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# Induction and Modification of Rat Liver Microsomal Arylamide *N*-Hydroxylase by Various Pretreatments

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SUMMARY

3-Methycholantrene, benzoanthracene, benzo[*e*]pyrene, and pyrene induce *N*-hydroxylase activity and modify the enzyme by increasing its apparent $K_m$. As exemplified by the effect of 3-methylcholanthrene, the polycyclic aromatic hydrocarbons also induce other mixed-function oxidases such as aryl hydrocarbon hydroxylase and the various arylamide *C*-hydroxylases. Acute or chronic pretreatment of rats with acetylaminofluorenes induces *N*-hydroxylase and modifies the enzyme affinity by decreasing its apparent $K_m$. Among the various-position isomers, 4-acetylaminofluorene is completely inactive and 2-acetylaminofluorene is the most potent. Its effect is both dose- and time-dependent, and it seems to be specific for *N*-hydroxylase, the same pretreatment having no effect on arylhydrocarbon hydroxylase or arylamide *C*-hydroxylases. After simultaneous treatment of rats with 3-methylcholanthrene and 2-acetylaminofluorene, even though *N*-hydroxylase activity as measured on hepatic microsomes *in vitro* is significantly induced, the urinary excretion of *N*-hydroxy-2-acetylaminofluorene is significantly reduced over a 24-hr period. This observation is discussed in relationship to the well-known inhibitory effect of 3-methylcholanthrene on the hepatocarcinogenicity of 2-acetylaminofluorene.

## INTRODUCTION

Microsomal arylamide *N*-hydroxylase is a cytochrome $P_{448}$-dependent (1) enzyme which catalyzes the first step in the metabolic activation of 2-AAF[1] (2). Like most of the cytochrome $P_{448}$-dependent mixed-function oxidases, it is largely inducible by pretreatment with 3-MC (3, 4) but not by phenobarbital (5). It is also inducible by acute and chronic pretreatment with 2-AAF (5–7). Although the induction of other mixed-function oxidase enzymes such as benzo[*a*]pyrene hydroxylase (8, 9), aniline hydroxylase (9, 10), and aminopyrine demethylase (9) has been studied, the induction of arylamide *N*-hydroxylase is less well documented. By applying a sensitive and highly specific gas-liquid chromatographic method (11, 12) developed in this laboratory for the quantitative assay of N-OH-2-AAF, we have analyzed the biochemical properties of rat liver microsomal arylamide *N*-hydroxylase after various pretreatments that could potentially induce the enzyme.

The present report is a presentation and discussion of those data.

[1] The abbreviations used are: 1-, 2-, 3-, or 4-AAF, 1-, 2-, 3-, or 4-acetylaminofluorene; 3-MC: 3-methylcholanthrene; N-OH-2-AAF, *N*-hydroxy-2-acetylaminofluorene; N-OH-4-AABP, *N*-hydroxy-4-acetylaminobiphenyl; $C_5$- or $C_7$-2-AAF, *N*-(5- or 7-)-hydroxy-2-acetylaminofluorene; PAH, polycyclic aromatic hydrocarbon.

## MATERIALS AND METHODS

7,8-Benzoflavone was obtained from Eastman Organic Chemicals (Rochester, N. Y.). 3-MC and chloroacetic anhydride were obtained from Fluka AG Chemische Fabrik, CH (Buchs, Switzerland); 2-AAF, benzanthracene, benzo[*e*]pyrene, benzo[*a*]pyrene, and pyrene from Aldrich-Europe (Beerse, Belgium); and 4-AAF from Suchard (München, Germany). All chemicals were of the purest grade available. β-Glucuronidase/arylsulfatase, NADH, and glucose 6-phosphate were obtained from Sigma Chemical Company (St. Louis, Mo.); $NADP^+$ and glucose 6-phosphate dehydrogenase were obtained from Boehringer GmbH Biochemicals (Mannheim, Germany); *N*-(5- and 7-) OH-2-AAF were kindly provided by Dr. J. N. Keith, National Cancer Institute, Chemical Depository, ITT Research Institute (Chicago, Ill.), and 1- and 3-AAF were kindly provided by Dr. H. R. Gutmann, Veterans Administration Hospital and Department of Biochemistry, University of Minnesota (Minneapolis, Minn.).

All of the other reagents of analytical grade were purchased from Merck (Darmstadt, Germany). N-OH-2-AFF and N-OH-4-AABP were synthetized and purified as previously described (13).

### *Animals and Treatments*

Male Wistar rats weighing 200–250 g were obtained from the Proefdierencentrum, Katolieke Universiteit te

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Leuven (Leuven, Belgium). They were fed a semisynthetic diet (AO3, UAR, Epinay-sur-Orge, France) and water ad libitum. Food was withdrawn 24 hr before sacrifice by decapitation.

The various pretreatments of the rats were as follows: (a) one i.p. injection of 2-AAF (5, 10, 20, or 40 mg/kg body weight in corn oil) 24 hr prior to sacrifice; (b) two i.p. injections of 3-MC, benzanthracene, benzo[*e*]pyrene, or pyrene (40 mg/kg body weight in corn oil) 48 hr and 24 hr prior to sacrifice; (c) one i.p. injection of 1,3- or 4-AAF (10 mg/kg body weight in corn oil 24 hr prior to sacrifice; (d) two i.p. injections of phenobarbital (75 mg/kg body weight in 0.9% isotonic saline, aqueous solution) 48 hr and 24 hr prior to sacrifice; and (e) one i.p. injection of 3-MC (40 mg/kg body weight in corn oil) together with one oral administration of 2-AAF (5 mg/kg body weight in corn oil) 24 hr prior to sacrifice.

For analysis of chronic effects, the rats were fed a diet containing 0.03% 2-AAF (the diet was prepared by UAR, Epinay-sur-Orge, France) for one, three, or five cycles. Each cycle consisted of 3 weeks of 2-AAF feeding followed by 1 week of normal diet. The rats were killed 4, 12, or 20 weeks after the beginning of treatment.

### *Liver Cell Fractionation*

The rat liver microsomes were prepared according to the method of de Duve, as described by Amar-Costesec *et al.* (14). The protein content of the microsomal fraction was determined by the method of Lowry *et al.* (15), with crystalline bovine serum albumin as a standard.

