was taken up into ethyl acetate and washed with water. The water layers were passed through an ion-exchange column (3.4 × 25 cm, Dowex 50W-8X, K^+ form, 50–100 mL of eluant). Freeze-drying left the potassium salts as yellow flaky residues. We were unable to obtain satisfactory field desorption or fast atom bombardment mass spectral data on any of these potassium salts.

Potassium [[3(*S*)-[2-(2-amino-4-thiazolyl)-2(*Z*)-(methoxyimino)acetamido]-2-oxo-1-azetidinyl]oxy]acetate (25) was prepared from **24g** by this method in 92% yield: ^{1}H NMR (D_2O) δ 3.9 (dd, 1 H), 4.0 (s, 3 H), 4.2 (t, 1 H), 4.5 (s, 2 H), 5.0 (m, 1 H), 7.1 (s, 1 H); IR (KBr) 3700–2800, 1760 cm^{-1}; reversed-phase TLC (2-propanol/water (1:1)) R_f 0.76; paper chromatography (2-propanol/water (7.5:2.5)) R_f 0.40.

Potassium [[4(*S*)-methyl-3(*S*)-[(2-amino-4-thiazolyl)-2-(*Z*)-(methoxyimino)acetamido]-2-oxo-1-azetidinyl]oxy]-acetate (26) was prepared from **24h** in 72% yield: ^{1}H NMR (D_2O) δ 1.5 (d, 3 H), 4.0 (s, 3 H), 4.35 (m, 1 H), 4.55 (s, 2 H), 4.6 (m, 1 H), 7.1 (s, 1 H); IR (KBr) 3700–2800, 1770 cm^{-1}.

Potassium [[4(*S*)-methyl-3(*S*)-[(2-amino-4-thiazolyl)-acetamido]-2-oxo-1-azetidinyl]oxy]acetate (31) was prepared from **30** in 91% yield: ^{1}H NMR (D_2O) δ 1.45 (d, 3 H), 3.55 (s, 2 H), 4.2 (m, 1 H), 4.35 (d, 1 H), 4.5 (s, 2 H), 6.6 (s, 1 H); IR (KBr) 3600–2800, 1760 cm^{-1}.

Potassium 2(*R*)-[[4(*S*)-methyl-3(*S*)-[2-(2-amino-4-thiazolyl)-2(*Z*)-(methoxyimino)acetamido]-2-oxo-1-azetidinyl]-oxy]-3-acetoxypropionate (37a) was prepared from **36a** in 55% yield: ^{1}H NMR (D_2O) δ 1.5 (d, 3 H), 2.1 (s, 3 H), 4.0 (s, 3 H), 4.35 (m, 1 H), 4.65 (d, 1 H), 4.70 (m, 3 H total), 7.1 (s, 1 H); IR (KBr) 3700–2800, 1760, 1730 cm^{-1}; reversed-phase TLC (2-propanol/water (1:1)) R_f 0.81; paper chromatography (2-propanol/water (7.5:2.5)) R_f 0.72.

Potassium 2(*R*)-[[4(*S*)-methyl-3(*S*)-[2-(2-amino-4-thiazolyl)-2(*Z*)-(methoxyimino)acetamido]-2-oxo-1-azetidinyl]-oxy]propionate (37b) was prepared from **36b** in 72% yield: ^{1}H NMR (D_2O) δ 1.6 (pair of d, 6 H total), 4.05 (s, 3 H), 4.3 (m, 1 H), 4.6 (d, 1 H), 4.8 (1 H under H_2O peak), 7.0 (s, 1 H); IR (KBr) 3600–2800, 1755 cm^{-1}; reversed-phase TLC (2-propanol/water (1:1)) R_f 0.70; paper chromatography (2-propanol/water (7.5:2.5)) R_f 0.75.

Acknowledgment. We gratefully acknowledge the support from the National Institutes of Health (NIH), Eli Lilly and Co., and a Reilly Fellowship (1983–1985) for S.R.W. We are grateful to the Lilly group, especially Dr. Robin D. G. Cooper, Byron Daugherty, and Dr. John L. Ott, for gifts of **21** and **28** and for the biological tests. Kathleen Peterson obtained the 300-MHz NMR spectra, using an NMR spectrometer made available by grants from the NIH and the University of Notre Dame.

Registry No. **7**, 1145-80-8; **8** (R = H), 645-88-5; **8a**, 25184-48-9; **8b**, 46230-31-3; **8b**·HCl, 97486-16-3; **8c**, 97486-17-4; **8c** tosylate, 97486-18-5; **9a**, 97486-19-6; **9b**, 97486-20-9; **9c**, 97486-21-0; **10a**, 97486-22-1; **10b**, 97486-23-2; **10c**, 97486-24-3; **10e**, 90849-46-0; **10f**, 90848-81-0; **10g**, 93589-32-3; **10h**, 97486-25-4; **13a**, 83105-75-3; **13b**, 93589-31-2; **18a**, 82933-25-3; **18b**, 82933-31-1; **19a**, 93589-28-7; **19b**, 93589-29-8; **20a**, 93589-30-1; **20b**, 93712-27-7; **21**, 71445-20-0; **23f**, 93589-33-4; **23g**, 93589-34-5; **23h**, 97486-32-3; **24a**, 97486-26-5; **24f**, 90857-56-0; **24g**, 93589-35-6; **24h**, 97486-27-6; **25**, 90849-05-1; **25** (acid), 97486-43-6; **26**, 93712-30-2; **26** (acid), 90898-90-1; **28**, 64220-26-4; **29**, 97486-33-4; **30**, 97486-34-5; **31**, 97486-40-3; **31** (acid), 97589-46-3; **32**, 97486-35-6; **32** (acid), 62076-21-5; **33**, 97486-36-7; **34a**, 97486-38-9; **34a** (acid), 97486-37-8; **34b**, 97486-39-0; **34b** (acid chloride), 22592-73-0; **35a**, 97486-29-8; **35b**, 97486-31-2; **36a**, 97486-28-7; **36b**, 97486-30-1; **37a**, 97486-41-4; **37a** (acid), 97588-27-7; **37b**, 97486-42-5; **37b** (acid), 97588-28-8; $BrCH_2CO_2CH_3$, 96-32-2; $BrCH_2CO_2CH_2Ph$, 5437-45-6; $BrCH_2CO_2C(CH_3)_3$, 5292-43-3; $BrCH_2CO_2CH_2CH_2Si(CH_3)_3$, 79414-13-4; $HOCH_2CH_2Si(CH_3)_3$, 2916-68-9; $(PhCH_2CO)_2O$, 1555-80-2; *O*-benzyl-L-serine, 4726-96-9; *O*-acetyl-L-serine, 5147-00-2.

Substituent Effects on the Bioactivation of 2-(*N*-Hydroxyacetamido)fluorenes by *N*-Arylhydroxamic Acid *N*,*O*-Acyltransferase

Adnan A. Elfarra[†] and Patrick E. Hanna*[‡]

Departments of Medicinal Chemistry and Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455.
Received January 22, 1985

A series of 7-substituted analogues of 2-(*N*-hydroxyacetamido)fluorene (1) was subjected to bioactivation by a partially purified preparation of hamster hepatic AHAT, and the rates of methylthio adduct formation resulting from the reaction of the activated intermediates with *N*-acetylmethionine were determined. Electronegative substituents enhanced the amount of adduct formed; this finding contrasted with the results of a previous study in which it was found that electron-donating substituents facilitated the mechanism-based inactivation of AHAT by analogues of 1. The structures of the adducts formed from reaction of the activated forms of several of the 7-substituted compounds with *N*-acetylmethionine and with 2′-deoxyguanosine were determined; the types of adducts formed were similar to those formed with electrophiles generated by the AHAT-catalyzed activation of 1. Electronegative substituents enhanced the amount of adducts formed in the reaction with 2′-deoxyguanosine as well as with *N*-acetylmethionine.

The relationship between the mutagenicity and/or carcinogenicity of numerous organic compounds and the covalent binding of such agents to cellular macromolecules has become well established during the past 25 years.[1] In addition to the role of covalent interactions in the production of genotoxicity, a variety of other toxic effects may be attributed to reactions between xenobiotics and critical functional groups on cellular macromolecules.[2]

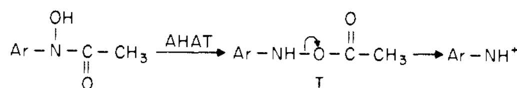
A number of arylamines and arylamides are included in that group of organic molecules that produce at least some of their untoward effects subsequent to covalent binding to cellular constituents. The metabolism and toxicological properties of arylamines and arylamides are of interest because of human exposure due to their industrial applications and to their presence as structural components of herbicides, pesticides, drugs, and hair dyes.[3-6] The

† Department of Medicinal Chemistry.
‡ Departments of Medicinal Chemistry and Pharmacology.

