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Some aspects of the metabolism of morphine-*N*-oxide

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After administration of morphine-*N*-oxide (MNO) to rats the opiates appearing in the urine were morphine (61%) and MNO (39%). After administration of morphine, the urinary opiates were morphine (80%) and normorphine (20%). When tacrine was given with morphine the urine also contained MNO (46% of total urinary opiates) and the amount of normorphine was much decreased (to 1%), the remainder being morphine (53%). Tacrine and amiphenazole inhibited demethylation of morphine and codeine by a rat liver fraction *in vitro*. MNO had weak inhibitory activity. Neither MNO nor codeine-*N*-oxide were demethylated *in vitro*.

Woo, Gaff & Fennessy (1968) showed that patients being treated with morphine in combination with either tacrine or amiphenazole excreted in their urine a substance resembling morphine-*N*-oxide (MNO), but this substance was not detected when morphine was administered alone. It was suggested that MNO might be an intermediate metabolite of morphine whose excretion was enhanced in the presence of tacrine or amiphenazole because of inhibition of further metabolism. Fennessy (1968) found that the analgesic activity of MNO was potentiated by tacrine and again suggested that this was due to inhibition of metabolism of MNO. The present investigation deals with the metabolism of morphine and MNO and the effects of tacrine on their metabolism in the rat.

METHODS AND MATERIALS

Drugs

Commercial samples were used of codeine (D.H.A.), morphine sulphate (D.H.A), normorphine (Merck Sharp & Dohme), tacrine (H. W. Woods) and amiphenazole (H. W. Woods). Morphine-*N*-oxide (MNO) and codeine-*N*-oxide (CNO) were synthesized by the method of Freund & Speyer (1910), all reagents employed being of analytical grade.

Administration of drugs

Male albino rats, 300–450 g, in groups of five were injected intraperitoneally with 2 ml of the following solutions: (1) 10 mg/ml of morphine (as the base); (2) 10 mg/ml of morphine (base) plus 1 mg/ml of tacrine; (3) 25 mg/ml MNO; (4) 0·9% (w/v) sodium chloride as control.

Each group was placed in a metabolic cage immediately after injection and the urine was collected for 18 h without contamination by faeces. The receiving vessels contained 1 ml of 11·6 M HCl to prevent bacterial growth.

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Extraction of opiate bases from urine

At the end of the collection period the urine volume was recorded and additional 11·6 M HCl was added to give a final concentration of 1·2 M. The vessel was plugged and autoclaved at 22 p.s.i. ($1·52 \times 10^5$ Nm^{-2}) to liberate free bases from conjugates. The bases were extracted into isopropanol–chloroform (1 : 3) after saturation of the aqueous phase with Na_2CO_3 (Milthers, 1961). The MNO, being poorly extracted, was converted to morphine quantitatively by reacting the acidified solution with zinc dust (100 mg) and cupric sulphate (10 μg/ml) for 60 min.

The autoclaved urine samples were divided into two equal portions; one was extracted 3 times with equal volumes of isopropanol–chloroform (1 : 3) to measure excreted morphine and normorphine, the other was similarly extracted after reduction with zinc to measure MNO as additional morphine (a step found not to interfere with the extraction of any morphine or normorphine).

The pooled organic phase extract was evaporated under vacuum in a Büchli Rotavapor to 5 ml. This was quantitatively transferred to a centrifuge tube at 60° and evaporated to dryness by using O_2 free N_2.

The residue was dissolved in 1 ml 0·1 M HCl and interfering substances were removed with 3 washes of n-butanol. An aliquot of 100 μl of the resulting solution was applied with an Agla microsyringe as a broad streak 5 cm in length to a t.l.c. plate of silica gel G (Merck) previously activated at 100° for 30 min. Another 5 μl was applied as a spot together with marker spots.

When the isopropanol–chloroform (1 : 3) solvent front was 17 cm from the origin, the plates were rapidly dried at 50° and redeveloped in the same direction using the same solvent system to provide adequate resolution of morphine, normorphine and MNO.

The plates were then dried and the centre section containing the two 100 μl streaks was covered with stiff paper leaving only the two 5 μl spots and the marker spots on either side uncovered. These were sprayed with iodoplatinate reagent and the plate was examined under ultraviolet light. The areas of the 100 μl streaks corresponding to the fluorescent iodoplatinate spots in the 5 μl urine sections were scraped into centrifuge tubes and those areas not already removed which corresponded to the marker spots of morphine, normorphine and MNO were collected. Background adsorbance was estimated from blank areas of the same plate. The scrapings in each tube were extracted with 5 ml of 0·1 M HCl. After centrifugation at 300 rev/min for 10 min to pack the gel, absorbance of the supernatant solution was measured at 285 nm to estimate opiates.

Preparation of rat liver

Rats were killed by a blow on the head. The liver was rapidly removed, weighed, cooled on ice, and homogenized in ice-cold 0·1 M phosphate buffer at pH 7·4. The homogenate was diluted with phosphate buffer to a final concentration of 500 mg liver/ml and centrifuged at 9000 rev/min for 20 min at 0°. The supernatant containing microsomes and soluble fraction was collected and kept at 0° until incubation.

