

Suppressive Role of Indole on 2-Acetylaminofluorene Hepatotoxicity¹

Martin L. Hopp,² Michio Matsumoto, Benjamin Wendell, Chung Lee, and Ryoichi Oyasu

Departments of Pathology [M. L. H., M. M., B. W., R. O.] and Urology [C. L.], Northwestern University Medical School, Chicago, Illinois 60611

SUMMARY

Indole is known to suppress the hepatotoxicity and carcinogenicity of 2-acetylaminofluorene (AAF) in rats and hamsters. For elucidation of the mechanism of its protective role, 2 experiments were conducted using young male rats. In the 1st experiment, the 24-hr biliary excretion of *N*-hydroxy-2-acetylaminofluorene (*N*-OH-AAF)-glucuronide was measured after 2 and 4 weeks of dietary administration of 0.03% AAF with or without 1.6% indole. The amount of [9-¹⁴C]*N*-OH-AAF that was excreted as the glucuronide following a single i.p. injection of [9-¹⁴C]AAF was lower after 2 weeks in animals fed AAF and indole, as compared to those fed AAF alone [$1.5 \pm 1.2\%$ versus $19.6 \pm 3.6\%$ S.E. ($p < 0.001$)]. After 4 weeks of AAF administration without indole, the biliary excretion fell to $4.8 \pm 2.1\%$. This was also significantly higher than that of the animals fed both AAF and indole [$1.8 \pm 1.2\%$ ($p < 0.025$)]. The suppressive role of indole on the conjugate excretion was also reflected in a decreased biliary excretion of all [9-¹⁴C]AAF metabolites in animals treated with indole alone.

In the 2nd experiment, the protective action of indole was assessed by survival following daily i.p. injections of *N*-OH-AAF and Na₂SO₄ solution. Na₂SO₄ increased the hepatotoxicity of *N*-OH-AAF. Indole suppressed the toxicity of *N*-OH-AAF even in the presence of Na₂SO₄. This protective role of indole was partially overcome only when excess sulfate was coadministered. These results indicate that indole suppresses the biliary excretion of the *O*-glucuronide of *N*-OH-AAF during the initial exposure of the animal to the carcinogen, possibly reflecting decreased *N*-OH-AAF formation. Indole also modifies the metabolism of AAF following *N*-hydroxylation, perhaps activating *N*-OH-AAF, depending upon the concentration of sulfate available.

INTRODUCTION

Indole has been repeatedly shown to play a role in decreasing the hepatotoxicity and hepatocarcinogenicity of AAF³ in rats and hamsters (13, 15, 16). In an experiment on the effect of indole on the AAF hepatotoxicity and carcinogenicity in hamsters, cholangiofibrosis, a nonmalignant proliferative glandular lesion with productive fibrosis, was ob-

served in all 26 animals treated with AAF alone, while only 7 of 27 animals treated with AAF and indole developed this lesion (13). In addition, none of the AAF- and the indole-treated animals developed cholangiocarcinoma, as did 4 of the AAF-treated animals.

It has been shown that AAF requires biochemical activation in the liver, in the form of hydroxylation of the nitrogen-yielding *N*-OH-AAF (2, 11). This derivative is more carcinogenic than the parent compound in sensitive species, and is active at the site of the injection (5, 12, 18). It is also active in species in which the parent compound is inactive (9). The ability of liver to *N*-hydroxylate AAF can be assessed *in vitro* (1, 8) and *in vivo* (8) by measuring the production of the *O*-glucuronide of *N*-OH-AAF, the major metabolite of *N*-OH-AAF in the rat liver (6). Irving *et al.* (6) have shown that rats, a species susceptible to hepatotoxicity and carcinogenicity by AAF, excrete a high percentage of a test dose as *N*-OGI-AAF on bile. In rabbits, a species resistant to AAF, the biliary excretion of *N*-OGI-AAF was much less.

Subsequent studies strongly indicate that *N*-OH-AAF must undergo further activation in order to manifest its hepatocarcinogenicity. At least one of these ultimate carcinogenic forms leading to liver cancer in rats has been shown to be the sulfate ester of *N*-OH-AAF (3, 17). This view was supported by increased hepatotoxicity and carcinogenicity of *N*-OH-AAF in the presence of acetanilide and excess dietary sulfate (4, 17). It was shown that susceptible species exhibit higher sulfotransferase activity than nonsusceptible species (3, 7). Indole has been shown to decrease [9-¹⁴C]*N*-OH-AAF binding to DNA, while increasing the binding to microsomal protein (9).