### *Gas Chromatography*

A PYE 104 isothermal oven equipped with a Chrompack 9000 solid injector for the capillary column, a WCOT column (12.5 m × 0.5 mm inner diameter) coated with SE 52, and a PYE Unicam Model 795012 electron-capture detector were used as reported previously (12).

### *Assays of 2-AAF Metabolites*

*N-OH-2-AAF.* The N-OH-2-AAF formed after incubation of 2-AAF in the presence of microsomes was assayed by applying the electron-capture gas chromatographic method previously described (11), and further adapted to capillary gas chromatography (12).

*$C_5$- and $C_7$-Hydroxy Metabolites.* The $C_5$- and $C_7$-hydroxy metabolites, either formed by incubating 2-AAF in the presence of liver microsomes or formed in urine, were assayed by electron-capture gas chromatography after derivatization by heptafluorobutyrile-imidazole as reported recently (16).

The final concentration of the substrate in the incubation media varied from 0.25 to 5 μM in a final volume of 3.35 ml. In all cases, incubations were carried out under strictly defined Michaelis-Menten conditions with regard to time and protein linearities, pH optimum, and substrate-to-enzyme concentration ratio.

The incubation media contained 20 mM phosphate buffer adjusted to the pH optimum; 8.7 mM glucose 6-phosphate, 0.38 mM $NADP^+$, 0.56 mM NADH, and 0.6 μl of glucose 6-phosphate dehydrogenase (350 units/ml) were preincubated in a capped tube for 15 min at 37° in a shaking bath. The $C_5$-, $C_7$-, and *N*-hydroxylation reactions were then initiated by adding 0.25 ml of microsomal protein suspension (0.06–0.3 mg/ml) and the various 2-AAF concentrations dissolved in 0.05 ml of methanol, an amount which was shown not to influence the 2-AAF hydroxylases. Urine from the rats was collected over a 24-hr period in containers cooled in solid $CO_2$/acetone.

The $C_5$-, $C_7$-, and *N*-hydroxy metabolites of 2-AAF excreted in the urine were evaluated as follows: 0.05 ml of urine was diluted to 2 ml with acetate buffer (pH 5.0). Conjugates were hydrolyzed by incubation in the presence of β-glucuronidase-arylsulfatase (800 units/ml) for 14 hr at 37°. An appropriate amount of either N-OH-4-AABP or 1,6-dibromonaphthol as internal standard for the *N*-hydroxy and $C_5$- and $C_7$-hydroxy metabolites, respectively, was added. The metabolites were then extracted, derivatized, and quantified by electron-capture gas chromatography as reported elsewhere (12, 16).

### *Benzo[a]pyrene Hydroxylase Assay*

Liver microsomal benzo[*a*]pyrene hydroxylase activity was measured by applying the spectrofluorimetric assay of Dhenen *et al.* (17), with slight modifications as previously reported (18).

### *Statistical Analysis*

The program of Cleland (19) as modified and completed by Cumps (20) was applied for quantitative estimation of enzymatic parameters. Each value reported here represents the mean ± standard deviation computerized from the enzyme velocities at six concentrations of the substrate using at least three different preparations of liver microsomes.

## RESULTS

### *Effects of Various Pretreatments in Vivo on Enzymatic Properties of Rat Liver Microsomal Arylamide N-Hydroxylase*

*Phenobarbital.* Phenobarbital is a classic inducer of the cytochrome $P_{450}$ (type *b*)-dependent monooxygenases (9). Pretreatment of rats with that drug did not have a significant effect on the enzymatic properties of liver microsomal arylamide *N*-hydroxylase as measured by time or protein linearity and apparent $K_m$. However, it did induce the $V_m$ (50% increase) slightly but significantly ($p < 0.05$) and caused a shift in the optimal pH value from 7.6 to 7.0 (Fig. 1).

*PAH.* 3-MC and other PAHs induced cytochrome $P_{448}$ ($P_{450}$ type *c*)-dependent mixed-function oxidase activities (21). Among the various PAHs, 3-MC, benzanthracene, benzo[*e*]pyrene, and pyrene were tested for their effects *in vivo* on rat liver microsomal arylamide *N*-hydroxylase. All significantly ($p < 0.05$) induced that enzyme, but the potency of their effects was variable, pyrene and benzo[*e*]pyrene being much less potent than 3-MC or benzanthracene (Fig. 1). The ratios of the induced over control activity were 3.5 or 6 for the former and 30 or 40 for the latter, respectively.

With 3-MC used as model compound in that series, it was also shown that PAH markedly affected the enzymatic properties of the enzymes by shortening both time and protein linearities and by shifting the optimal pH

