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EFFECTS OF NARCOTIC ANALGESICS ON THE UPTAKE AND RELEASE OF 5-HYDROXYTRYPTAMINE IN RAT SYNAPTOSOMAL PREPARATIONS

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1 The effects of various narcotic analgesics on the uptake and release of labelled 5-hydroxytryptamine (5-HT) in brain and spinal cord synaptosomes were investigated.

2 Methadone was most active in inhibiting 5-HT uptake (IC_{50} 2.5×10^{-7} M). Levorphanol also inhibited 5-HT uptake to a large extent (IC_{50} 8.8×10^{-7} M) while dextrorphan, pethidine and pentazocine showed much less activity. Etorphine and morphine had virtually no such activity, with IC_{50}s higher than 10^{-4} and 10^{-3} M respectively.

3 The same order of potency as '5-HT releasers' was found when radioactivity was measured in [^{3}H]-5-HT preloaded synaptosomal pellets incubated for 20 min with the various narcotics. Methadone, like chlorimipramine, showed a significant effect at a concentration of 10^{-7} M while morphine, at a concentration of 10^{-4} M, had no effect.

4 When 5-HT release was studied by a perfusion technique, which largely prevents reuptake of the released amine, only fenfluramine, an anorectic agent proposed as a 5-HT releaser, significantly increased spontaneous 5-HT release. These data suggest that the apparent 5-HT release induced by various narcotics in traditional incubation techniques may largely depend on their ability to interfere with neurotransmitter reuptake mechanisms.

5 The effects of the various narcotics on 5-HT uptake have no relationship to their relative potency as analgesics in the rat. In the light of their poor effectiveness as 5-HT releasers, it can be concluded that mechanisms other than 5-HT uptake inhibition and release are probably involved in the analgesic effects of these compounds in intact animals.

Introduction

Changes of brain 5-hydroxytryptamine (5-HT) synthesis and/or metabolism have been found by various authors after acute administration of morphine and other narcotic analgesics (Bowers & Kleber, 1971; Haubrich & Blake, 1973; Yarbrough, Buxbaum & Sanders-Bush, 1973; Goodlet & Sugrue, 1974; Papeschi, Theiss & Herz, 1975; Pérez-Cruet, Thoa & Ng, 1975; Samanin, Miranda & Mennini, 1978). These findings suggest that brain 5-HT might be involved in the pharmacological activity of these compounds. This hypothesis is supported by the fact that procedures affecting 5-hydroxytryptaminergic mechanisms have been found to modify the effect of narcotic analgesics in various animal species (Tenen, 1968; Samanin, Gumulka & Valzelli, 1970; Major & Pleuvry, 1971; Samanin & Valzelli, 1971; Flórez, Delgado & Armijo, 1972; Gorlitz & Frey, 1972; Genovese, Zonta & Mantegazza, 1973; Vogt, 1974; Garau, Mulas & Pepeu, 1975; Sewell & Spencer, 1975). However, other authors have failed to confirm these findings, thus casting doubt on the involvement of 5-HT in narcotic action (Buxbaum, Yarbrough & Carter, 1973; Reinhold, Bläsig & Herz, 1973; Lorens & Younger, 1974).

A few studies have been made with radioactively labelled 5-HT and brain preparations in an attempt to assess whether narcotic analgesics can directly affect 5-HT mechanisms such as uptake and release. Ciofalo (1974) reported that methadone markedly inhibits [^{3}H]-5-HT uptake in rabbit brain synaptosomes while morphine showed little such activity. Neither drug altered potassium-evoked release of 5-HT from preloaded synaptosomes. More recently, Moffat & Jhamandas (1976) found that methadone and levorphanol have a strong inhibitory action on the uptake of [^{3}H]-5-HT in rat hypothalamus slices while morphine and diacetylmorphine caused inhibition only at very high concentrations. These authors also found that morphine and naloxone inhibit 5-HT uptake to the same extent, a result recently confirmed by others

(Warwick & Schnell, 1976). Although these findings suggest that the effect of narcotic analgesics on 5-HT uptake is not related to their analgesic activity, interpretation of the results is difficult because of differences in animal species, brain region and methodology and the limited number of drugs studied.

