Proc. Nat. Acad. Sci. USA
Vol. 68, No. 8, pp. 1742–1747, August 1971

Stereospecific and Nonspecific Interactions of the Morphine Congener Levorphanol in Subcellular Fractions of Mouse Brain

AVRAM GOLDSTEIN, LOUISE I. LOWNEY, AND B. K. PAL

Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305

Communicated by Marshall Nirenberg, May 21, 1971

ABSTRACT **A method is described for analyzing the association of the opiate narcotic levorphanol with brain tissue into three components: nonsaturable, saturable nonspecific, and saturable stereospecific. The method may be of general applicability for the study of the interaction of drugs with body tissues. In mouse brain the stereospecific binding of levorphanol represents only 2% of the total association of drug with tissue, and it was found only in certain membrane fractions. The material responsible for the stereospecific binding might be the opiate receptor.**

Pharmacologic action of a drug presupposes interaction of drug molecules with tissue receptors. Progress has been made in identifying and isolating some drug receptors (1, 2), especially where specificity and affinity happened to be very high (3), or where a specific site-directed label could be attached irreversibly (4, 5). For most drugs, however (including the opiate narcotics), direct measurement of localization and binding in tissues is unlikely to yield useful information about cellular or subcellular receptor sites, until two methodologic obstacles are understood and surmounted.

1. Specificity

Many drugs interact nonspecifically with a wide variety of tissue components. Localization of drug in a particular organ, subcellular particle, or macromolecule does not necessarily imply a site of action there.

We distinguish between *nonsaturable* and *saturable* interactions. *Nonsaturable* interactions are of two kinds. First, particles surrounded by an osmotic membrane (e.g., synaptosomes) or having a spongy matrix can contain trapped drug in aqueous solution. Second, membranes will contain dissolved drug in amounts determined by the lipid/water partition coefficient and the ambient aqueous concentration. The mere finding that drug molecules are associated with some subcellular fractions cannot be interpreted as drug "binding".

Nonspecific saturable interaction arises through ionic bonds, hydrogen bonds, and hydrophobic forces. Cationic drugs like the opiate narcotics can be expected to interact nonspecifically with anionic groups of proteins, nucleic acids, phospholipids, sphingolipids, and mucopolysaccharides—interactions that are likely to be pharmacologically irrelevant. The problem is how to sort out the nonsaturable and nonspecific saturable interactions in order to measure a relatively small amount of *specific saturable* interaction, at the receptor sites, where drug binding triggers the chain of events that leads to the characteristic pharmacologic effect.

The opiate narcotics display an extraordinary degree of stereospecificity. Whereas the D(−) compounds are pharmacologically active, the L(+) isomers are neither agonists nor antagonists, and therefore presumably cannot enter the receptor sites. We take advantage of this by using radioactive levorphanol [a synthetic D(−) congener of morphine] and its nonradioactive enantiomer, dextrorphan, in the following way (Fig. 1). The association of [^{3}H]levorphanol (or [^{14}C]-levorphanol) with a given tissue fraction is measured under three conditions:

A, [^{3}H]Levorphanol alone is present. It will participate in all the possible kinds of interaction.

B, The system is first incubated with a 100-fold excess of nonradioactive dextrorphan. Then [^{3}H]levorphanol, at the same concentration as in *A*, should be largely blocked from entering the nonspecific saturable sites. But its nonsaturable associations ("trapped and dissolved") should not be hindered because here each isomer behaves independently. The difference *A* minus *B* measures nonspecific saturable binding.

C, The system is first incubated with a 100-fold excess of nonradioactive levorphanol. Then [^{3}H]levorphanol should be blocked from entering all the saturable sites, but should still participate in the nonsaturable associations. The difference *B* minus *C* measures stereospecific binding, which could be due to drug receptors. [^{3}H]Levorphanol that remains associated with the tissue fraction in condition *C* measures nonsaturable associations. (The results here and in condition *B* are, of course, corrected for 1/101 of the [^{3}H]levorphanol that will still occupy saturable sites.)

