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UPTAKE OF DIHYDROMORPHINE-^{3}H BY SYNAPTOSOMES*,†

C. C. Hug, Jr., and T. Oka‡

Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, Michigan 48104

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Summary

Tritium-labeled dihydromorphine (DHM-^{3}H) was accumulated *in vitro* by the synaptosomal fraction (nerve endings) of rat cerebral cortex. Particles derived from nerve endings by osmotic shock exhibited a higher concentration of DHM-^{3}H than did other subcellular fractions of brain when the isolated fractions were incubated with the drug *in vitro*. Nalorphine in large concentrations reduced the uptake of DHM-^{3}H by all fractions to the same degree. No difference was apparent in the uptake of DHM-^{3}H by subcellular fractions from non-tolerant and morphine-tolerant rats. The uptake of DHM-^{3}H by nerve endings was attributed to a binding phenomenon, although an active transport process may provide the drug an access to the binding sites in the intact tissue.

IDENTIFICATION of specific sites and types of interaction of narcotic analgesics with nervous tissue would facilitate studies of the nature of narcotic analgesic receptors, increase our understanding of the interactions of narcotic analgesics and their antagonists, and possibly provide clues to the cellular mechanisms affected by these drugs. Accordingly, we are studying the accumulation of narcotic analgesics by brain tissue *in vitro* where some of the variables affecting uptake of drugs *in vivo* (e. g., blood flow, plasma protein binding, pH) can be eliminated or better controlled.

Earlier reports from our laboratory indicated that narcotic analgesics are accumulated by brain tissue *in vitro* by a process with the characteristics

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‡ Present address of T. Oka: Department of Pharmacology, School of Medicine, Keio University, Tokyo, Japan.

of active transport[1, 2] and by binding of the drugs to tissue components.[3] In the present report we describe the accumulation of a narcotic analgesic, tritium-labeled dihydromorphine (DHM-^{3}H), by synaptosomes and other subcellular fractions of brain in vitro.

Methods

Isolation of synaptosomes from cerebral cortical slices incubated in vitro. Cerebral cortical slices from male Holtzman rats were prepared and incubated as described previously.[1] After incubation, each slice (20-40 mg wet weight) was homogenized in 0.7 ml of 0.32 M sucrose. One-half ml of the homogenate was layered over a 4.5 ml continuous (linear, 0.3-1.8 M)[4, 5] or a 4.5 ml discontinuous (1.5 ml each of 0.8, 1.2, 1.8 M)[6] gradient of sucrose. Continuous gradients were centrifuged at 0° C for 90 min at 125,700 x g (av) and discontinuous gradients at 51,000 x g (av). Continuous gradients were collected dropwise (0.15-0.2 ml portions) from the bottom of the tube. In the discontinuous gradients three tissue layers were evident. The three tissue layers and the four alternating layers of sucrose media were removed individually by pipette. The tissue layers were suspended in glass distilled water for subsequent analysis. All fractions were analysed for radioactivity and protein.

Incubation of isolated synaptosomes. Homogenates of rat whole brain in 0.32 M sucrose were centrifuged at 900 x g (max) x 10 min to remove nuclei and unbroken cells. The supernate was centrifuged at 11,500 x g (max) x 20 min to yield a pellet which was resuspended in 0.32 M sucrose, layered over a two-step gradient of sucrose (0.8 and 1.2 M), and centrifuged at 63,500 x g (av) x 90 min. The tissue layer at the interface of 0.8 and 1.2 M sucrose (synaptosomes) was mixed with an equal volume of distilled water and centrifuged at 105,000 x g (max) x 40 min. The pellet was resuspended in the Krebs-bicarbonate medium (pH 7.4) used for incubating tissue slices.[1] The suspension was incubated at 37° C (with or without an inhibitor) 10 min before and 10 min after the addition of DHM-^{3}H (6.7 Ci/mmol) or norepinephrine-^{3}H (10 Ci/mmol) (New England Nuclear Corp.). At the end of the incubation period the suspension was diluted with 3 volumes of ice-cold media and centrifuged at 105,000 x g (max) x 30 min at 0° C.

