Reactions *in Vitro* of Some Tissue Nucleophiles with the Glucuronide of the Carcinogen *N*-Hydroxy-2-acetylaminofluorene

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(Received September 11, 1967)

SUMMARY

The metabolically formed glucuronide of *N*-hydroxy-2-acetylaminofluorene (*N*-hydroxy-AAF) is cleaved at the *N*—*O* bond in reactions *in vitro* at pH 7 with methionine, tryptophan, and guanosine. These reactions of the glucuronide (*N*-GlO-AAF) are similar to, but considerably slower than, those with esters of *N*-hydroxy-AAF such as *N*-acetoxy-AAF.

At pH 7 the major product of either *N*-acetoxy-AAF or *N*-GlO-AAF with methionine is 3-CH_3S-AAF. At pH levels greater than 7, *N*-GlO-AAF also yields considerable amounts of 3-CH_3S-2-aminofluorene (3-CH_3S-AF). Neither *N*-hydroxy-AAF nor the triacetyl methyl ester of *N*-GlO-AAF gives significant reaction with methionine at pH 5–9; with *N*-hydroxy-2-aminofluorene the reaction with methionine to yield 3-CH_3S-AF increases markedly below pH 5.5.

The reaction of *N*-GlO-AAF with guanosine appears to yield a mixture of *N*-(guanosin-8-yl)-2-acetylaminofluorene (the predominant product with *N*-acetoxy-AAF) and *N*-(guanosin-8-yl)-2-aminofluorene. The uncharacterized products formed by reaction of *N*-GlO-AAF or *N*-acetoxy-AAF with tryptophan appear to be similar.

Tumor development did not occur within 12 months after repeated subcutaneous injections of either the sodium or cupric salt or of the triacetyl methyl ester of *N*-GlO-AAF into female rats. Under the same conditions *N*-hydroxy-AAF induced high incidences of tumors in the subcutaneous tissue, mammary glands, and ear duct glands.

Whether or not metabolically formed *N*-GlO-AAF is involved in the formation of protein- and nucleic acid-bound fluorene derivatives *in vivo* and in tumor induction by AAF and *N*-hydroxy-AAF requires further investigation.

INTRODUCTION

N-Hydroxy-2-acetylaminofluorene (*N*-hydroxy-AAF) is regarded as a proximate carcinogenic metabolite of 2-acetylaminofluorene (AAF) on the basis of its formation *in vivo* and its greater carcinogenic activity as compared to that of AAF (1–7). Recent findings have suggested that esterification *in vivo* of *N*-hydroxy-AAF may lead to the ultimate carcinogenic metab-

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olite(s). Thus, two synthetic esters of *N*-hydroxy-AAF, *N*-acetoxy-AAF and *N*-benzoyloxy-AAF, are more carcinogenic at the site of subcutaneous injection in the rat than is *N*-hydroxy-AAF (E. C. Miller and J. A. Miller, unpublished data). Furthermore, these compounds and the *N*-sulfate of AAF are very reactive *in vitro* at pH 7 with certain tissue nucleophiles (methionine, tryptophan, tyrosine, cysteine, and guanosine, either free or in macromolecules), while *N*-hydroxy-AAF has little or no reactivity under these conditions (8–11). Evidence that reactive esters or similarly reactive forms of *N*-hydroxy-AAF are formed *in vivo* was obtained from the observation that 3-methylmercapto-AAF (3-CH_3S-AAF), an *in vitro* product of the reaction of esters of *N*-hydroxy-AAF with methionine (10), also can be liberated from the liver protein of rats fed *N*-hydroxy-AAF (12). Similarly, a major product obtained by enzymatic degradation of the liver RNA from rats injected with *N*-hydroxy-AAF-9-^{14}C has the same chromatographic properties (12) as *N*-(guanosin-8-yl)-2-acetylaminofluorene, formed *in vitro* by the reaction of *N*-acetoxy-AAF and guanosine (8, 9).

