Studies on the Intracellular Distribution and Tissue Binding of Dihydromorphine-7,8-H^3 in the Rat.* (31036)

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Dihydromorphine-7,8-H^3 has been prepared by the catalytic tritiation of morphine. This method yields a radioactive analgesic, closely related to morphine, of high specific activity and labeled in chemically and metabolically stable positions. The present report deals with studies on the intracellular distribution of this drug after intravenous administration to rats, as well as with *in vitro* binding studies of the compound to tissue homogenates and serum. The effect of the narcotic antagonist nalorphine on the distribution and binding of dihydromorphine was also investigated. The ultimate goal of these studies is the detection of the putative receptor for narcotics frequently postulated to exist in the central nervous system. A tissue component with properties consistent with those of a possible receptor should be capable of binding morphine and other narcotics. This binding should be reversed readily by the addition of nalorphine.

Our present data on the distribution and binding of dihydromorphine-7,8-H^3 are similar to those reported for C^{14}-methyl labeled levorphanol by Mellet and Woods(1).

Materials and methods. Morphine sulfate and nalorphine were obtained through the generosity of Merck, Sharp & Dohme Research Laboratories.

Preparation and purification of labeled dihydromorphine. Dihydromorphine-7,8-H^3 was prepared by the catalytic tritiation of the 7,8 double bond of morphine. The tritiation was carried out by the New England Nuclear Corp. The reaction was run in ethyl acetate using a paladium catalyst adsorbed on charcoal. The product generally contained about 50-60% of impurities which were chromatographically separable from dihydromorphine. The main step in the purification procedure was preparative chromatography on Whatman 3MM paper, buffered at pH 7, using the solvent system of Misra and Woods(2). The dihydromorphine peak was eluted with methanol, evaporated to dryness and dissolved in HCl. Nonradioactive carrier dihydromorphine was added and further purification was achieved by precipitation of the free base with alkali. Dihydromorphine was recrystallized from 75% ethanol until its specific activity remained constant. It was isotopically pure in 2 paper chromatographic systems(2, 3). Before dilution with carrier, the material had a specific activity as high as 25 curies/mmol. The final material used for *in vivo* experiments had a specific activity of about 150 mC/mmol. The material was diluted further for binding studies.

Induction of tolerance in rats. Fourteen rats were divided into 2 groups of 7 each. One group was made tolerant to morphine sulfate by gradually increasing a single daily dose of morphine injected intraperitoneally from 15 mg/kg to 160 mg/kg daily over a period of 28 days. Thereafter for a period of 14 additional days the rats received a daily dose of 160 mg/kg of morphine sulfate before sacrifice(4). When 4 untreated rats of the same weight were injected with an equal dose of morphine sulfate, one of the 4 died after 1 hour and the remaining three were in a state of complete stupor for 14 hours after the injection. The tolerant rats remained alert and active and showed no sign of narcosis. The tolerant rats, as well as rats in the control group, were sacrificed by decapitation. On the day of sacrifice no morphine was administered.

Preparation of serum. Blood was removed by cardiac puncture from 5 rats anesthetized with ether. The blood was left to clot for 1 hour at room temperature and the serum obtained by centrifugation. For comparison, human serum was also obtained.

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Intracellular fractionation of liver and brain. Adult male rats of the Sherman strain weighing 150-200 g were injected intraperitoneally with 100 mg/kg of dihydromorphine-H³ calculated as free base. The total radioactivity administered per animal was 8 millicuries. Nalorphine(5), 30 mg/kg, in one experiment was added 30 minutes after dihydromorphine, in another, 100 mg/kg nalorphine was given in 3 portions, 30 minutes before, 30 minutes after, and 50 minutes after injection of the labeled dihydromorphine. The rats were sacrificed by decapitation 1 hour after administration of the isotopic material. The brain was removed quickly and weighed in ice-cold 0.25 M sucrose. The liver was excised after perfusion with cold 0.25 M sucrose and weighed. The brain was minced and homogenized in 0.25 M sucrose in the all glass homogenizer described by Dounce *et al*(6). The liver mince was homogenized in a conventional motor-driven Potter-Elvehjem tissue grinder. All homogenates were diluted to a final concentration of 10% (w/v). A modified Schneider-Hogeboom procedure(7) was used for the fractionation of both brain and liver.

