# Partial Purification of an Opiate Receptor from Mouse Brain

Abstract. *A proteolipid isolated from a lipid extract of mouse brain demonstrates stereospecific binding properties for levorphanol. It is present only in neuronal tissue and most abundant in the rhombencephalon. One component saturates at a concentration corresponding to maximum pharmacologic effect in vivo. The estimated mass is 60,000 daltons per bound opiate molecule.*

To understand fully the mode of action of opiate narcotics and the mechanisms responsible for tolerance and dependence, it will be necessary to identify specific sites of action and to isolate and characterize the narcotic receptors. We published a general method for detecting stereospecific opiate receptors (*1*). Using mouse brain homogenates, we showed that stereospecific binding (SSB) was present, largely in a membrane fraction, but it constituted less than 2 percent of the total opiate binding. In these experiments we used levorphanol, a D(−) congener of morphine, because it has close structural similarity to morphine and the pharmacologic actions of the two drugs are virtually identical. Dextrorphan, the pharmacologically inert L(+) enantiomer, is also available (*2*).

Several investigators modified and improved upon this general method. Pert and Snyder (*3*) used naloxone of high specific radioactivity to detect a set of stereospecific binding sites in rat brain. These sites became saturated at a low naloxone concentration and had an uneven regional distribution in brain, corpus striatum being richest and cerebellum poorest. Simon *et al.* (*4*) demonstrated stereospecific binding of the highly potent opiate etorphine in rat brain using similar procedures; some differences from the findings of Pert and Snyder made it uncertain whether the same receptor population was under study. Further reports of stereospecific opiate binding in brain tissue included those of Terenius (*5*) and of Lee *et al.* (*6*).

We described several other properties of opiate receptors (*7*). The fraction demonstrating SSB capacity in brain homogenates could be extracted into chloroform methanol (C-M) (*8*). When this total lipid extract was fractionated on LH-20 Sephadex as described by Soto *et al.* (*9*), numerous fractions across the entire elution diagram showed levorphanol binding capacity. But most of this binding was nonspecific and nonsaturable in the concentration range examined. We now report the partial purification of a proteolipid fraction that binds opiate narcotics stereospecifically.

We measured SSB capacity in extracts by a modification of the method of Weber *et al.* (*10*). The distribution of [$^{14}$C]levorphanol (or [$^{3}$H]dextrorphan) between an aqueous buffer layer and an organic layer was determined in the absence and presence of tissue extract. The increase in the apparent partition coefficient (organic/aqueous) caused by the extract was the measure of binding. We found *n*-heptane to be a more suitable solvent than chloroform because the levorphanol (or dextrorphan) partition coefficient was low in the absence of extract. Binding capacity was measured at aqueous phase levorphanol concentrations from $10^{-7}$ to $10^{-6}M$, which correspond to concentrations in brain water at which the drug produces analgesia and locomotor activity in nontolerant mice (*11*).

Because it seemed likely that much of the nonspecific interaction in the total C-M extract was due to various lipids, an ether precipitation step was introduced. An excess of ether precipitates many proteolipids (*9*). Sixty percent of the protein (*12*) in the C-M extract and virtually all of the SSB capacity was present in the ether precipitate. When it was redissolved in C-M 2 : 1 and fractionated on LH-20 Sephadex, there was a single major peak of binding capacity for levorphanol at fraction 63, 24 to 36 ml after the first emergence of the C-M 1 : 1 eluate from the column (Fig. 1). This did not correspond to any of the three principal protein peaks. Sometimes, the binding was completely stereospecific. In other experiments, variable degrees of dextrorphan binding were observed

