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Etorphine Binds to Multiple Opiate Receptors of the Caudate Nucleus with Equal Affinity but with Different Kinetics

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SUMMARY

The binding of [^{3}H]etorphine, a potent opiate agonist of the oripavine series, to membranes derived from sheep brain caudate nucleus is analyzed. Although the receptors are saturated by [^{3}H]etorphine in a homogeneous fashion with an apparent dissociation constant of 1.16 ± 0.3 nM, kinetic displacement studies reveal that at least two classes of sites are involved. All of the specific sites for [^{3}H]etorphine are blocked by morphine or naloxone if these ligands are added prior to, or simultaneously with, [^{3}H]etorphine. Otherwise, when [^{3}H]etorphine is added prior to morphine or naloxone, only one-third of the specific binding can be effectively displaced. The difference in the displacement patterns between the two classes of sites can be accounted for by the kinetics of the interaction between [^{3}H]etorphine and the receptors. At 37° [^{3}H]etorphine dissociates from one-third of the sites with a half-life of 2.3 min and from the remaining sites with a half-life of 70 min. The sites which release [^{3}H]etorphine slowly have a 10-fold higher apparent affinity for morphine and for naloxone as compared with the more rapidly reversible sites. The binding data are only partially compatible with a model which involves two independent classes of sites. The possibility of identifying the site from which [^{3}H]etorphine dissociates slowly with *mu*, *delta*, or *kappa* opiate receptors is explored in light of the fact that [^{3}H]etorphine is a mixture of D- and L-stereoisomers.

INTRODUCTION

Etorphine is a potent opiate agonist of the oripavine series (1, 2). In numerous binding studies it has been shown to bind with almost equal affinity to two prevalent classes of opiate receptors found in rat brain homogenate membrane preparations: the *mu* class, which binds morphine with a high affinity, and the *delta* class, which binds enkephalins with a high affinity (3–5). Recently it has also been shown to bind to *kappa* receptors with similar affinity (6). The lack of selectivity of opiate receptors for etorphine has been shown to correlate with a particularly high binding capacity of brain homogenates for etorphine as compared with other, more specific, radiolabeled opiates and opioid peptides (6, 7). A simple, saturable binding of etorphine to all of the binding sites was found (7).

Most of the evidence for the presence of a mixture of distinct opiate receptor subclasses in brain is derived from measurement of the efficacy (IC_{50} values) of a wide range of opiates and opioid peptides in displacing 50% of very low concentrations of radiolabeled ligands which have been selected for their specificity according to pharmacological activity profiles. Often the radioactive ligands are present at concentrations below their apparent half-saturation constants, and the IC_{50} values for cold ligands do not correspond to the half-saturation constant of their radioactive congeners.

The problem of quantifying multiple receptor distributions in different brain areas by binding studies has been approached indirectly by calculating the maximal extent of binding of a selected group of radioligands assayed at very low concentrations by applying the laws of mass action (8) or by examining the distribution of GTP-sensitive and GTP-insensitive opiate binding sites (9).

Etorphine would be ideally suited to examination of both the selectivity of opiate ligands to multiple classes of opiate receptors and the distribution of such receptors in specific brain regions, provided that the interaction of etorphine with each receptor subclass is indiscriminate, truly reversible, and kinetically equivalent. These features would allow binding studies to be conducted at saturating radiolabel concentrations, and IC_{50} values derived by careful fitting would then correspond more closely to thermodynamic half-saturation constants.

The striatum has been reported to possess multiple classes of opiate receptors (8, 10). This study presents an analysis of the kinetics of interaction of [^{3}H]etorphine with opiate receptors of the caudate nucleus and explores the applicability of simple molecular models in order to account for the complex kinetic behavior of this system.

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MATERIALS AND METHODS

[^{3}H]Etorphine and [^{3}H]diprenorphine were purchased from the Radiochemical Centre (Amersham, England) and had specific activities of 30.6 Ci/mmole and 6.1 Ci/mmole, respectively. The [^{3}H]etorphine was purified on silica gel with ethanol/acetic acid/water (6:3:1). [^{3}H]Diprenorphine was used without further purification. [^{3}H]Naloxone was purchased from New England Nuclear Corporation (Boston, Mass.) and was purified on silica gel with the etorphine solvent system. Its specific activity was 25.4 Ci/mmole. Naloxone was a gift from Endo Laboratories (Garden City, N. Y.). Morphine HCl was purchased from Verenigde Pharmaceutische Fabriken (Apeldoorn, Holland).

Membrane preparation. The head portion of the caudate nucleus was excised from sheep brains 5–10 min after slaughter and brought to the laboratory on ice. Material from five sheep brains was pooled, weighed, and homogenized in an Ultra-Turrax polytron set at 0.9 of maximal speed, in an ice-cold buffer consisting of 50 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, 1 mM Na_2EDTA, and 10% sucrose in a final volume 10 times the fresh weight. The homogenization consisted of four 30-sec runs in increasing volumes of the buffer until the desired volume was obtained. The homogenate was centrifuged for 5 min at 1,500 × *g*. The supernatant was saved, and the pellet was rehomogenized in 0.25 the volume of the first homogenization for 30 sec and centrifuged at 1,500 × *g* for 5 min. The two supernatants were combined and were centrifuged at 25,000 × *g* for 10 min. The pellet was collected in the 10% sucrose buffer, and 2 ml were layered in a discontinuous sucrose gradient which consisted of 3.5 ml of 41% sucrose and 3.5 ml of 27% sucrose, both of which were in 50 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, and 1 mM Na_2EDTA. The gradients were centrifuged for 45 min at 92,000 × *g* in a fixed-angle rotor. The material at the 27%–41% interface was collected, diluted in the 10% sucrose buffer, and recentrifuged at 25,000 × *g* for 10 min. Pellets were suspended in 10% sucrose buffer and frozen in liquid nitrogen. Prior to use, membranes were thawed at 37° and were washed twice by centrifugation at 25,000 × *g* for 10 min in 30 volumes of the ice-cold homogenization buffer lacking sucrose. Intermittent 5-min incubations at 37° were performed between washes. Protein was measured by the method of Lowry *et al.* (11), using bovine serum albumin as standard.