The glucuronide of *N*-hydroxy-AAF (*N*-GlO-AAF) (see Fig. 1) in the urine of rats and rabbits and the bile of rats fed *N*-hydroxy-AAF has been isolated and characterized by Irving and co-workers (13–17). In the view of its quantitative importance as a metabolite and its structural similarity to the synthetic esters of *N*-hydroxy-AAF, the reactivity and carcinogenicity of this glucuronide have been investigated. As reported in this paper, *N*-GlO-AAF has a definite, though low, reactivity toward several of the nucleophiles attacked by the esters, but it has not proved to be carcinogenic when administered to rats by subcutaneous injection.

MATERIALS AND METHODS

Chemicals.[2] *N*-Hydroxy-AAF (m.p. 151°) (3, 18), *N*-hydroxy-2-aminofluorene (*N*-hydroxy-AF) (18), *N*-acetoxy-AAF (m.p. 110°) (10), the triacetyl methyl ester of *N*-GlO-AAF[3] (m.p. 166°) (13), 3-CH_3S-AAF (m.p. 165°) (10, 19), and 3-CH_3S-AF[4] (m.p. 118°) (19) were synthesized by the methods indicated in the reference citations. *N*-GlO-AAF was isolated from the urine of rabbits given large oral doses of AAF or *N*-hydroxy-AAF (14, 15). The amorphous sodium salt of *N*-GlO-AAF (14) and the cupric salt of *N*-GlO-AAF were used for the carcinogenicity tests. The cupric salt of *N*-GlO-AAF was made by dissolving the sodium salt in methanol (100 ml/g) and adding a stoichiometric amount of a solution of cupric acetate monohydrate in methanol. The cupric salt was then precipitated by addition of 5 volumes of dry ether, collected, washed with ether and dried *in vacuo*. Crystalline sodium salt of *N*-GlO-AAF (15, 17) was used for the *in vitro* studies.

In vitro studies. All the incubations were carried out at 37° in a nitrogen atmosphere in a glove box with the media indicated in the tables. In all cases the fluorene compounds were dissolved, immediately before use, in solvent (water or ethanol for the glucuronide, ethanol or acetone for the other derivatives) which had been flushed with nitrogen. These solutions were added to nitrogen-flushed reaction media. When either radioactive guanosine (8-^{14}C, Schwarz BioResearch, Orangeburg, New York, or 8-^{3}H, Nuclear Chicago, Chicago, Illinois) or L-tryptophan-3-^{14}C (New England Nuclear, Boston, Massachusetts) was the nucleophile, 50–200 μl of each reaction medium was chromatographed on a cellu-

[2] The melting points were estimated to ±1° from the slopes of the melting curves obtained with the Accumelt apparatus (American Instrument Co.).

[3] Recent studies of the nuclear magnetic resonance spectrum of this compound indicate that it is a mixture of the methyl and ethyl esters. Apparently some transesterification occurred in the reaction of methyl(tri-*O*-acetyl-α-D-glucopyranosyl bromide) uronate with the potassium salt of *N*-hydroxy-AAF in absolute ethanol under slightly alkaline conditions (13).

[4] We are grateful to Dr. T. Lloyd Fletcher of the University of Washington, Seattle, Washington, for samples of 3-CH_3S-AF and 3-CH_3S-AAF.

lose (Brinkman MN300GF$_{254}$) thin-layer plate in *n*-butanol–acetic acid–water (50:11:25 by volume). After the plate was dried in a stream of warm air, each chromatogram was marked with visualization under ultraviolet light and each cellulose fraction (0.5–2 cm) was transferred quantitatively to 10 ml of scintillating fluid.[5] Media that contained all the ingredients except a fluorene derivative were similarly incubated and chromatographed; the radioactivity in these fractions was subtracted from that of the corresponding fractions of the chromatograms from samples that contained a fluorene derivative. Under these conditions the R_F ranges of the compounds (bottom to top of each spot) were as follows: tryptophan products, 0.67–1.0; guanosine, 0.35–0.44; and guanosine products, 0.55–0.70 (low R_F) and 0.75–0.95 (high R_F). Tryptophan migrated as a band about 8 mm wide, but the median R_F varied from about 0.42 to 0.55 on different lots of plates.

