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Pathobiologic and Metabolic Aspects of Mammary Gland Tumorigenesis by *N*-Substituted Aryl Compounds

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Metabolic or synthetic *N*-hydroxylation of *N*-arylamides yields *N*-arylacylhydroxamic acids considerably more carcinogenic for the rat mammary gland than the parent amides both by systemic and topical administration. The size of the aryl moiety, the position of the nitrogen relative to the aryl moiety and the type of acyl group are determinants of the carcinogenic potency of *N*-arylacylhydroxamic acids. Induction of mammary tumors required ovarian hormones. Receptors for estrogen, androgen and progesterone were shown in the *N*-hydroxy-*N*-2-fluorenylacetamide (N-OH-2-FAA)-induced mammary carcinoma. This tumor involved epithelial and stromal components of the mammary gland that were separated in culture and produced tumors of their respective origin in the isologous host. Both mammary epithelial cells and fibroblasts are capable of metabolism of carcinogens. The enzymes potentially involved in metabolic activation of *N*-arylamides and *N*-arylacylhydroxamic acids in the mammary gland include: a cytochrome P-450(P_1-450) system, UDP-glucuronyltransferase, *N,O*-acyltransferases and peroxidases. Mammary microsomes in which cytochrome P_1-450 was induced generated small amounts of N-OH-2-FAA from 2-FAA. N-OH-2-FAA and its carcinogenic isomer, N-OH-3-FAA, were oxidized by cytochrome c/H_2O_2 to the nitroxyl free radicals which dismutated to the respective acetate esters and nitrosofluorenes. The addition of unsaturated lipid to either the free radicals or to the nitrosofluorenes gave electron spin resonance signals characteristic of immobilized radicals. It is proposed that interactions of carcinogens with lipids and with DNA play a role in mammary tumorigenesis.

N-Hydroxylation of *N*-Arylamides: A Requirement for Mammary Gland Tumorigenicity

Mammary tumorigenesis by *N*-substituted aryl compounds has been intensely studied in the rat, the most susceptible species. Enhancement of carcinogenic activity by *N*-hydroxylation of *N*-arylamides was clearly demonstrated in immature female rats (Table 1). The examples include the *N*-hydroxy metabolites of *N*-2-fluorenylacetamide (2-FAA) and of N-4-biphenylacetamide (4-BPAA), discovered and tested 20 years ago by the Millers and co-workers (*1, 2*) and synthetic compounds obtained and tested by Gutmann and co-workers (*3, 4*). Metabolism of the *N*-arylamides and synthetic *N*-hydroxylation yielded hydroxamic acids with greater carcinogenic potency for the rat mammary gland. The size and shape of the aryl moiety was a determinant of its potency since the fluorenyl compounds even at lower doses were more carcinogenic than the biphenylyl compounds. The *N*-phenyl series was shown to be much less, if at all, carcinogenic (*3*). The type of *N*-acyl moiety also affected carcinogenic activity, since the *N*-formyl and *N*-propionyl compounds in the biphenylyl series were less carcinogenic than the *N*-acetyl compound (*5*). The effect of the position of nitrogen relative to the *N*-aryl moiety was also determined (Table 2) (*3, 6-8*). Although differences in the carcinogenicities of 2-FAA and *N*-hydroxy-2-FAA could be detected at about 6 months after administration (Table 1), these compounds produced similarly high incidences of mammary tumors when assessed 12 months after administration (*6*). In contrast to 2-FAA, the structural isomers, 1-, 3- and 4-FAA were weakly, if at all, carci-

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nogenic (*3, 7, 8*). *N*-Hydroxylation of 3-FAA and, to a lesser extent, of 1-FAA yielded hydroxamic acids with increased carcinogenicities for the rat mammary gland (*3*); however, *N*-hydroxylation of 4-FAA did not increase its carcinogenicity (*7*). In subsequent metabolic studies (Table 3) we showed that the weakly carcinogenic 3-FAA was not *N*-hydroxylated by the liver of the female rat *in vivo* (*9*) or *in vitro* (*10*). In contrast, substantial amounts of carcinogenic 2-FAA were converted to *N*-hydroxy-2-

Table 1. Carcinogenicities of *N*-arylamides vs. *N*-arylacylhydroxylamines for the rat mammary gland after systemic administration.[a]