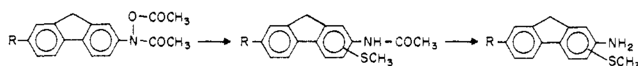
(1) Miller, E. C.; Miller, J. A. *Adv. Exp. Biol. Med.* **1982**, *136A*, 1.
(2) Nelson, S. D. *J. Med. Chem.* **1982**, *25*, 753.
(3) Cartright, R. A. *EHP, Environ. Health. Perspect.* **1983**, *49*, 13.
(4) Dybing, E.; Saxholm, H. J. K.; Aune, T.; Wirth, P. J.; Thorgeirsson, S. S. *Natl. Cancer Inst. Monogr.* **1981**, *No. 58*. 21.
(5) Clayson, D. B.; Garner, R. C. In "Chemical Carcinogens"; Searle, C. E., Ed.; American Chemical Society: Washington, DC, 1976; Chapter 8.

 © 1985 American Chemical Society

Scheme I



Scheme II



9, R = H
10, R = F
11, R = Br
12, R = $COCH_3$
13, R = OCH_3

14, R = H
15, R = F
16, R = Br
17, R = OCH_3

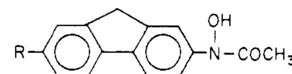
18, R = H
19, R = F
20, R = Br
21, R = OCH_3

pioneering work of the Miller laboratory provided incontrovertible evidence that carcinogenic arylamines and arylamides are metabolically activated by N-hydroxylation followed by conversion of the N-hydroxylated products to electrophilic species that react with nucleophilic sites on cellular constituents.[7] In particular, 2-aminofluorene and 2-acetamidofluorene have been used extensively as models in the investigation of the enzymatic and chemical mechanisms involved in the bioactivation of arylamines and arylamides.[8]

2-(*N*-Hydroxyacetamido)fluorene (1) is a prominent example of a carcinogenic *N*-arylhydroxamic acid that is converted enzymatically to an electrophilic reactant. One enzyme system that is capable of effecting this conversion is *N*-arylhydroxamic acid *N*,*O*-acyltransferase (AHAT), which is present in the cytosolic fraction of cells of many mammalian tissues.[9,10] The mechanism whereby AHAT catalyzes the production of electrophilic reactants is believed to involve the transfer of the acyl group of the *N*-arylhydroxamic acid from the nitrogen atom to the oxygen atom; the resulting *N*-acetoxy intermediate undergoes heterolytic cleavage of the N–O bond to produce an arylnitrenium ion (Scheme I).[9,10] Resonance-stabilized arylnitrenium–carbenium ions are believed to be the key reactive species in AHAT-catalyzed bioactivations as well as in certain related bioactivation processes.[11] Because these electrophilic reactants are positively charged, it would be expected that the presence of electronegative or electropositive aromatic ring substituents might influence the rate and extent of their reaction with nucleophilic functional groups. It is also possible that such substituents could influence the type of products formed.

This report describes the results of an investigation of the effects of various 7-substituents on the AHAT-catalyzed activation of 2-(*N*-hydroxyacetamido)fluorenes as measured by the reaction of the activated compounds with *N*-acetylmethionine and with 2′-deoxyguanosine (2′-dG). The structures of the products of the reaction with *N*-acetylmethionine and with 2′-dG were determined for several selected compounds.[12]

Chemistry. The synthesis and characterization of the 7-substituted 2-(*N*-hydroxyacetamido)fluorenes (1–8) have



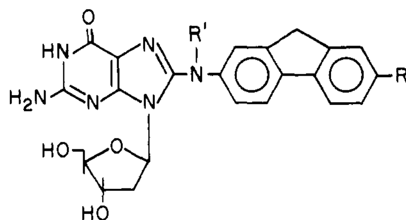
1, R = H
2, R = F
3, R = Br
4, R = I
5, R = CN
6, R = $COCH_3$
7, R = OCH_3
8, R = $O(CH_2)_3CH_3$

been reported.[13] The *N*-acetoxy derivatives (**9–13**; Scheme II) of compounds **1–3**, **6**, and **7** were prepared by acetylation of the appropriate *N*-arylhydroxamic acids with acetic anhydride in pyridine.[14] The *N*,*O*-diacetyl derivatives **9–11** and **13** were then used for the preparation of methylthio adducts of 7-substituted 2-aminofluorenes **18–21** (Scheme II).

The reaction of the *N*-acetoxy compounds **9–11** and **13** with *N*-acetylmethionine was carried out according to the method of Lotlikar et al.[15] This reaction resulted in the formation of mixtures of the 1- and 3-methylthio regioisomers of **14** and **15** in ratios of approximately 1:3; the 1- and 3-isomers of **17** were formed in equal amounts, and the 7-Br product **16** consisted of a mixture of 1- and 3-methylthio adducts in a ratio of approximately 1:8. The ratios of the isomers were readily estimated by integration of either the 1- and 3-SCH_3 peaks or the 9-CH_2 resonance bands of the NMR spectra as described by Westra et al. for **14**.[16] The 1-SCH_3 protons exhibit a smaller chemical shift than the 3-SCH_3 protons, and the 9-CH_2 protons of the 1-isomers appear at a position somewhat downfield relative to those of the 3-isomers. Treatment of the amides **14–17** under acidic conditions yielded the primary amines **18–21**. Compounds **18–21** were used as chromatographic standards for comparison with products formed from the reaction of electrophilic intermediates with *N*-acetylmethionine in large-scale experiments with AHAT.

The reaction of the *N*-acetoxy derivatives **9** and **10** with 2′-dG at neutral pH, as described by Kriek et al., afforded the amide adducts **22** and **23**, respectively.[17] Hydrolysis of **22** and **23** with NaOH yielded the expected secondary amines **24** and **25**. Others have reported that treatment of **22** with NaOH results in cleavage of the 7–8 guanine bond to produce significant quantities of pyrimidine derivatives.[17b] An 80% yield of **24** was obtained in the present study by using lower concentrations of NaOH and shorter reaction times than were used in previously reported experiments. Kriek and Westra also found that pyrimidine formation could be avoided by using 25% am-

(6) Lotlikar, P. D. In "Carcinogens in the Industry and the Environment"; Sontag, J. M., Ed.; Marcel Dekker: New York, 1981; Chapter 9.
(7) Miller, J. A.; Miller, E. C. *EHP, Environ. Health. Perspect.* **1983**, *49*, 3.
(8) Thorgeirsson, S. S.; Glowinski, I. B.; McManus, M. E. In "Reviews in Biochemical Toxicology"; Hodgson, E., Bend, J. R., Philpot, R. M., Eds.; Elsevier Biomedical: New York, 1983; Vol. 5, p 349.
(9) Bartsch, H.; Dworkin, M.; Miller, J. A., Miller, E. C. *Biochim. Biophys. Acta* **1972**, *286*, 272.
(10) King, C. M. *Cancer Res.* **1974**, *34*, 1503.
(11) Kriek, E. *Prog. Clin. Biol. Res.* **1983**, *132B*, 161.
(12) A preliminary report on portions of this work has been presented. Hanna, P. E.; Marhevka, V. C.; Elfarra, A. A.; Ebner, N. A.; Sehon, R. D. "Abstracts of Papers", 183rd American Chemical Society National Meeting, Las Vegas, NV, April, 1982; American Chemical Society: Washington, DC, 1982; Abstr MEDI 89.
(13) Marhevka, V. C.; Ebner, N. A.; Sehon, R. D.; Hanna, P. E. *J. Med. Chem.* **1985**, *28*, 18.
(14) Zieve, F.; Gutmann, H. *Cancer Res.* **1971**, *31*, 471.
(15) Lotlikar, P.; Scribner, J.; Miller, J. A.; Miller, E. C. *Life Sci.* **1966**, *5*, 1263.
(16) Westra, J. G.; Kriek, E., Hittenhausen, H. *Chem. Biol. Interact.* **1976**, *15*, 149.
(17) (a) Kriek, E.; Miller, J. A., Juhl, U.; Miller, E. C. *Biochemistry* **1967**, *6*, 177. (b) Kriek, E.; Westra, J. G. *Carcinogenesis (London)* **1980**, *1*, 459.