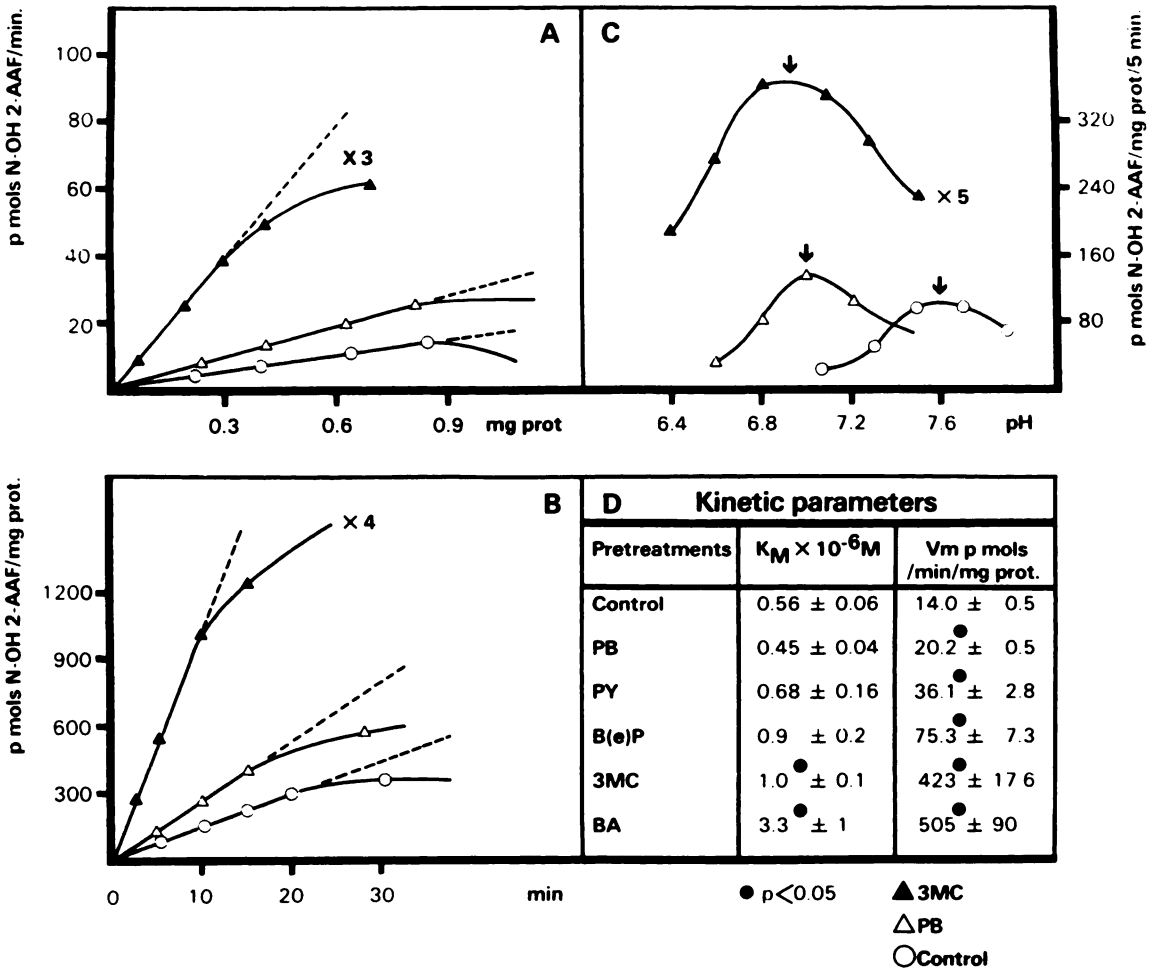


FIG. 1. *Effects of pyrene (PY), phenobarbital (PB), benzo[e]pyrene [B(e)P], 3-MC, and benzanthracene (BA) on the enzymatic properties of rat liver microsomal arylamide N-hydroxylase*

A. Effect of microsomal protein concentration on enzyme activity measured at 5 μM 2-AAF and optimal pH (cf. C).

B. Effect of time of incubation on enzyme activity measured at 5 μM 2-AAF, 0.06–0.3 mg of protein/ml, and optimal pH (cf. C).

C. Effect of pH of incubation on enzyme activity measured at 5 μM 2-AAF and 0.06–0.3 mg of protein/ml.

D. Kinetic parameters. Values were computerized as indicated under Materials and Methods.

value from 7.6 to 7.0. Pretreatment of the rats with the four PAHs tested also modified the apparent $K_m$ of the enzyme, which increased 1.5–2.0 times after pyrene, benzo[*e*]pyrene, or 3-MC and 6.0 times after benzanthracene. As compared with the control value, however, only the effects of 3-MC and benzanthracene were statistically significantly different ($p < 0.05$).

*Fluorene derivatives.* Previously published reports (22) have indicated that pretreatment of rats with 2-AAF induced liver microsomal *N*-hydroxylation without increasing the content of cytochrome P-450 or P-448. The present data (Fig. 2) confirm that such treatment does indeed induce *N*-hydroxylase activity, here by a factor of 3.5, 24 hr after a single dose of 2-AAF, 10 mg/kg. The induction by the position isomer 1,3- or 4-AAF, even though statistically significant ($p < 0.05$), corresponded only to a 25% or 45% increase. As compared with control microsomes, neither 2-AAF nor 4-AAF pretreatment caused changes in the time and protein linearities of *N*-hydroxylase activity. However, the microsomal enzyme of 2-AAF-pretreated rats showed a shift in the optimal pH value from 7.6 to 7.2. The pretreatment also increased the enzyme affinity as shown by the significant ($p < 0.01$) decreases (3-fold) in the value of the apparent $K_m$. The isomer in position 4, which is known not to be carcinogenic (23), did not produce such a qualitative modification of the microsomal enzyme, whereas the 3 and 1 isomers, which are considered to be weak carcinogens (23, 24) also modified the apparent $K_m$ value by reducing it to 57% and 41% of its control value, respectively ($p < 0.05$). However, the $K_m$ value of the liver microsomal *N*-hydroxylase of 2-AAF-pretreated rats was statistically different ($p < 0.05$) from that of the enzyme prepared from 1- or 3-AAF-pretreated rats. The modifying effect of pretreatment with 2-AAF was thus statistically ($p < 0.05$) significantly more pronounced than that of pretreatment with 1- or 3-AAF.

### *Comparative Effects of Pretreatment with Phenobarbital, 3-MC, and 2-AAF on Liver Microsomal Arylhydrocarbon Hydroxylase and Arylamide N-Hydroxylase*

Both arylhydrocarbon (benzo[*a*]pyrene) hydroxylase and arylamide (2-AAF) *N*-hydroxylase are considered