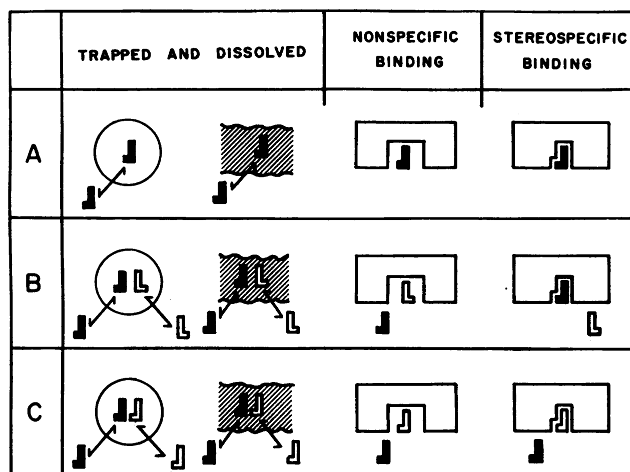

FIG. 1. Displacement of levorphanol from various types of binding in tissue. Solid symbols represent radioactive levorphanol, open symbols indicate a 100-fold excess of nonradioactive dextrorphan [the inert L(+) isomer] in *B* or the same excess of nonradioactive levorphanol in *C*.

2. Reversibility

Most pharmacologic effects are readily reversible, whence it is supposed that the corresponding drug–receptor interactions are also reversible. Dilution of a reversible system promotes ligand dissociation, and this may occur to a significant extent during manipulation of tissue fractions prior to analysis. The problem is analogous to the "dilution effect" whereby an enzyme inhibited reversibly *in vivo* recovers activity if diluted prior to assay (6).

Tissues are usually homogenized in several volumes of aqueous medium; but even if no water is added (7), the mixing of intracellular and interstitial fluids might cause dilution artifacts. Fractionation methods also entail dilution. In density-gradient centrifugation, as in simple sedimentation, drug molecules will dissociate from particles as they move down the tube, to be collected with components that band at higher levels, which will lead to spurious results. In molecular-sieving techniques, progressive dilution occurs down the length of the column. Acid precipitation of soluble macromolecules releases bound ligands to an unpredictable extent.

Seeing no meaningful way to assess the stereospecific binding of a drug *in vivo*, we decided to study the capacity of tissue fractions to bind *in vitro*. The essential precaution was to prevent dissociation of reversibly-bound drug by maintaining ambient drug concentrations constant throughout all procedures. Particulate material was fractionated (or simply sedimented) centrifugally in the presence of the appropriate radioactive and nonradioactive drugs (conditions *A*, *B*, and *C*), and the excess radioactivity associated with the bands and pellets was measured. For soluble and some particulate fractions, molecular sieving columns were used. Here the method of Hummel and Dreyer (8) suited our requirements perfectly, the column being first equilibrated with the appropriate drugs. Equilibrium dialysis was suitable for measuring the binding capacity of soluble fractions.

METHODS

Male Swiss-Webster mice (25–30 g) were used. Usually, 3–6 brains were pooled. After decapitation, whole brains or portions thereof were weighed quickly and homogenized in 10 volumes of cold 0.32 M sucrose in 0.01 M Tris·HCl, pH 7.0, with a loose-fitting motor-driven pestle, exactly as described by Whittaker and Dowe (9). In another procedure, tissue was homogenized by hand in 0.25 M sucrose–Tris in a Dounce homogenizer with 10 strokes of the A pestle (clearance 0.1 mm). $CaCl_2$ (10 μM) was added for further workup of the crude mitochondrial fraction (10).

For experiments with portions of brain, the cerebellum and cerebrum were dissected off and a cut was made at the rostral border of the pons, yielding a medulla–pons portion and a portion consisting mainly of diencephalic structures.

Levorphanol (3-hydroxy-*N*-methylmorphinan, L enantiomer) tartrate, dextrorphan (D-3-hydroxy-*N*-methylmorphinan) tartrate, [*methyl*-^{14}C]levorphanol (26.6 μCi/mg), and ditritiated [6,7-^{3}H]levorphanol, at 3.0 μCi/mg and at 36.7 μCi/mg) were generously donated by Hoffmann-La-Roche, Inc. The ^{14}C- and ^{3}H-labeled drugs were used interchangeably; no differences were observed. All drug concentrations are given in terms of free base.

Radioactivity was determined by liquid scintillation counting in a naphthalene–xylene–dioxane mixture (11), with Hyamine hydroxide (Packard) or ethanol, as appropriate for solubilization. Counting efficiencies were determined with $^{3}H_2O$ or [^{14}C]benzoate as internal standard. Protein was determined by the method of Lowry *et al.* (12).

