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THE INHIBITION OF THE LIVER MICROSOMAL N-DEMETHYLATION OF MORPHINE BY N-ALLYL NORMORPHINE AND ITS PHARMACOLOGICAL IMPLICATIONS

L. LEADBEATER and D. R. DAVIES

The Ministry of Defence, Chemical Defence Experimental Establishment, Porton Down, Wiltshire.

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Abstract—Theories of the mechanism of the pharmacological action of morphine imply that the N-demethylation of morphine by liver microsomal enzymes should be competitively inhibited by N-allyl normorphine. These have been challenged on the grounds that N-allyl normorphine is a non-competitive inhibitor.

The inhibition has been reinvestigated using rat liver microsomes and it appeared, from the application of the method of Lineweaver and Burk, to be neither entirely competitive nor non-competitive but a mixture of the two.

N-allyl normorphine was shown to be metabolised approximately twice as rapidly as morphine by the same microsomal preparations i.e. in the inhibition studies the inhibitor concentration was not constant, hence the simple Michaelis–Menton theory (on which the method of Lineweaver and Burk is based) is not applicable to this enzyme system. It is concluded that this type of experiment cannot be used either to support or challenge hypotheses of the pharmacological activity of morphine.

INTRODUCTION

N-ALLYL normorphine (Nalorphine) antagonises the pharmacological effects of morphine[1] and inhibits the liver microsomal enzyme system which N-demethylates morphine *in vitro*.[2] It has not been demonstrated that the antagonism of the pharmacological effects by nalorphine is caused by inhibition of morphine N-demethylation in the CNS. However, these two inhibitory actions of nalorphine have been related in two theories of the mechanism of morphine action by Beckett, Casy and Harper[3] and by Axelrod[4] and Cochin and Axelrod.[5]

Beckett, Casy and Harper[3] have suggested that analgesia is only produced after the N-demethylation of morphine at the receptor sites in the brain. Nalorphine, these authors believe, has a greater affinity for such sites than morphine and hence antagonises morphine action by displacing it from the site. In effect Beckett *et al.* believe that nalorphine is a competitive inhibitor of morphine N-demethylation.

Axelrod,[4] and Cochin and Axelrod[5] have suggested that the liver microsomal enzyme system which N-demethylates morphine may serve as a model for the morphine receptor sites in the central nervous system. Thus the inhibitory action of nalorphine on the liver microsomal M-demethylation of morphine should be produced by a similar mechanism to its antagonism of the pharmacological actions of morphine. This antagonism is thought to be competitive[6] and therefore on Cochin and Axelrod's

hypothesis nalorphine should be a competitive inhibitor of the microsomal N-demethylation of morphine.

These hypotheses have been criticised by Way and Adler.[7] One of their grounds of criticism is a report by Axelrod and Cochin[2] that nalorphine is a non-competitive inhibitor of morphine N-demethylation. More recently Elison, *et al.*[8] have confirmed this finding by two separate methods. They stated that their results failed to support either Beckett's theory or Axelrod's theory and concluded that the liver microsomal enzymes are not suitable as models by which these theories may be judged.

In a recent investigation in which the biochemical and pharmacological properties of morphine and related compounds were compared, the kinetic studies of Axelrod and Cochin[2] and of Elison *et al.*[8] were repeated. The results failed to confirm that nalorphine is a non-competitive inhibitor. From the present data its action appears to be a mixture of competitive and non-competitive inhibition and it is concluded that the hypotheses of Beckett *et al.* and of Axelrod and Cochin may be neither supported nor challenged on data obtained in these experiments.