Isolation and incubation of subcellular fractions from whole brain. Whole brains of decapitated rats were removed and homogenized in ice-chilled medium containing: 320 mM sucrose, 0.01 mM $CaCl_2$ and 25 mM Tris-buffer at pH 6.8. Subcellular fractions were prepared as described by De Robertis and his co-workers.[7-10] The individual fractions containing 1 to 5 mg protein were resuspended and incubated at 22-24° C for 30 minutes in 10 ml of the sucrose-$CaCl_2$-Tris medium containing DHM-^{3}H. After incubation, all tissue fractions were sedimented at 105,000 x g (max) x 30 min at 0° C.

Analysis of radioactivity and protein. Tubes containing tissue pellets were drained of media overnight and then the walls were rinsed with distilled water 4 times and the surface of the pellet once before resuspending the pellet in distilled water for analysis of radioactivity and protein.

Aliquots (0.05 to 0.2 ml) of each gradient fraction and tissue suspension were mixed with 3 ml absolute ethanol and 7 ml toluene-phosphor solution,[1] and the radioactivity was determined in a liquid scintillation spectrometer. An internal standard of toluene-^{3}H was used to determine quenching. Cerebral cortical slices and whole brain homogenates were incubated with DHM-^{3}H and then subjected to chromatographic analysis as described previously.[1] In each case, only unchanged DHM-^{3}H was detected.

Protein determinations were performed as described by Lowry et al.[11]

Characterization of the subcellular fractions. Deoxyribonucleic acid was estimated by the method of Burton.[12] Enzymatic activity was determined for succinate-INT-reductase,[13] NADPH-cytochrome C reductase[14] and acetylcholine esterase.[15] Accumulation of norepinephrine-^{3}H was determined for cerebral cortical slices[4,5] and for isolated synaptosomes.[6]

Analysis of data. Concentrations of DHM-^{3}H and norepinephrine-^{3}H were expressed as pmoles per mg of protein in the tissue fractions. The effect of a potential inhibitor was determined by comparing the concentration of DHM-^{3}H in a sample of tissue incubated without an inhibitor (control) to the concentration in another sample of the same tissue obtained from the same animal and incubated at the same time with the inhibitor. The results with the inhibitor were expressed as percent of control and were analysed statistically as a paired comparison. S.E.M. refers to standard error of the mean for the number of animals (n) studied.

Results

Uptake of DHM-^{3}H by cerebral cortical slices. When rat cerebral cortical slices were first incubated with DHM-^{3}H (1 μM) for 10 min at 37° C and then homogenized and centrifuged on a continuous gradient of sucrose, approximately 28% of the radioactivity was associated with the synaptosomal layer and most of the remainder of the radioactivity was recovered in the soluble fraction (Fig. 1). A similar distribution of radioactivity was seen when the slices were incubated with 0.04 μM norepinephrine-^{3}H (Fig. 1), in which case, about 53% of the tritium was in the synaptosomal fraction.

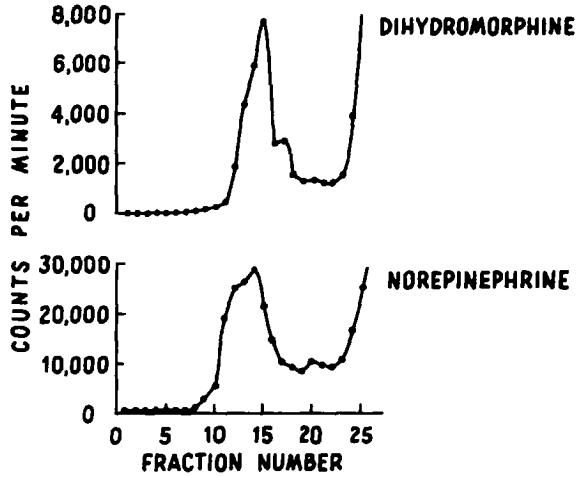

Fig. 1

Subcellular distribution of radioactivity in rat cerebral cortical slices incubated with DHM-^{3}H or norepinephrine-^{3}H. (Continuous gradient; see text for details.) Similar results were obtained in three other experiments.

