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AMINOPYRINE DEMETHYLASE—MULTIPLICITY SHOWN BY DIELDRIN AND DDT INHIBITION*

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Abstract—The inhibition of aminopyrine demethylase activity of rat liver microsomes by dieldrin and DDT suggests the presence of three components capable of demethylating aminopyrine. Two of these systems are sensitive to inhibition by DDT; however, not equally. One is sensitive to dieldrin inhibition. All three activities are present in microsomes from control animals. One component is extremely sensitive to DDT inhibition and is induced by pretreating the animals with phenobarbital. The activity of the component which is insensitive to inhibition by either dieldrin or DDT is slightly increased by 3-methylcholanthrene pretreatment.

THE SUBJECT of this study is the mechanism by which liver microsomal oxidases are capable of metabolizing such a wide variety of substrates. The substrate aminopyrine has been chosen as a model primarily because of earlier evidence for multiple enzymes capable of metabolizing aminopyrine. Kinetic evidence for the presence of multiple microsomal enzymes capable of demethylating aminopyrine has been presented by this laboratory.[1] Lineweaver–Burk plots of demethylase activities were not linear and were indicative of multiple activities with widely differing Michaelis constants for aminopyrine. Similar results were obtained with monomethylaminoantipyrine, suggesting that the results were not because of a dissociable monomethyl intermediate with altered kinetic properties. Other evidence indicated some additional basic differences in the enzymes. Their differences in susceptibility toward inhibition by SKF-525A and by benzpyrene suggested that the enzymes could be preferentially inhibited. Other evidence suggested that they could be preferentially induced.

This communication presents further evidence for multiple microsomal enzymes catalyzing the demethylation of aminopyrine. The tools of selective inhibition and induction have been used. The alternative substrate hypothesis, investigated by Rubin *et al.*[2] and Nakatsugawa *et al.*,[3] has been investigated, using the substrate aminopyrine and the inhibitors DDT and dieldrin. These experiments are based on the consideration that if two substrates are being metabolized by the same enzyme they must be competitive inhibitors.

MATERIALS AND METHODS

Materials. Aminopyrine was purchased from K & K Laboratories, Inc., Plainview, N. Y., and recrystallized before use. Phenobarbital was obtained from Merck & Co., Rahway, N. J.; benzpyrene from Aldrich Chemical Co., Milwaukee, Wisc.; and 3-methylcholanthrene, D,L-isocitrate, NADP, and NADP-isocitrate dehydrogenase all from Sigma Chemical Co., St. Louis, Mo.

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Purified dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4,5,8-dimethanonaphthalene) and *p,p'*-DDT (1,1,1-trichloro-2,2-tris(*p*-chlorophenyl)-ethane) were obtained from Drs. R. M. Cook and L. L. Bieber, respectively, of Michigan State University.

The rats (Holtzman strain, 250 g males) were exsanguinated and the livers perfused with cold 1·15% KCl before removal. The liver 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 and mitochondrial fraction were 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 0·05 M tris–HCl, pH 7·5, containing 50% glycerol to give a final concentration of 30–50 mg of protein per ml as assayed by the method of Lowry.[4] All operations were performed at 0–5°. The microsomes were either used immediately or stored under N_2 at −15°. No alteration in the various activities because of storage could be demonstrated. Similar results were obtained with fresh microsomes resuspended in 0·05 M tris–HCl, 1·15% KCl, 1·15% KCl containing 50% glycerol, 0·05 M phosphate buffer, pH 7·5, or 0·05 M phosphate buffer containing 50% glycerol. The above suspensions were also frozen under nitrogen and stored for various time intervals before use. In each case the presence of glycerol stabilized activity and the tris–HCl buffer containing 50% glycerol allows storage for at least 1 year with very little loss of enzymatic activity and no change in kinetic parameters.

