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KINETICS OF DRUG METABOLISM BY HEPATIC MICROSOMES*

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Abstract—The metabolism of drugs by hepatic microsomes is thought to proceed through a coupled reaction involving NADPH oxidase and one or more 'hydroxylases' of limited specificity. If this concept is correct, it can be predicted that numerous drugs would inhibit competitively the metabolism of another drug employed as a substrate for the microsomal system. This proved to be the case. The N-demethylation of ethylmorphine was competitively inhibited by hexobarbital, chlorpromazine, zoxazolamine, phenylbutazone, and acetanilide; and ethylmorphine, and chlorpromazine were mutually inhibitory, each retarding the metabolism of the other. All these drugs are known to be oxidized by microsomal enzymes. Barbital and acetazoleamide, drugs which are not metabolized, failed to act as inhibitors. Evidence is presented to show that certain of the observed inhibitions may be explained on a basis of interaction of alternative substrates. Kinetic data were obtained on both phenobarbital-induced and noninduced microsomal systems. The close similarity of the Michaelis and inhibitor constants seen in the two systems, coupled with the increased maximal velocity values observed for the oxidation of a given drug after induction, argue strongly that a quantitative rather than a qualitative change is involved in the inductive process.

GILLETTE *et al.*[1] have presented evidence to show that an oxidase in hepatic microsomes catalyzes the oxidation of NADPH to provide hydrogen peroxide or a peroxide derivative which, in the presence of nonspecific peroxidases, is responsible for the metabolism of many drugs. Brodie[2] has formulated this coupled reaction as follows:

$$\text{(1)}\quad \text{NADPH} + \text{O}_2 \xrightarrow[\text{'hydroxylase'}]{\text{E}} \text{E} \cdot \text{HO}_2^- + \text{NADP}$$

$$\text{(2)}\quad \text{E} \cdot \text{HO}_2^- + \text{drug substrate} \longrightarrow \text{hydroxylated drug substrate} + \text{E} + \text{OH}^-$$

where E is NADPH oxidase.

If the second step is rate limiting and the 'hydroxylase' is nonspecific such that it will catalyze the oxidation of a number of drugs, then one could predict that each of these drugs would competitively inhibit the oxidation of the others. This being the

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case, it is extremely likely that the Michaelis constant (K_m) for the second reaction would vary with the drug employed as a substrate. The current kinetic studies were performed with these premises in mind.

The administration of phenobarbital to rats is known to enhance the microsomal metabolism of many drugs.[3, 4] The inductive process could implicate the enzymes involved in either or both steps of the reaction sequence. The kinetics of drug metabolism by microsomes from phenobarbital-treated and untreated rats were compared to determine which of the coupled reactions are involved in the inductive process.

METHODS

Experimental animals

Male Holtzman rats weighing 70–100 g were used. When sodium phenobarbital was employed to engender increased microsomal drug metabolism, it was dissolved in 0·9% NaCl solution and was administered intraperitoneally (40 mg/kg) once daily for 5 days. Rats injected with saline served as controls. Livers were removed 24 hr after the last injection.

Tissue preparation

Liver was homogenized in 1·15% KCl solution for studies of the N-demethylation of ethylmorphine, or in 0·1 M Na_2HPO_4–KH_2PO_4 buffer, pH 7·4, for studies of the side-chain oxidation of hexobarbital and the sulfoxidation of chlorpromazine. The 9,000 *g* fraction of the homogenate was employed when hexobarbital and chlorpromazine were the substrates. When ethylmorphine was the substrate, washed microsomes were employed. These were prepared by centrifugation of the 9,000 *g* fraction for 1 hr at 100,000 *g* in a refrigerated Spinco ultracentrifuge, model L. The microsomes were washed with 1·15% KCl solution, recentrifuged at 100,000 *g* for 30 min and resuspended in KCl solution. Early studies showed that the kinetic values obtained from Lineweaver–Burk curves of the metabolism of ethylmorphine and chlorpromazine were the same whether washed microsomes or the 9,000 *g* fraction was used.

