Covalent Binding of *N*-Hydroxy-*N*-acetyl-2-aminofluorene and *N*-Hydroxy-*N*-glycolyl-2-aminofluorene to Rat Hepatocyte DNA: In Vitro and Cell-Suspension Studies

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Two 2-aminofluorene-derived hydroxamic acids that differ only in the nature of the *N*-acyl group were examined for their relative abilities to undergo covalent binding to nucleic acids. Studies of the bioactivation of *N*-hydroxy-*N*-acetyl-2-aminofluorene (*N*-OH-AAF) and *N*-hydroxy-*N*-glycolyl-2-aminofluorene (*N*-OH-GAF) were conducted with hepatocyte suspensions and subcellular fractions prepared from male Sprague–Dawley rats. Both hydroxamic acid substrates displayed equal binding to both DNA and RNA after incubations with hepatocyte suspensions. The extent of binding of each substrate was approximately the same for DNA and RNA. Investigations with subcellular fractions revealed some major differences between the probable mechanisms by which the two substrates were covalently bound to exogenous DNA. In agreement with the prior literature reports, *N*-OH-AAF was extensively bound to DNA through the action of cytosol enzymes, including both *N*,*O*-acyltransferase and sulfotransferase. The microsomal enzyme fraction also catalyzed binding to DNA, and this process was completely inhibited by paraoxon. The covalent binding of *N*-OH-GAF to DNA was catalyzed by cytosol enzymes to a significant extent only in the presence of 3′-phosphoadenosine-5′-phosphosulfate, which suggests the action of sulfotransferase. Covalent binding of *N*-OH-GAF to DNA was minimal through the action of cytosolic *N*,*O*-acyltransferase, which confirms our earlier observation that *N*-OH-GAF is a potent suicide inhibitor of this enzyme. The microsomal fraction catalyzed the binding of *N*-OH-GAF to DNA at a rate that was about twice that observed for *N*-OH-AAF. As in the case of *N*-OH-AAF binding, this microsomal-catalyzed binding of *N*-OH-GAF was prevented by paraoxon. On the basis of these results and from a knowledge of the enzymatic mechanisms involved in such binding, it was proposed that the nucleic acid adducts from *N*-OH-GAF should possess a much higher percent of adducts which retain the acyl group relative to those adducts resulting from *N*-OH-AAF bioactivation.

Introduction

The role of hydroxamic acids as mediators of the toxicity of arylamines and related chemicals has been well documented (*1–3*). Certain hydroxamic acids are proximate genotoxicants that require further bioactivation to electrophilic metabolites that can then react with nucleic acids. These bioactivation reactions include such processes as O-sulfation and N,O-acyltransfer (*2*, *3*). Most aromatic hydroxamic acids produced by metabolic reactions are *N*-acetyl derivatives of an arylhydroxylamine; however, we have reported on the biological production of certain unusual hydroxamic acids that possess acyl groups other than acetyl (*4*). Of particular interest is the type of hydroxamic acid in which the acyl group is glycolic acid. *N*-Glycolyl type hydroxamic acids are produced by the action of transketolase on *C*-nitroso aromatics as illustrated in Figure 1 (*4*, *5*). This adventitious metabolic reaction has been shown to occur within unicellular organisms (*6*), in liver homogenates (*7*), and most recently in mammalian cell cultures (unpublished data).

In view of the fact that the *N*-glycolyl type of hydroxamic acid is a metabolite of aromatic nitroso compounds, albeit a minor metabolite in most cases, it was of interest to determine the genotoxic potential of a representative *N*-glycolyl hydroxamic acid relative to the analogous *N*-acetyl hydroxamic acid. This comparative study was conducted with the *N*-glycolyl (*N*-OH-GAF)[1] and *N*-acetyl (*N*-OH-AAF) hydroxamic acid derivatives of the known carcinogen *N*-hydroxy-2-aminofluorene in isolated rat hepatocyte cultures and in subcellular fractions. We now report that *N*-OH-GAF gave the same degree of covalent binding to nucleic acid as did *N*-OH-AAF upon incubation in hepatocyte cultures; however, studies with subcellular fractions indicated that the two hydroxamic acids are probably bound to DNA through different pathways.