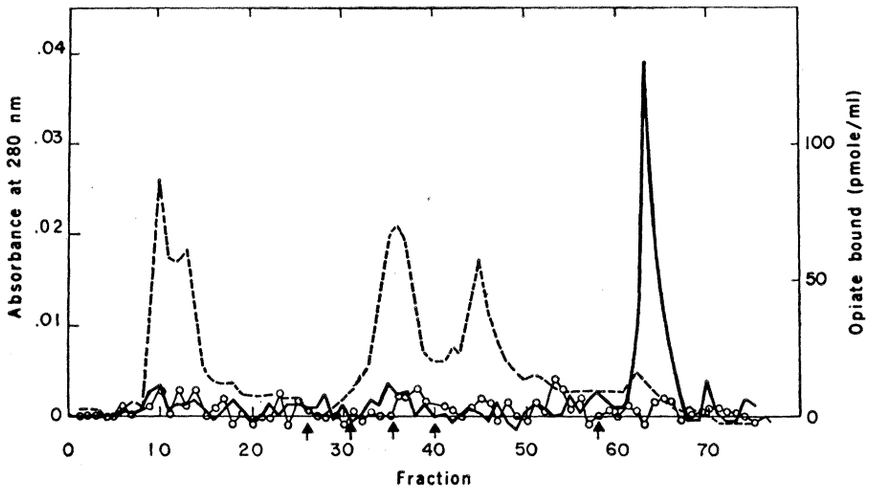


Fig. 1. Fractionation of opiate binding capacity of brain extract on Sephadex LH-20. Each mouse brain (about 500 mg of wet tissue) was homogenized at room temperature in 9.5 ml of a mixture containing two volumes of chloroform (C) and one volume of methanol (M). After centrifugation, the extract was washed once with 0.2 volume of distilled water, then precipitated with four volumes of cold ether. The precipitate was centrifuged at 8000*g* for 5 minutes, redissolved in C-M 2 : 1, and applied to a Sephadex LH-20 column (2 by 20 cm) that had been equilibrated overnight with C. Fractions (4 ml) were collected at 60 drops per minute (about 0.8 ml/min). Elution was with C (80 ml); C-M 15 : 1, 10 : 1, and 6 : 1 (20 ml each); and C-M 4 : 1 and 1 : 1 (80 ml each). Data have been corrected for column void volume (20 ml, or five fractions). Arrows indicate fractions in which successive eluting mixtures first emerge. Each fraction was tested for capacity to bind opiate by drying 0.1-ml portions under $N_2$, redissolving in 50 $\mu$l of C-M 2 : 1 and 1 ml of *n*-heptane, and determining distribution of [$^{14}$C]levorphanol or [$^{3}$H]dextrorphan (separately, $4 \times 10^{-7}M$ each in aqueous phase and $2 \times 10^{-9}M$ in equilibrium in organic phase) between 1 ml of 0.1*M* tris(hydroxymethyl)aminomethane (*p*H 7.4) and the heptane phase. Binding was determined by the increase in apparent partition coefficient in presence of brain extract (*10, 23*). Radioactivity was assayed in a Packard scintillation counter, with corrections for quenching by internal standards. Absorbance at 280 nm was monitored continuously as a rough measure of protein (– – –), levorphanol binding (——), and dextrorphan binding (○–○–○). Five brains were pooled in this experiment, data are normalized to one brain equivalent (*24*).

in this peak. In the experiment in Fig. 1, SSB was 512 pmole per gram of fresh total brain tissue at an aqueous levorphanol concentration of $4 \times 10^{-7}M$.

Instability of the binding sites is responsible for some variability between experiments. For example, fractions from brainstem, eluted in C-M 1 : 1 and stored in the same solvent mixture, lost 30 percent of the total opiate binding capacity and 40 percent of the SSB after 24 hours. Stability differed considerably according to brain region; whole brain extracts were most stable. The rate of degradation was identical at 25° and 2°C. Various methods for stabilizing the SSB capacity should be investigated.

We reported that SSB in brain particulates was not saturated with increasing opiate concentration, although Scatchard plots pointed to the presence of high-affinity, low-capacity sites that were obscured at high opiate concentrations (*7*). But in partially purified extracts of whole brain there are at least three distinct SSB components (Fig. 2). One, with a dissociation constant around $1.5 \times 10^{-7}M$, becomes saturated at 250 pmole/g; this concentration corresponds to that in mouse brain water at which levorphanol causes analgesia and locomotor activity (*11*). Another set of sites has a dissociation constant in the vicinity of $6 \times 10^{-7}M$ and becomes saturated at 430 pmole/g. Binding components of lower affinity and higher capacity are also evident. High-affinity sites, with dissociation constants below $10^{-7}M$, could not be detected by our method without levorphanol of much higher specific radioactivity than was available to us (*2*).

Table 1. Receptor binding in extracts from different tissues and brain regions. Tissues were fractionated and tested exactly as described for Figs. 1 and 2. For preparation of brain regions, see (*25*). Data are means and standard errors for the binding capacity in the pooled fractions of the C-M 1:1 eluate. Data are also given for two experiments with longitudinal muscle and attached myenteric plexus from guinea pig ileum; 4 g of fresh tissue was used in each experiment (*26*); *N*, number of determinations.