METHODS

Microsomal enzyme preparation

The method of preparation was based on that described by Axelrod.[9] Five male rats of the Porton strain were used for each preparation. The animals were killed and their livers excised as quickly as possible, and placed in ice-cold 0·10 M Tris-HCl buffer (pH 7·50), in an ice bath. The livers were dried between filter paper and dropped into a 100 ml cylinder containing 50 mls of the same buffer and their volumes determined. The livers were then homogenized in 2 vol of the buffer in a 500 ml head of the M.S.E. Atomix blender for 10 sec. at full speed. All these manipulations were carried out in a cold room (0–3°) using pre-cooled apparatus and solutions. The homogenate was centrifuged in a refrigerated Model L Spinco Centrifuge in the No. 30 head (stored in the cold overnight) at 12,000 rev/min for 20 min total running time. The microsomal enzymes, contained in the supernatant together with the soluble fraction of the liver cell, were assayed immediately after preparation.

Incubation conditions

A modification of the incubation medium of Axelrod[9] was used:

Microsome plus soluble fraction equivalent to 250 mgm liver

NADP	4×10^{-5} M
Nicotinamide	10^{-2} M
Semicarbazide HCl (Adjusted to pH 7·5)	2×10^{-2} M
$MgCl_2$	5×10^{-3} M
Tris-HCl Buffer (pH 7·50)	5×10^{-2} M

The incubations were carried out in a total volume of 5·0 ml contained in a 50 ml tube which was shaken in air 37·5°.

The rate of metabolism of morphine and nalorphine was linear for at least 30 min. For the inhibition studies the N-demethylation of morphine was estimated from the amount of formaldehyde produced in 20 min. The relative rates of metabolism of

nalorphine and morphine were determined by estimating the amount of normorphine formed during a 30 min incubation.

The rates of metabolism are expressed as μmoles/gm liver/hour and the results are given as the mean value obtained from the four preparations ± standard deviation. It was assumed in the calculation that the Specific Gravity of liver tissue is 1·0.

Estimation of formaldehyde

Formaldehyde was determined colorimetrically as diacetyldihydrolutidine by the method of Cochin and Axelrod.[5]

Estimation of Normorphine

Normorphine was assayed colorimetrically as its cupric dithiocarbamate derivative using a modification of the method described by Umbreit[10] for the estimation of secondary amines in the presence of tertiary and primary amines.

4·0 ml of a solution made up of 35 ml carbon disulphide, 25 ml pyridine and 65 ml iso-propanol were added to the 5·0 ml incubation medium, followed by 2·0 mls 0·0013 M cupric chloride in 50% (w/v) pyridine. The tube was shaken vigorously for 1 min. Exactly 15 min later 3·0 ml 10% (w/v) acetic acid and 6·0 ml benzene were added and the tubes shaken again for 2 min. The tubes were centrifuged and 1·0 ml of the benzene layer was diluted with 4·0 ml 20% (w/v) isopropanol in benzene. The extinction of the solution was measured at 440 mμ in a Unicam SP 500 spectrophotometer. Standard curves were obtained by putting known amounts of normorphine through the procedure.

RESULTS

Four different preparations of microsomes, each isolated from the pooled livers from five rats, were used in these experiments. The rate of N-demethylation of morphine was determined at various substrate concentrations in the presence and absence of 3×10^{-4} M nalorphine and the data fitted to the Lineweaver and Burk equation.[11] The substrate concentrations were chosen so that the points were uniformly distributed along the $^1/_S$ axis. The equation of the best fitting line was determined by the method of least squares and the values of K_m (Michaelis Constant) and V (maximum velocity of the reaction) calculated from the equation. The results are shown in Table 1.

TABLE 1. THE INHIBITION OF MORPHINE N-DEMETHYLATION BY NALORPHINE

	Preparation No.	Control	In presence of 3×10^{-4} M nalorphine	Significance of the change
K_m (10^{-4} M)	1	6·22	18·21	
	2	8·25	19·40	
	3	4·63	42·28	
	4	3·33	27·58	
	Mean	5·61 ± 1·83	27·87 ± 9·62	$p < 0.01$
V (μmoles/gm/liver/hr)	1	16·95	10·24	
	2	23·02	7·78	
	3	19·32	11·45	
	4	10·76	10·08	
	Mean	19·51 ± 2·20	9·89 ± 1·32	$p < 0.01$

Both K_m and V, determined in the presence of 3×10^{-4} M nalorphine, were significantly different from the constants determined in the absence of the inhibitor.