Reaction mixtures

The complete reaction mixtures (5 ml), which were incubated in open 25-ml Erlenmeyer flasks in a Dubnoff shaker at 37°, contained the following constituents adjusted to pH 7·4 with phosphate buffer (micromoles): NADP (2), G6P* (20), nicotinamide (40), magnesium chloride (20), various amounts of substrate and inhibitor, and 2 ml of microsomal preparation. Seventy-five micromoles of semicarbazide and 2 enzyme units of G6PDH* were added to the mixture when the N-demethylation of ethylmorphine was being measured, When ethylmorphine and chlorpromazine were employed as substrates, an amount of microsomal preparation equivalent to 250 mg of fresh liver was employed; four times this amount was used when hexobarbital was the substrate. Induced microsomal preparations were diluted so that reaction rates approximated those found in noninduced microsomal preparations. Kinetic values obtained with diluted and nondiluted preparations were compared and found not to differ.

* G6P = glucose-6-phosphate. G6PDH = glucose-6-phosphate dehydrogenase. These agents were purchased from the California Corp. for Biochemical Research, Los Angeles, Calif.

Determination of enzyme activity

The oxidative N-demethylation of ethylmorphine was measured as described by Takemori and Mannering.[5] The methods of Cooper and Brodie,[6] Salzman and Brodie,[7] and Gillette *et al.*[1] were used to determine the side-chain oxidation of hexobarbital, the sulfoxidation of chlorpromazine, and the oxidation of NADPH respectively. It was shown that the presence of one drug did not interfere with the assay of another, either before or after incubation. Under the conditions employed in these studies the N-demethylation of chlorpromazine or hexobarbital was negligible. All observations were made during a time interval when reaction rates were linear, which was 15 min when ethylmorphine and hexobarbital were the substrates, and 12 min when chlorpromazine was the substrate.

Statistical methods

The data, plotted according to the method of Lineweaver and Burk,[8] were analyzed statistically by the method of least squares[9] in order to facilitate drawing the line of best fit, and in some cases by the method of Wilkinson.[10]

RESULTS

Competitive inhibition of N-demethylation of ethylmorphine by various drugs

The inhibitory effects of hexobarbital, chlorpromazine, zoxazolamine, phenylbutazone, and acetanilide on the N-demethylation of ethylmorphine by hepatic

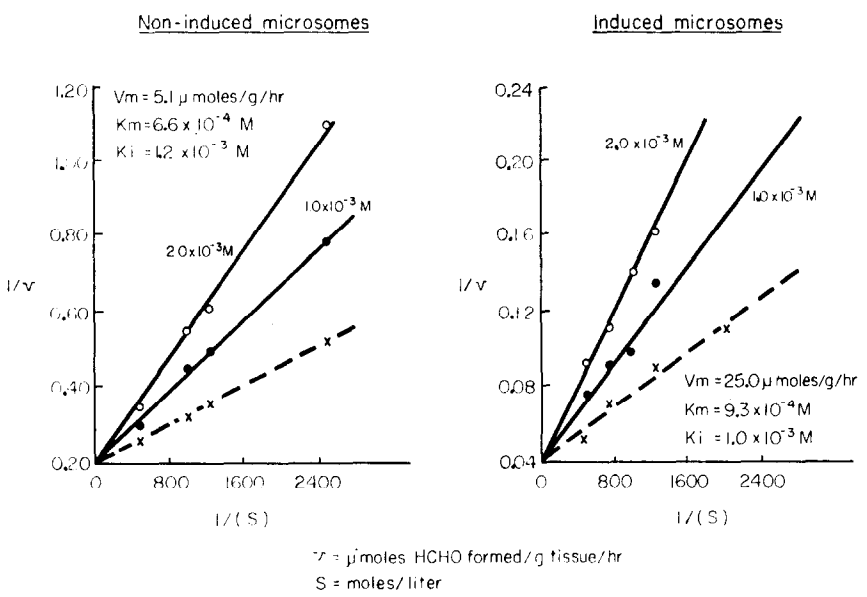

FIG. 1. Effect of hexobarbital on the N-demethylation of ethylmorphine by noninduced and phenobarbital-induced microsomal systems.