Discontinuous sucrose gradients were used to improve the separation of the fractions of cerebral cortical slices incubated with DHM-^{3}H. About 25% of the radioactivity was associated with the synaptosomal fraction (L-2), 5% with the nuclear-mitochondrial fraction (L-1) and 65% with the soluble fraction (Fig. 2). Although a third tissue fraction (L-3, microsomes and myelin) could be distinguished visually we did not attempt to separate its content of radioactivity from that of the soluble fraction. The total recovery of radioactivity from the gradient was 99.9% of that originally present in the slice.

In Figure 3, the distribution of DHM-^{3}H, norepinephrine-^{3}H, and several

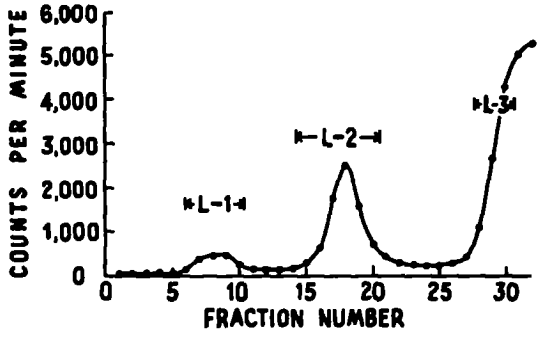

Fig. 2

Subcellular distribution of DHM-^{3}H in rat cerebral cortical slices. (A discontinuous gradient collected dropwise from the bottom of the tube. See text for details.) The contents of the fractions L-1, L-2 and L-3 are shown in Fig. 3. Similar patterns of distribution were seen in two additional experiments.

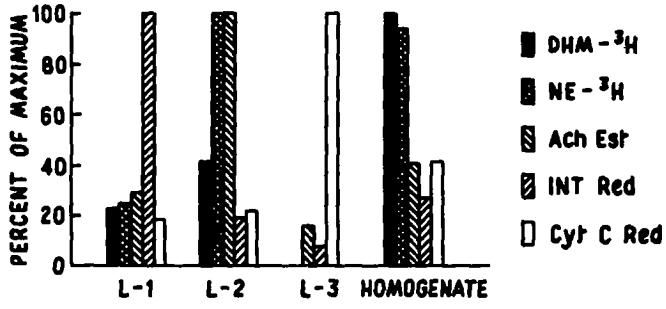

Fig. 3.

Distribution of radioactivity and enzymatic activity in subcellular fractions of rat cerebral cortical slices. The fractions designated L-1, L-2 and L-3 in Fig. 2 were analysed for DHM-^{3}H, norepinephrine-^{3}H (NE-^{3}H) acetylcholinesterase (Ach Est), succinate-INT-reductase (INT Red) and NADPH-cytochrome C reductase (Cyt C Red) activities. In each case, the fraction showing the highest activity (counts or OD per min per mg protein) was assigned a value of 100% and the other fractions were compared to it. Each bar represents the mean of values obtained in three different experiments.

enzyme activities are shown. The fractions are compared on the basis of activity per mg of protein for each constituent. There is about a 2-fold greater concentration of DHM-^{3}H in the synaptosomal layer compared to the nuclear-mitochondrial layer. The very high values for the whole homogenate are due

to its large content of free DHM-^{3}H. * The distribution of enzymatic activities among the fractions is an indication of the separation and identity of the fractions. Succinate-INT†-reductase was used as a mitochondrial marker,[16] NADPH-cytochrome C reductase as a microsomal marker,[17] and acetylcholine esterase[7] and norepinephrine uptake[4, 5] as synaptosomal markers.

When cerebral cortical slices were incubated in the presence of nalorphine or ouabain the uptake of DHM-^{3}H by the slices and their constituent subcellular fractions was reduced significantly (Table 1). In the case of nalorphine, the degree of reduction was similar for the slice and its constituent fractions.

Uptake of DHM-^{3}H and norepinephrine-^{3}H by isolated synaptosomes. Synaptosomes were isolated from rat whole brain and then incubated with DHM-^{3}H and norepinephrine-^{3}H (Table 2). DHM-^{3}H accumulation was depressed by the low temperature and in the presence of either nalorphine or naloxone; it was unaffected by ouabain. The uptake of norepinephrine was reduced at 0° C and by ouabain.

