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AMINOPYRINE DEMETHYLASE

KINETIC EVIDENCE FOR MULTIPLE MICROSOMAL ACTIVITIES*†

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Abstract—The Lineweaver–Burk plots of rat liver microsomal aminopyrine demethylase activity are nonlinear. The curve is characteristic of a reaction catalyzed by two enzymes. Pretreating animals with phenobarbital stimulates the demethylase activity and produces a linear reciprocal plot with an apparent K_m for aminopyrine of 7×10^{-4} M. Pretreatment with 3-methylcholanthrene causes no stimulation, but increases the apparent K_m for aminopyrine by an order of magnitude or more. 3-Methylcholanthrene *in vitro* has no effect on the aminopyrine demethylase activity and the changes in kinetic behavior following 3-methylcholanthrene treatment are inhibited by administration of ethionine. The inhibitor SKF-525A, at a concentration of 4×10^{-5} M, differentiated between the demethylase activities present in the two types of induced animals, inhibited the activity found in microsomes of phenobarbital-induced rats but had little effect on the activity in microsomes from 3-methylcholanthrene-treated rats. These results and the results of other investigators which suggest the existence of multiple drug-metabolizing activities are discussed.

THE SUGGESTION that the endoplasmic reticulum of mammalian liver contains more than one enzyme system for the oxidation of drugs was first proposed to explain the differential induction of drug metabolism by phenobarbital and polycyclic hydrocarbons. Phenobarbital (PB) stimulates the metabolism of many compounds, whereas induction by 3-methylcholanthrene (3-MC) stimulates the metabolism of relatively few compounds.[1] Subsequent studies by various investigators have produced several lines of evidence supporting this hypothesis.[2–7]

The studies presented in this paper began with the observation that aminopyrine demethylase activity continued to increase at high substrate concentrations. Attempts to explain the data nonenzymatically were without success. In this paper, we wish to report a kinetic analysis of the demethylation of aminopyrine which supports the existence of more than one system that can demethylate aminopyrine. The effects of induction by PB and 3-MC on the kinetic parameters of aminopyrine demethylase activity and the effects of SKF-525A inhibition have been investigated.

MATERIALS AND METHODS

Materials

Aminopyrine was purchased from K & K Laboratories, Inc., Plainview, N.Y.,

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and was either recrystallized or used as received, since identical results were obtained. Phenobarbital was purchased from Merck & Company, Inc., Rahway, N.J. Benzpyrene was purchased from Aldrich Chemical Company, Milwaukee, Wisc. SKF-525A (2-diethylaminoethyl-2,2-diphenylvalerate HCl) was a gift of the Smith, Kline & French Laboratory, Philadelphia, Pa. 3-Methylcholanthrene, D,L-ethionine, D,L-isocitrate, $NADP^+$, NADPH, NADH, and NADP-isocitrate dehydrogenase were all purchased from Sigma Chemical Company, St. Louis, Mo. 4-Monomethylaminoantipyrine was received as a gift from the Sterling Winthrop Drug Company, New York, N.Y., and purified by column chromatography on silica gel-G. Carbon monoxide was obtained from the Matheson Company, Inc., Joliet, Ill.

Animals

Initial experiments were done with microsomes isolated from the livers of male goats which have a high P-450 content and aminopyrine demethylase activity.* The majority of experiments, however, have been done with microsomes from male rats of the Holtzman strain weighing between 200 and 250 g. Animals induced with PB were given daily i.p. injections of 50 mg/kg in water for 5 days prior to being sacrificed. Animals treated with 3-MC were given a single injection, i.p., of 20 mg/kg in corn oil 24 hr prior to being sacrificed. Rats were treated with ethionine according to the method of Alvares *et al.*[4]

Preparation of microsomes

The animals were exsanguinated, and the livers were perfused with cold 1·15% KCl and removed. The tissue was homogenized in 4 vol. of 1·15% KCl containing 0·2% nicotinamide using a Potter–Elvehjem homogenizer with a Teflon pestle. The nuclear-mitochondrial fraction was removed by centrifugation at 15,000 *g* for 20 min. The microsomal pellet was isolated by centrifugation at 105,000 *g* for 90 min and resuspended in Tris-HCl buffer (0·05 M, pH 7·5) containing 50% glycerol. The final concentration varied from 30 to 50 mg protein per ml. Protein was assayed by the method of Lowry *et al.*[8] All operations were performed at 0–5°. The microsomes were either used immediately or stored under N_2 at −15°. No effect of storage on results was observed.

