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Acetylation of the Carcinogen *N*-Hydroxy-2-acetylaminofluorene by Acetyl Coenzyme A to Form a Reactive Ester

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SUMMARY

Nonenzymatic acetylation of various carcinogenic hydroxamic acids was studied with acetyl-1-^{14}C-CoA as the acetyl donor. Formation of acetate esters was studied by determination of the benzene-extractable radioactivity. Interaction of these esters *in vitro* at pH 7.5 with methionine and guanosine was used as a measure of their reactivity.

N-Hydroxy-2-acetylaminofluorene was acetylated to a much greater extent than 2-aminofluorene, 2-acetylaminofluorene, or its ring-hydroxylated derivatives. The reaction was dependent on pH and the type of buffer, period of incubation, and the concentrations of acetyl-CoA and *N*-hydroxy-2-acetylaminofluorene. With 5 min of incubation and $KHCO_3$–KOH buffer, the pH optimum was found to be 10. On the basis of its ultraviolet absorption spectrum, R_F value after paper chromatography, lability to alkali, and characteristic reaction products with methionine and guanosine, the acetylated reaction product of *N*-hydroxy-2-acetylaminofluorene has been characterized as *N*-acetoxy-2-acetylaminofluorene. Among various *N*-hydroxy derivatives tested, *N*-hydroxyurethane and its *N*-methyl derivatives were acetylated most rapidly.

It is suggested that acetate esters of hydroxamic acids may also be some of the ultimate carcinogenic metabolites of carcinogenic hydroxamic acids.

INTRODUCTION

Various mammalian species detoxify many foreign aromatic amines via acetylation (1). Acetyl-CoA was first demonstrated to be the acetyl donor in the acetylation of histamine and numerous other amines by pigeon liver preparations (2). Subsequently, various *N*-acetyltransferases have been partially purified from the livers of several mammalian species and have been shown to acetylate many foreign compounds (3).

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The carcinogenic aromatic hydroxamic acids are converted by rat liver cytosol to their corresponding phosphate and sulfate esters, which are probably some of the ultimate active metabolites (4–8). The synthetic acetate esters of aromatic hydroxamic acids have been shown to be more carcinogenic than the parent hydroxamic acids (9). Studies *in vitro* at physiological pH have indicated that synthetic acetate esters of these aromatic hydroxamic acids are reactive with several tissue nucleophiles, including methionine and guanosine, whereas the parent compounds are unreactive (7, 9–16).

The present paper describes acetylation of carcinogenic hydroxamic acids with acetyl-CoA as the acetyl donor. Acetylation of hy-

droxamic acids by acetyl-1-^{14}C-CoA was studied by determination of the benzene-extractable radioactivity. Interaction of these acetate esters *in vitro* at pH 7.5 with methionine and guanosine was used as a measure of their reactivity. A preliminary report of some of this work has appeared (17).

MATERIALS AND METHODS

Chemicals

2-Aminofluorene was purchased from K & K Laboratories, and 2-acetylaminofluorene was obtained from Mann Research Laboratories. The *N*-hydroxy (18) and *N*-acetoxy (12) derivatives of 2-acetylaminofluorene were prepared in this laboratory by the procedures described in the references cited. The following compounds were generously supplied by Dr. J. A. Miller of the McArdle Laboratory, Madison, Wis.: 1-, 3-, and 7-hydroxy-AAF;[2] 3-methylmercapto-AAF; 3-methylmercapto-AF; and 7-fluoro-AAF, 4-acetylaminobiphenyl, 2-acetylaminophenanthrene, 4-acetylaminostilbene, 2-acetylaminonaphthalene, and their respective *N*-hydroxy derivatives. 2-Benzoylaminofluorene and its *N*-hydroxy derivative were kindly provided by Dr. H. R. Gutmann of the Veterans Administration Hospital, Minneapolis. *N*-Hydroxyurethane and its *N*-methyl derivative were gifts of Dr. D. Swern of this institute. Unlabeled acetyl-CoA was obtained as the lithium salt from P-L Biochemicals. Acetyl-1-^{14}C-CoA (58.1 mCi/mmole) was purchased from New England Nuclear Corporation. L-Methionine and guanosine dihydrate were obtained from Sigma Chemical Company and Calbiochem, respectively. Guanosine-8-^{14}C (34.1 mCi/mmole) and L-methionine-^{35}S (25–30 mCi/mmole) were purchased from Schwarz BioResearch. *N*-Hydroxy-AAF-9-^{14}C (12.7 mCi/mmole) was purchased from Tracerlab. All other chemicals were of reagent grade.