After reaction with L-methionine (Sigma), the medium was extracted 3 times with 15% benzene in hexane (Skelly Solve B); these extracts were washed twice with 2 N NaOH and twice with water and evaporated to dryness. The residue was then dissolved in ethyl acetate for gas chromatography (10), which provided a very sensitive and specific assay. In experiments with L-methionine-methyl-^{3}H (New England Nuclear, Boston, Massachusetts) the residue was dissolved in 15% benzene in hexane and a 0.5-ml aliquot was added to 10 ml of scintillation fluid (Liquifluor, Pilot Chemicals, Watertown, Massachusetts) for radioactivity determinations.

In several experiments homoserine was determined after reaction of *N*-GlO-AAF with methionine at pH 12.5–13 (KOH) in water or 20% acetone for 20–24 hr. For this purpose the reaction medium was neutralized with $HClO_4$, the resultant $KClO_4$ was removed by centrifugation, and the supernatant solution was made 6 N with respect to HCl. After incubation at room temperature for 2 hr, the solution was evaporated to dryness and, after electrophoresis on paper, analyzed for homoserine lactone by the ninhydrin reaction (10).

Carcinogenicity assay. Female rats with initial weights of 110–130 g of the Charles River CD-random-bred stock were injected subcutaneously in the right hind leg twice weekly for 8 weeks with either 3.0 mg of *N*-hydroxy-AAF or an equimolar amount of the sodium or cupric salt or of the triacetyl methyl ester of *N*-GlO-AAF in 0.2 ml of trioctanoin (Eastman Organic Chemicals). Each of the compounds was ground in a Mullite mortar and suspended with the aid of a magnetic stirrer, without heat, immediately prior to use. The rats were housed in pairs in screen-bottomed cages, given Wayne Breeder Blox pellets (Allied Mills, Inc., Chicago, Illinois) and water ad libitum, and weighed and examined for external tumors at monthly intervals. At death all animals were subjected to careful gross examination of the mammary tissue, subcutaneous injection site, ear duct glands, and abdominal and thoracic organs. All gross tumors or other abnormal tissues were fixed in neutral formalin, sectioned at 6 μ, and stained with hematoxylin and eosin. We are grateful to Dr. Henry Pitot of the University of Wisconsin for the histological diagnoses. The tumor incidences are presented as of 12 months. All the animals injected with *N*-hydroxy-AAF died with tumors prior to this time, whereas 45 of the 48 injected with glucuronide preparations and all the controls injected only with trioctanoin were still alive. The livers of the surviving rats were examined by laparotomy at 12 months.

RESULTS

As much as 1.3% of *N*-GlO-AAF was converted by reaction with methionine to 3-CH_3S-AAF when the reaction was carried out in the absence of an organic solvent (Table 1). Addition of either ethanol to a concentration of 13% or of acetone to a concentration of 30% reduced the yields to 0.7 and 0.1%, respectively. This reaction was independent of the pH from pH 5–9. Under the same conditions little or no 3-CH_3S-AAF was formed from the tri-

[5] Naphthalene, 738 g; diphenyloxazole, 46 g; α-naphthylphenyloxazole, 0.46 g; xylene, 3500 ml; dioxane, 3500 ml; and absolute ethanol, 2100 ml.

TABLE 1

Reactions of N-GlO-AAF, N-acetoxy-AAF, N-hydroxy-AAF, and N-hydroxy-AF with methionine at pH 5–9

Eight micromoles of fluorene derivative, 200 μmoles of L-methionine, and 200 μmoles of sodium acetate buffer (pH 4.5–5.5) or tris(hydroxymethyl)aminomethane buffer (pH 7–9) were incubated under nitrogen in 3.0 ml of water, 13% ethanol or 30% acetone for 22 hr at 37°. 3-CH_3S-AAF and 3-CH_3S-AF were determined gas chromatographically after extraction into benzene–hexane. A 0.01% reaction could be detected under these conditions; "0" indicates that less than this percentage of reaction occurred. The data reported for each solvent system were obtained in a single experiment. The data for *N*-GlO-AAF in water, for all the compounds in 13% ethanol, and for *N*-GlO-AAF and *N*-AcO-AAF in 30% acetone have been replicated in at least one other experiment.

