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HEPATIC *N*-ACETYLTRANSFERASES: SELECTIVE INACTIVATION *IN VIVO* BY A CARCINOGENIC *N*-ARYLHYDROXAMIC ACID

TIMOTHY J. SMITH* and PATRICK E. HANNA*†‡

Departments of †Medicinal Chemistry and *Pharmacology, University of Minnesota, Minneapolis, MN 55455, U.S.A.

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Abstract—*N*-Hydroxy-2-acetamidofluorene (*N*-OH-AAF), a carcinogenic *N*-arylhydroxamic acid, is a selective and irreversible inhibitor of arylamine *N*-acetyltransferase (NAT) activity *in vitro*. The present study demonstrates that intraperitoneal administration of *N*-OH-AAF to hamsters caused an irreversible reduction of the hepatic transacetylase activity that catalyzes the transfer of the acetyl group from *N*-OH-AAF to 4-aminoazobenzene (AAB), but did not affect the acetyl coenzyme A (CoASAc) dependent NAT that is responsible for acetylation of *p*-aminobenzoic acid (PABA). A 40% loss of *N*-OH-AAF:AAB transacetylase activity occurred 4 hr after administration of 50 mg/kg of *N*-OH-AAF. To determine whether biotransformation of *N*-OH-AAF is a factor in determining its ability to inactivate *N*-OH-AAF:AAB transacetylase activity *in vivo*, the enzyme-inducing agent phenobarbital and the esterase/acylamidase inhibitor bis(*p*-nitrophenyl)phosphate (BNPP) were administered to the animals prior to the administration of *N*-OH-AAF. The loss of *N*-OH-AAF:AAB transacetylase activity was prevented by treatment of the animals with either phenobarbital or with BNPP. The ability of the esterase/acylamidase inhibitor, BNPP, to prevent the *N*-OH-AAF-mediated loss of transacetylase activity indicates that, in contrast to the inactivation process *in vitro*, esterase-catalyzed deacetylation of *N*-OH-AAF may be required for transacetylase inactivation *in vivo*. It is proposed that *in vivo* the endogenous acetyl donor, CoASAc, acetylates the enzyme and prevents the deacetylation of *N*-OH-AAF by NAT, thereby impeding the *N*-OH-AAF-mediated inactivation process, but facilitating enzyme inactivation by *N*-hydroxy-2-aminofluorene. The latter proposal was supported by the demonstration that CoASAc inhibited the *in vitro* inactivation of *N*-OH-AAF:AAB transacetylase activity by *N*-OH-AAF.

An abundance of evidence supports the conclusion that the ability of a number of arylamides to induce tumor formation depends upon their metabolic conversion to *N*-arylhydroxamic acids [1]. The latter compounds are proximate carcinogenic metabolites that require further transformation to the reactive electrophilic intermediates that are considered to be the ultimate carcinogens [2]. *N*-Arylhydroxamic acid *N,O*-acyltransferase (AHAT)§ is a cytosolic enzyme that is present in a variety of mammalian tissues, and is one of several enzymes that are capable of converting *N*-arylhydroxamic acids into electrophilic reactants [3]. Bartsch *et al.* [4] and King [5] provided early evidence that AHAT activity is associated with the enzyme that is responsible for the catalysis of the transfer of the acetyl group from *N*-arylhydroxamic acids to arylamines [6], and Glowinski *et al.* [7] found that rabbit liver AHAT activity and the genetically polymorphic arylamine *N*-acetyltransferase (NAT) (EC 2.3.1.5) are associated with the same enzyme. Because arylamine NAT is an acetyl coenzyme A (CoASAc) dependent enzyme that exists in multiple forms that have different substrate specificities [8], and because arylamine NAT plays an important role in the metabolism of xenobiotic compounds, its role in the bioactivation of carcinogenic chemicals is of particular interest.

An investigation of the *in vitro* bioactivation of *N*-arylhydroxamic acids by hamster and rat hepatic AHAT revealed that certain *N*-arylhydroxamic acids, including the carcinogen *N*-hydroxy-2-acetamidofluorene (*N*-OH-AAF), are mechanism based inactivators (suicide inhibitors) of AHAT [9]. The mechanism of the inactivation involves transfer of the acetyl group of the *N*-arylhydroxamic acid to the enzyme to form an acyl–enzyme intermediate (Fig. 1). *O*-Acetylation of the resulting *N*-arylhydroxylamine produces the *N*-acetoxyarylamine (I, pathway b) which undergoes heterolytic cleavage of the N—O bond to yield the resonance-stabilized aryl nitrenium-carbenium ion that reacts with nucleophilic centers on the enzyme [9, 10]. This mechanism is based on the earlier proposals [4, 5] for the AHAT-catalyzed bioactivation of *N*-arylhydroxamic acids and on the results of structure–activity relationship studies [10].

