ELECTRON SPIN RESONANCE STUDY OF THE SYNAPTOSOME OPIATE RECEPTOR

KINETICS OF STEREOSPECIFIC BINDING OF SPIN LABELED MORPHINE

EDMUND S. COPELAND *and* LARISSA DEBAARE

From the Division of Biochemistry, Walter Reed Army Institute of Research, Washington, D. C. 20012. Dr. Copeland's present address is Westwood Building 353, National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT Morphine, spin labeled on the 3- or 6-position has been used as the opiate ligand in a study of the time course of stereospecific opiate binding to intact synaptosomes isolated from non-cerebellar rat brain. The broadening of electron spin resonance lines induced by immobilization of the ligand on binding has been used to determine the concentration of bound opiate. The stereospecificity of the reaction was measured by comparing ligand binding in the presence of thousand-fold molar excesses of dextrorphan or levorphanol. Using both static and flow techniques, the binding process has been continuously monitored at times greater than 4.8 s after mixing spin labeled morphine with synaptosomes. It is shown that for this ligand and receptor preparation, binding takes place primarily during a delayed, abrupt process whose rate and time of onset are temperature dependent and reflect the presence of added opiate agonist or antagonist.

INTRODUCTION

We have recently reported certain benefits to be gained from the use of a spin labeled opiate probe in studies of the opiate receptor (1). Thus, the ability of the technique to differentiate bound and free probe simultaneously, without sample destruction or modification, seems to circumvent one great limitation of conventional radiolabel probe studies of the opiate receptor. Although the potential perturbation of the molecular behavior of an opiate probe caused by the spin label moiety remains a feature of the technique which must be kept in mind, its positive features appear well suited to study the kinetics of the opiate-receptor interaction. In those instances where opiate-receptor binding vs. time has been determined using radiolabel techniques (2–4), sampling of the reaction mixture at various incubation times, quenching with cold, and separating bound from unbound radioactive opiate in the cold have been required. Difficulties of such procedures have been recently enumerated (5). The immensity of the task required to obtain extensive details of the binding curve might deter attempts using conventional methods to obtain the information to be gleaned from such a study.

The use of a spin labeled opiate probe to study opiate-receptor binding kinetics

seems a good example of how this biophysical technique can complement conventional procedures. We have used the displacement procedure (levorphanol vs. dextrorphan) elaborated by Goldstein et al. (6) to determine both the *rate* of stereospecific binding (SSB) of spin labeled morphine (SLM) continuously during incubation in the electron spin resonance (ESR) cavity and the *extent* of SSB following incubation. We have monitored the binding reaction after about 2 min with a static analysis and after 4.8 s using flow and stop-flow procedures. The results indicate that opiate-receptor binding is not continuous during an incubation but takes place primarily in one abrupt event preceded and followed by periods of little binding.

MATERIALS AND METHODS

The two spin labeled morphine analogs, 3-SLM with the 3-(acetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl moiety attached via an ether linkage to the 3-position of morphine (1) and 6-SLM where 4-amino-2,2,6,6-tetramethyl-1-piperidinyloxyl was linked directly to the 6-position by a halogen displacement reaction with the 6-chloromorphide (7), were prepared and characterized as previously described (1, 7).

Dextrorphan tartrate (*d*-3-hydroxy-N-methylmorphinan tartrate) and levorphanol tartrate (*l*-3-hydroxy-N-methylmorphinan tartrate) were donated by Hoffmann-LaRoche, Nutley, N.J. Nalorphine HCl was obtained from Applied Science Laboratories, State College, Pa.

Noncerebellar synaptosomes were isolated in the cold as previously described (1, 8) from rat brain homogenate.[1] The crude mitochondrial fraction in 0.32 M sucrose was layered on a discontinuous gradient (15 ml 0.8 M sucrose, 10 ml 1.2 M sucrose) and centrifuged in a Beckman SW27 rotor at 100,000 g_{max} for 90 min (Beckman Instruments, Inc., Fullerton, Calif.). The band between 0.8 M and 1.2 M sucrose (synaptosomes) was collected, separated, and resuspended in 0.32 M sucrose of the desired pH (0.05 M Tris buffer) before determining protein content (9).

