ACTIVE UPTAKE OF DIHYDROMORPHINE AND OTHER NARCOTIC ANALGESICS BY CEREBRAL CORTICAL SLICES*†

J. T. SCRAFANI and C. C. HUG, JR.

Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Mich., U.S.A.

Abstract—Narcotic analgesics have been shown to be accumulated in cerebral cortical slices by a process having the characteristics of an active transport system. In addition, diffusion and binding contributed to the total uptake of the analgesics into brain slices. The total uptake of dihydromorphine (1×10^{-6} M in medium) was found to reach steady state equilibrium in 30 min at 37° with a maximum tissue to medium concentration ratio (T/M) of 2·3. Accumulation *in vitro* of the narcotic analgesics in brain differed from their accumulation in the kidney and in the choroid plexus in that there was a lower T/M ratio at equilibrium for brain and the uptake by brain was not inhibited by quaternary organic bases. The narcotic analgesic antagonist, nalorphine, consistently depressed the uptake of dihydromorphine in a competitive manner.

NARCOTIC analgesics have been shown to be taken up by the kidney[1–3] and choroid plexus[2, 4] by active transport processes. It has been suggested that transport of these drugs by the choroid plexus may facilitate their removal from cerebrospinal fluid,[2, 4] just as their transport by the kidney promotes their elimination from the body.[1, 3]

Upon administration of an analgesic dose, the central nervous system is the site of the most prominent effects of the narcotic analgesics. The central nervous system is known to have transport processes for the uptake and elimination of a variety of endogenous and exogenous substances.[5, 6] The transport systems are thought to affect the concentration of these substances in certain cellular and subcellular compartments.[7] The present study was undertaken to determine if the narcotic analgesics interact with the transport systems of brain tissue. Such interaction could have significant implications for the distribution of these drugs in nervous tissue and for their pharmacological alteration of central nervous system function.

MATERIALS AND METHODS

Preparation of slices. Male Holtzman rats (150–200 g) were sacrificed by decapitation. The brain was removed, cut longitudinally into hemispheres and cerebral cortical slices (10–40 mg) were cut freehand starting at the outer cortical surfaces by the method of McIlwain and Rodnight.[8] In order to avoid inclusion of white matter, only two slices were taken from each hemicortex of the rat; thus, in some experiments each

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slice was divided into 2 equal parts in order to provide 8 slices from the same animal. As each slice was cut, it was floated in ice-cold medium.

In a few experiments cerebral cortical slices were prepared as described above from species other than the rat. Monkeys were anesthetized with diethyl ether, rabbits were sacrificed with i.v. air and mice by decapitation before removal of the brain.

Incubation of slices. Each slice was placed in 5 ml of medium of the following composition (mM): NaCl, 134; KCl, 2·7; MgCl₂, 0·48; CaCl₂, 0·92; NaHCO₃, 14·8; Na₂HPO₄ (adjusted to pH 7·4 with HCl), 3·6; and glucose, 5·5. Before incubation a mixture of oxygen and carbon dioxide (95:5, v/v) or of nitrogen and carbon dioxide (95:5, v/v) was bubbled through the medium to adjust the pH. Unless otherwise specified, all incubations were performed in a Dubnoff metabolic shaker at 37° under a rapid flow of the gas mixture which always maintained the pH of the medium between 7·3 and 7·5.

All tissues were preincubated in medium for 15 min. They were then transferred to fresh medium containing a radioactive narcotic analgesic with or without a potential transport inhibitor. When metabolic inhibitors were studied, they were added both to the preincubation medium and to the medium containing the narcotic analgesic. All other inhibitors were added as chloride salts only to the second medium. Uptake in the presence of an inhibitor was always compared to uptake without an inhibitor control by tissue from the same animal.

After incubation the tissue was removed from the incubation medium, blotted on glass, tightly wrapped in aluminium foil on dry ice and stored at —10° until it was weighed and analyzed within the succeeding 24 hr.