Our present studies were undertaken to investigate the protective role of indole and its relation to alterations of the metabolism of AAF *in vivo*. In the 1st experiment, the biliary excretion of conjugated *N*-OH-AAF was measured following a short period of dietary carcinogen treatment with or without indole. In the 2nd experiment, the effect of indole on *N*-OH-AAF toxicity was assessed.

MATERIALS AND METHODS

Chemicals

Anhydrous granular sodium sulfate, reagent grade, was purchased from J. T. Baker Chemical Co., Phillipsburg, N. J., and AAF was from Aldrich Chemical Co., Milwaukee, Wis. *N*-OH-AAF was obtained as a gift from Dr. Harry B. Wood, Jr., Drug Research and Development Branch, Na-

¹ Supported in part by USPHS CA 14649 through the National Bladder Cancer Project and the Northwestern Memorial Hospital Research Fund.

² Predoctoral fellow, NIH Grant DM 00131.

³ The abbreviations used are: AAF, 2-acetylaminofluorene; *N*-OH-AAF, *N*-hydroxy-2-acetylaminofluorene; *N*-OGI-AAF, *O*-glucuronide of *N*-OH-AAF.

Received March 27, 1975; accepted October 1, 1975.

tional Cancer Institute, Bethesda, Md. [9-¹⁴C]AAF (11.8 mCi/mmole) and [9-¹⁴C]N-OH-AAF (16.43 mCi/mmole) were purchased from ICN Nutritional Biochemicals Division, Cleveland, Ohio. [9-¹⁴C]N-OGI-AAF (0.25 mCi/mmole) was a gift of Dr. Charles King, Michael Reese Hospital, Chicago, Ill.

Diets

The dietary items were purchased from Nutritional Biochemical Corp., Cleveland, Ohio, except for dextrin and casein, which were obtained from A. E. Staley Co. and Nutritional Casein Co., Chicago, Ill., respectively. The synthetic diet with indole and carcinogen was mixed by General Biochemicals, Inc., Chagrin Falls, Ohio, as previously described (11, 15).

Animals

Young male albino CD rats weighing 150 to 250 g were purchased from Charles River Breeding Laboratory, Wilmington, Mass.

Procedures

Experiment A. The animals were divided into 4 dietary groups, 0.03% AAF, 0.03% AAF plus 1.6% indole, 1.6% indole, and synthetic diet without additives. They had free access to diet and water. Those animals treated with indole-containing diets were fed an 0.8% indole diet for 3 days, in order that they would be adjusted to eating an indole diet upon commencement of the experiment. After 2 and 4 weeks of dietary treatment the bile duct was cannulated, using polyethylene tubing (PE-10, Clay-Adams, Parsippany, N. J.) during ether anesthesia. A catheter (PE-205) was placed in the peritoneal cavity to facilitate the injection of the test dose. The test dose was injected within 2 hr after surgery. The [9-¹⁴C]AAF was first dissolved in 95% ethanol (100 μCi/0.2 ml) and then suspended in water to make the total volume 10 ml. Each animal was given an injection of 2 ml 0.9% NaCl solution to prepare the abdominal cavity, followed by the test dose of 10 μCi of [9-¹⁴C]AAF, 4 mg of nonradioactive AAF per 100 g body weight suspended in 0.9% NaCl solution, and 10 ml of 5% dextrose to ensure mixing of the carcinogen in the abdomen. An additional 10 ml of dextrose solution were instilled during the subsequent 24 hr to maintain adequate hydration and ensure carcinogen absorption. The animals were restrained by twine in metal metabolism cages. The urine and bile were collected in containers chilled with ice water over a 24-hr period and stored frozen until analysis.

Analysis of Biliary [9-¹⁴C]N-OGI-AAF. A 2-ml portion of bile adjusted to pH 6 by adding 10 ml of 1 M sodium acetate buffer was digested with 10 mg of β-glucuronidase (Sigma Chemical Co., St. Louis, Mo.) for 3 hr at 37°. The mixture was then extracted 2 times with an equal volume of ethyl ether. The ether fraction was dried and the residue was dissolved in 7 ml of 0.5 N NaOH and washed 2 times with 20 ml of ethyl ether. The water phase was quickly acidified to pH 6 with 0.5 N HCl and extracted 2 times with ethyl ether.