Aminopyrine demethylase assay

Rate assays of *N*-demethylase activity were obtained by assaying the formaldehyde production by the Nash method[9] in fixed-point assays at 2- or 3-min intervals over a 10-min period. All activities were then calculated from initial linear velocities. Reaction mixtures were incubated at 37° under air in a Dubnoff metabolic shaker and, unless stated otherwise, contained microsomes (0·8 mg/ml), $MgCl_2$ (7 mM), NADPH (0·5 mM), NADH (0·3 mM), Tris-HCl (0·05 M, pH 7·5), and the desired concentrations of substrates and inhibitors. 3-MC was added in 25 μl acetone to the 5-ml incubation mixture. In several experiments, the NADPH was provided by a generating system containing: D,L-isocitrate (2 mM), $NADP^+$ (0·1 mM) and NADP-isocitrate dehydrogenase (0·05 unit/ml). At all levels of substrate incubation, blanks containing everything except microsomes or boiled microsomes were included.

* Unpublished observations.

Difference spectroscopy

The carbon monoxide difference spectra were obtained with microsomes resuspended at a concentration of 2 mg/ml in 1·0 M phosphate buffer (pH 7·5) containing 50% glycerol. Microsomes in both reference and sample cuvettes were reduced with dithionite and then CO was added to the sample cuvette. The spectra were recorded by a Coleman–Hitachi model 124 spectrophotometer.

RESULTS

The *N*-demethylase activity of goat liver microsomes at various concentrations of aminopyrine (AP) is shown in the Lineweaver–Burk plot in Fig. 1. The reciprocal plot deviates considerably from linearity at high substrate concentrations and the

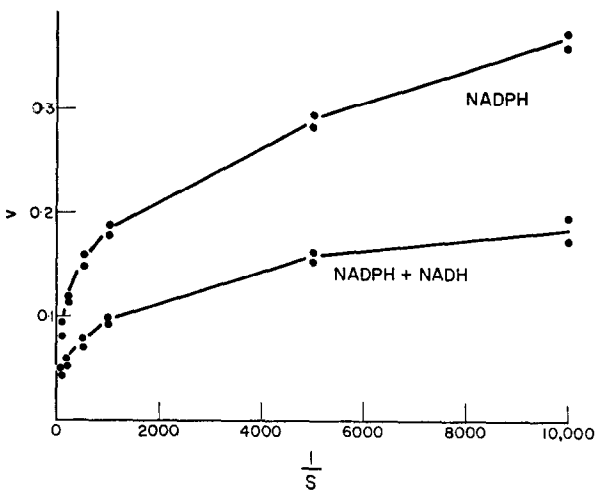

FIG. 1. Lineweaver–Burk plot for the *N*-demethylation of aminopyrine by goat liver microsomes. Velocities are given as millimicromoles of formaldehyde formed min^{-1}, mg^{-1} of microsomal protein. Substrate concentration is in moles $liter^{-1}$. The microsomal protein concentration in these assays was 0·72 mg/ml.

activity is increased by the addition of NADH. The addition of NADH to the incubation mixture had no effect on the results, except for a stimulation of total activity. Identical results were obtained with NADPH or NADP and the generating system. The requirement for both NADPH and NADH for maximal activity has been reported by other investigators.[10] No activity is observed with NADH alone, which indicates the effect of NADH is not due to transhydrogenase activity. Since the demethylation of aminopyrine involves the removal of two methyl groups, it was conceivable that the nonlinear reciprocal plot could be explained by the existence of monomethylaminoantipyrine (MAP) as a dissociable intermediate with altered kinetic parameters. The Lineweaver–Burk plot of MAP demethylase activity in goat liver microsomes is shown in Fig. 2. The nonlinear reciprocal plot, similar to that obtained with AP, indicates that the non-Michaelis–Menten kinetics must be a property of the microsomal enzymes.