22, R = H; R' = $COCH_3$
23, R = F; R' = $COCH_3$
24, R = H; R' = H
25, R = F; R' = H
26, R = $COCH_3$; R' = H

monia to effect the conversion of 22 to 24.[17b]

The structure of 22 was established unequivocally by Kriek et al., and comparisons of the ^{13}C NMR spectra of 22 and 23 with those of 2′-dG and 2-acetamidofluorene permitted assignment of structure 23 in the present study. With the exception of the C-8 atom, the chemical shifts of the 2′-dG carbons of 22 and 23 are similar to those of 2′-dG itself. In 2′-dG the C-8 resonance band appears as a doublet at 135.12 ppm whereas the analogous carbon atoms in 22 and 23 appear as singlets at 143.67 and 138.5 ppm, respectively. The chemical shifts and multiplicities of the ^{13}C NMR spectra of 22, 23, 2′-dG, and 2-acetamidofluorene are tabulated in the supplementary material.

The 250-MHz ^1H NMR spectrum of 24 was identical with that reported by Beland et al.[18] The only differences between the spectra of 24 and the 7-F derivative 25 are those expected due to the 7-F substituent. Thus, the spectrum of 24 exhibits a triplet at 7.21 ppm due to the 7-H resonance whereas such a triplet is not present in the NMR spectrum of 25. Also, the chemical shift of the proton at the 6-position of 25 appeared at 7.17 ppm whereas the corresponding resonance band appeared at 7.36 ppm in the spectrum of 24. These data are consistent with attachment of the fluorenyl nitrogen atom to the C-8 of guanine in 22–25.

When attempts were made to react 2-(N-acetoxyacetamido)-7-acetylfluorene (12) with 2′-dG under the same conditions used for the reactions with 9 and 10, only unreacted starting material and the hydrolysis product 6 were detected. When the reaction mixture was heated to 55–60 °C, unidentified decomposition products were recovered. Presumably, the poor reactivity of 12 is due to the strong electron-withdrawing effect of the 7-acetyl group.

Identification of Methylthio Adducts from AHAT-Catalyzed Reactions. The *N*-arylhydroxamic acids 1–3 were subjected to large-scale incubations under anaerobic conditions with partially purified hamster hepatic AHAT in the presence of *N*-acetylmethionine. The isolated products had TLC properties that were identical with those of the synthetic methylthio adducts 18–20. Additionally, the mass spectra of each of the products exhibited molecular ions, M − 15, and M − 47 peaks, analogous to the spectra of the synthetic standards. Incubation of 7-methoxy-2-(*N*-hydroxyacetamido)fluorene (7) with the AHAT preparation did not result in the isolation of any identifiable products.

When the large-scale enzymatic incubations of 1–3 were conducted under aerobic conditions, the mass spectra of the isolated products contained prominent M + 16 and M + 32 peaks. It was determined that these peaks represented products of air oxidation by carrying out the incubations under anaerobic conditions, which resulted in products that had mass spectra that did not contain the M + 16 and M + 32 peaks. Also, when an authentic sample of 1- and 3-(methylthio)-7-bromo-2-aminofluorene (20) was incubated in the presence of denatured enzyme under aerobic conditions, the mass spectrum of the isolated product contained M + 16 and M + 32 peaks. The structures of the oxidized materials were not determined.

Table I. Rate of Methylthio Adduct Formation, Quantitative Structure-Activity Results, and Physicochemical Constants

no.	rate,[a] nmol (mg of protein)$^{-1}$ (10 min)$^{-1}$	log rate obsd	log rate calcd[b]	σ^c	π	0.1MR
1	7.5 ± 0.1	0.87	0.67	0	0	0.103
2	15.2 ± 1.3	1.18	0.76	0.06	0.14	0.092
3	10.5 ± 1.1	1.02	1.01	0.23	0.86	0.890
4	2.4 ± 0.4	0.37	0.94	0.18	1.12	1.390
5	24.9 ± 3.9	1.40	1.65	0.66	−0.57	0.630
6	61.0 ± 4.8	1.79	1.41	0.50	−0.55	1.120
7	1.2 ± 0.3	0.09	0.27	−0.27	−0.02	0.790
8	0		0.20	−0.32	1.03	2.170

[a] Activity was measured as the rate of methylthio adduct formation (mean ± SE; $N = 3$) catalyzed by partially purified hamster hepatic enzyme. The procedure is described in the Experimental Section. [b] Calculated with eq 1. [c] Physicochemical constants were taken from: Martin, Y. C. "Quantitative Drug Design"; Marcel Dekker: New York, 1978; p 377. The squared correlation coefficients between variables were 0.26, 0.06, and 0.30 for σ–π, σ–0.1MR, and 0.1MR–π, respectively.

Electrophile Generation Assay Results. Having established for the representative 7-substituted compounds 2 and 3 that the methylthio adducts formed in AHAT-catalyzed reactions were the same types of products formed when the electrophiles produced from 1 were trapped with *N*-acetylmethionine, it was decided to compare the abilities of 1–8 to react with *N*-acetylmethionine in AHAT-catalyzed processes. The method of Bartsch et al., which involves the use of radiolabeled *N*-acetylmethionine to trap the electrophilic reactants, was used for this purpose.[9]

The *N*-arylhydroxamic acids 1–8, at a concentration of 1 mM, were incubated for 10 min with the partially purified hamster hepatic AHAT preparation in the presence of 10 mM *N*-acetylmethionine. The methylthio adducts were extracted and quantified by liquid scintillation counting as described in the experimental section. The results listed in Table I show that the rates of methylthio adduct formation varied over a range of 61-fold. Surprisingly, those 7-substituted *N*-arylhydroxamic acids having electron-withdrawing substituents (2, 3, 5, 6) exhibited the highest extent of methylthio adduct formation. An exception to the finding of higher activities with compounds having electronegative 7-substituents is the 7-iodo derivative (4). It should be noted, however, that 4 is a very poor substrate for AHAT as determined by the aminoazobenzene transacetylation assay and, therefore, would not be expected to be converted efficiently by AHAT to electrophilic reactants.[13]

The 7-methoxy analogue (7) formed very little methylthio adduct subsequent to interaction with AHAT, and the 7-*n*-butoxy compound (8) produced no detectable products. The inactivity of 8 can be readily rationalized on the basis of the earlier finding that 8 is a poor substrate for AHAT-catalyzed reactions.[13] In contrast, the 7-methoxy compound (7) is a good substrate for hamster hepatic AHAT, and additionally, the electron-donating properties of the methoxy substituent would be expected to favor the formation of the positively charged *N*-arylnitrenium ion,

(18) Beland, F. A.; Allaben, W. T.; Evans, F. E. *Cancer Res.* 1980, *40*, 834.

thereby enhancing the extent of reaction with nucleophiles such as *N*-acetylmethionine. Thus, the very small amount of methylthio product produced in these experiments with **7** was unexpected, but the result is consistent with the comparatively low yield obtained in the reaction of the *N*-acetoxy derivative (**13**) with *N*-acetylmethionine and the absence of detectable products from the large-scale AHAT-catalyzed activation of **7** in the presence of this nucleophile.

Identification of the 2′-dG Adducts from AHAT-Catalyzed Reactions. The 7-fluoro and 7-acetyl compounds (**2**, **6**) were selected for preparative scale incubations with AHAT in the presence of 2′-dG in order to compare the chemical structures of the adducts with those formed from **1**. Compound **2** was selected because it is one of the compounds that was used for the characterization of methylthio adducts and because it exhibited relatively high activity in the electrophile trapping assay with radiolabeled *N*-acetylmethionine. Compound **6** was used because of its unexpected high activity in the electrophile generation assay (Table I). Large-scale incubation of **1** and **2** with the partially purified hamster hepatic AHAT preparation and 2′-dG led to the isolation of products that had TLC and HPLC properties identical with those of synthetically derived samples of **24** and **25**. Sufficient quantities of **24** and **25** were obtained for determination of their 300-MHz ^{1}H NMR spectra by combining the products isolated from three experiments; the ^{1}H NMR spectra matched those of the synthetic samples of **24** and **25**.

As discussed above, the attempted preparation of an authentic sample of the 7-acetyl adduct **26** from the *N*-acetoxy derivative **12** was not successful. However, a sufficient quantity of **26** was obtained from a single preparative scale incubation with AHAT and 2′-dG to permit its structural characterization by 300-MHz ^{1}H NMR and mass spectral analysis. The assignments for the protons were based on intensity measurements, proton exchange with D_2O, homonuclear decoupling, and comparison with the proton chemical shifts observed for **24** and **25**. A tabulation of the chemical shifts of the protons of the enzymatically generated samples of **24**–**26** is presented in the supplementary material. In the following discussion, F and G refer to the protons of the 2-aminofluorene and the 2′-dG moieties, respectively.

