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LOCALIZATION IN BRAIN PARTICULATE FRACTIONS OF NARCOTIC ANALGESIC DRUGS ADMINISTERED INTRACISTERNALLY TO RATS*

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Abstract—Radio-labeled dihydromorphine, morphine, methadone and levorphanol were administered to rats in pharmacologically active doses by the intracisternal route of administration, and the rats were killed 0·5 or 1 hr later. The drugs were localized in the fraction of brain containing the pinched-off presynaptic nerve-endings, the synaptosomes, as well as in the soluble portion of the tissue. The ^{14}C-labeled narcotic antagonists, naloxone and nalorphine, were also localized in the same particulate fraction of brain. The amount of drug in the synaptosomal fraction was dependent on the dose of drug, on the time between drug administration and sacrifice, and on the region of brain, and was specific for each narcotic agonist and antagonist. The relevance of this localization of narcotic analgesic drugs in the synaptosomal fractions of rat brain to the mechanism of action of the drugs remains to be explored.

THE INCUBATION of brain slices or homogenates with narcotic analgesic drugs prior to subcellular fractionation of the tissue preparation leads to an accumulation of the drug in the nerve-ending particles, the synaptosomal fraction.[1,2] The relevance of this accumulation of drug to the localization of narcotic drugs in the central nervous system after the administration of the drugs to rats in pharmacologically active doses has been investigated in the studies reported in this paper. Narcotic agonists and antagonists labeled with carbon-14 or tritium were injected in pharmacologically effective doses into rats, usually by the intracisternal route of administration, and the subcellular localization of the drugs in whole brain, or in regions of brain, was examined after density gradient fractionation of tissue homogenates.[3]

MATERIALS AND METHODS

Drugs. Morphine was tritiated at the 7,8 double bond with tritium gas by the New England Nuclear Corp. The labeled dihydromorphine was removed from contaminating radioactivity by chromatography on Whatman 3 MM filter paper in an *n*-butyl ether–tertiary amyl alcohol–water system (60:7:13), elution with methanol and an initial dilution with unlabeled dihydromorphine to a specific radioactivity of 152·2 μCi/μmole. ^{14}C-Levo-methadone (28·8 μCi/μmole) was obtained through the courtesy of V. P. Dole of Rockefeller University and ^{3}H-levorphanol (26·7 μCi/μmole) through the courtesy of W. E. Scott of Hoffmann–LaRoche, Inc. ^{14}C-Morphine (16·5 μCi/μmole), ^{14}C-meperidine (5 μCi/μmole), ^{3}H-levo-methadone (92·5 μCi/μmole), ^{14}C-nalorphine (11·3 μCi/μmole) and ^{14}C-naloxone (6·3 μCi/μmole) were

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obtained commercially. The radiopurity of these drugs was examined by a thin-layer chromatography system in use in this laboratory.[4] All drug preparations were neutralized to pH 7 and diluted, with or without added unlabeled drug, so that the injected dose was contained in a volume of 0·05 ml or less.

Animals. The experimental animals were male Wistar rats weighing approximately 100 g, housed six to a cage, with food and water available at all times. The labeled drugs were administered under light ether anesthesia by way of the cisterna magna in doses approximately equianalgesic with 60 mg/kg of morphine administered by the intraperitoneal route. In the Wistar strain, this dose of morphine induces analgesia to a hot-plate at 55° for 3 hr. The doses injected intracisternally were: 50 or 52 μg of dihydromorphine, 50 μg of morphine, 100 μg of meperidine, 25 or 34 μg of levomethadone, 40 μg of levorphanol, and intraperitoneally 5·2 mg of dihydromorphine, each dose to rats weighing about 100 g. The analgesic response to these doses of narcotic agonists was blocked by the intraperitoneal injection of 0·2 mg of naloxone or 2 mg of nalorphine. Carbon-14 labeled naloxone and nalorphine were injected intracisternally in doses of 100 μg and 40 μg, respectively, without producing analgesia.

Tissue fractionations. At 30 or 60 min after the administration of the labeled drugs, the rats were killed by decapitation, and the brains were quickly removed and rinsed in 0·32 M sucrose buffered to pH 7·0 with 0·05 M Tris–HCl buffer, and homogenized in 10 volumes of the buffered sucrose solution. In some experiments the brains were dissected according to the procedure described by Glowinski and Iversen[5] before homogenization. One-ml aliquots of the homogenates were layered over linear sucrose gradients of 0·32 M to 1·5 M sucrose, which in turn had been layered over 5 ml of 1·5 M sucrose, and centrifuged for 20 hr in a model L-2 Spinco ultracentrifuge at 95,000 *g* in the six-place swinging bucket rotor, SW-27. The gradients were removed in one-ml serial fractions using an Isco gradient fractionator. Aliquots of each serial fraction were examined for radioactivity in a Nuclear-Chicago scintillation spectrometer, as previously described.[6] The protein content was measured in aliquots from the same serial fractions by the method of Lowry *et al.*[7] after the proteins were precipitated by trichloroacetic acid.

