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Scatchard Analysis of Opiate Receptor Binding

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SUMMARY

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The study of [^{3}H]naltrexone binding in a membrane preparation from rat brain revealed that experimental conditions of the opiate receptor assay markedly influenced the outcome of the corresponding Scatchard analysis. The use of inappropriate concentrations of the unlabeled displacing drugs to assess stereospecific [^{3}H]naltrexone interaction resulted in Scatchard plots which mimicked cooperativity in binding. These monophasic plots also indicated that sodium affects antagonist binding by changing receptor affinity (K_D) but not the density of sites. When assessed under correct experimental conditions the Scatchard plots for specific [^{3}H]naltrexone binding were biphasic. Sodium, without affecting the K_D, increased and decreased the number of high- and low-affinity sites, respectively. Thus, at 25° the total number of opiate receptor sites for [^{3}H]naltrexone binding in the absence and presence of sodium was statistically indistinguishable. Initial membrane incubation competed with sodium in increasing the density of high-affinity [^{3}H]naltrexone-binding sites. After kinetic resolution and computer analysis considering several binding models, the data for specific [^{3}H]naltrexone binding provided best fit for two saturable sites. At 25° and in the absence of sodium the respective approximate K_D values were 0.4 and 30 nM, with densities of 200 and 350 fmoles/mg of protein. Under a variety of experimental conditions, including different temperatures and exposure of the membranes to trypsin or freezing, some of these binding parameters differed, but the biphasic nature of specific [^{3}H]naltrexone binding was unaltered. Identical biphasic Scatchard plots were obtained if specific binding of [^{3}H]naltrexone was determined with excess unlabeled naltrexone, morphine, ethylketocyclazocine, or SKF 10047; with dextrorphan and levorphanol; or with enantiomers of naloxone as displacing ligands. Terminating the binding assay by rapid centrifugation yielded results identical with those obtained when quick filtration was used. The dissociation of bound [^{3}H]naltrexone was resolved into a rapid component and a slow component. In the presence of high concentrations of the drug an additional rapid component of dissociation became apparent. The K_D values calculated from the two rapid components of dissociation and the corresponding rate constants of association agreed well with those determined for the high- and low-affinity [^{3}H]naltrexone-binding sites by Scatchard analysis. The nature of the slow dissociation component with markedly high affinity has to await further clarification. The results of this study characterize the interaction of naltrexone with opiate receptor, contribute to the understanding of the mechanism of the sodium effect, and describe the role of methodology in evaluating ligand binding.

INTRODUCTION

Scatchard analysis of ligand-receptor interactions provides information on binding parameters, i.e., affinity and site density (1, 2). In addition, Scatchard plots can detect heterogeneity in ligand binding. Scatchard analysis of opiate receptor binding has been carried out in numerous studies, and is of particular interest in view of the recently presented evidence for multiple forms of the receptor (3-6). However, previously reported Scatchard analyses for opiate receptor binding were, in part, equivocal. For example, for radiolabeled naltrexone (7-9) and naloxone (10-13), both monophasic and biphasic plots were described, yielding differing binding parameters. After it was shown that the binding to opiate receptor of agonists and antagonists is differentially influenced by sodium (14), Scatchard analyses were used to support two opposing hypotheses on the mechanism underlying the phenomenon. According to one viewpoint, based on biphasic Scatchard plots, sodium unmasks additional antagonist-binding sites with identical affinity (10, 13, 14).

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The other hypothesis proposed that in the presence of the ion a constant number of binding sites has increased affinity for antagonists (7, 8). Furthermore, in the latter studies the shape of the obtained monophasic Scatchard plots was forwarded as evidence for the allosteric, positively cooperative nature of opiate receptor binding.

In the course of our studies on the opiate receptor binding of narcotic drugs (15), we became aware of the marked influence of experimental conditions on the outcome of corresponding Scatchard analyses (16, 17). Although the principles of applying Scatchard analysis in assessing ligand binding to membrane receptors have been reviewed (2, 18), the opiate receptor assay requires special considerations because of its inherent complexity, e.g., employment of displacing drugs to determine stereospecific interaction, occurrence of substantial nonspecific binding at higher drug concentrations, and marked effects of temperature and ions. Additional difficulty is generated by the apparent heterogeneity of opiate receptor sites (3–6). The contribution of experimental artifacts to equivocal evaluations of ligand-receptor binding *in vitro* has recently been highlighted (19). Although effects of specific assay conditions on opiate receptor binding have been described previously (11, 12, 14), the above-outlined circumstances warranted a comprehensive study on methodological aspects of opiate receptor binding, focusing thereby on the link between experimental approaches and the results of respective Scatchard analyses. Concurrently, this study provided the opportunity to assess the lingering controversy (8) on the mechanism by which sodium affects interaction of opiates with the receptor.

MATERIALS AND METHODS

Materials. [15,16-^{3}H]Naltrexone, specific radioactivity 9.8 Ci/mmole, was kindly provided by the National Institute on Drug Abuse, Bethesda, Md. The chemical purity of the radiolabeled compound was listed as higher than 95%. This claim was confirmed in our laboratory by thin-layer chromatography in two different solvent systems. The unlabeled opiates were obtained through the Drug Abuse Basic Research Center at the University of Michigan. *d*-Naloxone·HCl was a generous gift of Dr. A. Jacobson, National Institutes of Health, Bethesda, Md. Trypsin, its soybean inhibitor, and other biochemicals were purchased from Sigma Chemical Company, St. Louis, Mo.

Membrane preparation. Male Sprague-Dawley rats weighing 200 g were decapitated and the brains were excised at 4°. The cerebrum was dissected, washed in Tris-HCl (pH 7.4), blotted, and cleaned of adhering blood vessels. The weighed tissue was disrupted for 1 min in 100 volumes of ice-cold 50 mM Tris (pH 7.4), using a Polytron homogenizer (Model PT 10, Brinkmann Instruments, Inc., Westbury, N. Y.) at a power output 6.5. The homogenate was centrifuged at 20,000 × *g* for 15 min in the cold. The obtained pellet was resuspended with the original amount of buffer using a Dounce all-glass homogenizer. Aliquots of this suspension, sufficient for experiments on 1 given day, were frozen at −70°. Prior to use, the suspension was quickly thawed in a water bath and briefly dispersed in a Dounce homogenizer. The protein concentration in the latter preparation was approximately 0.6 mg/ml.

In certain experiments, the brain membranes were initially treated to remove bound endogenous ligand from opiate receptor sites. Aliquots of the membrane suspension in 50 mM Tris-HCl, pH 7.4 at 25°, approximate protein concentration 0.6 mg/ml, were centrifuged at 20,000 × *g* for 15 min at 4°. The pellet was suspended with the original volume of the buffer, pH 7.4 at 37°, using a Dounce homogenizer. After incubation at 37° for 40 min, the suspension was again centrifuged as described above. The pellet was suspended in 50 mM Tris-HCl, adjusted to pH 7.4 at temperatures of the subsequent opiate receptor assay.

Determination of protein. The method of Lowry *et al.* (20) was applied for determination of protein. Tris buffer, at appropriate concentrations, was included in the blanks and in the solutions of standards for which crystallized and lyophilized bovine serum albumin was used.