microsomes from untreated and phenobarbital-treated rats were found to be competitive (Figs. 1–5). In all cases the inhibition was not reversed by additional amounts of the NADPH-generating system (NADP, G6P, and G6PDH) or NADPH itself, nor was the maximal velocity (Vm) altered. The mean of at least six determinations of

the Michaelis constant (K_m) of the demethylation of ethylmorphine by noninduced microsomes (5·8 × 10^{-4} M) is not significantly different ($P > 0.30$) from the mean value obtained with the induced microsomes (6·9 × 10^{-4} M). The increase in Vm

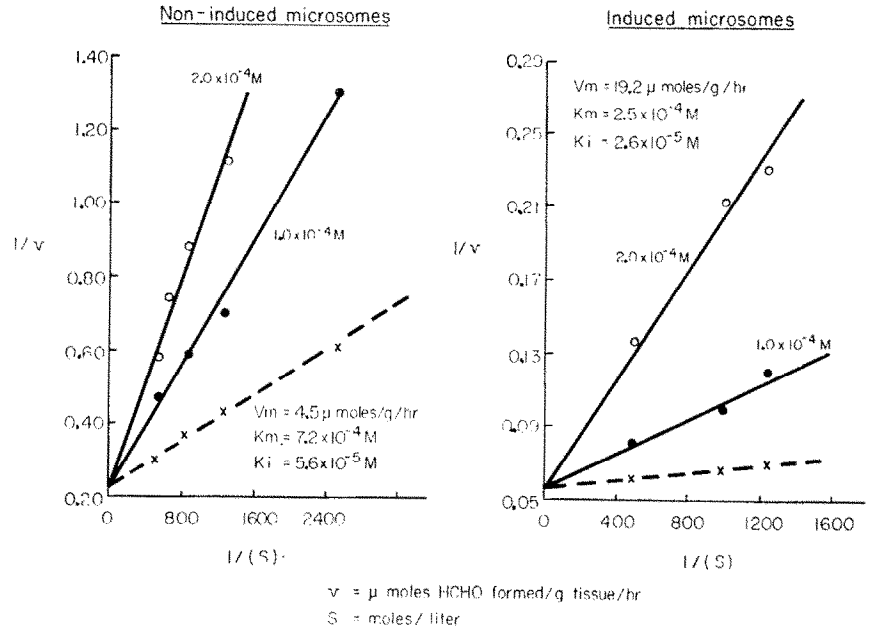

FIG. 2. Effect of chlorpromazine on the N-demethylation of ethylmorphine by noninduced and phenobarbital-induced microsomal systems.