Uptake of DHM-^{3}H by isolated subcellular fractions. Subcellular fractions isolated according to the methods of De Robertis et al[7-10] were characterized biochemically (Table 3). Studies of the uptake of DHM-^{3}H by the individual fractions incubated separately with the drug are summarized in Table 4. The data are expressed as the specific binding ratio (SBR) as defined by Azcurra and De Robertis.[7] Relatively higher concentrations of DHM-^{3}H were found in the M_1 0.8, M_1 0.9, M_1 1.0 and M_2 fractions. Although all fractions released DHM-^{3}H when resuspended in fresh media, these four fractions retained relatively more DHM-^{3}H as indicated by the increase in their SBR values (Table 4).

Preliminary studies with subcellular fractions from morphine-tolerant and dependent rats (which received morphine 33 mg/kg subcutaneously or intraperitoneally every 8 hours for 1-3 months) did not reveal any striking change in the pattern of DHM-^{3}H distribution among the fractions when they were incubated individually with the radioactive drug (Table 4).

Nalorphine in a concentration of 1 mM in the medium reduced the binding

* Approximately 85% of the dihydromorphine in the soluble fraction of a homogenate of cerebral cortical slices incubated with the drug (1 μM) was present in an ultrafiltrate of the homogenate.

† INT, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride.

TABLE 1

Uptake of DHM-^{3}H by rat cerebral cortical slices

Fraction*	n	Control (pmol/mg of protein)	Percent of Control ± S. E. M.	
			Nalorphine (1 mM)	Ouabain (1 mM)
Whole homogenate	4	94 ± 8**	80 ± 7***	79 ± 6^{+}
Synaptosomes	4	41 ± 2	79 ± 5^{+}	56 ± 6^{++}
Mitochondria + Nuclei	4	24 ± 1	79 ± 6^{+}	56 ± 3^{+++}

$^{+}p < .05$, $^{++}p < .01$, $^{+++}p < .001$

* Intact cerebral cortical slices were incubated with DHM-^{3}H (1 μM) and then homogenized and applied to a discontinuous sucrose gradient.

** This value is for total DHM-^{3}H (i. e., free and bound) present in an aliquot taken from the slice homogenate before it was layered over the sucrose gradient.

*** We have previously demonstrated a significant reduction of DHM-^{3}H uptake by whole cerebral cortical slices incubated in the presence of nalorphine under the same experimental conditions (86 ± 2% of control, n = 18, $p < .001$).[1]

TABLE 2

Uptake of DHM-^{3}H and norepinephrine-^{3}H by isolated synaptosomes

Inhibitor	Percent of Control ± S. E. M.			
	DHM-^{3}H*	n	Norepinephrine-^{3}H**	n
Temperature (0° C)	72 ± 1^{++}	8	14 ± 2^{++}	3
Ouabain 1 mM	102 ± 3	3	20 ± 1^{++}	3
Nalorphine 1 mM	87 ± 1^{+}	3	---	-
Naloxone 1 mM	86, 87	2	---	-

$^{+}p < .02$, $^{++}p < .001$

* The concentration of DHM-^{3}H was 1 μM in the media and 123 ± 8 pmols/mg of protein in the synaptosomal fraction (n = 8).

** The concentration of norepinephrine-^{3}H was 0.04 μM in the media and 42 ± 3 pmols/mg of protein in the synaptosomal fraction (n = 3).

TABLE 3

Characterization of subcellular fractions of rat whole brain

Subcellular Fraction	DNA (μg/mg of Protein)	Specific Activity Ratio ± S. E. M.* Succinate-INT-Reductase	NADPH-Cytochrome C Reductase	Acetylcholine Esterase
TP	12.7 ± 0.8	1	1	1
NUC	18.1 ± 0.6	1.1 ± 0.02	1.4 ± 0.1	0.8 ± 0.1
MIC	1.6 ± 0.3	0.4 ± 0.01	10.3 ± 2.2	2.0 ± 0.1
MIT	2.0 ± 0.2	4.7 ± 0.5	2.3 ± 0.4	1.6 ± 0.2
	Contents**			
M_1	Mitochondria, myelin, synaptic membranes			1.6 ± 0.1
0.8	Myelin			1.8 ± 0.5
0.9	Synaptic membranes, myelin			4.0 ± 0.2
1.0	Synaptic membranes (cholinergic)			3.2 ± 0.1
1.2	Synaptic membranes (non-cholinergic)			2.1 ± 0.1
P	Mitochondria			0.7 ± 0.1
M_2	Synaptic vesicles			2.6 ± 0.2

* Specific activity ratio = enzymatic activity per mg protein in the specific fraction/activity per mg protein in the total particulate fraction (TP).

