MOLECULAR PHARMACOLOGY, 6, 206–212

Two-Substrate Kinetics of Drug-Metabolizing Enzyme Systems of Hepatic Microsomes

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(Received January 3, 1970)

SUMMARY

Two-substrate kinetics was employed to show that more than one microsomal system functions in the dealkylation of drugs. Evidence is also presented to show that in some dealkylation reactions a common enzyme system or rate-limiting component is involved. Employing microsomes from the male rat, it was concluded that the same enzyme system or rate-limiting component is involved in the *N*-demethylation of the *l*- and *d*-forms of 3-methoxy-*N*-methylmorphinan and in the *N*-deethylation of the tertiary amine, 2-diethylaminoethyl 2,2-diphenylvalerate·HCl (SKF 525-A), and its analogue, the secondary amine, 2-ethylaminoethyl 2,2-diphenylvalerate·HBr (SKF 8742-A). Using microsomes from untreated rats or from rats treated with phenobarbital or 3-methylcholanthrene, it was shown that the same enzyme system *N*-demethylates ethylmorphine and *N*-methylaniline. Different enzyme systems or rate-limiting components appear to be involved in the *N*-demethylation of morphine and the *O*-demethylation of norcodeine. Using microsomes from untreated rats, different *N*-demethylase systems metabolized ethylmorphine and morphine, but microsomes from phenobarbital-treated rats employed the same system.

INTRODUCTION

The question whether drugs are metabolized by more than one microsomal enzyme system involving cytochrome P-450 is the cause of much speculation. Evidence for more than one system has been based on several kinds of observations (1). (a) There are marked species differences in the relative rates at which different drugs are metabolized. For example, the ether linkage of *p*-ethoxyacetanilide is cleaved equally well by rabbit and guinea pig hepatic microsomes, but guinea pig microsomes are only about one-tenth as effective as those of the rabbit in the *O*-demethylation of codeine (2). (b) There are marked sex differences in the relative rates at which different drugs are metabolized. Microsomes from male rats metabolize aminopyrine, pentobarbital, and hexobarbital 3 times more rapidly than microsomes from female rats, but no sex difference in the hydroxylations of aniline and zoxazolamine have been noted (3). (c) A variety of treatments of animals produce relative changes in the rates of metabolism of different drugs. Deprivation of food alters selectively the rates at which drugs are metabolized. The effects of fasting of male rats ranged from impairment of the sex-dependent enzymes which metabolize aminopyrine and hexobarbital to the enhancement of the sex-independent enzymes that hydroxylate aniline (3). Although the metabolism of ami-

This research was supported by United States Public Health Service Grants GM 12543 and GM 15477. Part of this material appeared in abstract form [*Pharmacologist* 9, 203 (1967)].

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nopyrine and hexobarbital by liver microsomes from male rats is impaired by adrenalectomy, castration, hypoxia, and the administration of ACTH, formaldehyde, epinephrine, morphine, alloxan, or thyroxine, the metabolism of aniline and zoxazolamine is not decreased appreciably by these treatments. Chronic administration of morphine to mice depresses the *N*-demethylation of narcotic compounds but not that of 3 methyl-4-methylaminoazobenzene (4). (d) Specificity is shown in the kinds of drug metabolism stimulated or inhibited by certain agents (1, 5). None of these observations in itself proves the existence of more than a single drug-metabolizing system; these findings may simply reflect relatively minor qualitative differences in a single enzyme system. In fact, this might be expected to be the case. Both the physical and biochemical properties of cytochrome P-450 appear to depend to a considerable degree upon the phospholipid environment with which it is associated in the membrane. It is reasonable to expect that this phospholipid environment may not be identical in all species, in both sexes, or under all environmental conditions to which animals are exposed.