F(Fluorenyl) series[a]			BP (Biphenylyl) series[b]		
Compound	R	Tumor incidence	Compound	R	Tumor incidence
2-FAA	$-NH-CO-CH_3$	5/26[b] 26/18[b]	4-BPAA	$-NH-CO-CH_3$	3/16[b] 10/16[b]
N-OH-2-FAA	$-NH-CO-CH_3$ | OH	12/12[c] 17/29[d]	N-OH-4-BPAA	$-N-CO-CH_3$ | OH	9/30[d]
2-FBS	$-NH-SO_2-C_6H_5$	1/12[c]	4-BPBS	$-NH-SO_2-C_6H_5$	1/12[c]
N-OH-2-FBS	$-NH-SO_2-C_6H_5$ | OH	12/12[c]	N-OH-4-BPBS	$-NH-SO_2-C_6H_5$ | OH	5/11[c]
7-OH-2-FAA		0/10[e]	N-OH-4-BPFA	-N-CO-H | OH	2/28[d]
N-OH-7-OH-2-FAA		6/12[e]	N-OH-4-BPPA	$-N-CO-CH_2CH_3$ | OH	6/30[d]

[a]*Abbreviations: 2-FAA = N-2-fluorenylacetamide; N-OH-, 7-OH- and N-OH-7-OH-2-FAA = N-hydroxy-, 7-hydroxy- and N-*hydroxy-7-hydroxy-*N*-2-fluorenylacetamide; 2-FBS = *N*-2-fluorenylbenzenesulfonamide; N-OH-2-FBS = *N*-hydroxy-N-2-fluorenylbenzene-sulfonamide; 4-BPAA = *N*-4-biphenylylacetamide; N-OH-4-BPAA = *N*-hydroxy-*N*-4-biphenylylacetamide; 4-BPBS = *N*-4-biphenylylbenzenesulfonamide; N-OH-4-BPBS = *N*-hydroxy-*N*-4-biphenylylbenzenesulfonamide; N-OH-4-BPFA = *N*-hydroxy-*N*-4-biphenylylformamide; N-OH-4-BPPA = *N*-hydroxy-*N*-4-biphenylylpropionamide.

[b]12 IP injections at a dose level of 0.2 (F-series) (*1*) or 0.3 (BP-series) (*2*) mmole/kg of body weight.

[c]12 IP injections at a dose level of 0.1 (F-series) or 0.2 (BP-series) mmole/kg of body weight (*3*).

[d]8 IP injections at a dose level of 0.1 mmole/kg of body weight (*5*).

[e]12 IP injections at a dose level of 0.2 mmole/kg of body weight (*4*).

Table 2. Carcinogenicities of isomeric *N*-fluorenylacetamides vs. *N*-fluorenylacetohydroxamic acids for the rat mammary gland after systemic administration[a]

N-Fluorenylacetamides		*N*-Fluorenylacetohydroxamic acids	
Isomer	Tumor incidence[b]	Isomer	Tumor incidence[b]
1-FAA	4/12	N-OH-1-FAA	8/12 (12)
2-FAA	11/12 (79)	N-OH-2-FAA	12/12 (80)
3-FAA	3/12	N-OH-3-FAA	12/12 (63)
4-FAA	1/13[c]	N-OH-4-FAA	0/12

[a]12 IP injections at a dose level of 0.1 mmole/kg of body weight (*3, 6, 7*).

[b]Numbers in parentheses are the percent of malignant tumors in the mammary gland.

[c]0.025% in diet for 13.3 months (*8*).

Table 3. *N*-Hydroxylation of 2-FAA and 3-FAA by the female rat.

		N-Hydroxylation (%)	
Pretreatment	Substrate	*In vivo*[a]	*In vitro*[b]
–	2-[9-^{14}C]FAA	17.3	1.72
3-MC			4.89
–	3-[G-^{3}H]FAA	0.03	0.01
2-FAA		0.05	0.02

[a]Biliary excretion of the *N*-hydroxy metabolite after one IP injection of the substrate (*9*).

[b]*By hepatic microsomes (10, 11).*

[a]3-MC = 3-methylcholanthrene.

FAA (*9, 11*). Hence, we postulated that only those arylamides that are metabolically converted to hydroxamic acids are carcinogenic for the rat mammary gland.