| Tissue | *N* | Levorphanol binding (pmole/g) | |
|---|---|---|---|
| | | Total | Stereospecific |
| Kidney | 10 | 214 ± 26 | 2 ± 6 |
| Liver | 8 | 31 ± 6 | − 9 ± 4 |
| Skeletal muscle | 10 | 62 ± 10 | − 18 ± 16 |
| Whole brain | 16 | 1880 ± 140 | 224 ± 28 |
| Cerebellum | 5 | 623 ± 105 | − 5 ± 5 |
| Cerebrum | 8 | 1280 ± 140 | 472 ± 42 |
| Brainstem | 8 | 6590 ± 410 | 1980 ± 110 |
| Brainstem sections | | | |
| 1 | 7 | 4230 ± 760 | 847 ± 69 |
| 2 | 7 | 2560 ± 200 | 307 ± 33 |
| 3 | 7 | 536 ± 120 | 28 ± 25 |
| Myenteric plexus | 2 | 78, 58 | 49, 18 |

Table 1 shows the total binding and SSB for several mouse tissues and brain regions. There was no SSB in kidney, liver, or skeletal muscle. In brain, SSB showed a pronounced regional localization, primarily in the rhombencephalic portion of brainstem. This is of interest in view of the localization of sites of analgesic action to the structures surrounding the fourth ventricle (*13*). Preparations of longitudinal muscle with attached myenteric plexus from guinea pig ileum, which contain opiate receptors (*14*), yielded similar elution diagrams, with SSB of approximately 35 pmole/g. With brain and myenteric plexus, binding capacity of levorphanol at $4 \times 10^{-7}M$ was only observed in the C-M 1 : 1 fraction in the elution pattern.

We also examined the subcellular distribution of the receptor in the portion of brainstem with greatest binding capacity, the region of the medulla and pons. The receptor was fairly uniformly distributed in the membranes of the crude nuclear, crude mitochondrial, and crude microsomal fractions, with a slight preponderance in the crude mitochondrial fraction containing nerve-terminal particles. This distribution is similar to that reported by us for particulate tissue fractions at a low concentration of levorphanol (*1*), but very different from that observed at a much higher levorphanol concentration. It is also similar to that obtained by Pert and Snyder (*3*) for binding of naloxone at $5 \times 10^{-9}M$ by particulate fractions. Various subcellular fractions appear to contain high- and low-affinity stereospecific receptors in different proportions.

It has been assumed that antagonists like naloxone bind competitively with opiate agonists at the same receptor sites (*15*). Using equimolar aqueous phase concentrations ($4 \times 10^{-7}M$), we found that the peak of binding capacity for naloxone in the 1 : 1 eluate from a Sephadex LH-20 column coincides with that for levorphanol. Naloxone can prevent levorphanol binding, reducing it by 50 percent at a concentration of $3 \times 10^{-5}M$. Naloxone binding capacity was also found in earlier parts of the elution pattern (*16*), in which no levorphanol binding capacity was demonstrable except at higher concen-

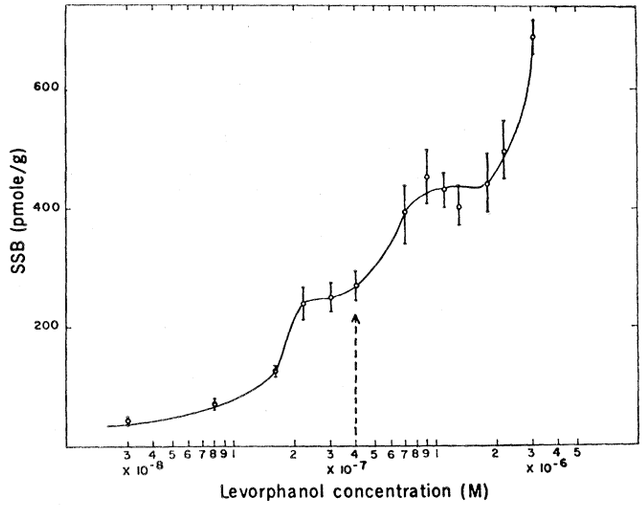


Fig. 2. Effect of opiate concentration on stereospecific binding in extracts of whole brain. For each experiment, brains of two mice were pooled and fractionated as for Fig. 1. Fractions (4 ml each) capable of binding levorphanol in the C-M 1 : 1 eluate (always fractions 6, 7, 8, and sometimes 9 after correction for 20-ml column void volume) were pooled. C-M 2 : 1 was added to make 20 ml per gram of original tissue, and 0.5-ml samples (25-mg tissue equivalent) were dried under $N_2$. Residues were dissolved in 50 μl of C-M 2 : 1 before addition of *n*-heptane, and binding was measured by the phase distribution procedure with [$^{14}$C]levorphanol and [$^{3}$H]dextrorphan present simultaneously. Partition coefficients (heptane/buffer) for both drugs without extract present were the same (0.0074) over the entire concentration range studied. Data represent three independent experiments, with four or five replications at each concentration (19 replications at $4 \times 10^{-7}M$). Data are means and standard errors for stereospecific binding (SSB), expressed as picomoles per gram of tissue equivalent in the extract. The arrow at $4 \times 10^{-7}M$ indicates the concentration used in most experiments in this report. Opiate concentration is that measured in the buffer phase at equilibrium.

trations. According to our observations, the sites with greatest affinity for naloxone are not the same as those with the highest affinity for levorphanol. This may be relevant to the identification of the site of high-affinity naloxone binding in tissue homogenates as "the opiate receptor" by Pert and Snyder (*3*).