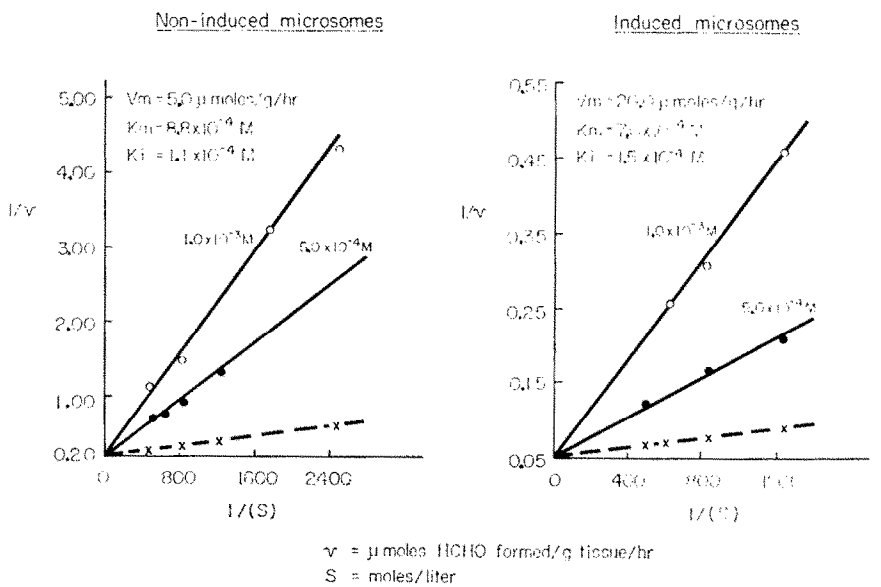

FIG. 3. Effect of zoxazolamine on the N-demethylation of ethylmorphine by noninduced and phenobarbital-induced microsomal systems.

without a change in K_m seen when induced microsomes were used is compatible with the view that induction reflects an increased synthesis of enzyme. The mean inhibitor constants (K_i) for each drug were not significantly different ($P > 0.05$) when non-induced or induced microsomes were employed, which indicates that the inhibitory process occurs at the same step in both preparations.

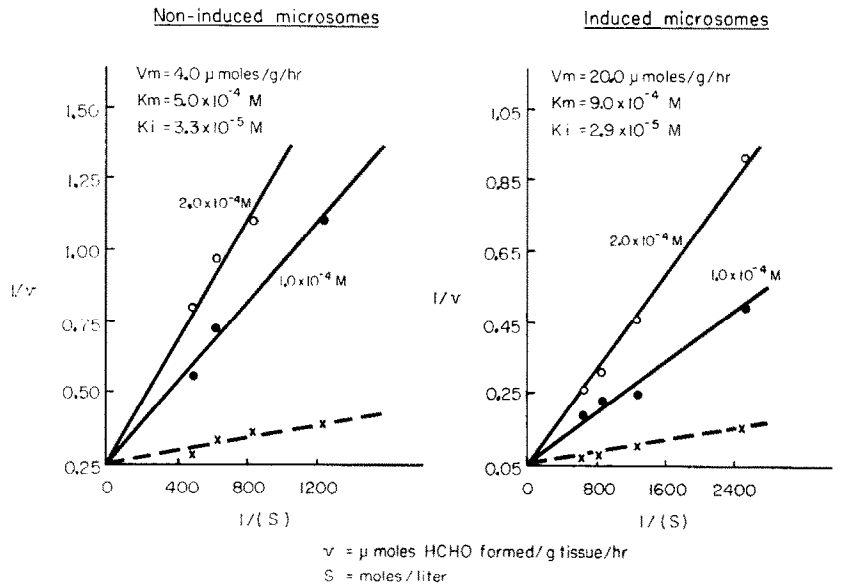

FIG. 4. Effect of phenylbutazone on the N-demethylation of ethylmorphine by noninduced and phenobarbital-induced microsomal systems.