In some experiments, a crude mitochondrial fraction was shocked hypo-osmotically in order to rupture the synaptosomes. Brain homogenates were centrifuged at 1000 *g* for 10 min to remove the unbroken cells and debris, and then at 10,000 *g* for 20 min to sediment the crude mitochondrial fraction which contained most of the nerve-ending particles. Half of the mitochondrial pellets were homogenized in water, and the rest in buffered sucrose. Four-ml aliquots of both preparations were centrifuged through a discontinuous sucrose gradient, as described by Bosmann and Hemsworth.[8] In this fractionation the synaptic vesicles remain in the upper layer, the synaptosomal membrane "ghosts" at the interface, and the intact mitochondria packed at the bottom of the tube.[8]

For a comparison of the binding of ^{3}H-dihydromorphine after intracisternal injection and the binding in brain homogenates incubated with ^{3}H-dihydromorphine *in vitro*, three rats were injected with 52 μg of ^{3}H-dihydromorphine (0·5 μCi/μg) intracisternally and three rats were injected with a like volume of saline by the same route. All rats were killed 0·5 hr after the injections, and the brains were removed and rinsed with 0·32 M sucrose buffered with 0·01 M Tris–HCl buffer, pH 7·2. The brains

from the drug-injected rats were homogenized individually in 10 vol. of buffered sucrose and an aliquot from each homogenate was examined for radioactivity. An equal amount of ^{3}H-dihydromorphine was added to the buffered sucrose in which the brains from the saline-injected rats were homogenized and all homogenates were incubated at room temperature for 5 min. Crude mitochondrial fractions were sedimented as described above. One set of mitochondrial pellets was not washed, one set washed once, and the third set washed twice. Washing involved rehomogenization in buffered sucrose and recentrifugation at 15,000 *g* for 20 min. All six mitochondrial preparations were then centrifuged through a continuous sucrose gradient, fractionated and counted as described above.

Lipid solubilities. The partition of the labeled drugs between an organic and an aqueous phase was evaluated by adding each drug at a final concentration of 10^{-6} M to a two-phase system composed of 10 ml of ethylene dichloride and 10 ml of 0·001 M Tris–HCl buffer, pH 7·0, shaking the vessel for 30 min at room temperature and separating the layers for measurement of the radioactivity in each phase.

Electron microscopic examination. For electron micrographs, the particulate fractions were pelleted in osmium tetroxide, dehydrated and sectioned. The sections were stained with lead nitrate–uranyl acetate, and examined in a RCA-EMU 3G electron microscope.*

Recovery of ^{3}H-dihydromorphine. The identification of tritiated dihydromorphine after recovery from gradient fractions was made by paper chromatographic examination of organic extracts of the aqueous fractions. Three tubes from the synaptosomal area of each of six gradients were combined and brought to pH 10 with NaOH. The aqueous sample was extracted with 2 volumes of ethylene dichloride with shaking at room temperature for 1 hr. The organic phase was evaporated under nitrogen and dissolved in 0·1 ml of methanol for spotting on 3 MM filter paper. The solvent system described in the isolation of dihydromorphine was used to fractionate the sample. Over 95 per cent of the radioactivity in the aqueous sample from rats injected intracisternally with ^{3}H-dihydromorphine was found as a single spot at the R_f of dihydromorphine.

RESULTS

When whole tissue is fractionated by centrifugation of a tissue homogenate through a continuous linear sucrose density gradient, the nuclei and unbroken cells are sedimented to the bottom of the centrifuge tube, while the myelin, microsomes, mitochondria and nerve-ending particles are found as discrete bands in the gradient.[9] After centrifugation of a homogenate of whole brain, proteins were distributed throughout the gradient, with broad bands in the upper and lower halves of the gradient, and, in the lower band, a narrow peak of protein centered in one or two of the serial samples usually located at samples 25–28, at a sucrose molarity of approximately 1·0 M. This peak coincided with the fraction rich in nerve-ending particles, the synaptosomes, which has been described by DeRobertis *et al.*[10] and Whittaker.[11] When homogenates of brain prepared from rats killed 30 min after the intracisternal injection of 52 μg ^{3}H-dihydromorphine were centrifuged through this

* The authors wish to thank Mr. Martin Beson of the Albert Einstein College of Medicine for preparing the electron micrographs.

gradient, the distribution of labeled dihydromorphine was uneven. Peaks of high specific radioactivity were found in the soluble fraction (samples 1–8), in the myelin fraction (samples 10–18) and in the synaptosomal fraction (samples 23–28) (Fig. 1). The highest amount of ^{3}H-dihydromorphine in any particulate fraction was in the latter fraction, and was shown by electron micrographs to be rich in nerve-ending particles (Fig. 2).

