# Effect of 3-Amino-1,2,4-Triazole Pretreatment on *N*- and Ring-Hydroxylation of 2-Acetylaminofluorene by the Rat[1] (37609)

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*N*-Hydroxylation is an initial activation step in the carcinogenesis by 2-acetylaminofluorene (AAF) and several other aromatic amines and amides. However, ring-hydroxylation of AAF is an inactivation process (1, 2). Administration of 3-methylcholanthrene (MC) inhibits the liver tumor induction by AAF in the rat (3, 4) apparently by MC's induction of the inactivation process (5, 6).

The herbicide 3-amino-1,2,4-triazole (aminotriazole) inhibits catalase (7) and heme biosynthesis (8) in the rat liver. The present studies were undertaken to investigate the effects of aminotriazole pretreatment on both *N*- and ring-hydroxylation of AAF *in vitro* and *in vivo* in the rat.

*Materials and Methods. Animals.* Sprague–Dawley strain male rats, purchased from Charles River Breeding Laboratories, Wilmington, Mass., were maintained on Rockland rat/mouse diet and water *ad libitum* before use.

*Chemicals.* *N*-Hydroxy-AAF was synthesized by a known method (9). Other compounds were obtained as follows: AAF, Mann Research Laboratories; MC and aminotriazole, Eastman Organic Chemical Co.; L-[$^{35}$S]-methionine (24–30 mCi/mmole), Schwarz Bioresearch Inc.; ATP, NADH, NADPH and β-glucuronidase, Sigma Chemical Co.; and takadiastase, Park, Davis and Co. Ring-hydroxy derivatives of AAF, 3-methylmercapto-AAF (3-$CH_3S$-AAF) and 3-methylmercapto-2-aminofluorene (3-$CH_3S$-AF), were kindly supplied by Dr. James A. Miller of the McArdle Laboratory, University of Wisconsin, Madison, Wis.

*Administration of chemicals for experiments* in vivo. Adult male rats (140–170 g body wt.) were injected intraperitoneally for 9 days with aminotriazole (1 g/kg body wt./day) dissolved in 0.9% NaCl. Control group was injected with 0.9% NaCl. In some experiments, the common bile duct of each of the control group and aminotriazole-treated group was ligated according to the procedure of Cameron and Oakley (10). Such ligations, done 24 hr before AAF administration, were kindly performed by Mrs. Margot Gruenstein of the Fels Research Institute. At 4 hr after the last injection of aminotriazole all animals received intraperitoneal injection of a freshly prepared suspension of AAF (30 mg/kg body wt.) in 1.75% gum acacia–0.9% NaCl solution. No food was available during urine collection but water was available *ad libitum*. Feces were separated by a wire screen and the urine samples were collected in dry-ice cooled tubes for 24 hr after AAF injection.

*Urine analysis.* An aliquot of urine was incubated with takadiastase and β-glucuronidase as described previously (5, 11). After extraction of fluorene derivatives with ethyl ether, the ether extract was washed successively with 0.5 *N* HCl and water. Acidic metabolites were chromatographed on Whatman No. 1 paper with a solvent system composed of cyclohexane:*t*-butanal:acetic acid:water in ratios of 16:4:2:1 (11) by volume. The metabolites were quantitated by uv spectrophotometry (12).

*Administration of chemicals for studies in vitro.* Adult male rats (175 ± 20 g body wt.) were used for these studies. For some experiments aminotriazole (1 g/kg body wt.)

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was injected intraperitoneally for 3 days. Control group was injected with 0.9% NaCl. Aminotriazole group was sacrificed 24 hr after their third injection. Another group of animals was given an intraperitoneal injection of MC (100 mg/kg body wt.) suspended in corn oil 24 hr before sacrifice.