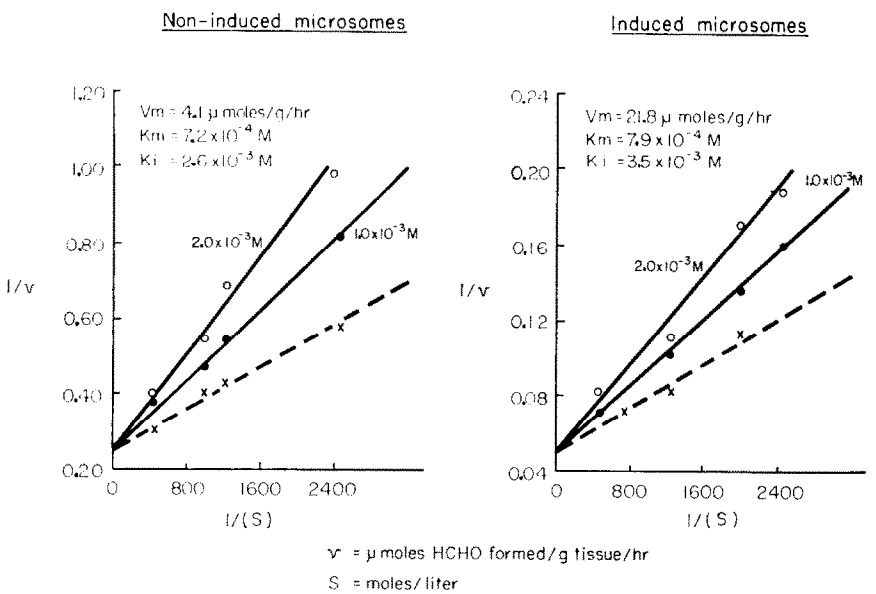

FIG. 5. Effect of acetanilide on the N-demethylation of ethylmorphine by noninduced and phenobarbital-induced microsomal systems.

All the drugs that inhibit the demethylation reaction are known to be oxidized by microsomes. Because barbital and acetazoleamide are not metabolized, the inhibitory properties of these compounds were tested. Concentrations of barbital and acetazoleamide as high as $2{\cdot}0 \times 10^{-2}$ M and $2{\cdot}0 \times 10^{-3}$ M, respectively, had no effect on the rate of metabolism of ethylmorphine.

Kinetics of the metabolism of hexobarbital and chlorpromazine

Lineweaver–Burk plots for the metabolism of hexobarbital and chlorpromazine by noninduced and induced microsomes are shown in Figs. 6 and 7. When the means of at least three determinations were analyzed, the K_m values for the metabolism of the

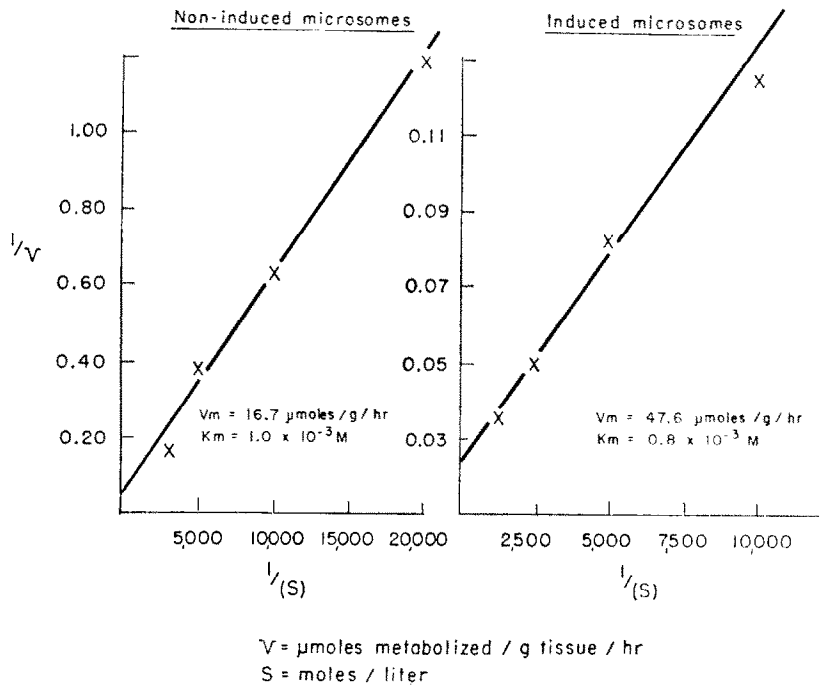

FIG. 6. Oxidation of hexobarbital by noninduced and phenobarbital-induced microsomal systems.

three drugs were significantly different ($P < 0{\cdot}05$) from each other regardless of whether noninduced or induced microsomes were used. The K_m values for the metabolism of each drug were not different ($P > 0{\cdot}05$) when determined in noninduced and induced systems. In accordance with expectations, the Vm values were increased after induction.

I_{50} values in noninduced and phenobarbital-induced microsomes

The concentration of one drug necessary to effect a 50% inhibition of the metabolism of another drug (I_{50}) was determined in both noninduced and phenobarbital-induced microsomal systems (Table 1). There is general agreement between the I_{50} values obtained in induced and noninduced systems. This reaffirms the view that a quantitative, not a qualitative, alteration is involved in the inductive process. However,

the inhibition of the sulfoxidation reaction by hexobarbital differed in the two systems. Because of the insolubility of hexobarbital at pH 7·4, it was not possible to attain a concentration that could effect a 50% inhibition of chlorpromazine sulfoxidation by noninduced systems.