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The rates of N-dealkylation of morphine and nalorphine, at a substrate concentration of 5×10^{-4} M, were determined for each of the microsomal preparations after storing overnight at −40°. Morphine was N-demethylated at $10{\cdot}12 \pm 1{\cdot}52$ μmoles/gm liver/hour and nalorphine was N-dealkylated at $18{\cdot}75 \pm 3{\cdot}48$ μmoles/gm liver/hour. These results suggested that the inhibitor concentration might decrease at almost twice the rate of the substrate during incubation therefore the extent of the inhibition of the demethylation of morphine (0·5 mM) produced by 0·1 mM nalorphine was determined after various periods of incubation. The results are illustrated in Fig. 1 where it is seen that the percentage inhibition decreases as the incubation proceeds.

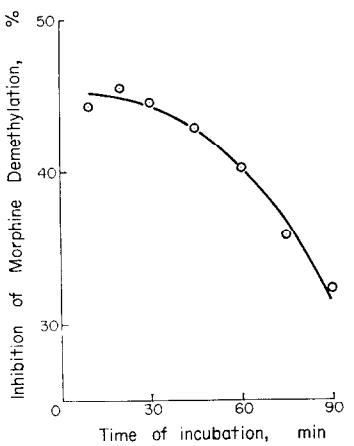

FIG. 1. The change in the extent of the inhibition of morphine demethylation by nalorphine during the incubation period. The incubation conditions are described in the text, the concentration of morphine was 0·5 mM and of nalorphine 0·1 mM.

DISCUSSION

If an inhibitor combines with the active centre of an enzyme, it must compete with the substrate for that site. This is competitive inhibition and the Michaelis Constant K_m is increased whilst the maximum velocity of the reaction V is unaltered. If, on the other hand, the inhibitor combines with the enzyme at a site removed from the active centre, there is no competition with the substrate. This is non-competitive inhibition, K_m is unchanged and V is decreased. However, K_m and V may both change and the inhibition is then neither wholly competitive nor wholly non-competitive.

Analysis of the present data (Table 1) shows that in the presence of nalorphine K_m is increased and V decreased and thus the inhibition is neither entirely competitive nor non-competitive.

Only two studies of the inhibitory action of nalorphine on morphine N-demethylation have been published previously. The first was by Axelrod and Cochin.[2] Their data, in the form of the Lineweaver and Burk equation, are shown in Fig. 2a, together with data from one of the experiments reported in this paper for comparison (Fig. 2b). Only

the part of the graph in full lines was published. Axelrod and Cochin concluded that nalorphine was a non-competitive inhibitor although they suggested that the reaction might be "an example of a slow pseudo-irreversible inhibition mimicking non-competitive inhibition but occurring at the same site".

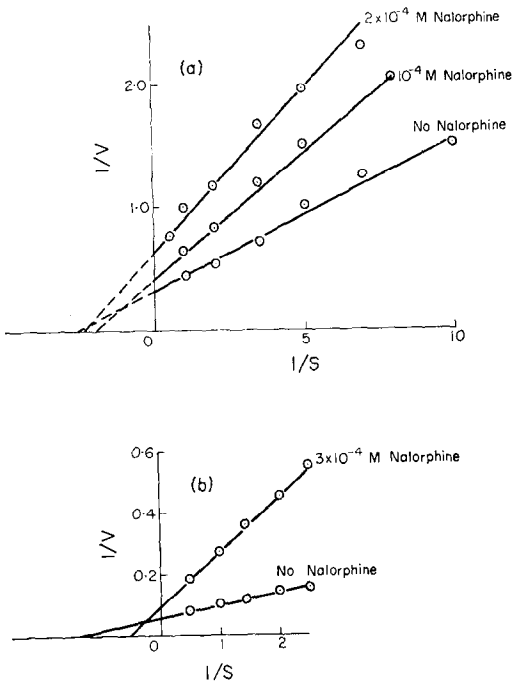

FIG. 2. The Lineweaver and Burk plot: (a) The data of Axelrod and Cochin (b) Data from the present experiment (Microsome preparation 1).
S = morphine concentration (mM)
V = rate of demethylation of morphine (μmoles/gm/liver/hour).