Binding assay. The basic assay procedure was similar to that previously described (21). The assay mixture, in 8-ml polypropylene tubes, consisted of 400 μl of membrane suspension, 50-μl aliquots of various unlabeled drugs or buffer, 50 μl of NaCl solution (or buffer) to give a final concentration of 150 mM sodium, and 25 μl of [^{3}H]naltrexone. The final volume of the assay was 525 μl. Constant pH during the incubation was ascertained. After incubation for a given length of time and at a specific temperature, the samples were filtered through glass-fiber discs (Whatman GF/C). Initially, the filters were repeatedly washed by swirling in water and decanting, and were treated on the filter assembly with water saturated at room temperature with *n*-amyl alcohol. The filtered samples were quickly washed as described previously (21) and placed into polyethylene counting vials. After the addition of 1 ml of absolute ethanol followed by 10 ml of xylene-dioxane-naphthalene-based scintillation fluid, the vials were subjected to liquid scintillation counting. The average counting efficiency, determined by the use of ^3H_2O, was 43%. In the course of this work the opiate receptor assay was carried out under different experimental conditions. These specific variations of the above-outlined basic assay procedure are described in the legends of the corresponding illustrations.

In the experiments investigating the effects of trypsin, membrane freezing, and of different opiates, the membranes were initially incubated as described above. For trypsinization the membrane suspension was incubated with the enzyme, 15 μg/ml, for 30 min at 25°. The interaction was terminated by the addition of 170 μg of soybean trypsin inhibitor. The suspension was centrifuged at 20,000 × *g* for 15 min, and the membranes were resuspended in 50 mM Tris-HCl, pH 7.4 at 25°; aliquots of this suspension were used in the binding assay. In experiments comparing properties of freshly prepared and frozen-thawed membranes, the binding assay was carried out with cerebral membranes which were either freshly isolated or stored at −70°. To investigate the effects of different opiates, the membranes were incubated at 25° as described above with 0.1–100 nM [^{3}H]naltrexone in the absence and presence of 100 μM naltrexone, morphine, ethylketocyclazocine, or SKF 10047. Specific interaction of [^{3}H]naltrexone was defined

as the difference in binding obtained in the absence and presence of the indicated opiates.

In selected experiments the opiate receptor assay was terminated by centrifugation instead of filtration. Although the principle of the assay remained the same, several modifications in the procedure had to be implemented. The volumes were reduced proportionately and the assay was carried out in 105 μl. The membrane suspension was concentrated by centrifugation at 20,000 × *g* for 15 min. The pellet was suspended with 50 mM Tris-HCl buffer, pH 7.4, and aliquots corresponding to 200 μg of protein were added to each assay tube. Incubation was performed in 250-μl polyallomer tubes at 25°. Subsequently, the tubes were centrifuged at 28 psi (155,000 × *g*) for 30 sec (Beckman Airfuge), and 60 μl of the supernatant were removed by pipetting. After the addition of 15 μl of buffer to the remaining supernatant and pellet (pellet fraction), 100 μl of 20% sodium dodecyl sulfate were added to both supernatant and pellet fractions. The preparation of the samples for liquid scintillation counting followed the procedure described by Albers and Krishnan (22) with the exception that the samples were solubilized overnight with the detergent before the scintillation fluid was added. Both supernatant and pellet fractions from triplicate samples were counted, and specific binding was determined, defined as $P - (F \times S)$, where P = counts in the pellet fraction, S = counts in the supernatant fraction, and $F = Po/So$ (Po and So representing the counts in the pellet and soluble fractions in the presence of excess unlabeled ligand).

Throughout the study, the total concentration of added [^{3}H]naltrexone in the assay medium was calculated from the radioactivity determined in an aliquot of the stock solution of the radiolabeled drug. Free [^{3}H]naltrexone was defined as the difference between total added and bound drug.

RESULTS

Mechanism of the sodium effect in [^{3}H]naltrexone binding. The effects of increasing concentrations of dextrorphan, levorphanol, and unlabeled naltrexone on the binding of [^{3}H]naltrexone, present at two different concentrations, was initially investigated (Fig. 1). The two chosen concentrations of radiolabeled naltrexone represent limits of the range with which the Scatchard analysis was carried out. The indicated range was initially selected to allow a comparison of our data with those reported previously (7, 8). In subsequent experiments it was shown that complete Scatchard analysis of specific [^{3}H]naltrexone binding requires the use of even higher concentrations of the radiolabeled ligand (Figs. 4 and 7, *left inset*). The effect of each of the displacing drugs was determined in the absence and presence of 150 mM NaCl. Without sodium, the binding of 0.15 nM [^{3}H]naltrexone was unaffected by 0.01 μM dextrorphan but was inhibited 36% at 1 μM concentration of the dextrorotatory isomer. This interference was not noticeable if the concentration of [^{3}H]naltrexone was 30 nM and was less pronounced in the presence of sodium. On the other hand, 1 μM levorphanol completely displaced [^{3}H]naltrexone binding in the absence but not in the presence of NaCl (Fig. 1). With sodium present a considerably higher concentration

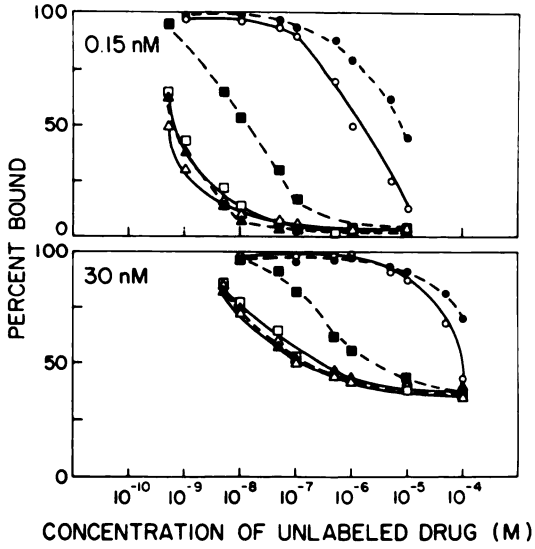

FIG. 1. *Effect of displacing ligands on binding of [^{3}H]naltrexone to brain membranes*

[^{3}H]Naltrexone, at concentrations of 0.15 nM or 30 nM, was incubated with aliquots of a suspension of membranes from rat cerebrum, corresponding to approximately 250 μg of protein, in the absence (*open symbols* and *solid lines*) and presence (*solid symbols* and *broken lines*) of 150 mM NaCl and various concentrations of dextrorphan (○, ●), levorphanol (□, ■), or naltrexone (△, ▲). The membranes were first incubated with unlabeled drug, without or with NaCl, for 15 min at 25°, then [^{3}H]naltrexone was added and the incubation was continued for an additional 15 min at which time the tubes were placed in ice. The samples were further treated as described under Materials and Methods. Plotted on the ordinate is the extent of [^{3}H]naltrexone binding. Results of representative experiments are presented.

of levorphanol was required for maximal displacement of 30 nM [^{3}H]naltrexone. Unlabeled naltrexone as displacing agent did not display such differential effects in the absence and presence of NaCl. Under both conditions, a 1 μM concentration was sufficient for full displacement of [^{3}H]naltrexone (Fig. 1).