In this study the effects of various narcotic analgesics on 5-HT uptake in rat brain synaptosomal preparations were compared with those of chlorimipramine, a potent 5-HT uptake inhibitor (Carlsson, Corrodi, Fuxe & Hökfelt, 1969a). The effects on 5-HT release in the same preparations using a traditional incubation or a superfusion technique were also investigated, and compared with fenfluramine, a supposed 5-HT releaser (Garattini, Buczko, Jori & Samanin, 1975). In addition, synaptosomes isolated from spinal cord were used since recent physiological and biochemical studies suggest that narcotic analgesics and 5-HT might interact at this level (Shiomi, Murakami & Takagi, 1974; Proudfit & Anderson, 1975).

Methods

Animals

Male CD-COBS Sprague Dawley (Charles River, Italy) rats weighing 175 to 200 g, housed in Makrolon cages and maintained at constant room temperature (21 ± 1°C) and relative humidity (60%), with free access to water and food, were used in these experiments.

Preparation of synaptosomes

Animals were killed by decapitation, the brain and spinal cord were quickly removed, weighed and homogenized in 10 volumes of ice-chilled 0.32 M sucrose, pH 7.4, in a glass homogenizer with teflon pestle (average clearance 0.010 to 0.015 cm), using 12 full up and down strokes in a 90 s period.

Synaptosomes were obtained as described by Gray & Whittaker (1962). The homogenate was centrifuged in a Sorvall RC-2B centrifuge at 4°C for 10 min at 1000 *g*. The resulting pellet was resuspended twice with 10 ml 0.32 M sucrose and centrifuged again as described above to obtain the crude nuclear pellet (P1). The supernatants of the three centrifugations at 1000 *g* were pooled and centrifuged at 17000 *g* for 60 min to yield the crude mitochondrial pellet (P2). The P2 pellet was resuspended in 0.32 M sucrose and layered on a discontinuous sucrose density gradient consisting of equal layers of 1.2 and 0.2 M sucrose, and centrifuged at 53,500 *g* for 2 h in a Beckman Spinco L 2 65B ultracentrifuge with SW 27 rotor. All operations were performed at 4°C.

The fractions recovered were: myelin (A) in the top layer, synaptosomes (B) in the middle layer and mitochondria (C) in the pellet. Electron microscopy indicated that the fractions were homogeneous including spinal cord preparations. Lactate dehydrogenase (LDH) (Johnson, 1960) and monoamine oxidase (MAO) (Krajl, 1965) determinations served as biochemical markers of the fractions. The relative specific activity (i.e. % of enzyme activity in fraction/% of protein in fraction) for brain and spinal cord were as follows: LDH (A) 0.50, 0.59; (B) 1.28, 1.86; (C) 1.29, 1.19; MAO (A) 0.15, 0.22, (B) 0.61, 0.69, (C) 3.21, 2.76 (the first figure refers to the brain, the second to spinal cord).

Uptake of [^{14}C]-5-hydroxytryptamine

Synaptosomes obtained from the gradient were diluted with Krebs & Henseleit (1932) buffer, half calcium concentration, containing 0.25 mM pargyline, to give a final concentration of 0.5 to 1 mg/ml of protein (Lowry, Rosebrough, Farr & Randall, 1951). Samples of 0.6 ml were incubated at 30°C in a water bath, with or without drugs. After 5 min preincubation, uptake was started, by the addition of [^{14}C]-5-HT. Control samples were incubated at 0°C to determine diffusion through membranes. The difference between [^{14}C]-5-HT accumulated at 30°C and 0°C was taken as a measure of the active transport system. The reaction was stopped by cooling the tubes in ice and adding 0.5 ml of ice-chilled Krebs-Henseleit buffer. Samples were filtered through Millipore filters (0.65 μm pore size) and washed twice with 1 ml of Krebs-Henseleit buffer. The filters were dissolved in Bray's solution (Bray 1960) and counted for radioactivity in a Packard Tri-Carb 3002 liquid scintillation spectrometer. Counting efficiency, determined by internal standardization on randomly chosen samples, averaged 75%. Data were calculated as percentage inhibition of accumulation according to the formula:

$$\% \text{ inhibition} = \frac{\text{ct/min control} - \text{ct/min drug}}{\text{ct/min control}} \times 100$$