The assignment of the F1 and F6 protons of **26** were based on the reported chemical shifts of 7.95 and 7.37 ppm for the F3 and F6 protons of 2-acetamidofluorene and the expected ortho and meta couplings of the F3 and F6 protons.[19] Saturation of the proton at F3 was used to identify the F1 and F4 protons, and saturation of the F6 proton allowed the assignment of the F5 and F8 protons. Because all of the 2-aminofluorene ring protons could be assigned for **26**, it was concluded that covalent binding to 2′-dG is through the nitrogen atom of the 2-aminofluorene moiety.

The resonance peaks at 10.45, 6.34, and 8.82 ppm readily exchanged with D_2O and were assigned to the G(N)1, G(N)2ab and the fluorenylamine protons, respectively. All the 2′-dG protons could be assigned except the C-8 proton, indicating that covalent linkage between 2′-dG and 7-acetyl-2-aminofluorene must be through the C-8 position of 2′-dG.

Discussion. The chemical and biochemical factors that determine the extent and types of covalent interactions between the activated forms of *N*-arylhydroxamic acids and biological nucleophiles are of considerable interest and importance.[11] Indeed, those processes that result in the conversion of N-arylhydroxamic acids to reactive species that form arylamine adducts with nucleic acid residues may have particular relevance to the production of mutagenic and carcinogenic effects.[20–22]

N-Arylhydroxamic acid *N*,*O*-acyltransferase (AHAT) constitutes one of the means whereby *N*-arylhydroxamic acids may be converted to intermediates that form arylamine adducts with biological nucleophiles. Thus, AHAT is a useful system for the generation of electrophilic species for the purpose of studying their fate and reactivity. In addition, AHAT has received considerable attention because of its potential relevance to carcinogenic and mutagenic processes and because it appears to be identical with certain forms of an important drug metabolizing enzyme, acetylcoenzyme A: arylamine *N*-acetyltransferase (EC 2.3.1.5).[23,24]

Only limited information is available regarding the influence of aromatic ring substituents on the reactivity or fate of activated forms of *N*-arylhydroxamic acids. Kriek and Hengeveld found that the potassium salt of 4-[(*N*-sulfonoxy)acetamido]-4′-fluorobiphenyl is at least twice as reactive toward methionine and guanosine as the 4′-H compound.[25] Lefevre et al. reported that the 7-iodo and 7-fluoro derivatives of 2-(*N*-acetoxyacetamido)fluorene showed reactivities with guanosine 5′-monophosphate and DNA that were similar to that of the parent compound **9**; the principal reaction products appeared to be the 7-substituted 2-[*N*-(deoxyguanosine-8-yl)acetamido]fluorenes.[26] In related investigations, 2-(*N*-acetoxyacetamido)-7-ethylfluorene exhibited greater in vitro reactivity than **9** with 2′-dG and with DNA, but the 7-*n*-butyl derivative was less reactive than **9**.[27] 2-[*N*-(Myristoyloxy)acetamido]-7-iodofluorene solvolyzed in aqueous solution approximately half as rapidly as 2-[*N*-(myristoyloxy)acetamido]fluorene, and the 7-iodo analogue was less than 10% as reactive with DNA as the 7-H compound.[28]

Gassman and Granrud studied the rates of thermal rearrangement of a series of ring-substituted *N*-arylhydroxamic acid *O*-methanesulfonates and found a relationship between the electron-donating properties of the substituents and the reaction rates, indicating heterolytic cleavage of the N–O bond and the generation of *N*-acyl-*N*-arylnitrenium ion intermediates.[29] Novak and coworkers found that the solvolysis of several ring-substituted *N*-(sulfonoxy)acetanilides in aqueous media proceeds

(19) Mathien, P.; Milano, J.; Douris, J. *Bull. Soc. Chim. Fr.* **1974**, 299.
(20) Beranek, D. T.; White, G. L.; Heflich, R. H.; Beland, F. A. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 5175.
(21) Scribner, J. D.; Scribner, N. K.; Koponen, G. *Chem. Biol. Interact.* **1982**, *40*, 27.
(22) Allaben, W. T.; Weis, C. C.; Fullerton, N. F.; Beland, F. A. *Carcinogenesis (London)* **1983**, *4*, 1067.
(23) Glowinski, I. B.; Weber, W. W.; Fysh, J. M.; Vaught, J. B.; King, C. M. *J. Biol. Chem.* **1980**, *255*, 7883.
(24) Hanna, P. E.; Banks, R. B.; Marhevka, V. C. *Mol. Pharmacol.* **1982**, *21*, 159.
(25) Kriek, E.; Hengeveld, G. M. *Chem. Biol. Interact.* **1978**, *21*, 179.
(26) Lefevre, J. F.; Fuchs, R. P. P.; Daune, M. P. *Biochemistry* **1978**, *17*, 2561.
(27) (a) Saint-Ruf, G.; Spodheim-Maurizot, M.; Loukakou, P. E.; Coic, J. P. *Carcinogenesis (London)* **1981**, *2*, 1919. (b) Saint-Ruf, G.; Loukakou, P. E.; Spodheim-Maurizot, M. *Cancer Biochem. Biophys.* **1984**, *7*, 89.
(28) Fuchs, R. P. P.; Lang, M.-C. E.; Miller, E. C.; Miller, J. A. *Carcinogenesis (London)* **1981**, *2*, 655.
(29) Gassman, P. G.; Granrud, J. E. *J. Am. Chem. Soc.* **1984**, *106*, 1498.

via cleavage of the N–O bond with the generation of nitrenium ion intermediates; evidence was presented that electrophilic quinone imine derivatives were generated from the nitrenium ions.[30] A striking example of the importance of electronic properties was described by Cole et al., who reported that derivatives of 3-(*N*-acetoxyacetamido)fluorene that contain electron-donating groups in positions conjugated with the 3-position exhibited both increased electrophilicity and mutagenicity.[31]

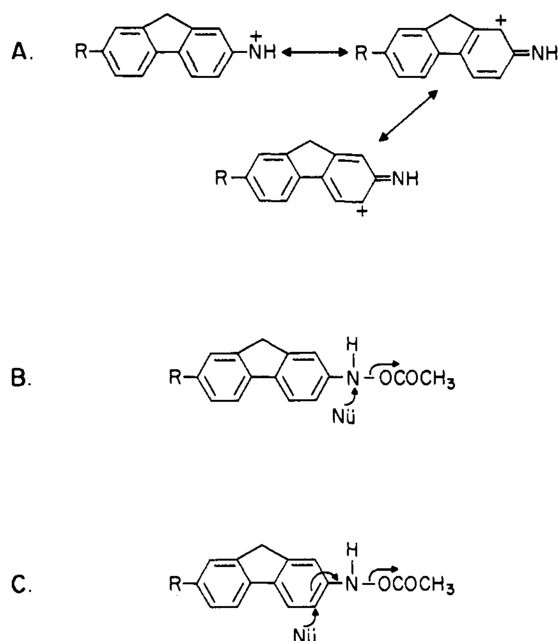
Each of the just described results was derived from experiments in which the presumed electrophilic reactants are *N*-acyl-*N*-arylnitrenium ions rather than *N*-arylnitrenium ions, which are believed to be the reactive species generated in AHAT-catalyzed processes. In the present investigation, substituent effects on the reaction of AHAT-activated 7-substituted 2-(*N*-hydroxyacetamido)fluorenes with *N*-acetylmethionine and 2′-dG were investigated. Bartsch et al. found that incubation of 1 and *N*-acetylmethionine with rat liver cytosol resulted in the formation of 3-(methylthio)-2-aminofluorene, although the possible presence of 1-(methylthio)-2-aminofluorene could not be excluded.[9] Similarly, preparative scale incubations of partially purified hamster hepatic AHAT, *N*-acetylmethionine, and compounds **1**–**3** led to the isolation of products whose TLC and mass spectral properties indicated that they were 7-substituted 1- and 3-(methylthio)-2-aminofluorenes. Thus, the presence of the electronegative 7-F and 7-Br substituents did not alter the type of product formed in the reaction of AHAT-generated intermediates with *N*-acetylmethionine. In contrast, the strongly electron-donating 7-methoxy group of **7** appeared to have a profound effect in that no identifiable methylthio adducts could be isolated when **7** was subjected to the same large-scale incubation conditions as **1**–**3**. If the formation and stabilization of positively charged intermediates such as resonance-stabilized nitrenium ions were the rate-limiting factors in the production of covalent adducts between AHAT-generated reactants and biological nucleophiles, it would be expected that electron-donating substituents such as the 7-methoxy group would facilitate the process.

