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Methods Used for the Study of Opioid Receptors*,†

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I. Introduction

OPIOID receptors exhibit a widespread distribution throughout the brain and periphery (38, 332, 380, 397, 598) and have been implicated in control of numerous physiological systems (7, 38, 149, 156, 221, 265, 384, 399, 579). These receptors mediate the pharmacological actions of morphine-like analgesics, a class of drugs which are important clinical agents and which are widely abused socially as a result of their euphorogenic and addictive properties (301, 302). They also mediate the physiological effects of three related families of endogenous opioid peptide (7, 38, 205, 271, 334, 607). Within the last two decades, an extensive research effort has been undertaken to determine the molecular properties and functional roles of opioid receptors and to develop novel synthetic opiates. Such studies are of both fundamental physiological and clinical significance, since they will not only elucidate basic neurochemical mechanisms but may also lead to the development of more specific therapeutic agents with fewer side effects. A wide range of methodologies are currently being used to characterize the properties of opioid receptors. The present article will review some of the more commonly used techniques and will attempt to evaluate the strengths and limitations of each approach.

The existence of a specific opioid receptor was first suggested by early behavioral and clinical studies, in which morphine-like analgesics were shown to exhibit a high degree of structural and steric specificity (26, 479, 625). By 1967, Martin had suggested that the complex clinical profiles of morphine-like drugs were best explained by postulating interaction with more than one type of opioid receptor (383, 386), a concept which he and his colleagues later extended to include multiple receptor types (193, 383). The development of in vitro methodologies, such as peripheral tissue bioassay (227, 281, 343) and receptor binding assay (209, 471, 555, 578), has since permitted a more rigorous analysis of the properties of opioid receptors and has provided confirmation of the multiple receptor hypothesis (72, 289, 373). Thus, convergent lines of evidence have indicated the existence of several different types of opioid receptor, each with subtle variations in pharmacological response. The data from these studies have been summarized in a number of reviews (208, 334, 384, 462, 642, 643).

As the methods for analysis of receptors have become more refined, the complexities of opioid pharmacology have appeared to increase. Opioid receptors have been classified into three main types (μ, δ, and K) (373, 385), with the tentative identification of others (ϵ and λ) which have not been as clearly differentiated (71, 220, 535, 540, 635). Recent reports indicate that subclasses of each receptor type may also exist (18, 67, 459, 476, 542). While there is some evidence that the different classes of opioid receptor are independent and noninteracting (93, 129, 130, 294, 494, 559, 564), other studies have indicated the possibility of allosteric interactions between receptor types (46, 505, 506) and of multiple modes of ligand interaction with an individual receptor (359, 481).

Analysis of the properties and functions of opioid receptors is further complicated by the finding that multiple receptor types may coexist within a single tissue (362, 373) and even within a single cell (145, 617, 618). Of the numerous endogenous opioids which have been identified (7, 38, 607) none has absolute pharmacological specificity for a given receptor type (271, 334). Although more specific synthetic ligands have recently become available for use (75, 103, 175, 186, 234, 350, 351, 423, 550, 637), many of the opioid drugs in current experimental use interact with several receptor types. Thus, in view of the heterogeneous properties of both biological tissues and the drugs which are used to study them, considerable care should be taken in the design of experiments and the interpretation of results.

The present paper will address the major methodological issues associated with the pharmacological analysis of opioid receptor properties. Although numerous approaches have been used for the study of the pharmacological characteristics and functional roles of opioid receptors, these generally fall into two broad categories, (*a*) determination of the biological activity of opioid agonists (bioassay) and (*b*) determination of the receptor binding properties of radiolabeled drugs (radioreceptor assay). The theoretical principles and methodological considerations associated with each approach will be considered separately, although considerable overlap does exist.

A. Terminology

Within recent years the definition of the term "opioid receptor" has become a matter of some controversy. At present, the most widely used criterion for classification of an "opioid" action is that of reversibility of the opioid antagonist, naloxone. This is the definition which will be used in the present review (see section II A1 for further discussion).

Using this criterion, some of the pharmacological effects of both synthetic and endogenous opioids may be considered to be "non-opioid." For example, the synthetic analgesic, cyclazocine, displays a wide range of behavioral effects, only some of which are blocked by naloxone (51, 105, 385, 588, 642). Although the nonnaloxone-reversible effects of this drug are defined as "non-opioid," they are mediated via a specific receptor (σ) which has distinct pharmacological properties. Thus, "non-opioid" does not necessarily equate to "nonspecific."

II. Bioassay

The fundamental principle of biological assay is that interaction of an agonist with a receptor induces a biological response. By comparing the responses elicited by varying doses and combinations of drugs, inferences may be made as to the nature of the drugs and the receptors upon which they act. The advantage of bioassay, as compared to receptor binding assay, is that the former involves measurement of a physiological endpoint; thus the receptor may be examined in its native, functional state. The disadvantage of bioassay is that the measured response may represent the culmination of a complex series of biochemical events; thus pharmacological constants derived in this way are not a direct measure of drug-receptor interaction. Bioassay systems for measurement of opioid activity range in complexity from biochemical measures in single cells to behavioral measures in whole animals. In the following sections the strengths and limitations of each methodological approach will be addressed.

A. General Principles

1. Determination of agonist potency. A necessary first step in measurement of the agonist activity of a test compound is to establish that the observed biological response is mediated via specific opioid receptors. This is particularly important when determining the activity of new drugs or of well-established drugs in new assay systems. While this has been an issue of some controversy, two criteria are generally used for classification of an "opioid" effect—naloxone reversibility and stereoselectivity. The agonist actions of a test compound should be blocked by naloxone in a concentration range which reflects the affinity of the antagonist for μ, K, or δ opioid receptors (equilibrium dissociation constant, $K_B = 5 \times 10^{-8}$ M, or lower). It should be noted that, while naloxone exhibits high affinity and selectivity for multiple opioid receptors, this drug does have additional pharmacological actions which are unrelated to opioid receptor blockade (for review, see refs. 261 and 521). Thus, antagonism by naloxone is a necessary, but not sufficient, criterion for defining an opioid agonist action. In order to confirm opioid receptor involvement, agonist effects must be shown to be blocked by other known opioid antagonists (e.g., dipenorphine, MR 2266). Drug-receptor interactions should also be shown to be stereospecific, with the (−) isomer of a test compound active in a dose range which is at least one order of magnitude lower than that of the corresponding (+) derivative. Since agonist stereoisomers may not always be available for use, stereospecificity may be demonstrated by showing that the agonist response is competitively blocked by low concentrations of (−), but not (+), isomers of opioid antagonists such as naloxone.

Having established that the observed biological response reflects interaction of the drug with an opioid receptor, agonist potency may then be determined. The agonist activity of a drug is usually characterized by the concentration which produces a 50% maximal response (ED_{50} or IC_{50}). The most common method for calculating agonist potency is to measure the effects of several doses of drug and to construct a log dose-response curve. Alternatively, bracketing methods may be employed in which the effects of one or two doses of test drug are compared with those of a standard agonist; ED_{50} values are then determined by extrapolation (204, 281, 343). This latter approach, although useful, is only valid if the slope of the log dose-response curve of the unknown is parallel to that of the standard.

Although an ED_{50} value is a measure of agonist potency (i.e., the ability of a drug to produce a biological response), it is not a direct measure of agonist affinity (i.e., the ability of a drug to bind to its receptor). Depending on the complexity of the test system, the measured biological response may represent the end point of a cascade of biochemical reactions initiated by receptor activation. Numerous factors may interfere at a secondary stage and affect the expression of the response. Additional factors may influence drug availability, such that the concentration of agonist added to the test system does not reflect the concentration at the receptors. These, and other, methodological considerations associated with measurement of agonist potency have been the subject of excellent reviews (172, 322) and will be dealt with extensively in later sections of this paper.

On theoretical grounds alone, it may be assumed that agonist potency is not equivalent to agonist affinity (47, 172, 321, 322, 511, 571, 605). The model most commonly used as the basis for analysis of pharmacological data is the "occupation" theory of drug-receptor interaction (95). In the original model, it is assumed that the magnitude of the biological response is linearly proportional to fractional receptor occupancy such that

$$\frac{E_A}{E_M} = \frac{[RA]}{[R_t]} \qquad \text{equation 1}$$

where E_A is the observed response to agonist A, E_M is the maximal response obtainable, $[RA]$ is the concentration of drug-receptor complex, and $[R_t]$ is the total concentration of receptors. Assuming a simple bimolecular interaction, the binding of the drug to its receptor may be described by the Law of Mass Action, such that

$$\frac{[RA]}{[R_t]} = \frac{[A]}{[A] + K_A} \qquad \text{equation 2}$$

where $[A]$ is the concentration of agonist, and K_A is the equilibrium dissociation constant of the drug for its receptor.

Although an implicit assumption of the original model is that 100% receptor occupancy is required to elicit a maximal response, later modifications of receptor theory have recognized that a nonlinear relationship may exist between receptor occupation and tissue response (14, 170, 566). According to Stephenson (566), the measured biological response is some function of stimulus (s), which is generated by interaction of ligand and receptor. The relationship between stimulus and response has been arbitrarily defined such that $s = 1$ when the response is 50% of the maximal response achieved by a full agonist. Thus

$$\frac{E_A}{E_M} = f(s) = f\left(\frac{e[RA]}{[R_t]}\right) = f\left(\frac{e[A]}{[A] + K_A}\right) \qquad \text{equation 3}$$

where e is efficacy, the parameter that relates stimulus to occupancy. This efficacy value may range from 0 to values much greater than 1 and characterizes the capacity of a drug to induce a biological response. By substituting in equation 3, it can be seen that if $e = 0$, $f(s) = 0$; in this case, the drug will bind to the receptor but will not initiate a response (i.e., it is an antagonist). If $e > 0$, $f(s) > 0$; in this case the drug will have agonist activity which will vary according to the value of e (fig. 1a). The concept of receptor reserve (or "spare" receptors) is implicit in this model, since an agonist with a high efficacy value need occupy only a fraction of the total receptor population to elicit a maximal response. When this occurs, $ED_{50} < K_A$; that is, the concentration which produces a 50% maximal response is less than the concentration which occupies 50% of the total receptor pool (fig. 1a). The relationship between potency and affinity is not a constant, but varies with each agonist tested, since agonists with different efficacies will occupy different proportions of the total receptor population to produce an equivalent response (fig. 1b).

Recent experimental evidence has provided support for the concept of "spare" opioid receptors (80, 82, 108, 151, 354, 467, 477, 572, 619). In vitro exposure of isolated tissue preparations to low concentrations of irreversible opioid receptor antagonists has been shown to produce large parallel rightward shifts in the dose-response curves of agonists, with little or no depression of the maximum

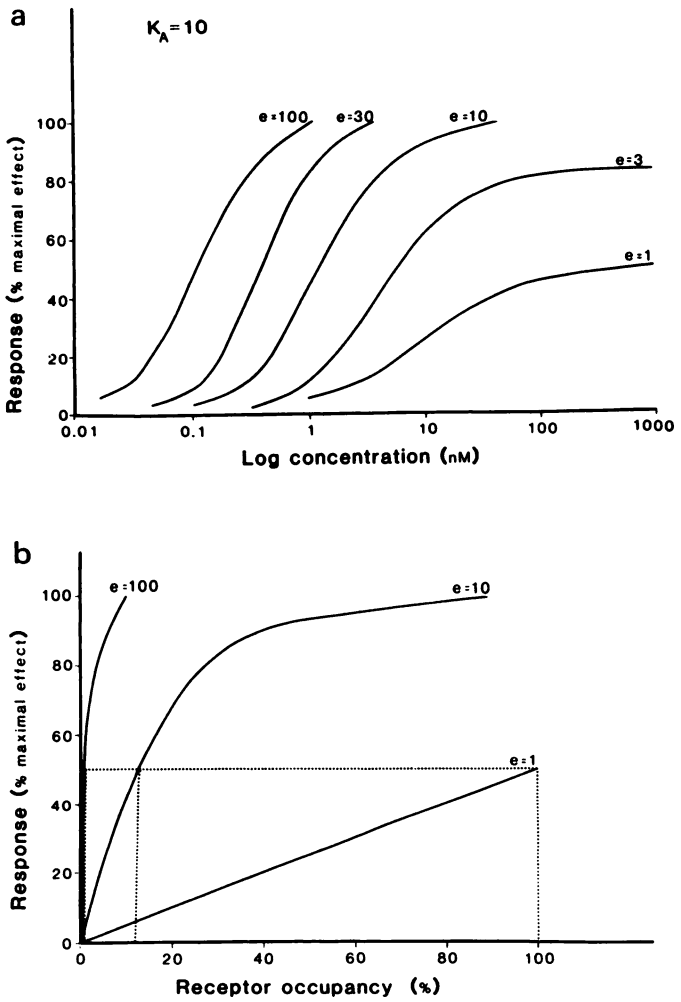

FIG. 1. a, relationship between response and concentration of agonists having the same dissociation constant (K_A = 10 nM) but different values of efficacy (e). b, relationship between response and percentage of receptor occupancy of agonists having the same dissociation constant (K_A = 10 nM) but different values of e. Data represent theoretical curves derived from equation 3.

response (80, 82, 151, 619); thus an agonist may produce a maximal effect, even when a significant proportion of total receptor pool has been irreversibly inactivated. This experimental approach has been used to demonstrate receptor reserve in preparations which contain a single opioid receptor type (locus coeruleus and NG108-15 hybrid cells) (151, 619), as well as in a preparation which contains a mixed population of μ and K receptors (guinea-pig ileum) (80, 82, 477). In the latter preparation, partial inactivation of the total receptor pool does not induce a change in the pharmacological selectivity of either μ or K agonists, suggesting that spare μ and K receptors exist within the same tissue (82).

In vivo pharmacological studies have indicated that opiate agonists may induce a full analgesic response while occupying only a fraction of the available receptors (467, 572). Partial receptor occlusion with low doses of buprenorphine, a slowly dissociating opiate antagonist, produces parallel rightward shifts in the dose-response curve

for morphine in the rat tail flick test, prior to reduction of the maximum analgesic response (572). Using a different pharmacological approach, Perry and coworkers (467) have directly measured the equilibrium binding constant of radiolabelled etorphine in vivo and have compared receptor occupation with analgesic effect in the hot-plate test. On the basis of these data, these authors have concluded that the dose of etorphine which produces a maximum analgesic effect occupies only 2% of the total receptor pool.

The efficacies of a number of opioid agonists in neuroblastoma-glioma NG108-15 hybrid cells have been determined by comparing, under identical incubation conditions, agonist radioligand binding constants with the subsequent agonist effect, inhibition of cyclic AMP accumulation (354). In this cell culture system, efficacy values were found to vary widely among the agonists studied. Opioid peptides, as a group, had higher efficacies than nonpeptide agonists. Thus, the full biological effect of peptide agonists was observed with minimum occupancy of binding sites.

These combined experimental data provide strong evidence that the potency of an opioid agonist is a function of both its affinity for the receptor and its efficacy. Furchgott (170) has argued that efficacy, as defined in equation 3, is a drug *and* tissue-dependent term, which reflects both the ability of the agonist to induce an active receptor-effector complex and the total number of receptors in the system. Thus

$$e = E[R_t] \qquad \text{equation 4}$$

where E is "intrinsic efficacy," a strictly drug-related property, and $[R_t]$ is the total concentration of receptors. Whereas intrinsic efficacy (E) should be constant for a given drug-receptor pair across species and tissues (321), efficacy (e) varies with receptor density. The theoretical relationship between agonist potency and receptor density is illustrated in fig. 2. When receptor density is high, the value of e will be high, and an agonist may produce a maximal effect by occupying a small proportion of the total receptor population (i.e, $ED_{50} \ll K_A$). Where receptor density is low, the value of e will be low, and the same agonist may occupy the total receptor population without producing a maximal response (i.e., behave as a partial agonist). Thus, both quantitative *and* qualitative differences may be observed in the activity of the same agonist acting on the same receptor in two tissues with differing receptor densities.

Experimental evidence has shown that, for a given opioid agonist, the degree of receptor reserve is dependent upon the tissue in which it is studied. Based on analysis of agonist dose-response curves in the presence and absence of the irreversible antagonist β-chlornaltrexamine (β-CNA), Cox and Chavkin (108) have concluded that mouse vas deferens (MVD) contains a smaller active pool of both μ and K receptors than does guinea-pig ileum (GPI). This observation may well explain the reported potency differences between opioid agonists in GPI and MVD (281, 362, 373; see table 2), as well as qualitative differences in the activity of partial agonists (213, 281, 411).

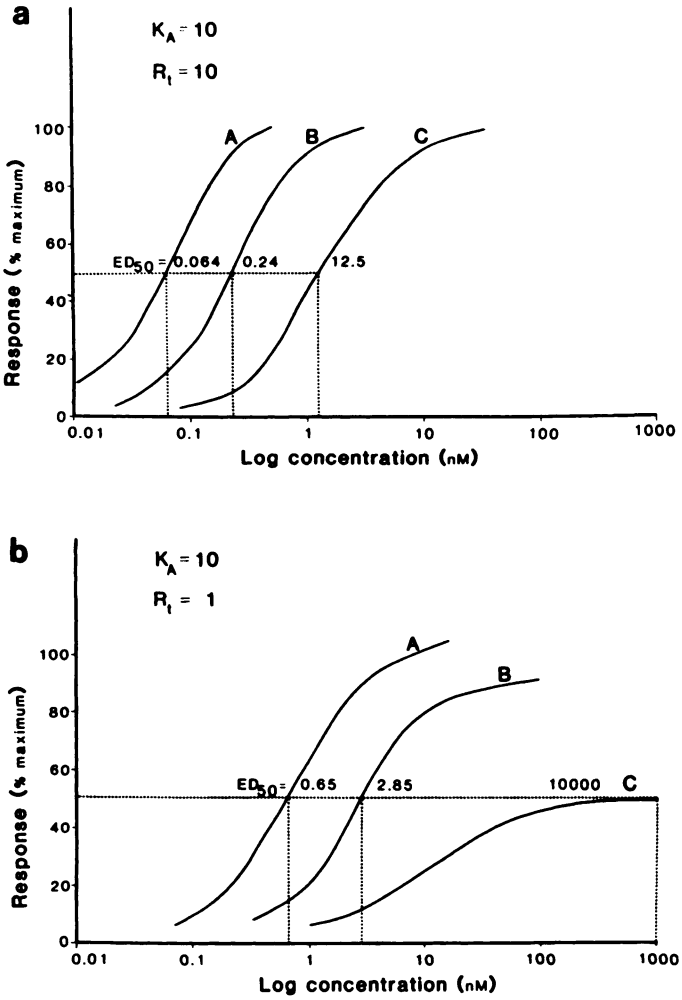

FIG. 2. Relationship between tissue receptor density and agonist response. *a* and *b* compare the response of three agonists (*A*, *B*, and *C*) with the same dissociation constant (K_A = 10 nM) in two tissues in which receptor density (R_t) differs by a factor of 10. Data represent theoretical curves derived from equations 3 and 5.

Within a given tissue, receptor reserve may be modified by pretreatment with an irreversibly acting antagonist (29, 82, 173, 477). The theoretical implications of this for the analysis of receptor selectivity will be discussed in a later section (II A3c). Receptor reserve may also be modified by prior chronic exposure to opioid agonists (82, 477). Since absolute receptor number does not appear to decrease in morphine-tolerant peripheral tissue preparations (110), this decline in receptor reserve may reflect a change in the function relating stimulus to response.

The various tissue and drug-related factors associated with agonist response may be summarized as follows.

$$\frac{E_A}{E_M} = f\left(\frac{E[R_t][A]}{[A] + K_A}\right) \qquad \text{equation 5}$$

Agonist activity is dependent upon factors which are specific to the drug (K_A, agonist dissociation constant, and E, intrinsic efficacy) as well as upon factors which are specific to the target tissue (f, the function relating stimulus to response, and $[R_t]$, total receptor concentration). Given these considerations, it is clear the ED_{50} values derived from simple dose-response curves may not be an accurate reflection of agonist affinity. A number of bioassay methodologies have been devised to measure the equilibrium dissociation constant (K_A) of an agonist for its receptor (for review, see ref. 322). Of these, the most commonly used is the Furchgott method which involves partial inactivation of the receptor pool by an irreversible antagonist (29, 173).

The basic premise of this approach is that equiactive concentrations of agonist before ($[A]$) and after ($[A']$) receptor inactivation produce an equivalent biological stimulus. Thus, if

$$\frac{E_A}{E_M} = \frac{E_{A'}}{E_M} \qquad \text{equation 6}$$

then

$$f(s) = f(s') \qquad \text{equation 7}$$

Assuming that only receptor number is changed by irreversible receptor blockade, then

$$\frac{e[A]}{K_A + [A]} = \frac{qe[A']}{K_A + [A']} \qquad \text{equation 8}$$

where q is the fraction of free receptors (i.e., those which have not been inactivated). This equation can be simplified to the following

$$\frac{1}{[A]} = \frac{1}{q[A']} + \frac{1-q}{qK_A} \qquad \text{equation 9}$$

Thus, a plot of the reciprocals of equieffective concentrations of agonist before ($1/[A]$) and after ($1/[A']$) receptor inactivation will yield a straight line with a slope of $1/q$ and an intercept of $\frac{1-q}{qK_A}$. The K_A of the agonist may then be determined as follows.

$$K_A = \frac{\text{Slope} - 1}{\text{intercept}} \qquad \text{equation 10}$$

It is important to note that the Furchgott method for determination of the agonist K_A value is only valid when the agonist acts on a homogeneous population of receptors (29). This approach has been used elegantly by Williams and North (619) to determine the affinities of opioid agonists for μ receptors in rat locus coeruleus.

2. Determination of antagonist potency. Determination of the dissociation constant of an antagonist (K_B) is not subject to the same theoretical limitations as is the case for that of an agonist. No assumptions need to be made as to the relationship between agonist occupancy and response, since a null detection method is used to measure antagonist affinity. According to classical theory, dose-response curves for agonist in the presence of a competitive antagonist should be shifted to the right in a parallel manner, regardless of whether tissue response is a linear or nonlinear function of agonist concentration (178, 529–531). Furthermore, equal responses to an agonist in the absence and presence of a competitive antagonist should occur only when the agonist occupies equal proportions of the receptor population. Receptor occupation of an agonist in the presence of a competitive antagonist is described mathematically as follows (177, 178)

$$\frac{[RA']}{[R_t]} = \frac{[A']}{[A'] + K_A\left(1 + \frac{[B]}{K_B}\right)} \qquad \text{equation 11}$$

where $[A']$ is the concentration of agonist in the presence of competitive antagonist, B, and the dissociation constants of agonist and antagonist are K_A and K_B, respectively.

The K_B of the antagonist can be calculated by comparing equiactive concentrations of agonist in the absence $[A]$ and presence $[A']$ of antagonist, since (from equations 3 and 11)

$$\frac{[A]}{[A] + K_A} = \frac{[A']}{[A'] + K_A\left(1 + \frac{[B]}{K_B}\right)} \qquad \text{equation 12}$$

Algebraic simplification yields the following relationship

$$\frac{[A']}{[A]} - 1 = \frac{[B]}{K_B} \qquad \text{equation 13}$$

where $[A']/[A]$ is the ratio of equiactive antagonist concentrations in the presence and absence of antagonist, more commonly known as the dose ratio (DR). If antagonism is competitive, a plot of log (DR − 1) against log $[B]$ will give a straight line with a slope of unity (15). In this Schild plot, the intercept along the abscissa is −log K_B and is commonly referred to as the pA_2, i.e., the negative logarithm of the molar concentration of antagonist that causes a 2-fold rightward shift in the agonist dose-response curve. Since pA_2 is a measure of antagonist affinity, it is characteristic of a particular antagonist and receptor type and is independent of the agonist used or the tissue in which the response is measured. Thus, the pA_2 of a competitive antagonist is a powerful tool for receptor classification (320).