Metabolic Capacity of Mammary Mixed Function Oxidase in Relation to Tumorigenesis

We investigated whether or not *N*-hydroxylation of 2-FAA also occurs in the mammary gland. In rat liver, *N*-hydroxylation, as well as hydroxylations at carbon atoms of the florene moiety, is catalyzed by microsomal cytochrome P-450, in particular by the 3-methylcholanthrene (3-MC)-inducible cytochrome P_1-450 (*9, 12-14*). However, detection of cytochrome P-450 or P_1-450 in the mammary gland was technically difficult (*11, 15*) due to the presence of hemoglobin and mitochondrial cytochrome aa_3 in the microsomes (*16*). Hemoglobin was removed by washing microsomes with hypotonic buffer (*17*). Spectral interference by cytochrome aa_3 was eliminated by anaerobic reduction prior to the reduction of cytochrome P-450 (P_1-450) which was then complexed with carbon monoxide (*16*). With improved assay conditions, we were able to detect 1 pmole of cytochrome P-450 and 14 pmole of cytochrome P_1-450/mg of protein in mammary microsomes of corn oil- and 3-MC- or β-naphthoflavone (β-NF)-treated lactating rats, respectively (Table 4). The levels of cytochrome P-450 and P_1-450 in hepatic microsomes were about 500- and 100-fold greater, respectively, than in mammary microsomes. Microsomes of both tissues were capable of hydroxylating benzo(*a*)pyrene (B[*a*]P) and 2-FAA. The amounts of the hydroxy metabolites of these two carcinogens were substantially increased by pretreatment of lactating rats with 3-MC or β-NF. In the liver the magnitude of induction of the hydroxylating activities was independent of the dose of inducer since three injections of 20 or 40 mg compound/kg of body weight gave similar increases (*16*). However, in the mammary gland, induction of hydroxylating enzyme activities was greater after treatment of lactating rats with the larger doses of inducers. The stage of lactation had no effect on the magnitude of induction since similar increases were observed with treatments from the fourth through fourteenth day of lactation, Hydroxylations of benzo(*a*)pyrene and 2-FAA induced by 3-methylcholanthrene or β-naphthoflavone in both hepatic and mammary microsomes were inhibited by 0.1 *mM* α-naphthoflavone (α-NF) *in vitro*. The α-NF had no significant effect on basal hydroxylating activities (*16*). Because α-NF is an inhibitor of cytochrome P_1-450-dependent aryl hydrocarbon hydroxylase in hepatic microsomes (*18*), we concluded that the increased hydroxylating activities of mammary microsomes were indeed cytochrome P_1-450-dependent.

In more recent experiments, we determined the level of each metabolite of 2-FAA formed by mammary microsomes of β-NF-treated lactating and nonlactating (50-day-old) female rats using high performance liquid chromatography (*19, 20*). Mammary microsomes from β-NF-treated lactating rats had a

Table 4. Microsomal and mitochondrial enzymes of mammary gland and liver of lactating rats.

	Mammary gland		Liver	
Enzyme[a]	Corn oil	β-NF[b]	Corn oil	β-NF[b]
Microsomes[c]				
Cytochrome P-450 (P_1-450)	1.3 ± 0.8	13.9 ± 6.1	533 ± 191	1046 ± 161
NADPH-cytochrome *c* reductase	18.8 ± 3.2	18.1 ± 2.0	160 ± 90	203 ± 82
Cytochrome b_5	19.7 ± 9.6	27.1 ± 10.2	362 ± 74	440 ± 76
NADH-cytochrome *c* reductase	27.6 ± 11.4	31.6 ± 13.0	1310 ± 437	1418 ± 652
NADH-$K_3Fe(CN)_6$ reductase	779 ± 212	759 ± 233	5350 ± 1336	4884 ± 1249
Total heme	44.4 ± 25.1	180 ± 77	1181 ± 183	1620 ± 184
Mitochondria[d]				
Cytochrome aa_3	125 ± 17	133 ± 21	66 ± 6	52 ± 16
Cytochrome *b*	47 ± 10	80 ± 32	212 ± 50	177 ± 30
Cytochrome cc_1	58 ± 16	96 ± 37	140 ± 14	145 ± 17
Cytochrome *c* oxidase[c]	667 ± 100	638 ± 306	435 ± 244	640 ± 175

[a]The units for cytochromes and total heme are in pmole/mg protein. The units for other enzymes are in nmole substrate (reduced or oxidized)/mg protein/min.

[b]β-NF, 40 mg/kg of body weight, in corn oil was injected IP on days 5 through 7 of lactation.

[c]These enzyme assays were described previously (*16*).

