Is the uptake of narcotic analgesics by cerebral cortical slices mediated in part by active transport?

Scrafani & Hug (1968) reported that the uptake of a variety of morphine and morphinan derivatives by cerebral cortical slices from several species was reduced in an anaerobic atmosphere or at a low temperature. They showed that the accumulation of tritium-labelled dihydromorphine [^{3}H]DHM by rat cerebral cortex *in vitro* occurred against an apparent concentration gradient, was saturable, was dependent on tissue metabolism, and was competitively inhibited by nalorphine. They concluded that the overall accumulation of narcotic analgesics such as [^{3}H]DHM by brain slices involved both an unsaturated and a saturated component, the latter having the characteristics of an active transport system. Their conclusions are not in agreement with those of Miller & Elliott (1954), Bell (1958) and Kayan, Misra & Woods (1970). We have attempted to resolve the major points of controversy.

Cerebral cortical slices from male Holtzman rats (150–200g) were incubated at 37° in an atmosphere of 5% carbon dioxide in oxygen with the pH of the medium between 7·3 and 7·5 according to Scrafani & Hug (1968). All tissues were preincubated for 15 min before the addition of [^{3}H]DHM (20 m Ci mmol^{-1}). Metabolic inhibitors were added just before pre-incubation and the tissues were incubated for 30 min. Nalorphine was added just before the [^{3}H]DHM and the tissues incubated for 10 min. Control tissues incubated with [^{3}H]DHM alone were included in each experiment.

Tissues and media were analysed for radioactivity as described previously except that 0·6 ml of Soluene (Packard Instrument Co.) was used in place of KOH in 95% ethanol, and 10 instead of 7 ml of the toluene phosphor solution was added to each tissue-sample vial. Results were expressed either as the concentration of [^{3}H]DHM per gram of tissue or per ml of medium or as the tissue to medium concentration ratio (T/M). "Percent of control" refers to the uptake of [^{3}H]DHM by a slice incubated in the presence of a potential inhibitor compared to the uptake by a slice from the same animal incubated simultaneously in the absence of that inhibitor (control).

As in the earlier study, descending paper chromatography confirmed the stability of [^{3}H]DHM during the incubation periods.

There are three major questions to be considered.

1. *Saturability.* If total tissue uptake of [^{3}H]DHM in 30 min is plotted against media concentrations above 1 mM on an arithmetic scale as was done by Kayan & others, the resulting slight curvilinear form of the line does not clearly indicate the participation of a saturable component in the uptake process. Yet in favour of the hypothesis that a saturable component participates in the overall uptake of [^{3}H]DHM are: (i) that it is necessary to use initial rates to determine kinetic parameters reliably; at 30 min the accumulation of [^{3}H]DHM has already reached the steady state (Scrafani & Hug, 1968; Kayan & others, 1970). While the measurement of true initial rates poses difficulties, approximate values can be obtained by using short periods of incubation. With 10 min incubation periods we consistently found that the T/M ratios declined from a mean of 2·8 to 1·7 as the media concentrations of [^{3}H]DHM were increased from 0·05 to 1·0 μM or more (Table 1). Kayan & others (1970) noted that higher T/M ratios were evident at 4 than at 8 μM and above even at the steady state, indicating the participation of a saturable component in the overall accumulation of [^{3}H]DHM. (ii) The finding of a steady state T/M value of approximately 1·7 at media concentrations of greater than 5 μM (Scrafani & Hug, 1968) indicates the presence of a significant unsaturated component. When an estimate of this component is subtracted from the total uptake, the saturable component becomes clearly evident (Fig. 1). (iii) Because of the uncertainty of estimating the contribution of the

Table 1. *Accumulation of* [^{3}H]*DHM by rat cerebral cortical slices incubated in different concentrations of* [^{3}H]*DHM.*