The Schild analysis is the most rigorous method for determining the affinity of antagonist-receptor complexes. For the analysis to be considered valid, it is essential that two criteria be fulfilled, (*a*) that the regression is linear over a wide range of antagonist concentrations and (*b*) that the slope of the line is unity. If these criteria are not met, the interaction of the antagonist with the receptor may not obey simple competitive kinetics. Alternatively, and more commonly, such devia-

tions may reflect nonequilibrium experimental conditions (see section II C2 below).

A simpler, "single-dose" method has frequently been used for determination of opioid antagonist K_B (or K_e) values (343). This method, which involves determination of equiactive concentrations of agonist in the absence and presence of a single dose of antagonist, is particularly useful for measurement of the antagoniost potency of compounds which have agonist activity (see fig. 3). Assuming competitive antagonism and ideal experimental conditions, then (464, 566)

$$K_B = \frac{[B]}{DR - 1} \qquad \text{equation 14}$$

where K_B is the equilibrium constant of the antagonist, $[B]$ is the molar concentration of antagonist, and DR is the dose ratio, i.e., the ratio of equieffective agonist concentrations in the absence and presence of antagonist. It is important to note that the assumptions upon which this method is based, i.e., competitive antagonism and equilibrium binding conditions, may not always be valid. Thus, although this method provides a short-cut for determination of antagonist potency, it is not as rigorous as the Schild analysis.

3. Determination of receptor selectivity. A combination of pharmacological approaches has been used for classification of opioid receptor types and the receptor selectivities of opioid drugs. Those which involve measurement of biological response include: (*a*) comparison of the qualitative and quantitative effects of agonists; (*b*) determination of the affinity constants of competitive antagonists, such as naloxone; and (*c*) selective receptor inactivation. It is worthwhile to briefly consider the theoretical principles associated with each approach.

a. AGONIST ACTIVITY. Comparison of the relative potencies of a series of agonists to elicit different biological responses has long been used as a means of receptor classification (5, 22) and continues to be a useful approach for classification of opioid receptors (373). However, care must be taken in the use of this approach. As has been discussed in previous sections, agonist activity is dependent upon parameters which are specific to the target tissue as well as to the drug-receptor complex (see equation 5); thus, the absolute potency of an agonist to elicit a response is influenced by the tissue in which it is tested. Tissue factors can be eliminated by comparing the potency ratio (pr) of two full agonists ($A1$ and $A2$) to elicit equal responses. Assuming that $K_A \gg ED_{50}$, i.e., that there is adequate receptor reserve, then (322)

$$pr = \frac{[A1]}{[A2]} = \frac{E_2 \cdot K_{A1}}{E_1 \cdot K_{A2}} \qquad \text{equation 15}$$

Since the potency ratio of two full agonists reflects only the drug parameters K_A and E and is tissue independent, comparison of the relative potencies of a series of agonists represents a powerful means of receptor classification.

It is important to note that this approach is theoretically valid only when the potencies of full agonists are compared. As was discussed in section II A1 and is illustrated in fig. 2, the potency ratio of a full and partial agonist is not tissue independent, but varies with receptor number. Furthermore, an agonist with a low intrinsic efficacy may act as a full agonist in tissues in which receptor density is high, but as a weak partial agonist in tissues in which receptor density is low. Thus, care must be taken in interpreting qualitative differences in agonist activity in different target tissues in terms of activation of different receptor subtypes. This point has recently been emphasized by Miller et al. (411), who have compared the pharmacological properties of μ and K opioid receptors in a number of peripheral tissue preparations. These authors have concluded that qualitative differences in the pharmacological actions of the prototype μ agonist, morphine, reflect tissue differences in the degree of receptor reserve, rather than in the binding properties of the receptor.

b. COMPETITIVE ANTAGONISM. As was discussed in section II A2, since a null detection method is used to determine the potency of a competive antagonist, this measure is less influenced by tissue factors than is agonist potency. Consequently, determination of antagonist pA_2 values has been considered to be a definitive means of receptor classification (172, 322, 531). Provided that the Schild plot is linear over a wide range of concentrations and has a slope of unity, then the intercept along the abscissa provides a good measure of the affinity of an antagonist for a receptor. It is important to note, however, that receptor heterogeneity within a tissue may confound this analysis. If both agonist and antagonist recognize more than one receptor within a tissue preparation, a Schild plot may be expected to show significant deviations from linearity (172, 322, 323). Theoretical calculations indicate that, while slopes less than unity may be expected at low dose ratios, larger dose ratios

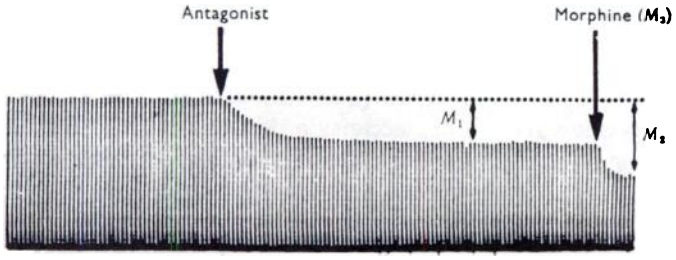

FIG. 3. "Single dose" method for determination of the antagonist activity of a partial agonist. Isometric recording of the contractions of GPI induced by electrical coaxial stimulation at a frequency of 0.1 Hz. At the *arrow* marked "antagonist," the partial agonist was added and produced a depression of the twitch equal to that caused by morphine in concentration M_1. Morphine was added 20 min later at a concentration of M_2; the total depression of twitch was equal to that caused by morphine in a concentration of M_2 in the absence of antagonist. The antagonist dissociation constant, $K_e = a/(DR - 1)$, where a is the concentration of antagonist, and dose ratio, $DR = M_2/(M_2 - M_1)$. Reproduced with permission from ref. 343.

may provide regressions in which nonlinearity may be difficult to detect (172, 322). Thus, the intercept of a Schild regression may provide an artifactual pA_2 value which reflects the weighted average of the pA_2 values at two different receptors.

The influence of receptor heterogeneity may be particularly significant when a "single-dose" method is used to determine the affinity of an antagonist for a receptor. This approach is not as rigorous as the Schild analysis and is based on assumptions as to the competitive kinetics of the antagonist and the homogeneity of the receptor population. It has the advantage, however, of being fast and easy and is commonly used to determine the K_e values for opioid antagonists in preparations, such as GPI and MVD, which have mixed receptor populations. In such tissues, the K_e value for naloxone antagonism of μ opioid effects is 2 to 3 nM, while that for antagonism of responses mediated by K or δ receptors is 20 to 30 nM (362, 373; see table 2). Intermediate K_e values have been reported for naloxone antagonism of agonists which have affinity for more than one receptor type (81, 305).

c. SELECTIVE RECEPTOR INACTIVATION. Another useful method for receptor and drug classification is to examine responses to agonists before and after selective inactivation of a given receptor type (80, 83, 84, 240, 241, 290, 541, 544, 619). Selective receptor inactivation can be achieved in a number of ways, including use of selective irreversible antagonists, selective receptor protection, and selective tolerance techniques (80, 84, 480, 541, 544, 600). Of these, the simplest approach is to selectively inactivate one type of receptor through the use of a specific irreversible antagonist. An example of such an agent is β-funaltrexamine (β-FNA), an irreversible antagonist which has been reported to have a high degree of selectivity for μ opioid receptors (570, 600). A number of opioid effects have been shown to be eliminated by prior exposure to this antagonist and have thus been classified as mediated via μ receptors (83, 240, 241, 290, 601, 602, 619). Additional studies have shown that another irreversible μ-receptor antagonist, naloxonazine, selectively blocks certain morphine-induced responses, such as supraspinal analgesia, without affecting others (459). Such data have provided preliminary evidence to suggest that μ opioid receptors may be subclassified into two distinct subtypes, a naloxonazine-sensitive μ_1 receptor, and an insensitive μ_2 receptor (459).

Since there are few irreversible antagonists currently available which have high selectivity for a single type of opioid receptor (490, 570), other approaches must be used to classify K or δ receptor-mediated effects. One approach which has been used successfully is that of selective receptor protection, in which tissues are incubated with high concentrations of a specific, competitive drug during exposure to an irreversible antagonist (80, 84). The rationale for this approach is that occupation of a receptor binding site by an agonist or antagonist will protect that site from alkylation by the irreversible reagent. If the protecting ligand is selective for a given receptor type, then the responsiveness of that receptor will be maintained while that of other receptors will be abolished. In contrast to the previous approach, which relies on the selectivity of the alkylating agent, this approach relies on the selectivity of the protecting ligand and the nonselectivity of the irreversible reagent. β-CNA is particularly useful in this regard, since it irreversibly inactivates μ, δ, and K opioid receptors (304, 480, 600).

The desensitization phenomenon following chronic exposure to opioid agonists is used as the basis for a third approach to selective receptor inactivation (544). Prior long-term exposure of tissues to agonists with selectivity for one class of opioid receptors will induce cross-tolerance to all agonists with selectivity for that receptor type. In contrast, tissue responsiveness to opioids with affinity for other classes of opioid receptor will not be changed. This technique has been used to demonstrate receptor selectivity in both intact animals (541) and isolated tissue preparations (539, 544, 547). In the latter case, the best results are obtained when tissues are taken from animals which have been pretreated for several days with a selective agonist, and when a low concentration of this agonist is included in the in vitro incubation buffer (544).

Although all of these techniques have been used successfully to differentiate classes of opioid receptors, there are two major drawbacks associated with their use. First, they are very dependent on the selectivity of the agents which are used either to inactivate or to protect the receptor. For instance, although β-FNA was initially reported to be a specific, irreversible antagonist of the μ opioid receptor (600), later reports have suggested that higher concentrations will also block K and δ receptors (240, 408, 562). Thus, although elimination of a response by prior exposure to β-FNA constitutes good evidence that the response is mediated via μ opioid receptors, it does not constitute definitive proof. Both selective protection and selective tolerance techniques are dependent on the specificity of the ligands which are used to, respectively, selectively protect and selectively desensitize a given class of receptors. Thus, although a ligand may show receptor selectivity at low doses, this specificity may be lost at the high concentrations of drug which are used to protect and/or desensitize the receptor.

A second consideration associated with selective inactivation techniques is that each approach will reduce the size of the active receptor pool and, consequently, the degree of receptor reserve. Agonists with equal affinity and selectivity for a given type of receptor may be differentially affected by this reduction in receptor number, depending on their intrinsic efficacies (see fig. 2); compounds with low intrinsic efficacy and a small receptor reserve will be more sensitive to partial removal of the receptor pool than will agonists with high intrinsic effi-

cacy and a large receptor reserve. Thus, although selective receptor inactivation may eliminate the response of one agonist but not another, this does not necessarily imply that the two compounds activate separate receptors (322, 605).

4. Control of experimental conditions. The equations from which the pharmacological constants of agonists and antagonists are derived are based on a number of assumptions (172) that include the following: (*a*) The response of the test preparation is the result of agonist A reacting with only one type of receptor. This is a reversible, bimolecular reaction which is governed by the Law of Mass Action. (*b*) When a response is measured, the concentration of free agonist in the region of the receptors is in thermodynamic equilibrium with both agonist bound to receptors and free agonist in the bathing fluid. (*c*) The antagonist B combines reversibly with the same type of receptor as does agonist A, in a bimolecular reaction governed by the Law of Mass Action. Antagonist B alters the response to agonist A only by competing with agonist A for occupancy of receptors of this type. (*d*) When a response is measured, the concentration of antagonist B in the region of the receptors is in thermodynamic equilibrium with both antagonist B bound to receptors and with free antagonist B in the bathing or perfusion medium.

In view of these assumptions, Furchgott (172) has provided the following list of optimal experimental conditions for determination of pharmacological constants.

(*a*) The response to an agonist should be due solely to the direct action of an agonist on one type of receptor. It should not be the result of actions on more than one type of receptor, nor should it be due, even partially, to indirect action at a site other than the target receptor.

(*b*) The altered sensitivity to an agonist in the presence of a competitive antagonist should be due solely to competition between the antagonist and the agonist for the receptor. The altered sensitivity after treatment with an irreversible antagonist should be due solely to inactivation of the receptor.

(*c*) The response elicited by a given dose of agonist should be measured under steady-state conditions, i.e., at a maximal level, which should be maintained for a reasonable length of time.

(*d*) In the case of either an agonist or competitive antagonist, the free concentration in the external solution should be maintained at a steady level at the time a response is measured, and should be known. In the case of an irreversible antagonist, the concentration in the solution should be essentially zero during measurement of responses.

(*e*) In the case of either an agonist or competitive antagonist, the concentration in the region of the receptors should be in diffusion-equilibrium with that in the external solution at the time a response is measured. To meet this condition, the rate of removal of drug from this region due to enzymatic action, transport into cells, and binding should be negligible compared with that due to diffusion back to the outside solution. In the case of an irreversible antagonist, the fraction of the receptor pool which is not inactivated should remain constant over the period in which responses are measured.

(*f*) The experimental design should include proper controls to permit measurements of, and corrections for, any changes in sensitivity to agonists during the course of an experiment that are not due to addition of an antagonist.

B. In Vivo Bioassay

Administration of opioid agonists to the intact animal may elicit a number of behavioral responses including antinociception, depression of respiratory, cardiovascular, and gastrointestinal function, and locomotor and endocrine disturbances (7, 384). Chronic exposure to agonists may result in tolerance to some of these effects and in a physical dependence syndrome which is characterized by withdrawal symptoms upon administration of an opioid antagonist (193, 384, 385, 609, 627). A number of behavioral tests have been used to examine the properties of the receptors mediating these pharmacological effects. These have been extensively reviewed in a previous article in this series (384). The present article will not attempt to review each test on an individual basis, but will rather examine the general methodological principles associated with in vivo pharmacology.

A combination of the following approaches has been used for characterization of opioid receptors in vivo (105). These include: (*a*) comparison of the qualitative and quantitative effects of agonists; (*b*) determination of the antagonist potency of naloxone and other opioid antagonists; (*c*) determination of the effectiveness of (+)-enantiomers of agonists and/or antagonists; (*d*) interactional studies with other prototype agonists of postulated opioid receptors; and (*e*) tolerance and cross-tolerance studies with appropriate agonists.

Given the complexity of drug action in the intact animal, it is clearly difficult to approach the desired optimal conditions for receptor characterization in vivo. In this type of study there may always be uncertainty as to whether potency measurements accurately reflect the dose of drug which was initially administered. A wide variety of factors may differentially influence the absorption, distribution, metabolism, or excretion of the drugs and therefore affect their concentrations at the receptors. The following are some of the important methodological considerations which must be taken into account in the design of in vivo experments and in the interpretation of results.

1. Access to receptor sites. Although opioid drugs have some peripheral sites of action (280), the majority of the behavioral effects of these compounds are mediated via receptors within the CNS (471). The investigation of the

effects of drugs on the CNS poses problems which are not associated with other organs, since the blood-brain barrier impedes the penetration of drugs with low lipid solubility into many areas of the brain (524). Pharmacokinetic studies have shown that the passage of morphine-like analgesics across the blood-brain barrier is directly related to their lipophilic properties (259, 298, 595). Following i.v. administration, lipophilic drugs such as etorphine are transported across the blood-brain barrier more rapidly than hydrophilic compounds such as morphine. Within the brain, lipophilic drugs may be bound to a large extent, resulting in concentrations far above plasma levels; conversely, the peak brain concentrations of hydrophilic drugs may represent a small fraction of the plasma level (259, 449, 595).

Differential transport across the blood-brain barrier is particularly relevant with respect to opioid peptides. In general, the permeability of the blood-brain barrier to peptides is low (455). The body of available evidence indicates that the first-pass extraction of enkephalin-like peptides is 1 to 2% or less (102, 316, 456, 638), similar to the extraction for monoamines and acetylcholine (448). Systemic administration of β-[^{3}H]endorphin has been shown to result in substantial tritium labeling of brain tissue (276); however, all of the radioactivity recovered is in the form of [^{3}H]tyrosine, which may have originated from metabolism of the peptide in plasma or at the blood-brain barrier. Both plasma and brain capillaries have been shown to be enriched in degradative enzymes which cleave endogenous opioid peptides (230, 456, 638). Thus, degradative mechanisms and low capillary permeability represent a dual barrier for penetration of peripherally administered peptides into the CNS.

In order to circumvent such difficulties, drugs may be administered intracerebroventricularly (i.c.v.) or by direct injection into brain tissue. Following i.c.v. application, the maximum analgesic activities of a series of morphine-like drugs show no correlation with lipid solubility (259). Thus, hydrophilic substances are much more potent when administered centrally than peripherally, their activities by this route much more closely reflecting their receptor affinities (258, 259, 349).

In contrast, the rate of onset of opioid action following i.c.v. administration is positively correlated with lipophilicity (259). Hydrophilic compounds may exert more rapid effects when administered peripherally than centrally (259). These differential rates of action may reflect differences in diffusion to the primary site of action within the brain. Following peripheral administration, opioids pass into brain tissue through capillaries which are located within 50 μm of most neurons (525). In contrast, the ventricular system may be located several millimeters from the relevant target neurones. Since there are no diffusion barriers for lipophilic drugs, they will rapidly reach their site of action following either central or peripheral administration. Hydrophilic drugs, however, will diffuse more slowly, such that the distance from the ventricle to the site of action becomes a significant factor in determining the speed of onset (259). This consideration is particularly important in the case of opioid peptides, which may be both hydrophilic and rapidly metabolized by enzymes in the brain (230). In this regard, it should be noted that the analgesic actions of several opioid peptides are greatly enhanced by administration into the cerebral aqueduct or periventricular grey, rather than into the lateral ventricle (254, 299, 300). Thus, quantitative analysis of opioid peptide activity in vivo may best be achieved by direct injection at the site of action.

2. *Removal from receptor sites.* In the intact animal a number of factors have been shown to influence the rate of removal of opioids from their receptor sites, including K_D, local receptor density and the drug concentration gradient between the receptor site and the surrounding medium (165, 259). Of these, the most critical determinant appears to be the concentration of drug in the vicinity of the receptor. Following either i.v. or i.c.v. administration, the duration of action of morphine-like analgesics has been shown to be inversely correlated with lipid solubility (259, 449). Whereas lipophilic drugs have a more rapid onset of action than hydrophilic drugs, this action is, in general, less prolonged. The short duration of action of lipophilic drugs probably reflects their rapid diffusion away from the receptor site and redistribution into other tissues, where they are stored, metabolized, or excreted (203, 259). Most opioid peptides, though not highly lipophilic, may also have a brief duration of action as a result of their rapid metabolism. Neural tissue, cerebrospinal fluid (CSF), and plasma all contain a wide variety of degradative enzymes which may cleave opioid peptides and rapidly reduce the concentration of intact drug at the receptor (230).

Given these pharmacokinetic considerations, it is clear that optimal conditions for pharmacological characterization of opioid receptors can rarely be achieved following acute in vivo administration of agonist or antagonist drugs. As a result of diffusion and/or metabolism, the concentration of free drug in the region surrounding the receptors may not be maintained at a steady level at the time a response is measured. As a consequence, the maximal response may not be maintained for a prolonged period of time and may not accurately reflect the affinity of a drug for its receptor. This may lead to confusing experimental results (636).

In order to minimize enzymatic degradation of opioid peptides, synthetic stable analogs may be used (73). In such cases, care should be taken to ensure that the synthetic derivative has a similar pharmacological profile to that of the parent compound, since minor changes in structure have been found to radically alter the receptor affinity and selectivity of certain opioid peptides (81). Sustained release preparations or multiple injection strategies may also be used to achieve a steady drug concentration at the site of action. Such constant deliv-

ery techniques have been used extensively in studies of tolerance and physical dependence, where it is essential that the concentration of drug at the receptor be maintained at a constant level for prolonged periods of time (539, 541, 544, 609).

3. Multiple actions of drugs. Agonist-receptor interaction initiates a complex sequence of events prior to the manifestation of a biological effect. Many factors, such as reflex activity, may interfere at a secondary stage and complicate interpretation of the results. Depending on its pharmacological selectivity and route of administration, a given drug may also interact with receptor sites in several different brain areas and initiate several different biological responses concomitantly. Manifestion of one response (e.g., catatonia) may thus significantly interfere with expression of another (e.g., stimulation of feeding).

Secondary effects, mediated via metabolites, may also complicate data interpretation. Whereas most metabolic processes result in inactivation of opioid drugs, some may change the structural conformation to potentiate or otherwise alter the biological activity of these compounds. Many examples of this phenomenon have been documented, as in the case of codeine which is transformed in vivo to the more potent compound, morphine (2). Some "active metabolites" may mediate their actions via non-opioid receptor sites, as in the case of Des-Tyr-dynorphin (597), while others may exhibit an altered opioid receptor selectivity (271, 410).

In order to reduce both pharmacokinetic variability and multiple drug actions, drugs may be delivered directly to the postulated site of action by microinjection into brain tissue. This approach has been increasingly used for characterization of the receptors mediating a variety of behavioral responses (49, 202). Although pharmacokinetic factors have less influence on the activity of drugs administered by this route, they must still be taken into consideration. Autoradiographic studies have shown that lipophilic drugs may diffuse several millimeters from the site of injection and may rapidly enter the ventricular system and the blood stream (259). This diffusion process results in reduced concentrations of drug at the target site, complicating quantitative comparisons of drug potency. It may also elicit secondary responses by stimulation of receptors in a nontarget area. Such pharmacokinetic complications may be minimized by using small injection volumes and nonlipophilic compounds.

The preceding discussion has emphasized a number of factors which may result in quantitative differences in biological activity following administration of a single drug by different routes. These same factors may also produce qualitative differences in drug effect. Such qualitative differences in biological activity have been reported for both peptide and nonpeptide agonists (3, 266, 581). For instance, systemically administered ethylketocyclazocine (EKC) lowers body temperature, while i.c.v. administration raises it (3). In practice, endogenous opioid peptides and their analogs are most often administered i.c.v. in order to circumvent the blood-brain barrier and to conserve drug. These data may then be routinely compared to those for standard or prototypic agents given by a systemic route of administration. Given the potential for both quantitative and qualitative differences in the effects of centrally and peripherally administered drugs, such comparisons may be expected to lead to erroneous conclusions.

C. *In Vitro Bioassay*

A primary advantage of investigating opioid receptor mechanisms in an isolated system is that the analysis of drug effect is less likely to be complicated by secondary factors such as distribution, metabolism, and excretion. Thus, in vitro bioassay systems have been used extensively for rigorous pharmacological characterization of opioid-receptor interactions (280, 281, 335, 340, 343, 443, 619). Peripheral tissue preparations, in particular, have proven to be invaluable models for in vitro analysis of opioid effects. More recently, brain slice and dissociated cell preparations have also been used for this purpose.

1. Assay methodologies. a. PERIPHERAL TISSUES. Opiates depress impulse transmission at certain peripheral junctions of the autonomic nervous system. These peripheral sites of action are not found consistently across species, however. At most neuroeffector junctions, opioid agonists act on receptors localized on presynaptic nerve terminals to inhibit electrically stimulated neurotransmitter release (64, 106, 111, 246, 443, 463, 583). Recent studies have, however, indicated that opioid receptors may also be associated with nonneural elements in some tissue preparations (36, 174, 513–515). A summary of opioid-sensitive peripheral tissues is included in table 1.

