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STUDIES ON THE METABOLISM OF AMINOPYRINE, ANTIPYRINE AND THEOPHYLLINE USING MONOCLONAL ANTIBODIES TO CYTOCHROME P-450 ISOZYMES PURIFIED FROM RAT LIVER

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Abstract—We investigated the role played by monoclonal antibody defined classes of cytochrome P-450 in the metabolism of antipyrine, aminopyrine and theophylline. Two enzyme inhibitory monoclonal antibodies (MAb 1-7-1 and MAb 2-66-3) raised to two forms of cytochrome P-450 were used. Microsomes were prepared from the livers of untreated, 3-methylcholanthrene (MC)-treated, and phenobarbital (PB)-treated male Wistar rats. Addition of either monoclonal antibody to hepatic microsomes from untreated rats had a negligible effect on the metabolism of aminopyrine, antipyrine or theophylline. These results indicate that the constitutive enzymes responsible for metabolism of these three drugs differ from the MAb inhibitable enzymes responsible for transformation of these drugs in induced microsomes. In microsomes from MC- and PB-treated rats, however, the two MAbs differentially inhibited individual pathways. For example, at 20 mM aminopyrine, as much as 55% of 4-amino-antipyrine (4-AA) formation arose from the family of cytochrome P-450 isozymes that were not inhibited for (1) 4-AA formation at 4 mM aminopyrine and (2) 4-methylaminoantipyrine (4-MAA) formation at either concentration. Thus, the enzyme that functions at 20 mM aminopyrine in 4-AA formation differs from that which functions at 4 mM aminopyrine in formation of 4-AA or 4-MAA. Addition of MAbs to induced microsomes revealed at least four isozymes with overlapping specificities involved in antipyrine and theophylline metabolism. Each MAb-inhibitable pathway and the isozymes associated with it were classified into one of three epitope families: those pathways inhibited by both MAbs, those inhibited only by the MAb raised against PB-inducible P-450 isozymes, and those inhibited only by the MAb raised against 3-MC-inducible P-450 isozymes. A fourth group of pathways consisted of those unaffected by addition of either monoclonal antibody. Analysis of metabolism with these two MAbs suggests more extensive heterogeneity of the isozymes that biotransform these drugs than previously recognized.

As an increasing number of cytochrome P-450 isozymes are purified and the extent of the molecular heterogeneity of the system is better appreciated, clarification of the precise role played by individual isozymes or isozyme families in the metabolism of various substrates is desirable. Defining the relationship between the cytochrome P-450 phenotype in a tissue or individual and its responsiveness and sensitivity to drugs and carcinogens would help to elucidate processes of substrate detoxification and activation [1, 2]. However, the extensive heterogeneity of the cytochrome P-450 system, coupled with its overlapping regio- and stereoselectivity for different substrates, has limited progress in understanding the precise role of individual cytochrome P-450 isozymes in the metabolism of specific substrates [1, 3]. Families of isozymes may participate together, but to differing extents, in catalyzing individual reactions. While current understanding of the structure and function of the hepatic cytochrome P-450 system has progressed greatly, it remains incomplete.

One major problem involves determining the exact contribution of individual cytochrome P-450 isozymes to the overall metabolism of a substrate [1–4]. Currently available *in vivo* techniques do not allow direct measurement of the participation of individual isozymes [1]. Rather, they provide a composite profile of all isozymes that contribute to formation of a metabolite. Moreover each isozyme may participate to markedly different extents. Conversely, studies with purified cytochrome P-450 isozymes reveal much about the structure of individual isozymes and their phylogeny, but little about *in vivo* function [1, 4]. Monoclonal antibodies (MAbs), however, recognize a specific epitope and thus bind to epitopes specific for single, or classes of, cytochrome P-450 isozyme. In some cases, the binding of the MAb to the epitope results in inhibition of enzyme activity. The use of these inhibitory MAbs permits the determination of the minimum contribution of an antibody-sensitive cytochrome P-450 isozyme(s) to a particular reaction [1, 4–10]. Thus, MAbs specific for single, or classes of, cytochrome P-450 isozyme can be viewed as chemically "pure" reagents that may

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be used *in vitro* to identify cytochrome P-450 isozymes that are responsible for a particular reaction in crude enzyme preparations [1, 4–10].

The present report described the use of two MAbs: (1) MAb 1-7-1, prepared to a highly purified rat liver cytochrome P-450 inducible by 3-methylcholanthrene (3-MC) and (2) MAb 2-66-3, prepared to the major cytochrome P-450 isozyme induced by administration of phenobarbital (PB) [5, 6]. These MAbs were used to investigate the metabolism of three drugs: aminopyrine, antipyrine, and theophylline. The metabolism of each drug has been intensively studied and well defined [11–20]. Results from both *in vivo* and *in vitro* studies indicate that multiple isozymes of cytochrome P-450 participate in the metabolism of antipyrine, aminopyrine and theophylline [15–20]. Using enzyme inhibitory MAbs 1-7-1 and 2-66-3, we defined the epitope-specific cytochrome P-450 isozymes involved in the metabolism of these individual drugs, as well as those that may participate in the metabolism of all three drugs.