Analysis of radioactivity. The frozen tissues were weighed to the nearest microgram on a Cahn M-10 Electrobalance. They were then transferred to scintillation-grade glass vials and allowed to dry overnight in the open vials at room temperature. After drying, 3 ml of 2N KOH in 95% ethanol were added to each vial to solubilize the tissue. The vials were tightly capped and placed in a covered, slowly oscillating shaker overnight at room temperature. Three ml of the KOH–ethanol solution were added to 2 empty vials to serve as blanks. Care was taken to prevent contact of the solution with the aluminum liner of the vial cap, since considerable quenching was observed when alkali dissolution of the liner occurred. In addition, care was taken to avoid exposure of the solution to light, which turned the solution yellow. After dissolution of the tissue, 7 ml toluene phosphor solution* were added to each vial and the vials were swirled carefully to mix the contents.

Duplicate 0·2-ml aliquots of the medium from each beaker (one slice per beaker) were pipetted into counting vials and 10 ml of a 3:7 (v/v) absolute ethanol : toluene phosphor solution were added.

Vials containing medium or tissue were then placed in a refrigerated liquid scintillation spectrometer (Packard Tri-Carb, model 3315) for 1 hr prior to counting. This was done to bring the solution to — 4° and also to eliminate any light activation of the glass which may have occurred.[9] Quenching was determined by the use of a radioactive toluene internal standard. Background counts due to the usual sources and to

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* Toluene phosphor solution was prepared by dissolving 3 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-*bis*-[2-(4-methyl-5-phenyloxazolyl)]—benzene (Dimethyl POPOP, Packard Instrument Co.) in 1 liter of reagent grade toluene.

the ^{40}K present in the alkali-solubilized tissue were subtracted to give net counts per minute.

The results were expressed either as the concentration of narcotic analgesic per gram of tissue or per milliliter of medium or as the tissue to medium concentration ratio (T/M). Under Results, "per cent of control" refers to the uptake of the narcotic analgesic by a slice incubated in the presence of a potentially inhibitory compound compared to the uptake by a slice from the same animal incubated simultaneously in the absence of that inhibitor (control).

It should be noted that throughout this investigation, 5 ml of medium were used for incubation of each slice of tissue (10–40 mg). The total amount of drug in the medium represented an excess such that the final concentration of the analgesic in the medium never differed from the initial concentration by more than 1 per cent. Total recovery of radioactivity from tissue and medium was equal to the quantity initially present in the medium.

Identification of dihydromorphine. Tissues were incubated exactly as described above for 90 min with either 10^{-3} or 10^{-6} M dihydromorphine. Then, one-half of the tissue was homogenized in methanol and the other half in 0·01 N HCl. The homogenates were centrifuged at 1000 g for 10 min. Both supernatants were concentrated under a stream of nitrogen at room temperature and then applied to Whatman No. 3 chromatography paper. The medium was also concentrated and applied to the paper. Nonradioactive dihydromorphine was applied to each paper as a standard. Descending chromatography was performed in the solvent systems given in Table 1.

TABLE 1. CHROMATOGRAPHIC ANALYSIS OF RADIOACTIVITY IN CEREBRAL CORTICAL SLICES AND MEDIA AFTER INCUBATION FOR 90 MIN WITH 10^{-3} AND 10^{-6} M DIHYDRO-MORPHINE-^{3}H

Solvent system	Nonradioactive dihydromorphine standard	R_f Values* Radioactive areas			
		10^{-3}M		10^{-6}M	
		Tissue	Medium	Tissue	Medium
1. *n*-Butanol–glacial acetic acid–water (4:1:2, v/v)	0·58	0·58	0·59	0·55	0·56
2. *tert*—Amyl alcohol–*n*-butyl ether–water (80:7:13, v/v); paper previously buffered to pH 8 by dipping in 0·2 M potassium phosphate	0·77	0·78	0·70		
3. *n*-Propanol–*n*-butanol–0·1 N ammonium hydroxide (2:1:1, v/v)	0·83			0·83	0·87

* See text for details.