Table 1

Injection schedule by groups (Experiment B, Part 1)

Fifteen rats in each group given 2 i.p. injections daily, 2 hr apart, for 2 weeks. The 1st injection contained either 0.5 M Na₂SO₄ or H₂O, 0.5 ml/100 g body weight. The 2nd injection contained N-OH-AAF (50 mg/kg body weight) and/or 1.0 M Na₂SO₄, 0.5 ml/100 g body weight suspended in 1% gum acacia.

Group	N-OH-AAF	Na ₂ SO ₄	Diet
A	+	-	Normal
B	+	-	Indole
C	+	+	Indole
D	-	+	Normal
E	+	+	Normal
F	-	+	Indole

The ether extract was dried and metabolites were separated by silica gel thin-layer chromatography. N-OH-AAF appears on silica gel plate as a spot distinct from other AAF metabolites as described previously (13). The N-OH-AAF spot was located under UV and scraped into a counting vial. To this were added 10 ml of scintillation fluid which consisted of 5.5 g PPO, 0.1 g POPOP, 333 ml Triton X-100, and 667 ml toluene. The radioactivity was measured by a Packard Model 3385 liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). An internal standardization procedure was used, with ¹⁴C toluene as the marker, to determine the efficiency of the counting for each sample. Results were expressed as dpm. With each test a 2-ml portion of normal bile, to which were added 3.9 μg of [9-¹⁴C]N-OGI-AAF, was analyzed to determine the efficiency of each analysis group. The results were expressed based on each group-recovery efficiency. The urine collected was analyzed by our previously described thin-layer chromatography method (14).

Experiment B. Young male albino CD rats weighing 150 to 200 g were divided into 6 groups, each group consisting of 15 animals. Animals were housed 3/cage and given water and synthetic diet *ad libitum*. Dietary consumption was not measured. The indole groups were first treated with 0.3% indole diet for 3 days as in Experiment A. Two series of experiments were made.⁴ In both, a large amount of N-OH-AAF (50 mg/kg body weight) was administered i.p. For Part 1, each animal received daily i.p. injections according to Table 1. Each animal received 2 injections daily, 2 hr apart. The experiment was terminated after 2 weeks by killing the surviving animals.

In Part 1, the N-OH-AAF was dissolved in 95% ethanol (250 mg/0.75 ml) and then diluted to 10 mg/ml by adding 1% gum

⁴ A preliminary study was conducted to determine the daily tolerable dose of Na₂SO₄. Injection i.p. of 2 M Na₂SO₄, 2 ml/100 g body weights was invariably followed by death. Autopsy examination of such rats revealed a copious amount of clear fluid in the abdominal cavity. Death was ascribed to hypovolemic shock secondary to sudden accumulation of ascitic fluid. No animals when given i.p. injections of 1 M Na₂SO₄, 2 ml/100 g body weight, died of hypovolemic shock. Daily dietary consumption was measured using the rats of Group C [dietary indole, daily injections of N-OH-AAF (50 mg/kg) and 1.0 M Na₂SO₄ (0.25 mmole/100 g body weight)]. Each rat during the observation period of 10 days consumed approximately 10 g of indole diet per day, per 100 g body weight. Thus, it was calculated that in those animals treated with indole and Na₂SO₄ concurrently, the molar ratio of indole ingested to Na₂SO₄ was 1.8:1.0, and indole excess. For animals to be treated in Part 2 of the study, the volume of Na₂SO₄ solution was increased so that the ratio of indole to sulfate was 1:1.5, a sulfate excess.

acacia and also 1 M Na₂SO₄ when appropriate.

For Part 2 of this experiment, the N-OH-AAF was dissolved in ethanol (200 mg/0.75 ml) and then diluted to 5 mg/ml by adding 1% gum acacia, including 1 M Na₂SO₄ when appropriate. The rats were treated identically to those in Part 1, except that the amount of Na₂SO₄ was increased as shown in Table 2.

In both parts of Experiment B, all animals were weighed daily. An autopsy was performed on all animals as early as possible following death. Routine microscopic examination was limited to the liver and other organs that appeared grossly abnormal.

RESULTS

Experiment A.

Following the administration of [9-¹⁴C]AAF, the mean volume of bile produced in all groups over a 24-hr period was 17.9 ml. Those animals that produced less than 10 ml of bile were considered moribund and were not included in the study. No group had an excessive number of such animals.