		Percent fluorene derivative converted to					
		3-CH_3S-AAF in			3-CH_3S-AF in		
Fluorene derivative	pH	Water	13% ethanol	30% acetone	Water	13% ethanol	30% acetone
N-GlO-AAF	5	—	0.6	0.1	—	0.01	0.01
	7	1.3	0.7	0.1	0.05	0.01	0
	8	1.3	0.7	—	2.5	2.5	—
	9	1.1	0.7	0.1	5.6	5.3	1.2
Triacetyl methyl ester of *N*-GlO-AAF	5	—	0	0	—	0	0
	7	—	0	0	—	0	0
	9	—	0	0	—	0	0
N-Acetoxy-AAF	5	—	64	66	—	0	0
	7	—	56	72	—	0	0
	9	—	30	24	—	0.3	0.1
N-Hydroxy-AAF	5	—	0	0	—	0	0
	7	—	0	0	—	0	0
	9	—	0.1	0	—	0.1	0.2
N-Hydroxy-AF	4.5	—	—	0	—	—	3.9
	5	—	0	0	—	1.0	1.0
	5.5	—	—	0	—	—	0.4
	7	—	0	0	—	0.2	0.2
	9	—	0	0	—	0.1	0.5

acetyl methyl ester of *N*-GlO-AAF, *N*-hydroxy-AAF, or *N*-hydroxy-AF. On the other hand, *N*-acetoxy-AAF reacted much more strongly than the glucuronide, so that 24–66% was converted to 3-CH_3S-AAF. The relative reactivity of *N*-acetoxy-AAF, as compared to that of the glucuronide, is even greater than is indicated by this comparison, since the reaction with the unstable *N*-acetoxy-AAF was linear for about 2 hr in 13% ethanol, while the reaction with the stable glucuronide was nearly linear for 2–3 days under these conditions. The three solvent systems were used to accommodate the different solubilities of the fluorene derivatives. None of the compounds except *N*-GlO-AAF was sufficiently soluble to carry out the reaction in water, and the triacetyl methyl ester of *N*-GlO-AAF and *N*-hydroxy-AF were not completely in solution in the 13% ethanol system.

At pH 5 or 7 very little 3-CH_3S-AF was formed by incubation of *N*-GlO-AAF with methionine, whereas 2.5 and 5.5% of the glucuronide reacted to yield this product at pH 8 and 9, respectively. In other experiments in which the reaction was carried out at pH 12.5–13, 10–20% of the glucuronide was converted to 3-CH_3S-AF; in these experiments equimolar amounts of homoserine also were liberated. These data indicate that the 3-CH_3S-AF is released from a sulfonium intermediate in the same manner that 3-CH_3S-AAF is released from the

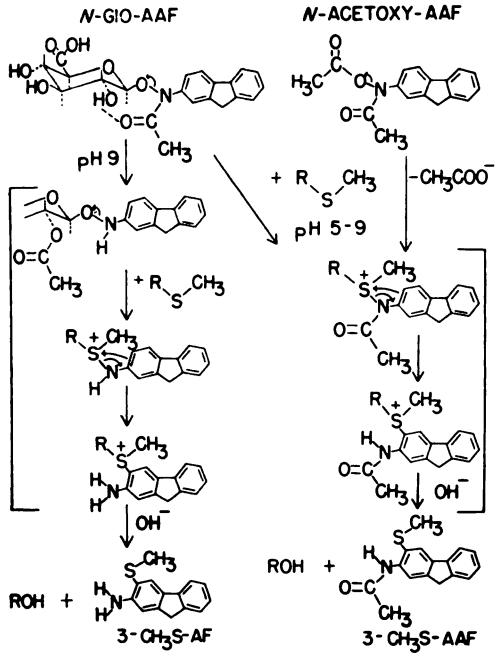

FIG. 1. Probable mechanism for the formation of 3-CH_3S-AF, 3-CH_3S-AAF, and homoserine (ROH) from *N*-GlO-AAF and methionine (R-S-CH_3) at pH values of 5–9. The reactions of methionine and its peptides with *N*-acetoxy-AAF are discussed by Lotlikar *et al.* (10).

intermediate formed by reaction of *N*-acetoxy-AAF with methionine (10) (Fig. 1). With short-term incubations decomposition of the sulfonium derivative with alkali is required to obtain maximum yields of 3-CH_3S-AAF (10). Treatment with alkali was avoided in the experiments reported in Table 1, since decomposition of the sulfonium derivatives was not a limiting factor with incubation times of 22 hr and since work-up of reaction mixtures containing *N*-GlO-AAF in the presence of alkali tended to generate small amounts of 3-CH_3S-AF.