MATERIALS AND METHODS

Animals. Male Wistar rats (75–100 g) obtained from Charles River Laboratories (Wilmington, MA) were housed six per cage in a temperature- and humidity-controlled environment (12 hr light–dark cycle: 7:00 a.m. lights on). Food and water were available *ad lib.* until 18 hr prior to killing the animals.

Rats induced with PB were given sodium PB (40 mg/kg of body weight) in two injections (i.p.) 12 hr apart, followed by a single dose (80 mg/kg, i.p.) for 3 consecutive days. Animals were killed 16 hr after the last dose. 3-MC (80 mg/kg of body weight) was given as a single dose (i.p.) 72 hr before sacrifice. Untreated rats received an equivalent volume of vehicle alone.

Rats were exsanguinated, and livers were removed. Liver microsomes were prepared as previously published [21]. The protein concentration of the microsomal suspensions was determined by the method of Lowry *et al.* [22]. These suspensions were all diluted with buffer to contain 10 mg protein/ml.

Preparation of MAbs. The two MAbs, 1-7-1 and 2-66-3, used in this study were prepared by the hybridoma method of Köhler and Milstein [23]. MAb 1-7-1 was raised in mice against a purified 3-MC-induced rat liver cytochrome P-450 (MCP-450) [24]. The detailed preparation and characterization of this MAb have been described previously [5]. MAb 2-66-3, raised in mice against a purified PB-induced cytochrome P-450, has also been characterized previously [6]. In addition, MAb (nonspecific HyHel-9 p14) was obtained from mice injected with an anti-lysozyme hybridoma. This MAb was used to control for nonspecific inhibition or stimulation of enzyme activity by the ascites fluid. All antibody preparations were standardized with 50 mM Tris–HCl, pH 7.4, to 30 mg protein/ml of ascites fluid.

MAb inhibition studies. [3-^{14}C]Antipyrine (sp. act. 9.6 mCi/mmol) and [8-^{14}C]theophylline (sp. act. 50 mCi/mol) were synthesized by the Amersham Corp. (Arlington Heights, IL). Radiolabeled substrates were dissolved in ethanol. Aliquots containing 1 μCi were added to incubation vials and reduced to dryness at 25° under nitrogen. Unlabeled drug was dissolved in 50 mM Tris–HCl, pH 7.4, and appropriate aliquots were added to the labeled substrate bringing the final concentration of antipyrine to 18 mM and theophylline to 0.25 mM. Aminopyrine was used unlabeled at final concentrations of 4 and 20 mM.

Rat microsomal suspensions (final concentration: 1 mg protein/ml of incubation) were preincubated with suitable dilutions (in 50 mM Tris–HCl, pH 7.4) of antibody-containing ascites fluid for 15 min at 37° in a shaking water bath. The microsome-antibody incubation was then transferred to the drug-containing incubation tube, and a cofactor solution (containing NADPH and $MgCl_2$ to yield final concentrations of 2 and 6 mM respectively) was added to initiate the reaction. The final reaction volume was 0.5 ml. Control reactions were run in which the antibody was replaced by an equal volume of 50 mM Tris–HCl, pH 7.4. Reactions were incubated in a shaking water bath at 37° for 15 min (antipyrine) or 30 min (aminopyrine and theophylline).

Analysis of antipyrine metabolites. Antipyrine reactions were terminated by addition of 1.5 ml of ice-cold sodium acetate buffer, pH 4.5. Twenty micrograms of each unlabeled metabolite, 3-hydroxymethylantipyrine (3-OHMAP), *N*-demethylantipyrine (NDMAP) and 4-hydroxyantipyrine (4-OHAP), were added in 100 μl methanol to improve the chromatography of the labeled metabolite. The metabolite 3-OHMAP was a gift of D. D. Breimer, University of Leiden, The Netherlands, and H. Yoshimura, Maidashi, Japan. Phenacetin (10 μg) was added as a recovery standard to 0.1 ml of methanol. In addition, 5 mg of sodium metabisulfite was added to the reaction mixture to prevent oxidative breakdown of antipyrine metabolites. Samples were then extracted as described by Danhof *et al.* [25], with the following modifications. Prior to extraction, samples were not incubated with β-glucuronidase-aryl sulfatase. In addition, the entire 2.0 ml was first acid extracted with 5.0 ml of pentane–dichloromethane (70:30, v:v). Then, after removal of the organic layer, the sample was made basic with 0.1 ml of 4 N NaOH and re-extracted with 10.0 ml of dichloromethane. Antipyrine and its major metabolites were separated according to methods developed by Teunissen *et al.* [26]. The sample was reconstituted in 0.75 ml of mobile phase, and 0.5 ml was injected onto a spherical 5 μm MOS-Hypersil column (4.6 mm i.d. × 10.0 cm) obtained from Shandon Southern Instruments Inc. (Sewickly, PA). The mobile phase was 0.05 M sodium phosphate buffer, pH 7.2, containing 15% acetonitrile run isocratically at 2.0 ml/min. The HPLC system consisted of a Waters Automatic Gradient Controller with two Waters 510 solvent delivery pumps and a Waters U6K injector. Detection of 3-OHMAP, NDMAP and 4-OHAP was accomplished by a Waters model 441 absorbance detector in tandem with a Berthold HPLC Radioactivity Monitor interfaced with an Apple IIe computer system. A Hewlett–Packard 3390A integrator was used to record changes in u.v. absorbance. Metabolite peaks were

identified by the relative mobility of the pure standards. Eluted counts were quantitated by the Berthold L 504 M Radio-Chromatography Program and corrected for recovery by comparison of the u.v. recovery of the internal standard to the average peak area for 10 μg of phenacetin. The extent to which parent drug was converted to metabolite approximated 10%, whereas more than 90% of the total radioactivity added was recovered from the incubations.