Analysis of the bile containing exogenously added [9-¹⁴C]N-OGI-AAF without β-glucuronidase demonstrated that less than 0.1% of the radioactivity was recovered as N-OH-AAF. The bile constituents from animals treated with indole did not alter the analysis efficiency, which ranged from 30 to 50% for each analysis procedure.

As shown in Table 3, 19.6 ± 3.6% (S. E.) of the test dose of [9-¹⁴C]AAF was excreted in the bile as the glucuronide of N-OH-AAF in those animals treated for 2 weeks with 0.03% AAF diet. When indole was added to the diet for 2 weeks the biliary excretion of N-OGI-AAF was significantly decreased to 1.5 ± 1.2% of the test dose (unpaired *t* test; *p* < 0.001). When treated with AAF alone for 4 weeks, the animals did not excrete as well as those at 2 weeks (4.8 ± 2.1%). However, the biliary conjugate excretion of N-OH-AAF was still significantly more than that of the animals fed AAF and indole diet for 4 weeks (1.8 ± 1.0%, *p* < 0.025). Untreated animals excreted 3.1 ± 2.8% of the test dose, while the groups of animals fed a normal diet with added indole excreted 0.5 ± 0.4 and 2.4 ± 1.0% respectively, of the test dose as the glucuronide of N-OH-AAF after 2- and 4-week treatment. The total radioactivity recovered in the bile and urine during the 24-hr period is shown in Table 4. Animals treated for 2 weeks with AAF diet excreted more of the test

Table 2

Injection schedule by groups (Experiment B, Part 2)

Treatment of 15 rats in each group was identical to those described in Table 1, except that the Na₂SO₄ volume was increased (1st injection, 2.0 ml/100 g body weight; 2nd injection, 1.25 ml/100 g body weight).

Group	N-OH-AAF	Na ₂ SO ₄	Diet
A'	+	-	Normal
B'	+	-	Indole
C'	+	+	Indole
D'	-	+	Normal
E'	+	+	Normal

Table 3

Biliary excretion of [9-¹⁴C]N-OGI-AAF in 24 hr following a test dose of [9-¹⁴C]AAF

Following specific dietary treatment, a bile duct fistula was made and 10 μCi of [9-¹⁴C]AAF were injected i.p. with AAF (4 mg/100 g body weight). Bile collected over the subsequent 24 hr was analyzed for [9-¹⁴C]N-OGI-AAF content.

Treatment	No. of animals	% [9- ¹⁴ C]N-OGI-AAF excreted in bile
2 wk		
AAF	6	19.6 ± 3.6 ^a
AAF + indole	5	1.5 ± 1.2
4 wk		
AAF	4	4.8 ± 2.1
AAF + indole	6	1.8 ± 1.2
None	6	3.1 ± 2.8
2 wk		
Indole	5	0.5 ± 0.4
4 wk		
Indole	5	2.4 ± 1.0

^a Mean ± S.E.

dose than those on the combination diet (80.1 ± 13.0 versus 51.1 ± 14.0%; *p* < 0.01). This difference is apparently due to a larger part of the dose excreted by way of the bile duct (62.4 versus 33.4%).

After 4 weeks of treatment, no difference could be demonstrated in the urinary radioactivity as well as total radioactivity in the bile and urine excreted between the groups (AAF, AAF + indole). The inhibiting effect of indole was demonstrated in animals fed a normal diet with indole added and not carcinogen. These animals excreted 22.3 ± 5.4% of the radioactivity in the bile, while the rats fed a normal diet excreted 63.0 ± 6.5% of the test dose (*p* < 0.01). While both the normal and the 2-week AAF treatment groups excreted approximately 63% of the test dose in the bile, in the AAF group approximately 32% of these excreted biliary metabolites were N-OGI-AAF, while the 5.4% were in the *N*-hydroxylated form in the normal rats. This selective enhancement of N-OH-AAF biliary excretion by dietary AAF administration was blocked when the AAF diet was fortified with 1.6% indole (4.7%). After 4 weeks of treatment, the AAF group still excreted more radioactivity as N-OGI-AAF (16.0%) than did the AAF-indole group (6.2%), despite the fact that the total biliary radioactivity in the former dropped to the same level as that of the combination-diet group, probably due to hepatotoxicity of AAF (Table 5). Subsequent studies on this surgical procedure in our laboratory demonstrated that less than 1% of the radioactive test dose remains in the abdominal cavity after 24 hr regardless of a dietary treatment. The urinary radioactivity ranged from 17 to 26% of the injected dose, although most of the groups excreted approximately 18% of the dose. The *O*-glucuronide of N-OH-AAF in the urine ranged from 1% (normal diet) to 3.3% (4 weeks AAF + indole) of the test dose. There were no statistical differences between any of the groups (2 weeks AAF, 2.1%; 2 weeks AAF + indole 2.9%; 4 weeks AAF, 3.2%; 2 weeks indole, 2.8%; 4 weeks indole 1.8%).

