Metabolism of \textit{N}-Hydroxy-2-acetylaminofluorene and \textit{N}-Hydroxy-2-aminofluorene by Guinea Pig Liver Microsomes\textsuperscript{1}

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\textbf{ABSTRACT}

The guinea pig is resistant to the hepatocarcinogenic effects of 2-acetylaminofluorene and 2-aminofluorene. This resistance, however, is not due to the lack of a \textit{N}-hydroxylating enzyme in the liver which catalyzes the first and rate-limiting step to the activation of these chemicals to proximal carcinogens. It is shown that guinea pig liver microsomes can \textit{N}-hydroxylate both of these compounds. The \textit{N}-hydroxylation of 2-acetylaminofluorene but not 2-aminofluorene is inducible by pretreating the guinea pigs with benz(a)anthracene. The microsomal reaction is inhibited by 3-methylcholanthrene, miconazole, or 7,8-benzoflavone. 7-Iodo-2-acetylaminofluorene is \textit{N}-hydroxylated by guinea pig liver microsomes at approximately the same rate as 2-acetylaminofluorene. The \textit{N}-hydroxylation of 7-fluoro-2-acetylaminofluorene occurs at a much faster rate.

The resistance of the guinea pig liver to the carcinogenic effect of the arylamides and arylamines may actually be due to the ability to further convert the \textit{N}-hydroxylated metabolites to the inactive \textit{C7}-hydroxylated product. The conversion of \textit{N}-hydroxy-2-acetylaminofluorene to \textit{C7}-hydroxy-2-aminofluorene by guinea pig liver microsomes is inhibited by 8-hydroxyquinoline or miconazole.

The microsomal metabolic activation of the 7-iodo-2-acetylaminofluorene used to confirm this new metabolic pathway proceeds via a deacetylation step which could explain the resistance of the rat to the carcinogenic effect of that chemical. The high yield of the \textit{N}-hydroxy-7-fluoro-2-acetylaminofluorene produced by liver microsomes could be responsible for its high carcinogenic potency.

\textbf{INTRODUCTION}

Both 2-AAF\textsuperscript{3} and 2-AF and their proximal active metabolites, N-OH-2-AAF and N-OH-2-AF, are known to be hepatocarcinogens when administered chronically (p.o. or i.p. injections) to rats, hamsters, or mice (18, 21, 32). Neither the arylamide nor the arylamine are, however, hepatocarcinogens when given p.o. to guinea pigs (4, 18, 31).

Moreover, if in this particular species, the p.o. injection of the \textit{N}-hydroxylated metabolites induces the development of adenocarcinomas of the small intestine or sarcomas at the site of injection, respectively (18), neither N-OH-2-AAF nor N-OH-2-AF is carcinogenic or even toxic to the guinea pig liver (18).

This resistance of the guinea pig liver to the carcinogenic effects of 2-AAF has been explained by assuming that hepatocytes in this species lack the mixed-function oxidase catalyzing the \textit{N}-hydroxylation of arylamides and arylamines (9, 18, 33). Such an hypothesis has, however, failed to explain the absence of hepatocarcinogenic effects of N-OH-2-AAF or N-OH-2-AF when injected i.p. into this species (18). It was also contradicted by the capacity of guinea pig liver S-9 fractions to activate 2-AAF to a mutagen in the Ames test (17, 22, 29).

Guttmann and Bell (7) and Kiese et al. (12) showed that guinea pig liver microsomes actually do contain a \textit{N}-hydroxylating mixed-function oxidase.

In a recent publication (26), we reported the application of a very sensitive and highly specific method suitable to assay both N-OH-2-AAF and N-OH-2-AF, which also demonstrates unequivocally that guinea pig liver microsomal enzymes \textit{N}-hydroxylate both 2-AAF and 2-AF. The rate of \textit{N}-hydroxylation of 2-AAF in guinea pig liver microsomes is about the same as in the rat whereas the \textit{N}-hydroxylation of 2-AF occurs about 25 times faster in the guinea pig microsomes (25-27).

