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Spin-Labeled Narcotics

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SUMMARY

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We have synthesized a series of spin-labeled derivatives of morphine and codeine and have used electron spin resonance spectroscopy to examine some of the interactions of these molecules. Animal studies were conducted to determine the relative analgesic potencies of these drugs. A series of experiments was conducted in which one of the spin-labeled drugs was isolated from the brains of rats and ESR was used to show that enzymatic hydrolysis of the drug was a relatively slow process. These experiments indicate that the pharmacological effects of these drugs are due to the spin-labeled molecules and not to a hydrolysis product (morphine or codeine). ESR was also used to demonstrate that the spin-labeled drugs bind to isolated synaptic membrane preparations. These experiments indicate that ESR will be a valuable tool for the study of the interaction of spin-labeled analgesic drugs with membranes.

INTRODUCTION

Spin-labeled molecules have been used as biochemical probes for the study of a variety of types of molecular interactions (1-3). The electron spin resonance spectrum of the labeled molecule undergoes characteristic changes as the motion of the molecule is restricted. Investigation of the ESR spectra of spin labels bound to substrate molecules can provide valuable insight into the mechanism of binding in some instances. We have synthesized and characterized three new spin-labeled morphine derivatives and one codeine derivative (Fig. 1). This paper reports both the physiological activity of these new spin-labeled drugs and results of ESR investigations.

METHODS

Syntheses

Codeine TMPO[1] *ester (IV).* A suspension of 1.008 g of 3-carboxy-2,2,5,5-tetramethylpyrroline-1-oxyl in 20 ml of dry benzene was treated with 1.2 ml of pyridine and cooled, and 0.54 ml of thionyl chloride was stirred in dropwise (4). The reaction mixture was stirred at room temperature for 1 hr. The solvent was removed under diminished pressure at room temperature. The yellow residue was further evacuated with a mechanical pump for 0.5 hr to ensure complete removal of thionyl chloride. The dark brown residue was used without further purification. A solution of 2 g of codeine in 20 ml of benzene was added to the carboxyl chloride in 2 ml of pyridine, and the reaction mixture was stirred for 2 days, filtered, and washed with benzene. The filtrate and washings were concentrated under diminished pressure. The crude residue was purified by column chromatography (grade 3 alumina) and eluted with benzene, a mixture of benzene and dichloromethane, and dichloromethane. The solid codeine ester was collected from

[1] The abbreviation used is: TMPO, 2,2,5,5-tetramethylpyrroline-1-oxyl 3-carboxylate.

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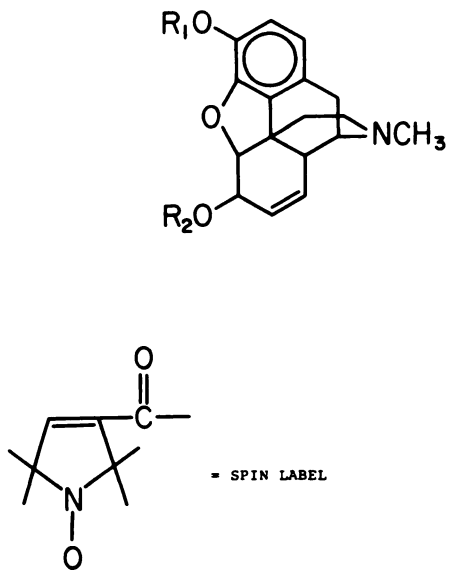

Compound	R_1	R_2
I	SPIN LABEL	H
II	SPIN LABEL	SPIN LABEL
III	H	SPIN LABEL
IV	CH_3	SPIN LABEL

FIG. 1. *Structures of spin labels*

the dichloromethane fraction. Recrystallization from benzene–hexane gave an analytical sample (yellow), m.p. 148–150°; yield, 1.5 g (50%); *m/e* 465; infrared peak at 1726 cm^{-1}.

$C_{27}H_{33}N_2O_5$

Calculated: C 69.66, H 7.14, N 6.02
Found: C 69.95, H 7.50, N 6.21

Morphine 3,5-TMPO diester (II). 3-Chloroformyl-2,2,5,5-tetramethylpyrroline-1-oxyl was prepared as previously described. Morphine (920 mg) in 30 ml of pyridine was added to the crude chloride prepared from 1.84 g of carboxylic acid. The resulting solution was stirred at room temperature for 1.5 days and treated as before. The morphine diester was recrystallized from benzene–hexane; yield, 1.4 g (70%); m.p. 206–208°; *m/e* 617. Its infrared spectrum showed a strong, broad peak at 1710–1750 cm^{-1}.