The ligand-free receptor adheres to Sephadex LH-20 until the eluting solvent becomes quite polar (Fig. 1). This property of the receptor changes when it forms a complex with an opiate molecule. The fractions capable of binding levorphanol in the C-M 1 : 1 eluates were pooled and reduced in volume, [$^{14}$C]levorphanol ($1 \times 10^{-6}M$) was added, and the material was fractionated again on Sephadex LH-20. Two peaks of radioactivity were obtained (Fig. 3). The major one, around fraction 40, corresponded to free levorphanol, which was also eluted at this position in the absence of tissue extract. The minor peak, around fraction 21, near the end of the chloroform elution, is obviously a complex of levorphanol with the binding component demonstrated in Fig. 1. This complex was only formed with material from the C-M 1 : 1 eulate; when those fractions eluted earlier were treated in the same manner, only the single peak of radioactivity corresponding to free levorphanol was observed.

The altered behavior of the receptor when combined with levorphanol could be due to neutralization of charge at the combining site or, simply, to the addition of a more lipophilic molecule to a less lipophilic one. It could also be due, at least in part, to an induced change in receptor conformation. Here we merely summarize the observations indicating that the change in elution behavior is indeed due to the binding of levorphanol. (i) Recycling the C-M 1 : 1 eluate on Sephadex LH-20 without added levorphanol did not change the elution behavior. The ligand-free receptor reappeared in the 1 : 1 eluate. (ii) The 1 : 1 eluate in the experiment in Fig. 3 contained no radioactivity, although some capacity to bind added levorphanol remained in these fractions (*17*). Thus, most of the receptor molecules, which combined with levorphanol at $10^{-6}M$, became more lipophilic (higher affinity for chloroform) after binding the ligand. (iii) The change in elution behavior was reversible. Dialysis for 3 days at 25°C in C-M 2 : 1 with frequent replacement of dialyzate removed 92 to 98 percent of the bound levorphanol. The receptor, free of drug, again emerged from Sephadex LH-20 in the 1 : 1 eluate and retained its capacity to complex with added levorphanol, giving rise again to a peak of radioactivity at fraction 21 when refractionated with drug as in Fig. 3. The dialysis result also demonstrated that the receptor was of high molecular weight, although exact limits for the passage of macromolecules through cellulose nitrate membranes in C-M solutions are not known.

The considerable shift in position of the receptor on the elution diagram as a consequence of its interaction with ligand provided the key to purification. To establish the ratio of bound levor-

Table 2. Protein content of the levorphanol-receptor complex. The peak containing the levorphanol-receptor complex, obtained from a Sephadex LH-20 column first equilibrated with $10^{-6}M$ [$^{14}$C]levorphanol, was collected and divided into two aliquots. One part was dried, resuspended in 0.5 ml of 0.5*N* NaOH in 0.5 percent sodium lauryl sulfate (Sigma), and left at room temperature for 3 hours with frequent mixing. Another part was subjected to acid hydrolysis in 6*N* HCl at 100°C for 18 hours in a sealed tube. The HCl was removed in vacuo over $P_2O_5$ and NaOH, and the dried residue was resuspended in 0.5 ml of 0.1*N* HCl. Samples (0.1 ml) of each preparation were assayed in duplicate by the fluorescamine procedure [1.5 ml of borate buffer, 0.2*M*, *p*H 9.0; 0.5 ml of fluorescamine reagent (Hoffmann-LaRoche), 0.1 mg/ml in acetone]. Relative fluorescence was read in an Aminco-Bowman fluorometer (excitation, 390 nm; emission, 475 nm) against a quinine standard (10 mg/ml in 0.1*N* $H_2SO_4$) set to 100. Leucine (10 nmole) gave a reading of 20. Levorphanol (10 nmole) gave no fluorescence greater than background. Relative fluorescence data are already corrected for the following solvent blanks: nonhydrolyzed procedure, 1.8; 6*N* HCl hydrolysis, 7.2. In these experiments the receptor was obtained from mouse brainstem. In experiment 1, 140 mg of original tissue was used, and levorphanol bound was 986 pmole/g. In experiment 2, 93 mg of tissue was used, and levorphanol bound was 1220 pmole/g.

| Hydrolysis | Relative fluorescence of complex | Amine, leucine equivalent (nmole) | Amine per gram of tissue (nmole) | Amine per levorphanol (mole/mole) | Combining weight* (daltons) |
|---|---|---|---|---|---|
| | | *Experiment 1* | | | |
| Before | 1.8, 2.1 | 4.9 | 35 | 35 | 4,600 |
| After | 27.5, 27.5 | 68.8 | 491 | 498 | 65,200 |
| | | *Experiment 2* | | | |
| Before | 0.5, 0.5 | 1.3 | 14 | 12 | 1,600 |
| After | 19.0, 19.3 | 47.9 | 515 | 422 | 55,300 |

* Leucine equivalent per molecule of levorphanol.