Acetylation Reaction

Incubation medium and extraction. Unless otherwise indicated, the complete incubation medium contained $KHCO_3$–KOH buffer, pH 9.0, 100 mM; acetyl-1-^{14}C-CoA (0.1 μCi), 0.4 mM; *N*-hydroxy-AAF or other substrate, 4 mM; dimethyl sulfoxide, used for dissolving substrates, 1410 mM; and water to a final volume of 0.5 ml. Duplicate tubes were incubated in air for 5 min at 37°. After incubation, 2.5 ml of 0.5 M HCl were added and the contents were extracted immediately with 5 ml of benzene. The benzene extract was washed several times with water. A 2-ml sample of the benzene extract was added to 10 ml of Bray's solution (19) for radioactivity measurements in a Packard Tri-Carb spectrometer.

Chromatography. Samples of the benzene extract were chromatographed on silicic acid-impregnated paper (Whatman No. SG-81) in the benzene–chloroform (2:3, v/v) solvent system described previously (8). After chromatography, 1-cm^2 zones were cut out and eluted with 1 ml of methanol, and radioactivity was measured using Bray's solution (19).

Separation of Methionine Reaction Products

After reaction with ^{35}S-methionine, the reaction mixture was made 1 M with respect to KOH, and 15 min later the mixture was extracted with benzene. Paper chromatography on silicic acid paper and radioactivity measurements on the benzene extracts were carried out as described previously (8). In the benzene–chloroform (2:3, v/v) solvent system, synthetic 3-methylmercapto-AAF and 3-methylmercapto-AF showed R_F values of 0.35–0.44 and 0.67–0.77, respectively. Radioactive zones corresponding to these regions were used for calculation of the amounts of these two products formed when *N*-hydroxy-AAF was the substrate. It has been demonstrated that the reaction of synthetic *N*-acetoxy-AAF with methionine yields a mixture of 1- and 3-methylmercapto-AAF; however, these two isomers have similar thin-layer and gas chromatographic properties (7). In the present studies, a mixture of these two isomers was probably formed; however, it is considered as 3-methylmercapto-AAF. When other hydroxamic acids were used as substrates, the radioactive zones with R_F values similar to those described above for *N*-hydroxy-AAF were used to calculate the amounts of the respec-

[2] The abbreviations used are: AAF, 2-acetylaminofluorene, AF, 2-aminofluorene.

tive *o*-methylmercaptoamide and *o*-methylmercaptoamine formed. Recently one such *o*-methylmercaptoamide, 3-methylmercapto-4-acetylaminobiphenyl, has been characterized as the reaction product of esters of *N*-hydroxy-4-acetylaminobiphenyl with methionine (7). 3-Methylmercapto-AAF formation was also determined by gas-liquid chromatography as described previously (8).

Separation of Guanosine Reaction Product by Thin-Layer Chromatography

After incubation with guanosine-8-^{14}C, an aliquot (usually about 100–150 μl) of the reaction mixture was chromatographed on a cellulose (Brinkmann, No. MN 300 UV254) thin-layer plate (250 μ in thickness) in 1-butanol–acetic acid–water (50:11:25 by volume) as described previously (14). Chromatography was terminated after the solvent front had traveled about 15 cm from the origin. Under ultraviolet light, guanosine showed R_F values of 0.23–0.45. Zones 1 cm square were scraped quantitatively into vials containing 10 ml of Bray's solution (19) for radioactivity measurements. The radioactive peak with R_F values of 0.73–0.93 was used to assay the formation of the guanosine reaction product.

