Lack of N-Hydroxy-2-acetylaminofluorene Sulfotransferase Activity in the Mammary Gland and Zymbal's Gland of the Rat

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SUMMARY

The O-sulfonate of N-hydroxy-2-acetylaminofluorene was not formed in vitro in the mammary gland or in the sebaceous gland of the external auditory canal (Zymbal's gland), tissues of the rat that are highly susceptible to carcinogenesis by 2-acetylaminofluorene and N-hydroxy-2-acetylaminofluorene. Consequently, the O-sulfonate of N-hydroxy-2-acetylaminofluorene, reported to be an ultimate reactive metabolite of these carcinogens in male rat liver, does not appear to be involved in the mechanism of carcinogenesis in all tissues of the rat in which tumors are induced by these compounds.

INTRODUCTION

In 1960, Cramer et al. (2) reported a new type of metabolic reaction, N-hydroxylation, undergone by carcinogetic aromatic acetylamines, such as AAF. Although a great deal of subsequent evidence clearly implicated N-hydroxy metabolites of carcinogetic aromatic amines in the mechanism of action of these compounds (reviewed in Ref. 15), the fact that the N-hydroxy derivatives showed only limited reactivity in vitro with biological macromolecules led to the speculation that further metabolic activation of the N-hydroxy metabolites might be necessary. Recent evidence has indicated that O-conjugates of N-hydroxy metabolites of carcinogetic aromatic acetylamines are the reactive metabolites in the liver (reviewed in Refs. 7 and 15). AAF-N-sulfate appears to be one of the ultimate reactive metabolites of the carcinogens AAF and N-hydroxy-AAF in rat liver. AAF-N-sulfate is formed in vitro (3) and probably in vivo (4) by the action of a liver sulfotransferase, the sulfate group being transferred to N-hydroxy-AAF from PAPS. AAF-N-sulfate is very reactive and is unstable in aqueous solutions, with a half-life of less than 1 min (15). The formation of this sulfonate conjugate in vitro has been detected indirectly by trapping it in situ by reaction with methionine or GMP, or with proteins and nucleic acids containing these nucleoephiles (3, 12).

AAF and N-hydroxy-AAF induce tumors in tissues other than the liver of the rat. Because of the reactivity and instability of AAF-N-sulfate, it is extremely unlikely that this metabolite is transported from the liver to extrahepatic tissues. Consequently, the question of whether AAF-N-sulfate is formed in other tissues becomes an important one. We wish to report results of studies on 2 extrahepatic tissues of the rat which are most highly susceptible to carcinogenesis by AAF and N-hydroxy-AAF, namely the mammary gland and the sebaceous glands associated with the external auditory meatus (Zymbal's gland (20)).

MATERIALS AND METHODS

Animals

Albino rats were obtained from the Holtzman Company, Madison, Wis. Male rats weighing 200 to 300 g were used in all experiments except those involving mammary gland, for which female rats weighing 160 to 180 g were used. The rats were maintained on Purina laboratory chow and water.

Compounds

N-Hydroxy-AAF-9,[14C]-2-[3H] (specific radioactivity, 45 mCi [3H]/mmole and 10 mCi [14C]/mmole) and N-hydroxy-AAF-9-[14C] (specific radioactivity, 4.51 mCi/mmole) were synthesized as described by Irving and Veazey (9). The radiochemical purities of these compounds were established by thin-layer chromatography (9).

Authentic 1- and 3-methylmercapto-AAF and the corresponding -AF compounds were generously provided by Dr. James Miller, University of Wisconsin, Madison, Wis., and by Dr. T. Lloyd Fletcher of the University of Washington, Seattle, Wash.

PAPS was prepared biosynthetically. Sulfate-activating enzymes were purified from liver as described by Robbins (17), except that rat liver (approximately 100 g) was used. For the synthesis of PAPS, the incubation mixture contained, in a final volume of 60 ml, Tris-HCl, pH 7.7, 0.75 mmole; ATP, 0.9 mmole; MgCl2, 0.75 mmole; K2SO4, 1.2 mmoles; cysteine, 0.6 mmole; and 240 mg of protein from the rat liver enzyme fraction obtained as described above. Solutions of ATP and cysteine were prepared fresh and adjusted to pH 7 with saturated KHCO3 just before use. After incubation at 37° for 2 hr, the mixture was heated in a boiling water bath for 3 min and then cooled and filtered. The precipitate was washed with 100 ml of 0.05 M ammonium bicarbonate. The filtrate and washings were combined and applied to a column (2.5 x