The amount of drug localized in the synaptosomal area of the gradient was to some extent dependent on the dose of drug administered, as both the amount of drug remaining in brain 30 min after its injection and the amount of drug in the synaptosomal fraction were higher with increasing doses of dihydromorphine (Table 1).

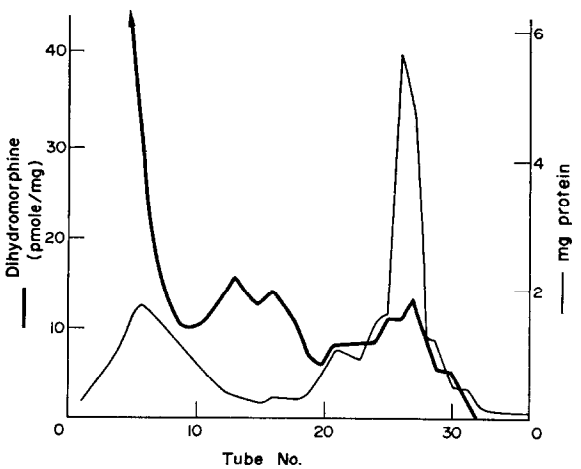

FIG. 1. Localization of ^{3}H-dihydromorphine in particulate fractions from rat brain. Three rats were injected with ^{3}H-dihydromorphine (50 μg/2·5 μCi) intracisternally and killed 1 hr later. Brain homogenates were fractionated by centrifugation through linear sucrose gradients with collection of 36 serial samples from the gradients. The average levels of drug and protein are shown for each sample tube.

The amount of drug remaining in brain varied with the interval of time between the injection and the sacrifice of the animal (Table 2). The highest amount of drug in the brain was found 10 min after the intracisternal injection, amounting to 38·5 per cent of the injected dose. The amount of drug in the synaptosomal fraction was 5·1 per cent of the total drug in brain 1 min after the injection, and 8·5 per cent after 30 min. However, the highest amount of drug in the synaptosomal fraction was found 10 min after the injection of drug (Table 2).

The distribution of ^{3}H-dihydromorphine in the synaptosomal bands of gradients prepared from different regions of the brains of rats killed 60 min after the injection of 50 μg of the drug into the CSF ranged from 3·23 nmoles/g wet wt in the hypothalamus to 0·41 nmole/g in the cortex (Table 3). The ratios of drug in synaptosomal fraction to total drug in the tissue homogenate varied from 0·14 in medulla to 0·25 in hypothalamus. In the same table, the levels and the regional distribution of particulate-localized ^{14}C-morphine, ^{14}C-meperidine and ^{3}H-levo-methadone are shown for brain preparations from rats killed 60 min after the injection of the labeled