Spectra

A Coleman-Hitachi model 124 spectrophotometer with a recorder was used for determination of ultraviolet absorption spectra between 350 and 270 nm.

RESULTS

Incubation of *N*-hydroxy-AAF with acetyl-1-^{14}C-CoA in a bicarbonate buffer at pH 9.0 for 5 min yielded about 7% of radioactivity extractable into benzene (Table 1). The yields of product from 7-hydroxy-AAF and 1-hydroxy-AAF were about 12% and 4%, respectively, of that obtained from *N*-hydroxy-AAF. Incubation of AF or AAF under similar conditions produced negligible amounts of acetylated products.

The results in Table 2 indicate that the acetylated reaction product of *N*-hydroxy-AAF extractable into benzene (steps 1 and 2) was *N*-acetoxy-AAF. (a) The peak of the radioactivity profile of the benzene extract (step 2) after silicic acid paper chromatography coincided with the R_F value (0.26–0.36) of synthetic *N*-acetoxy-AAF. Acetyl-CoA when chromatographed in this solvent system had a low R_F (0–0.03). (b) The ultraviolet absorption spectrum in ethanol (step 3), after benzene extraction and washing with alkali to remove *N*-hydroxy-AAF, was almost identical with that of synthetic *N*-acetoxy-AAF. Thus, the absorption maxima at 302, 289, and 275 nm and the minimum at 298 nm were the same for both compounds. A_{302}:A_{275} ratios were 0.696 and 0.714 for synthetic *N*-acetoxy-AAF and the isolated compound, respectively. (c) The amount of *N*-acetoxy-AAF formed after 5 min of incubation, calculated from the ultraviolet absorption spectrum (step 3), was almost equal to the amount of radioactivity extractable into benzene (steps 1 and 2). (d) The radioactive product was labile to alkali, and the radioactivity was quantitatively retained in the alkali (step 4).

TABLE 1

Acetylation of various fluorene derivatives by acetyl-CoA

The incubation medium and other details are described in MATERIALS AND METHODS. Results are averages of duplicate analyses.

Substrate (4 mM)	Benzene-extractable radioactivity
	nmoles/5 min
None	0.05
N-Hydroxy-AAF	0.08[a]
N-Hydroxy-AAF	13.4
1-Hydroxy-AAF	0.61
3-Hydroxy-AAF	0.27
7-Hydroxy-AAF	1.59
AF or AAF	0.12

[a] Zero time.

Rate of Acetylation

Acetylation, when followed over a period of 2 hr, was found to be linear for the first 5 min (Fig. 1). Therefore 5-min incubation periods have routinely been used.

Effect of pH and Buffers

The acetylation of *N*-hydroxy-AAF was dependent on the pH and the type of buffer

TABLE 2

Acetylation of N-hydroxy-AAF by acetyl-CoA

The incubation medium contained 4 μmoles of *N*-hydroxy-AAF dissolved in 0.1 ml of dimethyl sulfoxide, 100 μmoles of $KHCO_3$–KOH buffer (pH 9.0), and 2 μmoles of acetyl-1-^{14}C-CoA (3×10^5 dpm) in a total volume of 1.0 ml. After incubation in air at 37°, 2.5 ml of 0.5 M HCl were added, and the contents were extracted with benzene. The benzene extract was washed successively with water (step 1) and with 0.5 M KOH and water (step 2). A sample of the benzene extract (after step 2) was chromatographed on silicic acid-impregnated paper with a solvent system composed of benzene–chloroform (2:3, v/v) as described in MATERIALS AND METHODS. Benzene was removed under N_2, and the residue was dissolved in ethanol for measurements of ultraviolet absorption (350–270 nm) (step 3). The molar extinction coefficient of *N*-acetoxy-AAF in ethanol at 275 nm was about 25,000. This value was used for calculation of the total amount of *N*-acetoxy-AAF formed. Finally ethanol was removed under N_2, and 0.5 M KOH was added to the residue. After 5 min, the contents were extracted with benzene (step 4). Radioactivity was measured in samples of benzene (steps 1, 2, and 4) and 0.5 M KOH (step 4) extracts using 10 ml of Bray's solution (19).