Major subfractions

All procedures were carried out at 2–5°C. The homogenate was centrifuged at 1000 × *g* for 10 min. The sediment was resuspended twice in sucrose–Tris with a Vortex mixer and centrifuged as before, to obtain the crude nuclear fraction. The supernatant fluids and washes from the crude nuclear fraction were combined and centrifuged for 20 min at 12,000 × *g*, and the pellet was washed once as above, to obtain the crude mitochondrial fraction. The microsomal and soluble fractions were obtained by combining the supernatant and wash from the crude mitochondrial fraction and centrifuging for 1 hr at 105,000 × *g*.

Subfractionation of nuclear fraction

To isolate nuclei we used the method of Lovtrup-Rein and McEwen (13), without detergent. The nuclear fraction was resuspended in 2 M sucrose (10 ml/g brain tissue) and centrifuged for 30 min at 93,000 × *g*. The supernatant fluid, including a parchment-like floating layer, was removed, mixed vigorously, and centrifuged again. The floating material ("floated membranes") was collected and resuspended in 0.32 M sucrose–Tris. The pink jelly-like pellets containing whole nuclei were pooled and resuspended in 0.32 M sucrose–Tris. In another procedure we obtained membranes by subjecting 1 or 2 ml of the crude nuclear suspension to three 20-sec bursts in the MSE-Mullard ultrasonic disintegrator (19-mm probe, 9.5-mm tip, 0°C) in a 5 × 1.2 cm cellulose nitrate tube. No whole nuclei were visible microscopically after this treatment. The nuclear membranes (including nucleoli) were diluted in 0.32 M sucrose–Tris and sedimented for 1 hr at 105,000 × *g*.

Subfractionation of mitochondrial fraction

Nerve-ending fractions were isolated from the mitochondrial fraction by discontinuous gradient centrifugation (14). The mitochondria were resuspended in 0.32 M sucrose–Tris (1 ml per brain) containing 10 μM $CaCl_2$, and layered on a discontinuous sucrose gradient containing 1 ml each of 1.4, 1.2, 1.0, and 0.8 M sucrose–Tris–calcium. The gradient was centrifuged for 1 hr at 105,000 × *g* in the SW 50 rotor of the Spinco ultracentrifuge. This yields a myelin layer, three synaptosome bands, and a mitochondrial pellet. Each band was aspirated together with half the clear layer separating it from the band below. These fractions and the resuspended pellet were finally diluted to 0.32 M sucrose.

Synaptic membranes and vesicles were isolated according to De Robertis *et al.* (10, 15). The mitochondrial fraction was resuspended in distilled water containing 10 μM $CaCl_2$ (10 ml/g brain tissue), stirred on the Vortex mixer for 1 min, stored on ice for 10 min, stirred again, and centrifuged for 30 min at 20,000 × *g* to obtain synaptic membranes (M_1). The supernatant was centrifuged for 1 hr at 105, 000 × *g* to obtain synaptic vesicles (M_2) and the soluble protein (M_3) released by osmotic lysis of synaptosomes. M_1 was further fractionated on a discontinuous sucrose gradient containing 1 ml each of 1.2, 1.0, 0.9, and 0.8 M sucrose–Tris–calcium. This yields a myelin layer, three bands of synaptosome membranes, and a pellet of swollen mitochondria.

