

Acetylation, Deacetylation and Acyltransfer

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N-Substituted aromatic compounds can be metabolized in most species to *N*-acetylated derivatives that are themselves subject to further enzymatic transformations, including hydrolysis and *N,O*-acyltransfer. These processes can either potentiate or ameliorate the biological responses to these *N*-substituted derivatives. Decreasing the levels of metabolites, such as arylhydroxylamines may, in some systems, reduce the probability of eliciting adverse biological effects. In others, arylhydroxamic acids produced by the acetylation of arylhydroxylamines may increase their potential for metabolic activation by *N,O*-acyltransfer. In the rabbit, rat and perhaps other species, the acetyl CoA-dependent *N*-acetyltransferase is also capable of activating arylhydroxamic acids by *N,O*-acyltransfer. These cytosolic organotriphosphate ester-resistant enzymes can utilize arylhydroxamic acid as a donor of the acetyl moiety in the acetyl transferase reaction and apparently are capable of activating arylhydroxamic acids because of their ability to *O*-acetylate the arylhydroxamine. In mice, *N*-acetyltransferase and *N,O*-acetyltransferase seem not to exhibit this relationship. Enzymes from the microsomes of a number of species are also capable of activating arylhydroxamic acids. The particulate-bound enzymes are organotriphosphate ester-sensitive deacylases that are unable to form nucleic acid adducts on incubation with *N*-methoxy-*N*-acetylaminobenzenes, substrates that are not capable of activation by *N,O*-acyltransfer. Thus, depending on the specificity of the enzymes involved, *N*-substituted aromatic compounds may be activated by *N,O*-acyltransfer during both the acetylation and deacetylation process. The influence of this activation in the carcinogenic process is the object of continuing investigation.

Introduction

N-Substituted aromatic compounds are subject to enzymatic *N*-acetylation and further metabolism of the *N*-acetylated derivatives. Interest in the formation and disposition of these compounds comes from recognition that metabolism may modify the biological response to these agents by serving to reduce the levels of metabolites with undesirable properties (e.g., arylhydroxylamines) or to provide substrates (e.g., arylhydroxamic acids) for metabolic pathways which generate products that can adversely affect biological systems. Although metabolic oxidation of the methyl group of the *N*-acetyl moiety does occur, this pathway will not be addressed here, since the glycolamide does not appear to be involved in the tumorigenicity of arylamines (1). The purpose of this communication is to relate recent progress in our understanding of the interre-

relationships between *N*-acetylation, arylhydroxamic acid *N,O*-acyltransfer and deacetylation of *N*-substituted compounds, as these three reactions are capable of producing carcinogenic and mutagenic aromatic amine derivatives.

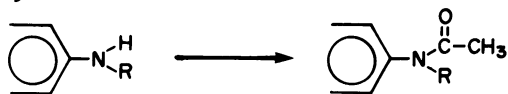
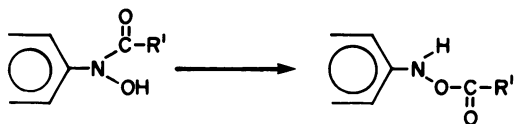
N-Acetylation

Mechanism of Action and Distribution

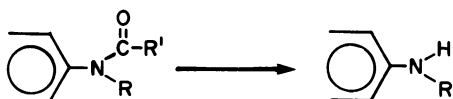
N-Acetylation of aromatic amine carcinogens was first observed in rat liver slices using aminofluorene as substrate (2) (Fig. 1). Subsequent studies showed that other species were also capable of *N*-acetylating 2-aminofluorene (AF) as well as 4-aminobiphenyl and 2-naphthylamine (3, 4). The enzyme responsible for this metabolic step, acetyl CoA-dependent *N*-acetyltransferase (NAT), is the same enzyme as that responsible for the *N*-acetylation of aromatic amine drugs such as isoniazid and sulfamethazine (5). This is important in that Poirier et al. (6) postulated that *N*-arylhydroxylamines were involved in bladder cancer induction, whereas the acetylated derivatives were required for carcinogenesis in liver. The dog, unable to acetylate aromatic amines

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N-Acetylation*N,O*-Acyltransfer

Deacylation



Where R = -H or -OH
R' = -H, -CH₃, -CH₂CH₃, etc.