Pretreatment of animals with phenobarbital (PB) was accomplished by including 0·1% PB in their drinking water for at least 1 week while 3-methylcholanthrene (3-MC) (20 mg/kg), dissolved in corn oil was given by a single i.p. injection 48 hr before sacrificing.

Incubations (Dubnoff metabolic shaker) were carried out at 37° under air with 5 ml of total volume. Most studies involved 1–10 mg microsomal protein, 7 mM $MgCl_2$, 0·05 M tris–HCl, pH 7·5, $NADP^+$, added either in the reduced form (0·5 mM) or by a generating system and varying levels of aminopyrine. The NADPH generating system consisted of 0·1 mM $NADP^+$, 2 mM isocitrate and 0·05 units of isocitrate dehydrogenase. Demethylase activity is expressed in mμmoles of formaldehyde,[5] min^{-1}, mg^{-1} protein. Rates were linear and subsequently all assays were for 7 min. All assays and experiments were done at least in duplicate and incubated blanks (minus substrate) were used as controls. Water-insoluble inhibitors were added in a minimum volume of acetone directly into the incubation flask followed by removal of the acetone under a stream of nitrogen.

RESULTS

Lineweaver–Burk plots of aminopyrine demethylase activity in microsomes from untreated animals (control-microsomes) were not linear (Fig. 1). However, similar plots of aminopyrine demethylase activity in microsomes obtained from animals pretreated with PB (PB-microsomes) were essentially linear (Fig. 1). PB-pretreatment resulted in induction of enzymatic activity and a decrease in the apparent K_m for aminopyrine (5×10^{-4} M vs. $1·7 \times 10^{-3}$ M). 3-MC pretreatment resulted in slightly greater activity at high substrate concentrations and reciprocal plots were linear at these concentrations (Fig. 1). Extrapolation of the Lineweaver–Burk plot at high substrate concentrations gave a K_m for aminopyrine of about 2×10^{-3} M.

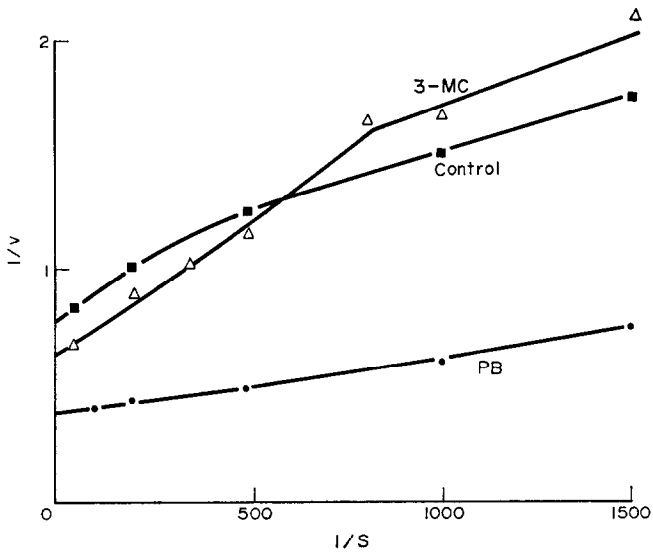

FIG. 1. Lineweaver–Burk plots of aminopyrine demethylase activity in microsomes from control (Control), phenobarbital (PB) and 3-methylcholanthrene (3-MC) pretreated animals. Velocity is in mμmoles of formaldehyde, min^{-1}, mg^{-1} protein; substrate concentrations are molar.