The above-described effects were reflected in the corresponding Scatchard plots. If specific binding of [^{3}H]naltrexone in the absence of sodium was determined with 1 μM levorphanol and 1 μM dextrorphan, the Scatchard plots were inwardly bent toward the abscissa (Fig. 2). Omitting dextrorphan, or decreasing its concentration to 0.1 μM, converted the bent shape of the plot into a linear one (Fig. 2). Under the latter assay conditions, the Scatchard plots were bent only at very low values of bound [^{3}H]naltrexone (Fig. 2, *inset*). The data in this region of the plot were characterized by lack of precision owing to the unfavorable ratio of stereospecific drug binding to radioactivity associated with the filter discs (filter blanks). The onset of the inward bend depended on the specific radioactivity of the ligand. With [^{3}H]naltrexone of 10 Ci/mmole, deviation from linearity occurred below 20 fmoles of bound drug, corresponding to a ratio of stereospecific binding to filter blank of less than 1.25.

In the presence of NaCl, the Scatchard plots for specific [^{3}H]naltrexone binding assessed with 0.1 μM dextrorphan and 0.1 or 1.0 μM levorphanol were inwardly bent at the abscissa (Fig. 3). With increasing concentra-

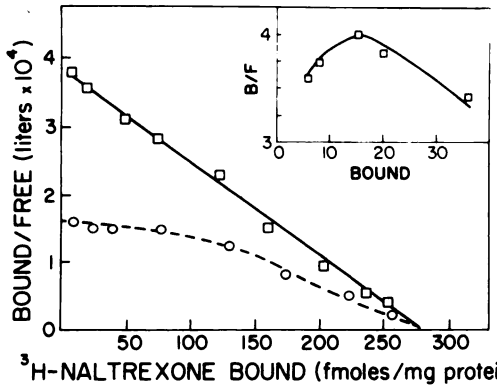

FIG. 2. *Effect of dextrorphan concentration on stereospecific binding of [³H]naltrexone*

Cerebral membranes corresponding to 250 μg of protein were incubated with various concentrations of [³H]naltrexone ranging from 0.1 nM to 20 nM, with 1 μM levorphanol and in the absence (□) or presence (○) of 1 μM dextrorphan. Stereospecific interaction was defined as the difference between the binding of [³H]naltrexone either in the presence of dextrorphan or levorphanol (○), or in the absence and presence of levorphanol (□). The *inset* depicts the enlarged segment of the Scatchard plot obtained at low values of [³H]naltrexone binding. The assays were carried out as described in the legend to Fig. 1 and under Materials and Methods. Shown are the averages of values obtained in two experiments. The range of individual values around the averages was in all cases less than ±6%. Within one experiment, duplicate values differed by less than 4%.

tions of levorphanol the curvature diminished, and at 10 μM concentration of the drug the plot became linear. In the absence of NaCl, the shape of the Scatchard plot was independent of levorphanol concentrations ranging from 1 to 10 μM. Binding of up to 30 nM [³H]naltrexone in the presence of 1.0 μM–10 μM unlabeled naltrexone resulted in Scatchard plots identical with those obtained with 10 μM levorphanol. In addition, similar results were obtained with 10 μM levallorphan.

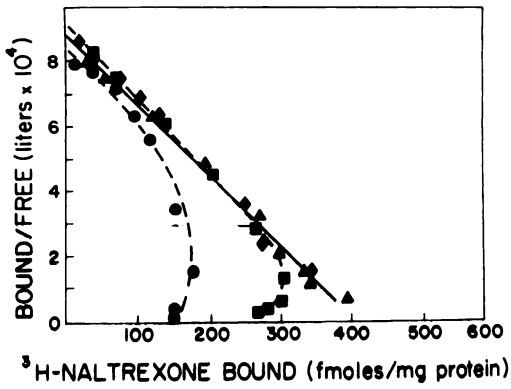

FIG. 3. *Effect of levorphanol concentration on stereospecific binding of [³H]naltrexone*

Cerebral membranes corresponding to 250 μg of protein were incubated with 150 mM NaCl, 0.1–20 nM [³H]naltrexone, and either 0.1 μM dextrorphan or 0.1 (●), 1.0 (■), or 10.0 μM (▲) levorphanol, or 10 μM unlabeled naltrexone (◆). Various concentrations of the latter drug ranging between 1 and 10 μM had identical effects on the stereospecific binding of [³H]naltrexone. Stereospecific interaction was defined as the difference between the binding of [³H]naltrexone either in the presence of dextrorphan and levorphanol, respectively, or in the absence and presence of excess unlabeled naltrexone. The assays were carried out as described in the legend to Fig. 1 and under Materials and Methods. Statistical aspects were as stated in the legend to Fig. 2.

If stereospecific binding of [³H]naltrexone was determined by the use of dextrorphan and levorphanol under experimental conditions described previously (7), the corresponding Scatchard plots were inverted at the ordinate and the abscissa in the absence and presence of NaCl, respectively (Fig. 4, *left graph*), reflecting the combined effects described above (Figs. 2 and 3). The intercepts of the curves indicated identical binding site densities but different equilibrium binding constants (K_D values) in the absence and presence of sodium. However, if specific binding of [³H]naltrexone was assessed by an appropriate excess of the unlabeled drugs, the shapes of the corresponding Scatchard plots, in both the absence and presence of sodium, were biphasic (Fig. 4, *right graph*). These plots indicated essentially unchanged K_D values, but an increased number of high-affinity binding sites in the presence of NaCl. It should also be noted that the assays in the latter experiments were terminated at 15 min in order to replicate previously described conditions (7). As shown in Fig. 5, this time point is at the very beginning of the equilibrium in [³H]naltrexone binding. Results obtained under such conditions are subject to greater variability than those determined within the plateau of the time-dependence curve, as carried out in all subsequent experiments of this work.

Time dependence of [³H]naltrexone binding at various temperatures. The temperature-dependent time

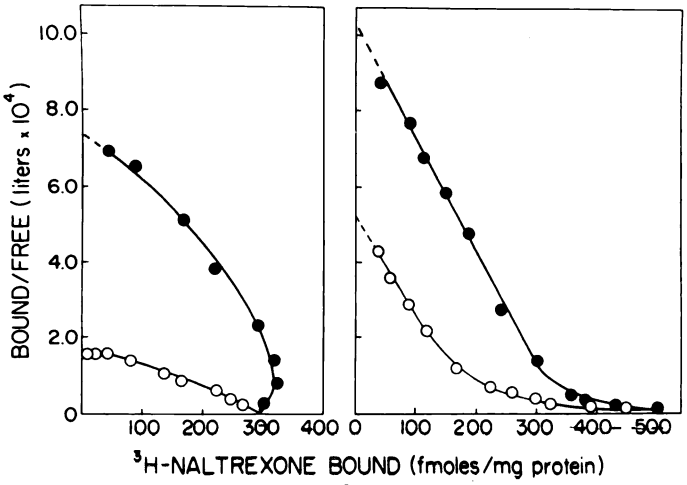

FIG. 4. *Scatchard analysis of [³H]naltrexone binding at different excesses of displacing ligands*

All samples in these experiments were incubated for 15 min at 25°, then placed in ice prior to filtering. In addition, two different sets of conditions were applied.