Not all of the opioid-sensitive tissues listed in table 1 are suitable for use as in vitro bioassay models. Such factors as expense, ease of dissection, and long-term stability in vitro have been important factors in determining which preparations are suitable for routine use. To date, the most commonly used tissue preparations have been GPI and MVD (281, 343). More recently, a number of tissues which contain a single type of opioid receptor have also proven useful (395, 447, 535).

i. Guinea-pig ileum. The isolated ileum of the guinea-pig has been used successfully to study not only the acute effects of opioids, but also the long-term effects of tolerance and dependence (68, 107, 210, 377, 537, 538). The specific action of opioids in this tissue is to inhibit electrically evoked longitudinal muscle concentrations by presynaptically depressing acetylcholine (ACh) release from the myenteric plexus (106, 111, 463). The magnitude of the opioid effect is dependent on the electrical stimulation parameters which are used; ACh release by high frequency electrical stimulation is much less sensitive to inhibition by morphine than is low

TABLE 1

Opioid actions at some peripheral neuroeffector junctions in vitro

Tissue	Species	Opioid effect	Site of action	Receptor type	Ref.
Vasculature					
Atria	Rat	↓ NE-induced chronotropic response and $^{45}Ca^{2+}$ accumulation	Postsynaptic		513
		↓ electrically induced cholinergic response	Presynaptic		339, 623
Atria	Guinea pig	↑ NE-induced chronotropic response and $^{45}Ca^{2+}$ accumulation	Postsynaptic		514
		↓ NE release	Presynaptic		167, 358
Atria	Rabbit	↓ electrically induced cholinergic response and [^{3}H]ACh release	Presynaptic	μ, δ	324, 339, 611, 623
Aorta	Rat	↓ NE-induced vascoconstriction	Postsynaptic		515
Ear artery	Rabbit	↓ field-stimulated vasoconstriction and [^{3}H]NE release	Presynaptic	δ, K	169, 291, 331, 499
Mesenteric artery	Rabbit	↓ stimulus-evoked extrajunctional potentials	Presynaptic	δ	292
Pial artery	Cat	↓ Prostaglandin $F_{2\alpha}$-induced vasoconstriction	Postsynaptic		235
Vas deferens					
Vas deferens	Hamster	↓ electrically induced contraction	Presynaptic	δ	395
Vas deferens	Mouse	↓ electrically induced contraction and NE release	Presynaptic	μ, δ, K	245, 281, 289, 373
Vas deferens	Rat	↓ electrically induced contraction	Presynaptic	μ, (ε)	188, 360, 369, 535, 563
		↓ adenylate cyclase activity			30
Vas deferens	Rabbit	↓ electrically induced contraction	Presynaptic	K	447
Gastrointestinal system					
Contractile activity					
Ileum (myenteric plexus-longitudinal muscle)	Guinea pig	↓ field stimulated, neurotensin- and substance P-induced muscle contraction and ACh release	Presynaptic	μ, K	86, 106, 111, 227, 273, 283, 463, 592, 593
		↓ substance P release	Presynaptic		200
Ileum (longitudinal muscle)	Rabbit	↓ field-stimulated contraction	Presynaptic	δ	446
Ileum	Dog	Contraction mediated via 5-HT release	Presynaptic		59
Rectum	Rat	Contraction	Postsynaptic/presynaptic		438
Colon	Rat	Contraction	Postsynaptic/presynaptic	μ, K	197, 421, 439, 528
Colon	Mouse	Contraction	Presynaptic		157
Taenia caeci	Rat	↓ electrically induced relaxation	Presynaptic		293
Duodenum	Rat	Relaxation	Postsynaptic		174
Duodenum	Guinea pig	Relaxation	Presynaptic	K	174
Esophagus	Guinea pig	↓ electrically induced contractions of the submucous plexus-longitudinal muscularis mucosae	Presynaptic		314
Secretory activity					
Stomach	Rat	↓ basal and gastrointestinal peptide-stimulated somatostatin secretion			87, 390
Ileal mucosa	Guinea pig	↓ short circuit current and transepithelial potential difference		δ	312, 313
		↑ Cl^- absorption		δ	312, 313

TABLE 1—Continued

Tissue	Species	Opioid effect	Site of action	Receptor type	Ref.
Ileal mucosa	Rabbit	↓ short circuit current and transepithelial potential difference		δ	35, 135, 391, 392
		↑ Cl^- & Na^+ absorption		δ	35, 135, 391, 392
Ileal mucosa	Rat	↓ Prostaglandin E_2-stimulated adenylate cyclase activity			603
Caecum submucous plexus	Guinea pig	↑ K^+ conductance	Presynaptic	δ	408
Colon submucous plexus	Rat	↓ ACh release	Presynaptic		179
Jejunum	Rat	↑ Na^+, H_2O, Cl^-, & glucose absorption			520
Miscellaneous					
Trachea	Dog	↓ electrically induced contraction	Presynaptic		512
Bile duct	Guinea pig	↓ electrically induced contraction	Presynaptic		17, 451
Spleen	Cat	↓ electrically induced [^{3}H] NE release	Presynaptic		176
Iris sphincter	Rabbit	↑ electrically induced cholinergic transmission	Presynaptic	K	584
		↓ electrically induced cholinergic transmission	Presynaptic	(δ)	584
		↓ electrically induced substance P transmission	Presynaptic	K, (δ)	584
Nictitating membrane	Cat	↓ electrically induced NE release & muscle contraction	Presynaptic		140, 246
Retina	Chicken	↓ K^+-stimulated [^{3}H] GABA release	Presynaptic		604
Skeletal muscle	Frog	↓ ACh release	Presynaptic		37, 161
		↓ Late Na^+ conductance	Postsynaptic		16
Electric organ	Torpedo	↓ ACh release	Presynaptic		407

frequency-induced release (106). It has recently been shown that neurotensin-induced contractures of guinea-pig ileal smooth muscle are also inhibited by opioids (283, 284). The mechanism of this effect appears to be an opioid inhibition of neurotensin-induced ACh release (283).

The activity of opioids to inhibit neurotransmitter release in GPI is usually measured by isometric recording of the longitudinal muscle contraction induced by electrical field stimulation of the transmural nerves (227, 343). Myenteric plexus-longitudinal muscle strips are prepared by a modification (337) of the method of Rang (488) and suspended in oxygenated Krebs buffer at 37°C. The preparation is stimulated by an electrical field at 0.1 Hz, 0.5- to 1.0-ms pulse duration, and supramaximal voltage. Application of opioid agonists inhibits the electrically stimulated longitudinal muscle contractions in a dose-dependent, naloxone-reversible manner. Once stabilized, this preparation can maintain consistent pharmacological responses for a period of several hours (281).

Pharmacological data indicate that separate populations of μ and K opioid receptors are localized on cholinergic neurons in GPI myenteric plexus (84, 362, 373, 600). Activation of either μ or K receptors by prototypic agonists will inhibit ACh release and, consequently, longitudinal muscle contractions (see table 2). Although there is both biochemical (362) and electrophysiological (145) evidence for the presence of δ receptors in GPI myenteric plexus-longitudinal muscle, opioids do not modulate ACh release by acting on this receptor. Prototypic δ agonists appear to inhibit cholinergic transmission in this preparation by acting upon μ receptors (see table 2; 84, 362, 373).

The guinea-pig isolated ileum myenteric plexus-longitudinal muscle preparation has been used extensively as an in vitro model for analysis of opioid receptor interactions (340). The structure-activity relationships of numerous synthetic compounds have been assayed in this test preparation (132, 340, 422, 452, 589), providing considerable data as to the properties of the drugs and the receptors with which they interact. Since there are considerable pharmacological similarities between opioid receptors in GPI and those in the CNS (120, 336, 340, 362, 373), activity in this preparation may predict activity in certain behavioral tests. For example, there is a high correlation between agonist potency at μ receptors in GPI and clinical analgesic activity (340). GPI has also been extensively used to monitor the activity of tissue extracts throughout the isolation and purification of endogenous opioids (109, 206, 211). Dynorphin, in par-

TABLE 2
Potencies of opioid agonists in GPI and MVD preparations and sensitivity to antagonism by naloxone

Drug	IC_{50} values (nM)			Naloxone K_e (nM)			Ref.
	GPI	MVD	GPI/MVD	GPI	MVD	GPI/MVD	
μ-agonists							
DHM*	38.7	156	0.25	2.8	2.6	1.1	362
PLO17	19.6	238	0.08	1.6	2.4	0.67	288
δ-agonists							
DADLE	26.6	0.42	63	2.6	27.3	0.09	362
DSLET	41.1	1.03	40	2.3	23.2	0.10	288
K agonists							
EKC	0.18	4.4	0.04	14.9	11.0	1.4	289
Dynorphin(1-17)	0.31	1.5	0.21	25.0	16.7	1.5	288

* DHM, dihydromorphine; PLO17, [N-MePhe^3, D-Pro^4]-morphiceptin; DSLET, [D-Ser^2]-Leu-enkephalin-Thr^6

ticular, exhibits extremely high potency as a K agonist in GPI (84, 211) and can be detected by this assay system in extremely low concentrations.

GPI has proven to be a useful peripheral tissue model for examination of the mechanisms of opioid tolerance and dependence (82, 107, 110, 377, 477, 537, 538). In order to produce morphine-tolerant preparations, guinea pigs are generally implanted with morphine pellets for a period of 3 to 5 days prior to sacrifice and excision of tissue (210). Tissues from chronically treated guinea pigs exhibit a marked decrease in sensitivity to opioid agonists and, when maintained in the presence of morphine, decreased maximum response (110, 537). They also exhibit a "quasidependence" phenomenon, in that acute challenge with naloxone will induce a "withdrawal" contracture (537).

Overall, GPI is an invaluable bioassay system in that it is a relatively inexpensive, easily dissected preparation which maintains stable responses for many hours. There are few limitations associated with its use. The primary disadvantage of the GPI as a predictive model of opioid activity is the absence of a δ receptor-mediated inhibition of contraction. Thus, this assay preparation is unsuitable for examination of the pharmacological properties of δ-selective drugs. A second limitation is the relative insensitivity of GPI to the antagonist properties of compounds with partial agonist activity. Whereas the antagonist activities of relatively weak agonists, such as nalorphine, can be readily demonstrated in this tissue, those of potent agonists, such as cyclorphan, are barely detectable (342).

ii. Mouse vas deferens. The primary action of opioids in this preparation is to inhibit stimulus-evoked norepinephrine (NE) release and, consequently, longitudinal muscle contraction (244, 245, 247). Although less robust and consistent in its responses than is GPI, MVD may maintain stable responses to electrical field stimulation at 0.1 Hz for long periods of time, provided that magnesium ions are omitted from the bathing fluid (281). It can also maintain consistent responses to opioid drugs for periods of several hours (F. M. Leslie, unpublished observations). It has thus been used extensively, in conjunction with GPI, for analysis of opioid receptor interactions (108, 281, 289, 362, 373, 600).

Striking variations have been observed in the relative potencies of different opioids to inhibit electrically evoked contractions of GPI and MVD (see table 2). Whereas GPI is relatively insensitive to the actions of enkephalins, these endogenous opioids potently inhibit longitudinal muscle contractions of MVD. The potency of naloxone to antagonize enkephalin actions in the two peripheral tissues has been shown to differ by more than an order of magnitude (362, 373). Such findings have led to the classification of MVD as containing predominantly enkephalin-selective δ receptors (373). Both μ and K receptors have also been shown to mediate presynaptic inhibition of neurotransmitter release in MVD, although the efficacies of selective μ and K agonists are somewhat lower in this preparation than in GPI (108, 289, 373, 411).

Unlike GPI, MVD is extremely sensitive to the antagonist properties of compounds with dual agonist-antagonist activity (281). Since partial agonists exhibit very flat dose-response curves in this preparation, it is possible to increase drug concentration in the bathing fluid sufficiently to obtain an accurate measurement of antagonist potency (281). The very shallowness of the agonist dose-response curves of these compounds does, however, impede the accurate quanitification of their agonist activity in this tissue (281).

Since MVD is highly sensitive to the agonist actions of δ-selective peptides, it has proven to be an invaluable assay system for analysis of the opioid activity of endogenous tissue extracts. This bioassay preparation was used exclusively by Hughes and coworkers for the initial purification and characterization of enkephalin (279, 282). MVD has also been used extensively as an in vitro model for analysis of mechanisms of opioid tolerance (538, 544, 627). Using osmotic minipumps, mice are chronically infused for periods of several days with receptor-selective agonists (544). When maintained in vitro in buffer containing the appropriate agonist, vasa deferentia from these animals exhibit a high degree of tolerance (544). This desensitization is receptor specific in that cross-tolerance to other prototypic opioid agonists is not seen (544). Since MVD does not exhibit a "withdrawal" contracture when challenged with naloxone (196), this preparation is not a suitable model for analysis of mechanisms of opioid dependence. This lack of withdrawal contracture does, however, make this preparation a good model for examining changes in receptor sensitivity to antagonists during tolerance (108).

iii. Other peripheral tissue preparations. Although GPI and MVD have proven to be invaluable as in vitro models of opioid-receptor interaction, neither tissue contains a homogeneous population of receptors

(289, 362, 373). Since, in both preparations, activation of more than one type of opioid receptor induces the same response, difficulties may arise in interpretation of pharmacological data when nonselective ligands are tested (see section II C2a). Within recent years, a number of peripheral tissues have been identified which contain a single type of opioid receptor (see table 1). Many of these fulfill the criteria for useful bioassay preparations and are now in routine use.

Vas deferens is a particularly useful tissue preparation in this respect. Vasa deferentia from different species exhibit marked variations in their sensitivities to opioid agonists. Whereas noradrenergic transmission in the rabbit vas deferens is selectively inhibited by K receptor agonists (239, 447), that of hamster vas deferens is sensitive only to drugs with δ opioid receptor activity (395). Although several reports have suggested that rat vas deferens contains a homogeneous population of ϵ receptors, which have high affinity for β-endorphin (188, 360, 535, 540), this is still a matter of some controversy (195, 369, 411, 563). Current data suggest that rat vas deferens contains receptors which have properties similar to that of μ receptors in other peripheral tissues (195, 369). Anomalous actions of morphine, and other μ opioid agonists, in this assay system may thus be explained by a low degree of μ opioid receptor reserve. A recent study has provided additional evidence, however, that rat vas deferens may also contain a novel receptor, which has high affinity for β-endorphin and its analogs (188).

b. BRAIN AND SPINAL CORD PREPARATIONS. Since the properties of peripheral opioid receptors resemble those of brain (120, 340, 362, 373), peripheral tissue preparations have proven to be invaluable as in vitro models of opioid-receptor interaction (280, 340, 443). Ultimately, however, analysis of opioid modulation of CNS function does necessitate direct examination of opioid receptor characteristics within the brain. Although analysis of the binding properties of central opioid receptors is quite straightforward (see section III), it has proven to be more difficult to measure a biological effect of opioids in isolated brain or spinal cord preparations. Whereas contractile response may serve as a physiological end point in peripheral tissues, in central tissues it is necessary to use a biochemical or electrophysiological measure. Despite such obstacles, considerable progress has recently been made towards analysis of the properties and function of central opioid receptors using in vitro assay systems.

i. **Tissue slices.** Since it was first shown, more than 20 yr ago, that synaptic field potentials could be reliably evoked in isolated brain slices (262, 629, 630), this in vitro preparation has gained steady recognition as a model for analysis of neural function (9, 144, 236). The brain or spinal cord slice offers several advantages for pharmacological characterization of opioid actions within the CNS (9, 133, 144, 236). Since there is no blood-brain barrier in such a preparation, there are fewer limitations of access to the receptor than in the intact animal. Although diffusion barriers may still exist to limit drug access to receptors within the interior of the tissue section, it is more feasible to apply drugs in known concentrations to circumscribed targets in vitro. In contrast to brain homogenates and cultured cells, the tissue slice also maintains a high degree of structural integrity; although major afferent and efferent pathways may be disrupted, local circuitry remains intact. Whereas the advantages of this tissue preparation are generally considered to outweigh the disadvantages, it should not be forgotten that the tissue slice is an isolate which is bathed in an artificial medium and which lacks normal synaptic inputs. Neuronal physiology within the slice may therefore be expected to differ significantly from that in the intact animal (9).

A wide variety of methodologies are currently being used for the preparation and maintenance of tissue slices. These have been reviewed in a number of articles (9, 12, 134, 236, 378, 454). In general, animals are decapitated, and their brains or spinal cords are removed and placed in cold, oxygenated, physiological buffer. The anatomical region of interest is then dissected out and carefully cut into slices. Slices may be prepared in a number of ways, including hand cutting, mechanical chopping, or vibratome sectioning, the care with which this procedure is undertaken being a critical determinant of physiological viability (9, 236). Sections are normally cut to a thickness of 200 to 400 μm. Histological studies have indicated that a 40- to 50-μm layer of a mechanically damaged tissue is usually present at each cut edge of the slice (9, 438); thus, if slices are cut too thin, there may be few undamaged cells within the interior of the section. On the other hand, the diffusion of oxygen throughout the slice is limited by section thickness (438); thus, slices with thicknesses in excess of 300 to 400 μm may become significantly anoxic (21, 168, 438). While slices from adult CNS have primarily been used for most acute physiological studies, many experimenters have preferred to use neonatal tissue for analysis of spinal cord opioid mechanisms (249, 433, 568). Although neonatal tissue may be less susceptible to anoxia than that of adult, it may also differ significantly in both synaptic organization and opioid receptor properties (114, 620).

Following sectioning, slices are transferred to an incubation chamber where they are bathed or superfused with physiological buffer. The composition of this ionic medium is not standardized, but varies with experimenter and experimental conditions (9). The temperature at which brain slices have been successfully maintained also varies over a wide range, from room temperature (25°C) to normal physiological temperatures (37–39°C) (9). Although more normal physiological responses may be expected to occur at the higher incubation temperatures, the survival of the preparation may be prolonged by maintenance of the tissue at a slightly lower temperature (9).

Over the last decade, brain and spinal cord slice preparations have been increasingly adopted for analysis of the properties of central opioid receptors. Both biochemical and electrophysiological techniques have been used to measure the pharmacological effects of opioid agonists and antagonists, as outlined in the following sections.

α. Transmitter release studies. One well-characterized effect of opioid agonists is to inhibit transmitter release from certain neurons of the peripheral autonomic nervous system (see section II C1a). Recent studies indicate that opioids may play a similar functional role within the brain. Opioid agonists have been shown to inhibit the release of a number of neuroactive substances from brain slices in a dose-dependent, stereospecific, and naloxone-reversible manner. Only certain neurons within the CNS appear to be targets for opioid action, however (see table 3). Such neurons are not representative of any specific chemical or neuroanatomical class. Thus, for example, opioid agonists inhibit cholecystokinin (CCK) release from hypothalamus but not cerebral cortex (403–406). Within the cortex, opioids exert a selective effect to inhibit the release of NE (229, 310, 370, 613, 614) and amino acid neurotransmitters (50), but not serotonin (5-HT) (229).

The identification of opioid-sensitive neurochemical release mechanisms within the brain has permitted systematic examination of the pharmacological properties of functionally coupled central opioid receptors (229, 296, 370, 613, 614). Although the detailed experimental design varies for different studies, the following general protocol has been used. Following sacrifice, brains are rapidly removed and dissected into specific anatomical subregions. Semithin brain slices are then cut and transferred to a tissue bath, where they are incubated (13, 50, 507, 613) or perfused (404, 419, 427, 576) with warm, oxygenated, physiological saline. Following an initial preincubation period, the effects of drugs on spontaneous or stimulated transmitter outflow are examined.

Several different approaches may be used for measuring the release of neuroactive substances. (*a*) For these

TABLE 3

Opioid effects on neurotransmitter release from brain slices

Neurochemical system	Brain area	Species	Opioid effect	Putative receptor type	Ref.
Monoamines					
NE	Cerebral cortex	Rat	↓ electrically, K^+ and Ca^{2+}-evoked release	μ	13, 217, 229, 310, 419, 450, 534, 575
NE	Cerebral cortex	Guinea pig	↓ K^+-evoked release	μ, K, δ	613, 614
NE	Cerebral cortex	Rabbit	↓ electrically evoked release	K	370
NE	Hypothalamus	Rat	↓ electrically and K^+-evoked release	μ	217, 427, 576
NE	Hippocampus	Rat	↓ K^+-evoked release	μ	296, 614
NE	Hippocampus	Guinea pig	↓ K^+-evoked release	μ, K, δ	614
NE	Hippocampus	Rabbit	↓ electrically evoked release	K	296
NE	Cerebellum	Rat	↓ electrically evoked release		418, 576, 614
DA	Caudate-putamen	Rat	↓ spontaneous & K^+-evoked release	K	427, 567
ACh	Caudate-putamen	Rat	↓ K^+-evoked release	δ	427
ACh	Cerebral cortex	Mouse	↑ K^+-evoked release	(μ)	147
ACh	Cerebral cortex	Rat	↓ K^+-evoked release	μ_1	148
ACh	Hippocampus	Rat	↓ K^+-evoked release		567
Amino acids					
Aspartate	Cerebral cortex	Rat	↓ Veratrine-evoked release	μ, K, (δ)	50
Glutamate	Cerebral cortex	Rat	↓ Veratrine-evoked release	μ, K, (δ)	50
GABA	Cerebral cortex	Rat	↓ Veratrine-evoked release	μ, K, (δ)	50
Peptides					
TRH*	Mediobasal hypothalamus	Rat	↓ K^+-evoked release	K	574
CCK	Hypothalamus	Cat	↓ K^+-evoked release	μ, (δ)	404, 405
CCK	Hypothalamus	Rat	↓ K^+-evoked release	μ, (δ)	406
VIP	Cerebral cortex	Cat	↓ K^+-evoked release	μ, (δ)	403
Substance P	Hypothalamus	Cat	↓ K^+-evoked release	μ, (δ)	405
LHRH	Mediobasal hypothalamus	Male rat	↓ DA- & K^+-evoked release	K	138, 139, 507
Somatostatin	Mediobasal hypothalamus	Rat	↓ K^+-evoked release	K	138

* TRH, thyrotropin-releasing hormone; VIP, vasoactive intestinal peptide; LHRH, luteinizing hormone-releasing hormone.

systems with presynaptic transmitter reuptake mechanisms [e.g., NE, dopamine (DA), 5-HT, γ-aminobutyric acid (GABA)], intraneuronal stores may be labeled by preincubating the tissue with radiolabeled transmitter (153, 419, 576). Subsequent spontaneous and stimulus-evoked outflow of radioisotope has been shown to represent a mixture of intact transmitters and associated metabolites (576). These may be further separated by chromatography (159, 219, 318, 576). It is important to note that, in those systems with presynaptic reuptake mechanisms, the overflow of radioisotope (usually tritium) represents the difference between tritium released and that taken back into the neurons. Data interpretation may be greatly simplified if, following preincubation with radiolabeled transmitters, a specific inhibitor of presynaptic reuptake is included within the assay buffer (576). (*b*) An alternative approach to labeling presynaptic transmitter stores is to preincubate tissues with a radiolabeled synthetic precursor (428). This approach has been used for analysis of ACh release (147, 427). Previous studies have shown that, in slices preincubated with [^{3}H]choline, the subsequent outflow of tritium represents a mixture of [^{3}H]choline and [^{3}H]ACh (428). Tissue depolarization, with high concentrations of K^+, has been reported to result in a selective increase in [^{3}H]ACh release, with little change in the release of [^{3}H]choline (428). (*c*) In contrast to the tracer methodologies outlined above, an alternative approach is the direct measurement of endogenously released neuroactive substances. This method is particularly applicable to the study of peptides, whose intraneuronal stores cannot easily be labeled by preincubation with radioactive tracers. In order to detect low levels of endogenously released material, it is necessary to include metabolic enzyme inhibitors within the assay buffer and to use a highly sensitive assay methodology, such as radioimmunoassay (403–406, 507, 574). When using radioimmunoassay, antisera should be used which have minimum cross-reactivity for other endogenous substances. The specificity of the technique can be further increased by combination with chromatography, although this fractionation of immunoreactive material may somewhat reduce detection sensitivity.