Analysis of theophylline metabolites. Theophylline reactions were terminated by addition of 0.1 ml of 2.5 N perchloric acid. We added 50 μg each of 1-methyluric acid (1MU), 3-methylxanthine (3MX), 1-methylxanthine (1MX), and 1,3-dimethyl uric acid (1,3MU) to facilitate chromatography of labeled metabolite. Samples kept on ice for 30 min were then centrifuged at 10,000 *g* for 15 min to remove precipitated protein. The sample (0.4 ml) was injected onto a Dupont 5 μm Zorbax ODS (4.5 mm i.d. × 25.0 cm). The mobile phase was 0.5% acetic acid and acetonitrile. The concentration of acetonitrile was increased linearly from 3.0% at the time of injection to 9.0% 10.0 min after injection. Chromatography was performed on the same HPLC system as for antipyrine metabolites. Counts under a metabolite peak were expressed as a percentage of the total counts recovered and then as nmol of theophylline converted/mg protein/30 min. The extent to which parent drug was converted to metabolite approximated 10%, whereas more than 90% of the total radioactivity was recovered from the incubations.

Analysis of aminopyrine metabolites. Aminopyrine reactions were terminated by the addition of 0.25 ml of 8.9% $ZnSO_4$. We added 10 μg of acetanilide as a recovery standard. The protein was precipitated by addition of 0.75 ml of saturated barium–borate solution (3:1) as previously described [27]. The sample was centrifuged at 10,000 *g* for 15 min to remove precipitated protein. The aminopyrine metabolites, 4-MAA and 4-AA, were separated using the same column and conditions as described for antipyrine [26].

Quantitation of the metabolite was accomplished by comparison of the u.v. absorbance of 4-aminoantipyrine (4-AA) and 4-methylaminoantipyrine (4-MAA) to the u.v. absorbance of the internal standard. The actual amount of metabolite produced was determined by comparison of the peak area ratio to a standard curve of peak area ratios that was linear from 200 ng to 15 μg/0.5 ml incubation volume. The extent to which parent drug was converted to metabolite approximated 30%.

For all three drugs, the reaction rates are expressed as a percent of control. Control incubations contained an equivalent volume of buffer in place of antibody. Cytochrome P-450 isozymes were determined to be saturated, and inhibition was considered maximal when addition of another 2 mg of antibody protein produced no further decrease in metabolite formation. For most assays, this endpoint occurred at 2 mg MAb protein (a 4:1 ratio of MAb protein to microsomal protein). No more than 10 mg MAb protein was used (a 20:1 ratio of MAb protein to microsomal protein) in any incubation.

Statistical analysis. The data represent the mean ± SD of four independent determinations, each assayed in duplicate. Significant inhibition of a sample as compared to control was determined using Student's *t*-test.

RESULTS

Effects of MAbs 1-7-1 *and* 2-66-3 *on aminopyrine metabolism.* The effects of MAbs 1-7-1 and 2-66-3 on formation of aminopyrine metabolites are shown in Fig. 1. Addition of MAbs to untreated microsomes had no inhibitory effect on formation of aminopyrine metabolites. Both specific MAbs as well as the nonspecific control MAbs stimulated 4-MAA production to levels higher than those in control microsomes. Thus, stimulation of aminopyrine metabolism produced by addition of cytochrome P-450 MAbs to control microsomes was considered to be nonspecific. The enhancement of microsomal enzyme activity by nonspecific proteins such as bovine serum albumin is commonly reported in the literature. Possibly, addition of the MAb protein enhances association of the components of the enzyme system, thereby increasing enzyme efficiency and activity.

Aminopyrine is biotransformed rapidly by sequential demethylation reactions [14, 15]. Each demethylation is thought to be performed by a different cytochrome P-450 isozyme. The first cytochrome P-450 catalyzed reaction is a demethylation to yield 4-MAA. A second demethylation, also mediated by the cytochrome P-450 system, then ensues to form 4-AA. In rat liver, the K_m values for these two sequential reactions are 4 and 20 mM respectively [14, 15].

At 4 mM aminopyrine in PB-induced microsomes, MAbs did not affect the formation of either 4-MAA or 4-AA. At 20 mM aminopyrine, however, addition of MAb 2-66-3 inhibited 4-AA production 55%, increasing the recovery of 4-MAA. MAb 1-7-1 had an almost identical effect, inhibiting 4-AA 54%. These results clearly demonstrate that cytochrome P-450 isozymes active at 20 mM substrate levels are not active at the lower 4 mM substrate concentration. This isozyme class was sensitive to both MAbs.