The resistance of the guinea pig liver to the carcinogenic effect of 2-AAF is thus not due to the absence or inefficiency of \textit{N}-hydroxylating enzymes. Another possible explanation for this resistance could be the presence of an enzyme in guinea pig liver which would further metabolize N-OH-2-AAF or N-OH-2-AF to inactive products. Miller et al. (18) reported that guinea pigs given i.p. injections of N-OH-2-AAF excreted mainly 7-OH-2-AAF which is inactive as a carcinogen (21). Therefore, it has been postulated (13, 18, 26) that liver enzymes in this species can transform the carcinogenic \textit{N}-hydroxylated metabolites of 2-AAF and 2-AF to inactive phenols (C7). The presence of that metabolic pathway would also explain the resistance of the guinea pig liver to both the toxic and the carcinogenic effects of i.p.-injected N-OH-2-AAF and N-OH-2-AF.

In this report, we present evidence for a new metabolic pathway in guinea pig liver microsomes for the transformation of N-OH-2-AAF and N-OH-2-AF to the inactive C7-OH-2-AAF and C7-hydroxy-2-aminofluorene products, respectively.

\textbf{MATERIALS AND METHODS}

\textbf{Chemicals.} 2-AAF, 2-AF, and PX were obtained from Aldrich-Europe, Beersel, Belgium. 7,8-BF was purchased from Eastman Organic Chemicals, Rochester, N. Y. Glucose 6-phosphate and 3-MC were obtained from Sigma Chemical Co., St. Louis, Mo. NADP* and glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim, Mannheim, Germany. All the above were of the purest grade available.
All other reagents of analytical grade were purchased from Merck, Darmstadt, Germany. SKF 525 A was a gift from Smith, Kline & French, Recherche et Industrie Thérapeutiques Laboratoires, Genval, Belgium. MN was kindly supplied by Janssen Pharmaceutica, Beerse, Belgium. 7-OH-2-AAF was kindly provided by Dr. J. N. Keith, National Cancer Institute Chemical Depository, IIT Research Institute, Chicago, III. N-OH-2-AAF was synthesized and purified as described previously (19). 2-AAIF and 2-AAFF were supplied by Dr. R. Fuchs, Institut de Biologie Moleculaire du Centre National de la Recherche Scientifique, Laboratoires de Biophysique, Strasbourg, France. Salmonella typhimurium strain TA 1538 was kindly supplied by Dr. B. N. Ames.

Animals. Male guinea pigs, weighing 400 to 500 g, and male Wistar rats, weighing 200 to 250 g, were obtained from the Proefdieren-Centrum, Katholiek Universiteit te Leuven, Belgium. They were fed a semisynthetic diet (A03; Uisse d’Alimentation Rationnelle, Epinay-sur-Orge, France) and water ad libitum. Food was withdrawn 24 hr before sacrifice. Guinea pig pretreatment consisted of 2 i.p. injections of BA (40 mg/kg body weight in corn oil) 48 and 24 hr prior to sacrifice. Controls received an equal amount of corn oil only 48 and 24 hr prior to sacrifice.

Liver Microsome Preparation. Liver microsomes were prepared according to the method of de Duve as described by Amar-Costesec et al. (1). This procedure was applied without modification for the subcellular fractionation of both rat and guinea pig livers.

Protein Determination. Protein content was determined by the method of Lowry et al. (16) with crystalline bovine serum albumin as a standard.

Microsomal Incubations. Substrates were added to the incubation medium in 50 µl of methanol after a 15-min preincubation period for the generation of NADPH. Immediately following this, microsomes were added to a final volume of 3.35 ml.

All the inhibitors were added at the same time with the substrate as follows: SKF 525 A and MN as a water solution; and 3-MC, 7,8-BF, PX, and 8-OH-Q in 50 µl of methanol. The final concentration of microsomal proteins, substrates, and inhibitors is indicated in the legend of each chart.

Incubations were carried out under strictly defined Michaelis-Menten conditions with regard to protein concentration, pH optimum, and substrate/enzyme concentration ratio.