Binding. Binding was performed essentially as described by Pert and Snyder (12). Membranes (1.5–2.5 mg/ml final concentration in assay) were preincubated in the presence of buffer or cold ligand 5 min prior to the addition of the radiolabeled ligand. The label was also incubated separately to the desired temperature before its addition to the membranes. Binding time was calculated from the moment of addition of radiolabel unless otherwise indicated in the figure legends. At the desired times, 100-μl aliquots were removed onto GF/B glass-fiber filters, which were washed four times with 4 ml of an ice-cold buffer containing 50 mM Tris-HCl (pH 7.4). All four washes of the filters were completed within 10 sec. At all ligand concentrations, 90–95% of the nonspecific binding was found to be due to the filter retention as assessed by a parallel experiment in which no membranes were added. No specific binding to GF/B filters was found. This method yielded results identical with those obtained with the decantation method, where the contents of the tube are poured onto the filter, and enabled multiple sampling from a single tube as well as the exact measurement of the ligand concentration in the assay. Total binding is defined as the amount of radiolabeled ligand bound in the absence of any cold opiate ligand. Nonspecific binding was measured in the presence of a 1000-fold excess of a nonlabeled opiate which was added before the addition of the label. The specific binding is the difference between binding measured in the two systems.

Fitting functions. Competition curves were simulated, or the data were fitted, assuming that the labeled and the nonlabeled opiates compete for the same sites in a reversible manner. Under equilibrium conditions the function assumes the following form:

$$[LR] = \frac{[L] \times [R_T]}{[L] + K_L(1 + [D]/K_D)} \quad (1)$$

where L is the label, R_T is the total receptor concentration, LR is the label-receptor complex, D is the free concentration of the competing ligand, and K_L and K_D are the equilibrium dissociation constants for the ligands L and D, respectively. When D and R equilibrate rapidly with DR but the binding of L is slow and is not at equilibrium, the function assumes the following form:

$$[LR]_t = \frac{[L] \times [R_T]}{[L] + K_L(1 + [D]/K_D)} e^{-(k_1[L]/(1 + [D]/K_D) + k_2)t} \quad (2)$$

where k_1 is the association rate constant of L, and k_2 is the dissociation rate constant of L. At infinite time t, the exponential term vanishes; k_2/k_1 is equal to K_L.

RESULTS

Binding of [^{3}H]etorphine and its displacement by opiates. The results shown in Fig. 1 represent the specific binding of various [^{3}H]etorphine concentrations as measured in five different membrane preparations which were derived during 1 year. An analysis of the binding by a linearization of the saturation function reveals only one class of high-affinity sites. The values of the apparent dissociation constant and the maximal capacity of each preparation are given in the legend to Fig. 1. The common average capacity of the membranes for [^{3}H]etorphine was 988 ± 293 fmoles per milligram of protein, and the common apparent dissociation constant was 1.16 ± 0.3 nM. The specific binding of [^{3}H]naloxone, up to a concentration of 20 nM, is also presented for comparison. Since the etorphine data were collected by determining the nonspecific binding in the presence of 10 μM naloxone, it is clear that high concentrations of naloxone can displace [^{3}H]etorphine from more sites than the number of sites which can be saturated by 20 nM [^{3}H]naloxone. Thus, one would expect the displacement of [^{3}H]etorphine by naloxone to reveal site heterogeneity.

The displacement of [^{3}H]etorphine by naloxone or by morphine is shown in Figs. 2 and 3. The binding of [^{3}H]etorphine was measured in the presence of mor-

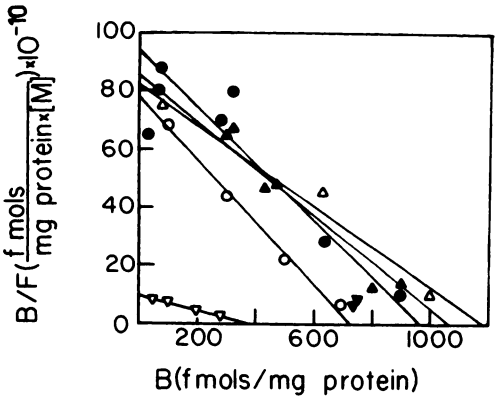

FIG. 1. *Binding of [^{3}H]etorphine to caudate nucleus membranes*

[^{3}H]etorphine (0.3–30 nM) was incubated with various preparations of caudate nucleus membranes (1.5–2.5 mg of protein per milliliter) for 30 min at 37° in the presence or in the absence of 10 μM naloxone in order to determine specific binding. Each point represents an average of duplicate determinations, where the error is indicated by the size of the point. Specific binding (B) (▲, △, ○, ●) is plotted as a function of free [^{3}H]etorphine concentration (F) in the form of a Scatchard plot. Linear regression yielded the following parameters for half-saturation constants and maximal capacities of each membrane preparation, respectively: ○, 0.93 ± 0.05 nM, 728 ± 43 fmoles per milligram of protein (these data are derived from the experiment shown in Fig. 5); ●, 1.23 ± 0.2 nM, 1074 ± 204 fmoles per milligram of protein; ▲, 1.02 ± 0.085 nM, 949 ± 78 fmoles per milligram of protein; △, 1.45 ± 0.2 nM, 1201 ± 191 fmoles per milligram of protein. The points ▼ represent the extent of specific etorphine binding taken as 100% in the experiment presented in Fig. 2. In the *left corner* the binding of [^{3}H]naloxone to membranes to which [^{3}H]etorphine binding is represented by 0 is shown (▽).

phine, which was added either 10 min before the addition of the label or 10 min after the addition of the label (Fig. 2A). The total incubation time was 30 min. The pattern of displacement changed as a function of the order of addition of the ligands. When [^{3}H]etorphine was added first, only one-third of the sites was displaced within 20 min. Displacement from these sites could be fitted by a function which assumed competition for one class of sites. A half-saturation constant of 600 ± 150 nM was derived for the binding of morphine to these sites (r = 0.90). After preincubation of the membranes with morphine, a second class of sites emerged to which morphine appeared to bind with a half-saturation constant of 30 nM. This constant was derived by the incorporation of a low-affinity site for morphine (half-saturation constant 600 nM, capacity 34%) into a fitting function that was composed of two classes of sites (r = 0.98) which were in equilibrium with the ligands. The difference in the half-saturation constants for morphine appeared to be 20-fold, and the difference in capacity about 1:2. The *dotted line* represents a fit according to a similar model except that the high-affinity site for morphine was not assumed to be in equilibrium with [^{3}H]etorphine (Eq. 2). This model generated a similar pattern of displacement, but the half-saturation constant for morphine was shifted to 60 nM, only 10-fold lower than the value derived for the low-affinity site. The relative contribution of each class of sites can be seen by following the simulation of the predicted displacement according to Eq. 2 (60 nM, morphine half-saturation constant) to a homogeneous population of sites (*dashed line*). Similarly, the predicted displacement from all of the sites according to Eq. 2 is shown, where the half-saturation constant for morphine was 600 nM.