Because the *N*-OH-AAF-mediated inactivation of AHAT, as reflected by the rate of *N*-OH-AAF-dependent 4-aminoazobenzene (AAB) acetylation (Fig. 1, pathway a) was much more extensive than the inactivation of CoASAc-dependent *p*-aminobenzoic acid (PABA) NAT activity, it was proposed

‡ To whom correspondence should be addressed.

§ Abbreviations: AHAT, *N*-arylhydroxamic acid *N,O*-acyltransferase; NAT, *N*-acetyltransferase; CoASAc, acetyl coenzyme A; *N*-OH-AAF, *N*-hydroxy-2-acetamidofluorene; *N*-OH-AF, *N*-hydroxy-2-aminofluorene; AAB, 4-aminoazobenzene; PABA, *p*-aminobenzoic acid; DTT, dithiothreitol; BNPP, bis(*p*-nitrophenyl)phosphate; and DMSO, dimethyl sulfoxide.

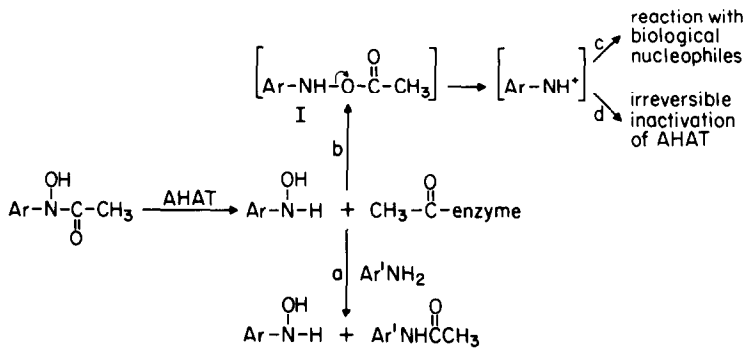

Fig. 1. Transacetylation (a) and bioactivation (b) reactions catalyzed by arylhydroxamic acid *N,O*-acyltransferase.

that AHAT and PABA NAT activities are not associated with the same transacetylase enzyme in hamster liver [9]. The validity of this proposal was substantiated by the successful separation of hamster hepatic AHAT and PABA NAT activities and by the demonstration that AHAT activity, but not PABA NAT activity, is inactivated upon incubation with *N*-arylhydroxamic acids [11]. Thus, the mechanism based inactivation of *N*-acetyltransferases by *N*-arylhydroxamic acids is a useful approach to the detection and characterization of multiple forms of the enzymes in mammalian tissues.

The objective of the present work was to determine whether the administration of *N*-OH-AAF on an acute dosage basis would result in a decrease in hamster hepatic *N*-OH-AAF:AAB transacetylation activity and to investigate the *in vivo* selectivity of the inactivation process with regard to PABA NAT activity.

EXPERIMENTAL PROCEDURES

Materials. *N*-OH-AAF was prepared as previously described [10]. The following reagents were obtained from the sources listed: 2-nitrofluorene and acetyl chloride (Aldrich Chemical Co., Milwaukee, WI); *N*-1-(naphthyl)ethylenediamine dihydrochloride and 4-aminoazobenzene (Eastman Kodak Co., Rochester, NY); sodium pyrophosphate (Mallinckrodt Inc., St. Louis, MO); *p*-aminobenzoic acid, acetyl coenzyme A (trilithium salt), bovine serum albumin, dithiothreitol (DTT), and cellulose dialysis tubing (10 in. × 0.62 in.) (Sigma Chemical Co., St. Louis, MO); bis(*p*-nitrophenyl)phosphate (Calbiochem, San Diego, CA). The dialysis tubing was rinsed in distilled water and in 0.05 M sodium pyrophosphate buffer (pH 7) containing 1 mM DTT prior to use.

Enzymatic studies. Male Golden Syrian hamsters (70–100 g) were obtained from either Charles River Laboratories (Wilmington, MA) or Harlan ARS/Sprague Dawley (Indianapolis, IN) and were fed Purina Laboratory Chow and water *ad lib*. The animals were kept on a 12 hr light/dark cycle. Ultracentrifugation was performed with a refrigerated Beckman L5-65 ultracentrifuge, and a Beckman J-21B instrument was used for low spin centrifugation. Incubations were performed in a Dubnoff metabolic shaking incubator, and enzyme activities were measured with a Beckman model 34 spectrophotometer. The hepatic *N*-acetyltransferase activities were purified 2- to 3-fold from hamster liver cytosol as described by King [5].