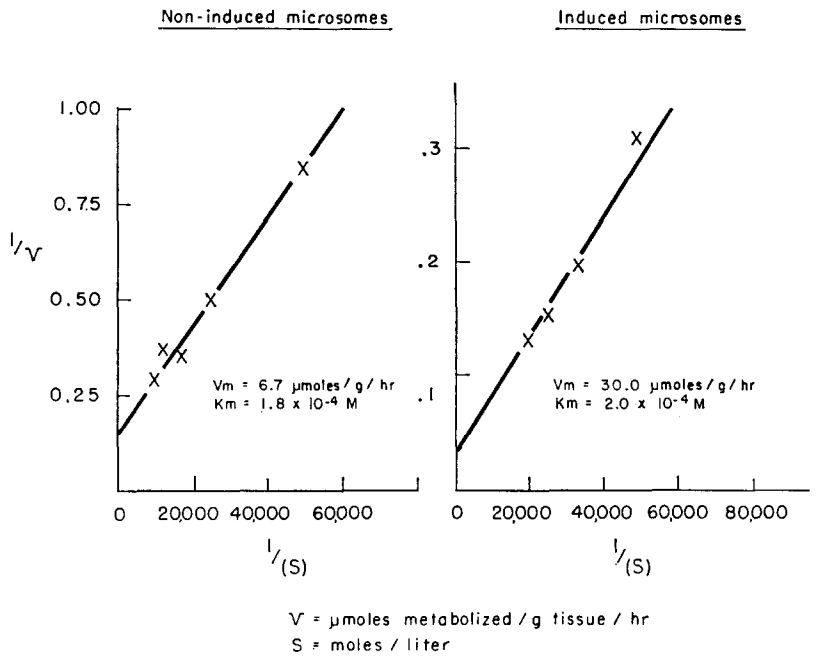

FIG. 7. Sulfoxidation of chlorpromazine by noninduced and phenobarbital-induced microsomal systems.

Two of the six inhibitory relationships listed in Table 1 have been shown to be competitive—namely, the effects of hexobarbital and chlorpromazine on the N-

TABLE 1. MUTUAL INHIBITION OF THE OXIDATION OF ETHYLMORPHINE, HEXOBARBITAL, AND CHLORPROMAZINE USING NONINDUCED AND PHENOBARBITAL-INDUCED MICROSOMAL SYSTEMS

		I_{50}*	
Substrate	Inhibitor	Noninduced	Induced
Ethylmorphine†	Hexobarbital	$1·2 \times 10^{-3}$	$1·2 \times 10^{-3}$
	Chlorpromazine	$1·3 \times 10^{-4}$	$1·0 \times 10^{-4}$
Chlorpromazine‡	Ethylmorphine	$1·5 \times 10^{-3}$	$1·7 \times 10^{-3}$
	Hexobarbital	¶	$6·0 \times 10^{-4}$
Hexobarbital§	Ethylmorphine	$2·8 \times 10^{-4}$	$2·6 \times 10^{-4}$
	Chlorpromazine	$1·5 \times 10^{-4}$	$1·3 \times 10^{-4}$

* Concentration (M) of inhibitor effecting 50% inhibition.
† $8·0 \times 10^{-4}$ M.
‡ $6·0 \times 10^{-5}$ M.
§ $2·0 \times 10^{-4}$ M.
¶ At a concentration of $4·0 \times 10^{-3}$ M only a 22% inhibition was observed.

demethylation of ethylmorphine (Figs. 1 and 2). Figure 8 illustrates the competitive nature of the inhibitory effect of ethylmorphine on the oxidation of chlorpromazine.