Because of the substituent effects on the formation of methylthio adducts when **1**–**3** and **7** were incubated on a large scale with AHAT, it was decided to study the rate of the AHAT-catalyzed formation of methylthio adducts with **1**–**8** under a standard set of experimental conditions. The use of radiolabeled *N*-acetylmethionine permits accurate quantification of the extent of methylthio adduct formation (Table I).[9] The finding that the two analogues with the strongest electron-withdrawing 7-substituents (**5**, **6**) formed the largest amounts of product, whereas the 7-alkoxy derivatives (**7**, **8**) formed the smallest amounts of adducts, was somewhat suprising because the alkoxy groups would be expected to facilitate the generation of the positively charged intermediates, which are presumed to be responsible for reaction with nucleophiles.

Previously, it was demonstrated that 7-substituted 2-(*N*-hydroxyacetamido)fluorenes function as mechanism-based irreversible inactivators of hamster hepatic AHAT and that **7** exhibited the largest inactivation rate constant of the compounds studied.[13] A quantitative structure–activity analysis provided support for the conclusion that positively charged species are involved in the inactivation of AHAT by compounds such as **1**–**8**. When the physicochemical properties of the substituents contained in

(30) Novak, M.; Pelecanou, M.; Roy, A. K.; Andronico, A. F.; Plourde, F. M.; Olefirowicz, T. M.; Curtin, T. J. *J. Am. Chem. Soc.* **1984**, *106*, 5623.
(31) Cole, C.; Pan, H.; Fletcher, T. L. *Cancer Lett.* **1980**, *9*, 61.

Scheme III



compounds **1**–**7** were correlated by multiple regression analysis with the rates of methylthio adduct formation (A) shown in Table I, eq 1 was obtained.

$$\log A = 1.476\ (\pm 1.327)\sigma + 0.673\ (\pm 0.462) \quad (1)$$

$$n = 7;\ r = 0.79;\ s = 0.39$$

In eq 1, n is the number of data points, r is the correlation coefficient, and s is the standard deviation. The numbers in parentheses are the 95% confidence intervals, and σ is the Hammett constant that describes the electronic properties of the substituents. Compound **8** was not included in the analysis because it was inactive in the assay for methylthio adduct formation. The use of either the hydrophobic constant π or the molar refractivity (MR) of the substituents in the analyses did not yield statistically significant equations.

The positive coefficient for σ in eq 1 indicates that electron-withdrawing substituents favor formation of adducts with intermediates produced by the AHAT-catalyzed activation of **1**–**7**. The conclusion that the substituent effects described by eq 1 reflect factors that influence the extent of reaction of activated intermediates with the nucleophile is supported by the previous finding that there are no significant electronic effects of the 7-substituents on the ability of compounds **1**–**8** to serve as acetyl donors in AHAT-catalyzed transacetylations and by the strong evidence that electron-donating groups enhance the rate of mechanism-based inactivation of AHAT by this group of compounds.[13] Further support for the conclusion that electronegative 7-substituents facilitate the formation of adducts between nucleophiles and AHAT-generated intermediates comes from the observation that the incubation of the 7-acetyl compound (**6**) with AHAT in the presence of 2′-dG yielded at least 10-fold larger quantities of adduct than did either the 7-fluoro (**2**) or the 7-H (**1**) compounds.

The pronounced enhancement of arylamine adduct formation by electronegative substituents may be interpreted in terms of the stabilizing effect that such functional groups would be expected to exert on the presumed *N*-acetoxy intermediate that is produced in the AHAT-catalyzed *N*,*O*-acyltransferase process (I; Scheme I). Stabilization of the *N*-acetoxy intermediate should increase the probability that it will diffuse away from the active site

of AHAT and subsequently react with nucleophiles in solution or elsewhere in the cell. The present data do not, however, permit a determination of whether the formation of arylamine adducts is the result of the decomposition of the N-acetoxy compound to N-arylnitrenium–carbenium ions (A; Scheme III) that react with nucleophiles or whether S_N2 (B; Scheme III) and S_N2' (C; Scheme III) mechanisms may be involved.

The data presented herein provide definitive experimental evidence of a role for the electronic properties of aromatic ring substituents in the determination of the extent of arylamine adduct formation with biological nucleophiles. The development of an understanding of the physicochemical properties that influence the formation of such adducts is of particular importance in light of the evidence that arylamine adducts, rather than arylamide adducts, with nucleic acid residues may be critical lesions for mutagenesis and/or the initiation of carcinogenesis subsequent to exposure to arylamines, arylamides, or arylhydroxamic acids.[20-22]

Experimental Section

Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. IR spectra were obtained with a Perkin-Elmer 281 recording spectrophotometer. NMR spectra were obtained with either a Varian T-60A, A-60D, HFT-80, or XL-100A, JEOL FX-900, or a Bruker WM-250 spectrometer; the samples were dissolved in either $CDCl_3$ or Me_2SO-d_6; proton chemical shifts were either relative to the internal reference standard tetramethylsilane (Me_4Si) or set to Me_2SO = 4041 Hz; ^{13}C chemical shifts were converted to the Me_4Si scale by setting δ (Me_2SO-d_6) = 39.5. Mass spectra were obtained with an Associated Electronic Industries (AEI) MS-30 (electron impact) or a Finnigan 4000 (chemical ionization) instrument in the University of Minnesota Mass Spectrometry Laboratory, Department of Chemistry; samples were introduced by direct inlet. Liquid scintillation counting was accomplished with a Beckman LS-100 instrument. Analytical TLC plates were plastic-backed Eastman 13181 silica gel with fluorescent indicator, No. 6060, or Eastman 13254 cellulose with fluorescent indicator, No. 6065; compounds were visualized with UV light and I_2; arylhydroxamic acids were visualized with a 2.5% $FeCl_3$ in 0.05 N HCl spray reagent. Elemental analyses were performed by Midwest Microlab Inc., Indianapolis, IN, and were within 0.4% of theoretical values except where noted.

7-Substituted 2-(N-Acetoxyacetamido)fluorenes (9–13). The appropriate hydroxamic acid (1–3, 6, 7) (2.1 mmol) was dissolved in 20 mL of pyridine. To the solution of the hydroxamic acid was added acetic anhydride (6 mmol) dropwise, and the reaction mixture was stirred at room temperature for 45 min. For compounds 9–11 the crude products were obtained by adding cold water to the reaction mixture until the solution became turbid and a precipitate was formed. However, for 12 and 13 the crude products were obtained by removing the solvent under reduced pressure.

2-(N-Acetoxyacetamido)fluorene (9): yield 80%; recrystallized from Et_2O; mp 108–110 °C (lit.[15] mp 109–111 °C).

7-Fluoro-2-(N-acetoxyacetamido)fluorene (10): yield 66%; recrystallized from acetone–H_2O; mp 113–114 °C (lit.[26] mp 113–114 °C). Anal. ($C_{17}H_{14}NO_3F$) C, H, N.

7-Bromo-2-(N-acetoxyacetamido)fluorene (11): yield 80%; recrystallized from acetone–H_2O; mp 101–102 °C. Anal. ($C_{17}H_{14}NO_3Br$) C, H, N, Br.

7-Acetyl-2-(N-acetoxyacetamido)fluorene (12): yield 70%; recrystallized from Et_2O; mp 128–130 °C. Anal. ($C_{19}H_{17}NO_4$) C, H, N.

7-Methoxy-2-(N-acetoxyacetamido)fluorene (13): yield 60%; recrystallized from Et_2O; mp 92–94 °C. Anal. ($C_{18}H_{17}NO_4$) C, H, N.

7-Substituted 1- and 3-(Methylthio)-2-acetamidofluorenes (14–17). The appropriate N-acetoxy derivative (9–11, 13) (1 mmol), dissolved in 10 mL of 95% ethanol, was added to a preheated solution (48 °C) of DL-N-acetylmethionine (25 mmol) in 475 mL of 0.1 M phosphate buffer (pH 7.8). The reaction mixture was stirred for 2.5 h under N_2 (5 h for the solvolysis of 11); it was then cooled to room temperature. KOH (25 mL, 11 N) was added, and the reaction mixture was extracted with two 100-mL portions of benzene–hexanes (1:1). The combined benzene–hexanes extracts were dried ($MgSO_4$); evaporation of the organic solvents under reduced pressure afforded the crude product.