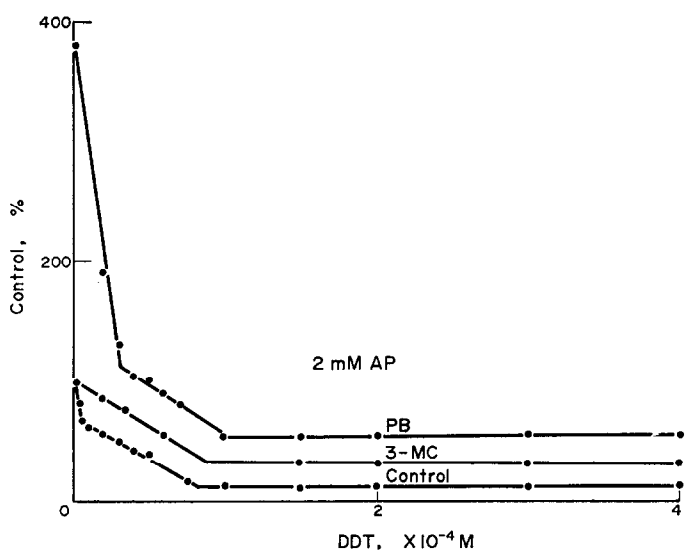

FIG. 2. Inhibition of microsomal aminopyrine demethylase activity by DDT. Activity is expressed as per cent of control activity (100 per cent) in the absence of inhibitor. Microsomes were obtained from control animals (Control) and from animals pretreated with phenobarbital (PB) and 3-methylcholanthrene (3-MC). Aminopyrine (AP) concentration was 2 mM. Control activity (no inhibitor) was 0·8 mμmoles formaldehyde, min^{-1}, mg^{-1} protein.

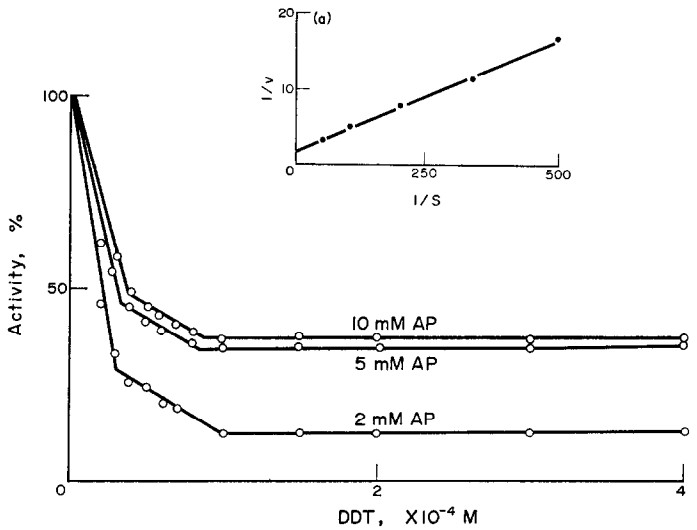

FIG. 3. The inhibition of aminopyrine demethylase in microsomes from phenobarbital pretreated animals by DDT at various concentrations of aminopyrine (AP). Control activities (no inhibitor) at 2, 5 and 10 mM aminopyrine were 1·4, 1·9 and 2·3 mμmoles formaldehyde, min^{-1}, mg^{-1} protein respectively.

(a) Lineweaver–Burk plot of aminopyrine demethylase activity in microsomes from phenobarbital (PB) pretreated animals at 0·2 mM DDT. Activity is expressed as mμmoles of formaldehyde, min^{-1}, mg^{-1} protein; substrate concentrations are molar.