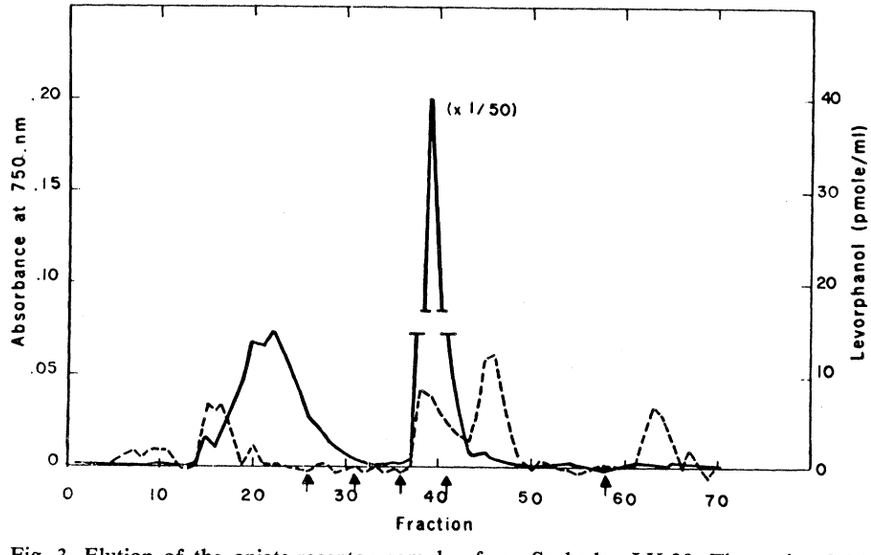


Fig. 3. Elution of the opiate-receptor complex from Sephadex LH-20. The entire C-M 1 : 1 eluate from an experiment like that shown in Fig. 1 was pooled, a half volume of chloroform added, and the volume was reduced to 2 ml under $N_2$. The sample was then incubated for 5 minutes at room temperature with $1 \times 10^{-6}M$ [$^{14}$C]levorphanol. This mixture was fractionated exactly as in Fig. 1. Protein was measured by a modification of the Lowry procedure (*12*); the resulting absorbance values at 750 nm are given by the broken line. The levorphanol-receptor complex, around fraction 21 in the C eluate, represents 390 pmole per brain (approximately 800 pmole/g). Note scale change by factor of 50 to show free levorphanol peak.

phanol to protein in the high-affinity complex, two precautions were necessary: (i) to use a small enough levorphanol concentration to avoid occupancy of lower-affinity sites; and (ii) to prevent dissociation of levorphanol during fractionation, since the free levorphanol concentration in the immediate environment of the complex decreases continuously during passage through the column. The use of a preequilibrated column, as described by Hummel and Dreyer (*18*), provided a solution to this problem. It was necessary first to saturate nonspecific sites on the dextran gel with nonradioactive dextrorphan. The column was washed free of unbound dextrorphan and then equilibrated with $1 \times 10^{-8} M$ [$^{14}$C]levorphanol. This ambient concentration was maintained throughout the subsequent procedures. The same concentration of [$^{14}$C]levorphanol was added to the concentrated 1 : 1 eluate from a brainstem extract, and the resulting complex was fractionated on the prepared column. Virtually all the radioactivity greater than the baseline (10 pmole or 100 count/min per milliliter) emerged in a single sharp peak in fractions 21 to 26 of the chloroform elution. Ligand-free receptor remained on the column until much later in the elution pattern, as shown previously. We believe, therefore, that the sharp peak of radioactivity in this position represented a stoichiometric complex of high-affinity receptor sites with levorphanol.