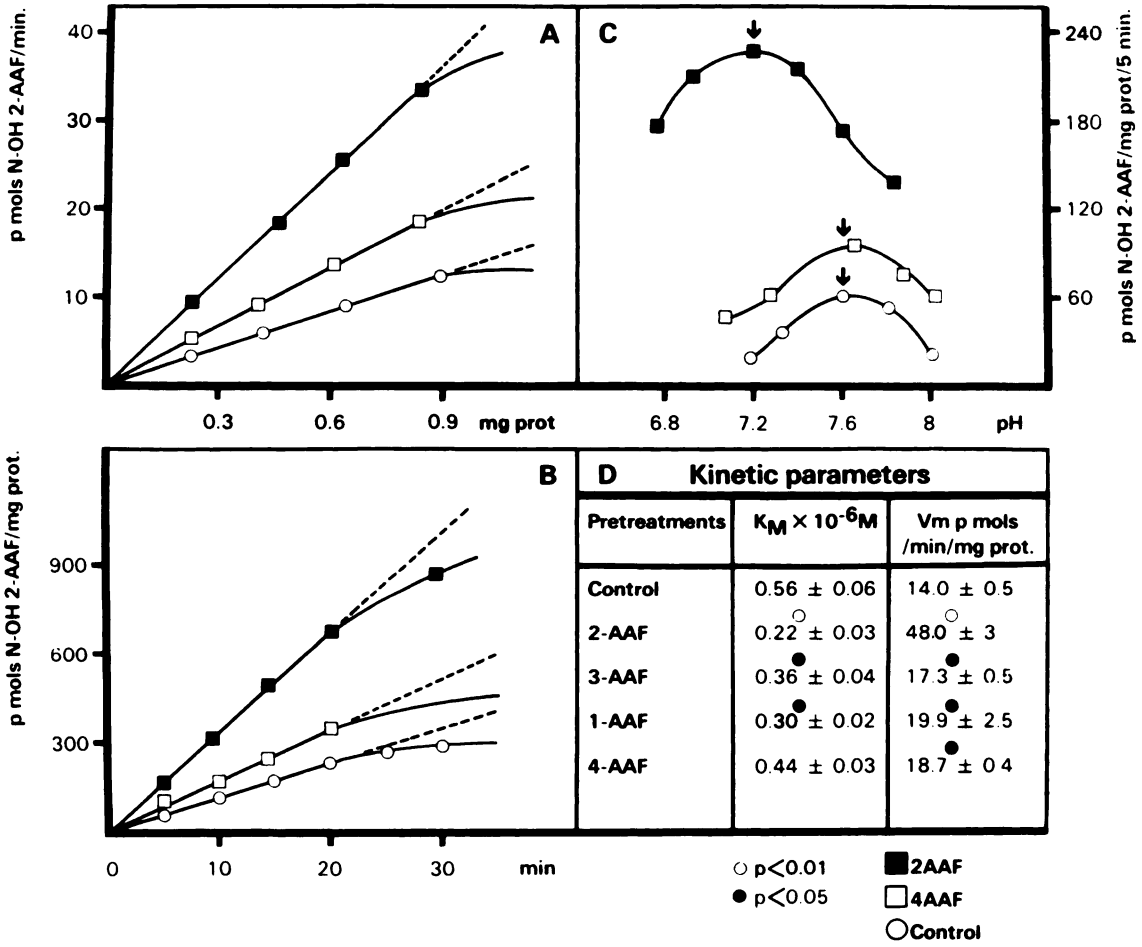


FIG. 2. *Effects of the various-position isomers of AAF on enzymatic properties of rat liver microsomal arylamide N-hydroxylase*
A. Effect of microsomal protein concentration on enzyme activity measured at 5 μM 2-AAF.
B. Effect of time of incubation on enzyme activity measured at 5 μM 2-AAF and 0.06–0.3 mg of protein/ml.
C. Effect of pH of incubation on enzyme activity measured at 5 μM 2-AAF and 0.06–0.3 mg of protein/ml.
D. Kinetic parameters. Values were computerized as indicated under Materials and Methods.

cytochrome P-448 (type *c*)-dependent mixed-function oxidases (21). The data presented show how pretreatment of rats with phenobarbital, 3-MC (as a model PAH), or 2-AAF affects the enzymatic properties of liver microsomal *N*-hydroxylase in different ways.

It was of interest to compare the effects of those three treatments on two microsomal enzymatic activities, namely benzo[*a*]pyrene hydroxylase and 2-AAF *N*-hydroxylase as measured *in vitro* under strictly defined Michaelis-Menten conditions. As shown in Fig. 3, phenobarbital, which induces cytochrome $P_{450}$ (type *b*)-dependent monooxygenase, did not have a significant effect on the enzymatic properties of hydrocarbon hydroxylase. After 3-MC pretreatment, the apparent $K_m$ of benzo[*a*] pyrene hydroxylase was significantly ($p < 0.01$) modified (more than 10-fold decrease) and its $V_m$ was induced more than 3 times, whereas after 2-AAF pretreatment (10 mg/kg) none of those parameters was significantly changed. Those effects are thus clearly different from those on 2-AAF *N*-hydroxylase, for which 3-MC caused a very large induction (30-fold increase in $V_m$) together with a significant increase in apparent $K_m$ (2 times). 2-AAF induced the enzyme activity by only a factor of 3.5 while decreasing the apparent $K_m$ to one-third of its control value.

### *Dose and Time Effect Relationship of In Vivo Pretreatment with 2-AAF on Kinetic Parameters of Rat Liver Microsomal N-Hydroxylase*

Since 2-AAF appeared to cause specific changes in the enzymatic properties of microsomal arylamide *N*-hydroxylase, the conditions of that effect were further analyzed. Figure 4 demonstrates that the inducing effect on $V_m$ was directly related to the dose of 2-AAF, which was between 5 and 40 mg/kg body weight injected i.p. 24 hr before sacrifice. All of the $V_m$ values were significantly ($p < 0.01$) different from the control value.

Values measured after pretreatment with 20 or 40 mg/kg were significantly different from values obtained after a dose of 5 or 10 mg/kg. However, there were no statistically significant differences between the increase in $V_m$ induced by doses of 5 and 10 or 20 and 40 mg/kg, respectively. The same doses of 2-AAF also modified the *N*-hydroxylase by significantly reducing its apparent $K_m$ value. The differences between the decrease after 5, 10, or 20 mg/kg were not statistically significant. However,