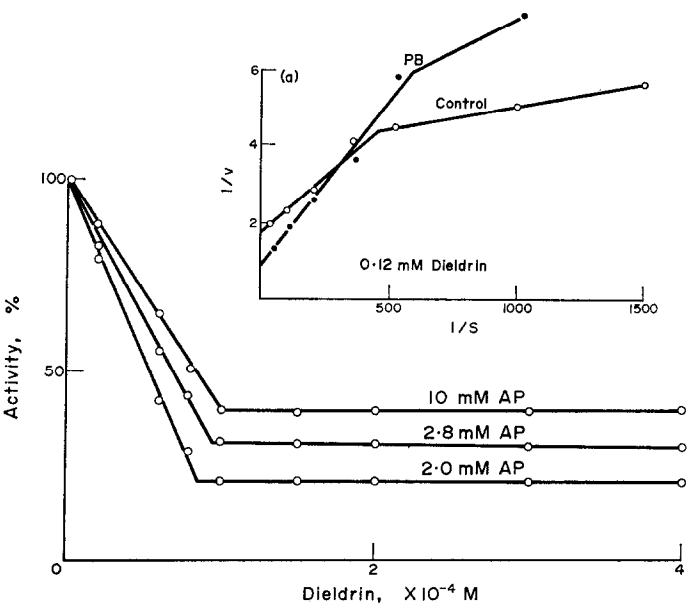

FIG. 4. The inhibition of aminopyrine demethylase in microsomes from phenobarbital pretreated animals by dieldrin at various concentrations of aminopyrine (AP). Control activities (no inhibitor) at 2·0, 2·8 and 10 mM aminopyrine were 1·4, 1·6 and 2·3 mμmoles formaldehyde, min^{-1}, mg^{-1} protein respectively.

(a) Lineweaver–Burk plots of aminopyrine demethylase activity in microsomes from control (Control) and phenobarbital (PB) pretreated animals at 0·12 mM dieldrin. Activity is expressed as mμmoles of formaldehyde, min^{-1}, mg^{-1} protein.

The inhibition of aminopyrine demethylation in control-, PB- and 3-MC-microsomes at increasing concentrations of DDT is shown in Fig. 2. The data was presented as the per cent of control microsomal activity in the absence of inhibitor. The curves are not hyperbolic but rather seem to be made up of three linear segments which divided total activity into three components, one not inhibitid by DDT, one moderately inhibited and a third which is extremely sensitive to DDT inhibition. In PB-microsomes the component which is most sensitive to DDT inhibition seems about ten times as active as in control-microsomes. This component is essentially completely inhibited at 50 μM DDT. The second component, also inhibited by DDT, is more active in control- and 3-MC-microsomes and is inhibited from 50 to 150 μM DDT. At 2×10^{-3} M aminopyrine, the component which is not inhibited by DDT does not contribute significantly to the overall activity. However, at concentrations of DDT over 150 μM, both of the DDT sensitive components are completely inhibited and it is possible to

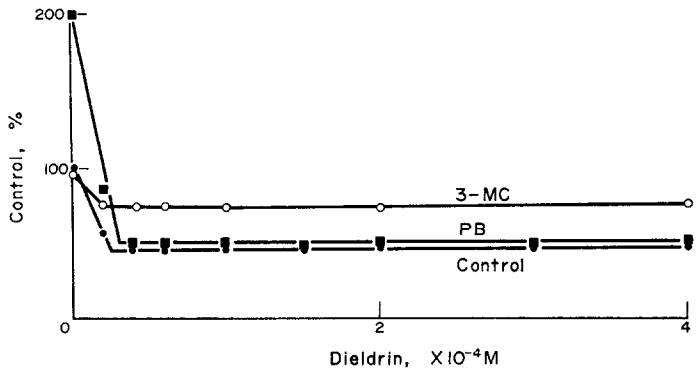

FIG. 5. Inhibition of aminopyrine demethylation in microsomes from control (Control) and phenobarbital (PB) pretreated animals by dieldrin. Activity is expressed as per cent of control activity in the absence of inhibitor. Control activity (no inhibitor) was 0·87 mμmoles formaldehyde, min^{-1}, mg^{-1} protein.

study the DDT-insensitive component. The activity of this component is related to substrate concentration as described by the Lineweaver–Burk plot (Figs. 3 and 3a). If the activities at various levels of substrate and 200 μM DDT are plotted in a reciprocal plot, a K_m of $1{\cdot}3 \times 10^{-2}$ M is obtained (Fig. 3a), which is about an order of magnitude greater than the K_m obtained by extrapolation of the Lineweaver–Burk plot for 3-MC-microsomes shown in Fig. 1.