The nuclear fraction was obtained by centrifugation at 2,000 $\times$ *g* for 10 minutes in the International Refrigerated Centrifuge. The mitochondrial fraction was sedimented at 20,000 $\times$ *g* for 10 minutes. The microsomal fraction was obtained by centrifugation at 100,000 $\times$ *g* for 60 minutes in the Spinco Preparative Ultracentrifuge. Each fraction was washed by recentrifugation at the respective original speed. All particulate fractions were suspended in 5 ml of water for counting and protein determination. The soluble fraction was assayed without further treatment. The proteins were determined by the method of Lowry *et al*(8). For determination of radioactivity 50 or 100 μl samples were treated with 0.5 ml of Hydroxide of Hyamine. Samples were left to stand at room temperature with occasional shaking to achieve a clear solution, then 18 ml of Bray solution(9) were added. Counting was done in the Packard Tricarb Liquid Scintillation Counter. Efficiency of counting was determined by adding a toluene-H³ standard to the counting vials.

Binding studies. Brain, liver and serum were removed from normal and tolerant animals in the manner already described. The homogenates were prepared in Tris buffer pH 7.4, ionic strength 0.04 (unless stated otherwise) and homogenates and serum were dialyzed against the same buffer for 48 hours. For *in vitro* binding studies, equilibrium dialysis(10,11) was carried out by adding 5 ml of homogenate to a Visking dialysis bag which was placed in a stoppered glass vial containing 5 ml of Tris buffer. Dihydromorphine-7,8-H³ hydrochloride (20 mC/mmol) was added to the dialysis bag or to the medium bathing it. The vials were agitated for 24 hours on a rotating wheel in the cold room. The bathing medium and the contents of the dialysis bag were then analyzed separately for radioactivity. The homogenate was also analyzed for protein. The results of binding experiments are expressed as per cent of drug bound, calculated by the formula:

$$\frac{\text{C inside} - \text{C outside}}{\text{C inside}} = \text{fraction bound (C} = \text{drug concentration)}.$$

Per cent of binding of dihydromorphine is given after subtraction of any apparent binding resulting from the Donnan equilibrium. The extent of the Donnan effect in the system was determined by adding 0.05 milliequivalents of KCl and determining the K^+ distribution with a flame photometer after equilibrium dialysis. At ionic strength of .01 the K^+ concentration inside the dialysis bag was 7-10% higher than on the outside. The observed binding of dihydromorphine was corrected for this amount of apparent binding contributed by the Donnan effect. As the ionic strength of the buffer was raised, the Donnan effect decreased rapidly. At ionic strength .04, and above, it was within the error of our measurements.

All homogenates represent pools of brains and of portions of livers from 7-12 rats. They were used immediately or after storage in the deep freeze with identical results.

Results. Effect of nalorphine on intracellular distribution of radioactivity after injection of dihydromorphine-H³. Experiments showing the intracellular distribution of radioactivity

in rat brain and liver 1 hour after administration of dihydromorphine-H³ and the effect of nalorphine upon this distribution are summarized in Table I.

Both in the brain and in the liver the bulk of the radioactivity was found in the soluble fraction. A rather high level of isotopic material in the nuclear fraction of the brain (10-20%) was a consistent finding. Relatively little (2-8%) was present in the mitochondrial and microsomal fractions while 65-70% appeared in the supernatant. In the liver virtually all of the recovered radioactivity (90-95%) was generally found in the soluble fraction. The injection of nalorphine in amounts sufficient to rouse the rat from stupor had no detectable effect on the distribution of radioactivity in the cells of brain or liver. The organ radioactivity which for the brain averaged about 0.01-0.02% and for the liver 8-12% of the total injected dose was also unaffected by nalorphine, in agreement with reports from other laboratories(12).