The reaction of *N*-hydroxy-AF with methionine to yield 3-CH_3S-AF is strongly dependent on the hydrogen ion concentration. The extent of the reaction increased from 0.4% at pH 5.5 to 3.9% at pH 4.5 (Table 1). This marked effect of small changes in pH probably explains our failure to detect the reaction in our earlier test (10). Only low reactions of *N*-hydroxy-AF with methionine were found at pH 7 or 9. Low levels of 3-CH_3S-AF were formed on incubation of *N*-hydroxy-AAF or *N*-acetoxy-AAF at pH 9; none was detected at pH 5 or 7.

Like *N*-acetoxy-AAF (8, 9, 11), *N*-GlO-AAF also reacted with guanosine and tryptophan (Table 2), but to a much smaller extent. The products obtained with tryptophan had the same R_F range as those observed for the four products obtained by reaction with *N*-acetoxy-AAF.[6] In the case of guanosine, approximately one-third of the product migrated with an R_F of 0.75–0.95, while two-thirds migrated with a R_F of 0.55–0.70. Comparative assays with guanosine-8-^{14}C and guanosine-8-^{3}H showed that neither product retained the hydrogen in the 8-position of guanosine (Table 3). This finding and their R_F values indicate that the high R_F product is *N*-(guanosin-8-yl)-AAF, the major product obtained on reaction with *N*-acetoxy-AAF, and the low R_F product is *N*-(guanosin-8-yl)-AF (9). The identification of the low R_F product is further substantiated by its bright blue fluorescence, which is also exhibited by *N*-(guanosin-8-yl)-AF (9).

The reactivity *in vitro* of *N*-GlO-AAF and its formation as a major metabolite of *N*-hydroxy-AAF in the rat prompted tests of its carcinogenicity. For this purpose the sodium and cupric salts and the triacetyl methyl ester of *N*-GlO-AAF were injected subcutaneously twice weekly for 8 weeks into female rats. While *N*-hydroxy-AAF induced malignant tumors in all rats (mammary and ear duct gland carcinomas and sarcomas at the injection site), the salts of *N*-GlO-AAF and its triacetyl methyl ester have not yielded significant tumor incidences by 12 months (Table 4). The livers

[6] The reaction of *N*-acetoxy-AAF with L-tryptophan yields four ninhydrin-positive products, which are not completely separated on cellulose in the butanol–acetic acid–water system. Thin-layer chromatography on silica gel with 40% methanol in benzene resolves the mixture into three components. On rechromatography on silica gel with absolute methanol, one of these fractions is resolved into two products. The characterization of these products is in progress.

TABLE 2

Reactions of N-GlO-AAF, N-hydroxy-AAF, and N-hydroxy-AF with methionine, tryptophan, and guanosine at pH 7

One micromole of nucleophile, the designated amount of fluorene derivative, and 5 μmoles of pH 7.0 sodium citrate buffer were incubated in 0.6 ml of 16% ethanol at 37° under nitrogen. The percentage of reaction was based on the limiting reactant; i.e., the fluorene derivative in experiments 1–5 and the nucleophile in experiment 6.

			Percent reaction with 1 μmole of			
					Guanosine	
Expt. No.	Fluorene derivative (μmoles)	Incubation time (hr)	Methionine (%)	Tryptophan (%)	High R_f (%)	Low R_f (%)
1–5	*N*-GlO-AAF, 0.2	20	0.4 ± 0.2	1.5 ± 0.4	0.5 ± 0.2	1.4 ± 0.4
	N-Acetoxy-AAF, 0.2	20	30 ± 1.0	59 ± 5	65 ± 5	1.0 ± 0.5
6	*N*-GlO-AAF, 4	70	—	9.0	2.4	3.5
	N-Hydroxy-AAF, 4	70	—	<0.5	<0.15	<0.3
	N-Hydroxy-AF, 4	70	—	<0.5	<0.15	<0.3

of the rats injected with the sodium or cupric salts of *N*-GlO-AAF were grossly normal. The livers of most of the rats injected with the triacetyl methyl ester of *N*-GlO-AAF contained multiple biliary cysts; similar cysts have been observed in the livers of rats that were injected with low levels of *N*-hydroxy-AAF and that survived for one year or longer (E. C. Miller and J. A. Miller, unpublished data).