When dieldrin was used as the inhibitor of aminopyrine demethylation by PB-microsomes, only two components were immediately evident. At substrate concentrations above 2×10^{-3} M, demethylase activity at increasing levels of dieldrin did not give a hyperbolic curve but seemed to be divided into two linear portions representing two components (Fig. 4); one having a low K_i for dieldrin and the second not inhibited by dieldrin. Repeating an experiment like that shown in Fig. 3, using dieldrin as the inhibitor at concentration greater than 100 μM, resulted in a nonlinear Lineweaver–Burk plot (Fig. 4a).

Comparison of the relative amounts of the components in control-, 3-MC- and PB-microsomes suggests that the dieldrin-sensitive component is that which is induced by PB (Fig. 5). The dieldrin-insensitive activity therefore must be made up of

two components, that which is moderately sensitive to DDT inhibition and that which is insensitive to DDT inhibition. Thus the PB-induced component is not the same as the low K_m component in control-microsomes. The K_m of the PB-induced component could only be estimated by assuming that the low K_m component of control-microsomes does not contribute significantly to the activity in "highly" induced PB-microsomes. Using this assumption, a K_m for aminopyrine of 5×10^{-4}M was obtained (Fig. 1).

Pretreatment of animals with 3-MC consistently decreased the maximum degree of inhibition obtainable by either DDT or dieldrin (Figs. 2 and 5). These results could be explained if 3-MC pretreatment induced the synthesis of the DDT-insensitive component. This was investigated by comparing the aminopyrine demethylase activity in control- and 3-MC-microsomes in the presence of 200 μM DDT. The results (Fig. 6) showed a 2-fold increase in maximum velocity with no change in K_m.

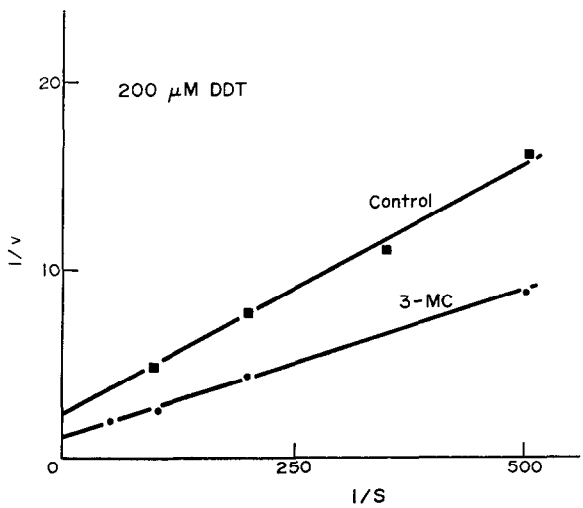

FIG. 6. Lineweaver–Burk plot of aminopyrine demethylase activity in microsomes from control (Control) and 3-methylcholanthrene (3-MC) pretreated animals in the presence of 200 μM DDT. Velocity is in mμmoles of formaldehyde, min^{-1}, mg^{-1} protein, substrate concentrations are molar.

DISCUSSION

Evidence, based on kinetic analysis of the inhibition of aminopyrine demethylation by dieldrin and DDT, is submitted for multiple microsomal enzymes capable of demethylating aminopyrine. Although other explanations for these results could be presented, the best explanation, in the view of the authors, is one involving the existence of multiple enzymes capable of demethylating aminopyrine. Multiplicity is indicated in microsomes from control animals by conventional Lineweaver–Burk plots of enzymatic activity. However, such plots were not useful for a number of reasons. In addition to the difficulty in obtaining a linear or useful Lineweaver–Burk plot in the absence of inhibitor, the curves became much more complex in the presence of inhibitor. In many cases the plots were misleading. The difficulties arise from the presence of multiple enzyme activities, their differences in susceptibility to inhibition and the fact that true competitive inhibition of a mixed-function oxidase has not been defined.