TABLE I. Effect of Nalorphine on Intracellular Distribution of Radioactivity 1 Hour After Injection of Dihydromorphine-H³.*

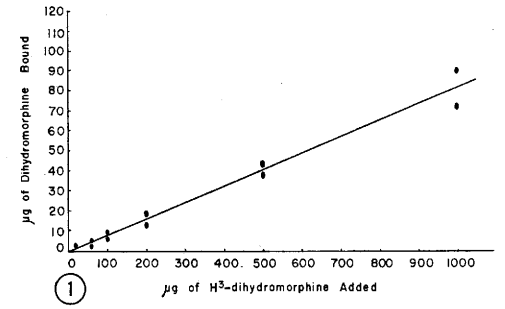
	Control		+Nalorphine†		Control		+Nalorphine‡	
	DPM × 10⁻⁴/ mg protein	% of homogenate	DPM × 10⁻⁴/ mg protein	% of homogenate	DPM × 10⁻⁴/ mg protein	% of homogenate	DPM × 10⁻⁴/ mg protein	% of homogenate
Brain fraction:								
Homogenate	1.24	100.0	1.72	100.0	2.32	100.0	2.36	100.0
Nuclear	.74	18.1	.86	16.0	1.20	19.5	1.04	8.9
Mitochondrial	.29	5.8	.35	4.1	.67	7.9	.80	11.5
Microsomal	.35	1.9	.27	1.1	.65	2.0	.92	2.9
Soluble	2.68	73.5	3.36	68.7	5.6	71.0	5.45	69.3
Liver fraction:								
Homogenate					88.5	100.0	139.0	100.0
Nuclear					15.5	4.4	25.5	4.2
Mitochondrial					25.4	5.9	24.7	3.9
Microsomal					30.8	2.9	56.9	3.4
Soluble					240.0	92.0	320.0	93.0

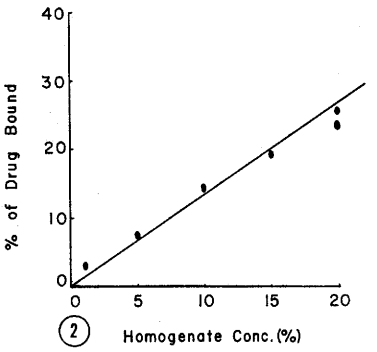
* Similar results were obtained in 2 additional experiments.
† 30 mg/kg of nalorphine administered 30 min after dihydromorphine-H³.
‡ 100 mg/kg of nalorphine administered in 3 portions: 30 min before, 30 min after, and 50 min after dihydromorphine-H³.

The total radioactivity found in the brain represents a concentration of dihydromorphine of approximately 3-5 μg/g wet weight of tissue 1 hour after administration of 100 mg of drug per kg of rat. Sanner and Woods (13) have studied the distribution of tritium-labeled dihydromorphine between maternal and fetal tissues of pregnant rats. One hour after administration of 1, 2 and 4 mg/kg, they found brain levels of dihydromorphine of 20, 55 and 105 ng/g, respectively. A comparison of these data indicates that the proportionality between dose of dihydromorphine injected and drug concentration in the brain holds between doses of 1 and 100 mg/kg. This appears to be true for liver as well.

Binding studies. It was possible to demonstrate binding of dihydromorphine to tissue homogenates by the use of equilibrium dialysis. After correction for the Donnan effect, the extent of binding to a 20% homogenate of brain or liver at ionic strength = 0.04 and pH 7.4 was 15-20%. In dialyzed rat and human serum the binding was 8%. In a 2% solution of bovine albumin the binding was 7%. The binding was weak, as shown by the fact that exhaustive dialysis removed virtually all of the radioactivity. Varying the amount of drug added from 20 to 1,000 μg, while the homogenate concentration, pH and ionic strength were kept constant, resulted in a linear increase of binding as shown in Fig. 1. No maximum was reached. Fig. 2 indicates that an increase in the homogenate concentration from 5-20% also produced a linear increase in binding. Binding decreased as the

ionic strength was raised, as shown in Fig. 3. The pH optimum of binding was pH 7.4 with a shallow decline on both sides. When similar experiments were carried out with tissue homogenates from rats made tolerant to morphine, no difference was observed in the degree of binding of dihydromorphine-7,8-H³ as shown in Table II.