FIGURE 1. Metabolism of *N*-acyl substituents.

(4), is still susceptible to bladder cancer by these compounds and recent work has shown that this process is probably mediated by the *N*-glucuronide of the *N*-arylhydroxylamine (7, 8).

NAT is a cytosolic enzyme found in many tissues of a number of species. In the rabbit, greater than 80% of the total acetylating capacity of the animal is in the liver and gut. Low yet detectable levels have been found in blood, brain, kidney, lung, spleen, testes and thymus of this species (9). Liver cytosols of hamster, guinea pig, mouse, rat and man are also capable of *N*-acetylating carcinogenic arylamines such as AF, 4-aminobiphenyl and 2-naphthylamine (4, 10). Recent studies in inbred mice have shown that in the A/J and C57BL/6J strains, small intestine, kidney and blood, as well as liver, are capable of *N*-acetylating AF and benzidine (11). In rabbit, the NAT of liver and gut reflects a genetic polymorphism (see below) that is not evident in kidney and spleen (9). In contrast, blood, liver, kidney and small intestine reflect the animal's phenotype in inbred mice (11).

Genetic Regulation

One of the most interesting aspects of *N*-acetylation in man is the existence of a hereditary polymorphism in the rate of this reaction. As a consequence, persons are classified as either rapid or slow acetylators of certain arylamine drugs and carcinogens (5). With respect to drugs such as isoniazid, these genetic differences in the rate of *N*-acetylation play an important role in determining the variability seen in

the incidence of toxic responses (12, 13). Similarly, it is possible that the capacity for *N*-acetylation may regulate both the occurrence of tumors in specific organs and the susceptibility to carcinogenesis from aromatic amines. Lower (14) has suggested a possible correlation between acetylator phenotype and urinary bladder cancer induction. Studies to determine if such a correlation exists are now in progress (15).

In addition to the human polymorphism, large genetic variation in *N*-acetylation of both aromatic amine drugs and carcinogens is also observed in rabbit (5). A comparative study of the capacity of rabbit liver to carry out *N*-acetylation and to activate arylhydroxamic acids by *N,O*-acyltransfer (Fig. 1) revealed large genetic variations in both enzymes in this species (Table 1). Thus, both acetyl transfer

Table 1. Liver NAT and AHAT from rabbits of specified acetylator phenotype.^a

Rabbit phenotype ^b	AHAT, nmole/min/mg	NAT, nmole/min/mg
Rapid		
(RR)	0.802	1.74
(RR)	0.626	2.64
(RR)	0.343	3.20
(RR)	0.330	3.33
(Rr)	0.669	3.65
Slow		
(rr)	0.00040	0.075
(rr)	0.00033	—
(rr)	0.00021	0.071

^aEnzyme activities were determined in rabbit liver cytosol with the use of *N*-hydroxy-AAF or AF as substrates (16).

^bRabbits were classified according to their arylamine acetylator phenotype: RR = homozygous rapid acetylator; Rr = heterozygous rapid acetylator; rr = slow acetylator

steps in the biotransformation of arylamines are under common genetic control in rabbit liver. Further studies showed that the two enzyme activities could not be resolved by protein purification techniques which exploited differences in solubility, charge and size. In addition, both enzymes migrated as a single symmetrical protein band after polyacrylamide gel electrophoresis and both had the same molecular weight (33,000) as determined by electrophoresis on SDS-polyacrylamide gels (16). The relationship between NAT and arylhydroxamic acid *N,O*-acyltransferase (AHAT) was further strengthened by recognition that arylhydroxamic acids could replace acetyl CoA as an acetyl donor for NAT. Thus, with respect to function, rabbit liver recognizes arylhydroxamic acids as the donor of acetyl groups. The identity of NAT and AHAT has not yet been established for other species.