$C_{35}H_{43}N_3O_7$

Calculated: C 68.05, H 7.02, N 6.80
Found: C 68.06, H 7.07, N 6.86

Morphine 3-TMPO ester (I). The preparation of this compound was similar to that of the codeine ester except that excess morphine was used (1.2 Eq). The methylene chloride fraction from column chromatography (grade 3 alumina) gave crystalline ester. Recrystallization from hexane–acetone gave an analytical sample, m.p. 113.5–115°. Its ultraviolet spectrum showed an immediate bathochromic shift on addition of a drop of 1% KOH solution. Its infrared spectrum showed C=O absorption at 1723 cm^{-1}, OH at 3423 cm^{-1}; *m/e* 451.

$C_{26}H_{31}N_2O_5$

Calculated: C 69.16, H 6.92, N 6.20
Found: C 69.09, H 7.16, N 6.16

Morphine 6-TMPO ester (III). Aqueous sodium carbonate (5 ml, 0.5 M) was added to a solution of 100 mg of morphine diester in 25 ml of absolute ethanol. The reaction mixture was stirred at room temperature for approximately 10 hr. The ethanol was removed under diminished pressure, and the residue was taken into chloroform, washed with water and 5% HCl solution, dried over Na_2SO_4, and concentrated and purified by column chromatography (grade 4 alumina, eluted with dichloromethane and chloroform). Recrystallization from benzene–hexane gave an analytical sample, m.p. 223–225°; *m/e* 451; infrared peak at 1715 cm^{-1} (sharp).

$C_{26}H_{30}N_2O_5$

Calculated: C 69.16, H 6.92, N 6.20
Found: C 69.10, H 6.89, N 6.48

ESR Measurements

All ESR measurements were made on a JEOL 3BSX X-band ESR spectrometer, employing a 100-KHz field modulation. Samples in toluene were placed in 5-mm sample tubes, and samples in water, in capillary tubes. The capillary tubes held about 20 μl of sample. The volumes were measured with a microsyringe. The area of the ESR signals is directly proportional to the concentration of the radical. Concentration measurements of unknown samples were made by comparing the area of the signal from the unknown sample with the area of the signal from a known sample.

Experiments to determine the concentration of spin-labeled drugs bound to synaptic membrane preparations were carried out by comparing the area of the ESR signal from the label-membrane mixture with the area of the ESR signal from a sample of identical label concentration in Tris buffer. The concentration determined by this technique was the concentration of unbound radical in the label-membrane mixture. The concentration of bound spin label was determined by subtracting the unbound concentration from the original concentration.

Pharmacological Evaluation of Spin-Labeled Derivatives

The analgesic potencies of the compounds were evaluated by the hot plate test, utilizing both mice and rats (5), and by the writhing method, involving the use of phenylquinone in mice (6). Male Sprague-Dawley rats weighing between 150 and 200 g were used. The mice were of the Carworth strain and weighed between 20 and 25 g. The doses employed ranged from 5 to 25 mg/kg, and all drugs were administered intraperitoneally as aqueous solutions of the hydrochloride salts. Analgesic potency was evaluated relative to morphine. Usually four rodents were used for both tests on each drug, and the data from the two tests were averaged. The Straub tail response in mice was also evaluated relative to a morphine value of 1.0. Six to eight mice were used to determine the relative dose needed to produce a Straub tail response.

Measurement of opiate binding to membranes. The technique for measuring stereospecific binding of [^{3}H]dihydromorphine to various membrane preparations was essentially that of Pert and Snyder (7). A typical incubation medium contained the following in a final volume of 1.2 ml: 0.05 M Tris (pH 7.5), 0.05 μCi of [^{3}H]dihydromorphine (70 Ci/mole), either 0.1 μM levorphanol or 0.1 μM dextrorphan, and a rat brain neural membrane preparation (1 mg of protein), consisting mainly of synaptic membranes (8). The spin-labeled opiates were used at a concentration of 0.1 μM. After the tissue had been incubated for 15 min at 37° with either levorphanol or dextrorphan in the presence of the spin label, [^{3}H]dihydromorphine was added and the incubation was continued for another 30 min. The tubes were then immersed in an ice bath, and the contents were filtered through 2.1-cm Whatman GF/B glass fiber filters and washed twice with 5 ml of ice-cold Tris buffer. Radioactivity was measured by liquid scintillation spectrometry.