Left graph. Cerebral membranes were incubated with 0.1–20 nM [³H]naltrexone and 1 μM dextrorphan or levorphanol in the absence (○) or presence (●) of 150 mM NaCl. Stereospecific interaction was defined as the difference between [³H]naltrexone binding obtained in the presence of dextrorphan and levorphanol, respectively.

Right graph. Cerebral membranes were incubated with 0.1–100 nM [³H]naltrexone in the absence or presence of 10 μM unlabeled naltrexone, and in the absence (○) or presence (●) of 150 mM NaCl. Stereospecific interaction was defined as the difference between [³H]-naltrexone binding obtained in the absence and presence of unlabeled drug. Shown are mean values of two to four experiments. The standard deviation around the means was less than ±6%.

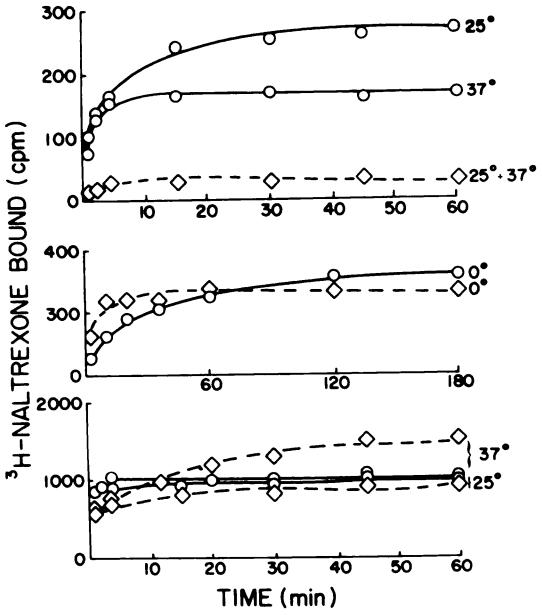

FIG. 5. *Time course of [^{3}H]naltrexone binding*

Aliquots of the membrane suspension were incubated with either 0.5 nM (*upper graph*), 3 nM (*middle graph*), or 50 nM (*lower graph*) [^{3}H]naltrexone for various lengths of time and at different temperatures as indicated. The assay was terminated by rapid filtration at room temperature, and the samples were washed with ice-cold 50 mM Tris buffer as described under Materials and Methods. Specific binding (○——○) was expressed as the difference between values obtained in the absence and presence of 10 μM unlabeled naltrexone. Nonspecific interaction (◇- - -◇) was defined as the binding of [^{3}H]naltrexone in the presence of excess unlabeled drug. Shown are results of representative experiments.

course of [^{3}H]naltrexone binding was investigated at three different concentrations (Fig. 5). Specific binding of 0.5 nM drug at 25° and 37° reached equilibrium after 40 and 15 min, respectively, and remained constant for 60 min. Nonspecific binding at both temperatures was low and steady between 5 and 60 min. At 0° the extent of specific and nonspecific binding of 3 nM [^{3}H]naltrexone was similar and reached equilibrium after 3 and 2 hr, respectively. Specific binding of 50 nM [^{3}H]naltrexone at 25° and 37° remained constant between 5 and 60 min, but nonspecific binding increased with time and was particularly pronounced at 37°.

Dependence of [^{3}H]naltrexone binding on pH. Specific binding of naltrexone at 25°, in both the absence and presence of 150 mM NaCl, was constant between pH 7.0 and 8.0.

Scatchard analysis of [^{3}H]naltrexone binding: effect of assay temperatures and of initial membrane incubation. Assayed at 25°, sodium increased the number of high-affinity binding sites with an unaltered K_D of approximately 0.35 nM, relative to corresponding values obtained in the absence of the ion (Fig. 6, *left graph*, and Table 1). Placing the tubes in ice after the completed incubation at 25° and prior to filtration had no significant effect on [^{3}H]naltrexone binding if the assay was carried out without sodium. However, under this condition of 25°/0° the effect of sodium in revealing additional high-affinity binding sites was more pronounced than if the entire assay procedure was conducted at 25°. If assessed at 0° the K_D for the high-affinity sites increased slightly in the absence of sodium and was considerably lower in its presence, relative to the values obtained at 25°. The number of high-affinity binding sites in the presence of sodium followed the trend observed at 25° (Table 1).

If assayed without sodium at 25° or 0°, initial incubation of the membranes resulted in an increased number of high-affinity [^{3}H]naltrexone binding sites wth unaltered K_D. The additional effect of sodium at 25° was marginal, but at 0° the ion reduced the high-affinity K_D in both incubated and untreated membranes (Fig. 6 and Table 1).

The K_D values for the low-affinity binding component of [^{3}H]naltrexone were similar at 25° or 25°/0°, and lower if determined at 0° (Table 1). Regardless of the assay temperature, initial incubation of the membranes increased the density of these sites, while the presence of sodium decreased their number. Thus, if assayed at 25° or 25°/0° but not at 0°, the total number of specific [^{3}H]naltrexone-binding sites in the absence and presence of sodium remained relatively constant in both untreated and pretreated membranes (Tables 1 and 2).

The temperature of the buffer medium used to wash the samples on the filter assembly had no effect on the binding parameters of [^{3}H]naltrexone. In these experiments the assay was carried out at 25°, and the filtered samples were subsequently washed with Tris-HCl buffer of either 25° (pH adjusted to 7.4 at 25°), or 0° (pH adjusted to 7.4 either at 0° or at 25°).

Resolution of the biphasic Scatchard plots. Binding of [^{3}H]naltrexone at high concentrations was influenced by an additional apparent binding site of low affinity (Fig. 7, *left inset*). Its interference in assessing specific [^{3}H]naltrexone binding was decreased by limiting the concentration of the radiolabeled drug to 100 nM. The validity of this approach was reflected in increased precision of the binding data relating to the low-affinity component of specific [^{3}H]naltrexone binding which, in general, were more variable than those describing the high-affinity site (Table 2). Binding of [^{3}H]naltrexone up to 100 nM gave best fit to a computer model of two saturable binding sites (24). Models depicting two or one saturable sites, each with an additional linear component, were less satisfactory (Table 2). Initial estimates for the respective K_D values and number of sites required for computer analysis were obtained from double-reciprocal plots of the binding data (Fig. 6, *left inset*) after first considering and resolving the mutual interference of the two processes (23). The computer-assisted resolution of the obtained biphasic Scatchard plots (Fig. 6, *right inset*) led to the values of the binding parameters listed in Table 1.

Biphasic Scatchard plots of [^{3}H]naltrexone binding: effect of trypsin and membrane freezing. Initial exposure of cerebral membranes to trypsin markedly decreased the number of high-affinity binding sites without significantly affecting the K_D (Table 1). Similarly, membrane freezing resulted in some loss of specific binding sites but had no effect on the affinity of [^{3}H]naltrexone binding.

