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OPIATE TARGET SITE N-DEMETHYLASE ENZYMES: DIFFERENCES FROM THE LIVER N-DEMETHYLASE

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SUMMARY

Mixtures of morphine-6^{3}H and morphine-N-$^{14}CH_3$ were incubated with rat brain subcellular fractions. Isotope ratio measurements served as the marker for identification, purification and quantitation of N-nor products which were shown to consist almost solely of N-normorphine. The microsomal, synaptosomal and mitochondrial, but not the supernatant brain preparations yielded N-normorphine. The microsomal incubations were then repeated in the presence of cytochrome P-450 inhibitors which suppressed the liver reaction but did not affect the brain biotransformation. The brain N-dealkylase is therefore different from the one in the liver and is not a cytochrome P-450 linked enzyme.

A major biotransformation of narcotics is the loss of their N-methyl group via N-demethylation (1-3). Abundant analogies now exist in other systems where metabolic alterations are prerequisite to the expression of biological action and our studies were directed to the possibility that a similar situation exists in the case of narcotics and that their N-dealkylation is involved in their mechanism of action. The current state of knowledge concerning N-demethylation of morphine and its congeners which is exclusively derived from studies of the reaction in the liver does not allow for any firm conclusions to be drawn regarding its significance in the expression of activity of these substances. On the logical assumption that N-demethylation at the site of action rather than in the liver has the greatest potential for pharmacological relevance we studied morphine N-dealkylation in the central nervous system. We confirmed by direct means the previous indirect evidence of the existence of this reaction in the CNS (4) and showed that N-demethylation of morphine in the brain is localized within those regions which contain high opiate receptor concentrations (5) and that it is decreased in the presence of naloxone (to be published). Further, we found that while castration of male rats produces a

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a large decrease in liver N-demethylation of morphine it produces an opposite effect on the reaction in the brain which is substantially increased (6). This observation suggested that the N-demethylase in the brain was different from that in the liver which is generally acknowledged to be a cytochrome P-450 linked mixed function oxidase (7). In this communication we report on the subcellular localization of the brain enzyme and present evidence that the brain and liver opiate N-demethylases are indeed different and that the former does not involve a cytochrome P-450 mediated process. The special nature of the N-demethylase in the CNS in conjunction with our observation that acute naloxone inhibits N-demethylation of morphine in the brain but not in the liver constitutes strong experimental support for the concept that this biotransformation in opiate target sites is involved in the expression of some of the biological actions of the narcotics.

MATERIALS AND METHODS

1) Incubation of radiolabelled morphine with subcellular fractions. Morphine-6-^{3}H (7.95 x 10^{6} cpm) (New England Nuclear) and morphine-N-$^{14}CH_3$ (9.01 x 10^{5} cpm) (Amersham Corporation) as a mixture were incubated in duplicate with male rat brain microsomal, synaptosomal and mitochondrial homogenates, and cytosol (8); and similarly with liver microsomal and mitochondrial homogenates. The NADPH generating system consisted of NADP (5 µM), glucose-6-phosphate (40 µM) and glucose-6-phosphate dehydrogenase (5 units) (Sigma Chemical Corporation). The incubations were allowed to proceed for 15 minutes under air at 37° after which morphine (5 mg) and normorphine (5 mg) were added. Delsals reagent (10 ml) was added to the incubation tubes to precipitate proteins, and the mixture was evaporated in vacuo. The residue was mixed with 10 ml water, the pH was adjusted to 9.4, and the water was extracted three times with chloroform: ethanol (2:1). The residue was purified by thin layer chromatoraphy (tlc) on silica gel using chloroform:methanol:ammonia (60:30:5). The areas corresponding to morphine and normorphine were eluted in ethanol and counted. Protein determinations were made in triplicate by the method of Lowry et al (9).

2) Incubation of radiolabelled morphine with brain and liver microsomes in the presence of CO and metopyrone (MP). Brain and liver microsomes were prepared and preincubated in duplicate with MP (10^{-3}M) for 15 min at 37°C. In other samples, CO was bubbled through a rubber septum for 15 min into microsomal homogenates kept in the dark. Morphine-6-^{3}H (9.64 x 10^{6} cpm) and morphine-N-$^{14}CH_3$ (9.79 x 10^{5} cpm) were added to each of the tubes after the 15 min preincubation period, and the samples were then allowed to incubate at 37°C for an additional 15 min. Control incubations were run identically except for the absence of cytochrome P-450 inhibitors. The samples were treated as described in section 1 to isolate morphine and normorphine.