1- and 3-(Methylthio)-2-acetamidofluorene (14): yield 80%; recrystallized from ethyl acetate; mp 140–144 °C dec (lit.[15] mp 140 °C); ^1H NMR (250 MHz, Me_2SO-d_6) δ 9.35 (br s, NH), 7.96–7.27 (m, aromatic H), 4.02, 3.87 (s, s, CH_2), 2.50, 2.34 (s, s, SCH_3), 2.14, 2.07 (s, s, $COCH_3$).

1- and 3-(Methylthio)-7-fluoro-2-acetamidofluorene (15): yield 66%; recrystallized from ethyl acetate mp 168–170 °C dec; ^1H NMR (250 MHz, Me_2SO-d_6) δ 9.38, 9.33 (s, s, NH), 7.99–7.17 (m, aromatic H), 4.03, 3.88 (s, s, CH_2), 2.49, 2.33 (s, s, SCH_3), 2.14, 2.07 (s, s, $COCH_3$). Anal. ($C_{16}H_{14}FNOS$) C, H, N, F.

1- and 3-(Methylthio)-7-bromo-2-acetamidofluorene (16): yield 90%; recrystallized from ethyl acetate; mp 203 °C dec; ^1H NMR (250 MHz, Me_2SO-d_6) δ 9.4, 9.35 (s, s, NH), 7.93–7.54 (m, aromatic H), 4.06, 3.89 (s, s, CH_2), 2.50, 2.33 (s, s, SCH_3), 2.15, 2.07 (s, s, $COCH_3$). Anal. ($C_{16}H_{14}BrNOS$) C, H, N, Br, S.

1- and 3-(Methylthio)-7-methoxy-2-acetamidofluorene (17): yield 45%; recrystallized from ethyl acetate; mp 172 °C dec; ^1H NMR (250 MHz, Me_2SO-d_6) δ 9.30 (br, NH), 7.85–6.92 (m, aromatic H), 3.83, 3.78 (s, s, CH_2), 3.80 (s, OCH_3), 2.48 (s, SCH_3), 2.06, 2.05 (s, s, $COCH_3$). Anal. ($C_{17}H_{17}NO_2S$) C, H, N, S.

7-Substituted 1- and 3-(Methylthio)-2-aminofluorenes (18–21). The appropriate amide (14–17) (200 mg) was added to 7 N HCl (30 mL) and was heated under reflux for 6–8 h on a steam bath. The reaction mixture was cooled to room temperature, was made slightly alkaline by the addition of 11 N KOH, and was extracted with two 50-mL portions of Et_2O. The combined Et_2O extracts were evaporated under reduced pressure to afford the crude product.

1- and 3-(Methylthio)-2-aminofluorene (18): yield 30%; recrystallized from EtOH–H_2O; mp 104–106 °C; mass spectrum (70 eV) m/e (relative intensity) 230.07 (1.23), 229.9 (4.15), 229.07 (7.18), 228.08 (16.70), 227.1 (5.4), 227.07 (M^+, 100), 226.07 (3.18), 212.05 (60.86), 180.08 (45.09), 152.05 (20.35), 76.03 (2.74); ^1H NMR (80 MHz, $CDCl_3$) δ 7.76–6.83 (m, aromatic H), 4.40 (br, NH_2), 3.95, 3.73 (s, s, CH_2), 2.36, 2.25 (s, s, SCH_3). Anal. ($C_{14}H_{13}NS$) C, H, N.

1- and 3-(Methylthio)-7-fluoro-2-aminofluorene (19): yield 20%; recrystallized from EtOH–H_2O; mp 97–100 °C; mass spectrum (70 eV) m/e (relative intensity) 247.1 (5.4), 246.1 (17.5), 245.1 (M^+, 100), 230 (57.3), 198 (34.7), 170 (22.6), 28.1 (12.5); ^1H NMR (100 MHz, $CDCl_3$) δ 7.66–6.74 (m, aromatic H), 4.32 (br, NH_2), 3.86, 3.67 (s, s, CH_2), 2.35, 2.24 (s, s, SCH_3). Anal. ($C_{14}H_{12}FNS$) C, H, F, N, S.

1- and 3-(Methylthio)-7-bromo-2-aminofluorene (20): yield 20%; recrystallized from EtOH–H_2O; mp 72–73 °C; mass spectrum (70 eV) m/e (relative intensity) 309 (7.1), 308 (16.8), 307 (M^+, 100), 306 (18.5), 305 (99), 291.9 (33.4), 289.9 (34.1), 261 (7.1), 259.9 (18.3), 259 (8.2), 258 (19.1), 83.6 (36.2), 28.1 (35.9); ^1H NMR (100 MHz, $CDCl_3$) δ 7.71–6.70 (m, aromatic H), 4.39 (br, NH_2), 3.89, 3.71 (s, s, CH_2), 2.37, 2.27 (s, s, SCH_3). Anal. ($C_{12}H_{14}BrNS$) C, H, N, S.

1- and 3-(Methylthio)-7-methoxy-2-aminofluorene (21): yield 25%; recrystallized from EtOH–H_2O; mp 126–128 °C; mass spectrum (70 eV) m/e (relative intensity) 259 (8.13), 258.1 (19.22), 257.1 (M^+, 100), 244.1 (5.36), 243.1 (13.86), 242.1 (76.52), 227 (19.41), 91.0 (44.18), 44.1 (78.56), 28.1 (45.84); ^1H NMR (100 MHz, $CDCl_3$) δ 7.67–6.80 (m, aromatic H), 4.23 (br, NH_2), 3.84, 3.72 (s, s, CH_2), 3.82 (s, OCH_3), 2.4, 2.37 (s, s, SCH_3). Anal. ($C_{15}H_{15}NOS$) C, H, N, S.

N-(Deoxyguanosin-8-yl)-2-acetamidofluorene (22). The N-acetoxy derivative (9) (2.14 mmol), dissolved in 80 mL of 95% EtOH, was added to a preheated (37 °C) solution of 2′-dG (Sigma, grade II, 2.14 mmol) in 200 mL of sodium citrate buffer (2 mM, pH 7). The reaction mixture was stirred for 3 h under N_2; it was then cooled to room temperature and was extracted with three 100-mL portions of Et_2O. The aqueous phase was concentrated under reduced pressure at 50 °C to approximately 10% of its original volume. The precipitate that formed was collected by filtration; it was then washed with a few milliliters of cold water and dried in air to give the crude product, which was recrystallized

from EtOH–H_2O: yield 33%; mp above 200 °C dec; ^{13}C NMR (Me_2SO-d_6, 62.9 MHz) tabulated in the supplementary material. Anal. ($C_{25}H_{24}N_6O_5 \cdot H_2O$) H, N; C: calcd, 59.28; found, 59.74.

N-(Deoxyguanosin-8-yl)-7-fluoro-2-acetamidofluorene (23). Compound **23** was prepared from **10** by the method described above for the synthesis of **22**: yield 30%; recrystallized from EtOH–H_2O; mp above 200 °C dec; ^{13}C NMR (Me_2SO-d_6, 62.9 MHz) tabulated in the supplementary material. Anal. ($C_{25}H_{23}FN_6O_5 \cdot 0.5H_2O$) C, H, F; N: calcd, 16.30; found, 16.84.

N-(Deoxyguanosin-8-yl)-2-aminofluorene (24). The amide **22** (100 mg) was dissolved in 30 mL of 0.01 N NaOH and 20 mL of EtOH. The reaction mixture was heated at 100 °C for 30 min; it was then cooled to room temperature and was neutralized (pH 7.0) with 0.1 N HCl. The precipitate that formed was collected by centrifugation, washed with H_2O, and recrystallized from EtOH–H_2O: yield 80%; mp above 200 °C dec; ^1H NMR (Me_2SO-d_6, 250 MHz; F refers to the protons of the 2-aminofluorene ring; G refers to the protons of the deoxyguanosine moiety) δ 8.74 (s, FN2), 8.08 (s, F1), 7.77 (d, F5), 7.73 (d, F4), 7.66 (d, F3), 7.51 (d, F8), 7.32 (t, F6), 7.21 (t, F7), 6.39 (br, GN2a,b), 6.34 (q, G1′), 6.08 (br, G05′), 5.38 (s, G03′), 4.43 (br, G3′), 3.94 (s, G4′), 3.88 (s, F9a,b), 3.78 (s, G5′a,b), 2.56 (br, G2′b), 2.06 (br, G2′a). Anal. ($C_{23}H_{22}N_6O_4 \cdot 0.5H_2O$) C, H; N: calcd, 18.45; found, 17.66.