A parallel experiment is shown in Fig. 2B, in which naloxone was the competing ligand. The displacement curve was generated by a model which contained two sites (r = 0.967). Naloxone appeared to bind to 63% of the sites with a half-saturation constant of 6 nM, a value derived directly from an analysis of the binding of [^{3}H]naloxone in the 1–20 nM range (Fig. 1, for example). The half-saturation constant derived for naloxone binding to the remaining sites was 140 nM. The *dotted line* represents a fit according to the sum of Eq. 1 (the low-affinity site) and Eq. 2 (the high-affinity site). The half-saturation constant for naloxone was shifted from 6 nM

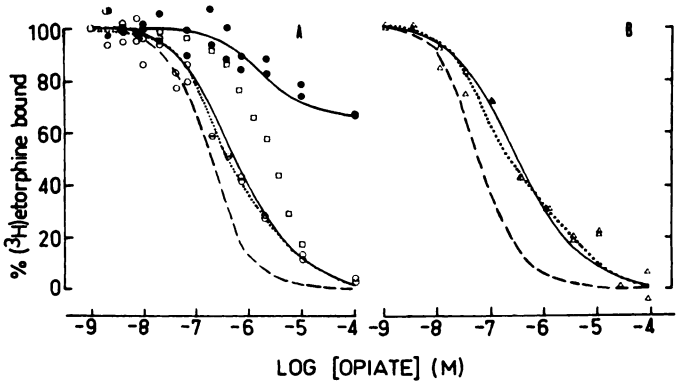

FIG. 2. *Displacement of [^{3}H]etorphine binding by morphine and naloxone*

A. Caudate nucleus membranes (2 mg of protein per milliliter) were incubated for 10 min at 37° in a volume of 250 μl in the presence of [^{3}H]etorphine (8 nM final concentration) in duplicate (●). After 10 min, 20 μl of morphine were added to each tube followed by 20 min of additional incubation in the presence of both ligands. After a total of 30 min two samples of 100 μl were removed to assess binding. An analogous experiment was performed in which morphine (○) was added first, followed by etorphine. One hundred per cent specific binding is equivalent to 588 fmoles per milligram of protein, which constitutes 86% of the total capacity of these membranes for [^{3}H]etorphine. The *lines* through the *points* are theoretical, using the following functions and parameters: When [^{3}H]etorphine preceded morphine (●): Eq. 1; [L] 8 nM, K_L 1.2 nM, K_D 600 nM, [R_T] 34%. When morphine preceded [^{3}H]etorphine (○): ——, sum of two expressions according to Eq. 1; [L] 8 nM, K_L 1.2 nM, low-affinity K_D 600 nM, [R_T] 34%, high affinity K_D 30 nM, [R_T] 66%. · · · · ·, Sum of low-affinity site according to Eq. 1 and of high-affinity site according to Eq. 2; [L], 8 nM, K_L 1.2 nM, low-affinity K_D 600 nM, [R_T] 34%, high-affinity K_D 60 nM, k_1 $1 \cdot 10^7$ M^{-1} min^{-1}, k_2 0.01 min^{-1}, [R_T] 66%, t 20 min. – – –, One class of high-affinity sites according to Eq. 2. The parameters of the high-affinity site are those indicated by · · · · ·; only R_T is 100%. □, One class of low-affinity sites according to Eq. 2; the parameters for etorphine are indicated by · · · · ·; the K_D is 600 nM, [R_T] 100%.

B. A similar experiment was conducted with the addition of naloxone preceding the addition of [^{3}H]etorphine (27 nM final concentration); 100% binding is equivalent to 506 fmoles per milligram of protein. ——, Sum of two expressions according to Eq. 1: [L] 27 nM, K_L 1.2 nM, low-affinity K_D 140 nM, [R_T] 37%, high-affinity K_D 6 nM [R_T] 63%. · · · · ·, Sum of two expressions according to Eq. 1 (low-affinity) and Eq. 2 (high-affinity): [L] 27 nM, K_L 1.2 nM, low-affinity K_D 140 nM, [R_T] 37%, high-affinity K_D 14 nM, [R_T] 63%; k_1, k_2, and t are given in A. – – –, One class of high-affinity sites according to Eq. 2; the parameters used are identical with those of the high-affinity site indicated by · · · · ·, except that [R_T] is 100%.