Experimental Section

Chemicals. [9-^{14}C]*N*-OH-2-AAF (sp act. 4.8 mCi/mmol) was obtained from Chemsyn Labs (Lenexa, KS) and purified by HPLC (*8*). [9-^{14}C]*N*-OH-GAF (sp act. = 3.84 mCi/mmol) was synthesized

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[1] Abbreviations: *N*-OH-AAF, *N*-hydroxy-*N*-acetyl-2-aminofluorene; *N*-OH-FAF, *N*-hydroxy-*N*-formyl-2-aminofluorene; *N*-OH-GAF, *N*-hydroxy-*N*-glycolyl-2-aminofluorene; PAPS, 3′-phosphoadenosine-5′-phosphosulfate.

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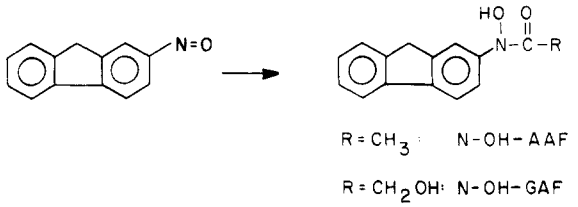

Figure 1. Conversion of 2-nitrosofluorene to the hydroxamic acids *N*-OH-AAF and *N*-OH-GAF.

and purified as previously described (*8*).

Preparation of Hepatocyte Suspensions. Suspensions of hepatocytes were prepared from livers of male Sprague–Dawley rats (80–100 g) by a modification of the procedure of Wiebkin et al. (*9*). Following the second incubation with phosphate buffered saline (PBS) and 0.5 mM EGTA, the liver slices were incubated with collagenase (CLS II, Cooper Biomedical, Malvern, PA) as previously described, except that hyaluronidase was excluded and the incubation time was 50 min. The cell suspension was filtered through a Nitex nylon screen (125 μm, Tetko Inc., Elmsford, NY) and washed three times with Leibowitz L-15 medium (Gibco Labs, Grand Island, NY). The cells were suspended in Leibowitz L-15 medium fortified with 10% (v/v) fetal calf serum (Gibco Labs) and counted with a hemocytometer. Viability was determined by trypan blue dye exclusion (*10*), and only those preparations with viabilities exceeding 90% were employed in subsequent studies.

Covalent Binding to Hepatocyte DNA and RNA. Incubations were conducted by the addition of 100 nmol of ^{14}C-labeled substrates as a solution in 30 μL of DMSO to 5 mL of L-15 medium containing hepatocytes at a concentration of 1.5×10^6 cells/mL. The suspensions were contained in a 50-mL polypropylene tube and incubated with gentle agitation at 37 °C for 2 h. After incubation, the cell suspension was immediately centrifuged at 1500*g* for 3 min. The cell pellet was washed once with 2 mL of PBS and centrifuged. The cell pellet was lysed at 50 °C for 1 h in 2 mL of lysis buffer (1% sodium lauryl sulfate [SDS] in 0.01 M Tris·HCl, 0.01 M EDTA, and 0.1 M NaCl, pH 7) which contained 0.5 mg of proteinase K (Sigma Chemical Co., St. Louis, MO). The suspension was extracted twice (30 and 15 min) with 2 mL of phenol reagent (phenol, chloroform, isoamyl alcohol, and 8-hydroxyquinoline, 25/24/1/0.05), which was saturated with 0.1 M Tris·HCl buffer, pH 8.0, containing 0.2% dithiothreitol). The upper aqueous phase was then extracted twice with 2 mL of chloroform reagent (chloroform and isoamyl alcohol, 24/1). The aqueous layer was further extracted with 2 mL of H_2O-saturated diethyl ether, and residual ether was removed with a N_2 stream. Nucleic acids were precipitated by the addition of 0.10 mL of 3 M NaCl solution followed by 4.0 mL of ice-cold ethanol. The fibrous DNA was retrieved with a glass rod, dried with a stream of N_2, and then redissolved in 1.74 mL of H_2O (DNA fraction). The aqueous ethanol solution containing mostly RNA was centrifuged at 12000*g* for 60 min. The pellet was washed with 70% ethanol and centrifuged at 12000*g* for 60 min. The pellet was dried with a stream of N_2 and redissolved in 1.74 mL of H_2O (RNA fraction). Both the DNA and RNA fractions were treated with 0.26 mL of LiCl solution (0.6 g/mL of H_2O), allowed to stand overnight at 4 °C, and then centrifuged at 17000*g* for 20 min at 4 °C to precipitate RNA. The resulting supernatant from the DNA fraction was transferred to a new centrifuge tube and treated with 4.0 mL of ethanol to precipitate the DNA. After centrifugation at 12000*g* for 60 min, the pellet was dried and dissolved in 1.2 mL of H_2O. The pellet from the LiCl treatment of the RNA fraction was dried and dissolved in 1.2 mL of H_2O. Radioactivity of a 1.0-mL aliquot was determined by liquid scintillation counting, and the amount of nucleic acid was determined from A_{260} (*11*). Nonspecific binding (blank) was determined by the addition of ^{14}C-labeled substrates to untreated hepatocytes after the cell lysis step.