Standards were located on the chromatogram under an ultraviolet light and by spraying with an iodoplatinate reagent.[10] The chromatogram was scanned for radioactivity by cutting out the path of migration of the radioactive material and dividing it lengthwise into 1 × 2 cm rectangles, each of which was placed at the bottom of a

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counting vial. One-half ml methanol was added to each vial to elute any radioactive material from the paper. After 10 min, 10 ml toluene phosphor solution were added and the vials were swirled to mix the contents. Care was taken to see that the paper strips were lying flat at the bottom of the vials. The samples were counted and R_f values were determined and compared to standards.

Each chromatogram contained a single peak of radioactivity with an R_f value corresponding to that of standard dihydromorphine (Table 1), indicating that there was no metabolic alteration of dihydromorphine. If as little as 3 per cent of the radioactivity had been present as another compound, it should have been detected by this procedure.

Materials. Tritium-labeled dihydromorphine was prepared as described by Hug and Mellett[11] and the sp. act. was adjusted to 20 mc/m-mole by dilution with non-radioactive dihydromorphine. Levorphan (0·9 mc/m-mole), dextrorphan (1·7 mc/m-mole), d-methorphan (3·7 mc/m-mole) and l-methorphan (0·6 mc/m-mole) were tritiated by New England Nuclear Corp. by the Wilzbach procedure and then purified in our laboratories.[12] Tritium-labeled nalorphine (1·1 mc/m-mole) was prepared as described by Hug and Woods.[13] N-methyl-^{14}C-labeled morphine (14·5 mc/m-mole) was purchased from the Nuclear-Chicago Corp.

RESULTS

Uptake of dihydromorphine by rat cerebral cortical slices. The uptake of dihydromorphine (1×10^{-6} M in medium) by rat cerebral cortical slices was rapid initially and reached a maximum (T/M = $2·3 \pm 0·01$) at 30 min (Fig. 1). Beyond 30 min, an essentially constant T/M of 2·1 was found for incubation periods lasting up to 120 min.
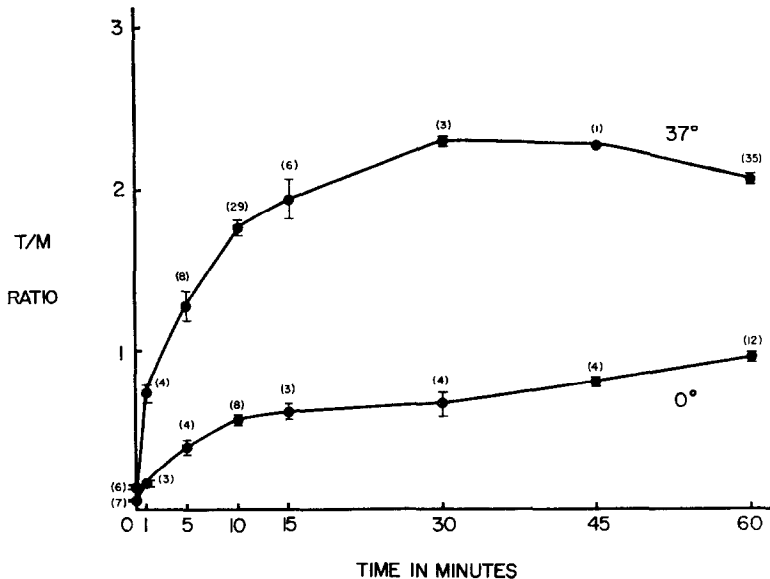

Fig. 1. Accumulation of dihydromorphine with time by cerebral cortical slices from rats. The slices were incubated in Krebs–Ringer bicarbonate medium containing 1×10^{-6} M dihydromorphine in an atmosphere of $O_2:CO_2$ (95:5) at 37°. All tissues were preincubated for 15 min in medium without dihydromorphine. Brackets indicate $\pm$ S.E.M. for the number of animals indicated in parentheses.