The methanol added to the incubations was shown not to influence N-hydroxylation activity under the experimental conditions of the assay. Assay of 7-OH-2-AAF. 7-OH-2-AAF was assayed by applying the electron capture gas chromatographic method developed recently by M. Batardy-Grégoire et al. (3). The composition of the incubation medium was essentially the same as that used for the N-hydroxylase assay (24). N-OH-2-AAF was incubated in the presence of 0.2 mg of microsomal proteins per ml at 37°C in open Erlenmeyer flasks. The reaction was stopped by adding 0.1 ml of 1 M CaCl2 and heating at 100°C for 5 min. After centrifugation at 3000 rpm for 10 min, an internal standard (1,6-dibromo-2-naphthol) was added to the supernatant along with 0.2 ml of 1 N NaOH. After 2 washings with 3 ml of hexane, the aqueous phase was acidified to pH 1.5 by adding 0.25 ml of 1 N HCl and extracted 3 times with 2 ml of tert-butyl methyl ether. The organic extract was then evaporated to dryness under nitrogen. After derivatization in the presence of 5 µl of heptafluorobutyrlimidazole (3), the residue was dissolved in 1 ml of cyclohexane, and 1 µl was injected into the gas chromatograph.

Gas Chromatography. A Pye 104 isothermal oven equipped with a Chrompack 9.000 solid injector for capillary columns was used along with a 6.5-m x 0.5-mm (inside diameter) wall coated open tube column coated with SE 52 or 14-m x 0.5-mm (inside diameter) wall coated open tube column coated with 3% OVI for the assay of N-hydroxy derivatives or 7-OH-2-AAF, respectively. A Pye Unicam Model 795012 electron capture detector was also used.

N-Hydroxyarylamine and N-Hydroxyarylamide Assays. The N-hydroxy metabolites formed after the incubation of 2-AAF, 2-AF, 2-AAIF, or 2-AAFF in the presence of microsomes were assayed by applying the electron capture gas chromatographic method described previously (24) and as further adapted to capillary gas chromatography (23). In order to distinguish N-OH-2-AAF, the proximal metabolite of 2-AAF, from N-OH-2-AF, formed by the deacetylation of N-OH-2-AAF, the assay was modified as suggested previously (25).

Mutagenicity Assays of 2-AF and 2-AAIF. The mutagenicity test was the classic plate assay described by Ames et al. (2) with the following modifications. (a) For the mutagenicity of 2-AF, asceptically prepared guinea pig liver microsomes were first incubated for various lengths of time with 2-AF at a final concentration of 0.25 µM. The mixture was then layered together with the suspension of Salmonella on minimal glucose agar. (b) For the mutagenicity of 2-AAIF, asceptically prepared guinea pig or rat liver microsomes were incubated for various lengths of time with 2-AAIF at a final concentration of 1 µM together with the suspension of Salmonella. The complete mixture (0.3 mg protein/plate) was then layered on minimal glucose agar.

After incubation for 48 hr at 37°C in the dark, the number of his revertants was counted on the plates. Since it was difficult to assess the true background revertant rate which varied from 10 to 20, it was decided not to subtract any background mutation rate and to illustrate all data simply as number of his revertants per plate. The results are the average of duplicate determinations which always varied by less than 10% in a given experiment.

Statistical Analysis. The program of Cleland (5) as modified and completed by Cumps (6) has been applied for the quantitative estimation of the enzyme parameters with their standard deviations.

RESULTS

Enzyme Properties of Liver Microsomal Arylamine (2-AF) and Arylamide (2-AAIF) N-Hydroxylyase from Control and BA-pretreated Guinea Pigs. As reported previously (26), guinea pig liver microsomes N-hydroxylate both 2-AF and 2-AAIF. Table 1 gives the values of the kinetic parameters which we have determined for the N-hydroxylation reactions in microsomes isolated from control and BA-pretreated animals. Since guinea pigs have a very active deacetylase (10, 11), the N-hydroxylation of 2-AAIF was measured in the presence of fluoride ion. This ion can also inhibit N-hydroxylation to a lesser extent (27), but a concentration of 10 mM was shown, in preliminary tests, to be sufficient in our experimental conditions to fully inhibit the deacetylase without interfering with the N-hydroxylation.

BA pretreatment of guinea pig induces the liver microsomal arylamine N-hydroxylyase by a factor of 2 (Table 1). It does not, however, increase the activity of the arylamine N-hydroxylyating enzyme.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>K_m (× 10^{-5} M)</th>
<th>V_max (pmol N-hydroxy metabolite/mg protein/min)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.99 ± 0.1^a</td>
<td>4.9 ± 0.6</td>
</tr>
<tr>
<td>BA</td>
<td>0.44 ± 0.17</td>
<td>6.2 ± 1.5</td>
</tr>
</tbody>
</table>

^a Mean ± S.D.