Until ways are found to solubilize and purify the microsomal oxidases in enzymatically active forms, indirect methods must be employed to determine whether different enzyme systems exist for the metabolism of drugs and other foreign compounds. Two-substrate kinetics can be employed for this purpose. The kinetics for the competitive interaction of drugs with receptor sites has been described by Ariëns *et al.* (6), and the mathematical concepts of those studies have been extended by Cha (7) to include substrate reactions with enzymes. If substrates *A* and *B* are incubated together with one enzyme such that substrate *B* in a single concentration is employed with several concentrations of *A*, and both produce a common product (such as formaldehyde in *N*-demethylation reactions) that is used to measure the sum of the velocities (v) of the two reactions, a hyperbolic curve will be obtained when $1/v$ is plotted against $1/[A]$ and it will intersect a similarly plotted linear curve obtained when varied amounts of substrate *A* are incubated under the same conditions without substrate *B*. The intercept can be predicted using the equation $S = V'K/(V - V')$, where S is the concentration of substrate *A* at the intercept of the two curves, K is the Michaelis constant[2] of substrate *A*, and V and V' are the maximum velocities[2] of the metabolism of substrates *A* and *B*, respectively. If two enzymes are involved, one for the metabolism of substrate *A* and the other for the metabolism of substrate *B*, the curves will not intersect or merge at any concentration of substrate *A*; that is, total product formation will always be greater when substrate *B* is also present. Sladek and Mannering (8) employed this method to show that the administration of 3-methylcholanthrene to rats caused a change in the microsomal enzyme system responsible for the *N*-demethylation of 3-methyl-4-methylaminoazobenzene. This kinetic analysis was employed in the current study to show that although many dealkylation reactions employ a common enzyme system, more than one system for dealkylation exists.

MATERIALS AND METHODS

Male Holtzman rats (90–120 g) were decapitated, and their livers were removed and placed in ice-cold 1.15% KCl solution. A 25% homogenate in 1.15% KCl solution was prepared using a Dounce homogenizer (15 strokes). The homogenate was centrifuged at 9000 × *g* for 20 min in a Lourdes centrifuge, model LRA (rotor No. 9RA). The 9000 × *g* supernatant fraction was centrifuged at 100,000 × *g* in a Spinco model L ultracentrifuge (rotor No. 50) for 60 min. The pellet was suspended in 1.15% KCl solution so that 1 ml contained the equivalent of 250 mg of wet liver. The microsomes were stored at −20° and used within 4 days of their preparation.

[2] Throughout the text, kinetic constants are given as the Michaelis constant (K_m) and the maximum velocity (V_{max}); it is to be understood that because the microsomal enzymes involved in these studies were not soluble and purified, these values are to be regarded as "apparent" kinetic constants.

Enzyme assays. Compounds were selected so that *N*- and *O*-demethylation and *N*-deethylation could be studied. The following compounds were studied: ethylmorphine·HCl, *N*-methylaniline·HCl, morphine sulfate, norcodeine·HCl, *l*-3-methoxy-*N*-methylmorphinan·HBr (levomethorphan), *d*-3-methoxy-*N*-methylmorphinan·HBr (dextromethorphan), 2-diethylaminoethyl 2,2-diphenylvalerate·HCl (SKF 525-A), and 2-ethylaminoethyl 2,2-diphenylvalerate·HCl (SKF 8742-A). The following reaction mixture, adjusted to pH 7.4, was used: NADP, 2 μmoles; KH_2PO_4–Na_2HPO_4 buffer, pH 7.4, 0.2 mmole; semicarbazide hydrochloride, 37.5 μmoles; nicotinamide, 20 μmoles; magnesium chloride, 10 μmoles; glucose 6-phosphate (Sigma), 20 μmoles; 2 enzyme units of yeast glucose 6-phosphate dehydrogenase (Sigma); 1 ml of various concentrations of the substrate dissolved in 1.15% KCl solution; 1 ml of microsomal suspension equivalent to 250 mg of wet liver; and sufficient 1.15% KCl solution to give a final volume of 5 ml. The mixture was incubated in a Dubnoff metabolic shaker (120 oscillations/min) at 37° in open, 25-ml Erlenmeyer flasks. Incubation times were 5 min when SKF 525-A and SKF 8742-A were used as substrates, and 15 min for all the other substrates. Reaction rates were linear during these time intervals.

Formaldehyde produced from demethylation reactions was measured by the method of Nash (9) as modified by Anders and Mannering (10). Acetaldehyde formed from *N*-deethylation of SKF 525-A or SKF 8742-A was determined by the method of Stotz (11) as described by Anders and Mannering (10).