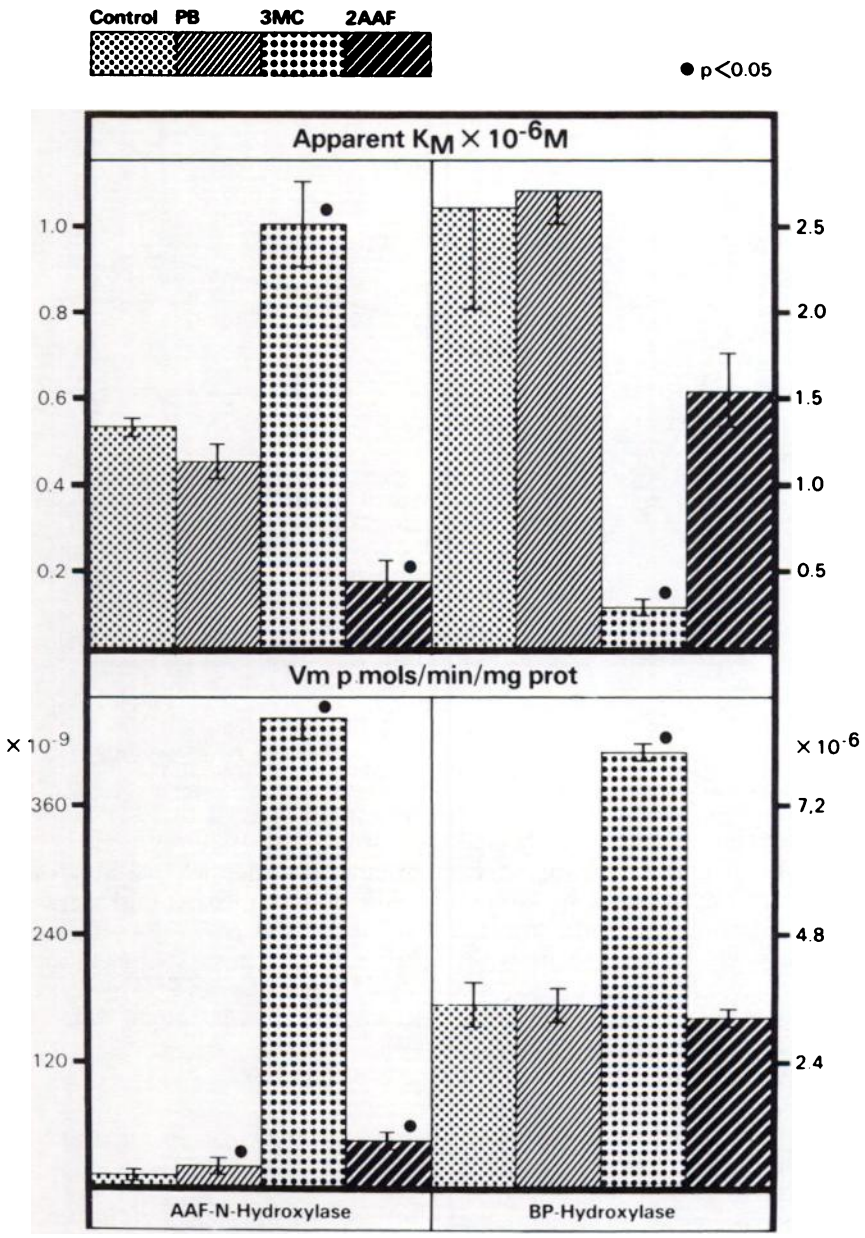


FIG. 3. *Effects of phenobarbital (PB), 3-MC, and 2-AAF on the kinetic parameters of benzo[a]pyrene hydroxylase and 2-AAF N-hydroxylase* The apparent $K_m$ and $V_m$ values are computerized as indicated under Materials and Methods.

there was a slight difference between the effect obtained with doses of 10 and 40 mg/kg.

The modifying effect on the apparent $K_m$ was already apparent and statistically significant ($p < 0.01$) 9 hr after one i.p. injection of 2-AAF (5 mg/kg) and it lasted for at least 72 hr. The inducing effect on $V_m$ was also statistically significant ($p < 0.05$) 9 hr after injection of 2-AAF; it reached its maximal value after 24 hr and was no longer present at 72 hr (Fig. 5).

Chronic feeding of rats with 2-AAF (0.03% in a standard diet) also markedly modified and induced the microsomal arylamide *N*-hydroxylase (Fig. 6). The $V_m$ increased progressively with duration of treatment, reaching a maximal value of 120 pmoles of N-OH-2-AAF formed per minute per milligram of protein (±10-fold induction) after 12 weeks, and decreasing thereafter to a 4-fold increase after 20 weeks. During the same period the apparent $K_m$ value decreased significantly ($p < 0.01$) to a minimal value which was reached after 4 weeks of feeding and lasted at least to 12 weeks. However, 8 weeks thereafter it was back to the normal value of 0.5–$10^{-6}$ M.

### *Effect of Combined Treatments (3-MC + 2-AAF) on Kinetic Parameters of Rat Liver Microsomal C- and N-Hydroxylases*

Both 3-MC and 2-AAF injected i.p. induced and modified rat liver microsomal arylamide *N*-hydroxylase as measured 24 hr after injection. However, their effects were not identical. It was thus of interest to analyze the effect of their sequential or simultaneous administration.

As compared with control values, 3-MC injected i.p. 24 hr before or simultaneously with an oral dose of 2-AAF

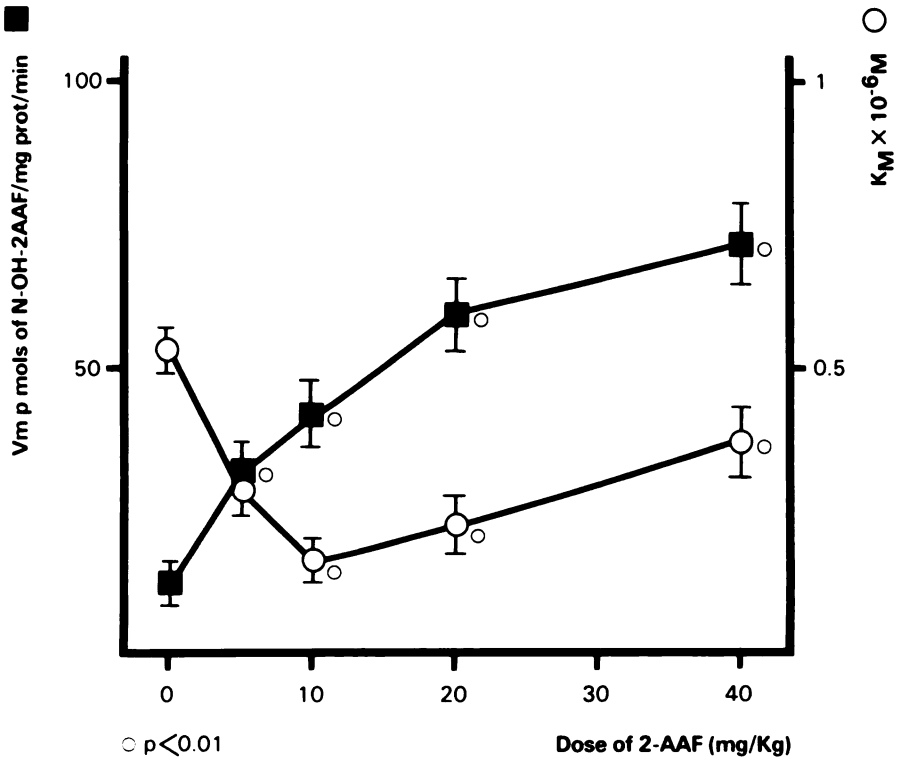


FIG. 4. *Effects of doses of 2-AAF injected i.p. on the kinetic parameters of rat liver microsomal arylamide N-hydroxylase*
The apparent $K_m$ and $V_m$ values are computerized as indicated under Materials and Methods.

largely induced the $V_m$ (Table 1). Those inducing effects were identical with that which appeared 24 hr after 3-MC treatment only (cf. Fig. 1). Similarly, 3-MC injected 24 hr before or simultaneously with 2-AAF significantly increased the $K_m$ value of the *N*-hydroxylating enzyme. However, this modifying effect in both cases was more pronounced than when 3-MC was injected alone (4- to 5-fold compared with 2-fold increase).