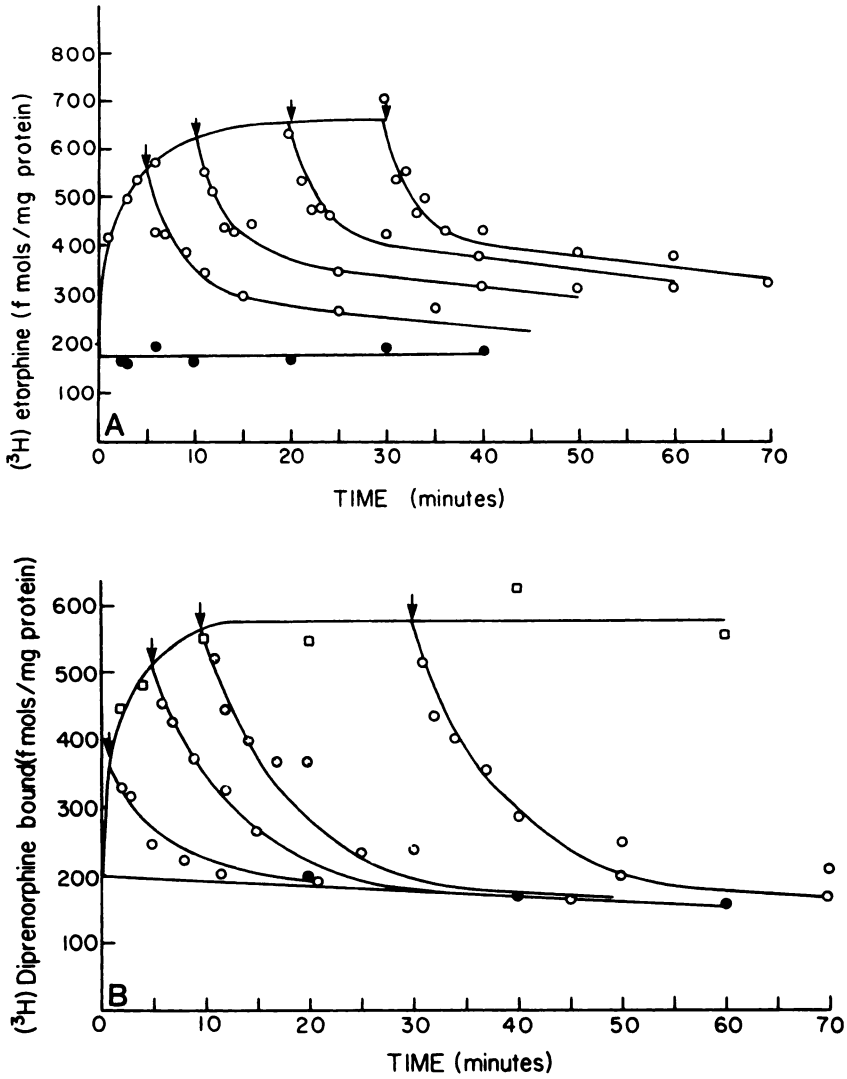

FIG. 3. *Kinetics of displacement of [^{3}H]etorphine and [^{3}H]diprenorphine by naloxone*

The binding of [^{3}H]etorphine and of [^{3}H]diprenorphine to caudate nucleus membranes (2.4 mg of protein per milliliter) at 37° was initiated by the addition of either 5 nM [^{3}H]etorphine (A) or of 20 nM [^{3}H]diprenorphine (B) in the absence of naloxone (○) or simultaneously with 10 μM naloxone (●). At the times indicated by the *arrows*, 10 μM naloxone was added to the systems which contained no naloxone and the remaining extent of label-receptor complex was measured. The *lines* through the *points* are theoretical simulations of the following function. A, Association was simulated by a first-order rate constant of 0.544 min^{-1}; the dissociation process was composed of two first-order rate constants, 0.315 min^{-1} and 0.014 min^{-1}. The rapidly reversible sites had a capacity of 200 fmoles/mg of protein and were equivalent for all of the dissociation curves. B, The association (□) was simulated by a first-order process with a rate constant of 0.73 min^{-1}. The dissociation was simulated at all times by a first-order process with a rate constant of 0.115 min^{-1} (○), and was equivalent for all of the dissociation curves.

to 14 nM. The *dashed line* follows a simulation according to Eq. 2, assuming a homogeneous population of sites. Similar deviations of this model from the data were observed for naloxone and for morphine.

The difference between the fit according to a sum of two terms which follow Eq. 1 and a sum of two terms which follow Eq. 1 (low-affinity sites) and Eq. 2 (high-affinity sites) is very small and statistically insignificant (13). The implication for the derivation of the apparent affinities for the displacing ligands is substantial, the ratio of affinities being reduced from 20-fold to 10-fold.

Kinetics of displacement. The competition pattern between [^{3}H]etorphine and either naloxone or morphine indicates the presence of a heterogeneous population of sites (Fig. 2). The pattern of competition between morphine and [^{3}H]etorphine also appears to change as a function of time. These results indicate that the displacement experiments were not performed under conditions of equilibrium. A kinetic experiment in which naloxone was added either simultaneously with [^{3}H]etorphine or at various times after the initiation of binding by [^{3}H]etorphine is presented in Fig. 3A. Naloxone could not displace all of the [^{3}H]etorphine once the label was bound. A similar experiment conducted with the analogue antagonist of etorphine, [^{3}H]diprenorphine, is presented in Fig. 3B. In contrast to etorphine binding, the complex of the receptors with [^{3}H]diprenorphine remained almost completely reversible, whereas the extent of binding was similar for the two analogues.

The ability of high concentrations of naloxone to

prevent all of the etorphine binding when naloxone was added simultaneously with etorphine would suggest that naloxone binding occurs very rapidly as compared with the rate of etorphine binding. The difference in the kinetics of the interaction between [^{3}H]etorphine and [^{3}H]naloxone and the receptors can also be observed directly in Fig. 4. In this experiment the effect of $MgCl_2$ and of NaCl on the association and the dissociation patterns of these ligands as well as on their reversibility

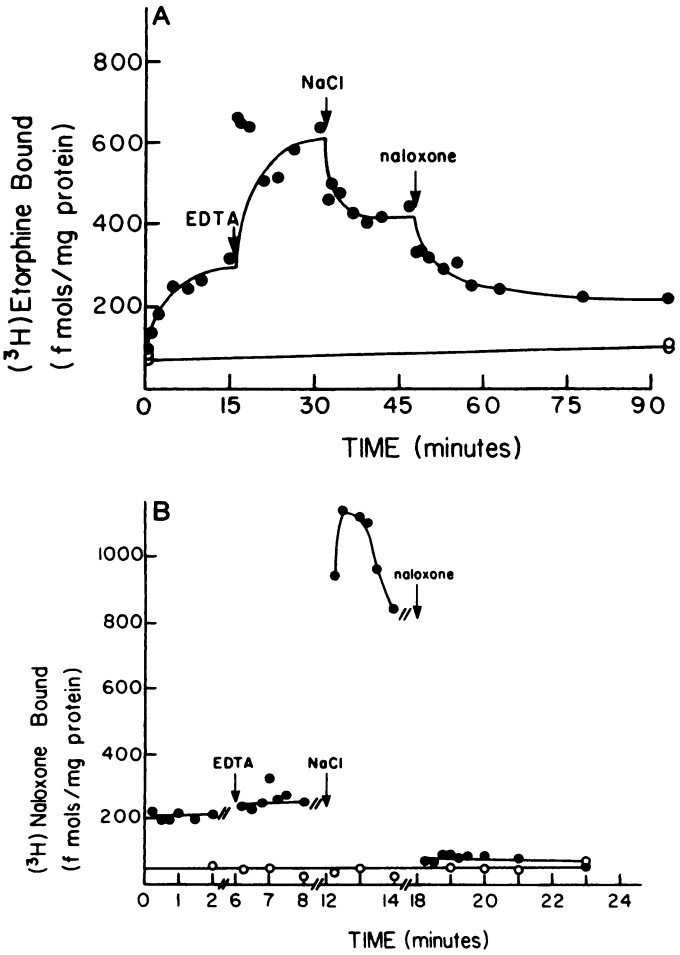

FIG. 4. *Redistribution of [^{3}H]etorphine and of [^{3}H]naloxone between free and bound forms following perturbations by EDTA and NaCl*

A. Membranes (1.5 mg of protein per milliliter) were incubated with 7 nM [^{3}H]etorphine for 15 min at 21°. At times indicated by the *points*, 100-μl samples were removed to measure binding. At 16 min, 120 μl of 100 mM Na_2EDTA (pH 7.4) were added (5 mM final concentration), and 105-μl aliquots were removed at the times indicated by the *points*. After an additional 16 min of sampling, 180 μl of 1 M NaCl (100 mM final concentration) were added and 110-μl aliquots were removed. Sixteen minutes after the addition of NaCl, 12 μl of 1 mM naloxone were added (10 μM final concentration) and sampling was completed as indicated.