Measurement of diester spin label in rat brain. Rats were injected intraperitoneally with 5 mg/kg of the morphine diester; after 30 min, when they had developed analgesia, they were killed by a sharp blow to the lumbar region. After removal of the meninges the brain was washed extensively in 0.9% NaCl to remove blood. A blood sample and the brain were extracted with dichloromethane containing 1% trimethylamine, and the extract was evaporated to dryness. The residue was then redissolved in toluene, a known volume of the toluene solution was placed in a sample tube, and the ESR spectrum was recorded. The concentration of the diester was determined by comparing the area of the ESR signal with the signal from a sample of the diester of known concentration.

RESULTS AND DISCUSSION

ESR Spectra

ESR spectra of the three monoradicals and the biradical were taken with toluene as the solvent. Spectra of the hydrochloride salts were taken in water. The nitrogen coupling constants of the free bases and the salts are listed in Table 1. The ESR spectra of the monoradicals (compounds I, III, and IV) showed coupling to a single nitrogen, while the biradical II exhibited a five-line ESR spectrum, characteristic of coupling to 2 equivalent nitrogen atoms. The ESR spectra of monoradical III and biradical II are shown in Fig. 2. The two unpaired spins in the biradical must interact with both nitroxide nitrogen atoms to produce an ESR spectrum of this type. The spin Hamiltonian used to describe the hyperfine and exchange interactions of biradicals of this type is given by (9–11).

TABLE 1

ESR data

Radical	A_N (toluene)	I_C/I_H[a]	$A_N(H_2O)$	I_C/I_H
	G		*G*	
I (3-ester)	14.2	1.06	15.8	1.38
II (diester)	7.1			
III (6-ester)	14.2	1.1	15.8	1.37
IV (codeine ester)	14.3	1.07	15.8	1.38
TMPO			16.1	1.08

[a] Intensity ratio of center line (I_C) to high-field line (I_H).

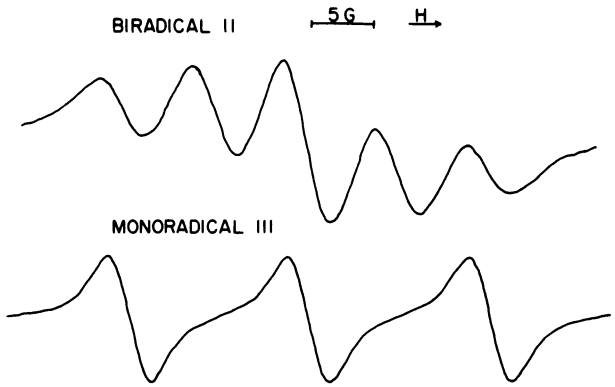

FIG. 2. *ESR spectra of monoradical III and biradical II*

$$H = A(S_1 \cdot I_1 + S_2 \cdot I_2) + JS_1 \cdot S_2$$

where A is the hyperfine coupling constant, J is the exchange integral, and I_1, I_2, S_1, and S_2 are the nuclear and electron spin operators. When $J >> A$, each of the electrons interacts with both nitrogen atoms and the ESR spectrum of the biradical exhibits coupling to 2 equivalent nitrogen atoms. The exchange interaction decreases exponentially with the separation of the orbitals containing the unpaired spins (12), and the ESR spectrum of the biradical indicates that the nitroxide groups are held relatively close to one another. The spectrum of the salt of the biradical consists of a single, unresolved line. The broadening of the ESR spectrum of the salt into a single line reflects an increase in the rotational correlation time for the protonated species in aqueous medium.