Aminopyrine demethylase activity in microsomes from controls and PB-treated or

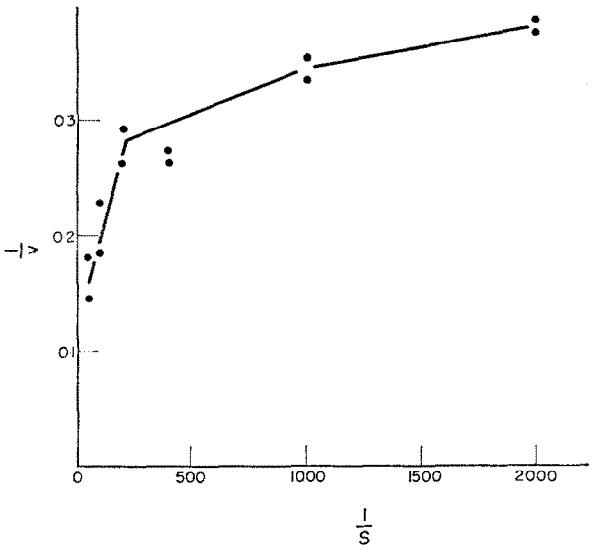

FIG. 2. Lineweaver–Burk plot for the *N*-demethylation of 4-monomethylaminoantipyrine by goat liver microsomes. The microsomal protein concentration in these assays was 0·75 mg/ml.

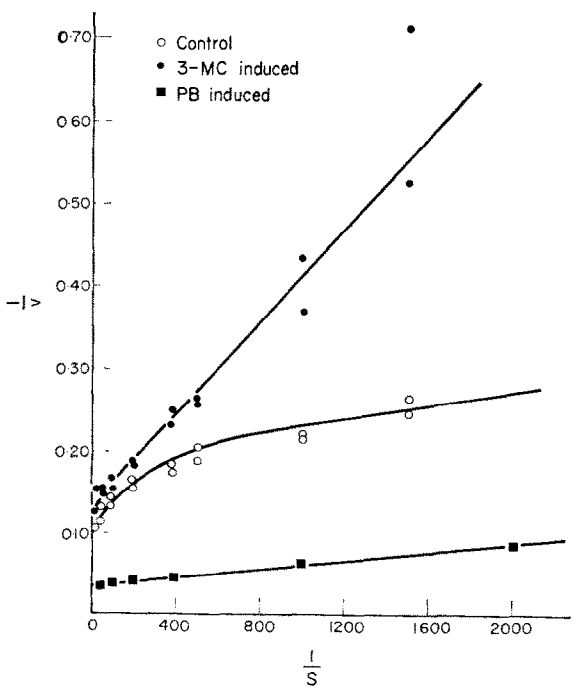

FIG. 3. Lineweaver–Burk plots for the *N*-demethylation of aminopyrine by male rat liver microsomes. Rats induced with 3-MC were given a single injection i.p. of 20 mg/kg in corn oil 24 hr before being sacrificed, and rats induced with PB were given daily injections, i.p., of 50 mg/kg in water 5 days prior to being sacrificed. Control rats were untreated.

3-MC-treated rats is shown in Fig. 3. The curve obtained with microsomes from PB-treated animals is linear and corresponds to an apparent K_m of 7×10^{-4} M, in agreement with the K_m of 8×10^{-4} M reported by Ernster and Orrenius.[11] The portion of the curve for control rat microsomes obtained at low substrate concentrations of AP yields a similar apparent K_m. The apparent K_m for aminopyrine obtained with microsomes from 3-MC-treated animals is at least an order of magnitude greater. Although AP demethylase activity is not stimulated by 3-MC treatment, benzypyrene hydroxylase activity in these microsomes is stimulated about 5-fold. The effect of 3-MC treatment, benzpyrene hydroxylase activity in these microsomes is stimulated about 5-fold. The effect of 3-MC on benzpyrene hydroxylation has previously been reported by Alvares *et al.*[5]

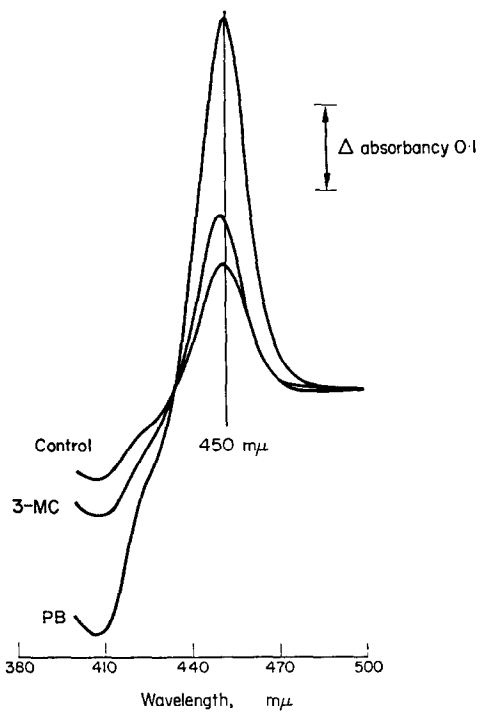

FIG. 4. CO difference spectra of dithionite-reduced rat liver microsomes. The microsomal protein concentration in each case is 2 mg ml^{-1}. The microsomal suspension was clarified by using 1·0 M phosphate buffer (pH 7·7) containing 50% glycerol.