B. An identical experiment was conducted with [^{3}H]naloxone (5.5 nM final concentration). The time scale is reduced such that the first sample was taken 15 sec after the addition of [^{3}H]naloxone or EDTA, NaCl, or cold naloxone. Sampling was terminated after 2 min, and filters were changed. The interval between the last sample point of one run and the addition of the next perturbant was 4 min, during which no samples were removed.

was also examined. The experiments were conducted at 21° in order to slow the interaction time. No effect of temperature on the final levels of binding for either ligand was observed.

At similar concentrations, [^{3}H]naloxone equilibrated with its binding sites within 15 sec, whereas the interaction of [^{3}H]etorphine with its binding sites was much slower. Upon perturbation of the system with EDTA, new equilibria were established. The change was much faster for naloxone than for etorphine. EDTA appeared to cause an increase in the binding of both ligands. The addition of NaCl after EDTA caused a fast dissociation of [^{3}H]etorphine close to the initial level of binding obtained in the presence of $MgCl_2$. When naloxone was added after NaCl, the dissociation of [^{3}H]etorphine still followed a rapid and a slow process. Thus, although NaCl caused the dissociation of [^{3}H]etorphine, neither the rapidly dissociating component nor the slow component was completely abolished by sodium. The binding of [^{3}H]naloxone, on the other hand, was completely reversible under all experimental conditions examined. The transient increase in [^{3}H]naloxone binding caused by NaCl was measured only up to 2 min. Within the next 4 min, the levels declined further (data not shown). Therefore, the level of [^{3}H]naloxone bound when cold naloxone was added was not identical with the level measured at 2 min. This finding explains why such increases were not seen after 30 min of binding.

Modeling the kinetics of association and dissociation of [^{3}H]etorphine. A simple, saturable binding of a ligand together with a heterogeneous dissociation pattern is not compatible with a model in which there is only one class of receptors (Model 1):

$$L + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} LR \qquad (3)$$

Such kinetic behavior is predicted, on the other hand, by the following two-state model (Model 2):

$$L + R \underset{k_3}{\overset{k_1}{\rightleftharpoons}} LR \underset{k_4}{\overset{k_2}{\rightleftharpoons}} LR^* \qquad (4)$$

where LR is the first thermodynamically stable complex which undergoes isomerization to another form, LR^*. Slow isomerization may be a function of agonist activation, especially since the binding of [^{3}H]diprenorphine, an antagonist, did not seem to change as a function of time whereas the competition experiments between naloxone or morphine and [^{3}H]etorphine indicate that precisely such a change in time was occurring subsequent to the formation of the etorphine-receptor complex. Also, if the isomerization step is slow as compared with the rate of formation of LR, the rate of binding will appear to be independent of the concentration of label.

The kinetic equations for dissociation, association, and equilibrium assume the following forms: at t_∞ the total binding to the LR and LR^* forms is

$$\frac{[LR] + [LR^*]}{[R_T]} = \frac{[L]}{[L] + \dfrac{k_4 \cdot k_2/k_3 \cdot k_1}{k_2/k_1 + 1}} \qquad (5)$$

Binding will appear a simple saturable function of $[L]$.

The association pattern is

$$\frac{[LR]_t + [LR^*]_t}{[R_T]} = [LR^*]_{t_\infty}\left(1 - \frac{a}{a-b}e^{-bt} + \frac{b}{a-b}e^{-at}\right) + [LR]_{t_\infty}\left(\frac{a(b-k_4)}{k_3(a-b)}e^{-bt} + \frac{b(k_4-a)}{k_3(a-b)}e^{-at} + \frac{k_4}{k_3}\right) \quad (6)$$

where

$$2a = k_1[L] + k_2 + k_3 + k_4 + \sqrt{(k_1[L] + k_2 + k_3 + k_4)^2 - 4(k_1[L]k_3 + k_1[L]k_4 + k_2k_4)} \quad (7)$$

and

$$2b = k_1[L] + k_2 + k_3 + k_4 - \sqrt{(k_1[L] + k_2 + k_3 + k_4)^2 - 4(k_1[L]k_3 + k_1[L]k_4 + k_2k_4)} \quad (8)$$

When dissociation is initiated after equilibrium is obtained (by infinite dilution or by dilution into a large excess of cold antagonist which prevents reassociation of the label), the pattern of dissociation is as follows:

$$\frac{[LR]_t + [LR^*]_t}{[R_T]} = [LR^*]_{t_0}\left(\frac{a}{a-b}e^{-bt} - \frac{b}{a-b}e^{-at}\right) + [LR]_{t_0}\left(\frac{a(b-k_4)}{k_3(a-b)}e^{-bt} + \frac{b(k_4-a)}{k_3(a-b)}e^{-at}\right) \quad (9)$$

where

$$2a = k_2 + k_3 + k_4 + \sqrt{(k_2 + k_3 + k_4)^2 - 4k_2k_4} \quad (10)$$

$$2b = k_2 + k_3 + k_4 - \sqrt{(k_2 + k_3 + k_4)^2 - 4k_2k_4} \quad (11)$$

This model predicts a deviation from pseudo-first order kinetics of ligand association as well as a change in the distribution of L between the LR and the LR^* forms as a function of time, especially at times shorter than equilibrium, if k_4 is much smaller than k_2.