The X-band ESR spectrometer (E-9, Varian Associates, Palo Alto, Calif.) used has been described elsewhere (1). The magnetic field was modulated at 100 kHz using 2.5 G amplitude and a microwave power of 16 mW incident on the TE_{104} dual cavity. SLM concentrations were determined using an on-line computer as previously reported (1). Most data were collected with the field sweep reduced to zero ($\Delta H = 0$), monitoring relative line amplitude vs. time (vide infra). Control of sample temperature was accomplished with a variable temperature controller (Varian) coupled to either a variable temperature insertion Dewar (S-821, James F. Scanlon Co., Solvang, Calif.) or a variable temperature liquid flow cell (Scanlon, S-865). Samples held in 100 μl capillary pipettes were placed in a 3 mm ID quartz ESR tube filled with a low dielectric oil and suspended in the insertion Dewar. Alternatively (vide infra), synaptosomes and reagents were pumped independently from reservoirs (0–4°) at 0.23 ml/min to the controlled temperature mixing chamber and flat cell where they were mixed and observed at 37°. The sample temperature was continuously monitored by a thermocouple positioned in the sample stream just outside the cavity sensitive volume. Samples were observed during flow, 4.8 s after mixing or as a function of time after flow was stopped. Incubations were thus performed in the controlled temperature ESR cavity during observation.

For each experiment, a calibration curve relating the change in observed line amplitude ($M = -1$ or $+1$) to the change in free SLM concentration was determined. It was found that

[1] In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

the *slope* of such curves (centimeters per picomole SLM per milliliter) was independent of the concentration of synaptosomal protein in the range used when 5 mM nalorphine was also present. The slope could thus be validly determined without synaptosomes. A calibration factor, k, was then derived from the slope and the synaptosomal protein concentration,

$$k = [(\text{cm/pmol/ml})(\text{mg/ml})]^{-1} = \text{pmol/mg/cm}, \quad (1)$$

for use in expressing line amplitude decreases in terms of picomoles SLM bound per milligram of synaptosomal protein (vide infra). The calibration factor was dependent on observation temperature and on the nature of the spin label moiety on morphine. This factor was determined for stopped flow conditions by mixing SLM concentration standards with 0.32 M sucrose in the liquid flow cell and measuring absolute line amplitudes during flow and stop-flow conditions.

RESULTS

Binding Kinetics—Static System

The capillary pipette system just described was used to obtain the binding curve of 3-SLM with synaptosomes shown in Fig. 1. This curve, whose general shape is repre-