Table 4
Total radioactivity excreted in 24-hr collection period
 Following specific dietary treatment, a bile duct cannula was made as described, and all bile and urine were collected for 24 hr at 0°.

Treatment	% of test dose excreted		Total
	Bile	Urine	
2 wk			
AAF	62.4 ± 9.4 ^a	17.7 ± 8.1	80.1 ± 13.0
AAF + Indole	33.4 ± 12.5	17.7 ± 5.8	51.1 ± 14.0
4 wk			
AAF	33.1 ± 15.7	21.9 ± 1.2	54.7 ± 15.1
AAF + Indole	30.7 ± 5.1	19.4 ± 3.1	50.7 ± 7.7
None	63.0 ± 6.5	16.9 ± 7.1	79.9 ± 11.4
2 wk			
Indole	22.3 ± 5.4	26.4 ± 10.8	48.5 ± 7.6
4 wk			
Indole	24.5 ± 3	13.3 ± 8.0	37.8 ± 9.0

^a Mean ± S.E.

Table 5
Biliary metabolites in the form of [9-¹⁴C]N-OGI-AAF
 Bile collected over 24 hr was analyzed for [9-¹⁴C]N-OGI-AAF. This metabolite made up only part of the metabolites excreted after an i.p. test dose of [9-¹⁴C]AAF.

	% of bile
Normal	5.4
2 wk	
AAF	32.0
AAF + indole	4.7
4 wk	
AAF	16.0
AAF + indole	6.2

Experiment B

Part 1. As shown in Chart 1, the toxicity of N-OH-AAF was well demonstrated by mortality in Group A (N-OH-AAF). Addition of Na₂SO₄ to the injection solution apparently increased the toxic effect of N-OH-AAF, as was shown by the accelerated mortality of animals in Group E. All animals in this group were dead by Day 11, with toxicity demonstrated by Day 3. The survival rates of these 2 groups (A and E) were significantly different by covariant analysis (*p* < 0.01). The presence of indole in the diet (Groups B and C) protected the animals. The administration of sulfate in these indole-treated animals (Group B) did not restore the toxicity of N-OH-AAF.

Part 2. The survival rate for these animals is shown in Chart 2. The toxicity of N-OH-AAF, as shown by Group A', was greatly enhanced by the large amount of Na₂SO₄ given concurrently in Group E'. In the presence of indole, the toxicity of N-OH-AAF was decreased (Group B', N-OH-AAF and indole). This protective effect of indole could be only partially overcome with excessive sulfate added to the injection (Group C', N-OH-AAF, indole, and Na₂SO₄). However,

this toxicity, shown by Group C', could not approach that of animals treated with N-OH-AAF alone (Group A').

Microscopic examination of the liver revealed hemorrhagic necrosis involving the perilobular parenchyma. The involvement was most pronounced in the groups of animals treated with N-OH-AAF with or without Na₂SO₄. Dietary indole clearly protected the liver and the periportal changes were minimal. No peritonitis was detected in any animals.

DISCUSSION

From the present investigation 2 findings emerged: (a) dietary indole suppressed the biliary excretion of N-OGI-AAF, the major rat liver metabolite of N-OH-AAF; (b) indole suppressed the hepatotoxicity of N-OH-AAF, with this action only partially overcome by an excessive amount of sulfate. In animals treated for 2 weeks with AAF diet, the biliary excretion of N-OGI-AAF was 19.6% of the test dose. The presence of indole in the AAF diet suppressed this biliary excretion to 1.5%, reflecting a significant difference in handling of the AAF (Table 4). Although less striking, the biliary excretion of conjugated N-OH-AAF at 4 weeks was still higher in the AAF group than the AAF-indole group (*p* < 0.025).