[^{3}H]DHM concentration in medium (μM)	[^{3}H]DHM concentration in tissue (μM)	T/M	n
0·05	0·14	2·82	8
0·1	0·22	2·19	8
0·2	0·40	2·00	15
0·5	0·98	1·96	12
0·8	1·46	1·83	4
1·0	1·78	1·78	8
3·0	5·33	1·77	4
3·75	6·60	1·76	4

unsaturated component, we attempted to calculate kinetic parameters only for the overall uptake process. With data from three separate experiments, we determined K_m valves of 5·9, 14·2, and 17·6 μm with corresponding V_{max} values of 9·3, 28·7 and 35·6 nmol g^{-1} 10 min^{-1}. Similarly determined values for the overall uptake of the narcotic antagonist, nalorphine-^{3}H, were $K_m = 0{\cdot}9\mu$M and $V_{max} = 5$ nmol g^{-1} 10 min^{-1}. (iv) Examination of the data of Bell (1958) for morphine and of Miller & Elliott (1954) for methadone reveals that there was a progressive decline in the T/M ratios for the uptake of these drugs by rat cerebral cortical slices as the media concentrations were increased from 1·0 to 10 mM. Such observations, even with the relatively high substrate concentrations in the media, support the concept of the participation of a saturable process in the overall uptake of these narcotic analgesics.

2. *Inhibition by narcotic antagonists.* In concentrations of 1 mM, the narcotic analgesic antagonists, nalorphine and naloxone, as well as the morphine analogue codeine, consistently inhibit the 10 min accumulation of [^{3}H]DHM (1 μM in medium) by approximately 23%. The degree of inhibition appeared to be proportional to the concentration of the antagonists between 0·01 and 1·0 mM. Inhibition of [^{3}H]DHM uptake by nalorphine was NOT evident with incubation periods of 30 min or longer (i.e. at the steady state of [^{3}H]DHM accumulation).

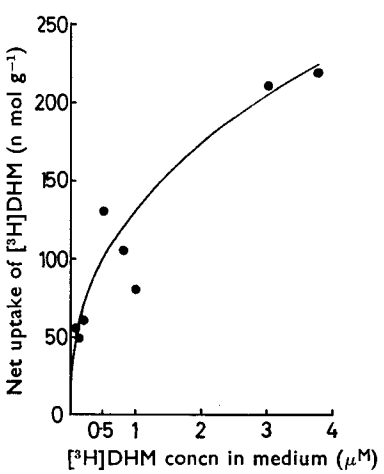

FIG. 1. Estimation of the saturable uptake of [^{3}H]DHM by rat cerebral cortical slices incubated for 10 min. The T/M of the unsaturated component was estimated as 1·7 which was subtracted from the overall T/M value in order to calculate the net uptake.

Nalorphine and dihydromorphine showed mutual competitive inhibition of their accumulation when the substrate concentration was in the range of 0·1 to 2 μM and the inhibitor concentration was 1 mM. Inhibitory constants (K_I) calculated for the overall uptake processes were 3·7 mM for nalorphine (against [^{3}H]DHM) and 6·8 mM for dihydromorphine against [^{3}H]nalorphine). Thus, there was a 300-fold difference in the K_m and K_I values for dihydromorphine. Such a large difference is not expected for competitive interactions at a single binding or transport site (Christensen, 1966 Webb, 1963). The divergence in K_m and K_I values for dihydromorphine interactions with nalorphine indicates that these drugs are interacting at more than one site.

Miller & Elliott (1954) did not demonstrate significant inhibition of methadone (0·3 mM) uptake after 1 h incubation in the presence of nalorphine (1·4 mM). Kayan & others (1970) reported that nalorphine (8 mM) did not affect the 30 min accumulation of [^{3}H]DHM (8·3 mM). In contrast, our observations were made after short periods of incubation before the steady state was reached; there was no inhibitory effect of nalorphine on [^{3}H]DHM uptake at the steady state. We also used concentrations of nalorphine or naloxone 100 to 1000 fold greater than those of [^{3}H]DHM; the other investigators used concentrations of nalorphine less than 5-fold greater than that of methadone and equal to that of [^{3}H]DHM. We have been unable to detect inhibition of [^{3}H]DHM uptake when the concentration of nalorphine was equal to that of [^{3}H]DHM (1 μM or 1 mM). Interestingly, Kayan & others (1970) found a significant depression of [^{3}H]DHM uptake *in vitro* when a large dose of nalorphine (20 mg kg^{-1}) was administered to the rat before it was killed.