The following model, in which two parallel sites are proposed, also conforms to the criteria of simple, saturable binding and non-first order dissociation kinetics if $k_3/k_4 = k_1/k_2$ (Model 3):

$$L + R_1 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} LR_1 \quad (12)$$

$$L + R_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} LR_2 \quad (13)$$

The association is composed of two exponentials:

$$[LR_1]_t + [LR_2]_t = \frac{[L][R_{1T}]}{[L] + k_2/k_1}(1 - e^{-(k_1[L]+k_2)t}) + \frac{[L][R_{2T}]}{[L] + k_4/k_3}(1 - e^{-(k_3[L]+k_4)t}) \quad (14)$$

The binding at infinite time (t_∞) is equal to the sum of the two pre-exponential terms. The dissociation pattern after equilibrium has been obtianed and the rebinding of the label is prevented is as follows:

$$[LR_1]_t + [LR_2]_t = [LR_1]_{t_0}e^{-k_2t} + [LR_2]t_0e^{-k_4t} \quad (15)$$

The kinetics of association and dissociation are different for Models 2 and 3.

In light of these models, the kinetics of [^{3}H]etorphine-receptor complex formation was followed as a function of time and of etorphine concentration (Fig. 5). Each curve through the experimental points was derived separately for each etorphine concentration and represents a first-order process of complex formation. The following may be observed: (a) At all four [^{3}H]etorphine concentrations the binding process appears to be first-order except for a minor component of rapid binding at the highest concentration. (b) The rate of complex formation changes 1.3-fold whereas the ligand concentration increases 60-fold. (c) At 15 min, stationary levels of binding are obtained, even at the lowest concentration tested. (d) The addition of naloxone at 15 min causes a dissociation which proceeds through a rapid and a slow phase. (e) The extent of complex formation as a function of [^{3}H]etorphine concentration is determined by a simple, saturable process (Fig. 1).

A simulation of the two-state model (Eqs. 4–11) and of the parallel-site model (Eqs. 12–15) is also presented in Fig. 5. The parameters used in the simulation of Model 2 were derived by introducing the dissociation constants which were derived from the experiment presented in Fig. 3: 0.3 min^{-1} and 0.014 min^{-1}. The half-saturation constant was derived from Fig. 1 (1.2 nM). Since the slow site was 1.5 times more abundant than the fast site (Figs. 2 and 3), k_3 was given the value of 0.021 min^{-1}, and k_1 was calculated from Eq. 5 as 6×10^7 M^{-1} min^{-1}.

Identical dissociation rate constants were used for the simulation of Model 3: $k_2 = 0.3$ min^{-1} (fast site, R_1) and $k_4 = 0.01$ min^{-1} (slow site, R_2), The ratio R_2/R_1 was 1.5. When k_1 and k_3 values were chosen so that $k_4/k_3 = k_2/k_1$ ($K_1 = 3 \times 10^8$ M^{-1} min^{-1} and $K_2 = 1.0 \times 10^7$ M^{-1} min^{-1}), the pattern deviated substantially from that observed, so that no slowly dissociating component would be occupied after 60 min at the lowest etorphine concentration tested. Therefore, in order to compensate for the slowness of Site 2, the association constant k_3 was increased slightly, to 3×10^7 M^{-1} min^{-1}. This shifted the apparent affinity of [^{3}H]etorphine to the slow site about 3-fold ($k_4/k_3 = 0.3$ nM, $k_2/k_1 = 1$ nM). Such a 3-fold difference in affinities would not be observed in the Scatchard analysis of two sites which are present in nearly equal amounts.

One can observe that the simulated parallel-site model (Model 3) is much more similar, in general, to the experimentally derived pattern of [^{3}H]etorphine binding and dissociation than is the two-state model (Model 2). The dissociation pattern is nearly identical, with the rapidly dissociable component appearing only at the higher concentrations of ligand because of the relatively lower affinity of the ligand for R_1. A discrepancy between the data and this model is apparent at low concentrations of ligand, where stable binding levels are achieved much more rapidly than is predicted by this model.

[^{3}H]Etorphine association and dissociation to rat brain membranes. The particular pattern of etorphine

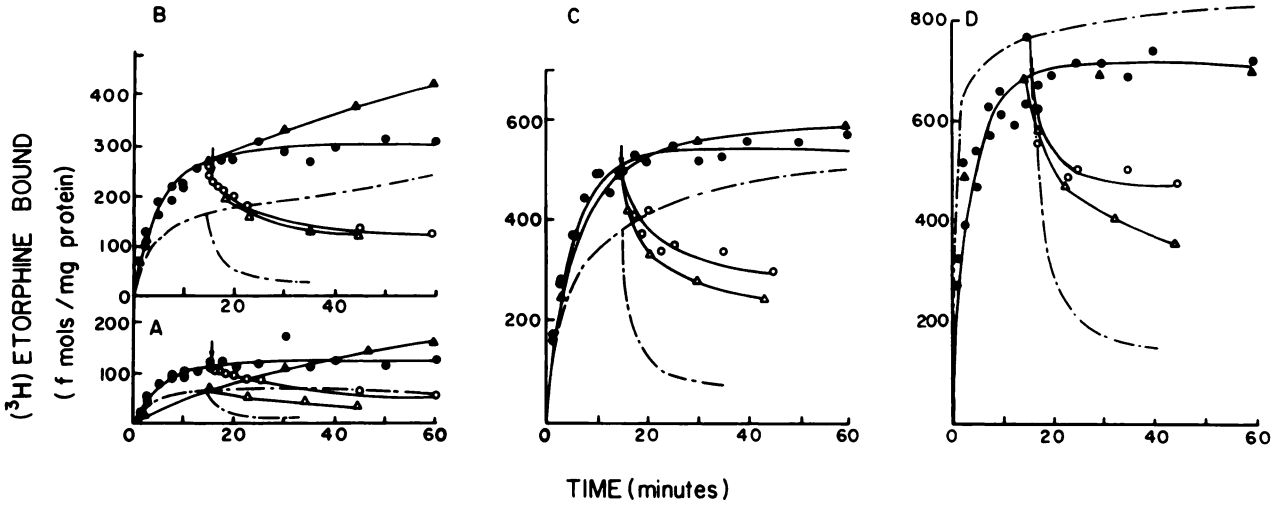

FIG. 5. *Kinetics of [³H]etorphine binding as a function of [³H]etorphine concentration*