Table 2 compares the effects of 2-AAF, 3-MC, and 3-MC + 2-AAF given 24 hr before sacrifice on liver microsomal 2-AAF $C_5$- and $C_7$-hydroxylase activities. Pretreatment with 2-AAF alone did not induce those enzyme

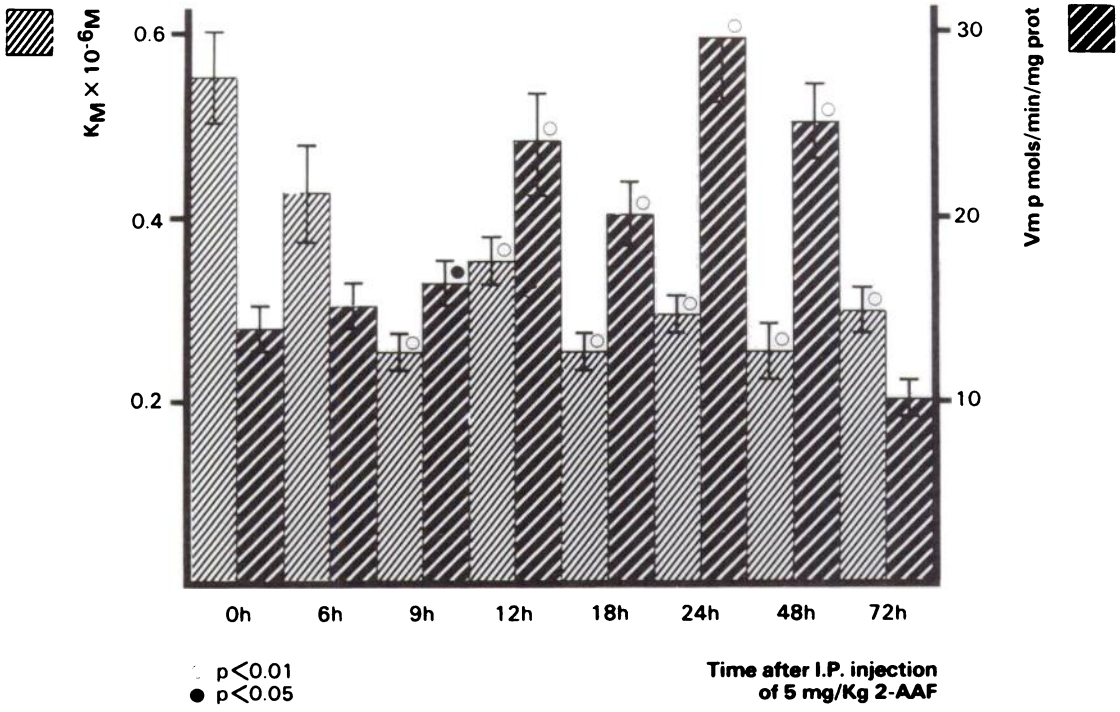


FIG. 5. *Effects of time on induction and modification of rat liver arylamide N-hydroxylase activity by one i.p. injection of 2-AAF (5 mg/kg)*
The apparent $K_m$ and $V_m$ values are computerized as indicated under Materials and Methods.

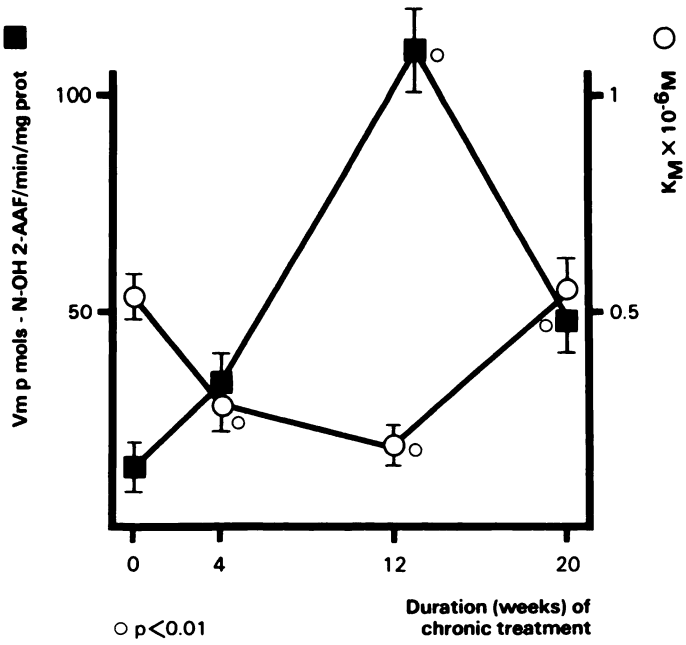


FIG. 6. *Effects of chronic feeding of 2-AAF (0.03%) on the kinetic parameters of rat liver microsomal arylamide N-hydroxylase*

The apparent $K_m$ and $V_m$ values are computerized as indicated under Materials and Methods.

activities; it even lowered the activity of $C_5$-hydroxylase. In contrast, pretreatment with 3-MC alone induced both $C_5$- and $C_7$-hydroxylases by factors of 17 and 3, respectively. When given simultaneously with 2-AAF, the inducing effect of 3-MC was reinforced, $C_5$- and $C_7$-hydroxylations being multiplied by 28 and 7.5, respectively.

### *Effect of 2-AAF and 3-MC + 2-AAF Pretreatment of Rats on Urinary Excretion of $C_5$-, $C_7$-, and N-Hydroxy Metabolites of 2-AAF*

All of the previous results concerned the measurement *in vitro* of rat liver microsomal 2-AAF and *N*- and *C*-hydroxylases, the activities of which were analyzed under strictly defined conditions of both protein and time linearity.

Figure 7 reports data on the 24-hr urinary excretion of $C_5$- and $C_7$-hydroxylations and N-OH-2-AAF after the i.p. injection of 2-AAF and 3-MC plus the simultaneous oral administration of 2-AAF or 3-MC and the oral administration of 2-AAF 24 hr later. Simultaneous or previous treatment with 3-MC reduced the excretion of N- and $C_7$-OH-2-AAF without significantly modifying the elimination of the $C_5$-OH metabolite. However, the urinary excretion of the ring-hydroxylated products was at least 250 times higher than that of the N-OH derivative.