3. Incubation of radiolabelled ethylmorphine (EM) with brain and liver microsomes with and without CO. Radiolabelled ethylmorphine-6-^{3}H and ethylmorphine-N-$^{14}CH_3$ were prepared by reacting morphine-6-^{3}H or morphine-N-

$^{14}CH_3$ with ethyl bromide (10). The radiohomogeneity of the compounds as determined by reverse isotope dilution was at least 95%. Norethylmorphine (norEM) standard was prepared by reaction of ethyl morphine (Merck Chemical Company) with phenylchloroformate (11). The identity of the norEM was verified by NMR and IR spectroscopy.

The incubations were performed as described in section 2 for radiolabelled morphine. Ethylmorphine-6^{3}H (6.52×10^6 cpm) and ethylmorphine-N-$^{14}CH_3$ (7.12×10^5 cpm) were added to liver and brain homogenates after preincubation with the inhibitor. The samples were incubated in duplicate for 15 min at 37°C, after which time carrier EM (5 mg), norEM (5 mg) and normorphine were added. Areas corresponding to EM, norEM and normorphine were isolated by tlc, dissolved in ethanol, and counted to determine their radioactive content.

RESULTS AND DISCUSSION

The experimental procedures used in the present study were a modification of the double isotope method originally developed for *in vivo* studies (5). N-demethylation of the radiolabelled substrate results in the loss of the ^{14}C isotope but not of the ^{3}H which is retained in the product. Metabolites lacking the N-methyl group are therefore distinguished by a large increase in the ^{3}H to ^{14}C isotope ratio due to the loss of their ^{14}C content. Such disproportionate isotope ratios serve as a definite marker for the detection of a N-nor derivative(s) which is otherwise notoriously difficult to separate and purify particularly in the small quantities generated in the CNS. The double isotope technique also has the important advantage that it can detect the presence of all N-nor metabolites, even those of unknown structure. Normorphine generated from the reaction exhibited the expected loss of ^{14}C content and its radiohomogeneity insofar as ^{3}H content was concerned was confirmed by conversion to its 3,N-bistertiarybutoxy derivative and also to morphine, both of which retained undiminished ^{3}H specific activity. During the isolation and purification of normorphine careful analysis of other areas on the thin layer chromatography plates did not reveal the presence of any other substances whose ^{3}H to ^{14}C isotope ratio was greater than that of the dose. The absence of other nor products ensured that in the extractable portion of the incubate (> 90% of the substrate used) normorphine was the only N-demethylated product formed, and its quantitation can serve as a measure of N-demethylase activity.

TABLE I

SUBCELLULAR LOCALIZATION OF MORPHINE N-DEMETHYLATION IN RAT BRAIN AND LIVER

	nmole Normorphine / mg protein	% Dose Conversion
BRAIN		
Microsomes (P-3)	1.49 ± 0.17	4.8
Synaptosomes	0.25 ± 0.08	0.8
Mitochondria	0.16 ± 0.03	0.5
Cytosol	-	-
LIVER		
Microsomes	1.51 ± 0.16	5.7
Mitochondria	-	-

The subcellular distribution of morphine N-demethylation in the rat brain and liver as determined by our procedure is given in Table 1. The reaction is present in only the particulate fraction of the brain and in that sense corresponds to the distribution of the opiate receptors (12, 13). While the microsomes of both liver and brain are the richest source of the enzyme, brain mitochondria but not that of the liver contain the enzyme as well.