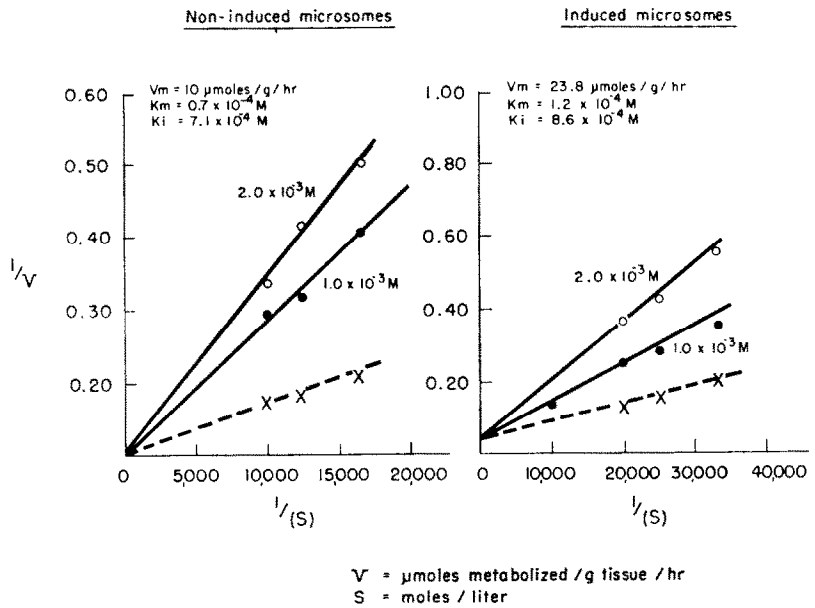

FIG. 8. Effect of ethylmorphine on the sulfoxidation of chlorpromazine by noninduced and phenobarbital-induced microsomal systems.

Kinetics of the oxidation of NADPH

The means (± S.E.) of three determinations of the Vm of the oxidation of NADPH by noninduced and induced microsomal preparations were 37·4 (±1·7) and 51·0 (±2·4) μmoles/g tissue per hr respectively. The K_m value of the oxidation of the reduced nucleotide (2·0 × 10^{-4} M) remained unchanged in the presence of ethylmorphine (8·0 × 10^{-4} M), hexobarbital (2·0 × 10^{-4} M), and chlorpromazine (3·3 × 10^{-5} M).

DISCUSSION

In accordance with predictions, a variety of drugs known to be metabolized by hepatic microsomes competitively inhibited the N-demethylation of ethylmorphine. Furthermore, ethylmorphine, hexobarbital, and chlorpromazine were mutually inhibitory, each retarding the metabolism of the other. All the drugs that exhibited inhibitory effects in this study are known to be oxidized by microsomes, whereas two drugs, barbital and acetazoleamide, which are not metabolized, failed to act as inhibitors. The inhibitions can be explained if the drugs are reacting with a common intermediate, or with a single enzyme of such minimal specificity that it will combine with a variety of structurally unrelated drugs. In the latter case, one drug could inhibit the metabolism of another either by serving as an alternative substrate or by combining with the enzyme without yielding a product. When an enzyme combines with a compound that serves both as a substrate of that enzyme and an inhibitor (alternative substrate) of other reactions catalyzed by that enzyme, one can expect the K_i to equal the K_m. A comparison of K_m and K_i is given in Table 2. Where hexobarbital is acting

as an inhibitor of demethylation the K_i value is seen to equal the K_m value, and the K_m and K_i values are quite similar where ethylmorphine is employed as an inhibitor of sulfoxidation. However, when chlorpromazine acts as an inhibitor of demethylation, the K_m and K_i values are dissimilar. This suggests that inhibition may be occurring as a result of alternative substrate competition in some cases and on a strictly competitive basis in other cases.