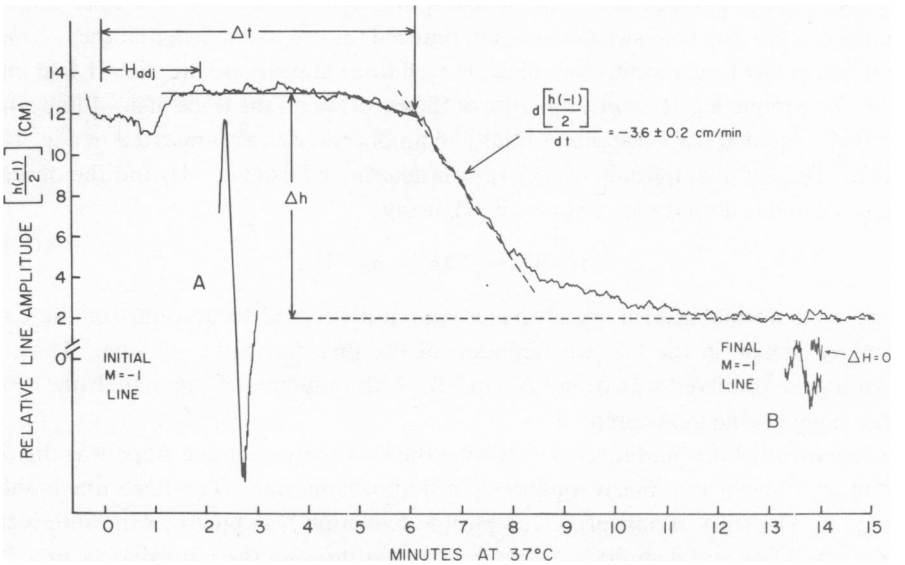

FIGURE 1 Binding curve of 3-SLM with rat brain synaptosomes. Using the static capillary pipette system described in the text, a sample containing 10.1 mg/ml synaptosomal protein and 1 μM 3-SLM in 0.32 M sucrose (pH 7.5) was incubated at 37° in the spectrometer cavity and the relative amplitude of the −1 line maximum was monitored as a function of time. Decreases of line amplitude are interpreted as due to loss of free 3-SLM by binding (see text). ESR observation characteristics are described in the text. *Inserts A* and *B* show the −1 line before and after the incubation depicted. Determination of the binding curve parameters here illustrated (H_{adj}, magnetic field adjustment; abscissa origin; Δt, time before abrupt decrease, and Δh, total observed line amplitude decrease) is described in the text. After completing the final sweep of the whole −1 line (*insert B*), the $\Delta H = 0$ field location was confirmed to be at the line maximum. The trace is included in *insert B*. Relative spectrometer gain, binding curve/inserts = 5/3.2 using 10 and 1 s filter time constants, respectively.

sentative of those observed in this study when an opiate agonist or antagonist was also present, was obtained when 20 μl 0.32 M sucrose (pH 7.5) (or "buffer") was combined with 20 μl 3-SLM and 60 μl of a freshly prepared synaptosomal suspension producing a sample that contained 10.1 mg/ml synaptosomal protein and 1 μM 3-SLM. Mixing was carried out at 0–4° and the sample was immediately withdrawn into a capillary pipette and placed in the spectrometer cavity at 37° with 75 s elapsing between mixing and beginning the ESR trace. The magnetic field of the spectrometer had been previously set at the maximum of the $M = -1$ line of 3-SLM. The time at which the sample reached 37° (zero time) was marked when the spectrometer detector current became constant after sample introduction. The final 20 s of this temperature equilibration can be seen as the initial amplitude decrease in Fig. 1. The magnetic field was then adjusted to confirm that the -1 maximum was being monitored. On the trace illustrated, this adjustment required 1.9 min (usually < 1 min). Consequently, not until after this time could binding (reduction in line amplitude) be observed.[2] After the sample had been at 37° for about 4 min, the line amplitude began to fall, the rate of decrease being maximal from 6 to 8 min and no further decrease being noted after 11 to 12 min. Immediately after the 15 min trace, the complete -1 line was scanned and is depicted in Fig. 1 (insert B). The notation, $h(-1)_f$, is used for this final absolute line amplitude. The field sweep was again reduced to zero to confirm that the -1 maximum had in fact been monitored. The $\Delta H = 0$ trace appears beside the -1 line maximum. To complete a descriptive picture of the experiment, the trace of a -1 line whose amplitude equaled the calculated initial line amplitude was also inserted in Fig. 1 (insert A). The initial amplitude or $h(-1)_i$ was determined from $h(-1)_f$ and the observed reduction in line amplitude, Δh (see Fig. 1), using

$$h(-1)_i = 2\Delta h + h(-1)_f, \tag{2}$$

where Δh is normalized for spectrometer gain and doubled to account for the corresponding change in the unseen minimum of the first derivative -1 line. Thus, the transition we observed was from "A" to "B" with only half of the actual line amplitude changes being monitored.

The portion of the binding curve showing the maximum negative slope was digitized and fit by a linear least mean squares (LMS) approximation. The LMS line is shown in Fig. 1. The start of abrupt line amplitude reduction was taken as the intersection of the LMS line and a similar straight line drawn through the immediately preceding data and is indicated by the vertical arrow in Fig. 1. The time elapsed at the incubation temperature before abrupt reduction, Δt, varied for a given synaptosome preparation according to added agonist or antagonist concentration, incubation temperature and the storage time at 0–4° before observation (vide infra).