The relative widths of the hyperfine lines from the monoradicals depend on the magnitude of the anisotropic hyperfine coupling constant and the rotational correlation time of the molecules (1). Changes in the widths of the lines with solvent may reflect either a solvent dependence of the anisotropic coupling constant or a change in the rotational correlation time. As the rotational correlation time for nitroxide radicals increases in magnitude, one generally observes a broadening of the high-field transition. Broadening of this line is readily measured by monitoring the decrease in intensity of this signal with respect to the center and low-field transitions. The ratios of intensity of the center to high-field lines of the labeled monoradicals are listed in Table 1 along with the ratio for the TMPO nitroxide radical. The widths of the lines from the free bases in toluene are almost identical, indicating rapid rotation of the labeled molecules. The high-field lines of the salts in aqueous solutions are broader than those of the corresponding free bases and broader than that of the TMPO radical in aqueous solution. The anisotropic coupling of the TMPO radical is probably similar in magnitude to those of the labeled monoradicals in aqueous medium. If this is the case, the

broadening of the high-field transition of the spin labels in aqueous solution can be accounted for by an increase in the rotational correlation time of the spin-labeled salts in this medium. The increase in the rotational correlation time of the salts probably reflects hydration of the cations.

Pharmacological and Opiate Binding Studies

The analgesic potency, as evaluated by two techniques, of the 6-ester (III) is somewhat higher than that of morphine; the 3-ester (I) is a little less effective (Table 2). The codeine monoester (IV) possesses about $^1/_{10}$, and the morphine diester (II), $^3/_{10}$, the analgesic potency of morphine. A comparable potency relative to morphine was observed with the analogues on the basis of the Straub tail response in mice. Both the 6- and 3-esters produced a spectrum of behavioral effects resembling those of morphine in rats, including catatonia, postural rigidity, and marked lethargy. With the diester derivative of morphine, considerably less postural rigidity and narcosis were observed, and then only at 5–10 times the dose needed for the monoesters. The codeine monoester possessed only slight narcotic action and at doses above 20 mg/kg produced severe, prolonged myoclonic seizures.

A comparison of the relative binding affinities of the various analogues for a specific site on synaptic membranes revealed that the 6-ester was comparable to morphine; the 3-ester and codeine monoester had less than half, and the morphine diester, 18%, of the activity of morphine (Table 2).

Spin Label from Animal Brain

The spin-labeled narcotics are hydrolyzed very slowly at physiological pH values, but enzymatic hydrolysis *in vivo* could be much more rapid. If the spin labels are rapidly removed by enzymatic hydrolysis to yield free morphine or codeine, the experiments on analgesic potency *in vivo* would reflect only the relative rates at which the labeled molecules are hydrolyzed. If this were the case, the physiological effect of the spin labels should be identical with that of either morphine or codeine. The myoclonic seizures produced by the codeine monoester (IV) cannot be due to the hydrolysis product (codeine or TMPO) and indicate that this molecule is probably intact when it is bound to a receptor site within the animal's brain. It is also possible for part of the physiological activity of this drug to be produced by the hydrolysis product (codeine, analgesia) and part by the intact spin label (myoclonic seizures).

It is necessary to determine the relative rates of enzymatic hydrolysis of the spin-labeled drugs *in vivo* for an understanding of the mechanism by which these molecules produce analgesia. The ester group at position 3 is normally hydrolyzed more readily than the ester group at position 6 in molecules of this type. The rate of hydrolysis of the 3-ester group in either spin label I or II will therefore give an upper limit on the relative rate of hydrolysis of any of the spin labels. To determine the

TABLE 2

Morphinomimetic potencies of various spin-labeled derivatives of opiates

Opiate	Relative analgesic potency	Straub tail	Inhibition of [^{3}H]dihydromorphine binding[a]	Comments
			%	
Morphine	1.0	1.0	100	10 mg/kg: catatonia
Morphine 6-TMPO ester (III)	1.2	1.4	85	10 mg/kg: catatonia
Morphine 3-TMPO ester (I)	0.7	1.0	40	10 mg/kg: catatonia
Codeine TMPO ester (IV)	0.1	0.1	38	15–20 mg/kg: strong convulsions
Morphine 3,6-diTMPO ester (II)	0.3	0.2	18	Weak catatonia

[a] Inhibition of 0.01 μM [^{3}H]dihydromorphine binding to synaptic membranes by 0.1 μM test opiate.

relative rate of enzymatic hydrolysis *in vivo*, we conducted a series of ESR experiments with the TMPO diester (compound II). If this molecule is hydrolyzed to lose the ester group at position 3, it is transformed into monoradical III, and the ESR spectrum will change from the five-line pattern characteristic of the biradical to a three-line spectrum characteristic of the monoradicals. If the ester group is also removed from position 6, only the ESR spectrum from the TMPO radical will be observed (three lines).