The urinary excretion of radioactivity remained relatively constant, regardless of the type of treatment. This suggests that dietary addition of indole may result in slower metabolism and longer retention of the carcinogen for a longer time in the body. The mechanism involved is not clear; however, it might affect glucuronide conjugation, thereby delaying excretion.

It has been clearly demonstrated that animals fed the AAF diet will develop malignant hepatic tumors in high incidence, while those fed indole concurrently will show a low incidence (12, 15, 16). Although increased biliary excretion of N-OH-AAF observed during the 1st 4 weeks in the animals treated with AAF alone does not necessarily mean more long-term active synthesis of N-OH-AAF, this is a distinct

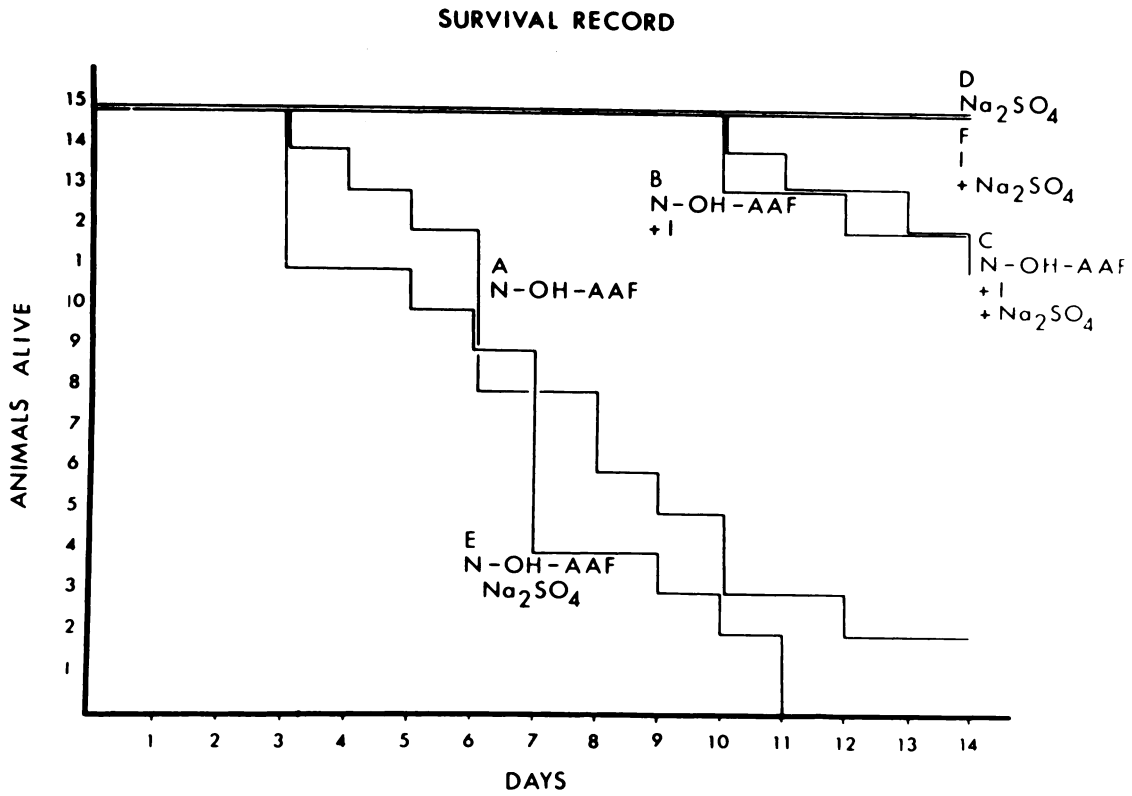


Chart 1. Animals were given daily injections (Table 1). Survival data were collected according to the number of animals alive for the 1st injection each day.