Hamsters were lightly anesthetized with diethyl ether prior to decapitation. The livers were removed immediately and the gallbladders were separated from the livers. Each liver was rinsed with cold (4°) 0.05 M pyrophosphate/NaCl buffer (pH 7.0) containing 1 mM DTT and was homogenized with 1 ml of this buffer per g of liver. Homogenization was performed with a motor driven (1500 rpm) Teflon on glass homogenizer. The resulting homogenate was centrifuged at 105,000 *g* (7°) for 60 min. The 105,000 *g* supernatant fraction was diluted with an equal volume of the buffer. The resulting 25% solution of the 105,000 *g* supernatant fraction was placed in an ice bath and brought to 35% saturation with ammonium sulfate by addition, with stirring (20 min), of an ice-cold saturated solution of ammonium sulfate in 0.05 M pyrophosphate buffer (pH 7.0) containing 1 mM DTT. The precipitate was removed by centrifugation at 9000 *g* for 10 min (4°). The resulting supernatant fraction was brought to 50% saturation by further addition of the cold saturated ammonium sulfate solution, as described above. The 50% saturated solution was centrifuged at 9000 *g* for 10 min (4°). The precipitate was washed twice with cold 50% saturated ammonium sulfate solution. The precipitate was stored at −70°. Protein concentrations were determined by the method of Lowry *et al.* [12].

Aminoazobenzene transacetylation assay. Transacetylation of 4-aminoazobenzene (AAB) was assayed by the method of Booth [6]. Incubation flasks (25 ml) contained 0.1 to 0.5 ml of enzyme preparation (2.5 mg protein), sodium pyrophosphate (50 μmol, pH 7.0), DTT (1.0 μmol), *N*-OH-AAF (2.5 μmol), AAB (0.375 μmol) and sufficient 1.15% KCl to give a final volume of 2.5 ml. *N*-OH-AAF was omitted from incubation mixtures used for reference standards. Reactions were started by addition of substrates (*N*-OH-AAF/AAB dissolved in 0.05 ml of 95% ethanol) and were carried out at 37° in air for 2 min. After termination of the reactions by addition of 2.5 ml of cold 20% trichloroacetic acid (in ethanol : water, 1 : 1), the precipitated protein was removed by centrifugation. The supernatant frac-

tions were analyzed spectrophotometrically at 497 nm.

PABA NAT assay. The acetylation rate of PABA was determined by Weber's modification [13] of the Bratton–Marshall procedure [14]. Reaction mixtures contained 0.05 ml of enzyme preparation (0.25 mg protein), PABA (0.0225 μmol), CoASAc (0.150 μmol), and sufficient 0.05 M pyrophosphate-NaCl buffer (pH 7.0) (1 mM DTT) to give a final volume of 0.25 ml. PABA, dissolved in 0.075 ml of water, was added to the incubation mixtures to initiate the reactions, which were carried out at 37° in air. Incubation mixtures without CoASAc were included as reference standards. The incubations were terminated after 1 min by addition of 0.5 ml of cold (4°) 5% trichloroacetic acid. Precipitated protein was removed by centrifugation. Supernatant fractions (0.5 ml/incubation) were treated sequentially with 0.05 ml of $NaNO_2$ (0.1% aqueous), 0.05 ml of ammonium sulfamate (0.5% aqueous), and 0.25 ml of *N*-1-(naphthyl)ethylenediamine dihydrochloride (0.05% aqueous) to form a colored complex with an A_{max} at 540 nm. This color was analyzed spectrophotometrically by comparison with water as a blank.

In vivo *experiments with* N-*OH-AAF.* Male Golden Syrian hamsters (80–100 g) were treated with *N*-OH-AAF (50 mg/kg) or with vehicle. The vehicle was an emulsion (dimethy sulfoxide:corn oil, 1:6, v:v) which was administered at 0.5 ml/100 g body weight. Treatments were intraperitoneal injections at 2, 4, 8, 10, and 12 hr prior to killing the animals. Control animals received only vehicle. The 4-hr time point was used for a dose–response study with 25, 50, and 60 mg/kg treatments. Ammonium sulfate fractions were prepared from the livers of these animals as described previously. Pooled protein from the livers of three animals was fractionated. The ammonium sulfate fractions were suspended in 4 ml of cold (4°) 0.05 M pyrophosphate-NaCl buffer (pH 7.0) containing 1 mM DTT and then placed in cellulose dialysis tubing and subjected to dialysis. Dialysis was performed for 5 hr at 4° against 500 ml of cold (4°) 0.05 M pyrophosphate-NaCl buffer (pH 7.0) (1 mM DTT) which was changed twice (1.5 and 3 hr from the beginning of dialysis). Nitrogen gas was bubbled constantly through the chambers. The protein was removed from the tubing, and its concentration was determined. Protein solutions were assayed for activity (*N*-OH-AAF:AAB and CoASAc:PABA transacetylation) as described previously.