At 4 mM aminopyrine in 3-MC-induced microsomes, MAb 1-7-1 inhibited production of 4-MAA by 38%. At 20 mM aminopyrine, MAb 1-7-1 did not inhibit 4-MAA production, but instead inhibited 4-AA formation by 25%. This isozyme was not recognised by MAb 2-66-3; therefore, it differs not only from the isozyme(s) responsible for 4-MAA production in untreated and PB-induced microsomes, but also from that responsible for 4-AA production at 20 mM aminopyrine. In 3-MC-induced microsomes, MAb 2-66-3 recognized and inhibited the isozyme responsible for 25% of 4-AA production at 20 mM aminopyrine.

To summarize experiments with aminopyrine, the two MAbs did not affect metabolism by untreated microsomes, demonstrating that metabolism in the latter is performed by cytochrome P-450 isozymes lacking the MAb 1-7-1 and MAb 2-66-3 sensitive epitopes. In contrast, in the induced state, at high aminopyrine concentrations, between 30 and 55% of 4-AA formation arose from isozymes of cyto-

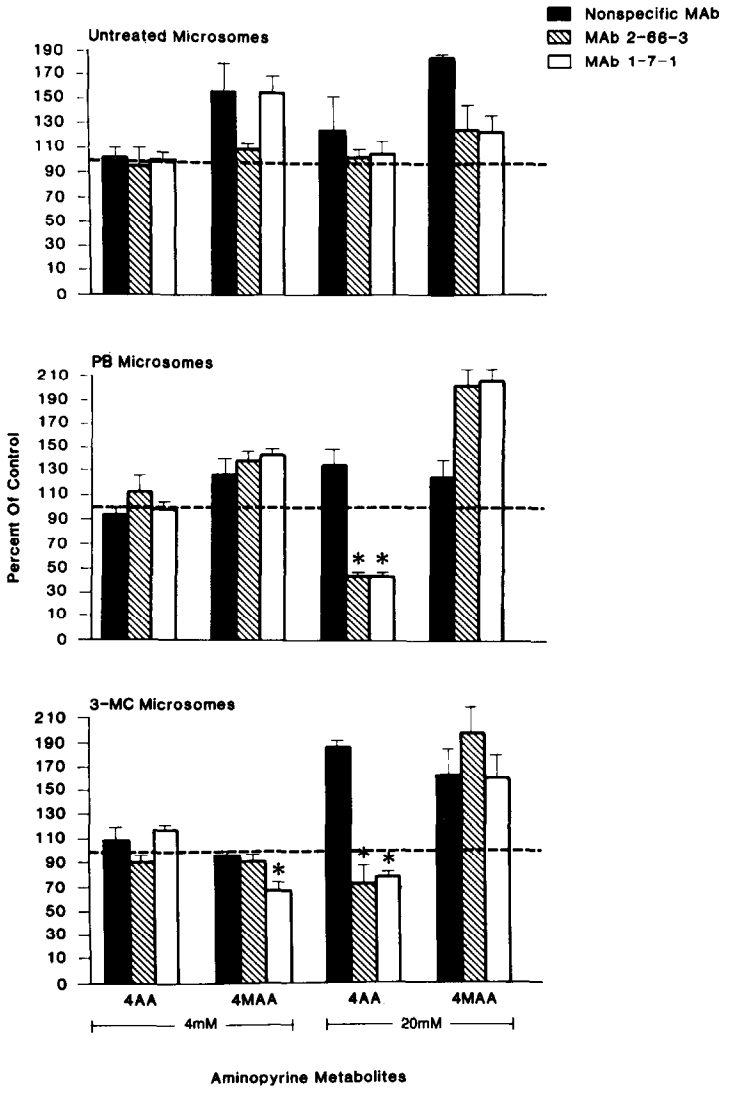

Fig. 1. Effects of two MAbs on aminopyrine metabolism by hepatic microsomes from untreated, PB-induced and 3-MC-induced rats. Data are expressed as percent of control incubation. For untreated microsomes at 4 mM aminopyrine, the control values equivalent to 100% of enzyme activity were: 4-AA, 2.0 ± 0.01 μg, 4-MAA, 0.06 ± 0.01 μg. For untreated microsomes at 20 mM, the control values were: 4-AA, 4.0 ± 0.03 μg; 4-MAA, 3.1 ± 0.02 μg. For PB-induced microsomes at 4 mM aminopyrine, the control values equivalent to 100% of enzyme activity were: 4-AA, 5.4 ± 1.1 μg, 4-MAA, 1.1 ± 0.06 μg. For PB-induced microsomes at 20 mM aminopyrine, the control values were: 4-AA, 9.7 ± 0.06 μg; 4-MAA, 2.1 ± 0.01 μg. For 3-MC-induced microsomes at 4 mM aminopyrine, the control values equivalent to 100% of enzyme activity were: 4-AA, 1.5 ± 0.09 μg; 4-MAA, 0.06 ± 0.05 μg. For 3-MC-induced microsomes at 20 mM aminopyrine, the control values were: 4-AA, 3.8 ± 0.04 μg; 4-MAA, 3.0 ± 0.009 μg. Significant inhibition of metabolite formation ($P < 0.05$) is indicated by an asterisk.

chrome P-450 that contain the MAb-sensitive epitopes and that (1) are not responsible for 4-AA formation at 4 mM aminopyrine and (2) are not involved in 4-MAA formation at either 4 or 20 mM aminopyrine. Thus, the latter is dependent on a P-450 isozyme lacking the epitope for both MAbs.