Biphasic Scatchard plots of [^{3}H]naltrexone binding: effect of different opiates. If an excess of morphine,

TABLE 1

Parameters of specific [^{3}H]naltrexone binding carried out under different experimental conditions

The listed values are results of computer analysis for best fit, considering a model of two saturable binding sites (Table 2). The experimental conditions and statistical information are described in the legend to Fig. 6 and under Materials and Methods. The experiments with trypsin and different opiates were repeated twice, each run in duplicate, and the averages of the data were plotted. Individual values varied less than 5% around the presented average.

Experimental conditions			Binding site 1		Binding site 2	
Temperature	Preincubation	Sodium	K_D	n	K_D	n
			nM	fmoles/mg protein	nM	fmoles/mg protein
25°	−	−	0.36	206	31	362
	−	+	0.36	295	38	285
	+	−	0.33	293	46	779
	+	+	0.39	346	63	751
25/0	−	−	0.30	217	32	384
	−	+	0.29	343	33	229
0	−	−	0.51	142	6.2	134
	−	+	0.14	248	2.1	117
	+	−	0.82	250	25	332
	+	+	0.26	307	5.7	64
25	+ (trypsin treatment)	−	0.59	106	55	842
25	+ (excess morphine)	−	0.43	300	40	709
25	+ (excess ethylketocyclazocine)	−	0.29	236	35	588
25	+ (excess SKF 10047)	−	0.50	303	74	814

ethylketocyclazocine, or SKF 10047 was used to displace [^{3}H]naltrexone, the corresponding binding parameters obtained from Scatchard plots of specific binding were virtually indistinguishable from those obtained with excess unlabeled naltrexone (Table 1).

Assessment of [^{3}H]naltrexone binding with naloxone enantiomers using both filtration and centrifugation. Initially, the "displacement window" for [^{3}H]naltrexone binding in the presence of *d*- and *l*-naloxone was established (Fig. 7, *right inset*). Specific binding of [^{3}H]naltrexone approached saturation at approximately 50 nM, however, at higher concentrations of the drug an additional low-affinity binding component became apparent (Fig. 7, *left inset*). Using either technique for terminating the binding assay, virtually identical biphasic Scatchard plots were obtained if specific [^{3}H]naltrexone interaction was defined as the difference between binding obtained in the absence and presence of excess unlabeled naltrexone, or as the difference between binding in the presence of *d*- and *l*-naloxone (Fig. 7).

If specific binding of [^{3}H]naltrexone was determined with an excess of the unlabeled drug, the corresponding filter blanks increased substantially with rising concentration of [^{3}H]naltrexone. At 100 nM of the radiolabeled drug the blank almost reached the extent of specific binding (Fig. 7, *left inset*). This occurred despite initial washing of the glass-fiber discs with a saturated aqueous solution of amyl alcohol, which reduces drug binding to the filters (21). Treatments with bovine serum albumin, protamine, polyethylenimine, and siliconizing reagents, respectively, did not substantially decrease the filter binding of [^{3}H]naltrexone. However, binding to filters but not to brain membranes was considerably lower if the enantiomers of naloxone, rather than excess unlabeled drug, were used to determine the specific interaction of [^{3}H]naltrexone (Fig. 7, *left inset*).

Dissociation of bound [^{3}H]naltrexone. The kinetics of [^{3}H]naltrexone dissociation from its membrane binding sites was primarily assessed by the method of drug displacement (Fig. 8). Additionally, in control experiments[1] carried out in the absence of sodium and at 25°, the approach of infinite dilution (28) was applied using 100-fold dilution of membranes previously incubated with [^{3}H]naltrexone until binding equilibrium at 25°. Adequate dilution was ascertained by the close similarity of results obtained with and without the presence of 10 μM unlabeled naltrexone in the diluting medium. The values for the dissociation rates constants (k_{-1}) obtained by both methods agreed closely.

The multiphasic dissociation curves were resolved into linear components, and the kinetic constants were obtained by computer analysis. In the absence of NaCl (Fig. 8, *left graph*), the first-order rate constants at 25° were 2.7×10^{-2} and 23×10^{-2} min^{-1}. At 37° these values changed to 3.9×10^{-2} and 51×10^{-2} min^{-1}. At 0° the dissociation of [^{3}H]naltrexone occurred by a linear process with a k_{-1} value of 0.94×10^{-2} min^{-1}. In the presence of NaCl and at 25°, the first-order rate constants were

[1] S. V. Fischel and F. Medzihradsky, unpublished observations.

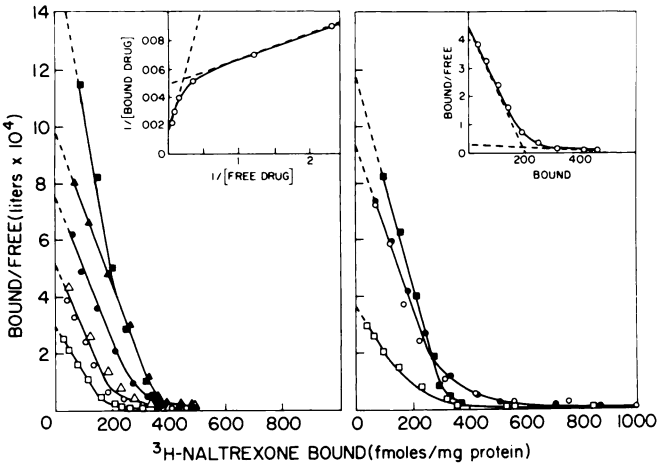

FIG. 6. *Effect of assay temperatures and of initial membrane incubation on [³H]naltrexone binding*

The binding assay was carried out in the absence (*open symbols*) and presence (*solid symbols*) of 150 mM NaCl, using thawed cerebral membranes without additional treatment (*left graph*) or after their initial incubation as described under Materials and Methods (*right graph*). In these experiments the opiate receptor assay was carried out at 25° or 0° for 40 min and 180 min, respectively. After the incubation at 25°, the samples were either filtered at that temperature (○, ●) or the tubes were placed on ice for an average of 15 min prior to filtration (△, ▲). The tubes incubated at 0° were filtered at that temperature (□, ■). In all cases, the samples on the filter were rapidly washed with ice-cold 50 mM Tris-HCl (pH 7.4) as described under Materials and Methods. Opiate receptor related interaction of [³H]naltrexone was defined as the difference between binding obtained in the absence and presence of 10 μM unlabeled naltrexone. Using the results obtained with untreated membranes at 25°, the two *insets* illustrate the resolution of the data for purposes of Scatchard analysis. The *broken lines* in the double-reciprocal plot (*left inset*) represent the two unresolved components of [³H]naltrexone binding. These intercepts and slopes were corrected for the mutual interference of the two processes (23), to obtain initial estimates for K_D and the number of binding sites. Final values (Table 1) were derived through the use of a nonlinear least-squares regression analysis program, examining the data for best fit to a model of two saturable binding sites (24, 25), and yielded Scatchard plots as depicted in the right *inset*. Shown are mean values obtained in three to six experiments for each condition, except for 0°, when experiments were repeated twice. The standard deviation around the means was ±5%.