Preparation of Subcellular Fractions from Rat Liver. Male Sprague–Dawley rats (100–200 g) were sacrificed by cervical dislocation after halothane anesthesia. Livers were removed and homogenized at 4 °C with a Potter-Elvehjem homogenizer in 3 vol of 20 mM Tris·HCl, pH 7.2, containing 0.15 M KCl, 3 mM $MgCl_2$, and 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 10000*g* for 20 min at 4 °C. The supernatant was then centrifuged at 100000*g* for 60 min at 4 °C to obtain the cytosolic and microsomal preparations in the supernatant and pellet fractions, respectively. Protein concentration was determined by the Bradford method (*12*) using bovine serum albumin as a standard. Partially purified arylhydroxamic acid *N,O*-acyltransferase was prepared according to the method of Mangold and Hanna (*13*). The protein in this preparation was determined by assuming that 1 mg/mL of protein gives an A_{280} of 1.0 (*14*).

Metabolism of *N*-OH-AAF and *N*-OH-GAF by Subcellular Fractions. The metabolism of *N*-OH-AAF and *N*-OH-GAF was determined as substrate disappearance by use of HPLC methods. Incubations consisted of 5-mL volumes in 25-mL polycarbonate flasks of 30 mM Tris·HCl and 9 mM $MgCl_2$, pH 7.4, cytosol enzymes (4 mg/mL) or microsomal enzymes (1 mg/mL) and 20 μM substrate. In the case of cytosol enzymes, incubations were conducted in the presence and absence of 3′-phosphoadenosine-5′-phosphosulfate (PAPS, 0.2 mM). The reactions were conducted at 37 °C in a shaking incubator. Aliquots of 0.80 mL each were taken at predetermined times and combined with an equal volume of methanol. Following centrifugation to deposit protein, the methanol-quenched aliquots were analyzed by HPLC employing a μBondapak C_{18} column (3.9 mm × 30 cm, Waters Assoc., Milford, MA) and a solvent of 55% methanol buffered to pH 3.5 with 0.01 M KH_2PO_4/H_3PO_4 and containing 0.01% desferal mesylate (*8*). The amount of substrate which remained was calculated on the basis of peak heights in the chromatograms generated with a UV detector at 280 nm.