Demethylating activity of the liver fraction

The demethylating activity of the microsomal + soluble fraction was determined by

a modification of the method of Axelrod (1956), in which formaldehyde production is measured.

The incubation mixtures had the following composition: liver microsomal + soluble fraction (corresponding to 500 mg liver/ml), 0·2 ml; substrate 10 mM, 1 ml; inhibitor 10 mM, 1 ml; NADPH regenerating system 1 ml (NADP 0·2 mM, trisodium isocitrate 15 mM, pig heart isocitrate dehydrogenase (Sigma) 40 μg protein/ml); buffer solution 1 ml ($MgCl_2$, 25 mM, and nicotinamide, 50 mM in 0·5 M phosphate buffer at pH 7·4); Nash B solution (Nash, 1953), 1 ml; water, to make 6·2 ml. The activity of the NADPH regenerating system was checked and found to be satisfactory before use; all mixtures were incubated at 37° with constant shaking.

After incubating for 120 min, the reaction was stopped by adding 2 ml of 40% trichloroacetic acid and the mixture was centrifuged at 3000 rev/min for 10 min. The adsorbance of the supernatant was measured at 412 nm to estimate formaldehyde produced by demethylation of the substrate.

A standard curve using monomethylol dimethyl hydantoin (0·03–0·3 μmol) to replace the substrate in the incubation mixture was prepared daily.

RESULTS

Excretion of opiates in the urine after administration of MNO to rats

Thin-layer chromatographs of the organic extract of both reduced and unreduced samples of rat urine contained a single spot with an R_F of 0·29 corresponding to that of morphine. This was extracted from an unreduced sample and was positively identified as morphine by mass spectrographic analysis; both the m/e peak positions and the disintegration pattern were distinguishable from those expected for MNO, and agreed with the published mass spectrographic data for morphine (Audier, Fetizon & others, 1965).

Normorphine was not detected, but taking into account the sensitivity of the detection method and extraction procedure, less than 70 μg of normorphine, accounting for 0·2% of excreted metabolites, could be present in the urine.

As pointed out in Methods, the difference in the morphine content of reduced and unreduced samples was equivalent to the MNO content. Table 1 shows that 61% of opiates collected in the urine was accounted for as morphine while the remainder was excreted as unchanged MNO.

Table 1. *Total excretion of morphine, normorphine and MNO in urine of a group of 5 rats 18 h after injection of 50 mg MNO to each rat.*

	Morphine	Normorphine	MNO
Concentration of opiate in urine (μg/ml)	382	—	246
Total of each opiate excreted (mg) ..	15·2	0	9·8
Proportion of each opiate in urine ..	61%	<0·2%	39%

The effect of tacrine on the excretion of morphine metabolites in the urine.

Thin-layer chromatograms of the extracts of urine from rats treated with morphine contained two spots the R_F values of which coincided with those of authentic morphine

Table 2. *Total excretion of morphine, normorphine and MNO in the urine of a group of 5 rats 18 h after intraperitoneal injection of 20 mg morphine or 20 mg morphine plus 2 mg tacrine to each rat.*

	Morphine alone			Morphine plus tacrine		
	Morphine	Normorphine	MNO	Morphine	Normorphine	MNO
Concentration of opiate in urine (μg/ml)	318	82	—	260	10·2	254
Total of each opiate excreted (mg)	2·21	0·54	0	5·20	0·09	4·43
Proportion of each opiate in urine	80·4%	19·6%	<0·1%	53·5%	0·9%	45·6%

(R_F 0·29) and normorphine (R_F 0·11). No MNO was detected; the sensitivity was 35 μg, which is equivalent to 0·1% of recovered opiates.

When morphine and tacrine were administered together, chromatograms of the unreduced urine extracts contained spots corresponding to morphine and MNO (R_F 0·05) but not to normorphine. The spot with the R_F of 0·05 was extracted from the plate and analysed by mass spectroscopy. The m/e value of the primary peak of 301 and the disintegration pattern were indistinguishable from those of a reference sample of MNO. This spot was not present after reduction of the urine sample.

The amounts of morphine, MNO and normorphine excreted in the urine following administration of morphine, or morphine plus tacrine are shown in Table 2. It can be seen that not only was the excretion of normorphine virtually abolished by the administration of tacrine but that MNO then accounted for a large proportion (45%) of the total opiates excreted. In view of these results it was decided to investigate the effect of tacrine on *N*-demethylation *in vitro*.

Demethylation of opiates in vitro

Axelrod (1956) showed that morphine and other opiates may be demethylated *in vitro* and that the products of the reaction were the corresponding demethylated opiate and formaldehyde. Table 3 shows the amounts of formaldehyde formed from codeine, morphine, codeine-*N*-oxide and MNO when these opiates were incubated with a rat liver microsomal + soluble fraction. Codeine was found to be the most reactive substrate; the amount of formaldehyde was barely detectable when morphine was used as the substrate, and no formaldehyde was detected when either codeine-*N*-oxide or MNO were used as substrates.