Although opioid agonists have been reported to modulate the spontaneous outflow of some neuroactive substances (427), their predominant effect is to inhibit stimulus-evoked release (see table 3). Transmitter outflow may be evoked by electrical field stimulation (229, 418, 419, 575, 576), as has been described above for peripheral tissues. Alternatively, release may be evoked by K^+-induced depolarization (13, 403–406, 427, 567) or by pharmacological stimuli (50, 507). Since intraneuronal transmitter stores can be depleted by repeated stimulation (576), experiments may be designed such that each tissue is exposed to a single concentration of test drug (403, 576). Alternatively, tissues may be subjected to continuous stimulation, and a cumulative drug dose-response curve constructed (159).

β. Electrophysiological studies. The use of electrophysiological techniques has proven to be a valuable tool for studying the functional and pharmacological properties of central opioid receptors (79, 141, 243). Since electrophysiological recording from brain and spinal cord slices provides an opportunity to examine the immediate physiological consequences of opioid receptor activation, it represents a powerful means of analysis of the molecular mechanisms underlying opioid action (442). As drugs may be applied and tested in a relatively quantitative manner, a classical pharmacological approach may also be used for analysis of drug response. Thus, rigorous examination of the pharmacological characteristics of central opioid receptors is possible (45, 83, 144, 162, 435, 586, 619).

The techniques used for brain slice preparation and electrophysiological recording have been discussed extensively elsewhere (9). Briefly, tissue slices are prepared as described above and mounted in an electrophysiological recording chamber. Tissues are bathed, or superfused, with warm physiological buffer and maintained in a humidified atmosphere of 95% O_2/5% CO_2. Spontaneous or evoked electrical potentials may be recorded with either extracellular or intracellular electrodes. Whereas extracellular recording permits analysis of the synaptic field potentials generated within a population of neurons, intracellular recording allows more detailed analysis of the response characteristics of an individual cell. In the absence of drug, stable base line measurements may be obtained over periods of several hours using either recording technique.

A number of methods can be used for application of drugs to the target neurons (9, 319, 484). Of these, addition of drug to the superfusion medium is the only method in which the extracellular concentration of drug can be accurately known. Since the tissue is uniformly bathed in drug solution, the superfusion method also ultimately allows a homogeneous distribution of drug within the slice (9). For these reasons, it is the method of choice for quantitative pharmacological studies. It should be noted, however, that the rate of diffusion into the slice is a function of slice thickness, and that a finite time is required for a homogeneous distribution to be achieved (438). Thus, the concentration of drug within the slice will not reach equilibrium until some time after the addition of drug to the bathing fluid.

Other methods, although less quantitative, allow more temporal and spatial control over drug administration (9, 319, 484). In microionotophoresis, concentrated drug solutions are placed in the barrel of a micropipet. When direct current is passed through the pipet, charged drug molecules are ejected and diffuse towards the target cell. The onset of drug action is more rapid than with the superfusion method, and the effect is limited to a smaller number of cells. Using this technique, however, the absolute concentration of drug at the receptor is unknown. The transport number, which describes the relationship

between the amount of current applied and the amount of drug ejected, not only differs from drug to drug but also from pipet to pipet. Thus, the absolute amount of drug discharged from a given pipet can only be estimated. Another limitation is that the current which is used to discharge drugs can result in the hydrolysis of drug within the pipet (218). The current may also exert direct effects on the target tissue which are not easily distinguishable from drug effects.

The pressure ejection method is similar to ionotophoresis except that pressure, rather than current, is used to discharge drug. This technique circumvents the adverse effects of discharge currents and eliminates some of the uncertainties as to the amount of drug which is released (9). However, since the distribution of drug within the tissue is nonhomogeneous, the concentration of drug at the target site can only be estimated. Since assumptions must be made as to the distance between the pipet tip and the target cell, as well as to the diffusion properties of the drug (619), this method of drug application is not optimal for quantitative pharmacological analysis. It may be very useful, however, for qualitative analysis of molecular mechanisms of drug action.

Over the past decade, a combination of these electrophysiological approaches has been used sucessfully for characterization of the primary actions of opioids in the brain and spinal cord (79, 141, 243, 442). By recording from opioid-sensitive neurons in vitro, receptor properties have been classified using standard pharmacological techniques, including use of selective agonists, determination of potencies of antagonists, and selective receptor inactivation (see section II A). The pharmacological characteristics of opioid receptors in hippocampus and locus coeruleus, in particular, have been studied extensively in this manner (53, 83, 162, 185, 435, 586, 619). Electrophysiological methods have also proven to be invaluable for analysis of signal transduction mechanisms in the brain and periphery (4, 86, 117, 442).

ii. Organotypic cultures. Considerable progress on analysis of neural mechanisms of opioid action has been made using chronic organotypic slice cultures (114, 115, 180). When maintained under culture conditions, slices from fetal or neonatal nervous tissue can remain viable for periods of up to several months (183). Two principal experimental approaches have been used for the development of organotypic cultures in vitro. In the first approach, slices are maintained in a stationary environment in Maximow depression slide chambers. The resulting cultures are many cell layers thick, with neuronal morphology and electrophysiological properties reminiscent of those seen in situ (114, 115). Cultured slices develop complex synaptic interactions as early as 3 days after explantation (118) and form functionally active contacts when cocultured with appropriate target regions (137). This Maximow-type culture was first applied to neuronal tissue by Crain and coworkers (112, 113), and has been used extensively by this group to examine opioid actions in spinal cord with attached dorsal root ganglion (114, 115, 117).

If slices are cultured in a roller-tube, rather than maintained in a stationary position, the tissue will spread out and flatten to a single cell layer (182, 183). These monolayer cultures still retain a high degree of intrinsic organization (182, 183) and can form synaptic contacts when cultured with appropriate target regions (184). In contrast to thicker slices, individual cells in thin roller-tube cultures can be easily identified and are more accessible to experimental manipulation. Such monolayer cultures also present fewer diffusion barriers than do fresh or cultured thick slices. While these have not been used as extensively as thicker slice cultures, organotypic monolayer cultures have been examined electrophysiologically for analysis of the actions of opioids in the hippocampus (180, 181).

The use of thick or thin organotypic slice cultures for electrophysiological or neurochemical studies offers certain advantages over that of freshly isolated slices. The long-term maintenance of the tissue slice in culture permits adequate recovery from the trauma of dissection and allows for cellular adaptation to the in vitro environment. The maintenance of neuronal slices under stable culture conditions also permits detailed analysis of mechanisms underlying adaptive tissue responses to chronic opioid exposure (116, 181). Such cultures are, however, derived from perinatal tissue which may have properties differing from that of the adult (114, 325, 332, 366, 445, 620). Synaptic reorganization may also occur as a result of long-term culture, as well as selective loss of certain subpopulations of cells (182).

iii. Tissue homogenates. Since the majority of opioid receptor binding studies involve the use of whole brain membranes, it is logically attractive to examine the pharmacological consequences of opioid receptor activation in the same type of assay preparation. Following the original report by Collier and Roy (98), that morphine inhibits prostaglandin-stimulated adenylate cyclase activity in brain homogenates, a number of investigators have examined opioid actions in brain membrane and synaptosomal preparations (see below). Unfortunately, however, such studies have often yielded inconsistent and confusing results.

Although some laboratories have confirmed that opioids inhibit adenylate cyclase activity in brain homogenates (88, 94, 238, 315, 357, 596), others have failed to replicate this finding and have reported that opioids are either ineffective (317, 577) or actually stimulate enzyme activity (295, 573). Other investigators have reported that opioids inhibit Ca^{2+} binding and uptake into brain synaptosomes (224, 503). Such findings have been confirmed by some (50, 131, 517), but not by other laboratories (25). Most recently it has been reported that opioids stimulate GTPase activity in brain membranes (160, 274) and also modulate protein phosphorylation (96, 97). There are a number of factors which may

influence detection of an opioid effect in brain homogenates. When studying opioid modulation of basic cellular processes, it is important to consider that the number of opioid-sensitive cells may be a small fraction of the total number of cells within the sample. Thus, any opioid action may be difficult to detect against a high background. This difficulty may be partially circumvented by using only those brain areas which contain high concentrations of opioid receptors, such as caudate-putamen or thalamus. Other technical factors, such as method of tissue preparation, buffer composition, drug concentration, and incubation parameters, may also be critical. For instance, opioid-sensitive adenylate cyclase activity has been shown to be influenced by a number of experimental variables (88, 99, 238, 315, 357, 596). In monkey amygdala, the inhibitory action of opioids on DA-stimulated adenylate cyclase is eliminated by prior freezing of the tissue or by prolonged incubation (>90 min) at 0°C (596). The opioid effect is also diminished if the interval between sacrifice and homogenization exceeds 5 min (596). Other studies have shown that opioid activity is influenced by nucleotides and ions (88, 99, 238, 315, 357). Whereas guanosine triphosphate (GTP) is an essential requirement for maximum opioid inhibition of adenylate cyclase in rat striatum (99, 315, 357), opioid activity is diminished in the presence of high concentrations of adenosine (99, 238). Opioid sensitivity is also modulated by the addition of Na^+ and K^+ ions (99, 238, 357) and by pretreatment of brain membranes with a low pH buffer (88).

Since minor changes in experimental conditions may induce major alterations in the activity of opioids in homogenate preparations, it is not surprising that variable and conflicting data have often been obtained by different laboratories. Given these considerations, it is important that detailed experimental protocols be included within publications. Utmost caution should also be exercised in the interpretation, and generalization, of results.

c. DISSOCIATED CELL PREPARATIONS. Since the original observation that opioid agonists inhibit adenylate cyclase activity in neuroblastoma-glioma hybrid cells (326, 327, 548, 582), dissociated cell preparations have been widely used for characterization of opioid receptor properties. Intact cells offer several advantages over other types of tissue preparation. Diffusion limitations, which are inherent in isolated organ and brain slice preparations, are obviated. Indirect actions mediated via neighboring cells are also reduced or completely eliminated. The use of cell preparations which contain a homogeneous population of receptors may further reduce the complexity of data interpretation, particularly when nonselective agonists are used.

Whereas the complexity and heterogeneity of intact tissue preparations may sometimes limit the identification of opioid-sensitive mechanisms, opioid actions are more easily detectable in homogeneous cell preparations, such as clonal cell lines. Using equivalent assay conditions for measurement of receptor binding and biological effect, direct comparisons may also be made between receptor occupancy and pharmacological response (354). Thus, homogeneous cell preparations represent a powerful tool for analysis of receptor properties and of receptor-effector mechanisms. Within recent years, a number of neuroblastoma-glioma hybrid cell lines (NG108-15, N4TG-1, and N18TG-2) have been identified which have homogeneous populations of δ opioid receptors (table 4). Another neuroblastoma-glioma hybrid (NCB-20) has been reported to contain a mixed population of δ and K receptors (374, 396), while a pituitary tumor cell line (7315c) has been tentatively identified as containing a pure population of μ receptors (163).

Although clonal cell lines are particularly useful models for analysis of opioid receptor mechanisms, it must be recognized that the properties of such transformed cells may not be identical to those of cells in normal, intact tissue (52, 228). Furthermore, receptor properties and effector coupling mechanisms, as characterized in one cell line, may be different in others (355). Thus, some caution should be exercised in generalizing results from one clonal cell system to that of all opioid-sensitive cells.

Primary dissociated cell cultures of both neural and nonneural tissue are also widely used for biochemical and electrophysiological analysis of opioid receptor properties and function (table 4). Such cultures are prepared by dissociating intact tissues into individual cells using enzymatic digestion or mechanical disruption (222, 551). By using various preparative procedures (248, 389), it is possible to obtain cell cultures which are relatively enriched in one particular cell type. Unlike clonal cell lines, however, such homogeneous populations of cells may not be expected to be chemically identical in all respects. The major advantage of primary cultures is that, since the cells are not tumor derived, their physiological properties may be more closely related to those of the intact tissue than is the case for neuroblastoma-glioma clones. It has been suggested, however, that the methods which are used to dissociate the cellular matrix, i.e., enzymatic digestion with trypsin and/or collagenase, may alter opioid receptor properties (222). Furthermore, such cultures are often derived from embryonic tissue, which may differ significantly in its receptor properties as compared to adult (325, 332, 366, 445).

Certain naturally occurring single cell preparations, including amoeba and blood cells, have also been shown to be sensitive to the actions of opioids (see table 4). Although these have not been used widely as model systems for analysis of opioid receptor mechanisms, they may be suitable for this purpose since they lack some of the drawbacks of both cloned and dissociated cells.

2. Methodological considerations and data interpretation. Although it is easier to control experimental conditions in vitro than in vivo, a number of confounding variables may still influence data interpretation. Such

TABLE 4
Opioids actions in some dissociated cell preparations

Cell	Species	Opioid effect	Receptor type	Ref.
Clonal cells				
NG108-15	Mouse/rat	↓ basal and PGE_1*-stimulated adenylate cyclase activity and ganglioside biosynthesis	δ	42, 123, 326, 354, 355, 548, 582
		↑ GTP hydrolysis	δ	333
N4TG1	Mouse	↓ adenylate cyclase activity and ganglioside biosynthesis	δ	123
		↑ cGMP accumulation	δ	226
N18TG2	Mouse	↓ basal and PGE_1-stimulated adenylate cyclase activity	δ	355
NCB-20	Mouse/hamster	↓ PGE_1-stimulated adenylate cyclase activity	δ, K	374
7315c	Rat	↓ adenylate cyclase activity and prolactin release	μ	163
Dissociated cells				
Cortical astrocytes	Rat	↑ NE-induced glycogen turnover		465
		↑ NE-induced adenylate cyclase activity		508
Dorsal root ganglion	Chicken	↓ substance P release and action potential duration		426
Dorsal root ganglion	Mouse	↓ action potential duration	μ, δ, K	617, 618
Adrenal cortex	Rat	↓ ACTH-induced corticosterone release		223
Cardiac myocytes	Chicken	Positive inotropic action		353
Gastric smooth muscle	Guinea pig	Contraction		36
Umbilical vein endothelium	Human	↑ arachadonic acid- or thrombin-induced prostacyclin production		44
Spleen	Mouse	↓ antibody production		309
Other				
Lymphocytes	Human	↓ T-cell rosette formation	(μ)	628
		↑ T-cell rosette formation	(δ)	409, 628
Granulocytes	Human	Morphological changes		150
		↑ superoxide production		549
Monocytes	Human	↑ chemotaxis		510, 587
Natural killer cells	Human	↑ NK activity		387
Erythrocytes	Rat	↓ Ca^{2+}-ATPase	K	633
		↓ Ca^{2+} efflux	K	632
Mast cells	Rat	↓ PGE_1-induced inhibition of IgE-mediated serotonin release		631
Amoeba		↓ pinocytosis		311

* PGE_1, prostaglandin E_1; cGMP, cyclic GMP; ACTH, adrenocorticotropic hormone; NL, natural killer.

factors have already been the subject of excellent reviews (172, 320–323) and will be discussed at present only to the extent that they affect analysis of opioid-receptor interactions.

a. AGONIST ACTION ON MORE THAN ONE TYPE OF OPIOID RECEPTOR. Certain tissue preparations, such as GPI and MVD, may contain a mixture of opioid receptors, all of which mediate the same response (see tables 1 to 4). Errors in pharmacological characterization of an opioid receptor may thus occur when the measured response of a test agonist reflects action at more than one type of receptor. As was discussed in section II A2, receptor heterogeneity within a tissue may seriously confound measurement of antagonist pA_2 values, such that the calculated value reflects a weighted average of the pA_2 values at two different receptors. Since antagonist pA_2 values are used as a primary means of receptor classification, measurement of "intermediate" values may result in the erroneous identification of novel types of receptor.

Such difficulties may be circumvented, to a certain extent, by using highly selective agonists to analyze the properties of receptors. The ligand selectivity of a number of opioid drugs is listed in table 5. It should be noted that even those ligands which show selectivity for a given opioid receptor may, in high concentrations, interact

TABLE 5

Binding selectivity profile of opioid ligands in guinea-pig brain membranes

Competing ligand	K_i (nM)			Ref.
	μ	δ	K	
Endogenous opioids				
Proenkephalin A derivatives				
[Met⁵]-enkephalin	9.5	**0.9**	4440	606
[Met⁵]-enkephalin-Arg⁶	24	**17**	540	606
[Met⁵]-enkephalin-Arg⁶-Phe⁷	**27**	30	145	460
[Met⁵]-enkephalin-Arg⁶-Gly⁷-Leu⁸	6.4	**4.8**	89	460
Metorphamide	**0.06**	1.8	0.2	288
BAM 18	**0.3**	3.2	0.7	288
Proenkephalin B derivatives				
Dynorphin(1-8)	**3.4**	4.4	9	101
Dynorphin(1-9)	3.8	5.0	**1.3**	101
Dynorphin(1-17)	0.7	2.4	**0.12**	101
Dynorphin B	0.7	3.2	**0.12**	335
α-Neoendorphin	1.3	0.57	**0.20**	335
POMC* derivatives				
β-Endorphin	**2.0**	2.7	57	335
Synthetic opioids				
Agonists with highest affinity for μ sites				
PL107	**16**	>1000	>1000	288
Morphiceptin	**107**	29,000	9,200	306
DAGO	**1.9**	345	6090	100
Normorphine	**4.0**	310	149	379
Sufentanyl	**1.6**	23	125	379
Agonists with highest affinity for δ sites				
DPDPE	710	**2.7**	>15,000	100
DPLPE	660	**2.8**	>15,000	100
DSLET	39	**1.8**	6,040	100
DTLET	34	**2.6**	14,500	100
DADLE	3.2	**1.5**	9,600	306
Agonists with highest affinity for K sites				
U50,4884	941	8690	**0.72**	306
Tifluadom	7.7	111.2	**0.08**	306
Etorphine	1.02	0.56	**0.23**	379
EKC	1.00	5.5	**0.52**	379
MR 2034	0.66	5.8	**0.45**	379
Bremazocine	0.62	0.72	0.41	379
[D-Pro¹⁰]dynorphin(1-11)	2.00	7.47	**0.03**	186
Monoiodo[D-Pro¹⁰]dynorphnin(1-11)	18.4	52.8	**0.38**	186
Antagonists				
Naloxone	**1.78**	27.0	17.2	379
Naltrexone	**1.08**	6.6	8.5	379
Diprenorphine	**0.84**	1.42	2.24	379
MR 2266	1.37	6.0	**0.69**	379
ICI 154,129	10,100	**778**	>50,000	100
ICI 174,864	27,200	**193**	>69,000	100

* POMC, proopiomelanocortin; DPLPE, [D-Pen², L-Pen⁵]enkephalin; DTLET, [D-Thr²]-Leu-enkephalin-Thr⁶.

with other receptor types. This is an important consideration when agonist concentration must be raised to overcome competitive antagonist blockade or receptor desensitization. It has been suggested that, even when selective agonists are used, it is a worthwhile precaution to include antagonists of the other receptor types in the incubation buffer (172). As yet, however, few highly selective opioid antagonists are presently available for this purpose.

For analysis of structure-activity relationships, problems of ligand cross-reactivity may be circumvented by assaying drug activity in preparations which contain homogeneous populations of receptors (see tables 1, 3, and 4). By assaying in a number of parallel preparations, the full spectrum of activity of a drug may be identified.

b. AGONIST ACTION AT A SITE OTHER THAN OPIOID RECEPTOR. In some tissues, measurement of agonist dose-response relationships may be complicated by an action at a site other than an opioid receptor. Such effects may become particularly significant when high concentrations of agonist are used. For example, high concentrations of morphine potentiate electrically stimulated contractions of GPI (341, 463). Although it has been suggested that this phenomenon represents "acute tol-

erance" (463), the effect is unrelated to opioid receptor activation, but, rather, reflects an inhibition of acetylcholinesterase (341). Other agonists, such as meptazinol, have anticholinesterase or cholinergic agonist activities which may interfere with measurement of their opioid effects in cholinergic tissues (34, 187, 352). Several opioid peptides have also been reported to have nonnaloxone-reversible actions, in addition to their opioid activity (124, 199, 597).

In order to confirm that an observed biological response reflects interaction with an opioid receptor, all agonist effects should be shown to be blocked by (−), but not (+), isomers of opioid antagonists, such as naloxone. Since nonspecific effects may only become significant when agonist concentration is raised to overcome competitive antagonist blockade, these may significantly interfere with determination of antagonist pA_2 values. Such non-opioid effects may be detected by significant deviations in the linearity and/or slope of the Schild regression.

c. CHANGES IN TISSUE SENSITIVITY. Changes in tissue sensitivity should always be considered as a possible source of error in pharmacological experiments. Spontaneous changes in the sensitivity of isolated tissue preparations to agonists may be expected to occur throughout an experiment, particularly during the initial phase, while a tissue is equilibrating in its novel environment (394). Sensitivity changes are also commonly observed in the final phase of experiments of long duration, as tissues undergo physiological deterioration (172, 281). Drug-induced desensitization is another well-established phenomenon, which may result from allowing insufficient intervals for recovery between drug doses (227, 463).

The design of an experiment should allow for the detection of, and correction for, changes in sensitivity to agonists throughout the course of an experiment. In experiments to compare the potencies of different agonists, a "bracketing" procedure has been recommended (171, 204, 343), in which concentration-response data for a "standard" agonist are always obtained prior to, and after, obtaining such data for the "test" agonist. In experiments to measure the potency of a competitive antagonist, a similar bracketing procedure may be used, in which "standard" agonist dose-response curves are constructed prior to administration of antagonist and after its complete removal (281, 343). Alternatively, a "paired control" preparation may be used to correct for any sensitivity change in the test preparation not caused by the antagonist. In this case, a well-matched control preparation is treated in an identical manner to the experimental preparation, except that it is not exposed to the antagonist.

d. REMOVAL OF AGONIST FROM THE BATHING FLUID. For measurements of agonist potency, the concentration of drug at the receptor should be in diffusion-equilibrium with that in the external bathing solution (172). If some process results in the continuous removal of agonist from the region of the receptor, then equilibrium cannot be achieved, and errors in determination of agonist potency will result (172). If the rate of removal differs for different agonists, the potencies of those agonists which are most rapidly removed will be underestimated, leading to gross distortions in measurements of relative activity. A saturable agonist removal process may also significantly interfere with determination of competitive antagonist potencies, yielding Schild plots with slopes which deviate significantly from unity (172, 323). Since determination of relative agonist potencies and antagonist pA_2 values are the primary means of receptor characterization, active agonist removal processes, if not recognized and abolished, may lead to the erroneous classification of novel receptor types.

It is widely recognized that many opioid peptides are highly sensitive to enzymatic degradation (255–257, 363, 383, 393, 545). Although peptidase inactivation has long been acknowledged as a source of error in receptor binding assays (198, 356, 363, 412, 470), it has generally been assumed to be of little significance in in vitro bioassay because of the short duration of drug exposure. Recent studies have indicated, however, that inhibition of peptidase activity with either bacitracin or a combination of bestatin, captopril, thiorphan, and L-leucyl, L-leucine results in a striking increase in the agonist potency of some opioid peptides in peripheral tissue preparations (8, 394). These effects of enzyme inhibitors are tissue dependent, in that greater potency changes are seen in rat and rabbit vas deferens than in GPI or MVD (394, 518). Within a single tissue, the rate of inactivation differs for different agonists; whereas the potencies of short-chain endogenous peptides are markedly increased by peptidase inhibition, those of certain longer-chain peptides, stabilized synthetic analogs, and nonpeptides are unaffected (394, 519).