Release of [^{3}H]-5-hydroxytryptamine

Synaptosome suspensions in Krebs-Henseleit buffer were obtained as described above. Pooled samples were incubated with [^{3}H]-5-HT, 10^{-7} M, for 15 min at 30°C and uptake was stopped by cooling them in ice. After centrifugation at 17,000 *g* for 10 min at 4°C, the synaptosomal pellet was gently resuspended in fresh buffer. Aliquots of 0.6 ml were incubated at 37°C for 5 min to allow equilibration. At this point (zero time) drugs were added. Control samples received solvent alone. After a further 20 min incubation at 37°C, samples were filtered and analyzed for

radioactivity as described for the uptake studies. Counting efficiency for ^3H was 13%.

Data were calculated as percentage of the release induced by drugs, according to the formula:

$$\% \text{ release} = \frac{\text{ct/min control 20 min} - \text{ct/min drug 20 min}}{\text{ct/min control 0 min}} \times 100$$

Total radioactivity was taken as a measure of 5-HT uptake and release since more than 85% of radioactivity in the sample was identified as authentic 5-HT by thin layer chromatography.

Superfusion study

Pooled synaptosome suspensions were preincubated in the presence of 0.1 μM [^{14}C]-5-HT as described before. Aliquots of 1 ml of the suspension were filtered through Millipore filters, washed and placed on the bottom of a superfusion chamber (Raiteri, Angelini & Levi, 1974). Drugs were dissolved in Krebs-Henseleit solution and superfused from 0 time to 20 min, at a constant rate of 0.5 ml/min. The effluent was collected directly into liquid scintillation vials every 5 min. The radioactivity remaining on the filters at the end of the superfusion was also counted, and was used for calculation of the percentage of total radioactivity released (i.e. total fractions recovered plus filter).

Drugs

The drugs used were: morphine hydrochoride (Carlo Erba, Italy); etorphine hydrochloride (Reckitt & Colman); methadone hydrochloride (Reckitt & Colman); pethidine hydrochloride (Carlo Erba, Italy); pentazocine lactate (Fortral, Winthrop); dextrorphan (+)-tartrate monohydrate and levorphanol (−)-tartrate dihydrate (kindly donated by Zambon, Italy); chlorimipramine hydrochloride (Anafranil, Ciba Geigy, Switzerland); (±)-fenfluramine (Servier, France). All drug concentrations refer to the free base. [^{14}C]-5-HT (58 mCi/mmol) and [^{3}H]-5-HT (8.5 Ci/mmol) were obtained from the Radiochemical Centre, Amersham. The radiochemical purity, determined by thin-layer chromatography on Merck cellulose plates, solvent acetic acid:butanol:water (3:12:5) was found to be over 80% in our laboratory.

All reagents were of analytical grade.

Results

Uptake of [^{14}C]-5-hydroxytryptamine

Brain and spinal cord synaptosomes showed a very rapid, linear uptake of 5-HT during the first 5 min and a slower accumulation rate for 20 min thereafter. The time of linear uptake chosen for the present studies was 5 min. Experiments at 30°C and 0°C, with or without ouabain 10^{-4} M, showed that [^{14}C]-5-HT is actively taken up by brain and spinal synaptosomes. The *Km* for the high affinity uptake, determined by the Lineweaver-Burk plot, was 0.14 μM and 0.16 μM for brain and spinal cord respectively. In experiments with drugs a 5-HT concentration equal to *Km* was used i.e. 0.1 μM for both preparations. Kinetic analysis in spinal synaptosomes of animals which had received an intraventricular injection of 75 μg of 5,6-dihydroxytryptamine (5,6-DHT) showed that the high affinity uptake is markedly reduced in these animals, while the low affinity uptake is not affected by 5,6-DHT treatment (unpublished results). Similar results have been reported for brain synaptosome

