration of nalorphine as well as its greater affinity for the narcotic receptor (three times morphine [24]) should prevent specific binding of 3-SLM. However, nonspecific binding of 3-SLM would be expected to occur under these conditions, so that the amplitude of the $M = -1$ line cannot be equated with the total concentration of 3-SLM in the sample. Studies in model systems have indicated that the nonspecific binding of 3-SLM is primarily associated with lipids, is minimal with proteins and is not influenced by nalorphine at these relative concentrations (unpublished results, this laboratory).

Since previous studies (21, 24) had demonstrated that a significant part of specific opiate binding in brain subfractions can occur at 0–4°, the specific binding both at 0° and after incubation at 23° was measured. The extent of specific binding is expressed both in terms of percent of 3-SLM available for such binding and as picomoles per milligram protein using an estimate of nonspecific binding.[8] Thus, the following comments on the interaction of 3-SLM with synaptosomes can be made on the basis of the data presented in Table I. About 38% of the 3-SLM that does not experience nonspecific binding was specifically bound after incubation for 10 min (26.1 pmol/mg). Since 14% of this 3-SLM pool available for receptor site binding interacts at 0° (9.6 pmol/mg), the remaining 24% must bind in a temperature dependent process (16.5 pmol/mg). Preincubation of synaptosomes with 1 mM nalorphine prevents essentially all temperature-dependent 3-SLM binding (16.5 ± 2.6 vs. 14.9 ± 3.8 pmol/mg).

No spectral indications of strongly immobilized 3-SLM as previously demonstrated with morphine antibody (5, 19) were observed. Unequivocal ESR identification of 3-SLM bound by the synaptosome opiate receptors requires that greater than about 85% of the label be immobilized. When less label than this is bound, the signal of the free label can dominate the composite spectrum (27).

Selective reduction of the nitroxide label during the course of these studies was not indicated. Spin labeled morphine bound to synaptosomes could be displaced into solution by addition of a 10^2–10^3 molar excess of levorphanol or nalorphine directly to the ESR flat cell containing the synaptosome+3-SLM complex. The increase in ESR line amplitude then observed approximately corresponded to the amount of 3-SLM specifically bound.

A complete kinetic study to delineate fully the effects of incubation time and tem-

[8] Both binding and viscosity changes can cause an alteration in line amplitude. If synaptosomes were omitted from samples made up in 0.32 M sucrose to measure line amplitude in the absence of binding, the viscosity change caused by synaptosomes would not be accounted for. It is feasible that the zero-binding limit can be evaluated by extrapolating "B" line amplitudes to zero protein concentration. An analysis of this nature showed that the logarithm of the $M = +1$ line amplitude varied linearly with synaptosomal protein concentration and on extrapolation to zero protein concentration, gave a value equal to that determined in 0.32 M sucrose. The logarithm of the $M = -1$ line amplitude did not vary linearly with protein concentration as might be expected from its considerably greater sensitivity to viscosity compared to the $M = +1$ line (27). By assuming that the $M = +1$ line amplitude reflected binding only, we obtained an *estimate* for maximum nonspecific binding. At the mean synaptosomal protein concentration of the four runs, 9.5 mg/ml, a maximum of about 36% of the total 3-SLM was nonspecifically bound. Since about 38% of the remaining 3-SLM or 24% of the total was specifically bound, the nonspecific to specific binding ratio was about 1.5/1.

perature and synaptosome concentration for this model system was not within the scope of the preliminary study reported here. At temperatures approaching 37°, a less stable interaction was noted. Also, as might be expected, when synaptosomes were heated at 100° for 5 min, no subsequent specific binding of 3-SLM could be demonstrated. Specific binding could also be completely eliminated by hypotonic lysis of the synaptosomes in 3 mM Tris buffer prior to analysis. As was found for the model lipid and protein systems mentioned above, nalorphine had no effect on free 3-SLM concentration in such lysed preparations at 0°, 23° or when added directly to the flat cell. The amount of specifically bound 3-SLM increased with synaptosomal protein concentration up to 5 mg/ml above which point a constant maximum was obtained. The data reported in Table I were obtained in this region of maximum 3-SLM binding.