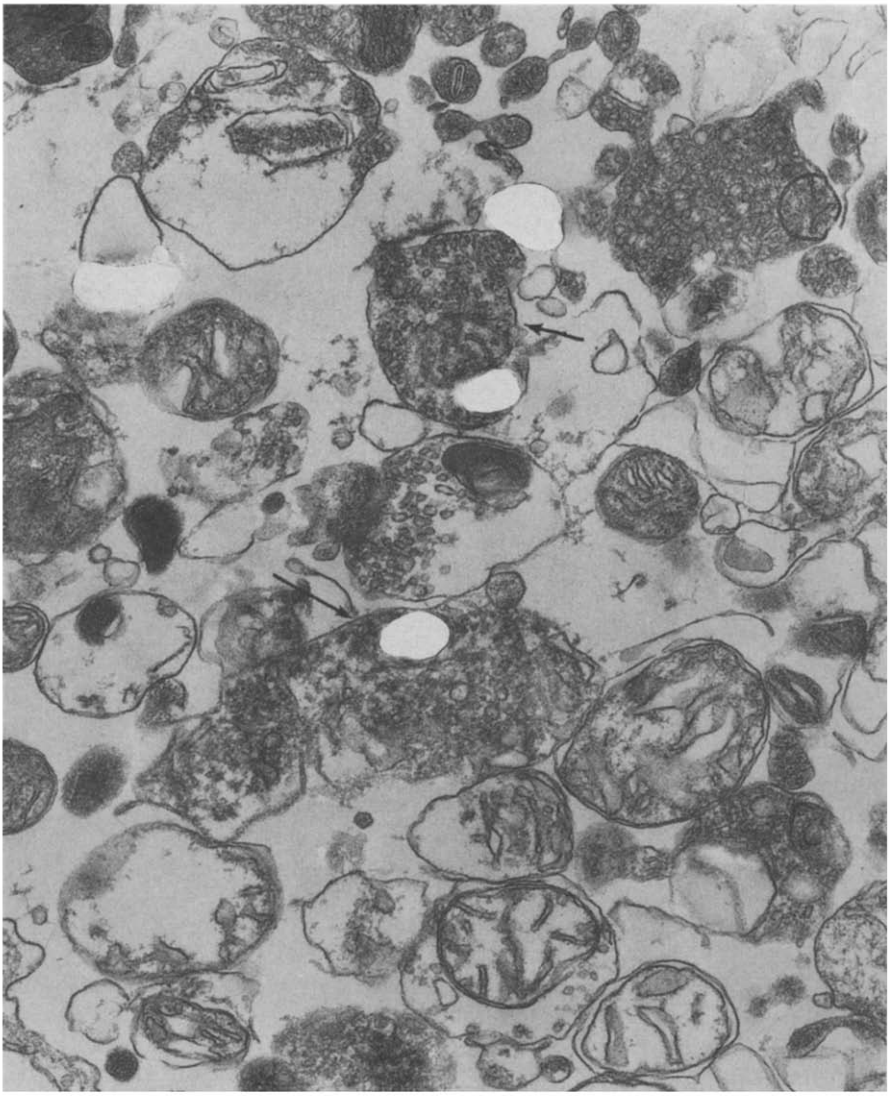

FIG. 2. Electron micrographs of tissue pellets from sample tubes 25 and 26 of the gradients shown in Fig. 1. There are a number of intact synaptosomes in the micrograph (arrows) (×54,000).

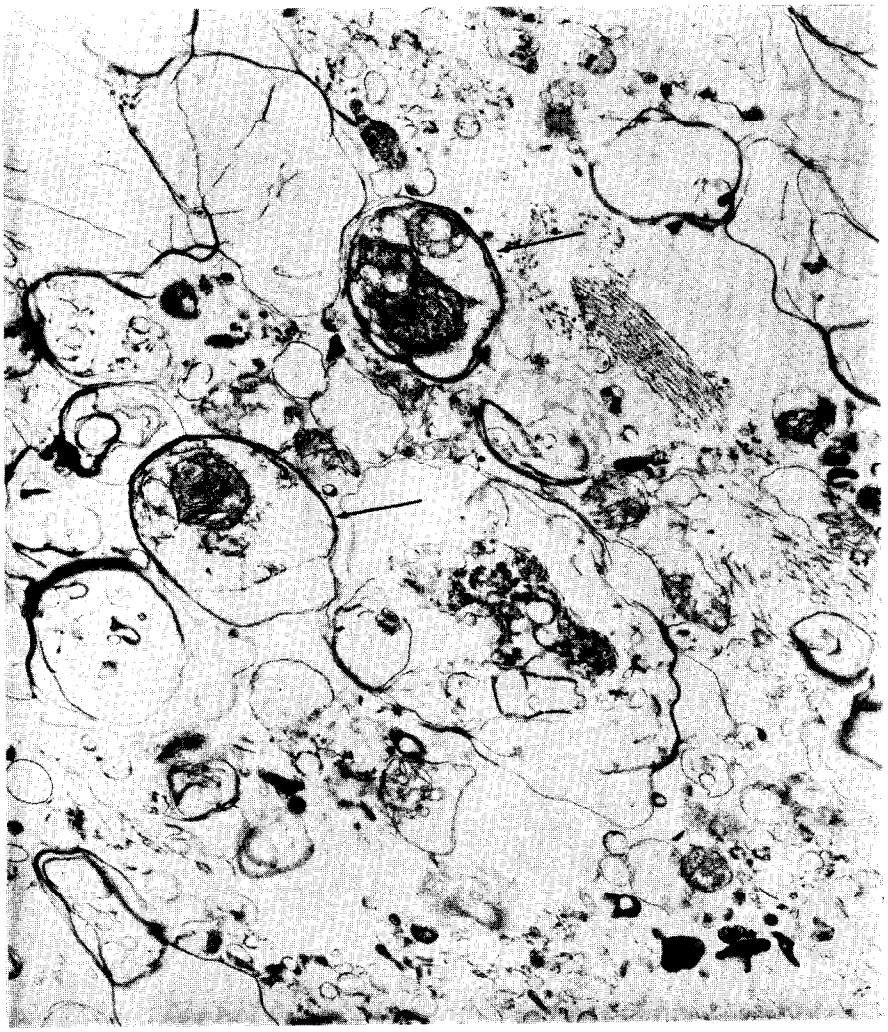

FIG. 4. Electron micrographs of tissue pellets from sample tubes 24–26 after centrifugal fractionation of hypo-osmotically shocked synaptosomal fraction. Disrupted synaptosomes and membrane "ghosts" are shown (arrows) (×43,200).