The pharmacological selectivity of a given agonist may also be affected by tissue peptidase activity. Significant differences have been reported in the K_e values for naloxone antagonism of the agonist actions of certain dynorphin analogs in the absence and presence of enzyme inhibitors (305). These data suggest that some peptides may be cleaved by tissue peptidases to yield agonists with receptor selectivities different from that of the parent compound (271). Such findings have recently been confirmed by biochemical analysis of radioligand metabolism by MVD (410); [^{3}H]dynorphin(1–9), a peptide which has pharmacological selectivity for the K receptor (81, 101), is rapidly degraded by tissue peptidases to yield [^{3}H]Leu–enkephalin, a peptide with δ-receptor selectivity (373). Although metabolic activity was shown to be reduced by the addition of an enzyme inhibitor cocktail, it was not completely abolished (410).

Given these findings, it is clear that rigorous pharmacological analysis of opioid peptide activity in vitro requires the presence of appropriate peptidase inhibitors

in the bathing fluid. Since endogenous peptides may act as substrates for a number of enzymes of differing specificities (255–257, 363, 381, 393, 394, 545), it may be necessary to incubate with several peptidase inhibitors simultaneously (394, 410). Although the potency of an unstable peptide may be significantly enhanced by addition of an enzyme inhibitor mixture, this does not constitute evidence that peptide metabolism has been completely blocked. The only definitive means of determining whether peptide breakdown has occurred is by chromatographic analysis of tissue perfusate. It should also be remembered that the agents which are used to inhibit peptidase activity may have additional actions on the tissue which interfere with the testing of agonist and of antagonist activity. These secondary actions should be recognized and avoided if possible.

III. Radioreceptor Assay

The fundamental principle of radioreceptor assay is that radiolabeled drugs, in low concentrations, selectively bind to the site for which they have highest affinity. Since this simple methodology permits the direct measurement of drug-receptor interaction, without the complications of intervening stimulus transduction mechanisms, radioligand binding has been used extensively to examine the properties of opioid receptors and the drugs with which they interact. Although the advantages of the technique are clear, there are certain limitations to this pharmacological approach. In most instances, radioligand-receptor interactions are examined under nonphysiological conditions. Since measurements do not involve a physiological endpoint, many criteria must be fulfilled in order to establish that the radioligand binding site under investigation represents a functional receptor (60–62, 269). An understanding of the factors which influence radioligand-receptor interactions is essential for proper experimental design and interpretation of binding data. These will be outlined in the following sections. For a more detailed review of the theoretical principles of radioreceptor binding, the reader is referred to the following additional texts (27, 43, 122, 268, 417, 610).

A. Criteria for Receptor Identification

The major problem of interpretation in any binding study is identification of the binding site as a receptor. The key property of a binding site which allows definition as a receptor is an association with function. Under ideal circumstances, ligand binding should be correlated with a measurement of drug response within the same tissue. If this is not possible, it is essential that the properties of the ligand binding site are extensively characterized pharmacologically and are shown to be consistent with those of an identified receptor in an intact preparation.

A minimum requirement for identification of a radioligand binding site as a receptor is that it is saturable, i.e., that binding can be displaced by increasing concentrations of nonradioactive ligand. "Specific" binding is thus defined as the difference in radioactivity bound in the absence and presence of an excess of unlabeled, competing ligand (209, 471, 555, 578). For classification of opioid binding sites, a second criterion which should be fulfilled is that of stereospecificity. Thus, radioligand binding should be displaced by the pharmacologically active (−) isomer of an opioid drug in a dose range which is at least one order of magnitude lower than that of the corresponding (+) derivative.

Although essential, these basic criteria are not sufficient for identification of a radioligand binding site as an opioid receptor. A number of investigators have demonstrated saturable binding of radioligands to sites other than receptors (33, 121). In particular, saturable, stereospecific binding of radiolabeled opioids to both glass-fiber filters (565) and cerebrosides (372) has been reported. Given these findings, it is essential that the pharmacological properties of the site under investigation are thoroughly characterized and shown to be similar to those of an identified, functionally active receptor.

B. General Principles

The binding equations which are outlined in the following sections are based on the principle of the Law of Mass Action, i.e., a simple, bimolecular interaction between a drug and its binding site. Thus, their derivation is identical to that of the equations, outlined in section II A, which govern measurement of biological response. In contrast to bioassay, however, radioligand binding assay permits direct measurement of both agonist and antagonist equilibrium dissociation constants.

1. Determination of radioligand binding constants. Assuming that the binding of a ligand (L) to its receptor (R) follows the Law of Mass Action, then, at equilibrium

$$R + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RL \qquad \text{equation 16}$$

The equilibrium dissociation constant, K_D, provides a measure of the affinity of a radioligand for its binding site and is characterized by

$$K_D = \frac{k_2}{k_1} = \frac{[R][L]}{[RL]} \qquad \text{equation 17}$$

Radioligand K_D may be measured in one of two ways: (a) saturation experiments in which total radioligand concentration, $[L_t]$, is increased and $[RL]$ is determined at equilibrium; and (b) kinetic experiments in which $[L_t]$ is held constant and $[RL]$ is determined as a function of time.

a. SATURATION EXPERIMENTS. In a saturation experiment, receptor concentration, $[R_t]$, is held constant and $[RL]$ is determined at equilibrium as a function of $[L]$. Since R_t is equal to $R + RL$, then

$$\frac{[R_t - RL][L]}{[RL]} = K_D \qquad \text{equation 18}$$

Rearranging equation 18

$$[RL] = \frac{[R_t][L]}{K_D + [L]} \qquad \text{equation 19}$$

Equation 19 describes a rectangular hyperbola (see fig. 4*a*) and is mathematically equivalent to both the Langmuir absorption isotherm (equation 2) and the Michaelis-Menton equation. From this equation, it can be seen that, when $K_D = [L]$, $[RL] = [R_t]/2$. Thus, K_D is equal to the concentration of radioligand which occupies 50% of the binding sites.

i. Scatchard analysis. One popular means of analysis of equilibrium binding data is the Scatchard or Rosenthal plot (502, 522). The mathematical basis of this approach depends upon rearrangement of equation 19.

$$\frac{[RL]}{[L]} = \frac{([R_t] - [RL])}{K_D} \qquad \text{equation 20}$$

The parameters of this equation are more usually represented by the following symbols, $[RL] = B$ (bound), $[L] = F$ (free), and $[R_t] = B_{max}$ (maximal number of binding sites). Thus

$$\frac{B}{F} = \frac{B_{max}}{K_D} - \frac{B}{K_D} \qquad \text{equation 21}$$

A plot of B/F versus B gives a straight line with a slope of $1/K_D$ and an intercept with the abscissa of B_{max} (fig. 4*b*).

The primary advantage of the Scatchard plot is that it provides a linear transformation of the hyperbolic saturation curve, allowing K_D and B_{max} to be calculated easily. It is therefore attractive in its apparent visual simplicity. As has been pointed out by many authors, however, there are several disadvantages associated with its use (43, 57, 58, 69, 329, 330, 429, 431, 441, 527, 552). Many factors may contribute to inaccurate parameter estimates as determined by Scatchard analysis, including the following.

(*a*) The Scatchard transformation contains a term for "*B*" on both axes; thus errors in "*B*" are magnified in two directions. This increases the scatter of the data points, particularly at either axis, where measurement of "*B*" is subject to the greatest error (431, 432).

(*b*) Data points which are evenly spaced in a nontransformed plot are clustered in a Scatchard plot; thus, at increasing values of *F*, there is an enormous compression of data. For this reason, the Scatchard graph is deceptive in that it may lead experimenters to conclude that ligand saturation has been achieved when it has not, and encourage inaccurate extrapolation of B_{max} (58, 329, 330).

(*c*) Theoretically, a linear Scatchard plot reflects interaction of a ligand in a simple bimolecular manner with a single class of binding sites, or with multiple classes of binding sites with equal affinity (502, 522). Nonlinear Scatchard plots may reflect more complex models, including cooperative interactions between binding sites or the presence of multiple classes of binding site for which the radioligand has differing affinity (see fig. 5; 43, 58, 297, 608). A number of methodological artifacts may, however, cause nonlinearity in Scatchard plots and complicate data interpretation, leading experimenters to propose a more complex model where none exists (43, 122, 429, 432).

(*d*) Conversely, Scatchard plots may not reveal the true complexity of a model. Several studies have shown that the probability of significantly resolving two binding sites is dependent upon several factors (57, 58, 208, 608); these include the scatter, and number, of data points, the concentration range of the radioligand, the ratio of affinity constants, and the proportion of high and low affinity sites. If optimal conditions are not achieved, a Scatchard plot may be inaccurately interpreted as being monophasic, rather than biphasic.

(*e*) Calculation of binding parameters from Scatchard graphs is also fraught with difficulties. Although determination of K_D and B_{max} from a linear Scatchard plot may seem straightforward, the simplicity is deceptive.

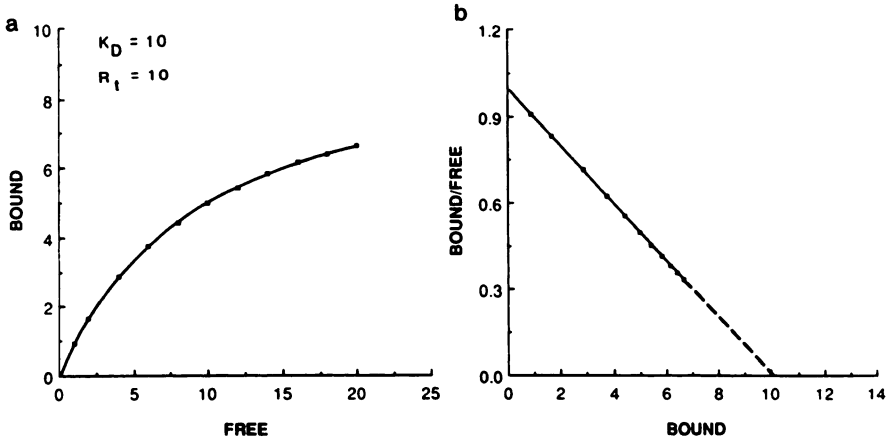

FIG. 4. Saturation analysis of radioligand binding constants. *a*, saturation curve of radioligand binding with an equilibrium dissociation constant, K_D, of 10 nM, to a homogeneous population of receptor sites at a density, R_t, of 10 pmol/g. *b*, Scatchard transformation of the same data. Data are theoretical curves derived from equations 19 and 21.

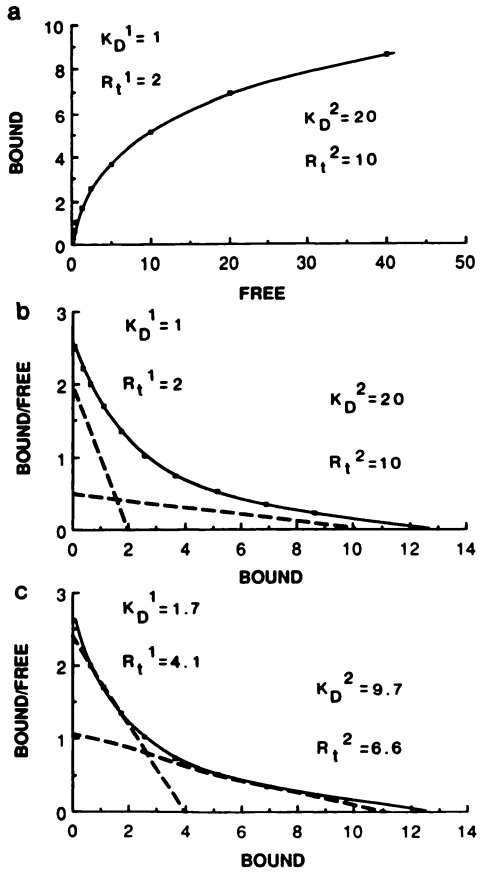

FIG. 5. Saturation analysis of radioligand binding constants. *a*, saturation curve of radioligand binding to two noninteracting sites with K_Ds of 1 and 20 nM and densities of 2 and 10 pmol/g, respectively. *b*, Scatchard analysis of saturation data. *Dashed lines*, radioligand binding to each site as determined by nonlinear regression analysis. *c*, Scatchard analysis of saturation data. *Dashed lines*, visual extrapolation of binding constants. Note that this method significantly overestimates the K_D and B_{max} values for the high affinity site and underestimates these values for the low affinity sites. Data are theoretical curves derived from equations 19 and 21.

Because of the correlated, nonuniform errors in both of the coordinates of the Scatchard plot, it is statistically invalid to use nonweighted linear regression for the analysis. Indeed, Munson and Rodbard (431) have shown that a more accurate estimation of binding parameters may be achieved by visual inspection than by simple linear regression. These problems of analysis become compounded when Scatchard plots are nonlinear. Although this procedure has been widely used, it is incorrect to simply draw two lines through the transformed data points (see fig. 5, *b* and *c*); this will result in a significant overestimation of both K_D and B_{max} values for the high affinity site, and underestimation of the same parameters for the low affinity site (441). The correct binding parameters may be calculated from accurate measurements of the slope at the extremes of the curve (286, 580). However, this method is limited by the large variance of these extreme data points and the complexity of the mathematical analysis (430). Alternatively, iterative curve-fitting procedures may be used to determine these binding parameters, such that corrections are made for the contribution of one binding component to the other (414, 417). However, such procedures suffer from the same disadvantage as that described for the one-site case, in that transformed data require a complicated weighting function because of the unequal, correlated errors in either axis.

ii. Computer-assisted analysis of saturation data. Given these considerations, it would appear that Scatchard plots may provide a useful means of graphic display of the data, but are not the method of choice for determination of binding parameters. An alternative approach, which has recently been shown to have greater statistical validity, is computerized, nonlinear regression analysis of untransformed data (375, 398, 431, 497). Convenient computer programs, such as LIGAND (431), have recently been developed for analysis of ligand saturation curves, based on the mathematical framework for complex binding models formulated by Feldman (154). When used appropriately, these represent powerful tools for estimating parameters of binding affinity and capacity, having several advantages over other methods of data analysis.

(*a*) "Total" binding can be examined as the dependent variable, rather than "specific," thus eliminating additional errors introduced into the analysis by subtraction of "nonspecific."

(*b*) Weighting is provided to compensate for the nonuniformity of variance of the dependent variable, *B*. Thus, data points at the extremes of the curve, which are inherently more variable, are not weighted equally with those in the middle.

(*c*) Such programs provide statistical methods for evaluating the "goodness of fit" of the binding data to a number of different mathematical models. Thus, the statistical validity of a multiple-site model can be compared directly with that of a one-site model.

(*d*) It is possible to simultaneously analyze curves from several different experiments, by introducing factors to correct for interexperimental differences in binding capacity. By combining the information from several experiments, binding parameters can be estimated more precisely.

The major disadvantage of computerized analysis of receptor binding data is that it lacks the intuitive simplicity of graphic techniques. Because of the complexity and power of the analysis, an experimenter may be tempted to unquestioningly accept computer-generated parameter estimates. This can, however, lead to erroneous conclusions, particularly when there is excessive data scatter. The best approach to analysis of receptor binding data is therefore to use graphic methods for a preliminary, subjective understanding of the data, in combination with computerized analysis techniques (580).

Although computerized analysis increases the preci-

sion of parameter estimates, the accuracy of the technique is limited by the quality of the initial data. Burgisser (58) has recently outlined the minimal criteria for accurate analysis of a saturation binding experiment. These include the following.

α. Minimum data scatter. The scatter of data is the most critical experimental parameter. Excessive scatter cannot be overcome by increasing the number of data points or by increasing ligand concentration. Although computerized analyses take account of the error distribution of the original data and avoid transformation biases and artifacts, the weighting functions which are used are based on the predicted variance and not the actual variance of the data (431). Parameter estimates may therefore be seriously distorted by spurious data points.

β. Radioligand concentration. The highest concentrations of radioligand which are used should be sufficient to occupy 90% of receptor sites (i.e., 9- to 10-fold higher than the K_D value). Saturation curves up to only half-maximal receptor occupancy are not satisfactory (329, 330). In order to reduce the expense of this procedure, higher concentrations of radioligand can be achieved by dilution with appropriate concentrations of unlabeled drug. For this procedure to be valid, however, it is essential that the labeled and unlabeled drugs are chemically identical.

Ψ. Number of data points. Although not as critical a determinant of data quality as data scatter or ligand concentration, the number of data points is also an important consideration. While 6 to 10 points are generally sufficient for analysis of a simple bimolecular reaction, more complex models require that the number of data points be at least doubled (57, 58).

Even if all of the above-mentioned criteria are fulfilled, however, there are limits to the power of computer-assisted resolution of radioligand binding data. A number of experimental artifacts may influence the analysis and increase the uncertainties in parameter estimates (122, 429, 430, 432). These will be discussed in more detail in section III B2. Computer models, such as LIGAND, are also based on a number of theoretical assumptions which may not always be valid. Such models assume simple receptor-ligand interactions and do not encompass the type of allosteric interaction which may typically be found (127, 297, 495, 526).

While computerized analysis of saturation data may distinguish radioligand binding to two or more noninteracting sites, the ability to resolve multiple binding sites is dependent not only on the quality of the data, but also on the ratio of K_D values and the proportion of high to low affinity sites (57, 58, 208, 608). If a radioligand binds to multiple classes of site with similar affinity, these cannot be distinguished by any type of mathematical analysis of radioligand saturation curves. Under such circumstances, heterogeneous binding sites may be resolved by either kinetic analysis of radioligand binding or analysis of log dose-response curves for inhibition of radioligand binding (332, 338, 416, 417, 462; see section III B1, b and c).

b. KINETIC EXPERIMENTS. In a kinetic experiment, receptor concentration, $[R_t]$, and ligand concentration, $[L_t]$, are held constant, and radioligand binding, $[RL]$, is determined as a function of time. When R and L are added together, the net rate at which RL is formed is equal to the difference in the rate of formation of RL from R to L minus the rate of dissociation of the RL complex.

$$\frac{d[RL]}{dt} = k_1[R][L] - k_2[RL] \qquad \text{equation 22}$$

where k_1 is the association rate constant, and k_2 the dissociation rate constant.

At equilibrium, the net rate of formation of RL is zero. Thus

$$\frac{d[RL]}{dt} = 0 = k_1[R][L] - k_2[RL] \qquad \text{equation 23}$$

and

$$\frac{k_2}{k_1} = \frac{[R][L]}{[RL]} = K_D \qquad \text{equation 24}$$

If radioligand binding obeys the Law of Mass Action, the kinetically derived K_D should be equivalent to that determined by saturation analysis (see section III B1a). A finding that these two values are not equivalent suggests that a more complex hypothetical model may apply, as has been suggested for the binding of opioid agonists to μ and δ receptors (526). Alternatively, such discrepancies may reflect methodological factors (see section III B2).

In addition to the determination of K_D, kinetic experiments are used for determining the time for apparent equilibrium to be reached, so that saturation and inhibition experiments may be carried out properly. It is important to note that, for a simple bimolecular reaction, the time to reach equilibrium is not only dependent on the kinetic rate constants, k_1 and k_2, but also on the concentrations of ligand and receptor $[L_t]$ and $[R_t]$. Thus, the lower the concentration of L or R, the longer the time for equilibrium to be reached (27, 63, 610, 621).

i. Measurement of k_1. For a simple bimolecular reaction, the rate of association between a ligand and its binding site may be described by the following second-order equation

$$\ln\left(\frac{[RL_{eq}]([L_t] - [RL][RL_{eq}]/[R_t])}{[L_t]([RL_{eq}] - [RL])}\right) = k_1 t\left(\frac{[L_t][R_t]}{[RL_{eq}]} - [RL_{eq}]\right) \qquad \text{equation 25}$$

where $[RL]$ is the amount bound at time, t, and $[RL_{eq}]$ is the amount bound at equilibrium. The kinetic association constant, k_1, can be calculated from the slope of a plot

of the natural log (ln) on the left side of the above equation versus time. While the use of this full second-order equation to determine k_1 has the advantage that it makes no assumptions regarding the relative concentrations of ligand and receptor ($[L_t]$ and $[R_t]$, respectively), it does necessitate an independent determination of $[R_t]$. In practice, this involves measurement of the density of binding sites within the same tissue sample by saturation analysis (see section III B1a).

If the experiment is designed such that $[L_t] \gg [R_t]$, the concentration of free radioligand, $[L]$, will not change appreciably as the reaction proceeds and can, for all practical purposes, be considered to be constant. Under these conditions, the reaction is "pseudo first-order." As the reaction proceeds to equilibrium, the amount bound, $[RL]$, at any time is related to the amount bound at equilibrium, $[RL_{eq}]$, by the following equation (27, 621)

$$\ln\left(\frac{[RL_{eq}]}{[RL_{eq}] - [RL]}\right) = (k_1' + k_2)t = k_{obs}t \qquad \text{equation 26}$$

where $k_1' = k_1[L]$, and k_{obs} is the experimentally observed rate constant. For a simple, bimolecular reaction, a plot of $\ln([RL_{eq}]/[RL_{eq}] - [RL])$ versus t will yield a straight line with a slope of k_{obs}. If k_2 is known from independent experiments, k_1 can be calculated from the equation

$$k_1 = \frac{(k_{obs} - k_2)}{L} \qquad \text{equation 27}$$

While this simplified method of calculating k_1 does not require concurrent measurement of $[R_t]$, it is only valid if $[R_t] \leq 10\%\ [L_t]$. If higher concentrations of ligand are bound, the value of k_1 will be significantly underestimated (610).

An alternative method for calculation of k_1, which does not require prior knowledge of the value of k_2, is to repeat the experiment at different radioligand concentrations and to determine k_{obs} for each L_t. Then a plot of k_{obs} against L_t will have a slope of k_1 and an intercept with the ordinate of k_2. Alternatively, the half-time ($t_{1/2}$) to reach equilibrium binding may be calculated at several values of L_t. Substituting these values into equation 26 gives

$$\frac{\ln 2}{t_{1/2}} = k_1 + k_2 \qquad \text{equation 28}$$

Thus, a plot of ($\ln 2/t_{1/2}$) as a function of $[L]$ yields a straight line with a slope of k_1 and an intercept of k_2.

ii. Measurement of k_2. The dissociation rate constant, k_2, can be calculated either as described above or by direct measurement. Experimentally, the association between R and L can be made negligible by "infinitely" diluting a preequilibrated mixture of R and L, or by adding an "excess" of competing ligand. (In practice, "infinite" dilution is defined as a 50-fold or greater dilution of the reaction mixture, while an "excess" of competing ligand is defined as a concentration which is 100-fold greater than that required to occupy 50% of the binding sites.) Under these circumstances, $k_1[R][L] = 0$, and the measured rate of change in the amount bound is the dissociation rate of the ligand-receptor complex

$$\frac{d[RL]}{dt} = -k_2[RL] \qquad \text{equation 29}$$

Integration and rearrangement of equation 28 give

$$\ln\left(\frac{[RL]}{[RL]_0}\right) = -k_2t \qquad \text{equation 30}$$

where $[RL]_0$ is the concentration of radioligand bound at $t = 0$, immediately prior to dilution of the ligand-receptor complex. For a simple bimolecular reaction, a plot of $\ln([RL]/[RL]_0)$ versus t yields a straight line with a slope of $-k_2$.