Specific binding of 3-SLM could also be demonstrated in whole brain homogenates using a similar experimental regimen. In parallel experiments we found that temperature dependent specific binding at 23° was about 10% of that exhibited by synaptosomes with approximately the same protein concentration, although specific binding at 0° was nearly the same. Specific binding could also be completely eliminated by hypotonic lysis of the brain homogenate.

DISCUSSION

This study demonstrates that an ESR spin label technique can complement radiotracer studies of the narcotic receptor site. The concentration of unbound spin label can be determined in the presence of a wide range of bound spin label concentrations (3–5, 19) and specific opiate binding can be examined in vitro without separating the bound and free labeled opiate.[9] In the case of 3-SLM binding by morphine antibody, strong immobilization of the label causes line broadening (5, 19) with a more than 40-fold reduction of the $M = 0$ line amplitude and essential disappearance of absorption at the magnetic fields of $M = \pm 1$. If the spin label is only partially immobilized by binding, a similar 20-fold decrease in the $M = 0$ line amplitude is noted (27). Thus, the decrease in concentration of free spin labeled material can easily be monitored, although definitive analysis of the bound spin label requires that greater than about 85% of the label be immobilized. The above phenomena have been used to demonstrate that the extent of specific binding of 3-SLM in vitro is temperature dependent in whole brain synaptosomes and whole brain homogenate and that this binding can be prevented or the bound 3-SLM can be displaced by an opiate agonist (levorphanol) or antagonist (nalorphine).

In vivo investigations suggest that 3-SLM cannot cross the blood-brain barrier, but has a pharmacologic effect is injected intracerebrally. Goldstein et al. (26) have re-

[9] The disparity between the concentration of specific opiate receptors reported here (~26 pmol/mg protein; assuming a 1.5/1 nonspecific to specific binding ratio) and those determined previously (22, 24, 30, 31) using radioactive labels (~0.1 pmol/mg protein) should be noted. It may in part be due to the fact that postincubation washing to remove unbound radioactive opiate, which may destroy evidence of weak binding (1,21), is not required in our assay.

ported that when the whole brain concentration of levorphanol is about 4 μg/g brain in nontolerant mice, the depressant actions of the opiate are dominant. When mice in the current study were given intracerebral injections of 3-SLM at a dose in morphine equivalents of 12.5 μg/g brain, lethargy and respiratory depression were observed. Although any comparison is at best qualitative, the observed effect is that which might be expected for an opiate with about 4–10 times less pharmacological activity than levorphanol.

In addition to the general findings of the synaptosome studies just mentioned, some specific discussion is warranted. The lack of an observed change in rotational correlation time in the various incubation conditions indicates that free 3-SLM dominates our spectra. We cannot determine the degree of immobilization of the spin label when 3-SLM is bound. Even moderate immobilization of the label, as mentioned above, would cause the changes in line amplitude (and therefore concentration) of unbound 3-SLM reported. We can rule out, however, complete binding of the 3-SLM with only very slight immobilization of the label as this would have caused a detectable increase in rotational correlation time.

In this study about 37% of the total specific binding took place at 0°. Hug and Oka (21), who did not distinguish between specific and nonspecific binding, found that 72% of the total [^{3}H]dihydromorphine was bound by isolated synaptosomes at 0°. Pert and Snyder (24), on the other hand, observed that in whole brain homogenate, only 10% of the total [^{3}H]naloxone specific binding occurred at 4°. Our studies with whole brain homogenate are insufficient for a quantitative comparison but indicate that about 75% of the total specific binding observed occurred at 0°.