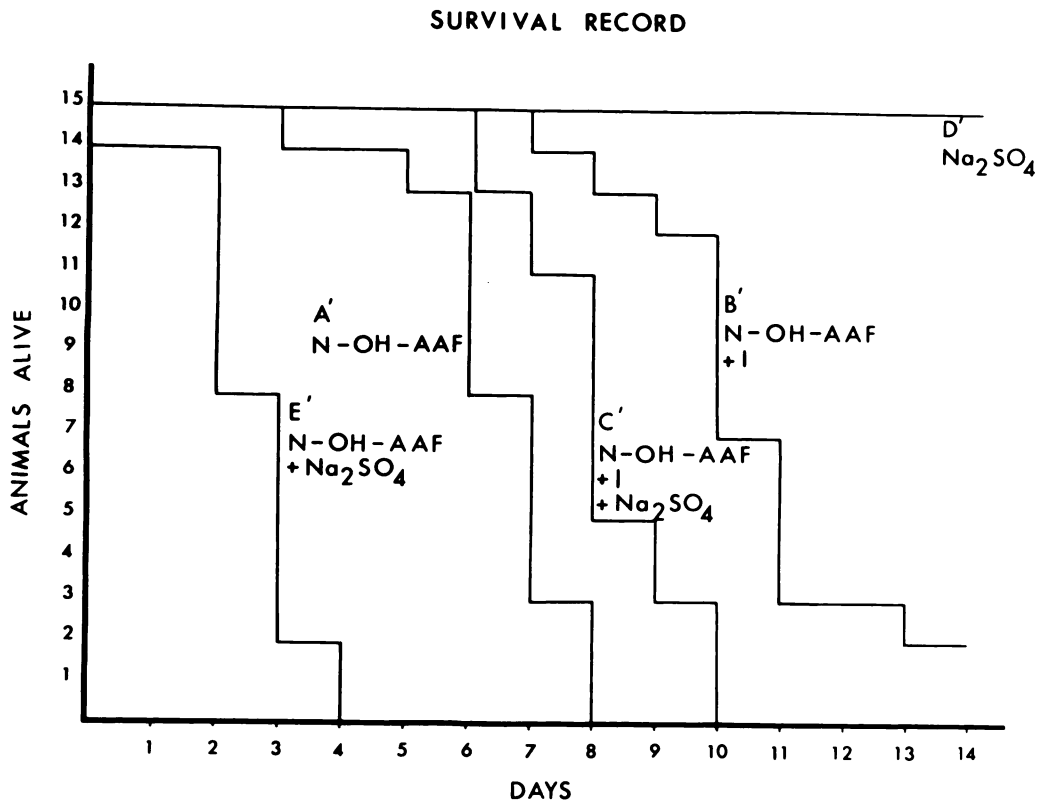


Chart 2. In Group E', one rat died shortly after the 1st injection from undetermined cause. Animals were injected daily (Table 2). Survival data were collected according to the number of animals alive for the first injection each day.

possibility. This may increase the initial incidence of, or susceptibility of animals to, malignant transformations, resulting in a high incidence of hepatic cancers.

The presence of excess sulfate has previously been shown to enhance N-OH-AAF hepatotoxicity and carcinogenicity while being treated with dietary acetanilide (4, 17). The difference in mortality rates demonstrated in Experiment B may indicate that indole plays an inhibitory role in the activation step beyond *N*-hydroxylation of AAF. The mechanisms involved are unknown, however. A possible inhibitory role of indole is that of depletion of the sulfate ions required for the final activation step. This is the mechanism offered by Weisburger *et al.* (17) to explain the inhibitory role of *p*-hydroxyacetanilide on N-OH-AAF hepatocarcinogenesis. In Experiment B, Part 1, treatment with dietary indole completely dominated over the effect of added sulfate. In Experiment B, Part 2, the amount of sulfate injected was increased, in order for it to be in excess of the amount of indole ingested by each animal. Indole again greatly decreased the mortality of the animals treated with N-OH-AAF and normal diet. However, now the protective role of indole could be partially overcome when this excess sulfate was available. There may be a competition between indole and N-OH-AAF for sulfate ions, or possibly a competition for enzyme usage. Since indole is normally excreted in the urine as the potassium salt of indoxyl sulfate (indican), the metabolism of indole could alter the sulfonation and thus the activation of N-OH-AAF.

ACKNOWLEDGMENTS

We wish to thank Dr. Charles King and Dr. Ray Cardona for consultation in the development of our analytical procedures.