In contrast to the nonlinear Lineweaver–Burk plots obtained with the system, however, plots of activity vs. the concentration of inhibitor were observed to be linear.

The fact that complete inhibition could not be obtained at very high concentrations of inhibitor could not be explained by partial competitive inhibition, as described by Palmer.[6] In partial competitive inhibition, the fraction of activity remaining at high inhibtior concentration is independent of enzyme concentration. In the systems described above, however, the fraction of total activity remaining at high inhibitor concentrations is altered by induction. It was increased by 3-MC induction and decreased by PB induction. This observation would appear to argue strongly against the hypothesis that a single enzyme is responsible for the demethylation of aminopyrine. The only alternative to a multiple enzyme hypothesis would appear to be that the induced enzyme is not a different enzyme but the same enzyme in a different environment, which affects its kinetic parameters. Such an explanation, however, appears less likely than that of multiple enzymes.

The strongest evidence that these activities involve separate proteins with different specificities is the observation that the individual activities can be preferentially induced by PB or 3-MC, and that the activities induced by these compounds have completely different kinetic properties. Upon induction by PB-pretreatment, only one component seems evident in a Lineweaver–Burk plot because of the low K_m and high activity of this component. However, upon the addition of increasing levels of DDT to the incubation mixture, three components are evident. One, induced by PB, is extremely active and easily inhibited by DDT and dieldrin. A second, present in all types of microsomes, is responsible for most of the activity in control-microsomes and is moderately sensitive to DDT and insensitive to dieldrin. The third component is slightly increased upon 3-MC pretreatment and is insensitive to DDT and dieldrin. The latter component has a very high K_m for aminopyrine and thus can be observed only at high substrate concentrations. This activity seems to be present in all types of microsomes.

The K_m of the phenobarbital-induced enzyme (5×10^{-4} M) can be fairly accurately estimated from a Lineweaver–Burk plot of activity in "highly" induced PB-microsomes because of its low K_m and high activity. The K_m of the very high K_m component can be obtained from a Lineweaver–Burk plot of aminopyrine demethylase activity in the presence of sufficient DDT to inhibit completely the other components. By this method a K_m of $1{\cdot}3 \times 10^{-2}$ M was obtained. The K_m of the third component cannot be accurately determined; it must, however, be close to the K_m of the PB-induced enzyme.

By the use of increasing levels of the inhibitor DDT, it is possible to show that the degree of induction of aminopyrine demethylase cannot be accurately assessed by assaying for total activity. Since total activity appears to be made up of three individual activities, it is necessary to find which activity has been induced. For example (Fig. 2), phenobarbital induction causes approximately a 4-fold increase in total activity (at 2 mM aminopyrine); however, in actuality it induced about a 10-fold increase in one of the three apparent activities.

The inhibition of aminopyrine demethylation by increased levels of dieldrin separated the activity into two fractions, one sensitive to dieldrin inhibition and the other insensitive. The insensitive fraction could be shown to consist of two activities by a Lineweaver–Burk plot of demethylase activity in the presence of 100 μM dieldrin. Such a plot is biphasic, presumably because of the presence of two substrate-binding

sites with large differences in their K_m for aminopyrine. The fact that the dieldrin-sensitive component was that induced by PB could be shown by comparing the activity of these two components in control and PB-microsomes. This latter finding confirms that the PB-induced component is present, although difficult to observe, in control microsomes. These data further support the hypothesis of the existence of multiple microsomal enzymes capable of metabolizing aminopyrine.