5.6×10^{-2} and 24×10^{-2} min^{-1} (Fig. 8, *middle graph*). When corrected for the slower component, the rates of rapid dissociation in the absence and presence of sodium were identical (Fig. 8, *middle inset*). Such a relationship was also obtained with preincubated membranes. Dissociation of [³H]naltrexone in the presence of 150 mM KCl was similar to that obtained in the absence of NaCl. If 50 nM [³H]naltrexone was incubated to binding equilibrium, the dissociation kinetics revealed an additional rapid component (Fig. 8, *right inset*). The first-rate order constants of the three dissociation components were 2.8×10^{-2}, 32×10^{-2}, and 350×10^{-2} min^{-1}. Considering the above-listed values for k_{-1} and the association constants (k_1), calculated from equilibrium binding of [³H]naltrexone at various temperatures (Fig. 5), the rapidly dissociating component corresponded to a K_D of 0.4 nM at 25°, and 2.5 nM at 37° and 0°. For the even faster dissociating component of [³H]naltrexone, revealed after equilibrium binding at 50 nM of the drug, a K_D of 8 nM was calculated. To the latter case, the k_1 value of the other rapid dissociation component was assumed, since measurement of the actual k_1 by kinetic resolution of the two binding components of 50 nM [³H]naltrexone was not possible because of the rapid rates of association at this concentration of the drug (Fig. 5). The slow component of dissociation, obtained at both 25° and 37°, corresponded to a K_D of 0.03 nM. In calculating this value, the unresolved association rate constant for [³H]naltrexone was again used.

DISCUSSION

The actions of opiates, including their binding to receptor, are characterized by stereospecificity. To distinguish specific, i.e., opiate receptor-related, binding from other drug-tissue associations, the use of enantiomers such as dextrorphan and levorphanol has been suggested (29). According to that principle, resolution of opiate receptor binding of radiolabeled ligands is achieved by saturating nonspecific and specific binding sites with an excess of a pharmacologically inactive and active isomer, respectively. The appropriate application of this approach requires that under conditions of the assay the inactive isomer does not bind to receptor, and that the binding of the radiolabeled opiate is completely displaced by the employed concentration of unlabeled active isomer. These optimal concentrations of the two displacing drugs ("binding windows") have to be determined for the binding of each radiolabeled ligand in any given tissue preparation. In view of the differential effect of sodium on agonist and antagonist binding (14), the setting of the displacement window in the presence of NaCl should also be established. Furthermore, in light of recent evidence for multiple opiate receptor sites (3–6), the structure and pharmacological nature of the displacing drug becomes of importance. It seems inappropriate to use a given pair of enantiomers, e.g., dextrorphan and levorphanol, to determine the stereospecific interaction of both putative μ and κ opiates which potentially interact with different receptor sites. The employment of an excess of the unlabeled form of the radioactive ligand to assess specificity avoids the criticism raised above, but this approach does not distinguish between receptor binding and saturable non-specific interactions of the ligand. Assuming the unavailability of specific enantiomers which bind to the same site as the radiolabeled ligand under investigation, opiate receptor binding has to be resolved by a combination of approaches serving as mutual controls.

The data of this study show that a neglect of the above outlined requirements has marked consequences for the Scatchard analysis of opiate receptor binding. Excessive concentrations of dextrorphan relative to that of the employed [³H]naltrexone resulted in Scatchard plots inverted toward the ordinate, reflecting significant binding of the pharmacologically inactive isomer to the receptor. On the other hand, concentrations of levorphanol insufficient to displace completely the radiolabeled antagonist yielded Scatchard plots which were inwardly bent at the abscissa. The latter phenomenon was observed only in

TABLE 2

Parameters of specific [^{3}H]naltrexone binding obtained by considering different models of ligand-receptor interaction

The listed values are results of computer analyses for best fit (24, 25), considering models of one saturable binding site + one linear binding component, two saturable binding sites, and two saturable binding sites + one linear binding component, respectively. All of the experiments were carried out at 25° in the absence and presence of 150 mM NaCl using untreated and initially incubated membranes, respectively. The concentration of [^{3}H]naltrexone in the assay ranged up to 100 nM. Significance, indicated by results of *F*-ratio tests, pertains to the statistical difference of the data relative to those for the model with two saturable sites. Shown are the computer-estimated mean values and the corresponding standard deviations. The estimation was based on 22–46 data points obtained in 3–6 separate experiments as indicated in the legend to Fig. 6.

Binding parameters	Binding site models		
	1 saturable + 1 linear	2 saturable	2 saturable + 1 linear
−Preincubation, −Na			
K_{D1} (nM)	0.62 ± 0.10	0.36 ± 0.08	0.37 ± 0.08
n_1 (fmoles/mg protein)	269 ± 14	206 ± 23	202 ± 23
K_{D2} (nM)	—	31 ± 14	34 ± 14
n_2 (fmoles/mg protein)	—	362 ± 40	356 ± 45
k_1 (fmoles/mg protein · nM)[a]	2.2 ± 0.3	—	0.0002 ± 0.005
SWSD[b]	104.3	65.4	65.4
Significance (p value)	<0.001	—	NS[c]
−Preincubation, +Na			
K_{D1} (nM)	0.50 ± 0.03	0.36 ± 0.03	0.32 ± 0.06
n_1 (fmoles/mg protein)	339 ± 7	295 ± 11	258 ± 28
K_{D2} (nM)	—	38 ± 12	7.3 ± 4.8
n_2 (fmoles/mg protein)	—	285 ± 23	154 ± 25
k_1 (fmoles/mg protein · nM)[a]	1.8 ± 0.1	—	1.1 ± 0.3
SWSD	54.1	34.1	30.2
Significance (p value)	<0.001	—	NS
+Preincubation, −Na			
K_{D1} (nM)	0.68 ± 0.18	0.33 ± 0.10	0.29 ± 0.13
n_1 (fmoles/mg protein)	436 ± 41	293 ± 33	281 ± 70
K_{D2} (nM)	—	46 ± 15	36 ± 36
n_2 (fmoles/mg protein)	—	779 ±101	752 ± 593
k_1 (fmoles/mg protein · nM)[a]	4.2 ± 0.8	—	0.02 ± 3.3
SWSD	526.3	366.5	372.7
Significance (p value)	$0.001 < p < 0.005$	—	NS
+Preincubation, +Na			
K_{D1} (nM)	0.56 ± 0.98	0.39 ± 0.09	0.39 ± 0.12
n_1 (fmoles/mg protein)	421 ± 25	346 ± 39	345 ± 56
K_{D2} (nM)	—	63 ± 37	58 ± 137
n_2 (fmoles/mg protein)	—	751 ± 172	689 ± 1523
k_1 (fmoles/mg protein · nM)[a]	4.3 ± 0.5	—	0.3 ± 6.4
SWSD	108.0	79.9	80.0
Significance (p value)	$0.01 < p < 0.02$	—	NS