^Significantly different (p < 0.05).
After BA treatment, the values of the apparent $K_{\text{m}}$s are not significantly modified.

Like the rat and hamster liver enzymes (27), the guinea pig liver microsomal arylamine $N$-hydroxylase is inhibited in vitro (80 to 90%) by $\mu M$ concentrations of 3-MC, 7,8-BF, and MN and to a lesser extent (25 to 50%) by $\mu M$ concentrations of PX, SKF 525 A, and 8-OH-Q (Chart 1). BA treatment does not significantly modify these patterns of inhibition.

**Guinea Pig Liver Microsomal Metabolism of N-OH-2-AF and N-OH-2-AAF.** As shown in Chart 2, the production of N-OH-2-AF by guinea pig liver microsomes increases with time of incubation up to 10 min. This metabolite then disappears progressively from the medium. BA pretreatment does not affect that pattern. One likely explanation for such a disappearance of N-OH-2-AF would be, as suggested previously (26), that guinea pig liver microsomes further metabolize that compound. Moreover, a similar phenomenon is observed when a modified Ames test with S. typhimurium strain TA 1538 is used to reveal the mutagenic activation of 2-AF (Chart 2).

This activation process is known to require $N$-hydroxylation as the primary rate-limiting step (19). In this experiment, the number of his* revertant colonies increases with the time of preincubation of the 2-AF with guinea pig liver microsomes up to 10 min and declines thereafter. Liver microsomes from BA-pretreated guinea pigs again behave similarly. These experiments, together with those reported previously (25, 26), thus confirm that guinea pig liver microsomes are able to $N$-hydroxylate arylamines or arylamides but also that, at least when the substrate concentration is in the $\mu M$ range, they further metabolize these active metabolites to nongenotoxic product(s). Moreover, this further metabolism of either 2-AF or 2-AAF is inhibited by $\mu M$ concentrations of 8-OH-Q. In the presence of 50 $\mu M$ of that compound, the $N$-hydroxy metabolites no longer disappear from the medium when the incubation time is prolonged even up to 30 min (Chart 3).

Such an hypothesis is confirmed by the results reported in Chart 4, which demonstrate that N-OH-2-AAF disappears rap-
Guinea Pig Microsomal Detoxification of N-OH-2-AAF and N-OH-2-AAF

...indly (95% in 20 min) from the medium when it is incubated in the presence of guinea pig liver microsomes supplemented with a NADPH-generating system. The metabolism of N-OH-2-AAF does not require deacetylation to N-OH-2-AF since the addition of NaF (0.1 mM) does not significantly inhibit it. This reaction is at least partly NADPH dependent and completely inhibited by 50 μM MN.

In a previous report, Miller et al. (18) indicated that, after p.o. administration of N-OH-2-AAF, guinea pigs excrete mainly C7-OH-2-AAF in their urines. It was therefore of interest to see whether liver microsomes from this species can transform N-OH-2-AAF to C7-OH-2-AAF. As shown in Chart 5A, upon incubation of N-OH-2-AAF in the presence of guinea pig liver microsomes and NADPH, C7-OH-2-AAF is indeed formed at a rate of approximately 50 pmol/min/mg protein. Such a metabolic pathway is furthermore inhibited by MN or 8-OH-Q (Chart 5B). It must also be pointed out that 7-OH-2-AAF was not found when N-OH-2-AAF was incubated in the presence of control rat liver microsomes.4

Guinea Pig and Rat Liver Microsomal N-Hydroxylation of 2-AAIF and 2-AAFF. Guinea pig liver microsomal metabolism of N-OH-2-AAF proceeds via transformation to C7-OH-2-AAF. Then compounds like 2-AAIF or 2-AAFF, which have a blocked C-7 position in the fluorenic ring, must be N-hydroxylated without further metabolism. When incubated in the presence of guinea pig liver microsomes, 2-AAIF is N-hydroxylated at a rate of 20 pmol/min/mg protein (Chart 6). This is about the same rate as that found, for 2-AAF metabolism in the presence of 8-OH-Q (Chart 3), to inhibit further metabolism of the N-OH-2-AAF product. As compared to guinea pig, rat liver microsomes N-hydroxylate this substrate at a much lower rate of approximately 2 pmol/min/mg protein (Chart 6).