Membranes (2 mg of protein per milliliter) were incubated with [³H]etorphine at 37° in two parallel sets. At the times indicated by the *points* (●), 100-μl aliquots were removed and analyzed for binding. After 15 min (*arrow*), 10 μl of naloxone (10 μM final concentration) were added to one set (○) and the incubation was continued for an additional 45 min. The line through ● follows a first-order process with the following parameters. A, Free [³H]etorphine concentration at the end of the binding process was 0.18 nM, which was 65% of the total ligand added at zero time; the rate constant was 0.173 min^{-1}. B, Free [³H]etorphine concentration at the end of run was 0.76 nM, which was 62% of the total ligand added at zero time; the rate constant was 0.165 min^{-1}. C, Free [³H]etorphine concentration at the end of run was 3 nM, which was 67% of the total ligand added; the rate constant was 0.2 min^{-1}. D, Free [³H]etorphine concentration was 12.5 nM, which was equivalent to the total ligand added; the rate constant was 0.23 min^{-1}. Models 2 and 3 are compared as follows: ▲ represents theoretically derived points according to the following equation (Eq. 13 in text):

$$\text{Bound} = \frac{0.67R_TL(1 - e^{-(0.01+3\times10^7L)t})}{0.01/3 \times 10^7 + L} + \frac{0.33R_TL(1 - e^{-(0.3+3\times10^8L)t})}{0.3/3 \times 10^8 + L}$$

where R_T is the total receptor concentration and L is the free label concentration. The value of R_T was derived from the Scatchard plot (Fig. 1) and is equal to 720 fmoles per milligram of protein. A simulation of the dissociation after 15 min of binding according to these parameters is given by △; - · - · - represents a simulation of Model 2 (Eqs. 6 and 9), using the following parameters: k_1, 6×10^7 M^{-1} min^{-1}; k_2, 0.3 min^{-1}; k_3, 0.021 min^{-1}; k_4, 0.014 min^{-1}; and R_T, 720 fmoles per milligram of protein.

binding exhibited in Fig. 5 could be exclusive for the sheep caudate nucleus preparation. Therefore, a similar kinetic experiment was conducted on a membrane preparation derived from rat brain (Fig. 6). The pattern of association of [³H]etorphine to rat brain membranes is very similar to that obtained in the sheep preparation: the binding is apparently first-order at each ligand concentration, and the rate of binding increases slowly relative to the concentration of the ligand. Also, the complex between [³H]etorphine and the membranes is composed of two distinct classes of dissociable sites as detected by the dilution of the membranes into 50 μM naloxone. When naloxone was present from zero time at the same concentration, full protection of both sites was achieved.

DISCUSSION

[³H]Etorphine appears to bind with similar high affinity to at least two classes of binding sites which exist in the caudate nucleus. These sites, which are indistinguishable from one another when etorphine binding is measured after apparently stationary levels of binding are obtained, are not equivalent acceptors of etorphine. The kinetics of interaction between etorphine and its binding sites seems to be distinguished by a 20- to 60-fold difference in the association as well as the dissociation rate constants.

The property of kinetic non-equivalence between the two sites is reflected clearly in the pattern of displacement of [³H]etorphine by naloxone or by morphine. Only one-third of the sites occupied by [³H]etorphine can be displaced by naloxone or by morphine after 30 min at 37° when [³H]etorphine is allowed access to its binding sites prior to the addition of the opiate displacing ligand (Fig. 2). This is due to the almost irreversible nature of the binding of [³H]etorphine to two-thirds of the sites. On the other hand, when morphine or naloxone is incubated with the membranes before the addition of [³H]etorphine, a 2- to 3-fold increase in the extent of the "specific" binding can be observed. This effective blocking of etorphine binding by the opiates is due to the rapid kinetics of the opiate binding, which slows considerably the rate of etorphine interaction with the receptors (Eq. 2). The sites which remain reversible to [³H]etorphine seem to possess a relatively low affinity to morphine and to naloxone as compared with a 10-fold higher affinity of these ligands for the slow-release sites. This value is derived from an analysis of displacement according to the simplified pre-equilibrium kinetic equation (Eq. 2), where it is assumed that the displacer equilibrates rapidly with its binding sites. This assumption is based on the rapid kinetics observed for naloxone directly (Fig. 4b) and on the observation that morphine added together with [³H]etorphine also protects all of the binding sites.

The homogeneous binding pattern of [³H]naloxone to

a fraction of the sites occupied by [^{3}H]etorphine can be accounted for by the 10-fold difference in the naloxone affinities to the two classes of sites. This difference allows 83% saturation of the high-affinity site with about 7% contribution from the binding to the lower-affinity sites.

The site which releases [^{3}H]etorphine slowly is also occupied by etorphine more slowly than the fast-release sites. Such kinetics would be compatible with a two-state model, Model 2 (Eq. 4). This model could not be fitted to the data presented in Fig. 5 and therefore must be rejected. Model 3, which contains the same number of rate constants as Model 2 but 1 more degree of freedom (due to the assumed existence of two independent receptor classes), obtains a much better fit to the data, especially at higher concentrations, but only if two values for the affinity of etorphine to the two receptor classes are introduced. Even then deviations from the predicted behavior appear at the lower concentrations of etorphine. At this concentration range, the binding is much faster than predicted by either model. This behavior could be rationalized in part by the considerable depletion of [^{3}H]etorphine from solution during the binding process. since L_{free} was considered to be equal to L_{total} in solving the equations, the initial rate of binding will be faster than the predicted rate, which was calculated on the basis of the final free etorphine concentration measured after binding was completed. Nevertheless, such a correlation will not alter the deviations seen at longer times (between 15 and 60 min; Fig. 5A) and will not account for the relative insensitivity of the rate of binding to the concentration of the label. Thus it is likely that the mechanism of interaction between [^{3}H]etorphine and its binding sites involves more complex models than those tested here (for instance, a combination of Models 2 and 3). Models more complex than those analyzed in the present study are difficult to resolve experimentally unless one has a specific ligand for either the R or the R^* form of the receptor (14).