Table 1 Effect of narcotic drugs on uptake of [^{14}C]-5-hydroxytryptamine by brain and spinal synaptosomes

Drug	IC_{50} M	
	Brain	Spinal cord
Methadone	$2.5 \pm 0.2 \times 10^{-7}$	$1.5 \pm 0.1 \times 10^{-7}$
Levorphanol	$8.8 \pm 1.3 \times 10^{-7}$	$3.6 \pm 0.3 \times 10^{-7}$
Pethidine	$1.5 \pm 0.2 \times 10^{-6}$	$1.2 \pm 0.1 \times 10^{-6}$
Dextrorphan	$4.4 \pm 0.5 \times 10^{-6}$	$4.5 \pm 1.0 \times 10^{-6}$
Pentazocine	$3.4 \pm 0.6 \times 10^{-5}$	$2.0 \pm 0.1 \times 10^{-5}$
Etorphine	$1.8 \pm 0.1 \times 10^{-4}$	$1.7 \pm 0.1 \times 10^{-4}$
Morphine	$> 10^{-3}$	$\geq 10^{-3}$
Chlorimipramine	$2.8 \pm 1.8 \times 10^{-7}$	$4.6 \pm 0.5 \times 10^{-8}$
(±)-Fenfluramine	$2.8 \pm 0.3 \times 10^{-6}$	—

Data are calculated as the concentration (M) of drug causing 50% inhibition of [^{14}C]-5-hydroxytrypramine uptake from a log dose-effect plot based on 3 points. Each value represents the mean ± s.d. of 4 experiments.