[d]Mitochondrial fractions were suspended in 0.25 *M* sucrose, 0.025 *M* Tris-Cl buffer, pH 7.4, to 5 mg/mL protein and were clarified with 1% desoxycholate. Cytochrome *c* (cc_1) and aa_3 were determined from change in absorbance ΔA 550-540 nm (ε = 19/*mM* cm) and 605-630 nm (ε = 16/*mM* cm), respectively, of the difference spectrum of 6 μ*M* antimycin A-treated and 7.4 μ*M* PES, 7.5 *mM* succinate, 20 *mM* ascorbate reduced minus 86 μ*M* $K_3Fe(CN)_6$ oxidized complex. Cytochrome *b* was calculated from the increase in absorbance at 562-575 nm (ε = 25.7/*mM* cm) upon addition of dithionite to the reduced sample.

higher metabolic capacity than those of nonlactating rats (Fig. 1). This may be explained by enzymatic changes associated with the initiation and maintenance of lactation in the rat mammary gland (*21, 22*). Mammary microsomes of lactating rats metabolized 2-FAA to 7-, 5- and 3-hydroxy-2-FAA, as well as to 9-hydroxy- and *N*-hydroxy-2-FAA. However, no detectable amounts of *N*-hydroxy-2-FAA were formed by mammary microsomes of nonlactating rats in which 9-hydroxy-2-FAA was a major metabolite. Comparison of 2-FAA-metabolizing capacities of mammary and hepatic microsomes of the induced lactating and nonlactating rats clearly shows that the liver is the chief site of metabolism of 2-FAA and of its activation *via* *N*-hydroxylation (Table 5). The critical role of hepatic metabolism is supported by the results of our earlier studies with disulfiram which inhibited hepatic *N*-hydroxylation of 2-FAA, thereby reducing its tumorigenicity in the mammary gland (*23*). In lactating rats, *N*-hydroxy-2-FAA produced by mammary microsomes could potentially be excreted in milk contributing to tumorigenicity in the suckling young. In preliminary studies, we found that IP administration of 2-FAA to lactating rats produced low to moderate tumor incidences in both male and female offspring (D. Malejka-Giganti and R. E. Rydell, unpublished experiments). The lack of capacity of mammary microsomes from 50-day-old female rats to *N*-hydroxylate 2-FAA may explain its weak carcinogenicity on direct application to the mammary gland (*11, 24*). It was found in two independent laboratories that 2-FAA, carcinogenic on systemic administration, was only marginally active after one topical administration, whereas *N*-hydroxy-2-FAA was strongly carcinogenic (Table 6). As expected from metabolic studies (Table 3), a weak systemic carcinogen, 3-FAA, was only marginally active after topical application. In contrast, its hydroxamic acid, *N*-hydroxy-3-FAA, was considerably more potent (Table 6).

Hormone Responsiveness and Cellular Composition of Mammary Tumors

Induction of mammary gland tumors by *N*-hydroxy-2-FAA and *N*-hydroxy-3-FAA was dependent on the presence of ovarian hormones in the host. Ovariectomized rats did not develop tumors, and administration of estrogen starting one week after ovariectomy did not restore carcinogenicity of the hydroxamic acids (*11*). The observed relationship between hormone responsiveness and the presence of hormone receptors in some endocrine tumors prompted us to analyze the *N*-hydroxy-2-FAA-in-