In theory, assuming that the reaction follows the Law of Mass Action, "infinite" dilution and addition of excess labeled ligand should result in identical dissociation curves. A finding that the dissociation rates calculated by the two methods differ significantly may indicate cooperativity (127, 128, 495).

c. DETERMINATION OF INHIBITOR BINDING CONSTANTS. In an inhibition experiment, receptor concentration, $[R_t]$, and radioligand concentration, $[L]$, are kept constant, while the concentration of a competing ligand, $[I]$, is varied. Thus, $[RL]$ is determined at equilibrium as a function of $[I]$. The reaction can be described by the following equation

$$[RL] = \frac{[L][R_t]}{K_D(1 + [I]/K_i) + [L]} \qquad \text{equation 31}$$

where $[I]$ is the concentration of the free unlabeled ligand, and K_i is the equilibrium dissociation constant for interaction of I with the receptor, defined as $K_i = [R][I]/[RI]$.

i. Measurement of K_i. Inhibition studies provide a useful means of measuring the affinity of a given drug for a radioligand binding site. The K_i of a competing ligand, I, can be determined by measuring the concentration of I, (IC_{50}), which produces a 50% inhibition of the specific binding of radioligand in concentration, $[L]$. The simplest method for determining the IC_{50} of a given inhibitor is to construct a standard dose-response semilogarithmic plot. Alternatively, the data may be linearized using a logit or a Hill transformation (27, 63, 260, 496). Having determined the value of IC_{50}, the K_i may then be calculated by the Cheng-Prusoff estimation (85).

$$K_i = \frac{IC_{50}}{1 + [L]/K_D} \qquad \text{equation 32}$$

It should be noted that the concentrations of I and L in equation 32 refer to the concentrations of *free* I and L at equilibrium. Since the concentration of free I may be difficult to determine experimentally, it may be approximated by the value for the concentration of total I, provided that the amount of I bound is low as compared

to K_i, i.e., if $R_t \ll K_i$. If $R_t \geq K_i$, the value of K_i from equation 32 will be an overestimate of the true value (371, 431). This problem may be circumvented by using computerized regression analysis programs, such as LIGAND, to analyze the data (126, 233, 398, 431, 492). Such programs fit the data directly to a mathematical equation and do not use the Cheng-Prusoff approximation. They are therefore valid for all levels of receptor occupancy. Unlike the Cheng-Prusoff estimation, which assumes that both labeled and unlabeled ligands interact with a homogeneous population of binding sites (85, 371), such programs may also be used for analysis of displacement of radioligand binding to heterogeneous sites (see below).

ii. Multiple classes of binding site. Shallow or biphasic inhibition curves with low slope factors (Hill coefficients less than 1) may indicate the existence of multiple binding sites which have differing affinities for the competing ligand. A number of methods have been formulated for analysis of such data to obtain a measure of the affinity of the competing drug for each of the binding sites and of the relative proportions of the different sites (417). In each case, the analysis is greatly facilitated if the radioligand has equal affinity for each of the multiple sites; under such circumstances, the radioligand saturation curves should best fit a one-site model, have a Hill slope of 1, and yield linear Scatchard plots (see fig. 6*a*). It is also assumed that the multiple binding sites are noninteracting and that the binding of both labeled and nonlabeled ligands obeys the Law of Mass Action.

The binding properties and relative densities of heterogeneous sites may be determined by either direct analysis of log dose-response curves for inhibition of ligand binding by competing drug, or analysis of transformed data. One means of graphic analysis of such data is the modified Scatchard or Hofstee plot (264, 414). The Hofstee plot (B versus B/I) will be nonlinear if the competing drug interacts with more than one class of binding sites with differing affinities (see fig. 6*b*). Although attractive in its simplicity, this method of data transformation suffers from the same drawbacks as those described previously for Scatchard analysis (see section III B1ai).

Computer-assisted analysis of nontransformed data represents a powerful means of differentiating multiple classes of binding site (126, 233, 398, 430, 431). This method has been used by a number of investigators to differentiate opioid receptor subtypes in brain and peripheral tissues (376, 388, 475). As has been discussed in section III B1aii, the accuracy of computer-generated parameter estimates is limited by the quality of the experimental data. In order to minimize experimental error, it is therefore necessary to perform replicate determinations at each of a large number of concentrations of competing drug.

It is important to note that, although shallow dose-response curves may indicate the presence of multiple classes of opioid receptor, there are other possible explanations of such data (24, 77, 127, 417, 495). Low Hill coefficients may indicate the existence of interconverting high and low affinity forms of the same binding site. A number of ions and nucleotides have been reported to act as allosteric effectors at opioid binding sites (40–42, 66, 67, 70, 89, 90, 158, 457, 472, 473, 485, 504, 612, 615, 640). Na^+ and GTP stabilize agonist binding sites in a low affinity state, while divalent cations such as Mn^{2+} and Mg^{2+} stabilize an agonist high-affinity state (24, 77). Thus, the complexity of analysis of receptor binding data is increased if assays are performed under conditions which permit the coexistence of both high and low affinity forms of *multiple* classes of receptor. An alternative explanation of shallow inhibition curves, with a low Hill

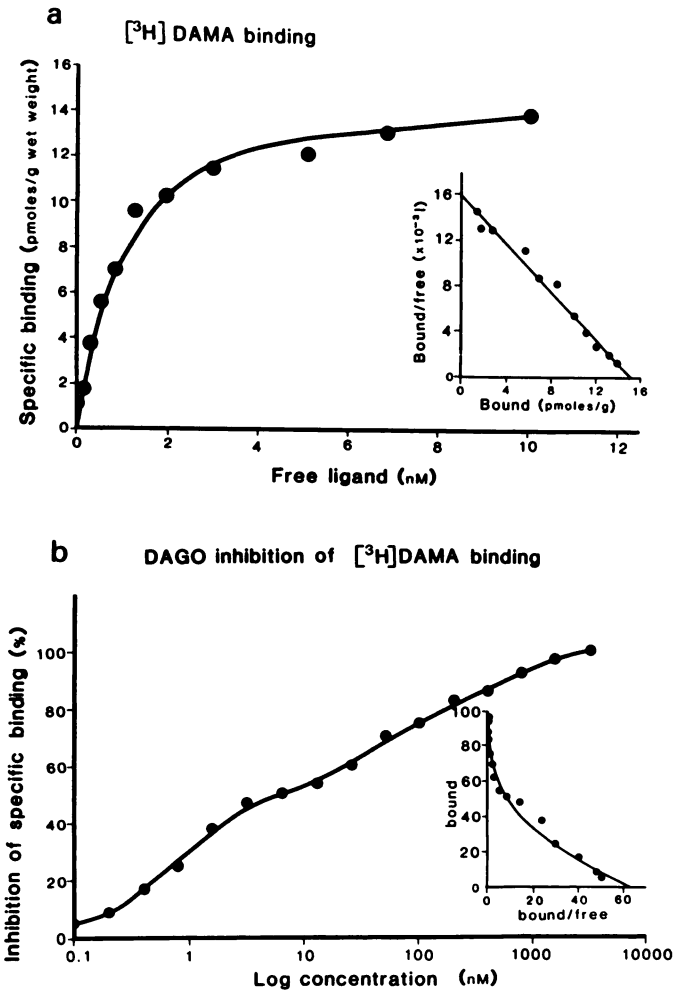

FIG. 6. Analysis of tritiated [D-Ala²]Met-enkephalinamide (*DAMA*) binding to μ and δ sites in guinea-pig brain membranes. *a*, saturation analysis of specific [³H]DAMA binding. Nonlinear regression analysis of the saturation curve indicates radioligand binding to an apparently homogeneous population of sites: K_D = 0.92 nM, R_t = 15.2 pmol/g tissue. *Inset*, Scatchard plot of the same data. *b*, inhibition of [³H]DAMA binding by the μ-selective agonist, *DAGO*. Nonlinear regression analysis of the DAGO inhibition curve indicates a nonhomogeneous population of sites: site 1 (μ), IC_{50} = 0.75 nM, 55%; site 2 (δ), IC_{50} = 110 nM, 45%. *Inset*, Hofstee plot of the same data.

coefficient, is negative cooperativity between binding sites. Appropriate experiments for demonstrating the existence of cooperativity have been described elsewhere (127, 495).

iii. Receptor selectivity. Multiple classes of opioid receptor have been distinguished on the basis of their differential binding properties in vitro (72, 208, 362, 373, 462). The relative affinities of a given drug at each opioid receptor type may be easily characterized as the ratio of K_i values for inhibition of "prototypic" μ, δ, and K ligands. In this way, a receptor selectivity profile may be generated for each competing ligand (100, 101, 194, 306, 338, 373, 379, 462, 624). The success of this approach is dependent on the selectivity of the "prototypic" radioligands which are used. For accurate characterization of the selectivity profile of competing ligands, it is necessary to use radioligands which have a much higher affinity for the preferred site than for the next preferred site (at least 100-fold, preferably 1000-fold). The binding isotherms for the "prototypic" radioligands should fit a single-site model, although such a fit is not, by itself, sufficient evidence for binding site homogeneity (see section III B1a). More critically, a wide range of competing ligands, with selectivities for sites other than that preferred by the radioligand, should yield inhibition curves with Hill slopes of unity.

In practice, many "prototypic" radioligands do not fulfill these selectivity criteria. Opioids like naloxone, [D-Ala^2,-D-Leu^5] enkephalin (DADLE), and EKC have been used extensively as prototypic ligands for μ, δ, and K receptors, respectively, even though these ligands exhibit less than 10-fold selectivity for their preferred sites (207, 208; see table 5). It is possible to direct the binding of a poorly selective radioligand to its preferred site by "blocking" the other sites with competing or noncompeting opioid ligands (208). In one approach, the radioligand is incubated in the presence of high concentrations of selective, competing ligands, which occupy the nonpreferred sites (194, 379). In another approach, the receptor preparation is exposed to an alkylating agent in the presence of a ligand which selectively protects one binding site while others are inactivated. Following washout of the alkylating agent, the resulting preparation is selectively enriched in the protected site (304, 306).

Although each of these approaches has been used successfully, they are critically dependent on the selectivity of the "blocking" or "protecting" drugs. It is therefore clearly preferable to use highly selective radioligands, if these are available commerically. To date, no receptor-selective antagonists are available in radioactive form. A number of selective agonists are currently available, however. Tritiated [D-Ala^2, Me Phe^4, Gly-ol^5] enkephalin (DAGO) fulfills the criterion as a prototypic ligand for the μ receptor (194, 208, 234), while tritiated [D-Pen^2, D-Pen^5] enkephalin (DPDPE) selectively labels δ sites (104, 125, 225). U69,593, a highly selective K receptor agonist (350), has recently become commercially available in radiolabeled form and may serve as a selective radiolabel for K sites. ^{125}I-[D-Pro^{10}] dynorphin(1–11) also exhibits a high degree of selectivity for the K receptor and has recently been used as a radiolabeled probe for this receptor type (186).

2. Control of experimental conditions. The preceding theoretical analysis of drug-receptor binding is predicated on a number of assumptions. It is assumed that the binding reaction is a simple, bimolecular reaction and that measurements of binding, other than kinetic measurements, are made at equilibrium. In view of these assumptions, a list of optimal experimental conditions, analogous to that defined by Furchgott for bioassay (172), may be derived. (*a*) Radioligand binding should be to a homogeneous population of binding sites. If binding is to multiple classes of sites, these should be noninteracting. (*b*) The altered binding of a radioligand in the presence of unlabeled drug should be due solely to competition for the same binding site. (*c*) Except for kinetic measurements, the binding of radioligand and competing drugs should be measured at equilibrium. (*d*) In the case of either radioligand or competing drug, the free concentration in external solution should be constant and should be known. (*e*) For comparison with pharmacological response, receptor binding should be determined under physiological conditions.

Although the measurement of pharmacological constants by radioligand binding assay is simpler than by bioassay, there are a number of methodological factors which should be taken into consideration in the design of experiments and interpretation of data. These include the following.

a. CHOICE OF RADIOLIGAND. In most tissues, the opioid receptor content represents a small fraction of total protein. In order to obtain a good signal/noise ratio (i.e., ratio of specific to nonspecific binding), it is therefore essential that the radiolabeled drug exhibit a high affinity and selectivity for opioid binding sites. In practical terms, this means that the radioligand should have a K_D of <10 nM. Since very small concentrations of radioligand are bound to the biologically relevant site, specific activity is also an important consideration; the higher the specific activity of the radioligand, the lower the minimum level of detection of the amount bound.

Relatively high specific activities can be obtained with tritium, making tritiated ligands extremely useful for receptor binding studies. Although higher specific activities can be obtained with ^{125}I, there are a number of limitations to the use of this isotope. The radiochemical half-life of ^{125}I is only 2 mo, as compared to 12 yr for tritium, necessitating the regular synthesis of new batches of radioligand. The substitution of bulky iodine atoms onto the NH_2-terminal tyrosine may also significantly alter the pharmacological properties of the radiolabeled drug (413). Careful pharmacological studies must therefore be undertaken to ensure the pharmacological identity of iodinated ligands.

It is important to ascertain the chemical structure of the radioligand and to ensure that it is homogeneous and pure. Radiochemical impurities may introduce a significant source of error into the analysis of binding data. Even when labeled ligands are prepared free of radioactive contaminants, ligands may undergo radiochemical decomposition upon storage (270, 599). The presence of a radioactive contaminant that is not bound can lead to errors in the determination of bound and unbound ligand and, consequently, to errors in estimation of the binding parameters (270, 429). Contaminants may also lead to distortions of Scatchard plots which may lead investigators to postulate unnecessarily complicated binding models (56, 429, 489). One way of assessing radiochemical purity is to vary the concentration of receptor protein at a fixed concentration of radioligand. If the radioligand contains a nonbinding contaminant, "saturating" receptor concentrations will not bind all of the radiolabel. This procedure may detect some types of impurity which may be missed by conventional chromatographic analyses (56, 270).

b. CHOICE OF INHIBITORS. Specific binding is normally defined as the difference in the amount of radioligand bound in the absence and presence of an excess of competing ligand (27, 60–62, 269, 610). Thus, the choice of inhibitor and the concentration in which it is used are critical determinants of binding. The inhibitor which is used to define "nonspecific" binding should have a high affinity for the radioligand binding site, and yet have a chemical structure which differs from that of the radioligand (610).

The competing ligand should be used in a concentration range which is sufficiently high to occupy all specific binding sites, yet which does not displace "nonspecific" binding. Too low a concentration of inhibitor will result in incomplete displacement of radioligand, particularly at higher levels of receptor occupancy. Under these conditions, saturation isotherms may yield convex Scatchard plots which bend in towards the abscissa. Such data may be mistakenly interpreted as evidence for positive cooperativity (155). Use of too high a concentration of inhibitor may result in displacement of "nonspecific" binding (60, 417, 610). Under such circumstances, saturation isotherms may yield biphasic Scatchard plots which may be mistakenly interpreted as evidence for negative cooperativity or binding site heterogeneity.

It is important to remember that alteration of incubation conditions by addition of ions and/or nucleotides may significantly alter the affinity of the competing ligand for the radioligand binding site (77). When altering binding conditions, it is therefore necessary to redetermine the optimal concentration of inhibitors for definition of blank. As has been shown by Fischel and Medzihradsky (155), failure to do so may result in serious methodological artifacts.

In those cases in which the radioligand binds specifically to more than one site, the choice of inhibitors to define "nonspecific" binding is particularly important. This will be discussed in greater detail in section III B2f below.

c. INCUBATION CONDITIONS. Opioid interactions at the receptor level are extremely complex. The binding characteristics of opioid receptors may be modified by a number of factors, including changes in temperature (119, 553), changes in pH (472), incubation with ions and nucleotides (24, 77), and tissue preincubation (70, 553). In view of the sensitivity of opioid receptors to modifying influences such as these, it is important to define incubation conditions exactly.

Under ideal circumstances, radioligand assays should be carried out under physiological conditions, so that receptor binding may be compared directly with pharmacological response. Thus, binding assays should be carried out using tissue preparation and buffer conditions identical to those for bioassay. While the use of physiological binding conditions is, in principle, an excellent approach, which has been used elegantly for analysis of opioid receptor properties in clonal cell lines (354), there are a number of relevant methodological considerations. First, as described by Motulsky et al. (425), there are a number of difficulties associated with the use of intact cells, rather than membrane preparations, for analysis of radioligand binding properties. In intact tissues, a number of factors may disrupt equilibrium binding and complicate the interpretation of data, including ligand internalization and receptor down-regulation. Although homogenization of the tissue may reduce such problems, this totally disrupts the normal cellular environment and exposes all parts of the receptor protein to ions and nucleotides within the bathing solution, eliminating the normal polarity. In the presence of physiological buffers, which contain high concentrations of Na^+ ions, opioid receptors undergo a conformational change such that the affinity of agonist binding is greatly reduced (24, 77, 472, 557). Thus, when a radioligand is an agonist, the fraction of total binding represented by specific binding may be much reduced in physiological buffer. Since most of the receptor-selective opioids which are currently available in radiolabeled form are agonists, binding assays have been routinely conducted in Tris-HCl buffer in the absence of added ions. Radiolabeled agonist binding is detectable in the presence of added ions, however (46, 65, 616).

Incubation temperature is another important variable which must be considered in the design of receptor binding experiments. Although it is preferable to carry out incubations at physiological temperatures, i.e., at 37°C, some ligands are unstable at this temperature (198, 356, 554), thus invalidating estimates of potency as measures of receptor properties. Significant losses of receptor may also occur after prolonged incubation at 37°C (270). For practical reasons, it is therefore more usual to conduct receptor binding experiments at lower incubation temperatures, such as 22 or 4°C. It should be noted,

however, that the binding properties of opioid receptors are markedly temperature dependent. At lower temperatures, the ligand-receptor association rate is decreased, such that longer time periods are required to reach equilibrium binding conditions (27, 554, 610). Furthermore, the pharmacological characteristics of opioid binding sites may be significantly altered by reduction of incubation temperature (119, 155).

It has been previously reported that preincubation of tissue samples at 37°C in the absence of added ions, or at 0°C in the presence of Na^+ and/or GTP, greatly increases the specific binding of radiolabeled opioids (553). This increase in binding appears to reflect an increase in B_{max} rather than K_D, and has been proposed to result from facilitated dissociation of endogenous ligand from receptors (553). Alternatively, the "unmasking" of opioid binding sites may reflect a conformational change in the receptor, such that low affinity sites are interconverted to a high affinity state (70).

d. SEPARATION OF "BOUND" FROM "FREE." An important step in any receptor binding assay is to terminate the reaction by separating the receptor bound ligand from that which is free in the incubation medium. The fundamental consideration for this procedure is the rate of dissociation of the ligand from the receptor. If the rate of dissociation of ligand from receptor is low, the tissue may be washed extensively with ligand-free medium without significant loss of specific binding. Since the rate of dissociation of ligand from nonspecific binding sites is usually high, such extensive washing will greatly increase the ratio of specific to nonspecific binding (27, 146). Alternatively, if the rate of ligand-receptor dissociation is high, extensive rinsing of the tissue following separation from the incubation buffer will produce a significant loss of specific binding.

In general, the radioligand dissociation rate is inversely proportional to K_D. Those ligands with a low affinity for the receptor will dissociate more rapidly than those with high affinity, making them less suitable candidates for separation procedures which involve extensive washes (27). The rate of radioligand dissociation is also a temperature-dependent process, such that ligands dissociate more rapidly at elevated temperatures (27). For this reason, it is common to cool samples on ice prior to separation of free ligand, even if the assay incubation temperature is 22 or 37°C. It should be recognized, however, that cooling the samples prior to filtering may alter receptor conformation, with a resulting shift in equilibrium to that characteristic of 4°C (119, 155). An alternative approach, which does not disrupt equilibrium binding, is to filter the samples without precooling and then rinse with ice-cold buffer.

e. FREE LIGAND CONCENTRATION. Although accurate estimation of free ligand concentration is essential for proper analysis and interpretation of ligand binding data, factors affecting this variable are frequently overlooked. Free ligand concentration is often determined by calculating the difference between total ligand concentration and the corresponding value for specific binding, rather than by direct measurement. Such estimations are invalid, however, if the ligand binds extensively to any component of the system, or if some active removal process exists.

Numerous factors may influence the concentration of free ligand in solution, including the following.

i. **Adsorptive losses.** When a radioligand is added to a complex biological sample, significant "nonspecific" binding to contaminating cellular components may occur and may markedly alter the concentration of free ligand at the receptor binding site (552). Such nonspecific binding may go undetected, however, since the ligand will dissociate rapidly from these low affinity sites during separation of bound from free. Nonspecific adsorption to container surfaces may also occur, further lowering the concentration of free ligand available for binding to the receptor (263, 287). For opioid peptides, such adsorptive losses are particularly significant at low concentrations of ligand and in the presence of ions (263). Reduction of free ligand concentration by adsorption has been shown to be a particularly significant problem for certain opioid peptides, such as dynorphin, and may lead to serious underestimations of absolute potency and of potency relative to other ligands (263). While adsorptive losses to vessel walls may be reduced by addition of proteins, such as bovine serum albumin (BSA) to the assay buffer, care should be taken to ensure that the ligand does not bind significantly to the added protein. Such "nonspecific" binding will markedly alter the concentration of free ligand available to bind at the receptor site and will complicate interpretation of data.

ii. **Enzymatic degradation.** Metabolic processes may provide another significant source of error in the estimation of free ligand concentration (363). Many opioid peptides are extremely susceptible to degradation by tissue peptidases (255–257, 381, 393, 545). The continual breakdown of active peptides into inactive fragments leads to nonequilibrium binding conditions, as well as to gross overestimates of the concentration of free ligand. A number of strategies may be adopted to decrease peptidase activity. These include the use of metabolic inhibitors in the assay buffer, lowering of the incubation temperature to 0°C, or the use of peptide analogs in which the critical bonds have been protected from enzymatic cleavage (198, 356, 363, 412, 470, 554, 594).

Although each of these approaches may provide significant protection from enzymatic attack, they each offer certain disadvantages. Since opioid peptides may act as substrates for numerous enzymes for differing specificities, it may be necessary to include several peptidase inhibitors in the incubation medium (28, 198). Such agents frequently have additional actions on the tissue which alter the binding properties of the receptor (363). The binding properties of opioid receptors may also be

significantly altered by lowering the assay incubation temperature to 0°C (119, 155). The benefits of decreased enzymatic activity may also be partially offset by the prolonged incubation periods which are required to reach equilibrium at this lower temperature (27, 554). "Stable" peptide analogs, in which novel amino acids are substituted at critical bonds, may have a significantly different pharmacological profile from that of the parent compound. Both affinity and receptor selectivity may be radically altered by such manipulations of the peptide structure (81, 461, 543, 626).

While the rapid enzymatic degradation of free ligand will usually result in significant underestimation of drug potency, this is not always the case. It has been shown that stabilization of dynorphin(1–13) in the radioreceptor assay does not markedly enhance its potency as an inhibitor of [^{3}H]naloxone binding (363). This finding may reflect a rapid interaction of the peptide with the receptor, where it is presumably protected from enzymatic attack.

iii. Receptor concentration. Even under ideal circumstances, in which adsorptive or metabolic losses of ligand do not occur, the concentration of free ligand is directly proportional to the concentration of receptors in the incubation medium. When the concentration of receptors is low in comparison to the equilibrium dissociation constant of the ligand, the effect of receptor binding on free ligand concentration is negligible, and the calculation of pharmacological constants is straightfoward. If the concentration of receptor is not 10 times lower than the true affinity constant, however, the apparent K_D obtained from direct concentration binding curves will increase as a linear function of receptor concentration (74, 122, 269). At high receptor concentrations, free ligand concentration may be so significantly reduced that saturability becomes difficult to demonstrate experimentally. In this case, binding data may yield sigmoid curves which may be mistakenly interpreted as indicating cooperative interactions (74).