For the studies of the effects of enzyme inducers and inhibitors on the effects of *N*-OH-AAF *in vivo*, sodium phenobarbital (80 mg/kg) was administered intraperitoneally on 4 consecutive days prior to the administration of *N*-OH-AAF. SKF 525-A (50 mg/kg) was administered intraperitoneally immediately prior to the administration of *N*-OH-AAF, and BNPP (100 mg/kg) was administered intraperitoneally 1 hr prior to the administration of *N*-OH-AAF. Control animals received only sodium phenobarbital, SKF 525-A, or BNPP.

Inactivation in vitro *of* N-*OH-AAF:AAB transacetylation activity: Effect of CoASAc.* Incubation mixtures contained protein from ammonium sulfate fractionation (5 mg/ml), CoASAc in 0.1 ml of distilled water, *N*-OH-AAF in 0.02 ml of ethanol, and sufficient 0.05 M pyrophosphate-NaCl buffer (pH 7.0) containing 1 mM DTT to make a 2.0-ml incubation mixture.

Buffer was added to each 25-ml flask, and the flasks were placed on a Dubnoff incubator at 37°. Enzyme was added to the flasks 5 min prior to addition of the CoASAc solutions. Two minutes after CoASAc solution addition, *N*-OH-AAF solutions were added. Incubations were carried out for 5 min in air. At the end of the incubation period, the flasks were placed on ice. The contents of each flask were transferred to cellulose dialysis tubing and were dialyzed for 3 hr at 4° against 100 ml of buffer which was changed hourly. The buffer contained 10 ml of 95% ethanol, 50 ml of distilled water, and sufficient 0.05 M pyrophosphate-NaCl buffer (pH 7.0) containing 1 mM DTT to make 1 liter of solution. Nitrogen gas was bubbled through the buffer during the dialysis procedure. After dialysis, the protein was removed from the dialysis tubing, and 0.5-ml aliquots were assayed for *N*-OH-AAF:AAB transacetylation activity.

Inactivation of AHAT by N-*OH-AAF* in vitro*: Effect of bis(p-nitrophenyl) phosphate (BNPP).* These experiments were similar in design to the experiments with CoASAc. BNPP (0.2 μmol) was dissolved in 0.02 ml of 10% DMSO in distilled water. A 2-ml incubation mixture was used. The dialysis procedure was identical to that described above,

Table 1. Effect of *N*-OH-AAF on hamster hepatic transacetylase activities *in vivo**

N-OH-AAF (mg/kg)	Transacetylase activity					
	N-OH-AAF:AAB (nmol·mg^{-1}·min^{-1})†			PABA:CoASAc (nmol·mg^{-1}·min^{-1})‡		
	Treated	Control	% Decrease	Treated	Control	% Decrease
25	18.3 ± 0.8	19.9 ± 0.3	9	45.6 ± 5.3	46.5 ± 4.1	2
50	9.9 ± 1.3	16.2 ± 1.4	39	32.6 ± 2.9	31.3 ± 0.8	0
60	12.2 ± 0.6§	17.4 ± 1.3	30	50.9 ± 6.1	48.4 ± 5.7	0

* Hamsters were injected intraperitoneally with *N*-OH-AAF, and 4 hr later the animals were killed. The enzyme activities were determined as described in Experimental Procedures.

† Activities are expressed as transacetylation rates (mean ± range; N = 2).

‡ Activities are expressed as transacetylation rates (mean ± SEM; N = 3).

§ Mean ± SEM, N = 3; significantly different from control, $P < 0.05$.

except that the dialysis buffer was composed of 10 ml of ethanol, 1 ml of DMSO, 9 ml of distilled water, and sufficient 0.05 M pyrophosphate–NaCl buffer (pH 7.0) containing 1 mM DTT to make 1 liter of dialysis solution.

RESULTS

Inactivation of N-*OH-AAF:AAB transacetylation activity following intraperitoneal injection of* N-*OH-AAF.* The assay that was used to monitor AHAT activity in this study measures the enzyme-catalyzed transfer of the acetyl group of *N*-OH-AAF to AAB. The evidence that supports the association of AHAT activity with *N*-OH-AAF:AAB transacetylase activity has been reviewed recently [11].

A significant decrease in the hamster hepatic *N*-OH-AAF:AAB transacetylase activity was evident 4 hr after injection of *N*-OH-AAF, but there was no decrease in PABA:CoASAc acetyltransferase activity (Table 1). The maximal decrease in *N*-OH-AAF:AAB transacetylase activity was approximately 40%. Greater reductions in activity were not achieved by the administration of doses larger than 75 mg/kg (data not presented), and it was noted that doses larger than 60 mg/kg were toxic to the animals. Based on these results, a dose of 50 mg (0.2 mmol) per kg was selected for use in subsequent experiments.