Statistical analysis. When single substrates were employed, the data for the double-reciprocal plots were analyzed by the method of Wilkinson (12), using a digital computer with a FORTRAN program written by Cleland (13). When two substrates were employed, the double-reciprocal plots were drawn without benefit of statistical analysis. Student's *t*-distribution was used as a test of the null hypothesis, using a level of significance of p equal to or less than 0.05.

RESULTS

Two-substrate kinetics of N-demethylation of l- and d-isomers of 3-methoxy-N-methylmorphinan. The pharmacologically active *l*-isomer of 3-methoxy-*N*-methylmorphinan is more rapidly *N*-demethylated than the *d*-form (14). To determine whether the same enzyme system demethylates both isomers (levomethorphan and dextromethorphan), two-substrate kinetic studies of their *N*-demethylations were performed (Fig. 1). At each of three concentrations of dextromethorphan, the substrate used in fixed concentration, the experimentally determined point of intersection agreed well with the calculated point of intersection. In four other experiments, in which a 0.2 mM concentration of dextromethorphan was used, the mean calculated point of intersection [0.62 ± 0.03 (SE) $\times 10^{-4}$ M] was not significantly different ($p > 0.6$) from the mean of experimentally determined points of intersection [0.58 ± 0.05 (SE) $\times 10^{-4}$ M].

Two-substrate kinetics of N-demethylation of morphine and O-demethylation of norcodeine. Hepatic microsomes from rats possess greater *O*- than *N*-demethylating capacities for morphine-type substrates, but the reverse is true of microsomes from mice (14). This suggests that different enzymes are responsible for the two types of demethylation. Figure 2 supports this view; the two reciprocal plots do not intersect. This is in agreement with Elison and Elliott (15) and George and Tephly (16), who also concluded that *O*- and *N*-demethylation of morphine are catalyzed by different enzymes.

Two-substrate kinetics of N-deethylation of SKF 525-A and SKF 8742-A. SKF 525-A, a tertiary amine, and SKF 8742-A, a secondary amine, are *N*-deethylated by rat liver microsomes (10). Two-substrate kinetic analysis of the *N*-deethylation of these amines (Fig. 3) showed that the experimentally determined point of intersection (3.48×10^{-5} M) was not significantly different ($p > 0.30$) from the mathematically predicted point of intersection (4.15×10^{-5} M), thus indicating that the same enzyme system metabolizes both substrates. The problem that might have arisen because SKF 8742-A is the product of the *N*-demethylation of

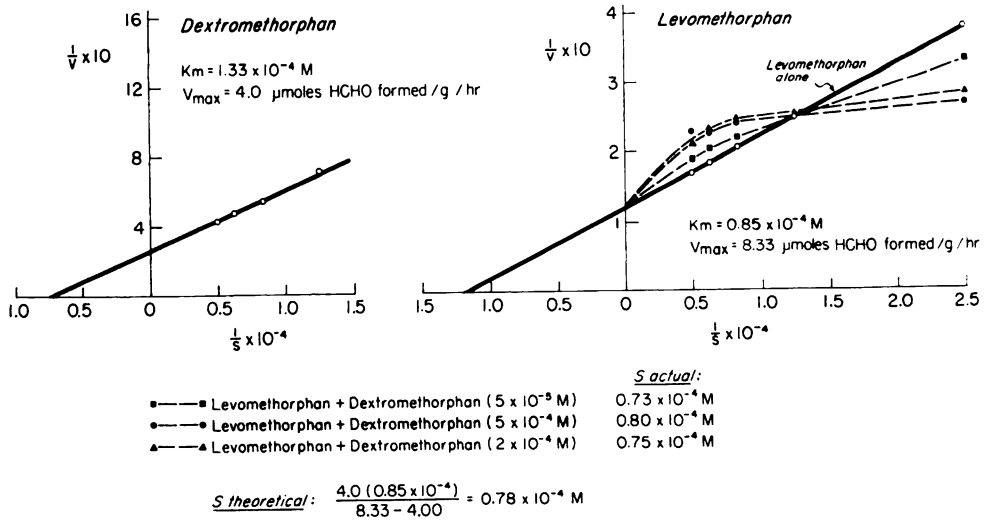


FIG. 1. *Two-substrate kinetics of N-demethylation of levomethorphan and dextromethorphan, the l- and d-isomers of 3-methoxy-N-methylmorphinan*