Effects of MAbs 1-7-1 *and* 2-66-3 *on antipyrine metabolism.* The effects of MAbs 1-7-1 and 2-66-3 on antipyrine metabolism are shown in Fig. 2. In untreated microsomes, antipyrine metabolite formation was unaffected by addition of either MAb 1-7-1 or MAb 2-66-3. Thus, the cytochrome P-450 isozymes responsible for antipyrine metabolism in control microsomes do not contain MAb 1-7-1 or MAb 2-66-3 sensitive epitopes. Previously published *in vivo* and *in vitro* studies suggested involvement of at least three different cytochrome P-450 isozymes in antipyrine metabolism by both uninduced rats and normal human subjects under basal environmental

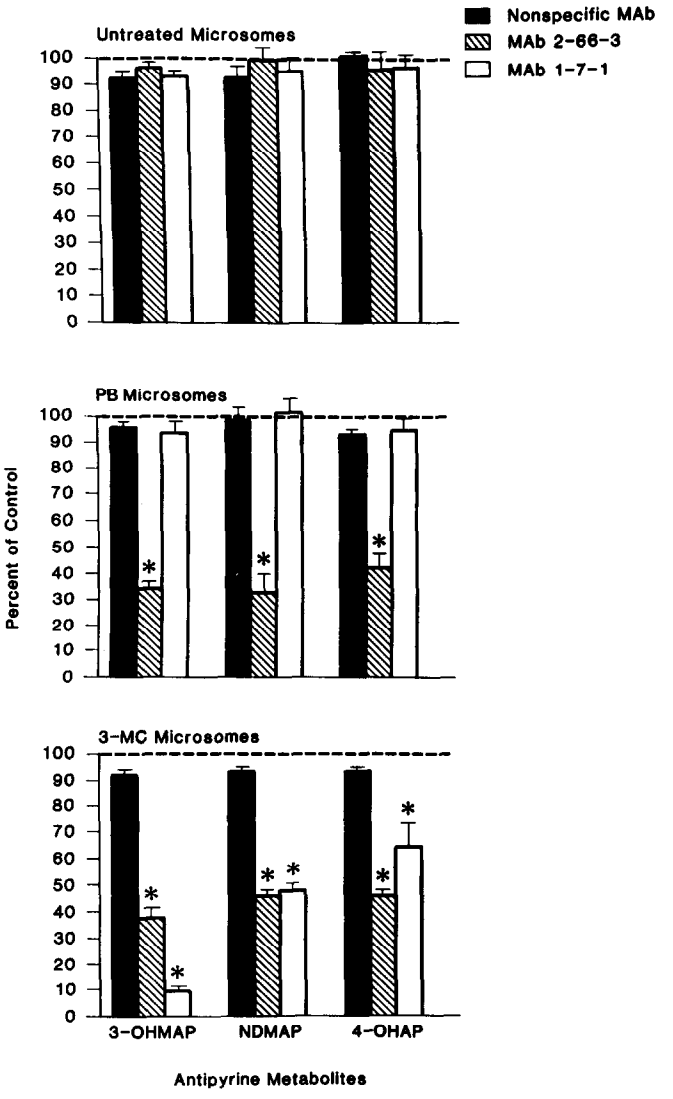

Fig. 2. Effects of two MAbs on antipyrine metabolism by hepatic microsomes from untreated, PB-induced and 3-MC-induced rats. Data are expressed as percent of control incubation. For untreated microsomes, the control values equivalent to 100% of activity were: 3-OHMAP, 14 ± 1.0 nmol; NDMAP, 10 ± 0.2 nmol; 4-OHAP, 8 ± 0.4 nmol. For PB-induced microsomes, the control values equivalent to 100% of activity were: 3-OHMAP, 27 ± 1.3 nmol. NDMAP, 19.5 ± 1.2 nmol; 4-OHAP, 14 ± 0.9 nmol. For 3-MC-induced microsomes, the control values equivalent to 100% of activity were: 3-OHMAP, 12.0 ± 0.8 nmol; NDMAP, 10.5 ± 0.8 nmol; 4-OHAP, 8.0 ± 0.3 nmol. Significant inhibition of metabolite formation ($P < 0.05$) is indicated by an asterisk.

conditions [16, 17, 19, 20]. Since MAbs 1-7-1 and 2-66-3 do not inhibit the constitutive isozymes responsible for antipyrine metabolism, the latter enzymes must be different from those sensitive to MAb 1-7-1 and 2-66-3 in MC- and PB-treated microsomes.

In PB-induced microsomes, MAb 1-7-1 exerted no effect on antipyrine metabolite formation. By contrast, MAb 2-66-3 significantly inhibited production of all three major antipyrine metabolites ($P < 0.01$). Formation of 3-OHMAP was inhibited 65%, whereas formation of NDMAP and 4-OHAP was inhibited 63% and 58% respectively. The pattern of inhibition was similar for each of the three metabolites, suggesting that antipyrine metabolite formation by PB microsomes may be catalyzed by only two forms or classes of cytochrome P-450. The first form or class of isozymes, responsible for approximately 60% of metabolite formation, was sensitive to inhibition by MAb 2-66-3, whereas the second class was uninhibitable. It accounts for the remaining 40% of metabolite production. Within these two classes there are P-450 isozymes capable of catalyzing both demethylation and hydroxylation reactions.