The time course of *N*-OH-AAF:AAB transacetylase inactivation and recovery was investigated by injecting a single dose of *N*-OH-AAF and measuring the hepatic transacetylase activities at various times after the injection. The maximum depression of *N*-OH-AAF:AAB transacetylase activity occurred 4 hr after administration of *N*-OH-AAF, and the activity had returned to near control values by 12 hr (Table 2). No inhibition of PABA:CoASAc activity was observed. Thus, the results presented in Tables 1 and 2 are consistent with the previously reported high degree of selectivity of transacetylase inactivation *in vitro* by *N*-OH-AAF [9, 11].

The irreversibility of the *in vivo* inactivation was established by the failure to recover *N*-OH-AAF:AAB transacetylation activity after extensive dialysis of the enzyme preparations obtained from animals that had been treated with *N*-OH-AAF. The data presented in Tables 1–3 were obtained with enzyme preparations that had been dialyzed as described in Experimental Procedures. When the *N*-OH-AAF:AAB transacetylation activity was measured both before and after dialysis, there was no significant recovery of activity after dialysis (data not presented). This result also is consistent with

Table 2. Inhibition and recovery of hamster hepatic transacetylase activities after intraperitoneal injection of *N*-OH-AAF*

Hours post injection	Transacetylase activity					
	N-OH-AAF:AAB ($nmol \cdot mg^{-1} \cdot min^{-1}$)†			PABA:CoASAc ($nmol \cdot mg^{-1} \cdot min^{-1}$)‡		
	Treated	Control	% Decrease	Treated	Control	% Decrease
2	17.1 ± 0.6	18.9 ± 1.4	10	32.7 ± 3.4	30.9 ± 1.2	0
4	9.9 ± 1.3	16.2 ± 1.4	39	32.6 ± 2.9	31.3 ± 0.8	0
8	13.1 ± 0.8	18.2 ± 1.9	28	28.2 ± 3.0	30.8 ± 4.2	9
10	14.5 ± 1.2	18.5 ± 0.2	22	29.3 ± 1.6	27.9 ± 3.1	0
12	16.7 ± 0.5	19.6 ± 0.3	15	30.8 ± 3.4	32.9 ± 2.0	6

* Hamsters were injected intraperitoneally with *N*-OH-AAF (50 mg/kg). The animals were killed at the indicated times, and the enzyme activities were determined as described in Experimental Procedures.

† Activities are expressed as transacetylation rates (mean ± range; N = 2).

‡ Activities are expressed as transacetylation rates (mean ± SEM; N = 3).

Table 3. Effect of administration of enzyme inducers and inhibitors on the inactivation of hamster hepatic *N*-OH-AAF:AAB transacetylase activity *in vivo* by *N*-OH-AAF*

Treatment	*N*-OH-AAF:AAB transacetylase activity ($nmol \cdot mg^{-1} \cdot min^{-1}$)	% Decrease
Phenobarbital (80 mg/kg)	18.5 ± 0.9†	
Phenobarbital + *N*-OH-AAF	18.3 ± 1.4	1
SKF 525-A (50 mg/kg)	22.0 ± 0.6†	
SKF 525-A + *N*-OH-AAF	16.2 ± 1.7	25
BNPP (100 mg/kg)	20.7 ± 1.0‡	
BNPP + *N*-OH-AAF	19.4 ± 2.9	6

* Hamsters were injected intraperitoneally with the agents according to the schedules described in Experimental Procedures. The animals were killed 4 hr after administration of *N*-OH-AAF (50 mg/kg), and enzyme activities were measured as described in Experimental Procedures.

† Activities are expressed as transacetylation rates (mean ± range; N = 2).

‡ Activities are expressed as transacetylation rates (mean ± SEM; N = 3).

those obtained from inactivation experiments with several *N*-arylhydroxamic acids *in vitro* [15].

Effect of phenobarbital, SKF-525A, and bis(p-nitrophenyl)phosphate (BNPP) on the inactivation of N-*OH-AAF:AAB transacetylase activity* in vivo *by* N-*OH-AAF.* To study the effect of induction of hepatic drug-metabolizing enzymes on the *N*-OH-AAF-mediated inactivation of *N*-OH-AAF:AAB transacetylase activity, hamsters were injected with phenobarbital on 4 consecutive days prior to treatment with *N*-OH-AAF. It was anticipated that enzyme induction might enhance the rate of oxidative and conjugative metabolism of *N*-OH-AAF and thereby reduce the quantity of the agent available for bioactivation by AHAT. The *N*-OH-AAF:AAB transacetylase activity of hepatic enzyme preparations obtained from animals that had been treated with both phenobarbital and *N*-OH-AAF was virtually identical with that of enzyme preparations obtained from hamsters that had received only phenobarbital (Table 3). Thus, prior treatment with phenobarbital prevented the inactivation of *N*-OH-AAF:AAB transacetylase activity by *N*-OH-AAF *in vivo*. The administration of phenobarbital alone did not affect the *N*-OH-AAF:AAB transacetylase activity. The conclusion that the effect of phenobarbital was the result of an enhanced conversion of *N*-OH-AAF to unreactive metabolites rather than to a direct interaction between phenobarbital and the transacetylase enzyme was supported by the inability of phenobarbital to inhibit either the inactivation of the enzyme by *N*-OH-AAF or the *N*-OH-AAF:AAB transacetylation reaction *in vitro* (data not presented).