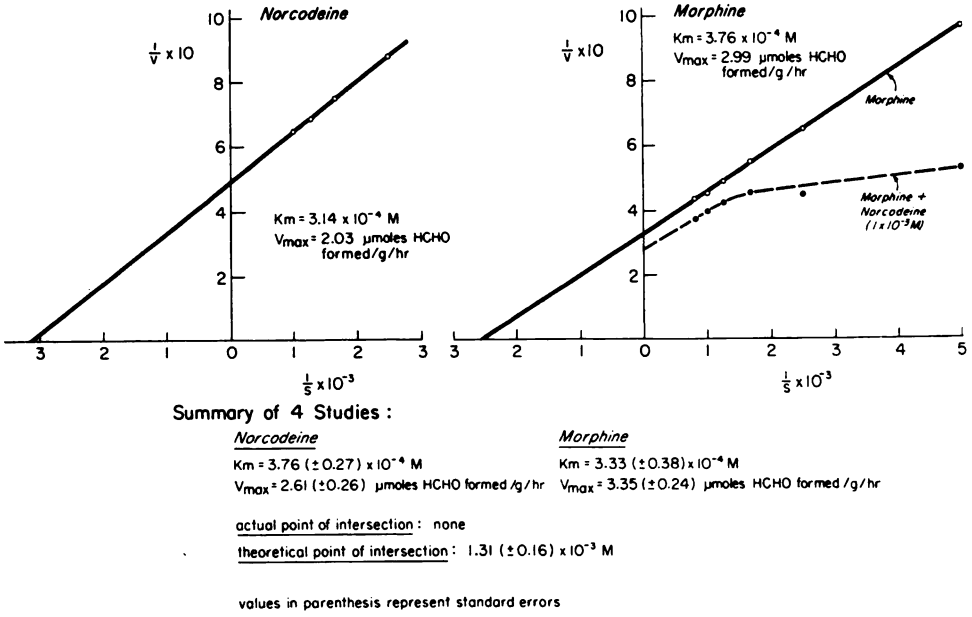
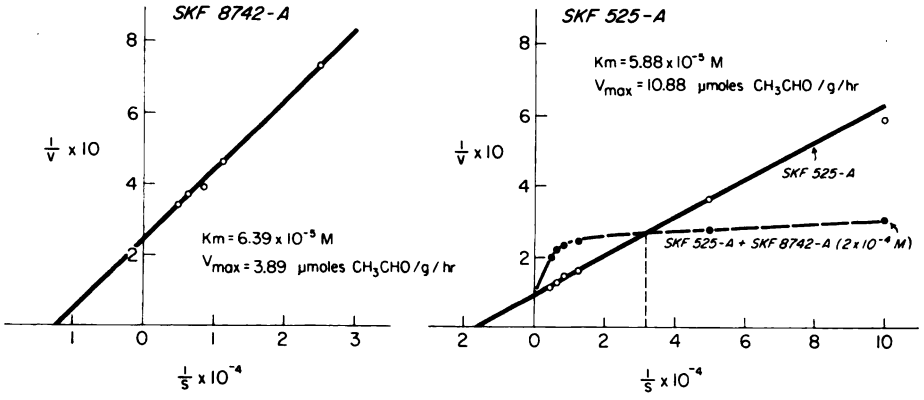


FIG. 2. *Two-substrate kinetics of N-demethylation of morphine and the O-demethylation of norcodeine*

SKF 525-A was minimized by the short incubation time; the amount of SKF 8742-A formed as a product was insufficient to change appreciably the kinetics of the reaction by serving as a substrate for the second *N*-deethylation.