Covalent Binding of *N*-OH-AAF and *N*-OH-GAF to DNA Mediated by Subcellular Fractions. Calf thymus DNA (Type I, Sigma) was denatured and purified as previously described (*8*). Incubations consisted of 5-mL volumes in 25-mL polycarbonate flasks of 30 mM Tris·HCl and 9 mM $MgCl_2$, pH 7.4, cytosol enzymes (4 mg/mL), microsomal enzymes (1 mg/mL) or *N,O*-acyltransferase (2.7 mg/mL), 20 μM ^{14}C-labeled substrate (*N*-OH-AAF or *N*-OH-GAF), and DNA (1 mg/mL for cytosol enzymes and 2 mg/mL for microsomal enzymes). The reactions were conducted at 37 °C in a shaking incubator and were initiated by the addition of the hydroxamic acid substrate. In the case of cytosol enzymes, incubations were conducted in the presence and absence of PAPS (0.2 mM); microsomal incubations were conducted in the presence and absence of paraoxon (0.5 mM); *N,O*-acyltransferase incubations were conducted under a N_2 atmosphere and with 0.05 M pyrophosphate/1 mM dithiothreitol buffer, pH 7.0, instead of the Tris·HCl buffer. At predetermined times, 1.0-mL aliquots were combined with 4 mL of ice-cold ethyl acetate (H_2O-saturated) and 1 mL of the Tris·HCl buffer and vigorously mixed. Following centrifugation, the aqueous layer was extracted with 2 mL of diethyl ether (H_2O-saturated). Residual ether was removed with a stream of N_2, and then the aliquots were treated with 100 μL of (20% w/v) aqueous SDS and 40 μL of an aqueous solution of proteinase K (10 mg/mL), followed by incubation at 50 °C for 1 h. The aliquots were then extracted twice with 2 mL of phenol reagent for 15 min each, followed by 2 mL of chloroform reagent and then 2 mL of diethyl ether (H_2O-saturated). Following the removal of residual ether with a stream of N_2, the aliquots were transferred to 10-mL polycarbonate centrifuge tubes and treated with 100 μL of 3M NaCl and 5 mL of cold ethanol to precipitate the DNA. After standing overnight at 4 °C, the samples were centrifuged (12000*g* for 60 min at 4 °C), and the DNA pellets were dried with a stream of N_2. Each pellet was dissolved in 1.0 mL of H_2O, and then aliquots (100 μL for cytosol enzymes and 300 μL for microsomal enzymes) were analyzed by liquid scintillation counting in 15 mL of ScintiVerse Bio-HP (Fisher Scientific). DNA was determined from the A_{260} of a 1:20 dilution of the DNA solution.

Results

Preliminary studies with unlabeled hydroxamic acid substrates were conducted to determine the approximate time required for the two substrates to be consumed by the hepatocyte suspensions. These studies indicated that both substrates were metabolized at similar rates (data not shown). On the basis of such studies, it was determined that 2-h incubation times would be employed for studies

Table I. Covalent Binding of Hydroxamic Acids to Nucleic Acids of Rat Liver Hepatocytes

expt[a]	% cell viability	binding of *N*-OH-AAF[b] DNA	binding of *N*-OH-AAF[b] RNA	binding of *N*-OH-GAF[b] DNA	binding of *N*-OH-GAF[b] RNA
1	93	955	927	898	992
2	92	1572	1081	1355	1197
3	96	813	757	824	852
mean ± SD					
(n = 3)		1113 ± 404	922 ± 162	1026 ± 288	1014 ± 174

[a] Both substrates were incubated simultaneously with identical hepatocyte suspensions prepared fresh for each experiment. [b] Nucleic acid binding is expressed as pmol of substrate bound per mg of nucleic acid.

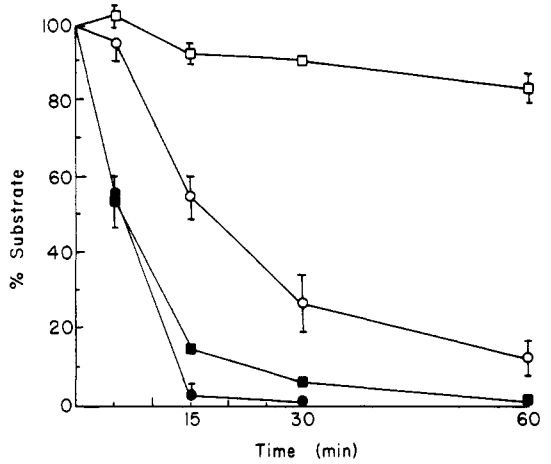

Figure 2. Progress curves for the metabolism of *N*-OH-AAF and *N*-OH-GAF by rat liver cytosol. Each substrate (20 μM) was incubated with rat liver cytosol (4 mg of protein/mL) in the presence or absence of PAPS (0.2 mM). Aliquots were taken at the indicated times for analysis of substrate disappearance by HPLC. The values plotted are the means (±SD) from two experiments: (○) *N*-OH-AAF; (●) *N*-OH-AAF plus PAPS; (□) *N*-OH-GAF; (■) *N*-OH-GAF plus PAPS.

with the radiolabeled substrates, since metabolism of both substrates (20 μM) was nearly complete by this time. Additional studies indicated that neither substrate was obviously toxic to the hepatocytes under the conditions employed. Cell viability was found to decrease to about 70% by the end of the 2-h incubations either in the presence or absence of the substrates. The amounts of covalent binding of *N*-OH-AAF and *N*-OH-GAF to DNA and RNA are shown in Table I for three paired and independent experiments. These results show no significant difference in the extent of binding of the two substrates to nucleic acids. Furthermore, there were no significant differences in the binding to either DNA or RNA. The extensive binding of both hydroxamic acid substrates to DNA is similar to that which has been reported for *N*-OH-AAF in rat hepatocytes (*15*, *16*). We did not observe any significant differences in the binding of either *N*-OH-AAF or *N*-OH-GAF between DNA and RNA, although a 3-fold greater binding of *N*-OH-AAF to RNA has been noted (*15*). In that study, hepatocytes in monolayer culture were employed (*15*). Our results show that incorporation of labeled substrate into total recovered nucleic acid was about 0.13% of the starting amount.