The explanation for this species difference is that the metabolism of the 7-iodo derivative of 2-AAF proceeds via a deacetylation step. In the presence of either guinea pig or rat liver microsomes, the amount of N-OH-2-AAIF which is formed is indeed very low (±2 pmol/min/mg protein). It is only by measuring the sum of the N-OH-2-AAIF + N-OH-2-AIF products that the high rate of N-hydroxylation by the guinea pig liver microsomes can be demonstrated, due to their high deacetylase activity (Chart 6B). The same species differences in metabolism are evident by comparing the capacity of rat and guinea pig liver microsomes to activate 2-AAIF to a mutagen in the modified Ames test (Chart 7).

When incubated for up to 30 min in the presence of 2-AAIF and S. typhimurium, rat liver microsomes are unable to activate 2-AAIF in our conditions. Incubation of 2-AAIF with guinea pig liver microsomes, however, produces a significant time-dependent increase in the number of his+ revertants per plate. As shown in Chart 8, 2-AAF is N-hydroxylated by guinea pig liver microsomes at a rate of 500 pmol/min/mg protein. Like 2-AAIF, 2-AAFF N-hydroxylation is not followed by disappearance of the product, as in the case of the metabolism of 2-AAF (25, 26) or 2-AF (Chart 2). Rat liver microsomes N-
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Chart 6. A, time-dependent formation of N-OH-2-AAIF and N-OH-2-AIF from 2-AAIF (5 μM) in the presence of guinea pig or rat liver microsomes (0.2 mg/ml). B and C, deacetylase activity of guinea pig and rat liver microsomes incubated with 2-AAIF as estimated by measuring both N-OH-2-AAIF and the sum of the N-OH-2-AIF + N-OH-2-AIF products. Incubation time was 10 min.

Chart 7. Effect of preincubation time of 2-AAIF (1 μM) with rat or guinea pig liver microsomes (0.3 mg/plate) on the mutagenicity of 2-AAIF in the Ames test.

Chart 8. Time-dependent formation of N-OH-2-AAFF from 2-AAFF (5 μM) incubated with rat or guinea pig liver microsomes (0.2 mg/ml).

AAFF by either guinea pig or rat liver microsomes is surprisingly high as compared to the rate observed for 2-AAF or 2-AAF (10 to 20 pmol/min/mg protein).

DISCUSSION

This report confirms the presence of both arylamine and arylamide N-hydroxylase activities in guinea pig liver microsomes. Like the rat or hamster liver enzyme (27), guinea pig liver microsomal 2-AF N-hydroxylase is inhibited very efficiently by nm concentrations of 3-MC, 7,8-BF, or MN.

Their activity is, however, only partly (30 to 50%) reduced in the presence of 50 μM PX, SKF 525 A, or 8-OH-Q. Beyond these similarities, however, the guinea pig liver microsomal N-hydroxylases differ significantly from those of the rat or hamster. Their activity is only slightly (2-fold for N-hydroxylation of 2-AAF) or not at all (N-hydroxylation of 2-AF) induced by pretreatment with BA or other polycyclic aromatic hydrocar-
Guinea Pig Microsomal Detoxification of N-OH-2-AAF and N-OH-2-AF

Chart 9. Possible metabolism detoxification pathway of N-OH-2-AAF by the guinea pig liver microsomes.

Hypothetical Pathways

We have found that the concentration of N-OH-2-AF in incubation of guinea pig microsomes with 2-AF increases for 2–3 min and then decreases, indicating further metabolism of the N-hydroxylated product. This second metabolic step is effectively inhibited by 8-OH-Q since the secondary decrease in N-OH-2-AF concentration does not occur in microsomal incubations containing this compound. Moreover, when 2-AF is preincubated in the presence of guinea pig liver microsomes for various lengths of time and then plated on minimal glucose agar with Salmo nella, the number of his^+ revertant colonies increases with time of preincubation up to 10 min and then decreases progressively back to the spontaneous reversion rate.