At an incubation temperature of $0°$, a constant tissue to medium concentration ratio (T/M) of 0.92 ± 0.04 S.E.M. ($n = 12$) was found after 60 min. This ratio approximates that expected for simple diffusion of dihydromorphine into total tissue water, which represents 85–90 per cent of the tissue wetweight.[14]

The accumulation of dihydromorphine in tissue slices during 10 min of incubation at $37°$ was dependent on the initial concentration of dihydromorphine in the medium (Fig. 2). The T/M ratio declined with increasing concentrations of dihydromorphine up to 5×10^{-6} M, suggesting that a portion of the uptake process was saturable.
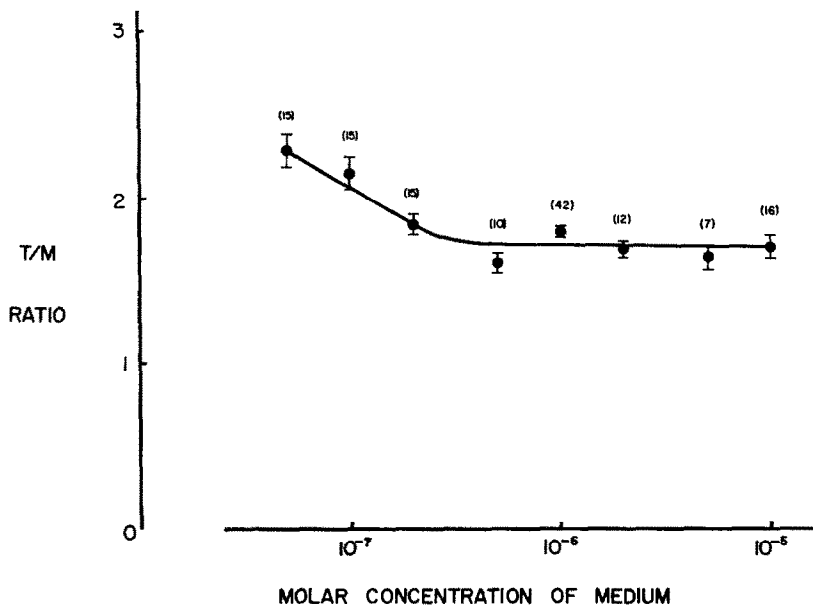

FIG. 2. Effect of the concentration of dihydromorphine in medium on its uptake by cerebral cortical slices. Slices prepared from rats were incubated for 10 min with different concentrations of the drug in the medium. Conditions of incubation and graphic representation were as described for Fig. 1.

Above this concentration, the T/M ratio was fairly constant around a ratio of 1.7, suggesting a nonsaturable component in the total uptake. The apparent K_m for the *total* uptake process was determined to be approximately 2×10^{-6} M with an apparent V_{max} of 21 n-mole/g tissue/hr. At $0°$ the T/M was constant at 0.5 to 0.6 after 10 min of incubation for all concentrations studied (5×10^{-7} to 1×10^{-4} M).

The steady state accumulation of dihydromorphine by cerebral cortical tissue was dependent on aerobic metabolism (Table 2). It may be noted that significant depression of uptake occurred when glucose was omitted from the medium or when a nitrogen atmosphere was employed. Neither of these methods of inhibition would be expected to alter simple diffusion and physicochemical binding of dihydromorphine to tissue components.

The inhibitor of active transport ATPase, ouabain,[15] reduced the steady state accumulation of dihydromorphine (Table 2). Ouabain (1×10^{-4} M) also depressed the initial rate of uptake (measured at 10 min) by 15.8 ± 4.2 per cent ($n = 7$; $P < 0.001$).

Table 2. Effect of metabolic inhibitors on the steady state accumulation
of dihydromorphine by rat cerebral cortical slices*

Inhibitor	Concn (M)	n	% of Control† (±S.E.M.)	P value
Dinitrophenol	1×10^{-3}	3	71 ± 3	$<0 \cdot 02$
Iodoacetate	2×10^{-4}	6	71 ± 6	$<0 \cdot 01$
Glucose omitted		3	79 ± 4	$<0 \cdot 05$
Nitrogen atmosphere		6	75 ± 1	$<0 \cdot 001$
Ouabain	2×10^{-4}	5	86 ± 10	NS
Ouabain	1×10^{-3}	3	71 ± 1	$<0 \cdot 01$
Temperature (0°)		4	50 ± 5	$<0 \cdot 01$

* Tissues were preincubated 15 min with or without the inhibitor and then incubated with 1×10^{-6} M dihydromorphine in the presence or absence of the inhibitor for 60 min at 37° under an atmosphere of $O_2:CO_2$ (95:5) except as noted. The number of animals is indicated by n, and each animal served as its own control. NS = not significant.