TABLE 1. EFFECT OF DOSE ON THE AMOUNT OF ^{3}H-DIHYDROMORPHINE IN THE SYNAPTOSOMAL FRACTION OF RAT BRAIN*

Dose (μg/rat)	^{3}H-dihydromorphine		
	Brain (μg/brain)	Synaptosomal fraction	
		(μg/brain)	(nmoles/g wet wt)
5·22	0·54	0·097	0·196
10·44	1·41	0·177	0·354
15·66	2·11	0·321	0·641
20·88	3·36	0·408	0·816
26·10	4·13 ± 0·54	0·485 ± 0·069	0·970
31·32	5·07	0·703	1·406
52·20	6·29 ± 0·63	0·772 ± 0·102	1·544

* The rats were injected intracisternally with the specified doses of ^{3}H-dihydromorphine (498 μCi/mg) and killed 30 min later. There were two rats in each group except for the groups with doses of 26·1 μg and 52·2 μg, which were composed of fifteen and eight rats respectively. The standard deviations are shown for these latter groups.

drugs. Meperidine and methadone had two sites of particulate localization, one in samples 10–15, an area rich in myelin and membrane fragments, and the other in the synaptosomal fraction. The uptake of methadone and meperidine in these sites was combined to show particulate accumulation in Table 3. The amounts of morphine and dihydromorphine remaining in brains of rats killed 60 min after drug administration were much higher than the amounts of methadone and meperidine, and consequently, the amounts of synaptosomal-bound drug were also higher for morphine and dihydromorphine, although the ratios of drug in synaptosomal fraction to total drug were as high as 0·40 for methadone in midbrain and medulla (Table 3). In general, the levels of morphine and dihydromorphine were higher in regions rich in

TABLE 2. EFFECT OF TIME ON THE AMOUNT OF ^{3}H-DIHYDROMORPHINE IN THE SYNAPTOSOMAL FRACTION OF RAT BRAIN*

Time (min)	Total drug (nmoles/g brain)	Drug in synaptosomal fraction (nmoles/g brain)
1	33·72 ± 6·28	1·73 ± 0·14
5	35·44 ± 3·17	1·93 ± 0·25
10	39·46 ± 2·21	2·63 ± 0·20
15	33·62 ± 5·73	2·47 ± 0·24
30	21·08 ± 6·28	1·80 ± 0·16
60	15·89 ± 3·25	1·35 ± 0·09

* The rats were injected with 50 μg of ^{3}H-dihydromorphine (498 μCi/mg) intracisternally and killed at intervals thereafter. There were three rats in each group with the drug levels in brain and in the synaptosomal fraction from the density gradient of whole brain measured separately, with standard deviations.

cell bodies such as cerebellum and hypothalamus, and lower in areas containing many lipid structures such as midbrain and medulla. There seemed to be a positive correlation between the relative lipid solubility of the drugs (Table 4) and their abundance in anatomical and subcellular lipid-rich areas of brain, and a negative

TABLE 3. ACCUMULATION OF NARCOTIC DRUGS IN PARTICULATE FRACTIONS OF REGIONS OF RAT BRAIN*

Uptake (nmoles drug in fraction/g wet wt region)					
Cerebellum	Medulla	Hypothalamus	Striatum	Midbrain	Cortex
		^{3}H-dihydromorphine (50 μg)			
3·03 ± 0·22	1·62 ± 0·22	3·23 ± 0·33	0·52 ± 0·06	0·97 ± 0·18	0·41 ± 0·03
		^{14}C-morphine (50 μg)			
1·65 ± 0·07	1·21 ± 0·18	1·85 ± 0·22	0·61 ± 0·12	0·86 ± 0·21	0·75 ± 0·09
		^{3}H-levo-methadone (34 μg)			
0·25 ± 0·03	0·84 ± 0·16	0·28 ± 0·01	0·32 ± 0·06	1·76 ± 0·08	0·30 ± 0·02
		^{14}C-meperidine (100 μg)			
0·31 ± 0·01	0·45 ± 0·05	0·36 ± 0·12	0·26 ± 0·05	1·13 ± 0·26	0·29 ± 0·03

* The rats were killed 1 hr after the intracisternal injection of the amounts of drugs specified. There were six animals measured in pairs for each drug except meperidine, in which two brain pools from two rats each were dissected and fractionated by gradient centrifugation.

correlation between relative lipid solubility and the amount of drug remaining in the synaptosomal fraction 30 min (Table 4) or 60 min (Table 3) after the injection of drug.