Fractions 21 to 26, containing bound levorphanol, were pooled and dried, and protein was determined by the fluorescamine method (*19*), which detects free primary amines. In two experiments, material derived from brainstem was analyzed (Table 2). The large difference in free amine content before and after acid hydrolysis is consistent with the behavior of a protein, especially one with a low content of diamino acids, as might be found in a lipophilic membrane protein (*20*), and as indicated here by the relative solubility of the receptor in water and organic solvents. The data on total amine content after hydrolysis, based on a leucine standard, yield a combining weight of approximately 60,000 daltons per molecule of levorphanol. This estimate may be regarded as an upper bound; an exact value will have to await amino acid analysis and demonstration of purity by physical means. The receptor comprises about 0.5 $\mu$mole of leucine equivalent per gram of brainstem, or about 65 $\mu$g of protein. Our determinations on mouse brain, in agreement with those of Lapetina *et al.* (*21*) on rat brain, indicate 80 mg of total protein per gram of tissue, of which 2 mg is proteolipid. Thus, the opiate receptor isolated here represents only 8 parts per 10,000 of the total protein, and 3 percent of the proteolipid.

We expected that the opiate receptors probably comprised a class of related macromolecules, with differences of affinity corresponding to the different effective doses (and brain concentrations) required for the several distinct pharmacologic actions. We therefore directed our efforts to isolation and purification of the receptors rather than to exhaustive studies on opiate binding in tissue fragments. The work described here shows the following:

1) Opiate receptors can be extracted from membranes of brain tissue by procedures used to isolate proteolipids. They are found only in neuronal tissue and are most abundant in the brainstem, especially in the rhombencephalon (medulla and pons), which has been implicated as the site of antiociceptive action of the opiates (*13*). One such receptor proteolipid from mouse brain has a mass of approximately 60,000 daltons per bound opiate molecule. It comprises only 3 percent of the total proteolipid, or less than 1 part per 1000 of the total brain protein.

2) The binding is stereospecific—in some experiments completely so, in others only partly so.

3) The partially purified receptor binds the typical opiate agonist, levorphanol, in concentrations corresponding to those associated with antinociceptive and locomotor activity in this species. A high-affinity stereospecific binding component becomes saturated at a concentration which, in brain water, produces maximum pharmacologic effect in vivo.

4) Higher concentrations of levorphanol lead to more total binding and also to more stereospecific binding. Several modes of binding, with different affinities, may be associated with the same receptors; or a heterogeneous population of receptors may be present. In a single species like the mouse, various agonist effects are manifested at widely different doses and brain concentrations. We reported a stereospecific lethal effect in the mouse which could be prevented by naloxone and required a levorphanol dose 30 times greater than that associated with analgesia and locomotor activity (*22*). Thus, the pharmacologic evidence suggests that opiate receptors with different affinities may mediate different pharmacologic action. We see no reason, a priori, why those with the highest affinities should necessarily be more important than those with lower affinities.

5) The binding is prevented, in a dose-dependent manner, by naloxone. We do not yet know how this phenomenon relates to opiate antagonism in vivo. As argued elsewhere (*7*), if competitive exclusion of agonists by antagonists at the same receptor sites suffices to prevent opiate actions, it follows that site occupancy alone cannot account for the agonist effects—a conformation change would probably be required. It should be possible to ascertain directly with the isolated receptors whether opiate agonists cause conformation change, and whether opiate antagonists prevent such change.

LOUISE I. LOWNEY
KARIN SCHULZ
PATRICIA J. LOWERY
AVRAM GOLDSTEIN

*Department of Pharmacology, Stanford University Medical Center, Stanford, California 94305, and Addiction Research Foundation, Palo Alto, California 94304*

### References and Notes

1. A. Goldstein, L. I. Lowney, B. K. Pal, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1742 (1971).
2. We are grateful to Hoffman-LaRoche, Inc., for generous gifts of levorphanol tartrate, dextrorphan tartrate, [*N-methyl*-$^{14}$C]levorphanol (26.6 $\mu$c/mg), and [6,7-$^{3}$H]dextrorphan (50 $\mu$c/mg).
3. C. B. Pert and S. H. Snyder, *Science* **179**, 1011 (1973); *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2243 (1973).
4. E. J. Simon, J. M. Hiller, I. Edelman, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1947 (1973).
5. L. Terenius, *Acta Pharmacol. Toxicol.* **32**, 317 (1973).
6. C. Y. Lee, S. Stolman, T. Akera, T. M. Brody, *Pharmacologist* **15**, 202 (1973).
7. A. Goldstein, in *Pharmacology and the Future of Man* (proceedings, Fifth International Congress of Pharmacology, San Francisco, 1972) (Karger, Basel, 1973), pp. 140–150; B. K. Pal, L. I. Lowney, A. Goldstein, in *Agonist and Antagonist Actions of Narcotic Analgesic Drugs* (proceedings, symposium of the British Pharmacological Society, Aberdeen, July 1971), H. W. Kosterlitz, H. O. J. Collier, J. E. Villarreal, Eds. (Macmillan, London, 1973), pp. 62–69; A. Goldstein, in *New Concepts in Neurotransmitter Regulation*, A. J. Mandell Ed. (Plenum, New York, 1973), pp. 297–309; in *Narcotic Antagonists* (proceedings, First International Conference on Narcotic Antagonists (November 1972), M. C. Braude, Ed. (Raven, New York, in press).
8. J. Folch, M. Lees, G. H. S. Stanley, *J. Biol. Chem.* **226**, 497 (1957).
9. E. F. Soto, J. M. Pasquini, R. Placido, *J. Chromatogr.* **41**, 400 (1969).
10. G. Weber, D. P. Borris, E. de Robertis, F. J. Barrantes, J. L. LaTorre, M. de Carlin, *Mol. Pharmacol.* **7**, 530 (1971).
11. J. A. Richter and A. Goldstein, *Proc. Natl. Acad. Sci. U.S.A.* **66**, 944 (1970); A. Goldstein and P. Sheehan, *J. Pharmacol. Exp. Ther.* **169**, 175 (1969); A. Goldstein, B. A. Judson, P. Sheehan, *Br. J. Pharmacol.* **47**, 138 (1973).