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Measurement of PAPS-dependent Activation of N-Hydroxy-AAF

Livers of male rats were homogenized in 0.1 M Tris-HCl, pH 7.4 (3.3 ml/g), and the homogenate was centrifuged at 38,000 rpm in a Spinc No. 40 rotor for 1 hr. The supernatant (soluble fraction) was used for assay of sulfotransferase activity (3). Zymbal’s glands were carefully dissected from male rats. Tissue from 14 to 16 animals was pooled (420 to 480 mg tissue) and homogenized in 2 to 3 ml of Tris buffer; a soluble fraction of the homogenate was prepared as described above. The whole mammary gland (abdominal-inguinal glands) was obtained from immature female rats (50 to 60 days old) or from pregnant rats on the 15 to 18th day of pregnancy. Mammary gland parenchymal cells, free of adipose tissue cells, were prepared from whole mammary gland by the procedure of Moon et al. (16). Whole mammary gland tissue or the mammary gland parenchymal cells were homogenized in Tris buffer (2 to 3 ml/g tissue), and the soluble fraction was obtained as above.

Method A: Use of Methionine to Trap the Intermediate AAF-N-sulfate. Determination of sulfotransferase activity with N-hydroxy-AAF as a substrate was carried out by the procedure in which the unstable AAF-N-sulfate which is formed is trapped by reaction with methionine, yielding as an end product a mixture of isomeric 1- and 3-methylmercapto-AAF, designated hereinafter as o-methylmercapto-AAF (3). Each tube contained, in a final volume of 1.0 ml, 40 ¡/mole Tris-HCl, pH 7.4; 40 ¡/mole methionine; 0.65 ¡mole PAPS; 5.0 ¡mole MgCl2; and 0.4 ¡mole N-hydroxy-AAF or N-hydroxy-AAF-9-14C. Soluble fraction containing 1 mg of protein (liver, 0.05 ml; mammary gland, 0.40 ml; and Zymbal’s gland, 0.25 ml) was added, the mixture was incubated at 37° for 30 min (liver) or for 1 hr (other tissues), and the tubes were then processed as described by DeBaun et al. (3). Actual protein concentrations were determined by analysis in each experiment. o-Methylmercapto-AAF, if formed, was extracted into benzene:hexane (30:70, v/v) and was determined by gas-liquid chromatography on 3% OV 1 on GasChrom Q (60 to 80 mesh) (Applied Science Laboratories, Inc., State College, Pa.) at 220° with a flame ionization detector. The retention time of o-methylmercapto-AAF was 9 min. Because of the relatively larger amounts of extraneous material in the soluble fraction of the mammary gland and Zymbal’s gland, which interfered in the gas-liquid chromatography analysis, and the need to retain or increase the sensitivity of the method, gas-liquid radiochromatography (19) was used to examine the extracts of the incubation mixtures from these tissues. For these analyses, a programmed temperature increase from 160—220° (3°/min), followed by isothermal operation at 220° for 15 min, was used. The retention time of o-methylmercapto-AAF was 26 min under these conditions. The effluent stream from the gas-liquid chromatography column was combusted over CuO at 75° in a Packard Model 325P furnace to yield 14CO2 and water. The water was trapped by Aquasorb (Mallinckrodt Chemical Works, St. Louis, Mo.), and the 14CO2 was measured in a proportional counter with a Packard Model 893 ratemeter. With this modification in the procedure in which gas-liquid radiochromatography was used, the sensitivity of the method was such that 0.05 ¡g of o-methylmercapto-AAF could have been detected in the injected sample of the extracts of mammary gland and Zymbal’s gland.