When studying high affinity drug-receptor interactions, the ability to lower the concentration of receptors is limited by the specific activity of the radioligand. Thus it is difficult to achieve optimal experimental conditions for measurement of true binding constants. Under such circumstances, an accurate measure of true K_D may be obtained experimentally by determining the apparent K_D at various tissue dilutions. By plotting apparent K_D against the reciprocal of receptor concentration, the true value of K_D may be determined by extrapolation to the ordinate intercept (60).

f. MULTIPLE BINDING SITES. Another confounding variable in receptor binding studies is the interaction of radioligand with multiple classes of binding sites. This greatly increases the complexity of data analysis, particularly when there are allosteric interactions between the different classes of binding site. This is a particularly serious problem for pharmacological analysis of opioid receptors, given that multiple types of opioid receptor may exist within a single tissue, and the lack of specificity of many of the drugs which are currently available for experimental use.

As has been discussed in section III B1c, these difficulties may be circumvented in a number of ways. Radioligands may be used which have high selectivity for a given receptor type. Although receptor-selective antagonists are not yet available in radiolabeled form, a number of highly selective radiolabeled agonists are now, or will be soon, available for use. Alternatively, receptor properties may be examined in tissues which contain a homogeneous population of binding sites. A number of preparations with a predominance of one type of opioid receptor have recently been identified (76, 401, 478, 493). These include NG 108 cells (δ), human placenta (K), guinea-pig cerebellum (K), and rabbit cerebellum (μ).

If preparations with heterogeneous receptor populations are to be used, a number of methods may be used to examine the site of interest. When using a radioactive ligand which binds to multiple classes of sites, a competing drug with high selectivity for the site of interest may be used to define nonspecific binding. In this case, all other saturable sites will be treated as nonspecific binding. When using this approach, an important control is to test other, competing ligands for additivity with the blank. Lack of additivity suggests that the test drug does not compete for a second class of sites which have been defined as "nonspecific" (60).

Another common approach is to direct the binding of a nonselective radioligand to the site of interest (208). This may be achieved by including within the assay buffer high concentrations of competing ligands which are selective for the nonpreferred sites (194, 379). Alternatively, the tissue preparation may be enriched in the site of interest using selective protection techniques (304, 306). One obvious drawback of any of these approaches is that they depend on the selectivity of the ligands which are chosen as "blocking" or "protecting" agents. Although numerous drugs are receptor selective in low concentrations (see table 5), significant cross-reactivity may occur at the higher concentrations which are required to occupy 100% of that receptor type (208, 304, 306, 379).

C. Assay Methodologies

1. Membrane binding assay. The most common method for direct analysis of opioid-receptor interactions is the measurement of ligand binding to membrane preparations. Although studies have been largely limited to homogenates of brain tissue and neuroblastoma-glioma cell lines, a similar experimental approach may be used for analysis of the binding properties of opioid receptors in peripheral tissues (120, 362, 365, 640).

Membranes are prepared by homogenizing tissues in ice-cold buffer. Peripheral organs, which contain a high proportion of tough, connective tissue, should be minced

thoroughly prior to homogenization. After homogenization, tissue debris may be removed by low speed centrifugation (10 min, 1000 × *g*) or by filtration through 100-μm nylon mesh (336, 362). Homogenized membranes are then washed extensively by high-speed centrifugation and resuspension in fresh buffer in order to remove soluble proteins. A preincubation step may also be included to facilitate dissociation of endogenous ligands (553; see section III B2c).

Tissue aliquots are then incubated with radiolabeled opioids in the presence or absence of competing ligands. As described in detail in section II B2, particular attention should be paid to a number of factors to ensure that optimal binding conditions are achieved. These include receptor concentration, radioligand concentration, and incubation time. Special precautions should also be taken to reduce adsorptive and metabolic losses, particularly when opioid peptides are to be included in the assay. Adsorptive losses to test-tube walls may be reduced by carrying out reactions in plastic rather than glass, by siliconizing adsorptive surfaces, by addition of protein such as BSA to assay buffers, and by standardizing the order of addition of reactive components such that membranes are added before radioactive or competing drug (242, 263, 275, 287). Metabolic losses may be reduced by lowering the assay temperature, including metabolic inhibitors in the assay buffer, or by using stable peptide analogs (198, 363, 412, 470, 554; see section III B2e).

Following incubation, a number of different approaches may be used to separate tissue-bound ligand from that in the surrounding medium (146, 270). The simplest, and most commonly used, method is filtration under vacuum through glass-fiber filters. Tissue is then washed free of loosely bound radioactivity by rinsing the filters 2 to 3 times with 5 to 10 ml of ice-cold buffer. Provided that the dissociation rate of the ligand-receptor complex is low, this rinsing procedure will greatly reduce nonspecific binding without significant loss of specific binding, and will thus provide optimal signal/noise ratios (146, 270). In order to minimize nonspecific binding of radioligand to the fiber glass surface, filters may be pretreated with BSA/polylysine (0.4% BSA/0.01% polylysine), polyethyleneimine (0.1%), or *t*-amyl alcohol prior to tissue filtration. Such filter pretreatment has been shown to be essential for the successful separation of certain radiolabeled peptides (6, 275, 400).

An alternative method for assay termination is the centrifugation technique (146, 155, 242, 270). Following centrifugation, the supernatant is decanted, and the pellet is rinsed superficially with ice-cold buffer. The bottom of the tube containing the pellet is then severed with a scalpel, and the tissue is solubilized prior to determination of bound radioactivity. Since the membrane pellet is not thoroughly washed after centrifugation, a significant amount of radioactivity will remain trapped within the tissue pellet, resulting in a higher blank than is usually seen with filtration. The centrifugation technique does, however, offer some advantages over the filtration method. During centrifugation, the tissue is continually exposed to radioligand, and equilibrium is not disturbed. Thus, for rapidly dissociating ligands, there will be no significant loss of specific binding. Furthermore, by counting the radioactivity in aliquots of supernatant, "free" ligand concentration can be measured directly, rather than estimated by subtraction.

2. Solubilized receptors. In order to characterize the physicochemical properties of multiple opioid receptors, it is necessary to first solubilize and purify their component proteins. Despite considerable effort, progress towards this goal has been hampered by a number of methodological difficulties. Although a thorough analysis of this complex field is beyond the scope of the present review, I will outline some of the basic methodological approaches which are used. For further review of this topic, the reader is referred to the following additional texts (24, 270, 558, 590, 591, 639).

Two basic approaches have been used for solubilization and purification of opioid receptors. Membrane receptors are either prelabeled with radioligand prior to detergent solubilization, or are characterized by their binding activity following extraction.

a. LABELING PRIOR TO SOLUBILIZATION. Prelabeling of receptor binding sites with radioligand prior to detergent solubilization provides a useful means of identification of opioid receptors during subsequent stages of purification. By extracting membranes which have been preincubated with radioligand in the absence and presence of an excess of competing ligand, proteins with specific binding activity may be characterized. While reversibly bound radioligands have been used to label receptors throughout the extraction and purification process (232, 307, 308, 474, 483, 556, 641), this approach is subject to limitations. Under nonequilibrium binding conditions, and particularly in nonaqueous solvents, such reversible receptor-ligand complexes may dissociate, thus reducing chromatographic resolution. Such difficulties may be minimized by the use of high affinity ligands which dissociate slowly, such as [^{3}H]buprenorphine or [^{3}H]diprenorphine (307, 308, 474, 483). Alternatively, the problem may be circumvented by using bifunctional reagents to irreversibly cross-link the reversible ligand-receptor complex prior to detergent extraction (277, 641). This latter method has proven useful for analysis of the subunit structure of multiple opioid receptors (277), although the efficiency of receptor labeling by this technique is sometimes low (641). An alternative approach, which has recently been employed, is the use of radioligands which interact covalently with the receptor binding site (436, 560). Such irreversible ligands offer several advantages in that their labeling efficiency is high, and they remain covalently attached to the receptor binding site, even under denaturing conditions. They therefore provide a useful means of identification of the receptor-ligand complex throughout all stages of receptor purifi-

cation and characterization. One such affinity ligand, 3-[^{3}H]methylfentanylisothiocyanate (super-FIT), has recently been used for purification of the δ opioid receptor from NG108-15 neuroblastoma-glioma hybrid cells (560). Another, ^{3}H-labeled Tyr–D–Ala–Gly–Phe–Leu–CH_2Cl (DALECK), binds irreversibly to the μ opioid receptor and has been used as a label for purification of this binding site from rat brain membranes (436).

b. LABELING AFTER SOLUBILIZATION. While irreversible labeling of the ligand binding site prior to detergent extraction is an invaluable method for opioid receptor identification during purification and structural analysis, this approach does not permit investigation of the binding properties of the solubilized receptor. For molecular analysis of receptor binding mechanisms, the receptor protein must be extracted in its native, unlabeled form and its binding activity monitored in solution. Since disruption of the membrane environment may profoundly alter the binding properties of opioid receptors (458, 556), serious difficulties have been encountered in obtaining these receptors in solubilized form with good retention of binding activity.

i. Receptor solubilization. In 1975, Simon and coworkers (556) first reported that several types of detergent, including Triton X-100, lubrol, and deoxycholate, inhibited opioid receptor binding. Membranes solubilized with these detergents did not yield extracts which could bind opiates in solution. While similar observations have been made by other laboratories (136), slight modifications of the detergent extraction method have subsequently proven to be useful. It has been found, for example, that if the excess of detergent is removed prior to assay, either by dilution (278, 509, 561) or by adsorption to a hydrophobic matrix (31), opioid binding activity may be detected in solubilized membrane extracts. With the introduction of novel detergents such as the non-ionic, zwitterionic steroid, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), soluble opioid binding activity has also been extracted in moderately high yields (561).

Using digitonin, Ruegg et al. (509) were initially successful in solubilizing opioid receptors from amphibian, but not rodent, brain. In subsequent studies, however, it has been shown that digitonin or deoxycholate may also be used for extracting opioid binding sites from mammalian tissue in high yield, provided that the membranes are preincubated with Na^+ or Mg^{2+} prior to detergent exposure (129, 130, 201, 278, 559). Since the protective effects of preincubation are specific to these ions, it may be presumed that these induce a conformational change in the receptor to a state which is more resistant to inactivation. Such protection by allosteric effectors appears to exhibit some receptor subtype specificity, however, since K sites, unlike μ or δ, can be solubilized in good yield in the absence of added ions (294).

Although detergent extraction is the most widely used method for solubilizing membrane receptors, a number of alternative approaches have also been adopted. Loh and coworkers (92) have solubilized a fraction containing opioid binding activity by sonication treatment of rat brain membranes in the absence of added detergents. This "soluble" extract, which may consist of finely dispersed membrane fragments (278), has not been further characterized. However, sonication in combination with detergent extraction has yielded promising results (91). A novel method, using lysophosphatide and acyl-CoA as "natural" detergents, has also recently been used to solubilize opiate receptors from rat brain membranes (152). This technique is interesting in that it provides a basis for subsequent reconstitution of extracted opioid receptors into artificial membranes, by treatment with acetyltransferase. By altering the composition of the reconstituted membrane bilayer, the effect of membrane environment on opioid receptor binding characteristics may be examined in detail.

ii. Measurement of binding activity. For detailed characterization and purification of solubilized opioid receptors, it is essential to have a quick, efficient assay for monitoring binding activity in solution. The principles of radioligand binding to solubilized receptors are the same as those outlined above (see section III B). However, the assay of receptor binding in solution does present some special technical problems. As discussed previously, the presence of detergent, in the high concentrations required to solubilize membrane proteins, may interfere with the binding activity of the receptor. In order to optimize binding, it is therefore necessary to reduce detergent concentrations in the assay buffer by dilution or by adsorption (31, 278, 509, 561). Improvements in binding may also be obtained by reducing the assay incubation temperature to 0°C (278). The most important methodological issue in assaying the binding activity of solubilized receptors is, however, the rapid separation of "free" from "bound" ligand. As outlined below, a number of different methodological approaches have been used, each of which takes advantage of the physicochemical differences between free ligand and the soluble ligand-receptor complex.

α: Gel filtration. Separation of free from bound ligand in a solubilized receptor preparation may be achieved using gel filtration chromatography (285). This technique was that initially used for quantitative analysis of the binding activity of solubilized opioid receptors (556, 641). In this approach, differences in the molecular weights of the free ligand and the ligand-receptor complex provide the basis for their differential elution from Sephadex G-50 columns. Whereas the ligand-receptor complex is too large to be retained by the gel matrix and elutes in the void volume, the smaller free ligand molecules are adsorbed and elute in later fractions. Optimal "signal/noise" ratios are obtained when ligand-free buffer is used for elution of the column. Although the resulting disturbance of equilibrium binding conditions will promote dissociation of the ligand-receptor complex,

this may be negligible for high affinity binding sites at 0–4°C. Alternatively, equilibrium binding conditions may be maintained by inclusion of the radioligand in the elution buffer. This does result, however, in a large diminution in the detection sensitivity of the assay.

β. Equilibrium dialysis. The fundamental principle of equilibrium dialysis is that receptor protein included within a dialysis sac will bind significant quantities of radioligand, thus generating a ligand concentration gradient across the membrane. By measuring the difference in radioligand concentration inside and outside the dialysis tubing, a quantitative measurement of the binding activity of the soluble protein may be obtained. While very useful for analysis of the binding properties of low affinity sites, this method is extremely limited in its sensitivity of detection (270, 402, 420). The technique has therefore not been generally applied to the study of solubilized opioid receptors.

ψ. Precipitation of the ligand-receptor complex. As the molecular weight of a protein increases, its solubility generally declines. Selective precipitation of the ligand-receptor complex may therefore be used to assay the binding activity of soluble receptor proteins, provided that the complex is not significantly dissociated during the precipitation reaction. Polyethylene glycol and ammonium sulfate were both initially introduced as precipitating agents for separation of free from antibody-bound ligand in radioimmunoassay (78). These methods have subsequently been adopted for analysis of the binding activity of a number of solubilized receptor proteins, including opioid receptors (93, 129, 130, 294). The general principle of the approach is that the ligand-receptor complex is precipitated out of solution and then separated from free ligand by filtration or centrifugation. These precipitation and separation steps are generally carried out at 0–4°C, in order to minimize dissociation of free ligand. Optimal precipitation conditions must be carefully determined for each ligand and receptor studied (270). If the concentration of precipitating agent is too low, the receptor complex will be incompletely precipitated, while excessive concentrations of precipitating agents may produce significant precipitation of free ligand (270). A number of factors, including buffer composition and pH and the addition of carrier proteins, may influence the precipitation reaction. In particular, it should be noted that Tris-HCl buffer has been found to significantly impair the polyethylene glycol precipitation reaction (270).

δ. Adsorption of the ligand-receptor complex. Differences in the net ionic charge of unbound ligand and ligand-receptor complexes have also been exploited as a means of quantitating binding activity in soluble receptor assays. In one approach, samples are filtered over DEAE-cellulose disks (328, 532). Provided that the appropriate buffer conditions are used, such ion-exhange filters will selectively adsorb the ligand-receptor complex but not the unbound ligand. While this method has been used successfully for the analysis of soluble opioid receptor binding (93, 303), its application has been limited by factors such as expense. More recently, however, Bruns and coworkers (54) have demonstrated that soluble ligand-receptor complexes are also selectively retained by glass-fiber filters, which have been pretreated with the cationic polymer, polyethyleneimine. Retention of soluble receptors to polyethyleneimine-treated filters is stable to washing, insensitive to changes in ionic strength, and of relatively high capacity. This simple, rapid procedure has been shown to be applicable to the study of solubilized opioid receptors (54, 482, 559).

ε. Adsorption of free radioligand. Certain ligands, particularly aromatic molecules, are selectively adsorbed by materials such as activated charcoal. Thus, provided that equilibrium binding of the protein-ligand complex is not disturbed, charcoal adsorption may be used for separation of "bound" from "free" radioligand. This method has been used extensively in radioimmunoassay for removal of radioligand from the assay mixture with minimal dissociation of the ligand-antibody complex (192, 444). While it is also the most effective method of quantitating binding activity in certain soluble receptor assays (216), the charcoal adsorption technique has not been applied to the analysis of solubilized opioid receptors.

3. In Vitro Autoradiography. It is now widely recognized that a detailed understanding of the anatomical localization of radioligand binding sites may provide a framework for the formulation of hypotheses as to their possible functional roles (361, 598). Homogenate binding techniques, however, do not provide a sufficiently high degree of anatomical resolution to achieve this goal. With this approach, the characterization of receptor properties in small brain regions is limited by the requirement that milligram quantities of tissue must be used for each assay. Studies of individual brain nuclei are therefore impossible, unless tissues from several animals are pooled.

In vitro autoradiographic techniques have recently been developed, however, which do permit a detailed analysis of the properties of opioid binding sites in anatomically defined regions of brain and peripheral tissues (46, 142, 190, 214, 215, 231, 252, 325, 332, 380, 397, 598, 634). In this approach, slide-mounted tissue sections are incubated with radioligand, in the absence or presence of competing drugs, in a manner analogous to tissue homogenates. After incubation, sections are washed briefly with ice-cold buffer, then dried, and exposed to tritium-sensitive film for autoradiographic visualization of radioligand binding sites. Depending on the autoradiographic method used, the resulting autoradiograms may be analyzed qualitatively for high resolution, anatomical localization of binding sites. Alternatively, autoradiographic grain densities in discrete anatomical regions may be quantified using densitometric techniques. The methodological factors which must be considered for optimal qualitative and quantitative analysis of receptor

distribution have been outlined in a number of recent reviews (250, 251, 344, 345, 347, 364, 380, 434, 598). These are summarized in the following sections.

a. PREPARATIVE PROCEDURES. **i. Tissue preparation.** Preserved tissue morphology is an important consideration for the accurate anatomical localization of receptor binding sites. Although fixation by aldehyde perfusion improves tissue histology, this procedure may partially denature opioid receptors, significantly reducing specific binding or otherwise altering binding properties (367, 634). Young and Kuhar (634) have shown that, while fixation with very dilute solutions of formaldehyde does not significantly alter specific binding compared to unfixed tissues, fixation with 2% formaldehyde causes significant loss of binding. The binding of some ligands is more sensitive to the deleterious effects of tissue fixation than others, however. Whereas the binding of many opioid agonists is markedly reduced by prior fixation with 4% formaldehyde (367; F. Leslie, unpublished observations), there is only partial loss of [^{3}H] naloxone binding (252, 367, 368).

Herkenham and Pert (252) have outlined a detailed alternative protocol for maintaining tissue quality without prior aldehyde fixation. The method involves rapid freezing of unfixed tissue, followed by cryostat sectioning of slices. Sections are then melted onto gelatin-coated slides and dried at or below 0°C before freezing. Tissue prepared in this manner may be stored for prolonged periods at −20°C without significant deterioration. Such sections have a dry, glassy appearance and remain securely attached to slides, even during prolonged incubations at physiological temperatures or in nonisotonic solutions (252; F. Leslie, unpublished observations).

ii. Incubation. In order to obtain a clear autoradiographic image, it is important to optimize binding conditions such that a high ratio of specific to nonspecific binding is attained. In practice, at least 50% specific binding is required for adequate receptor visualization (434). Initial preincubation of sections in ligand-free buffer may be used to dissociate endogenous ligand and to stabilize binding sites in a high affinity conformation (46, 70, 252, 554). Subsequent incubation with radioligand should be in a neutral, buffered solution. Tissue quality may be preserved, particularly at higher temperatures, by incubation in isotonic buffers and by inclusion of protease inhibitors and/or bovine serum albumin (250, 252).

Radioligands should be chosen which have high affinity ($K_D \leq 10^{-9}$ M) and selectivity for the receptors of interest. [If sufficiently selective radioligands are not available for use, selective "blockers" of the nonpreferred sites should be included within the assay buffers (see section III B2f).] Low concentrations (K_D or below) of radioligand are generally used, since these yield the highest ratio of specific to nonspecific binding (see fig. 8). Incubation times and temperatures are chosen empirically, such that equilibrium is achieved and specific binding maximized, while maintaining a good signal/noise ratio.

Following incubation with radioligand, sections are washed in ice-cold buffer to remove nonspecific binding. For high affinity ligands with slow dissociation rates, successive immersions in cold, nonradioactive buffers will greatly improve the ratio of specific to nonspecific binding. For those ligands with faster dissociation rates, the immersions must be done rapidly in order to prevent significant loss of specific binding.

Following termination of the binding procedure, tissues must be dried as quickly and completely as possible, in order to minimize diffusion of reversibly bound ligand. Tissue sections should be dried initially with an ambient temperature airstream from a blow dryer. The drying process may then be completed by storage with desiccant overnight. During prolonged autoradiographic exposure periods, desiccant should also be included within the cassettes to keep the tissue sections dry.

iii. Autoradiographic techniques. A number of approaches may be used for the autoradiographic visualization of radioligand binding sites. The simplest method is to appose the slide-mounted sections to tritium-sensitive film (LKB Ultrofilm [^{3}H] or Amersham) in standard X-ray cassettes. The cassettes are kept at room temperature, or below, for an appropriate exposure period prior to development of the film. The developed film may then be viewed directly or analyzed by densitometry (11, 252, 466). The advantages of the LKB film method are its simplicity and the ease of densitometric grain quantitation (see below). The disadvantage of this approach is that it provides a lower degree of anatomical resolution than other autoradiographic techniques, and it does not permit detailed analysis of the cellular localization of autoradiographic grains.

An alternative method for receptor visualization, which permits a higher degree of anatomical resolution, is the emulsion-coated coverslip technique (634). Following incubation and drying, tissue sections are apposed to coverslips which have been previously coated with photographic emulsion. Slides and coverslips are attached with a drop of glue and held together with binder clips. After exposure, the coverslips are gently bent away from the tissue sections, the latent image on the coverslip is developed, and the underlying tissue is treated with a standard histological stain. The coverslips are then permanently reattached to the slide with Permount. Using this technique, the autoradiographic grain distribution of reversibly bound ligands may be compared directly with the underlying tissue morphology.

An alternative approach, which permits an even higher degree of anatomical resolution, is the classical "wet" emulsion technique, in which tissue sections are defatted and coated directly with photographic emulsion (250, 252, 397). Since the photographic emulsion is in direct

apposition to the radiolabeled tissue section, the density of autoradiographic labeling cannot be altered by gaps in apposition or differential pressure, as is the case with film or predried emulsion methods (498). Problems of alignment between tissue morphology and autoradiographic grain distribution are also avoided. The limitation of this methodology, however, is that it is only useful for the visualization of those ligands which are covalently linked to their receptors. Those ligands which are reversibly bound will diffuse from their binding sites during the defatting and emulsion dipping procedures.

Reversible ligand-receptor complexes have been covalently cross-linked by a number of methods, including immersion fixation in glutaraldehyde (142), exposure to UV light (142), or exposure to hot paraformaldehyde vapor (252). These techniques permit only qualitative analysis of receptor labeling, however, since in no case has 100% retention of specific binding been achieved. These cross-linking methodologies may also be more successfully applied to some radioligands than to others; whereas paraformaldehyde vapor exposure produces significant covalent linkage of [^{3}H]naloxone to μ-opioid receptors, it does not significantly cross-link the μ-opioid agonist, [^{3}H]dihydromorphine (346). Before routinely using any procedure to covalently link drug-receptor complexes, it is important to ascertain that this procedure does not induce any selective loss or redistribution of radiolabel. This can be achieved by detailed densitometric comparison of film autoradiographs generated prior to, and following, ligand fixation and defatting steps (252).

b. QUALITATIVE ANALYSIS. Although qualitative analysis, at the light microscopic level, provides a good first approximation as to the regional distribution of radioligand binding sites, several factors must be considered which may bias interpretation of the data. When analyzing autoradiograms generated on both X-ray and emulsion films, it is important to remember that the function relating autoradiographic grain density and the radioactive content of the underlying tissue is nonlinear (11, 347; see fig. 7). In general, grain density does not increase as rapidly as does tissue radioactivity. Thus, qualitative analysis of the distribution of autoradiographic grains within a single tissue section may provide a misleading impression as to the relative densities of ligand binding sites in different anatomical regions (see fig. 8).