The CO difference spectra of the reduced cytochrome P-450 present in the microsomes of the untreated, PB-treated and 3-MC-treated rats are shown in Fig. 4. As was originally reported by Alvares *et al.*,[4] there is an increase in the amount of P-450 present in microsomes from both PB-treated and 3-MC-treated rats and, in addition, the absorption maximum of the P-450 from the 3-MC-treated animals has shifted to about 448 mμ.

Since the increase in apparent K_m after treatment with 3-MC could be the result of inhibition by traces of 3-MC or its metabolites remaining in the isolated microsomes,

3-MC was added to incubation mixtures containing microsomes from untreated animals. The results, shown in Fig. 5, demonstrate that 3-MC at a concentration of 5×10^{-5} M, failed to inhibit aminopyrine demethylation. The same results were obtained when the microsomes were preincubated with the 3-MC for 5 min. If all the 3-MC injected into the animals were still present in the isolated microsomes, the concentration in subsequent incubation mixtures would be about 7×10^{-5} M; however, 5×10^{-5} M had no effect in the assays *in vitro*.

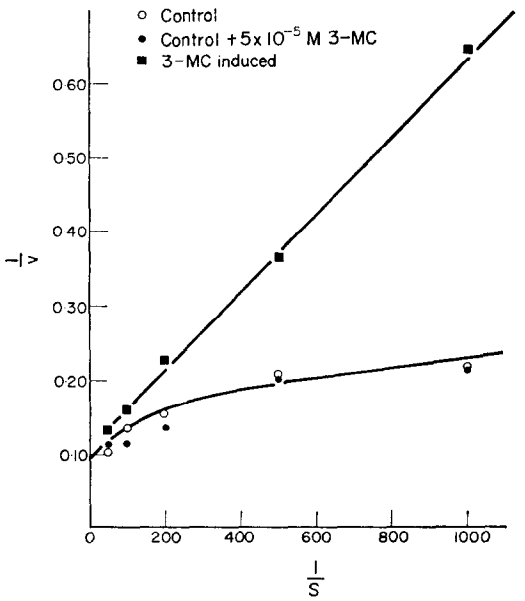

FIG. 5. Lineweaver–Burk plot for the *N*-demethylation of aminopyrine by rat liver microsomes demonstrating the effect of 3-MC *in vivo* and *in vitro*. The 3-MC was added to the incubation mixture in 25 μl acetone. The same experiment was performed, adding the 3-MC to the microsomes and incubating at 32° for 5 min before being added to the assay incubation mixtures. Identical results were obtained.

Attempts to demonstrate the presence of 3-MC or its metabolites in microsomes from 3-MC-treated rats were not successful. The thin-layer chromatogram of benzene–hexane extracts of 3-MC microsomes viewed under ultraviolet light is shown in Fig. 6. It can be seen that the extract from the microsomes from the incubation mixtures to which 3-MC was added produced, in addition to a fluorescent spot migrating like 3-MC, another spot near the origin. The spot at the origin could also be visualized by spraying the plate with $FeCl_3$, suggesting that it contains hydroxylated metabolites of 3-MC. Extracts from the microsomes of untreated and 3-MC-treated animals produced no visible spots, indicating that 3-MC is not present at levels detectable by this method.

The association between the change in apparent K_m and induction was investigated by using ethionine to block induction. Alvares *et al.*[12] had previously shown that ethionine and actinomycin D prevent the changes in spectral properties of P-450 following 3-MC treatment. The results of this experiment, shown in Fig. 7, indicate that induction is closely associated with the change in kinetic properties.