TABLE 2. MICHAELIS (K_m) AND INHIBITOR (K_i) CONSTANTS FOR ETHYLMORPHINE, HEXOBARBITAL, AND CHLORPROMAZINE*

Drug	K_m (M)	K_i (M)	Reaction inhibited
Ethylmorphine	$5{\cdot}8 \times 10^{-4}$†	$8{\cdot}3 \times 10^{-4}$†	Sulfoxidation
Hexobarbital	$1{\cdot}2 \times 10^{-3}$‡	$1{\cdot}2 \times 10^{-3}$‡	N-Demethylation
Chlorpromazine	$12{\cdot}0 \times 10^{-5}$§	$4{\cdot}4 \times 10^{-5}$§	N-Demethylation

* Noninduced microsomes were employed.
† Values not significantly different from each other ($P > 0{\cdot}05$).
‡ Values not significantly different from each other ($P > 0{\cdot}05$).
§ Values significantly different from each other ($P < 0{\cdot}05$).

This aspect of the study might have been clarified if all six of the possible substrate–inhibitor combinations involving ethylmorphine, hexobarbital, and chlorpromazine had been investigated. However, certain of these combinations present experimental difficulties. For example, the insolubility of hexobarbital at pH 7·4 and its relatively high K_m value make difficult the determination of experimental K_i values when it is used in combination with the other two drugs.

The implication that a single enzyme is mediating the metabolism of ethylmorphine and hexobarbital is supported by the observations of others. Quantitative alterations in the rates of metabolism of narcotic drugs and barbiturates, whether increased or decreased, are affected similarly by sex,[6, 11] starvation,[12, 13] disease,[14–16] and the administration of thyroxin,[17, 18] phenobarbital,[3, 4] or SKF-525A.[19, 20] One argument against a single enzyme being responsible for the oxidation of hexobarbital and the N-demethylation of narcotic drugs was the finding that nalorphine effected about a 50% inhibition of N-demethylation but only a 7% inhibition of hexobarbital oxidation.[21] The concentration of nalorphine used in that study was not given, but in this laboratory when 5×10^{-4} M nalorphine was incubated with 5×10^{-4} M ethylmorphine or hexobarbital for 15 rather than 180 min, thus ensuring a linear reaction rate throughout the period of measurement, a 90% inhibition of the metabolism of both drugs was observed.

The literature suggests that the competitive inhibition of the demethylation of ethylmorphine by acetanilide and zoxazolamine is not based on the interaction of alternative substrates with a single enzyme. Benzpyrene stimulates the metabolism of zoxazolamine and acetanilide by hepatic microsomes but has no such effect on the N-demethylation of narcotic drugs. This suggests that the enzyme that catalyzes the demethylation of ethylmorphine is not the same as that responsible for acetanilide or zoxazolamine oxidation. Competitive inhibition can still be explained when two enzymes are involved, if the competition is for a common intermediate or if the drug can combine with both enzymes but yield a product with only one.

Phenobarbital induction effected a greater increase in the V_m for the oxidation of drugs than for the oxidation of NADPH. It is also to be noted that the V_m for the oxidation of NADPH was greater than that for any of the drugs studied regardless of whether induced or noninduced microsomes were employed. From these two observations it would be tempting to conclude that the second step in the reaction sequence proposed by Gillette *et al.*[1] is rate limiting. However, for this conclusion to be valid it must be assumed that essentially all the hydroxyl donor supplied by the oxidation of NADPH is utilized by the second reaction, and this need not be the case. At any rate, these observations challenge the suggestion that the salient feature of the inductive process is the increase in NADPH activity.[22, 23]

The close similarity of the K_m and K_i values seen in phenobarbital-induced and noninduced systems, coupled with the increased V_m values observed for the oxidation of a given drug after induction, argue strongly that a quantitative rather than a qualitative change is involved in the inductive process. Further evidence for the view that the same enzyme is involved in noninduced and induced systems is provided by the I_{50} values. With but one exception they are identical in both systems. The exception, where the inhibition of the sulfoxidation of chlorpromazine by hexobarbital was favored in the induced system, is not readily explained in view of the other kinetic data relating to hexobarbital and chlorpromazine. Because of the relative crudeness of the enzyme systems employed, it is perhaps more surprising that discrepancies did not appear more frequently.

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