12. M. B. Lees and S. Paxman, *Anal. Biochem.* **47**, 184 (1972).
13. H. Teschemacher, P. Schubert, A. Herz, *Neuropharmacology* **12**, 123 (19[illegible] M. Satoh and H. Takagi, *Eur. J. Pharmacol.* **14**, 60 (1971).
14. E. A. Gyang and H. W. Kosterlitz, *Br. J. Pharmacol.* **27**, 514 (1966); W. D. M. Paton, *ibid.* **12**, 119 (1957); R. Schulz and A. Goldstein, *ibid.* **48**, 655 (1973).
15. H. W. Kosterlitz and A. J. Watt, *ibid.* **33**, 266 (1968); A. E. Takemori, H. J. Kupferberg, J. W. Miller, *J. Pharmacol. Exp. Ther.* **169**, 39 (1969); W. R. Martin, *Pharmacol. Rev.* **19**, 463 (1967); A. L. Cowie, H. W. Kosterlitz, A. J. Watt, *Nature (Lond.)* **220**, 1040 (1968).
16. A. Goldstein, unpublished observations.
17. This was not surprising. Levorphanol is complexed reversibly with the receptors. In the refractionation, levorphanol dissociates from the drug-receptor complex as it passes through the column. This process is promoted by the considerable retentive capacity of the dextran gel for free levorphanol. The resulting free receptor molecules are retarded, and finally emerge during the C-M 1 : 1 elution. Typical data were 2000 pmole per gram of tissue for total levorphanol binding capacity in the C-M 1 : 1 eluate from the first column; 1100 pmole/g in the levorphanol-receptor complex refractionated on the second column; and residual binding capacity of 400 pmole/g in the C-M 1 : 1 eluate of the second column.
18. J. P. Hummel and W. J. Dreyer, *Biochim. Biophys. Acta* **63**, 530 (1962).
19. S. Udenfriend, S. Stein, P. Böhlen, W. Dairman, W. Leingruber, M. Weigele, *Science* **178**, 871 (1972).
20. J. Folch, *Prog. Brain Res.* **29**, 1 (1968).
21. E. G. Lapetina, E. F. Soto, E. de Robertis, *J. Neurochem.* **15**, 437 (1968).
22. R. Dingledine and A. Goldstein, *Br. J. Pharmacol.* **48**, 718 (1973).
23. The two phases were equilibrated by three treatments on a Vortex mixer for 10 seconds each. Free drug equilibrated completely between the phases in less than 5 seconds. The tubes were centrifuged for 1 minute at 1000*g* at room temperature to separate the phases before sampling for radioactivity counting. Prolonged or vigorous mixing caused a loss of binding capacity in the organic phase, accompanied by the appearance of a fluffy layer at the interface, which retained bound opiate. The amount of extract used in the phase distribution procedure has a pronounced effect upon the total binding; with extract from increasing quantities of tissue, the binding per gram of original tissue increases greatly. However, SSB per gram does not change significantly; thus, the amount of tissue used is not critical for the measurement of SSB. Opiates labeled with $^{14}C$ or $^{3}H$ were used interchangeably with identical results. Since the $^{14}C$ compounds were labeled in the *N*-methyl group whereas the $^{3}H$ compounds were labeled in positions 7 and 8 of the ring system, the results indicate that binding involves the intact opiate molecule.
24. Except as otherwise noted, all reagents were obtained from J. T. Baker Co. Sephadex LH-20 was obtained from Pharmacia Fine Chemicals, Inc.; *n*-heptane (spectroscopic AR grade) and [*allyl*-1,3-$^{14}C$]naloxone (19.2 μg/mg) from Mallinckrodt Chemical Works; and L-leucine (A grade) from Calbiochem, Inc. Naloxone was a gift from Endo Laboratories.
25. Cerebellar and cerebral hemispheres were first removed. Then the brainstem was cut transversely just caudal to the posterior colliculus, so that section 1 contained the pons and medulla with most of the fourth ventricle. A second transverse cut just rostral to the pituitary stalk placed the mesencephalon and part of the diencephalon with all of the third ventricle in section 2. Finally, the olfactory lobes were removed, which left most of the diencephalic structures and the telencephalon in section 3. Material from eight mice was pooled, and 5 to 16 determinations were made on each pool. Mean weights of wet tissue (in milligrams) per brain were as follows: whole brain, 485; cerebellum, 53; cerebrum, 233; brainstem, 199; section 1, 45; section 2, 102; and section 3, 61.
26. We are indebted to R. Schulz for permission to use information derived from his experiments on the isolation of opiate receptor from guinea pig myenteric plexus.
27. Supported by NIMH Drug Abuse Research Center grant DA-00249 and grant DA-00026. Generous support from the Drug Abuse Council to the Addiction Research Foundation is also acknowledged.