The identical degree of nucleic acid binding that was observed for the two hydroxamic acids in hepatocyte cultures suggested that both substrates undergo bioactivation via the same metabolic processes. However, subsequent in vitro studies revealed some major differences in the probable pathways for the bioactivation of these two hydroxamic acids.

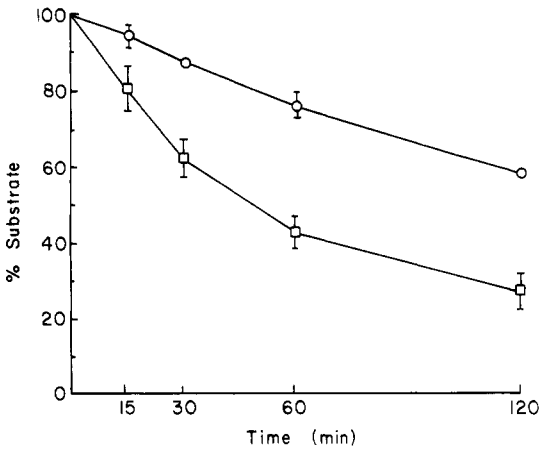

Figure 3. Progress curves for the metabolism of *N*-OH-AAF and *N*-OH-GAF by rat liver microsomes. Each substrate (20 μM) was incubated with rat liver microsomes (1 mg of protein/mL) in the absence of an NADPH-generating system. Aliquots were taken at the indicated times for analysis of substrate disappearance by HPLC. The values plotted are the means (±SD) from two experiments: (○) *N*-OH-AAF; (□) *N*-OH-GAF.

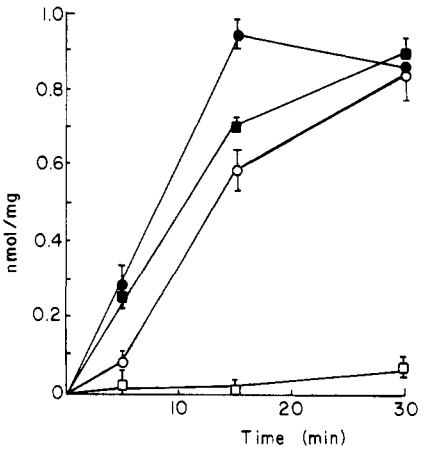

Figure 4. Progress curves for DNA binding by *N*-OH-AAF and *N*-OH-GAF by rat liver cytosol. Each ^{14}C-labeled substrate (20 μM) was incubated with rat liver cytosol (4 mg of protein/mL) and denatured calf thymus DNA (1 mg/mL) in the presence or absence of PAPS (0.2 mM). Aliquots were taken at the indicated times and the DNA processed as described in the experimental. The values plotted are the means (±SD) from three experiments: (○) *N*-OH-AAF; (●) *N*-OH-AAF plus PAPS; (□) *N*-OH-GAF; (■) *N*-OH-GAF plus PAPS.

Figures 2 and 3 illustrate the relative rates of consumption of the two hydroxamic acids by rat liver cytosol and microsomal fractions, respectively. Particularly striking was the much greater rate and extent of substrate disappearance for *N*-OH-AAF compared to *N*-OH-GAF in cytosol incubations in the absence of any added cofactors (Figure 2). The addition of PAPS nearly eliminated this large difference in the kinetics of metabolism of the two hydroxamic acids by cytosolic enzymes. The addition of PAPS also increased by several fold the rate of disappearance of *N*-OH-AAF (Figure 2). The microsomal fraction metabolized both substrates, but *N*-OH-GAF disappeared more rapidly than did *N*-OH-AAF (Figure 3).