Recent studies (10) have now shown that inbred mouse strains also exhibit a genetic polymorphism

in their capacity for *N*-acetylation of the arylamine carcinogens aminofluorene and benzidine (Tables 2 and 3). Of 20 strains surveyed, three are classified as slow acetylators (A/J, AHe/J and X/Gf) and 17 as rapid acetylators based on either blood benzidine or liver benzidine NAT activities (Table 2). The rapid

Table 2. NAT activities in liver and blood of inbred mouse strains.^a

Strain	Benzidine NAT activity, nmole/min/mg	
	Blood	Liver
A/J	ND ^b	0.058
AHe/J	ND	0.032
X/Gf	ND	0.178
AuSsJ	0.019	0.361
Castaneous		0.277
ST/bJ		0.123
C57BL/6J	0.036	0.364
Molossinus		0.209
SF	0.033	0.215
SWR/J	0.040	0.294
129/SV		0.287
RF/J	0.039	0.338
RIII/2J		0.114
IsCam		0.411
SJL/J		0.247
Balb/cJ	0.060	0.314
C3H/HeJ	0.064	0.362
CBA/J	0.048	0.314
AKR/J	0.052	0.345
DBA/J	0.064	0.320

^aFrom Glowinski and Weber (10).

^bND, not detectable.

acetylator strains possessed approximately 10 times greater liver benzidine NAT specific activity than the slow acetylator strains. Intercross and backcross matings of A/J (slow acetylator) and C57BL/6J (rapid acetylator) mice indicate that a single gene with two major alleles is responsible for the differences in NAT activity in both tissues for benzidine or AF (10). The analysis of recombinant inbred strains derived from matings of A/J with C57BL/6J mice (Table 3) supports this conclusion (10).

Studies of other mouse tissues showed that kidneys from these strains exhibited the genetic polymorphism in *N*-acetylation of AF, unlike the situation in rabbit. Interesting sex differences were apparent in C57BL/6J mouse kidney with males exhibiting approximately 2- to 5-fold higher AF-NAT activity than do females. Similar sex differences have been observed in the mutagenic activation of AAF by mouse kidney microsomes (17). Studies to determine if AHAT activity was genetically controlled in a manner similar to that for NAT in inbred mice revealed that this was not the case and that only marginal AHAT activity was detected (18).

Thus, it is clear that there are distinct species differences between the mouse and rabbit models of

Table 3. Liver and blood AF NAT activities in A/J, C57BL/6J, AC57F1 and A/JxC57BL/6J recombinant inbred mouse strains.^a

Mouse strain	AF NAT activity, nmole/min/mg		Genotype ^b
	Liver	Blood	
A/J	0.134	<0.016	A
C57BL/6J	1.26	0.110	B
AC57F1	0.703	0.065	
BXA-1	1.37	0.163	B
BXA-3	0.126	<0.016	A
BXA-6	0.120	<0.016	A
BXA-15	0.113	<0.016	A
AXB-2	1.31	0.206	B
AXB-3	0.060	<0.016	A
AXB-4	0.098	<0.016	A
AXB-5	1.10	0.165	B
AXB-6	1.24	0.162	B
AXB-9	0.168	<0.016	A
AXB-17	1.30	0.173	B

^aFrom Glowinski and Weber (10).

^bA, A/J; B, C56BL/6J.

N-acetylation in both substrate and tissue specificity, as well as in the AHAT-mediated metabolic activation pathway for AF. Rapid and slow acetylator rabbits that display large genetic differences in AHAT activity and rapid and slow acetylator mice that do not may both be helpful in studying the effect of acetylator phenotype on aromatic amine-induced carcinogenesis.

N,O-Acyltransfer Cytosolic AHAT

Incubation of arylhydroxamic acids with nucleic acids and rat liver cytosol results in adduct formation with reaction at carbon-8 of guanine and loss of the *N*-acetyl moiety (19-21). The metabolic pathway responsible for this activation results from the formation of reactive *N*-acyloxyarylamines (22, 23). The instability of these metabolites has thus far precluded their direct synthesis. Evidence for their metabolic formation has come from (1) the report that the addition of arylhydroxylamine increases adduct formation (22), (2) the inability of the enzyme to activate *N*-methoxy-*N*-acetyl-2-aminofluorene (23), and (3) the association of adduct formation with the ability of the enzyme to transfer the *N*-acetyl moiety of arylhydroxamic acids to primary arylamines (22-24). This latter reaction, first described by Booth (24), is analogous to the NAT pathway as the hydroxamic acid serves as acetyl donor to yield arylacetamides. Thus, the association between NAT and AHAT in the rabbit (Table 1), as noted above, appears to hold for the rat since both species can utilize arylhydroxamic acids for the acetylation of primary arylamines although extreme genetic variation has not been observed in the rat (25).