Incubation period	Radioactivity in extract				*N*-Acetoxy-AAF formed
	Benzene (step 1)	Benzene (step 2)	Benzene (step 4)	KOH (step 4)	(ethanol, step 3)
(*min*)	*nmoles*				*nmoles*
0	0.26	0.26	0.3	1.1	14
5	191	192	1.1	193	208

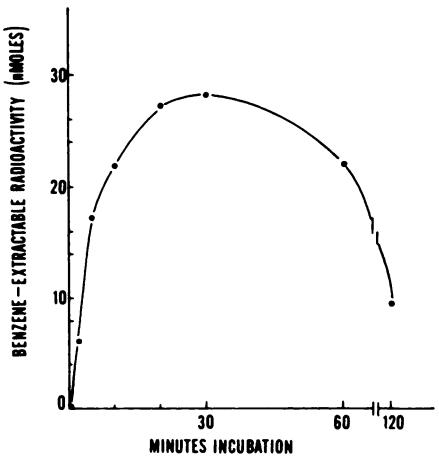

FIG. 1. *Rate of acetylation of N-hydroxy-AAF with acetyl-CoA*

The incubation medium was the same as described in MATERIALS AND METHODS, except that the total volume was 5.0 ml and the substrate was *N*-hydroxy-AAF. At various time intervals, 0.5-ml samples were withdrawn from the medium, and extraction and radioactivity measurements were carried out as described in the text.

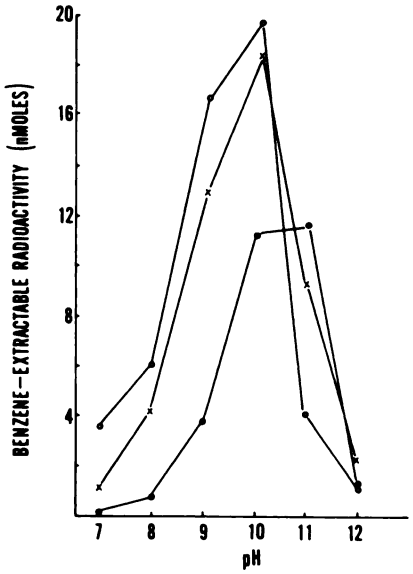

FIG. 2. *Effect of pH on acetylation of N-hydroxy-AAF by acetyl-CoA*

All details were the same as described in MATERIALS AND METHODS, except for the type of buffer and pH used. ●——●, Tris-HCl buffer; ○——○, $KHCO_3$–KOH buffer; ×——×, boric acid–NaOH buffer.

used (Fig. 2). Potassium bicarbonate–KOH buffer was found to be better than Tris-HCl and boric acid–NaOH buffers. With bicarbonate–KOH and boric acid–NaOH buffers, pH 10 was found to be optimal for the reaction. In routine studies, bicarbonate–KOH buffer, pH 9.0, has been used.

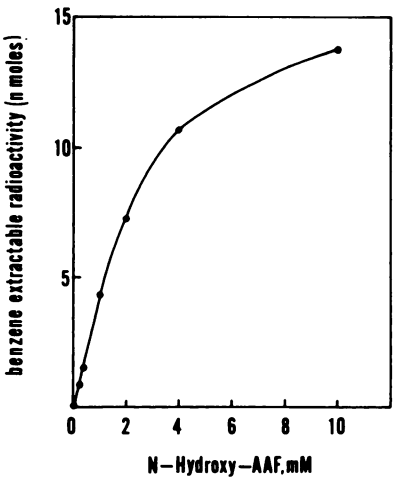

FIG. 3. *Effect of N-hydroxy-AAF concentration on its acetylation by acetyl-CoA*

All conditions were the same as described in MATERIALS AND METHODS, except for the concentration of *N*-hydroxy-AAF.

N-Hydroxy-AAF Concentration

At 0.4 mM acetyl-CoA, acetylation was directly proportional to the *N*-hydroxy-AAF concentration between 0.2 and 1.0 mM (Fig. 3).