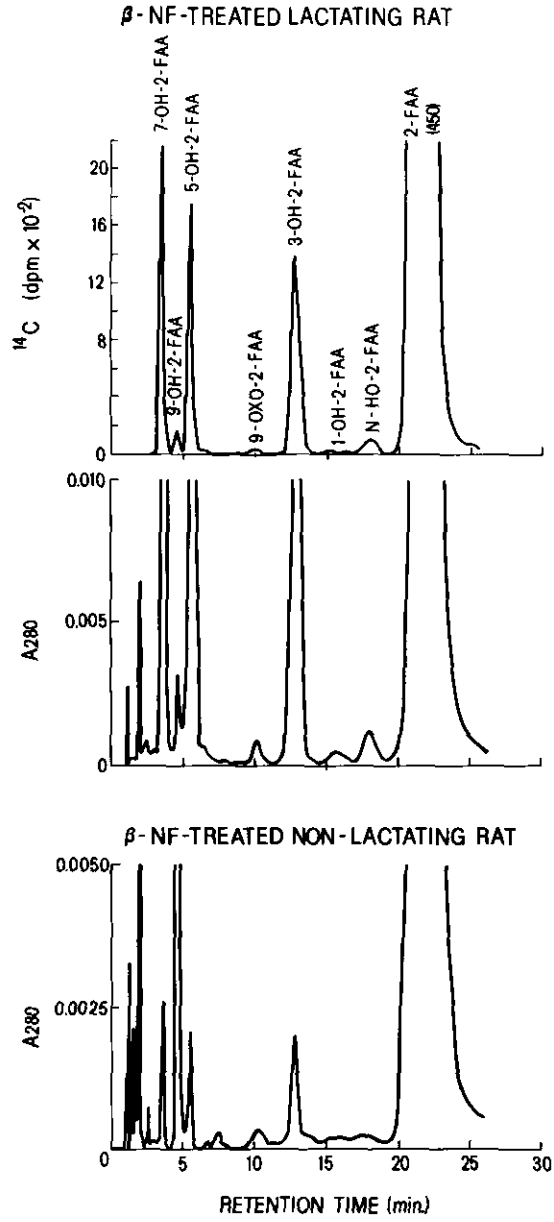

FIGURE 1. HPLC analyses of metabolites of 2-FAA formed by mammary microsomes of β-NF-treated female rats. Separations were carried out on a 150 × 4.6 mm ID Zorbax C_8 column with a 35.4 mm ID guard column of Perisorb RP-2 operated at 30°C and a flow rate of 2 mL/min. The mobile phase contained DMSO (9.5%, v/v), isopropanol (18%, v/v), tetrahydrofuran (1.5%, v/v), 0.5 *M* sodium chloroacetate, pH 3.0 (10%, v/v) and acetohydroxamic acid (1.25%, w/v) (*19*). A Varian Instruments Liquid Chromatograph, Model 5060, and a UV-50 variable-wavelength detector were used. When 2-[9-^{14}C]FAA (1.35 mCi/nmole) was used as the substrate, the eluate was collected in scintillation vials every 10 sec for the first 10 min and then every 15 sec for the remaining 15 min. A 10-mL portion of scintillation liquid was added to each vial and the radioactivity was determined using Tri-Carb Liquid Scintillation Spectrometer, Model 3255.

Table 5. Metabolites of 2-FAA formed by mammary and hepatic microsomes of β-NF-treated female rats.

Rat[a]	Source of microsomes	Metabolites of 2-FAA, nmole/6.0 mg protein ± S.D.[b]						
		7-OH-2-FAA	9-OH-2-FAA	5-OH-2-FAA	2-FA	3-OH-2-FAA	1-OH-2-FAA	N-OH-2-FAA
Nonlactating	Mammary gland	0.130 ± 0.127	0.581 ± 0.20	0.155 ± 0.042	—[c]	0.360 ± 0.111	—	—
	Liver	33.1 ± 0.13	5.98 ± 0.30	33.2 ± 0.52	2.29 ± 1.53	26.5 ± 0.62	0.378 ± 0.110	7.78 ± 1.22
Lactating	Mammary gland	2.26 ± 0.17	0.144 ± 0.016	3.00 ± 0.60	—	2.34 ± 0.70	—	0.282 ± 0.161
	Liver	38.2 ± 2.35	7.89 ± 1.79	35.9 ± 1.49	3.79 ± 0.95	27.3 ± 1.05	0.303 ± 0.088	9.15 ± 0.25

[a]β-NF (40 mg/kg of body weight) in corn oil was injected IP on days 48 through 50 of age or on days 5 through 7 of lactation.

[b]Mammary or hepatic microsomes, 6.0 mg protein in a total of 5.0 mL 0.1 *M* potassium phosphate buffer, pH 7.4, were incubated with 200 nmole 2-FAA in 0.1 mL methanol for 30 or 10 min, respectively; NADPH, 4.2 nmole, and 5.0 mL 1 *M* sodium acetate were added to start and to end the reaction, respectively. The control incubation mixtures contained heat-inactivated microsomes. The mixtures were extracted twice with diethyl ether, the combined either extracts washed with water and evaporated to dryness under a stream of nitrogen. The residues were dissolved in methanol and analyzed by HPLC (Fig. 1). Metabolite quantitation was by peak area determination relative to standard curves. The values were corrected for losses encountered during extraction.

[c]The limit of detection was 0.053 to 0.075 nmole of metabolite in the extract of the incubation mixture dissolved in 25 μL methanol of which 10μL was analyzed by HPLC.

Table 6. Carcinogenicities of *N*-fluorenylamides vs. *N*-fluorenylacetohydroxamic acids for the rat mammary gland after one topical application.