The simultaneous administration of an inhibitor of drug metabolism, SKF 525-A, and *N*-OH-AAF did not affect significantly the extent of inactivation of *N*-OH-AAF:AAB transacetylase activity *in vivo* by *N*-OH-AAF (Table 3). The 25% decrease in enzyme activity was approximately the same as that which occurred when only *N*-OH-AAF was administered (Tables 1 and 2).

One of the principal routes of metabolism of *N*-OH-AAF involves deacetylation by microsomal esterases [16, 17]. Therefore, hamsters were treated with an inhibitor of microsomal deacetylases prior to the administration of *N*-OH-AAF. It was anticipated that the inhibition of this metabolic pathway might increase the availability of *N*-OH-AAF for bioactivation by AHAT and that the extent of inactivation of the enzyme would be increased. The agent selected for these experiments was bis(*p*-nitrophenyl)phosphate (BNPP), a widely used and well characterized carboxylesterase/acylamidase inhibitor that is known to inhibit the deacetylation of arylhydroxamic acids [18–22]. As shown in Table 3, treatment of hamsters with BNPP 1 hr prior to the administration of *N*-OH-AAF prevented the expected reduction in *N*-OH-AAF:AAB transacetylase activity. Thus, rather than causing the expected increase in the extent of transacetylase inactivation, treatment of the animals with BNPP appeared to provide a protective effect against *N*-OH-AAF-induced inactivation.

Effects of BNPP and CoASAc on inactivation of N-*OH-AAF:AAB transacetylase activity* in vitro *by* N-*OH-AAF.* To determine whether the ability of BNPP to prevent the inactivation of *N*-OH-AAF:AAB transacetylation *in vivo* by *N*-OH-AAF is the result of a direct interaction with the enzyme, *in vitro* experiments were conducted in which the effects of BNPP on the *N*-OH-AAF:AAB transacetylation reaction and on the inactivation process were examined. The presence of 0.1 mM BNPP, a concentration that significantly inhibits esterases and amidases [19], did not inhibit the transacetylation reaction or the inactivation of the enzyme by *N*-OH-AAF (Table 4). These results are consistent with those of Weeks *et al.* [23] who found that diethyl *p*-nitrophenylphosphate, an organophosphate inhibitor of esterases, did not inhibit the AHAT-catalyzed bioactivation of *N*-OH-AAF.

Because of the evidence supporting the association of AHAT and CoASAc dependent arylamine NAT activities with the same enzyme in both rabbit [6] and hamster [24] liver, the effect of CoASAc on the inactivation of *N*-OH-AAF:AAB transacetylase activity by *N*-OH-AAF *in vitro* was investigated. It was anticipated that acetylation of NAT by the endogenous acetyl donor, CoASAc, might prevent the deacetylation of *N*-OH-AAF by NAT (Fig. 1), thereby impeding the *N*-OH-AAF-mediated inactivation of the enzyme. The results, shown in Fig. 2, demonstrate that the presence of CoASAc prevented the inactivation of the transacetylase activity by *N*-OH-AAF *in vitro*, and that the magnitude of the protective effect was dependent upon the concentration of CoASAc.

Table 4. Effect of bis(4-nitrophenyl)phosphate (BNPP) on the inactivation of hamster hepatic *N*-OH-AAF:AAB transacetylation activity *in vitro* by *N*-OH-AAF*

Incubation system	*N*-OH-AAF:AAB transacetylase activity† ($nmol \cdot mg^{-1} \cdot min^{-1}$)	% Inhibition
Control	17.9 ± 0.11	
N-OH-AAF (0.1 mM)	0.0	100
N-OH-AAF + BNPP (0.1 mM)	0.36 ± 0.36	100

* Enzyme preparations were incubated with the indicated agents. The incubation mixtures were dialyzed, and activities were measured as described in Experimental Procedures.

† Activities are expressed as transacetylation rates (mean ± range; N = 2). Transacetylation rate in the presence of BNPP: 18.0 ± 0.1 $nmol \cdot mg^{-1} \cdot min^{-1}$.