† The mean control T/M was $2 \cdot 0 \pm 0 \cdot 1$ (S.E.M.) for all 18 animals used in these experiments.

The influence of pH on the uptake of dihydromorphine is illustrated in Fig. 3. Increased uptake occurred with decreased hydrogen ion concentration of the medium. A maximum T/M ratio of $3 \cdot 6 \pm 0 \cdot 2$ was reached at pH 8·2; above this pH the T/M declined. However, it is not possible with these data to state whether the decreased
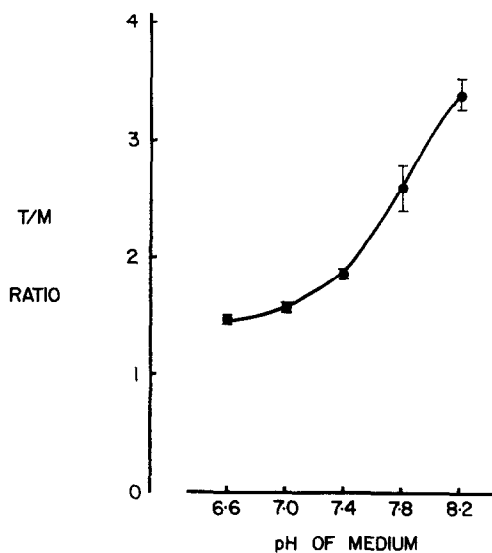

Fig. 3. Effect of pH of the medium on the steady state accumulation (60 min at 37°) of dihydromorphine (1×10^{-6} M) by cerebral cortical slices taken from 4 rats. The pH was altered by varying the concentration of $NaHCO_3$ and NaCl while maintaining a constant sodium ion concentration in the medium under an atmosphere of $O_2:CO_2$ (95:5).

uptake at higher pH values was due to a true pH effect or to the higher sodium ion concentrations which occurred with the use of $NaHCO_3$ in place of NaCl to produce a pH of 8·6 (i.e. 230 mEquiv/l. Na ion vs. 155 mEquiv/l. used routinely).

Dihydromorphine (pK_a 8·55)[16] is approximately 93 per cent ionized as an organic base at pH 7·4. Quaternary organic bases are known to compete with narcotic

analgesics for transport in both the kidney[1-3] and choroid plexus.[2] However, none of the quaternary bases (all used at a concentration of 10^{-3} M) appeared to affect the uptake of dihydromorphine into brain slices (Table 3). Note, however, that the uptake was depressed by the two morphine derivatives, codeine and nalorphine.

TABLE 3. EFFECT OF QUATERNARY ORGANIC BASES AND OF MORPHINE DERIVATIVES ON THE ACCUMULATION OF DIHYDROMORPHINE BY RAT CEREBRAL CORTICAL SLICES*

Compound	Concn (M)	n	% of Control† (± S.E.M.)	P value
Quaternary organic bases				
Carbachol	1×10^{-3}	4	105 ± 14	NS
Choline	1×10^{-3}	4	112 ± 11	NS
Decamethonium	1×10^{-3}	9	99 ± 5	NS
Hexamethonium	1×10^{-3}	12	95 ± 5	NS
Mepiperphenidol	1×10^{-3}	4	111 ± 4	NS
Morphine derivatives				
Codeine	1×10^{-3}	4	89 ± 3	<0·05
Nalorphine	1×10^{-3}	18	86 ± 2	<0·001

* Tissues were incubated for 10 min with 1×10^{-6} M dihydromorphine in the presence and absence of the compounds listed. Each animal (n) served as its own control. NS = not significant.
† The mean control T/M was 1·8 ± 0·1 (S.E.M.) for all 26 animals used in these experiments.