Acetyl-CoA Concentration

Similarly, at 4 mM *N*-hydroxy-AAF, the reaction was proportional to the acetyl-CoA concentration between 0.1 and 2 mM (Fig. 4).

Like *N*-hydroxy-AAF, various other *N*-hydroxy compounds, except for derivatives of 2-benzoylaminofluorene and stilbene, were also acetylated (Table 3). The corresponding unsubstituted amides of many of these *N*-hydroxy compounds, however, were not acetylated.

It has been demonstrated that synthetic *N*-acetoxy-AAF reacts nonenzymatically at neutral pH with the sulfur atom of methionine and cysteine (13, 20). It was also shown that *N*-acetoxy-AAF reacts with orthophosphate in a phosphate buffer at pH 7 to yield a large quantity of water-soluble fluorene derivatives (13, 21). It was apparent that *N*-acetoxy-AAF was unstable (see Fig. 1) and that it probably reacted with either CoASH or acetyl-CoA or both. Interaction with CoA could be demonstrated by using *N*-hydroxy-AAF-9-^{14}C (Table 4). In the presence of acetyl-CoA, a large amount of radioactivity remained in the aqueous phase. On the basis of previous studies (13, 20, 21), it would appear that in the present experiments *N*-acetoxy-AAF-9-^{14}C reacted with the phosphate moiety of either CoASH or

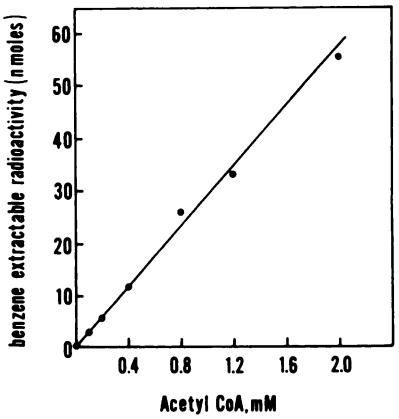

FIG. 4. *Effect of acetyl-CoA concentration on acetylation of N-hydroxy-AAF*

All conditions were the same as described in MATERIALS AND METHODS, except for the concentration of acetyl-CoA.

TABLE 3

Acetylation of various N-hydroxy compounds by acetyl-CoA

All conditions were as described in MATERIALS AND METHODS. Results are averages of duplicate analyses.

Substrate (4 mM)	Benzene-extractable radioactivity
	nmoles 5 min
None	0.05
N-Hydroxy-AAF	16.5
7-Fluoro-*N*-hydroxy-AAF	21.7
N-Hydroxy-2-benzoylaminofluorene	0.25
N-Hydroxy-4-acetylaminobiphenyl	26.8
N-Hydroxy-2-acetylaminophenanthrene	8.0
N-Hydroxy-4-acetylaminostilbene	0.9
N-Hydroxy-2-acetylaminonaphthalene	29.5
N-Hydroxyurethane	39.0
N-Methyl-*N*-hydroxyurethane	41.4

TABLE 4

Formation of water-soluble fluorene derivative by interaction of N-hydroxy-AAF with acetyl-CoA

Unless otherwise indicated, the complete incubation medium contained 50 μmoles of $KHCO_3$-KOH buffer (pH 9.0), 1 μmole of *N*-hydroxy-AAF-9-^{14}C (10 μCi) dissolved in 0.05 ml of ethanol, and 2 μmoles of acetyl-CoA in a total volume of 1.0 ml. After incubation in air for 4 hr at 37°, samples were extracted several times at neutrality with benzene. Samples of the aqueous phase were taken for radioactivity measurements using Bray's solution (19).

Addition of acetyl CoA	Radioactivity in aqueous phase
	% total
−	0.41
+	11.3

acetyl-CoA or with the sulfur atom of CoASH to form polar fluorene derivatives. Chromatography of the aqueous phase on Whatman No. 1 paper in a 0.1 M sodium acetate (pH 4.5)–ethanol (1:1, v/v) solvent system gave radioactivity peaks with R_F 0.80–0.87 and 0.66–0.73 for samples without and with acetyl-CoA, respectively. Chromatography of acetyl-1-^{14}C-CoA alone in this solvent system showed a radioactive peak with an R_F of 0.47–0.53.