Problems of interpretation are compounded when the relative distributions of two different radioligand binding sites are to be compared. A number of factors (including the specific activity of the ligand, the degree of receptor occupancy, and autoradiographic exposure time) may influence the degree of radioactive exposure to the photographic emulsion and thus influence the resulting autoradiographic image. If, in comparisons of different radioligands, these factors are not kept constant, qualitatively different autoradiographic patterns may emerge, even if common populations of sites are labeled (48).

Another technical limitation which may influence data interpretation is the problem of tissue absorption (or quenching) of the low energy β-rays emitted by tritiated

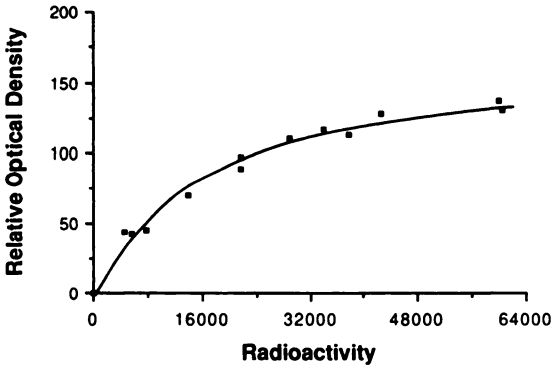

FIG. 7. Relationship between autoradiographic grain density and underlying radioactivity. Data represent computer-derived absorbance values generated on ^{3}H-labeled Ultrofilm by standards of known radioactivity. *Ordinate*, grey value; *abscissa*, cpm × days of exposure.

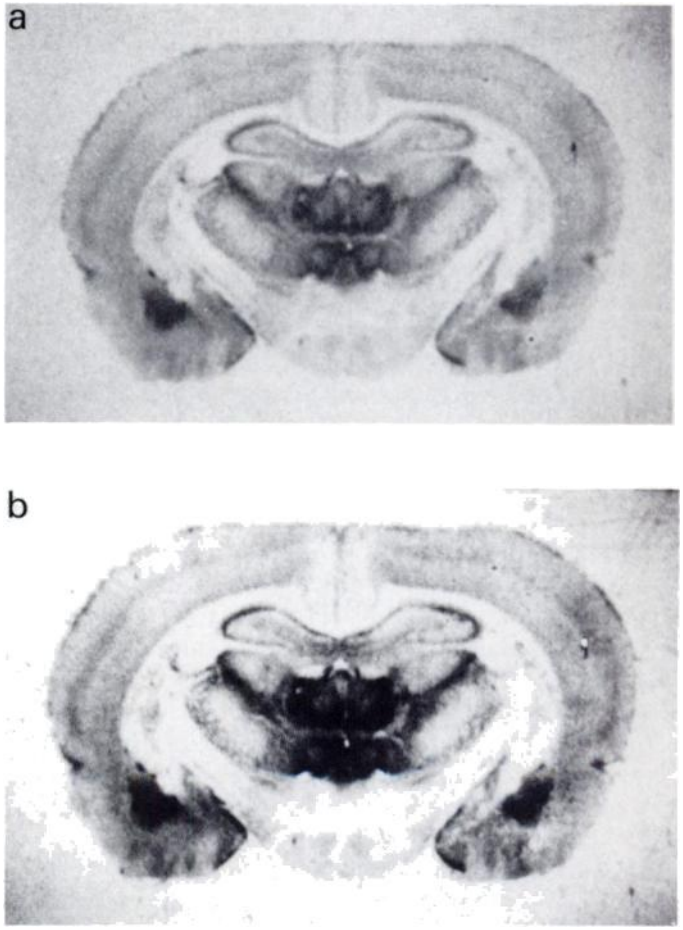

FIG. 8. Autoradiographic distribution of [^{3}H]DAGO binding to μ receptors in a coronal section through rat brain. *a*, nonlinearized autoradiographic image. *b*, computer-derived image of the same autoradiogram following correction for the nonlinear relationship between grain density and underlying radioactivity.

ligands. The amount of energy which reaches the overlying emulsion has been shown to be inversely related to the density of the material from which the β-particle is emitted (347). Since lipid-rich, white matter is relatively denser than grey matter, it will absorb a correspondingly greater amount of the emitted β-energy. This differential regional quenching results in autoradiographic images in which the grain densities overlying areas rich in white matter are artifactually low (191, 252, 253, 347). Since grey matter and white matter are mixed in varying proportions in different brain regions, this differential grey/white matter quenching represents a particularly serious problem for interpretation of autoradiographic data.

Since differential tissue absorption does not occur with higher energy isotopes, such as ^{125}I (347), iodinated peptides may be used in preference to tritiated ligands for receptor labeling. An additional advantage to the use of ^{125}I-labeled drugs is that shorter autoradiographic exposure times are required [in the order of hours rather than weeks (347)]. These advantages are partially offset, however, by a slight decrease in the resulting anatomical resolution of autoradiographic grain distribution (347). Additional limitations to the use of iodinated ligands have been discussed elsewhere (347; see section III B2a). Since radioligands are most readily available in tritiated form, alternative approaches to the problem of tissue quenching have also been explored (191, 253, 347). One effective approach has been to defat labeled, slide-mounted tissues by immersion in aqueous alcohol and xylene or chloroform (252, 387). Although removal of tissue lipids has been shown to eliminate, or greatly reduce, differential grey/white matter quenching (252, 253), this procedure can be used only for those ligands which are covalently linked to the receptor. As discussed above, efforts to cross-link reversible ligand-receptor complexes have, to date, met with only limited success (346). In view of the 20 to 80% loss of radioligand binding associated with defatting of paraformaldehyde vapor-fixed tissue, this technique is not suitable for quantitative autoradiographic analysis of receptor properties (see below). This method may be useful, however, for qualitative analysis of binding site distribution, provided that tne loss of label is demonstrated to be uniform across brain regions.

c. QUANTITATIVE ANALYSIS. Quantitative analysis of autoradiograms may be achieved by comparing the density of autoradiographic grains generated by labeled tissue sections with that generated by standards of known radioactivity (11, 190, 212, 466, 487, 585). The concentration of radioligand bound per unit tissue is then calculated. By measuring bound radioactivity in alternate sections which have been incubated in the absence and presence of an excess of competing ligand, specific receptor labeling may be quantified (1, 190, 332, 424; see fig. 9).

Although quantitative analysis of individual densities in photographic emulsion is possible (424), the counting of individual autoradiographic grains is a time-consuming process. For quantitative studies, tritium-sensitive film offers major advantages in reproducibility and ease of densitometric analysis (190, 332). Using this approach, tissue sections are mounted in an X-ray cassette in apposition to a sheet of tritium-sensitive film. Standards of known radioactivity, prepared by the individual investigator or obtained commercially, are included within each cassette (189). [If plastic standards are to be used, these must be precalibrated to correct for differences in self-absorption between plastic and tissue (see section III C3b; 189, 190, 347).] Following an appropriate exposure period, films are developed and analyzed.

Techniques for quantitative analysis of the exposed film range from simple measurement of absorbance to more elaborate, computer-assisted image processing (11, 212, 466, 487, 585). In a simple system described by Penney et al. (466), the autoradiographic image is projected in a photographic enlarger. Absorbance measurements are made by a photosensitive diode placed at the center of the enlarger's image plane. Tissue radioactivity is then calculated by interpolation from a standard curve generated on the same sheet of film. A more sophisticated means of data analysis, which is becoming increasingly available for routine use, is computer-assisted image processing (11, 212, 585). In this approach, the illuminated image of each autoradiogram is photographed by a video camera. The video signal is fed into an image array processor, which converts analog to digital display. Computer processing corrects for shading distortions of the video image and for the nonlinear relationship between tissue radioactivity and autoradiographic grain density. The resulting, "linearized" image represents an accurate reflection of the concentration of bound radioligand in different anatomical regions (see fig. 8). Using digital subtraction, the image of a section incubated with radioligand plus a competitive inhibitor may be subtracted from a superimposed image of total radioligand binding in an adjacent section, to produce a quantitative difference image that represents the binding displaced by the competitor (10, 214). Specific binding values may be automatically color coded and displayed as a computer-derived image. Alternatively, they may be determined for individual brain regions under the manual control of the operator.

Since data are calculated as concentration of radioligand bound per unit tissue, quantitative analysis of autoradiograms corrects for experimental variables such as specific activity and autoradiographic exposure time. Thus, autoradiographic data from different experiments and for different radioligands may be compared, provided that the percentage of receptor occupancy is equivalent in all cases (i.e., that radioligand concentration is a fixed ratio of K_D). Within a single experiment, receptor density and affinity (B_{max} and K_D) within specified anatomical regions may be calculated by incubating consecutive sections in the presence of increasing concentrations of

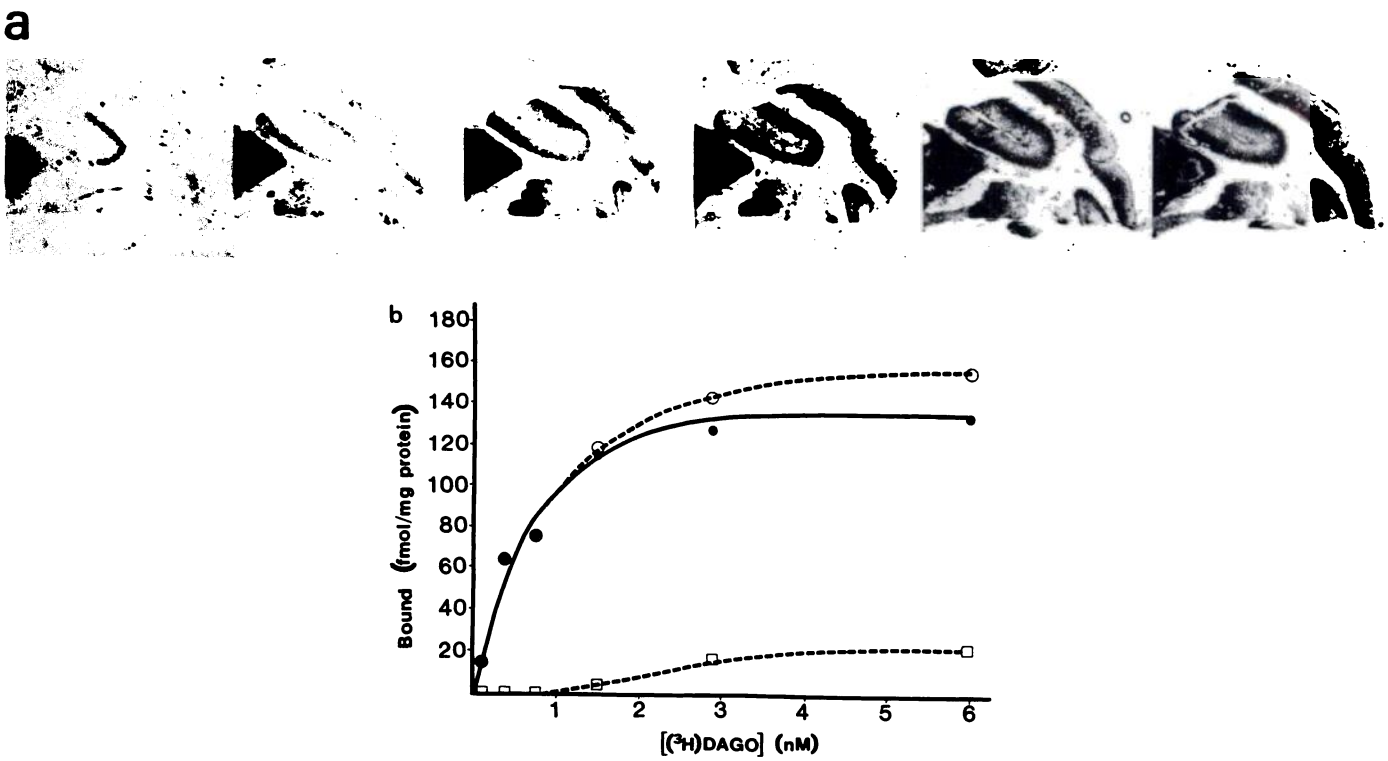

FIG. 9. Quantitative analysis of [^{3}H]DAGO binding to μ opioid receptors in layer VI of monkey temporal cortex. *a*, computer-derived linearized images of [^{3}H]DAGO binding at increasing concentrations of radioligand (0.1 to 6 nM). *Left*, cursor boxes used for quantitative analysis of radioligand binding. *b*, radioligand binding values derived by quantitative autoradiographic analysis. Specific binding (●) was defined as the difference in binding in the absence (○) and presence (□) of an excess of competing ligand (levallorphan, 1 μM). Nonlinear least-squares analysis of the saturation curve for [^{3}H]DAGO specific binding (●) yielded the binding parameters, K_D = 0.83 nM and R_t = 165 fmol/mg protein. (Courtesy of Diana E. Hurlbut.)

radioligand (1, 190). If tritiated ligands are used, calculated B_{max} values may represent an underestimate of the true receptor density because of differential quenching of the emitted radioactivity in different brain regions (see section III C3b; 191, 253). This inaccuracy may be eliminated by the use of iodinated ligands, which have a higher energy of emission. Alternatively, tritium quench correction factors may be applied, which reflect the differing energy-absorptive properties of each brain area (191, 253, 347).

Quantitative autoradiography of tissue sections labeled in vitro represents a powerful tool for analysis of receptor properties in defined anatomical regions, which may not be amenable to investigation using standard membrane binding techniques. By incubating alternate sections under different conditions, structure-activity relationships for displacement of radioligand binding by competitive inhibitors may be determined (10, 48). Possible allosteric interactions of ions and nucleotides may also be examined. Rigorous quantitative analysis of anatomically defined binding sites may thus provide an additional means of resolving the pharmacological complexities of a heterogeneous population of opioid binding sites.

d. ULTRASTRUCTURAL LOCALIZATION. Whereas the anatomical distribution of opioid receptors has been studied extensively at the light microscopic level (20, 190, 253, 397, 424, 589), little is known about the ultrastructural localization of these sites. Recently, however, electron microscopy (EM) has been used for higher resolution analysis of opioid binding site localization (231). Although a powerful technique, EM autoradiography of diffusible radioligands is subject to a number of limitations. Since tissue fixation, prior to and following the radioligand binding step, is an essential component of this type of analysis, possible fixation artifacts must be carefully considered and controlled for. Furthermore, since an autoradiographic grain may not directly overlie the point source of radioactive emission, it is necessary to use statistical analysis to calculate the probability of binding site distribution (23).

In view of the inherent limitations of EM autoradiography, this technique must be used in combination with other approaches to define the cellular localization of receptor sites. With the development of antibodies directed against opioid receptor epitopes (32, 437, 536), it may soon be possible to use immunohistochemical methods to define the ultrastructural localization of opioid binding sites. While this technique permits a higher degree of anatomical resolution than does autoradiography and has been used successfully for the localization of other receptor types (533, 569), it is critically dependent on the selectivity of antibody probes. Thus, as with

radioligand binding, it is essential to demonstrate the chemical specificity of antibody labeling.

An alternative approach, which is currently being used to define the cellular localization of opioid receptors, is to lesion afferent inputs into a specific brain region and to examine resultant changes in receptor density using quantitative autoradiography (1). A decline in receptor number following lesioning of input pathways may be interpreted as evidence for a presynaptic localization of receptors on afferent terminals. While such data are highly suggestive, they should be viewed with caution. Appropriate controls are necessary to ensure that diminished receptor densities do not reflect nonspecific transsection of fibers of passage (523) or transneuronal degeneration (237). Conversely, false negatives may occur when receptors are localized on both pre- and postsynaptic elements, with up-regulation of the deafferented postsynaptic receptor population masking a concomitant decline in presynaptic receptor number (382).

4. Receptor labeling in vivo. a. METHODOLOGICAL CONSIDERATIONS. Opioid receptors may be labeled in vivo by administration of tracer amounts of radiolabeled opioid ligand into the systemic circulation, or into the cerebral ventricle, of an intact animal (19, 143, 267, 272, 434, 453, 467–469, 500, 501, 516, 598). An important prerequisite for such studies is that the radioligand is of high specific activity and high affinity. Under such circumstances, a high ratio of specific to nonspecific binding may be achieved (165). The availability of high affinity radioligands, such as diprenorphine, has thus facilitated the analysis of opioid receptor occupation in vivo using autoradiographic and biochemical techniques.

The major advantage of in vivo labeling is that the tissue is physiologically intact, avoiding artifacts due to disruption of the normal receptor microenvironment. Analysis of radioligand binding in vivo allows direct correlations to be made between receptor occupation and pharmacological or physiological response (143, 272, 453, 467, 501, 546) and is an approach which is fundamental to the visualization of opioid receptors in the living animal by positron emission tomography (166, 468). It may also be useful for visual analysis of receptor occupation by endogenous opioid ligands (453). In vivo labeling of receptors does suffer, however, from several major disadvantages. As described in detail in section II B, numerous pharmacokinetic factors influence the disposition of radioligand in vivo and prevent the establishment of equilibrium binding conditions. Thus, measurement of pharmacological constants are only semiquantitative. Ratios of specific to nonspecific binding are also generally lower than those obtained in vitro, and indirect methods must be used to determine levels of nonspecific binding (see below). The cost of the in vivo approach is another disadvantage, in that each data point must be measured in a separate animal. Since drug is distributed throughout the entire body volume, large amounts of costly radioligand must be administered to each animal for specific binding to be detectable. Finally, the choice of suitable radioligand is restricted to those which are metabolically stable and which can, preferably, cross the blood-brain barrier.

A number of factors must be considered in the design of in vivo receptor labeling experiments. The first consideration is choice of radioactive tracer. As has been discussed already, the radioligand should be of high affinity and specific activity and should be metabolically stable. Since radiolabeled antagonists dissociate more slowly from the receptor than do agonists, these are generally more useful for in vivo labeling (345, 348, 598). Although it is not essential for the radioligand to be capable of crossing the blood-brain barrier, this is highly preferable. The blood-brain barrier may be bypassed by intraventricular administration, but the resulting tissue distribution is generally less even than when radioligand is administered via the peripheral circulation (434). Another important variable is the interval between administration of drug and sacrifice. The time of peak accumulation of radioligand within the brain is dependent upon both the pharmacokinetic properties of the individual drug and the route of administration (see section II B). Animals may be sacrificed when the concentration of radioligand within the brain has reached a maximum. Since radioligand clearance rates are higher in brain regions in which receptor density is low (166), longer survival times can also be used in order to improve the ratio of specific to nonspecific binding.

Several different approaches may be used to differentiate specific from nonspecific binding. Structures within the brain which contain few opioid receptors (such as cerebellum, in rat) may be used as an internal standard for nonspecific binding. Thus, the brain/cerebellum ratio is used as a measure of receptor occupation (272). Tissue blanks may also be generated in separate animals by administering an excess of unlabeled opioid drug before the administration of radioligand (598). Using a rapid filtration technique, Perry et al. (467) have separated membrane-bound radioactivity from that in the aqueous fraction and have determined that, for [^{3}H]etorphine, >95% of the membrane-bound material represents specifically bound drug.

The pharmacological properties of the specifically labeled sites may be determined by coadministering increasing doses of competing ligands with the radioactive tracer (166, 272). Caution should be exercised in interpreting such data, however, since differences in the pharmacokinetic characteristics of tracer and displacer substances may provide misleading conclusions (272). Particular care should be taken when agonists are used as displacing drugs, since these may induce regional changes in cerebral blood flow which can confound data interpretation (39, 55). In order to circumvent such pharmacokinetic problems, Richards and Sadée (491) have recently developed an ex vivo approach in which unlabeled drug is administered to the intact animal prior to sacrifice.

Tissues are then homogenized and incubated with radiolabel.

b. RECEPTOR VISUALIZATION. The earliest autoradiographic maps of opioid receptor distribution were generated following in vivo labeling of receptors by systemically administered radioligand, with subsequent tissue processing in vitro. The fundamentals of this autoradiographic approach are similar to those outlined for in vitro autoradiography (see section III C3), except that prelabeled tissue slices are dried immediately following cryostat sectioning and mounted in the dark onto emulsion-coated slides. Following an appropriate exposure period, the emulsion is developed and the data analyzed by light microscopy (see refs. 344, 434, and 598 for review). While this in vivo method has provided detailed information as to patterns of opioid receptor distribution (20), it is subject to a number of limitations as discussed above and has been largely superceded by in vitro autoradiographic techniques as a means of analyzing the pharmacological properties of receptors within discrete brain regions.

One limitation of the in vivo approach is that interpretation of autoradiographic data is complicated by competitive interactions between the exogenously administered radiotracer and endogenous opioid peptides. Thus, radioligand binding to receptors is inversely correlated with that of endogenous competitors. This competitive relationship between exogenous and endogenous ligands has recently been exploited as a means of detecting local release of endogenous opioid peptides in response to various physiological stimuli (453, 546). Given the many caveats associated with the in vivo autoradiographic approach, however, one must be cautious in interpreting decreases in binding of exogenously administered radioligand as reflecting receptor occupancy by endogenous opioid ligands (453). Validation of this as a measure of receptor occupancy does require independent confirmation using other neurochemical methods for monitoring local neuropeptide release.

In vivo labeling techniques have most recently been applied to the noninvasive imaging of receptors in the living animal using positron emission tomography (PET) (166, 468, 486, 622). In this approach, drugs labeled with short half-life positron emitters, such as ^{18}F, are administered by i.v. injection and monitored externally by an array of signal detectors. Radioligand binding to brain (or other tissues) is then determined by computerized reconstruction of the resulting autoradiographic images from selected tomographic slices. While this methodology has obvious clinical applications, it is presently limited by the degree of autoradiographic resolution which may be achieved, as well as by the complexities of quantitative analysis of radioligand binding in vivo. Detailed mathematical models are currently being developed, however, which describe the complete pharmacokinetic profiles of injected drugs (164, 165, 622), and which should permit a more quantitative assessment of ligand-receptor interactions in discrete brain regions. Although PET technology has been applied successfully for imaging of opioid receptor distribution in vivo (166, 468), the radioligands which are in current use predominantly label μ receptors. PET imaging of δ and K receptor distributions will require the development of antagonists selective for these receptor types which can cross the blood-brain barrier.

IV. Conclusion

As should be evident from the preceding discussion, rigorous analysis of the pharmacological properties of opioid receptors is complex. The design and interpretation of experiments is complicated by a number of factors, including the existence of multiple opioid receptors and the use of drugs which do not exhibit absolute pharmacological selectivity for one receptor type. Problems of interpretation are further compounded by factors, such as peptide degradation, which preclude a steady-state interaction between ligand and receptor. Thus, without a fundamental understanding of both the theoretical and methodological principles underlying each experimental approach, data may be misinterpreted, and unnecessarily complex theoretical models may be developed. Since data from any single experimental system may be biased by a number of artifacts, it is essential that conclusions be based on converging lines of evidence derived from multiple systems. The use of parallel assays is particularly important in the characterization of the receptor selectivity profiles of test drugs and in the classification of novel receptor types.

While considerable progress has been made over the last two decades in the characterization of opioid receptors, there is much yet to be discovered. Within the next decade, the methodologies which have been discussed in the present review will be used to examine the fundamental properties of opioid receptors, as well as their functional interrelationships with endogenous opioid peptides. Novel approaches, such as the molecular biological techniques which have been applied to the study of other receptors (415, 440), will also be applied to the study of opioids. Through the combined application of all of these techniques, both the structural properties of opioid receptors and the nature of their interactions with other membrane components should be better understood. Thus, the molecular basis of opioid receptor signal transduction will eventually be elucidated, as will mechanisms of receptor regulation, including tolerance and dependence.

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