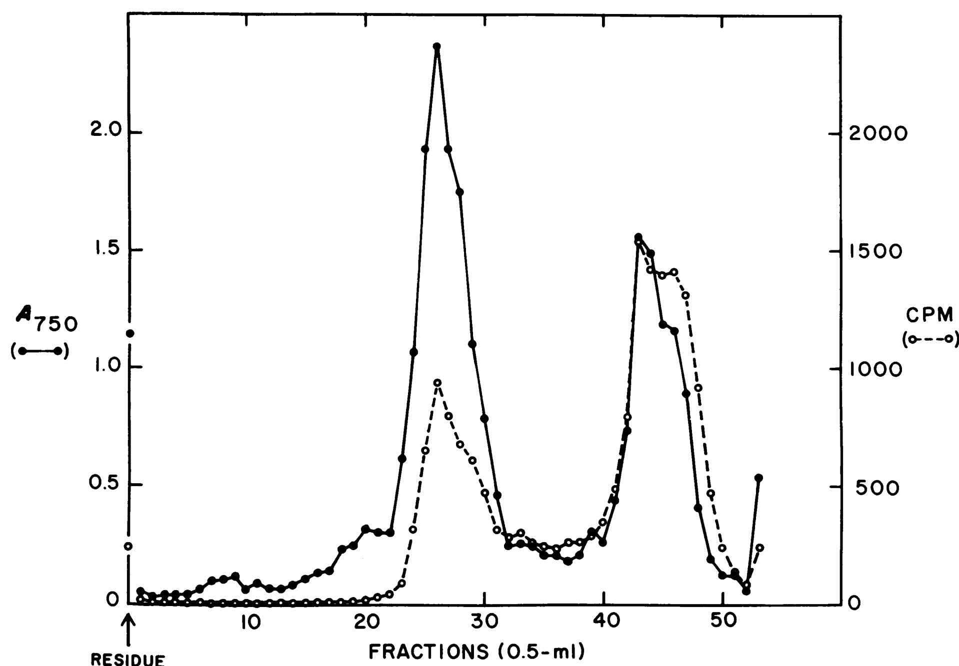

FIG. 2. Distribution of levorphanol binding in fractions of mouse brain homogenate on linear sucrose gradient. Homogenate (2 ml) containing 40 μg/ml [^{3}H]levorphanol was layered on a 24-ml linear sucrose gradient (0.8–1.75 M) and centrifuged in the SW 25 rotor for 3 hr at 50,000 × *g*. The gradient was overlaid with 3 ml of Tris buffer, and 0.5-ml fractions were collected dropwise from the punctured bottom of the tube. Protein (*solid circles*) and counts (*open circles*) were determined on each fraction and the resuspended pellet.

Binding studies

Pellets. Particulate fractions were diluted with 0.32 M sucrose–Tris to 50–150 μg protein per ml. 5-ml aliquots were used for drug incubation at 25°C in 10-ml polypropylene tubes under conditions *A*, *B*, and *C* (Fig. 1). Levorphanol or dextrorphan (50 μg/ml) was added to the appropriate tube (*B* or *C*), and 5 min later [^{3}H]- or [^{14}C]levorphanol (0.5 μg/ml) was added to all three tubes. After a further 15 min of incubation, the tubes were centrifuged for 1 hr at 105,000 × *g* or, in some experiments with crude nuclei and mitochondria, for 15 min at 20,000 × *g*. The supernatant fluids were aspirated, and adhering moisture was removed with absorbent cotton.

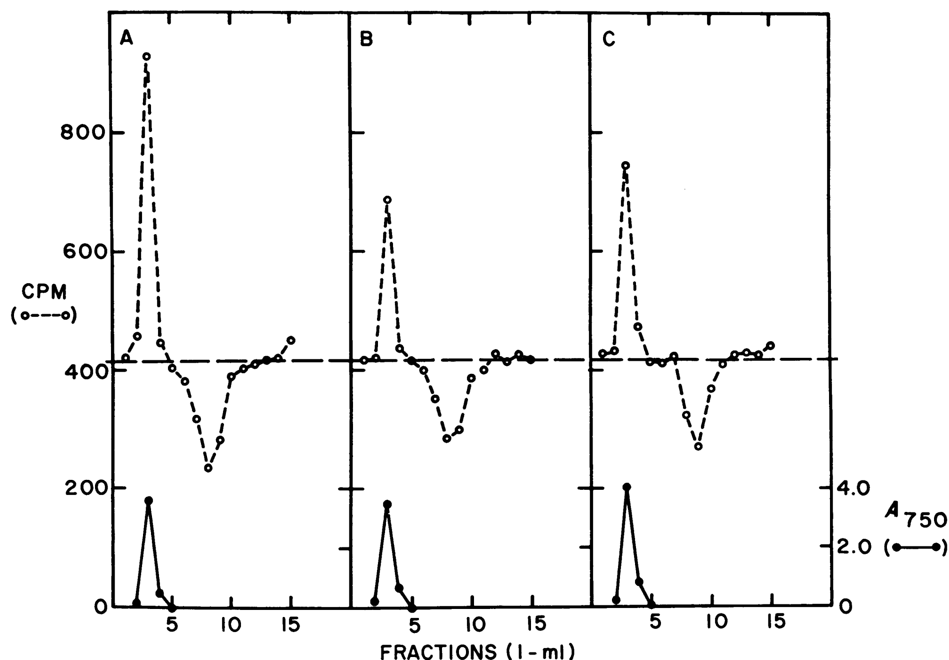

FIG. 3 Binding of levorphanol to the crude mitochondrial fraction of mouse brain on equilibrated Sephadex G-50 columns. Columns were equilibrated with 0.5 μg/ml [^{3}H]levorphanol. Equilibration medium for column B also contained 50 μg/ml dextrorphan; that for column C, 50 μg/ml nonradioactive levorphanol. 0.5-ml aliquots of crude mitochondrial fraction from mouse brain were equilibrated with the respective drug solutions and passed through the correspondng columns. Counts (*open circles*) and protein (*solid circles*) were determined for each 1-ml fraction. Horizontal broken line at 415 cpm represents the concentration of [^{3}H]levorphanol equilibrated with the column.