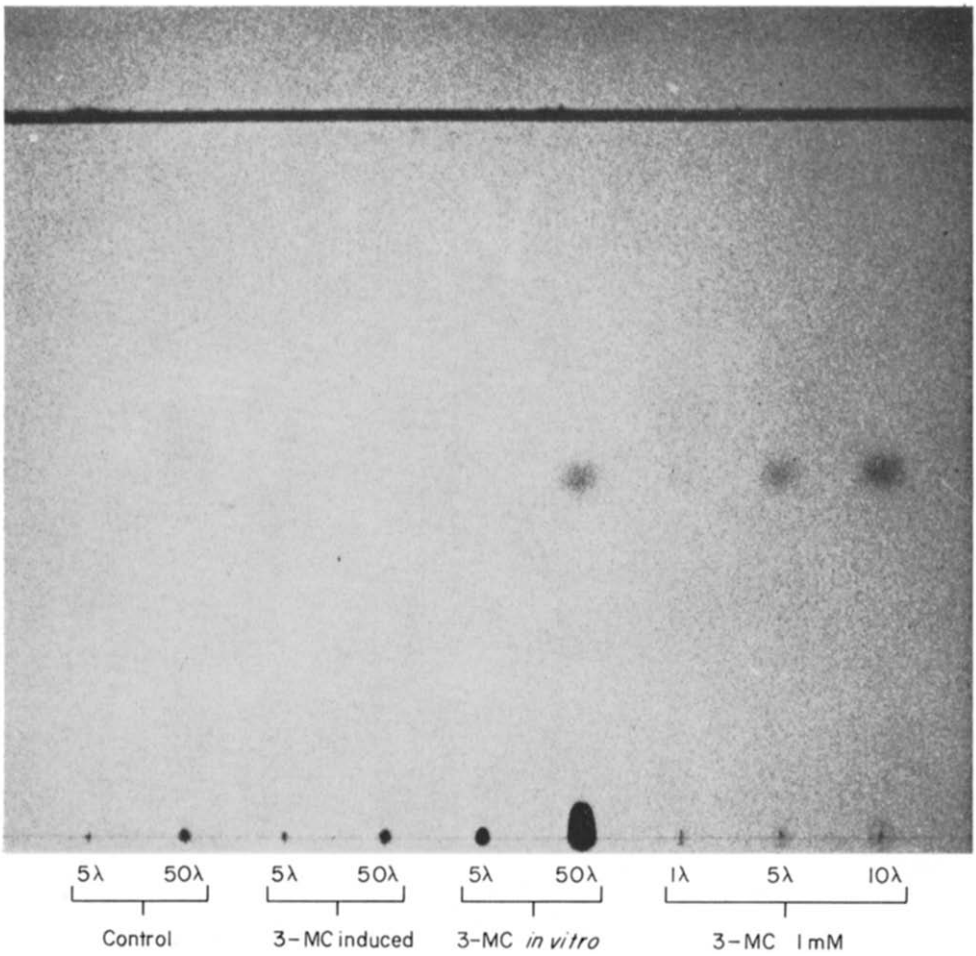

FIG. 6. Thin-layer chromatograms of microsome extracts viewed under u.v. light. Microsomes were extracted with benzene and benzene–hexane (1:1) and the amounts indicated were spotted from concentrated solutions containing the extracted material from 10 mg microsomes ml^{-1}. The chromatographic solvent used was benzene–hexane (1:1) and the plates were layered with silica gel G containing a fluorescent indicator.

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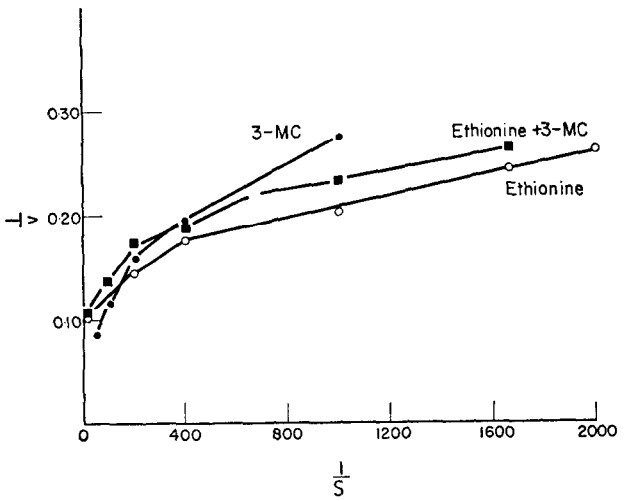

FIG. 7. Lineweaver–Burk plots for the *N*-demethylation of aminopyrine by rat liver microsomes showing the effect of ethionine on the changes produced by treatment with 3-MC. The animals were injected i.p. with ethionine, 500 mg/kg, 60 and 30 min before being injected with 3-MC, 20 mg/kg in corn oil, and sacrificed 24 hr after the last injection.