N-(Deoxyguanosin-8-yl)-7-fluoro-2-aminofluorene (25). Compound **25** was prepared from **23** as described above for the synthesis of **24** except that 0.05 N NaOH was used for the hydrolysis of the amide: yield 66%; recrystallized from EtOH–H_2O; mp above 200 °C dec; ^1H NMR (Me_2SO-d_6, 250 MHz, F refers to the protons on the 2-aminofluorene ring; G refers to the protons on the deoxyguanosine moiety) δ 8.72 (s, FN2), 8.07 (s, F1), 7.79 (d, F5), 7.74 (d, F4), 7.66 (d, F3), 7.38 (d, F8), 7.16 (t, F6), 6.39 (s, GN2a,b), 6.34 (br, G1′), 6.00 (br, G05′), 5.34 (br, G03′), 4.43 (br, G3′), 3.95 (s, G4′), 3.92 (s, F9a,b), 2.56 (br, G2′b), 2.04 (br, G2′a). Anal. ($C_{23}H_{21}FN_6O_4 \cdot H_2O$) H, F, N; C: calcd, 57.26; found, 57.77.

Enzymatic Studies. Male golden Syrian hamsters were purchased from Charles River (Wilmington, MA). Ultracentrifugation was performed on a Beckman L5-65 ultracentrifuge and low-spin centrifugation on a Beckman J-21-B or a J2-21 centrifuge. A Beckman 24 or 24/25 or DU-8 spectrophotometer was used. Incubations were performed in a Dubnoff, Blue M, or Eberbach shaker bath. Dithiothreitol (DTT), N-acetylmethionine (NAM), Grade III NAD^+, Grade II and Sigma Grade 2′-deoxyguanosine (2′-dG), and Sephadex LH-20-100 were obtained from Sigma Chemical Co. N-Acetyl-L-[^{14}C-CH_3]methionine (0.2–0.3 mCi/mmol) was prepared from L-[^{14}C-CH_3]methionine (New England Nuclear) and acetic anhydride according to the procedure of Wheeler and Ingersoll.[32] Preparative TLC was performed on 500 μm silica gel plates (Analtech, silica gel Gf) or 500 μm cellulose plates (Quantum Industries, microcrystalline cellulose). 2′-Deoxyguanosine adducts were visualized with UV light. Methylthio adducts were visualized as yellow spots on thin-layer chromatograms by using a 1% p-(dimethylamino)benzaldehyde in 1 N HCl spray reagent.[33] HPLC was accomplished with a 4.6 × 150 mm ultrasphere ODS 5-μm column attached to a 3.2 × 40 mm C-18 10-μm precolumn (Altex Scientific, Inc.) on a Waters M-6000 liquid chromatograph (Waters Associates, Milford, MA) equipped with a Rheodyne 7120 syringe loading sample injector (Rheodyne, Inc.), Altex 153 analytical UV detector (Altex Scientific, Inc.), and a Sargent-Welch SRG Recorder. Proton NMR spectra of **24**–**26** were obtained with a Nicolet NT-300 spectrometer in the Department of Chemistry, University of Minnesota. Mass spectra were obtained with an Associated Electronic Industries (AEI) MS-30 in the Department of Chemistry, University of Minnesota; samples were introduced by direct inlet.

Tissue Preparation. Animals were lightly etherized before decapitation. Livers were excised and placed in cold 0.05 M sodium pyrophosphate buffer (pH 7.0) containing 1 mM DTT. Livers were blotted dry, weighed, minced, and homogenized with 1 mL of cold buffer per gram of liver in a Potter-type homogenizer with a motor driven pestle. This 50% homogenate was centrifuged at 105000g for 60 min in a refrigerated Beckman preparative ultracentrifuge. The resultant supernatant was diluted with an equal volume of cold sodium pyrophosphate buffer to afford a 25% solution.

Enzyme Preparation. AHAT was partially purified (2–3-fold) from hamster hepatic cytosol by minor modification of the ammonium sulfate fractionation method described by King.[10] The cytosol (25%) from hamster liver was placed in an ice bath and brought to 35% saturation with ammonium sulfate by addition with stirring of an ice-cold saturated solution of ammonium sulfate in 0.05 M sodium pyrophosphate buffer (pH 7.0) containing 1 mM DTT. The cold 35% ammonium sulfate solution was stirred in an ice bath for an additional 20–30 min before removing the precipitate by centrifugation at 9500g for 15 min. The supernatant was then brought to 50% saturation by further addition of the cold saturated ammonium sulfate solution. The 50% saturated solution was stirred an additional 20–30 min at ice bath temperatures before centrifugation. The precipitate contained the majority of AHAT activity and was washed twice with cold 50% saturated ammonium sulfate.

The enzyme pellets were stored at −70 °C and were reconstituted in enough cold 0.05 M sodium pyrophosphate buffer (pH 7.0, 1 mM DTT) to give approximately 30 mg/mL of protein. Protein concentration was measured by the method of Lowry et al. with bovine serum albumin as the standard.[34]

Electrophile Generation Assay. The production of electrophiles was measured by the procedure of Bartsch et al.[9] Incubation flasks contained sodium phosphate buffer (41 μmol, pH 6.8), 0.8 μmol of NAD^+, 10 μmol of N-acetyl-L-[^{14}C-CH_3]-methionine, 1 mg of partially purified hamster liver enzyme, 1 μmol of hydroxamic acid substrate, and sufficient H_2O to give a final volume of 1.0 mL. Substrates were dissolved in 0.05 mL of solvent [**1**–**3**, **7**, **8**, 95% EtOH; **4**–**6**, Me_2SO–95% EtOH (1:1)]. Reactions were initiated by addition of substrates and were carried out for 10 min at 37 °C in air. At the end of the incubation period, the flasks were placed on ice and 2.5 mL of Et_2O was added. The contents of the flasks were transferred to test tubes and mixed thoroughly with a vortex mixer. The test tubes were then immersed in a dry ice–acetone bath until the aqueous layer was frozen. After the Et_2O layer was decanted, the test tube was heated in a water bath (90 °C) for 45 min. Benzene–petroleum ether (60–70 °C) or benzene–heptane (5 mL, 3:7) was added, and the two phases were mixed thoroughly. The organic layer was separated, washed with 2 mL of water, and was dried ($MgSO_4$). One milliliter of the organic extract was disolved in 10 mL of Econoflour scintillation fluid. The disintegrations per minute of ^{14}C present in each sample were determined by liquid scintillation counting. Control experiments were carried out with heat-denatured enzyme, and all results were adjusted for any nonenzymatic adduct formation.

Methylthio Adduct Formation and Isolation. The enzymatic reaction was run on a scale 150 times that of a standard incubation. The incubation mixture consisted of 60 mL of 0.05 M sodium pyrophosphate buffer (pH 6.8) containing 1 mM DTT, 0.12 mmol of NAD^+, 1.5 mmol of NAM, AHAT solution (130–150 mg of protein), 0.15 mmol of arylhydroxamic acid dissolved in 10 mL of 95% ethanol, and enough 1.5% KCl to bring the final volume to 150 mL. The reaction was carried out for 1 h in air at 37 °C. At the end of the incubation period, the incubation mixture was placed on ice and extracted with two 100-mL portions of Et_2O. The aqueous layer was frozen by immersing the incubation flask in a dry ice–acetone bath for 5 min, and any remaining Et_2O was decanted. The aqueous layer was heated in a water bath (90 °C) for 45 min; it was then cooled to room temperature and extracted with two 100-mL portions of benzene–hexanes (3:7). The benzene–hexanes extract was washed with three 100-mL portions of water and was dried ($MgSO_4$). The solvent was then evaporated to dryness; the residue was spotted on analytical silica gel TLC plates and compared to synthetic standards in order to identify the methylthio adducts; it was streaked on a 10 × 20 cm 500-μm silica gel preparative TLC plate and eluted with

(32) Wheeler, G.; Ingersoll, A. *J. Am. Chem. Soc.* **1951**, *73*, 4604.
(33) Cramer, J.; Miller, J. A.; Miller, E. C. *J. Biol. Chem.* **1960**, *235*, 885.
(34) Lowry, O.; Rosebrough, N.; Farr, A.; Randall, R. *J. Biol. Chem.* **1951**, *193*, 265.