Progress curves for the cytosol-catalyzed covalent binding of *N*-OH-AAF and *N*-OH-GAF to DNA are illustrated in Figure 4. Most notable was the much greater cytosol-catalyzed binding to DNA of *N*-OH-AAF relative to *N*-OH-GAF in the absence of added cofactors (Figure 4). The addition of PAPS to cytosol incubations increased the amount of DNA binding by both hydroxamic acids.

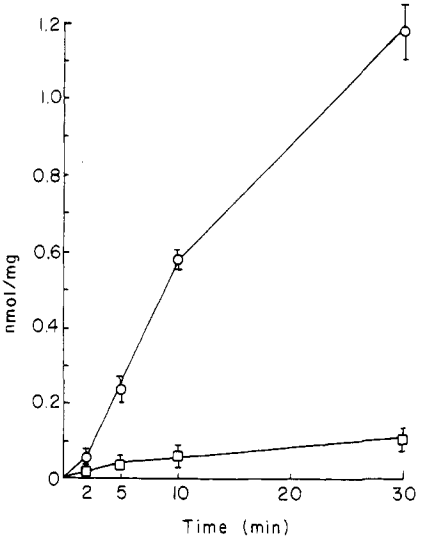

Figure 5. Progress curves for DNA binding by *N*-OH-AAF and *N*-OH-GAF by partially purified *N,O*-acyltransferase from rat liver cytosol. Each ^{14}C-labeled substrate (20 μM) was incubated with *N,O*-acyltransferase (2.7 mg of protein/mL) and denatured calf thymus DNA (1 mg/mL). Aliquots were taken at the indicated times and the DNA processed as described in the experimental. The values plotted are the means (±SD) from two experiments: (○) *N*-OH-AAF; (□) *N*-OH-GAF.

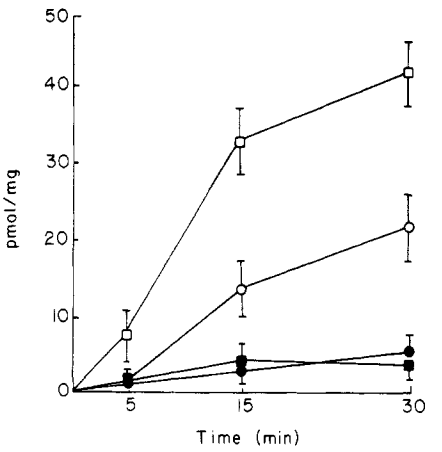

Figure 6. Progress curves for DNA binding by *N*-OH-AAF and *N*-OH-GAF by rat liver microsomes. Each ^{14}C-labeled substrate (20 μM) was incubated with rat liver microsomes (1 mg of protein/mL) and denatured calf thymus DNA (2 mg/mL) in the presence or absence of paraoxon (0.5 mM). Aliquots were taken at the indicated times and the DNA processed as described in the experimental. The values plotted are the means (±SD) from three experiments: (○) *N*-OH-AAF; (●) *N*-OH-AAF plus paraoxon; (□) *N*-OH-GAF; (■) *N*-OH-GAF plus paraoxon.

The PAPS-stimulated increase in DNA binding was particularly large for *N*-OH-GAF, an observation which parallels that reported above for the effect of PAPS on the metabolism of *N*-OH-GAF by the cytosolic fraction. It was observed during these studies that the substitution of a PAPS-generating system (*17*) for preformed PAPS gave erroneous results in the case of *N*-OH-GAF (data not shown). The PAPS-generating system resulted in only about 25% as much total DNA binding of *N*-OH-GAF compared to the use of PAPS. We suspect that *N*-OH-GAF, but not *N*-OH-AAF, is an inhibitor of the PAPS-generating system; therefore, subsequent studies were conducted with preformed PAPS.

The cytosol was partially purified to yield an *N,O*-acyltransferase-containing fraction (*13*), which was then tested for its ability to catalyze covalent binding of *N*-OH-AAF and *N*-OH-GAF to DNA. As shown in Figure 5, the relative degree of DNA binding of the two hydroxamic acids by the *N,O*-acyltransferase-containing fraction was the same as that observed for the crude cytosol fraction. The initial rate of binding of *N*-OH-AAF to DNA was about 3 times faster for the partially purified *N,O*-acyltransferase than for the cytosol fraction, which is most likely due to the higher specific activity of the acyltransferase in the former.