TABLE 1. *Interactions of levorphanol (low concentration) with particulate fraction of homogenate of whole mouse brain*

	cpm			Picomoles per brain		
Brain	A	B	C	Nonspecific saturable (A − B)	Trapped and dissolved (C)	Stereospecific binding (B − C)
1	5528	2521	2298			
	5247	2441	2352	31,900	25,500	1710
2	4793	2037	2113			
	4713	2014	1929	29,900	22,200	55
3	4807	2080	1983			
	4252	2069	2088	27,300	22,400	417
4	4560	1994	1980			
	4474	2116	1935	27,000	21,500	1060
5	4841	2454	2347			
	5150	2445	2382	28,000	26,000	944
6	4239	2177	1904			
	4289	2178	2196	22,900	22,500	1400
7	4146	1994	2079			
	4321	2210	1973	23,400	22,200	834
8	3778	1880	1881			
	3807	1896	1786	20,900	20,200	549
			Mean binding	26,400	22,800	871
			Percent of total binding	53	46	2

Each brain was homogenized in 5 ml of Tris–sucrose buffer, then diluted 1:10 with the same buffer. Six 1-ml aliquots were incubated under conditions A, B, and C (see text) with [^{14}C]levorphanol (0.5 μg/ml, 26.6 μCi/mg). After centrifuging at 105,000 × g for 1 hr the pellets were resuspended in 1 ml of 0.1 N NaOH, and 0.5 ml was counted. Counting efficiency = 60%, determined by internal standard. Raw data (cpm) are based on triplicate 10-min counts. Duplicate results for each brain represent independent incubations and workups. Levorphanol mol wt = 257. Picomoles per brain = cpm × 100 × 1/0.6 × 0.0659. Protein in this fraction: 29.0 mg per brain.

Pellets were dissolved in 1 ml of 0.1 N NaOH, and samples were taken for radioactivity counting.

Sephadex and DEAE-Cellulose Columns. The methods of Marchbanks (16) and Hummel and Dreyer (8) were used. Three Sephadex G-50 columns (1 × 10 cm) were equilibrated with 15 ml of radioactive levorphanol (0.5 μg/ml) in 0.32 M sucrose–Tris (A), or the same medium containing also 50 μg/ml nonradioactive dextrorphan (B) or levorphanol (C). Aliquots from a suspension of mitochondrial fraction (10 ml/g brain tissue) were incubated at 25°C for 15 min at the same drug concentrations used on the columns. Then 0.5 ml from each incubation mixture was placed on the corresponding column. Elution was with 15 ml of additional equilibration medium, and 1-ml fractions were collected and assayed for radioactivity and protein. The soluble supernatant from homogenate was fractionated on DEAE-cellulose as described elsewhere (17), with the exceptions noted in the text.

Equilibrium Dialysis of Soluble Fractions. 1-ml aliquots of soluble fractions or buffer alone in 6-mm Viscose dialysis tubing bags were placed in capped 10-ml polypropylene tubes containing 4 ml of 0.32 M sucrose–Tris. Dextrorphan was added to the medium bathing the bag in B tubes, levorphanol in C tubes, and radioactive levorphanol in A, B, and C tubes. After vigorous agitation on a reciprocal shaker for 18 hr at 2°C, the bathing media and bag contents were assayed for radioactivity. The excess of inside over outside radioactivity for each experimental tube was corrected by subtracting the result in the corresponding buffer control.

Continuous Sucrose Gradient Fractionation. A whole brain homogenate (5 volumes per g brain tissue, 0.25 M sucrose–Tris with 10 μM $CaCl_2$, Dounce homogenizer) was incubated with [^{3}H]levorphanol (40 μg/ml) for 15 min at 25°C. One brain equivalent of this mixture was fractionated on a linear sucrose gradient, as described by Potter and Axelrod (18).

RESULTS

We first show how the methodology described above clarifies an otherwise misleading result. Fig. 2 shows some levorphanol associated with the crude mitochondrial peak (fraction 25), a finding that has been interpreted by others (19) as a synaptosomal "binding" of opiate molecules. However, when this interaction was studied by the A, B, and C systems described, a different conclusion was reached. As shown in Fig. 3, levorphanol associated with the particles in A was reduced by about one-half in B, and there was no further reduction in C. Thus, about half the total "binding" was of the nonspecific saturable kind, the remainder was due to trapped and dissolved drug; there was no detectable stereospecific binding.