Experiments were conducted in which 5 mg/kg of the morphine diester was administered to rats. After the animals had developed pronounced analgesia (about 30 min), a sample of blood and the brain were removed and the spin label was extracted from these samples. Large ESR signals were observed from both samples, with the intensity of the signal from blood being about 100 times larger than that from brain. The five-line pattern from the biradical was observed for both types of samples. Except for very small changes in the relative intensities of the lines, the spectra were identical with the biradical spectrum shown in Fig. 2. If the spin-labeled biradical were partially hydrolyzed, one would observe a superposition of ESR spectra from the biradical, monoradical III, and TMPO. The three lines in the spectra of monoradical III and TMPO would overlap with the center and outer lines in the spectrum of the biradical, but not with the two middle lines. Measurement of the intensity of the outer relative to the middle lines in the experimental spectrum indicated that at least 96% of the signal came from the biradical. These experiments indicate that enzymatic hydrolysis of the spin-labeled esters is a relatively slow process and that the physiological effects of these drugs are probably due to the intact molecules rather than to a hydrolysis product. Even if it is assumed that a small percentage of the diester was converted to morphine, the amount of morphine would probably have been insufficient to produce analgesia. It has been reported that 1 hr after the injection of 5 mg/kg of [^{14}C]morphine the concentration of morphine in total rat brain was about 0.2 μg/g, wet weight (13). From the ESR data it was calculated that the amount of morphine diester in the brain was about 0.05 μg/g. It is therefore highly unlikely that the hydrolysis product, if any, was present in an amount sufficient to have a pharmacological action.

ESR Studies of Binding to Synaptic Membrane

The experiments with the biradical *in vivo* show that spin labels of this type may be a useful supplement to radioactively labeled molecules to determine the disposition of the labeled drug within various parts of the animal's body. A second use of the spin-labeled drugs involves studies of their binding to nerves or nerve components *in vitro*. We carried out a series of ESR experiments with mixtures of the spin-labeled 6-ester (compound III) and isolated synaptic membrane. When the spin-labeled drug is bound to the membrane its motion is restricted, and there should be a characteristic change in the ESR spectrum. If all of the spin-labeled drug is bound and its motion is completely restricted, an ESR spectrum characteristic of the rigid lattice limit should be observed. In this case anisotropic splitting of the nitroxide radical will be seen, and the ESR lines will be shifted and broadened with respect to a freely rotating radical in solution. If the motion of the radical is only partially restricted, a characteristic broadening of lines in the ESR spectrum, indicative of hindered rotation, is expected. If there is an equilibrium between bound and unbound spin label, superposition of spectra from molecules in the bound and unbound states will occur.

Our experiments involved investigation of samples of isolated synaptic membrane (28 mg of protein per milliliter) with varying concentrations of spin label III The ESR spectra of these mixtures were taken and compared with spectra from spin label III at the same concentration. A third set of samples containing the same concentration of membrane and

spin label plus about a 10-fold excess of naloxone were also monitored. The intensities of the ESR lines from samples of membrane and spin label were smaller in each instance than the signals from standard samples of the spin label alone. Addition of naloxone to the mixtures of spin label and membrane led to an increase in the intensity of the ESR signal, but the signal did not completely recover to the intensity of the standard. Figure 3 shows characteristic changes in the intensities of the low-field transitions of the three types of samples.

The ESR spectra of mixtures of the spin label and membrane showed lines only from the unbound radical, indicating that the lines from the bound species were broadened and shifted so that they could not be observed in the presence of the relatively high concentration of unbound spin label. This type of behavior is predicted if there is an equilibrium between bound and unbound spin label, with immobilization of the bound species. The decrease in the intensity of the signal from the unbound label with respect to the standard can be used to calculate the concentrations of both the bound and unbound spin labels. The partial recovery of the signal intensity when naloxone was added to the membrane-label mixture indicates that naloxone molecules replaced the spin label at some but not all of the binding sites. Plots of the concentration of bound spin label per gram of membrane protein against the concentration of unbound spin label for the membrane-label and membrane-label-naloxone samples are shown in Fig. 4.