TABLE 1

*Influence of in vivo sequential or simultaneous administration of 3-MC + 2-AAF on the kinetic parameters of rat liver microsomal arylamide N-hydroxylase*

The concentration of 2-AAF varied from 0.25 to 5.0 μM. The values of the parameters are computerized means ± standard error.

| | Pretreatment | | |
|---|---|---|---|
| | Control | 3-MC (40 mg/kg) at 48 hr + 2-AAF (5 mg/kg) at 24 hr | 3-MC (40 mg/kg) at 24 hr + 2-AAF (5 mg/kg) at 24 hr |
| $V_m$ (pmoles N-OH-2-AAF/mg of protein/min) | 13.9 ± 0.44 | 428 ± 20[a] | 458 ± 52[a] |
| $K_m$ ($\times 10^{-6}$ M 2-AAF) | 0.53 ± 0.02 | 2.3 ± 0.3[a] | 2.5 ± 0.5[a] |

[a] Value significantly different ($p < 0.01$).

## DISCUSSION

Acute pretreatment of the rat with PAH affects the enzymatic properties of the liver mixed-function oxidases quite differently from similar pretreatment with the fluorene derivatives. 3-MC and the various PAHs tested in the present work (benzo[*a*]pyrene, pyrene, and benzanthracene) induce *N*-hydroxylase activity and modify the enzyme by increasing its apparent $K_m$. Those effects are most probably a consequence of the well-known inducing capacity of those chemicals on multiple microsomal enzymatic activities, most of which are directly related to the concentration of cytochrome $P_{448}$ ($P_{450}$ type *c*) (21). As previously suggested (25), the *in vitro* increase in apparent $K_m$ could be an indirect effect due to competitive inhibition of the *N*-hydroxylase by the C-OH metabolites since, at least after 3-MC (3) and benzanthracene,[2] those metabolic pathways (monooxygenase and transoxygenase) are significantly induced. However, 3-MC was shown to be a very potent competitive inhibitor of rat liver microsomal arylamide *N*-hydroxylase (25). It could also be possible that the increase in $K_m$ after 3-MC or benzanthracene treatment is due to residual PAH in the microsomes. However, the fact that, after the same treatment (3-MC), the $K_m$ of hamster liver microsomal arylamide *N*-hydroxylase is significantly reduced (25) precludes such an explanation.

To confirm the multiplicity of the induction by 3-MC, microsomal benzo[*a*]pyrene hydroxylase was also enzymatically analyzed, and this showed the well-documented (18–26) increased $V_m$ and decreased apparent $K_m$.

Many reports in the literature have already demonstrated that i.p. injection of 3-MC to rats induces liver microsomal *N*-hydroxylase by a factor of 3–7 (3, 6, 21). We have already reported (4) that under our experimental conditions 3-MC induces the same enzyme activity by a factor of 30. As shown in this publication, 3-MC pretreatment significantly shortens both time and protein linearities and reduces the optimal pH. Measuring the initial velocity under optimal experimental conditions probably explains why such a large inducing effect of 3-MC could be demonstrated. Moreover, to the best of our knowledge, this effect is the largest which has been reported for an induction by 3-MC of microsomal cytochrome $P_{448}$ ($P_{450}$ type *c*)-dependent mixed-function oxidase.

As compared with 3-MC, benzanthracene appears to act as a potent inducer and modifier of microsomal *N*-hydroxylase activity. Its modifying effect on the $K_m$ is even more pronounced (6-fold increase) than that of 3-MC (2-fold increase). This could indicate that benzanthracene is a more potent inducer of *C*-hydroxylation and N-OH-2-AAF transoxygenase than is 3-MC. Such data are in agreement with the previously reported in-

[2] M. Batardy-Grégoire, personal communication.

TABLE 2

*Influence of in vivo administration of 2-AAF, 3-MC or 3-MC + 2-AAF on activities of liver microsomal 2-AAF $C_5$- and $C_7$-hydroxylase*

The values are means ± standard error of at least three different determinations. The concentration of 2-AAF was 7 μM. The incubation medium contained 0.07 mg or 0.01 mg of microsomal proteins per milliliter for control or 2-AAF-treated and 3-MC-treated rats, respectively. The incubation time was 10 min for microsomes from control rats and 2-AAF-treated rats and 5 min for microsomes from 3-MC-treated rats.

| | Control | 2-AAF (10 mg/kg) | 3-MC (40 mg/kg) | 3-MC 40 (mg/kg) + 2-AAF (5 mg/kg) |
|---|---|---|---|---|
| $C_5$-OH-2-AAF (pmoles/min/mg of protein) | 30 ± 1.5 | 22 ± 0.6[a] | 352 ± 11[a] | 787 ± 37[a] |
| $C_7$-OH-2-AAF (pmoles/min/mg of protein) | 112 ± 5 | 93 ± 15 | 511 ± 12[a] | 946 ± 23[a] |

[a] Value significantly different ($p < 0.01$).

formation showing that 2,3-benzanthracene was more effective than 3-MC in inducing aryl hydrocarbon hydroxylase activity (27).

Another characteristic of PAH pretreatment is that it produces a concomitant increase in both $V_m$ and apparent $K_m$ (cf. Fig. 1). Davies *et al.* (28) have suggested that such changes in the enzymatic properties of microsomal enzymes could reflect a situation in which the rates of reaction become diffusion-limited. They have proposed the following equation to express such a correlation: $V_m/2K$ (diffusion) = $K_m$ (apparent) − $K_m$ (true) (28). That equation was applied to the data reported in Fig. 1 by assuming that the $K_m$ of control microsomes = $K_m$ (true), an assumption which probably still overestimated the value. The calculated values of $K$ (diffusion) are then 120, 104, and 90 for microsomes from animals pretreated with pyrene, benzo[*e*]pyrene, and benzanthracene and 480 for microsomes from 3-MC-pretreated rats. As compared with other PAHs, it could thus be concluded that 3-MC induces specific changes in the microsomal *N*-hydroxylase, but since the validity of that equation has never been tested and since the enzymatic activity for which it was developed (ethylmorphine *N*-demethylase) is quite different from *N*-hydroxylase, it is difficult to comment further on the significance of that finding. Moreover, if a peculiar effect of 3-MC on the microsomal system cannot be excluded *a priori*, it is highly improbable that it should correspond to changes in the diffusion process. With the exception of immobilized enzymes, there have been few reported cases for which enzyme reactions occur at diffusion-controlled rates (29).