(3) *Metabolic dependence.* Experiments in which the oxygen/carbon dioxide (95:5%) atmosphere was replaced by a similar nitrogen/carbon dioxide atmosphere showed [^{3}H]DHM accumulation to be dependent in part on aerobic metabolism (Table 2). Kayan & others (1970) made similar observations for [^{3}H]DHM and Miller & Elliott (1954) reported an 18% reduction of methadone uptake in a N_2 atmosphere. When glucose was omitted from the medium the uptake of [^{3}H]DHM was 15% less than control and the data of Miller and Elliott (1954) show about a 9% reduction in the accumulation of methadone. The report of Kayan & others (1970) indicates that omission of glucose by itself had little effect on the uptake of [^{3}H]DHM, but when combined with other inhibitors (e.g. N_2 atmosphere, KCl) the omission of glucose increased their effectiveness. These observations support the hypothesis that a metabolically dependent process participates in the uptake of narcotic analgesics by brain slices. Additional support comes from the findings that ouabain, an inhibitor of Na, K-activated ATPase, and dinitrophenol, an uncoupler of oxidative phorphorylation, reduced the uptake of [^{3}H]DHM (Table 2). The latter observation has been confirmed by Kayan & others (1970).

Table 2. *Effects of metabolic inhibitors on the steady state accumulation of* [^{3}H]*DHM by rat cerebral cortical slices.*

Inhibitor	Concentration	n	% of Control* ± s.e.	$P \leqslant$
N_2/CO_2	95/5	7	88 ± 3	0·05
Glucose omitted ..	—	12	85 ± 3	0·001
Ouabain	1 mM	8	80 ± 2	0·001
Dinitrophenol ..	1 mM	7	75 ± 4	0·01
Iodoacetate	1 mM	8	82 ± 2	0·001

* The mean control T/M was 1·52 ± 0·03 (s.e.) for all 23 animals used in these experiments. Each animal served as its own control.

In agreement with Kayan & others, we observed no inhibition of [^{3}H]DHM uptake when slices were incubated in the presence of cyanide, malonate or fluoroacetate. These agents are relatively ineffective in reducing metabolism of unstimulated cerebral cortical slices although they are potent inhibitors of oxidative metabolism in electrically stimulated slices (Webb, 1963). No one has yet reported studies of the accumulation of narcotic analgesics by electrically stimulated slices.

Kayan & others (1970) stated that iodoacetic acid (1 mM) did not alter the uptake of [^{3}H]DHM by rat cerebral cortical slices, but we have consistently observed significant degrees of inhibition (see below and Scrafani & Hug, 1968). Webb (1963) has reported that in these concentrations, iodoacetate inhibits a variety of biochemical processes.

The narcotic analgesic antagonists were additive with either dinitrophenol or iodoacetate in producing inhibition of [^{3}H]DHM uptake. Slices were preincubated for 15 min in the presence or absence of the metabolic inhibitor (1 mM). When nalorphine (1 mM) was used it was added at the end of the preincubation period. Incubation with [^{3}H]DHM (1 μM) lasted 10 min. [% inhibition ($\pm$ s.e. n = 4, each animal serving as its own control): nalorphine 14 $\pm$ 6, iodoacetate 14 $\pm$ 7, nalorphine + iodoacetate 23 $\pm$ 9; nalorphine 12 $\pm$ 7, dinitrophenol 30 $\pm$ 4, nalorphine + dinitrophenol 43 $\pm$ 4]. Thus, it appears that narcotic antagonists and metabolic inhibitors were acting at separate sites to inhibit the uptake process. This is to be expected for active transport processes in which substrate analogues compete for carrier sites while metabolic inhibitors interfere with the production or utilization of energy stores (e.g. ATP) which activate the carrier mechanism.

Thus, the above data offer substantial support for the hypothesis that [^{3}H]DHM and other narcotic analgesics are taken up into rat cerebral cortical slices by a process having many of the characteristics of active transport, including uptake against an apparent concentration gradient, saturability, metabolic dependence and substrate competition.

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REFERENCES

BELL, J. L. (1958). *J. Neurochem.*, **2**, 265–282.
CHRISTENSEN, H. N. (1966). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **25**, 850–853.
KAYAN, S., MISRA, A. L. & WOODS, L. A. (1970). *J. Pharm. Pharmac.*, **22**, 941–943.
MILLER, J. W. & ELLIOTT, H. W. (1954). *J. Pharmac. exp. Ther.*, **110**, 106–114.
SCRAFANI, J. T. & HUG, C. C., Jr. (1968). *Biochem. Pharmac.*, **17**, 1557–1566.
WEBB, J. L. (1963). *Enzyme and Metabolic Inhibitors*, Vol. 1, p. 96–98 and 480, New York: *Academic Press.*