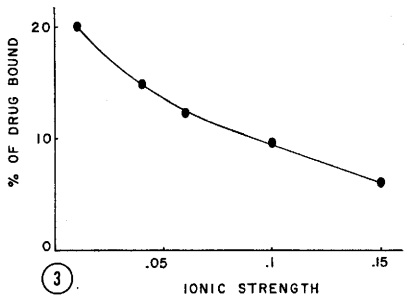


FIG. 1. The effect of drug concentration on the amount of drug bound by rat brain homogenate. The homogenate concentration was 20% (w/v). Total volume was 10 ml. Ionic strength was 0.04 and pH 7.4.

FIG. 2. Effect of homogenate concentration on the percent added dihydromorphine bound. These experiments were carried out with rat brain homogenate at ionic strength 0.01 and pH 7.4. 100 μg of dihydromorphine were added. Total volume was 10 ml.

FIG. 3. The effect of ionic strength on the percent of added dihydromorphine bound by rat brain homogenate. The experiments were carried out with a 20% (w/v) homogenate at pH 7.4. 100 μg of dihydromorphine were added. Total volume was 10 ml.

TABLE II. Binding of Radioactive Dihydromorphine in Brain and Liver Homogenates from Control and Tolerant Rats.

	% Binding	
	Control	Tolerant
Brain	13.6	16.5
	19.4	15.4
Liver	18.6	19.9
	20.3	16.1

20% homogenate in Tris buffer pH 7.5, ionic strength = .04. Production of tolerance is described in Experimental Section. Homogenates represent the pooled brains and portions of the livers from 7 control and 7 tolerant animals, respectively.

The effect of nalorphine and unlabeled dihydromorphine on the tissue binding of dihydromorphine-H³ was studied as shown in Table III. Replicate samples of rat brain homogenate were placed in dialysis bags and allowed to equilibrate with radioactive dihydromorphine for 18 hours. At this time the dialysis bags were transferred to fresh buffer containing no additions, nalorphine, and unlabeled dihydromorphine, respectively. Dialysis was continued for 30 hours at which time the amount of binding was measured. As seen from the Table, the degree of binding was not affected by the presence of nalorphine or of unlabeled dihydromorphine. An experiment in which the homogenates were preincubated with nalorphine or dihydromorphine for 18 hours followed by addition of labeled drug gave similar results. (The per cent of drug bound was 20%, 22% and 21%, respectively.)

Experiments were also carried out to determine the tissue binding of labeled dihydromorphine after injection into rats. When homogenates of brain and liver from injected rats were subjected to equilibrium dialysis, the radioactivity distributed itself in a manner analogous to that observed when the drug was added to the tissue homogenates *in vitro*.

Discussion. Dihydromorphine resembles morphine closely in its pharmacological properties. It was chosen for this study because the catalytic tritiation of the double bond of morphine results in a product of very high

TABLE III. Binding of Dihydromorphine by Brain Homogenate in Presence of Nalorphine or Unlabeled Dihydromorphine.

Brain homogenate	cpm outside of bag at 18 hr	Transferred to buffer plus:	% Drug bound* at 48 hr
1	2128	no additions	24.5
2	2138	nalorphine (40 μg)	27.0
3	2170	dihydromorphine (40 μg)	25.6

The experiment was performed with 10% rat brain homogenate at pH 7.4 and ionic strength = .01. 40 μg of dihydromorphine-H^3, containing 2 microcuries of radioactivity, were added to each sample. 0.1 ml aliquots were counted.

* Not corrected for Donnan effect.

specific activity which is readily purified. Furthermore, this method yields a ring-labeled compound of high chemical and metabolic stability.