Cytochrome P-450 mixed function oxidase has been securely identified as the enzyme responsible for the N-demethylation of morphine in the rat liver (7) and initially it had been assumed that the same applied to the brain reaction. In our studies we evaluated the impact of carbon monoxide (CO) and metopyrone (MP) on brain microsomal N-demethylation of morphine and contrasted the results with those obtained with liver microsomes. The results of these experiments are presented in Fig. 1 and show that while both of these P-450 inhibitors decreased the yield of normorphine from liver microsomes, they either had no effect on brain N-demethylation or actually increased it. It can therefore be concluded that the brain enzyme responsible for the formation of normorphine from morphine is not a cytochrome P-450 enzyme, and that it is,

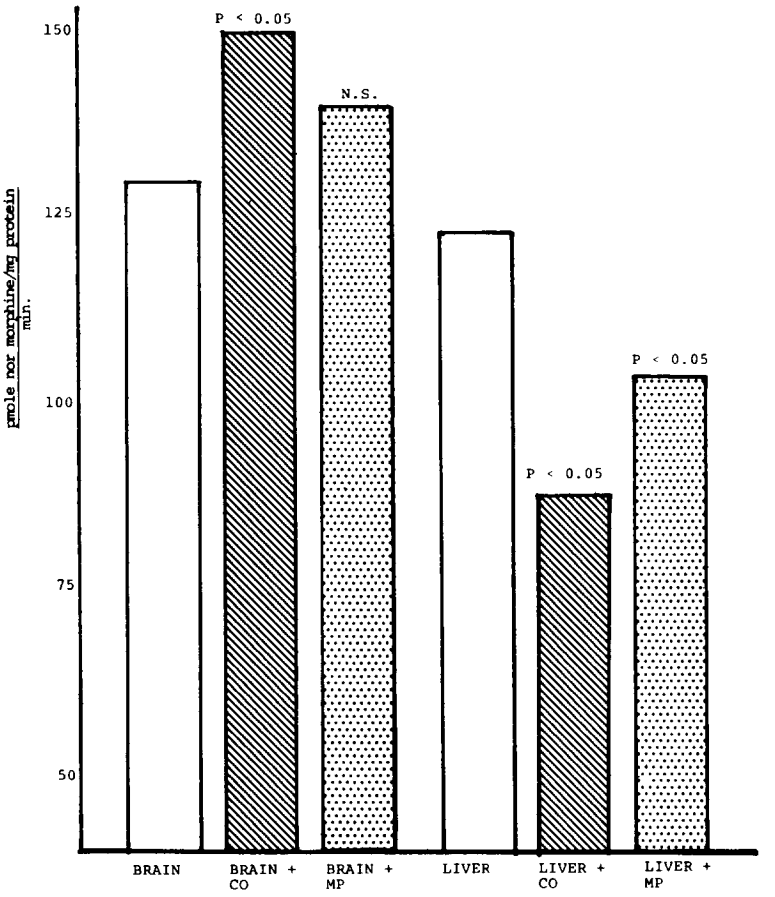

Fig. 1. Effect of CO and MP on Morphine N-Demethylation.

therefore, different from the enzyme performing the same function in the rat liver. It remains to be determined whether the mitochondrial and synaptosomal N-demethylases of the brain also conform to the character of the brain microsomal enzyme. In a majority of liver N-demethylase studies in the literature the substrate used was ethyl morphine and recently it has been reported that the liver enzymes responsible for the N-demethylation of the morphine and ethyl morphine may be different (14). We have therefore included ethyl morphine in out studies of brain N-demethylation. Mixtures of ethyl morphine-6-^{3}H and ethyl morphine-N-$^{14}CH_3$, gave N-nor ethyl morphine-^{3}H (146-180 pmol per mg protein per min) as the sole N-demethylation product after incubation with liver and brain microsomes. In the presence of carbon monoxide the yield of N-norethyl morphine

decreased by about 25% in the liver incubation, confirming the results obtained by other methods (15,16) but the reaction was unaffected in the brain microsomes. Thus both ethyl morphine and morphine are N-demethylated in the brain by an enzyme or enzymes which are different from those in the liver microsomes.

The difference of the brain microsomal N-demethylase from that in the liver supports the thesis that target site N-demethylation is a factor in the action of the narcotics. It must be emphasized that the pharmacological effects of normorphine generated peripherally or given exogenously need not reflect those of the material generated in situ in target tissues, particularly since the narcotic activity may not result from the N-demethylation product but from transient intermediates in this biotransformation such as carbinolamines. There is virtually no information available about the events subsequent to or coincident with opiate receptor binding which are responsible for the activity of these agents. The results obtained by us argue that N-demethylation may represent such an event. The activity of the opiate peptides which contain primary amino groups and indeed that of normorphine itself can be accomodated by postulating either prior N-methylation (17) or binding to the relevant enzyme, both of which are subject to experimental tests currently in progress.

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