The localization of dihydromorphine in the synaptosomal fraction of brain was not dependent on the intracisternal route of drug administration. When an equi-analgesic dose of ^{3}H-dihydromorphine was injected into rats by the intraperitoneal route, the

TABLE 4. LIPID SOLUBILITY AND UPTAKE OF NARCOTIC DRUGS IN THE SYNAPTOSOMAL FRACTION OF RAT BRAIN*

Drug (dose)	Solubility (% in organic phase)	Uptake (nmoles/g brain)	Ratio (particulate/total)
Methadone (34 μg)	96	0·57	0·34
Meperidine (100 μg)	92	0·76	0·23
Naloxone (100 μg)	80	0·81	0·24
Nalorphine (40 μg)	28	1·07	0·24
Levorphanol (40 μg)	11	1·10	0·27
Morphine (50 μg)	1·7	1·23	0·10
Dihydromorphine (50 μg)	0·6	1·29	0·14

* The relative solubility was measured by partitioning 10^{-6} M radio-labeled drug between ethylene dichloride and 0·001 M Tris–HCl buffer, pH 7·0 (1:1), and determining the radioactivity in the aqueous and organic phases. The uptake is the amount of labeled drug found in the synaptosomal area of the gradient after centrifugal fractionation of whole brain homogenates prepared from rats killed 30 min after drug administration. The ratio is the amount of drug in the synaptosomal fraction divided by the total drug in the brain homogenate.

amount of drug in brain after 30 min (6·37 ± 0·63 nmoles/g), and the amount of drug localized in the synaptosomal fraction (0·90 ± 0·11 nmoles/g), was only slightly lower than the corresponding levels after drug administration by the intracisternal route (7·11 ± 0·16 and 1·29 ± 0·02 nmoles/g respectively).

The chemical and pharmacological specificity of narcotic localization in brain particulate fractions was tested by pretreating rats with inactive isomers of active narcotic agonists, or with narcotic antagonists, before injecting a labeled narcotic agonist, and comparing the uptake with that in animals receiving the agonists alone. The administration of an inactive isomer, dextrorphan, at a dose of 10 mg/kg by the intraperitoneal route had no effect on the subsequent binding of ^{3}H-levorphanol

TABLE 5. EFFECT OF ANTAGONISTS AND INACTIVE ISOMERS ON THE UPTAKE OF NARCOTIC AGONISTS *in vivo**

Drug pairs	Uptake (nmoles/g brain)
^{3}H-levorphanol†	1·01 ± 0·08
+ dextrorphan‡	1·10 + 0·13
^{3}H-levo-methadone†	0·46 ± 0·04
+ dextromethadone‡	0·52 ± 0·08
^{3}H-dihydromorphine†	0·61 ± 0·07
+ naloxone‡	0·70 ± 0·06
^{14}C-naloxone†	0·71 ± 0·03
+ dihydromorphine‡	0·56 ± 0·01§
^{3}H-dihydromorphine‡	0·88 ± 0·02
+ naloxone†	0·74 ± 0·06§

* The rats were killed 30 min after the administration of the radiolabeled drugs, and 40 min after the intraperitoneal injection of naloxone, dextromethadone or dextrorphan. There were three rats in each group, measured separately.

† These drugs were administered intracisternally in doses of: 40, 25, 15·66 and 100 μg for levorphanol, levo-methadone, dihydromorphine and naloxone respectively.

‡ These drugs were administered intraperitoneally in doses of 10, 6, 0·2 and 2·6 mg of dextrorphan, dextromethadone, naloxone and dihydromorphine respectively.

§ The difference in uptake in the absence and presence of a second drug is significant at $P < 0·01$.

(Fig. 3). Similarly, the amount of ^{3}H-levo-methadone found in the synaptosomal fraction was not affected by the prior administration of the dextro isomer (Table 5). The systemic administration of the narcotic antagonist, naloxone, blocked the pharmacological response to intracisternally administered ^{3}H-dihydromorphine, but did not alter the amount of drug in the synaptosomal fractions (Table 5). In the reverse experiment, in which ^{14}C-naloxone was injected into the CSF and ^{3}H-dihydromorphine was administered intraperitoneally at the same time, there was a significant decrease in the amount of each drug in the synaptosomal fraction of brain when the other drug was also administered (Table 5).

The distribution of ^{3}H-dihydromorphine in the various components of the synaptosomes after rupture of the particles by hypo-osmotic shock was examined by subjecting