Compound	Dose, μmole/no. of glands	Tumor incidence	Total no. of tumors at the site
2-FAA	20/3	3/18	3 (3)[a]
N-OH-2-FAA		13/19	13 (9)
3-FAA		2/18	2 (1)
N-OH-3-FAA		15/18	34 (6)
2-FAA	15/6	3/25	1 (1)
N-OH-2-FAA		15/27	15 (13)
N-OH-2-FAA		12/27	11 (6)
N-OH-2-FPA		9/27	8 (3)

[a]No. of malignant tumors in parentheses.

duced mammary adenocarcinoma. We found receptors for estrogen, androgen and progesterone; the progesterone receptor content was increased 8-fold after treatment of ovariectomized rats with diethylstilbestrol (*25*). The sedimentation values, the association constants of cytoplasmic hormone receptor complexes, and the levels of estradiol and progesterone receptors were comparable to those of mammary carcinomas induced by 7,12-dimethylben(a)anthracene (DMBA) or by methylnitrosourea. The level of 5-α-dihydrotestosterone receptor in the *N*-hydroxy-2-FAA-induced tumor was twice that in the methylnitrosourea-induced tumor, but was considerably lower than the level of 7S androgen binder in the DMBA-induced tumor. The most prominent difference between the carcinoma induced by *N*-hydroxy-2-FAA and those induced by the other compounds was the absence of significant amounts of glucocorticoid receptor. This finding may be useful in studies of receptor interactions, e.g., those in which glucocorticoid binding would interfere with that of synthetic progestins.

With cell culture we showed that mammary adenocarcinoma induced in the rat with *N*-hydroxy-2-FAA involved epithelial and stromal cells of the mammary gland (*26*). Malignant transformation of both cell types was confirmed by induction of the respective tumor types after inoculation of subcloned cells into isologous hosts. Our findings are consistent with those of current studies showing that tumors have a heterogeneous cellular composition (*27*). Therefore, it will be necessary to assess the role of each type of cell in a target tissue in carcinogen metabolism and activation and to determine if intercellular regulation of metabolic activities occurs. Recent reports indicate that both cell types, rat mammary epithelial cells and mammary fibroblasts grown *in vitro*, metabolized B[*a*]P (*28, 30*). However, the levels of basal aryl hydrocarbon hydroxylase activity and the patterns of its inducibility by various agents *in vitro* differed in the two cell types.

Activation of N-Arylacylhydroxamic Acids to Ultimate Carcinogens

The mechanism by which *N*-hydroxy-2-FAA and other *N*-arylacylhydroxamic acids initiate tumorigenesis in the rat mammary gland is under investigation in several laboratories. Conversion to an electrophile is still considered the probable mechanism for activation of *N*-hydroxy-2-FAA. In theory, electrophilic species in the rat mammary gland may be generated from *N*-hydroxy-2-FAA *via* an intramolecular *N-O*-acetyltransfer catalyzed by *N-O*-acyltransferase, one-electron oxidation to the nitroxyl radical catalyzed by a peroxidase/peroxide system,

or conjugation to *N-O*-glucuronide catalyzed by UDP-glucuronyltransferase (*31*). Activation of *N*-hydroxy-2-FAA *via* all three mechanisms occurs to a significant extent in the livers of species susceptible and nonsusceptible to hepatocarcinogenesis (*32-34*). Therefore, carcinogenicity does not appear to be determined solely by these activation mechanisms. In the susceptible mammary gland, *N-O*-acyltransferase-mediated activation of *N*-hydroxy-2-FAA and of other related carcinogenic hydroxamic acids (Table 6) to *N*-acyloxyarylamines has been demonstrated (*24, 35, 36*). In the case of *N*-hydroxy-2-FAA, the most carcinogenic compound, this activation was insensitive to paraoxon, a deacylase inhibitor (*24*). Activation of the *N*-propionyl compound was partially inhibited and that of the *N*-formyl compound was completely inhibited by paraoxon. The mammary gland cytosol was subsequently shown to contain two separable enzymes, one specific for the *N*-acetyl and *N*-propionyl compounds and the other specific for the *N*-formyl compound. Since the *N*-formyl compound induced several sarcomas in the stromal component of the mammary gland (*24*), the distribution of the two *N-O*-acyltransferase activities among epithelial and stromal cells warrants further investigation. After administration of *N*-hydroxy-2-FAA to lactating rats, a majority of adducts isolated with ribosomal RNA from the mammary gland lacked the *N*-acetyl group of the hydroxamic acid (*36*). A similar result was obtained with nonlactating rats (*37*). These results indicate, although indirectly, that activation of *N*-hydroxy-2-FAA in the mammary gland can occur *via* the *N-O*-acyltransferase mechanism. Activation of the isomeric carcinogenic hydroxamic acid, *N*-hydroxy-3-FAA, by this mechanism has not been reported.