We have also found that N-OH-2-AAF is further metabolized by guinea pig liver microsomes. When N-OH-2-AAF is added directly to microsomal incubations, the concentration of this toxic substrate decreases rapidly. This pathway is not dependent on deacetylation, since it is not inhibited by NaF. At least one product of this microsomal metabolism of N-OH-2-AAF is the inactive (21) C7-OH-2-AAF derivative. Both the decrease in concentration of N-OH-2-AAF and the formation of C7-OH-2-AAF are comparably inhibited by MN. We have also shown that 8-OH-Q inhibits the formation of C7-OH-2-AAF.

As shown in Chart 9, various metabolic pathways could be involved in the transformation of N-OH-2-AAF to C7-OH-2-AAF. The possible reactions include: N-hydroxy reduction as first described by Lotlikar et al. (15), acting either on the N-OH-2-AAF or on the N-hydroxy-C7-hydroxy-2-acetylaminofluorene; N-hydroxy-C7-hydroxylase activity either on N-OH-2-AAF to form the N-hydroxy-C7-hydroxy metabolite or on 2-AAF formed by reduction of N-OH-2-AAF and a hypothetical N-hydroxy → C7-hydroxy transoxygenase. Such a transoxygenase has been shown to metabolize N-OH-2-AAF to either C7- or C8-hydroxy-2-acetylaminofluorene (8).

With the results obtained so far, it is not yet possible to say which is correct or the most important. Guinea pig liver microsomes are very active in C7-hydroxylating 2-AAF [5] which implies that the reductase → hydroxylase pathway is possible, but reduction of the N-hydroxy metabolites requires an oxygen-free incubation protocol (15, 34) which was not applied in our experiments. The high efficiency of the N-OH-2-AAF → C7-OH-2-AAF transformation in guinea pig microsomal incubations points to a direct conversion via a transoxygenase. The findings related to 2-AAFF metabolism also tend to support the transoxygenase pathway. N-Hydroxylation of this substrate by guinea pig microsomes is linear for a much longer time than the same reaction with 2-AAF, indicating that N-OH-2-AAFF is not further metabolized. Further metabolism of N-OH-2-AAFF would not be hindered if the N-hydroxy reduction step could occur separately.

Our results also show that the metabolism of 2-AAFF is very peculiar, requiring a deacetylation step. The requirement for such a metabolism could explain the difference between rat and guinea pig in hydroxylating this p-iodo substrate. It could also explain why 2-AAFF is less carcinogenic in the rat than 2-AAF or 2-AAFF whereas N-OH-2-AAF is more mutagenic in the Ames test than both N-OH-2-AAF or N-OH-2-AAFF (28).

5 M. Batardy-Grégoire, personal communication.
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Our data showing that rat liver microsomes are, in our conditions, unable to activate 2-AAIF as a promutagen in the Ames test as compared to guinea pig liver microsomes support such an hypothesis. The rate of N-hydroxylation of 2-AAFF in both guinea pig and rat microsomes is much higher than that of 2-AAF or 2-AAIF. This could explain the particularly high susceptibility of the rat to the carcinogenicity of that fluoro derivative (20, 21), which has never been assayed in the guinea pig. When incubated with rat liver microsomes, the N-hydroxylation of 2-AAFF proceeds at a constant rate for only 5 min. It then reaches a plateau. With guinea pig liver microsomes, the rate of that reaction is constant for at least 20 min. Such a difference could be due to the fact that C7-hydroxy-2-acetylaminofluorene is a major metabolite of 2-AAF in the rat, whereas it is only a minor one in the guinea pig (18), which is then unable to detoxify 2-AAFF.

In summary, although the rate of N-hydroxylation, the affinity of the cytochrome P-450-dependent N-hydroxylase, the enzymatic reduction of N-OH-2-AAF (15, 34), and the absence of cytosolic sulfate conjugation must also be considered, it is proposed that the ability of guinea pig liver to efficiently detoxify the N-hydroxy metabolite of amine and amide may be one major factor responsible for their low or nonhepatocarcinogenic activities in guinea pigs. In the case of 2-AAF metabolism, N-OH-2-AAF is converted to the inactive C7-OH-2-AAF, a detoxification pathway which is absent in rat liver.

REFERENCES

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