AHAT is widely distributed, as detected by nu-

Table 4. Relative cytosolic AHAT activities.^a

Tissue	AF ($\times 10^{-11}$ mole) bound to tRNA on incubation with cytosol from various species									
	Rat	Hamster	Rabbit	Guinea pig	Monkey	Baboon	Pig	Human	Mouse	Dog and goat
Liver	111 ^b	278 ^b	371	9	56	58	32	12	5 ^b	<2 ^b
Kidney	29	11	4	12					<2	<2
Small intestine	36 ^b	118 ^b	43	12 ^b	20			17	<2	<2
Colon	38	31	6	10	<2			5	<2	<2
Stomach	24 ^b	36 ^b	2	14	<2				<2	<2
Lung	13	18	3	3	<2			2	<2	
Mammary gland	10 ^b									
Zymbal's gland	10 ^b									
Spleen	7	4	2	3	<2				<2	<2
Brain	3	6	2	4						
Uterus			10 ^b							
Bladder			20 ^b							

^aThese data were obtained by use of standardized assay conditions (23, 25, 39, 40-42). The assay involved incubation of *N*-hydroxy-AAF (0.042 μ mole) and tRNA (15 A_{260} units) with cytosol equivalent to 20 mg of tissue in pyrophosphate buffer (0.05M, pH 7.0) at 37°C for 20 min. (43).

^bTissue in which AF derivatives most commonly induce tumors.

cleic acid adduct formation on incubation of substrates with tissue cytosols (Table 4). Although activity is usually greatest in liver, a wide variety of other organs have demonstrable levels of AHAT. Importantly, dog tissues have neither AHAT nor NAT. Cytosolic AHATs with molecular weights of approximately 28,000 to 33,000 daltons are inhibited by reagents that react with sulfhydryl groups. Both *N*-acetylated and *N*-propionylated arylhydroxylamines can serve as substrates for the cytosolic AHAT and these reactions are not affected by organophosphate esterase inhibitors; *N*-formylated derivatives are poor substrates at best (1, 18, 26, 27). Mono-, di- and tricyclic arylhydroxamic acids, including derivatives of phenacetin, naphthalene, biphenyl, stilbene, fluorene and phenanthrene, can also serve as substrates such as phenacetin, the rats of adduct formation are considerably less than those for *N*-acetyltransfer as measured by the Booth assay (29).

Although cytosolic AHAT can transform its substrate to products that are mutagenic for *Salmonella* (28-30), it has been shown that the reactive product of the AHAT reaction is not responsible for this activity (26, 30). The mutagenicity may result from small quantities of hydroxylamines produced as a consequence of hydrolytic uncoupling of the acyltransfer process.

Studies of the structure-activity relationships have provided evidence that the organophosphate-insensitive AHAT of mammary gland of Sprague-Dawley derived female rats is involved in arylamine-induced tumors in this organ. Giganti and her colleagues have shown that direct treatment of the mammary gland with the hydroxamic acid *N*-hydroxy-*N*-acetyl-2-aminofluorene (*N*-hydroxy-AAF) is more effective in producing tumors than is the ad-

ministration of the hydroxylamine, nitroso or amide derivatives (31). Subsequent experiments have employed arylhydroxamic acids that differ in their *N*-acyl moieties and, consequently, differ in their potential for activation by AHAT. The results suggest that rat mammary gland tumor susceptibility is related to the ability of arylhydroxamic acids to serve as substrates for AHAT, i.e., acetylated compounds are more active than formylated compounds (1, 27, 32). While the evidence of a causal role for AHAT in other tissues is less convincing, the major DNA adducts formed in all target tissues studied thus far are compatible with their formation via an acyltransfer mechanism.

Microsomal AHAT

Recent observations have disclosed that liver microsomes from a number of species have the ability to generate nucleic acid adducts on incubation with arylhydroxamic acids (Table 5) (1, 18). This activity yields arylamine-substituted guanine derivatives at carbon-8 without retention of the acyl group to give the same product as that produced by the cytosolic acyltransferases. However, unlike the case for the cytosolic enzymes, diethyl-*p*-nitrophenylphosphate (Paraoxon) is a potent inhibitor of the microsome-catalyzed activation. Resolution of solubilized guinea pig liver microsome preparations disclosed that two proteins are responsible for adduct formation (Table 6). These enzymes are readily distinguishable by differences in size and by their relative abilities to hydrolyze arylhydroxamic acids and amides. Substitution of the acidic hydrogen of the hydroxamic acid with a methyl group leads to reduction in adduct formation and provides evidence for activation by an acyltransfer mechanism (18).