Curves similar to that shown in Fig. 1 were observed for samples containing 1 mM dextrorphan, levorphanol, or nalorphine in 0.32 M sucrose (pH 7.5) with synap-

[2] Reduction in line amplitude and binding are used interchangeably according to hypotheses developed in Ref. 1.

TABLE I

STEREOSPECIFIC BINDING OF 3-SLM BY RAT BRAIN SYNAPTOSOMES*

Additive	$h(-1)_f$†	Δt§	$d[h(-1)/2]/dt$‖	BR¶
	cm	*min*	*cm/min*	*pmol/mg/min*
Buffer	2.6	6.1	-3.65 ± 0.24	45.2 ± 3.0
Nalorphine (1 mM)	8.5	8.0	-1.93 ± 0.23	23.9 ± 2.8
Dextrorphan (1 mM)	6.6	6.3	-2.95 ± 0.19	36.5 ± 2.4
Levorphanol (1 mM)	7.2	6.6	-2.21 ± 0.17	27.4 ± 2.1

Sample calculations

$SSB = k\{[h(-1)_f]_{lev} - [h(-1)_f]_{dex}\} = 9.68(0.6) = 5.8$ pmol/mg

$SSBR = BR_{dex} - BR_{lev} = 36.5 - 27.4 = 9.1$ pmol/mg/min

*Data taken from the experiment described in Fig. 1. Synaptosomal protein concentration was 10.1 mg/ml, the 3-SLM ligand was 1 μM. These and the additives were suspended in 0.32 M sucrose (pH 7.5; 0.05 M Tris) with no exogenous sodium being added. The incubation during ESR observation was at 37° and lasted 16 min (see text).

†The final line amplitude (see Fig. 1 and text).

§Incubation time before abrupt line amplitude decrease.

‖The maximum slope of the binding curve (see Fig. 2 and text).

¶The observed binding rate (see text).

tosomes and 3-SLM. The portion of each binding curve containing the abrupt reduction of line amplitude was fitted by linear LMS approximation and is shown in Fig. 2. The relative vertical displacement of the curves was determined from $h(-1)_f$ values which are listed in Table I. It can be seen from Fig. 2 that levorphanol and nalorphine prolonged the time before abrupt binding began and reduced the rate of that binding. Quantitative binding data are presented in Table I. The $h(-1)_f$ values show that the greatest amount of 3-SLM was bound when both stereospecific and nonspecific binding (NSB) occurred (buffer). Nalorphine was more effective than levorphanol at the same concentration (1 mM) in preventing 3-SLM binding. Dextrorphan allowed more binding than its active enantiomer (levorphanol) but prevented a significant fraction of the 3-SLM binding occurring in buffer alone. This latter observation suggests that introduction of the spin label moiety at the 3-position may lower the receptor affinity of the resulting molecule sufficiently that dextrorphan at 1,000 times molar excess can compete for the receptor even though its affinity is more than three orders of magnitude lower than that of morphine (2, 10). Final line amplitudes have been converted to picomoles of 3-SLM bound per milligram of synaptosomal protein using the relationships:

$$SSB_{total} = k\{[h(-1)_f]_{lev} - [h(-1)_f]_{dex}\} = 5.8 \text{ pmol/mg}, \quad (3)$$

where SSB_{total} is the stereospecific binding observed after the 16 min incubation illustrated in Fig. 1, and k, determined as previously described, was 9.68 pmol/mg per cm for this study; and,