DISCUSSION

The attack of *N*-GlO-AAF on methionine, tryptophan, and guanosine at pH 7 appears to yield the same products as those formed by esters of *N*-hydroxy-AAF (8, 11), although the reaction of the glucuronide occurs at a considerably slower rate. In the reaction between *N*-GlO-AAF and guanosine, *N*-(guanosin-8-yl)-AAF and *N*-(guanosin-8-yl)-AF are formed in the proportion of about 1:2, while in the reaction of *N*-acetoxy-AAF, *N*-(guanosin-8-yl)-AAF constitutes more than 95% of the product. The *N*-acetyl group of the glucuronide is readily labilized in weak alkali (17), and it is possible that steric factors in the approach of the two relatively large molecules, *N*-GlO-AAF and guanosine, also increase the lability of the *N*-acetyl group.

As in the case of the esters of *N*-hydroxy-AAF (8–10), the reactions of the glucuronide do not appear to depend on its degradation to *N*-hydroxy-AAF and *N*-hydroxy-AF. *N*-Hydroxy-AAF gives little or no reaction at pH 5–9. *N*-Hydroxy-AF

TABLE 3

Reaction of N-GlO-AAF with guanosine-8-^{3}H and guanosine-8-^{14}C at pH 7

Each reaction mixture contained 1 μmole of guanosine and 5 μmoles of pH 7.0 sodium citrate buffer in 0.6 ml of 16% ethanol. Incubations were carried out at 37° under nitrogen; the percentage of reaction was based on the limiting reactant.

			Percent reaction			
			High R_F product		Low R_F product	
Expt. No.	*N*-GlO-AAF (μmoles)	Incubation time (hr)	8-^{14}C	8-^{3}H	8-^{14}C	8-^{3}H
1	0.2	66	1.5	0.2	3.0	0.1
2	4.0	64	2.6	0.1	4.3	0.5

TABLE 4

Assay of carcinogenicity of N-GlO-AAF by repeated subcutaneous injections in female rats

Trioctanoin (0.2 ml) containing 3.0 mg of *N*-hydroxy-AAF or an equimolar amount of a glucuronide derivative was injected subcutaneously twice weekly for 8 weeks. Each group contained 16 female rats with initial weights of 110–130 g.

Injection	Number of rats with: Mammary carcinomas, 7 mo	Mammary carcinomas, 12 mo	Sarcomas at injection site, 7 mo	Sarcomas at injection site, 12 mo	Ear duct gland carcinomas, 7 mo	Ear duct gland carcinomas, 12 mo	Number of rats alive and tumor-free 12 mo
N-Hydroxy-AAF	4	6	6	9	5	7	0
N-GlO-AAF (Na salt)	0	0	0	0	0	0	16
N-GlO-AAF (Cu salt)	1	1	0	0	0	0	14[b]
N-GlO-AAF triacetyl methyl ester	0	0[a]	0	0	0	0	15
Solvent only	0	0	0	0	0	0	16

[a] One benign mammary tumor was found at 11 months.

[b] One died with generalized lymphoma at 12 months.

is moderately reactive with methionine (Table 1) and with nucleic acid-guanine (20), at pH levels of 5 or less, but shows much less reaction at pH 7 or at the alkaline pH levels that favor the formation of 3-CH_3S-AF from *N*-GlO-AAF and methionine. The most probable explanation of the latter reaction appears to be an alkali-catalyzed migration of the *N*-acetyl group of *N*-GlO-AAF to the glucuronic acid portion, probably to the 2′ position (17), with the 2′-*O*-acetyl glucuronide of *N*-hydroxy-AF serving as the intermediate in the reaction with methionine (Fig. 1).