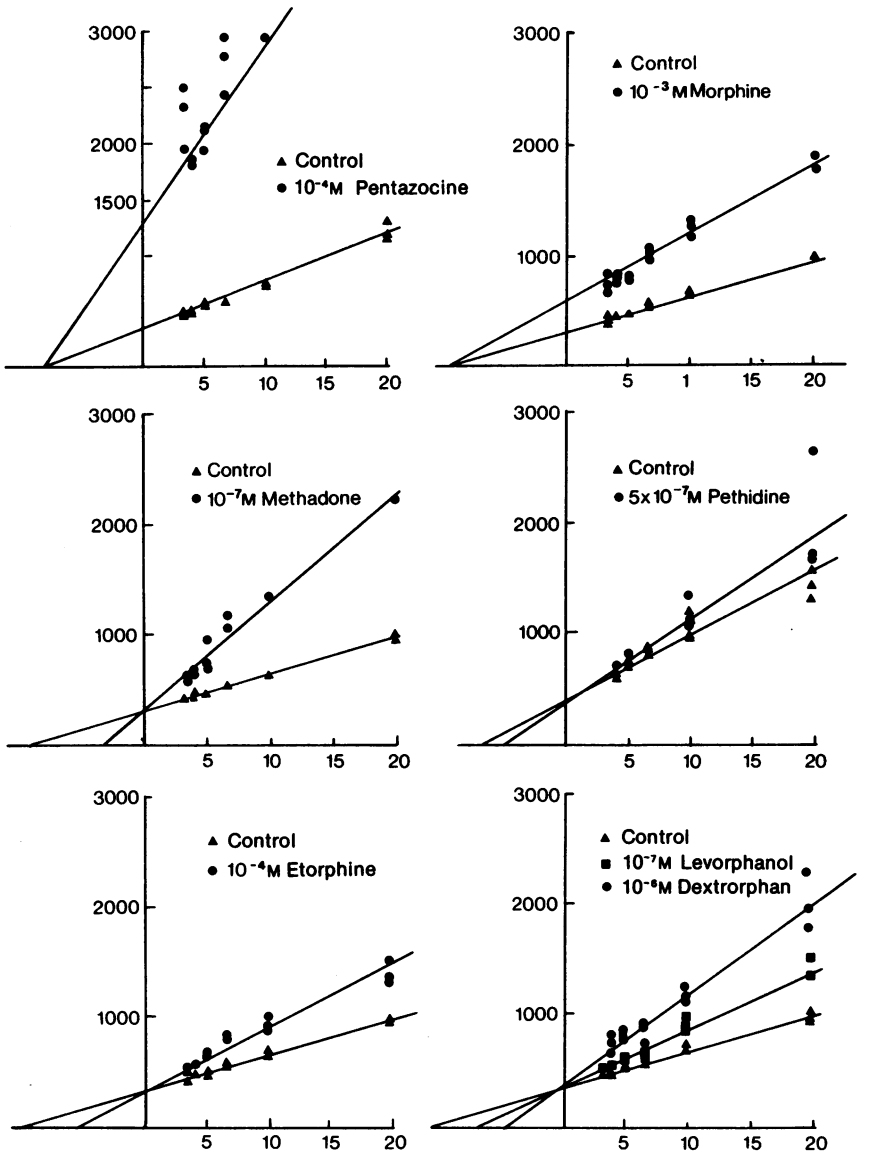

Figure 1 Lineweaver-Burk plots of [^{14}C]-5-hydroxytryptamine ([^{14}C]-5-HT) uptake kinetics: reciprocals of [^{14}C]-5-HT concentrations (μM) were plotted against reciprocals of uptake velocity (nmol min^{-1} mg^{-1} protein)

preparations (Belin, Kouyoumdjian, Bardakdjian & Gonnard, 1975).

Drug effects on uptake of [^{14}C]*-5-hydroxytryptamine*

IC_{50} values of the various drugs on the uptake of [^{14}C]-5-HT in brain synaptosomes are shown in Table 1. Methadone was the most active compound with an IC_{50} of 2.5×10^{-7} M very close to that shown by chlorimipramine (2.8×10^{-7} M) in this study. Levorphanol also markedly inhibited 5-HT uptake (IC_{50} 8.8×10^{-7} M) while the (+)-isomer, dextrorphan, showed less activity. A 50% inhibition at 10^{-6} and 10^{-5} M respectively was obtained with pethidine and pentazocine, while etorphine inhibited 5-HT uptake by 50% only at a concentration higher than 10^{-4} M. The least active compound was morphine with an IC_{50} higher than 10^{-3} M. IC_{50} values for

compounds in spinal synaptosomes were of the same order of magnitude as in brain synaptosomes (Table 1).

Figure 1 shows Lineweaver-Burk plots for inhibition kinetics; with the exception of morphine and pentazocine, all the compounds inhibited 5-HT uptake in a competitive manner.

Release of [^{3}H]-5-hydroxytryptamine

The efflux of [^{3}H]-5-HT was followed in control samples for 40 min. The apparent release amounted to approximately 10% after 20 min incubation, the time chosen for these experiments. A further 20 min incubation did not significantly increase the rate of spontaneous efflux, in either brain or spinal cord preparations. No appreciable leakage of endogenous LDH was observed in either preparation.

Drug effects on release of [^{3}H]-5-hydroxytryptamine

As shown in Table 2, chlorimipramine, a compound reported to act primarily by inhibiting 5-HT reuptake (Carlsson, Jonason & Lindqvist, 1969b) was very active in releasing [^{3}H]-5-HT, showing a significant effect at a concentration of 10^{-7} M. The various narcotics also had significant effects, with an order of potency as '5-HT releasers' corresponding closely to their ability to inhibit the uptake. Similar results were found when spinal synaptosomes were investigated. Since with this incubation technique the concentration of the labelled amine found in the sample is the net result of release and reuptake of the released neurotransmitter, it was difficult to establish whether a drug acted by increasing release, inhibiting reuptake or both (Johnson, Ho & Dewey, 1976; Kellar, Elliott, Holman, Vernikos-Danellis & Barchas, 1976). To

Table 2 Effect of narcotic drugs on release of [^{3}H]-5-hydroxytryptamine by brain synaptosomes