$$NSB = TB - SSB, \quad \text{where } TB = k[2(\Delta h)_{buf}], \quad (4)$$

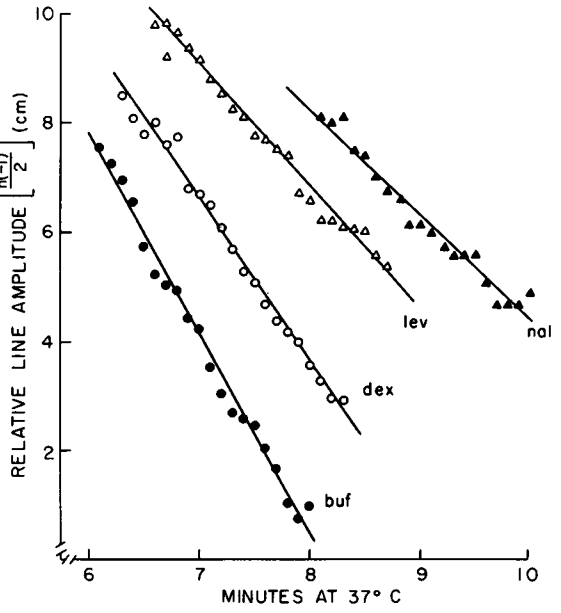

FIGURE 2 Abrupt line amplitude decreases in the presence and absence of SSB. Binding curves similar to Fig. 1 were obtained in the presence of 1mM dextrorphan (dex), levorphanol (lev), or nalorphine (nal) using the procedures described for buffer (buf). That portion of each curve having maximal negative slope was digitized at 0.1 min intervals and fitted by linear LMS approximation (see text). The slopes of these curves are tabulated in Table I and used to determine the rate of SSB. •, Buffer; ○, dextrorphan; △, levorphanol; ▲, nalorphine.

TB or total binding being the sum of NSB and SSB, and $(\Delta h)_{buf}$ is as illustrated in Fig. 1. Nonspecific binding was approximately 139 pmol/mg for this synaptosome preparation. Thus, at the high concentrations necessary for this spin label investigation, the NSB/SSB ratio was 23/1, a considerably higher value than previously estimated (1) when levorphanol and dextrorphan were not used to measure SSB.

It must be shown that the abrupt decreases in line amplitude observed in Figs. 1 and 2 are related to SSB and that they represent a significant event in this binding phenomenon. Findings which substantiate this hypothesis are the variations in the presence of buffer, dextrorphan, levorphanol, and nalorphine of the abrupt decrease parameters, (*a*) incubation time, Δt, and (*b*) the rate of line amplitude change, $d[h(-1)/2]dt$, and the correlation of these parameters with the total binding values just calculated. In buffer, where both NSB and SSB occur, Δt was 6.1 min and in dextrorphan where similar binding types should occur, Δt was 6.3 min. With levorphanol and nalorphine, this time increased to 6.6 and 8.0 min, respectively. That the time of binding is not the more important parameter was demonstrated when this experiment was repeated about 7 h after isolation of synaptosomes which had been stored at 0–4°. Although there was still evidence for SSB (2.9 pmol/mg), only 0.7 min separated the four Δt values (see Table II).

The slope of the abrupt line amplitude decrease, on the other hand, was found in every case investigated to parallel the magnitude of the binding measured after incubation. The slopes of the lines in Fig. 2 are listed in Table I with standard errors derived from LMS analysis. Although it is clear that the binding process of which the segment analyzed is a part probably follows exponential kinetics, a linear regression model fits this segment about as well as parabolic, power function, or exponential models. Hence, for simplicity and because of the fairly low signal to noise ratio encountered, the linear model was adopted. Thus,

$$\mathrm{BR} = 2k\{\mathrm{d}[h(-1)/2]/\mathrm{d}t\} \quad \text{pmol/mg/min}, \tag{5}$$

where BR is the binding rate, $\mathrm{d}[h(-1)/2]/\mathrm{d}t$ is the LMS slope of the abrupt decrease (see Figs. 1 and 2) in centimeters per min, 2 is the doubling term, and k is as previously defined. The standard errors of the slope determination were used to estimate the minimum error associated with the binding rates listed in Table I. By an argument analogous to that underlying Eq. 1, these data were used to evaluate stereospecific and nonspecific binding rates:

$$\mathrm{SSBR} = \mathrm{BR}_{\mathrm{dex}} - \mathrm{BR}_{\mathrm{lev}} = 36.5 - 27.4 = 9.1 \text{ pmol/mg/min}, \tag{6}$$

and similarly,

$$\mathrm{NSBR} = \mathrm{BR}_{\mathrm{total}} - \mathrm{SSBR} = 45.2 - 9.1 = 36.1 \text{ pmol/mg/min}, \tag{7}$$

giving a binding *rate* ratio (NSBR/SSBR) of 4/1. When binding rates were measured after synaptosome storage for 7 h, those for buffer and dextrorphan remained approximately the same but those for nalorphine and levorphanol fell to about half of the initial values. Although these data imply that some change in the synaptosomal opiate receptor or in its environment had occurred and inversely affected the rates of SSB and NSB, reducing the binding rate ratio to unity (see Table II), they are insufficient to clarify the phenomenon.