The ability to interact with the narcotic receptor apparently parallels opiate pharmacological activity (22, 24, 30). Thus preparation of spin labeled opiates with high pharmacologic potency would be advantageous for using these compounds as probes to characterize receptor sites. In this regard, the 6-, 7-, or 8-SLM derivatives might be more pharmacologically potent than 3-SLM for two reasons. First, if the Beckett-Casy model is essentially correct, then even though the spin label moiety can assume a "back-side" position in 3-SLM, there is an alteration at the edge of the proposed π-orbital binding group. Introduction of the label at a greater distance from this region would be more favorable. Second, codeine, which is similar to 3-SLM in that the phenolic hydroxy group is altered, has a markedly lower affinity for the opiate receptor than does morphine (24, 31).

An intriguing opportunity which spin label techniques offer to studies of the narcotic receptor is to analyze the ESR spectrum of bound spin labeled opiate probes to characterize the binding interaction and the geometry of the receptor site.

A cursory description of the synthetic technique was presented at the International Conference on Electron Spin Resonance and Nuclear Magnetic Resonance in Biological Systems, December 1972, New York, and appeared in (1973) *Ann. N. Y. Acad. Sci.* **222**:1087.

The authors wish to acknowledge the assistance and advice given during the various stages of this study by Doctors N. E. Weber, J. A. Cella, and D. L. Klayman and Mssrs. E. Matusik, Jr., W. Earl, R. Barber,

D. Gettman, G. Catchen, and W. Ceresa. We thank Doctors A. Goldstein, C. Chignell, and A. Keith for their critical reading of the manuscript.

Received for publication 2 April 1975.

REFERENCES

1. GOLDSTEIN, A. 1974. Opiate receptors. *Life Sci.* **14**:615.
2. BECKETT, A. H., and A. F. CASY. 1954. Synthetic analgesics: sterochemical considerations. *J. Pharm. Pharmacol.* **6**:986.
3. LEUTE, R. K., E. F. ULLMANN, A. GOLDSTEIN, and L. A. HERZENBERG. 1972. FRAT*, a rapid new immunoassay technique: determination of morphine by electron spin resonance spectroscopy. *Nat. (New Biol.).* **236**:93.
4. LEUTE, R. K., E. F. ULLMANN, and A. GOLDSTEIN. 1972. Instant determination of opiate narcotics in urine and saliva by spin immunoassay. *J. Am. Med. Assoc.* **221**:1231.
5. LEUTE, R. K. 1973. Experiences with ESR as a tool for surveying narcotic addiction. *Ann. N.Y. Acad. Sci.* **222**:1087.
6. MACKAY, M., and D. C. HODGKIN. 1955. A crystallographic examination of the structure of morphine. *J. Chem. Soc. (Lond.).* 3261.
7. JANZEN, E. G., C. A. EVANS, and J. I-P. LIU. 1973. Factors influencing hyperfine splitting in the ESR spectra of five-membered ring nitroxides. *J. Magn. Res.* **9**:513.
8. ELFSKIND, L. 1973. MO-calculations on nitroxide radicals. *Acta Chem. Scand.* **27**:71.
9. NOBLE, E. P., R. J. WURTMAN, and J. AXELROD. 1967. A simple and rapid method for injecting ^{3}H-norepinephrine into the lateral ventricle of the rat brain. *Life Sci.* **6**:281.
10. GRAY, E. G., and V. P. WHITTAKER. 1962. The isolation of nerve endings from brain: an electron-microscope study of cell fragments derived by homogenation and centrifugation. *J. Anat.* **96**:79.
11. BOYKIN, M. E., and R. H. MARTIN. 1973. Preliminary observations on the uptake of catecholamines in synaptosomes of opiate-treated animals. *J. Pharm. Pharmacol.* **25**:484.
12. GORNALL, A. G., C. J. BARDAWILL, and M. M. DAVID. 1949. Determination of serum proteins by means of biuret reaction. *J. Biol. Chem.* **177**:751.
13. MORRISON, A., and A. P. DAVIES. 1970. Mass spectrometry of piperidine nitroxides—a class of stable free radicals. *Org. Mass Spectrom.* **3**:353.
14. DAVIES, A. P., A. MORRISON, and M. D. BARRATT. 1974. Mass spectrometry of stable free radicals. II. Pyrrolidine nitroxides. *Org. Mass Spectrom.* **8**:43.
15. WHEELER, D. M. S., T. H. KINSTLE, and K. L. RINEHART. 1967. Mass spectral studies of alkaloids related to morphine. *J. Am. Chem. Soc.* **89**:4494.
16. SMITH, I. C. P. 1972. The spin label method. *In* Biological Applications of Electron Spin Resonance. H. M. Swartz, J. R. Bolton, and D. C. Borg, editors. Wiley-Interscience, New York. 493.
17. SNIPES, W., J. CUPP, G. COHN, and A. KEITH. 1974. Electron spin resonance analysis of the nitroxide spin label 2,2,6,6-tetramethylpiperidone-*N*-oxyl (tempone) in single crystals of the reduced tempone matrix. *Biophys. J.* **14**:20.
18. VAN VUNAKIS, H., E. WASSERMAN, and R. LEVINE. 1972. Specificities of antibodies to morphine. *J. Pharmacol. Exp. Ther.* **180**:514.
19. COPELAND, E. S. 1973. The effect of ambient temperature on spin-label immunoassay for morphine. *Ann. N.Y. Acad. Sci.* **222**:1097.
20. ADLER, T. K. 1963. Comparative potencies of codeine and its demethylated metabolites after intraventricular injection in the mouse. *J. Pharmacol. Exp. Ther.* **140**:155.
21. HUG, C. C., JR., and T. OKA. 1971. Uptake of dihydromorphine-^{3}H by synaptosomes. *Life Sci.* **10**: 201.
22. WONG, D. T., and J. S. HORNG. 1973. Stereospecific interaction of opiate narcotics in binding of ^{3}H-dihydromorphine to membranes of rat brain. *Life Sci.* **13**:1543.
23. TERENIUS, L. 1973. Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction from rat brain. *Acta Pharmacol. Toxicol.* **32**:317.
24. PERT, C. B., and S. H. SNYDER. 1973. Opiate receptor: demonstration in nervous tissue. *Science (Wash. D.C.).* **179**:1011.