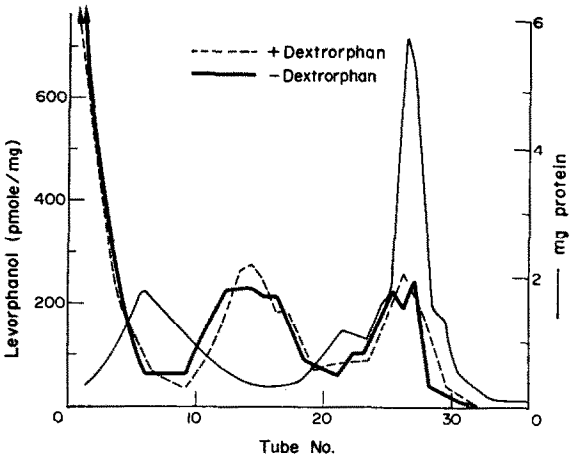

FIG. 3. Localization of ^{3}H-levorphanol in particulate fractions of rat brain. Two groups of three rats each were injected intracisternally with 40 μg of labeled levorphanol 10 min after one group had been pretreated with 10 mg of dextrorphan intraperitoneally. All rats were killed 30 min after the injection of levorphanol. The uptake of levorphanol in the particulate fractions is shown for the rats without (——) and rats with (- - - -) dextrorphan pretreatment, with the protein curve from Fig. 1 as reference.

ruptured and intact synaptosomal preparations to discontinuous sucrose gradient fractionation. A comparison of the gradients showed that the amount of drug in the synaptosomal area of the gradient (tubes 25–30 in the discontinuous gradient) decreased from 0·339 to 0·105 nmole/g of brain, while the amount of drug at the top of the gradient (tubes 1–6) increased from 0·214 to 0·455 nmole/g of brain after synaptosomal rupture. Since the synaptic vesicles equilibrate at 0·4 M sucrose (tube 5 or 6), the released drug could be either entirely soluble (or bound to soluble

TABLE 6. BINDING OF ^{3}H-DIHYDROMORPHINE TO SYNAPTOSOMAL FRACTIONS *in vivo* AND *in vitro**

	In vivo	*In vitro*
Homogenate	$1{\cdot}56 \times 10^6 \pm 0{\cdot}19$†	$1{\cdot}33 \times 10^6 \pm 0{\cdot}07$
Supernatant 15,000 *g*	$0{\cdot}80 \times 10^6 \pm 0{\cdot}10$	$0{\cdot}79 \times 10^6 \pm 0{\cdot}01$
Crude mitochondria	$52{\cdot}47 \times 10^3 \pm 6{\cdot}99$	$44{\cdot}07 \times 10^3 \pm 7{\cdot}16$
Washed mitochondria	$12{\cdot}60 \times 10^3 \pm 1{\cdot}16$	$5{\cdot}83 \times 10^3 \pm 1{\cdot}21$
Twice washed mitochondria	$6{\cdot}10 \times 10^3$	$2{\cdot}35 \times 10^3$
Synaptosomal fraction	$3{\cdot}31 \times 10^3 = 8{\cdot}3\%$	$1{\cdot}19 \times 10^3 = 4{\cdot}0\%$
Synaptosomal fraction washed once	$1{\cdot}73 \times 10^3 = 17{\cdot}4\%$	$0{\cdot}16 \times 10^3 = 4{\cdot}9\%$
Synaptosomal fraction washed twice	$0{\cdot}57 \times 10^3 = 16{\cdot}2\%$	$0{\cdot}05 \times 10^3 = 3{\cdot}9\%$

* Three rats were injected intracisternally with 52 μg of ^{3}H-dihydromorphine (0·5 μCi/μg) and killed 0·5 hr later and three rats were injected with saline intracisternally and killed 0·5 hr later. The brains were removed, rinsed and homogenized in 10 ml of buffered sucrose and ^{3}H-dihydromorphine was added to the homogenates of the brains of the saline-injected rats to bring the radioactivity in the homogenates equal to that in the three preparations from drug-treated rats. All six brains were incubated at room temperature for 5 min, and then fractionated as described in the Methods section.

† The radioactivity is expressed as counts per min per fraction derived from one rat brain. The ratio of synaptosomal:crude mitochondrial radioactivity is expressed as per cent.

components) or partially vesicle-bound. The localization of the drug remaining in tubes 25–30 at the interface between 0·6 M and 1·5 M sucrose coincided with that of the synaptosomal membrane "ghosts" which accumulate at the interface (Fig. 4).

A difference was found in the binding of ^{3}H-dihydromorphine to brain nerve-ending fractions *in vitro* and that after the intracisternal injection of the drug. There was more drug, and a higher ratio of synaptosomally-bound to total mitochondrial drug, in preparations from injected animals than after incubations *in vitro* (Table 6). After two washes of the crude mitochondrial preparations, there was twelve times more drug in the nerve-ending fraction labeled *in vivo*, and 17 per cent of the drug initially in the synaptosomal fraction remained after two washings in contrast to 4 per cent left of the drug bound *in vitro* (Table 6).