REFERENCES

- Baetcke, K. P., Gough, B. J., and Shellenberger, T. E. Modified System *in vitro* for the Metabolism of 2-Acetylaminofluorene. *Biochem. Pharmacol.*, 23: 1745-1752, 1974.
- Cramer, J. W., Miller, J. A., and Miller, E. C. *N*-Hydroxylation: A New Metabolic Reaction Observed in the Rat with the Carcinogen 2-Acetylaminofluorene. *J. Biol. Chem.*, 235: 885-888, 1960.
- DeBaun, J. R., Miller, E. C., and Miller, J. A. *N*-Hydroxy-2-acetylaminofluorene Sulfotransferase: Its Probable Role in Carcinogenesis and in Protein-(methionine-S-yl) Binding in Rat Liver. *Cancer Res.*, 30: 577-595, 1970.
- DeBaun, J. R., Smith, J. Y. R., Miller, E. C., and Miller, J. A. Reactivity *In Vivo* of the Carcinogen *N*-Hydroxy-2-acetylaminofluorene: Increase by Sulfate Ion. *Science*, 167: 184-186, 1970.
- Irving, C. C. Conjugates of *N*-Hydroxy Compounds. In: W. H. Fishman (ed.), *Metabolic Conjugation and Metabolic Hydrolysis*, Vol. 1, pp. 53-119. New York: Academic Press, Inc., 1970.
- Irving, C. C., Wiseman, R. Jr., and Hill, J. T. Biliary Excretion of the O-Glucuronide of *N*-Hydroxy-2-acetylaminofluorene by the Rat and Rabbit. *Cancer Res.*, 27: 2309-2317, 1967.
- Jackson, C., and Irving, C. Sex Difference in Cell Proliferation and *N*-Hydroxy-2-acetylaminofluorene Sulfotransferase Levels in Rat Liver during 2-Acetylaminofluorene Administration. *Cancer Res.*, 32: 1590-1594, 1972.
- Lotlikar, P. D., Enomoto, M., Miller, J. A., and Miller, E. C. Species Variations in the *N*- and Ring-hydroxylation of 2-Acetylaminofluorene and Effects of 3-Methylcholanthrene Pretreatment. *Proc. Soc. Exptl. Biol. Med.*, 125: 341-346, 1967.
- Matsushima, T., and Weisburger, J. H. Inhibition of Chemical Carcinogen as Probes for Molecular Targets: DNA as Decisive Receptor for Metabolite from *N*-Hydroxy-*N*-2-fluorenylacetylamine. *Chem.-Biol. Interactions* 2: 211-221, 1969-1970.
- Miller, J. A. Carcinogenesis by Chemicals: An Overview—G. H. A. Clowes Memorial Lecture. *Cancer Res.*, 30: 559-576, 1970.
- Miller, J. A., Cramer, J. W., and Miller, E. C. The *N*- and Ring-hydroxylation of 2-Acetylaminofluorene during Carcinogenesis in the Rat. *Cancer Res.*, 20: 950-962, 1960.
- Miller, J. A., and Miller, E. C. The Metabolic Activation of Carcinogenic Aromatic Amines and Amides. *Prog. Exptl. Tumor Res.*, 11: 273-301, 1969.
- Oyasu, R., Kitajima, T., Hopp, M. L., and Sumie, H. Enhancement of Urinary Bladder Tumorigenesis in Hamsters by Coadministration of 2-Acetylaminofluorene and Indole. *Cancer Res.*, 32: 2023-2027, 1972.
- Oyasu, R., Kitajima, T., Hopp, M. L., and Sumie, H. Induction of Bladder Cancer in Hamsters by Repeated Intratracheal Administrations of 2-Acetylaminofluorene. *J. Natl. Cancer Inst.*, 50: 503-506, 1973.
- Oyasu, R., Miller, D. A., McDonald, J. H., and Hass, G. M. Neoplasms of Rat Urinary Bladder and Liver. *A. M. A. Arch. Pathol.*, 75: 184-190, 1963.
- Oyasu, R., Sumie, H., and Burg, H. E. Neoplasms of Urinary Bladder of Hamsters Treated with 2-Acetylaminofluorene and Indole. *J. Natl. Cancer Inst.*, 45: 853-860, 1970.
- Weisburger, J. H., Yamamoto, R. S., Williams, G. M., Grantham, P. H., and Matsushima, T. On the Sulfate Ester of *N*-Hydroxy-*N*-2-fluorenylacetylamine as a Key Ultimate Hepatocarcinogen in the Rat. *Cancer Res.*, 32: 491-500, 1972.
- Weisburger, J. H., and Weisburger, E. K. *N*-Oxidation Enzymes. In: B. B. Brodie and J. R. Gillette (eds.), *Handbook of Experimental Pharmacology*, Vol. 28, Part 2, pp. 312-333. Berlin: Springer-Verlag, 1971.