We are presently investigating one-electron oxidation of the fluorenylhydroxamic acids as a possible route to electrophilic reactants in the mammary gland. Oxidation of carcinogenic aromatic amines to nitroxide free radicals by hepatic microsomal enzymes, presumably the cytochrome P-450 system, has been reported (*33, 38*). Since mammary microsomes contain a considerably less powerful mixed-function oxidase than hepatic microsomes (*16*), we looked for other cytochrome systems that might participate in this oxidation. In contrast to the differences in microsomal enzymes, the content of mitochondrial cytochromes and the cytochrome *c* oxidase activity were similar in the liver and mammary gland (Table 6). As mentioned earlier, we found significant amounts of cytochrome aa_3, hence, of cytochrome *c* oxidase activity, in mammary microsomes whose electron micrographs did not show the presence of mitochondrial structures (*16*). We attempted to generate nitroxide free radicals of fluorenylhydroxamic acids by the cytochromes present in amounts sufficient for activity in the mammary gland. The ESR spectra of nitroxyl radicals of *N*-hydroxy-2-FAA and *N*-hydroxy-3-FAA generated by cytochrome *c* and hydrogen peroxide are shown in Figure 2. Generation of these radicals necessitated incubation of cytochrome *c* with Triton X-100 and peroxide. The nitrogen-splitting constants (8 G) and the *g* values (2.0067) for the nitroxyl free radicals of these two isomeric hydroxamic acids were similar to each other and to the values reported for the nitroxide radical of *N*-hydroxy-2-FAA (*39*). The initial

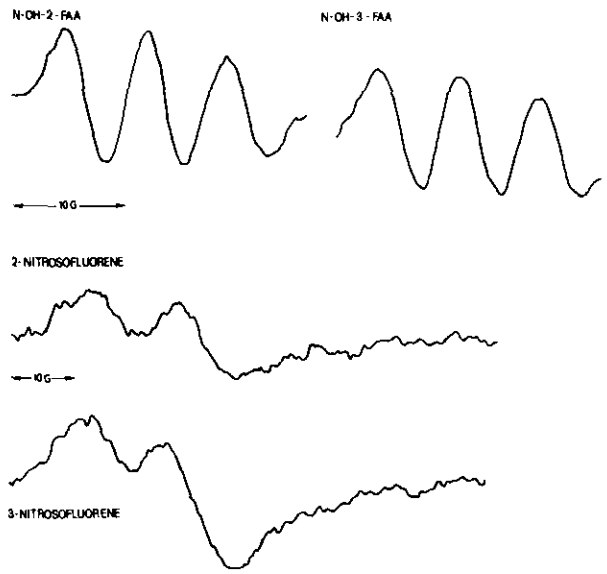

FIGURE 2. ESR spectra of nitroxyl radicals generated from *N*-fluorenylacetohydroxamic acids and nitrosofluorenes. N-OH-2-FAA or N-OH-3-FAA (a final concentration of 0.03 *mM*) was added in 0.02 mL methanol to 0.05 *M* potassium phosphate buffer, pH 7.5, containing 0.015% (v/v) Triton X-100 and 0.41 mg/mL of horse heart cytochrome *c* in a total volume of 3.0 mL. H_2O_2 was added with stirring to give a final concentration of 0.6 *mM*. The signal amplitude increased with time and the spectrum was recorded at ambient temperature after about 12 min. N-OH-2-FAA, at final concentrations of 0.03-0.33 *mM*, with 0.015 to 1.5% (v/v) Triton X-100 and 0.6 *mM* H_2O_2 did not give a signal even after 90 min. A Varian E-4 spectrometer with modulation frequency of 100 KHz at an 8 G amplitude was used. The scan rate was 20 G/min with an instrument response of 3 sec. The incident power was 20mW at a frequency of 9.442 GHz. 2-Nitrosofluorene or 3-nitrosofluorene (a final concentration of 0.33 *mM*) was added in 0.2 mL of methanol to the above buffer in a final volume of 3.0 mL. Hepatic microsomes were added to give a final protein concentration of 0.33 mg/mL. Solutions of L-α-lecithin (Sigma type V-E from egg yolk) in chloroform: methanol were evaporated under a stream of nitrogen and the lecithin was dissolved in buffer to a final concentraion of 10 mg/mL. The spectra were recorded after reaching maximal signal amplitudes at 16 min at ambient temperature. The instrument parameters were as above except the scan rate was 6.25 G/min with an instrument response time of 10 sec.