*Preparation of liver microsomal fraction and cytosol.* After the animals were decapitated, the livers were immediately removed and chilled in ice-cold 0.25 *M* sucrose solution. A 25% liver homogenate prepared in 0.25 *M* sucrose solution was sedimented at 10,000*g* for 15 min to remove nuclei and mitochondria, after which the microsomal pellet was sedimented at 105,000*g* for 60 min. The microsomal supernatant designated as cytosol was used for assay of enzymatic esterification of *N*-hydroxy-AAF. The surface of the microsomal pellet was washed twice with 0.25 *M* sucrose before resuspension in the same medium.

*Assay for N- and ring-hydroxylation of AAF.* The incubation medium, extraction, separation, and quantitation of hydroxy metabolites of AAF were as described previously (12). Content of 9–18 flasks were combined for each analysis. The acidic metabolites were chromatographed on Whatman No. 1 paper with a solvent system composed of cyclohexane:*t*-butanol:acetic acid:water in ratios of 18:2:2:1 by volume. This solvent system could not separate 5- and 7-hydroxy-AAF; these were eluted together ($R_f$, 0–0.27) and calculated as 7-hydroxy-AAF. This solvent system, however, gave better separation of *N*-hydroxy-AAF from the *ortho*-hydroxy metabolites. Whenever values of 10 nmoles or less/g liver/20 min are given for any metabolite, it indicates that a small amount of uv light absorbing material with a noncharacteristic spectrum was eluted from the paper.

*Assay for enzymic esterification of N-hydroxy-AAF.* The incubation medium and assay procedure were the same as described previously (13) except that liver cytosol equivalent to 50 mg instead of 100 mg wet wt. of tissue was used in the present experiments.

*Results.* Adult rats excreted about 0.5% of administered dose of AAF as *N*-hydroxy-AAF (Table I). However, pretreatment of rats with aminotriazole for 9 days caused about threefold increase in the urinary excretion of the *N*-hydroxy metabolite. Similarly, aminotriazole pretreatment also caused appreciable increases in the urinary excretion of ring-hydroxy derivatives.

Only a small fraction of the *N*-hydroxy-

TABLE I. Effects of Bile-Duct Ligation on the Urinary Metabolites of 2-Acetylaminofluorene in Control and Aminotriazole Treated Rats.

| Treatment[a] | Bile-duct ligation | No. of analyses | Urinary excretion (% of dose, mean ± SEM) | | | |
|---|---|---|---|---|---|---|
| | | | *N*-Hydroxy-AAF | 3-Hydroxy-AAF | 5-Hydroxy-AAF | 7-Hydroxy-AAF |
| Control | — | 7 | 0.47 ± 0.18 | 2.6 ± 1.4 | 5.3 ± 1.5 | 14 ± 4.9 |
| Aminotriazole | — | 7 | 1.4 ± 0.33[b] | 6.7 ± 2.9[b] | 8.6 ± 1.2[b] | 26 ± 8.5[b] |
| Control | + | 5 | 2.4 ± 0.6 | 3.1 ± 0.5 | 3.2 ± 0.6 | 21 ± 3.0 |
| Aminotriazole | + | 5 | 1.9 ± 0.3 | 3.1 ± 0.4 | 3.8 ± 0.8 | 20 ± 7.1 |

[a] Adult male rats (155 ± 15 g body wt) were used for these studies. Aminotriazole (1 g/kg body wt/day) dissolved in 0.9% NaCl was injected intraperitoneally for 9 days. Control animals were injected with 0.9% NaCl. Where indicated on 8th day, the common bile duct of each of the control and aminotriazole treated groups was ligated. At 4–5 hr after bile-duct ligation, both groups received their respective injections. On 9th day at 4 hr after the last injection of either 0.9% NaCl or aminotriazole, all animals received ip injections of AAF (30 mg/kg body wt) and 24 hr urine samples were collected. One rat was used for each urine collection and analysis.

[b] Statistical comparisons between control and aminotriazole treated groups with $p < 0.01$ are considered significant. Statistical comparisons between ligated control and ligated aminotriazole treated groups have $p > 0.20$ which are considered not significant.