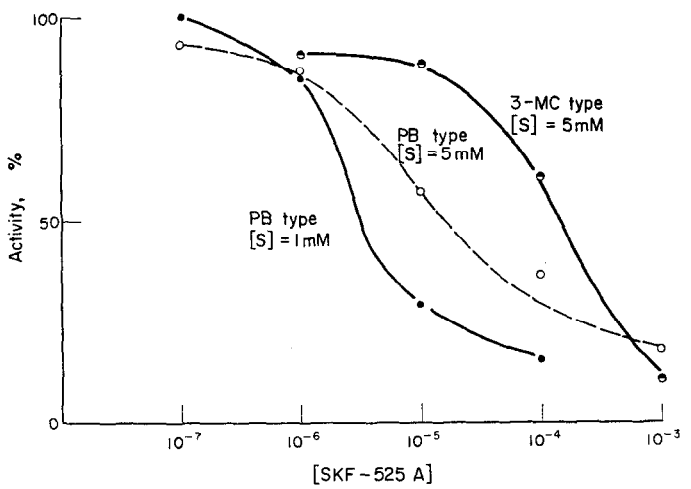

FIG. 8. Inhibition of aminopyrine demethylation in rat liver microsomes by SKF-525A. The concentrations of aminopyrine used, 1 and 5 mM, are approximately the respective concentrations at half maximal velocity in PB type and 3-MC microsomes.

Sladek and Mannering[13] reported that the inhibitor SKF-525A at a concentration of 4×10^{-5} M inhibited the demethylation of 3-methyl-4-monomethyl-aminoazo benzene in liver microsomes from PB-induced or untreated rats, but not in microsomes from 3-MC-induced rats. We similarly found (Fig. 8) that SKF-525A strongly inhibits AP demethylase activity in microsomes from untreated or PB-induced animals, but not in microsomes from 3-MC-induced animals.

DISCUSSION

The results presented in this paper are consistent with the proposal that liver microsomes contain more than one system capable of oxidatively demethylating aminopyrine. The curvature of the Lineweaver–Burk plots is characteristic of a reaction catalyzed by two distinct enzymes with different K_m values.[14] Figure 9 demonstrates the correlation between the observed values and a theoretical Lineweaver–Burk plot for two enzymes with K_m values of 4×10^{-4} and 2×10^{-2} M. Similar kinetic evidence for multiple drug-metabolizing enzymes has recently been reported by two other group of investigators. Wada *et al.*[15] observed nonlinear reciprocal plots of aniline hydroxylase

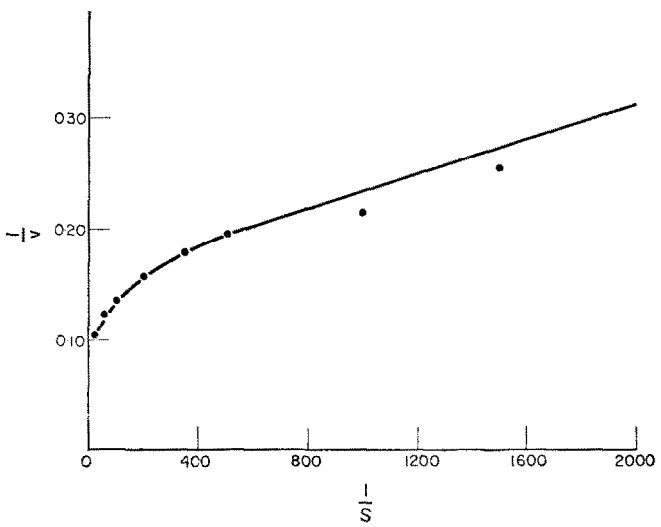

FIG. 9. Theoretical Lineweaver–Burk plot for aminopyrine demethylation catalyzed by two enzymes with K_m values of 4×10^{-4} and 2×10^{-2}. Theoretical, ——; observed values in rat liver microsomes, ●.

activity in mouse and rat liver microsomes and furthermore found that the curvature was increased in the presence of the inhibitor prednisolone. Likewise, Lewis *et al.*,[16] reported that reciprocal plots of aldrin epoxidation by pig liver microsomes showed similar departures from linearity at high substrate concentrations in the presence of the inhibitors 1,3-benzodioxoles.