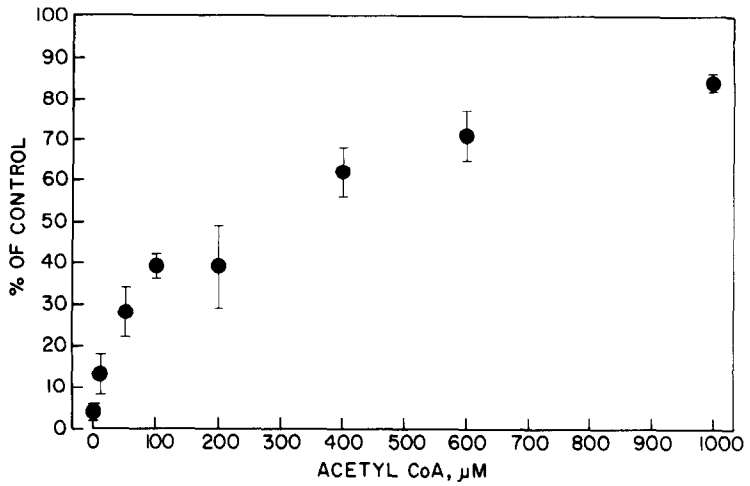

Fig. 2. Effect of CoASAc on the inactivation of *N*-OH-AAF:AAB transacetylation activity by *N*-OH-AAF *in vitro*. The enzyme preparation was incubated with *N*-OH-AAF (0.1 mM) and various concentrations of CoASAc. At the end of the incubation period, the reaction mixture was dialyzed, and the *N*-OH-AAF:AAB transacetylation activity was measured as described under Experimental Procedures. The ordinate indicates the percentage of *N*-OH-AAF:AAB transacetylase activity remaining after incubation and dialysis. The results are expressed as the mean ± SEM for three to seven experiments. Control activity: 21.1 ± 0.8 nmol·mg^{-1}·min^{-1}.

DISCUSSION

Hamster hepatic *N*-OH-AAF:AAB transacetylase activity is completely and irreversibly inactivated by *N*-OH-AAF *in vitro* (Table 4) [11], and the intraperitoneal administration of *N*-OH-AAF to hamsters resulted in a loss of approximately 40% of the hepatic *N*-OH-AAF:AAB transacetylase activity. Bartsch *et al.* [4] found that the hepatic transacetylase activity of male rats decreases to 20–30% of control levels within 4–8 days of administration of 0.027% of *N*-OH-AAF in the diet. Intraperitoneal administration of *N*-OH-AAF did not cause a reduction in PABA:CoASAc NAT activity (Tables 1 and 2), a result that is consistent with the previously observed selectivity of transacetylase inactivation by *N*-OH-AAF *in vitro* [9, 10].

The metabolism of *N*-OH-AAF by various animal species includes the formation of the *O*-glucuronide, deacetylation, and ring hydroxylation [17, 25, 26]. *N*-OH-AAF also undergoes sulfate conjugation, but hamsters have little capacity to catalyze sulfate formation [27]. Although treatment of hamsters with the drug metabolism inhibitor SKF 525-A did not alter significantly the extent of inactivation of *N*-OH-AAF:AAB transacetylase activity by *N*-OH-AAF, prior administration of phenobarbital prevented the inactivation of the transacetylase activity *in vivo* by *N*-OH-AAF (Table 3). Apparently, under the conditions of these experiments, SKF 525-A did not inhibit to a significant extent the enzymes involved in the biotransformation of *N*-OH-AAF, whereas the result obtained with phenobarbital is presumably due to the induction of enzymes involved in one or more of the pathways of metabolism. As O-glucuronidation is a major route of metabolism of *N*-OH-AAF, it is quite possible that the prevention of the effects of *N*-OH-AAF on transacetylase activity by phenobarbital is due to induction of glucuronyl transferase activity.

The principal results of this investigation are the findings that the selectivity of the *N*-OH-AAF mediated transacetylase inactivation that was observed *in vitro* [9, 11] also occurs *in vivo* (Tables 1 and 2) and that the organophosphate esterase inhibitor, BNPP, completely prevents the inactivation of *N*-OH-AAF:AAB transacetylase activity *in vivo*. The latter result was surprising because it was anticipated that the ability of an esterase inhibitor such as BNPP to prevent the deacetylation of *N*-OH-AAF would cause an increase in the amount of the arylhydroxamic acid that would be available to participate in the AHAT-catalyzed inactivation process. *In vitro* experiments were conducted to determine whether the prevention of transacetylase inactivation by BNPP may be due to a direct interaction with the transacetylase itself. The results shown in Table 4 demonstrate that BNPP has no effect on the inactivation of *N*-OH-AAF:AAB transacetylase activity *in vitro*.