Rat liver microsome preparations were also found to catalyze the binding of both *N*-OH-AAF and *N*-OH-GAF to DNA (Figure 6). *N*-OH-GAF gave significantly more binding to DNA than did *N*-OH-AAF ($p < 0.01$). In the presence of paraoxon, neither *N*-OH-AAF nor *N*-OH-GAF gave significant binding to DNA after a 30-min incubation period ($p < 0.05$).

Discussion

Glycolic acid derived hydroxamic acids constitute a novel class of metabolites that can be produced from the corresponding *C*-nitroso compound (*4, 5*). Nitroso compounds in turn are known in many cases to be intermediary metabolites of arylamines and aryl nitro compounds (*1, 2, 18, 19*). The role of the nitroso metabolite in the expression of the genotoxic properties of arylamines and aryl nitro compounds is poorly understood, yet most evidence suggests a close association with genotoxicity (*19*). The facile interconversion of the nitroso and hydroxylamine oxidation states (*19, 20*) makes it difficult to determine which of these two classes of reactive metabolites is most responsible for the toxic effects of the parent arylamines and aryl nitro compounds (*21, 22*). The facile reaction of nitroso compounds with protein sulfhydryl groups (*23, 24*) probably prevents high concentrations of nitroso metabolites from accumulating, although such protein binding in itself could result in toxicity. Our interest in the biochemistry of nitroso metabolites has centered about the unusual but general ability for nitroso groups to interact with thiamine-dependent enzymes to produce several types of hydroxamic acids including glycolic acid derived types (*4, 5*). Because the nitroso and hydroxylamine functional groups are much more reactive than hydroxamic acids, it is possible that the latter might serve as relatively-stable transport forms of these reactive metabolites. Thus, the toxic N-oxidation metabolites produced in the liver, lung, or certain other organs could be latent in the form of hydroxamic acids, which are more likely to be sufficiently stable so that their distribution to distant organs could occur. Depending upon the metabolic capabilities of the cells within such organs, the potential toxicity of the hydroxamic acids could be released by one or several possible reactions. An example of such latency by hydroxamic acids is the ability of rat mammary gland to cause nucleic acid binding of the procarcinogen *N*-OH-AAF by one or more activation pathways (*25, 26*). The question arises as to whether glycolic acid derived hydroxamic acids might also serve as latent forms of genotoxic metabolites of arylamines and related chemicals.

In the present study, we found that the glycolic acid derived hydroxamic acid, *N*-OH-GAF, was covalently bound to rat hepatocyte nucleic acid to the same extent as the acetic acid derived hydroxamic acid, *N*-OH-AAF. This observation suggests that *N*-OH-GAF might be genotoxic in a manner similar to *N*-OH-AAF, which is a widely accepted model for arylamine carcinogenesis. Investigations of the carcinogenicity of *N*-OH-GAF have not been conducted; however, *N*-OH-GAF has been found to be as potent as *N*-OH-AAF in the Ames mutagen assay (unpublished results).

In a recent in vitro study which employed peroxidative bioactivation conditions, we found that *N*-OH-GAF actually gave much higher covalent binding to DNA than did *N*-OH-AAF (*8*). This marked difference in reactivity prompted us to investigate other known pathways for the bioactivation of hydroxamic acids in order to determine if major differences might exist between these two types of hydroxamic acids. Our results with subcellular fractions of rat liver indicate that there are similarities in bioactivation pathways for the two substrates, but at least one major difference.

Our results demonstrate that O-sulfation occurs with *N*-OH-GAF and causes extensive DNA binding in a manner analogous to that with *N*-OH-AAF. The conversion of *N*-OH-AAF to its *O*-sulfate ester by the action of PAPS and a sulfotransferase is a well-known bioactivation reaction in the liver of male rats (*3*). Thorgeirsson and co-workers have proposed that this activation pathway, which results in extensive protein and nucleic acid binding, is probably of greatest importance to the promotion step of *N*-OH-AAF carcinogenesis (*27*). There is considerable evidence to suggest that the O-sulfation reaction is the predominant process leading to DNA binding by *N*-OH-AAF in isolated rat hepatocytes, which is in contrast to *N,O*-acyltransferase bioactivation of *N*-OH-AAF thought to predominate under in vivo conditions (*15*).