The cell fractionation studies carried out with brains and livers of rats injected with dihydromorphine-7,8-H^3 show most of the radioactivity to be in the soluble fraction. It is of interest, however, that in brain the nuclear fraction was found to contain consistently about 10-20% of the radioactivity of the homogenate. This fraction contains cell membranes in addition to nuclei which raises the possibility that a portion of the drug may have been associated with the cell membranes. The lack of effect of nalorphine administration on the distribution of isotope and, particularly, on the amount of radioactivity found in the nuclear-membrane fraction makes it unlikely that this is the pharmacologically active material. The possibility that radioactivity bound to a particulate fraction of the cells in the intact tissue was released during homogenization cannot be excluded. This would, of course, explain the lack of effect of nalorphine. Other explanations such as the possibility that the active portion of narcotic is exceedingly small and, perhaps, concentrated in a relatively small area of the brain are equally plausible.

The equilibrium dialysis experiments demonstrated that tissue constituents can bind dihydromorphine. However, the binding is quite weak since all the radioactivity can be removed by exhaustive dialysis. The fact that liver and brain homogenates, as well as serum, bind similar amounts of dihydromorphine and the lack of effect of nalorphine on the binding suggest that most, if not all, of the observed binding is nonspecific. The degree of binding was the same when tissues from morphine-tolerant rats were used.

The present results utilizing ring-labeled dihydromorphine of very high specific activity are very similar to those of Mellet and Woods(1) using C^{14}-methyl labeled levorphanol, indicating the similarities of distribution and binding between dihydromorphine and levorphanol.

Summary. Dihydromorphine-7,8-H^3 of high specific activity was prepared by the catalytic tritriation of the double bond of morphine and purified. Studies of the intracellular distribution of radioactivity in the brains and livers of rats injected with the labeled drug showed that most of it was in the soluble fraction. However, the brain nuclear fraction consistently contained 10-20% of the radioactivity of the homogenate. The administration of nalorphine had no effect on tissue levels or intracellular distribution. Binding of dihydromorphine to brain and liver homogenates and to serum proteins was demonstrated by equilibrium dialysis. The degree of binding was similar in the different tissues and not affected by the addition of nalorphine. Tissue homogenates from morphine-tolerant rats exhibited the same amount of binding as those from control rats.

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Cytological Virological and Chromosomal Studies of Cell Strains From Aborted Human Fetuses.* (31037)

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Spontaneous abortion, usually without obvious cause, is a frequent occurrence in human pregnancies. To test the hypothesis that viral infections may play a part in the development of spontaneous abortion, a technique was sought to obtain dividing cells from human embryos that might be carrying latent viruses. We used a method developed by Jensen *et al,* for studying mouse tissues, in which cells could be obtained readily from organ explants. In the course of this work we collected cytological and chromosomal data on human fibroblast cell strains.

Materials and methods. Collection and preparation of specimens. Embryos were obtained from 2 sources: (A) surgical abortions performed in Scandinavia for social and psychiatric reasons, and (B) spontaneous abortions that occurred at the Philadelphia General Hospital and the Hospital of the University of Pennsylvania. The surgically removed embryos were placed in antibiotics containing Hanks' solution and shipped to us by air at a temperature of approximately 0°C. The spontaneous abortions were refrigerated in plastic bags without solution or antibiotics until collected, usually within 12 hours. Only those embryos which were expected to have viable tissues were studied. Aside from the decomposed external appearance, one of the best indicators of the embryo's condition appeared to be the physical aspect of the liver. All assays performed on embryos with friable and discolored livers were discarded, because the cells failed to grow.

Organ culture technique. The organ culture technique described by Jensen *et al*(1) was used: a grid of stainless steel mesh‖ was enclosed in a small Petri dish containing 10 ml of double strength Eagle's Basal Medium in isotonic Earle's solution with 10% calf serum; a small disc of open mesh paper (tea bag paper)** was moistened in the medium and applied to the top of the grid. Fragments of organs were cut into pieces about one cubic mm with a surgical blade and placed directly on the tea bag paper without being washed. Two explants were placed on top of each paper; the volume of the individual explants did not exceed 2 cu mm. The

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