Malejka-Giganti and Ritter (22) recently demonstrated that acute 2-AAF pretreatment of rats induces *N*-hydroxylase activity. We have also published such results (5). As shown in the present report, acute pretreatment of rats with acetylaminofluorene induces *N*-hydroxylase and modifies the enzyme affinity by decreasing the apparent $K_m$. Among the various-position isomers, 4-AAF is completely inactive whereas 2-AAF is the most potent. The effect of 2-AAF on the $V_m$ is not necessarily paralleled by its effect on the $K_m$ as shown both by the time- and dose-effect relationships. In contrast to PAH, 2-AAF seems to affect selectively the monooxygenase which catalyzes its *N*-hydoxylation without changing other enzymatic activities or the nature or the amount of the cytochrome (6). The absence of induction and modification of microsomal benzo[*a*]pyrene hydroxylase is another argument for that selectivity. Moreover, 2-AAF pretreatment does not induce microsomal $C_5$- or $C_7$-hydroxylation. Chronic feeding of rats with 0.03% 2-AAF for up to 12 weeks similarly induces and modifies liver microsomal arylamide *N*-hydroxylase. Previous reports

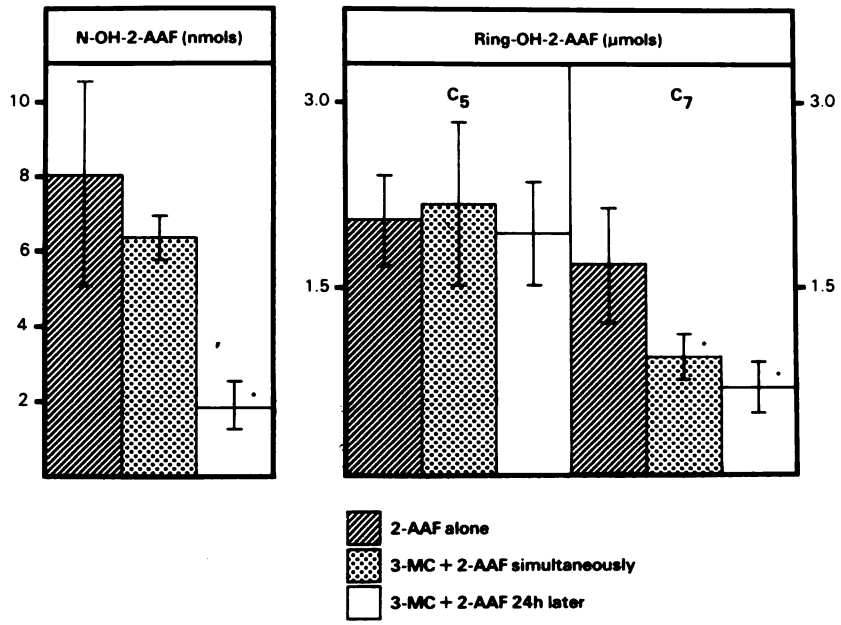


FIG. 7. *Effects of i.p. injection of 3-MC (40 mg/kg body weight) on the urinary excretion of N-, $C_5$-, and $C_7$-OH-2-AAF after one simultaneous or 24-hr delayed oral administration of 2-AAF (5 mg/kg)*

The values are means of two different experiments which never differed by more than 10%.

from Miller *et al.* (7) have shown that, during chronic administration of 2-AAF to rats, urinary excretion of the ring-hydroxylated metabolites does not increase whereas elimination of *N*-hydroxyacetylaminofluorene is drastically enhanced. It could thus be possible that the rat is a peculiar species in which chronic feeding of 2-AAF specifically induces and modifies microsomal *N*-hydroxylase activity. One argument in favor of this thesis is the fact that, in mice, urinary excretion of N-OH-2-AAF does not increase upon chronic treatment with 2-AAF (7).

It has been reported previously that the administration of 3-MC simultaneously with 2-AAF prevents the hepatocarcinogenic effect of 2-AAF. This inhibition has been explained by the large inducing effect of 3-MC on the ring hydroxylase which detoxifies 2-AAF (30). Our experiments confirm those data (Table 2). By showing *in vitro* that 3-MC is a very potent competitive inhibitor of liver microsomal arylamide *N*-hydroxylase, we previously suggested that the prevention of hepatocarcinogenesis could also be due to a competitive inhibition *in vivo* between 3-MC and 2-AAF when they reach the endoplasmic reticulum simultaneously (4).

We report here that such a combined treatment (3-MC + 2-AAF) given acutely induces hepatic microsomal *N*-, $C_5$-, and $C_7$-hydroxylases. The inducing effect on *N*-hydroxylation is identical with that of pretreatment with 3-MC only. In contrast, the combined treatment is twice as potent as treatment with 3-MC alone in inducing the ring hydroxylation (30- versus 15-fold increase for $C_5$-hydroxylase and 7- versus 3-fold increase for $C_7$-hydroxylase). As compared with 3-MC, the acute simultaneous pretreatment by 3-MC + 2-AAF also modifies *N*-hydroxylase activity by increasing its $K_m$ value. However, that increase is 2.5 times higher. Those data seem thus to confirm the importance of the competitive inhibitory effects of $C_5$-OH-2-AAF on *N*-hydroxylase activity. A larger production of the metabolite is indeed accompanied by an increased value of the $K_m$ of that enzyme.

Besides the large inducing effect of 3-MC or 3-MC + 2-AAF on the hepatic microsomal enzymes which catalyze the primary metabolism of 2-AAF, the 24-hr urinary excretion of the *N*- and ring hydroxy metabolites is either not modified or significantly reduced after such pretreatments. Moreover, even though the activities of those hepatic microsomal enzymes are of the same order of magnitude (picomoles per minute per milligram of protein), the amount of ring-hydroxylated metabolites in urine is at least 250 times higher than that of N-OH-2-AAF. It is thus difficult to correlate urinary excretion with hepatic microsomal metabolism. Various other parameters, such as covalent binding of N-OH metabolites to macromolecules, biliary excretion, enterohepatic recirculation, and pharmacokinetic behavior, could explain such discrepancies.

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