Effect of Temperature

When the experiment just described was conducted at a 30° incubation temperature, both the time of abrupt binding and the binding rates were affected (see Table II). Binding rate data treated with the Arrhenius relationship,

$$k'(T) = A_o \exp(-E_a/RT) \tag{8}$$

where A_o is a constant, E_a is the energy of activation in kilocalories per mole, R is the Rydberg gas constant, T is the absolute temperature, and BR is used as the rate constant, k', gave the energies of activation listed in Table II. The data suggest that the nonspecific binding process had no measurable energy of activation whereas about 18 kcal/mol were associated with stereospecific binding. However, because these data were obtained using synaptosomes stored at 0–4° for 7–8 h and the extent of SSB had changed from its initial value (Table I) and because only two temperatures were used in the derivation, the activation energies must be considered only as order of magni-

TABLE II

EFFECT OF INCUBATION TEMPERATURE ON STEREOSPECIFIC BINDING*

Additive	Temp.	$h(-1)_f$†	Δt	BR†
	°C	*cm*	*min*	*pmol/mg/min*
Buffer	37	2.4	7.4	42.7
	30	3.0	11.8	33.0
Nalorphine	37	10.9	8.0	12.7
	30	10.8	12.4	6.8
Levorphanol	37	7.9	7.3	13.3
	30	7.9	—	—
Dextrorphan	37	7.6	7.5	34.6
	30	7.1	14.8	17.7
		TBR	SSBR§	NSBR
	37	42.7	21.3	21.5
	30	33.0	10.9	22.1
E_a(kcal/mol)		6.9	17.8	−0.7

*Continuation of the experiment of Fig. 1 using synaptosomes which had been stored at 0–4°C for 7–8 h. Incubations at 30° lasted 20 min. All other conditions as in Fig. 1 and Table I.

†Tabulated values have been corrected for the temperature effect on line amplitude.

§Data for SSB calculated using nalorphine.

tude estimates. When the incubation temperature was lowered to 2°, there was no change in −1 line amplitude from a buffer sample during a 45 min incubation, suggesting that both SSB and NSB have positive activation energies.

Results with 6-SLM as Ligand

Concurrently with these experiments, similar studies were performed using 6-SLM as the ligand (not illustrated). While the SSBR was about the same as found with 3-SLM (cf. 29.4 ± 7.2 vs. 21.3 ± 3.6 pmol/mg per min for 6-SLM and 3-SLM, respectively), there was a complete loss of the −1 line in all samples after 10 min at 37°. Even with 5 mM nalorphine, there was no evidence for free 6-SLM after incubation. Thus, the extent of SSB could not be evaluated. That the binding rates of 6- and 3-SLM varied in parallel in the presence of the various additives suggests that the same pharmacologic process was being monitored but it is very likely that some process other than binding contributed to the complete loss of the −1 line. The binding rates with each additive were greater for 6-SLM. The fact that 6-SLM binding rates in levorphanol and nalorphine had the greatest increase over 3-SLM data when the ligand should have remained free, suggests that reduction of the label rather than enhanced binding was responsible. A high affinity for the receptor would have been expected to cause the greatest binding rate increase for dextrorphan and buffer samples. Further data are required for the 6-SLM results to be understood.