However, if the curves are extrapolated backwards (broken lines in Figures 2a) they do not intersect on the $^1/S$ and the K_m of the reaction was changed in the presence of the inhibitor. The data of Axelrod and Cochin are in fact consistent with those reported in the present paper, namely that the inhibitory action of nalorphine is not wholly non-competitive.

The second study was by Elison, *et al.*[8] They used the method of Hunter and Downs[12] to determine the nature of the inhibition. In this method the ratio of inhibited to reaction velocities is inserted into the Michaelis–Menton relationships to give the uninhibited following equation:

$$I \frac{V_i}{V\text{-}V_i} = K_i + \frac{K_i}{K_m} S$$

for the competitive inhibition and

$$I \frac{V_i}{V\text{-}V_i} = K_i$$

for non-competitive inhibition, where V is the velocity of the reaction in the absence of inhibitor, V_i the velocity in the presence of inhibitor, I the concentration of the inhibitor, S the concentration of the substrate, K_i the inhibitor constant and K_m the Michaelis Constant. Thus the plot of $[I\ (V_i/V - V_i)]$ against S has zero slope in the non-competitive case and a slope of K_i/K_m in the competitive. The data of Elison *et al.* are shown in Fig. 3(a) and the data from the present experiment in Fig. 3(b). There is no doubt that Elison's data indicates non-competitive inhibition whereas the data in Fig. 3(b) shows that the inhibition depends in some way upon the substrate concentration.

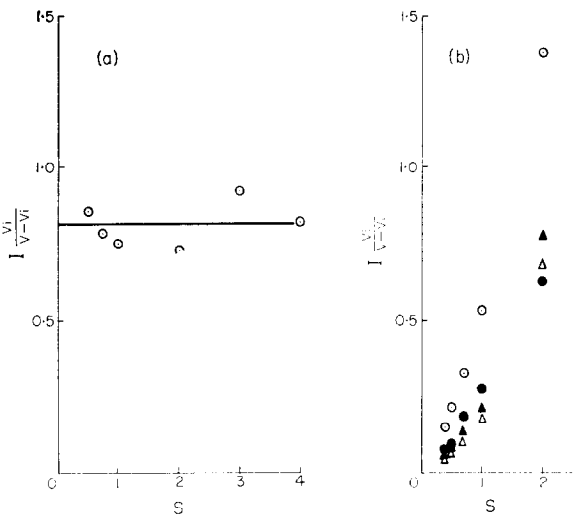

FIG. 3. The Hunter and Downs plot: (a) the data of Elison *et al.* (b) The data from the present experiment.

In B the four preparations are shown (see Table 1).
I = nalorphine concentration (mM), S = morphine concentration (mM),
v = rate of metabolism of morphine (μmoles/gm liver/hour) and v_i = rate of metabolism of morphine in the presence of nalorphine (μmoles/gm liver/hour).

Using deuteromorphine (M-NCD_3) Elison *et al.* found further evidence that the inhibition of morphine demethylation by nalorphine was not competitive. The N-demethylation of deuteromorphine, which they showed to have a higher K_m than morphine, was inhibited less strongly by nalorphine than that of morphine. This result is not consistent with competitive inhibition since, if this were the case, the N-demethylation of deuteromorphine would have been inhibited more strongly because it has a lower affinity for the enzyme than morphine. In as much as one experiment differed significantly from the mean inhibited values quoted (by more than four standard variations) it is difficult to assess the importance of these observations.

The values of K_m and V for the microsomal N-demethylation of morphine, obtained in the three laboratories, are summarised in Table 2.