Two-substrate kinetics of N-demethylation of ethylmorphine and morphine. Phenobarbital markedly stimulates the *N*-demethylation of ethylmorphine (17), but causes no increase in the *N*-demethylation of morphine (18). This suggests that more than a single enzyme system for the *N*-demethylation of these two narcotic drugs may exist. Two-substrate kinetic studies (Fig. 4 and Table 1) indicated the involvement of two *N*-demethylating enzymes when microsomes from untreated rats were used. However, when



Summary of 3 Studies:

SKF 8742-A	SKF 525-A
Km = 1.07 (± 0.15) x 10⁻⁴ M	Km = 7.44 (± 1.02) x 10⁻⁵ M
Vmax = 4.17 (± 0.01) µmoles CH_3CHO/g/hr	Vmax = 11.56 (± 0.47) µmoles CH_3CHO/g/hr

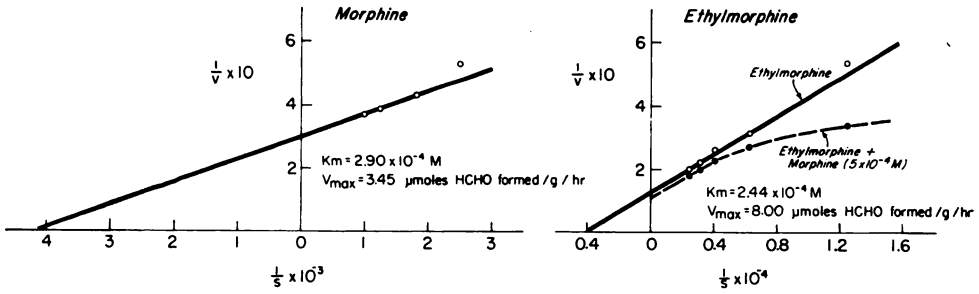
actual point of intersection: 3.48 (± 0.22) x 10⁻⁵ M*

theoretical point of intersection: 4.15 (± 0.47) x 10⁻⁵ M*

*values not significantly different from each other (P > 0.3)

values in parenthesis represent standard errors

FIG. 3. *Two-substrate kinetics of N-deethylation of SKF 525-A and SKF 8742-A*



Summary of 4 Studies:

Morphine	Ethylmorphine
Km = 4.59 (± 0.98) x 10⁻⁴ M	Km = 2.61 (± 0.08) x 10⁻⁴ M
Vmax = 3.50 (± 0.2) µmoles HCHO formed/g/hr	Vmax = 8.08 (± 0.15) µmoles HCHO formed/g/hr

actual point of intersection: none

theoretical point of intersection: 2.02 (± 0.26) x 10⁻⁴ M

values in parenthesis represent standard errors

FIG. 4. *Two-substrate kinetics of N-demethylation of ethylmorphine and morphine*

similar kinetic studies were performed with microsomes obtained from rats treated with phenobarbital (40 mg/kg/day administered intraperitoneally for 4 days), a single enzyme system or rate-limiting component appeared to be operative in the *N*-demethylation of both substrates (Table 1).

Two-substrate kinetics of N-demethylation of ethylmorphine and N-methylaniline. During the course of these studies, it was found that the *N*-demethylation of *N*-methylaniline, like that of ethylmorphine, was stimulated by phenobarbital, but not by 3-methylcholanthrene. Two-substrate kinetic studies were performed on the *N*-demethylation of *N*-methylaniline and ethylmorphine using

TABLE 1

Two-substrate kinetics of N-demethylation of ethylmorphine and morphine in untreated and phenobarbital-treated rats

Phenobarbital sodium, 40 mg/kg, was administered intraperitoneally daily for 4 days. Each value represents the mean ± standard error of four rats. Experimental and calculated intercepts were obtained as described in the text. When both substrates were employed together, morphine was present at a concentration of 5×10^{-4} M.

Treatment	Morphine		Ethylmorphine		Calculated point of intersection	Experimental point of intersection
	K_m	V_{max}[a]	K_m	V_{max}[a]		
	$M \times 10^4$		$M \times 10^4$		$M \times 10^4$	$M \times 10^4$
None	4.59 ± 0.98	3.50 ± 0.21	2.61 ± 0.08	8.08 ± 0.15	2.02 ± 0.26	—[b]
Phenobarbital	9.40 ± 2.10	3.17 ± 0.32	3.53 ± 0.50	30.50 ± 3.57	0.42 ± 0.08	0.53 ± 0.11[c]

[a] Micromoles of HCHO formed per gram of liver per hour.

[b] No experimental intercept was obtained, since the $1/v$ vs. $1/S$ plot obtained when ethylmorphine and morphine were present together did not intersect the $1/v$ vs. $1/S$ plot obtained with ethylmorphine alone.

[c] Statistical comparison of calculated and experimental intercepts: $p > 0.05$.