In view of these results, it would appear that the two components seen in Lineweaver–Burk plots of aminopyrine demethylase activity in control-microsomes are made up of two enzymes as postulated earlier.[1] However, the low K_m component does not seem to be a single activity, that induced by PB, as concluded earlier.[1] It would appear that the high K_m component is not primarily responsible for the demethylation of aminopyrine but is capable of doing so at high substrate concentrations. The low K_m enzyme present in control-microsomes seems to have a fair degree of specificity for aminopyrine, for it is not inhibited by dieldrin and only moderately inhibited by DDT. The specificity of the PB-induced enzyme is difficult to understand, however, since although it has a low K_m for aminopyrine, it is very susceptible to both dieldrin and DDT inhibition.

The ramifications of such findings are not completely understood but some predictions can be made and some precautions should be observed in experiments involving these enzymes. For example, it is extremely difficult to saturate the high K_m enzyme with substrate. Therefore, unless high levels of substrate are used, assays for aminopyrine demethylase activity would not adequately reflect the activity of the high K_m enzyme. Unless extremely high substrate concentratons are used, there is the danger of working with activities which are highly dependent on substrate concentration. Also, the degree of inhibition by such pesticides depends on such parameters as the treatment of the animals and the concentrations of inhibitor and substrate used in the experiments. All of these factors must be considered when studying the effect of such compounds on the metabolism of others. That is, the interactions that exist in the liver microsomal oxidases are many and complex. This complexity has been shown by other investigators.

Lewis *et al.*[7] found that the variation in the ability of the 1,3-benzodioxoles to inhibit lipid peroxidation is in marked contrast to their more uniform action on aldrin epoxidation. However, it was impossible to determine if the inhibition was competitive or noncompetitive. The curvilinear trend of reciprocal plots is characteristic of systems in which a common substrate is attacked by two distinct enzymes. No curvilinearity was noted with substrate alone; however, this may be because of similarities in Michaelis constants, for in the presence of inhibitors curvilinearity was seen, presumably because of differences in susceptibility to inhibition.

The data published by Wada *et al.*[8] are very good evidence for more than one enzyme for the hydroxylation of aniline. Essentially linear Lineweaver–Burk plots were found for the hydroxylation of aniline, however, nonlinearity was evident when the inhibitor prednisolone was included in the incubation mixture. The K_m's of the two enzymes appear to be similar; however, the differences in inhibition by prednisolone show up by kinetic evaluation.

Perhaps some of the best evidence for a multiple enzyme system or a system with some form of selectivity comes from induction studies. Conney[9] has reviewed the evidence for more than one mechanism for the stimulation of drug metabolism by

two types of inducers. Alvares *et al.*[10] were able to show a difference in Michaelis constants for the hydroxylation of benzpyrene by microsomes obtained from animals induced by phenobarbital and 3-methylcholanthrene.

The data submitted in this report strengthens the hypothesis of multiple microsomal enzymes, at least for one substrate, and points out differences in these enzymes. How these results might relate to the evidence for multiple enzymes for the metabolism of other substrates has yet to be ascertained. Preliminary experiments on the inhibition of ethylmorphine demethylase by DDT also suggest multiple activities; with this substrate, however, DDT is a better inhibitor of the activity in control-microsomes than of the activity in PB-microsomes. How these results might correspond to the different forms of P_{450}[11] will also have to be investigated. The different activities could possibly reflect different forms of P_{450} if the two forms are interconverted by substrate, as postulated by Schenkman *et al.*[12,13] However, these results would be difficult to understand unless the conversion is caused by DDT or dieldrin, and even then the conversion would have to be highly concentration dependent. The results could also be explained by one cytochrome P_{450} having multiple substrate-binding sites which have different Michaelis constants for aminopyrine and different dissociation constants for inhibitors. However, both of these possibilities are difficult to accept if one considers the preferential induction of the individial activities. Since the different activities were preferentially induced, one could argue for multiple, independent forms of P_{450}, if this cytochrome is the substrate-binding component. Alternatives would include some other inducible substrate-binding fraction or an inducible component which would be capable of affecting the substrate specificity of certain fractions of cytochrome P_{450}, i.e. some type of K_m determining factor. Such possibilities are currently under investigation.

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