17 August 1973; revised 2 November 1973

## Gating Currents of the Sodium Channels: Three Ways to Block Them

Abstract. *Preceding the opening of the sodium channels of axon membrane there is a small outward current, gating current, that is probably associated with the molecular rearrangements that open the channels. Gating current is reversibly blocked by three procedures that block the sodium current: (i) internal perfusion with zinc ions, (ii) inactivation of sodium conductance by brief depolarization, and (iii) prolonged depolarization.*

The sodium and potassium channels of nerve membrane open and close in response to electric field changes within the membrane. This almost certainly means that charged gating structures change conformation in response to the field changes, and in theory the charge movement or "gating current" associated with these changes should be measurable. Currents with many of the properties expected of Na channel gating current have been reported by Armstrong and Bezanilla (*1*), and their measurements have since been repeated by Keynes and Rojas (*2*). Though quite suggestive, none of the previously reported evidence conclusively links the observed currents with the gating structures. Perhaps the strongest evidence of such a linkage would be the discovery of a way to block both sodium current ($I_{Na}$) and the presumed gating current simultaneously. Rather disappointingly, tetrodotoxin (TTX), which selectively blocks $I_{Na}$, does not affect the presumed gating currents: apparently it blocks the channels without significantly altering the gating process. We report here three procedures that reversibly suppress both $I_{Na}$ and gating current, greatly strengthening the evidence for association of the two.

In order to see gating current, it is necessary to suppress ionic current and subtract from the records the linear component of capacitative current, both of which are large compared to gating current. Our procedure for doing this has been described (*1*), and we repeat here only its essential features. Most of our experiments were performed on axons perfused internally with CsF, which is essentially impermeant, and bathed in a medium in which the impermeant tris(hydroxymethyl)methyl ammonium ion (tris+) was substituted for all or most of the $Na^+$. The ionic current with $Cs^+$ inside and no external $Na^+$ is a time-invariant "leakage" current, which can easily be subtracted from the transient gating current. Ionic current through the sodium channels was further suppressed in some experiments by addition of $3 \times 10^{-7} M$ TTX to the external medium. To eliminate the linear portion of membrane capacitative current we algebraically summed the current produced by successive positive and negative pulses of exactly equal magnitude. Summation and averaging were performed by a digital averager of new design, which will be described elsewhere (*3*). The new averager made it possible to measure gating current with a small number of pulses.

Using these procedures, we recorded a transient current from axon membrane which from the electrical point of view is a slow and nonlinear component of capacitative current, and from the physiological point of view is, we think, gating current. The first way of blocking gating current is by internal perfusion with $Zn^{2+}$. Begenisich and Lynch (*4*) have reported that 10 m*M* $ZnCl_2$ in the internal medium suppresses both $I_{Na}$ and $I_K$ (potassium current) and, surprisingly, the effect is reversible. Figure 1 shows that $ZnCl_2$ also suppresses gating current. In this experiment, $I_{Na}$ was completely eliminated by adding TTX to the external medium, and there was no $Na^+$ inside or out. The control record shows an outward current that continues until the termination of the pulses. This is what we have interpreted as the charge movement associated with the opening of the Na channel activation gates. When the pulses end, there is a tail of inward current which we associate with the return of the gating charge from "open" to "closed" position (*1*). Both inward and outward gating current were suppressed almost completely when 10 m*M* $ZnCl_2$ was added to the medium (Fig. 1b) and both recovered