Amiphenazole and tacrine were found to inhibit significantly the formation of formaldehyde when codeine was used as the substrate (Table 3). A weaker inhibition was observed using MNO as inhibitor. Although only small amounts of formaldehyde were formed when normorphine was used as substrate, amiphenazole, tacrine and MNO reduced this amount to below the minimum detectable level.

DISCUSSION

MNO has weak analgesic activity, being less than one-tenth as potent as morphine (Fennessy, 1968). However, the lipid solubility of MNO is low and it seems unlikely that it would enter the brain. Bickel & Weder (1969) found that after the administration of imipramine-*N*-oxide to rats, none could be detected in the brain. After

Table 3. *The formation of formaldehyde by demethylation of opiates after incubation with rat liver microsomal plus soluble fractions.* Formaldehyde was measured spectrophotometrically after 2 h incubation at 37°. Each entry is the mean of at least five determinations.

Substrate	Amount of substrate added (μmol)	Inhibitor	Amount of inhibitor added (μmol)	Mol formaldehyde produced (mean)‡	Standard error
Codeine	10	—	—	0·31	0·04
Codeine	10	Amiphenazole	10	0·08*	0·02
Codeine	10	Tacrine	10	0·07*	0·02
Codeine	10	MNO	10	0·24†	0·04
Morphine	10	—	—	0·08	0·02
Morphine	10	Amiphenazole	10	$<0·05$	
Morphine	10	Tacrine	10	$<0·05$	
Morphine	10	MNO	10	$<0·05$	
Codeine-*N*-oxide ..	10	—	—	$<0·05$	
Codeine-*N*-oxide ..	10	Amiphenazole	10	$<0·05$	
Codeine-*N*-oxide ..	10	Tacrine	10	$<0·05$	
Codeine-*N*-oxide ..	10	MNO	10	$<0·05$	
Morphine-*N*-oxide (MNO)	10	—	—	$<0·05$	
MNO	10	Amiphenazole	10	$<0·05$	
MNO	10	Tacrine	10	$<0·05$	

* Significantly different from corresponding inhibitor-free incubation mixture $P < 0·01$ (*t*-test).
† Significantly different from corresponding inhibitor-free mixture $P < 0·05$ (*t*-test).
‡ Each entry is the mean of seven determinations.

administration of MNO to rats, morphine appeared in the urine in an amount equivalent to 61% of the amount of MNO administered. This finding suggests that the analgesic activity of MNO may be due to the morphine produced in the metabolism of MNO rather than to a direct action of MNO itself. Reduction of *N*-oxides to the corresponding tertiary base is an established metabolic pathway for *N*-oxides, many of those that have been studied in detail do not involve the liver microsomal system (Bickel, 1969).

The absence of normorphine in the urine of rats given MNO, even though appreciable quantities of morphine were excreted, might be explained by the inhibitory effect of MNO on demethylation observed *in vitro*.

The excretion of MNO in urine after administration of morphine together with tacrine confirms in rats the work of Woo, Gaff & Fennessy (1968) who showed that MNO was excreted in patients treated with a mixture of morphine and tacrine. Ziegler, Mitchell & Jollow (1969) have also shown that morphine-*N*-oxide can be formed from morphine in a solubilized preparation of pig liver microsomes; this reaction was NADPH- and oxygen-dependent.

Combination of tacrine with morphine led to a marked reduction in normorphine excretion. This effect is probably related to the inhibitory action of tacrine on the demethylating activity of the soluble and microsomal fraction of liver. In addition, the MNO that was formed has inhibiting activity on demethylation.

A number of other tertiary amines have been shown to be metabolized to form *N*-oxides. The best known examples are imipramine (Bickel, 1969), chlorcyclizine (Burns & Phillips, 1966), chlorpromazine (Beckett, 1968), dimethylamine (Ziegler &

Pettitt, 1964), nicotinamide (Chaykin & Block, 1959) and trimethylamine (Baker & Chaykin, 1962).

Our findings and the work of Ziegler & others (1969) indicate that MNO is a normal metabolite of morphine. Presumably, MNO accumulates in the presence of tacrine because tacrine inhibits further metabolism of MNO or it inhibits an alternative pathway of morphine metabolism; for example, glucuronide conjugation or *N*-demethylation.

Reduction was the only metabolic pathway demonstrated for MNO *in vivo*. This was confirmed *in vitro* by the absence of formaldehyde production when MNO was incubated with rat liver microsomes. MNO, being highly polar, may not be able to penetrate into the microsomes which are necessary for its metabolism. However MNO produced within the microsomes might undergo demethylation at a significant rate.

Though the demethylation of morphine and codeine were shown to be inhibited *in vitro* by tacrine, it seems difficult to account for the amount of MNO which is excreted following morphine and tacrine administration even if the demethylation of morphine was completely inhibited. The present data are not sufficient to draw a firm conclusion concerning the mechanism by which tacrine causes accumulation of MNO.

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