Method B: Use of Yeast tRNA to Trap the Intermediate AAF-N-sulfate. The PAPS-dependent binding of N-hydroxy-AAF-9-14C-2'-3H to tRNA by the soluble fraction of liver, Zymbal’s gland, and mammary gland was also studied. Soluble fraction containing approximately 1 mg of protein was added to a solution containing 100 ¡/mole of Tris-HCl, pH 7.4; 2 ¡mole of yeast tRNA; and 100 ¡/mole) of N-hydroxy-AAF-9-14C-2'-3H, in a final volume of 1 ml. Other additions to this basic incubation system, where indicated, were ATP, 10 ¡/mole; MgCl2, 5 ¡/mole; Na2SO4, 10 ¡/mole; and PAPS, 0.65 ¡/mole. The mixture was incubated at 37° for 1 hr, then it was extracted twice with an equal volume of phenol (saturated with water), and the nucleic acid was precipitated from the aqueous phase by the addition of 3 ml of ice-cold ethanol. After standing at —20° for 3 hr, the mixture was centrifuged and the precipitate was collected and dissolved in 1 ml of 0.1 M Tris-HCl, pH 7.4. One ml of 0.2 ¡g sodium acetate and 1 ml of 1% cetyltrimethylammonium bromide were added and the mixture was left at 2° overnight. The cetyltrimethylammonium salt of the RNA was collected by centrifugation, washed twice with ice-water, and then dissolved in 1 ml of methanol. Three ml of cold 3% potassium acetate in methanol were added, and the potassium salt of the RNA that precipitated was collected and dissolved in 1 ml of 0.1 M Tris-HCl, pH 7.4. One ml of 0.2 ¡g sodium acetate and 1 ml of 1% cetyltrimethylammonium bromide were added and the mixture was left at 2° overnight. The cetyltrimethylammonium salt of the RNA was collected by centrifugation, washed twice with ice-water, and then dissolved in 1 ml of methanol. Three ml of cold 3% potassium acetate in methanol were added, and the potassium salt of the RNA that precipitated was collected and dissolved in 1 ml of 0.1 M Tris-HCl, pH 7.4. Nucleic acid concentration was determined from the absorbance at 260 nm, and radioactivity was determined by liquid scintillation counting in the Triton X-100 system (8). Use of the procedure described was essential in order to obtain tRNA with a specific radioactivity. After purification of the tRNA by this procedure, the specific radioactivity did not change when the sample was further purified by chromatography on Sephadex G-25.
**N-Hydroxy-AAF Sulfotransferase**

**Recovery of PAPS from Incubation Mixtures**

The extent of disappearance of PAPS in complete incubation mixtures (Method B) containing soluble fraction of liver, mammary gland parenchymal cells, or Zymbal's gland was determined. Following incubation at 37°, the contents of 6 tubes (for each tissue) were combined and heated at 100° for 3 min. After cooling and centrifugation, the amount of PAPS in the supernatant was determined by column chromatography on a QAE-Sephadex A-25 column (0.9 x 15 cm) with a linear gradient of 100 ml each of 0.05 M and 1 M ammonium bicarbonate as described above.

**Recovery of N-Hydroxy-AAF-9-14C-2'-3H from Incubation Mixtures**

The amount of N-hydroxy-AAF-9-14C-2'-3H remaining in complete incubation mixtures (Method B) was determined by inverse isotope dilution with carrier N-hydroxy-AAF. After incubation at 37°, the contents of single tubes were added to a solution containing 50 mg of N-hydroxy-AAF in 3 ml of ethanol. The N-hydroxy-AAF was isolated, and the specific radioactivity was determined as previously described (6).

**Protein Analyses**

Protein was determined by the procedure of Lowry et al. (14) with bovine serum albumin as a standard.

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**RESULTS AND DISCUSSION**

The N-hydroxy-AAF sulfotransferase activities found in male rat liver were in agreement with data reported earlier by DeBaun et al. (3). In the assay used, the O-sulfonate of N-hydroxy-AAF that is formed is trapped by reaction with methionine to yield a mixture of 1- and 3-(methion-S-yl)-AAF, which is later decomposed by heat and alkali to give 1- and 3-methylmercapto-AAF. With the soluble fraction from male rat liver, 20 to 30 μg of o-methylmercapto-AAF were formed in 30 min/mg of protein (Table 1). The formation of o-methylmercapto-AAF from N-hydroxy-AAF and methionine by rat liver was completely dependent upon the addition of either PAPS or a PAPS-generating system (ATP, magnesium ion, sulfate ion) as described previously (3). However, the formation of o-methylmercapto-AAF from N-hydroxy-AAF, PAPS, and methionine by the soluble fraction of whole mammary gland from immature rats or from mammary gland parenchymal cells isolated from this tissue or by the soluble fraction of Zymbal's gland was not detectable (Table 1). The sensitivity of our method was such to permit detection of sulfotransferase activity in which 0.05 μg of o-methylmercapto-AAF/mg protein/30 min was formed. This is less than 1/500th the activity in male rat liver and is approximately 1/100th the activity reported for female rat liver (3). The liver of the male rat is highly susceptible to carcinogenesis by AAF and N-hydroxy-AAF, whereas the liver of the female rat is resistant to the carcinogenic activity of these compounds.