It was also possible to demonstrate that the acetylation product of *N*-hydroxy-AAF could react with methionine and guanosine (Table 5). The reaction product with methionine had the same R_F (0.33–0.47) as the synthetic 3-methylmercapto-AAF (R_F 0.35–0.44). Similarly, the reaction product obtained from guanosine gave about the same R_F (0.73–0.93) as the product that has been characterized as *N*-(guanosin-8-yl)-AAF (6, 16). *N*-Hydroxy-AAF in the absence of acetyl-CoA did not react to an appreciable extent with either methionine or guanosine.

Although various other aromatic hydroxamic acids were also acetylated by acetyl-CoA (see Table 3), it was important to investigate whether their *N*-acetoxy products would react with methionine. These results are summarized in Table 6. With the exception of 7-fluoro-*N*-hydroxy-AAF, other *N*-hydroxyamides in the presence of acetyl-CoA did not react with methionine to an appreciable extent.

Several attempts to demonstrate enzymatic acetylation of *N*-hydroxy-AAF and the other hydroxamic acids with acetyl-CoA by liver cytosol from various rodent species have been unsuccessful.

DISCUSSION

Although *N*-hydroxy derivatives are more carcinogenic than their corresponding aromatic amines and amides (22–27), these *N*-hydroxy compounds are unreactive with tissue macromolecules or their constituents at physiological pH *in vitro* (12, 14, 15, 28–31). However, the synthetic *N*-acetoxyamides react with proteins, RNA, DNA, or their methionine, tryptophan, tyrosine, cysteine, guanosine, or deoxyguanosine constituents *in vitro* (10, 12, 14, 16, 32–34) and are more carcinogenic than the corresponding *N*-hydroxyamides (9, 10). Whereas the *N*-acetylation of many nitrogen-containing foreign compounds is usually considered a detoxification process (1), the synthetic acetylation of many carcinogenic *N*-hydroxyamides appears to be an activation process.

The results presented in this paper indicate that many *N*-hydroxy compounds can

TABLE 5

Reaction of N-hydroxy-AAF with methionine and guanosine in the presence of acetyl-CoA

Unless otherwise indicated, the complete incubation medium contained 100 μmoles of Tris-HCl buffer (pH 7.5), 4 μmoles of *N*-hydroxy-AAF dissolved in 0.1 ml of dimethyl sulfoxide, 4 μmoles of acetyl-CoA, and 10 μmoles of L-methionine-^{35}S (4×10^6 dpm) or 2 μmoles of guanosine-8-^{14}C (2×10^6 dpm) in a total volume of 1.0 ml. After incubation in air for 19 hr at 37°, analyses of the reaction products of methionine and guanosine were performed as described in MATERIALS AND METHODS.

Addition of acetyl CoA	Reaction product formed with	
	Methionine (3-methylmercapto-AAF)	Guanosine (R_F 0.73–0.93)
	nmoles	
−	0.5	5.0
+	332	349

TABLE 6

Reaction of hydroxamic acids with methionine in the presence of acetyl-CoA

The complete incubation medium contained 50 μmoles of Tris-HCl buffer (pH 7.5), 2 μmoles of hydroxamic acid dissolved in 0.05 ml of dimethyl sulfoxide, 2 μmoles of acetyl-CoA, and 5 μmoles of L-methionine-^{35}S (1 μCi) in a total volume of 0.5 ml. After incubation in air for 19 hr at 37°, the reaction products, *o*-methylmercaptoamide and *o*-methylmercaptoamine, were separated by paper chromatography and determined quantitatively by radioactivity measurements as described for *N*-hydroxy-AAF in MATERIALS AND METHODS.