The type of nucleophilic substitution involved in the reactions of the glucuronide has not been determined. The reduction of the reaction rate with methionine with increases in the concentration of organic solvent suggests, however, that an ionization mechanism dependent on the dielectric constant and the ion-solvating power of the solvent is involved.

The reactivity of *N*-GlO-AAF with tissue nucleophiles is of particular interest in view of its role as a major metabolite of *N*-hydroxy-AAF in species susceptible to carcinogenesis by AAF or *N*-hydroxy-AAF (1, 6, 13, 18). While the reactivity of *N*-GlO-AAF is low, as compared to that of esters of *N*-hydroxy-AAF, the stability of the glucuronide and its formation in large amounts *in vivo* suggest that the reaction of the glucuronide with tissue constituents could be of importance in carcinogenesis by *N*-hydroxy-AAF. This possible role of the glucuronide as an ultimate carcinogenic metabolite is not supported, however, by the available data on the carcinogenicity of the glucuronide (Table 4); nevertheless, these negative data also could result from too rapid absorption and excretion of the administered glucuronide, and further tests are needed.

Note added in proof: During months 14 and 15 after the initial injection, sarcomas were found at the injection sites in 4 rats which received the triacetyl methyl ester of *N*-GlO-AAF and in 1 rat in each group treated with the sodium or cupric salt of *N*-GlO-AAF.

ACKNOWLEDGMENTS

This research was supported at the University of Wisconsin by Grants CA-07175 and CRTY-5002 of the National Cancer Institute, U.S. Public Health Service, by a grant from the Jane Coffin Childs Memorial Fund for Medical Research, and by the Alexander and Margaret Stewart Trust Fund. It was supported in Memphis by the Veterans Administration Hospital and in part, by

USPHS Research Grant CA-05490 from the National Cancer Institute.

REFERENCES

1. J. W. Cramer, J. A. Miller and E. C. Miller, *J. Biol. Chem.* **235**, 885 (1960).
2. J. A. Miller, J. W. Cramer and E. C. Miller, *Cancer Res.* **20**, 950 (1960).
3. E. C. Miller, J. A. Miller and H. A. Hartmann, *Cancer Res.* **21**, 815 (1961).
4. E. C. Miller, J. A. Miller and M. Enomoto, *Cancer Res.* **24**, 2018 (1964).
5. J. A. Miller, M. Enomoto and E. C. Miller, *Cancer Res.* **22**, 1381 (1962).
6. C. C. Irving, *Cancer Res.* **22**, 867 (1962).
7. C. C. Irving, R. Wiseman, Jr. and J. M. Young, *Cancer Res.* **27**, 838 (1967).
8. E. C. Miller, U. Juhl and J. A. Miller, *Science* **153**, 1125 (1966).
9. E. Kriek, J. A. Miller, U. Juhl and E. C. Miller, *Biochemistry* **6**, 177 (1967).
10. P. D. Lotlikar, J. D. Scribner, J. A. Miller and E. C. Miller, *Life Sci.* **5**, 1263 (1966).
11. P. D. Lotlikar, C. C. Irving, E. C. Miller and J. A. Miller, *Proc. Am. Assoc. Cancer Res.* **8**, 42 (1967).
12. J. R. DeBaun, E. C. Miller and J. A. Miller, *Proc. Am. Assoc. Cancer Res.* **8**, 12 (1967).
13. C. C. Irving, *J. Biol. Chem.* **240**, 1011 (1965).
14. J. T. Hill and C. C. Irving, *Federation Proc.* **25**, 743 (1966).
15. J. T. Hill and C. C. Irving, *Proc. Am. Assoc. Cancer Res.* **8**, 28 (1967).
16. R. Wiseman, Jr., J. T. Hill and C. C. Irving, *Proc. Am. Assoc. Cancer Res.* **7**, 76 (1966).
17. J. T. Hill and C. C. Irving, *Biochemistry* in press.
18. L. A. Poirier, J. A. Miller and E. C. Miller, *Cancer Res.* **23**, 790 (1963).
19. T. L. Fletcher, M. J. Namkung and H.-L. Pan, *J. Med. Chem.* **10**, 936 (1967).
20. E. Kriek, *Biochem. Biophys. Res. Commun.* **20**, 793 (1965).