** According to Azcurra and De Robertis.[7]

of DHM-^{3}H (0.1 μM in the medium) to the total particulate fraction of rat whole brain by 22 percent ± 3 S. E. M. ($n = 5$, $p < .01$). The degree of reduction was approximately the same (range of 14 to 24 percent) for each of the 10 subcellular fractions listed in Table 4. No difference in the pattern of distribution of DHM-^{3}H among the subcellular fractions was apparent in the presence of nalorphine. Nalorphine in a concentration of 10 μM had no effect on the uptake of DHM-^{3}H (0.1 μM) by any of the subcellular fractions.

Discussion

Tritium-labeled dihydromorphine (DHM-^{3}H) is a narcotic analgesic very similar to morphine in its physical, chemical and pharmacological properties as well as in its disposition in the animal body.[18-23] In the present study we have demonstrated that DHM-^{3}H was preferentially accumulated *in vitro* by nerve endings (synaptosomes) as compared to other subcellular fractions of brain. The preferential uptake was evident when either cerebral cortical slices or isolated subcellular fractions were incubated with the drug. A pre-

TABLE 4

Binding of dihydromorphine to isolated subcellular fractions of whole brain of non-dependent and morphine-dependent rats

Subcellular Fraction	Specific Binding Ratio ± S. E. M. *					
	Non-dependent				Morphine-dependent	
	n	Unwashed	n	Washed†	n	Washed†
TP	-	1 **	-	1 ***	-	1 ****
NUC	4	1.0 ± .04	3	0.9 ± .00	2	0.9 ± .06
MIC	11	1.1 ± .11	3	1.1 ± .20	2	0.7 ± .02
MIT	6	1.1 ± .06	2	1.2 ± .01	-	---
M_1	5	0.9 ± .05	2	1.1 ± .13	2	1.2 ± .01
0.8	7	1.7 ± .08	5	2.7 ± .54	4	1.4 ± .06
0.9	7	1.8 ± .16	2	2.4 ± .03‡	2	2.0 ± .13
1.0	7	1.7 ± .14	3	2.8 ± .15‡	7	2.2 ± .08
1.2	7	1.1 ± .10	2	1.3 ± .41	2	1.3 ± .05
P	7	0.7 ± .11	2	1.1 ± .50	2	1.6 ± .18
M_2	11	1.5 ± .05	7	2.1 ± .34	7	2.4 ± .34

* Specific binding ratio = cpm per mg protein in the specific fraction/cpm per mg protein in total particulate fraction (TP). Where n = 2, mean SBR ± range is listed. The concentration of DHM-^{3}H in the medium was 0.1 μM.

** 7.2 pmol DHM-^{3}H per mg of protein (n = 7).

*** 1.9 pmol DHM-^{3}H per mg of protein (n = 7).

**** 2.0 pmol DHM-^{3}H per mg of protein (n = 7).

† Each fraction was twice resuspended in fresh medium and sedimented. The final pellet was resuspended in distilled water and analysed for radioactivity and protein.

‡ In four additional experiments the M_1 0.9 and 1.0 fractions were analysed together and the average SBR value was 2.9 ± .59.

liminary report by Scrafani et al [24] indicates that accumulation of DHM-^{3}H by synaptosomes is also evident when the drug is administered to rats in vivo.