In 3-MC-induced microsomes, results of the addition of MAb 1-7-1 and MAb 2-66-3 suggest participation by at least three distinct classes of P-

450 in formation of 3-OHMAP alone. An isozyme responsible for 61% of 3-OHMAP formation contains epitopes recognized by both MAb 1-7-1 and 2-66-3. A second isozyme or isozyme family containing only the epitope recognized by MAb 1-7-1 is responsible for 26% of 3-OHMAP formation. The remaining 13% of 3-OHMAP was formed by a third isozyme(s) possessing neither epitope and inhibited by neither MAb.

In 3-MC-induced microsomes, MAb 2-66-3 also inhibited NDMAP and 4-OHAP formation to 45% of control, a level similar to that produced by MAb 2-66-3 in PB-induced microsomes. MAb 1-7-1 did not recognize the PB-induced isozyme, but recognized and inhibited the 3-MC-induced isozyme, suggesting that it is, in fact, a different isozymic form(s).

In summary, it appears that a minimum of four isozymes or classes of isozymes with overlapping specificities catalyzed formation of the major antipyrine metabolites by untreated, PB-induced, and 3-MC-induced rat liver microsomes. This estimate may prove conservative, since our results allow for the possibility that under various conditions of induction many more isozymes may participate in antipyrine metabolism.

Effects of MAbs 1-7-1 *and* 2-66-3 *upon theophylline metabolism.* The effects of MAbs 1-7-1 and 2-66-3 on theophylline metabolism are shown in Fig. 3. Unlike aminopyrine and antipyrine whose metabolism was unaffected by addition of MAb, addition of MAb 2-66-3 to untreated microsomes produced a small but significant inhibition (20%) in 1MX formation ($P < 0.05$). This observation is consistent with an earlier report of a small but significant amount of PB-inducible mRNA in uninduced rat liver [28]. Production of 1,3MU, 3MX and a polar unknown metabolite, however, was unaffected by MAb 2-66-3. In untreated microsomes, MAb 1-7-1 did not inhibit formation of any of the major theophylline metabolites.

In untreated microsomes, the cytochrome P-450 isozymes responsible for 20% of 1MX formation may also have been involved in formation of 1,3MU and the unknown polar metabolite by PB-induced microsomes. The patterns of inhibition were similar; the cytochrome P-450 isozyme responsible for 25% of 1,3MU production and 64% of polar unknown metabolite formation was inhibited by MAb 2-66-3 but unaffected by MAb 1-7-1. In contrast, the isozyme that catalyzed 1MX formation in PB-induced microsomes was sensitive to both MAb 2-66-3 and MAb 1-7-1. These results suggest that the isozymic form(s) of cytochrome P-450 responsible for 1MX production in PB-induced microsomes differed from that which catalyzed its formation in untreated microsomes.

In 3-MC-induced microsomes, MAb 1-7-1 inhibited 1,3MU formation by 73%, whereas MAb 2-66-3 did not affect 1,3MU production. This isozyme clearly differed from those responsible for 1,3MU production by either PB-induced or untreated microsomes. In 3-MC microsomes, the isozyme responsible for 50% of 1MX production may have been similar to the isozymic forms that catalyzed 1MX formation by untreated microsomes but different from the isozymic forms in PB-induced microsomes. Both 3MX and the polar unknown were inhibited to a similar extent by MAbs 1-7-1 and 2-66-3. Therefore, in 3-MC microsomes at least four different cytochrome P-450 isozymes appeared to participate in theophylline metabolism.

DISCUSSION

In this study MAbs were utilized as reagents to investigate the principal metabolic pathways of aminopyrine, antipyrine and theophylline. Even though we used only two MAbs, the results suggested more extensive isozymic heterogeneity than previously recognized. If additional antibodies had been employed, even more heterogeneity might have appeared.

MAbs are chemically defined reagents that provide a new approach to investigating both structure and function of the cytochrome P-450 system [1, 3, 5–8]. MAb specificity resides in the ability of a MAb to recognize and bind to a specific epitope on the enzyme surface [1]. If the epitope is unique to a single isozyme of cytochrome P-450, the MAb will recognize and inhibit only that isozyme [1]. If the epitope occurs on more than one isozyme, a MAb specific for that epitope will recognize and bind all isozymes of cytochrome P-450 containing that epitope [1]. Accordingly, biochemically distinguishable isozymes that bear the same epitope to which the MAb is directed will not be differentiated by this approach. MAbs can be used to classify cytochrome P-450 isozymes either as unique forms or as classes defined by their epitopes [1]. Thus, MAbs offer an alternative classification system for, and a fresh insight into, the structure and function of the cytochrome P-450 system [1].