[a] Linear binding constant, k_1 = bound drug (femtomoles per milligram of protein)/free drug (nanomolar).
[b] Sum of the weighted squared deviations (criterion of the variance ratio, or *F*-test).
[c] Not significant ($p > 0.1$).

the presence of sodium, a condition which favors antagonist binding and therefore requires a greater excess of levorphanol for complete displacement. Previously, inwardly bent Scatchard plots, obtained under the above outlined experimental conditions (Figs. 2 and 3), were compared with saturation curves displayed by allosteric enzyme systems and interpreted as evidence for positive cooperativity in opiate receptor binding (7, 8). Furthermore, the intercepts of these plots at the coordinates indicated that sodium lowers the K_D for antagonists without altering the density of the binding sites (Fig. 4, *left*). However, as shown by our data, inwardly bent Scatchard plots for [^{3}H]naltrexone binding with and without sodium are the consequences of inappropriate concentrations of the displacing drugs, and can be rectified by suitable excess of these compounds relative to the amount of [^{3}H]naltrexone in the assay medium. A slight bend of the Scatchard plots in the immediate vicinity of the ordinate (Fig. 2, *inset*) was identified as a consequence of an unfavorable ratio of specific binding to filter blanks, occurring at low values of bound ligand. If binding of [^{3}H]naltrexone to opiate receptor over a sufficiently wide range of concentrations was determined by an appropriate excess of either dextrorphan and levorphanol or of unlabeled naltrexone as the displacing ligands, the corresponding Scatchard plots were biphasic in both the absence and presence of NaCl. With sodium, an increased number of high-affinity binding sites with unaltered affinity was obtained. Since concurrently fewer low-affinity sites were present, the total number of binding sites with and without NaCl was statistically indistinguishable (Tables 1 and 2).

The competing effects of sodium and of initial membrane incubation to unmask high-affinity binding sites with unaltered K_D point to a similar underlying mechanism, i.e., the displacement of bound endogenous ligand

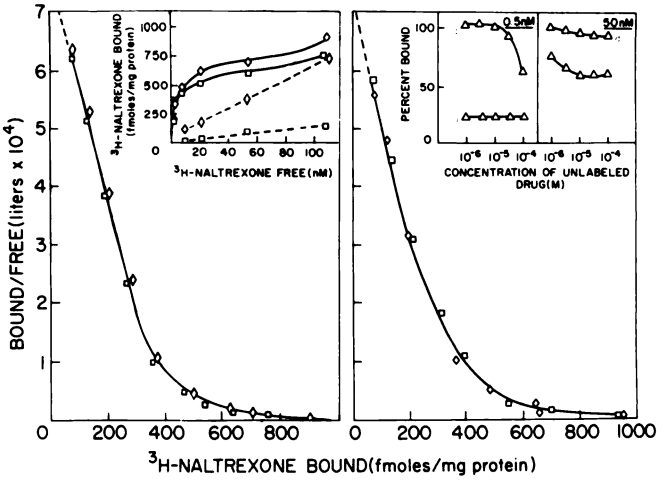

FIG. 7. *[³H]naltrexone binding in the presence of naloxone enantiomers, assessed by filtration or centrifugation*

The membranes were initially incubated as described under Materials and Methods. Binding of [³H]naltrexone, present at concentrations reflecting the range of the Scatchard analysis, in the presence of different concentrations of *d*- and *l*-naloxone was investigated (*inset, right graph*). Subsequently, [³H]naltrexone binding was assessed in the presence of 10 μM *d*- or *l*-naloxone (□) using either quick filtration (*left graph*) or rapid centrifugation (*right graph*) to terminate the assay. Binding of [³H]naltrexone was also determined in the absence and presence of 10 μM naltrexone (◇, *left* and *right graphs*). Details of the binding assay if centrifugation was used are described under Materials and Methods. The concentration-dependent specific binding of [³H]naltrexone to membranes (*solid lines*) and filters (*broken lines*), determined with naloxone enantiomers (□) or excess naltrexone (◇), is shown in the *left inset*. Plotted are the averages of values obtained in two experiments. The ranges of individual values around the averages were ±6% and ±7% in the experiments using filtration and centrifugation, respectively.

(12). The enhanced affinity of specific [³H]naltrexone binding in the presence of sodium, observed at 0°, was unrelated to initial membrane treatment, in agreement with a similar observation reported for [³H]naloxone (12). In the course of investigating the effects of temperature on the outcome of Scatchard analyses, its significant role in assessing opiate receptor binding was confirmed (11, 12). The evaluation of the binding equilibria at different temperatures and concentrations of [³H]naltrexone revealed a changing ratio of specific to nonspecific binding. A practical consequence of this finding, particularly significant if [³H]naltrexone binding is assayed at high concentrations of the drug and at 25° and 37°, is the requirement to filter sequentially and in close succession the tubes representing total and nonspecific binding at each given concentration of the radiolabeled drug.

In several previous reports Scatchard plots of specific [³H]naloxone binding were monophasic (10–12), but linearity toward the abscissa was poorly defined since insufficient data were presented at high concentrations of the radiolabeled drug, a frequent occurrence in presenting Scatchard plots for ligand binding. On the other hand, biphasic Scatchard plots for [³H]naloxone binding were also reported (13). Scatchard plots for [³H]naltrexone binding, in both the absence and presence of NaCl, were either inwardly bent (refs. 7 and 8; see also Fig. 4, *left graph*) or linear (9). Our data show that specific [³H]naltrexone binding, determined either by excess unlabeled drug or within a binding window defined by enantiomers, yields biphasic Scatchard plots. Given the biphasic nature, several possible binding models were considered. The fit of the experimental data to each model was analyzed by computer (24) considering two criteria (25). The requirement that the experimental values be scattered randomly about the theoretical curve was fulfilled by the models depicting two saturable binding sites, without and with an additional linear binding component. However, after considering the second criterion, i.e., a minimal value for the sum of the weighted squared deviations, our data did not justify the postulation of a linear component in addition to the two saturable binding sites (Table 2). Thus, the simplest binding model which offered the most accurate fit for the obtained results represented two saturable binding sites for the specific interaction of [³H]naltrexone with receptor. In computing vaues for the binding parameters (Tables 1 and 2) appropriate corrections (23) were applied to account for the frequently substantial (27) mutual interference of the two processes. In most of our experiments, limiting the concentration of [³H]naltrexone to 100 nM improved the statistical significance of the binding data. This interference by an additional apparent isotherm of low affinity (Fig. 7, *left inset*) was also reflected in the generally higher variability of the results pertaining to the low affinity site of specific [³H]naltrexone binding (Table 2).

Considering the potential problems in ascertaining specificity of low-affinity/high-capacity binding sites on the basis of biphasic Scatchard plots (30), a number of experimental approaches were undertaken to probe the heterogeneity of specific [³H]naltrexone binding. Neither membrane freezing nor limited exposure to trypsin changed the biphasic nature of the respective Scatchard plots. The effect of these treatments was limited to decreasing the number of binding sites. Previously, diminished [³H]naloxone binding after the enzyme treatment was described (10). In addition to confirming the effect of trypsin, our data identify that the decrease in specific [³H]naltrexone binding was primarily due to the loss of high-affinity sites, and that the K_D was unaltered by the enzyme.