The kinetic behavior of AP demethylase after pretreatment with PB or 3-MC reflects the established difference in induction by barbiturates and polycyclic hydrocarbons; that is, PB will induce AP demethylase while 3-MC does not.[1] However, a qualitative change in the enzyme does occur after 3-MC pretreatment and this change must involve protein synthesis or turnover. There was no clearcut separation of activities, but a shift in the activity from control type to a PB or 3-MC type of activity upon induction. The ability of ethionine to prevent partially the effects of 3-MC suggests that 3-MC induces the synthesis of enzyme(s) with altered substrate specificity which can demethylate AP if AP is present at high concentrations. The reciprocal plot for AP demethylase activity in control rat microsomes indicates that both PB and 3-MC type enzymes are present, but at lower levels.

Similar changes in hepatic drug-metabolizing activity after induction by polycyclic hydrocarbons have been reported by several investigators. Kuntzman *et al.*,[17] have shown in the 6β-, 7α-, and 16α-hydroxylation of testosterone by rat liver microsomes that PB induction stimulates the hydroxylation at all three positions, but causes the largest increase in 16α-hydroxylation. 3-MC, in contrast, stimulates only 7α-hydroxylation. Alvares *et al.*,[5] have demonstrated that the stimulation of benzpyrene hydroxylation by 3-MC is accompanied by a decrease in the apparent K_m. The stimulation by PB shows no such decrease in K_m. Recent studies by Nebert and Gelboin[18] of arylhydroxylase activity in hamster fetal cell cultures show that only polycyclic hydrocarbons are capable of inducing the hydroxylase activity.

The inhibition of SKF-525A is further evidence for the difference in substrate specificity in PB- and 3-MC induced animals. It should be noted that the differences in sensitivity to inhibition of SKF-525A represent a difference in affinity for the inhibitor and not a consequence of the difference in affinity for AP, which would have produced opposite results. Preliminary results in this laboratory indicate that benzpyrene is able to inhibit AP demethylase activity in PB-induced microsomes but not in 3-MC-induced microsomes. These results were not expected; however, it was found that most inhibitors of AP demethylase were more effective in microsomes from PB-induced animals than in microsomes from control animals.

It must be stated that the experimental results presented in this paper are not conclusive proof for the existence of multiple drug-metabolizing enzymes. The possibility still exists that 3-MC or its metabolites alter the properties of the drug-metabolizing enzymes present by some method which has not been reproduced *in vitro*. There is a possibility that two populations of microsomes exist with either different enzymes or enzymes in different environments. It is well known that PB treatment produces proliferation of the endoplasmic reticulum and size of the liver, whereas 3-MC causes little or no increase in microsomal protein.[1] Wold and Steele[19] have recently reported that PB primarily affects the transcription of ribosomal RNA, whereas 3-MC produces an increase in the transcription of RNA resembling messenger RNA.

There are several other types of evidence which may indicate the nature of the qualitative changes effected by polycyclic hydrocarbons. Daly *et al.*,[20] have recently reported that the retention of deuterium in *p*-deuteroacetanilide after *p*-hydroxylation by liver microsomes varies with species, sex and type of induction. Retention of the *p*-deuterium occurs when the deuterium atom migrates during hydroxylation to the meta-position. The degree of retention increases after the animals are pretreated with PB and decreases after pretreatment with 3-MC, suggesting there has been a change in the environment in which the acetanilide is located during hydroxylation. There is also considerable evidence, based on spectral studies, suggesting the existence of multiple forms of P-450 in liver microsomes.[2–4,6] Conney *et al.*,[21] have recently reported that there is considerable difference in the degree of inhibition of the 6β-, 7α-, and 16α-hydroxylations of testosterone by carbon monoxide. Levine and Kuntzman[22] have presented evidence for two P-450's or two pools of P-450 showing a biphasic decrease in radioactivity incorporated into P-450. Treatment with 3-MC increased the ratio of the slow phase component to the fast-decaying component.

The results reported here present kinetic evidence for multiple systems for the metabolism of AP. This evidence, together with that presented elsewhere, strongly supports the existence of multiple drug-metabolizing enzymes in mammalian liver. However, the

identification of the components that convey substrate specificity and are altered by induction will require the separation and identification of the cytochromes and other components which constitute the drug metabolizing system.

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