TABLE 2

Two-substrate kinetics of N-demethylation of ethylmorphine and N-methylaniline in untreated, phenobarbital-treated, and 3-methylcholanthrene-treated rats

Phenobarbital sodium, 40 mg/kg, or 3-methylcholanthrene, 20 mg/kg, was administered intraperitoneally once daily for 4 days. Each value represents the mean ± standard error of five rats. Experimental and calculated intercepts were obtained as described in the text. When both substrates were employed together, *N*-methylaniline was present at a concentration of 4×10^{-3} M.

Treatment	*N*-Methylaniline		Ethylmorphine		Calculated point of intersection	Experimental point of intersection
	K_m	V_{max}[a]	K_m	V_{max}[a]		
	$M \times 10^3$		$M \times 10^4$		$M \times 10^4$	$M \times 10^4$
None	1.13 ± 0.06	5.13 ± 0.60	3.54 ± 0.75	8.78 ± 0.93	4.79 ± 0.54	6.50 ± 1.33[b]
Phenobarbital	0.83 ± 0.08	9.62 ± 0.49	4.14 ± 0.18	29.61 ± 2.2	2.69 ± 0.76	2.35 ± 0.21[b]
3-Methylcholanthrene	1.28 ± 0.19	4.53 ± 0.24	4.71 ± 0.20	8.52 ± 0.61	5.63 ± 0.69	6.61 ± 1.39[b]

[a] Micromoles of HCHO formed per gram of liver per hour.

[b] Statistical comparison of calculated and experimental intercepts: $p > 0.05$.

microsomes from untreated and phenobarbital- and 3-methylcholanthrene-treated rats. Phenobarbital sodium (in 0.9% NaCl), 40 mg/kg/day, or 3-methylcholanthrene (in corn oil), 20 mg/kg/day, was administered intraperitoneally for 4 days. A comparison of the experimental and calculated intercepts shows that *N*-methylaniline and ethylmorphine are *N*-demethylated by the same enzyme system in microsomes from untreated, phenobarbital-treated, and 3-methylcholanthrene-treated rats (Table 2).

DISCUSSION

The two-substrate kinetic studies employed in this study were intended to provide a more direct answer to the question whether more than one microsomal drug-metabolizing system exists in hepatic microsomes than can be obtained by comparatively indirect means, such as the observation of species and sex differences in the relative rates of metabolism of drugs or the possible effect of certain environmental conditions in causing

differential changes in rates of metabolism of selected drugs. The results indicate that the following pairs of drugs utilize a single enzyme system: *l*- and *d*-3-methoxy-*N*-methylmorphinan, SKF 525-A and SKF 8742-A, and ethylmorphine and *N*-methylaniline. This does not mean that the same *N*-demethylase is employed in the metabolism of all three pairs of drugs, but there is also no reason to suggest that this is not the case. It is necessary to qualify these remarks with the consideration that if two or more *N*-demethylases shared a single rate-limiting cofactor, the two-substrate kinetic studies would have yielded the same results as those obtained with a single enzyme capable of metabolizing two substrates. Thus, the question as to the number of microsomal enzymes that comprise the hepatic microsomal drug-metabolizing system has not been answered with absolute definitiveness, and it probably will not be until all the components of the system have been solubilized, purified, and characterized; the current studies, however, narrow the range of speculation.

Henderson and Mazel (18) showed that phenobarbital does not induce increased *N*-demethylation of morphine, an observation which has been confirmed in our laboratory, but the *N*-demethylation of ethylmorphine is greatly enhanced (17). In the current studies it was shown that microsomes from untreated rats appeared to metabolize ethylmorphine and morphine by different enzyme systems whereas only a single enzyme system seemed to be utilized when microsomes from phenobarbital-treated animals were employed. Both observations suggest that qualitative as well as quantitative changes occur in the drug-metabolizing system after the administration of phenobarbital. However, the possibility exists that these changes that occur as a result of phenobarbital administration may simply reflect a disparity in the relative degrees of induction of different components of the enzyme complex in such a way that the rate-limiting component is not the same as the one that controlled the over-all reaction rate when microsomes from untreated animals were used.

ACKNOWLEDGMENT

The authors gratefully acknowledge the able technical assistance of Mrs. Janice Shoeman.

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