Hydroxamic acid	Amount formed	
	o-Methylmercaptoamide	*o*-Methylmercaptoamine
	nmoles	
None	0.5	0.03
N-Hydroxy-AAF	247	0.78
7-Fluoro-*N*-hydroxy-AAF	70	0.32
N-Hydroxy-4-acetylaminostilbene	0.28	0.23
N-Hydroxy-4-acetylaminobiphenyl	2.62	0.83
N-Hydroxy-2-acetylaminophenanthrene	0.27	0.21
N-Hydroxy-2-acetylaminonaphthalene	0.42	0.23

be nonenzymatically acetylated to different extents by acetyl-CoA. Although such a reaction is not surprising, it is significant that it occurs under physiological conditions under which no *N*-acetylation is observed (see Table 1). Based on several criteria, the acetylated product of *N*-hydroxy-AAF has been characterized as *N*-acetoxy-AAF. Like the synthetic *N*-acetoxy-AAF (12, 14, 16), the reaction product of *N*-hydroxy-AAF and acetyl-CoA reacted with methionine and guanosine to give similar products (Table 5). Similarly, the acetylated product of 7-fluoro-*N*-hydroxy-AAF was reactive with methionine, but to only about 28% of the extent observed with *N*-hydroxy-AAF (Table 6). It has been found that synthetic 7-fluoro-*N*-acetoxy-AAF was as reactive with methionine as *N*-acetoxy-AAF.[3] No explanation is evident to account for the decreased activity observed in the present studies with the 7-fluoro derivative.

Maher *et al.* (33) have shown that synthetic *N*-acetoxy-AAF caused more inactivation of the transforming activity of *Bacillus subtilis* DNA and was a stronger mutagen than were the *N*-acetoxy compounds of other aromatic amides. Similar results were obtained in studies of the interaction of these compounds with methionine and guanosine (11). In accordance with the findings of Scribner *et al.* (11), the present studies have revealed that the *N*-acetoxy derivatives of 4-acetylaminobiphenyl, 4-acetylaminostilbene, 2-acetylaminophenanthrene, and 2-acetylaminonaphthalene were much less reactive than *N*-acetoxy-AAF with methionine (Table 6).

Acyl esters of *N*-hydroxyurethane react *in vitro* at neutral pH with cytosine, cysteine, glutathione, and methionine more readily than does *N*-hydroxyurethane (35). Among several hydroxamic acids tested in the present studies, *N*-hydroxyurethane and its *N*-methyl derivative were acetylated most extensively by acetyl-CoA (Table 3). Such an acetylation reaction may be important in its carcinogenic activity.

Chloramphenicol is *O*-acetylated enzymatically on the hydroxyl group attached to the carbon atom by extracts of *Escherichia coli* (36). There is no evidence to indicate that such enzymatic *O*-acetylation of the hydroxyl group attached directly to either the carbon or nitrogen atom of a foreign compound takes place in mammalian systems. In the present studies, we were unable to demonstrate enzymatic *O*-acetylation of *N*-hydroxy-AAF or other carcinogenic hydroxamic acids, in confirmation of the work of Miller (9).

N-Oxides of guanine and xanthine are oncogenic (37, 38), and recent studies have demonstrated that their synthetic acetate esters are very reactive with methionine and chloride at neutral pH *in vitro* (39). Preliminary studies have suggested that 3-hydroxyxanthine was *O*-acetylated by acetyl-CoA in the presence of acetone powders of rat liver cytosol (40). However, those results are inconclusive with respect to the enzymatic nature of the reaction, because acetylation

[3] P. D. Lotlikar, J. A. Miller, and E. C. Miller, unpublished observations.

was not investigated either in the absence of enzyme or in the presence of boiled enzyme.

Recently the nonenzymatic nature of histone acetylation by acetyl-CoA has also been demonstrated (41). The results reported in the present paper furnish another example of the nonenzymatic nature of some acetylations.

Acetyl-CoA is present in most mammalian tissues, even though its concentration in these tissues is variable under different conditions (42). The results presented in this paper indicate that hydroxamic acids might be *O*-acetylated nonenzymatically *in vivo*. If this occurs, such acetate esters might also be some of the ultimate carcinogenic metabolites of the carcinogenic hydroxamic acids.

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