Binding Kinetics—Stop-Flow System

Binding which might have taken place between the time of mixing at 0–4° and the completion of field adjustment when the samples had been at incubation temperature for 1–2 min, i.e., the "hidden time," could not have been examined with the static sample system. Hence, in order to confirm that the static results relate to the actual binding phenomenon, we examined the events taking place in this hidden time. The flow system previously described was used to introduce 3-SLM and levorphanol or dextrorphan into the mixing chamber together with synaptosomes and then to the flat cell at 230 μl/min such that the synaptosomes had been exposed to the ligand for 4.8 s when the center of the cavity sensitive volume was reached. Each reactant had been exposed to ambient temperature (20°) for about 5 min and to 37° for about 30 s at the time of mixing. Thus, the flow experiment examined all but the first 4.8 s of the "hidden" time.

The data (not illustrated) revealed no consistent variations in line amplitude from that during flow until 2 to 3 min after flow had been stopped. When the mixed sample contained 1 mM dextrorphan and 7.25 mg/ml synaptosomal protein in 0.32 M sucrose (pH 8.0), there was a slow line amplitude decrease from 2 min to about 6 min, a rapid decrease from 6 to 7 min and then a slow decline from 10 to 15 min after stop-flow (cf. Fig. 1). Levorphanol at the same concentration prolonged the onset of abrupt line amplitude decrease until about 8 min and after 9 min following stop-flow, there was little additional decrease. SSB could first be discerned about 11 min after stop-flow. Discontinuous monitoring precluded determination of binding rates. Total stereospecific binding calculated as before was about 20.1 pmol/mg after a 17 min, 37° incubation. Thus, within the limits of experimental error, there was no evidence for any binding until about 2 min after mixing incubation temperature reagents. Since all the static experiments produced on-line real-time data by this time, their relevance is strengthened. The fact that final line amplitudes, when adjusted for the amplitude reduction actually observed, gave values for amplitude before binding (see Eq. 2) which agreed with concentration standards, indicates that only minimal binding could have occurred in the "hidden" 4.8 s. The nearly four times greater SSB measured during the stop-flow study at pH 8, 7.25 mg/ml protein is consistent with results (unpublished) showing that these two experimental conditions are closer to optimal for spin labeled ligand and synaptosome receptor (pH 8.0, 160/1 ligand to protein ratio, picomoles per milligram).

DISCUSSION

The events of the stereospecific opiate binding process reported here are probably inaccessible using conventional radiolabel procedures (see, for example, ref. 5). The fact that use of radiolabeled antagonists or agonists requires a washing and filtering step to remove unbound material not only precludes the continuous sampling desired for a kinetic experiment but also may remove weakly bound material, leave unbound

radioactive material trapped in cytoplasmic spaces (e.g., refs. 5, 11, 12) or allow the binding reaction to proceed beyond the nominal sampling time. The curve describing the time course of 2 nM [^{3}H]dihydromorphine binding to corpus striatum homogenate obtained by Wong and Horng (3) differs considerably from our results (Fig. 1). At 37°, they found that SSB had reached its half-maximum level in about 3 min. Moreover, the binding rate appeared constant over this time. There was no evidence for a delayed, abrupt binding. Pert and Snyder (2) have reported that 5 nM [^{3}H]naloxone SSB at 37° to rat brain homogenate reached its half-maximum level after a 2 min incubation. It is conceivable, then, that the prolonged period prior to an abrupt binding seen in our study could be due to the modification of the opiate ligand.[3] It could also be considered that the ability of the spin label method to distinguish bound and free ligand continuously without the possible errors introduced during washing and filtering may have been sufficient to detect events not previously found.

The principal limitation of the spin label method employed is the comparatively high ligand concentration required for ESR detection. Thus, the 1 μM ligand concentration used was at least 200 times higher than that below which nonspecific ligand binding becomes insignificant (10). Consequently, we have used the displacement technique of Goldstein et al. (6) to distinguish SSB in our system where less than 5% of the total binding observed was stereospecific. The ratio of NSB/SSB (23/1) observed is similar to that obtained by Goldstein et al. in earlier studies using a radiolabeled ligand at a comparable concentration (2 μM [^{3}H]levorphanol; NSB/SSB = 49/1) (6). There have been no previous reports where either the binding rate or activation energy of SSB has been specifically estimated. Creese et al. (5) have extensively studied the effect of temperature on agonist vs. antagonist binding after incubation to equilibrium, but did not specifically consider the activation energy of the binding reaction. The Q_{10} of SSB ([^{3}H]naloxone, whole brain homogenate) from 25 to 35° has been reported to be 1.5 (2) or approximately 7.4 kcal/mol (calculated from Q_{10} with Eq. 8). The order of magnitude estimate of SSB activation energy, 18 kcal/mol (Table II), is somewhat higher as might be expected for a ligand with increased bulk and decreased receptor affinity.