In sharp contrast to *N*-OH-AAF, *N*-OH-GAF was poorly metabolized by the postmicrosomal fraction (in the absence of PAPS) and gave very little DNA binding with this enzyme fraction or with partially purified *N,O*-acyltransferase. In fact, the glycolyl analogue, *N*-OH-GAF, has since been shown to be a rather potent irreversible inhibitor of the *N,O*-acyltransferase from rat liver cytosol (*28*).

The DNA-binding catalyzed by the microsomal fraction was about twice as large for *N*-OH-GAF than for *N*-OH-AAF. Other investigators have reported greater microsomal-catalyzed binding for another unusual hydroxamic acid type relative to *N*-OH-AAF. The formic acid derived hydroxamic acid derivative of *N*-hydroxy-2-aminofluorene (*N*-OH-FAF) has been shown to bind to nucleic acid to an extent of nearly 10 times greater than that obtained with *N*-OH-AAF by rat liver microsomes (*29*, *30*). The microsomal enzyme responsible for this much greater binding of *N*-OH-FAF is thought to be an *N,O*-acyltransferase that is different from the rat liver cytosolic *N,O*-acyltransferase, which displays greater specificity for *N*-OH-AAF as a substrate (*29*, *30*). The ability of the microsomal enzyme(s) to catalyze nucleic acid binding of either *N*-OH-FAF or *N*-OH-AAF is readily inhibited by the carboxyesterase/amidase inhibitor, paraoxon, at 0.1 mM (*27*), a property that is not characteristic of cytosolic *N,O*-acyltransferase. Based on the results of our studies, it is probable that the same activity in microsomes is responsible for catalyzing the binding of *N*-OH-GAF to DNA. Evidently, *N*-OH-GAF is between *N*-OH-AAF and *N*-OH-FAF with respect to relative reactivity toward the microsomal enzyme(s).

The apparent inability of *N,O*-acyltransferase to catalyze the covalent binding of *N*-OH-GAF to DNA creates somewhat of a dilemma in attempting to explain why both *N*-OH-GAF and *N*-OH-AAF give essentially the same amount of nucleic acid binding in rat hepatocyte cultures. One might expect that the high reactivity of *N*-OH-AAF in both the *N,O*-acyltransferase and sulfotransferase activation reactions would lead to much more nucleic acid binding than was seen for *N*-OH-GAF, since the latter is refractory to DNA binding catalyzed by cytosolic *N,O*-acyltransferase. Perhaps the cytosolic *N,O*-acyltransferase pathway is partially suppressed in isolated hepatocytes as suggested by Howard et al. (*15*); thus the similar reactivity of *N*-OH-AAF and *N*-OH-GAF with sulfotransferase could explain the nearly identical amounts of nucleic acid binding by these two substrates in isolated hepatocytes.

The chemical structures of the adducts of *N*-OH-GAF to DNA have not yet been determined. Since our results suggest that the sulfotransferase pathway is the predominate bioactivation reaction for *N*-OH-GAF, it is to be expected that base adducts which possess the glycolyl group will be abundant (*31*, *32*). Attempts to synthesize such an expected adduct of guanosine (e.g., *N*-guanin-8-yl-*N*-glycolyl-2-aminofluorene) by standard methods (*33*) have not been successful to date. The major problem has been the extreme instability of the requisite intermediate, *N*-acetoxy-*N*-glycolyl-2-aminofluorene. This intermediate probably decomposes by transfer of the acetyl group to the glycolyl hydroxyl group (unpublished results). An attempt will be made to isolate sufficient glycolyl-containing base adducts from rat hepatocyte suspensions, so that necessary structural determinations can be made. It is our hypothesis that, in vivo, *N*-OH-GAF should produce a high proportion of glycolyl-containing adducts in contrast to the major deacylated adduct produced by *N*-OH-AAF. The reasons for this proposal are the results presented in this study and our observation that *N*-OH-GAF is a strong inhibitor of cytosolic *N,O*-acyltransferase.

This and other studies (*8*, *29*, *34*, *35*) indicate that the nature of the acyl group exerts a major effect on the susceptibility of an aromatic hydroxamic acid to various bioactivation processes. Thus, hydroxamic acids derived from acids other than acetic might be expected to display biological activity that is somewhat different from their acetyl analogue. Such differences in toxicity could include not only potency but even a complete change in the target organ.

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