Each MAb used in this study recognizes, inhibits, and immunoprecipitates multiple isozymic forms of cytochrome P-450 [1, 5, 6]. Therefore, reduction of metabolite formation could be due to inhibition by each MAb not of a single isozyme but rather of multiple isozymes bearing the same epitope to which the MAb is directed. Accordingly, for purposes of this discussion, isozyme is used to refer not just to a biochemically pure form of cytochrome P-450, but rather to all such forms that bear an identical epitope inhibitable by a particular MAb. Since our objective was not to investigate the substrate specificity of biochemically purified isozymes, but rather to obtain qualitative information on the extent of isozyme heterogeneity and its functional consequences with respect to individual metabolic pathways, the specificity of each MAb to possibly more than one P-450 isozyme did not prevent pursuit of this goal. Stated otherwise, the MAbs are probes for a specific epitope, not for a chemically pure isozyme [1].

The results of our study permitted classification of each metabolic pathway examined into one of the four groups listed in Table 1. In the first group, pathways were unaffected by the presence of either MAb 1-7-1 or MAb 2-66-3. Both MAbs used in our study were formed in response to cytochrome P-450 isozymes purified from induced rats. Generally, these MAbs failed to recognize isozymes responsible for drug metabolism by untreated microsomes. Thus, the induced cytochrome P-450s that were inhibited

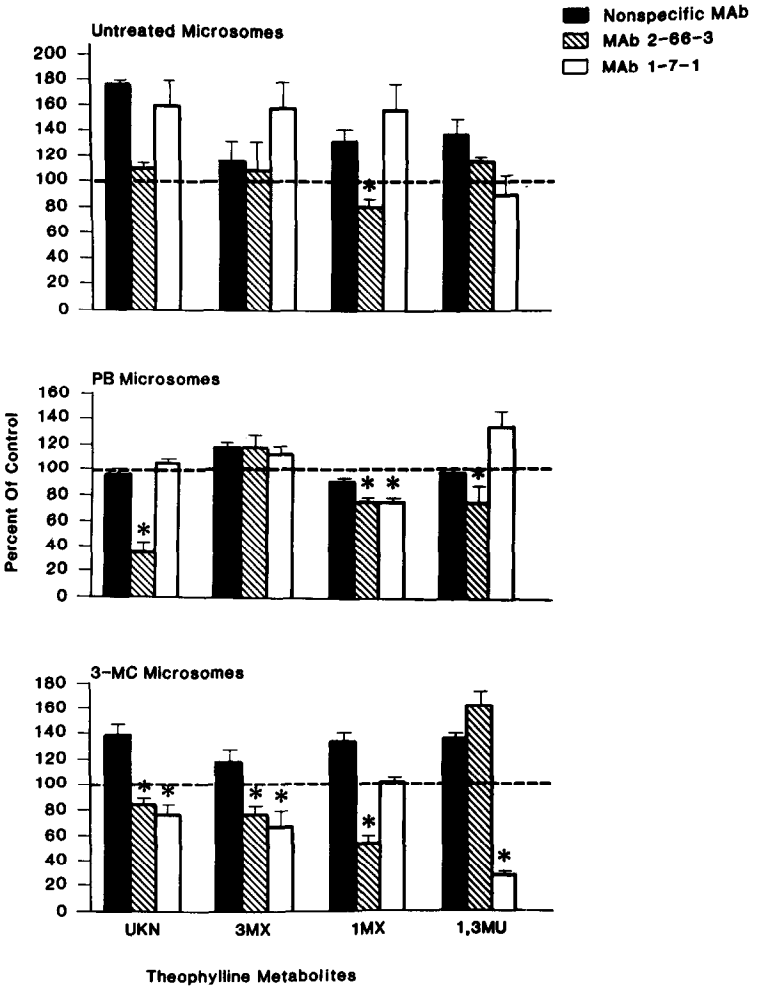

Fig. 3. Effects of two MAbs on theophylline metabolism by hepatic microsomes from untreated, PB-induced and 3-MC-induced rats. Data are expressed as percent of control incubation. For untreated microsomes, the control values equivalent to 100% of enzyme activity were: Ukn, 1.4 ± 0.11 nmol; 3MX, 0.24 ± 0.028 nmol; 1MX, 0.30 ± 0.02 nmol; 1,3MU, 1.0 ± 0.08 nmol. For PB-induced microsomes, the control values equivalent to 100% of enzyme activity were: Ukn, 5.5 ± 0.98 nmol; 3MX, 1.7 ± 0.22 nmol; 1MX, 2.8 ± 0.095 nmol; 1,3MU, 3.8 ± 0.09 nmol. For 3-MC-induced microsomes, the control values equivalent to 100% of enzyme activity were: Ukn, 2.2 ± 0.132 nmol; 3MX, 1 ± 0.7 nmol; 1MX, 1.8 ± 0.132 nmol; 1,3M, 7.2 ± 0.40 nmol. Significant inhibition of metabolite formation ($P < 0.05$) is indicated by an asterisk.

by MAb differ from constitutive isozymes. Previously published studies indicate that induction of the cytochrome P-450 system results in expression of genetically distinct groups of isozymes, each group characteristic of only a specific inducer [7–9]. Our studies show that the cytochrome P-450 isozyme profile from uninduced liver differed from the induced forms that were sensitive to MAbs. These results shed light on the composition and extent of heterogeneity of constitutive isozymes only to the extent that the P-450 isozymes responsible for the metabolism of these drugs did not contain the MAb-sensitive epitopes. In addition, not all isozymes present in induced microsomes were recognized by our two MAbs, such as the isozyme(s) responsible for 3MX production by PB-induced microsomes or for aminopyrine metabolism at 4 mM by either PB- or 3-MC-induced microsomes.