Whole-brain homogenate was incubated with radioactive levorphanol under conditions A, B, and C. Then the particulate material was sedimented to obtain the data of Table 1. The concentration of radioactive levorphanol (0.5 μg/ml, 1.95 μM) was well within the pharmacologically relevant range of brain levels determined *in vivo* (ref. 7 and Goldstein and Judson, to be published). In each of eight brains about half the total interaction was due to nonspecific binding, and nearly half to trapped and dissolved drug. There was also a significant, though small, B minus C difference, representing stereospecific binding, about 2% of the total.

The same technique was applied to the various particulate fractions and subfractions (Table 2). Significant stereospecific binding was found in the crude nuclear fraction and in membranes from that fraction after lysis, in membranes from the lysed crude mitochondrial fraction, and in the crude micro-

TABLE 2. *Stereospecific binding of levorphanol (low concentration) in subcellular fractions of mouse brain*

Fraction	Subfraction	n	Picomoles per brain	Protein content per brain (mg)	Picomoles per mg protein	*P*
Homogenate	Particulate	17	953 ± 139	29.0	32.9 ± 4.8	<0.01
Crude nuclear		24	182 ± 82	13.9	13.1 ± 5.9	<0.05
	Membranes	6	99.3 ± 35.8	5.7	17.4 ± 6.3	<0.01
	Nuclei	8	−25.2 ± 34.0	1.2	−21.0 ± 28.3	N.S.
Crude mitochondrial		11	253 ± 166	9.3	27.2 ± 17.8	N.S.
	Membranes (M_1)	5	208 ± 88	5.6	37.1 ± 15.8	<0.05
	Vesicles (M_2)	3	−17.0, −11.0, 5.0	1.2	−13.7, −8.9, 4.0	N.S.
	Soluble (M_3)	1	0	3.0	0	—
Crude microsomal		4	109 ± 22	3.6	30.3 ± 6.2	<0.01
Soluble supernatant		1	−19.0	8.3	−2.3	—

Data are means ±SE, based on *B* minus *C* differences, as described in text. For fractionation procedures see *Methods*. Membranes of the crude nuclear fraction were obtained by sedimenting at 105,000 × *g* for 1 hr after ultrasonic treatment. n is number of separate experiments, usually 3–6 pooled mouse brains in each. Entries under mg protein are for the given fraction in a single brain; total brain protein is sum of homogenate particulate and soluble supernatant. *P* is significance of difference from zero, by *t* test; N.S. = $P > 0.05$. Except as noted below, concentration of radioactive levorphanol was 0.5 μg/ml (1.95 μM).

Equilibrium dialysis of soluble fractions: M_3 = 6 pooled brains, protein 0.3 mg/ml inside bag, [^{3}H]levorphanol 0.5 μg/ml at equilibrium. Soluble supernatant = 14 pooled brains, protein 8.6 mg/ml inside bag, [^{3}H]levorphanol 0.1 μg/ml at equilibrium. Each soluble fraction run as three aliquots. Data based on mean dpm inside minus mean dpm outside, corrected for buffer controls (see *Methods*).

somal fraction. In other experiments it was found that the major regions of brain (cerebrum, cerebellum, medulla, pons, diencephalon) did not differ greatly in binding capacity of this type. The crude mitochondrial fraction from whole brain and separate regions was subfractionated by discontinuous sucrose gradient centrifugation. The myelin layer and the mitochondrial sediment in both intact and lysed preparations were devoid of stereospecific-binding capacity. The several bands of synaptosomes and synaptosome membranes gave variable results; more experiments are required.

Soluble fractions were studied by equilibrium dialysis (Table 2). Soluble material from osmotically lysed synaptosomes (M_3) gave no indication of stereospecific binding. The soluble supernatant fraction from homogenate of 14 brains was pooled and lyophilized to achieve a high protein concentration inside the dialysis bag, and a range of levorphanol concentrations was explored. Stereospecific binding should have been detected most readily with high protein and low drug concentration. The result in Table 2 was obtained at very low concentration of radioactive levorphanol; a similar outcome—virtually no stereospecific binding—was seen at all concentrations up to 10 μg/ml.