The spin-labeled drug can be bound by both lipids and proteins within the synaptic membrane, giving multiple equilibria for binding. Because our samples were tested at higher concentrations than are normally used for binding studies with radioactively labeled molecules, equilibria which are unimportant in samples of lower concentration should be observed. The difference between the concentration of bound spin label in the absence and presence of naloxone permits estimation of the number of binding sites preferentially occupied by a naloxone molecule. We estimate that naloxone replaced the

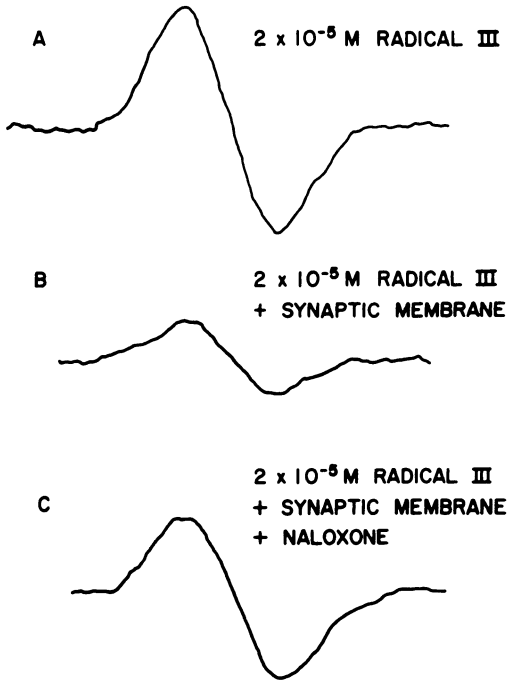

FIG. 3. *Low-field ESR lines*

A. Radical III, 20 μM solution. B. Mixture of 20 μM radical III and the synaptic membrane preparation containing 28 mg of protein per milliliter. C. Mixture of 20 μM radical III, synaptic membrane, and excess naloxone.

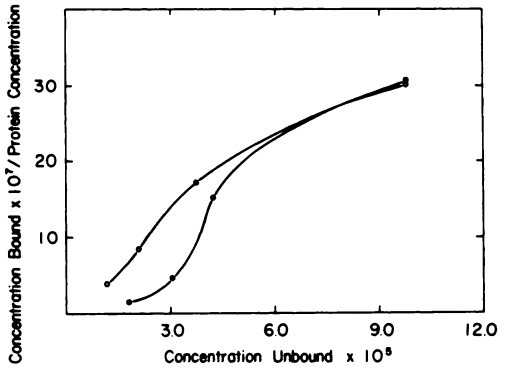

FIG. 4. *Molar concentration of bound radical, divided by protein concentration (grams per liter), plotted against molar concentration of unbound radical*

O, mixtures of radical and synaptic membrane; ●, mixtures of radical, synaptic membrane, and excess naloxone.

spin-labeled drug at about 10^{16} binding sites per gram of brain, wet weight. Experiments with radioactively labeled samples have indicated that there are about 10^{13} stereospecific binding sites per gram (7). The difference in these numbers is probably due to differences in the relative importance of the various possible equilibria for binding in the two concentration regions. Our experiments were carried out down to the limit of sensitivity of an older, relatively insensitive ESR spectrometer. Experiments with a newer spectrometer would enable the concentration range of these investigations to be extended by a factor of about 100.[2] Experiments of this type should allow precise determination of binding constants and numbers of binding sites.

The experiments reported in this paper indicate that ESR studies of spin-labeled analgesics will be a valuable technique for the investigation of binding to various types of substrate molecules. This technique can also be used to study the motion of complexes of the spin-labeled molecule and lower molecular weight substrates. We are currently studying the binding of the labeled molecules to a series of phospholipids and some small proteins. We hope to be able to extend the study of binding to synaptic membrane to samples of lower concentration.

[2] The ESR spectrometer used in these studies had a sensitivity of about 5×10^{11} spins/G. Newer instruments can detect about 5×10^{9} spins/G.

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ADDENDUM

Through use of a newer, more sensitive Varian E-12 spectrometer, it has been possible to observe binding of opiate spin labels at concentration levels normally used in the radiotracer method. The ESR determination indicated that there were 5×10^{-13} moles bound l^{-} mg membrane protein. This value is almost identical to that obtained with radioactive binding studies involving similar membrane preparations.