In further attempts to determine whether there was metabolic activation of N-hydroxy-AAF by esterification in mammary gland and Zymbal's gland, the question of the PAPS-dependent binding of N-hydroxy-AAF to tRNA by the soluble fraction of these tissues was examined. The use of N-hydroxy-AAF-9-14C-2'-3H in these experiments afforded a means of discriminating between covalent binding of AAF residues (due to reaction with N-hydroxy-AF, arising by deacetylation of N-hydroxy-AAF) and the binding of AAF residues (due to activation of N-hydroxy-AAF by esterification) [see King and Phillips (12) for further discussion]. With male rat liver soluble fraction, conditions that favored conjugation of N-hydroxy-AAF with sulfate (addition of ATP, magnesium ion, sulfate ion, or addition of PAPS and mag...
nesium ion) led to 30- to 35-fold increase in the binding of \( N \)-AAF residues to tRNA (Table 2). The binding of AF residues to tRNA by rat liver soluble fraction was not influenced under conditions favoring esterification (Table 2). On the other hand, there was no detectable PAPS-dependent activation of \( N \)-hydroxy-AAF by the soluble fraction of mammary gland parenchymal cells (immature rats) or by Zymbal's gland (Table 2). Additional experiments (data not shown) revealed similar results, with whole mammary gland (immature rats) or with mammary gland tissue or mammary gland parenchymal cells from pregnant rats, i.e., no activation of \( N \)-hydroxy-AAF.

Most known sulfotransferases are found in the soluble fraction of homogenates of tissues containing these enzymes (1, 5). However, in order to examine the possibility that sulfotransferase activity might be localized in some particulate fraction of homogenates of cells of mammary gland or Zymbal's gland, the experiments described in Table 2 were repeated with whole-tissue homogenates instead of the soluble fraction. Similar results as reported in Table 2 were obtained.

The inability of mammary gland or Zymbal's gland of the rat to form the \( O \)-sulfonate of \( N \)-hydroxy-AAF appears to be due to lack of a sulfotransferase in these tissues, which can use \( N \)-hydroxy-AAF as a substrate rather than some other reason such as a high rate of destruction of \( N \)-hydroxy-AAF or PAPS. Thus, there was no inhibitor of \( N \)-hydroxy-AAF sulfotransferase present in these tissues, since we found PAPS-dependent activation of \( N \)-hydroxy-AAF by soluble fraction of male rat liver in the presence of equivalent amounts (in terms of mg of protein) of either Zymbal's gland or mammary gland. PAPS was not limiting in the incubation mixtures containing soluble fraction from mammary gland or Zymbal's gland since we were able to recover 85% of the added PAPS after 1 hr incubation. Furthermore, addition of 9-fold amounts of PAPS compared to the usual concentration of PAPS did not change the results reported in Table 2. Finally, failure to detect \( N \)-hydroxy-AAF sulfotransferase activity in these tissues was not due to a rapid disappearance of the \( N \)-hydroxy-AAF by other metabolic pathways, since 85 to 100% of the \( N \)-hydroxy-AAF added could be recovered after 1 hr incubation with the soluble fraction of mammary gland or Zymbal's gland homogenates.

These data indicate that the \( O \)-sulfonate of \( N \)-hydroxy-AAF is not the ultimate reactive metabolite of AAF and \( N \)-hydroxy-AAF in all tissues in which tumors are induced by these carcinogens. On the basis of current knowledge of the metabolism of AAF and \( N \)-hydroxy-AAF (7, 15), several candidates for the proximate metabolite of these carcinogens come to mind: (a) \( N \)-hydroxy-AAF itself, which has been reported to have limited but detectable reactivity with nucleic acids in vitro (10); (b) the glucuronide of \( N \)-hydroxy-AAF (10, 11) or \( N \)-hydroxy-AF (8); and (c) \( N \)-hydroxy-AF (12, 13). The need for further study of the metabolism of these carcinogens in extrahepatic tissues is obvious.

REFERENCES


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