DISCUSSION

The localization of labeled narcotic analgesics in the synaptosomal fraction of rat brain isolated by gradient centrifugation after an incubation *in vitro* of brain homogenate and radioactive drug may be attributed to a physiologically unimportant ionic binding of basic drugs to acidic tissue constituents.[12] The accumulation of labeled drug in the synaptosomal fraction after the intracisternal administration of the drug may also be ascribed to this kind of post-mortem binding, since homogenization of the brain brings organelles into contact with free drug. Evidence suggesting that some of the labeled drug found in the synaptosomal fraction gained this site during technical manipulations of the tissue preparation includes: (1) the correlation of the level of ^{3}H-dihydromorphine in the synaptosomal fraction with the amount in the brain homogenate, when the time of drug exposure *in vivo* and doses of drug were varied, and when the route of administration was varied, and (2) the lack of effect of inactive isomers and antagonists of the pharmacologically active radio-labeled agonists on localization of the agonists. The same data may also be used to support the idea of a pre-sacrifice localization of drug in the nerve-endings. The ratio of synaptosomally-bound to total ^{3}H-dihydromorphine varies with time and dose, as do the ratios in brain regions. The wide spread in ratios of bound:total drug for other agonists and antagonists suggests that the chemical characteristics of the drugs govern the rates of entrance and exit of the drugs from the CNS, and only indirectly affect drug localization in the particulate fraction. The inverse correlation between relative lipid solubility of a drug and the amount of the drug in the nerve-ending fraction after a 30-min drug exposure supports the concept of a blood:brain barrier (or a CSF:brain barrier) for narcotic analgesic drugs,[13] since the more hydrophilic drugs are present in higher concentration in brain at this time than are the more lipophilic drugs.

It has been noted in this laboratory that the nerve-ending fractions labeled *in vivo* retained more labeled drug during technical manipulations such as rehomogenization and recentrifugation than brain preparations labeled by incubations *in vitro*. An experiment designed to test this observation supported it: there was more ^{3}H-dihydromorphine in the synaptosomal area of the gradient whether the crude mitochondrial fraction was fractionated without washing or with two washings, when the drug was introduced *in vivo* (Table 6). These results suggest that no more than half of the synaptosomally localized ^{3}H-dihydromorphine can be attributed to binding *in vitro* after sacrifice of the injected rats, and that the drug reaching the nerve-ending after

injection is less easily removed from the nerve-ending fraction than drug bound *in vitro*.

The question of specificity of the localization of narcotic analgesic drugs in the particulate fractions of brain which contain the nerve-ending particles is pertinent to the pharmacological importance of the localization. Only a small proportion of the ^{3}H-levorphanol bound to brain membrane fractions *in vitro* was found to be unsusceptible to replacement by an inactive isomer.[12] If such a small proportion of the drug bound *in vivo* is specific pharmacologically, the effects of inactive isomers and/or antagonists would be within the experimental error, and thus not detected. In these experiments, only when the agonist and antagonist were administered simultaneously, the first by a systemic route and the second by the intracisternal route, was there an effect on the uptake of both drugs in the brain synaptosomal fraction. It is possible that the blocking effects are exquisitely sensitive to the duration of exposure to each drug, and that the optimal timing for this effect was not attained.

The nature of the organelles which bind the labeled narcotics, or whether more than one type of organelle binds the drugs, cannot be determined solely by the molarity of sucrose at which the particles equilibrate. While nerve-ending particles predominate in the electron micrographs prepared from tubes 24–48, there were also small mitochondria and synaptic vesicles within the synaptosomal membrane as well as some membrane fragments. The division of synaptosomally bound ^{3}H-dihydromorphine between the soluble and the membrane fractions after hypo-osmotic shock of the synaptosomal fractions is evidence that at least half of the bound drug was freed by the rupture of the synaptosomal membrane, either because the drug was intrasynaptosomal, or because it was bound to membranal elements disturbed by the rupture. The most likely site for the rest of the synaptosomally bound drug is the membrane "ghosts." In other studies in which the synaptosomal membranes derived from the brains of rats injected with ^{3}H-levo-methadone were further fractionated by the method of Azcurra and DeRobertis,[14] a specific localization in one membrane fraction, the M_1 (1·0) fraction, was found, amounting to 45 pmoles drug/mg of membrane protein.[15]

The large amount of drug which is localized in the soluble portion of the gradient may be free drug, or freed drug, or soluble protein-bound drug. There was also labeled drug at the bottom of the gradient where unbroken cells and the nuclei are sedimented. In the present experiments, this radioactivity was not examined systematically, although in earlier experiments, ^{3}H-dihydromorphine was found in fractions of RNA derived from brain nuclei.[16]

The present data suggest that labeled narcotic analgesic drugs are accumulated in the synaptosomal fraction of brain in a time-, dose- and region-dependent manner after the administration of the drugs in pharmacologically effective doses to rats, and that the characteristics of the synaptosomal uptake vary for the individual drugs. The synaptosomal boundary membrane is suggested as one site of drug accumulation. The question of the relevance of the localization of narcotic drugs in the nerve-endings to the site of action of the drugs remains to be explored by a refined methodology.

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