It may be seen that the values of K_m agree satisfactorily and hence the differences in the experimental technique employed did not modify the enzyme. However, the

value of V obtained by Axelrod and Cochin was about one sixth the values reported by the other two groups of workers. It is possible that this difference is due to the different strains of rats which have been employed (Axelrod and Cochin used black NIH animals, Elison *et al.* Long–Evans rats and in these experiments albino rats of the Porton strain, which is derived from the Wistar strain, were used) since Adler, Elliott and George[13] have reported differences in the metabolism of morphine by different strains of rats.

TABLE 2. THE VALUES OF K_m AND V, OBTAINED IN DIFFERENT LABORATORIES, FOR THE N-DEMETHYLATION OF MORPHINE

Laboratory	K_m (10^{-4} M)	V (μmoles/gm/liver/hr)
Leadbeater and Davies	5·61 ± 1·83†	19·51 ± 2·20†
Elison *et al.**	3·95 ± 1·44‡	23·55 ± 11·75‡
Axelrod and Cochin (obtained from Fig. 1a)	3·92	3·13

* Elison *et al.* quote $V = 0{\cdot}589$ μmoles HCHO/15 min. It is not clear whether this refers to the whole incubation mixture (10 ml) or the volume of mixture (2·0 ml) used for the actual assay. However, in Fig. 1 of their paper they show that, with an initial concentration of 5×10^{-4} M morphine, 1·05 μmole HCHO were produced per 500 mg liver in 15 min. Therefore it has been assumed that their V data refers to HCHO formed per 2·0 ml assay since this makes their V consistent with the data of their Fig. 1.

† These values are not significantly different ($0{\cdot}2 > p > 0{\cdot}1$).

‡ The values are not significantly different ($0{\cdot}7 > p > 0{\cdot}6$).

In view of the apparently inexplicable discrepancy between the data presented here and by Axelrod and Cochin and those of Elison *et al.* the question arose as to whether the Michaelis–Menton theory (in the form of the Lineweaver–Burk and Hunter-Downs equations) could be applied to the data. These equations require that the inhibitor shall be unchanged during the reaction (see Dixon and Webb[14]). In the enzyme preparations used nalorphine was in fact metabolised at 18·75 μmoles/gm liver/hour, or 1·85 times faster than the substrate morphine, confirming the observation of Axelrod and Cochin[2] who reported a relative rate of 2·3 for the dealkylation of the two compounds. If this is so the extent of inhibition should decrease during the incubation as the ratio of inhibitor to substrate decreases. This does in fact occur as may be seen from Fig. 1 and the simple Michaelis–Menton equations are not applicable to this enzyme system.

The 10,000g supernatant of a liver homogenate, used as the enzyme system in the work, discussed in this paper, contains the microsomal and soluble fractions of the liver cell and is probably contaminated with sub-mitochondrial particles and mitochondrial enzymes. In order to confirm that the data reported apply to the microsomal enzymes and are not modified by the presence of other cellular components the experiments were repeated with microsomes which were isolated and washed twice with buffer by successive centrifuging at 100,000 g for 60 min. In these experiments NADP and the soluble fraction, which contains enzymes and substrates capable of reducing it, were replaced by 5×10^{-4} M $NADPH_2$. The results obtained were similar to those

reported above except that V was reduced, this was probably due to the inactivation of the enzyme during the isolation and washing of the microsomes. Thus the kinetic data reported with the crude 10,000 g supernatant apply to the purified microsomal fraction.

To determine the inhibitory action of nalorphine unequivocally the enzyme systems, present in the microsomal fraction of the liver cell, which are involved in the N-dealkylation of morphine and nalorphine must be separated. Until these definitive experiments have been performed the hypotheses of Beckett, Casy and Harper[3] and of Axelrod[4] and Cochin[5] may neither be supported nor challenged on the basis of the inhibitory action of nalorphine on the liver microsomal enzyme system which N-demethylates morphine *in vitro*.

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