To see if there might nevertheless be stereospecific-binding capacity limited to one or a few kinds of soluble protein, we fractionated soluble supernatant from 10 pooled brains on DEAE-cellulose by means of discontinuous NaCl gradient elution (0.01–2 M), Hummel–Dreyer (8) technique (compare Fig. 3) in the presence of dextrorphan (condition *B*). Recovery of protein was 98%, and 11 well-defined peaks were obtained. In none of the 152 fractions was there significant increase in radioactivity above the equilibrium level in the eluting solutions, with the following exception. A protein peak eluted by shifting from 2 M NaCl back to salt-free Tris buffer (0.005 M) had twice the equilibrium level of radioactivity. When this peak was further studied on Sephadex G-10, exactly as in Fig. 3, no *B* minus *C* difference was found; thus the binding was not stereospecific.

Since stereospecific binding could be measured only as a small difference between large numbers (compare Table 1), variability was high. We found, however, that a 20-fold higher concentration of radioactive levorphanol (with the same 100:1 ratio of nonradioactive to radioactive drug in conditions *B* and *C*) yielded larger and more consistent values for the binding (Table 3). Most of the stereospecific-binding

TABLE 3. *Stereospecific binding of levorphanol (high concentration) in subcellular fractions of mouse brain*

Fraction	Subfraction	n	Picomoles per per brain	Protein content per brain (mg)	Picomoles per mg protein	*P*
Homogenate	Particulate	9	6470 ± 1600	29.4	220 ± 54	<0.01
Crude nuclear		11	5420 ± 1890	16.3	332 ± 116	<0.05
	Floated membranes	6	5930 ± 732	10.5	565 ± 70	<0.01
	Nuclei	6	527 ± 238	1.4	376 ± 170	N.S.
Crude mitochondrial		10	1510 ± 542	8.3	182 ± 65	<0.05
Crude microsomal		7	157 ± 368	2.9	54 ± 127	N.S.

Methods and presentation of data as in Table 2, except as follows. Concentration of radioactive levorphanol was 10 μg/ml (39 μM). Crude nuclear and crude mitochondrial pellets were sedimented (after incubation with levorphanol) at 20,000 × *g* for 15 min. Floated membranes were obtained from the crude nuclear fraction as described under *Methods*.

capacity in homogenate appeared in the crude nuclear fraction, and this was nearly all accounted for in membranes floated on 2 M sucrose after sedimentation of nuclei. Little of this capacity was in the crude mitochondrial, and none in the crude microsomal fraction. The floated membranes were enriched in stereospecific-binding capacity per mg protein as compared with homogenate or any other fraction.

As reported in a preliminary communication (20), the floated membranes retain their stereospecific-binding capacity after extraction of 70% of the protein by Triton X-100 or sodium dodecyl sulfate, provided the detergent is removed by dialysis. This binding in such preparations has a pH optimum in the neutral region, is enhanced by EDTA, is greatly diminished by Ca^{2+} or Mg^{2+}, and is largely abolished by treatwith neuraminidase or pronase but not trypsin. *p*-Chloromercuribenzoate, mercaptoethanol, and iodoacetic acid do not affect stereospecific-binding capacity. Of greatest interest, the capacity is retained nearly quantitatively in material extracted into chloroform–methanol (21).

DISCUSSION

It may be assumed that the receptor for the D(−) opiates is stereospecific, because the L(+) isomers are neither agonists nor antagonists. Therefore, in order to identify and try to isolate opiate receptors, we developed a method for measuring stereospecific binding in the presence of nonspecific interactions. We found that about half the total association of levorphanol with brain tissue is of the nonsaturable kind (trapped and dissolved drug), nearly half is of the nonspecific saturable kind, and only about 2% is stereospecific. Consequently, it is clear that neither measurements of total "binding" (19, 22–24) nor even a direct comparison of the distributions of a D(−) narcotic and its L(+) enantiomer in brain tissue (25) could be relevant to the identification of receptor sites.

The subcellular distribution of stereospecific-binding capacity was interesting. Virtually none was found in soluble supernatant of whole homogenate, soluble axoplasmic material from nerve-ending particles, synaptic vesicles, purified nuclei or mitochondria, or myelin fractions. Stereospecific binding occurred mainly in membranes separated by flotation from the crude nuclear fraction, but also to some extent in crude mitochondrial (synaptosomal) and microsomal membrane fractions. Major regions of brain did not differ significantly in stereospecific binding, a not unexpected finding in view of the multiplicity of narcotic sites of action (26).