	Concentration (M)			
Drug	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Methadone	9.7 ± 0.3**	21.7 ± 0.7**	39.5 ± 1.6**	79.0 ± 13.0**
Levorphanol	—	9.0 ± 0.3	20.7 ± 3.0**	60.7 ± 0.3**
Pethidine	—	8.9 ± 0.3	22.7 ± 1.3**	38.9 ± 9.8**
Dextrorphan	3.1 ± 0.4	8.8 ± 0.7	36.5 ± 3.6**	68.0 ± 4.7**
Pentazocine	—	—	19.6 ± 1.0**	59.4 ± 18.0**
Etorphine	—	5.0 ± 0.6	20.6 ± 1.0**	47.9 ± 2.1**
Morphine	—	—	—	—
Chlorimipramine	30.4 ± 4.0**	36.5 ± 1.1**	50.1 ± 12.0**	93.2 ± 28.0**
(±)-Fenfluramine	4.4 ± 0.5	37.2 ± 1.8**	54.1 ± 4.1**	69.4 ± 8.0**

Data are calculated as % release induced by drug as described in the text. Each value represents the mean ± s.d. of 5 experiments.
**$P < 0.01$, statistical difference from controls, Dunett's test.

Table 3 Effect of superfused narcotic drugs on [^{14}C]-5-hydroxytryptamine release by brain synaptosomes

Drug (10^{-5} M)	% [^{14}C]-5-HT released during 20 min superfusion	% *stimulation*
None	45.4 ± 8.8	
Methadone	55.2 ± 1.8	22
Levorphanol	52.4 ± 4.2	15
Pethidine	55.5 ± 1.1	22
Dextrorphan	48.3 ± 3.4	6
Pentazocine	48.5 ± 4.0	7
Etorphine	43.3 ± 0.7	0
Morphine	49.1 ± 1.4	8
Chlorimipramine	53.5 ± 3.2	18
(±)-Fenfluramine	66.1 ± 3.5**	46

Data are mean values ± s.d. of 3 experiments. See Methods for experimental details.
**$P < 0.01$, Dunnett's test.
% stimulation calculated as follows: (% release with drug − % release with control/% release with control) × 100.

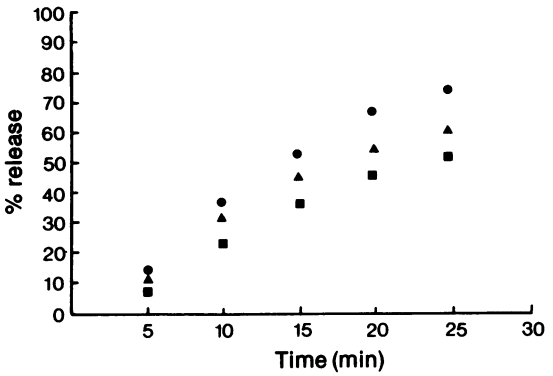

Figure 2 Percentage release of [^{14}C]-5-hydroxytryptamine is plotted versus time (min) in a superfusion system which avoids reuptake. The % release at each time is expressed as cumulative percentage of total radioactivity recovered (see Methods for details). Each point represents the mean of 3 experiments; the s.e. was in all cases less than 10%: (■) control; (▲) chlorimipramine 10^{-5} M; (●) fenfluramine 10^{-5} M. At 15, 20 and 25 min fenfluramine is significantly different from controls, $P < 0.01$, Dunnett's test.

overcome this difficulty the same drugs were studied in a superfusion system described by Raiteri *et al.* (1974) which minimizes interference of the reuptake mechanism in release studies with synaptosomes. As shown in Figure 2, fenfluramine, which releases 5-HT and inhibits its reuptake (Garattini *et al.*, 1975) significantly increases spontaneous 5-HT release in this system at 10^{-5} M while chlorimpramine, at the same concentration, produced no significant increase above the release control values. Like chlorimipramine, none of the narcotics studied significantly increased the release of 5-HT in this superfusion system (Table 3).

Discussion

Morphine and other narcotic analgesics such as methadone, heroin and etorphine have been found to increase the metabolism of brain 5-HT (Bowers & Kleber, 1971; Haubrich & Blake, 1973; Yarbrough *et al.*, 1973; Papeschi *et al.*, 1975; Samanin *et al.*, 1978). Increased 5-HT metabolism in the spinal cord has also been reported (Shiomi *et al.*, 1974). However, some authors found an increase of brain 5-hydroxyindoleacetic acid concentrations with morphine but not with other drugs such as pethidine, methadone and pentazocine (Goodlet & Sugrue, 1974; Sawa & Oka, 1976) suggesting that the interaction with 5-HT is not applicable to all narcotic analgesics. More recently Pérez-Cruet *et al.* (1975) reported an increase of brain tryptophan hydroxylase activity in rats treated with morphine and heroin.