Any attempt to interpret the binding of etorphine and the kinetic displacement profiles in terms of multiple receptor populations is made complicated by the fact that [^{3}H]etorphine is a mixture of D- and L-stereoisomers. This raises three alternative possibilities: (a) D- and L-[^{3}H]etorphine compete with each other for the binding to a homogeneous population of sites in a kinetically nonequivalent fashion; one isomer would be interacting slowly, the other would interact rapidly. This would generate a heterogeneous pattern of displacement of the label, and the binding pattern would be qualitatively similar to that predicted by Model 2. (b) D- and L-labeled stereoisomers are competing with each other for more than one class of sites. (c) Either a D- or an L-isomer is not involved in the binding to a heterogeneous population of sites. The following evidence makes it highly unlikely that the reason for the kinetic heterogeneity in the binding of etorphine is due solely to the presence of a racemic mixture of stereoisomers: (a) There is direct evidence that at least two classes of opiate receptors exist in rat striatum (8, 10, 15). These sites, which discriminate between enkephalins and dihydromorphine, exist in a 1:1 ratio (8) or a 1:2 ratio (10), similar to the ratio of fast- and slow-release sites which bound [^{3}H]etorphine in the present study. (b) Blume (16) has shown that [^{3}H]etorphine is released in a homogenous manner from opiate receptors of membranes of the cell line NG 108-15. The rate constant for dissociation is 0.013 min^{-1} (at 32°), similar to the rate constant found for the slow release sites in the present study (Figs. 3, 5, and 6). These sites have been identified by Chang *et al.* (8) as a pure population of *delta* sites. (c) The steady-state data (Fig. 1) indicate that two stereoisomers, which presumably generate such different kinetic patterns, bind with similar apparent affinities (linear Scatchard plots). In contrast, recent studies show that the presence of a labeled racemic mixture generates a heterogeneous pattern of equilibrium binding (nonlinear Scatchard plots), although the binding is to one class of sites (17). (d) Widely different affinities (by 3 orders of magnitude) have been found for D- and L-opiate stereoisomers such as dextrorphan and levorphanol (12, 18), whereas almost equal affinities are generated by the putative stereoisomers of etorphine in the present study. This may be due to the fact that the

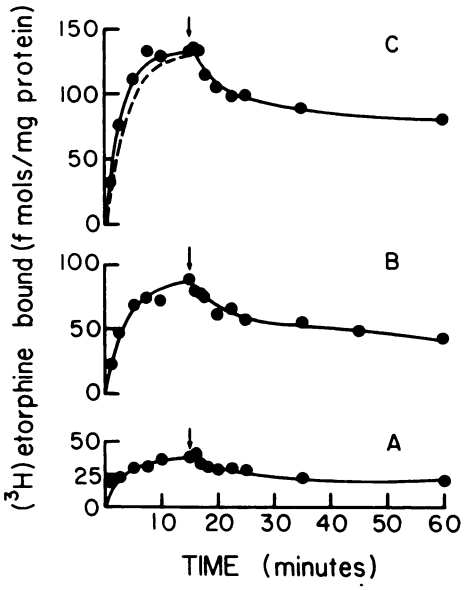

FIG. 6. *Specific binding of [^{3}H]etorphine to rat brain membranes*
A crude preparation of rat brain membranes was used. Eight rats (mature males and females, Sabra strain of the Hebrew University) were stunned and bled, and the brains minus cerebella were rapidly removed and placed in ice-cold homogenization buffer which contained 10% sucrose. The process of membrane preparation was identical with that described under Materials and Methods, but the discontinuous sucrose gradient step was eliminated. The crude 25,000 × g pellet was diluted in the ice-cold assay buffer and homogenized with five strokes in a glass/glass homogenizer before use. The concentration of membranes in the assay was 2 mg/ml. A, The final free [^{3}H]etorphine concentration was 0.345 nM. The rate of complex formation followed a first-order process, the rate constant being 0.266 min^{-1}. The dissociation process was not fitted. B, The final free [^{3}H]etorphine concentration was 1.5 nM; the rate of association was fitted according to a rate constant of 0.266 min^{-1}. C, The final free [^{3}H]etorphine concentration was 4.15 nM; the rate of association was fitted according to a rate constant of 0.3465 min^{-1} (a process governed by a rate constant of 0.266 min^{-1}; the value obtained for A and B is shown by - - - for comparison). At 15.5 min, 10 μl of naloxone (10 μM final concentration) were added to initiate dissociation. The maximal specific binding capacity of these membranes (determined by the prior addition of naloxone) was 397 fmoles per milligram of protein.

asymmetrical carbon atom of etorphine, which involves substituents of carbon atom 19, does not exist on the morphinan backbone. Indeed, [^{3}H]diprenorphine, which is a pure oripavine-derived isomer but also an antagonist, seems to occupy in a kinetically equivalent fashion as many sites as [^{3}H]etorphine (Fig. 3).

Thus, although it is tempting to speculate about the nature of the slow- and fast-release sites described in this study in terms of *mu*, *delta*, or *kappa* receptors, such clarification awaits the purification and separation of the etorphine stereoisomers. Since [^{3}H]etorphine binding is relatively irreversible to a fraction of the sites, the method of isomer depletion which has been used by Bürgisser *et al.* (17) could be applied here as well.

Should the kinetic anomaly described in the present study be ascribed to the presence of a D-isomer, D-isomers which bind strongly should not be discarded. Fennessy and Laska (19) have shown that dextrorphan, which binds weakly to *mu* and *delta* sites, can elicit acute effects and cause withdrawal symptoms similar to those produced by cyclazocine. It would be interesting to determine whether some effects induced by etorphine remain after the administration of naloxone *in vivo*.

APPENDIX

Competition between a slowly interacting ligand and a rapidly equilibrating ligand to a homogeneous population of sites (Eq. 2): when

$$L + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} LR \qquad \text{(A1)}$$

$$D + R \underset{K_D}{\rightleftharpoons} DR \qquad \text{(A2)}$$

where L interacts slowly with R, and D interacts very rapidly, so that essentially at the shortest time D and R are in equilibrium with DR, the rate of LR formation (where $[L] > [R]$) is

$$\frac{d[LR]}{dt} = k_1[L][R] - k_2[LR] \qquad \text{(A3)}$$

The conservation equation for R is

$$[R_T] = [R] + [LR] + [DR] \qquad \text{(A4)}$$

or

$$[R_T] = [R](1 + [D]/K_D) + [LR] \qquad \text{(A5)}$$

Inserting Eq. A5 into Eq. A3, one obtains

$$\frac{d[LR]}{dt} = \frac{k_1[L][R_T]}{1 + [D]/K_D} - \left(\frac{k_1[L]}{1 + [D]/K_D} + k_2\right)[LR] \qquad \text{(A6)}$$

Integrating with respect to time, one obtains

$$[LR]_t = \frac{[L][R_T]}{[L] + \frac{k_2}{k_1}(1 + [D]/K_D} \exp - \left(\frac{k_1[L]}{1 + [D]/K_D} + k_2\right)t \qquad \text{(A7)}$$

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