A second group of pathways encompassed isozymes inhibited by addition of MAb 2-66-3 but unaffected by MAb 1-7-1. Examples are isozymes present in PB-induced microsomes responsible for production of approximately 50% of the major antipyrine metabolites.

A third group is comprised of metabolic pathways inhibited by MAb 1-7-1 and unaffected by MAb 2-66-3. Only two pathways are represented: (1) formation of 1,3MU by 3-MC-induced microsomes and (2) at least 35% of 3-OHMAP production by 3-MC-induced microsomes. Formation of these two metabolites represents the major routes of theophylline and antipyrine metabolism not only in 3-

Table 1. Metabolism of aminopyrine, antipyrine, and theophylline: four groups according to MAb response

	Aminopyrine	Antipyrine	Theophylline
Group 1: Pathways uninhibited by either MAb			
Untreated	1. 4-AA (4 mM, 20 mM)	1. 3-OHMAP	1. Polar unknown
	2. 4-MAA (4 mM, 20 mM)	2. NDMAP	2. 3MX
		3. 4-OHAP	3. 1,3MU
PB-induced	1. 4-AA (4 mM)		1. 3MX
	2. 4-MAA (4 mM, 20 mM)		
3-MC-induced	1. 4-AA (4 mM)		
	2. 4-MAA (20 mM)		
Group 2: Pathways inhibited by MAb 2-66-3 only			
Untreated	—		1. 1MX
PB-induced	—	1. 3-OHMAP	1. Polar unknown
		2. NDMAP	2. 1,3MU
		3. 4-OHAP	
3-MC-induced	—	1. 4-OHAP (28%)	1. 1MX
Group 3: Pathways inhibited by MAb 1-7-1 only			
Untreated	—		
PB-induced	—		
3-MC-induced	1. 4-MAA (4 mM)	1. 3-OHMAP (37%)	1. 1,3MU
Group 4: Pathways inhibited by both MAbs			
Untreated	—		
PB-induced	1. 4-AA (20 mM)		1. 1MX
3-MC-induced	1. 4-AA (20 mM)	1. 3-OHMAP (61%)	1. Polar unknown
		2. NDMAP	2. 3MX
		3. 4-OHAP (37%)	

MC-induced microsomes but also in PB-induced and control rat liver microsomes [16, 19, 20].

The final group is composed of isozymes inhibited by both MAb 1-7-1 and MAb 2-66-3. Pathways displaying this type of inhibition occurred for all three drugs: (1) NDMAP formation from antipyrine by 3-MC-induced microsomes; (2) 4-AA production by both 3-MC- and PB-induced microsomes at 20 mM aminopyrine; and (3) production of 1MX from theophylline by PB-induced microsomes. With the exception of the polar unknown metabolite and 4-OHAP formation, each of these pathways represents a demethylation reaction. Apparently, a family of cytochrome P-450 isozymes is capable of catalyzing a broad range of demethylation reactions. This isozyme family contains either a single epitope recognized by both MAbs or two distinct sites, each recognized by a different MAb. Demethylation reactions, however, are not catalyzed solely by this family of cytochrome P-450. Cytochrome P-450 isozymes catalyzing sequential demethylations of aminopyrine at the 4 mM concentration were unrecognized by our MAbs as members of this group. In addition, at least one isozyme remained unaffected by the MAbs we used; it was responsible for the uninhibitable portion of metabolite formation observed for every metabolite examined. Thus, at least two subclasses of isozymes appeared to demethylate antipyrine, aminopyrine and theophylline: one class that was inhibitable by the MAbs used and another class that was uninhibitable.

The picture of the cytochrome P-450 system offered by these studies using only two MAbs, though only partial, is nonetheless one of a complex network of multiple isozymes. It suggests involvement of more isozymes of cytochrome P-450 than previously documented in the metabolism of aminopyrine, antipyrine, and theophylline [15–20]. At least four different isozymes of cytochrome P-450 participated in the *in vitro* metabolism of aminopyrine. At 4 mM aminopyrine, the cytochrome P-450 isozymes responsible for the *in vitro* demethylation of 4-MAA to 4-AA appeared to differ from those that catalyzed this reaction at 20 mM aminopyrine. For antipyrine, at least four cytochrome P-450 isozymes catalyzed formation of the three major metabolites. Also, at least four isozymes appeared to be involved in theophylline metabolism. These estimates of isozyme heterogeneity are conservative; our data are also compatible with direct participation of many more isozymes in the *in vitro* metabolism of aminopyrine, antipyrine and theophylline.

It should be recognized that these results were all obtained under limited *in vitro* conditions. As new methodologies develop, these conclusions need to be tested and validated under the more complex and challenging situations that exist *in vivo*. Our results contain several functional implications for cytochrome P-450 isozymes. For example, metabolism of a drug biotransformed by multiple pathways appears to depend not on only one or a few, but rather on many cytochrome P-450 isozymes, each subject to regulation by different genetic and environmental factors. Multiple families of iso-

zymes provide diverse opportunities for an organism to adjust to changing concentrations of both endogenous and foreign chemicals.

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