In an earlier study, Mule et al[25] did not observe preferential accumulation of morphine-^{3}H by nerve endings in the brains of guinea pigs injected with the radioactive drug. Rather, they recovered the major portion of the drug (68%) from the soluble fraction and found the remainder distributed rather evenly among the various particulate fractions of whole brain. How-

ever, the degree of washout and redistribution of morphine during the isolation procedure was not evaluated. It is likely that washout was greater for nerve ending particles than for other fractions since these particles were subjected to a greater number of cycles of centrifugation and resuspension and to osmotic shock. Several different investigators[3, 19, 26, 27] have found that the binding of morphine-like drugs to subcellular fractions of brain is reduced by washing the tissue (i. e., by resuspension or dialysis). In the present study, 75 percent of the DHM-^{3}H was removed by just two cycles of resuspension and centrifugation of the tissue fractions. However, in the present study, all tissues were simultaneously subjected to exactly the same manipulations and thus would be expected to be affected to the same degree. The fact that certain nerve ending fractions (M_1 0.8, M_1 0.9, M_1 1.0 and M_2) retained relatively more DHM-^{3}H after successive washings may indicate that these particular fractions have a relatively greater affinity for the narcotic analgesic. A careful kinetic analysis is required to evaluate fully the latter possibility.

The mechanism of accumulation of DHM-^{3}H by nerve endings is not yet totally clear. Participation of a passive process, probably binding to tissue components, is suggested on the basis of the relatively large accumulation of DHM-^{3}H in the synaptosomal fraction at low temperatures and because of the preferential uptake of the drug by particles derived from nerve endings by osmotic shock. Also, ouabain, in concentrations which markedly depressed the uptake of norepinephrine, failed to reduce the uptake of DHM-^{3}H by isolated synaptosomes. However, ouabain did depress the accumulation of DHM-^{3}H in cerebral cortical slices[1] and in the synaptosomal, nuclear and mitochondrial fractions derived from slices incubated with the drug. Thus, in the tissue slice, and possibly in the intact brain, a transport process may provide the drug an access to the binding sites.

Nalorphine, the narcotic analgesic antagonist, consistently reduced the uptake of DHM-^{3}H by all tissue fractions, and the degree of reduction was similar for the various fractions. However, very large concentrations of nalorphine were required in order to reduce the uptake of DHM-^{3}H and the degree of reduction was small. Hence, several questions may be raised concerning the biological significance of the findings. 1) Does the accumulation of DHM-^{3}H represent binding to specific receptor sites? The fact that nal-

orphine reduced the binding of DHM^{3}H to brain tissue but not to liver, kidney or muscle[3] may indicate an element of specificity, but it does not rule out a large contribution of non-specific sites to the overall uptake by brain. 2) Do the narcotic analgesic antagonists and DHM-^{3}H bind and act at different sites? This possibility has been examined by a number of investigators[28] and cannot be ruled in or out with the data presented here. 3) Do the affinities of DHM-^{3}H and narcotic antagonists for the binding sites differ? A careful kinetic analysis of the binding of DHM-^{3}H in the presence and absence of the antagonists is required to answer these questions.

Although the data are insufficient to rule out small quantitative changes in the binding of narcotic analgesics to nerve endings with the development of tolerance and physical dependence, the results indicate that dramatic diffences are not likely to be found with the techniques applied in the present study.

The biological significance of preferential accumulation of narcotic analgesics by nerve endings may be considerable in light of a number of reports relating the effects of these drugs to their actions on synaptic functions. To cite but a few examples: The turnover (i. e., synthesis, release or degradation) of catecholamines[29, 30] and serotonin[31] in the central nervous system of rodents has been altered by the administration of morphine and other narcotic analgesics. Morphine-like drugs interfere with the release of acetylcholine from nerve endings in the gut, [32] from the brain of the cat[33] and rat[34] in vivo and from cerebral cortical slices of rat[35] and mouse[36] in vitro. The findings related to acetylcholine are especially interesting since DHM-^{3}H appeared to be preferentially accumulated in the M_1 1.0 fraction which was identified by Azcurra and De Robertis[7] as the one containing cholinergic synaptic membranes. However, as of the present time, none of the changes in nerve ending function have been established as the basis of analgesia, tolerance, physical dependence or any other effect produced by narcotic analgesics. Thus an understanding of the biological significance of the uptake of narcotic analgesics by nerve endings awaits further studies.

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