Biphasic Scatchard plots were also obtained if specific [³H]naltrexone binding was determined by the use of *d*- and *l*-naloxone, selected considering the unavailability of naltrexone enantiomers. Naltrexone and naloxone displayed similar properties in their interaction with opiate receptor in the guinea pig ileum and in brain membranes (31), and in our studies on the resolution of μ and κ drugs.[2] The findings obtained with naloxone enantiomers assert the analogous outcome of Scatchard analysis of specific [³H]naltrexone binding, determined independently by two different pairs of enantiomers or by excess unlabeled drug (Figs. 4, 6, and 7). An additional benefit of the use of *d*- and *l*-naloxone was the markedly reduced stereospecific filter blank relative to values obtained with excess unlabeled naltrexone. This finding can be attributed to the displacement of [³H]naltrexone from nonspecific sites on the filter by excess *d*-naloxone. The ability

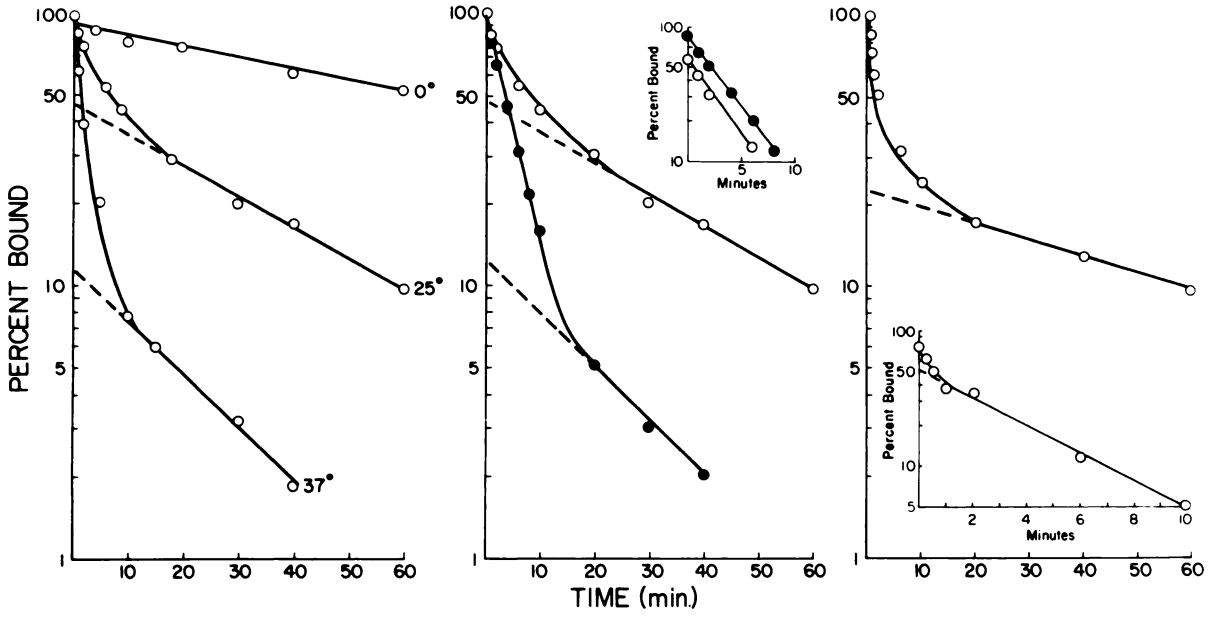

FIG. 8. *Dissociation of bound [^{3}H]naltrexone*

Untreated membranes were incubated with 1 nM [^{3}H]naltrexone in the absence (○) of 150 mM NaCl at different temperatures to reach equilibrium in binding. Then, unlabeled naltrexone was added to give a final concentration of 10 μM. Subsequently, at the indicated times, the samples were rapidly filtered and washed as described under Materials and Methods. Plotted is the percentage of specific [^{3}H]naltrexone binding remaining after a given time of incubation. Nonspecific interaction of naltrexone was determined by incubating membranes with the radiolabeled drug in the presence of 10 μM unlabeled naltrexone until the indicated times. Multiphasic dissociation was resolved by kinetic analysis (26), and the kinetic parameters were computed by data fit (24, 25). The experiments depicted in the *middle graph* were carried out as described above, but at 25° and in the absence (○) and presence (●) of 150 mM NaCl. The *inset* shows the early kinetics of the rapid dissociation component obtained by subtracting the slow component from total dissociation. The *right graph* shows the dissociation after binding of 50 nM [^{3}H]naltrexone at 25° in the absence of sodium. Also depicted are the residual components obtained after subtracting the slow component from total dissociation (*inset*). The data represent the average of values obtained in two experiments. Variability of individual data points was less then 3% around the average.

of glass-fiber filters to bind stereospecifically opiates with high affinity has previously been demonstrated (32). As emphasized earlier in this paper, the extent of radioactive ligand binding to these filter discs represents a troublesome aspect of the opiate receptor assay (Fig. 2), and the artifactual consequences of inaccurate assessment of nonspecific ligand interaction on Scatchard analysis of receptor binding have been described (30). Drug interaction with glass-fiber filters was the focus of a recent controversy on the specificity of [^{3}H]phencyclidine binding to receptor (19). In view of these critical assessments of the quick-filtration technique, [^{3}H]naltrexone binding was also investigated by rapid microcentrifugation using both excess unlabeled drug and enantiomers of naloxone to determine the specific component of the drug-membrane interaction. All of these approaches yielded biphasic Scatchard plots with similar binding parameters (Fig. 7).

Evidence for more than one type of specific binding site for [^{3}H]naltrexone was also provided by its dissociation kinetics, and by the good agreement of K_D values obtained from Scatchard analysis on one hand and by calculation from the observed rate constants of association (Fig. 5) and dissociation (Fig. 8) on the other. At the two higher temperatures, [^{3}H]naltrexone dissociation was biphasic, and the kinetic characteristics of the rapid component corresponded to the high-affinity binding process. An additional rapid dissociation component of low affinity was revealed after equilibrium binding of high concentrations of the radiolabeled antagonist. On the basis of its K_D, this component was related to the low-affinity binding site for [^{3}H]naltrexone. The nature of the ubiquitous slow-dissociation component, corresponding to very high binding affinity, has not yet been identified, although a similar phenomenon was observed previously in other binding systems (33), including the opiate receptor (34).

No differences in the interaction of putative μ, κ, or σ opiates (3) with the two binding sites for [^{3}H]naltrexone were observed (Table 1). It is possible that brain membranes represent a preparation unsuitable for the study of the multiple opiate receptor binding sites owing to the perturbing effect of the isolation procedure. Furthermore, in considering cross-recognition of opiates at these sites, the employed concentration of the displacing agents might have been excessive to detect limited differences in binding affinity. In recently conducted experiments, putative μ and κ opiates were separated on the basis of their different binding properties displayed in brain membranes in the absence and presence of sodium.[2]

[2] P. J. Dahlstrom, S. V. Fischel, and F. Medzihradsky, unpublished observations.

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