Is the magnitude of stereospecific binding consistent with the possibility that it represents opiate receptor sites? After an ED_{50} (median effective dose) of the very potent opiate etorphine in rats, the brain concentration was 3 nM (27). In the mouse this would be 7×10^{11} molecules per brain. That is clearly an upper limit for the number of receptor sites that could be occupied at the ED_{50}. In our low-concentration experiments we used a concentration of radioactive levorphanol equivalent to that established in brain at about five times the ED_{50} (to be published), and we found stereospecific binding to be about 1 nmol (6×10^{14} molecules) per brain. Moreover, with 20-fold higher drug concentration, we found about 10 times as much stereospecifically bound. Consequently, if these binding sites are indeed receptor sites, pharmacologic action of an opiate must require only a very low fractional occupancy of the receptors, and equal effects must not necessarily imply equal receptor occupancies. Both these postulates are already inherent in certain theories of drug action (28, 29).

We are proceeding to purify and characterize the material responsible for the stereospecific binding. Preliminary results indicate it has some properties characteristic of proteolipids. This work was supported by research grant MH13963 from the National Institute of Mental Health.

1. Ehrenpreis, S., J. H. Fleisch, and T. W. Mittag, *Pharmacol. Rev.*, **21**, 131 (1969).
2. Alivisatos, G. A., and P. K. Seth, *Methods and Techniques of Neuroscience*, ed. R. Fried (Marcel Dekker Inc., New York, in press).
3. DeRobertis, E., *Science*, **171**, 963 (1971).
4. Miledi, R., P. Molinoff, and L. T. Potter, *Nature*, **229**, 523 and 554 (1971).
5. Karlin, A., *J. Gen. Physiol.*, **54**, 245S (1969).
6. Straus, O. H., and A. Goldstein, *J. Gen. Physiol.*, **26**, 559 (1943).
7. Richter, J. A., and A. Goldstein, *Proc. Nat. Acad. Sci. USA* **66**, 944 (1970).
8. Hummel, J. P., and W. J. Dreyer, *Biochim. Biophys. Acta*, **63**, 530 (1962).
9. Whittaker, V. P., and G. H. C. Dowe, *Biochem. Pharmacol.*, **14**, 194 (1965).
10. DeRobertis, E., G. R. de Lores Arnaiz, L. Salganicoff, A. P. de Iraldi, and L. M. Zieher, *J. Neurochem.*, **10**, 225 (1963).
11. Carey, N. H., and A. Goldstein, *Biochim. Biophys. Acta*, **55**, 346 (1962).
12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
13. Lovtrup-Rein, H., and B. S. McEwen, *J. Cell Biol.*, **30**, 405 (1966).
14. DeRobertis, E., A. P. de Iraldi, G. R. de Lores Arnaiz, and L. Salganicoff, *J. Neurochem.*, **9**, 23 (1962).
15. DeRobertis, E., M. Alberici, G. R. de Lores Arnaiz, and J. M. Azcurra, *Life Sci.*, **5**, 577 (1966).
16. Marchbanks, R. M., *Biochem. J.*, **104**, 148 (1967).
17. Hahn, D. L., and A. Goldstein, *J. Neurochem.* (1971), in press.
18. Potter, L. T., and J. Axelrod, *J. Pharmacol. Exp. Ther.*, **142**, 291 (1962).
19. Scrafani, J. T., N. Williams, and D. H. Clouet, *Pharmacologist*, **12**, 230 (1970).
20. Pal, B. K., A. Goldstein, and L. I. Lowney, *Fed. Proc.*, **30**, A272 (1971) (abstract).
21. Folch, J., and M. Lees, *J. Biol. Chem.*, **191**, 807 (1951).
22. Van Praag, D., and E. J. Simon, *Proc. Soc. Exp. Biol. Med.*, **122**, 6 (1966).
23. Mule, S. J., C. M. Redman, and J. W. Flesher, *J. Pharmacol. Exp. Ther.*, **157**, 459 (1967).
24. Navon, S., and A. Lajtha, *Brain Res.*, **24**, 534 (1970).
25. Ingoglia, N. A., and V. P. Dole, *J. Pharmacol. Exp. Ther.*, **175**, 84 (1970).
26. Seevers, M. H., and G. A. Deneau, *Physiol. Pharmacol.*, **1**, 595 (1963).
27. Dole, V. P., *Annu. Rev. Biochem.*, **39**, 821 (1970), (see p. 826.)
28. Stephenson, R. P., *Brit. J. Pharmacol. Chemother.*, **11**, 379 (1956).
29. Paton, W. D. M., *Proc. Roy. Soc. Ser. B*, **154**, 21 (1961).