The characteristic shape of the binding curves we have observed seems consistent with the concept that the opiate receptor has two conformations, agonist and antagonist, which are in equilibrium and that the position of the equilibrium may be shifted by factors such as sodium ion concentration, temperature, and the presence of agonists or antagonists (4, 5, 13, 14). In isolated synaptosomes, the equilibrium might be shifted toward the "antagonist" conformation. Initial binding of the ligand to the few "agonist" receptors might shift the equilibrium toward that conformation, precipitating the binding cascade observed. The lower affinity of the spin labeled ligand

[3] Similar very slow initial binding has apparently been observed using standard procedures (i.e., radiolabeled ligand, washing and filtration) giving much higher temperature coefficients than for simple binding interactions and suggesting that a conformation change of some sort is required as part of the binding process. Such conformation change might be related to some kind of aggregation of receptors in the fluid membrane as is now suggested for various antibody receptors. (A. Goldstein, 1976, personal communication).

may expand the time scale of the phenomenon, exaggerating events which occur before the first sampling in a radiolabeled ligand experiment.

The authors thank Doctors P. Siu, C. Chignell, and A. Goldstein for helpful discussions and for their critical reading of the manuscript.

Received for publication 2 June 1976.

REFERENCES

1. COPELAND, E. S., M. E. BOYKIN, J. A. KELLEY, and M. P. KULLBERG. 1975. An electron spin resonance study of synaptosome opiate receptors. The preparation and use of a spin labeled morphine. *Biophys. J.* **15**:1125.
2. PERT, C. B., and S. H. SNYDER. 1973. Opiate receptor: demonstration in nervous tissue. *Science (Wash. D.C.).* **179**:1011.
3. 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.
4. SIMON, E. J., J. M. HILLER, J. GROTH, and I. EDELMAN. 1975. Further properties of stereospecific opiate binding in rat brain: on the nature of the sodium effect. *J. Pharmacol. Exp. Ther.* **192**:531.
5. CREESE, I., G. W. PASTERNAK, C. B. PERT, and S. H. SNYDER. 1975. Discrimination by temperature of opiate agonist and antagonist receptor binding. *In* The Opiate Narcotics. Neurochemical Mechanisms in Analgesia and Dependence. A. Goldstein, editor. Pergamon Press, New York. 85.
6. GOLDSTEIN, A., L. I. LOWNEY, and B. K. PAL. 1971. Stereospecific and nonspecific interactions of the morphine congener levorphanol in subcellular fractions of mouse brain. *Proc. Natl. Acad. Sci. U.S.A.* **68**:1742.
7. CELLA, J. A., and J. A. KELLEY. 1976. Preparation of spin labeled opiates: morphine and codeine. *J. Pharm. Sci.* In press.
8. 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.
9. 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.
10. 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.
11. GOLDSTEIN, A. 1974. Opiate receptors. *Life Sci.* **14**:615.
12. HUG, C. C., JR., and T. OKA. 1971. Uptake of dihydromorphine-^{3}H by synaptosomes. *Life Sci.* **10**: 201.
13. PASTERNAK, G. W., and S. H. SNYDER. 1975. Identification of novel high affinity opiate receptor binding in rat brain. *Nature New Biol.* **253**:563.
14. KLEE, W. A., S. K. SHARMA, and M. NIRENBERG. 1975. Opiate receptors as regulators of adenylate cyclase. *In* The Opiate Narcotics. Neurochemical Mechanisms in Analgesia and Dependence. A. Goldstein, editor. Pergamon Press, New York. 117.