rate of formulation of the nitroxyl free radical from *N*-hydroxy-2-FAA was six times that from *N*-hydroxy-3-FAA, measured at two concentrations of the hydroxamic acids. The radical from *N*-hydroxy-3-FAA persisted for 29 min or 8 min at concentrations of 0.17 *mM* or 0.03 *mM*, respectively, about twice as long as did the radical from *N*-hydroxy-2-FAA. Using thin-layer chromatography and ultraviolet spectroscopy, we showed that the nitroxyl radicals of both isomeric fluorenylhydroxamic acids dismutated to their respective acetate esters and nitrosofluorenes (Fig. 3) (*40*). Both 2- and 3-nitrosofluorene were found to interact with double bonds in lecithin or in hepatic microsomal lipids and yielded ESR signals characteristic of immobilized free radicals (Fig. 2). Similar signals were obtained on interaction of the nitroxyl radicals with hepatic microsomes or mitochondria. In contrast, mammary microsomes or mitochondria rapidly quenched the signals of added nitroxyl radicals. As yet, we do not have a satisfactory explanation for the differences in behavior between mammary and hepatic subcellular fractions. We suspect that the lower contents of cytochrome P-450 (P_1-450) and of other hemes in the mammary gland, compared to the liver, may result in the accumulation of reducing equivalents and the consequent reduction of radicals. Work is in progress to define conditions under which the nitroxyl radicals could be generated in the mammary gland and be bound to its lipid membranes: a mechanism of considerable interest because of the high lipid content of the mammary gland.

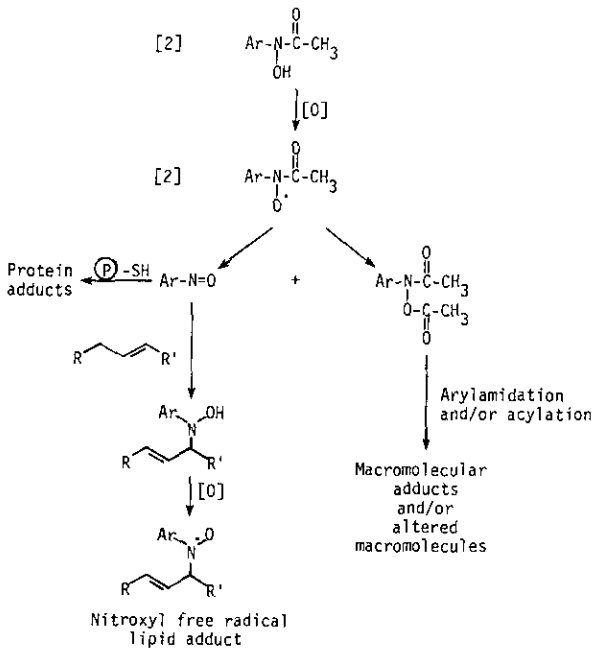

FIGURE 3. Activation of *N*-arylacetohydroxamic acid *via* nitroxide radical.

The dismutation product, 2-nitrosofluorene, interacts with -SH groups on proteins to yield fluorene-amine-adducts (Fig. 3) (*41*). 2-Nitrosofluorene may also add to double bonds of lipids in an Alder-ene fashion to form a hydroxylamine derivative which undergoes oxidation to yield a fairly stable nitroxyl free radical-lipid adduct (*42*). The other dismutation product, an acetate ester of fluorenylhydroxamic acid, may permanently alter DNA (*43*) or other macromolecules (*44, 45*), thus affecting the transcription process.

It is possible that both carcinogen-lipid and carcinogen-DNA interactions are critical in mammary tumorigenesis. Additions of aryl-nitroso compounds to double bonds of lipids (*42*) or spin-trapping of nitroxyl free radicals of hydroxylamines by membrane components (*33*) may cause membrane perturbations or structural membrane changes thereby affecting transport. Conceivably, transport of DNA-reactive carcinogens could be facilitated through carcinogen-primed membranes. Furthermore, interactions of carcinogens with lipids may be affected by dietary factors which have been shown to alter the lipid content of the mammary gland and to modify chemically induced mammary tumorigenesis in the rat (*46, 47*).

Conclusions

Activation of a systemic mammary gland carcinogen, 2-FAA, involves its N-hydroxylation by hepatic mixed function oxidase. Mammary mixed-function oxidase and activation of carcinogens, e.g., *N*-hydroxylation of 2-FAA, may be induced in response to mammary gland development or to specific environmental factors.

Of the *N*-arylacylhydroxamic acids tested heretofore, *N*-hydroxy-2-FAA is the most potent systemic and local mammary carcinogen in the rat.

N-hydroxy-2-FAA-induced mammary adenocarcinoma is hormone-responsive and involves epithelial and stromal components of the rat mammary gland.

Mammary tumorigenesis by *N*-hydroxy-2-FAA and related *N*-arylacylhydroxamic acids may involve the interaction of the products of oxidative metabolism with lipids and the interaction of the *N*-acyloxyarylamine, the *N-O*-ester or the *N-O*-glucuronide with DNA or chromatin.

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