Table 5. Species variation in the deacylation and AHAT activities of liver microsomes.

Species/strain	Deacylase, nmole/min/mg protein ^a		AHAT, nmole/min/mg protein ^a	
	With <i>N</i> -Hydroxy-AAF	With <i>N</i> -Hydroxy-FAF	With <i>N</i> -Hydroxy-AAF	With <i>N</i> -Hydroxy-FAF
Guinea pig (11) ^b	59	31	0.01	0.32
Hamster (2)	14.3	4.8	0.45	0.26
Rabbit ^c				
RR (1)	1.5	2.7	0.08	0.17
Rr (1)	3.9	5.0	0.09	0.18
rr (1)	7.5	11.6	0.14	0.64
Mouse				
A/J (2)	3.4	3.7	0.02	0.09
C57BL/6J (2)	4.0	3.2	0.03	0.21
Dog (3)	2.3	1.5	0.003	0.05
Rat (3)	1.9	1.3	0.02	0.18

^aMeans of the initial rates (18).

^bThe number of animals employed are given in parentheses.

^cRabbits were classified according to their arylamine acetylase phenotype (16): RR = homozygous rapid acetylase; Rr = heterozygous rapid acetylase; rr = slow acetylase.

Table 6. Relationship of AHAT from cytosols and microsomes.

Enzyme source	Type of AHAT ^a	
Rat		
Liver cytosol	II ^F	III ^{Ac}
Liver microsomes	II ^F & Ac	
Mammary gland cytosol	II ^F	III ^{Ac}
Guinea pig		
Liver cytosol	I ^F & Ac	III ^{Ac}
Liver microsomes	I ^F & Ac	II ^F & Ac

^aThe AHATs are classified according to the resolution of cytosols and solubilized microsomal preparations by gel filtration on Sephacryl S-200. The relative elution volumes of types I, II and III are 1.3 to 1.4, 1.55 to 1.65, and 1.8 to 1.9, respectively. These values, which are inversely related to molecular weight, are defined as the ratio of the volume required for elution of the peak enzyme activity to the volume at which excluded macromolecules first emerge from the column. Rat liver AHAT migrates with an apparent molecular weight of 28,000 on Sephadex G-100 (23). The AHAT activities were detected by assay of nucleic acid adduct formation on incubation with either the *N*-formylated (F) or *N*-acetylated (Ac) *N*-2-fluorenylhydroxylamine. Type I and II AHAT are inhibited by diethyl-*p*-nitrophenylphosphate; Type III is resistant to this organophosphate (1, 18).

Deacylation

Hydroxamic Acids

Both *N*-hydroxy-AAF (33, 34) and *N*-hydroxy-*N*-formyl-2-aminofluorene (*N*-hydroxy-FAF) can be deacylated by enzymes present in the liver microsomal fractions of guinea pig, hamster, rabbit, mouse, dog and rat (Table 5) to produce the hydroxylamine, *N*-hydroxy-AF. As previously shown for *N*-hydroxy-AAF (33), guinea pig possesses the highest microsomal deacylase activity. All species tested, except guinea pig, have very low or nondetectable cytosolic deacylase activity for *N*-hydroxy-AAF. In contrast, *N*-hydroxy-FAF can be deacylated by cytosols of all species except hamster.

Guinea pig liver microsomes contain two enzymes capable of these reactions that differ in molecular weight as demonstrated by gel filtration (18, 35). A wide range of acylated compounds are substrates for both enzymes (35). With respect to aromatic amines, the larger enzyme hydrolyzes *N*-hydroxy-AAF approximately 200 times faster than *N*-acetyl-2-aminofluorene (AAF), whereas the activities for hydrolysis of both compounds are about the same with the smaller enzyme (18, 35). Recent work has shown that these purified microsomal deacylases are also capable of transforming *N*-hydroxy-AAF and *N*-hydroxy-FAF to derivatives that are capable of reacting with nucleic acid (see above). Both enzymes are inhibited by paraoxon. Guinea pig liver microsomes are also capable of deacetylating the *O*-glucuronide of *N*-hydroxy-AAF to yield metabolites that react with nucleic acids (36).