We conclude from our studies that the effects of the various drugs on 5-HT uptake are not correlated with their potency *in vivo* as analgesics. Thus, etorphine showed very little activity in inhibiting 5-HT uptake while *in vivo* it is over 1000 times more potent than methadone (Blane, Boura, Fitzgerald & Lister, 1967) which, however, is very active in inhibiting 5-HT uptake. The effect of methadone found by others (Ciofalo, 1974; Moffat & Jhamandas, 1976) with one exception (Cahill & Medzihradsky, 1976) is particularly striking since it is of the same order of magnitude as that of chlorimipramine, which is one of the most potent 5-HT uptake inhibitors (Carlsson *et al.*, 1969a).

It has been noted that methadone but not morphine inhibits decapitation convulsions in rats (Moffat & Jhamandas, 1976) and it was suggested that an activation of descending 5-HT neurones to the spinal cord could be involved in this effect (Kamat & Sheth, 1971; Moffat & Jhamandas, 1976). The present findings showing inhibition of spinal 5-HT uptake by methadone but not by morphine could support this hypothesis, although various other factors might contribute to the reduction of decapitation convulsions. It would be interesting to investigate whether the effect on 5-HT uptake contributes to any other pharmacological actions of methadone not shown by morphine.

Levorphanol and pethidine also markedly inhibited 5-HT uptake. The effect of levorphanol was previously reported in experiments with rat hypothalamus slices (Moffat & Jhamandas, 1976). These authors found dextrorphan ten times less active than levorphanol, a finding which agrees with the present results but not with those of Cahill & Medzihradsky, (1976). While differences in methodologies and other factors could account for these discrepancies, it is clear that inhibition of 5-HT uptake is not related to the pharmacological effects of these compounds since dextrorphan has practically no activity in intact animals (Goldstein & Sheehan, 1969). That pethidine can inhibit 5-HT uptake was previously suggested on the basis of its ability to potentiate the '5-hydroxytryptophan syndrome' and to counteract the decrease of brain 5-HT induced by H 75/12 in mice (Carlsson & Lindqvist, 1969). However, these effects were observed at doses well above those required to cause analgesia, indicating a dissociation between the biochemical and analgesic effects.

The present study indicates that the apparent effect of narcotics on spontaneous release is mainly due to an inhibition of 5-HT uptake. This is borne out by the fact that none of the narcotics studied was effective (up to 10^{-5} M) as a 5-HT releaser in superfusion studies in which interference from the reuptake mech-

anism is minimized. Conversely, fenfluramine, a 5-HT releaser (Garattini *et al.*, 1975) raised 5-HT release well above control values in this system.

It was recently shown by Ciofalo (1974) that methadone increased spontaneous release of [^{3}H]-5-HT in brain synaptosomes by 38%. He found that neither methadone nor morphine significantly modify KCl-induced release of 5-HT, suggesting that the apparent increase of release could be due to inhibition of reuptake.

In conclusion, the scant effect of morphine and its potent congener, etorphine, as 5-HT uptake inhibitors makes it unlikely that this mechanism plays an important role in the interaction of these compounds with 5-HT in the brain and spinal cord of the rat. The effects observed *in vivo* could be due to other mechanisms such as changes in tryptophan concentrations (Goodlet & Sugrue, 1974), tryptophan hydroxylase activity (Knapp & Mandel, 1972; Pérez-Cruet *et al.*, 1975) and others.

Methadone and, to a lesser extent, pethidine and levorphanol, can be considered effective inhibitors of 5-HT uptake *in vitro* and probably *in vivo* too, but the significance of these effects in relation to the pharmacological actions of these compounds remains to be elucidated. However, our present results clearly indicate that an ability to inhibit 5-HT uptake is not related to analgesic activity in these compounds.

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