The narcotic analgesic antagonist, nalorphine, consistently depressed the uptake of dihydromorphine by cerebral cortical slices. When the data were plotted according to the method of Lineweaver and Burk,[17] the inhibition appeared to be competitive in nature (Fig. 4).
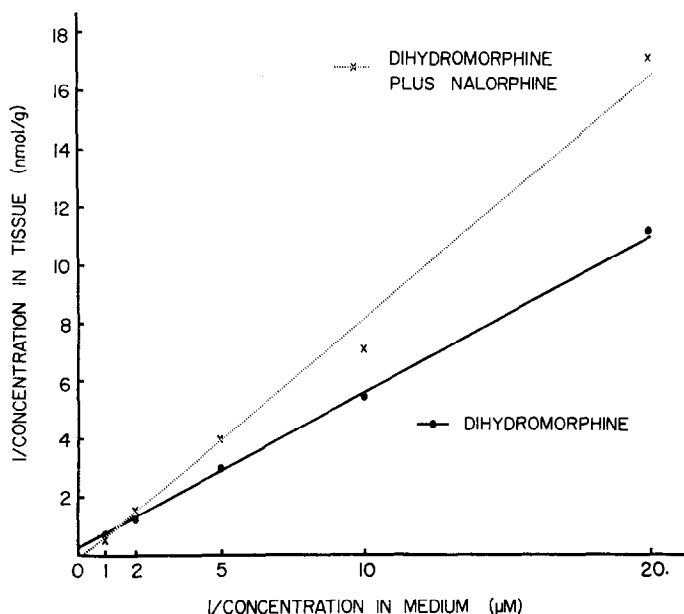

FIG. 4. Effect of nalorphine (1×10^{-3} M) on the accumulation of dihydromorphine by cerebral cortical slices. The slices were prepared from rats and were incubated for 10 min at 37° in media containing different concentrations of dihydromorphine (0·05 to 1·0 $\times 10^{-6}$ M). In the Lineweaver–Burk plot, each point is the mean value for 8 animals and the lines are fitted by the linear regression method.

Uptake of other narcotic analgesics. All morphine and morphinan derivatives studied thus far (Table 4) were found to be accumulated in brain by a metabolically dependent process. In general, the relative total uptake of the compounds appeared to correlate with their degree of lipid solubility.

At a concentration of 5×10^{-6} M, there was little difference between the uptake of the stereoisomers, as can be seen in Table 4. However, at lower concentrations the levo-isomer, levorphan, was accumulated more rapidly than the corresponding dextro-rotatary compound, dextrorphan.

TABLE 4. METABOLICALLY DEPENDENT UPTAKE OF MORPHINE DERIVATIVES AND MOR-PHINAN DERIVATIVES BY RAT CEREBRAL CORTICAL SLICES*

Compound	D.R.†	T/M ratio ± S.E.M.	
		37°	0°
Dihydromorphine	1·0	1·6 ± 0·1	0·52 ± 0·05
Morphine	1·5	2·1 ± 0·1	0·89 ± 0·09
Nalorphine	1·8	2·9 ± 0·3	1·08 ± 0·10
Levophan	3·1	5·7 ± 1·0	1·46 ± 0·21
Dextrorphan	3·0	5·5 ± 0·4	1·22 ± 0·06
Codeine		7·0 ± 0·8	2·53 ± 0·42
l-Methorphan	7·4	8·7 ± 0·8	2·07 ± 0·11
d-Methorphan	7·4	7·6 ± 0·8	2·16 ± 0·18

* Tissues were incubated for 10 min with the radioactive narcotic analgesic (5×10^{-6} M). Tissues from 4 animals were used for each compound and each animal served as its own control.

† Solvent–water distribution ratios (D.R.) were determined for ethylene dichloride and an equal volume of 0·2 M aqueous potassium phosphate at pH 7·4. The values are relative to that of dihydromorphine, which is arbitrarily designated as 1·0 (L. B. Mellett, personal communication).