AAF formed by the rat was excreted in the urine whereas a large fraction was excreted in the bile (14). The bile excretion of *N*-hydroxy-AAF by the rat could be shunted to the urine by ligation of the common bile duct (14, 15). It was therefore of interest to study the effects of bile-duct ligation on the urinary excretion of hydroxy metabolites of AAF. Bile-duct ligation caused severalfold increase in the urinary excretion of *N*-hydroxy-AAF (Table I). It also increased the excretion of 7-hydroxy metabolite. The present results are in agreement with those reported previously (14, 15). Urinary excretion of hydroxy metabolites of AAF was about the same in both bile-duct ligated groups with or without aminotriazole pretreatment.

Both *N*- and ring-hydroxylation of AAF occur in the liver microsomes in the presence of NADPH and molecular oxygen (6, 16-18). Pretreatment of rat swith MC causes severalfold increases both in *N*- and ring-hydroxylation of AAF by liver microsomes (6, 17). Our present data with MC (Table II) are in agreement with our previous studies (6). In the present studies ring-hydroxylation of AAF was decreased by about 25% by aminotriazole pretreatment of rats (Table II). The chromatography solvent system used in these experiments could not resolve 5- and 7-hydroxy-AAF. Therefore, it is difficult to indicate whether aminotriazole treatment produced specific inhibition of either 7-hydroxy or 5-hydroxy metabolite or both. Levels of *N*- and 3-hydroxy-AAF were too low in the control to observe any inhibitory effect on their levels by aminotriazole treatment.

Results of esterification of *N*-hydroxy-AAF by rat liver preparations are summarized in Table III. Pretreatment of adult rats with MC caused a small increase in the formation of 3-$CH_3S$-AAF by liver cytosol preparations whereas aminotriazole treatment produced about 25% decrease in the formation of 3-$CH_3S$-AAF compared to control levels. Similar results were obtained on treatment of young male rats (79–80 g body wt.) with aminotriazole.

*Discussion.* 2-Acetylaminofluorene (AAF) is a useful model compound for studying the biochemical mechanisms of hydroxylation since it could undergo hydroxylations at several carbon atoms in the ring as well as on the nitrogen atom. These hydroxylation reactions are important due to the fact that *N*-hydroxylation is an activation step in the carcinogenic process whereas ring-hydroxylation is an inactivation step (1, 2).

In the present studies aminotriazole pretreatment of rats for several days produced severalfold increase compared to control group in the urinary excretion of *N*-hydroxy-AAF after administration of a test dose of

TABLE II. Effects of Aminotriazole and 3-Methylcholanthrene Pretreatment on *N*- and Ring-Hydroxylation of AAF by Rat Liver Microsomes.

| Treatment[a] | Hydroxylation (nmoles formed/g liver/20 min) | | |
|---|---|---|---|
| | *N*-Hydroxy-AAF | 3-Hydroxy-AAF | 7-Hydroxy-AAF[b] |
| Control | 10 ± 7[c] | 9 ± 4 | 158 ± 53 |
| 3-Methylcholanthrene | 72 ± 30[d] | 104 ± 30[d] | 1233 ± 132[d] |
| Aminotriazole | 6 ± 5 | 10 ± 4 | 119 ± 45 |

[a] Four adult (175 ± 20 g body wt) male rats were used for each group. All injections were given intraperitoneally. Aminotriazole (1 g/kg body wt/day) dissolved in 0.9% NaCl was injected for 3 days. Control animals were injected with 0.9% NaCl. 3-Methylcholanthrene (100 mg/kg body wt) suspended in corn oil was injected 24 hr before sacrifice. Control and aminotriazole groups were sacrificed 24 hr after their third injection. Liver from each rat was used per analysis.

[b] 7- and 5-Hydroxy-AAF could not be separated with the solvent system used; these were eluted together and calculated as 7-hydroxy-AAF.

[c] Values are expressed as the mean ± SEM.

[d] Statistical comparisons between control and treated groups with $p < 0.05$ are considered significant.