25. RICHTER, J. A., and A. GOLDSTEIN. 1970. Tolerance to opioid narcotics. II. Cellular tolerance to levorphanol in mouse brain. *Proc. Natl. Acad. Sci. U.S.A.* **66**:944.
26. GOLDSTEIN, A., B. A. JUDSON, and P. SHEEHAN. 1973. Cellular and metabolic tolerance to an opioid narcotic in mouse brain. *Br. J. Pharmacol.* **47**:138.
27. JOST, P., and O. H. GRIFFITH. 1973. Electron spin resonance and the spin labeling method. *In* Methods in Pharmacology. C. Chignell, editor. Appleton-Century-Crofts, New York. 255.
28. WEINER, H. 1969. Interaction of a spin-labeled analog of nicotinamide-adenine dinucleotide with alcohol dehydrogenase. I. Synthesis, kinetics, and electron paramagnetic resonance studies. *Biochemistry.* **8**:526.
29. MULÉ, S. J., G. CASELLA, and D. H. CLOUET. 1974. Localization of narcotic analgesics in synaptic membranes of rat brain. *Res. Commun. Chem. Pathol. Pharmacol.* **9**:55.
30. SIMON, E. J., J. M. HILLER, and I. EDELMAN. 1973. Stereospecific binding of the potent narcotic analgesic [^{3}H] etorphine to rat brain homogenate. *Proc. Natl. Acad. Sci. U.S.A.* **70**:1947.
31. KLEE, W. A., and M. NIRENBERG. 1974. A neuroblastoma x glioma hybrid line with morphine receptors. *Proc. Natl. Acad. Sci. U.S.A.* **71**:3474.