The prevention of the *N*-OH-AAF-mediated inactivation of *N*-OH-AAF:AAB transacetylase activity *in vivo* by BNPP and the failure of BNPP to prevent the inactivation *in vitro* indicates that the effect of BNPP is due to the inhibition of esterase catalyzed deacetylation of *N*-OH-AAF and that deacetylation of the arylhydroxamic acid may be essential for the enzyme inactivation process to occur *in vivo*. According to this conclusion, acetyl group transfer from *N*-OH-AAF to the transacetylase enzyme (Fig. 1) is not involved in the enzyme inactivation process *in vivo*.

It is reasonable to expect that the principal acetyl group donor in the formation of the acetyl–enzyme complex (Fig. 1) *in vivo* could be the natural cofactor, CoASAc. The occupation of the enzyme by an acetyl group derived from CoASAc would prevent the transfer of the acetyl group from *N*-OH-AAF to

the enzyme and would require that the *N*-arylhydroxamic acid undergo deacetylation in order to generate the *N*-arylhydroxylamine that is needed for the formation of the *N*-acetoxyarylamine, I (Fig. 1). Therefore, the ability of CoASAc to prevent the inactivation of *N*-OH-AAF:AAB transacetylase activity by *N*-OH-AAF *in vitro* was examined. The concentration-dependent inhibition of the inactivation process exhibited by CoASAc (Fig. 2) indicates that formation of an acyl–enzyme complex by CoASAc may be responsible for protection of the enzyme from inactivation by *N*-OH-AAF *in vivo* whereas it would be expected to facilitate O-acetylation and the inactivation mediated by *N*-hydroxy-2-aminofluorene (*N*-OH-AF), the deacetylation product of *N*-OH-AAF.

The 15% inactivation of transacetylase activity that occurred even in the presence of 1.0 mM CoASAc (Fig. 2) may reflect the limited ability of *N*-OH-AAF to compete with CoASAc as an acetyl donor, thereby producing a small amount of the arylhydroxylamine that is required for the formation of the *N*-acetoxy intermediate, I (Fig. 1). It is also possible that some of the inactivation of transacetylase activity that occurred in the presence of CoASAc *in vitro* was the result of reaction of the enzyme with *N*-acetoxy-2-acetamidofluorene, an electrophilic compound that can be formed as the result of nonenzymatic transfer of the acetyl group from CoASAc to *N*-OH-AAF [28].

The bioactivation of mutagenic and carcinogenic arylhydroxylamines by CoASAc-dependent acetyltransferases has been reported [29–32]. The data indicate that the latter bioactivation processes involve the formation of *N*-acetoxyarylamine intermediates (I, Fig. 1) and, therefore, are identical to the events proposed herein to be involved in the *in vivo* inactivation of *N*-OH-AAF:AAB transacetylase activity by *N*-OH-AAF. Thus, it might be expected that incubation of hepatic acetyltransferase enzymes with *N*-OH-AF and CoASAc *in vitro* would result in the irreversible inactivation of transacetylase activity. Unfortunately, such an experiment would not provide definitive evidence for a mechanism involving the direct formation of an *N*-acetoxyarylamine intermediate because arylhydroxylamines such as *N*-OH-AF and *N'*-hydroxy-*N*-acetylbenzidine are known to undergo not only O-acetylation, but also N-acetylation to yield the corresponding *N*-arylhydroxamic acids [33, 34]. The latter compounds would be expected to participate in the inactivation of the transacetylases via the *N,O*-acyltransfer process depicted in Fig. 1. Certain other arylhydroxylamines, however, are activated by NAT-catalyzed O-acetylation, whereas the corresponding arylhydroxamic acids are not substrates for the *N,O*-acyltransferase reaction [31]. Inactivation of acyltransferases by such an arylhydroxylamine in the presence of CoASAc might be expected to occur solely through the direct formation of an N-acetoxy intermediate.

Both the importance of hereditary factors in the determination of N-acetylation capacity and the relationships between AHAT activity and various forms of CoASAc-dependent arylamine *N*-acetyltransferases are well established [7, 9, 11, 24, 35, 36]. Hein *et al.* [37, 38] and Smith and Hanna [11] demonstrated the existence of at least two distinct transacetylase activities in hamster liver by the use of chromatographic methods, and the transacetylase activity that was established by Hein *et al.* as being expressed monomorphically is the activity that is inactivated by *N*-OH-AAF *in vitro* and *in vivo*. The demonstration that *N*-arylhydroxamic acids can cause selective inactivation of acetyltransferase enzymes both *in vitro* and *in vivo* and the development of an understanding of the chemical mechanisms [10] and biochemical processes involved indicate that it should be possible to design other, less toxic, transacetylase inactivators that will be useful probes of acetyl transferase multiplicity and function in mammalian tissues.

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