$CHCl_3$–EtOAc (4:1). The region corresponding to the adduct was scraped off the plate. The product was extracted by stirring the silica gel with 50 mL of CH_2Cl_2. The silica gel was filtered from the solvent, which was dried ($MgSO_4$), and was evaporated in vacuo. The residue was again chromatographed as before to afford the adduct sample, which was analyzed by mass spectrometry. The mass spectrum was corrected for the background of the TLC plate and was compared to the mass spectrum obtained with the synthetic material.

For the anaerobic incubation, the reaction mixture was stirred under N_2 for 1 h before the addition of the enzyme solution. After the addition of the AHAT solution, the N_2 purge was continued, while the incubation flask was at ice-bath temperature, for another 30 min. The flask was then closed with a rubber cap and incubated at 37 °C. The hydroxamic acid solution was introduced into the flask with a syringe. The incubation and the workup procedure were carried out as before, except the heating of the aqueous layer at 90 °C for 45 min was done under N_2.

As a control experiment, 1- and 3-(methylthio)-7-bromo-2-aminofluorene (**20**) (0.035 mmol), dissolved in 10 mL of 95% ethanol, was incubated in air at 37 °C for 1 h with the heat-denatured AHAT preparation (150 mg), 0.05 M sodium pyrophosphate buffer (pH 6.8, containing 1 mM DTT), 0.12 mmol of NAD^+, andd 1.5 mmol of NAM. The reaction mixture was treated as before except no Et_2O extraction was done before heating the aqueous layer at 90 °C; the methylthio adduct was purified by preparative chromatography and was then analyzed by mass spectrometry.

2′-Deoxyguanosine Adduct Formation and Isolation. The incubation mixture consisted of 60 mL of 0.05 M sodium pyrophosphate buffer (pH 6.8) containing 1 mM DTT, 0.12 mmol of NAD^+, 1.5 mmol of 2′-dG (Grade II), hamster hepatic AHAT solution (95–125 mg of protein), 0.15 mmol of the hydroxamic acid (**1**, **2**) dissolved in 10 mL of 95% ethanol [**6** was dissolved in 10 mL of Me_2SO–95% ethanol (1:1)], and enough 1.5% KCl to bring the final volume to 150 mL. The enzyme was allowed to equilibrate at 37 °C for 10 min before adding the hydroxamic acid; the reaction was carried out, with continuous shaking, for 1 h in air at 37 °C. At the end of the incubation period, the incubation mixture was cooled on ice and extracted with two 100-mL portions of Et_2O. The aqueous layer was frozen by immersing the incubation flask in a dry ice–acetone bath for 5 min, and any remaining Et_2O was decanted.

Preliminary purification of the adducts was accomplished by passing the aqueous mixture through a Sephadex LH-20 (45 g; 50 × 2.5 cm) column at room temperature. Nonconjugated 2′-dG, proteins, and salts were eluted with water (500 mL), and then the adducts were eluted with methanol. The methanol fractions were concentrated under reduced pressure, and samples were spotted on analytical cellulose TLC plates, eluted with 1-butanol–glacial acetic acid–H_2O (50:11:25), and compared to synthetic standards.

For hydroxamic acids **1** and **2**, the residues obtained by evaporating the methanol (fractions 150–450 mL) were collected; the residues were streaked on 20 × 20 cm 500 μm cellulose preparative TLC plates and eluted with 1-butanol–glacial acetic acid–water (50:11:25). The region corresponding to the 2′-dG adduct was scraped off the plate. The product was extracted by stirring the cellulose with 50 mL of 95% ethanol. The cellulose was filtered from the solvent, which was then evaporated under reduced pressure at 37 °C. For HPLC analysis, the residue was redissolved in CH_3CN–H_2O (4:6) and then filtered through 0.5-μm Millipore FHLP filters (Millipore Corp., Bedford, MA). However, for ^1H NMR analyses, the residues, obtained from three experiments, were collected; they were again chromatographed on a Sephadex LH-20 (7.5 g; 15 × 1 cm) column and were eluted with methanol. The adduct fraction was concentrated under vacuum to afford 1.0 and 0.8 mg of **24** and **25**, respectively; they were dissolved in Me_2SO-d_6 for ^1H NMR analyses.

In some experiments, HPLC analyses of the products formed as a result of the bioactivation of **2** in the presence of 2′-dG indicated the presence of a major component that had a retention time of 2 min in both solvent systems (see below) rather than a retention time that was identical with that of the synthetic reference sample of **25**. Heating solutions of this component at 60–70 °C resulted in a concomitant decrease in the intensity of the peak that had a 2-min retention time and the appearance of a component that had a retention time identical with that of authentic **25**. Thus, the formation of **25** may be the result of rearrangement of a relatively labile intermediate.

For the hydroxamic acid **6**, the adduct **26** (4 mg) was crystallized by concentrating the 225–435-mL methanol fractions; 2 mg of **26** was dissolved in Me_2SO-d_6 for ^1H NMR analysis. Treatment of **26** (1 mg) with 10 mL of 0.1 N HCl for 4 h at 80 °C afforded a solid product (**27**) that was collected by centrifugation and washed with H_2O, 1% $(NH_4)_2CO_3$, and H_2O before it was analyzed by mass spectrometry.

TLC of the solution obtained by Sephadex LH-20 chromatography of the 2′-dG adduct obtained with **6** (cellulose plastic-backed plates; 1-butanol–glacial acetic acid–water (50:11:25)) revealed the presence of a minor component (R_f 0.94). The R_f value of **26** was 0.66. The minor component was not characterized.

***N*-(Guanin-8-yl)-7-acetyl-2-aminofluorene (27):** mass spectrum (EI 70 eV) m/e (relative intensity) 371.8 (M^+, 1.9), 294.8 (0.7), 147 (0.9), 107.1 (1.2), 72.1 (1.2), 64.0 (1.9), 44.1 (100), 32.0 (17.2), 28.1 (96.0), 26.3 (1.6).

HPLC Experiments. The solvent systems used in these experiments were CH_3CN–H_2O (3:7) (solvent A) and 4:6 (solvent B); all solvents were filtered through 0.45-μm Millipore HA filters (Millipore Corp., Bedford, MA); they were also degassed by stirring under reduced pressure or by the use of ultrasonic vibrations. The flow rate of the solvents was 1 mL/min, and UV detection was at 254 nm. The retention times of the chemically synthesized reference compounds **24** and **25** were 22 and 19 min in solvent A and 5 and 4.5 min in solvent B, respectively. The retention times of the amide adducts **22** and **23** were 16 and 13 min in solvent A and 3.5 and 4 min in solvent B, respectively.

Acknowledgment. This investigation was supported in part by NIH Grant CA-24427. The authors are grateful to Gerald Bratt, Department of Biochemistry, University of Minnesota, School of Medicine, for valuable assistance in determining several of the NMR spectra.

Registry No. **1**, 53-95-2; **2**, 2508-18-1; **3**, 92901-05-8; **4**, 70954-93-7; **5**, 92901-06-9; **6**, 92901-07-0; **7**, 92901-08-1; **8**, 92901-10-5; **9**, 6098-44-8; **10**, 6344-58-7; **11**, 97235-33-1; **12**, 97235-34-2; **13**, 97235-35-3; **14** (1-MeS), 21879-09-4; **14** (3-MeS), 21879-09-4; **15** (1-MeS), 97235-36-4; **15** (3-MeS), 16233-02-6; **16** (1-MeS), 97235-37-5; **16** (3-MeS), 97235-38-6; **17** (1-MeS), 97235-39-7; **17** (3-MeS), 97235-40-0; **18** (1-MeS), 21321-55-1; **18** (3-MeS), 13111-11-0; **19** (1-MeS), 97235-41-1; **19** (3-MeS), 16233-01-5; **20** (1-MeS), 97235-42-2; **20** (3-MeS), 97235-43-3; **21** (1-MeS), 97235-44-4; **21** (3-MeS), 97235-45-5; **22**, 37819-60-6; **23**, 97235-46-6; **24**, 73051-69-1; **25**, 97235-47-7; **26**, 97235-48-8; **27**, 97235-49-9; AHAT, 52660-15-8; 2′-dG, 961-07-9; *N*-acetylmethionine, 65-82-7.

Supplementary Material Available: Tabulations of chemical shifts and multiplicities of ^{13}C NMR data for **22**, **23**, 2′-deoxyguanosine, and 2-acetamidofluorene and of the protons of enzymatically produced samples of **24**–**26** (3 pages). Ordering information is given on any current masthead page.