Jarvinen et al. (35) showed that in addition to microsomes from guinea pig liver, other tissues such as kidney and brain contain *N*-hydroxy-AAF deacylase activity. In an extensive survey of rat tissues, Irving (37) demonstrated that by far the highest *N*-hydroxy-AAF deacylation in this species occurs in the Zymbal's gland, an organ that is highly susceptible to carcinogenesis by AAF, *N*-hydroxy-AAF and other aromatic amines and amides. In contrast, the mammary gland, another rat tissue highly susceptible to carcinogenesis from these compounds, has very low deacylase activity.

Amides

Liver microsomes of many species also possess the ability to deacylate carcinogenic arylacetamides. Lower (34) showed that this is both substrate- and species-specific. Hamster and dog have relatively good activity for the deacylation of acetylaminobi-

phenyl and acetylaminofluorene; mouse has very high activity for acetylaminobiphenyl deacylation; and rat and guinea pig have very low activities for both. We have recently shown that guinea pig liver microsomes are also capable of deacylating *N*-formyl-2-aminofluorene as well as AAF (18). In addition to rat liver, rat intestine, a target organ for AAF-induced tumorigenesis, is capable of deacylating AAF as well as other acylaminofluorenes (38).

Relationship of *N*-Acetylation, *N,O*-Acyltransfer and Deacylation

As noted above, NAT and AHAT activities are identical in rabbit, but may not be related in mouse. While definitive experiments have not been carried out in rat, the association of cytosolic AHAT with the ability of arylhydroxamic acids to serve as acetyl donors in this species suggests that such a relationship does exist. It is probable that evolutionary changes have resulted in the development of a series of related NATs that differ both in their abilities to utilize hydroxamic acids as acetyl donors, and in their abilities to transfer the putative NAT-bound acyl group to the oxygen of the hydroxylamine, to produce a reactive *N*-acyloxyarylamine.

Recognition of the presence of other AHATs that are unrelated to the NAT enzymes came from experiments in which *N*-formylated hydroxamic acids were used to induce mammary gland tumors (1, 27). These studies identified a rat cytosolic enzyme that was capable of transforming *N*-formyl, but not *N*-acetyl or *N*-propionyl, derivatives to reactive products. The enzyme that activated the formyl compounds differed from the previously studied enzyme in that it was larger and was inhibited by diethyl-*p*-nitrophenylphosphate. Inhibition by an organophosphate prompted experiments to examine the possibility that this enzyme was a microsomal deacylaselike component that had been solubilized during the homogenization procedure (18). These efforts produced evidence (Table 6) that the organophosphate-sensitive enzyme had been released from microsomes with a concomitant loss of ability to activate *N*-acetylated derivatives. Similar experiments with guinea pig liver preparations demonstrated that the two microsomal AHATs of this organ also appear in cytosol. The sizes, sensitivities to inhibitors, and structure-activity relationships of these enzymes support the conclusion that microsomal deacylases can activate arylhydroxamic acids by *N,O*-acyltransfer. The great species variation in the structure-activity relationships of liver microsomal AHATs and deacylases suggests that evolution has resulted in families of enzymes that are analogous to those of NAT.

The presence of both deacylase and AHAT activities in the same enzyme preparation raises the question of the relative contributions of these two pathways to nucleic acid adduct formation. In rat microsomes, the rate of adduct formation after *N*-hydroxy-FAF metabolism is only 14% that of hydroxylamine formation (Table 5). However, in guinea pig liver microsomes, adduct formation after *N*-hydroxy-AAF metabolism is less than 0.02% that of the rate of hydrolysis, and most of the adduct formation potential is chromatographically separable from the deacylase activity. These data suggest that, while reaction of arylhydroxylamines with nucleic acid can occur at neutral pH, adduct formation in these enzyme-mediated systems occurs primarily as a consequence of the formation of products that are more reactive than the hydroxylamine. However, based on the results of studies with cytosolic AHAT, mutagenicity in *Salmonella* would be expected to be more influenced by hydroxylamine production than by the potential for adduct formation in cell-free systems. It is hoped that recognition of the relationship of these three metabolic pathways will be of aid in better defining their role in the induction of tumors by arylamines.

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