TABLE III. Effects of Aminotriazole and 3-Methylcholanthrene Pretreatment on the Esterification of *N*-Hydroxy-AAF by Rat Liver.

| Treatment[a] | nmoles formed/50 mg liver/2 hr | |
|---|---|---|
| | 3-$CH_3S$-AAF | 3-$CH_3S$-AF |
| Control | 45 ± 3.0 | 4.6 ± 1.2 |
| 3-Methylcholanthrene | 52 ± 1.5[b] | 2.9 ± 0.3 |
| Aminotriazole | 33 ± 2[b] | 2.7 ± 0.7 |

[a] Three adult (175 ± 20 g body wt) male rats were used for each group. Injection schedule was as described in Table II. One rat liver was used for each analysis.

[b] Statistical comparisons between control and treated groups with $p < 0.05$ are considered significant.

AAF to these animals. Such a treatment also increased the urinary excretion of ring-hydroxy metabolites. These results would suggest that aminotriazole pretreatment might have enhanced the metabolism of AAF in the rat.

Irving *et al.* (14) have shown that a large fraction of *N*-hydroxy-AAF formed by the rat liver is retained in the bile. The bile excretion of *N*-hydroxy-AAF by the rat could be shunted to the urine by ligation of the bile-duct (14, 15). In the present experiments after bile-duct ligation of control rats, the *N*-hydroxy-AAF excretion in the urine increased severalfold. These results are in agreement with those reported previously (14, 15). Aminotriazole pretreatment however, had no appreciable effect on the urinary excretion of either *N*- or ring-hydroxy metabolites of AAF in a rat whose bile ducts had been ligated. Data obtained so far suggest that increased urinary excretion of hydroxy derivatives of AAF after aminotriazole pretreatment was not due to an increased rate of metabolism of AAF but rather due to an increase in urinary excretion of metabolites from the bile.

Reportedly, pretreatment of rats with a large dose of aminotriazole (3 g/kg body wt.) decreased the activities of drug metabolizing enzymes of liver microsomes by about 50% (8, 19). The lesser inhibition (25%) obtained in the present study might be due to use of a smaller dose (1 g/kg body wt.). Matushima and Weisburger (20) have reported similar inhibition of ring-hydroxylation of AAF by hamster liver microsomes even though aminotriazole dose for these animals was 3 g/kg body wt. In their study, they observed increased *N*-hydroxylation of AAF by microsomes from aminotriazole pretreated hamster. In the present study no conclusions could be drawn about effect of aminotriazole pretreatment on *N*-hydroxylation by liver microsomes due to low levels of formation of this metabolite in *in vitro* studies.

In addition to *N*-hydroxylation, esterification of *N*-hydroxy-AAF is another activation step in the liver carcinogenesis by AAF (1, 2). Apparently aminotriazole pretreatment of rats has some inhibitory effect on this activation step (Table III). Such an aminotriazole effect may protect rats against liver carcinogenesis by AAF and its *N*-hydroxy derivative.

*Summary.* (1) Pretreatment of adult male rats with aminotriazole (1 g/kg body wt./day) for 9 days produced severalfold increase compared to control group in the urinary excretion of *N*-hydroxy-2-acetylaminofluorene (*N*-hydroxy-AAF) after ip administration of a test dose of AAF. Such a treatment also increased appreciably the urinary excretion of ring-hydroxy metabolites of AAF. (2) Effects of bile-duct ligation of adult male rats on the urinary excretion of hydroxy metabolites of AAF were similar to the effects of aminotriazole pretreatment. The urinary excretion pattern of these metabolites was about the same in both bile-duct ligated groups with or without aminotriazole treatment. (3) Ring-hydroxylation of AAF by liver microsomes was inhibited by 25% in aminotriazole treated group compared to the controls. (4) Aminotriazole (1 g/kg body wt./day) treatment of rats for 3 days inhibited enzymatic esterification of *N*-hydroxy-AAF by liver cytosol preparations by about 25%.

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