Uptake of dihydromorphine by cerebral cortical slices from different species. In all other species studied, including the monkey, mouse and rabbit, the uptake of dihydromorphine was found to be metabolically dependent (Table 5).

TABLE 5. METABOLICALLY DEPENDENT UPTAKE OF DIHYDROMORPHINE BY CEREBRAL CORTICAL SLICES OF VARIOUS ANIMAL SPECIES*

Species	T/M ratio ± S.E.M.		
	Control	N₂ atmosphere	Temperature (0°)
Monkey	2·1 (1)	1·5 (1)	0·99 (1)
Rabbit	1·6 ± 0·1 (10)	1·4 ± 0·1 (3)	0·85 ± 0·05 (3)
Rat	2·1 ± 0·1 (35)	1·6 ± 0·1 (6)	0·95 ± 0·06 (8)
Mouse	2·2 ± 0·1 (5)	1·4 ± 0·1 (6)	

* Tissues were incubated for 60 min with 1×10^{-6} M dihydromorphine at 37° or 0° under an atmosphere of $O_2:CO_2$ (95:5) or $N_2: CO_2$ (95:5). The number of animals used for each value is indicated in parentheses.

DISCUSSION

Narcotic analgesics have been shown to be taken up into brain slices by a process having many of the characteristics of active transport, including uptake against an

apparent concentration gradient, saturability, metabolic dependence, structural specificity, stereospecificity and substrate competition.

A nonsaturable component was also observed in the accumulation of narcotic analgesics in cortical slices, as evidenced by a constant T/M ratio of about 1·7 above a concentration of 5×10^{-6} M in the medium. The relative contribution of simple diffusion and a binding process with a high capacity cannot be distinguished by these experiments.

The results discussed above are in disagreement with those obtained by Bell[18] and by Miller and Elliott,[19] who could not reduce uptake of morphine and methadone, respectively, by metabolic inhibition, by omission of glucose from the medium or by the addition of nalorphine. Although methadone was not included in our studies, it might be predicted that transport would be observed only at low concentrations of these drugs, and that at the higher concentrations of drug used by these investigators (i.e. 0·1 to 1 mM) only binding and diffusion contributed significantly to the total uptake.

In comparison to results obtained in the kidney[2] and choroid plexus,[2, 4] the system transporting the narcotic analgesics into brain slices was found to have a lower capacity for uptake of dihydromorphine, as evidenced by the low T/M ratio at equilibrium.

In addition, unlike the earlier studies with the kidney and choroid plexus, quaternary organic bases did not appear to compete with dihydromorphine for uptake into brain slices. These results with brain slices were not unexpected, since Tochino and Schanker[20] found no evidence for transport of the organic base, hexamethonium, into brain slices against a concentration gradient.

On the other hand, the accumulation of choline into brain slices as reported by Schuberth et al.[21] and of carbachol as reported by Creese and Taylor[22] may be due to uptake by a system other than that transporting the narcotic analgesics.

The effects of some tertiary and secondary bases on dihydromorphine uptake are currently being studied. Preliminary results by Oka and Hug (unpublished) suggest that histamine, norepinephrine and serotonin all inhibit dihydromorphine uptake into brain slices. Investigation is continuing in our laboratory to determine if narcotic analgesics are transported by one of the systems normally operating to accumulate these or other constituents of brain tissue.

Codeine, an analgesic, and nalorphine, an analgesic antagonist with analgesic action,[23] both depressed the uptake of dihydromorphine by brain slices. The inhibition of uptake by nalorphine was shown to be a competitive one. Such depression may represent competition for transport or tissue binding sites or for both, competition which is to be expected for such structurally similar analogues, which are also accumulated by the slices.

Finally, it should be emphasized that demonstration of active transport in tissue slices provides no indication of the direction of the transport in the intact animal (e.g. blood to brain vs. brain to blood). However, since many of the narcotic analgesics enter the central nervous system to a very limited degree,[24–26] it is tempting to speculate that the transport mechanism demonstrated in vitro may function to limit the penetration of these drugs into brain.[6]

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